FISH riboprobe synthesis Entered by Kevin Janes
Janes Lab Protocols 6/7/07

When referring to this protocol, please cite: Janes KA, Wang CC, Holmberg KJ, Cabral K, Brugge JS. (2010) Identifying single-cell molecular programs by stochastic profiling. *Nat Methods*, 7, 311-7.

Before starting:
- Clone a sequence-verified gene fragment into an appropriate expression vector (e.g., pcDNA3 or pBluescript)
- Tissue penetration is optimal with probes ~175-225 bp in size (nonspecific binding is also less problematic), and our RNA FISH protocol (see Janes_RNAFISHcryo.pdf) seems to work best with probes that have a GC content 40–50% (any region of the mRNA with these characteristics should work)

I. Plasmid linearization and purification

1. Mix 5 μg probe construct (20 μl at 0.25 μg/μl), 5 μl 10× restriction enzyme buffer, 0.5 μl 100× BSA, 2.5 μl restriction enzyme, and 22 μl H_2O
   - *For each construct, prepare two digestions, one at the 5' and another at the 3' end of the probe, to act as sense and antisense templates*
2. Incubate 2.5 hr at 37°C
   - *T7, T3, and Sp6 are very processive enzymes and template binding is the rate-limiting step; thus, it is crucial to linearize the template completely*
3. Add 150 μl H_2O, then 200 μl phenol-chloroform in a fume hood. Vortex thoroughly and spin at max speed on a benchtop centrifuge for 1 min
4. Transfer 180 μl of the aqueous (top) fraction to a new tube, add 20 μl of 3 M NaOAc (pH 5.2), and 1 μl 20 mg/ml glycogen (Invitrogen #10814-010). Vortex
5. Add 500 μl ice-cold 100% EtOH, vortex, and incubate at –20°C for at least 30 min
   - *Keep EtOH stock 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. Carefully aspirate supernatant and remove residual EtOH by hand with a pipette tip
10. Air dry pellets for 5–10 min at room temperature
11. Resuspend in 10 μl EB and incubate for 15 min at 37°C to redissolve
12. Measure DNA concentration by spectrophotometry on a Nanodrop
13. Dilute linearized template to a convenient concentration (ideally, 0.25 μg/μl or 0.2 μg/μl)

Before starting:
- From the MAXIscript kit (Ambion #AM1322), mix the ATP, CTP, and GTP stocks to prepare (ACG)TP at 3.33 mM, and dilute the UTP stock to 2 mM in nuclease-free H_2O
- Dilute aaUTP (Ambion #AM8437) and digUTP (Roche #11209256910) to 2 mM in nuclease-free H_2O
- (These dilutions make it easier to calculate labeled:unlabeled nucleotide ratios)

II. Riboprobe synthesis

1. Set up the following reaction at room temperature
   - 4 μl linearized template at 0.25 μg/μl (adjust volume for 1 μg template)
   - 2 μl 10× in vitro transcription buffer
   - 3 μl 3.33 mM (ACG)TP
   - 4 μl 2 mM aaUTP + 1 μl 2 mM UTP (if aminoallyl labeling)
   - 1.75 μl 2 mM DIG-UTP + 3.25 μl 2 mM UTP (if DIG labeling)
   - 0.35 μl 10 mM DNP-UTP + 1.4 μl nuclease-free H_2O + 3.25 μl 2 mM UTP (if DNP labeling)
   - 3.5 μl nuclease-free H_2O (adjust volume for 1 μg template)

- 0.5 μl RNAsin Plus (or other RNAse inhibitor)
- 2 μl T7 or Sp6 polymerase

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  20 μl total volume

2. Mix by flicking the tube, spin down, and incubate 2 hr at 37°C (for T7) or 40°C (for Sp6)
   - *For a target sequence cloned 5' to 3' in pcDNA3 using BamH1 and EcoR1 sites, the sense probe uses EcoR1-digested template and T7 polymerase, and the antisense probe uses BamH1-digested template and Sp6 polymerase*
   - *Increasing the temperature of the Sp6 transcription reaction provides yields similar to T3 and T7*
3. Add 1 μl DNAse and incubate 15 min at 37°C
   - *Digesting the template makes absorbance readings more accurate at the end*
4. Add 1 μl 0.5 M EDTA (pH 8.0) to stop the DNAse digestion and inhibit RNA hydrolysis
5. Add 22.5 μl nuclease-free H_2O, 5 μl NaOAc (pH 5.2), and 0.5 μl 20 mg/ml glycogen. Vortex
   - *Do not substitute ammonium acetate if preparing aminoallyl riboprobes (free amines inhibit subsequent labeling)*
6. Add 150 μl ice-cold 100% EtOH, vortex, and incubate at –20°C for at least 30 min
   - *Keep EtOH stock at –20°C to speed the precipitation to completion*
   - *Longer incubations at –20°C are fine*
7. Spin for 10 min at max speed on a benchtop centrifuge
8. Carefully aspirate supernatant and wash pellet with 500 μl 70% EtOH at room temperature
9. Spin for 1 min at max speed on a benchtop centrifuge
10. Carefully aspirate supernatant and wash pellet with 500 μl 70% EtOH at room temperature
11. Spin for 1 min at max speed on a benchtop centrifuge
12. Carefully aspirate supernatant and remove residual EtOH by hand with a pipette tip
13. Air dry pellets for 5–10 min at room temperature
14. Resuspend in 10 μl nuclease-free H_2O and incubate for 15 min at 37°C to redissolve
15. Determine RNA concentration by spectrophotometry on a NanoDrop
16. Dilute riboprobe to a convenient concentration (ideally, 0.2 μg/μl)
17. Store riboprobes in small aliquots at –80°C

When referring to this protocol, please cite: Wang L, Brugge JS, Janes KA. (2011) Intersection of FOXO and RUNX1 gene-expression programs in single breast epithelial cells during morphogenesis and tumor progression. *Proc Natl Acad Sci*, 108, E803-12.

III. Amine-labeling of riboprobes

1. Mix 1 μg of aaRNA and 3 μl of 1M $NaHCO_3$ in a total volume of 8 μl
   - *Adding more aaRNA will not increase yield at the end and will decrease coupling efficiency*
   - *Perform each labeling reaction in duplicate*
2. Dissolve one vial of Alexa 647 succinimidyl ester (Invitrogen #A32757) in 2 μl DMSO
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. Combine the duplicate labeling reactions and add 10 μl NaOAc (pH 5.2), 70 μl nuclease-free H_2O, and 400 μl PureLink Binding Buffer (Invitrogen #K3100)
   - *Adding NaOAc neutralizes the $NaHCO_3$ and can improve the yield off the PureLink column*
   - *Do **not** use the High-Cutoff Binding Buffer, which allows nucleotides <300 bp to flow through*
6. Apply the entire solution to a PureLink column and spin at 10,000 rcf for 1 min
7. Collect the flow through, re-apply the entire solution to the PureLink column, and spin at 10,000 rcf for 1 min

- *RNA does not bind to these columns as well as DNA, so a second pass through the column improves the yield of the purification*

8. Discard the flow through, wash the column with 650 µl Wash Buffer, and spin at 10,000 rcf for 1 min
9. Transfer the column to a clean elution tube and add 50 µl Elution Buffer prewarmed to 37°C
10. Cut off the cap from the old tube, seal the colum with the cap, and incubate at 37°C for 10 min
    - *Warming the elution ensures complete release of the purified RNA from the column*
11. Spin at 10,000 rcf for 1 min
12. Add another 50 µl Elution Buffer prewarmed to 37°C, and repeat Steps #10-11
13. Add 10 µl NaOAc (pH 5.2) and 1 µl 20 mg/ml glycogen and vortex.
14. Add 300 µl ice-cold EtOH, vortex, and incubate at –20°C for at least 30 min
    - *RNA precipitations require three equivalents of EtOH*
    - *Keep EtOH stock at –20°C to speed the precipitation to completion*
    - *Longer incubations at –20°C are fine*
15. Spin for 10 min at max speed on a benchtop centrifuge
16. Carefully aspirate supernatant and wash pellet with 500 µl 70% EtOH at room temperature
17. Spin for 1 min at max speed on a benchtop centrifuge
18. Carefully aspirate supernatant and remove residual EtOH by hand with a pipette tip
19. Air dry pellets for 5–10 min at room temperature
20. Resuspend in 5 µl nuclease-free H_2O and incubate for 15 min at 37°C to redissolve
21. Determine RNA concentration and degree of labeling by spectrophotometry on a NanoDrop
    - *A good labeling reaction should yield 1.5–2 dye molecules per 100 bases*
22. Dilute riboprobes to a convenient concentration (ideally, 0.2 µg/µl)
23. Store riboprobes in small aliquots at –80°C