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3' mRNA-Seq Library Prep Trial Kit (REV)

Instruction Manual

Catalog Numbers:
016 (QuantSeq 3' mRNA-Seq Library Prep Kit for Illumina (REV) with Custom Sequencing Primer)
020 (PCR Add-on Kit for Illumina)
022 (Purification Module with Magnetic Beads)
047 (i5 Dual Indexing Add-on Kit for QuantSeq/SENSE for Illumina)
070 (RS-Globin Block, *Homo sapiens*)
071 (RS-Globin Block, *Sus scrofa*)

016IM051V0130

1. Overview

This instruction manual outlines the protocol for the QuantSeq Reverse (REV) Trial Kit (016.04). For more detailed information please refer to the complete User Guide (015UG009), which is available for download at our website (www.lexogen.com).

Lexogen's QuantSeq kit provides a library preparation protocol designed to yield sequences close to the 3' end of polyadenylated transcripts. As this is a 3' mRNA-Seq protocol the quality of the RNA input is not as critical as for other RNA-Seq applications and even lower quality RNA (including FFPE samples) can yield good RNA-Seq results. With QuantSeq REV it is possible to exactly pinpoint the 3' end of transcripts during Read 1. Libraries are compatible with both single-read and paired-end sequencing. However, for Read 1 a Custom Sequencing Primer Version 2 (**CSP ●**, included in the kit) is required for sequencing (see Appendix E, p.14).

ATTENTION: QuantSeq REV (016) libraries should not be multiplexed with other Illumina libraries and the **CSP ●**, should never be mixed with the standard Illumina Read 1 Sequencing Primer (see also Appendix D, p.13).

The amount of total RNA required for QuantSeq depends on the poly(A) RNA content of the sample in question. This protocol was tested extensively with various cell cultures, animal and plant tissues, fungi, and human reference RNA (Universal Human Reference RNA (UHRR) and Brain Reference RNA). Typically we recommend using 500 ng total RNA input as starting material, but with minor protocol adjustments the input amount can even be reduced to 10 ng for QuantSeq REV. The maximum input amount is 2 µg of total RNA.
A list of cycle numbers and respective yields for different input RNAs can be found in Appendix A (p.11) or, in our online Frequently Asked Questions (FAQ) at www.lexogen.com.

This trial kit contains enough reagents to perform a qPCR and an endpoint PCR for each sample. We highly recommend performing the qPCR to determine the exact cycle number required for your RNA input.

2. Kit Components and Storage Conditions

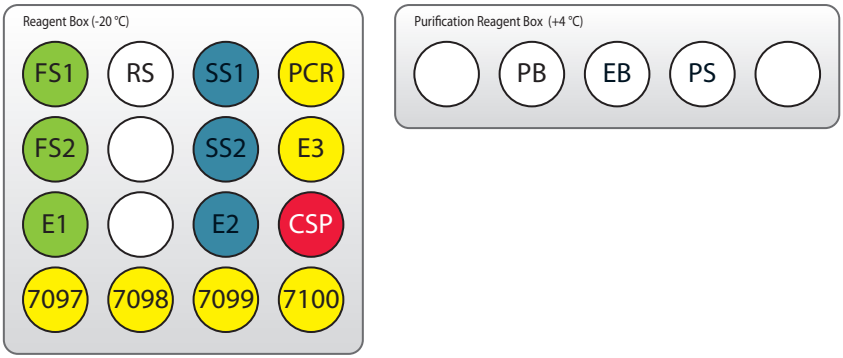

Figure 1. Location of kit contents.

Kit Component	Tube Label	Volume*	Storage
First Strand cDNA Synthesis Mix 1	FS1	22 µl	-20 °C
First Strand cDNA Synthesis Mix 2	FS2	41.8 µl	-20 °C
Enzyme Mix 1	E1	2.2 µl	-20 °C
RNA Removal Solution	RS	22 µl	-20 °C
Second Strand Synthesis Mix 1	SS1	66 µl	-20 °C
Second Strand Synthesis Mix 2	SS2	17.6 µl	-20 °C
Enzyme Mix 2	E2	4.4 µl	-20 °C
PCR Mix	PCR	61.6 µl	-20 °C
Enzyme Mix 3	E3	8.8 µl	-20 °C
i7 Index Primer	7097-7100	11 µl each	-20 °C
Custom Sequencing Primer Version 2 (100 µM)	CSP	6.6 µl	-20 °C
Purification Beads	PB	220 µl	+4 °C
Purification Solution	PS	352 µl	+4 °C
Elution Buffer	EB	11616 µl	+4 °C

*including 10 % surplus

ATTENTION: The Purification Module is shipped at -20 °C, however **PB, PS should be stored at +4 °C**. The rest of the kit (Reagent Box) should be stored in a -20 °C freezer. **EB** can be stored either at +4 °C or -20 °C. Before use, check the contents of **PS** 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. 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	**RS** ○ – thawed at RT	**SS1** ● – thawed at 37 °C **SS2** ● – 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, then 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 Illumina-compatible sequence at its 5'end is hybridized to the RNA and reverse transcription is performed.

ATTENTION: The minimum recommended input amount for QuantSeq REV Library Prep is 10 ng.

NOTE: Protocol modifications are recommended for low quality, degraded, and FFPE RNA samples. These are indicated as **"REMARK"** in the respective protocol steps.

1. Mix 10 ng - 2 µg 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.
2. Denature the RNA / **FS1** mix for 3 minutes at 85 °C in a thermocycler and then cool down to 42 °C. **REMARK:** 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:** For 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.

5 Add 5 µl RNA Removal Solution (**RS** ○) 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.

Second Strand Synthesis

During this step the library is converted to dsDNA. Second strand synthesis is initiated by a random primer containing an Illumina-compatible linker sequence at its 5' end. A reverse complement prevents the linker sequence from taking part in the hybridization.

ATTENTION: Second Strand Synthesis Mix 1 (**SS1** ●) is a viscous solution and needs to be mixed thoroughly before use. Thaw for 5 minutes at 37 °C. If a precipitate is visible, incubate for a further 5 minutes at 37 °C and mix well until buffer components dissolve completely. Thereafter, keep **SS1** ● at room temperature until use. Pipette slowly and carefully to ensure the correct volume is dispensed.

NOTE: At this point we recommend placing the purification components (**PB**, **PS**, **EB**) for step 12 at room temperature to give them enough time to equilibrate.

7 Add 10 µl Second Strand Synthesis Mix 1 (**SS1** ●) to the reaction. Mix well by pipetting, and seal the plate. **REMARK:** Use a pipette set to 30 µl for efficient mixing.

8	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.
9	Prepare a mastermix containing 4 µl Second Strand Synthesis Mix 2 (**SS2** ●) and 1 µl Enzyme Mix 2 (**E2** ●). Mix well.
10	Add 5 µl of the **SS2** / **E2** mastermix per reaction. Mix well. **REMARK:** Use a pipette set to 30 µl for efficient mixing.
11	Incubate the reaction at 25 °C for 15 minutes. ☞ 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 components (**PB**, **PS**, **EB**) 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.

12	Add 16 µl of properly resuspended Purification Beads (**PB**) to each reaction, mix well, and incubate for 5 minutes at room temperature.
13	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).
14	Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.
15	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.
16	Add 56 µl of Purification Solution (**PS**) to the beads / **EB** mix to reprecipitate the library. Mix thoroughly, and incubate for 5 minutes at room temperature. **REMARK:** For low quality, degraded, and FFPE RNA samples add only 48 µl **PS**.
17	Place the plate onto a magnetic plate, and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
18	Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.
19	Add 120 µl of 80 % EtOH, and incubate 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.

20	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.
21	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), as this will negatively influence the elution and the resulting library yield.
22	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.
23	Place the plate onto a magnetic plate and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
24	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.

4. Library Amplification

Preparation

PCR	Purification
PCR ● – thawed at RT **E3** ● – keep on ice or at -20 °C **7097-7100** ● – thawed at RT; **spin down before opening!** SYBR Green I (Sigma-Aldrich, Cat. No. S9430), 10,000 x in DMSO for qPCR - provided by user	**PB** – 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 } 30-35x for qPCR 72 °C, 30 sec } Endpoint as determined by qPCR 72 °C, 1 min 10 °C, ∞	Equilibrate all reagents to room temperature for 30 minutes prior to use.

qPCR

A qPCR assay is recommended to determine the exact number of PCR cycles for the endpoint PCR to avoid any under- or overcycling of your samples.

NOTE: If you are confident about the number of cycles for your endpoint PCR (e.g., because your RNA is listed in Appendix A, p.20), you can skip the qPCR.

NOTE: If you do not have a qPCR available, skip step 25 - 29 and proceed directly with step 30, initially conduct 13 cycles of PCR (for 500 ng RNA input) with half of your sample, and check the yield. If the libraries show a second peak in high molecular regions your sample is overcycled and you should use less PCR cycles for the remaining half of your sample. If the yield is too low, add 1 - 5 PCR cycles (yield doubles with each cycle).

25	Dilute the double-stranded library from step 24 to 19 µl by adding 2 µl Elution Buffer (**EB**) or molecular biology-grade water (H_2O).
26	Prepare a 1:4,000 dilution of SYBR Green I dye in DMSO, for a 2.5x working stock concentration. **ATTENTION:** The final concentration in the reaction should be 0.1x. Higher concentrations of SYBR Green I will inhibit the amplification.
27	For each reaction combine: 1.7 µl of the diluted cDNA library, 7 µl of PCR Mix (**PCR** ●), 5 µl of the respective i7 Index Primer (**7097-7100** ●), 1 µl of Enzyme Mix (**E** ●), and 1.2 µl of 2.5x SYBR Green I nucleic acid dye. Make the total reaction volume up to 30 µl by adding 14.1 µl of Elution Buffer (**EB**) or molecular biology-grade water (**H_2O**). **ATTENTION:** Include a no template control!
28	Perform 40 - 50 cycles of PCR with the following program: Initial denaturation at 98 °C for 30 seconds, 45 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 **REMARK:** There is no need to purify or analyze the overcycled PCR reaction on a Bioanalyzer.
29	Using the amplification curves in linear scale, determine the value at which the fluorescence reaches the plateau. Calculate 50 % of this maximum fluorescence value and determine at which cycle this value is reached. As the endpoint PCR will contain 10x more cDNA compared to the qPCR, subtract three from this cycle number. This is then the final cycle number you should use for the endpoint PCR with the remaining 17 µl of the template.

Example for Endpoint Calculation

500 ng total RNA input was used for generating libraries. Using 1.7 µl of cDNA for a qPCR, the cycle number corresponding to 50 % of the maximum fluorescence was 15 cycles. The remaining 17 µl of the template should therefore be amplified with 12 cycles (15-3 cycles =12 cycles).

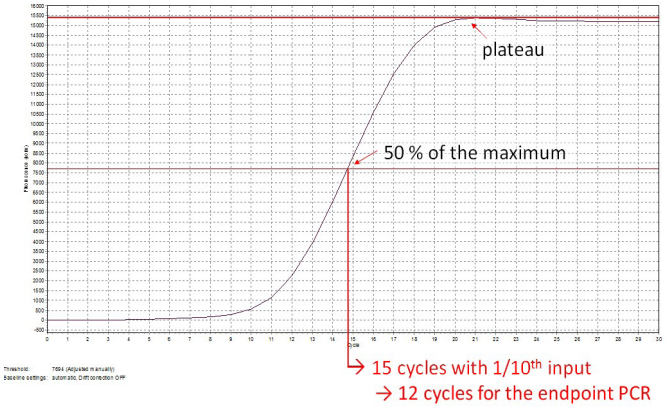

Figure 3. Calculation of the number of cycles for the endpoint PCR.

NOTE: Once the number of cycles for the endpoint PCR is established for one type of sample (same input amount, tissue, and RNA quality), there is no need for further qPCRs. The entire cDNA can be used for the endpoint PCRs.

Endpoint PCR

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

NOTE: At this point we recommend placing the purification solutions (**PB**, **PS**, **EB**) for step 34 at room temperature to give them enough time to equilibrate.

30	Prepare a mastermix containing 7 µl of PCR Mix (**PCR** ●) and 1 µl Enzyme Mix 3 (**E3** ●) per reaction.
31	Add 8 µl of this **PCR / E3** mastermix to 17 µl of the eluted library.
32	Add 5 µl of the respective i7 index primer (**7097-7100** ●, in microtubes). Ensure the total volume of the PCR is 30 µl. If necessary, adjust the total volume using Elution Buffer (**EB**) or molecular biology-grade water (**H_2O**). Mix well by pipetting. Seal the plate and quickly spin down to make sure all liquid is collected at the bottom of the well.
33	Use the cycle number that was determined in step 29: Initial denaturation at 98 °C for 30 seconds, 12 cycles (or whatever cycle number was determined in step 29) 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 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.

34	Add 30 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 samples add only 27 µl **PB**.
35	Place the plate onto a magnetic plate, and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
36	Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.

37	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.
38	Add 30 µl of Purification Solution (**PS**) to the beads / **EB** mix to reprecipitate the library. Mix thoroughly, and incubate for 5 minutes at room temperature.
39	Place the plate onto a magnetic plate, and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
40	Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.
41	Add 120 µl of 80 % EtOH, and incubate 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.
42	Repeat this washing step once for a total of two washes. Make sure to remove the supernatant completely.
43	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), as this will negatively influence the elution and the resulting library yield.
44	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.
45	Place the plate onto a magnetic plate and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
46	Transfer 15 - 17 µl of the supernatant into a fresh PCR plate. Make sure not to transfer any beads.
47	At this point, the libraries are finished and ready for quality control, pooling, and cluster generation (see QuantSeq 3′ mRNA-Seq User Guide for Illumina). ☞ Safe stopping point. Libraries can be stored at -20 °C at this point.

5. Appendix A: Input RNA

QuantSeq has been tested with different input materials, e.g., different tissues, cells, plants, and yeast. Depending on the mRNA content of your sample, the number of cycles for your endpoint PCR will vary. If using lower RNA input amounts further cycles need to be added. The table below depicts some examples of higher and lower input RNA amounts using Universal Human Reference (UHR) RNA.

Input RNA (UHRR)	Step 6: RNA Removal 95°C	Step 16: PS Addition	Library*			Insert				Library Yield		PCR Cycles
			Start [bp]	End [bp]	Mean Size*	Mean Size	≥ 50 nt	≥ 100 nt	≥ 200 nt	ng/µl	nM	
2,000 ng	10 min	56 µl	132	2000	456	324	97 %	80 %	31 %	2.0	10.2	11
500 ng	10 min	56 µl	132	2000	364	232	98 %	78 %	27 %	1.8	9.8	12
100 ng	10 min	56 µl	132	2000	350	218	97 %	74 %	21 %	2.1	11.3	14
50 ng	10 min	56 µl	132	2000	389	257	96 %	70 %	20 %	2.4	12.7	15
10 ng	10 min	48 µl	132	2000	350	218	96 %	70 %	24 %	2.6	14.1	18

*All libraries were prepared with dual indexing. Linker sequences are 132 bp including 6 nt long i5 and i7 indices, respectively. For single indexing (i7 only) adapter sequences are 122 bp.

In the following table some reference values (cycles numbers and approximate yields) for 500 ng high quality input RNA from other RNA sources are depicted:

500 ng Input RNA from	Library Yield		PCR Cycles	500 ng Input RNA from	Library Yield		PCR Cycles
	ng/µl	nM			ng/µl	nM	
UHRR	1.8	9.8	12	Mm myoblast	0.9	5.2	12
HBRR	2.0	13.8	13	Mm fibroblast	1.0	5.6	14
Mm heart	1.9	10.5	13	Mm myoblast progenitors	2.1	11.5	11
Mm brain	2.9	15.6	13	Mm neural progenitors	1.2	7.0	12
Mm liver	1.3	6.7	12	Arabidopsis thaliana	1.7	9.8	13
Mm kidney	2.3	12.2	12	Tomato seeds	1.7	9.4	16
Mm spleen	1.4	8.0	13	Fungi RNA	1.24	7.1	13
Mm lung	2.6	15.5	14	Yeast RNA (Sc)	1.2	7.7	12
Mm embryonic stem cells	1.3	7.5	11	Drosophila melanogaster	1.6	7.9	13

Mm: Mus musculus; Sc: Saccharomyces cerevisiae

REMARK: Tables should be used as a guideline only! Use the qPCR assay to determine the optimal PCR cycle number for your sample type.

6. Appendix B: Low Quality RNA - FFPE

QuantSeq was tested with 500 pg - 50 ng FFPE or degraded RNA input (see table below). The table comprises some results obtained from using different amounts of mouse brain FFPE RNA input with a RIN of 1.8 (DV_{200} of 51 %). The DV_{200} value is the percentage of RNA fragments larger than 200 nucleotides. The lower the DV_{200}, the more degraded the RNA is. Other FFPE RNAs or RNAs with lower DV_{200} values may require more PCR cycles, hence we strongly recommend taking advantage of the qPCR assay.

ATTENTION: FFPE RNA is degraded RNA and hence the insert sizes are smaller than for non-degraded RNA samples. Keep this in mind when choosing your sequencing length.

Input RNA (UHRR)	Step 6: RNA Removal 95°C	Step 16: PS Addition	Library*			Insert				Library Yield		PCR Cycles
			Start [bp]	End [bp]	Mean Size*	Mean Size	≥ 50 nt	≥ 100 nt	≥ 200 nt	ng/µl	nM	
50 ng	10 min	48 µl	132	600	264	132	96 %	52 %	5 %	2.3	14.2	15
10 ng	10 min	48 µl	132	600	265	133	95 %	56 %	5 %	2.1	13.1	18

*All libraries were prepared with dual indexing. Linker sequences are 132 bp including 6 nt long i5 and i7 indices, respectively. For single indexing (i7 only) adapter sequences are 122 bp.

If you see a lot of linker-linker products in your sample, an additional purification of the lane mix with 0.9x **PB** (e.g., 50 µl lane mix plus 45 µl **PB**), incubating 5 minutes at room temperature, and following the protocol from step 35 on again may be necessary.

Please also refer to the QuantSeq Frequently Asked Questions (FAQs) page at www.lexogen.com for continuous updates on other RNA sources.

7. Appendix C: Multiplexing

i7 indices are 6 nt long and require an additional index-specific sequencing reaction.
This trial kit contains four i7 indices (**7097-7100** ●) which are perfectly color balanced to be run on a lane of an Illumina sequencer.

7097: CGTGAT
7098: GAACTA
7099: ACGTCG
7100: TTCAGC

ATTENTION: QuantSeq REV (016) should not be multiplexed with other Illumina libraries as a Custom Sequencing Primer **CSP** ● Version 2) is required for Read 1 (see Appendix D, p.13).

8. Appendix D: Sequencing

The amount of library loaded onto the flow cell will greatly influence the number of clusters generated. Each sequencing facility has slightly different preferences of how much to load. Machine-specific loading instructions can be found at www.lexogen.com under QuantSeq Frequently Asked Questions (FAQs 1.26 (REV)).

A schematic representation of QuantSeq FWD libraries is shown below. The required sequencing primers are listed as well.

ATTENTION: QuantSeq REV requires a Custom Sequencing Primer (**CSP** ● Version 2) for Read 1. For instructions on CSP usage for different Illumina Platforms please refer to Appendix E, p.14). QuantSeq REV libraries are not supported on NextSeq™ and MiniSeq™ Illumina sequencers!

QuantSeq REV Libraries with i7 Indexing

i7 indices (6 nt) are introduced during PCR (step 32).
For QuantSeq REV libraries, Read 1 corresponds to the cDNA sequence.

```
                                          5'-(Read 1 Custom Sequencing Primer)-3'
5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-TTTTTTTTTTTTTTTTT-Insert…
3'TTACTATGCCGCTGGTGGCTCTAGATGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-AAAAAAAAAAAAAAAAA-Insert…

            5'-(Index Read Sequencing Primer)-3'
   …Insert- AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-i7-ATCTCGTATGCCGTCTTCTGCTTG 3'
   …Insert- TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-i7-TAGAGCATACGGCAGAAGACGAAC 5'
             3'-(Read 2 Sequencing Primer)-5'
```

Read 1: Custom Sequencing Primer (included):
5' CCCTACACGACGCTCTTCCGATCTTTTTTTTTTTTTTTTTT 3'

Index 1 Read (i7): Multiplexing Index 1 Sequencing Primer (not supplied):
5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC 3'

Read 2: Multiplexing Read 2 Sequencing Primer (not supplied):
5' GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

ATTENTION: Do not use Multiplex Read 1 Sequencing Primer for QuantSeq REV (Cat. No. 016). Multiplex Read 1 Sequencing primer would result in a failed sequencing run as cluster calling would be impossible due to the poly(T) stretch.

ATTENTION: Do not mix **CSP** ● Version 2 and Read 1 Sequencing Primer! A primer mixture would result in low clusters calls and the resulting reads would be contaminated by poly(T) stretches.

*Note: Some nucleotide sequences shown in Appendix D may be copyrighted by Illumina, Inc.

9. Appendix E: Usage of the Customized Sequencing Primer CSP ●

For QuantSeq REV (Cat. No. 016) the Read 1 linker sequence is located at the 5′ end of the oligodT primer. Here a Custom Sequencing Primer (**CSP ●** Version 2, included in the kit) is required for Read 1. The Custom Sequencing Primer covers the poly(T) stretch. Without the Custom Sequencing Primer cluster calling is not possible.

ATTENTION: Do not mix CSP ● and Read 1 Sequencing Primer! Do not mix **CSP ●** into HP10! A primer mixture would result in low clusters calls and the resulting reads would be contaminated by poly(T) stretches.

HiSeq® 2000, HiSeq® 2500 (CSP ● Version 2 added on cBot)

CSP ● Version 2 should be provided in a tube strip at 0.5 µM final concentration in a volume of 120 µl (final concentration 0.5 µM, to be diluted in HT1 = Hybridization buffer). Take 0.6 µl of 100 µM **CSP ●** Version 2 and add 119.4 µl of HT1 buffer per sequencing lane. Place the 8-tube strip into the cBot position labeled primers.

HiSeq® 2500 (CSP ● Version 2 replaces HP10 in cBot Cluster Generation Reagent Plate)

Alternatively, **CSP ●** Version 2 can be placed directly into the cBot Cluster Generation Reagent Plate. **ATTENTION:** The standard Illumina Multiplex Read 1 Sequencing Primer solution HP10 (for V4 chemistry located in row 2) provided in the cBot Cluster Generation Reagent Plate has to be **REMOVED** first! The Illumina V4 chemistry cBot Cluster Generation Reagent Plate only has 8 rows filled. A simple trick is to have the empty rows facing towards you, this way if you want to use a CSP in lane 1, you have to remove the HP10 solution from well 1 (first one on the far left) of the second row, rinse the well a couple of times with HT1 and then add the diluted **CSP ●** Version 2. For this take 1.25 µl of 100 µM **CSP ●** Version 2 and add 248.75 µl of HT1 buffer per sequencing lane. The **CSP ●** should be at 0.5 µM final concentration in a volume of 250 µl (final concentration 0.5 µM, to be diluted in HT1 = Hybridization buffer). **ATTENTION:** Do not add the CSP to the Standard Illumina Multiplex Read 1 Sequencing Primer = HP10 solution! Always use fresh HT1 and add the **CSP ●** / HT1 dilution to the empty and rinsed well.

HiSeq® 2500 - Rapid Run

Add 12.5 µl of 100 µM **CSP ●** Version 2 to 2487.5 µl HT1 = Hybridization buffer, resulting in a total volume of 2.5 ml and a final **CSP ●** concentration of 0.5 µM. In a rapid run, both lanes will use the same sequencing primer. It is not possible to run the two lanes with different sequencing primers.

MiSeq®

Clustering is performed on the machine, not on the c-Bot. The MiSeq® uses a reservoir of 600 µl with 0.5 µM sequencing primer final concentration, i.e., 3 µl of 100 µM **CSP ●** Version 2 in 597 µl HT1.

HiSeq® 3000, HiSeq® 4000 (CSP ● Version 2 replaces HP10 in cBot Cluster Generation Reagent Plate)

Usage of a custom sequencing primer is currently not supported on HiSeq® 3000 and 4000 machines. A work around as described for the HiSeq® 2500 (**CSP ●** Version 2 **REPLACES** HP10 in the cBot Cluster Generation Reagent Plate) is possible though. **ATTENTION:** Do not add the **CSP ●**Version 2 to the HP10 solution! A primer mixture would result in low clusters calls and the resulting reads would be contaminated by poly(T) stretches. Always use fresh HT1 and add the **CSP ●**Version 2 / HT1 dilution to the empty and rinsed well.

NextSeq™, MiniSeq™, and NovaSeq™

Please contact info@lexogen.com if you wish to run QuantSeq REV on 2-channel instruments, including: NextSeq™ 500/550, MiniSeq™, or NovaSeq™.

ATTENTION: FORWARD THIS INFORMATION along with **CSP ●** Version 2 and the lane mix to **YOUR SEQUENCING FACILITY** before starting an NGS run.

10. Appendix F: Automated Data Analysis

Each QuantSeq Trial Kit comes with an Activation Code to access the automated QuantSeq Data Analysis Pipeline, available on the Bluebee® Genomics Platform. The Activation Code is provided on your order confirmation and on the delivery / packing slip. Each code allows you to perform four pipeline runs, meaning you can analyze four fastq files (i.e., one file for each library prepared with the trial kit).

Instructional videos showing how to register on Bluebee, setup your connector, upload data, and run the analysis pipeline are provided on the Lexogen - Bluebee webpage at www.bluebee.com/lexogen.

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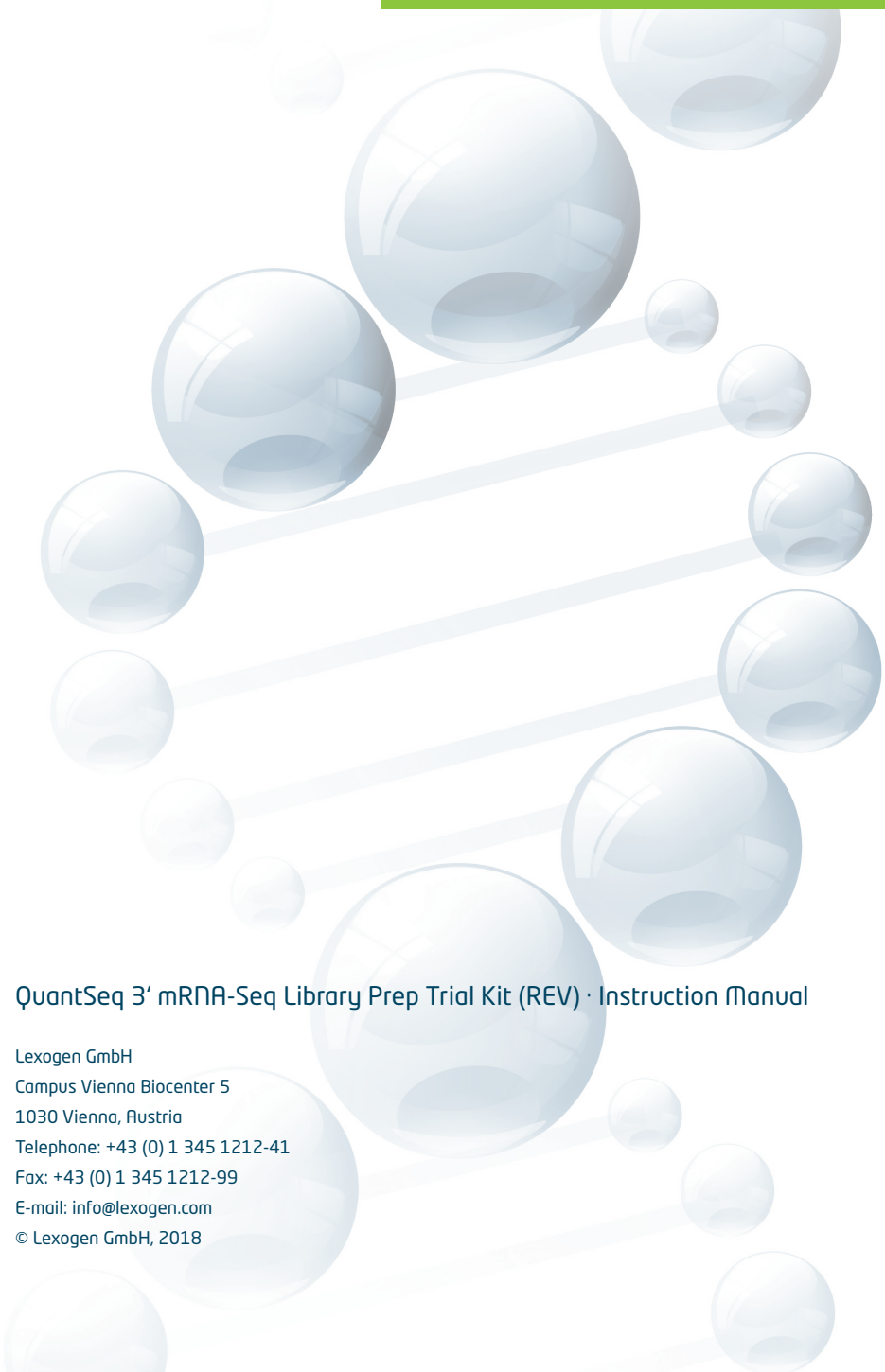
Enabling complete transcriptome sequencing



QuantSeq 3′ mRNA-Seq Library Prep Trial Kit (REV) · Instruction Manual

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