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Single Cell -ATAC-Sequencing Validation Report, version 1.1

This report describes the analytical validation of single cell ATAC sequencing of cryopreserved, characterized melanoma tumor cells and corresponding Tumor-Infiltrating Lymphocytes (TIL) isolated from the two metastatic melanoma patients.

Table 1. Summary of analytical validation findings for scATAC-seq profiling

Accuracy	Accurate assay performance determined by fragment size/peak distribution of libraries (Figure 1) and QC plots of sequenced data (Figure 4) Obtained QC metrics compared to reference sample (10x Genomics).
Precision: Inter-assay	Technical reproducibility across analyzed samples. Peak intensities between technical replicates (n=2), and assessment of ability to detect the relevant information in 1:10 dilution of TIL in mixed sample (Figure 5-12, also see Table 3).
Precision: Intra-assay	Plots and detection of cellular characteristic pattern (Figure 5 and 6). Differential motif analysis shows common patterns of chromatin accessibility (Figure 5-12, also see Table 3).
Analytical sensitivity	Targeting 500 – 10,000 isolated nuclei, depending on collection and preparation method and recovery rate. Here, we start with 1mln cells for nuclei isolation and target 3000 nuclei for library preparation.
Analytical sensitivity including interfering substances	Preservation and storage condition (fresh vs frozen) of samples as well as storage and dilution of Nuclei Buffer (PN-2000153, 10x Genomics) could affect transposase binding and subsequent activity (10x Genomics technical notes). Here we processed cryopreserved samples for nuclei isolation according to optimized protocol with 10x Genomics recommended 1x diluted Nuclei Buffer.

Reportable range	Relative chromatin accessibility over the whole genome as read out by level of transposition. Sample dependent.
Reference interval (normal range)	Generated data represent the reference ranges of QC metrics of publicly available PBMC data reported in technical note “CG000202” by 10x Genomics), see Table 3.
Standardization, harmonization, reproducibility, and ruggedness	Standardized with a pooled library of unique indexed samples, sequenced on NovaSeq6000 Sequencer using 1 sequencing lane to minimize technical variation. All analysis for a given sample set performed against the same reference data base (Table 3 additionally includes comparison of QC metrics of counts of data aligned either to hg19 or hg38 reference genome pointing out the systematic version dependent differences).
Quality control and improvement procedures	Experiments carried out with 2 technical and 2 biological replicates (depending on the cells accessibility). Processing of scATAC sequencing data was done with the cell ranger atac (10x Genomics) pipeline. The QC metrics are summarized in Table 3 and Figure 4.
Any other performance	N/A

1. Purpose of the assay.

Profiling sequencing techniques at the single cell level [1], greatly exceeded the standard bulk sequencing [2], allowing to dissect the extremely heterogenous nature of tumors and the equally diverse immune repertoire in a population [3]. Moreover, it enables clonal evolution tracking, assessing tumor microenvironment remodeling status and development of cancer therapy resistance. The application of single-cell sequencing technologies, in combination with existing genomics methods, has greatly facilitated high-resolution interrogation of the genome [4], transcriptome, proteome [5, 6] and epigenome [7, 8]. 10x Genomics (https://www.10xgenomics.com) is one of the leading single cell platforms for integrated solution for RNA and DNA analysis currently extensively applied by the scientific community.

Here, our focus was to validate 10x Genomics Chromium Single Cell ATAC (Assay for Transposase Accessible Chromatin) Solution (https://www.10xgenomics.com/products/single-cell-atac/) to perform single cell Assay for Transposase-Accessible Chromatin with high-throughput sequencing (scATAC-seq) technique for dissecting epigenetic variability. We utilized primary tumor cell lines expanded from metastatic melanoma resections and the corresponding Tumor-Infiltrating Lymphocytes (TIL) to assess cell type unique patterns underlying chromatin accessibility and the regulatory landscape of their genome at the single cell level. The scATAC-seq is a method relying on next-generation sequencing (NGS) library construction using the hyperactive transposase Tn5, an enzyme to preferentially insert and tag accessible DNA fragments with adaptors, to get sequencing ready libraries. The library that is generated can be sequenced by NGS and the regions of the genome with open or accessible chromatin are analyzed using 10x Genomics developed pipeline and downstream bioinformatic analysis. scATAC-seq is widely used for studying the regulatory landscape of the genome at high resolution of tens of thousands of single cells in parallel.

2. Materials & Methods.

Materials:

Single cell ATAC-seq assay was performed on 2 sets of cryopreserved primary melanoma cell lines and their corresponding TIL expanded from 2 metastatic melanoma patients (#2559 and #2765), obtained and expanded from each tumor as previously described [9]. In order to test whether it's possible to detect a small fraction of TIL in heterogenous samples, the assay validation was performed by spiking TIL with melanoma cells lines (10% TIL + 90% tumor cell line) (see Table 2).

Table 2. Samples

Batch Number	Sample #	Sample Name
Set 1	1.	Mel 2559 (100%)
	2.	TIL 2559 (100%)
	3.	Mel 2559 (90%) + TIL 2559 (10%)
Set 2	4.	Mel 2765 (100%)
	5.	TIL 2765 (100%)
	6.	Mel 2765 (90%) + TIL 2765 (10%)

Methods: detailed methodology, equipment and reagents details can be found within following documents:

- CG000053_CellPrepGuide_RevC.pdf,
- CG000168_ChromiumSignleCell_ATAC_ReagentsKits_UserGuide_RevC,
- CG000169 Demonstrated Protocol Nuclei Isolation ATAC Sequencing RevD guide,

and other related documentation deposited on https://support.10xgenomics.com/single-cell-atac.

The Chromium Single Cell ATAC Solution provides a comprehensive, scalable approach to determine the regulatory landscape of chromatin in hundreds to thousands of cells in a single sample. This is achieved by transposing nuclei in a bulk solution; then using a micro fluidic chip, the nuclei are partitioned into nanoliter-scale Gel Beads-in-emulsion (GEMs). GemCode Technology samples a pool of ~750,000 10x Barcodes to separately and uniquely index the transposed DNA of each individual nucleus. Libraries are generated and sequenced, and 10x Barcodes are used to associate individual reads back to the individual partitions, and thereby, to each individual nucleus.

Briefly, each sample was thawed according to the optimized protocol and assessed for the cell count and viability (expected >= 80%) using Trypan Blue staining in the Automated Cell Count. Next, we used 1mln cells of each 100% sample of each sample for nuclei isolation. After isolation nuclei were resuspended with targeted volume (see CG000169 user guide) of 1x Nuclei Buffer followed by nuclei count and viability check (expected =<5%). The mixture of nuclei of TIL and of cancer cells in 1:10 ratio was prepared and used into the Chromium Single Cell ATAC assay along with 100% pure nuclei of cancer cells and 100% pure nuclei of corresponding TIL.

(1) **Transposition** – nuclei suspensions are incubated in a Transposition Mix that includes a Transposase. The Transposase enters the nuclei and preferentially fragments the DNA in open regions of the chromatin. Simultaneously, adapter sequences are added to the ends of the DNA fragments.

(2) **GEM Generation & Barcoding** – EMs are generated by combining barcoded Gel Beads, transposed nuclei, a Master Mix, and Partitioning Oil on a Chromium Chip E. To achieve single nuclei resolution, the nuclei are delivered at a limiting dilution, such that the majority (~90-99%) of generated GEMs contains no nuclei, while the remainder largely contain a single nucleus. Upon GEM generation, the Gel Bead is dissolved. Oligonucleotides containing (i) an Illumina® P5 sequence, (ii) a 16 nt 10x Barcode and (iii) a Read 1 (Read 1N) sequence are released and mixed with DNA fragments and Master Mix. Thermal cycling of the GEMs produces 10x barcoded single- stranded DNA. After incubation, the GEMs are broken and pooled fractions are recovered.

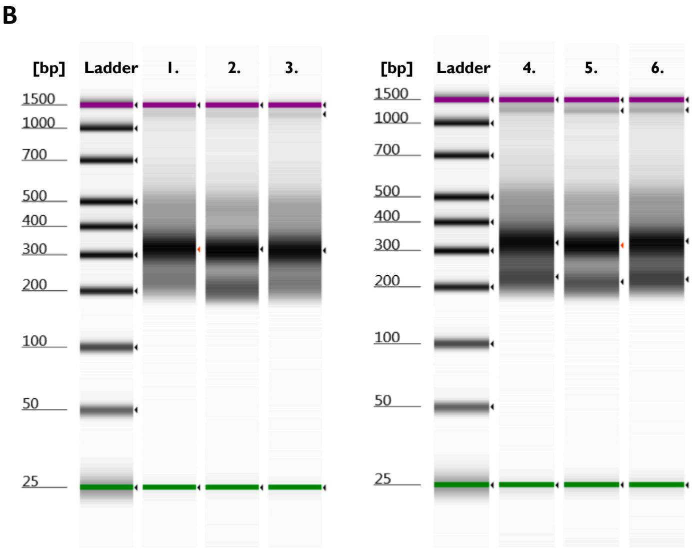
(3) **Post GEM Incubation Cleanup** – Silane magnetic beads are used to remove leftover biochemical reagents from the post GEM reaction mixture. Solid Phase Reversible Immobilization (SPRI) beads are used to eliminate unused barcodes from the sample.

(4) **Library Construction** – P7 and a sample index are added during library construction via PCR. The final libraries contain the P5 and P7 sequences used in Illumina® bridge amplification.

The QC of prepared libraries is indicated in the Figure 1 and it showed the reference guide criteria.

A

	Sample #	Sample Name
Set 1	1.	Mel 2559 (100%)
	2.	TIL 2559 (100%)
	3.	Mel 2559 (90%) + TIL 2559 (10%)
Set 2	4.	Mel 2765 (100%)
	5.	TIL 2765 (100%)
	6.	Mel 2765 (90%) + TIL 2765 (10%)



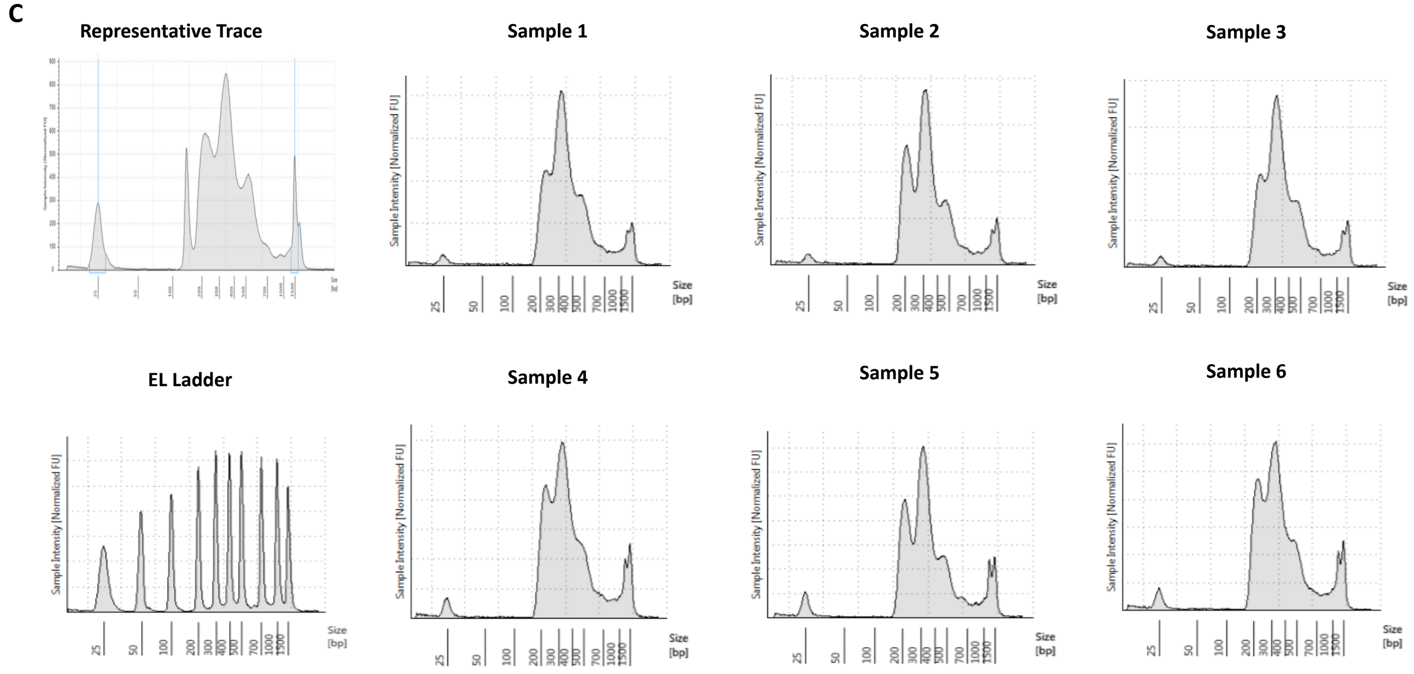


Figure 1. QC of scATAC libraries. (A) Sample annotation. (B) Fragment length distribution pattern and (C) peaks distribution using Agilent 4200 Tape Station HS D1000 assay.

(5) **Sequencing** – The Chromium Single Cell ATAC protocol produces Illumina®-ready sequencing libraries. For Illumina® sequencer compatibility, sample indices, sequencing depth & run parameters, library loading and pooling see CG000168_ChromiumSignleCell_ATAC_ReagentsKits_UserGuide_RevC file.
The generated here libraries (Figure 2) contain unique indexes which allowed to pool all libraries together maintaining equal molarity ratio and sequence on the same sequencing lane of the used here sequencer (Novaseq 6000, ATGC MDACC core facility; NIH 1S10OD024977-01) to minimize technical variation. Libraries were sequenced using the following read length format: 50 bp Read 1N, 8 bp i7 Index, 16 bp i5 Index and 50 bp Read 2N. Considering the minimum sequencing depth of 25,000 read pairs per nucleus, we targeted 50,000 read pairs per nucleus. Chromium Single Cell ATAC libraries comprise double stranded DNA fragments which begin with P5 and end with P7. Sequencing these libraries produces a standard Illumina® BCL data output folder.

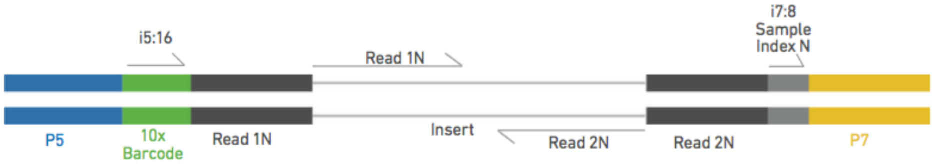


Figure 2. Chromium Single Cell ATAC Library.

3. **Data Analysis and Assay Performance Assessment.**

The Cell Ranger ATAC pipeline developed by 10x Genomics (see: https://support.10xgenomics.com/single-cell-atac/software/pipelines/latest/what-is-cell-ranger-atac) was used to processed all raw sequencing files.
The BCL data for Single Cell ATAC libraries includes: Paired-end Read 1N containing insert sequence only; Read 2N containing insert sequence, starting from the opposite end of fragment; 8bp sample index in the i7 read; 16bp 10X barcode sequence in the i5 read.

Main steps (Figure 3) of cell ranger atac pipeline (v1.1.0) relevant to single cell chromatin accessibility experiments includes:

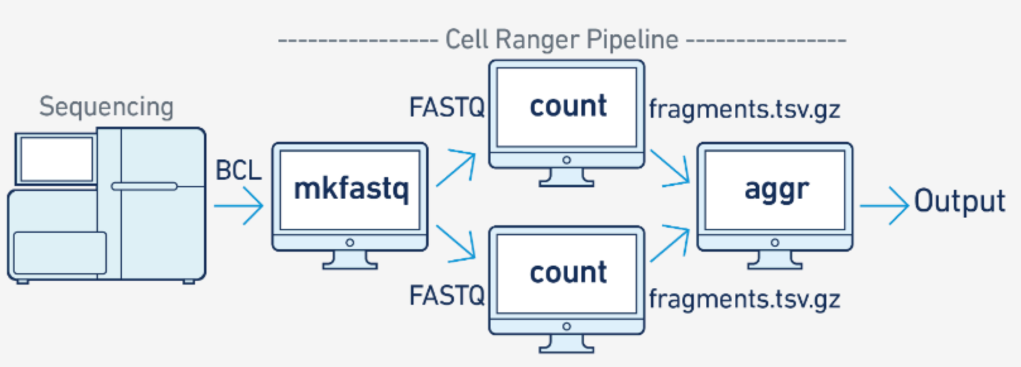


Figure 3. Data analysis processing steps using cell ranger atac pipeline.

(1) **cellranger-atac mkfastq** demultiplexes raw base call (BCL) files generated by Illumina® sequencers into FASTQ files. It is a wrapper around bcl2fastq from Illumina®, with additional useful features that are specific to 10x Genomics libraries and a simplified sample sheet format.

(2) **cellranger-atac count** takes FASTQ files from cellranger-atac mkfastq and performs ATAC analysis, including: Read filtering and alignment to the reference human genome, Barcode counting, Identification of transposase cut sites, Detection of accessible chromatin peaks, Cell calling, Count matrix generation for peaks and transcription factors, Dimensionality reduction, Cell clustering, Cluster differential accessibility.

For each step a web summary file is generated with the QC metrics which are **the initial point of reference for determining sample performance and sensitivity** of the Single Cell ATAC assay. The QC parameters for each sample are summarized in Table 3, along with a reference PBMC sample (fresh PBMC - 5K nuclei retrieved from CG000202_TechnicalNote_InterpretingCellRangerATACWebSummaryFiles_Rev-A, 10x Genomics) with indicated reference ranges of each parameter.

Table 3. QC metrics in the scATAC web summary files.

	Reference PBMC-5K (fresh)	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
CELLS PARAMETERS							
Estimated number of cells	500-10,000	5,059 (5,058)	5,509 (5,512)	4,894 (4,892)	3,658 (3,670)	4,357 (4,356)	2,974 (2,973)
Median fragments per cell	>500	11,897 (11,910)	19,738 (19,760)	12,754 (12,781)	24,129 (24,128)	19,221 (19,241)	24,315 (24,370)
Fraction of fragments overlapping any targeted region	Dependent on cell type and sequencing depth	84.2% (73.0%)	86.6% (78.1%)	84.3% (73.3%)	85.9% (75.8%)	87.9% (79.7%)	86.0% (76.2%)
Fraction of transposition events in peaks in cell barcodes	>25%	59.0% (59.0%)	60.7% (60.6%)	57.9% (57.8%)	61.2% (61.2%)	64.3% (64.3%)	60.6% (60.6%)
SEQUENCING PARAMETERS							
Total number of read pairs	User defined	214,496,958 (214,496,958)	230,995,104 (230,995,104)	213,932,438 (213,932,438)	209,721,486 (209,721,486)	188,607,464 (188,607,464)	186,415,483 (186,415,483)
Fraction of read pairs with a valid barcode	>75%	97.6% (97.6%)	97.6% (97.6%)	97.6% (97.6%)	97.6% (97.6%)	97.5% (97.5%)	97.6% (97.6%)
Q30 bases in Read 1	ideally >65%	94.4% (94.4%)	94.5% (94.5%)	94.5% (94.5%)	94.4% (94.4%)	94.6% (94.6%)	94.4% (94.4%)
Q30 bases in Read 2	ideally >65%	94.1% (94.1%)	94.3% (94.3%)	94.2% (94.2%)	94.1% (94.1%)	94.3% (94.3%)	94.1% (94.1%)
Q30 bases in Barcode	ideally >65%	88.3% (88.3%)	88.5% (88.5%)	88.4% (88.4%)	88.5% (88.5%)	88.5% (88.5%)	88.5% (88.5%)
Q30 bases in Sample Index	ideally >90%	92.2% (92.2%)	89.6% (89.6%)	90.5% (90.5%)	90.9% (90.9%)	90.3% (90.3%)	91.1% (91.1%)
TARGETING							
Enrichment score of TSS	>5%	6.35 (6.42)	8.14 (8.22)	6.82 (6.85)	5.75 (5.81)	8.66 (8.78)	6.20 (6.24)
Fraction of fragments overlapping TSS	NA	26.6% (26.3%)	47.2% (47.2%)	28.5% (28.3%)	30.4% (30.2%)	48.3% (48.1%)	31.5% (31.3%)
Fraction of fragments overlapping called peaks	NA	61.7% (61.7%)	62.5% (62.5%)	60.6% (60.6%)	64.2% (64.2%)	66.1% (66.1%)	63.6% (63.6%)
Fraction of fragments overlapping any targeted region	>55%	84.2% (73.0%)	86.6% (78.1%)	84.3% (73.3%)	85.9% (75.8%)	87.9% (79.7%)	86.0% (76.2%)

Fraction of total read pairs mapped confidently to genome (>30 mapq)	>80%	86.3% (86.5%)	87.1% (87.2%)	85.3% (85.5%)	85.9% (80.9%)	87.3% (87.4%)	79.5% (79.7%)
Fraction of total read pairs that are unmapped and in cell barcodes	NA	0.6% (0.6%)	0.7% (0.7%)	0.6% (0.6%)	0.6% (0.6%)	0.8% (0.8%)	0.7% (0.7%)
Fraction of total read pairs in mitochondria and in cell barcodes	<40%	0.9% (0.9%)	0.6% (0.6%)	1.5% (1.5%)	3.8% (3.8%)	0.8% (0.8%)	3.8% (3.8%)
INSERT SIZES							
Fragments in nucleosome-free regions	>40%	36.9% (36.9%)	42.4% (42.4%)	37.0% (37.0%)	40.9% (40.9%)	42.9% (42.9%)	41.6% (41.6%)
Fragments flanking a single nucleosome	Dependent on sample type	45.0% (45.0%)	41.3% (41.3%)	45.1% (45.1%)	40.4% (40.4%)	40.4% (40.4%)	40.2% (40.2%)

*Red numbers within brackets indicate results from alignment performed to hg38.

Moreover, key plots helping assessing assay performance (standardization and reproducibility) are summarized in Figure 4 below:

- **Barcode Rank Plot:** The Barcode Rank (or knee plot) for fragments overlapping peaks marks the barcodes that were inferred to be associated with cells.

- **Fragment Distribution Plot**: The distribution of the number of fragments per barcode for the non-cell and cell groups is displayed in the Fragment Distribution plot.

- **Insert Size Distribution Plot:** Insert size distribution of transposase accessible fragments sequenced is displayed in the Insert Size Distribution plot.

- **Enrichment Around (TSS) Plot:** The Transcription Start Site (TSS) profile, which is computed as the number of cut sites per base, of all the barcodes irrespective of cell versus non-cell assignment in a window of 2,000 bases around the full set of annotated TSSs is displayed in the Transcription Start Site plot. The y-axis scale is normalized by the minimum signal in the window.

(3) **cellranger-atac aggr** aggregates and analyzes the outputs from multiple runs of cellranger-atac count (such as from multiple samples from one experiment) by performing the following steps: Normalization of input runs to same median fragments per cell (sensitivity) – by depth, Detection of accessible chromatin peaks, Count matrix generation for peaks and transcription factors for the aggregate data, Dimensionality reduction, Cell clustering, Cluster differential accessibility.

The Loupe Cell Browser (v 4.2.0) was used for downstream analysis and visualization of 10x Chromium™ Single Cell ATAC data. It is optimized for finding significant peaks and distinguishing transcription factor motifs, identifying cell types and subtypes, comparing chromatin accessibility between groups of cells, and exploring substructure within cell clusters.

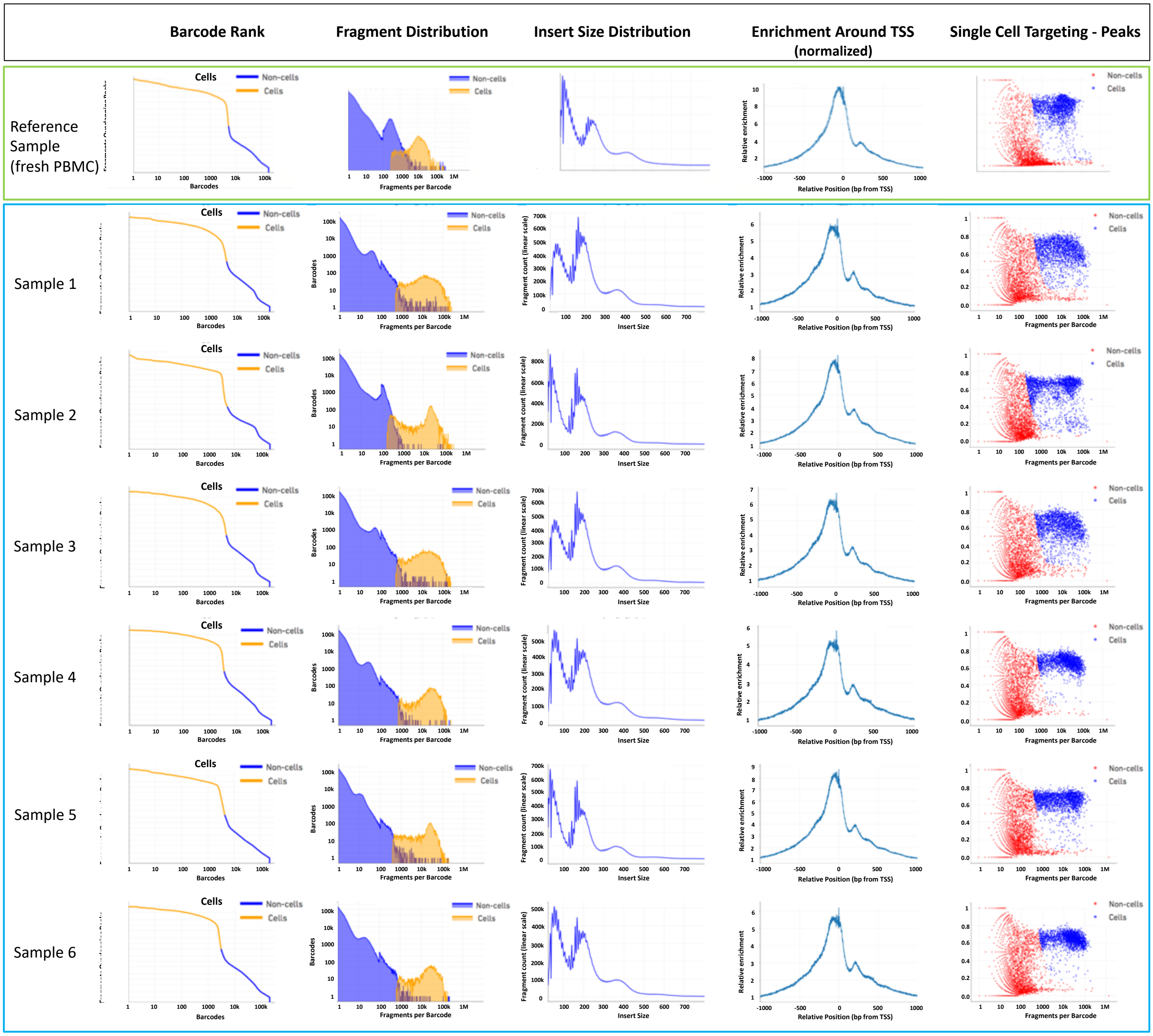


Figure 4. QC Plots displaying the assay performance.

The precision of intra- and inter- assay of pooled samples across different batches (set 1 and set 2), was assessed within further analysis. We applied t-distributed stochastic neighbor embedding (t-SNE) method [10], an unsupervised, non-linear technique for data exploration and visualizing high-dimensional data across all of processed six samples. The t-SNE clustering based on the samples name (library ID) is displayed on Figure 5 which indicates separate clustering for TIL from both sets when comparing to melanoma cancer cells, and additional separation within two sets of melanoma cancer cells. The Figure 5B displays the clustering of each sample (library ID) within a separate, split view, which clearly shows the population of TIL within the mixed samples (sample #3 and #6) of melanoma cancer cells with TIL. The TILs percentage (10% for set 1 and 7.4% for set 2) and cells are marked within the figure. Further on, we performed graph-based clustering (Figure 6), an algorithm which seeks to find highly-connected "modules" in the graph, dividing the samples in 13 clusters. Based on the distribution of the clusters within mixed samples of cancer cells and TIL, we identify a similar TIL pattern within the TIL subpopulation (Figure 6B).

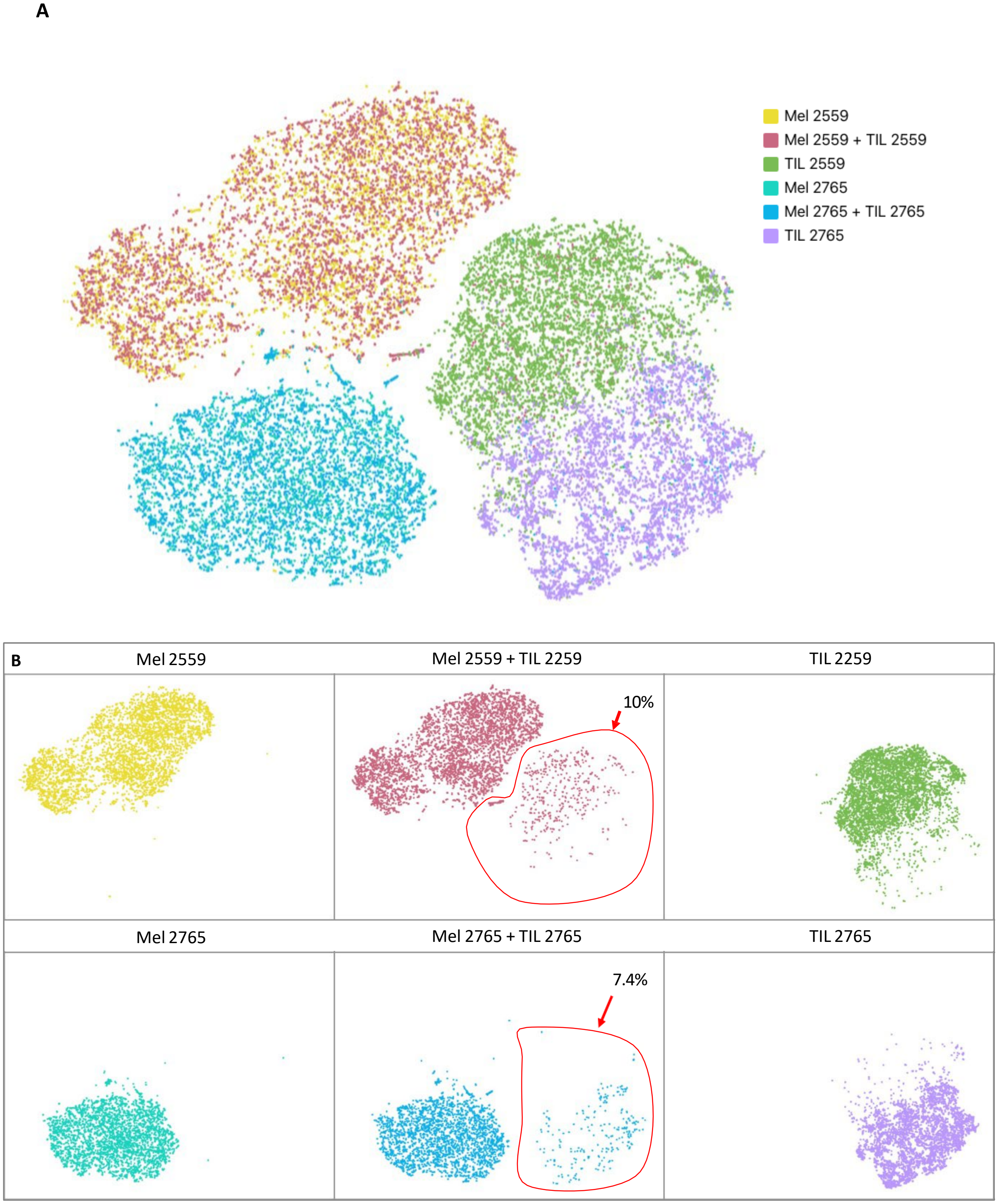


Figure 5. t-SNE plot of aggregated six samples based on sample name (library ID). A - t-SNE plot of aggregated six libraries, displayed together colored based on library ID. B – The split view of each library with indication of TILs percentage in mixed samples are indicated in the frame with calculated percentage based on cellular count out of total sample count.

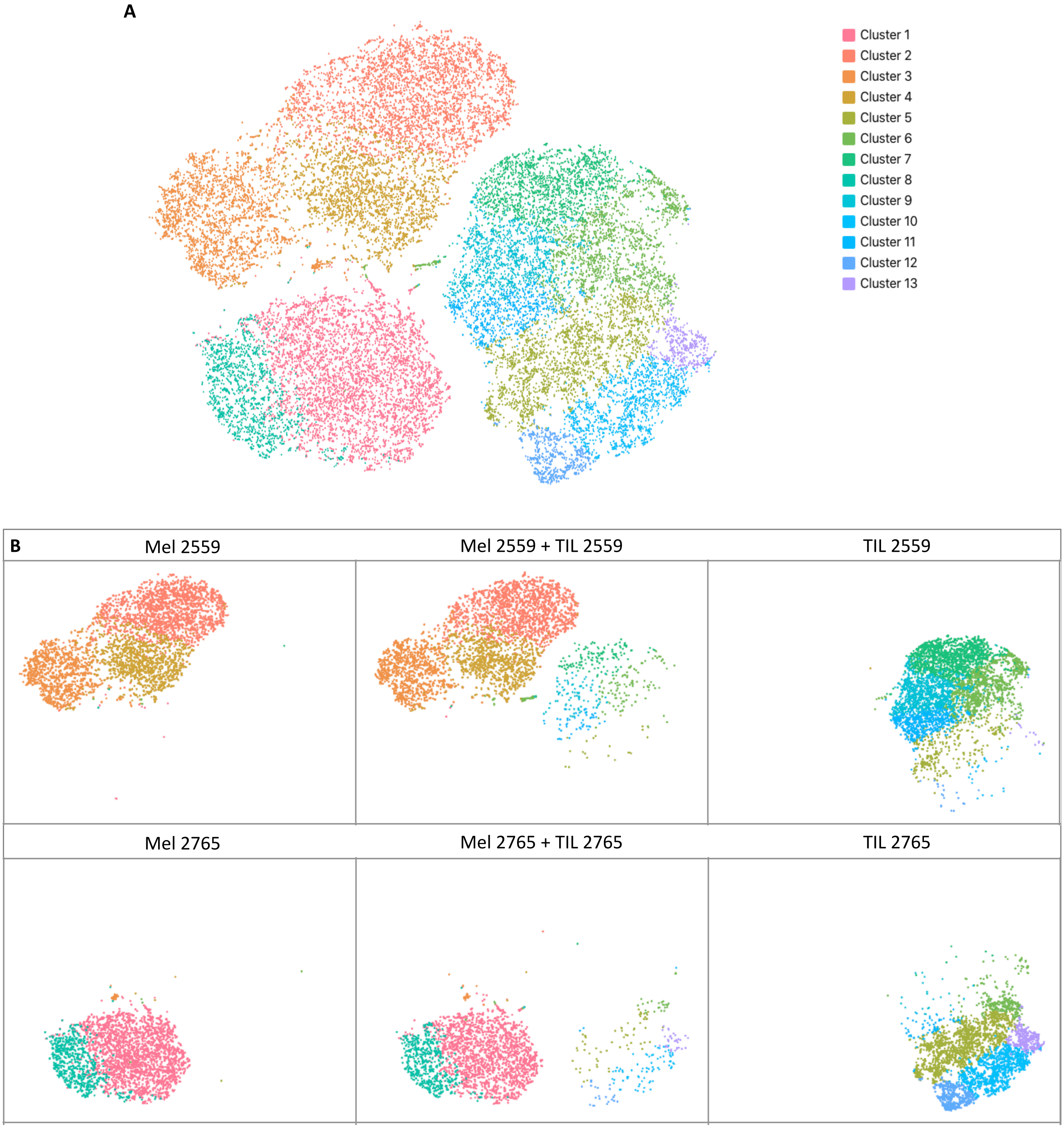


Figure 6. t-SNE plot of aggregated libraries with marked clusters 1-13 generated using graph-based clustering. A- aggregated view, B- split view for each sample.

The peak distribution reflecting the chromatin accessibility for **selected** genes characteristic for analyzed groups additionally confirmed **differential** pattern among analyzed samples (Figure 7) and reproducibility of the results.

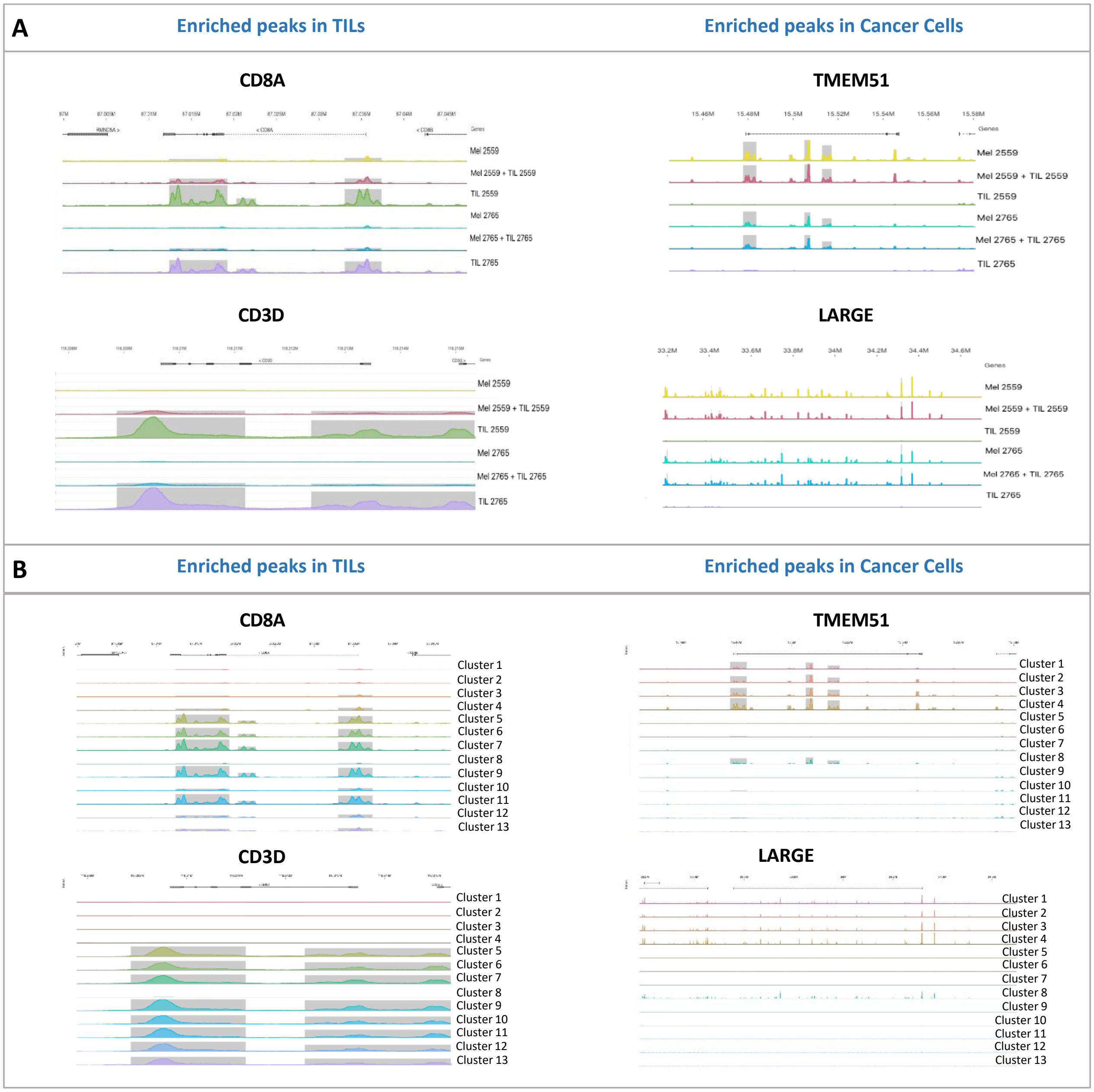


Figure 7. The Peak Viewer panel of selected genes displays differential chromatin accessibility across genomic regions of interest (genes), showing the sizes of called peaks, and the relative number of cells per cluster within those peaks and cut site tracks. The height of each peak is proportional to the number of cells within cluster that had cut sites falling within the called peak. A – displayed per library ID, B- displayed by clusters generated using graph-based clustering.

The precision of intra- and inter- assay and biological reproducibility was additionally assessed by performing Spearman's correlation analysis between identified 13 graph-based clusters using all significant nearby genes from differential analysis (Figure 8). The correlation plot unbiasedly shows the differences between analyzed groups: melanoma cancer cells and TIL and similarity between two sets within those groups.

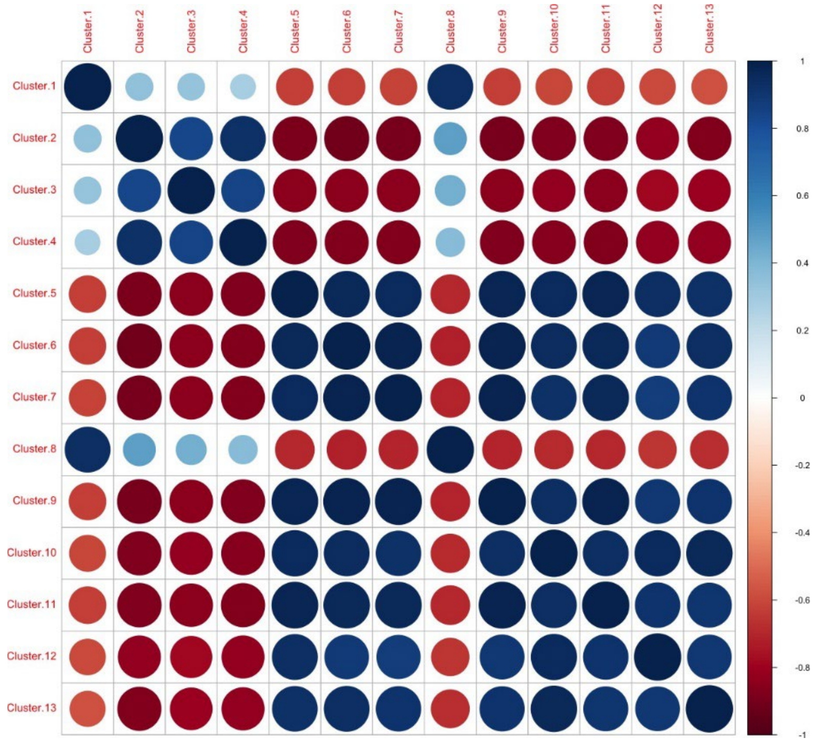


Figure 8. Spearman's correlation plot of Log2FC values of all significant nearby genes (features expressed highly within clusters, relative to the entire dataset) found within clusters. Plot generated using 'corrplot' package in R v3.6.3.

Next, to see profiles of each replicate within each set, we split cells of the mixed sample into two separate parts (part A – corresponding to melanoma cancer cells and part B – corresponding to TIL, see Figure 9) and displayed the examples of top enriched genes of selected clusters 1, 2, 5, and 7 across samples before and after splitting (Figure 10).

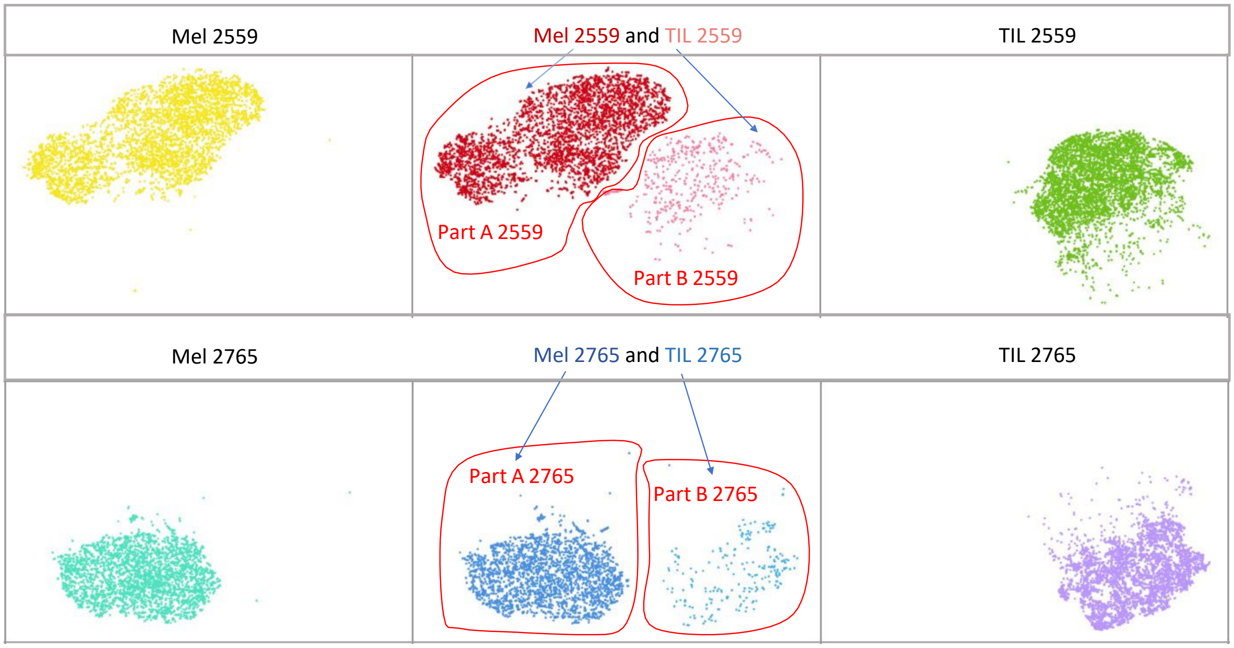
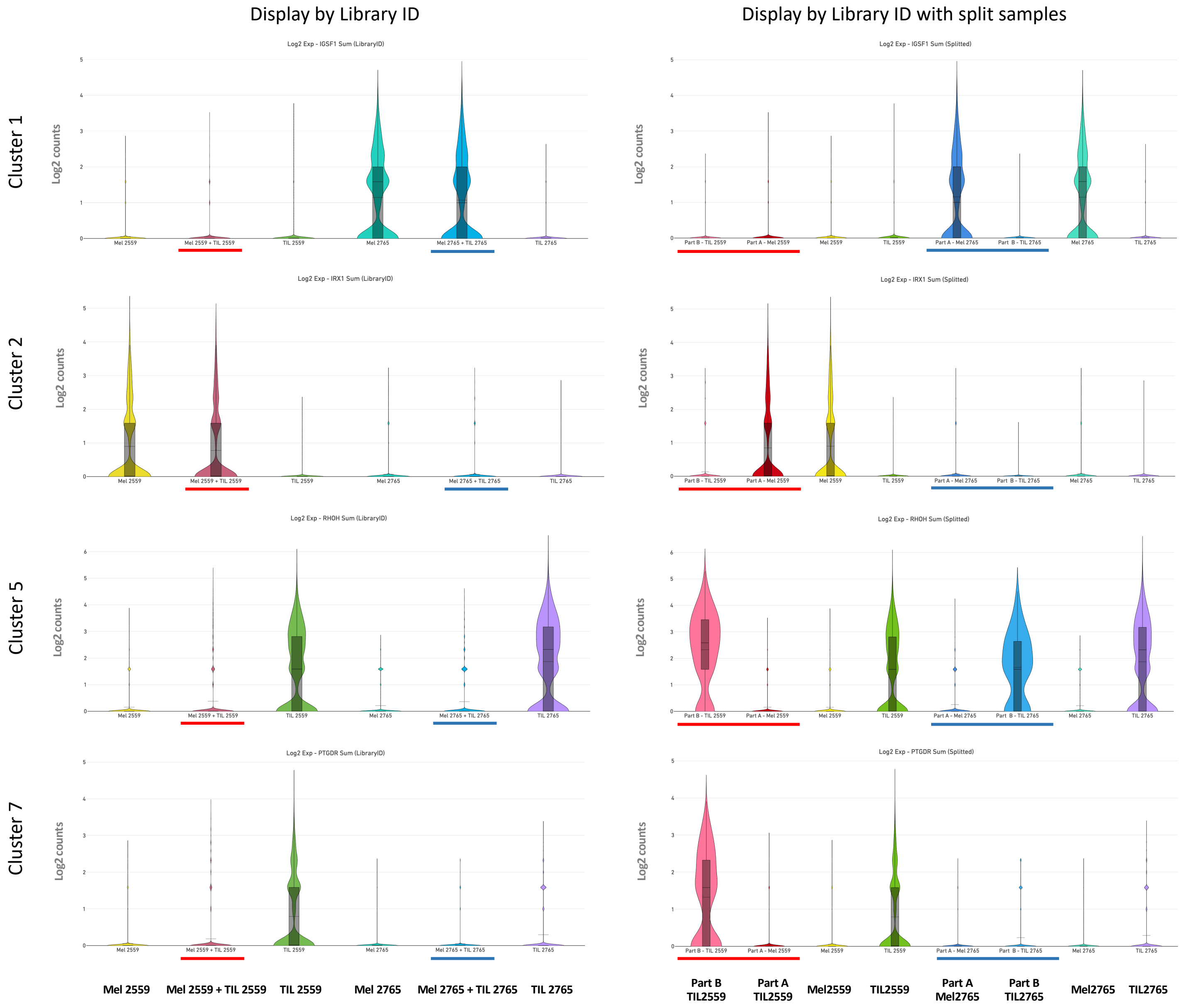


Figure 9. The split view of each library with indication of melanoma cancer cells (part A) and TIL (part B) as separate samples taken for further analysis.



	Gene	*pvalue*	log2FC
Cluster 1	*IGSF1*	0	3.78
Cluster 2	*IRX1*	9.51E-231	2.41
Cluster 5	*RHOH*	2.34E-112	2.07
Cluster 7	*PTGDR*	3.44E-107	2.4

Figure 10. Violin plots of top significant nearby gene identified within selected clusters 1, 2, 5 and 7; data displayed by library ID of samples without (left panel) and with split (right panel) of the mixed samples into melanoma cancer cells (part A) and TIL (part B) for each set. The y axis displays log2 count. The significance of each gene is summarized in adjacent table.

Additionally, we displayed the overall chromatin accessibility profiles across different immune cell subtypes (Figure 11) and peak distribution with percentage coverage (Figure 12) for selected corresponding genes of those immune cell subtypes across split view Part B of samples and their corresponding positive TIL control.

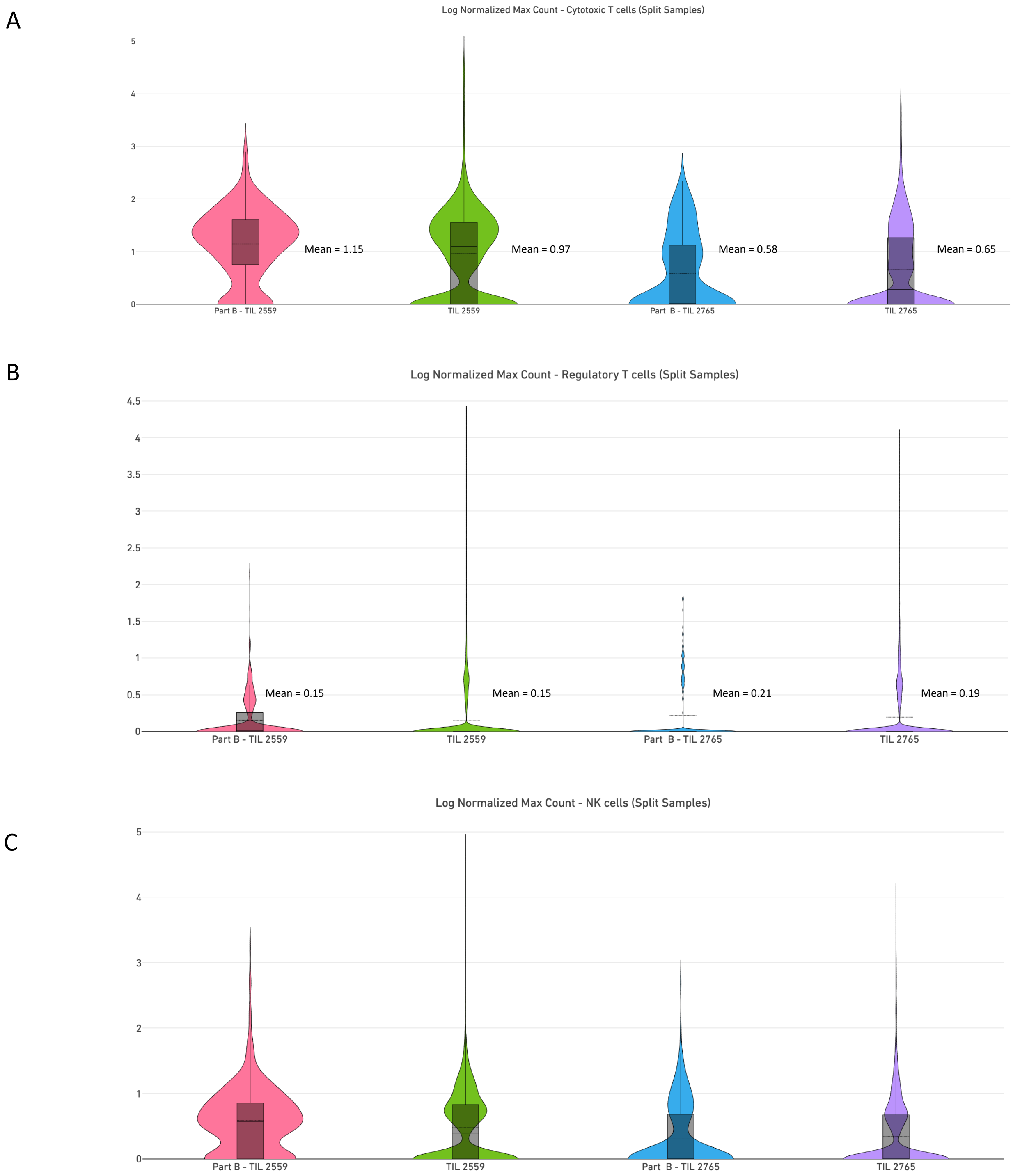
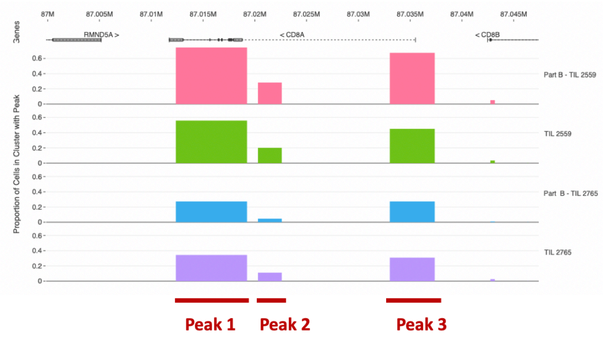


Figure 11. Violin plots representing the chromatin accessibility profile for selected immune subtypes: A - cytotoxic T cells (Promoter Sum of *CD8A, CD8B, PTPRC*), B - regulatory T cells (Promoter Sum of *CD4*, *FOXP3, IL2RA*) and C - NK cells (Promoter Sum of *NKG7, KLRC1, NCAM1, PRF1*) across split view Part B of samples and their corresponding positive TIL control. The y axis displays the Log Normalized Max Count, mean indicated for each plot.

A

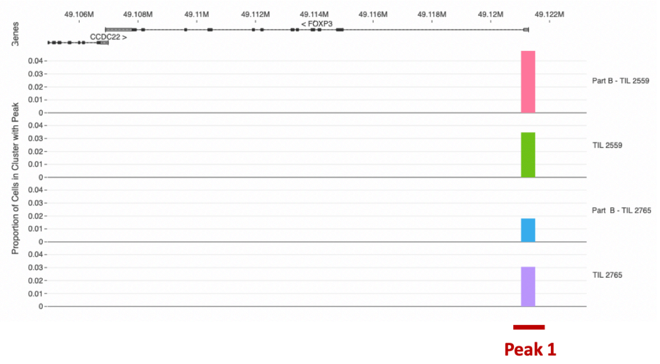
CD8A



Sample	Peak 1	Peak 2	Peak 3 (promoter)
Part B – TIL2559	374/502 (54.50%)	142/502 (28.29%)	338/502 (67.33%)
TIL 2559	3,082/5,509 (55.94%)	1,104/5,509 (20.04%)	2,487/5,509 (45.14%)
Part B – TIL 2765	60/221 (27.15%)	10/221 (4.52%)	60/221 (27.15%)
TIL 2765	1,501/4,357 (34.45%)	493/4,357 (11.32%)	1,353/4,357 (31.05%)

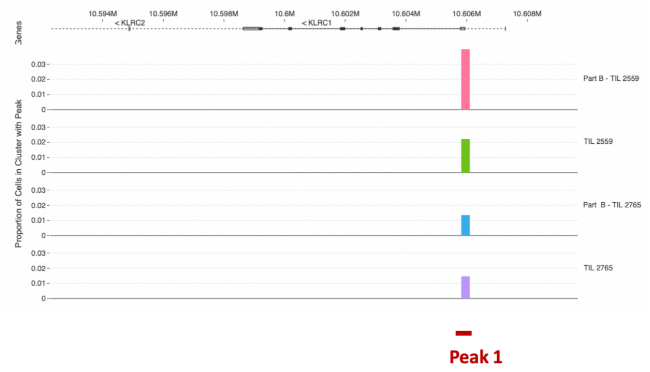
B

FoxP3



Sample	Peak 1 (promoter)
Part B – TIL2559	24/502 (4.78%)
TIL 2559	191/5,509 (3.47%)
Part B – TIL 2765	4/221 (1.81%)
TIL 2765	133/4,357 (3.05%)

KLRC1



Sample	Peak 1 (promoter)
Part B – TIL2559	20/502 (3.98%)
TIL 2559	122/5,509 (2.21%)
Part B – TIL 2765	3/221 (1.36%)
TIL 2765	64/4,357 (1.47%)

Figure 12. The Peak Viewer (left panel) of proportion of cells in cluster with peak of selected genes (A-C) associated with different immune subpopulation across split view Part B of samples and their corresponding positive TIL control. Summary table of cell contribution relative to the total count per sample, with indication in brackets as percentage is presented within right panel of each figure.

Appendix:

- CG000053_CellPrepGuide_RevC,
- CG000168_ChromiumSignleCell_ATAC_ReagentsKits_UserGuide_RevC,
- CG000169 Demonstrated Protocol Nuclei Isolation ATAC Sequencing RevD guide

References:

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