SUPPLEMENTARY PROTOCOL 1

Nm-seq

REAGENTS AND MATERIALS

- Total RNA, DNase-treated
- Molecular biology grade, RNase-free water (Biological Industries, cat. no. 01-866-1B)
- Sodium periodate (Sigma-Aldrich, cat. No. 311448)
- L-Lysine monohydrochloride (Sigma-Aldrich, cat. no. 62929)
- Ethylene glycol (Sigma-Aldrich, cat. no. 03747)
- RNA Fragmentation Reagents: Fragmentation Reagent (10X) and Stop Solution (Thermo Fischer Scientific, cat. no. AM8740)
- RNA Clean & Concentrator (Zymo Research, cat. no. R1015)
- Shrimp Alkaline Phosphatase and CutSmart Buffer (10X) (New England Biolabs, cat. no. M0371S)
- Antarctic Phosphatase and Reaction Buffer (10X) (New England Biolabs, cat. no. M0289S)
- T4 Polynucleotide Kinase (3' phosphatase minus) and Reaction Buffer (10X) (New England Biolabs, cat. no. M0236S)
- Adenosine 5'-Triphosphate (New England Biolabs, cat. no. P0756S)
- NEBNext® Small RNA Library Prep Set for Illumina (New England Biolabs, cat. no. E7330S)
- Light-protected tubes

REAGENT SETUP

Lysine-HCl buffer, 2 M, pH 8.5: Dissolve 3.653 g of L-Lysine monohydrochloride in 10 ml of molecular biology grade, RNase-free water. Titrate to pH 8.5 with Sodium hydroxide.
Sodium periodate solution, 200 mM: Dissolve 42.778 mg of sodium periodate in 1 ml of molecular biology grade, RNase-free water. Protect from light.

PROCEDURE

RNA FRAGMENTATION

1| Set up the following fragmentation reaction in a thin-walled 200 µl PCR tube. Mix well by vortex and spin down.

Component	Volume (µl)	Final
Total RNA	18	10 µg
10x fragmentation reagent	2	1X
Total volume	20	

2| Incubate at 95 °C for 5 min in a pre-heated thermal cycler block with the heated lid closed. Remove tubes from block and immediately add 2 µl of stop solution. Mix well by vortex and spin down, and place on ice.

3| Purify fragmented RNA using RNA Clean & Concentrator-5 kit, according to the manufacturer's instructions. Elute in 15 µl molecular biology grade, RNase-free water. Note that each RNA Clean & Concentrator-5 column is suitable for purification of up to 10 µg RNA.

3' END REPAIR

4|Set up the following 3' end repair reaction in a thin-walled 200 µl PCR tube. Mix well by gentle tapping and spin down.

Component	Volume (µl)	Final
Fragmented total RNA	43 (adjust with water)	-
Reaction Buffer (10X)	5	1X
Antarctic Phosphatase	2	10 units
Total volume	50	

5| Incubate at 37 °C for 30 minutes.

6| Purify 3' end-repaired fragmented RNA using RNA Clean & Concentraton-5 kit, according to the manufacturer's instructions. Elute in 15 µl molecular biology grade, RNase-free water.

OXIDATION-ELIMINATION-DEPHOSPHORYLATION CYCLES

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

Component	Volume (µl)	Final
3' end repaired fragmented RNA	32 (adjust with water)	-
Lysine-HCl buffer, 2 M, pH 8.5	4	200 mM
Sodium periodate solution, 200 mM	4	20 mM
Total volume	40	

8| Incubate at 37 °C for 30 minutes with shaking.

9| Quench the reaction by adding 2 µl of ethylene glycol. Mix well and spin down.

10| Dephosphorylate by adding 5 µl of CutSmart Buffer (10X), 2 µl of rSAP enzyme (2 units) and 1 µl of molecular biology grade, RNase-free water (total reaction volume of 50 µl), mix well and spin down.

11| Incubate at 37 °C for 30 minutes with shaking.

12| Purify RNA using RNA Clean & Concentraton-5 kit, according to the manufacturer's instructions. Elute in 32 µl molecular biology grade, RNase-free water.

13| Repeat steps 7-12 seven more times.

FINAL OXIDATION-ELIMINATION

14| Repeat steps 7-9 once. Make sure not perform dephosphorylation (steps 10-11).

15| Purify RNA using RNA Clean & Concentraton-5 kit, according to the manufacturer's instructions. Elute in 32 µl molecular biology grade, RNase-free water.

5' PHOSPHORYLATION

16|Set up the following 5' dephosphorylation reaction. Mix well by gentle tapping and spin down.

Component	**Volume (µl)**	**Final**
RNA from step 15	39 (adjust with water)	-
T4 PNK Reaction Buffer (10X)	5	1X
ATP, 10 mM	5	1 mM
T4 PNK (3' phosphatase minus)	1	10 units
Total volume	50	

17| Incubate at 37 °C for 30 minutes.

18| Purify RNA using RNA Clean & Concentraton-5 kit, according to the manufacturer's instructions. Elute in 8 µl molecular biology grade, RNase-free water.

LIBRARY PREPARATION FOR MASSIVELY PARALLEL SEQUENCING

19| Use RNA from step 18 to construct a small RNA library using NEBNext® Small RNA Library Prep Set for Illumina, according to the manufacturer's instruction, with the exception that 3' adaptor ligation should be performed overnight at 16 °C.