mSTARR-seq protocol\* (6-Jun-17 version)

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Starting material

1. Starting material = 1-5 ug of size selected DNA of interest
   1. In the original protocol, we used a mixture of Msp1 digested and randomly sheared genomic DNA from the GM12878 cell line. However, a wide variety of starting materials could be used (e.g., DNA derived from an enzyme digest, random shearing, capture, ChIP-seq, etc.)
   2. In the original protocol, DNA was size selected to 300-800bp by running the DNA on a 1% agarose gel, cutting out the desired size range, and purifying the size selected DNA with the QIAquick Gel Extraction Kit (QIAGEN).

Ligate adaptors to DNA to prepare for large-scale cloning

1. The following protocol describes a single replicate library prep. In the original protocol, 7 replicate library preps were performed (3 using 1-2ug of size selected, sheared GM12878 DNA and 4 using 1-2ug of size selected, Msp1 digested genomic DNA). This section of the protocol largely follows the manufacturer’s instructions for the NEBNext DNA Library Prep Master Mix Set for Illumina.
2. Mix the following components in a sterile microfuge tube or PCR plate:
   1. Fragmented DNA 85 μl
   2. (green) NEBNext End Repair Reaction Buffer (10X) 10 μl
   3. (green) NEBNext End Repair Enzyme Mix 5 μl
   4. Sterile H2O to 100 ul
3. Incubate in a thermal cycler for 30 minutes at 20°C. Now is a good time to take the AMPure XP beads (Beckman Coulter) out of the fridge to warm to room temperature.
4. Do an AMPure cleanup:
   1. Transfer the reaction to a sterile 1.5mL tube.
   2. Add 160 μl (1.6X) of resuspended AMPure XP beads to the ligation reaction. Mix thoroughly on a vortex mixer or by pipetting up and down at least 10 times.
   3. Incubate for 5 minutes at room temperature.
   4. Put the tube on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the DNA targets.
   5. Add 200 μl of 80% freshly prepared ethanol to the tube while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Avoid discarding any beads.
   6. Repeat the above step once.
   7. Air dry beads for approximately 5 minutes while the tube is on the magnetic stand with the lid open. When beads are appropriately dry they will appear matte instead of glossy.
      1. Caution: Do not overdry the beads (apparent by cracked appearance). This may result in lower recovery of DNA target.
   8. Remove the tube from the magnet. Elute the DNA target from the beads by adding 44 μl of 0.1X TE (this is the same as Qiagen EB buffer).
   9. Mix well on a vortex mixer or by pipetting up and down and incubate for 2 minutes at room temperature.
   10. Put the tube in the magnetic stand until the solution is clear. Without disturbing the bead pellet, carefully transfer 42 μl of the supernatant to a fresh, sterile microfuge tube or PCR plate.
5. Mix the following components in a sterile microfuge tube or PCR plate:
   1. End Repaired, Blunt DNA 42 μl (from step above)
   2. (yellow) NEBNext dA-Tailing Reaction Buffer (10X) 5 μl
   3. (yellow) Klenow Fragment (3´→ 5´ exo–) 3 μl
6. Incubate in a thermal cycler for 30 minutes at 37°C.
7. Do an AMPure cleanup:
   1. Transfer each sample to a clean 1.5mL tube.
   2. Add 90 μl (1.8X) of resuspended AMPure XP Beads to the ligation reaction. Mix thoroughly on a vortex mixer or by pipetting up and down at least 10 times.
   3. Incubate for 5 minutes at room temperature.
   4. Put the tube on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the DNA targets.
   5. Add 200 μl of 80% freshly prepared ethanol to the tube while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
   6. Repeat the above step once.
   7. Air dry beads for approximately 5 minutes while the tube is on the magnetic stand with the lid open. When beads are appropriately dry they will appear matte instead of glossy.
      1. Caution: Do not overdry the beads (apparent by cracked appearance). This may result in lower recovery of DNA target.
   8. Remove the tube from the magnet. Elute the DNA target from the beads by adding 27 μl of 0.1X TE.
   9. Mix well on a vortex mixer or by pipetting up and down and incubate for 2 minutes at room temperature.
   10. Put the tube in the magnetic stand until the solution is clear. Without disturbing the bead pellet, carefully transfer 25 μl of the supernatant to a fresh, sterile microfuge tube.
8. Mix the following components in a sterile PCR tube or plate:
   1. dA-Tailed DNA 25 μl
   2. (red) Quick Ligation Reaction Buffer (5X) 10 μl
   3. NEBNext adapter 10 μl
      1. Sequence = 5´-/5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGT C/ideoxyU/ACACTCTTTCCCTACACGACGCTCTTCCGATC\*T-3´
   4. (red) Quick T4 DNA Ligase 5 μl
9. Incubate in a thermal cycler for 30 minutes at 20°C.
10. Without removing tubes from thermocycler, add 3 μl of USER™ Enzyme Mix by pipetting up and down, and incubate at 37°C for 15 minutes.
11. Do an AMPure cleanup:
    1. Transfer each sample to a sterile 1.5mL tube.
    2. Add 90 μl of resuspended AMPure XP Beads to the ligation reaction (~53 μl). Mix thoroughly on a vortex mixer or by pipetting up and down at least 10 times.
    3. Incubate for 5 minutes at room temperature.
    4. Put the tube plate on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the DNA targets.
    5. Add 200 μl of 80% freshly prepared ethanol to the sample while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
    6. Repeat the above step once.
    7. Air dry beads for approximately 5 minutes while the tube is on the magnetic stand with the lid open. When beads are appropriately dry they will appear matte instead of glossy.
       1. Caution: Do not overdry the beads (apparent by cracked appearance). This may result in lower recovery of DNA target.
    8. Remove the tube from the magnet. Elute the DNA target by adding 17 µl EB.
    9. Mix well on a vortex mixer or by pipetting up and down and incubate for 2 minutes at room temperature.
    10. Put the tube in the magnetic stand until the solution is clear. Transfer 15 μl of supernatant (or desired volume) to a new tube/well.
12. Do a PCR; setup as follows:
    1. NEB Q5 hot start reaction mix 25 μl
    2. mSTARR\_primerF2 (10umol) 2.5 ul = actaaagtctagagcttgtaACACTCTTTCCCTACACG
    3. mSTARR\_primerR2 (10umol) 2.5 ul = gaagtggctggctgaattccGTGACTGGAGTTCAGACG
    4. Adaptor Ligated DNA Fragments (from above step) 15 μl
    5. Water 5 μl
13. Cycling is as follows:

98C for 30s

12 cycles:

98C for 10s

62C for 30s annealing/extension

72C for 60s

Final extension:

72C for 5 min

1. Do an AMPure cleanup:
   1. Add 45 μl (0.9X) of resuspended AMPure XP Beads to the PCR reactions (~50 μl). Mix well on a vortex mixer or by pipetting up and down at least 10 times. Transfer to a sterile 1.5mL tube.
   2. Incubate for 5 minutes at room temperature.
   3. Put the tube on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the DNA targets.
   4. Add 200 μl of freshly prepared 80% ethanol to the tube while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
   5. Repeat the above step once.
   6. Air dry beads for approximately 5 minutes while the tube is on the magnetic stand with the lid open. When beads are appropriately dry they will appear matte instead of glossy.
      1. Caution: Do not overdry the beads (apparent by cracked appearance). This may result in lower recovery of DNA target.
   7. Remove the tube from the magnet. Elute the DNA target from the beads by adding 30 μl of 0.1X TE.
   8. Mix well on a vortex mixer or by pipetting up and down and incubate for 2 minutes at room temperature.
   9. Incubate at 37C for 5 min in EB buffer.
   10. Put the tube in the magnetic stand until the solution is clear. Without disturbing the bead pellet, carefully transfer 29 μl of the supernatant to a clean tube.
   11. Visualize and quantity the final library (e.g., using a Bioanalyzer).

Generate linearized mSTARR backbone for large-scale cloning

1. First, make large quantities of the mSTARR plasmid. To do so, transform 1-3ul of mSTARR plasmid DNA into 50ul of GT115 chemically competent cells (Invivogen). Do 2-4 replicates. For each replicate, do this:
   1. Thaw 50ul aliquots of cells on ice for 2-5 minutes. If aliquots are larger, gently pipette 50ul of cells into a prechilled tube.
   2. Add 1-3ul of plasmid DNA. Tap/gently flick to mix.
   3. Incubate on ice for 30min.
   4. Heat shock in a water bath set to 42C for 30sec exactly. Make sure to immerse the cells into the region of the water bath that is 42C.
   5. Place the tube back on ice for 1-2 minutes.
   6. Add 450ul SOC and allow the cells to recover at 37C for 1hr (with shaking at 250rpm).
2. After recovery, add all replicate cultures to a flask containing 200-300mL LB with Zeocin (50ug/mL). Incubate overnight (8-12hr maximum) at 37C (with shaking at 250rpm).
3. In the morning, extract the plasmid DNA using the QIAGEN Plasmid Plus Maxi Kit, following the manufacturer’s instructions.
   1. Note that it is advisable to confirm the identity of the extracted plasmid before proceeding, for example using a series of restriction enzyme digests or Sanger sequencing of targeted regions in the plasmid backbone.
4. Make cleaned, linearized mSTARR backbone for Gibson assembly:
   1. Linearize the mSTARR plasmid by digesting with NcoI and SpeI
      1. An example reaction: add 2ul NEB SpeI-HF, 2ul NEB NcoI-HF, 3.22ul cutsmart buffer, 25ul plasmid DNA (~5ug)
      2. Incubate at 37C for 2 hours
   2. Run a gel and extract the large, ~3.5kb fragment
   3. Clean up with a column cleanup kit such as Zymo Gel Extraction or QIAquick Gel Extraction Kit (QIAGEN)
   4. Quantify the linearized backbone (e.g., with a Qubit).

Clone libraries with putative enhancer elements into mSTARR plasmid via Gibson assembly

1. The following protocol describes assembly and transformation for a single replicate. In the original protocol, 7 replicates were performed (3 using a library made from 1-2ug of size selected, sheared GM12878 DNA and 4 using a library made from 1-2ug of size selected, Msp1 digested genomic DNA).
2. Determine the number of moles you will use of (i) the cleaned backbone from the above section and (ii) the cleaned NEB library that you will be cloning into the backbone for Gibson assembly.
3. NEB recommends a total of 0.1–2.5 picomoles of DNA fragments should go into the Gibson assembly in total, and we want there to be 2x picomoles of the library relative to the backbone. We suggest using 1ug of the 3.875kb backbone, which is ~0.397 picomoles.
4. Next, determine how many picomoles of library to add. For example, for a library with an average size of ~550bp, add 0.794 pmols of library (which is 283.88ng) to 1ug of backbone.
5. Combine the appropriate amounts of plasmid backbone and NEB library, for example:

|  |  |
| --- | --- |
| DNA library | 283.88ng |
| Clean, linearized mSTARR backbone | 1 ug |
| NEBuilder HiFi DNA Assembly Master Mix | 50 ul |
| Water | X ul |
| Total volume | 100 ul |

1. Incubate the reaction at 50C for 60 min.
2. Do an AMPure cleanup:
   1. Add 100 μl (1 X) of resuspended AMPure XP Beads to each assembled/digested reaction. Mix thoroughly on a vortex mixer or by pipetting up and down at least 10 times.
   2. Incubate for 5 minutes at room temperature.
   3. Put the tube on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the DNA targets.
   4. Add 200 μl of 80% freshly prepared ethanol to the tube while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
   5. Repeat the above step once.
   6. Air dry beads for approximately 5 minutes while the tube is on the magnetic stand with the lid open. When beads are appropriately dry they will appear matte instead of glossy.
      1. Caution: Do not overdry the beads (apparent by cracked appearance). This may result in lower recovery of DNA target.
   7. Remove the tube from the magnet. Elute the DNA target from the beads by adding 8 μl of 0.1X TE (this is the same as Qiagen EB buffer).
   8. Mix well on a vortex mixer or by pipetting up and down and incubate for 2 minutes at room temperature.
   9. Put the tube in the magnetic stand until the solution is clear. Without disturbing the bead pellet, carefully transfer 7.5 μl of the supernatant to a fresh, sterile microfuge tube.

Transform assembled plasmids into electrocompetent GT115 cells (purchased from Intact Genomics)

1. For each transformation (using 7.5ul of assembled plasmid as the input), do the following using a Gene Pulser Xcell:
   1. Thaw cells on ice for 2-5 minutes.
   2. Put as many 2mm cuvettes as you plan to use on ice (1 cuvette = 1 transformation).
   3. Add 7.5 ul assembled plasmid DNA to the 2mm cuvette. Add 300 ul thawed cells to the cuvette and mix by tapping gently.
      1. While transferring cells to the cuvette make sure not to warm up the cells, keep your hands away from the bottom of the tube.
   4. From the home screen on the Gene Pulser Xcell, go to preset protocols > bacteria > E.coli 2mm gap 3kv.
   5. Insert the cuvette in the shock pod and press ‘pulse’.
   6. Immediately add 1mL of SOC media to the cuvette. It is essential to quickly resuspend the electroporated cells in media.
   7. Continue adding SOC with a pipette, mixing the SOC around the cuvette, and transferring the SOC with cells to two clean 10mL cell culture tubes (i.e., put 5mL of SOC with cells into each cell culture tube). Use 10mL of SOC per 300ul rxn total.
   8. Incubate the cells in SOC (with shaking at 250rpm) at 37C for 1hr.
   9. Transfer the 10mL of SOC (containing transformed cells) into 300mL of LB + Zeocin (50ug/mL). Incubate overnight, but for no more than 12 hours. Ideally, stop when the OD 600nm absorbance reaches 0.9-1.
2. In the morning, extract plasmids using the QIAGEN Plasmid Plus Maxi Kit following the manufacturers instructions.
   1. Quantify the extracted plasmid DNA (e.g., using a Qubit).
3. OPTIONAL: Make an NEB library and sequence the inserts in the extracted plasmid DNA pool to check diversity.
   1. To do so, PCR the extracted plasmid DNA (for each replicate transformation) using the protocol below:

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| --- | --- |
| 2X NEB Q5 Hot start master mix | 25 ul |
| 10 µM Indexed Primer (NEB) | 1 µl |
| 10 µM Universal Primer (NEB) | 1 µl |
| Template DNA | ~10ng |
| Total volume | 50 ul |

* 1. Cycling is as follows:

98C for 30s

12 cycles:

98C for 10s

62C for 30s annealing/extension

72C for 60s

Final extension:

72C for 5 min

* 1. Do an AMPure cleanup on the PCR product:
     1. Add 60 μl (1.2 X) of resuspended AMPure XP Beads to each assembled/digested reaction. Mix thoroughly on a vortex mixer or by pipetting up and down at least 10 times.
     2. Incubate for 5 minutes at room temperature.
     3. Put the tube on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the DNA targets.
     4. Add 200 μl of 80% freshly prepared ethanol to the tube while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
     5. Repeat the above step once.
     6. Air dry beads for approximately 5 minutes while the tube is on the magnetic stand with the lid open. When beads are appropriately dry they will appear matte instead of glossy.
        1. Caution: Do not overdry the beads (apparent by cracked appearance). This may result in lower recovery of DNA target.
     7. Remove the tube from the magnet. Elute the DNA target from the beads by adding 25 μl of 0.1X TE (this is the same as Qiagen EB buffer).
     8. Mix well on a vortex mixer or by pipetting up and down and incubate for 2 minutes at room temperature.
     9. Put the tube in the magnetic stand until the solution is clear. Without disturbing the bead pellet, carefully transfer 24 μl of the supernatant to a fresh, sterile microfuge tube.
  2. Visualize and quantify the resulting library (e.g., on a Bioanalyzer) and sequence (e.g., on an Illumina MiSeq; note, we recommend using paired end sequencing).

Methylate or mock treat plasmid DNA

1. Pool all plasmid DNA to be used and then split into two treatment conditions. Methylate half of the plasmid library with M.SssI (NEB) and mock treat the other half (using the same reaction protocol, but replacing the enzyme with water).
2. To mock treat or methylate DNA (50ug at a time), add these reagents in order and incubate overnight (ideally) or for at least 3 hours at 37C.
3. With the following protocol, we have achieved methylation levels of ~90-95%. Note that it may be advisable to do multiple methyltransferase reactions, or add new methyltransferase after a few hours, to increase the efficiency of the reaction (see https://www.researchgate.net/post/How\_can\_one\_increase\_efficiency\_of\_DNA\_methylation\_by\_MSssI).

|  |  |
| --- | --- |
| Nuclease free water | X ul |
| 10x NEB buffer 2 | 50 ul |
| SAM (32 μM) | 5 ul |
| Genomic DNA (50ug) | X ul |
| SssI methylase (20 U/μl; 3x excess) or water | 7.5 ul |
| Total volume | 500 ul |

1. Cleanup the DNA following the methyltransferase reaction using AMPure beads:
   * 1. Add 400 μl (0.8 X) of resuspended AMPure XP Beads to each assembled/digested reaction. Mix thoroughly on a vortex mixer or by pipetting up and down at least 10 times.
     2. Incubate for 5 minutes at room temperature.
     3. Put the tube on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the DNA targets.
     4. Add 1000 μl of 80% freshly prepared ethanol to the tube while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
     5. Repeat the above step once.
     6. Air dry beads for approximately 5 minutes while the tube is on the magnetic stand with the lid open. When beads are appropriately dry they will appear matte instead of glossy.
        1. Caution: Do not overdry the beads (apparent by cracked appearance). This may result in lower recovery of DNA target.
     7. Remove the tube from the magnet. Elute the DNA target from the beads by adding 50 μl of 0.1X TE (this is the same as Qiagen EB buffer).
     8. Mix well on a vortex mixer or by pipetting up and down and incubate for 2 minutes at room temperature.
     9. Put the tube in the magnetic stand until the solution is clear. Without disturbing the bead pellet, carefully transfer 49 μl of the supernatant to a fresh, sterile microfuge tube.
2. Quantify the DNA (e.g., with a Qubit).

Transfect the methylated or mock treated plasmid DNA

1. The following protocol describes transfection procedures for a single replicate of K562 cells. Follow the manufacturer’s instructions for performing Lipofectamine transfection according to your cell type, scaling accordingly to transfect ~20 x 106 cells per replicate. In the original protocol, 12 replicates were performed (6 using methylated plasmid DNA and 6 using mock treated plasmid DNA).
2. The day of transfection, seed 20 x 106 cells in a T150 flasks (1 flask = 1 replicate).
   1. Cell density should be ~60-80% confluent at the time of transfection. For K562 cells this requires splitting cells the day before transfection.
3. To transfect, do the following for each replicate:
   1. Label a tube and add 2mL opti-MEM media to the tube.
   2. Add 40µg of DNA to the tube and mix by flicking.
   3. Mix P3000 (Thermo Fischer Scientific) reagent gently, add 80µL to the DNA mixture tube. Flick to mix. Incubate at RT for 5 min.
   4. In a new tube, add 120µL Lipofectamine 3000 (Thermo Fischer Scientific) containing 2mL opti-MEM. Mix by flicking.
   5. Add diluted DNA and P3000 to diluted Lipofectamine 3000 and incubate for 15 min at RT.
   6. Add entire volume of transfection mixture drop-wise to cells.
   7. Gently rock the flasks back and forth and from side to side. Place flasks back in the incubator.
4. Incubate the cells at 37C (with 5% carbon dioxide) for 48hrs.

Harvest the transfected cells and perform a plasmid extraction

1. Prepare a DNase treatment solution by diluting a 0.05-0.1ml aliquot of Turbo DNase I (Ambion) in 1mL of cell culture media (such as RPMI, DMEM, etc.; whatever is appropriate for the cell line you are using).
2. Pellet transfected cells via centrifugation at 300g for 5min.
3. Aspirate culture medium from each aliquot of cells.
4. Add the 1ml DNase solution to the cells. Flick to resuspend.
5. Incubate at 37C for 5-10 min.
6. Add 5mL of PBS to resuspended mixture and centrifuge at 300g for 5min.
7. Aspirate supernatant
8. Repeat steps 6 and 7.
9. Resuspend cells in 5mL of PBS and aliquot a small number of cells in PBS for plasmid extraction (we use ~2 million cells for plasmid extraction, but the desired number will depend on transfection efficiency).
10. Pellet the remaining cells by centrifugation for 5min at 300g.
11. Aspirate the supernatant.
12. Add 2 ml QIAGEN RLT buffer containing 20ul beta mercaptoethanol to each replicate. Fully resuspend the cell pellet and freeze the lysed cells at -80C for RNA extraction.
13. When you are done collecting cells, immediately perform a plasmid extraction on the small aliquot of unlysed cells, following QIAGEN’s recommendations for isolating plasmids from mammalian cells: https://www.qiagen.com/us/resources/faq?id=b3a99f9f-150b-44a4-90dc-3f55f5856f54&lang=en

Extract total RNA from lysed cells and isolate the mRNA

1. Total RNA extraction. Extract total RNA from each replicate (consisting of cells in 2mL of RLT) with the QIAGEN RNeasy midi prep kit. Use 1 column per sample and follow the manufacturer’s instructions with the following modifications:
   1. Homogenize the lysate using QIAGEN QIAshredder columns (use 3-4 QIAshredder columns per sample).
      1. Pipet ~700 ul of the lysate directly into a QIAshredder spin column placed in a 2 ml collection tube, and centrifuge for 2 min at maximum speed.
      2. Pool the homogenized lysates for each sample and use as the input for the RNeasy midi extraction.
   2. During the RNA purification, make sure to do the optional on column DNase treatment.
   3. Elute in 200 and 250 ul of RNase free water (do 2 rounds of elution into the same tube for a total of 450ul).
2. Quantify the resulting total RNA (e.g., using a Qubit RNA assay kit).
3. mRNA purification. Start with 70 µg of DNA-free total RNA per replicate.
4. Note, for best results, keep all the reagents used during the Poly(A) isolation except the NEBNext Oligo d(T)25 beads, on ice when not in use.
5. Also note, the buffers used in the following protocol can be ‘home made’ following the recipes described here: <https://www.neb.com/~/media/Catalog/All-Products/5682470F049B443D8F9EB5B1B7F46260/Datacards%20or%20Manuals/manualE7490.pdf>
   1. Dilute 70ug of total RNA with nuclease-free water to a final volume of 500 μl in a nuclease-free 0.2 ml PCR tube
   2. In a second nuclease-free 0.2 ml PCR tube aliquot 200 μl of fully resuspended NEBNext Magnetic Oligo d(T)25 Beads.
   3. Wash the beads by adding 1000 µl of RNA binding buffer to the beads. Pipette the entire volume up and down 6 times to mix thoroughly.
   4. Place the tube on the magnetic rack at room temperature for 2 minutes or until the solution is clear.
   5. Remove and discard all of the supernatant from the tube. Take care not to disturb the beads.
   6. Remove the tube from the magnetic rack.
   7. Repeat steps c-f once for a total of 2 washes.
   8. Resuspend the beads in 500 μl of RNA Binding Buffer and add the 500 μl of total RNA sample from step 1. Pipette the entire volume up and down to mix thoroughly. Incubate on roller/rotator for 5 min at 4C.
   9. Place the tubes on the thermal cycler and heat the sample at 65°C for 5 minutes and hold at 4°C to denature the RNA and facilitate binding of the poly-A-RNA to the beads.
   10. Remove tubes from the thermal cycler when the temperature reaches 4°C.
   11. Resuspend the beads. Pipette up and down slowly 6 times to mix thoroughly.
   12. Place the tubes on the bench and incubate at room temperature for 5 minutes to allow the RNA to bind to the beads.
   13. Resuspend the beads. Pipette up and down slowly 6 times to mix thoroughly.
   14. Incubate for 5 more minutes on the bench at room temperature to allow the RNA to bind to the beads.
   15. Place the tubes on the magnetic rack at room temperature for 2 minutes or until the solution is clear to separate the poly-A RNA bound to the beads from the solution.
   16. Remove and discard all of the supernatant. Take care not to disturb the beads.
   17. Remove the tubes from the magnetic rack.
   18. Wash the beads by adding 1mL of Wash Buffer to remove unbound RNA. Pipette the entire volume up and down 6 times to mix thoroughly.
   19. Place the tubes on the magnetic rack at room temperature for 2 minutes or until the solution is clear.
   20. Remove and discard all the supernatant from each well of the plate. Take care not to disturb the beads.
   21. Remove the tubes from the magnetic rack.
   22. Repeat Steps r-u once for a total of 2 washes.
   23. Add 500 μl of Tris Buffer to each tube. Gently pipette the entire volume up and down 6 times to mix thoroughly.
   24. Place the tubes on the thermal cycler. Close the lid and heat the sample at 80°C for 2 minutes, then hold at 25°C to elute the poly-A RNA from the beads.
   25. Remove the tubes from the thermal cycler when the temperature reaches 25°C.
   26. Add 500 μl of RNA Binding Buffer to each sample to allow the RNA to bind to the same beads. Gently pipette the entire volume up and down 6 times to mix thoroughly.
   27. Incubate the tubes on the bench at room temperature for 5 minutes.
   28. Resuspend the beads. Pipette up and down slowly 6 times to mix thoroughly.
   29. Incubate the tubes on the bench at room temperature for 5 more minutes to allow the RNA to bind to the beads.
   30. Place the tubes on the magnetic stand at room temperature for 2 minutes or until the solution is clear.
   31. Remove and discard all of the supernatant from each tube. Take care not to disturb the beads.
   32. Remove the tubes from the magnetic rack.
   33. Wash the beads once with 1mL of Wash Buffer. Gently pipette the entire volume up and down 6 times to mix thoroughly.
   34. Place the tubes on the magnetic rack at room temperature for 2 minutes or until the solution is clear.
   35. Remove and discard all of the supernatant from each tube. Take care not to disturb the beads. Note: It is important to remove all of the supernatant to successfully use the RNA in downstream steps. Spin down the tube. Place the tube on the magnetic rack and with a 10 µl tip remove all of the wash buffer. (Caution: Do not disturb beads that contain the mRNA).
   36. Remove the tubes from the magnetic rack.
   37. Elute the mRNA from the beads by adding 200 µl of the Tris Buffer, mix by pipetting 6 times and incubating the sample at 80°C for 2 minutes, then hold at 25°C to elute the polyA RNA from the beads. Immediately, place the tubes on the magnetic rack for 2 minutes or until the solution is clear.
   38. Collect the purified mRNA by transferring the supernatant to a clean nuclease-free PCR Tube.
6. Purified mRNA concentration can be measured by Qubit RNA HS Assay kit. Normally, mRNA accounts for 1% – 5% of total RNA population.
7. Turbo DNase I treatment.
   1. Prepare the following mixture in a PCR tube:
      1. mRNA: all from step 5
      2. 10X Buffer: 25 μL
      3. Dnase I (Ambion): 3 μL
      4. H2O: up to 250 μL
      5. Total volume: 250 μL
   2. Incubate the mixture at 37 °C for 30 min then place on ice.
8. Perform a Qiagen RNA MiniElute cleanup as per the manufacturers instructions.
   1. Elute in 2 rounds of 14ul into the same tube (for a total of what will be ~26ul).
   2. Quantify final mRNA concentration by Qubit RNA HS Assay Kit.
9. Targeted cDNA synthesis. Perform reverse transcription reactions following the SuperScript® III recommended protocol for higher yield. Double the reaction to accommodate ~1ug of mRNA:
   1. Perform reverse transcription in a total volume of 45 ul.
      1. To each ~26 ul of mRNA, add 4 pMol of RT\_polyA primer (this is 1 ul of 1:25 dilution of 100nM primer) and 2 ul of 10uM dNTPs.
      2. RT\_polyA: CAAACTCATCAATGTATCTTATCATG
      3. Incubate at 65 degrees for 5 minutes and then place on ice.
   2. To the above mixture, add:
      1. 9 ul first strand buffer
      2. 2.25 ul DTT
      3. 4ul super script III
      4. Adjust the total volume to 45 ul with RNase free water as needed
10. Incubate for 2 hours at 50C, then 70C for 15 minutes, then hold at 4C.
11. Add 1 μL of Rnase H to each tube and incubate for 20 min at 37C.
12. Purify the reaction with AMPure beads:
    1. Add 90 μl (2 X) of resuspended AMPure XP Beads to each reaction. Mix thoroughly on a vortex mixer or by pipetting up and down at least 10 times.
    2. Incubate for 5 minutes at room temperature.
    3. Put the tube on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the DNA targets.
    4. Add 200 μl of 80% freshly prepared ethanol to the tube while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
    5. Repeat the above step once.
    6. Air dry beads for 5 minutes while the tube is on the magnetic stand with the lid open. Caution: Do not overdry the beads. This may result in lower recovery of DNA target.
    7. Remove the tube from the magnet. Elute the DNA target from the beads by adding 25 μl of 0.1X TE (this is the same as Qiagen EB buffer).
    8. Mix well on a vortex mixer or by pipetting up and down and incubate for 2 minutes at room temperature.
    9. Put the tube in the magnetic stand until the solution is clear. Without disturbing the bead pellet, carefully transfer 24 μl of the supernatant to a fresh, sterile microfuge tube.

Final preparation of DNA input and RNA libraries

1. Targeted PCR. For each replicate, mix the following reaction:
   1. cDNA: ~250ng in 25ul (all of the sample from the above AMPure cleanup)
   2. F primer (GGGCCAGCTGTTGGGGTG\*T\*C\*C\*A\*C) at 10uM: 2.5 μL
   3. R primer (RT\_polyA primer) at 10uM: 2.5 μL
   4. KAPA HiFi HotStart Readymix DNA Polymerase OR NEB Q5 Hot start mix: 25 μL
   5. Sterile H2O: up to 50 μL
   6. Total volume: 50 μL
2. Place the tubes in Thermocycler and run the following program:
   1. 98 °C for 2 min
   2. 98 °C for 20 s, 62 °C for 30 s, 72 °C for 2 min (10 cycles)
   3. 72 °C for 5 min
   4. Hold at 4 °C
3. Purify the reaction with AMPure beads:
   1. Add 100 μl (2 X) of resuspended AMPure XP Beads to each reaction. Mix thoroughly on a vortex mixer or by pipetting up and down at least 10 times.
   2. Incubate for 5 minutes at room temperature.
   3. Put the tube on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the DNA targets.
   4. Add 200 μl of 80% freshly prepared ethanol to the tube while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
   5. Repeat the above step once.
   6. Air dry beads for 5 minutes while the tube is on the magnetic stand with the lid open. Caution: Do not overdry the beads. This may result in lower recovery of DNA target.
   7. Remove the tube from the magnet. Elute the DNA target from the beads by adding 26 μl of 0.1X TE (this is the same as Qiagen EB buffer).
   8. Mix well on a vortex mixer or by pipetting up and down and incubate for 2 minutes at room temperature.
   9. Put the tube in the magnetic stand until the solution is clear. Without disturbing the bead pellet, carefully transfer 25 μl of the supernatant to a fresh, sterile microfuge tube.
4. Index PCR. For each post-PCR, cleaned cDNA sample, mix the following reaction:
   1. DNA input (from above AMPure cleanup): 25ul
   2. NEB universal primer: 2.5 μL
   3. NEB indexed primer : 2.5 μL
      1. Record which index is used with what sample
   4. KAPA HiFi HotStart Readymix DNA Polymerase OR NEB Q5 Hot start mix: 25 μL
   5. Sterile H2O: up to 50 μL
5. Also, PCR the plasmid DNA extracted from the transfected cells (Step 13 under “Harvesting the transfected cells and preform a plasmid extraction”) as an input control, using the following mixture:
   1. Plasmid DNA (from miniprep): ~10 ng
   2. NEB universal primer: 2.5 μL
   3. NEB indexed primer : 2.5 μL
      1. Record which index is used with what sample
   4. KAPA HiFi HotStart Readymix DNA Polymerase OR Q5 Hot start mix: 25 μL
   5. Sterile H2O: up to 50 μL
6. Use following cycling conditions to add NEB indexes onto the input plasmid DNA library and the cDNA library:
   * 1. 98 °C for 2 min
     2. 98 °C for 15s, 62 °C for 30 s, 72 °C for 2 min (do 15 cycles for cDNA/RNA; 10 cycles for input plasmid DNA)
     3. 72 °C for 5 min
     4. Keep at 4 °C
7. Purify the reactions with AMPure beads:
   1. Add 100 μl (2 X) of resuspended AMPure XP Beads to each reaction. Mix thoroughly on a vortex mixer or by pipetting up and down at least 10 times.
   2. Incubate for 5 minutes at room temperature.
   3. Put the tube on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the DNA targets.
   4. Add 200 μl of 80% freshly prepared ethanol to the tube while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
   5. Repeat the above step once.
      1. Air dry beads for approximately 5 minutes while the tube is on the magnetic stand with the lid open. When beads are appropriately dry they will appear matte instead of glossy.
   6. Caution: Do not overdry the beads (apparent by cracked appearance). This may result in lower recovery of DNA target.
   7. Remove the tube from the magnet. Elute the DNA target from the beads by adding 25 μl of 0.1X TE (this is the same as Qiagen EB buffer).
   8. Mix well on a vortex mixer or by pipetting up and down and incubate for 2 minutes at room temperature.
   9. Put the tube in the magnetic stand until the solution is clear. Without disturbing the bead pellet, carefully transfer 24 μl of the supernatant to a fresh, sterile microfuge tube.
8. Quantify and visualize the libraries (e.g., on a Bioanalyzer).

Preparation of bisulfite sequencing libraries to confirm plasmid methylation levels

DAY 1 – ENZYME DIGESTS

1. For each replicate, aliquot 200ng of plasmid DNA extracted from transfected cells (Step 13 under “Harvesting the transfected cells and preform a plasmid extraction”) and add the following:
   1. 10ng of sheared (to 200-500bp) unmethylated lambda phage DNA (Promega). We use a Diagenode BioRuptor for DNA shearing, but any sonicator will do.
   2. 3ul of 10X NEB Cutsmart buffer
   3. 1ul BfaI + 1ul HindIII + 1ul HpaI (NEB)
   4. Enough water to bring total the volume to 30ul
2. Digest the DNA at 37C overnight (do not heat the lid higher than 37C if using a thermocycler).

DAY 2 – FILLING IN AND A-TAILING, BEAD CLEANUP, ADAPTOR LIGATION

1. Transfer each sample to a PCR tube. Perform end repair and A-tailing by adding: 1ul Klenow fragment + 1ul dNTPs (10mM) to each sample.
2. Incubate at 30C for 20min then 37C for 20min in a thermocycler without the heated lid (this will inactivate the enzyme!).
3. Once the program is complete, remove PCR tubes from thermocycler and spin down. Transfer each sample to a 1.5mL centrifuge tube for cleanup.
4. Add 64ul AMPure beads (2x the 32ul sample volume) to each sample and mix by pipette or short vortex.
5. Incubate at room temperature for 10min.
6. Place plate on magnetic stand until the solution is clear.
7. Remove supernatant.
8. Wash beads twice with freshly prepared 80% ethanol (leaving each wash on for approximately 30sec).
9. After removing the ethanol from the second wash, let the beads dry for ~5-10min or until beads are no longer shiny (do not overdry the beads, this will reduce yield).
10. Add 20ul EB buffer to each sample.
11. Cap samples, vortex very briefly to mix, and spin down.
12. Put the tubes back on the magnet and transfer the ~20ul of sample to a new tube.
13. Dilute each adaptor (Bioo Scientific NEXTfle Bisulfite-Seq Barcodes) 1:20 or get stock of already diluted adaptors.
14. Ligate adaptors by adding: 3ul T4 ligation buffer + 2ul T4 ligase (2,000,000 units/ml) + 4ul H20 + 2ul diluted adaptor to each well.
15. Incubate reaction overnight at 16C (use the thermocycler without the heated lid so you do not inactivate the enzyme).

DAY 3 – SAMPLE POOLING, BEAD CLEANUP

1. Pool all samples according to the lane they will be sequenced on.
2. Add a 1.5x concentration of beads to each pool of samples. Mix and incubate at room temperature for 10 min to ensure binding.
3. Put samples on magnetic stand and incubate until solution is clear.
4. Remove supernatant.
5. Wash beads twice with freshly prepared 80% ethanol.
6. After removing the second ethanol wash, dry the beads (~5-10min).
7. Elute DNA from the beads by adding 25ul EB buffer.
8. Vortex to mix and spin down. Wait 3-5 min.
9. Place tubes back on the magnetic stand and transfer elutant to a new microcentrifuge tube.

DAYS 3 and 4 – BISULFITE CONVERSIONS

1. Using Qiagen EpiTect Bisulfite Kit, perform the bisulfite conversion as specified for FFPE samples. A few notes on the Qiagen protocol:
   1. Do the optional/recommended steps.
   2. Check the date on the tube of bisulfite mix (they only last for 3-4 weeks after being mixed with water and stored at -20 C)
   3. Ensure that DNA, bisulfite mix, and DNA protect buffer are added in the order indicated.
   4. When you are ready for the elution, do this:
      1. Get EB preheated to 50-60C
      2. Add 24ul of EB directly to the column membrane (be careful to pipette directly onto the membrane).
      3. Incubate for 5min.
      4. Spin at full speed for 1min.
      5. Take the flow through (~21ul) and put it back on the membrane for a second elution.
      6. Incubate for 3-5min.
      7. Spin at full speed for 1min.
   5. Note, be very careful with bisulfite converted DNA. Do not vortex it or freeze thaw it. The DNA is fragile and will break apart easily.
2. Perform another round of bisulfite conversion as specified for FFPE samples, using ~20ul of sample and following the same tips provided above.

DAY 4 – PCR, FINAL LIBRARY QC

1. Perform 4 PCRs for each sample as follows: 5ul Pfu buffer + 0.5ul 100mM dNTP mix (or 5ul of 10mM dNTP mix, containing all 4 dNTPs) + 2 ul of the F primer at 10uM + 2 ul of the R primer at 10uM + 1ul Pfu DNA polymerase (Agilent) + 5 ul DNA + water to 50ul
   1. The forward and reverse primers are AATGATACGGCGACCACCGA\*G and CAAGCAGAAGACGGCATACGA\*G
   2. Cycling conditions are as follows:
      1. 95C for 2min
      2. 16 cycles of 95C for 30s, 60C for 30s, 72C for 45s
      3. 72 for 7min
2. Pool all 4 PCRs for each sample into a 1.5ml tube.
3. Add 260ul AMPure beads to each 200ul pool and mix.
4. Incubate at room temperature for 10 min.
5. Place samples on magnetic stand until supernatant is clear.
6. Remove supernatant.
7. Wash beads twice with freshly prepared 80% ethanol.
8. After removing the second ethanol was, dry the beads for ~5-10min. Be careful not to overdry the beads.
9. Elute in 40ul EB buffer by adding EB buffer to dried beads, mixing, waiting a few minutes, placing tube back on the magnetic stand, and removing the elutant.
10. Do a second cleanup, by mixing the 40ul of sample with 60ul of beads and repeating steps 32-36. Elute the final library in 20ul of EB.
11. Quantify and visualize the library (e.g., on a Bioanalyzer).