OTTR cDNA library purification, size selection, and PCR indexing for Illumina NGS

The steps below were performed in published work, but other workflows could be developed. For example, library cDNA could be purified using SPRI selection. If using SPRI selection without prior cDNA clean-up, dilute the finished reaction ~15 fold to reduce the concentration of salts for proper bead performance.

cDNA clean-up

1. Remove enzyme from product.

   a. Denature OTTR duplexes by heating to 95 °C.

   b. Add 1 μL of RNase A. Optional: also add 1 μL of thermostable RNase H (NEB, M0523S).

   c. Incubate at 50 °C for 10 minutes.

   d. Add 35-80 μL of stop solution (50 mM Tris pH 8, 20 mM EDTA, 0.2% SDS) and mix (volume will vary to adapt to downstream purification options below). Recommended: add 1 μL of proteinase K and incubate again at 50 °C for 10 minutes.

   e. Incubate at 95 °C for 5 minutes.

   f. Cool the samples to room temperature. Placing the samples on ice can induce SDS precipitation; if this happens, place tubes at 50 °C for 5 minutes or longer to reverse it.

2. Purify DNA (two tested options given below).

A. Use a spin column such as Oligo clean & concentrator (Zymo)

B. Organic extraction and precipitation:

   a. Extract with an equal volume phenol:chloroform:isoamyl alcohol (25:24:1 at near pH 6.7), move the upper aqueous phase to a new tube containing 10 μL 3 M NaOAc pH 5.2 and 20 μg glycogen, mix, add 350 μL 100% ethanol, mix. Chill in dry ice bath for 2-3 min or flash freeze in liquid nitrogen or place at -80 °C for an hour.

   b. Spin 15 min at maximum microfuge speed. Remove supernatant and wash pellet with 1 mL 70% ethanol. Spin again. Remove supernatant and let pellet dry for 5 min.

3. Resuspend in 5 – 25 μL of nuclease free water or, if proceeding to gel purification, in 5 μL of 2X formamide loading buffer. Optional: allow 10 minutes for resuspension, then vortex and briefly spin to collect liquid at the bottom of the tube.

Recipe:

2X formamide loading buffer: 95% formamide, 5 mM EDTA (pH 8.0), 0.05% (w/v) bromophenol blue and/or 0.05% (w/v) xylene cyanol

cDNA size selection using denaturing PAGE

1. Pre-run an approximately 8% denaturing urea polyacrylamide gel for 15 minutes.
2. Rinse the wells of the gel using a needle and syringe. Load the gel. Ideally, load a positive control OTTR library in a middle lane to have a cDNA size marker. Also it is useful if at least one well has dilute xylene-cyanol and bromophenol blue as migration markers.
3. Run the gel until the xylene cyanol dye front is near the bottom of the gel. In a 1X TBE 8% denaturing urea polyacrylamide gel, the xylene cyanol migrates at roughly the same rate as the unwanted adapter dimer cDNA.
4. Disassemble gel and transfer to saran wrap. Carefully cut the gel above the xylene cyanol dye front and discard the bottom portion of the gel to reduce adapter dimer contamination.
5. Options for detecting cDNA depend on which fluorescent dye is present at a primer 5′ end (original OTTR primers have 5′ Cy5, others have IR800).
    a. **Direct Cy5 detection**: capture Cy5 using an emission filter of 655 – 685 nm. Warning: xylene cyanol remaining on the gel will also be detected in this emission spectra.
    b. **Direct IR800 detection**: capture IR-long using an emission filter of 810 – 840 nm.
    c. **SYBR Gold nucleic acid staining**: Combine 5 μL of SYBR Gold nucleic acid stain with 5 – 10 mL of 1X TBE and cover the gel with this buffer for no more than 3 minutes. Agitate the gel while it stains by gentle shaking. Rinse briefly with 1X TBE and remove as much residual buffer from the gel as possible before proceeding. Transfer gel to the scanner and image using a Cy2 emission filter of 515 – 535 nm.
6. Print the scanned gel image at actual size with signal adjusted so that it is easy to see cDNA products. Transfer the gel to the printed image and perfectly align.
7. Using a clean razor blade, excise the desired size range of cDNA. Use a clean edge of the blade for each cDNA sample. Transfer gel slice to a pre-labeled tube.
8. Crush the gel slices with a clean 1 mL pipette tip. Add 500 μL of cDNA elution buffer (300 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA). Incubate at 70 °C for 1 hour.
9. Centrifuge for 10 minutes at maximum speed.
10. Transfer 450 μL of eluate to a new tube. If necessary, centrifuge and transfer again to completely remove residual gel fragments.
11. Add 1 μL of 20 mg/mL glycogen, vortex, then add 3 volumes of 100% ethanol. Split across two tubes if necessary. After adding ethanol and vortexing, any remaining gel fragments will immediately become opaque and flocculate. Typically, a pipette tip can be used to fish these bits out before centrifuging.
12. Incubate at -20 °C for 30 minutes.
13. Pellet the cDNA by centrifugation at 4 °C at 20,000 × g for 15 minutes.
14. Aspirate 90% of the solution and add 1 mL of -20 °C chilled 70% ethanol and briefly vortex. Optional: Incubate at -20 °C for 15 minutes.
15. Pellet the cDNA by centrifugation at 4 °C at 20,000 × g for 15 minutes.
16. Carefully remove liquid, briefly spin to collect any remaining liquid to the bottom of the tube, and carefully remove any remaining liquid using a 10-20 μL pipette.
17. Air dry, ideally for no more than 10 minutes, to evaporate any remaining 70% ethanol.
18. Resuspend cDNA pellet in nuclease-free water. Optional: quantify yield by qPCR.

Multiplexing PCR and recommended post-PCR NGS library size selection

Template should be up no more than 10% volume of the PCR reaction to avoid contaminant carry over. For multiplexing libraries, select a unique i5 and i7 index for each library. PCR reaction components can be premixed before adding to cDNA.

1. Combine cDNA and nuclease-free water to 36.5 μL.
2. Add 1 μL 25 μM i5 primer*
3. Add 1 μL 25 μM i7 primer*
4. Add 1 μL 10 μM dNTPs.
5. Add 10 μL 5X Q5 reaction buffer (NEB).
6. Add 0.5 μL of Q5 polymerase (NEB).
7. Pre-heat thermocycler to 95 °C.
8. Perform PCR with the following cycling parameters, where X is cycle number:
    a. 98°C, 1 min
        i. 98°C, 20 sec
        ii. 65°C, 20 sec
        iii. 72°C, 10 sec
        iv. Repeat no more than 13 times.
    b. 72°C, 2 min
9. Transfer 50 μL reaction to a 1.5 mL tube and add 50 μL nuclease-free water, 1 μL 20 mg/ml glycogen, 10 μL 7.5 M ammonium acetate, 100 μL 100% isopropanol.
10. Incubate at -20 °C for 30 minutes and pellet the DNA by centrifuging for 15 minutes at 20,000 × g.
11. Wash the pellet once by adding 1 mL of 75% ethanol. Optional: Incubate at -20 °C for 15 minutes.
12. Pellet the DNA by centrifuging for 15 minutes at 20,000 × g.
13. Aspirate the liquid from the pellet and let it air dry for 10 minutes.

*IMPORTANT INFORMATION about OTTR cDNA PRIMER sequence compatibility.

OTTR Buffer 4B2 primer:
5IRD800/GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
OTTR cDNA primer in Buffer 4B2 has no incompatibilities.

OTTR Buffer 4B primers (early kit version):
5Cy5/GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
5Cy5/GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC**C**
Some commercial i7 (Read 2) PCR primers will have a terminal mismatch with the lower-concentration 3'C cDNA primer in the two-primer mix of original Buffer 4B. If using original OTTR Buffer 4B, use recommended PCR primers:
i5: AATGATACGGCGACCACCGAGATCTACAC[i5]ACACTCTTTCCCTACACGAC
i7: CAAGCAGAAGACGGCATACGAGAT[i7]GTGACTGGAGTTCAGACGTG

Standard Illumina Multiplexing Read 1 Sequencing Primer
5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT
Standard Illumina Multiplexing Read 2 Sequencing Primer
5' GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

Post-PCR size selection

14. Resuspend DNA pellet in 2X formamide loading dye (95% formamide, 5 mM EDTA (pH 8.0), 0.05% (w/v) bromophenol blue and/or 0.05% (w/v) xylene cyanol).
15. Pre-run 9% native polyacrylamide gel for 15 minutes.
16. Rinse the wells with 1X TBE before loading samples. Use appropriate size markers.
17. Run the gel until the bromophenol blue dye reaches the bottom of the gel.
18. Combine 5 μL of SYBR Gold nucleic acid stain with 5 – 10 mL of 1X TBE and cover the gel with this buffer for no more than 3 minutes. Agitate by gentle shaking. Rinse briefly with 1X TBE and remove as much residual buffer from the gel as possible before proceeding.
19. Transfer gel to a new Saran Wrap. Image using Cy2 emission filter of 515 – 535 nm.
20. Using a Blue-Box transilluminator, illuminate the gel and excise the size region of interest. Transfer gel slice to a pre-labeled tube.
19. Crush the gel slices with a clean 1 mL pipette tip. Add 500 μL of cDNA elution buffer (300 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA). Incubate at room temp overnight with gentle rotation.
20. Centrifuge for 10 minutes at maximum speed.
21. Transfer 450 μL of eluate to a new tube. If necessary, centrifuge and transfer again to completely remove residual gel fragments.
22. Add 1 μL of 20 mg/mL glycogen, vortex, then add 3 volumes of 100% ethanol. Split across two tubes if necessary. After adding ethanol and vortexing, any remaining gel fragments will immediately become opaque and flocculate. Typically, a pipette tip can be used to fish these bits out before centrifuging.
23. Incubate at -20 °C for 30 minutes.
24. Pellet the cDNA by centrifugation at 4 °C at 20,000 × g for 15 minutes.
25. Aspirate 90% of the solution and add 1 mL of -20 °C chilled 70% ethanol and briefly vortex. Optional: Incubate at -20 °C for 15 minutes.
26. Pellet the cDNA by centrifugation at 4 °C at 20,000 × g for 15 minutes.
27. Carefully remove liquid, briefly spin to collect any remaining liquid to the bottom of the tube, and carefully remove any remaining liquid using a 10-20 μL pipette.
28. Air dry, ideally for no more than 10 minutes, to evaporate any remaining 70% ethanol.
29. Resuspend cDNA pellet in nuclease-free water.