Small-sample cDNA reamplification and labeling Entered by Lixin Wang
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Before starting:

- Have single-cell cDNA material as template for reamplification

I. Identification of optimum cycle threshold for reamplification

1. Set up a 19 μl master mix-1 on ice
2. Add 19 μl of the master mix to a well on a qPCR plate
3. Add 1 μl of 5×-diluted amplified cDNA (one well for each sample)
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*
   - *If there is a great disparity in the apparent abundances of cDNA material for different samples, multiple reamplifications of the same low-concentration cDNA template can be run in parallel and pooled during purification*
   - *High-Fidelity gives the highest yield at the end of the reamplification and the highest aminoallyl incorporation for labeling (do not substitute AmpliTaq)*

II. Reamplification and purification of amine-modified single-cell cDNA

1. Set up a 100 μl master mix-2 on ice
2. 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
3. Purify the PCR reaction on a PureLink column to remove unincorporated primer and dNTPs
   i. Add 400 μl PureLink Binding Buffer (**not** High-Cutoff)
   ii. Apply entire solution to a PureLink column and spin at 10,000 × g for 1 min
   iii. Discard flow through, wash column with 650 μl Wash Buffer, and spin at 10,000 × g for 1 min
   iv. Discard flow through, and spin again at 10,000 × g for 1 min
   v. Transfer column to a clean elution tube and add 50 μl elution buffer
   vi. Seal the column with the cap, and incubate at 65°C for 10 min
      - *High-temperature incubations of the column ensure maximum yield*
      - *Be sure to seal the cap to prevent excessive evaporation*
      - *Eluting in water does not significantly improve the labeling*
   vii. Spin at 10,000 × g for 1 min
   viii. Add another 50 μl elution buffer, reseal the column with the cap, and incubate at 65°C for 10 min
   ix. Spin at 10,000 × g for 1 min
4. Add 10 μl NaOAc (pH 5.2) and 1 μl 20 mg/ml glycogen. Vortex
   - *Do not substitute ammonium acetate (free amines inhibit subsequent labeling)*
5. Add 250 μl ice-cold 100% EtOH, vortex, and incubate at –20°C for at least 30 min
   - *Keep a tube of EtOH at –20°C to speed the precipitation to completion*
   - *Longer incubations at –20°C are fine*
6. Spin for 10 min at max speed on a benchtop centrifuge
7. Carefully aspirate supernatant and wash pellet with 500 μl 70% EtOH at room temperature
8. Spin for 1 min at max speed on a benchtop centrifuge
9. Repeat Steps 7 and 8
10. Carefully aspirate supernatant and remove residual EtOH by hand with a pipette tip
11. Air dry pellets for 5–10 min at room temperature

12. Resuspend in 5 μl nuclease-free H_2O and incubate for 15 min at 37°C to redissolve
    - *Performing a second EtOH precipitation may slightly increase labeling efficiency, but yield will be lower*
13. Determine aa-cDNA concentration by spectrophotometry on a NanoDrop
14. Dilute aa-cDNA to 0.2 μg/μl
15. Freeze aa-cDNA at –20°C or proceed to Part III

III. Amine-labeling of single-cell cDNA

1. Mix 1 μg of aa-cDNA and 3 μl of 1M $NaHCO_3$ in a total volume of 8 μl
   - *Adding more aa-cDNA will not increase yield at the end and will decrease coupling efficiency*
2. Dissolve Alexa Fluor 555 succinimidyl ester dye aliquot in 2 μl DMSO
   - *Add DMSO to the side of each tube, then spin all the tubes together to minimize the time the dye is sitting in DMSO outside of the reaction*
3. Add 2 μl resuspended dye to the mixture and vortex a max speed for 15 sec
   - *Vortexing time is critical to ensure high coupling efficiencies*
4. Spin down and incubate 1 hr at room temperature
5. Proceed to Part IV

IV. Purification of labeled cDNA

1. Add 10 μl NaOAc (pH 5.2) and 80 μl water to each coupling reaction
   - *Adding NaOAc here to neutralize the $NaHCO_3$ helps improve the yield off the PureLink column*
2. Purify the labeling reaction on a PureLink column to remove unincorporated dye, repeat part II step 3
3. Add 10 μl NaOAc (pH 5.2) and 1 μl 20 mg/ml glycogen. Vortex
4. Add 250 μl ice-cold 100% EtOH, vortex, and incubate at –20°C for at least 30 min
   - *Keep a tube of EtOH at –20°C to speed the precipitation to completion*
   - *Longer incubations at –20°C are fine*
5. Spin for 10 min at max speed on a benchtop centrifuge
6. Carefully aspirate supernatant and wash pellet with 500 μl 70% EtOH at room temperature
7. Spin for 1 min at max speed on a benchtop centrifuge
8. Carefully aspirate supernatant and remove residual EtOH by hand with a pipette tip
9. Air dry pellets for 5–10 min at room temperature
10. Resuspend in 5 μl nuclease-free H_2O and incubate for 15 min at 37°C to redissolve
11. Determine labeled cDNA concentration and degree of labeling by spectrophotometry on a NanoDrop
    - *Degree of labeling should be at least 1.5 and preferably closer to 2.0 dyes per 100 bases*
12. Dilute 555-cDNA to 0.2 μg/μl
13. Samples (5 μl) are now ready for hybridization

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
  0.064 μl 50 mM aminoallyl dUTP
  0.08 μl 10 mM dTTP (note 10-fold lower concentration than other dNTPs)
  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
  13.53 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
  0.32 μl 50 mM aminoallyl dUTP
  0.4 μl 10 mM dTTP (note 10-fold lower concentration than other dNTPs)
  0.5 μl 20 mg/ml BSA
  0.3 μl 15 mg/ml AL1 primer
  1 μl amplified cDNA
  71.88 μl PCR-grade H_2O
  100 μl total volume

- **1 M $NaHCO_3$**
  Dissolve 12.6 g of $NaHCO_3$ in 100 mL of H_2O. Adjust the volume to 150 mL to yield a final concentration of 1 M. Filter-sterilize the solution, and store it at room temperature.