FISH riboprobe synthesis Entered by Kevin Janes
Janes Lab Protocols 8/29/23

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

  ---

  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 Qubit RNA BR assay (Section IV)
    - *We have learned that A260 readings of these ethanol precipitates are unreliable readouts of RNA concentration because of ribonucleotide carryover from the in vitro transcription*
16. Dilute riboprobe to a convenient concentration
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

IV. Riboprobe quantification by Qubit assay (Invitrogen #Q10210) on the CFX96 instrument

1. Prepare a seven-point standard curve by serially diluting the Qubit RNA BR Standard #2 (at 100 ng/µl) twofold in Qubit RNA Standard #1 (at 0 ng/µl) to yield 100, 50, 25, 12.5, 6.25, 3.1, and 1.6 ng/µl
   - *Working stocks of the RNA Standards are stored at 4ºC in the deli fridge with the betaine aliquots*
   - *Backup aliquots of RNA BR Standard #2 and RNA BR Reagent are stored at –80ºC with the RNA FISH riboprobes undergoing testing*
2. Add 1 µl of each serial dilution or 1 µl purified riboprobe to the base of an optically clear PCR strip tube (Bio-Rad #TLS0801 and #TCS0803) or hard-shell PCR plate (BioRad #HSP9601 and #MSB1001) (one well for each sample)
   - *1 µl riboprobe from the MAXIscribe kit should fall within the linear range of the assay, but if there are concerns that the concentration will be above 100 ng/µl, 1/3 of the final reaction (6.7 µl) can be split from the final reaction, diluted with 6.7 µl Qubit working solution, and the entire plate run at 13.4 µl total volume instead of 20 µl total volume*
3. Prepare Qubit Working Solution by diluting Qubit RNA BR Reagent 200-fold in Qubit RNA BR Buffer
   - *199 µl of Buffer + 1 µl of Reagent is enough for 8-9 samples*
4. Add 19 µl of Qubit working solution to each well and mix by pipetting 10 times
5. Seal the strip tube or plate and read once on the CFX96 instrument at 22ºC after a hold at 22ºC for 2 min
   - *The Qubit detection reagent has an excitation maximum at 647 nm and an emission maximum at 665 nm, which is indistinguishable from Cy5 (Ex/Em = 647/665 nm) to use any CFX96 qPCR detection instrument*
   - *Our saved protocol is named "QUBIT"; <u>be sure to change the detection from “SYBR/FAM” to “All Channels” before starting the run</u>*
6. Export the ZPCR file from the instrument and load into the CFX Manager Software
7. Once the PCRD file had been created, deselect all empty wells on the plate

- *Having only the active wells selected makes data export easier*

8. Go to Settings > Baseline Setting > No Baseline Subtraction to turn off the default background subtraction settings
   - *It is crucial to export the raw RFU values or else the data will be uninterpretable*
9. Go to Export > Export All Data Sheets > Excel 2003
   - *The file ending in "End Point Results.xls" will contain the end RFUs from the instrument for the selected wells*
   - *Export > Custom Export will export only one file of RFUs if set up correctly, although all the wells of the 96-well plate will be exported*
   - *One can also select the End Point tab and copy-paste from the table*
10. Open the exported RFU data in Excel, perform linear regression on the standard curve with zero intercept after background subtraction, and back calculate the DNA concentrations of the riboprobe
    - *Slopes of 10–15 (ng/µl) per background-subtracted Cy5 RFU are typical*
11.