

The background of the entire page is decorated with a network of light blue lines and numerous translucent blue spheres of varying sizes, creating a molecular or network-like aesthetic.

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PCR Add-on Kit for Illumina User Guide

Catalog Number:
020 (PCR Add-on Kit for Illumina)

020UG064V0132

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

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1. Overview

This User Guide outlines the protocol for the PCR Add-on Kit for Illumina (Cat. No. 020). This kit can be used for performing a qPCR Assay to determine the optimal number of cycles to use for the Endpoint PCR for single- or dual-indexed QuantSeq, LUTHOR, and CORALL RNA-Seq protocols. The qPCR Assay is strongly recommended to prevent under- or overcycling of your library.

The PCR Add-on Kit for Illumina is suitable for all QuantSeq, LUTHOR, and CORALL RNA-Seq Kits for Illumina.

The PCR Add-on Kit contains PCR Mix (**PCR** ●) that includes a non-indexed, Illumina P5 primer, Enzyme Mix (**E** ●) containing a thermostable polymerase, and the non-indexed P7 Primer (**7000** ●), which is required for the qPCR Assay. Enzyme Mix 3 (**E3** ●) supplied in the QuantSeq and CORALL Kits is interchangeable with **E** ● from the PCR Add-on Kit. **PCR** ● is also interchangeable between the PCR Add-on, CORALL (Cat. No. 095) and QuantSeq Kits with single indexing (Cat. No. 015, 016).

The PCR Add-on Kit also contains a Reamplification Primer (**i7-RE** ○) that can be used to reamplify undercycled, single-indexed libraries in order to obtain enough material for sequencing. Dual-indexed libraries cannot be reamplified using this kit. For reamplification of dual-indexed libraries, the Reamplification Add-on Kit for Illumina (Cat. No. 080) is available on request. Please contact Lexogen at support@lexogen.com (see also p.13).

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 Kit 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

PCR Add-on Kit (-20 °C)

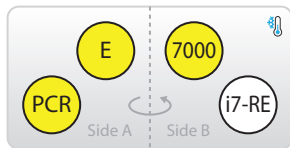


Figure 1. Location of kit components

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: Enzyme Mix (E ●) is the same as Enzyme Mix 3 (E3 ●) in CORALL (Cat. No. 095, 117 - 119, 132 - 134) and QuantSeq Kits (Cat. No. 015, 016, 113 -115, 129 - 131). These enzyme mixes can be used interchangeably.

Enzyme Mix (E ●) must not be exchanged with PCR Enzyme Mix (PE ○) provided in QuantSeq with UDIs V2 (Cat. No. 191 - 196), QuantSeq-Pool (Cat. No. 139), and CORALL V2 Kits (Cat. No. 171-176).

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 cDNA after completion of second strand synthesis, and purification from the following steps and protocols: step 27 for CORALL Total RNA-Seq, step 28 in CORALL RNA-Seq V2, or step 24 in QuantSeq 3' mRNA-Seq.

REMARK: Please refer to the respective User Guide appendices for LUTHOR 3' mRNA-Seq library prep (143UG290, Appendix B, p.27) and QuantSeq-Pool 3' mRNA-Seq library prep (139UG272, Appendix C, p.23), for the precise qPCR protocols and Endpoint Cycle Calculation methods to use in combination with the reagents provided in this PCR Add-on Kit (Cat. No. 020).

ATTENTION: Do not use the i7 Reamplification Primer (**i7-RE** ○) for the qPCR Assay on the cDNA-library, as non-amplified cDNA lacks binding sites for the Reamplification Primer. The Reamplification Primer can only be used on already amplified (single-indexed) cDNA libraries.

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 nucleic acid dye (e.g., Sigma-Aldrich, Cat. No. S9430) - provided by user		
Thermocycler	98 °C, 30 sec	
	98 °C, 10 sec	} 35 - 50x
	65 °C, 20 sec	
	72 °C, 30 sec	
	72 °C, 1 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.

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. Add 14.1 µl of Elution Buffer (**EB**) or molecular biology-grade water (**H₂O**) to bring the total volume to 30 µl. **ATTENTION:** Include a no template control!

4

Perform 35 cycles of PCR with the following program: Initial denaturation for 30 seconds at 98 °C, 35 cycles of 10 seconds at 98 °C, 20 seconds at 65 °C and 30 seconds at 72 °C, and a final extension for 1 minute at 72 °C, hold at 10 °C. **REMARK:** Use up to 50 cycles for QuantSeq-Flex libraries when targeting low abundant transcripts.

5

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 µl of the template (see section 4.2, p.8). **REMARK:** There is no need to purify or analyze the overcycled PCR product.

6

For the remaining 17 µl of cDNA library, continue from step 28 for CORALL Total RNA-Seq, step 29 for CORALL RNA-Seq V2, or step 25 for QuantSeq 3' mRNA-Seq.

4.2 Example for Endpoint Calculation

ATTENTION: The below method of endpoint cycle number calculation does **not** apply to LUTHOR 3' mRNA-Seq libraries. For details of endpoint calculation for LUTHOR, please refer to the LUTHOR 3' mRNA-Seq User Guide (143UG290, Appendix B, p.27).

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).

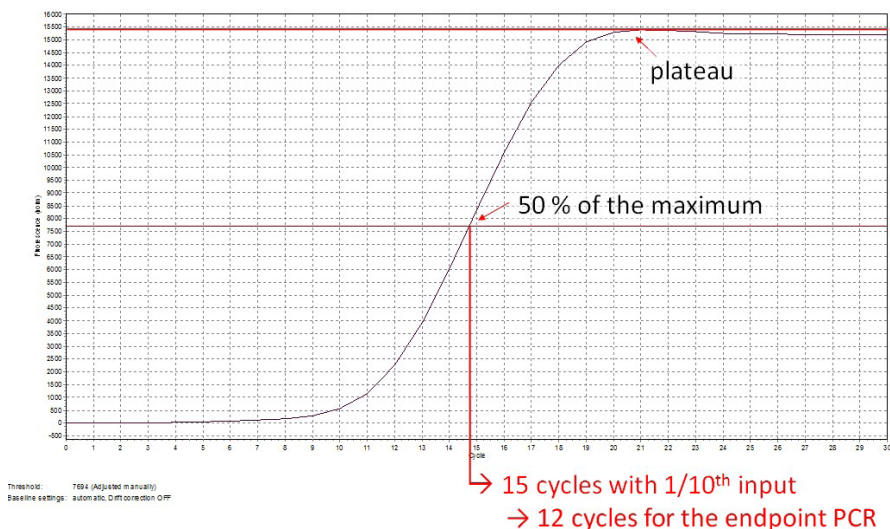


Figure 2. Calculation of the number of cycles for the endpoint PCR.

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 Kit for Illumina (Cat. No. 020) 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 can **not** be used for Endpoint PCR testing for LUTHOR, QuantSeq-Pool 3' mRNA-Seq, QuantSeq V2, or CORALL RNA-Seq V2 libraries. QuantSeq-Pool 3' mRNA-Seq, QuantSeq V2, and CORALL RNA-Seq V2 use a different Endpoint PCR reagent system (see 139UG272, Library Amplification, p.16, 191UG444, Library Amplification, p.13, and 171UG394, Library Amplification, p.13 and p.22, respectively). Trial Reactions are instead recommended for LUTHOR 3' mRNA-Seq, using the PCR Master Mix (**PCR** ●) provided in the LUTHOR kit, and are detailed in the User Guide (143UG290, Library Amplification, p.19).

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

Endpoint PCR		Purification
PCR ●	– thawed at RT	PB – stored at +4 °C
E ●	– keep on ice or at -20 °C	PS – stored at +4 °C
7000 ●	– thawed at RT	80 % EtOH – provided by user; prepare fresh!
	spin down before opening!	EB – stored at +4 °C
Thermocycler	98 °C, 30 sec 98 °C, 10 sec 65 °C, 20 sec 72 °C, 30 sec 72 °C, 1 min 10 °C, ∞ } 11 - 25x (see 5.3)	Equilibrate all reagents to room temperature for 30 minutes prior to use!

For this protocol, use cDNA from step 27 in CORALL Total RNA-Seq, or step 24 in QuantSeq 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 (**PCR** ●), 5 µl P7 Primer (**7000** ●), and 1 µl Enzyme Mix (**E** ●). Mix well and spin down.
- 3 In a fresh PCR tube or plate, add 13 µl of the **PCR / 7000 / E** 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 30 µl.

4

Conduct **11 - 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 30 seconds at 98 °C, 11 - 25 cycles of 10 seconds at 98 °C, 20 seconds at 65 °C and 30 seconds at 72 °C, and a final extension for 1 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 **32** in CORALL Total RNA-Seq, or step **29** in QuantSeq 3'mRNA-Seq, 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 µl of cDNA for the Endpoint PCR Test, versus 17 µl (10x more) that is used for standard Endpoint PCR.

Total RNA Input Amount	QuantSeq 3' mRNA-Seq*	CORALL Total RNA-Seq**
0.5 - 1ng	24 - 28	20 - 22
10 - 50 ng	20 - 24	18 - 20
100 - 200 ng	17 - 20	14 - 16
500 - 1000 ng	14 - 17	13 - 15

* Input range for QuantSeq 3' mRNA-Seq FWD is 0.5 - 500 ng of total RNA (or 10 - 500 ng for REV). 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 library prep. Recommended input range is 1 - 1000 ng. Cycle numbers will also vary according to size selection conditions.

6. Library Reamplification

6.1 Reamplification of Single-Indexed Libraries (i7 only)

Single-indexed libraries prepared with Lexogen's 6 nt i7 indices 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 can be used to check whether you have sufficient library for pooling.

ATTENTION: Do not use the P7 Primer **7000** 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
PCR ● E ● i7-RE ○	– thawed at RT – keep on ice or at -20 °C – 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	98 °C, 30 sec 98 °C, 10 sec 65 °C, 20 sec 72 °C, 30 sec 72 °C, 1 min 10 °C, ∞ } 3 - 10x	Equilibrate all reagents to room temperature for 30 minutes prior to use!

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

Conduct 3 - 10 additional PCR cycles with the following program: initial denaturation for 30 seconds at 98 °C, 3 - 10 cycles of 10 seconds at 98 °C, 20 seconds at 65 °C, 30 seconds at 72 °C, and a final extension for 1 minute at 72 °C, hold at 10 °C.

At this point, the libraries are finished and should be purified according to the respective User Guide Purification Protocols. Follow step 32 in CORALL Total RNA-Seq, or step 29 in QuantSeq 3' mRNA-Seq, using the Purification Module with Magnetic Beads (Cat. No. 022).

6.2 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.

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

For reamplification of dual-indexed libraries, including those prepared with Lexogen's 12 nt Unique Dual Indices (UDIs), the Reamplification Add-on Kit for Illumina (Cat. No. 080) is available on request. Please contact Lexogen at support@lexogen.com for further information.

7. Appendix: Primer Sequences

P7 Primer (7000)

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

...Insert)- AGATCGGAAGAGCACACGTCTGAACTCCAGTCACATCTCGTATGCCGTCTTCTGCTTG 3'
...Insert)- TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTGTAGAGCATACGGCAGAAGACGAA 5'
3'-P7 Primer (7000) sequence-5'
```

ATTENTION: Libraries amplified with P7 Primer **7000** do not have an i7 index and cannot be multiplexed for sequencing!

i7 Reamplification Primer (i7-RE)

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

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

ATTENTION: The Reamplification Primer can only be used on already amplified single-indexed (i7 only) PCR libraries.

8. Appendix: Revision History

Publication No. / Revision Date	Change	Page
020UG064V0132 Jan. 3, 2023	Changed Instruction Manual to User Guide throughout the document.	
	Updated legal information.	2
	Updated product references within the document. Added CORALL RNA-Seq V2 and QuantSeq 3' mRNA-Seq V2. Removed QuantSeq-FLEX Cat. No. and SENSE mRNA-Seq V2 references throughout.	4 - 12
	Updated Kit Components Figure 1 and Table to reflect current packaging and storage requirements.	5
020IM064V0131 Nov. 4, 2020	Legal information including terms and conditions, and table of contents added.	2 - 3
	Added reference information for LUTHOR and QuantSeq-Pool libraries to qPCR Assay (section 4.1) and Endpoint PCR (section 5.1)	6 - 8
020IM064V0100 Jun. 19, 2015	Initial Release.	

Associated Products:

015 (QuantSeq 3' mRNA-Seq Library Prep Kit for Illumina (FWD))
015 (QuantSeq 3' mRNA-Seq Library Prep Kit for Illumina (FWD) HT including Lexogen i5 6 nt Dual Indexing Add-on Kit (5001-5004))
016 (QuantSeq 3' mRNA-Seq Library Prep Kit for Illumina (REV) with Custom Sequencing Primer)
022 (Purification Module with Magnetic Beads)
044 (Lexogen i7 6 nt Index Set (7001 - 7096))
080 (Reamplification Add-on Kit for Illumina)
095 (Version 1: CORALL Total RNA-Seq Prep Kit)
113 - 115, 129 - 131 (Version 1: QuantSeq 3' mRNA-Seq Library Prep Kit FWD with UDIs)
117 - 119, 132 - 134 (Version 1: CORALL Total RNA-Seq Library Prep Kit with UDIs)
139 (QuantSeq-Pool Sample-Barcoded 3' mRNA-Seq Library Prep Kit)
143 (LUTHOR 3' mRNA-Seq Library Prep Kit for Illumina)
146 (Version 1: CORALL Total RNA-Seq Library Prep Kit with RiboCop (HMR))
147 (Version 1: CORALL Total RNA-Seq Library Prep Kit with RiboCop (HMR+Globin))
158 - 163 (Version 1: CORALL mRNA-Seq)
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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