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Reamplification Add-on Kit for Illumina
User Guide

Catalog Numbers:
080 (Reamplification Add-on Kit for Illumina)

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When describing a procedure for publication using this product, please refer to it as the Reamplification Add-on Kit for Illumina.

CONTACT INFORMATION

Lexogen GmbH
Campus Vienna Biocenter 5
1030 Vienna, Austria
www.lexogen.com
E-mail: support@lexogen.com

Support
E-mail: support@lexogen.com
Tel. +43 (0) 1 3451212-41
Fax. +43 (0) 1 3451212-99

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1. Overview

This User Guide outlines the protocol for the Reamplification Add-on Kit for Illumina (Cat. No. 080.96). The kit contains reagents sufficient for 96 PCRs and is suitable for all CORALL (Cat. No. 095, 117 -119, 132 -134, 171 - 186), QuantSeq (Cat. No. 015, 016, 113 - 115, 129 - 131) including QuantSeq-Flex (Cat. No. 028 and 166), QuantSeq-Pool (Cat. No. 139), LUTHOR (Cat. No. 143) and Small RNA-Seq (Cat. No. 052 and 058) Kits for Illumina.

The Reamplification Add-on Kit for Illumina includes, a Reamplification PCR Mix (**RE-PCR** ●) that contains the Illumina P5- and P7-specific reamplification primers, plus a thermostable polymerase (**E** ●).

The Reamplification Add-on Kit for Illumina can be used for reamplification of undercycled cDNA libraries or lane mixes and is compatible with both single- and dual-indexed libraries. Reamplification can only be performed on already amplified libraries that contain Lexogen i7 and / or i5 6 nt indices (i.e., indexed libraries). Reamplification of indexed libraries should only be performed if the library (or lane mix) yields are extremely low and insufficient for pooling (or sequencing). A Library Quantification Calculation File is available from the Downloads section of the product pages for CORALL, QuantSeq (https://www.lexogen.com/support-tools/lane-mix-calculation/), and can be used to determine whether the library yield is sufficient for pooling and sequencing.

Please note, additional purification reagents are required for post-PCR purification of reamplified libraries. The Purification Module with Magnetic Beads (Cat. No. 022) is required for purification of reamplified CORALL, QuantSeq, and Small RNA-Seq libraries. Alternatively, the Gel Extraction Module (Cat. No. 054) can be used for purification of reamplified Small RNA-Seq libraries.

2. Kit Components and Storage Conditions

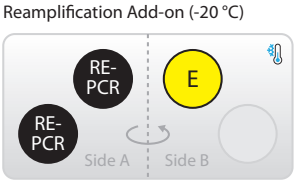

Figure 2. Location of kit components.

Kit Component	Tube Label	Volume*	Storage
Reamplification PCR Mix	**RE-PCR** ●	2x 898 µl	-20 °C
Enzyme Mix	**E** ●	106 µl	-20 °C

*including 10 % surplus

NOTE: The Enzyme Mix (**E** ●) is the same as Enzyme Mix 3 (**E3** ●) in the CORALL and QuantSeq Kits, and Enzyme Mix (**E** ●) from the PCR Add-on Kit for Illumina (Cat. No. 20). All of these enzymes can be used interchangeably for reamplification PCRs.

PCR Enzyme (**PE** ○) which is provided in QuantSeq-Pool and CORALL RNA-Seq V2 Library Prep Kits is not interchangeable with Enzyme Mix (**E** ●).

3. User-Supplied Consumables and Equipment

Check to ensure that you have all the necessary materials and equipment available before starting the reamplification.

Reagents / Solutions

- Purification Module with Magnetic Beads (Lexogen, Cat. No. 022) - For CORALL, QuantSeq, and Small RNA-Seq libraries.
- Gel Extraction Module (Lexogen, Cat. No. 054) - for Small RNA-Seq libraries.
- Fresh 80 % ethanol (for magnetic bead purification).
- Molecular biology-grade water (**H_2O**), or Elution Buffer (**EB**) from the CORALL/QuantSeq/QuantSeq-Pool/LUTHOR/Small RNA-Seq Kits.

Equipment and Labware

- Thermocycler.
- Magnetic plate (for magnetic bead purification), e.g., 96S Super Magnet Plate (Alpaqua, Cat. No. A001322).
- Suitable certified ribonuclease-free low binding pipette tips (pipette tips with aerosol barriers recommended).
- 200 µl PCR tubes or 96-well plates with caps and sealing foils.

NOTE: The complete set of materials, reagents, and labware required for post-PCR purification and library quality control is not listed.

4. Reamplification of Libraries

PCR

PCR		Purification (Cat. No. 022.96)	
RE-PCR ●	– thawed at RT	**PB**	– stored at +4°C
E ●	– keep on ice or at -20 °C	**PS**	– stored at +4°C
H_2O or **EB**	– **user supplied!**	**80 % EtOH**	– provided by user **prepare fresh!**
ATTENTION: Spin down all solutions before opening tubes!		**EB**	– stored at +4°C
Thermocycler	98 °C, 30 sec 98 °C, 10 sec 65 °C, 20 sec } 3 - 10x 72 °C, 30 sec 72 °C, 1 min 10 °C, ∞		

ATTENTION: We recommend performing a minimum of 3 PCR cycles for reamplification. The yield doubles with each cycle, take care not to overcycle your libraries!

NOTE: If your libraries are undercycled but have measurable yields, perform 3 - 6 cycles of reamplification PCR, calculating approximate doubling of the original yield per cycle, or 10-fold increase per 3 cycles. For example, if your library concentration is e.g., 0.05 ng/µl, perform 3 - 5 cycles to obtain a concentration in the range of 0.5 - 2 ng/µl. If you do not see a library at all e.g., on a Bioanalyzer or similar and / or have no measurable library concentration, use 6 - 10 cycles for reamplification. For further PCR cycle recommendations please contact support@lexogen.com.

1. Prepare a mastermix containing 17 µl Reamplification PCR Mix (**RE-PCR** ●) and 1 µl Enzyme Mix (**E** ●).
2. Add 18 µl of the **RE-PCR** / **E** mastermix to up to 17 µl of the eluted library. Bring the total reaction volume to 35 µl with molecular biology-grade water (**H_2O**) or Elution Buffer (**EB,** e.g., from the CORALL/QuantSeq/Small RNA-Seq 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.
3. Conduct 3 - 10 cycles of PCR with the following program: Initial denaturation at 98 °C for 30 seconds; 3 - 10 cycles of 98 °C for 10 seconds, 65 °C for 20 seconds, 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.

5. Purification

5.1 Purification of Reamplified CORALL/QuantSeq libraries

The Purification Module with Magnetic Beads (Cat. No. 022) and a magnetic stand compatible with PCR tubes or plates (96-well format) is required for magnetic bead purification.

ATTENTION: Important notes for magnetic bead purifications!

- The Purification Module (**PB, PS, EB**) should equilibrate for 30 minutes at room temperature before use. The Purification Beads (**PB**) may have settled and must be thoroughly resuspended before adding them to the reaction.
- Prepare fresh 80 % ethanol (**EtOH**) for bead washing.
- If PCR products were stored at -20 °C, ensure these are thawed and equilibrated to room temperature before Purification Beads (**PB**) are added.

4	Add 35 µl of fully resuspended Purification Beads (**PB**) to each reaction, mix thoroughly, and incubate for 5 minutes at room temperature. **REMARK:** For CORALL Total RNA-Seq, and for QuantSeq libraries generated from low RNA input or degraded RNA, add only 31.5 µl **PB**.
5	Place the plate onto a magnet and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
6	Remove and discard the clear supernatant without removing the PCR plate from the magnet. Do not disturb the beads!
7	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.
8	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.
9	Place the plate onto a magnet and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
10	Remove and discard the clear supernatant without removing the PCR plate from the magnet. Do not disturb the beads!
11	Add 120 µl of 80 % EtOH and incubate 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.
12	Repeat this washing step once for a total of two washes. **ATTENTION:** Remove the supernatant completely.

13	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 only and do not let the beads dry too long (visible cracks appear). This will negatively influence the elution and hence the resulting library yield.
14	Add 20 µl of Elution Buffer (**EB**) per well, remove the plate from the magnet, and resuspend the beads fully in **EB**. Incubate for 2 minutes at room temperature.
15	Place the plate onto a magnet and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
16	Transfer 15 - 17 µl of the supernatant into a fresh PCR plate. Do not transfer any beads.
17	At this point, the libraries are finished and ready for quality control, pooling, and cluster generation. For more details please refer to the respective CORALL and QuantSeq User Guides.

5.2 Purification of Reamplified Small RNA-Seq Libraries

A simple column- or bead-based purification is sufficient to remove PCR components from reamplified Small RNA-Seq libraries. Ideally, Small RNA-Seq libraries should already have been size selected using magnetic beads or gel purification (see Appendices G and H, Small RNA-Seq Library Prep Kit User Guide (052UG128), p.25-28) before reamplification. Samples without previous size selection might have linker-linker side products or long library fragments (200 - 1,000 bp) that will also be reamplified in the additional PCR cycles. For further information and size-selection protocol details, please see the Small RNA-Seq Kit User Guide (052UG128).

Workflow	Purification Method	Kit
5.2.1	Silica-Column	054.24
5.2.2	Magnetic Beads	022.96

5.2.1 Silica-Column Purification of Reamplified Small RNA-Seq Libraries

The Gel Extraction Module (Cat. No. 054) is needed for the silica-column purification.

ATTENTION: Ensure that absolute ethanol (100 % EtOH) has been added to the bottle of Column Wash Buffer (**CW**)! Avoid contamination! When pipetting liquids from bottles, take special care that only the sterile pipette tip touches the bottle opening to prevent introducing RNases or other contaminants. Tips are sterile, whereas the pipette itself is not. If necessary, tilt the bottle to bring the liquid closer to the opening to facilitate pipetting!

4. Transfer the finished PCR product into a 1.5 ml tube and add molecular biology-grade water (H_2O) to bring the total volume to 100 µl. Add 300 µl Column Binding Buffer (**CB**) and 50 µl 100 % EtOH to the reaction, mix well. Transfer the solution onto a Purification Column placed in a 2 ml Collection Tube.
5. Centrifuge for 1 minute at 3,500 x g (6,000 rpm) at 18 °C. Discard the flow-through.
6. Apply 600 µl of Column Wash Buffer (**CW**) to the column and centrifuge for 1 minute at 14,000 x g (~12,000 rpm) at 18 °C. Discard the flow-through.
7. Repeat this washing step once (for a total of two washes).
8. Discard the flow-through. Centrifuge for 2 minutes at 14,000 x g (~12,000 rpm) at 18 °C to dry the column.
9. Transfer the column to a new 1.5 ml tube and apply 20 µl of Elution Buffer (**EB**) to the column. Centrifuge for 1 minute at 200 x g (~1,400 rpm) and 2 minutes at 14,000 x g (~12,000 rpm) at 18 °C to elute the library.
10. At this point, the Small RNA-Seq library is finished and ready for quality control, size selection, pooling, and cluster generation. For more details refer to the Small RNA-Seq Kit User Guide (052UG128).

5.2.2 Magnetic Bead Purification of Reamplified, Size-Selected Small RNA-Seq Libraries

The Purification Module with Magnetic Beads (Cat. No. 022) is needed for the magnetic bead purification.

ATTENTION: Important notes for magnetic bead purifications!

- The Purification Module (**PB, PS, EB**) should equilibrate for 30 minutes at room temperature before use. The Purification Beads (**PB**) may have settled and must be properly resuspended before adding them to the reaction.
- Prepare fresh 80 % ethanol (**EtOH**) for bead washing.
- If PCR products were stored at -20 °C, ensure these are thawed and equilibrated to room temperature before Purification Beads (**PB**) are added.

NOTE: The Purification Solution (**PS**) is not used in this protocol.

REMARK: The amount of **PB** added at step 4 differs, depending on whether the Small RNA-Seq libraries have already undergone size-selection or repurification to remove linker-linker (See Appendices G and H, p.25-28, of the Small RNA-Seq Kit User Guide: 052UG128).

4	For Small RNA-Seq libraries that were size-selected before reamplification, add 2 volumes of properly resuspended Purification Beads (**PB**) to each reamplified Small RNA-Seq library PCR product. Mix well, and incubate for 5 minutes at room temperature. **EXAMPLE:** Add 70 µl **PB** to 35 µl of reamplified Small RNA-Seq library. **REMARK:** If Small RNA-Seq libraries were not size-selected before reamplification, add 1.3 volumes of **PB** (i.e., 45.5 µl) to each reamplified Small RNA-seq library PCR product (i.e., 35 µl).
5	Place the plate onto a magnet and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
6	Remove but save the clear supernatant in a fresh tube or well, without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed as the library is bound to the beads at this stage! **ATTENTION:** We strongly recommend saving the supernatant in a separate tube until you have analyzed the final lane mix.
7	Add 120 µl of 80 % EtOH to the beads and incubate for 30 seconds. Leave the plate in contact with the magnet as the beads should not be resuspended during this washing step. Remove and discard the supernatant.
8	Repeat this washing step once for a total of two washes. **ATTENTION:** Remove the supernatant completely.
9	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 only and do not let the beads dry too long (visible cracks appear). This will negatively influence the elution and hence the resulting library yield.

10	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.
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	Transfer 15 - 17 µl of the supernatant into a fresh PCR plate. Make sure not to transfer any beads.
13	At this point, the libraries are finished and ready for quality control, pooling (for multiplexing), and cluster generation. For more details refer to the Small RNA-Seq Kit User Guide (052UG128).

6. Appendix A: Primer Sequences

NOTE: The Reamplification Mix containing the P5 and P7 reamplification primers can only be used on already amplified (i.e., indexed) libraries or lane mixes.

Reamplified QuantSeq and CORALL Libraries

```
5'-(P5 Reamplification Primer)-3'
5'AATGATACGGCGACCACCGAGATCT-i5-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-(Insert…
3'TTACTATGCCGCTGGTGGCTCTAGA-i5-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-(Insert…

             5'-(Index Read Sequencing Primer)-3'
   …Insert)- AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-i7-ATCTCGTATGCCGTCTTCTGCTTG 3'
   …Insert)- TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-i7-TAGAGCATACGGCAGAAGACGAAC 5'
                                                  3'- P7 Reamplification Primer-5'
```

Reamplified Small RNA-Seq Libraries

```
5'-(P5 Reamplification Primer)-3'
5'AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGACGATC -(Insert…
3'TTACTATGCCGCTGGTGGCTCTAGATGTGCAAGTCTCAAGATGTCAGGCTGCTAG -(Insert…


   …Insert)- TGGAATTCTCGGGTGCCAAGGAACTCCAGTCAC-i7-ATCTCGTATGCCGTCTTCTGCTTG 3'
   …Insert)- ACCTTAAGAGCCCACGGTTCCTTGAGGTCAGTG-i7-TAGAGCATACGGCAGAAGACGAAC 5'
                                                  3'- P7 Reamplification Primer-5'
```

NOTE: Indicated sequencing primers are for illustrative purposes only and do not reflect the actual primer start and/or end sites. Some sequences depicted in Appendix A may be copyrighted by Illumina.

7. Appendix B: Revision History

Publication No. / Revision Date	Change	Page
080UG169V0103 Jan. 26, 2023	Updated Kit Components Figure 2 and Table to reflect current packaging and storage requirements.	5
	Added information about PE enzyme.	5
080IM169V0102 Aug. 25, 2020	Legal terms and conditions statements updated.	2
	Added table of contents.	3
	Page numbers adjusted and minor corrections	2-16
080IM169V0101 Apr. 30, 2019	References to CORALL library reamplification added and minor corrections.	2-10
080IM169V0100 Oct. 09, 2018	Initial Release.	

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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)
047 (Lexogen i5 6 nt Dual Indexing Add-on Kits (5001-5096))
052 (Small RNA-Seq Library Prep Kit for Illumina)
054 (Gel Extraction Module)
058 (Small RNA-Seq Library Prep Kit for Illumina including Purification Module with Magnetic Beads)
095 (CORALL Total RNA-Seq Library Prep Kit)
096 (CORALL Total RNA-Seq Library Prep Kit with RiboCop)

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Lexogen GmbH
Campus Vienna Biocenter 5
1030 Vienna, Austria
Telephone: +43 (0) 1 345 1212-41
Fax: +43 (0) 1 345 1212-99
E-mail: support@lexogen.com

Lexogen, Inc.
51 Autumn Pond Park
Greenland, NH 03840, USA
Telephone: +1-603-431-4300
Fax: +1-603-431-4333
www.lexogen.com