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Enabling complete transcriptome sequencing

i5 Dual Indexing Add-on Kits for QuantSeq/SENSE (5001-5096) **Instruction Manual**

Catalog Numbers:

047 (i5 Dual Indexing Add-on Kits for QuantSeq/SENSE (5001-5096))

- 001 (SENSE mRNA-Seq Library Prep Kit V2 for Illumina)
- 009 (SENSE Total RNA-Seq Library Prep Kit for Illumina)
- 015 (QuantSeq 3' mRNA-Seq Library Prep Kit for Illumina (FWD))
- 016 (QuantSeq 3' mRNA-Seq Library Prep Kit for Illumina (REV) with Custom Sequencing Primer)
- 020 (PCR Add-on Kit for Illumina)
- 022 (Purification Module with Magnetic Beads)
- 033 (QuantSeq-Flex Targeted RNA-Seq Library Prep Kit V2 with First Strand Synthesis (Nodule)
- 034 (QuantSeq-Flex Targeted RNA-Seq Library Prep Kit V2 with Second Strand Synthesis (Module V2)
- 035 (QuantSeq-Flex Targeted RNA-Seq Library Prep Kit V2 with First and Second Strand Synthesis Modules)

080 (Reamplification Add-on Kit for Illumina)

1. Overview

This instruction manual outlines the protocol for Lexogen's i5 Dual Indexing Add-on Kits for QuantSeq/SENSE.

The i5 Dual Indexing Add-on Kit for QuantSeq/SENSE (5001 - 5004, Cat. No. 047.4x24, 047.4x96) contain four perfectly balanced i5 indices **5001 - 5004**. This kit is designed for non-unique dual indexing.

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

Lexogen's i7 (Index 1) and i5 (Index 2) indices are 6 nt 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), SENSE mRNA-Seq V2 for Illumina (Cat. No. 001), and SENSE Total RNA-Seq for Illumina (Cat. No. 009, 042). The i7 indices are always included in the standard library prep kits or can be purchase separately (Cat. No. 044.96). Using the i5 and i7 Index Plates together, up to 9,216 different i5 / i7 index combinations are possible (see Appendix A, p.7).

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

- The **Dual PCR Mix** (**Dual PCR** ●) supplied with these i5 Dual Indexing Add-on Kits **must** be used instead of the PCR Mix from the standard library prep kits (**PCR** from QuantSeq and SENSE mRNA or **PCR** O from SENSE Total).
- 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.6). Please follow the purification protocol steps listed on p.5-6 of this instruction manual to purify dual-indexed libraries.
- Each well of the i5 Index Plate (Cat. No. 047.96) contains a sufficient volume for one library prep per index and is intended for **single use only!**
- Spin down the i5 and i7 Index Plates 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 SENSE 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.7). For sequencing details including i5 read orientation and dual index read-out workflows for different flow cells and instruments see Appendix B (p.10).

2. Kit Components and Storage Conditions

2.1 i5 Dual Indexing Add-on Kit for QuantSeq/SENSE (5001 - 5004), Cat. No. 047.4x24, 047.4x96



Figure 1. Location of kit contents for Cat. No. 047.4. The dotted PCR tube is only included for 047.4x96.

Kit Component (Cat. No. 047.4)	Tube Label	Volume* 047.4x24	Volume* 047.4x96	Storage
Dual PCR Mix	Dual PCR	740 µl	2,957 µl	-20 °C
i5 Index 01	5001 🔍	132 µl	528 µl	-20 °C
i5 Index 02	5002 🔵	132 µl	528 µl	-20 °C
i5 Index 03	5003 🔵	132 µl	528 µl	-20 °C
i5 Index 04	5004 🔍	132 µl	528 µl	-20 °C

*including 10 % surplus

2.2 i5 Unique Dual Indexing Add-on Kit for QuantSeq/SENSE (5001 - 5096), Cat. No. 047.96





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

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

*including 10 % surplus

NOTE: Each i5 Index Plate contains sufficient volume for one library prep per index and 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, 015UG058, 015UG110), steps 35 - 38 of the SENSE mRNA-Seq V2 Kit User Guide (001UG004), or steps 23 - 26 of the SENSE Total RNA-Seq Kit User Guides (009UG013, 009UG102), respectively.

Preparation

PCR	Purification (Cat. No. 022)
From i5 Dual Indexing Add-on Kit: - thawed at RT Dual PCR - thawed at RT 5001 - 5004 or 5001 - 5096 (i5 Index Plate) - thawed at RT From standard library prep kits: - thawed at RT pening! 7001 - 7096 (i7 Index Plate) - thawed at RT pening! Enzyme Mix - keep on ice or at -20 °C	from standard library prep kits:PB- stored at +4°CPS- stored at +4°C80 % EtOH - provided by userprepare 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, ∞	

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. 020.96) and see Instruction Manual 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** O) from the standard library prep kit with the **Dual PCR Mix** (**Dual PCR** •) supplied in the i5 Dual Indexing Add-on Kits!
- The Enzyme Mix needed for the PCR is provided in the standard library prep kits: E3
 from QuantSeq Kits, E2
 from SENSE mRNA-Seq V2 Kit, E2 O from SENSE Total RNA-Seq Kit,
 or E
 from the PCR Add-on Kit. Do not use E2
 from QuantSeq Kits for this PCR!
- Each i5 Index Plate contains sufficient volume for one library prep per index and is intended for **single use only!**
- Spin down the i5 and i7 Index Plates 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**, **EB**, included in the QuantSeq / SENSE Kits) for step ⁶ at room temperature, to give them enough time to equilibrate.

Prepare a mastermix containing 7 µl PCR Mix from the i5 Dual Indexing Add-on Kit (**Dual PCR •**) and 1 µl **Enzyme Mix** from the standard library prep kits per reaction (see **ATTENTION** note p.4). **ATTENTION**: Do not use **E2** • from QuantSeq for the PCR reaction! Do not use **PCR •** or **PCR** 0 from the standard kits if dual indexing is intended.

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

Add 5 µl of the respective i5 Index Primer (**5001 - 5004** •, in microtubes or **5001 - 5096**, in 96-well plate, from the i5 Dual Indexing Add-on Kits). **ATTENTION:** Spin down the i5 Index Plate before opening! Pierce or cut open the sealing foil of the wells containing the desired barcodes. Avoid cross contamination! Reseal opened wells of the barcode plate after use to prevent cross contamination!

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 i7 Index Plate before opening! Pierce or cut open the sealing foil of the wells containing the desired barcodes. Avoid cross contamination! Reseal opened wells of the barcode plate after use to prevent cross contamination!

Conduct 11 - 27 cycles of PCR (see recommendations in SENSE and QuantSeq User Guides, or determine the cycle number to use by qPCR) with the following program: Initial denaturation at 98 °C for 30 seconds, 11 - 27 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. Crew Safe stopping point. Libraries can be stored at -20 °C at this point.

Purification

3

4

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, steps 39 52 of the SENSE mRNA-Seq V2 Kit, or steps 27 40 of the SENSE Total RNA-Seq Kit.
- If PCR products were stored at -20 °C, ensure these are thawed and equilibrated to room temperature before Purification Beads (**PB**) are added.

Add 35 μl of properly resuspended Purification Beads (PB) to each reaction, mix well,

- and incubate for 5 minutes at room temperature. ATTENTION: For SENSE Total RNA-Seq and for QuantSeq libraries generated from low RNA input or degraded RNA, add only 31.5 μl PB. For SENSE FFPE Total RNA-Seq library preps add only 29 μl PB.
- Place the plate onto a magnetic plate 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.
- Add 30 μl of Elution Buffer (EB), remove the plate from the magnet, and resuspend the beads properly in EB. Incubate for 2 minutes at room temperature.
- 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. **ATTENTION:** Add
- only 29 μl **PS** for SENSE FFPE Total RNA-Seq library preps.
- 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.
- 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.
- Repeat this washing step once for a total of two washes. Make sure to remove the supernatant completely.
- 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.
- $\begin{tabular}{l} \label{eq:add} Add 20\,\mu l \mbox{ of Elution Buffer (EB) per well, remove the plate from the magnet, and resuspend the beads properly in EB. Incubate for 2 minutes at room temperature. \end{tabular}$
- Place the plate onto a magnetic plate and let the beads collect for 2 5 minutes or until the supernatant is completely clear.
- Transfer 15 17 μ l of the supernatant into a fresh PCR plate. Make sure not to transfer any beads.

At this point, the libraries are finished and ready for quality control, pooling (for multiplexing, see also Appendix A, p.7), and sequencing. For more details please refer to the respective SENSE and QuantSeq User Guides.

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4. Appendix A: Multiplexing

Libraries prepared with dual indexing using the i5 Dual Indexing Add-on Kits, in combination with Lexogen's i7 Index Plate (Cat. No. 044.96, or included in all full-sized QuantSeq 3'mRNA-Seq, QuantSeq-Flex, SENSE mRNA-Seq, and SENSE Total RNA-Seq Library Prep Kits, Cat. No. 001, 009, 015, 016, 033, 034, 035, 042), 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 (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 CGCCATAC.

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

Table 1. i5 index sequences - forward orientation

i5 Index Sequences (Reverse Complement Orientation)

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

- MiniSeq[™] (all (PE) flow cells)
- NextSeq[®] 500/550 (all (PE) flow cells)
- HiSeq® 3000/4000 (PE flow cells only)

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.10). 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 ATGGCGGT.

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

Table 2. i5rc index sequences - reverse complement orientation

NOTE: Using the full set of 96 i5 indices, together with the 96 i7 indices provided in Lexogen's i7 Index Plate (Cat. No. 044.96, also included in all full-sized QuantSeq/SENSE 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[®], MiniSeqTM, and NovaSeqTM), 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 reads separately when designing the multiplexing strategy for dual-indexed libraries. To determine the optimal combinations of indices (i5 and i7) for the specific number of libraries to be sequenced, we recommend using Lexogen's online Index Balance Checker tool, available at <u>www.lexogen.com/</u> <u>support-tools/index-balance-checker/</u>.

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 i5 Index Plate (indices 5001 5008).

For suggestions and guidelines regarding i7 index selection for multiplexing please also refer to the QuantSeq 3' mRNA-Seq, QuantSeq-Flex, SENSE mRNA-Seq, and SENSE 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 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®, and NovaSeq™ systems use PE flow cells only. In general there are four different workflows used for Index 2 Read (i5) read-out on Illumina instruments, which are summarised in the Table 3 below.

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	Multiplexing Index 2 (i5) Sequencing Primer (HP9)		0
	2	HiSeq® 3000/4000		-	
	3	HiSeq® 2000/2500 MiSeq® NovaSeq™ 6000	Grafted P5 Oligo	Forward (i5)	7
PE	4	MiniSeq™ NextSeq® 500/550	Multiplexing Index 2 (i5) Sequencing Primer (HP14)	Reverse	
		HiSeq® 3000/4000	Multiplexing Index 2 (i5) Sequencing Primer (dual- indexing primer mix)	complement (i5rc)	U

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

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 2 (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 2 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/SENSE 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 (included in HP9) is required for Index 2. A minimum of six cycles with imaging are required for the i5 read.

Index 2 Read (i5): Multiplexing Index 2 (i5) Sequencing Primer (not supplied): 5' AATGATACGGCGACCACCGAGATCTACAC 3'

QuantSeq FWD, QuantSeq-Flex, SENSE mRNA, and SENSE Total libraries:

```
5'-(Index 2 (i5) Sequencing Primer)-3'
5' AATGATACGGCGACACCGAGATCTACAC-i5-ACACTCTTCCCTACACGACGCTCTCCGATCT-(Insert...
3'TTACTATGCCGCGCGAGATCTACAC-i5-ACACTCTTCCCTACACGACGCGCTCTCGGAGAAGGCTAGA-(Insert...
5'-(Index 1 (i7) Sequencing Primer)-3'
...Insert)-AGATCGGAAGGCACACGTCTGAACTCCAGTCAC-i7-ATCTCGTATGCCGTCTTGGTG 3'
...Insert)-TCTAGCCTTCTCGTGTGCAGACTCGAGGCTAGTG-i7-TAGAGCATACGGCAGAAGACGAAC 5'
QuantSeq REV libraries:
5'-(Index 2 (i5) Sequencing Primer)-3'
5'-(Index 2 (i5) Sequencing Primer)-3'
5'-(Read 1 Custom Sequencing Primer)-3'
```

5 '	'AATGATACGGCGACCACCGAGATCTACAC-15-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-TTTTTTTTTT	Insert
3 '	'TTACTATGCCGCTGGTGGCTCTAGATGTG-i5-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-(Insert	
	5'-(Index 1 (i7) Sequencing Primer)-3'	
	Insert)-A <mark>GATCGGAAGAGCACACGTCTGAACTCCAGTCAC</mark> -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, SENSE mRNA, and SENSE Total libraries:



Workflow 3: Paired-End Flow Cells - HiSeq $^{\rm o}$ 2000 / 2500, MiSeq $^{\rm o}$, and NovaSeq $^{\rm m}$

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'

3'-(Read 2 Sequencing Primer)-5'

QuantSeq FWD, QuantSeq-Flex, SENSE mRNA, and SENSE Total libraries:

5'-(Grafted P5 Oligo)-3' 5'-(Read	1 Sequencing Primer)-3'
5' AATGATACGGCGACCACCGAGATCTACAC-i5-ACACTCTT	CCCTACACGACGCTCTTCCGATCT-(Insert
3'TTACTATGCCGCTGGTGGCTCTAGATGTG-i5-TGTGAGAAA	GGGATGTGCTGCGAGAAGGCTAGA-(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' AATGATACGGCGACCACCGAGATCTACAC-i5-ACACTCTTT	CCCTACACGACGCTCTTCCGATCT-TTTTTTTTTTTTTTT
3'TTACTATGCCGCTGGTGGCTCTAGATGTG-i5-TGTGAGAAA	GGGATGTGCTGCGAGAAGGCTAGA-(Insert
5'-(Index 1 (i7) Sequencing Primer)-3'	
Insert)-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-	i7-ATCTCGTATGCCGTCTTCTGCTTG 3'
Insert) -TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-	i7-TAGAGCATACGGCAGAAGACGAAC 5'

Workflow 4: Paired-End Flow Cells - MiniSeq^m, NextSeq^{\circ}, and HiSeq^{\circ} 3000 / 4000

All instruments use a Multiplexing Index 2 (i5) Sequencing Primer, which is included in the "Dual-Indexing Primer Mix" for 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.7).

Index 2 Read (i5): Multiplexing Index 2 (i5) Sequencing Primer (not supplied): 5' AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT 3'

QuantSeq FWD, QuantSeq-Flex, SENSE mRNA, and SENSE Total libraries:



QuantSeq REV libraries on HiSeq® 3000 / 4000:



QuantSeq REV libraries on MiniSeq[™] and NextSeq[®]:

ATTENTION: Please contact <u>info@lexogen.com</u> if you wish to run QuantSeq REV on 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 V2 (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 Multiplexing 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.11-13).

Read 1 for QuantSeq FWD, QuantSeq Flex, SENSE mRNA, and SENSE Total:

Multiplexing Read 1 Sequencing Primer (not supplied): 5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT 3'

Read 1 for QuantSeq REV:

Index 1 Read (i7): Multiplexing Index 1 (i7) Sequencing Primer (not supplied): 5'GATCGGAAGAGACACACGTCTGAACTCCAGTCAC 3'

Index 2 Read (i5):

<u>Workflow 1:</u> Multiplexing Index 2 (i5) Sequencing Primer (not supplied): 5' AATGATACGGCGACCACCGAGATCTACAC 3' <u>Workflows 2 and 3:</u> Grafted P5 Oligo on Flow Cell (not supplied): 5' AATGATACGGCGACCACCGAGA 3' <u>Workflow 4:</u> Multiplexing Index 2 (i5) Sequencing Primer (not supplied):

5' AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT 3'

Read 2: Multiplexing Read 2 Sequencing Primer (not supplied): 5'GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

6. Appendix C: Library Reamplification

If your library yields are extremely low and insufficient for pooling reamplification of the libraries can be done.

For reamplification of single- or dual-indexed libraries the Reamplification Add-on Kit for Illumina (080.96) is available on request. Please contact Lexogen at <u>info@lexogen.com</u>.

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 <u>www.lexogen.com</u>.

8. Appendix E: Revision History

Revisions made to this Instruction Manual are indicated from 2016 onwards. The most recent updates are highlighted in green. The complete Revision History is available from the Support Tools page at www.lexogen.com/support-tools/.

Publication No. / Revision Date	Change	Page
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 reamplifiaction 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.	



i5 Dual Indexing Add-on Kits for QuantSeq/SENSE (5001-5096) · Instruction Manual

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