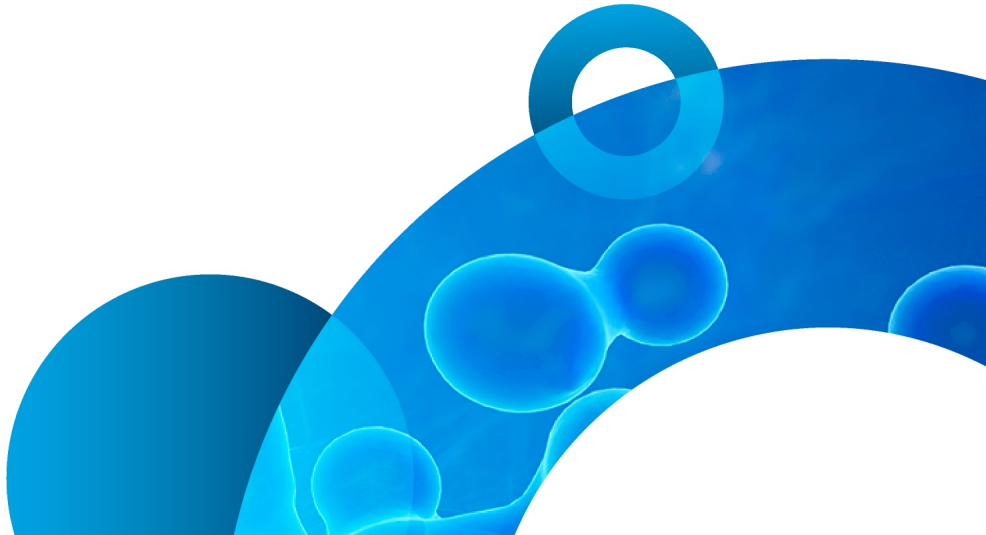
Single Cell Immune Profiling Solution with Feature Barcode technology

Workflow training



CG000359 Rev B

Chromium Single Cell Immune Profiling Solution with Feature Barcode technology

Agenda

- Single Cell Immune Profiling Solution with Feature Barcode technology Overview
- The Biochemistry Behind the Single Cell Immune Profiling Solution
- Sample preparation recommendations
- Chromium Single Cell 5' v2 Workflow
- Sequencing recommendations

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Overall training goals

Chromium Single Cell Immune Profiling Solution with Feature Barcode technology

- Discuss critical concepts for single cell immune profiling
  - Describe the relationship between input cell quality and output data quality
  - Review best practices for cell handling and counting
  - Review correct chip handling and loading processes
- Execute Immune Profiling Solution workflow steps
  - Run training samples and training kits on the Chromium Controller
  - Prepare emulsions from real samples
  - Perform key downstream protocol steps
- Introduce the fundamentals of Single Cell Immune Profiling data analysis
  - Review key steps in the Cell Ranger Pipelines and the usage of Loupe Browser and Loupe VDJ Browser

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Schedule highlights

2 day training agenda

- Please prepare the working area prior to FAS arrival, including receiving and locating all reagents
- Presentations can accommodate as many people as will comfortably fit in your conference room
- Up to 3 trainees may be present for lab training
  - Two hands-on trainees, one observer
  - Only one trainee runs real reagents
  - Up to 4 samples may be run during training (including optional control sample)
- Aim to have cells ready by ~2pm at ~1,000 cells/µl

	Day 1	Day 2
9:00 AM 9:30 AM 10:00 AM 10:30 AM	Chromium Controller Single Cell Application and Protocol Overview	Break Emulsions Dynabead Cleanup 5’ cDNA Amp SPRI Cleanup BioAnalyzer QC
11:00 AM 11:30 AM	Platform Technique Training	
12:00 PM 12:30 PM	Lunch	Lunch
1:00 PM 1:30 PM 2:00 PM	Cell Preparation	Software Overview Cell Ranger Loupe Cell/VDJ Browsers
2:30 PM 3:00 PM 3:30 PM 4:00 PM	Single Cell Setup Start Run	
4:30 PM	Review and Prep for Day 2	
5:00 PM		

Highlighted sessions require conference room with projector (white board optional)

Single Cell Immune Profiling Solution with Feature Barcode technology overview

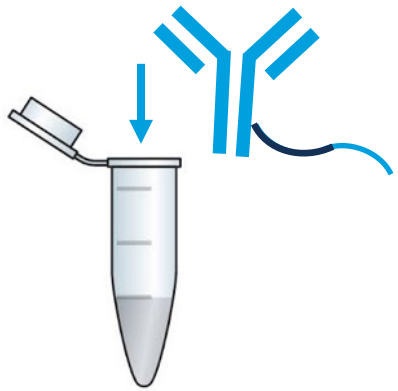
Link cell surface phenotype with transcriptome & TCR/ig

Cells are classified by proteins expressed on their surface: immune markers, cancer markers, maturation markers... Gene Expression can approximate the diversity, but doesn't tell the whole story

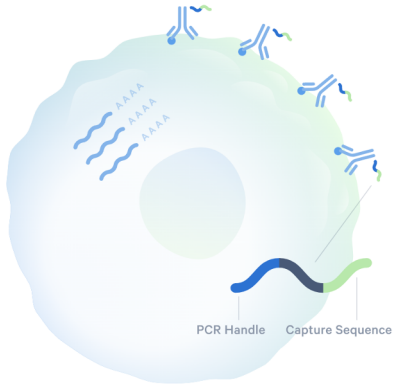
Applications of Feature Barcode technology

- Identify cell surface markers (oligo-barcoded antibody): TotalSeq™-C from BioLegend
- pMHC binding phenotype linked with TCR clonotype: Immudex Dextramer® reagents

Label cells with oligo-barcoded protein



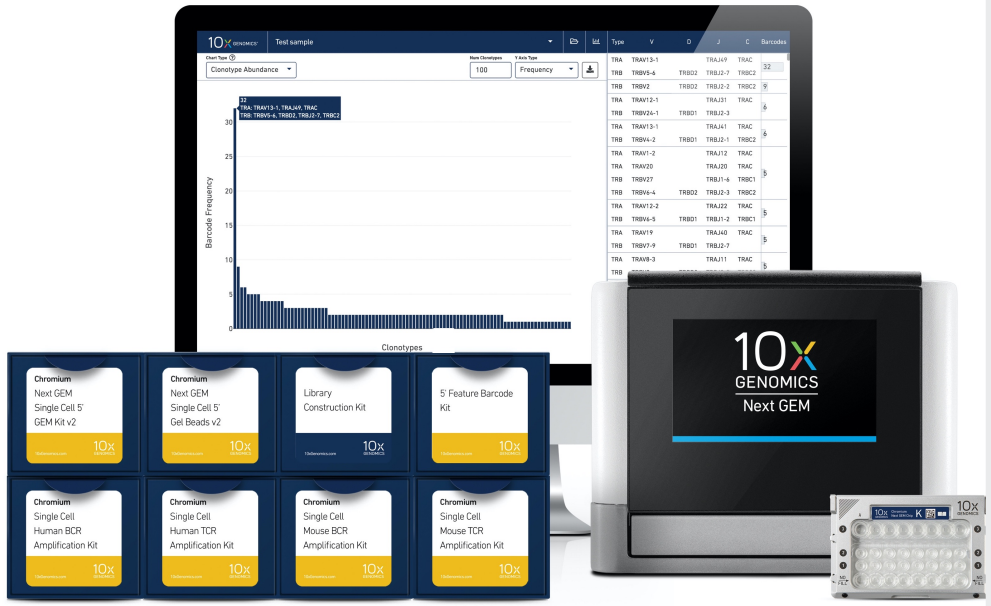
Link Feature Barcode reagents and cell barcodes in GEMs



Prepare & sequence GEX library & secondary library, analyze linked data

Single Cell Immune Profiling Solution with Feature Barcode technology

Solution highlights

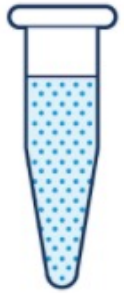


- Profile 100s to 10,000s of single cells to detect rare clonotypes and cell type sub-populations per sample
- Simultaneously characterize gene expression and enrich for single TCR and Ig cells providing paired, full length V(D)J receptor sequences in the same cells
- Simultaneously measure additional biological information with Feature Barcode technology
  - Link full-length, paired TCR α and β chain sequences with TCR-pMHC antigen specificity
  - Simultaneously measure cell surface protein expression
- Easy to install and use pipelines and visualization software
- Now on Next GEM technology*

Next GEM reagents are specific to Next GEM products and should not be used interchangeably with non-Next GEM reagents

Chromium Single Cell Immune Profiling Solution

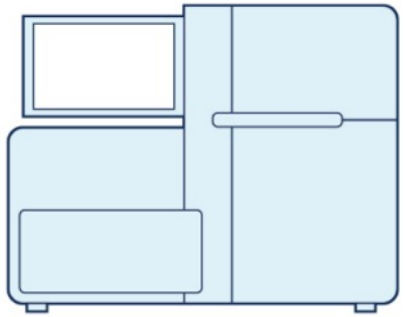
Input | Library creation | Sequence | Data analysis & visualization



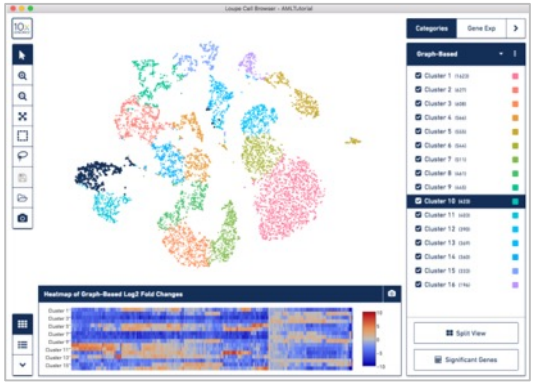
User-supplied cells

Chromium Controller and Next GEM Single Cell 5' v2 Reagents

- Cell Partitioning and Lysis
- Barcoding and Library Creation
- T Cell TCR or B Cell Ig Amplification
- Human or Mouse Species
- Feature Barcode Library Kit



Illumina sequencer compatibility



Cell Ranger Pipeline
Loupe Cell Browser
Loupe V(D)J Browser

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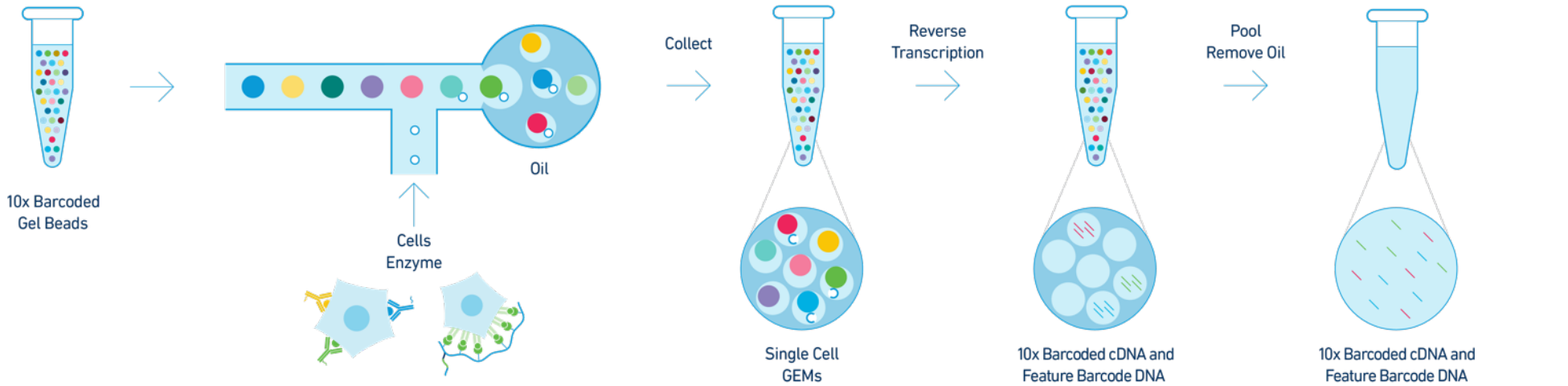
Single Cell Immune Profiling Solution with Feature Barcode technology

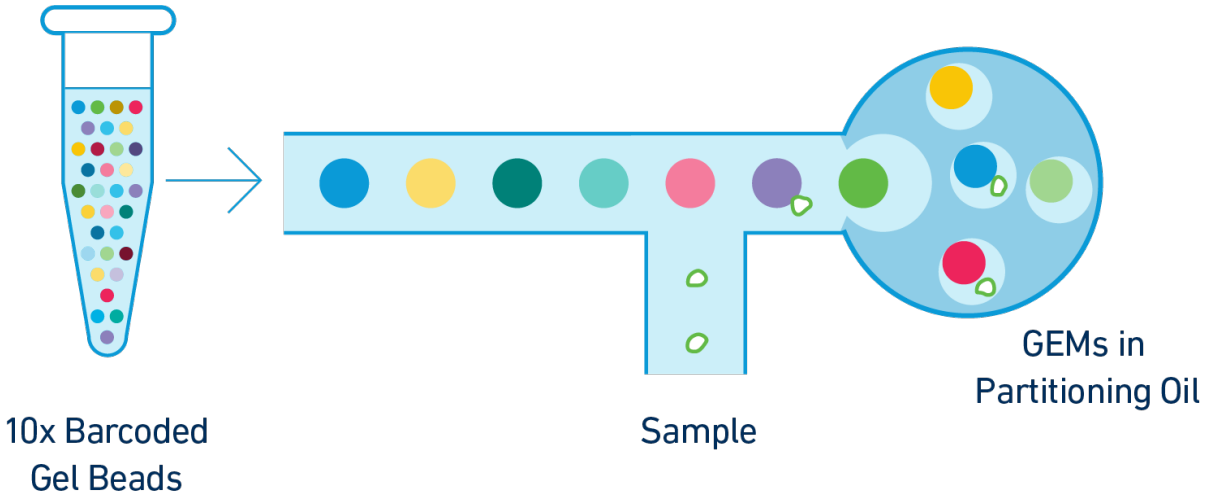
Workflow overview

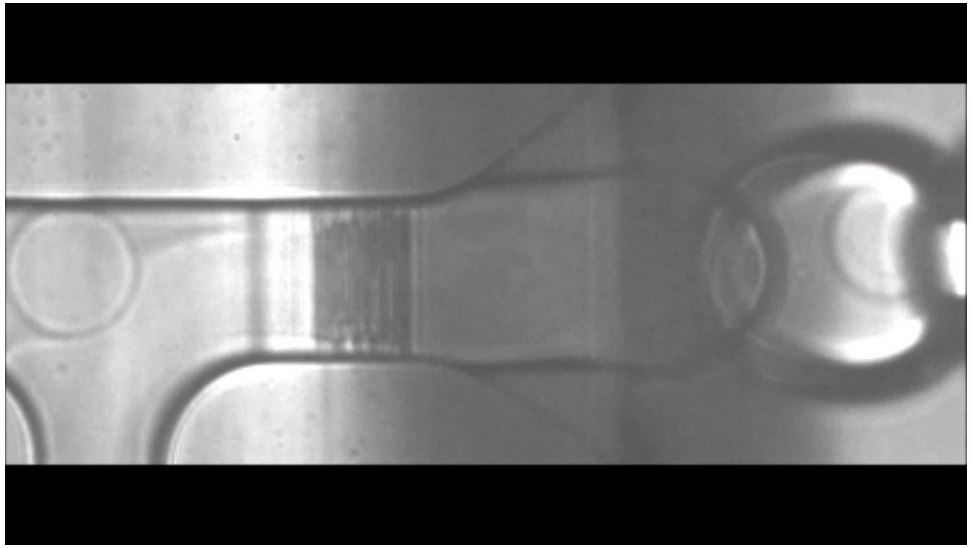


Input: Labeled single cells in suspension + 10x Gel Beads and Reagents

Output: Full-length paired V(D)J sequences, digital gene expression, cell surface protein expression profiles, and antigen binding specificities from single cells

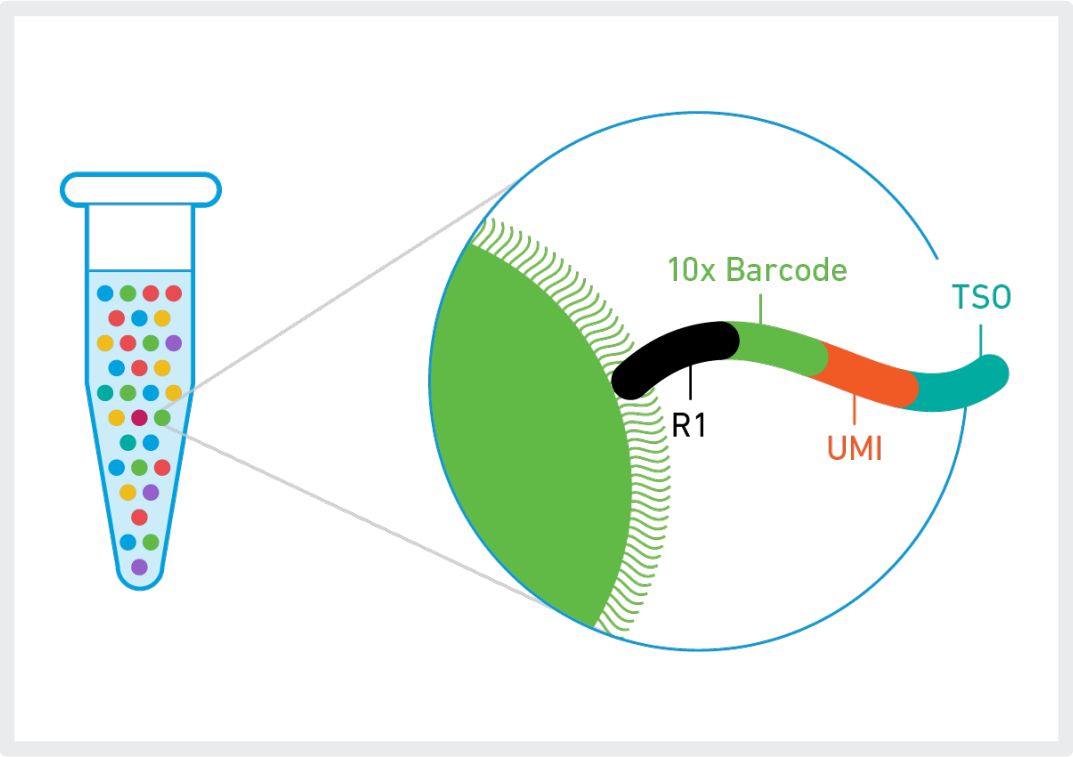
Next GEM technology – Creating Gel Beads in EMulsions (GEMs)





Single Cell Immune Profiling Solution with Feature Barcode technology

Capture of multiple targets enabled via single cell 5′ gel beads



i. **TruSeq Read 1**
22 nt Partial Illumina TruSeq Read 1 sequence

ii. **10x Genomics Barcode**
16 nt 10x Genomics Barcode
~750,000 defined barcode sequences

iii. **UMI**
10 nt Unique Molecular Identifier

iv. **TSO**
13 nt Template Switch Oligo sequence
Enables capture of poly-adenylated mRNA and cell surface protein Feature Barcode sequences

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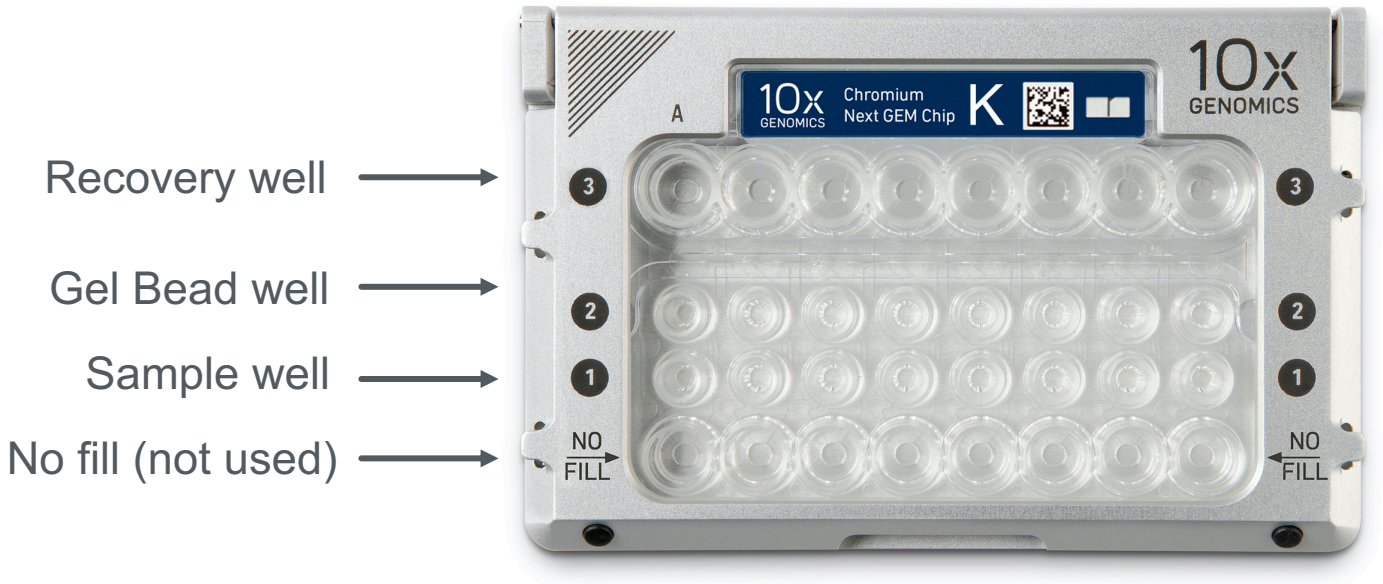
Single Cell Immune Profiling Solution with Feature Barcode technology

Chromium Next GEM Chip K Single Cell



- Up to 8 channels processed in parallel
- 500 to 10,000 cells per channel
- **18 min Chromium run time (per chip)**
- Up to 65% cell capture efficiency
- Up to 30 µm cell diameter tested
- Temperature range: 18-28^{0}C

Number of cells loaded	Number of cells recovered	Expected multiplet rate (%)*
~870	500	~0.4
~1,700	~1,000	~0.8
~8,700	~5,000	~3.9
~17,400	~10,000	~7.6

Firmware 4.00 or higher is required for running this assay

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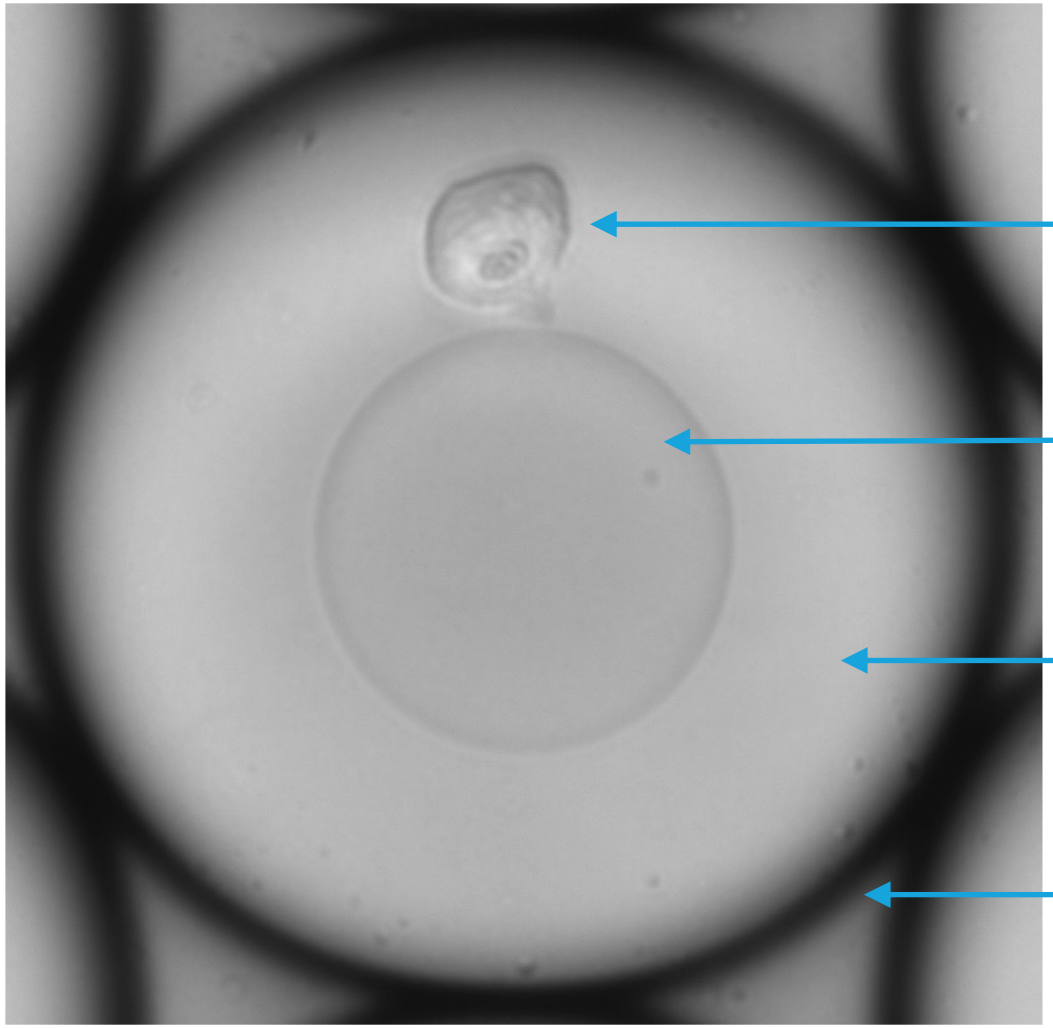
Gel Bead-in-Emulsion (GEM)



Next GEM Single Cell 5’ Kit v2 Configurations & Storage Conditions

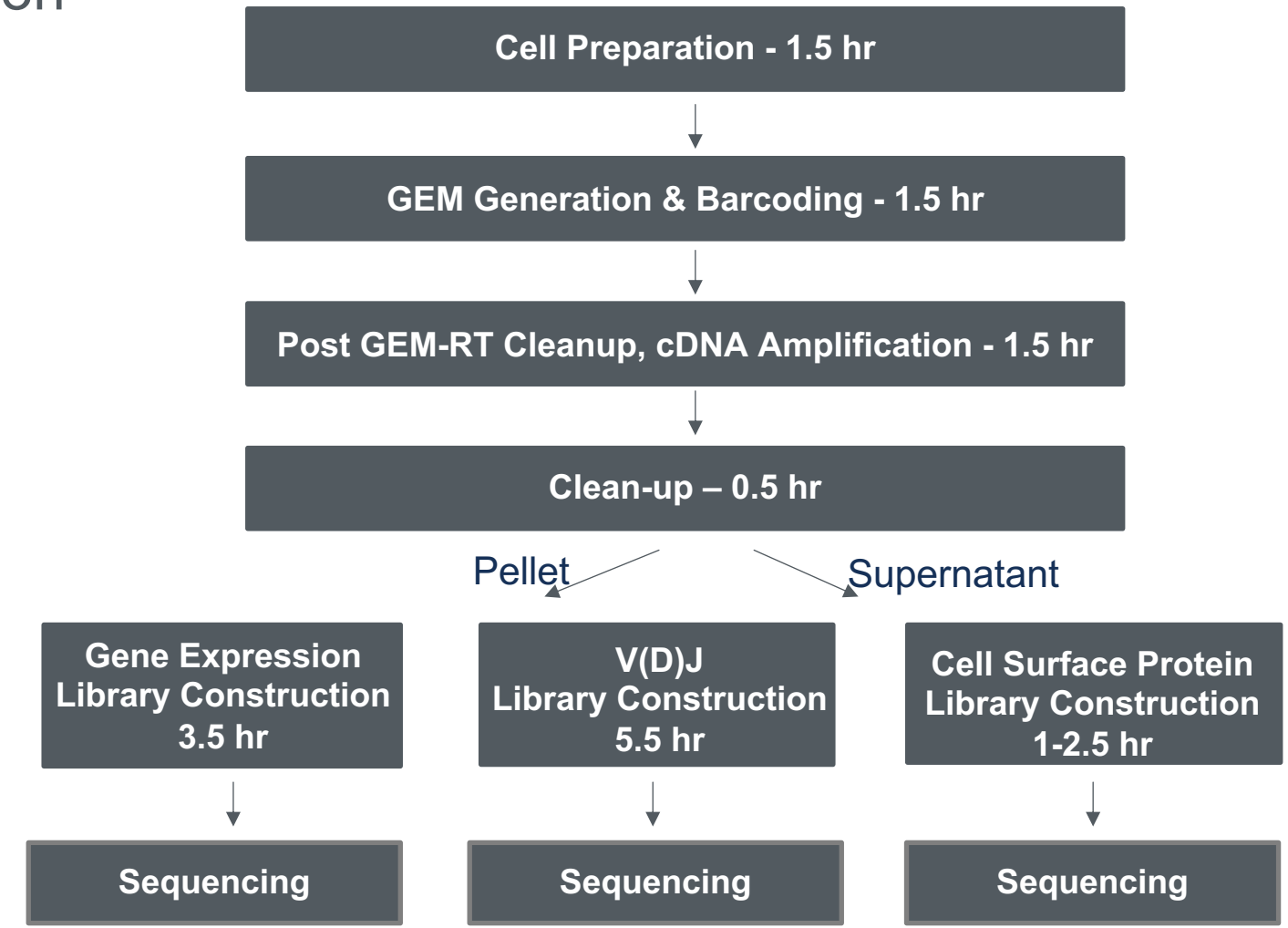
Component	Shipping conditions	Storage conditions
Chromium Next GEM Single Cell 5' Kit v2	Dry Ice	GEM Kit -20°C Library Kit -20°C Gel Beads -80°C
Chromium Single Cell V(D)J Amplification Kit, Human or Mouse, TCR or BCR	Dry Ice	-20°C
Dynabeads® MyOne™ Silane	Ambient	4°C
Dual Index Kit TT Set A	Dry Ice	-20°C
Library Construction Kit*	Dry Ice	-20°C
5′ Feature Barcode Kit**	Dry Ice	-20°C
Dual Index Kit TN Set A**	Dry Ice	-20°C
Chromium Next GEM Chip K Single Cell Kits	Ambient	Ambient

**with Feature Barcode technology

*The Next GEM Single Cell 5' Kit v2 contains reagents to generate one library type (Gene Expression, TCR or Ig) from one Gel Bead reaction. Each additional library type from the same Gel Bead reaction requires additional reactions from the 5′ Library Construction Kit.

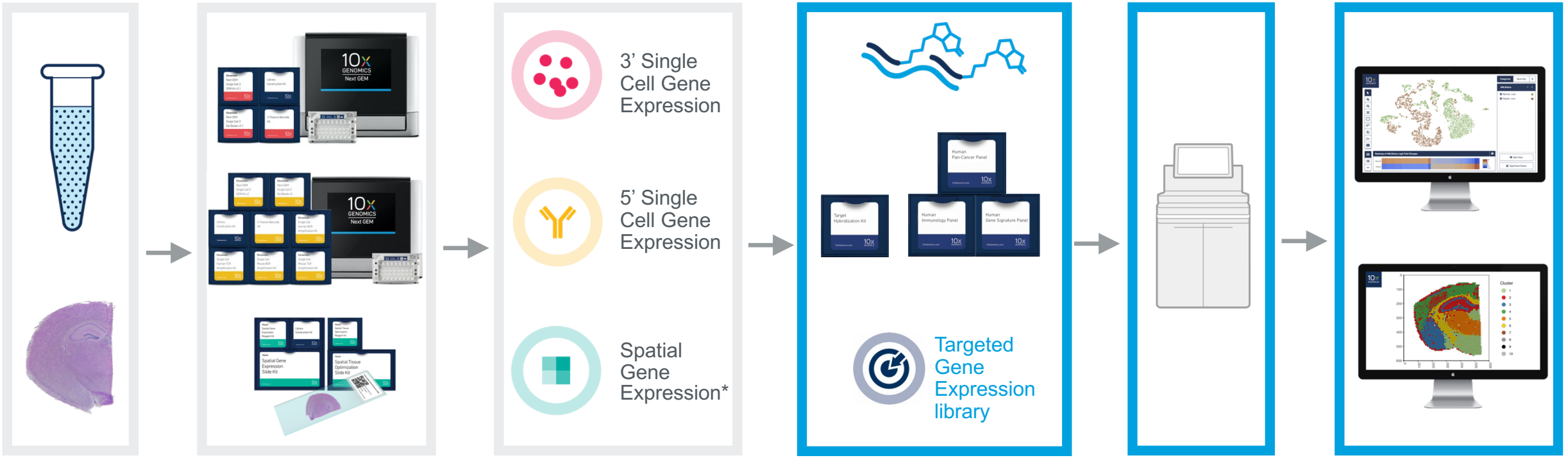
Single Cell Immune Profiling with Feature Barcode technology

Library preparation



Targeted Gene Expression

From discovery to focused transcriptomics

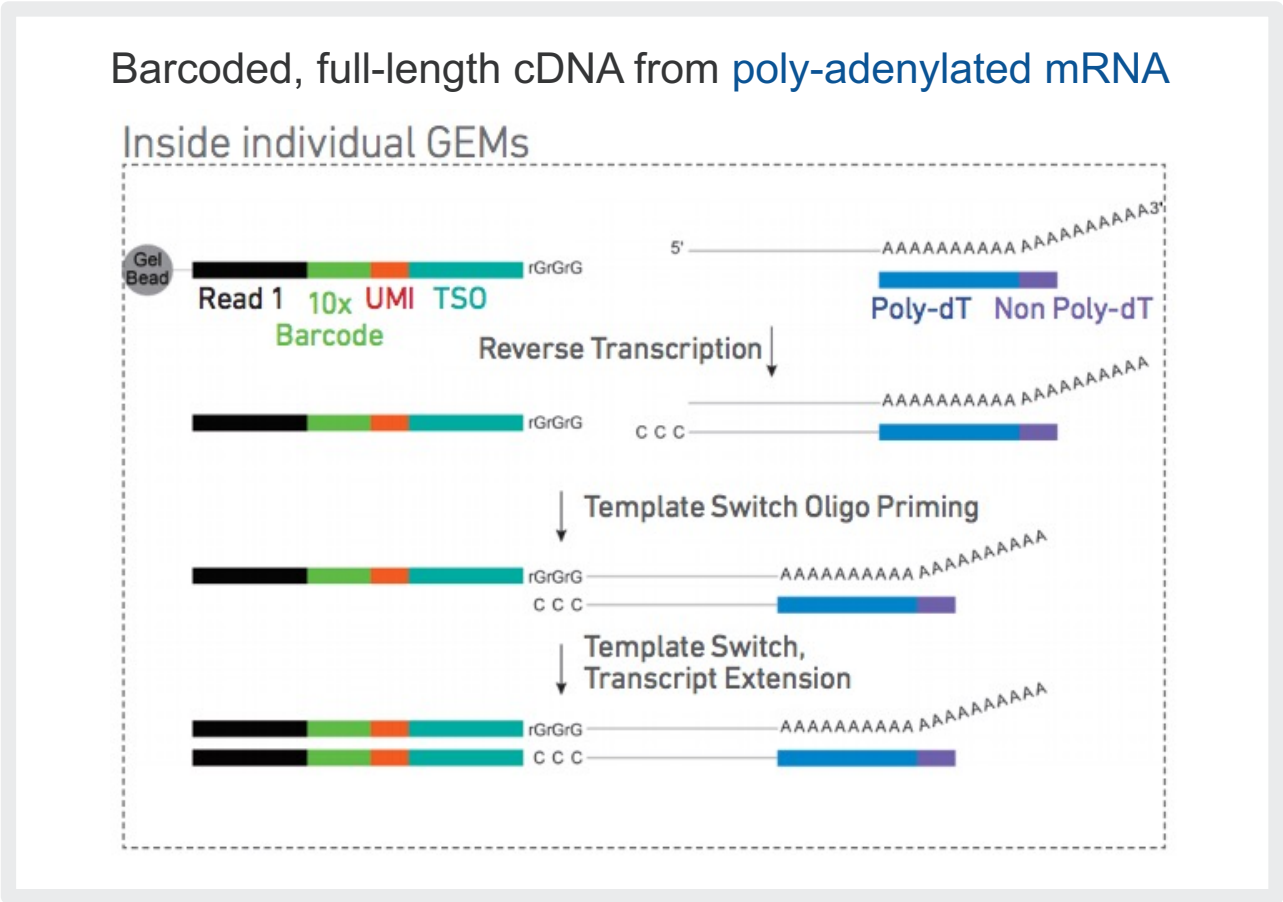


* Optimized protocol, support and software for targeted analysis of Spatial Gene Expression libraries coming soon

The biochemistry behind the Single Cell Immune Profiling Solution

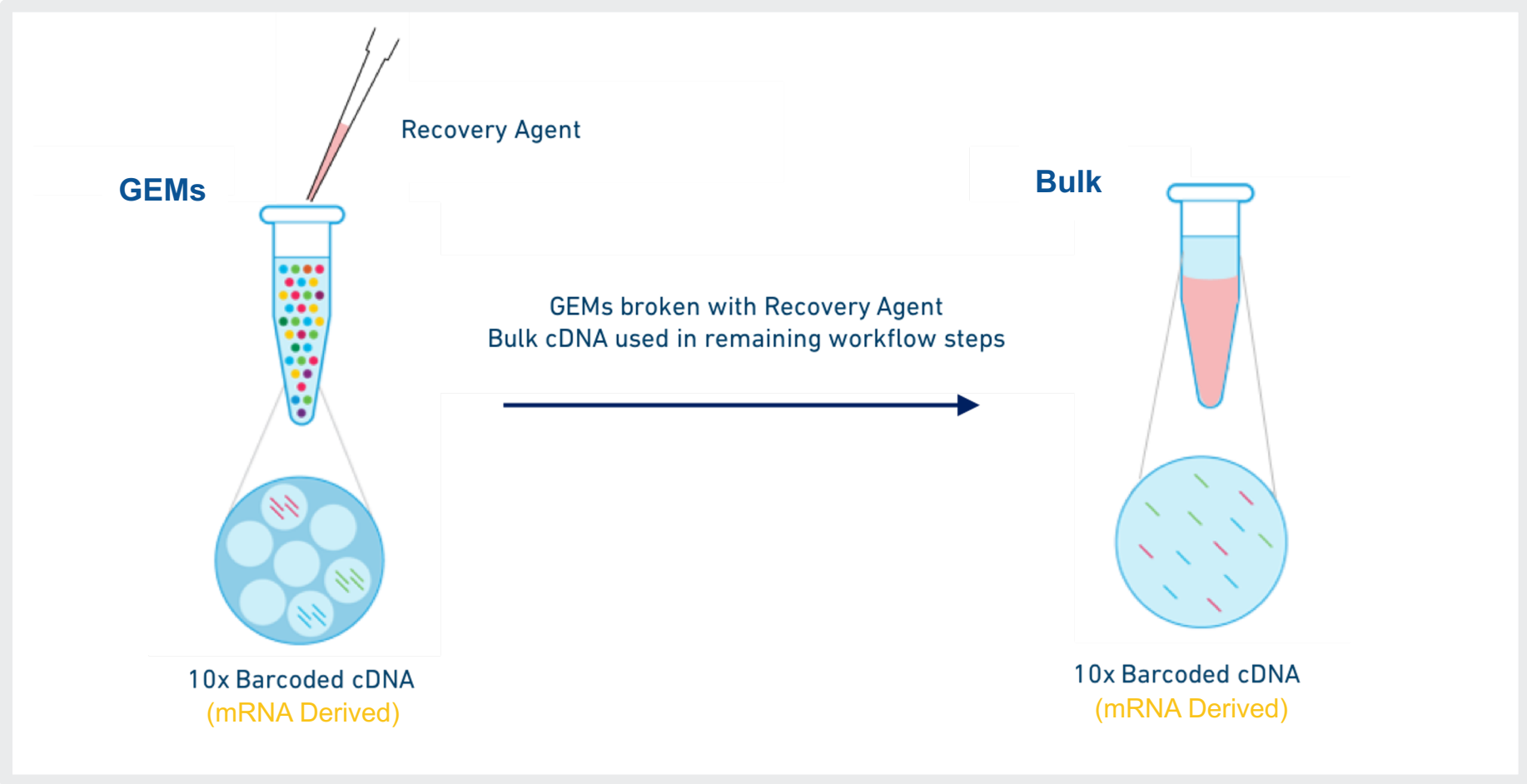
Single Cell Immune Profiling Solution: Target capture in GEMs

Direct capture of poly-adenylated mRNA inside individual GEMs



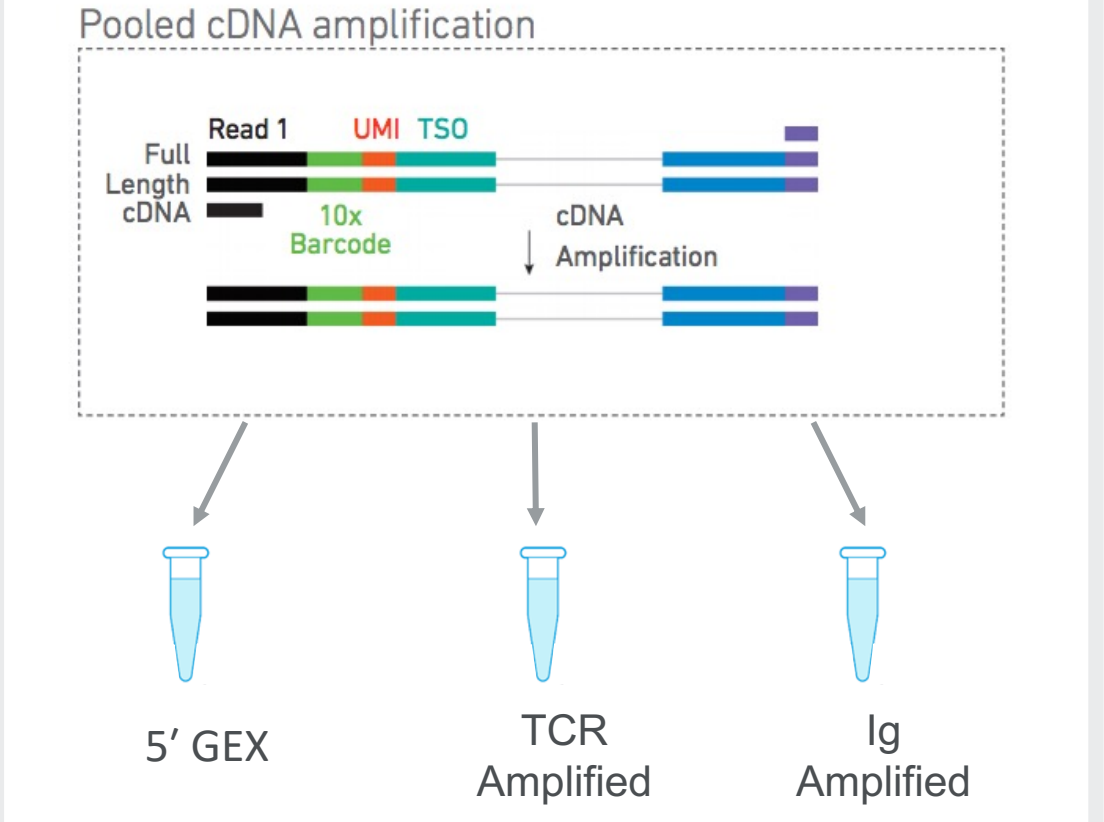
Note: Gene Expression poly-adenylated mRNA is captured by the corresponding gel bead oligo by Reverse Transcription and Template Switching

Single Cell Immune Profiling Solution: Breaking GEMs



Single Cell Immune Profiling Solution : cDNA amplification

In bulk amplification of cDNA



- Bulk first-strand cDNA was cleaned up after breaking GEMs
- cDNA amplification generates sufficient material to construct multiple libraries from the same cells

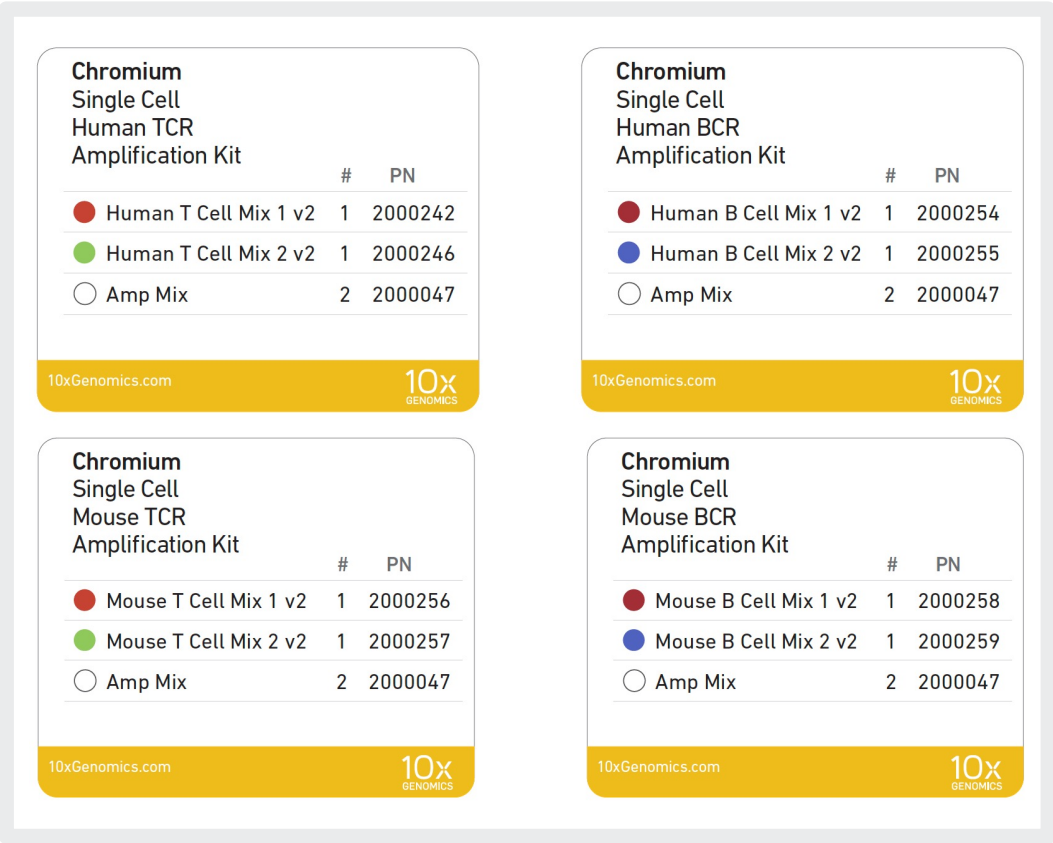
Note: For cDNA Amplification of poly-adenylated mRNA please use: cDNA Primers (PN-2000089)

Single Cell Immune Profiling Solution: Target amplification

Target amplification primers are species- and target cell-specific

Human or Mouse	
TCR Amplification Primers	**BCR Amplification Primers**
TRA	IGHA
TRB	IGHD
	IGHE
	IGHG
	IGHM
	IGL
	IGK

Oligonucleotide Sequences can be found in the User Guide



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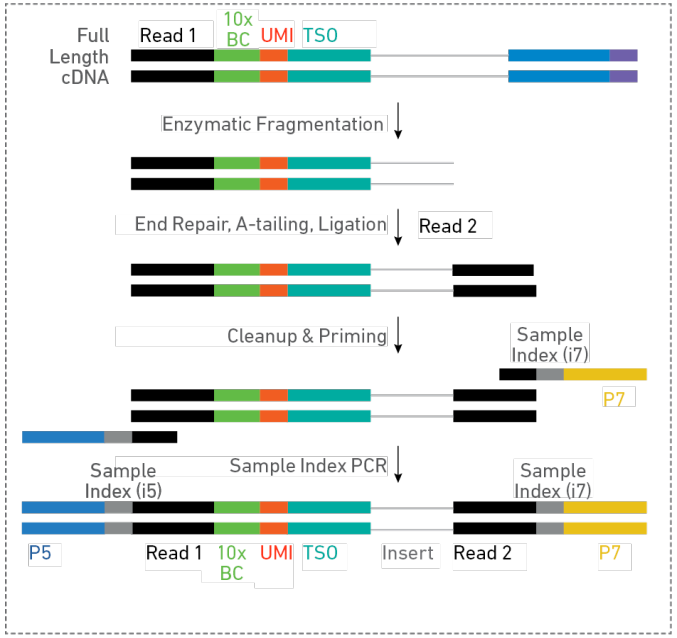
Single Cell Immune Profiling Solution: Library construction

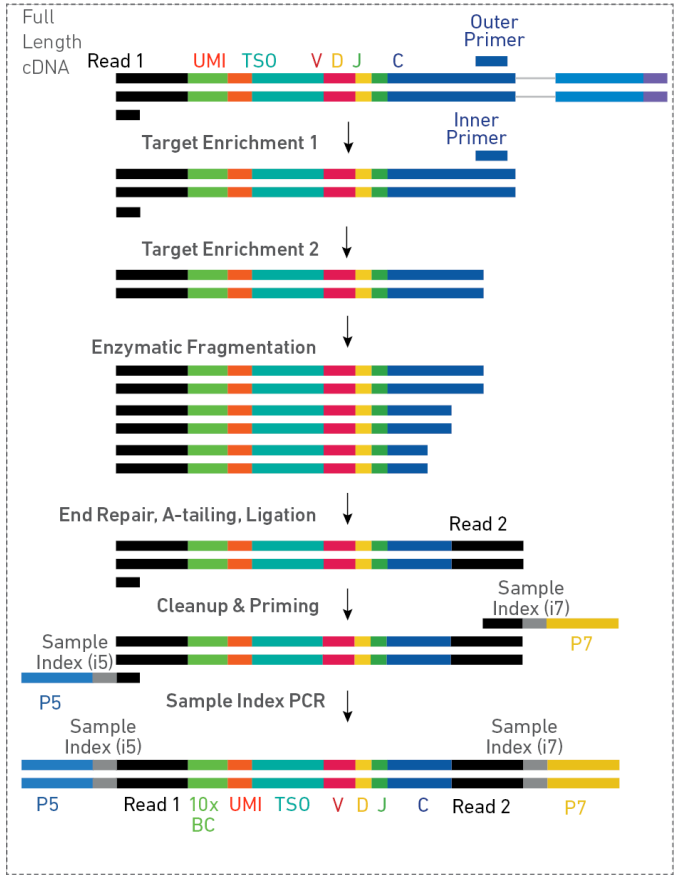
Amplified cDNA is split to generate multiple libraries for sequencing

Pooled amplified cDNA processed in bulk



*TCR and Ig libraries must be processed independently

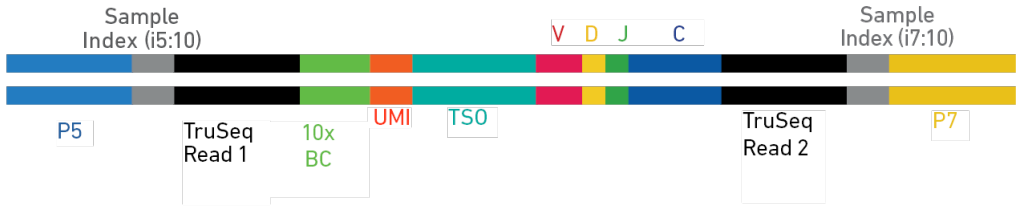
Pooled amplified cDNA processed in bulk



Sequencing V(D)J and 5′ Gene Expression Libraries

Libraries may be pooled in various configurations

Chromium Single Cell V(D)J Dual Index Library



Chromium Single Cell 5' Gene Expression Dual Index Library

Sequencing Depth	Minimum 5,000 read pairs per cell for V(D)J Dual Index library Minimum 20,000 read pairs per cell for 5' Gene Expression Dual Index library Minimum 5,000 read pairs per cell for Cell Surface Protein Dual Index library
Sequencing Type	Paired-end, Dual indexing
Sequencing Read	Read 1: 26 cycles i7 Index: 10 cycles i5 Index: 10 cycles Read 2: 90 cycles

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Libraries compatible with Illumina sequencers

Consider sequencing depth requirements when pooling libraries

- Recommended sequencing depths
  - V(D)J Amplified Library: 5,000 read pairs per cell
  - 5′ Gene Expression Library: 20,000 read pairs per cell

Library Pooling Examples:

Libraries	Sequencing Depth (read pairs per cell)	Library Pooling Ratio
Example 1		
V(D)J Enriched library	5,000	1
5' Gene Expression library	20,000	4
Example 2		
V(D)J Enriched library	5,000	1
5' Gene Expression library	50,000	10


MiSeq


NextSeq 500/550


HiSeq 2500

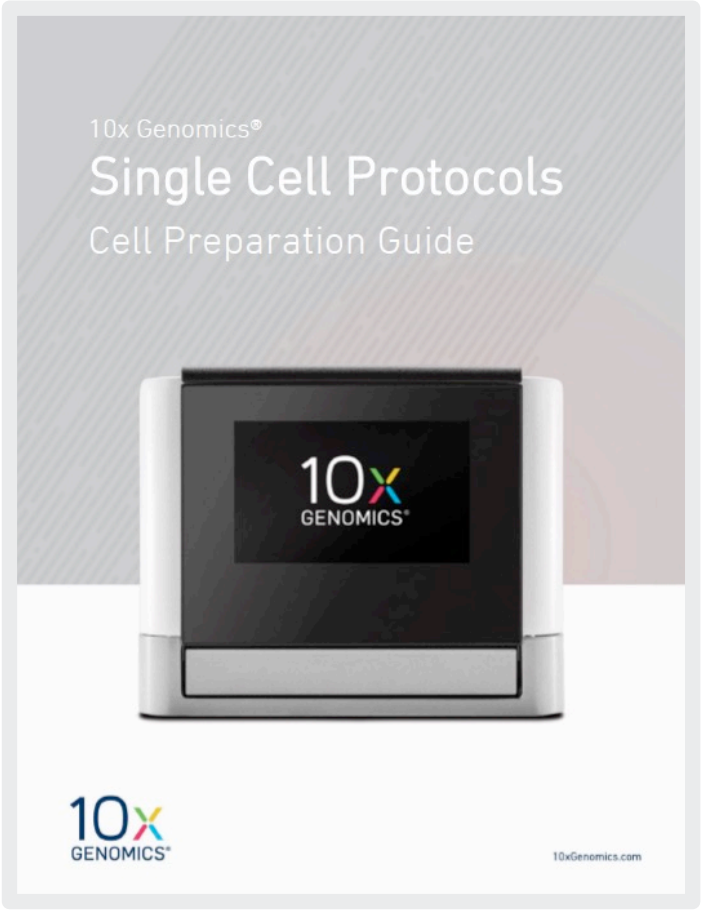

HiSeq 3000/4000


NovaSeq

Sample preparation recommendations

Cell preparation guide

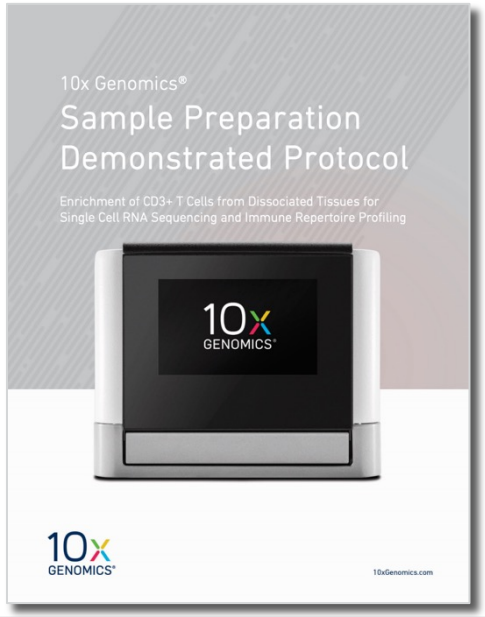
- Available on the 10x Genomics Support website
- Cell Preparation Guide includes best practices and general protocols for:
  - Washing and resuspension
  - Straining
  - Counting
  - Cell concentration
  - Working with limited cell suspensions (less than 100,000 total cells)



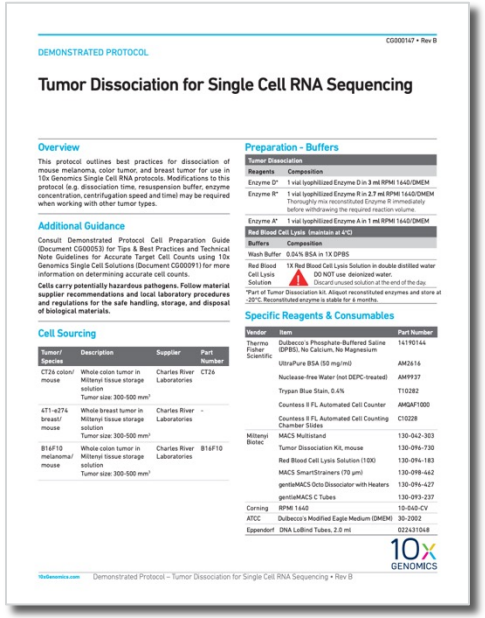
Single Cell Immune Profiling Solution Sample Preparation

Routine use of Demonstrated Protocols – Optimization may be required

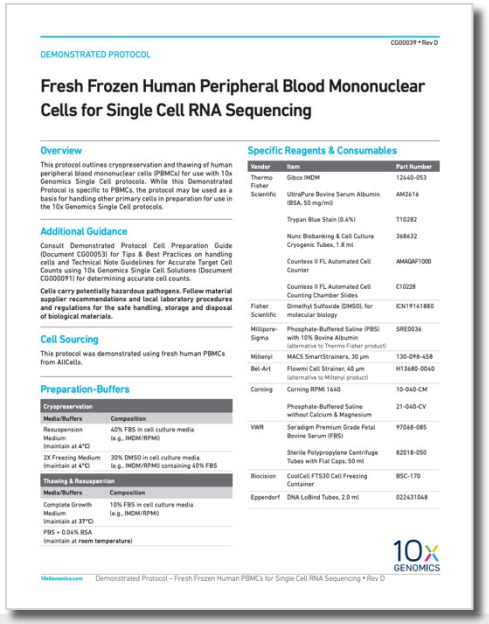
CG000123
Enrichment of CD3+ T Cells from Dissociated Tissues for Single Cell RNA Sequencing and Immune Repertoire Profiling



CG00147
Tumor Dissociation for Single Cell RNA Sequencing



CG00039
Fresh Frozen Human Peripheral Blood Mononuclear Cells for Single Cell RNA Sequencing



Single Cell Immune Profiling Solution

Demonstrated protocol compatibility table

✓ Compatible (tested in-house)
✓* Untested but low risk
✗* Untested but high risk
✗ Not compatible

Protocol	SC5′	SC5' with Feature Barcode technology
Single Cell Protocols – Cell Preparation Guide	✓	✓
Enrichment of CD3+ T Cells from Dissociated Tissues for Single Cell RNA Sequencing and Immune Repertoire Profiling	✓	✓*
Tumor Dissociation for Single Cell RNA Sequencing	✓*	✓*
Single Cell Suspensions from Cultured Cell Lines for Single Cell RNA Sequencing	✓	✓*
Removal of Dead Cells from Single Cell Suspensions for Single Cell RNA Sequencing	✓	✓*
Fresh Frozen Human-Mouse Cell Line Mixtures for Single Cell RNA Sequencing	✓	✓
Fresh Frozen Human Peripheral Blood Mononuclear Cells for Single Cell RNA Sequencing	✓	✓
Cell Surface Protein Labeling for Single Cell RNA Sequencing Protocols	✓	✓
Cell Labeling with Dextramers for Single Cell RNA Sequencing Protocols*	✓	✓
Methanol Fixation of Cells for Single Cell RNA Sequencing	✗*	✗
Isolation of Nuclei for Single Cell RNA Sequencing	✗*	✗

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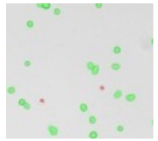
General cell handling recommendations

Analysis of single cell transcriptomes

- Input cell suspensions should contain more than 90% viable cells
- The presence of a high fraction of non-viable or dying cells may decrease recovery
  - The presence of ambient RNA and cellular debris may impact application performance metrics
- V(D)J pairing can be affected by the fraction of T or B cells and V(D)J transcript expression levels

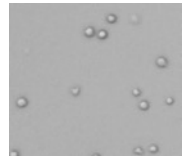
Importance of input cell quality

- Requires a fully dissociated, single cell suspension
- Minimizing the presence of cellular aggregates, dead cells, non-cellular nucleic acids and potential inhibitors of reverse transcription is critical to obtaining high quality data
- Suspension cell lines, bead-enriched and flow-sorted cells can be used directly after washing



Cell handling

- It is important to treat cells gently to minimize cell lysis and loss:
  - When cells lyse, the released ambient mRNA will contaminate other GEMs
  - Wash cells twice using a wide-bore pipette tip to remove ambient RNA and contaminants
  - Wash and resuspend in PBS + 0.04% non-acetylated BSA to minimize cell loss during handling



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General cell handling recommendations

Debris/aggregate removal

- Use a cell strainer to remove aggregates or debris from washed cells
- The presence of cell aggregates, debris and/or fibers can result in:
  - Inaccurate cell counts
  - GEM generation failures
    - GEM generation occurs in microfluidic channels that are narrower than the typical human hair (i.e. < 100 µm)

Cell counting

- Quantitate cells accurately before loading into the system
  - Approximately 65% loaded cells will be recovered
  - To maximize the likelihood of achieving the desired recovery target, the optimal input cell concentration is 700-1200 cells/µl
  - Recommended range: 500 to 10,000 recovered cells
  - Under- or over-loading may impact application performance

Storage of single cell suspensions

- Cell suspensions should always be kept on ice and where possible proceed with cell loading immediately after sample preparation
  - Ideally incubation time should be kept to a minimum (< 30 min)
- Some cell types are more fragile and cell viability may decrease significantly if not processed and loaded immediately

Rough pipetting leads to cell lysis and lower reads in cells

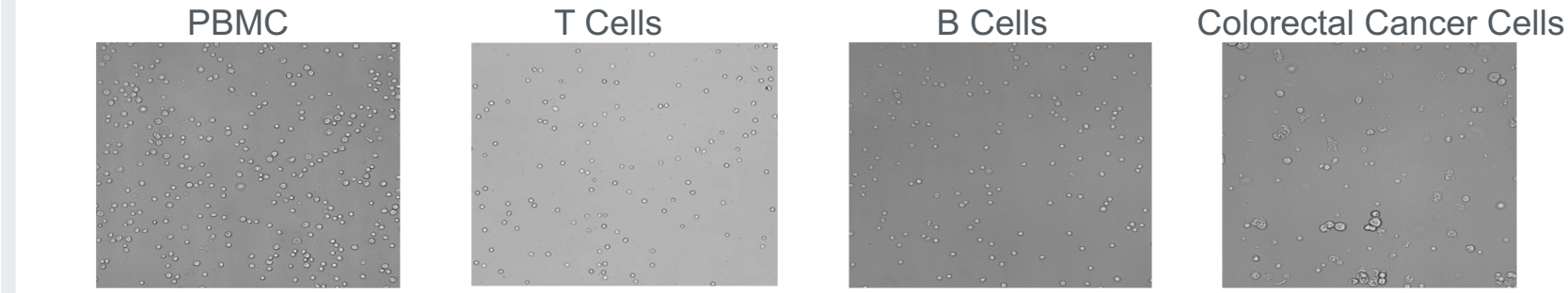
Application performance metrics reflect rough cell handling (SC3′ data)

- If cells are handled too roughly, many will lyse, releasing mRNA into the cell suspension buffer.
- The ambient RNA will be incorporated into the sequenced library, but will not be associated with cell-containing GEMs. This effectively increases the background, while decreasing the Fraction of Reads in Cells.

Metric	Control	Wide Bore (Rough)	Narrow Bore (Rough)	Vortex 5s
Number of Cells	1,118	846	1,012	983
Reads per Cell	50,000	50,000	50,000	50,000
Fraction Reads in Cells	79.40%	72.80%	54.00%	63.10%
Median Genes per Cell	3,137	3,180	2,833	2,934
Median UMI counts per Cell	10,726	11,053	8,832	9,503

Cell types validated by 10x Genomics for immune profiling

Cells Tested	Species	Cell Source
PBMC	Human and Mouse	Extracted from blood
Splenocytes	Mouse	Cryopreserved dissociated spleen
Primary T cells	Human and Mouse	MACS- or FACS-based enrichment of CD3 positive cells from PBMCs
Primary B cells	Human and Mouse	MACS- or FACS-based enrichment of CD19 positive cells from PBMCs
Dissociated tumor cells (10-80% T cells, depending on enrichment strategy)	Human	Cryopreserved dissociated tumor tissue with or without MACS-based CD3+ or CD45+ enrichment
In vitro expanded T cells	Human	Selected from primary T cells using antigen-specific proliferation
T cell Lines	Human and Mouse	Suspension cell culture and cryopreserved cell lines
B cell lines	Human and Mouse	Suspension cell culture and cryopreserved cell lines



Recommended control cells

Peripheral Blood Mononuclear Cells (PBMCs)*

- Human From AllCells (Catalog # PB003F)
  - Cryopreserved, 15 million cells
- Mouse, pooled C57BL/6 from C&M Lab Pro (Catalog # 11121912)
  - Cryopreserved, 1 million cells
- Mouse, pooled BALB/c from C&M Lab Pro (Catalog # 12121912)
  - Cryopreserved, 1 million cells

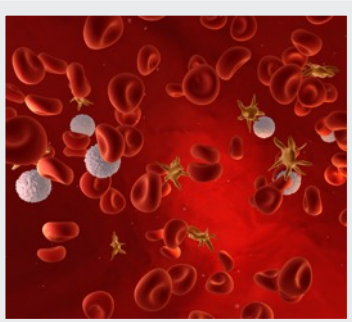
Please review the 10x Genomics – Demonstrated Protocol CG00039 – Fresh Frozen Human Peripheral Blood Mononuclear Cells for Single Cell RNA Sequencing for general guidelines on cell thawing, washing and preparation.

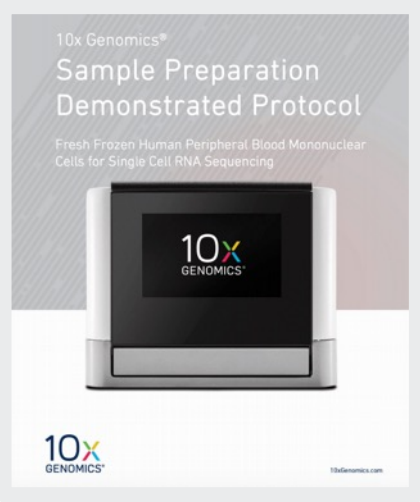
** Observed capture efficiency depends on the fraction of PBMCs that are T Cells and B cells*

Sample types

PBMCs (Peripheral Blood Mononuclear Cells)

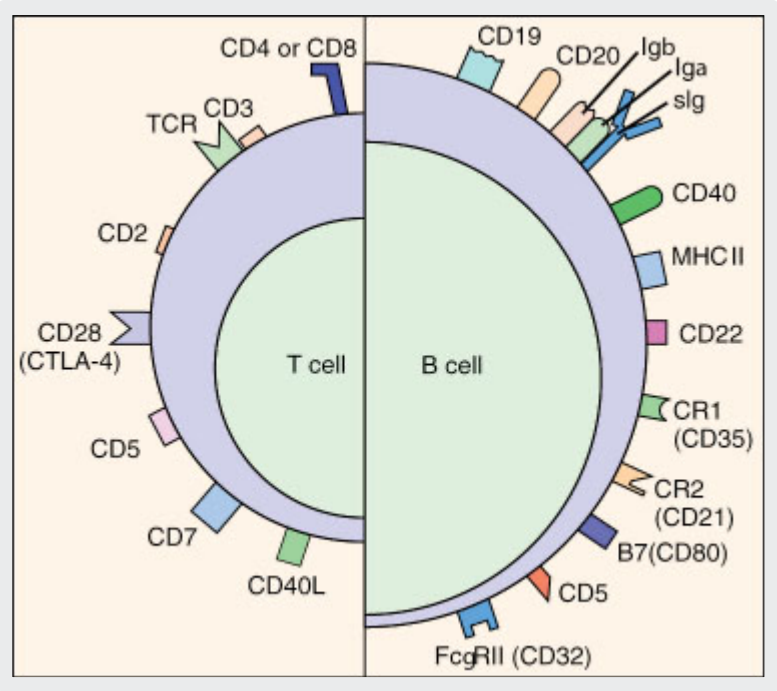
- Optimal freezing conditions for cryopreservation
  - 40 % FBS, 15% DMSO in media (eg IMDM); 1 – 10 million cells per cryotube
- Thawing protocol
  - Rapidly thaw cryovial (37 ºC water bath for 2-3 minutes)
  - Add 1 mL warm media dropwise (1 drop per 5 sec)
  - Sequentially dilute cells with thawing medium, with steps of 1:1 volume additions
  - Critical: dropwise addition of medium allows cells sufficient time for gradual loss of DMSO and therefore prevents osmotic lysis.
  - Wash with medium followed by PBS/BSA





Available enrichment protocols for T or B cells

- Magnetic beads
  - Easysep human T or B cell enrichment kit* (STEMCELL technologies)
  - MACS cell separation with microbeads** (MACS miltenyi biotec)
  - Magnisort™ human T or B cell enrichment kit* (thermo fisher scientific)
- Column based
  - T cell enrichment columns (R&D systems)*
- Fluorescence-activated cell sorting (FACS)*
- Any enrichment protocol that does not block the cell surface markers that are targeted in the feature barcoding workflow should be compatible

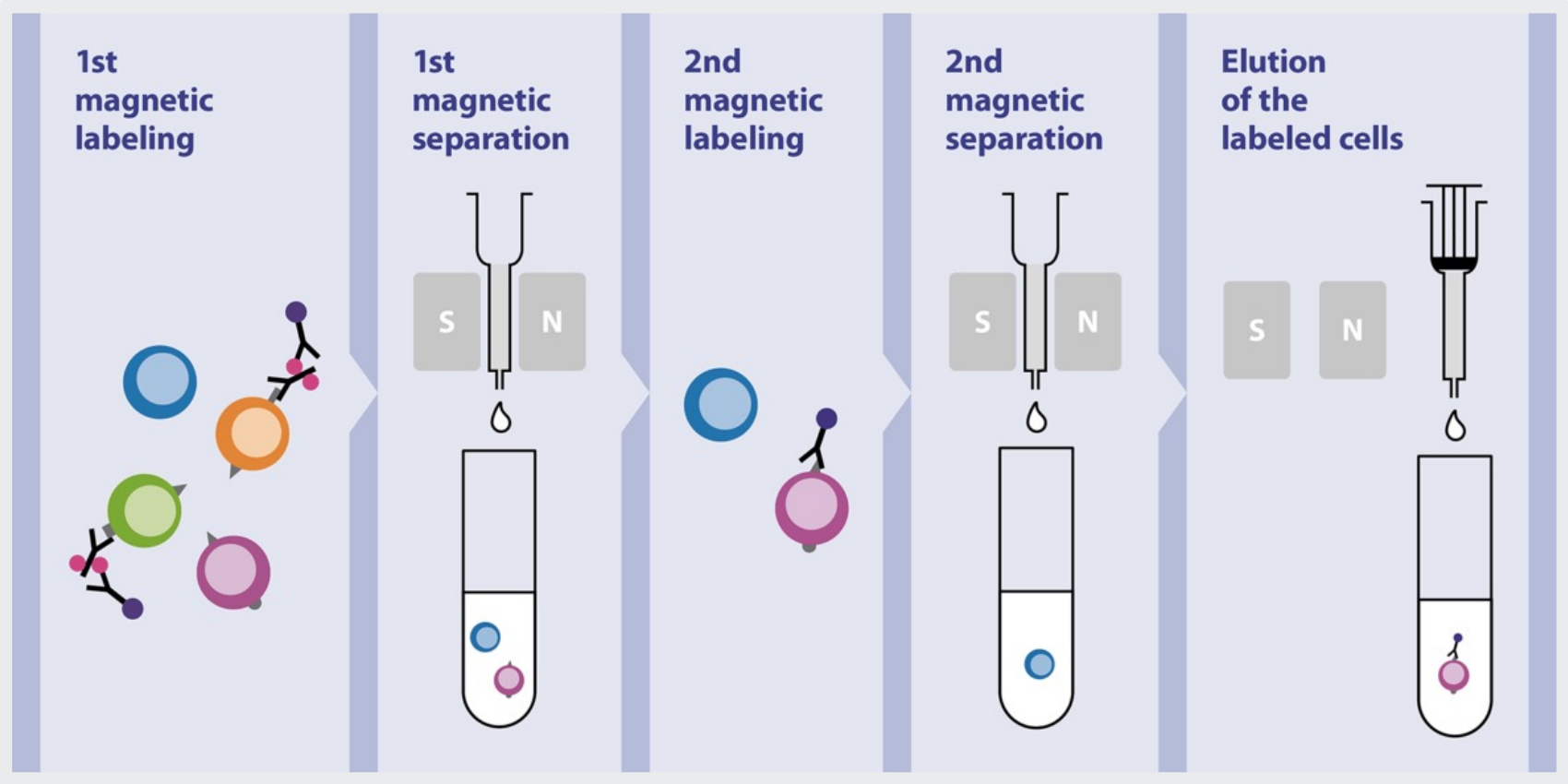


Medical dictionary. 2011.

** Protocols have not been tested by 10x Genomics for the Single Cell Immune Profiling Solution*

*** Magnetic MicroBeads are small (~50nm) and do not interfere during GEM generation.*

Considerations for enrichment of T or B cells



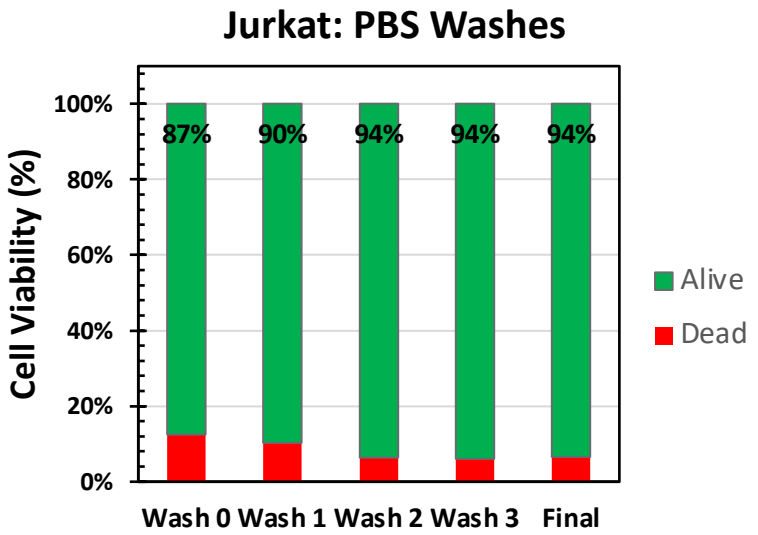
Miltenyi.com

- Any enrichment protocol that does not block the cell surface markers that are targeted in the Feature Barcode workflow should be compatible

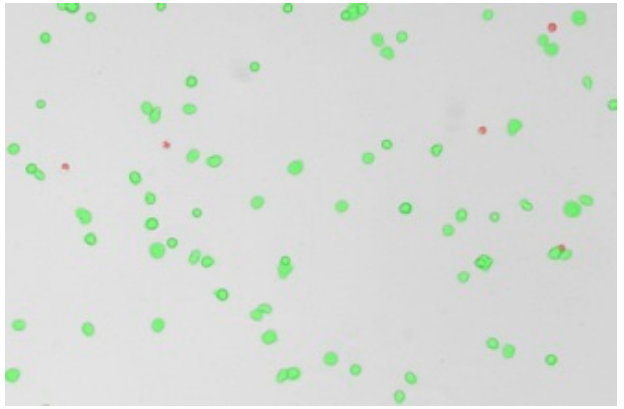
Cell Washing

Washing isolated cells

- Transfer cells in media to a 2 mL Eppendorf tube
- Spin down cells to form pellet
  - Depending on cell size and concentration, pellet size varies
- Remove supernatant
- Gently add 1x PBS + 0.04% BSA away from cell pellet
- Gently pipette mix with Wide Bore pipette tip
- Repeat the wash one more time
- Spin down cells to form pellet
- Remove supernatant
- Resuspend cells in 1x PBS + 0.04% BSA with gentle pipette mix
  - **For accurate cell counting, do not invert tubes**
- Adjust to desired cell concentration



Live/Dead Staining of Final Suspension



***Note**: PBS can be replaced with most common cell culture buffers and media if cells are unstable in PBS*

Recommendations for limited samples

Samples with low starting numbers of cells

- If using FACS, directly sort cells into the optimal media for the cell type
- If necessary, strain cells when cells are still dilute and in media
- Count cells before washing to estimate the approximate cell number
  - This step will minimize cells lost to counting
  - This step will allow one to add appropriate volume of PBS for resuspension to achieve a target concentration
- Spin down cells in 2mL Round bottom LoBind tubes
  - Inefficient centrifugation may lead to further loss of cells
  - Smaller cells: use higher speed and longer time for centrifugation
  - Important: know the expected position of the pellet as pellet may be invisible to naked eyes
- Washing may be skipped if the number of available cells are very small
  - Recommend washing cells once
  - Centrifuge once, remove supernatant but ~ 50 µl, and resuspend cells in the leftover supernatant
  - Important to remove residual Mg^{2+} and EDTA. A 2-fold change up or down in Mg2+ concentration will affect the efficiency of the RT step

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Factors influencing cell recovery

- Cell viability
  - The presence of a high fraction of non-viable cells may decrease cell recovery.
- Cell counting
  - Overestimation of cell concentration decreases the recovered cell number.
- Time that cells left on ice before loading into the chip
  - Some cells such as PBMC form clumps that cannot be suspended back into single cell when they are kept in PBS for a prolonged period of time (e.g. 2 or more hours). This lowers the effective concentration of suspended cells.
  - Depending on cell type, cell viability may decrease significantly.
- Cell type
  - Depends on the “stickiness” of cells.
  - Depends on the fraction of T or B cells in the sample.
- Pipetting volume of cell
  - e.g. 2.5 µl vs 15 µl may result in greater variance.
- User’s pipetting technique
  - This may impact the number of cells recovered.

Technical Note available on the 10x Genomics support website

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Alternative buffer and media tested in-house

Buffer and media tested with Single Cell 3′ v2 and expected to be compatible with the Immune Profiling Solution

- If cells are unstable in PBS, then most common cell culture buffers and media can be used as a replacement so long as it does not contain Mg^{2+} or EDTA.
  - Tested input volume: 2.5 µl and 33 µl
- Alternative Buffer*: no influence on performance
  - Dulbecco's Phosphate-Buffered Saline (DPBS)
  - Hank's Balanced Salt Solution (HBSS)
- Alternative Media*: minimal reduction or no loss in performance
  - Eagle's Minimum Essential Medium (EMEM) + 10% FBS
  - Dulbecco's Modified Eagle Medium (DMEM) + 10% FBS
  - Iscove's Modified Eagle Medium (IMEM) + 10% FBS
  - Roswell Park Memorial Institute (RPMI) + 10% FBS
  - Ham's F12 + 10% FBS
  - 1:1 DMEM/F12 +10% FBS
  - M199

**Note: these buffers have not been tested for compatibility with Cell Surface Protein profiling.*

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Debris removal

Filtering cell suspensions

- Strainers with appropriate pore sizes should be used to allow cells to pass through the filter while cellular debris and aggregates are retained

Pro: required sample volume is low

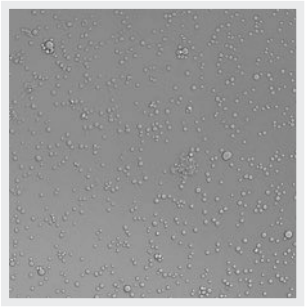
Con: sample concentration is decreased by 20 to 40% after straining

Flowmi™ Cell Strainer (Bel-Art Products)

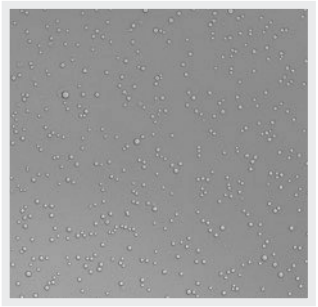
Pro: minimal change in sample concentration

Con: required minimal volume is ~ 500 µL + loss of sample by 100 -150 µL

MACS® SmartStrainer (Miltenyi Biotec)



Unfiltered Jurkat cells



Jurkat cells filtered with FlowMi (40 µm)

Jurkat cells filtered with MACS (30 µm)

Chromium Single Cell 5’ v2 workflow

Protocol steps & timing

- Entire workflow can be completed in less than 2 days
- Numerous safe stopping points throughout the protocol

Protocol Steps & Timing

	Steps	Timing	Stop & Store
3 h	**Cell Preparation and Labeling** Dependent on cell type and labeling protocol used	~1-2 h	
	Step 1 – GEM Generation & Barcoding		
	1.1 Prepare Reaction Mix	20 min	
	1.2 Load Chromium Next GEM Chip G	10 min	
	1.3 Run the Chromium Controller	18 min	
	1.4 Transfer GEMs	3 min	
	1.5 GEM-RT Incubation	55 min STOP	4°C ≤72 h or –20°C ≤1 week
	Step 2 – Post GEM RT Cleanup, cDNA Amplification & QC		
6 h	2.1 Post GEM-RT Cleanup – Dynabead	45 min	
	2.2 cDNA Amplification	50 min STOP	4°C ≤72 h or –20°C ≤1 week
	2.3 cDNA Cleanup		
	2.3A Pellet Cleanup	15 min STOP	4°C ≤72 h or –20°C ≤1 week
	2.3B Supernatant Cleanup	20 min STOP	4°C ≤72 h or –20°C ≤1 week
	2.4 cDNA Quantification & QC	50 min	
	Step 3 – V(D)J Amplification from cDNA		
	3.1 V(D)J Amplification 1	40 min STOP	4°C ≤72 h
	3.2 Post V(D)J Amplification 1 Double Sided Size Selection – SPRIselect	20 min STOP	4°C ≤72 h or –20°C ≤1 week
	3.3 V(D)J Amplification 2	40 min STOP	4°C ≤72 h
	3.4 Post V(D)J Amplification 2 Double Sided Size Selection – SPRIselect	30 min STOP	4°C ≤72 h or –20°C ≤1 week
	3.5 Post V(D)J Amplification QC & Quantification	50 min	
10 h plus* *Time dependent on Stop options used.	**Step 4 – V(D)J Library Construction**		
	4.1 Fragmentation, End Repair & A-tailing	45 min	
	4.2 Adaptor Ligation	25 min	
	4.3 Post Ligation Cleanup – SPRIselect	20 min	
	4.4 Sample Index PCR	40 min STOP	4°C ≤72 h
	4.5 Post Sample Index PCR Cleanup – SPRIselect	20 min STOP	4°C ≤72 h or –20°C long-term
	4.6 Post Library Construction QC	50 min	
	Step 5 – 5′ Gene Expression (GEX) Library Construction		
	5.1 GEX Fragmentation, End Repair & A-tailing	45 min	
	5.2 GEX Post Fragmentation, End Repair & A-tailing Double Sided Size Selection – SPRIselect	30 min	
	5.3 GEX Adaptor Ligation	25 min	
	5.4 GEX Post Ligation Cleanup – SPRIselect	20 min	
	5.5 GEX Sample Index PCR	40 min STOP	4°C ≤72 h
	5.6 GEX Post Sample Index PCR Double Sided Cleanup – SPRIselect	30 min STOP	4°C ≤72 h or –20°C long-term
	5.7 GEX Post Library Construction QC	50 min	
	Step 6 – Cell Surface Protein Library Construction		
	6.1 Sample Index PCR	30 min	
	6.2 Post Sample Index PCR Size Selection – SPRIselect	20 min STOP	4°C ≤72 h or –20°C long-term
	6.3 Post Library Construction QC	50 min	

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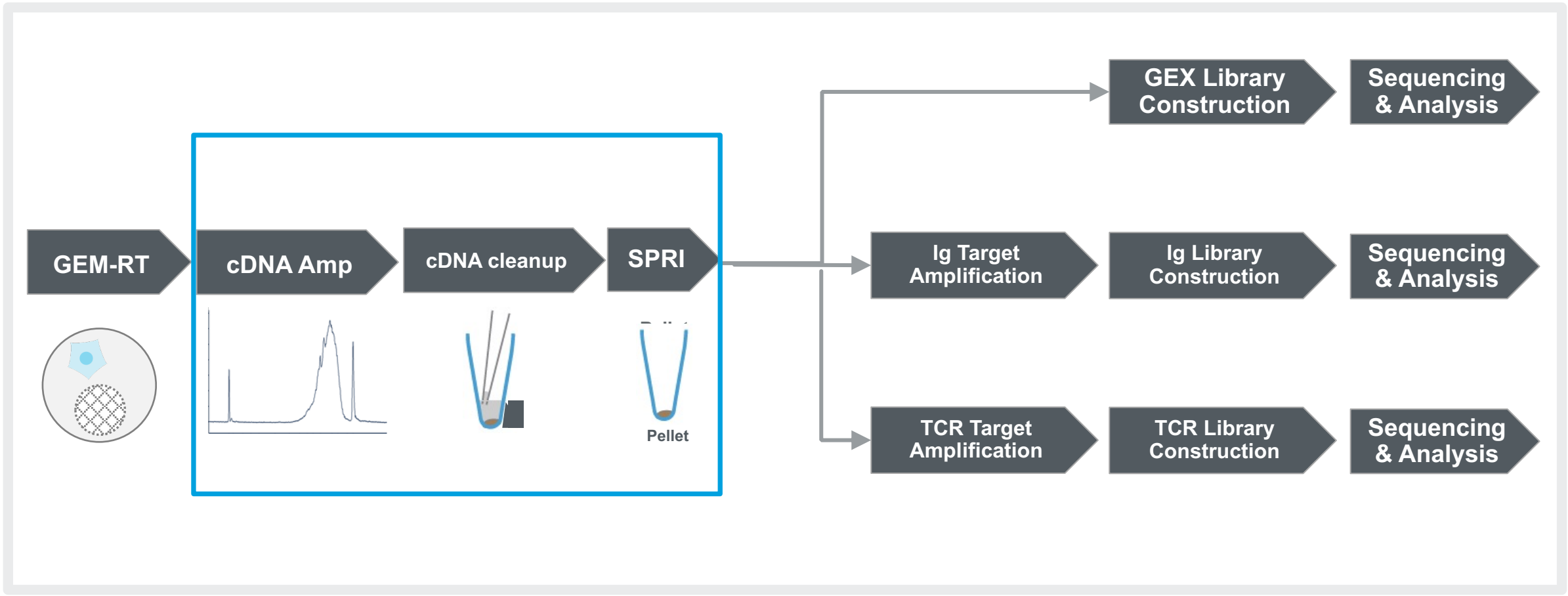
Single Cell 5’ v2 workflow summary



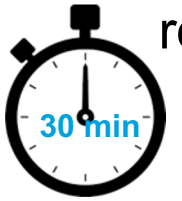
Note: TCR and Ig libraries require independent library constructions.

Chromium Single Cell 5’ v2 workflow

GEM generation, barcoding and
Post-GEM RT cleanup

Getting Started: Equilibrate Reagents

From -80 °C storage – Place at room temperature:



Remove the **Single Cell VDJ 5' Gel Beads** from -80 °C storage and equilibrate to room temperature for **30 min.**

Failure to equilibrate Gel Beads for 30 minutes will lead to run failure.

From -20 °C storage – Place at room temperature:

Remove the:

- **RT Reagent B (blue cap)**
- **Reducing Agent B (white cap)**
- **Poly-dT RT Primer (blue cap)**

from the **Next GEM Single Cell 5' GEM Kit v2** stored at -20 °C and equilibrate to room temperature for at least **10 min.**

From -20 °C storage – Place on ice:

Remove the:

- **RT Enzyme C (white cap)** from the **Next GEM Single Cell 5' GEM Kit v2** stored at -20°C and place on ice.

From room temperature storage:

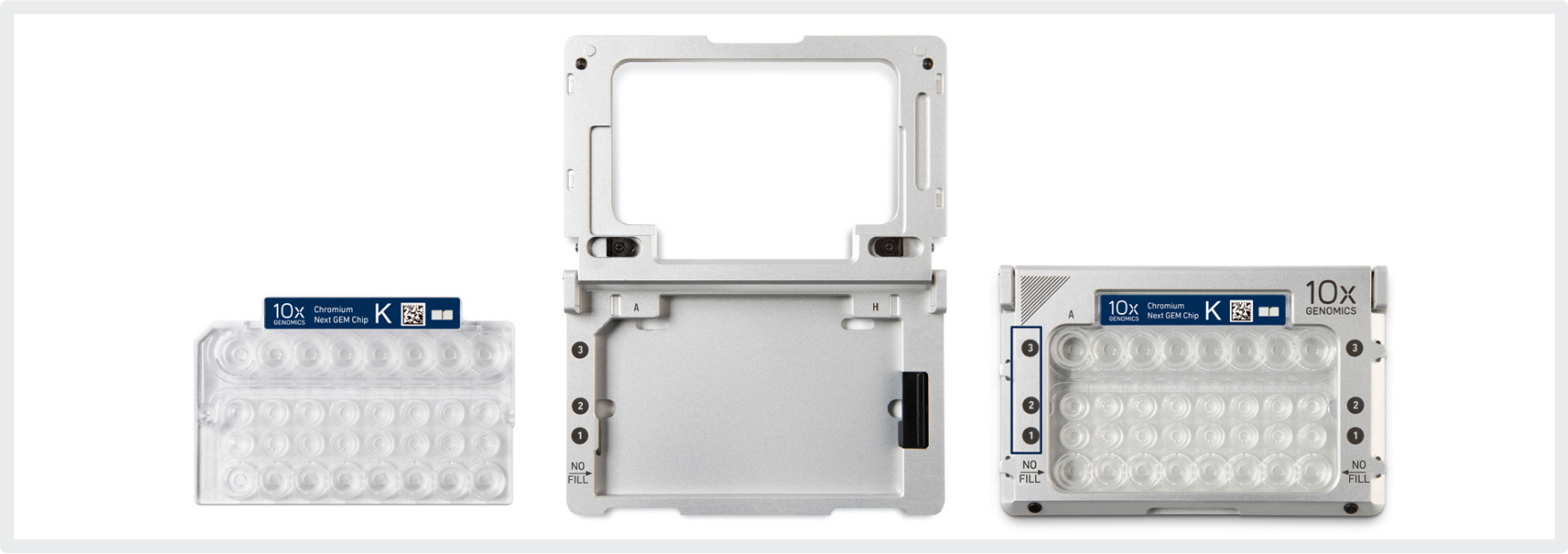
Remove the:

- **Partitioning Oil (clear cap)**
- **Next GEM Chip K, Gasket, Chip Holder** from room temperature storage.

Single Cell 5’ v2 workflow

Assemble Chromium Next GEM Chip K in a Chromium Next GEM Chip Holder

- Chips come packaged in mylar bags containing desiccant packet
  - Chip should be run within 24 hrs of opening the mylar bag
  - Shelf life if unopened: one year from date of packaging
- Insert the Chromium Next GEM Chip K into the Chromium Next GEM Chip Holder



Single Cell 5’ v2 workflow

Assemble Chromium Next GEM Chip K in a Chromium Next GEM Chip Holder

Single Cell 5’ v2 workflow

Chip loading considerations: unused channels must be loaded with 50% glycerol

- If running less than 8 samples per chip, dispense 50% Glycerol into the unused wells.
  - 70 µl to unused wells in Row 1
  - 50 µl to unused wells in Row 2
  - 45 µl to unused wells in Row 3
  - This should be done prior to adding cells to the aliquoted Master Mix.
- Failure to add glycerol to unused wells can lead to sample loss and may damage the Controller
- Do NOT add glycerol to the bottom row of NO FILL wells
- Do NOT use any substitute for 50% glycerol solution

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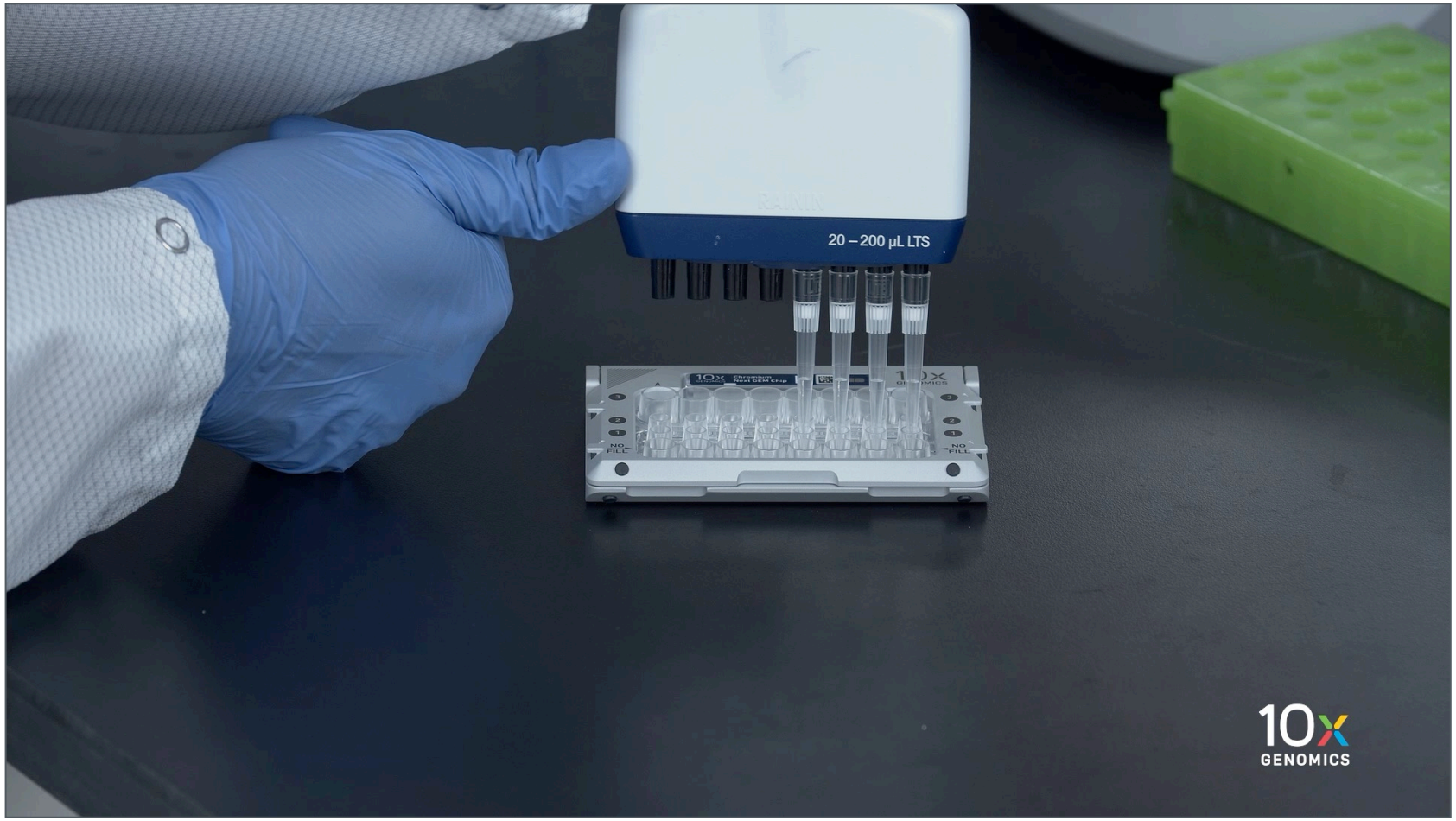
Single Cell 5’ v2 workflow

Chip loading considerations: unused channels must be loaded with 50% glycerol



Single Cell 5’ v2 workflow

Determine targeted number of cells

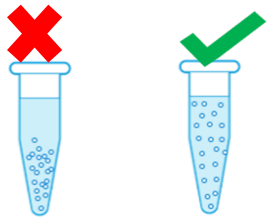
- Cell stock concentration is based on total cell count
- Observed cell recovery will decrease if sample contains non-viable cells or cells not expressing TCRs or Igs
- For Example: Targeting 5000 cells with a cell concentration of 1000 cells/µl:
  - Add 30.4 µl water to Master Mix aliquot
  - Add 8.3 µl cell suspension to combined Master Mix + water in strip tube
- Remember to resuspend the cells gently whenever taking aliquots to count or add to Master Mix

Cell Suspension Volume Calculator Table

(for step 1.2 of Chromium Next GEM Single Cell 5' v2 (Dual Index) protocol)

Volume of Cell Suspension Stock per reaction (µl) | Volume of Nuclease-free Water per reaction (µl)

Cell Stock Concentration (Cells/µl)	Targeted Cell Recovery										
	500	1000	2000	3000	4000	5000	6000	7000	8000	9000	10000
100	8.3 30.4	16.5 22.2	33.0 5.7	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
200	4.1 34.6	8.3 30.4	16.5 22.2	24.8 13.9	33.0 5.7	n/a	n/a	n/a	n/a	n/a	n/a
300	2.8 35.9	5.5 33.2	11.0 27.7	16.5 22.2	22.0 16.7	27.5 11.2	33.0 5.7	n/a	n/a	n/a	n/a
400	2.1 36.6	4.1 34.6	8.3 30.5	12.4 26.3	16.5 22.2	20.6 18.1	24.8 13.9	28.9 9.8	33.0 5.7	n/a	n/a
500	1.7 37.0	3.3 35.4	6.6 32.1	9.9 28.8	13.2 25.5	16.5 22.2	19.8 18.9	23.1 15.6	26.4 12.3	29.7 9.0	33.0 5.7
600	1.4 37.3	2.8 35.9	5.5 33.2	8.3 30.5	11.0 27.7	13.8 24.9	16.5 22.2	19.3 19.4	22.0 16.7	24.8 13.9	27.5 11.2
700	1.2 37.5	2.4 36.3	4.7 34.0	7.1 31.6	9.4 29.3	11.8 26.9	14.1 24.6	16.5 22.2	18.9 19.8	21.2 17.5	23.6 15.1
800	1.0 37.7	2.1 36.6	4.1 34.6	6.2 32.5	8.3 30.4	10.3 28.4	12.4 26.3	14.4 24.3	16.5 22.2	18.6 20.1	20.6 18.1
900	0.9 37.8	1.8 36.9	3.7 35.0	5.5 33.2	7.3 31.4	9.2 29.5	11.0 27.7	12.8 25.9	14.7 24.0	16.5 22.2	18.3 20.4
1000	0.8 37.9	1.7 37.0	3.3 35.4	5.0 33.7	6.6 32.1	8.3 30.4	9.9 28.8	11.6 27.1	13.2 25.5	14.9 23.8	16.5 22.2
1100	0.8 37.9	1.5 37.2	3.0 35.7	4.5 34.2	6.0 32.7	7.5 31.2	9.0 29.7	10.5 28.2	12.0 26.7	13.5 25.2	15.0 23.7
1200	0.7 38.0	1.4 37.3	2.8 35.9	4.1 34.6	5.5 33.2	6.9 31.8	8.3 30.4	9.6 29.1	11.0 27.7	12.4 26.3	13.8 24.9
1300	0.6 38.1	1.3 37.4	2.5 36.2	3.8 34.9	5.1 33.6	6.3 32.4	7.6 31.1	8.9 29.8	10.2 28.5	11.4 27.3	12.7 26.0
1400	0.6 38.1	1.2 37.5	2.4 36.3	3.5 35.2	4.7 34.0	5.9 32.8	7.1 31.6	8.3 30.4	9.4 29.3	10.6 28.1	11.8 26.9
1500	0.6 38.1	1.1 37.6	2.2 36.5	3.3 35.4	4.4 34.3	5.5 33.2	6.6 32.1	7.7 31.0	8.8 29.9	9.9 28.8	11.0 27.7
1600	0.5 38.2	1.0 37.7	2.1 36.6	3.1 35.6	4.1 34.6	5.2 33.5	6.2 32.5	7.2 31.5	8.3 30.4	9.3 29.4	10.3 28.4
1700	0.5 38.2	1.0 37.7	1.9 36.8	2.9 35.8	3.9 34.8	4.9 33.8	5.8 32.9	6.8 31.9	7.8 30.9	8.7 30.0	9.7 29.0
1800	0.5 38.2	0.9 37.8	1.8 36.9	2.8 35.9	3.7 35.0	4.6 34.1	5.5 33.2	6.4 32.3	7.3 31.4	8.3 30.5	9.2 29.5
1900	0.4 38.3	0.9 37.8	1.7 37.0	2.6 36.1	3.5 35.2	4.3 34.4	5.2 33.5	6.1 32.6	6.9 31.8	7.8 30.9	8.7 30.0
2000	0.4 38.3	0.8 37.9	1.7 37.0	2.5 36.2	3.3 35.4	4.1 34.6	5.0 33.7	5.8 32.9	6.6 32.1	7.4 31.3	8.3 30.4



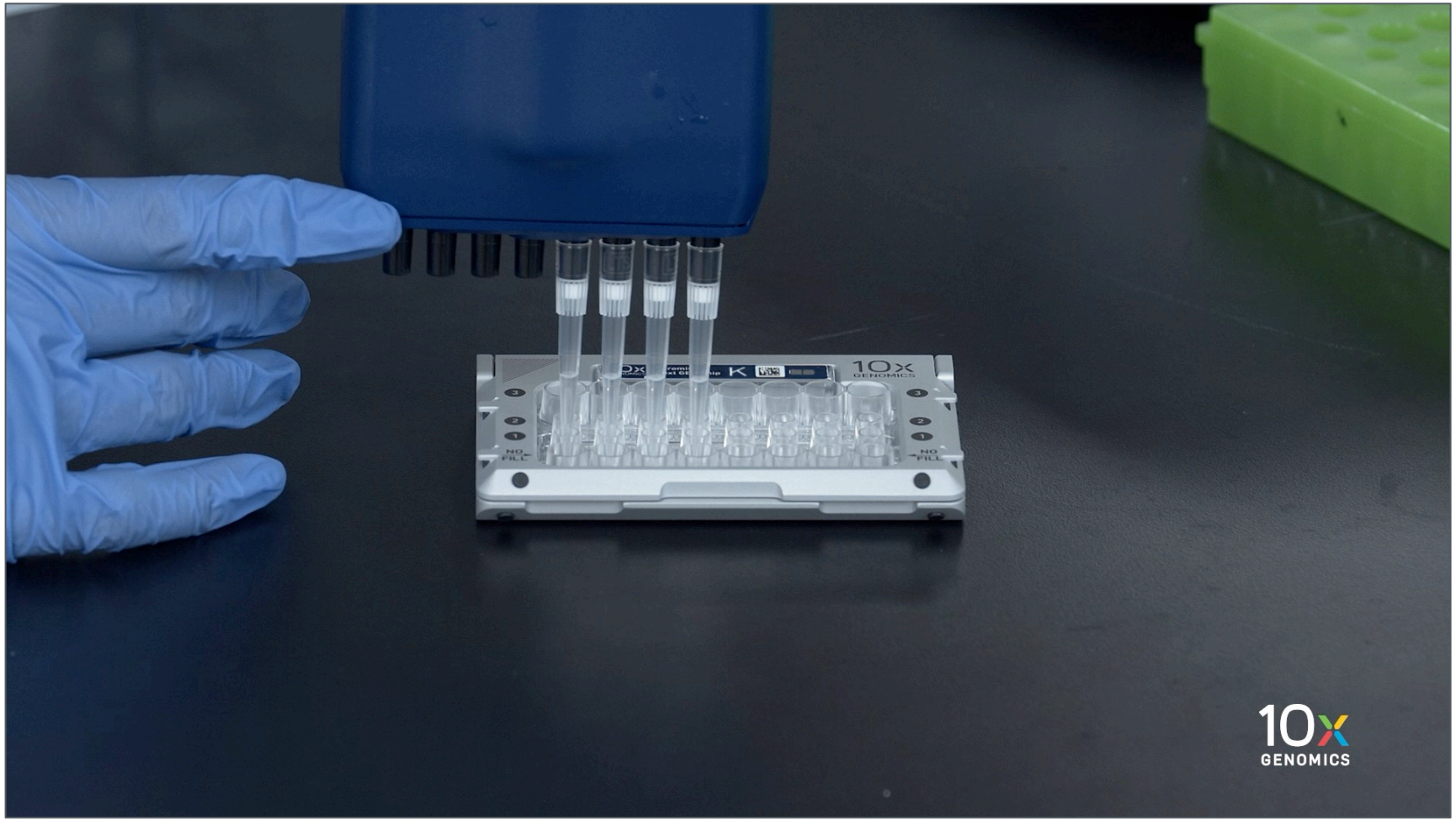
Single Cell 5’ v2 Workflow: Loading Chip K

Load sample into row 1

- Prepare Master Mix and aliquot into strip tube
- Add water and cell suspension for each sample to Master Mix aliquot(s)
- Gently pipette mix Master Mix + water + cell suspension and load 70 µl into Row labeled 1
- Proceed to prepare Gel Beads

Single Cell 5’ v2 Workflow: Loading Chip K

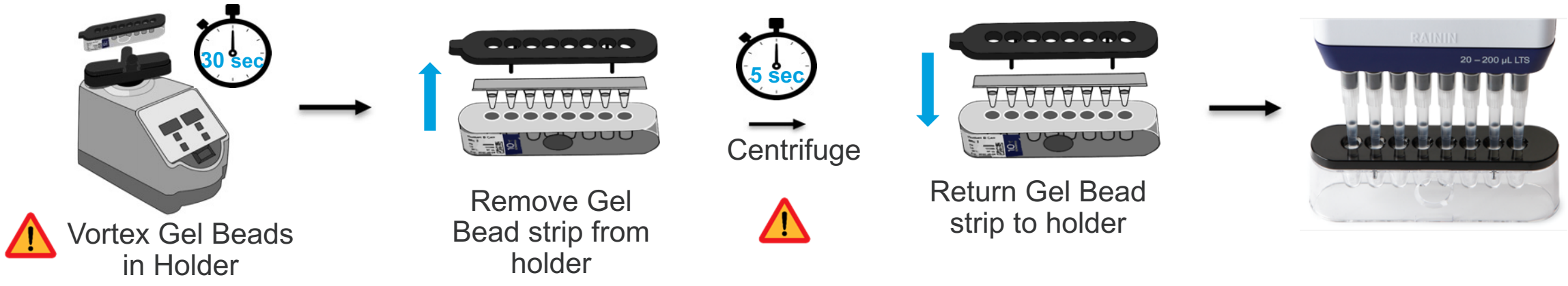
Load sample into row 1



Single Cell 5’ v2 Workflow: Loading Chip K

Preparing Gel Beads

- Snap the tube strip holder with the Gel Bead strip into a 10x Genomics Vortex Adapter
- After loading Row 1 with Master Mix and cells, simultaneously prime row 1 microfluidic channels, while vortexing Gel Beads for 30 sec at highest speed
- Remove the Gel Bead strip from the holder and centrifuge for ~5 sec. Confirm there are no bubbles at the bottom of the tubes and the liquid levels are even. Place the Gel Bead strip back in the holder. Secure the holder lid.
- Pierce foil and very slowly aspirate Gel Beads and dispense into row 2, avoiding bubbles



Single Cell 5’ v2 Workflow: Loading Chip K

Preparing Gel Beads

Single Cell 5’ v2 Workflow: Loading Chip K

Load Gel Beads into row 2

- Pierce foil seal on the required number of Gel Bead tubes
- Slowly aspirate 50 µl and dispense into row labeled 2
- **Wait 30 seconds** before proceeding to loading partitioning oil.

Single Cell 5’ v2 Workflow: Loading Chip K

Load Gel Beads into row 2

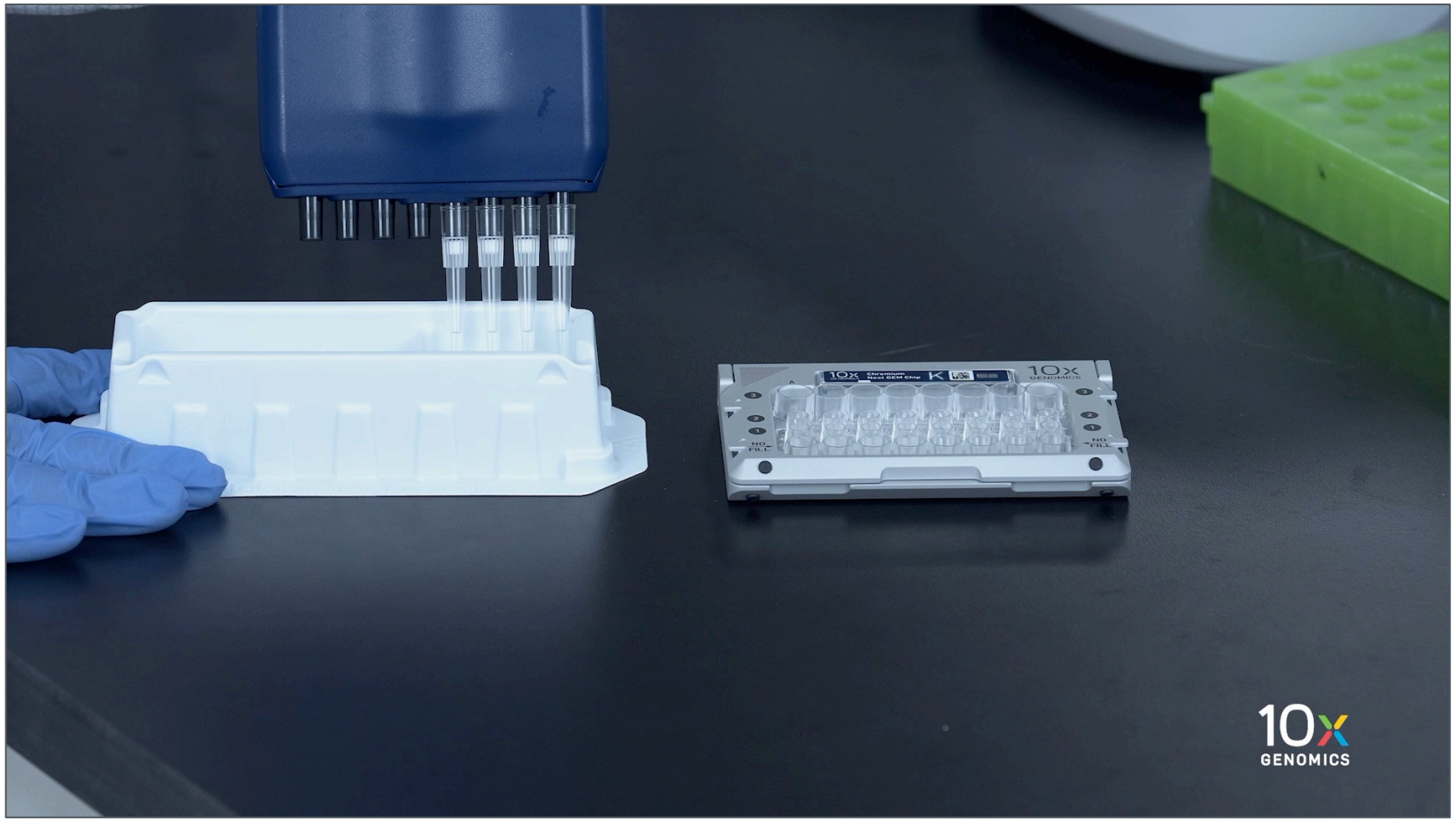
Single Cell 5’ v2 Workflow: Loading Chip K

Load partitioning oil into recovery wells (row 3)

- Load 45 µl partitioning oil from a reservoir into row labeled 3

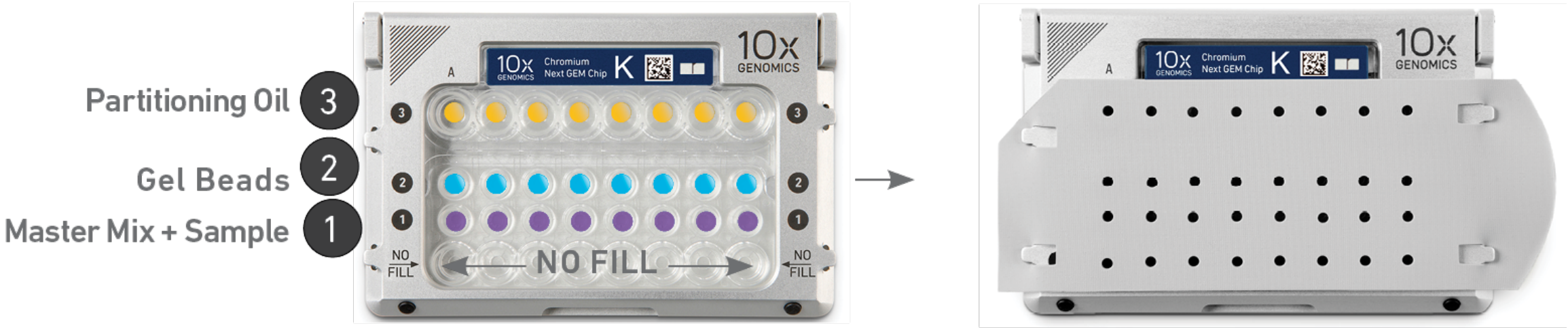
Single Cell 5’ v2 Workflow: Loading Chip K

Load partitioning oil into recovery wells (row 3)



Single Cell 5’ v2 Workflow: Loading Chip K

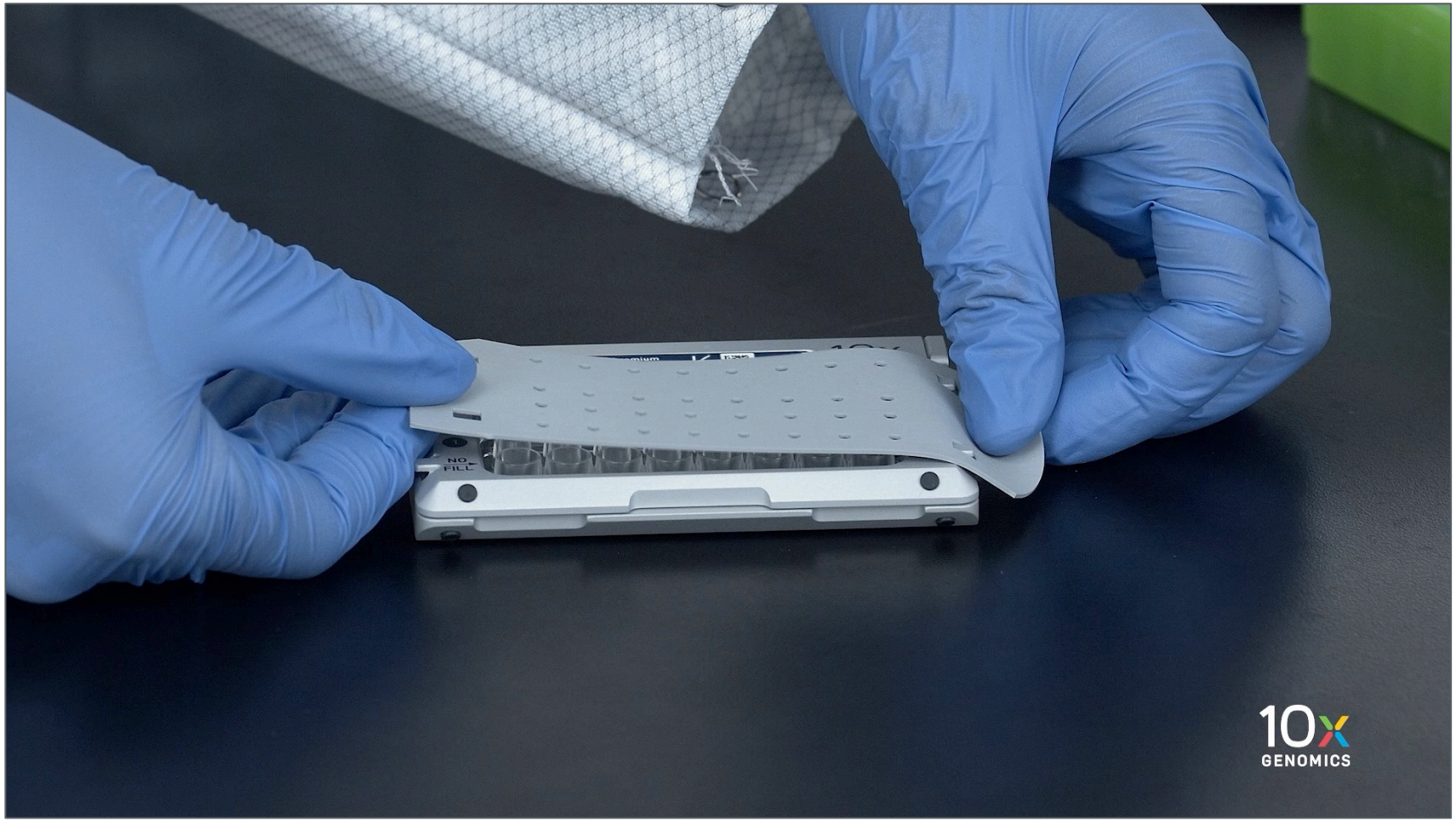
Attach gasket



Keep horizontal to avoid wetting the gasket. DO NOT press down on the gasket.

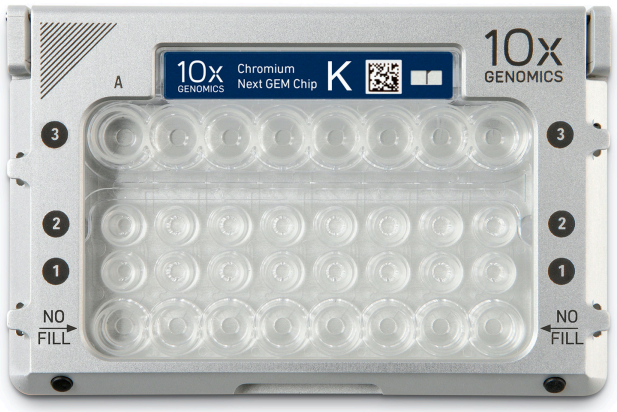
Single Cell 5’ v2 Workflow: Loading Chip K

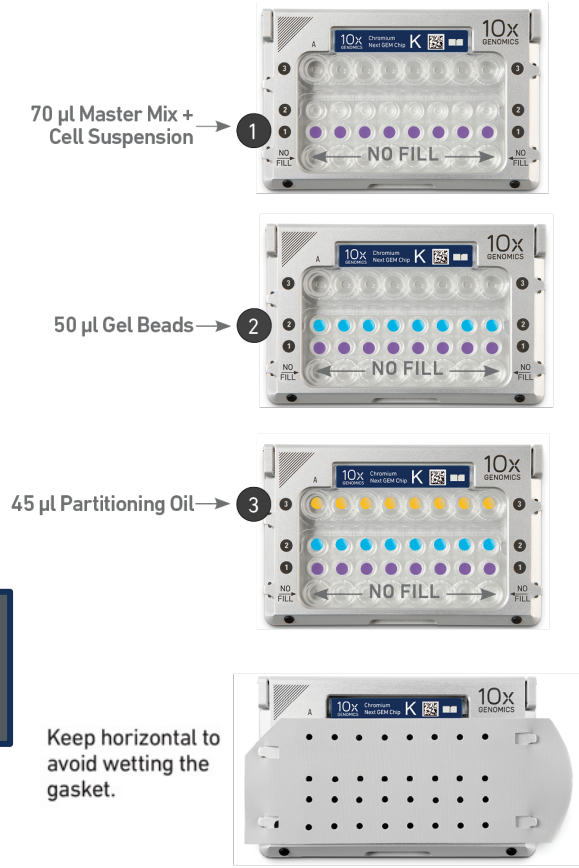
Attach gasket



Single Cell 5’ v2 workflow

Chip K loading summary





Firmware 4.00 or higher is required for running this assay

- Assemble a chip into a Chip holder and load all unused wells in rows 1, 2 & 3 with 50% Glycerol
- Prepare and aliquot Master Mix
  - Add water to Master Mix
  - Add cells to Master Mix
    - **Gently resuspend each cell sample immediately before adding to master mix
- Load Chromium Chip K:
  - Load Row 1: 70 µl Cells and Master Mix
  - Prime Chip and Vortex Gel Beads for 30 seconds
  - Load Row 2: 50 µl Gel beads and wait 30 seconds
  - Load Row 3: 45 µl Partitioning Oil
  - DO NOT load reagents in the bottom row labeled NO FILL
- Attach 10x Gasket
- Run on Chromium Controller immediately after loading the Partitioning Oil (Run time ~18 mins)

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Updates for Users Transitioning to Next GEM Chemistry

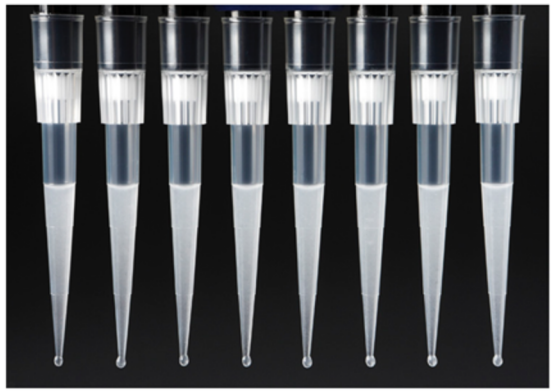
Generating GEMs

- Chips should be run immediately after loading
- **Chip runtime is ~18 minutes**
- Recover emulsions immediately after run is complete
- After transferring to a strip tube on ice, GEMs may be stored on ice for up to an hour before beginning the GEM-RT reaction

Single Cell 5’ v2 workflow

Recovering GEMs

Expose wells at 45 Degrees



GEMs should appear opaque and uniform across all channels

Single Cell 5’ v2 workflow

Recovering GEMs

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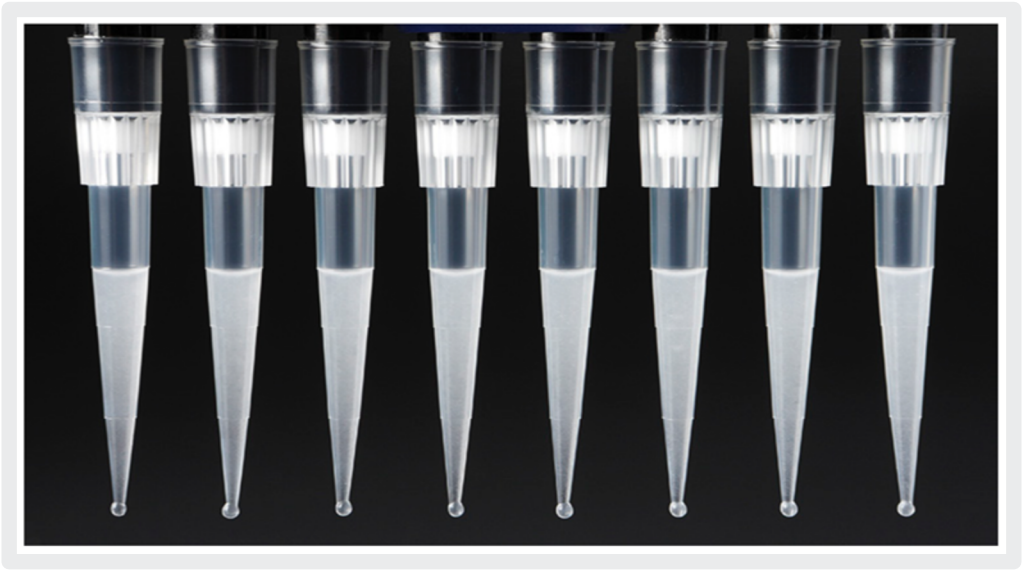
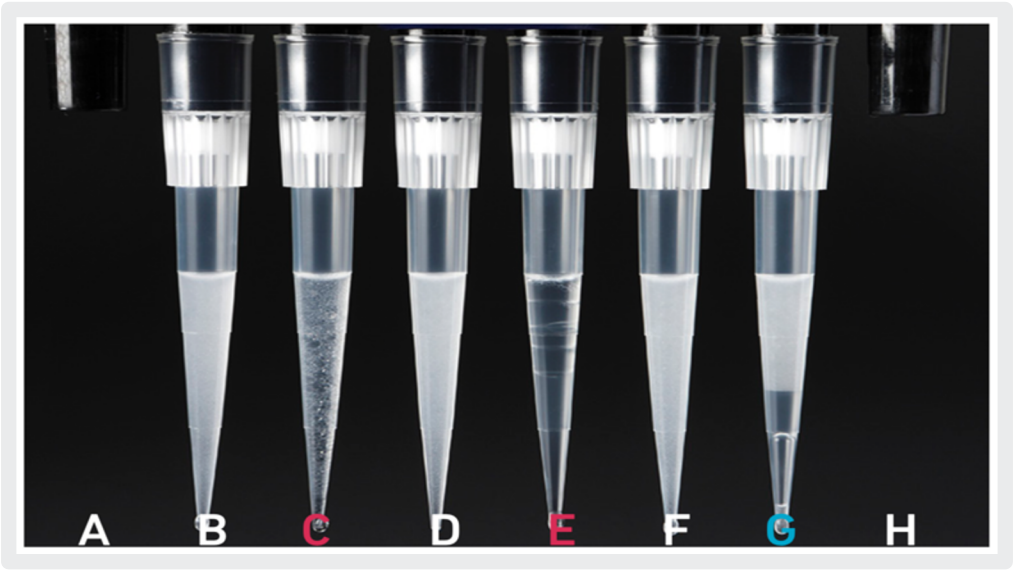
Single Cell 5’ v2 workflow

Visually inspect GEMs to confirm the presence of a uniform emulsion



All liquid levels are similar in volume and opacity without air trapped in the pipette tips.



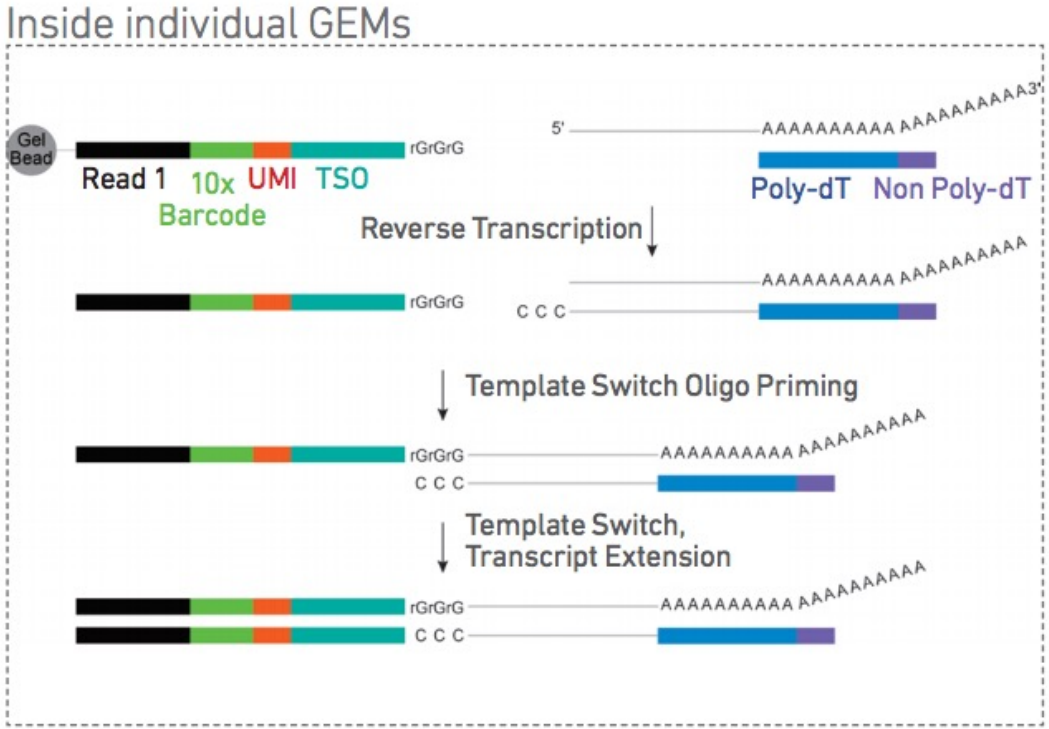
Pipette tips C and E indicate a wetting failure. Pipette tip C contains partially emulsified GEMs. Emulsion is absent in pipette tip E. Pipette tip G indicates a reagent clog.

Single Cell 5’ v2 workflow

GEM RT incubation

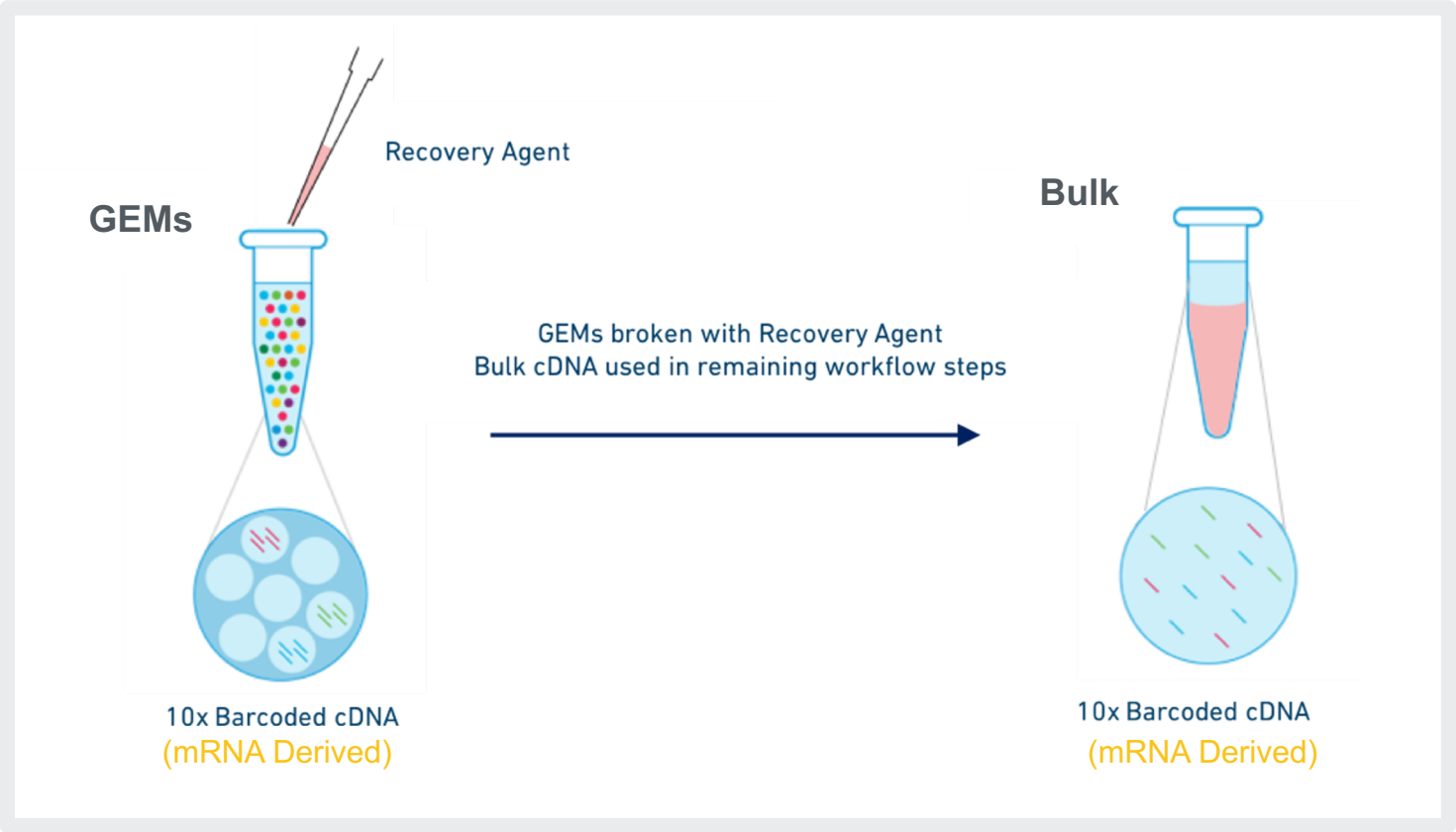
- Transfer emulsions to an approved plastic strip tube and incubate to generate first-strand cDNA
- After incubation, GEMs may be held at 4°C up to 72 h or at -20°C for up to one week.

Lid Temperature	Reaction Volume	Run Time
53°C	125 µl	~55 min
Step	**Temperature**	**Time**
1	53°C	00:45:00
2	85°C	00:05:00
3	4°C	Hold



Single Cell 5’ v2 workflow

Breaking GEMs



- Wait 2 minutes after adding Recovery Agent to each sample
- If biphasic separation is incomplete: firmly cap strip tubes, gently invert 5x, and centrifuge briefly

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Single Cell 5’ v2 workflow

Breaking GEMs

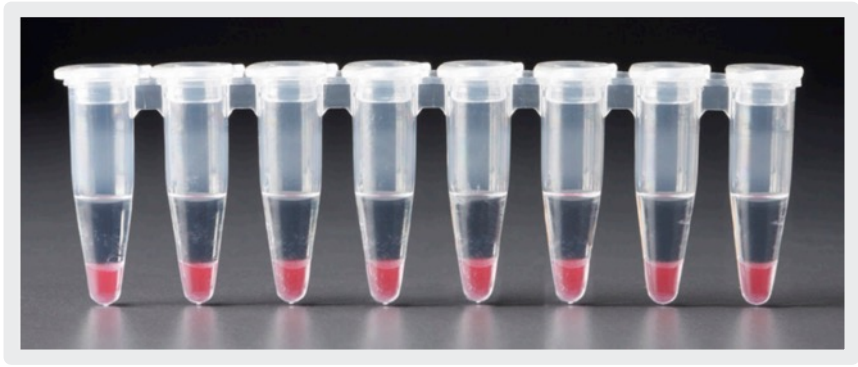
10x GENOMICS

Single Cell 5’ v2 workflow

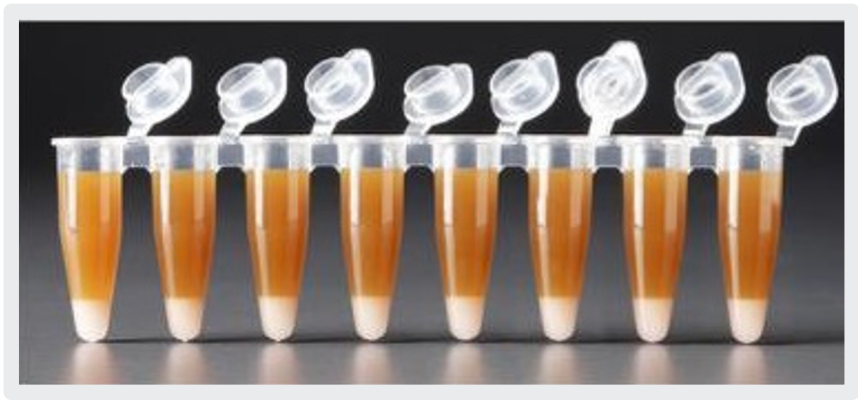
Post GEM incubation cleanup – Dynabeads

- Prepare Dynabeads Cleanup Mix
  - Vortex Dynabeads thoroughly for 30 sec
    - Confirm that Dynabeads have been fully resuspended by aspirating the full liquid volume with a pipette tip. If clumps are present, pipette mix to resuspend completely.
- Always use fresh preparations of 80% Ethanol
- Elute in 35 µl of Elution Solution I

After removing Recovery Agent (pink) from Aqueous Phase



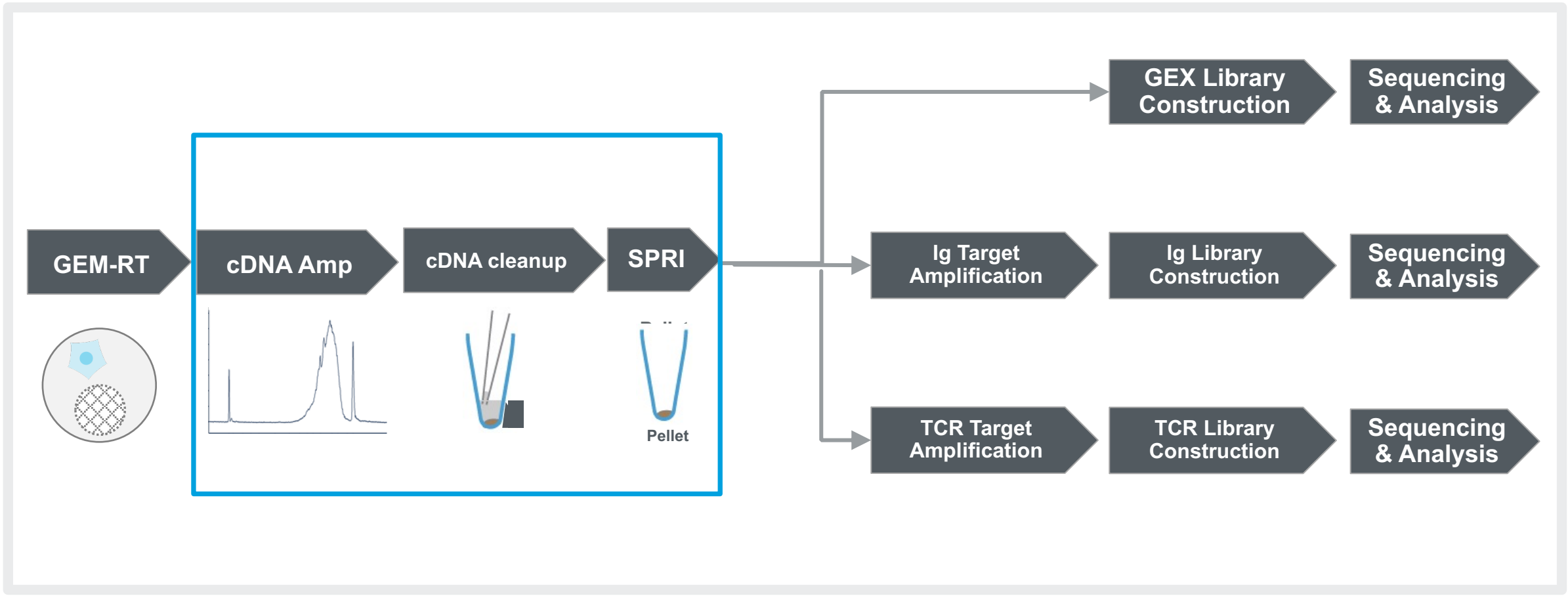
After adding Dynabeads Cleanup Mix to Aqueous Phase



Chromium Single Cell 5’ v2 workflow

cDNA Amplification

Single Cell 5’ v2 workflow summary



Note: TCR and Ig amplified libraries require independent library constructions.

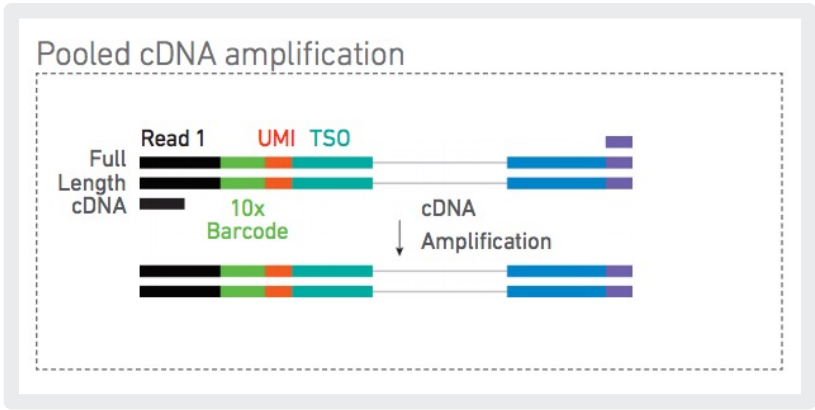
Single Cell 5' v2 workflow

cDNA amplification in bulk

- Prepare cDNA Amplification Reaction Mix
  - Primers must be selected based on whether generating only Gene Expression and V(D)J Libraries, or Gene Expression, V(D)J and Cell Surface Protein Libraries.
  - Confirm annealing temperature; temperatures are different than 5' v1/v1.1 (5' v2: 63°C, 5' v1/v1.1: 67°C)
- Add 65 µl cDNA Amplification Reaction Mix to 35 µl of sample

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	~25-50 min
Step	**Temperature**	**Time**
1	98°C	00:00:45
2	98°C	00:00:20
3	63°C	00:00:30
4	72°C	00:01:00
5	Go to Step 2, see table below for total # of cycles	
6	72°C	00:01:00
7	4°C	Hold

- Use Cell Load to determine number of total cycles for cDNA amplification



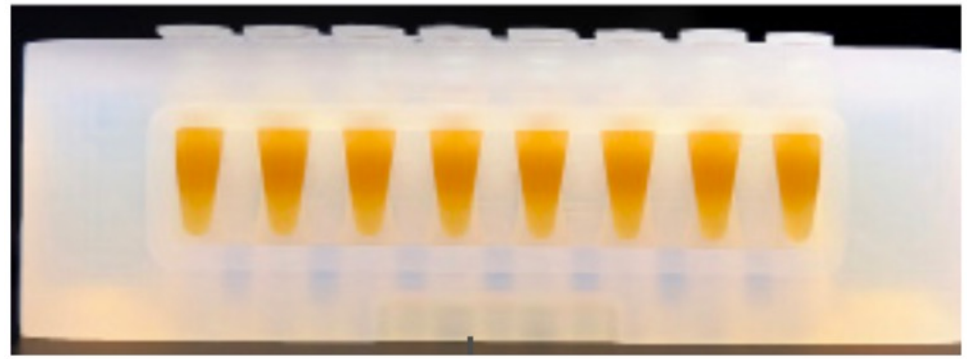
Recommended starting point for cycle number optimization.

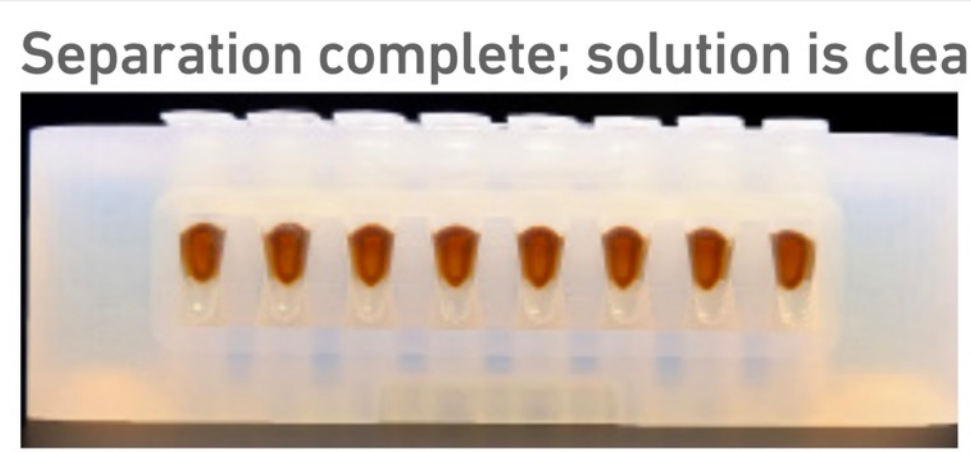
Targeted Cell Recovery	Low RNA Content Cells e.g., Primary Cells Total Cycles	High RNA Content Cells e.g., Cell Lines Total Cycles
500-2,000	16	14
2,001-6,000	14	12
6,001-10,000	13	11

Single Cell 5' v2 workflow

SPRIselect cDNA cleanup

- Vortex SPRIselect Reagent thoroughly before adding to samples and ensure samples are fully mixed with beads
- If generating Cell Surface Protein libraries, **DO NOT** discard supernatant after the first bead incubation. See appropriate User Guide for application-specific guidance.
- Always use fresh preparations of 80% Ethanol
- Elute in 45.5 µl of Buffer EB

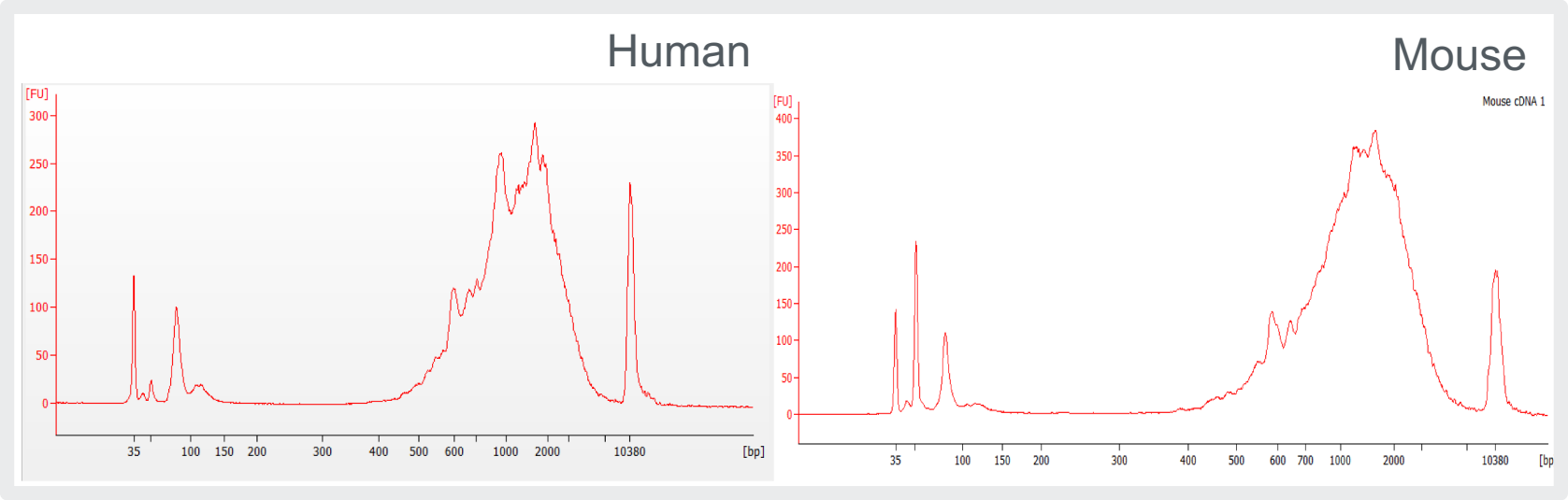




Single Cell 5' v2 workflow

cDNA QC: representative traces from PBMCs

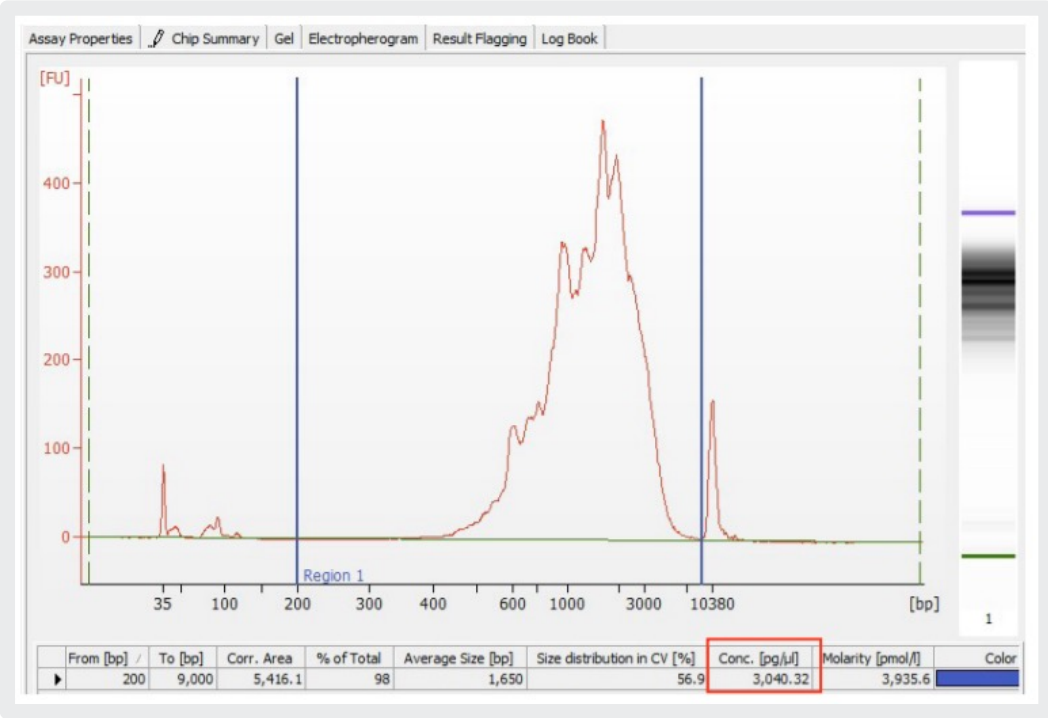
- 1 ul of undiluted sample (High Sensitivity BioAnalyzer chip)
- Lower molecular weight product (35 – 150 bp) may be present. This is normal and does not affect sequencing or application performance.
- Specific yield and shape of the curve may vary based on sample, regardless of species



Single Cell 5' v2 workflow

Determine cDNA amplification product yield

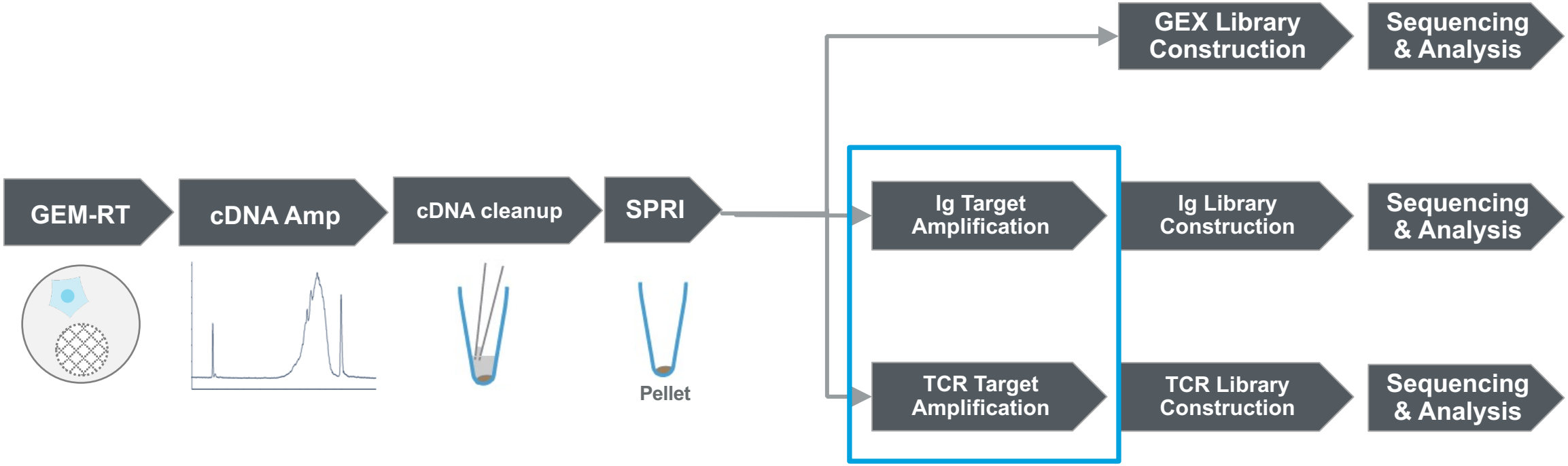
- Determine the amplified cDNA yield per sample via the “Electropherogram” view
  - Choose the “Region Table” tab on the Agilent 2100 Expert Software
  - Manually select the region encompassing 200 – 9000 bp, note Concentration reported
- cDNA amplification reaction generates sufficient material to construct multiple libraries from the same cells, including both TCR and/or Ig libraries and 5′ gene expression libraries.
  - 50 ng of amplified cDNA is carried forward into 5′ Gene Expression library construction.
  - If less than 50 ng of amplified cDNA is generated, carry 20 ul into library construction
- 2 µl of amplified cDNA is carried forward into Target Amplification Reactions



Chromium Single Cell 5' v2 workflow

Target amplification from cDNA

Single Cell 5' v2 Workflow Summary



Note: TCR and Ig amplified libraries require independent library constructions.

Single Cell 5’ v2 workflow

TCR and Ig target amplification

- Series of target amplification reactions (1 and 2)
- Check reagents and settings carefully:
  - TCR and Ig amplification must be performed separately. Each uses different primers and different numbers of PCR cycles
  - Confirm amplification primer species – primers are human or mouse specific
  - Target Amplification 1 and Target Amplification 2 use different numbers of cycles – check your PCR settings carefully!
  - Confirm annealing temperature; temperatures are different than 5’ v1/v1.1 (5’ v2: 63°C, 5’ v1/v1.1: 67°C)
- Double Sided SPRI cleanup after Target Amplification 1
- Double Sided SPRI cleanup after Target Amplification 2

Target Amplification 1

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	~20-30 min
Step	**Temperature**	**Time**
1	98°C	00:00:45
2	98°C	00:00:20
3	62°C	00:00:30
4	72°C	00:01:00
5 Different cycle numbers for T & B cells	T Cell: Go to Step 2, 11x (total 12 cycles) B Cell Go to Step 2, 7x (total 8 cycles)	
6	72°C	00:01:00
7	4°C	Hold

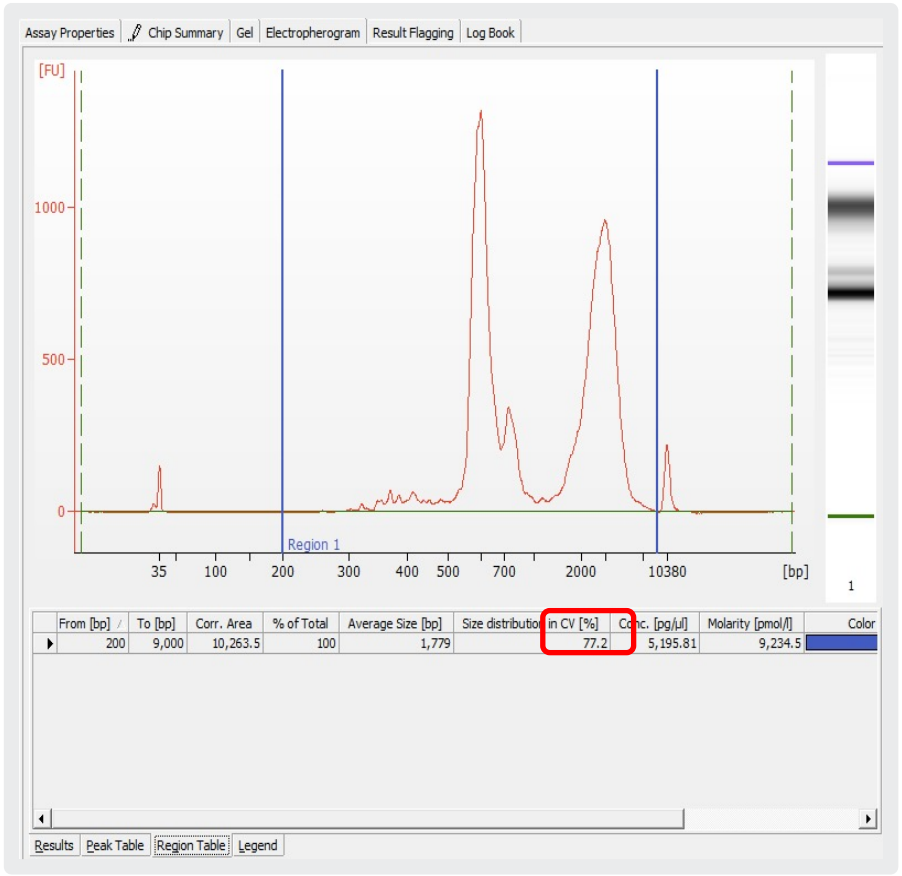
Target Amplification 2

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	~25-30 min
Step	**Temperature**	**Time**
1	98°C	00:00:45
2	98°C	00:00:20
3	62°C	00:00:30
4	72°C	00:01:00
5 Different cycle numbers for T & B cells	T Cell: Go to Step 2, 9x (total 10 cycles) B Cell: Go to Step 2, 7x (total 8 cycles)	
6	72°C	00:01:00
7	4°C	Hold

Single Cell 5' v2 workflow

Post target amplification QC & quantification

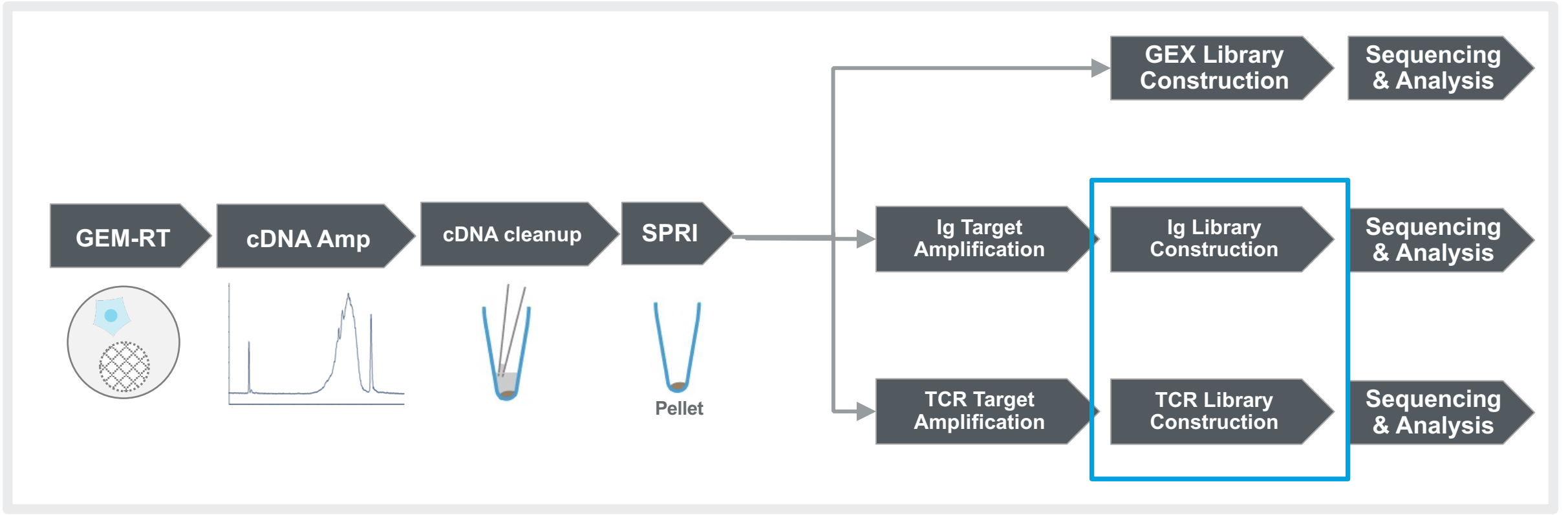
- Determine the Amplification Product yield per sample via the “Electropherogram” view
  - Choose the “Region Table” tab on the Agilent 2100 Expert Software.
  - Manually select the region encompassing 200 – 9000 bp.
- 50 ng of Amplification Product is carried forward into library construction
- If less than 50ng Amplification Product is generated, only carry 20 µl into library construction (2-50 ng is supported)



Chromium Single Cell 5' v2 workflow

Amplification library construction

Single Cell 5' v2 workflow summary

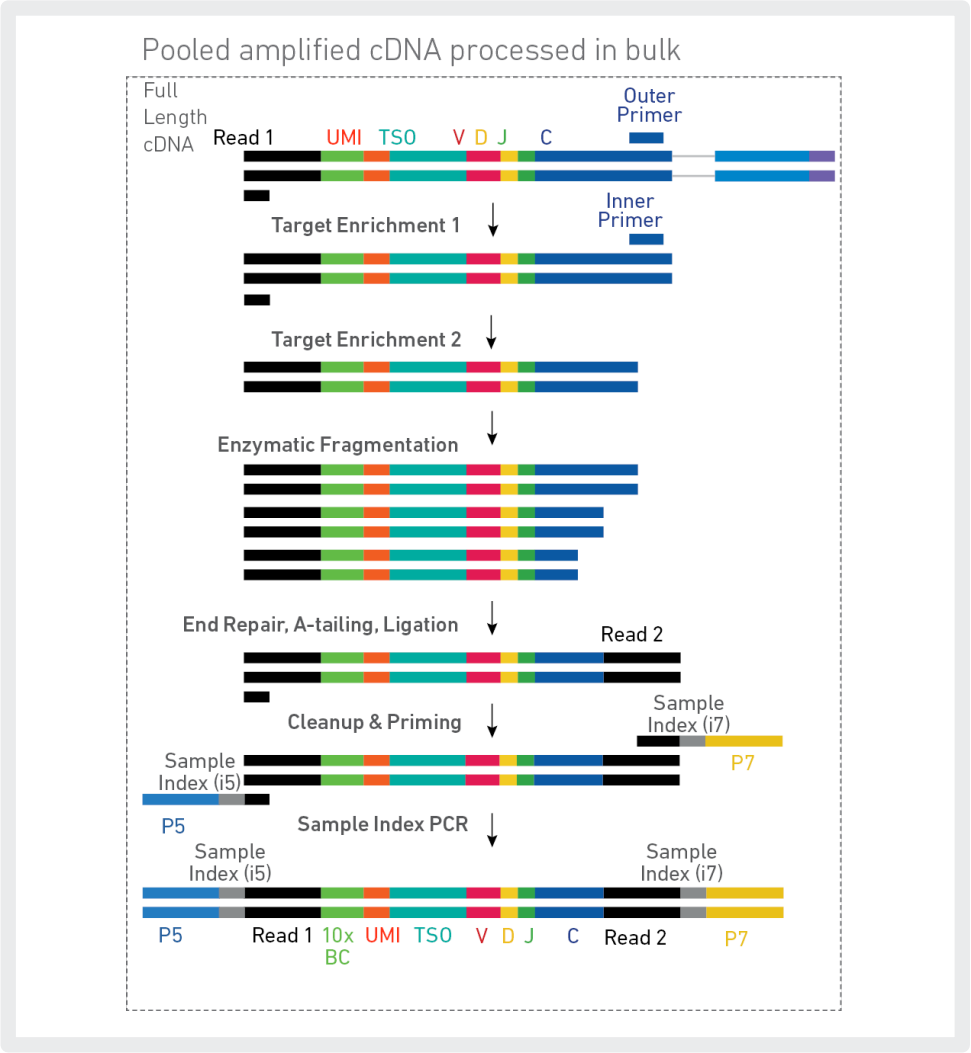


Note: TCR and Ig libraries require independent library constructions.

Single Cell 5' v2 workflow

TCR and Ig library construction

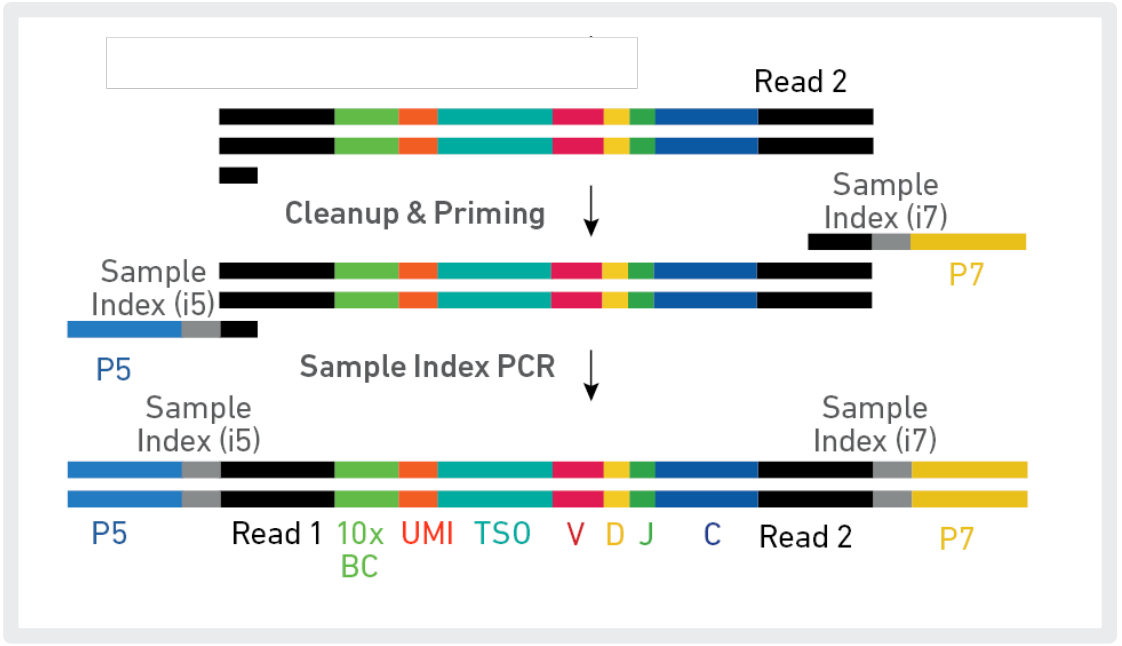
- Fragmentation, end repair and A-tailing
  - It is critical to pre-chill the thermal cycler block to 4°C prior to assembling the reaction
  - Check thermal cycler program for correct length
    - 2 minutes for Amplified Library fragmentation
- Adaptor ligation
- Post ligation cleanup - SPRIselect
- Sample Index PCR
- Post sample index PCR cleanup - SPRIselect



Single Cell 5' v2 workflow

TCR and Ig library sample index PCR

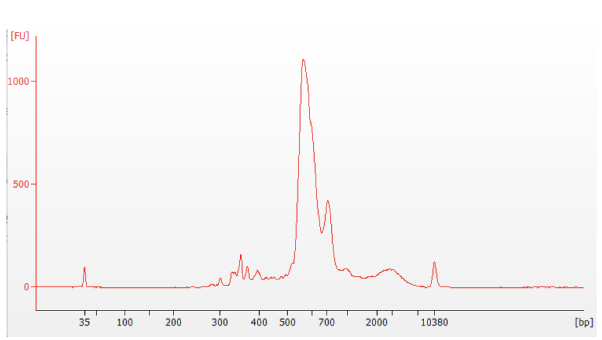
- Uses Dual Index Kit TT Set A
  - Record the Well ID used for each sample. This is needed for demultiplexing the sequencing data.
  - Choose the appropriate sample index sets to ensure that no sample indices overlap in a multiplexed sequencing run
- Fixed number of sample index PCR cycles
- Size selection with SPRIselect to remove excess primers and optimize final library size distribution
  - Measure and transfer bead volumes carefully



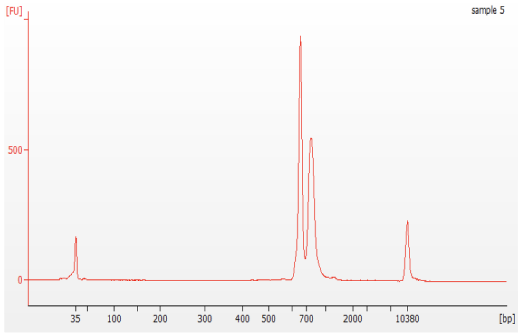
Single Cell 5’ v2 workflow

Target amplification QC: Representative traces from human PBMCs

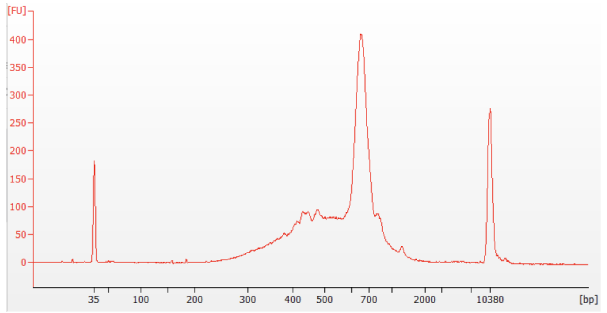
- 1:10 dilution of amplification product run on BioAnalyzer High Sensitivity chip
- HMW product (2,000-9,000bp) in the QC traces may be present. This is normal and does not affect sequencing or application performance
  - These fragments represent off-target product; if excessive, more sequencing may be required to capture enriched constructs
- Specific yield and shape of the curve may vary based on sample, regardless of species
- Low yield may be indicative of a low total number of T or B cells in the sample



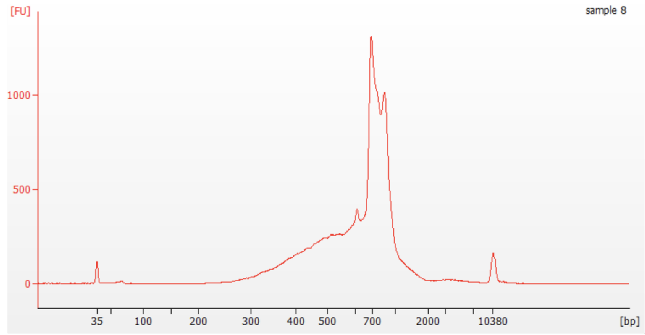
TCR Amplification



BCR Amplification



TCR Final Library Trace

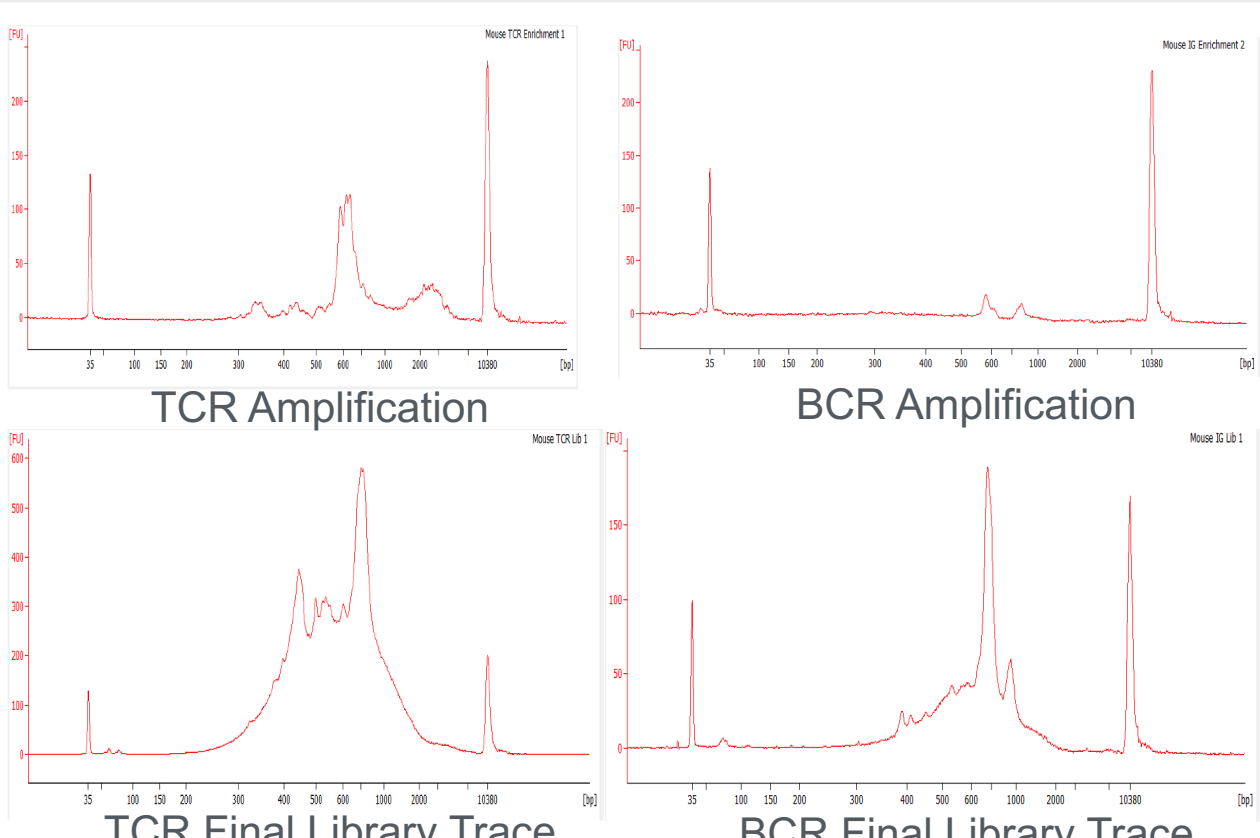


BCR Final Library Trace

Single Cell 5’ v2 workflow

Target amplification and final library QC: Representative traces from mouse splenocytes

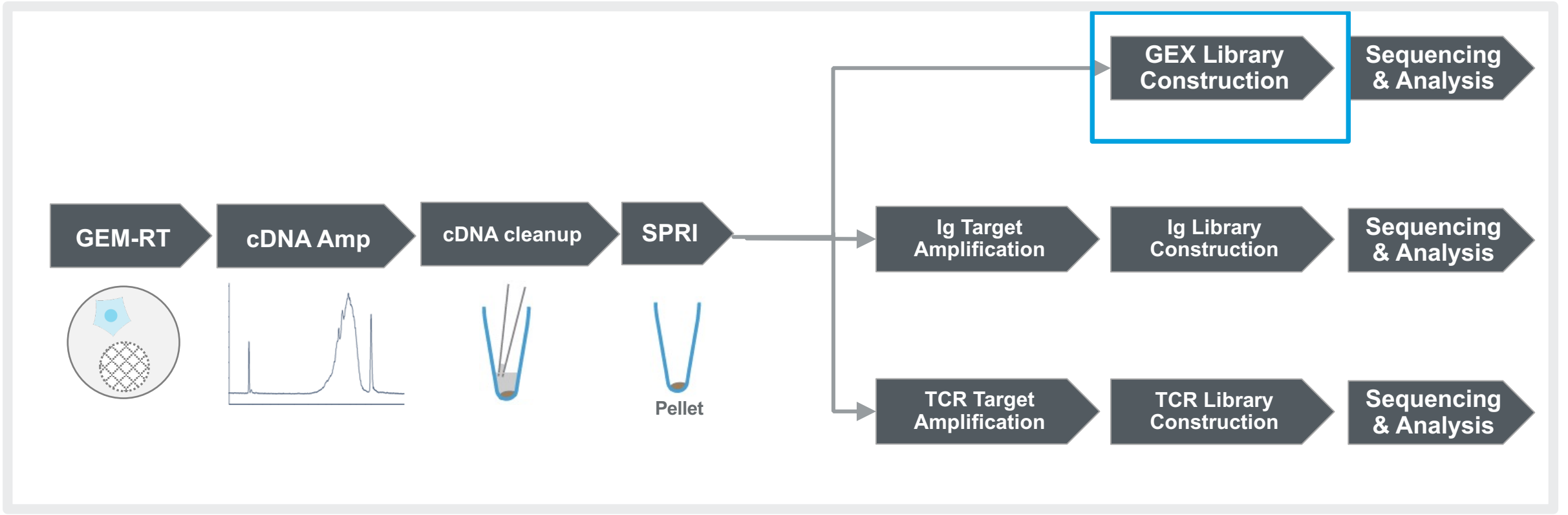
- 1:10 dilution of amplification product run on BioAnalyzer High Sensitivity chip
- HMW product (2,000-9,000bp) in the QC traces may be present. This is normal and does not affect sequencing or application performance
  - These fragments represent off-target product; if excessive, more sequencing may be required to capture enriched constructs
- Specific yield and shape of the curve may vary based on sample, regardless of species
- Low yield may be indicative of a low total number of T or B cells in the sample



Chromium Single Cell 5' v2 workflow

Gene expression library construction

Single Cell 5' v2 workflow summary

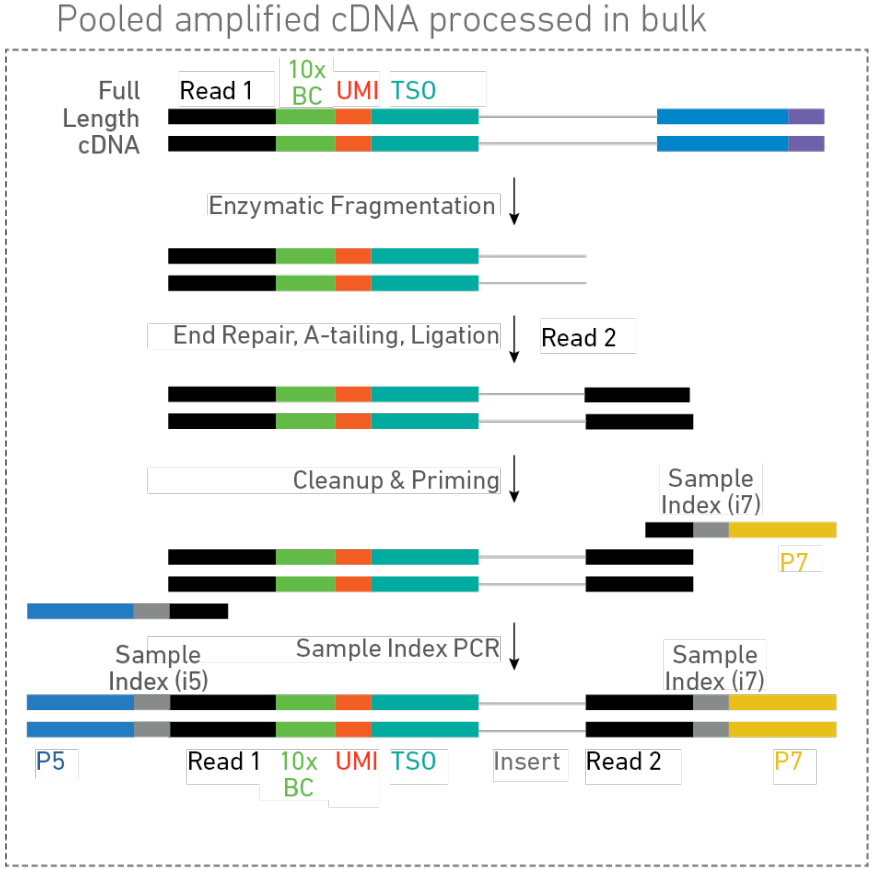


Note: TCR and Ig libraries require independent library constructions.

Single Cell 5' v2 workflow

Gene expression library construction

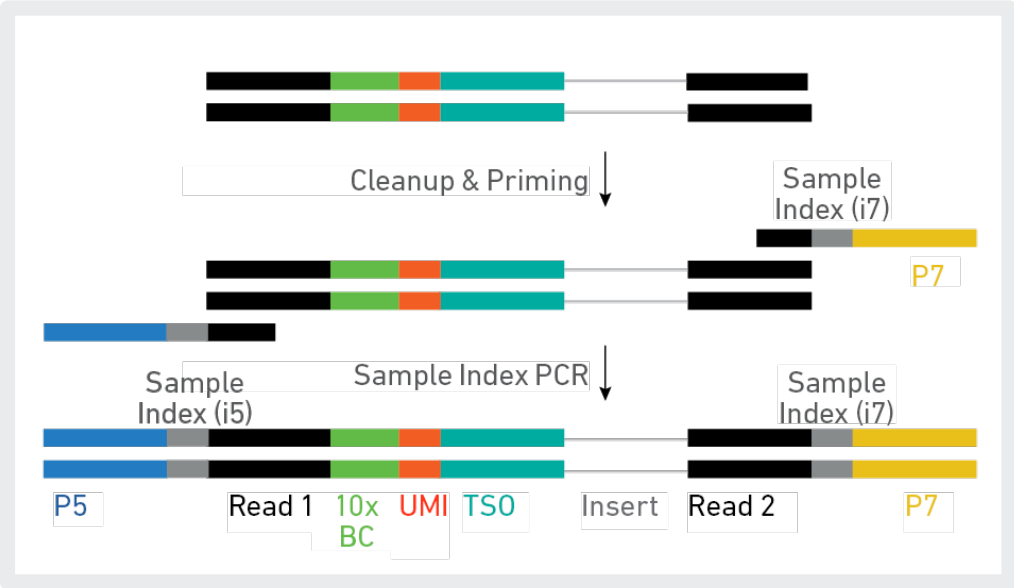
- Fragmentation, end repair and A-tailing
  - It is critical to pre-chill the thermal cycler block to 4°C prior to assembling the reaction
  - Check thermal cycler program for correct length
    - 5 minutes for GEX library fragmentation
- Post fragmentation, end repair & A-tailing Double Sided Size Selection – SPRIselect
- Adaptor ligation
- Post ligation cleanup – SPRIselect
- Sample index PCR
- Post sample index PCR double sided size selection – SPRIselect



Single Cell 5' v2 workflow

Gene expression library sample index PCR

- Uses Dual Index Kit TT Set A
  - Record the Well ID used for each sample. This is needed for demultiplexing the sequencing data.
  - Choose the appropriate sample index sets to ensure that no sample indices overlap in a multiplexed sequencing run
- Number of Sample Index PCR cycles depends on mass of cDNA used for library construction
- Size selection with Double Sided SPRIselect to remove excess primers and optimize final library size distribution
  - Measure and transfer bead volumes carefully



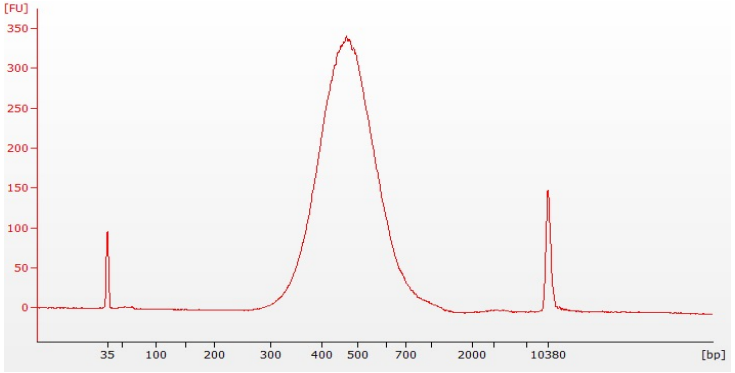
Input into Library Construction	Total Sample Index Cycles
1-25 ng	16
26-50 ng	14

Single Cell 5' v2 workflow

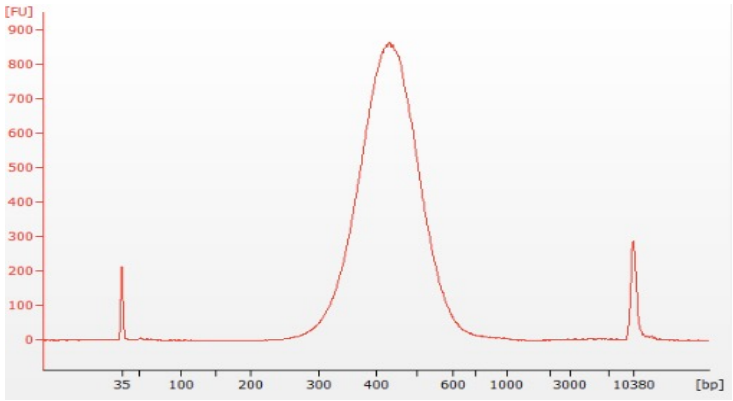
Gene expression library QC: Representative traces from PBMCs

- 1:10 dilution of final library run on BioAnalyzer High Sensitivity chip
- Fragment sizes range from 300 – 700 bp (average 450 bp)
- Specific yield and shape of the curve may vary based on sample, regardless of species

Human



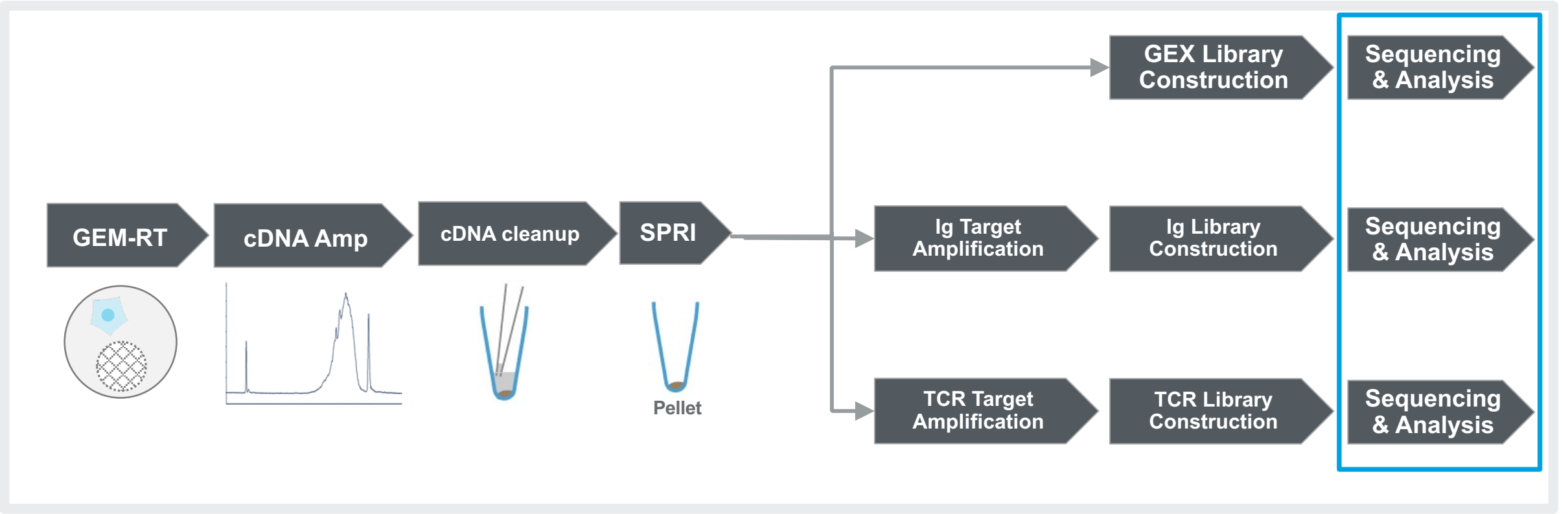
Mouse



Sequencing recommendations

5′ Gene expression and V(D)J libraries

Single Cell 5' v2 workflow summary

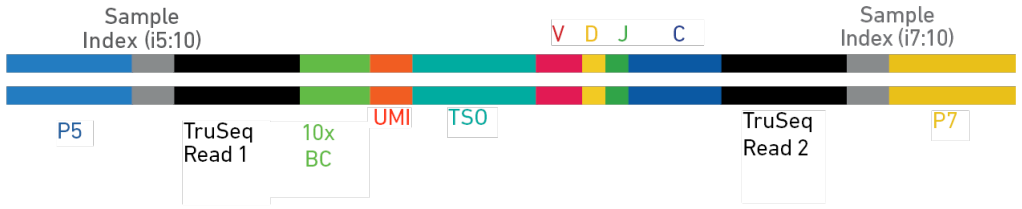


Note: TCR and Ig libraries require independent library constructions.

Sequencing V(D)J and 5′ Gene Expression Libraries

Libraries may be pooled in various configurations

Chromium Single Cell V(D)J Dual Index Library



Chromium Single Cell 5' Gene Expression Dual Index Library

Sequencing Depth	Minimum 5,000 read pairs per cell for V(D)J Dual Index library Minimum 20,000 read pairs per cell for 5' Gene Expression Dual Index library Minimum 5,000 read pairs per cell for Cell Surface Protein Dual Index library
Sequencing Type	Paired-end, Dual indexing
Sequencing Read	Read 1: 26 cycles i7 Index: 10 cycles i5 Index: 10 cycles Read 2: 90 cycles

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Sequencing recommendations: Pooling guidelines

The following **dual index** libraries can be pooled in any combination for Illumina sequencing:

Single Cell 3’ v3.1 Dual Index Gene Expression Libraries*

Single Cell 3’ v3.1 Dual Index Cell Surface Protein or CRISPR Screening Libraries*

Single Cell 5’ v2 Gene Expression or V(D)J Libraries

Single Cell 5’ v2 Cell Surface Protein Libraries

Spatial Gene Expression Libraries*

We have not tested the compatibility of pooling for sequencing with:

Single Cell ATAC libraries

Single Cell CNV libraries

* If run with 28 cycles for Read 1

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Sequencing V(D)J and 5′ Gene Expression libraries

Consider sequencing depth requirements when pooling libraries

- Recommended sequencing depths
  - V(D)J Library: 5,000 read pairs per cell
  - 5′ Gene Expression Library: 20,000 read pairs per cell

Library Pooling Examples:

Libraries	Sequencing Depth (read pairs per cell)	Library Pooling Ratio
Example 1		
V(D)J Enriched library	5,000	1
5' Gene Expression library	20,000	4
Example 2		
V(D)J Enriched library	5,000	1
5' Gene Expression library	50,000	10

MiSeq

NextSeq 500/550

HiSeq 2500

HiSeq 3000/4000

NovaSeq

Chromium Single Cell Immune Profiling Solution with Feature Barcode technology

Chromium Single Cell Immune Profiling Solution with Feature Barcode technology

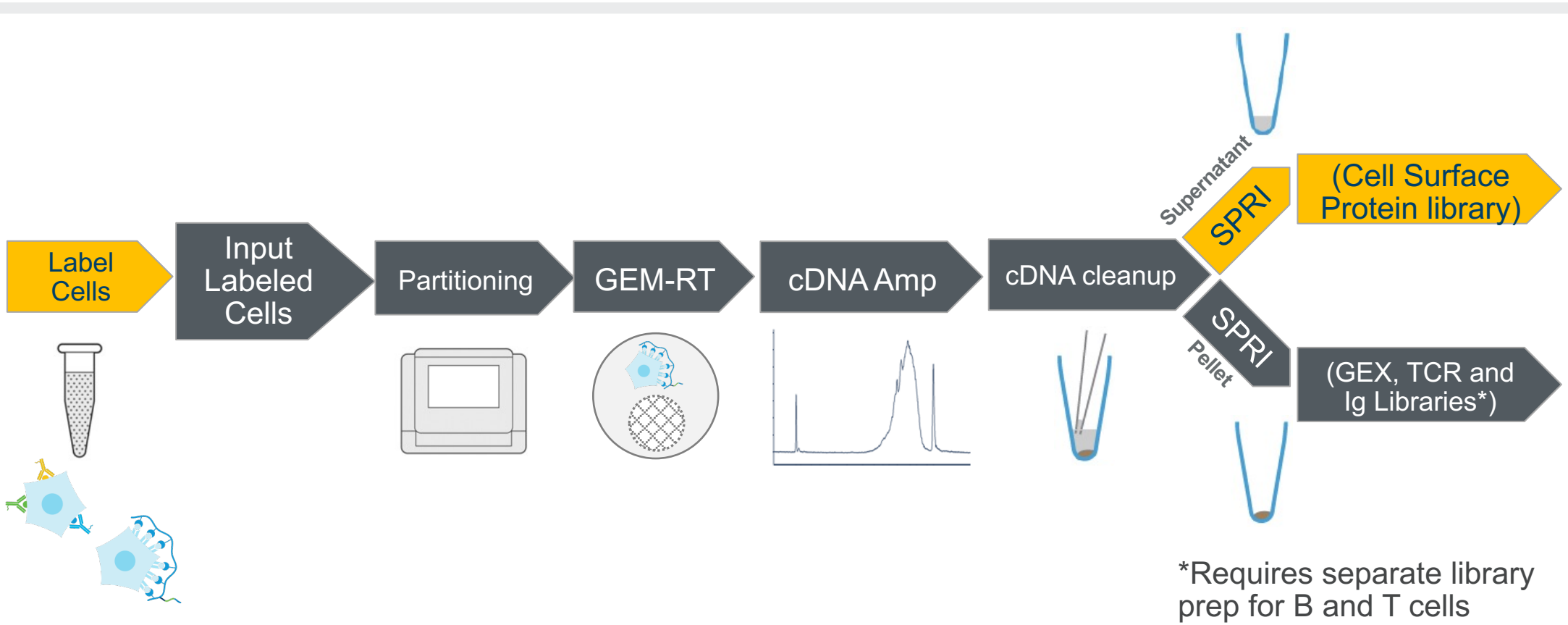
Agenda

- Single Cell Immune Profiling Solution with Feature Barcode technology overview
- The biochemistry and workflow for the Single Cell Immune Profiling Solution with Feature Barcode technology
- Sample preparation recommendations for cell surface protein profiling
- Sequencing cell surface protein libraries

The biochemistry and workflow for the Single Cell Immune Profiling Solution with Feature Barcode technology

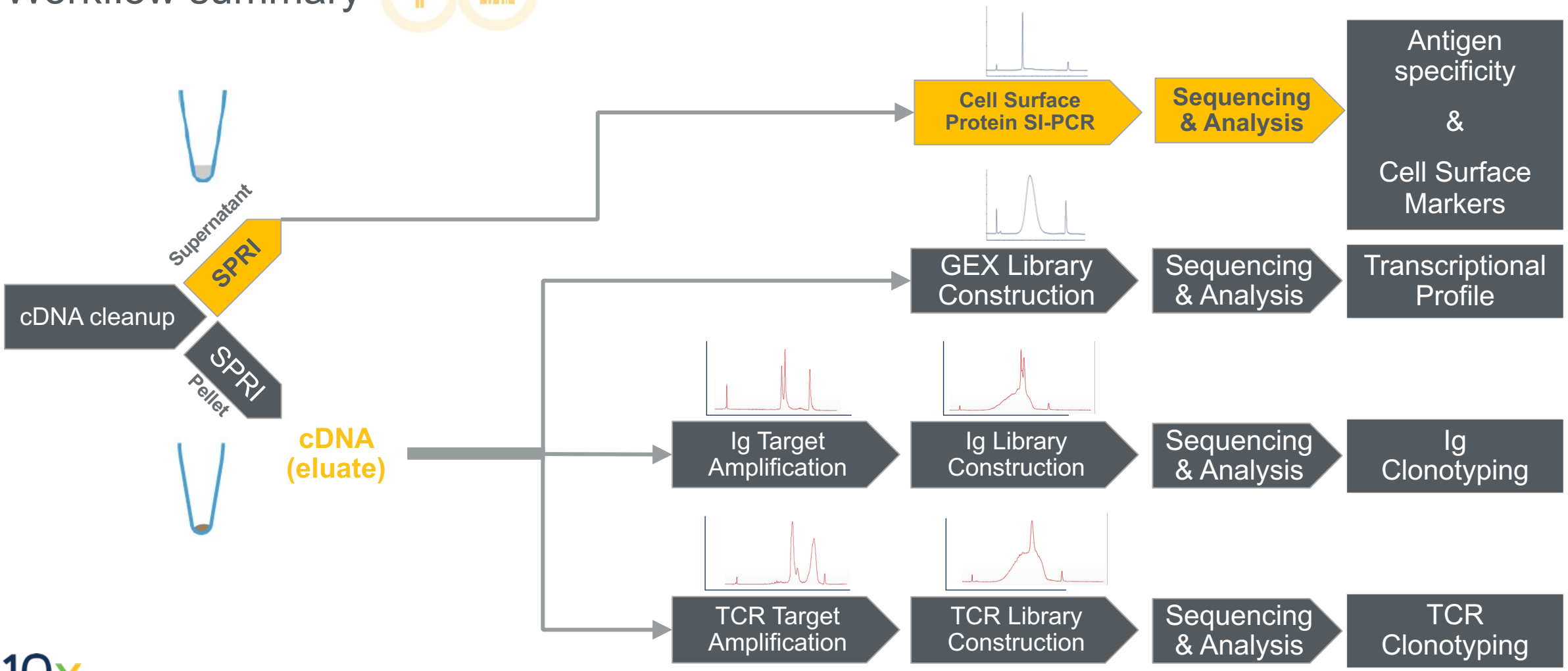
Immune profiling with Feature Barcode technology

Workflow summary



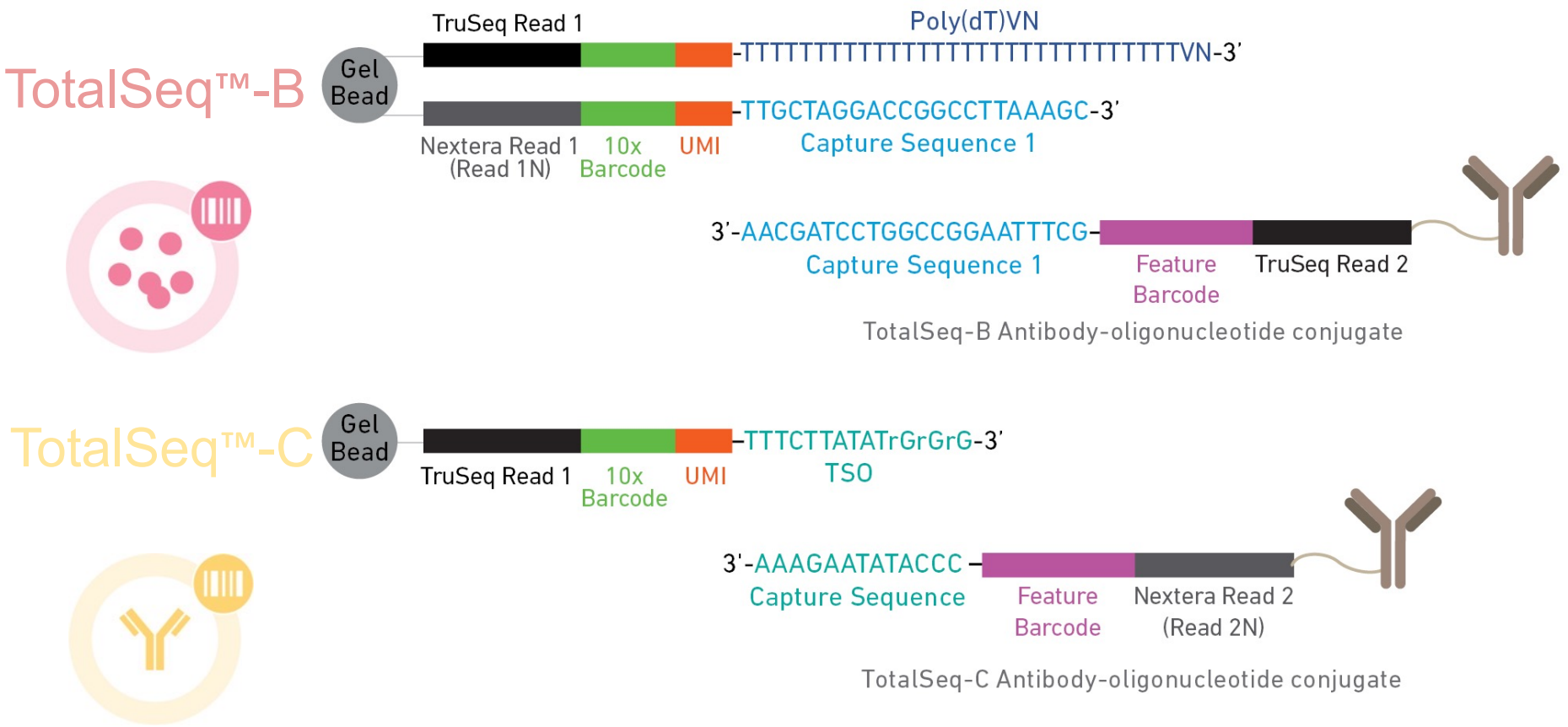
Immune profiling with Feature Barcode technology

Workflow summary



BioLegend's TotalSeq™- Antibody reagents

10x Genomics compatible products



Compatible with Immune Profiling Solution?

✘ No

✔ Yes

Feature Barcode sequence structure

- Oligonucleotide sequence can be found in User Guide Appendices and the Cell Labeling / Custom Conjugation Demonstrated Protocol

10x Genomics Protocol	Feature Barcode Oligonucleotide Sequence
Single Cell 3' v3 or v3.1 – Cell Surface Protein (CG000185 or CG000206)	/5AmMC12/GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNN-NNNNNNNNNNNNNNN-NNNNNNNNNGCTTTAAGGCCGGTCCTAGCAA TruSeq Read 2 — 10 nt — Feature Barcode (15 nt) — 9 nt — Capture Sequence 1
Single Cell V(D)J or V(D)J v1.1 – Cell Surface Protein (CG000186 or CG000208)	/5AmMC12/CGGAGATGTGTATAAGAGACAGNNNNNNNNNN-NNNNNNNNNNNNNNN-NNNNNNNNNCCCATATAAGAAA Nextera partial Read 2 — 10 nt — Feature Barcode (15 nt) — 9 nt — Capture Sequence

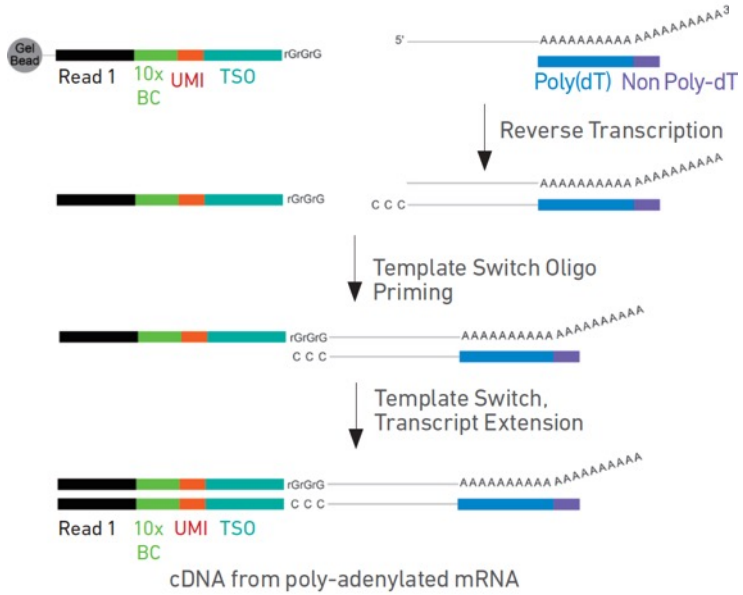
Link to protein --- 5′ - Read 2 - 10N - Feature Barcode (15N) - 9N - Capture sequence

- The diversity sequences flanking the Feature Barcode sequence increase the sequencing quality of the Feature Barcode bases
- Is not directly used by CR3.0, but is flagged in the Feature Reference File

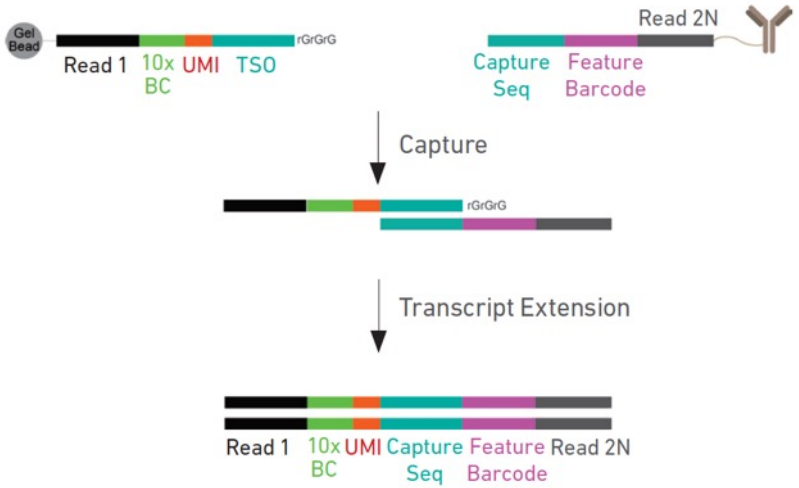
Feature Barcode technology workflow: Target capture

Multiple targets captured simultaneously inside individual GEMs

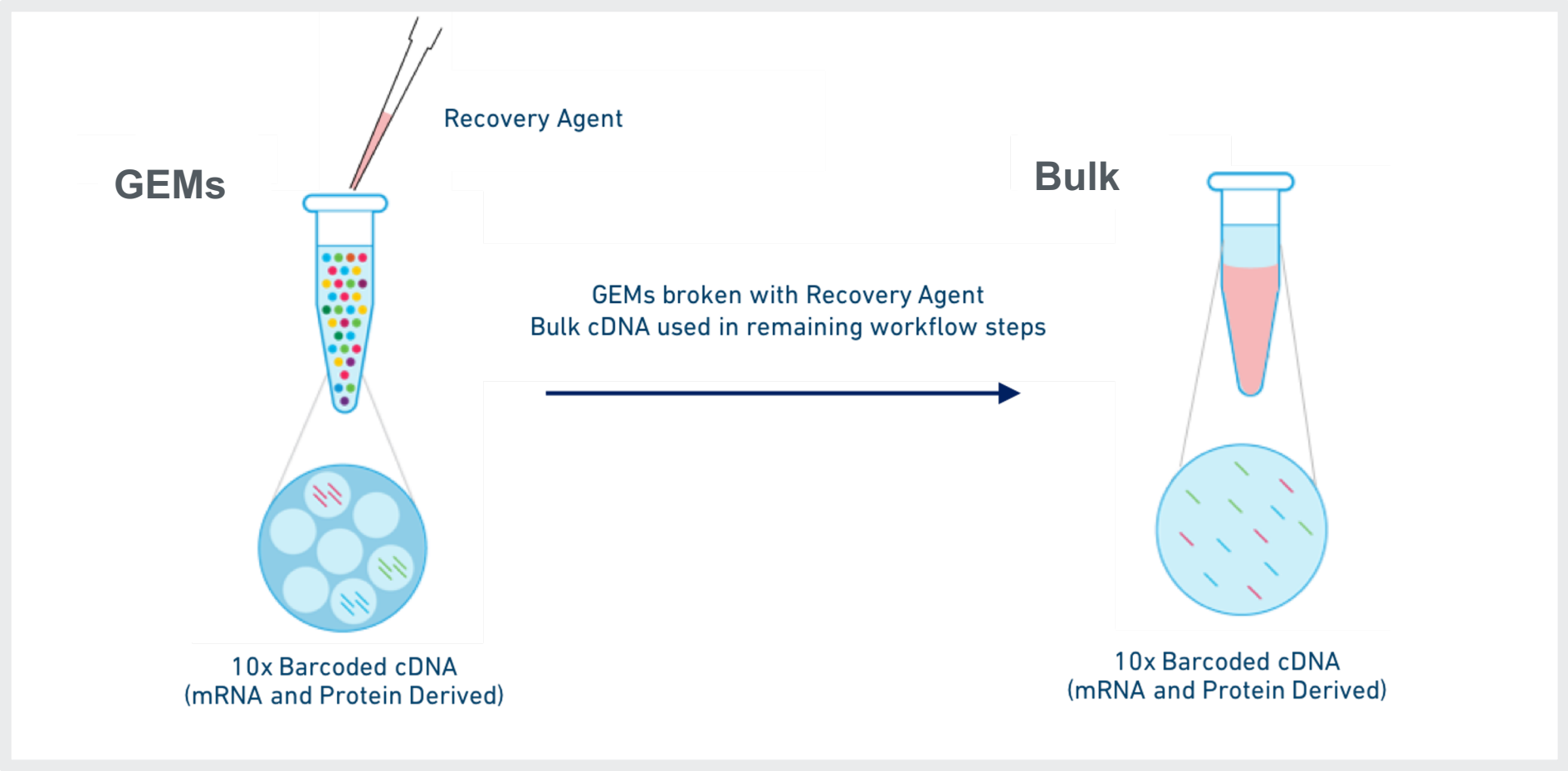
Barcoded, full-length cDNA from poly-adenylated mRNA



Barcoded, cell surface protein conjugated oligonucleotide



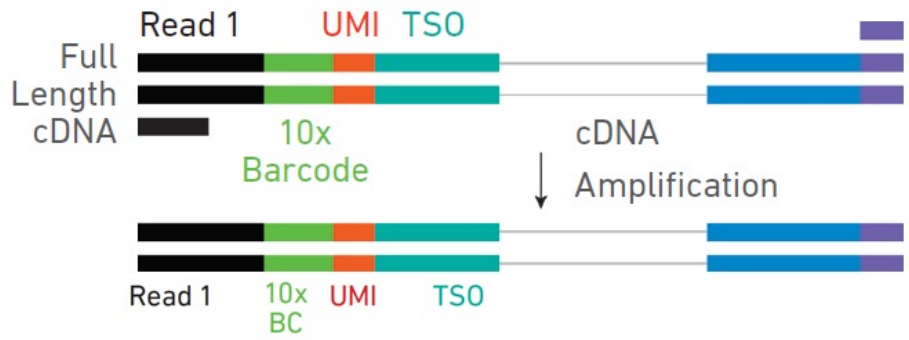
Feature Barcode technology workflow: Breaking GEMs



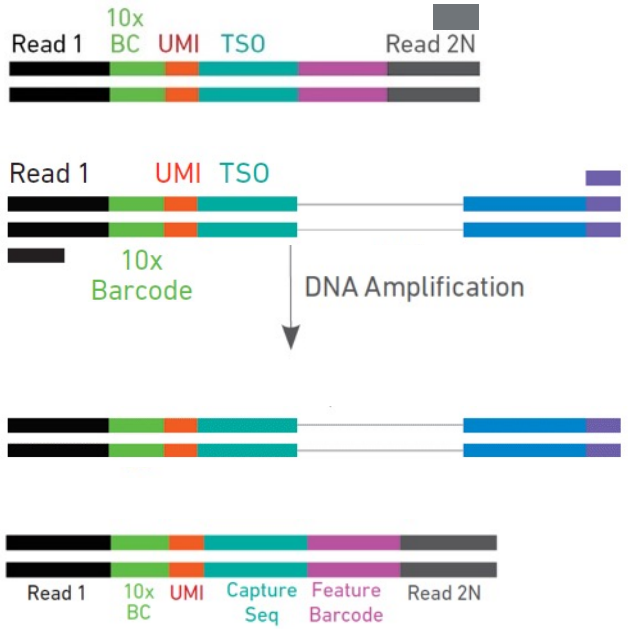
Feature Barcode technology workflow: cDNA Amplification

Amplification of mRNA and Cell Surface Protein Feature Barcode Targets in Bulk

cDNA Amplification of poly-adenylated mRNA only



cDNA Amplification of poly-adenylated mRNA and DNA from protein Feature Barcode



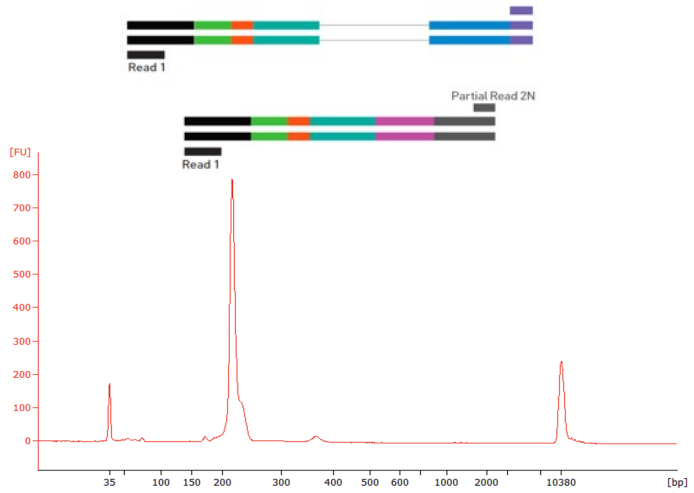
Note: For cDNA Amplification of poly-adenylated mRNA only please use: cDNA Primers (PN-2000089)
For cDNA Amplification of poly-adenylated mRNA and protein please use: Feature cDNA Primers 4 (PN 2000277)

Feature Barcode workflow: cDNA amplification

Correct cDNA primer choice is critical for amplification of Feature Barcode sequences

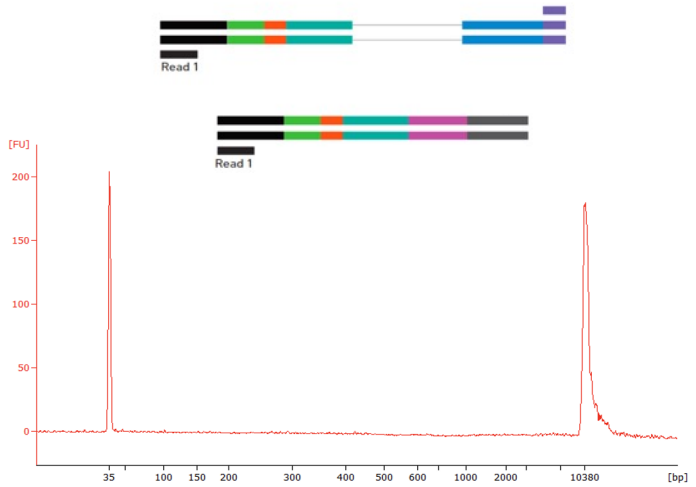
Correct cDNA Primers Used
Cell Surface Protein Library Product Present

Feature cDNA Primers 4 (PN 2000277)



Incorrect cDNA Primers Used
No Cell Surface Protein Library Product

cDNA Primers (PN-2000089)

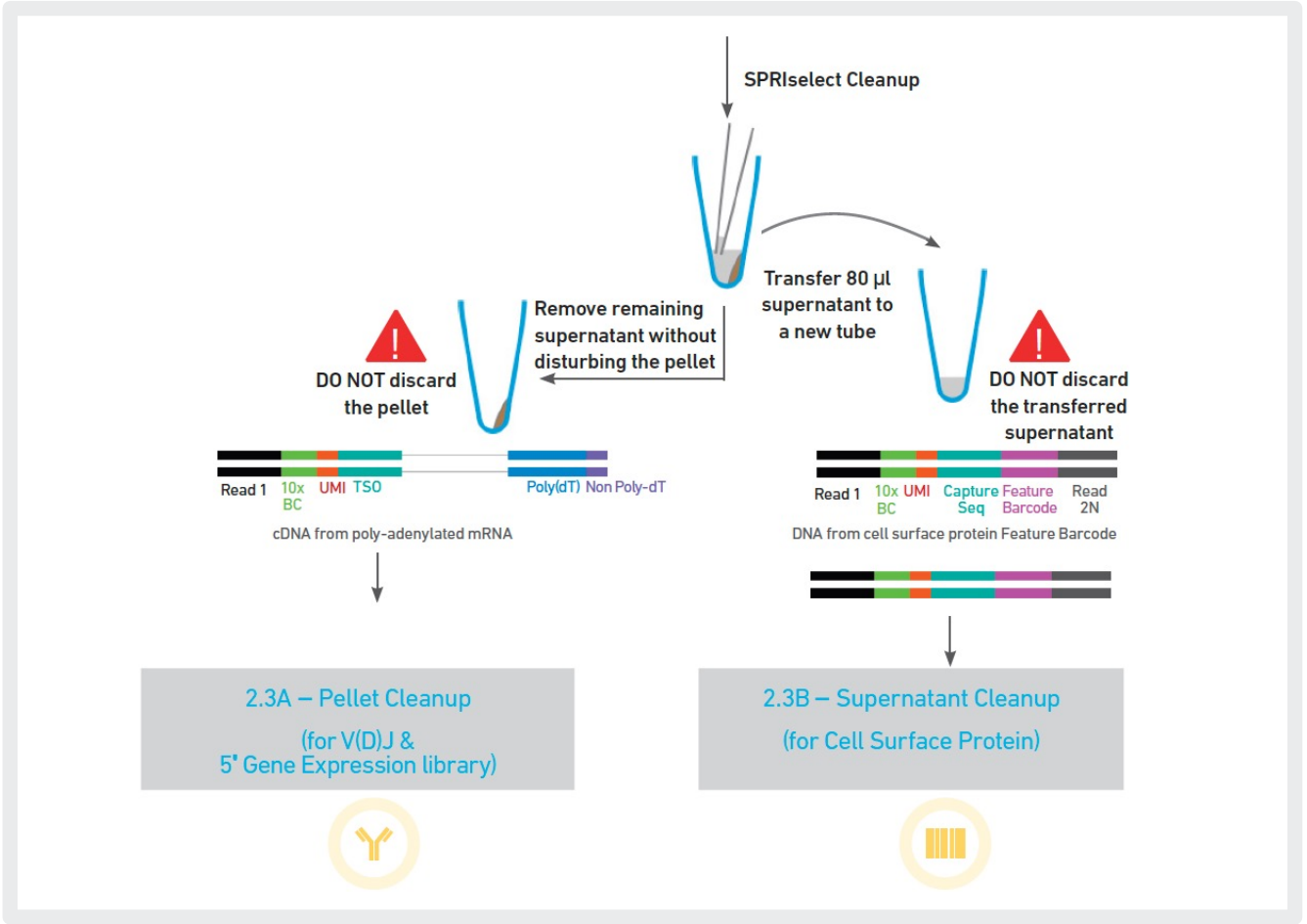


Bioanalyzer traces from Post Library Construction QC

Feature Barcode workflow: cDNA cleanup

Sample bifurcation enables cell surface protein library construction

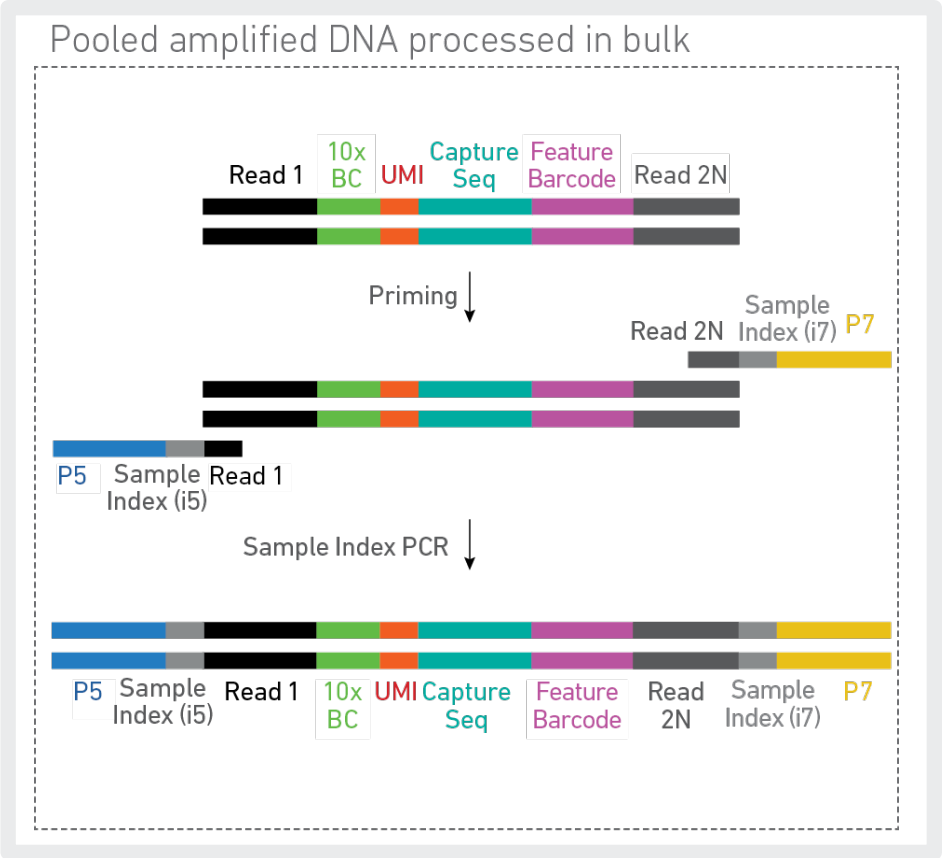
- Immune Profiling with Feature Barcode workflow
  - Transfer and save 80 µl of supernatant
  - Discard the remaining supernatant (~70 µl)
- Immune Profiling only Workflow
  - Remove the supernatant (and discard)



Feature Barcode workflow: Sample index PCR

Cell surface protein library sample index PCR

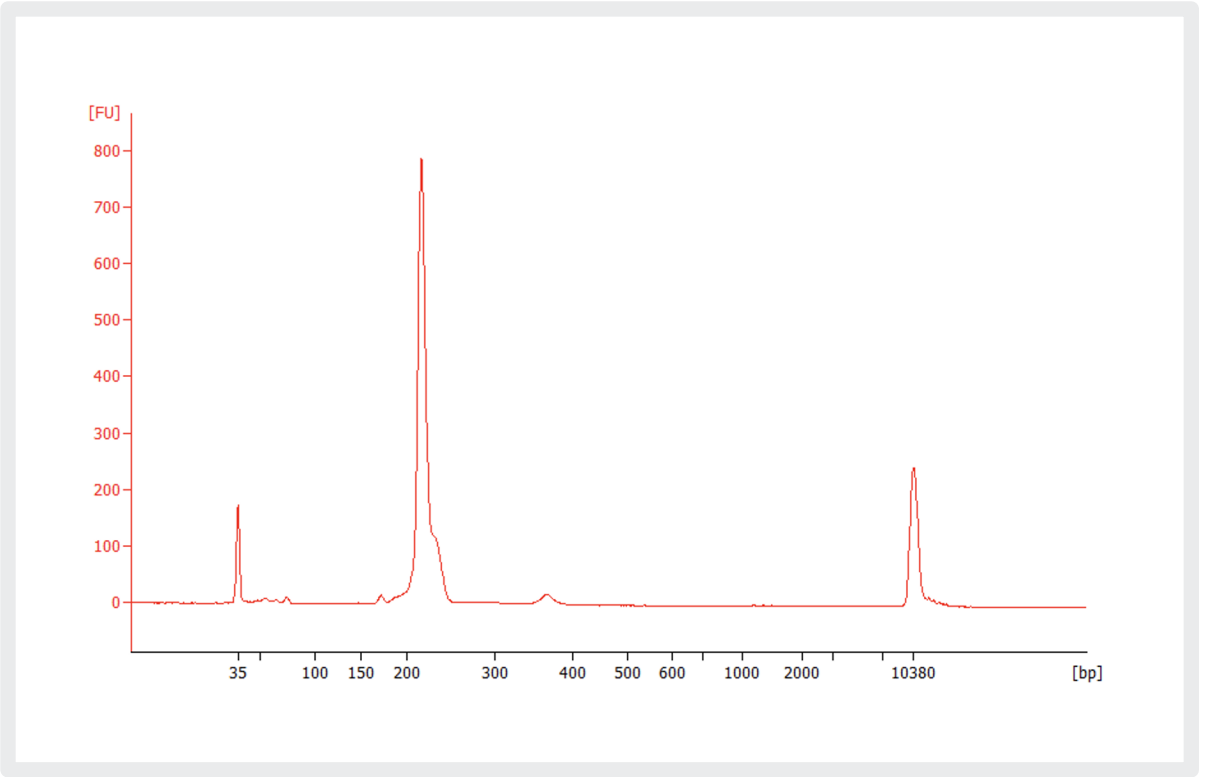
- Uses Dual Index Kit TN Set A
  - This is different than the index plate used for the Gene Expression and V(D)J libraries
  - Record the Well ID used for each sample. This is needed for demultiplexing the sequencing data.
  - Choose the appropriate sample index sets to ensure that no sample indices overlap in a multiplexed sequencing run
- Number of Sample Index PCR cycles is fixed
- Single sided SPRIselect cleanup to remove excess primers and optimize final library size distribution



Feature Barcode workflow

Cell surface protein library QC: Representative trace from labeled PBMCs

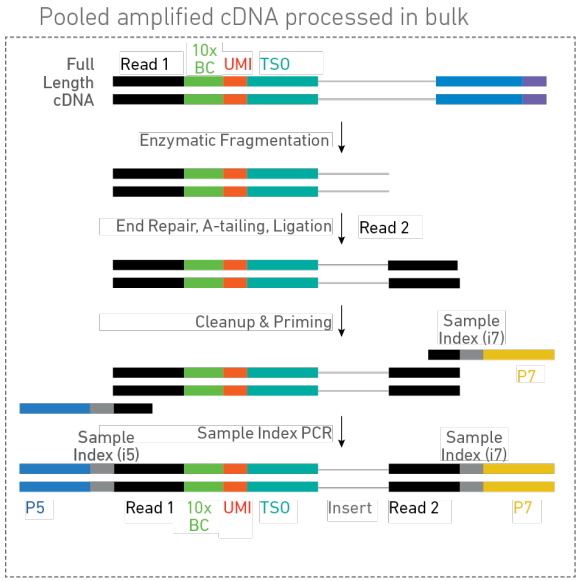
- 1:10 dilution of final library run on BioAnalyzer High Sensitivity chip
- Sharp peak with narrow size range expected at ~200 bp



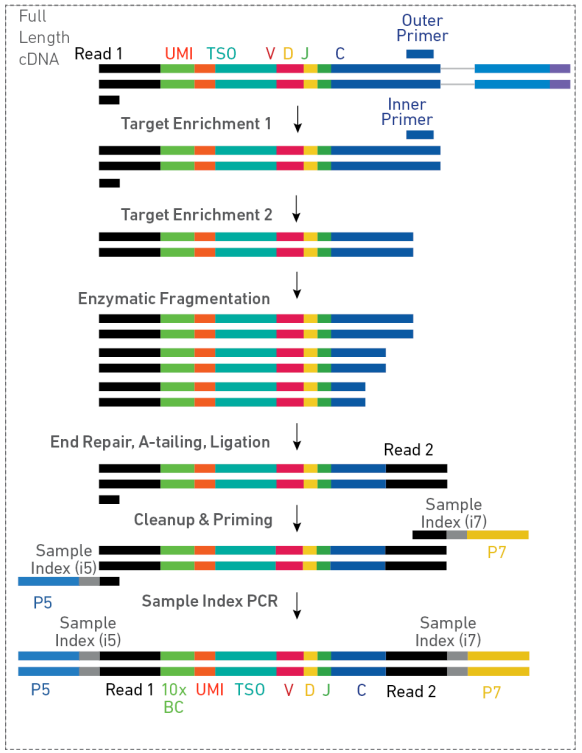
Feature Barcode workflow: Library construction

Amplified cDNA is split to generate multiple libraries for sequencing

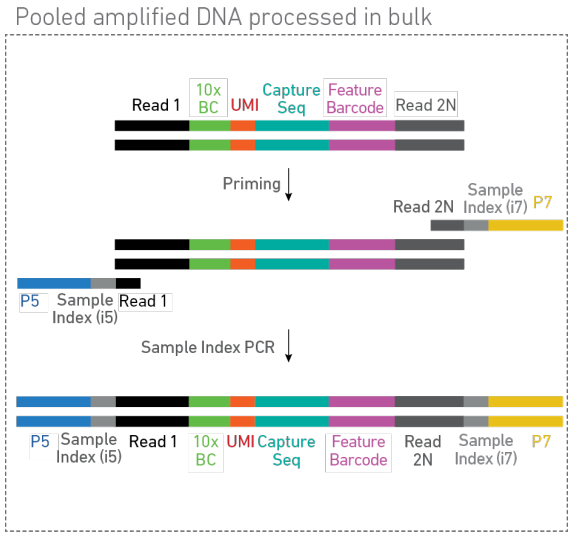
5′ Gene Expression Library Construction (from SPRI pellet)



V(D)J Library Construction* (from SPRI pellet)



5′ Cell Surface Protein/Antigen Specificity Library Construction (from SPRI supernatant)

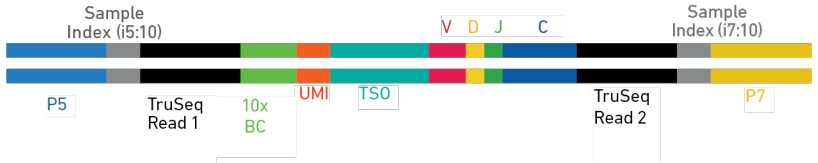


**TCR and Ig libraries must be processed independently*

Feature Barcode workflow: sequencing-ready libraries

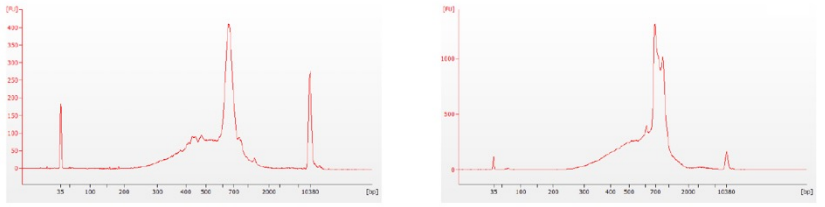
Final library constructs

Chromium Single Cell V(D)J Dual Index Library

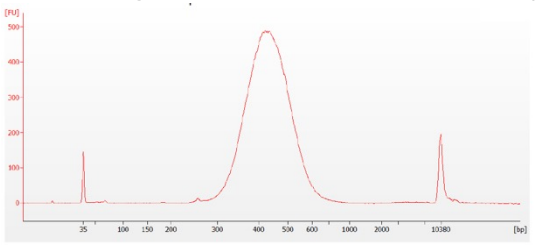


Chromium Single Cell 5' Gene Expression Dual Index Library

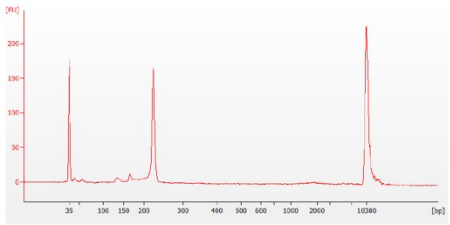
Chromium Single Cell 5' Cell Surface Protein Dual Index Library*



TCR/Ig libraries - 300-1000 bp size (average 650 bp)



5′ GEX libraries – 300-700 bp size (average 450 bp)



Cell Surface Protein libraries – ~200bp

Sample preparation recommendations for cell surface protein profiling

Single Cell Immune Profiling Solution with Feature Barcode technology

Diverse sample types validated at 10x Genomics

Cells Tested	Species	Cell Source	5′ GEX + V(D)J	5′ GEX + V(D)J + Cell Surface Protein	Surface Labels Tested
PBMC	Human	Extracted from blood from healthy donors	✓	✓	Antibody and Dextramer®
PBMC	Human	Extracted from blood from donors with Lupus or Rheumatoid Arthritis	✓	✓	Antibody
PBMC	Mouse	Extracted from blood from C57BL/6 and BALB/c mice	✓		
Splenocytes	Mouse	Cryopreserved dissociated spleen	✓		
Primary T and B cells	Human and Mouse	MACS- or FACS-based enrichment of CD19 or CD3 positive cells from PBMCs	✓	✓	Antibody
Dissociated tumor cells (10-80% T cells, depending on enrichment strategy)	Human	Cryopreserved dissociated tumor tissue (with or without enrichment) e.g. CCRC, NSCLC, MALT and Glioblastoma	✓	✓	Antibody
In vitro expanded T cells	Human	Selected from primary T cells using antigen-specific proliferation	✓	✓	Antibody and Dextramer®
T cell Lines	Human	Suspension cell culture and cryopreserved cell lines	✓	✓	Antibody
B cell lines	Human	Suspension cell culture and cryopreserved cell lines	✓	✓	Antibody

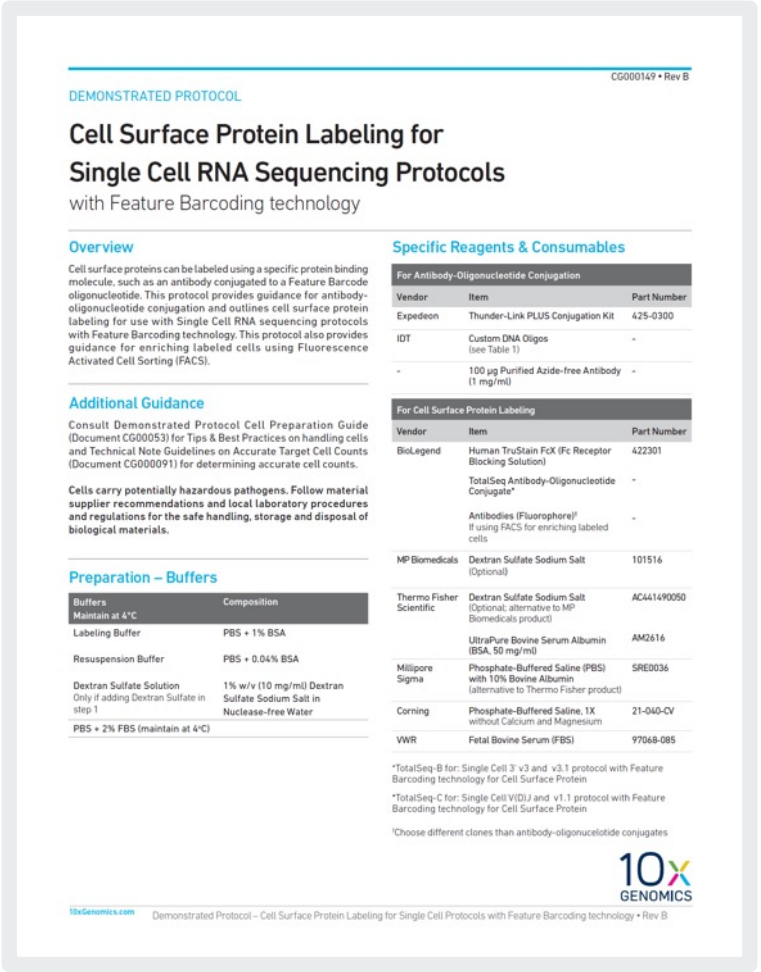
10x GENOMICS

Demonstrated protocol available from 10x Genomics

Cell Surface Protein Labeling

Key Workflow Steps

1. Block cells (optional), on ice
   a. FcX: block cell Fc receptors
2. Prepare antibody pool
   a. TotalSeq™-C antibodies from BioLegend, and/or custom oligo-conjugated antibodies
3. Remove antibody aggregates (14,000xg, 10 min)
4. Label cells by incubating with antibody pool
5. Wash cells to remove unbound antibodies
6. Count cells and proceed to making GEMs



CG000149 • Rev B

DEMONSTRATED PROTOCOL

Cell Surface Protein Labeling for Single Cell RNA Sequencing Protocols

with Feature Barcoding technology

Overview

Cell surface proteins can be labeled using a specific protein binding molecule, such as an antibody conjugated to a Feature Barcode oligonucleotide. This protocol provides guidance for antibody-oligonucleotide conjugation and outlines cell surface protein labeling for use with Single Cell RNA sequencing protocols with Feature Barcoding technology. This protocol also provides guidance for enriching labeled cells using Fluorescence Activated Cell Sorting (FACS).

Additional Guidance

Consult Demonstrated Protocol Cell Preparation Guide (Document CG00053) for Tips & Best Practices on handling cells and Technical Note Guidelines on Accurate Target Cell Counts (Document CG000091) for determining accurate cell counts.

Cells carry potentially hazardous pathogens. Follow material supplier recommendations and local laboratory procedures and regulations for the safe handling, storage and disposal of biological materials.

Preparation – Buffers

Buffers Maintain at 4°C	Composition
Labeling Buffer	PBS + 1% BSA
Resuspension Buffer	PBS + 0.04% BSA
Dextran Sulfate Solution Only if adding Dextran Sulfate in step 1	1% w/v (10 mg/ml) Dextran Sulfate Sodium Salt in Nuclease-free Water
PBS + 2% FBS (maintain at 4°C)	

Specific Reagents & Consumables

For Antibody-Oligonucleotide Conjugation		
Vendor	Item	Part Number
Expedeon	Thunder-Link PLUS Conjugation Kit	425-0300
IDT	Custom DNA Oligos (see Table 1)	-
-	100 µg Purified Azide-free Antibody (1 mg/ml)	-

For Cell Surface Protein Labeling		
Vendor	Item	Part Number
BioLegend	Human TruStain FcX (Fc Receptor Blocking Solution)	422301
	TotalSeq Antibody-Oligonucleotide Conjugate*	-
	Antibodies (Fluorophore)† If using FACS for enriching labeled cells	-
MP Biomedicals	Dextran Sulfate Sodium Salt (Optional)	101516
Thermo Fisher Scientific	Dextran Sulfate Sodium Salt (Optional; alternative to MP Biomedicals product)	AC441490050
	UltraPure Bovine Serum Albumin (BSA, 50 mg/ml)	AM2616
Millipore Sigma	Phosphate-Buffered Saline (PBS) with 10% Bovine Albumin (alternative to Thermo Fisher product)	SRE0036
Corning	Phosphate-Buffered Saline, 1X without Calcium and Magnesium	21-040-CV
VWR	Fetal Bovine Serum (FBS)	97068-085

*TotalSeq-B for: Single Cell 3' v3 and v3.1 protocol with Feature Barcoding technology for Cell Surface Protein

*TotalSeq-C for: Single Cell V(D)J and v1.1 protocol with Feature Barcoding technology for Cell Surface Protein

†Choose different clones than antibody-oligonucelotide conjugates

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10xGenomics.com Demonstrated Protocol – Cell Surface Protein Labeling for Single Cell Protocols with Feature Barcoding technology • Rev B

Prepare the antibody pool

- Target compatibility
  - Very common markers (ie, CD45) can overtake a library when pooled with rare markers
  - Can compensate by sequencing deeper
- Antibody titration
  - BioLegend recommendation: 1 µg per Ab/ 100 µL
  - 10x Verification and Validation data collected at 0.25 µg / 100 µL
  - Recommend using flow cytometry titrated concentrations

Target	Clone	Cell Type Target
CD3	UCHT1	T Cells
CD4	RPA-T4	CD4 T Cells
CD8a	RPA-T8	CD8 T Cells
CD14	M5E2	Monocytes
CD15	W6D3	Haematopoietic Cells
CD16	3G8	Natural Killer Cells/ Monocytes
CD56	QA17A16	Natural Killer Cells
CD19	HIB19	B Cells
CD25	BC96	Regulatory T Cells
CD45	HI30	Lymphocytes
CD45RA	HI100	Naive T Cells
CD45RO	UCHL1	Experienced T Cells
PD-1	EH12.2H7	Exhausted T Cells
TIGIT	A15153G	Exhausted T Cells
CD127	A019D5	Immature B and T Cells
Isotype control IgG1	MOPC-21	IgG1
Isotype control IgG2a	MOPC-173	IgG2a
Isotype control IgG2b	MPC-11	IgG2b

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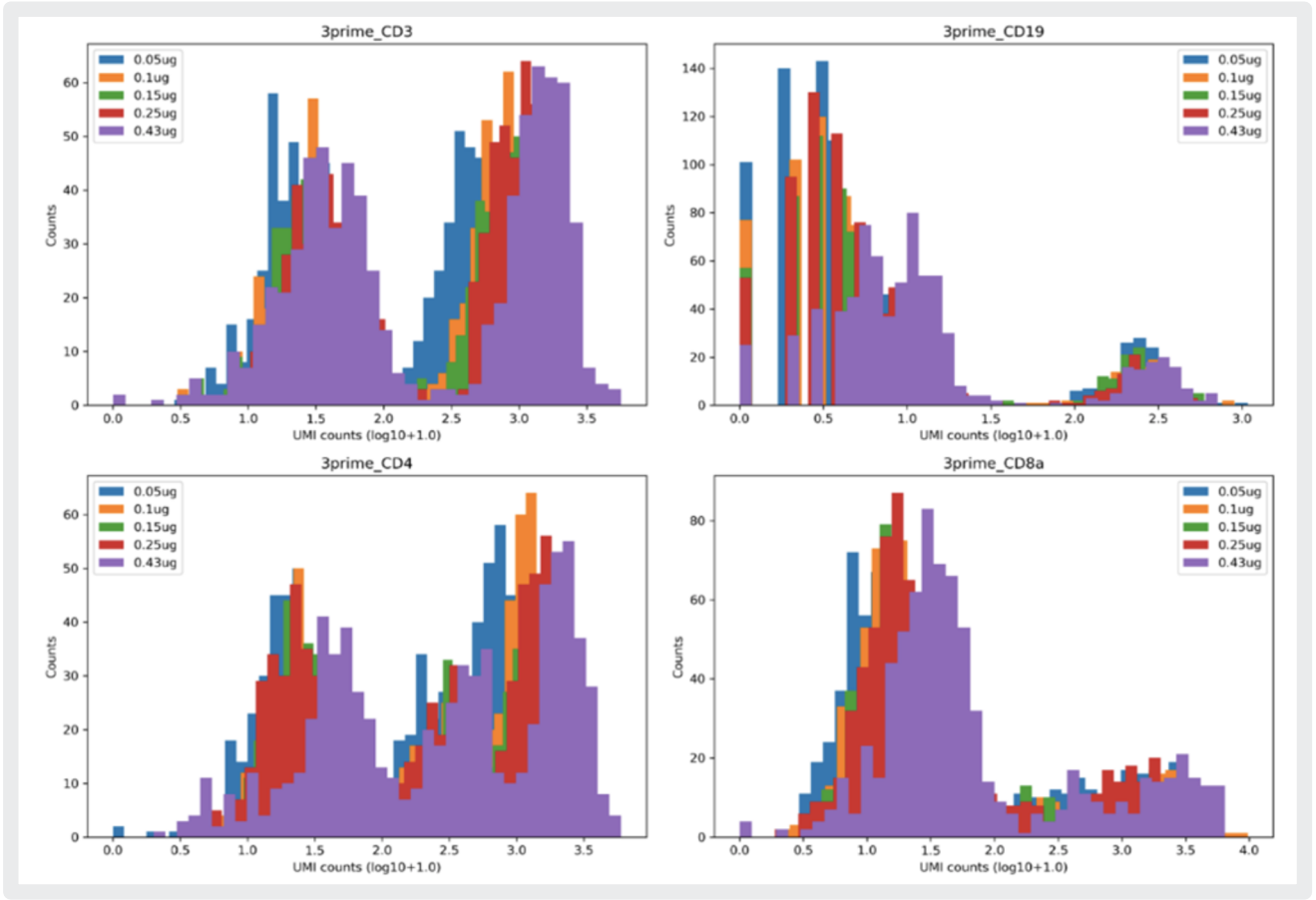
Titrating antibody concentrations for cell surface protein labeling

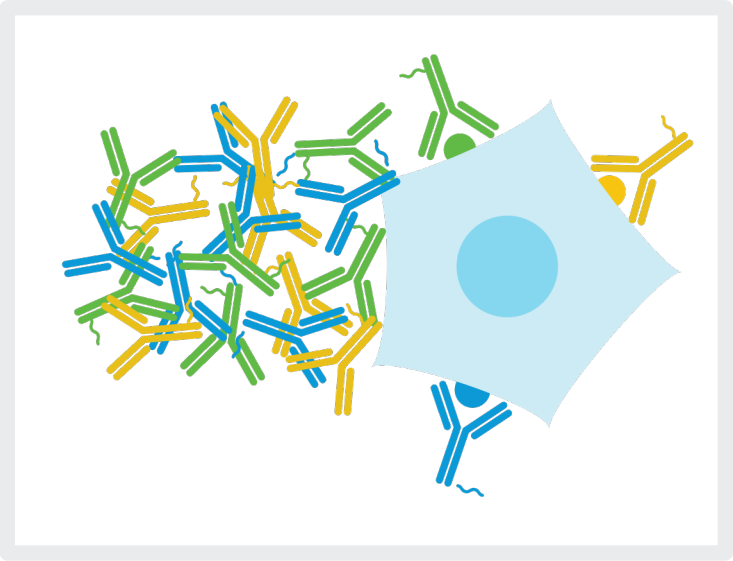
Specificity observed over ~1 log range of concentration

- Tested antibodies are broadly resilient to input concentration
- Matching flow cytometry concentrations is a good starting place
- BioLegend can provide additional support on titration.



All antibodies in suspension form aggregates over time

Remove aggregates before staining

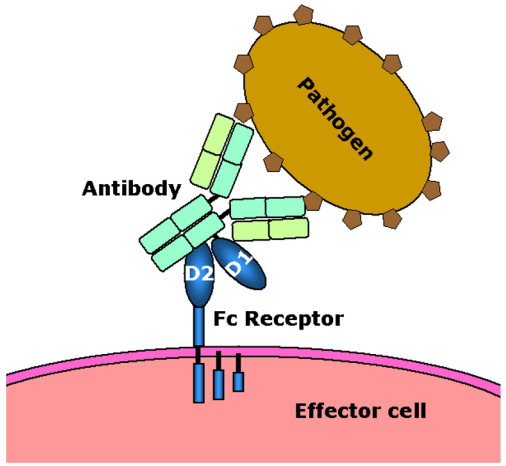


1. Make a pool of the desired antibodies for staining, allowing ~10% excess volume
2. Centrifuge at 14,000 rcf for 10 minutes at 4°C
3. Carefully remove the supernatant for use
4. Stain with appropriate volume of antibody pool
   - Alternate: filter antibody pool through 0.2 um filter
   - 10x have not validated storage of pooled antibodies, refer to BioLegend for recommendations

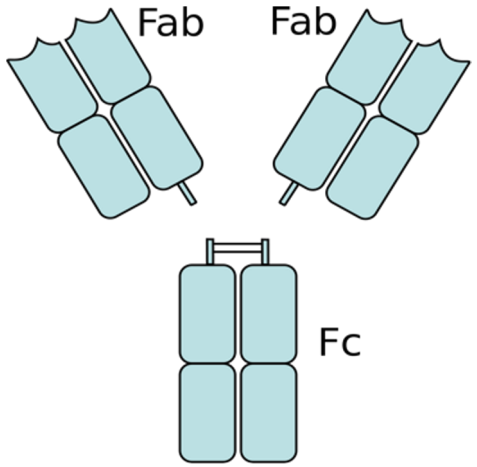
Fcx (Fc block) decreases nonspecific binding to Fc receptors

Optional, but recommended, step prior to labeling cells with specific antibodies

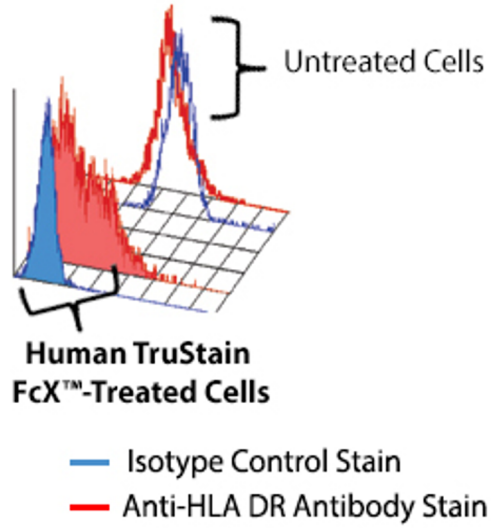
FC Receptor binds antibodies



We want to capture specific interaction via the Fab region



FcX helps Feature Barcode technology in the same way it helps Flow Cytometry



https://www.biolegend.com/en-us/products/human-trustain-fcx-fc-receptor-blocking-solution-6462

Labeling and washing cells

Buffers

- Recommend PBS + 1% BSA for labeling, washing and final resuspension
- Delicate cell types
  - Staining can be done in media, buffer + FBS, or any cell-compatible buffer
  - Recommended: final resuspension in PBS + 1% BSA

Centrifugation conditions

- Select cell centrifugation speed and time based on cell type

Sample Types	Speed (rcf)	Time (min)
Cell Lines	300	5
PBMCs	400	5
Dissociated Tumor Cells	150	10

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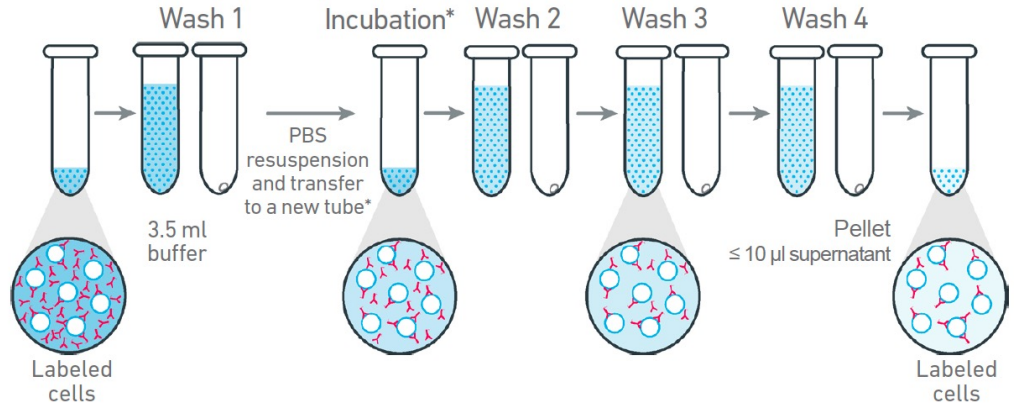
Labeling and washing cells

- Adequate washing is essential to reduce background
- Wash 4 times in 5 ml tubes
- Transfer to a new tube after wash 1
  - To reduce background from antibodies adhering to tube
- Optional PBS incubation step
  - 100 µl PBS, 5 min, room temp
  - Helps reduce background from unbound antibodies
  - Can skip this step if working with fragile cells

Wash Steps in 5-ml Microcentrifuge Tubes



*Exclude these steps for fragile cells and proceed directly to Wash 2 and transfer the cells to a new tube after adding 3.5 ml buffer.

Leaving behind excess supernatant can lead to increased background from unbound antibodies

10X GENOMICS

Cell surface protein labeling for single cell immune profiling with Feature Barcode technology

Do's

- Make antibody pools fresh every experiment
- Perform optimization for antibody pooling to determine the optimal concentration for your sample type
- Concentrations that show good staining and low background in flow cytometry are readily portable into the Feature Barcode workflow
- During washing steps (esp first wash), remove as much supernatant as possible to prevent carryover of unbound antibodies
- Obtain high quality cells

Don'ts

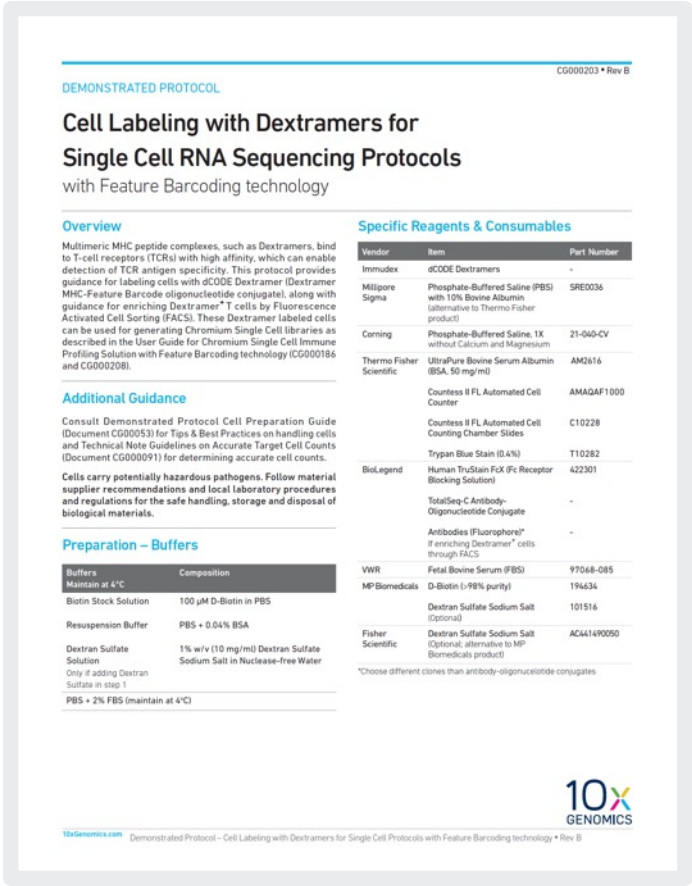
- Don't rush! Depending on the cell type and number of cells, the pellet may be difficult to see. Avoid disturbing the pellet
- Don't forget to centrifuge antibodies before use to avoid aggregates
- Don't resuspend cells too roughly. Ambient RNA will get partitioned across all GEMs—increasing background

10x GENOMICS

Demonstrated protocol available from 10x Genomics

Cell labeling with Dextramer® Reagents

- Demonstrated protocol optimized using dCODE™ Dextramer® Reagents from Immudex
- Cells may also be labeled with
  - TotalSeq™-C antibodies from BioLegend
  - custom oligonucleotide-conjugated antibodies
  - and/or antibodies for FACS
- Includes suggestions for enriching for Dextramer®-positive T-cells by FACS to enable identification of low frequency TCR:antigen binding events
- Validated using 3-20 x 10^6 cells



CG000203 • Rev B

DEMONSTRATED PROTOCOL

Cell Labeling with Dextramers for Single Cell RNA Sequencing Protocols

with Feature Barcoding technology

Overview

Multimeric MHC peptide complexes, such as Dextramers, bind to T-cell receptors (TCRs) with high affinity, which can enable detection of TCR antigen specificity. This protocol provides guidance for labeling cells with dCODE Dextramer (Dextramer MHC-Feature Barcode oligonucleotide conjugate), along with guidance for enriching Dextramer® T cells by Fluorescence Activated Cell Sorting (FACS). These Dextramer labeled cells can be used for generating Chromium Single Cell libraries as described in the User Guide for Chromium Single Cell Immune Profiling Solution with Feature Barcoding technology (CG000186 and CG000208).

Additional Guidance

Consult Demonstrated Protocol Cell Preparation Guide (Document CG00053) for Tips & Best Practices on handling cells and Technical Note Guidelines on Accurate Target Cell Counts (Document CG000091) for determining accurate cell counts.

Cells carry potentially hazardous pathogens. Follow material supplier recommendations and local laboratory procedures and regulations for the safe handling, storage and disposal of biological materials.

Preparation – Buffers

Buffers Maintain at 4°C	Composition
Biotin Stock Solution	100 µM D-Biotin in PBS
Resuspension Buffer	PBS + 0.04% BSA
Dextran Sulfate Solution Only if adding Dextran Sulfate in step 1	1% w/v (10 mg/ml) Dextran Sulfate Sodium Salt in Nuclease-free Water
PBS + 2% FBS (maintain at 4°C)	

Specific Reagents & Consumables

Vendor	Item	Part Number
Immudex	dCODE Dextramers	-
Millipore Sigma	Phosphate-Buffered Saline (PBS) with 10% Bovine Albumin (alternative to Thermo Fisher product)	SRE0036
Corning	Phosphate-Buffered Saline, 1X without Calcium and Magnesium	21-040-CV
Thermo Fisher Scientific	UltraPure Bovine Serum Albumin (BSA, 50 mg/ml)	AM2616
	Countess II FL Automated Cell Counter	AMAQAF1000
	Countess II FL Automated Cell Counting Chamber Slides	C10228
	Trypan Blue Stain (0.4%)	T10282
BioLegend	Human TruStain FcX (Fc Receptor Blocking Solution)	422301
	TotalSeq-C Antibody-Oligonucleotide Conjugate	-
	Antibodies (Fluorophore)* If enriching Dextramer® cells through FACS	-
VWR	Fetal Bovine Serum (FBS)	97068-085
MP Biomedicals	D-Biotin (>98% purity)	194634
	Dextran Sulfate Sodium Salt (Optional)	101516
Fisher Scientific	Dextran Sulfate Sodium Salt (Optional; alternative to MP Biomedicals product)	AC441490050

*Choose different clones than antibody-oligonucleotide conjugates

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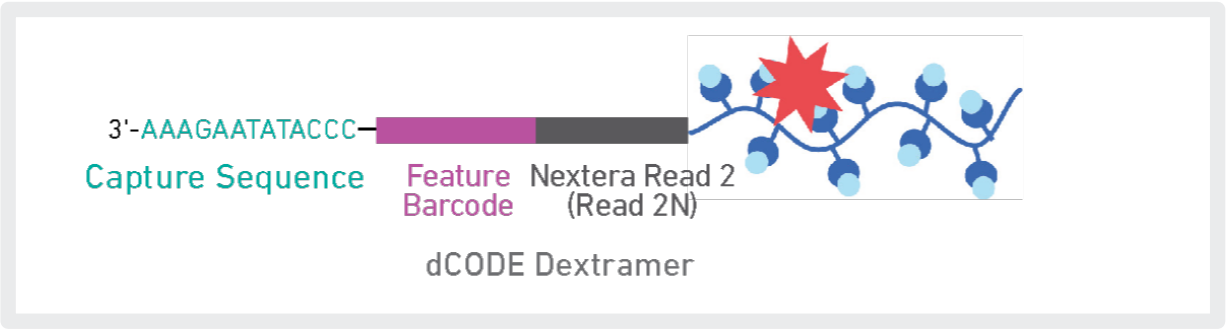
10xGenomics.com Demonstrated Protocol – Cell Labeling with Dextramers for Single Cell Protocols with Feature Barcoding technology • Rev B

Immudex dCODE™ Dextramer® Reagents

- Supplied by Immudex
- No need for titrating concentrations — use 2 µl of each Dextramer® per sample
- We have successfully multiplexed panels of varying sizes:
  - 12 Dextramers® and 14 TotalSeq™-C antibodies (plus isotype controls)
  - 44 Dextramers® and 11 TotalSeq™-C antibodies (plus isotype controls)



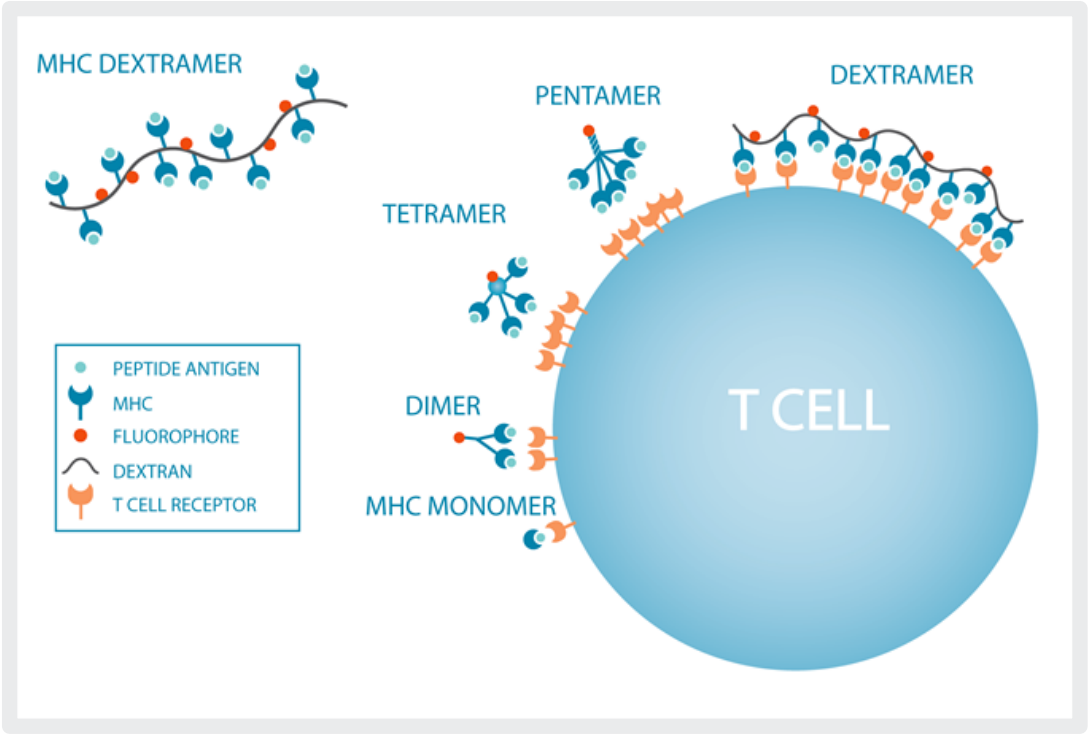


Image from Immudex: immudex.com

Differences & similarities with cell surface protein labeling protocol

Side-by-side comparison

	Cell Surface Protein	Dextramer® & Cell Surface Protein
Blocking reagents	TruStain FcX	TruStain FcX & Biotin
Labeling and wash buffers	PBS + 1% BSA	PBS + 2% FBS*
Wash steps	3 washes	3 washes

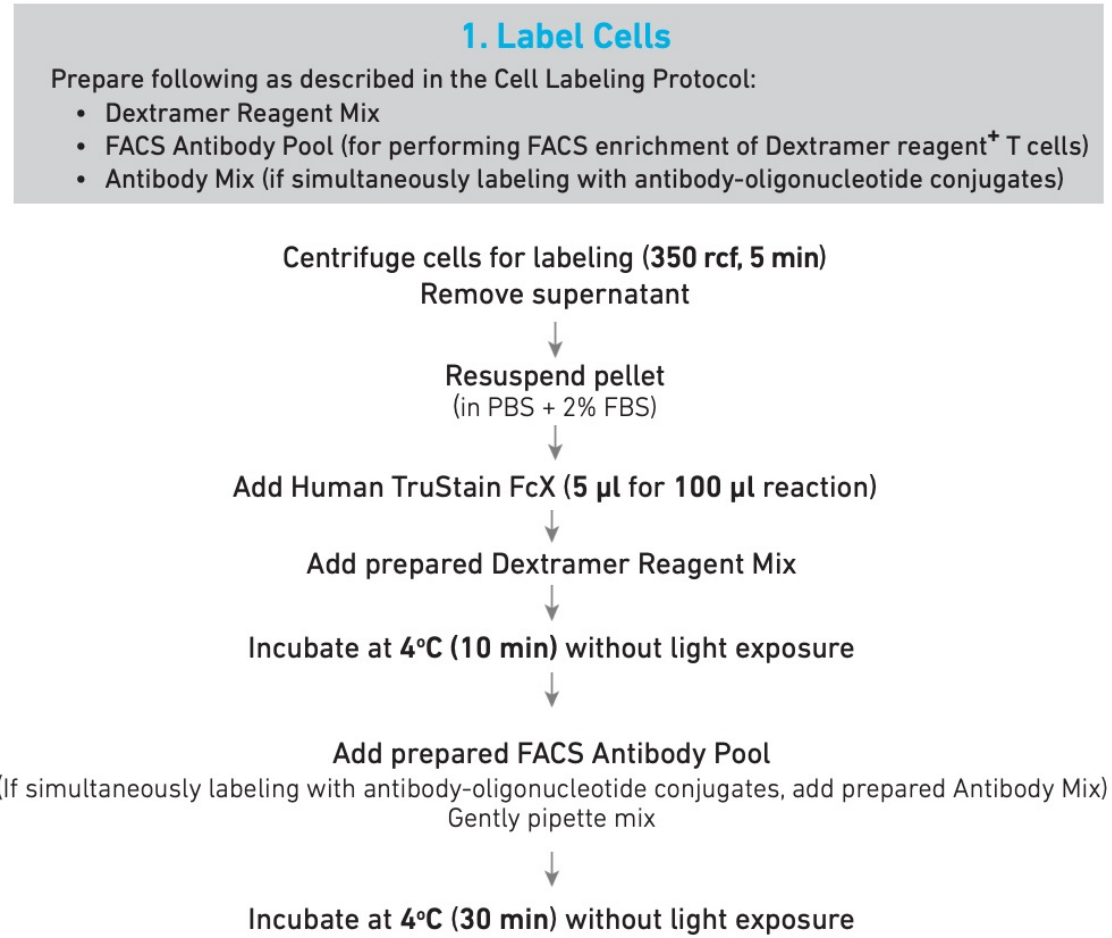
**Cells are happier in FBS when going through the flow sorter*

Sample preparation: Labeling with Dextramers® and antibodies

Label cells: Key workflow steps

1. Prepare Dextramer® mix
   a) Add Biotin Stock Solution
   b) Add Dextramers®
   c) Avoid light exposure to the mix
2. Prepare FACS Antibody Pool and Antibody Mix if using.
3. Spin down cells and resuspend in <100 µl of PBS + 2% FBS. .
4. Add prepared Dextramer® Mix.

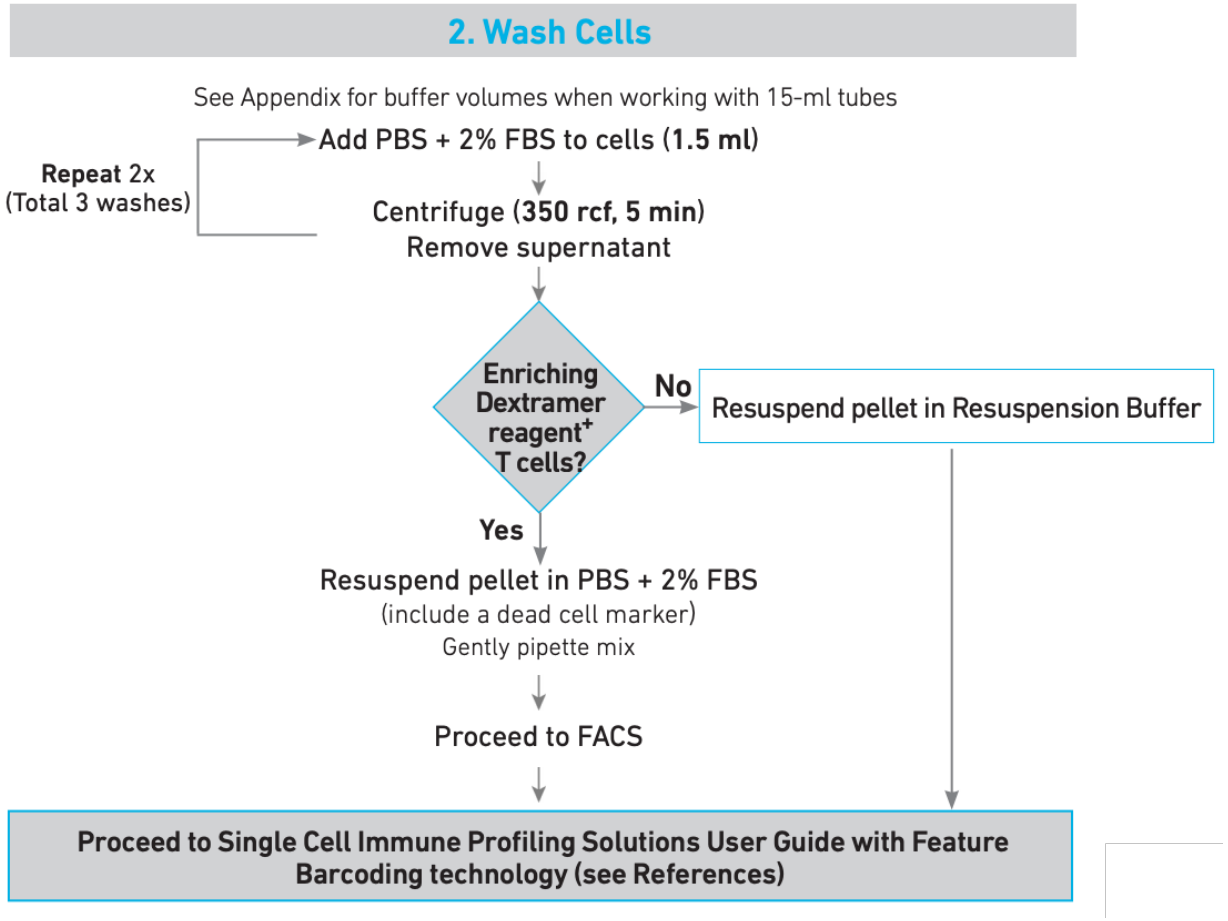
   WARNING: DO NOT add FACS Antibody Pool and/or Antibody Mix at this step.



Sample preparation: Wash steps

Wash cells: Key workflow steps

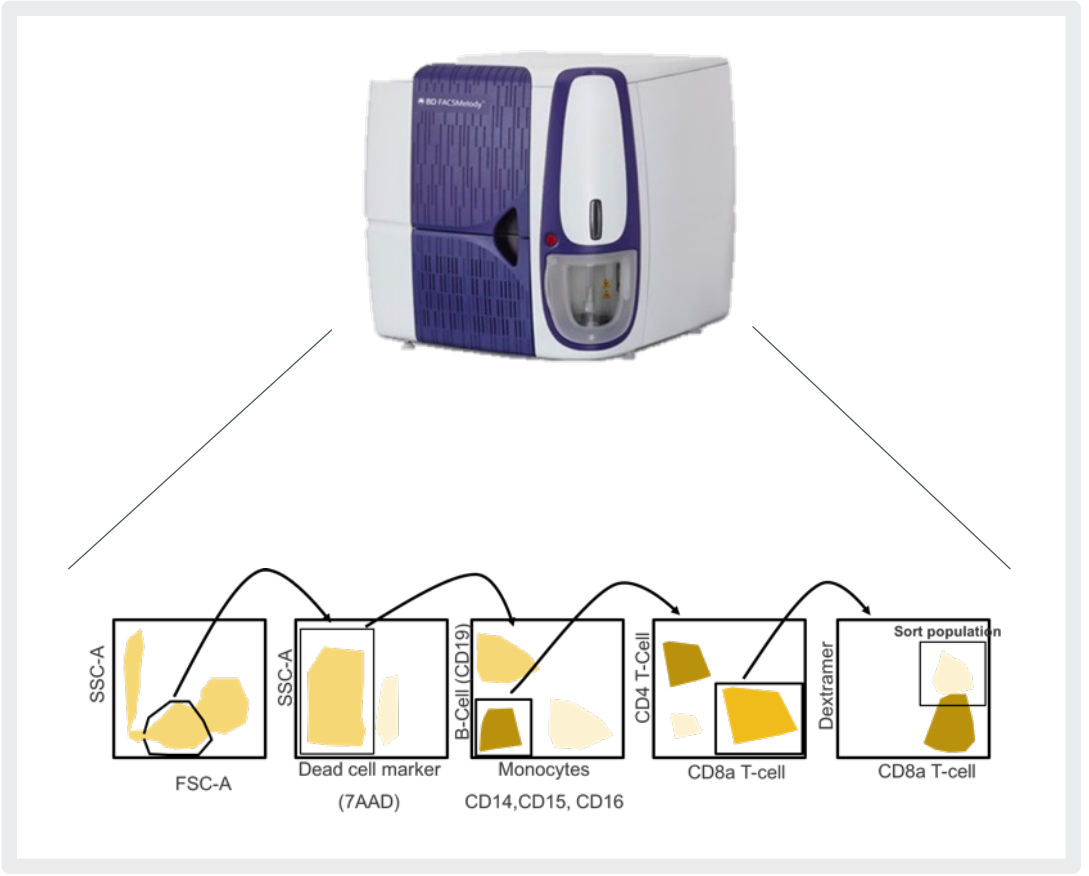
1. Wash in 1.5 ml PBS + 2% FBS
2. Wash 3X using a wide-bore pipette tip.
3. Optional (recommended): Enrich for Dextramer®-positive cells by FACS.
4. If not performing FACS, proceed directly to Chromium Single Cell Immune Profiling Solutions User Guide with Feature Barcode technology.



Appendix: Flow sorting strategy

Enrich for Dextramer®-positive cells post-labeling

- Sorting is recommended to increase specificity of signal
- Assume at least 50% cell loss
  - Cell loss depends on starting cell numbers. Lower numbers = more loss
- Collect FACS enriched cells in PBS + 10% FBS
- **Always count cells after flow sorting**
- Potential gating strategy shown



Cell Labeling with Dextramer® Reagents for Single Cell RNA Sequencing Protocols with Feature Barcode technology

Dos

- Make buffers fresh every time
- Add biotin to tube before adding Dextramers®
- Add blocking reagents prior to labeling
- Label cells with Dextramer® Mix prior to adding antibodies
- Perform 3 thorough wash steps to remove background
- Be gentle when handling cells
- Count cells with a cell counter prior to loading

Don’ts

- Don’t rush! Handle cells gently and avoid disturbing the pellet
- But also don’t waste time, especially post flow-sorting. Cells that have been sorted should be moved quickly into the 10x workflow.
- Don’t add FACS Antibody Pool or Antibody Mix concurrently with the Dextramer® Mix.
- Don’t rely on FACS count for accurate cell counting

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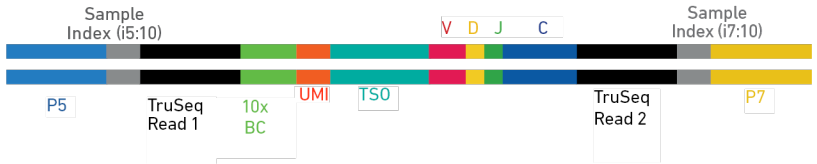
Sequencing Cell Surface Protein Libraries

Sequencing V(D)J, 5′ Gene Expression, and Cell Surface Protein Libraries

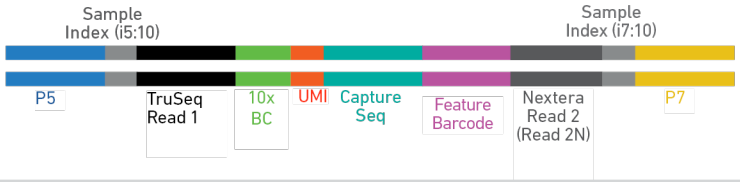
Libraries may be pooled in various configurations

Chromium Single Cell V(D)J Dual Index Library



Chromium Single Cell 5' Gene Expression Dual Index Library

Chromium Single Cell 5' Cell Surface Protein Dual Index Library*



Sequencing Depth	Minimum 5,000 read pairs per cell for V(D)J Dual Index library Minimum 20,000 read pairs per cell for 5' Gene Expression Dual Index library Minimum 5,000 read pairs per cell for Cell Surface Protein Dual Index library
Sequencing Type	Paired-end, Dual indexing
Sequencing Read	Read 1: 26 cycles i7 Index: 10 cycles i5 Index: 10 cycles Read 2: 90 cycles

All libraries are sequenced with 10 bp sample indices (i5 and i7)

**Detects antigen specificity in cells labeled with antibodies and Dextramer reagents.*

Sequencing recommendations: Pooling guidelines

The following **dual index** libraries can be pooled in any combination for Illumina sequencing:

Single Cell 3’ v3.1 Dual Index Gene Expression Libraries*

Single Cell 3’ v3.1 Dual Index Cell Surface Protein or CRISPR Screening Libraries*

Single Cell 5’ v2 Gene Expression or V(D)J Libraries

Single Cell 5’ v2 Cell Surface Protein Libraries

Spatial Gene Expression Libraries*

We have not tested the compatibility of pooling for sequencing with:

Single Cell ATAC libraries

Single Cell CNV libraries

* If run with 28 cycles for Read 1

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Sequencing V(D)J and 5′ Gene Expression Libraries

Consider sequencing depth requirements when pooling libraries

- Recommended sequencing depths
  - V(D)J Library: 5,000 read pairs per cell
  - 5′ Gene Expression Library: 20,000 read pairs per cell
  - Cell Surface Protein Library: 5,000 read pairs per cell

Library Pooling Examples:

Libraries	Sequencing Depth (read pairs per cell)	Library Pooling Ratio
Example 1		
V(D)J Enriched library	5,000	1
5' Gene Expression library	20,000	4
Cell Surface Protein library	5,000	1
Example 2		
V(D)J Enriched library	5,000	1
5' Gene Expression library	50,000	10
Cell Surface Protein library	5,000	1

MiSeq

NextSeq 500/550

HiSeq 2500

HiSeq 3000/4000

NovaSeq

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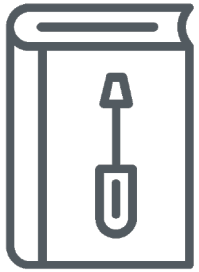
Reference materials and resources

Available at support.10xgenomics.com

Specifications

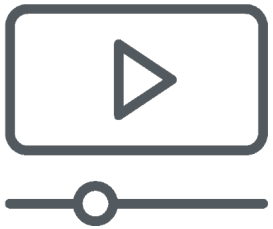


User guides

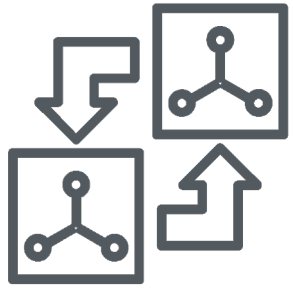
Product sheet

Technical notes

Public data sets



How-to-videos



Demonstrated protocols

Contact us: support@10xgenomics.com

10x Genomics Support contact information

Support overview:

http://support.10xgenomics.com

Q&A knowledgebase:

https://kb.10xgenomics.com/hc/en-us

Please send questions, comments, and feedback to:

support@10xgenomics.com

