For LCM captured cells:

- Thaw samples (LCM cap in Trizol) on ice
- Move Trizol/cell solution into 1.5 ml microtube and add 100 µl of Chloroform (Sigma C- 2432)
- Shake vigorously for 15 sec and let sit at RT 1-2 min
- Spin down at 12,000g for 5–10 min (old protocol 15 min) @ 4º C
- Aspirate SN to fresh tube and add 250 µl of isopropanol (Sigma I-9516) and 2 µl of Linear Acrylamide (Ambion 9520 5mg/ml)
- Mix and spin @ 12,000g for 20 min @ 4º C
- Rinse pellet with 80% EtOH and air dry (not more than 5 min) inverted and resuspend pellet in 5.5 µl of ddH2O (Rnase free)

RNA Amplification protocol:

- Transfer samples to 0.5ml thin wall PCR tubes (usually volume is 6 µl, otherwise adjust to 6 µl) and add:
    - 1 µl Poly T primer (100 ng/ µl) (primer sequence TTT TTT TTT TTT TTT TTT TT)
- Mix samples and incubate in PCR machine: 65 ºC for 2 min, then 45º C for 1 min
- While samples in PCR machine, heat denature TC primer (5T7C 100 ng/ µl) to 70 º C for 1-2 min and put on ice.(primer sequence: AAA CGA CGG CCA GTG AAT TGT AAT ACG ACT CAC TAT AGG CGC GAG AGG CCC)
- Set up 1st strand amplification, add these ingredients to rxn above (can make master mix):
    - 5X First strand buffer -- 4 µl per reaction
    - 0.1M DTT -- 1 µl per reaction
    - 10mM dNTPs (A,T,C,G) -- 1 µl per reaction
    - First strand enzyme mix -- 2 µl per reaction
    - (Superscript III {Invitrogen #50575 200U/ul and SuperRNase Inhibitor Ambion #2696 20 U/ul 1:1 ratio)
    - TC primer (5T7C 100 ng/µl)-- 1 µl per reaction
    - ddH2O to 20µl reaction total volume
- Incubate reaction at 50 ºC 60 min {if using SSII, lower temp to 42 ºC}, then 65 ºC 15 min
- Set up "2nd strand synthesis rxn" (don't actually do 2nd strand synthesis), add to reaction above:
    - 0X taq PCR buffer -- 10 µl per reaction
    - RNase H (Ambion 2293 10 U/ul) -- 1 µl per reaction
    - ddH2O -- 69 µl per reaction (100 µl reaction volume total)
- Incubate reaction 37 ºC 30 min, then 95 ºC 3 min, then 60 ºC 3 min
- **All above reactions done in PCR machine
- Use Sartorius columns vivaspin 500 10kD MW spin column (VS0101) down to 13 µl, adjust volume if below 13 µl with ddH2O
- Set up T7 Transcription reaction for 40 µl total reaction volume:
    - cDNA -- 13 µl
    - 5X Transcription buffer (Epicentre BP1001) -- 8 µl
    - 2.5 mM NTPs (A,G,C) -- 2 µl
    - 100 µM UTP (cold) -- 1 µl
    - 0.1M DTT -- 1 µl
    - SuperRNase Inhibitor -- 1 µl
    - go behind shield then add:/li>
    - 33P-UTP (PE: NEG307H001MC) -- 12 µl/li>

  - T7 RNA polymerase (Epicentre TH950K 1KU/ul) -- 2 µl
- Mix up and incubate at least 4 hrs at 37 ºC (max of 6 hrs incubation)
- While incubating T7 Transcription reaction, mix up pre-hybridization buffer
  - 100% Formamide (Fisher) -- 10 ml/rxn
  - 50X Denhart's solution (AmResCo) -- 2 ml/rxn
  - 20X SSPE (Ambion 9767) -- 6 ml/rxn
  - 10% SDS (Ambion 9823) -- 200 µl /rxn
  - MilliQ H2O -- 1.4ml/rxn
  - Salmon Sperm DNA (Eppendorf 955155629) -- 400µl /rxn
  - (denatured 84* 4 min, 200 ug/ml) Add 20 ml to each blot in hybridization tubes (wrap blot in mesh, label tube with blot # and date of blot and reaction)
  - Incubate at 42 ºC for 4 hrs on rotator(until T7 reaction done)
- Add T7 reaction to correct tubes and incubate on rotator o/n at 42 ºC
- Next day: 16-18 hr incubation
- Discard hybridization solution behind shield in bottle
- Wash blots with 20 ml of 2X SSC buffer (2X SSC {Ambion9763} + 0.1% SDS) for 15 minutes at 37*C on rotator
- Discard buffer behind shield
- Wash blots with 20 ml of 1X SSC buffer (1X SSC + 0.1% SDS) for 15 minutes at 37*C on rotator
- Discard buffer behind shield
- Wash blots with 20 ml of 0.5X SSC buffer (0.5X SSC + 0.1% SDS) for 15 minutes at 37*C on rotator
- After done, put blots between 2 pieces of plastic wrap and expose to phosphoscreens
- Incubate mesh, caps and bottles with MilliQ H2O and add 200 µl of 10% SDS o/n (wash 3 times total)
- Next day: 24-30 hr exposure develop phosphoscreens using Storm 840 phosphoimager, save each image and go onto image analysis