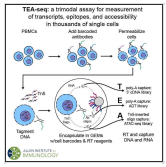

Version 4

Aug 18, 2021

TEA-seq V.4

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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-A) 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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KEYWORDS

10x Genomics, Trimodal, Triple, scATAC-seq, scRNA-seq, ADT, Allen Institute, Allen Institute for Immunology, AIFI, BioLegend, TotalSeq

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51934

GUIDELINES

10X Genomics Multiome ATAC + Gene Expression User Guide
Protocol steps often refer to the Chromium Next GEM Single Cell Multiome ATAC + Gene Expression User Guide for additional instructions or details. The User Guide referenced in this protocol is CG000338 RevD. Later versions of the User Guide may be inconsistent with the step numbers specified.

Cell Hashing
When performing Cell Hashing as part of TEA-seq please note that Hashtag Oligos (HTO) and Antibody Derived Tags (ADT) have different PCR handles on their 5' ends and thus require different primers, both during the Pre-Amplification (step 47) and cDNA Amplification steps (Step 57), and during indexing PCR. BioLegend refers to both types of antibodies as "TotalSeq-A" which can lead to confusion. Different PCR handles allow ADT and HTO libraries to be prepared separately and sequenced at different read numbers.

RNase Inhibitor
Following release of Version 2 we have updated the TEA-seq protocol to use Roche's Protector RNase Inhibitor instead of Lucigen's NxGen RNAse Inhibitor. We have found that we get higher and more consistent RNA UMI counts with the Protector reagent. This change is also supported by a 10x Genomics technical support Q&A.
https://kb.10xgenomics.com/hc/en-us/articles/360049543672-Can-I-use-an-alternative-RNase-inhibitor-part-number-?
mkt_tok=eyJpIjoiWTJVelpEZ3habVJrWkRneSIsInQiOiJPdk9kR0g0XC9kZG1JYnFJbm81dmhzTGdTWEpDMnhkMURDM2VpXC9pYzVxaFNIMkNjeGxSaGZtd3VWOUkyV1BUTHRQNlY3bjMrY2VJNVAzbmlzMDY1MlM4R2JSOUx2QzIxeG5vd003cG1xeW5lN1ZKeGRodGpMRFVXSmdaT3pWanFjIn0%3D

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:

Citation: Elliott Swanson, Lucas Graybuck, Peter J Skene (08/18/2021). TEA-seq. https://dx.doi.org/10.17504/protocols.io.bwx6pfre

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

Citation: Elliott Swanson, Lucas Graybuck, Peter J Skene (08/18/2021). TEA-seq. https://dx.doi.org/10.17504/protocols.io.bwx6pfre

^ 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

Protector RNase Inhibitor **Millipore Sigma Catalog #3335399001**

FC blocking reagent (FcX) **BioLegend**

SPRIselect Reagent **Beckman Coulter Catalog #B23317**

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

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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 85

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:

A	B	C
Name	**Sequence (5'->3')**	**Length**
SI-PCR-Oligo	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTC	49
ADT-Rev-AMP	CCTTGGCACCCGAGAATTCC	20
ADT-i7	CAAGCAGAAGACGGCATACGAGATXXXXXXXXGTGACTGGAGTTCCTTGGCACCCGAGAATTCC*A	65
Additive HTO Primer	GTGACTGGAGTTCAGACGTGTGCTC	25
HTO-i7 Primer	CAAGCAGAAGACGGCATACGAGATXXXXXXXXGTGACTGGAGTTCAGACGTGTGC	55

Oligonucleotides required for ADT and HTO amplification. Note that for ADT-i7 and HTO-i7 Primers an 8nt index sequence must be added in place of the stretch of 'X' nucleotides. * designates the location of a Phosphorothioate bond.

External equipment:

10X Genomics Chromium Controller
Illumina NovaSeq 6000

Citation: Elliott Swanson, Lucas Graybuck, Peter J Skene (08/18/2021). TEA-seq. https://dx.doi.org/10.17504/protocols.io.bwx6pfre

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:

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 Protector RNase Inhibitor at a final concentration of 1U / µl.

Sample preparation 1h

Citation: Elliott Swanson, Lucas Graybuck, Peter J Skene (08/18/2021). TEA-seq. https://dx.doi.org/10.17504/protocols.io.bwx6pfre

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 ✱ 10m

Centrifuge antibodies at **14000 x g, 4°C, 00:10:00** to remove antibody aggregates. Avoid pipetting from the

Citation: Elliott Swanson, Lucas Graybuck, Peter J Skene (08/18/2021). TEA-seq. https://dx.doi.org/10.17504/protocols.io.bwx6pfre

bottom of the tube when aspirating antibodies following centrifugation.

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

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

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

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

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

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

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

Permeabilization 30m

20 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

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

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

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

24 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.

25

Citation: Elliott Swanson, Lucas Graybuck, Peter J Skene (08/18/2021). TEA-seq. https://dx.doi.org/10.17504/protocols.io.bwx6pfre

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

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

Tagmentation and GEM generation 3h

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

28 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.

29 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 33). 1h

Perform steps 30 - 32 during the incubation time.

30 ⚠

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

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

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

32 Create a barcoding master mix on ice as detailed in the 10x Multiome ATAC + Gene Expression user guide (step 2.1 of the guide).

33 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

34 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.

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

Citation: Elliott Swanson, Lucas Graybuck, Peter J Skene (08/18/2021). TEA-seq. https://dx.doi.org/10.17504/protocols.io.bwx6pfre

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

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

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

39 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).

40 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

41 During the barcoding reaction remove the Quenching Agent from storage and equilibrate to **Room temperature** .

42

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

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

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

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

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

46 Perform a 2.0x bead:sample SPRIselect cleanup. 10m

Citation: Elliott Swanson, Lucas Graybuck, Peter J Skene (08/18/2021). TEA-seq. https://dx.doi.org/10.17504/protocols.io.bwx6pfre

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

47 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 and/or Additive HTO Primer in Low TE Buffer.

When processing both HTOs and ADTs in parallel create an equal mixture of both the ADT-Rev-AMP and Additive HTO Primer. ***Each*** primer in the mixture should be at a concentration of 0.2 uM.

> Primers can be prepared ahead of time and frozen at **-20 °C** .

48 **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 and/or Additive HTO Primer solution per sample

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

50

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

Lid Temperature: 105°C
Reaction Volume: 100 µl

Citation: Elliott Swanson, Lucas Graybuck, Peter J Skene (08/18/2021). TEA-seq. https://dx.doi.org/10.17504/protocols.io.bwx6pfre

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.

51 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

52 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.

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

54 **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.

Citation: Elliott Swanson, Lucas Graybuck, Peter J Skene (08/18/2021). TEA-seq. https://dx.doi.org/10.17504/protocols.io.bwx6pfre

55

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.

56

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

57 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 and/or Additive HTO Primer in Low TE Buffer.

When processing both HTOs and ADTs in parallel create an equal mixture of both the ADT-Rev-AMP and Additive HTO Primer. ***Each*** primer in the mixture should be at a concentration of 2 uM.

Citation: Elliott Swanson, Lucas Graybuck, Peter J Skene (08/18/2021). TEA-seq. https://dx.doi.org/10.17504/protocols.io.bwx6pfre

Primers can be prepared ahead of time and frozen at **-20 °C** .

58 **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 and/or Additive HTO Primer solution per sample.

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

60

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.

61

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

Add 60 µl of SPRIselect beads to each reaction, mix thoroughly, and incubate the plate at room temperature on the bench for **5 minutes**.
Transfer the plate to a magnet and incubate on a magnet for 5 minutes or until the solution is clear.
Transfer the supernatant to a new half-skirt plate. **The supernatant contains the ADT & HTO fragments and is retained for further processing.**

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

Citation: Elliott Swanson, Lucas Graybuck, Peter J Skene (08/18/2021). TEA-seq. https://dx.doi.org/10.17504/protocols.io.bwx6pfre

Add an additional 140 µl of SPRIselect beads to the ADT/HTO containing supernatant from the first cleanup. Carefully pipette mix ten times or until the solution is fully homogenous.
Incubate the plate at room temperature on the bench for **10 minutes**.
Transfer the plate to a magnet and incubate on a magnet for 5 minutes or until the solution is clear.
Remove and discard the supernatant.
Perform two washes with 80% ethanol.

ADT or HTO only
Elute the ADTs or HTOs off the beads by resuspending in 45.5 µl of Buffer EB.
Transfer 45 µl of ADT solution to a new plate.

ADT & HTO
Elute the ADTs or HTOs off the beads by resuspending in 90.5 µl of Buffer EB.
Transfer 45 µl of the HTO/ADT solution to each of two new plates. HTO and ADT fragments will be amplified in separate indexing PCRs using 45 µl of starting material each.

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

62 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 (step 6.1) if adjusting the number of PCR cycles in the Gene Expression indexing reaction is necessary.

63 Proceed to step 64 for ADT indexing PCR or step 68 for HTO indexing PCR. When preparing both ADT and HTO libraries these sections can be performed in either order.

ADT indexing PCR 2h

64 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.

65 **On ice** , add the following to each 45 µl **ADT** sample. Mix thoroughly.

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

66

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

Lid Temperature: 105°C
Reaction Volume: 100 µl

Citation: Elliott Swanson, Lucas Graybuck, Peter J Skene (08/18/2021). TEA-seq. https://dx.doi.org/10.17504/protocols.io.bwx6pfre

A	B	C
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.

67

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** .

HTO indexing PCR

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

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

69 **On ice** , add the following to each 45 µl **HTO** sample. Mix thoroughly.

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

70

Incubate reactions on a thermal cycler using the following 10 cycle PCR protocol:

Lid Temperature: 105°C
Reaction Volume: 100 µl

Citation: Elliott Swanson, Lucas Graybuck, Peter J Skene (08/18/2021). TEA-seq. https://dx.doi.org/10.17504/protocols.io.bwx6pfre

A	B	C
Step	**Temperature**	**Time**
1	95°C	3 min
2	95°C	20 sec
3	64°C	30 sec
4	72°C	20 sec
5	-	Go to step 2 x9 (total of 10 cycles)
6	72°C	5 min
7	4°C	Hold

10 cycle **HTO** indexing PCR protocol

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

71

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 HTO library to a new plate.

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

Gene Expression Library Prep 4h

72 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** .

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

74 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

75 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.

Citation: Elliott Swanson, Lucas Graybuck, Peter J Skene (08/18/2021). TEA-seq. https://dx.doi.org/10.17504/protocols.io.bwx6pfre

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 7.2 of the guide).

77 **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.

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

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

80 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).

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

Mix thoroughly.

82

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.

Citation: Elliott Swanson, Lucas Graybuck, Peter J Skene (08/18/2021). TEA-seq. https://dx.doi.org/10.17504/protocols.io.bwx6pfre

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

83

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 .

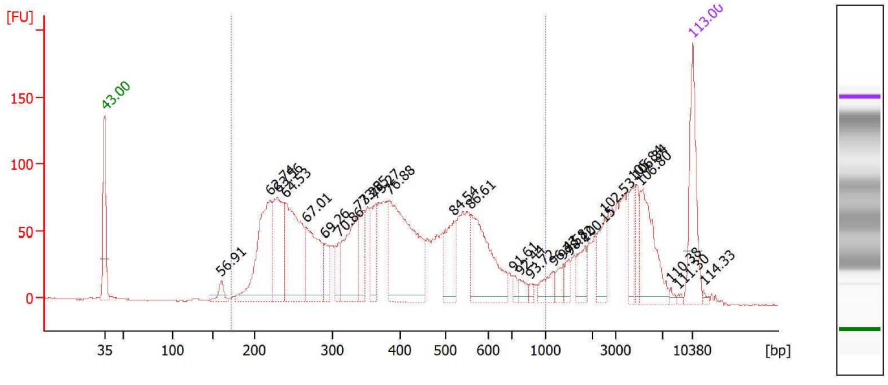
Final Library QC 2h

84 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.

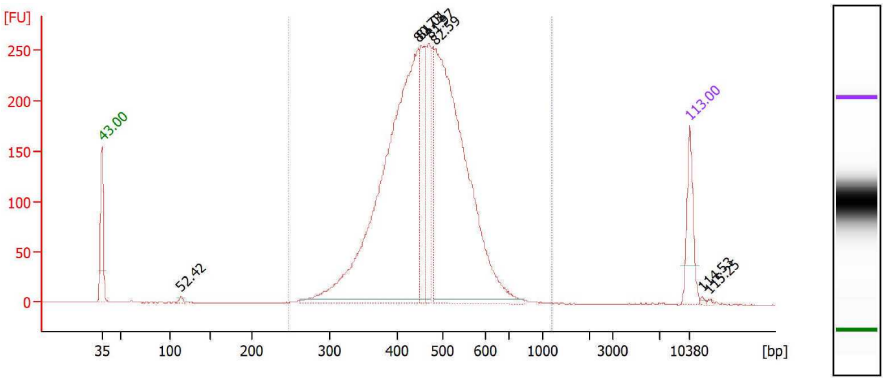
Set regions around each library trace to estimate the average base-pair size of the library. Evaluate the traces for presence of primer-dimer of other processing artifacts.

85 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.

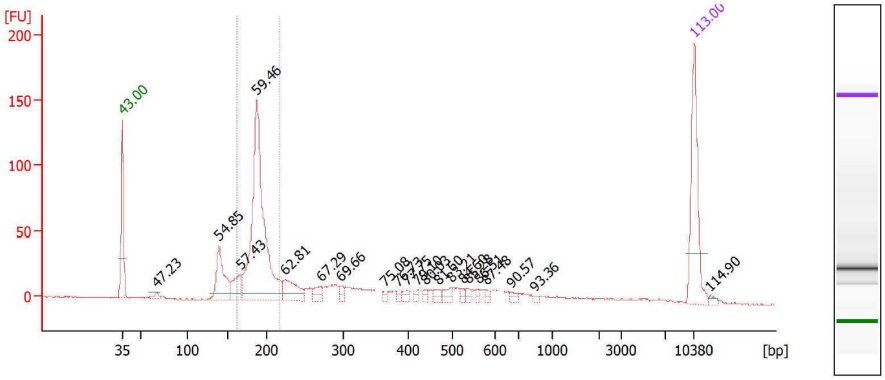


ATAC final library Bioanalyzer trace (1:10 dilution), above.

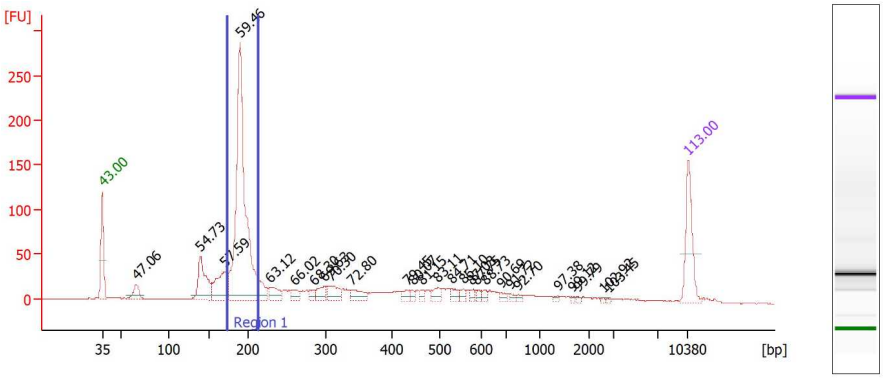
Citation: Elliott Swanson, Lucas Graybuck, Peter J Skene (08/18/2021). TEA-seq. https://dx.doi.org/10.17504/protocols.io.bwx6pfre



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



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



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

86 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 & HTO libraries.

Multiply the calculated average concentration with the following factor:

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

Citation: Elliott Swanson, Lucas Graybuck, Peter J Skene (08/18/2021). TEA-seq. https://dx.doi.org/10.17504/protocols.io.bwx6pfre

Sequencing

87 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 Strand 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.

Reference the 10x Genomics Q&A for instrument specific use of the Forward and Reverse indexing workflows.
https://kb.10xgenomics.com/hc/en-us/articles/360056364852-Should-I-select-Workflow-A-or-Workflow-B-for-the-i5-index-sequence-

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 flow cell (with or without ADT or HTO)
Read 1: 28 bp
i7 Index: 10 bp
i5 Index: 10 bp
Read 2: 90 bp

Read length for ADT and/or HTO 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, 12k for ADT, and 2k for HTO, per cell when sequencing PBMCs. Other cell types may require additional sequencing. In our experience the required sequencing depth of ADT libraries 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

88 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 a combination of ATAC, Gene Expression, ADT, or HTO libraries, must use bcl2fastq2 for demultiplexing.

Citation: Elliott Swanson, Lucas Graybuck, Peter J Skene (08/18/2021). TEA-seq. https://dx.doi.org/10.17504/protocols.io.bwx6pfre

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*,n8Y16,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 and HTO data (can use the same sample sheet).

```
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}
```


Citation: Elliott Swanson, Lucas Graybuck, Peter J Skene (08/18/2021). TEA-seq. https://dx.doi.org/10.17504/protocols.io.bwx6pfre

When demultiplexing adjust the read2 bases mask to be no longer than the read2 length (i.e. Y15n* for a 15 bp read 2). This applies to ADT and.or HTO only flow cells with a read2 length less than 90 bp.

ATAC demultiplexing requires a different --use-bases-mask for the i5 read depending on if the flow cell was sequenced using the Forward Strand or Reverse Complement indexing workflow. The bcl2fastq2 command above uses the base mask ***n8Y16*** and assumes the flow cell was sequenced using the Reverse Complement workflow, consistent with the commonly used NovaSeq 6000 v1.5 platform. The Forward strand workflow will require the --use-bases-mask ***Y16*** for the i5 read.

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

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

Citation: Elliott Swanson, Lucas Graybuck, Peter J Skene (08/18/2021). TEA-seq. https://dx.doi.org/10.17504/protocols.io.bwx6pfre