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Enabling complete transcriptome sequencing

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PCR Add-on Kit for Illumina

Instruction Manual

Catalog Numbers:
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)
020 (PCR Add-on Kit for Illumina)
022 (Purification Module with Magnetic Beads)
033 (QuantSeq-Flex Targeted RNA-Seq Library Prep Kit with First Strand Synthesis Module)
034 (QuantSeq-Flex Targeted RNA-Seq Library Prep Kit with Second Strand Synthesis Module)
035 (QuantSeq-Flex Targeted RNA-Seq Library Prep Kit with First and Second Strand Synthesis Modules)

020IM064V0112

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 and 009) and QuantSeq (Cat. No. 015 and 016) 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 a barcode referred to as Barcode 00 (**BC00** ●) for 96 PCR reactions. By adding SYBR Green I to the PCR reaction a qPCR assay to determine the optimal number of cycles for the endpoint PCR of your QuantSeq or SENSE cDNA libraries can be determined.
The qPCR assay is recommended to determine the exact number of cycles for the endpoint PCR in order to prevent any under- or overcycling of your library. Undercycling may result in too little library and overcycling can lead to significant distortions in gene expression values.

The kit furthermore contains a Reamplification Primer (**RE** ❍) that can be used to reamplify already barcoded libraries if they were undercycled to get enough material for sequencing.

ATTENTION: Do not use the **BC00** ● for the reamplification of already barcoded libraries!! This will lead to a loss of barcodes and to a mixed and not assignable sequence pool in an NGS run.

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

2. Kit Components and Storage Conditions

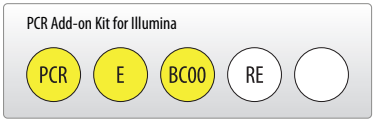

Figure 1. Location of kit components.

Kit Component	Tube Label	Volume*	Storage
PCR Mix	**PCR** ●	1267.2 µl	-20 °C
Enzyme Mix	**E** ●	105.6 µl	-20 °C
Barcode 00	**BC00** ●	528 µl	-20 °C
Reamplification Primer	**RE** ○	528 µl	-20 °C

*including a 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 and can be interchangeably used.

3. User-supplied Reagents

SYBR Green I (e.g., Sigma-Aldrich S9430-.5ML) has to be provided by the user.

4. qPCR

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

qPCR	
PCR **E** **BC00**	– thawed at RT – keep on ice or at -20 °C – thawed at RT; **ATTENTION**: Spin down all solutions before opening tubes!
Thermocycler	98 °C, 30 sec 98 °C, 10 sec 65 °C, 20 sec } 40 x 72 °C, 30 sec 72 °C, 1 min 10 °C, ∞

1. Dilute the double-stranded cDNA library with 17 µl of **H_2O** ● after the second-strand purification has been finished. (Step 21 for SENSE Total RNA-Seq, step 33 in SENSE mRNA-Seq, step 25 in QuantSeq 3' mRNA-Seq, or step 26 in QuantSeq-Flex Targeted RNA-Seq.)

2. Prepare a mastermix containing 7 µl of PCR Mix (**PCR** ●), 5 µl of Barcode 00 (**BC00** ●), and 1 µl Enzyme Mix (**E** ●) per reaction, add SYBR Green I (or an equivalent fluorophore) to the PCR reaction to a final concentration of 0.1x. **EXAMPLE:** For 0.1x SYBR Green I add 1.2 µl 2.5x SYBR Green I solution (1:4,000 SYBR Green I dilution, diluted in DMSO). The total PCR reaction volume will be 31.2 µl.

   Alternatively, if 8 qPCRs are run at the same time, best practice would be to prepare a mastermix with 0.15 µl of a 20x SYBR Green I solution per reaction. SYBR Green I has an emission maximum at 520 nm, which for some qPCR machines has to be adjusted manually.

3. Add 14.2 µl of this **PCR / BC00 / E** / SYBR Green I mastermix to 17 µl of the eluted library.

4. Conduct 40 cycles of PCR with the following program: Initial denaturation at 98 °C for 30 seconds, 40 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.

5. Determine the fluorescence value at which the fluorescence reaches the plateau. Calculate where the fluorescence is 33 % of the maximum, and determine at which cycle these 33 % of fluorescence are reached (see Figure 2, p.5). This is the cycle number you should use for the endpoint PCR using the remaining 17 µl of the template. There is no need to purify or analyze the overcycled PCR reaction.

6. For the remaining half of the template follow step 23 for SENSE Total RNA-Seq, step 35 for SENSE mRNA-Seq, step 26 for QuantSeq 3' mRNA-Seq, or step 27 for QuantSeq-Flex Targeted RNA-Seq for the endpoint PCRs.

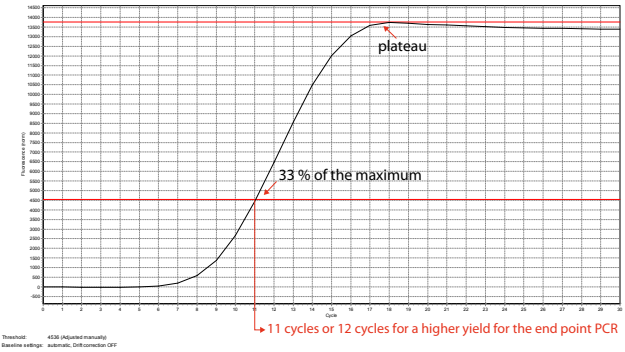

Figure 2. Calculation of the number of cycles for the endpoint PCR.

NOTE: For similar samples that have not been diluted for the qPCR assay the cycle number determined in the overcycled qPCR has to be reduced by 1 cycle. 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 inserted into the endpoint PCRs.

4. Reamplification

If your libraries are undercycled you can add some more cycles using the Reamplification Primer.

qPCR	
PCR **E** **RE**	– thawed at RT – keep on ice or at -20 °C – thawed at RT; **ATTENTION**: Spin down all solutions before opening tubes!
Thermocycler	98 °C, 30 sec 98 °C, 10 sec 65 °C, 20 sec } 3-10 x 72 °C, 30 sec 72 °C, 1 min 10 °C, ∞

1. Take 17 µl of your PCR amplified double-stranded library.
2. Prepare a mastermix containing 7 µl of PCR Mix (**PCR** ●), 5 µl of Reamplification Primer, (**RE** ❍), and 1 µl Enzyme Mix (**E** ●) per reaction,
3. Add 13 µl of this **PCR / E** / **RE** to 17 µl of the eluted library.
4. 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.
5. 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, step 30 for QuantSeq 3′ mRNA-Seq, or step 31 for QuantSeq-Flex Targeted RNA-Seq using the Purification Module with Magnetic Beads (Cat. No. 022.96).

5. Appendix: Primer Sequences

Libraries with Barcode 00 (BC00)

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

   …Insert)- AGATCGGAAGAGCACACGTCTGAACTCCAGTCACATCTCGTATGCCGTCTTCTGCTTG 3'
   …Insert)- TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTGTAGAGCATACGGCAGAAGACGAAC 5'
                          3'-Barcode 00-5'
```

NOTE: Libraries with BC00 don´t have an Index and cannot be multiplexed.

Libraries with Reamplification Primer (RE)

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

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

NOTE: The reamplification primer can only be used on already amplified libraries.

6. Appendix: Revision History

Publication No.	Change	Page
020IM064V0112	For reamplification no SYBR Green I is needed	6
020IM064V0111	Corrected step numbers for SENSE mRNA-Seq	4, 6
	Endpoint PCR set at 33 % of the maximum qPCR fluorescence.	4, 6
020IM064V0110	Addition of Reamplification Primer (RE).	1, 3, 6
	Addition of primer sequences.	7
020IM064V0100	Initial Release PCR Add-on Kit for Illumina.	

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