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i5 Dual Indexing Add-on Kit for QuantSeq/SENSE for Illumina

Instruction Manual

Catalog Numbers:
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 with First Strand Synthesis Module)
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)
047 (i5 Dual Indexing Add-on Kit for QuantSeq/SENSE for Illumina)

047IM109V0101

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ATTENTION: Barcodes rearranged!

047UI124V0100

The i5 Dual Indexing Add-on Kit for QuantSeq/SENSE has been updated!

ATTENTION: This Instruction Manual is only compatible with QuantSeq 3' mRNA-Seq kits bought after February 17th, 2017. If you are using the i5 Dual Indexing Add-on Kit with an older kit version, please contact info@lexogen.com to get the respective Instruction Manual.

Major changes of the update:

- New arrangement and renaming of the barcode plate to improve the nucleotide balance → i7 Index Plate (7001-7096), unique set of barcodes – no overlap with Illumina-specific indices (BC05 removed). An evaluation tool to check the color balance of index subsets is available on the Lexogen website.
- Barcode 00 (BC00) renamed to P7 Primer 7000.
- References to the QuantSeq protocols adjusted to be compatible with the upgraded version.

For more details please have a look at the Revision History at the end of this Instruction Manual or contact us under support@lexogen.com or +43 1 3451212 41.

1. Overview

This instruction manual outlines the protocol for Lexogen's i5 Dual Indexing Add-on Kit for Illumina including four perfectly balanced i5 Indices **5001-5004** (Cat. No. 047.4x24 and Cat. No. 047.4x96).

Lexogen's i7 (Index 1) and i5 (Index 2) indices can be introduced at the PCR step of SENSE mRNA-Seq V2 for Illumina (Cat. No. 001), SENSE Total RNA-Seq for Illumina (Cat. No. 009), QuantSeq 3' mRNA-Seq (Cat. No. 015 and Cat. No. 016), and QuantSeq-Flex Targeted RNA-Seq Library Prep Kits (Cat. No. 033-035). i7 indices are always included in the basic kits (i7 Index Plate, 96-well plate).
ATTENTION: When using the i5 Dual Indexing Add-on Kit for i5 indexing the PCR Mix from the basic kit (**PCR** ● or **PCR** ○) has to be exchanged with the PCR mix (**PCR** ●) supplied with the i5 Dual Indexing Add-on Kit. Be aware that Dual Indexing also uses a larger PCR volume than the standard single indexing PCR (i7 indices only) and hence the Post PCR Purification needs to be adjusted as outlined in the protocol on page 5 - 6.

Each of the four i5 indices can be combined with any of the 96 i7 indices (**7001-7096,** in a 96-well plate) included in Lexogen's SENSE mRNA-Seq V2, SENSE Total RNA-Seq, QuantSeq 3' mRNA-Seq, and QuantSeq-Flex Targeted RNA-Seq Library Prep Kits for Illumina, enabling up to 384 different barcoding options (see Appendix A, p.7 for multiplexing). Lexogen's i5 and i7 indices are 6 nt long.

Dual Indices are read out with the standard Illumina workflows (for details see Appendix B, p.8).

2. Kit Components and Storage Conditions

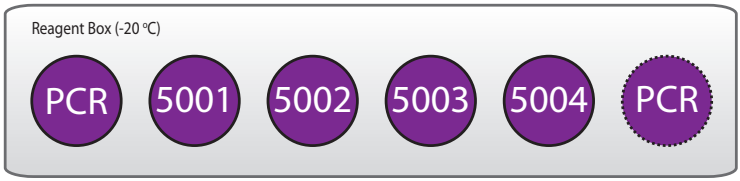

Figure 1. Location of kit contents. The dotted PCR tube is only included for 047.4x96.

Kit Component	Tube Label	Volume* in 047.4x24	Volume* in 047.4x96	Storage
PCR Mix	**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

ATTENTION: For dual indexing **REPLACE** the **PCR Mix** (**PCR** ● or **PCR** ○) from the basic kit with the **PCR Mix** (**PCR** ●) supplied in the i5 Dual Indexing Add-on Kit!

For index sequences and sample sheet entry see Appendix A, p.7. Details on index read-out on various Illumina sequencing machines can be found in Appendix B, p.8.

3. Detailed Protocol - Library Amplification

Preparation

PCR		Purification (Cat. No. 022)	
PCR ● (from **i5 Dual Indexing Add-on Kit**)	– thawed at RT	**PB**	– stored at +4°C
5001-5004 ● (from **i5 Dual Indexing Add-on Kit**)	– thawed at RT, **spin down before opening!**	**PS**	– stored at +4°C
7001-7096 (i7 Barcode Plate from basic kit)	– thawed at RT, **spin down before opening!**	**80 % EtOH**	– provided by user **prepare fresh!**
E2 ○ **or E2** ● **(from SENSE kits)**	– keep on ice or at -20 °C	**EB**	– stored at +4°C
E3 ● **(from QuantSeq kits)**	– keep on ice or at -20 °C		
Thermocycler 98 °C, 30 sec 98 °C, 10 sec 65 °C, 20 sec } 11- 27x 72 °C, 30 sec } see SENSE or QuantSeq User Guide recommendations 72 °C, 1 min or endpoint as determined by qPCR (Cat. No. 020.96) 10 °C, ∞ 96-well PCR plate or 8-well strip PCR sealing films Plate centrifuge		96-well magnetic plate 96-well PCR plate PCR sealing films Plate centrifuge	

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. The following PCR replaces the single indexing PCR described in steps 25 - 28 of the QuantSeq 3' mRNA-Seq and QuantSeq-Flex Targeted RNA-Seq Kits, steps 35 - 38 of the SENSE mRNA-Seq V2 Kit, or steps 23 - 26 of the SENSE Total RNA-Seq Kit, respectively. **ATTENTION:** The PCR Enzyme in SENSE Kits is called **E2** ● (SENSE mRNA-Seq V2) or **E2** ○ (SENSE Total RNA-Seq), and for QuantSeq Kits **E3** ●. Do not use **E2** ● from QuantSeq for the PCR reaction!

NOTE: For qPCR determination of the appropriate endpoint PCR cycle number use the PCR Add-on Kit (Cat. No. 020.96) and follow the instructions of 020IM064. The single indexing PCR (i7 only) of the PCR Add-on Kit and the dual indexing PCR (i5 and i7) run with the same efficiency, so there is no need to exchange any solutions.

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.

1 Prepare a mastermix containing 7 µl PCR Mix from the i5 Dual Indexing Add-on Kit (**PCR** ●) and 1 µl Enzyme Mix 3 (**E3** ●) from the QuantSeq basic kits, 1 µl Enzyme Mix 2 (**E2** ●) from SENSE mRNA-Seq V2, or 1 µl Enzyme Mix 2 (**E2** ○) from SENSE Total RNA-Seq per reaction. **ATTENTION:** Do not use **E2** ● from QuantSeq for the PCR reaction! Do not use **PCR** ● or **PCR** ○ from the basic kits if dual indexing is intended.

2	Add 8 µl of this **PCR** / Enzyme mastermix to 17 µl of the eluted library.
3	Add 5 µl of the respective i5 Index Primer (**5001-5004** ●, provided in microtubes with the i5 Dual Indexing Add-on Kit).
4	Add 5 µl of the respective i7 index primer (**7001-7096,** provided in 96-well plate, supplied with the basic kits). 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 barcode 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 usage to prevent cross contamination!
5	Conduct 11 - 27 cycles of PCR (see recommendations in SENSE and QuantSeq User Guides, or determine the optimal number of cycles by qPCR using the PCR Add-on Kit (Cat. No. 020.96)) 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. ☞ 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.
The following purification replaces the Post PCR Purification described in steps 29 - 41 of the QuantSeq 3' mRNA-Seq and QuantSeq-Flex Targeted RNA-Seq, steps 39 - 52 of the SENSE mRNA-Seq V2, or steps 27 - 40 of the SENSE Total RNA-Seq Kit.

6	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, SENSE mRNA-Seq, and for QuantSeq libraries generated from low RNA input and/or degraded RNA, add only 31.5 µl **PB**. For SENSE FFPE Total RNA-Seq library preps add only 29 µl **PB**.
7	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.
9	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.
10	Add 30 µl of Purification Solution (**PS**) to the beads / **EB** mix to reprecipitate 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.

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:** Do not let the beads dry too long (visible cracks appear) as 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.7), and cluster generation. For more details please refer to the respective SENSE and QuantSeq User Guides.

4. Appendix A: Multiplexing

The i5 Dual Indexing Add-on Kit contains 4 perfectly balanced i5 indices (**5001-5004** ●) suitable for every Illumina sequencing machine. Each nucleotide (A, C, G, and T) is present at each position of the 6 nt long index. This is of particular importance for NextSeq and MiniSeq sequencing platforms, where only a two-channel readout is used: T is labeled with green, C is labeled with red, half of A is a different red and the other half is a different green, and G remains unlabeled.

i5 read-out is performed during the Index 2-specific sequencing reaction. Depending on the Illumina sequencing machine there are differences when and how Index 2 is read (for details see Appendix B, p. 8). This affects the way the i5 indices have to be entered into the corresponding sample sheets. **ATTENTION:** A dual-indexed sequencing run on the MiniSeq, NextSeq, HiSeq 3000, or HiSeq 4000 performs the Index 2 Read after the Read 2 resynthesis step, hence here the reverse complement of the index is read. Details on how to enter the i5 indices on the respective sequencing machines are shown in the table below (see also Appendix B, p.8). **EXAMPLE: 5001** is read as CGCCAT on a MiSeq but as ATGGCG on a NextSeq.
REMARK: Some Illumina sample sheets may require 8 nt to be entered for Index 2. In this case, add two nucleotides from the Illumina Adapter sequence to the 3' end of the index e.g., **5001** becomes CGCCAT**AC** on a MiSeq and ATGGCG**GT** on a Next Seq, respectively.

Index 2 (i5) Primer	i5 Bases for Sample Sheet	
	MiSeq, HiSeq 2000/2500, HiSeq 3000/4000 (SR Flow Cell)	MiniSeq, NextSeq, HiSeq 3000/4000 (PE Flow Cell)
5001 ●	CGCCAT (AC)	ATGGCG (GT)
5002 ●	ATTTTA (AC)	TAAAAT (GT)
5003 ●	GCAACG (AC)	CGTTGC (GT)
5004 ●	TAGGGC (AC)	GCCCTA (GT)

Nucleotides in brackets (AC) or (GT) are derived from the Illumina Adapters and are not actually part of the index. They are listed only for completeness if the sample sheet requires 8 nt to be entered. PE: Paired-End SR: Single-Read.

The individual libraries within a lane should be mixed at an equimolar ratio to ensure the perfect nucleotide balance. **EXAMPLE:** For 4 libraries use all four i5 indices (**5001-5004** ●). For 8 libraries use **5001** ● for 2 libraries in combination with two different i7 indices (from the i7 Index Plate, 96-well plate), included in Cat. No. 001, 009, 015, and 016), **5002** ● for 2 libraries in combination with two different i7 indices, **5003** ● for 2 libraries in combination with two different i7 indices, and **5004** ● for 2 libraries in combination with two different i7 indices.

Each i5 index can be combined with any of the i7 indices provided within the i7 Index Plate of the SENSE mRNA-Seq V2 Kit for Illumina (Cat. No. 001), SENSE Total RNA-Seq Kit for Illumina (Cat. No. 009), QuantSeq 3' mRNA-Seq Kits for Illumina (Cat. No. 015, Cat. No. 016), and the QuantSeq-Flex Targeted RNA-Seq Kits for Illumina (Cat. No. 033-035). Please refer to the respective SENSE and QuantSeq User Guides for more information on i7 multiplexing.
By combining the four different i5 indices with the 96 different i7 indices, 384 different barcode combinations can be achieved.

5. Appendix B: Sequencing*

Single Indexing (i7)

Index 1 (i7) is read out directly after Read 1. i7 indices (6 nt) are provided in the i7 Index Plate (**7001-7096**, 96-well plate) included in all Illumina-compatible SENSE (Cat. No. 001, 009) and QuantSeq Kits (Cat. No. 015, 016, 033-035).
After Read 1, Index 1 (i7), and after Read 2 resynthesis, Read 2 is being sequenced.

```
                              5'-(Read 1 Sequencing Primer)-3'
5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-(Insert…
3'TTACTATGCCGCTGGTGGCTCTAGATGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-(Insert…

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

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

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

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

Dual Indexing (i7 and i5)

Dual Index read-out differs depending on the Illumina platform and flow cell (Single-Read or Paired-End flow cell) used. Single-Read flow cells and Paired-End flow cells are available for HiSeq systems. MiniSeq, NextSeq, and MiSeq systems only include Paired-End flow cells. The following sections describe the dual indexing read out in more detail.

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

Dual Indexing on Single-Read Flow Cell - HiSeq 3000 and HiSeq 4000

Index 1 (i7) is read out directly after Read 1. i7 indices (6 nt) are provided in the i7 Index Plate (**7001-7096**) included in all Illumina-compatible SENSE and QuantSeq Kits (Cat. No. 001, 009, 015, 016, 033-035). The i5 indices are available in the i5 Dual Indexing Add-on Kit (Cat. No. 047). Seven additional chemistry-only cycles are required to read the i5 index, as here the grafted P5 oligo is used to initiate Index 2 (i5) read-out. This step uses the resynthesis mix during the Index 2 Read process.

```
   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'
```

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

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

Index 2 Read (i5): Grafted P5 Oligo on Flow Cell (not supplied):
5' AATGATACGGCGACCACCGAGA 3'

Dual Indexing on Single-Read Flow Cell - HiSeq 2000 and HiSeq 2500

Index 1 (i7) is read out directly after Read 1. i7 indices (6 nt) are provided in the provided in the i7 Index Plate (**7001-7096**, 96-well plate) included in all Illumina-compatible SENSE (Cat. No. 001, 009) and QuantSeq Kits (Cat. No. 015, 016, 033-035). The i5 indices are available in the i5 Dual Indexing Add-on Kit (Cat. No. 047). Here, Index 2 sequencing primer (included in HP9) is required for Index 2 read-out. After Read 1, Index 1 (i7) and Index 2 (i5) are being sequenced.

```
   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'
```

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

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

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

Dual Indexing on Paired-End Flow Cell - MiniSeq, NextSeq, HiSeq 3000, and HiSeq 4000

Index 1 (i7) is read out directly after Read 1. i7 indices (6 nt) are provided in the i7 Index Plate (**7001-7096**, 96-well plate) included in all Illumina-compatible SENSE (Cat. No. 001, 009) and QuantSeq Kits (Cat. No. 015, 016, 033-035). The i5 indices (6 nt) are available in the i5 Dual Indexing Add-on Kit (Cat. No. 047).

ATTENTION: MiniSeq, NextSeq, HiSeq 3000, and HiSeq 4000 perform the Index 2 Read after the Read 2 resynthesis step, hence a reverse complement of the Index 2 (i5) primer sequence is read-out here compared on other Illumina platforms (see also Appendix A, p.7).

The Index 2 sequencing primer is part of the dual indexing primer mix for MiniSeq and NextSeq. For HiSeq 3000 and HiSeq 4000, the Index 2 sequencing primer is part of HP14, an indexing primer mix that contains primers for both index reads.

After Read 1, Index 1 (i7), and after Read 2 resynthesis, Index 2 (i5) and Read 2 are being sequenced.

```
                                    5'-(Read 1 Sequencing Primer)-3'
5'AATGATACGGCGACCACCGAGATCTACAC-i5-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-(Insert…
3'TTACTATGCCGCTGGTGGCTCTAGATGTG-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'
```

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

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

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

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

Dual Indexing on Paired-End Flow Cell - MiSeq, HiSeq 2500, and HiSeq 2000

Index 1 (i7) is read out directly after Read 1. i7 Indices (6 nt) are provided in the i7 Index Plate (**7001-7096**, 96-well plate) included in all Illumina-compatible SENSE (Cat. No. 001, 009) and QuantSeq Kits (Cat. No. 015, 016, 033-035). The i5 indices (6 nt) are available in the i5 Dual Indexing Add-on Kit (Cat. No. 047). Seven additional chemistry-only cycles are required to read the i5 index as here the grafted P5 oligo is used to initiate Index 2 read-out. After Read 1, Index 1 (i7), Index 2 (i5), and after Read 2 resynthesis, Read 2 is being sequenced.

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

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

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

Index 2 Read (i5): Grafted P5 Oligo on Flow Cell (not supplied):
5' AATGATACGGCGACCACCGAGA 3'

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

6. Appendix C: Reamplification

For reamplification of dual indexed libraries contact Lexogen at info@lexogen.com.

7. Appendix D: Revision History

Publication No.	Change	Page
047IM109V101	Renaming of i5 Kit Components. Rearreanged i7 Index Plate 7001-7096 replaces Barcode Plate.	2-11
	Inclusion of QuantSeq-Flex.	1-11
047IM109V100	Initial Release.	

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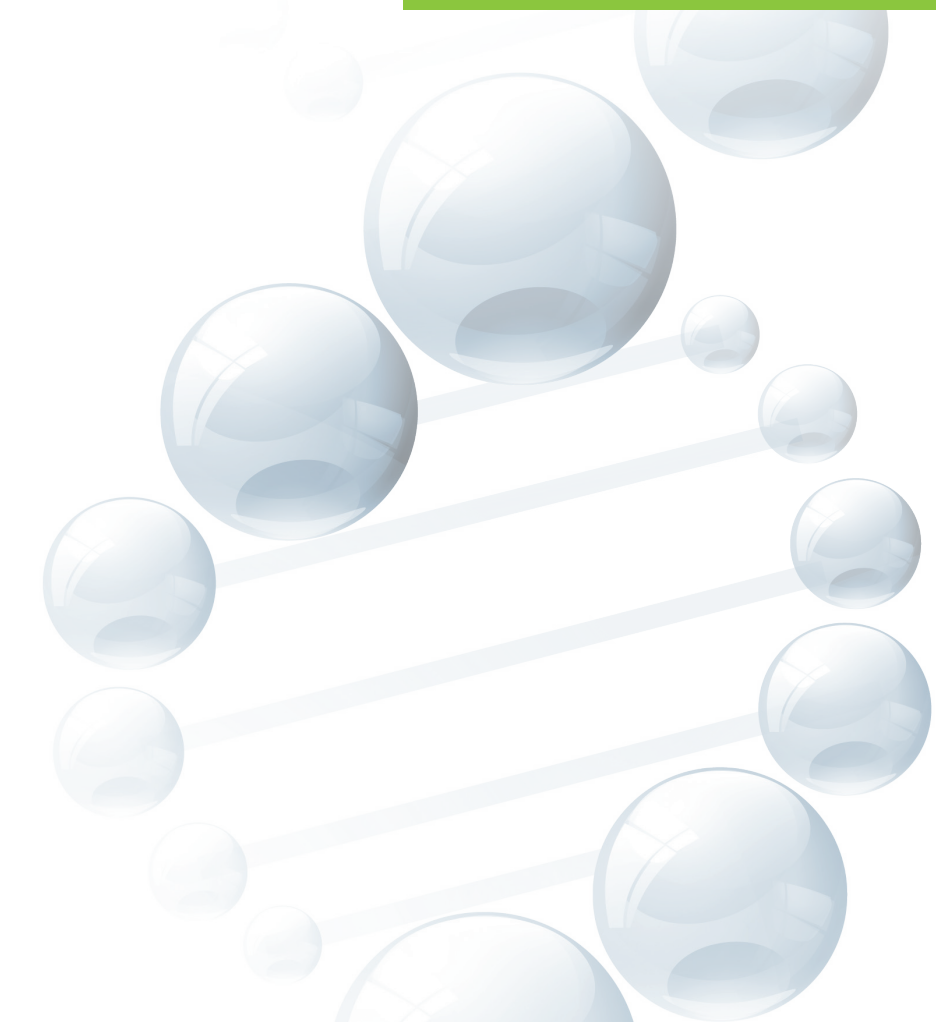

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