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Single Cell RNA Multi-Omics Sequencing with TCR & BCR Repertoire Enrichment (BD Rhapsody system) Validation Report, version 1.2

This report describes the analytical validation of BD Rhapsody single cell RNA multi-omics sequencing with matched single cell TCR and BCR sequencing of different types of cryopreserved human or mouse T cell lines, B cell line, PBMCs, sorted cells, and bone marrow cells.

Accuracy	Accurate assay performance was determined by cartridge cell capture rate (Table 1) and high correlation between cell types was seen with the BD Rhapsody system and flow cytometry (Figure 1)
Precision: Inter-assay	Gene expression patterns between technical replicates, and assessment of ability to detect the relevant information in 3,000 cells from 40,000 cells loaded is shown in Figure 2.
Precision: Intra-assay	T cells were multiplexed with different sample tags, then captured with two cartridges. Virtually identical cell phenotypes were recovered from

	each cartridge through sequencing (Figure 3). No batch effect for gene expression profiles was observed between the two cartridges as shown in tSNE plots (Figure 4).
Analytical sensitivity	We targeted 10,000, 20,000, and 40,000 cells loaded onto three cartridges for captures and library preparation. There is excellent correlation of theoretical multiplet rate and actual values, as well as a high capture rate of up to 90% (Figure 5).
Analytical sensitivity and reproducibility including interfering substances	Sample preparations, storage conditions (fresh vs frozen) and processing by different users at different sites could affect cell quality. Here we utilized the same frozen PMBC samples and performed the assay at different sites to compare reproducibility. There is a strong correlation between mRNA (R^2=0.952) and Abseq (R^2=0.973) markers between the two sites (Figure 6a). Fresh samples have higher capture rate than frozen samples (Figure 6b).
Analytical Specificity	We used Rhapsody Abseq to validate strong correlation (R^2=0.75) of mRNA and protein expression in resting and activated T cell between cartridges (Figure 7).
Reference interval (normal range)	BD Rhapsody is used to optimize whole assay workflow. The scanner metrics are highly correlated with recovery by sequencing. ~75% of cells loaded onto cartridge are retrieved by sequencing (Figure 8). This scanner is unique to BD Rhapsody vs. other single cell platforms, such as 10x Genomics or Tapestri.

Standardization, harmonization, reproducibility, and ruggedness	Standardized with a pooled library as a batch of unique indexed samples, sequenced on one flow cell type on NovaSeq X plus Sequencer to minimize technical variation.
Quality control and improvement procedures	The Scanner is an excellent tool for workflow QC so that cell loading density is verified before proceeding with library preparation and sequencing. The subsampled beads can generate similar proportions of each cell type using either whole transcriptome or targeted mRNAseq approach (Figure 9).
Any other performance: VDJ reproducibility and sensitivity.	We have compared the performance of VDJ targeting of TCR and BCR on different cell types to determine sensitivity (% of TCR/BCR calls on relevant cell types) and specificity (% of "background" TCR/BCR calls on irrelevant cell types). Results in Figure 10 show very good sensitivity (>80% of relevant cell types yield paired-chain calls for TCR; >90% for BCR). Specificity is also very good, in that <5% of B cells yield calls for TCR, and <1% of T cells yield calls for BCR (Figure 11).

1. ***Purpose of the assay***.

Single-cell RNA sequencing (scRNA-seq) technology has become the state-of the-art approach for unravelling the heterogeneity and complexity of RNA transcripts within individual cells, as well as revealing the composition of different cell types and functions within highly organized tissues/organs/organisms. Studies based on scRNA-seq provide

massive information across different fields making exciting new discoveries in better understanding the composition and interaction of cells within humans, model animals and plants. In general, different scRNAseq methodologies can robustly generate high-quality data for single-cell gene expression profiling. Individual cells are captured separately and lysed, then reverse transcription is performed to select mRNA (by polyT priming) and to obtain cDNA. The amplified cDNA is used for sequencing library preparation. scRNAseq technologies can simultaneously measure cell-surface proteins, which are often reliable indicators of cellular activity and function, is critical to understanding the unique characteristics of the various cell types. Using this to measure both gene and cell surface protein expression in the same cell can identify protein isoforms and proteins with low abundance transcripts, further increasing the phenotypic specificity. Cell hashing allows multiplexing of many different samples or experimental conditions and driving down the per-experiment cost of single-cell genomic studies. Sample multiplexing technology enables the use of high throughput platforms, such as BD Rhapsody and 10x Genomics.

Analyzing paired chain information of T cell receptors (TCR) and B cell receptors (BCR) at the single-cell level is a powerful tool for probing T and B cell diversity and function in lymphoid malignancies, infectious diseases, autoimmune disorders, and tumor immunology. Both T cells and B cells are activated in the immune response to viral infection at early stage by killing infected cells and by producing effective neutralizing antibodies. At the early stage of T cell development, TCR is generated by somatic rearrangement of variable (V), diversity (D), and joining (J) gene segments, known as V(D) J recombination. When T cell receptors (TCR), expressed on the surface of T cells, become engaged by peptide-MHC on antigen-presenting cells, the T cells proliferate and expand rapidly and produce a clone of T cells with the same TCR specificity.

The BD Rhapsody system is a commercial platform for single-cell multi-omic analysis, including targeted or whole-transcriptome RNAseq, with options for AbSeq (quantitation of nucleic acid-tagged antibodies by sequencing), B cell and/or T cell receptor targeting, scATACseq, and sample multiplexing using differentially tagged CD45 (mouse) or MHC (mouse)/HLA (human) antibodies (SampleTags, SMKs). The system uses a microwell cartridge which is loaded with up to ~50,000 cells, plus an excess of

capture beads, which fit one per microwell. An imager allows visualization of beads and cells and can quantitate the frequency of bead-cell co-occupied wells. A lysis reagent is added to allow each cell's mRNA to be captured on the beads, the beads are pooled, and cDNA is synthesized. Index sequences from each bead allow identification of RNA's derived from the same cell. Libraries are prepared separately for mRNA (whole transcriptome or targeted), TCR/BCR, AbSeq, ATACseq (from nuclei), and Sample Tags. Sequencing is completed on AVITI and/or NovaSeq XP (Illumina) and data analyzed using a BD single cell sequencing analysis pipeline hosted in the Seven Bridges Genomics cloud platform. After processing, data are imported into Cellisimo (BD) for secondary analysis and visualization. The output files are formatted to H5AD and SEURAT for further analysis.

We at Stanford CIMAC/HIMC have adapted BD Rhapsody assays since early 2020 and performed over 300 captures for multiple big projects with various cell types. We validated assays on multiple parameters, including but not limited to cell capture rate, cell types, cell size, cell numbers, cell-to-cell cross talk, batch effect, biological replicates, inter-and intra assays variations, sensitivity and specificity, etc.

2. ***Materials and methods***:

Human/mouse PBMCs, Jurkat/Ramos/k562/BT546 cells lines, mouse bone marrow cells, FSCS-sorted immune cells were used to validate the system for sensitivity, specificity, accuracy, and inter-/intra- variations. Standard BD Rhapsody multi-omics kits were used, followed by the manufacturer's instruction and protocols (see the link below).

https://www.bdbiosciences.com/en-us/resources/protocols/single-cell-multiomics.

3. ***Major validation results***:

For an accurate assay, multiple types of cells were captured with cartridges. the performance of capture rate was observed consistently by cartridge cell capture rate

(Table 1). By using mouse immune cells, high correlation between cell types were seen with the BD Rhapsody system and those seen using flow cytometry (Figure 1)

We also used human and mouse cells which were mixed before loading onto the cartridge at a 40k cell load. Even at this high cell load, the data is very clean, with low doublet rates and little crosstalk between the human/mouse genes (Figure 2). Gene expression patterns between technical replicates (n=2), and assessment of ability to detect the relevant information in 3000 cells from a 40,000 cell load (1:1 mixture of human and mouse cell lines) were assessed.

For Intra-assay Precision: T-Cells activated for 3-14 days using ThermoFisher Dynabeads® CD3/CD28 and IL2, then sample tagged and pooled. Pooled cells were split into two different cartridges (20,000 cells loaded onto each). BD® SMK/AbSeq/Targeted Libraries were prepared. Here, similar proportions of each sample tag were recovered from cartridges (note that proportions of cells from each sample tag may differ due to differences in viability at different timepoints) (Figure 3). No batch effect with the gene expression profiles was observed between the two cartridges as shown in tSNE plots (Figure 4).

For analytical sensitivity, we targeted 10,000, 20,000, and 40,000 cells loaded onto three cartridges for captures and library preparation. There is excellent correlation of theoretical multiplet rate and actual values, as well as a high capture rate of up to 90% (Figure 5).

We also looked at concordance across users and instruments, as sample preparation and processing by different users at different sites could affect cell quality. Here we utilized the same frozen PMBC samples and performed the assay at different sites to compare reproducibility. There is a strong correlation of mRNA and Abseq markers between the two sites (Figure 6a). Fresh sample with a single lysis has greater than 100% capture efficiency (due to imprecise hemocytometer measurement—in some runs the hemocytometer reading underestimated the actual cell concentration). Fresh samples had a higher capture rate than frozen samples (Figure 6b).

We also used Rhapsody Abseq to validate strong correlation of mRNA and protein expression in resting and activated T cells between cartridges. We saw a strong correlation of all Abseq markers between cartridges (Figure 7).

The BD Rhapsody scanner is used to optimize the assay workflow. The scanner metrics are highly correlated with recovery by sequencing. ~75% of cells loaded onto a cartridge are retrieved by sequencing (Figure 8). This scanner is unique to this single cell platform versus others like 10x Genomics or Tapestri. There is better correlation of cell recovery to cells captured on cartridge than to cells loaded onto the cartridge. Having the information about the number of cells captured allows researchers to make better decisions downstream (e.g., need to capture another round of cells to get higher cell numbers, or how big a sequencing run is needed for the cells captured, etc.).

In another quality control experiment, we loaded 20,000 PBMCs into a cartridge. Half were taken through WTA and half were taken through a targeted workflow. Similar cell types were seen with each assay. The subsampled beads can generate similar proportions of each cell type using either whole transcriptome or targeted mRNAseq approach (Figure 9).

Finally, for single T cell and B cell immune repertoire detections, we have compared the performance of VDJ targeting of TCR and BCR on different cell types to determine sensitivity (% of TCR/BCR calls on relevant cell types) and specificity (% of "background" TCR/BCR calls on irrelevant cell types). Results in Figure 10 show very good sensitivity (>80% of relevant cell types yield paired-chain calls for TCR; >90% for BCR). Specificity is also very good, in that <5% of B cells yield calls for TCR, and <1% of T cells yield calls for BCR. We further compared, for CD4+ and CD8+ T cells and B cells, the stability of TCR/BCR calls across varying numbers of sequencing reads. The results, in Figure 11, show excellent retention of paired- and individual-chain detection between 500 and 5000 sequencing reads.

In conclusion, this system's performance has been validated by multiple assays without significant inter-assay or intra-assay variation. This system has a high capacity to profile up to 50,000 single cells per cartridge with high specificity and sensitivity. Overall, the BD Rhapsody single cell multi-omics platform has reliable, robust, consistent results with minimal variation across biological replicates, site-to site, and

user-to-user. This system also has minimum sample bias, and ability to integrate large date sets without correction of batch effects.

Table 1. Cartridge capture rate for various cell types

Cell type	Tissue	Sample prep	Live cells loaded to cartridge*	Cartridge Capture rate**
CAR-T cells	Blood	Cell in-house manufacturing	25,000	72%
Mesenchymal stem cells (MSC)	Fatty tissue	Frozen, ON grown and trypsinised cells	25,000	73%
Tumor xenograft	Head and neck cancer	Dissociated tumor on Mice (PDX - paw)	25,000	67%
Total CD4+ T cells	Blood	Magnetic isolation (negative selection of total CD4 T cells)	25,000	74%
CD45+ immune cells	Duodenal biopsies	FACS sorted	25,000	68%
FACS sorted NK and T cell subsets	Blood	FACS sorted	15,000	66%
MSC (cyropreserved)	Cell lines	Cryopreserved	11,443	80%
iPSC, Adipocyte (primary fresh), GABA Neurons (cell line), Hepatocytes (primary)	Primary cells/Cell lines	Live/Cryopreserved	22,885	60%
Myeloma cell lines	Cell lines	Flask grown	11,000	73%

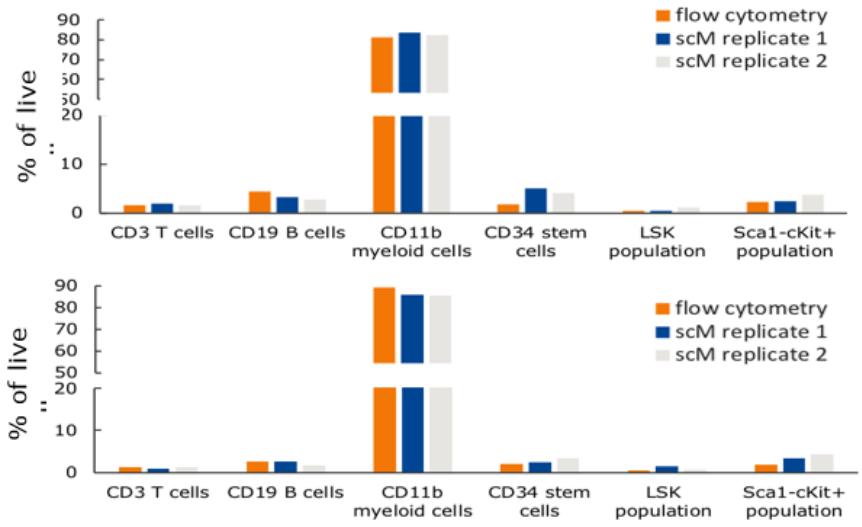


Figure 1. correlation between cell types seen between Rhapsody and flow cytometry

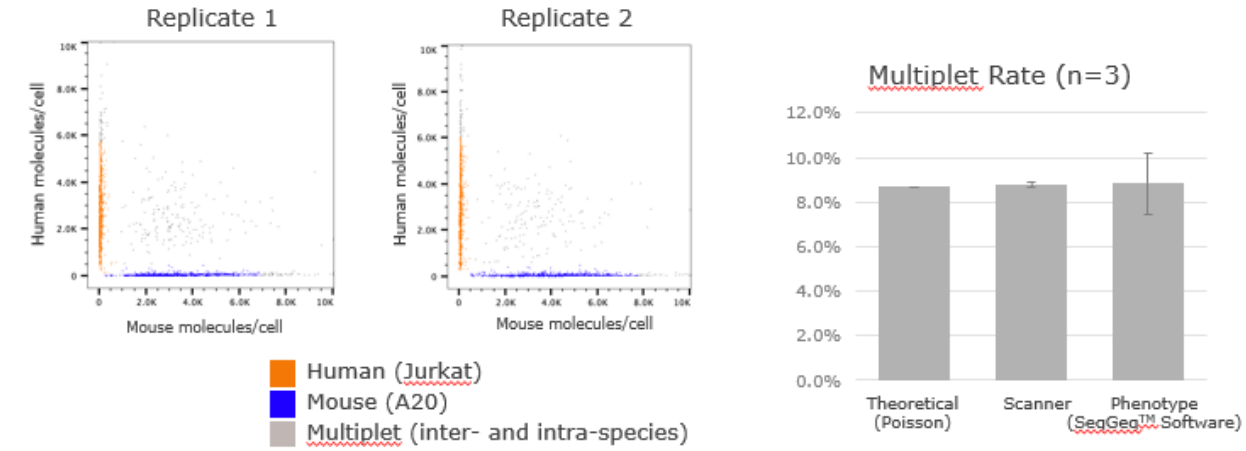


Figure 2. Reproducible data with minimal cell-cell crosstalk from high cell inputs.

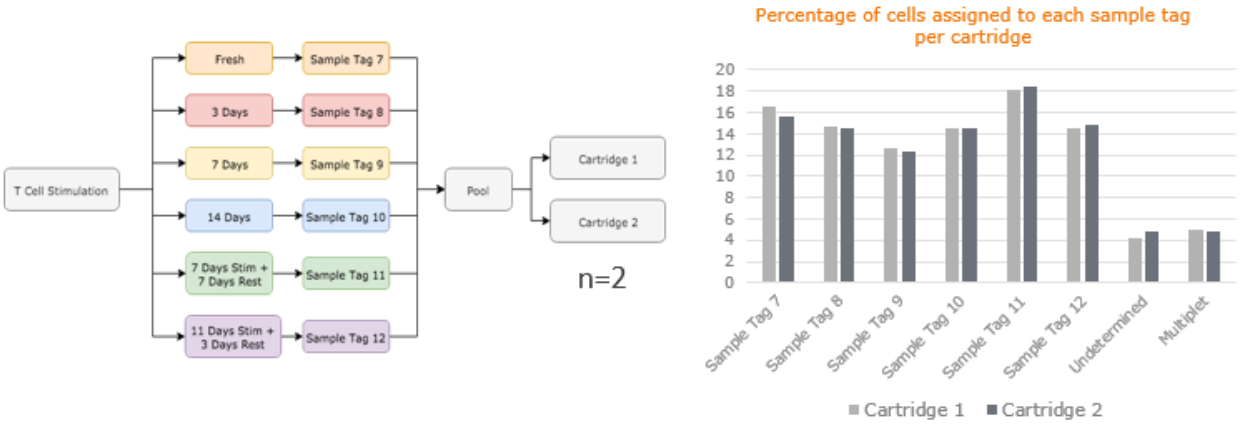


Figure 3. T cell activation time-course examined across technical replicates. Multiplet rates determined from Rhapsody scanner.

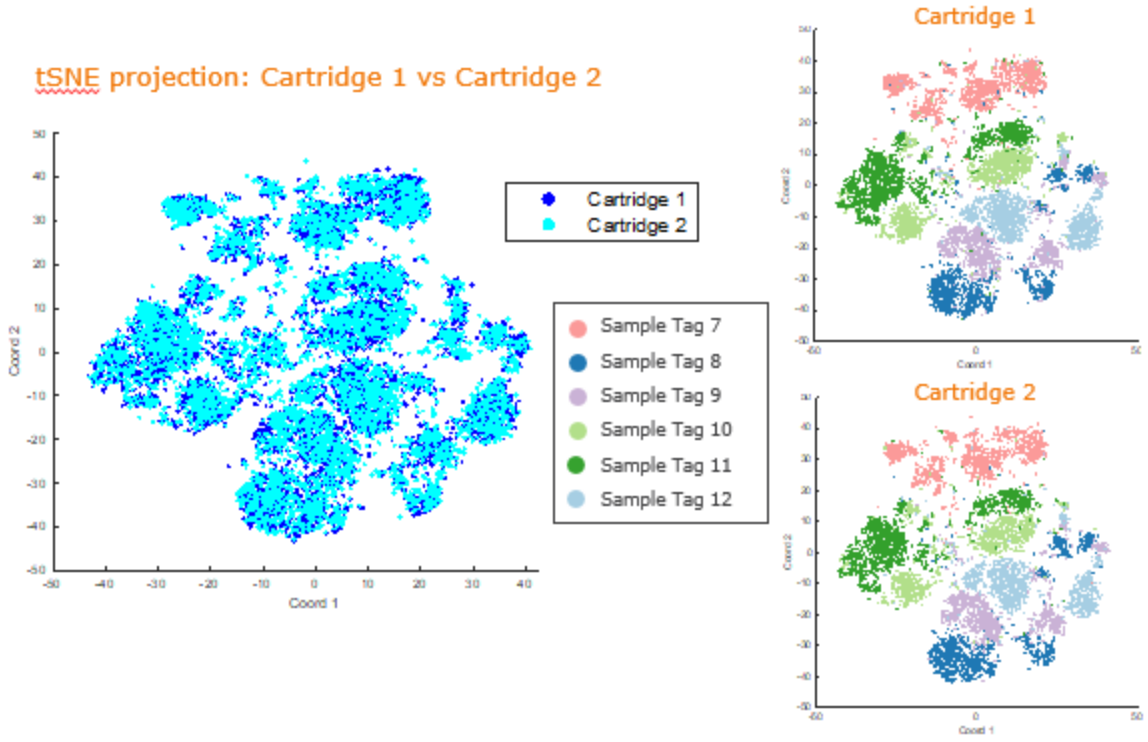
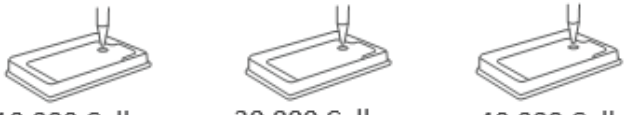


Figure 4. Minimal batch effect from technical replicates at the gene expression level. Each cartridge represents a separate experiment.



Target cell input	Live cells loaded (hemocytometer count)	Viable cells captured in wells with a bead	Capture rate
*40,000	52352	43432	0.83
*20,000	26176	22949	0.88
*10,000	13088	11789	0.90

*Mix of PBMC, Jurkat, Ramos and THP1 cells

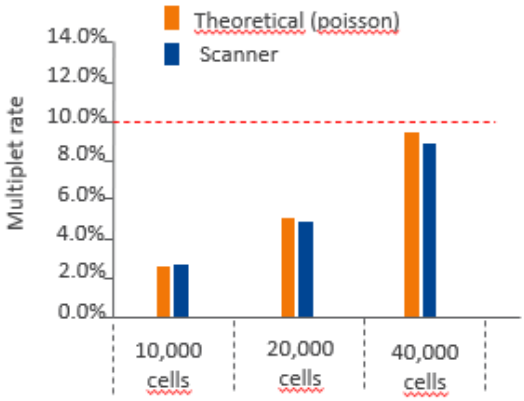


Figure 5. High cell capture rate and low multiplet rate across cell input numbers.

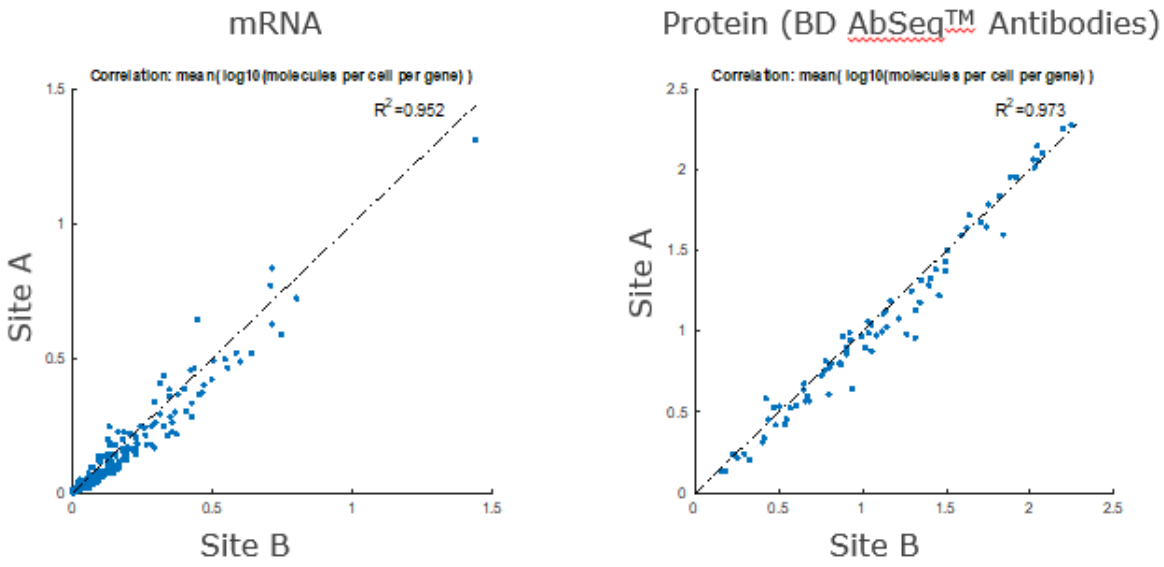


Figure 6a. Strong correlation of mRNA and Abseq markers on samples run at two sites.

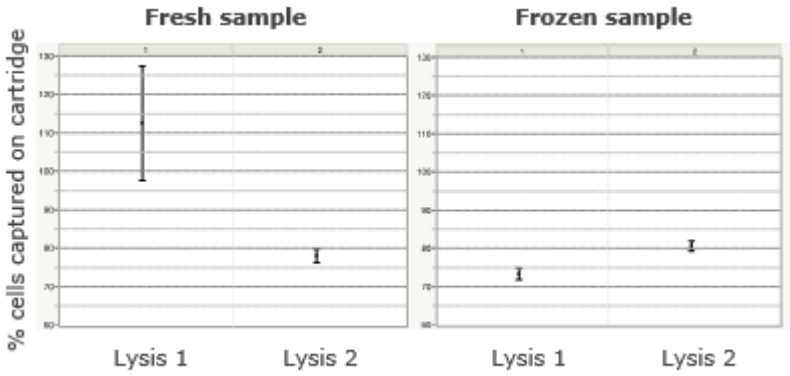


Figure 6b. Cell capture rate with fresh and frozen samples.

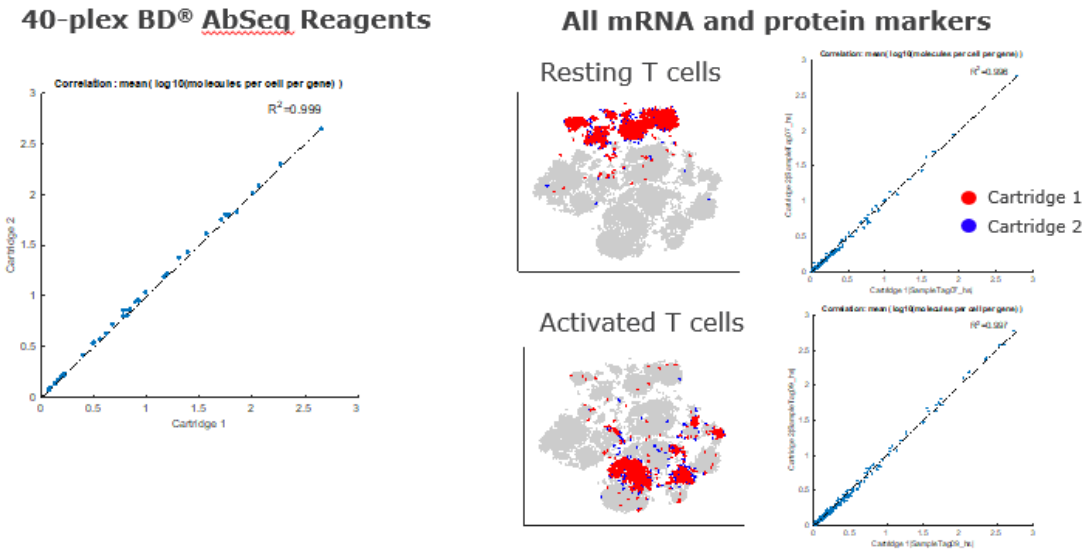


Figure 7. Strong correlation of mRNA and protein expression in resting and activated T cell between cartridges.

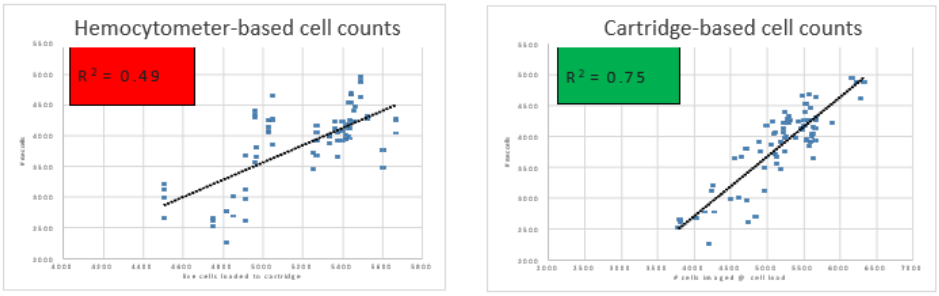


Figure 8. The scanner metrics are highly correlated with recovery by sequencing.

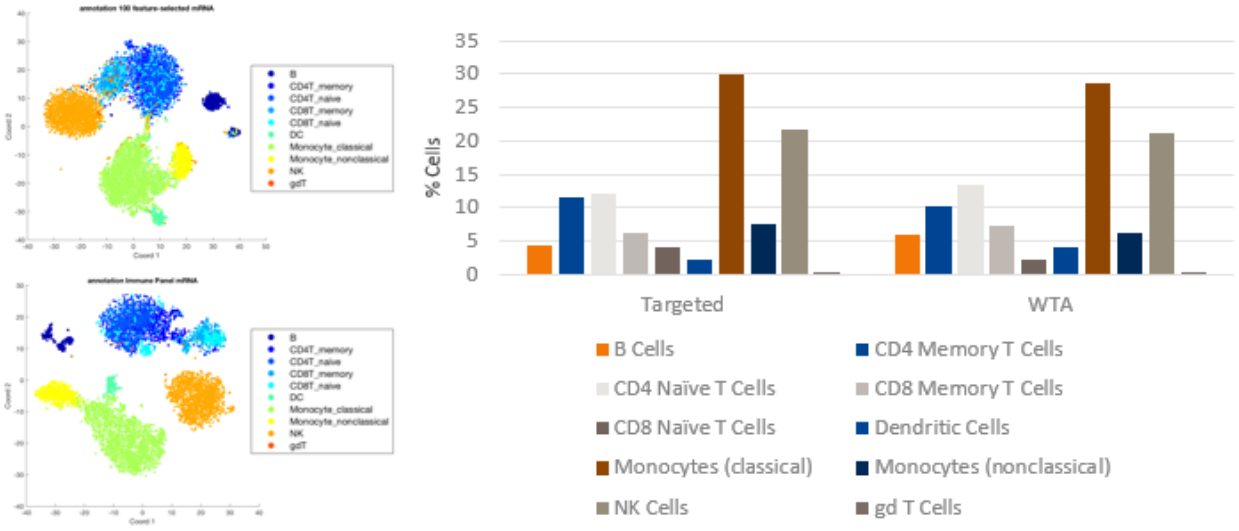


Figure 9. Similar proportions of each cell type are recovered from WTA and Targeted assays performed on subsampled beads.

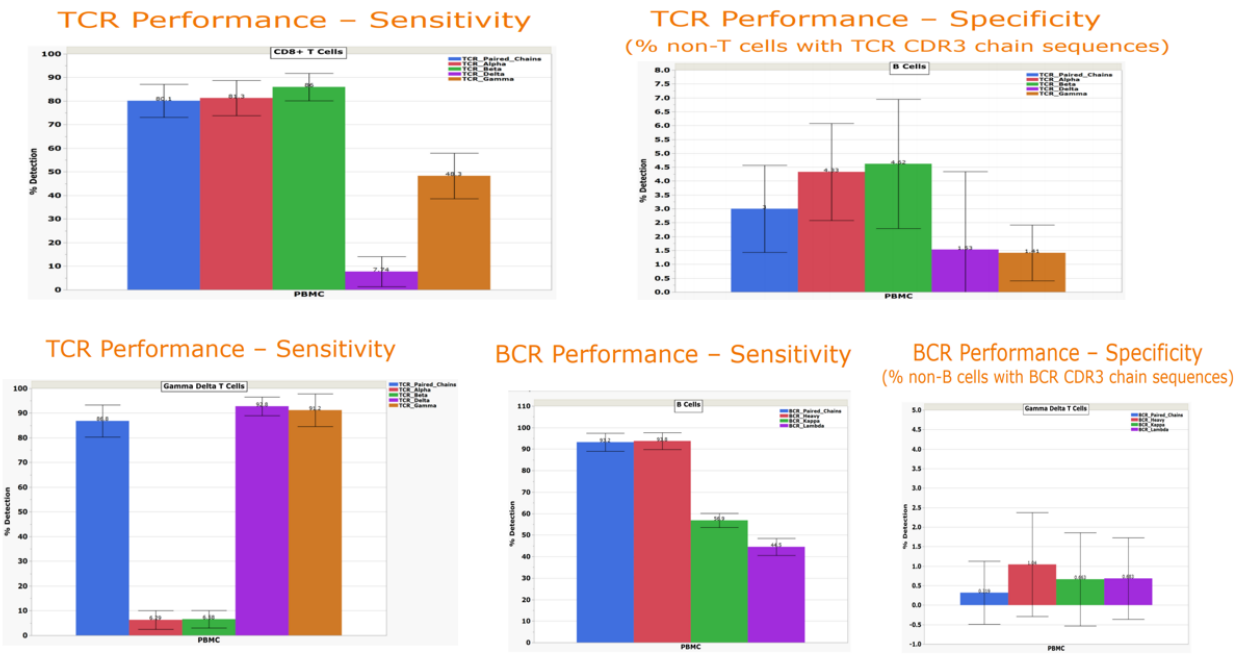


Figure 10. high sensitivity (>80% of relevant cell types yield paired-chain calls for TCR; >90% for BCR) and specificity (<5% of B cells yield calls for TCR, and <1% of T cells yield calls for BCR) for the VDJ profiles.

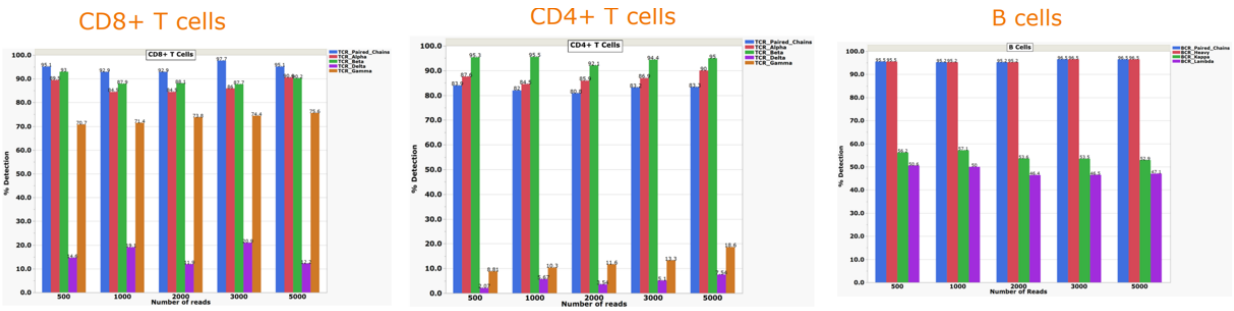


Figure 11. Retention of paired- and individual-chain detection between 500 and 5000 sequencing reads.

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