**ACT2 Protocol**

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**REQUIRED REAGENTS**

**Recombinant PA-Tnp protein** (see Appendix A)

**Oligonucleotide complex adapters** (see Appendix B)

**Library preparation adapters** (see Appendix B)

**2X Complex Buffer (CB)**  For 50 mL:

0.1 M Tris pH 8.0 5 mL of 1 M Tris pH 8.0

0.3 M NaCl 3 mL of 5 M NaCl

0.1% Triton X-100 50 L of 100% Triton X-100

25% Glycerol 12.5 mL of 100% Glycerol

*Add protease inhibitors to a 500 L aliquot immediately before use.*

*For enzyme storage in freezer stocks, add 2 mM DTT.*

**Wash Buffer**  For 50 mL:

50 mM Tris pH 8.0 2.5 mL of 1 M Tris pH 8.0

150 mM NaCl 1.5 mL of 5 M NaCl

0.05% Triton X-100 25 L of 100% Triton X-100

2 mM EDTA 200 L of 0.5 M EDTA

**RIPA Buffer** For 10 mL:

Prepare in 1X TE pH 7.5

150 mM NaCl 300 L of 5 M NaCl

0.2% SDS 200 L of 10% SDS

0.1% Na deoxycholate 10 mg of solid Na deoxycholate

1% Triton X-100 100 L of 100% Triton X-100

*Prepare fresh each time. Deoxycholate is light sensitive.*

**Materials:**

Phusion® High-Fidelity PCR Master Mix with HF Buffer (NEB, cat. #M0531S)

MinElute PCR Purification Kit (Qiagen cat. #28004, or equivalent)

MinElute Gel Extraction Kit (Qiagen cat. #28604, or equivalent)

**PROCEDURE**

*See Appendix A for expression and purification of recombinant PA-Tnp.*

*See Appendix B for instructions on preparing the annealed complex adapters.*

**Antibody-PA-Tnp Complex Formation (1 hour)**

1. For each PA-Tn5 complex adapter pair, mix the following in a microcentrifuge tube and incubate at room temp. for 10 min:

4.5 L of 2X CB + PI

2 L of 50 M annealed complex adapter A

2 L of 50 annealed complex adapter B

2.5 L of 1 g/L recombinant PA-Tn5 enzyme

1. Label a set of microcentrifuge tubes for each antibody/PA-Tn5 complex combination. To each of these tubes, add 1.5 L of the matching complex from step 1, 1.5 L of 2X CB + PI, and 1.5 L of the desired antibody. (Multiply these volumes for the number of replicates, if applicable.) Mix by pipetting and incubate at room temperature for 30 min.

**Cell Permeabilization (30 min)**

*See Appendix C for instructions on preparing crosslinked cells. If using frozen crosslinked cells, thaw a cell pellet equivalent to 1 million cells on ice and proceed immediately to step 4 below.*

*If starting with fewer than ~1 million cells, scale the final volume used in step 7 appropriately.*

1. Transfer 1 million cells to a clean 1.5 mL tube and centrifuge for 2 min at 500 \*g.
2. Remove the supernatant and suspend the pellet in 1 mL of freshly prepared RIPA Buffer. Incubate the tube at room T for 10 min to lyse the cells and decondense the chromatin.

*Note: a swinging-bucket rotor is strongly recommended for the remaining centrifugations!*

1. Spin down the cells at 850 \*g for 2 min. Carefully remove the supernatant, leaving ~50 L at the bottom to avoid loss of cells.
2. Suspend the cells in 1 mL of Wash Buffer. Repeat the centrifugation and carefully remove the supernatant down to ~50 L
3. Suspend the cells in 1 mL of Wash Buffer. If you started with fewer than ~1 million cells, make sure the final cell concentration is equivalent.

**Complex Binding and Tagmentation (2 hours)**

*Note: the use of a swinging-bucket rotor is strongly recommended for these steps.*

1. Label a 1.5 mL tube for each sample. Transfer a 50 L cell aliquot (~50 thousand cells) into each tube. Add 4.5 L of the matched AB-Tnp complexes from step 2 for the first binding step. Mix each sample by pipetting gently. Incubate the samples at room temperature for 30 min.
2. Add 1 mL of Wash Buffer to each tube. Rotate the tubes for 5 min at room temperature. Centrifuge the cells at 850 \*g. Remove the supernatant, leaving ~50 L at the bottom to avoid loss of cells.
3. Add 3 L of 50 M annealed blocking adapter to the sample and mix by pipetting. Incubate for 10 min at room temperature.
4. Add 4.5 L of the next AB-Tnp complex to each sample and mix by pipetting gently. Incubate at room temperature for 30 min.
5. If probing more than two marks per sample, repeat steps 9 through 11 for each remaining complex.
6. Add 500 L of Wash Buffer to each tube and rotate the tubes for 5 min at room temperature. Centrifuge the cells at 850 \*g and remove the supernatant to ~50 L.
7. Repeat step 13.
8. Dilute the samples to ~100 L with Wash Buffer using the volume markings on the sides of the tubes. Add 1.5 L of 1 M MgCl2 to each tube of cells and gently suspend the cells by pipetting. Incubate the cells at 37°C for 60 min to allow targeted tagmentation to occur.

*Note: extending this reaction time is not recommended as it may increase the background signal at accessible chromatin regions due to nonspecific transposase activity.*

**Sample Purification (1.5 hours)**

1. Stop the reactions by adding 8 L of 0.5 M EDTA. Vortex thoroughly to mix. Incubate samples at 80 °C for 5 min.
2. Add 2 L of 10% SDS and 1 L of 20 mg/mL Proteinase K. Incubate at 55 °C for 60 min.
3. Purify the DNA using a MinElute PCR Purification kit (or equivalent) and an elution volume of 20 L.
4. Store the purified samples at -20°C or -80°C.

**Library Preparation (~1 hour)**

*Note: the library PCR adapters are not the same as the complex adapters used in step 1! Use barcoded Illumina NextEra PCR primers or equivalent.*

1. Transfer each sample to a PCR tube. To each tube, add 0.5 L of 50 PCR adapter #1, 0.5 L of 50 PCR adapter #2. Add 20 L of 2X NEB Phusion HF Master Mix to each tube, quickly mix, and amplify immediately using the following program:

72 °C for 5 min

98 °C for 10 s |

65 °C for 30 s | 15 to 18 cycles

72 °C for 15 s |

1. °C for 5 min
2. Visualize the PCR products using gel electrophoresis (e.g. on a 2% E-gel run for 20 min). Visible smears and bands above ~250 bp indicate a detectable signal.
3. Excise gel slices corresponding to DNA fragment sizes between ~250 to 800 bp. Purify the PCR products using a MinElute Gel Purification Kit (or equivalent). The DNA libraries are ready for quantification, multiplexing, and sequencing.

*Note: check with your sequencing facility or technician to determine the baseline sample concentration needed for sequencing. If your Qubit sample quantification (or equivalent) indicates that your samples are too dilute, consider passing two or more dissolved gel slices from the same PCR reaction through a single MinElute column. This should increase the resulting concentration without increasing elution volume.*

**Appendix A: Expression and Purification of PA-Tnp**

**REQUIRED REAGENTS**

**Protease Inhibitors Stock (1000X)**

10 μg/mL Pepstatin A (calbiochem cat# 516481)

10 μg/mL Leupeptin Hemisulfate (calbiochem cat# 108975)

10 μg/mL Chymostatin (calbiochem cat# 230790)

6 μg/mL Antipain dihydrochloride (Sigma cat# A6191)

*Freeze in aliquots for future use.*

***E. Coli* Lysis Buffer** For 50 mL (one use):

50 mM Tris pH 8.0 2.5 mL of 1 M Tris pH 8.0

300 mM NaCl 3 mL of 5 M NaCl

20 mM Imidazole 250 L of 4 M Imidazole

0.1% Triton X-100 50 L of Triton X-100

1X Protease Inhibitors 50 L of 1000X stock

1 mg/mL Lysozyme 50 mg of Lysozyme powder

Adjust the pH to 7.5 using HCl.

**Ni-NTA Wash Buffer** For 50 mL:

50 mM Tris pH 8.0 2.5 mL of 1 M Tris pH 8.0

1 M NaCl 10 mL of 5 M NaCl

20 mM Imidazole 250 L of 4 M Imidazole

0.1% Triton X-100 50 L of Triton X-100

1X Protease Inhibitors 50 L of 1000X stock

Adjust pH to 7.5 using HCl.

**Ni-NTA Elution Buffer** For 50 mL:

50 mM Tris pH 8.0 2.5 mL of 1 M Tris pH 8.0

1 M NaCl 10 mL of 5 M NaCl

250 mM Imidazole 3.12 mL of 4 M Imidazole

0.1% Triton X-100 50 L of Triton X-100

1X Protease Inhibitors 50 L of 1000X stock

Adjust the pH to 7.5 using HCl.

**Materials:**

PA-Tnp Expression Construct (Addgene acc# 121137)

BL21-Gold DE3 Competent Cells (Agilent cat# 230132)

Ni-NTA Agarose Beads (Qiagen cat# 1018244, strongly suggest this brand to avoid stickiness)

**PROCEDURE**

1. Inoculate 20 to 50 mL of LB containing 100 g/mL of ampicillin/carbenicillin with a glycerol stock of BL21 (DE3) Gold transformed with the PA-Tnp T7 expression construct. Shake at 37 °C overnight.
2. Transfer four 5 mL aliquots of the starter culture into four 1-2 L flasks containing 600 mL of LB each. Continue shaking at 37 °C until the cultures reach an OD600 of 0.6.
3. Cool the flasks in an ice-water bath to room temperature. Add 0.4 mL of 1 M IPTG to each flask (final concentration of 0.3 mM). Shake the cultures at room temp. for 4 h.
4. Harvest bacteria by centrifugation in 250 mL conical centrifuge tubes using the following settings: 3,700 rpm, 4 °C, 15 min. *If desired the resulting pellets can be flash frozen in liquid nitrogen and stored at -80 °C.*
5. Suspend the cell pellets in 50 mL of Lysis Buffer in total and incubate on ice for 30 min. The solution will be viscous.
6. Divide the solution into tubes containing 5 mL each. Perform sonication with a microtip on a Misonix S-4000 sonicator (or equivalent) using a 15 s pulse of 90% amplitude (~35 W) on each sample. Repeat this sonication for a total of four bursts for each 5 mL aliquot.
7. Combine the lysates into two balanced centrifuge tubes and spin them at 20,000 \*g for 10 min at 4 °C. Transfer the supernatant to a new tube and add 2-ME to a concentration of 5 mM (~17 L per 50 mL solution), 1 mM PMSF, and 1 M NaCl.
8. Mix with 3 mL of 50% Ni-NTA agarose bead slurry and rotate for 1 hour at 4 °C.
9. Transfer the beads to a 10 mL syringe with glass wool on the bottom and allow the lysate solution to flow through. Wash the beads by passing 20 mL of Ni-NTA Wash Buffer through the syringe.
10. Elute the bound PA-Tnp by loading 5 mL of Ni-NTA Elution Buffer and collecting aliquots of 500 L of eluate into 1.5 mL tubes. Add a final concentration of 1 mM dithiothreitol and 50% glycerol to the eluates and mix by pipetting until there is no turbidity.
11. Check the purity and concentrations of the eluates (~0.1 to 1 L of eluate per lane) using SDS-PAGE alongside a protein standard such as BSA of known concentration.
12. Store protein in 20 to 50 L aliquots at -80 °C. Once removed for use, store at -20 °C to avoid freeze-thaw. Aliquots of enzyme remain good at -20 °C for several weeks.

**Appendix B: Design and Preparation of Oligonucleotide Adapters**

*PA-Tn5 Complex Adapters*

As in ACT-seq, the oligonucleotide adapters that are bound by the PA-Tn5 enzyme are partially double stranded after being annealed to the pMENTS (“mosaic-end, non-transfer strand”) oligonucleotide. In ACT2, however, each adapter bears a distinct barcode sequence to enable the signals from each antibody to be distinguishable in the resulting sequence data. The general sequences are provided in the following table, given in 5′ to 3′ orientation:

|  |  |
| --- | --- |
| **Oligo Name** | **Sequence** |
| pMENTS | 5′Phos-CTGTCTCTTATACACATCT |
| Complex Adapter A | CCTACACGACGCTCTTCCGATCTNNNNNNNNAGATGTGTATAAGAGACAG |
| Complex Adapter B | TTCAGACGTGTGCTCTTCCGATCTNNNNNNNNAGATGTGTATAAGAGACAG |

Where the poly-N region represents the variable barcode sequences of 7 or 8 nucleotides in length. The sequences upstream (left, 5′) of the barcodes are designed to anneal to either the i5 or i7 PCR primers that are used later in the protocol during library preparation. The sequence downstream (right, 3′) of the barcodes is the mosaic end (ME) sequence that is bound by the Tn5 enzyme. We recommend varying the lengths of the barcodes between 7 and 8 bp to help promote sequence complexity in the resulting libraries.

Prior to use, complex adapters A and B must be annealed to pMENTS using the following procedure:

1. Mix 25 l of 100 M pMENTS with 25 l of 100 M complex adapter.
2. Heat to 99 °C for 5 min. Turn off the heat source and allow the solutions to cool to room temperature over the course of two hours or overnight.
3. Store the resulting 50 M oligos at in the refrigerator or at -20 °C.

*PCR Library Adapters*

The adapters used in the library preparation steps are distinct from the complex adapters detailed above. The library adapters are based on Illumina NextEra designs and are barcoded to enable different samples to be distinguished from one another after multiplexing. The general sequences are provided in the following table, given in 5′ to 3′ orientation:

|  |  |
| --- | --- |
| **Oligo Name** | **Sequence** |
| Library Adapter A | AATGATACGGCGACCACCGAGATCT-  ACACTCTTTCCCTACACGACGCTCTTCCGATCT |
| Library Adapter B | CAAGCAGAAGACGGCATACGAGAT-  NNNNNNNNGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT |

For this study, a single universal Library Adapter A sequence was used in all samples, whereas each sample received a variant of Library Adapter B containing a unique i7 index sequence (represented by the 8 bp poly-N region in the table). This enables the samples to be distinguished from one another after multiplexing.

**Appendix C: Preparing Crosslinked Cells**

1. Harvest ~1 to 5 million cells in a 15 mL conical vial. Pellet the cells and aspirate the supernatant completely. If the cells were treated with trypsin or the like, pellet and wash the cells twice with PBS.
2. Suspend the cell pellet in 10 mL of room-temperature culture medium supplemented immediately prior to use with fresh methanol-free formaldehyde to a concentration of 1% v/v. Rotate the tube for 10 min at room T.
3. Add 1 mL (~1/10 volume) of 1.25 M glycine. Rotate the tube for an additional 5 min at room temperature. Centrifuge at 500 \*g for 3 min at 4 °C.
4. Aspirate the supernatant and suspend the cells in 10 mL of PBS. Repeat centrifugation.
5. Repeat step 4. Suspend the cells in 1 mL of PBS and obtain a cell count.
6. Proceed using the cells immediately or flash freeze the pellets in liquid nitrogen for storage.