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Enabling complete transcriptome sequencing

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Lexogen UDI 12 nt Unique Dual Indexing Add-on Kits User Guide

Catalog Numbers:

107 - 110 (Lexogen UDI 12 nt Unique Dual Indexing Add-on Kit, Set A1, A2, A3, or A4 (UDI12A_0001-0384))

111 (Lexogen UDI 12 nt Unique Dual Indexing Add-on Kit, Set B1 (UDI12B_0001-0096))

120 (Lexogen UDI 12 nt Unique Dual Indexing Add-on Kit, Sets A1-A4 (UDI12A_0001-0384))

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For any publication using these products, please refer to them as the Lexogen UDI 12 nt Unique Dual Indexing Sets.

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Enabling complete transcriptome sequencing

1. Overview

This User Guide outlines the protocol for preparing RNA-Seq libraries with the Lexogen UDI 12 nt Unique Dual Indexing Add-on Kits. The 12 nt UDIs are designed to provide superior error correction, accuracy, and highest quality sequencing data and are introduced at the PCR step of library preparation for QuantSeq 3' mRNA-Seq FWD and REV, QuantSeq-Flex, and CORALL RNA-Seq kits. For convenience, QuantSeq-FWD and CORALL with UDI kits are available which directly include the 12 nt Unique Dual Indexing Sets and their introduction into the libraries is described in the respective User Guide.

The Lexogen UDI 12 nt Unique Dual Indexing Add-on Kits are available in different sets (A1 - A4 and B1) for optimal compatibility with different Illumina instruments. These Add-on Kits are also suitable for unique dual indexing of sequencing libraries from other vendors containing TruSeq - compatible partial adapters.

We recommend using Sets A1 - A4 (Cat. No. 107 - 110, and 120) for all Illumina sequencing machines using the Forward Strand Workflow (A) for i5 index read-out. Set B1 (Cat. No. 111) is recommended for all Illumina sequencing machines using the Reverse Complement Workflow (B) for i5 index read-out (see Table below, for more information see Appendix A, p.10).

Illumina Instruments	Flow Cell Type	Workflow	Lexogen UDI 12 nt Unique Dual Indexing
HiSeq 2000/2500 HiSeq 3000/4000	SR	Forward Strand (A)	Add-on Kits, Set A1, A2, A3, and/or A4 (UDI12A_0001-0384): Cat. No. 107 – 110.96 or 120.384
HiSeq 2000/2500 MiSeq NovaSeq 6000 (v1.0 reagent kits)	PE		
iSeq 100 MiniSeq NextSeq 500 - 2000 HiSeq 3000/4000 NovaSeq 6000 (v1.5 reagent kits)	PE	Reverse Complement (B)	Add-on Kit, Set B1 (UDI12B_0001-0096): Cat. No. 111.96

Lexogen UDI 12 nt Unique Dual Indexing Sets contain a unique nested feature enabling adjustable index read-out lengths. Longer index read-out lengths enable higher error correction capability. The UDIs are 12 nucleotides long, however, the superior error correction feature can also be used if only 8 or 10 nucleotides of the index are read out. To use this nested feature it is essential to use Set A1 - A4 for Illumina machines using the Forward Strand Workflow (A) for i5 index read-out and Set B for Illumina machines using the Reverse Complement Workflow (B) for i5 index read-out.

If Set A1 - A4 is used for Workflow B index read-out, all 12 nucleotides of the i5 index must be read out for error correction.

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

- The **Dual PCR Mix (Dual PCR ●)** supplied with the Library Amplification Module **must** be used instead of the PCR Mix from the standard library prep kits (**PCR ●** from QuantSeq or 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 (step 5, p.8). Please follow the purification protocol steps listed on p.8 - 9 of this User Guide to purify unique dual-indexed libraries.
- Each well of the Lexogen UDI 12 nt Sets contains sufficient volume for one library prep per UDI and is intended for **single use only!**
- **Spin down the plates containing UDIs before opening!** Pierce or cut open the sealing foil of the wells containing only the desired UDI. Reseal opened wells of the UDI plate after use with a fresh sealing foil to prevent cross contamination!

Dual-indexed QuantSeq and CORALL libraries prepared with these Add-on Kits are compatible with all Illumina sequencing instruments. For index sequences and multiplexing guidelines see Appendix A, p.10. For sequencing details including i5 read orientation and dual index read-out workflows for different flow cells and instruments, see Appendix B, p.12.

ATTENTION: The Lexogen UDI 12 nt Unique Dual Indexing Sets are not compatible with Small RNA library preparation kits, e.g., Lexogen's Small RNA library preparation kit (Cat. No. 052) or library preparation kits using Nextera adapters.

2. Kit Components and Storage Conditions

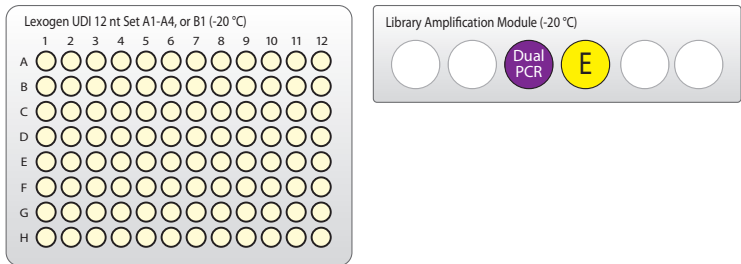


Figure 1. Location of kit components. Each Lexogen UDI 12 nt Unique Dual Indexing Add-on Kit is provided with one UDI 12 nt Set (96-well plate) and the Library Amplification Module.

There are 5 different Lexogen UDI 12 nt Sets available: Set A1 (UDI12A_0001-0096) in Cat. No. 107.96; Set A2 (UDI12A_0097-0192) in Cat. No. 108.96; Set A3 (UDI12A_0193-0288) in Cat. No. 109.96; Set A4 (UDI12A_0289-0384) in Cat. No. 110.96; and Set B1 (UDI12B_0001-0096) in Cat. No. 111.96. Set A1-A4 (Cat. No. 120.384) contains the four Sets A1 to A4 and four Library Amplification Modules.

Kit Component	Label	Volume* provided	Storage
Lexogen UDI 12 nt Set A1, A2, A3, A4, or B1	UDI12A_0001-0096, UDI12A_0097-0192, UDI12A_0193-0288, UDI12A_0289-0384, UDI12B_0001-0096	10 µl / reaction	-20 °C
Library Amplification Module included in each kit			
Dual PCR Mix	Dual PCR ●	740 µl	-20 °C
Enzyme Mix	E ●	106 µl	-20 °C

*including ≥10 % surplus

NOTE: Each well of the Lexogen UDI 12 nt Sets contains sufficient volume for one library prep per UDI and is intended for **single use only!**

Spin down the plates containing UDIs before opening! Pierce or cut open the sealing foil of the wells containing only the desired UDI. Reseal opened wells of the UDI plate after use with a fresh sealing foil to prevent cross contamination!

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, or 015UG110) or steps 28 - 31 of the CORALL Total RNA-Seq Kit User Guide (095UG1090).

Preparation

PCR		Purification (Cat. No. 022)*
Dual PCR ● Lexogen UDI 12 nt Sets (A1 - A4 or B1) E ●	<div>- thawed at RT - thawed at RT - keep on ice or at -20 °C</div> <div>} spin down before opening!</div>	from standard library prep kits: PB – stored at +4 °C PS – stored at +4 °C 80 % EtOH – provided by user prepare fresh! EB – stored at +4 °C
Thermocycler	<div>98 °C, 30 sec 98 °C, 10 sec 65 °C, 20 sec 72 °C, 30 sec 72 °C, 1 min 10 °C, ∞</div> <div>} 11- 25x Endpoint cycle number as determined by qPCR (Cat. No. 020), or see CORALL or QuantSeq User Guides.</div>	Equilibrate all reagents to room temperature for 30 minutes prior to use.

*The Purification Module (Cat. No. 022) is included in the QuantSeq and CORALL Kits.

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. The PCR Add-on Kit (Cat. No. 020) is required. 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 ●) from the standard library prep kit with the Dual PCR Mix (Dual PCR ●) supplied in the Lexogen UDI 12 nt Unique Dual Indexing Add-on Kits. Do not use PCR ● from the standard kits if dual indexing is intended.
- The Enzyme Mix (E ●), provided in the Lexogen UDI 12 nt Unique Dual Indexing Add-on Kits, is interchangeable with E3 ● from QuantSeq and CORALL or E ● from the PCR Add-on Kit.
Do not use E1 ●, E2 ● / E1 ●, E2 ● from the QuantSeq or CORALL Kits, respectively for this PCR!

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 equilibrate for at least 30 minutes.

1 Prepare a mastermix containing 7 µl Dual PCR Mix (**Dual PCR** ●) and 1 µl Enzyme Mix (**E** ●) per reaction.

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

Add 10 µl of the respective Unique Dual Index Primer pair (UDI12A_0001-0384, or UDI12B_0001-0096) to each sample. Use only one UDI per sample! **ATTENTION:** Spin down the plates containing the UDIs before opening! Pierce or cut open the sealing foil of the wells containing only the desired UDIs. Reseal opened wells of the UDI plate after use with a fresh sealing foil to prevent cross contamination!

4 Conduct 11 - 25 cycles of PCR (determine the required cycle number by qPCR, see also CORALL or QuantSeq User Guides) 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.

Purification

The finished library is purified from PCR components that can interfere with quantification. The Purification Beads (**PB**) must be fully resuspended before use. Thorough mixing by pipetting or vortexing is recommended.

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 or 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.

5 For **QuantSeq (standard input >10 ng)** libraries, add 35 µl of thoroughly resuspended Purification Beads (**PB**) to each reaction. For **QuantSeq libraries generated from low input (≤10 ng) / low quality / FFPE RNA**, add only 31.5 µl **PB**.

For **CORALL** libraries add 31.5 µl of thoroughly resuspended **PB** to each reaction and for **CORALL libraries generated from FFPE RNA**, add 42 µl **PB**. Mix well, and incubate for 5 minutes at room temperature.

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

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

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

9 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.

- 10 Place the plate onto a magnet and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
- 11 Remove and discard the clear supernatant without removing the PCR plate from the magnet. Make sure that accumulated beads are not disturbed.
- 12 Add 120 μ l 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.
- 13 Repeat this washing step once for a total of two washes. Make sure to remove the supernatant completely.
- 14 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 at room temperature and do not let the beads dry too long (visible cracks appear), this will negatively influence the elution and the resulting library yield.
- 15 Add 20 μ l of Elution Buffer (**EB**) per well, remove the plate from the magnet, and resuspend the beads thoroughly in **EB**. Incubate for 2 minutes at room temperature.
- 16 Place the plate onto a magnet and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
- 17 Transfer 15 - 17 μ l of the supernatant into a fresh PCR plate. Make sure not to transfer any beads.
- 18 At this point, the libraries are finished and ready for quality control, pooling (for multiplexing, see also Appendix A, p.10), and sequencing. For more details please refer to the respective QuantSeq or CORALL User Guides.

4. Appendix A: Multiplexing

Libraries prepared with the Lexogen UDI 12 nt Unique Dual Indexing Add-on Kits are suitable for sequencing on all Illumina instruments listed below.

The Lexogen UDI 12 nt Sets enable adjustable read-out of 8, 10, or the full 12 nucleotides long UDI sequence while maintaining superior error correction features. The longer the UDI read-out the higher is the error correction capability.

The complete lists of i5 and i7 index sequences for all Lexogen UDI 12 nt Sets are available at www.lexogen.com/docs/indexing.

Depending on the instrument workflow, flow cell type (paired-end, PE; single-read, SR), and chemistry, i5 indices are sequenced on the forward or the reverse complement strand (see also Appendix B, p.12).

Sets A1 - A4 (UDI12A_0001-0384) for Forward Strand Workflow (A)

For instruments using the Forward Strand workflow, the Index 2 Read (i5) is primed using the Grafted P5 Oligo on the flow cell (or the Index 2 (i5) Sequencing Primer (HP9) for SR HiSeq 2000 / 2500 flow cells).

Set B1 (UDI12B_0001-0096) for Reverse Complement Workflow (B)

For instruments using the Reverse Complement workflow, the Index 2 Read (i5) is performed after Read 2 Resynthesis, using the Index 2 (i5) Sequencing Primer.

ATTENTION: If library preps with UDI Set A (UDI12A_0001-0384) are sequenced on Illumina machines using the Reverse Complement Workflow (B), or *vice versa*, the i5 Index will be read out as reverse complement. **In this case, all 12 nt of the i5 Index must be read out if error correction shall be applied.** Additionally, the reverse complement of the i5 index read out needs to be analyzed.

EXAMPLE: i512_0001 is read as GTCTTTGGCCCT instead of AGGGCCAAAGAC. The read out in reverse complement (GTCTTTGGCCCT) shall be used for demultiplexing and error correction.

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.

In general, using the UDIs 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 smaller numbers of samples we can also suggest the following:

- **Four libraries:** Use UDI12A / B_0001 - 0004 as these contain almost perfect nucleotide balance at each position of the index read.
- **Eight libraries:** Use column 1 of the Lexogen UDI 12 nt Set A or B (UDI12A / B_0001 - 0008).

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.

Lane Mix Preparation

Libraries should ideally be pooled in an equimolar ratio for multiplexed sequencing. It is important to ensure accurate quantification of the individual libraries prior to pooling, as well as for the library pool (lane mix). To quantify your libraries:

1 Measure the concentration of each library, using either qPCR or fluorescence-based assays (e.g., QuBit, Thermo Fisher Scientific Inc.).

2 Determine the average library size, using microcapillary electrophoresis analysis (e.g., Bioanalyzer, Agilent Technologies, Inc.). Set the range to 175 - 1,000 bp to include the whole library size distribution, and to exclude any linker-linker artifacts or overcycling bumps (>1,000 bp).

Molarity is then calculated from the average library size and the concentration (ng/μl) using the following equation:

$$\text{Molarity} = (\text{library concentration (ng/}\mu\text{l)} \times 10^6) / (660 \times \text{average library size (bp)})$$

A template for molarity calculation is also available for download from www.lexogen.com.

After pooling the libraries, the prepared lane mix and any dilutions made for denaturing (e.g., 2 nM), should be reanalyzed to ensure the accuracy of the concentration. This can be performed according to steps **1** and **2** as above.

5. Appendix B: Sequencing*

Dual-Indexed Library Sequencing Workflows

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. Illumina defines Forward Strand (A) and Reverse Complement (B) Workflows for dual indexing read-out, which refer to the order of Index 2 read-out in relation to Read 2 Resynthesis.

Illumina defines four different workflows used for Index 2 Read (i5) read-out on Illumina instruments, which take into account also the differences in flow cell types and sequencing primers used for Index 2 (i5) read-out. These are summarized in the table below along with the compatible UDI 12 nt sets recommended for each.

Work-flow	Illumina Instrument(s)	Flow Cell Type	Illumina Workflow (i5 Index Read Orientation)	Index 2 (i5) Sequencing Primer	No. of Chemistry-Only Cycles ¹	Lexogen 12 nt UDI Set
1	HiSeq 2000/2500	SR	Forward Strand (i5)	i5 Index Primer	0	Set A1 - A4
2	HiSeq 3000/4000			Grafted P5 Oligo	7	
3	HiSeq 2000/2500 MiSeq NovaSeq 6000 (v1.0 reagent kits)	PE	Reverse Complement (i5rc)	i5 Index Primer	0	Set B1
4	HiSeq 3000/4000 iSeq 100 MiniSeq NextSeq 500 - 2000 NovaSeq 6000 (v1.5 reagent kits)					

¹Additional chemistry-only (no-imaging) cycles are performed before the i5 index is read-out.

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

The order of sequencing for Workflow 4 (Illumina Reverse Complement Workflow) is: Read 1, index read preparation, Index 1 Read (i7), Read 2 Resynthesis, and Index 2 Read (i5), and 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.

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

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.

Sequencing Workflows for Dual-Indexed Libraries

The following section depicts 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.

NOTE: All CORALL libraries include 12 nt Unique Molecular Identifiers (UMIs) at the beginning of Read 1 as depicted for workflow 1, below:

```
5'-(Index 2 (i5) Sequencing Primer)-3'      5'-(Read 1 Sequencing Primer)-3'      UMI
5'AATGATACGGCGACCAACCGAGATCTACAC-i5-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-N(12)-(Insert...
3'TTACTATGCCGCTGGTGGCTCTAGATGTG-i5-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-N(12)-(Insert...

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

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

The i5 Index Primer (included in HP9) is required for Index 2. A minimum of eight cycles with imaging are required for i5 Index read-out, provided the correct UDI set is used for the correct i5 Index read out workflow. UDIs are 12 nt long. 12 nt, 10 nt, or 8 nt can be read out optionally.

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

5' AATGATACGGCGACCAACCGAGATCTACAC 3'

QuantSeq FWD, QuantSeq-Flex, and CORALL libraries:

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

5'-(Index 1 (i7) Sequencing Primer)-3'
...Insert)-AGATCGGAAGAGCACACGCTCTGAACCTCCAGTCAC-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'AATGATACGGCGACCAACCGAGATCTACAC-i5-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-TTTTTTTTTTTTTTTTTT-(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 eight cycles with imaging are required for i5 Index read-out, provided the correct UDI set is used for the correct i5 Index read out workflow. UDIs are 12 nt long. Optionally, 12 nt, 10 nt, or 8 nt can be read out.

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' TTACTATCCGCTGGTGGCTCTAGATGTG-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' AATGATACGGCGACCACCGAGA TCTACAC-i5-ACACTCTTTCCCTACACGACGCTCTTCCGATCT- TTTTTTTTTTTTTTTTTT- (Insert...
3' TTACTATCCGCTGGTGGCTCTAGATGTG-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 (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 eight cycles with imaging are required for i5 Index read-out, provided the correct UDI set is used for the correct i5 Index read out workflow. UDIs are 12 nt long. Optionally, 12 nt, 10 nt, or 8 nt can be read out.

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' TTACTATCCGCTGGTGGCTCTAGATGTG-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'AATGATACGGCGACCACCGAGATCTACAC-i5-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-TTTTTTTTTTTTTTTTTT-(Insert...
3'TTACTATGCGCGCTGGTGGCTCTAGATGTG-i5-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-(Insert...

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

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

All instruments use an i5 Index Primer, which is included in the “Dual-Indexing Primer Mix” for iSeq, MiniSeq (with standard reagent kits), NextSeq Systems, in the v1.5 reagent kits for NovaSeq, and in HP14 for HiSeq 3000 / 4000. A minimum of eight cycles with imaging are required for i5 Index read-out, provided the correct UDI set is used for the correct i5 Index read out workflow. UDIs are 12 nt long. Optionally, 12 nt, 10 nt, or 8 nt can be read out.

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.10).

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

5' AGATCGGAAGAGCGCTGTAGGGAAAGAGTGT 3'

QuantSeq FWD, QuantSeq-Flex, and CORALL libraries:

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

5'-(Index 1 (i7) Sequencing Primer)-3'
...Insert)-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-i7-ATCTCGTATGCCGTCTTCTGCTTG 3'
...Insert)-TCTAGCCTTCTCGTGTGCAGACTTGAAGTCAGTG-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-TTTTTTTTTTTTTTTTTT-(Insert...
3'TTACTATGCGCGCTGGTGGCTCTAGATGTG-i5-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-(Insert...
3'-(Index 2 (i5) Sequencing Primer)-5'

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

QuantSeq REV libraries on MiniSeq and NextSeq:

ATTENTION: Please contact support@lexogen.com 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 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 i7 Index 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.

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

i7 Index Primer (not supplied):

5' ACACTCTTCCCTACACGACGCTCTCCGATCT 3'

Read 1 for QuantSeq REV:

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

5' ACACTCTTCCCTACACGACGCTCTCCGATCTTTTTTTTTTTTTTTTTTTT 3'

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

5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC 3'

Index 2 Read (i5):

Workflow 1: i5 Index Primer (not supplied):

5' AATGATACGGCGACCACCGAGATCTACAC 3'

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

5' AATGATACGGCGACCACCGAGA 3'

Workflow 4: i5 Index Primer (not supplied):

5' AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT 3'

Read 2: i5 Index Primer (not supplied):

5' GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

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 for more information.

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 (UDI12A_0001-0384 and UDI12B_0001-0096) are available for download at www.lexogen.com/docs/indexing.

Additionally to the standard error-correction included in the Illumina pipeline, Lexogen's idemux tool is freely available on github: <https://github.com/Lexogen-Tools> and can be used for higher accuracy in error correction. Please contact support@lexogen.com for more information.

8. Appendix E: Revision History

Publication No. / Revision Date	Change	Page
107UG223V0200 Feb. 23, 2021	Renamed the document to User Guide.	
	Minor text edits and typo corrections.	
	Inserted Table of Contents.	3
	Updated information in Appendix Sections.	10 - 17
	Removed SENSE mRNA-Seq V2 from the protocol.	all
107IM223V0100 Dec. 27, 2019	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)
022 (Purification Module with Magnetic Beads)
033 - 035 (QuantSeq-Flex Targeted RNA-Seq Library Prep Kits)
080 (Reamplification Add-on Kit for Illumina)
095 (CORALL Total RNA-Seq Library Prep Kit)
113, 129 - 131, 114, 115 (QuantSeq 3' mRNA-Seq Library Prep Kit FWD with UDI 12 nt Sets A1-A4, or B1)
117, 132 - 134, 118, 119 (CORALL Total RNA-Seq Library Prep Kit with UDI 12 nt Set A1-A4, or B1)
146 (CORALL Total RNA-Seq Library Prep Kit with RiboCop (HMR))
147 (CORALL Total RNA-Seq Library Prep Kit with RiboCop (HMR+Globin))
158 - 163 (CORALL mRNA-Seq Library Prep Kits)

Lexogen 12 nt UDI Add-on Kits · User Guide

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