

1. Add 4  $\mu$ l of digestion buffer to the ExtracSure adaptor
  - *To include control ERCC spike-in transcripts, use recipe for “digestion buffer with ERCC spike-in mix”*
  - *Make sure that the digestion buffer completely covers the dissecting area of the LCM cap (lightly tap the base of the LCM cap, if needed)*
  - *Do not add more digestion buffer if planning on amplifying multiple samples from the same LCM cap (dilute afterwards in digestion buffer + digestion stop buffer)*
2. Cover the ExtracSure adaptor with a 0.5 ml thin-walled PCR tube (Applied Biosystems #N8010611)
  - *Some other 0.5 ml PCR tubes will fit tightly with the ExtracSure adaptor, but not all tubes (check for a tight fit beforehand with one of the spare ExtracSure adaptors)*
3. Incubate both the LCM cap and the remaining digestion buffer at 42°C for 1 hr
  - *Can use a dry-air incubator or a heat block that holds 15 ml tubes*
  - *For a heat block, make sure to cover the top of the block to maintain temperature*
  - *The proteinase K in the digestion buffer self cleaves at 42°C, so the incubation will keep the digestion buffer similar to what is in the LCM cap, if dilutions of the samples are required*
4. Spin PCR tubes for 2 min at 2500 rpm on a benchtop centrifuge at room temperature
  - *Higher centrifuge speeds will cause the ExtracSure adaptor to fly off from the PCR tube*
  - *Prepare digestion stop buffer during Step 4*
5. Quickly add 1  $\mu$ l of digestion stop buffer to the sample and mix by pipetting
  - *Make sure that PMSF has not precipitated from the digestion stop buffer*
  - *After stopping all the LCM caps, add the remaining digestion buffer to the remaining digestion stop buffer for the blank sample and any sample dilutions*
6. Vortex and centrifuge briefly
7. For pool-and-split control samples, dilute sample with digestion buffer + digestion stop buffer (mixed at a 4:1 ratio immediately beforehand)
  - *It is critical that the digestion buffer incubate for 1 hr at 42°C before adding digestion stop buffer and using to dilute*
8. Transfer 4.5  $\mu$ l of the sample to a 0.2 ml thin-walled PCR tube (Applied Biosystems # N8010612) or 96-well plate (Bio-Rad #MLL9601) and place on ice
9. Prepare a blank tube for the amplification by adding 4  $\mu$ l of digestion buffer + 1  $\mu$ l digestion stop buffer
  - *It is critical that the digestion buffer incubate for 1 hr at 42°C before adding digestion stop buffer and using as a blank*
10. Add 0.5  $\mu$ l of Maxima Reverse Transcriptase (200 U/ $\mu$ L) (Thermo Scientific #EP0741), pipet thoroughly, and incubate at 50°C for 30 min
  - *For all incubations, use a thermocycler for more-accurate temperature control*
  - *Mix Maxima Reverse Transcriptase in the sample by pipetting up and down the 0.5  $\mu$ l as best as possible and then vortexing at medium speed if unsure about the extent of mixing*
  - *Do not use the buffer that comes with the Maxima Reverse Transcriptase as it contains DDT (causes precipitation of the  $\text{CoCl}_2$  in the subsequent tailing reaction)*
11. Heat-inactivate by incubating at 70°C for 15 min
12. Place samples on ice and spin for 2 min at 14,000 rpm on a benchtop centrifuge at 4°C
13. (Mouse samples only) Transfer Streptavidin Magnetic Beads (Pierce #88817) to a 1.5 ml tube (5  $\mu$ l per sample + 5% extra)
14. (Mouse samples only) Place the tube on a magnetic stand, wait 5 min, remove the liquid, remove the tube from the magnetic stand, and resuspend beads in Binding Buffer (5  $\mu$ l per sample + 5% extra)
15. (Mouse samples only) Add 5  $\mu$ l of Streptavidin Magnetic Beads in Binding Buffer to each sample and mix thoroughly
16. (Mouse samples only) Incubate for 30 min at room temperature
17. (Mouse samples only) Place samples on a magnetic stand, incubate for 5 min, and remove the liquid
18. (Mouse samples only) Wash samples with 100  $\mu$ l High-Salt Buffer and then with 100  $\mu$ l Low-Salt Buffer
  - *Wash by removing samples from the magnetic stand, resuspending the beads, placing samples back on magnetic stand, incubating for 5 min and removing the liquid*

- *When procesing many samples at the same time be careful not to dry the beads; process only a few samples at the time or use a multichannel pipette to speed up the washes*
19. (Mouse samples only) Resuspend samples in 5  $\mu$ l of 1x First Strand Buffer.
  20. Add 1  $\mu$ l of RNase H–Mg<sup>2+</sup> mix and incubate at 37°C for 15 min
    - *Mix the RNase H–Mg<sup>2+</sup> mix in the sample by pipetting up and down the 1  $\mu$ l as best as possible*
    - *Do not use the reaction buffer that comes with the RNase H*
  21. Place samples on ice and spin for 2 min at 14,000 rpm on a benchtop centrifuge at 4°C
  22. Add 3.5  $\mu$ l of 2.6 $\times$  tailing buffer containing 0.2  $\mu$ l of 400 U/ $\mu$ l terminal transferase per sample and incubate at 37°C for 15 min
    - *Mix the 2.6 $\times$  tailing buffer + terminal transferase in the sample by pipetting up and down the 3.5  $\mu$ l as best as possible*
    - *Label tubes for Step 18*
  23. Heat-inactivate by incubating at 65°C for 10 min
    - *Prepare ThermoPol PCR buffer with everything except Taq, Pfusion, and AL1 primer*
  24. Place samples on ice and spin for 2 min at 14,000 rpm on a benchtop centrifuge at 4°C
    - *While samples are spinning, add Taq, Phusion, and AL1 primer*
  25. Add 90  $\mu$ l of ThermoPol PCR buffer and split sample into three 33  $\mu$ l aliquots
    - *Mix the ThermoPol PCR buffer in the sample by pipetting up and down the 90  $\mu$ l as best as possible*
    - *Add 33  $\mu$ l to two new 0.2 ml thin-walled PCR tubes and use the remainder as the third aliquot*
  26. Run 25 cycles of PCR on a thermocycler containing a heated lid:
    - 4 cycles of 94°C denaturation (1 min), 32°C annealing (2 min), 72°C extension (2 min, with 10 sec increase at each cycle)
    - 21 cycles of 94°C denaturation (1 min), 42°C annealing (2 min), 72°C extension (2 min 40 sec, with 10 sec increase at each cycle)
    - Indefinite hold at 4°C
  27. Pool the three splits into the original 0.2 ml PCR tube, vortex and centrifuge briefly, and run 5 cycles of PCR on a thermocycler containing a heated lid:
    - 5 cycles of 94°C denaturation (1 min), 42°C annealing (2 min), 72°C extension (2 min)
    - Indefinite hold at 4°C
  28. Samples can be frozen at –20°C for months to years

## Buffer recipes

- **Digestion buffer with ERCC spike-in mix**

- 17  $\mu$ l nuclease-free H<sub>2</sub>O
  - 40  $\mu$ l 1:2x10<sup>6</sup> dilution of ERCC spike-in mix #1 (Ambion #4456740)
  - 20  $\mu$ l 5 $\times$  MMLV RT buffer ("First-strand buffer") (included in Invitrogen #18080-044)
  - 2  $\mu$ l 1 $\times$  stock primer mix
  - 1  $\mu$ l 20 mg/ml proteinase K (Sigma #P2308)
- 80  $\mu$ l total volume

Each 10-cell sample requires 4  $\mu$ l per LCM cap, each 100-cell pool-and-split control requires 20  $\mu$ l per LCM cap, and the blank samples requires 4  $\mu$ l

Prepare digestion buffer on ice and use immediately

Prepare proteinase K solution in nuclease-free H<sub>2</sub>O and store in 20  $\mu$ l aliquots at -20°C

Do not add CaCl<sub>2</sub> to proteinase K solution

After thawing proteinase K solution, keep at 4°C for up to one month

Dilute 25 $\times$  stock primer mix to 1 $\times$  in nuclease-free H<sub>2</sub>O before adding to digestion buffer

ERCC dilutions:

ERCC spike-in mix #1 (Ambion #4456740) is diluted 1:1000 in nuclease free water and stored at -80°C in 5uL aliquots

1:1000 stock is further diluted 0.5uL in 999.5uL nuclease free water to 1:2x10<sup>6</sup> for each use, yielding a final dilution of 1:4x10<sup>6</sup> dilution in digestion buffer (~ 6.34x10<sup>4</sup> spike-in molecules per 4  $\mu$ l reaction).

- **Digestion stop buffer**

- 17  $\mu$ l nuclease-free H<sub>2</sub>O
  - 2  $\mu$ l SuperAse In (Invitrogen #AM2696)
  - 1  $\mu$ l 100 mM PMSF (17.42 mg/ml in ethanol) (Sigma #P7626)
- 20  $\mu$ l total volume

Each 10-cell sample requires 1  $\mu$ l per LCM cap, each 100-cell pool-and-split control requires 5  $\mu$ l per LCM cap, and the blank samples requires 1  $\mu$ l

Prepare digestion stop buffer at room temperature

Add reagents in the order listed, mix very briefly, and use immediately (do not use if PMSF has visibly precipitated)

Prepare PMSF solution in 100% EtOH shortly before use

AEBSF cannot substitute for PMSF

- Add 1 $\mu$ l digestion stop buffer per 4  $\mu$ l digestion buffer

- **25 $\times$  stock primer mix** Store as 5  $\mu$ l aliquots at -20°C

- 15  $\mu$ l nuclease-free H<sub>2</sub>O
  - 5  $\mu$ l 100 mM dATP (dNTP set: Roche #11277049001)
  - 5  $\mu$ l 100 mM dCTP
  - 5  $\mu$ l 100 mM dGTP
  - 5  $\mu$ l 100 mM dTTP
  - 5  $\mu$ l 80 OD/ml T<sub>24</sub> (25 nmol synthesis from IDT)
- 40  $\mu$ l total volume

Good for 6 months at -20°C

Dilute 25 $\times$  stock primer mix to 1 $\times$  in nuclease-free H<sub>2</sub>O before adding to cDNA-lysis buffer

- **Binding Buffer** Store in room temperature

- 1 ml 5 $\times$  MMLV RT buffer ("First-strand buffer") (included in Invitrogen #18080-044)
- 4 ml 5 M NaCl solution (final concentration: 4 M)
- 2  $\mu$ l Tween-20 (final concentration: 0.02%)

5 ml total volume

Good indefinitely at room temperature

- **High-Salt Buffer** Store in room temperature

23.6 ml nuclease-free H<sub>2</sub>O

2.5 ml 1 M Tris buffer [pH 8.3] (final concentration: 50 mM)

20 ml 5 M NaCl solution (final concentration: 2 M)

3.75 ml 1 M KCl solution (final concentration: 75 mM)

0.15 ml 1 M MgCl<sub>2</sub> solution (final concentration: 3 mM)

5 µl Tween-20 (final concentration: 0.01%)

50 ml total volume

Good indefinitely at room temperature

- **Low Salt Buffer** Store in room temperature

43.6 ml nuclease-free H<sub>2</sub>O

2.5 ml 1 M Tris buffer [pH 8.3] (final concentration: 50 mM)

3.75 ml 1 M KCl solution (final concentration: 75 mM)

0.15 ml 1 M MgCl<sub>2</sub> solution (final concentration: 3 mM)

50 ml total volume

Good indefinitely at room temperature

- **RNAse H–Mg<sup>2+</sup> mix**

5 µl 5 U/ml RNAse H (Amersham #E70054Z)

5 µl 25 mM MgCl<sub>2</sub>

10 µl total volume

Each samples requires 1 µl

Prepare RNAseH–Mg<sup>2+</sup> mix on ice and use immediately

- **2.6× tailing buffer** Store as 100 µl aliquots at –20°C

363 µl nuclease-free H<sub>2</sub>O

400 µl 5× Invitrogen terminal transferase buffer (Invitrogen #16314-015)

15 µl 100 mM dATP (dNTP set: Roche #11277049001)

778 µl total volume

Each sample requires 3.5 µl

Do not use the Roche 5× TdT reaction buffer that comes with the terminal transferase (lacks CoCl<sub>2</sub>)

Add 0.2 µl of 400 U/µl terminal transferase (Roche #03333574001) per 3.5 µl 2.6× tailing buffer before adding to PCR tubes

- **ThermoPol PCR buffer:**

71.35 µl nuclease-free H<sub>2</sub>O

10 µl 10× ThermoPol buffer (New England Biolabs #B9005S)

2.5 µl 100 mM MgSO<sub>4</sub>

0.5 µl 20 mg/ml BSA (Roche #10711454001)

1 µl 100 mM dATP (dNTP set: Roche #11277049001)

1 µl 100 mM dCTP

1 µl 100 mM dGTP

1 µl 100 mM dTTP

0.75 µl Taq polymerase (NEB #M0273L)

0.75 µl Phusion polymerase (NEB #M0530L)

0.15 µl 15 µg/µl AL1 primer (1 µmol synthesis from IDT)

90 µl total volume / sample

Prepare ThermoPol PCR buffer on ice in the order of reagents listed above

Add polymerases (first) and AL1 primer (second) just before starting PCR