Prepare the internal standard for RNA extractions.

Solutions/supplies needed

- pFN18a plasmid
- BamHI
- Buffer E (for BamHI)
- BSA (10ug/uL)
- Water bath at 37˚C
- QIAquick PCR purification
- Mung bean nuclease
- Mung bean nuclease buffer
- Glycerol (100%)
- Riboprobe System T7
    - Transcription Optimized 5X buffer
    - 100mM DTT
    - Recombinant RNasin Ribonuclease Inhibitor (100u total)
    - Nucleotide mix
    - RNA polymerase
    - DEPC water
- Qiagen RNeasy Mini kit
    - Buffer RLT
    - 100% ethanol
    - RNeasy spin column
    - Buffer RW1
    - DNase i
    - RPE buffer

Protocol

1. Linearize the pFN18a plasmid with PmeI

- Mix
    - 7 uL H2O
    - 2 uL CutSmart Buffer (10x)
    - 10 uL pFN18A plasmid (100 ng/uL)
    - 1 uL PmeI
- Incubate at 37˚C for 90 minutes, then inactivate at 65˚C for 20 minutes

2. Extract and purify DNA by phenol:chloroform extraction and ethanol precipitation

- Move 20μl of digestion mix to a 1.5ml Eppendorf microcentrifuge tube
- Add 50μl of phenol:chloroform:IAA (pH~8)
- Invert or shake sample for 2 minutes
- Centrifuge at max speed for 5 minutes
- Remove upper aqueous layer into a new 1.5ml microcentrifuge tube

- Add 2μl of filter-sterilized 3M NaAc (pH 5.5) and mix
- Add 200μl of 100% EtOH and invert to mix
- Precipitate DNA for 2hr at -20˚C.
- Spin down the sample for 30 minutes at top speed
- Pipette off the supernatant
- Rinse with 300μl of 70% EtOH, invert to mix
- Spin down for 25 minutes at max speed
- Pipette off the supernatant
- Allow DNA to dry
- Resuspend in 20μl of NF-H2O

3. Run in vitro transcription kit (Riboprobe System T7)

- Recipe:
    - 20μl Transcription Optimized 5X buffer
    - 10μl 100mM DTT
    - 2.5μl Recombinant RNasin Ribonuclease Inhibitor (100u total)
    - 20μl Nucleotide mix
        - To prep this, mix equal parts of the four different 10mM rNTPs stocks
    - 2μl Linearized template DNA (1-2.5mg/ml)
    - 2μl T7 RNA polymerase (40u total)
    - 43.5 DEPC-treated water (bringing up to 100μl)
- Incubate mix for 1-2 hours at 37-40˚C

4. DNA removal and RNA purification (Qiagen RNeasy Mini kit)

- Prep solutions:
    - Add 4 volumes of 100% EtOH to Buffer RPE (only needs to be done once)
- Add 350μl Buffer RLT and mix
- Add 250μl 100% ethanol and mix well
- Transfer sample to RNeasy spin column
- Centrifuge 15s at >10,000rpm then discard flow-through
- Add 350μl of Buffer RW1 to column, spin at >10,000rpm for 15s
- Add 10μl DNase I to 70μl Buffer RDD, mix and centrifuge
- Add 80μl DNase/RDD mix to RNeasy spin column.
- Incubate at room temperature for 15 min.
- Add 350μl RW1 buffer to RNeasy spin column, spin at >10,000rpm for 15s, discard flow-through
- Add 500μl Buffer RPE to RNeasy spin column, spin at >10,000rpm for 15s, discard flow-through
- Spin RNeasy spin column at >10,000rpm for 1min
- Place RNeasy spin column in new 1.5ml Eppendorf centrifuge tube
- Add 50μl of DEPC-treated water directly to RNeasy spin column, spin at >10,000rpm for 1min

9. Qubit quantification + run in gel