OPTIMIZATION OF FLOW CYTOMETRIC SORTING IN THE HEMATOPATHOLOGY LABORATORY

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ABSTRACT

A subset of neoplasms in Hematopathology show rare neoplastic cells in a background of abundant non-neoplastic reactive cells. Developing a method of enriching for neoplastic cells, in in combination with efficient DNA extraction for molecular applications, could be a valuable tool for evaluation of the rare cell neoplasm (RCN). The purpose of this study is to optimize the procedures for flow cytometric cell sorting, affording enrichment of neoplastic cells of RCN. This project also addressed validation of an effective and automated DNA extraction method using minimal numbers of sorted cells. The detection level limit for clonality based molecular assay was also established used abnormal and normal sorted cells.

In this study, we considered different technical aspects of the flow sorting such as how nozzle sizes will affect recovery of the various sorted cells. For this experiment, we used a megakaryocytic lineage induced cell ine, a Hodgkin lymphoma cell line (L428), and normal T lymphocytes. Another technical consideration evaluated, compared different precision modes using various percentage of the spiked cell to calculate the efficiency of sorting. Finally, these studies used sorted cells for comparison of two DNA extraction technique to determine which method afforded a greater yield of DNA for subsequent molecular studies; the B cell clonality assay platform was used for sorting abnormal and normal cells to establish detection levels.

The studies suggest that it is necessary to have larger nozzle sizes for rare cell neoplastic sorting if large neoplastic cells from RCN. The precision modes study demonstrated that the combination of yield mode and purity mode is the preferable method for rare cells sorting. Comparison of DNA extraction methods proved that the EZ viral kit methods are more effective

and sensitive when using the sorted cells than other DNA extraction methodologies; the limit of detection with B cell clonality assay determined that the level of detection using sorted cells could be hundred fold better than with unsorted bulk specimen.

These studies demonstrated that by combining a reliable enrichment method with an effectve DNA extraction (for downstream molecular techniques) would improve the diagnostic value of these tests.

CHAPTER I

PURPOSE AND RELEVANCE OF THE PROJECT

Introduction:

In this new age of molecular sequencing and personalized medicine, the concept of reduction of background noise from non-neoplastic reactive cells holds an extremely important place in molecular studies. One method for reduction of background is by using enrichment of the cells of interest and this could be done using fluorescence activated cell sorting (FACS). The technique is flow cytometry derived application that uses unique features, which allows the cells to be sorted (purified), based on the size, granularity and phenotypic markers.

One of the purposes of this project is to optimize the process of flow cell sorting using 100 μm and 130 μm nozzles, so that it will be available for clinical sorting of large neoplastic cell populations and fragile cell populations. Another objective is to validate molecular DNA extraction methods for the selection of better and sensitive method for sorted cells. A third purpose is to define the minimum number of cells for clinical molecular clonality assay using the sorted abnormal and normal cells.

The optimization of cell sorter using the 100 μm and 130 μm nozzles is done by setting up different experiments that pertains to cell sizes and fragility, compensation setting, precision mode setting and sort recovery with different percentage of spiked cells. When dealing with neoplasms in which neoplastic cells are rare, rare cell neoplasms (RCN), the methodology requires fine-tuning the settings that are appropriate to effectively result

in the maximum yield with high purity. The cell size is directly proportional to the nozzle size, meaning that as the cell size increases the nozzle size must increase. Currently the clinical lab at Hematopathology lab only use the 70 μm nozzle for clinical sorts, which could include majority of leukocytes. The 70 μm nozzle may not be appropriate for neoplasms comprised of large cells since the pressure level at this nozzle is higher (70 psi) may cause shear stress and thus damage large cells. The experiment designed to find the percentage of recovery of various cell type of different sizes using different nozzle types. Compensation setup experiments were setup to open up the clinical sorter for multicolored sorting, that would need for separation of target cells from RCN. Different precision modes were compared with various percentages of spiked cells using 100 μm and 130 μm nozzle to find the preferable precision modes that could be used for rare cell sorting.

Most of the current molecular downstream applications, whether it is RNA based or DNA based, and the detection method requires at least 1% of neoplastic population in the sample. If the abnormal cell population is below this threshold, the technique may not detect the neoplastic population. It is necessary to have an efficient way to extract DNA when dealing with minimal amount of starting material, which is why the comparing extraction to a new more efficient method is needed. In this new age with treatment options, it is extremely important to create a methodology that could be used monitor the cell of interest even when the percentage is very low. This requires the combination of enrichment method and highly sensitive molecular techniques work hand in hand. The optimizations of larger nozzle sizes were necessary for the enrichment of RCN and larger cell neoplasms. The cell size and the nozzle size correlation studies were done using two different cell lines using different nozzle type. The sort recovery studies will evaluate

correlation between number of sorted cells indicated by the instrument and the actual number in relation to the number of cell sorted. The mask adjustment and the time required for sorting could be crucial especially when dealing with the lower percentages of cells.

Any molecular downstream application needs a good yield of DNA; the two methods compared were Puregene and EZ viral DNA extraction method. Two DNA extraction methods were compared and the range of sensitivity is created using the sorted cells. Sorted cells are used to determine the efficiency of two extractions methods. Sorted abnormal and normal cells are used with B cell clonality platform to determine the limit of detection.

CHAPTER II

OPTIMIZATION OF NOZZLES

Introduction:

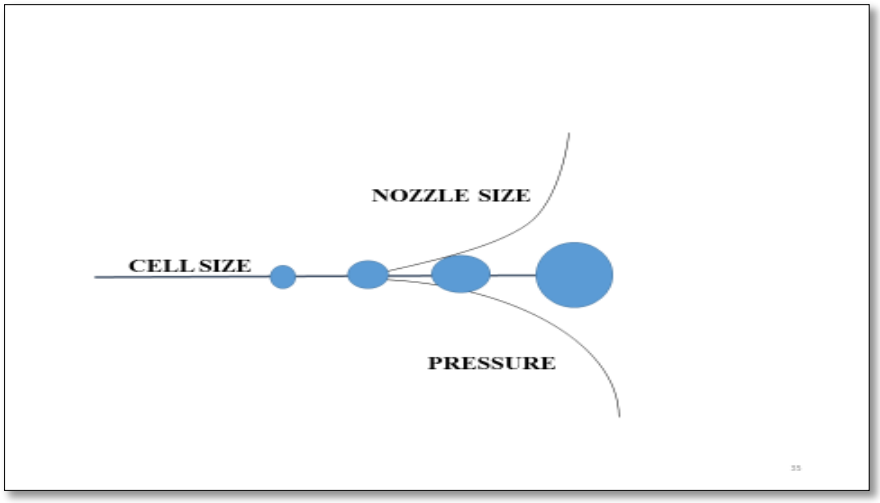
Currently at the University of Washington Hematopathology lab (Seattle, WA) the clinical use of the flow sorter is restricted to 70-micron (μm) nozzle for clinical sorts. The usage of 70-μm nozzle only allows for efficient sorting of the cellular components that are relatively small (including majority of leukocytes). This project is an attempt to optimize the use of other larger nozzles by allowing the fluorescence activated cell sorter (FACS) to be used in full capacity. In case of malignant neoplasms with large cells the needs for a larger diameter nozzle is an absolute necessity for sorting intact cells. This project addresses the necessary validation steps that are needed for 100 μm and 130 μm nozzle optimization for clinical use.

The initial experiment explores the relationship of cell size with the nozzle sizes. The technical notes from the vendor BD Biosciences (31), suggests the use of nozzle that are double the diameter of the cells that are being sorted. In this experiment setup different types of cells and cell lines are used to evaluate the relationship between the nozzle size and the efficiency of cell sorting for a given cell size, as shown in Figure 1. This experiment also explores the percentage of cell recovery while using different nozzle sizes. During the cell sorting experiment, the different nozzle size require different pressures as shown in Figure1; and in the case of fragile cells the pressure difference and the size of nozzle could increase the percentage of recovery. As the nozzle size gets bigger the required pressure decreases and shown in Figure 1. Two sets of experiment are setup to address the possibility whether the nozzle size increase would affect the recovery of bigger

cells and whether the pressure differences that are provided with nozzle affect the recovery percentage of fragile these cells.

Figure 0-1: Relationship between cell size, nozzle size and pressure.

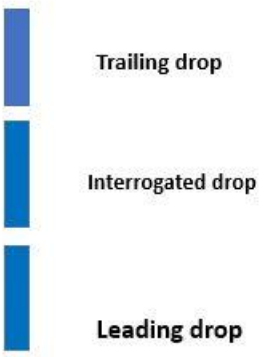
There is directly proportional relationship between cell size and nozzle size and inversely proportional relationship between nozzle size and required pressure



The next step is the selection of an appropriate precision mode for sorting the rare cells. The sorting decision of cells of interest is also made dependent on the property of the sorting mask.

Figure 0-2: Sorting decision.

Sorting decision using by the sorter using the trailing, interrogation and trailing drop



For this purpose, the sorter identifies the drops as the drop that is being interrogated, the drop that is trailing and the leading drop as shown in Figure 2. This interrogated drop is further divided in the 32 increments and it is monitored for any contaminant particles. The sort decision is then based on how far the contaminant is, how close to the center of the interrogated drop is the particle of interest by using different types of masks. There are three different types of masks that could be applied separately and in combination with each other, these includes yield mask, purity mask and phase mask.

The yield mask used to enrich the cell of interest at cost of the purity. For example, if the yield mask is set at 16, if the cell is in the center of the interrogation droplet the sorter will sort one drop, but if the cells are on the edge of the drop, it will sort two drops thereby increasing the chances for the cell of interest to be sorted. If yield mask is set as zero, only the drop interrogated will be sorted no matter where the location of cell of interest is. The purity mask has a coincidence zone that includes half increments of 32 of the leading drop and the trailing drop, if there are contaminant cells present in the coincidence zone the

drop interrogated will not be sorted. When phase mask is applied the drop interrogated will only be sorted if the particle of interest is in the middle of the interrogated drop. By combining different masks different precision modes are created for increasing the purity of the sort as shown in Table 1.

Table 1: Summary of different precision mode setting appropriate for different sorting needs.

Sort Mode	Settings	When to Use
Purity	Yield =32 (2-drop sort) Purity = 32 Phase = 0	Highest combination of purity and yield
4-way Purity	Yield = 0 Purity = 32 Phase = 0	Precise side streams needed with highest purity and yield
Yield	Yield = 32 Purity = 0 Phase = 0	Maximum yield of cell of interest at the cost of purity

Another consideration is sort recovery at different percentages of spiked cells. This phase of the experiments was setup to measure the precision and accuracy of the predetermined cells that could be selected from the sorting window. The category of sorted cells tested was 100, 500, and 1000. The setup used L428 CHL cell that is stained with monoclonal antibodies and fixed using 0.25% formaldehyde and then spiked to the normal lymph node. Each specific category of cells are sorted repeatedly into pre-quantified normal lymph node and then these specimens were fed back to cytometer to determine the percentage of sort recovery. From the observed value and the target value the percentage of recovery is then calculated.

Materials and Methods:

For all the experiment setup FACS™ Aria II sorter at University of Washington Hematopathology Lab with 3 lasers that includes violet, blue and red were used. The L428 (CHL cell line) were grown in RPMI reconstituted with 10% fetal calf serum (FCS) at 37C in 5% CO2 and HEL (Megakaryocytic lineage induced cell line) was a generous gift from the Loeb lab. The normal lymph node used was morphologically and flow cytometry confirmed reactive lymph node that was stored in liquid nitrogen tank. All the frozen tissues were thawed using a prewarmed medium constituting of 60% RPMI and 40% FCS. Monoclonal antibodies used to identify L428 cells were CD95 PB, CD30 PE, CD40 PE Cy5.5 and CD15 APC and the normal lymphocytes is separated using CD20 PC 7, CD5 PE-TR and CD45 APC- H7. Initial gating strategy includes separating cell aggregates using FSC-A and FSC-H, and then viable cells gated using FSC-A and SSC-H. The viable cells are then further categorized based on the expression of CD30, CD15, CD40, CD95 to identify L428 cells,

CD20 is used to identify B lymphocyte and CD5 is used to detect T lymphocytes and CD64 is used to identify macrophages. The initial gating strategy is used to separate out singlets and viable cells, and from viable cell the T cells are identified using CD5 PE-TR (T lymphocytes), CD45 APC-H7 and CD20 PC 7 is used to identify B lymphocytes, while for HEL cells CD45 APC-H7, CD41 PE, CD14 PE Cy5.5, and CD34 APC were used. All the specimens were stained with appropriate antibodies for 15 minutes in the dark and then washed once with PBS/0.3% BSA then reconstituted with RPMI and strained to get rid of the cell clumps. Collection medium for all the sorted cells in this experiment was a mixture of FCS and Hepes buffer. For fragile cell sorting a clinical specimen with 1.1% of abnormal plasma neoplasm was used. The cells were bulked lysed using NH4Cl (1X) for 10-15 minutes at room temperature. The lysed specimen is washed twice using PBS/0.3%BSA then reconstituted with PBS/0.3% BSA. A white cell count of 9.7*10^6 was obtained using Horiba ABX automated cell counter. The cells were stained with CD38 PE, CD19 PE-TR, CD56 PC 7, CD138 APC and CD45 APC-H7. The flow cytometric patient report was used to create the hierarchical gating strategies to separate the abnormal plasma cell from the rest of the population. Initial gating strategy includes separating cell aggregates using FSC-A and FSC-H, and then viable cells gated using FSC-A and SSC-H. The viable cells are then further categorized based on the expression of CD45, CD38, CD138, CD19 and CD56. The cells were sorted in a mixture of FCS and Hepes buffer and reacquired through the cytometer. Three replicates of 1000 plasma cells were sorted using each nozzle sizes and sorted cells were reacquired through the cell sorter. The percentage of recovery is calculated as a number of acquired events by the number of sorted cells.

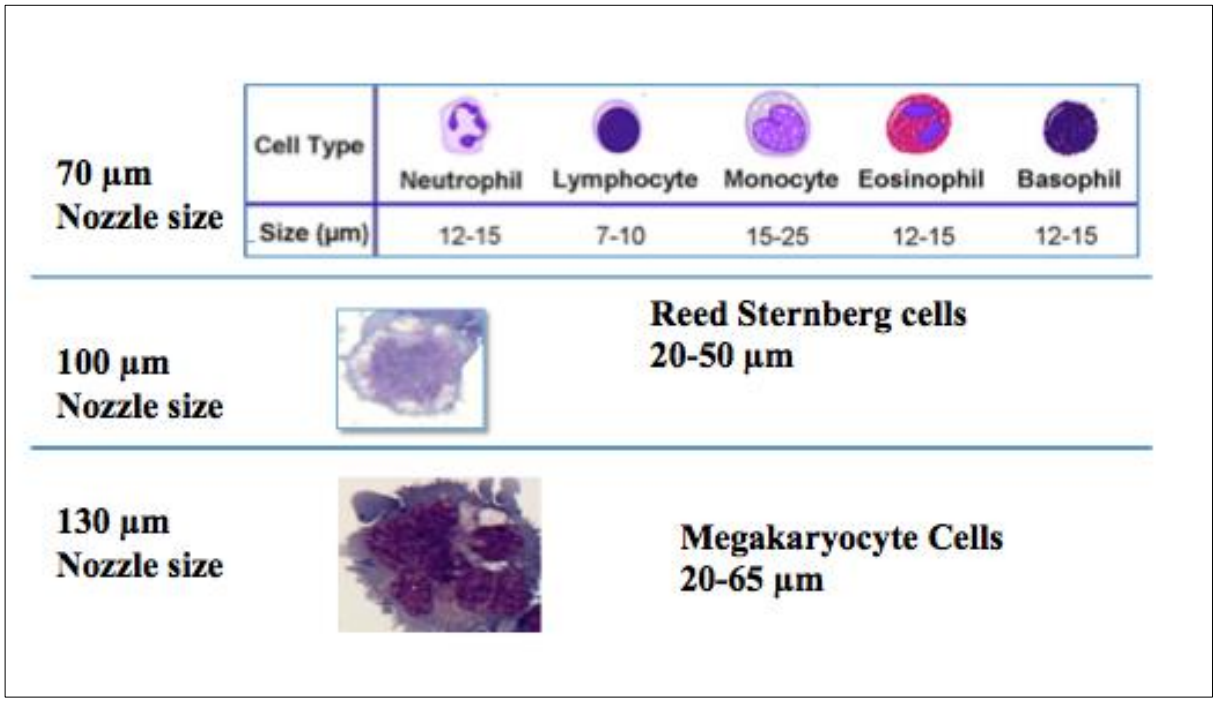
For sort recovery experiment L428 cells were stained and fixed and then spiked into a morphologically confirmed normal lymph node. For the initial experiment different category (in triplicates) of L428 cells were sorted into FCS and Hepes buffer mix and then reacquired through the sorter to calculate the rate of recovery. The next phase of the experiment used different percentage of spiked cells ranging from 0.1% to 2% of L428 cells, sorted into a pre-quantified normal lymph node mixture and reacquired through the sorter to calculate the percentage of recovery of sorted cells of interest. Horiba ABX automated cell counter was used to obtain the cells count, the cell count obtained was used further for dilutions and spiking. All the experiments were setup in triplicates.

Results:

2.1.The effect of nozzle size on the efficiency of cell sorting variably sized cells: Experiment setup and Results

Figure 0-3: Cell sizes and nozzle sizes.

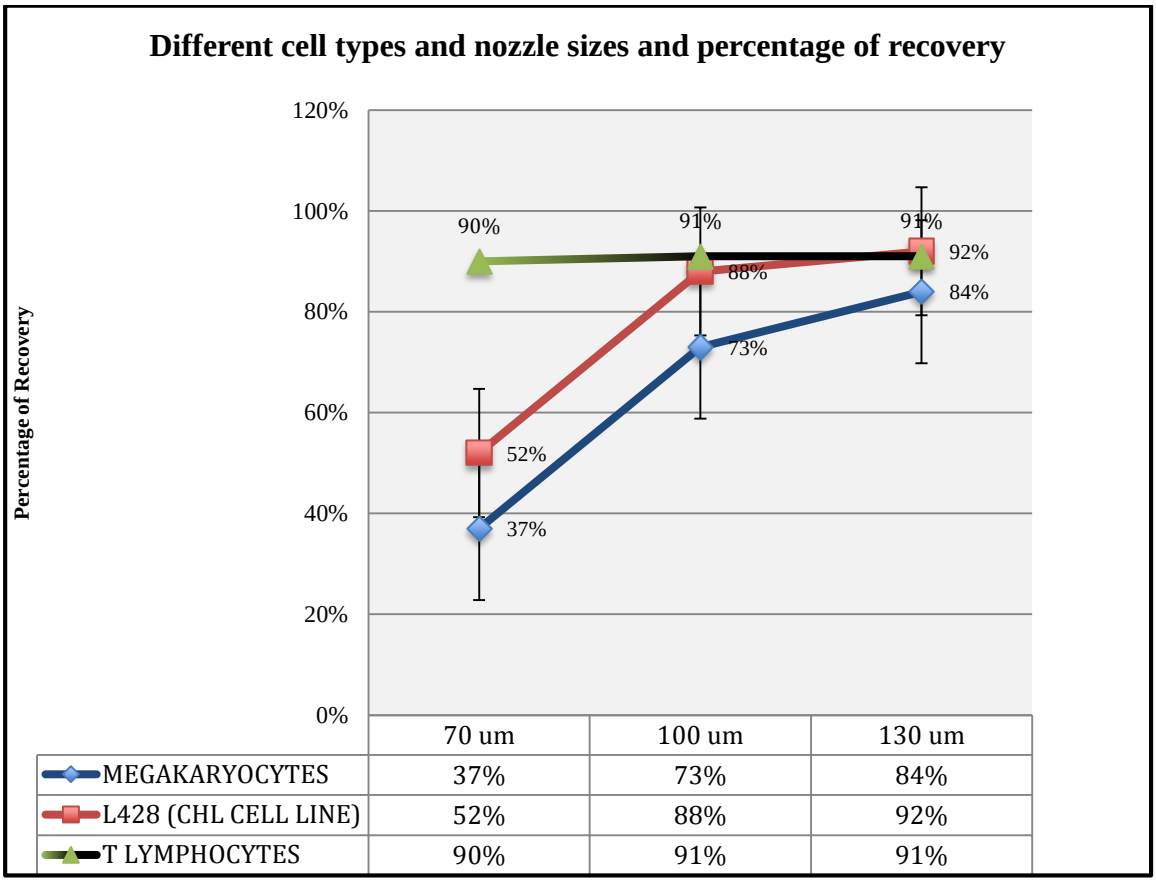
The chart showing the relative diameters of different cells types and the nozzle size used for sorting



Approximately 5% of L428 cell line and HEL cell line were each separately spiked in to normal lymph node and stained with antibodies described in material and method section. Using the gating strategies (6,7,8,9), 10,000 cells of interest were identified that express the appropriate immunophenotypic markers using the purity mask and with three different nozzle 70 μm, 100 μm and 130 μm. The sorted cells are then evaluated using the cell sorter into a template that allows for the differentiation of viable cells and identifies cell of interest. The percentage of purity is determined by taking ratio of the number of the cells of interest divided by the number of viable cells.

Figure 0-4: Cell size and nozzle size results.

Results shown from the sorting efficiency using different nozzle and cell types with the table with triplicate values and average±standard deviation.



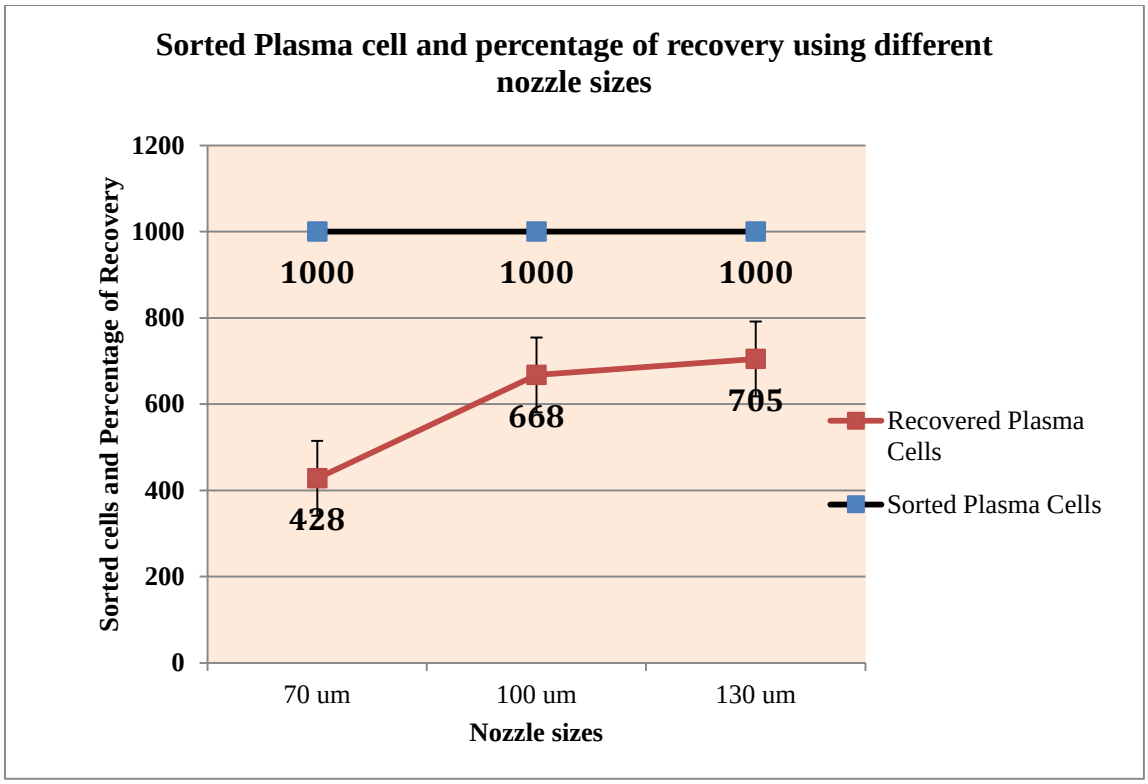
Cell type	**70 um**	**1**	2	3	Average±StdDev
Megakaryocytes		35.6	38.1	37.0	37±1.3
L428 cells		51.2	54.5	50.2	52±2.3
Lymphocytes		91.2	90.5	89.2	90±1.0
Cell types	**100 um**	**1**	2	3	Average±StdDev
Megakaryocytes		72.5	71.8	74.8	73±1.6
L428 cells		87.7	86.5	90.7	88±2.2
Lymphocytes		92.5	90.8	91	91±0.9
Cell types	**130 um**	**1**	2	3	Average±StdDev
Megakaryocytes		83.9	84.2	84.6	84±0.4
L428 cells		91.8	92.6	92.4	92±0.4
Lymphocytes		89.5	92.3	91	91±1.4

The differences in nozzle size and pressure differences and plate voltage are shown in Table 2. The experiment results show that as the cell size gets bigger, the greater nozzle size is associated with better cell recovery suggesting that the nozzle size has to bigger to accommodate the large sorted cell size. The HEL cells which has the approximate diameter of anywhere from 20 μm to 65 μm, the rate of recovery using the 70 μm nozzle is only 37±1.3% (average±StdDev for triplicates) compared with 84±0.4% when using the 130 μm nozzle. Statistical analysis were carrried out using the Microsoft Excel's t-test and found that the p value of 0.0002 which is statistically significant for the replicates from 70 μm and 130 μm nozzles and it shows the substantial difference using 130 μm nozzle when sorting megarkaryoctyes. The p value of 0.001 which is also statistically significant was obtained for comparision of 70 μm nozzle and 100 μm nozzle for megarkaryocytic sorting.

The diameter of the nozzle affects the recovery of the sorted cells, as in L428 (CHL cell line) the 70 μm nozzle only recovered 52±2.3% of the sorted cells while the 100 μm and 130 μm nozzle were able to recover 88±2.2 and 92±0.4% of the sorted cells, respectively. The p-value of 0.09 which is less of statistically significant shows that L428 sorting using either 100 μm and 130 μm the recovery percentage difference is minimal. The p values were 0.005 for 70 μm and 100 μm comparison with L428 and is statically significant and same as for the comparison with 70 μm and 130 μm (p value of 0.0009). The normal T lymphocyte was not affected by the size difference of nozzle and the p values for 70 μm and 130 μm (0.6), 70 μm versus 100 μm of 0.1, and 100 μm and 130 μm of 0.7, prove that the difference between recovery percentate with various nozzle sizes is not statistically significant. This is likely because, the size of normal lymphocyte is only 7-10 μm in diameter, and could easily pass through any of the given nozzle

Figure 0-5: Recovery of plasma cells with different nozzle sizes.

Sorted Plasma cells and percentage of recovery using different nozzle sizes.



In the sorted plasma cell experiment using different nozzle sizes, it the experiment showed that the pressure differences and the nozzle size differences affect the recovery percentage of plasma cells. The pressure differences shown in Table 2 also could contribute to the loss of recovery. At 70 μm nozzle the pressure of the sorter is at 70 psi and as the bigger cells pass through the smaller nozzle the chances of them getting damaged are higher.

Table 2: Summarizes the pressure (PSI) and plate voltages with different nozzle sizes as provided from the manufacture's manual (31)

NOZZLES	PLATE VOLTAGE	PRESSURE (PSI)
70 micron	4500	70
100 micron	2500	22
130 micron	2000	12

With the 70 µm nozzle the recovery was approximately 43±6.5% (average of triplicates ± StdDev) of the 1000 cells. While increasing the nozzle diameter (with a corresponding decrease in the pressure) shows an increase in the recovery percentage of 67±7.51% and 71±10.26% respectively.

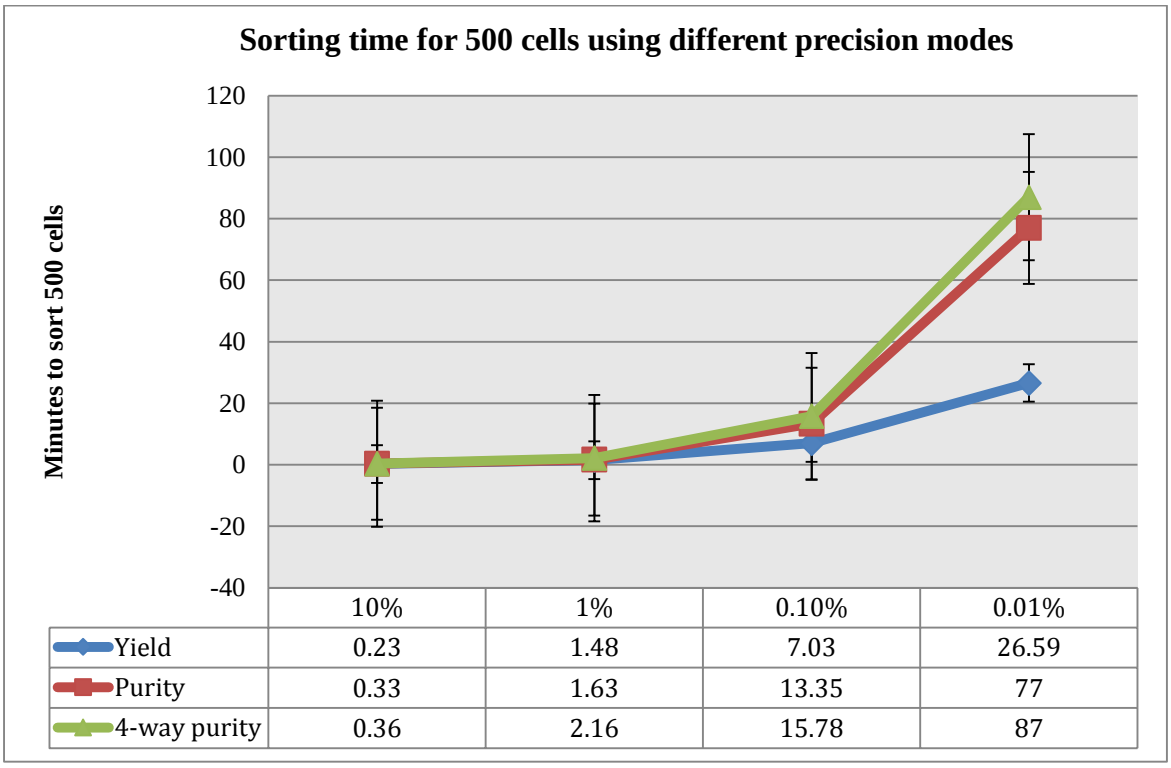
2.2 Sort Masks Experiment setup and Results

Experiment setup: Three different precision modes were tested using different percentage of cells of interest. The precision mode tested includes yield, purity and 4-way purity. For this experiment, different percentages of L428 cells were spiked into the morphologically confirmed normal lymph node. The different percentage of spiked cells ranged from 10%, 1%, 0.1% and 0.01%, which are representative of the percentage of abnormal cells in a rare cell neoplasm. The time consumed using different precision modes with various spike percentages are calculated for 500 cells and is listed in Figure 6. The conflict count is the number of events that met the sort criteria but were not sorted because of the conflicts. Conflict count increases with purity masks because of the stringency of the mask applied. The conflict count (loss of number of cells of interest) with

different precision modes at various percentages of spiked cells was also calculated using different precision mode for various percentages of spiked L428 cells were calculated, as shown in Figure 7.

Figure 0-6: Different precision modes and time for sort with different percentage of spiked cells.

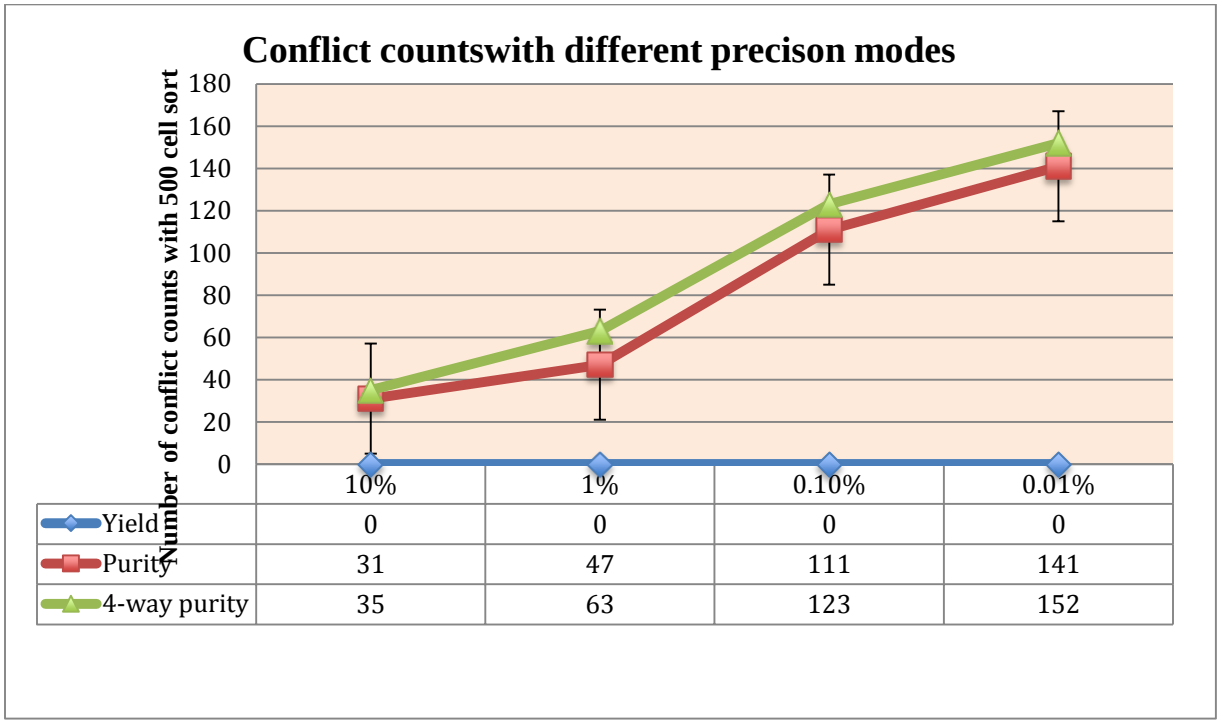
Time needed to sort 500 cells using various precision modes with different percentage of abnormal cells is displayed



	10%	1%	0.10%	0.01%
Yield	0.23	1.48	7.03	26.59
Purity	0.33	1.63	13.35	77
4-way purity	0.36	2.16	15.78	87

Figure 0-7: Conflict count rate with different precision modes.

Represents number of conflict counts per 500 cells sorted with different percentages of abnormal cells

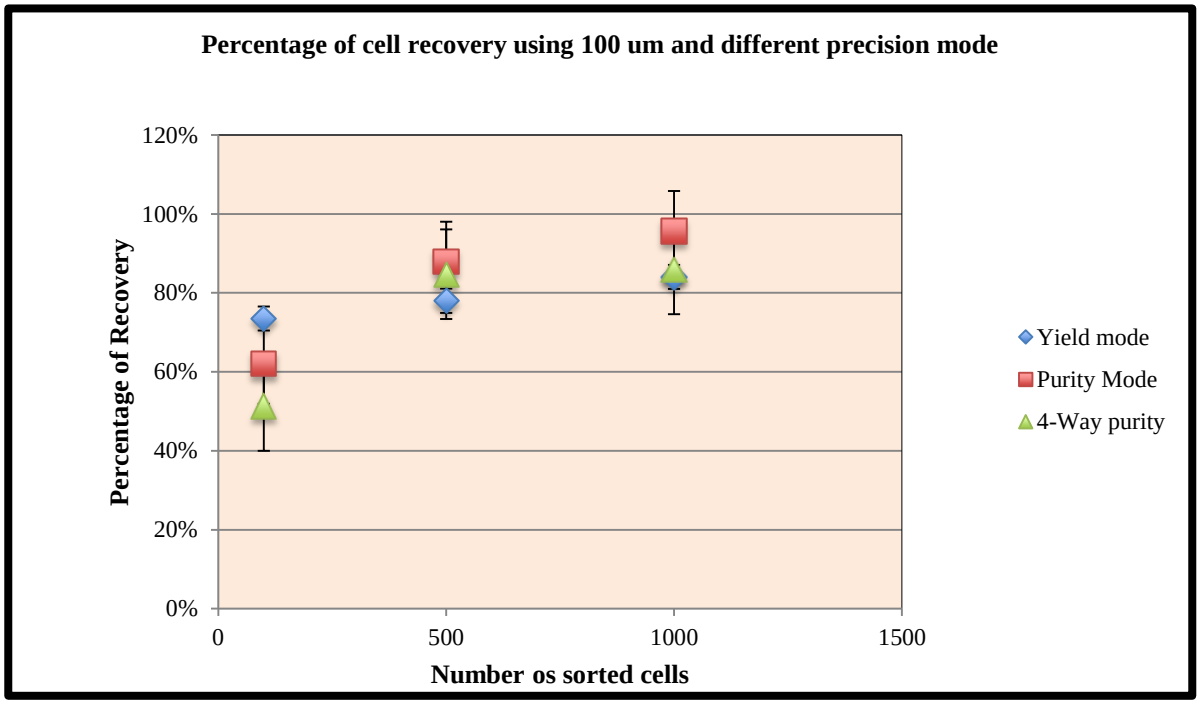


	10%	1%	0.10%	0.01%
Yield	0	0	0	0
Purity	31	47	111	141
4-way purity	35	63	123	152

For this experiment setup the cell mixture is stained with CHL cocktail mixture with all the monoclonal antibodies, which is described in material and method section for 15 minutes at room temperature. This stained mixture is washed once with PBS/BSA and strained before sorting. The cells of interest are gated to separate them from the rest of the background cells using the hierarchical gating strategy (as described in the materials and methods section).

Figure 0-8: Recovery using precision mode with 0.1% spiked cells.

This figure summarizes the results from a 0.1% spike using all three precision mode setting

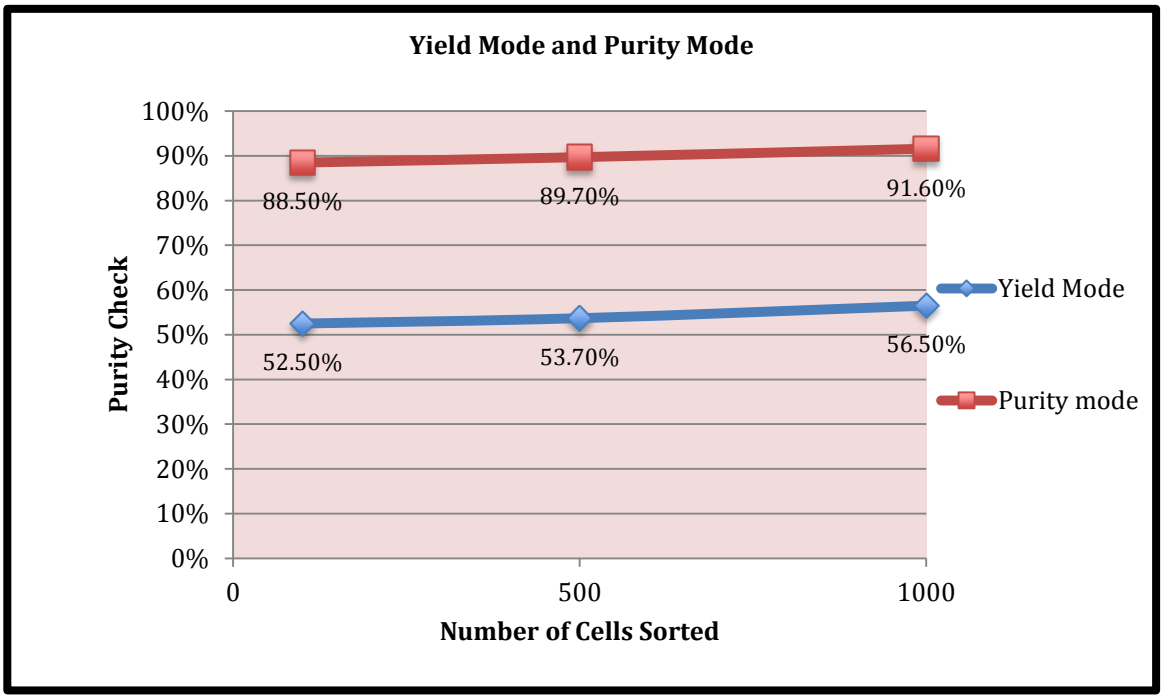


The three different precision mode settings are use to sort 100, 500 and 1000 cells, for 0.01% spike only; 100 and 500 cells are sorted due time constrains. Reacquiring the sorted events through the sorter and recording the sorted events calculated the purity. The percentage is calculated by dividing the number of sorted cells of interest by the number of cells in the viable gate.

The results from the 0.1% spiking experiments demonstrate that the purity precision mode is the choice for the rare cell neoplasms.

Figure 0-9: Plasma cell sort recovery with yield and purity mode.

The summarized results for yield and purity mode combined sorting a plasma cell sample with 1.1% of abnormal plasma cells.



When the cells of interest are rare, the conflict count increases as the sorter has to process more background cells and the cell of interest constitute extremely small percentages. The conflict count rate is also increased due to the fact with lower percentage of spiked rare cells the chances of background cells contaminant are to close to target cell is higher.

2.3. Sort recovery experiment setup and results:

Experiment setup:

L428 cell line spiked into a normal lymph node: 5% spiked, stained with CHL cocktail and sorted into the sorting medium (FCS and Hepes buffer).

Sorting window: 100, 500, 1000

In this experiment cells were not fixed and so chances of unbound antibodies were higher. Table 3 summarizes the results from the experiments with 100, 500, and 1000 sorted cells.

Table 3: Number of sorted cells (in triplicates) specified in sort window of FACS Aria sorter and observed average and the rate of recovery.

Number of sorted cells	1	2	3	Observed Average	Recovery
1000	761	797	768	775	77.5%
500	405	395	394	398	79.6%
100	75	81	77	78	78%

This experiment show that with 100 cells sorted the percentage of recovery was 78% and with 500 cells it was around 79.6% and 1000 sorted cells the recovery 77.5%. These results imply percentage recovery is similar whether 100, 500, or 1000 cells are sorted. Although this experiment shows that percentage recovery is similar across different number of cells, it doesn't account for the non-specific losses. The next set of experiment was setup to address non-specific losses.

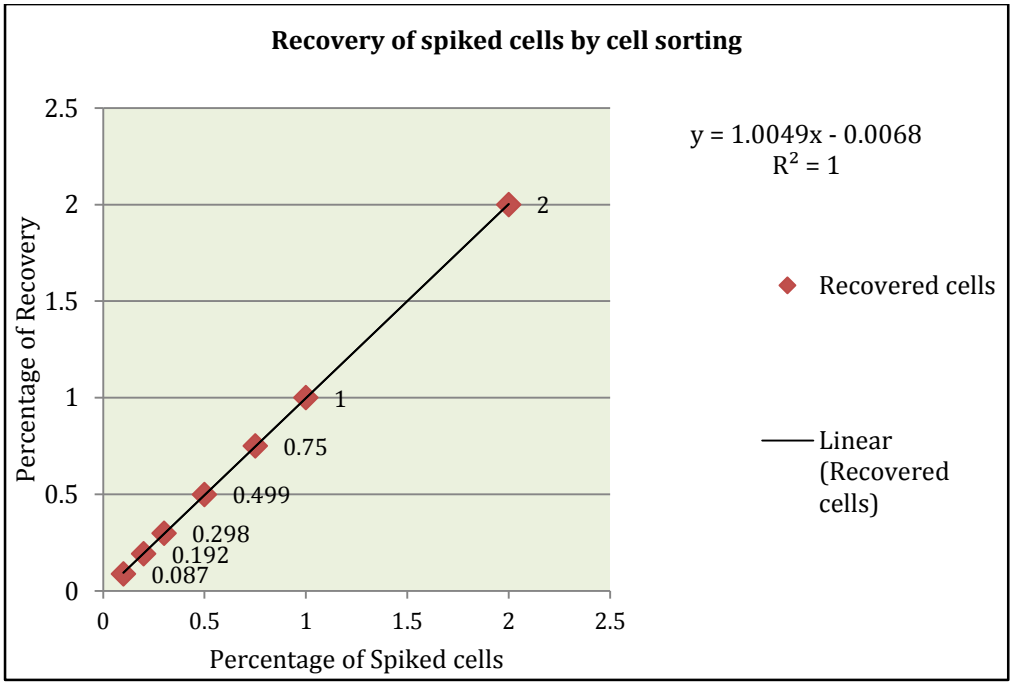
L428 (CHL) cell line is sorted into normal lymph node. L428 cells are stained with CD45, CD40, CD30, CD95 and CD15 and then fixed and washed. Normal lymph node cells are stained and fixed with CD5, CD20, CD45 and CD64 and washed. The fixed cells are counted and then appropriate dilutions are made using sorting medium. Then different percentages of stained and fixed L428 cell lines are sorted into the normal stained and fixed lymph node cells. These mixture were then flow sorted, the percentage of observed values are obtained from the FACS Diva software. The spike level of L428 cells the percentage of spiked and the recovery is shown in Table 4.

Table 4: Summary of the spike and recovery results from FACS Aria sorter.

Spike Level	1	2	3	Observed Average±Std Dev	Recovery
0.10%	0.092	0.079	0.089	0.087±0.007	87%
0.20%	0.185	0.192	0.198	0.192±0.007	96%
0.30%	0.298	0.3	0.296	0.298±0.002	99%
0.50%	0.497	0.5	0.5	0.499±0.002	99.8%
0.75%	0.75	0.751	0.749	0.750±0.001	100%
1.00%	0.997	1.003	1	1.00±0.003	100%
2.00%	2	2	2	2.00±0.00	100%

Figure 0-10: Percentage of recovery with different percentage of spiked cells.

Summary of the spike and recovery results from FACS Aria sorter with different percentages of spiked cells and it correlation coefficient.



For low level (0.1%) of sorted cells the recovery was 87%, compared to the medium (99.8%), and with higher percentages (0.75%, 1% and 2%) with 100% recovery. This

shows that even with dilution factor of 1:1000 the percentage of recovery of sorted cells are extremely reliable.

Discussion:

The purpose of the experiment was to explore the sorting capabilities or the UW Hematopathology flow sorter and also to optimize the sorter for more RCN and CHL sorting that is complex and time consuming. The initial experiments cell size and nozzle size with pressure showed that when sorting bigger cells it is necessary to have a larger nozzle. For HEL cells the recovery using the 70 μm nozzle was less than half around 37% and using the bigger nozzle significantly improved the recovery rate. The L428 cells had the highest recovery using the 100 μm and 130 μm nozzles, while the 70 μm nozzle only recovered around 52% of the cells. The different size nozzle did not affect the T lymphocyte, the smallest of the three. In case of cells like plasma cells the 70 μm nozzle only had a recovery of 43%, while the bigger nozzle 100 and 130 μm had a substantial increase in the recovery percentage of 67% and 71% respectively.

By testing the different types of precision mode setting for sorting decisions, it was clear with the both the 100 μm and 130 μm nozzles the purity mode was more favorable for downstream application. Even though the purity mode imparts the purity of particle of interest, it could cost the loss of target particles due to purity mode due to conflict count. As shown in Figure 7 the conflict count increases as the percentage of neoplastic cells decreases. When choosing a yield mode the conflict count is zero because it allows all the target particles to be sorted irrespective of its closeness to any contaminant. This experiment shows that the percentage of recovery of the cells are highest when the yield mode is used, because it minimize the sort aborts due to the position uncertainty. The yield mode could eliminate the loss of target cells which otherwise would be eliminated on positional basis, re-sorting with purity mode is recommended to enrich the sample.

The sort recovery experiments show that at the lower fraction of neoplastic cell, the recovery is about 87%, while at higher neoplastic fraction of recovery increased to 100%. For any molecular applications, especially the downstream application that requires minimal sorted cells; it is necessary to know the percentage recovery of sorted cells. Future molecular techniques may require single or few cells for complex downstream applications. These applications require knowledge regarding the number of the cells sorted and this sort recovery experiments addresses those concerns and questions.

CHAPTER III

COMPARISON OF DNA EXTRACTION METHODS AND SENSITIVITY DETERMINATION OF CLONALITY ASSAYS USING SORTED CELLS

Introduction:

Non-neoplastic cell contamination and cellular heterogeneity within the samples from tumor biopsies could interfere with results in molecular testing (5). In rare cell neoplasms (RCN) and in the setting of minimal residual disease (MRD), the percentage of neoplastic cells ranges from 1-0.001% of the sample. Enrichment of cell of interest is extremely important with RCN such as Hodgkin lymphoma and MRD where high levels of background cells would otherwise mask clonal peaks (as present in diagnostic molecular assay, for instance) or interfere with diagnostic tests (13). Adding steps of enrichment should improve the likelihood that downstream molecular biology techniques will be successful. The enrichment method of flow cytometric cell sorting, in combination with an effective and reliable DNA extraction methods will improve the sensitivity of molecular studies thereby will increase the diagnostic certainty of the evaluation of RCN and MRD.

In this experiment, flow sorting is used as an enrichment method. This technology, utilizes the immunophenotype characteristics to purify cells of interest. The flow cytometric sorted cells needs efficient DNA extraction method for providing a good starting material for molecular testing. That is where the comparison of two DNA extraction methods was necessary. Currently at Molecular Hemepathology lab (MolHP) at University of Washington (UW) the method of use is Puregene (Gentra, Qiagen) method, which is open and manual and prone to contaminants. This method also requires multiple washing steps and decanting of the supernatant, which could cause a substantial amount of material loss.

The method compared is using EZ Viral DNA extraction method, which requires an automated machine the EZ1 Advanced XL (Qiagen). The MolHP at UW currently owns this machine and is in need of validation of the EZ Viral method.

The B -cell clonality studies were done on all the cell lines to show different types of clonal population present in these cell lines. Based on the results the cell lines were selected to use for spiking experiments into normal lymph node that could mimic a rare cell neoplasm or MRD specimen. The detectable range for B-cell clonality in the Molecular Hematopathology (University of Washington) requires at least 1% of malignant cells present in the specimen to detect the clonal peak in the polyclonal background for IGH clonality assay and 5% of neoplastic cells for IGK clonality assay. According to World Health Organization (WHO), approximately 90% of lymphomas that are non-Hodgkin lymphoma are B cell related and recent studies also suggests Hodgkin lymphoma is a type of B cell lymphoma (11,13,17). This project's primary objective is towards determining the level of sensitivity of the DNA extraction method and B cell clonality assays using minimal number of sorted abnormal and normal back ground cells. The InvivoScribe kit for B cell clonality claims 95% sensitivity when the primers for both IGH and IGK are used (19), suggesting that RCN and MRD may be missed by this method. It is extremely important to detect the level of B cell clonality studies, which could be clinically utilized to create a range of sorted cells needed for RCN and MRD.

Combined usage of multiple techniques might be the answer for the efficiently and effectively detecting the clonal population in RCN and MRD. In this project the flow sorting enrichment method is used with EZ viral DNA extraction method to create a better product

that could be used for molecular techniques with higher diagnostic certainty for RCN and MRD.

Techniques Used:

1. Fluorescence Activated Cell Sorting (FACS):

Based on the same principle as flow cytometry, the cells of interest are sorted based on intrinsic and extrinsic properties. The intrinsic properties include the light scattering ability such as granularity and cell size. The extrinsic characteristics are presence or absence of antigen to which the multicolor fluorescently labeled antibodies are directed. The labeled cells hydrodynamically focused and presented to the lasers. During the laser interrogation different types of signals are created, these signals are then categorized in to groups based on the characteristics of each cells. The hierarchical gating structure allows to the operator to select individual cells based on the chosen parameters. These selected cells in the stream are then formed into droplets by the vibration of the nozzles. Selected particles are charged when it reaches the break-off point. The electrostatic field from plates with high voltages deflects droplets. The cells are collected in collection medium and processed further based on the downstream applications.

2. Puregene DNA extraction method:

The cells that are sorted into RPMI medium were centrifuge for 3 minutes at 14000xg, and the supernatant discarded. Cell lysis solution with an anionic detergent in the presence of DNA stabilizer is added to the cell pellet to lyse the cell walls to release DNA. The next step is to remove the contaminants by salt precipitation. The genomic DNA is

recovered by precipitation with alcohol and eluted out using a hydration solution. The eluted DNA is can be stored in 2–8°C, –20°C, or –80°C.

3. EZ Viral DNA extraction method:

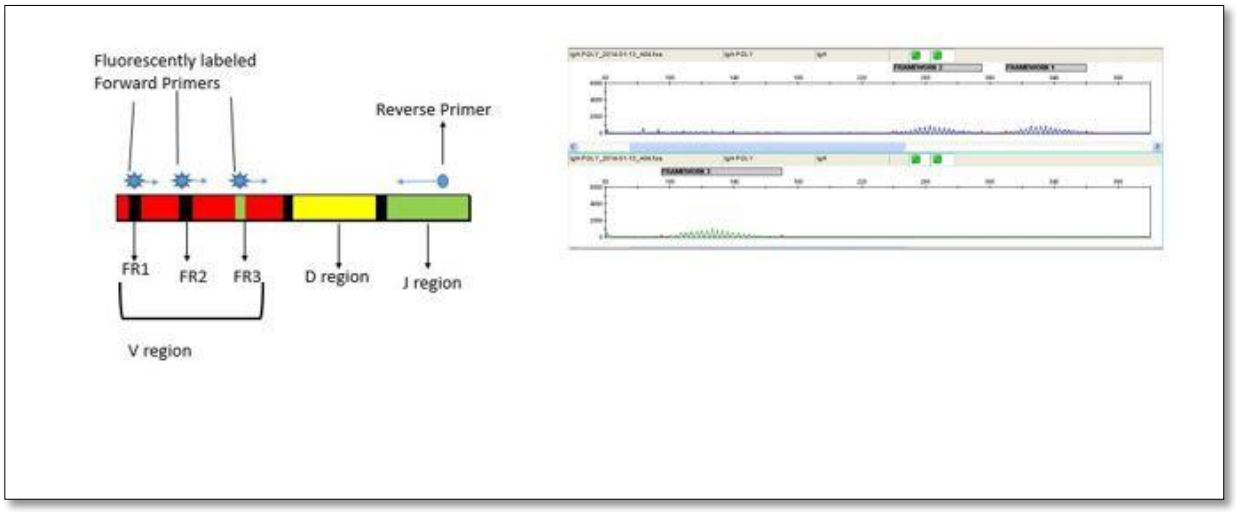
Qiagen EZ1 Advanced XL with EZ1 Advanced XL Virus Card v2.0 is an automated closed system that is used for DNA extraction. The cell of interest are sorted into RPMI are spun down, and the supernatant discarded. Cell lysis solution with anionic detergent and DNA stabilizer is added to cell pellet. This solution is loaded into the cartridge that uses a proprietary silica coated-magnetic particle technology for the extraction of DNA. The extracted DNA can be stored in 2–8°C, –20°C, or –80°C.

4. B Cell Clonality Assay:

B cell clonality assays (IGH and IGK) are PCR based testing for B-cell neoplasms. The IGH and IGK are located on chromosome 14 and chromosome 2 respectively. The IGH and IGK under goes gene rearrangement during the programmed somatic hypermutation, which allows it to produce a diverse variety of antibody against the foreign particles. During this rearrangement the V-(D)-J segments, sections are excised or added to form rearranged DNA. This testing relies primarily on the introduced addition or deletion of V-(D)-J segments during the rearrangement processing of B-cells. This assay include multiplex primers that detect the size differences V-(D)-J segments to determine the clonality and uses forward primers that targets the conserved framework region of (FR1), FR2 and FR3 all in the IGH J region. The multiplex primer sets for IGH clonality each recognize 1 of 3 conserved "Framework Regions" (FR1, FR2, or FR3) of IgH variable gene segments (VH) as shown in Figure 11.

Figure 0-1: Immunoglobulin and primers with normal polyclonal B pattern.

B cell clonality assay that targets IgH V(D) J region with Framework 1, 2 and 3 with fluorescently labeled forward primer to V region and unlabeled reverse primer for J region. The resulting product from normal B cell shows a Gaussian distribution pattern due to different size amplicons.



Each multiplex IGH primer set contains 6 or 7 VH primers in combination with a single consensus joining region (JH) primer. IGK testing consists of two multiplex primer sets that bind to conserved regions on either side of the V-J region where programmed genetic rearrangements occur during maturation of B cells. The IGK "A" multiplex primer set contains six kappa variable (VK) region primers paired with two joining (JK) region primers. The IGK "B" multiplex primer set contains the same six kappa variable region primers and an intronic recombination signal sequence (RSS) primer which are paired with a kappa deleting element (KDE) reverse primer. Multiple size segments of PCR amplicons are produced when normal B cell population is amplified and produce Gaussian/normal distribution pattern. The neoplastic or monoclonal B-cell population only produces PCR products or amplicons of same size that produce a single peak or two different peaks. One of the primers in each multiplex primer set is labeled with a fluorescent dye, which allows the amplicons to be analyzed on the ABI 3130 Sequence Detector by capillary electrophoresis.

5. T cell Clonality Testing:

The TCR-γ gene is a favorite target for T cell clonality testing because of it is rearranged in most of α/β and γ/δ T cells gene and in its germline state it only has only has of 6 functional V gene segments, 8 V pseudogene segments and 5 J segments in contrast of TCR-β locus which has 64 to 67 V segments, 2 D segments, and 13 J segments. There is enough homology among the V region segments and among the J region segments to allow use of small numbers of consensus primers to amplify across the V-J join N region. In normal scenario, polyclonal T cell population produce a Gaussian distribution pattern of PCR products on capillary electrophoresis, while a clonal or neoplastic cells will produce a

discrete peak. The primer sets for TCRγ rearrangement assay used at Molecular Hematopathology (University of Washington) has a level of detection of 95% of T cell lymphomas, although it requires at least 1% of neoplastic T cell to detect clonality peak in the polyclonal background.

Materials and Methods:

Specimens used:

- L1236 – Classical Hodgkins lymphoma cell line
- L428 – Classical Hodgkins lymphoma cell line
- Raji – Burkitt lymphoma cell line
- SUP B 15 – B-Lymphoblastic Leukemia
- DEV – Nodular Lymphocyte Predominant Hodgkins lymphoma cell line
- Morphologically confirmed reactive lymph node

Culture Conditions: The CHL cell lines were obtained from DSMZ (Germany) included L428 and L1236. Raji (Burkitt Lymphoma) was a gift from the Sabath lab, and SUP-B15 (Acute Lymphoblastic Leukemia) was brought from ATCC (Manass, VA, USA). DEV (Lymphocyte Predominant Hodgkins Lymphoma) cell line was a generous gift from Drs. van den Berg and Poppema (University Medical Center, Netherlands). All CHL cell lines and Raji were grown in RPMI-1640 medium supplemented with 10% fetal calf serum and 100U/mL penicillin/streptomycin at 37°C in an atmosphere containing 5% CO2. DEV was cultured in RPMI-1640 with 20% fetal bovine serum, 1% glutamine, 1% penicillin, and 1% streptomycin at 37 °C in 5% CO2. SUP-B 15 was cultured in Iscove's modified Dulbecco's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and supplemented with 0.05 mM 2-mercaptoethanol, 80%; fetal bovine serum, 20%, 1% penicillin, and 1% streptomycin at 37 °C in 5% CO2.

BD FACS Aria™ cell sorting: Cryopreseved morphologically confirmed normal lymph nodes were thawed using a pre-warmed mixture of 60% fetal bovine serum and 40% RPMI. L428 cultured according to previously explained conditions, are harvested spun down and

reconstituted with RPMI. Cell counts on both lymph node and the cell lines were obtained using Horiba ABX automated cell counter. The cell lines are spiked into normal lymph node to obtain a total 5% of L428 cells. This mixture is stained at room temperature for 15 minutes with the multicolor (8 color) antibodies specified below as in Table 5 (7,8,9)

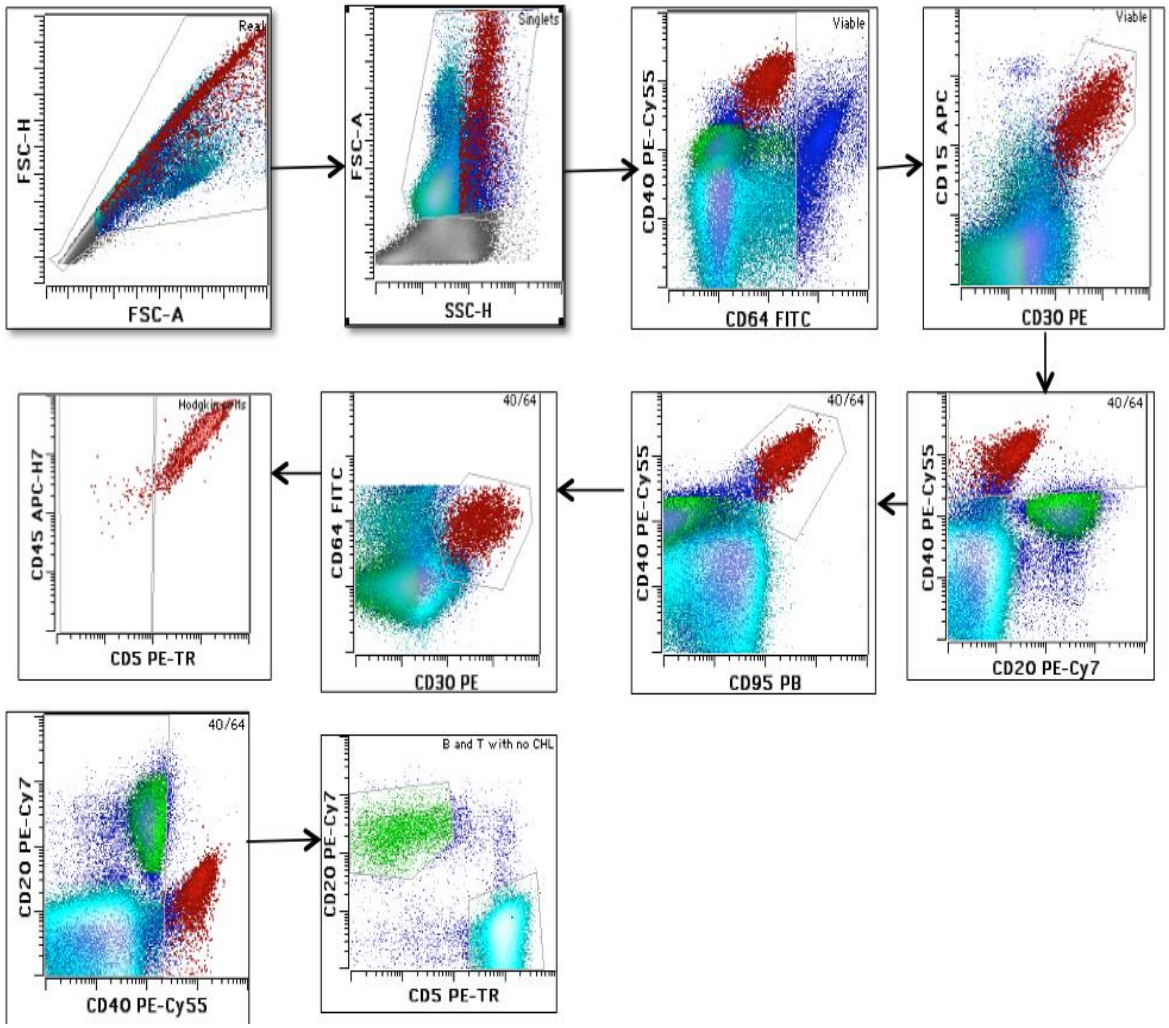
Table 5: List of antibodies, vendors, fluorochromes, lasers used to flow sort spiked L428 cells in normal lymph node

Antibody	Vendor	Fluorochrome/Laser Used
CD 95	Caltag (Invitrogen)	PacificBlue (PB)/Violet Laser
CD 64	Beckman Coulter	Fluroscein Thiocynate (FITC)/ Blue Laser
CD30	BD Biosciences	Phycoerythin (PE)/ Blue Laser
CD5	Beckman Coulter	ECD/Blue Laser
CD40	Beckman Coulter	PE-Cy5.5/Blue Laser
CD20	Beckman Coulter	PE-CY7/Blue Laser
CD15	BD Biosciences	APC/Red Laser
CD45	BD Biosciences	APC-CY7/Red Laser

The stained cells are washed once with PBS/BSA, the cell pellet then reconstituted with PBS/BSA and strained using BD Falcon cell strainer (40 microns). The gating strategies used are forward scatter area and height to eliminate the doublets and cell aggregates (8,9,10,11). The side scatter (SSC) and forward scatter (FSC) were used then to differentiate based on size and granularity. L428 cells are identified from the normal B and T cells using FSC and SSC in combination with CD95, CD30, CD40 and CD15 (positive on the cells of interest) as shown in Figure 12. The normal B and T cell and macrophages cells are separated using the FSC and SSC in combination with CD20 (identifies B cells), CD5 (identifies T cells), CD64 (identifies macrophages) and CD45.

Figure 0-2: Gating strategies for separation of CHL cells from the background.

See text for a description of the gating strategy to identify the different cell populations. Hodgkin cells are in red, T cells in teal and B cells in green.



PureGene DNA Extraction Method: The sorted cells are spun down and sorting medium discarded. The Puregene DNA Isolation Kit (Qiagen, Catalog #158489), Isopropanol (2-propanol) and 70% Ethanol (Qiagen, Catalog # 158930) are used for lysis and precipitation of DNA. Cell lysis solution containing RNase is added to the re-suspended cells with protein precipitation solution and centrifuged, discard the supernatant. The supernatant containing the DNA is mixed with 100 % isopropanol (to precipitate DNA), collected by centrifugation. The retenate is allowed to dry. 70% ethanol is added and mixed by inverting the tube several times to wash the pellet. The mixture is centrifuged and the supernatant is removed. DNA Hydration solution is added. DNA is allowed to rehydrate overnight at room temperature or by heating at 56o C for 1 hour. The product is stored at 2-8 o C.

EZ viral DNA Extraction method: The manufactures procedure is followed. Briefly, the sorted cells are spun down, the supernatant discarded and Qiagen Cell Lysis Solution (Catalog# 158908) is added. The closed reagent rack from EZ1 DNA Virus Kit (Qiagen, Catalog# 955134) is loaded with the cells in lysis solution with Rnase (Qiagen RNase A Solution (5ml) Catalog# 158924, Qiagen Buffer ATL Catalog# 19076), solution with Pre-Clear Columns (Fisher Scientific Catalog# 2302610) and 1.5 ml elution tubes. The automated closed system is Qiagen EZ1 Advanced XL, which uses EZ1 Advanced DNA Virus Card that has pre-programmed steps for the extraction of DNA.

B cell clonality testing: ABI 3130xl Genetic Analyzer and Gene Mapper ® Software:

Method Description: The bulk cells lines were spun down twice discarding the supernatant and the cell pellets were reconstituted in RPMI. The cell lines counts were determined using a Horiba ABX automated cell counter. The number of cells needs to be in the normal

range (which is 1-10*10^6 cells), 5.7*10^6 cells were achieved by diluting in RPMI. DNA extraction is done using the PureGene extraction methods and quantitated by the Nanodrop Technology. The B cell clonality assay was setup using the IGH and IGK Gene Rearrangement Assay Kit for ABI Fluorescence Detection (InvivoScribe Technologies Catalog# 11000041, 6330 Nancy Ridge Drive, Suite 106, San Diego, CA 92121 USA, store in the -80o C freezer), IGK Gene Clonality Assay for ABI Fluorescence Detection (InvivoScribe Technologies Catalog# 11000041, 6330 Nancy Ridge Drive, Suite 106, San Diego, CA 92121 USA), Amplitaq Gold (Applied Biosystems # 4311814). A positive control DNA (IVS 0019), size ladder control, a negative control, and a polyclonal control DNA (IVS 0000, InvivoScribe Technologies) is used on each PCR run. A NTC control using the sorting medium (RPMI) to replace for each sets of primer set on each PCR run consists of all of the reagents.

IGH Tubes:

- IGH Tube A: FAM (blue) labeled--- FR1-JH
- IGH Tube B: FAM (blue) labeled--- FR2-JH
- IGH Tube C: HEX (green) labeled--- FR3-JH

Programs for the DNA Thermal Cyclers for IGH assay is as follows: pre-activation for 7 minutes at 95°C, followed by 35 cycles of 45 seconds denaturation at 95°C, 45 seconds annealing at 60°C, and 90 seconds extension at 72°C. After the last cycle, a final extension step of at least 10 minutes at 72°C was performed and then kept at 12˚C indefinitely. The amplicons were then analyzed on the ABI 3130 Sequence Detector by capillary electrophoresis. A mixture of PCR products is made from tubes IGH A, B and C and 1 µl of the mixture is combined with 10 µl of Hi-Di formamide and 0.5 µl of ROX 500 base pair (bp)

markers. The samples are run with the IGH panel using the instrument protocol MDX_HP60 that has an oven temperature of 60o C, an injection time of 60 seconds, an injection voltage of 1.6, a run voltage of 15, and a run time of 1800 seconds. The size standards used are GS500 (-35-100-250) and the analysis method is microsatellite default. Electropherograms are analyzed using the GeneMapper® software.

IGK Gene Clonality Assay for ABI Fluorescence Detection (InvivoScribe Technologies Catalog# 11000041, San Diego, CA 92121 USA), Amplitaq Gold (Applied Biosystems # 4311814), a positive control DNA (IVS 0019), a polyclonal control (IVS0000, InvivoScribe Technologies), size ladder control, a negative control.

Programs for thermal cycler for IGK assays were similar to the IGH assay except the amplicons is kept at 15˚C indefinitely. A mixture of PCR products is made from tubes IGK A and B, 1 µl of the mixture is combined with 10 µl of Hi-Di formamide and 0.5 µl of ROX 500 base pair (bp) markers. The samples were run using a pre-programmed set of conditions, MDX_HP30 that has an oven temperature of 60o C, an injection time of 30 seconds, an injection voltage of 1.6, a run voltage of 15, and a run time of 1800 seconds. The IGK A product is run using the Vk-Jk panel and the IGK B product is run using the Vk-Kde panel. Electropherograms are analyzed using the GeneMapper® software.

IGK Tubes:

- IGK Tube A: IgK A FAM labeled (blue) Vk-JK
- IGK Tube B: IgK B FAM labeled (blue) Vk-Kde and RSS-Kde

T cell Clonality Assay: ABI 3130xl Genetic Analyzer and Gene Mapper ® Software

Method Description: The bulk cells lines were spun down twice discarding the supernatant and the cell pellets were reconstituted in RPMI. The cell lines counts were determined

using the Horiba ABX automated cell counter. An appropriate amount of cells are achieved by diluting in RPMI. DNA extraction is done using the EZ viral extraction methods and quantitated by the Nanodrop Technology. The T cell clonality assay was setup using AmpliTaq Recombinant Taq DNA (Applied Biosystems# N808-0153), TaqStart Antibody, Clontech Laboratories, Inc. Catalog #: 5400-1, -2, dATP, dCTP, dGTP, and dTTP. Ultrapure dNTP Set, GE Healthcare Cat. #28406552, PCR 10X Buffer II (Applied Biosystems #N808-0153), 25 mM MgCl2 (Applied Biosystems, P/N 4306898, a positive control DNA which is a mix of Jurkat, Molt3 and Invivoscribe positive control (IVS-0021) which produce a clonal peak in three out of four primer sets, a polyclonal control DNA (IVS 0000, Invivoscribe Technologies). A NTC control using the sorting medium (RPMI) to replace for each sets of primer set on each PCR run consists of all of the reagents. The PCR final volume of 50 µl which include the DNA on Thermal Cyclers for IGH assay is as follows: The cycling (50 cycles) parameters were as follows: Denaturation for 15 seconds at 95°C, followed annealing and extension at 60°C for a minutes and final extension at 72°C for 10 minutes before on hold at 12°C. The amplicons will be analyzed on the ABI 3130 Sequence Detector by capillary electrophoresis (see ABI 3130 Capillary Electrophoresis procedure). A dilution of the PCR product are made by adding 1 µl of the product with 10 µl of Hi-Di formamide and 0.5 µl of ROX 500 base pair (bp) markers. The samples are run with the pre-programmed program using the instrument protocol MDX_HP30 that has an oven temperature of 60°C, an injection time of 30 seconds, an injection voltage of 1.6, a run voltage of 15, and a run time of 1800 seconds. The size standards used are GS500 (-35-250). Electropherograms are analyzed using the GeneMapper® software.

Results:

a) 3.1. Selection of cell lines to represent abnormal cell sorting and clonality study

The clonality studies on bulk cell lines results are summarized on Table 6 based on the presence of clonal peaks on different frameworks of IGH and IGH gene. Table 7 summarizes the results based on the base pair of different clonal peaks in the cell lines and Figure 13 shows the clonal peaks all the frameworks. L428 cell lines are selected for all the next phase experiments, since it expresses clonal population in the entire framework in both IGH and IGK.

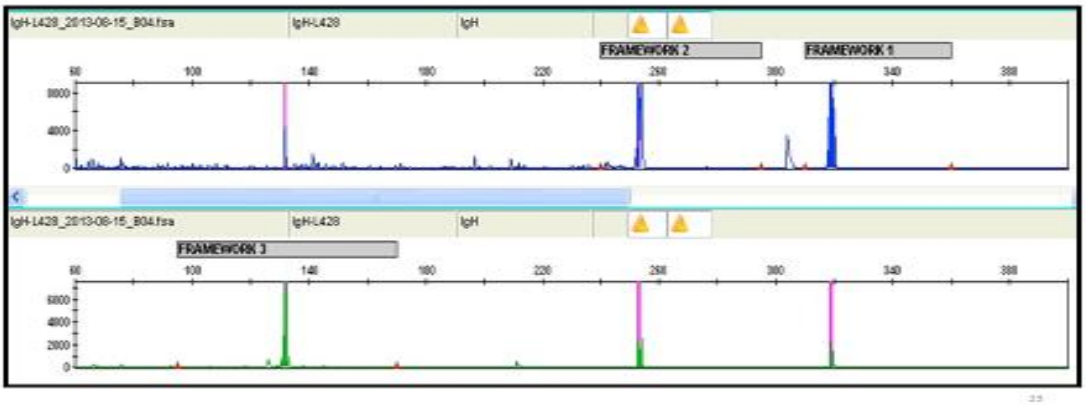
Table 6: Results from the B cell clonality testing of cell lines

	IGH Heavy Chain			IGK Light Chain	
Cell line	FR 1	FR 2	FR 3	Kappa A	Kappa B
L1236	+	+	-	+	+
L428	+	+	+	+	+
RAJI	+	+	+	-	+
SUP B 15	+	+	+	+	+
DEV	-	-	-	+	+

Table 7: Clonal peaks base pair for cell lines from IGH and IGK assay.

	IGH (Clonal peaks base pair)			IGK Light Chain (Clonal peaks base pair)	
Cell line	FR 1	FR 2	FR 3	Kappa A	Kappa B
L1236	324.38	263.01	-	260.83	276.99
L428	318.52	252.81	132.84	151.36	272.48
RAJI	343.75	277.45	142.39	-	235.97
SUP B 15	343.87	277.50	146.76	279.47	282.18
DEV	-	-	-	260.86	277.01

Figure 0-3: L428 cell line showing the clonal peaks on all the frameworks in IGH assay



b) 3.2. Comparison of different DNA extraction methods and determination of sensitivity of B cell clonality and DNA extraction methods using sorted cells

The next step of the project was comparison of DNA extraction methods, using sorted cells. The proven method for DNA extraction at UW Mol Hp lab was using Puregene extraction method using the anion detergent and salt precipitation to extract DNA and involves multiple steps of centrifugation and decanting of the supernatant. The new method that is being compared is EZ viral kit (Qiagen) used closed system and used magnetic beads to extract DNA using a silica-based system. For this experiment, L428 cells are sorted in duplicates that ranged from 10 cells to 10,000 cells in to RPMI and the negative control was RPMI. The L428 cells are extracted using both methods and B cell clonality assays for IGH and IGK are done. Table 4 summarizes the result of comparison and the sensitivity levels of each extraction method. A positive result is when any of the frameworks in IGH and IGK show a clonal peak. The clonal peak base pair values are compared to values from the bulk L428 studies in the prior experiments. The Puregene method looses the sensitivity around 5000-sorted cells; by using EZ viral method extraction the sensitivity level is 100-sorted cells.

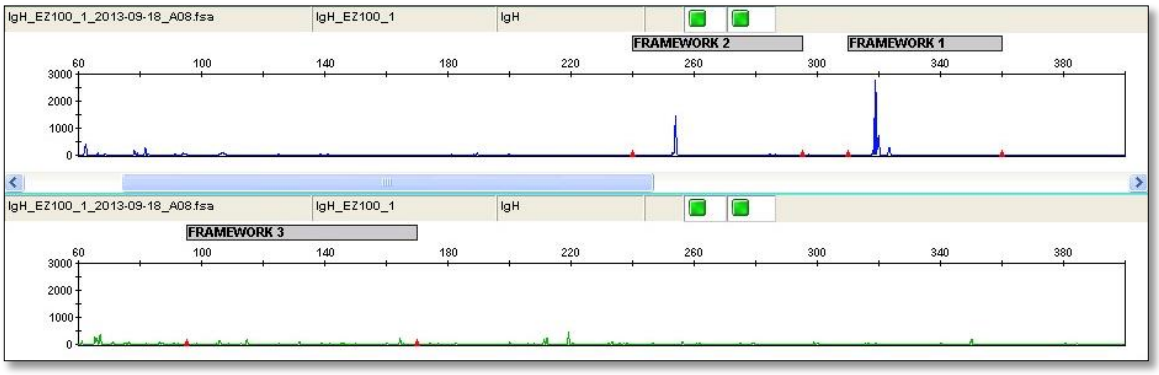
Table 8: Sorted L428 cells comparison of Puregene versus EZ Viral DNA extraction method

	Pure Gene Method		**EZ Viral Method**	
Number of Sorted cells	**IGH**	**IGK**	**IGH**	**IGK**
10,000 cells	+	+	+	+
5000 cells	+	+	+	+
1000 cells	-	-	+	+
500 cells	-	-	+	+
100 cells	-	-	+	+
50 cells	-	-	+	-
Negative Control	-	-	-	-

c) Determination of sensitivity for sorted abnormal and normal cell fractions using clonality studies

This phase of the experiment uses spiked L428 and EZ viral extraction steps that have been proven to have a better sensitivity for minimal cell load (see above).

Figure 0-4: EZ Viral Kit (top)and Puregene (bottom) DNA Extraction Method Comparison- 100 sorted cells with their clonal peaks base pair



Sample File Name	Size	Height	Area
IgH_EZ100_1_2013-09-18_A08.fsa	252.94	100	683
IgH_EZ100_1_2013-09-18_A08.fsa	318.86	2774	21279

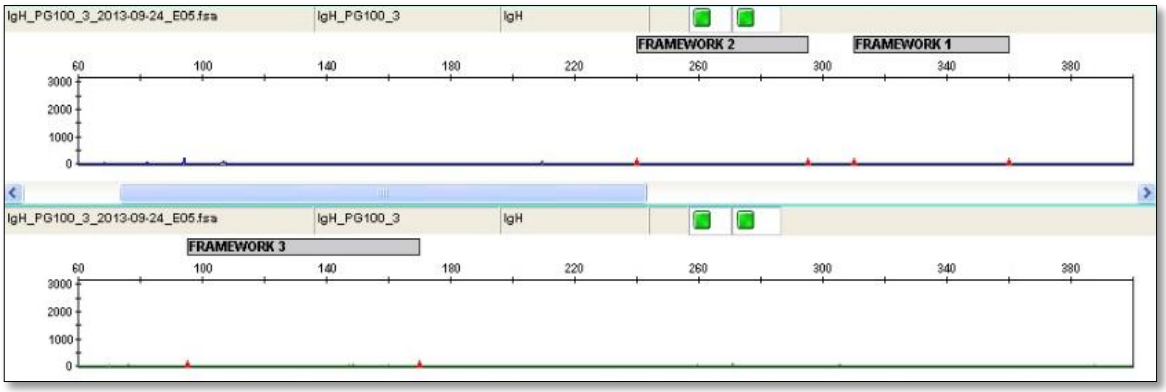
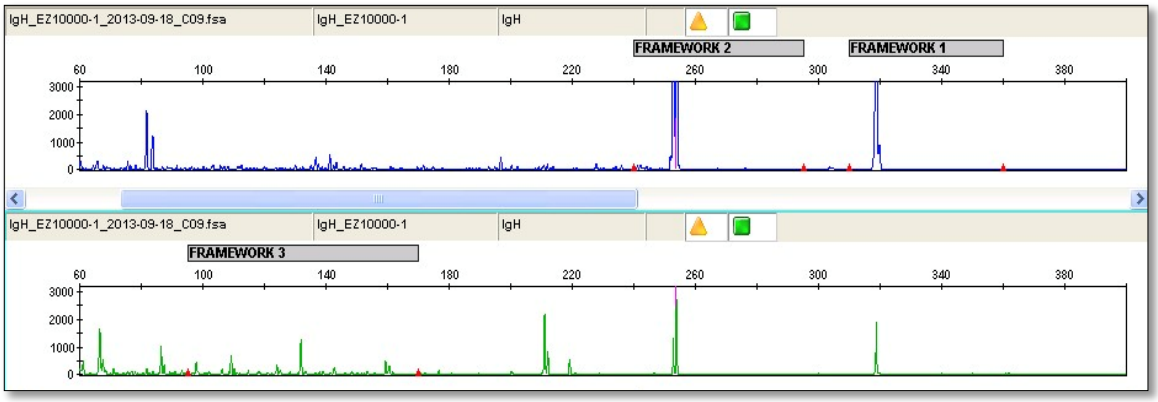
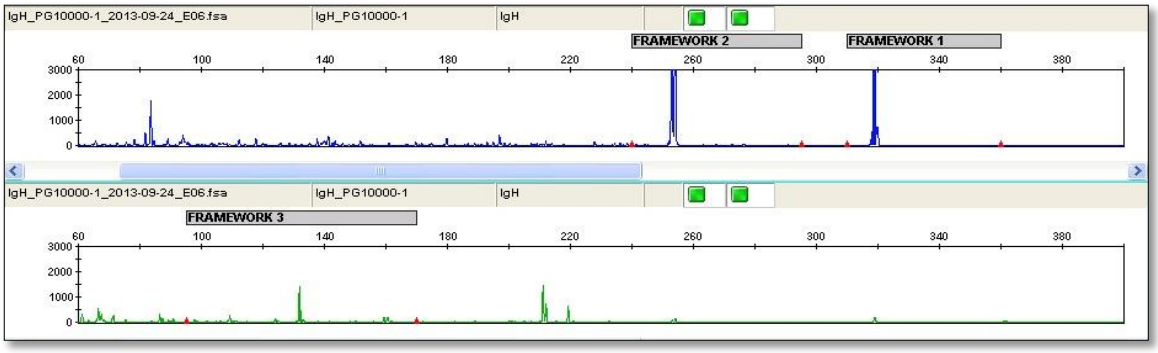


Figure 0-5: EZ Viral Kit (top) and Puregene (bottom) DNA Extraction Method Comparison- 10000 sorted L428 cells with clonal peaks and the base pair values



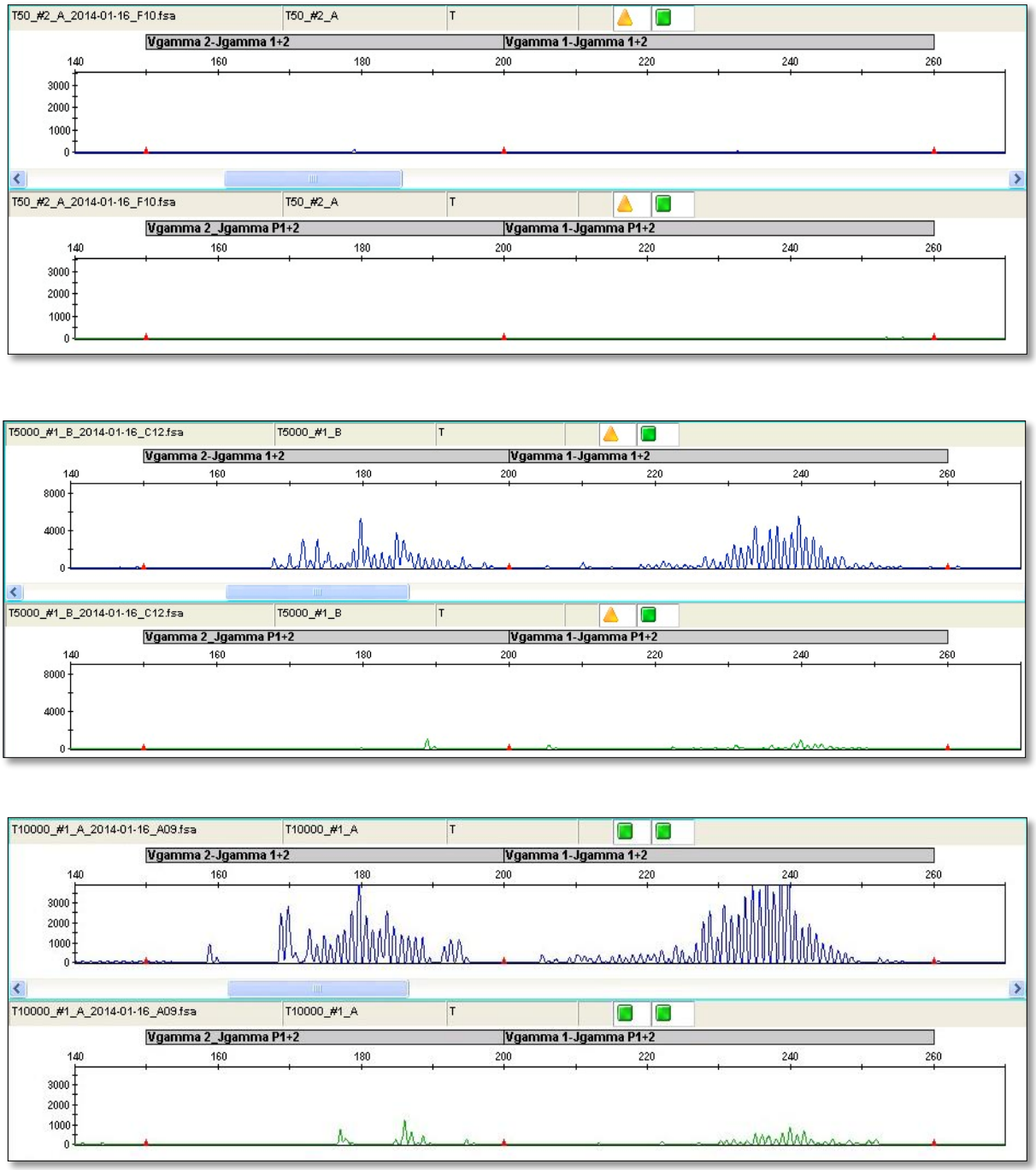
	Sample File Name	Size	Height	Area
	IgH_EZ10000-1_2013-09-18_C09.fsa	252.82	7734	56830
	IgH_EZ10000-1_2013-09-18_C09.fsa	318.71	7932	76196
	IgH_EZ10000-1_2013-09-18_C09.fsa	131.84	1270	6338



	Sample File Name	Size	Height	Area
	IgH_PG10000-1_2013-09-24_E06.fsa	252.03	215	1282
	IgH_PG10000-1_2013-09-24_E06.fsa	318.79	6644	54923
	IgH_PG10000-1_2013-09-24_E06.fsa	131.92	1407	7310

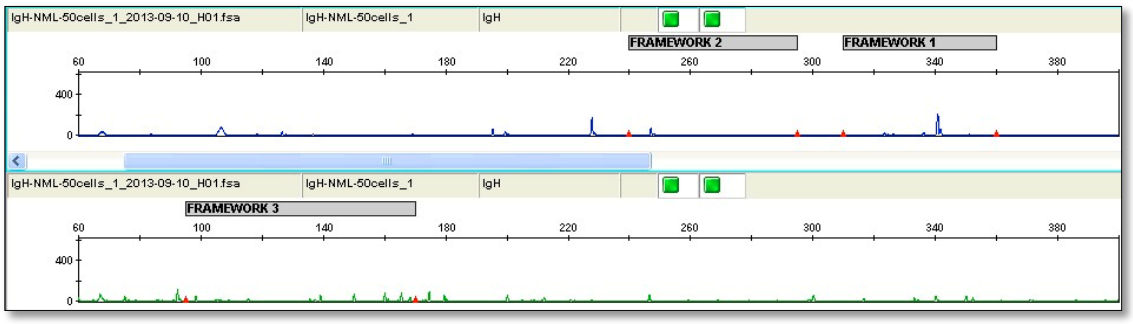
The sensitivity of B cell clonality assays using the two extraction methods as shown in Figure 14 and 15. The EZ viral extraction methods allow the detection of clonal peaks from 100 sorted L428 cells, while the Puregene extraction method lose the sensitivity around 5000 sorted cells. The range of sorted cells using both extraction method using IGH and IGK testing were from 100 to 10,000 sorted. The next task was the comparison of sorted normal B and T cells detection using the EZ viral extraction method

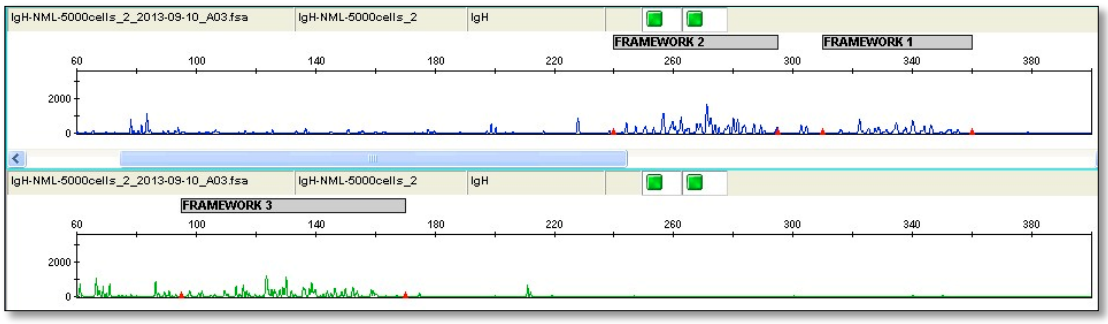
Figure 0-6: Sensitivity detection using EZ Viral Kit with sorted 50 cells (top), 5000 cells (middle) and 10000 (bottom) sorted normal T cells

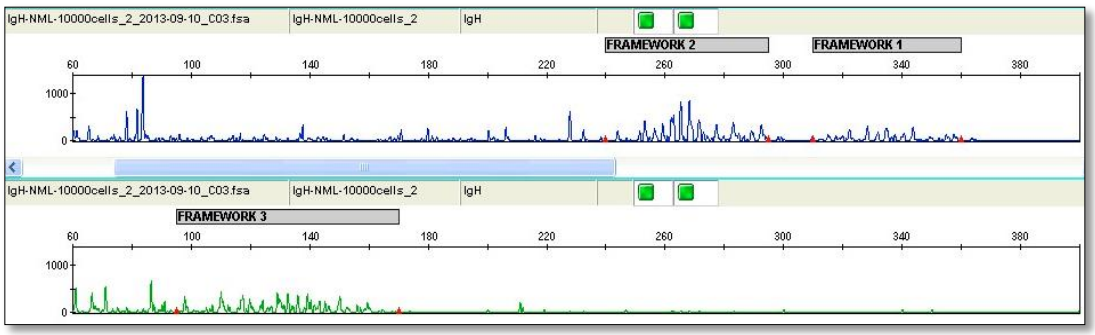


The T cell clonality testing results in the Figure 6 that the polyclonal pattern with normal T cells are only seen with at least 10,000 normal T cells. At least 10,000-sorted B cells are needed to give the normal polyclonal pattern.

Figure 0-7: Sensitivity detection using EZ Viral Kit with sorted 100 (top)and 5000 (middle)-sorted and 10000 (bottom)cells







Discussion:

The level of detection using the B cell clonality assays currently requires at least 1% of the abnormal populations. If the enrichment method is combined with an effective DNA extraction method, the detection limit could be set lower.

Initial experiment setup, the L428 (CHL cell line) was selected for the spiking experiment due to the fact that it had clonal peak in different framework of IGH and IGK testing. The base pairs of the clonal peaks on the bulk L428 cell line were recorded and then compared with the results especially with low number of sorted L428 cells to confirm the findings. The L428 cell lines were used for all the consequent testing.

The Puregene DNA extraction method is labor intensive and has multiple centrifugation steps and decanting especially working with minimal number of cells. The EZ viral extraction method on the other hand is automated and closed system that works well with minimal or sorted cells. In the experiment the results shows that when Puregene technique lost the sensitivity around 5000 cells, EZ viral kit was proven effective even with 100 sorted cells (when the peak sizes are known in advance).

The clonal peak detection limit for abnormal sorted cells is at 100 cells. The study also proved the limit of detection when using sorted normal B or T cells. For the normal B and T cells, the assays gives a Gaussian pattern with variety of fragment sizes, and with sorted cells this experiment set the limit at 5000-sorted cells for a polyclonal pattern.

The data suggests that B clonality assays sensitivity increases if the cells of interest are enriched and effective DNA extraction methods are used. The study proves that the using the an efficient enrichment method and DNA extraction method could improve the

sensitivity of assays, this could be used in clinical setting especially in case with specimens with minimum number of cells like cerebral spinal fluid (CSF) or MRD's. In the case of MRD or a rare cell neoplasm enrichment steps could improve the detection of clonal peaks for diagnostic purposes.

CHAPTER IV

SUMMARY

As stated in Chapter I, the purpose of this project was to optimize flow sorting in the Hematopathology laboratory. In addition, it was necessary to determine the sensitivity of DNA extraction methods so that minimum number of sorted cells could be used for downstream molecular techniques. Another purpose of the project was to create a range of sorted abnormal cells for B cell clonality assay and create a range of normal sorted to obtain a valid result when using the B cell clonality assay and T cell clonality assays.

The optimization of sorter using 100 μm and 130 μm nozzle involved setting up a set of experiment to measure the rate of recovery using different methods of sorting. The initial experiment was setup-using variety of sorted cells using 70 μm, 100 μm and 130 μm nozzles. The experiment shows that as the cell size increases the smaller nozzle fail to recover the cells, only 37% of the megakaryocytic cell line was recovered using the 70 μm nozzle, while a substantial increase in rate of recovery was noted after using the bigger nozzle. This was also seen in the case of L428 (CHL cell line), and in the case of lymphocyte, which measures 7-10 μm the nozzle, size difference did not make much difference the rate of recovery. Another part of this experiment was to measure whether the pressure difference associated with the nozzle size had any effect on recovery of plasma cells and the results showed a difference in percentage of recovery, with lower recover around 43% using 70 μm nozzle and increase to 67-71% when you using 100 μm and 130 μm nozzle respectively. The precision modes were tested using various percentages of L428 cells added to normal lymph node cells. Even though the purity mode results in the greatest purity, it may result in the loss of target particles due to conflict rate. In the studies, it is

clear that percentage of recovery of the cells are highest when the yield mode is used, because it minimizes the sorting aborts due to the position uncertainty. In the experiment setup using the plasma cells using a yield and purity mode together was faster that using a purity mode alone. The yield mode allows one to increase the rate of sorting without compromising the sort aborts. By combining both yield mode and purity mode allows sorting faster with a higher efficiency. The sort efficiency experiment gives us an insight into percentage of recovery after sorting. For this experiment, different percentages of stained and fixed cells were sorted into a premeasured mixture of cells then the whole mixture is reacquired through the sorter. The percentage of recovery is calculated using ratio of number of target cells by the number of all events collected. As expected the 0.1% cells only produced 87% recovery, while 2% had 100% recovery. This experiment helps to determine the rate of recovery when using the sorter.

The comparison of EZ viral DNA extraction method with Puregene method showed that the EZ viral DNA extraction method was more efficient and effective when dealing with sorted cells. The validation studies using EZ viral extraction kit has shown that the DNA extraction product from even a 100 cells could produce detectable results while the comparison method lost it efficiency around 5000 sorted cells. In clinical setting, the validation of EZ viral DNA extraction method will help the low-input DNA studies.

While the normal detectable range for B cell clonality testing needs at least 1% of abnormal cell population to diagnostically call the clonal peak from polyclonal background. The experiment showed that the sorted abnormal cells limit of detection B cell clonality even with 100 sorted cells, if the bulk cells from the neoplasm had been previously tested. The normal T and B cells are usually used as normal controls when running molecular assays.

By implementing all the necessary technical considerations for using 100 μm and 130 μm nozzles, that contributed to major research studies encompassing 5 different neoplasms. This study also helps contribute to the validation of low-input DNA extractions that could be used hypocelluar samples like CSF, body fluids or skin biopsies. This project defined the minimal input of sorted cell DNA for clonality studies, which would help to diagnostically decide a clonal population and polyclonal background.

Table 9: Summary of the list of neoplasm sorted using the optimized 100 µm and 130 µm nozzles.

The types of diseases included Classical Hodgkin lymphoma (CHL), Nodular Lymphocyte Predominant Hodgkin lymphoma (NLPHL), T cell rich B cell lymphoma (TCRBCL), composite lymphomas with Follicular lymphoma (FL) and Diffuse large B Cell lymphoma (DLBCL). Application for which the sorted cells were used includes Whole Genomic Amplification (WGA), Next Generation Sequencing (NGS), and Clonality studies.

Type of Disease	Number of cases	Initial percentage of abnormal cells	Purity	Applications
CHL	**53**	**0.001%-6.2%**	**42.90 - 96.50%**	**Clonality studies WGA NGS**
NLPHL	**11**	**0.0004%-0.21%**	**55.60% - 88.34%**	**Clonality studies WGA NGS**
TCRBCL	**3**		**74.42-97.05%**	**Clonality studies**
COMPOSITE LYMPHOMA	**2**	**CHL-1.0% DLBCL- CHL-0.4% FL-9.1%**	**DLBCL-98.91%, CHL-91.17% FL-97.3%, CHL-86.40%**	**Clonality studies**

Overall, implementing both the enrichment and molecular technique together will gain efficiencies in diagnostic purposes and clear the background noise issues. The platform used in this experiment is clonality assays, but in this new age of next generation sequencing and personalized medicine, the combination of both enrichment and molecular testing could be used in different setting like NGS.

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