Introduction

This protocol outlines a sample enhancement method using extracted non-polyadenylated RNA to produce a clean 3' polyadenylated sample that is suitable for downstream sequencing applications, such as direct RNA (SQK-RNA002) and cDNA sequencing (SQK-PCS111, SQK-PCB111.24 and SQK-DCS109) with a short preparation time of approximately 20 minutes.

Tip: *E. coli* poly(A) polymerase will indiscriminately add an adenosine homopolymer to the 3’ end of any RNA transcript, therefore it is important to consider isolating the transcript(s) of interest before polyadenylating. For example, when targeting prokaryotic protein-coding mRNA transcriptomes for sequencing, we strongly recommend removing all prokaryotic rRNA and tRNA transcripts, which comprise 97-98% of a total RNA extraction before polyadenylating the remaining 2-3% of non-polyadenylated mRNAs. For prokaryote ribodepletion enrichment, we recommend using the ThermoFisher RiboMinus™ Transcriptome Isolation Kit, for bacteria (Cat No: K155004).

While this ribodepletion kit is similar in scope and procedure to theEnrichment of polyadenylated molecules from a sample of total RNA by depletion of background rRNA protocol, please follow the specific instructions for the bacterial kit if starting with prokaryotic total RNA.

Protocol for polyadenylating with *E. coli* poly(A) polymerase

Prep time: ~20 minutes

Input sample: prokaryote ribodepleted RNA; other non-polyadenylated RNA (≤ 10 µg)

Enhanced end-product: 3’ polyadenylated RNA transcripts (e.g. prokaryotic mRNA, eukaryotic lncRNA, etc.)

Materials

- *E. coli* Poly(A) Polymerase (NEB: M0276L)
- 50 mM RNase-free EDTA (Invitrogen: AM9260G)
- Nuclease-free or DEPC-treated water, or 10 mM Tris-HCL, pH7.5
- Freshly-prepared 70% ethanol
- Agencourt RNAClean XP beads (Beckman Coulter: A63987)
- Nuclease-free 1.5 ml microcentrifuge tubes
- Heat block set to 37°C
- Centrifuge fitted for microcentrifuge tubes
- Vortex mixer
- Magnetic rack
- RNA HS Qubit kit (Invitrogen: 10320093)
- Qubit fluorometer (Invitrogen: 16223001)
- Hula mixer

Recommendations to reduce RNase digestion or RNA degradation:
1. Wear clean gloves at all times to avoid degradation of RNA. Change to fresh gloves if they are suspected to be contaminated.
2. Clean the bench and equipment (pipettes, racks, heat-block, etc) with a RNase-neutralising chemical such as RNase-Zap.
3. Keep the RNA sample on ice unless otherwise indicated.
4. Use clean, RNase-free solutions.

Method

1. Start with clean, non-polyadenylated RNA of interest. The sample should be EDTA-free, with no salts present.
2. In a 1.5 ml microcentrifuge tube, set up 3’ polyadenylation reaction as follows:

Reagent	Voulme	Final
Non-polyadenylated RNA	X µl	≤ 10 µg
10X *E. coli* poly(A) polymerase buffer	2 µl	1X
ATP (10 mM)	2 µl	1 mM
Nuclease-free water	15-X µl	
E. coli poly(A) polymerase (5 U/µl)	1 µl	5U
TOTAL	**20 µl**	

3. Incubate at 37°C for 0.5–1.5 minutes.
4. After 0.5–1.5 minutes of incubation, stop the reaction by adding 5 µl of 50 mM EDTA (to a final concentration of 10 mM EDTA). Final volume will be 25 µl.

   Note: We recommend an incubation time of 0.5–1.5 minutes to add a 3’ adenosine homopolymer (poly(A) tail) of approximately 50-100 nucleotides in length. An increase in incubation time will result in longer poly(A) tails. The maximum incubation time is 5 minutes and longer timings may result in a lower total sequencing yield. For more information, please see our polyadenylation Know-how page.

   Tip: We recommend removing EDTA before starting any sequencing kit protocols.
5. Add 45 µl of RNase-free SPRI beads to the reaction.
6. Incubate on a Hula Mixer for 5 minutes at room temperature.
7. Spin down the sample and pellet on a magnet. Keep the tube on the magnet and pipette off and discard the supernatant.
8. Keep the tube on the magnet and wash the beads with 200 µl of freshly-prepared 70% ethanol. Carefully turn the tube 180° twice in the rack to wash pelleted beads. Pippette off and discard the supernatant.
9. Repeat the previous step for a total of two washes.
10. Briefly spin down and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for 30 seconds, but do not dry the pellet to the point of cracking.
11. Remove the tube from the magnetic rack and resuspend the pellet in 12 µl of nuclease-free water. Incubate on ice for 5 minutes.
12. Pellet the beads on a magnet until the eluate is clear and colourless.
13. Remove and retain 12 µl of eluate containing the 3’-polyadenylated RNA in a clean 1.5 ml microcentrifuge tube.
14. Remove 1 µl of final eluate and quantify the final concentration using HS RNA Qubit kit.

    Optional step: Dilute the final eluate to 100 ng/µl and quantify 1 µl using Agilent 6000 Nano RNA kit, using total RNA or mRNA setting depending on the sample.
15. Store the final product at -80°C or proceed immediately with the library preparation, keeping your sample on ice.

Change log

Version	Change
v4, March 2023	Updated URL link and product code for RNase-free EDTA
v3, April 2022	Grammatical corrections
v2, March 2022	Updated product code error
v1, March 2022	Initial publication