CircAID-p-seq for Oxford Nanopore Technologies

Product	Catalog no	Rxns.
CircAID-p-seq for phospho-RNA-seq	#CA001	6

Shipping: Dry ice and 4°C

Storage Conditions: store components according to this manual

Shelf Life: 12 months

Description: CircAID-p-seq kit (Circular Amplification and IDentification of short RNA sequences bearing a 3 Phosphate) is designed for quick (1-day) high quality library preparation for short RNAs (20-50 nt) bearing a 3' -phosphate/2',3'-cyclic phosphate (3'-P/2',3'-cP) end. The protocol, suitable for the characterization of cP-forming endoribonucleases, is also applicable to ribosome profiling experiments and transcriptome analysis.
CircAID-p-seq is suitable for the Oxford Nanopore platform (Direct cDNA Sequencing Kit).

CircAID-p-seq for Oxford Nanopore Technologies

List of components

Product (label)	Cap Color	Catalog no.	Store Conditions	Quantity
CircAID-p-seq kit		#CA001	according to manual	1kit - 6 rxns
Buffer PK (BPK)	Red	#CA001-1	-20°C	50 µL
PK enzyme (PK)	Red	#CA001-2	-20°C	8 µL
ATP 10 mM	Red	#CA001-3	-20°C	50 µL
Buffer A (BA)	Blue	#CA001-4	-20°C	50 µL
Enzyme Mix A (mix A)	Blue	#CA001-5	-20°C	14 µL
Linker R ™(R)	Blue	#CA001-8	**-80°C**	20 µL
$MnCl_2$	Blue	#CA001-6	-20°C	50 µL
GTP 1 mM	Blue	#CA001-7	-20°C	50 µL
Buffer B (BB)	Yellow	#CA001-9	-20°C	50 µL
Enzyme Mix B (mix B)	Yellow	#CA001-10	-20°C	10 µL
PEG 8000	Yellow	#CA001-11	-20°C	170 µL
P1 oligo (P1) 20 µM	Green	#CA001-14	-20°C	25 µL
Buffer RT (BRT)	Green	#CA001-15	-20°C	35 µL
RT enzyme (RT)	Green	#CA001-16	-20°C	8 µL
dNTPs 10 mM	Green	#CA001-17	-20°C	30 µL
HI solution (HI)	Green	#CA001-18	-20°C	150 µL
Enhanced Buffer (EnB)	Green	#CA001-19	-20°C	150 µL
Buffer Taq (BT)	Clear	#CA001-20	-20°C	85 µL
Taq	Clear	#CA001-21	-20°C	8 µL
P2 oligo (P2) 20 µM	Clear	#CA001-22	-20°C	25 µL
Control (CTRL)	Clear	#CA001-23	**-80°C**	20 µL

Additional Materials Required:

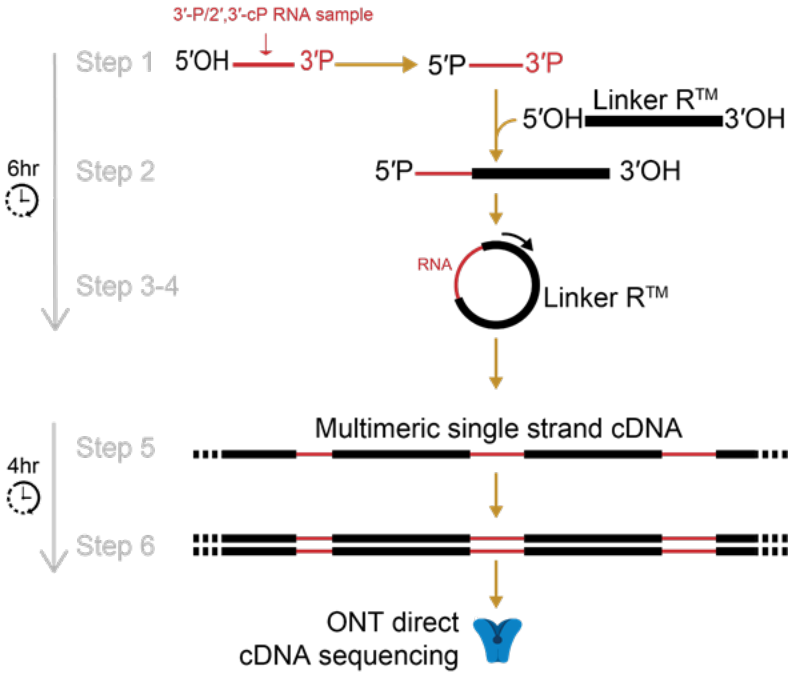
- RNA Clean & Concentrator™-5 (Zymo catalog. no. R1015 & R1016)
- Sodium Acetate 3M
- Nuclease-free water
- GlycoBlue (Ambion catalog no. AM9515)
- Isopropanol (Sigma catalog no. 278475)
- Microcentrifuge and nonstick RNase-free microfuge tubes (0.2 mL and 1.5 mL)
- Automatic wheel (rotator mixer)
- Magnetic stand for 1.5mL tube
- High-Sensitivity DNA chip (Agilent Tech. catalog no. 5067-4626)
- Direct-cDNA Sequencing kit (SQK-DCS109) or Ligation Sequencing Kit (SQK-LSK109) from Oxford Nanopore Technologies
- Qubit Fluorometer
- Qubit miRNA Qubit (Thermo, catalog no.Q32880)

Optional:

- PAGExt Gel extraction kit (IMMAGINA catalog no. #KGE-002)

Work always in an RNase-free environment.

Principle and Procedure



Sample Recommendations

- **Input RNA amount:** ≥ 30 ng of small RNA fragments (miRNA QuBit quantification).
- **Input RNA quality:** high RNA purity and integrity is recommended in order to ensure optimal downstream result.

If you are using this kit for the first time, we recommend to perform a positive control reaction. The positive control (Control, clear cap) is an RNA fragment with a 5'OH and 3'P (10 µM, see sequence at the end of the protocol). For library preparation of the positive control, use 2 µl of the RNA fragment. At the end of the library preparation add 1 µL of step 6 - second strand synthesis - to your library sample and proceed with cDNA direct ONT sequencing.

Step 1
5' phosphorylation

This step is essential when starting with short RNAs bearing 5'-OH ends. If RNA inputs already harbour 5'-P ends, the step can be omitted

- Mix the following reagents in a 0.2 mL nuclease-free PCR tube:

Buffer PK	5µL
10mM ATP	5 µL
PK	1 µL
RNA 3'-P/2'3' -cP	30 pmol
Nuclease free water	Up to 50 µL

- Incubate the reaction for 1h at 37 °C in a thermal cycler.
- Purify the reaction through the RNA Clean & Concentrator™-5 kit, following the protocol for small RNAs and performing the final elution in a volume of 6 µL of nuclease-free water. For more information, see appendix 1.

Step 2
3'-P/2',3'-cP capture

- Mix the following reagents in a 0.2 mL nuclease-free PCR tube:

	RPFs amount			
	30 ng	**50 ng**	**100 ng**	**300 ng**
RNA (from step 1)	6 µL	6 µL	6 µL	6 µL
Buffer A	1 µL	1 µL	1 µL	1 µL
GTP 3 mM	0.5 µL	0.5 µL	0.5 µL	0.5 µL
$MnCl_2$	0.6 µL	0.6 µL	0.6 µL	0.6 µL
Enzyme Mix A	1 µL	1 µL	1 µL	1 µL
Linker_R 10 µM*	0.15 µL	0.25 µL	0.5 µL	1.5 µL
H2O	0.85 µL	0.75 µL	0.5 µL	-

*use always the following ratio between smRNA fragments and linker: 100 ng RPFs (quantified by miRNA Qubit), 0.5 µL of linker_R. If it is necessary, you can dilute the Linker_R in nuclease-free water (e.g instead of pipetting 0.15 µL , you can diluite the linker_MC 1:10 and add 1.5 µL of diluited Linker_R into the reaction).

- Incubate the reaction for 2h at 37 °C in a thermal cycler.
- Add nuclease free water up to 50 µL final volume, then purify the reaction through the RNA Clean & Concentrator™-5 kit, following the protocol for small RNAs and performing the final elution in a volume of 8 µL of nuclease free water.

Step 3
Circularization

- Prepare a 1 mM ATP solution by diluting the 10 mM ATP stock in nuclease-free water. Assemble the following reaction in a 0.2 mL nuclease-free PCR tube:

RNA (from step 2)	**8 µL**
Buffer B	2 µL
ATP 1 mM	1 µL
PEG8000	8 µL
Enzyme Mix B	1 µL

- Incubate the reaction for 2h at 25 °C in a thermal cycler.
- Add nuclease free water up to 50 µL final volume, then purify the reaction through RNA Clean & Concentrator™-5 kit, following the protocol for small RNAs and performing the final elution in a volume of 10 µL of nuclease free water.

OPTIONAL STOPPING POINT *(store at -80°C).*

Step 4
Reverse Transcription

- For the generation of multimeric single-stranded cDNA, combine the following reagents:

Circular RNA (from step 4)	**10 µL**
dNTPs 10 mM	1 µL
P1	2.5 µL
Nuclease-free water	Up to 13 µL

- Heat the circular RNA-primer mix at 65°C for 5 minutes, then incubate on ice for at least 1 minute.
- Add the following reagents to the annealed RNA:

Buffer RT	4 µL
Enhanced Buffer	2 µL
RT enzyme	1 µL

- Incubate 4 h at 42 °C, then add 2.2 µL of HI and heat the mix for 20 min at 70 °C.
- Transfer the reaction to a new 1.5 mL tube.
- Add 156 µL nuclease-free water, 20 µL sodium acetate (3M), 300 µL isopropanol and 2 µL GlycoBlue.
- Store at -80°C for at least 2 hours.
- Pellet the RNA by centrifugation (20000 g) for 30 min.
- Resuspend the pellet in 20 µL of nuclease-free water.

Step 5
Second strand synthesis

- Set up the following PCR reaction in a 0.2 mL nuclease-free PCR tube:

cDNA (from step 5)	**20 µL**
Buffer T	10 µL
dNTPs10mM	1 µL
P2	2.5 µL
Taq	0.3 µL
Nuclease-free water	Up to 50 µL

Cycling conditions:

Step	**Temperature**	**Time**
Initial denaturation	94°C	3 min
1 Cycle	94°C	30 secs
	55°C	30 secs
	68°C	1 min
Hold	4°C	

- Purify the reaction by adding 40 µL of resuspended Agencourt AMPure XP beads and mix by flicking the tube.
- Incubate on a rotator mixer for 5 minutes at RT.
- Prepare 500 µL of fresh 70% ethanol in nuclease-free water.
- Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.

- Keep on magnet, wash beads with 200 µL of freshly prepared 70% ethanol without disturbing the pellet. Remove the 70% ethanol using a pipette and discard.
- Repeat the previous step.
- Spin down and place the tube back on the magnet.
- Pipette off any residual ethanol. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking.
- Remove the tube from the magnetic rack and resuspend pellet in 25 µL nuclease-free water. Incubate on a rotator mixer for 10 minutes at RT.
- Pellet beads on magnet until the eluate is clear and colourless.
- Remove and retain 20 µL of eluate into a clean nuclease-free 1.5 mL tube.

Step 6
ONT Library preparation

- Use the purified double-stranded cDNA for ONT library preparation, using the following kit:
- Ligation sequencing kit (SQK-LSK109)
- Direct-cDNA Sequencing kit (SQK-DCS109), starting from the “End Prep Step”.

Appendix 1
smRNA column purification

Zymo column purification for smRNA (RNA Clean & Concentrator™-5)

Perform all steps at room temperature and centrifugation at 10,000-16,000 x g for 30 seconds, unless otherwise specified.

1. Prepare adjusted RNA Binding Buffer (as needed). Mix an equal volume of buffer and ethanol (95-100%). Example: Mix 50 µl buffer and 50 µl ethanol.
2. Add 2 volumes of the adjusted buffer to the sample and mix. Example: Mix 100 µl adjusted buffer and 50 µl sample.
3. Transfer the mixture to the Zymo-Spin™ Column and centrifuge. Save the flow-through!
4. Small RNAs (17-200 nt) are in the flow-through.
    - Add 1 volume ethanol and mix. Example: Add 150 µl ethanol to 150 µl sample.
    - Transfer the mixture to a new column and centrifuge. Discard the flow-through.
5. Add 400 µl RNA Prep Buffer to the column and centrifuge. Discard the flow-through.
6. Add 700 µl RNA Wash Buffer to the column and centrifuge. Discard the flow-through.
7. Add 400 µl RNA Wash Buffer to the column and centrifuge for 1 minute ensure complete removal of the wash buffer. Carefully, transfer the column into a RNase-free tube (not provided).
8. Add up to 12 µl DNase/RNase-Free Water directly to the column matrix and centrifuge

Appendix 2
Sequences

Sequences

Control RNA: 5' OH-CUGAGAAAGUAGAGCAAGAAGAAAUAGAGC-3'Phosp
Linker R: 5'- ACACAUCGUAUGCGCUGCUAGUAG -3'
P1: 5'- CTACTAGCAGCGCATACGATG -3'
P2: 5'- CACATCGTATGCGCTGCTAGT -3'

Notes:

Contacts

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