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Working

Bulk Calling Cards Library Preparation [↗](#)Arnav Moudgil<sup>1</sup>, Michael N. Wilkinson<sup>1</sup>, Xuhua Chen<sup>1</sup>, Robi D. Mitra<sup>1</sup><sup>1</sup>Washington University, Saint Louis

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Transposon Calling Cards



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## ABSTRACT

This protocol describes how to create calling card libraries from bulk RNA. This protocol assumes you have successfully transformed cells with *piggyBac* self-reporting transposons and either undirected *piggyBac* transposase or your favorite transcription factor (YTF) fused to *piggyBac*. Your cells are now ready for RNA extraction, SRT amplification, and library preparation.

## 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 with the listed settings.

## MATERIALS

NAME	CATALOG #	VENDOR
Agencourt Ampure XP	A63880	Beckman Coulter
dNTP	639125	Takara
2x Kapa HiFi Hotstart Readymix	KK2602	Kapa Biosystems
Maxima H Minus Reverse Transcriptase (200 U/uL)	EP0752	Thermo Fisher Scientific
Qubit dsDNA HS Assay Kit	Q32851	Thermo Fisher Scientific
Capillary electrophoresis instrument (e.g. Agilent Tapestation 4200)		
RNEasy Plus Mini Kit	74134	Qiagen
2-mercaptoethanol	21985023	Gibco - Thermo Fisher
Qubit RNA HS Assay Kit	Q32852	Thermo Fisher Scientific
RNaseOUT™ Recombinant Ribonuclease Inhibitor	10777019	Thermo Fisher Scientific
RNase H	M0297S	New England Biolabs
Nextera XT DNA Library Preparation Kit	FC-131-1024	Illumina, Inc.
High Sensitivity D1000 Reagents	5067-5585	Agilent Technologies

NAME ▾	CATALOG # ▾	VENDOR ▾
High Sensitivity D1000 ScreenTape	5067-5584	Agilent Technologies

#### MATERIALS TEXT

##### Primers

>SMART\_dT18VN

AAGCAGTGGTATCAACGCAGAGTACGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN

>SRT\_PAC\_F1

CAACCTCCCCTTCTACGAGC

>SRT\_tdTomato\_F1

TCCTGTACGGCATGGACGAG

>SMART

AAGCAGTGGTATCAACGCAGAGT

>Raff\_ACTB\_F

CCTCGCCTTTGCCGATCCG

>Raff\_ACTB\_R

GGATCTTCATGAGGTAGTCAGTCAGGTCC

Barcoded *piggyBac* primers, for example:

>OM-PB-ACG (barcode sequence is underlined)

AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTACGTTTACGCAGACTATCTTTCTAG

You should have multiple barcoded primers. See the linked preprint (Supplemental Table 4) for more examples.

Indexed Nextera N7 primers, for example:

>Nextera\_N701 (index sequence is underlined)

CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGG

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.

These primers can be ordered purified by standard desalting.

##### Other reagents

- Ethanol (96-100%)
- Ethanol (70%)
- Molecular biology grade water (ddH<sub>2</sub>O)

#### SAFETY WARNINGS

- Add 10 µl 2-mercaptoethanol to 1 ml Buffer RLT Plus (1% final concentration). 2-mercaptoethanol should be handled in a fume hood with appropriate protective clothing. This solution is stable at room temperature for 1 month.
- Add 4 volumes of ethanol (96-100%) to Buffer RPE before first use.
- If Buffer RLT Plus has formed a precipitate, redissolve by warming.
- Buffers RLT Plus and RW1 contain guanidine salts and cannot be cleaned using disinfectants containing bleach.

#### BEFORE STARTING

Please read and familiarize yourself with the manuals for the QIAGEN RNEasy Plus 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.

Ensure that you have performed multiple (i.e. 8-12), independent replicates of your experiment before proceeding. The calling card

assay relies on the clustering of multiple nearby insertions to identify TF binding sites. Some regions of the genome may have relatively few insertion sites for the transposase. Therefore, doing multiple independent replicates increases the statistical power to discriminate between a true binding site and background noise.

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.

#### RNA Extraction with QIAGEN's RNEasy Plus Mini Kit

- 1 Harvest cells. Process each replicate independently. Do not overload gDNA Eliminator columns. If you have more than  $10^7$  cells, split cells in half and process on two columns, then merge the RNA pools. Adherent cells may have to be dissociated using trypsin or a cell scraper.

Pellet cells by centrifuging at 300g for 5 minutes. Aspirate all of the supernatant.

- 2 Add Buffer RLT Plus (with added 2-mercaptoethanol) to the pellet. Use the following table as a guide.

# cells	Buffer RLT Plus
< 5e6	350 $\mu$ l
5e6 to 1e7	600 $\mu$ l



Note the volume used here

Mix by vortexing or pipetting.

- 3 Homogenize the lysate by vortexing briefly, then letting rest on bench for 1 minute. Alternatively, cells can be homogenized using QIAshredder spin columns or by repeatedly passing through a 20-gauge needle.
- 4 Transfer lysate to a gDNA Eliminator spin column placed in a 2 ml collection tube. Centrifuge for 30 seconds at  $\geq 8,000g$ . Ensure no liquid remains on the column membrane. Repeat centrifugation if necessary. Keep the flow-through and discard the column.
- 5 Add 1 volume (i.e. 350 or 600  $\mu$ l) 70% ethanol to the flow-through and mix by pipetting.
- 6 Transfer up to 700  $\mu$ l of the sample to an RNEasy spin column placed in a 2 ml collection tube. Spin for 15 seconds at  $\geq 8,000g$ . Discard the flow-through. If sample volume was greater than 700  $\mu$ l, centrifuge sample in successive batches on the same column, discarding the flow-through at every step.
- 7 Add 700  $\mu$ l of Buffer RW1 to the column and spin for 15 seconds at  $\geq 8,000g$  to wash. Discard flow-through.
- 8 Prepare DNase solution by adding 10  $\mu$ l of DNaseI to 70  $\mu$ l of Buffer RDD for each sample. Add 80  $\mu$ l of DNase solution to each column. Incubate at room temperature for 15 minutes.
- 9 Add 350  $\mu$ l of Buffer RW1 to the column and spin for 15 seconds at  $\geq 8,000g$  to wash. Discard flow-through.
- 10 Add 500  $\mu$ l of Buffer RPE to the column. Spin for 15 seconds at  $\geq 8,000g$ . Discard flow-through.
- 11 Repeat Step 8 but spin for 2 minutes. Discard the flow-through and the collection tube.
- 12 Place the spin column in a new collection tube and centrifuge for 1 minute at  $\geq 8000g$ .



- 13 Place the spin column in a new 1.5 ml collection tube. Add 40 µl RNase-free water to the column and spin for 1 minute at  $\geq 8,000g$  to elute RNA. RNA can be stored at  $-80^{\circ}\text{C}$ .
- 14 Dilute 1 µl of RNA in 9 µl of ddH<sub>2</sub>O and quantitate using the Qubit RNA HS Assay Kit.

### cDNA Synthesis

- 15 For cDNA synthesis, continue processing each replicate separately. Prepare the reverse transcription (RT) reaction mix:
- 2 µg total RNA
  - 1 µl of 50 µM SMART\_dT18VN primer
  - 1 µl of 10 mM dNTPs
  - Raise to 14 µl with ddH<sub>2</sub>O
- Incubate RT mix at  **65 °C** for  **00:05:00**
- Place on ice for 1 minute
- 16 Create 1x Maxima RT buffer:
- For 5 or fewer samples, combine 1 uL of 5X Maxima RT buffer with 4 uL of ddH<sub>2</sub>O.
  - Mix by pipetting and store on ice.
- 17 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 step 16 (e.g. 2 uL of Enzyme + 2 uL of 1x buffer).

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

- 18 Add the following to the RT mix:
- 4 µl 5X Maxima RT Buffer
  - 1 µl RNaseOUT
  - 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)

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

- 19 Heat inactivate the reaction by incubating at  **85 °C** for  **00:10:00**

- 20 Clean up reaction using 1 µl RNase H and incubating at  **37 °C** for  **00:30:00**



Digestion with RNase H removes the complementary RNA strand from the DNA-RNA first strand duplex. This is thought to aid amplification of longer cDNA molecules (> 1 kb)

- 21 cDNA can be stored at  $-20^{\circ}\text{C}$

### Amplification of Self-Reporting Transcripts

- 22 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 (SMART)
  - 2 µl of cDNA
  - 21 µl of ddH<sub>2</sub>O
  - 1 µl of Forward Primer, either:
    - 25 µM SRT\_PAC\_F1 primer, if using PB-SRT-Puro

- 25  $\mu$ M SRT\_tdTomato\_F1, if using PB-SRT-tdTomato



This PCR can be run as half-size reactions by halving each of the listed volumes. If you find yourself doing this PCR repeatedly, this can be a way to decrease costs.

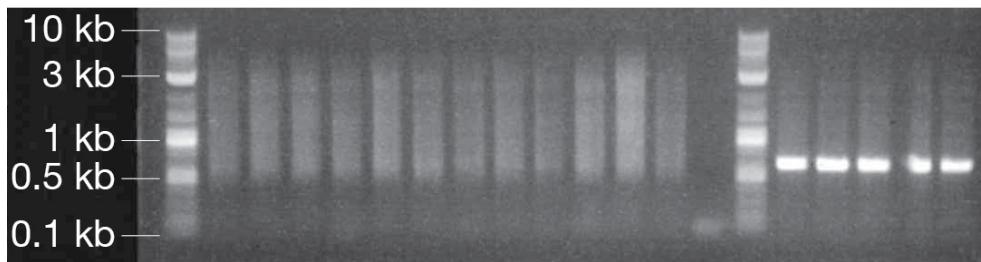
If you have multiple replicates, amplify them separately.

## 23 Perform PCR using the following thermocycling parameters:

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

## 24 At this point, gel electrophoresis can be performed to check the quality of amplification. We recommend running 5 $\mu$ L of the PCR product from step 23 on 1% TAE agarose gel. The expected product is a smear extending from ~1 kb up to 5 kb.


As a control, we recommend that the constitutive  $\beta$ -actin gene be amplified in parallel to the calling card libraries in steps 22 and 23. The control amplification uses the same PCR mix and thermocycler settings as step 22 and 23, but replaces the calling card forward and reverse primers with human  $\beta$ -actin primers (sequence provided in Materials as Raff\_ACTB\_F and Raff\_ACTB\_R). The expected product of the  $\beta$ -actin amplification is 626 bp (see Figure 1 in <https://doi.org/10.2144/97233st02>).



Representative products of SRT amplification

1% TAE gel showing expected products of SRT amplification. Left: the first 12 lanes are biological replicates of a calling card experiment, while the thirteenth is a no template control. The calling card libraries appear as smears extending up to 5 kb. Right: amplification of  $\beta$ -actin with Raff\_ACTB\_F and Raff\_ACTB\_R from the same RT product as the SRT samples produces the expected 626-bp product. The ladder is NEB's 1 kb Plus (previously, 2-Log) DNA Ladder (#N3200S).

## Purification of PCR Products





- 25 Vortex AMPure XP beads to resuspend them. Beads should be brought to room temperature for at least 30 minutes prior to use.
- 26 Add 30  $\mu$ L beads to each 50  $\mu$ L PCR mixture (0.6x ratio; if you did a half-size PCR, add 15  $\mu$ L beads). Mix by pipetting 10 times until evenly dispersed.
- 27 Incubate at room temperature for  00:05:00
- 28 Place on a magnetic rack for 2 minutes. Aspirate supernatant and discard.

- 29 Add 200 µl of freshly-prepared 70% ethanol and incubate  $\geq$  30 seconds. Aspirate supernatant and discard.
- 30 Repeat Step #27.
- 31 Air dry the pellet at room temperature for 2 minutes.
- 32 Remove the tube from the magnetic rack. Add 20 µl ddH<sub>2</sub>O to elute PCR products. Mix by pipetting until evenly dispersed. Incubate off the rack for 2 minutes.
- 33 Place on magnetic rack for 1 minute, or until supernatant is clear.
- 34 Transfer supernatant to new tube. Create a 1:10 dilution and quantitate using the Qubit dsDNA HS Assay Kit.



Expected concentration of product should be 10-20 ng/µl.


#### Generation of Bulk Calling Card Libraries

- 35 The tagmentation protocol fragments the long PCR products into libraries suitable for sequencing. This protocol is based on the standard Drop-seq library preparation workflow. Continue processing each replicate independently.
- 36 Preheat thermocycler to  55 °C
- 37 Take 1 ng of PCR product and resuspend in a total of 5 µl ddH<sub>2</sub>O 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 Add the following to each PCR tube in order:
  - 15 µl Nextera PCR Mix (NPM)
  - 8 µl ddH<sub>2</sub>O
  - 1 µl of 10 µM barcoded *piggyBac* primer (e.g. OM-PB-ACG)
  - 1 µl of 10 µM indexed Nextera N7 primer (e.g. Nextera\_N701)

Each replicate should be identifiable by its barcode-index combination. It would be ideal if each replicate had a unique barcode *and* a unique index assigned to it. For some experimental setups, that may not be feasible. One option might be to assign a different index for different conditions/treatments, and within a condition/treatment, assign different barcodes to each replicate.

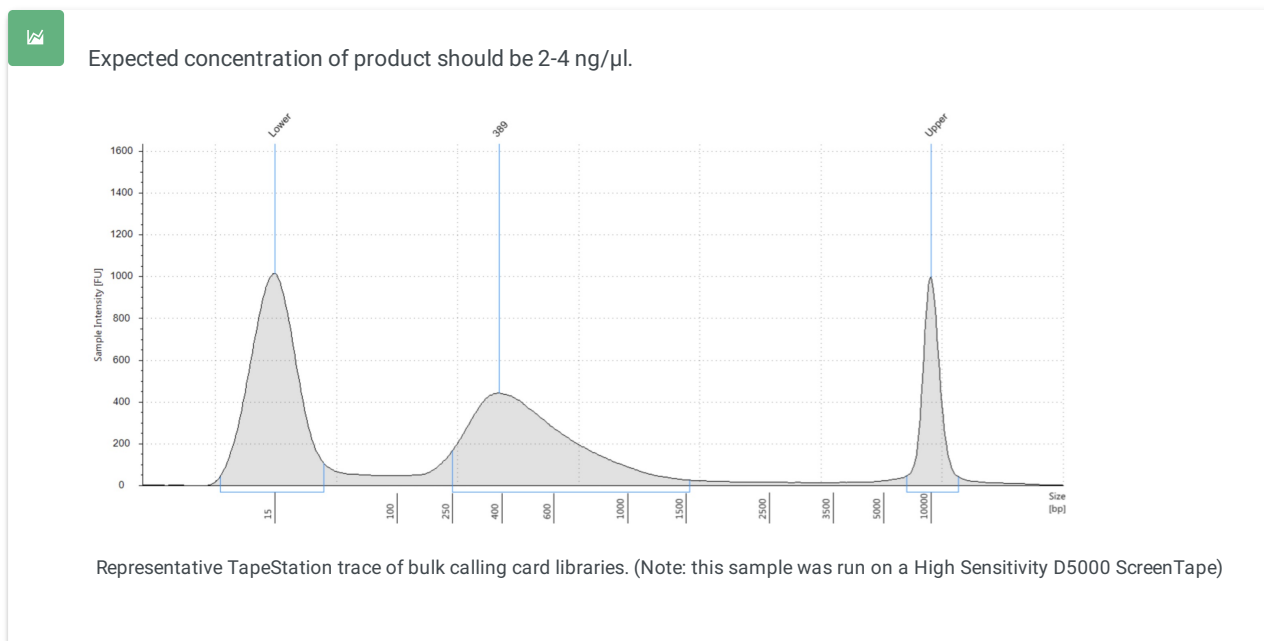
- 41 Perform PCR using the following thermocycling parameters:
  - 95°C for 3 minutes
  - 13 cycles of:
    - 95°C for 10 seconds
    - 50°C for 30 seconds
    - 72°C for 30 seconds

- 72°C for 5 minutes
- 4°C forever

- 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 and discard.
- 46 Add 200 µl of freshly-prepared 70% ethanol and incubate ≥ 30 seconds. Aspirate supernatant and discard.
- 47 Repeat Step #27.
- 48 Air dry the pellet at room temperature for 2 minutes.
- 49 Remove the tube from the magnetic rack. Add 11 µl ddH<sub>2</sub>O 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.

#### Final Quantitation and Sequencing

- 51 Create a 1:10 dilution of each final library. Measure concentrations using the Qubit dsDNA HS Assay Kit or on a TapeStation device with a High Sensitivity D1000 ScreenTape. Libraries should be smoothly distributed between 300-60 bp.



52 Libraries can be sequenced on any Illumina sequencing platform. Due to the low complexity nature of calling card libraries, we recommend adding PhiX at a final concentration of 50%.



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.



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