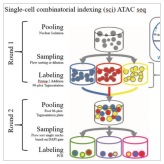

Version 2

Jan 24, 2021

sciMAP-ATAC V.2

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1 Works for me dx.doi.org/10.17504/protocols.io.brhxm37n

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ABSTRACT

High-throughput single cell genomic assays resolve the heterogeneity of cell states in complex tissues, however, the spatial orientation within the network of interconnected cells is lost. As cell localization is a necessary dimension in understanding complex tissues and disease states, we present a tool for highly scalable spatially-resolved single cell profiling of chromatin state. We use high density multiregional sampling to perform single-cell combinatorial indexing on Microbiopsies Assigned to Positions for the Assay for Transposase Accessible Chromatin (sciMAP-ATAC) to produce single-cell data of equivalent quality to non-spatial single-cell ATAC-seq.

EXTERNAL LINK

doi: https://doi.org/10.1101/407668

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Thornton, C. A., Mulqueen, R. M., Nishida, A., Torkenczy, K. A., Lowenstein, E. G., Fields, A. J., ... Adey, A. C. (2020). Spatially mapped single-cell chromatin accessibility. BioRxiv, 815720. https://doi.org/10.1101/815720

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KEYWORDS

Genomics, Epigenomics, Single Cell, Biotechnology

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MATERIALS TEXT

MATERIALS

Magnesium Chloride **Fisher Scientific Catalog #AC223210010**

IGEPAL-CA630 **Sigma Aldrich Catalog #I3021 SIGMA-ALDRICH**

Triton X-100 **Sigma Aldrich Catalog #T8787-50ML**

Tween-20 **Sigma-aldrich Catalog #P-7949**

Sodium Chloride **Fisher Scientific Catalog #S271-3**

Agencourt Ampure XP **Beckman Coulter Catalog #A63880**

4,6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) **Thermo Fisher Scientific Catalog #D1306**

Embedding base molds **Fisher Scientific Catalog #22-363-553**

Jung tissue freezing medium (Leica Microsystems) or OCT compound (TissueTek) **Contributed by users**

Cell strainer, 35 µm **Corning Catalog #352235**

Pierce Preotease Inhibitor Tablets, EDTA-Free **Thermo Fisher Scientific Catalog #A32955**

Tris-HCl **Life Technologies Catalog #AM9855**

Superfrost Plus Microscope Slies **Thermo Fisher Scientific Catalog #4951PLUS4**

1X PBS, cell culture grade **Thermo Fisher Scientific**

Potassium Chloride **Sigma Aldrich Catalog #P9541**

EDTA **Invitrogen - Thermo Fisher Catalog #AM9261**

Qiagen Protease **Fisher Scientific Catalog #NC9221823**

Citation: Andrew Adey, Casey Thornton (01/24/2021). sciMAP-ATAC. https://dx.doi.org/10.17504/protocols.io.brhxm37n

Pitstop 2 **Sigma**

Aldrich Catalog #SML1169-5MG

Nextera DNA Flex Library Prep **Illumina,**

Inc. Catalog #20018705

QIAquick PCR Purification

Kit **Qiagen Catalog #28106**

Uniquely Indexed Transposomes **Contributed by users**

Sci- Barcoded PCR Primers **Contributed by users**

Pitstop 2 **Sigma**

Aldrich Catalog #SML1169

Tween-20: working stock is 10% (100X). Aliquots are stored at 4C.
IGEPAL-630: Prepare 10% (v/v) stock made with diH20, store at Room Temperature (RT).
DAPI: Resuspend to 5 mg/mL in diH20. Aliquot and store at -20C.
Pitstop2: Resuspend in 3mM in DMSO. Aliquot and store at -20C.

Supplies List:

- 96-well PCR plates (Eppendorf, 951020427)
- 35 um cell strainer (VWR, 21008-948)
- High Sensitivity DNA Chip (Agilent, 5067-4627)

Instrument List:

- Table top centrifuge cooled to 4C with rotors for spinning 1) 96-well plates, and 2) 15 mL falcon tubes at 600 rcf
- Fluorescence Activated Cell Sorter (FACS), we use Sony SH800S
- Thermomixer with 96 well plate adapter (55C incubations at 300 rpm), we use Eppendorf Themomixer C
- Real-Time PCR instrument (Bio-Rad CFX Connect)
- DNA fluorometer or spectrophotometer (Qubit Fluorometer 2.0 is used in this protocol)
- Agilent Bioanalyzer
- Sequencing: NextSeq 500 using custom chemistry protocol

Loading Tn5 Enzyme with sci-protocol Oligonucleotides

1 Prepare Reagents

Prepare **50 mL 2.125X Tn5 Dilution buffer** for protein dilution.

Reagent	Stock Concentration	Final Concentration	Amount of Stock
HEPES-KOH (pH 7.2)	1M	100mM	5mL
NaCl	5M	200mM	2mL
Glycerol	100%	25%	12.5mL
Triton-X100	100%	0.2%	100uL
ddH2O			30.4mL (to 50mL)
DTT	Dry	2mM	15.4 mg

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Tn5 Dilution buffer can be stored at 4C for up to 2 months.

2 Prepare Mosaic End reverse compliment (ME'), i7, i5 oligonucleotides at
[M]**100 Micromolar (µM) Tris-HCl buffer (pH 8.0)**

See attached spreadsheet for oligonucleotide sequences.

Three sets of oligonucleotides are listed for both i5 and i7 Tn5 loading.
This yields (3 i5 sets) x (3 i7 sets)=9 uniquely identifiable 96 well plates or 864 unique well barcode combinations.

Mosaic End oligonucleotide sequence used for Tn5 loading is also listed within the spreadsheet.

Example_sciTn5_Oligos.xlsx

Synthesis quality of these oligonucleotides is critical. HPLC purification is essential. We find that Eurofins oligos outperform IDT by roughly 10 fold in library complexity.

All indexes are designed to be 2 or greater Hamming distance from all others to allow for sequencing errors.

3 **Anneal Indexed Oligoes to Mosaic End Reverse Compliment**

Preparation of dsDNA through annealing. Volumes are adjusted for a single 96-well plate loading.

1. For each i5 barcoded oligo prepare the following reaction (8 total):

A	B
12.5 uL	100 uM i5 Tn5 Indexed oligo
12.5 uL	100 uM Mosaic End Reverse Compliment oligo
53.125 uL	2.125x Tn5 Dilution Buffer

Henceforth referred to as **i5/ME'**

2. For each i7 barcoded oligo prepare the following reaction (12 total):

Citation: Andrew Adey, Casey Thornton (01/24/2021). sciMAP-ATAC. https://dx.doi.org/10.17504/protocols.io.brhxm37n

8.5 uL	100 uM i7 Tn5 Indexed oligo
8.5 uL	100 uM Mosaic End Reverse Compliment oligo
36.125 uL	2.125x Tn5 Dilution Buffer

Henceforth referred to as **i7/ME'**

4 Anneal Oligo mixtures within a Thermocycler with the following reaction.

5m

- **95 °C** **00:05:00**
- Slow ramp down to **20 °C at a rate of -2.5C/min**
- **20 °C hold**

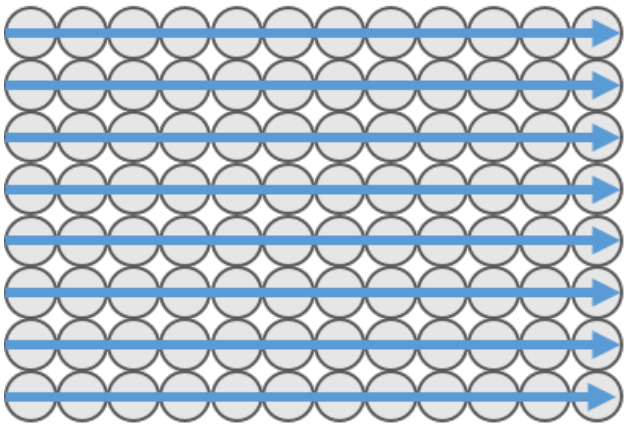
This results in **16 Micromolar (µM)** annealed oligo species per reaction (**i7/ME'** and i5/ME').

Oligoes should be freshly annealed prior to loading Tn5 transposome

5 Plate Annealed Oligos

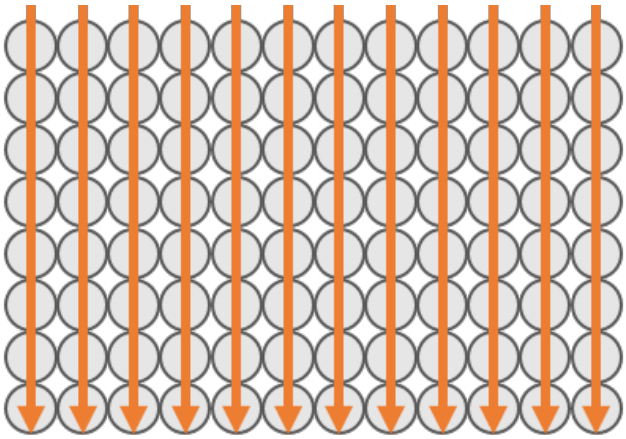
Prepare a 96-well plate with the following loading schema.

1. Add **5 µl** of i5/ME' (**16 Micromolar (µM)**) to each respective wells in a row-wise fashion.



2. Add **5 µl** of i7/ME' (**16 Micromolar (µM)**) to each respective wells in a column-wise fasion.

Citation: Andrew Adey, Casey Thornton (01/24/2021). sciMAP-ATAC. https://dx.doi.org/10.17504/protocols.io.brhxm37n



This results in **10 µl i5/ME' and i7/ME' Indexed Oligos** at **8 Micromolar (µM)** /well

6 Adjust Salt Concentration on Tn5 and Load

Prepare Tn5 protein as described in *"Generation and Purification of pTXB1.Tn5"* protocol.

Prior to loading Tn5 protein adjust NaCl concentration. Combine:

A	B
1152 uL	1152 uL
144 uL	144 uL

This adjusts salt to a final concentration of **555.55 Milimolar (mM)** NaCl

1h

7 Add **12 µl** of salt-corrected Tn5 to each well of the 96 well plate.

Assemble the Tn5/oligo mixture via incubation at **25 °C** for **01:00:00** .

Store at -20C for no more than 8 months.

Prepare Nuclei Isolation Buffer

8

Construct 50mL **Nuclei Isolation Buffer** (NIB):

Citation: Andrew Adey, Casey Thornton (01/24/2021). sciMAP-ATAC. https://dx.doi.org/10.17504/protocols.io.brhxm37n

Final Concentration	Stock Concentration	Volume of Stock
10 mM Tris HCl, pH 7.5	1M Tris-HCl, pH7.5	500 uL
10 mM NaCl	5M NaCl	100 uL
3mM MgCl2	1M MgCl2	150 uL
0.1 % Igepal	10% Igepal	500 uL
0.1 % Tween	10% Tween	500 uL
ddH20		to 50mL (add 48.25mL)

OPTIONAL: To prevent protease degradation, we also add 2 tablets of Pierce Preotease Inhibitor Tablets, EDTA-Free to NIB following construction. Vortex to fully dissolve tablets.

NIB is stable at 4 °C for at least 1 month without noticeable degradation in library quality or nuclei dissociation ability.

Store NIB on ice throughout nuclei dissociation and preparation of tagmentation plates.

Isolate nuclei

9 Nuclei isolation from cryopreserved histological sections

If sample is sourced from microbiopsy of a cryopreserved histological section, dissociate cells using NIB incubation and trituration (described below).

Note
Isolation of nuclei is dependent on the sample being used. And optimization should be performed. Below we list two example nuclei isolation protocols to act as general use for cell culture and primary tissue samples. Tissue should follow a dounce homogenization protocol, while liquid cell cultures can be pelleted and resuspended directly in NIB.

This protocol is optimized for brain tissue microbiopsies. Additional optimization may need to be performed for other tissues.

1. Prepare 96-well plate(s) for microbiopsy punches
   - Pipette 100 uL NIB into each well. Number of wells corresponds to number of punches to be collected.
   - Seal plate and store on ice until ready to collect microbiopsies

2. Prepare instruments & tissue for collecting microbiopsies.
   - Transfer cryopreserved tissue sections from -80C freezer on to dry ice in an insulated container

Citation: Andrew Adey, Casey Thornton (01/24/2021). sciMAP-ATAC. https://dx.doi.org/10.17504/protocols.io.brhxm37n

- Load Palkovitz punch handle with selected diameter punch (options: 250 um, 500 um, 750 um, 1 mm, 1.25 mm)
- Prechill Palkovitz punch by placing the punch in dry ice

3. Collect microbiopsies **in a cryostat at -20C**
- Place tissue cryosection slide in cryostat and allow ~1 min to acclimate
- Locate region of interest and collect punch
- Deposit punch in well of 96-well plate by depressing punch plunger. (Ensure that punch enters well)
- Repeat for each region to be resected. Place each new punch in new well
- Reseal 96-well plate(s)

Note: Keep a record of slide number, punch location, and well ID for each punch.

4. Dissociate and wash microbiopsies
- Shake plate on ice for 1 hour at 80 rpm
- Using a multi-channel pipettor, triturate each well 30x.

Note: Pipette gently in order to reduce bubbles and to prevent nuclei shearing

- Spin down plate for 10 min at 500 rcf at 4C
- Using a multi-channel pipettor, aspirate 90 uL of supernatant.

Note: Pellet will not be visible. Be careful to not touch sides of bottom while drawing off supernatant.

5. Dilute microbiopsy nuclei to desired concentration

Note: We find that for microbiopsy punches from 200 um thick tissue /250 um biopsy punch results in (thousand nuclei):
Min: 6, 1st Q: 12, Median: 15, Mean: 16.85, 3rd Q: 22.25, Max: 29
We want 10 µL nuclei well. Each punch dissociation can be split into 4 wells (4.2K nuclei/reaction).
Therefore, we want 40 µL of 4,200 nuclei/10 µL:
C1V1 = C2V2
(1,685 nuclei/µL)(10µL) = (421.25 nuclei/µL)(**x** µL)
x = 40 µL
Volume to add: 40 µL - 10 µL (residual volume) = **30 µL**

Final concentration of Pitstop 2 should be 70 µM in 40 µL of resuspended nuclei. Therefore:
C1V1 = C2V2
(3000 µM)(**x** µL) = (70 µM)(30 µL); x = 1.43 µL

Therefore, for each well, add: (1.4 µL 3 mM Pitstop 2 + 28.6 µL NIB) = 30 µL of 70 µM Pitstop 2 NIB

This should be done by making a master-mix. Given 1 plate (96 wells), prepare a master-mix for 120 wells:
(168 µL 3mM Pitstop 2 + 3,432 µL NIB) = 3600 µL of 70 µM Pitstop 2 NIB, for one plate.

- Prepare 70 uM Pitstop 2 + NIB master mix: For one plate, combine 168 uL 3 mM Pitstop2 & 3,432 uL NIB
- Add 30 uL of 70 uM Pitstop 2 NIB master mix to each well and triturate to resuspend cells

Citation: Andrew Adey, Casey Thornton (01/24/2021). sciMAP-ATAC. https://dx.doi.org/10.17504/protocols.io.brhxm37n

6. Split punches into multiple plates
- Split 40 uL of resuspend cells into 4 new 96-well (DNA/protein Lo-bind) plates with 10uL diluted cells/well.

Note: Make sure to keep well ID consistent between plates.

96-plex Tagmentation

10 Prepare tagmentation plate

Add the following reagents to diluted nuclei in 96-well plate(s) (DNA and Protein Lo-bind):

Prepare 70 µM Pitstop 2 + 2X TD buffer Master Mix for one plate: 28 µL 3mM Pitstop2 + 1,200 µL 2X TD

Add **10 µl 70uM Pitstop 2/TD buffer (2X)** to each well

Add **1 µl 8uM uniquely indexed transposase** to each well

Spin down plate for **00:01:00 min** at 500 rcf at **4 °C**

Tagmentation can be performed by the addition of 1 µL of 2.5 µM barcoded transposome (EZ-Tn5 variant) (Amini et al. 2014)

11 Tagmentation

Seal plate and incubate at **55 °C with gentle shaking (300 rpm on themomixer)** for **00:15:00**

Place plate on ice immediately to stop reaction.
- Keep samples on ice to prevent over-transposistion and nuclei lysis.

12 Pool all wells for second sort

Pool all wells into 15mL conical tube, while maintaining everything on ice.

Add 2uL/per mL pooled sample of DAPI (5mg/mL) and bring to sorter for second sort.

96-plex PCR

13 Preparing Second Plate of Transposase Neutralization Buffer (8.5uL/well):

Citation: Andrew Adey, Casey Thornton (01/24/2021). sciMAP-ATAC. https://dx.doi.org/10.17504/protocols.io.brhxm37n

Final Concentration	Stock Concentration	Volume of Stock
0.59 mg/mL	20 mg/mL BSA	0.25 uL
0.059% (w/v)	1% SDS (w/v)	0.5 uL
to 8.5 uL	ddH2O	7.75 uL

Per well reagent volumes.

Add 2.5 uL of 10 uM i5 Indexed PCR Primer and 2.5 uL of 10 uM i7 Indexed PCR Primer prior to sort.

2nd Sort Protocol

14 Flow sort single nuclei based on DAPI gate

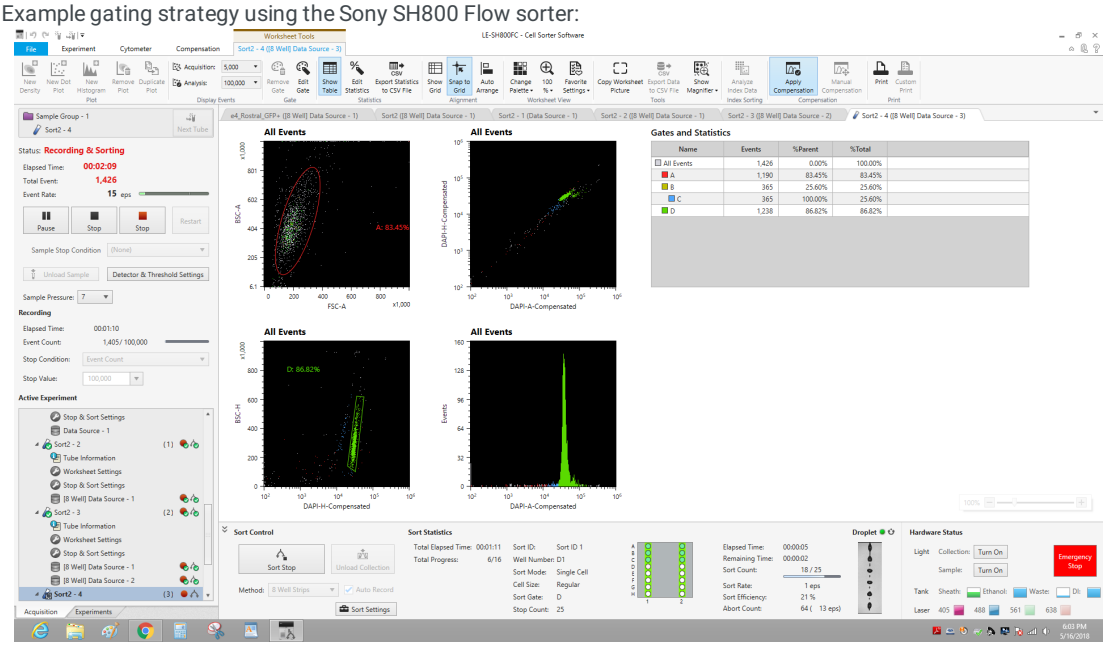
Sort **X** nuclei per well (**X** is dependent on number of wells tagmented in first sort, as a linear trend)
• 96 wells (1 plate) = 22 nuclei/well for PCR
• 144 wells (1 and 1/2 plates) = 33 nuclei/well for PCR
• 192 wells (2 plates) = 44 nuclei/well for PCR etc...

Using the same gates as first sort, sort **X** nuclei per well into prepared second plate with modified sort settings:

- "Single cell" rather than "Normal"
  This leads to a higher abort count (less efficient sorting) but is more precise in quantification

- Keep sorted samples on ice to prevent transposases cross-reacting with other nuclei.

Example gating strategy using the Sony SH800 Flow sorter:



Spin down plate at 500 rcf for **00:03:00 min** at **4 °C** to ensure nuclei are properly suspended in solution.

Volume added, even by sorting 100 nuclei is minimal in our hands and does not require concentration adjustments.

Citation: Andrew Adey, Casey Thornton (01/24/2021). sciMAP-ATAC. https://dx.doi.org/10.17504/protocols.io.brhxm37n

Transposase Denaturation

15 Transposase Denaturation

Denature remaining transposase in sorted nuclei using SDS mixture on Eppendorf Thermocyclers.

55 °C for **00:20:00 min**

96-plex PCR

16 Amplifying single cell libraries

Nextera PCR Mater Mix currently produces the highest quality libraries. An alternative master mix using Kapa Hifi Non-Hotstart has been developed and produces good results.

Step 16 includes a Step case.
Using Nextera PCR Master Mix
Using Kapa Hifi Non-Hotstart

step case

Using Nextera PCR Master Mix

17 Add 13.5 uL PCR Master Mix to each well

7.5 µl NPM

0.25 µl 100X SYBR Green I

5.5 µl dH2O

Perform Real-time PCR on the Bio-Rad CFX Connect:

Temperature (C)	Time	
72	5 min	
98	30 sec	
98	30 s	
55	30 s	
72	1 min	x30 Cycles
	PLATE READ	
72	20 sec	

PCR protocol for Kapa Hifi Non-Hotstart Library Amplification

Pull once majority of well begin to plateau. Sci-ATAC libraries amplify between 14-22 cycles dependent on nuclei per well.

Store libraries at **4 °C for 6 months** or **-20 °C forever**

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Library Clean-up and Quantification

18 Pool post-PCR Product

Pool 10 uL from each well into 15mL conical tube.

19 Concentrate DNA via column clean up

Run full pool volume through Qiaquick PCR purification column following manufacturer's protocol.

Elute in **50 µl 10 mM Tris-HCl pH 8.0**

20 Clean by size selection with SPRI beads

Perform a 1X SPRI bead size selection (selecting for DNA > 200 bp).

Add **50 µl 18% PEG SPRI Beads** to column elution, once beads are at room temperature.
Let mixture incubate at room temperature for **00:05:00 min**

Place tube on magnetic rack and wait for magnetic beads to pellet and elution to fully clear (roughly **00:02:00 min**)
Remove full volume of elution without disrupting bead pellet.

Resuspend bead pellet in freshly prepared **100 µl 80% ethanol (v/v)**
Remove full volume of elution without disrupting bead pellet.
Let beads fully air dry (roughly **00:08:00 min**)

- Beads will first lose sheen, and then begin to form cracks.

Resuspend beads off magnetic rack in **31 µl 10 mM Tris-HCl pH 8.0**
Let mixture incubate at room temperature for **00:05:00 min** for DNA to fully become suspended.

Place tube on magnetic rack and wait for magnetic beads to pellet and elution to fully clear (roughly **00:20:00 min**)
Remove full volume of elution without disrupting bead pellet and move to clean tube.

21 Qubit DNA HS Quantification

Quantify DNA concentration with 1uL eluted sample on Qubit DNA High-sensitivity kit following manufacturer's protocol.

22 Agilent DNA HS Bioanalyzer Quantification

Dilute sample to 4 ng/uL based on read out of Qubit by addition of 10mM Tris-HCl pH 8.0.
Run 1 uL sample on Agilent DNA HS Bioanalyzer following manufacturer's protocol.

Sequencing

23 Custom Nextseq500 Chemistry Protocol

Sequence libraries on a NextSeq^{TM} 500 sequencer (Illumina Inc.) running NextSeq500 NCS (v4.0) software loaded within a range of 1.2-1.6 pM with a custom sequencing chemistry protocol (Read 1: 50 imaged cycles; Index Read 1: 8

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imaged cycles, 27 dark cycles, 10 imaged cycles; Index Read 2: 8 imaged cycles, 21 dark cycles, 10 imaged cycles; Read 2: 50 imaged cycles) using custom sequencing primers.

A	B	C	D	E	F	G	H	I
Oligonucleotide ID	**Oligonucleotide Set**	**Sequence 5'>3'**						
Read1_sequencing_Primer	Read1_IlluminaNextSeq500_SequencingPrimer	GCGATCGAGGACGGCAGATGTGTATAAGAGACAG						
Read2_sequencing_Primer	Read2_IlluminaNextSeq500_SequencingPrimer	CACCGTCTCCGCCTCAGATGTGTATAAGAGACAG						
Index1_sequencing_Primer	Index1_IlluminaNextSeq500_SequencingPrimer	CTGTCTCTTATACACATCTGAGGCGGAGACGGTG						
Index2_sequencing_Primer	Index2_IlluminaNextSeq500_SequencingPrimer	CTGTCTCTTATACACATCTGCCGTCCTCGATCGC						

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