LEXOGEN
The RNA Experts

General Guidelines User Information

001UI375V0100

Table of Contents

1. General Guidelines

1.1 RNA Handling

- RNases are ubiquitous, and special care should be taken throughout library preparation procedures 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. Well 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. **ATTENTION:** Do not forget to rinse off any RNaseZap residue with RNase-free water after usage. Residues of RNaseZap may damage the RNA.
- 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.

1.2 Purification Bead Handling

- Purification Beads (**PB**) 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 or storage bottle.
- 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 plate or stand. The time required for complete separation will vary depending on the strength of your magnets, wall thickness of the wells / tubes, viscosity of the solution, and the proximity of the well / 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 well / tube, depending on the magnet that was used.

- Do not remove the plate / tube from the magnetic plate / stand when removing the supernatant, as the absence of the magnet will cause the beads to go into suspension again. To remove the supernatant the plate / tube containing the beads has to stay in close contact with the magnet.
- Always store beads in an upright position to ensure that they are covered by liquid.
- When using a multichannel pipette to remove the supernatant, make sure not to disturb the beads. If beads were disturbed, ensure that no beads are stuck to the pipette tip opening and leave the multichannel pipette in the well for an extra 30 seconds before removing the supernatant. This way all beads can be recollected at the magnet and the clear supernatant can be removed.
- In general, beads should not be centrifuged during the protocol. However, should drops of fluid stay on the wall of the reaction tube / plate (e.g., after mixing by vortexing), centrifugation at 2,000 x g for 30 seconds should be carried out before placing the tube / plate on the magnetic stand / plate.
- Allowing the beads to dry out can damage them. Always keep the beads in suspension except for the short period after withdrawing the supernatant, and before adding the next reagent. Beads can be resuspended by vortexing, but make sure that beads are not deposited on the well / tube walls above the level of the liquid, where they can dry during incubation. If necessary, stuck beads can be collected by centrifuging the plate / tube briefly with a suitable benchtop centrifuge.

1.3 General Guidelines: Pre-processing and Library Prep

- Always spin down solutions before opening caps or seals to ensure the reagents are collected at the bottom of the provided tubes or plates. This prevents spillage and cross contamination.
- The library generation and purification protocols are primarily designed to be performed in PCR plates or strips but can also be carried out in 1.5 ml tubes and thermoblocks or thermomixers. This may increase the time requirements for bead separation in purifications steps.
- Depletion and enrichment protocols are primarily designed to be performed in 1.5 ml tubes and thermoblocks but can also be performed in PCR plates or strips and thermocyclers. In this case, make sure the total volume fits into the chosen reaction vessel.
- Unless explicitly mentioned, all centrifugation 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.
- Steps requiring a thermocycler have been tested with a maximum ramp speed of 5 °C/sec before denaturation and extension, and 2.5 °C/sec during primer annealing. While these

ramp speeds are typical for most modern thermocyclers, some models can exceed these rates, and ramp speed may need to be decreased to ensure efficient annealing. Ramp speeds may be reduced even further in some steps of the protocol to ensure better hybridization. Preheat lid to 105 °C, in case this has to be adjusted manually.

- Pre-heat thermomixers (dry bath incubators) well in advance.
- Ensure that adequate volumes of all reagents and the necessary equipment are available and set to the proper temperatures before beginning the protocol.
- Perform all pipetting steps with calibrated pipettes, and always use fresh tips. Pipette carefully to avoid foaming as some solutions contain detergents.
- Thaw or equilibrate 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.
- Enzyme Mixes should not be vortexed! Centrifuge briefly with a benchtop centrifuge to collect contents before use.
- Keep Enzyme Mixes at -20 °C until just before use or store in a -20 °C benchtop cooler.
- Enzyme Mixes are viscous and require care to pipette accurately (see next section).
- When mixing by pipetting, set the pipette to a larger volume. For example after adding 1 µl of enzyme to a mix of 19 µl use a pipette set to 15 µl to ensure proper mixing.
- Reaction mixes must be mixed thoroughly by pipetting or vortexing.
- To maximize reproducibility and avoid cross contamination spin down the reactions both after mixing, and after incubations at elevated temperatures (i.e., before removing the sealing foil from PCR plates or tubes).

1.4 Pipetting and Handling of (Viscous) Solutions

- Several components of the library prep kits such as Enzyme Mixes are viscous solutions which require care to pipette accurately. Quickly spin down the tubes to collect all liquid at the bottom of the tube. Be sure to pipette slowly and check the graduation marks on your pipette tips when removing an aliquot.
- Ensure that any viscous solutions are not cooled below room temperature (20 - 25 °C) before use (excluding Enzme Mixes!). Cooler temperatures can enhance viscosity and may increase the time and mixing effort required to achieve homogeneity. Follow the specified temperature instructions for thawing and equilibration of these solutions before use!
- Ensure that pipetting of viscous solutions into mixes is performed swiftly, while avoiding bubble formation. Ensure the tip remains under the surface of the liquid. Mix thoroughly until the samples are homogeneous.

- When drawing up liquid, the tip should be dipped 3 to 5 mm below the surface of the liquid, always at a 90 degree angle. Do not dip the tip in any further as viscous solutions tend to stick to the outside of the pipette tip.
- Any residual liquid adhering to the tip should be removed by sliding the tip up the wall or edge of the tube from which the liquid was taken. Spin down the tube afterwards again to ensure that all liquid is collected at the bottom of the tube for further storage.
- When dispensing, the pipette should be held at a 45 degree angle, and the tip placed against the side of the receiving vessel.
- When pipetting liquids from bottles, take special care that only the sterile pipette tip touches the bottle opening to prevent introducing RNases or other contaminants. Tips are sterile, whereas the pipette itself is not. If necessary, tilt the bottle to bring the liquid closer to the opening and facilitate pipetting.

1.5 Preparation of Mastermixes and Pipetting with Multi-Channel Pipettes

In several steps of Lexogen kit protocols, mastermixes of several different components should be prepared. When preparing mastermixes and when using multi-channel pipettes, always include a 10 % surplus per reaction in order to have enough solution available for all reactions.

EXAMPLE:

From QuantSeq FWD: "Prepare a mastermix containing 9.5 µl First Strand cDNA Synthesis Mix 2 (**FS2**) and 0.5 µl Enzyme Mix 1 (**E1**) per reaction."

For 24 preps use 250.8 µl **FS2** (= 9.5 µl x 24 rxn x 1.1)
+ 13.2 µl **E1** (= 0.5 µl x 24 rxn x 1.1)
resulting in a total of 264 µl, which is sufficient for multi-channel pipetting.

All reagents of Lexogen's library prep kits include at least 10 % surplus.

2. General RNA Requirements

2.1 RNA Purity and Chemical 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 a 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 has an additional 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. These contaminants may have a negative impact on the efficiency of the protocols.

2.2 Genomic DNA Contamination

Depending on the RNA extraction protocol used, samples may also contain significant amounts of genomic DNA (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 more intensively 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. RNA-Seq libraries generated from samples containing gDNA may have an increased number of intergenic reads or lower strandedness.

The best way to avoid gDNA contamination is to use an RNA extraction protocol that does not co-isolate gDNA. However, DNA can be removed from irreplaceable samples by acidic phenol extraction or DNase I digestion. DNase I treatment is highly recommended for FFPE RNA and for RNA preparations from blood samples. If samples must be DNase treated, heat inactivation should be avoided, and the enzyme should be deactivated by other means such as phenol / chloroform extraction or silica column purification.

2.3 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). RIN or RQN values above 8 are considered good quality RNA.

2.4 RNA Integrity - FFPE RNA

RNA extracted from formalin-fixed, paraffin-embedded (FFPE) tissue is often heavily degraded due to the fixation process. As a result of this degradation 28S and 18S rRNA peaks are often completely absent from such RNA samples and hence the calculation of a meaningful RIN value is not possible anymore. A more commonly used reference value for FFPE and degraded RNA is the Distribution Value 200 (DV_{200}), which is the percentage of RNA fragments larger than 200 nucleotides. The lower the DV_{200}, the more degraded the RNA is. This typically leads to smaller insert sizes which should be kept in mind when choosing read lengths for the sequencing experiment. For processing of FFPE samples and samples with lower RIN, the QuantSeq 3′ mRNA-Seq Kits or CORALL Total RNA-Seq with RiboCop are recommended.

2.5 Mitochondrial Ribosomal RNA

Mitochondrial ribosomal RNAs (mt-rRNAs) are polyadenylated and hence will also be reverse transcribed and converted into a cDNA library using poly(A) selection or oligo(dT) priming. mt-rRNAs can make up 1 - 2 % of the reads when using a 3′ mRNA-Seq protocol, such as QuantSeq, as only one fragment will be generated for each transcript. Optionally, an rRNA depletion method, which also removes mt-rRNAs, such as Lexogen's RiboCop rRNA Depletion Kits, can be used before starting the library preparation if it is essential to remove mt-rRNA transcripts.

2.6 RNA Storage

If immediate RNA extraction is not possible, tissue samples can be either flash-frozen with liquid nitrogen or submerged in RNA*later* (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. Addition of RNasin or an equivalent RNase inhibitor is recommended. Avoid frequent freeze / thaw cycles as RNA might be sheared.

2.7 SIRV Spike-in RNA Variant Control Mixes

The Lexogen SIRV (Spike-In RNA Variant) controls are artificial spike in transcripts that serve as a control and anchor set for the comparison of RNA-Seq experiments. The SIRVs consist of 69 artificial RNA transcripts with no identity to any known genomic sequences, hence they can be spiked into any RNA. SIRVs are available in four sets. SIRV-Set 1 (Cat. No 025) contains the Isoform Mixes E0, E1, and E2 of the isoform module, SIRV-Set 2 (Cat. No. 050) provides the Isoform Mix E0 only, whereas SIRV-Set 3 (Cat. No. 051) has the SIRV Isoform Mix E0 in a mixture with the ERCC RNA Spike-in controls (Thermo Fisher Scientific Inc., see below). SIRV-Set 4 contains the SIRV Isoform Mix E0 and the ERCC RNA Spike-in controls, as well as 15 additional long SIRVs, ranging in size from 4 - 12 kb (Cat. No. 141).

The SIRVs are polyadenylated mRNAs and therefore are efficiently captured during QuantSeq 3′ mRNA-Seq or CORALL mRNA-Seq library preparation. SIRVs are not recommended for use with Lexogen's TeloPrime Full-Length cDNA Amplification Kit, as they do not have a 5′ cap and therefore, are not efficiently captured by the 5′ cap-specific method used by TeloPrime.

ERCC RNA Spike-in Controls

To enable the hypothesis-neutral calculation of strandedness, to assess internal oligo(dT) priming events, and as a true reference on detection limit and preservation of dynamic range, we highly recommend the addition of artificial transcripts of known strand orientation and concentration such as the ERCC RNA Spike-in controls (Thermo Fisher Scientific Inc.). For general use and QuantSeq library preparation we recommend using SIRV-Set 3 (Cat. No. 051), which contains ERCCs together with the SIRV isoform controls. ERCCs have a known strand orientation and no antisense transcripts, so the calculation of strandedness based on ERCC sequences is more accurate than calculations based on reads aligned to the genome. The input-output correlation can be computed by comparing the given concentrations of the ERCC RNA Spike-in transcripts with their expression value in the sequenced library. Any potential overcycling of the libraries can be detected. Transcripts may have different and not yet annotated 3' ends, which might be mistaken for internal priming events of the oligo(dT) primer, when in fact those are true 3' ends. As ERCC transcripts only have one defined 3' end, this provides the only true measure to determine internal priming.

3. Appendix A: Revision History

Publication No. / Revision Date	Change	Page
001UI375V0100 Nov. 16, 2021	Initial Release.	

LEXOGEN
The RNA Experts

General Guidelines · User Information

Lexogen GmbH
Campus Vienna Biocenter 5
1030 Vienna, Austria
Telephone: +43 (0) 1 345 1212-41
Fax: +43 (0) 1 345 1212-99
E-mail: support@lexogen.com

Lexogen, Inc.
51 Autumn Pond Park
Greenland, NH 03840, USA
Telephone: +1-603-431-4300
Fax: +1-603-431-4333
www.lexogen.com