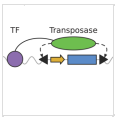

Apr 03, 2019

Working

Mammalian Calling Cards Quick Start Guide

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Transposon Calling Cards

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ABSTRACT

Transposon calling cards can identify transcription factor (TF) binding sites. This involves fusing your favorite TF (YFTF) to the hyperactive *piggyBac* transposase (HyPBase). This is delivered to cells in conjunction with a *piggyBac* transposon. The TF will visit sites in the genome and YFTF-HyPBase will deposit transposons near binding sites. We then generate sequencing libraries to map the genome-wide localization of transposons. Finally, we identify significant clusters of insertions to identify TF binding sites.

PROTOCOL STATUS

Working

We use this protocol in our group and it is working

GUIDELINES

These experiments are intended to introduce you to the calling cards assay. We recommend following them to establish baseline confidence in your transcription factor constructs before proceeding to your favorite model system. Alternatively, if you just wish to use the directed *piggyBac* transposase, you can use this protocol to familiarize yourself with our molecular workflow.

MATERIALS

NAME	CATALOG #	VENDOR
Lipofectamine 3000	L3000015	Thermo Fisher Scientific

MATERIALS TEXT

- **pRM1258/pENTR_myc-hypPBase:** This Gateway entry vector contains the HyPBase gene. It is convenient to make the YFTF-HyPBase fusions in this vector because you can easily port the fusion into a variety of other plasmids for AAV packaging, homologous recombination into the *Rosa26* locus, etc.
- **pRM1114/CMV_HyPBase:** This is our standard positive control transposase plasmid. For some experiments it may be more convenient to make your YFTF-HyPB fusion in this construct.
- **pRM1304/PB_SRT_Rz_Puro:** This is a *piggyBac* self-reporting transposon (SRT) encoding a puromycin resistance gene. Cells transfected with this plasmid and HyPBase survive puromycin selection.
- **pRM1535/SRT_tdTtomato**. This is a *piggyBac* self-reporting transposon (SRT) donor encoding a tdTomato fluorescent reporter. Cells transfected with this plasmid and HyPBase can be sorted for based on high fluorescence signal.
- **pRM1294/BrokenHeart:** This plasmid is a reporter of *piggyBac* transposase activity. It encodes the DsRed fluorescent protein gene interrupted by a *piggyBac* transposon. When cells are co-transfected with BrokenHeart and *piggyBac*, the transposon is removed and the DsRed reading frame is restored. These cells fluoresce brightly, while cells transfected with BrokenHeart alone will not fluoresce.
- A non-fluorescent control, or "empty," plasmid. This can be something you use regularly in-house or a commercially available plasmid, such as NEB's pUC19 vector (#N3041S).
- Optional: a GFP expression plasmid as a transfection control. This can be something used routinely in your lab or a commercially available vector, such as Addgene #54767.

- HCT-116 cells. Stocks may be obtained from ATCC (#CCL-247) if necessary.

SAFETY WARNINGS

Cloning and Sequence Validation of YFTF-HyPBase Fusions

1 *Make C- and N- terminal fusions of your favorite transcription factor (YFTF) with HyPBase*. It is important to include a linker sequence between these genes. We strongly recommend the following amino acid linker sequence: KLGGGAPAVGGGPKAADK. We have tested many and have found this sequence works best. It is often convenient to make these fusion constructs by using In-Fusion (Clontech/Takara) or Gibson (NEB) cloning to drop YFTF into pRM1258/pENTR_myc-hypPBase. We do not have good antibodies to the *piggyBac* transposase, so we recommend designing your construct so that the chimeric protein is tagged with *myc*.

2 *Validate the constructs*. Perform restriction digest analysis on the plasmid with at least 3 restriction enzymes to make sure there were no gross rearrangements. Next, Sanger sequence the full chimeric gene, or alternatively perform Illumina sequencing on the whole plasmid. It is important to do the restriction digest and EITHER of the sequencing stratgies.

3 *(Only required for Gateway strategy)*. Move the chimeric gene from the pENTR vector to an expression vector.

Functional Validation of YFTF-HyPBase Fusions

4 After creating the YFTF-HyPBase and HyPBase-YFTF fusions, the next steps are to validate them. First we will assess whether the fusions retain *piggyBac* transposase activity. We recommend transforming HCT-116 cells with the YFTF fusions and the BrokenHeart transposon along with appropriate controls. We recommend using Lipofectamine 3000 (following manufacturer's instructions) to deliver 1 µg total DNA to approximately 200,000 cells in each well of a 6-well plate. The following table summarizes each condition and the expected results.

Condition	Empty plasmid	pRM1294 BrokenHeart	pRM1114 CMV_HyPBase	YFTF-HyPBase	HyPBase-YFTF	Red cells?	Notes
Negative control	*0.5 µg*	*0.5 µg*	NA	NA	NA	None	
Positive control	NA	*0.5 µg*	*0.5 µg*	NA	NA	Many	
YFTF-HyPBase	NA	*0.5 µg*	NA	*0.5 µg*	NA	Some	Perform in duplicate
HyPBase-YFTF	NA	*0.5 µg*	NA	NA	*0.5 µg*	Some	Perform in duplicate

Optional control #1 -- a "lipofection only" negative control. This is not a bad idea, particularly if you are new to lipofections or are testing a new cell line and are concerned about toxicity. These cells should show high viability and no fluorescence signal. If these cells are viable but cells transfected with DNA are not, it may indicate issues with plasmid isolation (e.g. endotoxin contamination).

Optional control #2 -- a GFP expression plasmid could be transfected in parallel to estimate overall transfection efficiencies.

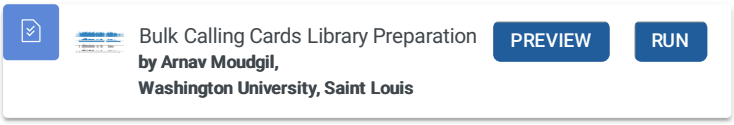
5 The second validation will test whether the YFTF-fusions successfully redirect *piggyBac* insertions near YFTF binding sites. Since *piggyBac* inserts into TTAAs, we would like to be able to distinguish unique insertions into the same TTAA. For this reason, we recommend 6 replicates per condition, at least for your two “test” samples, and the unfused *piggyBac* (1 6-well plate each, 3 total). In addition, we recommend running one well as a transposon-only negative control and one well as a mock lipofection negative control. Here we will use puromycin selection to obtain cells with transpositions. Once again, we will work with HCT-116 cells.

Condition	**Empty plasmid**	**pRM1304 PB_SRT_Rz_Puro**	**pRM1114 CMV_HyPBase**	**YFTF-HyPBase**	**HyPBase-YFTF**	**Alive cells?**	**Notes**
No transfection control	1 µg	NA	NA	NA	NA	None	No colonies after selection
SRT only control	0.5 µg	0.5 µg	NA	NA	NA	None	No colonies after a few days
Positive control	NA	0.5 µg	0.5 µg	NA	NA	Many	Perform 6 replicates
YFTF-HyPBase	NA	0.5 µg	NA	0.5 µg	NA	Some	Perform 6 replicates
HyPBase-YFTF	NA	0.5 µg	NA	NA	0.5 µg	Some	Perform 6 replicates

We typically split each well 1:1 and transfer to a 10 cm dish after 24 hours. We add puromycin to a final concentration of 2 µg/ml after cells have seeded, typically 6-8 hours after transferring. Media is replenished every two days. All replicates are cultured separately. Cells are harvested after the SRT-only control transfectants are dead.

Calling Card Library Preparation

6 Calling card libraries can now be made from successfully selected cells. For first-time users, we recommend following the bulk calling card protocol for making libraries.



6.1 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.

6.2 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.

6.3 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.

6.4 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.

6.5 Add 1 volume (i.e. 350 or 600 µl) 70% ethanol to the flow-through and mix by pipetting.

6.6 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.

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

6.8 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.

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

6.10 Add 500 µl of Buffer RPE to the column. Spin for 15 seconds at ≥ 8,000g. Discard flow-through.

6.11 Repeat Step 8 but spin for 2 minutes. Discard the flow-through and the collection tube.

6.12 Place the spin column in a new collection tube and centrifuge for 1 minute at ≥ 8000g.

6.13 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.

6.14 Dilute 1 µl of RNA in 9 µl of ddH_2O and quantitate using the Qubit RNA HS Assay Kit.

6.15 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

6.16 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.

6.17 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.

6.18 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**

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

6.20 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)

6.21 cDNA can be stored at –20ºC

6.22 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

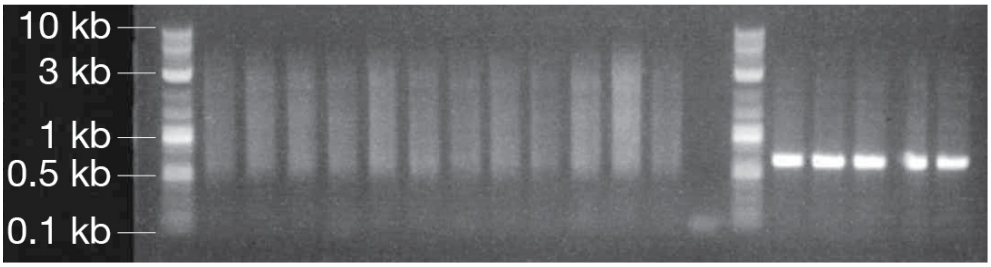
This PCR can be run as half-size reactions by halving each of the listed volumes. If you find yourself doing this PCR repeatedly, this can be a way to decrease costs.

If you have multiple replicates, amplify them separately.

6.23 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

6.24 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).



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).

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

6.26 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.

6.27 Incubate at room temperature for 00:05:00

6.28 Place on a magnetic rack for 2 minutes. Aspirate supernatant and discard.

6.29 Add 200 µl of freshly-prepared 70% ethanol and incubate ≥ 30 seconds. Aspirate supernatant and discard.

6.30 Repeat Step #27.

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

6.32 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.

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

6.34 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.

6.35 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.

6.36 Preheat thermocycler to **55 °C**

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

6.38 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**

6.39 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**

6.40 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.

6.41 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

6.42 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.

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

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

6.45 Place on a magnetic rack for 2 minutes. Aspirate supernatant and discard.

6.46 Add 200 µl of freshly-prepared 70% ethanol and incubate ≥ 30 seconds. Aspirate supernatant and discard.

6.47 Repeat Step #27.

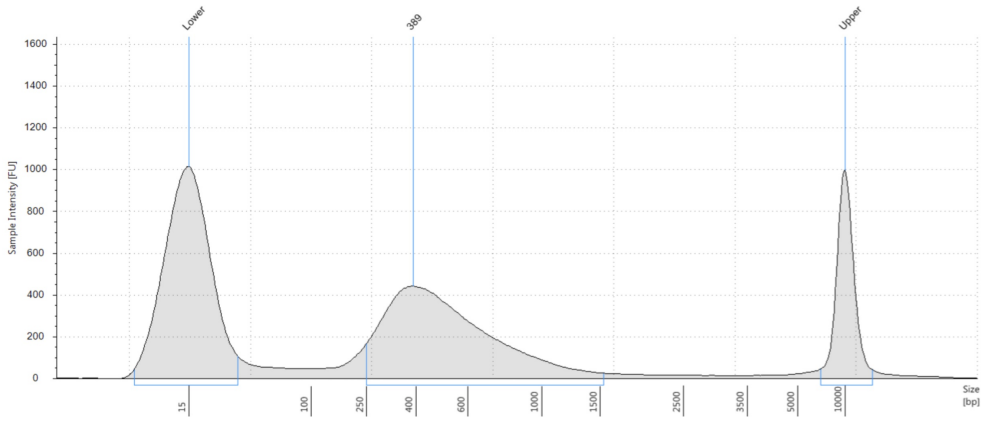
6.48 Air dry the pellet at room temperature for 2 minutes.

6.49 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.

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

6.51 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 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)

6.52 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%.

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.

7 *(Advanced)* Depending on your application, you may also be interested in making single cell calling card libraries.

Single Cell Calling Cards Library Preparation PREVIEW RUN
by Arnav Moudgil,
Washington University, Saint Louis

7.1 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.

7.2 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**

7.3 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.

7.4 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.

7.5 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.

7.6 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

7.7 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.

7.8 Incubate:

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

7.9 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.

7.10 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.

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

7.12 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.

7.13 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.

7.14 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.

7.15 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.

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

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

7.18 Incubate at room temperature for 00:05:00

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

Aspirate supernatant and discard.

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

7.21 Repeat Step #20

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

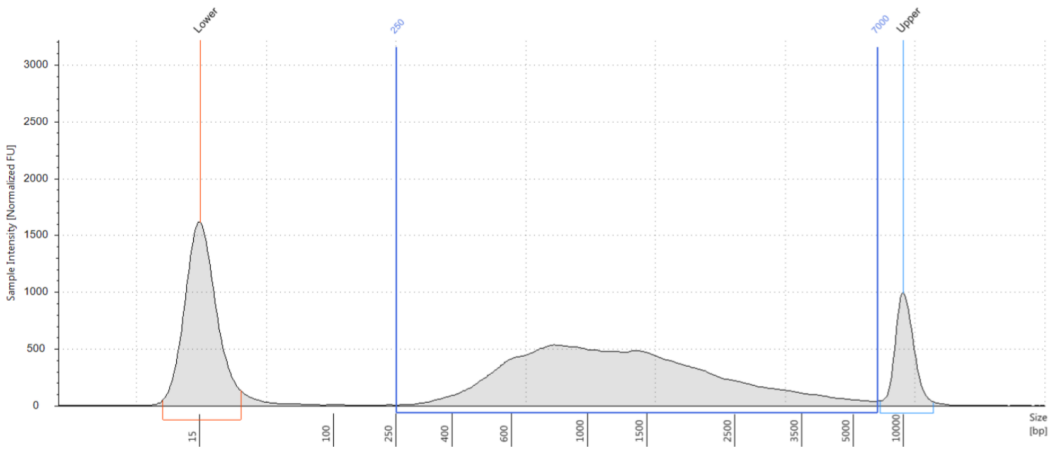
7.23 Remove the tube from the magnetic rack. Add 40 µl QIAGEN Elution Buffer to elute PCR products. Mix by pipetting until evenly dispersed.

Incubate off the rack for **00:05:00**

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

7.25 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

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

7.27 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).

7.28 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**

7.29 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

7.30 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

7.31 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.

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.

7.32 Resuspend Dynabeads M-280 by vortexing briefly.

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

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

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

7.36 Repeat Step #35.

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

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

7.39 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.

7.40 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

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

7.42 Incubate **20 °C** **00:15:00**

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

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

7.44 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

7.45 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.

7.46 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

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

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

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

7.50 Incubate **30 °C** **00:30:00**

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

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

7.52 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

7.53 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.

7.54 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.

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

7.56 Incubate 37 °C 00:30:00

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

7.57 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).

7.58 Incubate 30 °C 00:10:00

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

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

7.61 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

7.62 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.

7.63 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)

7.64 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.

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

7.66 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.

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

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

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

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

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

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

7.73 Repeat Step #72

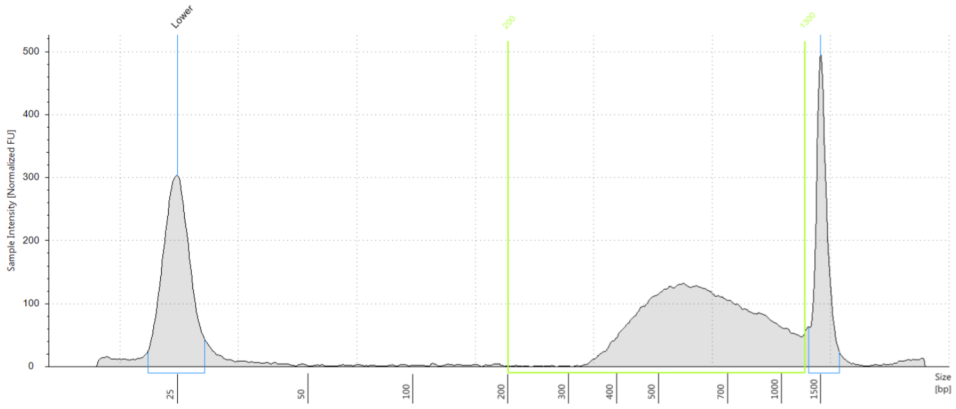
7.74 Air dry the pellet at room temperature for **00:02:00**

7.75 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**

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

7.77 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

7.78 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%.

7.79 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.

Sequencing, Analyzing, and Visualizing Calling Card Data

8 If your library preparation has been successful, you are ready to sequence your calling card libraries. We have successfully sequenced libraries on the Illumina MiSeq, MiniSeq, HiSeq, and NextSeq platforms. Due to relatively low sequence complexity, we typically run our libraries with 50% PhiX genome spiked in.

Next Steps

9 If you've made it this far and your data look great, congratulations! One or both of the YFTF fusions have worked and successfully enriched for insertions near YFTF binding sites. You may now wish to repeat the above experimental workflow with your model systems, or try different transgenesis techniques (e.g. electroporation, nucleofection, viral transduction). Otherwise, you are now ready to move into your model system and study YFTF binding. Please let us know if you have any difficulties and we will do our best to provide assistance.