nanoSHAPE (In vitro - HE, 1AI)

Introduction

This protocol describes the nanoSHAPE method for in vitro RNA structural probing with a high-efficiency (HE) step for optimal direct RNA nanopore sequencing. This involves blocking the 3′-end of an RNA prior to exposure to the SHAPE reagent such that efficient polyA tailing and ligation can be performed during nanopore library preparation. The overall experimental workflow can be summarized as follows:

1) Oxidize RNA and Beta-elimination
2) RNA folding and Modification with SHAPE reagent (1AI)
3) De-phosphorylation and Poly(A) tailing
4) Nanopore library preparation

Materials

- RNA (> 5 ug)
- 1AI [25 - 400 mM]
- Folding buffer
  - 333mM HEPES (pH 8.0)
  - 333mM NaCl
  - 33mM $MgCl_2$
- HEPES [800mM] (pH 8.0)
- Lysine-HCl buffer, [2M] (pH 8.5)
- $NaIO_4$ solution [200mM]
- Ethylene glycol [99%]
- Beta-elimination buffer (pH = 9.5)
  - 33.75 mM Sodium borate
  - 50 mM Boric acid
- CutSmart buffer [5X]
- Phosphatase (AnP [5k U/mL] or rSAP [1k U/mL])
- RNase Inhibitor (Superase IN)
- EPAP buffer [10X]
- ATP [10mM]
- E. coli polyA polymerase enzyme (EPAP)

Procedure

RNA oxidation and beta-elimination

1. Set up the following oxidation-elimination reaction in a **dark tube**. Mix well by vortex and spin down.

Oxidation

	A	B	C	D
1	Component	Volume (ul)	Final	Prep
2	RNA (end repaired if fragmented)	32 (adjust with H2O)	-	-
3	Lysine-HCl buffer, [2M], pH 8.5	4	200mM	-
4	NaIO4 solution [200mM]	4	20mM	21 mg into 500 ul mol H2O
5	*Total volume*	40		

2. Incubate at 37°C for 30 minutes with shaking.
3. Quench the reaction by adding 2 µl of ethylene glycol. Mix well and spin down.
4. CRITICAL Purify using SPRI (~1.0-1.5X) and **elute into Beta-elimination buffer (42ul or less? 30 ul?)**
5. Incubate in beta-elimination buffer at 45°C for 45 mins.
6. Purify using SPRI (~1.0-1.5X) and elute into ~7 ul mol H_2O.

RNA folding and modification

7. For each sample (i.e. control or modifed) dilute ~2-3 ug RNA into 7ul mol H2O.
8. Heat the RNA to 95°C for 2 min and then place it immediately on ice for at least 2 mins.
9. Add 6ul of Folding buffer [3.3X] and 5ul of HEPES [800mM] (pH 8.0) to each sample, mix well by pipetting.
10. Allow the RNA to fold at 37°C for 15 mins.
11. CRITICAL In a separate set of tubes, place 2ul of either DMSO (control) or *freshly prepared* 1AI (modified) at [10X] concentration of desired final concentraiton (i.e. for a 100mM final concentration in the 20ul rxn, use 2ul of 1M 1AI).
12. Transfer 18ul of RNA (control or modified) to the respective DMSO or 1AI tubes for modificaiton. Incubate at 37°C for at least 15 mins.

RNA dephosphroylation and poly(A) tailing

13. Dephosphorylate the modified RNA by setting up the following reaction:

Dephosphorylation

	A	B	C	D
1	Component	Volume (ul)	Final	Notes
2	RNA (oxidized/modified)	20 (from prev rxn)	-	
3	Molecular water	22	-	
4	AnP rxn Buffer [10X] or CutSmart buffer [5X]	5	1X	IMPORTANT: If using AnP, AnP reaction buffer is required to 1X due to Zinc required for the reaction.
5	Phosphatase (AnP [5k U/mL] or rSAP [1k U/mL])	2	10U /2 U	
6	RNase Inhibitor (Superase IN)	1	-	
7	***Total volume***	50		

14. Incubate at 37°C for 30 minutes with shaking.
15. Inactivate the phosphatase by placing the rxn at 65°C for 5 mins.
16. Purify RNA using SPRI (~0.6X-1.0X), elute into 16 ul mol H_2O
17. CRITICAL Quantify the RNA and prepare a dilution of ~1 ug in 15 ul.
18. Setup the poly(A) tailing rxn:

poly(A) tailing

	A	B	C
1	Component	Volume (ul)	Final
2	RNA (dephosphorylated, ~1 ug)	15	-
3	EPAP buffer [10X]	2	1X
4	ATP [10mM]	2	1mM
5	E. coli polyA polymerase enzyme (EPAP)	1	5 U
6	***Total volume***	20	

19. Incubate the reaction at 37°C for 20 minutes.
20. Quench the reaction with 0.5 ul EDTA [0.5M], mix well and spin down.
21. Purify using 0.6X-1.5X SPRI and elute into ~9-13ul mol H_2O.
22. PAUSE Poly(A) tailed RNA can be stored at -80°C overnight or the RNA can be carried directly into nanopore library preparation.

Nanopore library preparation

23. CRITICAL Follow the most up-to-date Direct RNA library preparation protocol. Using only the RNA from one poly(A) tailing reaction (~500 ng - 1 ug). Perform RT using SuperScript III as RT will improve yield and SSIII is most tolerant of SHAPE adducts on RNA.