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Enabling complete transcriptome sequencing

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Poly(A) RNA Selection Kit User Guide

039 (Poly(A) RNA Selection Kit)

009 (SENSE Total RNA-Seq Library Prep Kit for Illumina, including Barcodes)

020 (PCR Add-on Kit for Illumina)

022 (Purification Module with Magnetic Beads)

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1. Overview

Lexogen's Poly(A) RNA Selection Kit is suited for rapid and highly specific isolation of polyadenylated RNA from total RNA samples.

Total RNA is briefly denatured and the polyadenylated 3' ends present in most mRNAs are hybridized to oligodT beads. Any RNA without poly(A) stretches, such as 28S and 18S ribosomal RNAs and tRNAs, will not be captured by the oligodT beads and hence be removed during subsequent washing steps.

Polyadenylated RNA can either be eluted from the oligodT beads or directly inserted into downstream applications while still being bound to the oligodT beads, as the beads do not interfere with downstream applications such as cDNA synthesis. In fact the oligodT attached to the beads can be directly used to prime first strand cDNA synthesis. Be aware, however, that the first strand cDNA is then covalently attached to the beads.

Another downstream application for polyadenylated RNAs is Next Generation Sequencing (NGS). This poly(A) Selection Module is derived from the SENSE mRNA-Seq V2 library preparation protocol. Here, an elution step is added to free the poly(A) RNA from the beads, for e.g., inserting the RNA into the SENSE Total RNA-Seq library prep or any other downstream application for poly(A) selected RNA.

The protocol was extensively tested with 100 µg - 500 ng of total RNA input. In general, we recommend using 5 µg of total RNA input per 10 µl Magnosphere MS150 / OligodT Beads. With this kit a total of 500 µg total RNA (100 x 5 µg) or 1000 µg Total RNA (10 x 100 µg) can be purified.

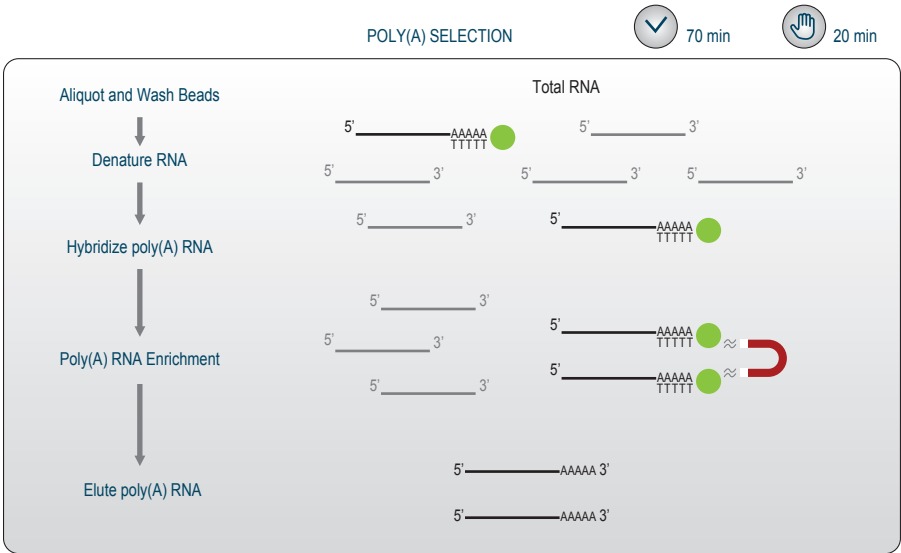


Figure 1. Schematic overview of the poly(A) selection.

2. Kit Components and Storage Conditions

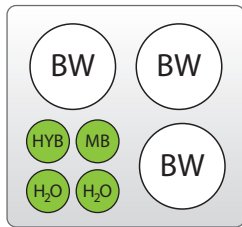


Figure 2. Location of kit components

Kit Component	Tube Label	Volume*	Storage
Magnosphere MS150 / OligodT Beads	MB ●	1100 µl	+4 °C
RNA Hybridization Buffer	HYB ●	1100 µl	+4 °C
Molecular Biology Grade Water	H ₂ O ●	2750 µl	+4 °C
Bead Wash Buffer	BW ○	39600 µl	+4 °C

*including a 10 % surplus

The kit is shipped at room temperature, upon receiving the kit please it store at +4 °C. Before use, check the contents of **BW** ○ which may precipitate during shipping. If a precipitate is visible or the content appears milky, incubate at 37 °C until buffer components dissolve completely.

3. User-supplied Consumables and Equipment

Check to ensure that you have all of the necessary materials and equipment before beginning library preparation. All reagents, equipment, and labware must be free of nucleases and nucleic acid contamination.

Equipment

- Magnetic rack / plate.
- Benchtop centrifuge (rotor compatible with 1.5 ml tubes).
- Calibrated single-channel pipettes for handling 1 μ l to 1000 μ l volumes.
- Thermomixer for 1.5 ml tubes or 96-well plates (dry bath incubator with shaking function).
- UV-spectrophotometer to quantify RNA.

Optional Equipment

- Automated microfluidic electrophoresis station (Agilent Technologies 2100 Bioanalyzer).
- Benchtop fluorometer and appropriate assays (for RNA quality control).
- Agarose gels, dyes, and electrophoresis rig (for RNA quality control).

Labware

- Suitable pipette tips (pipette tips with aerosol barriers recommended).
- 1.5 ml reaction tubes, low binding, certified ribonuclease-free.
- Vortex mixer.

The complete set of materials, reagents and labware necessary for RNA extraction and quality control is not listed. Consult Appendix A (p.12) for more information on RNA quality.

Consult Appendix B (p.15) for information on RNA quantification methods.

4. Guidelines

RNA Handling

- RNases are ubiquitous, and special care should be taken throughout the procedure to avoid RNase contamination.
- Use commercial ribonuclease inhibitors (i.e., RNasin, Promega Corp.) to maintain RNA integrity when storing samples.
- Use a sterile and RNase-free workstation or laminar flow hood if available. Please note that RNases may still be present on sterile surfaces, and that autoclaving does not completely eliminate RNase contamination. Before starting a library preparation, clean your work space, pipettes, and other equipment with RNase removal spray (such as RNaseZap, Ambion Inc.) as per the manufacturer's instructions.
- Protect all reagents and your RNA samples from RNases on your skin by wearing a clean lab coat and fresh gloves. Change gloves after making contact with equipment or surfaces outside of the RNase-free zone.
- Avoid speaking above opened tubes. Keep reagents closed when not in use to avoid airborne RNase contamination.

Bead Handling

- Beads are stored at +4 °C and must be resuspended before usage. Beads can be resuspended by pipetting up and down several times or by vortexing. When properly resuspended, the solution should have a uniform brown color with no visible clumping on the walls or bottom of the tube.
- Beads may stick to certain pipette tips, in which case removing the beads from the inside of the tip may be impossible. Avoid resuspending by repeated pipetting, and instead resuspend by vortexing if this occurs with your tips.
- Beads are superparamagnetic and are collected by placing the plate/tube in a magnetic 96-well plate or a magnetic stand. The time required for complete separation will vary depending on the strength of your magnets, tube thickness, viscosity of the solution, and the proximity of the tube to the magnet. Separation time may need to be adjusted accordingly. When fully separated, the supernatant should be completely clear, and the beads collected at one point or as a ring along the wall of the tube, depending on the magnet that was used.
- To remove the supernatant, the plate/tube containing the beads has to stay in close contact with the magnet. Do not remove the plate/tube from the magnetic stand when removing the supernatant, as the absence of the magnet will cause the beads to go into suspension again.

- In general, beads should not be centrifuged during the protocol. However, should liquid become entrapped in the cap or drops of fluid stay on the side of the reaction tube, centrifugation at 2,000 x g for 30 seconds should be carried out before placing the tube on the magnetic rack.
- Allowing the beads to dry out can damage them. Always keep the beads in suspension except for the short period after withdrawing the supernatant but before adding the next reagent. Beads can be resuspended by vortexing, but make sure that beads are not deposited on the tube walls above the level of the liquid, where they can dry during incubation. If necessary, stuck beads can be collected by centrifuging the tube briefly with a benchtop centrifuge.

General

- Always spin down the microtubes before opening! This prevents spillage.
- Unless explicitly mentioned, all steps should be carried out at room temperature (RT) between 20 °C and 25 °C. Results may be negatively impacted if the protocol is performed at temperatures outside of this range. While reaction set-up is often performed at RT, incubation temperatures are explicitly defined and must be strictly adhered to.
- Ensure that adequate volumes of all reagents and the necessary equipment are available and set to the proper temperatures before beginning the protocol.
- Make sure to pre-heat thermomixers (dry bath incubators) well in advance.
- Perform all pipetting steps with calibrated pipettes and always use fresh tips. Pipette carefully to avoid foaming as some solutions may contain detergents.
- Thaw all necessary buffers at room temperature or as indicated in the preparation tables at the beginning of each step of the detailed protocol. Mix reagents well by vortexing or pipetting repeatedly, and centrifuge briefly with a benchtop centrifuge to collect contents before use.

5. Detailed Protocol

Preparation

Aliquot and Wash Beads	Denature RNA	Hybridize poly(A) RNA	Elute poly(A) RNA
MB – stored at 4 °C HYB – stored at 4 °C BW – stored at 4 °C	Total RNA – thawed on ice H₂O – stored at 4 °C	BW – stored at 4 °C	H₂O – stored at 4 °C
Magnetic rack / plate	Thermocycler 60 °C, 1 min 25 °C, ∞	Thermomixer set to 25 °C, 1,250 rpm	Magnetic rack / plate Thermomixer 70 °C, 1 min

Aliquot and Wash Beads

Lexogen's Poly(A) RNA Selection Kit uses Magnosphere MS150/oligodT beads from JSR Life Sciences. Magnetic beads must be washed before use. All steps are performed at room temperature.

- 1 Mix the beads (**MB** ●) well. Transfer 10 µl of the resuspended beads per poly(A) selection for up to 5 µg total RNA into a new 1.5 ml tube. Beads can be washed as a batch if multiple poly(A) selections are required. For higher total RNA input amounts see Appendix A, p.12.
- 2 Place the tube in a magnetic rack and let the beads collect for 5 minutes or until the supernatant is completely clear (depends on the magnet strength). Remove and discard the supernatant with a pipette while the tube remains in contact with the magnet..
- 3 Remove the tube from the magnetic rack and add 75 µl Bead Wash Buffer (**BW** ○) per poly(A) selection. Resuspend the beads and transfer the tube to the magnetic rack. Let the beads collect for 5 minutes; remove and discard the supernatant.
- 4 Repeat this washing step once (for a total of two washes).
- 5 Resuspend the beads in 10 µl RNA Hybridization Buffer (**HYB** ●) per sample. Pipette and mix carefully to avoid introducing air bubbles.

Denature RNA

RNA samples are briefly heated to resolve secondary structures and promote efficient hybridization. For information on appropriate amounts of total RNA input as well as RNA quantification and quality control see Appendix A, (p.12).

- 6 Dilute 5 µg of total RNA to a volume of 10 µl with RNase-free Water.
- 7 Denature RNA samples using a thermocycler at 60 °C for 1 minute and then hold at 25 °C. Do not cool samples excessively or place denatured RNA on ice.

Hybridize Poly(A) RNA

The denatured total RNA is incubated with the washed beads, which specifically bind polyadenylated RNAs. RNAs lacking a poly(A) tail are then washed away, leaving only purified poly(A) RNA hybridized to the beads.

8 Add the 10 µl of denatured RNA to 10 µl of washed beads and incubate using a thermomixer at 25 °C for 20 minutes with 1,250 rpm agitation.

9 Transfer the tube onto a magnetic rack and let the beads collect for 5 minutes (or until the supernatant is clear). Remove and discard the supernatant.

Remove the tube from the magnetic rack and add 100 µl Bead Wash Buffer (**BW** ○). Resuspend the beads and mix well. Incubate using a thermomixer at 25 °C for 5 minutes with 1,250 rpm agitation. Collect the beads by placing the tube onto a magnetic stand for 5 minutes or until the supernatant is completely clear. Remove and discard the supernatant.

11 Repeat this washing step once (for a total of two washes).

RNA Elution

The poly(A) RNA is eluted from the oligodT beads by heating in water.

12 Completely remove the supernatant from the last wash and add 25 µl of RNase-free Water (**H₂O** ●) to the beads. **OPTIONAL:** If a higher mRNA concentration is desired the elution volume can be reduced to 12 µl.

13 Incubate for 1 minute at 70 °C.

14 Immediately transfer the tube onto a magnetic rack and let the beads collect for 5 minutes (or until the supernatant is clear).

15 Transfer the supernatant into a fresh tube. The supernatant now contains the poly(A) selected RNA that can be used for quality control, concentration determination, and further downstream applications such as a SENSE Total RNA-Seq library preparation for instance.

16 For long term storage we recommend adding 1/10 th (i.e., 2.5 µl if the RNA was eluted in 25 µl) of 100 mM Tris, pH7.0 to the isolated poly(A) RNA to final concentration of 10 mM Tris, pH7.0. Furthermore, addition of 0.2 units of an RNase inhibitor is recommended for long term storage of poly(A) RNA.

6. Short Procedure

ATTENTION: Spin down solutions before opening tubes or plates!

70 min Poly(A) Selection

<input type="checkbox"/>	Wash 10 µl beads (MB ●) twice with 75 µl BW ○.	Aliquot and Wash Beads
<input type="checkbox"/>	Resuspend beads with 10 µl HYB ●.	
<input type="checkbox"/>	Dilute 5 µg total RNA in a volume of 10 µl e.g., RNase-free water.	Denature RNA
<input type="checkbox"/>	Incubate for 1 min at 60 °C, hold at 25 °C.	
<input type="checkbox"/>	Add denatured RNA (10 µl) to washed beads (10 µl).	
<input type="checkbox"/>	Incubate for 20 min at 25 °C / 1,250 rpm.	Hybridize Poly(A) RNA
<input type="checkbox"/>	Wash 2 x for 5 min at 25 °C / 1,250 rpm with 100 µl BW ○.	
<input type="checkbox"/>	Withdraw supernatant.	Elute pPoly(A) RNA
<input type="checkbox"/>	Add 25 µl of RNase-free water..	
<input type="checkbox"/>	Incubate for 1 min at 70 °C.	
<input type="checkbox"/>	Place on magnet for 2 - 5 min or until supernatant is clear.	
<input type="checkbox"/>	Transfer the clear supernatant into a fresh tube.	

7. Appendix A: RNA Requirements

RNA Amount

High quality mRNA isolation relies on high-quality input RNA. The poly(A) content of an RNA sample or tissue varies, but is usually between 1 and 3 % of the total RNA amount. This protocol was tested extensively with various mouse tissues and human reference RNA. Total RNA isolated from kidney, liver, and brain for instance has a higher poly(A) content than total RNA isolated from lung or heart.

An important factor in determining the % of poly(A) RNA recovered is using the same method for concentration measurement before and after poly(A) enrichment. Determining the total RNA input by Nandorop measurements and the recovered poly(A) RNA using Bioanalyzer determined concentrations may result in the impression of a low mRNA recovery as the two methods differ significantly in the concentration determination, with the Nandodrop usually giving much higher concentration values.

We typically recommend using 5 µg of total RNA input with 10 µl of beads (**MB** ●). However, we have also tested 100 µg and 50 µg of total RNA (Universal Human Reference RNA) with a few protocol adjustments (more oligodT beads (**MB** ●), changed RNA and **HYB** ● volumes, see table below) and achieved good results.

The lowest RNA input with good recovery of poly(A) RNA was 500 ng total RNA. Here, the elution volume in step 12 was reduced to 12 µl RNase-free Water (**H₂O** ●) for a higher final poly(A) RNA concentration.

For Universal Human Reference RNA the amount of poly(A) RNA recovered is about 2 % of the total RNA input.

Universal Human Reference RNA input	Step 1 Amount of MB	Step 5 HYB added to MB	Step 6 RNA in (volume)	Step 12 Elution volume
100 µg UHR	100 µl	100 µl HYB	100 µl	50 µl
50 µg UHR	50 µl	50 µl HYB	50 µl	25 µl
5 µg UHR	10 µl	10 µl HYB	10 µl	
2 µg UHR				
500 ng UHR				

RNA Integrity

The integrity of an RNA sample can be assessed with a variety of methods. We recommend the use of a microfluidics assay such as the RNA6000 series for the 2100 Bioanalyzer (Agilent Technologies Inc.), although RNA quality can also be assessed with denaturing agarose gel electrophoresis if such a device is not available. Most microfluidics platforms will carry out an automated peak analysis and generate a quality score (RIN or RQN), and we recommend a RIN score of 8 or greater for optimal full-length poly(A) RNA selection. Typically such samples have easily detectable rRNA peaks and a comparatively low abundance of short RNAs, which can arise from

both intact short transcripts as well as from RNA degradation. Poly(A) RNA can also be isolated from lower quality RNA, but this may lead to 3'-bias in subsequent reactions.

Potential Contaminants

RNA samples should be free of salts, metal ions, and organic solvents which can be carried over from RNA extraction. Several sources of contamination can be detected with an UV-Vis spectrophotometer. An acceptably pure RNA sample should have an A260/A280 ratio between 1.8 and 2.1. The A260/A230 ratio should be approximately 2. Several common contaminants including proteins, chaotropic salts, and phenol absorb strongly between 220 and 230 nm and can often be identified as peaks in this region. Contamination with any of these generates a lower A260/230 ratio. Phenol also has an absorption maximum between 250 and 280 nm which overlaps that of nucleic acid, so high 230 nm absorbance combined with a biphasic or broad peak between 250 and 280 nm may indicate contamination with phenol rather than chaotropic salts.

Genomic DNA Contamination

Depending on the RNA extraction protocol used, samples may also contain significant amounts of gDNA, which is indistinguishable from RNA on a spectrophotometer. Furthermore, as many of the dyes used in RNA microfluidics assays stain single-stranded nucleic acids much more intensely than double-stranded, low to moderate amounts of gDNA may not be readily visible with an RNA-specific microfluidics assay. We highly recommend examining all RNA samples on a denaturing agarose gel or using a fluorometric assay with DNA- and RNA-specific dyes to check samples for DNA contamination. On an agarose gel, gDNA can appear as either a dark mass which remains in the slot if relatively intact, or as a high molecular weight smear if it has been sheared during extraction. Poly(A) RNA selection can also be performed on samples containing gDNA but some genomic A stretches may also be bound by the oligodT beads.

The best way to avoid gDNA contamination is to use an RNA extraction protocol that does not co-isolate gDNA such as Lexogen's SPLIT RNA Extraction Kit (Cat. No. 008.48). However, DNA can be removed from irreplaceable samples by acidic phenol extraction or DNase I digestion. We do not recommend DNase treatment as the extended incubation with divalent cations can lead to RNA hydrolysis and decrease RNA integrity. If samples must be DNase treated, heat inactivation should be avoided, and the enzyme deactivated by other means such as phenol/chloroform extraction or silica column purification.

RNA Storage

If immediate RNA extraction is not possible, tissue samples can be either flash-frozen with liquid nitrogen or submerged in RNAlater (Life Technologies Inc.) and stored at -80 °C. After extraction, RNA can be stored at -20 °C or -80 °C in 10 mM Tris pH 7.0. Avoid frequent freeze/thaw cycles as RNA might be sheared.

RNA Spike-in Controls Mixes

RNA spike-in control mixes such as the ERCC RNA Spike-in Controls (Ambion Inc.) and / or Lexogen's set of artificial spike in transcripts called SIRVs (Spike-in RNA Variant Control Mixes, Cat. No. 025.03), can be spiked into the Total RNA before poly(A) selection. As both ERCC control RNAs and SIRVs are polyadenylated they will be isolated with the oligodT beads along with the mRNA. Such control mixes serve as a control and anchor set for the comparison of experiments. Also for some applications such as RNA-Seq they enable the hypothesis-neutral calculation of strandedness, isoform quantitation and the validation of the performance of isoform-specific RNA-Seq workflows.

9. Appendix B: Quality Control

Quality Control Methods

For quality control we recommend microcapillary electrophoresis. Several electrophoresis platforms are available from various manufacturers. For low- to medium-throughput applications, we recommend the Agilent Bioanalyzer 2100 and RNA 6000 Pico chips (Agilent Technologies, Inc.). Typically, 1 µl of eluted poly(A) RNA produced according to the directions in this manual can be analyzed directly on a Pico RNA chip. For high throughput applications instruments such as LabChip GX II (Perkin Elmer) or 2200 TapeStation (Agilent Technologies, Inc.) are recommended. Depending on the minimum sample loading requirements for each instrument, 1 µl of the eluted poly(A) RNA may be diluted to the required volume (e.g., 2 µl sample for TapeStation RNA 6000 Pico and High Sensitivity R6K kits and 10 µl for LabChip GX II).

ATTENTION: Be advised that poly(A) RNA does not contain 18S and 28S rRNA peaks any more so an RNA integrity number (RIN) or RNA Quality Number (RQN) cannot be calculated anymore.

If microcapillary electrophoresis platforms are not available, very basic quality control can also be performed by separating a small aliquot of the RNA on a polyacrylamide or a denaturing agarose gel. RNA quantification can also be performed with an inexpensive benchtop fluorometer using one of several commercially available assays. We recommend using a Qubit HS RNA assay (Life Technologies, Inc). Most UV-Vis spectrophotometers are not sensitive enough at these concentrations to accurately quantify low RNA amounts and should be avoided.

Typical results

The poly(A) content of an RNA sample or tissue varies, but is usually between 1 and 3 % of the total RNA amount. From 5 µg Universal Human Reference RNA (UHRR) around 100 ng poly(A) RNA can be isolated (~2 %).

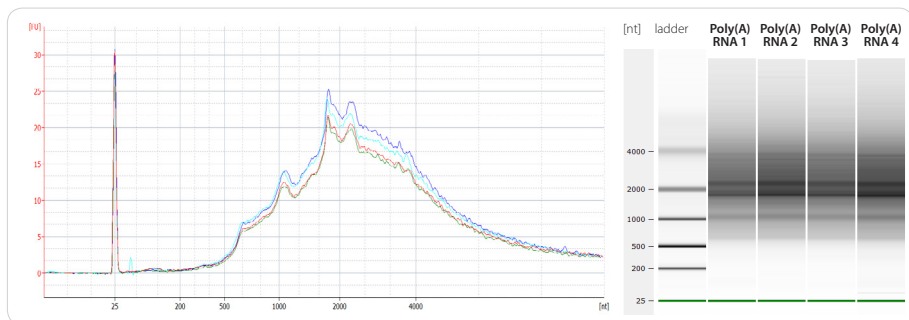


Figure 3. Bioanalyzer traces of 4 independent poly(A) selections from 5 µg Universal Human Reference RNA. Poly(A) RNA was eluted in 25 µl RNase-free water and 1 µl was loaded onto an RNA 6000 Pico chip (Agilent Technologies, Inc). The concentration of all 4 samples was around 4 ng/µl poly(A) RNA (3.8 ng/µl - 4.1 ng/µl).

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