RiboLace Ribo-Seq library preparation

Product	Catalog no	Rxns
RiboLace Ribo-Seq - Module 2	#RL001_mod2	9

Shipping: Dry ice and 4°C

Storage Conditions: store components as indicated on datasheet

Shelf Life: 5 months

Description: RiboLace Ribo-Seq kit module 2 is used to prepare libraries for Illumina platform starting from Ribosome Protected Fragments (RPFs) isolated using RiboLace Ribo-Seq kit module 1 or other protocols.

Reagents provided

Product (label)	Catalog no	Store Conditions	Quantity
filters and vials	#RL001-8	RT	For 40 rxns
Sodium acetate (NaAc)	#RL001-12	4°C	2 ml
TR buffer (TR)	#RL001-13	4°C	2 mL
HI-solution (HI)	#RL001-21	4°C	2 mL
Buffer I (BI)	#RL001-10	4°C	15 mL
Buffer II (BII)	#RL001-11	4°C	15 mL
Marker M1 (20 - 100 nt)	#RL001-15	-20°C	10 µL
Marker M2	#RL001-16	-20°C	10 µL
PR oligo (PR)	#RL001-18	-20°C	15 µL
RT oligo (RT)	#RL001-19	-20°C	25 µL
FR oligo (FR)	#RL001-20	-20°C	25 µL
RiboLace RNA control	#RL001-26	-80°C	10 µL

Shelf life: 5 months from the delivery date

RiboLace Ribo-Seq library preparation

RiboLace kit code number.....

Number of samples (N).....

Reagents and equipment to be supplied by user:

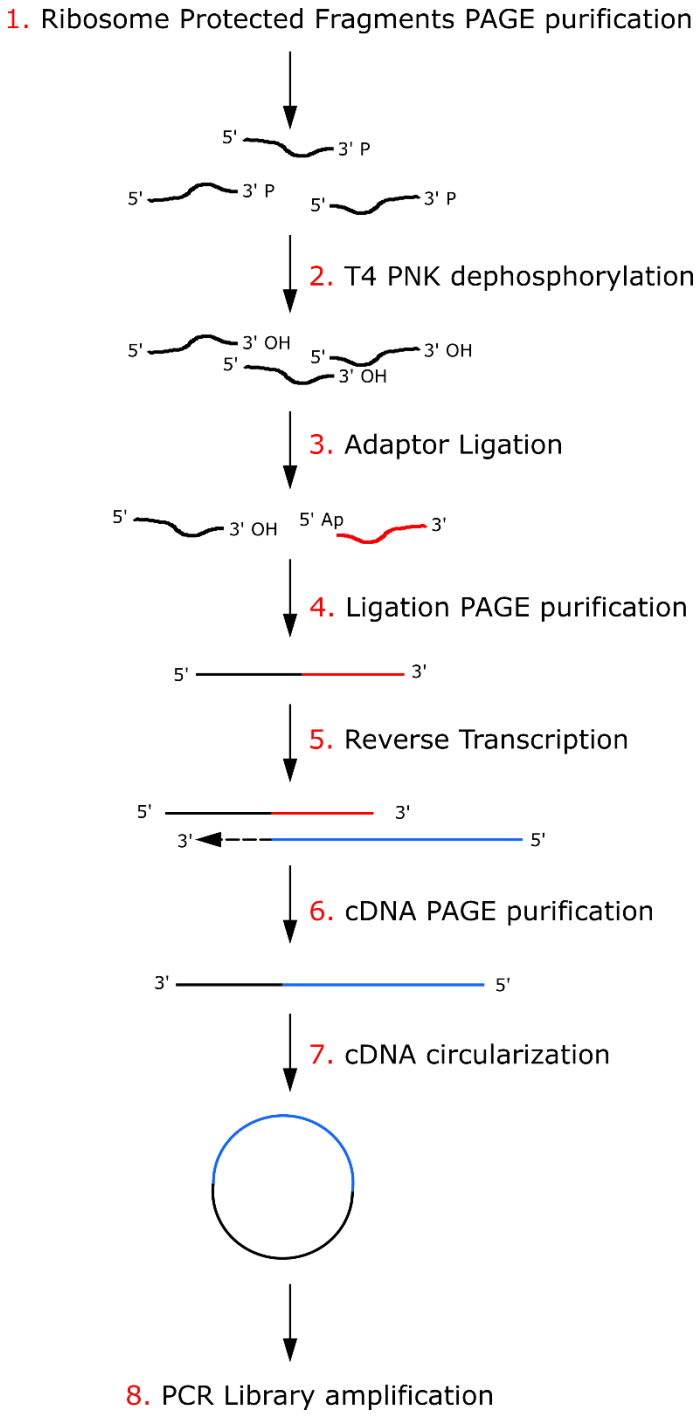
- T4 Polynucleotide Kinase (NEB catalog no. M0201S)
- 15% TBE-Urea polyacrylamide (BioRad catalog no. 450-6053 or Thermo Scientific catalog no. EC6885BOX)
- Gel Loading Buffer II (Denaturing PAGE) (Thermo Scientific catalog no. AM8546G)
- SYBR Gold (Thermo Scientific, catalog no. S11494)
- Isopropanol (Sigma catalog no. 278475)
- GlycoBlue (Invitrogen catalog no. AM9515)
- Nuclease free water
- SUPERaseIn (Invitrogen catalog no. AM2696)
- T4 RNA Ligase 2, truncated (NEB catalog no. M0242S)
- SuperScript™ III Reverse Transcriptase (Invitrogen, catalog no. 18080044)
- dNTP (Invitrogene, catalog no 18427088)
- CircLigase™ II ssDNA Ligase (Lucigen catalog no. CL9021K)
- Phusion® High-Fidelity PCR Master Mix (NEB, catalog no. M0531S)
- MgCl2 (Invitrogene, catalog no R0971)
- 6-8% TBE native gel (homemade or Invitrogen catalog no. EC6265BOX)
- DNA loading dye (Thermo Scientific, catalog no. R0611)
- Illumina Index reverse primers
- Microcentrifuge and nonstick RNase-free microfuge tubes (0.2 mL and 1.5 mL)
- Automatic wheel (rotator)
- Thermocycler

OPTIONAL

- Agilent 2100 Bioanalyzer
- High-Sensitivity DNA chip (Agilent Tech. catalog no. 5067-4626)
- Agencourt AMPure XP (optional) (Beckman Coulter, catalog no. A63881)

Work always in an RNase-free environment

Principle and Procedure

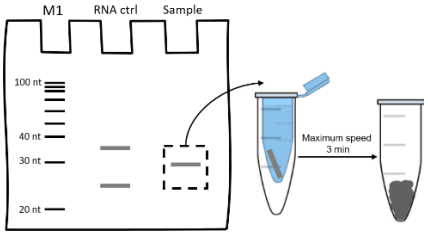


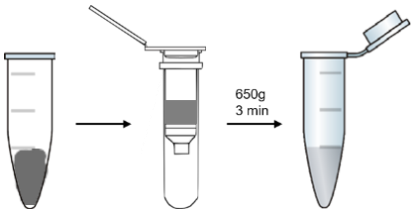
Step 1

RPFs PAGE Purification

Pre-run the gel at 200 V for 30 min in TBE prepared with nuclease-free water. Clean well the gel wells with a syringe to remove UREA residuals before to load the samples.

The RPFs will be ~25-35 nts in length. The IMMAGINA RNA control, provided in the kit, is a mix of oligos from 25 nt to 35 nt and it will be used as a size marker.





- Prepare samples: add 5 µL of Gel Loading Buffer II to 5 µL of RPFs obtained from 1-3 rxns of RiboLace Ribo-Seq kit module 1 or other protocols
- Prepare RNA control: mix 2 µL IMMAGINA RNA control, 3 µL nuclease-free water and 5 µL Gel Loading Buffer II
- Prepare M1 marker: mix 1 µL M1, 4 µL nuclease-free water and 5 µL of Gel Loading Buffer II
- Denature the samples, RNA control and marker M1 for 90 s at 80 °C. Place the tubes immediately on ice
- Load the samples, RNA control, and marker on 15% TBE-urea polyacrylamide gel and run the gel at 200V until the bromophenol blue band reaches the bottom of the gel
- Stain the gel with SYBR Gold and visualize the RNA using a Transilluminator
- Size select the ribosome protected fragments (RPF) between 25-nt and 35-nt according to the RNA control and the marker M1. Also, excise the “RNA control” oligo bands. You can use the “RNA control” as a reference sample for all next steps of the library prep (Fig. 1)
- Place each gel slice in a provided 1.5 mL blue-cup tubes. Spin at maximum speed for 3 min at 4°C. Collect the gel debris and discard the empty blue cupped tubes.
- Add 400 µL of Buffer I, close the vial with the provided cup, incubate the tubes for 1 hour at - 80°C, thaw them at RT and then place the samples on a wheel in slow motion (3 rpm), at RT overnight.
- With a 1 mL cut-tip, add the gel slurry to the provided filter and spin at 650g for 3 min at 4°C to remove the gel debris. Transfer the eluted solution to a new tube
- Add 700 µL of isopropanol and 1.5 µL GlycoBlue to the eluted sample
- Store at - 80°C for 2h (fast procedure) or overnight (safe procedure)
- Thaw the samples on ice and pellet the RNA by centrifugation (20000g) for 30 min at 4°C
- Resuspend the pellet in 10 µL TR buffer

Step 2

T4 PNK dephosphorylation

Tab. 1

Component	**µL**
RNA	10
Nuclease-free water	33
T4 PNK buffer	5
SUPER In (20U/ µL)	1
T4 PNK (10U/ µL)	1

- Thaw the samples on ice
- Pellet the RNA by centrifugation (20000g) for 30 min
- Resuspend the pellet in 10 µL TR buffer
- Transfer the reaction in a 0.2 mL RNase-free PCR tube suitable for your thermocycler
- Mix the reagents for the dephosphorylation reaction according to Table 1
- Incubate the reactions in a thermocycler for 60 min at 37°C, then for 10 min at 70°C, then cool the samples at RT
- Add 39 µL of nuclease-free water, 10 µL sodium acetate, 150 µL of isopropanol and 1 µL GlycoBlue
- Transfer the solution to a 1.5 mL tube
- Store at -80°C for:
    - 2 hours (fast procedure)
    - overnight (safe procedure, recommended for low input samples)

Step 3

Adaptor Ligation

Tab. 2

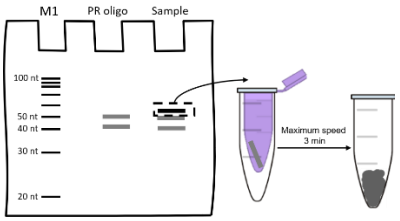
Component	µL
RNA + PR oligo	8
T4 Rnl2 Buffer 10X	2
Peg 8000	8
SUPER In (20U/ µL)	1
T4 Rln2 (200U/ µL)	1

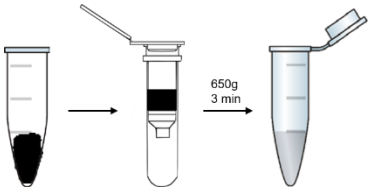
- Thaw the samples on ice and pellet the RNA by centrifugation at full speed for 30 min
- Resuspend the pellet in 7 µL TR buffer and transfer it to a 0.2 mL RNase-free PCR tube
- Add 1 µL PR oligo, denature the sample for 90 sec at 80°C in a thermocycler, then cool the reactions at RT
- Mix the reagents for the ligation reaction according to Table 2
- Incubate for 3 h at 22 °C in a thermocycler
- Transfer the reaction to a new 1.5 mL tube.
- Add 338 µL of nuclease-free water 40 µL sodium acetate, 500 µL of isopropanol and 1.5 µL GlycoBlue
- Store at -80°C for:
    - 2 hours (fast procedure)
    - overnight (safe procedure, recommended for low input samples)

Step 4

Ligation PAGE Purification

Pre-run the gel at 200 V for 30 min in TBE prepared with nuclease-free water. Clean well the gel wells with a syringe to remove UREA residuals before to load the samples.





- Pellet the RNA by centrifugation (20000 g) for 30 min
- Resuspend the pellet in 5 µL TR buffer, add 5 µL of TBE-urea sample loading, and load the samples on 15% TBE-urea polyacrylamide gel
- Load 1 µL of PR and M1 as a reference in separated wells of the gel
- Run the gel at 200V until the bromophenol blue band reaches the bottom of the gel
- Size select the fragments at ~ 55 nt according to the M1 and PR oligo. Proceed only if you see the ligation product. The efficiency depends on (i) the starting material and on (ii) the translational state of the cells (Figure 1, 2)
- Place each gel slice in a provided 1.5 mL violet-cup tubes. Spin at maximum speed for 3 min at 4°C. Collect the gel debris and discard the empty violet cupped tubes.
- Add 400 µL of Buffer I, close the vial with the provided cup, incubate the tubes for 1 hour at - 80°C, thaw them at RT and then place the samples on a wheel in slow motion (3 rpm), at RT overnight.
- With a 1 mL cut-tip, add the gel slurry to the provided filter and spin at 650g for 3 min at 4°C to remove the gel debris. Transfer the eluted solution to a new tube
- Add 700 µL of isopropanol and 1.5 µL GlycoBlue to the eluted sample
- Store at - 80°C for 2h (fast procedure) or overnight (safe procedure)
- Thaw the samples on ice and pellet the RNA by centrifugation (20000g) for 30 min at 4°C
- Resuspend the pellet in 10 µL TR buffer and transfer it to a new 0.2 RNase-free PCR tube

Figure 1

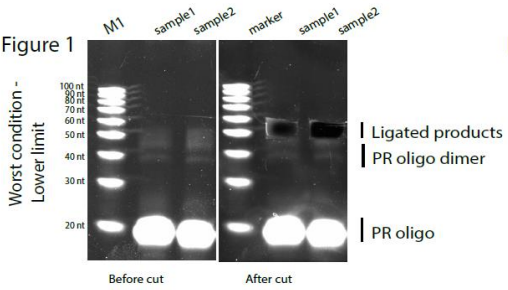
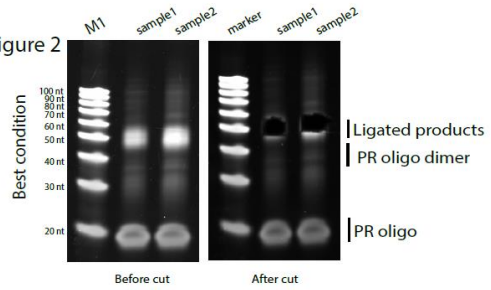


Figure 2



Step 5
Reverse Transcription

Tab. 3

Component	µL
RNA + RT oligo	12
SuperScript III buffer 5x	4
dNTPs (10 mM)	1
DTT	1
SUPER In (20U/ µL)	1
SuperScript III (200U/ µL)	1

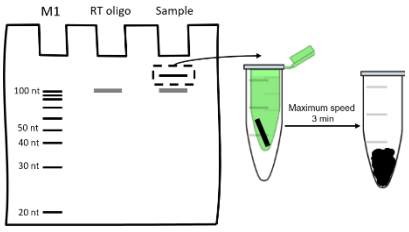
Note that Table 3 is referred to the product Invitrogen, catalog no. 18080044

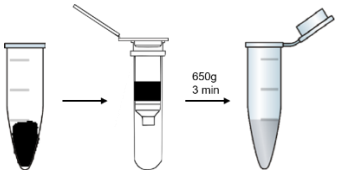
- Add 2 µL RT oligo, denature the sample for 2 min at 80°C in the thermocycler, then cool the reactions on ice
- Mix the reagents for the reverse transcription reaction according to Table 3
- Incubate for 30 min at 55 °C in a thermocycler
- Add 2.2 µL HI-solution, heat the sample at 70°C for 20 min and then cool it at 4°C
- Transfer the reaction to a new 1.5 mL tube.
- Add 156 µL of nuclease-free water, 20 µL sodium acetate, 300 µL of isopropanol and 2 µL GlycoBlue
- Store at -80°C for:
  - 2 hours (fast procedure)
  - overnight (safe procedure, recommended for low input samples)

OPTIONAL STOPPING POINT

Step 6
cDNA PAGE Purification

Pre-run the gel at 200 V for 30 min in TBE prepared with nuclease-free water. Clean well the gel wells with a syringe to remove UREA residuals before to load the samples.

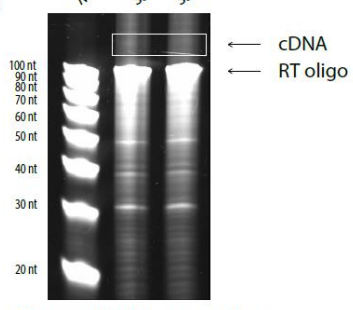




- Thaw the samples on ice, then pellet the cDNA by centrifugation at 20000g for 30 min at 4°C, and air-dry the pellet for 10 min
- Resuspend the pellet in 5 µL TR buffer, add 5 µL of TBE-urea sample loading, and load the samples on 15% TBE-urea polyacrylamide gel
- Load 1 µL of RT oligo and M1 as a reference in separated wells of the gel
- Run the gel at 200V until the bromophenol blue band reaches the bottom of the gel
- Size select the fragments at ~ 130 nt according to M1 and RT oligo (Figure 3, 4)
- Place each gel slice in a provided 1.5 mL green-cup tubes. Spin at maximum speed for 3 min at 4°C. Collect the gel debris and discard the empty green cupped tubes.
- Add 400 µL of Buffer II, close the vial with the provided cup, incubate the tubes for 1 hour at - 80°C, thaw them at RT and then place the samples on a wheel in slow motion (3 rpm) for 8-10 hours at 37°C
- With a 1 mL cut-tip, add the gel slurry to the provided filter and spin at 650g for 3 min, at 4°C to remove the gel debris. Transfer the eluted solution to a new tube
- Add 700 µL of isopropanol and 1.5 µL GlycoBlue to the eluted sample
- Store at - 80°C for 2h (fast procedure) or overnight (safe procedure)
- Thaw the samples on ice and pellet the cDNA by centrifugation (20000g) for 30 min at 4°C
- Resuspend the pellet in 12 µL TR buffer and transfer it to a new 0.2 RNase-free PCR

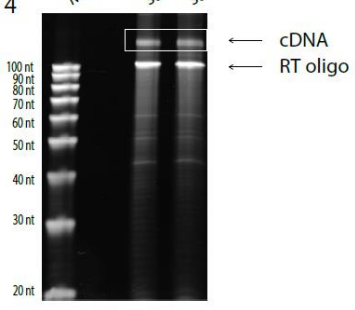
OPTIONAL STOPPING POINT

Figure 3



Worst condition - Lower limit

Figure 4



Best condition

Step 7
cDNA Circularization

Tab. 4

Component	µL
cDNA	12
CircLigase buffer 10x	2
Betaine	4
$MnCl_2$	1
CircLigase II	1

- Mix the reagents for the cDNA circularization reaction according to Table 4
- Incubate for 2 hours at 60 °C, followed by 10 min at 80°C in a thermocycler, then cool the samples at 4°C
- Store the samples at -20°C or proceed directly with PCR amplification

OPTIONAL STOPPING POINT

Step 8
PCR Library Amplification

For most samples, use 8 µl of the circularized cDNA from Step 7 and 10 PCR cycles that are typically enought to produce sufficient amounts of library. For low input samples use 10 µl of the circularized cDNA from Step 7 and increase the number of PCR cycles to 12-14 cycles. Avoid the use of too much cDNA or too many PCR cycles that can produce an "overamplification" of the library and adapter dimer-derived products.

Tab. 5

Component	µL
2X Phusion Master Mix	25
Circ cDNA	8-10
FR Oligo	2
Rev Index (not provided)	2
50 mM $MgCl_2$	0.5
Nuclease-free water	to 50

- Mix the reagents for the PCR Library amplification reaction according to Table 5
- Set up the PCR reaction conditions as follow:
  - 98°C, 30 sec
  - 98°C, 10 sec; 65°C, 10sec; 72°C, 5 sec (10-14 cycles)
  - 4°C hold
- The remaining cDNA (10-12 µl) can be stored at –20°C and can be used in case template input amount and PCR cycle number shoud be optimized.

OPTIONAL
Security Check Point
Quality-check of the Library

This step will help the understanding of the gel in Step 9

- Perform the purification of the 10 µL of PCR product with Agencourt AMPure XP according to the user's manual
- Elute the sample and load it on a BioAnalyzer High-Sensitivity DNA chip according to user's manual
- A single peak at ~ 176 nt indicates good quality (Figure 5). Adaptor peak at ~145 nt is acceptable if is less than 10-15% of the total library. If Adaptor peak at ~145 nt is more than 10-15% of the total library, proceed with Library PAGE Purification (Step 9).

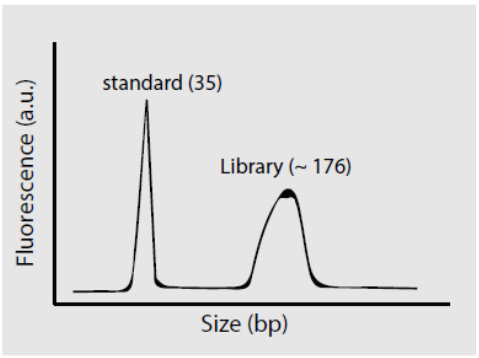
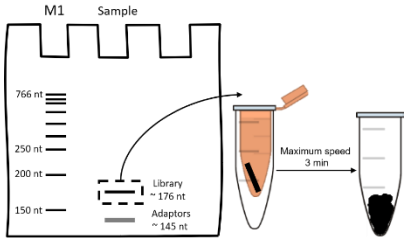
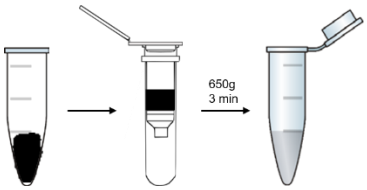


Figure 5

Step 9

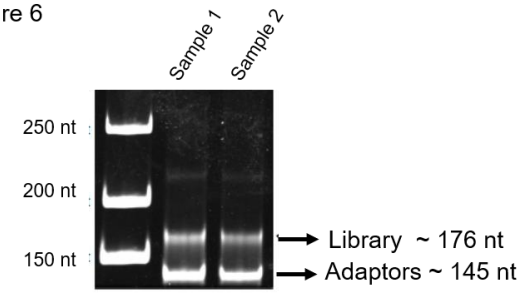
Library PAGE Purification





- Mix 10 µL PCR product and 3 µL DNA loading dye and load the sample on a native 6-8% TBE gel
- Run the gel at 200V until the blue dye reaches the bottom of the gel
- Size select the library at ~ 176 nt according to the marker M2 (Figure 6)
- Place each gel slice in a provided 1.5 mL orange-cup tubes. Spin at maximum speed for 3 min at 4°C. Collect the gel debris and discard the empty orange cupped tubes.
- Add 400 µL of Buffer II, close the vial with the provided cup, incubate the tubes for 1 hour at - 80°C, thaw them at RT and then place the samples on a wheel at RT in slow motion (3 rpm) overnight.
- With a 1 mL cut-tip, add the gel slurry to the provided filter and spin at 650g for 3 min, at 4°C to remove the gel debris. Transfer the eluted solution to a new tube
- Add 700 µL of isopropanol and 1.5 µL GlycoBlue to the eluted sample
- Store at - 80°C overnight
- Thaw the samples on ice and pellet the library by centrifugation (20000g) for 30 min at 4°C
- Resuspend the pellet in 11 µL TR buffer and transfer it to a new 0.2 RNase-free PCR
- Use 1 µL of the library of each sample for quality check using BioAnalyzer High-Sensitivity DNA chip
- Libraries are ready for sequencing and can be stored indefinitely at -20°C.

Figure 6



Appendix I (reverse primers)

Index 1 PCR Primer 5' CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

Index 2 PCR Primer 5' CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

Index 3 PCR Primer 5' CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

Index 4 PCR Primer 5' CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

Index 5 PCR Primer 5' CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

Index 6 PCR Primer 5' CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

Index 7 PCR Primer 5' CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

Index 8 PCR Primer 5' CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

Index 9 PCR Primer 5' CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

Index 10 PCR Primer 5' CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

Index 11 PCR Primer 5' CAAGCAGAAGACGGCATACGAGATGTAGCCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

Index 12 PCR Primer 5' CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

Recommended Index Combinations for Sequencing

Library Pools

- Duplex (two indices): Index 06 and Index 12
- Triplex (three indices): Index 04 and Index 08 - Add one: Index 01, 02, 05, 06, 09 or 10
- Triplex (three indices): Index 07 and Index 11 - Add one: Index 01, 02, 05, 06, 09 or 10
- Triplex (three indices): Index 02 and Index 09 - Add one: Index 03, 04, 07, 08, 11 or 12
- Four or more indices: Index 06 and Index 12 - Add any other indexes