1. Add 4 μl 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 μl 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. (optional) Dilute sample with digestion buffer + digestion stop buffer (mixed at a 4:1 ratio immediately beforehand)
8. Transfer 4.5 μl of the sample to a 0.2 ml thin-walled PCR tube (Applied Biosystems # N8010612) and place tube on ice
9. Prepare a blank tube for the amplification:
   Add 4 μl of digestion buffer + digestion stop buffer and 0.5 μl of nuclease-free water to a 0.2 ml thin-walled PCR tube
   Heat-denature at 65°C for 1 min
   Allow to cool at room temperature for 90 sec
   Spin for 2 min at 14,000 rpm on a benchtop centrifuge at 4°C
10. Add 0.5 μl of SuperScript III (Invitrogen #18080-044), pipet thoroughly, and incubate at 50°C for 30 min
    - *For all incubations, use a thermocycler for more-accurate temperature control*
    - *Mix SuperScript III in the sample by pipetting up and down the 0.5 μl as best as possible and then vortexing at medium speed if unsure about the extent of mixing*
    - *Do not use the DTT that comes with the SuperScript III (causes precipitation of the $CoCl_2$ 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. Add 1 μl of RNAse H–Mg^{2+} mix and incubate at 37°C for 15 min
    - *Mix the RNAse H–Mg^{2+} mix in the sample by pipetting up and down the 1 μl as best as possible*
    - *Do not use the reaction buffer that comes with the RNAse H*
14. Place samples on ice and spin for 2 min at 14,000 rpm on a benchtop centrifuge at 4°C
15. Add 3.5 μl of 2.6× tailing buffer containing 0.2 μl of 400 U/μl terminal transferase per sample and incubate at 37°C for 15 min
    - *Mix the 2.6× tailing buffer + terminal transferase in the sample by pipetting up and down the 3.5 μl as best as possible*
    - *Label tubes for Step 18*
16. Heat-inactivate by incubating at 65°C for 10 min
    - *Prepare ThermoPol PCR buffer with everything except Taq, Pfusion, and AL1 primer*

17. 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*
18. Add 90 μl of ThermoPol PCR buffer and split sample into three 33 μl aliquots
    - *Mix the ThermoPol PCR buffer in the sample by pipetting up and down the 90 μl as best as possible*
    - *Add 33 μl to two new 0.2 ml thin-walled PCR tubes and use the remainder as the third aliquot*
19. 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
20. 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
21. Samples can be frozen at –20°C for months to years

Buffer recipes

- **Digestion buffer with ERCC spike-in mix**
  17 μl nuclease-free H_2O
  40 μl 1:2x10^6 dilution of ERCC spike-in mix #1 (Ambion #4456740)
  20 μl 5× MMLV RT buffer ("First-strand buffer") (included in Invitrogen #18080-044)
  2 μl 1× stock primer mix
  1 μl 20 mg/ml proteinase K (Sigma #P2308)
  80 μl total volume
  Prepare digestion buffer on ice and use immediately
  Prepare proteinase K solution in nuclease-free H_2O and store in 20 μl aliquots at –20°C
  Do not add $CaCl_2$ to proteinase K solution
  After thawing proteinase K solution, keep at 4°C for up to one month
  Dilute 25× stock primer mix to 1× in nuclease-free H_2O 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 1:2x10^6 for each use, yielding a final dilution of 1:4x10^6 dilution in digestion buffer (~ 6.34x10^4 spike-in molecules per 4 μl reaction).

- **Digestion stop buffer**
  17 μl nuclease-free H_2O
  2 μl SuperAse In (Invitrogen #AM2696)
  1 μl 100 mM PMSF (17.42 mg/ml) (Sigma #P7626)
  20 μl total volume
  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 1μl digestion stop buffer per 4 μl digestion buffer

- **25× stock primer mix** Store as 5 μl aliquots at –20°C
  15 μl nuclease-free H_2O
  5 μl 100 mM dATP (dNTP set: Roche #11277049001)
  5 μl 100 mM dCTP
  5 μl 100 mM dGTP
  5 μl 100 mM dTTP
  5 μl 80 OD/ml T_{24} (25 nmol synthesis from IDT)
  40 μl total volume
  Good for 6 months at –20°C
  Dilute 25× stock primer mix to 1× in nuclease-free H_2O before adding to cDNA-lysis buffer

- **RNAse H–Mg^{2+} mix**
  5 μl 5 U/ml RNAse H (Amersham #E70054Z)
  5 μl 25 mM $MgCl_2$
  10 μl total volume
  Prepare RNAseH–Mg^{2+} mix on ice and use immediately

- **2.6× tailing buffer** Store as 100 μl aliquots at –20°C
  363 μl nuclease-free H_2O
  400 μl 5× Invitrogen terminal transferase buffer (Invitrogen #16314-015)
  15 μl 100 mM dATP (dNTP set: Roche #11277049001)
  778 μl total volume
  Do not use the Roche 5× TdT reaction buffer that comes with the terminal transferase (lacks $CoCl_2$)
  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_2O
  10 μl 10× ThermoPol buffer (New England Biolabs #B9005S)
  2.5 μl 100 mM $MgSO_4$
  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
  Prepare ThermoPol PCR buffer on ice in the order of reagents listed above
  Add polymerases (first) and AL1 primer (second) just before starting PCR