

©Copyright 2026

Tiernan Kennedy

Toward Robust DNA Strand Displacement Circuits through Systematic Multi-Objective Design Optimization

Tiernan Kennedy

A dissertation
submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy

University of Washington

2026

Reading Committee:

Chris Thachuk, Chair

Georg Seelig

Jeff Nivala

Paul Yager

Program Authorized to Offer Degree:
Paul G. Allen School of Computer Science & Engineering

University of Washington

Abstract

Toward Robust DNA Strand Displacement Circuits through Systematic Multi-Objective Design Optimization

Tiernan Kennedy

Chair of the Supervisory Committee:

Assistant Professor Chris Thachuk

Paul G. Allen School of Computer Science & Engineering

Molecular computation using biochemical components offers a path toward programming and control of matter at the molecular level. Nucleic acid strand displacement circuits have emerged as a powerful substrate for systems spanning molecular signal transduction, logic, and dynamic chemical reaction networks. Yet despite major advances, practical deployment remains constrained by off-target interactions and other operational failure modes.

This dissertation advances a robustness-by-design framework for molecular computation by addressing four major classes of failure modes through three complementary optimization strategies spanning network architecture, sequence-level encoding, and chemical alphabet choice. These include sequence-level optimization layered onto existing leak-suppressing circuit architectures to reduce spurious interactions, expanded chemical alphabets for tuning molecular orthogonality, and architectural strategies for scaling input selectivity. Across theory, simulation, and experiment, these strategies systematically improve the operational fidelity and robustness of DNA strand displacement circuits.

Collectively, this work provides concrete solutions to key failure modes and a unifying framework for organizing robust molecular circuit design in programmable polymers. More broadly, it establishes a foundation for extending robust design principles to programmable sequence-defined molecular systems.

TABLE OF CONTENTS

	Page
Glossary	iv
Chapter 1: Introduction	1
1.1 Aspirations and challenges in molecular computing	1
1.2 Applications of molecular computation	2
1.3 Barriers to advances	4
1.4 Optimization axes	6
1.5 Contributions	7
1.6 Thesis statement	8
1.7 Research questions	8
Chapter 2: Foundations	10
2.1 Biophysics of strand exchange	10
2.2 Architectures and applications in nucleic acid computation	13
2.3 Operational challenges in nucleic acid circuits	17
2.4 Improvements in strand displacement design	22
2.5 Expanded chemistry	26
2.6 Summary	30
Chapter 3: Sequence-level reaction landscape optimization in leakless circuits . . .	31
3.1 Introduction	31
3.2 Design Framework	35
3.3 Results	40
3.4 Discussion	47
Chapter 4: Systematic chemical reactivity modification to expand the sequence optimization landscape	54
4.1 Introduction	54
4.2 Results	59

4.3	Discussion	66
Chapter 5:	Network-level optimization of selectivity in TMSD	76
5.1	Depth as a scalable selectivity variable	80
5.2	Simulated scaling and architectural limits	85
5.3	Experimental characterization	88
5.4	Discussion	93
Chapter 6:	Toward systematic design and optimization of sequence-programmable strand displacement systems	102
6.1	Key takeaways from Chapters 3–5	103
6.2	A multi-axis framework for DSD optimization	105
6.3	General design principles for new robustness axes	107
6.4	Future work: toward compilers for multiply robust DSD systems	108
Appendix A:	Methods	112
A.1	Chapter 3 Methods (Sequence-Level Optimization)	112
A.2	Chapter 4 Methods (Chemical Alphabet Expansion)	116
A.3	Chapter 5 Methods (Network-Level Modeling)	121
Appendix B:	Chapter 3 Supplementary Information	124
B.1	Design	124
B.2	Supplementary methods	131
B.3	X to Y Experiments	141
B.4	X to X Amplifier	157
B.5	Sequences	160
Appendix C:	Chapter 4 Supplementary Information	161
C.1	Full cascade mechanism	162
C.2	Detailed statistical methods	163
C.3	isoG/isoC substitution thermodynamics	167
C.4	Estimating isoC mismatch energies	170
C.5	Exemplary Leak	172
C.6	Reporter rate constants, 3' toehold system	173
C.7	Rate constants for each complex in both 3' toehold cascades	175
C.8	Fitting of kinetic constants	178
C.9	Tables of all sequences	192

Appendix D: Chapter 5 Supplementary Information	194
D.1 Detailed architecture description	194
D.2 Re-calibrated kinetic experiments	198
D.3 Effective concentrations used in remaining experiments	198
D.4 Sequences	201
Appendix E: References and Contribution Statements	202
Bibliography	203

GLOSSARY

DNA STRAND DISPLACEMENT: A type of reaction in which an invading single-stranded sequence of DNA binds to a complementary duplex through a single-stranded region and displaces one of the incumbent signal strands from the duplex. This reaction is often considered irreversible.

DNA STRAND EXCHANGE: A generalization of the strand displacement reaction where the invading single-stranded molecule cannot fully displace the incumbent strand from the substrate duplex, requiring the displaced strand to melt-off by a number of base-pairs. These reactions are reversible.

NETWORK ARCHITECTURE: This work defines network architecture as the ideal CRN targeted to implement a specific behavior

SPECIES-LEVEL DESIGN: The species-level decision of a circuit determines how well individual species participate in/implement the desired network architecture

CHEMICAL ALPHABET: The set of bases/monomers used in the species-level design for a molecular circuit

SUBSTRATE STRAND: The strand in a strand displacement or exchange reaction that binds the input

FEED-FORWARD: A circuit is feed-forward if it does not contain any cycles (loops)

DIGITAL POLYMER: A polymeric molecule capable of storing information in the sequence of its monomers

FUEL: A molecular species maintained at high concentration to produce the driving force for a reaction. For example, given DNA strands X and Y , the conversion $X \rightarrow Y$ is often implemented as $X + F \rightarrow Y + W$, where F is a double-stranded fuel species that binds X to release Y , an initially sequestered output strand. W is a waste product consisting of the input and one strand of F . In catalytic system, single-stranded fuels are often used to return a catalytic species to its original state by displacing them from an intermediate

WASTE: A product of reaction not intended to react in downstream complexes. Some wastes might react with the output they released to initiate the reverse reaction

TRIGGER: A single-stranded reactant in a strand displacement or exchange reaction that upon addition to the fuel species will lead to the production of output

INVADER: A single-stranded nucleic acid in the process of toehold binding or branch migration with a complex in a nucleic acid circuit

OUTPUT: A species released from a strand displacement or exchange reaction intended for detection or to participate in downstream reactions

INPUT: A trigger molecule that serves as a logical bit. The presence of an input signifies the value 1 and the absence signifies the value 0

CHEMICAL REACTION NETWORK (CRN): A network formed from the composition of abstract chemical reactions. $X \rightarrow Y, Y \rightarrow Z + X$ is an example of a small CRN.

DOMAIN: A functional unit of a nucleic acid system with an abstracted sequence. A toehold— a single-stranded region adjacent to a duplex designed for an input strand to bind— is a classic example of a domain. Domains of the same type (like a toehold) can have different sequences. A single domain with a defined sequence might have multiple instances across the design

LEAK: The production of output in a circuit without the presence of input

SPURIOUS REACTION: An interaction in a molecular circuit that is detrimental to desired behavior and was not part of the set of designed reactions

SPURIOUS: A non-designed detrimental occurrence in a molecular system is spurious. For example, spurious displacement would be the undesired release of an output due to an undesigned invasion event

THRESHOLD : A species intended to preferentially consume a certain amount of signal before it can propagate elsewhere

CASCADE: A chain of reactions where the output of one reactions is the input for the next. The abstract CRN $X \rightarrow Y, Y \rightarrow Z$ as an example of a two-step cascade

FAN-IN: The conversion of a number of input signals to a lesser number of output signals

ENTHALPY NEUTRAL: A reaction where the only change in free energy is related to entropy. For strand displacement or exchange circuits that preserve the number of independent species, enthalpy neutral reactions have approximately zero change in free energy

CROSS-TALK: Reactions between molecules or pathways that were not designed to interact

LEAKLESS: A class of nucleic acid circuits that can systematically reduce the thermodynamic prevalence of leak by adding redundancy to each step in the computation

COOPERATIVE: Reactions that require simultaneously interaction of multiple reactants to proceed

FAN-OUT: The conversion of a number of input signals to a greater number of output signals

PRODUCTIVE: A productive reaction that leads to the release of an output molecule

UNPRODUCTIVE: An unproductive reaction that temporarily consumes/depletes reactants but does not lead to the production of output

TOEHOLD OCCLUSION: A reaction in which a toehold for a desired reaction is bound and blocked by an undesired species

SYSTEMATIC REDUNDANCY: A design motif by which the translation of one sequence to a distinct output is split into multiple redundant reactions where strands where intermediate signals have partially shared sequences

MISMATCH ELIMINATION: The process by which a mismatching base-pair in a strand displacement/exchange fuel is replaced by a Watson-Crick-Franklin base-pair between the substrate strand and fuel

MISMATCH INTRODUCTION: The process by which a Watson-Crick-Franklin in a strand displacement/exchange fuel is replaced by a non-complementary base-pair between the substrate strand and fuel

MISMATCH MAINTENANCE: The process by which a mismatching base-pair in a strand displacement/exchange fuel is replaced by a mismatching base-pair between the substrate strand and fuel. This could be the same mismatch or a different mismatch sequence

CLAMP: A domain at the distal end (relative to the invader) of a strand displacement reaction that must melt in order to provide a kinetic pathway for leak to occur. These can be designed by including slight sequence overlap between two complexes with a potential leak pathway (which provides a kinetic barrier only) or by adding a reverse toehold to the reaction, converting it to strand exchange. This adds a thermodynamic barrier to leak, but also a barrier to the desired reaction

UNIVERSAL TOEHOLD: A single toehold sequence used across all gates in a design

ROBUST: I consider a molecular circuit with minimal observed impact of an error mode to be robust to that error mode

MISMATCH: A site in a DNA duplex where two bases that are not Watson-Crick-Franklin pairs are placed across from each other as if they would pair

TOEHOLD: A single-stranded portion of a nucleic acid structure intended to bind a single-stranded invader, providing an initiation point for a reaction that leads to the eventual release of a distinct strand

REVERSE-TOEHOLD: A single-stranded region in the waste product of a strand exchange reaction where the output can rebind to initiate the reverse reaction

INCUMBENT: In a strand displacement or exchange reaction an incumbent strand is bound to the initial fuel complex, and released as an output during the reaction

BRANCH MIGRATION: The process by which multiple strands in a DNA complex dynamically ‘compete’ for pairing with the same bases. If three strand take place in this process it is termed three-way branch migration, and is termed four-way branch migration if four strands are involved

DETAILED BALANCE: Reactions that satisfy detailed balance has forward rates (k_f) and reverse rates (k_r) that satisfy $\frac{k_f}{k_r} = e^{-\frac{\Delta G}{RT}}$, where R is the ideal gas constant, T is the absolute temperature, and ΔG is the standard free energy of reaction

ACKNOWLEDGMENTS

I thank my PhD advisor, Chris Thachuk, for his guidance and support throughout this work. I am also grateful to my committee for their valuable feedback.

I thank my collaborators, especially Cadence Pearce and Thomas Mayer, with whom I co-first-authored two of the projects included in this dissertation.

I also thank my labmates Tracy Mallette, Lancelot Wathieu, Zoë Derauf, Kellen Rodriguez, and Chandler Petersen for their support.

Finally, I thank my friends and family for their continued encouragement.

DEDICATION

To my family, for always supporting my dreams.

Chapter 1

INTRODUCTION

1.1 Aspirations and challenges in molecular computing

The general-purpose electronic computer has revolutionized our ability to process information. However, many forms of information originate in molecular form, such as nucleic acids, metabolites, or environmental chemicals. More broadly, the behavior of many natural and engineered systems is ultimately determined by molecular interactions. These interactions not only encode information about system state, but also govern processes such as chemical production, environmental response, and biological regulation. Because many macroscopic failure modes ultimately arise from molecular processes, monitoring these interactions can also provide insight into phenomena beyond the molecular scale, such as degradation of catalysts or structural materials. As a result, the ability to engineer molecular information processing systems may ultimately enable new forms of monitoring, coordination, and control within complex molecular environments.

Converting molecular information into a form suitable for electronic computation typically requires complex chemical assays followed by analysis on conventional computers. Similar challenges arise whenever the molecular state of a system must be monitored and acted upon. In such settings, information is often extracted from the system, processed externally, and only then used to guide intervention.

Conversely, systems that process molecular information using molecules as sensors, computational elements, and actuators have the potential to simplify these workflows by integrating sensing, information processing, and response directly within the molecular environment (Zhang and Seelig [2011], Simmel et al. [2019]). As an example, low-resource diagnostic solutions can substantially reduce barriers to healthcare access and have enabled

important advances in point-of-care testing (Weigl et al. [2014], Bharadwaj et al. [2021]). However, many such assays simplify molecular information into easily interpretable signals prior to analysis, effectively restricting the class of computations that can be performed on the underlying molecular data.

Compared to traditional molecular analysis workflows, molecular computation explicitly tackles the long-term challenge of designing modular and scalable molecular information processing systems that are robust to self-interference and interference from the environment. This simultaneously preserves the original molecular information available as input to the computation, and streamlines implementation.

1.2 Applications of molecular computation

1.2.1 Streamlining medical care

Streamlining medical diagnostics shows tremendous potential for reducing barriers to care in both high-income and low- and middle-income countries (LMICs) (Weigl et al. [2014]). The World Health Organization identified novel low-resource molecular diagnostics as a key tool in combating uncontrolled neglected tropical diseases by reducing the resource burden in primary healthcare centers (Bharadwaj et al. [2021]). Molecular computation may simplify diagnostic pipelines that require more complex reasoning than simple yes/no outputs. In 2018, Lopez et al. demonstrated the ability of molecular computation to realize complicated medical computations such as cancer diagnostics and classification of pathogens by type using molecular implementations of machine learning (Lopez et al. [2018]).

1.2.2 Environmental monitoring

The field of environmental monitoring faces similar challenges, where the detection and quantification of relevant molecular data is typically expertise- and resource-intensive, and low-cost alternatives often show low reproducibility and limited practical use (Andreescu and Sadik [2004]). Emerging bio-sensing technologies such as cell-free biosensors integrated

with downstream molecular information processing show great potential for mitigating these issues (Jung et al. [2022]). Jung et al.’s system in particular uses transcription factors as the sensing agent to enable detection of chemically diverse analytes, while DNA-based circuits enable sophisticated downstream processing of signals. Future applications might see simplification of logistically complicated and resource-intensive monitoring pipelines and potentially even coordination in intricate bio-manufacturing processes.

1.2.3 Manufacturing

Beyond sensing and diagnostics, molecular computation could enable new forms of control within biochemical production systems. Modern bio-manufacturing pipelines typically rely on carefully tuned reaction conditions and periodic monitoring to maintain stable production regimes. In many cases, reaction samples must be removed for offline analysis, or measurements from specialized sensors must be interpreted by external computer systems that coordinate subsequent interventions.

Embedding molecular information processing directly within reaction environments could enable systems to dynamically monitor and regulate production *in situ*. Compared to offline sampling, which introduces contamination risk, or control strategies mediated by external electronic systems, molecular circuits could operate directly within the chemical environment they regulate. Because these circuits are themselves implemented as *chemical reaction networks*, they naturally interact with multiple molecular degrees of freedom and information sources, as well as the nonlinear relationships among molecular species, potentially enabling more integrated responses to complex chemical states.

Recent work has begun to explore molecular implementations of control theory concepts such as proportional–integral–derivative (PID) controllers using *DNA strand displacement* systems (Whitby et al. [2021], Zhang and Lv [2023], Yuan et al. [2021], Paulino et al. [2019]) (Figure 1.1). These demonstrations suggest the possibility of molecular control systems that operate directly within engineered biochemical reaction processes. Realizing

such applications, however, requires molecular circuits that are significantly more robust and scalable than those currently available.

1.2.4 *Implementing parallel computation*

Given recent advances in using DNA as a data storage medium (Bee et al. [2021]), molecular systems may offer advantages for specific classes of computation that benefit from extreme molecular parallelism or direct computation on data stored in DNA (Adleman [1994]).

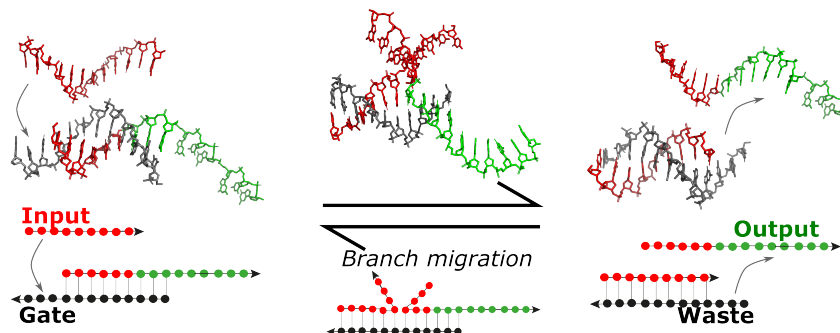
Because large numbers of molecular interactions can occur simultaneously within a reaction vessel, molecular computation operates in a fundamentally different computational regime than conventional electronic computing. When computing with chemical reactions, every copy of a molecular species can participate independently in reactions distributed across multiple competing computational pathways, allowing on the order of 10^{11} concurrent molecular interactions from a 1 nM input population. While conventional electronic systems also exploit parallelism, molecular systems may have the potential to extend parallelism to a fundamentally different scale, though with corresponding biophysical tradeoffs. While unlikely to replace general-purpose electronic computers, such systems may prove useful for specialized search, inference, or information-processing tasks that naturally operate on molecular data or benefit from massively parallel computation.

1.3 *Barriers to advances*

Despite these potential applications, and the emergence of the DSD reaction as a powerful primitive for molecular computation, large-scale implementations of molecular computation remain limited by practical constraints and scaling challenges. *Spurious reactions*, which are not part of the intended design but still impact circuit behavior, are one such limiting factor. These reactions often originate from architectural constraints when optimizing for a desired circuit behavior (Thachuk et al. [2015], Wang et al. [2023]).

Spurious reactions can also arise from the physical environment in which the circuit is implemented. Environmental factors, like degradation mechanisms (Mallette and Lakin

A DNA strand displacement is a powerful **primitive** for molecular computing



B The three common **failure modes** and paired **composable systematic solutions** presented in this thesis

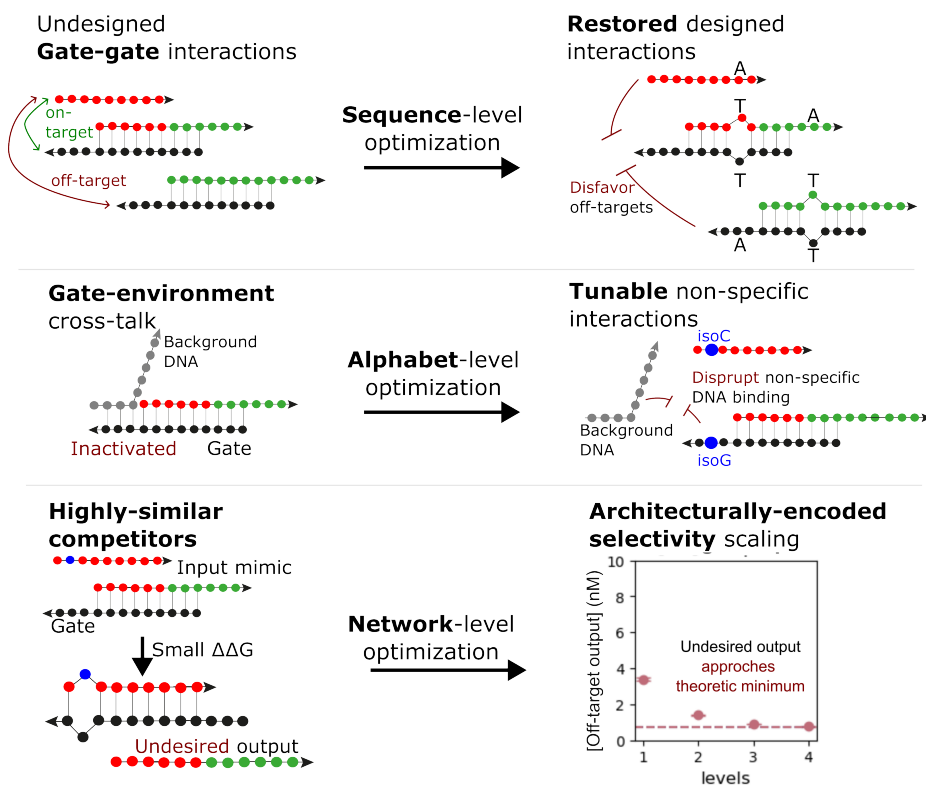


Figure 1.1: A) An overview of DNA strand displacement: a powerful primitive for implementing molecular computing. In the reaction, a single-stranded input binds a partially double-stranded duplex at the single-stranded toehold region. Then through branch migration, a competition between the invading and incumbent strands, the output may be displaced, providing an active input for downstream reactions. B) A summary of three major classes of circuit errors addressed in this work, paired with the systematic optimization strategies used to address them. The mechanisms underlying these error modes and optimization strategies are developed in Chapter 2.

[2022]) and interfering species (Mayer et al. [2022], Tang et al. [2020]), can alter the speed or correctness of computation.

Lastly, the ability to produce high-fidelity implementations of designed components at scale, including commonly used secondary structural motifs, remains an open challenge.

This thesis work systematically addresses the following two classes of failure modes resulting from off-target reactions with designed components (admitting active degradation and high-quality component synthesis as separate pursuits).

1. Unintended gate-gate interactions
2. Unintended gate-environment interactions

Each of these interaction types can lead to the erroneous production of output, which we call a *productive* reaction. Otherwise, we refer to the reaction as *unproductive*. Together, these give rise to four types of operational failure.

Systematic design strategies have emerged that address productive gate-gate interactions (called “leak”), but in the process slow kinetics and introduce new unproductive reactions (Thachuk et al. [2015], Wang et al. [2018, 2023]). This work extends this style of optimization to systematically address productive and unproductive variants of gate-gate interactions (Chapter 3), unproductive gate-environment interactions (Chapter 4), and productive gate-environment interactions (Chapter 5).

1.4 Optimization axes

This work classifies designs and optimization strategies for DSD circuits into three groups. At the most abstract level, *network architecture* determines the set of reactions used to implement a desired behavior. At a more detailed level, *species-level design* determines the sequences and structures used to implement those reactions and therefore controls their kinetic and thermodynamic properties. Depending on architectural restriction, not all target CRNs may have a faithful sequence-level implementation. Finally, the *chemical alphabet*

used to construct molecular species determines the underlying reactivity space available for circuit implementation.

While prior work has leveraged optimizations at the architectural (Thubagere et al. [2017], Thachuk et al. [2015], Wathieu et al. [2023]), sequence (Fornace et al. [2020], Irmisch et al. [2020]), and chemical alphabet (Ho et al. [2006], Mallette et al. [2020]) levels, this work provides strategies for systematically tuning circuit designs along these axes to achieve robustness to targeted failure modes by design.

1.5 Contributions

Molecular programming has produced a rich set of abstract circuit architectures, but translating these designs into reliable physical implementations remains challenging. In practice, these circuits often fail due to spurious reactions that were not intended in the design but nevertheless affect circuit behavior.

This thesis investigates systematic strategies for improving DNA-based molecular circuits across three complementary design axes: sequence-level optimization, chemical composition, and network architecture. This is demonstrated through a combination of theory, computational modeling, and experiment.

I begin this thesis by addressing the known kinetic drawbacks of “leakless” architectures by applying a complementary sequence-level optimization strategy. This strategy works by systematically biasing the reaction energy landscape toward designed reactions and against undesired reactions between circuit components. I then show that one can layer an additional, distinct sequence modification strategy over the first two to reduce the prevalence of a distinct class of spurious reactions, without negating the benefits of the first two strategies. Lastly, for a class of spurious reactions that resists sequence-level solutions, I develop a systematic network-level design strategy that does not preclude the use of any of the aforementioned sequence-level strategies. The promise of such composable layering of strategies, while not investigated across all possible operational drawbacks, is summarized in the following thesis statement.

1.6 Thesis statement

Composable and largely orthogonal optimization strategies targeted at specific error modes provide a systematic pathway for addressing spurious reactions in toehold-mediated strand displacement circuits.

1.7 Research questions

In this work, I address the following research questions.

1. Can sequence-level optimization mitigate existing kinetic drawbacks in systematically robust strand displacement circuits?
2. Can sequence-level optimization improve kinetic reliability of strand displacement circuits in the presence of competing background species?
3. Can network-level optimization provide systematically scalable input selectivity in constrained molecular circuits?
4. To what degree are these novel optimizations against spurious reactions orthogonal and composable?

Research questions 1, 2, and 3 are addressed in Chapters 3, 4, and 5 respectively. Research question 4 is partially addressed jointly in Chapters 3, 4, and 5, and summarized holistically in Chapter 6.

This work designs, implements, and characterizes composable, systematic optimization strategies for three highly operationally impactful classes of spurious interactions in toehold mediated strand displacement systems. While this work on its own does not fully bridge the gap between strand displacement circuits as an art form and a robust engineering technology, by demonstrating the efficacy of composable design strategies to address specific operational challenges, I hope this work encourages development of principled strategies for

systematically engineering robust strand displacement systems over a broader landscape of challenges.

Chapter 2

FOUNDATIONS

2.1 Biophysics of strand exchange

Due to the sequence programmability of nucleic acids, DNA computing emerged as a key strategy for implementing molecular computation (Zhang and Seelig [2011], Simmel et al. [2019]). DNA *strand displacement* reactions, where a DNA duplex binds a single-stranded DNA complementary to one of its strands and releases a single-stranded DNA with a distinct sequence, allow construction of molecular circuits by implementing chemical reaction networks (*CRNs*). These reactions offer a natural choice for building circuits of chemical reactions due to their ability to translate sequences to those with a distinct reactivity. Although presented as early as 1986 (Ellwood et al. [1986]), this reaction was popularized by Yurke and Mills (Yurke and Mills Jr [2003]) as well as Turberfield et al. (Turberfield et al. [2003]) in the early 2000s.

A strand displacement reaction begins when a single-stranded DNA *input* binds a *toehold*, a typically 3 to 7 nucleotide single-stranded region complementary to the input, on a double-stranded *fuel* molecule. The toehold provides a kinetic initiation point and thermodynamic drive for the reaction. The strand with the toehold is often called the *substrate strand*. Upon toehold binding, the invading strand can compete with and partially displace the *incumbent* strand opposite the toehold-containing strand. This competition between strands, termed *branch migration*, is often modeled as a random walk process over the number of nucleotides in the branch migration domain occupied by either strand. In an unsuccessful reaction, the invader might reach its initial toehold bound state, and dissociate with a probability inversely proportional to the binding energy. This dissociated strand is called an *output*. However, if the invading strand reaches the end of the helix, the incumbent

strand is displaced. Since the displaced strand does not have a toehold to rebind, the backward reaction is greatly kinetically and thermodynamically diminished. If the DNA strand does not completely displace the incumbent, such that a final melt-off step is required, this is termed *strand exchange* (Zhang and Winfree [2009]). This reverse toehold might serve in reactions with strands produced by other reactions. I present a schematic of a strand exchange reaction in Figure 2.1, including common vocabulary, and a representation of the kinetic reaction energy landscape. If the length of the *reverse-toehold* is zero, the diagram depicts a toehold mediated strand displacement.

Together, the lengths of the toehold, reverse-toehold, and branch migration domains provide powerful tools for tailoring the biophysics of strand displacement reactions. Yurke and Mills as well as Turberfield found an exponential increase in reaction rate constant with increasing toehold length up until approximately 7 nucleotides, at which point it plateaus. In a strand displacement reaction, thermodynamics and kinetics are linked. Increasing the toehold both increases the *rate constant* and the number of bound nucleotides in the product, leading to an overall decrease in free energy. While the output strand of a strand displacement reaction does not have a toehold to initiate the reverse reaction, the reverse reaction may proceed if initiated by transient fraying of 1-2 nucleotides at the end of the helix. However, this reaction is significantly slower with a typical rate constant of $10^0 - 10^1 M^{-1} s^{-1}$ compared to a maximal strand displacement rate constant of $10^6 - 10^7 M^{-1} s^{-1}$.

The addition of this clamping domain also decouples the kinetics and thermodynamics of the reaction, allowing for independent tuning of reaction free energy and rate constant. Zhang experimentally analyzed the biophysics of strand exchange reactions, including strand displacement as a special case (Zhang and Winfree [2009]). Zhang showed that the bimolecular rate constant approximation is a good approximation for modeling the kinetics of DNA strand displacement, so long as the reactant concentrations ensure that initial toehold binding is rate limiting. In situations where branch migration is limiting, more detailed models that explicitly include branch migration may be required.

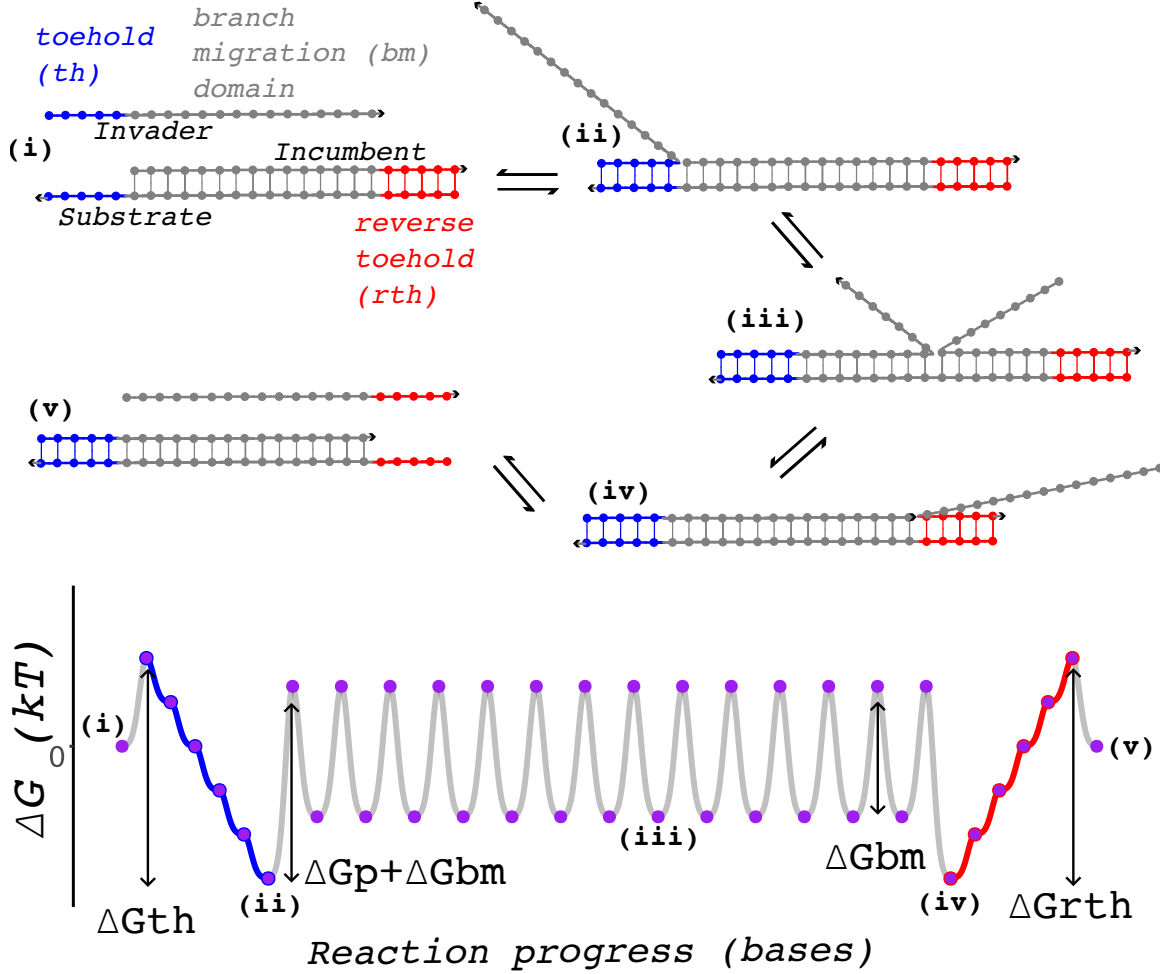


Figure 2.1: A general description of a strand exchange reaction. The reaction begins when an invader strand binds the single-stranded toehold domain on the substrate. The invader and incumbent then compete in a random walk process for binding of the incumbent strand. When the invader has fully invaded, the output/incumbent is released through melt-off of its bases bound to the reverse toehold domain. In a strand displacement reaction, this domain is not present and the incumbent is immediately displaced. The reaction coordinate energy plot shows the kinetic landscape for the reaction as the invader binds, progresses through branch migration, and displaces the incumbent. Visualized points in the reaction are labeled with roman numerals. The value ΔG_{bm} represents the activation energy barrier for the invader to exchange a bound base with the incumbent, and ΔG_p represents an energy penalty for initiating branch migration (Srinivas et al. [2013]). All reaction coordinate plots in this manuscript were created with the assistance of EveRplot (Bogdos and Morandi [2023]).

Srinivas et al. went on to develop a predictive ‘Intuitive Energy Landscape’ model of strand displacement, which models the system’s energy as a function of the number of base pairs bound by the invading strand (Srinivas et al. [2013]). In order to provide accurate predictions using this model, the authors found they needed to include an additional energy parameter ΔG_p , which serves as a penalty for initiating branch migration after the toehold is bound. Similar constructions based on the transitions between base pairs have been used for first passage time studies of strand displacement (Broadwater et al. [2021], Irmisch et al. [2020]).

More detailed models of DNA biophysics have been developed including the oxDNA model (Šulc et al. [2012]), which is a coarse-grained molecular dynamics framework for simulating DNA strand displacements and other DNA interactions.

2.2 Architectures and applications in nucleic acid computation

Since Lee Adleman’s first use of DNA in a paradigm to solve NP-Hard problems (Adleman [1994]), nucleic acids have gained significant traction for implementing logic at the molecular level, typically using strand displacement methods. Since then, researchers have developed a variety of circuit architectures for implementing arbitrary logic (Thachuk [2013], Qian and Winfree [2011a], Qian et al. [2011a], Thachuk and Condon [2012]) with varying degrees of space and energy efficiency. While a large set of architectures and motifs exist for nucleic acid computation, in this section I focus on a select set of architectures that provide a representative survey of widely used architectures, and a diverse set of accessible targets for further systematic optimization at the sequence level. In Chapters 3 and 4 I use “leakless” designs as a starting architecture for sequence and alphabet optimization. In Chapter 5, I present a motif that can be viewed as a modification of the seesaw or two-domain architectures.

2.2.1 Seesaw circuits

Seesaw circuits have enabled impressive applications including a binary square root circuit (Qian and Winfree [2011a]), associative memory through a Hopfield Network (Qian et al. [2011b]), and hand-written digit classification with both winner-take-all (Cherry and Qian [2018]) and loser-take-all (Rodriguez et al. [2021]) neural networks. Because they can implement a fundamental set of logic gates they are also Turing-Universal.

The network makes use of strand exchange *fuel* complexes with equal length forward and reverse toeholds. After initial strand exchange, a high concentration single-stranded *fuel* can carry out the reverse displacement instead of the output strand, leading to catalytic amplification by allowing the input to react with other fuel molecules. I review the structure of this reaction in detail in Chapter 4.

One downside of this architecture is that the long single-stranded fuels are complementary to the branch migration domain on the gate and can spuriously displace the output, leading to signal *leak*, or output in the absence of the input. To counteract this, the architecture also features a *threshold* species for each logic gate which preferentially consumes a certain amount of the output.

The seesaw motif easily handles *fan-in* and *fan-out* by including multiple signals compatible with a gate and using gates that consume the same input but distinct output signals respectively. The catalytic signal amplification ensures that circuits can pass signal independently of the ratio of fan-in to fan-out at a node in the network. Additionally, for a gate with fan-in, adjusting the amount of threshold for a gate tunes it to perform either 'and' or 'or' computation.

Seesaw circuits overcome the fundamental limitation in the design space of toehold sequences by using a *universal toeholds*. However, this leads to practical difficulties in scaling networks. Increasing the number of strands in the system which incorporate this universal sequence compounds the number of off-target interactions, slowing computation.

The strand exchange principles behind the motif have been extended to include temporal

logic gates and cooperative catalysts using the similar motifs (Lapteva et al. [2022], Taylor et al. [2021]). The authors proposed a route for array synthesis and enzymatic preparation of large-scale circuits (Qian et al. [2011a]) and have additionally explored methods to improve construction from low-cost unpurified DNA strands (Thubagere et al. [2017]).

2.2.2 Two domain architecture

The two-domain architecture, developed by Cardelli in 2013 (Cardelli [2013]) is another architecture for DNA computing arbitrary logic circuits. However, rather than implementing a fundamental set of logic gates like in the seesaw system, the two-domain architecture implements computation through networks of join and fork components. The two-domain architecture also uses a series of strand exchange reactions with a universal toehold to implement computation, however each signal is composed of two short domains that can be annealed to a single long strand of DNA or multiple shorter strands.

The design choices made for two-domain systems are extremely amenable to formal theoretical treatment, which has laid the groundwork for sophisticated molecular computing systems with theoretical grounding on par with electrical computers. Two domain circuits can be specified directly in a DNA programming language (Phillips and Cardelli [2009]) and are built on a robust set of abstractions (Lakin et al. [2012]).

In addition to the core computational components of the architecture, Cardelli also designed a sequence-able event reporter (Cardelli [2023]). This motif records the relative timing at which molecular events in the two-domain circuit occurred and can be read out in massively parallel fashion through high throughput sequencing. To assist in the design of large-scale two-domain circuits, users can leverage VisualDSD software which naturally interfaces with two-domain programming (Lakin et al. [2011]).

Despite its theoretical grounding, two-domain circuits suffer from the same signal leakage inherent to seesaw circuits due to complementarity of some of its components to entire domains of gates. The design additionally suffers from kinetic drawbacks from the use of a

single universal toehold.

2.2.3 *Leakless strand displacement designs*

Due to the prevalence of leak reactions in DNA circuit architectures, researchers developed a class of strand displacement designs that systematically reduce these undesired reactions (Thachuk et al. [2015], Wang et al. [2018]). These problems and solutions are discussed in detail in Sections 2.3.1 and 2.4.1 respectively. This design space was later modified with *enthalpy neutral* strand exchange reactions to impart further robustness. (Wang et al. [2022, 2023]).

Despite impressive guarantees in circuit robustness, *leakless* circuits pose their own challenges. Converting a 'leaky' design to a leakless strand displacement system generally requires increasing the number of DNA complexes implementing each reaction, which slows computation both by increasing the number of reactions and by introducing additional kinetically burdensome *cross-talk* between components.

2.2.4 *Dynamical nucleic acid systems*

Beyond digital logic, DNA strand displacement reactions can implement chemical reaction networks with other desirable dynamic properties. While some research focuses on rate-less circuits (Chen et al. [2014]), where computation is independent of reaction rates, other rate dependent circuits exhibit complex dynamic behaviors if the reaction rate constants are implemented precisely.

The power and limitations of DNA dynamical systems are summarized by the Srinivas et al. rationally designed rock-paper-scissors oscillator (Srinivas et al. [2017]), which physically implemented a three-reaction network which produces long-term stable oscillations between the concentrations of the three species.

2.2.5 *Enzyme-free catalysis*

Robust enzyme-free catalytic amplification has long been a goal for the field. In prior sections I addressed the catalytic amplification component of seesaw circuit, where the input serves as a true catalyst (Qian and Winfree [2011a]). In 2007, Zhang developed an entropy driven catalytic amplifier that could achieve linear signal amplification (Zhang et al. [2007]). When operated in an auto-catalytic setup, the amplifier could achieve exponential signal growth, however leak also increased exponentially leading to complete signal leak in under one hour. While they achieved reliable detection of 1 pM input, this rate of leak prevents hyper-sensitive detection and applications requiring long-term robustness of untriggered components. Catalytic hairpin assembly also achieves an exponential signal growth, but with a longer initial plateau (Dirks and Pierce [2004]). Fast and robust signal amplification remains a goal for the field.

2.3 *Operational challenges in nucleic acid circuits*

Widespread applications of complex molecular computation with nucleic acids has been historically hindered by the difficulty in implementing an idealized behavior in a realizable chemical system without side-reactions between engineered molecular components or between engineered components and the environment in which they are deployed. This thesis presents advances in systematically addressing both of these failure modes in DSD circuits.

Within a circuit, operational efficiency is reduced by undesigned or undesired reaction pathways that hinder circuit performance or introduce error.

2.3.1 *Productive error modes*

Regardless of the origin, productive error modes lead to the erroneous production of output. In this work, output that results from spurious interactions of designed components is termed “leak”, and output that stems from gate-environment interactions is termed “selectivity error”.

Productive reactions within circuits

Leak reactions, or those that produce output in the absence of input have been burdensome in strand displacement reactions throughout the field’s history. In Section 2.2, I highlighted multiple impactful strand displacement applications whose performance is limited by leak reactions.

Alistarh et al. highlighted that in addition to non-robust components, leak reactions may result from fundamental properties of chemistry (Alistarh et al. [2017]). For example, the authors highlighted that in any catalytic reaction $C + X \rightarrow C + Y$, an un-catalyzed reaction $X \rightarrow Y$ must exist at a lower rate. In many strand displacement designs with a catalytic reaction, the catalyst molecule C that is produced by the reaction is sequestered as part of the fuel. In this case, if there is leak by *spurious* invasion and release of the product, the leaked catalyst will cause a runaway reaction. Even in non-catalytic reactions, or those where the catalyst is not sequestered in fuel complexes, Alistarh et al. highlighted that it is common for two fuel complexes in a strand displacement to spuriously react and produce the output of one or both fuels. One prolific source of this type of leak in strand displacement implementations is the inclusion of components where single-stranded regions are sufficiently complementary to a duplex such that if there was base pair fraying, there would be a three-way *branch migration* pathway to leak (Thachuk et al. [2015]). This problem is often exacerbated when the spurious strand invasion is stabilized by stacking of the duplex on the invading complex against the incumbent complex (Qian and Winfree [2011a]). Olson et al. proposed the metric of availability (Olson et al. [2017]), the frequency in which a portion of a design is unpaired, as a metric for reducing leak by designing against spurious activation of fuels.

Productive interactions with the environment

In some cases, rather than output originating from interaction of designed components, an environmental nucleic acid might be so similar to the target input such that it leads to

the erroneous production of output. Biological examples of such input mimics include single nucleotide variants and epigenetic or post-translational base modifications. For single reversible bimolecular reactions, the selectivity between two competing reactant variants is bounded entirely by the difference in standard reaction free energies when considering either variant as input. Work in the field has improved the achievable selectivity in circuits by adding strategic modifications to the reaction network, like using competitive sink and competitive amplification together. However, an architecturally scalable solution, one where the design can be tuned to effectively suppress all off-target output even in the hardest practical edge-cases, remains a gap. Chapter 5 addresses this gap by expanding prior strategic composition network-level motifs, into a scalable, DSD-grounded proofreading strategy for systematically tuning circuit selectivity.

2.3.2 Unproductive interactions

Unproductive reactions within circuits

Some interactions temporarily sequester kinetically important intermediates, slowing or altering desired behavior, but do not lead to the production of output. One such example in the seesaw architecture, where the fixed toehold shared across numerous gates creates off-target interactions where signals temporarily bind the toehold of the wrong gates, preventing binding the correct input and slowing computation (Qian and Winfree [2011b]). Even leakless designs, which systematically disfavor productive spurious interactions, introduce new unproductive interactions between designed components in the process. This operational failure is addressed in Chapter 3.

Unproductive reactions with the environment

In addition to spurious reactions within a circuit, interactions between a circuit and the reaction environment can also significantly reduce operational efficiency. A major source of interference comes from nucleic acid strands in a sample mixture. This poses a significant

challenge in biomedical and environmental sensing applications. Mayer et al. highlight the impact of such interactions using pools of random nucleic acid background as a proxy for complicated biological environments (Mayer et al. [2022]). In their study they found significant kinetic slowdowns when single strand displacement reactions were operated in this random background. The analysis of their results found that these slowdowns likely stemmed from the small population of strands in the random mixtures with sufficient sequence complementary to kinetically vital regions of the circuit, such as the toehold. While they found that there were significant rate reductions when circuits were mixed directly with background, these slowdowns were increased when circuit components were incubated in the background before triggering. This operational failure is addressed in Chapter 4.

2.3.3 Additional implementation challenges

Precise rates in rate-dependent circuits

In Section 2.2, I highlighted the rock paper scissors oscillator as an impressive example of a rationally designed dynamical DNA system (Srinivas et al. [2017]). However, while this work demonstrated rationally designed oscillations, the behavior of the oscillator was not consistent with the rate constants they designed. These deviations from desired rate constants were confirmed by model fits. This is especially problematic in the case of the rock paper scissors oscillators because behavior is relatively insensitive to species concentrations, but sensitive to absolute rates. In situations like this, changing toehold length is a standard choice for rate modulation. However, this alters rates by orders of magnitude (Zhang and Seelig [2011]), which may be too coarse for rate-sensitive applications.

Designing precise domain affinities

In strand displacement circuits nucleic acid interactions are typically programmed through tuning nearest neighbor binding affinities. NUPACK is a leading software package for predicting such affinities in a variety of experimental conditions (Fornace et al. [2020]).

However, the increase in popularity of strand commutation circuits which use highly non-complementary domains has highlighted a failure of such tools to accurately predict affinities in these regimes (Nikitin [2023]). The authors hypothesize that because NUPACK was trained on experiments using highly similar nucleic acid strands, it under-performs in high dissimilar regimes.

In addition, non-standard nucleic acids, modified nucleic acids, and other digital polymers have gained popularity as a tool in nucleic acid engineering (Kabza et al. [2022]) (see Section 2.5). While robust consolidated models for DNA and RNA thermodynamics exist (Fornace et al. [2020]), it is resource intensive using existing methods to fit these parameters for non-standard nucleotides. This problem is compounded when considering base mismatches between classes of modified nucleotides. A research gap exists for high-throughput methods to quickly determine thermodynamic parameters for engineered digital polymers to an acceptable level for engineering applications.

Active degradation

Active degradation in biological or environmental regimes is a classic example of such a problem. When nucleic acid circuits are operated in samples containing nucleases, the components are degraded which can lead to signal leak or prevent circuit operation entirely (Fern and Schulman [2017]). Mallette et al. have previously demonstrated that end-capping strands with L-DNA, the enantiomers of the standard D-DNA bases can significantly reduce degradation of circuit components by exonucleases (Mallette and Lakin [2022]). Additionally, other strategies, like phosphorothioate backbone modification, can significantly inhibit cleavage by endonucleases (Eckstein [2014]). While this thesis does not focus on this failure mode, the strategies I present in subsequent chapters are compatible with these existing techniques.

Reliable and scalable gate production

The framing of this thesis does not ignore the current challenge of producing high quality DSD gates. At the time of writing, it typically takes significant labor to purify DNA strands and complexes for molecular circuits. And even with the best available purification, typical phosphoramidite synthesis methods still produce populations of strands with synthesis error or truncation (Filges et al. [2021]). However, throughout this thesis gates are explicitly designed with extra length to avoid side-reactions enabled by expected truncations. In addition, enzymatic preparation of components is another promising direction to achieve high quality strand and complexes.

2.4 Improvements in strand displacement design

2.4.1 Improving robustness

In Section 2.3.1, I highlighted signal leak in the absence of input as a pervasive source of non-robust behavior in nucleic acid circuits. Multiple strategies, ranging from the molecule level to the systems level have emerged to combat this.

Some early approaches suggested combating leak at the applications engineering level. Soloveichik et al. proposed that leak might be combated at the implementation level using well-mixed flow reactors to reduce the probability of leak events and their subsequent effects (Soloveichik et al. [2010]).

Leak can also be reduced at the architecture level. In Section 2.3.1, I highlighted undesigned three-way branch migration interactions as a pervasive source of leak pathways. This occurs when a single-stranded region is sufficiently complementary to the substrate strand of a duplex that fraying of base pairs at the edge are sufficient to allow spurious invasion and possible displacement. One early strategy for reducing leak added short ‘*clamp*’ domains at the end of helices that must melt, paying an enthalpy penalty, in order for a three-way strand migration leak pathway can occur (Qian and Winfree [2011a]). These clamps also prevent acceleration of leak pathways by additional stacking of helix ends between fuels. If

using reverse toehold as a clamp, one can scale this motif decrease the rate of leak exponentially by increasing the length of the clamps, which can reach up to a toehold length without disfavoring the desired reaction (Wang et al. [2022]) .

Olson et al. proposed the metric of minimizing mutual availability, the percent of time when domains are single-stranded, between complexes at possible initiation points for leak as a systematic strategy for leak reduction (Olson et al. [2017], Lysne [2023]).

While clamps introduce a bounded enthalpy barrier to leak, Thachuk et al. showed how one can use *systematic redundancy* to add an arbitrary entropic barrier to leak at the architecture level (Thachuk et al. [2015]). By splitting a single translation reaction between multiple complexes with domain-level redundancy, this ensures that more complexes are required to participate in a single leak event, and produces products with fewer total complexes, reducing the entropy of the system. This entropy barrier scales linearly with redundancy, providing a reduction in leak probability that scales exponentially with redundancy. This strategy was verified experimentally (Wang et al. [2018]). Wang et al. later showed how to combine the strategy with the enthalpy penalty gained from toehold sized clamps to create circuits with arbitrarily high signal-to-noise in addition to the energy efficiency imparted by the designs enthalpy neutral physically reversible architecture (Wang et al. [2023]).

Soloveichik later approached the problem of leak from a circuit level. They recognized that some forms of leak are inherent to the laws of chemistry. For example, in a catalytic system, the catalyzed reaction is always present, but proceeds at slower rate than the catalyzed version. Therefore they developed a circuit-level optimization for building leak-robust systems with components that have an inherent tendency to leak (Alistarh et al. [2017]). By ensuring the input species was a true catalyst (not sequestered by any fuel) they ensured any leak reactions could be recovered at the architecture level while still maintaining ‘fast’ detection.

While the field has made great progress toward robustness, in state-of the art circuit

level optimizations there exists a trade-off between robustness and fast kinetics (Wang et al. [2023]).

2.4.2 Improved kinetic and thermodynamic control

Mismatch introduction and elimination

Mutating DNA sequences to introduce or eliminate mismatched bases in strand displacement and other DNA interactions has emerged as a powerful mechanism for kinetic and thermodynamic control. This was proposed as early as the 1980s when Ellwood et al. investigated a single base pair mismatch in their synthetic DNA strand displacement assay (Ellwood et al. [1986]). Mismatches were also used to tailor dynamics during studies of branch migration in four-armed DNA junctions (Biswas et al. [1998]). As engineered strand displacement reaction gained popularity in the 2000s, this tool reemerged for performance optimization. For example in 2014, Jiang et al. showed that a strategic mismatch introduction in catalytic hairpin assembly circuits (a versatile and clinically relevant isothermal DNA amplification technique) improved performance by providing barriers to off-target reactions between components (Jiang et al. [2014]). These types of strategic mismatch optimization carried over generally to strand displacement circuit designs. As part of the development of the strand displacement architecture for DNA temporal logic circuits, Lapteva et al. used mismatches to reduce the design complexity in achieving their target behavior (Lapteva et al. [2022]). Strategically engineering a mismatch into one of their branch migrations domains allowed them to reduce cross-talk between signals without significantly altering displacement rates from their target. Apart from strand displacement, the thermodynamics of DNA mismatches are routinely employed in strategies for single nucleotide polymorphism (SNP) detection such as in Zhang’s 2023 paper (Zhang et al. [2023]). Beyond individual strategic applications of mismatched bases in individual application, I have recently published a general systematic strategy for fast and robust strand displacement with systematic mismatches (Kennedy et al. [2022]). I present this strategy in detail in Chapter 3.

Engineering and optimization works using mismatches in strand displacement circuits typically draw from a section of literature characterizing the biophysics of such modifications. Machinek et al. highlight that the energy landscape of strand displacement can be customized with DNA mismatches (Machinek et al. [2014]), which enables kinetic and thermodynamic programming. They demonstrated a decrease in strand displacement kinetics for an invader introducing a mismatch during strand displacement that decays exponentially with the distance from the toehold. Using this added control they were able to build a strand displacement system with independently tailored kinetics and thermodynamics, by simultaneously varying the position of an introduced mismatch and the number of bonds gained by an invader. Their results showed that when two invaders compete for binding of a duplex, an invader that introduces a mismatch early in invasion, but binds more strongly than the competitor has slower kinetics but a higher completion level compared to the competitor which introduces a mismatch later in invasion but binds less strongly.

Haley et al. later complemented and expanded this work by showing that *mismatch elimination* during strand displacement can provide a ‘hidden thermodynamic driving force’ and additional source of kinetic (Haley et al. [2020]) control. Inversely to the study of mismatch introductions, they found that an invader eliminating a mismatch in a strand displacement increases the rate of displacement with the effect dropping exponentially with distance from the start of branch migration. They were able to justify these results by showing how eliminating a mismatch increases kinetics by decreasing the chance the invader will dissociate early in invasion, whereas at longer distances from the toehold the probability of dissociation is too low for eliminating a mismatch to make a large difference in kinetics. Using their understanding, they showed how engineering a mismatch in a seesaw gate-style catalyst that is maintained by the invader, but eliminated by the fuel strand, greatly increases the completion level of the reaction. However, in this example having a fuel strand eliminate the mismatch also greatly increased the rate of leak.

Irmish et al. also published a detailed experimental and modeling study of mismatch

introduction and elimination (Irmisch et al. [2020]). In their model they expand the intuitive energy landscape model (Srinivas et al. [2013]) to include energy terms for the energy gain or reduction from mismatch introduction or replacement during branch migration. They found their fitted rate constants to agree closely with Machinek et al. (Machinek et al. [2014]) and expanded the experimental space of mismatch introduction and elimination to include a wider range of mismatch sites and toehold lengths. In support of their energetic model, they found that even if it was trained on data from single mismatches, it agreed with experimental results for multiple mismatch introductions. This work provides a powerful elementary model and experimental reference for practitioners engineering mismatches in strand displacement.

A complementary approach for modeling kinetics of mismatch interactions was taken by Broadwater et al. (Broadwater Jr and Kim [2016]) who developed a simplified linear Markov chain model that can use tuneable transition rates to model kinetics of the branch migration process through mean first passage time calculations.

Remote toehold strand displacement

Remote toehold strand displacement (Genot et al. [2011]) was introduced as an additional sequence level, kinetic modulator for strand displacement. By placing an unpaired sequence between the toehold and the start of branch migration, one can achieve an exponential rate decrease with the length of the spacer. This technique has been used to increase sensitivity for detecting single base mismatched with a probe (Li et al. [2016]). This technique can in theory also be implemented internally to a branch migration process by introducing a bulge loop.

2.5 Expanded chemistry

While nucleic acid computing is typically conducted with DNA, RNA, or DNA/RNA hybrids, non-natural bases, base modifications, and engineered *digital polymers* expand the chemical diversity of the sequence programming toolbox (Freund et al. [2022], Kabza et al.

[2022], Appella [2009]).

2.5.1 *Hetero-chiral nucleic acids*

While natural DNA exists in the D-isomer, researchers have investigated building strand displacement circuits that include the L-isomer, which is not found in biology (Mallette et al. [2020]). In fact Mallette et al. were able to build a robust strand displacement circuit using a leakless architecture that was partially composed of L-DNA and partially composed of D-DNA. The non-natural component of such systems confer robustness against biological exonuclease activity (Mallette and Lakin [2022]). One downside to such a strategy is that each transition between a section of L-DNA and a section of D-DNA in a circuit causes a kinetic slowdown.

2.5.2 *Modified nucleotides*

While diverse types of and applications for modified nucleotides exists, I present a small selection that highlights the breadth of potential for such modifications. Without modifying the base composition of the nucleic acid, it is possible to modify the backbone linkages. This has been found to confer additional nuclease resistance (Clavé et al. [2021]). Kabza et al. highlight a number of additional chemistries suggesting improved strand displacement robustness in harsh biological conditions (Kabza et al. [2022]).

One chemistry that Kabza et al. highlight is peptide nucleic acid, or PNA, which has gained a significant pool of research. This polymer uses a peptide-like backbone to join nucleobases (Demidov et al. [1995]). This modified polymer has even been synthesized with a number of standard techniques and applications in strand displacement including kinetic characterization (Demidov et al. [1995]), *cooperative* displacement (Kurakin et al. [1998]), and hetero-chiral strand displacement (Kundu et al. [2021]).

Molecules like PNA and LNA (locked nucleic acid), with differing binding affinities to DNA than DNA itself, have also emerged as an engineering tool for sensitive nucleic acid

probes (Mana et al. [2022]). Other nucleic acid analogues with acyclic base scaffold have been incorporated to provide pathways orthogonal to the presence of natural nucleic acids, but with some kinetic drawbacks (Murayama et al. [2023]).

2.5.3 *Orthogonal base pairs*

Orthogonal xeno-nucleic acid (XNA) bases mimic the base pairing properties of natural nucleic acid bases, but do so with human-engineered bases (Freund et al. [2022], Ding et al. [2020], Hirao [2006]).

While multiple non-natural base-pairing systems have been developed (Hirao [2006]), a select number have gained significant prominence and development. In the late 1990s Benner et al. developed a novel nucleic acid base (isoG: isoC) pair that could be incorporated enzymatically (Switzer et al. [1989]). He later led the development of a second non-natural base pair (P:Z) (Georgiadis et al. [2015]). Optimized variants of Benner’s base pairs were added to the set of natural nucleic acids to become the Hachimoji nucleotides, which were shown to be transcribeable and form a natural B-form helix (Hoshika et al. [2019]). Kawabe et al. recently expanded this alphabet to include all 12 possible nucleotides with standard topology and demonstrated enzymatic synthesis and nanopore sequencing (Kawabe et al. [2023]). The Romesburg Lab also developed a complementary pair of hydrophobic bases with transcriptional activity (Seo et al. [2009]), however there is difficulty with polymerase stalling and termination after incorporation.

The isoG:isoC pair has received significant experimental and theoretical study (Horn et al. [1995], Roberts [1997], Roberts et al. [1997], Johnson et al. [2004], Chaput and Switzer [2000], Sismour and Benner [2005]). At the time it is the only base in the expanded alphabet commercially available at scale from Integrated DNA Technologies. Firebird Biosciences additionally offers individual arrangements for synthesis of oligos containing P or Z nucleotides.

While the set of Benner-style bases have provided a valuable addition to the central dogma (Blas et al. [2004], Bande et al. [2015]), there are issues in fidelity during enzymatic

replication. This is likely due to the multiple tautomeric forms of the bases (Eberlein et al. [2020]), which can lead to stable pairing between isoG and T, as one example. This tautomeric instability decreases their viability as information carriers for a genetic code (Jaworski et al. [1985]) and creates a propensity for being substituted by natural bases (Switzer et al. [1989]).

Despite the promiscuity of these bases in enzymatic replication, the expanded chemical diversity and strand binding energy of isoG:isoC base has led to success in enhancing the fidelity and stability of DNA nanostructures with these XNAs (Liu et al. [2017], Ho et al. [2006]). The increased chemical diversity and orthogonality to natural bases has aided improvements for DNA-based sensing and diagnostic applications (Kimoto et al. [2011]). Hydrophobic bases that also act as a fluorophore quencher pair have been used for improved molecular beacons (Kimoto et al. [2010]). Since Benner-style bases are orthogonal to natural base pairs, they have been used to reduce false positive results and cross-talk with interfering natural sequences for molecular beacons, PCR primers, and sensitive hybridization probes (Sheng et al. [2008], Yang et al. [2010], Collins et al. [1997]).

I previously highlighted that strand displacement circuits show reduced efficiency when operated with interfering natural nucleic acid sequences. While bio-orthogonal DNA bases have shown significant promise for reducing cross-talk in nucleic acid diagnostic applications, researchers have yet to apply these additions within strand displacement reactions or circuits. Although orthogonal bases have been used in nucleic acid hairpin probes, the XNA was not designed to interact with an input and was instead placed in the hairpin duplex to reduce the chance of false-positive opening by background sequences. XNAs with altered backbones have been shown to exhibit orthogonality to binding DNA and RNA, however these modifications have yet to reach the commercial market, do not integrate with enzymatic machinery, and have shown kinetic disadvantages (Machinek et al. [2014]). In Chapter 3, I present a systematic sequence level strategy for improving reliability of fast and robust strand displacement circuitry using orthogonal base modifications. In this regime,

commercially available XNAs participate both in the circuit’s designed reactions as well as providing barriers to off-target reactions with background sequences.

2.6 Summary

In this chapter I’ve highlighted the great breadth of work in the area of nucleic acid-based molecular programming. The field has already developed a number of impressive demonstrations of its capabilities. However, the field still faces many operational challenges. I highlighted circuit robustness to “leak” reactions as one historical challenge with an existing systematic solution. However, I also highlighted that this solution leads to additional circuit complications that decrease kinetic performance. In Chapter 3, I’ll show how combining sequence level optimization and domain-level optimizations for robustness leads to a mutually beneficial synthesis of fast and robust molecular circuits. Systematic sequence level optimization allows us to maintain robust properties while reducing spurious interactions internal to the circuit. Another operational challenge I highlighted was circuit interference by components in the background. In Chapter 4 I show how I can systematically include non-standard nucleotides within our sequence-level strategy to discriminate against these external spurious interactions. Lastly, in Chapter 5 I show how paired network- and sequence-level optimization can together systematically enforce selectivity in DSD circuits.

Chapter 3

SEQUENCE-LEVEL REACTION LANDSCAPE OPTIMIZATION IN LEAKLESS CIRCUITS

This chapter is based on collaborative work co-first authored with Cadence Pearce and includes substantial experimental contributions from Zoë Derauf. Detailed contributions are described in Appendix E.

3.1 Introduction

Molecular programming researchers have long sought to design and build molecular architectures that 1) carry out complex computation, 2) operate robustly, and 3) have fast, reliable kinetics. This typically corresponds to choosing a chemical reaction network that computes the desired function or functions and accurately implementing the abstract network in a physical reaction system. Molecular circuit implementations often use nucleic acids (Zhang and Seelig [2011], Simmel et al. [2019], Hong and Šulc [2019]) and other synthetic digital polymers (Nielsen and Egholm [2001], Kurakin et al. [1998], Kundu et al. [2021]), due to the high programmability of these materials based on the predictive pairing of their monomers. DNA toehold-mediated strand displacement (TMSD) reactions have emerged as a dominant medium for molecular programming (Figure 3.1(A)). Using TMSD, researchers have successfully implemented large feed-forward circuits, like DNA neural networks (Qian et al. [2011b]), and cyclic circuits such as catalytic amplifiers capable of enzyme-free isothermal detection of low quantities of nucleic acids (Zhang et al. [2007], Jiang et al. [2013]).

Despite many impressive examples of DNA-based computations, spurious reactions within architectures often impact robustness and reliability, limiting widespread application of such circuits (Thachuk et al. [2015], Wang et al. [2018], Kennedy et al. [2022]). For example,

undesigned interactions between circuit components can lead to the output of signal in the absence of input, a computational error called “leak” (Thachuk et al. [2015]). Previous work addressed the tendency of these systems to “leak” output by adding scalable thermodynamic barriers to these undesired pathways (Thachuk et al. [2015], Wang et al. [2018]). While this work demonstrated a scalable entropic barrier to leak, increasing the number of DNA strand displacement reactions in the cascade slows the overall rate of output production in the system. This strategy also introduces unproductive spurious interactions between strands that do not impact robustness, but further slow reactions. Figure 3.1 illustrates how symmetric free-energy profiles imposed by systematic redundancy lead to kinetically unavoidable unproductive interactions. Converting each step in a leakless cascade to an approximately enthalpy-neutral strand exchange reaction adds additional enthalpic guarantees against leak (Wang et al. [2017, 2023]), but reduces the thermodynamic driving force for the designed reactions. As a result, systematic optimizations have suppressed leak, but at the cost of reaction kinetics, thermodynamic driving force, and the introduction of additional undesired reaction pathways, as an inevitable aspect of their structure.

Another design motif used in strand displacement systems is strategic use of mismatched (non-Watson-Crick-Franklin) base pairs. Mismatches alter the strand displacement reaction energy landscape (Irmisch et al. [2020], Broadwater Jr and Kim [2016], Haley et al. [2020], Machinek et al. [2014]), tailoring reaction thermodynamics (Machinek et al. [2014], Haley et al. [2020]) and tuning kinetics (Irmisch et al. [2020], Machinek et al. [2014]) with non-linear dependence on position. Introducing a mismatch during a strand displacement reaction requires the system to climb a local energy barrier which reduces the forward reaction rate constant and increases the reverse rate constant. Eliminating a mismatch during displacement provides an energy drop that produces the reciprocal kinetic effect on forward and reverse reactions. However, implementations of this motif have been carried out on a case-by-case basis (Jiang et al. [2014], Khodakov et al. [2015], Chen et al. [2013a], Wu et al. [2017]) for specific applications, rather than integrated into an existing composable

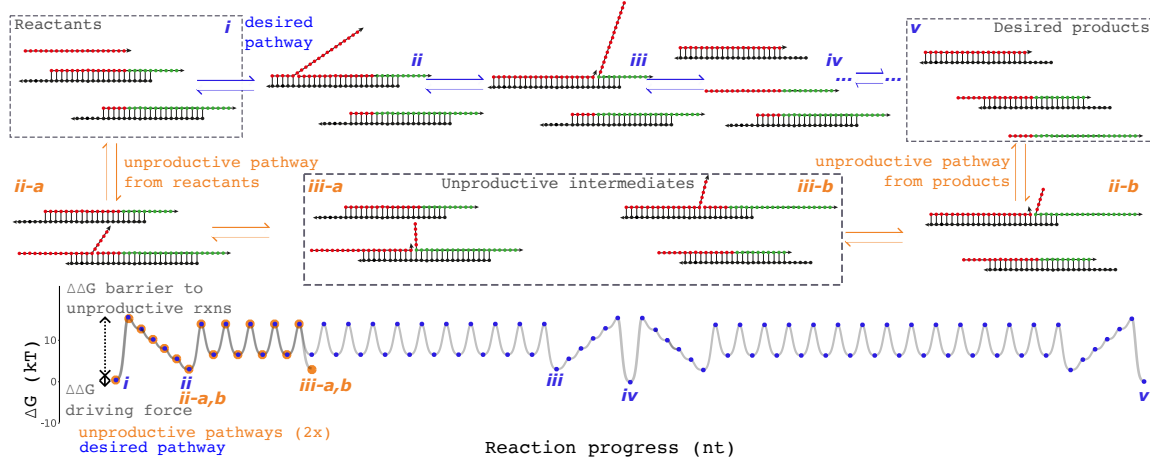
architecture in a way that scales arbitrarily with circuit size and complexity. This leaves a gap in how to best apply these motifs at scale across existing architectures to maximize circuit performance while avoiding off-target effects.

The challenges we have highlighted fall into two categories: 1) systematic strategies that suppress leak at the expense of kinetics (leakless designs), and 2) strategic motifs that can fine-tune kinetics but have been applied case-by-case rather than systematically (mismatches). A principled way to unify these approaches, maintaining systematic robustness while enabling performance optimization to mitigate operational drawbacks, remains an open question.

In this work, we show that the same structural feature (redundancy) that causes operational drawbacks actually provides the solution space. Specifically, domain-level redundancy, introduced to create barriers against leak, simultaneously creates sequence-level degrees of freedom that enable mismatch-based energy landscape optimization. We contribute a systematic implementation of mismatch optimization motifs that maintains the robustness of leakless design while effectively eliminating the operational drawbacks. By systematically introducing mismatches at redundant sequence positions, rather than requiring perfect complementarity throughout, we create pathway-specific energy landscapes that simultaneously favor desired reactions and disfavor spurious ones, as shown in Figure 3.1(B). By choosing mismatch optimizations that cause desired interactions to replace mismatches with matching pairs, and undesired interactions to introduce mismatches, we introduce ratchets that favor the designed pathway over competing pathways. We show that these mismatches can simultaneously add kinetic barriers to the reverse reactions of cascades, spurious interactions between signal strand and non-target fuel species, as well as signal strand and waste species, while simultaneously adding kinetic and thermodynamic drive to the desired reactions.

In addition to presenting a flexible design space with a feasible design approach and thermodynamic modeling of the strategy, we provide extensive experimental characterization of exemplary systems. We implement and model feed-forward four-step strand displace-

a. Robust design w/ burdensome unproductive reactions



b. Sequence-level kinetic/thermodynamic optimization

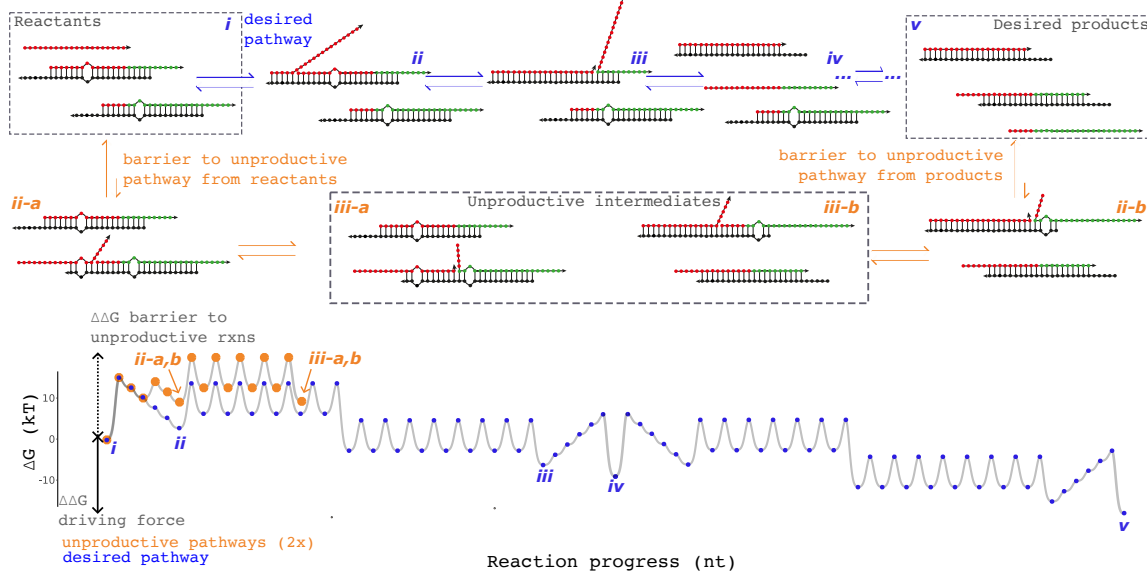


Figure 3.1: Energy landscapes of leakless strand exchange with and without systematic mismatch optimization. (a) Traditional leakless designs use domain redundancy to create barriers against leak, but symmetric sequence complementarity results in similar energy profiles for desired and spurious pathways, enabling unproductive interactions. (b) Systematic sequence-level optimization with mismatches provides preferential pathway-level optimization: desired invaders eliminate mismatches (kinetic ratchets favoring forward reaction and disfavoring reverse reactions), while spurious invaders introduce mismatches (thermodynamic barriers preventing invasion). This asymmetry maintains leak suppression while restoring performance. Thermodynamic simulations of this approach over concrete sequences are presented in SI Section B.3.3.

ment cascades (two toehold lengths with and without the strategy applied), showing that applying our systematic optimization improves bulk reaction rates and nearly eliminates reverse rate constants. Additionally, the optimization drastically reduces the measurable effect of kinetically burdensome spurious reactions between species. We also analyze the performance of the strategy in cyclic systems. This is an important area of consideration for leakless design motifs because leak is often a limiting factor in limit of detection for cyclic, amplifying TMSD systems (Zhang et al. [2007]). We compare the kinetic performance and limit of detection between a four-layer cyclic cascade built with only leakless enthalpy neutral principles, to one additionally optimized with our strategy, showing orders-of-magnitude improvements in kinetic performance and limit of detection. Finally, we demonstrate single-nucleotide discrimination in amplifying cascades, showing that diverse applications, such as fast signal translation to sensitive and selective detection, emerge naturally from different regions of the same unified design space.

This work establishes simultaneous optimization over the domain-level and sequence-level as a general methodology for constructing robust molecular circuits. Rather than choosing between robustness and kinetic performance, practitioners can now independently tune these properties across a Pareto frontier by systematically optimizing mismatch placement and sequence. This expanded design space provides a foundation for constructing faster, more robust, and more expressive molecular circuits than previously accessible by either strategy alone. More broadly, our results highlight that structural constraints introduced to solve one design challenge can provide concrete structural foundations for optimizing orthogonal objectives.

3.2 Design Framework

Systematic mismatch optimization in redundant leakless motifs

We first present our generalized strategy for simultaneously favoring on-target reactions, disfavoring reverse and unproductive spurious reactions, without significantly compromising

leak. In Figure 3.1, we showed how mismatches introduced at sites of systematic redundancy can break the energetic symmetry between on-target and off-target pathways. This can be implemented at any region of redundancy, starting from an existing sequence design with domain-level redundancy, through the following steps:

1. Set a maximum number of allowable mutation sites in the design
2. Restrict placement of mismatches to kinetically disfavor leak
3. Place optional restrictions on mutating input/output sequences
4. Constrain complementarity rules over reactants and products
5. Enumerate design space
6. Choose designs from the Pareto frontier under the desired design constraints

In Figure 3.2, we illustrate this general framework applied concretely to the 5nt toehold four-step feed-forward cascade we demonstrate experimentally in this work.

Firstly, this approach allows feasible enumeration (or other search techniques) over the design space to efficiently identify an acceptable candidate under a set of desired properties. This density of acceptable designs that underlies this feasibility is illustrated later in this chapter.

Under nearest-neighbor energetic models (SantaLucia Jr and Hicks [2004]), mismatch optimization can increase the thermodynamic favorability of leak relative to the unmodified design when mismatch replacement is possible between overlapping fuel complexes. However, it is possible to have a thermodynamic tendency to leak without any kinetically viable leak pathways. Under the common hypothesis that leak initiates through fraying of helices in reactants and products (Thachuk et al. [2015]), we impose design constraints (α and β) that exclude kinetically accessible leak pathways from the considered space. All mismatches must be introduced with at least α base-pairs to the nearest helix end, and at

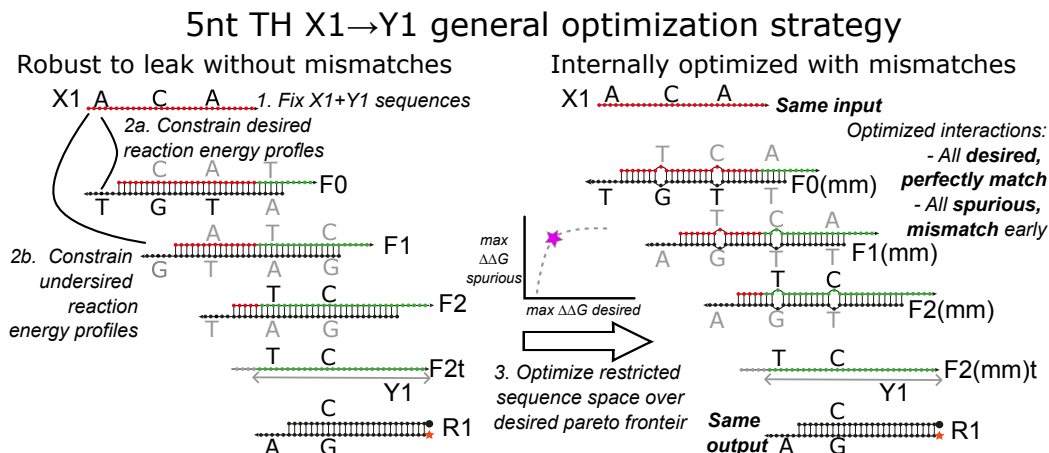


Figure 3.2: A general sequence-level strategy for kinetic optimization of “leakless” strand displacement designs (Thachuk et al. [2015], Wang et al. [2023]), illustrated over a four-step feed-forward cascade with 5 nucleotide toeholds (5ntTH X1 → Y1), with only key sequences illustrated. Bubbles in the illustrated duplexes represent the position of internal mismatches. Except for the marked positions, all positions within a vertical column share the same domain sequence. 1) To ensure the optimized cascade consumed the same input and produced the same output (compatible with the same reporter), we first fixed the sequences of the input (X1) and output (Y1). This provided strong energetic control for our demonstration, but may be relaxed depending on one’s specific goals. The sequences that were altered during optimization are colored in gray, with the remainder as black. 2) The reaction free energy profiles of desired and undesired reactions were optimized. While this step provides a flexible framework, we chose to enforce that all invading strands were completely complementary to their target incumbents, while all interactions between signal strands and off-target duplexes create mismatches in a toehold region and an additional position within 10 nucleotides of invasion. We applied this restriction for interactions between signals with both fuel and waste complexes to minimize kinetically burdensome sequestration of intermediate signals. 3) With these restrictions in place, the remaining positions were enumerated and the final design was chosen from the Pareto frontier for simultaneously desired properties of desired and undesired interactions. In our case we chose to compare the maximum $\Delta\Delta G$ of reaction for desired reactions (bounding the worst driving force) and the minimum $\Delta\Delta G$ of spurious interaction (bounding the most favorable undesired interaction). This constrained optimization yields the kinetic ratchet motif illustrated in Figure 3.1(B), but optimizes the mismatch energetics across sequence space; for example, ensuring that spurious invaders do not replace existing mismatches with one that is significantly more stable, which is possible across the set of mismatch sequences (SantaLucia Jr and Hicks [2004]). While here we illustrated the importance of optimizing the free energy landscape over a set of potential sequences, rather than arbitrarily choosing sequences by hand, in SI Figure B.1 we show how to rationally implement the constrained motif at specific sites of sequence-level redundancy. Full sequences for each strand are presented in SI Table B.12. A second cascade used in this study with additional mismatches and 7 nt toeholds is depicted in SI Figure B.2.

least β base-pairs between adjacent mismatches. This prevents helix fraying and formation of large internal bubbles which provide fast avenues to leak. These parameters can be estimated thermodynamically using modeling tools like NUPACK (Fornace et al. [2020]), or may need to be optimized empirically for reactant conditions not covered by existing models.

In this work, we do not change the input and output sequence of our feed-forward cascades during optimization. For our demonstration, this allows us to decouple the effects of our strategy from the net thermodynamics of the cascades. While this restriction is not generally necessary, it may be useful for those detecting biological sequences, wishing to use an existing reporter sequence, or applying the strategy at a subset of reactions in a larger network.

In this work, we use one set of complementarity constraints over reactants and products. We constrain that mismatches can exist in reactants if they do not both violate α and β , and that these mismatches must be converted to complementary base-pairs in the products. This set of constraints prioritizes increasing the driving force of the desired reactions, adding kinetic and thermodynamic barriers to reverse reactions, and providing modest energetic barriers to spurious interaction. Other complementarity restrictions are possible under this general strategy with distinct optimization tradeoffs, for example allowing on-target reactions to replace one mismatch with another.

With these algorithmic and physical constraints in place, we designed and implemented a set of exemplary systems which we experimentally characterize and validate in the subsequent sections. We used our design methodology to develop three distinct pairs of systems that demonstrate the flexibility of the framework to optimize different objectives. These designs probe two architectural contrasts related to our mechanistic hypothesis about the strategy. We designed two three-step 5 nucleotide toehold (5ntTH) feed-forward ($X \rightarrow Y$) leakless cascades, with and without systematic mismatch optimization, consuming the same input and producing outputs that trigger the same reporter, as a means to benchmark the

kinetic effect of the strategy. We then built a comparable set of cascades, but using longer 7 nt toeholds (7ntTH), where spurious toehold occlusion is expected to be more likely (see SI Figure B.16), to probe the specific kinetic impact of the strategy on unproductive spurious interactions. Lastly we constructed a paired set of control and mismatch optimized 5ntTH cyclic ($X \rightarrow X$) cascades, which implement a different overall reaction with distinct thermodynamic behavior.

The flexibility of the systematic mismatch optimization allowed us to tune model systems for these particular objectives through our choice of Pareto optimization. For example, in the 5ntTH $X \rightarrow Y$ cascades used to demonstrate the kinetic improvement with the strategy, we evaluated the space with respect to the most favorable $\Delta\Delta G$ of spurious invasion and the $\Delta\Delta G$ of the desired reactions. In contrast for the 7ntTH cascade where occlusion was central to the demonstration, we specifically optimized, at high gate concentrations, the thermodynamic difference in predicted spurious toehold occupancy resulting from the strategy. Compared to the 5ntTH feed-forward system, for the cyclic system we additionally optimized desired kinetics by placing the largest free energy decreases in the desired reactions closest to the site of initiation (Irmisch et al. [2020]). SI Section B.1.2 provides additional analysis of the enumeration of these design spaces.

Lastly, we found that across our designs, the strategy yielded dense sets of useful designs under the evaluated objectives for each optimization. For the 5ntTH $X \rightarrow Y$ cascade, the space includes 685,307 candidate designs. However, to illustrate the scope of acceptable designs induced by the strategy, for this cascade we did not choose a design on the Pareto frontier, but instead one that was approximately average in our objective (SI Section B.1.2). As we show subsequently, this design still achieved significant performance improvements.

3.3 Results

3.3.1 $X \rightarrow Y$ cascades: mechanistic verification

Systematic mismatches do not increase leak in feed-forward cascades

First, across both the 5ntTH and 7ntTH systems, we verified that despite increasing the thermodynamic drive toward leak, the strategy did not introduce substantial leak at typical experimental timescales. We present leak measurements and associated gate concentrations for each of the four cascades in SI Section B.3.1. Even at high gate concentrations (near $1\mu\text{M}$), we observed a maximum of 0.04% leak relative to the lowest gate concentration in either of the 5ntTH cascades over two hours. In the 7ntTH systems we observed two major timescales of leak. A small amount of leak was observed at early timepoints up to a maximum of 2% of the gate concentration over 2 hours for the mismatched variant and 0.52% for the control variant. Extended (24 h) measurements were performed for the 7ntTH mismatched system, which showed slower long-timescale leak of 0.35% from 2 hours to 24 hours.

Systematic mismatches offer kinetic advantages

We next tested whether the mismatch strategy increases the effective rate of translation, inferred through the simplified two-step model presented in Section A.1.4, and using a previously fit bimolecular rate constant for the reporting reaction in the second step (SI Section B.3.2). The fit kinetic curves used to produce an effective forward and reverse rate constant for each of the four cascades are illustrated in Figure 3.3(A). Associated statistical comparisons between fit rate constants across groups are presented in SI Table B.4.

For both the 5ntTH and 7ntTH cascades we measured a statistically significant increase in the effective forward rate constant and decrease in the reverse rate constant for the mismatched variant compared to the non-mismatched control. We also measured a statistically significant increase in the effective forward rate constant and decrease in the reverse rate

constant when comparing the same mismatch variant between 5ntTH and 7ntTH systems. However, we did not detect a significant difference between the forward rate constants for the 5ntTH mismatched and 7ntTH non-mismatched systems.

Systematic mismatches alter reaction network structure

While systematic mismatches improve the kinetics of the entire $X \rightarrow Y$ translation reaction, we also investigated the impact of the strategy on fitted forward and reverse rate constants at each level of all four $X \rightarrow Y$ cascades. The results and statistical analysis of the detailed rate modeling are presented in SI Section B.3.2. To probe system behavior under increased susceptibility to toehold occlusion and spurious invasion, we examined analogous cascades using 7nt toeholds at various levels of excess trigger concentration. The completion level analysis of this experiment is presented in Figure 3.3(B).

In the detailed model, inverse-variance pooled omnibus comparisons qualitatively agreed with the simplified modeling results. In both analyses, mismatch optimization significantly increased forward rate constants and substantially reduced reverse rate constants. Consistent with the simplified model, no significant difference was detected between the forward rate constants of the 5ntTH mismatched and 7ntTH non-mismatched cascades. The primary exception was that the detailed model did not detect a significant difference between the pooled forward rate constants of the 5ntTH and 7ntTH mismatched cascades, whereas the simplified model did.

Examination of individual gates revealed one notable exception to the overall trend. While mismatch optimization generally increased or did not significantly change fitted forward rate constants across the cascade, the 7ntTH F2 gate exhibited a statistically significant decrease in the forward rate constant following mismatch optimization. This exception is addressed in the discussion.

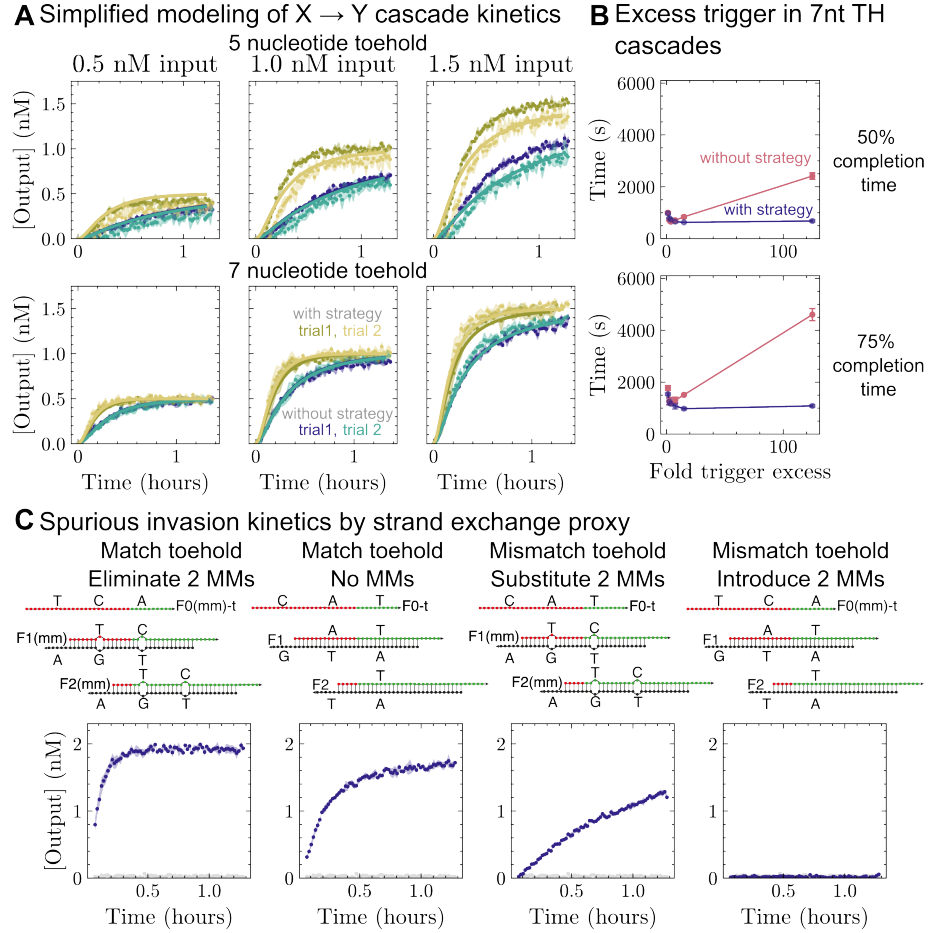


Figure 3.3: A) Simplified modeling of each of the feedforward cascades as an $X \rightarrow Y$ translation by a single fuel species, with Y consumed irreversibly by the reporter. The shaded regions around each curve represent the propagated point-wise standard deviations for each set of triplicates, the scatter points represent the corresponding mean, and the solid line represents the model fit to the curve of the same color. The non-mismatched 5nt toehold cascade was well fit to an effective forward rate constant of $1.8e05 \pm 2.3e04 \text{ M}^{-1}\text{s}^{-1}$ and a reverse rate constant of $5.2e03 \pm 6.8e02 \text{ M}^{-1}\text{s}^{-1}$. The non-mismatched 7nt toehold cascade was well fit to an effective forward rate constant of $5.4e05 \pm 3.2e04 \text{ M}^{-1}\text{s}^{-1}$ and a reverse rate constant of $9.3e-03 \pm 5.6e-04 \text{ M}^{-1}\text{s}^{-1}$. The mismatched 5nt toehold cascade was well fit to an effective forward rate constant of $6.7e05 \pm 1.4e05 \text{ M}^{-1}\text{s}^{-1}$ and a reverse rate constant of $1.4e-14 \pm 2.8e-15 \text{ M}^{-1}\text{s}^{-1}$. The mismatched 7nt toehold cascade was well fit to an effective forward rate constant of $1.3e06 \pm 1.2e05 \text{ M}^{-1}\text{s}^{-1}$ and a reverse rate constant of $1.3e-29 \pm 1.2e-30 \text{ M}^{-1}\text{s}^{-1}$. B) The 50% and 75% completion times, with associated standard deviations, versus trigger excess for the mismatched and non-mismatched 7nt toehold cascades. C) Directly testing spurious invasion kinetics using multiply mismatched triggers provides insight into the mechanism of spurious invasion reduction by systematic mismatches. We present the results of triggering gates at the F1 and F2 level of the 5ntTH cascades with 12.5X excess trigger strand, using both the correct trigger for each partial cascade as well as swapping triggers between the cascades. All cases were tested in the presence of 12 nM R1.

3.3.2 $X \rightarrow X$ cascades: systematic mismatches in cyclic, amplifying cascades

To this point we’ve presented data for feedforward cascades to verify the efficacy and mechanistic basis of our systematic mismatch optimization. However, cyclic circuits, where an output triggers an upstream gate in the cascade, represent an expressive circuit motif with distinct kinetic and thermodynamic behavior from the feed-forward case. In this section, we focus on using $X \rightarrow X$ cascades to illustrate behaviors made accessible by systematic mismatches.

Systematic mismatches introduce internal driving force to leakless cyclic circuits

We compared thermodynamic equilibria, simulated using NUPACK (Fornace et al. [2020]), between a control $X \rightarrow X$ amplifier built with balanced forward and reverse toeholds at each step and a systematically mismatch-optimized variant, and found qualitatively different predicted equilibrium distributions (SI Tables B.10, B.11). For the same concentration of input and fuel complexes, the equilibrium of the mismatch translator is predicted to be in the fully on state, while the non-mismatched translator is predicted to split between the starting complexes and the waste complexes.

Leak remains low in the mismatch-optimized $X \rightarrow X$ cascade

Although thermodynamic modeling predicts product-favored equilibria in the absence of input for the mismatch-optimized $X \rightarrow X$ cascade at infinite timescale, we observed low kinetic leak on experimental timescales. As in the $X \rightarrow Y$ systems, untriggered $X \rightarrow X$ cascades showed slow, approximately linear accumulation of output over time, with measured leak rates at 10 nM gate concentration of 0.0039 nM/h and 0.013 nM/h for the non-mismatched and mismatched systems respectively (Figure 3.4). This behavior was qualitatively consistent across both high and low gate concentration regimes tested.

Mismatch optimization enables rapid catalytic amplification

Analysis of the kinetics of the 5ntTH mismatched and non-mismatched amplifiers shows clear qualitative differences (Figure 3.4). For example, at 10 nM gate concentration, the non-mismatched cascade triggered with 10 nM input produced less than 1 nM output over 24 hours, while at the same gate concentration the mismatched cascade triggered with 500 pM input surpassed 5 nM output within minutes of initiating the reaction. At this cascade concentration the non-mismatched cascade did not produce a measurable output in response to a 100 pM input (SI Figure B.19).

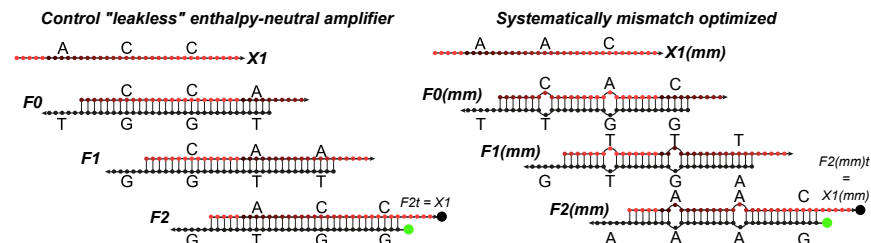
At 1 nM gate concentration, we found that the mismatch optimized cascade produced a measurable response to input signals as low as 10 pM. Additionally, the $X \rightarrow X$ translator amplified a 500 pM input signal up to an output concentration of 100 nM. This corresponds to approximately 600 individual strand exchange steps (three per catalytic cycle), or roughly two hundred full cycles of catalytic turnover.

3.3.3 Single-nucleotide discrimination in the $X \rightarrow X$ amplifiers

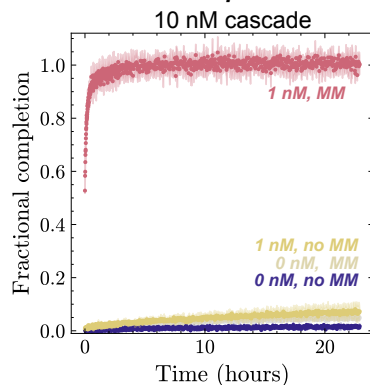
Given the catalytic behavior of the mismatch-optimized $X \rightarrow X$ cascade, we tested whether the amplifier exhibited distinguishable kinetics in response to single-nucleotide variants in the input strand (Figure 3.5). At 10 nM gate concentration, the mismatched amplifier showed distinguishable kinetics when triggered with 1 nM of the complementary input or 1 nM of an input with a single nucleotide mismatch in the toehold region. Under these conditions, the mismatched amplifier was able to kinetically distinguish between the same toehold-centered single nucleotide variants when present at 100 pM, whereas the non-mismatched variant did not show distinguishable kinetics.

We also tested whether the systems could distinguish between single nucleotide variants present in the branch migration region of the inputs. In this case, the mismatched amplifier with gates at 10 nM and inputs at 1 nM could distinguish the two variants kinetically, while the non-mismatched variant did not show distinguishable kinetics. For 100 pM input,

A $X \rightarrow X$ linear amplifier designs



B No MM and MM amplifier kinetics



C MM amplifier can detect 10 pM input

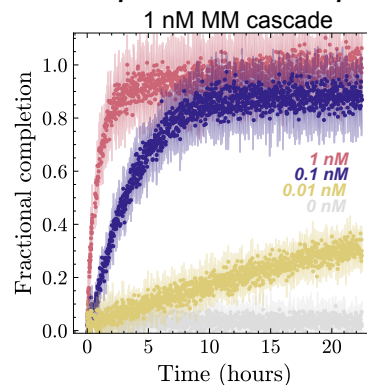
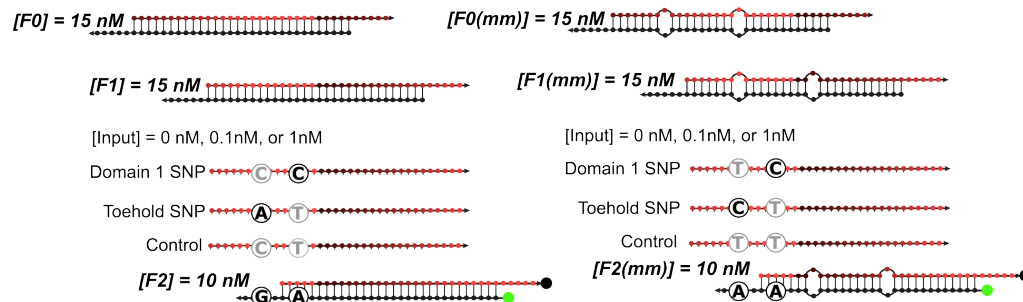


Figure 3.4: A) The $X \rightarrow X$ amplifier was modeled after the $X \rightarrow Y$, 5 nucleotide toehold cascade, replacing the Y domain with a second X domain. This design allows the output strand to serve as an input to the same cascade, amplifying the signal in a catalytic cycle. **a.** The no mismatch $X \rightarrow X$ amplifier design. **b.** The mismatch $X \rightarrow X$ amplifier design. Within each design, domains with the same sequence are given the same color. B) Kinetics of both mismatch and no mismatch amplifiers with F_2 complexes at 10 nM and all other gates at 15 nM. Amplifiers were triggered with their respective input strands at 1 nM. Cases with no input are shown to establish a baseline leak for each amplifier. The leak rates for the no mismatch cascade and mismatch cascade were calculated as 0.0039 nM/h and 0.013 nM/h ("0 nM, noMM" and "0nM, MM"), respectively. Fractional completion is defined relative to the endpoint of the highest output for the mismatched amplifier. C) The $X \rightarrow X$ mismatched amplifier with 1 nM complexes allows observable response to a 10 pM input signal. F_2 present at a concentration of 1 nanomolar with F_0 and F_1 complexes at 1.5 nanomolar were triggered by the input concentrations labeled in the respective color. The data for the un-triggered cascade ("0 nM cascade") is provided to distinguish observed output from leak. Fractional completion is defined relative to the highest output measured.

A SNP discrimination experiment overview



B SNP discrimination experimental results

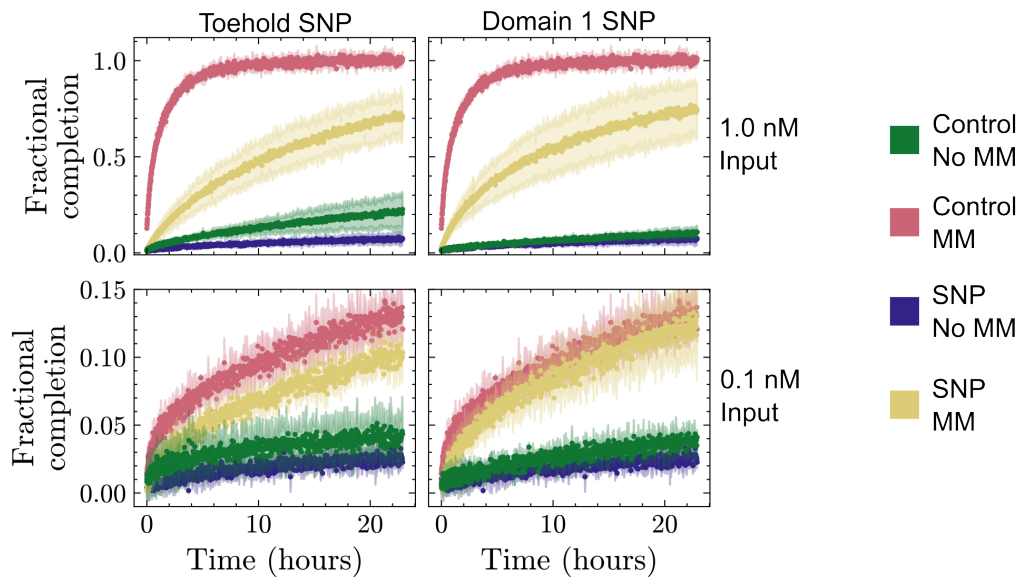


Figure 3.5: Standard vs mismatched inputs in the mismatch and no mismatch amplifiers. The mismatch amplifier allows the mismatched input to be distinguished from the standard input at 1 nM and 100 pM concentrations of input, while the comparable curves are not visually distinguishable for the no mismatch cascade with an input mismatch outside of the toehold region at 100 pM concentration.

neither amplifier variant showed clear kinetic discrimination between the branch migration domain variants across the observed timescales. However, the kinetic curves for the two variants in the mismatched system show a small amount of visible separation at early time points.

3.4 Discussion

In this work, we contributed a novel design framework that augments the leakless design space for faster and more expressive circuits without compromising robustness. We’ve presented a generalizable design strategy and concrete experimental characterization of key regions of design space. Using $X \rightarrow Y$ feedforward cascades we have analyzed the kinetic improvement of the strategy, and elucidated its mechanism. Using the $X \rightarrow X$ cascades, we’ve explored whether the strategy can qualitatively expand the useful regions of the underlying leakless design space. In both classes of circuits we verified whether the strategy preserved robustness to leak, as intended.

Our novel strategy frames the sites of systematic redundancy, not as drawbacks that necessitate spurious interactions between components, but as useful structural constraints. These constrained positions provide tunable latent degrees of freedom for using mismatches to break the energetic symmetry between kinetic pathways for designed and off-target interactions. Rather than an ad hoc approach, we present a systematic, tunable, and generalizable optimization procedure. Starting from any design with regions of domain-level redundancy, the procedure guides implementation of sequence constraints between species to balance properties of on-target and off-target reactions, while enforcing constraints that prevent re-introducing the leak pathways suppressed by the underlying leakless architecture. Because resolving mismatches in desired reactions also thermodynamically favors leak reactions, we parameterized the design space to avoid mismatch placements that would provide kinetically accessible leak pathways—namely fraying of helix edges and formation of multi-base internal bubbles.

Using this strategy, it is feasible for users to enumerate large portions of or even the

entire design space. They can then choose from candidates with specific predicted properties of on-target and off-target interactions. Apart from presenting the design strategy, through our construction of exemplary systems, we show the methodology can produce systems with different target behaviors depending on the Pareto frontier used in optimization. Lastly, we showed that kinetic improvements we achieved are not rare, but instead the design space is rich with acceptable designs— for example, choosing an approximately average candidate from hundreds of thousands of cascade designs still showed greatly improved kinetic performance.

The coherence of our design space is contingent upon preserving the robustness of the underlying leakless architecture. The lack of difference in leak between the mismatches and non-mismatched 5ntTH cascades is consistent with the existence of regions of design space where robustness is not compromised. For the 7ntTH mismatched cascade, which has the largest number of mismatches, closest spacing between mismatches, and highest gate-level thermodynamic drive toward leak, we measured leak of only 2% of the possible output over 24 hours. Furthermore, we found that only about 3.5% of the total observed leak occurred at the long-time scale, while the bulk occurred on short timescales more consistent with output resulting from a population of misinformed gates, as opposed to the slow first-order leak which the underlying leakless architecture is designed to suppress. Additionally, the near micromolar concentrations used in this analysis provide entropic conditions with low barrier to leak (Wang et al. [2018]). Because leak itself is amplified in cyclic circuits, the $X \rightarrow X$ provides a significant stress test for robustness across the design space. In the mismatched $X \rightarrow X$ system, at the lowest gate concentration used for the most sensitive detection, we did not observe significant leak over 24 hours. While some leak was observed at higher concentrations, it only represented a small fraction of the possible output at long timescales. Overall we observed some cases where robustness was not compromised and only small increases in leak relative to the possible output for systems with the highest pressure to leak.

In addition to preserving robustness, we found improved kinetic performance across the design landscape. Our investigation into feedforward cascades supports our mechanistic hypothesis for the efficacy of the strategy. Firstly, the fact that the strategy effectively eliminated reverse rates in both toehold length variants of the $X \rightarrow Y$ cascades is consistent with mismatch resolution in desired reactions providing kinetic ratchets that favor forward rates and provide strong energetic barriers to reverse rates. We observed comparatively low changes in forward rate constants with application of the strategy, which are consistent with the fact that starting forward rate constants were already close to the saturating value of 2×10^6 . Interestingly, across both simplified and detailed models of the cascades, we found that implementing our mismatch strategy over the 5ntTH toehold cascade yielded indistinguishable simplified and gate-pooled forward rates compared to the 7ntTH non-mismatched cascade. While toehold length is a classical parameter for modulating rates in TMSD systems (Zhang and Winfree [2009]), these results suggest that in some regions of design space, implementing systematic mismatches over a region of redundancy can give comparable kinetic improvement to lengthening the toehold. Even further, this kinetic improvement is expected to reduce the propensity for spurious invasion, rather than increasing it as lengthening the toehold does (SI Figure B.16).

One instructive case where we found that application of our strategy significantly reduced the forward rate is actually supportive of our mechanistic hypothesis. In our systematic design of a set of cascades with maximized difference in predicted spurious invasions, we selected a design where spurious invaders introduce multiple mismatches to the F2(mm) gate, but as a result the desired invader does not resolve two mismatches early in invasion, as occurs in the other gates of the complex. At the same time the mismatch optimization weakened the toehold of this gate. This weaker toehold, and absence of kinetic ratchets early in the reaction to compensate (Irmisch et al. [2020]) is consistent with the reduced rate. In contrast, the F0(mm) gate also had a weakened toehold, but had mismatches resolved early in the desired reaction. Consistent with this interpretation, we did not detect a significant

decrease in the forward rate constant for this gate, and the fitted forward rate estimate was instead higher than for the corresponding non-mismatched gate.

While we highlighted a case where the fit forward rate in the 7ntTH cascade slowed with application of our strategy, the 7ntTH system excelled in mechanistically demonstrating its design goal– the kinetic impact of unproductive spurious invasions. As we stated above, we specifically designed this pair of cascades to maximize the difference in predicted toehold occupancy from spurious invasion events. Under our experimental conditions the impact of spurious invasion is expected to be most pronounced at high trigger concentration (SI Figure B.16). While for a system without competing spurious invasions, increasing trigger concentration should monotonically increase reaction rate, by the law of mass action, in systems with spurious invasion the simultaneous increase in the prevalence of occlusion events for kinetically relevant species can result in non-monotonic behavior. We clearly observed this non-monotonic behavior for the non-mismatched variant, while the monotonic behavior in the mismatched variants suggests an effective elimination of the kinetic impact of spurious invasion even at more than 100-fold trigger excess. Detailed kinetic analyses of proxy spurious invasion trajectories further support this interpretation and are presented in SI Section B.3.2.

Our demonstration of the strategy over an $X \rightarrow X$ cyclic cascade highlights a distinct contribution of the strategy– improving the useful design space of leakless and enthalpy neutral designs. Since leak can be a critical failure model in cyclic systems, where it is amplified and self-reinforces, it makes sense to build architectures with as much systematic leak prevention as possible. A promising design strategy for this goal is to use systematic redundancy in combination with “toehold-sized clamps” (reverse toehold of the same size as the forward toehold) like we did in this work, rendering the reactions approximately enthalpy neutral but providing scalable entropic and enthalpic barriers to leak (Wang et al. [2018]). However, despite suppressing leak, the enthalpy neutral nature of this design provides a poor driving force in cyclic circuits, as indicated by the non-mismatched amplifier showing

not only ineffective amplification (Figure 3.4(B)), but even slower signal transduction than comparable feedforward cascades (Figure 3.3(A)). Independent of our previous kinetic analyses, our strategy restored internal driving force to the reactions without requiring changes to the toehold structure, preserving the same underlying structural guarantees against leak. This change, in turn, enables high-speed, sensitive amplification of signals, not possible in the starting leakless design (Figure 3.4(B,C)). While we did not iteratively optimize for application performance, we demonstrated desirable kinetic and thermodynamic properties, including the ability to distinguish single nucleotide variants (Figure 3.5) at multiple positions in an input strand. These emergent properties lay promising groundwork for using mismatch-optimized leakless cyclic circuits in biotechnological applications.

As previously demonstrated, mismatch optimization decouples driving force from toehold length (Haley et al. [2020]). We show experimentally that, in the context of leakless systems this allows leak-suppressing structural constraints to be preserved while expanding the accessible kinetic and thermodynamic design space. While it is possible to introduce driving force by lengthening the toehold, we have shown how to do so in a way that is not predicted to increase the prevalence of spurious invasions, which we’ve highlighted in our analysis of feed-forward systems.

In leakless enthalpy neutral designs, the enthalpic barrier to leak scales with the energy (typically via length) of the reverse toehold (clamp), while forward and reverse reactions are designed to be approximately enthalpy neutral, yielding equilibrium constants near unity for each reaction step. Chaining such reactions therefore preserves a constant lower bound on completion level, even with arbitrary cascade depth (Wang et al. [2023]). Our results suggest that systematic mismatch optimization could extend this enthalpy-neutral design space beyond restoring driving force in a cyclic circuit. In particular, it may enable architectures with stronger kinetic barriers to leak while maintaining sufficient thermodynamic driving force for effective signal propagation. For example, it is consistent with our results that reverse toeholds longer than the forward toehold could be used, in principle,

to further suppress leak, with introduced mismatches compensating to maintain favorable thermodynamics and without proportionally increasing observed leak.

While this strategy is applicable across systems with systematic redundancy, it is important to highlight the implications of restricting our analysis to a maximum redundancy level of three overlapping complexes. While this level of redundancy was demonstrated to suppress observable leak vastly across operational regimes for feedforward cascades (Wang et al. [2018]), applications like highly sensitive amplifiers may require a higher degree of redundancy to fully suppress leak. However, with a four-letter alphabet, at higher levels of redundancy it is no longer possible to simultaneously maintain certain sequence restrictions at a single column of redundancy in isolation—for example ensuring that all spurious invaders introduce a mismatch at that position. In this regime, it is possible to amortize the same guarantee across multiple modification sites, rather than a single position (SI Figure B.3). When such guarantees are not possible across a single column of redundancy, system-level guarantees—for example that all spurious invaders resolve a mismatch within a certain distance of the toehold, while spurious invaders mismatch the toehold and maintain the internal mismatches within the same range—are still possible.

We have presented a multifaceted systematic augmentation of the leakless design space that: 1) largely retains robustness to leak, 2) provides concrete kinetic performance improvements, 3) effectively prunes undesigned spurious reactions while favoring desired reactions, and 4) enhances expressivity of the leakless design space in translationally relevant regimes. We highlight the versatility in applying this strategy to TMSD designs with systematic redundancy in both feed-forward and cyclic circuits. However, the broad principles beyond this optimization may generalize across domains. It is worth considering whether this strategy could apply to domains across chemistry, biology, and engineering that study sequential networks of interactions, where regions are conserved or can promiscuously bind across components. Across diverse domains, such as *de novo* protein pathway engineering, this work suggests that it may be possible to use redundant motifs as structural handholds

for breaking undesired energetic symmetries.

In summary, we can maintain the leak-robustness of existing architectures while ameliorating their kinetic and thermodynamic drawbacks. We show how the redundancy constraint that slows kinetics in leakless cascades also provides the structure to augment the design space for faster and more expressive circuits. This design space emerged from the synthesis of two orthogonal optimization strategies: systematic leakless architectures and strategic mismatch optimization. We frame this strategy in a way where it is immediately applicable over existing designs with systematic redundancy or could be used for *de novo* construction of robust systems. In each case, the framework provides flexibility to tailor design constraints and evaluation criteria to the application of interest. Lastly, this layering of orthogonal optimization strategies may not be unique. Interrogating the constraints encoded across molecular network designs may provide the foundation for generalized and improved design spaces for diverse design objectives.

Chapter 4

**SYSTEMATIC CHEMICAL REACTIVITY MODIFICATION TO
EXPAND THE SEQUENCE OPTIMIZATION LANDSCAPE**

This chapter is based on collaborative work co-first authored with Thomas Mayer. Detailed contributions are described in Appendix E.

4.1 Introduction

Molecular computing has emerged as a promising field for information processing at the molecular level, with applications in synthetic biology, diagnostics, and biosensing (Jung and Ellington [2014], Simmel et al. [2019], Srinivas et al. [2017]). Toehold-mediated strand displacement (TMSD) circuits, in particular, have enabled impressive realizations of molecular computing *in vitro*, showcasing their potential for complex computations. Figure 4.1 highlights the principles of TMSD.

The simplicity and predictability of TMSD circuits have contributed to their widespread use and success in various applications (Zhang and Seelig [2011]). Though TMSD circuits have demonstrated success *in vitro*, operating them in complex environments poses unique challenges (Mayer et al. [2022], Oesinghaus and Simmel [2019], Green et al. [2014]). These environments include pools of random nucleic acids (Mayer et al. [2022]), cell extracts (Fern and Schulman [2017]), and living organisms. In these scenarios, TMSD circuits must contend with myriad factors, such as the presence of competing nucleic acid species, time-varying background compositions, and potential degradation mechanisms (Fern and Schulman [2017], Mallette and Lakin [2022], Chandrasekaran [2021], Rangarajan and Shankar [2001], Kühlmann et al. [1999], Liu et al. [2017]). The complexity and dynamic nature of these environments introduce additional kinetic and design constraints for TMSD circuits.

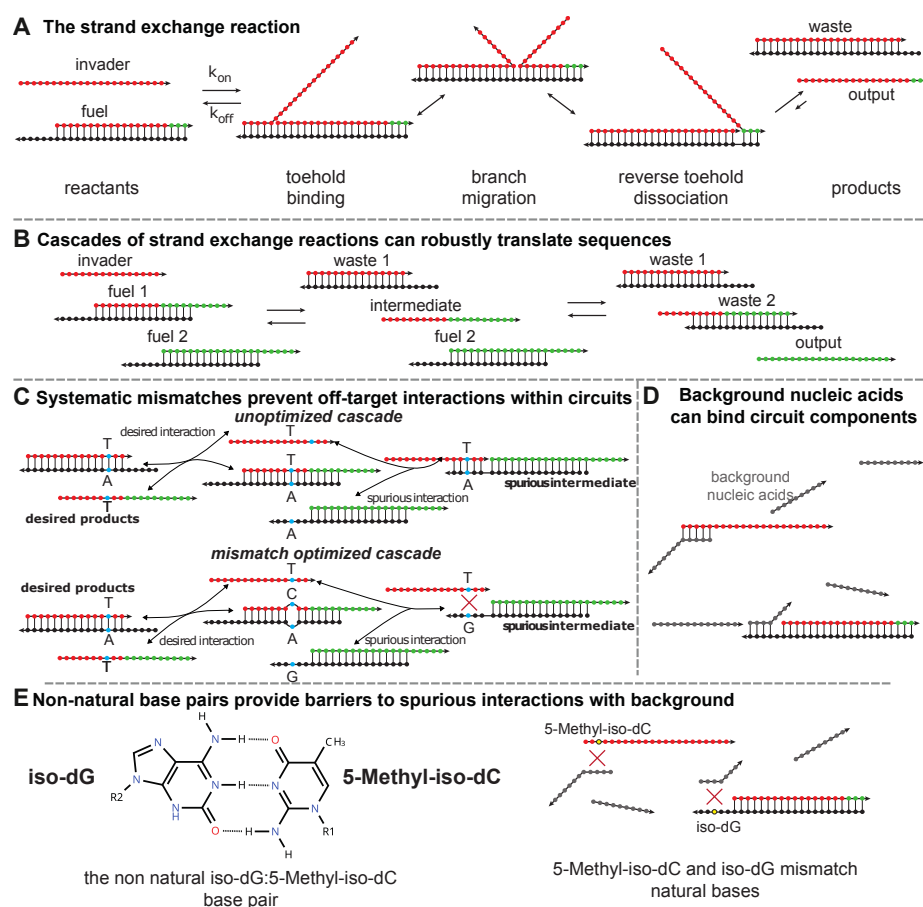


Figure 4.1: (A) The strand exchange reaction is a versatile tool for engineering chemical reaction networks. The reaction starts when a single-stranded invader binds a double-stranded complex (fuel) with a short single-stranded overhang, called the ‘toehold’. After toehold binding, the invader can begin to displace the incumbent strand, which initiates branch migration, i.e., a random walk process in which both the invader and incumbent compete for binding to the substrate strand. After the invader is completely bound, the last bases, called reverse toeholds, of the incumbent strand fray to release the output. If there is no reverse toehold, strand exchange is typically called TMSD. (B) Multiple TMSD or exchange reactions can chain together into a cascade. In a cascade, the incumbent strand displaced from a fuel species further reacts with a downstream fuel. By systematically sharing sequences between fuel complexes, cascades can translate an invader sequence to a distinct output while reducing the prevalence of signal leak (output in the absence of invader) (Wang et al. [2023]). (C) The systematic redundancy that reduces leak in cascades also introduces side reactions between signal strands and fuel complexes, such as that depicted between the invader and fuel 2. However, systematic modification with mismatching bases imposes barriers to these side reactions by ensuring that all spurious reactions introduce mismatches and all desired reactions replace mismatches with complementary pairs. (D) Even cascades that are kinetically optimized with mismatching bases face further kinetic drawbacks when operated in the face of competing background nucleic acids. These background sequences can bind, e.g., occlude toehold bases, causing operational slowdowns. (E) Xenonucleic acid (XNA) non-natural base pairs, like the iso-dG:5-Methyl-iso-dC pair, form a complementary base pairing system but substantially mismatch with natural bases. As a result, these bases confer protection against binding by natural nucleic acids. Synthesizing iso-dG:5-Methyl-iso-dC pairs with systematic mismatch optimization of strand exchange cascades ameliorates slowdowns from competing background nucleic acids by providing significant barriers to binding by any sequence without the complementary XNA.

Here we isolate one component: nonspecific background nucleic-acid binding, modeled with random DNA pools.

While the Watson-Crick-Franklin base pairings (A:T and G:C) leveraged to program complexity in these circuits make them susceptible to interference from other biomolecules, natural nucleic acids are not the only molecules that can implement such programming. Researchers have explored incorporating non-natural (XNA) bases and base-pairs as a way to maintain the desired properties of nucleic acids in engineered systems while imparting new and distinct chemical interactions to the components. These artificial base pairs offer distinct advantages, such as weakening interactions with natural sequences and slowing down natural circuit degradation mechanisms (Liu et al. [2017], Fern and Schulman [2017], Mallette and Lakin [2022]). Here we focus specifically on kinetic isolation from background nucleic acids, rather than resistance to enzymatic degradation. By incorporating non-natural base pairs, TMSD circuits can potentially mitigate unwanted interactions and increase their stability and reliability in the face of competing background DNA sequences.

This work focuses on using non-natural base pairs to combat one specific problem introduced by complex sample matrices: slowdowns resulting from spurious binding between TMSD circuit components and ‘background’ nucleic acids. Such binding between TMSD circuits and DNA background has been shown to lead to significant decreases in kinetics even *in vitro* (Mayer et al. [2022, 2023]). These background molecules might come from other components of extremely large DNA circuits, or from biological or environmental samples.

To probe such background interactions, we leverage Mayer et al.’s model of introducing randomly synthesized DNA oligonucleotides of specific lengths and concentrations into an *in vitro* circuit. While it does not capture the full complexity of a biological environment, this model does capture the combinatorial and entropic challenge of diverse interfering components in a way that provides a tractable initial study to probe design principles and lay the groundwork for progressively more complicated background interactions.

We focus our effort on one such non-natural base pair, iso-dG:5-Me-iso-dC (hereafter isoG:isoC). The isoG:isoC system has been incorporated into both the 8-letter ‘Hachimoji DNA’ (Hoshika et al. [2019]) alphabet and a larger 12-letter alphabet (Kawabe et al. [2023]). Hachimoji DNA includes the standard biological bases (A, T, C, G) and two non-natural pairs, P:Z (5-Aza-7-deazapurine : 6-Amino-5-nitropyridin-2-one) and B:S (isoG : isoC in RNA and isoG : 5-Me-iso-dC in DNA), allowing for a completely non-natural analogue to DNA. Because these were designed to mismatch strongly with natural base pairs, we hypothesized that strategic and systematic incorporation into TMSD circuits would reduce the effects of DNA background interactions (Figure 4.1(E)). Importantly, we chose this particular base pair because it is readily available as a modification from Integrated DNA Technologies, a widely used commercial supplier of oligonucleotides for TMSD circuits, enabling rapid integration into existing and future designs across the molecular computing and nucleic acid nanotechnology community.

This approach of using non-natural bases to reduce interactions between TMSD circuits and the environment is supported by a previous study which showed that building a TMSD complex with acyclic bases in the toehold reduced binding by natural sequences (Murayama et al. [2023]). However, in contrast to our approach which enables facile integration of the bases of interest into existing designs, the type of XNA used by Murayama et al. is not commercially available. It additionally does not interface with enzymatic machinery, and exhibits slower kinetics compared to ‘Hachimoji’-style XNA pairs.

Apart from the potential for bio-orthogonality, including non-natural base pairs in TMSD circuits has the immediate advantage of expanding the design space, easing the difficulty of sequence design for circuits with hundreds of components. For example, in such circuits, mostly short toeholds, e.g., of 5 nucleotides (nts), are used to minimize unintended binding to other sequences. However, for such toeholds, the total number of different sequences is only $4^5 = 1024$, and the number of perfectly orthogonal sequences is much lower. By incorporating two new bases, the number increases more than seven-fold to $6^5 = 7776$,

and the number of orthogonal sequences also substantially increases, simplifying the design process.

We implement non-natural base incorporation in TMSD circuits through a straightforward integration with an existing mismatch-based sequence-level optimization strategy for TMSD circuits that are architecturally robust to signal leak and kinetically robust to spurious strand-strand interactions between circuit components (Kennedy et al. [2022]). This strategy, illustrated simply in Figure 4.1(C) improves kinetics in TMSD cascades by favoring on-target reactions— which replace mismatches with a matching pair— and disfavoring off-target interactions between circuit components, by ensuring they must form new mismatches. Supplementary information (SI) Figure C.1 shows the reaction steps for an example TMSD cascade systematically optimized with this strategy at multiple sites carefully chosen to maximize kinetic discrimination between on-target and off-target reactions. The strategies proved to be fully compatible, providing additional benefits, beyond those provided by systematic mismatches alone, when operated in random DNA pools. While our current implementation requires non-natural bases in the invader strand, it may be adapted to use a natural strand for the first invader and apply the strategy only to downstream components.

Notably, though non-natural bases show promise for engineering bio-orthogonal nucleic acids, in biological conditions these expanded alphabets are not completely orthogonal to natural bases (Eberlein et al. [2020], Blas et al. [2004]). This motivated us to systematically characterize all possible base pairing interactions between natural bases A, T, C, G and the non-natural isoG and isoC as a prelude to our analysis of background interaction. Kinetics of different mismatch pairings were assayed by measuring their influence on TMSD kinetics when substituted in the central position of a 5 nt toehold. As a point of comparison, we performed the same experiments with the natural C and G interacting with all possible natural bases.

Our results show that even a single non-natural base in each toehold of a TMSD system

significantly reduces the negative impact of random background sequences. Interestingly, we found that an isoG:isoC base pair in the toehold region improved kinetics in the absence of background, even though it rendered toehold binding less energetically favorable. Our characterization of the relative orthogonality of different non-natural:natural pairs and additional benefits caused by non-natural bases in the branch migration domain provides a solid foundation for engineering TMSD circuits with optimized orthogonality to pools of background DNA. We hope this lays the foundation to expand this study to random and cell-specific RNA pools, cell extracts, and more complex backgrounds. The synthesis we show between strategic placement of non-natural bases and systematic mismatch optimization of TMSD circuits offers new design space for engineering molecular circuits that are architecturally robust to spurious reactions between circuit components and strategically orthogonal to specific interfering components in the environment.

4.2 Results

Influence of non-natural bases in the absence of background. While TMSD kinetics are highly correlated with increasing toehold binding strength (Zhang and Winfree [2009]), we found that replacing a G:C pair with an isoG:isoC pair at the center of the toehold in the 5'TH system led to improved kinetics despite making the toehold binding less thermodynamically favorable (Figure 4.2(A)). We present a detailed kinetic and thermodynamic analysis for the toehold of systems in Section C.3 alongside free energy differences (at 25°C and 29°C) for substituting any isoC or isoG base at the center of any trinucleotide sequence in Tables C.1, C.2, C.3, and C.4. We found that, if only considering free energy of toehold binding, both the 5'TH and 3'TH systems showed faster kinetics than expected based on toehold binding free energy alone.

Mismatches of natural and non-natural toeholds. We conducted displacement kinetic experiments on all possible combinations of the non-natural isoC and isoG bases with all natural bases, as well as for the natural counterparts C and G. For comparison, we added the matching isoG:isoC kinetics, which proved to be the fastest, followed by the combination

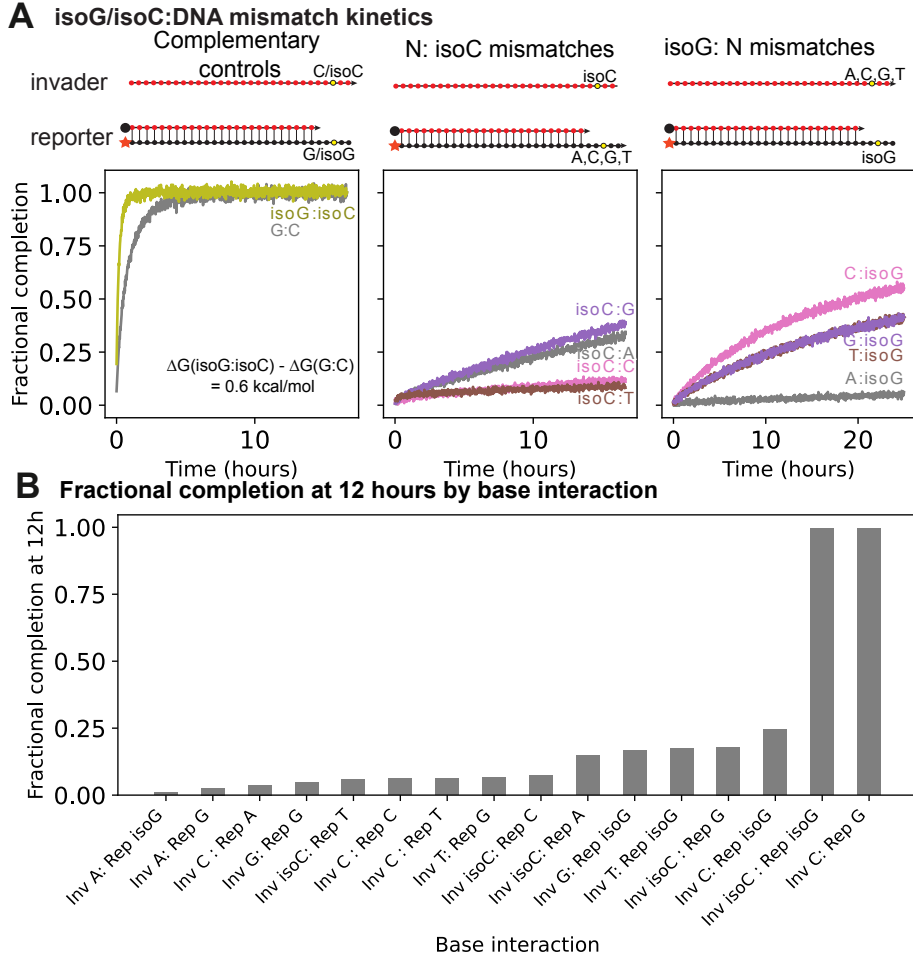


Figure 4.2: Substituting a G:C base pair in the toehold region for an isoG:isoC base pair can increase strand displacement kinetics even in the case that the toehold binding energy is slightly increased. Additionally, mismatches with isoC or isoG show increased promiscuity compared to natural-only mismatches. (A) Kinetic curves measured for the 40 nM reporter and 20 nM invader with various matching and mismatching bases in the center of the toeholds. (left) Data for a G:C pair compared to an isoG:isoC pair. The difference in toehold binding energies is marked on the plot as calculated from published nearest neighbor parameters (SantaLucia Jr and Hicks [2004], Hoshika et al. [2019]). While endpoints for both the natural and non-natural cases are normalized to a value of 1, we present the baseline adjusted fluorescent curves in SI Figure C.2 which differ by less than 5% at 12 hours. (center) Data for the isoC base in the invading strand mismatching to each of the standard A,T,C,G bases or matching the isoG base in the center of the reporter toehold. (right) Analogous data for an isoG base in the center of the reporter toehold. (B) The promiscuity of each mismatch of isoG and isoC, assessed by the completion level at twelve hours in the experiments from (A) compared to the analogous data for each possible mismatch of the natural C and G bases in the center of the toehold. We observed significantly increased promiscuity of isoC and isoG compared to natural C and G.

of the natural G:C pair. Figure 4.2(A) shows the kinetic curves for all combinations with the non-natural bases, and completion levels for all combinations including the natural mismatches are shown in Figure 4.2(B).

In our study of mismatches between isoC/isoG and natural bases, observable triggering kinetics are still possible since only the middle of the toehold is not matching, but the remaining 4 bases are still complementary. However, when the resulting sequence is weak (see SI Table C.15 for all sequences), one mismatch is sufficient to prevent initial, stable toehold binding. For the natural C base, only the matching G base significantly increases fluorescence intensity for the measured timescale. For isoC, the combination with the natural G base, as well as with the natural A base, increases the fluorescence signal.

For the combinations of G with the natural bases, the G:T and G:G mismatches show some interaction, although the latter is barely visible. For isoG, noteworthy interactions occur for several combinations. The strongest is for isoG:C, which shows slightly faster kinetics compared to G:isoC, which is the strongest interaction in the isoC mismatch set. This is consistent with the findings by Roberts et al. (Roberts [1997]).

Influence of random pools on single-step displacement kinetics. To gain insights into the effect of non-natural base pairs on the kinetics of TMSD circuits with random pool strands, we measured different combinations, varying the length (N25 and N50) and concentration (10 and 25 μ M) of the random pool strands tested against two variants of a single TMSD reaction with different toehold orientations and sequences. We present the results in Figure 4.3. In each condition we report an estimated single second-order rate constant as well as the ratio of rate constants between the non-natural and natural variants.

Across all background and incubation conditions in the one-step systems, the measured ratios were significantly greater than 1, indicating a global kinetic improvement from the introduction of the strategy. In contrast to the global increase in kinetics, we found that whether the rate constant ratio with background was significantly different from the control ratio in the absence of background (the intrinsic speedup from introducing the non-natural

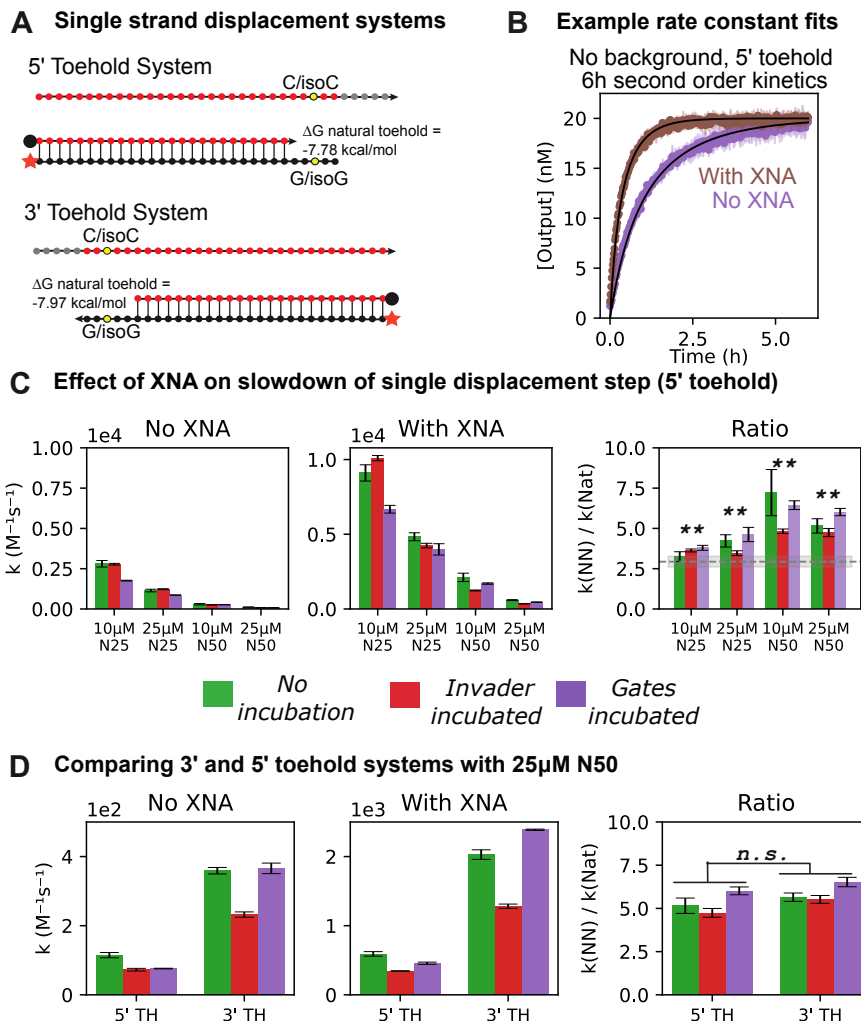


Figure 4.3: Non-natural base incorporation improves performance of a single TMSD in random pools of DNA. (A) Sequence depiction of the two different single displacements used for this set of experiments, including their toehold strength in the case with only natural bases. (B) Example kinetics for the 5' toehold system with and without XNA in the absence of background. Shading on the scatter points corresponds to the point-wise standard deviation on the experimental data. The fit second-order models are plotted as black lines. Detailed kinetic modeling for the 3' toehold one-step system can be found in SI Figure C.5. (C) Comparison of the fit second-order rate constants for the 5' toehold version and different background lengths and concentrations, as described in the Methods section. Error bars represent the standard error on the rate constant estimate. The dashed line corresponds to the ratio for the systems in the absence of any background, with the shading representing the standard error. For easier comparison, the rightmost graph shows the ratio of the non-natural rate constant to the natural rate constant for the same incubation and background conditions. The “**” markings correspond to $p < 0.01$ for Wald Z-tests comparing the pooled data for each background condition (across incubation types) to the ratio in the absence of background. (D) Comparison of the 5' and 3' toehold versions for one example background case (N50 at 25 μM). We found no statistically significant difference between the ratios presented here for the 5' and 3' toehold system tested separately across each incubation condition.

bases alone) depended on the specific incubation method, background length, and background concentration. We tabulate the results of all statistical analyses in SI Tables C.13 and C.12.

We observed a faster kinetic timescale for the 3'TH system than the 5'TH system, despite the similar predicted toehold binding energies for both reporters. In contrast, pooled across all background and incubation conditions, we did not find a significant difference in the rate constant ratios between the two reporter designs.

Influence of random pools on cascade displacement kinetics. We have established that non-natural bases can improve kinetics in single TMSD reactions in the presence and absence of a random DNA background. It is natural to ask whether this holds true for larger displacement systems with multiple displacement steps. In this set of experiments, we repeated the analysis from the previous section using four-step strand displacement cascades that feed into the same reporters previously tested.

While we started with a cascade architecture designed to be systematically robust to leak (Wang et al. [2023], Kennedy et al. [2022]), it is possible that changing the reaction energy landscape of the cascades with non-natural bases can introduce new leak pathways. We did not observe any leak, or output in the absence of invader, for either cascade and any of the concentrations of random background tested in these experiments. Example kinetic curves showing the absence of leak are included in the SI Figure C.3.

Second, as in the single TMSD reaction, the circuit containing isoG:isoC exhibited faster kinetics than the natural counterpart in almost all conditions. (We include fitted rate constants for each complex in both cascades for the 3' version in the SI Figure C.6.) As shown in Figure 4.4, we found that all ratios were significantly larger than 1 except for the control ratio and the cascade incubated ratio which were greater than 1 but not statistically significant due to a high baseline. Whether the ratio was significantly larger than the control ratio (ratio without background) depended on condition. The results of the statistical analysis are tabulated in SI Tables C.13 and C.12. As seen in SI Figure C.7, we once again

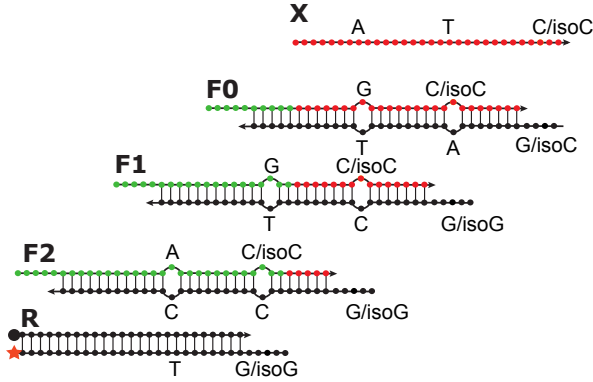
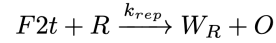
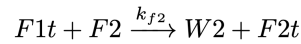
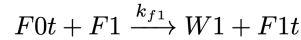
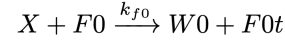
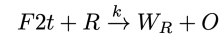
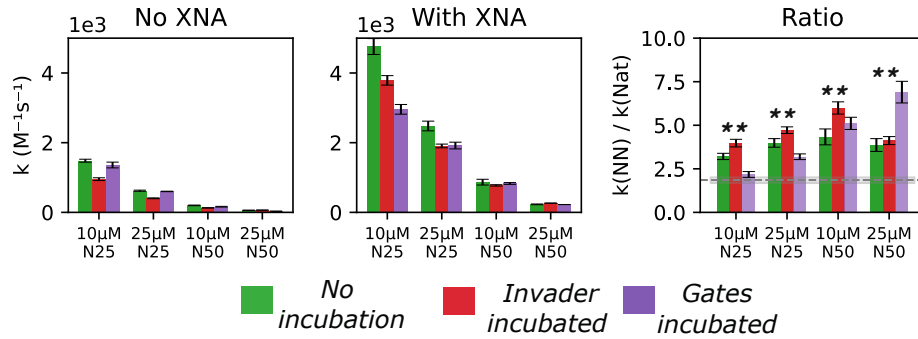
A 5' toehold TMSD cascade system**Expected reactions****Simplified empirical model****B Effect of XNA on slowdown of cascade**

Figure 4.4: Non-natural base incorporation improves performance of a multi-step cascade of TMSD reactions in random pools of DNA. (A) A schematic of the complete 5' toehold cascade. Each expected reaction in a cascade of this depth is depicted in SI Figure C.1. We list the chemical reactions expected in this mechanism alongside the simplified one-step model used for empirical comparison of the cascades. (B) Comparison of the fit second-order rate constants for the 5' toehold version and different background lengths and concentrations, as described in the Methods section. Error bars represent the standard error on the rate constant estimate. The dashed line corresponds to the ratio for the systems in the absence of any background, with the shading representing the standard error. For easier comparison, the rightmost graph shows the ratio of the non-natural rate constant to the natural rate constant for the same incubation and background conditions. The “**” markings correspond to $p < 0.01$ for Wald Z-tests comparing the pooled data for each background condition (across incubation types) to the ratio in the absence of background. Comparison of the 5' and 3' toehold cascades is shown in SI Figure C.7, but like the one-step case, showed no significant difference between ratios across the two cascade systems.

found no significant difference in ratios between the 5' toehold cascade and the 3' toehold cascade.

Influence of multiple non-standard bases on strand displacement kinetics We have shown that incorporating non-natural bases in the toehold region is beneficial for TMSD kinetics. We next expanded our investigation by adding non-natural bases in the branch migration domain of signal strands.

For this experiment, we designed a reporter with either an A or isoG base in the toehold region. To enable reuse of the reporter across multiple invader sequences, we introduced internal mismatches at two positions where isoC could be combinatorially added to invader variants without requiring additional mismatch introduction during strand displacement for different invaders. For natural bases, nearest-neighbor mismatch thermodynamics predict that substituting one mismatch for another can alter $\Delta\Delta G$ by several kcal/mol depending on local sequence context (SantaLucia Jr and Hicks [2004]). However, because comprehensive nearest-neighbor parameters do not exist for isoC mismatches, we instead used kinetic similarity to natural mismatches to bound the possible energetic effects (see SI Section C.4). Using this approach, we could disentangle kinetic differences arising from changes to the reporter free-energy landscape in the absence of background from those due to interactions with background arising from additional isoC bases in the branch-migration region. Figure 4.5 shows the results.

As expected, incorporating a non-natural base pair in the toehold region leads to a significant increase in reaction speed ($p < 0.01$). Including a second isoC base provides further benefit ($p < 0.01$) only when the isoC is positioned distal to the toehold; no measurable improvement is observed for the proximal case. In cases where the second isoC improved completion, the incremental gain was smaller than that achieved by introducing the first isoC in the toehold.

Because second-order fits showed large residuals even in the natural control cases, we quantify kinetic behavior in this set of experiments using fractional completion at fixed time

points; corresponding kinetic fits and normalized error are tabulated in Table C.11. The fits are shown graphically in SI Figure C.15 alongside a reproduction of the analysis displayed in Figure 4.5A, which supports the same qualitative statistical conclusions as the endpoint analysis with the exception of one additional difference between incubation conditions.

We investigated whether incorporating a non-natural base in the overhang of a gate is beneficial for displacement kinetics. Since the cascades investigated thus far use short 5-nucleotide overhangs with binding energies comparable to a toehold, we sought to determine whether inclusion of an isoC base in this longer region—which can bind more stably to background strands but may be less kinetically relevant than the toehold—could provide additional kinetic benefit.

We used the same reporter as described above to ensure that any observed differences were not caused by large thermodynamic changes independent of background interactions. To avoid using a costly isoG base in the bottom strand of the fuel complex with the long overhang, we designed the system such that the isoC base that binds the downstream toehold was initially mismatched. The invader replaces this mismatch with a matched base pair, providing an additional kinetic and thermodynamic driving force for the reaction (Irmisch et al. [2020], Haley et al. [2020]) and reducing the likelihood that the initial strand displacement step is rate limiting compared to interactions between the overhang and the background.

The results in Figure 4.5 show no significant improvement in reaction kinetics upon addition of isoC in the overhang.

4.3 Discussion

Much recent work in the field has kinetically and thermodynamically optimized TMSD circuits against spurious reactions between circuit components themselves (Wang et al. [2023], Kennedy et al. [2022]). However, that work does not address how to make circuit components robust against spurious interactions with the background environment. Our strategy complements these existing internal optimization techniques to provide additional

external (environment-facing) optimization.

A critical observation pertains to the tautomeric nature of isoG, which shows some interaction with natural bases, indicating that isoG does not provide perfect orthogonality (Eberlein et al. [2020], Blas et al. [2004]). The increased promiscuity of isoG compared to isoC is consistent with the increased number of theoretical low-energy tautomers capable of binding the natural bases. We also observed limited but kinetically measurable interactions involving isoC, indicating that neither non-natural base provides absolute orthogonality.

Surprisingly, we found that the incorporation of isoG:isoC pairs into the toehold regions of TMSD circuits improved kinetics even in the absence of any background. While this was true for both systems, it is particularly notable that isoG:isoC incorporation improved kinetics in a case where the base substitution made toehold binding less thermodynamically favorable based on nearest-neighbor predictions (SantaLucia Jr and Hicks [2004], Hoshika et al. [2019]). One potential explanation is that the orthogonal isoG:isoC pair prevents transient secondary structure within the invader strand, which could otherwise sterically hinder toehold association. This is consistent with prior work showing that incorporating non-natural bases can reduce off-target folding, as measured by ensemble defect (Pham et al. [2023]). Similarly, researchers seeking to improve co-transcriptional folding kinetics in riboswitch systems by reducing off-target folding states found that incorporating a single non-natural pair led to a significant rate increase (Manna et al. [2023]).

The fact that the results of the one-step system (Figure 4.3) generalize well to the four-step cascade (Figure 4.4 and SI Figure C.7) is promising for future applications. While the linear cascade may seem simple, it is a convenient model comparable in depth with sophisticated implementations of molecular computing (Qian and Winfree [2011a]). In these settings, kinetic performance with depth is a major operational consideration.

Despite the similar predicted toehold binding energies for both reporters, we observed a faster kinetic timescale for the 3'TH system than the 5'TH system. One plausible explanation is the directionality of phosphoramidite synthesis, which proceeds from 3' to 5';

truncations therefore occur preferentially at the 5' end, potentially shortening the effective toehold in the 5' system. A detailed study of these truncations found that a population of truncated strands will remain even after purification, especially those truncated 1-2 nucleotides (Filges et al. [2021]). This can shrink the population of full-length toeholds in the 5' system, slowing kinetics. One might expect that the complement would be true for the 3' system, where 5' truncations in invader that overlap the toehold would equivalently slow kinetics. However, because the invader for these systems were designed in the context of a larger cascade, they possess bases that overhang the toehold as an artifact from their sequestration by an upstream complex (depicted as gray in Figure 4.3(A)) and can be truncated without significantly reducing kinetics. The small population of truncated strands are unlikely to fully explain the kinetic differences, and the remaining variation may stem from changes in implementation details across the systems such as the exact methods used for PAGE purification of single strands and complexes, or the gate concentration calibration procedure.

In contrast to the difference in overall kinetic timescale, the absence of a statistically significant difference in ratios between the two toehold architectures for both the one-step and multi-step cascade systems suggests that the ameliorative effect of the non-natural base pair is robust to changes in implementation details between the 5' and 3' system designs.

Additionally, we demonstrated the effectiveness of our strategy whether the invader or the circuit itself is incubated with the background for a long period of time. This is promising for engineering and bio-sensing applications. For example, a designer may want to introduce information encoded as a DNA signal into a complicated environment for a long period of time with the intent of later adding a detection circuit to retrieve the information, e.g., for data storage or tagging applications. Or, a designer might engineer a biosensor that must wait in a complicated background for a long period of time for the correct signal to appear. However, one additional observation showed surprising deviations from previous work. Incubation of the reporter with the random pool showed almost no

influence for the long toehold used in (Mayer et al. [2022]) but a very pronounced influence in the cascade system with a weaker toehold of only 5 nucleotides. We hypothesize that this deviation could be caused by a combination of multiple factors. First, occlusion of only one or two bases of the short toehold significantly reduces the initial binding strength, whereas for long toeholds (exceeding 10 bases), one or two occluded bases have less impact, since binding kinetics saturate at a toehold length of approximately 7 bases, depending on the GC content (Zhang and Winfree [2009]). Second, finding a sequence stretch of up to 5 complementary bases in the random pool strands is much more likely than finding a subset of, e.g., 10, perfectly complementary bases for a longer toehold. Although binding of 5 bases should not enable a very stable interaction, a pre-incubated system with a large excess of the background could still lead to a favorable equilibrium for background binding.

We also tested whether incorporating additional non-natural bases outside the toehold region adds to the positive kinetic effect. Our results show a significant but slight kinetic benefit for additional bases added in the branch migration region that is minor compared to the speedup caused by the non-natural base pair in the toehold region. We did observe a position dependence of the impact of multiple isoC in an invader. We hypothesize that this dependence arises from local sequence context. For example, the isoC substitution may slightly increase the free energy of reaction for the proximal mismatch (see Supplementary Note C.4). Alternatively, inserting a proximal isoC creates the same trinucleotide sequence present over the toehold, which could increase transient toehold occlusion and slow kinetics. We found no significant improvement from introducing an additional base into a long non-toehold overhang region of the components. This suggests diminished returns for additional non-natural bases outside of the toehold. While we chose to investigate the isoG:isoC pair because it is widely commercially available, it is more expensive than a natural base pair. Because additional non-natural base pairs were found to yield diminishing returns, economical designs should prioritize placing one such base pair per toehold, with any additional non-natural residues optionally located within the kinetically impactful toehold region as

well.

The second-order rate model used to probe the kinetics of our one-step and multi-step cascade systems is a standard mechanistic model for single TMSD and strand exchange reactions in the absence of background, shown to hold without needing to explicitly model the branch migration process, under our experimental conditions (Zhang and Winfree [2009]). In the presence of background, since the rate constant is tied explicitly to the identity of the reactants under constant temperature, we do not expect the changes in the fit rate constant to hold mechanistically in a strict sense, but instead provide a convenient proxy for kinetic comparison. The same is true for the multi-step cascade system where a cascade with four expected steps can be effectively compressed into a one-reaction model without losing significant predictive power. We empirically verified the quality of the fits for these simplified models and present the quality of the fits graphically and numerically in SI Section C.8.

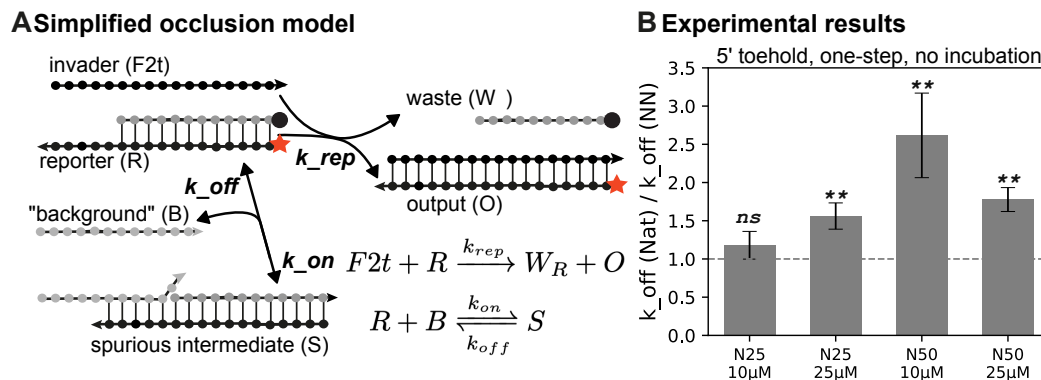


Figure 4.6: Kinetic speedup of TMSD reactions in the presence of random DNA background is consistent with an increased dissociation rate from spurious complexes with the background. A) A simplified model to capture the overall effect of the background species on kinetics. The invader and gate can react as intended, with rate constant k_{rep} , to form output, but otherwise may bind a representative background species (B) with rate k_{on} , that dissociates with unimolecular rate constant k_{off} . This encapsulates the effects of interaction with any background strand into a single representative reaction with the invader. B) Ratios of k_{off} with XNA to k_{off} without XNA for the 5' toehold, one-step system, added to background without incubation. All cases used $1 \times 10^7 M^{-1} s^{-1}$ as an estimate for k_{on} , which is consistent with reported associated constants for short DNA duplexes (Wyer et al. [2014]). The total concentration of background in each case was used as the concentration of B. An omnibus one-sample T-test comparing all the k_{off} ratio across all four cases to the null hypothesis of 1 was significant with a p-value less than 0.0002. The annotations above each bar indicate the significance of Wald Z-tests comparing each ratio to the null hypothesis of 1, where ns represents $p \geq 0.05$, * represents $0.01 \leq p < 0.05$, and ** represents $p < 0.01$.

Since we also observed increased kinetics in the absence of background in addition to the kinetic increase in the presence of background, it is natural to question whether the non-natural bases actually decrease interaction with background or if background-independent speed-up accounts entirely for the effect. This question is addressable given our data. For one, the speedup observed in the absence of background establishes a baseline, background-independent kinetic effect of the non-natural base pair. Any additional increase in the rate-constant ratio beyond this baseline under background conditions therefore reflects a background-specific contribution rather than altering fundamental kinetics of the reaction in isolation. Because control ratios in presence of background were never lower than the control ratio in the absence of background (Figures 4.3, 4.4), the system maintained at least the same speed-up with background added. Additionally, the statistically significant increase in ratio over the control suggests a speedup that goes beyond what can be explained by kinetic differences in the absence of background alone. The conclusion is reinforced by a hypothesized explanatory model presented in Figure 4.6. Similarly to the simplified model analysis, we found that for the N25 cases there was no significant difference in the dissociation rate constant between the natural and non-natural variants, but for the N50 cases we found that the dissociation rate constant significantly increased for the non-natural variant. This means the data is consistent with at least one mechanism in which the non-natural bases kinetically destabilize interaction between circuit components with the background. While this is not the only possible mechanistic model given the data, and neither the simplified or mechanistic model can be uniquely determined from the data, in C.8 where we provide in depth analysis of both models, we illustrated how our explanatory model is more mechanistically consistent than the one-step empirical model.

In our studied systems, every step contains non-natural base pairs. While this is most beneficial for the system kinetics it can only be used for pure *in vitro* designed TMSD circuits. Sensing applications however, rely on a natural initial invader. In this case, the first TMSD gate cannot have a non-natural base in the first step toehold, but it can introduce

them via mismatches for the downstream steps which in turn will kinetically benefit from it.

The random pool concentration used in our system might seem high initially, but it is more meaningful in the context of cells. The small volume of a cell or cellular compartment leads to large effective concentrations even for small numbers of molecules; further, an altered random strand background composition can lead to a more versatile distribution of displacement kinetics, including much slower displacements (Mayer et al. [2023]). For example, assuming a compartment with a volume of $1\text{ }\mu\text{m}^3 = 1 \times 10^{-15}\text{ L}$, a single DNA/RNA molecule (A) would result in a concentration of

$$[A] = \frac{1}{N_A \cdot 1 \times 10^{-15}\text{ L}} = 1.66\text{ nM}$$

with $N_A = 6.022 \times 10^{23}\text{ mol}^{-1}$, the Avogadro constant. This means that only 10,000 nucleic acid molecules lead to a concentration of $16.6\text{ }\mu\text{M}$, which has the same order of magnitude as our experiments.

Nevertheless, a clear deviation to real in vivo environments is our experimental limitation to DNA. We speculate that a full RNA based circuit should have the same qualitative trend, as all binding energies between components shift in the same way. However, while it is possible to commercially order modified RNA with the isoC and isoG bases, synthetic RNA is very expensive and was not considered in this study. The option of using the non-natural DNA-based TMSD system in a random RNA background was considered, but expected to have an overlap of different effects which are hard to disentangle. Beside the fact that the non-natural bases will have different interaction strengths with the natural RNA bases, the branch migration process is much more complex. Beyond nucleic acids pools, more complex environments like cell extracts and in vivo systems possess nucleic acid backgrounds, however avoiding non-specific interactions with non-nucleic acid components merits significant study, and avoiding degradation by cellular machinery is an active field of research.

Degradation resistance of TMSD circuits constructed from Hachimoji or other non-natural bases remains an area of future work. Previous work suggests that capping strands with non-natural nucleotides may provide exonuclease resistance; however, Kawabe et al. were able to successfully remove each of 8 non-natural bases with exonucleases as part of their sequencing process (Kawabe et al. [2023]), so a synthesis of our strategy with L-DNA caps (Mallette and Lakin [2022]) known to provide exonuclease resistance might provide a promising avenue forward. Non-natural nucleic acids with nonspecific nuclease resistance might be achieved through modified backbones or non-natural bases with significant differences in minor groove electron density (Hoshika et al. [2019]). Furthermore, even promiscuous endonucleases are known to have natural sequences with reduced endonuclease activity (Herrera and Chaires [1994]); therefore, directed evolution experiments with non-natural bases may increase the set of known low endonuclease activity sequences or even identify some with lower activity than their natural counterparts.

Tools for simulating DNA and RNA thermodynamics, like NUPACK (Fornace et al. [2020]), model each base in the alphabet using a collection of nearest-neighbor pair thermodynamic parameters. Further experimental characterization can establish these nearest-neighbor parameters for different non-natural base pairs. This was partially done for Hachimoji DNA (Hoshika et al. [2019]), but additional parameters such as those for mismatches are required for NUPACK integration. Tools like oxDNA (Ouldrige et al. [2011]) might include non-natural bases like the isoG:isoC pairs as coarse-grained fields in molecular dynamics simulations of DNA.

Within the scope of the sequence contexts, background models, and non-natural bases examined here, our results suggest several practical considerations for designing the incorporation of isoC and isoG bases in TMSD circuits:

1. Toehold substitution with a single isoG:isoC base pair consistently produced the largest kinetic benefit, both in the absence and presence of random DNA backgrounds.
2. Incorporating non-natural bases expands the available design space for orthogonal

toeholds, enabling multiplexing without relying solely on sequence length or composition.

3. Combining non-natural bases with mismatch-optimized designs can provide additive kinetic improvements across a range of background concentrations and lengths.
4. Additional non-natural bases outside the toehold region yielded diminishing returns in our experiments and may not justify their added synthesis cost.

In conclusion, the strategic and systematic incorporation of non-natural nucleic acids adds to the toolbox for designing and implementing TMSD circuits. We present promising experimental data in this direction. However, the detailed implications and possible challenges in larger experimental systems and more complex backgrounds, for example in cytosol, require thorough computational and experimental study.

Chapter 5

NETWORK-LEVEL OPTIMIZATION OF SELECTIVITY IN TMSD

Previous chapters have focused on systematic optimization against a combination of both productive and unproductive circuit-circuit interactions and unproductive circuit-environment interactions. This chapter now addresses the last error mode highlighted in Chapter 1—productive interactions between circuit components and the environment, which I term *selectivity error*. I ask whether cascade depth can serve as an architecturally scalable design variable for suppressing selectivity error under common DSD implementation constraints. I answer this question through theory, simulation, and experiment.

Conceptually, this chapter is the mirror of Chapter 3. In Chapter 3, I examined a network-level optimization whose physical implementation introduced off-target pathways. There, I showed how systematically relaxing sequence restrictions on “leakless” designs could strategically disfavor these spurious interactions, restoring desired behavior. In this chapter, I address the complementary error mode: selectivity error. While leak arises from productive interactions between designed components, selectivity error arises from unintended productive interactions with uncontrolled species in the reaction environment. Analogously to the leakless optimization strategies summarized in Chapters 2 and 3, the strategy developed here leverages the network structure of the designed CRN to suppress error beyond what is possible at the single-reaction level. However, rather than using redundant reactions to provide sites for the systematic sequence overlap, the additions to the network architecture are used to perform multi-round filtering to improve selectivity toward the target input. As a final parallel to the leak case, I conclude by showing that implementation of the intended behavior is limited by domain-level constraints of the DSD design space. This motivates returning to the sequence-level optimization strategies presented in Chapter 3 to enable

Simplified representation of depth-based selectivity scaling motif

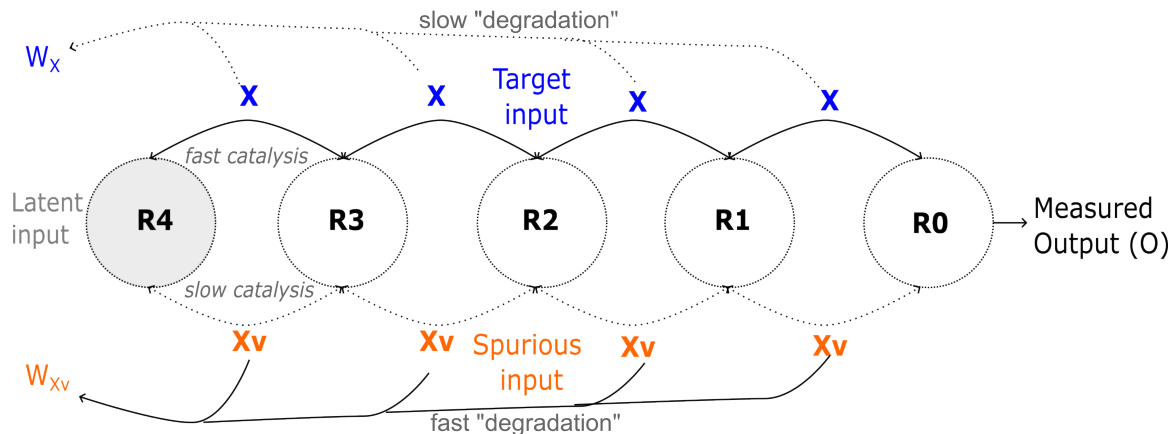


Figure 5.1: A simplified schematic of the multi-layer selectivity optimization introduced in this work. X represents a correct input, while X_v is a closely-related, but off-target input. In this chapter, X is a DNA strand and X_v is a single nucleotide variant of X . Discrimination is compounded beyond the single-reaction level by combining conditionally chained catalytic gates that are preferentially catalyzed by the on-target species with a competing sink tuned to prefer the off-target species for irreversible binding or removal from the productive pathway. A four-layer DSD implementation of this abstracted network is shown in Figure 5.4.

more faithful implementation of the target CRN.

Leak and selectivity error are distinct variants of productive error. In both cases, suppressing erroneous pathways at the level of a single reaction typically requires introducing kinetic or thermodynamic penalties, which can reduce the kinetic and thermodynamic performance of the desired pathways (Wang et al. [2018], Hopfield [1974], Ninio [1975]). Because both error modes are types of productive spurious interactions, it's natural to question whether the redundancy used in leakless design may also provide a foundation for suppressing selectivity error. This idea is theoretically plausible: from von Neumann's "Probabilistic Logics and the Synthesis of Reliable Organisms from Unreliable Components" (Von Neumann [1956]) to Hopfield/Ninio kinetic proofreading (Hopfield [1974], Ninio [1975]), prior work has demonstrated that compounding a fixed discrimination over multiple dependent steps can produce exponential improvements in effective selectivity.

Notably, Hopfield/Ninio proofreading has been implemented in isothermal DNA circuits

(Mukherjee et al. [2024]). However, these implementations rely on more complex and less characterized gate structures and reactions compared to the widespread architectures like the two-domain and seesaw circuits, and therefore do not directly address generalization when restricted to simpler gate implementations. To interface more easily with simpler but more widespread gate architectures, this chapter focuses on motifs found in these two-domain and seesaw architectures (Cardelli [2013], Qian and Winfree [2011a]) described in Chapter 2, but allowing flexibility of domain lengths. Interestingly, Chen et al.’s prior strategic combination of competition, amplification, and strand exchange motifs into a DSD system with improved scaling of selectivity with off-target affinity difference uses only structural motifs and reactions found in the aforementioned architectures of interest (Chen and Seelig [2016]). This makes it a natural starting point for the present work. The remaining question is whether these strategic network modifications can be transformed into an architectural property that scales systematically with depth, and if so whether this architectural variable provides discrimination scaling behavior not possible with a single layer.

In this chapter, I accomplish this by making two changes to the domain-level design of Chen et al.’s motif that allow the catalytic discrimination to be conditionally chained and compounded across N catalytic layers. This scalable DSD implementation, however, results in a distinct architectural analogue of proofreading rather than a direct implementation of Hopfield/Ninio proofreading. Hopfield/Ninio scales discrimination by compounding the difference in dissociation rate of two substrates as they irreversibly traverse a reaction network (Hopfield [1974], Ninio [1975]). In the design I present here, it is only guaranteed that downstream gates cannot be activated before at least one of the immediately upstream gates has been activated. Despite this weaker sequential coupling, mass-action simulations show that increasing cascade depth consistently improves both maximum and endpoint discrimination across the explored parameter domain. In many regimes, discrimination improves approximately exponentially with layer number.

However, simulations also highlight the true operational drawback of the CRN approxi-

mation needed to embed the desired behavior into the DSD system. When multiple competing analytes are present in the system at the same time—a case often not considered in prior work (Chen and Seelig [2016])—rapid unlocking of downstream gate layers by the on-target input could allow the off-target variant to “short-circuit” unlocking steps that would be required if it were in isolation. In the worst case, this could reduce discrimination to the single-layer case. This drawback highlights a possible necessity to produce more complex gates at scale to achieve different classes of scaling behavior.

Experimentally, I tested multiple variants of this strategy up to four layers and observed consistent increases in discrimination with increasing depth. Additionally, I found that a two-layer implementation strictly outperformed the corresponding one-layer implementation in discrimination across all tested sink concentrations. Notably, I observed reduced discrimination scaling with depth in the no-sink regime relative to simulation. Extending the model to incorporate leak and spurious toehold occlusion reproduces this reduced scaling and preserves the predicted co-optimization between sink concentration and the number of redundant layers. This observation brings the optimization philosophy proposed in this thesis full circle. While network-level optimization can expand the behaviors of a circuit beyond that possible at the sequence-level, embedding into a physical system under implementation constraints can lead to the introduction of undesigned spurious reactions. Later in this chapter, I highlight the future potential for sequence-level optimization to more closely approximate desired behavior. This is the same problem framing I addressed in Chapter 3.

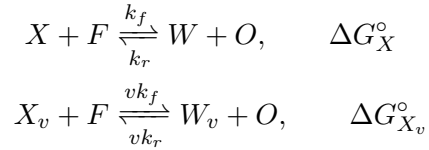
This chapter proceeds as follows. I first define discrimination metrics under mass-action kinetics and formalize a target selectivity-scaling behavior. I then show through mass-action simulation how this behavior can be approximated with a CRN that is well-suited to implementation under common architectural restrictions. Next, I experimentally characterize the system and reconcile deviations from simulation through modeling of leak and spurious occupancy. Finally, I show opportunities where sequence-level optimization and increased

gate complexity are expected to enable more faithful implementations of the desired scaling behavior.

5.1 Depth as a scalable selectivity variable

5.1.1 Single-reaction discrimination limit

I first consider the limitations of selectivity optimization when restricted to the level of a single reaction. Consider a single reversible reaction, like the strand exchange motifs used throughout this dissertation, with two competing inputs X, X_v :



where I define $\Delta\Delta G_{X_v}^\circ = \Delta G_{X_v}^\circ - \Delta G_X^\circ$. Using the identity $K = \frac{k_f}{k_r} = \exp\left(\frac{-\Delta G^\circ}{RT}\right)$, analogously to Hopfield (Hopfield [1974]), I define the thermodynamic error fraction as

$$\epsilon_{\Delta\Delta G_v^\circ} = \frac{K_v}{K} = \frac{[O][W_v]}{[X_v][F]} \cdot \frac{[X][F]}{[O][W]} = \frac{\alpha_v}{\alpha} = e^{-\frac{\Delta\Delta G_v^\circ}{RT}}$$

where $\alpha = \frac{[W]}{[X]}$, $\alpha_v = \frac{[W_v]}{[X_v]}$ are the yield/completion ratios for the on-target and off-target inputs added in isolation. As a result, although selectivity can be altered by changing experimental conditions that affect reaction free energies (for example temperature or salt concentration), for any fixed set of experimental conditions the thermodynamic error fraction is fixed by $\Delta\Delta G_{X_v}^\circ$. Therefore, the prevalence of selectivity error is directly tied to the equilibrium thermodynamics of the desired and undesired reactions. This motivates asking whether a network architecture can compound this fixed thermodynamic quantity to improve selectivity by making the observed output depend on the same fixed energetic difference multiple times in the critical path to output in order to compound the discrimination difference.

5.1.2 Target depth scaling behavior

To approach scalable selectivity in architectural depth, I first define an idealized target behavior: *perfect conditional gating*. Consider a mechanism, not necessarily physically realizable, that enforces that X and X_v interact with gate layers F_i only in strictly increasing order. In such a system, output can be produced only after the input has passed through N sequential discrimination steps.

If each layer imposes the same energetic discrimination $\Delta\Delta G_v^\circ$, then the thermodynamic error fraction compounds across layers:

$$\epsilon_N = \prod_{i=1}^N e^{-\frac{\Delta\Delta G_v^\circ}{RT}} = e^{-\frac{N\Delta\Delta G_v^\circ}{RT}}.$$

Thus, under perfect conditional gating, selectivity error as defined in this chapter decreases exponentially with architectural depth.

This scaling is useful because any fixed decrease in reaction-level discrimination can be compensated by a linear increase in the number of layers. For example, if an off-target differs from the target by a smaller $\Delta\Delta G_v^\circ$ than needed for the desired response, increasing N can restore the target error fraction without changing temperature or the single-reaction chemistry.

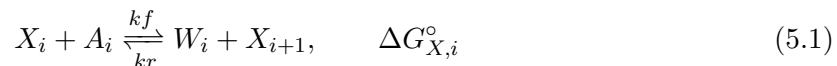
Perfect conditional gating is an abstract target rather than an immediately implementable CRN. This idealized target is not without precedent. Prior leakless architectures likewise began from an implementation-guiding target behavior, namely requiring productive leakage to traverse a larger architectural barrier than the desired pathway, before asking how imperfect DSD architectures could approximate that ideal. Here I adopt the same strategy for selectivity.

To realize or approximate perfect conditional gating, the system must contain some form of state information that determines the gate layer with which X or X_v are allowed to interact. The next section considers multiple CRN-level routes for encoding this state

information, and then specializes to the DSD-constrained frontier-unlocking motif used in this chapter to systematically extend Chen et al.’s network motif.

5.1.3 CRN-level routes to depth scaling

To kinetically or thermodynamically compound a fixed thermodynamic discrimination between target and variant across a network, proofreading architectures typically apply that difference across multiple reaction steps (Hopfield [1974], Ninio [1975]). One sufficient condition is for each successful reaction with a target gate to modify X in a way that encodes which gate can be accessed next, while preserving the same energetic discrimination at each step. One can abstract the motif as the reactions



where X_i and X_{i-1} differ in their affinity for amplifier gates G_i versus G_{i+1} , while maintaining a constant $\Delta\Delta G_v^\circ$ at each step.

Under the architectural restrictions considered here, converting an initially unstructured catalytic signal into a distinct chemical signal that meets these properties requires additional state to be stored somewhere in the reacting complex, which increases structural complexity beyond the design regime emphasized in this chapter.

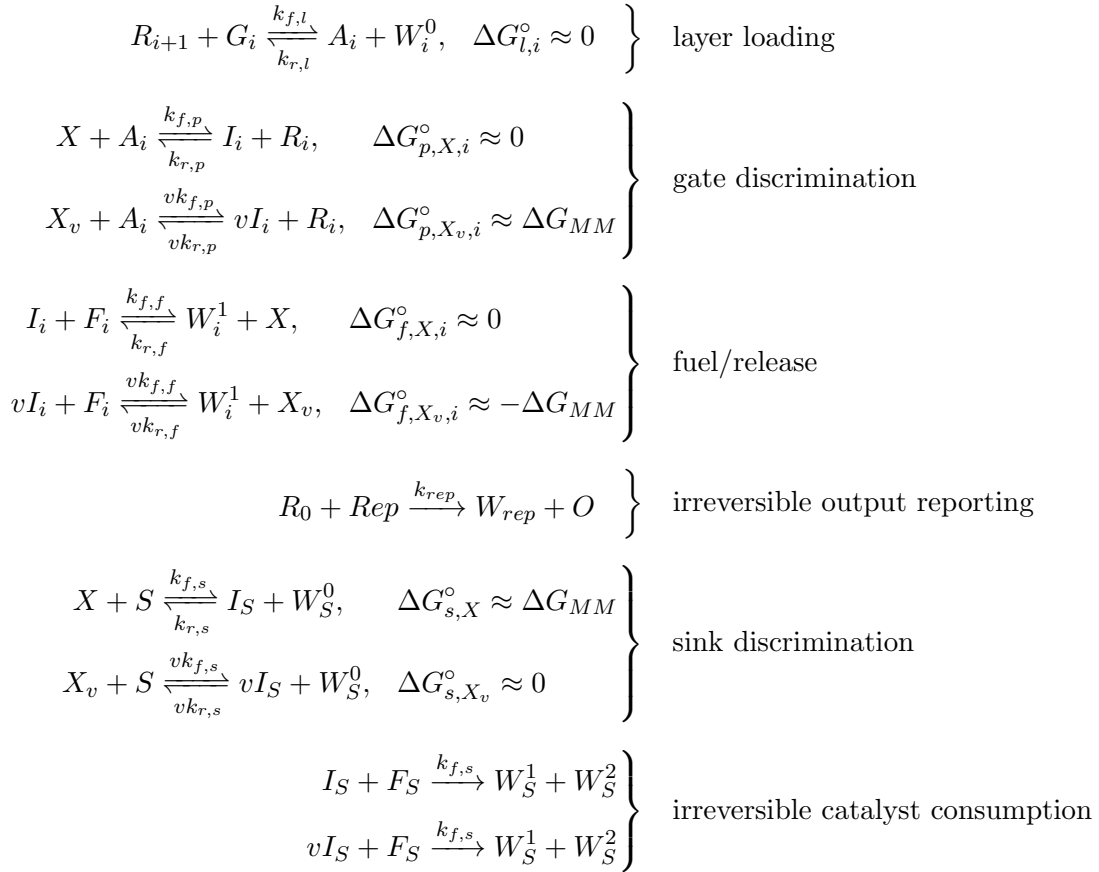
Another possibility is to encode the next accessible gate not at the level of the individual species, but as regulator species that can globally activate the next layer of the system. In this case, I introduce a second type of regulating species that allosterically unlocks a particular layer’s amplifying gate, which then reacts catalytically with X or X_v . While allosterically activated toeholds have been previously introduced in DSD literature (Yang et al. [2016]), this work leverages the motif in a novel way to achieve a network-level behavior beyond conditional activation. Requiring each amplifying gate to be unlocked by a previous

gate restricts X and X_v to explore the landscape along a frontier that expands in one direction. It does not, however, enforce that all X proceed through their interactions in a strict gate order. Thus, this motif approximates the target scaling behavior rather than exactly realizing perfect conditional gating. I analyze the consequences of this approximation in simulation and experiment in subsequent sections.

5.1.4 *DSD-constrained frontier unlocking motif*

As in prior domain-level optimizations such as leakless designs, I first restrict attention to a fixed complementary base-pairing pattern. This allows me to probe the limit of network-level optimization without simultaneously introducing sequence-level optimization. I additionally restrict the design to the functional motifs used in two-domain and seesaw architectures, in order to root the motif in architectures for which large implementations already exist. Finally, I focus the first generation of gate designs to those with at most three strands per component. More complex gates could enlarge the possible design space, for example by enabling simultaneous catalytic restoration and output release, but would expand the restricted implementation space beyond the simple and fundamental motifs on which this chapter intentionally focuses.

Under these restrictions, the frontier-unlocking motif introduced in the previous section becomes a natural architectural realization shown below. Unlike the ideal scaling behavior, in this architecture, layer state is stored in gate accessibility rather than encoded by modifications of X itself. Each layer contains a locked amplifying gate G_i and a regulator R_i . Reaction at layer $i + 1$ produces regulator R_i , which unlocks G_i . Once unlocked, the activated gate A_i can react catalytically with either X or X_v , producing the next downstream regulator while preserving the same target/variant discrimination at that layer.



As the reaction system evolves, the accessible reaction frontier therefore moves downstream as regulators are produced. This creates a DSD-realizable approximation to perfect conditional gating: downstream layers cannot be accessed before upstream layers have generated the corresponding regulators, but individual X molecules are not forced to traverse every layer in strict order. Additionally, because each catalytic cycle has approximately zero net free-energy change and the effectively irreversible reporter drives both competing pathways equally, the system lacks endpoint discrimination in the absence of a competitive sink that differentially consumes X and X_v .

The physical implementation imposes additional constraints beyond the abstract CRN. In particular, fuel-mediated catalytic restoration must be arranged so that the fuel cannot

directly substitute for the input, and regulator-mediated unlocking must precede catalytic reaction with X or X_v . Otherwise, the design admits bypass reactions that destroy the designed reactions. Under these restrictions, binding of the catalytic species is also not naturally coupled to a fast and strongly irreversible release step, making the resulting discrimination partly dependent on kinetic timing and sink-mediated stabilization. These constraints motivate the specific domain arrangement shown in Figure 5.2.

The resulting implemented motif is therefore neither a direct Hopfield/Ninio proofreading network nor a perfect conditional-gating cascade. It is a DSD-constrained approximation whose effective selectivity depends both on how much conditionally dependent depth survives under mass-action kinetics and on how strongly each variant is exposed to the competing sink. The next section investigates the power of this constrained motif using mass-action simulation.

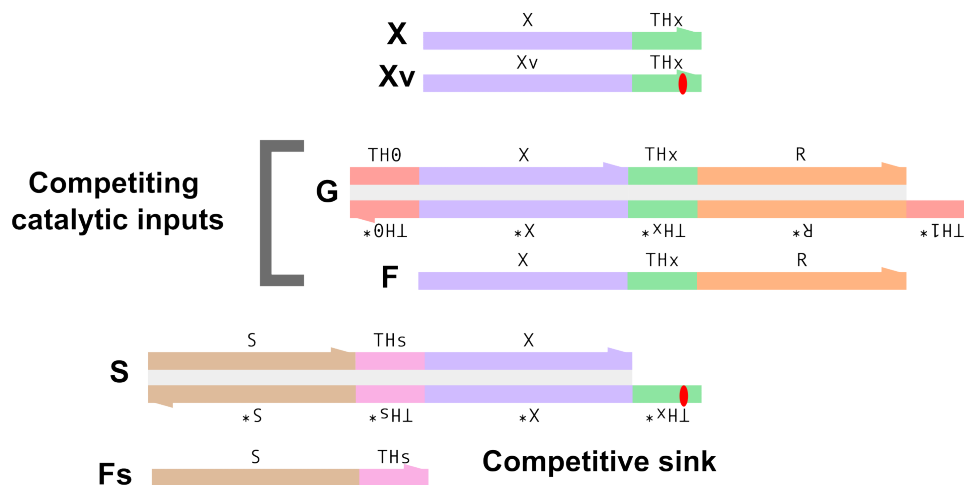
5.2 *Simulated scaling and architectural limits*

Although the frontier-unlocking motif only approximates perfect conditional gating, mass-action simulations show that substantial depth-dependent selectivity can survive under experimentally relevant conditions. I use these simulations (Figure 5.3) to test three predictions: whether increasing layer number improves discrimination, how sink concentration co-optimizes with depth, and how simultaneous exposure to target and off-target inputs can create short-circuit behavior that weakens ideal scaling.

Figure 5.3 shows that depth improves endpoint discrimination over an intermediate sink range, that time-dependent discrimination increases with layer number, and that simultaneous analyte exposure creates a short-circuit pathway. The results of these simulations suggest that, for a single analyte in the presence of competing sink, endpoint discrimination can still improve approximately exponentially with layer despite the theoretical differences between frontier unlocking and perfect conditional gating. Thus, much of the desired scaling behavior survives in the DSD-constrained implementation.

However, an architectural limitation immediately emerges when competing analytes are

A Starting strategic network motifs



B Conditionally chained frontier-unlocking architecture

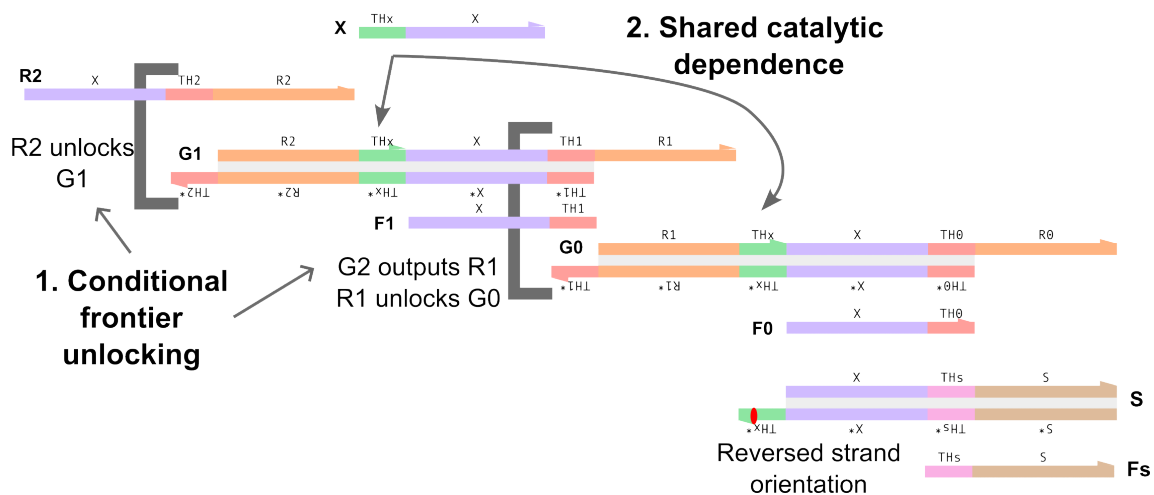


Figure 5.2: Domain-level implementation of the conditionally chained frontier-unlocking motif. The design preserves shared catalytic dependence on X or X_v while using regulator-mediated unlocking to restrict downstream gate access. Additional implementation constraints prevent fuel-only bypasses and premature catalytic activation. Red ovals are used to signify the single nucleotide variant distinguishing X_v from X and the complementary base in the sink, which preferentially favors binding of X_v .

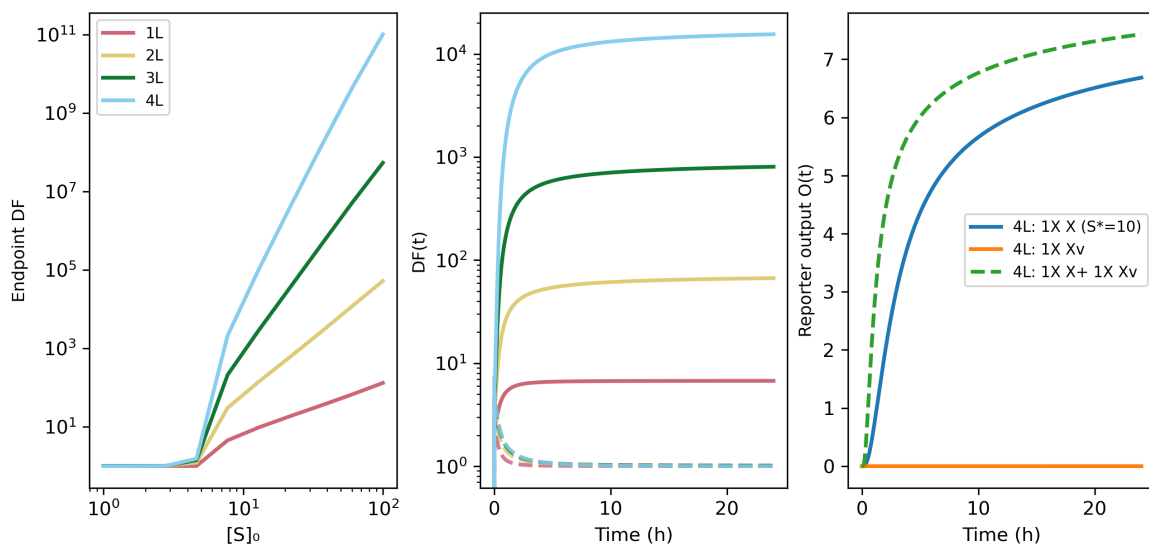


Figure 5.3: Numerical simulation of the DSD-constrained frontier-unlocking CRN. (left) The relationship between endpoint discrimination factor and sink concentration across experimentally characterized amplifier module layer numbers. (center) Comparing the discrimination factor over time across layers, with and without 10 nM sink. (right) Kinetic curves illustrating a short circuit mechanism, by which introducing both competing inputs to the system at the same time allows the off-target to skip amplifying layers, once downstream layers are unlocked by the correct input.

present simultaneously. Because both analytes share the same expanding frontier of unlocked gates, the off-target input can exploit downstream layers unlocked by the target, effectively short-circuiting discriminatory steps that would have been required in isolation. This mechanism reduces the effective depth experienced by the off-target and can, in the worst case, collapse discrimination toward the single-layer regime. One potential mitigation strategy might be a staged assay in which the first layer and sink are introduced initially, and downstream layers are added sequentially after sufficient kinetic evolution of the preceding stage has occurred. Such an approach may provide a practical mitigation for this short-circuiting effect, but detailed consideration falls outside the scope of the present work. Throughout this chapter, I focus exclusively on autonomous one-pot implementations. I address the persistence of this phenomenon in experimental implementation in the following section.

5.3 Experimental characterization

In the previous section, I presented a CRN architecture that approximates a targeted selectivity scaling behavior under DSD implementation constraints. Here, I experimentally test the resulting frontier-unlocking system. Specifically, I ask whether discrimination increases with layer number, whether layered discrimination behaves differently from one-layer discrimination, and which operational effects bound discrimination in practice.

The experimental system (Figure 5.4(A)) consists of up to four discrimination layers and a shared two-step differential sink implemented in the same style as Chen et al. (Chen et al. [2013b]). I analyze both maximum kinetic discrimination and endpoint discrimination across layers and sink concentrations. I also investigate the circuit behavior when both catalytic input variants are introduced simultaneously. This work uses the same experimental discrimination metric as Chen et al., defined as $\frac{[O]_X(t)}{[O]_{X_v}(t)}$. Endpoint discrimination refers to this quantity evaluated at long times and approximated experimentally using steady-state measurements. Expanded methods are presented in Section A.3.

5.3.1 Layer scaling behavior and sink dependence

I first measured output kinetics and discrimination factors across layer number, both with and without the differential sink. I also tested the hypothesized short-circuit pathway by measuring circuit output when both input variants were present simultaneously.

As expected, little endpoint discrimination was observed in the absence of sink (Figure 5.4B). Additionally, although I observed an increase in maximum discrimination between the one- and two-layer systems in the absence of sink, maximum discrimination decreased with additional layers. I analyze possible explanations for this deviation from CRN predictions, including thermodynamic and kinetic properties of the implemented system, in the Discussion.

In the presence of sink, however, increasing depth increased both kinetic and endpoint discrimination. Endpoint discrimination increased substantially with depth, primar-

ily through suppression of off-target output while maintaining high on-target output (Figure 5.4B,C). The majority of selectivity improvement occurred from one to three layers, with discrimination approaching a plateau in the three- to four-layer regime. In this regime, off-target output approached the observed level of leak while on-target output remained high. Thus, the layered architecture produced conditions in which off-target output was effectively suppressed to the level of leak without substantially reducing on-target output.

Mass-action simulations predicted that simultaneous addition of X and X_v could reduce discrimination by allowing X_v to access downstream gates unlocked by X . Consistent with this prediction, evidence of short-circuit behavior was observed in the absence of sink. Surprisingly, in the presence of sink, mixed-input conditions closely followed the on-target response, indicating that sink stabilization suppressed the predicted short-circuit effect under the tested conditions (Figure 5.4B).

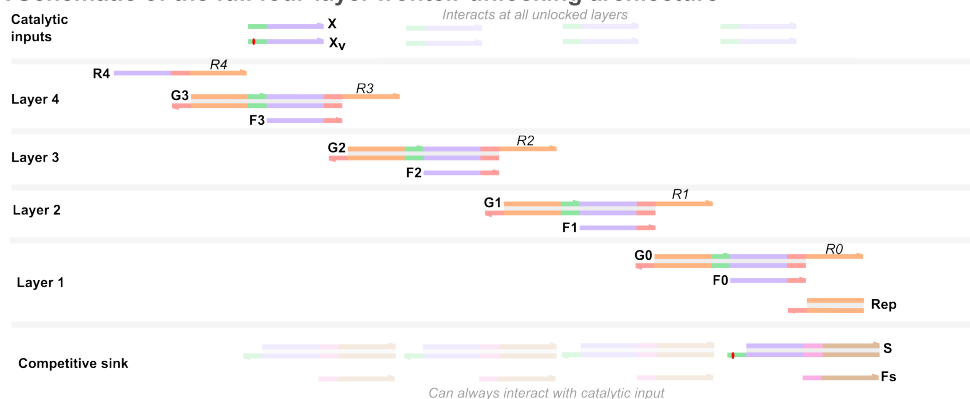
Reanalysis of the above results using recalibrated gate concentrations produced qualitatively similar behavior (SI Section D.2), including strong suppression of off-target output under sink stabilization and substantially increased discrimination with increasing depth. The recalibrated analysis incorporated substantial concentration adjustments for one gate species, but produced only modest quantitative differences in absolute output levels and the magnitude of short-circuit effects, particularly in the deepest cascades. It importantly did not alter the primary conclusions of this chapter.

5.3.2 Comparison to single-layer optimizations

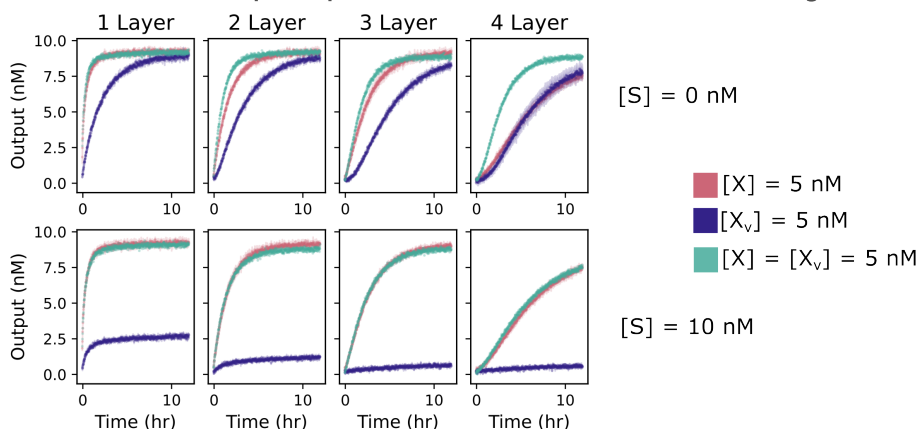
Although increasing the number of discriminatory layers increased discrimination, it is important to ask whether this behavior could be replicated by tuning a single-layer architecture alone. To test this, I compared layer-based scaling against three single-layer optimization strategies: increasing sink concentration, increasing target gate concentration, and increasing the concentration of an unproductive competing gate.

Comparing discrimination as a function of sink concentration in one- and two-layer

A Schematic of the full four-layer frontier unlocking architecture



B Effect of sink on depth-dependent discrimination and short-circuiting



C Endpoint discrimination increases with depth under sink stabilization

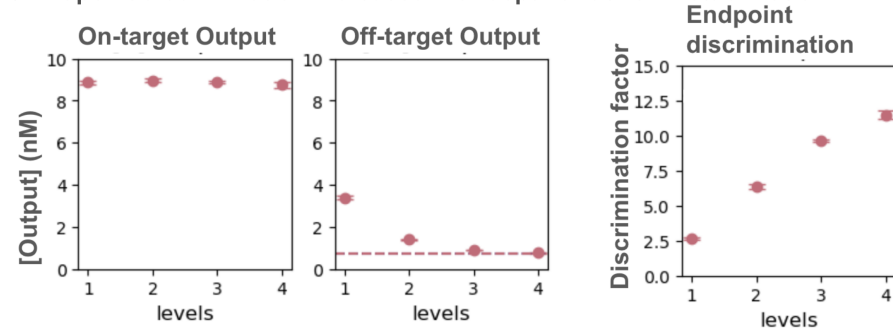
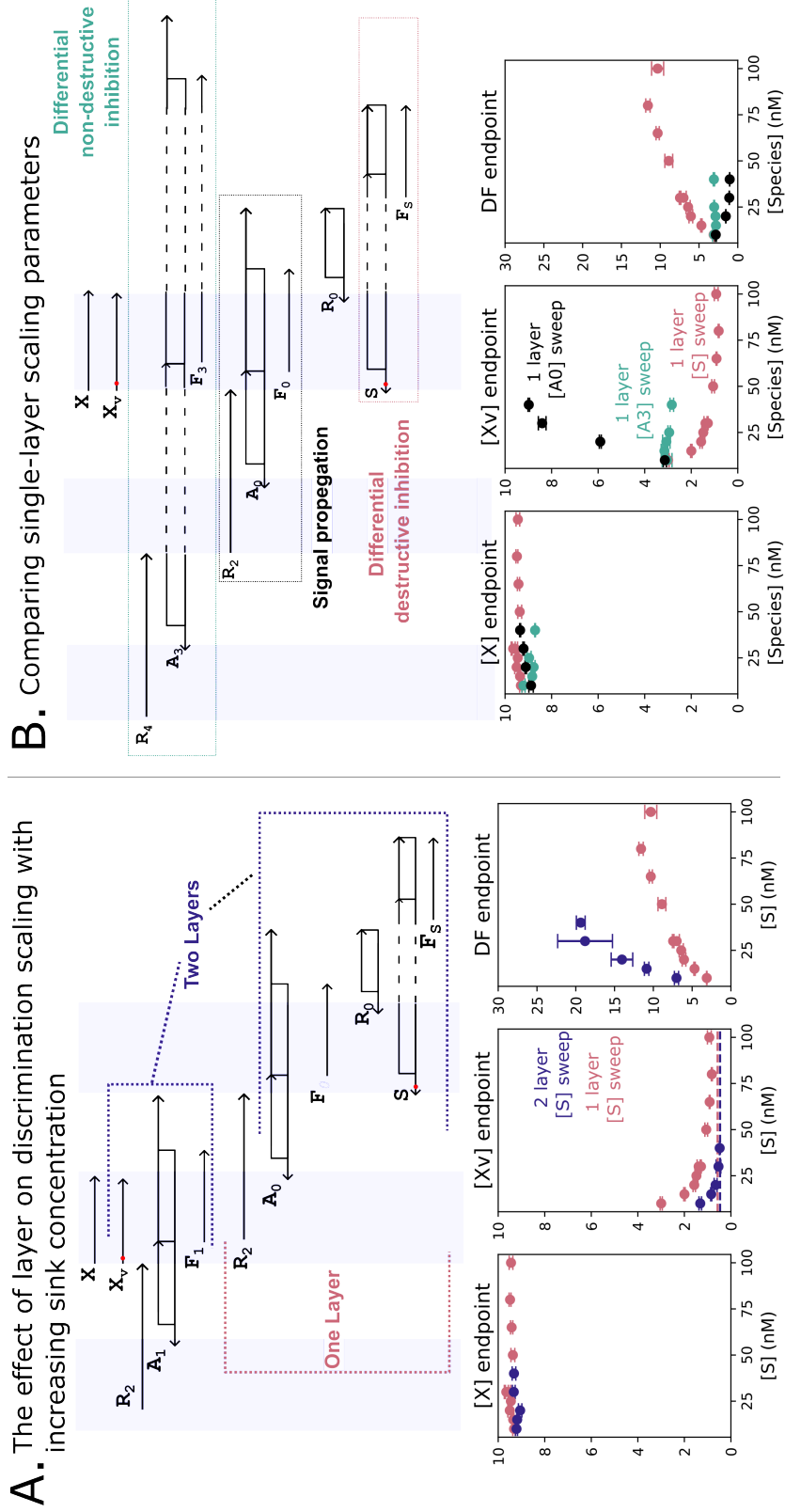


Figure 5.4: **Layered frontier-unlocking experimentally amplifies discrimination while suppressing short-circuit behavior under sink stabilization.** (A) Simplified schematic of the multilayer discrimination architecture. Detailed mechanistic diagrams of the intended reactions are presented in Appendix D.1. (B) Kinetic evolution of on-target, off-target, and mixed-input responses across cascade depth with and without sink stabilization, illustrating both depth-dependent discrimination and suppression of short-circuit behavior. (C) Endpoint on-target output, off-target output, and discrimination factor as functions of cascade depth. Discrimination increases substantially with depth primarily through progressive suppression of off-target completion while preserving high on-target output. The dashed line represents the endpoint leak value, which typically corresponds to the minimum observable output.



systems (Figure 5.5A) shows that the two-layer system achieved higher discrimination at every tested sink concentration. Furthermore, the single-layer system appeared to approach a fixed level that rested above the one-layer case, whereas the two-layer system approached the effective minimum output imposed by leak. I provide a theoretical argument for why this may be the case in Section 5.4.

As shown in Figure 5.5B, none of the tested single-layer concentration-based strategies reproduced the qualitative scaling observed with increasing network depth. Increasing target gate concentration reduced discrimination by increasing off-target output while leaving on-target output approximately unchanged. Increasing the concentration of unproductive competing species produced little apparent improvement in discrimination. Together, these results suggest that layered discrimination provides a qualitatively distinct optimization parameter rather than an unnecessary complication of one-layer parameter tuning.

5.3.3 Layering reshapes effective off-target rejection thresholds

In addition to increasing discrimination at fixed input concentration, increasing the number of layers increased the range of concentrations for which off-target input was indistinguishable from triggered baseline at the kinetic endpoint but the on-target input was distinguishable.

To characterize this effect, I compared one- and four-layer systems across increasing target (X) and off-target (X_v) input concentrations (Figure 5.6). In the one-layer system, X_v was detectable at endpoint (above the level of leak) for every tested concentration and X was detectable at all tested concentrations. In the four-layer system, X remained detectable at all concentrations, while X_v was not detected at 5 nM and below.

This shift was reflected both in the observed reaction kinetics and in endpoint completion. Across the tested concentration range, increasing the network depth expanded the regime over which off-target inputs effectively did not change the level of output beyond leak without simultaneously reducing on-target output to the same leak level.

These results suggest that layered discrimination changes not only the magnitude of discrimination at a fixed input, but also the effective input-output response of the system. I return to possible mechanistic interpretations of this behavior in the Discussion.

5.3.4 *Observed scaling deviates from idealized predictions*

The preceding results show that architectural depth can increase discrimination. However, a number of observations differed from those predicted by the idealized CRN. Firstly, I observed the presence of leak in the range of 5–10% of the maximum output, which was not included in the model. Secondly, I observed regimes where maximum kinetic discrimination is not monotonically increasing with layer as predicted by simulation. Lastly, I observed that in the presence of the stabilizing sink, predicted short-circuit pathways do not appear as prevalent as predicted in simulation (Figure 5.3).

5.4 *Discussion*

In this chapter, I presented an architectural proofreading strategy for strand displacement circuits based on conditionally chained frontier unlocking. Both simulations and experiments showed substantial increases in endpoint discrimination factor with increasing architectural depth in the presence of sink stabilization. Experimental discrimination approached a plateau in the deepest cascades, consistent with practical implementation constraints discussed below. I also showed that the two-layer system exceeded the one-layer system in endpoint discrimination across all tested sink concentrations. In this section, I discuss a possible kinetic mechanism underlying this discrimination scaling, then address sources of deviation from ideal behavior, including the short-circuit mechanism described previously in this chapter.

5.4.1 *Depth-dependent discrimination as kinetic filtering*

The results above suggest that layered frontier unlocking can amplify discrimination even when individual per-reaction discrimination is weak. Additionally, because each catalytic

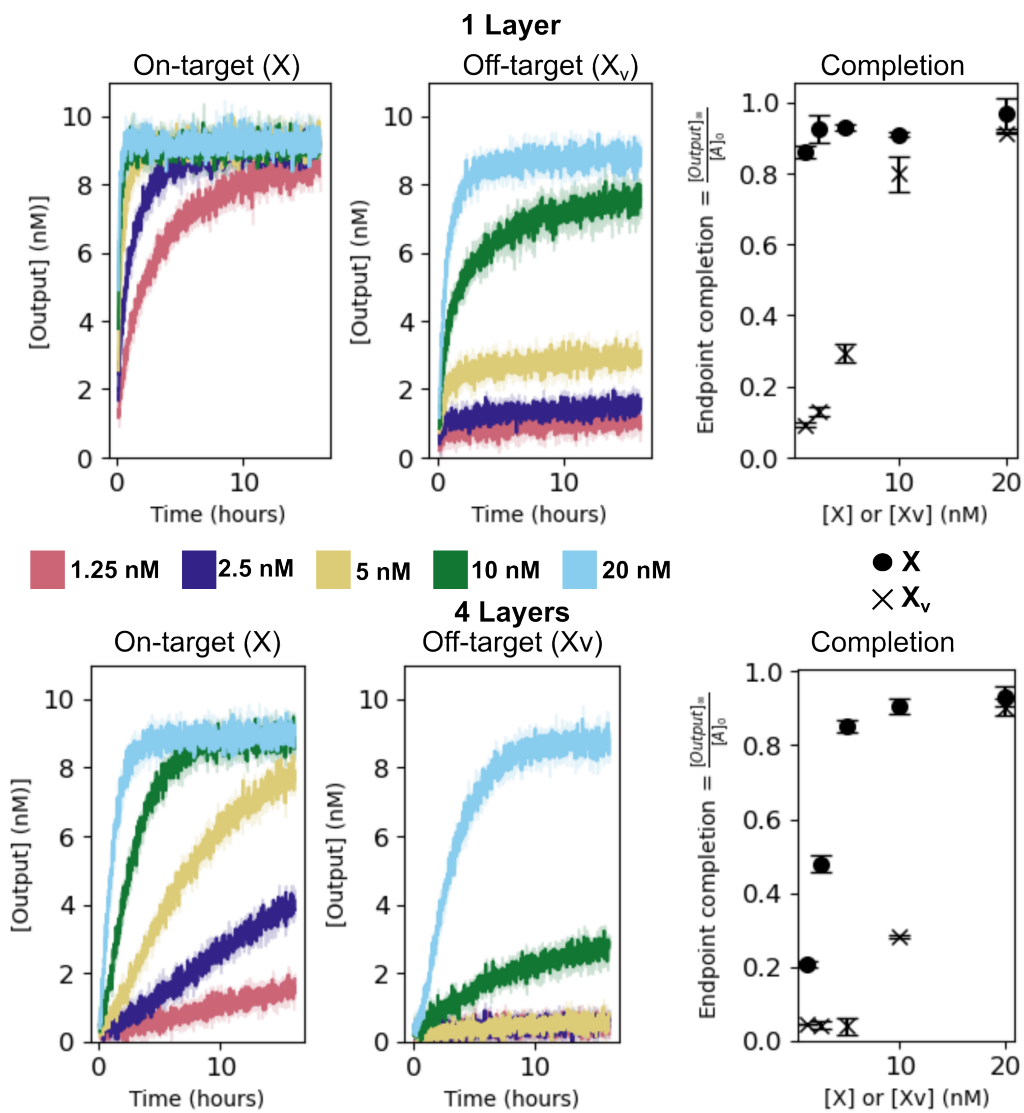


Figure 5.6: Multi-layer scaling changes the effective behavior of the competitive sink. Comparing the one-layer and four-layer implementation supports that layering may expand the effect concentration range over which off-target signal is suppressed, but on-target signal is detectable.

cycle is approximately enthalpy neutral and the network contains both effectively irreversible sink and reporter reactions, this discrimination is not simply an equilibrium thermodynamic effect.

The implemented motif also differs from Hopfield/Ninio kinetic proofreading (Hopfield [1974], Ninio [1975]). Classical kinetic proofreading compounds discrimination through dissociation-rate differences as substrates traverse irreversible intermediate states. In the frontier-unlocking architecture, X and X_v instead act catalytically to enable passage of regulatory molecules through a latent cascade of reactions terminating with an observed output. Simultaneously these catalytic inputs are also differentially consumed by a two-step sink. Discrimination therefore emerges from competition between productive regulator propagation and input loss to the sink.

A useful way to view this behavior is as repeated, but dynamic filtering of input molecules between the sink pathway and the pathway producing R_0 . In a one-layer system, an off-target input can produce output after a single successful productive collision. This is because a gate that produces output is already unlocked, providing a non-zero chance that the first round of collisions for every input molecule can produce an output molecule before ever having the chance to bind the competing sink. In a conditionally layered system, terminal output cannot be produced immediately because downstream layers are initially locked. Output production therefore requires multiple successful propagation events through the regulator cascade in a way that scales with layer.

In addition to precluding an initial path to output, layered frontier unlocking provides a structure where successful reactions in the path to output change make reaction with the sink pathway less likely and reaction with the output producing pathway more likely. Initially, X and X_v encounter a similar set of possible reactions. They can either participate in the regulator cascade or enter the sink pathway. However, each successful catalytic step in the regulator cascade unlocks additional downstream gates. This expands the set of productive reactions available to the catalyst without doing the same for the sink pathway. In this

sense, successful propagation commits the system toward output production. Importantly, this reinforcement is not symmetric between X and X_v . Because X_v is more likely to be removed by the sink early in the cascade, it is less likely to benefit from the expansion of accessible productive pathways that follows successful propagation. In contrast, X is more likely to survive initial filtering events and therefore to produce a progressively more favorable reaction landscape through frontier expansion. Thus, increasing depth therefore does not simply add repeated opportunities for discrimination, but preferentially amplifies early differences in kinetic discrimination into asymmetries in access to downstream productive pathways.

5.4.2 Architectural depth reshapes effective sink behavior

The kinetic interpretation in the previous subsection helps explain the thresholding behavior observed in Figure 5.6. If successful discrimination is defined operationally as preserving detectable on-target output while suppressing off-target output to the level of leak, then increasing depth expands the input concentration range over which the system avoids selectivity error. I observed that in the one-layer system, off-target output remained detectable even at the lowest tested input concentration (1.25 nM). While in the four-layer system, off-target output was suppressed near the leak floor for off-target concentrations up to 5 nM, while on-target output remained distinguishable from leak for all tested cases.

Thus, increasing layer number provides an effect on discrimination that goes beyond a single fixed input concentration. It also reshapes the effective input-output response of the system, making the sink behave more like an off-target-selective irreversible sink over a broader practical concentration range, without comparably suppressing on-target output.

5.4.3 Theoretical tradeoffs: ideal gating and frontier unlocking

The perfect conditional gating model assumes molecular state information can enforce strict progression through discriminatory layers. At the CRN level, this could be encoded either

by species-level state transformations, in which each productive step converts the catalyst into a species specific to the next layer, or by system-level encoding, such as using globally accessible regulators to control which layers are available. The frontier-unlocking architecture adopts the latter strategy because it is more naturally compatible with existing scalable DSD motifs.

This simplification comes with tradeoffs. Because layer state is encoded globally in gate accessibility rather than locally in the catalyst identity, all catalysts share a common frontier, introducing short-circuit pathways and weakening the strict sequential coupling of ideal proofreading (Figure 5.4(B)). Additionally, because productive advancement requires repeated bimolecular search events, the time required to find the next productive gate can match or exceed the kinetic discrimination between target and off-target pathways, especially at increasing depth. This is detrimental to kinetic scaling behavior of the system in the absence of sink.

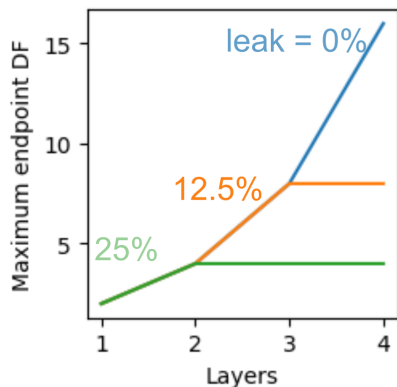
These tradeoffs reflect a general distinction between ideal CRN behavior and DSD-constrained implementations. More complex multi-stranded motifs, or architectures capable of partially irreversible off-target filtering, may approach stronger scaling regimes than those explored here, but at increased implementation complexity.

5.4.4 *Operational bounds on discrimination*

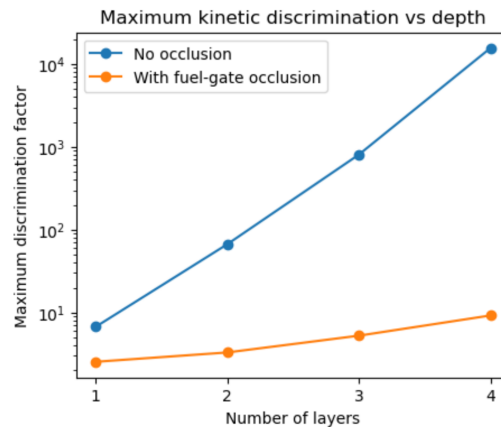
Apart from deviations arising from the chosen CRN implementation, additional off-target interactions are introduced when embedding the system in a physical architecture. This was illustrated clearly for the case of leakless redundancy in Chapter 3. My experimental results suggest that scaling can also be ultimately bounded by off-target interactions introduced by the physical implementation.

First, productive leak imposes an endpoint bound on observable discrimination. Once off-target output is suppressed to the leak floor, further increases in depth or sink strength cannot produce proportionally larger measured discrimination. A simple leak-limited model

A Leak limits endpoint discrimination



B Unproductive interactions limit



C Orthogonal optimization strategies have the potential to restore desired behavior

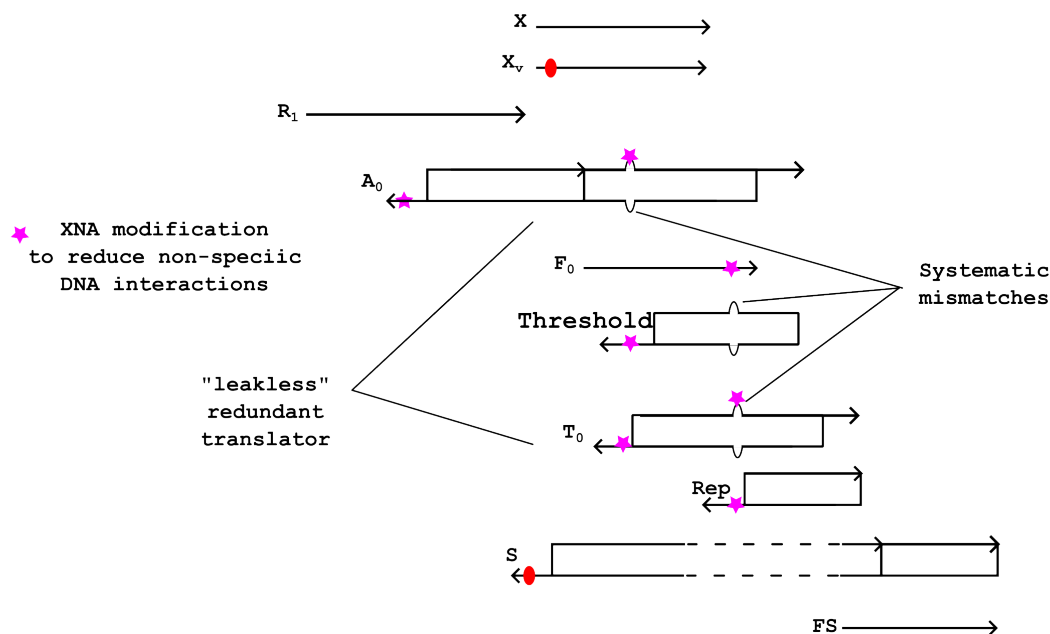


Figure 5.7: Leak and unproductive spurious interactions impact performance, but may be eliminated through largely orthogonal optimization strategies. A) Even in the ideal case of a true exponential suppression of leak per layer (2 fold reduction per layer pictured), with constant on-target output, leak provides a ceiling on the achievable endpoint discrimination. B) Mass action simulations including the presence of toehold occlusion between gates and single-stranded fuels, show how these off-target reactions can limit scaling of kinetic discrimination. The same forward rate constant as Chapter 4 was used for spurious association. C) An example design that combines network-level, sequence-level, and alphabet-level optimization to reduce such off-target interactions.

captures this effect (Figure 5.7(A)), predicting that discrimination increases with depth only until off-target output approaches the leak floor. Consistent with this interpretation, replicated experiments using recalibrated gate concentrations (Section D.2) exhibited discrimination that plateaued in the deepest cascades once off-target output approached the observable leak floor.

Second, unproductive interactions can impose a kinetic burden even when they do not directly produce output. Because deeper cascades require productive propagation through more gate layers, layer-dependent occlusion or spurious occupancy can reduce the maximum kinetic discrimination obtained before endpoint equilibration. A simplified kinetic model which includes occlusion between fuel complexes and gate toeholds illustrates how such interactions can suppress the increase in discrimination with depth (Figure 5.7(B)).

Together, these models explain why experimentally observed depth scaling can deviate from the ideal CRN prediction even when the intended frontier-unlocking architecture is only a proper subset of the implemented reactions. They also motivate the sequence-level optimization strategies discussed below as a route to make the physical implementation more faithfully approximate the target CRN. These departures from idealized CRN scaling suggest that additional implementation-level interactions shape observable discrimination in deeper cascades.

5.4.5 *Design routes toward improved scaling*

It may be possible to mitigate these off-target interactions discussed in the previous section through orthogonal strategies (Figure 5.7(C)), like in Chapter 3. In this case, the leak resulting from interaction between sequential gates can be eliminated through the application of leakless design, which should also be optimized at sequence-level for performance. While leak from the catalytic fuel fundamentally cannot be eliminated thermodynamically without comparable suppression of the desired reactions, as in seesaw systems, a threshold species can be used to consume regulator species at a low rate, with minimal impact on

on-target signal due to innate signal restoration in the architecture. Interestingly, only the terminal reaction between the fuel and gate can lead to leak, so the minimum number of thresholds to prevent leak with this strategy is one. However, using thresholds on each regulator domain can additionally prevent spurious production of intermediate regulators that can unlock downstream gates, reducing kinetic discrimination.

Secondly, to arrive at the sequential unlocking implementation, I first excluded a species-level encoding strategy that provides guaranteed thermodynamic exponential scaling. Whether or not existing multi-stranded motifs like the handhold mediated strand displacement used for kinetic proofreading in catalytic templating (Cabello-Garcia et al. [2025]) can be adapted to reliably implement this CRN remains an open question.

5.4.6 *Conclusions*

This chapter shows that multi-layer proofreading-like selectivity scaling can be encoded in simple and scalable strand displacement motifs by combining network-level redundancy with gate-level conditional activation and repeated catalytic dependence on the input, allowing discrimination to increase with depth beyond single-reaction limits. As in the case of leakless optimization, however, embedding the target CRN into a constrained physical implementation introduces off-target interactions that limit ideal behavior. In this sense, the chapter conceptually mirrors Chapter 3: architectural optimization expands behavior beyond single-reaction limits, while sequence-level optimization restores the fidelity lost under implementation constraints. This parallel suggests a broader design principle. Architecture can expand the achievable robust design space, while sequence-level optimization can make those architectural behaviors more faithfully realizable. Together, these results suggest a generalizable methodology for combining architectural and sequence-level optimization to expand the usable design space of molecular circuits. Lastly, it is intriguing to ask whether related catalytic discrimination strategies may exist in natural sequence-recognition systems (such as catalytic RNA guides), or could be engineered into systems such as guide-directed

nucleases to achieve tunable sequence selectivity in impactful applications like molecular diagnostics and gene therapies.

Chapter 6

**TOWARD SYSTEMATIC DESIGN AND OPTIMIZATION OF
SEQUENCE-PROGRAMMABLE STRAND DISPLACEMENT
SYSTEMS**

The previous chapters introduced several distinct optimization strategies for improving the robustness of strand displacement circuits against different classes of spurious interactions. In this chapter, I synthesize these results into a broader framework for reasoning about the design space of molecular computing systems.

Designing robust molecular circuits requires coordinated decisions across multiple layers of abstraction. Desired behaviors are typically first specified at the level of ideal reaction dynamics (or a derived system property), then approximated by architectures that are abstracted from the properties of the underlying physical, molecular implementation. These architectures must then be embedded into sequence-defined polymers, where sequence design and alphabet choice determine how faithfully the intended reactions are realized. Finally, I highlight that experimental design and operation determine the regimes in which the target behavior becomes observable, and the dynamics by which chemical signals propagate.

The results of this thesis highlight three structural axes along which strand displacement systems can be optimized: reaction network architecture, sequence-level implementation, and chemical alphabet. Designing new architectures or systematically augmenting existing designs guided by these structural axes can reveal new regions of the design space, and can produce qualitatively different optima for target behaviors.

Finally, reflecting on the experimental work underlying this thesis, I comment on an additional but less frequently discussed optimization axis: the design of experiments.

6.1 Key takeaways from Chapters 3–5

6.1.1 Chapter 3

Sequence-level redundancy creates thermodynamic bounds against leak, but introduces kinetic drawbacks as part of their imposed structure. Mismatch-based sequence-level optimization of leakless designs used the sequence-level degrees of freedom introduced by leakless optimization to optimize reaction energy landscapes to ameliorate kinetics and prune undesired network behaviors. In doing so this strategy preserved leak suppression, improved kinetics, and expanded the architectural expressivity of leakless designs.

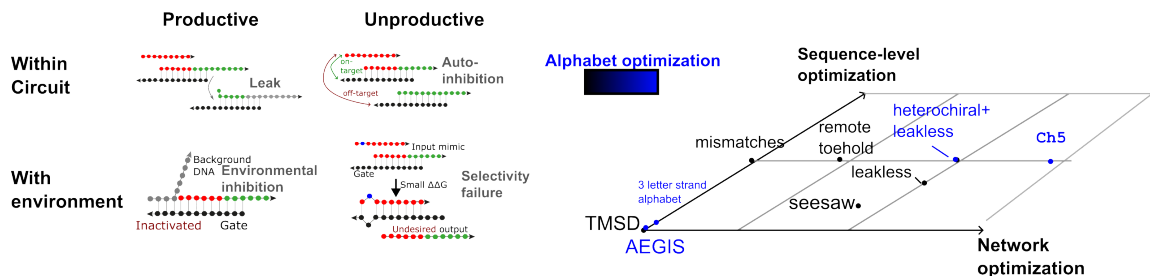
6.1.2 Chapter 4

Using the non-natural isoG:isoC base pair as an example, I showed that optimization can occur at the level of the chemical alphabet itself. Expanding the chemical alphabet for the circuit beyond natural DNA provides a basis for systematic kinetic optimization against burdensome binding by non-specific DNA components. The same could be accomplished for circuits in clean environments by removing a base pair from the design alphabet and systematically reintroducing it, but with a severely limited sequence space. While this reactivity optimization can be implemented orthogonally to mismatch-based sequence-level optimization, the sites chosen for each converge naturally due to their similar target kinetic behavior.

6.1.3 Chapter 5

In the case of selectivity, where optimizing individual species in a network may be inadequate, redundantly expanding the reaction network and encoding selectivity at the network-level provides exponential scaling of discrimination in theory. I showed that approximating this ideal CRN with a DSD-compatible architecture produces substantial increases in discrimination with architectural depth in both simulation and experiment.

A. Robustness challenges and optimization axes



B. Co-optimization under implementation constraints

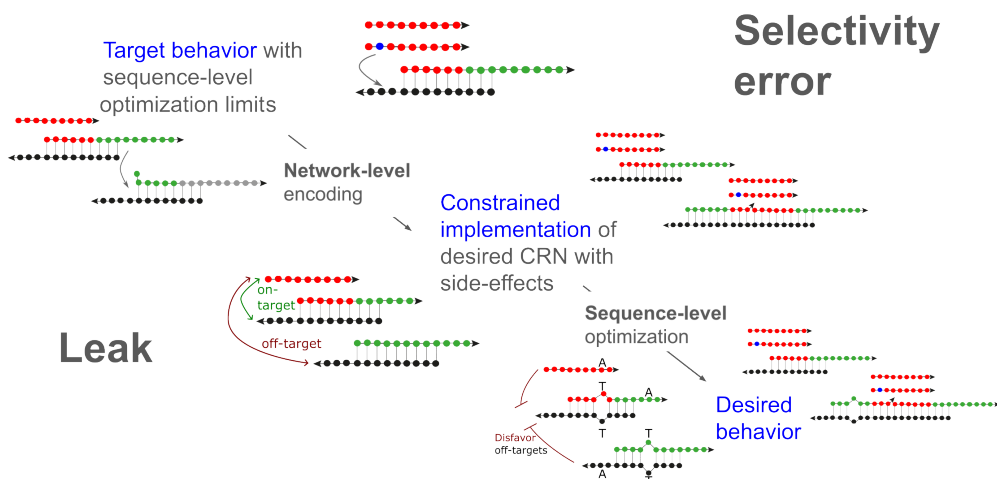


Figure 6.1: **Multi-axis optimization as a framework for robust molecular programming.** Robust molecular programming may be viewed as navigation of a constrained design space in which architectural, sequence-level, and alphabet-level optimization act as complementary levers for expanding robustness and restoring desired behavior under multiple error modes. (A) Representative error modes addressed in this dissertation and the corresponding optimization axes used to mitigate them. Architectural strategies expand achievable system behaviors beyond limits of sequence-level optimization alone, sequence-level optimization suppresses undesired interactions that emerge upon implementation, and alphabet expansion enlarges the underlying interaction space available for robust design. (B) A conceptual co-optimization workflow for extending the frontier of programmable strand-displacement architectures to new failure modes. Network-level design expands the space of achievable behaviors, physical implementation introduces side effects and operational constraints, and sequence-level optimization prunes undesired reactions through kinetic and thermodynamic tuning to restore target behavior. Together, these axes suggest a possible staged methodology for systematically expanding the robust design space of molecular circuits.

6.2 A multi-axis framework for DSD optimization

The results of Chapters 3–5 suggest that optimization of strand displacement systems naturally decomposes into three structural axes of optimization. These axes provide a useful coordinate system for structuring the space of existing strand displacement circuit designs. Chapters 3 and 5 suggest that, practically, when optimizing strand displacement circuits under architectural constraints, network-level optimization toward a desired network-level property can only be approximated when implemented in a constrained physical system and introduces off-target molecular pathways that must be optimized at the sequence level. Simultaneously network-level CRN strategies, if implemented faithfully, can optimize certain design targets more than sequence-level optimization could alone. Thus these two axes must often be co-optimized. Lastly, chemical alphabet modification determines the effective number of chemically orthogonal species, and defines the fundamental implementation rules of the implementing species.

1. **Network architecture:** determines the abstract reaction graph and the asymptotic scaling behavior of the system.
2. **Sequence-level implementation:** determines how accurately the physical chemistry realizes the intended reaction network.
3. **Chemical alphabet:** determines the underlying space of chemical potential for implementing molecular interactions.

Figure 6.1(B) organizes a selection of existing DSD optimizations highlighted in Chapter 2 along these three design axes. These axes are not strictly orthogonal, but they are separable enough to support staged optimization, and might be viewed as different layers of molecular design. While not strictly independent, they represent separable design decisions, and based on this work, appear to admit largely independent optimization strategies. This decomposition provides a conceptual framework for systematically engineering robust

molecular circuits. In the following sections I map these optimization axes and their modes of operations to specific error modes in DSD circuits and analyze their inherent tradeoffs.

6.2.1 *Error modes*

This thesis addresses the following error modes.

1. Leak (productive gate-gate interactions)
2. Selectivity error (productive gate-environment interactions)
3. Unproductive gate-gate interactions
4. Unproductive gate-environment interactions

Figure 6.1(A) highlights the structural and functional relationships between each of these four error modes.

6.2.2 *Representation limits under implementation constraints*

Although an abstract CRN might exist for a desired behavior, finite architectural constraints can limit which networks admit scalable and robust strand-displacement implementations of such behavior. This is clearly illustrated in Robert Johnson’s doctoral thesis, where they show how certain behaviors cannot be implemented under common architecture restrictions, analogous to those used in seesaw circuits (Johnson [2020]). In this sense, the ability of a CRN to encode a behavior does not mean an implementation exists under practical molecular constraints. This motivates treating implementability itself as part of the design problem. This thesis provided multiple examples of the power of a multi-stage approach to converge upon a desired robustness behavior when navigating such a design problem. I summarize these insights in the next section.

6.3 General design principles for new robustness axes

Principle 1 — Match the optimization to the biophysical origin of the failure

For example, off-target circuit interactions fundamentally occur because of sequence complementarity in nucleic acid systems, thus sequence-level optimization provides a powerful optimization landscape. Non-specific binding occurs because the components of the circuit share reactivity with the environment, the alphabet that determines the reactivity provides an optimization. Selectivity error occurs at the level of sequence interactions but the limits of selectivity optimization occur at the level of a single reaction, thus using the structure of the reaction network can optimize scaling behavior. Effective optimization therefore begins by matching the intervention axis to the best known origin of the failure mode.

Principle 2 — Higher-level optimization expands achievable behavior but introduces implementation burdens. Architectural redundancy can generate behaviors inaccessible through local parameter tuning alone, but implementation of those behaviors often introduces new side reactions and constraints. This recurring tradeoff appears in both leakless and layered selectivity architectures. However, at the same time, initially restricting to simplified “domain-level” designs improves the feasibility of design and reasoning about system behaviors, and may be restricted in such a way as not to prohibit downstream optimization at the sequence-level.

Principle 3 — Lower-level optimization restores fidelity. Once higher-level behaviors are encoded, sequence-level can prune undesired pathways and restore intended behavior. In this sense, lower-level optimization often acts as a repair layer for the limits of optimization over constrained architectural design. This mirrors a recurring pattern in this thesis: higher-level optimization expands reachable behaviors, while lower-level optimization restores fidelity under implementation constraints. Additionally, while various sequence-level motifs could be allowed in de novo design, initially restricting the design space and then systematically relaxing constraints (for example, in this work through Hamming-distance-level edits) can balance reliability with design scalability.

Principle 4 — Alphabet optimization defines possible sequence behavior Expanding the chemical alphabet used to implement sequence design can add new reactivity and chemical orthogonality to different reactive motifs, such as non-specific binding to DNA bases. At the same time, if complementary bases from an expanded alphabet are in similar proportion to the remainder of the alphabet, they can reintroduce non-specific interactions within a circuit. The inverse of this phenomenon underlies our approach to remove one base from the design of DNA signals to reduce folding and non-specific interactions. Thus the alphabet determines the possible chemical reactivity of a sequence design, and the distribution of bases in the design and the environment partially defines the landscape of non-specific binding.

These principles describe a separable methodology for expanding the design space to incorporate new robustness mechanisms. Once a jointly optimized combination of architecture and sequence-level tuning has been identified, however, this separation need not remain explicit. In *ab initio* design, the combined architectural and sequence-level degrees of freedom can instead be treated as a unified design space to search directly. The staged decomposition is therefore best viewed not as always a permanent separation of optimization problems, but as a practical strategy for discovering new robust architectures, and balancing the expressivity, robustness, and tractability of the molecular design space.

6.4 Future work: toward compilers for multiply robust DSD systems

6.4.1 Compiling multiply robust molecular circuits

The expanded design space introduced at the end of Chapter 5 requires a number of operational design parameters such as (i) an output-level target for each gate, (ii) a sink concentration for the regulators, (iii) a sink concentration for any specific off-target input, (iv) a degree of redundancy for selectively detecting each catalytic input, (v) a degree of sequence-level redundancy for suppressing leak between amplifying modules, (vi) an allowable budget of mismatch modifications, (vii) leak-prevention parameters for systematic

mismatch optimization, and (viii) an allowed budget of XNA or expanded alphabet modifications. In theory, from these specifications the resultant circuit could be compiled into a systematic specification, with the network-level degrees of freedom serving as a hyperparameter for this compilation. The resultant domain-level design can then be optimized further with a sequence-level compiler pass. The use of compilers in DSD circuit design has precedent in the literature (Thubagere et al. [2017], Wathieu et al. [2023], Srinivas et al. [2017]), but the scope and function of such compilers may be expanded.

This optimization does not preclude additional gate-level optimization strategies, for example selective inclusion of remote toeholds to add additional unimolecular interactions, from being included as a possible modification during compilation. This framework suggests that focusing computational efforts toward expanded multi-axis compilers for scalable DSD systems could be a fruitful path forward for the field.

6.4.2 *Experimental design as an optimization axis*

In addition to molecular design choices, circuit behavior can also be influenced by experimental operating conditions and measurement design. For example, initial conditions and instrumentation determine the regime in which the target circuit behavior can be observed. Designing experiments to maximize information about circuit behavior under practical constraints represents another potential optimization axis. Additionally, model-based experimental design may be able to accelerate both the construction and optimization of new molecular architectures and the extraction of information from them. By explicitly considering variables such as observed species, parameter identifiability, experimental duration, and material cost, systematic experimental design might improve the efficiency of constructing molecular systems. This could expand the future scope of optimization to explicitly include the number of experimental iterations that are required to construct an optimized circuit or answer a given question about a molecular input.

6.4.3 Conclusion

As summarized in Figure 6.1, this thesis frames robust molecular programming as a multi-axis optimization problem. Network architecture determines which behaviors can be specified and how sequence-level properties scale across the network, sequence-level implementation determines how faithfully those behaviors are realized by physical strand interactions, and chemical alphabet determines the space of interactions available for implementation. These axes are not strictly orthogonal, but separating them provides a practical framework for designing and analyzing robust strand displacement systems.

While this thesis focuses on toehold-mediated strand displacement systems implemented in DNA and non-natural alphabets, the design principles described here arise from more general properties of sequence-programmable polymers. Systems built from DNA, RNA, XNA, or other sequence-defined polymers can be viewed as part of the same conceptual design space defined by network architecture, sequence-level implementation, and chemical alphabet. In this view, strand displacement circuits represent one realization of a broader class of programmable reaction systems in which information is encoded in linear polymer sequences and computation emerges from designed interaction networks.

Winfree has compared the current state of molecular programming to computing before the VLSI revolution: a field rich with clever physical implementations by highly trained specialists, but still lacking sufficiently stable abstractions for routine design by non-specialists. In that analogy, scalable progress requires “Tall Thin Molecular Programmers” whose feet remain grounded in quantum mechanics, thermodynamics, and molecular biophysics, while their long-term vision remains oriented toward high-level applications (Winfree [2023]). Their role is not merely to exploit the depth of their expertise, but to help build the abstraction layers that make robust molecular systems scalable. The work in this thesis is motivated by that goal. Rather than treating every new circuit as a unique expert design problem, I have instead drawn inspiration from prior systematic architectural optimization strategies in the field and attempted to extract simple design principles for constructing

operationally robust architectures from sequence-programmable polymers. These principles do not eliminate the need for molecular expertise, but they help repackage that expertise into composable optimization strategies with clearer guarantees, tradeoffs, and application-facing design rules.

Appendix A

METHODS

A.1 Chapter 3 Methods (Sequence-Level Optimization)

Below we provide the detailed procedures for our experimental characterization and modeling of the $X \rightarrow Y$ and $X \rightarrow X$ cascades.

A.1.1 DNA Strand Design and Synthesis

DNA strands for non-mismatched cascades were designed using Nupack (Fornace et al. [2020]) dna04 model at 25°C, 50 mM Na^+ concentration and 12.5 mM Mg^{2+} to verify a set of optimal design conditions. All thermodynamic quantities reported in this work use the same model and buffer conditions unless otherwise stated. The exact constraints used for each system are detailed in SI Section B.1.1.

All strands for the $X \rightarrow Y$ cascades were produced by phosphoramidite synthesis and ordered from Integrated DNA Technologies with PAGE purification and formulated at 100 μM concentration in 1X TE (10 mM Tris, 1 mM EDTA, pH 8.0) buffer. Strands for the $X \rightarrow X$ cascade were ordered with HPLC purification.

A.1.2 Gate Purification and Stock Preparation

To prepare the gates for each cascade, the respective top and bottom strands were combined with 1.2X excess top strand. Magnesium chloride was then added from a 10X stock in 1X TE (125 mM MgCl_2 , 1 mM Tris, 1 mM EDTA, pH 8.0) to reach a final concentration of 1X = 12.5 mM Mg^{2+} . Each complex was annealed by heating to 90°C over two minutes then cooled at 1°C/min to 20°C in an Eppendorf Mastercycler Nexus thermocycler. Each gate was purified from the excess top strand by running the sample through a 12% polyacrylamide

gel with 6% stacking gel. The band corresponding to the gate was then excised, and eluted from the gel. The final stocks were simultaneously concentrated and buffer exchanged into 1X TEM with 0.01% Tween-20 (1X TEMT). See SI Section B.2.1 for a detailed description of the PAGE purification procedure.

A.1.3 Determining species concentrations

Concentrations of signal strands and complexes were determined via triplicate A260 measurements of each stock collected with a Nanodrop One blanked with 1X TEM. The extinction coefficient for each complex was calculated from the sum of the tabulated extinction coefficients for each dinucleotide in the complex (Kallansrud and Ward [1996]), with base assignments as single-stranded or double-stranded determined from NUPACK’s MFE proxy structure of each complex (Fornace et al. [2020]).

We find that concentration estimation from absorbance spectroscopy provided a reasonable estimate of species concentration. However, to provide additional control for kinetic modeling, we performed a concentration calibration procedure to determine the effective concentration of each gate in each cascade system. The exact procedure differs between the feedforward and cyclic systems (see SI Section B.2.2). However, in general the process involved triggering the cascade in excess over multiple experiments, verifying that each gate of interest is the limiting species in at least one experiment. The resulting fluorescence endpoint values are used with a calibration curve of the triggered reporter, collected concurrently, to determine the effective gate concentration. This process was repeated each time a new preparation or dilution of the gate stocks were made.

For detailed modeling, where the systems are triggered at multiple levels of the cascade, we performed additional internal validation of the relative trigger stock concentrations as described in SI Section B.2.3.

A.1.4 Fluorescence spectroscopy

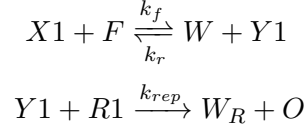
All fluorescence spectroscopy experiments were instrumented with either a BioTek Cytation 5 microplate reader or a BioTek Synergy H1 microplate reader. For each experiment presented in this work the microplate reader was set to read from the bottom of the plate using an excitation wavelength of 563 nm and an emission wavelength of 592 nm. Differences between instruments and day-to-day variation were accounted for by collecting a separate calibration curve for each microplate experiment. The desired components for each experiment were combined at the specified concentrations using an ECHO 525 Acoustic Liquid Handler (Beckman Coulter). Using the instrument, separate high concentration stocks of each of the circuit gates or triggers, suspended in 1X TEMT (1X TE, 12.5 mM Mg^{2+} , 0.01% Tween-20), and solvent (1X TEMT) were transferred and combined from source wells into a clear bottom destination plate (Corning® 384 well microplate, low volume). In every experiment presented, each experimental condition was prepared in triplicate. To facilitate accurate kinetic modeling, the time between when gate and trigger transfers began and the first read on the plate reader was measured and recorded for each experiment.

To average over possible batch effects and better distinguish underlying kinetic invariants in downstream modeling, two separate trials were collected on different days for all $X \rightarrow Y$ experiments used in kinetic modeling.

Kinetic modeling

Explicit kinetic models were fit to each of the feedforward $X \rightarrow Y$ cascades. For each of the three triggering concentrations and two replicated experiments, the point-wise average and standard deviation of triplicate measurements for each experimental condition were calculated to yield 6 curves with corresponding errors.

For each cascade we also fit the following empirical kinetic model:



where X is the input to the cascade, $Y1$ is the input for the reporter (the top strand of the last gate upstream of the reporter), F is a single fuel species representing the three translator complexes in the cascade, W and W_R are waste species, and O is a detectable fluorescent output. This corresponds to ODE dynamics

$$\begin{aligned} \frac{dO}{dt} &= \frac{dW_R}{dt} = -\frac{dR1}{dt} = k_{rep}[Y1][R1] \\ \frac{dW}{dt} &= -\frac{dX1}{dt} = -\frac{dF}{dt} = k_f[X1][F] - k_r[W][Y1] \\ \frac{dY1}{dt} &= k_f[X1][F] - k_r[W][Y1] \\ &\quad - k_{rep}[Y1][R1] \end{aligned}$$

This reduced model is used to extract effective rate constants and is not intended to capture intermediate species explicitly.

Rate constants were fit to the data by minimizing the sum of the error between the simulated mass-action ordinary differential equation (ODE) model of the reaction and the experimental curves. To ensure a single parameter to fit for each experiment, the value of k_{rep} was first fit in isolation. This point-estimate was then treated as a constant when fitting k_f and k_r . To determine this pair of constants, k_f was fit directly to the data then the principle of detailed balance, which enforces consistency between the free energy of the reaction and the forward and reverse rate constants

$$k_{r_i} = \frac{k_{f_i}}{e^{-\Delta\Delta G_{rxn_i}/RT}}$$

was used to compute k_r . Here R is the ideal gas constant, T is the Kelvin temperature, and

$\Delta\Delta G_{rxn}$ was computed as the difference in $\Delta G_{structure}$ between products and reactants for each designed reaction based on Nupack’s dna04 energy model (Fornace et al. [2020]).

The estimated means and associated standard errors were calculated using a leave-one-group-out jackknife procedure (described in detail in Section A.2.3).

A.1.5 Data normalization

To aid data interpretation the raw fluorescence values were normalized. Unless otherwise specified for the $X \rightarrow Y$ cascades the fluorescence values were converted to concentrations using the calibration curve for the triggered reporter collected in the same experiment. In $X \rightarrow X$ cascades, where kinetic models were not fit, and relative completion levels are more important, the data are presented as fractional completion values. These are calculated from the raw fluorescence by subtracting the baseline fluorescence of the reporter and dividing by the difference between the maximum endpoint fluorescence of the highest completion level in the experiment and the baseline fluorescence value.

A.1.6 Statistical analysis

Since the Jackknife procedure for model fitting provided point estimates of parameter values, we compared fit rates across cascades using Wald Z-tests using the Holm correction for multiple hypothesis testing (Holm [1979]). When performing omnibus tests, pooled across an experimental condition (for example comparing rate constants between systems pooled over all gates in the cascades), the estimates were combined using inverse-variance pooling. Precise methods for error propagation, statistical tests, pooling, and statistical assumptions are presented in SI Section A.2.3.

A.2 Chapter 4 Methods (Chemical Alphabet Expansion)

We were interested in evaluating different toehold placements, which led to each lab using different cascade designs and some differences in the methods used. One lab conducted all

experiments on the 3' toehold cascades, while the other conducted them on the 5' toehold cascades. The versions (3' or 5') are specified in the following paragraphs.

A.2.1 Reagents

Where applicable, existing designs from (Mayer et al. [2022]) for the 5' toehold and (Kennedy et al. [2022]) for the 3' toehold cascades were used. The reporter for the 5' version had an incumbent with a 5' Iowa Black[®] RQ and a substrate with a 3' ROX (NHS Ester) fluorophore. The 3' version used a 3' Black Hole Quencher modification on the incumbent strand and a 5' ATTO565 modification on the substrate strand.

All main TMSD-circuit components were ordered either PAGE purified or HPLC purified and lab ready at 100 μ M except for the natural variants for the multi-isoC and long overhang systems, which were ordered unpurified and were later PAGE purified by hand.

For single strand purification, each strand was run on a 12% polyacrylamide gel impregnated with 1X TBE buffer and 8.3 M urea, with a 6% stacking gel at 55 °C. After the run, the upper half of the desired band was excised, eluted into extraction buffer (500mM $\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$, 10mM $\text{Mg}(\text{C}_2\text{H}_3\text{O}_2)_2$, 2mM EDTA, pH 8.0), purified by ethanol precipitation, then re-suspended in 1X Tris-EDTA buffer with 12.5mM MgCl_2 and 0.01% Tween-20 (1X TEMT).

Sequences with random base compositions at certain lengths (N25 and N50) were ordered as dried DNA choosing the randomized machine mixing method provided by the manufacturer. We re-natured the dried DNA in 1 $\times$ TE buffer with 100 mM NaCl, 12.5 mM MgCl_2 and 0.01% Tween-20 for the 5' toehold system experiments and 1 $\times$ TE buffer with 12.5 mM MgCl_2 and 0.01% Tween-20 otherwise. The same buffers were used for all kinetics experiments conducted in the study. The reporters were annealed with 1.2 $\times$ excess top strand and used without further purification.

All upstream complexes were annealed with 1.2 $\times$ excess top strand and purified by native polyacrylamide gel electrophoresis to recover the band corresponding to the duplex.

For the annealing process, the mixed strands were heated up to 95 °C to break up any secondary structure and slowly cooled down to room temperature at a rate of 0.5 °C per minute. Complexes for the 5' toehold cascade were annealed with 1.2× excess top strand and purified using a “ZR small-RNA PAGE Recovery Kit” which can also be used for DNA according to the manufacturer specifications. Native 12% polyacrylamide gel was used and run at room temperature and post stained with SYBR[™] gold nucleic acid gel stain. Extraction and downstream purification of the DNA was performed according to the manufacturer's protocol. For the 3' toehold system, a 10% PAGE gel impregnated with 12.5 mM MgCl₂ with a 5% stacking gel, incubated at 25 °C was used. The bands corresponding to the duplex were excised from the gel, eluted overnight in 1X TEMT, then concentrated using an Amicon® 10 kDa MWCO Ultra Centrifugal Filter.

A.2.2 Experiment conditions

The reporter, consisting of substrate and incumbent strands, was always used at a final concentration of 40 nM, the gates at 30 nM, and the invader at 20 nM. For the 5' version, fluorescence measurements were performed at 29 °C using either a FLUOstar® or CLARIOstar® plate reader by BMG LABTECH and Corning® low volume, non-binding plates. The filters were chosen to be 584 nm for excitation and 640 nm for emission.

For the 3' version, reaction components and buffer were combined using a Labcyte Echo525 acoustic liquid handling robot into a Corning 384-well black clear bottom microplate. All fluorescence experiments for this version were conducted using BioTek Synergy H1 fluorescence or BioTek Cytation 5 plate readers set to 25 °C, with excitation and emission wavelengths optimized for ATTO565.

For all measurements containing random pool strands, we used three different incubation methods for both the natural and non-natural cases: none, invader, or cascade. For invader and cascade incubation, the system equilibrated overnight.

A.2.3 Statistical Analysis

Data normalization. To account for the different conditions and material used in the experiments, analyses were conducted only within subsets of experiments sharing identical conditions. To improve readability, we normalized the intensity data to match the signal for the reporter or cascade triggered with 20 nM invader in the absence of background. The concentrations of the invader strand stocks were determined through triplicate absorbance measurements taken at 260 nm using a Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometer. Since the per-dinucleotide extinction coefficients were not available for the isoC and isoG bases, we approximated the extinction coefficient of these non-natural strands as identical to their natural analogue because the sequence difference contributes only a small percentage to the overall extinction coefficient for the strand.

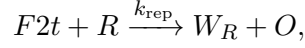
For each measurement, we subtracted the intensity value for the un-triggered reporter (when all fluorophores should be quenched) to set the zero value. We then normalized fluorescence values such that the difference in fluorescence between the reporter/cascade triggered at 20 nM in the absence of background and the baseline un-triggered reporter fluorescence equals 20. This scaling preserves the proportionality of signals but improves interpretability.

For the 5' toehold system, after purification, the concentrations of the gates in the cascade were estimated using their extinction coefficient values and absorbance measurements with an Implen Nanophotometer N50. For the 3' system, each gate's (including the reporter) stock concentration was determined by measuring the fluorescence of the fully triggered gate with the downstream gates in excess. The fluorescence was then converted to a concentration value using a calibration curve of the reporter, kept at the same concentration as that used in the excess triggering experiment.

Data modeling. All models were fit to data after normalization to the 20 nM control triggering. Two related mass-action ODE models were used across this study.

The first model represents a single irreversible reporter reaction, where $F2t$ is the invader

for the reporter, F is the fuel strand, W_R is waste, and O is the output:

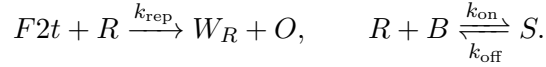


with dynamics

$$\frac{d[F2t]}{dt} = \frac{d[R]}{dt} = -\frac{d[W_R]}{dt} = -\frac{d[O]}{dt} = -k_{\text{rep}} [F2t] [R].$$

This model was used for all conditions except the single-step occlusion experiment.

For the results in Figure 4.6 we extended the reporter model to include a reversible competing reaction between the invader and a representative background strand B that forms a spurious complex S :



where B represents the ensemble of background species and S the respective spurious intermediate. To restrict the model to a single free parameter, k_{on} was fixed at $1e7 \text{ M}^{-1}\text{s}^{-1}$, which is typical for short DNA duplexes (Wyer et al. [2014]), and the value of k_{rep} was fixed at the estimated parameter value in the absence of background.

For each experimental condition, a set of three curves was generated by leaving out one entire replicate curve and averaging the remaining two at each time-point (replicates shared identical sampling time-points). Three fitted values of k (either k_{rep} or k_{off}) were obtained by fitting the appropriate ODE model using the implementation of the Levenberg–Marquardt algorithm for nonlinear least squares provided by the SciPy Python package (Virtanen et al. [2020]) and the experiment’s concentrations as initial conditions. Our estimator of k was defined by the average of these three leave-one-out estimates, and its standard error was computed using the jackknife formula:

$$SE = \frac{n-1}{\sqrt{n}} s$$

where n is the number of jackknife samples (3 in our case) and s is the standard deviation of the three leave-one-out fitted k values.

Significance testing. Two distinct statistical frameworks were used, corresponding to two different types of quantities. We summarize the overall procedure here and present the detailed procedure in SI Section C.2. (i) For kinetic analyses, where hypotheses involve functions of fitted rate constants (e.g., k_{nn}/k_{nat}), the estimators arise from nonlinear model fits with jackknife standard errors and were evaluated using Wald Z -tests. (ii) For endpoint fractional completion values at 6 hours (Figure 4.5), which were analyzed as direct sample means with empirical standard deviations, we used t -tests.

For both types of statistical analysis, standard deviations and standard errors were propagated from the point-wise standard deviations across triplicate measurements, using first-order error propagation, to errors on normalized data-points and averages across data-points. Uncertainty was pooled across conditions for omnibus hypothesis testing as described in the SI.

A.3 Chapter 5 Methods (Network-Level Modeling)

A.3.1 Mass-action modeling

All reactions were modeled under standard mass-action kinetics. Each reaction was parameterized by forward and reverse rate constants, k_f and k_r , which are related to the reaction equilibrium constant as follows:

$$K = \frac{k_f}{k_r} = e^{-\Delta G/RT}$$

Discrimination between target (X) and off-target (X_v) inputs corresponds to a free energy difference:

$$\Delta\Delta G_v := \Delta G_v - \Delta G$$

This defines the equilibrium error fraction:

$$\frac{K_v}{K} = e^{-\Delta\Delta G_v/RT}$$

I then related rate constants between discriminatory reactions multiplicatively

$$e_{vk_f} = \frac{vk_f}{k_f}, \quad e_{vk_r} = \frac{vk_r}{k_r}$$

such that

$$\frac{K_v}{K} = \frac{e_{vk_f}}{e_{vk_r}} = e^{-\Delta\Delta G_v/RT}$$

as a representative convention.

The experimental discrimination factor is quantified as the ratio of product formation under on-target versus off-target conditions:

$$\text{DF}(t) = \frac{O(X;t)}{O(X_v;t)}$$

Maximum kinetic discrimination is defined as the maximum value of $\text{DF}(t)$ over the simulation timecourse.

A.3.2 *Experimental conditions*

Unless otherwise specified, experiments were conducted with the following nominal experimental conditions (as determined by absorption spectroscopy): 10 nM gate, reporter, and sink complexes; 13 nM fuel complexes; 5 nM catalytic input; and 30 nM upstream regulator. All experimental conditions were conducted in triplicate measurements. At most one replicate was removed per condition when the observed kinetics indicated an obvious experimental artifact, including evaporation or incorrect/incomplete liquid transfer. Effective gate concentrations were also determined as detailed previously in this appendix.

A.3.3 Data analysis

Raw fluorescence time-course data were processed using a custom analysis pipeline implemented in Python. For each experimental condition, replicate wells were grouped and analyzed together.

Fluorescence signals were converted to effective concentrations using a linear calibration obtained from known input concentrations.

For visualization of each condition, replicate time-courses were averaged to obtain a mean trajectory, and the standard error of the mean (SEM) was computed across replicates. Error in $DF(t)$ was estimated using standard propagation of uncertainty from the replicate-level SEM values.

Endpoint discrimination was computed by averaging the final portion of each replicate time-course and forming the ratio of these endpoint values. Rather than first averaging the curves, endpoint discrimination was computed individually on each replicate to obtain a reliable approximation of the quantity and associated error for use in any downstream statistical analysis.

A.3.4 Fluorescence spectroscopy

All time-course fluorescence experiments were conducted on a BioTek Synergy H1 plate reader with excitation and emission settings optimized for the ATTO565 fluorophore. Experiments were performed at an incubation temperature of 25°C without active cooling.

Appendix B

CHAPTER 3 SUPPLEMENTARY INFORMATION

B.1 Design

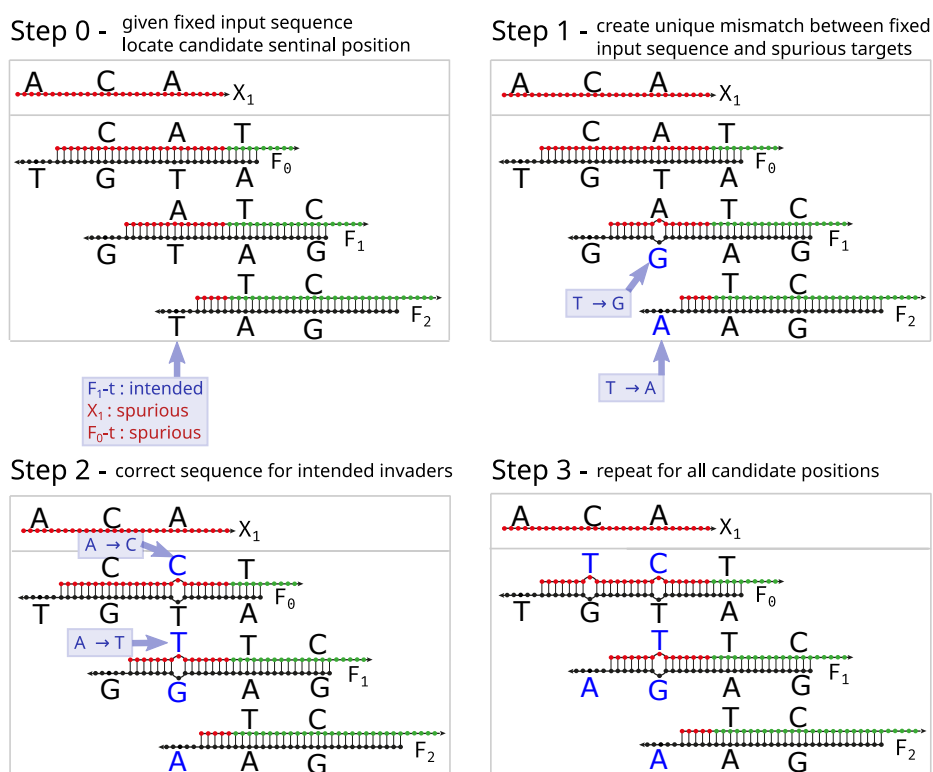


Figure B.1: A simple strategy for introducing kinetic ratchets within a column sequence-level redundancy in a “leakless” design.

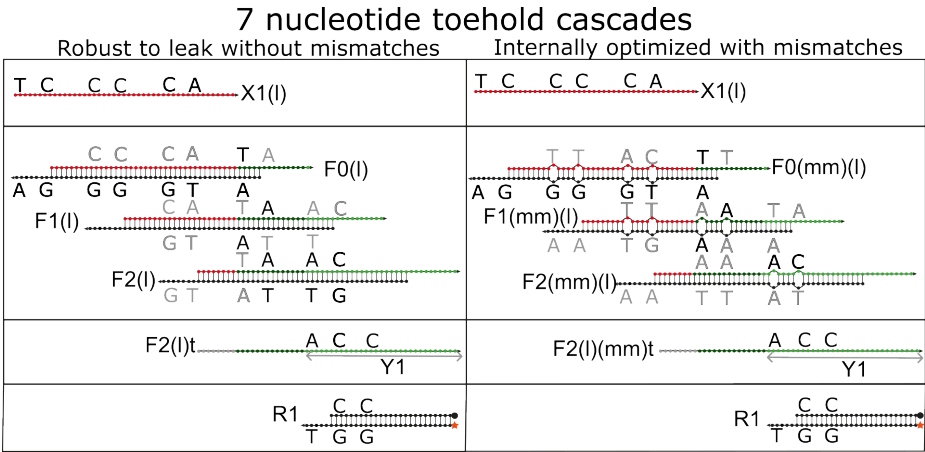


Figure B.2: Final domain-level and sequence-level designs for the seven nucleotide toehold non-mismatched and mismatch cascades. Both cascades consumed the same input and were designed to produce outputs compatible with the same reporter as the 5ntTH system. Bubbles in the illustrated duplexes represent the position of internal mismatches. Except for the marked positions, all positions within a vertical column share the same domain sequence. Sequences that change during optimization are marked in gray. Full sequences for each strand are presented in Table B.12.

X

F0

F1

F2

F3

F4

A

B

G

C

T

B*

Figure B.3: (left) At high levels of redundancy, there are not enough bases in the standard nucleotide alphabet to ensure some guarantees apply simultaneously for all spaces at a column of redundancy. For example, with five overlapping complexes in an input, it is impossible to make sure that, at the position, all desired interactions are complementary, but all spurious interactions are not. (right) In contrast, it is still possible to maintain system level guarantee even when redundancy is high. Here we illustrate that it is possible to keep all desired interaction complementary globally and have desired reaction resolve a mismatch within 10 bases of initiating branch migration, but ensure all spurious interactions introduce a toehold mismatch, and that spurious invaders do not resolve any mismatches within 12 bases of branch migration. As the kinetic effect of mismatch introduction and elimination are highly sequence dependent (Irmisch et al. [2020]), this kind of positional bounded global guarantee may be sufficient for desired kinetic behavior. In this panel we show a minimal example of a global guarantee, but note that it would still be possible to add additional mismatches at higher positions in columns to extend the range in which spurious invaders cannot resolve a mismatch.

The diagram illustrates the formation of a DNA hairpin loop over four generations (X, F0, F1, F2, F3, F4). The DNA sequence is T C G G G. In generation X, the sequence is shown as a single strand. In generation F0, the sequence is shown as a double strand with a hairpin loop. In generation F1, the sequence is shown as a double strand with a hairpin loop. In generation F2, the sequence is shown as a double strand with a hairpin loop. In generation F3, the sequence is shown as a double strand with a hairpin loop. In generation F4, the sequence is shown as a double strand with a hairpin loop.

Figure B.3: (left) At high levels of redundancy, there are not enough bases in the standard nucleotide alphabet to ensure some guarantees apply simultaneously for all spaces at a column of redundancy. For example, with five overlapping complexes in an input, it is impossible to make sure that, at the position, all desired interactions are complementary, but all spurious interactions are not. (right) In contrast, it is still possible to maintain system level guarantee even when redundancy is high. Here we illustrate that it is possible to keep all desired interaction complementary globally and have desired reaction resolve a mismatch within 10 bases of initiating branch migration, but ensure all spurious interactions introduce a toehold mismatch, and that spurious invaders do not resolve any mismatches within 12 bases of branch migration. As the kinetic effect of mismatch introduction and elimination are highly sequence dependent (Irmisch et al. [2020]), this kind of positional bounded global guarantee may be sufficient for desired kinetic behavior. In this panel we show a minimal example of a global guarantee, but note that it would still be possible to add additional mismatches at higher positions in columns to extend the range in which spurious invaders cannot resolve a mismatch.

B.1.1 Design constraints

Using the NUPACK dna04 model (Fornace et al. [2020]), we firstly verified that all signal strands had an unstructured minimum free energy (MFE) proxy structure at 1 μ M and a MFE greater than -1 kcal/mol. We also checked that all signal strands possessed less than 1% binding to themselves or any other signal strand under the same concentration conditions. Unstructured signal strands were also promoted by designing over an ‘ATC’ three-letter alphabet. Sequences were designed such that each toehold exchange reaction had a $\Delta\Delta G$ of less than or equal to 0 kcal/mol. To promote high fidelity synthesis and prevent formation of G-quadruplex structures in strands, we prevented runs of longer than three of the same nucleotide in a row. To avoid spurious quenching of the fluorophore on the reporter, we restricted guanine bases from within three nucleotides of the fluorophore modification in R1.

When introducing systematic mismatches into each of the non-mismatched cascades, we verified the same constraints under the same Nupack model as well as an additional set of optimizing conditions. For a given choice of mismatch sites in our design, we evaluated a subset of all sequence modifications that maintained complementary between each desired invader and incumbent strand pairing as well as preserving the input and output sequences, choosing those that also maintained a substantially product-favored $\Delta\Delta G$ of strand exchange for desired interactions and minimal predicted occupancy of spurious invading strands on fuel duplexes at 100 nM concentration.

B.1.2 Design metrics

$X \rightarrow Y$ cascades

Exploring the full mismatch design space, we evaluated all 685,307 possible mismatch designs for ($\alpha = \beta = 5$). Each design was evaluated for the $\Delta\Delta G$ of each designed reaction (Figure B.4(A)), as well as the $\Delta\Delta G$ values for spurious invasions were also calculated. We present a Pareto frontier, optimizing the minimum barrier to spurious invasion while minimizing the net $\Delta\Delta G$ for the translator in (Figure B.4(B)).

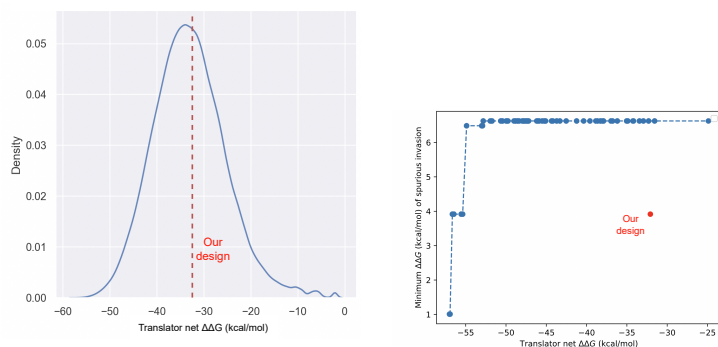


Figure B.4: **a.** Density distribution of all mismatch designs starting with our original sequences and adding all possible mismatching schemes that obey $\alpha = \beta = 5$ and disfavor spurious invasion. **b.** Pareto frontier of full design space, maximizing the minimum barrier to spurious invasion and minimizing the translator net $\Delta\Delta G$.

$X \rightarrow X$ cascades

The domain design for the X to X amplifier was modeled after the X to Y translator, with mismatches placed in the same sequence columns. Instead of using a separate reporter complex, the F2 complex was used as a reporter. Under these constraints we found a sequence space of 216 possible designs that satisfy our complementarity constraints across these modification sites.

To choose a candidate design, we examined the difference between the positive and negative design space. We defined the positive design space as the maximum $\Delta\Delta G$ of the 3 forward strand exchange reactions (X into F0, F0-top into F1, F1-top into F2). This value was chosen to favor forward speed of the reaction, assuming that having a reaction with a larger $\Delta\Delta G$ would help to kinetically ratchet the system forward, improving catalytic turnover. We specified that the negative design space was the minimum enthalpic barrier to a spurious strand invasion (the entropy term for bringing two strands together was excluded in the calculation of this value). The negative design term was also chosen to increase the overall catalytic turnover speed, with the goal of decreasing the dwell time of strands on their unintended target complex.

We additionally constrained the design space by requiring that the mismatch proximal to the toehold raises the complex energy by more than the distal mismatch in each complex, reducing the optimization landscape 42 possible designs (Figure B.5a, b). This is based on prior work showing that the effect of a mismatch on kinetics non-linearly increases with proximity to the start of branch migration (Haley et al. [2020]). We visualized the distribution of this contained design space using the positive design value subtracted from the negative design value as metrics (Figure B.5(A,B)).

Finally the space was further constrained to eight candidates, using the criteria that the minimum barrier to spurious strand invasion in the system must exceed 2 kcal/mol (Figure B.5(C)). Of these eight designs, we selected the candidate that minimized the maximum $\Delta\Delta G_{rxn}$ across the three forward TMSE reactions in the cascade. The $\Delta\Delta G_{rxn}$ values

for the sequence of TMSE steps in the final translator design were -7.55 kcal/mol, -10.31 kcal/mol, and -7.12 kcal/mol (calculated using the Nupack Python package v4.0 (Fornace et al. [2020])). The reaction coordinate diagrams show the desired (blue) and spurious invasions (red and green) for each complex in the mismatch amplifier (Figure B.5(D,E,F)).

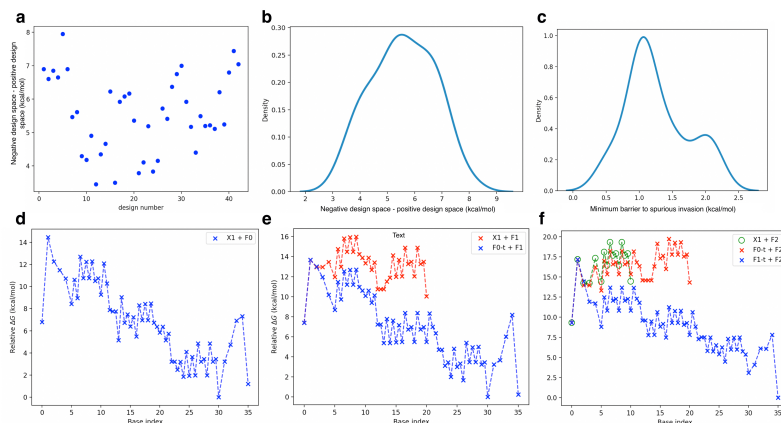


Figure B.5: **a.** Design space for X to X mismatch amplifier where the first mismatch is higher energy than the second mismatch in each complex (42 total designs). The X axis defines the number assigned to each design, the Y axis plots the difference between the the maximum $\Delta\Delta G$ of the 3 forward strand exchange reactions and the minimum enthalpic barrier to a spurious strand invasion. **b.** Design density plot of the difference between the the maximum $\Delta\Delta G$ of the 3 forward strand exchange reactions and the minimum enthalpic barrier to a spurious strand invasion for the 42 designs. **c.** Design density plot of the difference between the the maximum $\Delta\Delta G$ of the 3 forward strand exchange reactions and the minimum enthalpic barrier to a spurious strand invasion for the final 8 designs where the minimum enthalpic barrier to a spurious strand invasion is greater than 2 kcal/mol. **d,e,f.** Intended reaction energy pathways in the X to X mismatch amplifying translator (blue) versus spurious strand invader energy pathways (red and green).

B.2 Supplementary methods

B.2.1 PAGE purification and postprocessing

After annealing, Ficoll loading dye (25% Ficoll 400, 0.25% Bromophenol blue in milliQ water) was added to each sample at a 1:5 dye to sample ratio. The annealed complexes with excess top strand were loaded on a 12% polyacrylamide gel with 6% polyacrylamide stacking gel and separated into duplex and residual top strand by non-denaturing gel electrophoresis in sodium borate buffer (10 mM sodium boric acid) at 25°C maintained by a Thermo Scientific ARCTIC A10 Refrigerated Circulator in a Hoefer SE600X Chroma Deluxe Electrophoresis unit. Acrylamide for the PAGE purification as supplied from a 40% stock of 19:1 Acrylamide/Bis-acrylamide (Research Products International). After separation, the topmost band from each lane was excised from the gel, cut into 1 mm wide strips, and eluted into 1 mL TE buffer with 12.5mM magnesium (TEM) at room temperature for 12 hours

Following elutions, stock samples were buffer exchanged into pure 1X TEM and concentrated using Amicon 10k MWCO spin concentrators. The resultant volume was calculated by mass. The concentration was estimated from A260 measurements of each sample collected with a Nanodrop One blanked with 1X TEM. The extinction coefficient for each complex was calculated from the sum of the tabulated extinction coefficients for each dinucleotide in the complex (Kallansrud and Ward [1996]), with the single-stranded or double-strand dinucleotide value determined from the MFE proxy structure of each complex. High concentration stocks of each complex were finalized by adding Tween-20 (Sigma-Aldrich) to a final concentration of 0.01% from a stock of 0.1% Tween 20 in 1X TEM.

B.2.2 Determining gate concentrations

$X \rightarrow Y$ cascades

While the concentrations of each gate stock were estimated by absorbance spectroscopy, more accurate concentrations for kinetic modeling were determined by fluorescence spectroscopy with external calibration. First the fluorescence resulting from reporter R1 was calibrated to the concentration of its trigger through a linear fit of the averages of triplicated measurements of the fluorescence from the same amount of reporter without trigger and at three equally spaced trigger concentrations below the expected concentration of reporter. In the same experiment on the same plate, we collected triplicate measurements of fluorescence resulting from triggering the reporter in at least 3X the expected concentration. The calibration established from the lower concentration triggering was used to convert this raw fluorescence value to an effective reporter concentration. All calibration measurements took place at the same volume— either 4 μL or 10 μL depending on the experiment.

An analogous process was repeated for each gate upstream in the cascade. Once again, in each experiment, a new calibration curve was collected for the reporter to account from any day-to-day variation across the plate reader and acoustic liquid handler. To determine the effective concentration of the gate upstream of the reporter, the fluorescence from triggering the immediately upstream gate (with no other present) in the presence of excess reporter was measured and converted to an effective concentration using the calibration curve established in the same experiment. The same process was repeated from upstream gates, each time ensuring that all downstream gates were added in excess of that being measured.

$X \rightarrow X$ cascades

Effective concentrations in the $X \rightarrow X$ cascades were determined analogously to the $X \rightarrow Y$ cascades, but using F2 as the reporter species. The concentration of F2 was first validated by saturation titration with its upstream trigger, and a calibration curve relating fluorescence to triggered F2 concentration was established. Effective concentrations of upstream

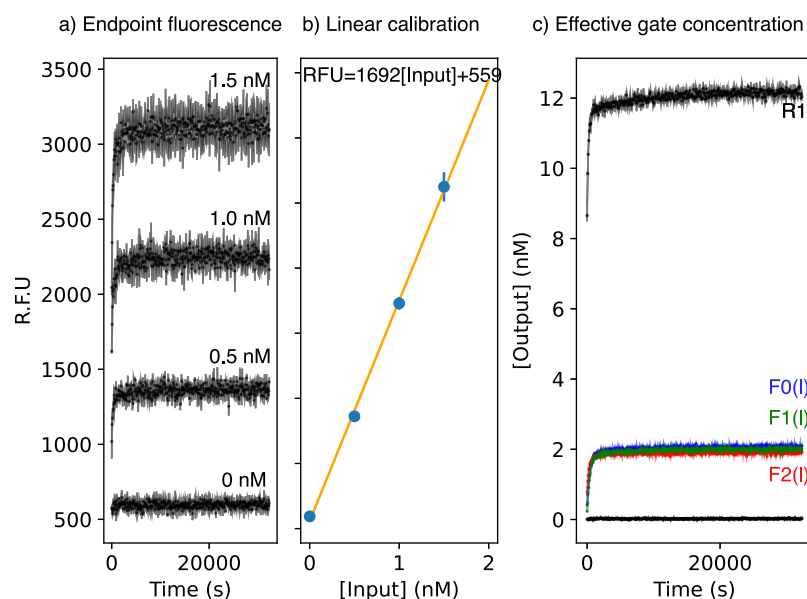


Figure B.6: The process for determining the effective concentration of each translator complex and reporter in the $X \rightarrow Y$ cascades. (a) The endpoint fluorescence value was determined for four different trigger concentrations. (b) The endpoint raw fluorescence values were linearly fit to the respective input concentration. (c) Under the same reaction conditions, reporter concentration, and in the same microplate each gate was triggered in excess (corresponding curves are labeled in the same color), with downstream gates in excess to ensure the gate of interest was the limiting reagent. The established calibration was used to convert the raw fluorescence values to an output concentration, and the endpoint values after 12 hours were taken as the effective gate concentrations.

Table B.1: Effective gate concentrations in $X \rightarrow Y$ cascades

Cascade	Gate	[Effective] (nM) trial1	[Effective] (nM) trial2
5ntTH noMM	R1	12	12
	F2	2.0	2.1
	F1	2.0	1.8
	F0	1.9	1.9
5ntTH MM	R1	12	12
	F2(mm)	2.0	2.0
	F1(mm)	2.0	1.8
	F0(mm)	1.9	1.8
7ntTH noMM	R1	12	13
	F2(l)	2.0	2.1
	F1(l)	2.1	2.0
	F0(l)	2.0	2.0
7ntTH MM	R1	12	12
	F2(l)(mm)	1.9	2.2
	F1(l)(mm)	2.1	2.2
	F0(l)(mm)	2.0	2.3

complexes were determined by triggering the cascade with downstream complexes in excess and converting endpoint fluorescence to concentration using the F2 calibration curve. Following re-purification of the mismatch amplifier, the calibration procedure was repeated. For the no-mismatch amplifier, reporter triggering was incomplete due to the reversibility of the strand exchange reaction, yielding an apparent conversion factor of approximately 0.56 between F1-top input and F2-top output, consistent with the weak thermodynamic driving force predicted for the system. To determine the effective concentration in the non-natural system, a full-length trigger that increases the thermodynamics driving force of the cascade was used for calibration.

B.2.3 Trigger concentration validation

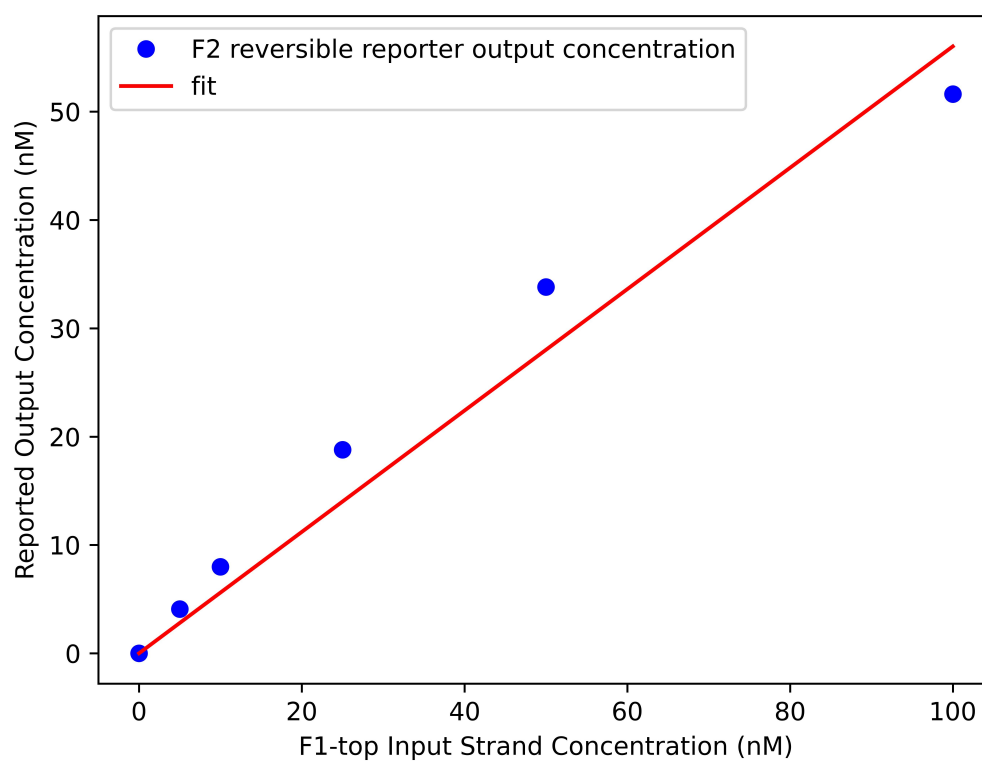


Figure B.7: F1(no-mm)-top + F2(no-mm) reversible reporter shows effective F2-top(no-mm) output concentration at which given F1-top(no-mm) input concentration trigger at. The slope fit to this line is 0.56 nM F1-t/ nM F2-t, or an approximately 50% triggering rate.

Table B.2: Trigger concentrations in $X \rightarrow Y$ detailed modeling

Cascade	Trigger	[Trigger]1 (nM)	[Trigger]2 (nM)	[Trigger]3 (nM)
5ntTH noMM	F1-t trial1	0.52	1.05	1.57
	F1-t trial2	0.52	1.05	1.57
	F0-t trial1	0.57	0.95	1.52
	F0-t trial2	0.57	0.95	1.52
	X1 trial1	0.52	1.03	1.55
	X1 trial2	0.52	1.03	1.55
5ntTH MM	F1(mm)-t trial1	0.64	1.06	1.49
	F1(mm)-t trial2	0.64	1.06	1.49
	F0(mm)-t	0.5	1.0	1.5
	X1 trial1	0.52	1.03	1.55
	X1 trial2	0.52	1.03	1.55
7ntTH noMM	F1(l)-t trial1	0.50	1.00	1.50
	F1(l)-t trial2	0.44	1.10	1.54
	F0(l)-t trial1	0.56	1.11	1.39
	X1(l) trial1	0.5	1.0	1.5
	X1(l) trial2	0.5	1.0	1.5
7ntTH MM	F1(l)(mm)-t trial1	0.5	1.0	1.5
	F1(l)(mm)-t trial2	0.5	1.0	1.5
	F0(l)(mm)-t trial1	0.5	1.0	1.5
	F0(l)(mm)-t trial2	0.5	1.0	1.5
	X1(l) trial1	0.5	1.0	1.5
	X1(l) trial2	0.5	1.0	1.5

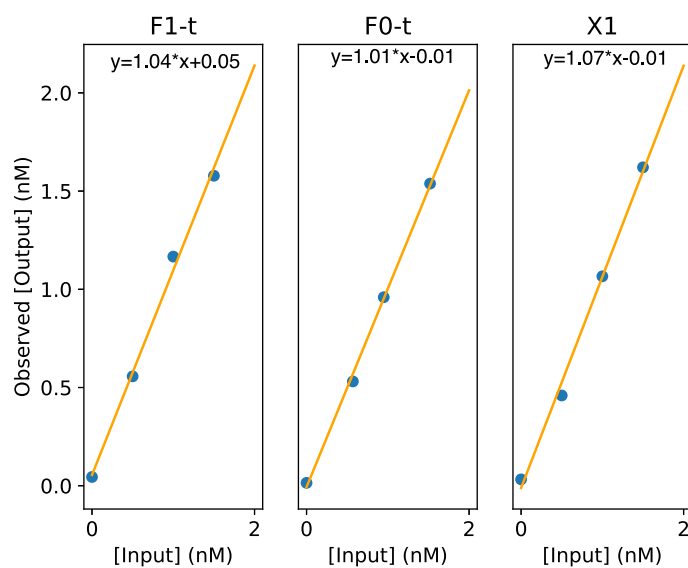
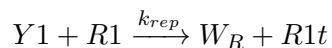


Figure B.8: The process of verifying stock trigger concentrations for upstream gates. We ensured that that the slope of the linear fit of the intended concentration versus observed endpoint concentration for triggering cascades with each trigger had a slope within 10% of 1.0.

B.2.4 Detailed kinetic modeling

Each of the four cascades were designed to share a common reporter (R1) which irreversibly consumes the output of the cascade. In order to measure the forward and reverse rate of each toehold exchange in each cascade, we first determined the rate constant for the reaction of the cascades output with R1. This reaction was modeled as the following a single irreversible molecular reaction



where R1 is the reporter, Y1 is the output of the cascade, WR is the resultant waste duplex, and R1t is the incumbent top strand displaced from R1. k_{rep} was determined by using least squares fitting of the ordinary differential equation associated with the above mass-action kinetic model. The model was fit to 16 separate kinetic curves, each the average of three triplicate measurements, across two effective R1 concentrations and 8 separate trigger concentrations for each R1 concentration. The effective concentration of each reporter was determined from the endpoint calibration curve for each effective R1 concentration and the fluorescence measured when the trigger was added in excess of nominal R1 concentration. We determined an effective forward rate constant of $3.4 * 10^5 M^{-1} s^{-1}$. This same synthesis and anneal of R1 was used in all subsequent kinetic experiments.

For each gate in the cascade, models were fit across data from two separate trials (collected on different days) of three curves computed from averages of triplicate replicates for triggering the gate of interest in the cascade with upstream gates omitted. Rate constants were determined by minimizing the sum of the error between the simulated mass-action ordinary differential equation (ODE) model of the reaction and the experimental curves. Specifically `scipy.optimize.least_squares` was used with a linear loss function to minimize the

following sum via the Levenberg-Marquardt algorithm:

$$Error = \sum_{i, curves} (f(t)_{pred} - f(t)_{obs})^2$$

were $f(t)_{pred}$ is ODE simulated data at time t and $f(t)_{obs}$ is the experimental trace at time t .

For each rate constant, the ODE model of a subset of the following chemical reaction network was used to compute $f(t)_{obs}$, with only a single unknown parameter fit at a time.



For example, to begin we optimized the ODE associated with the reporting reaction (0):

$$\frac{d[WR]}{dt} = \frac{d[R1t]}{dt} = -\frac{d[Y1]}{dt} = -\frac{d[R1]}{dt} = k_{rep}[Y1][R1]$$

$f(t)_{obs} = [R1t](t)$ was computed by solving the system of ODEs using `scipy.integrate.odeint`, and k_{rep} was varied to minimize the squared error between the simulated and observed curves as described above. To determine k_{f2}, k_{r2} we could then use the best fit value of k_{rep} as constant in model associated with reactions (0) and (1). By additionally using the principal of detailed balance, we linked k_{f2} and k_{r2} into a single parameter for least-squares fitting according to eq(0),

$$k_{r_i} = \frac{k_{f_i}}{e^{-\Delta\Delta G_{rxn_i}/RT}}$$

where R is the ideal gas constant, T is the Kelvin temperature, and $\Delta\Delta G_{rxn}$ was computed

as the difference in $\Delta G_{structure}$ between products and reactants for each designed reaction based of Nupack’s dna04 energy model (Fornace et al. [2020]). We continued as such to determine the rate constants for the remainder of the cascade, using the fit constants for downstream reaction and detailed balance to reduce each rate constant to a single parameter optimization.

For each cascade we also fit the following simplified model, by the same process as the detailed model, to the data for the the cascade triggered with the X input:



where F is a single fuel species representing the three translator complexes in the cascade, W and W_R are waste species, and O is a detectable fluorescent output. In modeling we used the average effective concentration of each complex in the translator as the concentration of the fuel species.

The uncertainty on each experimental rate constant within each trial was estimated within trials using the Leave One Group Out cross-validation method as performed in (Zhang and Winfree [2009]), and previously described in the methods appendix.

The effective concentrations of each gate used in the chemical reaction network model were determined as described in Section B.2.2. The intended trigger concentrations programmed for the acoustic liquid handler were used as the effective trigger concentrations. These trigger concentrations are provided in Table B.2. The accuracy of these concentrations were verified through a linear fit of the normalized concentration versus intended concentration for each trigger as described in Section B.2.3.

B.3 X to Y Experiments

B.3.1 Leak results

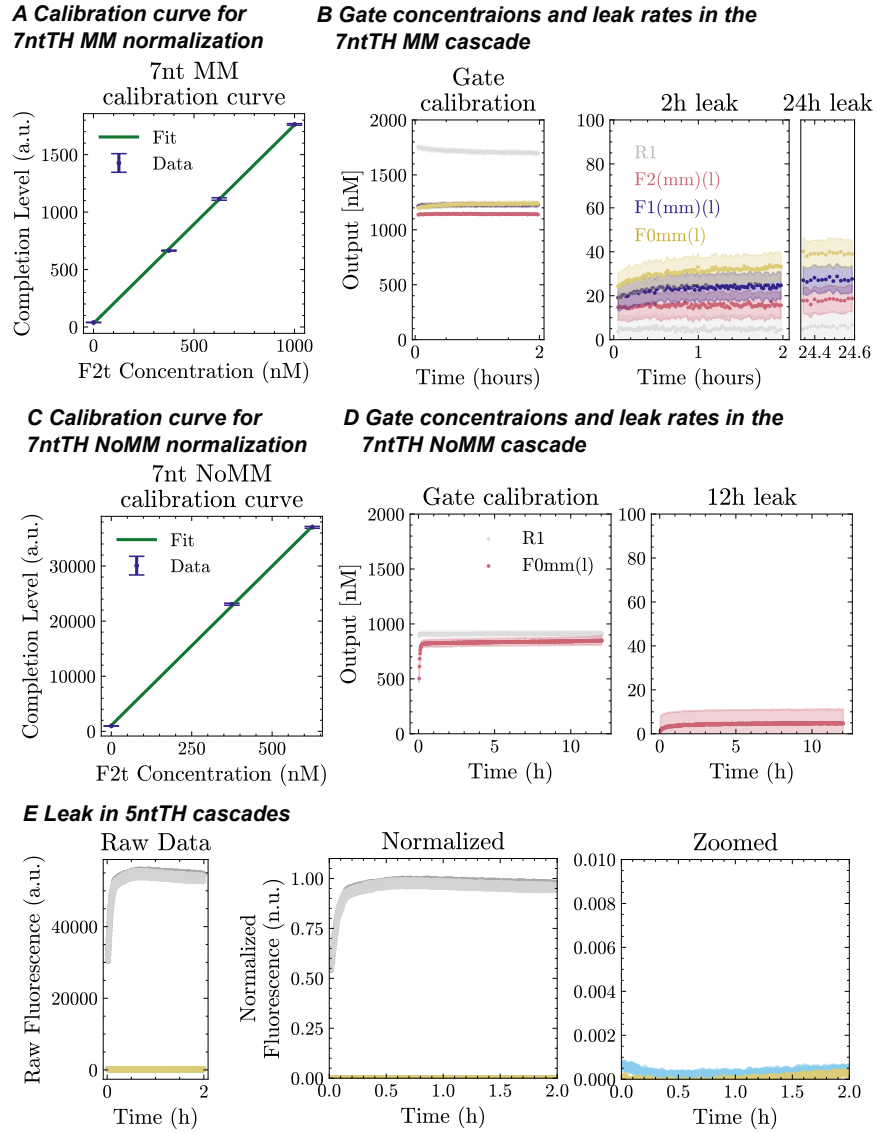


Figure B.9: A summary of the observed experimental leak across the systems tested in this chapter.

B.3.2 Kinetic experiments

Reporter characterization

Table B.3: Trigger concentrations for each reporter concentration used in fitting a second order rate constant for R1

[R1] (nM)	[F2-t] (nM)
4.00	0.00
	0.16
	0.31
	0.47
	0.62
	0.78
	0.94
	1.87
	3.75
1.45	0.00
	0.31
	1.25
	2.19
	2.81
	3.44
	4.06
	8.75
	18.12

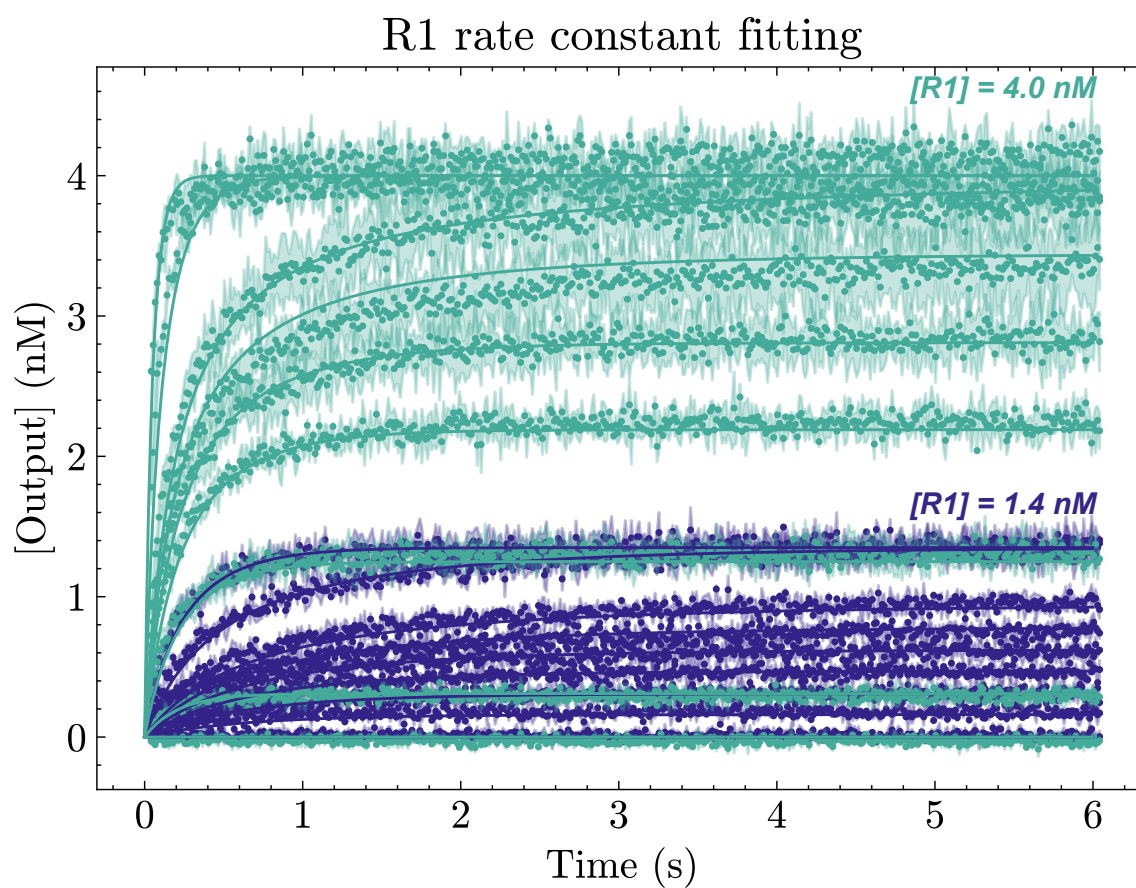


Figure B.10: Detailed fitting of the reporter rate constant. The rate constant was fit simultaneously to triggering the reporter at two gate concentrations, each with 9 respective trigger concentrations. Each curve corresponds to the average of three triplicate measurements of the same condition.

Simplified kinetic modeling of full cascades

The results of the simplified modeling analysis are presented in Figure 3.3(A). This section tabulates the values of the results of model fits and statistical comparisons between them.

Table B.4: Results of simplified rate constant fitting for for each combination of toehold length and mismatch variant. Rate constants are reporter in units of $\text{M}^{-1}\text{s}^{-1}$ with associated standard errors determined by the jackknife methods as described in Methods.

toehold length	optimization	kf	SE kf	kr	SE kr
5ntTH	NoMM	1.80e+05	2.34e+04	5.22e+03	6.78e+02
5ntTH	MM	6.66e+05	1.38e+05	1.35e-14	2.80e-15
7ntTH	NoMM	5.36e+05	3.25e+04	2.15e-02	1.30e-03
7ntTH	MM	1.26e+06	1.17e+05	7.27e-26	6.75e-27

Table B.5: Results of Holm-corrected Wald Z-tests for rate constant comparisons for the simplified model between combination of toehold length and mismatch variant.

rate type	group1	k1	se1	group2	k2	se2	z value	p value	holm-adjusted p value
kf	NoMM5nt	1.80e+05	2.34e+04	MM5nt	6.66e+05	1.38e+05	-3.47	0.000523	0.00157
kf	NoMM5nt	1.80e+05	2.34e+04	NoMM7nt	5.36e+05	3.25e+04	-8.90	0	0
kf	NoMM5nt	1.80e+05	2.34e+04	MM7nt	1.26e+06	1.17e+05	-9.05	0	0
kf	MM5nt	6.66e+05	1.38e+05	NoMM7nt	5.36e+05	3.25e+04	0.91	0.36	0.36
kf	MM5nt	6.66e+05	1.38e+05	MM7nt	1.26e+06	1.17e+05	-3.27	0.00108	0.00217
kf	NoMM7nt	5.36e+05	3.25e+04	MM7nt	1.26e+06	1.17e+05	-5.95	2.64e-09	1.06e-08
kr	NoMM5nt	5.22e+03	6.78e+02	MM5nt	1.35e-14	2.80e-15	7.71	1.27e-14	5.06e-14
kr	NoMM5nt	5.22e+03	6.78e+02	NoMM7nt	2.15e-02	1.30e-03	7.71	1.27e-14	5.06e-14
kr	NoMM5nt	5.22e+03	6.78e+02	MM7nt	7.27e-26	6.75e-27	7.71	1.27e-14	5.06e-14
kr	MM5nt	1.35e-14	2.80e-15	NoMM7nt	2.15e-02	1.30e-03	-16.53	0	0
kr	MM5nt	1.35e-14	2.80e-15	MM7nt	7.27e-26	6.75e-27	4.82	1.4e-06	1.4e-06
kr	NoMM7nt	2.15e-02	1.30e-03	MM7nt	7.27e-26	6.75e-27	16.53	0	0

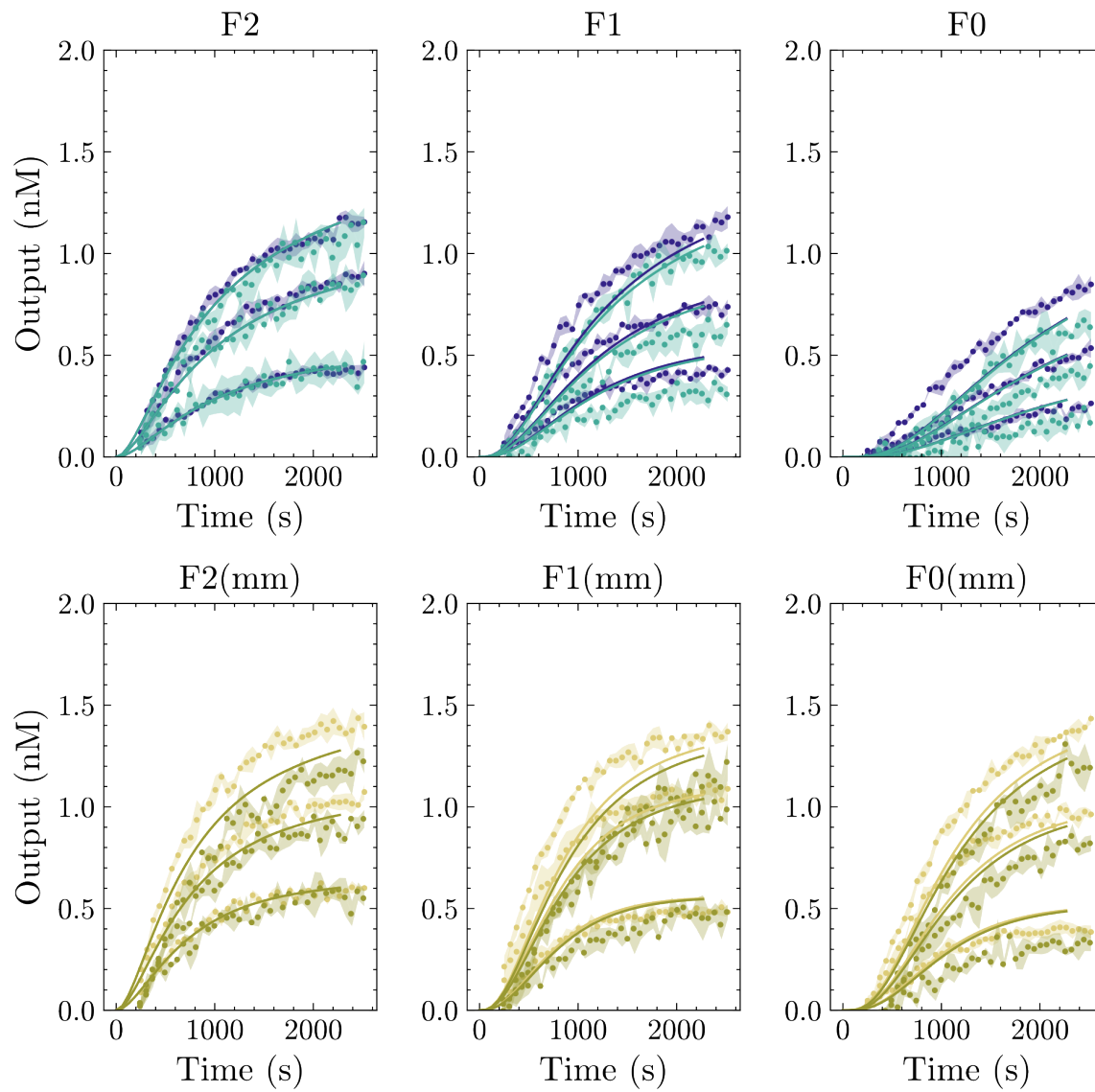
Detailed kinetic modeling of full cascades

Figure B.11: Detailed kinetic modeling data for both of the five nucleotide toehold cascades

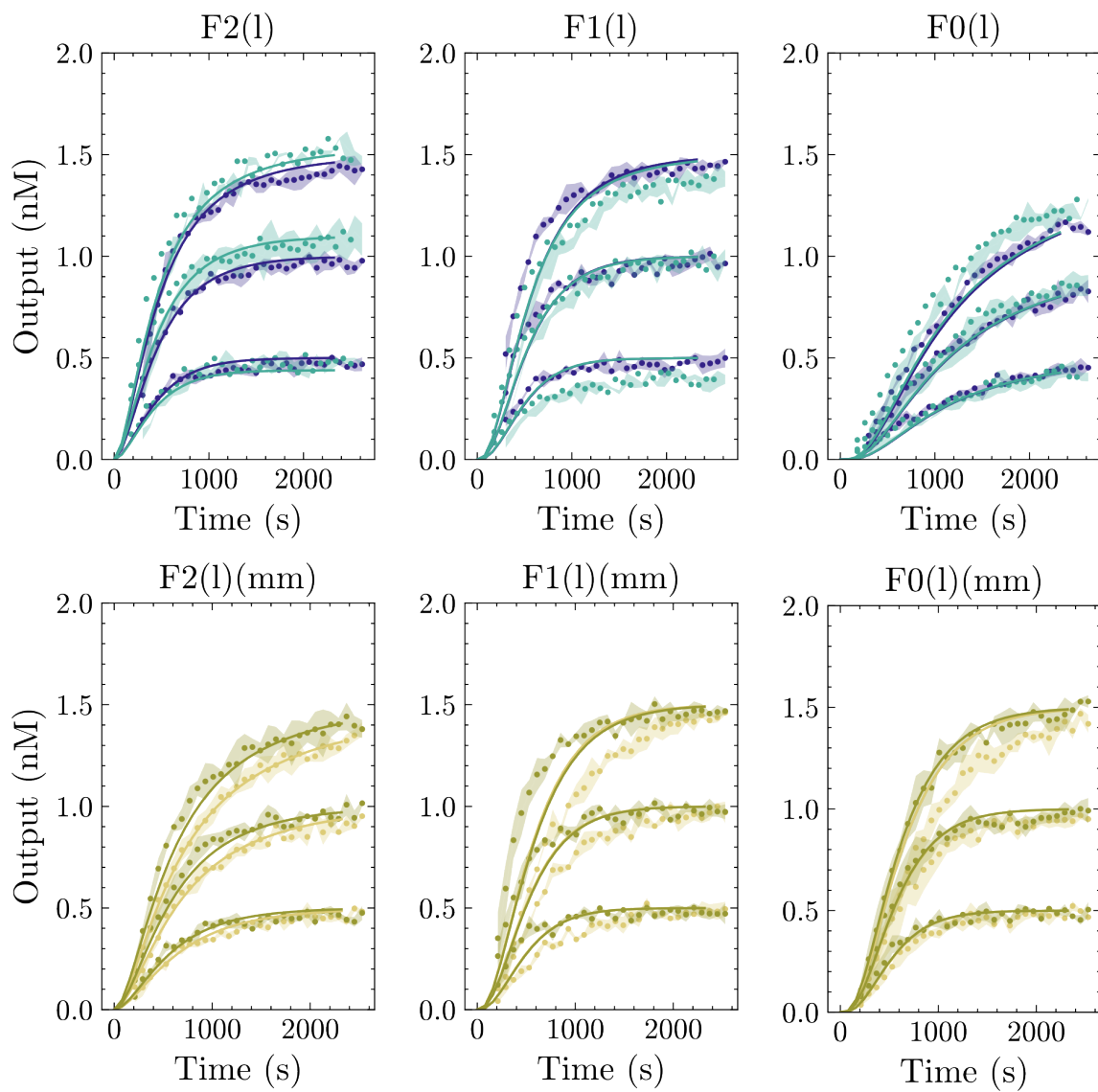


Figure B.12: Detailed kinetic modeling data for both of the seven nucleotide toehold cascades

Table B.6: Results of detailed rate constant fitting for each combination of toehold length and mismatch variant, at each complex in the cascade. Rate constants are reported in units of $M^{-1}s^{-1}$ with associated standard errors determined by the jackknife methods as described in Methods.

toehold length	optimization	complex	kf	SE kf	kr	SE kr
5nt TH	NoMM	F2	5.72e+05	4.49e+04	1.63e+05	1.28e+04
5nt TH	MM	F2	9.71e+05	1.95e+05	2.46e-01	4.93e-02
7nt TH	NoMM	F2	2.36e+06	1.83e+05	3.50e+04	2.72e+03
7nt TH	MM	F2	1.22e+06	5.66e+04	1.87e+00	8.64e-02
5nt TH	NoMM	F1	9.65e+05	1.52e+05	1.38e+05	2.17e+04
5nt TH	MM	F1	1.97e+06	7.76e+05	2.49e-01	9.83e-02
7nt TH	NoMM	F1	3.00e+06	8.19e+05	7.82e+02	2.13e+02
7nt TH	MM	F1	5.67e+06	6.18e+06	1.33e-06	1.45e-06
5nt TH	NoMM	F0	3.87e+05	7.55e+04	2.75e+05	5.37e+04
5nt TH	MM	F0	1.42e+06	3.62e+05	8.97e-01	2.29e-01
7nt TH	NoMM	F0	7.45e+05	6.44e+04	7.73e+03	6.68e+02
7nt TH	MM	F0	3.08e+06	6.10e+05	4.99e-07	9.87e-08

Table B.7: Results of omnibus Holm-corrected Wald Z-tests on rate constant comparisons, inverse-variance pooled across either the kf or kr for each cascade type, compared between all combinations of toehold length and mismatch variant.

rate type	group1	k1	se1	group2	k2	se2	z value	p value	holm-adjusted p value
kf	NoMM 5nt	5.50e+05	3.74e+04	MM 5nt	1.11e+06	1.68e+05	-3.28	0.00104	0.00311
kf	NoMM 5nt	5.50e+05	3.74e+04	NoMM 7nt	9.34e+05	6.06e+04	-5.38	7.37e-08	3.69e-07
kf	NoMM 5nt	5.50e+05	3.74e+04	MM 7nt	1.24e+06	5.64e+04	-10.20	0	0
kf	MM 5nt	1.11e+06	1.68e+05	NoMM 7nt	9.34e+05	6.06e+04	1.01	0.313	0.625
kf	MM 5nt	1.11e+06	1.68e+05	MM 7nt	1.24e+06	5.64e+04	-0.72	0.473	0.625
kf	NoMM 7nt	9.34e+05	6.06e+04	MM 7nt	1.24e+06	5.64e+04	-3.71	0.000211	0.000845
kr	NoMM 5nt	1.61e+05	1.08e+04	MM 5nt	2.70e-01	4.33e-02	14.94	0	0
kr	NoMM 5nt	1.61e+05	1.08e+04	NoMM 7nt	1.61e+03	2.02e+02	14.79	0	0
kr	NoMM 5nt	1.61e+05	1.08e+04	MM 7nt	5.03e-07	9.85e-08	14.94	0	0
kr	MM 5nt	2.70e-01	4.33e-02	NoMM 7nt	1.61e+03	2.02e+02	-7.95	1.78e-15	5.33e-15
kr	MM 5nt	2.70e-01	4.33e-02	MM 7nt	5.03e-07	9.85e-08	6.23	4.57e-10	4.57e-10
kr	NoMM 7nt	1.61e+03	2.02e+02	MM 7nt	5.03e-07	9.85e-08	7.95	1.78e-15	5.33e-15

Table B.8: Results of Holm-corrected Wald Z-tests for rate constant comparisons between all combination of toehold length and mismatch variant, for the same complex in the cascade.

rate type	complex	group1	k1	se1	group2	k2	se2	z value	p value	holm-adjusted p value
kf	F2	NoMM 5nt	5.72e+05	4.49e+04	MM 5nt	9.71e+05	1.95e+05	-1.99	0.0462	0.369
kf	F2	NoMM 5nt	5.72e+05	4.49e+04	NoMM 7nt	2.36e+06	1.83e+05	-9.47	0	0
kf	F2	NoMM 5nt	5.72e+05	4.49e+04	MM 7nt	1.22e+06	5.66e+04	-9.02	0	0
kf	F2	MM 5nt	9.71e+05	1.95e+05	NoMM 7nt	2.36e+06	1.83e+05	-5.19	2.1e-07	3.15e-06
kf	F2	MM 5nt	9.71e+05	1.95e+05	MM 7nt	1.22e+06	5.66e+04	-1.25	0.213	1
kf	F2	NoMM 7nt	2.36e+06	1.83e+05	MM 7nt	1.22e+06	5.66e+04	5.92	3.26e-09	5.21e-08
kf	F1	NoMM 5nt	9.65e+05	1.52e+05	MM 5nt	1.97e+06	7.76e+05	-1.27	0.204	1
kf	F1	NoMM 5nt	9.65e+05	1.52e+05	NoMM 7nt	3.00e+06	8.19e+05	-2.45	0.0143	0.143
kf	F1	NoMM 5nt	9.65e+05	1.52e+05	MM 7nt	5.67e+06	6.18e+06	-0.76	0.447	1
kf	F1	MM 5nt	1.97e+06	7.76e+05	NoMM 7nt	3.00e+06	8.19e+05	-0.92	0.358	1
kf	F1	MM 5nt	1.97e+06	7.76e+05	MM 7nt	5.67e+06	6.18e+06	-0.59	0.553	1
kf	F1	NoMM 7nt	3.00e+06	8.19e+05	MM 7nt	5.67e+06	6.18e+06	-0.43	0.669	1
kf	F0	NoMM 5nt	3.87e+05	7.55e+04	MM 5nt	1.42e+06	3.62e+05	-2.79	0.00525	0.0577
kf	F0	NoMM 5nt	3.87e+05	7.55e+04	NoMM 7nt	7.45e+05	6.44e+04	-3.61	0.000301	0.00362
kf	F0	NoMM 5nt	3.87e+05	7.55e+04	MM 7nt	3.08e+06	6.10e+05	-4.39	1.16e-05	0.000162
kf	F0	MM 5nt	1.42e+06	3.62e+05	NoMM 7nt	7.45e+05	6.44e+04	1.83	0.0669	0.468
kf	F0	MM 5nt	1.42e+06	3.62e+05	MM 7nt	3.08e+06	6.10e+05	-2.34	0.0191	0.172
kf	F0	NoMM 7nt	7.45e+05	6.44e+04	MM 7nt	3.08e+06	6.10e+05	-3.81	0.000139	0.00181
kr	F2	NoMM 5nt	1.63e+05	1.28e+04	MM 5nt	2.46e-01	4.93e-02	12.75	0	0
kr	F2	NoMM 5nt	1.63e+05	1.28e+04	NoMM 7nt	3.50e+04	2.72e+03	9.79	0	0
kr	F2	NoMM 5nt	1.63e+05	1.28e+04	MM 7nt	1.87e+00	8.64e-02	12.75	0	0
kr	F2	NoMM 5nt	1.63e+05	1.28e+04	MM 7nt	1.87e+00	8.64e-02	12.75	0	0
kr	F2	MM 5nt	2.46e-01	4.93e-02	NoMM 7nt	3.50e+04	2.72e+03	-12.87	0	0
kr	F2	MM 5nt	2.46e-01	4.93e-02	MM 7nt	1.87e+00	8.64e-02	-16.31	0	0
kr	F2	NoMM 7nt	3.50e+04	2.72e+03	MM 7nt	1.87e+00	8.64e-02	12.87	0	0
kr	F1	NoMM 5nt	1.38e+05	2.17e+04	MM 5nt	2.49e-01	9.83e-02	6.34	2.34e-10	2.34e-09
kr	F1	NoMM 5nt	1.38e+05	2.17e+04	NoMM 7nt	7.82e+02	2.13e+02	6.30	2.96e-10	2.37e-09
kr	F1	NoMM 5nt	1.38e+05	2.17e+04	MM 7nt	1.33e-06	1.45e-06	6.34	2.34e-10	2.34e-09
kr	F1	MM 5nt	2.49e-01	9.83e-02	NoMM 7nt	7.82e+02	2.13e+02	-3.67	0.000245	0.000731
kr	F1	MM 5nt	2.49e-01	9.83e-02	MM 7nt	1.33e-06	1.45e-06	2.54	0.0112	0.0112
kr	F1	NoMM 7nt	7.82e+02	2.13e+02	MM 7nt	1.33e-06	1.45e-06	3.67	0.000244	0.000731
kr	F0	NoMM 5nt	2.75e+05	5.37e+04	MM 5nt	8.97e-01	2.29e-01	5.12	3e-07	2.1e-06
kr	F0	NoMM 5nt	2.75e+05	5.37e+04	NoMM 7nt	7.73e+03	6.68e+02	4.98	6.39e-07	3.19e-06
kr	F0	NoMM 5nt	2.75e+05	5.37e+04	MM 7nt	4.99e-07	9.87e-08	5.12	3e-07	2.1e-06
kr	F0	MM 5nt	8.97e-01	2.29e-01	NoMM 7nt	7.73e+03	6.68e+02	-11.57	0	0
kr	F0	MM 5nt	8.97e-01	2.29e-01	MM 7nt	4.99e-07	9.87e-08	3.92	8.86e-05	0.000355
kr	F0	NoMM 7nt	7.73e+03	6.68e+02	MM 7nt	4.99e-07	9.87e-08	11.57	0	0

Proxy invasion measurements

Our proxy kinetic analysis of spurious invasion trajectories, reinforces our mechanistic conclusion overall. The fact that a strand exchange invader that resolves two mismatches was faster than one that was complementary but did not replace mismatches is consistent with literature (Irmisch et al. [2020]). However we found a reduction of rate for invader that mismatches the toehold, but only replaces mismatches with distinct mismatch sequences during invasion. A similar invader that mismatches the toehold, but introduces two mismatches did not produce any observable rate. The latter is consistent with (Irmisch et al. [2020]) which found very low reaction rates for introducing two mismatches, but demonstrates an elimination of reaction rate with a third. Comparing the fit bimolecular rates for the invasions to the toehold energies and free energies of reaction we found that for these sequences, the kinetic reduction for the invader with mismatch replacement can be attributed entirely to the different toehold strength, while the absence of an observed forward rate for the mismatch introducing invader is explained solely by the free energy change of reaction, for all feasible forward rates.

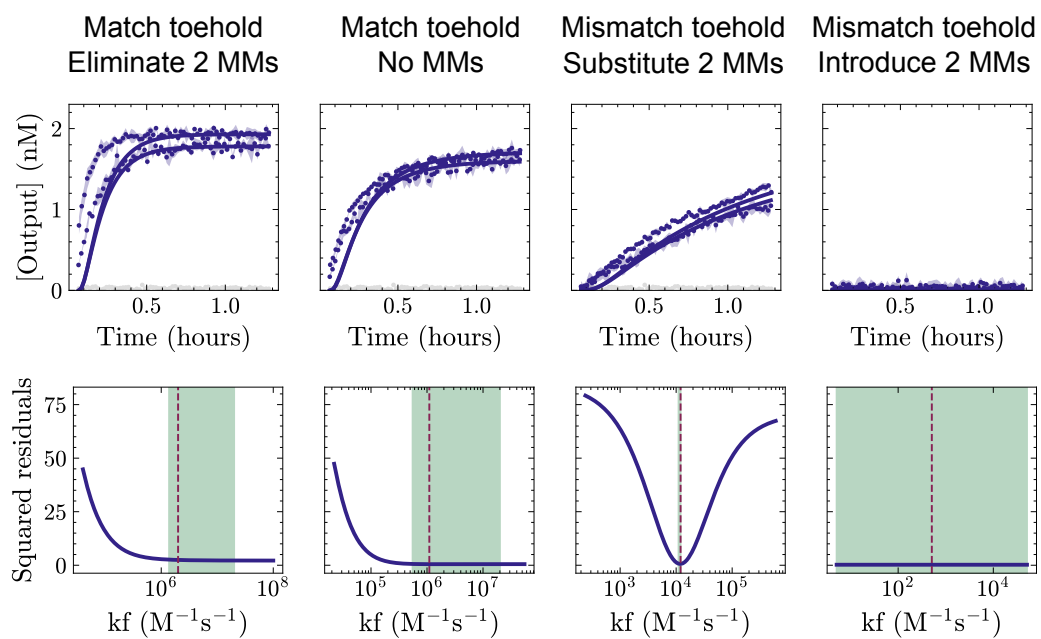


Figure B.13: Top: Best-fit bimolecular rate models to two trials across of each of the four spurious triggering conditions. Bottom: Comparing the residuals vs parameter value reveals only one model is well-constrained under these excess triggering conditions.

B.3.3 Thermodynamic modeling

5ntTH free energy landscape

Figures in this sections were created by Cady Pearce based on the concrete X to Y translator translator sequences I designed.

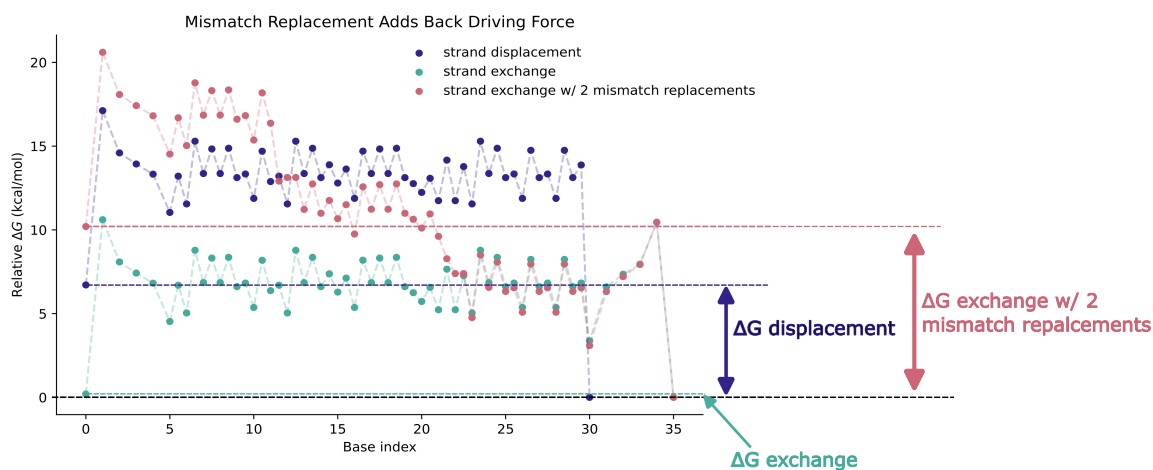


Figure B.14: Energy landscape of the 5 base pair toehold X to Y **F2** translator with mismatch strategy (pink) and without mismatch strategy (teal). The initial free energy of **F2mm** is higher than that of **F2**, since **F2mm** has fewer paired bases, as well as mismatches destabilizing its helix.

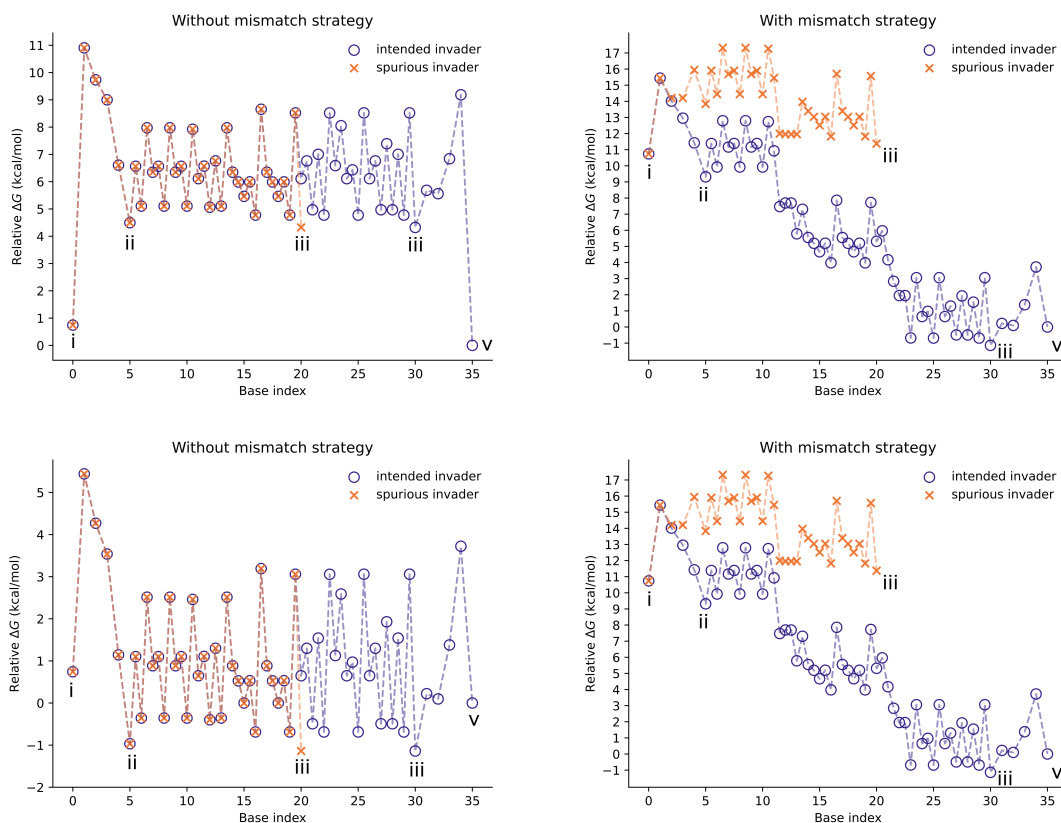


Figure B.15: Free energy differences (kcal/mol) of F2 translators without and with mismatches, relative to initial state of the system (base index = 0), at 1 nano molar (top row) and 10 micro molar (bottom row). In the system without the mismatch strategy, spurious invader, F0-t, follows same 1 pathway as the intended invader, F1-t, until base 20 (micro-state IV). At base 20 the spurious invasion system (blue) loses the branch migration penalty ΔG_p , since F0-t no longer has a single stranded dangle on its 3' end, and reaches a local energy minimum. All reactions pay a concentration-dependent entropic penalty upon binding of first base in toehold, shown as the energy jump from micro-state I to micro-state II. At higher concentrations (c) this penalty is smaller, and it becomes favorable for the no mismatch system to sit in a thermodynamic minimum during the branch migration portion of strand exchange. In contrast, even at high concentrations, the mismatch strategy provides an additional energy barrier to spurious invasion (d, micro-state III) while maintaining a net free energy drop for the desired strand exchange (orange, micro-state I to micro-state V).

Toehold binding energies

The table below specifies the toehold binding energy for each gate-trigger pair used in the X→Y cascades investigation.

Complex	ΔG_{struc} Toehold (kcal/mol)
F0	-8.0
F1	-9.4
F2	-8.5
F0(mm)	-8.0
F1(mm)	-8.3
F2(mm)	-8.3
F0(l)	-10.5
F1(l)	-12.1
F2(l)	-12.8
F0(l)(mm)	-10.5
F1(l)(mm)	-11.0
F2(l)(mm)	-10.2

Table B.9: The structural free energy difference between each complex in our four feed-forward cascades with and without the intended trigger. Structural energies were computed using Nupack’s dna04 model at 25°C, 0.05M Na⁺, 0.0125M Mg²⁺ assigning each structure such that each trigger strand was completely bound the to gate’s toehold and otherwise unstructured. Comparing the free energies of respective complexes across cascades it can be seen all toehold binding energies were left unchanged or weakened by systematic mismatch optimization. This suggests that any rate increase observed as a result of systematic mismatch optimization likely stemmed from factors beyond toehold binding energy.

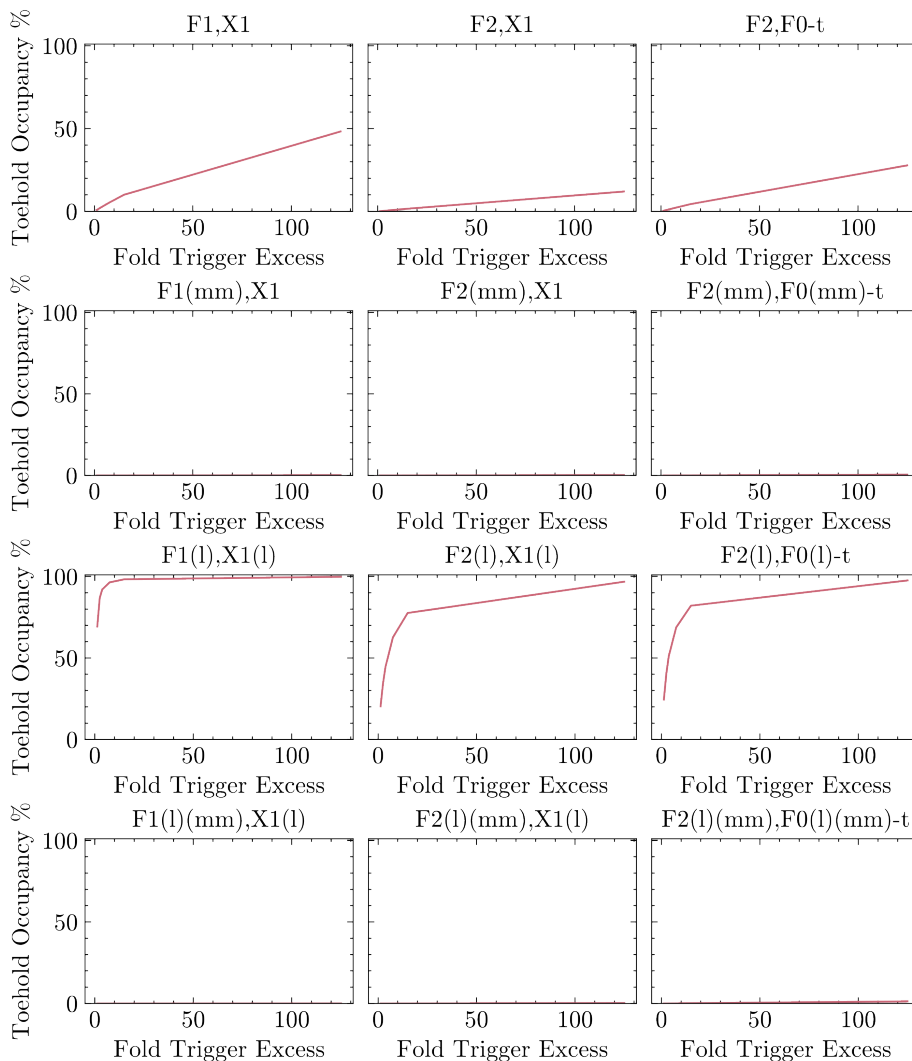
Spurious occupancy modeling

Figure B.16: Predicted occupancy for the principle spurious interaction between signals strands and fuel complexes in all feedforward complexes tested. Percent occupancy was modeled using Nupack's dna04 model at 25°C, 0.05M Na⁺, 0.0125M Mg²⁺, gate strand concentrations specified by curve color according to the figure legend, and trigger strand in fold-excess specified by the x-axis coordinate.

B.4 X to X Amplifier

B.4.1 Thermodynamic modeling

Without mismatches, an X to X toehold exchange translator where the output equals the input is not expected to have an equilibrium that favors the products since there is no net formation of DNA base pairs, and the products are not lower energy than the reactants. Adding two mismatch replacements to each fuel reaction in the translator gives each reaction a net driving force greater than 7 kcal/mol. This shifts the predicted equilibrium of the system to heavily favor the product state.

Mismatches are predicted to drive turnover of the X to X amplifier until all fuel complexes are exhausted. The mismatches in the X to X amplifier also disfavor spurious invasion (like the mismatches in the X to Y translator), protecting against leak in a system where any low level of leak will be amplified and consequently degrade the signal-to-noise ratio of the translator.

Complex	Final Concentration (nanomolar)
F1(no-mm)-t + F2(no-mm)-b	64.3
F2(no-mm)-t + F0(no-mm)-b	66.5
F0(no-mm)-t + F1(no-mm)-b	63.5
F0(no-mm)-t + F0(no-mm)-b	32.6
F1(no-mm)-t + F1(no-mm)-b	33.7
F2(no-mm)-t + F2(no-mm)-b	35.4

Table B.10: X to X no mismatch amplifier thermodynamic equilibrium at 100 nano molar initial concentration of all three complexes and 10 nano molar input. Complexes in red are product complexes and complexes in blue are reactant complexes.

B.4.2 Kinetics experiments

Kinetics experiments were carried out in a BioTek SynergyH1 plate reader or a BioTek Cytation5 plate reader. Experiments where the reporter was at 100 nM concentration were done with a plate reader gain of 100 and experiments where the reporter was at 1 nM

Complex	Final Concentration (nanomolar)
F1(mm)-t + F2(mm)-b	99.8
F2(mm)-t + F0(mm)-b	99.8
F0(mm)-t + F1(mm)-b	100.
F0(mm)-t + F0(mm)-b	0
F1(mm)-t + F1(mm)-b	0
F2(mm)-t + F2(mm)-b	0

Table B.11: X to X mismatch amplifier thermodynamic equilibrium at 100 nano molar initial concentration of all three complexes with 10 nano molar input. Complexes in red are product complexes and complexes in blue are reactant complexes.

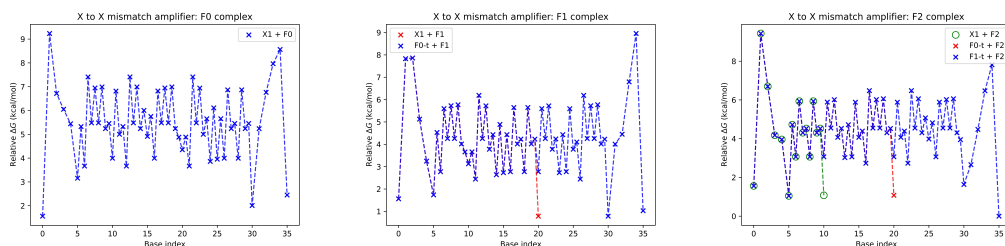


Figure B.17: Reaction coordinate energy graphs for X to X amplifier without mismatches.

concentration were done with a plate reader gain of 140. All experimental conditions were mixed in an ECHO 525 Acoustic Liquid Handler (Beckman Coulter).

Complex concentrations were calculated using the normalization procedures outlined in the previous sections. For in plate normalizations, an untriggered F2 reporter was used as background signal and subtracted from each averaged RFU trace. Additionally, each cascade was triggered with excess input to obtain a full cascade completion standard that was used to normalize the fractional completion of each condition based on the RFU output.

Comparison Between Mismatch and No Mismatch Amplifiers

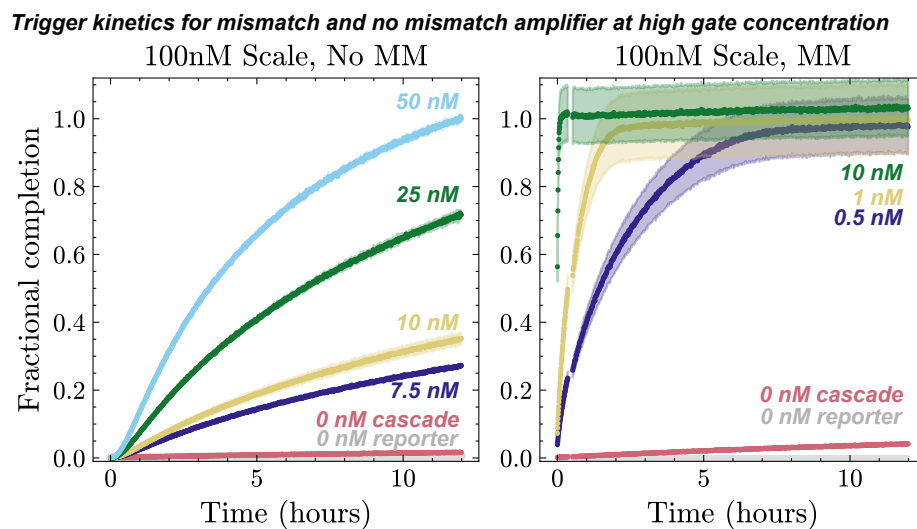


Figure B.18: Kinetics for X to X no mismatch and mismatch amplifiers. Each F2 gate is present at 100 nM and all other gates at 150 nM. Trigger concentrations range from 200 pico molar to 10 nanomolar with the untriggered amplifier plotted as a reference for the leak rate (teal). Uncertainty ranges are graphed as the standard deviation of the triplicate average for each time point.

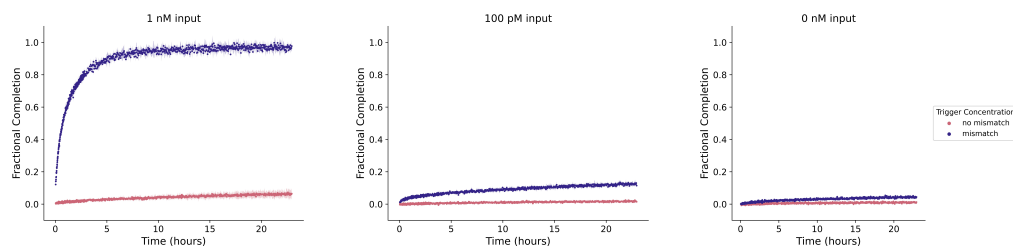


Figure B.19: Kinetics of both mismatch and no mismatch amplifiers with complexes at 10 nano molar. Amplifiers were triggered with respective input strands at 1 nano molar and 100 pico molar. Controls with no input are shown to establish a baseline leak for each amplifier.

B.5 Sequences

Table B.12: Sequences used for all experimental systems

System	Strand	Sequences
X to Y	R1b	TTGTTGGTATGTTTGTGGTTGTAGTAGATG
X to Y	R1t	ACTACAACCACAAACATACCAACAA
X to Y, 5ntTH	X1	CCATCACCCCTCACCTTCCCTAAACCTCTCT
X to Y, noMM, 5ntTH	F0b	AGATGAGAGAGGTTTAGGGAAGGTGAGGGTGATGG
X to Y, noMM, 5ntTH	F0t	ACCCTCACCTTCCCTAAACCTCTCTCATCTACTAC
X to Y, noMM, 5ntTH	F1b	TGGTTGTAGTAGATGAGAGAGGTTTAGGGAAGGTG
X to Y, noMM, 5ntTH	F1t	TCCCTAAACCTCTCTCATCTACTACAACCACAAAC
X to Y, noMM, 5ntTH	F2b	GGTATGTTTGTGGTTGTAGTAGATGAGAGAGGTTT
X to Y, noMM, 5ntTH	F2t	TCTCTCATCTACTACAACCACAAACATACCAACAA
X to Y, MM, 5ntTH	F0(mm)b	AGTTGAGAGAGGTTTAGGGAAGGTGAGGGTGATGG
X to Y, MM, 5ntTH	F0(mm)t	ACCCTCATCTTCCCTAACCCCTCTCTCAACTACTAC
X to Y, MM, 5ntTH	F1(mm)b	TGTTTGTAGTAGTTGAGAGAGGGTTAGGGAAGATG
X to Y, MM, 5ntTH	F1(mm)t	TCCCTAATCCTCTCTCACCTACTACAACACAAAC
X to Y, MM, 5ntTH	F2(mm)b	GGTATGTTTGTGGTTGTAGTAGGTGAGAGAGGATT
X to Y, MM, 5ntTH	F2(mm)t	TCTCTCATCTACTACAACCACAAACATACCAACAA
X to Y, 7ntTH	X1(l)	TTCACTCACTATTAAACCATCACCCCTCACCTACCCTAAACCTCTCT
X to Y, noMM, 7ntTH	F0(l)b	GGTAAAGAGAGGTTTAGGGTAGGTGAGGGTGATGGTTAATAGTGAGTGAA
X to Y, noMM, 7ntTH	F0(l)t	ACTATTAACCATCACCCCTCACCTACCCTAAACCTCTCTTTACCTATATCCTCT
X to Y, noMM, 7ntTH	F1(l)b	AGATGAGAGGATATAGGTAAGAGAGGTTTAGGGTAGGTGAGGGTGATGG
X to Y, noMM, 7ntTH	F1(l)t	CCTCACCTACCCTAAACCTCTCTTTACCTATATCCTCTCATCTACTACAACCA
X to Y, noMM, 7ntTH	F2(l)b	GTTTGTGGTTGTAGTAGATGAGAGGATATAGGTAAGAGAGGTTTAGGGT
X to Y, noMM, 7ntTH	F2(l)t	ACCTCTCTTTACCTATATCCTCTCATCTACTACAACCACAAACATACCAACAA
X to Y, MM, 7ntTH	F0(mm)(l)b	GGTAAAGAGAGGTTTAGGGTAGGTGAGGGTGATGGTTAATAGTGAGTGAA
X to Y, MM, 7ntTH	F0(mm)(l)t	ACTATTAACCTATCATCCTCACCTAACCTACACCTCTCTTTACCTTTATCCTCT
X to Y, MM, 7ntTH	F1(mm)(l)b	AGAAGAGAGGATAAAGGTAAGAGAGGTTGATGGTTAGGTGAGGATGATAG
X to Y, MM, 7ntTH	F1(mm)(l)t	CCTCACCTATCCTATACCTCTCTTAACCTATATCCTCTCTTCTAATACAACCA
X to Y, MM, 7ntTH	F2(mm)(l)b	GTTTGTGGTTGTATTAGAAGAGAGGATATAGGTTAAGAGAGGTATAGGAT
X to Y, MM, 7ntTH	F2(mm)(l)t	ACCTCTCTTAACCTATATCCTCTCATCTACTACAACCACAAACATACCAACAA
X to X, noMM	X1	TCTCTCCATCACCCCTCAACTCCCTACCAATCTCT
X to X, noMM	F0b	GATGGAGAGATTGGTAGGGAAGGTGAGGGTGATGG
X to X, noMM	F0t	ACCCTCACCTTCCCTACCAATCTCTCCATCACCCCT
X to X, noMM	F1b	AGGTGAGGGTGATGGAGAGATTGGTAGGGAAGGTG
X to X, noMM	F1t	TCCCTACCAATCTCTCCATCACCCCTCACCTTCCCT
X to X, noMM	F2b	/5ATT0565N/TTGGTAGGGAAGGTGAGGGTGATGGAGAGATTGGT
X to X, noMM	F2t	TCTCTCCATCACCCCTCACCTTCCCTACCAATCTCT/3BHQ_2/
X to X, noMM	F2_full.trigger	ACCAATCTCTCCATCACCCCTCACCTTCCCTACCAA
X to X, MM	X1(mm)	TCTCTCCATCACCCCTCACCTTCCCTACCAATCTCT
X to X, MM	F0(mm)b	GAGGGAGAGATTGGTAGGGAAGTTGAGGGTGATGG
X to X, MM	F0(mm)t	ACCCTCACCTTCCCTACAAATCTCTCCCTCACCCCT
X to X, MM	F1(mm)b	AGATGAGGGTGAGGGAGAGATTTGTAGGGAAGGTG
X to X, MM	F1(mm)t	TCCCTACTAATCTCTCCTTACCCTCATCTTCCCT
X to X, MM	F2(mm)b	/5ATT0565N/TTGGTAGGGAAGATGAGGGTGAAGGAGAGATTAGT
X to X, MM	F2(mm)t	TCTCTCCATCACCCCTCAACTTCCCTACCAATCTCT/3BHQ_2/
X to X, MM	F2(mm)_full.trigger	ACTAATCTCTCCTTACCCTCATCTTCCCTACCAA
SNP detection	F1-mm-t-toe-SNP	TCCCTACCAATCTCTCCTTACCCTCATCTTCCCT
SNP detection	F1-mm-t-mid-SNP	TCCCTACTAATCCCTCCTTACCCTCATCTTCCCT
SNP detection	F1-match-t-toe-SNP	TCCCTACAAATCTCTCCATCACCCCTCACCTTCCCT
SNP detection	F1-match-t-mid-SNP	TCCCTACCAATCCCTCCATCACCCCTCACCTTCCCT

Appendix C

CHAPTER 4 SUPPLEMENTARY INFORMATION

C.1 Full cascade mechanism

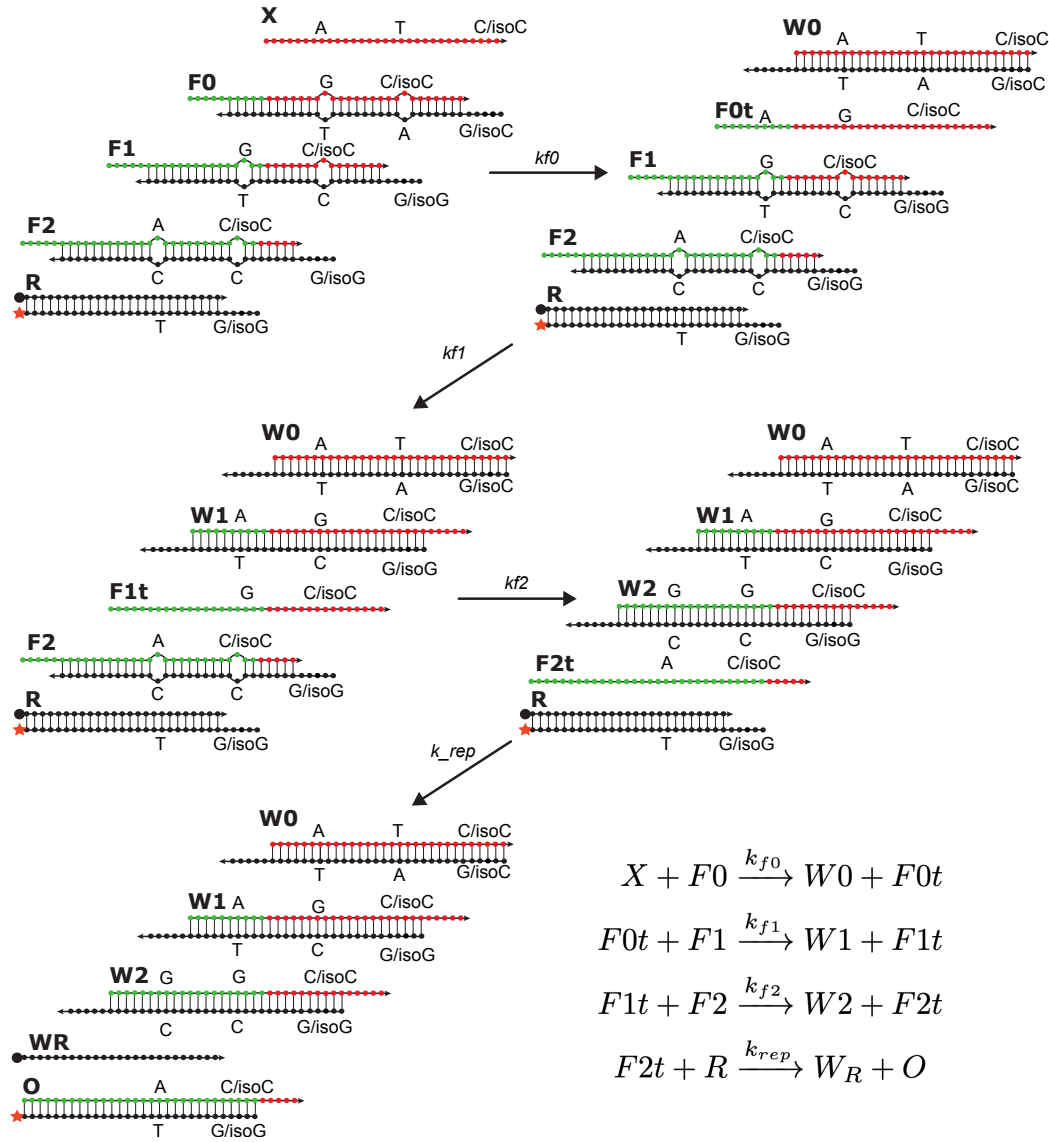


Figure C.1: A visual depiction and reaction-level description of the four-step CRN implemented by the 5' toehold and 3' toehold cascades. At each step an invader reacts with a fuel complex to produce a waste and an output. For example, in the first step X and $F0$ react with second order rate constant k_{f0} to produce $F0t$ and $W0$. At all but the last step of the cascade, this output reacts with a downstream fuel complex in the aforementioned manner (e.g. $F0t$ reacts with $F1$).

Baseline subtracted raw fluorescence values

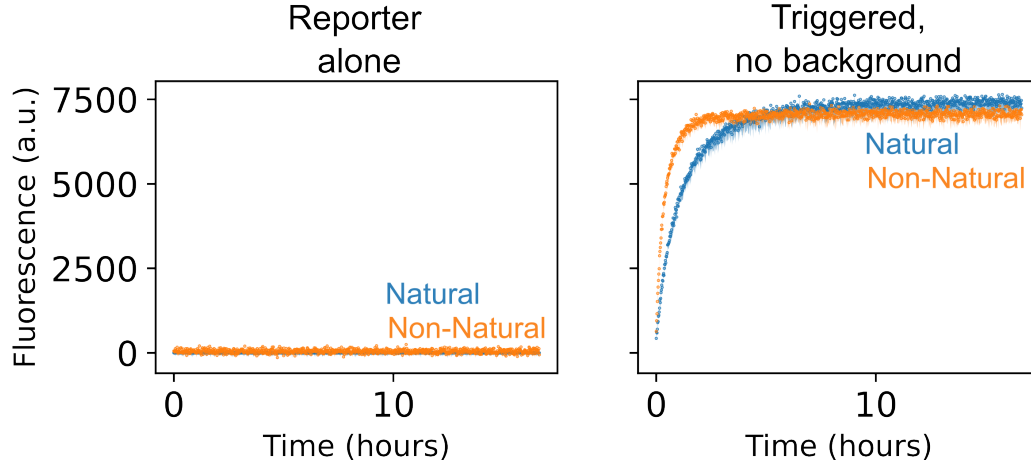


Figure C.2: Raw fluorescent unit (R.F.U) values for matching isoC:isoG and C:G base pairs in the toehold, adjusted with baseline subtraction of the R.F.U of the buffer alone. The comparison for the background resulting from the 40 nM reporters alone is presented in the left panel and the kinetics of the reporters triggered at 20 nM is presented in the right panel. At 12 hours, we observed a 4.2% difference in the adjusted R.F.U. readings between the natural and non-natural systems, averaged across the last 10 data points in each timeseries.

C.2 Detailed statistical methods

Wald Z-tests. For all but the data presented in Figure 4.5, hypothesis testing was conducted on rate constant ratios. Starting from estimates of k and their associated standard errors, the standard error on the ratio of rate constants with and without the non-natural base was propagated using first-order error propagation as follows:

$$SE_{\text{ratio}} = \sqrt{\left(\frac{\sigma_{nn}}{\mu_{nat}}\right)^2 + \left(\frac{\sigma_{nat} \mu_{nn}}{\mu_{nat}^2}\right)^2},$$

where μ_{nat} and μ_{nn} are the estimated rate constants for the natural and non-natural cases, σ_{nat} and σ_{nn} are the associated standard errors, and $\mu_{nat} \neq 0$.

When comparing two ratios with respective uncertainty (for example, comparing a ratio with background to a ratio without background), we computed the difference between the two ratios, assuming independence because each contrast involved non-overlapping ex-

periment sets, and compared this difference to a null hypothesis value of 0. In such cases the standard error on the difference of ratios was propagated, again using first-order error propagation, as

$$SE(\text{difference}) = \sqrt{SE_0^2 + SE_1^2},$$

where SE_0 and SE_1 are the propagated standard errors for each ratio.

Significance testing between ratios and propagated standard errors was conducted using Wald Z-tests (Casella and Berger [2024]), which are valid under the asymptotic normality of the propagated estimator. The resulting Z statistic is given by

$$Z = \frac{\theta_{\text{test}} - \theta_0}{SE_{\text{test}}},$$

where θ_{test} and SE_{test} are the calculated rate constant ratio and its standard error, and θ_0 is the null hypothesis ratio. Since the Z statistic is asymptotically normally distributed, the corresponding two-tailed p -value was calculated as

$$p = 2(1 - \Phi(|Z|)),$$

where Φ is the cumulative distribution function of the normal distribution.

It is advisable to conduct omnibus tests across groups of conditions, for example comparing all incubation conditions together across two background conditions, before investigating individual conditions. To conduct such omnibus tests we combined like conditions using inverse-variance pooling as follows:

$$\mu_{\text{pooled}} = \frac{\sum_i \mu_i / SE_i^2}{\sum_i 1 / SE_i^2}, \quad SE_{\text{pooled}} = \frac{1}{\sqrt{\sum_i 1 / SE_i^2}},$$

where μ_i and SE_i are each individual ratio and corresponding standard error. Omnibus tests were conducted by either (i) comparing pooled ratios to 1 within each background condition, or (ii) comparing pooled ratios between background conditions.

For each question of interest (e.g., “are any ratios different from the null hypothesis of 1?”), p -values were adjusted using the Bonferroni–Holm procedure (Holm [1979]). In our analysis we use $p < 0.05$ as the cutoff for marginal significance and $p < 0.01$ as the cutoff for high significance. In each figure we label marginally significant comparisons with “*” and highly significant results with “**”. Non-significant tests are marked with “n.s.”.

Welch t -tests. The 6-hour completion values shown in Figure 4.5 represent direct experimental measurements, not fitted model parameters. Each bar corresponds to the sample mean of three independent replicates, and its error bar reflects the sample standard deviation computed from those replicates.

For endpoint analyses, we quantified fractional completion at 6 hours using a procedure that propagates uncertainty from raw fluorescence traces. Let $V_j(t)$ denote the fluorescence of replicate j at time t , and let the baseline and a control triggered at 20 nM be denoted by B and T , respectively. For each control condition and each time point, we first computed the mean fluorescence across the three replicate wells and its sample standard deviation s_t . To obtain stable reference levels, we then defined the baseline and triggered reference intensities as the average of the last five time points of the corresponding mean fluorescence traces,

$$\bar{B} = \frac{1}{n} \sum_{t \in \mathcal{T}} \bar{V}_B(t), \quad \bar{T} = \frac{1}{n} \sum_{t \in \mathcal{T}} \bar{V}_T(t),$$

where $\mathcal{T}$ is the set of the last five time points ($n = 5$). The uncertainty on these averaged reference levels was propagated from the time-point-wise standard deviations using

$$\sigma_{\bar{B}} = \frac{1}{n} \sqrt{\sum_{t \in \mathcal{T}} s_{B,t}^2}, \quad \sigma_{\bar{T}} = \frac{1}{n} \sqrt{\sum_{t \in \mathcal{T}} s_{T,t}^2},$$

which corresponds to treating the last five time points as independent estimates of a common baseline or triggered level.

For each experimental condition, we identified the first recorded time point t^* such that $t^* \geq 6$ h, and extracted the fluorescence $V_j(t^*)$ for each replicate well j . The fractional

completion for that condition was then defined at the replicate level by

$$C_j = \frac{V_j(t^*) - \bar{B}}{\bar{T} - \bar{B}}.$$

The bar heights in the completion plots correspond to the sample mean $\bar{C}$ across the three replicate wells, and the error bars correspond to the sample standard deviation of the $\{C_j\}$. These means, standard deviations, and replicate counts were then used as inputs to the one-sample or two-sample t -tests described below.

One-sample t -tests were used to evaluate whether each completion-difference value differed from a null hypothesis of zero. When comparing between experimental conditions (e.g., N25 vs N50), two-sample Welch t -tests were used.

For the multi-isoC completion experiment (Figure 4.5), we also performed omnibus comparisons between sequence designs that pooled across incubation states. For each isoC configuration, let μ_{no} and σ_{no} denote the mean and sample standard deviation of the 6-hour fractional completion without prior incubation, and μ_{trig} and σ_{trig} the corresponding quantities with invader incubation. To obtain a single pooled completion level for each configuration, we defined

$$\mu_{\text{pooled}} = \frac{\mu_{\text{no}} + \mu_{\text{trig}}}{2}, \quad \sigma_{\text{pooled}} = \sqrt{\frac{\sigma_{\text{no}}^2 + \sigma_{\text{trig}}^2}{2}}.$$

These pooled summary statistics were then treated as arising from an effective sample size of $n_{\text{eff}} = 2n$ (with $n = 3$ replicates per incubation state) and compared between isoC configurations using two-sample Welch t -tests.

For all endpoint analyses, p -values were corrected for multiple testing using the Holm procedure, applied across the set of pre-specified comparisons for each figure.

C.3 *isoG/isoC substitution thermodynamics*

The one-step 5' toehold system substituted an 'ACA' trinucleotide for 'AisoCA' yielding a free energy difference of 0.60 kcal/mol, while the 3' toehold system substituted an 'ATC' trinucleotide for 'AisoCC' yielding a free energy change of -0.33 kcal/mol at 29°C for the 5' system and 25°C for the 3' system. However, the 5' and 3' systems showed approximately 2.9 and 5.4-fold increases in effective rate constant with isoC. Using Zhang et al.'s reported fit of $\log_{10}(k) \propto \frac{-\log_{10} e}{RT} \cdot \Delta G_{\text{toehold}}$, (Zhang and Winfree [2009]), these fold changes in rate constant should correspond to lower bounds on the magnitude of the effective free energy decrease (the true values may be more negative since $\log_{10}(k)$ saturates around 6) of approximately 0.6 kcal/mol and 0.5 kcal/mol for the 5' and 3' systems respectively. Thus while we did observe larger increases in rate for more negative free energy changes as expected, in the presence of isoC the systems behaved kinetically as if the toeholds possessed an apparent binding free energy advantage of at least ~ 0.2 -0.7 kcal/mol relative to predictions based on toehold binding thermodynamics alone.

Regardless of these estimated values, the fact that including an isoC in the 5' toehold system increased kinetics despite weakening the toehold is a surprising discovery.

Table C.1: Nearest neighbor stacking energies of all possible trinucleotide sequences with isoC (abbreviated S) at the center minus stacking energies for the same trinucleotide with each possible natural base at the center, reported in kcal/mol. Each trinucleotide free energy was computed from the two component di-nucleotide contributions calculated at 25°C. Nearest neighbor stacking enthalpies and entropies were taken from (SantaLucia Jr and Hicks [2004]) for natural nucleotides and from (Hoshika et al. [2019]) for the S bases. The average of each column represents the average free energy change for substituting each natural base with the isoC base.

Trinucleotide	$\Delta\Delta G$ (A-center)	$\Delta\Delta G$ (T-center)	$\Delta\Delta G$ (C-center)	$\Delta\Delta G$ (G-center)
ASA	-0.17	-0.70	0.78	0.45
AST	-0.17	-0.17	0.72	0.72
ASC	-0.03	-0.30	0.79	1.06
TSA	-0.63	-0.63	0.58	0.58
TST	-0.63	-0.10	0.52	0.85
TSC	-0.49	-0.23	0.59	1.20
CSA	0.17	-0.24	0.99	1.26
CST	0.17	0.29	0.93	1.53
CSC	0.31	0.16	0.99	1.88
Average	-0.16	-0.21	0.77	1.06

Table C.2: Nearest neighbor stacking energies of all possible trinucleotide sequences with isoG (abbreviated B) at the center minus stacking energies for the same trinucleotide with each possible natural base at the center, reported in kcal/mol. Each trinucleotide free energy was computed from the two component di-nucleotide contributions calculated at 25°C. Nearest neighbor stacking enthalpies and entropies were taken from (SantaLucia Jr and Hicks [2004]) for natural nucleotides and from (Hoshika et al. [2019]) for the B base. The average of each column represents the average free energy change for substituting each natural base with the isoG base.

Trinucleotide	$\Delta\Delta G$ (A-center)	$\Delta\Delta G$ (T-center)	$\Delta\Delta G$ (C-center)	$\Delta\Delta G$ (G-center)
ABA	-0.10	-0.63	0.85	0.52
ABT	-0.17	-0.17	0.72	0.72
ABC	-0.05	-0.32	0.77	1.04
TBA	-0.63	-0.63	0.58	0.58
TBT	-0.70	-0.17	0.45	0.78
TBC	-0.58	-0.32	0.50	1.11
CBA	-0.30	-0.71	0.52	0.79
CBT	-0.37	-0.25	0.39	0.99
CBC	-0.25	-0.40	0.43	1.32
Average	-0.35	-0.40	0.58	0.87

Table C.3: Nearest neighbor stacking energies of all possible trinucleotide sequences with isoC (abbreviated S) at the center minus stacking energies for the same trinucleotide with each possible natural base at the center, reported in kcal/mol. Each trinucleotide free energy was computed from the two component di-nucleotide contributions calculated at 29°C. Nearest neighbor stacking enthalpies and entropies were taken from (SantaLucia Jr and Hicks [2004]) for natural nucleotides and from (Hoshika et al. [2019]) for the S bases. The average of each column represents the average free energy change for substituting each natural base with the isoC base.

Trinucleotide	$\Delta\Delta G$ (A-center)	$\Delta\Delta G$ (T-center)	$\Delta\Delta G$ (C-center)	$\Delta\Delta G$ (G-center)
ASA	-0.34	-0.87	0.60	0.28
AST	-0.34	-0.34	0.55	0.55
ASC	-0.20	-0.47	0.62	0.88
TSA	-0.80	-0.80	0.40	0.40
TST	-0.80	-0.27	0.35	0.67
TSC	-0.66	-0.40	0.42	1.01
CSA	0.00	-0.41	0.82	1.06
CST	0.00	0.12	0.76	1.33
CSC	0.13	-0.02	0.83	1.67
Average	-0.34	-0.39	0.59	0.87

Table C.4: Nearest neighbor stacking energies of all possible trinucleotide sequences with isoG (abbreviated B) at the center minus stacking energies for the same trinucleotide with each possible natural base at the center, reported in kcal/mol. Each trinucleotide free energy was computed from the two component di-nucleotide contributions calculated at 29°C. Nearest neighbor stacking enthalpies and entropies were taken from (SantaLucia Jr and Hicks [2004]) for natural nucleotides and from (Hoshika et al. [2019]) for the B base. The average of each column represents the average free energy change for substituting each natural base with the isoG base.

Trinucleotide	$\Delta\Delta G$ (A-center)	$\Delta\Delta G$ (T-center)	$\Delta\Delta G$ (C-center)	$\Delta\Delta G$ (G-center)
ABA	-0.27	-0.80	0.67	0.35
ABT	-0.34	-0.34	0.55	0.55
ABC	-0.22	-0.49	0.60	0.86
TBA	-0.80	-0.80	0.40	0.40
TBT	-0.87	-0.34	0.28	0.60
TBC	-0.75	-0.49	0.33	0.92
CBA	-0.47	-0.88	0.35	0.59
CBT	-0.54	-0.42	0.22	0.79
CBC	-0.43	-0.58	0.27	1.11
Average	-0.52	-0.57	0.41	0.69

C.4 Estimating *isoC* mismatch energies

In the experiments presented in Figure 4.5, we used a reporter containing internal mismatches in order to reuse a single reporter across multiple invader sequences without introducing large free-energy penalties from additional mismatches during strand invasion. However, the thermodynamic consequences of mismatch substitution are highly sequence dependent and poorly constrained. Using published nearest-neighbor mismatch parameters (Allawi and SantaLucia Jr [1998], Peyret et al. [1999], SantaLucia Jr and Hicks [2004]), the nearest-neighbor contexts surrounding both mismatch sites, a temperature of 25°C, and an effective salt concentration of 1 M Na⁺, we find that the maximum possible variation in $\Delta\Delta G$ across natural mismatch sequences is approximately 3.4 kcal mol⁻¹ for the mismatch closest to the toehold and 2.6 kcal mol⁻¹ for the more distal mismatch.

In the case of natural invaders, these energy differences can be more tightly constrained by design. At one mismatch position, we ensured that the invading base was identical to the incumbent base, resulting in a $C:T \rightarrow C:T$ substitution and a $\Delta\Delta G$ contribution of 0 kcal mol⁻¹. At the second mismatch position, this choice led to undesired interactions between natural components of the circuit, so we instead selected a compromise that minimized secondary structural defects while keeping the $\Delta\Delta G$ contribution close to zero. This resulted in a $\Delta\Delta G$ contribution of approximately -1.7 kcal mol⁻¹ for the natural substitution at the toehold-proximal mismatch.

Because a complete nearest-neighbor parameterization does not exist for *isoC*-containing mismatches, the free-energy change associated with *isoC* substitution cannot be calculated directly. Instead, during the design of the mismatch positions and invader sequences for this set of experiments, we used kinetic measurements comparing non-natural and natural mismatches (Figure 4.2) as a first-order proxy for mismatches with similar effective free energies. In this experiment, the kinetics of invader *isoC*:reporter T were most similar to those of invader C:reporter T. We therefore used the invader C:reporter T mismatch at both reporter positions to minimize the kinetic perturbation associated with replacing a C base

with isoC during strand displacement. Although the nearest-neighbor contexts between the reporter mismatch sites and the site used for the kinetic proxy were not identical, the total range of ΔG values for C:T mismatches across all natural nearest-neighbor contexts is approximately $1.3 \text{ kcal mol}^{-1}$, substantially narrower than the full range of mismatch energies within a fixed nearest-neighbor context.

It is important to note that, in the natural case, the mismatch closest to the reporter changes from invader C:reporter T to invader T:reporter T during strand displacement, a design choice made to minimize off-target reactions unrelated to background. We do not have a direct kinetic comparison between invader isoC:reporter T and invader T:reporter T in our proxy measurements. However, as shown above, substituting a C base with a T base at this position leads to a more negative free energy. Thus, if invader isoC:reporter T and invader C:reporter T are kinetically similar, substituting invader T:reporter T for invader isoC:reporter T would raise the free energy. At worst, this would lead to an underestimation of the energetic effect of introducing additional isoC bases into the trigger.

C.5 Exemplary Leak

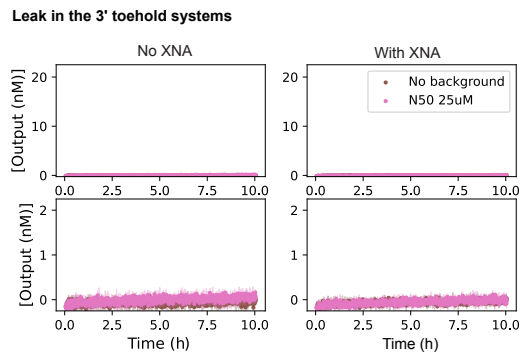


Figure C.3: Leak (output in the absence of input) for the 3' toehold cascade over a period of 10 hours. Data for the cascade without XNA is shown in the left column, and the variant with XNA on the right. The pink curves represent leak in the presence of N50 25 μM while the brown conditions are in the absence of background. Each row of the figure shows the same data, but the scale of the y-axis in the lower row is zoomed by a factor of ten. Values for quantifying the leak in each case is presented in Table C.5. When normalizing the R.F.U. values the reporter, baseline fluorescence without background was used for the cascade without background, and the reporter fluorescence with background was used for the cascade with background. This was done to decouple the increase in reporter auto-fluorescence with background from measuring leak resulting from the spurious interaction between gates.

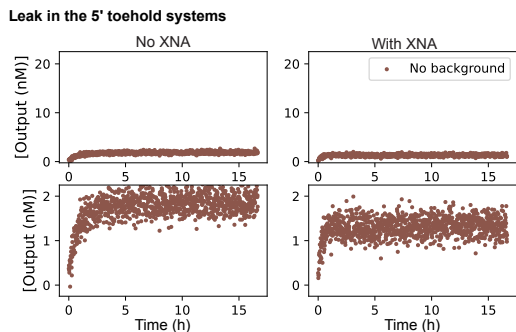


Figure C.4: Exemplary leak (output in the absence of input) for the 5' toehold cascade in the absence of background. Data for the cascade without XNA is shown in the left column, and the variant with XNA on the right. Each row of the figure shows the same data, but the scale of the y-axis in the lower row is zoomed by a factor of ten. Both systems show some initial fluorescence increase, which can result from slight inaccuracies in stoichiometry when annealing gates, however after plateauing the curves show a negligible further increase in signal. The inclusion of XNA in the circuit did not increase the initial fluorescence change or long-term leak.

Table C.5: Leak (output in the absence of input) for the 3' toehold system's reporters over a period of 10 hours. To differentiate leak resulting from the spurious interaction between gates and fluorescence from transient separation of the fluorophore and quencher on the reporter by background, the signal increase for each reporter in the presence of background was subtracted from the signal for the cascade in the presence of background.

Reporter type	Background condition	Completion increase over 10 hours
No XNA	No background	0.71%
No XNA	25 μ M N50	0.99%
With XNA	No background	1.02%
With XNA	25 μ M N50	0.64%

C.6 Reporter rate constants, 3' toehold system

We both analyzed the effect of a non-natural base substitution in the toehold and the effect of a natural:non-natural mismatch on the rate constant of a single strand displacement reaction. We fit data from both reporters, as well as the non-natural reporter triggered with the natural trigger, with a model for the following chemical reaction:



whose mass-action kinetics are defined by the following ordinary differential equation (ODE) model.

$$\frac{d[R]}{dt} = -\frac{d[WR]}{dt} = -\frac{d[Rt]}{dt} = -k_{rep}[F2t][R]$$

Firstly, the effective concentrations of each reporter was determined as described in Methods A.2.3. Then the above ODE was fit to kinetic curves for the reporter triggered at three sub-stoichiometric concentrations to determine k_{rep} . Since the reporter implements a strand

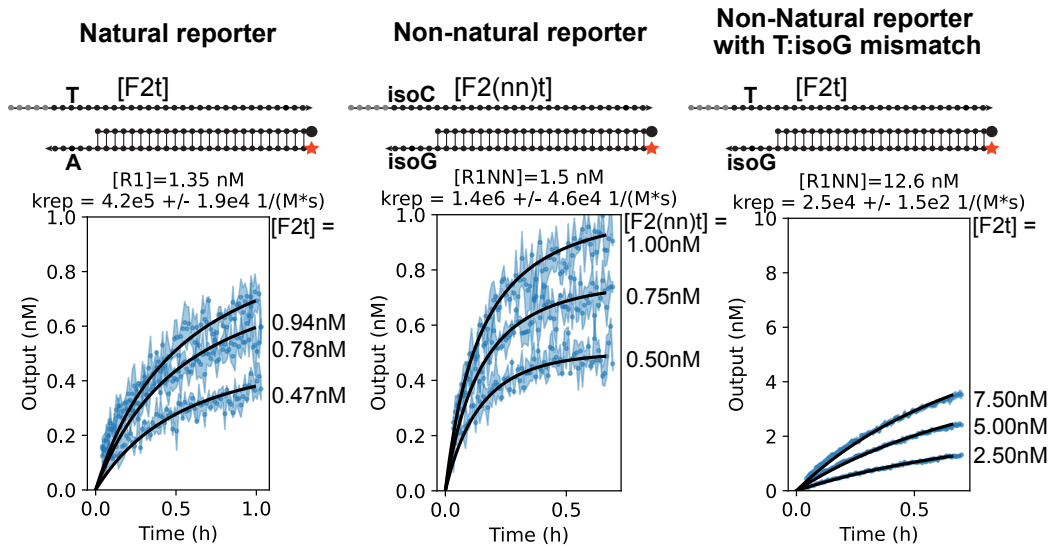
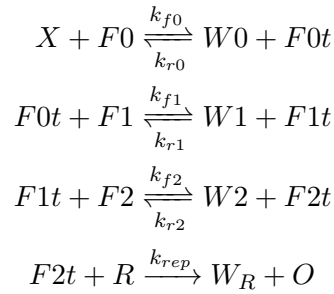


Figure C.5: Rate constants for the 3' toehold cascade's natural reporter (left), non-natural reporter (center), and non-natural reporter triggered with natural invader (right). The concentration of reporter and invader used in fitting the model are shown for each system. Black curves indicate the model fits.

displacement reaction rather than strand exchange, we approximate this to be an irreversible reaction. The experimental conditions and rate constant for each reporter analysis are presented in Figure C.5.

C.7 Rate constants for each complex in both 3' toehold cascades

To analyze the effect of XNA introduction on each gate in the cascade, we fit rate constants for each step of the natural and non-natural variants of the 5' and 3' toehold cascades. Firstly, as part of previous work, we fit both cascades to the following chemical reaction network (CRN) model:



whose mass-action kinetics are defined by the following ordinary differential equation (ODE) model.

$$\begin{aligned}
 \frac{d[X]}{dt} &= \frac{d[F0]}{dt} = -\frac{d[W0]}{dt} = k_{r0}[W0][F0t] - k_{f0}[X][F0] \\
 &\quad \frac{d[F0t]}{dt} = -\frac{d[F0]}{dt} - k_{f1}[F0t][F1] \\
 \frac{d[F1]}{dt} &= -\frac{d[W1]}{dt} = k_{r1}[W1][F1t] - k_{f1}[F0t][F1] \\
 &\quad \frac{d[F1t]}{dt} = -\frac{d[F1]}{dt} - k_{f2}[F1t][F2] \\
 \frac{d[F2]}{dt} &= -\frac{d[W2]}{dt} = k_{r2}[W2][F2t] - k_{f2}[F1t][F2] \\
 &\quad \frac{d[F2t]}{dt} = -\frac{d[F2]}{dt} - k_{rep}[F2t][R] \\
 \frac{d[R]}{dt} &= -\frac{d[W2]}{dt} = -\frac{d[O]}{dt} = -k_{rep}[F2t][R]
 \end{aligned}$$

Firstly, the effective concentrations of each gate in the systems were determined as de-

scribed in Methods A.2.3, which alongside absorbance measurements of the invader strands taken at 260nm using a Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometer were used to determine the concentration of each species in the starting system for fitting each rate. Next the F0 and R gates were triggered with F1-t at three sub-stoichiometric concentrations. Using the established k_{rep} it was possible to fit a single parameter k_{f2} , and use the detailed balance relationship $\frac{k_{f2}}{k_{r2}} = e^{\frac{-\Delta G_{rxn}}{RT}}$ – where ΔG_{rxn} is the free energy change of the reaction, R is the ideal gas constant, and T is the temperature – to determine k_{r2} . We continue this procedure for the remaining two gates, analyzing each reaction gate one at a time and using the previously fit rate constants and detailed balance to reduce the problem to a single parameter fit at each step. Since we fit reverse rate constants of effectively 0 for the natural system, and the non-natural additions are expected to provide even more thermodynamic drive – increasing the ratio of the forward rate constant to the reverse rate constant, we assumed all reverse rate constants to be zero for the non-natural system. For this supplementary detailed modeling analysis, k_{rep} was fit, but the kinetics for upstream gates were taken from the experimental data collected for Chapter 3. Because this value differs modestly from the k_{rep} used in the Chapter 3 detailed modeling, the fitted gate rates should be interpreted as effective parameters for qualitative comparison rather than as a re-analysis of the original Chapter 3 dataset. The fit-curves, rate constants, and experimental concentrations for each reaction are presented in Figure C.6.

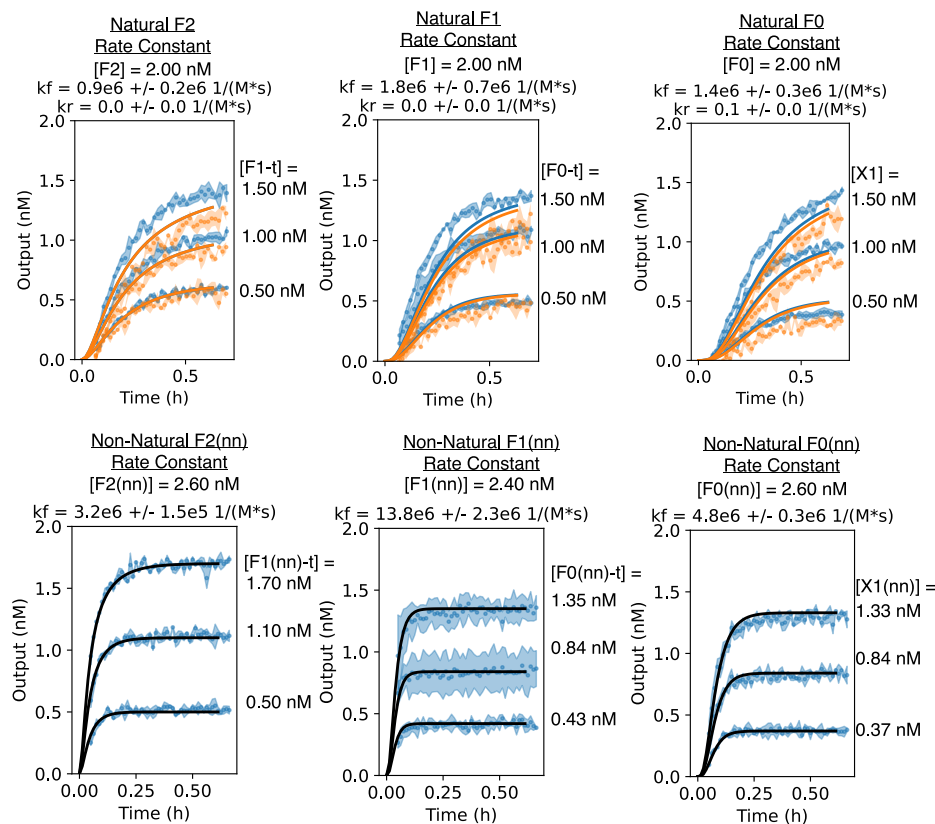


Figure C.6: Rate constants for each complex in the natural 3' toehold cascade (top row) and non-natural 3' toehold cascade (bottom row). Each column shows the rate constant for a different complex in the cascade. The data for the natural cascade displays two different colors of curves, which correspond to technical replicated collected on different days as part of a prior study. Otherwise each curve represents a different concentration of trigger, with the invader and concentration label. The concentration of gate of interest is labeled in the title of each plot. In determining the rate constant each gate, all upstream gates were omitted, the gate of interest was added at the concentration listed, and all downstream complexes were added at twice their listed concentration. The reporters were always added at 12nM for natural cascade and 17nM for the non-natural cascade.

C.8 *Fitting of kinetic constants*

For both the one-step and multi-step cascade systems we observed improved kinetics across nearly all cases. However, since we also observed increased kinetics in the absence of background it is natural to question whether the non-natural bases actually decrease interaction with background or if background-independent speed-up accounts entirely for the effect. This question is addressable given our data. We address this in two ways. For one, the speedup observed in the absence of background establishes a baseline, background-independent kinetic effect of the non-natural base pair. Any additional increase in the rate-constant ratio beyond this baseline under background conditions therefore reflects a background-specific contribution rather than altering fundamental kinetics of the reaction in isolation. In Figures 4.3 and 4.4, for each condition with background present, we compare the rate constant ratios between the non-natural and natural variants to the same ratio in the absence of background. We found that ratios were never lower than the control ratio (in the absence of background), so the system maintained at least the same speed-up. For shorter lengths and lower concentrations of background we found that the ratios were not significantly different, but for higher background pressure we did observe a significantly higher ratio, suggesting a speedup that goes beyond what can be explained by kinetic differences in the absence of background alone.

In Figure 4.6, we support this analysis by fitting the 5' toehold, one-step, no incubation data to a supportive mechanistic model that explicitly accounts for interaction with background (see Methods). The model treats the background as a singular averaged species and assigns all interaction with the background to the reporter. To maintain a single parameter to fit, this model assigns a reasonable value of $1 \times 10^7 M^{-1}s^{-1}$ to the association constants between the background and reporter (Wyer et al. [2014]), fixes the rate constant for interaction of the invader and reporter to that measured in the absence of background. This is equivalent to hypothesizing that rate of collision between background species and circuit components only changes based on their concentration, but the rate of dissociation

depends on their identity, such as background length or presence of isoG/isoC. Similarly to the above analysis, we found that for the N25 cases there was no significant difference in the dissociation rate constant between the natural and non-natural variants, but for the N50 cases we found that the dissociation rate constant significantly increased for the non-natural variant. This means the data is consistent with at least one mechanism in which the non-natural bases kinetically destabilize interaction between circuit components with the background.

As seen in the Supplementary Information (Compare Table C.6 and Figure C.10 to Table C.10 and Figure C.14), neither the empirical model nor this possible mechanistic model provide a better fit to the data, so both models are equally plausible statistically. However, when interpreted mechanistically, a changing rate constant at a given temperature implies a physical change in the structure of the reactants. So the fact that we see changes in the empirical rate constant when background is added without changing the reporter or invader identity suggests an interaction with the background that is encapsulated by the changing rate constant. While not the only possible model to explain the data, the multi-parameter mechanistic model shows that destabilizing interaction with the background is one hypothesis consistent with the data.

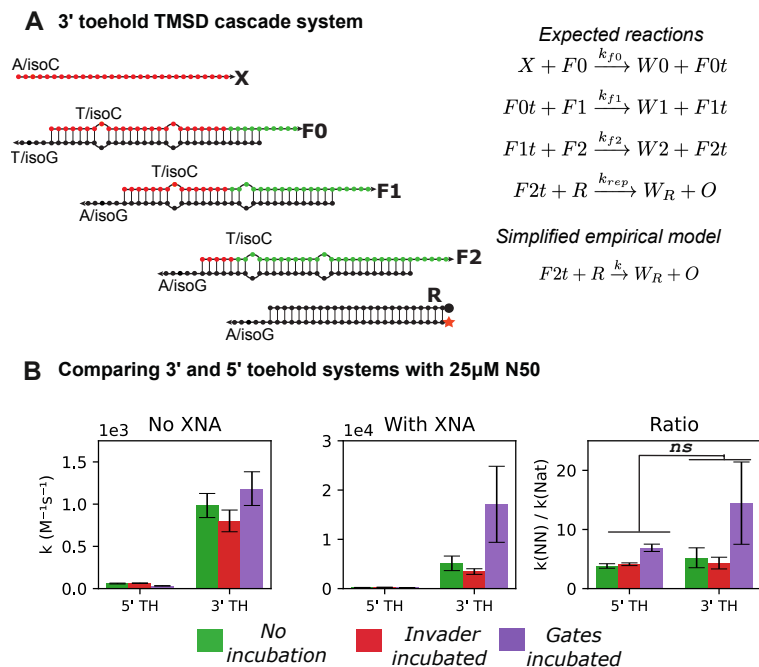


Figure C.7: (A) A schematic of the complete 5' toehold cascade. Each expected reaction in the cascade is depicted in Figure C.1. We list the chemical reactions expected in this mechanism alongside the simplified one-step model used for empirical comparison of the cascades. The decision to omit reverse reactions from the cascade is supported by detailed kinetic modeling of mismatch-optimized cascade systems in the absence of background as shown in Figure C.6 (B) Comparison of the 5' and 3' toehold multi-step cascades for one example background case (N50 at 25 μM). We found no statistically significant difference between the ratios presented here for the 5' and 3' toehold system tested separately across each incubation condition.

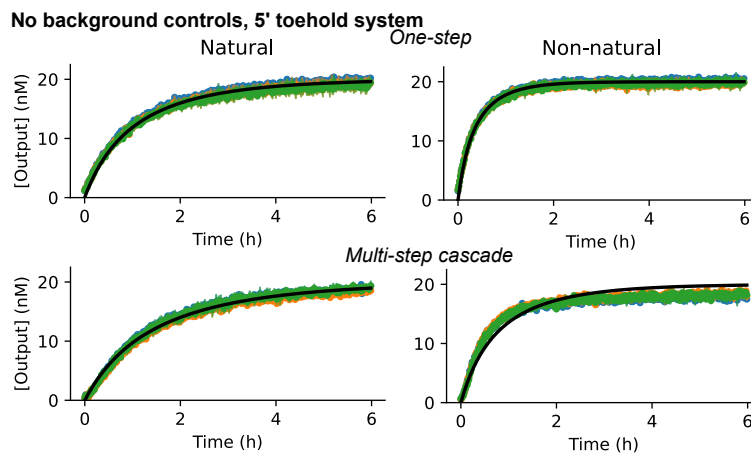


Figure C.8: Kinetic curves for control data (without background) for the one-step (top) and multi-step cascade (bottom) 5' toehold systems fit with an effective second-order rate constant. The three different colored curves represent the three sets of averaged duplicates used to estimate the variance of fit parameters by the jackknife method. The black curves represent the second-order models plotted with the average parameter for the fit determined through the jackknife method.

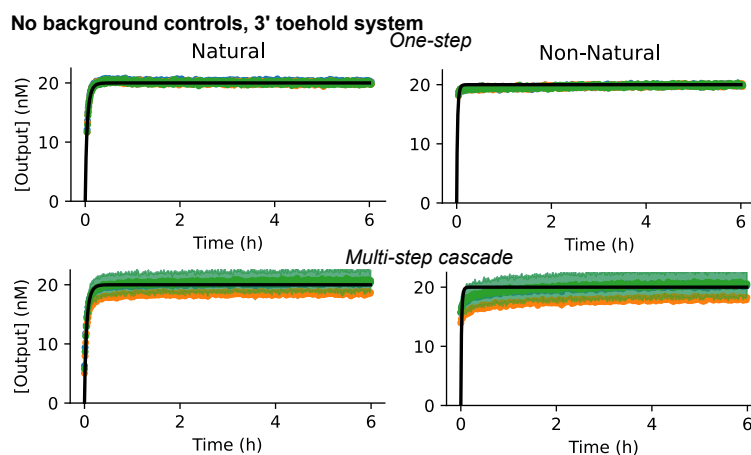


Figure C.9: Kinetic curves for control data (without background) for the one-step (top) and multi-step cascade (bottom) 3' toehold systems fit with an effective second-order rate constant. The three different colored curves represent the three sets of averaged duplicates used to estimate the variance of fit parameters by the jackknife method. The black curves represent the second-order models plotted with the average parameter for the fit determined through the jackknife method.

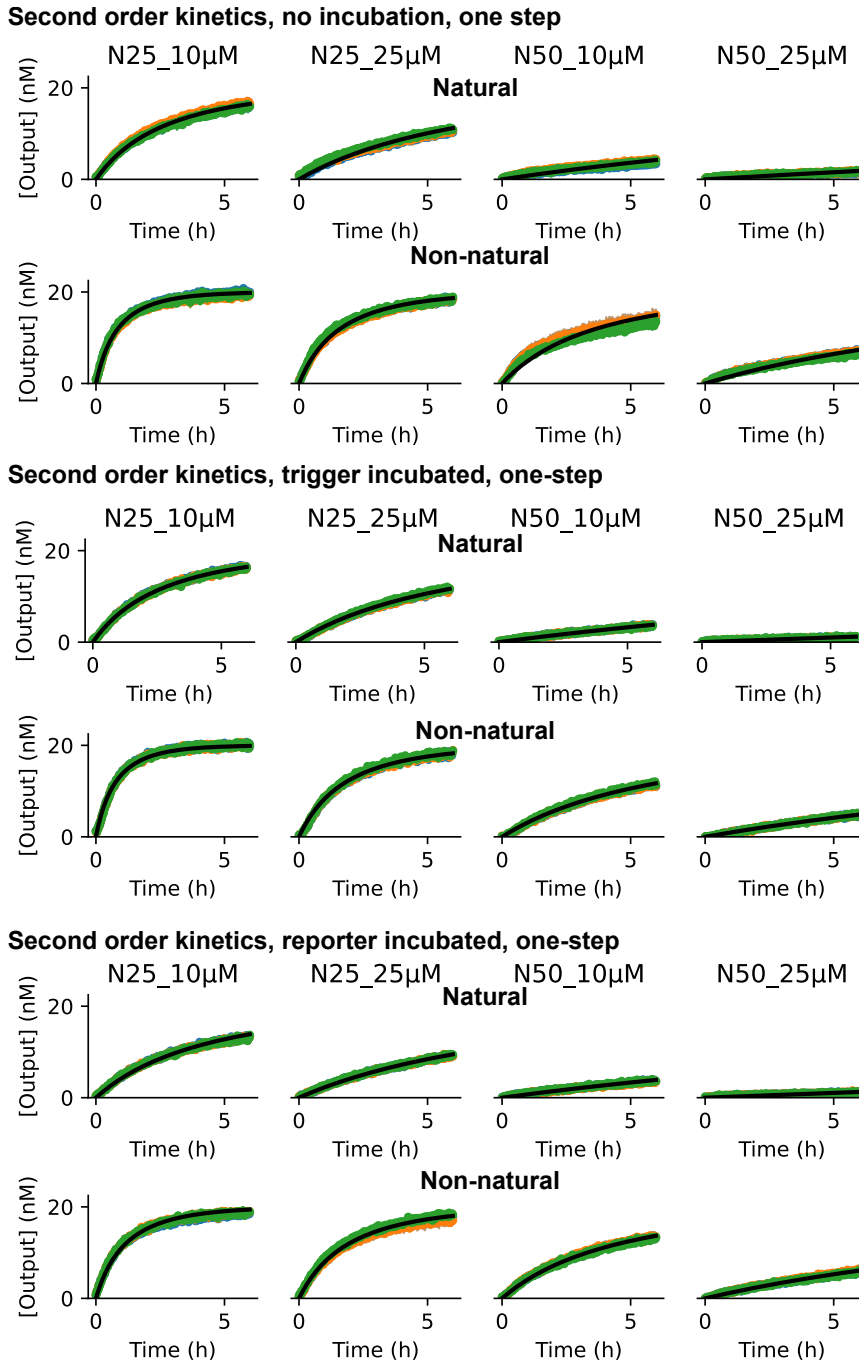


Figure C.10: Kinetic curves for the 5' toehold one-step system across incubation conditions, background concentrations and background lengths, fit with an effective second-order rate constant. The three different colored curves represent the three sets of averaged duplicates used to estimate the variance of fit parameters by the jackknife method. The black curves represent the second-order models plotted with the estimated parameter fit determined through the jackknife method.

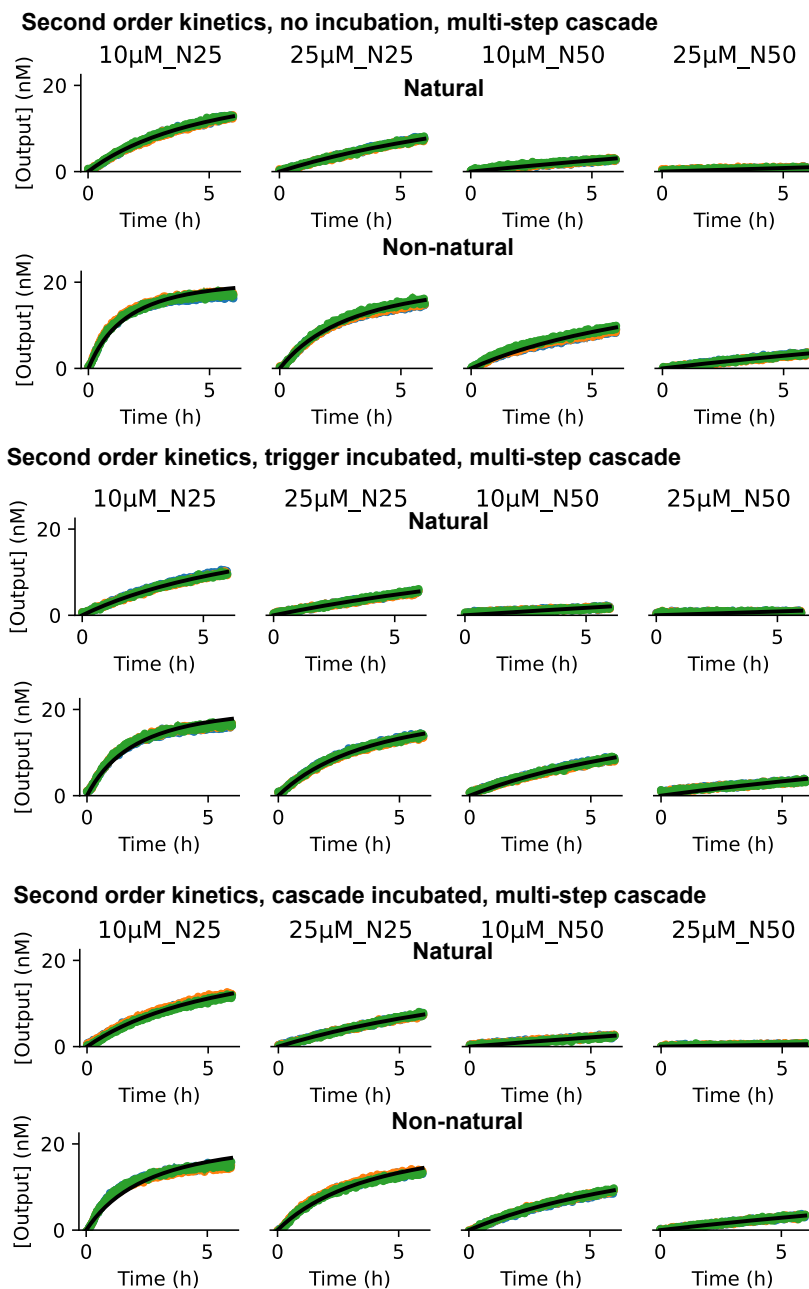


Figure C.11: Kinetic curves for the 5' toehold multi-step cascade system across incubation conditions, background concentrations and background lengths, fit with an effective second-order rate constant. The three different colored curves represent the three sets of averaged duplicates used to estimate the variance of fit parameters by the jackknife method. The black curves represent the second-order models plotted with the estimated parameter fit determined through the jackknife method.

3' toehold one-step system, N50 25 μ M

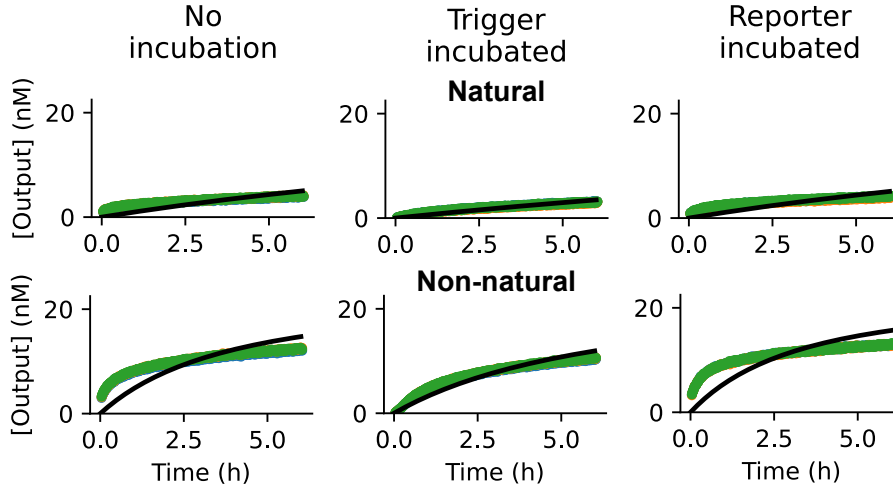


Figure C.12: Kinetic curves for the 3' toehold one-step system across incubation conditions with 25 μ M N50, fit with an effective second-order rate constant. The three different colored curves represent the three sets of averaged duplicates used to estimate the variance of fit parameters by the jackknife method. The black curves represent the second-order models plotted with the estimated parameter fit determined through the jackknife method.

3' toehold multi-step cascade, N50 25 μ M

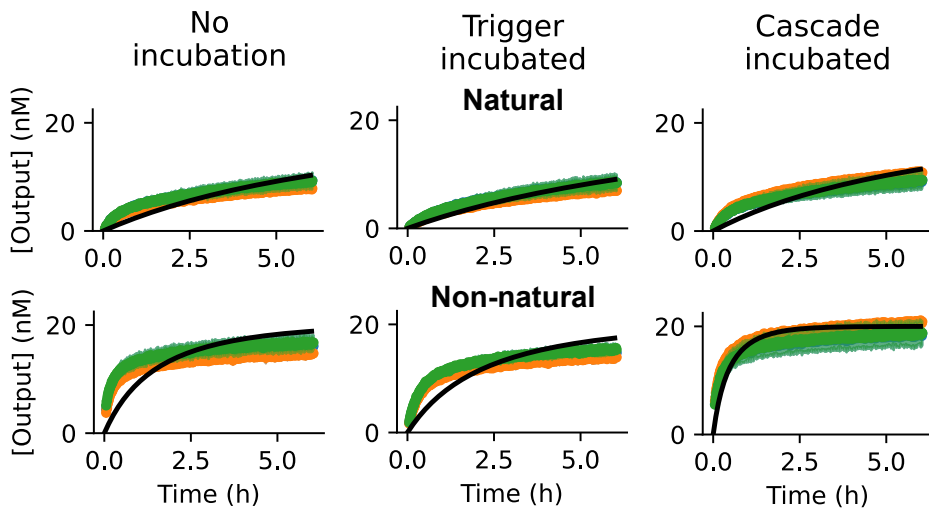


Figure C.13: Kinetic curves for the 3' toehold multi-step cascade system across incubation conditions with 25 μ M N50, fit with an effective second-order rate constant. The three different colored curves represent the three sets of averaged duplicates used to estimate the variance of fit parameters by the jackknife method. The black curves represent the second-order models plotted with the estimated parameter fit determined through the jackknife method.

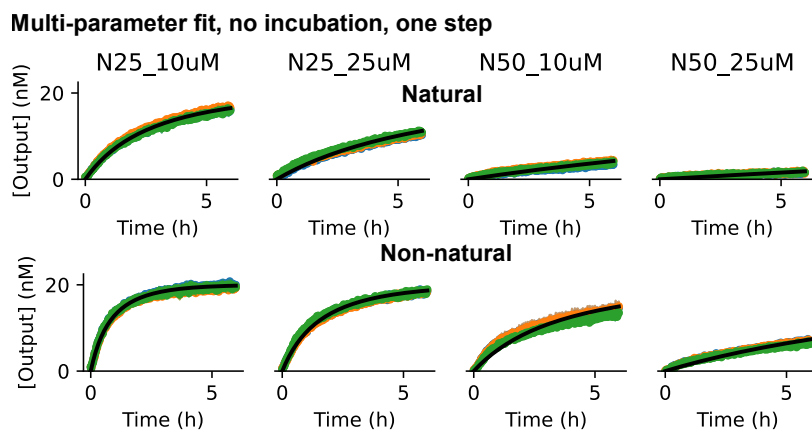


Figure C.14: Kinetic curves for the 3' toehold one-step system across incubation conditions, background concentrations and background lengths, fit with a the two parameter occlusion model as described in methods. This is an alternative mechanistic fit to the data presented in the top panel of Figure 4.6. The black curves represent the model fit.

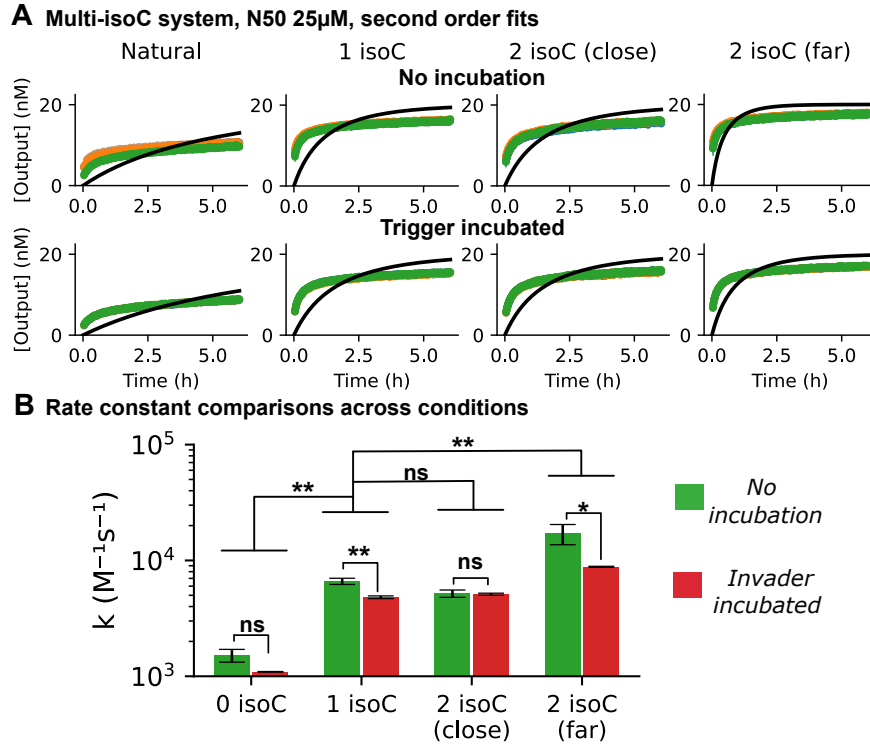


Figure C.15: A) Kinetic curves for the multi-iso system across incubation conditions, background concentrations and background lengths, fit with a second order rate model as described in methods. There is some qualitative deviation from the model even in the natural control. B) A consolidated summary of effective rate constant across conditions. The annotations above each bar indicate the significance of Wald Z-tests comparing differences between rate constants to the null hypothesis of 0 difference, where ns represents $p \geq 0.05$, * represents $0.01 \leq p < 0.05$, and ** represents $p < 0.01$. The annotations over groups of bars represent the results of inverse-variance pooled omnibus Wald test between the x-axis groupings, pooled across incubation conditions. The results agree with those in Figure 4.5A with the exception of identifying an additional marginally significant difference between the incubation conditions in the 2 isoC (far) case.

Table C.6: Effective rate constants and root mean squared error normalized to mean (nRMSE) for each condition tested in the one-step 5' toehold system.

Natural/ Non-natural	Background length (nt)	[background] (μM)	Incubation	k effective (M-1s-1)	nRMSE
Natural	0	0	None	$7.6\text{e}+03 \pm 7.6\text{e}+02$	0.033
Non-natural	0	0	None	$2.2\text{e}+04 \pm 1.1\text{e}+03$	0.023
Natural	25	10	None	$2.8\text{e}+03 \pm 2.0\text{e}+02$	0.038
Natural	25	25	None	$1.1\text{e}+03 \pm 8.2\text{e}+01$	0.083
Natural	50	10	None	$2.9\text{e}+02 \pm 4.3\text{e}+01$	0.174
Natural	50	25	None	$1.1\text{e}+02 \pm 7.6\text{e}+00$	0.243
Non-natural	25	10	None	$9.1\text{e}+03 \pm 5.4\text{e}+02$	0.028
Non-natural	25	25	None	$4.8\text{e}+03 \pm 2.7\text{e}+02$	0.035
Non-natural	50	10	None	$2.1\text{e}+03 \pm 2.8\text{e}+02$	0.101
Non-natural	50	25	None	$5.9\text{e}+02 \pm 3.3\text{e}+01$	0.107
Natural	25	10	Invader	$2.8\text{e}+03 \pm 5.0\text{e}+01$	0.023
Natural	25	25	Invader	$1.2\text{e}+03 \pm 3.1\text{e}+01$	0.031
Natural	50	10	Invader	$2.6\text{e}+02 \pm 2.7\text{e}+00$	0.091
Natural	50	25	Invader	$7.3\text{e}+01 \pm 3.8\text{e}+00$	0.262
Non-natural	25	10	Invader	$1.0\text{e}+04 \pm 1.8\text{e}+02$	0.023
Non-natural	25	25	Invader	$4.2\text{e}+03 \pm 1.5\text{e}+02$	0.030
Non-natural	50	10	Invader	$1.2\text{e}+03 \pm 3.3\text{e}+01$	0.036
Non-natural	50	25	Invader	$3.4\text{e}+02 \pm 3.3\text{e}+00$	0.070
Natural	25	10	Reporter	$1.8\text{e}+03 \pm 2.3\text{e}+01$	0.048
Natural	25	25	Reporter	$8.6\text{e}+02 \pm 9.2\text{e}+00$	0.042
Natural	50	10	Reporter	$2.6\text{e}+02 \pm 8.4\text{e}+00$	0.102
Natural	50	25	Reporter	$7.6\text{e}+01 \pm 9.7\text{e}-01$	0.245
Non-natural	25	10	Reporter	$6.7\text{e}+03 \pm 2.6\text{e}+02$	0.039
Non-natural	25	25	Reporter	$4.0\text{e}+03 \pm 3.8\text{e}+02$	0.038
Non-natural	50	10	Reporter	$1.7\text{e}+03 \pm 4.7\text{e}+01$	0.037
Non-natural	50	25	Reporter	$4.6\text{e}+02 \pm 1.6\text{e}+01$	0.071

Table C.7: Effective rate constants and root mean squared error normalized to mean (nRMSE) for each condition tested in the multi-step cascade 5' toehold system.

Natural/ Non-natural	Background length (nt)	[background] (μM)	Incubation	k effective (M-1s-1)	nRMSE
Natural	0	0	None	$5.4\text{e}+03 \pm 4.0\text{e}+02$	0.030
Non-natural	0	0	None	$1.0\text{e}+04 \pm 5.8\text{e}+02$	0.086
Natural	25	10	None	$1.5\text{e}+03 \pm 4.3\text{e}+01$	0.032
Natural	25	25	None	$6.2\text{e}+02 \pm 1.6\text{e}+01$	0.052
Natural	50	10	None	$2.0\text{e}+02 \pm 9.0\text{e}+00$	0.144
Natural	50	25	None	$5.9\text{e}+01 \pm 4.9\text{e}+00$	0.350
Non-natural	25	10	None	$4.8\text{e}+03 \pm 2.3\text{e}+02$	0.064
Non-natural	25	25	None	$2.5\text{e}+03 \pm 1.4\text{e}+02$	0.045
Non-natural	50	10	None	$8.7\text{e}+02 \pm 8.2\text{e}+01$	0.094
Non-natural	50	25	None	$2.3\text{e}+02 \pm 1.1\text{e}+01$	0.103
Natural	25	10	Invader	$9.5\text{e}+02 \pm 4.0\text{e}+01$	0.045
Natural	25	25	Invader	$4.0\text{e}+02 \pm 1.2\text{e}+01$	0.075
Natural	50	10	Invader	$1.3\text{e}+02 \pm 6.8\text{e}+00$	0.247
Natural	50	25	Invader	$6.4\text{e}+01 \pm 3.4\text{e}+00$	0.457
Non-natural	25	10	Invader	$3.8\text{e}+03 \pm 1.4\text{e}+02$	0.067
Non-natural	25	25	Invader	$1.9\text{e}+03 \pm 5.4\text{e}+01$	0.037
Non-natural	50	10	Invader	$7.7\text{e}+02 \pm 2.0\text{e}+01$	0.065
Non-natural	50	25	Invader	$2.6\text{e}+02 \pm 4.0\text{e}+00$	0.207
Natural	25	10	Reporter	$1.4\text{e}+03 \pm 8.3\text{e}+01$	0.046
Natural	25	25	Reporter	$6.0\text{e}+02 \pm 6.5\text{e}+00$	0.050
Natural	50	10	Reporter	$1.6\text{e}+02 \pm 9.9\text{e}+00$	0.163
Natural	50	25	Reporter	$3.3\text{e}+01 \pm 2.9\text{e}+00$	0.549
Non-natural	25	10	Reporter	$3.0\text{e}+03 \pm 1.4\text{e}+02$	0.093
Non-natural	25	25	Reporter	$1.9\text{e}+03 \pm 9.6\text{e}+01$	0.060
Non-natural	50	10	Reporter	$8.3\text{e}+02 \pm 2.6\text{e}+01$	0.050
Non-natural	50	25	Reporter	$2.2\text{e}+02 \pm 1.4\text{e}+00$	0.105

Table C.8: Effective rate constants and root mean squared error normalized to mean (nRMSE) for each condition tested in the one-step 3' toehold system.

Natural/ Non-natural	Background length (nt)	[background] (μM)	Incubation	k effective (M-1s-1)	nRMSE
Natural	0	0	None	$2.1\text{e}+05 \pm 1.2\text{e}+04$	0.012
Non-natural	0	0	None	$5.0\text{e}+05 \pm 7.0\text{e}+03$	0.019
Natural	50	25	None	$3.6\text{e}+02 \pm 9.4\text{e}+00$	0.284
Non-natural	50	25	None	$2.0\text{e}+03 \pm 6.9\text{e}+01$	0.214
Natural	50	25	Invader	$2.3\text{e}+02 \pm 7.7\text{e}+00$	0.162
Non-natural	50	25	Invader	$1.3\text{e}+03 \pm 3.1\text{e}+01$	0.129
Natural	50	25	Reporter	$3.7\text{e}+02 \pm 1.5\text{e}+01$	0.280
Non-natural	50	25	Reporter	$2.4\text{e}+03 \pm 9.3\text{e}+00$	0.224

Table C.9: Effective rate constants and root mean squared error normalized to mean (nRMSE) for each condition tested in the multi-step cascade 3' toehold system.

Natural/ Non-natural	Background length (nt)	[background] (μM)	Incubation	k effective (M-1s-1)	nRMSE
Natural	0	0	None	$2.0\text{e}+05 \pm 7.0\text{e}+04$	0.052
Non-natural	0	0	None	$6.1\text{e}+05 \pm 4.5\text{e}+05$	0.087
Natural	50	25	None	$9.8\text{e}+02 \pm 1.4\text{e}+02$	0.198
Non-natural	50	25	None	$5.1\text{e}+03 \pm 1.5\text{e}+03$	0.203
Natural	50	25	Invader	$8.0\text{e}+02 \pm 1.3\text{e}+02$	0.160
Non-natural	50	25	Invader	$3.5\text{e}+03 \pm 5.7\text{e}+02$	0.186
Natural	50	25	Reporter	$1.2\text{e}+03 \pm 2.0\text{e}+02$	0.193
Non-natural	50	25	Reporter	$1.7\text{e}+04 \pm 7.7\text{e}+03$	0.097

Table C.10: Effective k_{off} and root mean squared error normalized to mean (nRMSE) for the multi-parameter fitting experiment described in Figure 4.6 and Methods. The model is fit to each of the experiments across background lengths and concentrations for the 5' toehold one-step system.

Natural/ Non-natural	Background length (nt)	[background] (μM)	Incubation	k_{off} (s^{-1})	nRMSE
Natural	25	10	None	$5.8\text{e}+01 \pm 6.8\text{e}+00$	0.033
Natural	25	25	None	$4.4\text{e}+01 \pm 3.7\text{e}+00$	0.072
Natural	50	10	None	$4.0\text{e}+00 \pm 6.1\text{e}-01$	0.172
Natural	50	25	None	$3.8\text{e}+00 \pm 2.6\text{e}-01$	0.226
Non-natural	25	10	None	$6.9\text{e}+01 \pm 6.9\text{e}+00$	0.023
Non-natural	25	25	None	$5.0\text{e}+01 \pm 0.0\text{e}+00$	0.032
Non-natural	50	10	None	$1.0\text{e}+01 \pm 1.4\text{e}+00$	0.095
Non-natural	50	25	None	$6.9\text{e}+00 \pm 4.6\text{e}-01$	0.105

Table C.11: Effective rate constants and root mean squared error normalized to mean (nRMSE) for each condition tested in the multi-isoC system presented in Figure C.15.

Natural/ Non-natural	Background length (nt)	[background] (μM)	Incubation	k ($\text{M}^{-1}\text{s}^{-1}$)	nRMSE
Natural	50	25	None	$1.5\text{e}+03 \pm 1.9\text{e}+02$	0.283
1 isoC	50	25	None	$6.6\text{e}+03 \pm 4.1\text{e}+02$	0.215
2 isoC (close)	50	25	None	$5.2\text{e}+03 \pm 3.8\text{e}+02$	0.205
2 isoC (far)	50	25	None	$1.7\text{e}+04 \pm 3.4\text{e}+03$	0.161
Natural	50	25	Invader	$1.1\text{e}+03 \pm 6.5\text{e}+00$	0.267
1 isoC	50	25	Invader	$4.8\text{e}+03 \pm 1.3\text{e}+02$	0.218
2 isoC (close)	50	25	Invader	$6.9\text{e}+01 \pm 6.9\text{e}+00$	0.200
2 isoC (far)	50	25	Invader	$8.8\text{e}+03 \pm 9.0\text{e}+01$	0.177

Table C.12: Results of Holm adjusted Wald Z-tests comparing non-natural to natural all tested conditions across 5' and 3' single-step and multi-step cascade systems to the null hypothesis of 1.

System	Background Concentration (μM)	Incubation Mode	Test value	Comparison value	Test statistic	p-value	Holm adjusted p-value	Significance (ns/*/**)
One-step 5' N25	10	No incubation	3.247 $\pm$ 0.305	2.935 $\pm$ 0.330	0.694	4.88E-01	4.88E-01	n.s.
One-step 5' N25	25	No incubation	4.225 $\pm$ 0.381	2.935 $\pm$ 0.330	2.561	1.04E-02	5.22E-02	n.s.
One-step 5' N50	10	No incubation	7.222 $\pm$ 1.433	2.935 $\pm$ 0.330	2.916	3.55E-03	2.13E-02	*
One-step 5' N50	25	No incubation	5.155 $\pm$ 0.445	2.935 $\pm$ 0.330	4.009	6.10E-05	4.88E-04	**
One-step 5' N25	10	Invader incubated	3.633 $\pm$ 0.091	2.935 $\pm$ 0.330	2.04	4.14E-02	1.24E-01	n.s.
One-step 5' N25	25	Invader incubated	3.460 $\pm$ 0.152	2.935 $\pm$ 0.330	1.444	1.49E-01	2.97E-01	n.s.
One-step 5' N50	10	Invader incubated	4.826 $\pm$ 0.137	2.935 $\pm$ 0.330	5.295	1.19E-07	1.19E-06	**
One-step 5' N50	25	Invader incubated	4.742 $\pm$ 0.254	2.935 $\pm$ 0.330	4.34	1.43E-05	1.28E-04	**
One-step 5' N25	10	Reporter incubated	3.794 $\pm$ 0.155	2.935 $\pm$ 0.330	2.359	1.83E-02	7.33E-02	n.s.
One-step 5' N25	25	Reporter incubated	4.621 $\pm$ 0.444	2.935 $\pm$ 0.330	3.051	2.29E-03	1.60E-02	*
One-step 5' N50	10	Reporter incubated	6.442 $\pm$ 0.271	2.935 $\pm$ 0.330	8.212	2.22E-16	2.67E-15	**
One-step 5' N50	25	Reporter incubated	6.015 $\pm$ 0.226	2.935 $\pm$ 0.330	7.707	1.29E-14	1.42E-13	**
One-step 5' N25	10	No incubation	3.211 $\pm$ 0.179	1.857 $\pm$ 0.174	5.42	5.94E-08	2.38E-07	**
Cascade 5' N25	10	No incubation	3.986 $\pm$ 0.246	1.857 $\pm$ 0.174	7.07	1.55E-12	9.32E-12	**
Cascade 5' N25	25	No incubation	4.332 $\pm$ 0.456	1.857 $\pm$ 0.174	5.073	3.91E-07	1.17E-06	**
Cascade 5' N50	10	No incubation	3.828 $\pm$ 0.380	1.857 $\pm$ 0.174	4.723	2.32E-06	4.65E-06	**
Cascade 5' N25	10	Invader incubated	3.975 $\pm$ 0.218	1.857 $\pm$ 0.174	7.595	3.09E-14	2.16E-13	**
Cascade 5' N25	25	Invader incubated	4.717 $\pm$ 0.192	1.857 $\pm$ 0.174	11.056	0.00E+00	0.00E+00	**
Cascade 5' N50	10	Invader incubated	5.993 $\pm$ 0.352	1.857 $\pm$ 0.174	10.547	0.00E+00	0.00E+00	**
Cascade 5' N50	25	Invader incubated	4.120 $\pm$ 0.230	1.857 $\pm$ 0.174	7.853	4.00E-15	3.60E-14	**
Cascade 5' N25	10	Reporter incubated	2.175 $\pm$ 0.167	1.857 $\pm$ 0.174	1.321	1.87E-01	1.87E-01	n.s.
Cascade 5' N25	25	Reporter incubated	3.193 $\pm$ 0.164	1.857 $\pm$ 0.174	5.596	2.19E-08	1.10E-07	**
Cascade 5' N50	10	Reporter incubated	5.108 $\pm$ 0.350	1.857 $\pm$ 0.174	8.316	0.00E+00	0.00E+00	**
Cascade 5' N50	25	Reporter incubated	3.60E-14	1.857 $\pm$ 0.174	7.828	4.89E-15	3.91E-14	**
One-step 3' N50	25	No incubation	3.250 $\pm$ 0.278	1.400 $\pm$ 0.136	11.708	0.00E+00	0.00E+00	**
One-step 3' N50	25	Invader incubated	3.122 $\pm$ 0.264	1.400 $\pm$ 0.136	11.826	0.00E+00	0.00E+00	**
One-step 3' N50	25	Reporter incubated	4.126 $\pm$ 0.305	1.400 $\pm$ 0.136	13.521	0.00E+00	0.00E+00	**
Cascade 3' N50	25	No incubation	2.114 $\pm$ 3.046	2.100 $\pm$ 2.539	0.694	4.88E-01	9.75E-01	n.s.
Cascade 3' N50	25	Invader incubated	1.217 $\pm$ 2.726	2.100 $\pm$ 2.539	0.446	6.55E-01	9.75E-01	n.s.
Cascade 3' N50	25	Reporter incubated	11.355 $\pm$ 7.409	2.100 $\pm$ 2.539	1.533	1.25E-01	3.76E-01	n.s.

Table C.13: Results of Holm adjusted Wald Z-tests comparing non-natural to natural all tested conditions across 5' and 3' single-step and multi-step cascade systems to a null hypothesis ratio of 1.

System	Background Concentration (μM)	Incubation Mode	Test Type	Test statistic	p-value	Holm adjusted p-value	Significance (ns/*/**)
One-step 5'TH None	0	No incubation	Wald Z-test	5.871	4.34E-09	8.67E-09	**
One-step 5'TH N25	10	No incubation	Wald Z-test	7.361	1.83E-13	5.48E-13	**
One-step 5'TH N25	25	No incubation	Wald Z-test	8.464	0.00E+00	0.00E+00	**
One-step 5'TH N50	10	No incubation	Wald Z-test	4.343	1.41E-05	1.41E-05	**
One-step 5'TH N50	25	No incubation	Wald Z-test	9.337	0.00E+00	0.00E+00	**
One-step 5'TH N25	10	Invader incubated	Wald Z-test	28.863	0.00E+00	0.00E+00	**
One-step 5'TH N25	25	Invader incubated	Wald Z-test	16.142	0.00E+00	0.00E+00	**
One-step 5'TH N50	10	Invader incubated	Wald Z-test	27.877	0.00E+00	0.00E+00	**
One-step 5'TH N50	25	Invader incubated	Wald Z-test	14.706	0.00E+00	0.00E+00	**
One-step 5'TH N25	10	Reporter incubated	Wald Z-test	18.028	0.00E+00	0.00E+00	**
One-step 5'TH N25	25	Reporter incubated	Wald Z-test	8.163	2.22E-16	8.88E-16	**
One-step 5'TH N50	10	Reporter incubated	Wald Z-test	20.048	0.00E+00	0.00E+00	**
One-step 5'TH N50	25	Reporter incubated	Wald Z-test	22.187	4.34E-09	8.67E-09	**
Cascade 5'TH None	0	No incubation	Wald Z-test	4.927	8.35E-07	8.35E-07	**
Cascade 5'TH N25	10	No incubation	Wald Z-test	12.326	0.00E+00	0.00E+00	**
Cascade 5'TH N25	25	No incubation	Wald Z-test	12.139	0.00E+00	0.00E+00	**
Cascade 5'TH N50	10	No incubation	Wald Z-test	7.309	2.70E-13	8.10E-13	**
Cascade 5'TH N50	25	No incubation	Wald Z-test	7.452	9.17E-14	3.67E-13	**
Cascade 5'TH N25	10	Invader incubated	Wald Z-test	13.641	0.00E+00	0.00E+00	**
Cascade 5'TH N25	25	Invader incubated	Wald Z-test	19.402	0.00E+00	0.00E+00	**
Cascade 5'TH N50	10	Invader incubated	Wald Z-test	14.204	0.00E+00	0.00E+00	**
Cascade 5'TH N50	25	Invader incubated	Wald Z-test	13.57	0.00E+00	0.00E+00	**
Cascade 5'TH N25	10	Reporter incubated	Wald Z-test	7.025	2.15E-12	4.29E-12	**
Cascade 5'TH N25	25	Reporter incubated	Wald Z-test	13.403	0.00E+00	0.00E+00	**
Cascade 5'TH N50	10	Reporter incubated	Wald Z-test	11.731	0.00E+00	0.00E+00	**
Cascade 5'TH N50	25	Reporter incubated	Wald Z-test	9.51	0.00E+00	0.00E+00	**
One-step 3'TH None	0	No incubation	Wald Z-test	10.317	0.00E+00	0.00E+00	**
One-step 3'TH N50	25	No incubation	Wald Z-test	19.201	0.00E+00	0.00E+00	**
One-step 3'TH N50	25	Invader incubated	Wald Z-test	19.968	0.00E+00	0.00E+00	**
One-step 3'TH N50	25	Reporter incubated	Wald Z-test	20.216	0.00E+00	0.00E+00	**
Cascade 3'TH None	0	No incubation	Wald Z-test	0.827	4.08E-01	4.08E-01	n.s.
Cascade 3'TH N50	25	No incubation	Wald Z-test	2.505	1.23E-02	3.68E-02	*
Cascade 3'TH N50	25	Invader incubated	Wald Z-test	3.345	8.22E-04	3.29E-03	**
Cascade 3'TH N50	25	Reporter incubated	Wald Z-test	1.933	5.32E-02	1.06E-01	n.s.

C.9 Tables of all sequences

Table C.14: Promiscuity testing

System	Strand	Sequences
5'th	invader (A)	CGGAATAAGGCAAGATAAGAACAGATA A AT
5'th	invader (C)	CGGAATAAGGCAAGATAAGAACAGATA C AT
5'th	invader (G)	CGGAATAAGGCAAGATAAGAACAGATA G AT
5'th	invader (T)	CGGAATAAGGCAAGATAAGAACAGATA T AT
5'th	invader (isoC)	CGGAATAAGGCAAGATAAGAACAGATA/ iMe-isodC /AT
5'th	reporter top	/5IAbRQ/CGGAATAAGGCAAGATAAGAACAGA
5'th	reporter bottom (A)	AT A TATCTGTTCTTATCTTGCCTTATTCCG/3Rox N/
5'th	reporter bottom (C)	AT C TATCTGTTCTTATCTTGCCTTATTCCG/3Rox N/
5'th	reporter bottom (G)	AT G TATCTGTTCTTATCTTGCCTTATTCCG/3Rox N/
5'th	reporter bottom (T)	AT T TATCTGTTCTTATCTTGCCTTATTCCG/3Rox N/
5'th	reporter bottom (isoG)	AT/ iisodG /TATCTGTTCTTATCTTGCCTTATTCCG/3Rox N/

Table C.15: Displacement cascades

System	Strand	Sequences
5'th	X1	TCTCTCCAAATCCCTTCCACTCCCACTCAC
5'th	F0-t	ACAGATATATTCTCTCCGAATCCCTTCCACTCCCA
5'th	F0-b	GTGAGTGGGAGTAGAAGGGATTTGGAGAGAATATA
5'th	F1-t	CAAGATAGGAACAGATAGATTCTCTCCCAATCCCT
5'th	F1-b	GTGGAAGGGATTTCGAGAGAAATATATCTGTTCTTA
5'th	F2-t	CGGAATAAGGCAAGATAAGAACAGATACATTCTCT
5'th	F2-b	TTGGGAGAGAATCTATCTGTTCTCTATCTTGCCTTA
5'th	R-t	/5IAbRQ/CGGAATAAGGCAAGATAAGAACAGA
5'th	R-b	ATGTATCTGTTCTTATCTTGCCTTATTCCG/3Rox N/
5'th	X1(nn)	TCTCTCCAAATCCCTTCCACTCCCACT/iMe-isodC/AC
5'th	F0(nn)-t	ACAGATATATTCTCTCCGAATCCCTTC/iMe-isodC/ACTCCCA
5'th	F0(nn)-b	GT/iisodG/AGTGGGAGTGAAGGGATTTGGAGAGAATATA
5'th	F1(nn)-t	CAAGATAGGAACAGATAGATTCTCTCC/iMe-isodC/AATCCCT
5'th	F1(nn)-b	GT/iisodG/GAAGGGATTTCGAGAGAAATATATCTGTTCTTA
5'th	F2(nn)-t	CGGAATAAGGCAAGATAAGAACAGATA/iMe-isodC/ATTCTCT
5'th	F2(nn)-b	TT/iisodG/GGAGAGAATCTATCTGTTCTCTATCTTGCCTTA
5'th	R(nn)-t	/5IAbRQ/CGGAATAAGGCAAGATAAGAACAGA
5'th	R(nn)-b	AT/iisodG/TATCTGTTCTTATCTTGCCTTATTCCG/3Rox N/
3'th	X1	CCATCACCTCACCTTCCCTAAACCTCTCT
3'th	F0-t	ACCCTCACCTTCCCTAAACCTCTCTCATCTACTAC
3'th	F0-b	AGATGAGAGAGGTTTAGGGAAGGTGAGGGTGATGG
3'th	F1-t	TCCCTAAACCTCTCTCATCTACTACAACCACAAAC
3'th	F1-b	TGGTTGTAGTAGATGAGAGAGGTTTAGGGAAGGTG
3'th	F2-t	TCTCTCATCTACTACAACCACAAACATACCAACAA
3'th	F2-b	GGTATGTTTGTGGTTGTAGTAGATGAGAGAGGTTT
3'th	R1-t	ACTACAACCACAAACATACCAACAA/3BHQ_2/
3'th	R1-b	/5ATT0565N/TTGTTGGTATGTTTGTGGTTGTAGTAGATG
3'th	X1(nn)	CC/iMe-isodC/TCACCTCACCTTCCCTAAACCTCTCT
3'th	F0(nn)-t	ACCCTCA/iMe-isodC/CTTCCCTAACCTCTCTCACTACTAC
3'th	F0(nn)-b	AGTTGAGAGAGGTTTAGGGAAGGTGAGGGTGA/iisodG/GG
3'th	F1(nn)-t	TCCCTAA/iMe-isodC/CCTCTCTACCTACTACAAACACAAAC
3'th	F1(nn)-b	TGTTTGTAGTAGTTGAGAGAGGTTAGGGAAG/iisodG/TG
3'th	F2(nn)-t	TCTCTCA/iMe-isodC/CTACTACAACCACAAACATACCAACAA
3'th	F2(nn)-b	GGTATGTTTGTGGTTGTAGTAGGTGAGAGAGG/iisodG/TT
3'th	R1(nn)-t	ACTACAACCACAAACATACCAACAA/3BHQ_2/
3'th	R1(nn)-b	/5ATT0565N/TTGTTGGTATGTTTGTGGTTGTAGTAG/iisodG/TG
MultiIso	X2	TTTTCATCTTCTCCTCTCCAACTACTAC
MultiIso	W-t(nn2,far)	TTCTCCTCTCCA/iMe-isodC/CTACTACATCCACAAAC/iMe-isodC/TACCAACAA
MultiIso	W-t(nn2,close)	TTCTCCTCTCCA/iMe-isodC/CTACTACA/iMe-isodC/CCACAAACCTACCAACAA
MultiIso	W-t(nn1)	TTCTCCTCTCCA/iMe-isodC/CTACTACATCCACAAACCTACCAACAA
MultiIso	W-t	TTCTCCTCTCCATCTACTACATCCACAAACCTACCAACAA
MultiIso	W-b	TGGATGTAGTAGTTGGAGAGGAGAAGGATG
MultiIso	R2(nn)-t	ACTACACCCACAAACCTACCAACAA/3BHQ_2/
MultiIso	R2(nn)-b	/5ATT0565N/TTGTTGGTATGTTTGTGGTTGTAGTAG/iisodG/TG
MultiIso	R2-t	ACTACACCCACAAACCTACCAACAA/3BHQ_2/
MultiIso	R2-b	/5ATT0565N/TTGTTGGTATGTTTGTGGTTGTAGTAGATG

Appendix D

CHAPTER 5 SUPPLEMENTARY INFORMATION***D.1 Detailed architecture description***

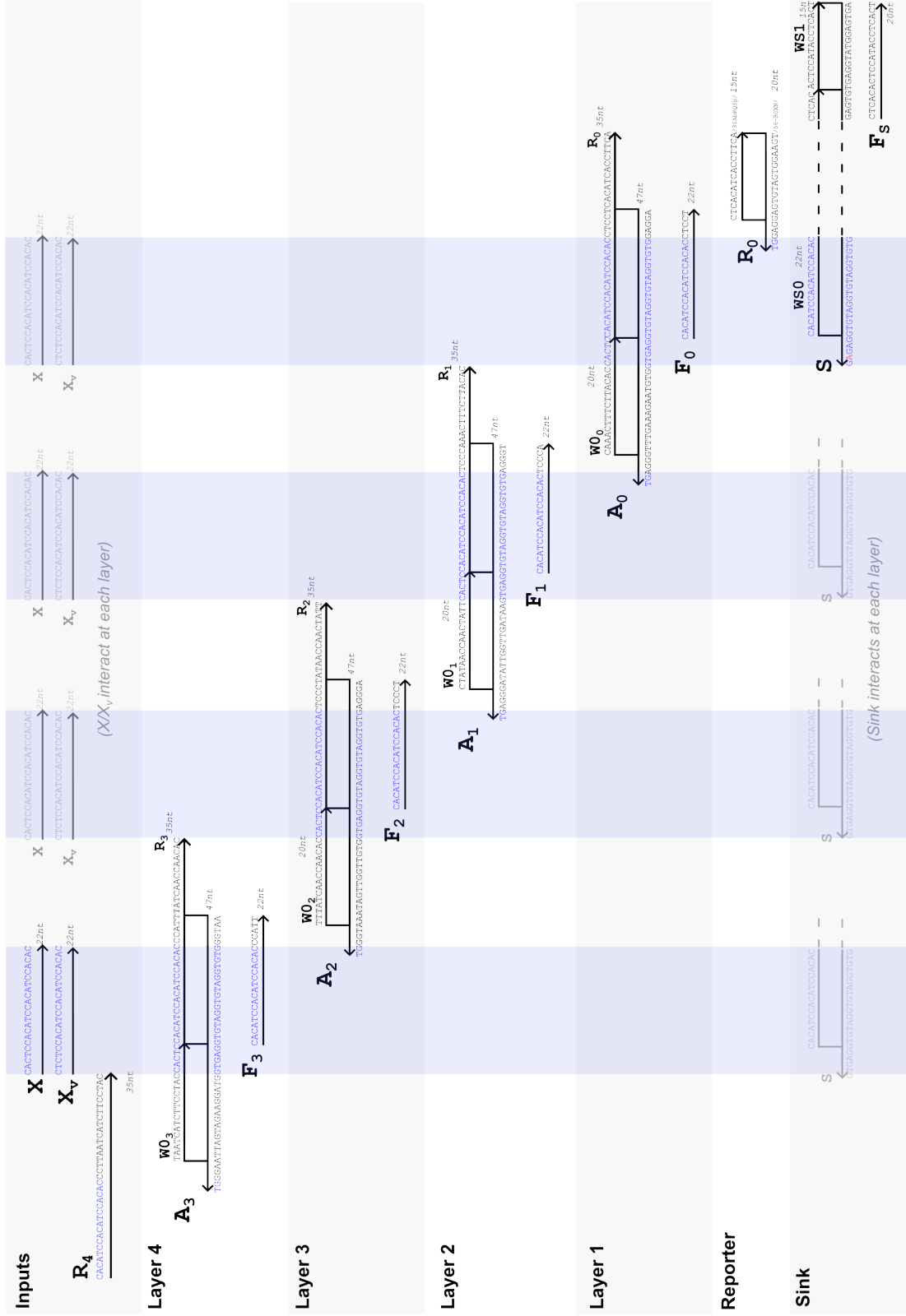
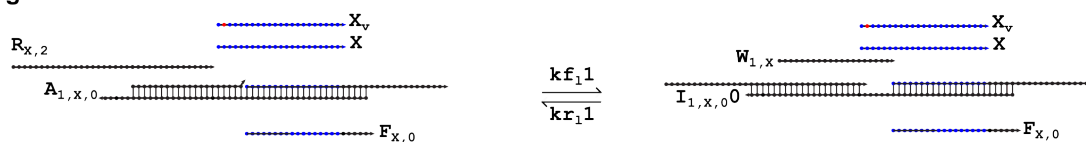
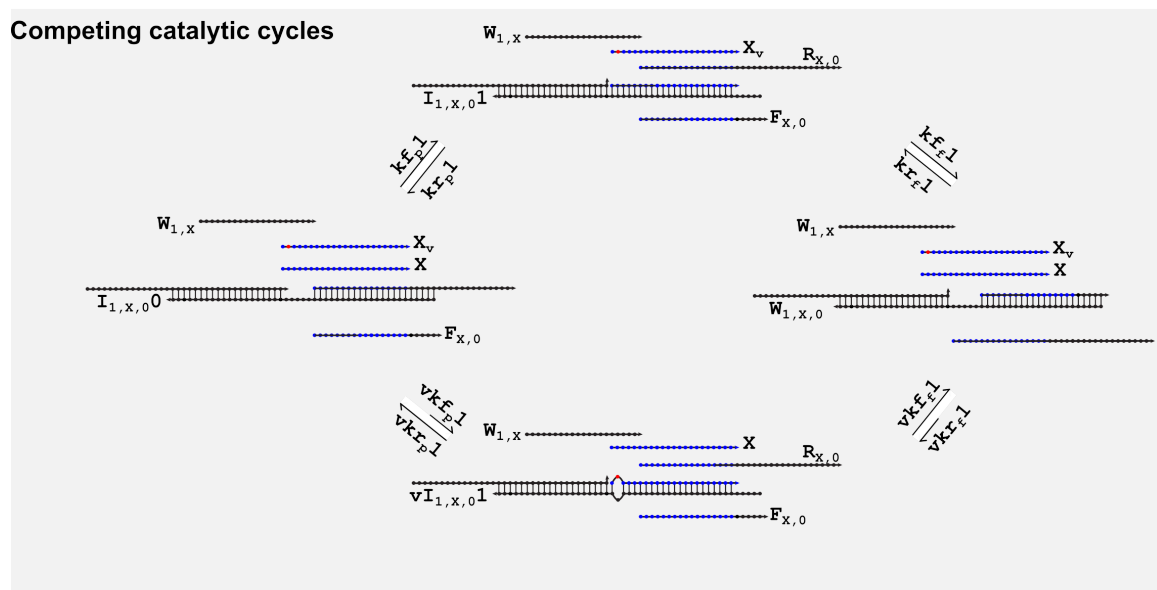


Figure D.1: A schematic of each reactant species used in the four layer conditionally gated amplifier system and their respective sequences.

Gating reaction



Competing catalytic cycles



Irreversible reporting reaction

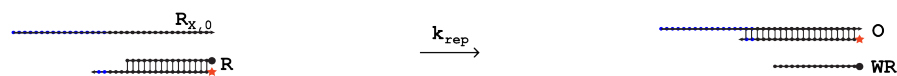
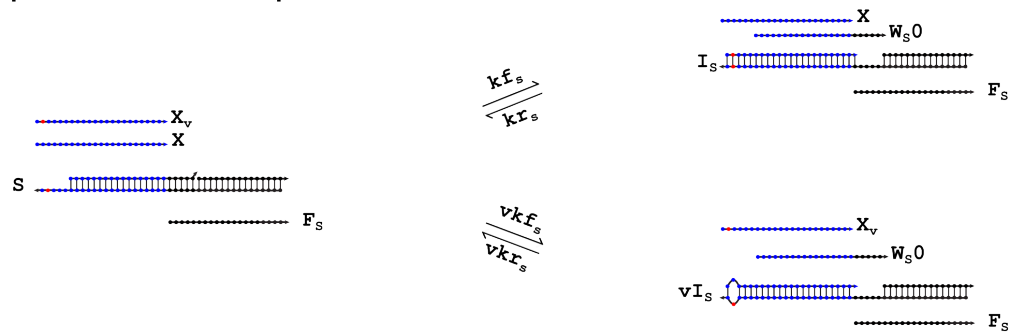


Figure D.2: Latent regulator cascade propagation reactions

Initial competitive reversible sequestration



Fuel-mediated irreversible sequestration

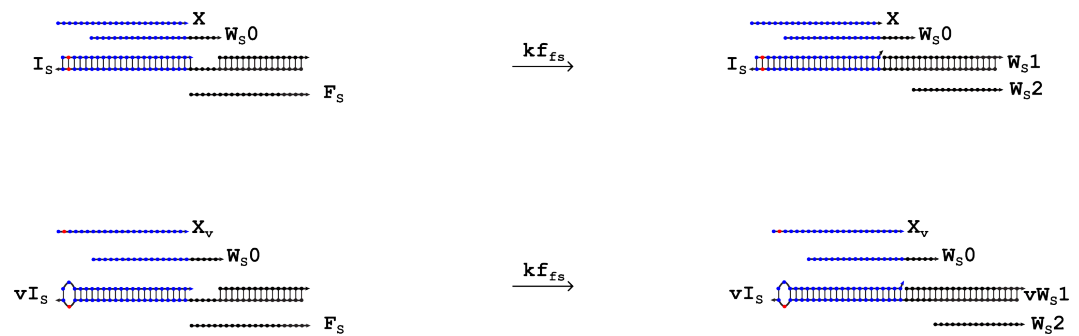


Figure D.3: Two step sink mechanism

D.2 Re-calibrated kinetic experiments

The kinetic experiments presented in Section 5.3 were initially analyzed using nominal gate concentrations. Subsequent calibration measurements revealed substantial variation in the effective concentrations of some gate complexes, particularly A1. The measured effective concentrations and the concentrations used for the recalibrated analysis are summarized in Table D.1.

To evaluate the sensitivity of the reported conclusions to these calibration differences, the experiments were replicated using concentrations adjusted according to the measured effective gate concentrations. The results of the replicated experiment are presented in Figure D.4.

In contrast to the nominal-concentration experiments, endpoint discrimination in the recalibrated systems appeared to plateau in the three- to four-layer regime. As an illustrative comparison, a direct test of endpoint discrimination between the three- and four-layer sink-stabilized systems did not detect a statistically significant difference (Welch's t-test, $p = 0.40$, $n = 3$). Despite this quantitative difference, the recalibrated experiments continued to support the conclusion that layered discrimination substantially increases off-target suppression relative to a single layer.

Taken together, these results support that the principal conclusions of Chapter 5.3 are robust to the observed calibration differences, while also highlighting the sensitivity of quantitative performance metrics to modest variations in effective gate concentration.

D.3 Effective concentrations used in remaining experiments

The effective concentrations measured for the remaining experiments presented in Chapter 5.3 are summarized below.

Table D.1: Measured effective concentrations of gate complexes used in the Figure 5.4 kinetic experiments. Recalibrated experiments were performed using concentrations adjusted according to the measured effective values to reduce discrepancies between target and measured concentrations.

Species	Nominal (nM)	Measured effective (nM)	Recalibrated (nM)
Rep	10	10.0	9.6
A0	10	9.9	9.7
A1	10	17.6	12.2
A2	10	9.3	10.6
A3	10	8.7	8.4

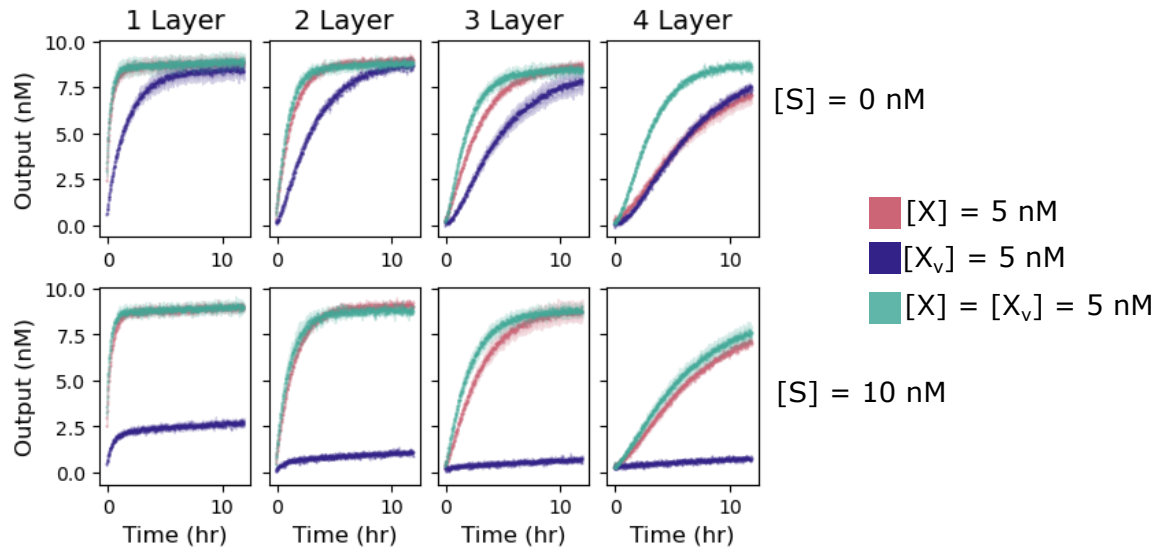
Table D.2: Measured effective concentrations used for Figure 5.5.

Species	Target concentration (nM)	Measured concentration (nM)
Rep	10	9.8
A0	10	9.9
A1	10	10.5
A2	10	9.3
A3	10	8.5

Table D.3: Measured effective concentrations used in Figure 5.6.

Species	Target concentration (nM)	Measured concentration (nM)
Rep	10	9.5
A0	10	9.7
A1	10	12.0
A2	10	10.6
A3	10	9.7

A Effect of sink on depth-dependent discrimination and short-circuiting



B Endpoint discrimination increases with depth under sink stabilization

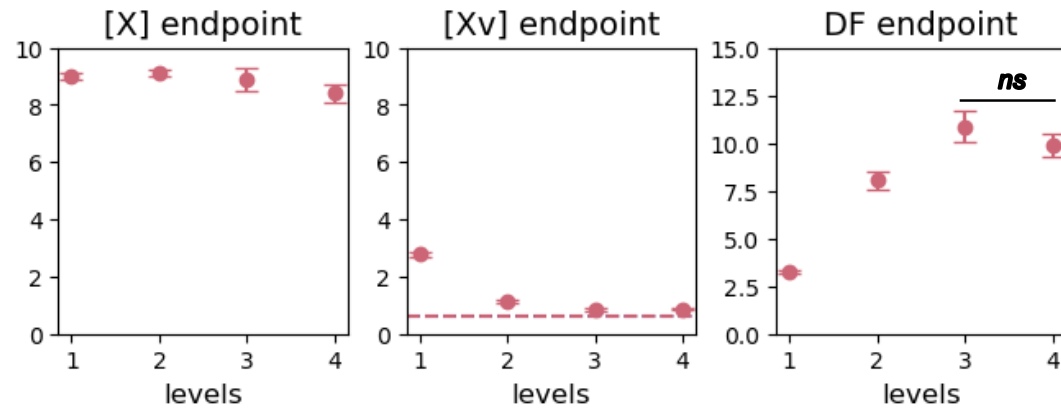


Figure D.4: Replication of Figure 5.4 using gate concentrations normalized to measured effective concentrations. Qualitative behaviors reported in the main text, including sink-mediated suppression of off-target completion and increased discrimination with increasing depth, were preserved following recalibration.

D.4 Sequences

Table D.4: Sequences used for all experimental systems

textbfStrand	Sequences
R4	CACATCCACATCCACACCCTTAATCATCTTCCTAC
R3	CACATCCACATCCACACCCATTTATCAACCAACAC
R2	CACATCCACATCCCACTCCCTATAACCAACTATT
R1	CACATCCACATCCCACTCCCAAACCTTTCTTACAC
R0	CACATCCACATCCACACCTCCTCACATCACCTTCA
F3	CACATCCACATCCACACCCATT
F2	CACATCCACATCCCACTCCCT
F1	CACATCCACATCCCACTCCCA
F0	CACATCCACATCCACACCTCCT
A3-b	AATGGGTGTGGATGTGGATGTGGAGTGGTAGGAAGATGATTAAGGGT
A2-b	AGGGAGTGTGGATGTGGATGTGGAGTGGTGTGGTTGATAAATGGGT
A1-b	TGGGAGTGTGGATGTGGATGTGGAGTGAATAGTTGGTTATAGGGAGT
A0-b	AGGAGGTGTGGATGTGGATGTGGAGTGGTGTAAAGAAAGTTTGGGAGT
W0 ₃	TAATCATCTTCCTACCACTC
W0 ₂	TTTATCAACCAACACCACTC
W0 ₁	CTATAACCAACTATTCACTC
W0 ₀	CAAACCTTTCTTACACCACTC
X	CACTCCACATCCACATCCACAC
X _v	CTCTCCACATCCACATCCACAC
Rep-b	/5ATT0565N/TGAAGGTGATGTGAGGAGGT
Rep-t	CTCACATCACCTTCA/3BHQ_2/
S-b	AGTGAGGTATGGAGTGTGAGGTGGATGTGGATGTGGAGAG
S-t0	CACATCCACATCCACCTCAC
S-t1	ACTCCATACCTCACT
F_S	CTCACACTCCATACCTCACT

Appendix E

REFERENCES AND CONTRIBUTION STATEMENTS***Author Contributions***

Cadence Pearce led the design, analysis, and drafting of the X-to-X amplifier presented in Chapter 3, as well as the thermodynamic modeling of the X-to-Y systems included in Appendix B. Zoë Derauf led the data collection for these X-to-X amplifier experiments. Thomas Mayer led the design, implementation, and analysis of the 5' toehold system and mismatch promiscuity studies presented in Chapter 4. Apart from these contributions, the author led the remaining intellectual development, experimentation, analysis, and drafting presented in this dissertation, including all work described in Chapter 5.

During dissertation preparation, generative AI tools were used for supplementary editorial assistance, limited to feedback on organization, clarity, and prose. All scientific concepts, experimental designs, analyses, interpretations, and conclusions presented in this dissertation were developed and verified by the author.

BIBLIOGRAPHY

- L. M. Adleman. Molecular computation of solutions to combinatorial problems. *science*, 266(5187):1021–1024, 1994.
- D. Alistarh, B. Dudek, A. Kosowski, D. Soloveichik, and P. Uznański. Robust detection in leak-prone population protocols. In *DNA Computing and Molecular Programming: 23rd International Conference, DNA 23, Austin, TX, USA, September 24–28, 2017, Proceedings 23*, pages 155–171. Springer, 2017.
- H. T. Allawi and J. SantaLucia Jr. Thermodynamics of internal c · t mismatches in dna. *Nucleic acids research*, 26(11):2694–2701, 1998.
- S. Andreescu and O. A. Sadik. Trends and challenges in biochemical sensors for clinical and environmental monitoring. *Pure and applied chemistry*, 76(4):861–878, 2004.
- D. H. Appella. Non-natural nucleic acids for synthetic biology. *Current opinion in chemical biology*, 13(5-6):687–696, 2009.
- O. Bande, R. Abu El Asrar, D. Braddick, S. Dumbre, V. Pezo, G. Schepers, V. B. Pinheiro, E. Lescrinier, P. Holliger, P. Marlière, et al. Isoguanine and 5-methyl-isocytosine bases, in vitro and in vivo. *Chemistry–A European Journal*, 21(13):5009–5022, 2015.
- C. Bee, Y.-J. Chen, M. Queen, D. Ward, X. Liu, L. Organick, G. Seelig, K. Strauss, and L. Ceze. Molecular-level similarity search brings computing to DNA data storage. *Nature Communications*, 12(1):4764, 2021.
- M. Bharadwaj, M. Bengtson, M. Golverdingen, L. Waling, and C. Dekker. Diagnosing point-of-care diagnostics for neglected tropical diseases. *PLoS neglected tropical diseases*, 15(6):e0009405, 2021.

- I. Biswas, A. Yamamoto, and P. Hsieh. Branch migration through DNA sequence heterology. *Journal of molecular biology*, 279(4):795–806, 1998.
- J. R. Blas, F. J. Luque, and M. Orozco. Unique tautomeric properties of isoguanine. *Journal of the American Chemical Society*, 126(1):154–164, 2004.
- M. K. Bogdos and B. Morandi. Everplot: A web-based shiny application for creating energy vs reaction coordinate diagrams, 2023.
- D. B. Broadwater, A. W. Cook, and H. D. Kim. First passage time study of DNA strand displacement. *Biophysical Journal*, 120(12):2400–2412, 2021.
- D. B. Broadwater Jr and H. D. Kim. The effect of basepair mismatch on DNA strand displacement. *Biophysical journal*, 110(7):1476–1484, 2016.
- J. Cabello-Garcia, R. Mukherjee, W. Bae, G.-B. V. Stan, and T. E. Ouldrige. Information propagation through enzyme-free catalytic templating of DNA dimerization with weak product inhibition. *Nature Chemistry*, 17(8):1179–1187, 2025.
- L. Cardelli. Two-domain DNA strand displacement. *Mathematical Structures in Computer Science*, 23(2):247–271, 2013.
- L. Cardelli. Sequenceable event recorders. In *Visions of DNA Nanotechnology at 40 for the Next 40: A Tribute to Nadrian C. Seeman*, pages 295–310. Springer, 2023.
- G. Casella and R. Berger. *Statistical inference*. Chapman and Hall/CRC, 2024.
- A. R. Chandrasekaran. Nuclease resistance of DNA nanostructures. *Nature Reviews Chemistry*, 5(4):225–239, 2021.
- J. C. Chaput and C. Switzer. Non-enzymatic transcription of an isog $\odot$ isoc base pair. *Journal of the American Chemical Society*, 122(51):12866–12867, 2000.

- H.-L. Chen, D. Doty, and D. Soloveichik. Rate-independent computation in continuous chemical reaction networks. In *Proceedings of the 5th conference on Innovations in theoretical computer science*, pages 313–326, 2014.
- S. X. Chen and G. Seelig. An engineered kinetic amplification mechanism for single nucleotide variant discrimination by DNA hybridization probes. *Journal of the American Chemical Society*, 138(15):5076–5086, 2016.
- S. X. Chen, D. Y. Zhang, and G. Seelig. Conditionally fluorescent molecular probes for detecting single base changes in double-stranded dna. *Nature chemistry*, 5(9):782–789, 2013a.
- Y.-J. Chen, N. Dalchau, N. Srinivas, A. Phillips, L. Cardelli, D. Soloveichik, and G. Seelig. Programmable chemical controllers made from dna. *Nature nanotechnology*, 8(10):755–762, 2013b.
- K. M. Cherry and L. Qian. Scaling up molecular pattern recognition with DNA-based winner-take-all neural networks. *Nature*, 559(7714):370–376, 2018.
- G. Clavé, M. Reverte, J.-J. Vasseur, and M. Smietana. Modified internucleoside linkages for nuclease-resistant oligonucleotides. *RSC Chemical Biology*, 2(1):94–150, 2021.
- M. L. Collins, B. Irvine, D. Tyner, E. Fine, C. Zayati, C.-a. Chang, T. Horn, D. Ahle, J. Detmer, L.-P. Shen, et al. A branched DNA signal amplification assay for quantification of nucleic acid targets below 100 molecules/ml. *Nucleic acids research*, 25(15):2979–2984, 1997.
- V. V. Demidov, M. V. Yavnilovich, B. P. Belotserkovskii, M. D. Frank-Kamenetskii, and P. E. Nielsen. Kinetics and mechanism of polyamide (” peptide”) nucleic acid binding to duplex DNA. *Proceedings of the National Academy of Sciences*, 92(7):2637–2641, 1995.
- T. Ding, F. Tang, G. Ni, J. Liu, H. Zhao, and Q. Chen. The development of isoguanosine:

- from discovery, synthesis, and modification to supramolecular structures and potential applications. *RSC advances*, 10(11):6223–6248, 2020.
- R. M. Dirks and N. A. Pierce. Triggered amplification by hybridization chain reaction. *Proceedings of the National Academy of Sciences*, 101(43):15275–15278, 2004.
- L. Eberlein, F. R. Beierlein, N. J. van Eikema Hommes, A. Radadiya, J. Heil, S. A. Benner, T. Clark, S. M. Kast, and N. G. Richards. Tautomeric equilibria of nucleobases in the hachimoji expanded genetic alphabet. *Journal of chemical theory and computation*, 16(4):2766–2777, 2020.
- F. Eckstein. Phosphorothioates, essential components of therapeutic oligonucleotides. *Nucleic acid therapeutics*, 24(6):374–387, 2014.
- M. Ellwood, M. Collins, E. Fritsch, J. Williams, S. Diamond, and J. Brewen. Strand displacement applied to assays with nucleic acid probes. *Clinical chemistry*, 32(9):1631–1636, 1986.
- J. Fern and R. Schulman. Design and characterization of DNA strand-displacement circuits in serum-supplemented cell medium. *ACS Synthetic Biology*, 6(9):1774–1783, 2017.
- S. Filges, P. Mouhanna, and A. Ståhlberg. Digital quantification of chemical oligonucleotide synthesis errors. *Clinical Chemistry*, 67(10):1384–1394, 2021.
- M. E. Fornace, N. J. Porubsky, and N. A. Pierce. A unified dynamic programming framework for the analysis of interacting nucleic acid strands: Enhanced models, scalability, and speed. *ACS Synthetic Biology*, 9:2665–2678, 10 2020. ISSN 21615063.
- N. Freund, M. J. L. J. Fürst, and P. Holliger. New chemistries and enzymes for synthetic genetics. *Current Opinion in Biotechnology*, 74:129–136, 2022.
- A. J. Genot, D. Y. Zhang, J. Bath, and A. J. Turberfield. Remote toehold: a mechanism for flexible control of DNA hybridization kinetics. *Journal of the American Chemical Society*, 133(7):2177–2182, 2011.

- M. M. Georgiadis, I. Singh, W. F. Kellett, S. Hoshika, S. A. Benner, and N. G. Richards. Structural basis for a six nucleotide genetic alphabet. *Journal of the American Chemical Society*, 137(21):6947–6955, 2015.
- A. A. Green, P. A. Silver, J. J. Collins, and P. Yin. Toehold switches: De-novo-designed regulators of gene expression. *Cell*, 159(4):925–939, 2014. ISSN 0092-8674. doi: <https://doi.org/10.1016/j.cell.2014.10.002>. URL <https://www.sciencedirect.com/science/article/pii/S0092867414012896>.
- N. E. Haley, T. E. Ouldrige, I. Mullor Ruiz, A. Geraldini, A. A. Louis, J. Bath, and A. J. Turberfield. Design of hidden thermodynamic driving for non-equilibrium systems via mismatch elimination during DNA strand displacement. *Nature Communications*, 11(1): 1–11, 2020.
- J. E. Herrera and J. B. Chaires. Characterization of preferred deoxyribonuclease I cleavage sites. *J. Mol. Biol.*, 236(2):405–411, Feb. 1994.
- I. Hirao. Unnatural base pair systems for DNA/RNA-based biotechnology. *Current opinion in chemical biology*, 10(6):622–627, 2006.
- K. Ho, H. Li, E. B. Roesch, C. B. Sherrill, J. R. Prudent, and T. H. LaBean. Increasing the thermal stability of self-assembling DNA nanostructures by incorporation of isog/isoc base pairs. In *2006 NSTI Nanotechnology Conference and Trade Show NSTI Nanotech*, 2006.
- S. Holm. A simple sequentially rejective multiple test procedure. *Scandinavian journal of statistics*, pages 65–70, 1979.
- F. Hong and P. Šulc. An emergent understanding of strand displacement in RNA biology. *Journal of structural biology*, 207(3):241–249, 2019.
- J. J. Hopfield. Kinetic proofreading: a new mechanism for reducing errors in biosynthetic

- processes requiring high specificity. *Proceedings of the National Academy of Sciences*, 71(10):4135–4139, 1974.
- T. Horn, C.-A. Chang, and M. L. Collins. Hybridization properties of the 5-methyl-isocytidine/isoguanosine base pair in synthetic oligodeoxynucleotides. *Tetrahedron letters*, 36(12):2033–2036, 1995.
- S. Hoshika, N. A. Leal, M.-J. Kim, M.-S. Kim, N. B. Karalkar, H.-J. Kim, A. M. Bates, N. E. Watkins Jr, H. A. SantaLucia, A. J. Meyer, et al. Hachimoji DNA and RNA: A genetic system with eight building blocks. *Science*, 363(6429):884–887, 2019.
- P. Irmisch, T. E. Ouldrige, and R. Seidel. Modeling DNA-strand displacement reactions in the presence of base-pair mismatches. *Journal of the American Chemical Society*, 142(26):11451–11463, 2020.
- A. Jaworski, J. S. Kwiatkowski, and B. Lesyng. Why isoguanine and isocytosine are not the components of the genetic code. *International Journal of Quantum Chemistry*, 28(S12):209–216, 1985.
- Y. Jiang, B. Li, J. N. Milligan, S. Bhadra, and A. D. Ellington. Real-time detection of isothermal amplification reactions with thermostable catalytic hairpin assembly. *Journal of the American Chemical Society*, 135(20):7430–7433, 2013.
- Y. S. Jiang, S. Bhadra, B. Li, and A. D. Ellington. Mismatches improve the performance of strand-displacement nucleic acid circuits. *Angewandte Chemie*, 126(7):1876–1879, 2014.
- R. F. Johnson. *Formal design and analysis for DNA implementations of Chemical Reaction Networks*. California Institute of Technology, 2020.
- S. C. Johnson, C. B. Sherrill, D. J. Marshall, M. J. Moser, and J. R. Prudent. A third base pair for the polymerase chain reaction: inserting isoc and isog. *Nucleic acids research*, 32(6):1937–1941, 2004.

- C. Jung and A. D. Ellington. Diagnostic applications of nucleic acid circuits. *Accounts of chemical research*, 47(6):1825–1835, 2014.
- J. K. Jung, C. M. Archuleta, K. K. Alam, and J. B. Lucks. Programming cell-free biosensors with DNA strand displacement circuits. *Nature chemical biology*, 18(4):385–393, 2022.
- A. M. Kabza, N. Kundu, W. Zhong, and J. T. Sczepanski. Integration of chemically modified nucleotides with DNA strand displacement reactions for applications in living systems. *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology*, 14(2):e1743, 2022.
- G. Kallansrud and B. Ward. A comparison of measured and calculated single-and double-stranded oligodeoxynucleotide extinction coefficients. *Analytical biochemistry*, 236(1):134–138, 1996.
- H. Kawabe, C. A. Thomas, S. Hoshika, M.-J. Kim, M.-S. Kim, L. Miessner, N. Kaplan, J. M. Craig, J. H. Gundlach, A. H. Laszlo, et al. Enzymatic synthesis and nanopore sequencing of 12-letter supernumerary dna. *Nature Communications*, 14(1):6820, 2023.
- T. Kennedy, C. Pearce, and C. Thachuk. Fast and robust strand displacement cascades via systematic design strategies. In *28th International Conference on DNA Computing and Molecular Programming (DNA 28)(2022)*. Schloss-Dagstuhl-Leibniz Zentrum für Informatik, 2022.
- D. A. Khodakov, A. S. Khodakova, D. M. Huang, A. Linacre, and A. V. Ellis. Protected DNA strand displacement for enhanced single nucleotide discrimination in double-stranded DNA. *Scientific reports*, 5(1):1–8, 2015.
- M. Kimoto, T. Mitsui, R. Yamashige, A. Sato, S. Yokoyama, and I. Hirao. A new unnatural base pair system between fluorophore and quencher base analogues for nucleic acid-based imaging technology. *Journal of the American Chemical Society*, 132(43):15418–15426, 2010.

- M. Kimoto, R. S. Cox 3Rd, and I. Hirao. Unnatural base pair systems for sensing and diagnostic applications. *Expert review of molecular diagnostics*, 11(3):321–331, 2011.
- U. C. Kühlmann, G. R. Moore, R. James, C. Kleanthous, and A. M. Hemmings. Structural parsimony in endonuclease active sites: should the number of homing endonuclease families be redefined? *FEBS letters*, 463(1-2):1–2, 1999.
- N. Kundu, B. E. Young, and J. T. Sczepanski. Kinetics of heterochiral strand displacement from PNA–DNA heteroduplexes. *Nucleic acids research*, 49(11):6114–6127, 2021.
- A. Kurakin, H. J. Larsen, and P. E. Nielsen. Cooperative strand displacement by peptide nucleic acid (PNA). *Chemistry & biology*, 5(2):81–89, 1998.
- M. R. Lakin, S. Youssef, F. Polo, S. Emmott, and A. Phillips. Visual dsd: a design and analysis tool for DNA strand displacement systems. *Bioinformatics*, 27(22):3211–3213, 2011.
- M. R. Lakin, S. Youssef, L. Cardelli, and A. Phillips. Abstractions for DNA circuit design. *Journal of The Royal Society Interface*, 9(68):470–486, 2012.
- A. P. Lapteva, N. Sarraf, and L. Qian. DNA strand-displacement temporal logic circuits. *Journal of the American Chemical Society*, 144(27):12443–12449, 2022.
- C. Li, Y. Li, Y. Chen, R. Lin, T. Li, F. Liu, and N. Li. Modulating the DNA strand-displacement kinetics with the one-sided remote toehold design for differentiation of single-base mismatched DNA. *RSC advances*, 6(78):74913–74916, 2016.
- Q. Liu, G. Liu, T. Wang, J. Fu, R. Li, L. Song, Z.-G. Wang, B. Ding, and F. Chen. Enhanced stability of DNA nanostructures by incorporation of unnatural base pairs. *ChemPhysChem*, 18(21):2977–2980, 2017.
- R. Lopez, R. Wang, and G. Seelig. A molecular multi-gene classifier for disease diagnostics. *Nature chemistry*, 10(7):746–754, 2018.

- D. Lysne. *Kinetic Barriers for Leakage Reduction in Autocatalytic Strand Displacement Networks*. PhD thesis, Boise State University, 2023.
- R. R. Machinek, T. E. Ouldridge, N. E. Haley, J. Bath, and A. J. Turberfield. Programmable energy landscapes for kinetic control of DNA strand displacement. *Nature Communications*, 5(1):1–9, 2014.
- T. L. Mallette and M. R. Lakin. Protecting heterochiral DNA nanostructures against exonuclease-mediated degradation. *ACS Synthetic Biology*, 11(7):2222–2228, 2022.
- T. L. Mallette, M. N. Stojanovic, D. Stefanovic, and M. R. Lakin. Robust heterochiral strand displacement using leakless translators. *ACS Synthetic Biology*, 9(7):1907–1910, 2020.
- T. Mana, B. Bhattacharya, H. Lahiri, and R. Mukhopadhyay. XNAs: A troubleshooter for nucleic acid sensing. *ACS Omega*, 7(18):15296–15307, 2022.
- S. Manna, M. Kimoto, J. Truong, P. Bommisetti, A. Peitz, I. Hirao, and M. C. Hammond. Systematic mutation and unnatural base pair incorporation improves riboswitch-based biosensor response time. *ACS sensors*, 8(12):4468–4472, 2023.
- T. Mayer, L. Oesinghaus, and F. C. Simmel. Toehold-mediated strand displacement in random sequence pools. *Journal of the American Chemical Society*, 145(1):634–644, 2022.
- T. Mayer, L. Givélet, and F. C. Simmel. Micro-compartmentalized strand displacement reactions with a random pool background. *Interface Focus*, 13(5):20230011, 2023.
- R. Mukherjee, A. Sengar, J. Cabello-García, and T. E. Ouldridge. Kinetic proofreading can enhance specificity in a nonenzymatic DNA strand displacement network. *Journal of the American Chemical Society*, 146(28):18916–18926, 2024.
- K. Murayama, H. Okita, and H. Asanuma. Highly functional acyclic xeno nucleic acids. *Bulletin of the Chemical Society of Japan*, 96(10):1179–1187, 2023.

- P. E. Nielsen and M. Egholm. Strand displacement recognition of mixed adenine–cytosine sequences in double stranded DNA by thymine–guanine PNA (peptide nucleic acid). *Bioorganic & medicinal chemistry*, 9(9):2429–2434, 2001.
- M. P. Nikitin. Non-complementary strand commutation as a fundamental alternative for information processing by DNA and gene regulation. *Nature Chemistry*, 15(1):70–82, 2023.
- J. Ninio. Kinetic amplification of enzyme discrimination. *Biochimie*, 57(5):587–595, 1975.
- L. Oesinghaus and F. C. Simmel. Switching the activity of Cas12a using guide RNA strand displacement circuits. *Nature Communications*, 10(1):2092, 5 2019. ISSN 2041-1723. doi: 10.1038/s41467-019-09953-w. URL <https://doi.org/10.1038/s41467-019-09953-w>.
- X. Olson, S. Kotani, J. E. Padilla, N. Hallstrom, S. Goltry, J. Lee, B. Yurke, W. L. Hughes, and E. Graugnard. Availability: a metric for nucleic acid strand displacement systems. *ACS Synthetic Biology*, 6(1):84–93, 2017.
- T. E. Ouldridge, A. A. Louis, and J. P. Doye. Structural, mechanical, and thermodynamic properties of a coarse-grained dna model. *The Journal of chemical physics*, 134(8), 2011.
- N. M. Paulino, M. Foo, J. Kim, and D. G. Bates. PID and state feedback controllers using DNA strand displacement reactions. *IEEE Control Systems Letters*, 3(4):805–810, 2019.
- N. Peyret, P. A. Seneviratne, H. T. Allawi, and J. SantaLucia. Nearest-neighbor thermodynamics and NMR of DNA sequences with internal a⊙ a, c⊙ c, g⊙ g, and t⊙ t mismatches. *Biochemistry*, 38(12):3468–3477, 1999.
- T. M. Pham, T. Miffin, H. Sun, K. K. Sharp, X. Wang, M. Zhu, S. Hoshika, R. J. Peterson, S. A. Benner, J. D. Kahn, et al. DNA structure design is improved using an artificially expanded alphabet of base pairs including loop and mismatch thermodynamic parameters. *ACS Synthetic Biology*, 12(9):2750–2763, 2023.

- A. Phillips and L. Cardelli. A programming language for composable DNA circuits. *Journal of the Royal Society Interface*, 6(suppl_4):S419–S436, 2009.
- L. Qian and E. Winfree. Scaling up digital circuit computation with DNA strand displacement cascades. *Science*, 332(6034):1196–1201, 2011a.
- L. Qian and E. Winfree. A simple DNA gate motif for synthesizing large-scale circuits. *Journal of the Royal Society Interface*, 8(62):1281–1297, 2011b.
- L. Qian, D. Soloveichik, and E. Winfree. Efficient turing-universal computation with DNA polymers. In *DNA Computing and Molecular Programming: 16th International Conference, DNA 16, Hong Kong, China, June 14-17, 2010, Revised Selected Papers 16*, pages 123–140. Springer, 2011a.
- L. Qian, E. Winfree, and J. Bruck. Neural network computation with DNA strand displacement cascades. *Nature*, 475(7356):368–372, 2011b.
- E. S. Rangarajan and V. Shankar. Sugar non-specific endonucleases. *FEMS microbiology reviews*, 25(5):583–613, 2001.
- C. Roberts, R. Bandaru, and C. Switzer. Theoretical and experimental study of isoguanine and isocytosine: base pairing in an expanded genetic system. *Journal of the American Chemical Society*, 119(20):4640–4649, 1997.
- C. D. Roberts. *The synthesis and biophysical evaluation of nucleic acids containing isoguanine and iso-cytosine, and, DNA bending and alkylation protection by zwitterionic DNA, and, Activated guanosine mononucleotide oligomerization on hairpin templates bearing a single 2', 5'-RNA of DNA defect*. University of California, Riverside, 1997.
- K. R. Rodriguez, N. Sarraf, and L. Qian. A loser-take-all DNA circuit. *ACS Synthetic Biology*, 10(11):2878–2885, 2021.
- J. SantaLucia Jr and D. Hicks. The thermodynamics of DNA structural motifs. *Annu. Rev. Biophys. Biomol. Struct.*, 33(1):415–440, 2004.

- Y. J. Seo, S. Matsuda, and F. E. Romesberg. Transcription of an expanded genetic alphabet. *Journal of the American Chemical Society*, 131(14):5046–5047, 2009.
- P. Sheng, Z. Yang, Y. Kim, Y. Wu, W. Tan, and S. A. Benner. Design of a novel molecular beacon: modification of the stem with artificially genetic alphabet. *Chemical communications*, (41):5128–5130, 2008.
- F. C. Simmel, B. Yurke, and H. R. Singh. Principles and applications of nucleic acid strand displacement reactions. *Chemical reviews*, 119(10):6326–6369, 2019.
- A. M. Sismour and S. A. Benner. The use of thymidine analogs to improve the replication of an extra DNA base pair: a synthetic biological system. *Nucleic Acids Research*, 33(17):5640–5646, 2005.
- D. Soloveichik, G. Seelig, and E. Winfree. DNA as a universal substrate for chemical kinetics. *Proceedings of the National Academy of Sciences*, 107(12):5393–5398, 2010.
- N. Srinivas, T. E. Ouldridge, P. Šulc, J. M. Schaeffer, B. Yurke, A. A. Louis, J. P. Doye, and E. Winfree. On the biophysics and kinetics of toehold-mediated DNA strand displacement. *Nucleic acids research*, 41(22):10641–10658, 2013.
- N. Srinivas, J. Parkin, G. Seelig, E. Winfree, and D. Soloveichik. Enzyme-free nucleic acid dynamical systems. *Science*, 358(6369):eaal2052, 2017.
- P. Šulc, F. Romano, T. E. Ouldridge, L. Rovigatti, J. P. Doye, and A. A. Louis. Sequence-dependent thermodynamics of a coarse-grained DNA model. *The Journal of chemical physics*, 137(13), 2012.
- C. Switzer, S. E. Moroney, and S. A. Benner. Enzymatic incorporation of a new base pair into DNA and RNA. *Journal of the American Chemical Society*, 111(21):8322–8323, 1989.
- W. Tang, W. Zhong, Y. Tan, G. A. Wang, F. Li, and Y. Liu. DNA strand displacement reaction: a powerful tool for discriminating single nucleotide variants. *DNA Nanotechnology: From Structure to Functionality*, pages 377–406, 2020.

- D. N. Taylor, S. R. Davidson, and L. Qian. A cooperative DNA catalyst. *Journal of the American Chemical Society*, 143(38):15567–15571, 2021.
- C. Thachuk. Logically and physically reversible natural computing: a tutorial. In *Reversible Computation: 5th International Conference, RC 2013, Victoria, BC, Canada, July 4-5, 2013. Proceedings 5*, pages 247–262. Springer, 2013.
- C. Thachuk and A. Condon. Space and energy efficient computation with DNA strand displacement systems. In *International Workshop on DNA-Based Computers*, pages 135–149. Springer, 2012.
- C. Thachuk, E. Winfree, and D. Soloveichik. Leakless DNA strand displacement systems. In *DNA Computing and Molecular Programming: 21st International Conference, DNA 21, Boston and Cambridge, MA, USA, August 17-21, 2015. Proceedings 21*, pages 133–153. Springer, 2015.
- A. J. Thubagere, C. Thachuk, J. Berleant, R. F. Johnson, D. A. Ardelean, K. M. Cherry, and L. Qian. Compiler-aided systematic construction of large-scale DNA strand displacement circuits using unpurified components. *Nature Communications*, 8(1):14373, 2017.
- A. J. Turberfield, J. Mitchell, B. Yurke, A. P. Mills Jr, M. Blakey, and F. C. Simmel. DNA fuel for free-running nanomachines. *Physical Review Letters*, 90(11):118102, 2003.
- P. Virtanen, R. Gommers, T. E. Oliphant, M. Haberland, T. Reddy, D. Cournapeau, E. Burovski, P. Peterson, W. Weckesser, J. Bright, et al. Scipy 1.0: fundamental algorithms for scientific computing in python. *Nature methods*, 17(3):261–272, 2020.
- J. Von Neumann. Probabilistic logics and the synthesis of reliable organisms from unreliable components. *Automata studies*, 34(34):43–98, 1956.
- B. Wang, C. Thachuk, A. D. Ellington, and D. Soloveichik. The design space of strand displacement cascades with toehold-size clamps. In *International Conference on DNA-Based Computers*, pages 64–81. Springer, 2017.

- B. Wang, C. Thachuk, A. D. Ellington, E. Winfree, and D. Soloveichik. Effective design principles for leakless strand displacement systems. *Proceedings of the National Academy of Sciences*, 115(52):E12182–E12191, 2018.
- B. Wang, C. Thachuk, and D. Soloveichik. Classifying enthalpy-neutral DNA strand displacement cascades: trading off desired and undesired signal propagation. *bioRxiv*, pages 2022–04, 2022.
- B. Wang, C. Thachuk, and D. Soloveichik. Speed and correctness guarantees for programmable enthalpy-neutral DNA reactions. *ACS Synthetic Biology*, 12(4):993–1006, 2023.
- L. Wathieu, G. Smith, L. Ceze, and C. Thachuk. Fridge compiler: Optimal circuits from molecular inventories. In *International Conference on Computational Methods in Systems Biology*, pages 236–252. Springer, 2023.
- B. H. Weigl, T. Neogi, and H. McGuire. Point-of-care diagnostics in low-resource settings and their impact on care in the age of the noncommunicable and chronic disease epidemic. *Journal of laboratory automation*, 19(3):248–257, 2014.
- M. Whitby, L. Cardelli, M. Kwiatkowska, L. Laurenti, M. Tribastone, and M. Tschaikowski. PID control of biochemical reaction networks. *IEEE Transactions on Automatic Control*, 67(2):1023–1030, 2021.
- E. Winfree. The tall thin molecular programmer. In *2023 IEEE International Solid-State Circuits Conference (ISSCC)*, pages 1–3. IEEE, 2023.
- L. R. Wu, S. X. Chen, Y. Wu, A. A. Patel, and D. Y. Zhang. Multiplexed enrichment of rare DNA variants via sequence-selective and temperature-robust amplification. *Nature biomedical engineering*, 1(9):714–723, 2017.
- J. A. Wyer, M. B. Kristensen, N. C. Jones, S. V. Hoffmann, and S. B. Nielsen. Kinetics of

- DNA duplex formation: A-tracts versus at-tracts. *Physical Chemistry Chemical Physics*, 16(35):18827–18839, 2014.
- X. Yang, Y. Tang, S. M. Traynor, and F. Li. Regulation of DNA strand displacement using an allosteric DNA toehold. *Journal of the American Chemical Society*, 138(42):14076–14082, 2016.
- Z. Yang, F. Chen, S. G. Chamberlin, and S. A. Benner. Expanded genetic alphabets in the polymerase chain reaction. *Angewandte Chemie (International Ed. in English)*, 49(1):177, 2010.
- Y. Yuan, H. Lv, and Q. Zhang. DNA strand displacement reactions to accomplish a two-degree-of-freedom PID controller and its application in subtraction gate. *IEEE transactions on nanobioscience*, 20(4):554–564, 2021.
- B. Yurke and A. P. Mills Jr. Using DNA to power nanostructures. *Genetic Programming and Evolvable Machines*, 4(2):111–122, 2003.
- D. Y. Zhang and G. Seelig. Dynamic DNA nanotechnology using strand-displacement reactions. *Nature chemistry*, 3(2):103–113, 2011.
- D. Y. Zhang and E. Winfree. Control of DNA strand displacement kinetics using toehold exchange. *Journal of the American Chemical Society*, 131(47):17303–17314, 2009.
- D. Y. Zhang, A. J. Turberfield, B. Yurke, and E. Winfree. Engineering entropy-driven reactions and networks catalyzed by dna. *Science*, 318(5853):1121–1125, 2007.
- W. Zhang and H. Lv. Disturbance observer-based PID control system using DNA strand displacement and its application in exponential gate. *IEEE Access*, 11:113160–113175, 2023.
- Y. Zhang, L. Wang, J. Ye, J. Chen, S. Xu, S. Bu, M. Deng, L. Bian, X. Zhao, C. Zhang, et al. Rationally designed dual base pair mismatch enables toehold-mediated strand

displacement to efficiently recognize single-nucleotide polymorphism without enzymes.
Analytical Chemistry, 96(1):554–563, 2023.