Published in Heyer EE, et al. *NAR* 2014; doi:10.1093/nar/gku1235

RNA Fragment DeepSeq Library Preparation Protocol

I) LIGATION

Recommended input: RNA between 0.05-2 pmol; must have 3' OH

1. Thaw 10X T4 RNA Ligase Reaction Buffer, 50% PEG8000, 20 mM DTT, 7 uM App Adaptor and keep on ice
2. Add the following to a PCR tube on ice:

7 uM App Adaptor	1.0 µl
RNA	x µl
Water	to 4.8 µl

3. Heat 65°C 10 min; 4°C hold
4. Transfer tubes to ice. Add the following to each tube for a total reaction volume of 15 µl:

	1X rxn	note – minimize pipetting error by
10X Buffer (from NEB)	1.5 µl	preparing a ligation mastermix
50% PEG8000	7.5 µl	
33 mM DTT	0.45 µl	
T4RNL2 Tr. K227Q	0.75 µl	
Total Volume added	10.2 ul	

5. Incubate 30°C for 6 hr; 65°C 20 min heat inactivation; 4°C hold

II) REVERSE TRANSCRIPTION

1. Thaw RT Primer, 10 mM dNTP mix, 5X First-Strand Buffer w/o MgCl2 (see recipe below), 100 mM DTT; store on ice
2. Add to 15 µl ligation on ice, same tube:

10 uM RT Primer	1.0 µl
10 mM dNTP mix	2.25 µl
Water	14.3 µl

3. Incubate 65°C 5 min; 4°C hold
4. Transfer to ice and add the following to each tube for a total reaction volume of 45 µl:

5X FS Buffer w/o $MgCl_2$	9.0 µl
100 mM DTT	2.25 µl
SSIII (200U/µl)	1.2 µl

5. Incubate 55°C 30-45 min; heat inactivate 70°C 15 min; 4°C hold
6. Add 25 µl 2X Denaturing Load Buffer (see recipe below)
7. Gel purify RT product on 10% denaturing PAGE
8. Cut RT product from gel, using a new razor blade for each sample
9. Crush gel fragment: place in a 0.5 ml eppy sitting inside a 2 ml eppy, having previously poked a 22 gauge needle hole in the bottom of the 0.5 ml eppy; spin 10,000xg for 3 min at RT to crush

10. Elute in DNA Elution Buffer (see recipe below) O/N at 37°C with constant rotation (add enough volume so that the gel fragments are free to move in the tube)
11. Transfer slurry to Spin-X Column and spin at 10,000xg for 3 min, RT
12. Concentrate eluate, either by SpeedVac or butanol extraction
13. Ethanol precipitate. Note: if the RT fragments are < 100 nts, you might improve precipitation efficiency by adding $MgCl_2$ to a final concentration of 10 mM before adding ethanol

III) Circularization

1. Thaw CircLigase I Reaction Buffer, 1 mM ATP, 50 mM $MnCl_2$; 5M Betaine stored @ 4°C
2. Spin down RT product at 12,000xg, 4°C, 30 min
3. Wash with 1 ml 75% EtOH (stored at -20°C)
4. Repeat spin for 10 min
5. Remove as much EtOH as possible; heat pellet at 37°C for 3-5 min to dry pellet
6. Resuspend DNA in 10 µl water;
7. Transfer DNA to PCR tube and prepare 20 µl Circularization Reaction:

RT Product	10.0 µl
CircLigaseI Rxn Buffer	2.0 µl
1 mM ATP	1.0 µl
50 mM $MnCl_2$	1.0 µl
5M Betaine	4.0 µl
Water	1.0 µl
CircLigase	1.0 µl

8. Incubate 60°C 3-4 hr; inactivate 80°C 10 min

IV) PCR Amplification

1. Thaw phosphorothioate PE1.0 and PE2.0 primers and KAPA 2X HiFi; store on ice once thawed
2. To determine the appropriate number of PCR cycles, prepare several small-scale reactions with phosphorothioate primers:

KAPA 2x HiFi	7.5 µl	Denaturation:	45 sec @ 98°C
10 uM PE 1.0 phos	0.75 µl	Cycling:	15 sec @ 98°C
10 uM PE 2.0 phos	0.75 µl		30 sec @ 65°C
Circularization Rxn	up to 3 µl		30 sec @ 72°C
Water	to 15 µl	Final Extension:	1 min @ 72°C

3. Analyze on normal length 8% nondenaturing PAGE to determine the optimal number of PCR cycles, ensuring that primers show no depletion (i.e. are still abundant in the rxn)
4. Repeat with a 50 µl PCR for fixed cycle number (determined in steps 2-3):

KAPA 2x HiFi	25 µl
10 uM PE 1.0	2.5 µl
10 uM PE 2.0	2.5 µl
Circularization Rxn	3.3*volume used in test
Water	to 50 µl

5. Purify PCR fragment on 8% nondenaturing PAGE run on double-wide apparatus to minimize heating and denaturation of double stranded library.

   Alternatively, replace steps 5-8 with purification on Pippin Prep/BluePippin
6. Stain gel in SYBR Gold, excise product band, crush as in II Step 8
7. Elute by nutating O/N in DNA Elution Buffer (see recipe below); the following morning, remove some buffer and keep on ice; add back an equal volume of DNA Elution Buffer for 2-4 more hours to elute as much material as possible from the gel fragments
8. Concentrate the eluate by SpeedVac or butanol extraction
9. Ethanol precipitate
10. Spin down libraries at 12,000 x g for 30 min, 4°C. Wash 2x 75% EtOH. Remove as much EtOH as possible by pipetting and immediately resuspend pellet in 20 µl ddH20
11. Quantify library by submitting 2 µl for Bioanalyzer analysis on High Sensitivity DNA Chip
12. Pool libraries (if necessary) to a final concentration of 10 nM (usually submit at least 15 µl). If necessary, SpeedVac samples on low setting, 3 min increments, to concentrate the libraries

GENERAL NOTES:

1. The inclusion of 4 degenerate bases (NNNN) at the beginning of the 5' adaptor allows us to determine if a sequence was captured multiple times or over-amplified during PCR. However, this benefit of NNNN only applies if your sequence read is longer than your RNA insert length, meaning you will read through to these bases. If you want the benefit of NNNN but are using a short sequencing run, move the NNNN to the RT primer, between the PE1.0 sequence and the barcode. The first 4 bases of the sequencing reaction will be the degenerate bases, then the 5 nt barcode, then GG, then your sample. Ex.

   BC1: 5' - pGGATCACNNNNAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT-SP18-
   CTCGGCATTCCTGCTGAACCGCTCTTCCGATCT-CCTTGGCACCCGAGAATTCCA – 3'

2. In an attempt to keep the sample free from contaminants, all pipetting should be done with filter tips (use low retention when possible, especially for handling viscous solutions like PEG8000)
3. The RT is done with a buffer lacking additional $MgCl_2$, since a 3-fold dilution of the ligation buffer yields $MgCl_2$ concentration optimal for SSIII
4. All primers should be gel purified or HPLC purified
5. IMPORTANT: CircLigase circularization efficiency can change depending on the lot (also see Epicentre's CircLigase patent #WO 2010094040 A1; Jendrisak, Decker and Dahl). 1Therefore, every tube of CircLigase should be checked for circularization efficiency before use in library preparation. To do this, perform an RT with N24 RNA and ^{32}P dCTP, gel purify the RT product and test circularization on a gel.
6. If you're having trouble with these reactions, your primers may not be fully 5' phosphorylated. A few other labs have occasionally run into this issue. If this is a problem, 5' phosphorylate with T4 PNK prior to use, ensuring both the RT primer and the 3' adaptor (before Mth Ligase pre-adenylation that requires 5' P; if manually pre-adenylating) are fully 5' phosphorylated.
7. Depending on the amount of RNA input, your RT product may not be visible with SYBR Gold staining. It is advisable to use a guide oligo in a separate ligation/RT reaction to indicate where to cut on the gel.

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PURCHASED REAGENTS:

T4 RNA Ligase 2, truncated K227Q – NEB #M0351L (PEG8000 currently comes with the enzyme)
Superscript III Reverse Transcriptase – Invitrogen #18080-044
CircLigase ssDNA Ligase – Epicentre Biotechnologies #CL4115K
5M Betaine – Sigma #B0300-5VL
KAPA HiFi Library Amplification Kit – KAPA Biosystems #KK2611 or #KK2612

PREPARED REAGENTS:

5X First-Strand Buffer w/o $MgCl_2$
250 mM Tris-HCl (pH 8.3 at room temp), 375 mM KCl

DNA Elution Buffer (TE)
10 mM Tris-HCl (pH 8.0 at room temp), 1 mM EDTA

2X Denaturing Load Buffer
2 mL 5X TBE, 1.2 g Ficoll Type 400, 4.2 g Urea, 2 mg bromophenol blue, 2 mg xylene cyanol,
up to 10 ml ddH20; store at 4°C
- heat to get into solution or nutate O/N
- add dyes after adjusting the volume to 10 mL

OLIGOS:

Preadenylated Adaptor for SE Sequencing:
5' – AppTGGAATTCTCGGGTGCCAAGGddC – 3' (miRCat-33® Conversion Kit from IDT)
or
5' – AppNNNNTGGAATTCTCGGGTGCCAAGGddC – 3' (order 5' phosphorylated; preadenylate with NEB 5' DNA Adenylation Kit - E2610; gel purify)

RT Primers (barcodes based upon TruSeq barcodes for Illumina sequencing) for SE Sequencing:
NOTE: the barcodes used in Heyer et al. Nucleic Acids Research 2014; doi: 10.1093/nar/gku1235 are the reverse of those used below due to an initial ordering mistake. The sequences below should be used, as the first few sequenced nucleotides are more base-balanced.

BC1: 5' - pGGCACTAAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT-SP18-
CTCGGCATTCCTGCTGAACCGCTCTTCCGATCT-CCTTGGCACCCGAGAATTCCA – 3'

BC2: 5' - pGGGTAGCAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT-SP18-
CTCGGCATTCCTGCTGAACCGCTCTTCCGATCT-CCTTGGCACCCGAGAATTCCA – 3'

BC3: 5' - pGGTCGATAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT-SP18-
CTCGGCATTCCTGCTGAACCGCTCTTCCGATCT-CCTTGGCACCCGAGAATTCCA – 3'

BC4: 5' - pGGCCTCGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT-SP18-
CTCGGCATTCCTGCTGAACCGCTCTTCCGATCT-CCTTGGCACCCGAGAATTCCA – 3'

BC5: 5' - pGGTGACAAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT-SP18-
CTCGGCATTCCTGCTGAACCGCTCTTCCGATCT-CCTTGGCACCCGAGAATTCCA – 3'

BC6: 5' - pGGTAGACAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT-SP18-
CTCGGCATTCCTGCTGAACCGCTCTTCCGATCT-CCTTGGCACCCGAGAATTCCA – 3'

BC7: 5' - pGGGCCCTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT-SP18-
CTCGGCATTCCTGCTGAACCGCTCTTCCGATCT-CCTTGGCACCCGAGAATTCCA – 3'

BC8: 5' - pGGATCGGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT-SP18-
CTCGGCATTCCTGCTGAACCGCTCTTCCGATCT-CCTTGGCACCCGAGAATTCCA – 3'

BC9: 5' - pGGACTGAAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT-SP18-
CTCGGCATTCCTGCTGAACCGCTCTTCCGATCT-CCTTGGCACCCGAGAATTCCA – 3'

BC10: 5' - pGGTGTTCAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT-SP18-
CTCGGCATTCCTGCTGAACCGCTCTTCCGATCT-CCTTGGCACCCGAGAATTCCA – 3'

BC11: 5' - pGGTAAGTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT-SP18-
CTCGGCATTCCTGCTGAACCGCTCTTCCGATCT-CCTTGGCACCCGAGAATTCCA – 3'

BC12: 5' - pGGAGATGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT-SP18-
CTCGGCATTCCTGCTGAACCGCTCTTCCGATCT-CCTTGGCACCCGAGAATTCCA – 3'

PCR Primers (PE DNA from Illumina):

PE PCR Primer 1.0:
5'–AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC*T–3'

PE PCR Primer 2.0:
5'–CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATC*T–3'

* indicates location of phosphorothioate bond