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3′ mRNA-Seq Library Prep Kit

User Guide

Catalog Number:
012 (QuantSeq 3′ mRNA-Seq Library Prep Kit for Ion Torrent)
021 (PCR Add-on Kit for Ion Torrent)
022 (Purification Module with Magnetic Beads)
025, 050, 051 (Spike-In RNA Variant Controls: SIRV-Set 1, SIRV-Set 2, SIRV-Set 3)
070 (RS-Globin Block, *Homo sapiens*)
071 (RS-Globin Block, *Sus scrofa*)

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Important recommendations for QuantSeq 3′ mRNA-Seq Library Prep for Ion Torrent

New changes to QuantSeq 3′ mRNA-Seq Library Prep Protocol!

- **The ☞ safe stopping point after reverse transcription, i.e., after step 4 has been removed!** – continue directly to RNA removal after reverse transcription of the libraries. Do not place samples on ice and do not store samples at this point. Cooling the samples below room temperature at this point can cause mishybridization.

Best practice handling would be as follows:

1. After the 42 °C incubation is complete spin down the plate/tubes briefly and place at room temperature.
2. Immediately add the RNA Removal Solution (RS, thawed at room temperature) to the samples and mix well.
3. Briefly spin down the plate / tubes at room temperature, then place on the thermocycler to commence the 10 minute incubation at 95 °C (step 6).

- **When storing libraries at -20 °C safe stopping points, ensure the samples are thawed and equilibrated to room temperature before re-starting the protocol.** This is particularly critical before purification steps. Cooler temperatures may negatively affect yields and enhance the presence of unwanted side products.

Critical Steps for QuantSeq 3′ mRNA-Seq Library Prep Kits!

First Strand cDNA Synthesis (p.11-12):

1. At step 3 pre-warm the FS2 / E1 mastermix for 2 - 3 minutes at 42 °C – Do not cool the mastermix on ice!
2. Have the RNA / FS1 samples at 42 °C when adding the pre-warmed FS2 / E1 mastermix (step 4) and mix properly when adding. Any drop in temperature at this point can cause mishybridization. Seal the plate or tubes and begin the 42 °C incubation.
3. Spin down the samples at room temperature before and after adding the FS2 / E1 mastermix.

If step 2 is skipped for low input or degraded samples (≤10 ng, or FFPE samples):

1. Prepare your RNA samples in 5 µl volumes.
2. Prepare a mastermix containing 5 µl FS1, 9.5 µl FS2, and 0.5 µl E1, mix well, spin down, and pre-warm at 42 °C on a thermocycler for 2 - 3 minutes.
3. Bring your RNA samples to room temperature while the mastermix is pre-warming.
4. Spin down the pre-warmed FS1 / FS2 / E1 mastermix and add 15 µl to each RNA sample. Mix, seal the plate or strip-tubes, spin down briefly at room temperature, and then commence the 42 °C incubation for 15 minutes (or 1 hour for low input RNA (≤ 10 ng)).

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For any publication using this product, please refer to it as Lexogen's QuantSeq 3′ mRNA-Seq Kit.

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1. Overview

Lexogen's QuantSeq Kit provides a library preparation protocol designed to generate Ion Torrent-compatible libraries from polyadenylated RNA within 4.5 hours. The QuantSeq protocol generates only one fragment per transcript, resulting in extremely accurate gene expression values. The kit is designed to yield sequences close to the 3' end of the transcripts.
QuantSeq contains the P1 adapter in the 5' part of the oligodT primer and the A adapter sequence in the 5' part of the second strand synthesis primer, hence NGS reads are generated towards the poly(A) tail. Reads directly reflect the mRNA sequence.

QuantSeq maintains strand-specificity and allows the mapping of reads to their corresponding strand on the genome, enabling the discovery and quantification of antisense transcripts and overlapping genes. The kit includes magnetic beads for the purification steps and hence is compatible with automation. Optional multiplexing of libraries can be carried out using up to 24 in-line barcodes up, when using a single kit, or up to 48 in-line barcodes, when combining the kits containing Barcode Ssets A (Cat. No. 012.24A) and B (Cat. No. 012.24B).

QuantSeq uses total RNA as input, hence no prior poly(A) enrichment or rRNA depletion is required. Information regarding input RNA requirements can be found in Appendix A (p.20).

Library generation is initiated by oligodT priming. The primer already contains Ion Torrent-compatible linker sequences. After first strand synthesis the RNA is removed and second strand synthesis is initiated by random priming and a DNA polymerase. The random primer also contains Ion Torrent-compatible linker sequences. No purification is required between first and second strand synthesis. The insert size is optimized for shorter reads (SR100). However, longer read lengths are also possible if a more detailed analysis of the very 3' end of transcripts is desired.

Second strand synthesis is followed by a magnetic bead-based purification step rendering the protocol compatible with automation. The library is then amplified, introducing the sequences required for colony formation (see Appendix E, p.28, for a schematic representation of the finished library). Library quantification can be performed with standard protocols and is further discussed in Appendix C (p.25).
Barcodes can be introduced as in-line barcodes at the beginning of each read (Appendix D, p.27). Data can be analyzed with a number of standard bioinformatic pipelines. Special considerations for the analysis of QuantSeq data, such as read orientation, are presented in Appendix F (p.29).

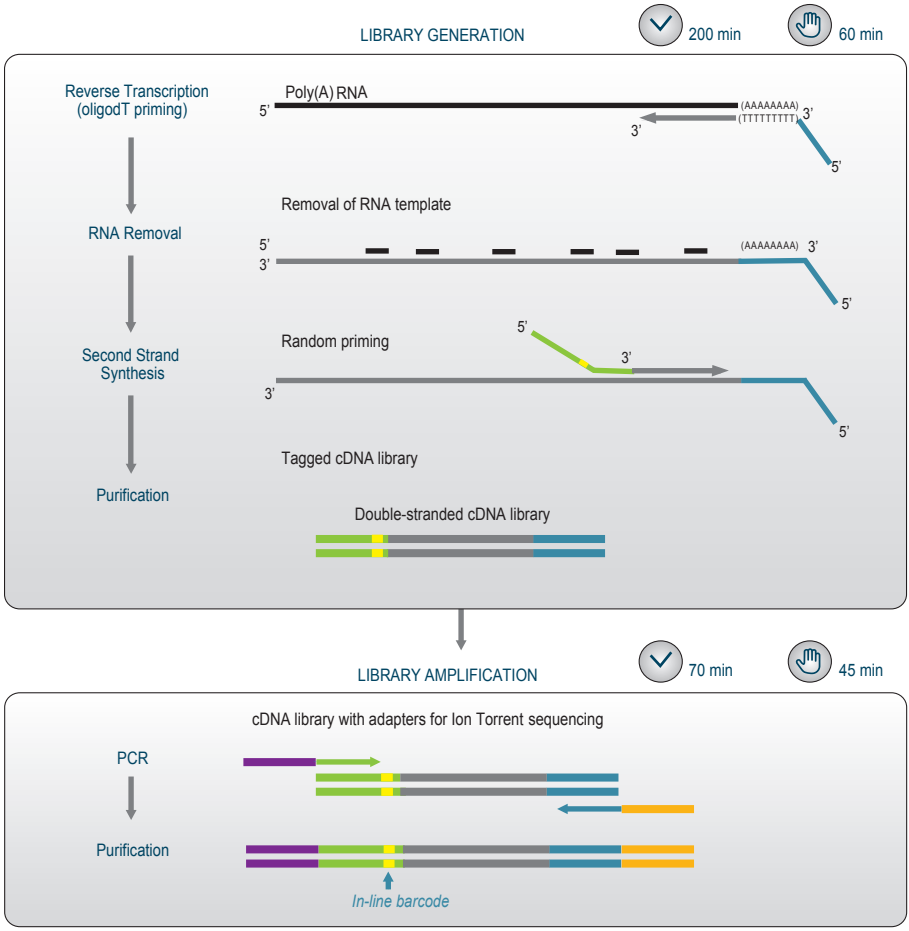

Figure 1. Schematic overview of the QuantSeq library preparation workflow. Barcodes are introduced during second strand synthesis.

2. Kit Components and Storage Conditions

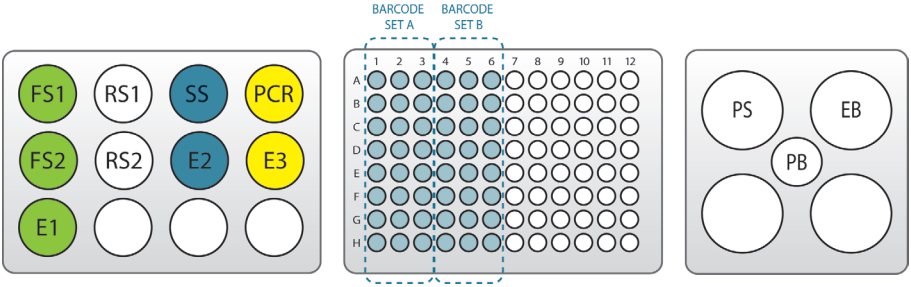

Figure 2. Location of kit components. In QuantSeq Cat. No. 012.24A the barcodes BC01-24 are located in the columns 1 to 3 (Barcode Set A). In QuantSeq Cat. No. 012.24B the barcodes BC25-48 are located in the columns 4 to 6 (Barcode Set B) and columns 1-3 are empty.

Kit Component	Tube Label	Volume* for 24 preps	Storage
First Strand cDNA Synthesis Mix 1	FS1 ●	132 µl	-20 °C
First Strand cDNA Synthesis Mix 2	FS2 ●	250.8 µl	-20 °C
Enzyme Mix 1	E1 ●	13.2 µl	-20 °C
RNA Removal Solution 1	RS1 ○	132 µl	-20 °C
RNA Removal Solution 2	RS2 ○	132 µl	-20 °C
Barcodes 01-24 or 25-48	BC Set A or Set B	15 µl / reaction	-20 °C
Second Strand Synthesis Mix	SS ●	105.6 µl	-20 °C
Enzyme Mix 2	E2 ●	26.4 µl	-20 °C
PCR Mix	PCR ●	316.8 µl	-20 °C
Enzyme Mix 3	E3 ●	26.4 µl	-20 °C
Purification Module (Cat. No. 022.24) included in the kit			
Purification Beads	PB	1478.4 µl	+4 °C
Purification Solution	PS	2429 µl	+4 °C
Elution Buffer	EB	2904 µl	+4 °C

*including a 10 % surplus

Upon receiving the QuantSeq kit, store the purification module containing **PB, PS** and **EB** at +4 °C and the rest of the kit in a -20 °C freezer. **OPTIONAL: EB** could also be stored at -20 °C. Before use, check the contents of **PS**. If a precipitate is visible, 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 material and equipment before beginning library preparation. All reagents, equipment, and labware must be free of nucleases and nucleic acid contamination.

Reagents / Solutions

- 80 % fresh ethanol (washing of Purification Beads **PB**).

Equipment

- Magnetic plate e.g., 96S Super Magnet Plate, article# A001322 from Alpaqua.
- Benchtop centrifuge (3,000 x g, rotor compatible with 96-well plates).
- Calibrated single-channel pipettes for handling 1 µl to 1000 µl volumes.
- Calibrated multi-channel pipettes for handling 1 µl to 200 µl volumes.
- Thermocycler.
- UV-spectrophotometer to quantify nucleic acids (RNA, DNA).
- Ice bath or ice box, ice pellets, benchtop cooler (-20 °C for enzymes).
- Vortex mixer.

Optional Equipment

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

Labware

- Suitable certified ribonuclease-free pipette tips (pipette tips with aerosol barriers recommended).
- 1.5 ml reaction tubes, low binding, certified ribonuclease-free.
- 200 µl PCR tubes or 96 well plates and caps or sealing foil.

The complete set of material, reagents, and labware necessary for RNA extraction and quality control is not listed. Consult Appendix A (p.20) for more information on RNA quality.

Consult Appendix C (p.25) for information on library 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 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.
- 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 plate / stand when removing the supernatant, as the absence of the magnet will cause the beads to go into

suspension again.

- 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 re-collected 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, but before adding the next reagent. Beads can be resuspended by vortexing, and 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 tube briefly with a plate centrifuge.

General

- 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.
- To further increase reproducibility and to avoid cross contamination a centrifugation step should be performed after incubations at elevated temperatures and before removing the sealing foil from PCR plates.
- Ensure that adequate volumes of all reagents and the necessary equipment are available 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 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.
- Keep enzyme mixes at -20 °C until right before use or store in a -20 °C benchtop cooler.
- 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.

- When mixing by pipetting, set the pipette to a larger volume. For example after adding 5 µl in steps 5 and 7 use a pipette set to 15 µl or 20 µl to ensure proper mixing.

Pipetting and Handling of (Viscous) Solutions

- Enzyme mixes, **BC01-48**, **PB**, and **PS** 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.
- 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.

Preparation of Mastermixes and Pipetting with Multi-Channel Pipettes

In steps 3, 10, and 26 of the QuantSeq protocol mastermixes of enzymes and reaction buffers 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: Step 3 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 the QuantSeq kit include a 10 % surplus.

Automation

QuantSeq is compatible with automation and Lexogen provides automated protocols and software for diverse platforms. If you are interested in an automated protocol or need help automating QuantSeq on your NGS workstation, please contact Lexogen at info@lexogen.com.

5. Detailed Protocol

5.1 Library Generation

Preparation

First Strand cDNA Synthesis	RNA Removal	Second Strand Synthesis	Purification
FS1 ● – thawed at RT **FS2** ● – thawed at RT **E1** ● – keep on ice or at -20 °C	**RS1** ○ – thawed at RT **RS2** ○ – thawed at RT	**BC** – thawed at 37 °C; **spin down before opening!** **SS** ● – thawed at RT **E2** ● – keep on ice or at -20 °C	**PB** – stored at +4 °C **PS** – stored at +4 °C **80 % EtOH** – provided by user **prepare fresh!** **EB** – stored at +4 °C
85 °C, 3 min 42 °C, 15 min	95 °C, 10 min cool down to 25 °C	98 °C, 1 min cool to 25 °C (0.5 °C/sec) 25 °C, 30 min 25 °C, 15 min	Equilibrate all reagents to room temperature for 30 minutes prior to use.

First Strand cDNA Synthesis - Reverse Transcription

An oligodT primer containing an Ion Torrent-compatible sequence at its 5' end is hybridized to the poly(A) RNA and reverse transcription is performed. **REMARK:** Keeping the reagents and reaction from step 2 to step 4 at 42 °C at all times (e.g., pipette on the heating block) prevents mishybridisation of the RT primer.

ATTENTION: The minimum recommended input amount is 5 ng of total RNA.

NOTE: Protocol modifications are recommended for low quality, degraded, and FFPE RNA samples. These are indicated as **"REMARK"** in the respective protocol steps (see also Appendix A, p.20).

1. Mix 5 ng - 500 ng of total RNA in a volume of 5 µl with 5 µl First Strand cDNA Synthesis Mix 1 (**FS1** ●) in a PCR plate or 8-well strip tubes. If necessary, adjust the total volume to 10 µl with RNase-free water. Mix well by pipetting. Ensure the plate or PCR strips / tubes are tightly sealed, and spin down to collect the liquid at the bottom of the wells. **REMARK:** For low quality, degraded, and FFPE RNA, do not add **FS1** to the RNA. Instead place the RNA samples at room temperature while preparing the mastermix (see below, and Appendix A, p.20 and Appendix B, p.23).

2. Denature the RNA / **FS1** mix for 3 minutes at 85 °C in a thermocycler and then cool down to 42 °C. **ATTENTION:** Leave the reactions at 42 °C until step 4. **REMARK:** Skip this step for low quality, degraded, and FFPE RNA.

3 Prepare a mastermix containing 9.5 µl First Strand cDNA Synthesis Mix 2 (**FS2** ●) and 0.5 µl Enzyme Mix 1 (**E1** ●) per reaction. Mix well, spin down, and pre-warm the mastermix for 2 - 3 minutes at 42 °C. **REMARK:** If step 2 is skipped, prepare a mastermix containing 5 µl First Strand cDNA Synthesis Mix 1 (**FS1** ●), 9.5 µl **FS2** ●, and 0.5 µl **E1** ● per sample. Mix well, spin down, and pre-warm for 2 - 3 minutes at 42 °C. **ATTENTION:** Do not cool mastermixes on ice!

4 Quickly spin down the denatured RNA / **FS1** samples from step 2 at room temperature to make sure all liquid is collected at the bottom of the wells. Place the samples back onto the thermocycler at 42 °C and carefully remove the sealing foil / tube caps. Add 10 µl of the **FS2** / **E1** mastermix to each reaction, mix well, and seal the plate / tubes. If necessary, quickly spin down the liquid at room temperature. Incubate the reactions for 15 minutes at 42 °C. **REMARK:** If step 2 is skipped, add 15 µl of the pre-warmed **FS1** / **FS2** / **E1** mastermix to each 5 µl RNA sample. Seal the plate / tubes and mix with gentle vortexing. Quickly spin down at room temperature and incubate the reactions for 15 minutes at 42 °C. **OPTIONAL:** Fow low quality, degraded, and FFPE RNA, extend the incubation time to 1 hour at 42 °C. **ATTENTION:** Proceed immediately to the RNA removal step! Do not cool the samples below room temperature after reverse transcription!

RNA Removal

During this step the RNA template is degraded, which is essential for efficient second strand synthesis. Before removing the sealing foil after the first strand synthesis reaction, quickly spin down the plate to make sure all liquid is collected at the bottom of the wells.

NOTE: At this point we recommend placing the Purification Module (**PB**, **PS**, **EB**) for step 13 at room temperature to give them enough time to equilibrate.

5 Add 5 µl RNA Removal Solution 1 (**RS1** ○) directly to the first strand cDNA synthesis reaction. Mix well and re-seal the plate using a fresh foil. **REMARK:** Use a pipette set to 15 µl for efficient mixing.

6 Incubate 10 minutes at 95 °C, then cool down to 25 °C. Spin down the plate at room temperature and carefully remove the sealing foil.

7 Add 5 µl of RNA Removal Solution 2 (**RS2** ○) and mix well. **REMARK:** Use a pipette set to 15 µl for efficient mixing.

Second Strand Synthesis

During this step the library is converted to dsDNA. Second strand synthesis is initiated by a random primer containing an Ion Torrent-compatible linker sequence at its 5' end. A reverse complement prevents the linker sequence from taking part in the hybridization. In-line barcodes (Barcode Set A: **BC01-24** or Barcode Set B: **BC25-48**) are introduced during this step.
ATTENTION: Avoid cross-contamination by spinning down the barcode plate (Barcode Set A: **BC01-24** or Barcode Set B: **BC25-48**) and cut open the sealing foil of the desired barcodes.

ATTENTION: Barcodes (**BC01-24** and **BC25-48**) are supplied in a viscous solution and need to be mixed thoroughly before use. Reseal all barcodes after usage to prevent crosscontamination!

8	Add 15 µl of the desired barcode (**BC01-24** or **BC25-48**, provided in a 96-well plate, see Appendix D, p.27). **ATTENTION:** Spin down before opening! Cut open the sealing foil of the wells containing the desired barcodes. Avoid cross contamination!). Mix well by pipetting, and seal the plate. **REMARK:** Use a pipette set to 40 µl for efficient mixing.
9	Incubate the plate for 1 minute at 98 °C in a thermocycler, and slowly cool down to 25 °C by setting the ramp speed to 10 % (0.5 °C/second). Incubate the reaction for 30 minutes at 25 °C. Quickly spin down the plate at room temperature before removing the sealing foil.
10	Prepare a mastermix containing 4 µl Second Strand Synthesis Mix (**SS** ●) and 1 µl Enzyme Mix 2 (**E2** ●). Mix well.
11	Add 5 µl of the **SS** / **E2** mastermix per reaction. Mix well. **REMARK:** Use a pipette set to 40 µl for efficient mixing.
12	Incubate the reaction for 15 minutes at 25 °C. Quickly spin down before proceeding with purification. ☞ Safe stopping point. Libraries can be stored at -20 °C at this point.

Purification

The double-stranded library is purified by using magnetic beads to remove all reaction components. The Purification Beads (**PB**) should equilibrate for 30 minutes at room temperature before use. **PB** may have settled and must be properly resuspended before adding them to the reaction.

ATTENTION: If the libraries were stored at -20 °C, ensure that they are thawed and equilibrated to room temperature before restarting the protocol.

13	Add 20 µl of properly resuspended Purification Beads (**PB**) to each reaction, mix well, and incubate for 5 minutes at room temperature.
14	Place the plate onto a magnetic plate, and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear (depends on the strength of your magnet).

15	Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.
16	Add 40 µl of Elution Buffer (**EB**), remove the plate from the magnet and resuspend the beads properly in **EB**. Incubate for 2 minutes at room temperature.
17	Add 56 µl of Purification Solution (**PS**) to the beads / **EB** mix to re-precipitate the library. Mix thoroughly, and incubate for 5 minutes at room temperature. **REMARK:** For low quality, degraded, and FFPE RNA add only 48 µl (**PS**).
18	Place the plate onto a magnetic plate, and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
19	Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.
20	Add 120 µl of 80 % EtOH, and wash the beads for 30 seconds. Leave the plate in contact with the magnet as beads should not be resuspended during this washing step. Remove and discard the supernatant.
21	Repeat this washing step once for a total of two washes. Make sure to remove the supernatant completely as traces of ethanol can inhibit subsequent PCR reactions.
22	Leave the plate in contact with the magnet, and let the beads dry for 5 - 10 minutes or until all ethanol has evaporated. **ATTENTION:** Dry the beads only at room temperature and do not let the beads dry too long (visible cracks appear), this will negatively influence the elution and the resulting library yield.
23	Add 20 µl of Elution Buffer (**EB**) per well, remove the plate from the magnet, and resuspend the beads properly in **EB**. Incubate for 2 minutes at room temperature.
24	Place the plate onto a magnetic plate and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
25	Transfer 17 µl of the clear supernatant into a fresh PCR plate. Make sure not to transfer any beads. ☞ Safe stopping point. Libraries can be stored at -20 °C at this point. See Appendix B, p.23 for more information on PCR Endpoint determination.

5.2 Library Amplification

Preparation

PCR	Purification
PCR ● – thawed at RT **E3** ● – keep on ice or at -20 °C	**PB** – stored at +4 °C **PS** – stored at +4 °C **80 % EtOH** – provided by user; **prepare fresh!** **EB** – stored at +4°C
Thermocycler 98 °C, 30 sec 98 °C, 10 sec 65 °C, 20 sec 72 °C, 30 sec } 11 - 17 x see Appendix B, p.23 72 °C, 1 min 10 °C, ∞	Equilibrate all reagents to room temperature for 30 minutes prior to use.

PCR

The library is amplified to add the complete adapter sequences required for colony formation and to generate sufficient material for quality control and sequencing.

ATTENTION: Lexogen also offers a PCR Add-on Kit for Ion Torrent (Cat. No. 021) which can be used for additional PCR reactions on diluted cDNA samples. For details see Appendix B (p.23), or the PCR Add-on Kit for Ion Torrent Instruction Manual (021IM066).

NOTE: At this point we recommend placing the Purification Beads (**PB**) for step 29 at room temperature to give them enough time to equilibrate.

26 Prepare a mastermix containing 12 µl of PCR Mix (**PCR** ●) and 1 µl Enzyme Mix 3 (**E3** ●) per reaction.

27 Add 13 µl of this **PCR / E3** mastermix to 17 µl of the eluted library. Mix well by pipetting. Seal the plate, and quickly spin down to make sure all liquid is collected at the bottom of the well.

28 Conduct 11 - 17 cycles of PCR (depending on the amount of input RNA used, see Appendix B, p.23) with the following program: Initial denaturation at 98 °C for 30 seconds, 11 - 17 cycles of 98 °C for 10 seconds, 65 °C for 20 seconds and 72 °C for 30 seconds, and a final extension at 72 °C for 1 minute, hold at 10 °C.
☞ Safe stopping point. Libraries can be stored at -20 °C at this point.

Purification

The finished library is purified from PCR components that can interfere with quantification. The Purification Beads (**PB**) may have settled and must be properly resuspended before adding them to the reaction.

ATTENTION: If the libraries were stored at -20 °C, ensure that they are thawed and equilibrated to room temperature before restarting the protocol.

29	Add 36 µl of properly resuspended Purification Beads (**PB**) to each reaction, mix well, and incubate for 5 minutes at room temperature. **REMARK:** For low quality, degraded, and FFPE RNA add only 30 µl Purification Beads (**PB**).
30	Place the plate onto a magnetic plate, and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
31	Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.
32	Add 30 µl of Elution Buffer (**EB**), remove the plate from the magnet and resuspend the beads properly in **EB**. Incubate for 2 minutes at room temperature.
33	Add 36 µl of Purification Solution (**PS**) to the beads / **EB** mix to re-precipitate the library. Mix thoroughly, and incubate for 5 minutes at room temperature.
34	Place the plate onto a magnetic plate, and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
35	Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.
36	Add 120 µl of 80 % EtOH, and wash the beads for 30 seconds. Leave the plate in contact with the magnet as beads should not be resuspended during this washing step. Remove and discard the supernatant.
37	Repeat this washing step once for a total of two washes. Make sure to remove the supernatant completely.
38	Leave the plate in contact with the magnet, and let the beads dry for 5 - 10 minutes or until all ethanol has evaporated. **ATTENTION:** Dry the beads only at room temperature and do not let the beads dry too long (visible cracks appear), this will negatively influence the elution and the resulting library yield.
39	Add 20 µl of Elution Buffer (**EB**) per well, remove the plate from the magnet, and resuspend the beads properly in **EB**. Incubate for 2 minutes at room temperature.
40	Place the plate onto a magnetic plate and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.

41 Transfer 15 - 17 µl of the supernatant into a fresh PCR plate. Make sure not to transfer any beads.

42 At this point, the libraries are finished and ready for quality control (Appendix C, p.25), pooling (for multiplexing; see Appendix D, p.27), and colony formation. ☞ Safe stopping point. Libraries can be stored at -20 °C at this point.

6. Short Procedure

ATTENTION: Spin down solutions before opening tubes or plates!

	200 min	Library Generation
☐	Mix 5 µl RNA and 5 µl **FS1** ●.	First Strand cDNA Synthesis
☐	Heat for 3 min at 85 °C, cool to 42 °C. Skip this step for low quality, degraded, and FFPE RNA. **ATTENTION:** Keep samples on thermocycler at 42 °C.	
☐	Prepare a mastermix with 9.5 µl **FS2** ● and 0.5 µl **E1** ● per reaction, mix well, and pre-warm for 2 - 3 min to 42 °C. **ATTENTION:** Do not cool the mastermix on ice!	
☐	Add 10 µl **FS2/ E1** mix per reaction.	
☐	Incubate for 15 min at 42 °C. **OPTIONAL:** May be increased to 1 hr for low quality, degraded, and FFPE RNA.	
☐	Add 5 µl **RS1** ○, mix well.	RNA Removal
☐	Incubate 10 min at 95 °C, then cool to 25 °C.	
☐	Add 5 µl **RS2** ○ mix well.	
☐	Add 15 µl **BC01-24** and/or **BC25-48** (see p.27), mix well.	2nd Strand Synthesis
☐	Incubate 1 min at 98 °C, slowly ramp down to 25 °C.	
☐	Incubate 30 min at 25 °C.	
☐	Pre-mix 4 µl **SS** ● and 1 µl **E2** ● per reaction.	
☐	Add 5 µl **SS / E2** mix per reaction.	
☐	Incubate 15 min at 25 °C. ☞ Safe stopping point.	
☐	Add 20 µl **PB** per reaction, mix well, incubate 5 min.	Purification
☐	Place on magnet for 2 - 5 min, remove and discard supernatant.	
☐	Add 40 µl **EB**, mix well, incubate 2 min at RT.	
☐	Add 56 µl **PS** (or 48 µl **PS** for low quality, degraded, and FFPE RNA), mix well, incubate 5 min at RT.	
☐	Place on magnet for 2 - 5 min, remove and discard supernatant.	
☐☐	Rinse the beads (**PB**) twice with 120 µl 80 % EtOH, 30 sec.	
☐	Air dry beads for 5 - 10 min. **ATTENTION:** Do not let the beads dry too long!	
☐	Add 20 µl **EB**, mix well, incubate 2 min at RT.	
☐	Place on magnet for 2 - 5 min, transfer 17 µl of the supernatant into a fresh PCR plate. ☞ Safe stopping point.	

70 min Library Amplification

☐	Pre-mix 12 µl **PCR** ● and 1 µl **E3** ● per reaction, mix well.	PCR
☐	Add 13 µl **PCR** / **E3** premix to 17 µl of each purified library.	
☐	PCR: 98 °C, 30 sec 98 °C, 10 sec 65 °C, 20 sec 72 °C, 30 sec } 11 - 17 x (see p.23) 72 °C, 1 min; 10 °C, ∞ ☞ Safe stopping point. **ATTENTION:** Increase cycle number for low input RNA and samples with low mRNA content! (see p.23)	
☐	Add 36 µl **PB** (or 30 µl **PB** for low quality, degraded, and FFPE RNA) per reaction, mix well, incubate 5 min.	Purification
☐	Place on magnet for 2 - 5 min, remove and discard supernatant.	
☐	Add 30 µl **EB**, mix well, incubate 2 min at RT.	
☐	Add 36 µl **PS**, mix well, incubate 5 min at RT.	
☐	Place on magnet for 2 - 5 min, remove and discard supernatant.	
☐☐	Rinse the beads twice with 120 µl 80 % EtOH, 30 sec.	
☐	Air dry beads for 5 - 10 min. **ATTENTION:** Do not let the beads dry too long!	
☐	Add 20 µl **EB**, mix well, incubate 2 min at RT.	
☐	Place on magnet for 2 - 5 min, transfer 15 - 17 µl of the supernatant into a fresh PCR plate. ☞ Safe stopping point.	

7. Appendix A: RNA Requirements

RNA Input

In general high quality mRNA-Seq data relies on high quality input RNA. However, as QuantSeq is a 3' mRNA-Seq protocol the quality of the RNA input is not as critical as for other RNA-Seq applications. With QuantSeq even lower quality RNA can yield good RNA-Seq results.

The recommended input amount is 5 ng - 500 ng. As a starting point, we recommend performing the protocol initially with 500 ng total RNA, especially for tissues with lower mRNA content (such as lung and heart). Please keep in mind that the cycle number reference values were generated using Universal Human Reference RNA (UHRR). The input requirements for your particular experiment may be different, as RNAs differ in their mRNA content. If the RNA input is not sufficient, either due to naturally low poly(A) RNA content or degraded RNA, cycle numbers need to be adjusted accordingly (see also Appendix B, p.23).

When working with low quality / degraded RNA, or RNA isolated from Formalin-Fixed Paraffin Embedded (FFPE) samples, only minor protocol modifications are recommended in order to maximise the consistency and yield of the libraries. The different protocol modifications are outlined in the table below alongside the standard protocol steps.

Protocol Step	Standard Input	Low Quality / Degraded / FFPE RNA
Step 2	Incubate for 3 minutes at 85 °C, then cool to 42 °C. Hold samples at 42 °C on the thermocycler.	**Skip!** Prepare pre-warmed **FS1 / FS2 / E1** mastermix! Place RNA samples at room temperature.
Step 3	Prepare **FS2 / E1** mastermix – pre-warm for 2 - 3 minutes at 42 °C.	Prepare **FS1 / FS2 / E1** mastermix – pre-warm for 2 - 3 minutes at 42 °C.
Step 4	Add pre-warmed mastermix to **RNA / FS1** samples at 42 °C. Incubate for 15 minutes at 42 °C.	Add pre-warmed mastermix to RNA samples at room temperature. **OPTIONAL:** Increase incubation time to 1 hour at 42 °C.
Step 17	Add 56 µl of Purification Solution (**PS**).	Reduce volume of Purification Solution (**PS**) to 48 µl.
Step 25	Optimization of endpoint PCR cycle numbers is strongly recommended for all low quality, FFPE, or degraded RNA library preps., or when processing samples from different or new sample types (e.g., species, tissue, cell type). This prevents under or over-cycling of the libraries.	
Step 29	Add 36 µl of Purification Beads (**PB**).	Reduce volume of Purification Beads (**PB**) to 30 µl.

Furthermore, an additional purification of the lane mix with 1 x **PB** (e.g., 50 µl lane mix plus 50 µl **PB**, incubating 5 minutes at room temperature) and following the protocol from step 30 on with only 30 µl **PS** added in step 33 may be necessary.

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). As QuantSeq specifically targets the 3' end of transcripts even RNAs with a low RIN are suitable as input material.

Mitochondrial Ribosomal RNA

Mitochondrial ribosomal RNAs (Mt rRNAs) are polyadenylated and hence will also be reverse transcribed and consequently converted into a cDNA library. 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.

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 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 protocol.

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 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. QuantSeq 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 (e.g., Lexogen's SPLIT RNA Extraction Kit, Cat. No. 008). 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 should be 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. Addition of RNasin or an equivalent RNase inhibitor is recommended. Avoid frequent freeze/thaw cycles as RNA might be sheared.

SIRVs Spike-In RNA Variant Control Mixes

Lexogen offers a set of artificial spike in transcripts called SIRVs (Spike-In RNA Variants), to 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 three 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 (Ambion Inc., see below). The SIRVs are polyadenylated mRNAs and therefore are efficiently captured during QuantSeq 3′ library preparation.

ERCC RNA Spike-in Controls

To enable the hypothesis-neutral calculation of strandedness, to assess internal oligodT 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 (Ambion Inc.). For QuantSeq 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 oligodT 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.

8. Appendix B: PCR Cycles - Low Input RNA

Typically we recommend using 500 ng total RNA input as starting material. If not enough total RNA is available, the input RNA amount can be reduced. However, we do not recommend reducing the total RNA input below 5 ng as this already causes an increase in inserts with poly(T) sequences and/or Ion Torrent linker sequences.

The table below comprises some results obtained from using different amounts of total RNA input. Reference values were generated using high quality, Universal Human Reference RNA (UHRR). Other RNAs with lower mRNA content, or samples with reduced RNA quality, may require a different number of PCR cycles.

Input RNA (UHRR)	PS Used in Step 17	Library*			Insert						Library Yield		PCR Cycles
		Start [bp]	End [bp]	Mean Size*	Mean Size	≥ 50 nt	≥ 100 nt	≥ 200 nt	≥ 300 nt	≥ 400 nt	ng/µl	nM	
500 ng	56 µl	100	1,500	331	260	98 %	76 %	29 %	11 %	4 %	2.2	13.0	11
50 ng	56 µl	100	1,500	298	227	97 %	71 %	24 %	8 %	2 %	1.6	10.0	14
10 ng	56 µl	100	1,500	290	219	94 %	70 %	23 %	6 %	2 %	1.8	11.4	17
5 ng	56 µl	100	1,500	294	223	94 %	70 %	24 %	7 %	2 %	1.2	7.9	17

*All libraries were prepared with in-line barcode BC01. Linker sequences are 84 bp including the 9 nt long in-line barcode.

REMARK: We strongly recommend performing an endpoint PCR test, to determine the optimal number of cycles for library amplification for your specific sample type (see p.24). The PCR Add-on Kit for Ion Torrent (Cat. No. 021) provides the necessary reagents for the additional endpoint PCR testing.

Optimising the PCR cycle numbers also prevents overcycling, which can generally lead to amplification bias and quantification errors.

Optimizing Endpoint PCR Cylcle Numbers

The PCR Add-on Kit for Ion Torrent (Cat. No. 021) contains additional PCR reactions to establish the correct cycle number for amplification. An endpoint test can be performed as follows:

NOTE: At this point we recommend placing the Purification Beads (**PB**) for step 29 at room temperature to give them enough time to equilibrate.

1	Dilute the cDNA from step 25 with 17 µl Elution Buffer (**EB**) or RNase-free water to a total volume of 34 µl.
2	Take 17 µl of the diluted cDNA into a fresh PCR tube and add 12 µl of PCR Mix (**PCR** ●) and 1 µl Enzyme Mix 3 (**E3** ●) per reaction.
3	Conduct **13 cycles** of PCR (or adjust depending on the amount of input RNA used, see table above) with the following program: Initial denaturation at 98 °C for 30 seconds, 13 cycles of 98 °C for 10 seconds, 65 °C for 20 seconds and 72 °C for 30 seconds, and a final extension at 72 °C for 1 minute, hold at 10 °C. ☞ Safe stopping point. Libraries can be stored at -20 °C at this point.
➡	Continue from step 29 onwards of the detailed protocol (p.16) to purify the library.

With each PCR cycle your yield doubles, therefore you can determine whether more or less cycles are required based on the yield of the libraries obtained from the endpoint PCR test.

- If you are content with the yield obtained with this initial PCR, you do not need to amplify the second half of the template. To amplify other libraries prepared from the same type of input RNA, use the same cycle number for diluted cDNA, or use 1 cycle less when using undiluted cDNA (i.e., to adjust for doubling the amount of the cDNA template).
- If you see that your library is already overcycled, use less cycles for the second half of the template. A second peak in high molecular weight regions (between 1,000 - 9,000 bp) is an indication of overcycling.
- If your sample is not amplified enough with 13 cycles, use more cycles for the remaining 17 µl of the cDNA.

Once an appropriate cycle number is established for your RNA, the undiluted cDNA can be used for the PCR. Please keep in mind that the cycle number can be reduced by one if undiluted cDNA is used.

If you need to amplify also the second half of the template with more or less PCR cycles, repeat the steps above adjusting the number of cycles. Lexogen's Purification Module with Magnetic Beads (Cat. No. 022) provides additional purification reagents required for purifying these PCR products.

9. Appendix C: Library Quality Control

Quality control of finished QuantSeq libraries is highly recommended and can be carried out with various methods depending on available equipment. A thorough quality control procedure should include the analysis of both the concentration and the size distribution of libraries.

Quality Control Methods

The analysis of a small volume of the amplified library with microcapillary electrophoresis has become the de facto standard for many NGS laboratories and generates information regarding library concentration and size distribution. Several electrophoresis platforms are available from various manufacturers. For low- to medium-throughput applications, we recommend the Agilent Bioanalyzer 2100 and High Sensitivity DNA chips (Agilent Technologies, Inc.). For high throughput applications instruments such as the Fragment Analyzer (Advanced Analytical Technologies, Inc.), LabChip GX II (Perkin Elmer), or 2200 TapeStation (Agilent Technologies, Inc.) are recommended. Typically, 1 µl of a QuantSeq library produced according to the directions in this manual is sufficient for analysis. Depending on the minimum sample loading requirements for each instrument, 1 µl of the finished library may be diluted to the required volume (e.g., 2 µl sample for TapeStation and 10 µl for LabChip GX II).

More accurate library quantification can be achieved with custom or commercially available qPCR assays. With these assays, the relative or absolute abundance of amplifiable fragments contained in a finished QuantSeq library is calculated by comparing Cq values to a set of known standards. While delivering a more accurate quantification, these assays do not supply the user with information regarding library size distribution. Unwanted side products such as linker-linker artifacts are not discernible from the actual library in the qPCR assay as both will be amplified. Hence, it is highly recommended to combine such an assay for quantification with microcapillary electrophoresis analysis for library size distribution.

If microcapillary electrophoresis platforms and qPCR machines are not available, very basic quality control can also be performed by separating a small aliquot of the library on a polyacrylamide or agarose gel. Library quantification can also be performed with an inexpensive benchtop fluorometer using one of several commercially available assays. Most UV-Vis spectrophotometers are not sensitive enough at these concentrations to accurately quantify NGS libraries and should be avoided.

Typical Results

QuantSeq libraries are intended for a high degree of multiplexing, and hence libraries do not need to be extensively amplified. 11 cycles of PCR amplification are usually enough when using 500 ng total RNA input. This will prevent overcycling and distorting expression values while at the same time providing enough material for quantification and subsequent colony formation.

Typical concentrations are between 10 - 13 nM (1.6 - 2.2 ng/µl) for 50 ng (14 cycles) and 500 ng (11 cycles) input RNA, respectively, with most inserts being between 80 - 190 nts in length (see also Appendix B, p.23).

A shorter side-product caused by priming of the second strand synthesis oligo on the oligodT primer is sometimes visible at ~100 bp, and should not compose more than 0 - 3 % of the total library. Higher proportions of this side-product can indicate problems during library preparation. Low input RNA for instance will result in an increase of this side product.

A second peak in high molecular weight regions (between 1,000 - 9,000 bp) is an indication of overcycling. This could occur if cycle numbers are increased by too much to compensate for lower input material.

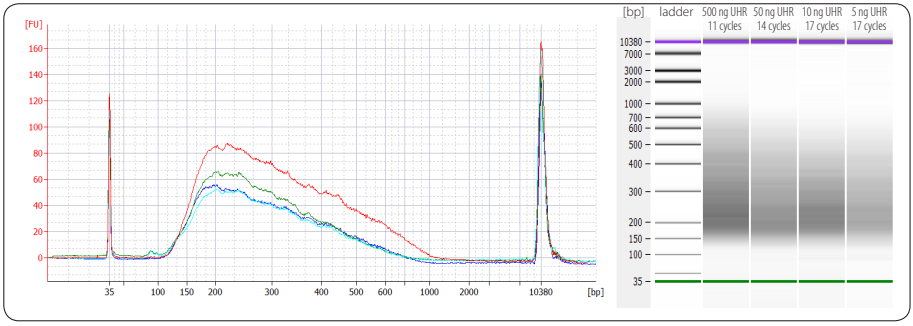

Figure 3. Bioanalyzer traces of QuantSeq libraries synthesized from 500 ng total RNA input (red trace) amplified with 11 cycles, from 50 ng total RNA input (dark blue trace) amplified with 14 cycles, from 10 ng total RNA input (green trace) amplified with 17 cycles, and from 5 ng total RNA input (light blue trace) also amplified with 17 cycles. Input RNA was Universal Human Reference RNA (UHR).

10. Appendix D: Multiplexing

QuantSeq libraries are designed for a high degree of multiplexing. Standard in-line barcodes are introduced during the second strand synthesis step (to be added in step 8).

In-Line Barcodes

QuantSeq Ion Torrent is offered in two sets of 24 preps: Cat. No. 012.24A contains Barcode Set A with in-line barcodes 01 - 24 (**BC01-24**). Cat. No. 012.24B contains Barcode Set B with in-line barcodes 25 - 48 (**BC25-48**). Barcodes are included in the kit in the Barcode Plate and with each QuantSeq kit up to 24 samples can be sequenced in one sequencing run. Please note for Cat. No. 012.24A barcodes are located in column 1 - 3 and for Cat. No. 012.24B barcodes are located in column 4 - 6 (for illustration see Figure 2, p.6).
By combining both sets (Cat. No. 012.24A and Cat. No. 012.24B) up to 48 samples can be sequenced in one sequencing run.
Barcodes are 9 - 11 nt long (+ 4 nt CGAT) and are located within the first 17 - 19 nt nucleotides of the read (TCAG - barcode - CGAT).

	1	2	3	4	5	6
A	BC01: CTAAGGTAA	BC09: TGAGCGGAA	BC17: TCTATTCGT	BC25: CCTGAGATA	BC33: TTCTCATTGAA	BC41: TTCCACTTCG
B	BC02: TAAGGAGAA	BC10: CTGACCGAA	BC18: AGGCAATTG	BC26: TTACAACCT	BC34: TCGCATCGTT	BC42: AGCACGAAT
C	BC03: AAGAGGATT	BC11: TCCTCGAAT	BC19: TTAGTCGGA	BC27: AACCATCCG	BC35: TAAGCCATTGT	BC43: CTTGACACCG
D	BC04: TACCAAGAT	BC12: TAGGTGGTT	BC20: CAGATCCAT	BC28: ATCCGGAAT	BC36: AAGGAATCGT	BC44: TTGGAGGCCAG
E	BC05: CAGAAGGAA	BC13: TCTAACGGA	BC21: TCGCAATTA	BC29: TCGACCACT	BC37: CTTGAGAATGT	BC45: TGGAGCTTCCT
F	BC06: CTGCAAGTT	BC14: TTGGAGTGT	BC22: TTCGAGACG	BC30: CGAGGTTAT	BC38: TGGAGGACGGA	BC46: TCAGTCCGAA
G	BC07: TTCGTGATT	BC15: TCTAGAGGT	BC23: TGCCACGAA	BC31: TCCAAGCTG	BC39: TAACAATCGG	BC47: TAAGGCAACCA
H	BC08: TTCCGATAA	BC16: TCTGGATGA	BC24: AACCTCATT	BC32: TCTTACACA	BC40: CTGACATAAT	BC48: TTCTAAGAGA

Barcode sequences are available for download at www.lexogen.com. **REMARK:** IonXpress barcodes can be selected from the machine. The only difference is that Lexogen considers the C a part of the constant region whereas Life Technologies considers it a part of the barcode. **EXAMPLE:** BC01: CTAAGGTAA + **C**GAT vs IonXpress01: CTAAGGTAA**C** + GAT

QuantSeq is specifically designed for multiplexing 24 or 48 samples per sequencing run (depending on the intended read depth).

Barcoded libraries should be mixed in an equimolar ratio. Ion Torrent sequencers do not have any prerequisites regarding barcode mixing. Hence, any barcode combination can be used if fewer than 24 samples are to be multiplexed.

11. Appendix E: Sequencing*

General

The amount of library required for the template preparation depends on which system is used for sequencing, the Ion PGM, Ion Proton, or Ion S5 systems.
We recommend to adhere to the template concentrations for loading, as recommended by Life Technologies.

Read lengths longer than SR100 can be chosen (e.g., SR200, SR300) if the exact 3'end is to be pinpointed for most transcripts. Be aware that adapter reads will increase with longer read length. In this case, trimming the adapter sequences before mapping is essential.

Libraries with In-line Barcodes

In-line barcodes are 9 - 11 nt long followed by an additional 4 nt constant sequence (CGAT) and compose the first 17 - 19 nucleotides of the read (TCAG - barcode - CGAT). These barcodes are introduced during second strand synthesis (step 8). 15 µl of the desired barcode (Cat. No. 012.24A: barcodes **BC01-24** and Cat. No. 012.24B: barcodes **BC25-48**, provided in 96-well plates) are added. For Barcode sequences please refer to Appendix D, p.27. No separate read-out of the index is required.

```
5'CCATCTCATCCCTGCGTGTCTCCGACTCAG- barcode- CGAT- Insert…
3'GGTAGAGTAGGGACGCACAGAGGCTGAGTC- barcode- GCTA- Insert…

   …Insert -ATCACCGACTGCCCATAGAGAGGAAAGCGGAGGCGTAGTGG 3'
   …Insert -TAGTGGCTGACGGGTATCTCTCCTTTCGCCTCCGCATCACC 5'
```

*Note: Some nucleotide sequences shown in Appendix E may be copyrighted by Life Technologies.

12. Appendix F: Data Analysis

This section describes a basic bioinformatics workflow for the analysis of QuantSeq data and is kept as general as possible for integration with your standard pipeline. More information about the principal data analysis can be found under www.lexogen.com (https://www.lexogen.com/quantseq-data-analysis/)
QuantSeq contains the P1 adapter in the 5′ part of the oligodT primer and the A adapter sequence in the 5′ part of the second strand synthesis primer, hence NGS reads are generated towards the polyA tail. The reads directly reflect the mRNA sequence.

De-Multiplexing

Barcodes are automatically demultiplexed by the machine.

The barcode is contained within the first 17 - 19 nucleotides of the read (4 nt lead sequence TCAG, 9 - 11 nt barcode plus 4 nt constant region CGAT), and the entire 17 - 19 nt are usually removed automatically by the machine during demultiplexing.

Quality Assessment

We recommend the use of a general fastq quality control tool such as FastQC or NGS QC Toolkit to examine the quality of the sequencing run. These tools can also identify over-represented sequences, which may optionally be removed from the dataset.

Processing Raw Reads - Trimming

As second strand synthesis is based on random priming, there may be a higher proportion of errors at the first nucleotides of the insert due to non-specific hybridization of the random primer to the cDNA template. These mismatches can lead to a lower percentage of mappable reads when using a stringent aligner (e.g., TopHat 2) in which case it may be beneficial to trim these nucleotides (nt 1 - 12). Alternatively a less stringent aligner (e.g., STAR Aligner) could be used with an increased number of allowed mismatches. While trimming the first nucleotides can decrease the number of reads of suitable length, the absolute number of mapping reads may increase due to the improved read quality. Reads which are too short or have generally low quality scores should be removed from the set.

For trimming we recommend using the trimming functions of the bbmap suite http://sourceforge.net/projects/bbmap/.

Alignment

At this point the filtered and trimmed reads can be aligned with a short read aligner to the reference genome.

STAR aligner or bbmap can be used for mapping. As QuantSeq is a 3′ Seq protocol, most sequences will originate from the last exon and the 3′ untranslated region (UTR).

Alternatively, the TMAP mapping program can be used, as this program is optimized for aligning reads of variable length. It includes three algorithms that may be run together (mapall) or individually (map1, map2, and map3). For RNA-Seq seed lengths of 18 nucleotides and employing the default number of allowable mismatches per seed are commonly used.

Annotation

Mapping only the 3′ end of transcripts requires an annotation which covers the 3′ untranslated region (UTR) accurately. Conservative annotations might decrease the power of correct gene quantification after mapping. For some gene annotations it might be an advantage to extend the 3′ UTR further downstream in order to assign the mapped read correctly to the 3′ UTR of the gene.

13. Appendix G: Revision History

Revision	Change	Page
012UG036V0130 Jun. 13, 2018	Added attention: Leave the reactions at 42 °C until step 4 (step 2).	11
	Added remark: Pre-warm mastermixes for first strand synthesis (step 3).	12
	Safe stopping point removed after step 4: Proceed immediately to RNA removal.	12
	Endpoint PCR test protocol reformatted in Appendix B and minor revisions of text in Appendices A, B, F.	24, 23-29
	Reduced Revision History table to show updates from 2016 onwards.	31
012UG036V0123 Jul. 5, 2017	Dry the beads only at room temperature.	13, 14
	Changed SIRVs text - Appendix A.	19
012UG036V0122 Mar. 28, 2017	Safe stopping point also added to finished library.	13, 16
	Fixed typo.	23
012UG036V0121 Aug. 3, 2016	Prevent internal priming by keeping RT at 42 °C (pipette on heating block, don't cool FS2/E1).	11
	Indication of safe stopping points.	11-14, 16
	Reseal Barcode Plate after usage to prevent crosscontamination.	12
	Possibility to select IonXpress barcodes from the machine.	23
012UG036V0120 Mar. 15, 2016	RT temperture raised from 37 °C to 42 °C.	11
	Protocol adjustments for or low input, FFPE, or low quality RNA in step 2, 6, 17, and 29.	11-13, 15-17
	Hyperlinks	14, 17, 20, 25
	Spike-in RNA Variant Control Mixes, Cat. No. 025.03.	19
012UG036V0100 Sep. 4, 2014	Initial Release.	

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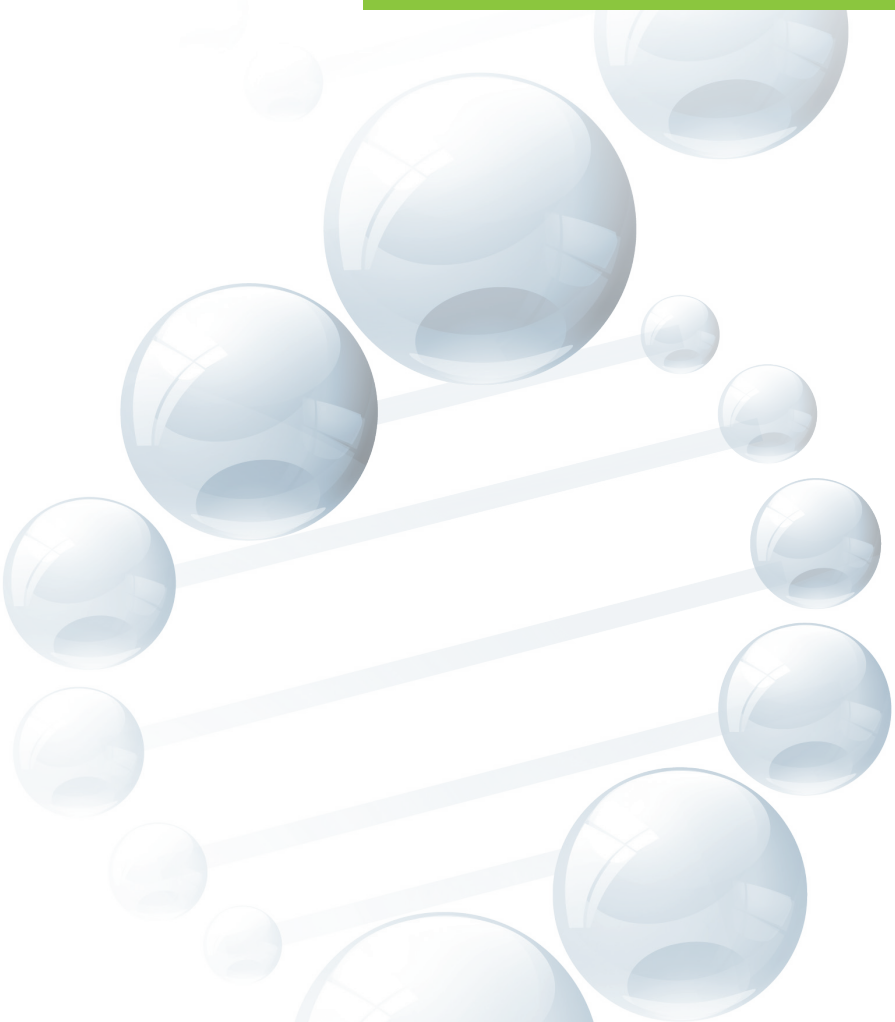
Enabling complete transcriptome sequencing



QuantSeq 3′ mRNA-Seq Library Prep Kit · User Guide

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