

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

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Lexogen i5 6 nt Dual Indexing Add-on Kits (5001-5096) User Guide

Catalog Numbers:
047 (Lexogen i5 6 nt Dual Indexing Add-on Kits (5001 - 5096))

047UG109V0300

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1. Overview

This User Guide outlines the protocol for dual indexed library preparation, using Lexogen i5 6 nt Dual Indexing Add-on Kits. These kits are compatible for use with Lexogen's QuantSeq, or CORALL Library Prep Kits. They are not compatible with Lexogen's Small RNA-Seq Library Prep Kit.

The Lexogen i5 6 nt Dual Indexing Add-on Kit (5001 - 5004, Cat. No. 047.4x96) contains four perfectly balanced i5 indices **5001 - 5004**. This kit is designed for combinatorial dual indexing.

The Lexogen i5 6 nt Unique Dual Indexing Add-on Kit (5001 - 5096, Cat. No. 047.96) provides one 96-well index plate containing 96 i5 indices (**5001 - 5096**, Lexogen's 6 nt i5 Index Set) and can be used together with Lexogen's 96 i7 indices (**7001 - 7096**, Lexogen's 6 nt i7 Index Set) for preparing up to 96 uniquely dual-indexed libraries.

Lexogen's i7 (Index 1) and i5 (Index 2) indices are 6 nucleotides long and are introduced at the PCR step of library preparation for the following kits: QuantSeq 3' mRNA-Seq (Cat. No. 015, 016), QuantSeq-Flex (Cat. No. 033, 034, 035) and CORALL Total RNA-Seq (Cat. No. 095, 096). The i7 indices are always included in the standard library prep kits or can be purchased separately (Cat. No. 044.96). Using the i5 and i7 index sets together, up to 9,216 different i5 / i7 index combinations are possible (see Appendix A, p.9).

ATTENTION: Important information for amplification of dual-indexed libraries!

- The **Dual PCR Mix (Dual PCR ●)** supplied with the Lexogen i5 6 nt Dual Indexing Add-on Kit **must** be used instead of the PCR Mix from the standard library prep kits (**PCR ●** from QuantSeq and CORALL).
- The total volume of the PCR is 35 µl (*versus* 30 µl for single-indexed library amplification).
- The volume of Purification Beads (**PB**) added for the post-PCR purification is also increased to 35 µl (step **6**, p.8). Please follow the purification protocol steps listed on p.7 - 8 of this User Guide to purify dual-indexed libraries.
- Each well of the Lexogen i5 6 nt Index Set (Cat. No. 047.96) contains a sufficient volume for one library prep per index and is intended for **single use only!**
- **Spin down the plates or tubes containing i5 and i7 indices before opening!** Pierce or cut open the sealing foil of the wells containing only the desired barcodes. Reseal opened wells of the barcode plate after use to prevent cross contamination!

Dual-indexed QuantSeq and CORALL libraries prepared with this Add-on Kit are compatible with all Illumina sequencing instruments. For i5 index sequences and multiplexing guidelines see Appendix A, p.9. For sequencing details including i5 read orientation and dual index read-out workflows for different flow cells and instruments, see Appendix B, p.12.

2. Kit Components and Storage Conditions

2.1 Lexogen i5 6 nt Dual Indexing Add-on Kit (5001 - 5004), Cat. No. 047.4x96

Lexogen i5 6 nt Dual Indexing Add-on Kit, 047.4x96 (-20 °C)

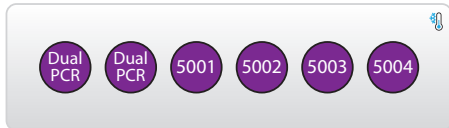


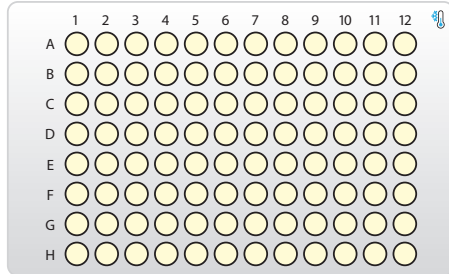
Figure 1. Location of kit components for Cat. No. 047.4x96.

Kit Component (Cat. No. 047.4)	Tube Label	Volume* 047.4x96	Storage
Dual PCR Mix	Dual PCR ●	2x 1,480 µl	-20 °C
i5 Index 01	5001 ●	528 µl	-20 °C
i5 Index 02	5002 ●	528 µl	-20 °C
i5 Index 03	5003 ●	528 µl	-20 °C
i5 Index 04	5004 ●	528 µl	-20 °C

*including ≥10 % surplus

2.2 Lexogen i5 6 nt Unique Dual Indexing Add-on Kit (5001 - 5096), Cat. No. 047.96

Lexogen i5 6 nt Index Set (-20 °C)



Dual PCR Mix Module (-20 °C)

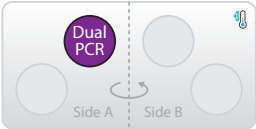


Figure 2. Location of kit components for Cat. No. 047.96

Kit Component (Cat. No. 047.96)	Tube Label	Volume* provided	Storage
Lexogen i5 6 nt Index Set (5001 - 5096)		5 µl / reaction	-20 °C
Dual PCR Mix	Dual PCR ●	740 µl	-20 °C

*including ≥10 % surplus

NOTE: Each 96-well Lexogen i5 6 nt Index Set contains sufficient volume for one library prep per index per well. Each well is intended for **single use only**!

3. Detailed Protocol - Library Amplification

This PCR protocol replaces the single indexing PCR protocols in steps 25 - 28 of the QuantSeq Kits User Guides (015UG009, 015UG110) or steps 28 - 31 of the CORALL Total RNA-Seq Kit User Guide (095UG190).

Preparation

PCR		Purification (Cat. No. 022)
<p>From i5 6 nt Dual Indexing Add-on Kits:</p> <p>Dual PCR ●</p> <p>5001 - 5004 ● or 5001 - 5096 (i5 6 nt Index Set)</p> <p>From standard library prep kits:</p> <p>7001 - 7096 (i7 6 nt Index Set)</p> <p>Enzyme Mix</p>		<p>from standard library prep kits:</p> <p>PB – stored at +4°C</p> <p>PS – stored at +4°C</p> <p>80 % EtOH – provided by user prepare fresh!</p> <p>EB – stored at +4°C</p>
	<p>– thawed at RT</p> <p>– thawed at RT</p> <p>– thawed at RT</p> <p>– keep on ice or at -20 °C</p>	<p>spin down before opening!</p>
Thermocycler	<p>98 °C, 30 sec</p> <p>98 °C, 10 sec</p> <p>65 °C, 20 sec</p> <p>72 °C, 30 sec</p> <p>72 °C, 1 min</p> <p>10 °C, ∞</p>	
	<p>11- 25x</p> <p>Endpoint cycle number as determined by qPCR (Cat. No. 020), or see CORALL or QuantSeq, User Guides.</p>	

PCR

The library is amplified to add the complete adapter sequences required for cluster generation, to introduce i5 and i7 indices, and to generate sufficient material for quality control and sequencing.

We strongly recommend performing a qPCR assay to determine the optimal number of PCR cycles for the endpoint PCR. Please use the PCR Add-on Kit (Cat. No. 020) and see User Guide 020IM064 for assay details. The qPCR assay is equally efficient for single- and dual-indexed libraries.

ATTENTION: Important information for dual-indexed library amplification!

- For dual indexing **REPLACE** the **PCR Mix** (**PCR** ● or **PCR** ○) from the standard library prep kit with the **Dual PCR Mix** (**Dual PCR** ●) supplied in the Lexogen i5 6 nt Dual Indexing Add-on Kits! Do not use **PCR** ● or **PCR** ○ from the standard kits if dual indexing is intended.
- The **Enzyme Mix** needed for the PCR is provided in the standard library prep kits: **E3** ● from QuantSeq and CORALL, or **E** ● from the PCR Add-on Kit. **Do not use E2** ● / **E2** ● from the **QuantSeq** or **CORALL** Kits, respectively for this PCR!
- Each 96-well Lexogen i5 6 nt Index Set contains sufficient volume for one library prep per index per well. Each well is intended for **single use only!**
- Spin down the plate or tubes containing i5 and i7 indices before opening!** Pierce or cut open the sealing foil of the wells containing only the desired barcodes. Reseal opened wells of the barcode plate after use to prevent cross contamination!

NOTE: At this point we recommend placing the purification components (**PB**, **PS**, and **EB**, included in the QuantSeq / CORALL Kits) for step 6 at room temperature, to give them enough time to equilibrate.

1 Prepare a mastermix containing 7 µl PCR Mix from the Lexogen i5 6 nt Dual Indexing Add-on Kit (**Dual PCR** ●) and 1 µl **Enzyme Mix** from the standard library prep kits per reaction (see **ATTENTION** note p.6).

2 Add 8 µl of the **Dual PCR** / Enzyme mastermix to 17 µl of the eluted library.

Add 5 µl of the respective i5 Index Primer (**5001 - 5004** ● in tubes, or **5001 - 5096** in 96-well plate, from the Lexogen i5 6 nt Dual Indexing Add-on Kits). **ATTENTION:**

3 Spin down tubes and plates before opening! For 96-well plates, pierce or cut open the sealing foil of the wells containing the desired barcodes. Reseal opened wells of the barcode plate after use to prevent cross contamination!

4 Add 5 µl of the respective i7 Index Primer (**7001 - 7096**, in 96-well plate, supplied with the standard kits). **REMARK:** Ensure the total PCR volume is 35 µl, if necessary adjust the volume with Elution Buffer (**EB**) or molecular biology-grade water. Mix well by pipetting. Seal the PCR plate and quickly spin down to make sure all liquid is collected at the bottom of the well. **ATTENTION:** Spin down the 96-well plate containing i7 indices before opening! Pierce or cut open the sealing foil of the wells containing the desired barcodes. Reseal opened wells of the barcode plate after use to prevent cross contamination!

5 Conduct 11 - 25 cycles of PCR (determine the required cycle number by qPCR, see also CORALL and QuantSeq User Guides) with the following program: Initial denaturation for 30 seconds at 98 °C, 11 - 27 cycles for 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.

Purification

The finished library is purified from PCR components that can interfere with quantification. The Purification Beads (**PB**) may have settled and must be properly resuspended before adding them to the reaction.

ATTENTION: Important information for purification of dual-indexed libraries!

- The following purification protocol replaces the post-PCR purification described in steps 29 - 41 of the QuantSeq Kits and steps 32 - 45 of the CORALL Kit.
- If PCR products were stored at -20 °C, ensure these are thawed and equilibrated to room temperature before Purification Beads (**PB**) are added.

For **QuantSeq (standard input >10 ng)** libraries, add 35 µl of properly resuspended Purification Beads (**PB**) to each reaction. For **CORALL** libraries, and **QuantSeq libraries generated from low input (≤10 g) / low quality / FFPE RNA**, add only 31.5 µl **PB**. Mix well, and incubate for 5 minutes at room temperature.

7 Place the plate onto a magnet and let the beads collect for 2 - 5 minutes, or until the supernatant is completely clear.

8 Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.

9 Add 30 µl of Elution Buffer (**EB**), remove the plate from the magnet, and resuspend the beads fully in **EB**. Incubate for 2 minutes at room temperature.

10 Add 30 µl of Purification Solution (**PS**) to the beads / **EB** mix to re-precipitate the library. Mix thoroughly and incubate for 5 minutes at room temperature.

11 Place the plate onto a magnetic plate and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.

12 Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.

13 Add 120 µl of 80 % **EtOH** and wash the beads for 30 seconds. Leave the plate in contact with the magnet as beads should not be resuspended during this washing step. Remove and discard the supernatant.

14 Repeat this washing step once for a total of two washes. Make sure to remove the supernatant completely.

15 Leave the plate in contact with the magnet and let the beads dry for 5 - 10 minutes or until all ethanol has evaporated. **ATTENTION:** Dry the beads only at room temperature and do not let the beads dry too long (visible cracks appear), this will negatively influence the elution and hence the resulting library yield.

16 Add 20 µl of Elution Buffer (**EB**) per well, remove the plate from the magnet, and resuspend the beads properly in **EB**. Incubate for 2 minutes at room temperature.

17 Place the plate onto a magnetic plate and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.

18 Transfer 15 - 17 µl of the supernatant into a fresh PCR plate. Make sure not to transfer any beads.

19 At this point, the libraries are finished and ready for quality control, pooling (for multiplexing, see also Appendix A, p.9), and sequencing. For more details please refer to the respective QuantSeq or CORALL User Guides.

4. Appendix A: Multiplexing

Libraries prepared with dual indexing using the Lexogen i5 6 nt Dual Indexing Add-on Kits, in combination with Lexogen i7 6 nt Index Set (Cat. No. 044.96, or included in all full-sized QuantSeq 3' mRNA-Seq, QuantSeq-Flex and CORALL Library Prep Kits, Cat. No. 001, 015, 016, 033, 034, 035, 095, 096), are suitable for sequencing on all Illumina instruments listed below.

Depending on the instrument workflow and flow cell type (paired-end, PE; single-read, SR), i5 indices are sequenced in either forward or reverse complement. A file containing the complete set of forward and reverse complement i5 index sequences is available for download from www.lexogen.com/support-tools/index-balance-checker/.

i5 Index Sequences (Forward Orientation)

The following Illumina instruments read the i5 index in forward orientation:

- HiSeq® 2000/2500 (all SR and PE flow cells)
- HiSeq® 3000/4000 (SR flow cells only)
- MiSeq® (all (PE) flow cells)
- NovaSeq™ 6000 with v1.0 reagent kits (all (PE) flow cells)

For these instruments the Index 2 Read (i5) is primed using the Grafted P5 Oligo on the flow cell (or the Index 2 (i5) Sequencing Primer for SR HiSeq® 2000/2500 flow cells). Therefore, the i5 index sequences must be entered into the sample sheet in the **forward** orientation (see Table 1). If an 8-cycle index read length is used, the next two nucleotides of the downstream adapter sequence (i.e., AC), should be added to the end of the index sequence. For example 5001 becomes CGCCAT**AC**.

Table 1. i5 index sequences - forward orientation

	1	2	3	4	5	6	7	8	9	10	11	12
A	5001: CGCCAT	5009: AGCGCC	5017: GAATTG	5025: GAGTCC	5033: ATGTGG	5041: TCATAA	5049: TCAGCC	5057: ATCAGA	5065: CTTCTT	5073: GCTCTG	5081: TATCAA	5089: CGGAGT
B	5002: ATTTTA	5010: GCACAT	5018: ATTCGG	5026: AGACTA	5034: CTCAAC	5042: CGAGGG	5050: GAAAAC	5058: TCGGTG	5066: GACTGA	5074: TAATCT	5082: CGTGCT	5090: TCCAGC
C	5003: GCAACG	5011: CGCGTA	5019: CTGCGC	5027: ATTAAT	5035: TCTCCT	5043: ATCGAG	5051: TACCGT	5059: CGCTGC	5067: ATAGCA	5075: TAGTTA	5083: GCGATC	5091: GGGTAG
D	5004: TAGGGC	5012: GATTAT	5020: CGAAAA	5028: TCCCTA	5036: CGGCTG	5044: GACACT	5052: AGTGTG	5060: TCAATT	5068: CGGGAC	5076: CTCCCA	5084: GCATGC	5092: AGATCG
E	5005: TAGCCG	5013: ATGACC	5021: TCGTGT	5029: CTCGGT	5037: GCCTTT	5045: CGTATC	5053: CTTTCC	5061: GAACCA	5069: TGTAAG	5077: AGCCGG	5085: GAGGAA	5093: GCAGTA
F	5006: GCTGAC	5014: CTGATA	5022: AGTACA	5030: GAGAGG	5038: TAGGCA	5046: TAACTC	5054: AGGCCT	5062: CGATTT	5070: GCTAGT	5078: CTACAG	5086: ATATAC	5094: GCCGGG
G	5007: AGCATT	5015: TCACGG	5023: GACGTC	5031: CCTTAG	5039: GATATA	5047: CTAACT	5055: CTGGCG	5063: TCGCAC	5071: AGCTAA	5079: GAAGGT	5087: TCGACA	5095: AGTCAC
H	5008: CTATGA	5016: TATTGG	5024: TCCGAT	5032: CGACCC	5040: AGAAGC	5048: GCGCGA	5056: GCCAAA	5064: GATGCG	5072: TACATG	5080: ATGGTT	5088: AGGTTC	5096: CTGTAT

i5 Index Sequences (Reverse Complement Orientation)

The following Illumina instruments read the i5 index in reverse complement orientation:

- iSeq 100™ (PE flow cell)
- MiniSeq™ (all (PE) flow cells)
- NextSeq® 500/550/2000 (all (PE) flow cells)
- HiSeq® 3000/4000 (PE flow cells only)
- NovaSeq™ 6000 with v1.5 reagent kits (all (PE) flow cells)

For these instruments the Index 2 Read (i5) is performed after Read 2 Resynthesis, using the Index 2 (i5) Sequencing Primer (see also Appendix B, p.12). Therefore, the i5 index sequences must be entered into the sample sheet in the **reverse complement** orientation (i5rc, see Table 2). If an 8-cycle index read length is used, the next two nucleotides of the downstream adapter sequence, (i.e., GT), should be added to the end of the index sequence. For example, 5001 becomes ATGGCG**GT**.

Table 2. i5rc index sequences - reverse complement orientation

	1	2	3	4	5	6	7	8	9	10	11	12
A	5001rc: ATGGCG	5009rc: GGCGCT	5017rc: CAATTC	5025rc: GGACTC	5033rc: CCACAT	5041rc: TTATGA	5049rc: GGCTGA	5057rc: TCTGAT	5065rc: AAGAAG	5073rc: CAGAGC	5081rc: TTGATA	5089rc: ACTCCG
B	5002rc: TAAAT	5010rc: ATGTGC	5018rc: CGGAAT	5026rc: TAGTCT	5034rc: GTTGAG	5042rc: CCCTCG	5050rc: GTTTTC	5058rc: CACCGA	5066rc: TCAGTC	5074rc: AGATTA	5082rc: AGCACG	5090rc: GCTGGA
C	5003rc: CGTTGC	5011rc: TACGCG	5019rc: GCGCAG	5027rc: ATTAAT	5035rc: AGGAGA	5043rc: CTCGAT	5051rc: ACGGTA	5059rc: GCAGCG	5067rc: TGCTAT	5075rc: TAACTA	5083rc: GATCGC	5091rc: CTACCC
D	5004rc: GCCCTA	5012rc: ATAATC	5020rc: TTTTCG	5028rc: TAGGGA	5036rc: CAGCCG	5044rc: AGTGTC	5052rc: CACACT	5060rc: AATTGA	5068rc: GTCCCG	5076rc: TGGGAG	5084rc: GCATGC	5092rc: CGATCT
E	5005rc: CGGCTA	5013rc: GGTCAT	5021rc: ACACGA	5029rc: ACCGAG	5037rc: AAAGGC	5045rc: GATACG	5053rc: GGAAAG	5061rc: TGGTTC	5069rc: CTTACA	5077rc: CCGGCT	5085rc: TTCCTC	5093rc: TACTGC
F	5006rc: GTCAGC	5014rc: TATCAG	5022rc: TGTAAT	5030rc: CCTCTC	5038rc: TGCGTA	5046rc: GAGTTA	5054rc: AGGCCT	5062rc: AAATCG	5050rc: ACTAGC	5078rc: CTGTAG	5086rc: GTATAT	5094rc: CCCCGC
G	5007rc: AATGCT	5015rc: CCGTGA	5023rc: GACGTC	5031rc: CTAAGG	5039rc: TATATC	5047rc: AGTTAG	5055rc: CGCCAG	5063rc: GTGCGA	5071rc: TTAGCT	5079rc: ACCTTC	5087rc: TGTCGA	5095rc: GTGACT
H	5008rc: TCATAG	5016rc: CCAATA	5024rc: ATCGGA	5032rc: GGGTGC	5040rc: GCTTCT	5048rc: TCGCGC	5056rc: TTTGCC	5064rc: CGCATC	5072rc: CATGTA	5080rc: AACCAT	5088rc: GAACCT	5096rc: ATACAG

NOTE: Using the full set of 96 i5 indices, together with the 96 i7 indices provided in Lexogen's i7 6 nt Index Set (Cat. No. 044.96, also included in all full-sized QuantSeq / CORALL Library Prep Kits), a maximum of 9,216 different i5 / i7 index combinations can be achieved. Alternatively, up to 96 uniquely dual-indexed libraries can be multiplexed for sequencing, without repeating the use of any of the i5 or i7 indices.

Index Balance

In general, it is important that each nucleotide (A, C, G, and T) is present at each position of the index reads (Index 1 Read, i7; and Index 2 Read, i5), and that the signal intensity of each nucleotide is perfectly balanced to maintain optimal base calling accuracy and read quality. This is particularly critical for instruments that use two-channel detection (e.g., NextSeq®, MiniSeq™, and NovaSeq™), in which: T is labeled with a green fluorophore, C is labeled with red, half of A is labeled red and the other half is labeled green, and G is unlabeled.

The index balance should be checked for both the i5 and i7 index sets separately when designing the multiplexing strategy for dual-indexed libraries. To determine the optimal balance for each index read (i5 and i7) for the specific number of libraries to be sequenced, we recommend using Lexogen's online Index Balance Checker tool, available at www.lexogen.com/support-tools/index-balance-checker/.

In general, using the i5 indices in numerical order as the number of libraries to multiplex increases, or column-wise for increasing multiples of 8 samples, will result in optimal nucleotide balance for Index 2 Read (i5). For smaller numbers of samples we can also suggest the following:

- **Four libraries:** Use indices **5001 - 5004** as these contain perfect nucleotide balance at each position of the index read.
- **Eight libraries:** Use column 1 of the Lexogen i5 6 nt Index Set (indices **5001 - 5008**).

For suggestions and guidelines regarding i7 index selection for multiplexing please also refer to the QuantSeq 3' mRNA-Seq, QuantSeq-Flex and CORALL Total RNA-Seq User Guides.

NOTE: Individual libraries within a lane or run should always be pooled at an equimolar ratio to preserve perfect nucleotide balance at each position of the index read. If non-equimolar library pooling is required, the relative molarity of each library can be adjusted using the Index Balance Checker tool, to ensure that optimal index balance is preserved.

5. Appendix B: Sequencing*

The workflow for dual-indexed library sequencing differs, depending on the Illumina instrument and flow cell type. Dual indexing can be performed on single-read (SR) and paired-end (PE) flow cells. All HiSeq® systems support SR and PE flow cells. NextSeq®, MiniSeq™, MiSeq®, iSeq™ and NovaSeq™ systems use PE flow cells only, which can also be used in single-read mode. In general there are four different workflows used for Index 2 Read (i5) read-out on Illumina instruments, which are summarized in the Table 3 below.

Table 3. Sequencing workflows for dual-indexed libraries on Illumina instruments

Flow Cell Type	Workflow ¹	Instrument(s)	i5 Index Read Primer	i5 Index Read Orientation	No. Chemistry-Only Cycles ²
SR	1	HiSeq® 2000/2500	Index 2 (i5) Sequencing Primer	Forward (i5)	0
	2	HiSeq® 3000/4000	Grafted P5 Oligo		7
PE	3	HiSeq® 2000/2500 MiSeq® NovaSeq™ 6000 (v1.0 reagent kits)			
	4	iSeq 100 MiniSeq™ NextSeq® 500-2000 HiSeq® 3000/4000 NovaSeq™ 6000 (v1.5 reagent kits)	Index 2 (i5) Sequencing Primer	Reverse complement (i5rc)	0

¹Workflows 3 and 4 correspond to Illumina's Dual-Indexed Workflows A and B on paired-end flow cells, respectively.

²Additional chemistry-only (no-imaging) cycles are performed before the i5 index is read-out.

The order of sequencing for Workflows 1 - 3 is: Read 1, index read preparation, Index 1 Read (i7), Index 2 Read (i5), plus Read 2 Resynthesis, and Read 2 for paired-end flow cells / runs.

The order of sequencing for Workflow 4 is: Read 1, index read preparation, Index 1 Read (i7), Read 2 Resynthesis, and Index 2 Read (i5), plus Read 2 for paired-end runs.

For paired-end runs, Read 2 is read-out after the Index 2 Read (i5). The order of Read 2 Resynthesis with respect to the Index 2 Read (i5) determines whether or not the i5 index is sequenced in forward or reverse complement orientation.

For QuantSeq REV libraries the Custom Sequencing Primer Version 5 (**CSP** ●, included with the QuantSeq 3' mRNA-Seq Kits for Illumina (REV, Cat. No. 016)) must be used for Read 1 instead of the Multiplexing Read 1 Sequencing Primer.

ATTENTION: Important information for QuantSeq REV library sequencing!

- Do not use Multiplex Read 1 Sequencing Primer for QuantSeq REV libraries! This will result in a failed sequencing run as cluster calling would be impossible due to the poly(T) stretch.
- Do not mix the **CSP** ● Version 5 and Read 1 Sequencing Primers! A primer mixture would result in low cluster calls and the resulting reads would be contaminated by poly(T) stretches.

* Note: Some nucleotide sequences shown in Appendix B may be copyrighted by Illumina, Inc.

Sequencing Workflows for Dual-Indexed Libraries

The following depict the library adapters and sequencing primer binding sites for QuantSeq / CORALL libraries. The sequences of the relevant Index 2 Read (i5) Sequencing Primers are also provided.

Workflow 1: Single-Read Flow Cells - HiSeq® 2000 / 2500

The Index 2 (i5) Sequencing Primer is required for Index 2. A minimum of six cycles with imaging are required for the i5 read.

Index 2 Read (i5): Index 2 (i5) Sequencing Primer (not supplied):

5' AATGATACGGCGACCACCGAGATCTACAC 3'

QuantSeq FWD, QuantSeq-Flex, and CORALL libraries:

```
5'-(Index 2 (i5) Sequencing Primer)-3'      5'-(Read 1 Sequencing Primer)-3'
5'AATGATACGGCGACCACCGAGATCTACAC-i5-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-(Insert...
3'TTACTATGCCGCTGGTGGCTCTAGATGTG-i5-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-(Insert...

5'-(Index 1 (i7) Sequencing Primer)-3'
...Insert)-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-i7-ATCTCGTATGCCGTCTTCTGCTTG 3'
...Insert)-TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-i7-TAGAGCATACGGCAGAAGACGAAC 5'
```

QuantSeq REV libraries:

```
5'-(Index 2 (i5) Sequencing Primer)-3'      5'-(Read 1 Custom Sequencing Primer)-3'
5'AATGATACGGCGACCACCGAGATCTACAC-i5-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-TTTTTTTTTTTTTTTTT-(Insert...
3'TTACTATGCCGCTGGTGGCTCTAGATGTG-i5-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-(Insert...

5'-(Index 1 (i7) Sequencing Primer)-3'
...Insert)-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-i7-ATCTCGTATGCCGTCTTCTGCTTG 3'
...Insert)-TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-i7-TAGAGCATACGGCAGAAGACGAAC 5'
```

Workflow 2: Single-Read Flow Cells - HiSeq® 3000 / 4000

The Grafted P5 Oligo is used to initiate index 2 (i5) read-out. Seven additional chemistry-only cycles (no imaging) plus a minimum of six cycles (with imaging) are required to read the i5 index.

Index 2 Read (i5): Grafted P5 Oligo on Flow Cell (not supplied):

5' AATGATACGGCGACCACCGAGA 3'

QuantSeq FWD, QuantSeq-Flex, and CORALL libraries:

```
5'-(Grafted P5 Oligo)-3'      5'-(Read 1 Sequencing Primer)-3'
5'AATGATACGGCGACCACCGAGATCTACAC-i5-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-(Insert...
3'TTACTATGCCGCTGGTGGCTCTAGATGTG-i5-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-(Insert...

5'-(Index 1 (i7) Sequencing Primer)-3'
...Insert)-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-i7-ATCTCGTATGCCGTCTTCTGCTTG 3'
...Insert)-TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-i7-TAGAGCATACGGCAGAAGACGAAC 5'
```

QuantSeq REV libraries:

```
5'-(Grafted P5 Oligo)-3'      5'-(Read 1 Custom Sequencing Primer)-3'
5'AATGATACGGCGACCACCGAGATCTACAC-i5-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-TTTTTTTTTTTTTTTTT-(Insert...
3'TTACTATGCCGCTGGTGGCTCTAGATGTG-i5-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-(Insert...

5'-(Index 1 (i7) Sequencing Primer)-3'
...Insert)-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-i7-ATCTCGTATGCCGTCTTCTGCTTG 3'
...Insert)-TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-i7-TAGAGCATACGGCAGAAGACGAAC 5'
```

Workflow 3: Paired-End Flow Cells - HiSeq® 2000 / 2500, MiSeq®, and NovaSeq™ 6000 (v1.0 reagent kit)

The Grafted P5 Oligo is used to initiate index 2 (i5) read-out. Seven additional chemistry-only cycles (no imaging) plus a minimum of six cycles (with imaging) are required to read the i5 index.

Index 2 Read (i5): Grafted P5 Oligo on Flow Cell (not supplied):

5' AATGATACGGCGACCACCGAGA 3'

QuantSeq FWD, QuantSeq-Flex, and CORALL libraries:

```
5'-(Grafted P5 Oligo)-3'          5'-(Read 1 Sequencing Primer)-3'
5' AATGATACGGCGACCACCGAGA TCTACAC-i5-ACACTCTTTCCCTACACGACGCTCTTCCGATCT- (Insert...
3' TTACTATGCGCTGGTGGCTCTAGATGTG-i5-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA- (Insert...

5'-(Index 1 (i7) Sequencing Primer)-3'
...Insert)-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-i7-ATCTCGTATGCCGTCTTCTGCTTG 3'
...Insert)-TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-i7-TAGAGCATACGGCAGAAGACGAAC 5'
3'-(Read 2 Sequencing Primer)-5'
```

QuantSeq REV libraries:

```
5'-(Grafted P5 Oligo)-3'          5'-(Read 1 Custom Sequencing Primer)-3'
5' AATGATACGGCGACCACCGAGA TCTACAC-i5-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-TTTTTTTTTTTTTTTT- (Insert...
3' TTACTATGCGCTGGTGGCTCTAGATGTG-i5-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA- (Insert...

5'-(Index 1 (i7) Sequencing Primer)-3'
...Insert)-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-i7-ATCTCGTATGCCGTCTTCTGCTTG 3'
...Insert)-TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-i7-TAGAGCATACGGCAGAAGACGAAC 5'
3'-(Read 2 Sequencing Primer)-5'
```

Workflow 4: Paired-End Flow Cells - iSeq™, MiniSeq™, NextSeq®, HiSeq® 3000 / 4000 and NovaSeq™ 6000 (v1.5 reagent kit)

All instruments use a Index 2 (i5) Sequencing Primer, which is included in the “Dual-Indexing Primer Mix” for iSeq™, MiniSeq™ and NextSeq®, and in HP14 for HiSeq® 3000 / 4000. A minimum of six cycles (with imaging) are required to read the i5 index.

ATTENTION: Index 2 (i5) is read-out after the Read 2 Resynthesis step, hence a reverse complement of the Index 2 (i5) primer sequence is produced (see also Appendix A, p.9).

Index 2 Read (i5): Index 2 (i5) Sequencing Primer (not supplied):

5' AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT 3'

QuantSeq FWD, QuantSeq-Flex, and CORALL libraries:

```
5'-(Read 1 Sequencing Primer)-3'
5' AATGATACGGCGACCACCGAGATCTACAC-i5-ACACTCTTTCCCTACACGACGCTCTTCCGATCT- (Insert...
3' TTACTATGCGCTGGTGGCTCTAGATGTG-i5-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA- (Insert...

3'-(Index 2 (i5) Sequencing Primer)-5'

5'-(Index 1 (i7) Sequencing Primer)-3'
...Insert)-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-i7-ATCTCGTATGCCGTCTTCTGCTTG 3'
...Insert)-TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-i7-TAGAGCATACGGCAGAAGACGAAC 5'
3'-(Read 2 Sequencing Primer)-5'
```

QuantSeq REV libraries on HiSeq® 3000 / 4000:

```
5'-(Read 1 Custom Sequencing Primer)-3'
5'AATGATACGGCGACCACCGAGATCTACAC-i5-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-TTTTTTTTTTTTTTTT-(Insert...
3'TTACTATGCCCTGGTGGCTCTAGATGTG-i5-FGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-(Insert...
3'-(Index 2 (i5) Sequencing Primer)-5'

5'-(Index 1 (i7) Sequencing Primer)-3'
...Insert)-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-i7-ATCTCGTATGCCGTCTTCTGCTTG 3'
...Insert)-TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-i7-TAGAGCATACGGCAGAAGACGAAC 5'
3'-(Read 2 Sequencing Primer)-5'
```

QuantSeq REV libraries on iSeq™, MiniSeq™ and NextSeq® and NovaSeq™ 6000:

ATTENTION: Please contact support@lexogen.com if you wish to run QuantSeq REV on iSeq™, MiniSeq™ and NextSeq®.

Sequencing Primers

Standard Illumina sequencing primers are used for all dual-indexed libraries, with the exception of QuantSeq REV libraries, which require the Custom Sequencing Primer V5 (**CSP** ●, included in the REV Kits, Cat. No. 016) for Read 1. For further details of **CSP** ● usage and instrument compatibility, please refer to the QuantSeq 3' mRNA-Seq User Guide 015UG009.

The Read 1 Sequencing Primer is always used for Read 1 sequencing, and the Index 1 (i7) Sequencing Primer is always used for Index 1 Read (i7) sequencing. The Index 2 Read (i5) is initiated using different sequencing primers specific to the instrument and flow cell type as outlined previously (see p.12).

Read 1 for QuantSeq FWD, QuantSeq Flex, and CORALL libraries:

Read 1 Sequencing Primer (not supplied):

5'ACACTCTTTCCCTACACGACGCTCTTCCGATCT 3'

Read 1 for QuantSeq REV:

Custom Sequencing Primer Version 5 (**CSP** ●, included in 016 QuantSeq REV Kit):

5'ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTTTTTTTTTTTTTTTTTT 3'

Index 1 Read (i7): Index 1 (i7) Sequencing Primer (not supplied):

5'GATCGGAAGAGCACACGTCTGAACTCCAGTCAC 3'

Index 2 Read (i5):

Workflow 1: Index 2 (i5) Sequencing Primer (not supplied):

5'AATGATACGGCGACCACCGAGATCTACAC 3'

Workflows 2 and 3: Grafted P5 Oligo on Flow Cell (not supplied):

5'AATGATACGGCGACCACCGAGA 3'

Workflow 4: Index 2 (i5) Sequencing Primer (not supplied):

5'AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT 3'

Read 2: Read 2 Sequencing Primer (not supplied):

5'GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

Multiplexing with Other Library Types

We do not recommend multiplexing Lexogen libraries with libraries from other vendors in the same sequencing lane.

Though this is possible in principle, specific optimization of index combinations, library pooling conditions, and loading amounts may be required, even for advanced users. Sequencing complex pools that include different library types at different lane shares may have unpredictable effects on sequencing run metrics, read quality, read outputs, and / or demultiplexing performance. Lexogen assumes no responsibility for the altered performance of Lexogen libraries sequenced in combination with external library types in the same lane (or run).

Due to size differences, libraries prepared with the Lexogen Small RNA-Seq Library Prep Kit (or any other small RNA library prep kit) should not be sequenced together with QuantSeq, QuantSeq-Flex or CORALL libraries. Please refer to the sequencing guidelines for each library type (library adapter details, loading amounts to use, and use of custom sequencing primers, etc), which are provided in our Library Prep Kit User Guides, and online Frequently Asked Questions (FAQs).

6. Appendix C: Library Reamplification

If your library yields are extremely low and insufficient for pooling, reamplification can be performed using the Reamplification Add-on Kit for Illumina (080.96). This kit is available only upon request. Please contact Lexogen at support@lexogen.com.

Please note that the PCR Add-on Kit (Cat. No. 020) **cannot** be used for reamplification of dual-indexed libraries.

7. Appendix D: Data Analysis

Demultiplexing

Demultiplexing can be carried out by the standard Illumina pipeline. Index sequences (i7: **7001 - 7096**, and i5: **5001 - 5096**) are available for download at www.lexogen.com.

8. Appendix E: Revision History

Publication No. / Revision Date	Change	Page
047UG109V0300 Jan. 3, 2023	Updated Kit Components Figure 2 and Table to reflect current packaging and storage requirements.	5
	Added iSeq 100 and NovaSeq 6000 (chemistry v 1.5) to Table 3.	12
047IM109V0211 Dec. 31, 2019	Legal terms and conditions statements updated.	2
	Overview text edited: not compatible with Small RNA-Seq Library Prep Kit.	3
	SENSE Total kit and 047.4x24 references removed.	3 - 17
047IM109V0210 Mar. 12, 2019	Kit naming changed to "Lexogen i5 6 nt Dual Indexing Add-on Kits (5001-5096)", and kit component naming changed to "Lexogen i5 6 nt Index Sets (5001 - 5004, and 5001 - 5096)".	
	Custom Sequencing Primer (CSP) changed to Version 5, sequence and schematics updated.	10 - 13
	Notes for dual indexing of CORALL Total RNA-Seq libraries added.	2, 4-7, 9, 11-13
	Updated sequencing guidelines for Lexogen libraries.	14
047IM109V0200 May 24, 2018	Addition of 047.96 i5 Index Plate, 96 indices (5001-5096), Kit Components.	2, 3
	Updated Appendix A text and sample multiplexing information.	7-9
	Updated Appendix B text for dual indexing sequencing workflows.	10-13
	Updated Appendix C text for reamplification of dual-indexed libraries.	14
047IM109V0101 Feb. 7, 2017	Renaming of i5 Kit Components, Rearranged i7 Index Plate reference added.	2-11
047IM109V0100 Dec. 5, 2016	Initial Release.	

Associated Products:

- 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)
- 080 (Reamplification Add-on Kit for Illumina)
- 095 (CORALL Total RNA-Seq Library Prep Kit)
- 096 (CORALL Total RNA-Seq Library Prep Kit with RiboCop)

Lexogen i5 6 nt Dual Indexing Add-on Kits (5001-5096) · User Guide

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