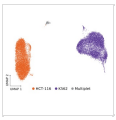

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Working

Single Cell Calling Cards Library Preparation

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Transposon Calling Cards

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ABSTRACT

This protocol describes how to create calling card libraries from single cell RNA. We assume you have successfully transformed cells with *piggyBac* self-reporting transposons and either undirected *piggyBac* transposase or your favorite transcription factor (YFTF) fused to *piggyBac*. We also assume you have optimized the dissociation protocol for your specific cells or tissues and can generate single cell suspensions.

EXTERNAL LINK

https://doi.org/10.1101/538553

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Self-reporting transposons enable simultaneous readout of gene expression and transcription factor binding in single cells

PROTOCOL STATUS

Working

We use this protocol in our group and it is working

GUIDELINES

Please read this protocol in its entirety before starting. For several steps, it may help to pre-program your thermocycler or heat block with the listed settings. While single cell calling card (scCC) libraries can, in principle, be generated from any poly(A)-based scRNA-seq method, this protocol specifically describes how to proceed from 10x Chromium 3' scRNA-seq libraries. Please obtain all additional kits, reagents, and equipment as specified in the 10x Chromium Single Cell 3' User Guide.

MATERIALS

NAME	CATALOG #	VENDOR
dNTP	639125	Takara
2x Kapa HiFi Hotstart Readymix	KK2602	Kapa Biosystems
Dynabeads M-280 Streptavidin	11205D	Thermo Fisher Scientific
Maxima RT 5X Buffer	Provided with EP0752	Thermo Fisher Scientific
Maxima H Minus Reverse Transcriptase (200 U/uL)	EP0752	Thermo Fisher Scientific
High Sensitivity D5000 ScreenTape	5067-5592	Agilent Technologies
High Sensitivity D5000 Reagents	5067-5593	Agilent Technologies
RNaseOUT™ Recombinant Ribonuclease Inhibitor	10777019	Thermo Fisher Scientific
High Sensitivity D1000 Reagents	5067-5585	Agilent Technologies
High Sensitivity D1000 ScreenTape	5067-5584	Agilent Technologies
Chromium Single Cell 3' Library & Gel Bead Kit v2	120267	10x Genomics

NAME	CATALOG #	VENDOR
Chromium Single Cell A Chip Kit	1000009	10x Genomics
Chromium i7 Multiplex Kit	120262	10x Genomics
Nextera Mate Pair Library Prep Kit	FC-132-1001	Illumina, Inc.
1.7 ml Axygen Maxymum Recovery Microcentrifuge Tubes	MCT-175-L-C	Axygen
Covaris T6 (6 x 32 mm) glass tubes	520031	Covaris
Covaris Snap-Cap - Teflon Silicone Septa 8 mm	520042	
Ficoll PM-400	17030010	Ge Healthcare
NEBuffer 2 (10X)	B7002S	New England Biolabs

MATERIALS TEXT

Primers

All primers should be resuspended in Low TE Buffer (10 mM Tris-HCl ph 8.0, 0.1 mM EDTA) at a final concentration of 100 µM and stored at –20ºC.

The following primers should be purified by HPLC and stored as 5 µl aliquots:

>Bio_Illumina_Seq1_scCC_10X_3xPT
/5Phos/ACACTCTTTCCC/iBiodT/ACACGACGCTCTTCCGA*T*C*T

>Bio_Long_PB_LTR_3xPT
/5Phos/GCGTCAATTTTACGCAGAC/iBiodT/ATCTTTC*T*A*G

These primers should be purified by PAGE:

>scCC_PB_CustomRead2
CGTGTAGGGAAAGAGTGTGCGTCAATTTTACGCAGACTATCTTTCTAG

>scCC_CustomIndex1
GAGACTGGCAAGTACACGTCGCACTCACCATGA

These primers can be purified by standard desalting:

>scCC_P5_adapter
AATGATACGGCGACCACCGAGATCTTCACTCATTCCACACGACTCCTTGCCAGTCTC*T

>scCC_P7_adapter
/5Phos/GAGACTGGCAAGTACACGTCGCACTCACCATGA[index]ATCTCGTATGCCGTCTTCTGCTTG

Note that you can replace [index] with an 8-10 bp sequence for multiplexing samples. For more guidance, consult the Illumina Adapter Sequences Document.

>scCC_P5_primer
AATGATACGGCGACCACCGAGATC

>scCC_P7_primer
CAAGCAGAAGACGGCATACGAGAT

>10x_TSO
AAGCAGTGGTATCAACGCAGAGTACATrGrGrG

Equipment

- 10x Chromium Controller

- Thermocycler for PCR
- Heat blocks or programmable thermoshaker
- Covaris AFA Ultrasonicator, model S2, S220, or E220.

Other reagents

- Consult the 10x Chromium Single Cell 3' User Guide for scRNA-specific consumables
- Ethanol (96-100%)
- ddH_2O

SAFETY WARNINGS

BEFORE STARTING

The components in this protocol are sensitive to repeated freeze-thaw cycles, specifically the modified primers for amplifying self-reporting transcripts and the components of the Nextera Mate Pair Library Prep kit. We recommend pipetting the primers (at 100 µM) and kit buffers (CB: Circularization Buffer 10X; ERP3: End Repair Mix; ATL2: A-Tailing Mix; LIG2: Ligation Mix; STL: Stop Ligation Buffer; EPM: Enhanced PCR Mix) into five-use aliquots and storing at –20ºC until needed. Sterilize the Axygen 1.7 ml tubes in an autoclave.

In bulk calling cards, we recommend collecting 8-12 independent biological replicates to ensure sufficient statistical power for identifying true binding sites. This is not necessary in single cell calling cards, where each cell is barcoded and is thus considered an independent replicate.

This protocol is meant to describe how we prepare calling card libraries. While it is possible that another kit or component could equally suffice, we have not tested any substitutions and do not officially support deviations from this protocol. This document enumerates what we have had success with and is a starting point from which we can best help troubleshoot.

Single Cell Barcoding and Reverse Transcription

1 Prepare cells for isolation and encapsulation in gel bead emulsions (GEMs). If your experiment involves a *piggyBac* transposase with PB-SRT-Puro transposons, cells that have survived sleection should be dissociated and resuspended in solution. If you are using *piggyBac* with PB-SRT-tdTomato, we recommend using FACS to isolate tdTomato-positive cells, running cells transfected with PB-SRT-tdTomato alone as a gating control.

2 Follow 10x's instructions for GEM Generation & Barcoding, with this modification:
- Step 1.1: Replace the RT Primer with an equivalent volume of Low TE Buffer

Proceed with Steps 1.2–1.5 as instructed: loading the Single Cell 3' chip, running the controller, transferring GEMs, and reverse transcription.

Incubate the RT reaction under standard conditions.
- Set lid temperature to **53 °C**
- **00:45:00** **53 °C**
- **00:05:00** **85 °C**
- Hold at **4 °C**

3 Step 2.1: clean the GEM-RT mixture using the Recovery Agent and DynaBeads MyOne Silane per 10x's instructions. At the final elution stage, add 36.5 µl Elution Solution I to the tube, mix by pipetting, and incubate at room temperature for 1 minute. Place the tube in a 10x Magnetic Separator in the Low position until the solution turns clear. Transfer 36 µl of the eluted sample to a new tube.

4 Divide the eluate into two 18-µl aliquots. These can be stored at –20ºC until needed. One aliquot will be used for scRNA-seq library preparation, while the other will be used to generate scCC libraries.

Single Cell RNA-seq Library Preparation and Sequencing

5 To continue preparing scRNA-seq libraries, we need to add the template switch oligonucleotide to first strand synthesis products from the RT reaction. Take one of the 18 µl aliquots and thaw on ice.

6 Prepare the following 1X master mix:
- 20 µl Maxima 5X RT buffer
- 20 µl 20% w/v Ficoll PM-400
- 10 µl 10 mM dNTPs
- 2.5 µl RNaseOUT
- 2.5 µl 100 µM 10x_TSO

7 To the mix, add 18 µl of first strand RT product and 22 µl H_2O. Add 5 µl Maxima H– RTase to the reaction, flick to the mix, and centrifuge briefly.

8 Incubate:
- 00:30:00 25 °C
- 01:30:00 50 °C
- 00:05:00 85 °C

9 Clean up following 10x's post GEM-RT Cleanup protocol, starting with the addition of DynaBeads MyOne Silane (Step 2.1, part D). Clean samples per manufacturer's instructions.

10 Complete cDNA amplification and library construction according to the 10x's instructions (Steps 2.2–3.7). For each sample, record which index sample index was used for the final PCR. Quantiate each library by running a 1:10 dilution on an Agilent TapeStation High Sensitivity D1000 ScreenTape.

11 Finished scRNA-seq libraries can be pooled and sequenced on Illumina MiSeq, NextSeq, HiSeq, and NovaSeq platforms.

Amplification of Self-Reporting Transcripts

12 To prepare single cell calling cards libraries, we start by amplifying self-reporting transcripts from the other aliquot of first-strand synthesis product. As before, thaw the remaining 18 µl aliquot on ice.

13 Prepare a PCR primer cocktail in a PCR tube:
- 5 µl of 100 µM Bio_Illumina_Seq1_scCC_10X_3xPT primer
- 5 µl of 100 µM Bio_Long_PB_LTR_3xPT primer
- 10 µl of Low TE Buffer

Mix by vortexing and spin down briefly. This cocktail can be stored at –20ºC.

14 Prepare the following PCR mix in PCR tube:
- 25 µl of 2X Kapa HiFi Hotstart Readymix
- 18 µl of first-strand synthesis product
- 6 µl of ddH_2O
- 1 µl of PCR primer cocktail

Keep on ice until ready for PCR.

15 Perform PCR using the following thermocycling parameters:
- 98ºC for 3 minutes
- 20 cycles of:
- 98ºC for 20 seconds
- 67ºC for 30 seconds
- 72ºC for 5 minutes
- 72ºC for 10 minutes
- 4ºC forever.

The number of cycles may need to be adjusted depending on the cell type and number of cells represented in the library. If uncertain, you can use 9 µl of first-strand synthesis product as template, reserving the other 9 µl for another round of PCR with more cycles as needed.

Purification of PCR Products

16 Vortex AMPure XP beads to resuspend them. Beads should be brought to room temperature for at least 30 minutes prior to use.

17 Add 30 µl beads to the 50 µl PCR mixture (0.6x ratio). Mix by pipetting 10 times until evenly dispersed.

18 Incubate at room temperature for **00:05:00**

19 Place on a magnetic rack for **00:05:00**
Aspirate supernatant and discard.

20 While the tube is still on the rack, add 200 µl of 70% ethanol and incubate ≥ 30 seconds. Aspirate supernatant and discard.

21 Repeat Step #20

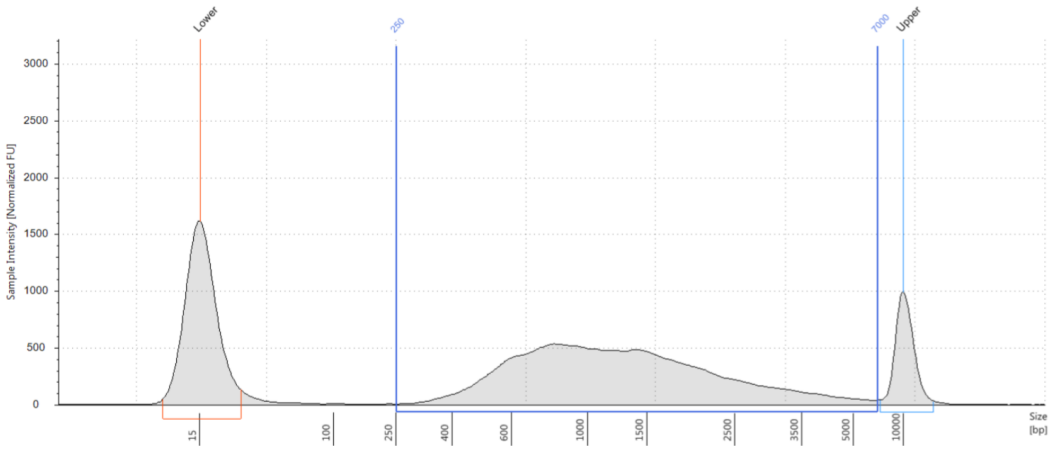
22 Air dry the pellet at room temperature for **00:02:00**

23 Remove the tube from the magnetic rack. Add 40 µl QIAGEN Elution Buffer to elute PCR products. Mix by pipetting until evenly dispersed.
Incubate off the rack for **00:05:00**

24 Place on magnetic rack for **00:05:00** or until supernatant is clear. Transfer supernatant to new 1.7 ml tube.

25 Take 1 µl of the eluate and dilute in 9 µl of ddH2O to make a 1:10 dilution. Quantitate on TapeStation using a High Sensitivity D5000 ScreenTape. Measure the molar concentration of the sample, taking everything from 250 bp to 7000 bp. Ideally, the diluted sample will be at least 750 pM, corresponding to 7.5 nM for the original eluate. If you kept half of the template aside, you can increase the number of PCR extension cycles until you get to a minimum of 7.5 nM of product.

This is what a representative TapeStation trace looks like. The library should be smooth and unimodal.



Region Table

From [bp]	To [bp]	Average Size [bp]	Conc. [pg/µl]	Region Molarity [pmol/l]	%of Total	Region Comment	Color
250	7000	1445	1170	1790	96.59	Everything	

Representative TapeStation trace of SRT amplification from 10x 3' scRNA-seq library

Single Cell Calling Cards – Circularization

26 Thaw an aliquot of CB – Circularization Buffer 10X on ice.

27 Add the following components to a new 1.7 ml tube in this order:

1. 300 fmol self-reporting transcripts from Step 25
2. ddH_2O up to a total of 268 µl
3. 30 µl CB
4. 2 µl Circularization Ligase

To calculate what volume of eluate corresponds to 300 fmol, divide 300 by the molar concentration (in nM) of the eluate. For example, if the concentration is 10 nM, 300 fmol/10 nM = 30 µl, and consequently you would need 238 µl ddH_2O. If you have less than 300 fmol total, you may proceed but might need to make adjustments at the final PCR step. If you do not have a way to quantitate the molarity of your solution, we have observed that 300 fmol of self-reporting transcripts is (very) approximately 200 ng.

We strongly encourage you to calculate the volume of SRT solution based on molarity, not by mass. The circularization reaction is sensitive to starting concentration. If overloaded, it can lead to excess intermolecular ligations and, subsequently, increased noise with respect to the assignment of insertions to cell types.

Mix by flicking the tube and spin down briefly. Incubate at **30 °C** overnight (12-16 hours).

Single Cell Calling Cards – Exonuclease and Setup

28 Add 9 µl of PS1 – Exonuclease directly to the overnight circularization mixture. Flick to mix, spin down, and incubate as following:

- **00:30:00** **37 °C**
- **00:30:00** **70 °C**

29 While the exonuclease digestion proceeds, prepare for the rest of the library preparation. Fill a large ice bucket with ice. Thaw, on ice, aliquots of:

- STL – Stop Ligation Buffer
- ERP3 – End Repair Mix
- ATL2 – A-tailing Mix

- LIG2 – Ligation Mix
- EPM – Enhanced PCR Mix

Also thaw the following oligonucleotides:
- scCC_P5_adapter (100 µM)
- scCC_P7_adapter (100 µM)
- scCC_P5_primer (25 µM)
- scCC_P7_primer (25 µM)

Finally, thaw NEBuffer 2

30 While the exonuclease incubates, anneal the scCC adapters. Prepare the following mixture in a PCR tube, using a different indexed scCC_P7_adapter for each sample:
- 4.5 µl scCC_P5_adapter
- 4.5 µl scCC_P7_adapter
- 1 µl NEBuffer 2

31 Anneal scCC adapters in a thermocycler using the following settings:
- 95ºC for 5 minutes
- 70ºC for 15 minutes
- Ramp down to 25ºC as slowly as possible
- 25ºC for 5 minutes
- 4ºC forever

scCC adapters can be kept on ice until needed.

Adapters should be prepared fresh. NEBuffer 2 contains magnesium salts which can promote DNase activity, leading to degradation of adapters.

32 Prepare the streptavidin-coated magnetic beads. These instructions are for 1 sample; up to 5 can be prepared in a single 1.7 ml tube. Resuspend Dynabeads M-280 by vortexing briefly.

33 Transfer 20 µl of beads to a clean 1.7 ml tube.

34 Place on a magnetic rack for 1 minute. Once clear, aspirate and discard supernatant.

35 Add 40 µl BBB – Bead Bind Buffer. Incubate for 1 minute, then aspirate and discard supernatant.

36 Repeat Step #35.

37 Remove from rack and add 300 µl BBB. Beads can be stored at room temperature until needed.

38 The exonuclease digestion should be complete by now. Add 12 µl STL – Stop Ligation Buffer. Flix to mix and centrifuge gently.

Single Cell Calling Cards – Shearing and Capture

39 Transfer the entire sample (now approximately 320 µl) to a Covaris T6 tube. Add ddH_2O as necessary to fill to the top, then cap the tube. Check to make sure there are no air bubbles.

40 Shear DNA on a Covaris ultrasonicator. Here are recommended settings for various models (we have tested this protocol on the E220):

Model	S2	S220	E220
Peak Power Intensity	N/A	240	200
Intensity	8	N/A	N/A
Duty Cycle/Factor	20%	20%	20%
Cycles Per Burst	200	200	200
Time	40	40	40
Temperature	6	6	6

Recommended shearing settings for preparing scCC libraries

41 Transfer the sample to a new 1.7 ml tube. Add 300 µl of bead solution to the sheared DNA.

42 Incubate **20 °C** **00:15:00**

If incubating on a thermoshaker, shake at 1000 RPM. Otherwise, flick to mix every 2 minutes.

43 Centrifuge briefly (5-10 seconds), then place on a magnetic rack for 1 minute. Discard the supernatant.

44 Wash 4 times with BWB – Bead Wash Buffer:
- Add 200 µl BWB
- Remove from rack, flick to mix, and spin down briefly (1-2 seconds)
- Place on rack for 30 seconds
- Discard supernatant

Repeat for a total for 4 washes

45 Wash 2 times with RSB – Resuspension Buffer:
- Add 200 µl RSB
- Remove from rack, flick to mix, and spin down briefly
- Place on rack for 30 seconds
- Discard supernatant

For the second wash, do not discard supernatant until ready to add the master mix in the next step.

Single Cell Calling Cards – End Repair, A-Tailing, and Adapter Ligation

46 Prepare master mixes for End Repair and A-Tailing as follows.

1X End Repair Master Mix:
- 40 µl ERP3 – End Repair Mix
- 60 µl ddH_2O

1X A-Tailing Master Mix:
- 12.5 µl ATL2 – A-Tailing Mix
- 17.5 µl ddH_2O

47 Discard all supernatant from the DNA sample. Centrifuge briefly, then place on a magnetic rack.

48 Use a 10 µl pipette to aspirate any residual supernatant.

49 Add 100 µl End Repair reaction mix, remove from the rack, flick to mix, and centrifuge briefly (do not allow beads to pellet).

50 Incubate 30 °C 00:30:00

If incubating on a thermoshaker, shake at 1000 RPM, to prevent beads from settling.

51 Centrifuge briefly (5-10 seconds), then place on a magnetic rack for 1 minute. Discard the supernatant.

52 Wash 4 times with BWB – Bead Wash Buffer:
- Add 200 µl BWB
- Remove from rack, flick to mix, and spin down briefly (1-2 seconds)
- Place on rack for 30 seconds
- Discard supernatant

Repeat for a total for 4 washes

53 Wash 2 times with RSB – Resuspension Buffer:
- Add 200 µl RSB
- Remove from rack, flick to mix, and spin down briefly
- Place on rack for 30 seconds
- Discard supernatant

For the second wash, do not discard supernatant until ready to add the master mix in the next step.

54 Discard all supernatant from the DNA sample. Centrifuge briefly, then place on a magnetic rack. Use a 10 µl pipette to aspirate any residual supernatant.

55 Add 30 µl A-Tailing reaction mix, remove from the rack, flick to mix, and centrifuge briefly (do not allow beads to pellet).

56 Incubate 37 °C 00:30:00

If incubating on a thermoshaker, shake at 1000 RPM, to prevent beads from settling.

57 Add the following components in order to the A-tailing mix:
- (30 µl A-tailing reaction)
- 2.5 µl LIG2 – Ligation Mix
- 4 µl ddH_2O
- 1 µl annealed scCC adapter

Flick to mix and centrifuge briefly (do not allow beads to pellet).

58 Incubate 30 °C 00:10:00

59 Add 5 µl STL – Stop Ligation Buffer. Flick to mix.

60 Centrifuge briefly (5-10 seconds), then place on a magnetic rack for 1 minute. Discard the supernatant.

61 Wash 4 times with BWB – Bead Wash Buffer:
- Add 200 µl BWB
- Remove from rack, flick to mix, and spin down briefly (1-2 seconds)
- Place on rack for 30 seconds

- Discard supernatant

Repeat for a total for 4 washes

62 Wash 2 times with RSB – Resuspension Buffer:
- Add 200 µl RSB
- Remove from rack, flick to mix, and spin down briefly
- Place on rack for 30 seconds
- Discard supernatant

For the second wash, do not discard supernatant until ready to add the master mix in the next step.

Single Cell Calling Cards – Final PCR and Purification

63 Prepare a 1X PCR master mix in a new 1.7 ml tube:
- 20 µl EPM – Enhanced PCR Mix
- 28 µl ddH_2O
- 1 µl scCC_P5_primer (25 µM)
- 1 µl scCC_P7_primer (25 µM)

64 Discard all supernatant from the DNA sample. Centrifuge briefly, then place on a magnetic rack. Use a 10 µl pipette to aspirate any residual supernatant.

65 Add 50 µl PCR reaction mix to the sample and pipette to mix. Transfer to PCR tubes.

66 Incubate in a thermocycler with the following settings:
- 98ºC for 30 seconds
- 15 cycles of:
- 98ºC for 10 seconds
- 60ºC for 30 seconds
- 72ºC for 2 minutes
- 72ºC for 5 minutes
- 4ºC forever.

If you started with less than 300 fmol of self-reporting transcripts, you can increase the number of extension cycles here. More cycles will increase the risk of artifacts, however, so we recommend increasing by the minimum necessary to obtain reasonable sequencing libraries. The most we have pushed this PCR is to 17 extension cycles.

67 Vortex AMPure XP beads to resuspend them. Beads should be brought to room temperature for at least 30 minutes prior to use.

68 Place PCR tubes on a magnetic rack for 1 minute. Transfer 50 µl of supernatant to new tubes.

69 Add 35 µl beads to the 50 µl PCR mixture (0.7x ratio). Flick to mix and centrifuge briefly.

70 Incubate at room temperature for **00:05:00**

71 Place on a magnetic rack for **00:05:00**

Aspirate supernatant and discard.

72 Add 200 µl of 70% ethanol and incubate ≥ 30 seconds. Aspirate supernatant and discard.

73 Repeat Step #72

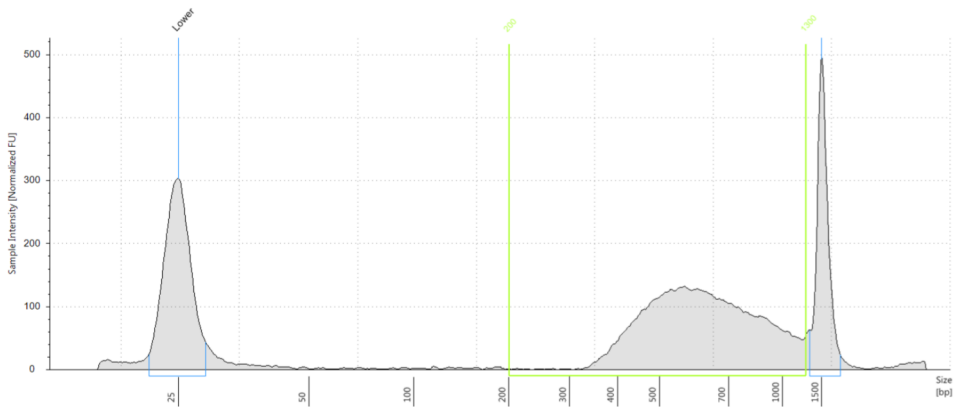
74 Air dry the pellet at room temperature for **00:02:00**

75 Remove the tube from the magnetic rack. Add 25 µl RSB – Resuspension Buffer to elute PCR products. Mix by pipetting until evenly dispersed. Incubate off the rack for **00:05:00**

76 Place on magnetic rack for **00:05:00** or until supernatant is clear. Transfer supernatant to new 1.7 ml tube.

77 Make a 1:10 dilution of the eluate and quantitate on TapeStation using a High Sensitivity D1000 ScreenTape. Measure the molar concentration of the sample, taking everything from 200 bp to 1300 bp.

This is what a representative TapeStation trace looks like. The library should be smooth and unimodal, peaking between 500-700 bp. Occasionally, you may see a primer-dimer peak. However, as scCC libraries are sequenced from the middle and not the ends, the primer-dimer product will not sequence on the Illumina flow cell.



Region Table

From [bp]	To [bp]	Average Size [bp]	Conc. [pg/µl]	Region Molarity [pmol/l]	% of Total	Region Comment	Color
200	1300	663	731	1870	88.88	scCC	

Representative TapeStation trace of scCC libraries

Single Cell Calling Cards – Sequencing

78 Single cell calling cards libraries use a mix of standard and custom primers for sequencing and rely on dual-indexing for proper demutiplexing. We have sequenced scCC libraries on Illumina NextSeq 500 machines, using v2 Reagent Cartridges. These libraries use the standard Illumina primers BP10 and BP14 for read 1 and index 2, respectively. Read 1 sequences the cell barcode and unique molecular index (UMI), while index 2 reads into the terminal repeat of the *piggyBac* transposon, confirming that molecules successfully circularized.

In addition, we use the custom sequencing primers scCC_PB_CustomRead2 and scCC_CustomIndex1 for read 2 and index 1, respectively. Read 2 anneals at the end of the transposon and sequences into the genome. The first six base pairs typically begin "GGTTAA", which are the terminal two base pairs of the *piggyBac* repeat followed by the insertion site tetramer. The remainder of the read is genomic DNA sequence. Index 1 sequences the sample-specific sequence on the scCC adapter and is used to demultiplex libraries.

Due to the low complexity nature of calling card libraries, we recommed adding PhiX at a final concentration of 50%.

79 While index 1 should be sufficient to demultiplex libraries, we have observed a biphasic response when sequencing scCC libraries at low and high concentrations. At low library concentrations, (e.g., 1-2%) the index 1 read generates high-quality reads and can demultiplex libraries; however at higher concentrations (i.e., 50%) the index 1 read can fail, yielding all N's. If this happens, libraries can be demultiplexed by the index 2 read alone: scCC reads that have successfully circularized will have "GCGTCAAT" as the index 2 sequence.

After this, scCC reads can be assigned to specific samples using the cell barcodes obtained from the corresponding scRNA-seq libraries. Different libraries may, by chance, have cells that share the same cell barcode. Typically, these represent a very small fraction of cells (< 1% per library) and we discard these reads and cells from downstream calling cards and scRNA-seq analyses, respectively.