

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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Enabling complete transcriptome sequencing

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# PCR Add-on Kit for Illumina Instruction Manual

Catalog Number:  
020IM064V0130

020IM064V0130



## The PCR Add-on Kit Instruction Manual has been updated!

### Changes to the Instruction Manual:

- New protocol for Endpoint PCR Test as an alternative to the qPCR Assay was added (p.6 - 7).
- Updated guidelines for reamplification of single-indexed libraries.
- Updated attention notes in protocol overview, including warnings against the use of qPCR mastermixes and non-SYBR Green I dyes for the qPCR Assay.
- Added references for use with the CORALL Total RNA-Seq Library Prep Kit (Cat. No's 095, 096).

**NOTE:** This Instruction Manual is only compatible with QuantSeq 3' mRNA-Seq kits bought after February 17<sup>th</sup>, 2017. If you are using the i5 Dual Indexing Add-on Kit with an older kit version, please contact [support@lexogen.com](mailto:support@lexogen.com) to get the respective Instruction Manual.

*For more details please have a look at the Revision History at the end of this Instruction Manual or contact us at [support@lexogen.com](mailto:support@lexogen.com).*

# 1. Overview

This Instruction Manual 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, CORALL, and SENSE mRNA V2 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 (Cat. No. 015, 016, 033, 034, and 035) CORALL (Cat. No. 095, 096), and SENSE mRNA V2 (Cat. No. 001) 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, QuantSeq-Flex, and CORALL Kits, and Enzyme Mix 2 (**E2** ●) from SENSE mRNA V2, are interchangeable with **E** ● from the PCR Add-on Kit. **PCR** ● is also interchangeable between the PCR Add-on, CORALL, SENSE mRNA V2, QuantSeq, and QuantSeq-Flex Kits.

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 [info@lexogen.com](mailto:info@lexogen.com) (see also p.9).

## **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.4). 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.6).

# 2. Kit Components and Storage Conditions

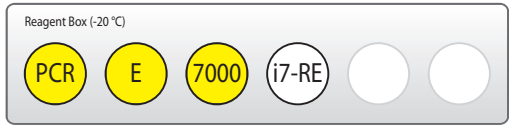


Figure 1. Location of kit components

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:** Enzyme Mix (E ●) is the same as Enzyme Mix 2 (E2 ●) in the SENSE mRNA V2 Kit and Enzyme Mix 3 (E3 ●) in the CORALL and QuantSeq Kits. These enzyme mixes can be used interchangeably.

# 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. For this assay use cDNA after completion of second strand synthesis, and purification, i.e., from step 27 for CORALL Total RNA-Seq, step 33 in SENSE mRNA-Seq V2, or step 24 in QuantSeq 3' mRNA-Seq and QuantSeq-Flex Targeted RNA-Seq

**ATTENTION:** Do not use the i7 Reamplification Primer (i7-RE O) 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.

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

qPCR		
PCR ●	– thawed at RT	<b>ATTENTION:</b> 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 (eq., Sigma-Aldrich, Cat. No. S9430) - provided by user		
Thermocycler	98 °C, 30 sec	
	98 °C, 10 sec	
	65 °C, 20 sec	
	72 °C, 30 sec	
	} 35 - 50x	
	72 °C, 1 min	
	10 °C, ∞	

1 Dilute the double-stranded library to 19 µl by adding 2 µl Elution Buffer (EB) or molecular biology-grade water (H<sub>2</sub>O).

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 (H<sub>2</sub>O) 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 at 98 °C for 30 seconds, 35 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:** Use up to 50 cycles for QuantSeq-Flex libraries when targeting low abundant transcripts.

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  $\mu$ l of the template (see section 4.2 below for example). **REMARK:** There is no need to purify or analyze the overcycled PCR product.

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For the remaining 17  $\mu$ l of cDNA library, continue from step 28 for CORALL Total RNA-Seq, step 35 for SENSE mRNA-Seq V2, step 25 for QuantSeq 3' mRNA-Seq and QuantSeq-Flex Targeted RNA-Seq for the Endpoint PCR.

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## 4.2 Example for Endpoint Calculation

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

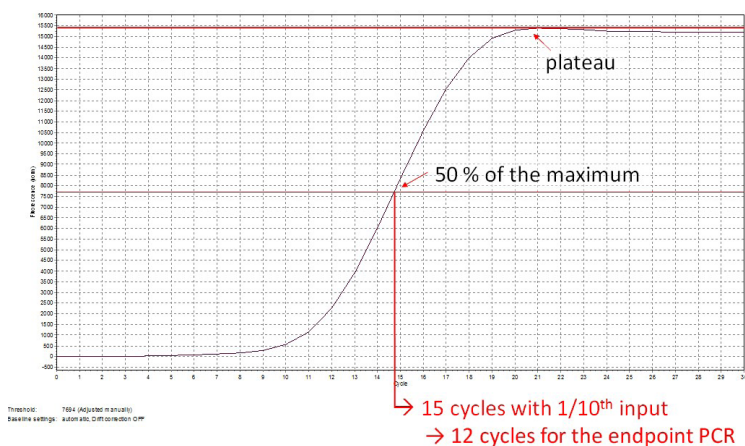


Figure 2. Calculation of the number of cycles for the endpoint PCR.

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


Endpoint PCR		Purification
<b>PCR</b> ●	– thawed at RT	<b>PB</b> – stored at +4 °C
<b>E</b> ●	– keep on ice or at -20 °C	<b>PS</b> – stored at +4 °C
<b>7000</b> ●	– thawed at RT <b>spin down before opening!</b>	<b>80 % EtOH</b> – provided by user; <b>prepare fresh!</b>
		<b>EB</b> – 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, ∞	Equilibrate all reagents to room temperature for 30 minutes prior to use!

For this protocol use cDNA from step 27 for CORALL Total RNA-Seq, step 33 in SENSE mRNA-Seq V2, or step 24 in QuantSeq 3' mRNA-Seq and QuantSeq-Flex Targeted RNA-Seq).

1 Add 2 µl Elution Buffer (**EB**) or molecular biology-grade water (H<sub>2</sub>O) 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 (H<sub>2</sub>O) 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.7) with the following program: Initial denaturation at 98 °C for 30 seconds, 11 - 25 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.  
 Safe stopping point. Libraries can be stored at -20 °C at this point.

 Purify the amplified cDNA according to: step 32 for CORALL Total RNA-Seq, step 39 in SENSE mRNA-Seq V2, or step 29 in QuantSeq 3' mRNA-Seq and QuantSeq-Flex Targeted RNA-Seq, and perform Quality Control to assess library size distribution and yield.

# 5.2 Calculating Endpoint PCR Cycle Number

When performing the 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 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 cycles.
- 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, 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 the 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.4).

**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**	SENSE mRNA-Seq V2***
0.5 - 1ng	24 - 28	20 - 22	25 - 28
10 - 50 ng	20 - 24	18 - 20	20 - 21
100 - 200 ng	17 - 20	14 - 16	14 - 21
500 - 1000 ng	14 - 17	13 - 15	11 - 17

\* 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. \*\*\* Input range for SENSE mRNA-Seq V2 is 1 - 2000 ng. Cycle numbers will also vary according to size selection conditions.



# 6. Reamplification of Undercycled Libraries

## 6.1 Reamplification of Single-Indexed Libraries (i7 only)

Single-indexed libraries (i7) that are undercycled, with yields insufficient for pooling and sequencing, can be reamplified using the i7 Reamplification Primer (**i7-RE** ○). 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](http://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
<b>PCR</b> ●	– thawed at RT	<b>PB</b> – stored at +4 °C
<b>E</b> ●	– keep on ice or at -20 °C	<b>PS</b> – stored at +4 °C
<b>i7-RE</b> ○	– thawed at RT	<b>80 % EtOH</b> – provided by user; <b>prepare fresh!</b>
	<b>spin down before opening!</b>	<b>EB</b> – 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, ∞	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 (H<sub>2</sub>O) 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 at 98 °C for 30 seconds, 3 - 10 cycles of 98 °C for 10 seconds, 65 °C for 20 seconds, 72 °C for 30 seconds, and a final extension at 72 °C for 1 minute, 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 for CORALL Total RNA-Seq, step 39 for SENSE mRNA-Seq V2, or step 29 for QuantSeq 3' mRNA-Seq and QuantSeq-Flex Targeted RNA-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](mailto:support@lexogen.com).

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

For reamplification of dual-indexed libraries the Reamplification Add-on Kit for Illumina (Cat. No. 080) is available on request. Please contact Lexogen at [info@lexogen.com](mailto:info@lexogen.com) for further information.

## 7. Appendix: Primer Sequences

### P7 Primer (7000)

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

...Insert)- AGATCGGAAGAGCACACGTCTGAACTCCAGTCACATCTCGTATGCCGTCTTCTGCTTG 3'
...Insert)- TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTGTAGAGCATACGGCAGAAGACGAAAC 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' TTACTATGCCGCTGTTGGCTCTAGATGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA- (Insert...

5'-(Index Read Sequencing Primer)-3'
...Insert)- AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-i7-ATCTCGTATGCCGTCTTCTGCTTG 3'
...Insert)- TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-i7-TA GAGCATAACGGCAGAAGACGAAAC 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
<b>020IM064V0130</b> Jul. 1, 2019	Included references to CORALL Total RNA-Seq Library Prep and updated attention notes in protocol overview.	2 - 6, 8
	Use 35 cycles for qPCR Assay (or up to 50 for QuantSeq-Flex).	5
	Maximum Fluorescence (MF) used in place of Fluorescence Units (FU)	5
	New Section 5: Endpoint PCR Testing as qPCR Assay alternative.	6 - 7
	Revised attention notes and cycle guidelines updated for reamplification PCR.	8 - 9
	Updated i5 / i7 index and kit naming.	12
<b>020IM064V0122</b> 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
<b>020IM064V0121</b> Feb. 24, 2017	Minor corrections.	4
<b>020IM064V0120</b> 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
<b>020IM064V0113</b> Oct. 12, 2016	PCR volume in Kit Contents Table corrected.	3
	Updates to schematic representation of libraries in Appendix.	7
<b>020IM064V0112</b> May 17, 2016	For reamplification no SYBR Green I is needed.	6
<b>020IM064V0111</b> Apr. 27, 2016	Corrected step numbers for SENSE mRNA-Seq.	4, 6
	Endpoint PCR set at 33 % of the maximum qPCR fluorescence.	4, 6
<b>020IM064V0100</b> Jun. 19, 2015	Initial Release.	

## Associated Products:

001 (SENSE mRNA-Seq Library Prep Kit V2 for Illumina)  
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)  
033 (QuantSeq-Flex Targeted RNA-Seq Library Prep Kit V2 with First Strand Synthesis Module for Illumina)  
034 (QuantSeq-Flex Targeted RNA-Seq Library Prep Kit V2 with Second Strand Synthesis Module for Illumina)  
035 (QuantSeq-Flex Targeted RNA-Seq Library Prep Kit V2 with First and Second Strand Synthesis Module for Illumina)  
044 (Lexogen i7 6 nt Index Set (7001 - 7096))  
047 (Lexogen i5 6 nt Dual Indexing Add-on Kits (5001 - 5096))  
080 (Reamplification Add-on Kit for Illumina)  
095 (CORALL Total RNA-Seq Prep Kit)  
096 (CORALL Total RNA-Seq Prep Kit with RiboCop)

## PCR Add-on Kit for Illumina · Instruction Manual

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