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Light-Seq Cell Barcoding V.3

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Light-Seq



Ninning Liu

ABSTRACT

We present Light-Seq, an approach for multiplexed spatial indexing of intact biological samples using light-directed DNA barcoding in fixed cells and tissues followed by ex situ sequencing. Light-Seq combines spatially targeted, rapid photocrosslinking of DNA barcodes onto complementary DNAs in situ with a one-step DNA stitching reaction to create pooled, spatially indexed sequencing libraries. This light-directed barcoding enables in situ selection of multiple cell populations in intact fixed tissue samples for full-transcriptome sequencing based on location, morphology or protein stains, without cellular dissociation. Applying Light-Seq to mouse retinal sections, we recovered thousands of differentially enriched transcripts from three cellular layers and discovered biomarkers for a very rare neuronal subtype, dopaminergic amacrine cells, from only 4–8 individual cells per section. Light-Seq provides an accessible workflow to combine in situ imaging and protein staining with next generation sequencing of the same cells, leaving the sample intact for further analysis post-sequencing.

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PROTOCOL CITATION

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COLLECTIONS ⓘ

 [Light-Seq Collection](#)

KEYWORDS

Light-Seq, Spatial Transcriptomics

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PARENT PROTOCOLS

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MATERIALS TEXT

General Buffers

 PBS - Phosphate-Buffered Saline (10X) pH 7.4 **Thermo Fisher Scientific Catalog #AM9625**

 DNase/RNase free distilled water **Invitrogen Catalog #10977023**

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

 Sodium Chloride (5M) **Invitrogen - Thermo Fisher Catalog #AM9760G**

 10 x 2 ml IDTE pH 7.5 (1X TE Solution) **IDT Catalog #11-01-02-02**

- ☒ SSC (20X), RNase-free **Thermo Fisher Catalog #AM9765**
- ☒ Tween 20 **Sigma Aldrich Catalog #P9416-50ML**
- ☒ Pierce™ 16% Formaldehyde (w/v), Methanol-free **Thermo Fisher Catalog #28908**
- ☒ Formamide (Deionized) **Thermo Fisher Catalog #AM9342**
- ☒ POLY-D-LYSINE HYDROBROMIDE MOL WT 70000 - 5MG **Sigma Aldrich Catalog #P6407-5MG**
- ☒ Pierce™ 20X Borate Buffer **Thermo Fisher Catalog #28341**

Cell culture

- ☒ DMEM, high glucose, GlutaMAX™ Supplement, pyruvate **Thermo Fisher Catalog #10569010**
- ☒ Penicillin Streptomycin **Invitrogen - Thermo Fisher Catalog #15140 122**
- ☒ Blasticidin S hydrochloride 10 mg/ml in HEPES buffer sterile-filtered **Thermo Scientific Catalog #J67216.XF**
- ☒ MEM Non-Essential Amino Acids Solution (100X) **Thermo Fisher Catalog #11140050**
- ☒ Calf Bovine Serum Iron Fortified **ATCC Catalog #30-2030**
- ☒ Fetal Bovine Serum, certified, heat inactivated, United States **Thermo Fisher Catalog #10082147**
- ☒ TrypLE™ Express **Thermo Fisher Scientific Catalog #12605010**
- ☒ DPBS with Ca and Mg **Gibco - Thermo Fischer Catalog #14040-133**

Enzymes and related reagents

- ☒ Maxima H Minus Reverse Transcriptase (200 U/μL) **Thermo Fisher Catalog #EP0753**
- ☒ RNaseOUT™ Recombinant Ribonuclease Inhibitor **Invitrogen - Thermo Fisher Catalog #10777019**
- ☒ Terminal Transferase - 2,500 units **New England Biolabs Catalog #M0315L**
- ☒ 2'3'-Dideoxyadenosine 5'-Triphosphate 100 mM solution **Sigma Aldrich Catalog #GE27-2051-01**
- ☒ Deoxynucleotide Solution Set - 25 umol of each **New England Biolabs Catalog #N0446S**
- ☒ ThermoPol Reaction Buffer Pack - 6.0 ml **New England Biolabs Catalog #B9004S**
- ☒ Bst DNA Polymerase Lg Frag - 8,000 units **New England Biolabs Catalog #M0275L**
- ☒ RNase H - 1,250 units **New England Biolabs Catalog #M0297L**
- ☒ Deoxynucleotide Solution Mix - 8 umol of each **New England Biolabs Catalog #N0447S**
- ☒ KAPA HiFi DNA Polymerase **Kapa Biosystems**

Gels

- ☒ GeneRuler Low Range DNA Ladder, ready-to-use **Thermo Fisher Catalog #SM1193**
- ☒ Novex™ TBE-Urea Gels, 15%, 15 well **Thermo Fisher Catalog #EC68855BOX**
- ☒ UltraPure™ TBE Buffer 10X **Thermo Fisher Scientific Catalog #15581044**
- ☒ E-Gel™ 1 Kb Plus Express DNA Ladder **Thermo Fisher Catalog #10488091**
- ☒ E-Gel™ Sample Loading Buffer, 1X **Thermo Fisher Catalog #10482055**
- ☒ E-Gel™ EX Agarose Gels, 1% **Thermo Fisher Catalog #G402001**
- ☒ SYBR™ Gold Nucleic Acid Gel Stain (10,000X Concentrate in DMSO) **Thermo Fisher Catalog #S11494**

qPCR and DNA purification

- ☒ SYBR™ Green I Nucleic Acid Gel Stain - 10,000X concentrate in DMSO **Thermo Fisher Catalog #S7563**
- ☒ Agencourt AMPure XP **Beckman Coulter Catalog #A63880**
- ☒ Qubit 1X dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q33230**

Cell chambers

 u-Slide 18 well Poly-L-Lysine (Custom order) **Ibidi Catalog #81814**

BEFORE STARTING

This protocol is for selective barcoding of cells seeded onto an Ibidi 18-well μ -slide. A separate protocol can be found for selective barcoding of tissue samples.

Clean workspace (bench, pipettes, etc.) with 70% ethanol before starting.

When the protocol calls for water, always use UltraPure water.

All reagents should be molecular-grade and RNase free.

All reactions with enzymes are prepared on ice.

Key Equipment:

Microscope

Light-Seq requires an optical system that can focus UV light onto specific regions of interest. In the publication, we employ a wide field microscope with a 365 nm LED and a digital micromirror device (DMD) to accomplish this, but other systems are also amenable. We have also tested a point-scanning confocal microscope with a 405 nm laser. We recommend consulting your microscopy core or representative to discuss the best solution at your institution.

Flat-top Thermocycler

The first few steps of the protocol require thermal cycling and incubations of tissue slides. For this, we recommend the Eppendorf® Mastercycler® nexus Flat Thermal Cyclers ([VWR Cat No. 71003-568](https://www.eppendorf.com/usa/products/thermal-cycling/mastercycler-nexus-flat-thermal-cyclers/)).

Protocol Overview

- 1 This protocol is for selective barcoding of cells seeded onto an Ibidi 18-well μ -slide for our Light-Seq paper.
<https://www.nature.com/articles/s41592-022-01604-1>

A separate protocol can be found for selective barcoding of tissue samples.

<https://www.protocols.io/view/light-seq-x54v9jno4g3e/v1>

The protocol will take roughly one week with some pause points. An example schedule can be:

Day 0: Cell seeding and chamber preparation

Cell seeding is an overnight process, should be performed the day before planned barcoding.

Chamber Preparation

An overnight process, should be performed in parallel with cell seeding

Day 1: Cell fixation, in situ reverse transcription, A-tailing

Cell fixation and permeabilization (10-20 minutes)

Reverse transcription (2.5 hours)

A-tailing (1 hour)

Day 2: Barcoding, cDNA displacement and Cross-junction synthesis

Barcoding (0.5 - 2 hours, depending on number of desired ROI's)

cDNA displacement (1 hour)
Cross-junction synthesis (1-2 hours)

Day 3: qPCR amplification

qPCR (5 hours)

Day 4: Library prep for NGS

Library Preparation for Next-Generation Sequencing (3 hours)

Cell line maintenance

2 For our cell mixing experiment, we used two different cell lines

- HEK-293-GFP (Human)
- 3T3 (Mouse)

Which must be cultured and maintained separately prior to a cell mixing experiment.

Cell lines

☒ HEK293 / GFP stable cells **Contributed by users Catalog #SC001**

☒ NIH/3T3 **ATCC Catalog #CRL-1658**

Reagents needed:

☒ DMEM, high glucose, GlutaMAX™ Supplement, pyruvate **Thermo Fisher Catalog #10569010**

☒ Penicillin Streptomycin **Invitrogen - Thermo Fisher Catalog #15140 122**

☒ Blasticidin S hydrochloride 10 mg/ml in HEPES buffer sterile-filtered **Thermo Scientific Catalog #J67216.XF**

☒ MEM Non-Essential Amino Acids Solution (100X) **Thermo Fisher Catalog #11140050**

☒ Calf Bovine Serum Iron Fortified **ATCC Catalog #30-2030**

☒ Fetal Bovine Serum, certified, heat inactivated, United States **Thermo Fisher Catalog #10082147**

2.1 HEK293-GFP Cell line growth media

DMEM, GlutaMax growth media supplemented with:

A	B
Reagent	Concentration
Fetal Bovine Serum (FBS)	10%
MEM non-essential amino acids	0.1 mM
Penicillin Streptomycin	1%
Blasticidin	10 µg/mL

The blasticidin is supplemented into the growth media at **10 µg/µL** in order to act as a selective marker for the eGFP expression cassette inserted into the HEK cells.

For growing the HEK-GFP cells after an initial thaw from a frozen aliquot, it is recommended to grow the cells in a flask with pre-warmed 37 C media **without** blasticidin for 1 night.

- The blasticidin can be too much of a stressor after the initial thaw and will stunt growth.

The following day, replace the media with pre-warmed 37C growth media **with** blasticidin.

2.2 3T3 Cell line growth media

DMEM, GlutaMax growth media supplemented with:

A	B
Reagent	Concentration
Calf Bovine Serum (BCS)	10%
Penicillin Streptomycin	1%

Note that the ATCC website recommends their 3T3 cells to be grown in Calf Bovine Serum rather than Fetal Bovine Serum. We have found that the cells do indeed grow far better in the calf serum than the FBS serum.

2.3 Cell splitting and subculture protocol

Our cell splitting and subculturing protocol generally follows the recommended ATCC protocols that are listed on their website for all their cell lines:

<https://www.atcc.org/products/crl-1658>

Reagents needed

TrypLE™ Express Thermo Fisher Scientific Catalog #12605010

DPBS with Ca and Mg Gibco - Thermo Fischer Catalog #14040-133

Cell Splitting

The following protocol assumes a standard T-75 corning flask for volumes.
Pre-warm all buffers to be used to 37C.

1. Aspirate out cell growth media with a pipette. Aspirate away from cells.
2. Wash cells with **10 mL of DPBS**.
3. Aspirate out the DPBS media
4. Add **2-3 mL of Trypsin** and coat the surface evenly.
5. Place flask in the 37C incubator for 5-10 min. Cells should detach in that time.
6. Add **7-8 mL of DMEM growth media** to inactivate the trypsin.
7. Pipette media up and down to de-clump cells. Wash the entire flask surface with media to get any cells that are still adhering to the flask.

Splitting and re-plate the cells.

To do a quick and dirty split without a cell counter, we typically dilute the cells to some volume in fresh media depending on how long we want to wait before the cells become confluent.

A rule of thumb that we use is:

1 mL in 10 ml (1:10) - 1-2 days
0.5 mL in 10 ml (1:20) - 3 days
0.4 mL in 10 ml (1:25) - 4 days

Incubate in a cell culture incubator at 37C and 5% CO₂

Preparing the ibidi Chamber (overnight)

- 3 While the cells are growing, it is good to start preparing the chamber the day before you plan to seed the cells.

The chamber itself will initially be coated with Poly-L-Lysine (PLL) from the vendor and it will be coated again with Poly-D-Lysine (PDL). We find that a double coating greatly improves cell adhesion, especially for HEK cells. This is important in order for the cells to be robust against the repeated washing steps with 60% formamide.

Reagents needed:

☒ u-Slide 18 well Poly-L-Lysine (Custom order) **Ibidi Catalog #81814**

☒ POLY-D-LYSINE HYDROBROMIDE MOL WT 70000 - 5MG **Sigma Aldrich Catalog #P6407-5MG**

☒ Pierce™ 20X Borate Buffer **Thermo Fisher Catalog #28341**

☒ DNase/RNase free distilled water **Invitrogen Catalog #10977023**

Prepare PDL aliquots

- Dissolve PDL to a working concentration of **1mM 0.3 mg/mL** in 2X Borate buffer (diluted in water)
- Store aliquots at **-20 °C**

Chamber Coating

All steps with the chamber open should be ideally performed in a sterile fume hood to prevent contamination during drying steps

1. Take a clean 18-well PLL chamber and pipette **100 µL** of the **PDL solution** into each well
2. Place the lid back on the chamber, cover and leave in **4C, overnight**.
3. In the hood, pipette off the PDL solution and dry in hood for **1 hour**.
4. Wash each well with UltraPure water (**100 µL**) X3.
5. Dry in hood for **1 hour**.

Note

Storing Chambers

After the final drying step, the chamber is ready for use. We typically use the chamber within a day of preparation. Some sites have suggested that a double-coated chamber can be stored for up to 2 weeks before use, however we have not tested this.

Cell Seeding

4 Cell seeding and co-culturing

The day before the barcoding experiment, we will then need to seed and co-culture both cell lines in a single ibidi well.

Co-culture growth media

DMEM, GlutaMax growth media supplemented with:

A	B
Reagent	Concentration
Calf Bovine Serum (BCS)	10%
MEM non-essential amino acids	0.1 mM
Penicillin Streptomycin	1%

The growth media here is a combination of the two different cell growth media.

We find that HEK can grow equally well in Calf Bovine Serum.

Blasticidin must be removed, since the 3T3 cells do not carry the resistance marker for it.

4.1 Detach cells from flask and re-suspend in 1 mL growth media

1. Aspirate out media with a pipette (2 mL glass) Aspirate away from cells.
2. Wash with 10 mL of DPBS (pre-warmed)
3. Aspirate out media (2 mL glass)
4. Add 2 mL of Trypsin, coat the well evenly. (5 mL pipette)
5. Place 2 min in incubator (**Minimize time in trypsin!, check if detached after 2 min**)
6. Add 8 mL of Co-culture media (w/ BCS), should inactivate rest of trypsin. (10 mL pipette)
7. Pipette media up and down, using the surface to remove adherent cells.
8. Put media in 10 mL tube and spin down for 5 min at 200 RCF.
9. Aspirate cell media and resuspend in 1 mL media.

Measure cells with a cell counter

This step is highly recommended in order to seed the cells to an optimal density in the wells.

1. Put 10 μ L of cell media with 10 μ L of dye for cell counter.

4.2 Seed Cells

Note

For a single well in a 18-well ibidi chamber, we find that seeding roughly 5000 3T3 cells and 4000 HEK cells together gives a good density of cells after an overnight growth.

We seed slightly more 3T3 cells because they tend to be larger than HEK and seeding more 3T3 gives better density of cells within a single field of view.

1. Pre-warm the co-culture growth media to **37C**.
2. Take the prepped 18-well ibidi chamber and add **80 µL** of pre-warmed media to all the wells that you want to seed the cells in.

1. 3. Using cell counter, seed with **10 µL** for **EACH** cell into the 18-well ibidis for a total of 100 µL.

If we want to seed 5k cells, then we need to dilute the cell culture to a density of 500 cells/µL

By pipetting a small volume of cells into a larger volume of media, we ensure a more even cell deposition.

4. Create a pre-mixture of both cell lines into a stock tube. ~200 µL.
5. Pipette **20 µL** into each well that requires the mixture. Pipette up and down to mix.
6. Grow overnight in 37C incubator.

3h

Fixation, Permeabilization, Reverse Transcription

5

Note

The protocol for *in situ* reverse transcription here is largely similar to what we did with the mouse retina tissue. There are some differences in the fixation and permeabilization steps for cell culture.

Reagents:

☒ PBS - Phosphate-Buffered Saline (10X) pH 7.4 **Thermo Fisher Scientific Catalog #AM9625**

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

☒ Tween 20 **Sigma Aldrich Catalog #P9416-50ML**

☒ Pierce™ 16% Formaldehyde (w/v), Methanol-free **Thermo Fisher Catalog #28908**

☒ Formamide (Deionized) **Thermo Fisher Catalog #AM9342**

☒ Sodium Chloride (5M) **Invitrogen - Thermo Fisher Catalog #AM9760G**

☒ DPBS with Ca and Mg **Gibco - Thermo Fischer Catalog #14040-133**

Reagents for Reverse Transcription

A	B	C
Reagents	Supplier	Cat. No.
Thermo Scientific™ Maxima H Minus Reverse Trans	Thermo Scientific	FEREP0753
Triton-X-100	Sigma Aldrich	T8787- 50ML
5M NaCl	Invitrogen	AM9760G
TWEEN® 20	Sigma Aldrich	P9416- 50ML
PBS	Invitrogen	AM9625
Formamide	Invitrogen	AM9342

A	B	C
UltraPure Water	Invitrogen	10977023
Deoxynucleotide (dNTP) Solution Mix - 8 µmol at 10	NEB	N0447S
RNaseOUT™ Recombinant Ribonuclease Inhibitor	Invitrogen	10777019

Reverse Transcription DNA Oligos

A	B	C	D	E
Name	Description	Sequence	Supplier	Purification
RT.5N.3G	RT Primer	TTTACACGATTGAGTTATNNNNNGGG	IDT	HPLC

Recommended to dilute this primer to a 10 µM stock.

Buffers

Fixation buffer (4% FA)

Prepare in fume hood if possible, formaldehyde is toxic.

- Take one ampule of 16% FA and dilute 1:4 in 1X PBS
- Store aliquots in -20C

Permeabilization buffer (0.25% Triton X-100)

4 mL 1X PBS

10 µL Triton X-100

1X PBS (40 mL)

4 mL 10X PBS

36 mL Ultrapure water

0.1% PBS-Tween (30 mL)

30 mL 1X PBS

30 µL Tween-20

Stringent Wash (0.1% PBS-Tw + 60% formamide):

3 mL 10X PBS

18 mL 100% formamide

9 mL UltraPure H₂O

30 µL Tween-20

Note: Store at 4C for use later during barcoding.

High-Salt Wash (1X PBS + 1 M NaCl + 0.1% Tween- 20):

4 mL 10X PBS

8 mL 5 M NaCl

28 mL UltraPure H₂O

40 µL Tween-20

Note: Store at room temperature for use later during barcoding.

10% Triton X-100 (100 µL):

10 μ L Triton X-100
90 μ L UltraPure water

Note: Mix very well by vortexing. Recommended to make this in a 2 mL tube for better mixing.

Reverse Transcription Master Mix (RTMM)

A	B	C
Reagent	Reaction concentration	μ L reagent per 50 μ L reaction
5X RT buffer	1x	10
10 mM dNTPs	300 μ M	1.5
UltraPure water		26
10% Triton X-100	0.5%	2.5
10 μ M RT.5N.3G primer	1 μ M	5
100 mM RnaseOUT	6 mM	3
Maxima RT H Minus (200 U/ μ L)	8 U/ μ L	2
Total		50

Note: Keep on ice. Leave out RnaseOUT and Maxima RT H Minus until final step.

5.1 Fixation and permeabilization

1. Remove ibidi chamber with cells from the incubator and carefully remove the cell media. Cells may not be super adherent prior to fixation.
2. Wash once with **pre-warmed** 37C DBPS.
3. Warm 4% FA to room temperature. Fix cells by adding **~75 μ L of 4% FA** into each well for 10 minutes at room temperature.
4. Wash wells 2x with **1X PBS**.
5. Permeabilize cells with **0.25% Triton** for 10 minutes at room temperature.
6. Wash wells 2x with **1X PBS**.
7. Immediately move to reverse transcription.

5.2 *In situ* Reverse transcription.

2h 30m

Add enzymes (Maxima RT H minus and RNase Out) to **Reverse Transcription Master Mix (RTMM)** and mix well.

1. After washing wells with 1XPBS, add **50 μ L** of RTMM to each well.
2. Place slide on a flat-top thermocycler.

Reverse Transcription Thermocycler Program

12-cycle ramp program (Lid: 60°C):

Phase 1:

22°C 30 mins

Phase 2 (12 cycles):

8°C 30 s
15°C 30 s
25°C 30 s
30°C 1 min
37°C 1 min
42°C 2 min

Phase 3:

42°C 30 min
4°C Forever

3. Wash each well **3 x 5 min in Stringent Wash.**
4. Wash each well **2 x 2 min in High Salt Wash.**
5. Wash each well **2 x 2 min in 0.1% PBS-Tw.**
6. Resuspend in **0.1% PBS-Tw.**

Optional Pause Point: Store at 4°C overnight before A-tailing.

45m

A-Tailing

6

Note

The A-tailing protocol for the cell-mixing is overall very similar to the one used for the retina barcoding protocol. The volumes of reagents used here will be reflective of the smaller 18-well ibidi chamber instead of the larger 8-well used for the retina barcoding protocol.

Reagents needed:

- ☒ ThermoPol Reaction Buffer Pack - 6.0 ml **New England Biolabs Catalog #B9004S**
- ☒ 2'3'-Dideoxyadenosine 5'-Triphosphate 100 mM solution **Sigma Aldrich Catalog #GE27-2051-01**
- ☒ Deoxynucleotide Solution Set - 25 umol of each **New England Biolabs Catalog #N0446S**

We are only using the dATP from the dNTP set

- ☒ Terminal Transferase - 2,500 units **New England Biolabs Catalog #M0315L**

- ☒ PBS - Phosphate-Buffered Saline (10X) pH 7.4 **Thermo Fisher Scientific Catalog #AM9625**

- ☒ Tween 20 **Sigma Aldrich Catalog #P9416-50ML**

Prepare Aliquots

1. Create aliquots of **10 mM dATP** (20 µL aliquots) and **25 mM ddATP** (~3 µL aliquots). Store at -20°C.
Both reagents are supplied in ultrapure water.
2. Dilute **25 mM ddATP** stock further to 250 µM (1:100 dilution from 25 mM stock).

Buffers

A-tailing master mix

Barcoding Reagents

A	B	C
Reagents	Supplier	Cat. No.
Dextran Sulfate 50% Solution (NOTE: Was not used in this protocol)	Sigma Aldrich	S4030
Salmon-sperm DNA (NOTE: Was not used in this protocol)	Thermo Fisher	AM9680
5 M NaCl	Invitrogen	AM9760G
TWEEN® 20	Sigma Aldrich	P9416- 50ML
PBS	Invitrogen	AM9625
Formamide	Invitrogen	AM9342
UltraPure Water	Invitrogen	10977023
Barcode DNA Oligos (see sequences table)	GeneLink	

From the buffers we prepared in **Step 5**. We will also need.

1X PBS (40 mL)

4 mL 10X PBS

36 mL Ultrapure water

0.1% PBS-Tween (30 mL)

30 mL 1X PBS

30 µL Tween-20

Stringent Wash (0.1% PBS-Tw + 60% formamide):

3 mL 10X PBS

18 mL 100% formamide

9 mL UltraPure H₂O

30 µL Tween-20

Note: Store at 4°C for use later during barcoding.

High-Salt Wash (1X PBS + 1 M NaCl + 0.1% Tween- 20):

4 mL 10X PBS

8 mL 5 M NaCl

28 mL UltraPure H₂O

40 µL Tween-20

Note: Store at room temperature for use later during barcoding.

Barcode Hybridization Solution (See note at top of section)

A	B	C
Reagent	Reaction concentration	µL per 50 µL w
10X PBS	1x	5
5M NaCl	500 mM	5
UltraPure water		38.7
TWEEN® 20	0.1%	0.05

A	B	C
Total		48.75

**Dextran sulfate makes this mix viscous, so it is recommended to make significant excess (~1.2X what is needed for the number of wells).

Barcoding Solution (250 nM final concentration)

Barcode 1 Solution (per 50 μ L well - make excess!):

1.25 μ L of 10 μ M Barcode Strand (GATE.D12.B1)

48.75 μ L hybridization master mix

Barcode 2 Solution (per 50 μ L well - make excess!):

1.25 μ L of 10 μ M Barcode Strand (GATE.D12.B2)

48.75 μ L hybridization master mix

7.1 Barcoding Workflow

1. Wash **1x with High Salt Wash**.
2. Aspirate out High-salt and add **50 μ L of Barcode 1 solution**.
Incubate for **30 minutes** at room temperature. Cover from light.
3. Aspirate to remove all liquid, then wash **3 x 1 min in High-Salt Wash**.
This removes most of the un-hybridized barcoding strands.
The barcoding strand has enough base-pairing to be relatively stable in high-salt buffer.
4. Sample is now ready to be barcoded, bring to instrument and draw ROI's around desired regions.
See supplemental in publication for proper alignment of the photomask.
5. Wash wells **2x with Stringent Wash**. Let sit in stringent wash for 2 minutes on last wash.
Repeat this step three more times, for a total of $4 \times 2 = 8$ washes.
6. Wash **2 x 2 min in High-Salt Wash**, then add fresh High-Salt Wash.
7. **Repeat** steps 1-6 for **Barcode 2**.

Transfer to 0.1% PBS-Tween and optionally image barcoded cDNAs to visualize.

Optional Pause Point: Store at 4C overnight in a humidified chamber.

Displacement of cDNAs and cross-junction synthesis

- 8 Now that cDNAs are barcoded and A-tailed, they must be extracted for library preparation and sequencing. To do this, the RNA template is digested by mild RNaseH treatment, which degrades the RNA bound to the barcoded cDNAs in situ, liberating the barcoded cDNAs for collection.

The workflow for cDNA displacement is largely similar to the one we used for the retina tissue.

Note: Use low retention tips.

Displacement Reagents

A	B	C
Reagents	Supplier	Cat. No.
ThermoPol® Reaction Buffer Pack	NEB	B9004S
RNase H - 1,250 units	NEB	M0297L
UltraPure Water	Invitrogen	10977023

Cross-Junction Synthesis Reagents

A	B	C
Reagents	Supplier	Cat. No.
Bst DNA Polymerase, Large Fragment- 8,000 units	NEB	M0275L
ThermoPol® Reaction Buffer Pack	NEB	B9004S
UltraPure Water	Invitrogen	10977023
Deoxynucleotide (dNTP) Solution Mix - 8 µmol at 10mM ea	NEB	N0447S

Cross-Junction Synthesis Primer

A	B	C	D	E
Name	Description	Sequence	Supplier	Purification
GATC.20T.p	Primer for Cros	GAGAATGTGAGTGAAGATGT	IDT	HPLC

Buffers

Displacement Mix

A	B	C
Reagent	Reaction concentration	µL reagent per 50 µL
10x ThermoPol Reaction Buffer	1x	5 µL
RNase H (from NEB) (5,000U/mL)	250 U/mL	2.5 µL
UltraPure Water		42.5 µL
Total		50

8.1 Displacement Workflow

Note

NOTE: The eluate must be collected in this step!

At this point we must collect the generated cDNA library after displacement with RNAase H treatment.

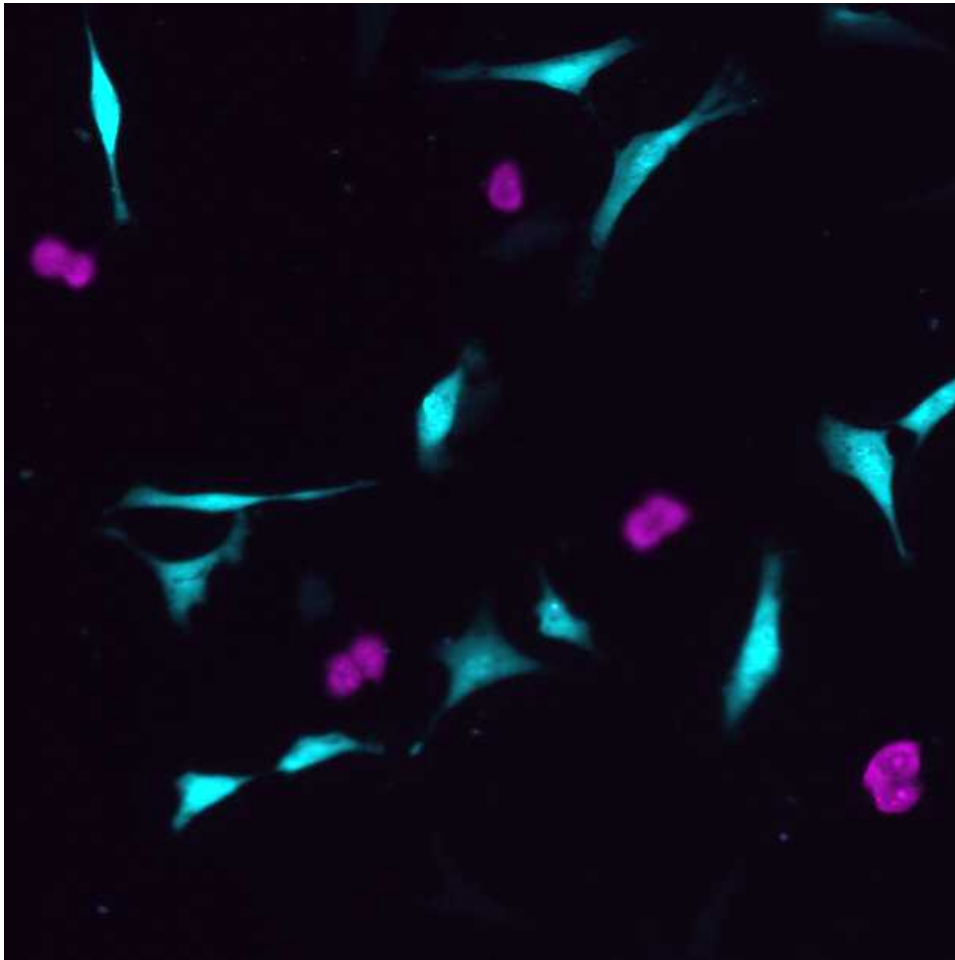
As a general rule, every step should use low-retention tips and lo-bind tubes, and care should be taken to minimize the number of pipetting steps.

Because the cDNA library has not been PCR amplified at this stage, any pipetting error, or contaminating samples will be amplified for all downstream steps.

1. Aspirate out buffer from previous step and add **67.5 μ L of Displacement Mix** to each well.
Incubate on flat-top incubator for **37C for 30 minutes**.
2. Prepare a set of lo-bind PCR tubes that will collect the eluate from each well by pipetting **1.6 μ L of 1 μ M GATC.20T.p** cross-junction synthesis primer into each tube.
Pre-pipetting the primer into the tube will minimize the number of pipette steps the cDNA library is subjected to. For example, adding the primer after collection will introduce another pipette tip into the solution which some cDNA's may stick to.
3. Prepare a set of **20 nM GATC.20T.p** primer solution. Take a low-retention tip and pipette this solution up and down and three times to "pre-block" it with the primer DNA.
Pipetting this solution with a tip helps to block the tip with some DNA to prevent any cDNA from sticking to the tip surface.
Take care not to generate bubbles during this step.
4. With **the same empty tip** from step 3, move to a well and carefully pipette the solution in the well up and down three times.
The well should still be in displacement mix along with the now displaced cDNA's. It is important to not over-pipette or introduce bubbles into the solution at this stage.
The mild agitation we introduce with pipetting up and down should help resuspend the cDNA library.
5. **Collect** the eluate and place in a collection tube that contains the **GATC.20T.p** primer. Pipette up and down a few times to mix the primer.
6. Repeat steps 1-5 with a fresh tip for all wells.
7. Heat inactivate the collection tubes in **75C for 20 min**.
8. Re-hydrate the chamber wells and wash wells **3 times with 0.1% PBS-Tw**.
The chamber containing the samples can be stored for follow-up immunostains.
9. Move on to **Cross-junction synthesis**.

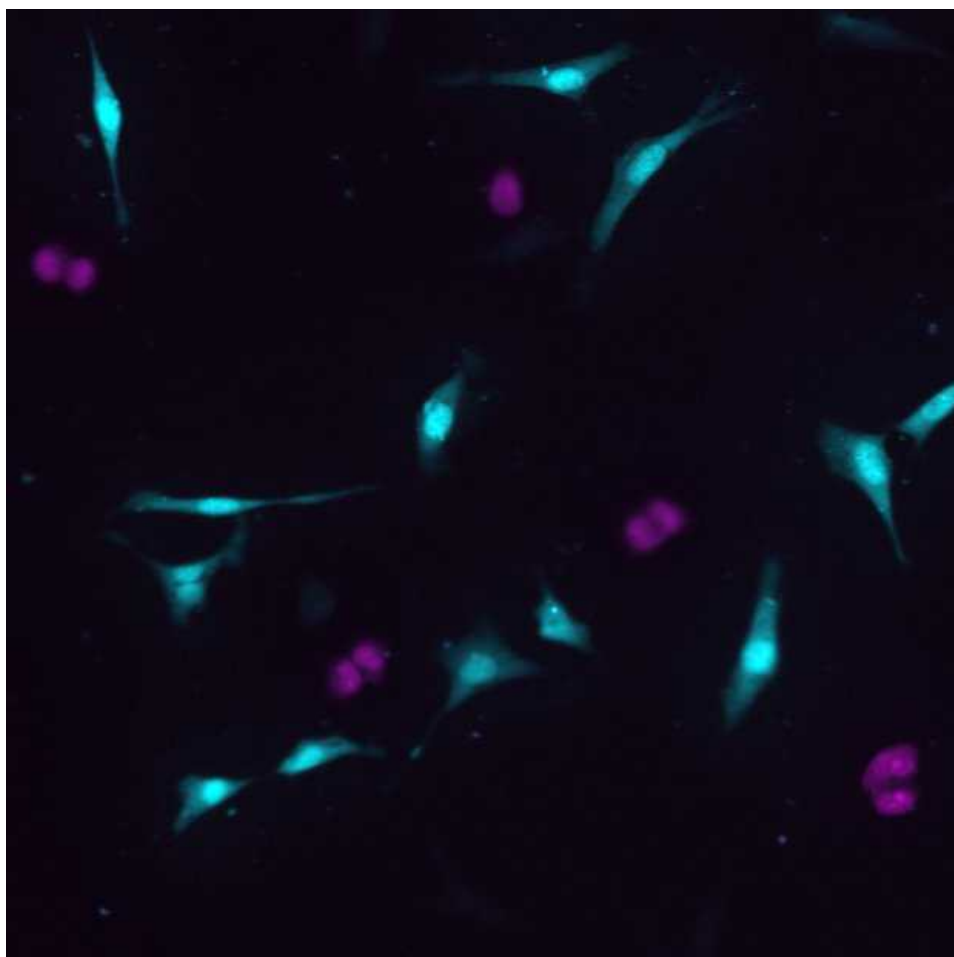
Optional: Samples can be imaged after displacement to verify that the fluorescent barcode signal is no longer present. At this point, follow-up stains can be performed on the sample including antibody stains, H&E, etc.

Barcoded sample



Barcoded HEK (magenta) and 3T3 cells (mouse)

Displaced samples



Fluorescent image of same cells after displacement of cDNA's. Note that most of the nuclear signal remained while the cytoplasmic signal decreased. Thus the collected cDNA library is mostly cytoplasmic RNA.

8.2 Cross-junction synthesis

1. Add 10.9 μL **Cross-Junction Synthesis Mix** to each tube:

Cross-Junction Synthesis Mix

A	B	C
Reagent	Final Reaction c	μL reagent per
10X ThermoPol buffer	1x	1.25
10mM dNTPs	100 μM	0.8
Ultrapure water		0.85
Bst LF polymerase (8,000U/mL)	800U/mL	8
Total		10.9

Note: These volumes are for 80 μL wells, and volumes should be scaled according to well volume. Note

that the primer (GATC.20T.p) was added in the previous step, before heat inactivation.

2. Vortex and spin briefly.
3. Incubate in a thermocycler for:
37C for 30 mins
80C for 20 mins (heat inactivation)
4. The resulting products are **CJS Samples**. Proceed to qPCR to amplify **CJS Samples**.

Optional Pause Point : Store at -20C overnight.

qPCR Amplification

9

Note

The qPCR protocol for the cell mixing is largely similar to the one for the retina tissue.

Cross-Junction Synthesis libraries of barcoded cDNAs are now amplified and prepared for sequencing. We tested both the Kapa Hifi HotStart and Taq polymerase for this step and saw similar results for both. Only the Kapa Hifi results were published in the paper.

We recommend performing qPCR with 30 cycles on a small subset of each sample (5 µL sample in a 10 µL PCR), to identify the appropriate cycle number where the yield displays an inflection point for optimal amplification.

qPCR Primers

A	B	C	D	E
Name	Description	Sequence	Supplier	Purification
GATE	PCR Primer 1	GGAGTTGGAGTGAGTGGATG	IDT	HPLC
GATC	PCR Primer 2	GAGAATGTGAGTGAAGATGT	IDT	HPLC

Reagents for qPCR

A	B	C
Reagents	Supplier	Cat. No.
SYBR™ Green I Nucleic Acid Gel Stain - 10,000X conc	Invitrogen	S7563
HiFi HotStart DNA Polymerase, KapaBiosystems	Roche	KK2502
UltraPure Water	Invitrogen	10977023

qPCR to Determine Amplification Cycle Number

1. Vortex **CJS Samples**.

2. Dilute Sybr Green I dye in water to make 5x solution (e.g. dilute 10,000X stock twice, 1:100 in water, then 1:20 in water).
3. Create PCR master mix:

A	B
Kapa qPCR Master Mix	
Reagent	μL reagent per
5x SYBR Green I	1
Kapa HiFi HotStart buffer (5x)	2
10 μM GATE primer	0.3
10 μM GATC primer	0.3
10 mM dNTPs from Kapa kit	0.3
UltraPure water	0.9
Kapa HiFi Hot Start polymerase	0.2
Total	5

The ratio of Kapa qPCR Master Mix to Cross-Junction Synthesis Solution is 1:1, so each sample will have a 10 μL total PCR with 5 μL of Kapa qPCR Master Mix and 5 μL of Cross-Junction Synthesis Solution.

4. Quickly vortex and spin down master mix, then add **5 μL** master mix into each tube.
5. Add **5 μL** of appropriate sample from Cross-Junction Synthesis reaction to each tube.
6. Quickly vortex and spin down reactions.
7. Place into qPCR machine for 30 cycles and run the **qPCR Machine Protocol**.

qPCR Machine Protocol

98C for 3 minutes

30 cycles of:

98C for 20 seconds

60C for 30 seconds

72C for 2 minutes

Plate read

72C for 5 minutes

Melting curve analysis

Hold at 4C

8. Check amplification graph to choose appropriate cycle number.

Note

NOTE: Cycle number for amplification of the remaining Cross-Junction Synthesis Product should be chosen to prevent over-amplification of the library. In general, the optimal cycle number will correlate with the size of the barcoded region, which directly impacts how much amplifiable cDNA exists within the reaction. Note that for technical replicates, the amplification curves are generally very consistent. In general, optimal cycle number will vary across experiments and we recommend that the full PCR is run at the optimal cycle number per experiment.

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After the test qPCR on a small amount of sample, a PCR is performed on all of the **CJS Samples** to amplify the entire sample for library preparation and sequencing. The cycle number for amplification is chosen based on the test qPCR in the previous step, to prevent over-amplification. Each Cross-Junction Synthesis product (1 per sample well) is amplified in a separate tube. This protocol is identical to the prior qPCR, but scaled to amplify the entire sample and with a reduced number of cycles.

All of the sample should be amplified. However, some qPCR machines have a limit of 50 µL/reaction. Therefore, the full volume should be split into multiple PCRs to ensure accurate amplification.

Amplification of Full Samples

1. Briefly vortex and spin **CJS Samples**.

2. Measure the volume of CJS sample in each tube and use to calculate volume of qPCR master mix needed for each sample (**1:1 ratio of qPCR master mix : CJS sample**).

Note: Ensure that PCR volumes do not exceed the limits for your machine and otherwise, split into multiple tubes.

3. Dilute Sybr Green I dye in water to make 5x solution (e.g. dilute 10,000X stock twice, 1:100 in water, then 1:20 in water).

4. **Create qPCR master mix:**

A	B
Kapa Full qPCR Master Mix	
Reagent	µL reagent per
5x SYBR Green I	10
Kapa HiFi HotStart buffer (5x)	20
10 µM GATE primer	3
10 µM GATC primer	3
10 mM dNTPs from Kapa kit	3
Ultrapure water	9
Kapa HiFi hot start polymerase	2
Total	50

5. Add the entire remaining sample with equal parts master mix to each tube.

6. Quickly vortex and spin down reactions.

7. Place into qPCR machine for exactly XX cycles and run the **Full qPCR Machine Thermocycler Program**.

Full qPCR Machine Thermocycler Program

98C for 3 minutes

XX cycles of:

98C for 20 seconds

60C for 30 seconds

72C for 2 minutes

Plate read
72C for 5 minutes
Hold at 4C

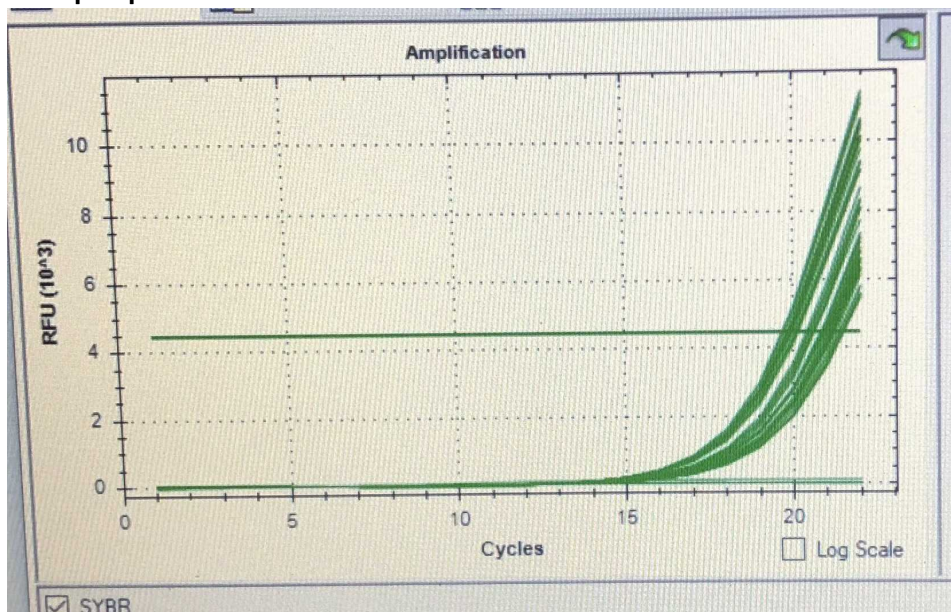
***XX is based on the amplification curves from the test qPCR. This number will likely range between 16 and 25.**

8. The resulting products are **Amplified Samples**. Store at -20C.

Ideally the amplification will stop right at the exponential phase of the signal.

An example is shown in a phone screenshot below where the amplification is stopped at cycle 22. Past this cycle would be the inflection point and the signal increase will start to slow and eventually plateau. There is generally little benefit to amplifying the library past this point and can also lead to over-amplification of the library.

Example qPCR curve



Note

As a general rule, we like to keep amplified libraries separate from non-amplified libraries. In our case, separate -20 freezers for amplified and non-amplified samples.

Library Preparation for Illumina Sequencing

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Light-Seq uses conventional tagmentation-based library preparation for Illumina sequencing, but with custom primers for the secondary PCR and for Read 1/i5 sequencing. The custom primers are necessary to specifically enrich for and sequence amplicons containing the light-directed barcode sequences. The first step is bead purification of the amplified libraries for each well, followed by tagmentation, secondary PCR amplification, and a second bead purification.

We generally like to use the magnetic stand rack from permagen

Equipment	
0.2 mL PCR Strip Magnetic Stand	NAME
Permagen	BRAND
MSR812	SKU
https://www.permagenlabware.com/0-2-ml-pcr-strip-magnetic-separator-rack/	LINK

Library Preparation Reagents

A	B	C
Reagents	Supplier	Cat. No.
Magnetic Separator (or equivalent)	Permagen	MSR812
Ampure XP Beads	Beckman	A63881
Ethyl alcohol, pure (200 proof)	Sigma Aldrich	E7023-1L
Qubit™ 1X dsDNA High Sensitivity (HS) and Broad Range (Invitrogen	Q33231
Nextera XT Library Preparation Kit	Illumina	FC-131-1096
UltraPure Water	Invitrogen	10977023

Library Preparation DNA Oligos

These primer sequences are used for unique indexing of samples for pooled sequencing. For each sample, a unique pair of S50X and Next.N70X are required. You do not need to order all primers, only enough pairs to uniquely index your samples of interest. Because the custom i5 index primer does not work well on all Illumina machines, we highly recommend each sample be prepared with a unique i7 index. We hope to adjust our recommendations on this front soon, so stay tuned for updated protocols.

The full table of index primers used can be found in the supplement of our publication

<https://www.nature.com/articles/s41592-022-01604-1>

as well as the partner protocols.io for the retina

<https://www.protocols.io/view/light-seq-x54v9jno4g3e/v1?step=12>

Bead purification of Amplified Samples

NOTE: Each well/replicate is processed separately.

The ampure bead purification is based off of the ratio of sample volume to beads. The ampure bead solution contains PEG. The premise is that the PEG will act as a crowding agent to induce DNA binding to the bead surface, and the concentration of PEG can be adjusted to size select for the desired DNA size. We use a **1.2x** ratio for bead purification which should get rid of most DNA <100 bp, <100bp should get rid of most primer dimers and fragmented DNA.

1. Make **50 mL** of 80% Ethanol, diluted in UltraPure water. (Over time the concentration will slowly decrease but even a 70% Ethanol mixture will also work).
2. For each well, combine **80 µL Amplified Sample** with **96 µL Ampure XP Beads** in a new PCR tube. **1.2x ratio**.
3. Mix well by pipetting (incubate at room temperature for **5 min**). DO NOT overextend this step.
4. Place on Magnetic Separator Stand for **2 min**.
5. Discard supernatant. Wash **3 x 30 sec** with **200 µL of 80% Ethanol**.
6. Discard ethanol and aspirate remainder with a small 10 µL tip.
7. Air dry for **~5 min**, until beads are dry. Beads will change color slightly when dry. Try not to overdry the beads, otherwise they will crack.
8. Add **30 µL of Ultrapure water** to resuspend the beads.
9. Remove from the magnetic stand. Mix well by pipetting or vortexing and incubate for 2 min at room temperature.
10. Place Magnetic Separator Stand for 1 min. **Collect supernatants** in new PCR tubes. These are **Purified Amplified Samples**.

Measure DNA concentrations of Amplified Samples with Qubit HS dsDNA assay.

It is *critical* that an accurate **dsDNA** concentration is obtained for the next step. A nanodrop is *not* sufficient for this purpose. Therefore a Qubit high sensitivity dsDNA assay must be performed on all samples.

This should be done according to the manufacturer's instructions, found here:

https://assets.fishersci.com/TFS-Assets/LSG/manuals/MAN0017455_Qubit_1X_dsDNA_HS_Assay_Kit_UG.pdf.

1. Measure concentrations using Qubit Fluorometer.
2. Record concentrations of each **Purified Amplified Sample**.

Perform Nextera tagmentation

Reagents in this section are from the Nextera XT Library Preparation Kit (Illumina cat. no. FC-131-1096).

Tagmentation will fragment the amplified PCR products into shorter sequences, and the length of the reaction time determines how much fragmentation occurs. Therefore, it is very important to follow the times strictly.

1. In a clean set of PCR tubes, aliquot **7 µL of Neutralize Tagment Buffer (NT)**. This will be used to stop the tagmentation and prevent over-tagmentation. One tube per sample/well is required. Set aside, next to the thermocycler.
2. To a new PCR tube for each **Purified Amplified Sample** (on ice):
 - a. Add **10 µL of Tagment DNA buffer (TD)**
 - b. Add **2 ng** of purified **Purified Amplified Samples** and add water up to **5 µL**. Calculated sample and water volume to add based on the Qubit concentrations from previous step.
 - c. Add **5 µL of Amplicon Tagment Mix (ATM)** to lid of tube. This mixture contains the tagmentation enzyme. We will spin the tube down to add all enzymes at the same time.
3. Briefly spin down tubes, vortex, and spin down. The tagmentation enzyme has now been added.
4. Place into PCR machine and incubate at **55°C for EXACTLY 5 minutes**.

5. Immediately stop reactions with **5 µL of Neutralize Tagment buffer (NT)**.
(Use a multichannel to pipette the buffer from the previously aliquoted PCR tubes in step 1 to mix quickly).
6. Vortex and spin to ensure full mixing. Hold **Tagmented Samples** on ice.

Sample indexing PCR with unique i5 and i7 primer pairs

To pool **Tagmented Samples** from each well/replicate for sequencing, each sample is assigned a unique pair of i7 and i5 primer sequences. The primer pairings should be decided and documented. Each **Tagmented Sample** will be amplified briefly in this step to attach the sample-specific i5 and i7 sequences.

1. Assign and record unique index primer pairs to each sample. For example:

A	B	C
Sample	i5 primer	i7 primer
Tagmented Sample 1 (Well 1)	S502.GATE	Next.N701
Tagmented Sample 2 (Well 2)	S503.GATE	Next.N702
Tagmented Sample 3 (Well 3)	S505.GATE	Next.N703
Tagmented Sample 4 (Well 4)	S506.GATE	Next.N704

Each sample will receive a different pair of primers in the indexing PCR.

2. To each **Tagmented Sample** tube from the previous tagmentation step:
 - a. Add 6.5 µL water
 - b. Add 1.75 µL of standard Nextera (i7: **Next.N70X**) primer (from 10 uM stock)
 - c. Add 1.75 µL of custom Nextera (GATE, i5: **S50X.GATE**) primer (from 10 uM stock)
 - d. Add 15 µL PCR mix (**NPM PCR master mix**)
3. Vortex and spin down reactions.
4. Place in thermocycler and start **Indexing PCR Program**.

Indexing PCR Program

72°C for 3 minutes
95°C for 15 seconds

12 cycles of:

95°C for 15 seconds
55°C for 15 seconds
72°C for 40 seconds
72°C for 1 minute
Hold at 10°C

5. The resulting tubes are the **Indexed Samples**.

Purify indexed samples with Ampure Beads

The final bead purification will remove primers and other reagents from the sample prior to sending off for NGS. We now use a ratio of **0.9x** as the indexed samples of interest should be a bit longer now.

- Make **50 mL** of 80% Ethanol, diluted in UltraPure water.
- For each well, combine **50 µL** of **Indexed Sample** and mix with **45 µL Ampure XP Beads** in a new PCR tube.
- Mix well by pipetting (incubate at room temperature for **5 min**). DO NOT overextend this step.
- Place on Magnetic Separator Stand for **2 min**.

- Discard supernatant. Wash **3 x 30 sec** with **200 µL of 80% Ethanol**.
- Discard ethanol and aspirate remainder with a small 10 µL tip.
- Air dry for **~5 min**, until beads are dry. Beads will change color slightly when dry.
- Add **30 µL** of water to resuspend the beads.
- Remove from the magnetic stand. Mix well by pipetting or vortexing and incubate for **2 min** at room temperature.
- Place on Magnetic Separator Stand for **1 min**.
- Collect supernatants in new tubes. These are the **Purified Indexed Samples**.

Measure DNA concentrations and length of Indexed Samples

1. Measure concentrations using Qubit HS dsDNA assay. This should be done according to the manufacturer's instructions, found here: https://assets.fishersci.com/TFS-Assets/LSG/manuals/MAN0017455_Qubit_1X_dsDNA_HS_Assay_Kit_UG.pdf.
2. Record concentrations of each **Purified Indexed Sample**.
3. Run 2 µL of each **Purified Indexed Sample** on a 1% agarose gel.

Sequencing

- 12** This library preparation is compatible with standard Illumina next-generation sequencing. Note that custom Read 1 and i5 index primers are required and are listed in the table below.

DNA Oligos for Sequencing

A	B	C	D
Description	Sequence	Supplier	Purification
Custom Read 1 Primer -required for	CGCCGGAGTT	IDT	HPLC
Custom i5 index primer -required for	CATCACTCAT	IDT	HPLC

We note that the custom index primer is compatible with HiSeq, but for NovaSeq, unique i7 indices were needed for de-convolution due to inefficient i5 index sequencing.

We have found adding 60% above the standard concentration of each custom primer works well on many of the Illumina sequencers and would recommend this as a starting point.

Pooling samples and prepping for sequencing.

When preparing a pooled sample for sequencing, the total amount of DNA should not exceed the recommended amount for the NGS platform you are using. For example MiSeq and other instruments typically recommend **4 nM** of DNA.

Below is an example prep we used for a MiSeq machine with a 30% PhiX spike in.

1. Prepare freshly diluted **0.2N NaOH**.
2. Dilute **PhiX** to 4nM: 1 µL 10nM PhiX stock + 1.5 µL water
3. Prepare a pooled sample at 4 nM concentration. The volume needed should be calculated based on the final Qubit reading and the size of the amplicon from the gel.
4. Mix 1.5 µL 4nM PhiX + 3.5 uL 4nM pool (30% spike in)

5. Add **5 µL diluted NaOH**, incubate for **5 minutes**.
6. Add **1,657 µL Hyb buffer (HT1)**, vortex to make 12 pM. (HT1 will come with the MiSeq kit).
7. Add **600 µL** of this sample to **Well 17**, tape over to avoid dust. This is the sample well.
8. Add **3.2 µL P5.GATE.p (Custom Read 1 Primer)** into **reservoir 12** (use syringe to pull out, spike primer, mix well, replace)

We have had good success with elevated custom primer concentrations on Illumina MiSeq and NextSeq machines. The standard custom primer concentrations worked for HiSeq. For NovaSeq, we haven't seen as good efficiency even with the elevated custom primer concentrations, particularly for the custom i5 index sequencing but hope to have updated recommendations soon. For now, we recommend having unique i7 index sequences for each sample to aid in sequence de-multiplexing, and that you check back here periodically or reach out for updated sequencer-specific recommendations.

Immunostaining

- 13** A final immunostaining step can be performed on the cells.

All antibodies were spun down at 10,000 g for 10 min at 4 °C before use.

Primary antibody mix (1x)

- (150 µL) 1x PBS, 0.3% Triton X-100, 5% BSA mixture
- (2 µL) Lamin B goat primary
- (2 µL) TFAM mouse primary
- (2 µL) alpha-tubulin rat primary

Secondary antibody mix (1x)

- (150 µL) 1x PBS, 0.3% Triton X-100, 5% BSA mixture
- (1 µL) secondary alexa 647 anti-mouse
- (1 µL) secondary Cy3b anti-goat
- (1 µL) secondary alexa 488 anti-rat

1. Aspirate, add **primary antibody solution** and incubate at **Room temp** for **1 hour**.
2. Wash 2 x 1 minute with 1xPBS
3. Aspirate, add **secondary antibody solution** and incubate at **Room temp** for **1 hour**.
4. Wash 2 x 1 minute with 1xPBS
5. Aspirate, add **DAPI solution** and incubate at **Room temp** for **5 minutes**.
6. Wash 2 x 1 minute with 1xPBS and resuspend in fresh 1xPBS
7. Image