In-cell UV cross-linking of photo-reactive ligands to RNA

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Author: Seth D. Veenbaas

Aim: Prepare ligand crosslinked total RNA for JuMP RT, and sequencing.

Logic: Treating cells with photo-reactive ligand and 365 nm UV cross-links ligand to RNA at direct binding sites, which can be subsequently mapped through JuMP RT and sequencing.

NOTE: All experiments should start fresh by streaking B. sub on plates.

- B. sub does not grow well from glycerol stocks or passaged cell pellets.
- B. sub does not store well at 4 °C because it enters a spore form.

Day 1: Plate cells

Materials:

- B. subtilis str. 168 glycerol stock
- LB Agar plate

Protocol:

1. Streak a plate with B. subtilis str. 168 to form single colonies.
2. Incubate plate at 30 °C overnight.

Notes:

Day 2: Overnight culture

Materials:

- LB media (5 mL)
- Culture tube

Protocol:

1. Inoculate 5 mL LB media with a single colony from plate.
2. Grow cells overnight at 30 °C, 225 rpm.

Day 3: Probe cells and extract RNA (~10 hours)

Materials:

- LB media (50 mL)
- PBS buffer (100 mL)

- 50 mM ligand stock in DMSO (20 uL per sample)
- Sterile 6-well plastic plate (1 well per sample)
- lysozyme (60 mg)
- 1M Tris pH 7 (180 uL)
- 0.5 M EDTA (120 uL)
- Chloroform
- 100% ethanol
- Qiogen RNAeasy Plus Universial Mini kit

Protocol:

3. Inoculate 60 mL LB media with 3 mL cultured cells and grow cells to 0.50 OD_{600}.

   NOTE: Expect cell growth to take ~2.5 hours.

Time point	#1 OD_{600}	#2 OD_{600}	#3 OD_{600}
	-	-	-

4. Buffer excahnge cells.
   - Gently pellet cells at 3200g for 10 min at 4 °C.
   - Carefully remove supernatant.
   - Wash cell pellet with 50 mL PBS buffer, repeat centrifugation, and carefully remove supernatant.
   - Resuspend cells in 50 mL PBS buffer.
5. Probe cells with functionalized ligands.
   - Combine 4,980 uL of cultured media with 20 uL of photo-reactive ligand (50 mM stock in DMSO) and pipette mix. Avoid prolonged light exposure.

     NOTE: final [ligand] is 200 uM. 250X dilution.

   - Incubate ligand-treated cells for 30 minutes at 30 °C and 225 rpm.

     NOTE: Keep solution in dark.
6. Cross-link functionalized ligands to RNA.
   - Transfer 5 mL cell solution to a sterile 6-well plastic plate.
   - Place uncover plate and place on ice
   - Irradiate with 365 nm light source and cross-link with 3 J/cm2.

     NOTE: This is nine minutes (or three max energy hits) 5-6 cm from the light source in a UVP crosslinker.
7. Create cell pellet.
   - Centrifuge to pellet the cells. 8,000xg for 10 min. at 4 °C.

- Discard supernatant.

8. Prepare fresh lysis buffer.
   - (30 mM Tris pH 7, 10 mM EDTA, 10 mg/mL lysozyme):

Reagent	Amount
lysozyme	50 mg
1M Tris pH 7	150 uL
0.5M EDTA	100 uL
RNase inhibitor murine	50 uL
NF H2O	4700 uL
TOTAL	**5,000 uL**

9. Lysis cell pellet with lysozyme buffer.
   - Add 200 uL of buffer lysis to each cell pellet.
   - Incubate for 30 min. at RT.
10. Extract and purify total RNA from cells.
    - Use 1000 uL of Tri-reagent
    - Mix aggressively to aid in lysis
    - Lyse for 15 minutes

      OPTIONAL PAUSE: store at 4 °C
    - Add 250 uL of chloroform
    - Vortex and incubate at RT for 3-5 minutes
    - Centrifuge at 15000xg for 15 minutes at 4 °C

      NOTE: Keep sample on ice until aqueous layer is recovered
    - Add 100% ethanol to each recovered aqueous layer in equal parts (1:1)
    - Follow Monarch total RNA miniprep kit instructions. Start at Part 2 step 4.
    - Elute from column with 51.5 uL of NFW

      NOTE: Yields of ~50-60 ug total RNA have been achieved per 5 mL culture probed.
11. Check RNA purity.df
    - Measure absorbance ratio (A260/280 and A260/230) on nanodrop.

Sample ID	Replicate	Ligand	Yield (µg)	[RNA] (ng/µL)	A260/280	A260/230	Vol NFW to 500 ng/µL (µL)

NOTES:

Storage:

- RNA can be stored overnight at 4 °C for ~ 1 week.
- RNA can be flash-frozen in aliquots and stored at -80 °C for ~1 year.

Next Steps:

- Partial RNA Fragmentation