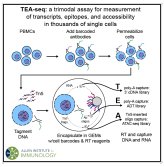

Version 2

Dec 01, 2020

TEA-seq V.2

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1 *Works for me* This protocol is published without a DOI.

Human Cell Atlas Method Development Community

Lucas Graybuck

ABSTRACT

TEA-seq is a method for Transcriptomic, Epitope, and Accessibility measurement from thousands of single cells on the 10x Genomics Multiome platform. It generates scRNA-seq, scATAC-seq, and cell surface epitope (BioLegend TotalSeq) sequencing libraries linked by 10x cell barcodes for coordinated analysis. TEA-seq has been optimized for use with peripheral blood mononuclear cells (PBMCs).

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Elliott Swanson, Cara Lord, Julian Reading, Alexander T. Heubeck, Adam K. Savage, Richard Green, Xiao-jun Li, Troy R. Torgerson, Thomas F. Bumol, Lucas T. Graybuck, and Peter J. Skene. *TEA-seq: a trimodal assay for integrated single cell measurement of transcripts, epitopes, and chromatin accessibility*. bioRxiv (2020). doi: https://doi.org/10.1101/2020.09.04.283887

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PROTOCOL CITATION

Elliott Swanson, Lucas Graybuck, Pete Skene 2020. TEA-seq. **protocols.io** https://protocols.io/view/tea-seq-bqagmsbw Version created by Lucas Graybuck

MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

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GUIDELINES

Permeabilization testing:

Cell permeabilization in this protocol utilizes Digitonin, a naturally-derived saponin molecule. Because it's a natural product, the precise concentration and permeabilization strength may vary somewhat from source to source and batch to batch. We recommend testing your digitonin permeabilization using your cell type of interest and a live/dead cell stain (e.g. AO/PI) to find the lowest concentration that provides reliable, reproducible permeabilization (100% of cells "dead" by AO/PI stain)

Cell Handling:

Following thaw, maintain cells **On ice** or at **4 °C** at all times.

Antibodies and titering:

Successful measurement of antibody-bound oligos depends on antibody titer and on retention of antibody binding after digitonin treatment. Because digitonin alters membrane composition, some membrane-bound proteins may be lost after permeabilization.

Antibodies for which we have noticed reduced binding after digitonin treatment:
CD20 Clone 2H7

Titrations we have used in our study:

Target	Clone	TEA-seq ug Ab / M cells
CD10	HI10a	0.5
CD11b	ICRF44	0.05
CD11c	S-HCL-3	0.025
CD123	6H6	0.1
CD127 (IL-7Rα)	A019D5	0.025
CD14	M5E2	0.2
CD141 (Thrombomodulin)	M80	0.1
CD16	3G8	0.05
CD172a (SIRPa)	15-414	0.25
CD185 (CXCR5)	J252D4	0.125
CD19	HIB19	0.2
CD192 (CCR2)	K036C2	0.5
CD197 (CCR7)	G043H7	0.5
CD21	Bu32	0.05
CD24	ML5	0.5
CD25	BC96	0.08
CD269 (BCMA)	19F2	0.5
CD27	LG.3A10	0.125
CD278 (ICOS)	C398.4A	0.01
CD279 (PD-1)	EH12.2H7	0.1
CD3	UCHT1	0.05
CD304 (Neuropilin-1)	12C2	0.1
CD319 (CRACC)	162.1	0.5
CD38	HB-7	0.05
CD39	A1	0.05
CD4	RPA-T4	0.1
CD40	5C3	0.25
CD45RA^	HI100	0.0625
CD45RO	UCHL1	0.1
CD56 (NCAM)	5.1H11	0.15
CD66b	6/40c	0.25
CD71	CY1G4	0.025
CD80	2D10	0.5
CD86	IT2.2	0.05
CD8a	RPA-T8	0.2
CD95 (Fas)	DX2	0.1
FcεRIα	AER-37 (CRA-1)	0.5
HLA-DR	L243	0.25
IgD	IA6-2	0.05
Mouse IgG1, κ Isotype Control	MOPC-21	0.5
IgM	MHM-88	0.05
KLRG1 (MAFA)	SA231A2	0.25
TCR Vα24-Jα18 (iNKT cell)	6B11	0.5
TCR Vα7.2	3C10	0.05
TCR α/β	IP26	0.125
TCR γ/δ	B1	0.5

^ Low in our experience - may want to increase for future studies

Plate Sealing:

We recommend using an automated microplate heat-sealer for all steps following the GEM barcoding reaction. We find that we get more consistent results and higher yield by heat-sealing and vortexing or mixing by inversion, than by pipette mixing. If you do not have access to a heat-sealer Bio-Rad Microseal B adhesive seals can be used in combination with pipette mixing.

Reagent Handling:
Primers and Buffers may be thawed at room temperature. With the exception of Cleanup Buffer, store buffers and primers on ice once thawed. Reagents containing enzymes should be removed from **-20 °C** immediately before use and maintained on ice. Return enzymes to **-20 °C** storage immediately after use. Buffers and primers may be vortexed. Do not vortex enzyme containing buffers.

MATERIALS TEXT

10x Genomics equipment and reagents:

10x Chromium Controller (10x Genomics)

Chromium Next GEM Chip J Single Cell **10x Genomics Catalog #1000230**

Chromium Next GEM Single Cell Multiome ATAC/Gene Expression Reagent Bundle **10x Genomics Catalog #1000285**

Dual Index Kit TT Set A **10x Genomics Catalog #1000215**

Single Index Kit N Set A **10x Genomics Catalog #1000212**

Other equipment (as tested - others may work):

Beckman Coulter Avanti J-15RIVD centrifuge with JS4.750 swinging bucket, B99516
C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module (Bio-rad #1851197)
CFX96 Touch Real-Time PCR Detection System (Bio-rad #1855195)
BioAnalyzer (Agilent G2939A)
Vortex Mixer

Vendor-specific Reagents:

BioLegend TotalSeq-A antibodies for desired cellular epitopes

AIM V™ Medium, liquid **Thermo Fisher Scientific Catalog #12055091** Step 3

NxGen RNAse Inhibitor **Lucigen Catalog #30281-2**

FC blocking reagent (FcX) **BioLegend**

SPRIselect Reagent **Beckman Coulter Catalog #B23317**

Dynabeads™ MyOne™ Silane **Thermo Fisher Catalog #37002D**

Buffer EB **Qiagen Catalog #19086**

Kapa HiFi HotStart ReadyMix **Kapa Biosystems Catalog #KM2602**

KAPA Library Quantification Kit for Illumina® Platforms **Kapa Biosystems Catalog #KK4835** Step 78

Agilent High Sensitivity DNA Kit **Agilent Technologies Catalog #5067-4626**

Generic Reagents:
Dulbecco's phosphate-buffered saline (DPBS)
Bovine serum albumin (BSA)
50% Glycerol
Digitonin
DMSO, anhydrous
$MgCl_2$
NaCl
Tris-HCl pH 7.4, a.k.a. Tris(hydroxymethyl)aminomethane hydrochloride
Low TE Buffer (10 mM Tris-HCl, 0.1 mM EDT, pH 8.0)

Labware (as tested - others may work):

Falcon® Round-Bottom Tubes Disposable Polystyrene Corning® 5 mL **Corning Catalog #352054**

Eppendorf Safe-Lock Tubes 1.5 mL PCR clean colorless 500 tubes **Eppendorf Catalog #022363212**

Falcon® 5 mL Round Bottom Polystyrene Test Tube, with Cell Strainer Snap Cap **Corning Catalog #352235**

Eppendorf® twin.tec 96-Well PCR Plate Semi-Skirted **Eppendorf Catalog #951020303**

Eppendorf twin.tec® PCR 96-well plate, skirted **Eppendorf Catalog #951020401**

Microseal® 'B' Adhesive Seals **BioRad Sciences Catalog #MSB-1001**

Additional Oligos:

Name	Sequence (5'->3')	Length
SI-PCR-Oligo	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTC	49
ADT-Rev-AMP	CCTTGGCACCCGAGAATTCC	20
ADT-i7	CAAGCAGAAGACGGCATACGAGATXXXXXXXXGTGACTGGAGTTCCTTGGCACCCGAGAATTCC*A	65

Oligonucleotides required for ADT amplification. Note that for ADT-i7 Primers, You'll need to add 8nt index sequences in place of the stretch of 'X' nucleotides. * designates the location of a Phosphorothioate bond.

External equipment:
Illumina NovaSeq 6000

SAFETY WARNINGS

Digitonin is an acutely toxic health hazard.

Handling of digitonin-containing solutions, especially at high concentration, should be performed with personal protective equipment and in a biosafety cabinet.

Please refer to an MSDS for your Digitonin product (e.g. this version from Fisher) before beginning permeabilization experiments, and take all necessary precautions.

DISCLAIMER:

ABSTRACT

TEA-seq is a method for Transcriptomic, Epitope, and Accessibility measurement from thousands of single cells on the 10x Genomics Multiome platform. It generates scRNA-seq, scATAC-seq, and cell surface epitope (BioLegend TotalSeq) sequencing libraries linked by 10x cell barcodes for coordinated analysis. TEA-seq has been optimized for use with peripheral blood mononuclear cells (PBMCs).

BEFORE STARTING

1. Pre-chill a swinging-bucket rotor centrifuge to **4 °C** for use throughout antibody staining protocol, with holders for 5 mL polystyrene round-bottom tubes.

2. If performing bead cleanup steps (36 and onward), remove Dynabeads MyOne SILANE reagent from storage at least **00:30:00** before beginning the cleanup to allow them to equilibrate to room temperature.

Buffer preparation 30m

1 **Stain Buffer** Dulbecco's phosphate-buffered saline (DPBS) supplemented with 2% w/v bovine serum albumin.

Wash Buffer Final composition of 20 mM Tris HCl (Tris(hydroxymethyl)aminomethane hydrochloride) pH 7.4, 150 mM NaCl, 3 mM $MgCl_2$.

Perm Buffer Wash Buffer (above) with the addition of digitonin to a final concentration of 0.01% w/v. Stocks of 5% w/v digitonin in DMSO can be prepared ahead of time and aliquots can be stored at **-20 °C** for future use. We recommend limiting the number of freeze-thaw cycles to five.

Digitonin concentration was optimized for PBMCs. Permeabilization of different sample types may require further optimization. See Guidelines for suggestions.

Tagmentation Buffer Wash Buffer (above) with the addition of NxGen RNase Inhibitor at a final concentration of 1U / µl.

Sample preparation 1h

2 ⚠

Remove cryopreserved cells from liquid nitrogen storage and thaw in a **37 °C** water bath for 3-5 minutes until no ice is visible.

This cell thaw protocol has been optimized for cryopreserved PBMCs. Alternate cell types or preservation methods may require different sample preparation procedures.

3 Slowly dilute cells in 10 mL of pre-warmed **37 °C**
AIM V™ Medium, liquid **Thermo Fisher Scientific Catalog #12055091** adding the first 3 mL dropwise.

4 Centrifuge the cells at **400 x g, 4°C, 00:05:00** and remove the supernatant.

5 Resuspend the cell pellet in 1 mL of ice cold Dulbecco's phosphate-buffered saline supplemented with 0.2% w/v bovine serum albumin (DPBS + 0.2% BSA) by pipette mixing. Bring the volume to 10 mL with DPBS + 0.2% BSA.

6 Centrifuge the cells at **400 x g, 4°C, 00:05:00** and remove the supernatant.

7 Resuspend the cell pellet in 1 mL of ice cold Dulbecco's phosphate-buffered saline supplemented with 0.2% w/v bovine serum albumin (DPBS + 0.2% BSA) by pipette mixing. Dilute the cell suspension in DPBS + 0.2% BSA as desired, targetting a cell concetration of 1 - 5 million cells / mL.

8 Count cells before proceeding to Antibody Staining.

Antibody staining 1h 30m

9 Centrifuge cells (recommended input of 1.5 - 5 million) at **400 x g, 4°C, 00:05:00** in a 5 mL polystyrene round bottom tube and remove the supernatant.

10 Resuspend the cell pellet in ice cold Stain Buffer (DPBS + 2% BSA) by gently pipette mixing.

11 Add 10 ul of TruStain FcX and pipette mix thoroughly. Incubate cells for **00:10:00 on ice** . 10m

12 Add TotalSeq-A antibodies and pipette mix thoroughly. Incubate cells for **00:30:00 on ice** . 30m

13 Dilute cells with 4 mL of ice cold Stain Buffer and gently mix.

14 Centrifuge cells at **400 x g, 4°C, 00:05:00** and remove the supernatant.

15 Resuspend the cell pellet in 4 mL of ice cold Stain Buffer by gently pipette mixing.

16 Centrifuge cells at **400 x g, 4°C, 00:05:00** and remove the supernatant. 5m

17 Repeat steps 15-16 for a total of three washes. 10m

18 Resuspend the cell pellet in 1 mL of ice cold Stain Buffer and count the cells.

Permeabilization 30m

19 Aliquot 1 million stained cells into a 1.5 mL low bind tube and centrifuge at **400 x g, 4°C, 00:05:00** . Remove the supernatant. 5m

20 Resuspend the cell pellet in 100 µl of ice cold Perm Buffer by pipette mixing. Incubate cells for **00:05:00 on ice** . 5m

21 Dilute cells with 1mL of ice cold Wash Buffer and gently mix.

22 Centrifuge cells at **400 x g, 4°C, 00:05:00** and remove the supernatant. 5m

23 Resuspend the cell pellet in 50 - 100 µl of ice cold Tagmentation Buffer depending on the desired cell input into the GEM barcoding reaction. An input of 15k cells requires a final concentration of 3k cells per µl.

24 ⊛

Filter cell suspension using a 35 µm cell strainer to remove cell clumps. Transfer filtered solution to a new tube.

25 Count the cells and calculate the volume of cell suspension required for the desired cell input.

Tagmentation and GEM generation 3h

26 Aliquot the desired number of cells to a new 96-well skirted PCR plate and bring volume to 5 µl with Tagmentation Buffer.

27 Create a master mix containing 7 µl of ATAC Buffer B and 3 µl of ATAC Enzyme B. Add 10 µl of master mix to each sample and gently pipette mix ten times.

28 Seal the plate and incubate on a thermal cycler for **01:00:00** at **37 °C** . Hold the reaction at **4 °C** upon completion. Allow samples to cool to **4 °C** , then proceed immediately to GEM generation (Step 28). 1h

Perform steps 29 - 31 during the incubation time.

29 ⚠

During the tagmentation reaction remove the Single Cell Multiome Gel Beads, Template Switch Oligo, Reducing Agent B, Barcoding Reagent Mix, and Quenching Agent from storage and allow them to thaw at **Room temperature** .

Note: First use of the Template Switch Oligo requires resuspension in 80 µl of Low TE Buffer.

30 Remove the Barcoding Enzyme Mix from storage 10 - 15 minutes before the end of the tagmentation reaction and maintain **On ice** .

31 Create a barcoding master mix on ice as detailed in the 10x Multiome ATAC + Gene Expression user guide.

32 Load a Chromium Next GEM Chip J into a secondary holder. Dispense 50% Glycerol into **unused** chip wells as follows:

Row 1: 70 µl
Row 2: 50 µl
Row 3: 40 µl

33 Remove the sample plate from the thermal cycler once it reaches **4 °C** and maintain **On ice** .

Add 60 µl of master mix to each sample well and gently pipette mix five times.

34 Load 70 µl of master mix plus sample into row 1 of the chip.

35 Vortex the Multiome Gel Beads for **00:00:30** and add 50 µl to row 2 of the chip. 30s

36 Add 45 µl of Partitioning Oil to row 3 of the chip.

37 Place a gasket over the loaded chip and load it onto the Chromium Controller. Initiate the run.

38 At completion of the run, slowly aspirate 100 µl of GEMs from the chip and slowly dispense into a new half-skirt plate on ice as detailed in the 10x Multiome ATAC + Gene Expression user guide (step 2.4 of the guide).

39 Incubate GEMs on a thermal cycler for **00:45:00** at **37 °C** , followed by **00:30:00** at **25 °C** , ending with a **4 °C** hold. 1h 15m

40 ⏸

Add 5 µl of Quenching Agent to each sample and mix.

Optional Stopping Point: GEMs can be stored at **-80 °C** for for up to four weeks.

GEM cleanup 1h 30m

41 Remove Dynabeads MyOne SILANE reagent from storage at least 30 minutes before beginning the cleanup and allow it to equilibrate to **Room temperature** .

20m

42 Remove the Cleanup Buffer from **-20 °C** storage.

Heat at **65 °C** and thoroughly vortex until no precipitates are visible (approx. **00:20:00**).

43 Perform the Post GEM Incubation Dynabead cleanup as detailed in the 10x Multiome ATAC + Gene Expression User Guide (step 3.1 of the guide).

10m

44 Perform a 2.0x bead:sample SPRIselect cleanup.

Bind beads for **00:10:00** at **Room temperature** .

Elute DNA off the beads by resuspending in 45.5 µl of Buffer EB.

Transfer 45 µl of sample to a new plate.

Pre-Amplification 1h 30m

45 Remove Pre-Amp Primers from storage and allow them to thaw at room temperature.

Remove Pre-Amp Mix from storage and thaw on ice.

Prepare a 0.2 µM solution of ADT-Rev-AMP primer in Low TE Buffer.
Note: can be prepared ahead of time and frozen at **-20 °C** .

46 **On ice** prepare a Pre-Amp master mix consisting of:
50 µl of Pre-Amp Mix
4 µl of Pre-Amp Primers
1 µl of 0.2 µM ADT-Rev-AMP primer
per sample

47 **On ice** add 55 µl of Pre-Amp master mix to 45 µl of sample and mix.

48 ⏸

Incubate reactions on a thermal cycler using the following 7 cycle PCR protocol:

Lid Temperature: 105°C
Reaction Volume: 100 µl

Step	Temperature	Time
1	72°C	5 min
2	98°C	3 min
3	98°C	20 sec
4	63°C	30 sec
5	72°C	1 min
6	-	Go to step 3 x6 (total of 7 cycles)
7	72°C	1 min
8	4°C	Hold

7 cycle Pre-Amp PCR protocol

Optional Stopping Point: Following amplification, reactions can be held at **4 °C** for up to 72 hours.

49 10m

Perform a 2.0x bead:sample SPRIselect cleanup.

Bind beads for **00:10:00** at **Room temperature** .

Elute DNA off the beads by resuspending in 160.5 µl of Buffer EB.

Transfer 160 µl of sample to a new plate.

Optional Stopping Point: Following cleanup, libraries can be held at **4 °C** for up to 72 hours or stored at **-20 °C** .

ATAC Library Prep 1h 30m

50 Remove SI-PCR Primer B and Sample Index plate N, Set A from **-20 °C** storage and allow them to thaw at room temperature.

Remove Amp Mix from **-20 °C** storage and thaw on ice.

51 **On ice** , prepare a PCR master mix consisting of 50 µl Amp Mix and 7.5 µl SI-PCR Pimer B per sample.

52 **On ice** , add 57.5 µl of PCR master mix and 2.5 µl of an individual index to 40 µl of each pre-amplified sample. Mix thoroughly.

53

Incubate reactions on a thermal cycler using the following 9 cycle PCR protocol:

Lid Temperature: 105°C
Reaction Volume: 100 µl

Step	Temperature	Time
1	98°C	45 sec
2	98°C	20 sec
3	67°C	30 sec
4	72°C	20 sec
5	-	Go to step 2 x8 (total of 9 cycles)
6	72°C	1 min
7	4°C	Hold

9 cycle ATAC indexing PCR protocol

Optional Stopping Point: Following amplification, reactions can be held at **4 °C** for up to 72 hours.

54

Perform a dual-sided 0.6x/1.6x bead:sample SPRIselect size-selection clean-up as detailed in the 10x Multiome ATAC + Gene Expression User Guide (step 5.2 of the guide).

Optional Stopping Point: Final ATAC libraries can be stored at **-20 °C** .

cDNA Amplification 2h

55 Remove cDNA Primers from storage and allow them to thaw at room temperature.

Remove Amp Mix from storage and thaw **On ice** .

Prepare a 2 µM solution of ADT-Rev-AMP primer in Low TE buffer.
Note: can be prepared ahead of time and frozen at **-20 °C** .

56 **On ice** , prepare a cDNA Amplification master mix consisting of:

50 µl of Amp Mix
15 µl of cDNA Primers
1 µl of 2 µM ADT-Rev-AMP primer
per sample.

57 **On ice** , add 66 µl of cDNA Amplification master mix to 35 µl of each pre-amplified sample. Mix thoroughly.

58

Incubate reactions on a thermal cycler using the following 8 cycle PCR protocol:

Lid Temperature: 105°C
Reaction Volume: 101 µl

Step	Temperature	Time
1	98°C	3 min
2	98°C	15 sec
3	63°C	20 sec
4	72°C	1 min
5	-	Go to step 2 x7 (total of 8 cycles)
6	72°C	1 min
7	4°C	Hold

8 cycle cDNA Amplification protocol

Optional Stopping Point: Following amplification, reactions can be held at **4 °C** for up to 72 hours.

59

Perform a dual-sided 0.6x/2.0x bead:sample SPRIselect size-selection clean-up.

Add 60 µl of SPRIselect beads to each reaction, incubate at room temperature for 5 minutes, incubate on a magnet for 5 minutes or until clear, transfer the supernatant to a new half-skirt plate.

Supernatant contains the ADT fragments and is retained for further processing.

Complete the SPRI cleanup on the large cDNA fragments by performing two washes with 80% ethanol.
Elute cDNA off the beads by resuspending in 40.5 µl of Buffer EB.
Transfer 40 µl of each cDNA library to a new plate.

Add an additional 140 µl of SPRI beads to the ADT-containing supernatant from the first cleanup.
Incubate at room temperature for 10 minutes, incubate on a magnet and remove the supernatant.
Perform two washes with 80% ethanol, elute ADTs off the beads by resuspending in 45.5 µl of Buffer EB.
Transfer 45 µl of ADT solution to a new plate.

Optional Stopping Point: Following cleanup, amplified cDNA can be held at **4 °C** for up to 72 hours or stored at **-20 °C** .

60 Run 1 ul of each amplified cDNA library on an Agilent Bioanalyzer High Sensitivity chip at a 1:10 dilution.

Set a region at 200 - 9000 bp and record the concentration in pg / µl. Calculate the total cDNA yield in ng.

Consult the 10x Multiome ATAC + Gene Expression user guide if adjusting the number of PCR cycles in the Gene Expression indexing reaction is necessary.

ADT indexing PCR 2h

61 Remove KAPA HiFi HotStart ReadyMix from **-20 °C** storage and thaw on ice.

Remove 10 µM SI-PCR Primer and 10 µM ADT-i7 primers from **-20 °C** storage and thaw at room temperature.

62 **On ice** , add

50 µl KAPA HiFi HotStart ReadyMix
2.5 µl 10 µM SI-PCR Primer
2.5 µl of an individual 10 µM ADT-i7 primer

to each 45 µl ADT sample. Mix thoroughly.

63

Incubate reactions on a thermal cycler using the following 15 cycle PCR protocol:

Lid Temperature: 105°C
Reaction Volume: 100 µl

Step	Temperature	Time
1	95°C	3 min
2	95°C	20 sec
3	60°C	30 sec
4	72°C	20 sec
5	-	Go to step 2 x14 (total of 15 cycles)
6	72°C	5 min
7	4°C	Hold

15 cycle ADT indexing PCR protocol

Optional Stopping Point: Following amplification, reactions can be held at **4 °C** for up to 72 hours.

64

Perform a 1.6x bead:sample SPRIselect cleanup.

Bind beads for 5 minutes at room temperature.

Elute DNA off the beads by resuspending in 30.5 µl of Buffer EB.

Transfer 30 µl of final ADT library to a new plate.

Optional Stopping Point: Final ADT libraries can be stored at **-20 °C** .

Gene Expression Library Prep 4h

65 Remove Fragmentation Buffer, Adapter Oligos, Ligation Buffer, and Dual Index Plate TT Set A from **-20 °C** storage and thaw at room temperature.

Remove Fragmentation Enzyme, DNA Ligase, and Amp Mix immediately before use and maintain **On ice** .

66 **On ice** , prepare a fragmentation master mix conisting of 5 µl of Fragmentation Buffer and 10 µl of Fragmentation Enzyme per sample.

67 Transfer 10 µl of each amplified cDNA library to a new plate. **On ice** , add 25 µl of Buffer EB and 15 µl of fragmentation master mix to each sample. Mix thoroughly.

35m

68 Incubate reactions on a pre-cooled thermal cycler holding at **4 °C** .

Skip to the next step to ramp to **32 °C** for **00:05:00** , followed by **65 °C** for **00:30:00** , ending with a **4 °C** hold.

69 Perform a dual-sided 0.6x/0.8x bead:sample SPRIselect size-selection clean-up as detailed in the 10x Multiome ATAC + Gene Expression User Guide (step 7.2 of the guide).

70 **On ice** , prepare an adapter ligation master mix consisting of
20 µl of Ligation Buffer
10 µl of DNA Ligase
20 µl of Adapter Oligos.
per sample.

71 **On ice** , add 50 µl of adapter ligation master mix to each sample well. Mix thoroughly.

15m

72 Incubate reactions on a thermal cycler at **20 °C** for **00:15:00** , ending with a hold at **4 °C** .

73 Perform a 0.8x bead:sample SPRIselect cleanup as detailed in the 10x Multiome ATAC + Gene Expression User Guide (step 7.4 of the guide).

74 **On ice** , add 50 µl of Amp Mix and 20 µl of an individual Dual Index TT Set A to each sample.

Mix thoroughly.

75

Incubate reactions on a thermal cycler using the following 14 cycle PCR protocol:

Lid Temperature: 105°C
Reaction Volume: 100 µl

Step	Temperature	Time
1	98°C	45 sec
2	98°C	20 sec
3	54°C	30 sec
4	72°C	20 sec
5	-	Go to step 2 x13 (total of 14 cycles)
6	72°C	1 min
7	4°C	Hold

14 cycle Gene Expression indexing PCR protocol

If desired, the number of PCR cycles can be altered depending on cDNA yield. Reference the 10x Multiome ATAC + Gene Expression User Guide (step 7.5 of the guide) for vendor recommendations.

Optional Stopping Point: Following amplification, reactions can be held at **4 °C** for up to 72 hours.

76

Perform a dual-sided 0.6x/0.8x bead:sample SPRIselect size-selection clean-up as detailed in the 10x Multiome ATAC + Gene Expression User Guide (step 7.6 of the guide).

Optional Stopping Point: Final Gene Expression libraries can be stored at **-20 °C** .

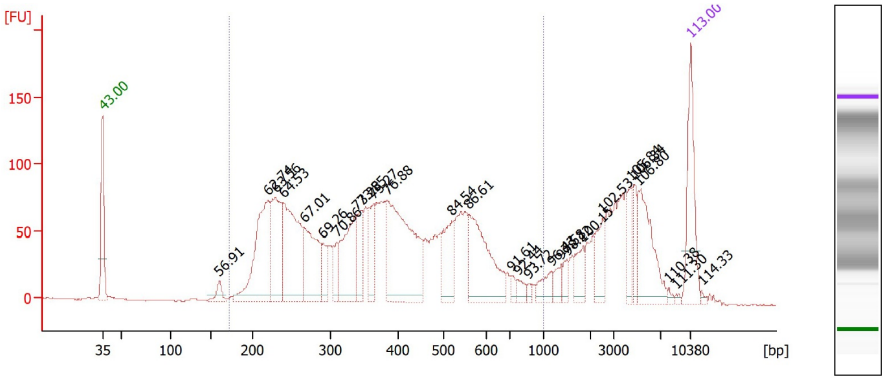
Final Library QC 2h

77 Run each ATAC, Gene Expression, and ADT final library on an Agilent Bioanalyzer High Sensitivity chip or equivalent electrophoresis assay.

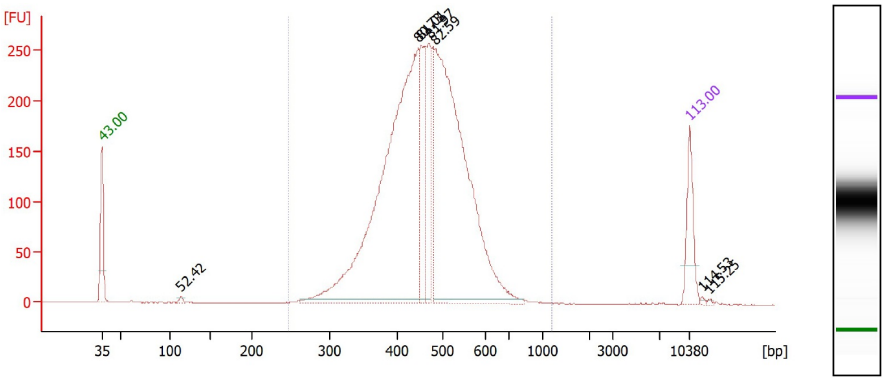
We recommend loading libraries at a 1:10 dilution for greater accuracy.

Set regions around each library trace to estimate the average base-pair size of the library. Evaluate the traces for presence of primer-dimer of other processing artifacts.

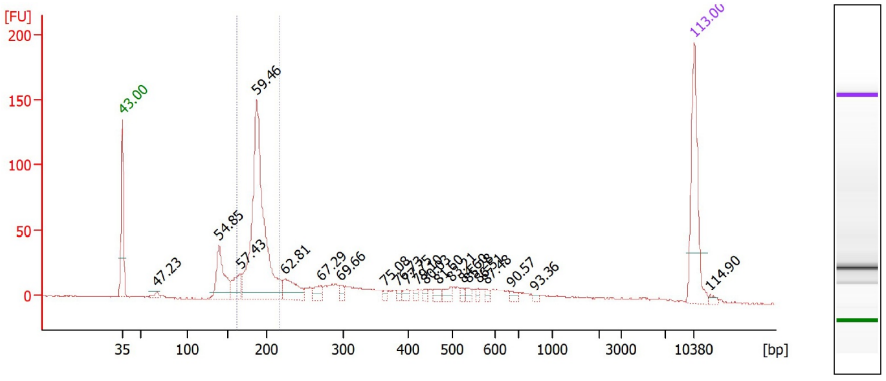
78 Quantify library concentration via qPCR using the KAPA Library Quantification Kit for Illumina® Platforms **Kapa Biosystems Catalog #KK4835** or equivalent kit. Specifications will vary by qPCR instrument.



ATAC final library Bioanalyzer trace (1:10 dilution), above.



Gene Expression final library Bioanalyzer trace (1:10 dilution), above.



ADT final library Bioanalyzer trace (1:10 dilution), above.

79 Calculate a size-adjusted concentration (in pM) for each library using the electropherogram and qPCR data.

Library concentrations determined using methods other than qPCR such as PicoGreen may be less accurate for this assay, especially for ADT libraries.

Multiply the calculated average concentration with the following factor:

Size of DNA Standard in bp (452) / Average fragment length of library in bp

Sequencing

80 Sequence libraries on the desired Illumina instrument platform. ATAC libraries will require different i5 index read lengths depending on whether the platform uses the Forward or Reverse Complement indexing workflow. The Reverse Complement workflow requires ATAC libraries to be sequenced on a separate flow cell with a longer i5 read length due to the spacer in the capture sequence.

The Forward Strand indexing workflow allows for all three library types to be sequenced on the same flowcell.

Read length for Forward Strand indexing workflow joint flow cell (ATAC, Gene Expression, ADT)
Read 1: 50 bp
i7 Index: 10 bp
i5 Index: 16 bp
Read 2: 90 bp

Read length for Forward Strand indexing workflow ATAC only flow cell
Read 1: 50 bp
i7 Index: 8 bp
i5 Index: 16 bp
Read 2: 50 bp

Read length for Reverse Strand indexing workflow ATAC only flow cell
Read 1: 50 bp
i7 Index: 8 bp
i5 Index: 24 bp
Read 2: 50 bp

Read length for Gene Expression plus ADT (or Gene Expression only) flow cell
Read 1: 28 bp
i7 Index: 10 bp
i5 Index: 10 bp
Read 2: 90 bp

Read length for ADT only flow cell
Read 1: 28 bp
i7 Index: 8 bp
Read 2: 15 bp

Sequencing Depth
We recommend a minimum read count of 30k for Gene Expression, 60k for ATAC, and 12k for ADT, per cell when sequencing PBMCs. Other cell types may require additional sequencing. In our experience the required sequencing depth of the ADT library tends to scale with the size of the panel, with the exception of rare markers.

Reference the 10x Genomics Sequencing Requirements for Single Cell Multiome ATAC + Gene Expression support page for more details.

https://support.10xgenomics.com/single-cell-multiome-atac-gex/sequencing/doc/specifications-sequencing-requirements-for-single-cell-multiome-atac-gene-expression

Demultiplexing

81 Demultiplexing of ATAC and Gene Expression data can be performed using either Cell Ranger mkfastq (10x Genomics) or bcl2fastq2 (Illumina). We prefer bcl2fastq2 as we have found that it significantly outperforms mkfastq in terms of processing time and flexibility. Flow cells containing both ATAC and Gene Expression libraries must use bcl2fastq2 for demultiplexing.

bcl2fastq 2.20
by Illumina

Cell Ranger 5.0
by 10x Genomics

The commands below may be used to demultiplex TEA-seq data by library type. Use of bcl2fastq2 requires a sample sheet in standard Illumina Experiment Manager format for each library type.

 # demultiplex gene expression data

```
bcl2fastq --use-bases-mask=Y28n*,I10,I10n*,Y90n* \
    --create-fastq-for-index-reads \
    --minimum-trimmed-read-length=8 \
    --mask-short-adapter-reads=8 \
    --ignore-missing-positions \
    --ignore-missing-filter \
    --ignore-missing-bcls \
    -r 24 -w 24 -p 80 \
    -R ${FLOWCELL_DIR} \
    --output-dir={OUTPUT_DIR} \
    --interop-dir={INTEROP_DIR} \
    --sample-sheet={GEX_SAMPLE_SHEET_PATH}
```

 # demultiplex ATAC data

```
bcl2fastq --use-bases-mask=Y50n*,I8n*,Y16,Y50n* \
    --create-fastq-for-index-reads \
    --minimum-trimmed-read-length=8 \
    --mask-short-adapter-reads=8 \
    --ignore-missing-positions \
    --ignore-missing-filter \
    --ignore-missing-bcls \
    -r 24 -w 24 -p 80 \
    -R ${FLOWCELL_DIR} \
    --output-dir={OUTPUT_DIR} \
    --interop-dir={INTEROP_DIR} \
    --sample-sheet={ATAC_SAMPLE_SHEET_PATH}
```

 # demultiplex ADT data

```
bcl2fastq --use-bases-mask=Y28n*,I8n*,n*,Y90n* \
    --create-fastq-for-index-reads \
    --minimum-trimmed-read-length=8 \
    --mask-short-adapter-reads=8 \
    --ignore-missing-positions \
    --ignore-missing-controls \
    --ignore-missing-filter \
    --ignore-missing-bcls \
    -r 24 -w 24 -p 80 \
    -R {FLOWCELL_DIR} \
```

```
--output-dir={OUTPUT_DIR} \
--interop-dir={INTEROP_DIR} \
--sample-sheet={ADT_SAMPLE_SHEET_PATH}
```

These commands have been written into a Bash script for ease of use. It is required to have bcl2fastq2 added to the environment path before use.

TEA-seq_Joint_Flowcell_bcl2fastq.sh

Data Preprocessing and analysis

82 Please see our Github repository for downstream data processing and analysis code:
https://github.com/AllenInstitute/aifi-swanson-teaseq/