mRNA Display Protocol

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1. Obtain DNA library

2. Using NEB T7 Quick High Yield RNA Synthesis Kit
    a. For transcripts > 0.3 kb in a 20 uL reaction volume:
        i. Nuclease-free water – X uL
        ii. NTP Buffer Mix – 10 uL (10 mM each NTP final)
        iii. Template DNA – X uL (1 ug)
        iv. T7 RNA Polymerase Mix – 2 uL
    b. Mix and incubate at 37C for 2 hrs. Recommended to use a dry air incubator or thermocycler to prevent evaporation
    c. Optional: DNAse treatment to remove DNA template
        i. Add 30 uL nuclease-free water to 20 uL RNA synthesis reaction.
        ii. Add 2 uL of DNase I (RNase-free)
        iii. Mix and incubate at 37C for 15 minutes

3. Purification of synthesized RNA
    a. Using Zymoclean RNA Clean and Concentrator -5 Kit, follow provided instructions. Ensure that sample has at least 50 uL volume. Resuspend sample in a low, but manageable volume in order to maximize the concentration of RNA.
    b. Take an OD measurement of the RNA. The RNA concentration in uM or pmoles/uL is approximately the absorbance at 260 nm (A-260) divided by 3.2

4. Ligation of splint/linker to RNA using NEB T4 DNA Ligase Kit
    a. Will need roughly 100-200 uL RXN to have sufficient amounts by the end of mRNA display. This can be broken into smaller volume RXNs.
    b. Prepare preliminary mix
        i. 8-10 uM RNA; 11 uM splint; 12 uM puromycin-linker; ddH2O as necessary
            1. Lower amounts (~8 uM) of RNA seemed to provide the greatest efficiency
    c. Heat at 65C for 1-3 minutes to remove RNA tertiary structures. DO NOT ADD T4 DNA LIGASE OR LIGASE BUFFER YET!
        i. Allow to cool for 1-3 minutes at RT
    d. Add T4 DNA Ligase Buffer and Ligase as appropriate
    e. Incubate at room temperature for 30-60 minutes

5. Analysis of ligated transcripts by gel electrophoresis
    a. Prepare 2X RNA Loading Buffer

        i. 95% Formamide; 0.025% SDS; 0.025% Bromophenol Blue; 0.025% Xylene Cyanol; 0.5 mM EDTA
        ii. Mix well and dissolve all SDS
    b. Run sample on agarose gel with ethidium bromide.
        i. Run at a lower voltage than with DNA

6. Gel Purification
    a. Extract appropriate band according to expected RNA size
    b. Several methods of purification are available such as LiCl/EtOH precipitation, PCR-cleanup column, PH:CL extraction/EtOH precipitation, or gel purification
        i. When high purity RNA transcript is desired, gel purification is recommended

7. Affinity purification using oligo dT cellulose to remove unligated transcripts
    a. Prepare oligo dT Binding Bufer
        i. 50 mM Tris, pH 8; 1 M NaCl; 10 mM EDTA; 0.1% Triton x-100
    b. Use 2 mg oligo dT cellulose per 100 uL product (~0.4 uM RNA)
    c. Dilute sample 10X in Binding Buffer
    d. Transfer to spin-x filter.
    e. Wash 3X with Binding Buffer at RT
    f. Wash 1X with ice cold TBS with 0.05% Tween
    g. Elute with RT or warm 5 mM Tris, 3 x 25 uL

8. Translation
    a. 50-100 uL Translation volume, using Ambion Retic Lysate for 0.4 uM ligated RNA template. Follow kit instructions
    b. Incubate for 1 hour at 30C
    c. Add salt to facilitate fusion formation. Per 50 uL RXN:
        i. 12 uL 3 M KCl; 4 uL 1 M MgCl2
        ii. Incubate for 30 minutes at RT

9. FLAG Pre-selection
    a. Wash 10 uL M2 anti-FLAG agarose beads with PBS with 0.02% Tween-20
    b. Add 10 uL 0.5 M EDTA to translation sample. Dilute to 500 uL with TBS, 0.02% Tween-20 and add to 10 uL M2 agarose beads
        i. Can do binding in spin-x cup
    c. Bind 1 hour at 4C, then wash 3X with cold TBS – tween
    d. Wash twice with cold 1XRT buffer (SSII First Strand Buffer)
    e. Elute off beads by incubating with 100 uL of 1XRT buffer with 0.2 mg/ml 3XFLAG peptide for 30 minutes at RT

10. Reverse Transcription
    a. 100 uL Reaction volume

i. 2 uL 25 mM dNTP mix; 150 pmoles [1.5 uL of 100 uM] of reverse primer; 1ng-5ug RNA
ii. Heat sample for 2 minutes at 42C, then add 1 uL of superscript II enzyme
iii. Incubate for one hour at 42C

b. Test on a gel and gel purify