Graveley Lab CRISPR Genome Editing followed by RNA-seq Library Preparation and Characterization Document

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Project: ENCORE
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Sample Description: CRISPR genome editing of PAN2 in HepG2 cells
Cell Line: HepG2
RNA ID: PAN2-BGHcLV22-35
Multiplex Barcode: ATGTAAGT+ACTCTATG
ENCODE BIOSAMPLE ACCESSION: ENCBS647PKG
ENCODE LIBRARY ACCESSION: ENCLB927SYC
ENCODE EXPERIMENT ACCESSION: ENCSR932GTJ

This document contains the protocols used for library preparation and characterization of RNA isolated from HepG2 cells depleted of PAN2.

RNA Quality Control

RNA was isolated from HepG2 cells transduced with lentiviral particles expressing a CRISPR genome editing the PAN2 mRNA as described in ENCODE Biosample Accession number ENCBS647PKG. The quality of the RNA was measured using an Agilent TapeStation Instrument with an RNA Screen Tape (Catlog Number 5067−5576).

1. Mix 1 μl of RNA and 5 μl RNA sample buffer.
2. Vortex and briefly spin to ensure sample is at the bottom of the tube.
3. Heat samples in a thermocycler to 72 °C for 3 minutes.
4. Place samples on ice, then briefly spin to ensure sample is at the bottom of the tube.
5. Run samples on the RNA Screen Tape.

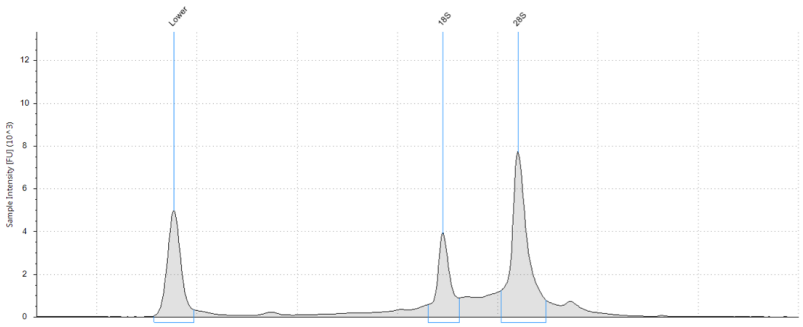

Figure 1: Agilent TapeStation image of 1 μl of the input total RNA sample.

Library Prep Protocol

RNA-Seq libraries were prepared following the Illumina the TruSeq Stranded mRNA Sample Preparation Kit (Catalog numbers RS−122−2101 or RS−122−2102). Spike-in RNAs are obtained from NIH (Catalog number ERCC−78A−11119).

Purify and Fragment mRNA

This process purifies the polyA containing mRNA molecules using poly−T oligo attached magnetic beads using two rounds of purification. During the second elution of the polyA RNA, the RNA is also fragmented and primed for cDNA synthesis.

1. Dilute 1.0 μg of the total RNA with nuclease-free ultra pure water to a final volume of 25 μL in a 0.3 ml PCR plate or tube with a 1:1000 ratio of spike-ins.
2. Vortex the RNA Purification Beads tube vigorously to resuspend the oligo−dT beads.
3. Add 25 μL of RNA Purification Beads to the sample to bind the polyA RNA to the oligo dT magnetic beads.
4. Gently pipette the entire volume up and down 6 times to mix thoroughly.
5. Incubate the sample in a thermal cycler at 65 °C for 5 minutes and then hold at 4 °C to denature the RNA and facilitate binding of the RNA to the beads.
6. When the thermal cycler reaches 4 °C, remove the sample from the thermal cycler and incubate it at room temperature for 5 minutes to allow the RNA to bind to the beads.
7. Place the sample on the magnetic stand at room temperature for 5 minutes to separate the polyA RNA bound beads from the solution.
8. Remove and discard all of the supernatant.
9. Remove the sample from the magnetic stand.
10. Wash the beads by adding 150 μl of Bead Washing Buffer to remove unbound RNA.
11. Gently pipette the entire volume up and down 6 times to mix thoroughly.
12. Place the sample on the magnetic stand at room temperature for 5 minutes.
13. Remove and discard all of the supernatant from the sample and remove the sample from the magnetic stand.
14. Add 25 μl of Elution Buffer to the sample and gently pipette the entire volume up and down 6 times to mix thoroughly.
15. Incubate the sample in a thermal cycler at 80 °C for 2 minutes, and then hold at 25 °C hold to elute the mRNA from the beads.
16. Remove the sample from the thermal cycler when it reaches 25 °C and place on the bench at room temperature.
17. Add 25 μl of Bead Binding Buffer to the sample to allow the mRNA to specifically rebind the beads, while reducing the amount of rRNA that non-specifically binds.
18. Gently pipette the entire volume up and down 6 times to mix thoroughly.
19. Incubate the sample at room temperature for 5 minutes.
20. Place the sample on the magnetic stand at room temperature for 5 minutes and then remove and discard all of the supernatant.

21. Remove the sample from the magnetic stand and wash the beads by adding 150 μl of Bead Washing Buffer.

22. Gently pipette the entire volume up and down 6 times to mix thoroughly and then place the sample on the magnetic stand at room temperature for 5 minutes.

23. Remove and discard all of the supernatant and then remove the sample from the magnetic stand.

24. Add 11.0 μl of Fragment, Prime, Finish Mix to the sample.

25. Gently pipette the entire volume up and down 6 times to mix thoroughly. The Fragment, Prime, Finish Mix contains random hexamers for RT priming and serves as the 1st strand cDNA synthesis reaction buffer.

26. Incubate the sample in a thermal cycler at 94 °C for 4 minutes and then hold at 4 °C to elute, fragment, and prime the RNA.

27. Remove the sample from the thermal cycler and centrifuge briefly.

Synthesize First Strand cDNA

This process reverse transcribes the cleaved RNA fragments that were primed with random hexamers into first strand cDNA using reverse transcriptase and random primers. The addition of Actinomycin D to the First Stand Synthesis Act D mix prevents spurious DNA−dependent synthesis, while allowing RNA-dependent synthesis, improving strand specificity.

1. Place the sample on the magnetic stand at room temperature for 5 minutes.

2. Transfer 8.5 μl of the supernatant to a new 0.3 ml plate or tube.

3. Add SuperScript II to the First Strand Synthesis Act D Mix tube at a ratio of 1 μl SuperScript II for each 9 μl First Strand Synthesis Act D Mix.

4. Add 4.0 μl of First Strand Synthesis Act D Mix and SuperScript II mix to the sample and gently pipette the entire volume up and down 6 times to mix thoroughly.

5. Place the sample in a thermal cycler and incubate at 25 °C for 10 min, 42 °C for 15 min, 70 °C for 15 min, and hold at 4 °C.

6. Remove the sample from the thermal cycler and proceed immediately to the second strand cDNA synthesis reaction.

Synthesize Second Strand cDNA

This process removes the RNA template and synthesizes a replacement strand, incorporating dUTP in place of dTTP to generate ds cDNA. The incorporation of dUTP quenches the second strand during amplification, because the polymerase does not incorporate past this nucleotide.

1. Add 2.5 μl of Resuspension Buffer to the reaction.
2. Add 10 μl of thawed Second Strand Marking Master Mix to the reaction.
3. Gently pipette the entire volume up and down 6 times to mix thoroughly.
4. Return the reaction to the pre-heated thermal cycler, close the lid and incubate at 16 °C for 1 hour.
5. Remove the reaction from the thermal cycler, place it on the bench and let it to come to room temperature.
6. Add 45 μl of well-mixed AMPure XP beads (Beckman Coulter, Catalog Number A63881) to the ds cDNA.
7. Gently pipette the entire volume up and down 10 times to mix thoroughly and incubate at room temperature for 15 minutes.
8. Place the reaction on the magnetic stand at room temperature, for 5 minutes to make sure that all of the beads are bound to the side of the wells.
9. Remove and discard 65.0 μl of supernatant from the reaction.
10. While leaving the tube on the magnetic stand add 150 μl freshly prepared 80% EtOH without disturbing the beads.
11. Incubate at room temperature for 30 seconds, and then remove and discard all of the supernatant.
12. Repeat once for a total of two 80% EtOH washes.
13. Let stand at room temperature for 15 minutes to dry, and then remove from the magnetic stand.
14. Add 11.25 μl Resuspension Buffer and gently pipette the entire volume up and down 10 times to mix thoroughly.
15. Incubate at room temperature for 2 minutes and then place on the magnetic stand at room temperature for 5 minutes.
16. Transfer 8.75 μl supernatant (ds cDNA) from the tube to the new 0.3 ml PCR plate or tube.

A Tailing

A single 'A' nucleotide is added to the 3' ends of the blunt fragments to prevent them from ligating to one another during the adapter ligation reaction. A corresponding single 'T' nucleotide on the 3' end of the adapter provides a complementary overhang for ligating the adapter to the fragment. This strategy ensures a low rate of chimera (concatenated template) formation.

1. Add 6.25 μl of thawed A-Tailing Mix to each reaction.
2. Gently pipette the entire volume up and down 10 times to mix thoroughly.

3. Gently pipette the entire volume up and down 10 times to mix thoroughly.
4. Place the tube in a thermal cycler with the lid pre-heated to 100 °C, close the lid, and incubate at 37 °C for 30 minutes, 70 °C for 5 minutes, and hold at 4 °C.
5. Proceed directly to ligating the adapters.

Ligate Adapters

This process ligates multiple indexing adapters to the ends of the ds cDNA, preparing them for hybridization onto a flow cell.

1. Add 1.25 μl of Resuspension Buffer to each reaction.
2. Add 1.25 μl of Ligation Mix to each reaction.
3. Add 1.25 μl of the thawed RNA Adapter Index to each reaction.
4. Gently pipette the entire volume up and down 10 times to mix thoroughly.
5. Place the tube in the pre-heated thermal cycler. Close the lid and incubate at 30 °C for 10 minutes.
6. Remove from the thermal cycler.
7. Add 2.5 μl of Stop Ligation Buffer to each reaction to inactivate the ligation.
8. Gently pipette the entire volume up and down 10 times to mix thoroughly.
9. Vortex the AMPure XP Beads for at least 1 minute or until they are well dispersed.
10. Add 21 μl of mixed AMPure XP Beads to each reaction.
11. Gently pipette the entire volume up and down 10 times to mix thoroughly.
12. Incubate the ALP plate at room temperature for 15 minutes.
13. Place the ALP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.
14. Remove and discard 38.0 μl of supernatant from the tube taking care not to disturb the beads.
15. With the tube on the magnetic stand, add 150 μl freshly prepared 80% EtOH without disturbing the beads.
16. Incubate at room temperature for 30 seconds, and then remove and discard all of the supernatant taking care not to disturb the beads.
17. Repeat twice for a total of two 80% EtOH washes.
18. While still on the magnetic stand, let the samples air-dry at room temperature for 15 minutes.

19. Remove the tube from the magnetic stand.
20. Add 27.5 μl Resuspension Buffer to each tube.
21. Gently pipette the entire volume up and down 10 times to mix thoroughly or until the beads are fully resuspended.
22. Incubate at room temperature for 2 minutes.
23. Place the reaction on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.
24. Transfer 25 μl of supernatant from the tube to a new 0.3 ml PCR plate or tube labeled with the CAP barcode. Take care not to disturb the beads.
25. Vortex the AMPure XP Beads until they are well dispersed.
26. Add 25 μl of mixed AMPure XP Beads to each tube for a second cleanup.
27. Gently pipette the entire volume up and down 10 times to mix thoroughly.
28. Incubate at room temperature for 15 minutes.
29. Place the tube on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.
30. Remove and discard 47.5 μl of supernatant taking care not to disturb the beads.
31. With the tube on the magnetic stand, add 150 μl freshly prepared 80% EtOH taking care not to disturb the beads.
32. Incubate at room temperature for 30 seconds, and then remove and discard all of the supernatant taking care not to disturb the beads.
33. Repeat one time for a total of two 80% EtOH washes.
34. With the tube on the magnetic stand, let the sample air-dry at room temperature for 15 minutes, and then remove the tube from the magnetic stand.
35. Add 12.5 μl Resuspension Buffer to the tube.
36. Gently pipette the entire volume up and down 10 times to mix thoroughly or until the beads are fully resuspended.
37. Incubate at room temperature for 2 minutes.
38. Place the tube on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.
39. Transfer 10 μl of supernatant to a new 0.3 ml PCR plate or tube taking care not to disturb the beads.

Enrich DNA Fragments

This process uses PCR to selectively enrich those DNA fragments that have adapter molecules on both ends and to amplify the amount of DNA in the library. The PCR is performed with a PCR Primer Cocktail that anneals to the ends of the adapters. Minimize the number of PCR cycles to avoid skewing the representation of the library.

1. Add 2.5 μl of thawed PCR Primer Cocktail to the sample.
2. Add 12.5 μl of thawed PCR Master Mix to the sample.
3. Gently pipette the entire volume up and down 10 times to mix thoroughly.
4. Place the tube in the pre-programmed thermal cycler with the lid pre-heated to 100 °C, close the lid, and incubate at 98 °C for 30 seconds, and then 15 cycles of 98 °C for 10 seconds, 60 °C for 30 seconds, 72 °C for 30 seconds, and then 72 °C for 5 minutes and hold at 4 °C.
5. Add 25 μl of AMPure XP Beads to the tube.
6. Gently pipette the entire volume up and down 10 times to mix thoroughly.
7. Incubate the PCR plate at room temperature for 15 minutes.
8. Place the tube on a magnetic stand at room temperature for 5 minutes or until the liquid is clear.
9. Remove and discard 47.5 μl of supernatant from the tube.
10. While on the magnetic stand, add 150 μl freshly prepared 80% EtOH without disturbing the beads.
11. Incubate at room temperature for 30 seconds, and then remove and discard all of the supernatant.
12. Repeat one time for a total of two 80% EtOH washes.
13. While on the magnetic stand, let the sample air-dry at room temperature for 15 minutes, and then remove from the magnetic stand.
14. Add 27.5 μl Resuspension Buffer to the sample.
15. Gently pipette the entire volume up and down 10 times to mix thoroughly.
16. Incubate at room temperature for 2 minutes.
17. Place the sample on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.
18. Transfer 25 μl of the supernatant from the tube to a new 0.3 ml PCR plate or tube.

Validate Library

1. Initially quantitate the libraries by Nanodrop or Tecan.

2. Further validate the libraries by running on an Agilent Tapestation D1000 screen tape (Catalog number 5067−5582). Dilute 1 μl of each library into 3 μl of sample buffer and run on the Tapestation.

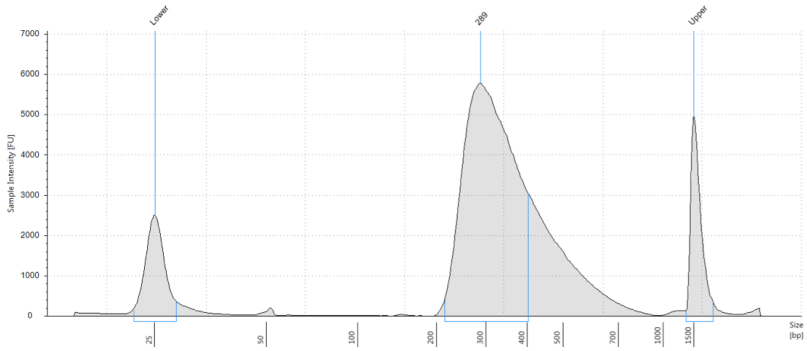

Figure 2: Agilent TapeStation image of 1 μl of the library.