Small-sample cDNA reamplification for RNA-seq Entered by Kevin Janes
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Before starting:

- Have small-sample cDNA material as template for reamplification
- Have access to a 96-well plate or SPRIPlate ring magnet (Beckman #A32782 or equivalent)

I. Identification of optimum cycle threshold for reamplification

1. Set up a 19 μl master mix-1 on ice
2. Add 1 μl of 5×-diluted amplified cDNA to each well of an optically clear PCR strip tube (Bio-Rad #TLS0801 and #TCS0803) or hard-shell PCR plate (BioRad #HSP9601 and #MSB1001) (one well for each sample)
3. Add 19 μl of the master mix to each well on ice and mix by pipetting
   - *Be sure to use barrier tips when mixing to avoid amplicon contamination of the pipette*
4. Run 40 cycles of qPCR:
   i. 1 min at 94°C
   ii. 2 min at 42°C
   iii. 3 min at 72°C (fluorescence read at the end of the extension)
5. The optimum cycle for reamplification is when the first sample hits the middle of its exponential phase
   - *Going longer than the middle of the exponential phase will overamplify the sample and cause loss of quantitative accuracy*
   - *The Nextera XT kit requires only 1 ng of cDNA, meaning that most samples will have acceptable yield even if there is a great disparity in the apparent abundances of cDNA material for different samples*
   - *High-Fidelity gives the highest yield at the end of the reamplification (do not substitute AmpliTaq)*

II. Reamplification and purification of small-sample cDNA

1. Set up a 99 μl master mix-2 on ice
2. Add 1 μl of amplified cDNA to each well of a PCR strip tube or PCR plate (one well for each sample)
   - *Any thin-walled strip tube or plate will suffice at this step*
3. Add 99 μl of the master mix to each well and mix by pipetting
   - *Be sure to use barrier tips when mixing to avoid amplicon contamination of the pipette*
4. Run the optimum number of cycles of PCR as determined in Part I:
   i. 1 min at 94°C
   ii. 2 min at 42°C
   iii. 3 min at 72°C
5. Purify and concentrate the PCR reaction with AMPure XP beads (Beckman #A63880)
   i. Equilibrate AMPure beads at room temperature for 30 min before starting
   ii. Add 70 μl AMPure beads per 100 μl reamplification (0.7× volume), mix by pipetting 10 times, and incubate at room temperature for 15 min
      - *The lower volume of AMPure beads compared to the manufacturer's protocol enables selective purification of amplicon >300 bp in size and the removal of AL1-oligo(dT) concatemers*
   iii. Magnetize the samples at room temperature for 5 min
      - *Depending on the magnet, some of the beads may stick to the upper walls of the tube; if using strip tubes, it is possible to tilt the strips and guide the beads to the bottom of the well*
      - *For the SPRIPlate ring magnet, the beads are supposed to stick to the walls of the tube*
   iv. On the magnet, aspirate the supernatant with a gel-loading pipette tip, leaving 5 μl in the well
      - *Leaving residual volume is important for plate magnets that pull the beads to the bottom of the well*
   v. On the magnet, wash with 200 μl of freshly prepared 80% EtOH for 30 sec and aspirate with a gel-loading pipette tip
      - *80% EtOH is defined here as 8 ml 100% EtOH + 2 ml H2O (no voluming up)*
      - *Add the 80% EtOH gently to the beads to avoid disrupting them*

vi. Repeat Step 3.v, making sure to remove all the residual EtOH
vii. Air dry the wells for 10 min at room temperature
- *It is important to dry the beads thoroughly to improve recovery, as EtOH will reduce the solubility of the bead-bound amplicon*
- *However, it is also important not to overdry the beads, as the amplicon will not elute off the bead surface; avoid a "cracked" appearance of dried beads on the tube walls*

viii. Resuspend the beads in 10 μl of elution buffer (10 mM Tris-HCl, pH 8.5) and incubate for 2 min at room temperature
- *When dissolving in this small of a volume, it is important to rinse all the beads to the bottom of the well for maximum recovery*
- *Be sure to use barrier tips when mixing to avoid amplicon contamination of the pipette*

ix. Magnetize the samples at room temperature for 1 min
x. Transfer the supernatant to a fresh PCR strip tube or PCR plate

6. Repurify the PCR reaction with AMPure XP beads (Beckman #A63880)
- *This second round of purification improves the amplicon purity from 85% to >95%*

i. Equilibrate AMPure beads at room temperature for 30 min before starting
ii. Add 7 μl AMPure beads per 10 μl purified sample (0.7× volume), mix by pipetting 10 times, and incubate at room temperature for 15 min
- *The lower volume of AMPure beads compared to the manufacturer's protocol enables selective purification of amplicon >300 bp in size and the removal of AL1-oligo(dT) concatemers*

iii. Magnetize the samples at room temperature for 5 min
- *Depending on the magnet, some of the beads may stick to the upper walls of the tube; if using strip tubes, it is possible to tilt the strips and guide the beads to the bottom of the well*
- *For the SPRIPlate ring magnet, the beads are supposed to stick to the walls of the tube*

iv. On the magnet, aspirate the supernatant with a gel-loading pipette tip, leaving 5 μl in the well
- *Leaving residual volume is important for plate magnets that pull the beads to the bottom of the well*

v. On the magnet, wash with 200 μl of freshly prepared 80% EtOH for 30 sec and aspirate with a gel-loading pipette tip
- *80% EtOH is defined here as 8 ml 100% EtOH + 2 ml H2O (no voluming up)*
- *Add the 80% EtOH gently to the beads to avoid disrupting them*

vi. Repeat Step 6.v, making sure to remove all the residual EtOH
vii. Air dry the wells for 10 min at room temperature
- *It is important to dry the beads thoroughly to improve recovery, as EtOH will reduce the solubility of the bead-bound amplicon*
- *However, it is also important not to overdry the beads, as the amplicon will not elute off the bead surface; avoid a "cracked" appearance of dried beads on the tube walls*

viii. Resuspend the beads in 10 μl of elution buffer (10 mM Tris-HCl, pH 8.5) and incubate for 2 min at room temperature
- *When dissolving in this small of a volume, it is important to rinse all the beads to the bottom of the well for maximum recovery*
- *Be sure to use barrier tips when mixing to avoid amplicon contamination of the pipette*

ix. Magnetize the samples at room temperature for 1 min
x. Transfer the supernatant to a fresh PCR strip tube or PCR plate

7. Use 1 μl of the 2x purified amplicon for quantification by Qubit assay, and store the remainder at –20ºC before Nextera XT tagmentation

III. Amplicon quantification by Qubit assay (Life Technologies #Q32850) on the CFX96 instrument

1. Prepare a seven-point standard curve by serially diluting the Qubit dsDNA BR Standard #2 (at 100 ng/μl) twofold in Qubit dsDNA Standard #1 (at 0 ng/μl) to yield 100, 50, 25, 12.5, 6.25, 3.1, and 1.6 ng/μl

- *Only 1 μl of each serial dilution will be used in the assay*
- *Also be sure to include a blank of 100% Qubit dsDNA BR Standard #1*

2. Add 1 ul of each serial dilution or 2x purified amplicon to the base of an optically clear PCR strip tube (Bio-Rad #TLS0801 and #TCS0803) or hard-shell PCR plate (BioRad #HSP9601 and #MSB1001) (one well for each sample)
3. Prepare Qubit Working Solution by diluting Qubit dsDNA BR Reagent 200-fold in Qubit dsDNA BR Buffer
   - *199 μl of Buffer + 1 μl of Reagent is enough for 8–9 samples*
4. Add 19 μl of Qubit working solution to each well and mix by pipetting 10 times
5. Seal the strip tube or plate and read once on the CFX96 instrument at 22ºC after a hold at 22ºC for 2 min
   - *The Qubit detection reagent has an excitation maximum at 510 nm and an emission maximum at 527 nm, which is close enough to SYBR Green (Ex/Em = 497/520 nm) to use any qPCR detection instrument*
   - *Our saved protocol is named "QUBIT"*
6. Export the ZPCR file from the instrument and load into the CFX Manager Software
7. Once the PCRD file had been created, deselect all empty wells on the plate
   - *Having only the active wells selected makes data export easier*
8. Go to Settings > Baseline Setting > No Baseline Subtraction to turn off the default background subtraction settings
   - *It is crucial to export the raw RFU values or else the data will be uninterpretable*
9. Go to Export > Export All Data Sheets > Excel 2003
   - *The file ending in "End Point Results.xls" will contain the end RFUs from the instrument for the selected wells*
   - *Export > Custom Export will export only one file of RFUs if set up correctly, although all the wells of the 96-well plate will be exported*
10. Open the exported RFU data in Excel, perform linear regression on the standard curve, and back calculate the DNA concentrations of the 2x purified amplicon samples
11. Dilute a fraction of the 2x purified amplicon samples to 0.2 ng/μl in H_2O for tagmentation with the Nextera XT kit.

Master mix and buffer recipes

- **Master mix-1** Prepare fresh on ice
  2 μl 10× High-Fidelity PCR buffer without Mg^{2+}
  2.8 μl 25 mM $MgCl_2$
  0.04 μl 100 mM dATP
  0.04 μl 100 mM dCTP
  0.04 μl 100 mM dGTP
  0.04 μl 100 mM dTTP
  0.1 μl 20 mg/ml BSA
  0.06 μl 15 mg/ml AL1 primer
  0.2 μl High-Fidelity polymerase
  0.05 μl 100× SYBR green
  13.63 μl PCR-grade H_2O
  19 μl total volume

- **Master mix-2** Prepare fresh on ice
  10 μl 10× High-Fidelity PCR buffer without Mg^{2+}
  14 μl 25 mM $MgCl_2$
  0.2 μl 100 mM dATP
  0.2 μl 100 mM dCTP
  0.2 μl 100 mM dGTP
  0.2 μl 100 mM dTTP
  0.5 μl 20 mg/ml BSA
  0.3 μl 15 mg/ml AL1 primer
  1 μl High-Fidelity polymerase
  72.4 μl PCR-grade H_2O
  99 μl total volume