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ATAC-STARR-seq

I. Materials

**Oligo sequences available in Hansen & Hodges NAR 2021.*

** Plasmids*

- hSTARR-Seq_ORI (Addgene #99296)
- pcDNA3.1-eGFP (Addgene #13031)

**Special Equipment:*

- Gene Pulser Xcell Microbial System (Bio-Rad, #1652662) – *the Cortez Lab has one.*
- Neon™ Transfection System (Invitrogen, #MPK5000)
- 4150 TapeStation System (Agilent, #G2992AA)
- DynaMag™-PCR Magnet (Invitrogen, #492025)
- DynaMag™-2 Magnet (Invitrogen, #12321D)

**Buffer Recipes:*

- STE Buffer - (10 mM Tris-HCl pH 8.0, 50 mM NaCl, 1mM EDTA, pH 8.0)
- Tn5 Storage Buffer - (50 mM Hepes pH 7.2, 100 mM NaCl, 0.1 mM EDTA, 1mM DTT, 0.1% Triton-X 100, 50% Glycerol)
- ATAC-Resuspension Buffer - (10mM Tris-HCl pH 7.4, 10mM NaCl, 3mM MgCl2)
- 5x Tris-DMF - (50 mM Tris-HCl pH 7.5, 25 mM $MgCl_2$, 50% DMF). *Pipette DMF with a glass pipette.*
- Flow cytometry buffer – (1xPBS + 1%BSA)
- Recovery Media – RPMI 1640, 20% FBS
- Maintenance Media – RPMI 1640, 15% FBS, 2mM GlutaMax, 1X Penn/Strep
- Binding Buffer – 20 mM Tris-HCl, pH 7.5, 1.0 M LiCl, 2 mM EDTA
- Wash Buffer A – 10 mM Tris-HCl, pH 7.5 ,0.15 M LiCl, 1 mM EDTA 10 mM Tris-HCl, pH 7.5

**Reagents and consumables:*

- 10% IGEPAL CA-630 – (Roche cat# 11332473001) *Also called NP-40 substitute, IGEPAL CA-630 is supplied at 10%. Store at 4°C.*
- Digitonin (Cayman Chemical, #14952) – *Prepared Digitonin at 2% in DMSO (20mg/mL). Dilute 1:1 with water to make a 1% (100x) stock solution. Avoid more than 5 freeze thaw cycles of 2%* solution. *1% can be kept at -20°C for up to 6 months and does not freeze at -20°C*.
- Tween-20 – (Roche cat# 11332465001) *Tween-20 is supplied at 10%. Store at 4°C.*
- DNA Clean & Concentrate-5 (Zymo Research, #D4004)
- NEBNext® High-Fidelity 2X PCR Master Mix (M0541L)
- High Sensitivity D5000 ScreenTape (Agilent, # 5067-5592)
- High Sensitivity D5000 Reagents (Agilent, #5067-5593)

- SPRIselect (Beckman-Coulter, #B23317)
- DynaMag™-PCR Magnet (Invitrogen, #492025)
- NEBNext® Ultra™ II Q5® Master Mix (NEB, #M0544L)
- NEBuilder® HiFi DNA Assembly Master Mix (NEB, #E2621L)
- GlycoBlue™ Coprecipitant (15 mg/mL) (Invitrogen, # AM9515)
- MegaX DH10B™ T1R Electrocomp™ Cells (Invitrogen, # C640003)
- Gene Pulser/MicroPulser Electroporation Cuvettes, 0.1 cm gap (Bio-Rad, #1652089), *these can be reused—clean with 70% EtOH and dry overnight.*
- ZymoPURE™ II Plasmid Gigaprep Kit (Zymo Research, #D4204)
- Neon™ Transfection System 100 µL Kit (Invitrogen, #MPK10096)
- Propidium iodide solution (1mg/mL in water) (Sigma-Aldrich, #P4864)
- TRIzol™ Reagent and Phasemaker™ Tubes Complete System (Invitrogen™, #A33251)
- ZymoPURE™ II Plasmid Midiprep Kit (Zymo Research, #D4200)
- Oligo d(T)25 Magnetic Beads (NEB, #S1419S)
- DNaseI (NEB, #M0303S)
- Zymo Research RNA Clean & Concentrator-25 kit (Zymo Research, #R1018)
- PrimeScript™ Reverse Transcriptase (Takara, #2680)
- KAPA dNTP Mix (10 mM each) (Roche, #KK1017)
- RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen, #10777019). We have also used RiboLock RNase Inhibitor (Thermo Scientific, #EO0382).
- RNase A, DNase and protease-free (10 mg/mL) (Thermo Scientific, #EN0531)

II. Annealing oligos:

**Many recommend adding Magnesium to help stabilize two annealing oligos. Do NOT do this for this Annealing oligo step.

Dilute oligos to 100 µM in STE buffer.

In a 200 µL PCR tube,
Mix (Adaptor A) [50µL]:
5µL Tn5MERV oligo (100uM)
5µL Tn5_1 Oligo (100uM)
40µL H_2O

In a separate 200µL PCR tube,
Mix (Adaptor B) [50µL]:
5µL Tn5MERV oligo (100uM)
5µL Tn5_2_ME_Comp Oligo (100uM)
40µL H_2O

Anneal Adaptor solutions separately with thermocycler conditions as follows:
95°C, 3 min
65°C, 3 min
Ramp to 24°C, -1ºC per cycle, 30 s
Hold at 24°C forever

After annealing mix:
50µL Adaptor solution A

50µL Adaptor solution B
100µL Glycerol (≥99.5%)
200 µL Adaptor mixture, which is now 5 µM.

III. Transposome assembly:
*Each sample uses 40µL. The recipe below can be scaled up if needed.

1. Mix the following:

25	µL	5µM Adaptors Mix
25	µL	Tn5 (3mg/mL in Tn5 storage buffer)
20	µL	Total Reaction Volume

2. Let stand at room temp for 30-60min, then immediately place on ice. Assembled transposome may be stored at -20°C for several months.

IV. ATAC reaction:

1. Turn on all equipment!!
2. Make ATAC-Resuspension Buffer (RSB):
    a. *For 50 ml ATAC-RSB, combine 1mL 500mM Tris-HCl pH 7.4, 100 ul 5M NaCl, 300 ul 0.5M MgCl2, and 48.6 ml sterile water.*
    b. *Keep on ice.*
3. Count cells in duplicate using a hemocytometer. *Note: It is important that cell viability is greater than 85%. If less than 85%, I strongly recommend trying again when viability is much better.* **Cell count:**

4. In two tubes, pellet 200,000 viable cells in each tube at 500 RCF at 4°C for 10 min in a fixed angle centrifuge. Use **low-bind Eppendorf tubes** to prevent pellets from sticking along the back of the tube (this is much more important for reproducibility than you might think).
5. While waiting, make lysis buffers.
    a. ATAC-Resuspension Buffer (RSB) containing 0.1% NP40, 0.1% Tween-20, and 0.01% digitonin (lysis buffer):
        i. *For 110µL, mix 1.1µL 10% Tween-20, 1.1µL 10% IGEPAL CA-630, 1.1µL 1% digitonin, 106.7µL 1x RSB.*
        ii. *Keep on ice.*
    b. ATAC-RSB containing only 0.1% Tween-20 (wash out buffer):
        i. *For 2.2mL, mix 220µL 10% Tween-20 and 2mL 1x RSB*
        ii. *Keep on ice.*
6. Aspirate supernatant and resuspend each cell pellet in 50µL cold ATAC-Resuspension Buffer (RSB) containing 0.1% IGEPAL CA-630, 0.1% Tween-20, and

0.01% Digitonin and pipette up and down 5 times. *The volume used here is a modification to the Omin-ATAC protocol. In my hands, 50µL for this number of cells works just as well as 200µL and is much more manageable.*

7. Incubate on ice for 3 minutes.
8. Wash out lysis with 1 ml of cold ATAC-RSB containing ONLY 0.1% Tween-20 and invert tube 3 times to mix. *The volume used here is a modification to the Omin-ATAC protocol. In my hands, 1mL for this number of cells works just as well as 4mL and is much more manageable.*
9. Immediately pellet nuclei at 500 RCF for 10min at 4°C in a fixed angle centrifuge. While waiting, make transposition mix.
10. Aspirate all supernatant, carefully avoiding visible cell pellet, using two pipetting steps (aspirate down to 100 ul with a p1000 pipette and remove final 100 ul with a p200 pipette).
11. Resuspend cell pellets in 180µL of transposition mix by pipetting up and down 10 times. Combine like samples for a total volume of 360µL.

Transposition mix:

Component	Per Sample (2 tubes)	X___	Master Mix
5x Tris-DMF buffer	80µL		
PBS	132µL		
1% digitonin	4µL		
10% Tween-20	4µL		
Nuclease free H2O	140µL		
TOTAL	*360µL*		

12. Add 40µL assembled Tn5 transposase and quickly vortex to mix well. *This is the equivalent to 5µL/reaction.*
13. Divide resuspension into 8-1.5mL tubes (50µL/tube).
14. Incubate reaction at 37°C for 30min in a thermomixer with 1000 RPM mixing.
15. After incubation, pool the eight like-reactions together in a 15mL conical tube containing 2mL DNA binding Buffer.
16. Purify the small-isolated DNA fragments using the Zymo C+C kit. Run all 2.4mL through one column, adding in 800µL increments. Elute in 22µl pre-warmed elution buffer. Incubate on column for 5min. Can stop here and store at -20°C.

V. PCR Amplification:

1. Make PCR mastermix:

Component	Per reaction	X___	Master Mix
NEBNext High-Fidelity 2x PCR MasterMix	25µL		
10µM Fwd_atac-starr_tag primer	1.5µL		
10µM Rev_atac-starr_tag primer	1.5µL		
Nuclease free H2O	2µL		
TOTAL	*30µL*		

**Note: do not use a hot-start polymerase here. The reaction requires polymerase activity in the initial extension step.*

2. Run PCR with the following thermocycler parameters:

   72 ºC, 5 min
   98ºC, 30 sec
   4 cycles of
   98ºC, 10 sec
   62ºC, 30 sec
   72ºC, 1 min
   Final extension 72ºC, 2 min

3. Purify PCR Product using Zymo Clean and Concentrator Kit. Mix 250µL Binding Buffer with 50µL PCR products. Elute in 32µl pre-warmed elution buffer. Incubate on column for 5min.
4. Analyze by running 2uL on High-Sensitivity D5000 Tapestation. Determine size distribution and molarity. If it looks good, move on to cloning. Need between 0.04pmols and 0.27pmols total—no more, no less. If there are too many large fragments, perform the SPRI bead size selection below.

VI. SPRI bead size-selection:

*Perform a 0.6X ratio right-side selection to remove fragments larger than 500bp. The volumes listed below are for that ratio specifically. Reference the SPRISelect User Manual if this needs to be altered.

1. Thoroughly shake the SPRIselect bottle to resuspend the SPRI beads.
2. Add 18µL of SPRIselect to the sample. Pipette up and down and vortex for at least 5 seconds to mix thoroughly. *Insufficient mixing of sample and SPRIselect will lead to inconsistent size selection results.*
3. Place the reaction vessel on DynaMag-PCR magnet and allow the SPRI beads to settle to the magnet for ~1min.
4. Transfer the clear supernatant, which contains the Right-Side Size Selected sample, to a new reaction vessel. The reaction vessel with the remaining beads can be discarded.
5. Add 36µL of SPRIselect to the Right-Side Size Selected sample. Pipette up and down and vortex for at least 5 seconds to mix thoroughly.
6. Place the reaction vessel on an appropriate magnetic stand or plate and allow the SPRI beads to settle to the magnet for ~1min.
7. Remove and discard the clear supernatant.
8. With the reaction vessel still on the magnet, add 180 µL of freshly-made 85% ethanol and incubate at RT for 30 seconds. Remove and discard ALL of the ethanol supernatant.
9. Let the beads dry but not over dry.

10. Once cracks are starting to form in the pellet, elute by removing the reaction vessel from the magnet and adding 12µL of 10mM Tris-HCl pH 8.0, elution buffer, or nuclease-free water.
11. Pipette up and down and vortex for at least 5 seconds to mix thoroughly.
12. Place the reaction vessel on an appropriate magnetic stand or plate and allow the SPRI beads to settle to the magnet for ~1min.
13. Transfer the eluate (size selected sample) to an appropriate storage vessel. *It is very important to avoid pipetting beads over to the new tube. They can cause the tape station to run inaccurately.*
14. Analyze size-selected sample by running on 2uL on High-Sensitivity D5000 Tapestation and analyze for concentration and size range.

VII. Linearization of hSTARR-Seq_ORI:

**Note: at this step, you will generate the vector required for the gibson cloning reaction. This linearized vector contains the homology arms for gibson. In the NAR paper we used an oligo to add the N504 barcode to the plasmid, this is not required for most purposes. For this reason, I've denoted the reverse primer as N5XX/universal because you can add whatever index you want OR not add an index at all, just like the forward primer.*

Component	Per reaction
Nuclease free H_2O	19µL
NEBNext **Ultra II** Q5 2x MasterMix	25µL
10µM Fwd_universal_STARR	2.5µL
10µM Rev_N5XX/universal_STARR	2.5µL
hSTARR-Seq_ORI plasmid (0.1ng/µL)	1µL
TOTAL	*50µL*

1. PCR amplify vector backbone from the hSTARR-Seq_ORI plasmid (Addgene #99296) with the above mixture and the following thermocycler conditions:

   98ºC, 30 sec
   2 cycles of
   98ºC, 10 sec
   59ºC, 30 sec
   72ºC, 90 sec
   28 cycles of
   98ºC, 10 sec
   72ºC, 2min
   Final extension 72ºC, 2 min

2. Purify PCR Product using Zymo Clean and Concentrator – 5 Kit. Elute in 20µL elution buffer.
3. Determine concentration via Nanodrop.
4. Analyze by running 0.1ng on 1% agarose gel with EtBr stain to determine purity.

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VIII. Clone fragments into linearized hSTARR-Seq_ORI via Gibson Cloning:

*This part of the protocol is intentionally flexible in order to add the entire volume of tagments to these gibson reactions. In this step, perform four Gibson reactions per sample at a 1:2 rato of vector:insert. *Note: this used to be a 1:3 ratio but was changed to 1:2 after learning that 1:2 would require fewer PCR cycles and is the recommended ratio for this enzyme cocktail.* The insert (tagments) should be at a total amount of 0.04pmols and 0.27pmols. Calculate the reaction molarity as follows:

_____ ÷ 4 = _____ ÷ 2 = _____

Insert amount (in pmols) *(# of pmol/reaction for insert)* *(# of pmol/reaction for vector)*

Volume of insert/reaction =

Volume of vector/reaction =

1. In 4 PCR tubes, combine the volumes of insert and vector as written above.
2. Bring up to a total volume of 5µL with water. *Note: The total volume cannot exceed 5µL*.
3. In a separate PCR tube, prepare a negative control where you substitute the inserts for water (only 1 reaction).
4. Add 5µL of NEBuilder Hifi DNA Assembly 2x MasterMix and mix well.
5. Incubate at 50°C for 1hr.

IX. Ethanol Precipitation of Gibson Reactions

*This is needed to prevent arching in the Electroporation step. DO NOT SKIP.

1. Pool all four like-reactions together into a **low-bind Eppendorf tube**.
2. Adjust volume to 222.5µL by adding 182.5µL elution buffer (212.5µL to negative control).
3. Add 25µL 3M NaAc pH5.2 and Vortex
4. Add 2.5µL GlycoBlue and Vortex.
5. Add 750µL cold (-20°C) 100% EtOH and vortex.
6. Incubate at -20°C for 30-120min.
7. Spin at full speed for 30min @ 4°C
8. Aspirate supernatant
9. Wash with 750µL cold 70% EtOH. Spin at full speed for 15min @ 4°C.
10. Aspirate supernatant and repeat wash.
11. Dry pellet at room temp until dry.
12. Resuspend pellet in 10µL 10mM Tris-HCl pH 8.0. Pipette up/down until reconstituted. Can stop here, store @ -20°C.

X. Electroporation of STARR-seq Plasmid Library into Bacterial Cells

*In addition to the negative control, do a positive control using the supplied pUC19. For the controls, only do one reaction and use refrozen cells if possible.

1. Pre-cool pipette tips, GenePulser cuvettes, and 1.5mL tubes at -20°C. Prewarm 2L LB+amp flasks and SOC @ 37°C.
2. Add all 10µL of each gibson sample to a pre-cooled tube (2.5µL for controls). Keep on ice.
3. Thaw MegaX DH10B cells on ice. *Each tube contains ~120µL*.
4. Add 80µL of thawed cells to each 10µL gibson sample and 20µL to each 2.5µL control using pre-cooled tips. Keep on ice.
5. Pipette SLOWLY up/down to mix with pre-cooled tips. Then add 20µL (max volume) to a GenePulser 0.1cm cuvette—may need to use 3-4 cuvettes for each sample, but only one for each control. Keep cuvettes on ice. *Make sure the volume is touching both metal plates, otherwise it will arc (the best technique here is to pipette into the corner of the cuvette at an angle so the cells reach the bottom). To help, gently tap to the bottom.*
6. Electroporate with "exponential decay" pulse type at 2kV, 200Ω, and 25µF - (Gap distance is 0.1cm for brown-capped cuvettes).
7. Immediately add 1mL pre-warmed SOC media to the 20µL DNA/bacteria mixture in the cuvette.
8. Combine like-reactions into a round bottom 14mL culture tube.
9. Recover at 37°C for 1 hour in shaker incubator.
10. After recovery make a dilution series:

Dilution	**Volume of Culture**	**Volume of LB**
1:10	20µL undiluted	180µL LB
1:100	20µL of 1:10 dilution	180µL LB
1:1,000	20µL of 1:100 dilution	180µL LB
1:10,000	20µL of 1:1000 dilution	180µL LB

11. Plate 100µL of each dilution onto a prewarmed LB+ampicillin(100µg/mL) plate. Incubate at 37°C for ~16hrs.
12. With the remaining undiluted culture, add directly to 1L pre-warmed liquid LB+ ampicillin(100µg/mL) media. Shake at 37°C O/N 225 RPM. Do not do this for the controls! ***DO NOT FORGET TO ADD THE AMPICILLIN TO THE 1L CULTURE!!!***

XI. Quantification of colony counts to estimate number of transformants

Count colonies on plates after overnight growth either manually or via automated means (imaging and particle counting with imageJ)
The equations below assume a plating of 100µL from a 1mL dilution preparation
(.01 colony count) * 1000 = # colonies in 1mL of recovery media
(.001 colony count) * 10000 = # colonies in 1mL of recovery media
(.0001 colony count) * 100000 = # colonies in 1mL of recovery media

XII. Purification of Large Scale Plasmid Library Growth

1. Record OD_{600}:
2. Pellet 1L culture at 3400xg for 10min.
3. Proceed with Zymo Plasmid GigaPrep Kit protocol according to manufacturer directions.
4. Elute with 2mL ZymoPURE Elution Buffer. Nanodrop for purity and concentration. Store DNA at -20°C.

XIII. Low-Read Sequencing of Plasmid Library to Determine Insert Complexity

*Before proceeding to electroporation, it is important to determine whether the library has sufficient complexity. To do this, perform limited-cycle PCR to add illumina adapters and amplify the insert off the plasmid. Then submit the sample sequencing and target 15 million reads. Must be PE, but the cycle number is irrelevant as long as reads can be mapped.

* To reduce rxn bias effect, perform two PCRs for each sample.

1. Prepare a mastermix:

Component	**Per Rxn**	**Mastermix (x___=2.2*#Samples)**
NEBNext Ultra II Q5 2x MasterMix	25µL	
10µM ____ P5 Nextera primer	5µL	
10µM ____ P7 Nextera primer	5µL	
1:2000 diluted AS Plasmid Library(_____ng/µL)	1µL	
NF-Water	14µL	

2. Aliquot **50**µL mix into PCR tubes.
3. Vortex and centrifuge, briefly. Run the following PCR reaction:

   98ºC, 30 sec
   8 cycles of
   98ºC, 10 sec
   68ºC, 30 sec
   72ºC, 30 sec
   Final extension 72ºC, 2 min

4. Pool like-reactions and then purify using Zymo Clean and Concentrate - 5 kit – elute in 30µL elution buffer (warm buffer to 55°C or 37°C prior to elution and incubate on column for 5min).
5. Determine concentration and size distribution via HS D5000 ScreenTape on the Agilent Tapestation. Concentration should be greater than 5nM but not much higher. Anything greater than 50nM is significantly overamplified.
6. Submit samples for paired end sequencing. In the NAR paper, we requested 25M reads at PE150 on NovaSeq.

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XIII. Electroporate ATAC-STARR Libraries into Mammalian Cells (~2-3hrs)

*Each transfection reaction has a max capacity of 5 x10^6 cells and 10µL DNA. Do 20 reactions to reach 100x10^6 cells per sample.
*To assess transfection efficiency, conduct an eGFP electroporation and no DNA electroporation (as unstained control). For this, only do one reaction of 5 million cells. The eGFP plasmid is ~2x the size of the ATAC-STARR library plasmids, so to match the required molarity, increase the concentration 2-fold. This control should be done for each individual cell type used in the experiment.

Prepare DNA samples:

1. Add 110µg ATAC-STARR library DNA into a 1.5 mL Eppendorf tube in a max volume of 220µL [This equates to 5µg DNA per 5 million cells].
2. For GFP control, add 11µg of pcDNA3-eGFP plasmid (endotoxin-free) to a 1.5mL Eppendorf tube in a max volume of 11µL. [This equates to 10µg DNA per million cells].
3. For no DNA control, add 11µL to a 1.5mL Eppendorf tube.

Prepare Suspension Cells:

4. Day 0: Transfer cells into a T-75 flask with fresh medium. Cell density should be between 400,000 and 800,000 cells/mL on Day 1 (counting with hemocytometer). At that density, each reaction will require 4-5 flasks.
5. Day 1: Perform cell count (with hemocytometer):

6. Per sample, add 50 mL of warm recovery medium (without antibiotics) into four T-75 culture flasks. Incubate at 37°C until use.
7. For each GFP or noDNA control reaction, add 10mL of media into a T-12.5 culture flasks.
8. Transfer 121 million cells to as many 50 mL falcon tube as necessary and centrifuge at 300xg for 5 minutes. Aspirate supernatant. Wash and resuspend with 45mL of DPBS or PBS, combine pellets. Centrifuge for 300xg for 5 min.
9. Set up the Neon Pipette Station:
    a. Fill a fresh neon tube with 3 mL of pre-warmed Buffer E2. The electrode on the side of the tube should be completely submerged in buffer.
    b. Insert tube into the pipette station until you hear a click.
    c. Replace neon tube between samples—only need to change between experimental and controls (can use the same tube for water and GFP if doing water first).

10. Resuspend cells in 2178µL Buffer R/T. (R for GM12878, GM11831, and SUDHL-6 and T for LCL8664). **Gently** pipette for a single cell suspension. (Do not leave cells resuspended in Buffer R/T for longer than 15-30 min).

Electroporation:

11. Add 1980µL cells to ATAC-STARR library DNA and **gently** mix.
12. Add 99µL cells to GFP plasmid. **Gently** mix.
13. Add 99µL cells to water. **Gently** mix.
14. Electroporate 20 ATAC-STARR reactions and both control reactions (100µL at a time) using the following conditions: 100µL Neon Tip, **1100V, 30ms, 2 pulses**. If the sample arcs, eject into waste and grab a new tip—19 good reactions are better than 19 good + 1 very dead. *Note: Each tip can be used 5 times. Reuse the tip only if doing an aliquot of the same sample. After use, save the tip and it can be regenerated to use for another application. Regenerated tips are somewhat less efficient and more prone to arcing than fresh tips, so **do not use regenerated tips in this protocol**. That said, these tips are quite expensive at ~$20 each, so keep that in mind when using.*
15. After each electroporation, remove the pipette from the station and expel contents into one of the prepared T-75 (or T-12.5 for control reactions) flasks from step 6 above. Repeat electroporation with remaining aliquots and expel 5 aliquots into each T-75 flask containing 50mL media—cell concentration will be ~500,000 cells/mL. Gently swirl flask after addition of each transfected aliquot. Place in incubator at 37°C for 24 hours.

IN:	OUT:

16. After ~24 hours: Check transformation efficiency via flow cytometry.
    We will use PI as a viability dye; PI will stain dead cells
    a. For both GFP and No DNA transfections:
        i. Spin down cells in 15mL conical at 300xg, 5min.
        ii. Remove supernatant and resuspend in 5mL 1x PBS or DPBS.
        iii. Spin at 300xg, 5min. Remove Supernatant.
        iv. Resuspend in 1000µL Flow Cytometry Buffer (1%BSA in 1xPBS) to achieve a cell density of ~$1.25x10^6$ cells/mL.
        v. Split each sample into two aliquots of 500µL each. Pass through the flow cytometry tube cap filter to obtain single-cell suspensions.
        vi. Stain one from each with propidium iodine:
            1. Make a 1:1 dilution of 1µg/µL PI solution with 1xPBS+1%BSA.
            2. Add 1.25µL of diluted PI to the desired tubes. Vortex.
    b. Analyze samples with 3-laser or 5-laser Flow Cytometer in core. Reserve assisted appointment in advance.
        i. Use no DNA as unstained compensation. No DNA+PI and GFP noPI as compensation controls.

ii. Analyze viable GFP+PI cells for GFP/no GFP.

XIV. Harvest RNA and plasmid DNA from ATAC-STARR transfected cells

1. Exactly 24 hours after electroporation (counting from last transfection added to media), mix all four T-75 flasks—200mL should all fit in one.
2. Divide into two 100mL volumes and process each separately. *One is for RNA harvest, the other is for isolated plasmid DNA harvest. Process the RNA aliquot first as it is the most time-sensitive.*
3. Before processing each, spin down in 50mL conical tubes, 300xg 5min. Remove sup and wash with 25mL 1x PBS or 1x DPBS. Spin down cells at 300xg, 5min and remove supernatant.

RNA extraction – Trizol and Chloroform steps should be done in a chemical fume hood. (~2hrs)

1. Add 5mL Trizol per sample (this equates to 1ml Trizol per 10e6 cells). Pipette up/down to homogenize. *Note: can stop here and store at -20°C overnight.*
2. Centrifuge 5 Invitrogen™ Phasemaker™ Tubes at 12,000xg for 30s.
3. Aliquot five volumes of 1mL into Invitrogen™ Phasemaker™ Tubes.
4. Incubate for 5 min at RT. *Note: can stop here and store at -20°c overnight.*
5. Add 0.2mL chloroform to each 1mL aliquot and mix vigorously by inversion.
6. Incubate for 2-3min at RT.
7. Spin at 12,000xg for 15min @4°C.
8. Transfer the aqueous layer to a 1.5mL Eppendorf tube—try to get ~600µL from each.
9. Add 2µL Glycoblue co-precipitant (15mg/ml) to each. Do not combine like-reactions yet.
10. Add 0.5mL isopropanol to each aqueous phase.
11. Incubate at RT for 10min.
12. Spin for 10min at 12,000xg @4°C.
13. Check for a blue/white gel-like precipitate. If there, remove sup and continue with protocol. If no pellet spin again.
14. Resuspend pellet in 1mL 75% EtOH. RNA can be stored in this solution at -20°C for up to a year.
15. Vortex briefly, and centrifuge 5min at 7500xg @4°C. Completely discard supernatant. It helps to use a P10 after briefly spinning.
16. Air-dry pellet for 5-10min.
17. Combine by suspending all 5 pellets in 100µL nuclease-free water. Incubate @ 55°C for 10min.
18. Determine RNA yield with Nanodrop (expected yield: 2.5-1.5µg/µL, 260/280 > 2, 260/230 > 1.8). *Note: Can stop here and store RNA at -70°C or -80°C overnight.*

Plasmid DNA extraction – Use ZymoPURE II midiprep to isolate plasmid DNA (~60min)

1. Follow manufactures protocol for kit. Add 8mL P1 buffer directly to cell pellet.
2. Elute in 50µL pre-warmed 10mM Tris pH 8.0. Incubate 5min on column.

3. Determine concentration and purity via nanodrop (expect ~300-400ng/µL). Store at -20°C. These samples will be amplified in a PCR reaction in the NGS PCR step.

XV. Poly(A)+ RNA enrichment with oligo(dT)$_{25}$ magnetic beads: (~90min)

*The magnetic beads are supplied at 5mg/mL and each mg can bind up to 5µg Poly(A)+ RNA. We will assume that Poly(A)+ RNA makes up about 5% (this is at the higher end because we are accounting for both mRNAs and the Reporter RNAs). Therefore, for 100µg Total RNA, we expect 5µg to be Poly(A)+ RNA. To capture all 5µg, we need 1mg of magnetic beads. Therefore, the ratio is 1µg Total RNA:10µg beads.

1. Determine the amount of beads to wash:
    a. ____µg Total RNA * 10 = ____µg beads ÷ (5µg/µL) = ___µL beads to add
    b. ___µL beads to add *1.05 = ____µL beads to wash
    Note: The 1.05 multiplier is to have extra to prevent running out.
2. Chill Wash Buffer B and 1.5mL tubes at 4°C.
3. Wash magnetic beads:
    a. Resuspend magnetic beads in the vial (vortex for 30s).
        i. Transfer the desired amount to a 1.5mL Eppendorf tube (use more than one tube if volume > 750µL).
        ii. Add an equal volume of Binding Buffer and resuspend.
        iii. Place tube on DynaMag-2 magnet and incubate for 1min. Remove supernatant.
        iv. Remove tube from magnet and resuspend beads in the same volume of binding buffer used in step ii.
    b. Isolate Poly(A)+ RNA from Total RNA:
        i. Add 100µL binding buffer to each 100µL Total RNA sample.
        ii. Heat to 65°C for 2min to disrupt secondary structures.
        iii. Immediately place on ice. Incubate for 1min.
        iv. Add the indicated amount of washed beads to add to the 200µL Total RNA solution in a pre-chilled 1.5mL tube.
        Note: Gently handle the beads for the remaining steps of the protocol.
        v. Mix well and incubate on a rotator for 5min at 4°C.
        vi. Place tube on the magnet and incubate for ~30s. Remove supernatant.
        vii. Remove tube from magnet and add the same volume used in step iv of Washing Buffer B. Mix by pipetting up/down gently.
        viii. Rotate for 2-3min at 4°C.
        ix. Incubate on magnet for ~30s and remove supernatant.
        x. Repeat wash for a total of 4 washes.
        xi. Add 82µL of 10mM Tris-HCl pH 7.5 to the beads.
        xii. Gently mix. Incubate at 75°C for 2min.
        xiii. Place on magnet and quickly transfer eluate to a 1.5mL tube. Determine concentration via nanodrop (should be ~500-250ng/µL).
        Note: Can stop here and store at -70°C.

XVI. DNaseI digestion: (20 units are sufficient to treat ~100µg RNA): (~45min)

1. Per sample, add 10µL DNaseI Reaction buffer and 10µL DNase I (RNase-free)(20 units)
2. Mix well and incubate @ 37°C for 10min.
3. Purify Poly(A)+ RNA with Zymo RNA Clean & Concentrator – 25 kit.
    a. Volumes: 200µL RNA binding buffer, 300µL 100% EtOH
    b. Elute in 55µL Nuclease-Free water.
4. Nanodrop to determine concentration.

XVII. Reverse Transcription: (From Takara PrimeScript Protocol): (~90min)

For each 20µL reaction, the input maximum is 1µg Poly(A)+ RNA. We expect to have ~25µg, so scale up 25-fold. Do ten 50µL reactions.

1. Anneal primer to template RNA:
    a. Make MasterMix following the recipe below:

Component	**Per 20µL rxn (for reference)**	**Per 50µL Rxn**	**Mastermix (x10)**
2µM STARR_GSP	1µL	2.5µL	25µL
10mM each dNTP mix	1µL	2.5µL	25µL
Template RNA	2µL	5µL	50µL
NF-water	6µL	15µL	150µL

    b. Aliquot 25µL into ten 0.2mL PCR tubes.
    c. Heat the RNA-primer mix at 65°C for 5min and then immediately incubate on ice for at least 1 min.
2. Prepare RT reaction mix:
    a. Vortex and briefly centrifuge the 5x PrimeScript Buffer
    b. Make MasterMix following the recipe below:

Note: While recommended, RNase inhibitor is not required. If forgone, compensate with NF-water.

Component	**Per 50µL Rxn**	**Mastermix (x10)**
5X PrimeScript Buffer	10µL	100µL
NF-water	12.5µL	125µL
RNase inhibitor (40 U/µL) (if desired)	1.25µL	12.5µL
PrimeScript Reverse Transcriptase (200U/µL)	1.25µL	12.5µL

3. Add 25µL of the MasterMix to each of the 10 RNA-Primer reactions. Mix well.
4. Incubate at 42°C for 60min.
5. To inactivate the reaction, heat at 70°C for 15min and then cool on ice.

XVIII. RNaseA Treatment: (~90min)

6. Pool like reactions for a total volume of ~500µL.
7. Add 1µL RNAseA (10mg/mL) and incubate 37°C for 1hr.

Note: final concentration is ~20µg/mL in low salt conditions [KCl] = 75mM and [$MgCl_2$] = 8mM. At low salt concentrations, RNase A cleaves single-stranded and double-stranded RNA as well the RNA strand in RNA-DNA hybrids.

8. Purify cDNA using Zymo DNA Clean and Concentrate-25 kit:
    a. Add 3.5mL Binding Buffer (7:1). Mix in a 15mL conical. Spin through column in 800µL increments.
    b. Wash with 200µL wash buffer twice.
    c. Elute in 28µL elution buffer.
9. Nanodrop to determine yield and purity. (Expected yield: ~10-50ng/µL).

<u>IXX. Next-Generation sequencing PCR:</u> (~60min)

* To reduce rxn bias effect I will perform two PCRs for each sample.

1. Prepare a mastermix:

Component	**Per 50µL rxn**	**Mastermix (x___=2.2*#Samples)**
NEBNext Ultra II Q5 2x MasterMix	25µL	
50µM ____ P5 Nextera primer	1µL	
NF-Water	13µL	

2. Aliquot **39**µL mix into PCR tubes and add 10µL of sample to each.
3. Add 1µL of the correct **50µM P7 Nextera primer (N7XX).**
4. Vortex and centrifuge, briefly. Run the following PCR reaction:

    98ºC, 30 sec
    <u>8 or 12 cycles</u> of → *plasmid DNA = 8 cycles, reporter RNA = 12 cycles*
    98ºC, 10 sec
    68ºC, 30 sec
    72ºC, 30 sec
    Final extension 72ºC, 2 min

5. Pool like-reactions and then purify cDNA using Zymo Clean and Concentrate -5 kit – elute in 30µL elution buffer.
6. Determine concentration and size distribution via HS D5000 ScreenTape on the Agilent Tapestation. Concentration should be greater than 5nM but not much higher. Anything greater than 50nM is significantly overamplified. *Note: you will see the template and initial amplification products in the Tapestation, these products are not capable of generating reads on the sequencer.*
7. Submit samples for paired end sequencing. In the NAR paper, we requested 50M reads for the DNA samples and 75M for the RNA samples at PE150 on NovaSeq.