Probing of RNA base-pairing using clickable psoralen

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The following protocol provides a method for studying RNA structure and RNA-RNA interactions inside cells. The method employs a clickable psoralen as a direct crosslinker of base-paired nucleotides, the identity of which is then revealed by proximity ligation and deep sequencing. The use of clickable psoralen provides a robust biotin-streptavidin based enrichment method for crosslinked RNA regions, while overcoming the limited cell permeability of psoralen-biotin. The protocol is applicable for studying the base-pairing properties of any RNA in any cellular context with an estimated time to completion of 1 week.

Reagents

- Psoralen-TEG-azide (Berry&Associates PS 5030)
- Phosphate buffered saline (Sigma P5493)
- Opti-MEM I Reduced Serum Medium, no phenol red (Gibco 11058021)
- RNeasy Mini Kit (Qiagen 74104)
- RNase III (Ambion AM2290)
- RNA Clean & Concentrator-5 (Zymo R1015)
- Click-IT Biotin DIBO Alkyne (Invitrogen C10412)
- SUPERase In RNase Inhibitor (Invitrogen AM2696)
- Dynabeads MyOne Streptavidin C1 (Invitrogen 65001)
- Formamide-deionised (Sigma F9037)
- Novex TBE-Urea Gels, 10% (Invitrogen EC6875BOX)
- SYBR Gold Nucleic Acid Gel Stain (Invitrogen S11494)

- T4 RNA Ligase 1, High concentration (NEB M0437)
- 2x Binding buffer: 200 mM TRIS-HCl pH 7.5, 20 mM EDTA, 2 M NaCl, 0.2% Tween-20
- Wash buffer: 2xBinding buffer diluted 1:1 with 5 M NaCl
- Elution buffer: 95% Formamide, 10mM EDTA, 10 mM TRIS-HCl pH 7.5, Orange G
- TEN250 buffer: 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 250 mM NaCl

Equipment

- Short wavelength UV crosslinker (254 nM)
- Long wavelength UV crosslinker (365 nM)

Procedure

RNA corsslinking

1. Grow cells in tissue culture dishes to 50-90% confluence.
2. Dissolve 5 mg psoralen-TEG-azide in 4.5 ml water. Incubate at 37 ºC for 5 min and vortex. Add 0.5 ml 10x PBS and 7.5 ml OptiMEM with no phenol red. This results in a concentration of 0.4 mg/ml.
3. Wash cells twice with 1x PBS.
4. Add psoralen-TEG-azide solution to cells, incubate for 20 min in a 37 ºC incubator.
5. Irradiate cells with 50 KJ/m^2 long wave length UV in an ice-water bath.
6. Aspirate psoralen carefully and add RNeasy lysis buffer supplemented with DTT. Scrap cells and collect lysate.
7. Extract total RNA following the RNeasy protocol.

RNA fragmentation

8. Dilute 5-10 μg RNA in water to 50-100 ng/μl and make 20 μl aliquots in PCR tubes.

9. Add 2.5 μl RNase III buffer and 2.5 μl RNase III to each tube.
10. Incubate at 37 ºC for 25 min.
11. Purify with RNA Clean & Concentrator-5. Elute with 40 μl. This should result in RNA size of ~ 100 nt.

Biotin Click reaction

12. Add 8 μl of 1.85 mM Click-IT Biotin DIBO Alkyne (dissolved in DMSO), 5 μl SUPERase-in, 1 μl Tris pH 7.5 and 46 μl water. Incubate at 37 ºC for 1.5 hours with gentle mixing (600 RPM).
13. Stop reaction by purifying with RNA Clean & Concentrator-5.

Pulldown of crosslinked RNA

14. Wash 100 μl Dynabeads MyOne Streptavidin C1 3 times with 1 ml 1x Binding buffer.
15. Resuspend beads in 200 μl 2x Binding buffer.
16. Add RNA diluted in 190 μl water + 10μl Superase-In.
17. Incubate for 1 hour at room temp. with gentle rotation.
18. Spin down beads and place on a magnet. Remove supernatant.
19. Wash beads 5 times with 1 ml 1x Wash buffer.
20. Add 60 μl Formamide elution buffer and incubate at 65 ºC for 5 min, gentle mixing.
21. Place on a magnet for 1 min and collect supernatant.
22. Load supernatant on a 10% TBE-Urea gel and run at 300V for 15min.
23. Cut RNA size 100-250nt. Use blue light and SYBR Gold Nucleic Acid Gel Stain (Avoid UV as it reverses the crosslink).
24. Crush gel and add 350 μl TEN250 buffer supplemented with 0.1% SDS. Rotate overnight at 4 ºC.

25. Clean with RNA Clean & Concentrator-5. Elute with 30 μl water.

Proximity ligation & crosslink reversal

26. Split sample to 2, irradiate one half (control sample) with 2.5 KJ/m^2 short wavelength UV on ice. The non-irradiated half is the interaction sample.
27. Add 35 μl water to both samples and warm to 85 ºC for 2 min. Cool down rapidly on ice.
28. Add 10 μl Superase-In, 20 μl 10x RNA ligase buffer, 10 μl 1mM ATP (final: 50μM), 6.8 μl T4 RNA Ligase 1 high concentration (final: 1 unit/μl) and 103 μl water.
29. Incubate at 16 ºC for 16 hours.
30. Add 22 μl 0.5M EDTA and 10 μl 1M Tris pH 7.
31. Inactivate ligase by heating to 65 ºC for 5 min.
32. Clean RNA with RNA Clean & Concentrator-5. Elute in 15 μl water.
33. Irradiate interaction sample (but not the control sample) with 2.5 KJ/m^2 short wavelength UV on ice.
34. Proceed to NGS directional RNA library preparation
35. Submit to sequencing, paired-end 100.

Trouble shooting

- Pre-test the highest psoralen concentration the cells can resist without detaching from the plate. For some cell lines that is 0.4 mg/ml.
- Avoid exposure of the crosslinked RNA to direct light or UV as it might reverse the crosslink.

Anticipated results

A starting material of 5 µg RNA typically results in ~50-100 ng crosslinked enriched RNA and can be prepared for sequencing with no more than 12-13 PCR cycles.