

The background features a series of blue, semi-transparent spheres of various sizes, some connected by thin, light blue lines, creating a molecular or network-like structure. The overall aesthetic is clean and scientific.

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PCR Add-on and Reamplification Kit V2 for Illumina User Guide

Catalog Number:
208 (PCR Add-on and Reamplification Kit V2 for Illumina)

208UG591V0101

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When describing a procedure using this product for publication, please refer to it as Lexogen's PCR Add-on and Reamplification Kit V2 for Illumina.

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1. Overview

This User Guide outlines the protocol for the PCR Add-on and Reamplification Kit V2 for Illumina (Cat. No. 208). This kit can be used for performing a qPCR Assay to determine the optimal number of cycles to use for the Endpoint PCR for QuantSeq V2, LUTHOR, and CORALL V2 RNA-Seq protocols. The qPCR Assay is strongly recommended to prevent under- or overcycling of your library.

The PCR Add-on and Reamplification Kit V2 for Illumina is suitable for reamplification of libraries generated with all QuantSeq, LUTHOR, and CORALL RNA-Seq Kits for Illumina.

ATTENTION: For qPCR assays for libraries prepared with previous versions of QuantSeq (Cat. No. 015, 016, 113 - 115, 129 -131) and CORALL (Cat. No. 095, 116 -119, 123 -134) please refer to the PCR Add-on Kit (Cat. No. 020). The PCR Add-on and Reamplification Kit V2 for Illumina (Cat. No. 208) is not recommended for qPCR assays for older kit versions of QuantSeq and CORALL.






The PCR Add-on and Reamplification Kit V2 contains PCR Mix (**PM** ○), non indexed Illumina P5 and P7 primers (**P5** ● and **P7** ●), for qPCR endpoint determinations, a Reamplification Primermix (**RE** ○) for reamplification of undercycled indexed libraries (outer nested primers) as well as PCR Enzyme Mix (**PE** ○).

Any single or dual indexed Illumina libraries can be reamplified using (**RE** ○).

ATTENTION: Important notes for the qPCR Assay and Endpoint PCR Test!

- Before starting, ensure the ramp rates of the thermocycler instruments used for qPCR and PCR are equal, or adjust accordingly. Specifically, the rate at which the thermocycler block cools from 98 °C to 65 °C for primer annealing in the PCR program (see p.6). This ensures equal efficiency of the PCR between qPCR and Endpoint PCR instruments.
- For the qPCR Assay, SYBR Green I nucleic acid dye (Sigma Aldrich, S9430; ThermoFisher S7563) is needed in addition to the PCR Add-on and Reamplification Kit V2 and must be supplied by the user.
- The use of SYBR Green I-containing qPCR mastermixes for the qPCR Assay is explicitly not recommended.
- The instructions provided here relate to the use of SYBR Green I dye only for endpoint cycle calculation via qPCR Assay. The use of EvaGreen or other similar dyes at 0.1x or 1x final concentration in the qPCR reaction will also produce amplification curves. However, the calculation of endpoint PCR cycle numbers using the instructions provided for SYBR Green I may not apply. Therefore, cycle number calculations must be validated by the user (i.e., via additional Endpoint PCR Test).
- If a qPCR Assay using SYBR Green I dye is not possible, the Endpoint PCR Test can be performed instead (see p.9).

2. Kit Components and Storage Conditions

Kit Component	Tube Label	Volume*	Storage
PCR Mix	PM ○	740 µl	 -20 °C
Enzyme Mix	PE ○	106 µl	 -20 °C
P5 Primer	P5 ●	528 µl	 -20 °C
P7 Primer	P7 ●	528 µl	 -20 °C
Reamplification Primer Mix	RE ○	1056 µl	 -20 °C

*including 10 % surplus

PCR Mix (**PM** ○) and Enzyme Mix (**PE** ○) provided in QuantSeq V2 with UDIs (Cat. No. 191 - 196), QuantSeq-Pool (Cat. No. 139), and CORALL V2 Kits (Cat. No. 171-176) are the same as provided in the PCR Add-on and Reamplification Kit V2 (Cat. No. 208) and can therefore be used interchangeably.

ATTENTION: PCR (**PCR** ●) and Enzyme Mix (**E** ●, from Cat. No. 020) or Enzyme Mix 3 (**E3** ●) in CORALL (Cat. No. 095, 117 - 119, 132 - 134) and QuantSeq Kits (Cat. No. 015, 016, 113 -115, 129 - 131) cannot be used interchangeably with the PCR components **PM** ○ and **PE** ○ provided with the PCR Add-on and Reamplification Kit V2 (Cat. No. 208).

3. User-Supplied Consumables and Equipment

Reagents

- SYBR Green I nucleic acid stain (10,000x in DMSO; e.g., Sigma-Aldrich S9430, or ThermoFisher S7563) has to be provided by the user.
- Purification Module with Magnetic Beads (Cat. No. 022) for purification of reamplified libraries, or purification of PCR products used instead of qPCR for testing optimal Endpoint PCR cycle numbers.

Equipment

- Quantitative Real-Time PCR Machine, capable of SYBR Green I detection (emission maximum at 520 nm) - ROX passive reference detection should be turned off.
- Calibrated single- and / or multi-channel pipettes for handling 1 µl to 1,000 µl volumes.
- Ice bath or ice box, ice pellets, benchtop cooler (-20 °C for enzymes).

4. qPCR Assay

4.1 Detailed Protocol

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. This assay uses purified cDNA of COR-ALL RNA-Seq V2, QuantSeq 3' mRNA-Seq V2 with UDIs, and QuantSeq-Pool Sample-Barcoded 3' mRNA-Seq kit. Please refer to the corresponding User Guides for indication at which step the qPCR assay is recommended.

REMARK: For older kit versions containing PCR (PCR ●) and (E3 ●) we recommend using the PCR Add-on Kit (Cat. No. 020) for qPCR.

ATTENTION: Do not use the Reamplification Primermix (RE ○) for the qPCR Assay on the cDNA library, as non-amplified cDNA lacks binding sites for the Reamplification Primers. The Reamplification Primer can only be used on already amplified (single or dual indexed) Illumina libraries.

qPCR		
PM ○	– thawed at RT	ATTENTION: Spin down all solutions before opening tubes!
PE ○	– keep on ice or at -20 °C	
P5 ●	– thawed at RT	
P7 ●	– thawed at RT	
	spin down before opening!	
	SYBR Green I nucleic acid dye (e.g., Sigma-Aldrich, Cat. No. S9430) - provided by user	
Thermocycler	95 °C, 60 sec	
	95 °C, 15 sec	} 35 - 50x
	60 °C, 15 sec	
	72 °C, 60 sec	
	72 °C, 6 min	
	10 °C, ∞	

NOTE: SYBR Green I has an emission maximum at 520 nm, which for some qPCR machines has to be adjusted manually. ROX passive reference dye detection is not required for this qPCR Assay and should be turned off to ensure correct calculation of SYBR Green fluorescence intensity.

- 1 Dilute the double-stranded library to 19 µl by adding 2 µl Elution Buffer (EB) or molecular biology-grade water (H₂O).
- 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.

For each reaction, combine: 1.7 μ l of the diluted cDNA library, 7 μ l of PCR Mix (**PM** ○), 5 μ l of P7 Primer (**P5** ●), 5 μ l of P7 Primer (**P7** ●), 1 μ l of Enzyme Mix (**PE** ○), and 1.4 μ l of 2.5x SYBR Green I nucleic acid dye. Add 13.9 μ l of Elution Buffer (**EB**) or molecular biology-grade water (**H₂O**) to bring the total volume to 35 μ l. **ATTENTION:** Include a no template control!

3

Perform 35 cycles of PCR with the following program: Initial denaturation at 95 °C for 60 seconds, 35 cycles of 95 °C for 15 seconds, 60 °C for 15 seconds and 72 °C for 60 seconds, and a final extension at 72 °C for 6 minutes, hold at 10 °C. **REMARK:** Use up to 50 cycles for QuantSeq-Flex libraries when targeting low abundant transcripts.

4

Using the amplification curves in linear scale, determine the value at which the fluorescence reaches the plateau. Calculate 50 % of this maximum fluorescence (50 % MF) 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 (see section 4.2, p.8). **REMARK:** There is no need to purify or analyze the overcycled PCR product.

5

For the remaining 17 μ l of cDNA library, continue from step **29** for CORALL RNA-Seq V2, step **25** for QuantSeq 3' mRNA-Seq V2 with UDIs, or step **34** for QuantSeq-Pool Sample-Barcoded 3' mRNA-Seq.

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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 qPCR, the cycle number corresponding to 50 % MF was 15 cycles. The remaining 17 μ l of the template should therefore be amplified with 12 cycles (15 - 3 cycles = 12 cycles).

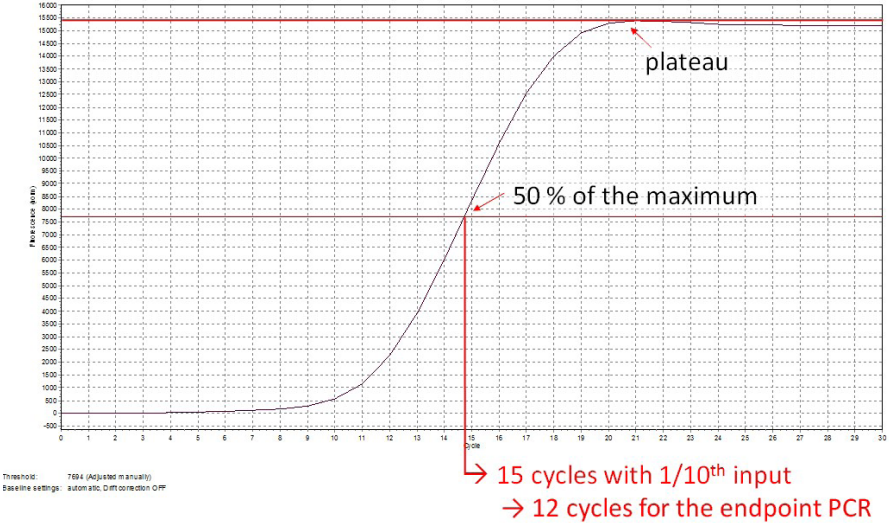


Figure 1. Calculation of the number of cycles for the endpoint PCR.

The optimal cycle number can also be determined using the amplification data from the qPCR software to calculate the 50 % MF value. Take the cycle number that is the next closest fluorescence value above the 50 % MF and subtract 3 to get the endpoint cycle number.

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. Endpoint PCR Test

5.1 Detailed Protocol

If a qPCR Assay cannot be performed, the PCR Add-on and Reamplification Kit V2 for Illumina (Cat. No. 208) can also be used to run additional Endpoint PCR tests, to establish the correct cycle number for library amplification. This Endpoint PCR Test omits the use of SYBR Green I and requires additional purification of the amplified libraries using the Purification Module with Magnetic Beads (Cat. No. 022).


ATTENTION: The protocol below should only be used for Endpoint PCR testing for Kits using the same for Endpoint PCR reagent system with PCR Mix (**PM** ○) and Enzyme Mix (**PE** ○) i.e., CORALL RNA-Seq V2, QuantSeq 3' mRNA-Seq V2, or QuantSeq-Pool Sample-Barcoded 3' mRNA-Seq Kit.

For older kit versions containing PCR (**PCR** ●) and (**E3** ●) use Cat. No. 020 for qPCR.

ATTENTION: The purification reagents (**PB**, **PS**, and **EB**) must be equilibrated to room temperature for at least 30 minutes before use!


Endpoint PCR	Purification
PM ○ – thawed at RT PE ○ – keep on ice or at -20 °C P5 ● – thawed at RT P7 ● – thawed at RT spin down before opening!	PB – stored at +4 °C PS – stored at +4 °C 80 % EtOH – provided by user; prepare fresh! EB – stored at +4 °C
Thermocycler 95 °C, 60 sec 95 °C, 15 sec } 11 - 25x 60 °C, 15 sec } (see 5.3) 72 °C, 60 sec } 72 °C, 6 min 10 °C, ∞	Equilibrate all reagents to room temperature for 30 minutes prior to use!

For this protocol, use cDNA from step 28 for CORALL RNA-Seq V2, step 24 for QuantSeq 3' mRNA-Seq V2 with UDIs, or step 33 for QuantSeq-Pool Sample-Barcoded 3' mRNA-Seq.

- 1 Add 2 µl Elution Buffer (**EB**) or molecular biology-grade water (**H₂O**) to dilute the cDNA to a total volume of 19 µl.  Store the remaining cDNA at -20 °C.
- 2 Prepare a mastermix containing 7 µl of PCR Mix (**PM** ○), 5 µl P5 Primer (**P5** ●), 5 µl P7 Primer (**P7** ●), and 1 µl Enzyme Mix (**PE** ○). Mix well and spin down.
- 3 In a fresh PCR tube or plate, add 18 µl of the **PM / P5 / P7 / PE** mastermix to 1.7 µl of cDNA, per sample. Add 15.3 µl of Elution Buffer (**EB**) or molecular biology-grade water (**H₂O**) to bring the total volume to 35 µl.

4

Conduct **6 - 25 cycles** of PCR (choose the cycle number depending on the amount of input RNA used, see 5.3, p.11) with the following program: initial denaturation for 60 seconds at 95 °C, 6 - 25 cycles of 15 seconds at 95 °C, 15 seconds at 60 °C and 60 seconds at 72 °C, and a final extension for 6 minute at 72 °C, hold at 10 °C.

 Safe stopping point. Libraries can be stored at -20 °C at this point.



Purify the amplified cDNA according to: step **33** in CORALL RNA-Seq V2, or step **29** in QuantSeq 3' mRNA-Seq V2, step **37** for QuantSeq-Pool Sample-Barcoded 3' mRNA-Seq and step **36** for High Definition Single Cell LUTHOR™ 3' mRNA-Seq Library Prep Kits, respectively and perform Quality Control to assess library size distribution and yield.

5.2 Calculating Endpoint PCR Cycle Number from Endpoint Testing

When performing Endpoint PCR with the remaining 17 µl of cDNA, the cycle number needs to be adjusted to account for using 10x more cDNA as template. The base rule to calculate the endpoint PCR cycle number is:

No. cycles for Endpoint PCR (17 µl cDNA) = No. cycles for Endpoint Test (1.7 µl cDNA) - 3 cycles

With each PCR cycle, the yield doubles. Therefore, you can determine whether more or less cycles are required based on the yield and library size distribution.

- If the yield is sufficient for sequencing, amplify the remaining 17 µl of cDNA with 3 less cycles than used for the Endpoint Test.
- If the library is overcycled, indicated by a second peak in high molecular weight regions (between 1,000 - 9,000 bp), reduce the number of cycles for Endpoint PCR by more than 3.
- If the library is undercycled and has insufficient yield for sequencing, use the obtained yield from the Endpoint Test to calculate how many additional cycles would be needed. Then subtract 3 to obtain the number of cycles to use when amplifying the remaining 17 µl of cDNA.

EXAMPLE: 500 ng total RNA input was used for library preparation. 15 cycles were chosen for the Endpoint PCR Test using 1.7 µl of cDNA. The yield was sufficient for sequencing and, therefore, the remaining 17 µl of cDNA should be amplified with $15 - 3 = 12$ cycles for Endpoint PCR.

Once an appropriate cycle number is established for a particular sample type, further Endpoint PCR Testing is not required.

5.3 PCR Cycle Numbers for Endpoint Testing

Please note the number of cycles to use for Endpoint PCR testing cannot be exactly advised as sample types vary. We strongly recommend determining the optimal cycle number using the qPCR Assay instead (see 4.1, p.6).

ATTENTION: The cycle number ranges below are not recommendations for the given input amounts for Endpoint PCR! These ranges are provided **as a guideline only** for choosing an approximate number of cycles for the Endpoint PCR Test. The ranges account for the 3 extra cycles needed when using 1.7 μ l of cDNA for the Endpoint PCR Test, versus 17 μ l (10x more) that is used for standard Endpoint PCR.

Total RNA Input Amount	QuantSeq V2 3' mRNA-Seq*	CORALL V2 Total RNA-Seq**	QuantSeq-Pool 3' mRNA-Seq***
100 cells - 1 cell			
1 ng - 10 pg			
1 - 0.5 ng	24 - 28	20 - 22	
50 - 10 ng	20 - 24	18 - 20	
200 - 100 ng	17 - 20	14 - 16	
1000 - 500 ng	14 - 17	13 - 15	
960 - 80 ng TOREQ			10 - 17

* Input range for QuantSeq V2 3' mRNA-Seq FWD is 1 - 500 ng of total RNA. Cycle numbers for QuantSeq-Flex depend entirely on the targeting strategy and should always be optimized using the qPCR Assay. ** Input range is indicated as total RNA input for RiboCop rRNA depletion, or Poly(A) selection prior to CORALL V2 library prep. Recommended input range is 1 - 1000 ng. Cycle numbers will also vary according to size selection conditions. *** TOREQ refers to the Total RNA Equivalent during Second Strand Synthesis, e.g.; TOREQ of 240 ng, may refer to pool of 24 RT reactions a 10 ng total input as well as 8 RT reactions a 30 ng total input or any variation of pools leading to a total RNA equivalent of 240 ng.

6. Library Reamplification

Single-indexed as well as dual indexed Illumina libraries can be reamplified using this kit. Even if only a small bump is visible on a quality control trace (Bioanalyzer or similar), the quantity is often sufficient for sequencing. The Library Quantification Calculation Sheet available from www.lexogen.com/support-tools/lane-mix-calculation can be used to check whether you have sufficient library for pooling.

ATTENTION: Do not use non indexed primers P5 Primer (**P5** ●) and P7 Primer (**P7** ●) for reamplification of already indexed libraries! This will lead to a loss of indices, and high levels of undetermined reads from the NGS run.

NOTE: Additional purification reagents (Cat. No. 022) will be required for purification of reamplified libraries.

Reamplification PCR		Purification	
PM ○	– thawed at RT	PB	– stored at +4 °C
PE ○	– keep on ice or at -20 °C	PS	– stored at +4 °C
RE ○	– thawed at RT spin down before opening!	80 % EtOH	– provided by user; prepare fresh!
		EB	– stored at +4 °C
Thermocycler	95 °C, 60 sec 95 °C, 15 sec 60 °C, 15 sec 72 °C, 60 sec 72 °C, 6 min 10 °C, ∞	Equilibrate all reagents to room temperature for 30 minutes prior to use!	
	} 3 - 10x		

For each reaction, combine: 7 µl of PCR Mix (**PM** ○), 10 µl of Reamplification Primer Mix (**RE** ○), 1 µl of Enzyme Mix (**PE** ○), and up to 17 µl of PCR amplified double-stranded Illumina single- or dual indexed library. If required, add Elution Buffer (**EB**) or molecular biology-grade water (**H₂O**) to bring the total volume to 35 µl. **ATTENTION:** Include a no template control!

1

Conduct 3 - 10 additional PCR cycles with the following program: initial denaturation for 60 seconds at 95 °C, 3 - 10 cycles of 15 seconds at 95 °C, 15 seconds at 60 °C, 60 seconds at 72 °C, and a final extension for 6 minute at 72 °C, hold at 10 °C.

4

At this point, the libraries are finished and should be purified according to the respective User Guide Purification Protocols. Follow step 33 in CORALL RNA-Seq V2, or step 29 in QuantSeq 3' mRNA-Seq V2, or step 37 for QuantSeq-Pool Sample-Barcoded 3' mRNA-Seq, using the Purification Module with Magnetic Beads (Cat. No. 022).

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6.1 PCR Cycle Number for Library Reamplification

If your libraries are undercycled but have measurable yields, perform 3 - 6 cycles of reamplification PCR, calculating approximate doubling of the original yield per cycle.

EXAMPLE: If your library concentration is 0.05 ng/μl, perform 3 - 5 cycles to obtain a concentration in the range of 0.4 - 1.5 ng/μl.

If you do not see a library at all e.g., on a Bioanalyzer trace (Agilent Technologies), and / or have no measurable library concentration, use 6 - 10 cycles for reamplification. For further PCR cycle recommendations for reamplification, please contact: support@lexogen.com.

7. Appendix: Primer Sequences

P5 Primer (P5) and P7 Primer (P7)

```
5'-(P5 Primer Sequence)-3'
5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTCCGATCT- (Insert...
3' TTAGCTATGCGCGCTGGTGGCTCTAGATGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA- (Insert...

...Insert)- AGATCGGAAGAGCACACGTCTGAACTCCAGTCACATCTCGTATGCCGTCTTCTGCTTG 3'
...Insert)- TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTGTAGAGCATAACGGCAGAAGACGAAC 5'
3'-(P7 Primer sequence)- 5'
```

ATTENTION: Libraries amplified with P5 Primer (P5 ●) and P7 Primer (P5 ●) do not contain any index or barcode and cannot be multiplexed for sequencing!

Reamplification Primer (RE)

```
5'-(Reamplification Primer)-3'
5' AATGATACGGCGACCACCGAGATCTACAC-i5-ACACTCTTTCCCTACACGACGCTCTCCGATCT- (Insert...
3' TTAGCTATGCGCGCTGGTGGCTCTAGATGTG-i5-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA- (Insert...

5'-(Index Read Sequencing Primer)-3'
...Insert)- AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-i7-ATCTCGTATGCCGTCTTCTGCTTG 3'
...Insert)- TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGT-i7-TAGAGCATAACGGCAGAAGACGAAC 5'
3'-(Reamplification Primer)-5'
```

ATTENTION: The Reamplification Primer can only be used on already amplified PCR libraries. Do not use it on non amplified cDNA as it lack primer binding sited for the Reamplification Primer Mix (RE ○)!

NOTE: RE ○ can be used on single-or dual indexing libraries. As an example, a dual indexed library is shown.

8. Appendix: Revision History

Publication No. / Revision Date	Change	Page
208UG591V0101 Aug. 08, 2023	Adjusted extension time in Reamplification PCR table to 60 seconds.	12
208UG591V0100 July 12, 2023	Initial Release.	

Associated Products:

022 (Purification Module with Magnetic Beads)
139 (QuantSeq-Pool Sample-Barcoded 3' mRNA-Seq Library Prep Kit)
171 - 176 (CORALL RNA-Seq V2)
177 - 182 (CORALL mRNA-Seq V2)
183 - 184 (RiboCop (HMR) and CORALL Total RNA-Seq V2 Library Prep Kit with UDIs)
185 - 186 (RiboCop (HMR+Globin) and CORALL Total RNA-Seq V2 Library Prep Kit with UDIs)
191 -196 (QuantSeq 3' mRNA-Seq V2 Library Prep Kit FWD with UDIs)

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