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Product Information

Product Name: Nextseq Small RNA Library Kit (with 12 indexes)
Catalog #: KIT97
Size: 20 Reactions

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Product Description:

This kit provides an easy & simple way to clone small RNA from 20 ng to 2 µg total RNA in a single reaction tube without buffer changes by phenol extraction and ethanol precipitation. The whole procedure can be done within 6-8 hours including the final gel purification step. The protocol can be used to efficiently clone triphosphorylated RNA, capped RNA and small RNA with a 2'-O modification at the 3' end. The resulting library is compatible with Illumina HiSeq and NEXTseq platforms with build-in 8nt barcodes in the 3' linker, thus allowing multiplexing. And the library can be used as template cDNAs for realtime PCR. The kit includes all the required enzymes/reagents, e.g. decapping enzyme, RNA Polyphosphatase (transfer pppRNA to pRNA), 3' and 5' RNA Ligases, Reverse transcriptase, and, HF PCR polymerase. Twelve 3' PCR primers with different barcodes (indexes) are also included in the kit for convenience. With addition of PNK, the kit can be used to clone mRNAs.

Components

25 µl 10x ligation buffer (without ATP)
120 µl 50% PEG-8000 (for cloning 2'-O-modified RNAs)
12 µl 20 mM ATP
6 µl Ligation enzyme mix A
6 µl Ligation enzyme mix B
6 µl EZdeCAP (for cloning capped small RNA, 10U/ µl)
6 µl RNA Polyphosphatase (for cloning pppRNA, 10 µM)
6 µl Reverse transcriptase
10 µl 20 µM RNA linker: ACACUCUUUCCCUACACGACGCUCUUCCGAUCU
5.5 µl 3' linker :
12 µl RT primer (in inactivation buffer) GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
15 µl 10µM 5' PCR primer: AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGA
15 µl 10 µM 3' PCR primer #1(Barcode #1):
15 µl 10 µM 3' PCR primer #2(Barcode #2):
15 µl 10 µM 3' PCR primer #3(Barcode #3):
15 µl 10 µM 3' PCR primer #4(Barcode #4):
15 µl 10 µM 3' PCR primer #5(Barcode #5):
15 µl 10 µM 3' PCR primer #6(Barcode #6):
15 µl 10 µM 3' PCR primer #7(Barcode #7):
15 µl 10 µM 3' PCR primer #8(Barcode #8):
15 µl 10 µM 3' PCR primer #9(Barcode #9):
15 µl 10 µM 3' PCR primer #10(Barcode #10):
15 µl 10 µM 3' PCR primer #11(Barcode #11):
15 µl 10 µM 3' PCR primer #12(Barcode #12):
35 µl 10 mM dNTP
25 µl 100 mM DTT
120 µl 10x HFPol buffer
12 µl 100x PCR enzyme (HFPol)
50 µl 10x RT dilution buffer
100 µl DMSO

Procedure:

NOTE: RNase inhibitor may be needed if samples have RNase contamination. Do not use RNA inhibitor if you don't have to. If PEG-8000 is used, please mix the sample well at each step using 20 µl tip by pipetting. Carefully watch it to make sure the sample is mixed well

Step 1: 3' ligation

Denature the RNA at 95 oC for 2 min and then chill it on ice. And then mix the following components in a clean tube and incubate at room temperature for 2 hours.

20 ng to 2 µg total RNA*	3.0 µl
Ligation Enzyme mixture A	0.25 µl
PEG-8000	5 µl
3' linker	0.25 µl
10x ligation buffer (without ATP)	1 µl

Note: If cloning pppRNA, 0.25ul RNA Polyphosphatase is required. PEG-8000 can be substituted with 10% DMSO if not cloning RNAs with 2'-O-modification at the 3' end. For cloning miRNAs, 1 hour incubation is sufficient. Please keep PEG-8000 at room temperature since PEG-8000 is very viscous. If a dried RNA pellet is used as RNA substrate, please make a master mixture without PEG-8000 to dissolve the RNA pellet first and then add PEG-8000. Glycogen may precipitate in reactions containing PEG-8000. However, it does not affect the ligation and can be spun down. It is not necessary to remove glycogen precipitates.
*Some RNA samples may need phosphatase treatment, please use FastAP Thermosensitive Alkaline Phosphatase (Thermo Scientific) instead of CIP (Calf Intestinal Phosphatase), which requires phenol extraction to inactivate the enzyme. Here is how: In step 1, add RNA, 10x ligation buffer (without ATP), and ddH_2O, follow the manufacture instruction by incubating the mixture at 37^0C for 10min, and heat inactivate at 75^0C for 5 minutes. Then add the rest of the components in step 1 to perform 3' ligation.

* RNA isolated by column, phenol, or Trizol-based methods are suitable for this kit. RNA samples should be EDTA-free.

Step 2: Inactivation

Add 0.5µl RT primer to each reaction and incubate at 65°C for 15 min and then chill on ice for 2 min.

Step3: 5' ligation.

Add the following components to step 2 and incubate at room temperature for 2hs or at 4 °C for overnight.

ATP	0.5 µl
RNA linker	0.4 µl
Ligation Enzyme mixture B	0.25 µl
H2O	8.35 µl

Note: If cloning capped small RNAs, 0.25µl EZdCAP is added to the reaction at this step.

Step 4: Reverse transcription (RT)

Add the following components to step 3 and incubate at 42°C for 20 minutes.

RT dilution buffer	2 µl
dNTP	1 µl
DTT	1 µl
Reverse transcriptase	0.25 µl

Step 5: PCR

H2O	35.5 µl
10x HFPol buffer	5.0 µl
DMSO	2.5 µl
dNTP	0.5 µl
5' PCR primer	0.5 µl
3' PCR primer (barcode #1-12)	0.5 µl
The above RT reaction	5.0 µl
HFPol PCR enzyme	0.5 µl

Mix above components and perform PCR:

1	denaturing cycle	94°C 1 min
16	PCR cycles	94°C 20 sec 53 °C 20 sec 68°C 30 sec

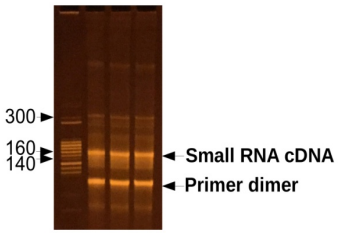
Add 3µl each of the same 5' primer and 3' primer and continue for 2 more PCR cycles at the same setting above.

Step 6: Gel purification of PCR Products

Each PCR reaction can be purified from 2-3 wells of 8% native PAGE gel (acrylamide:Bis is 29:1 or 19:1), and then dissolved in 7.5 µl Tris-Cl buffer (10 mM, pH 7.5). Alternatively and more conveniently, the samples can be roughly quantified using a page gel or bioanalyzer. And then the samples can be mixed, phenol/chloroform extracted and precipitated. The precipitated sample can be purified as a mixture. Please don't dry the sample at any step during precipitation and washing. Otherwise, DNA is denatured and becomes bulged products which do not affect sequencing but quantification using Bioanalyzer.

Purification of a sample PCR product:

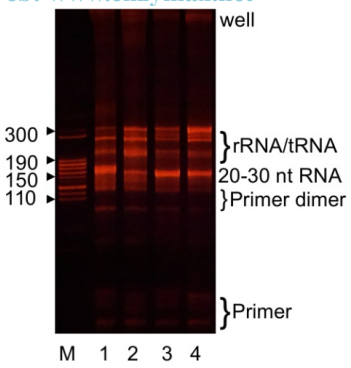
This library is made from 1 µg small RNA from *C. elegans* and RNA Polyphosphatase were used in the 5' ligation reaction to clone the triphosphorylated RNAs. The first lane is a 10bp marker. The cDNA product of 22mer RNA is 146 nucleotides (nt) long. Cut the PCR band, grind it in a 1.5 ml tube using a 200µl tip and elute in 0.75 ml buffer containing 0.3 M NaCl and 10 mM Tris (pH 7.5) overnight. Remove the gel pieces with filter columns (EZC109 or IP111), precipitate the flow-through with 20µg glycogen and 1.1 fold (v/v) isopropanol at -20 °C for 30 min. Spin at 4 °C at 15,000xg for 15 mins, wash once with 75% cold ethanol and dissolve in10µl Tris, pH 7.5. Now the samples are ready for (1) real time PCR with high specificity & sensitivity (due to PCR amplification and gel purification), and (2) Deep sequencing with Illumina HiSeq and NEXTseq platforms.



The final cDNA sequence including the linker:
AATGATACGGCGACC
ACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGAT
CT---i--n--s--e--r--t--AGATCGGAAGAGCACACGTCTGAA
CTCCAGTCAC -index-ATCTCGTATGCCGTCTTCTGCTTG

Other RNA Samples are also tested with this Kit:

This kit was also tested with RNA samples from different sources: mouse testis, mouse ovary, and *C. elegans* small RNA and total RNA.



M: 10 bp marker (110-200, 260 and 300)
1: 0.5 µg mouse testis total RNA
2: 0.4 µg mouse ovary total RNA
3: 0.5 µg *C. elegans* small RNA (<200 nt)
4: 1 µg *C. elegans* total RNA

Note for the marker: 10bp marker (load 5-10µl)
Range of 110-200 (140 is weaker, 200 is also very weak), 260 and 300 (weak). Since the amplicon runs slower than the marker and appears at position 155 instead of 146 for 22nt insert. It is suggested to cut the range of 140-180 to include everything but background noise.

Quality Control:

This kit was extensively tested and validated through manual and next-generation sequencing of *C.elegans* small RNA libraries and mRNA libraries (with a minor modification).

Appendix A (3' PCR primer barcode sequences)

3' PCR primer #1(Barcode #1):
CAAGCAGAAGACGGCATACGAGATAGTTCCACGTGACTGGAGTT
CAGACGTGT
3' PCR primer #2(Barcode #2):
CAAGCAGAAGACGGCATACGAGATCCG TCCCAGTGACTGGAGTT
CAGACGTGT
3' PCR primer #3(Barcode #3):
CAAGCAGAAGACGGCATACGAGATGTAGAGCAGTGACTGGAGTT
CAGACGTGT
3' PCR primer #4(Barcode #4):
CAAGCAGAAGACGGCATACGAGATGTCCGCTCGTGACTGGAGTT
CAGACGTGT
3' PCR primer #5(Barcode #5):
CAAGCAGAAGACGGCATACGAGATGTGAAACTGTGACTGGAGTT
CAGACGTGT
3' PCR primer #6(Barcode #6):
CAAGCAGAAGACGGCATACGAGATGTGGCCGGGTGACTGGAGTT
CAGACGTGT
3' PCR primer #7(Barcode #7):
CAAGCAGAAGACGGCATACGAGATGTTTCGCCGTGACTGGAGTT
CAGACGTGT
3' PCR primer #8(Barcode #8):
CAAGCAGAAGACGGCATACGAGATCGTACGTAGTGACTGGAGTT
CAGACGTGT
3' PCR primer #9(Barcode #9):
CAAGCAGAAGACGGCATACGAGATGAGTGGCGGTGACTGGAGTT
CAGACGTGT
3' PCR primer #10(Barcode #10):
CAAGCAGAAGACGGCATACGAGATGGTAGCTAGTGACTGGAGTT
CAGACGTGT
3' PCR primer #11(Barcode #11):
CAAGCAGAAGACGGCATACGAGATATGAGCGAGTGACTGGAGTT
CAGACGTGT
3' PCR primer #12(Barcode #12):
CAAGCAGAAGACGGCATACGAGATCA AAAGGCGTGACTGGAGTT
CAGACGTGT