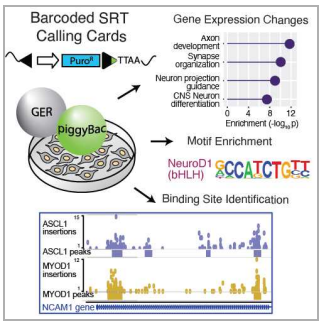

NOV 22, 2022

WORKS FOR ME 1

Barcoded Calling Cards and Transcriptomes: Library Preparation

Forked from Bulk Calling Cards Library Preparation

COMMENTS 0

DOI
dx.doi.org/10.17504/protocols.io.bp2l6nqokgqe/v1

Matthew Lalli[1], Urvashi Thopte[1], Arnav Moudgil[2], Michael N. Wilkinson[2], Xuhua Chen[2], Robi D. Mitra[2]

[1]Icahn School of Medicine at Mount Sinai; [2]Washington University, Saint Louis

Matthew Lalli
Icahn School of Medicine at Mount Sinai

DISCLAIMER

This protocol is a modfication of Moudgil *et al's* Bulk Calling Cards Library Preparation. Some instructions have not been modified, and are included verbatim from the original protocol.

ABSTRACT

This protocol is a modification of Moudgil *et al's* Bulk Calling Cards Library Preparation that uses barcoded self-reporting transposons for Calling Cards and barcoded oligos for transcriptome capture. This protocol describes how to create next-generation sequencing libraries from barcoded self-reporting transposon (SRT) Calling Cards experiments to measure transcription factor binding and gene expression in parallel from collected RNA.

This protocol assumes you have successfully transformed cells with barcoded *piggyBac* self-reporting transposons and either undirected *piggyBac* transposase or a gene expression regulator (GER) fused to *piggyBac*. Your cells are now ready for RNA extraction, SRT and transcriptome amplification, and library preparation.

DOI

dx.doi.org/10.17504/protocols.io.bp2l6nqokgqe/v1

EXTERNAL LINK

https://doi.org/10.1093/nargab/lqac061

PROTOCOL CITATION

Matthew Lalli, Urvashi Thopte, Arnav Moudgil, Michael N. Wilkinson, Xuhua Chen, Robi D. Mitra 2022. Barcoded Calling Cards and Transcriptomes: Library Preparation. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bp2l6nqokgqe/v1

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

Lalli M, Yen A, Thopte U, Dong F, Moudgil A, Chen X, Milbrandt J, Dougherty JD, Mitra RD (2022) Measuring transcription factor binding and gene expression using barcoded self-reporting transposon calling cards and transcriptomes. NAR Genomics and Bioinformatics 4:lqac061.

FORK NOTE

FORK FROM

Forked from Bulk Calling Cards Library Preparation, Arnav Moudgil

KEYWORDS

Calling cards, self-reporting transposons, SRTs, transcription factors, BRB-seq, transcriptome

CREATED

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PROTOCOL INTEGER ID

40296

GUIDELINES

Please read this protocol in its entirety before starting. For several steps, it may help to pre-program your thermocycler with the listed settings. Please also read the BRB-seq paper , especially the Maxima H protocol implementation, to become familiarized with the barcoded transcriptome capture.

MATERIALS TEXT

MATERIALS

RNasin(R) Plus RNase Inhibitor, 10,000u **Promega Catalog #N2615**

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

dNTP **Takara Catalog #639125**

2x Kapa HiFi Hotstart Readymix **Kapa Biosystems Catalog #KK2602**

Maxima H Minus Reverse Transcriptase (200 U/uL) **Thermo Fisher Scientific Catalog #EP0752**

Qubit dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q32851**

Direct-zol™ RNA MiniPrep kit **Zymo Research Catalog #R2070 - R2073**

NucleoSpin® Gel and PCR Clean-up **Macherey and Nagel Catalog #740609.10**

Capillary electrophoresis instrument (e.g. Agilent Tapestation 4200) **Contributed by users**

Qubit RNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q32852**

Nextera XT DNA Library Preparation Kit **Illumina, Inc. Catalog #FC-131-1024**

High Sensitivity D1000 Reagents **Agilent Technologies Catalog #5067-5585**

High Sensitivity D1000 ScreenTape **Agilent Technologies Catalog #5067-5584**

NanoDrop™ 2000 packaged with laptop computer **Thermo Fisher Catalog #ND2000LAPTOP**

D5000 ScreenTapes **Agilent Technologies Catalog #5067-5588**

Primers

>BRB-seq_dT30VN

CTACACGACGCTCTTCCGATCTCTGATAGCATGGTCATNNNNNVVVVVTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN

Citation: Matthew Lalli, Urvashi Thopte, Arnav Moudgil, Michael N. Wilkinson, Xuhua Chen, Robi D. Mitra Barcoded Calling Cards and Transcriptomes: Library Preparation https://dx.doi.org/10.17504/protocols.io.bp2l6nqokgqe/v1

This is a set of 12 BRB-seq Oligo (dT) reverse transcription primers compatible with 10x Genomics single cell 3’ v2 chemistry

A	B
pSeq1-BC1-UMI-dtVN	CTACACGACGCTCTTCCGATCTCTGATAGCATGGTCATNNNNN
pSeq1-BC2-UMI-dtVN	CTACACGACGCTCTTCCGATCTCACAGTAGTTAGGGTGNNNNN
pSeq1-BC3-UMI-dtVN	CTACACGACGCTCTTCCGATCTGTAACTGCATGGTCTANNNNN
pSeq1-BC4-UMI-dtVN	CTACACGACGCTCTTCCGATCTACTGAACCAGTGGGATNNNNN
pSeq1-BC5-UMI-dtVN	CTACACGACGCTCTTCCGATCTAACACGTTCAGTTCGANNNNN
pSeq1-BC6-UMI-dtVN	CTACACGACGCTCTTCCGATCTTATCAGGGTTTAGCTGNNNNN
pSeq1-BC7-UMI-dtVN	CTACACGACGCTCTTCCGATCTACTTTCATCGTAGGAGNNNNN
pSeq1-BC8-UMI-dtVN	CTACACGACGCTCTTCCGATCTAACGTTGGTAGCGTCCNNNNN
pSeq1-BC9-UMI-dtVN	CTACACGACGCTCTTCCGATCTCTCATTACAGACGCCTNNNNN
pSeq1-BC10-UMI-dtVN	CTACACGACGCTCTTCCGATCTTGACTAGCAGGGTTAGNNNNN
pSeq1-BC11-UMI-dtVN	CTACACGACGCTCTTCCGATCTGCGGGTTAGTAATCCCNNNNN
pSeq1-BC12-UMI-dtVN	CTACACGACGCTCTTCCGATCTTCTCATAGTTGTGGAGNNNNN

Generic
>SRT_PAC_F1
CAACCTCCCCTTCTACG*A*G*C

>SRT_tdTomato_F1
TCCTGTACGGCATGGAC*G*A*G

> SMART_TSO
AAGCAGTGGTATCAACGCAGAGTACrGrGrG

>SMART
AAGCAGTGGTATCAACGCAG*A*G*T

>Partial Seq1
CTACACGACGCTCTTCCGA*T*C*T

* = Phosphorothioate bonds

Staggered *piggyBac* Barcoded SRT Forward Primers (XXXXX is i5 index):

A	B
P5_BC_SRT_STAGGER1	AATGATACGGCGACCACCGAGATCTACAC XXXXXX ACACTC
P5_BC_SRT_STAGGER3	AATGATACGGCGACCACCGAGATCTACAC XXXXXX ACACTC
P5_BC_SRT_STAGGER5	AATGATACGGCGACCACCGAGATCTACAC XXXXXX ACACTC
P5_BC_SRT_STAGGER7	AATGATACGGCGACCACCGAGATCTACAC XXXXXX ACACTC

Citation: Matthew Lalli, Urvashi Thopte, Arnav Moudgil, Michael N. Wilkinson, Xuhua Chen, Robi D. Mitra Barcoded Calling Cards and Transcriptomes: Library Preparation https://dx.doi.org/10.17504/protocols.io.bp2l6nqokgqe/v1

A	B
P5_BC_SRT_STAGGER9	AATGATACGGCGACCACCGAGATCTACAC XXXXXX ACACTC
P5_BC_SRT_STAGGER11	AATGATACGGCGACCACCGAGATCTACAC XXXXXX ACACTC

Indexed P5-seq1 primer for transcriptomes, for example:

>P5-index1-Seq1 (i5 index sequence is underlined)
AATGATACGGCGACCACCGAGATCTACAC<u>AGGACAA</u>CACTCTTTCCCTACACGACGCTCTTCCGATCT

Indexed Nextera N7 primers, for example:
>Nextera_N701 (i7 index sequence is underlined)
CAAGCAGAAGACGGCATACGAGAT<u>TCGCCTTA</u>GTCTCGTGGGCTCGG

You should have multiple indexed primers. These can be either official Nextera indexes or custom, lab-specific indexes. For a comprehensive list of official Nextera indexes, consult the Illumina Adapter Sequences Document.

Sequencing primers are ordered as Ultramers for higher quality but with no additional purifications.

Other reagents

- Ethanol (96-100%)
- Ethanol (70%)
- Molecular biology grade water (ddH_2O)

SAFETY WARNINGS

- TRI Reagent should be used in a chemical fume hood
- TRI Reagent waste should be collected and discarded in accordance with EH&S policies

DISCLAIMER

This protocol is a modfication of Moudgil *et al's* Bulk Calling Cards Library Preparation. Some instructions have not been modified, and are included verbatim from the original protocol.

BEFORE STARTING

Please read and familiarize yourself with the manuals for the DirectZol RNA Mini Kit and the Nextera XT Tagmentation Kit. The instructions are meant to summarize those workflows; however, when in doubt, please refer to the manufacturer's instructions for guidance.

RNA Extraction wtih Direct-Zol RNA Miniprep

1 Harvest cells. Do not overload columns. If you have more than 10^7 cells, split cells in half and process on two columns, then merge the RNA pools.

Citation: Matthew Lalli, Urvashi Thopte, Arnav Moudgil, Michael N. Wilkinson, Xuhua Chen, Robi D. Mitra Barcoded Calling Cards and Transcriptomes: Library Preparation https://dx.doi.org/10.17504/protocols.io.bp2l6nqokgqe/v1

2 Add TRI Reagent directly to cells in a dish. Use the following table as a guide.

A	B
# cells	Add TRI Reagent
< 1e6	300 µl
< 5e6	600 µl

Lyse the cells and transfer into 1.7 mL tube.

3 Add an equal volume ethanol (95-100%). Homogenize the lysate by vortexing briefly.

4 Transfer 700 ul of this mixture into a Zymo-Spin IICR Column spin column placed in a 2 ml collection tube. Centrifuge for 30 seconds at ≥ 8,000g. Ensure no liquid remains on the column membrane. Repeat centrifugation for samples > 700 µl.

Safety information

Collect flow-through for proper disposal.

5 Add 400 µL RNA PreWash buffer to column and centrifuge. Discard the flow-through.

6 Add 700 µl RNA Wash Buffer to the column and centrifuge briefly. Discard flow-through and centrifuge for 2 minutes. Transfer column into an RNase-free tube.

7 Elute RNA by adding 50 µl DNase/RNase-free water or te buffer to column and centrifuging.

8 Measure RNA concentration using Nanodrop or Qubit.

Citation: Matthew Lalli, Urvashi Thopte, Arnav Moudgil, Michael N. Wilkinson, Xuhua Chen, Robi D. Mitra Barcoded Calling Cards and Transcriptomes: Library Preparation https://dx.doi.org/10.17504/protocols.io.bp2l6nqokgqe/v1

First Strand Synthesis (reverse transcription)

9 For first strand synthesis by reverse transcription, process samples according to experimental design. Use a unique BRB-seq primer for each transcriptome to be captured.

Prepare the reverse transcription (RT) reaction mix:

- 2 µg total RNA
- 2 µl of 25 µM BRB-seq_dT30VN primer (e.g., pSeq1-BC1-UMI-dtVN)
- 1 µl of 10 mM dNTPs
- Raise to 14 µl with ddH_2O

Incubate RT mix at 65 °C for 00:05:00

Place on ice for 1 minute.

10 Create 1x Maxima RT buffer:

- For 5 or fewer samples, combine 1 µL of 5X Maxima RT buffer with 4 µL of ddH_2O.
- Mix by pipetting and store on ice.

11 Create a 0.5x Maxima RT H Minus enzyme dilution:

- Mix an equal volume of Maxima RT H Minus Enzyme with the 1x Maxima RT buffer made in previous step (e.g. 2 µL of Enzyme + 2 µL of 1x buffer).

You will need 1 µL of the 0.5x enzyme dilution for every sample being processed. Avoid pipetting volumes < 1 µL.

12 Add the following to the RT mix:

- 4 µl 5X Maxima RT Buffer
- 1 µl RNasin RNase Inhibitor
- 1 µl of 0.5X Maxima RT H Minus enzyme (1:1 mixture of 1X Maxima RT Buffer and Maxima RT H Minus enzyme = 100 U)
- 1 uL SMART_TSO (25 µM)

Mix by pipetteing and incubate at 50 °C for 01:00:00

13 Heat inactivate the reaction by incubating at 85 °C for 00:10:00

14 Column purify using NucleoSpin Gel and PCR Clean-up kit to remove carry-over oligoDT primers.

Note

Citation: Matthew Lalli, Urvashi Thopte, Arnav Moudgil, Michael N. Wilkinson, Xuhua Chen, Robi D. Mitra Barcoded Calling Cards and Transcriptomes: Library Preparation https://dx.doi.org/10.17504/protocols.io.bp2l6nqokgqe/v1

Pooling cDNA from multiple samples (up to 12 samples tested) at this step enables ultra-low cost preparation of RNA-sequencing libraries.

Pooled cDNA can also be used for SRT library preparation if experimental replicates or conditions were transfected with unique barcode combinations.

Alternatively, samples can be pooled for transcriptomes and processed individually for SRT libraries.

- If pooling, combine 3-5 µl of each sample *before* column purification.

Bring total volume of pooled or individual samples to 100 µL by adding water.
Add 200 µl NT1 and proceed with column purification according to manufacturer's instructions.

Elute in 30-50 µl DNAse/RNAse free water.

15 cDNA can be stored at –20 ºC for long term, or 4 ºC until downstream processing

Amplification of Self-Reporting Transcripts

16 This PCR will specifically amplify self-reporting transcripts from cDNA libraries. Prepare the following solution:

- 25 µl 2X Kapa HiFi HotStart ReadyMix
- 1 µl of 25 µM Reverse Primer (partial seq1)
- 4 µl of purified cDNA*
- 19 µl of ddH_2O
- 1 µl of Forward Primer, either:

25 µM SRT_PAC_F1 primer, if using PB-SRT-Puro
25 µM SRT_tdTomato_F1, if using PB-SRT-tdTomato

Note

* For pooled SRT libraries, increasing the amount of input cDNA and reducing the number of PCR cycles in the next step are likely to improve library complexity

17 Perform PCR using the following thermocycling parameters:

Citation: Matthew Lalli, Urvashi Thopte, Arnav Moudgil, Michael N. Wilkinson, Xuhua Chen, Robi D. Mitra Barcoded Calling Cards and Transcriptomes: Library Preparation https://dx.doi.org/10.17504/protocols.io.bp2l6nqokgqe/v1

- 95ºC for 3 minutes
- 20 cycles of:
- 98ºC for 20 seconds
- 65ºC for 30 seconds
- 72ºC for 5 minutes
- 72ºC for 10 minutes
- 4ºC forever

Amplification of Full-length Barcoded Transcriptomes

18 If desired, this PCR will amplify full-length transcriptomes from cDNA libraries. Prepare the following solution:

- 25 µl 2X Kapa HiFi HotStart ReadyMix
- 1 µl of 25 µM Reverse Primer (partial seq1)
- 1 ul of 25 uM Forward Primer (SMART)
- 4 µl of pooled and purified cDNA
- 19 µl of ddH_2O

19 Perform PCR using the following thermocycling parameters:

- 95ºC for 3 minutes
- 10 cycles of:
- 98ºC for 20 seconds
- 60ºC for 30 seconds
- 72ºC for 6 minutes
- 72ºC for 10 minutes
- 4ºC forever

Purification of PCR Products

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

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

22 Incubate at room temperature for 00:05:00

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23 Place on a magnetic rack for 2 minutes. Aspirate supernatant.

24 Add 200 µl of freshly-prepared 70% ethanol off the magnetic rack and mix. Place on magnetic rack and incubate ≥ 30 seconds. Aspirate ethanol.

25 Repeat Step 24 on the magnetic rack.

26 Air dry the beads at room temperature for 2 minutes. Do not over dry.

27 Remove the tube from the magnetic rack. Add 20 µl ddH_2O to elute PCR products. Mix by pipetting until evenly dispersed. Incubate off the rack for 2 minutes.

28 Place on magnetic rack for 1 minute, or until supernatant is clear.

29 Transfer supernatant to new tube. Quantify product on Tapestation D5000.

Expected result

Expected concentration of product should be 10-20 ng/µl.
Product sizes should be distributions from 400 bp - 9 kb for SRTs and full transcriptomes alike.

Generation of Bulk Calling Card Libraries

30 The tagmentation protocol fragments the long PCR products into libraries suitable for sequencing.

Preheat thermocycler to 55 °C

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31 Take 1 ng of PCR product and resuspend in a total of 5 µl ddH_2O in a PCR strip tube.

Note

Tagmentation is very sensitive to the quantity of input DNA. Stay within a narrow range of 600 pg - 1.2 ng of PCR product.

32 Add 10 µl of Nextera Tagment DNA (TD) Buffer and 5 µl of Amplicon Tagment Mix (ATM). Pipette to mix and briefly spin down; bubbles are normal. Incubate at 55 °C for 00:05:00

33 Add 5 µl of Neutralization Tagment (NT) Buffer. Pipette to mix and briefly spin down; bubbles are normal. Incubate at room temperature for 00:05:00

34 For SRT libraries, add the following to each PCR tube in order:

- 15 µl Nextera PCR Mix (NPM)
- 8 µl ddH_2O
- 1 µl of 10 µM barcoded *piggyBac* primer (e.g., P5_BC_SRT_STAGGER1)
- 1 µl of 10 µM indexed Nextera N7 primer (e.g. Nextera_N701)

Note

Because SRT libraries have low diversity on Read1, we designed a set of 6 P5 primers with stagger regions of different lengths to introduce sequence diversity. We recommend sequencing at least 4 SRT libraries on the same flow cell and using 20% PhiX DNA spike-in.

35 Perform PCR using the following thermocycling parameters:

- 72ºC for 3 minutes
- 95ºC for 30 seconds
- 16 cycles of:
- 95ºC for 10 seconds
- 52ºC for 30 seconds

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- 72ºC for 30 seconds
- 72ºC for 5 minutes
- 4ºC forever

Generation of BRB-seq Libraries

36 In parallel with Calling Card libraries, prepare BRB-seq libaries with tagmentation.

Preheat thermocycler to 55 °C

37 Take 1 ng of PCR product and resuspend in a total of 5 µl ddH_2O in a PCR strip tube.

38 Add 10 µl of Nextera Tagment DNA (TD) Buffer and 5 µl of Amplicon Tagment Mix (ATM). Pipette to mix and briefly spin down; bubbles are normal. Incubate at 55 °C for 00:05:00

39 Add 5 µl of Neutralization Tagment (NT) Buffer. Pipette to mix and briefly spin down; bubbles are normal. Incubate at room temperature for 00:05:00

40 For BRB-seq library amplification, add the following to each PCR tube in order:

- 15 µl Nextera PCR Mix (NPM)
- 8 µl ddH_2O
- 1 µl of 10 µM P5-index-seq1 primer
- 1 µl of 10 µM indexed Nextera N7 primer (e.g. Nextera_N701)

41 Perform PCR using the following thermocycling parameters:

- 72ºC for 3 minutes
- 95ºC for 30 seconds
- 16 cycles of:
- 95ºC for 10 seconds
- 55ºC for 30 seconds
- 72ºC for 30 seconds
- 72ºC for 5 minutes
- 4ºC forever

Citation: Matthew Lalli, Urvashi Thopte, Arnav Moudgil, Michael N. Wilkinson, Xuhua Chen, Robi D. Mitra Barcoded Calling Cards and Transcriptomes: Library Preparation https://dx.doi.org/10.17504/protocols.io.bp2l6nqokgqe/v1

Purification of Sequencing Libaries

42 Purify PCR libraries using AMPure XP beads. Vortex AMPure XP beads to resuspend them. Beads should be brought to room temperature for at least 30 minutes prior to use.

43 Add 35 µl beads to each 50 µl PCR mixture (0.7x ratio). Mix by pipetting 10 times until evenly dispersed.

44 Incubate at room temperature for 00:05:00

45 Place on a magnetic rack for 2 minutes. Aspirate supernatant.

46 Add 200 µl of freshly-prepared 70% ethanol off the magnetic rack and mix. Place on magnetic rack and incubate ≥ 30 seconds. Aspirate ethanol.

47 Repeat Step #46 on the magnetic rack.

48 Air dry the pellet at room temperature for 2 minutes.

49 Remove the tube from the magnetic rack. Add 11 µl ddH_2O to elute PCR products. Mix by pipetting until evenly dispersed. Incubate off the rack for 2 minutes.

50 Place on magnetic rack for 1 minute, or until supernatant is clear. Transfer supernatant to new tube.

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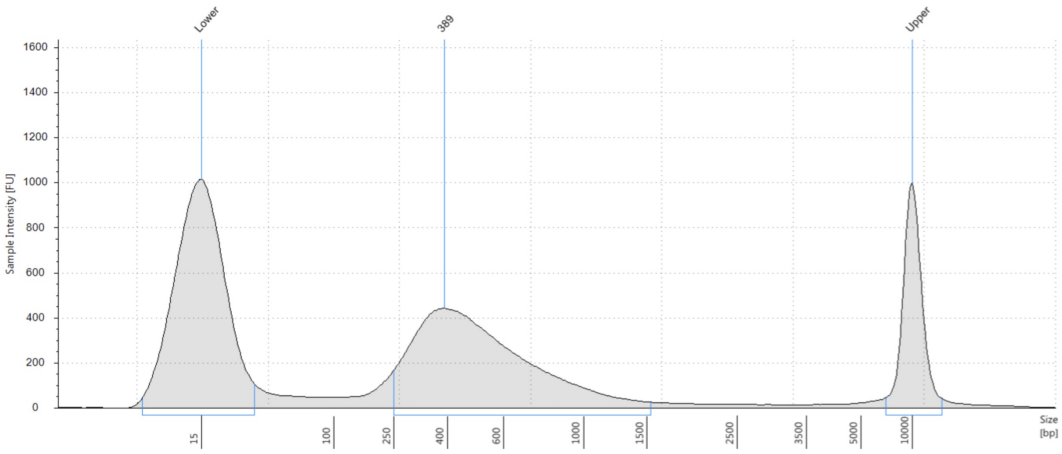
Final Quantitation and Sequencing

51 Measure library concentration on a TapeStation device with a High Sensitivity D1000 ScreenTape. Libraries should be smoothly distributed between 300-600 bp.

Expected result

Expected concentration of product should be 2-4 ng/µl.



Representative TapeStation trace of bulk calling card libraries. (Note: this sample was run on a High Sensitivity D5000 ScreenTape)

52 Libraries can be sequenced on any Illumina sequencing platform.

Note

Bulk calling card libraries only use the information from read 1 for mapping insertions. Therefore, single-end sequencing should be sufficient, with at least 75 bp for read 1. An index 1 read will also be necessary for demultiplexing samples.

We typically sequence 12-24 libraries on a NextSeq 500/550 Mid Output Kit v2.5 (150 Cycles) with the library configuration of 150x9x6x0 (read 1, index 1, index 2, read 2) to obtain 4-8 million reads per library. We use 20-30% PhiX DNA spike-in.

In sum, we recommend performing 3-4 experimental replicates per factor to recover between 500,000 and 1 million insertions. We recommend sequencing in sets of 6 libraries constructed using staggered oligos.

Citation: Matthew Lalli, Urvashi Thopte, Arnav Moudgil, Michael N. Wilkinson, Xuhua Chen, Robi D. Mitra Barcoded Calling Cards and Transcriptomes: Library Preparation https://dx.doi.org/10.17504/protocols.io.bp2l6nqokgqe/v1

We recommend generating a representative background set of PBase insertions for each cell line.

Note

BRB-seq libraries use the information from read 1 for demultiplexing samples and assigning unique molecular identifiers onto transcripts. As implemented here, read 1 mimics 10X Genomics Chromium Single Cell 3' Solution V2 with a 16 bp barcode and 10 bp UMI. Read 2 is used to map 3' transcriptomes.

Citation: Matthew Lalli, Urvashi Thopte, Arnav Moudgil, Michael N. Wilkinson, Xuhua Chen, Robi D. Mitra Barcoded Calling Cards and Transcriptomes: Library Preparation https://dx.doi.org/10.17504/protocols.io.bp2l6nqokgqe/v1