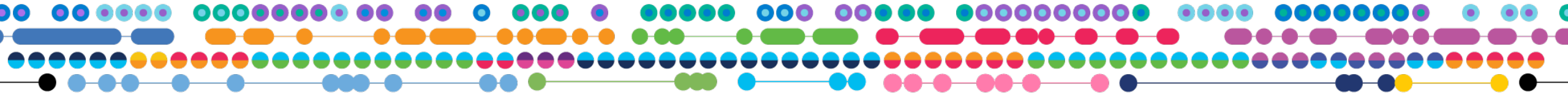
10x GENOMICS

Chromium Single Cell Gene Expression Solution with Feature Barcoding technology

Workflow Training

CG000191 Rev A



Chromium Single Cell Gene Expression Solution with Feature Barcoding technology

Agenda

- Single Cell Gene Expression Introduction
- Solution Overview
- Solution Biochemistry
- Sample Preparation Recommendations
- Chromium Single Cell Gene Expression Workflow
- Technical Specifications and Selected Data

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Chromium Single Cell Gene Expression Solution with Feature Barcoding technology

Solution Features and Overview

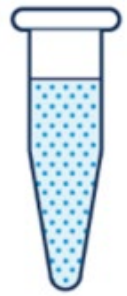
Chromium Single Cell Gene Expression Solution with Feature Barcoding technology

Solution Features

- Unbiased single cell transcriptome 3’ gene expression that enables discovery research
- Simultaneously assess perturbation phenotypes, protein abundance and gene expression from the same cell
- Ready-to-use, robust workflow, including demonstrated protocols for various sample types such as cell lines, primary cells, dissociated fresh tissue
- Compatible with whole cells and nuclei
- Latest improvements increase sensitivity enabling the detection of more unique transcripts per cell, potentially decreasing sequencing requirements
- Easy-to-use and convenient software with Cell Ranger Analysis Pipeline and Loupe Cell Browser visualization tools

Chromium Single Cell Gene Expression Solution with Feature Barcoding technology

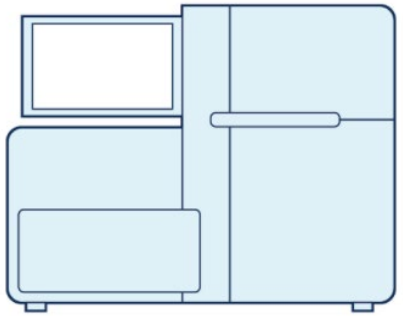
Input | **Library Creation** | **Sequence** | **Data Analysis and Visualization**



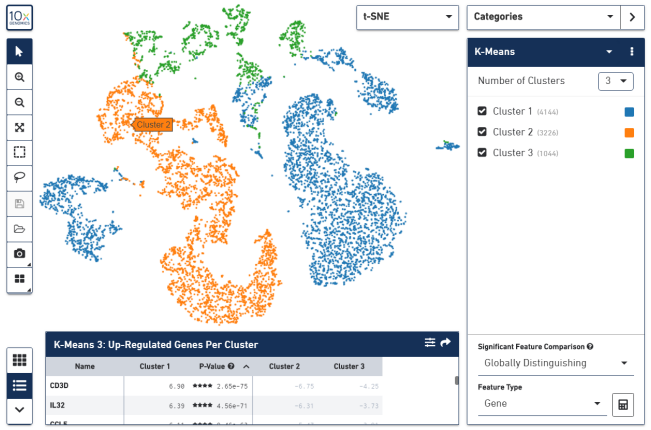
User-Supplied Cells or Nuclei

Chromium Controller and Single Cell 3' Reagents

- Chromium Single Cell 3' GEM, Library & Gel Bead Kit v3
- Chromium Single Cell 3' Feature Barcode Library Kit
- Chromium Chip B Single Cell Kit
- Chromium i7 Multiplex Kit



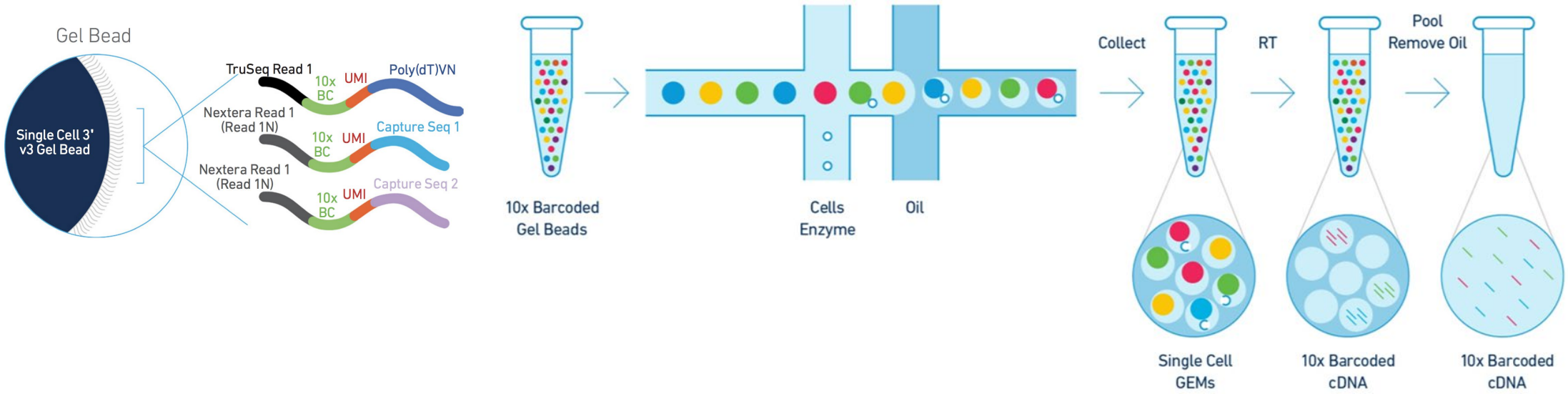
Sequencer



Cell Ranger Loupe Cell Browser

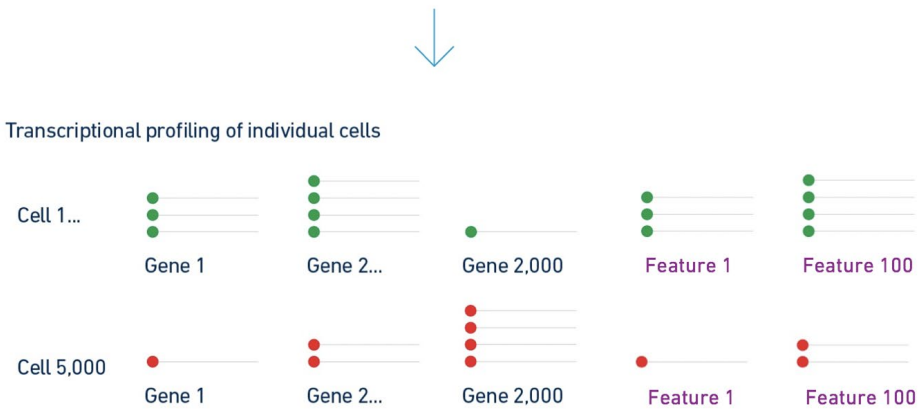
Single Cell Gene Expression with Feature Barcoding technology

Biochemistry Overview



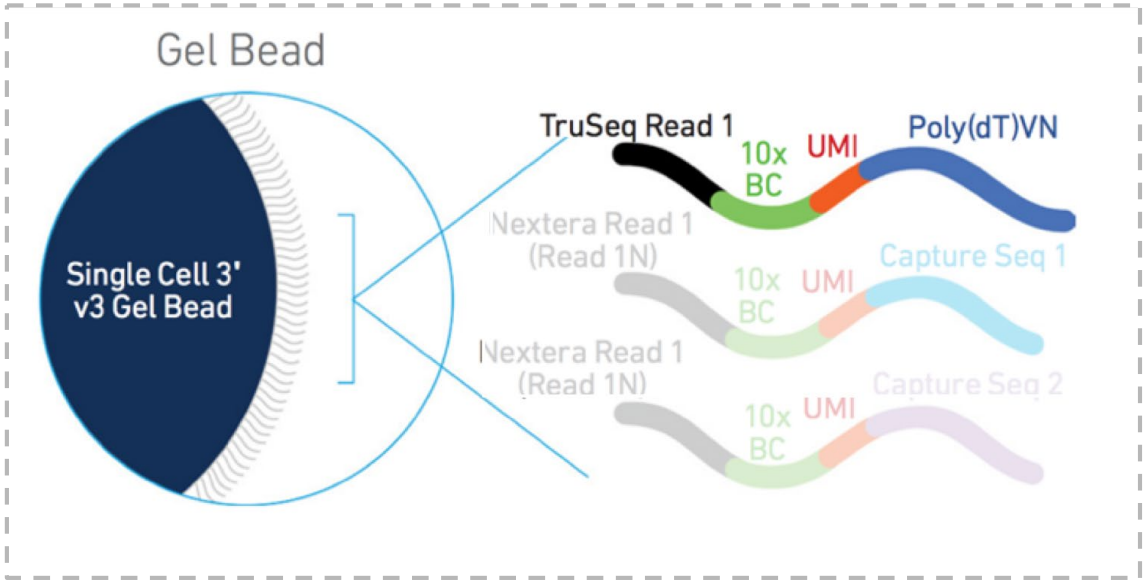
- Inputs:
  10x Gel Beads, Reagents and single cells in suspension

- Outputs:
  Digital gene expression and cell surface protein expression or CRISPR perturbation profiles from every partitioned cell



Single Cell Gene Expression with Feature Barcoding technology

Feature Barcoding technology enabled via Single Cell 3' v3 Gel Beads



i. **TruSeq Read 1**

22 nt Partial Illumina TruSeq Read 1 sequence

ii. **10x BC**

16 nt 10x Barcode
~3.6 M defined barcode sequences

iii. **UMI**

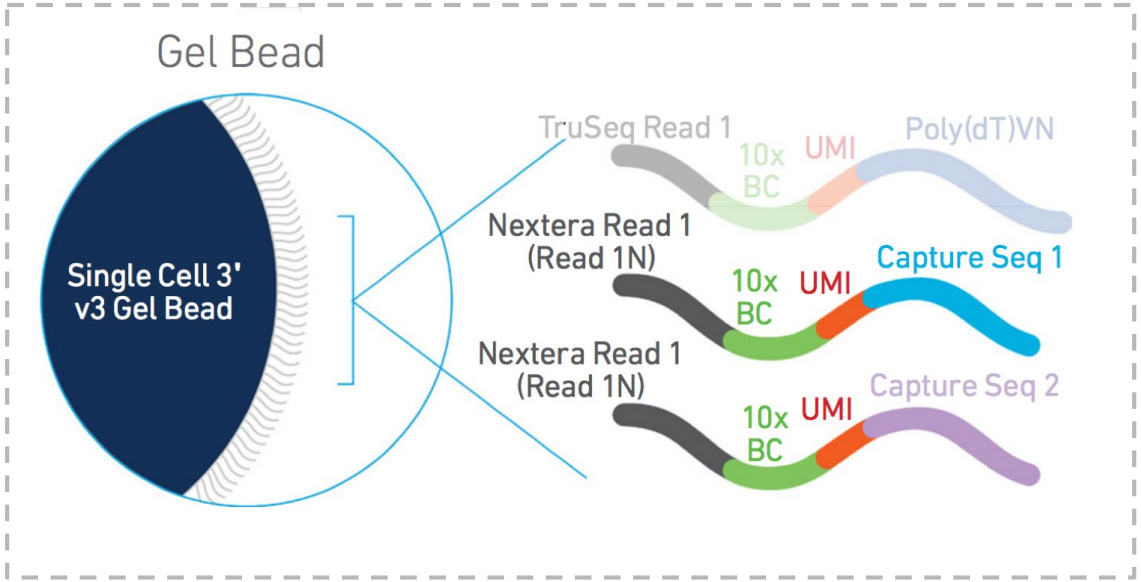
12 nt Unique Molecular Identifier

iv. **Poly(dT)VN**

30 nt Poly(dT) sequence
Enables capture of poly-adenylated mRNA molecules

Single Cell Gene Expression with Feature Barcoding technology

Feature Barcoding technology enabled via Single Cell 3' v3 Gel Beads



i. **Nextera Read 1 (Read 1N)**

22 nt Partial Illumina Nextera Read 1 sequence (Enables selective enrichment of the Feature Barcode construct)

ii. **10x BC**

16 nt 10x Barcode
~3.6 M defined barcode sequences

iii. **UMI**

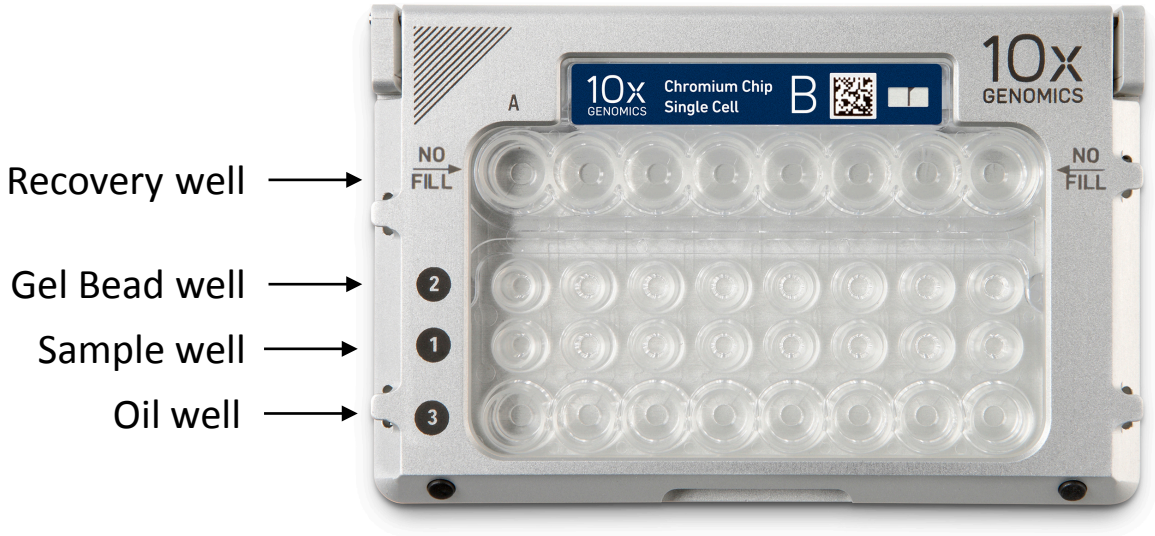
12 nt Unique Molecular Identifier

iv. **Capture Sequence 1** or **2**

22 nt sequence that is the reverse complement of the sequence inserted into the DNA (Antibody) or RNA (sgRNA) based Feature

Chromium Chip B Single Cell Performance

Single-use microfluidics chip



- Up to 8 channels processed in parallel
- 500 to 10000 cells per channel
- 8.5 minute run time per chip
- Up to 30 µm cell diameter tested
- Up to 65 % cell processing efficiency
- Temperature Range 18-28˚C

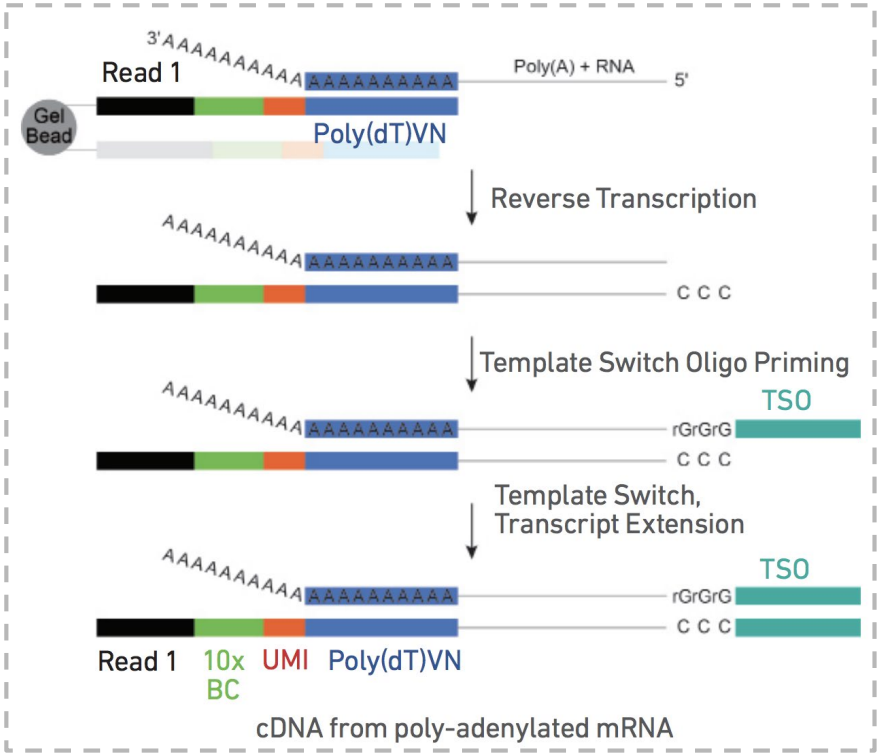
User controlled trade-off between cell numbers and doublet rate

Number of Cells	Expected Multiplet Rate (%)*
500	~0.4
1000	~0.8
5000	~3.9
10000	~7.6

Single Cell Gene Expression Workflow: Target Capture in GEMs

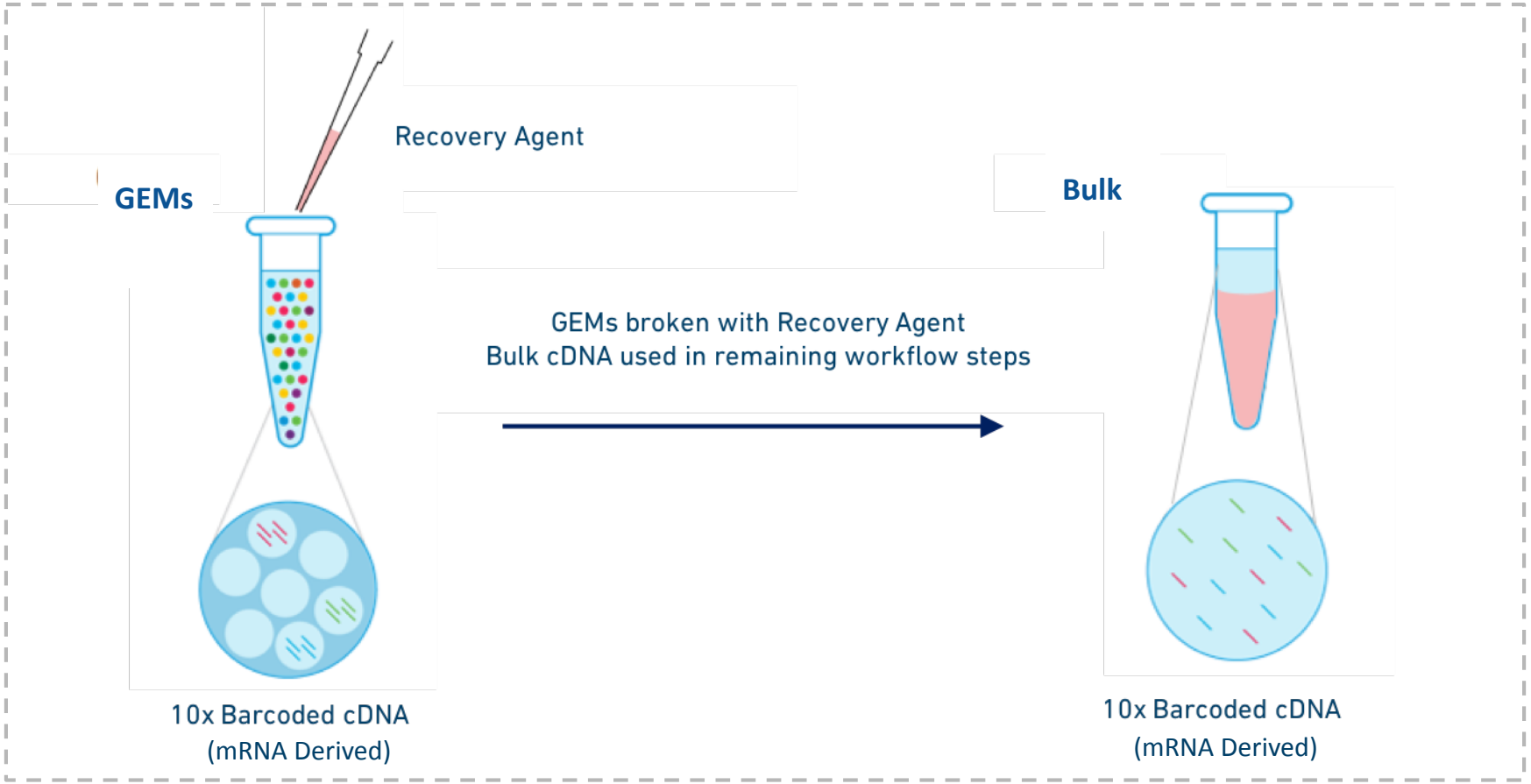
Direct capture of poly-adenylated mRNA inside individual GEMs

Barcoded, full-length cDNA from poly-adenylated mRNA



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

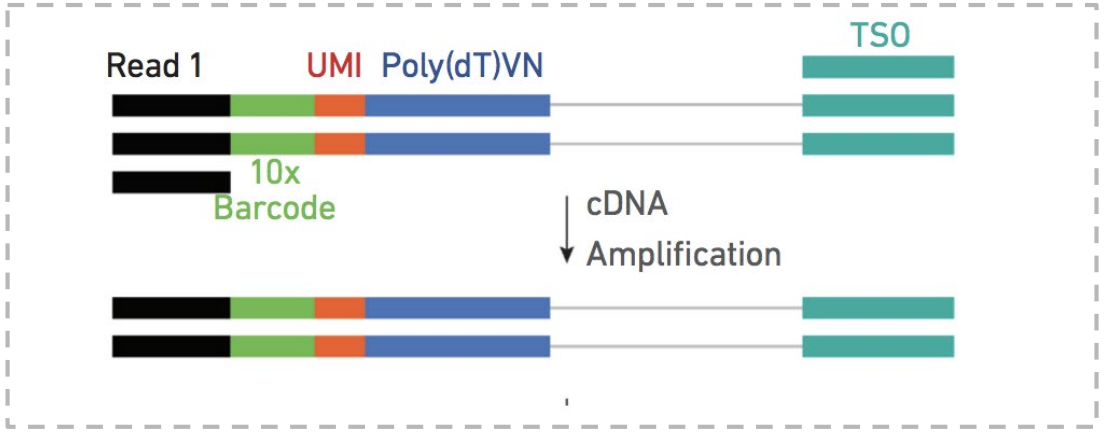
Single Cell Gene Expression Workflow: Breaking GEMs



Single Cell Gene Expression Workflow: cDNA Amplification

In Bulk Amplification of cDNA

cDNA Amplification of poly-adenylated mRNA

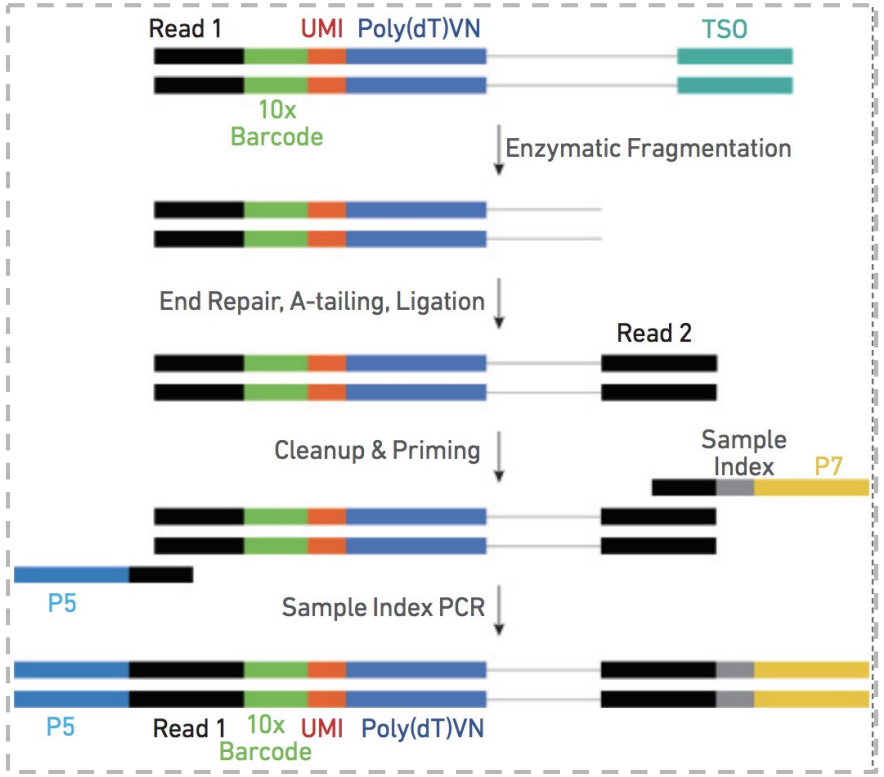


Note: For cDNA Amplification of poly-adenylated mRNA please use: cDNA Primers (PN 2000089)

Single Cell Gene Expression Workflow: Library Construction

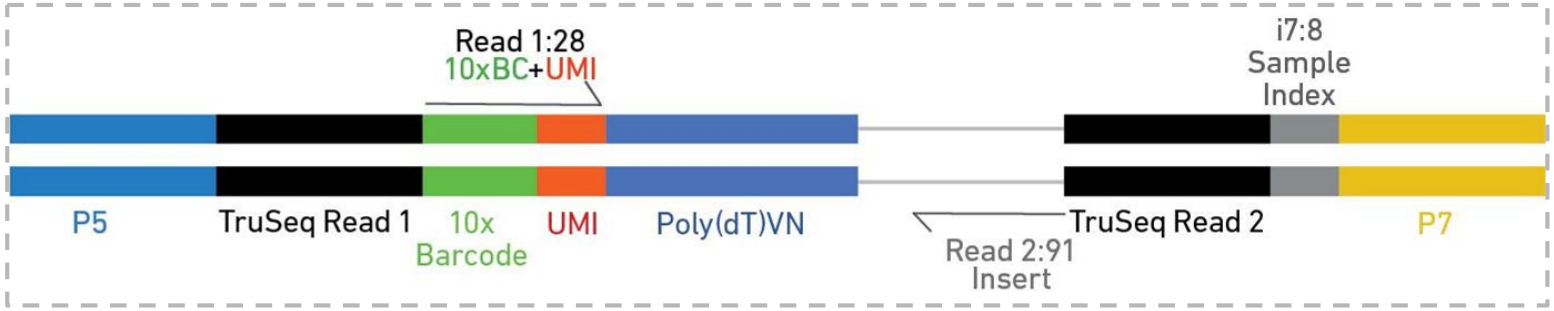
In Bulk Library Construction

Library construction of poly-adenylated mRNA



Recommended Read Length

Single Cell 3’ Gene Expression Library

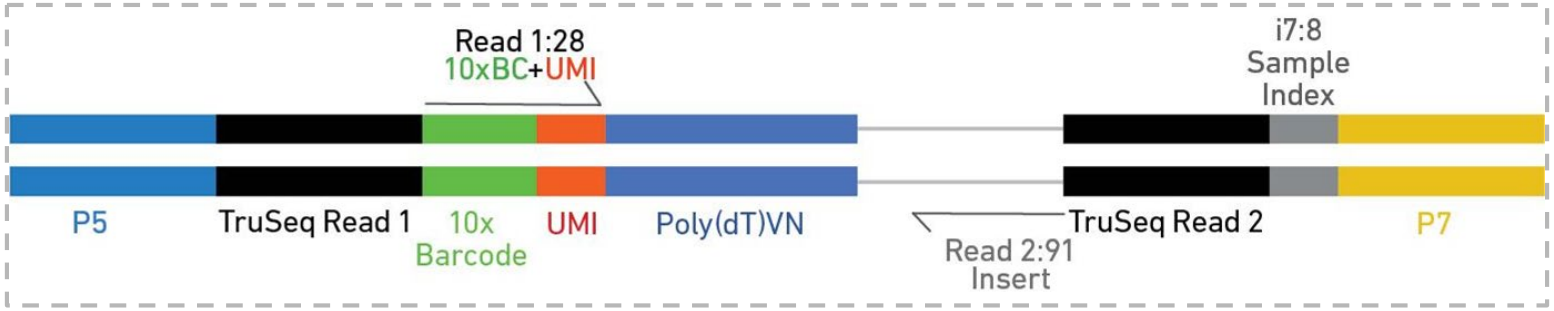


	Read 1	i7 Index	i5 Index	Read 2
Purpose	10x Barcode & UMI	Sample Index	N/A	Transcript
Length	28*	8	0	91

Note: * If pooled with Single Cell 3’ Gene Expression Libraries v2, ensure that the Read 1 length is adjusted to 28 bp

Supported Sequencers

Single Cell 3’ Gene Expression Library



	Single Cell 3’ Gene Expression
Raw Read Pairs per Cell	Minimum 20,000

MiSeq

NextSeq

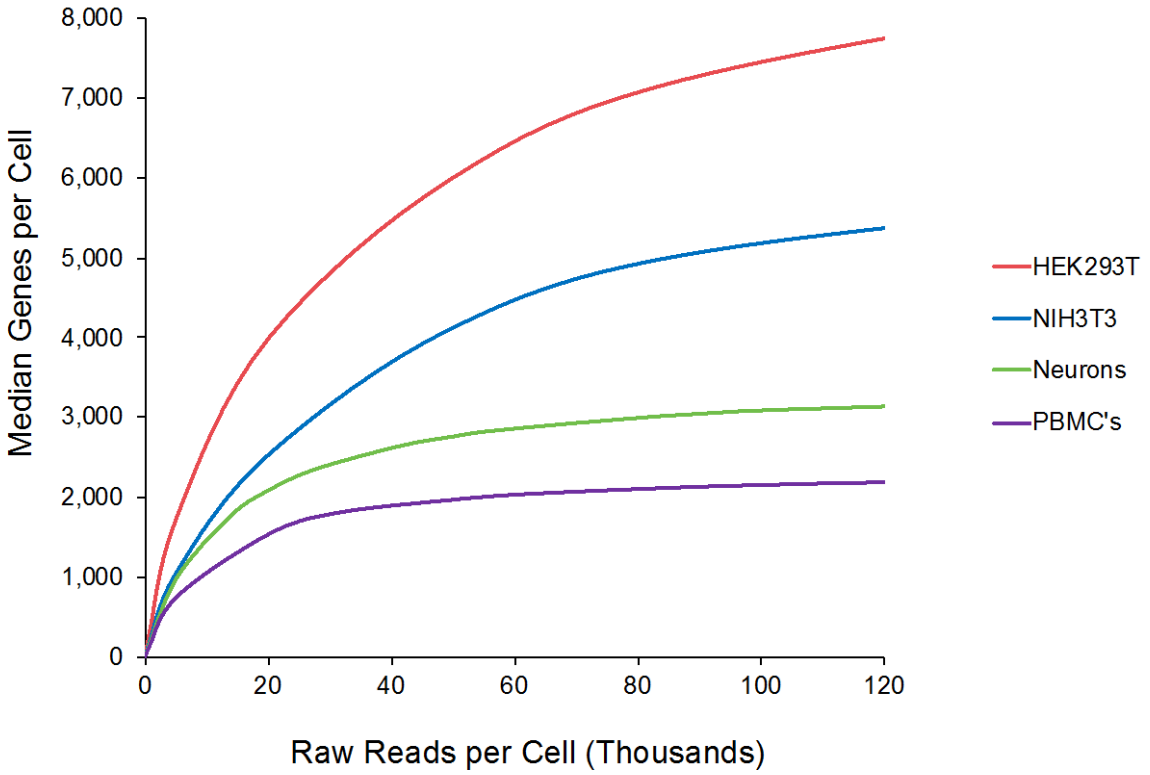
HiSeq2500

HiSeq 3000/4000

NovaSeq

Sequencing Depth for Typical Samples

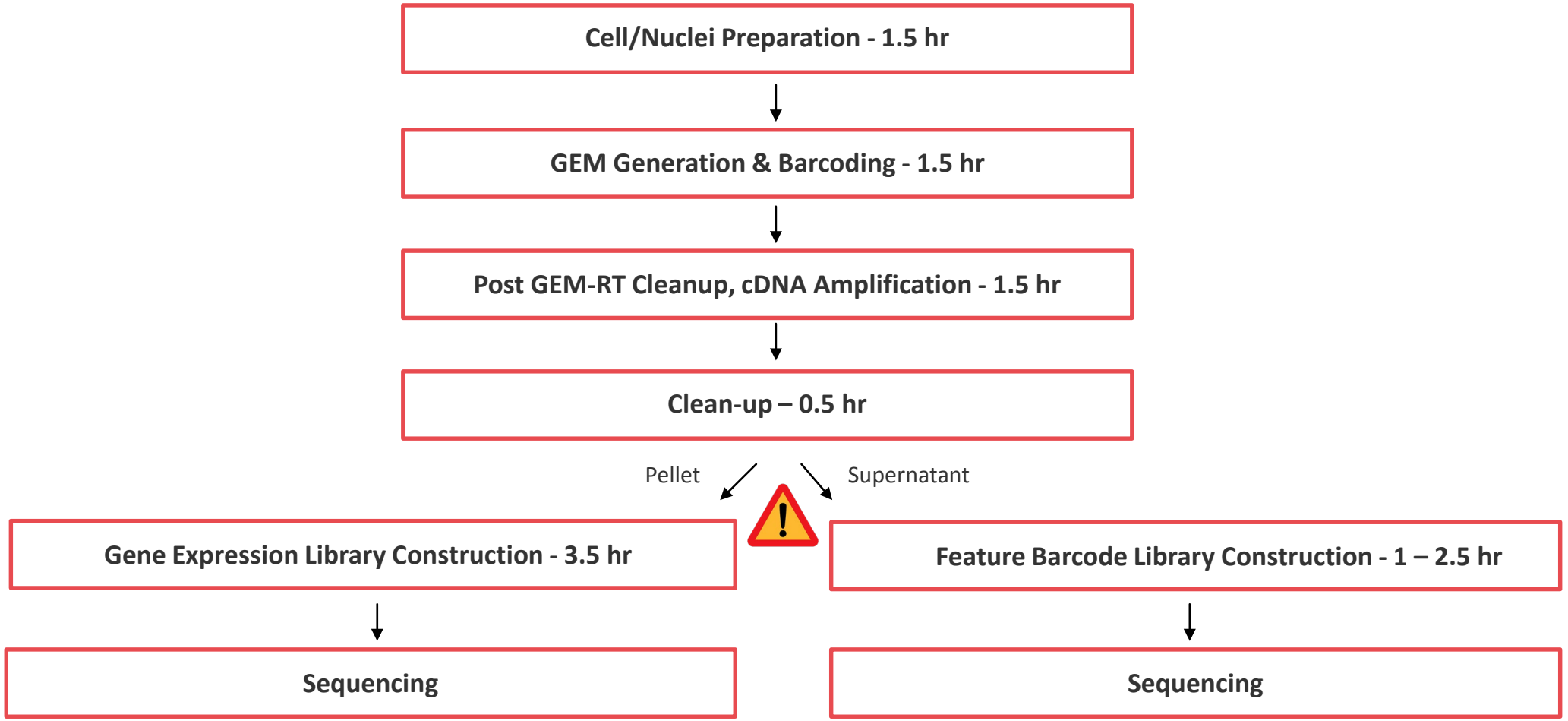
Gene Expression Libraries



- 20,000 raw read pairs per cell is the recommended minimum sequencing depth for typical samples.
- Given variability in cell counting and loading, extra sequencing may be required if the cell count is higher than anticipated.

Chromium Single Cell Gene Expression Solution with Feature Barcoding technology

Library Preparation



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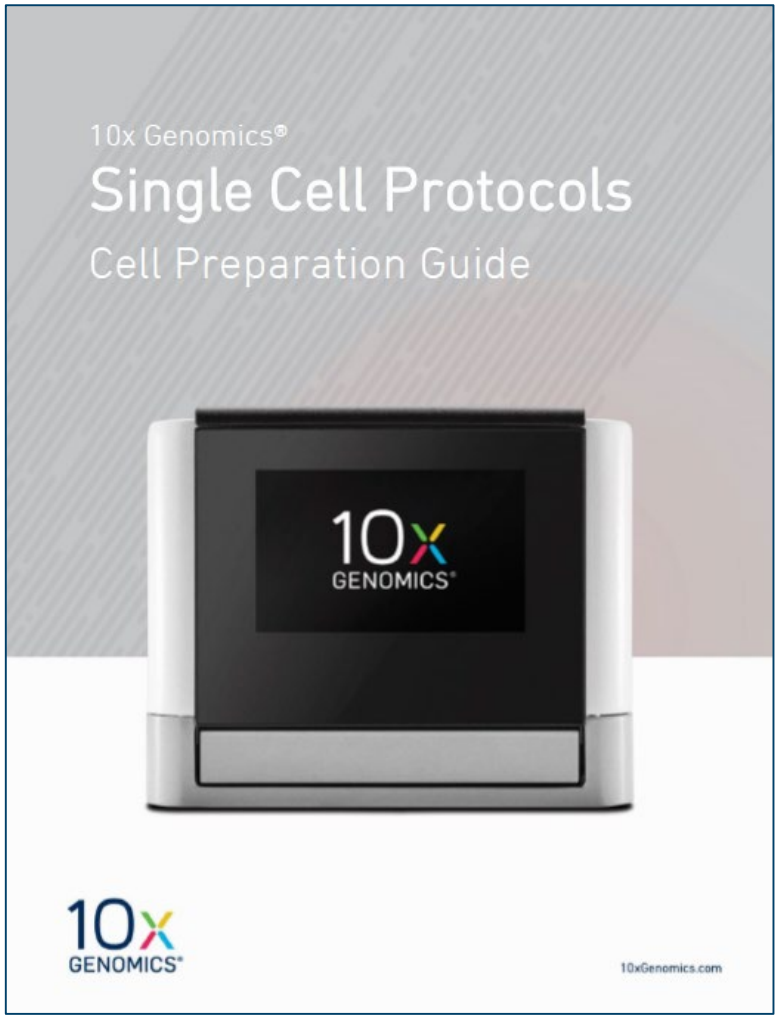
Sample Preparation Recommendations

Cell Preparation

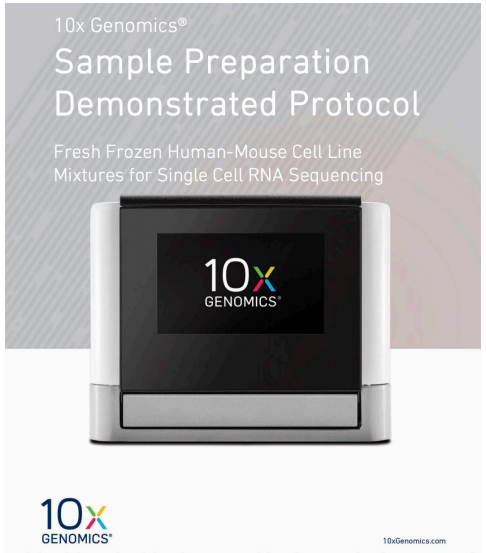
- Cell prep guide includes recommendations/tips for preparing cells.
- Demonstrated Protocols available on 10x support website.
  (e.g. fresh frozen PBMCs, neural tissue dissociation)
- Flow-sorted and primary white blood cells can generally be treated like suspension cell lines.



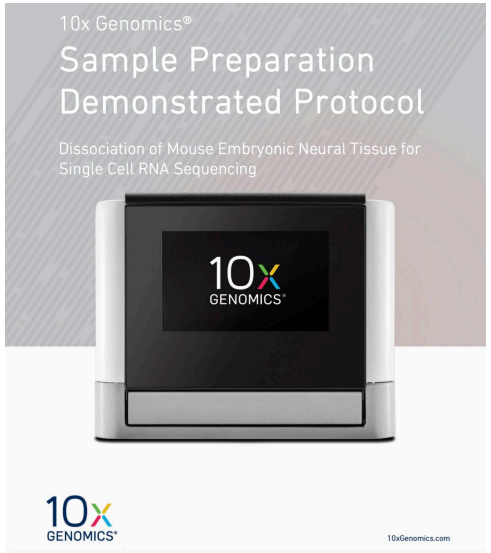
Single Cell 3’ Gene Expression and Feature Barcode technology

Routine use of demonstrated protocols – Optimization may be required

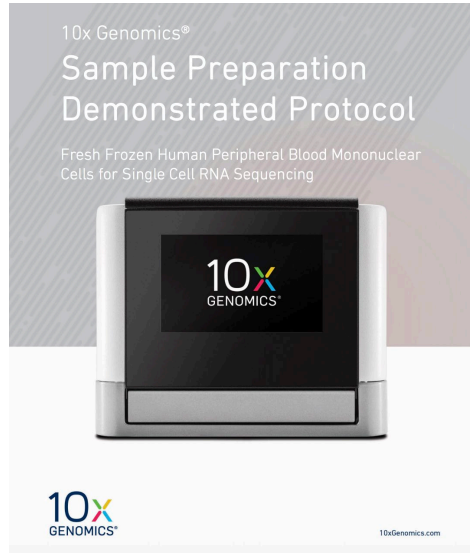
CG00014
Fresh Frozen Human-Mouse Cell Line Mixtures for Single Cell RNA Sequencing



CG00055
Dissociation of Mouse Embryonic Neural Tissue for Single Cell RNA Sequencing



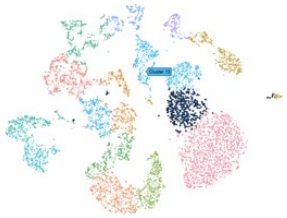
CG00039
Fresh Frozen Human Peripheral Blood Mononuclear Cells for Single Cell RNA Sequencing



General Cell Handling Recommendations

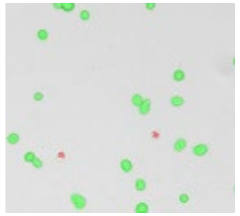
Analysis of Single Cell Transcriptomes

- 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.



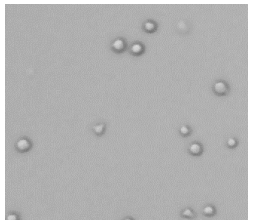
Importance of Input Cell Quality

- Ideally, 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 and negatively impact quality metrics reported by Cell Ranger.



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.



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 occurs in microfluidic channels that are narrower than the typical human hair (i.e. < 100 µm) and the presence of cell debris or large aggregates may clog or wet the chip.

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.

Single Cell 3’ Gene Expression and Feature Barcode technology

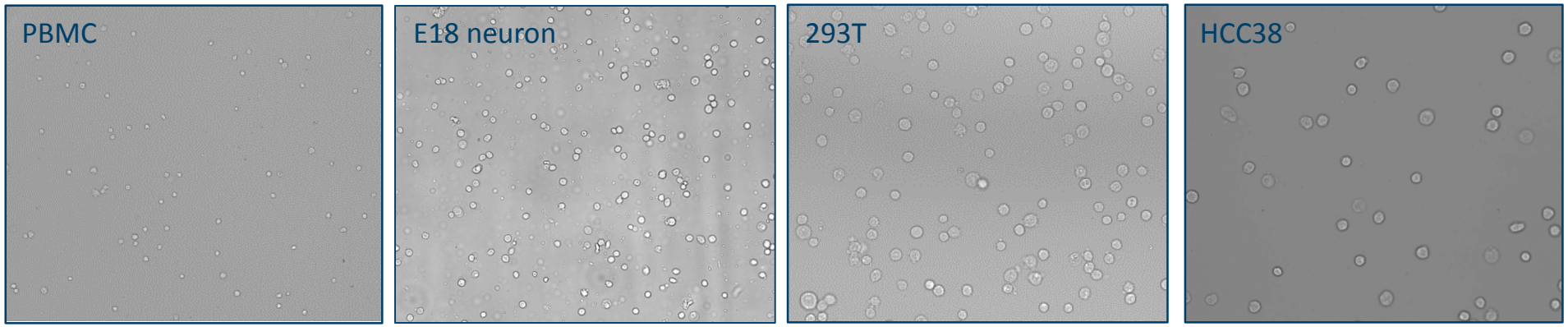
Diverse Sample Types Validated

Sample Type	Gene Expression	Feature Barcode Cell Surface Protein	Feature Barcode CRISPR Screening
HEK293T	✓	✓	
Jurkat	✓	✓	✓
K562	✓		✓
Raji	✓	✓	
NA12878	✓	✓	
NIH3T3	✓		
Embryonic Mouse Cortex, Hippocampus, Ventricular Zone	✓		
Embryonic Mouse Whole Heart (Pre-dissociated)	✓		
Glioblastoma Multiforme (Stage III-B)	✓	✓	
Non-Hodgkins Lymphoma (Mucosa Associated Lymphoid Tissue)	✓	✓	
Peripheral Blood Mononuclear Cells – Human	✓	✓	
Peripheral Blood Mononuclear Cells – Human : Pig	✓		
Peripheral Blood Mononuclear Cells – Human : Mouse	✓	✓	
Peripheral Blood Mononuclear Cells – Human (Multiple Myeloma)	✓	✓	
Peripheral Blood Mononuclear Cells – Human (Lupus)	✓	✓	
Bone Marrow Mononuclear Cells	✓	✓	

Cell Types Tested at 10x Genomics

Cells Tested	Species	Cell Source	Total RNA (pg/cell)*	Cell Size (µm)
PBMC	human	extracted from blood	~0.75	~5-10
E18 neuron	mouse	brain tissue	~ 2 - 3	~9
Jurkat	human	suspension	5.5	~12
Raji	human	suspension	7.3	~12
293T	human	adherent	14.2	~18
3T3	mouse	adherent	16.1	~18
HCC1954	human	adherent	15.7	~18
HCC38	human	adherent	21.6	~30

**Determined by Qubit assay & extracted by using Maxwell RSC SimplyRNA cells kit*



Wide Working Window of Total RNA Input

# of Cells Recovered	Total RNA Input*			
	PBMC's (RNA content per cell: 1 pg)	Jurkat Cells (RNA content per cell: 6 pg)	293T cells (RNA content per cell: 15 pg)	HCC38 cells (RNA content per cell: 22 pg)
500	0.5 ng	3 ng	7.5 ng	11 ng
1000	1 ng	6 ng	15 ng	22 ng
2000	2 ng	12 ng	30 ng	44 ng
3000	3 ng	18 ng	45 ng	66 ng
4000	4 ng	24 ng	60 ng	88 ng
5000	5 ng	30 ng	75 ng	110 ng
6000	6 ng	36 ng	90 ng	132 ng
7000	7 ng	42 ng	105 ng	154 ng
8000	8 ng	48 ng	120 ng	176 ng
9000	9 ng	54 ng	135 ng	198 ng
10000	10 ng	60 ng	150 ng	220 ng

**Cell lines we have worked with in-house*

- Wide working window of total RNA input drives several key workflow decisions.
- Supporting potentially 0.5 – 220 ng of total RNA input requires high ligation adaptor, sample index and SI-PCR primer concentrations.
- To ensure high quality sequencing libraries two double-sided SPRI's have been included into the workflow (1) after Fragmentation / End Repair and A-tailing and (2) after sample index PCR.

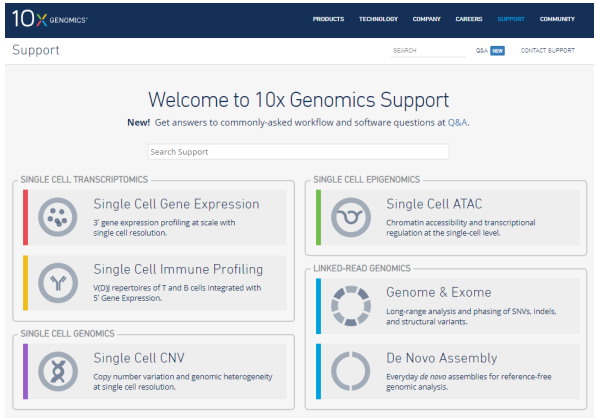
Rough Pipetting Leads to Cell Lysis and Lower Reads in Cells

Application Performance Metrics Reflect Rough Cell Handling

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

- 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.

Single Cell Sample Prep Resources from 10x Genomics



- support.10xgenomics.com
- Protocols are free to download
- Start with the flowchart “Guidelines for Optimal Sample Preparation”

General Sample Preparation Guidelines

- Guidelines for Optimal Sample Preparation
- Guidelines for Accurate Target Cell Counts
- General Cell Preparation Guide
- Preparation of Single Cell Suspensions from Cultured Cell Lines
- Isolation of Nuclei

Preparation of Specific Sample Types

- Fresh Frozen Human-Mouse Cell Line Mixtures
- Fresh Frozen Human Peripheral Blood Mononuclear Cells
- Dissociation of Mouse Embryonic Neural Tissue
- Tumor Dissociation
- Methanol Fixation of Cells
- Moss Protoplast Suspensions

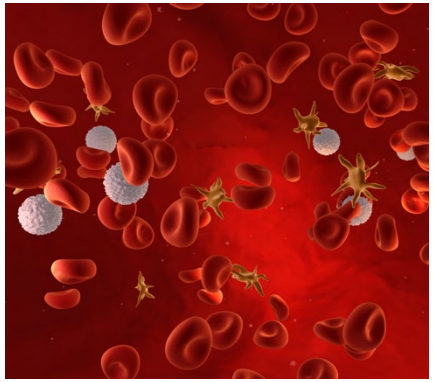
Sample Improvement

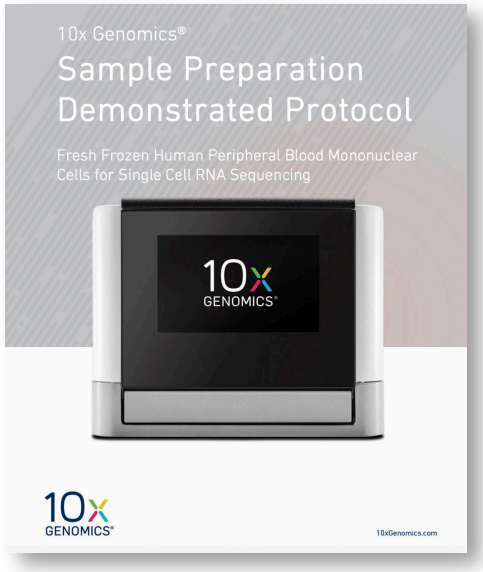
- Enrichment of CD3+ T Cells from Dissociated Tissues
- Removal of Dead Cells from Single Cell Suspensions

Sample Types

PBMCs (Peripheral Blood Mononuclear Cells)

- Optimal freezing conditions for cryopreservation
  - 40 % FBS, 10 or 15% DMSO in IMDM; 1 – 10 million cells per cryotube, 4 ^{o}C
- Thawing protocol
  - Rapidly thaw cryovial (37 ^{o}C water bath for 2-3 minutes)
  - Add 1 mL warm media dropwise (1 drop per 5 sec)
  - Serially 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



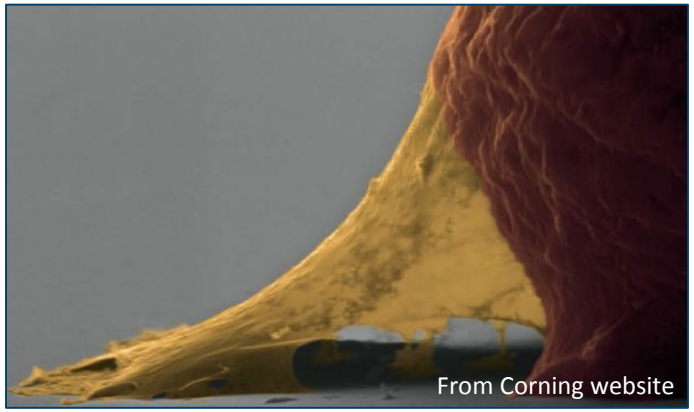


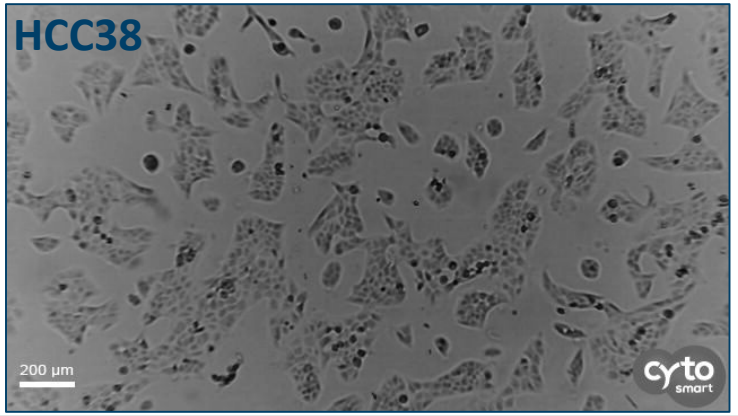
Sample Types

Adherent Cells Require Trypsin Treatment to Dissociate Cells from Culture Surface

- Incubation time varies with cell type: over-incubation may damage cells
- Confirm the complete digestion using a light microscope

Cell Type	Approximate Cell Dissociation Time
Embryonic Cells (293T, 3T3)	5 min
Breast Cancer HCC38, HCC1954	10 min
Breast Cancer HCC1143	15 min





Sample Types

Dissociating Cultured Cell Lines, Primary cells, and Solid tissue

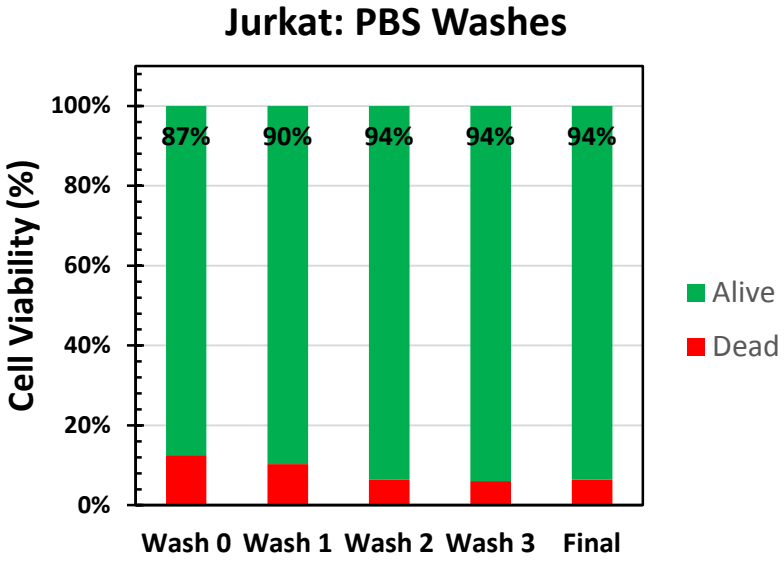
- Cultured cell lines
  - Enzymatic dissociation
    - Collagenase (e.g. differentiated cells in culture), Dispase (e.g. differentiated cells in culture), Accutase (e.g. iPSCs, hESCs) , Accumax (e.g. iPSCs, hESCs), Trypsin-EDTA (e.g. fibroblasts), TrypLE (e.g. fibroblasts)
- Primary cells
  - FAC-sorted
  - Magnetic-bead purified (e.g. Miltenyi Microbeads)
  - Gradient-purified (e.g. Percoll, Optiprep, Apheresis)
- Solid tissue
  - Best practices in 10x Genomics DP for mouse neuronal cells
  - Refer to publications (Worthington database)
  - Enzymatic dissociation
    - Papain (neurons), Collagenase, Dispase, Accutase, Accumax, Trypsin-EDTA
  - Mechanic dissociation (less frequent)
    - Cut, pipette, centrifugal mill

Cell Washing

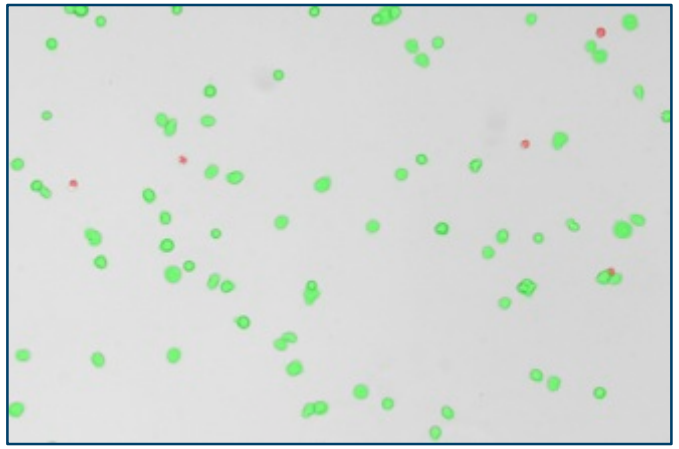
Washing isolated cells

- 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

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



Live/Dead Staining of Final Suspension



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 Mg2+ and EDTA. A 2-fold change up or down in Mg2+ concentration will affect the efficiency of the RT step.

Alternative Buffer and Media

Tested in-house

- Tested input volume: 2.5 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 to 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

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

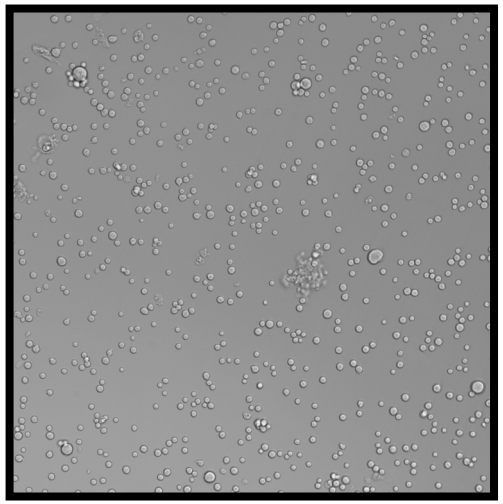
Con: sample concentration is decreased by 20 to 40% after straining

Flowmi™ Cell Strainer (Bel-Art Products)

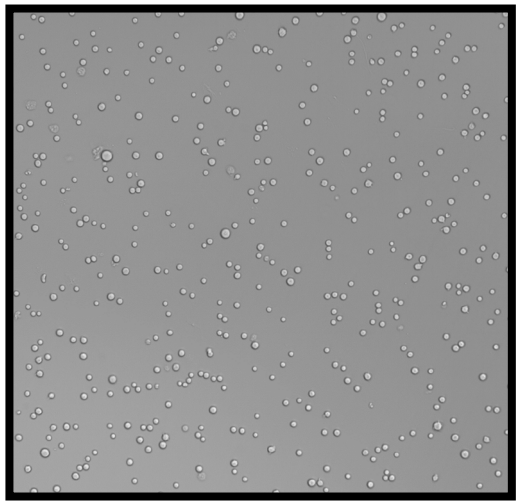
Pro: minimal change in sample concentration

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

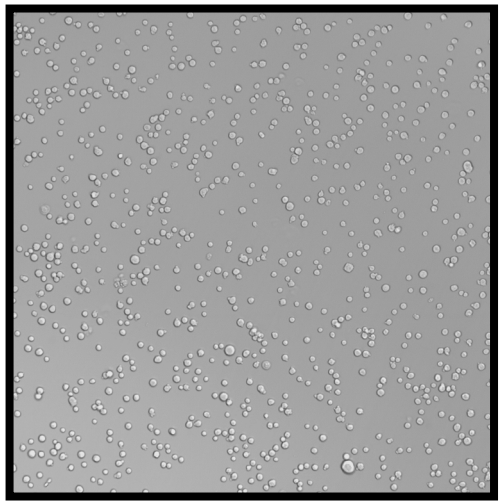
MACS® SmartStrainer (Miltenyi Biotec)



Unfiltered Jurkat cells



Jurkat cells filtered with FlowMi (40 µm)



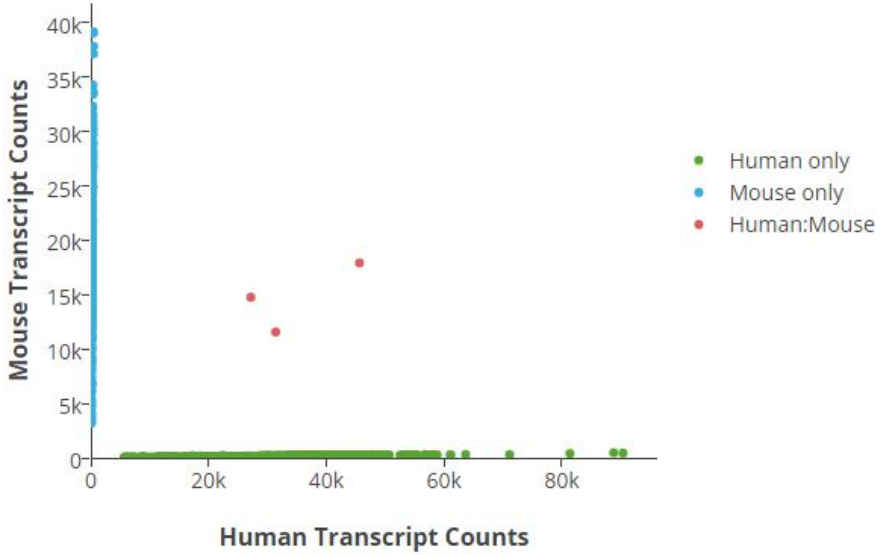
Jurkat cells filtered with MACS (30 µm)

Human-mouse mixture control (Optional)

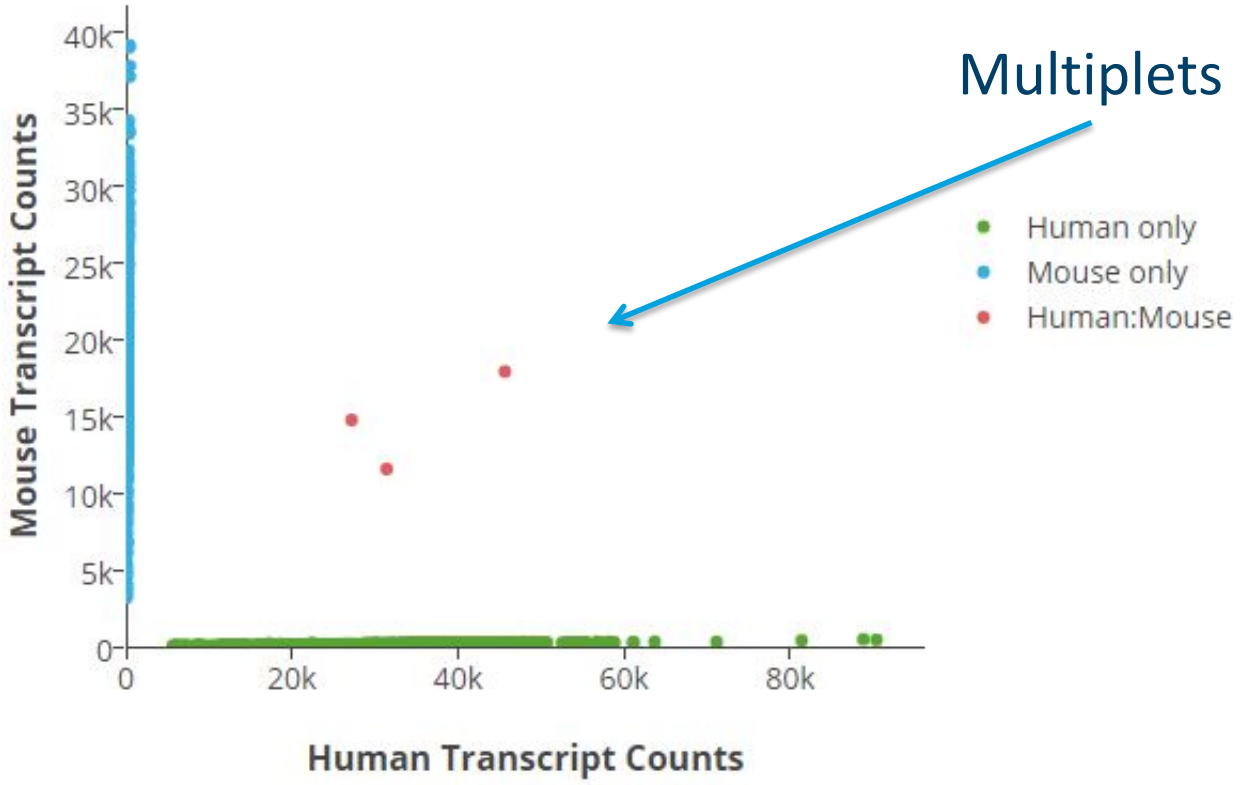
- 1:1 mixture of cryopreserved 293T (human) and 3T3 (mouse) cells
- No cell culture required
- Rapidly thaw cryovial (37 ^{o}C water bath for 2-3 minutes)
- Wash in PBS + 0.04% BSA and count cells
- Add to Master Mix for GEM generation

Human-mouse mixture control (Optional)

- Goal: Establish functionality of the system
- Mix ~500 human and ~500 mouse cells
- 5,000 reads per cell is sufficient sequencing depth for the human-mouse mixture and for library QC.
- Cell Ranger reports specific metrics when given a mouse-human mixture that cannot be obtained otherwise:
  - Multiplet rate
  - UMI count purity

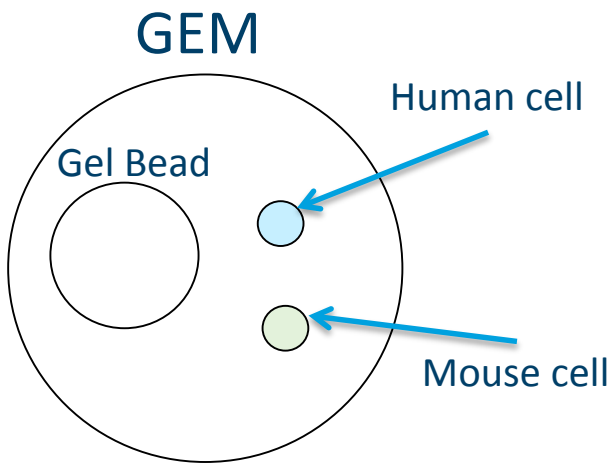


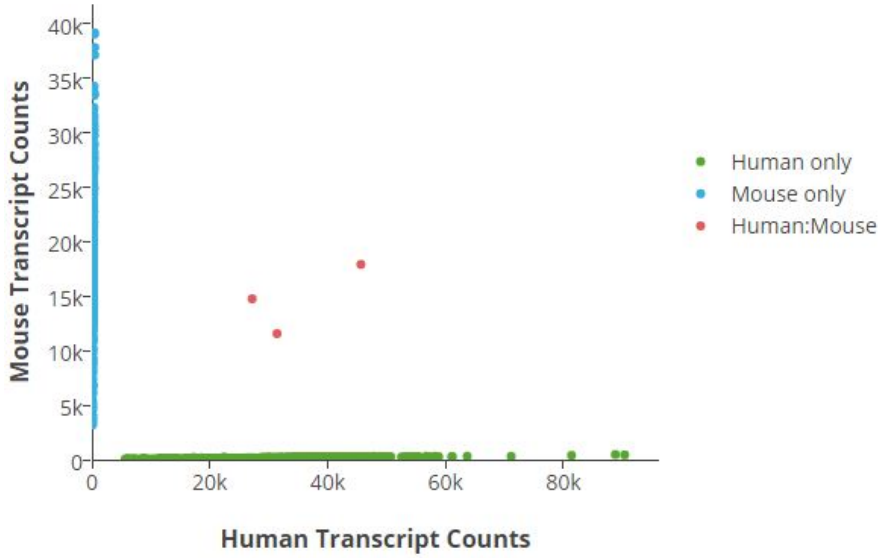
The human-mouse mixing experiment



Calculating the Multiplet Rate

- Count GEMs that are more likely to be multiplets than singletons
- Infer the number of unobserved multiplets
  - In a 1:1 mixing ratio, this should be equal to the number of observed multiplets
- The reported multiplet rate will be approximately twice the number of “Multiplet” dots seen on the plot.





10X GENOMICS

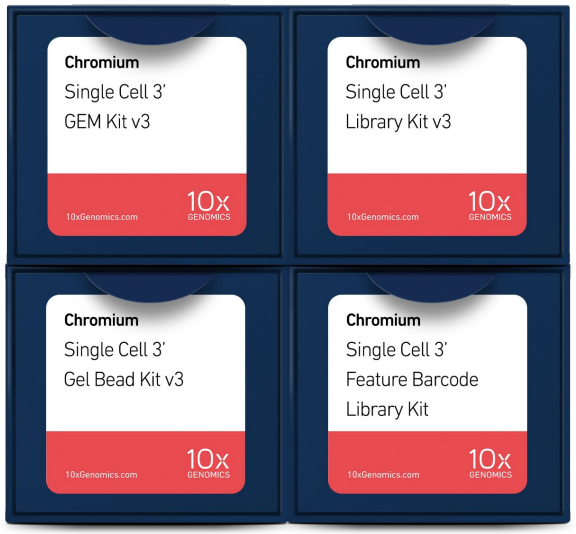
Chromium Single Cell Gene Expression Solution with Feature Barcoding technology

Workflow

Protocol Steps & Timing

Day	Steps		Timing	Stop & Store
2 h	**Cell Preparation**			
	Dependent on Cell Type		~1-1.5 h	
	Step 1 – GEM Generation & Barcoding			
4 h	1.1	Prepare Reaction Mix	20 min	
	1.2	Load Chromium Single Cell B Chip	10 min	
	1.3	Run the Chromium Controller	8.5 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			
6 h	2.1	Post GEM RT-Cleanup – Dynabead	45 min	
	2.2	cDNA Amplification	40 min	STOP 4°C ≤72 h or –20°C ≤1 week
	2.3	cDNA Cleanup – SPRIselect	20 min	STOP 4°C ≤72 h –20°C ≤4 weeks
	2.4	cDNA QC & Quantification	50 min	
8 h	**Step 3 – 3' Gene Expression Library Construction**			
	3.1	Fragmentation, End Repair & A-tailing	50 min	
	3.2	Post Fragmentation, End Repair & A-tailing Double Sided Size Selection – SPRIselect	30 min	
	3.3	Adaptor Ligation	25 min	
	3.4	Post Ligation Cleanup- SPRIselect	20 min	
	3.5	Sample Index PCR	40 min	STOP 4°C ≤72 h
	3.6	Post Sample Index PCR Double Sided Size Selection- SPRIselect	30 min	STOP 4°C ≤72 h or -20°C long term
	3.7	Post Library Construction QC	50 min	

Getting Started: Equilibrate Reagents



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

Remove the **Chromium Single Cell 3' v3 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 on ice:

Remove the:

- **RT Enzyme C (blue cap)**

from the **Single Cell 3' GEM Kit v3** stored at -20 °C and place on ice.

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

Remove the:

- **RT Reagent (blue cap)**
- **Reducing Agent B (white cap)**
- **Template Switch Oligo (blue cap)** provided as lyophilized oligos; after resuspension, *store unused primers at –80°C*

from the **Single Cell 3' GEM Kit v3** stored at -20 °C and equilibrate to room temperature for at least **10 min.**

From room temperature storage:

Remove the:

- **Partitioning Oil (clear cap)**
- **Chip B Single Cell, Gasket, Chip Holder**

from room temperature storage.

Targeted Number of Cells

Calculate Volumes of Cell suspension and Water Required to Achieve Targeted Cell Recovery

- Cell Suspension Volume Calculator Table is provided in the User Guide
- Targeted Cell Recovery numbers take into account a 65% cell processing efficiency
- For Example:
  - Targeting 5000 cells with a cell concentration of 1000 cells/µl:
    - Add 38.6 µl water
    - Add 8.0 µl cell suspension

Cell Suspension Volume Calculator Table (for step 1.2)

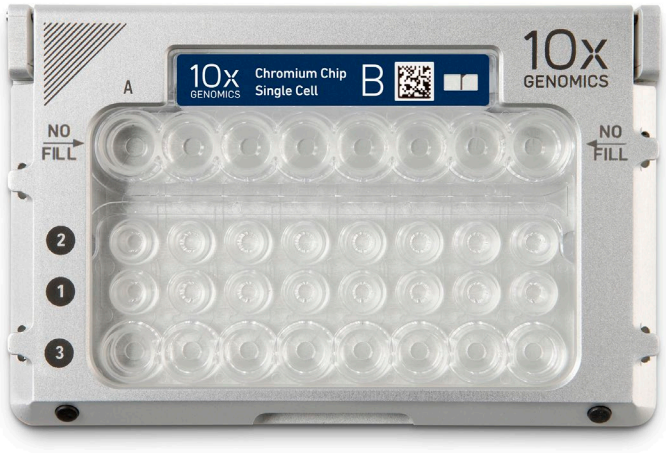
Volume of Cell Suspension Stock per reaction (µl) | Volume of Nuclease-free Water per reaction (µl)

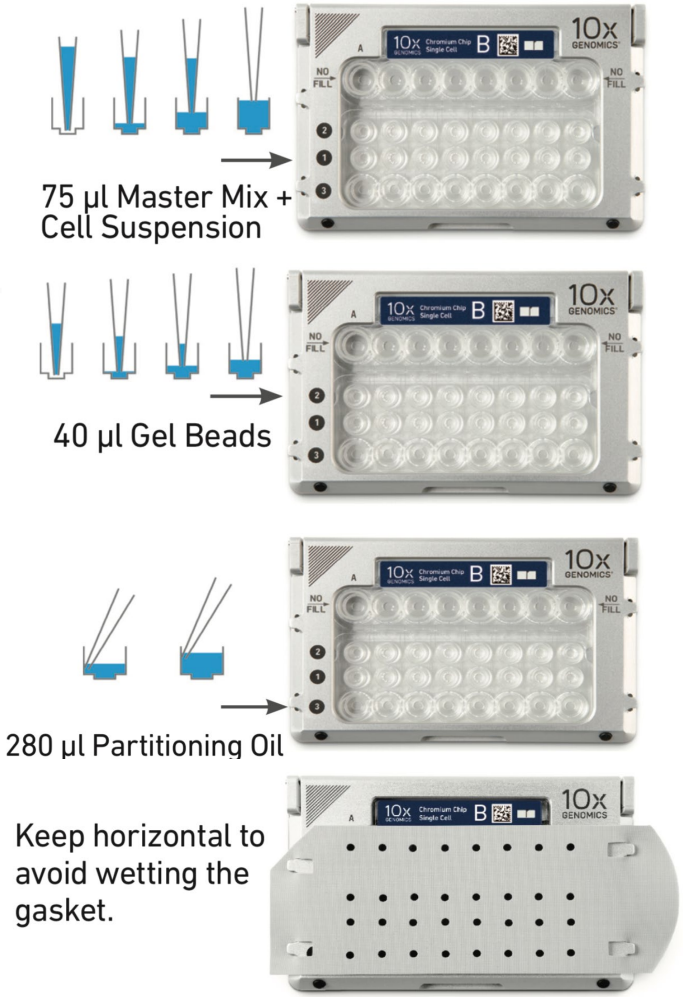
Cell Stock Concentration (Cells/µl)	Targeted Cell Recovery										
	500	1000	2000	3000	4000	5000	6000	7000	8000	9000	10000
100	8.0 38.6	16.0 30.6	32.0 14.6	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
200	4.0 42.6	8.0 38.6	16.0 30.6	24.0 22.6	32.0 14.6	40.0 6.6	n/a	n/a	n/a	n/a	n/a
300	2.7 43.9	5.3 41.3	10.7 35.9	16.0 30.6	21.3 25.3	26.7 19.9	32.0 14.6	37.3 9.3	42.7 3.9	n/a	n/a
400	2.0 44.6	4.0 42.6	8.0 38.6	12.0 34.6	16.0 30.6	20.0 26.6	24.0 22.6	28.0 18.6	32.0 14.6	36.0 10.6	40.0 6.6
500	1.6 45.0	3.2 43.4	6.4 40.2	9.6 37.0	12.8 33.8	16.0 30.6	19.2 27.4	22.4 24.2	25.6 21.0	28.8 17.8	32.0 14.6
600	1.3 45.3	2.7 43.9	5.3 41.3	8.0 38.6	10.7 35.9	13.3 33.3	16.0 30.6	18.7 27.9	21.3 25.3	24.0 22.6	26.7 19.9
700	1.1 45.5	2.3 44.3	4.6 42.0	6.9 39.7	9.1 37.5	11.4 35.2	13.7 32.9	16.0 30.6	18.3 28.3	20.6 26.0	22.9 23.7
800	1.0 45.6	2.0 44.6	4.0 42.6	6.0 40.6	8.0 38.6	10.0 36.6	12.0 34.6	14.0 32.6	16.0 30.6	18.0 28.6	20.0 26.6
900	0.9 45.7	1.8 44.8	3.6 43.0	5.3 41.3	7.1 39.5	8.9 37.7	10.7 35.9	12.4 34.2	14.2 32.4	16.0 30.6	17.8 28.8
1000	0.8 45.8	1.6 45.0	3.2 43.4	4.8 41.8	6.4 40.2	8.0 38.6	9.6 37.0	11.2 35.4	12.8 33.8	14.4 32.2	16.0 30.6
1100	0.7 45.9	1.5 45.1	2.9 43.7	4.4 42.2	5.8 40.8	7.3 39.3	8.7 37.9	10.2 36.4	11.6 35.0	13.1 33.5	14.5 32.1
1200	0.7 45.9	1.3 45.3	2.7 43.9	4.0 42.6	5.3 41.3	6.7 39.9	8.0 38.6	9.3 37.3	10.7 35.9	12.0 34.6	13.3 33.3
1300	0.6 46.0	1.2 45.4	2.5 44.1	3.7 42.9	4.9 41.7	6.2 40.4	7.4 39.2	8.6 38.0	9.8 36.8	11.1 35.5	12.3 34.3
1400	0.6 46.0	1.1 45.5	2.3 44.3	3.4 43.2	4.6 42.0	5.7 40.9	6.9 39.7	8.0 38.6	9.1 37.5	10.3 36.3	11.4 35.2
1500	0.5 46.1	1.1 45.5	2.1 44.5	3.2 43.4	4.3 42.3	5.3 41.3	6.4 40.2	7.5 39.1	8.5 38.1	9.6 37.0	10.7 35.9
1600	0.5 46.1	1.0 45.6	2.0 44.6	3.0 43.6	4.0 42.6	5.0 41.6	6.0 40.6	7.0 39.6	8.0 38.6	9.0 37.6	10.0 36.6
1700	0.5 46.1	0.9 45.7	1.9 44.7	2.8 43.8	3.8 42.8	4.7 41.9	5.6 41.0	6.6 40.0	7.5 39.1	8.5 38.1	9.4 37.2
1800	0.4 46.2	0.9 45.7	1.8 44.8	2.7 43.9	3.6 43.0	4.4 42.2	5.3 41.3	6.2 40.4	7.1 39.5	8.0 38.6	8.9 37.7
1900	0.4 46.2	0.8 45.8	1.7 44.9	2.5 44.1	3.4 43.2	4.2 42.4	5.1 41.5	5.9 40.7	6.7 39.9	7.6 39.0	8.4 38.2
2000	0.4 46.2	0.8 45.8	1.6 45.0	2.4 44.2	3.2 43.4	4.0 42.6	4.8 41.8	5.6 41.0	6.4 40.2	7.2 39.4	8.0 38.6

Grey boxes: Volumes that would exceed the allowable water volume in each reaction
Yellow boxes: Indicate a low transfer volume that may result in higher cell load variability
Blue boxes: Optimal range of cell stock concentration to maximize the likelihood of achieving the desired cell recovery target

Single Cell Gene Expression Workflow

Partitioning Cells into GEMs





- 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 B:
  - Load Row 1: 75 µl Cells and Master Mix
  - Prime Chip and Vortex Gel Beads for 30 seconds
  - Load Row 2: 40 µl Gel beads
  - Load Row 3: 280 µl Partitioning Oil
- Attach 10x Gasket
- Run on Chromium Controller within 2 minutes from loading (Run time ~8.5 mins)

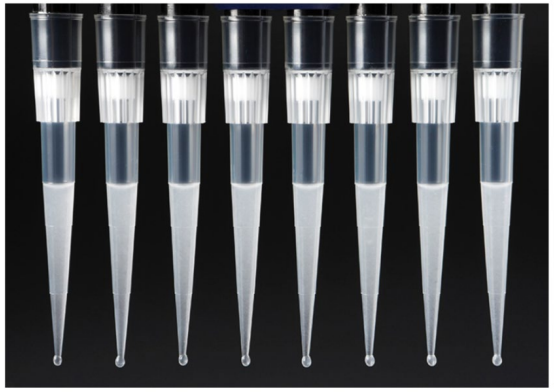
Firmware 3.16 is required for running this assay

Single Cell Gene Expression Workflow

Recovering GEMs

Expose wells at 45 Degrees

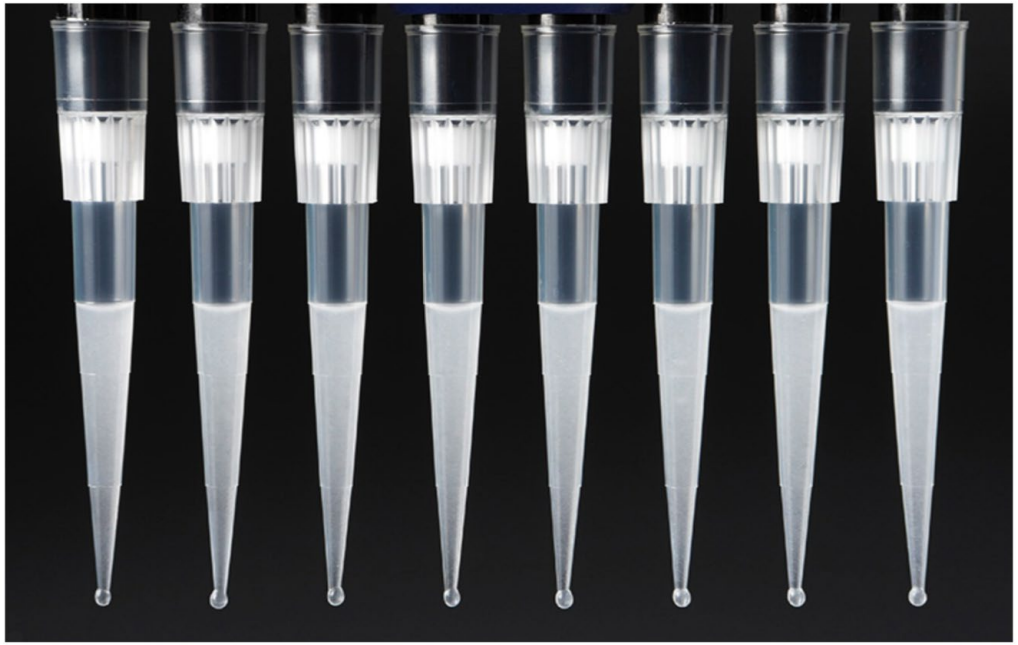
Slowly aspirate GEMs from the lowest points of the Recovery Wells



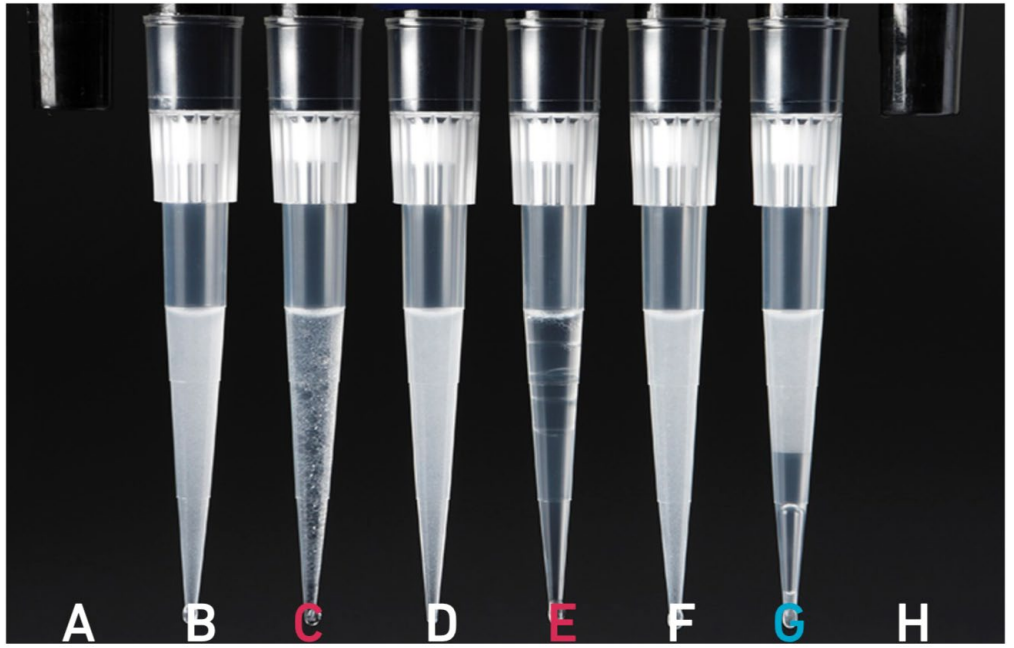
GEMs should appear opaque and uniform across all channels

Single Cell Gene Expression 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.



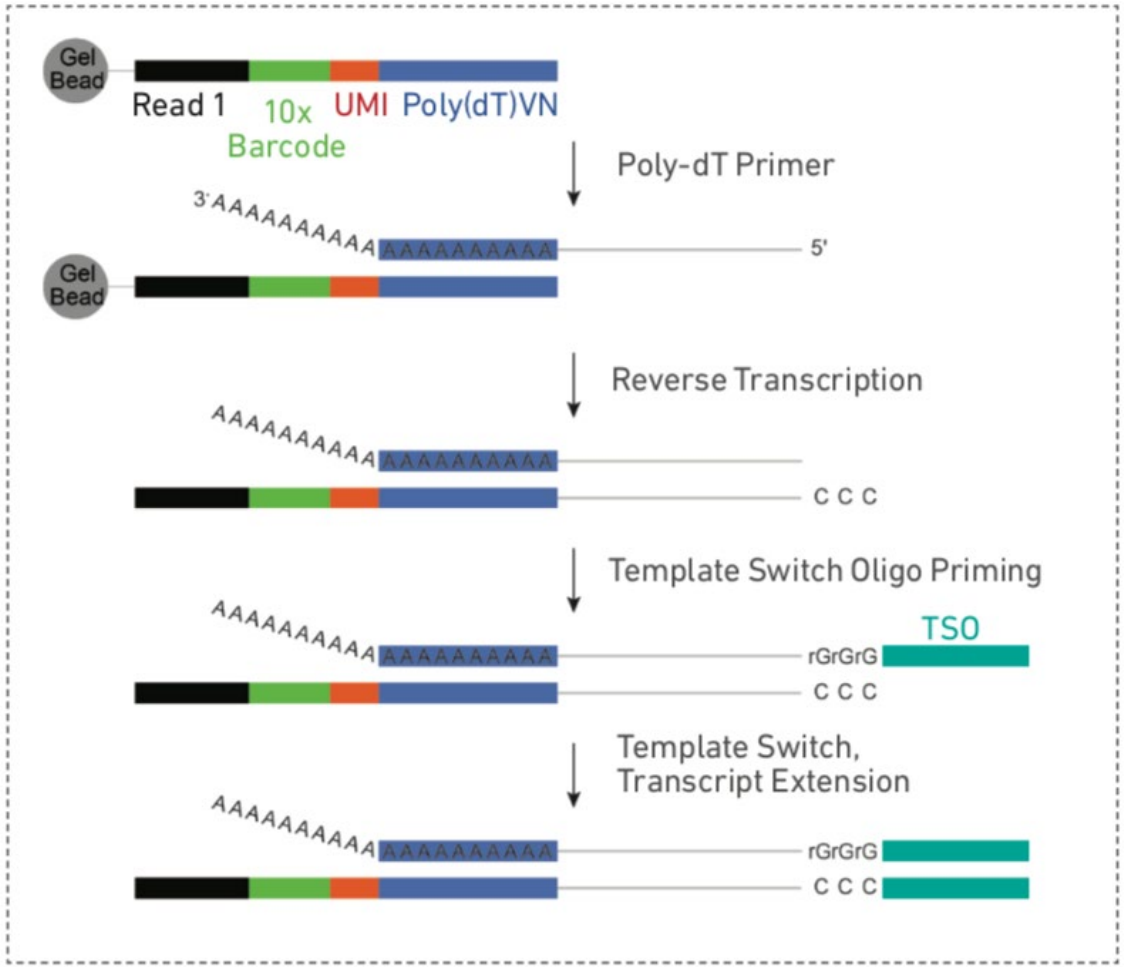
Pipette tips C and E indicate a wetting failure.
Pipette tip C contains partially emulsified GEMs.
Emulsion is absent in pipette tip E.
Pipette tip G indicates a reagent clog.

Single Cell Gene Expression Workflow

GEM RT Incubation

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

Inside individual GEMs



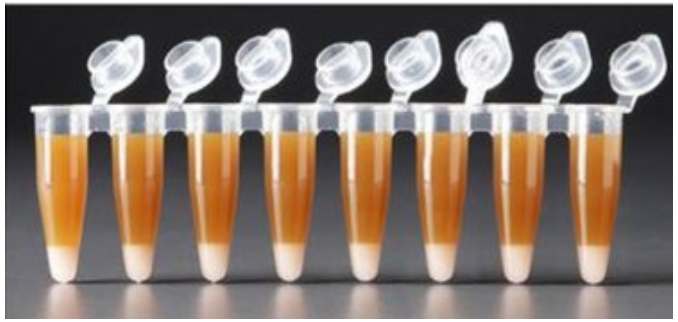
Single Cell Gene Expression Workflow

Breaking GEMs

Single Cell Gene Expression Workflow

Post GEM Incubation Cleanups – Dynabeads

- Prepare Dynabeads Cleanup Mix
  - Vortex Dynabeads thoroughly for 30 sec
  - Use only Reducing Agent B and Cleanup Buffer from Chromium Single Cell Gene Expression Reagent kits
- Always use fresh preparations of 80% Ethanol
- Elute in 35 µl of Elution Solution I



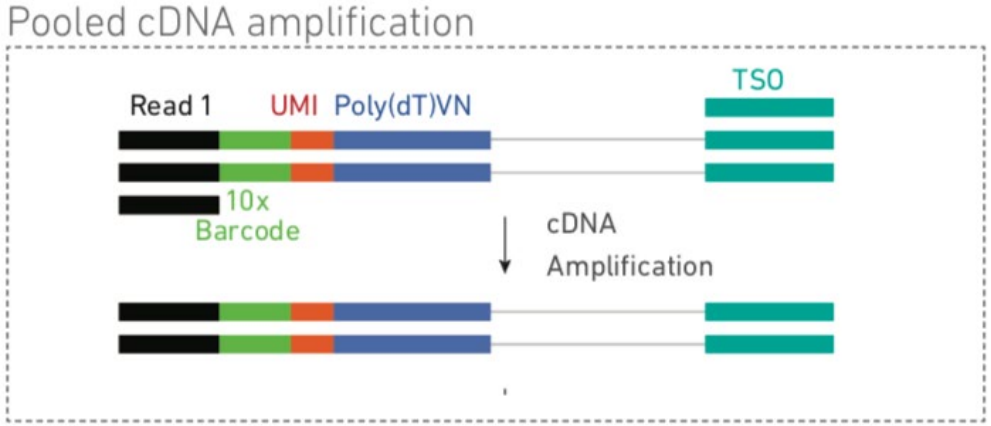
Single Cell Gene Expression Workflow

cDNA Amplification

- Prepare cDNA Amplification Reaction Mix
  - Primers must be selected based on whether generating only Gene Expression Libraries, or Gene Expression and Cell Surface Protein or CRISPR Libraries.
- Add 65 µl cDNA Amplification Reaction Mix to 35 µl of sample

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	~30-45 min
Step	**Temperature**	**Time**
1	98°C	00:03:00
2	98°C	00:00:15
3	63°C Version Specific Updated Temperature	00:00:20
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



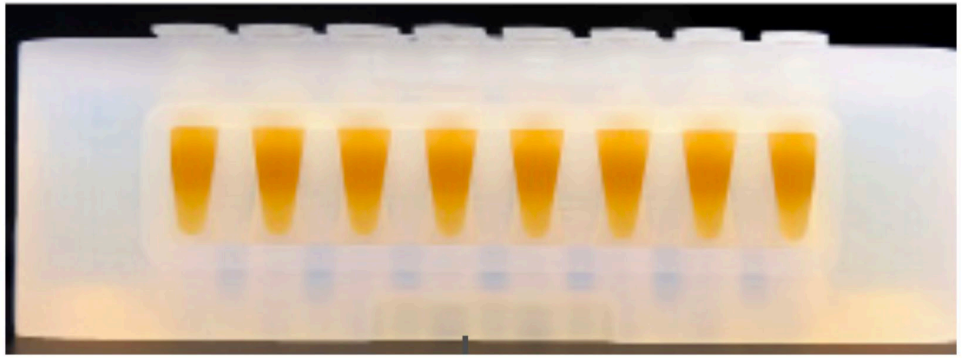
Cell Load	Total Cycles
<500	13
500–6,000	12
>6,000	11

Single Cell Gene Expression Workflow

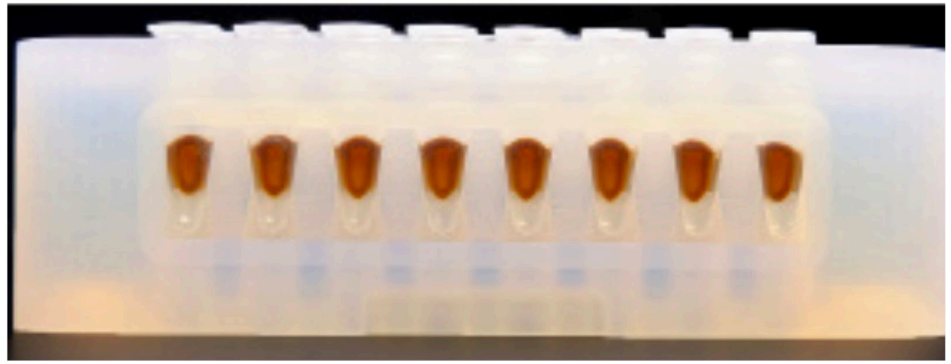
SPRIselect cDNA Cleanup

- Vortex SPRIselect Reagent thoroughly before adding to samples and ensure samples are fully mixed with beads
- If generating Cell Surface Protein or CRISPR screening 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
- Perform a 0.6x Single sided SPRI cleanup, eluting in 40 µl of Buffer EB

Magnetic beads mixed with reagent

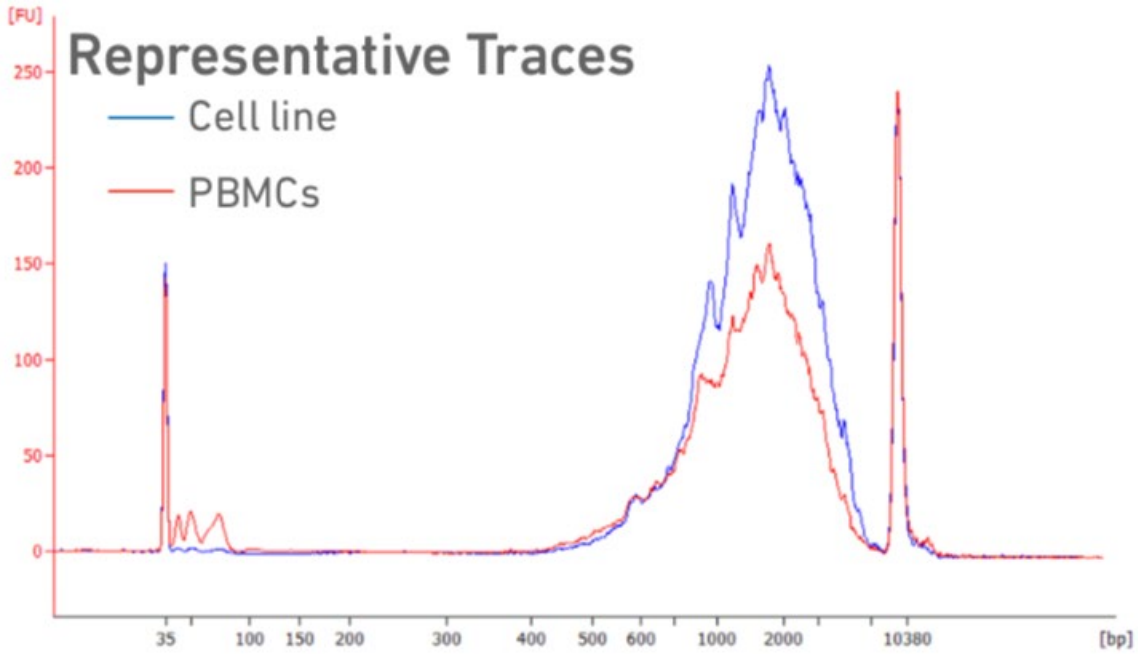


Separation complete; solution is clear



Single Cell Gene Expression Workflow

cDNA QC and Quantification



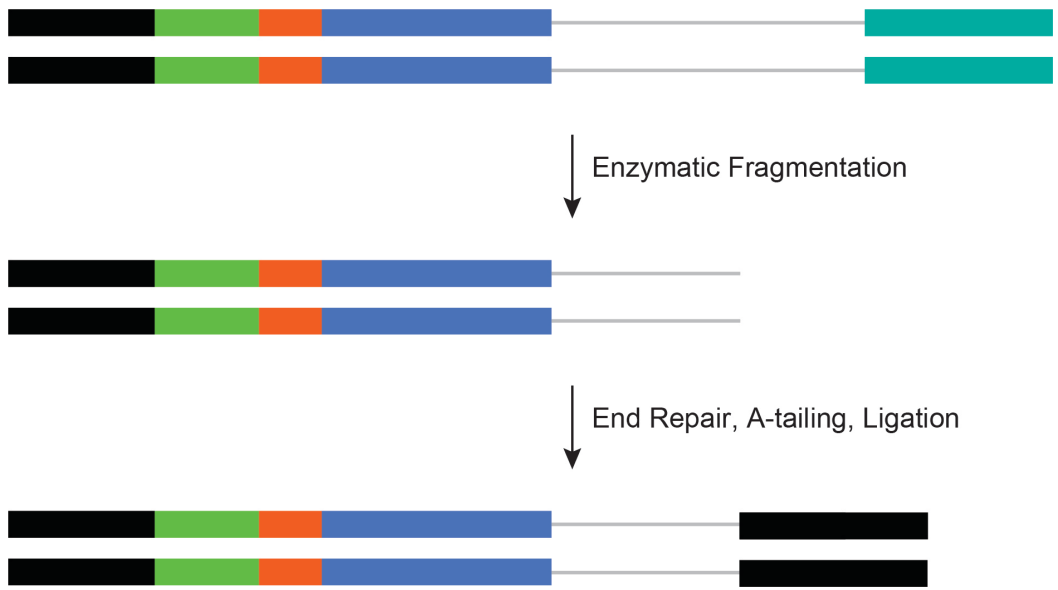
- For input cells with low RNA content (<1 pg total RNA/cell), 1 µl undiluted product may be run
- Lower molecular weight product (35-150 bp) may be present. This is normal and does not affect sequencing or application performance
- Carry forward **only 25%** of total cDNA yield into 3' Gene Expression Library Construction
  - i.e. use only 10 µl of total 40 µl elution volume
  - Quantification (based on BioAnalyzer estimate) will be used to determine number of Sample Index PCR cycles
  - You can save remaining cDNA for future uses if desired

Single Cell Gene Expression Workflow

Library Construction

- Fragmentation
  - It is critical to pre-chill the thermal cycler block to 4°C prior to assembling the reaction
- End Repair and A-tailing
- SPRIselect cleanup
- Adaptor Ligation
- SPRIselect cleanup

Pooled Library Construction

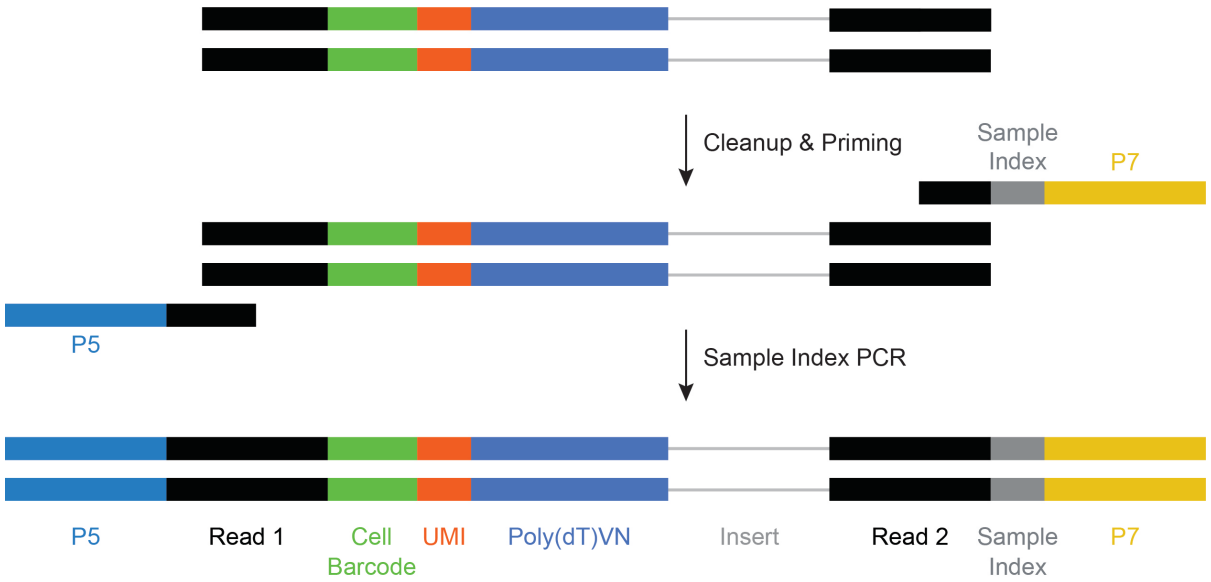


Single Cell Gene Expression Workflow

Sample Index PCR

- Uses Chromium i7 Sample Index Plate (PN 220103)
  - Record the Well ID used for each sample. This is needed for demultiplexing the sequencing data.
- Incubate on the thermal cycler using the appropriate number of cycles
  - cDNA input informs total cycles
  - Should be optimized based off of 25% carry forward
- Double-sided size selection with SPRIselect (0.6X, 0.8X) to remove excess primers and optimize final library size distribution

Pooled Library Construction



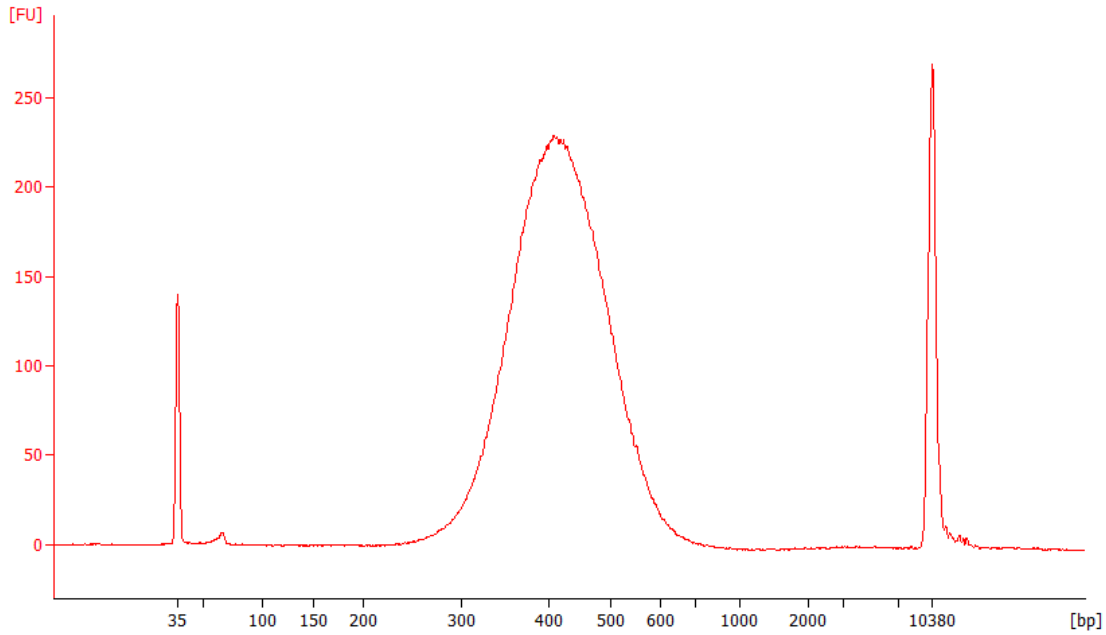
Single Cell Gene Expression Workflow

Post Library Construction QC and Quantification

- Final library shows size distribution between ~300-600 bp

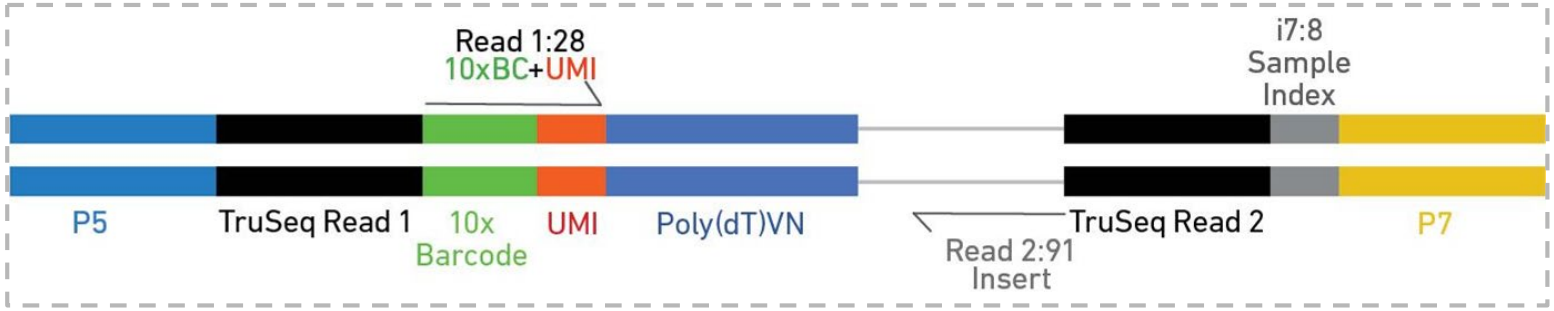
- Libraries should be quantified by qPCR, using the KAPA Library Quantification Kit for Illumina Platforms

Representative Trace



Recommended Read Length

Single Cell 3’ Gene Expression Library

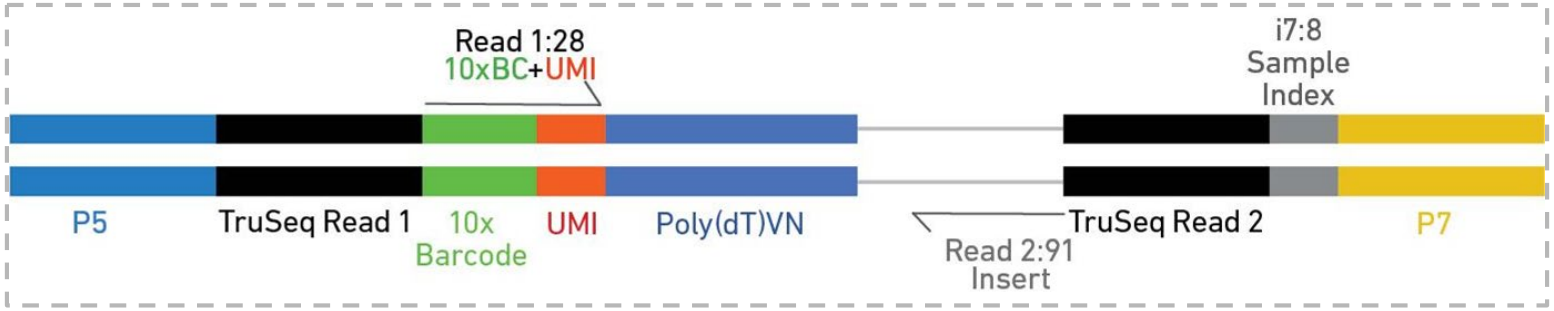


	Read 1	i7 Index	i5 Index	Read 2
Purpose	10x Barcode & UMI	Sample Index	N/A	Transcript
Length	28*	8	0	91

Note: * If pooled with Single Cell 3’ Gene Expression Libraries v2, ensure that the Read 1 length is adjusted to 28 bp

Supported Sequencers

Single Cell 3’ Gene Expression Library



	Single Cell 3’ Gene Expression
Raw Read Pairs per Cell	Minimum 20,000

MiSeq

NextSeq

HiSeq2500

HiSeq 3000/4000

NovaSeq

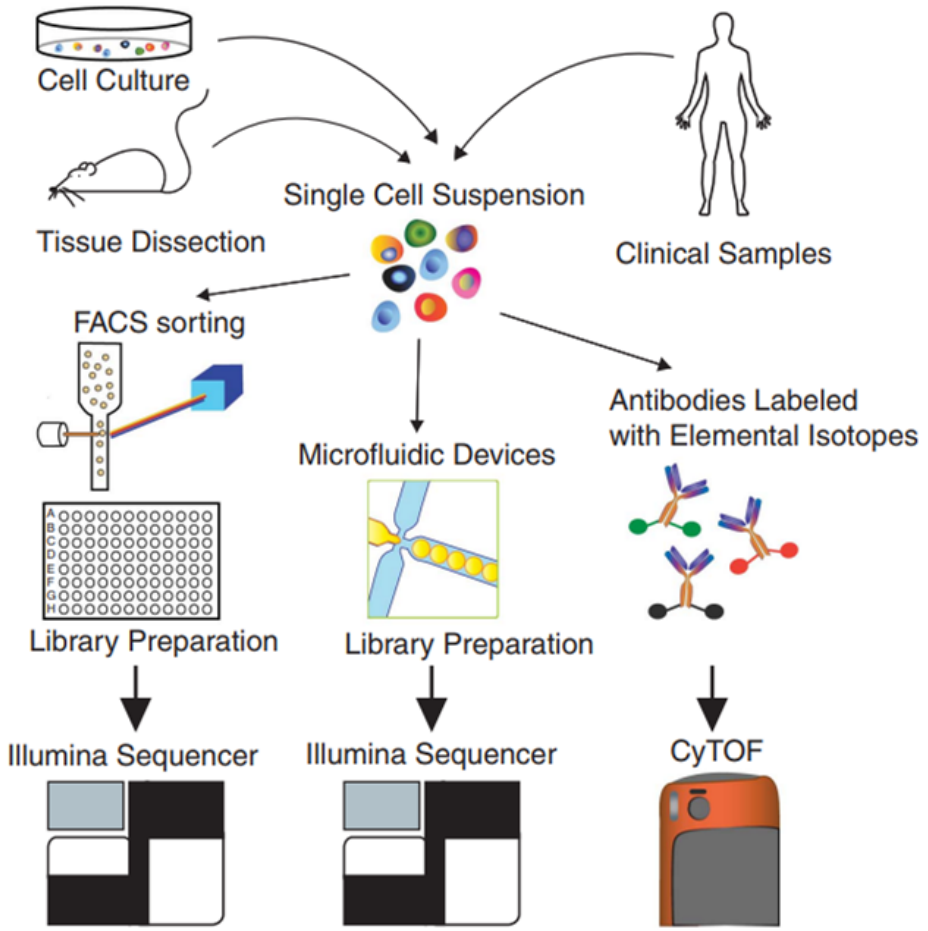
10X GENOMICS

Chromium Single Cell Gene Expression Solution with Feature Barcoding technology

Cell Surface Protein

Single Cell Gene Expression and Cell Surface Proteins

More Information from a Single Assay



- Measure cell surface protein markers, splice isoforms, and post-translational modifications, to identify cell types and states, and detect rare cell types, all in a single assay in single cells
- Detect markers that are difficult to measure at the RNA level
- Evaluate differences between mRNA and protein expression profiles
- Obtain a more detailed characterization of cellular phenotypes compared to transcriptome measurements alone

Single Cell Gene Expression and CRISPR Screening

Customer Demonstrations

Perturb-Seq

Resource

Cell

Perturb-Seq: Dissecting Molecular Circuits with Scalable Single-Cell RNA Profiling of Pooled Genetic Screens

Atray Dixit,[1,2,9] Oren Parnas,[1,3,10] Biyu Li,[1] Jenny Chen,[1,2] Charles P. Fulco,[1,4] Livnat Jerby-Arnon,[1] Nemanja D. Marjanovic,[1,2] Danielle Dionne,[1] Tyler Burks,[1] Raktima Raychowdhury,[1] Britt Adamson,[5] Thomas M. Norman,[6] Eric S. Lander,[1,4,8] Jonathan S. Weissman,[5,7] Nir Friedman,[1,8] and Aviv Regev[1,4,7,11,*]

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http://dx.doi.org/10.1016/j.cell.2016.11.038

SUMMARY

Genetic screens help infer gene function in mammalian cells, but it has remained difficult to assay complex phenotypes—such as transcriptional profiles—at scale. Here, we develop Perturb-seq, combining single-cell RNA sequencing (RNA-seq) and clustered regularly interspaced short palindromic repeats (CRISPR)-based perturbations to perform many such assays in a pool. We demonstrate Perturb-seq by analyzing 200,000 cells in immune cells and cell lines, focusing on transcription factors regulating the response of dendritic cells to lipopolysaccharide (LPS). Perturb-seq accurately identifies individual gene targets, gene signatures, and cell states affected by individual perturbations and their genetic interactions. We posit new functions for regulators of differentiation, the anti-viral response, and mitochondrial function during immune activation. By decomposing many high content measurements into the effects of perturbations, their interactions, and diverse cell metadata, Perturb-seq dramatically increases the scope of pooled genomic assays.

INTRODUCTION

Genetic screens systematically analyze gene function in mammalian cells. Such screens are designed in either: (1) an individual ("arrayed") format, where each perturbation is delivered and assessed separately; or (2) a pooled format, performed en masse. Pooled readouts measure cell autonomous phenotypes, such as growth, drug resistance, or marker expression. Pooled screens are more efficient and scalable, but have been limited to low-content readouts. Distinguishing between different molecular mechanisms that yield similar phenotypes requires time and labor intensive follow-up.

Bridging the gap between rich profiles and pooled screens has been challenging. In mammalian cells, a few studies transcriptionally profiled hundreds of individual perturbations (Berger et al., 2016; Parnas et al., 2015). In yeast (Hughes et al., 2000), up to ~1,500 knock out (KO) strains have been assessed (Kemmeren et al., 2014). Even signature screens were only performed in centralized efforts (Lamb et al., 2006).

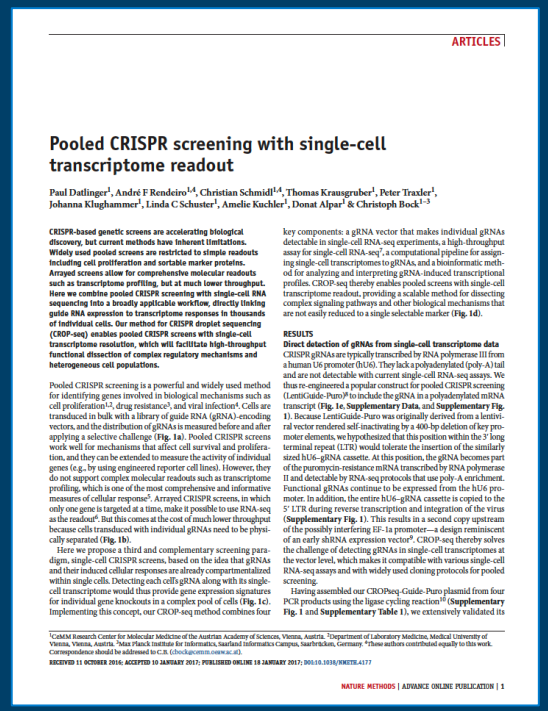
Profiling may particularly help interpret the combined nonlinear effects of multiple factors. Comprehensive analysis of genetic interactions in growth phenotype between pairs of genes has been performed in yeast (Costanzo et al., 2016). In mammals, only small sets of pre-selected pairs have been assessed for cell viability (Bassik et al., 2013) or morphology (Laufer et al., 2013). One yeast study determined the combined effects of regulators on expression profiles in a circuit of three to five genes (Capaldi et al., 2008). Very few studies have examined higher order interactions (Elena and Lenski, 1997; Haber et al., 2013) and none have coupled those with a high content scalable readout.

To address this challenge, we develop Perturb-seq, combining the modularity of clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 to perform multi-locus gene perturbation (Cong et al., 2013; Qi et al., 2013) with the scale of massively parallel single cell RNA sequencing

CrossMark

Cell 167, 1853–1866, December 15, 2016 © 2016 Elsevier Inc. 1853

CROP-Seq



ARTICLES

Pooled CRISPR screening with single-cell transcriptome readout

Paul Datlinger[1], André F Rendeiro[1,4], Christian Schmidl[1,4], Thomas Krausgruber[1], Peter Traxler[1], Johanna Klughammer[1], Linda C Schuster[1], Amelie Kuchler[1], Donat Alpar[1] & Christoph Bock[1–3]

CRISPR-based genetic screens are accelerating biological discovery, but current methods have inherent limitations. Widely used pooled screens are restricted to simple readouts including cell proliferation and sortable marker proteins. Arrayed screens allow for comprehensive molecular readouts such as transcriptome profiling, but at much lower throughput. Here we combine pooled CRISPR screening with single-cell RNA sequencing into a broadly applicable workflow, directly linking guide RNA expression to transcriptome responses in thousands of individual cells. Our method for CRISPR droplet sequencing (CROP-seq) enables pooled CRISPR screens with single-cell transcriptome resolution, which will facilitate high-throughput functional dissection of complex regulatory mechanisms and heterogeneous cell populations.

Pooled CRISPR screening is a powerful and widely used method for identifying genes involved in biological mechanisms such as cell proliferation[1,2], drug resistance[3], and viral infection[4]. Cells are transduced in bulk with a library of guide RNA (gRNA)-encoding vectors, and the distribution of gRNAs is measured before and after applying a selective challenge (**Fig. 1a**). Pooled CRISPR screens work well for mechanisms that affect cell survival and proliferation, and they can be extended to measure the activity of individual genes (e.g., by using engineered reporter cell lines). However, they do not support complex molecular readouts such as transcriptome profiling, which is one of the most comprehensive and informative measures of cellular response[5]. Arrayed CRISPR screens, in which only one gene is targeted at a time, make it possible to use RNA-seq as the readout[6]. But this comes at the cost of much lower throughput because cells transduced with individual gRNAs need to be physically separated (**Fig. 1b**).

Here we propose a third and complementary screening paradigm, single-cell CRISPR screens, based on the idea that gRNAs and their induced cellular responses are already compartmentalized within single cells. Detecting each cell's gRNA along with its single-cell transcriptome would thus provide gene expression signatures for individual gene knockouts in a complex pool of cells (**Fig. 1c**). Implementing this concept, our CROP-seq method combines four key components: a gRNA vector that makes individual gRNAs detectable in single-cell RNA-seq experiments, a high-throughput assay for single-cell RNA-seq[7], a computational pipeline for assigning single-cell transcriptomes to gRNAs, and a bioinformatic method for analyzing and interpreting gRNA-induced transcriptional profiles. CROP-seq thereby enables pooled screens with single-cell transcriptome readout, providing a scalable method for dissecting complex signaling pathways and other biological mechanisms that are not easily reduced to a single selectable marker (**Fig. 1d**).

RESULTS

Direct detection of gRNAs from single-cell transcriptome data

CRISPR gRNAs are typically transcribed by RNA polymerase III from a human U6 promoter (hU6). They lack a polyadenylated (poly-A) tail and are not detectable with current single-cell RNA-seq assays. We thus re-engineered a popular construct for pooled CRISPR screening (LentiGuide-Puro[8]) to include the gRNA in a polyadenylated mRNA transcript (**Fig. 1e**, **Supplementary Data**, and **Supplementary Fig. 1**). Because LentiGuide-Puro was originally derived from a lentiviral vector rendered self-inactivating by a 400-bp deletion of key promoter elements, we hypothesized that this position within the 3′ long terminal repeat (LTR) would tolerate the insertion of the similarly sized hU6–gRNA cassette. At this position, the gRNA becomes part of the puromycin-resistance mRNA transcribed by RNA polymerase II and detectable by RNA-seq protocols that use poly-A enrichment. Functional gRNAs continue to be expressed from the hU6 promoter. In addition, the entire hU6–gRNA cassette is copied to the 5′ LTR during reverse transcription and integration of the virus (**Supplementary Fig. 1**). This results in a second copy upstream of the possibly interfering EF-1α promoter—a design reminiscent of an early shRNA expression vector[9]. CROP-seq thereby solves the challenge of detecting gRNAs in single-cell transcriptomes at the vector level, which makes it compatible with various single-cell RNA-seq assays and with widely used cloning protocols for pooled screening.

Having assembled our CROPseq-Guide-Puro plasmid from four PCR products using the ligase cycling reaction[10] (**Supplementary Fig. 1** and **Supplementary Table 1**), we extensively validated its

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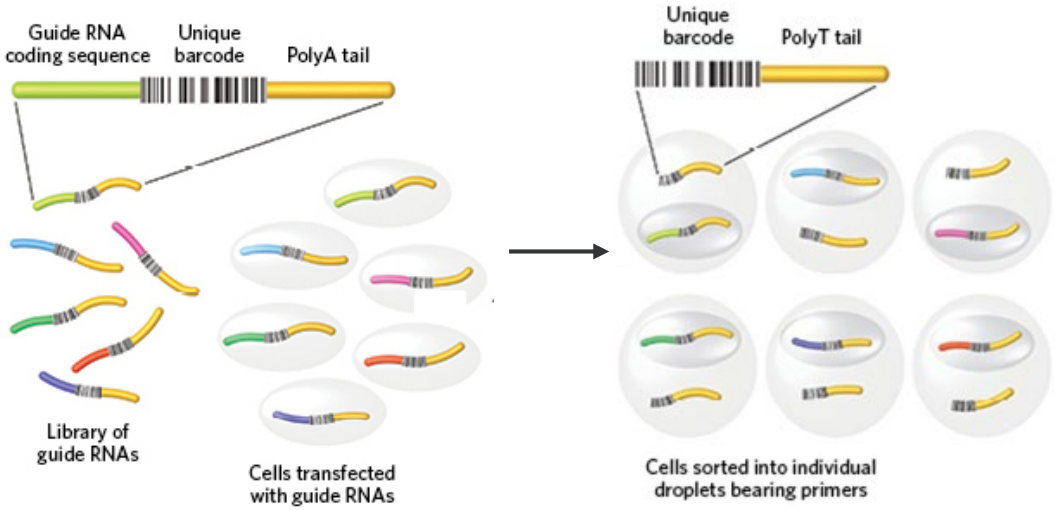
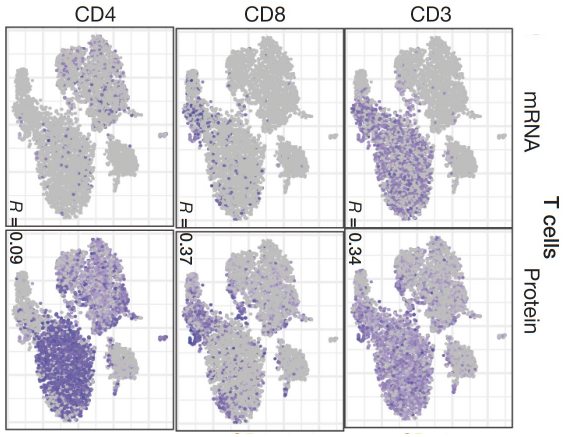
NATURE METHODS | ADVANCE ONLINE PUBLICATION | 1

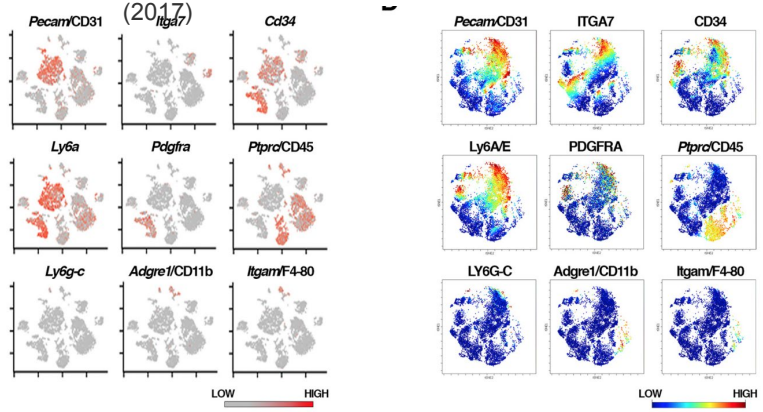


Single Cell Gene Expression and Cell Surface Proteins

Applications and Research Areas



Stoeckius M *et al.*, Nat Methods (2017)



Giordani L *et al.*, bioRxiv (2018)

Cancer

- Study cancer disease pathways and other complex biological systems
- Identify biomarkers for different types of cancers
- Protein and/or transcript based cancer drug development
- More comprehensive profiling of tumor microenvironment

Immunology

- Study mechanisms at the level of receptor, the cell, the population of responding cells
- Study dynamic interactions between lymphocytes and target cells
- T-cell genetic engineering & TCR-based fusion protein screens
- Develop effective vaccination (antibody screening)

Neuroscience & Infectious Disease

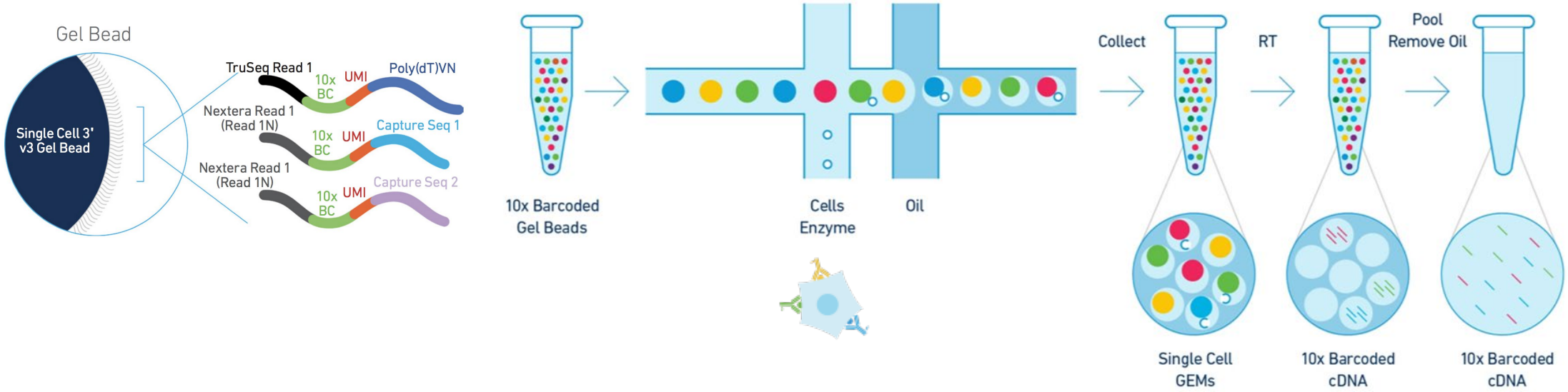
- Discover rare cell types and new biomarkers
- More complete pathway and cell signaling analysis

Human Cell Atlas/Tissue Cataloguing

- Enhanced phenotyping of tissues and organs
- Build more complete frameworks to better understand disease states

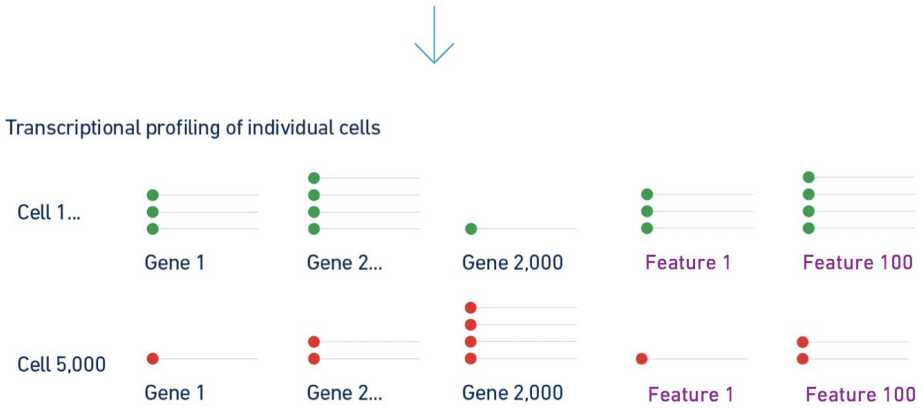
Single Cell Gene Expression and Cell Surface Protein Solution

Biochemistry Overview



- Inputs:
  10x Gel Beads, Reagents and single cells in suspension

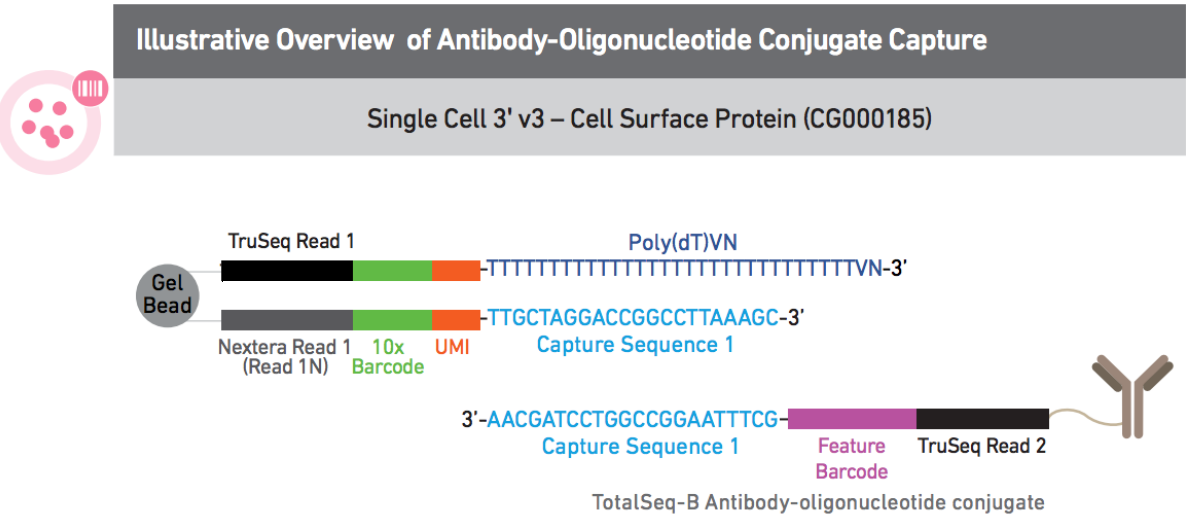
- Outputs:
  Digital gene expression and cell surface protein expression profiles from every partitioned cell



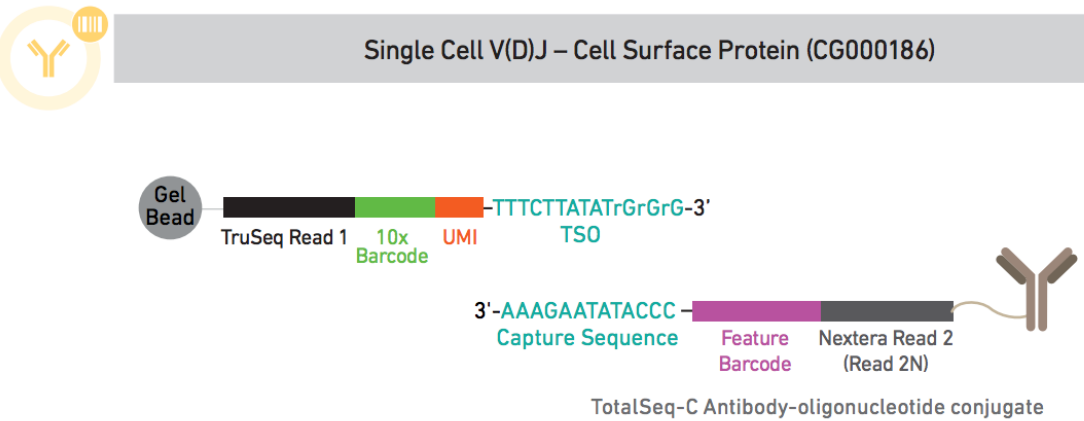
BioLegend's TotalSeq™- Antibody Reagents

10x Compatible Partner

TotalSeq-B



TotalSeq-C



Feature Barcode Structure

- Barcode structure can be found in User Guide Appendices and the Cell Labeling / Custom Conjugation DP

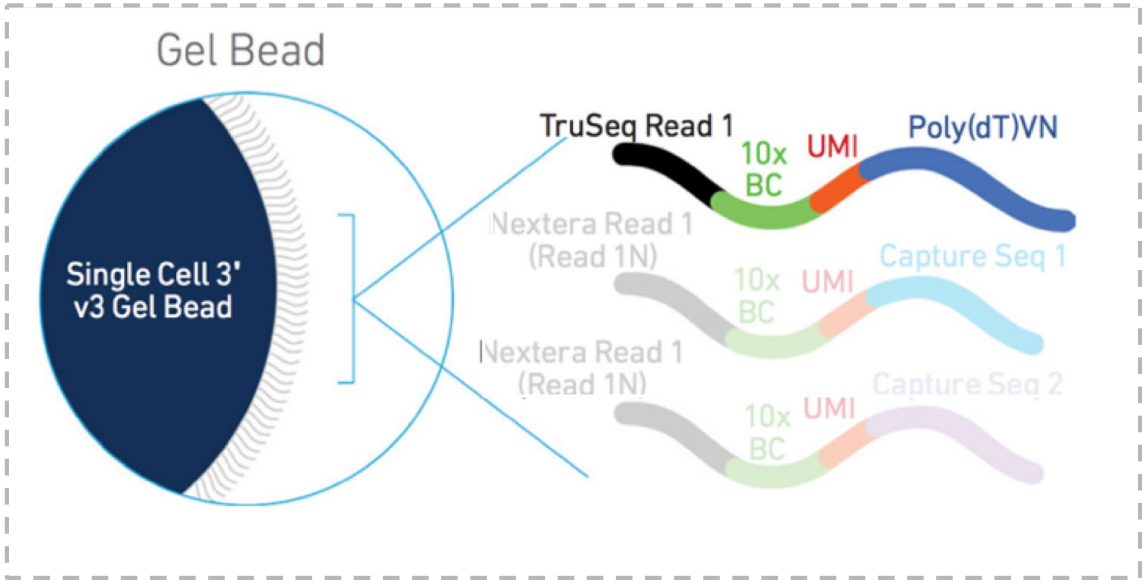
10x Genomics Protocol	Feature Barcode Oligonucleotide Sequence
Single Cell 3' v3 – Cell Surface Protein (CG000185)	/5AmMC12/GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNN-NNNNNNNNNNNNNNN-NNNNNNNNNGCTTTAAGGCCGGTCCTAGCAA TruSeq Read 2 — 10 nt — Feature Barcode (15 nt) — 9 nt — Capture Sequence 1
Single Cell V(D)J – Cell Surface Protein (CG000186)	/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 BC (15N) - 9N - Capture sequence

- The diversity sequence flanking the Feature Barcode is a UMI that can be used in custom analysis to demonstrate the absence of intra-GEM barcode exchange in Feature Barcode Libraries.
- Increases Feature Barcode R2 sequencing quality
- Is not directly used by CR3.0, but is flagged in the Feature Reference File

Single Cell Gene Expression with Feature Barcoding technology

Feature Barcoding technology Enabled via Single Cell 3' v3 Gel Beads



i. **TruSeq Read 1**

22 nt Partial Illumina TruSeq Read 1 sequence

ii. **10x BC**

16 nt 10x Barcode
~3.6 M defined barcode sequences

iii. **UMI**

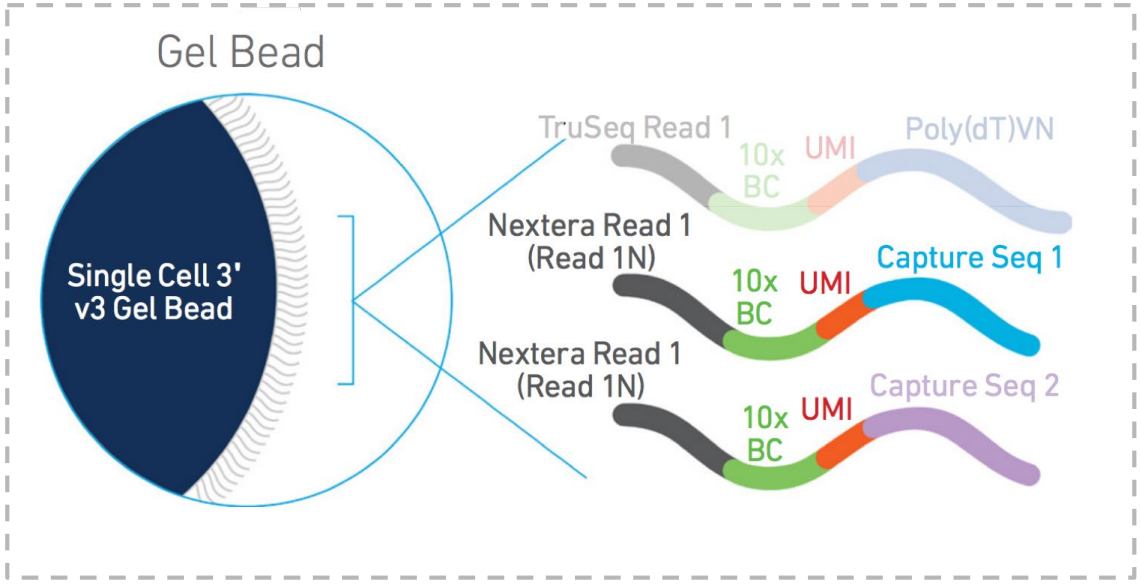
12 nt Unique Molecular Identifier

iv. **Poly(dT)VN**

30 nt Poly(dT) sequence
Enables capture of poly-adenylated mRNA molecules

Single Cell Gene Expression with Feature Barcoding technology

Feature Barcoding technology Enabled via Single Cell 3' v3 Gel Beads



i. **Nextera Read 1 (Read 1N)**

22 nt Partial Illumina Nextera Read 1 sequence
(Enables selective enrichment of the Feature Barcode construct)

ii. **10x BC**

16 nt 10x Barcode
~3.6 M defined barcode sequences

iii. **UMI**

12 nt Unique Molecular Identifier

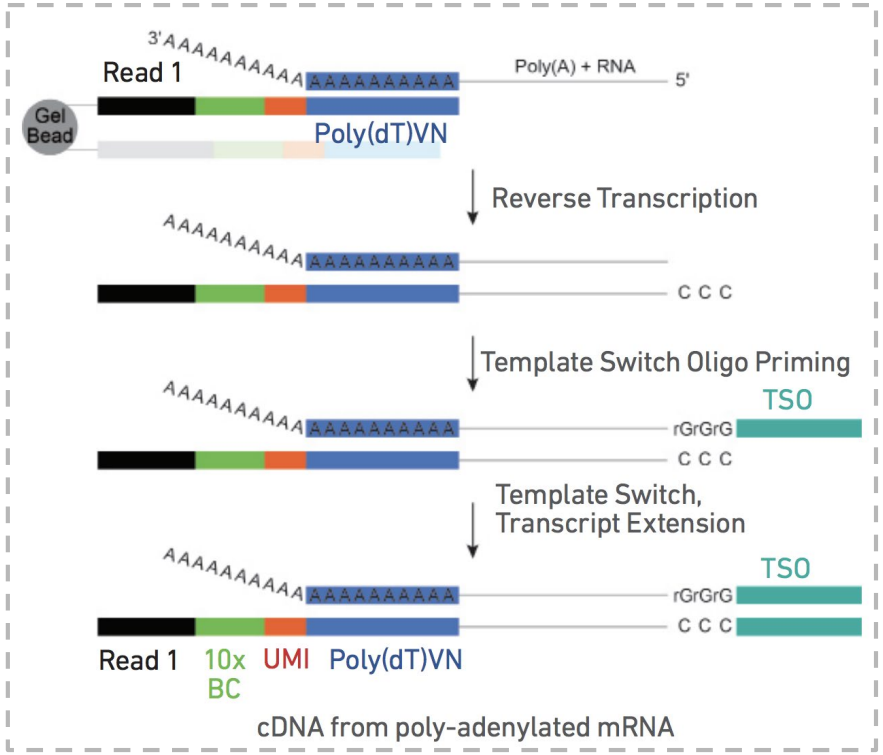
iv. **Capture Sequence 1** or **2**

22 nt sequence that is the reverse complement of the sequence inserted into the DNA (Antibody) or RNA (sgRNA) based Feature

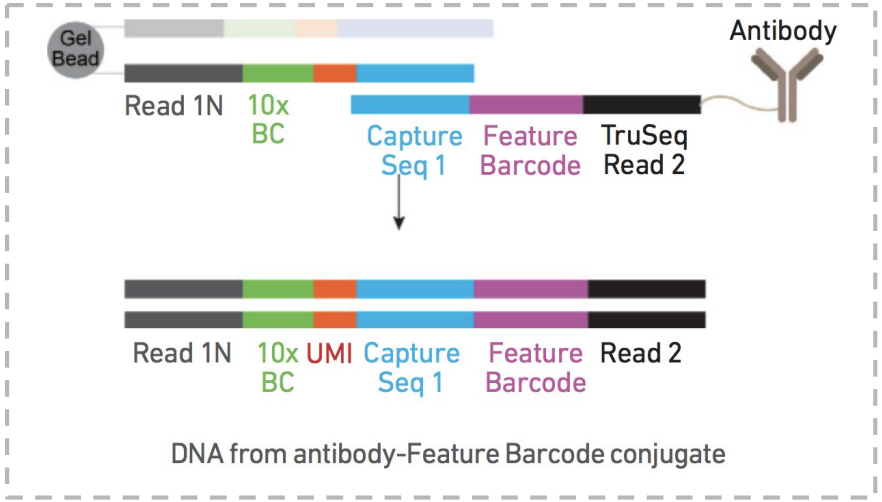
Feature Barcoding Workflow: In GEM Target Capture

Direct Capture of Multiple Targets Inside Individual GEMs

Barcoded, full-length cDNA from poly-adenylated mRNA



Barcoded, cell surface protein conjugated oligonucleotide

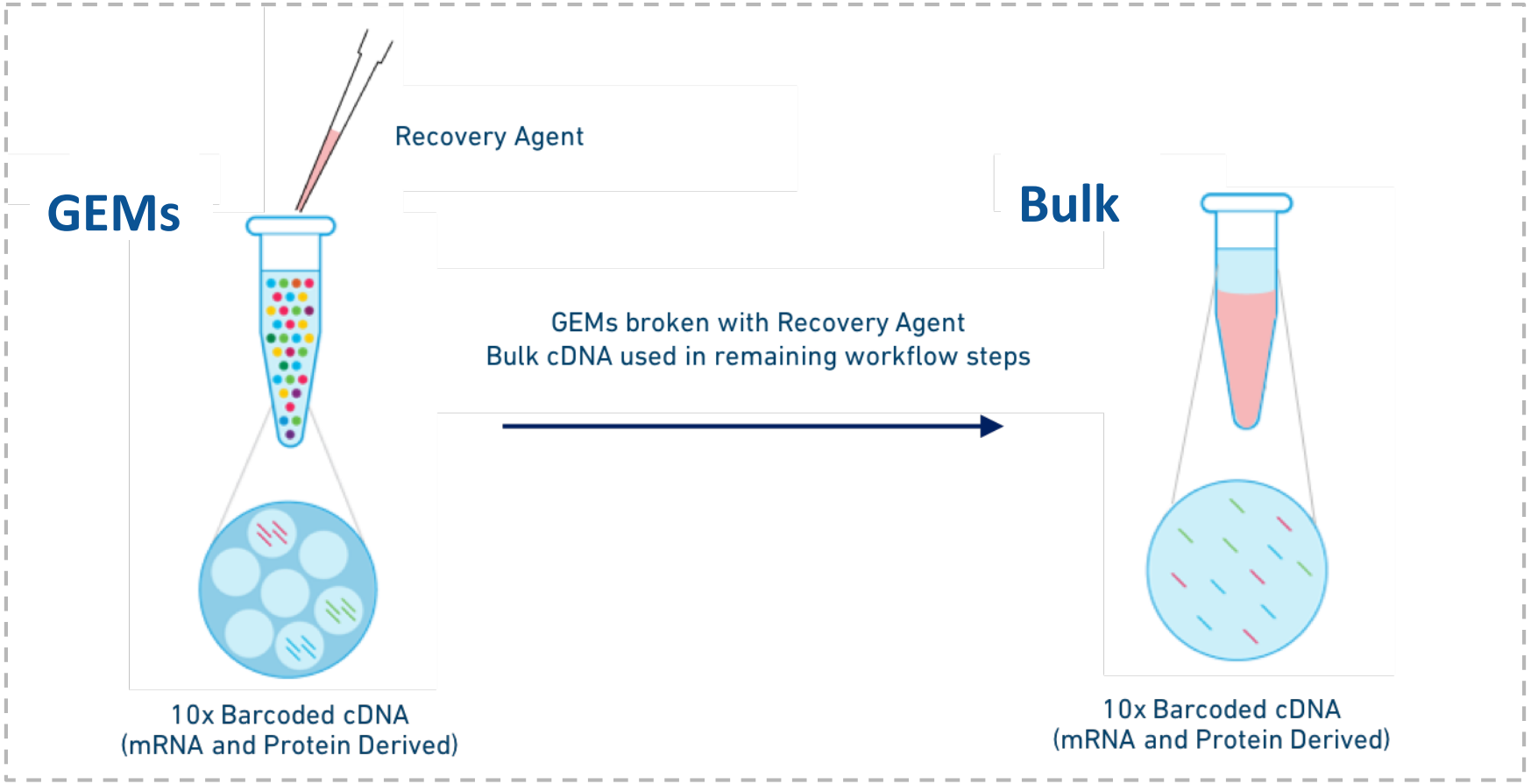


Note: Gene Expression and Feature Barcode molecules are all directly captured by the corresponding gel bead oligo, however the mechanism of capture is different.

Poly-adenylated mRNA: Reverse Transcription and Template Switching

Cell surface protein conjugated oligonucleotide: Prime and Extend

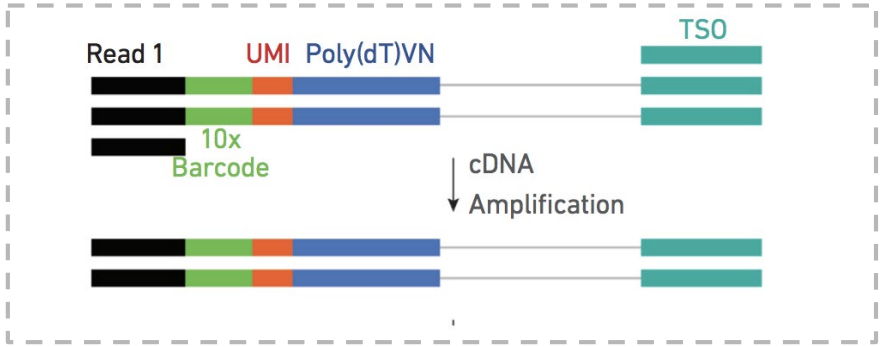
Feature Barcoding Workflow: Breaking GEM's



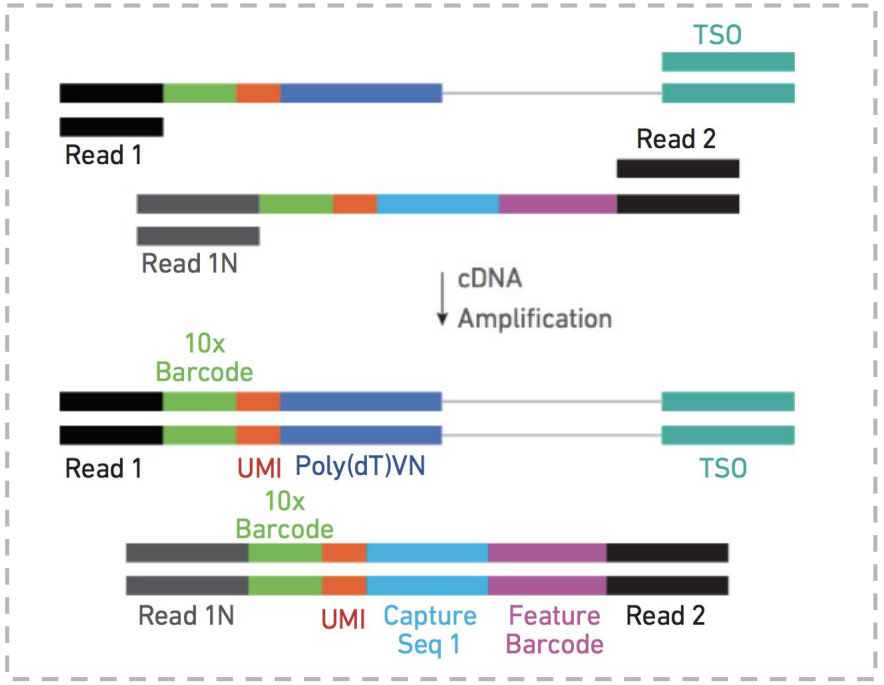
Feature Barcoding Workflow: cDNA Amplification

In Bulk Amplification of mRNA and Protein-Feature Barcode Targets

cDNA Amplification of poly-adenylated mRNA only



cDNA Amplification of poly-adenylated mRNA and cell surface protein conjugated oligonucleotide

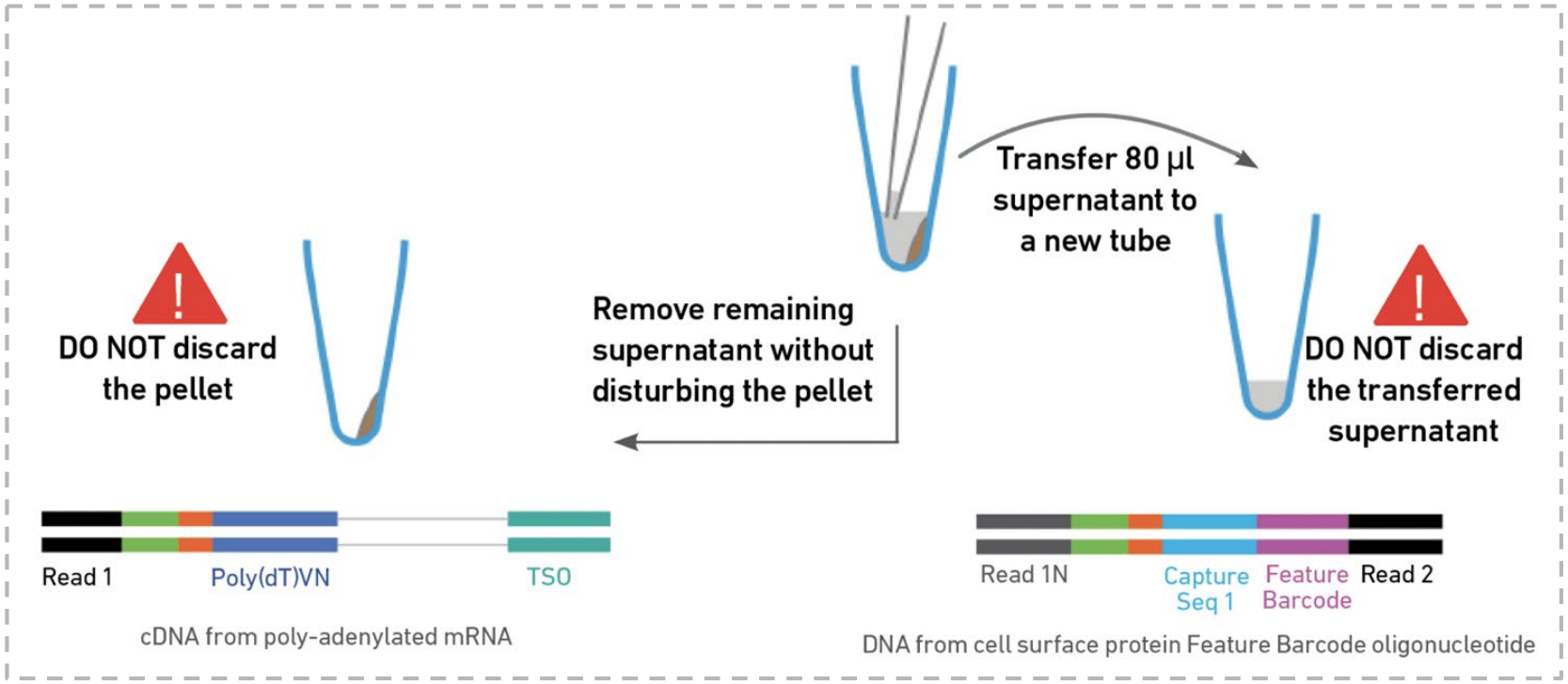


Note: For cDNA Amplification of poly-adenylated mRNA only please use:
For cDNA Amplification of poly-adenylated mRNA and protein please use:

- cDNA Primers (PN 2000089)
- Feature cDNA Primers 2 (PN 2000097)

Feature Barcoding Workflow: cDNA Cleanup

Sample Bifurcation Critical to Success

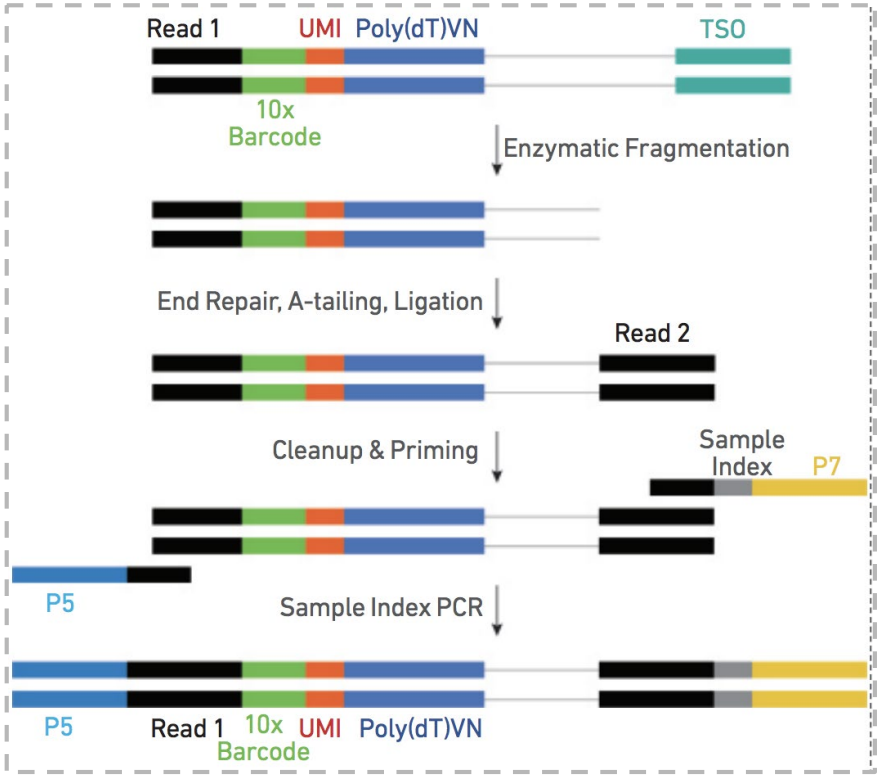


Feature Barcoding Workflow: Library Construction

Sample Bifurcation Enables Generation of Two Sequenceable Library Constructs

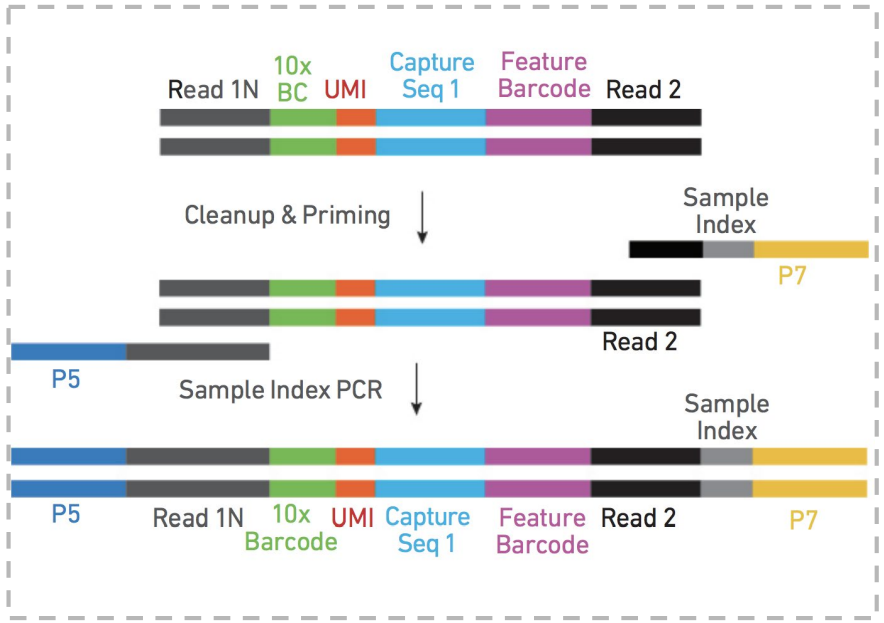
Pellet

Single Cell Gene Expression Library Construction



Transferred Supernatant

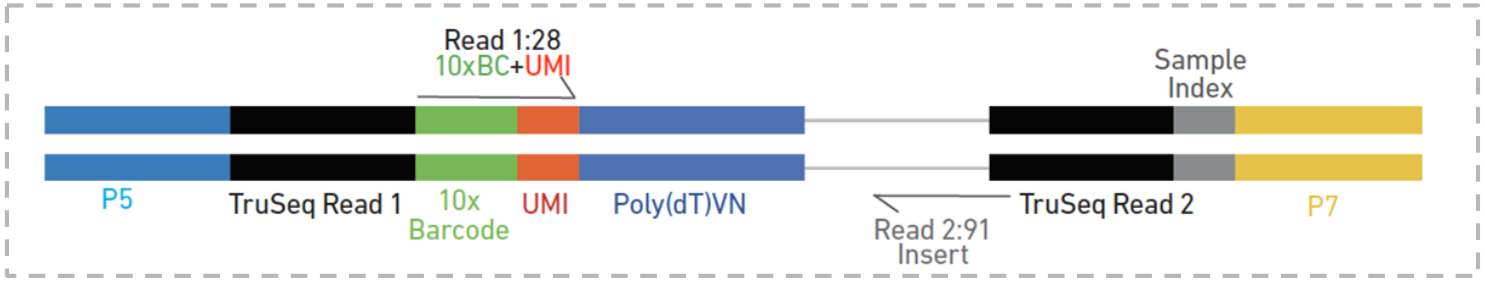
Single Cell 3’ Cell Surface Protein Library Construction



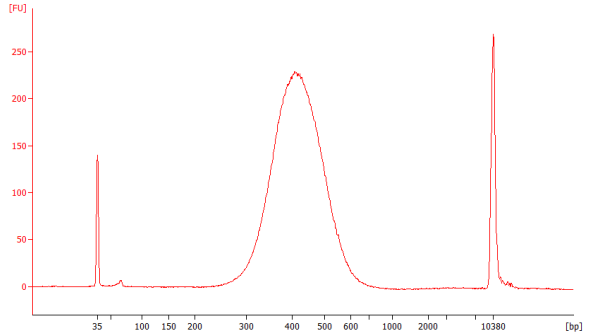
Feature Barcoding Workflow

Final Library Constructs

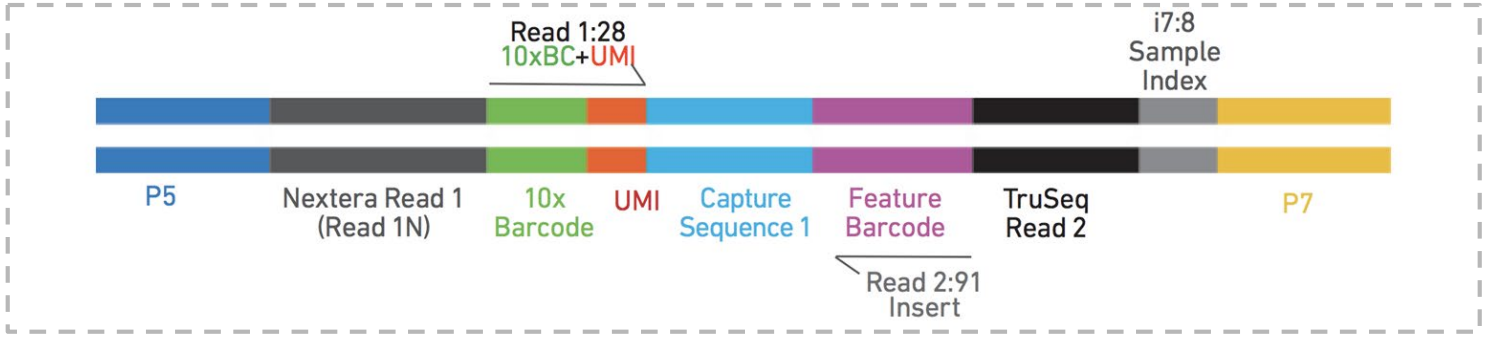
Chromium Single Cell 3’ Gene Expression Library



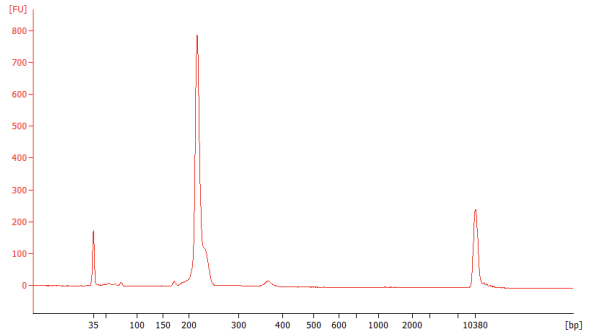
Representative Trace



Chromium Single Cell 3’ Cell Surface Protein Library



Representative Trace



10X GENOMICS

Chromium Single Cell Gene Expression Solution with Feature Barcoding technology

Sample Preparation

Antibody Preparation

Custom Conjugation

Specific Reagents and Consumables

Vendor	Item	Part Number
Expedeon	ThunderLink Plus	425-0300
IDT	Feature Barcode Oligonucleotide (see Table 1 & Appendix)	-
	100 µg Purified Azide-free Antibody	

To perform custom conjugation, a customer needs to acquire:

- ThunderLink Plus kit
- Feature Barcode Oligonucleotide
  - 10 nmoles, HPLC purified, lyophilized
  - **NOT** resuspended in TE buffer
  - The BC structure must be compatible with desired assay
  - The BC sequence should be selected from the provided whitelist
    - This ensures compatibility if pooled with commercially available Feature Barcoding technology compatible antibodies
- 100 µg Antibody

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

Note: Consult Barcode Whitelist for Custom Feature Barcoding conjugates (Document CG000193), for more information

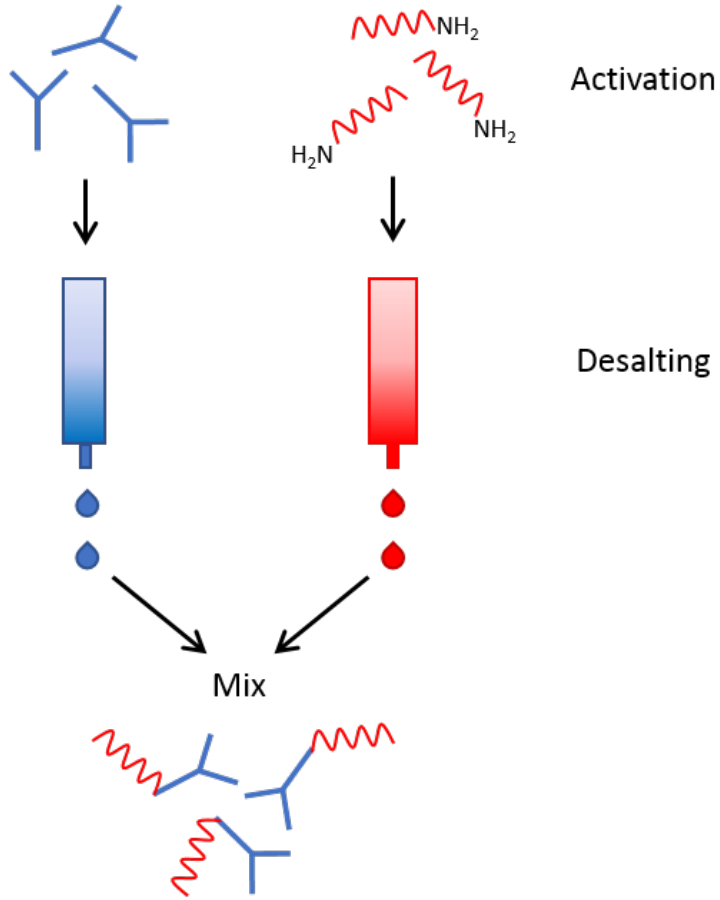
Antibody Preparation

Thunder-Link® PLUS Oligo Conjugation System

Innova Biosciences

Key Workflow Steps

1. Suspend oligo and antibody in compatible buffer
2. Activate antibody, activate oligo (mix with lyophilized activation reagent)
3. Purify activated antibody, purify activated oligo
4. Mix purified species together & incubate
5. Purify the conjugate from unconjugated oligo
6. QC using non-reducing SDS-PAGE



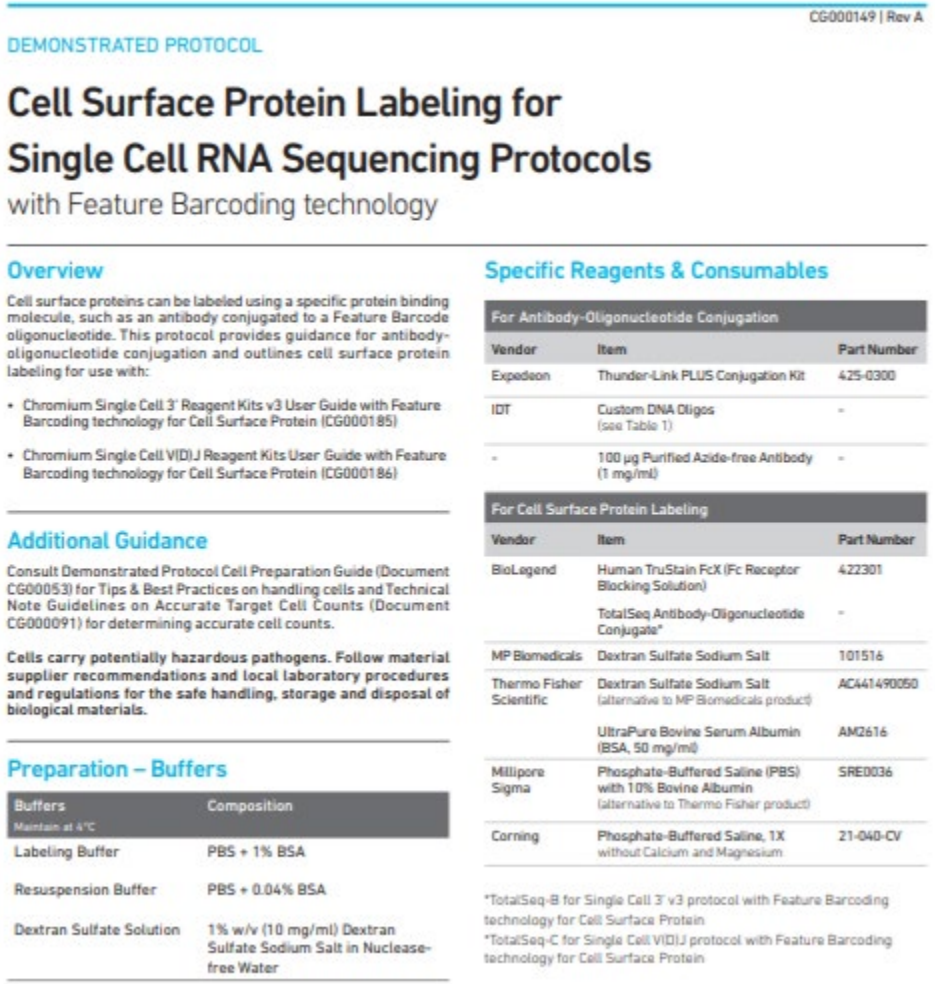
Thunderlink Plus Protocol: https://www.expedeon.com/images/stories/innova/pdfs/thunderlink_plus_oligo_protocol_r1.pdf

Sample Preparation

Cell Surface Protein Labeling Demonstrated Protocol

Key Workflow Steps

1. Block cells (optional), on ice
    a. FcX: block cell Fc receptors
    b. Dextran Sulfate: block positive charges
2. Prepare antibody pool
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 load GEMs



CG000149 | Rev A

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:

- Chromium Single Cell 3' Reagent Kits v3 User Guide with Feature Barcoding technology for Cell Surface Protein (CG000185)
- Chromium Single Cell V(D)J Reagent Kits User Guide with Feature Barcoding technology for Cell Surface Protein (CG000186)

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	1% w/v (10 mg/ml) Dextran Sulfate Sodium Salt in Nuclease-free Water

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)	-

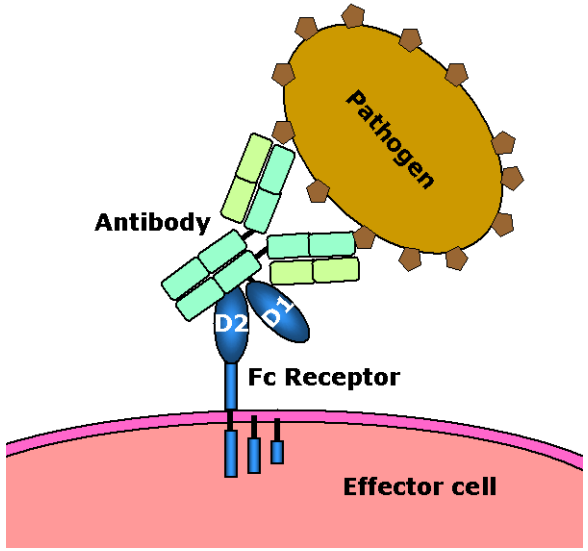
For Cell Surface Protein Labeling		
Vendor	**Item**	**Part Number**
BioLegend	Human TruStain FcX (Fc Receptor Blocking Solution)	422301
	TotalSeq Antibody-Oligonucleotide Conjugate*	-
MP Biomedicals	Dextran Sulfate Sodium Salt	101516
Thermo Fisher Scientific	Dextran Sulfate Sodium Salt (alternative to 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

*TotalSeq-B for Single Cell 3' v3 protocol with Feature Barcoding technology for Cell Surface Protein
*TotalSeq-C for Single Cell V(D)J protocol with Feature Barcoding technology for Cell Surface Protein

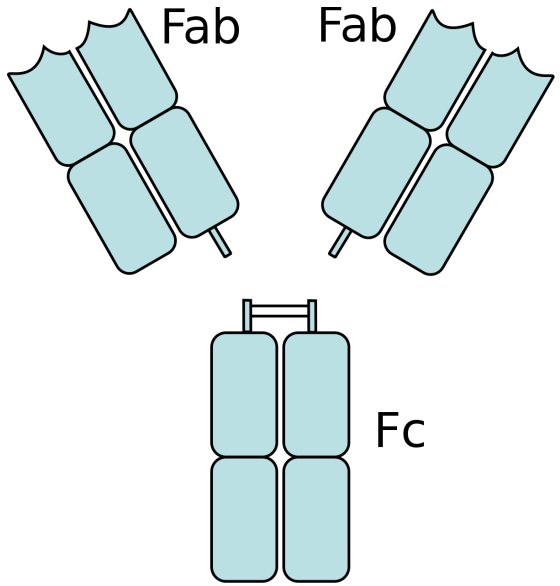
FcX (Fc block) decreases nonspecific binding to Fc receptors

Optional, but Recommended, Step Prior to Labeling Cells with Specific Antibodies

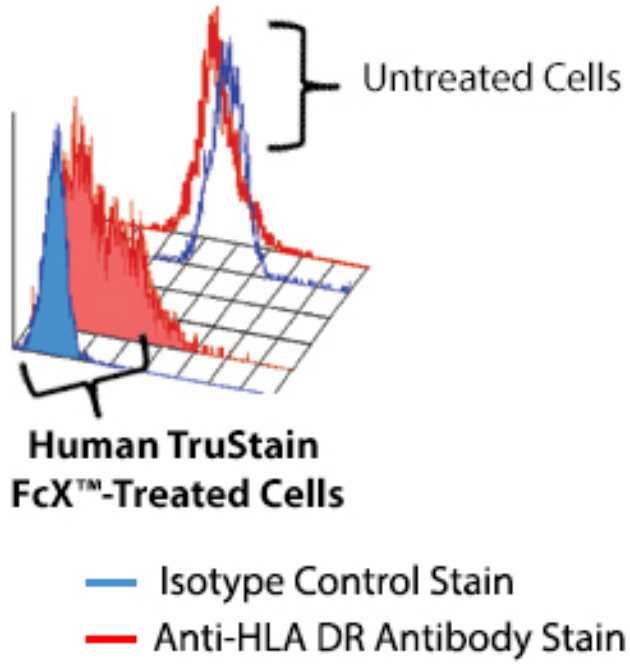
FC Receptor binds antibodies



We want to capture specific interaction via the Fab region



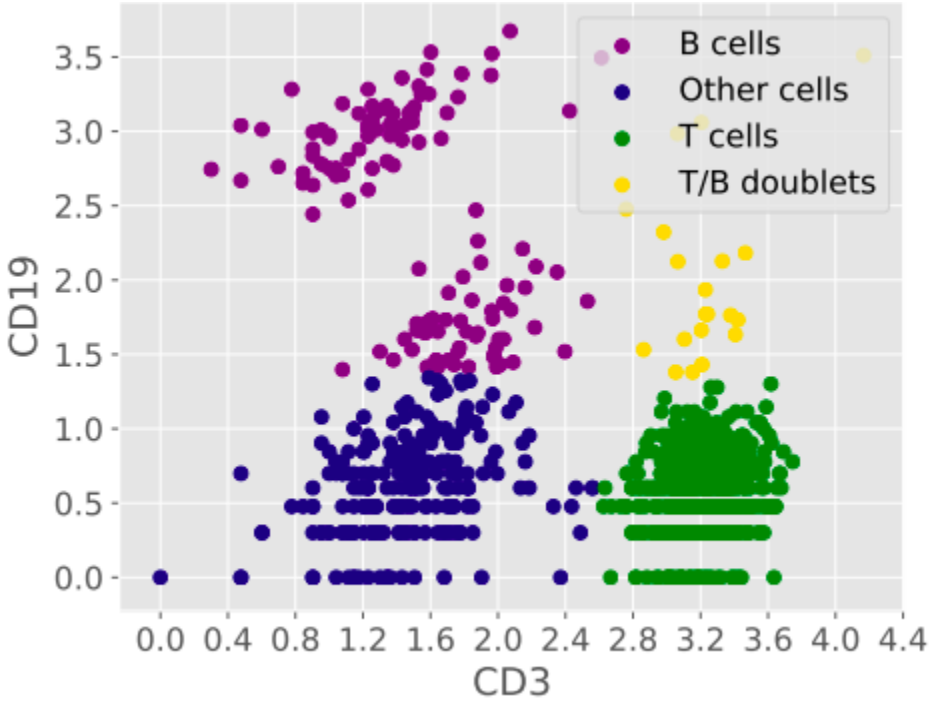
FcX helps Feature Barcoding technology in the same way it helps Flow Cytometry



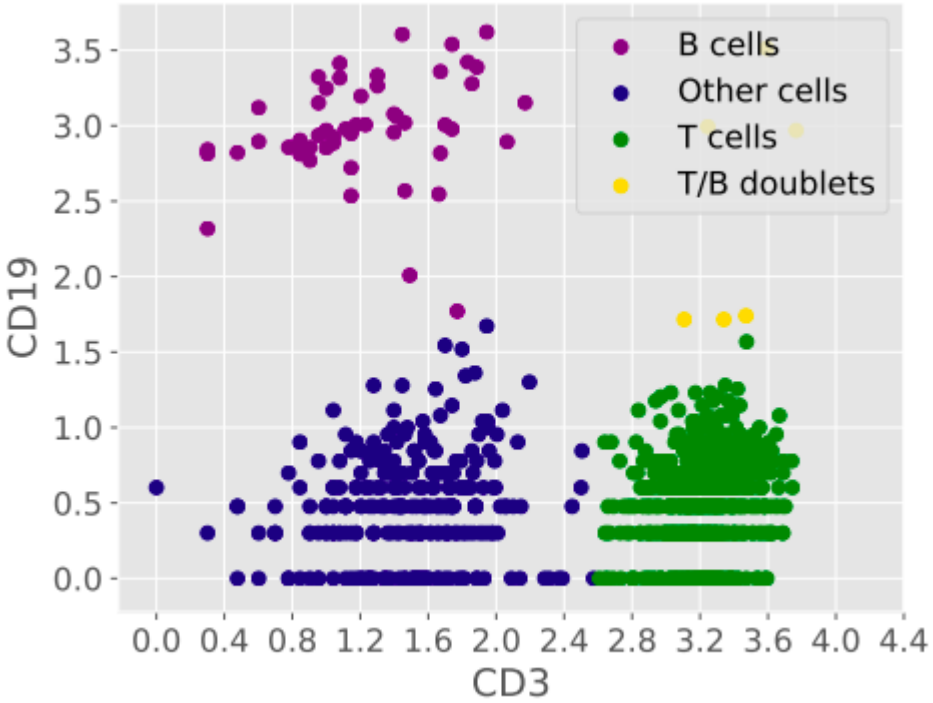
https://www.biolegend.com/en-us/products/human-trustain-fcx-fc-receptor-blocking-solution-6462

Dextran sulfate increases T/B cell purity

Without Dextran Sulfate
Increased Background staining on non T/B cells



With Dextran Sulfate: cleaner staining



Prepare the Antibody Pool

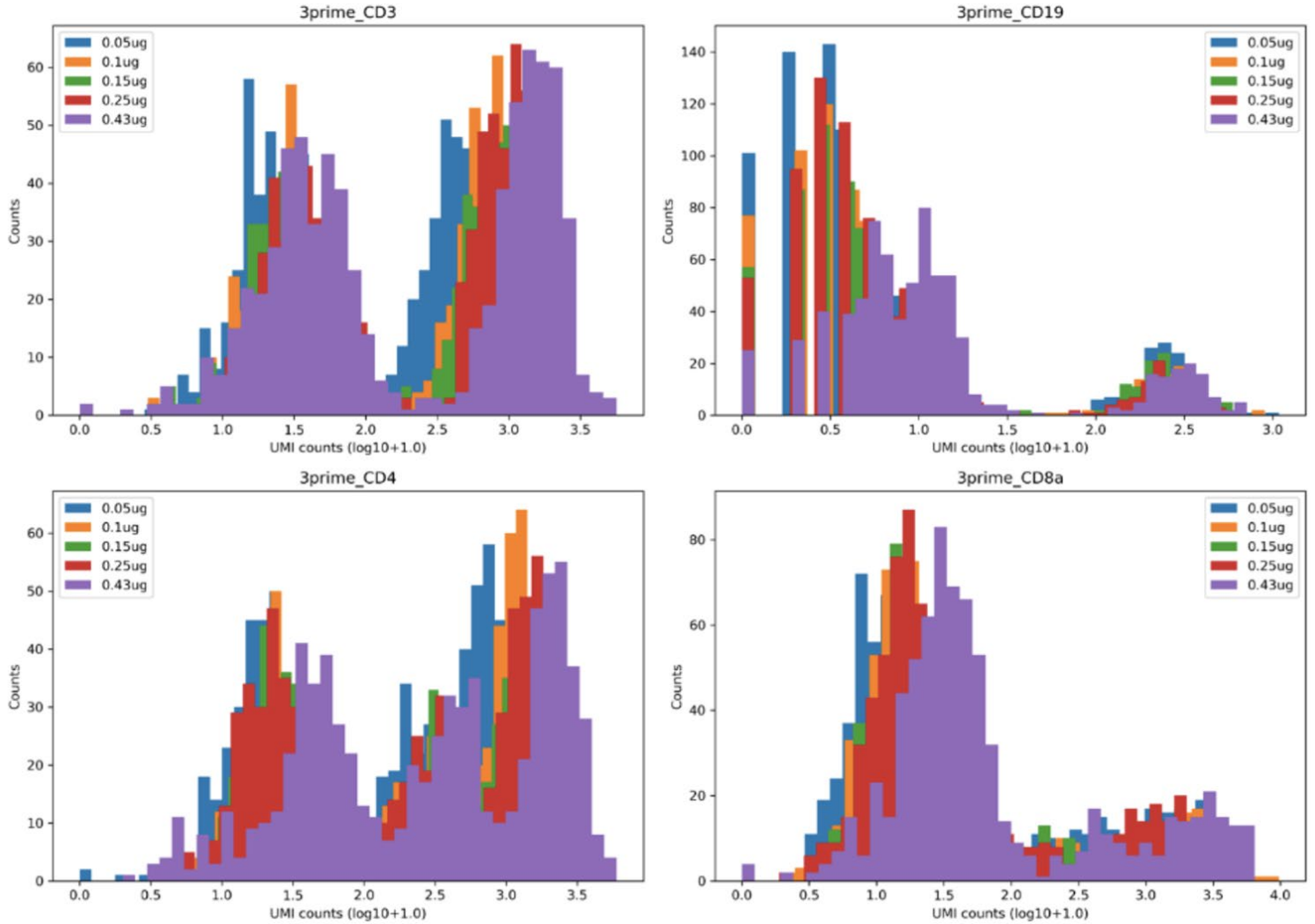
- Target compatibility
  - Very common markers (ie, CD45) can overtake a library when pooled with rare markers
  - Can compensate by sequencing deeper
- Antibody titration
  - BioLegend recommendation: 1 µg per Ab/ 100 µL
  - 10x Verification and Validation data collected at 0.25 µg / 100 µL
  - Recommend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

Feature Barcode Titration

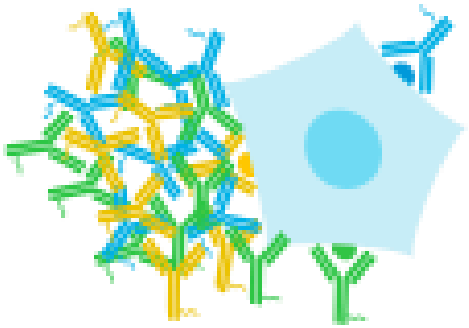
Specificity over ~1 log of concentration

- 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

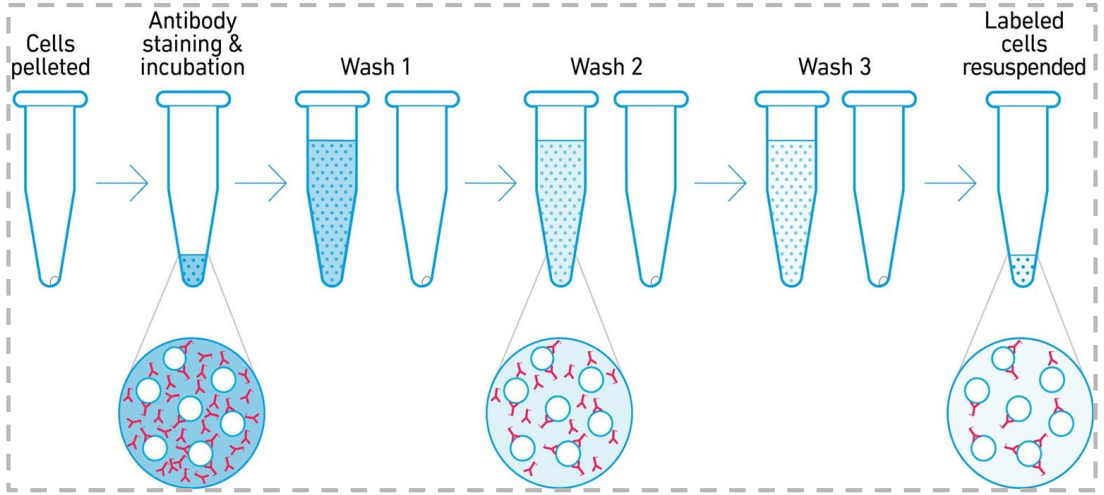
Remove Aggregates Before Staining



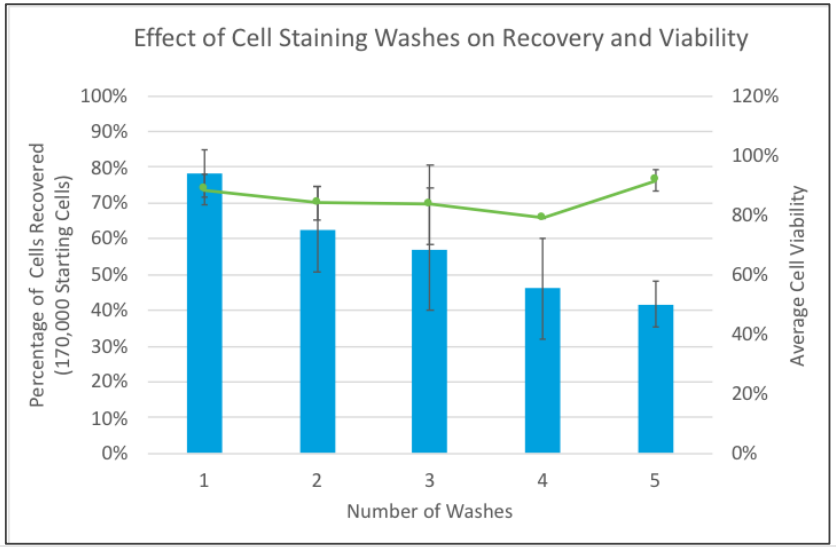
1. Make a pool of the desired antibodies for staining, allowing ~10% excess volume
2. Centrifuge at 14,000 rcf for 10 minutes (room temperature or 4°C)
3. Carefully remove the supernatant for use
4. Stain with appropriate volume of antibody pool
   - Alternate: filter antibody pool through 0.2 um filter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

Labeling and Washing Cells

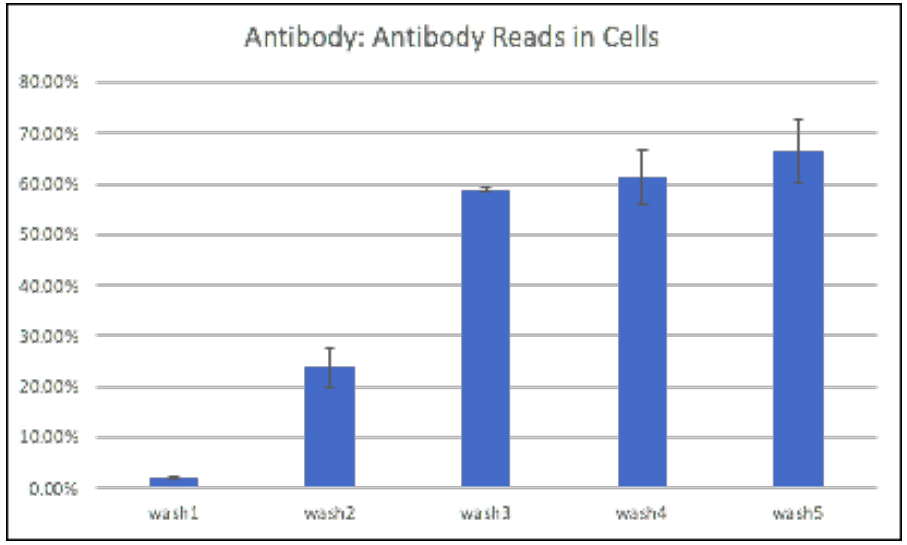
- For delicate cell types, staining can be done in media, buffer + FBS, or any cell-compatible buffer
- Cell centrifugation should be done at a speed appropriate for cell type
- Recommended: final resuspension in PBS + 0.04% BSA



Cells are lost with each subsequent wash



Three washes results in high Antibody Reads in Cells



TotalSeq-B Antibodies Tested In-House

Immune Based Panel

- Control cells will depend on the experimental design and the panel constituents.
- Peripheral Blood Mononuclear Cells (PBMCs)*
  From AllCells (Catalog # PB003F)
  Cryopreserved, 15 million cells
- Normal Peripheral Blood Pan T Cells
  From AllCells (Catalog # PB009-1F)
  Cryopreserved, 20 million cells
  Negatively isolated from mononuclear cells with magnetic beads
- Normal Peripheral Blood CD19+ B Cells
  From AllCells (Catalog # PB010F)
  Cryopreserved, 10 million cells
  Negatively isolated from mononuclear cells with magnetic beads

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

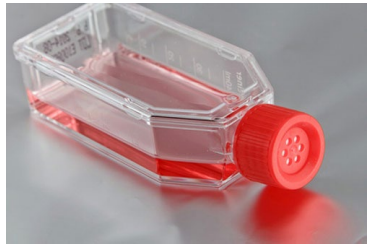
10X GENOMICS

Chromium Single Cell Gene Expression Solution with Feature Barcoding technology

Workflow Considerations and Sequencing Metrics

Samples Validated In-House

Human Cell Lines



Suspension Cells: Jurkat, Raji, GM12878

ATCC®

Primary Immune Cells

Human PBMC's
Human BMMC's
Mouse PBMC's

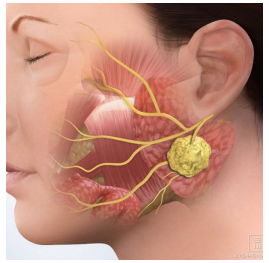
ALLCELLS®

iQ BIOSCIENCES®

STEMCELL™ TECHNOLOGIES

Dissociated Primary Tissues

Mouse Splenocytes



Mucosa Associated Lymphoid Tissue Lymphoma

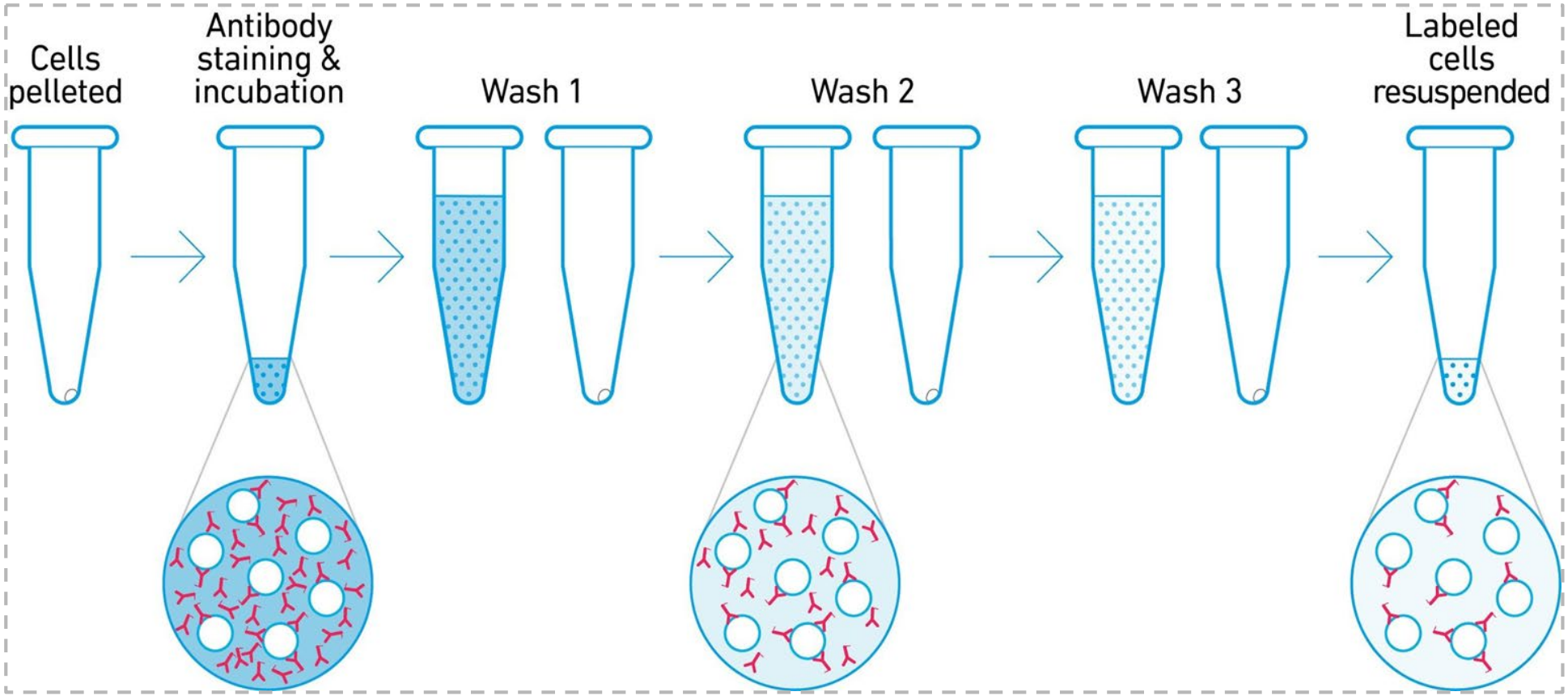
DISCOVERY LIFE SCIENCES

iQ BIOSCIENCES®

Workflow Considerations, Critical Steps and Performance Impacts

Staining and Washing Labeled Cells Reduces Non-specifically Bound Antibodies

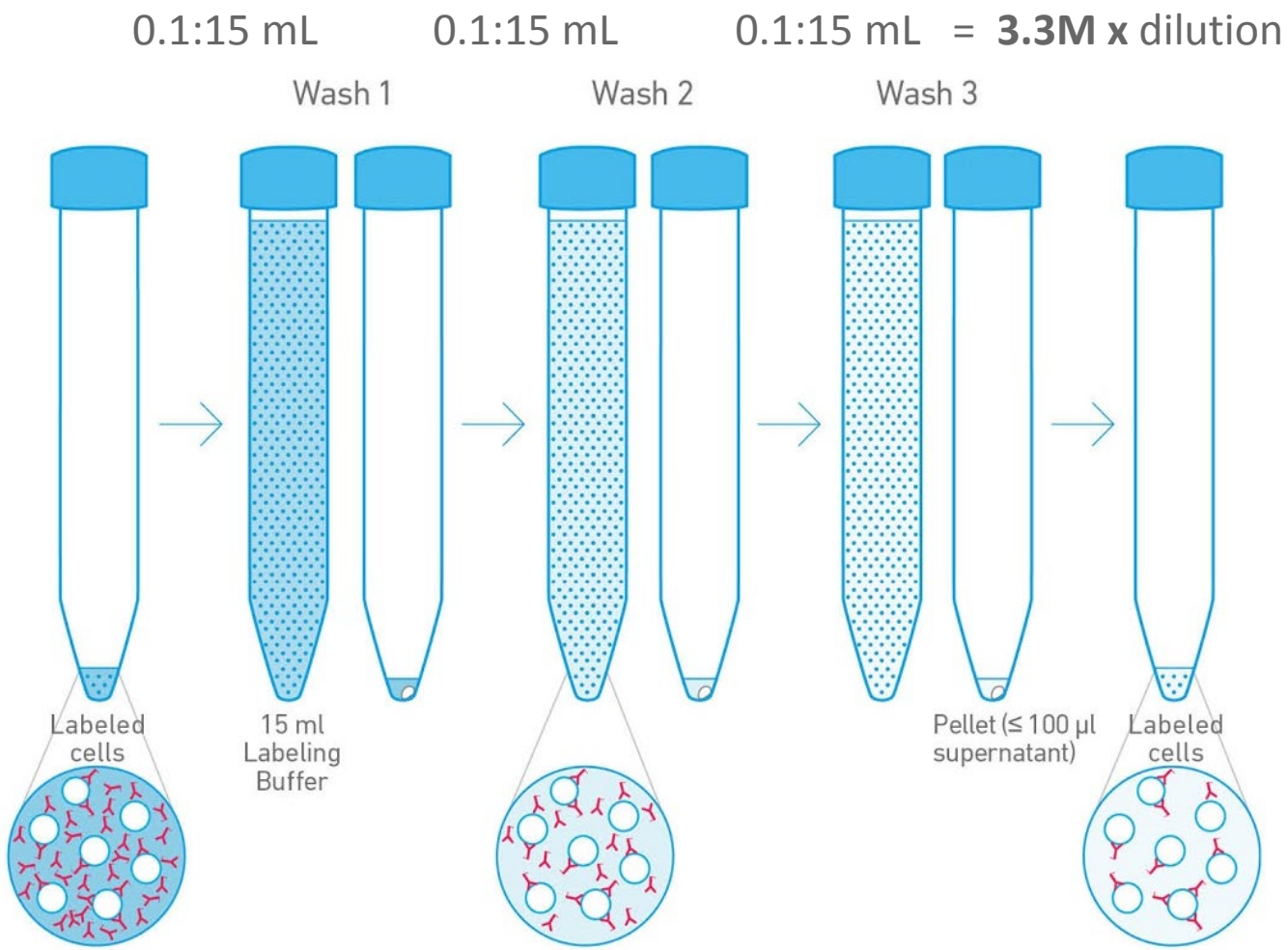
100:1500 µL 10:1500 µL 10:1500 µL 10:50 µL = ~**1.5M x** dilution



Larger Wash Volume is Compatible

Performing Wash in 15 mL Centrifuge

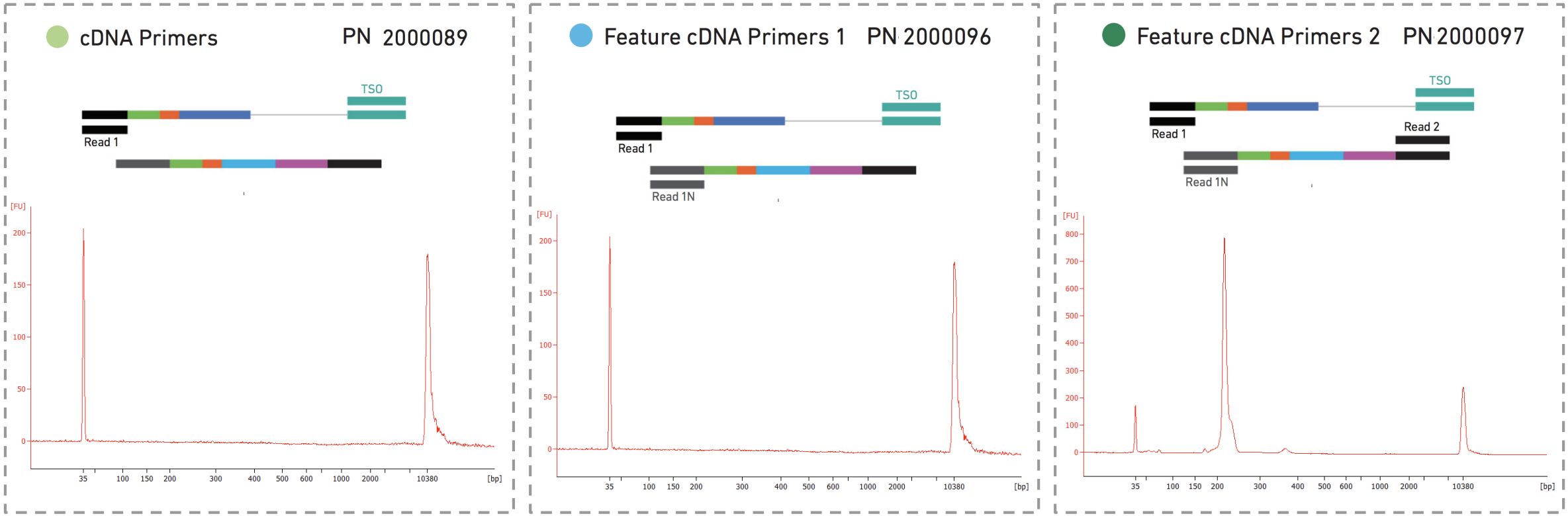
- Increasing the wash volume to 15 ml may be preferred by som customers
- Remove supernatant, and leave <100 µl of buffer behind with cell pellet
- Overall background dilution is the same as 1.5 ml wash with 1 µl pellet



Workflow Considerations, Critical Steps and Performance Impacts

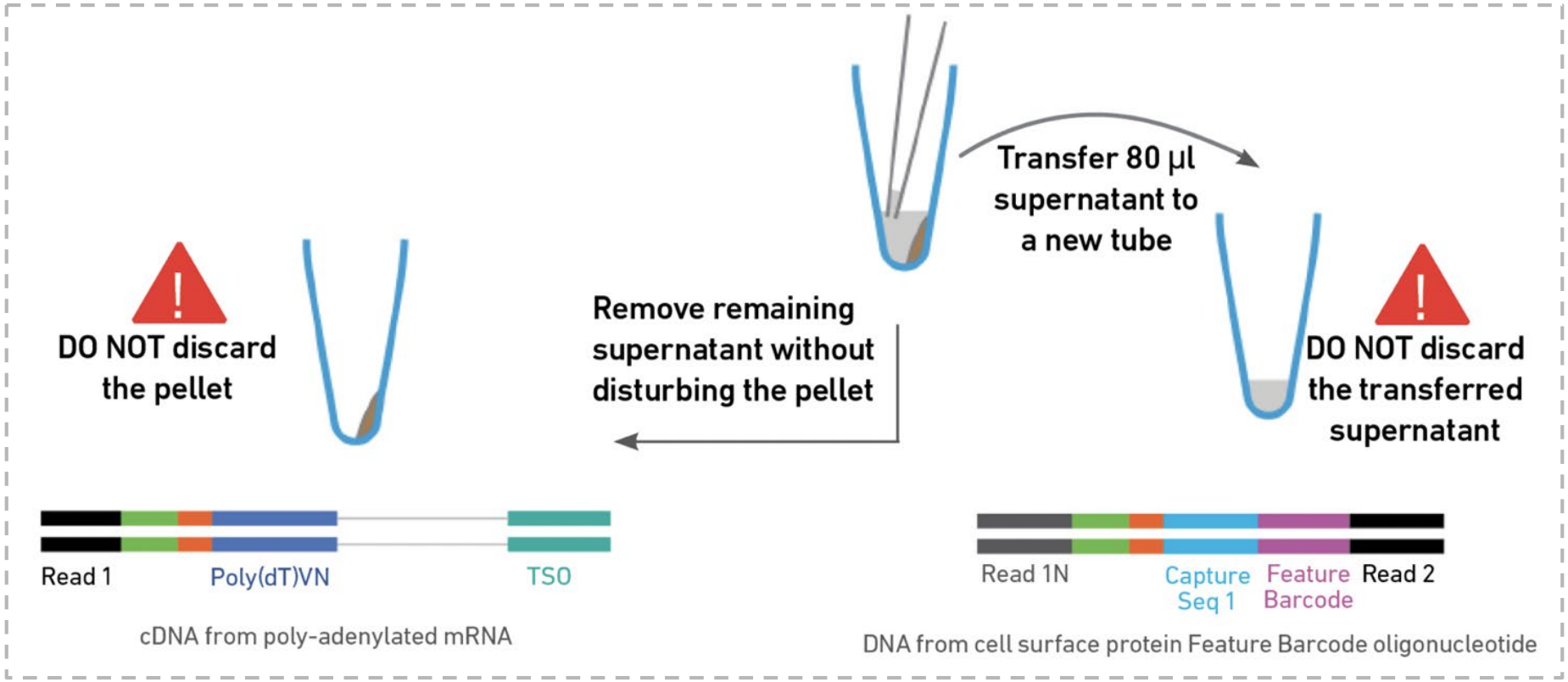
Correct cDNA Primer Choice Enables Successful Feature Barcode Amplification

Feature cDNA Primers 2 enables successful amplification of the Feature Barcode target



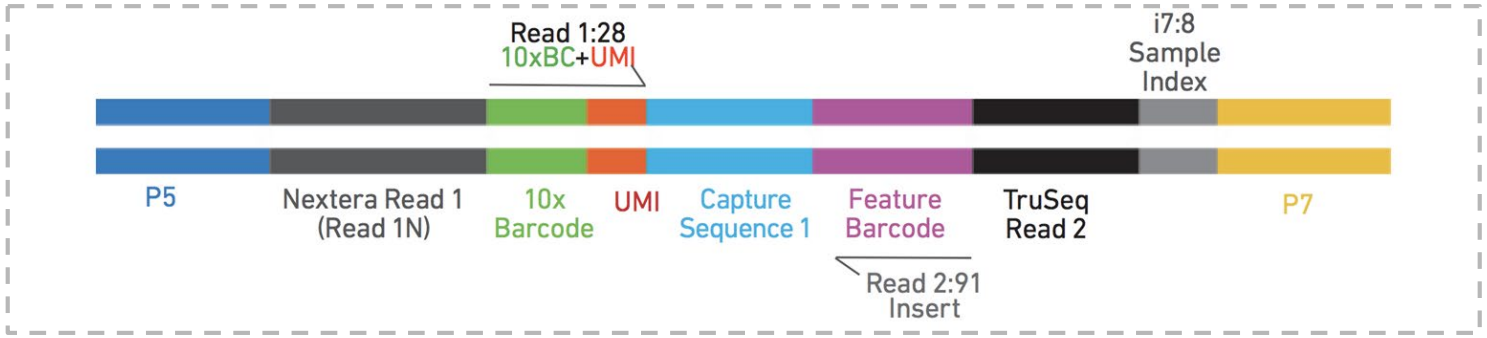
Workflow Considerations, Critical Steps and Performance Impacts

Save the Supernatant - Sample Bifurcation Enables Feature Barcode Library Construction



Supported Sequencers

Single Cell 3’ Cell Surface Protein Library



	Single Cell 3’ Gene Expression	Single Cell 3’ Cell Surface Protein
Raw Read Pairs per Cell	Minimum 20,000	Minimum 5,000

MiSeq

NextSeq

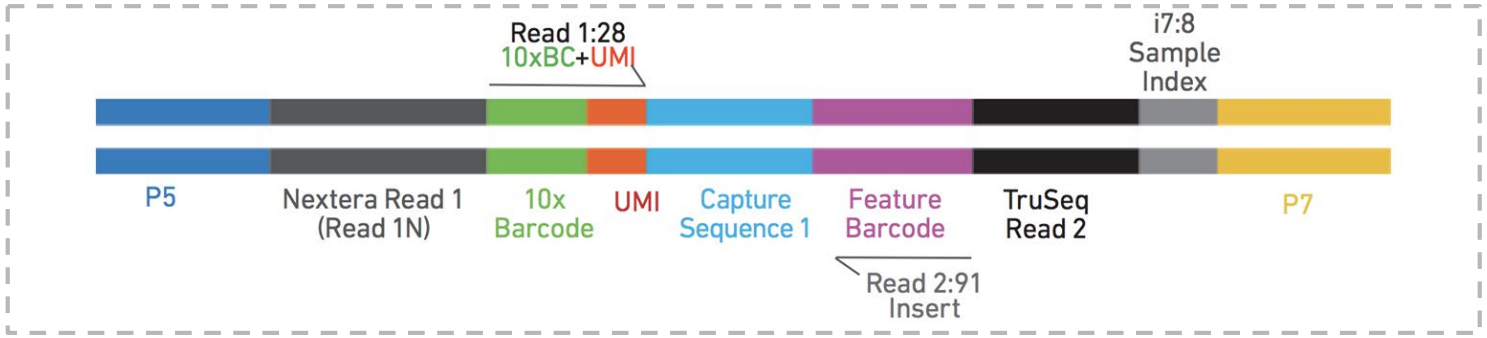
HiSeq2500

HiSeq 3000/4000

NovaSeq

Recommended Read Length

Single Cell 3’ Cell Surface Protein Library

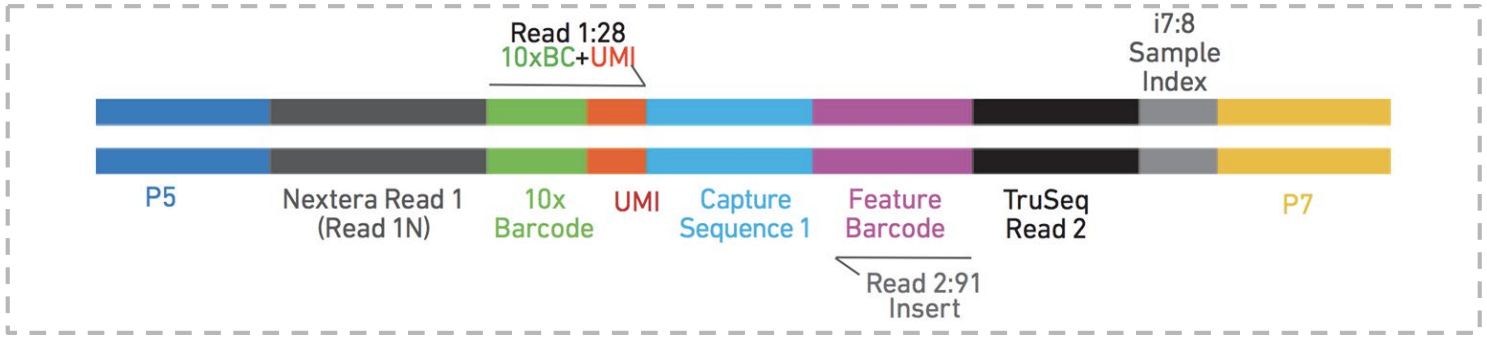


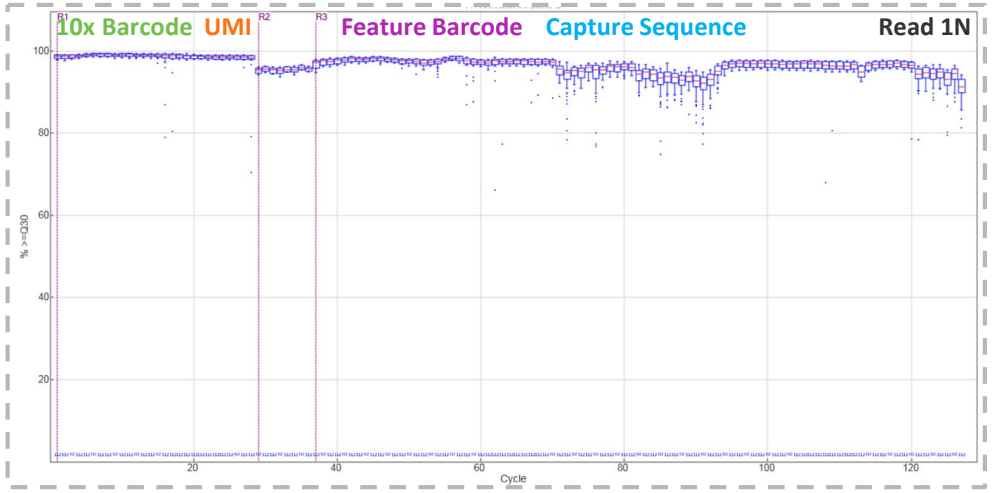
	Read 1	i7 Index	i5 Index	Read 2
Purpose	10x Barcode & UMI	Sample Index	N/A	Insert
Length	28	8	0	91*

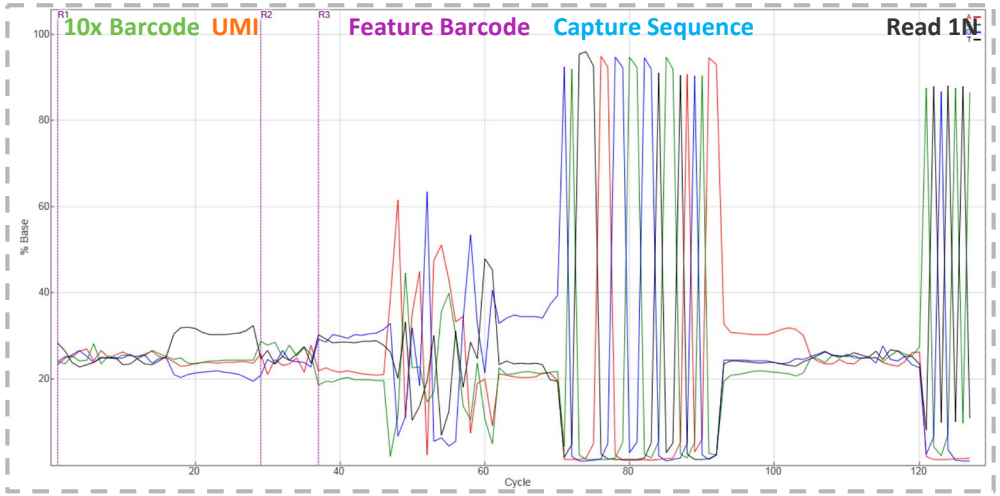
Note: * Single Cell 3’ Cell Surface Protein Libraries are typically pooled with Single Cell Gene Expression Libraries and sequenced using these parameters. The minimum required Read 2 length for Cell Surface Protein Libraries is 25 bp.

Base Balance Composition

Single Cell 3’ Cell Surface Protein Library



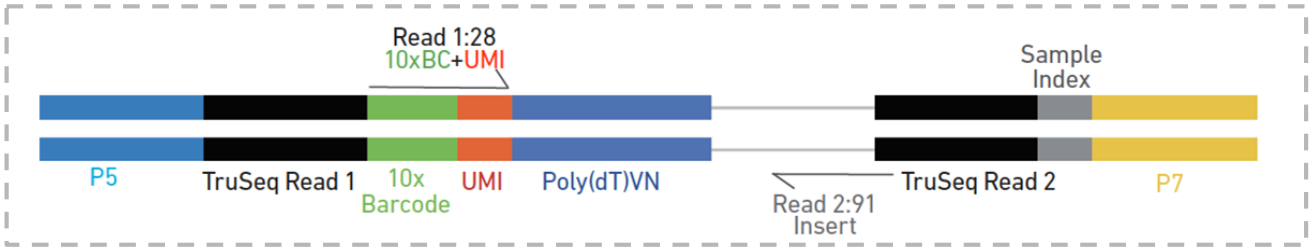


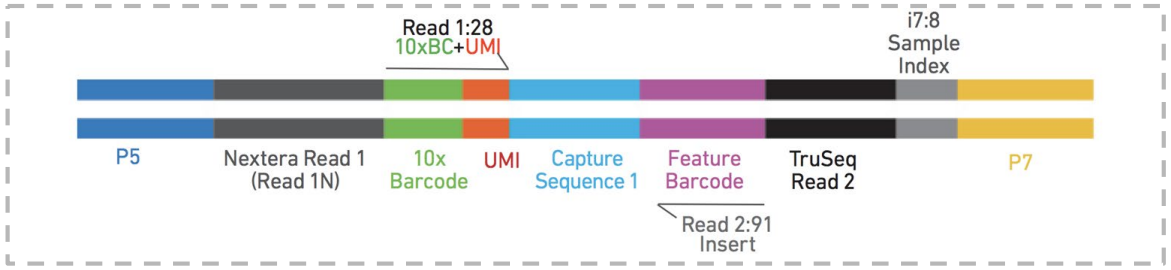


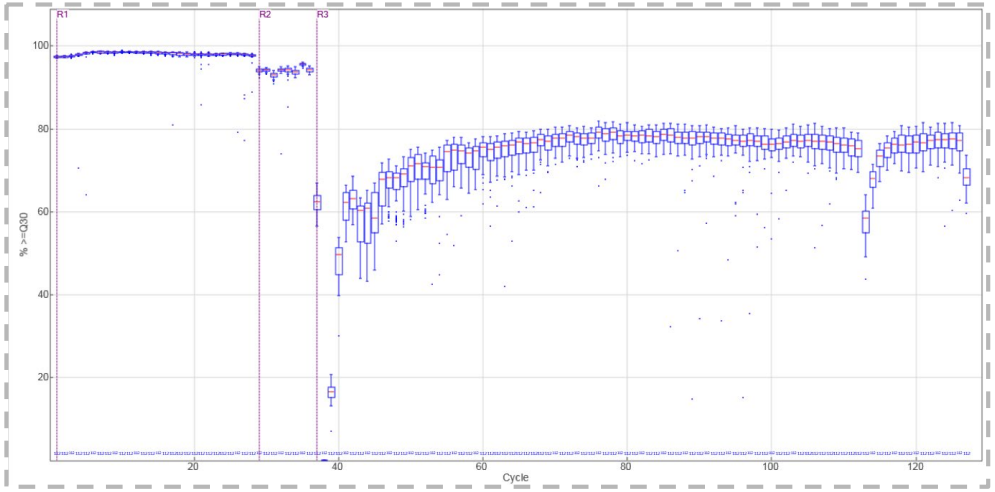
Note: * Pool of 4 Single Cell 3’ Cell Surface Protein Libraries run on one lane of a HiSeq 4000

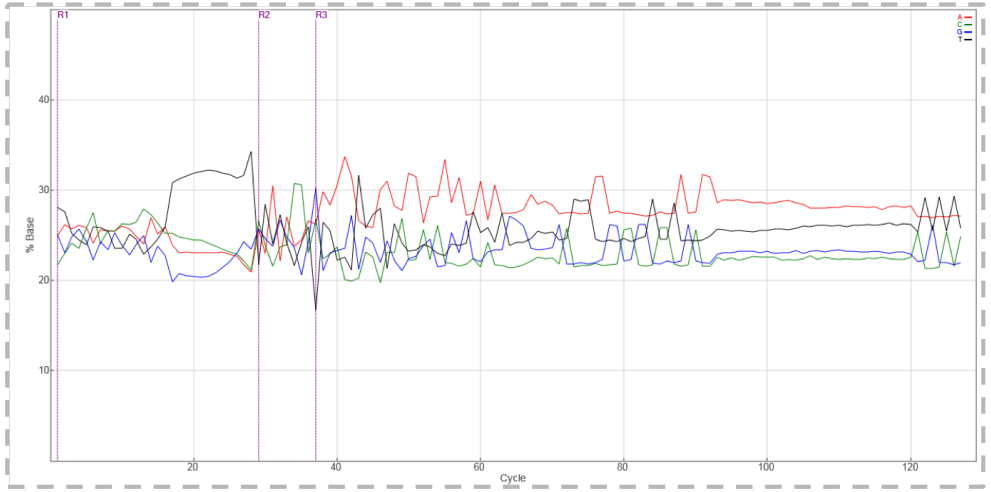
Base Balance Composition

Pooled Single Cell Gene Expression and Cell Surface Protein Libraries









Note: * Pool of 2 Single Cell 3’ Gene Expression and 2 Cell Surface Protein Libraries run on one lane of a HiSeq 4000

Sequencing Recommendations: Pooling Guidelines

Single Cell 3’ Cell Surface Protein Libraries

Single Cell 3’ Cell Surface Protein libraries may be pooled for sequencing with:

- Single Cell 3’ Gene Expression v3 libraries
- Single Cell 3’ Gene Expression v2 libraries (if run as a 28 x 91 read length configuration)
- Single Cell 5’ Gene Expression libraries (if run as a 28 x 91 read length configuration)
- Single Cell 5’ Cell Surface Protein libraries (if run as a 28 x 91 read length configuration)

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

- Single Cell ATAC libraries
- Single Cell CNV libraries

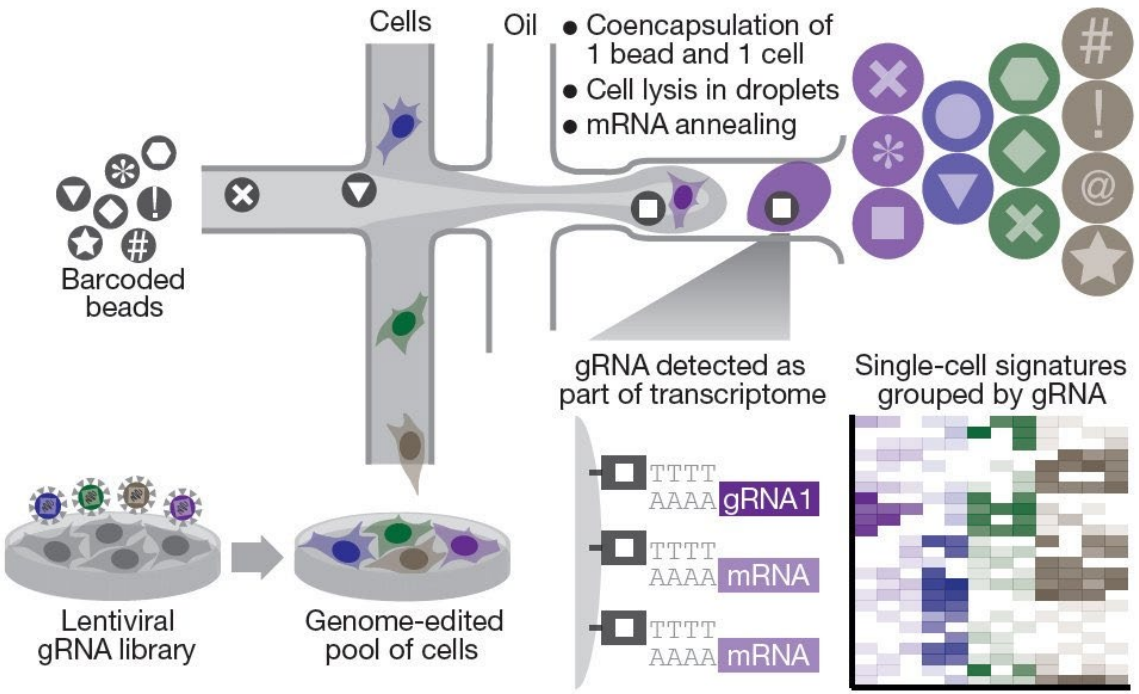
10X GENOMICS

Chromium Single Cell Gene Expression Solution with Feature Barcoding technology

CRISPR Screening

Single Cell Gene Expression and CRISPR Screening

More Information from a Single Assay



- Simultaneously assess perturbation phenotypes and gene expression from the same cell
- Enable high throughput and high resolution functional genetic screens in hundreds to tens of thousands of cells simultaneously
- Detect heterogeneity that would otherwise go undetected in analyses of pooled cells
- Determine comprehensive gene expression phenotypes for individual perturbations
- Directly capture and sequence gRNAs, eliminating the need for proxy barcodes

Single Cell Gene Expression and CRISPR Screening

Customer Demonstrations

Perturb-Seq

Resource

Cell

Perturb-Seq: Dissecting Molecular Circuits with Scalable Single-Cell RNA Profiling of Pooled Genetic Screens

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http://dx.doi.org/10.1016/j.cell.2016.11.038

SUMMARY

Genetic screens help infer gene function in mammalian cells, but it has remained difficult to assay complex phenotypes—such as transcriptional profiles—at scale. Here, we develop Perturb-seq, combining single-cell RNA sequencing (RNA-seq) and clustered regularly interspaced short palindromic repeats (CRISPR)-based perturbations to perform many such assays in a pool. We demonstrate Perturb-seq by analyzing 200,000 cells in immune cells and cell lines, focusing on transcription factors regulating the response of dendritic cells to lipopolysaccharide (LPS). Perturb-seq accurately identifies individual gene targets, gene signatures, and cell states affected by individual perturbations and their genetic interactions. We posit new functions for regulators of differentiation, the anti-viral response, and mitochondrial function during immune activation. By decomposing many high content measurements into the effects of perturbations, their interactions, and diverse cell metadata, Perturb-seq dramatically increases the scope of pooled genomic assays.

INTRODUCTION

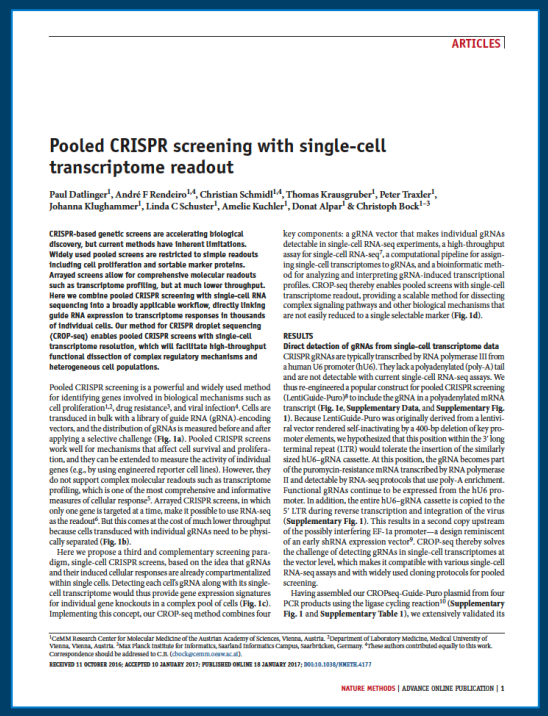
Genetic screens systematically analyze gene function in mammalian cells. Such screens are designed in either: (1) an individual ("arrayed") format, where each perturbation is delivered and assessed separately; or (2) a pooled format, performed en masse. Pooled readouts measure cell autonomous phenotypes, such as growth, drug resistance, or marker expression. Pooled screens are more efficient and scalable, but have been limited to low-content readouts. Distinguishing between different molecular mechanisms that yield similar phenotypes requires time and labor intensive follow-up.

Bridging the gap between rich profiles and pooled screens has been challenging. In mammalian cells, a few studies transcriptionally profiled hundreds of individual perturbations (Berger et al., 2016; Parnas et al., 2015). In yeast (Hughes et al., 2000), up to ~1,500 knock out (KO) strains have been assessed (Kemmeren et al., 2014). Even signature screens were only performed in centralized efforts (Lamb et al., 2006).

Profiling may particularly help interpret the combined nonlinear effects of multiple factors. Comprehensive analysis of genetic interactions in growth phenotype between pairs of genes has been performed in yeast (Costanzo et al., 2016). In mammals, only small sets of pre-selected pairs have been assessed for cell viability (Bassik et al., 2013) or morphology (Laufer et al., 2013). One yeast study determined the combined effects of regulators on expression profiles in a circuit of three to five genes (Capaldi et al., 2008). Very few studies have examined higher order interactions (Elena and Lenski, 1997; Haber et al., 2013) and none have coupled those with a high content scalable readout.

To address this challenge, we develop Perturb-seq, combining the modularity of clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 to perform multi-locus gene perturbation (Cong et al., 2013; Qi et al., 2013) with the scale of massively parallel single cell RNA sequencing

CROP-Seq



Pooled CRISPR screening with single-cell transcriptome readout

Paul Datlinger[1], André F Rendeiro[1,4], Christian Schmidl[1,4], Thomas Krausgruber[1], Peter Traxler[1], Johanna Klughammer[1], Linda C Schuster[1], Amelie Kuchler[1], Donat Alpar[1] & Christoph Bock[1–3]

CRISPR-based genetic screens are accelerating biological discovery, but current methods have inherent limitations. Widely used pooled screens are restricted to simple readouts including cell proliferation and sortable marker proteins. Arrayed screens allow for comprehensive molecular readouts such as transcriptome profiling, but at much lower throughput. Here we combine pooled CRISPR screening with single-cell RNA sequencing into a broadly applicable workflow, directly linking guide RNA expression to transcriptome responses in thousands of individual cells. Our method for CRISPR droplet sequencing (CROP-seq) enables pooled CRISPR screens with single-cell transcriptome resolution, which will facilitate high-throughput functional dissection of complex regulatory mechanisms and heterogeneous cell populations.

Pooled CRISPR screening is a powerful and widely used method for identifying genes involved in biological mechanisms such as cell proliferation[1,2], drug resistance[3], and viral infection[4]. Cells are transduced in bulk with a library of guide RNA (gRNA)-encoding vectors, and the distribution of gRNAs is measured before and after applying a selective challenge (**Fig. 1a**). Pooled CRISPR screens work well for mechanisms that affect cell survival and proliferation, and they can be extended to measure the activity of individual genes (e.g., by using engineered reporter cell lines). However, they do not support complex molecular readouts such as transcriptome profiling, which is one of the most comprehensive and informative measures of cellular response[5]. Arrayed CRISPR screens, in which only one gene is targeted at a time, make it possible to use RNA-seq as the readout[6]. But this comes at the cost of much lower throughput because cells transduced with individual gRNAs need to be physically separated (**Fig. 1b**).

Here we propose a third and complementary screening paradigm, single-cell CRISPR screens, based on the idea that gRNAs and their induced cellular responses are already compartmentalized within single cells. Detecting each cell's gRNA along with its single-cell transcriptome would thus provide gene expression signatures for individual gene knockouts in a complex pool of cells (**Fig. 1c**). Implementing this concept, our CROP-seq method combines four key components: a gRNA vector that makes individual gRNAs detectable in single-cell RNA-seq experiments, a high-throughput assay for single-cell RNA-seq[7], a computational pipeline for assigning single-cell transcriptomes to gRNAs, and a bioinformatic method for analyzing and interpreting gRNA-induced transcriptional profiles. CROP-seq thereby enables pooled screens with single-cell transcriptome readout, providing a scalable method for dissecting complex signaling pathways and other biological mechanisms that are not easily reduced to a single selectable marker (**Fig. 1d**).

RESULTS

Direct detection of gRNAs from single-cell transcriptome data

CRISPR gRNAs are typically transcribed by RNA polymerase III from a human U6 promoter (hU6). They lack a polyadenylated (poly-A) tail and are not detectable with current single-cell RNA-seq assays. We thus re-engineered a popular construct for pooled CRISPR screening (LentiGuide-Puro[8]) to include the gRNA in a polyadenylated mRNA transcript (**Fig. 1e**, **Supplementary Data**, and **Supplementary Fig. 1**). Because LentiGuide-Puro was originally derived from a lentiviral vector rendered self-inactivating by a 400-bp deletion of key promoter elements, we hypothesized that this position within the 3′ long terminal repeat (LTR) would tolerate the insertion of the similarly sized hU6–gRNA cassette. At this position, the gRNA becomes part of the puromycin-resistance mRNA transcribed by RNA polymerase II and detectable by RNA-seq protocols that use poly-A enrichment. Functional gRNAs continue to be expressed from the hU6 promoter. In addition, the entire hU6–gRNA cassette is copied to the 5′ LTR during reverse transcription and integration of the virus (**Supplementary Fig. 1**). This results in a second copy upstream of the possibly interfering EF-1α promoter—a design reminiscent of an early shRNA expression vector[9]. CROP-seq thereby solves the challenge of detecting gRNAs in single-cell transcriptomes at the vector level, which makes it compatible with various single-cell RNA-seq assays and with widely used cloning protocols for pooled screening.

Having assembled our CROPseq-Guide-Puro plasmid from four PCR products using the ligase cycling reaction[10] (**Supplementary Fig. 1** and **Supplementary Table 1**), we extensively validated its

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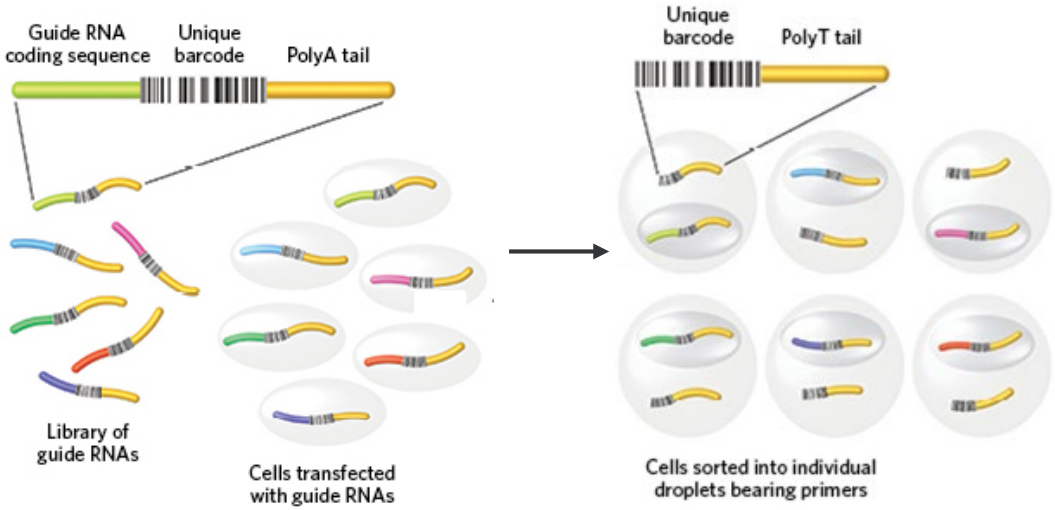
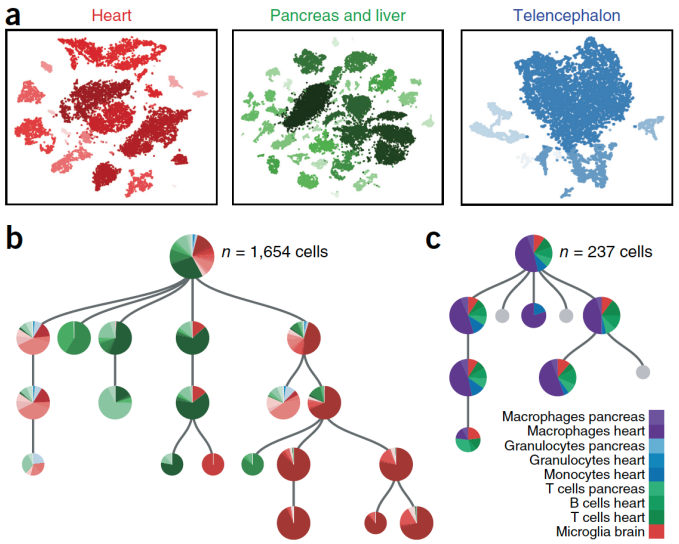
RECEIVED 11 OCTOBER 2016; ACCEPTED 10 JANUARY 2017; PUBLISHED ONLINE 16 JANUARY 2017; DOI:10.1038/NMETH.4177

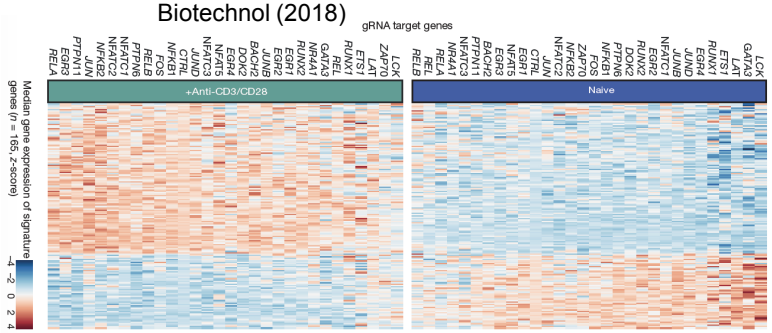


Single Cell Gene Expression and CRISPR Screening

Applications and Research Areas



Spanjaard B *et al.*, Nat Biotechnol (2018)



Datlinger P *et al.*, Nat Methods (2017)

Drug Screening

- Comprehensive characterization of large CRISPR libraries
- Study p53, kinase, apoptosis, Jak-Stat and other pathways
- Study impact of perturbation of many members of the same pathway

Stem Cell & Dev Biology

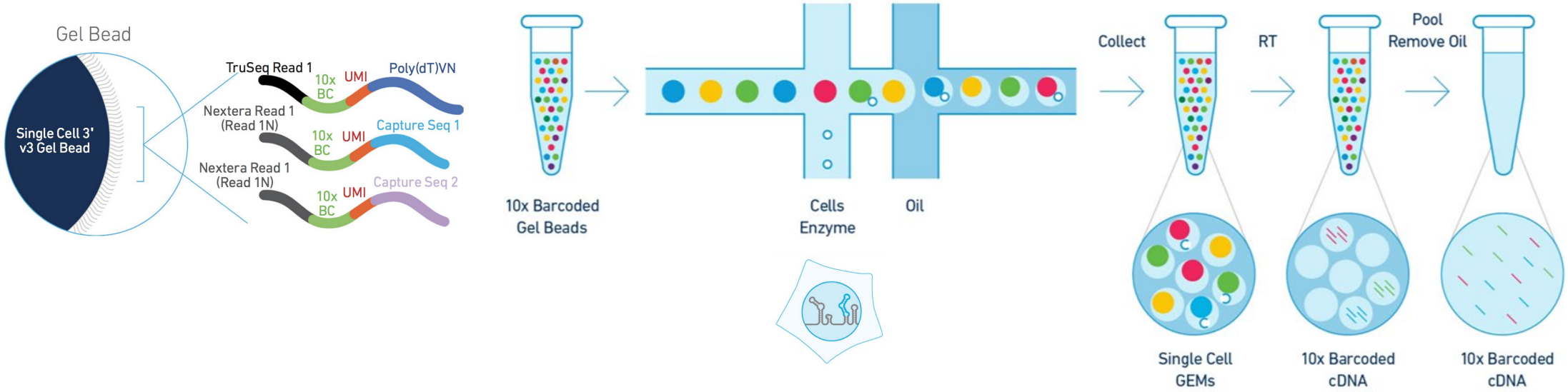
- Cell lineage tracing during development and disease
- Establish cellular differentiation hierarchies
- Adapt to many model organisms including mammalian, *Zebrafish*, *C. elegans*, *D. melanogaster*
- Resolve complex gene regulatory networks

Immunology and Immune-oncology

- Screen T cell receptor activation
- Study cellular signaling events

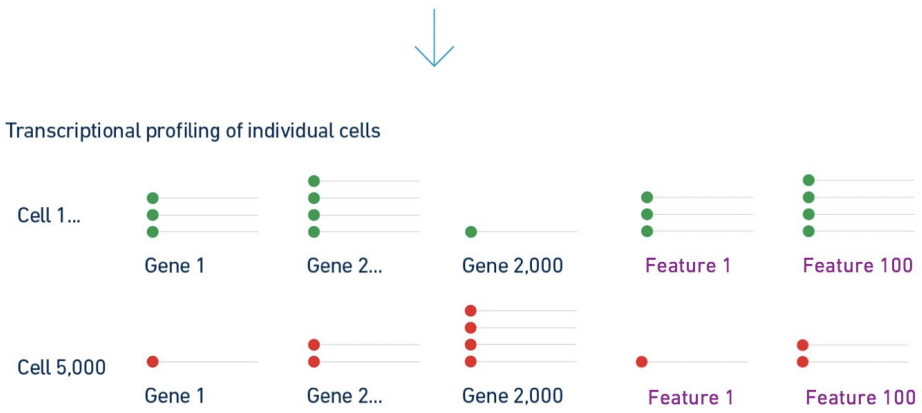
Single Cell Gene Expression and CRISPR Screening Solution

Biochemistry Overview



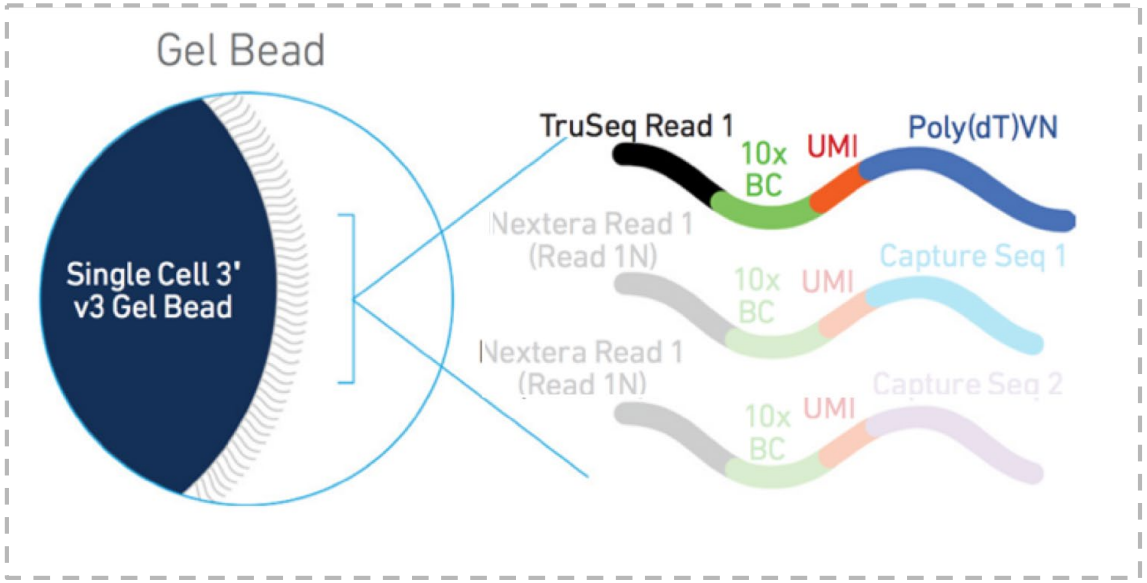
- Inputs:
  10x Gel Beads, Reagents and single cells in suspension

- Outputs:
  Digital gene expression and CRISPR perturbation profiles from every partitioned cell



Single Cell Gene Expression and CRISPR Screening Solution

Feature Barcoding technology Enabled via Single Cell 3' v3 Gel Beads



i. **TruSeq Read 1**

22 nt Partial Illumina TruSeq Read 1 sequence

ii. **10x BC**

16 nt 10x Barcode
~3.6 M defined barcode sequences

iii. **UMI**

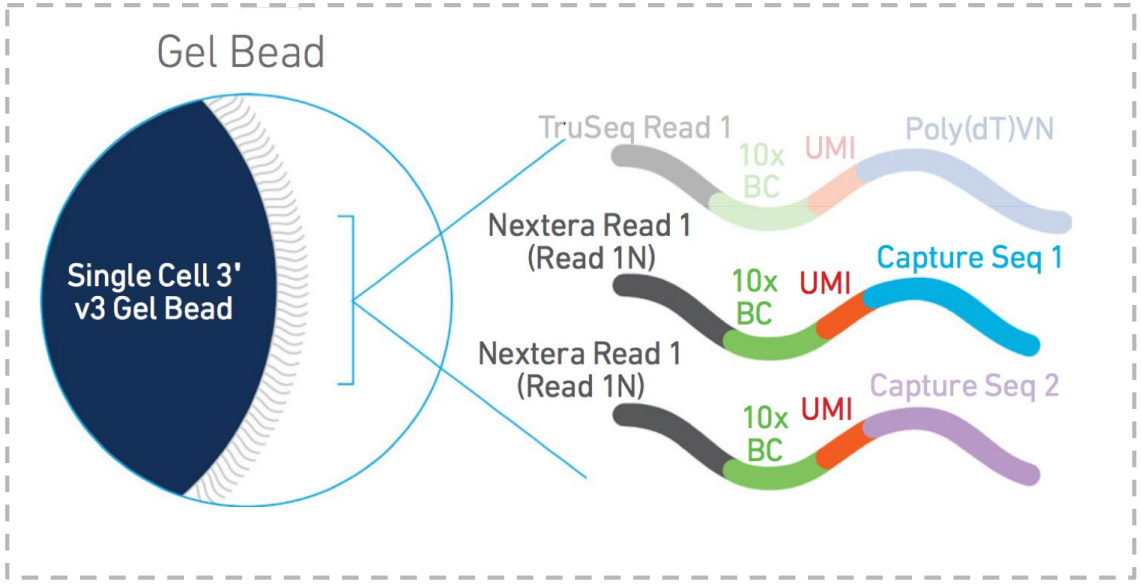
12 nt Unique Molecular Identifier

iv. **Poly(dT)VN**

30 nt Poly(dT) sequence
Enables capture of poly-adenylated mRNA molecules

Single Cell Gene Expression and CRISPR Screening Solution

Feature Barcoding technology Enabled via Single Cell 3' v3 Gel Beads



i. **Nextera Read 1 (Read 1N)**

22 nt Partial Illumina Nextera Read 1 sequence enables selective enrichment of the Feature Barcode construct

ii. **10x BC**

16 nt 10x Barcode
~3.6 M defined barcode sequences

iii. **UMI**

12 nt Unique Molecular Identifier

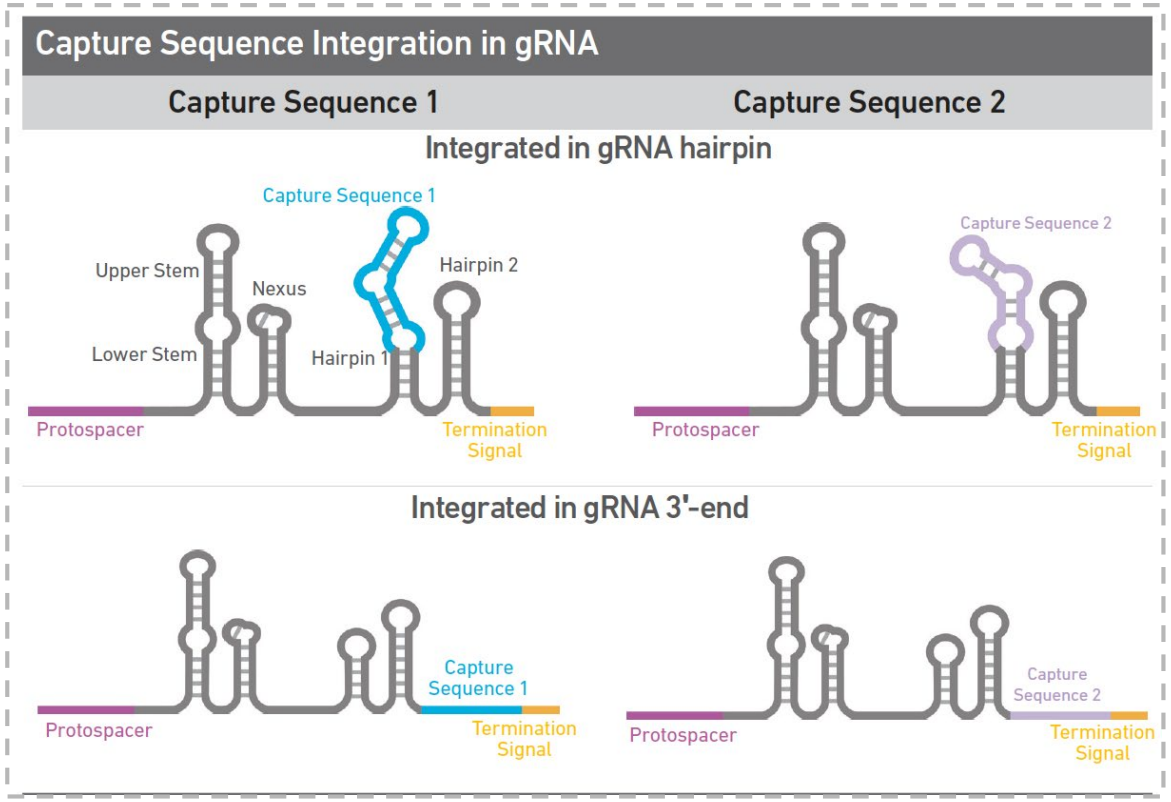
iv. **Capture Sequence 1** or **2**

22 nt sequence that is the reverse complement of the sequence inserted into the DNA (Antibody) or RNA (sgRNA) based Feature

Single Cell 3’ Gene Expression and Feature Barcoding Technology

Feature Barcoding technology for CRISPR Screening

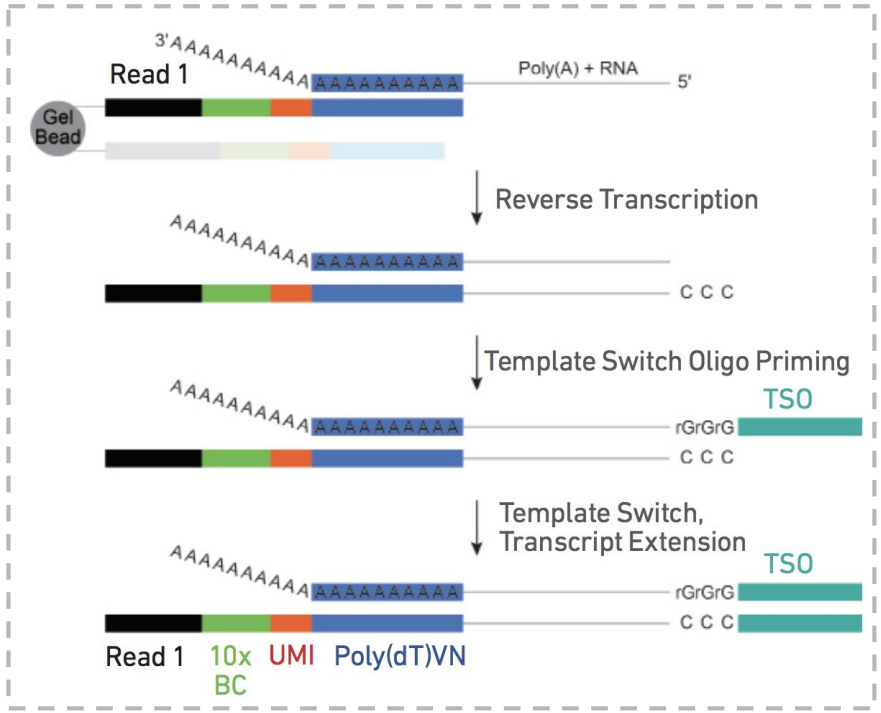
- sgRNA should incorporate one of the two Capture Sequences
  - 22 nt capture sequence inserted into the guide RNA backbone (reverse complement of the sequence on the Single Cell 3’ v3 Gel Beads)
  - Enables ‘direct capture’ of the guide which eliminates the need for proxy barcodes.
  - Integration site options:
    - 20 bp upstream of the 3’ termination signal
    - Immediately adjacent to the 3’ termination signal
- Performing sgRNA QC by qPCR, NGS or other methods is recommended prior to proceeding with the Single Cell Gene Expression and CRISPR Screening Solution



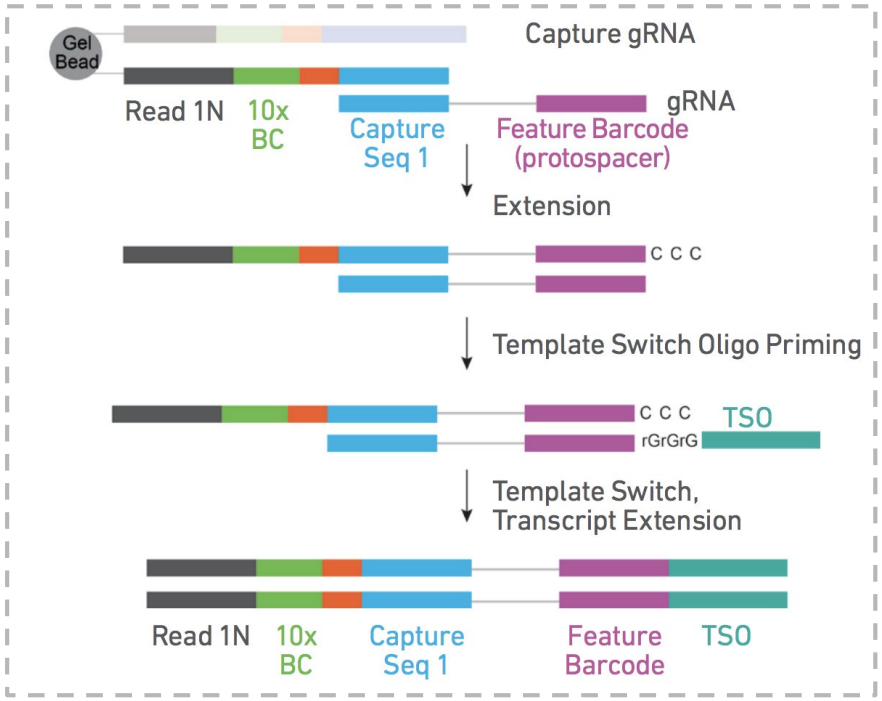
Feature Barcoding Workflow: In GEM Target Capture

Direct Capture of Multiple Targets Inside Individual GEMs

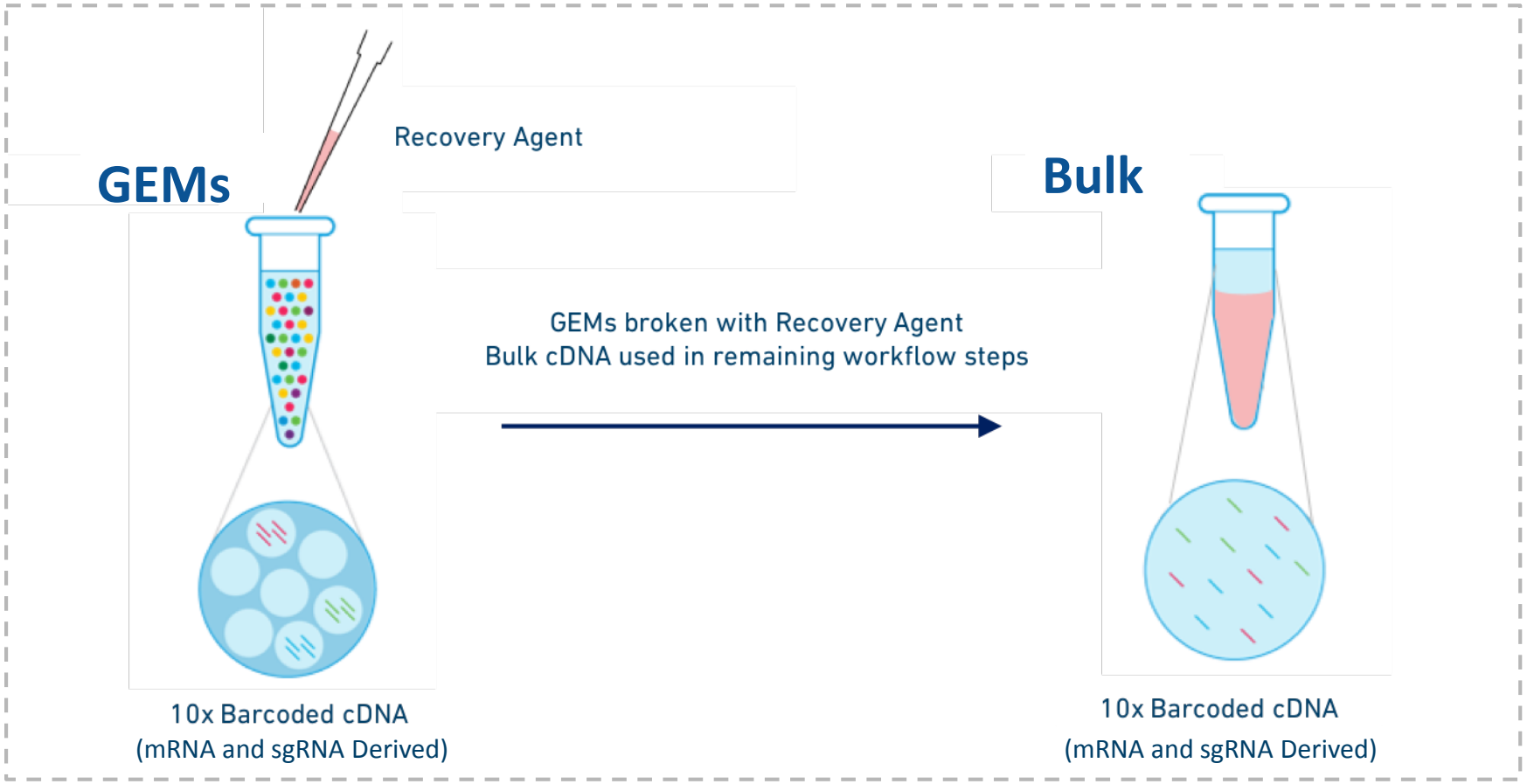
Barcoded, full-length cDNA from poly-adenylated mRNA



Barcoded cDNA from sgRNA



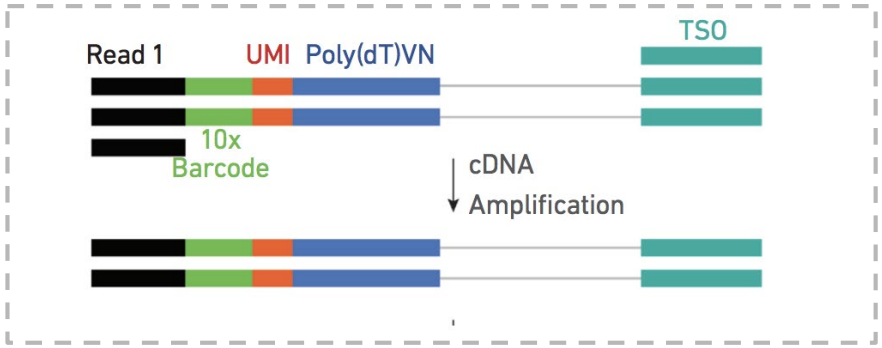
Feature Barcoding Workflow: Breaking GEMs



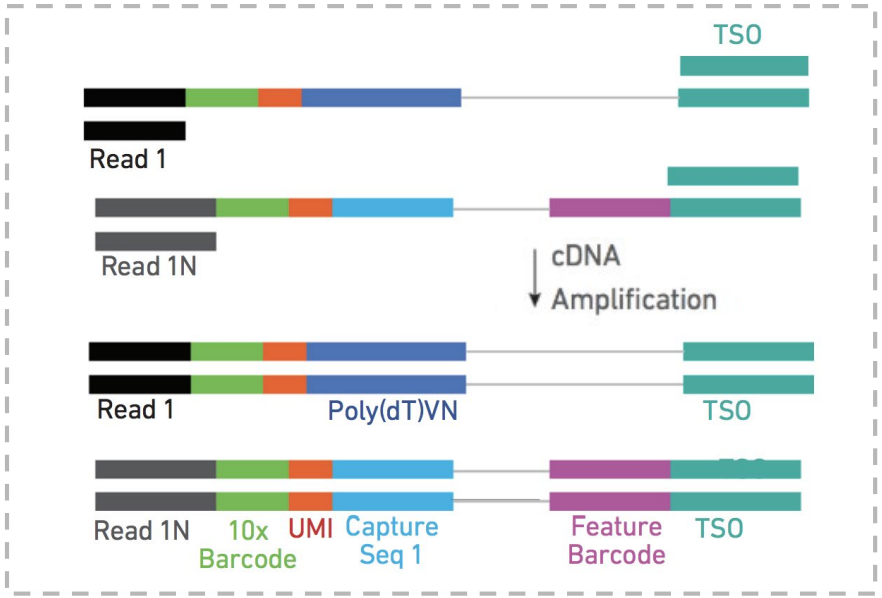
Feature Barcoding Workflow: cDNA Amplification

In Bulk Amplification of mRNA and sgRNA Feature Barcode Targets

cDNA Amplification of poly-adenylated mRNA **only** with cDNA Primers



cDNA Amplification of poly-adentylated mRNA and sgRNA with Feature cDNA Primers 1



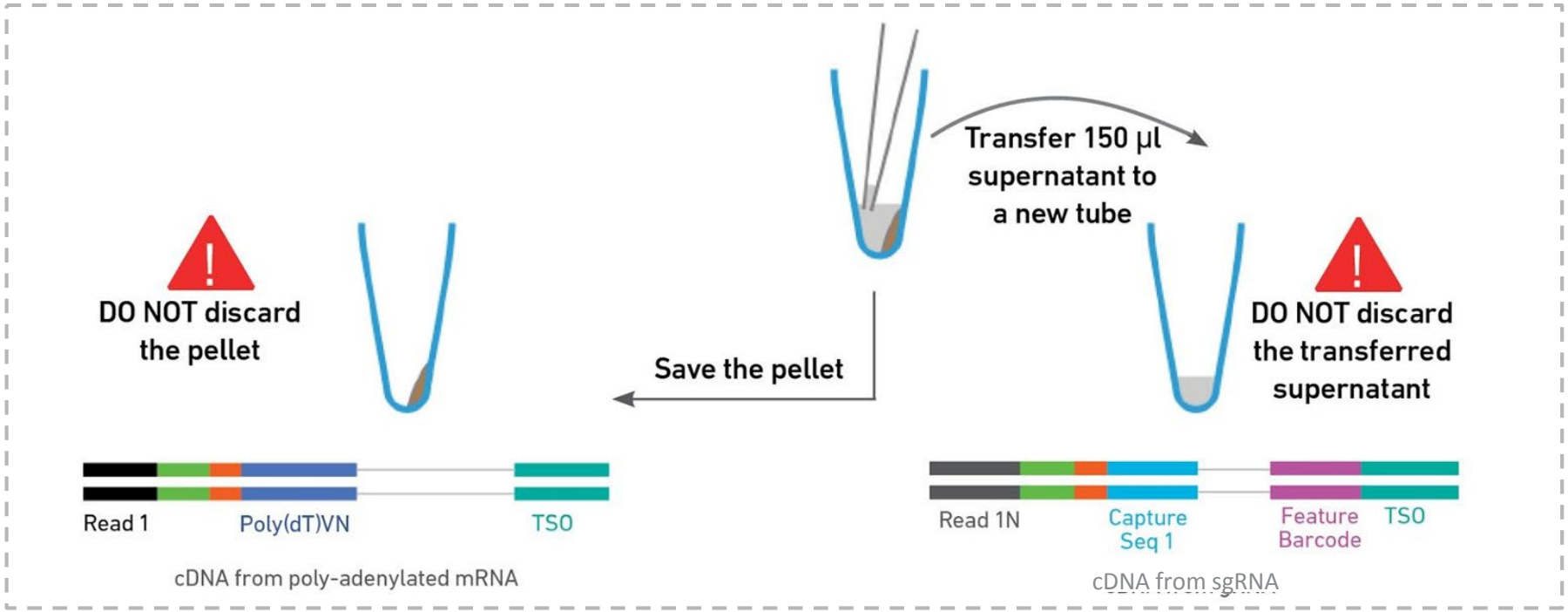
Note:

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

For cDNA Amplification of poly-adenylated mRNA and sgRNA please use: Feature cDNA Primers 1 (PN 2000096)

Feature Barcoding Workflow: cDNA Cleanup

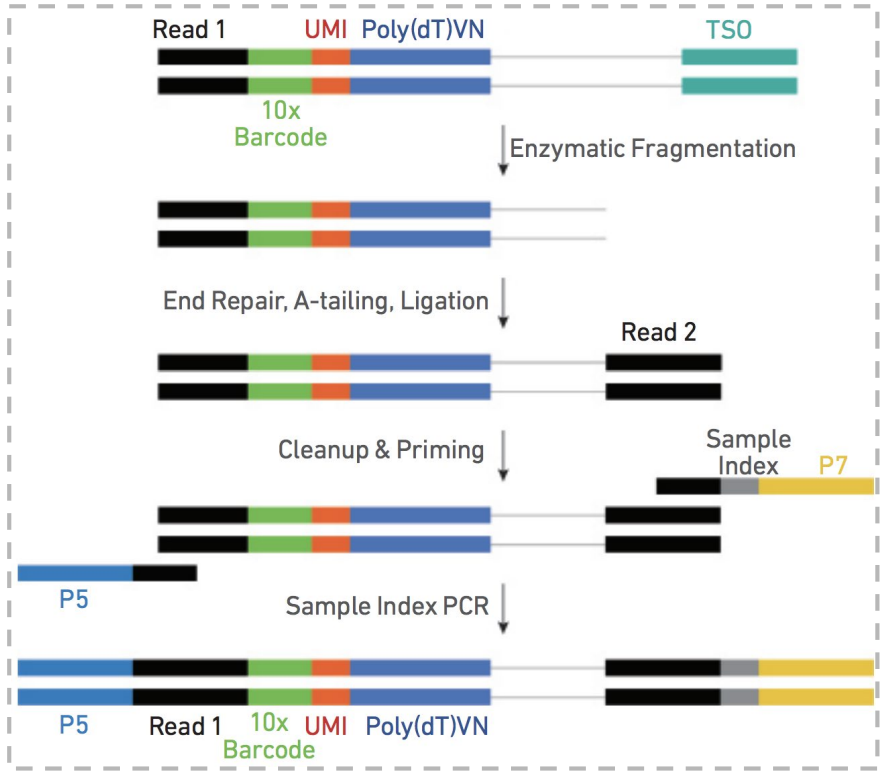
Sample Bifurcation Critical to Success



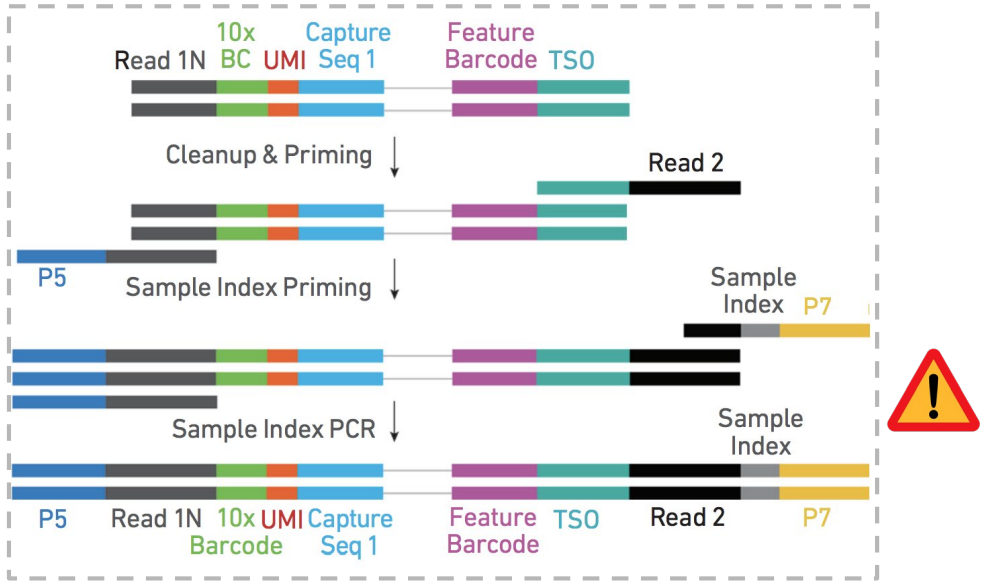
Feature Barcoding Workflow: Library Construction

Sample Bifurcation Enables Generation of Two Library Constructs

Single Cell Gene Expression Library Construction (from pellet)



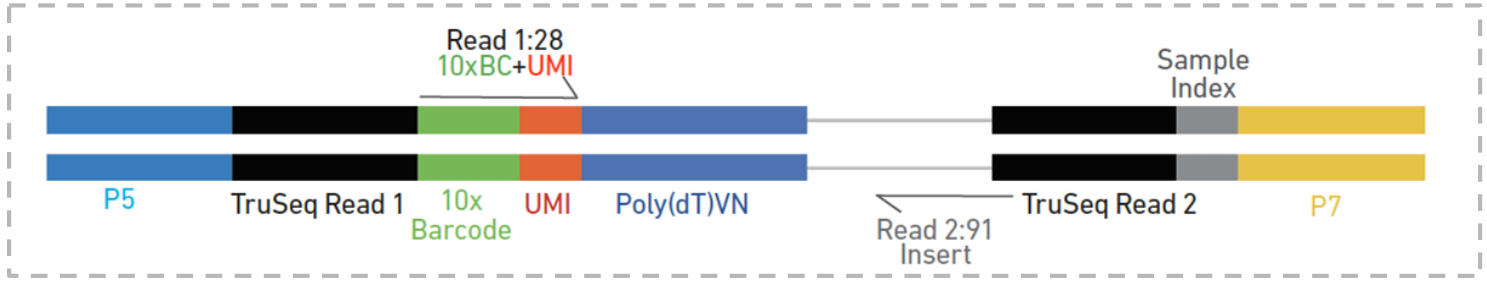
Single Cell 3’ CRISPR Screening Library Construction (from supernatant)



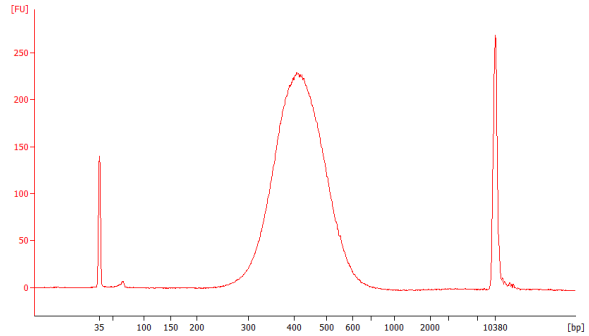
Feature Barcoding Workflow

Final Library Constructs

Chromium Single Cell 3’ Gene Expression Library

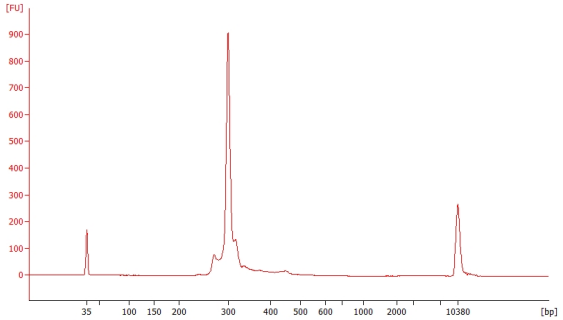


Representative Trace



Chromium Single Cell 3’ CRISPR Screening Library

Representative Trace



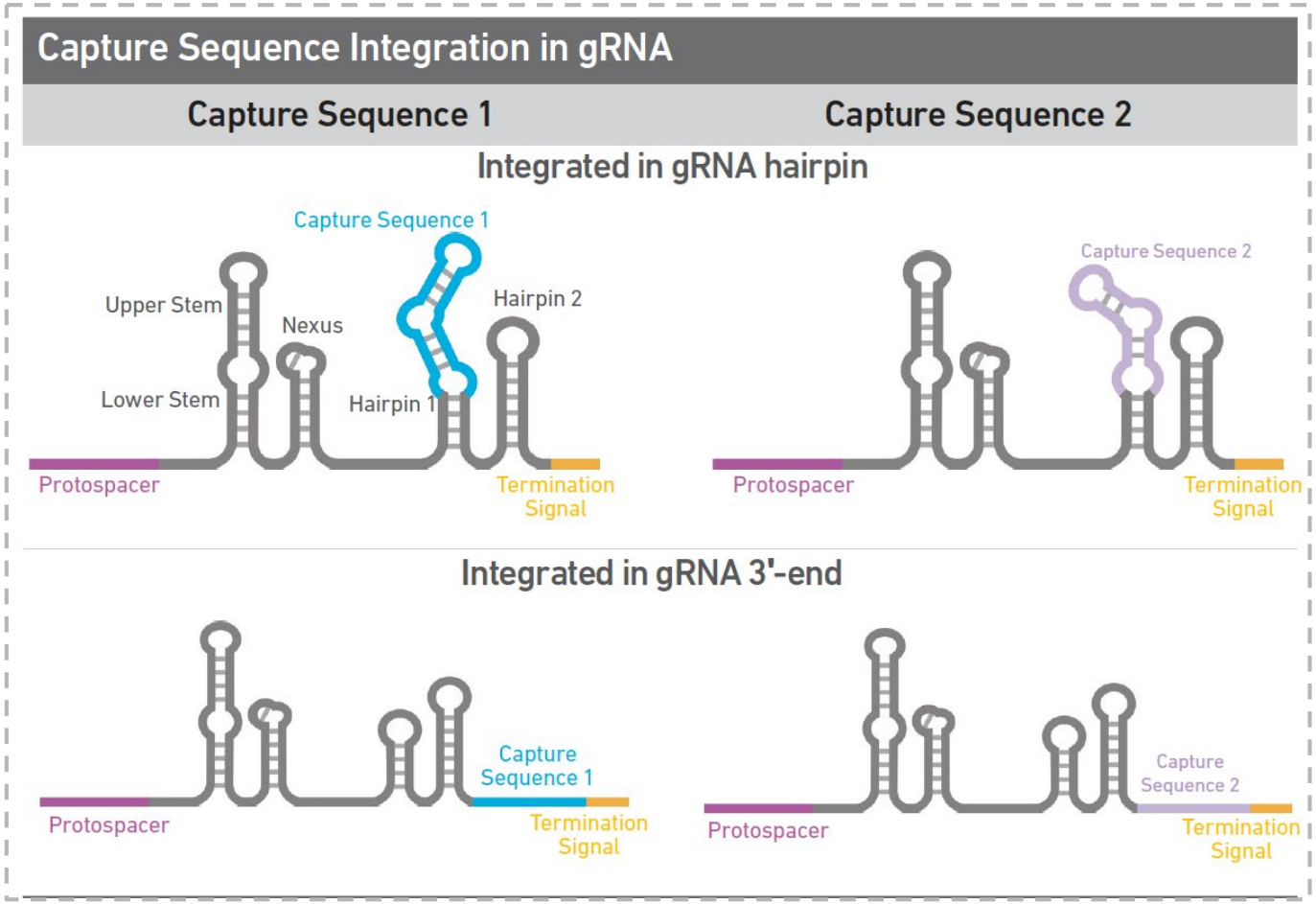
10X GENOMICS

Chromium Single Cell Gene Expression Solution with Feature Barcoding technology

Workflow Considerations

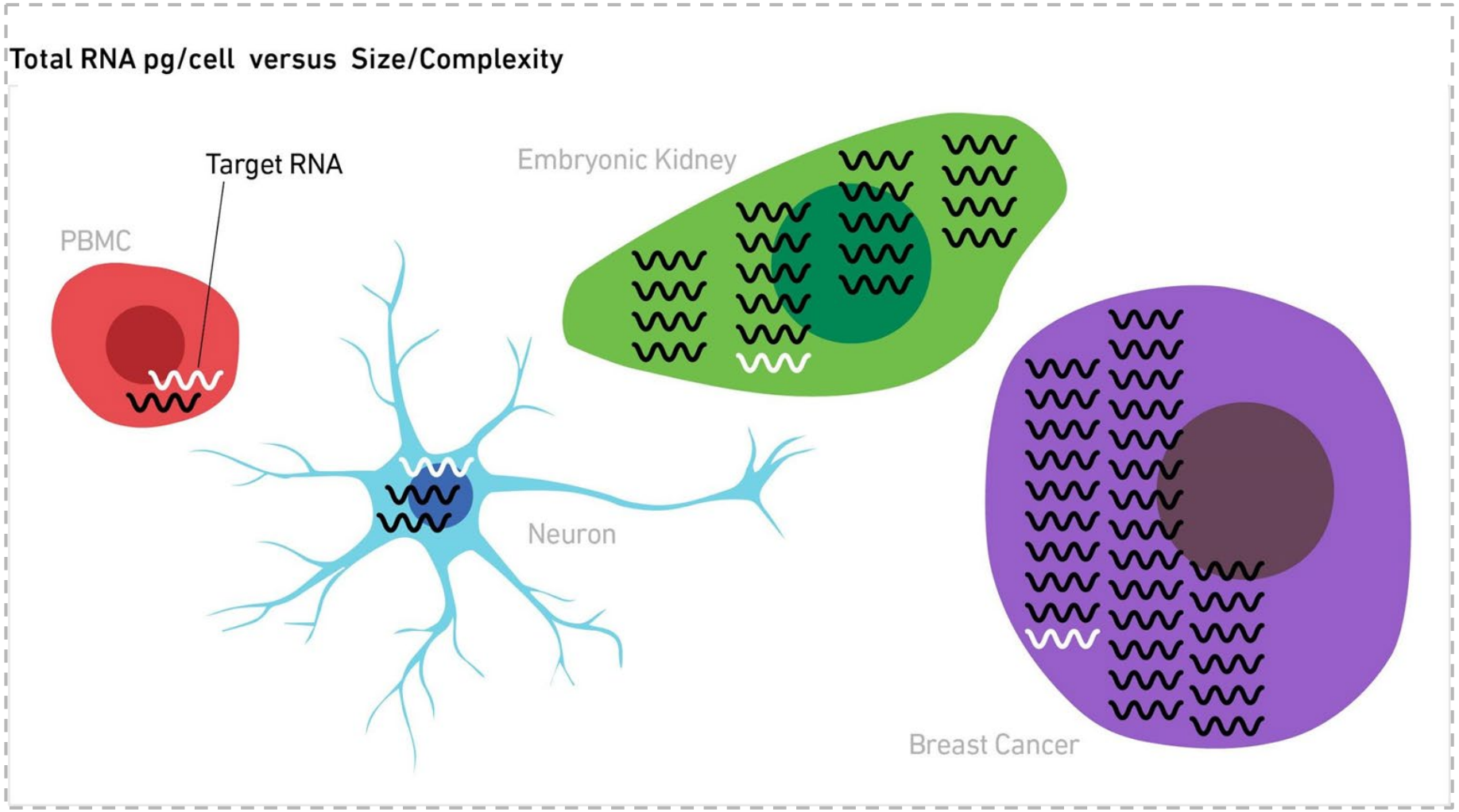
Workflow Considerations, Critical Steps and Performance Impacts

Integration of a Feature Barcode Compatible Capture Sequence is critical



Workflow Considerations, Critical Steps and Performance Impacts

Cell Type

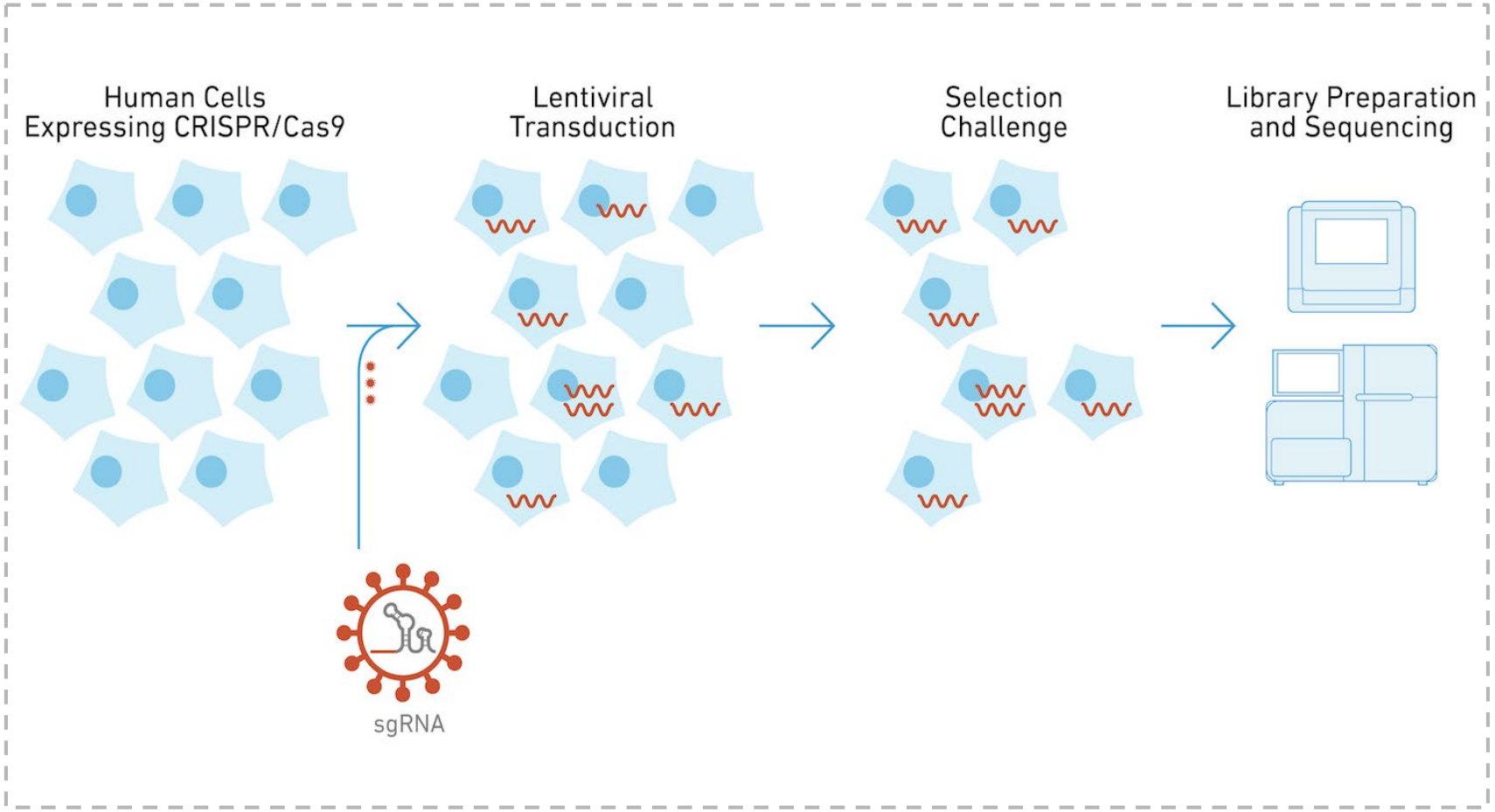


Workflow Considerations, Critical Steps and Performance Impacts

Multiplicity of Infection (MOI) and Selection

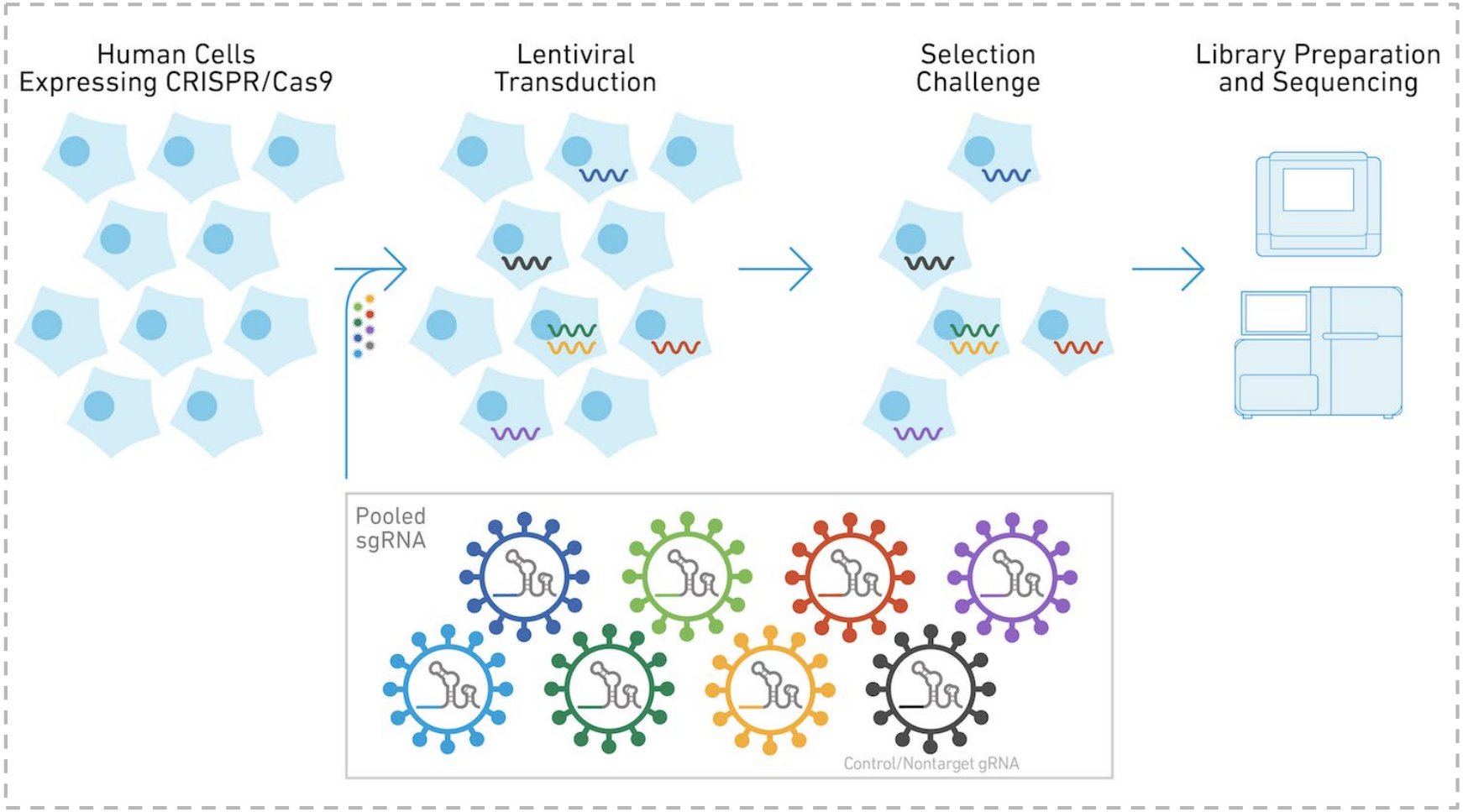
Workflow Considerations, Critical Steps and Performance Impacts

Single Guide



Workflow Considerations, Critical Steps and Performance Impacts

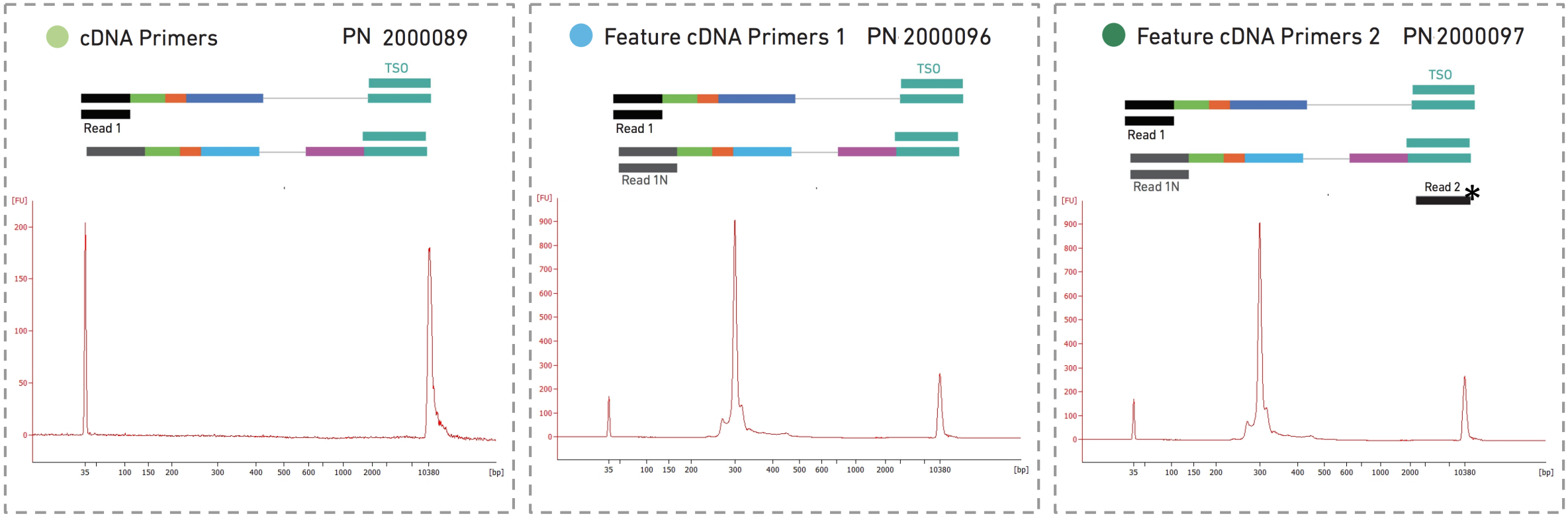
Pooled Library



Workflow Considerations, Critical Steps and Performance Impacts

Correct cDNA Primer Choice Enables Successful Feature Barcode Target Amplification

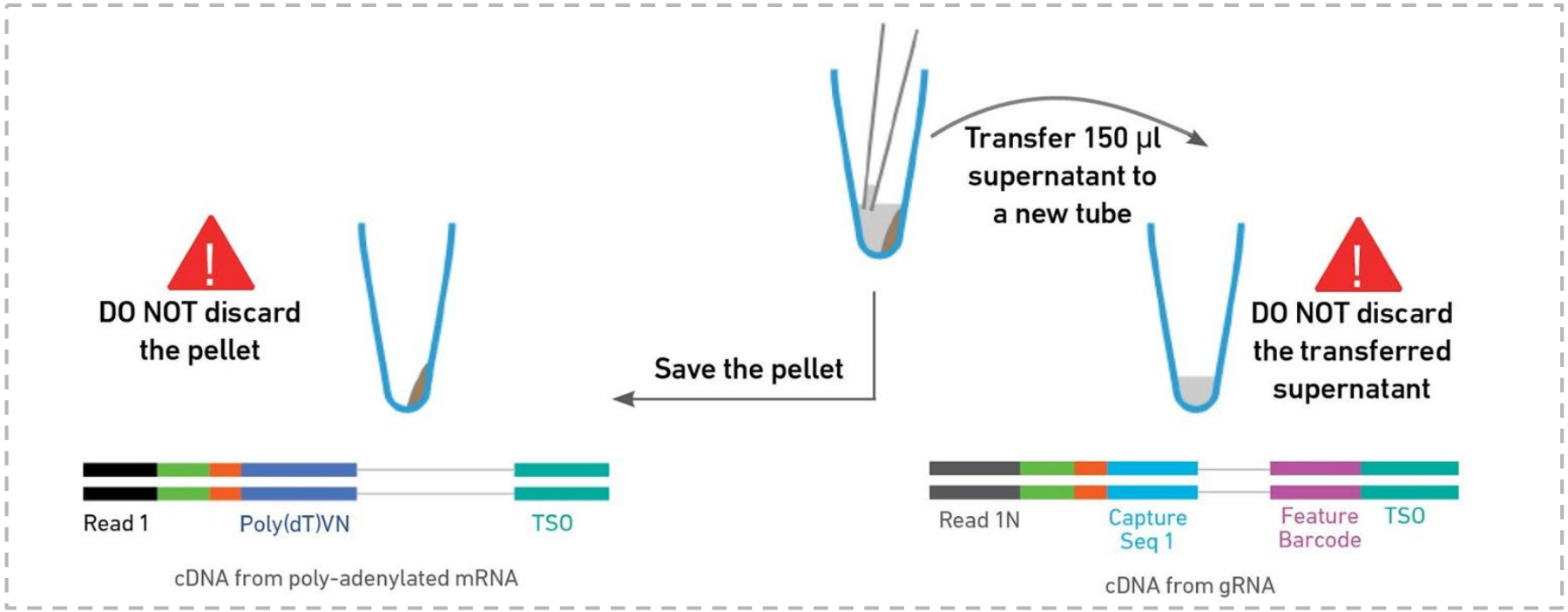
Feature cDNA Primers 1 (and 2) enable successful amplification of the Feature Barcode target



Note: * Feature cDNA Primers 2 contains a TruSeq Read 2 primer which is not required during sgRNA cDNA Amplification.

Workflow Considerations, Critical Steps and Performance Impacts

Save the Supernatant - Sample Bifurcation Enables Feature Barcode Library construction



10X GENOMICS

Chromium Single Cell Gene Expression Solution with Feature Barcoding technology

Technical Specifications and Selected Data

Supported Sequencers

Single Cell 3’ CRISPR Screening Library

	Single Cell 3’ Gene Expression	Single Cell 3’ CRISPR Screening
Raw Read Pairs per Cell	Minimum 20,000	5,000

MiSeq

NextSeq

HiSeq2500

HiSeq 3000/4000

NovaSeq

Recommended Read Length

Single Cell 3’ CRISPR Screening Library

	Read 1	i7 Index	i5 Index	Read 2
Purpose	10x Barcode & UMI	Sample Index	N/A	Insert
Length	28	8	0	91*

Note: * Single Cell 3’ CRISPR Screening Libraries are typically pooled with Single Cell Gene Expression Libraries and sequenced using these parameters, however it should be noted that the minimum required Read 2 length is 70 bp.

Recommended Loading Concentrations

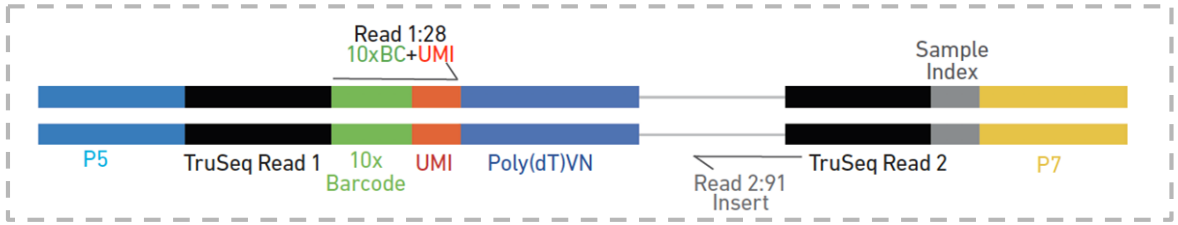
Single Cell 3’ CRISPR Screening Library

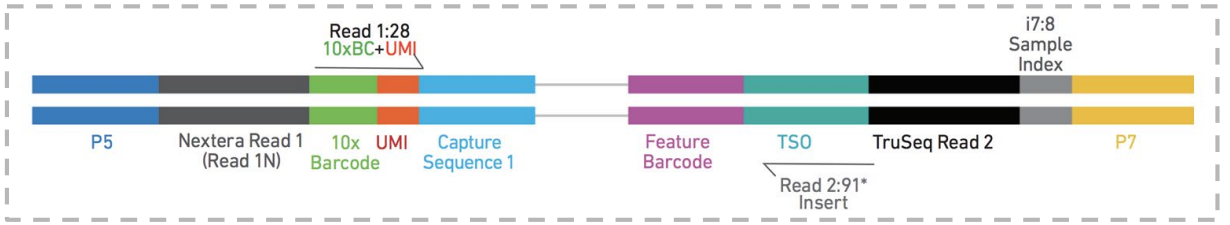
Platform	Loading Concentration (pM)	PhiX (%)
MiSeq™	11	1
NextSeq™ 500/550	1.8	20***
HiSeq™ 2500 (Rapid Run)	11	1
HiSeq™ 3000/4000	180*	1
NovaSeq™	300**	1

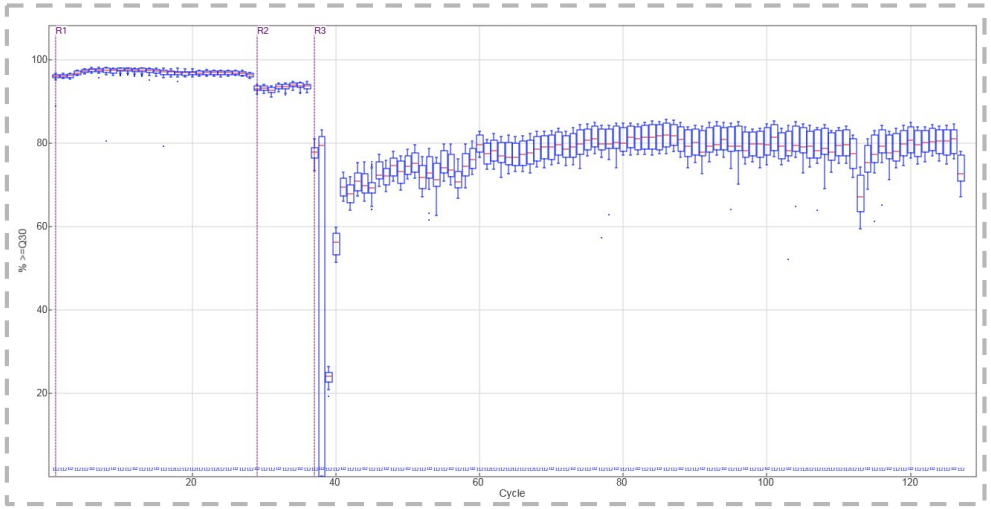
Note: * If Single Cell 3’ CRISPR Screening Libraries are pooled with Single Cell Gene Expression Libraries, adjust the loading concentration to 240 pM.
** If using XP Workflow, adjust the loading concentration to 150 pM.
*** if Single Cell 3’ CRISPR Screening Libraries are run by themselves on the NextSeq 500/550, to prevent a low Q30 score over the first 30 bases of Read 2, 20% PhiX can be added (the preference would be however to pool with Single Cell 3’ Gene Expression libraries)

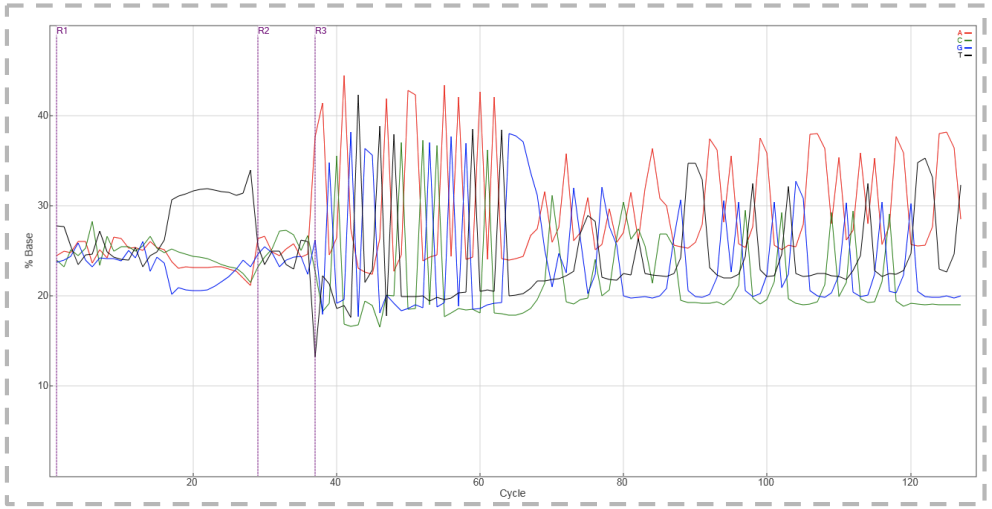
Base Balance Composition

Pooled Single Cell 3' Gene Expression and CRISPR Screening Libraries





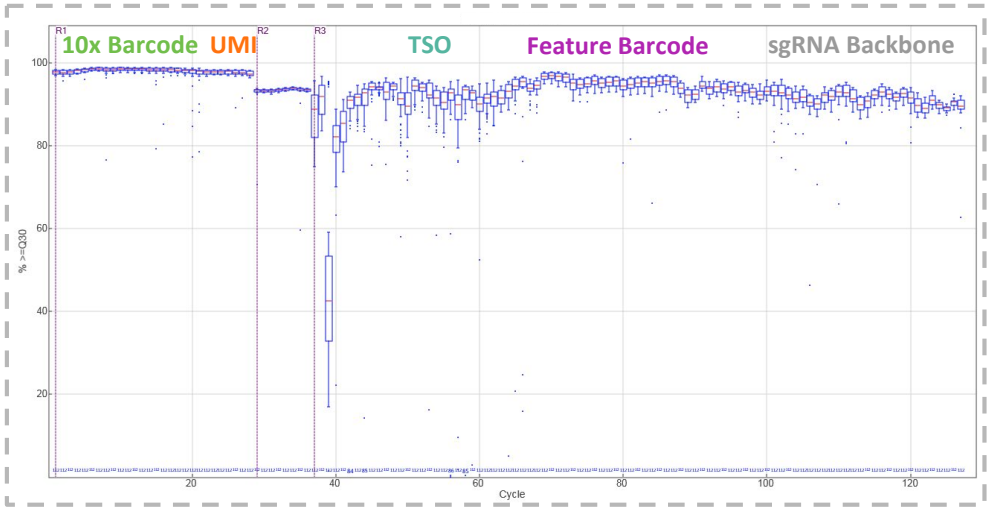


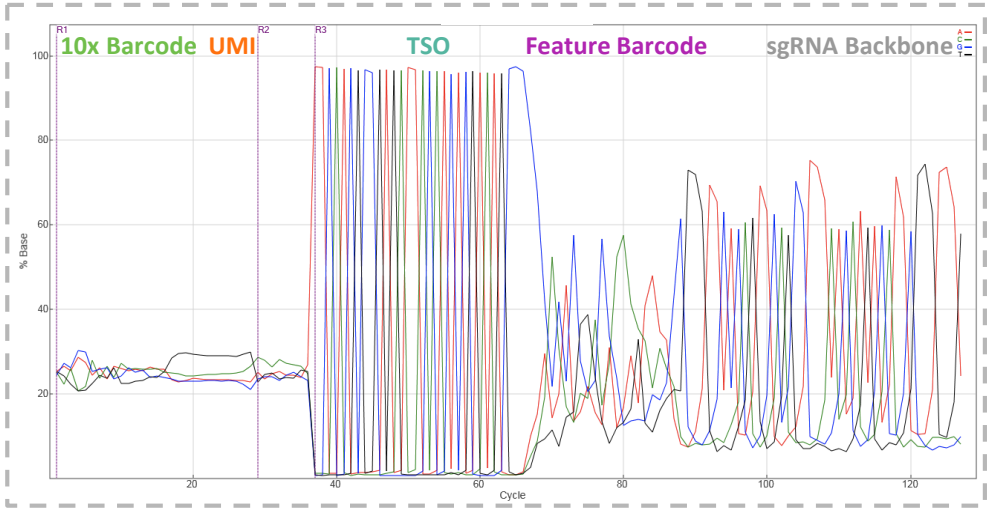


Note: * Pool of 2 Single Cell 3' Gene Expression and 2 CRISPR Screening Libraries run on one lane of a HiSeq 4000

Base Balance Composition

Single Cell 3’ CRISPR Screening Library





Note: * Pool of 4 Single Cell 3’ CRISPR Screening Libraries run on one lane of a HiSeq 4000

Sequencing Recommendations: Pooling Guidelines

Single Cell 3’ CRISPR Screening Libraries

Single Cell 3’ Cell CRISPR Screening libraries are may be pooled for sequencing with:

- Single Cell 3’ Gene Expression v3 libraries
- Single Cell 3’ Gene Expression v2 libraries (if run as a 28 x 91 read length configuration)
- Single Cell 5’ Gene Expression libraries (if run as a 28 x 91 read length configuration)
- Single Cell 5’ Cell Surface Protein libraries (if run as a 28 x 91 read length configuration)

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

- Single Cell ATAC libraries
- Single Cell CNV libraries

10x 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