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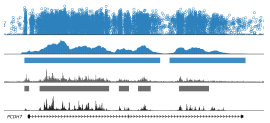
Bulk Calling Cards Library Preparation



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Transposon Calling Cards

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

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We use this protocol and it's working

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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 (YFTF) fused to *piggyBac*. Your cells are now ready for RNA extraction, SRT amplification, and library preparation.

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

MATERIALS

- Agencourt Ampure XP **Beckman Coulter** **Catalog #**A63880
- dNTP **Takara Bio Inc.** **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
- Capillary electrophoresis instrument (e.g. Agilent Tapestation 4200)
- RNEasy Plus Mini Kit **Qiagen** **Catalog #**74134
- 2-mercaptoethanol **Gibco - Thermo Fisher Scientific** **Catalog #**21985023
- Qubit RNA HS Assay Kit **Thermo Fisher Scientific** **Catalog #**Q32852
- RNaseOUT™ Recombinant Ribonuclease Inhibitor **Thermo Fisher Scientific** **Catalog #**10777019
- RNase H **New England Biolabs** **Catalog #**M0297S
- 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

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)

AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTACGTTTACGCAGACTATCTTT CTAG

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_2O)

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 start

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 µl
	5e6 to 1e7	600 µl

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 ≥ 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 µl) 70% ethanol to the flow-through and mix by pipetting.

6 Transfer up to 700 µl of the sample to an RNEasy spin column placed in a 2 ml collection tube. Spin for 15 seconds at ≥ 8,000g. Discard the flow-through. If sample volume was greater than 700 µl, centrifuge sample in successive batches on the same column, discarding the flow-through at every step.

7 Add 700 µl of Buffer RW1 to the column and spin for 15 seconds at ≥ 8,000g to wash. Discard flow-through.

8 Prepare DNase solution by adding 10 µl of DNaseI to 70 µl of Buffer RDD for each sample. Add 80ul of DNase solution to each column. Incubate at room temperature for 15

minutes.

9 Add 350 µl of Buffer RW1 to the column and spin for 15 seconds at ≥ 8,000g to wash. Discard flow-through.

10 Add 500 µl of Buffer RPE to the column. Spin for 15 seconds at ≥ 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 ≥ 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 ≥ 8,000g to elute RNA. RNA can be stored at –80ºC.

14 Dilute 1 µl of RNA in 9 µl of ddH_2O 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_2O

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_2O.
- 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

Note

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 –20ºC

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_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

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.

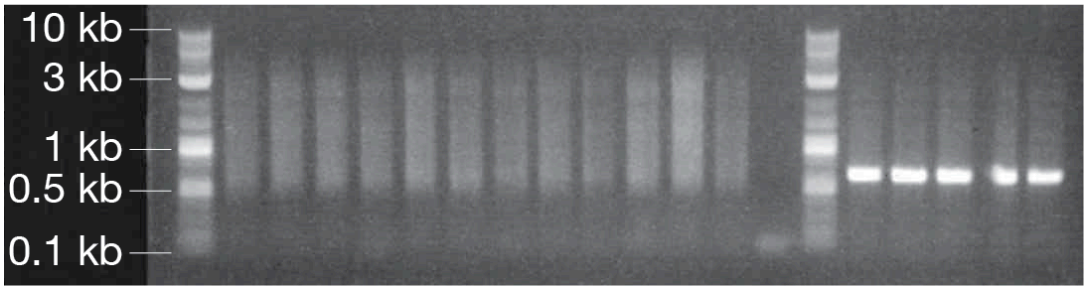
If you have multiple replicates, amplify them separately.

23 Perform PCR using the following thermocycling parameters:
- 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

24 At this point, gel electrophoresis can be performed to check the quality of amplification. We recommend running 5 uL 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 β-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 β-actin primers (sequence provided in Materials as Raff_ACTB_F and Raff_ACTB_R). The expected product of the B-actin amplification is 626 bp (see Figure 1 in https://doi.org/10.2144/97233st02).

Expected result



Representative products of SRT amplifcation

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 β-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 µl beads to each 50 µl PCR mixture (0.6x ratio; if you did a half-size PCR, add 15 µl 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 ≥ 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_2O 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 result

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_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 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. 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:
- 95ºC for 3 minutes
- 13 cycles of:
- 95ºC for 10 seconds
- 50ºC for 30 seconds
- 72ºC for 30 seconds
- 72ºC for 5 minutes
- 4ºC 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_2O to elute PCR products. Mix by pipetting until evenly dispersed. Incubate off the rack for 2 minutes.

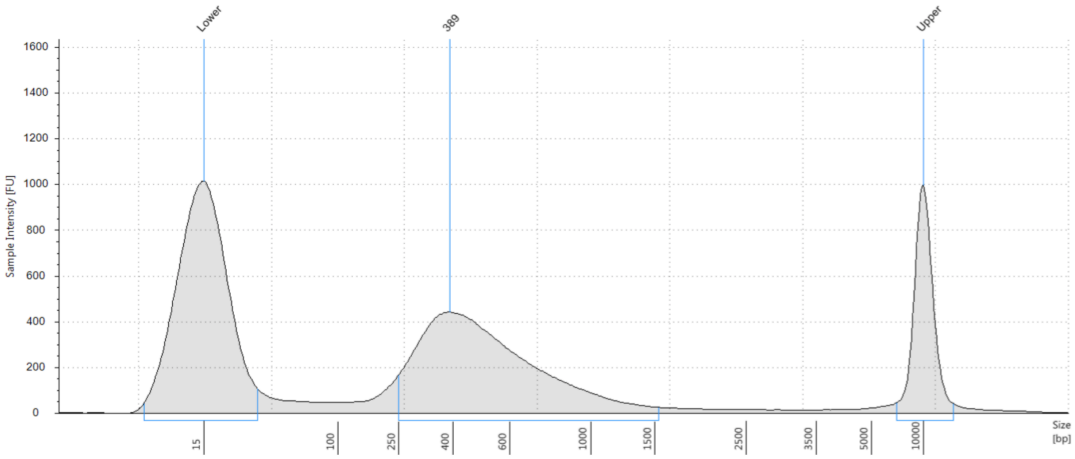
50 Place on magnetic rack for 1 minute, or until supernatant is clear. Transfer supernatant to new tube.

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.

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

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.