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LEXOGEN

Enabling complete transcriptome sequencing

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PCR Add-on Kit for Illumina Instruction Manual

Catalog Numbers:

020 (PCR Add-on Kit for Illumina)

001 (SENSE mRNA-Seq Library Prep Kit V2 for Illumina)

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

015 (QuantSeq 3' mRNA-Seq Library Prep Kit for Illumina (FWD))

016 (QuantSeq 3' mRNA-Seq Library Prep Kit for Illumina (REV) with Custom Sequencing Primer)

022 (Purification Module with Magnetic Beads)

026 (QuantSeq-Flex First Strand Synthesis Module for Illumina)

028 (QuantSeq-Flex Second Strand Synthesis Module V2 for Illumina)

047 (i5 Dual Indexing Add-on Kit for QuantSeq/SENSE for Illumina)

080 (Reamplification Add-on Kit for Illumina)

020IM064V0122

1. Overview

This instruction manual outlines the protocol for the PCR Add-on Kit for Illumina (Cat. No. 020.96).

The PCR Add-on Kit for Illumina is suitable for all SENSE (Cat. No. 001, 009, and 042) and QuantSeq (Cat. No. 015, 016, 033, 034, and 035) Kits for Illumina.

The PCR Add-on kit contains a PCR Mix (**PCR** ●) including an Illumina P5 specific primer, a thermostable polymerase (**E** ●), and an Illumina P7 Primer without an index (**7000** ●) for 96 PCR reactions. By adding SYBR Green I nucleic acid dye (Sigma-Aldrich, S9430, user-provided) to the PCR reaction, a qPCR assay to determine the optimal number of cycles for the endpoint PCR of your single or dual indexed QuantSeq or SENSE cDNA libraries can be performed.

The qPCR assay is recommended to prevent any under- or overcycling of your library. Over- or undercycling may bias your sequencing results (transcript abundance estimation and library quantification).

ATTENTION: The use of SYBR Green I-containing qPCR mastermixes is not recommended.

The PCR Add-on Kit also contains a Reamplification Primer (**i7-RE** ○) that can be used to reamplify undercycled i7-indexed libraries (single-indexed only) in order to obtain enough material for sequencing.

For reamplification of dual-indexed libraries the Reamplification Add-on Kit for Illumina (Cat. No. 080.96) is available on request. Please contact Lexogen at info@lexogen.com.

2. Kit Components and Storage Conditions

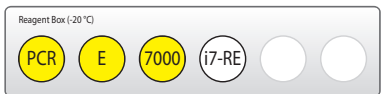


Figure 1. Location of kit components.

Kit Component	Tube Label	Volume*	Storage
PCR Mix	PCR ●	740 µl	-20 °C
Enzyme Mix	E ●	106 µl	-20 °C
P7 Primer	7000 ●	528 µl	-20 °C
i7 Reamplification Primer	i7-RE ○	528 µl	-20 °C

*including 10 % surplus

NOTE: The Enzyme Mix (E ●) is the same as the Enzyme Mix 2 (E2 ● or E2 ○) in the SENSE Kits and the Enzyme Mix 3 (E3 ●) in the QuantSeq Kits. These enzyme mixes can be interchangeably used.

3. User-Supplied Reagents

SYBR Green I (e.g., Sigma-Aldrich S9430) has to be provided by the user.

4. qPCR

4.1 qPCR Assay

The qPCR assay is recommended to determine the exact number of cycles for the endpoint PCR in order to prevent under- or overcycling of the library.

ATTENTION: Do not use the i7 Reamplification Primer (**i7-RE O**) for a qPCR assay on the cDNA-library as the cDNA lacks binding sites for the Reamplification Primer. The Reamplification Primer can only be used on already amplified (i7 indexed) PCR libraries.

NOTE: SYBR Green I has an emission maximum at 520 nm, which for some qPCR machines have to be adjusted manually.

qPCR	
PCR ● – thawed at RT	ATTENTION: Spin down all solutions before opening tubes!
E ● – keep on ice or at -20 °C	
7000 ● – thawed at RT	
SYBR Green I (Sigma-Aldrich, Cat. No. S9430), 10,000 x in DMSO for qPCR - provided by user	
Thermocycler	<div><div>98 °C, 30 sec 98 °C, 10 sec 65 °C, 20 sec 72 °C, 30 sec 72 °C, 1 min 10 °C, ∞</div><div>}</div><div>40 - 50x</div></div>

1 Dilute the double-stranded library to 19 µl by adding 2 µl Elution Buffer (**EB**) or molecular biology-grade water (H₂O) after the second-strand purification has been finished (i.e., step 22 for SENSE Total RNA-Seq, step 33 in SENSE mRNA-Seq, or step 24 in QuantSeq 3' mRNA-Seq and QuantSeq-Flex Targeted RNA-Seq).

2 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.

3 For each reaction combine: 1.7 µl of the diluted cDNA library, 7 µl of PCR Mix (**PCR ●**), 5 µl of P7 Primer (**7000 ●**), 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 (H₂O). **ATTENTION:** Include a no template control!

4 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.

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 than 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 μ l of the template. There is no need to purify or analyze the overcycled PCR product.

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For the remaining 17 μ l cDNA library remaining, continue from step 23 for SENSE Total RNA-Seq, step 35 for SENSE mRNA-Seq, step 25 for QuantSeq 3' mRNA-Seq and QuantSeq-Flex Targeted RNA-Seq for the endpoint PCRs.

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4.2 Example for Endpoint Calculation

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

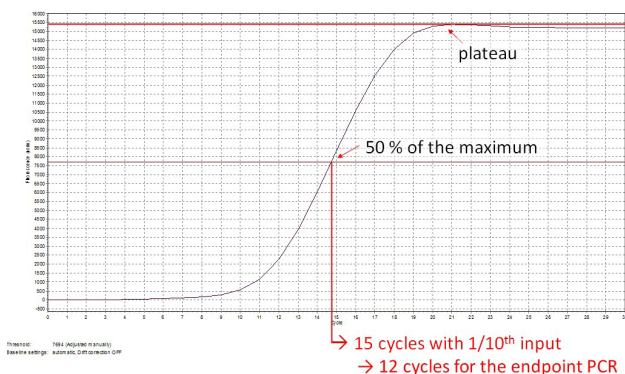


Figure 2. Calculation of the number of cycles for the endpoint PCR.

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 directly for the endpoint PCR.

5. Reamplification of Libraries

5.1 Reamplification of Single-Indexed Libraries (i7 only)

If your single indexed libraries (i7) are undercycled you perform a reamplification PCR using the i7 Reamplification Primer (**i7-RE O**). **ATTENTION:** Do not use the P7 Primer **7000 ●** for the reamplification of i7 indexed libraries! This will lead to a loss of indices and undetermined reads from the NGS run.

qPCR	
PCR ●	– thawed at RT
E ●	– keep on ice or at -20 °C
i7-RE O	– thawed at RT
ATTENTION: Spin down all solutions before opening tubes!	
Thermocycler	<div>98 °C, 30 sec</div> <div><div>98 °C, 10 sec</div><div>65 °C, 20 sec</div><div>72 °C, 30 sec</div></div> <div>72 °C, 1 min</div> <div>10 °C, ∞</div> <div>3 -10x</div>

- For each reaction combine: 7 µl of PCR Mix (**PCR ●**), 5 µl of i7 Reamplification Primer (**i7-RE O**), 1 µl Enzyme Mix (**E ●**) and up to 17 µl of the i7-indexed PCR amplified double-stranded library. Make the total reaction volume up to 30 µl by adding Elution Buffer (**EB**) or molecular biology-grade water (H₂O). **ATTENTION:** Include a no template control!

- Conduct 3 - 10 additional PCR cycles with the following program: Initial denaturation at 98 °C for 30 seconds, 12 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. **ATTENTION:** The yield doubles with each additional cycle, try to not overcycle your libraries. **NOTE:** If your libraries are undercycled use 3 - 6 additional cycles, e.g., if the maximum of your library is at 5 FU (analyzed by 2100 Bioanalyzer) you should add another 6 cycles. If you don't see a library at all, 8 - 10 cycles can be added.

- At this point the libraries are finished and need to be purified according to the respective User Guides. Follow step **27** for SENSE Total RNA-Seq, step **39** for SENSE mRNA-Seq, or step **29** for QuantSeq 3' mRNA-Seq and QuantSeq-Flex Targeted RNA-Seq, using the Purification Module with Magnetic Beads (Cat. No. 022.96).

5.2 Reamplification of Dual-Indexed Libraries (i5 and i7)

For reamplification of dual indexed libraries the Reamplification Add-on Kit for Illumina (Cat. No. 080.96) is available on request. Please contact Lexogen at info@lexogen.com.

6. Appendix: Primer Sequences

P7 Primer (7000)

```
5'-(Read 1 Sequencing Primer)-3'
5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT- (Insert...
3' TTAATAATGCGCTGGTGGCTCTAGATGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA- (Insert...

...Insert)- AGATCGGAAGAGCACACGTCTGAACTCCAGTCACATCTCGTATGCCGCTTCTGCTTG 3'
...Insert)- TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTGTAGAGCATACGGCAGAAGACGAAC 5'
3'-P7 Primer (7000) sequence-5'
```

NOTE: Libraries amplified with P7 Primer **7000** don't have an index and cannot be multiplexed.

i7 Reamplification Primer (i7-RE)

```
5'-(Read 1 Sequencing Primer)-3'
5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT- (Insert...
3' TTAATAATGCGCTGGTGGCTCTAGATGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA- (Insert...

5'-(Index Read Sequencing Primer)-3'
...Insert)- AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-i7-ATCTCGTATGCCGCTTCTGCTTG 3'
...Insert)- TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-i7-TAGAGCATACGGCAGAAGACGAAC 5'
3'-Reamplification Primer-5'
```

NOTE: The Reamplification Primer can only be used on already amplified PCR libraries.

7. Appendix: Revision History

Publication No. / Revision Date	Change	Page
020IM064V0122 Aug. 8, 2018	Reference to Reamplification Add-on Kit for the reamplification of dual-indexed libraries.	2
	RE renamed to i7-RE.	2, 3, 6, 7
	Restructuring of Section 4 and 5.	4-6
020IM064V0121 Feb. 24, 2017	Minor corrections.	4
020IM064V0120 Feb. 7, 2016	BC00 renamed to 7000.	3-5
	qPCR endpoint determination using only 1.7 µl template and set to 50 % FU (previously 33 %). Subtract 3 cycles from determined endpoint (EP) when using 10x as much template (17 µl in EP, 1.7 µl in qPCR). Figure 2.	4-5
020IM064V0113 Oct. 12, 2016	PCR volume in Kit Contents Table corrected.	3
	Updates to schematic representation of libraries in Appendix.	7
020IM064V0112 May 17, 2016	For reamplification no SYBR Green I is needed.	6
020IM064V0111 Apr. 27, 2016	Corrected step numbers for SENSE mRNA-Seq.	4, 6
	Endpoint PCR set at 33 % of the maximum qPCR fluorescence.	4, 6
020IM064V0100 Jun. 19, 2015	Initial Release.	

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