

CircAID-p-seq for Oxford Nanopore Technologies

Product	Catalog no	Rxns
CircAID-p-seq for phospho-RNA-seq	#CA001	7

Shipping: Dry ice

Storage Conditions: store components as indicated on data sheet

Shelf Life: 12 months

Description: CircAID-p-seq kit (**C**ircular **A**mplification and **I**Dentification of short RNA **s**equences bearing a 3 **P**hosphate) 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



Reagents provided

Product (label)	Cap Color	Cat. no.	Store condition	Quantity
CircAID-p-seq kit		#CA001	according to manual	1kit - 7 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
MnCl ₂	Blue	#CA001-6	-20°C	50 µL
GTP 1 mM	Blue	#CA001-7	-20°C	50 µL
Linker R™(R) 10 µM	Blue	#CA001-8	-80°C	20 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
Nuclease (N)	yellow	#CA001-12	-20°C	7 µL
Buffer N (BN)	yellow	#CA001-13	-20°C	20 µL
P1 oligo (P1)	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)	Clear	#CA001-22	-20°C	25 µL
Control (CTRL)	Clear	#CA001-23	-80°C	20 µL

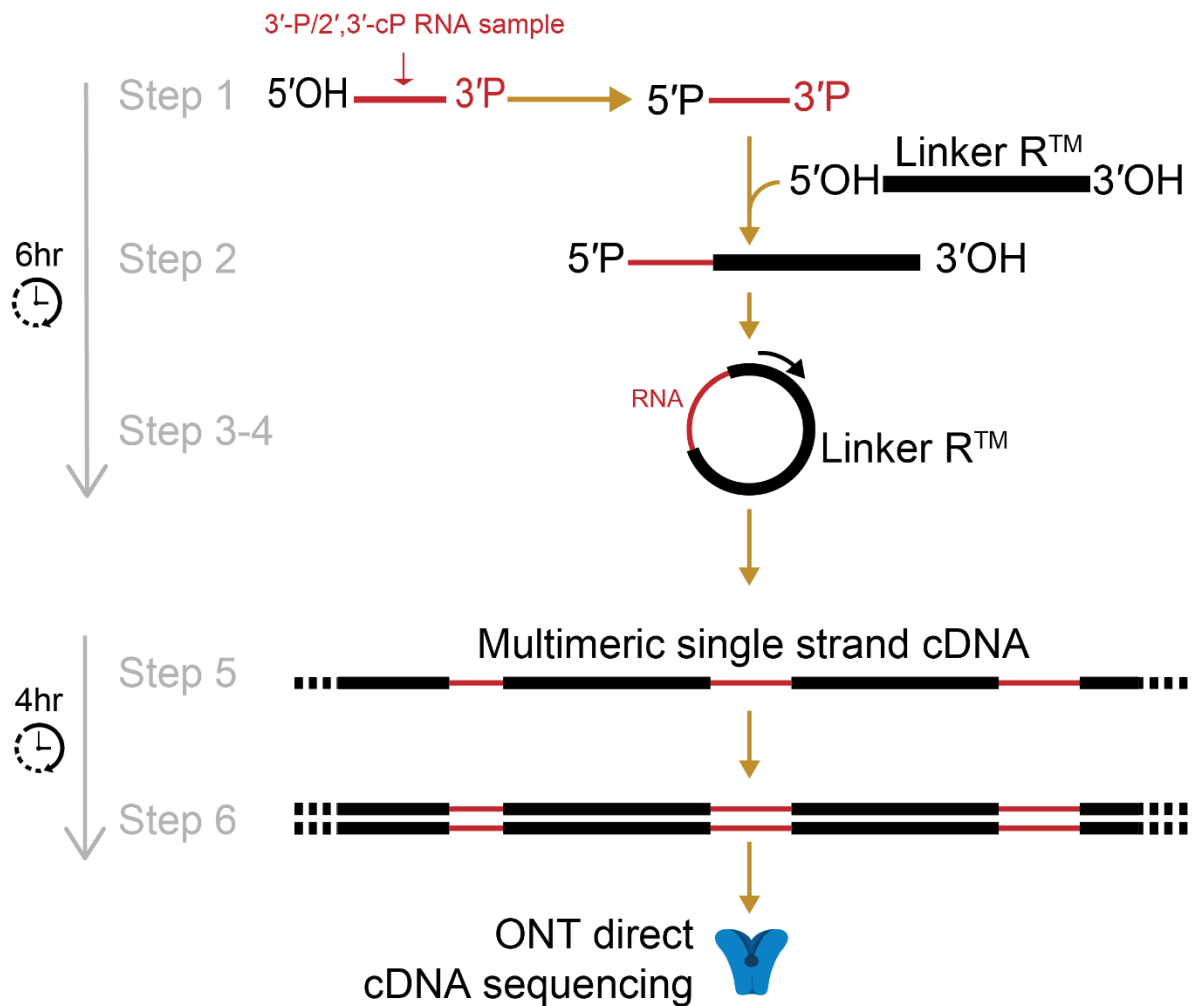
Shelf life: 12 months from the delivery date

Reagents and equipment to be supplied by user:

- 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
- Direct-cDNA Sequencing kit (SQK-DCS109) from Oxford Nanopore Technologies

Work always in an RNase-free environment!

Workflow



Sample Recommendations

Input RNA amount: the optimum RNA input amount is 30 pmol, but it is possible to scale down until 1 pmol of starting RNA material.

Input RNA quality: high RNA purity and integrity is recommended in order to ensure optimal downstream results

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 μ M, see sequence at the end of the protocol). For library preparation of the positive control, use 3 μ l of the RNA fragment. At the end of the library preparation add 1 μ L 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.

- **Step1:** 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
H ₂ O	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.

- **Step2:** Mix the following reagents in a 0.2 mL nuclease-free PCR tube:

RNA (from step 1)	6 µL
Buffer A	1 µL
GTP 1mM	1 µL
MnCl ₂	1 µL
Enzyme Mix A	1 µL
Linker R™ 10 µM	1 µL

- **Note:** for an input RNA amount between 30 and 10 pmol it is recommended to use 10 pmol of Linker R (1 µL). For lower RNA amount (down to 1 pmol) it is recommended to scale down the amount of Linker R (by diluting it in nuclease-free water), to a 1:1 ratio with the RNA sample (i.e. perform the ligation with 3 pmol of RNA and 3 pmol of Linker R).
- 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 3-4 Circularization

- **Step3:** 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.
- **Step 4:** at the end of the incubation, add the following reagents to the reaction mix:
 - 2.5 µL Buffer N
 - 1 µL Nuclease
 - H₂O up to 25 µL

- Incubate for 1 h at 37 °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 5

Reverse Transcription

- **Step 5:** For the generation of multimeric single-stranded cDNA, combine the following reagents:

Circular RNA (from step 4)	10 μL
dNTPs 10 mM	1 μ L
P1	2.5 μ L
H ₂ O	Up to 13 μ L

- 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	2 μ L
Enhanced Buffer	2 μ L
RT enzyme	1 μ L

- Incubate 4 h at 42 °C, then add 2.2 μ L of HI and heat the mix for 20 min at 70 °C.
- Transfer the reaction to a new 1.5 mL tube.
- Add 156 μ L nuclease-free water, 20 μ L sodium acetate (3M), 300 μ L isopropanol and 2 μ L 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 μ L of nuclease-free water.

- **Step 6:** Set up the following PCR reaction in a 0.2 mL nuclease-free PCR tube:

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

Cycling conditions:

Step	Temperature	Time
Initial denaturation	94°C	3 min
1 Cycle	94°C	30 secs
	54°C	30secs
	68°C	1 min
Hold	4°C	

- Purify the reaction by adding 45 μ L of resuspended Agencourt AMPure XP beads and mix by flicking the tube.
- Incubate on a rotator mixer for 5 minutes at RT.
- Prepare 500 μ L of fresh 70% ethanol in nuclease-free water.

Step 6

Second strand synthesis

Step 7
ONT Library preparation

- 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 25 µl of eluate into a clean nuclease-free 1.5 ml tube.

- **Step 7:** use the purified double-stranded cDNA for ONT library preparation, following the protocol Direct-cDNA Sequencing kit (SQK-DCS109), starting from the “**End Prep Step**”.

Appendix 1. Sequences

Control RNA: 5' OH-ATTCTTGTTGAATTAGATGGCGATGTTAAT 3'Phosp

Linker R: 5'- TCTCCTTTAATAATCACCAACCAT-3'

P1: 5'-GTTGGTGATTATTAAGGAG-3'

P2: 5'-TCTCCTTTAATAATCACCAACCAT-3'