BD Rhapsody™ Whole Transcriptome Analysis (WTA) Amplification Kit

Additional Performance Data

Introduction

Data demonstrating the use of BD WTA assay for analyzing the below experiments,

- Cell inputs as low as 100 cells
- Samples with 40,000 cells
- Whole transcriptome of nuclei preps
- Murine samples

Data set 1: Testing 100 cell load with the BD Rhapsody™ Whole Transcriptome Analysis Amplification Kit

Background

- BD WTA assay was released with 1,000 and 10,000 cell input claims
- Certain niche applications necessitate the need for processing cell numbers lower than 1000
- The smaller input number presents a challenge in detecting signal over noise

Experimental Design

- Cartridge loaded with 1000 cells
- Subsampled 100 cells* from the 1000 cells above
  - Reduce cycle number to reduce the noise relative to the target
  - Eliminate an ampure step after RPE PCR to retain more molecules
  - Because of reduced yield due to lower cycle number, must eliminate Bioanalyzer step after RPE PCR cleanup
  - Compared results from 1000 and 100 cells

Sample	Post RPE 1.8x Ampure	RPE PCR Cycles	RPE PCR 1x Ampure	Index PCR Cycles	Post Index 0.75x Ampure
1000 PBMCs	2	13	1	9	1
100 PBMCs	1	9	1	15	2

*Added RT-treated cDNA-free beads to mimic the ratio of a cartridge loaded with 100 cells

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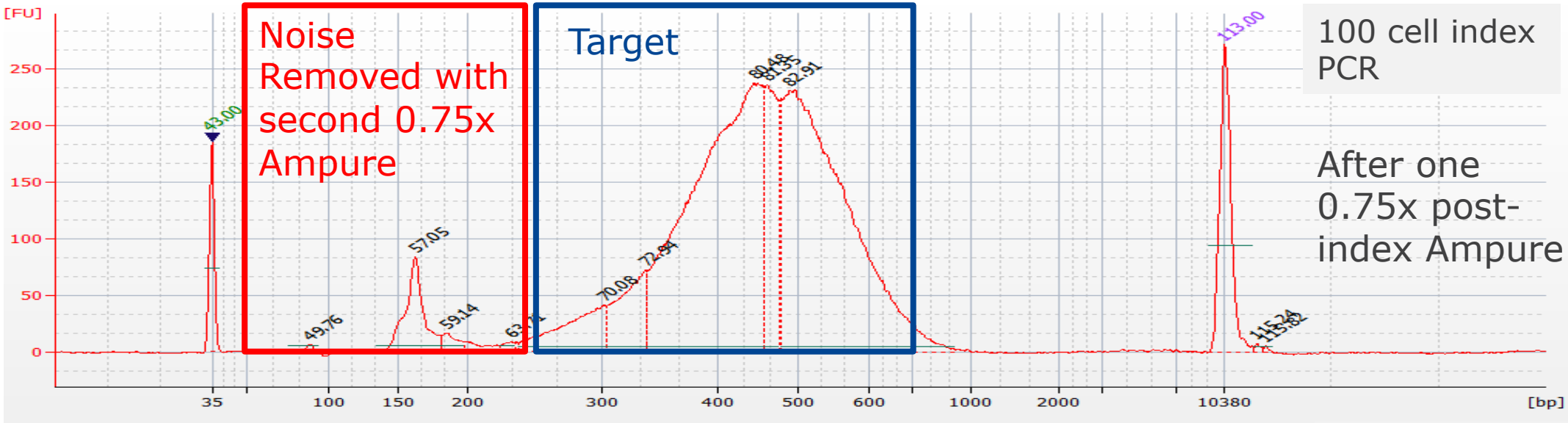
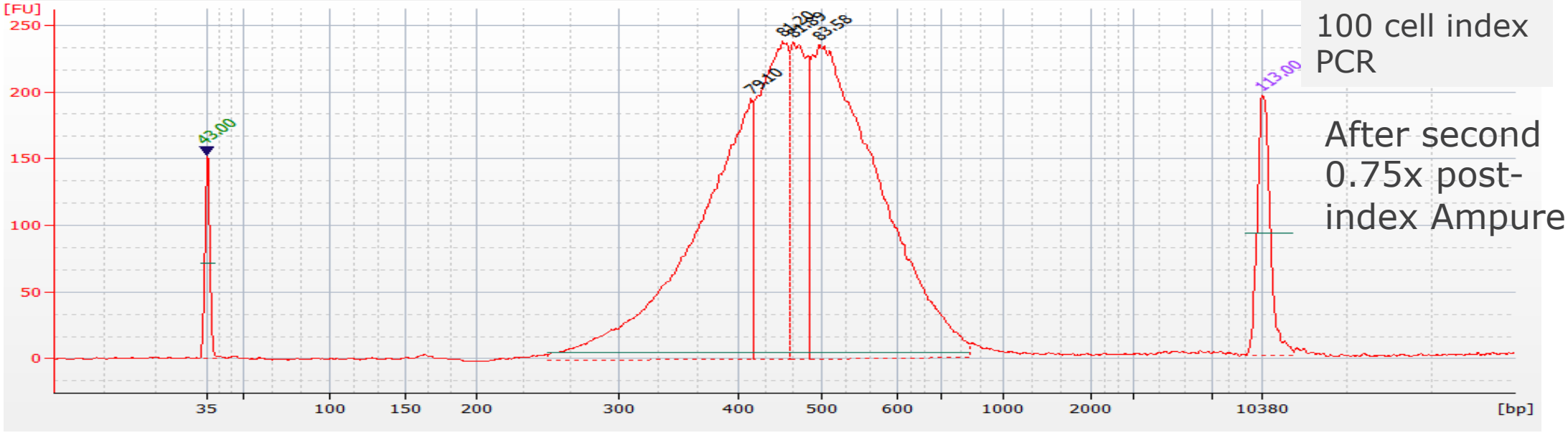
Results-100 cell BioAnalyzer



100 cell index PCR

After one 0.75x post-index Ampure



100 cell index PCR

After second 0.75x post-index Ampure

Eliminating the second post-RPE Ampure step increased the visible noise after index PCR. However, a second 0.75x cleanup removed the peak

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Comparison Between 100 and 1000 Cell Input Data

Metric	Specification	100 Cell	1000 Cell
Raw Reads Per Cell	N/A	11922	10387
Reads Per Cell	5,000	5,000	5043
Median Mol Per Cell	>1200	1445	1446
Median Targets Per Cell	>550	684	666
% Aligned to Transcriptome	>50%	55%	61%
% Q30	>75%	85%	84%

The 100 cell sample shows comparable sensitivity to the 1000 cell sample, despite a small increase in filtered reads.

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Summary

- Sensitivity of the 100 cell assay similar to 1000 cell input
- Workflow is almost unchanged and similar to current protocol in terms of time and reagents

- Limitations
  - No BioAnalyzer QC step to check progress after RPE PCR

Data set 2: Testing up to 40,000 cell load (super loading) using BD Rhapsody™ Whole Transcriptome Analysis Amplification Kit

Experimental Overview

- Combined three cell lines with PBMCs (to enable identification of multiplets, based on the presence of multiple cell types in a tSNE cluster)
- Aimed to capture ~10,000, 20,000, or 40,000 cells on a Rhapsody cartridge
- Followed the WTA protocol, with modifications made to the RPE PCR cycle number based on cell input
  - 10,000 cells = 12 cycles
  - 20,000 cells = 11 cycles
  - 40,000 cells = 10 cycles

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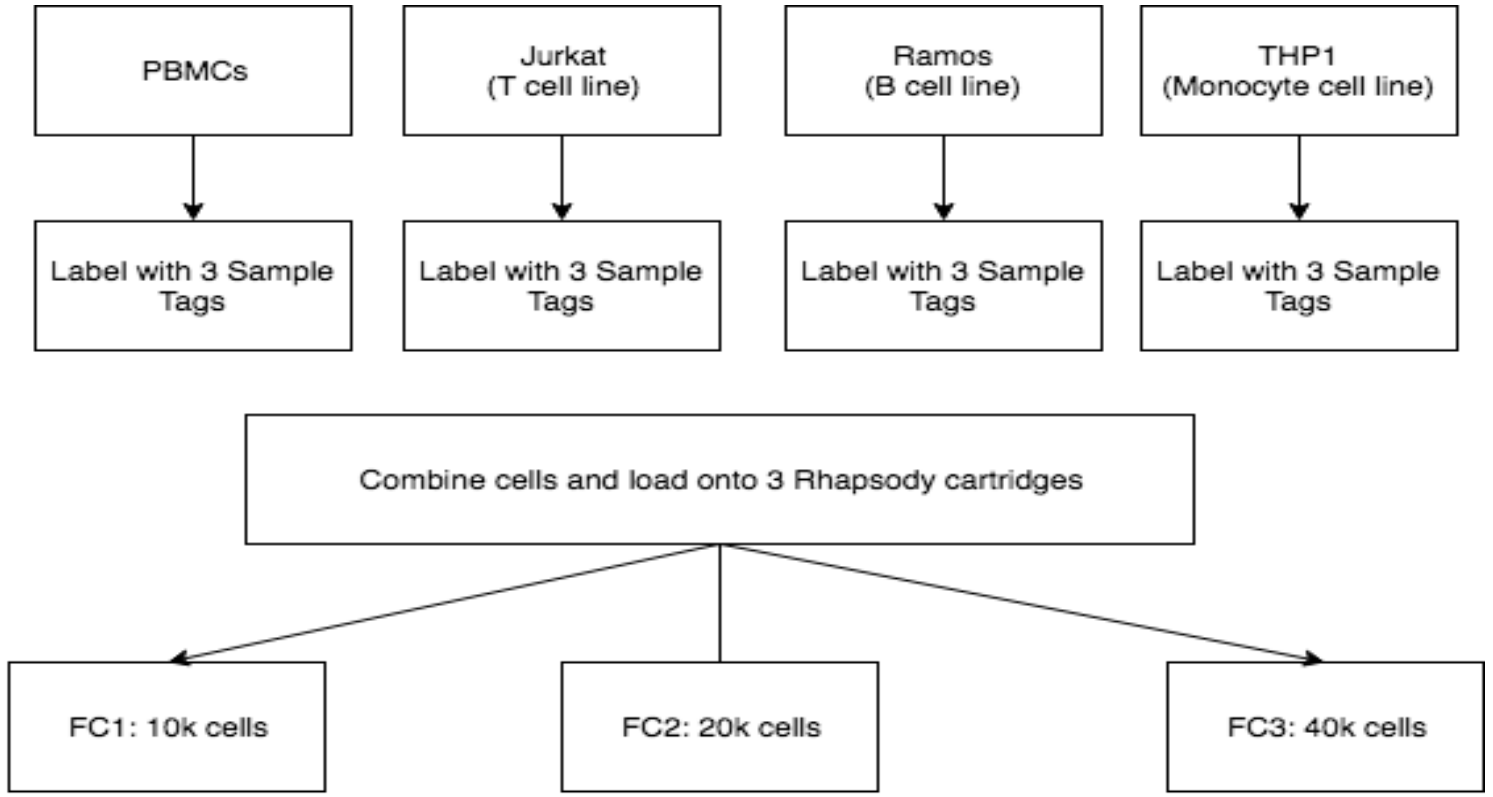
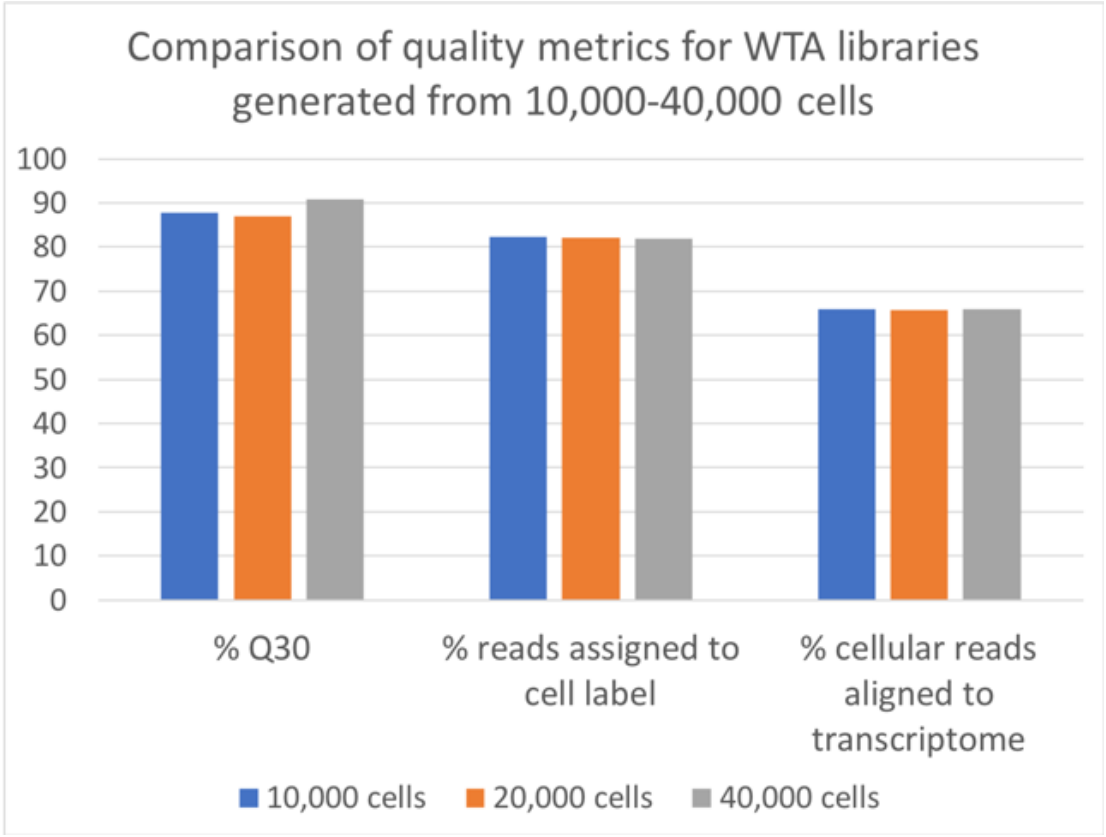
Experimental Overview (cont.)

Combined three cell lines with PBMCs (to enable identification of multiplets, based on the presence of multiple cell types in a tSNE cluster)

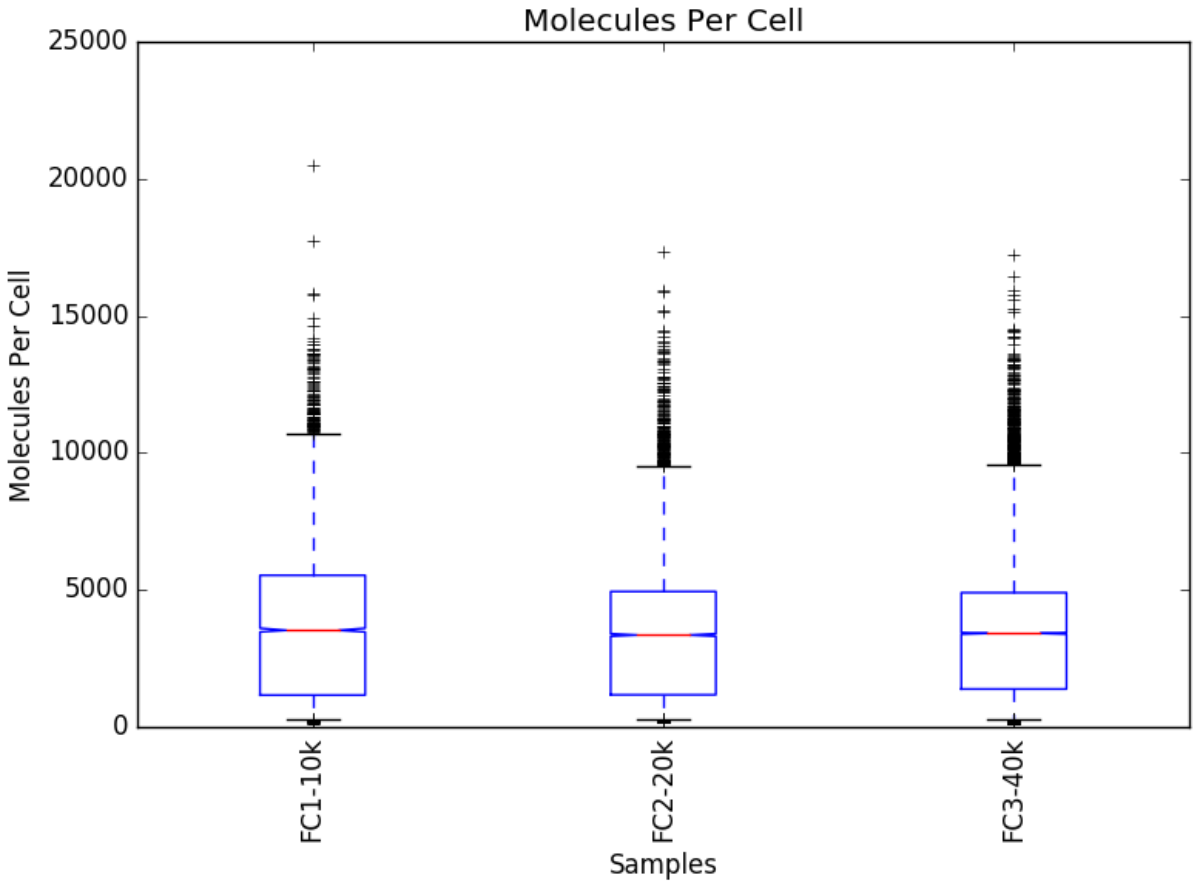


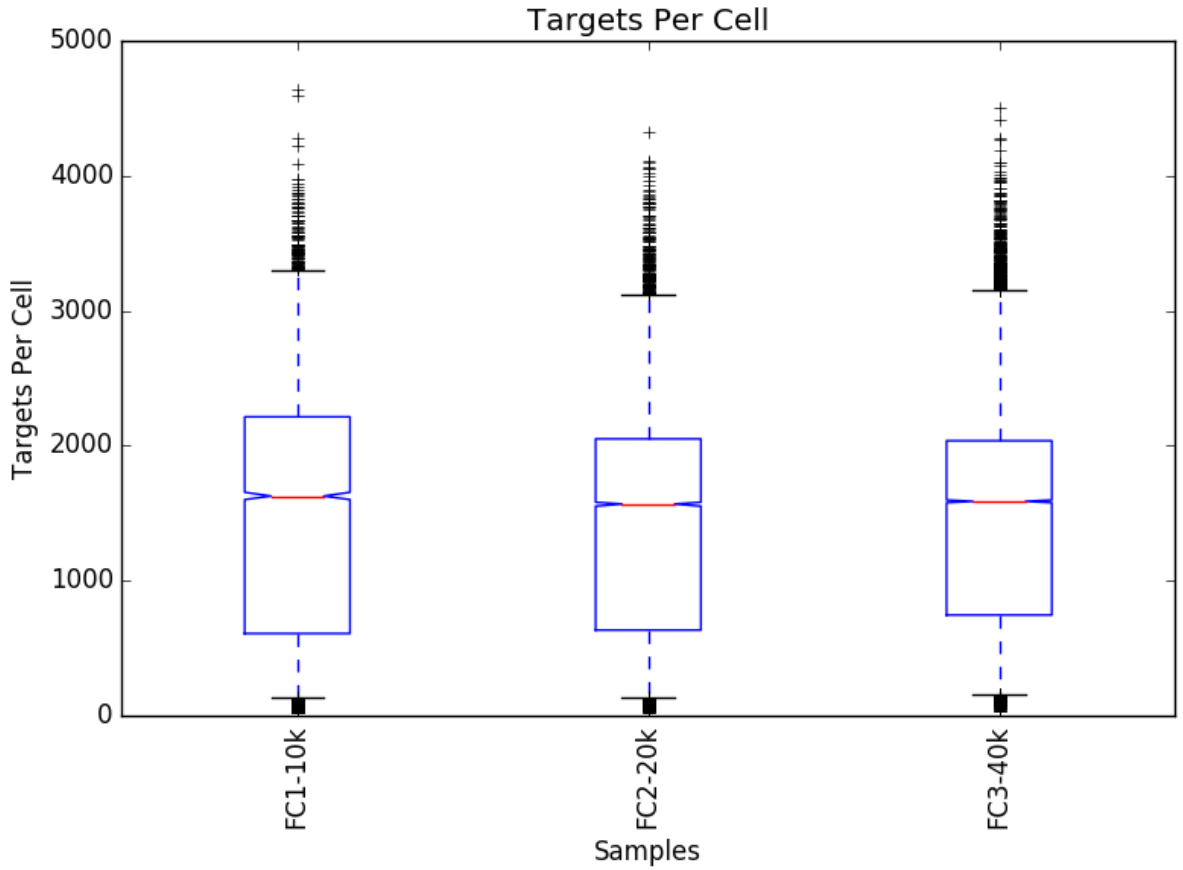
Perform WTA library prep

Quality Metrics Are Similar Across 10,000 to 40,000 Cell Inputs



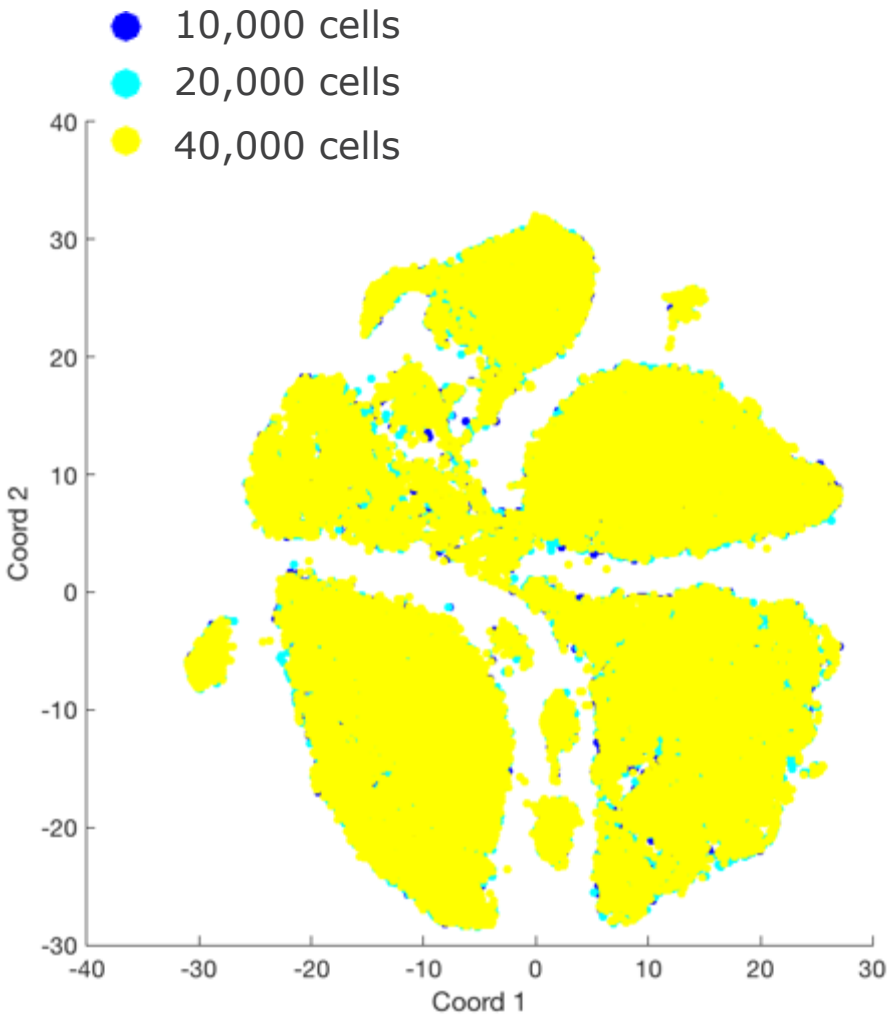
Similar Molecules/Cell and Genes/Cell Are Detected Across 10,000 - 40,000 Cells

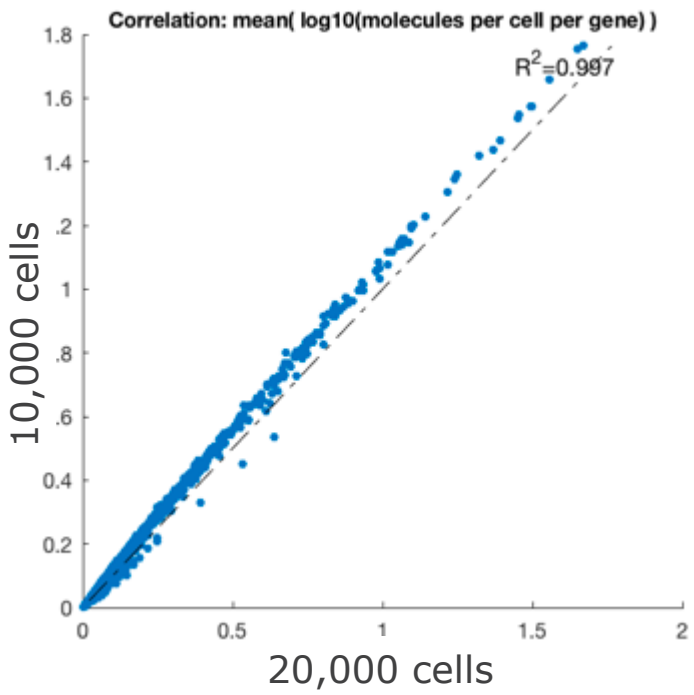


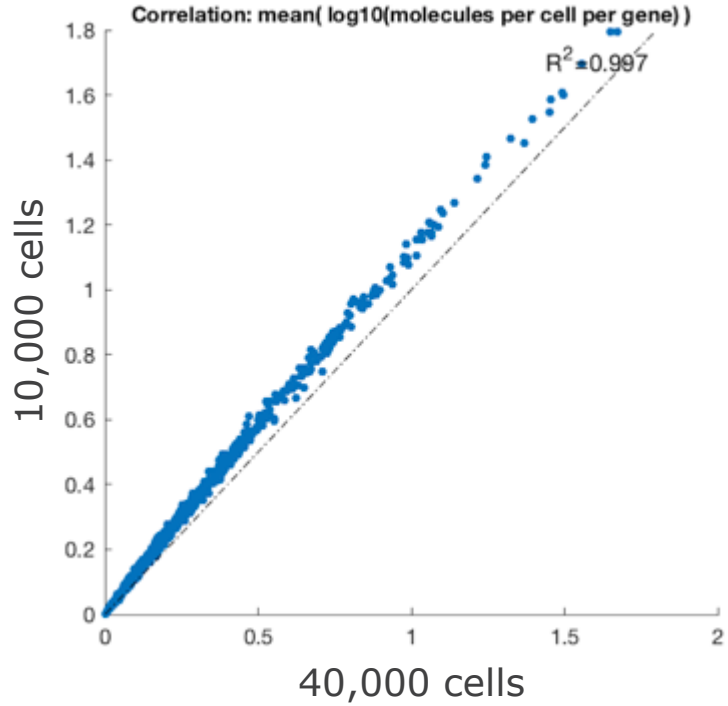


For ~4000-4500 RSEC reads per cell

Strong Correlation in Molecules Per Cell Across 10k, 20k and 40k Cells







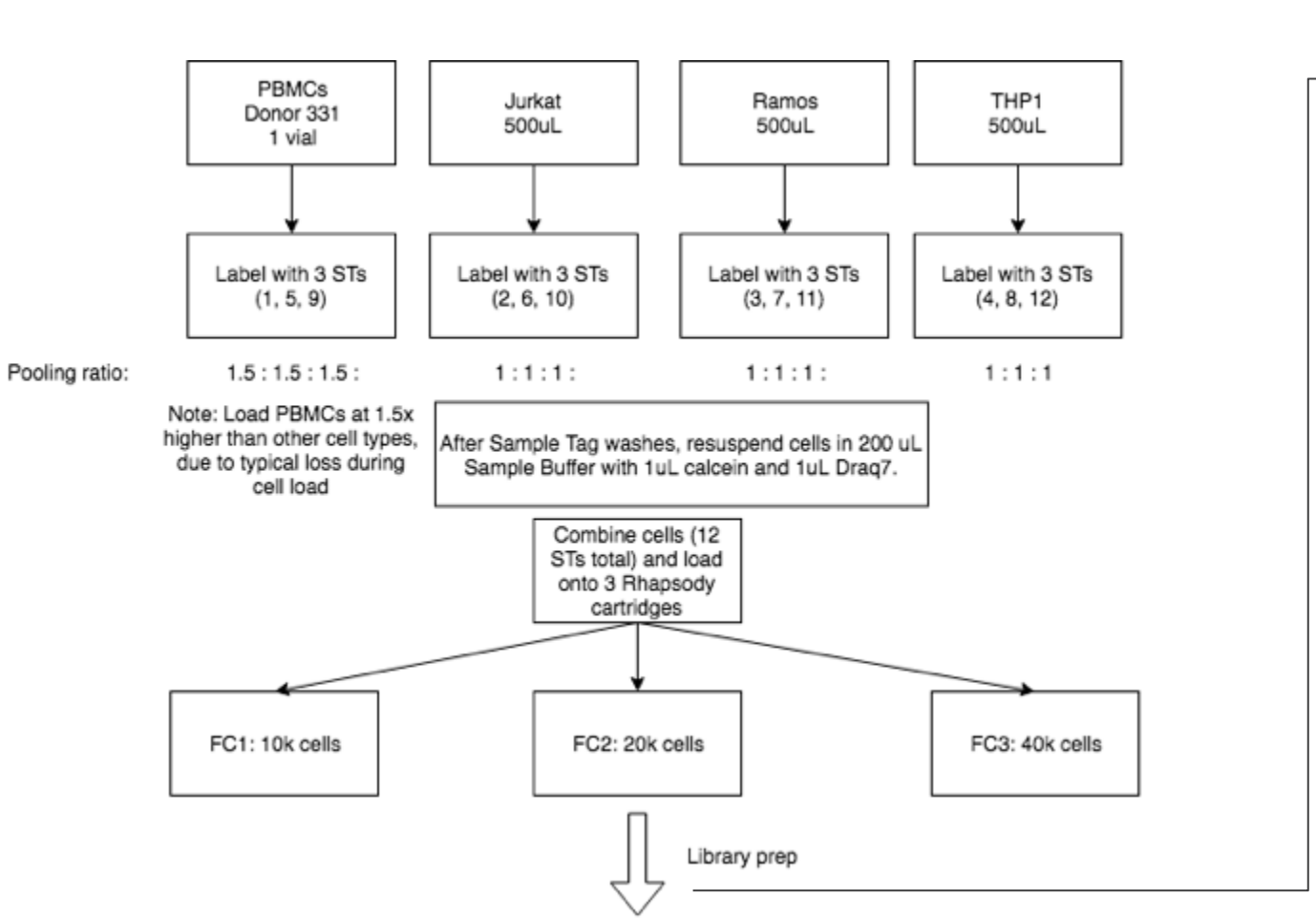
All three cell inputs show no batch effect (similar gene expression profiles)

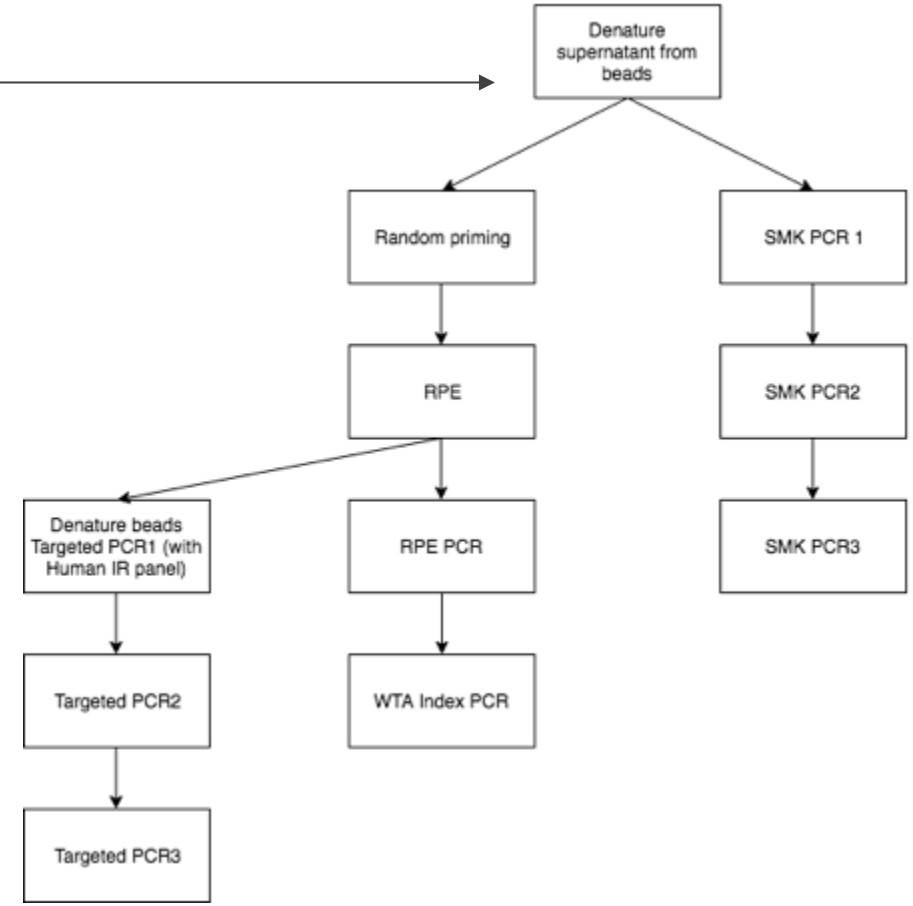
Conclusion

- Rhapsody WTA protocol generates similar quality and sensitivity metrics across 10,000, 20,000, and 40,000 cell inputs

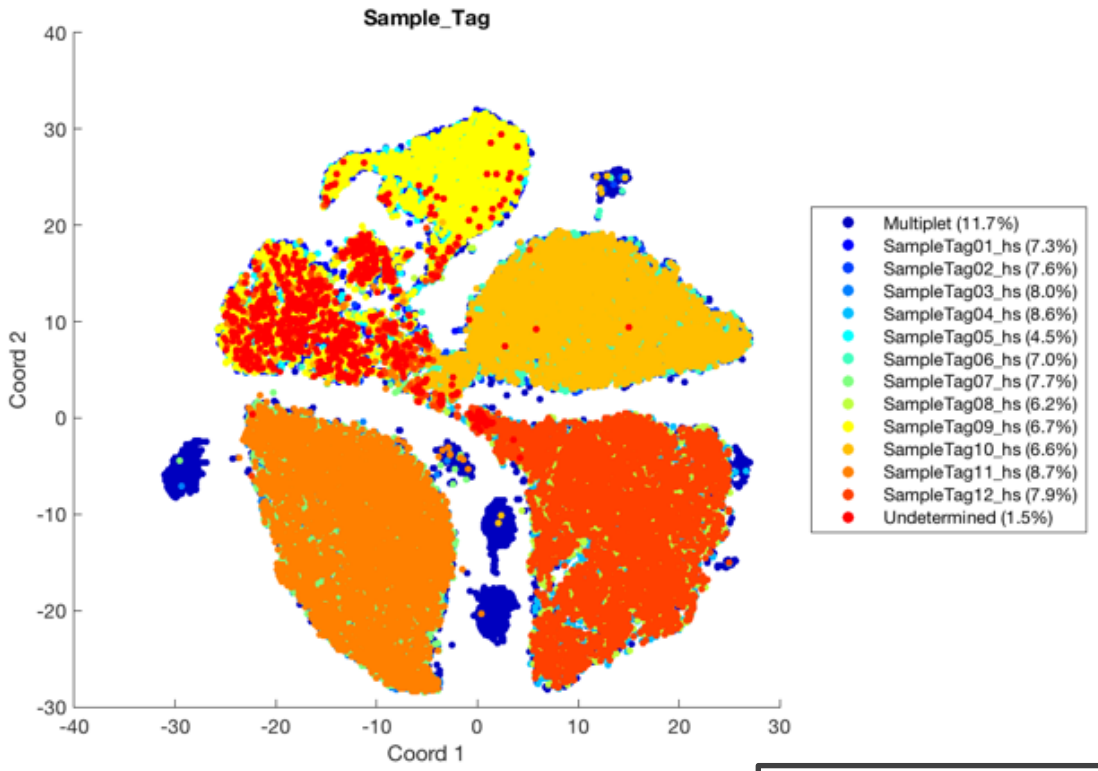
Extended Experimental Workflow

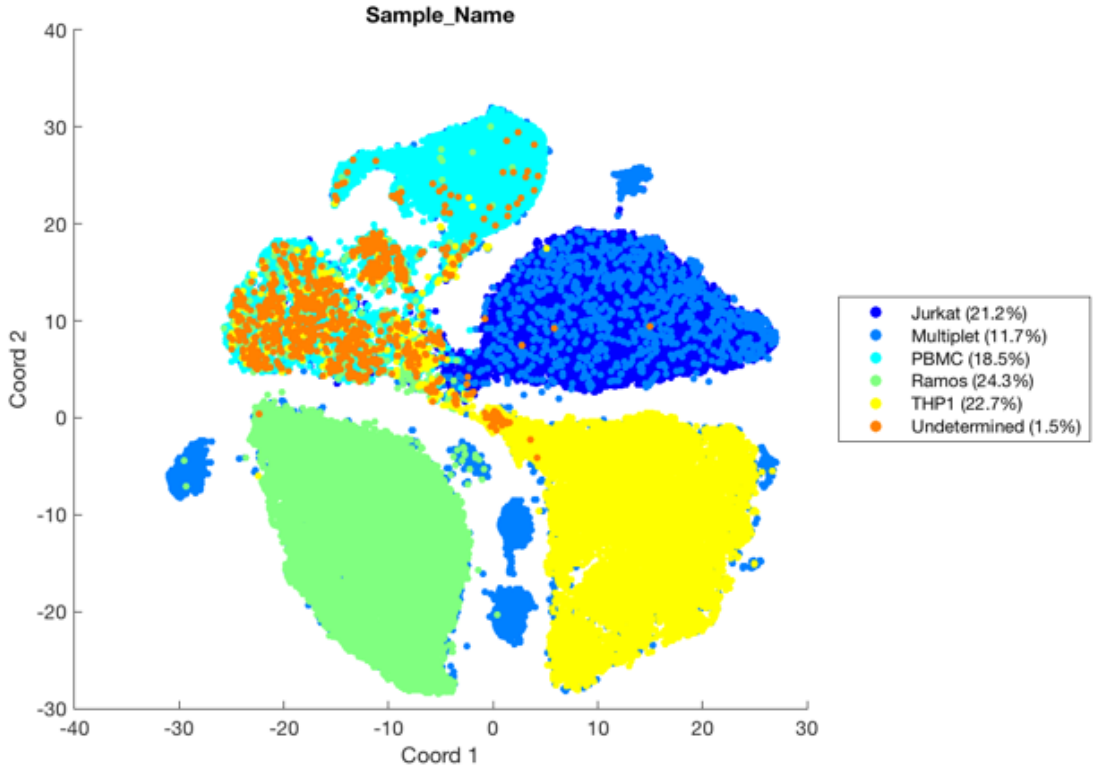
Experiment also included Sample Tags and targeted library prep to compare multiplet rates across cell inputs and between Targeted and WTA





Sample Tag and Cell Type Annotations



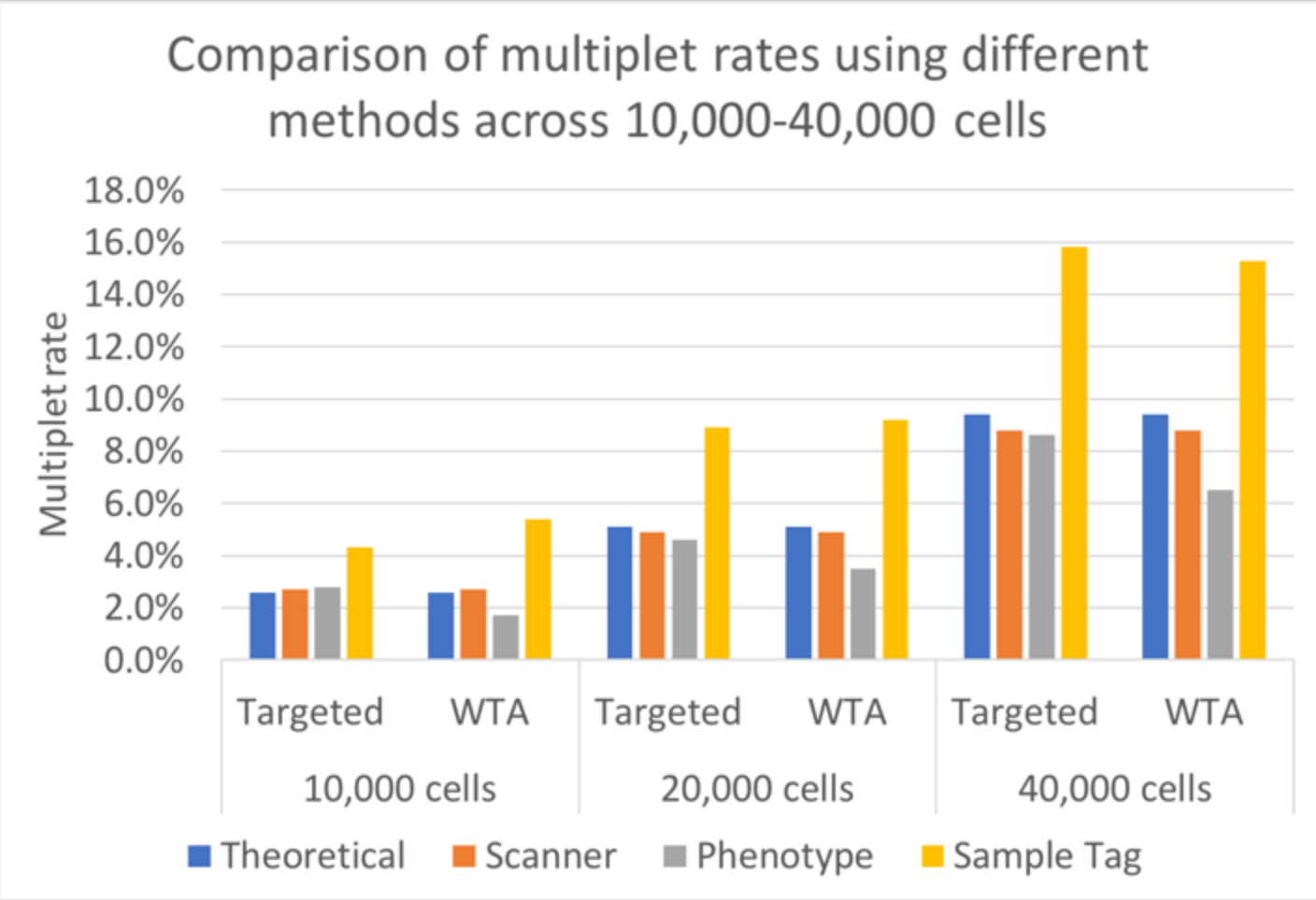


PBMCs = ST 1, 5, 9
Jurkat = ST 2, 6, 10
Ramos = ST 3, 7, 11
THP1 = ST 4, 8, 12

Explanation of Multiplet Rates

Multiplet rate	Method of calculation
Theoretical	Based on Poisson distribution for the particular # of cells See 20190919_Rhapsody MultipletsTable-fromPhil.xlsx
Scanner	Calculated from Rhapsody scanner images after BeadWash2 scan – based on number of cells that are captured together in a single well
Phenotype	Generate tSNE, identify clusters containing multiple cell types (based on Sample Tags and gene expression profile), get % of cells in these clusters with multiple cell types
Sample Tag	Sample Tag metrics calculate % of cells that have more than one Sample Tag called

Comparison of WTA and Targeted Multiplet Rates Across Cell Inputs



- Theoretical multiplet rate is similar to scanner multiplet rate
- Phenotype multiplet rate is similar to theoretical/scanner rate, but less than Sample Tag rate
  - Phenotype multiplets only include multiplets from different cell types
  - Sample Tag multiplets include multiplets from same cell type, but may be overestimated
- The multiplet rate at 40,000 cells seems to be around 8-10%, and can generally be removed based on clusters of multiple cell types. However, any WTA assay alone could underestimate the true number of multiplets

Data set 3: Testing the whole transcriptome of nuclei preps using the BD Rhapsody™ Whole Transcriptome Analysis (WTA) Amplification Kit

Rationale for Nuclei Isolation Method Selected

- Preference was to use a method that can isolate pure nuclei with standard reagents/equipment, while minimizing nuclei clumping (related to DNA leakage, which can occur during multiple centrifugation steps)
- Opted for Lysis Gradient Centrifugation (LGC) as it is published and used widely. In addition, LGC has the below features
  - Single centrifugation step – can be done with standard lab centrifuge
  - Iodioxanol-based gradient – no sucrose
  - Mild lysis, using IGEPAL surfactant
  - Works with a variety of cell types

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Changes to Rhapsody Cartridge Protocol Adapted for Nuclei Workflow

- Using DyeCycle Green to stain nuclei instead of Calcein AM and Draq7

Cell preparation (before isolation)

- Obtain ~1 million cells. Centrifuge 400g 5 min, wash in 1 mL media. Centrifuge 400 g 5 min, resuspend in 500 uL media.
- Staining: Add 2 uL of 5mM DyeCycle Green (ThermoFisher V35004) (to stain all nuclei – for imaging on cartridge after isolation) to the 500 uL of cells in media. Incubate at 37C in the dark for 5 min.
- Note: No additional staining will be performed after nuclei isolation and before cell loading on the cartridge, due to the need to keep nuclei at 4C to minimize degradation
- After incubation, filter cells through a cell strainer cap into a Falcon tube (Corning 352235). Place on ice.
- Count 10 uL of cells using the Rhapsody scanner and proceed further to next steps.

Changes to Rhapsody Cartridge Protocol Adapted for Nuclei Workflow

- For nuclei lysis, following changes were made to the lysis protocol:
  - Instead of Lysis Buffer + DTT, add Lysis Buffer + DTT + 1:20 Proteinase K
    - Proteinase K: NEB P8107S, 800 U/mL
  - Instead of performing lysis incubation at room temperature for 2 minutes, incubate at room temperature for 5 minutes

- **Recommendation**: Increase cell load incubation time from 15 minutes to 20-30 minutes (allow for increased settling of nuclei due to smaller size)

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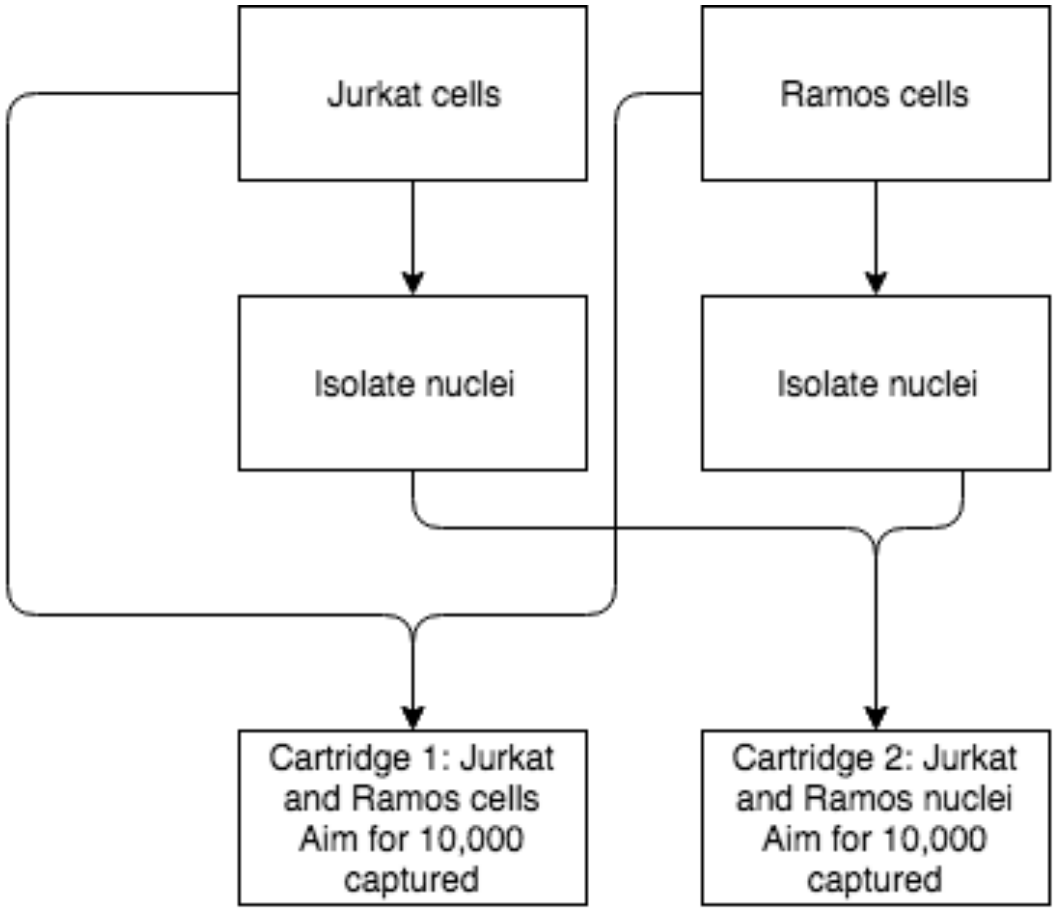
Experiment Overview

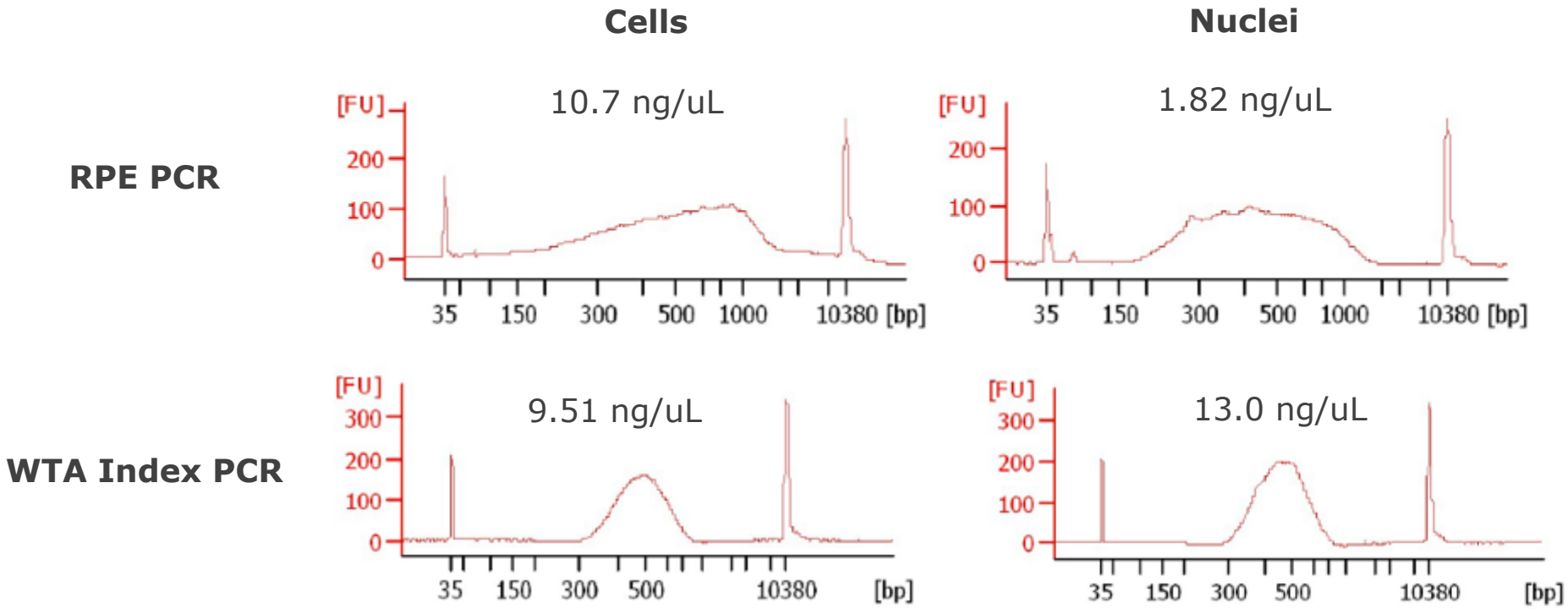


Only ~6000 nuclei captured.
Subsampled Cartridge 1 to ~6000 cells
Proceeded with WTA library prep with 6000 cells or nuclei, following WTA protocol as written

Nuclei Capture Rate Tends to be Lower Than Cells, Even When Aiming for Same Loading Number

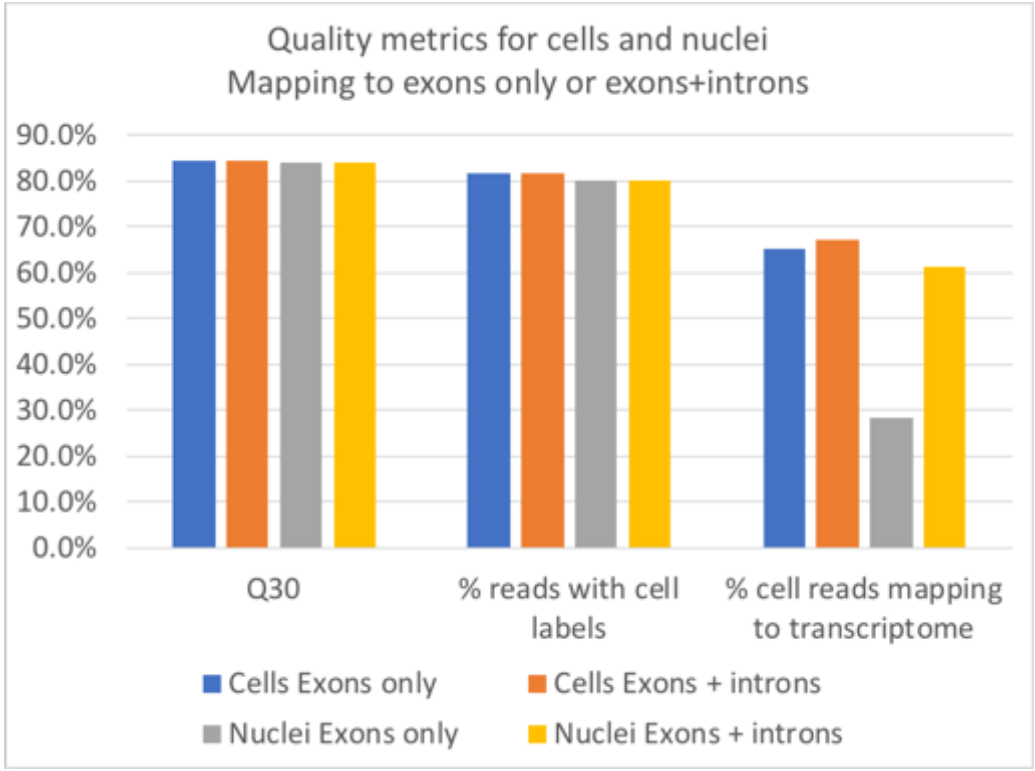
Scanner Metrics for Cartridges	Cartridge 1	Cartridge 2
Cells or nuclei	Cells	Nuclei
Number of cells aim to load	10000	10000
Number of cells aim to capture	10000	10000
Scanner	1006	1006
Number of wells with viable cells and a bead	11564	5942
Cell multiplet rate	2.30%	1.40%
Bead loading efficiency	PASS (95.40%)	PASS (94.60%)
Cell retention rate	PASS (89.10%)	PASS (92.40%)
Bead retrieval efficiency	PASS (95.00%)	PASS (98.10%)

Bioanalyzer Traces for RPE PCR and WTA Index PCR



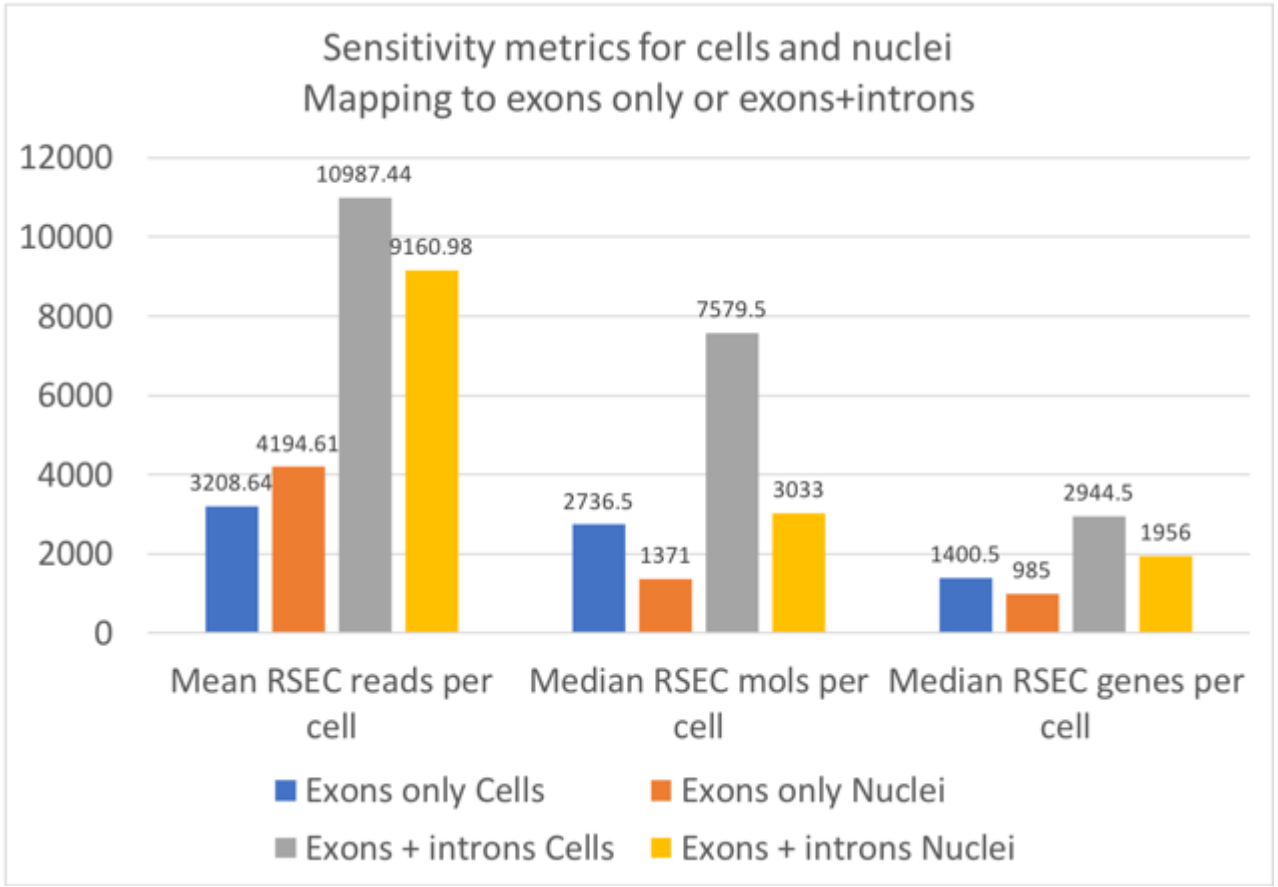
Note: All traces were normal, RPE PCR appears more symmetrical for nuclei

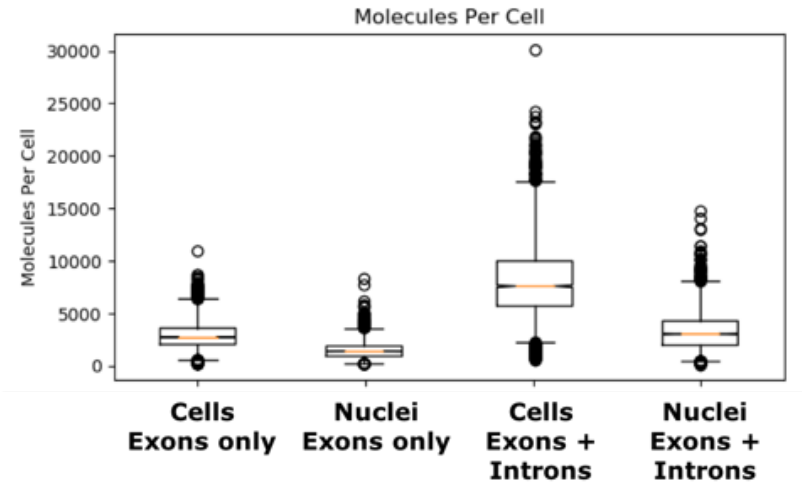
Quality Metrics

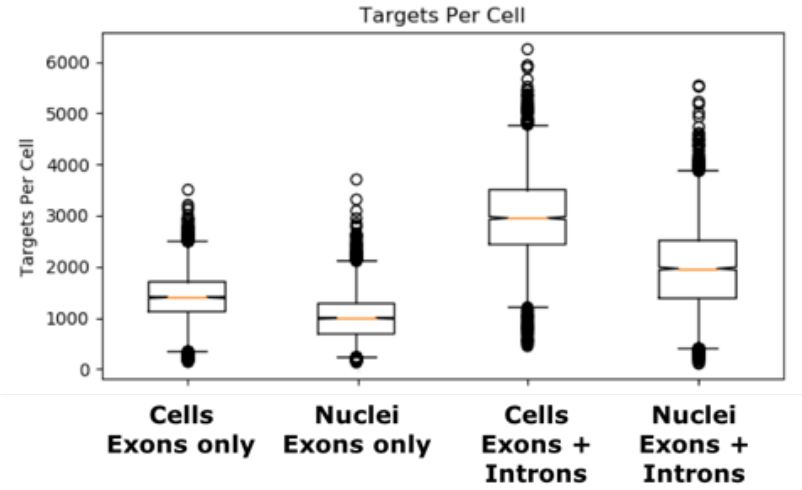


- Similar Q30 and % reads to cell labels across cells/nuclei, with and without mapping to introns
- Nuclei have lower % reads to transcriptome than cells, but increased mapping when include introns

Nuclei Exhibit Slightly Lower Sensitivity Than Whole Cells







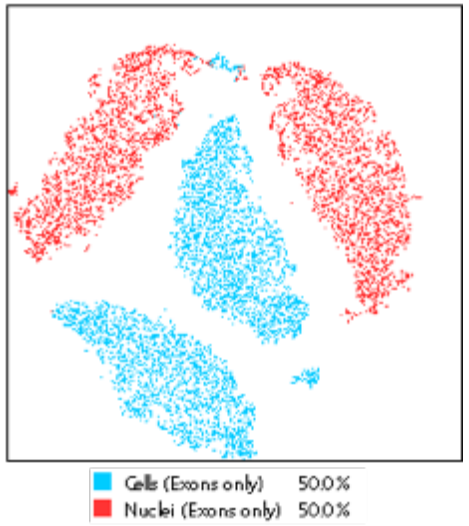
Nuclei and Cells Form Separate Clusters, Regardless of Whether Introns are Included

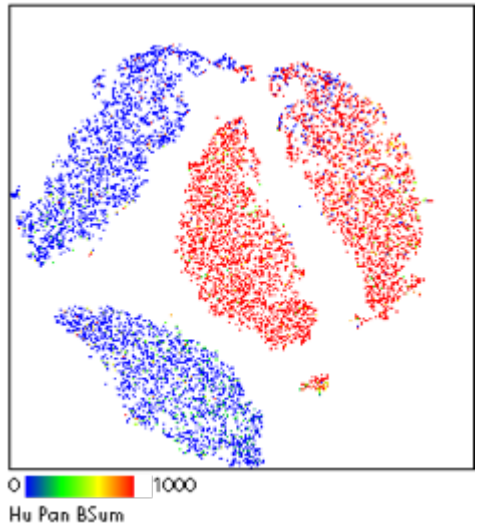
tSNE with file name annotation

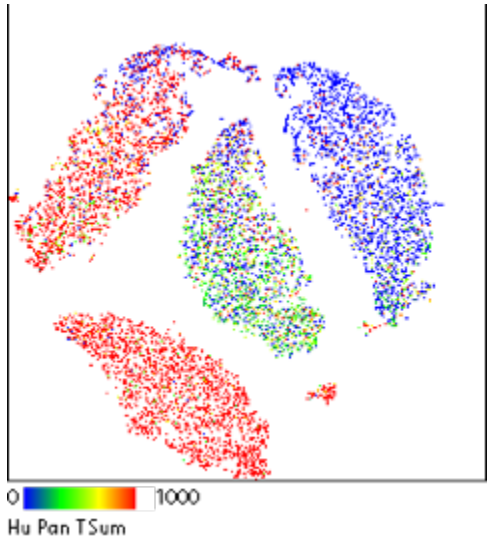
B cell expression (Ramos)

T cell expression (Jurkat)

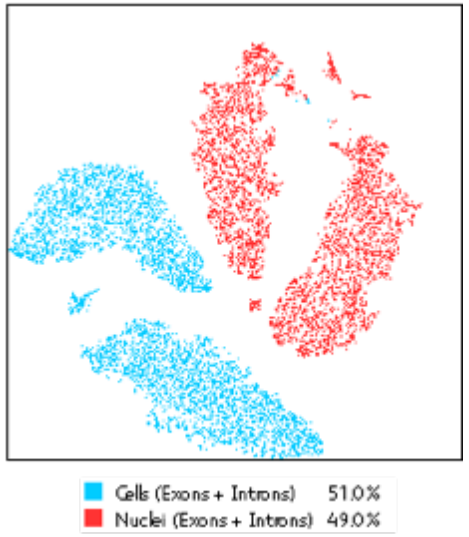
Exons only

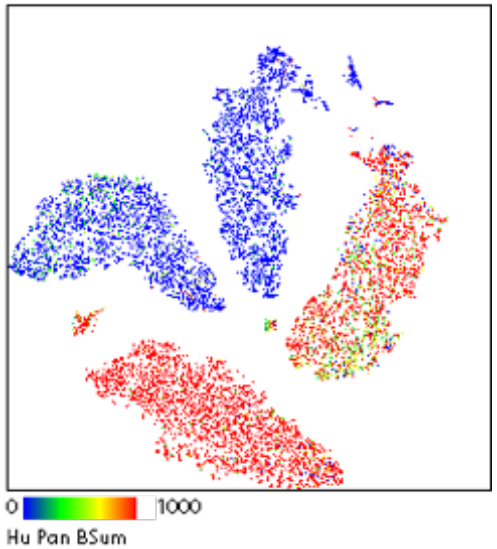


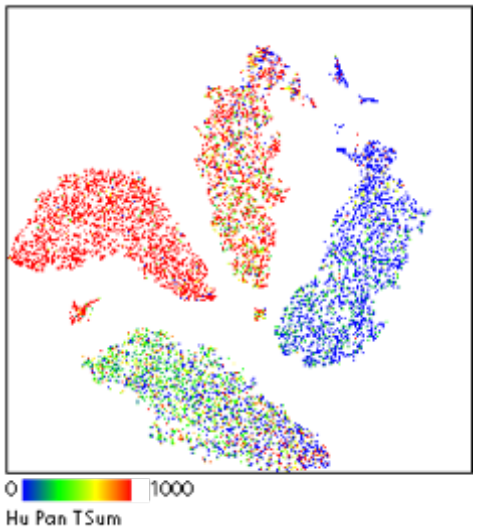




Exons + introns



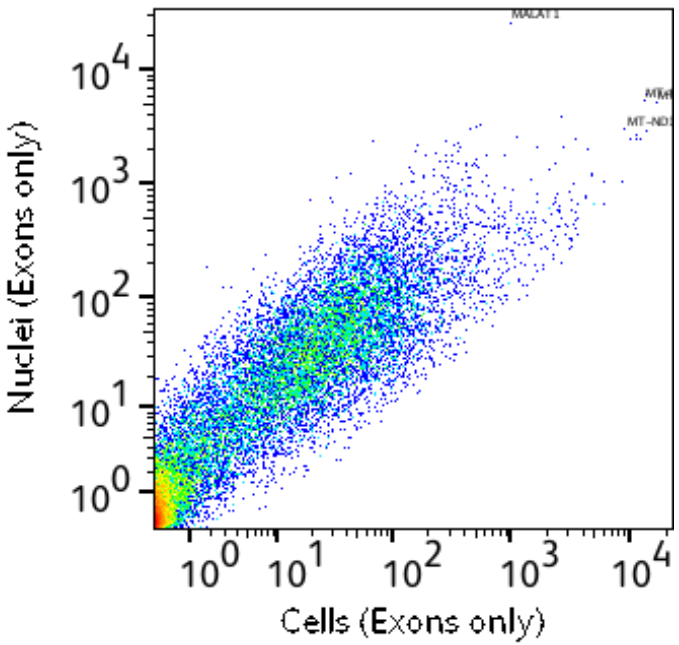




Generally High Correlation Between Nuclei and Cells for WTA (With and Without Introns)

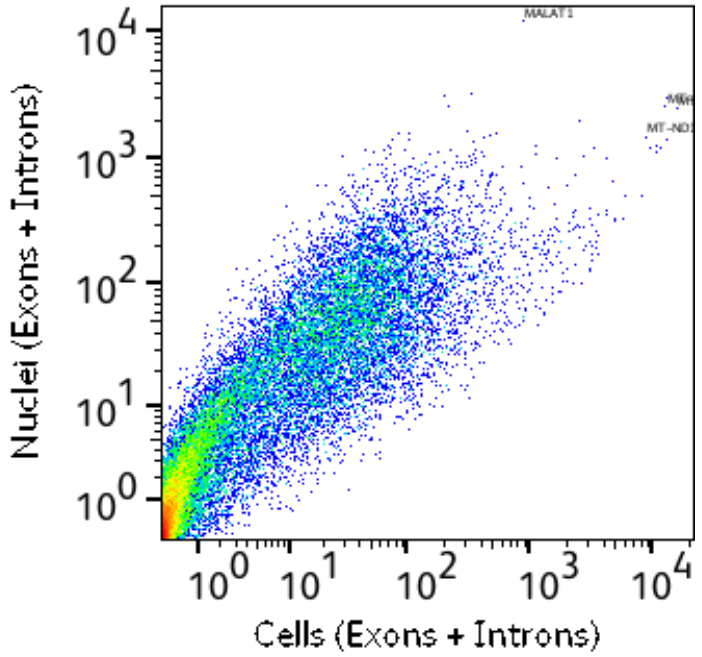
- High expressors in nuclei include MALAT1 (long non-coding RNA)
- High expressors in cells include mitochondrial genes

Exons only



Pearson coefficient
R = 0.90

Exons + introns



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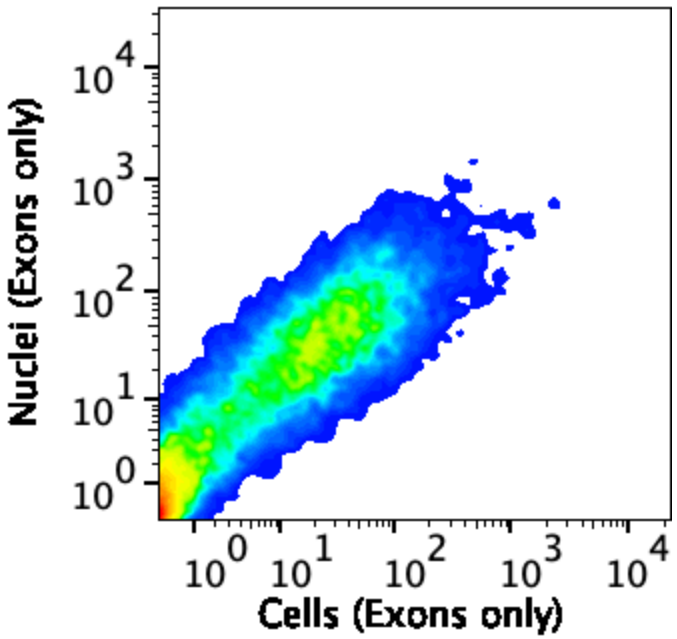
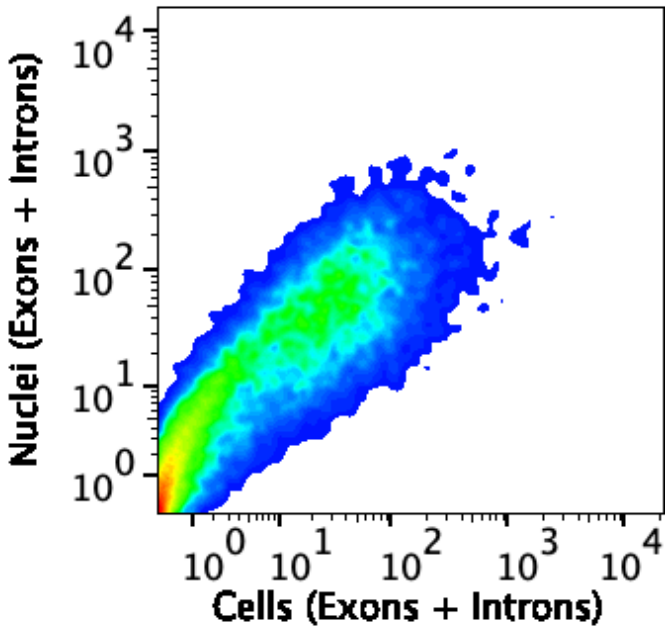


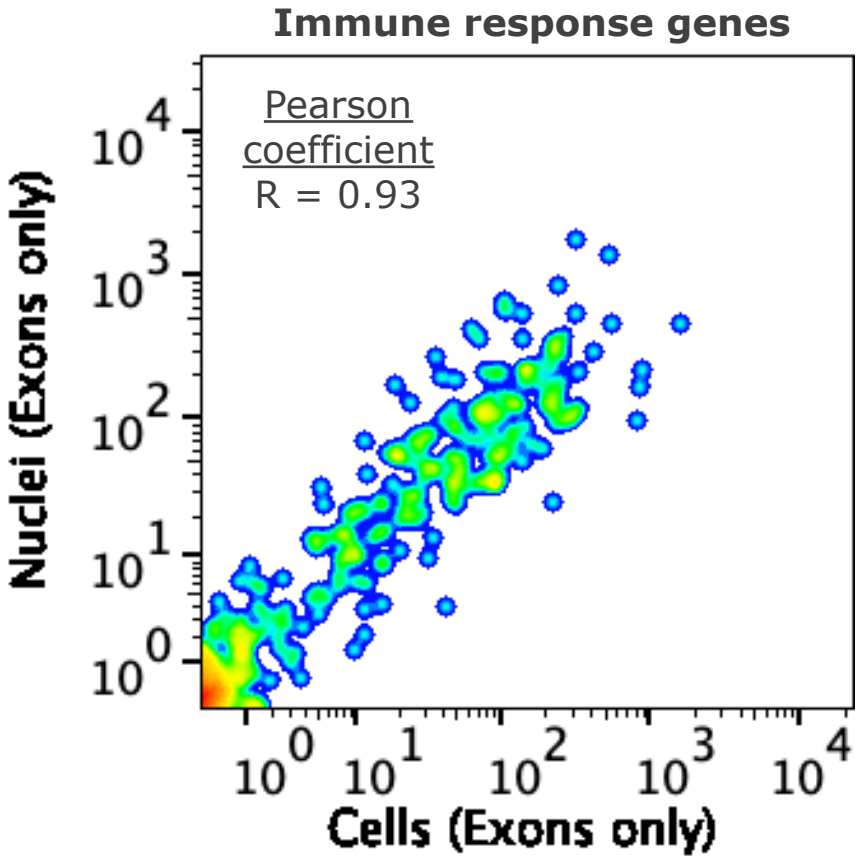
Pearson coefficient
R = 0.90

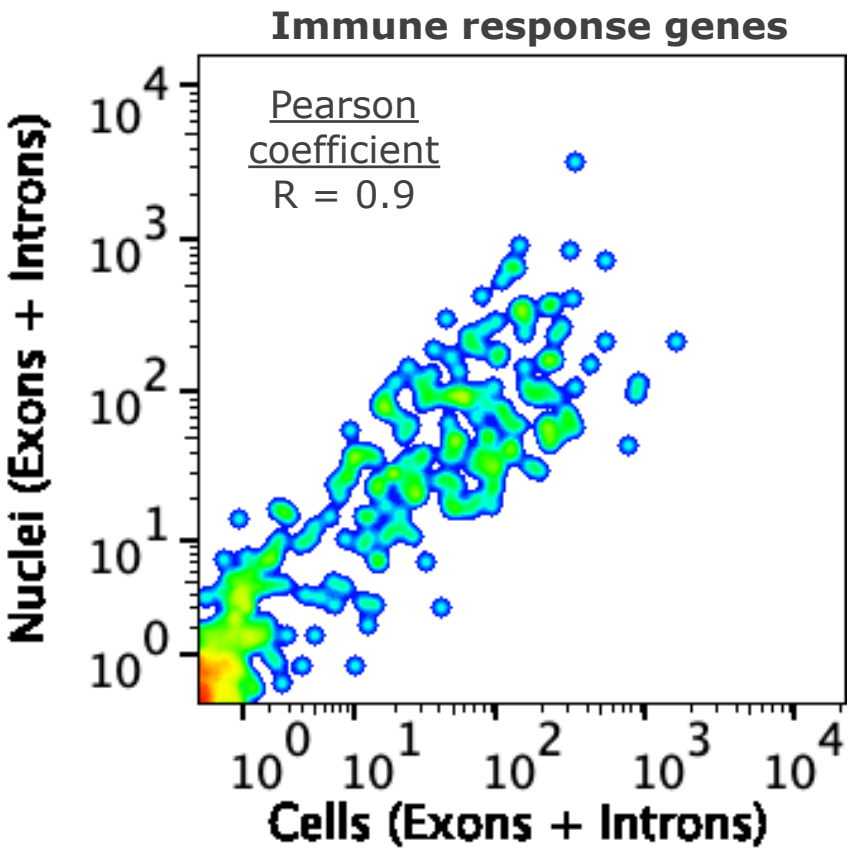
Exons + introns



Pearson coefficient
R = 0.89

Strong Correlation for Immune Response Genes Between WTA Results From Nuclei Preps vs. Whole Cell Preps





Summary

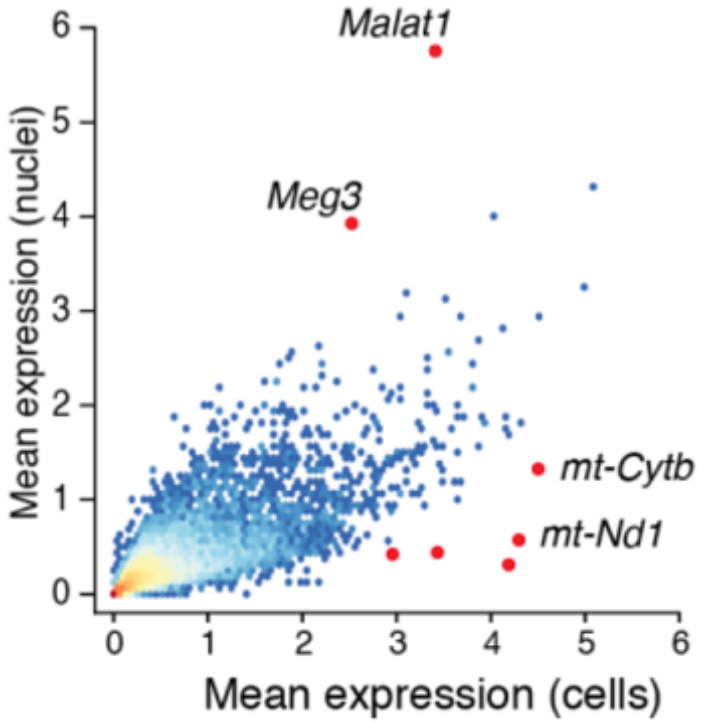
- Nuclei isolation using lysis gradient centrifugation works with human Jurkat and Ramos cells

- Nuclei expresses fewer molecules than intact cells but high correlation in gene expression observed between WTA results from nuclei and intact cells

References for Nuclei vs Cell Comparison Data

- Habib, et al, Nature Methods (2017)

- Supp Fig 2d: Comparison of 3T3 cells and nuclei (mouse cell line). Calculated Pearson coefficient of r = 0.81 ± s.d. = 0.0024

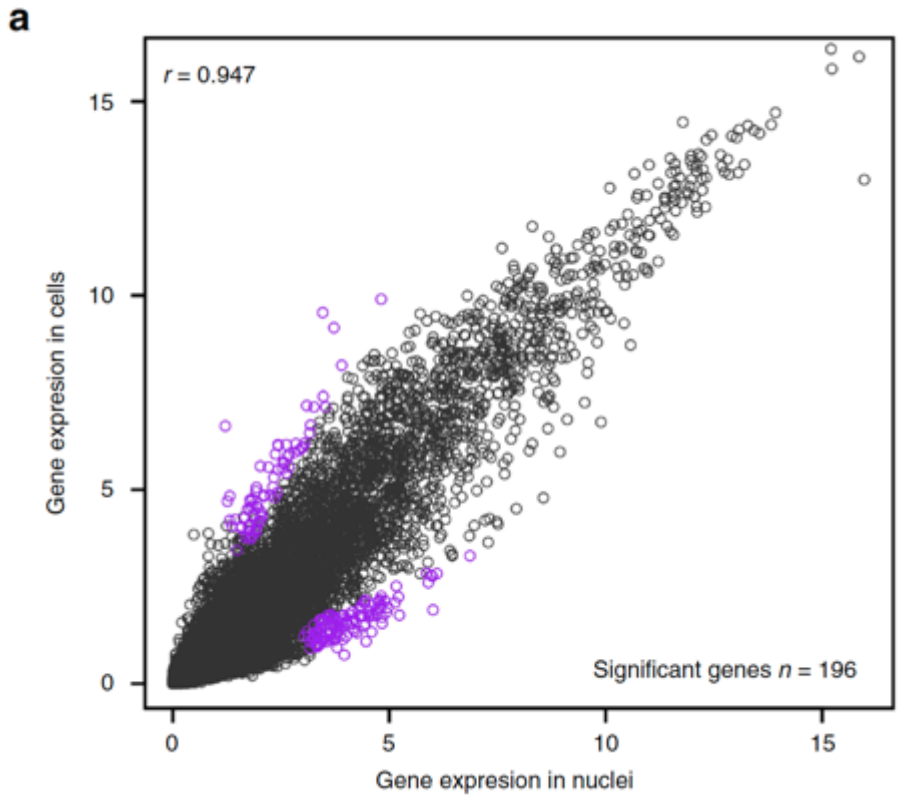


References for Nuclei vs Cell Comparison Data

Gao, et al, Nature Communications (2017)

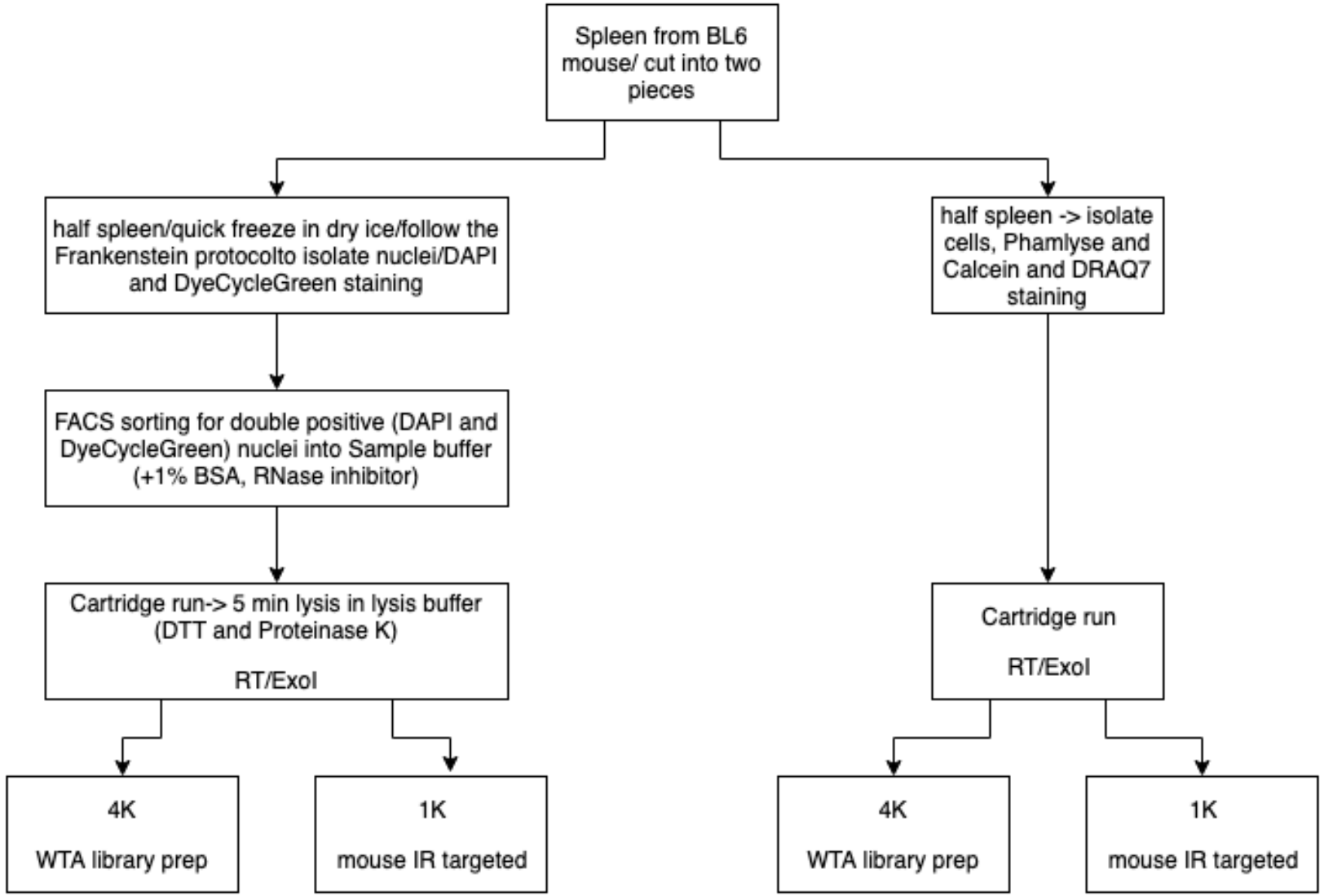
Fig 4a: Scatter plot of average gene expression [log2(count + 1)] of 485 single nuclei and 424 single cells, with 196 significantly differential genes labeled in purple and Spearman's correlation values indicated.

Used breast cancer cells



Data set 4: Evaluating the whole transcriptome of samples of murine origin using the BD Rhapsody™ Whole Transcriptome Analysis (WTA) Amplification Kit

Workflow

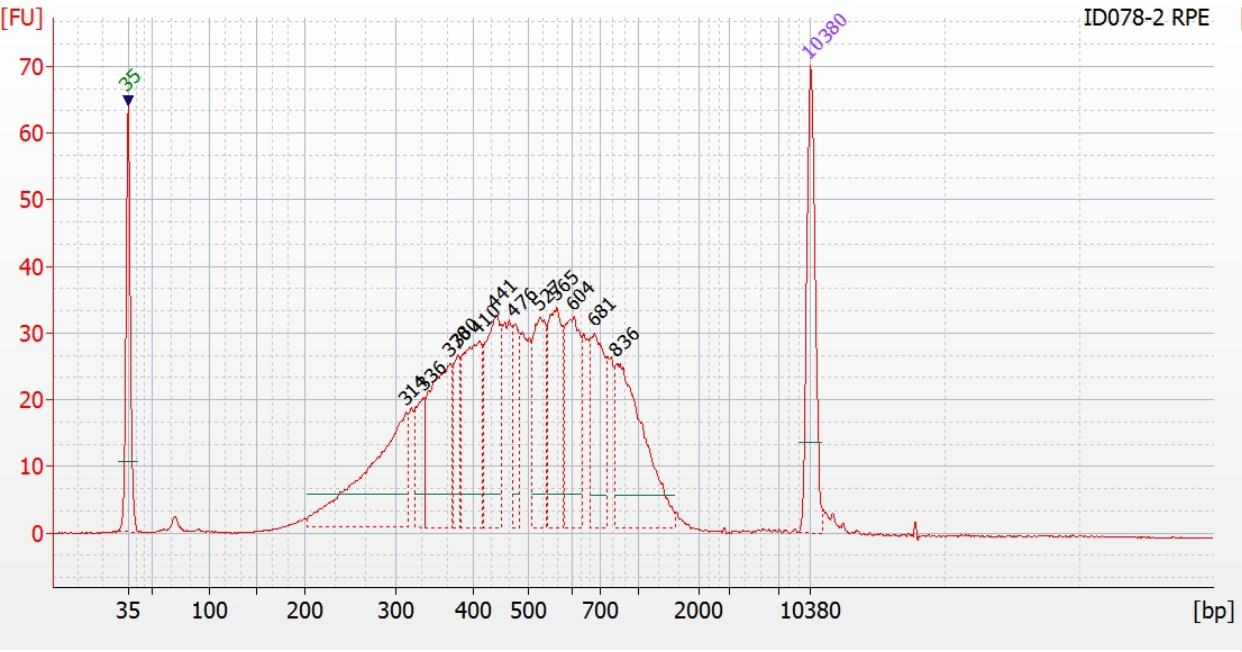

Spleen from BL6 mouse/ cut into two pieces
half spleen/quick freeze in dry ice/follow the Frankenstein protocolto isolate nuclei/DAPI and DyeCycleGreen staining
half spleen -> isolate cells, Phamlyse and Calcein and DRAQ7 staining
FACS sorting for double positive (DAPI and DyeCycleGreen) nuclei into Sample buffer (+1% BSA, RNase inhibitor)
Cartridge run-> 5 min lysis in lysis buffer (DTT and Proteinase K)
RT/ExoI
Cartridge run
RT/ExoI
4K
WTA library prep
1K
mouse IR targeted
4K
WTA library prep
1K
mouse IR targeted

Cartridge Metrics

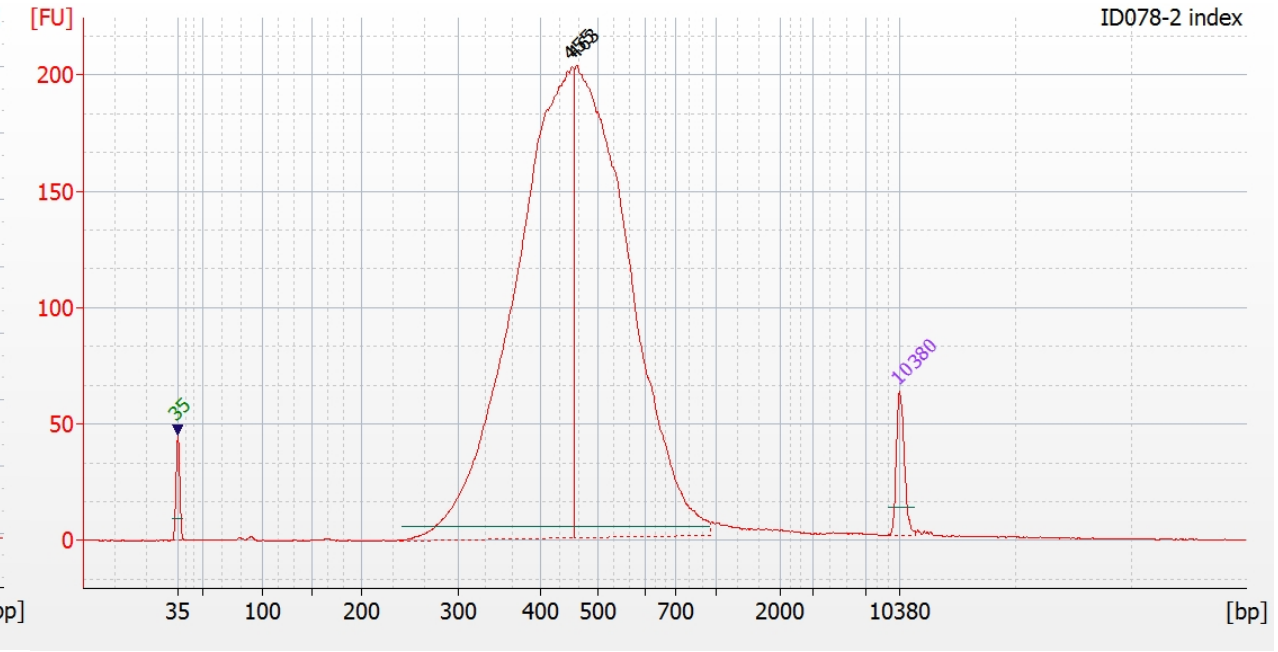
Metrics	Cell
Number of cells aim to capture	15,000
Sample name	C
Viability from cell count	77.14
Number of wells with viable cells captured with a bead	7689
Cell multiplet rate	1.8
Bead loading efficiency	PASS
Cell retention rate	PASS
Bead retrieval efficiency	PASS
Subsample	26uL/1000 cell

Bioanalyzer Trace

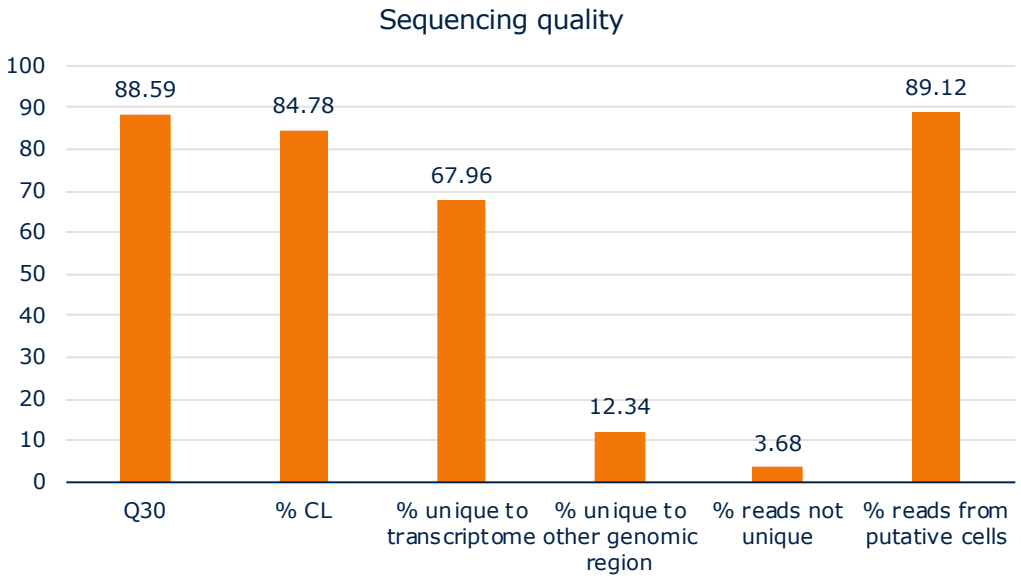
RPE-PCR

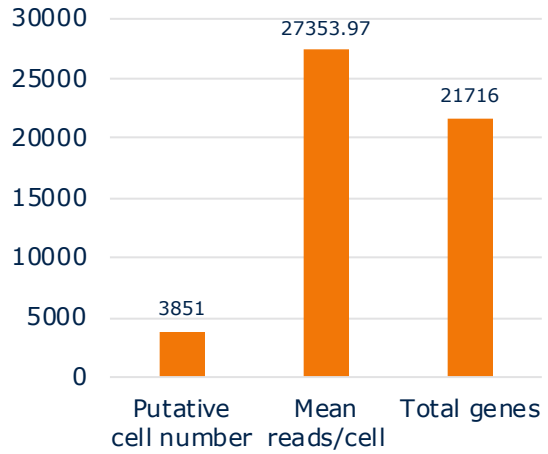


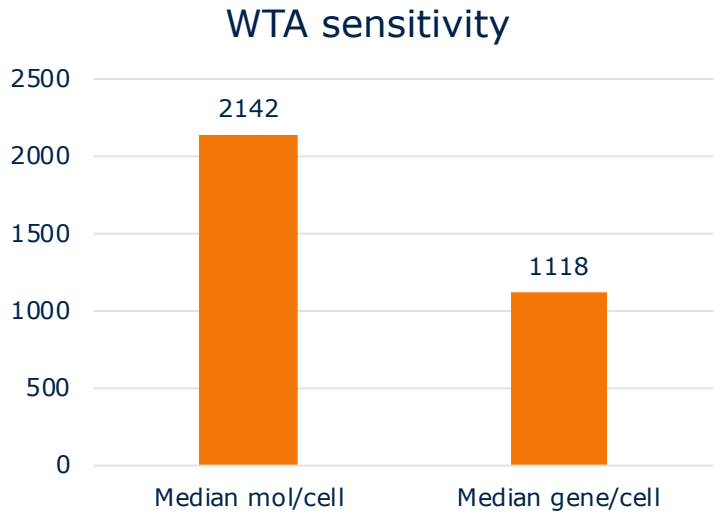
Index-PCR



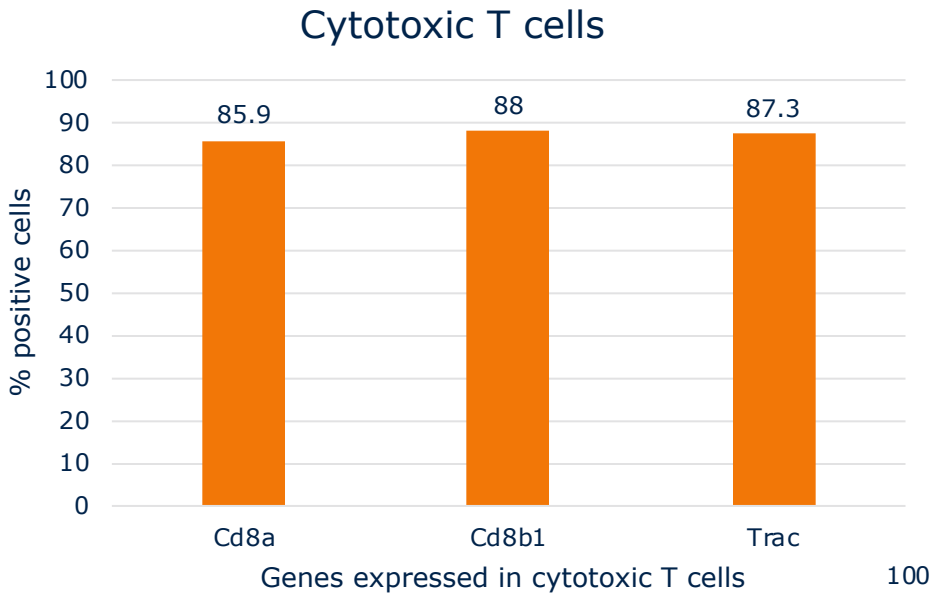
Sequencing Quality Metrics and Sensitivity Data

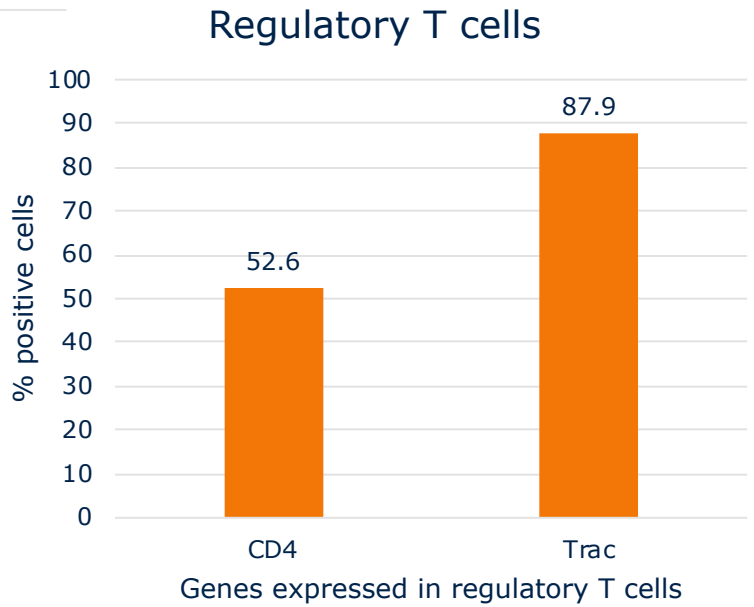


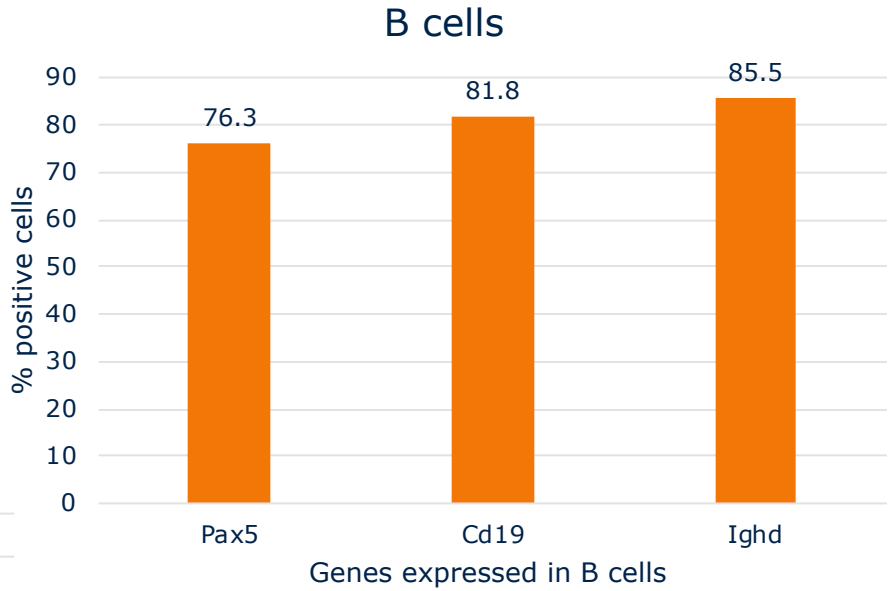




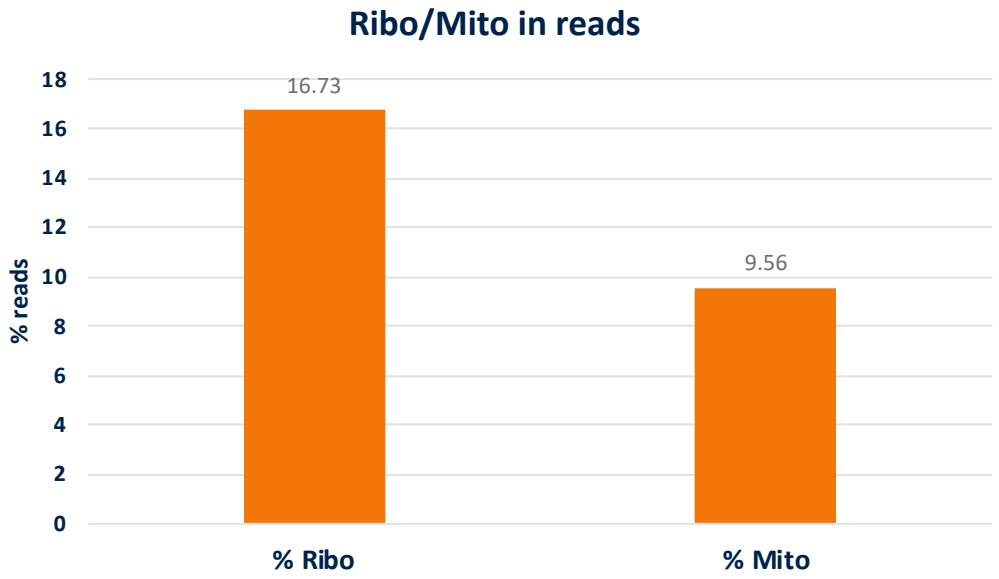
WTA Sensitivity Metrics (cont.)

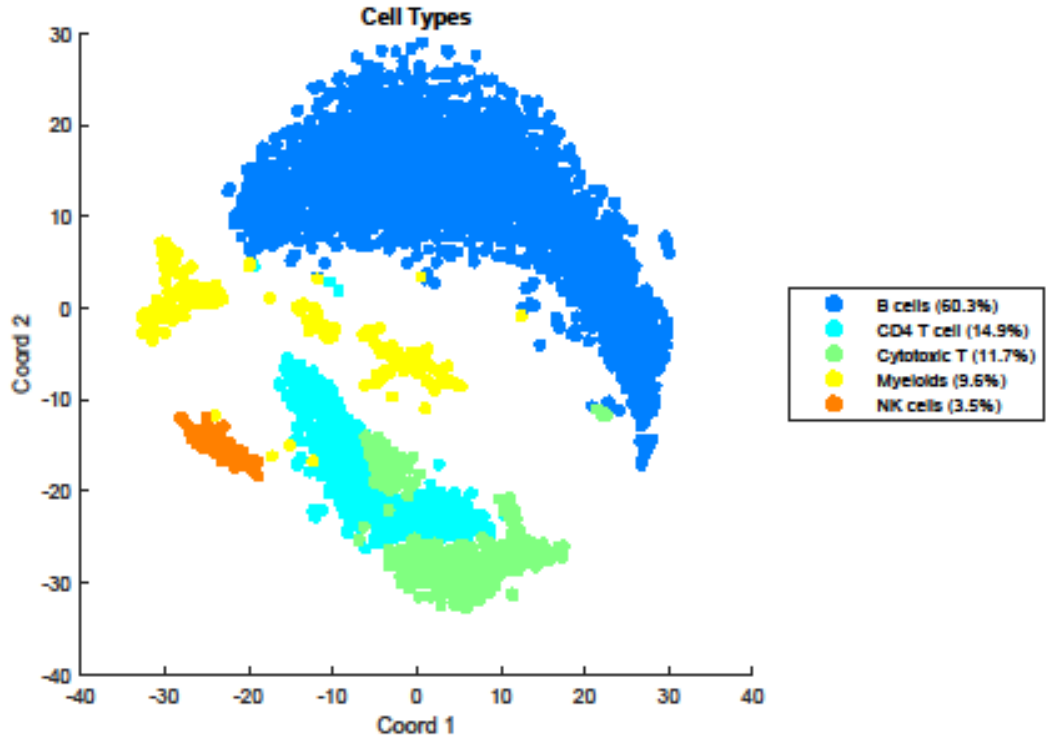






Data (Reads Aligned and Cell Types)





Conclusions

- WTA analyses using BD WTA assay was performed using mouse splenocytes
- Overall data performance similar to human samples
- WTA sensitivity was on par to our observations with human PBMCs (assessed by # median mol/cell)
- Percentage positive cells for specific markers in specific cell types was within expected range with minimal drop-outs

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