CircAID-p-seq for Oxford Nanopore Technologies

Product	Catalog no	Rxns
CircAID-p-seq	#CA001	6

Shipping: Dry ice and 4°C

Storage Conditions: store components as indicated on data sheet

Shelf Life: 5 months

Description: CircAID-p-seq kit (**Circ**ular **A**mplification and **ID**entification of short RNA **seq**uences 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

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The Ribosome Company

Reagents provided

Poduct (label)	Cap Color	Cat. no.	Store condition	Quantity
CircAID-p-seq kit		**#CA001**	**according to manual**	**1kit - 6 rxns**
Buffer PK (BPK)	Red	**#CA001-1**	-20°C	40 µL
PK enzyme (PK)	Red	**#CA001-2**	-20°C	7 µL
ATP 10 mM	Red	**#CA001-3**	-20°C	45 µL
Buffer A (BA)	Blue	**#CA001-4**	-20°C	21 µL
Enzyme Mix A (mix A)	Blue	**#CA001-5**	-20°C	21 µL
MnCl2	Blue	**#CA001-6**	-20°C	21 µL
GTP 1 mM	Blue	**#CA001-7**	-20°C	21 µL
Linker R ™(R)	Blue	**#CA001-8**	**-80°C**	10.5 µL
Buffer B (BB)	yellow	**#CA001-9**	-20°C	14 µL
Enzyme Mix B (mix B)	yellow	**#CA001-10**	-20°C	7 µL
PEG 8000	yellow	**#CA001-11**	-20°C	56 µL
Nuclease (N)	yellow	**#CA001-12**	-20°C	14 µL
Buffer N (BN)	yellow	**#CA001-13**	-20°C	18 µL
P1 oligo (P1) 20 µM	Green	**#CA001-14**	-20°C	19 µL
Buffer RT (BRT)	Green	**#CA001-15**	-20°C	14 µL
RT enzyme (RT)	Green	**#CA001-16**	-20°C	7 µL
dNTPs 10 mM	Green	**#CA001-17**	-20°C	7 µL
HI solution (HI)	Green	**#CA001-18**	-20°C	16 µL
Enhanced Buffer (EnB)	Green	**#CA001-19**	**RT**	50 µL
Buffer Taq (BT)	Clear	**#CA001-20**	-20°C	3.5 µL
Taq	Clear	**#CA001-21**	-20°C	70 µL
P2 oligo (P2) 20 µM	Clear	**#CA001-22**	-20°C	18 µL

Shelf life: *5 months from the delivery date*

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kit code number.....

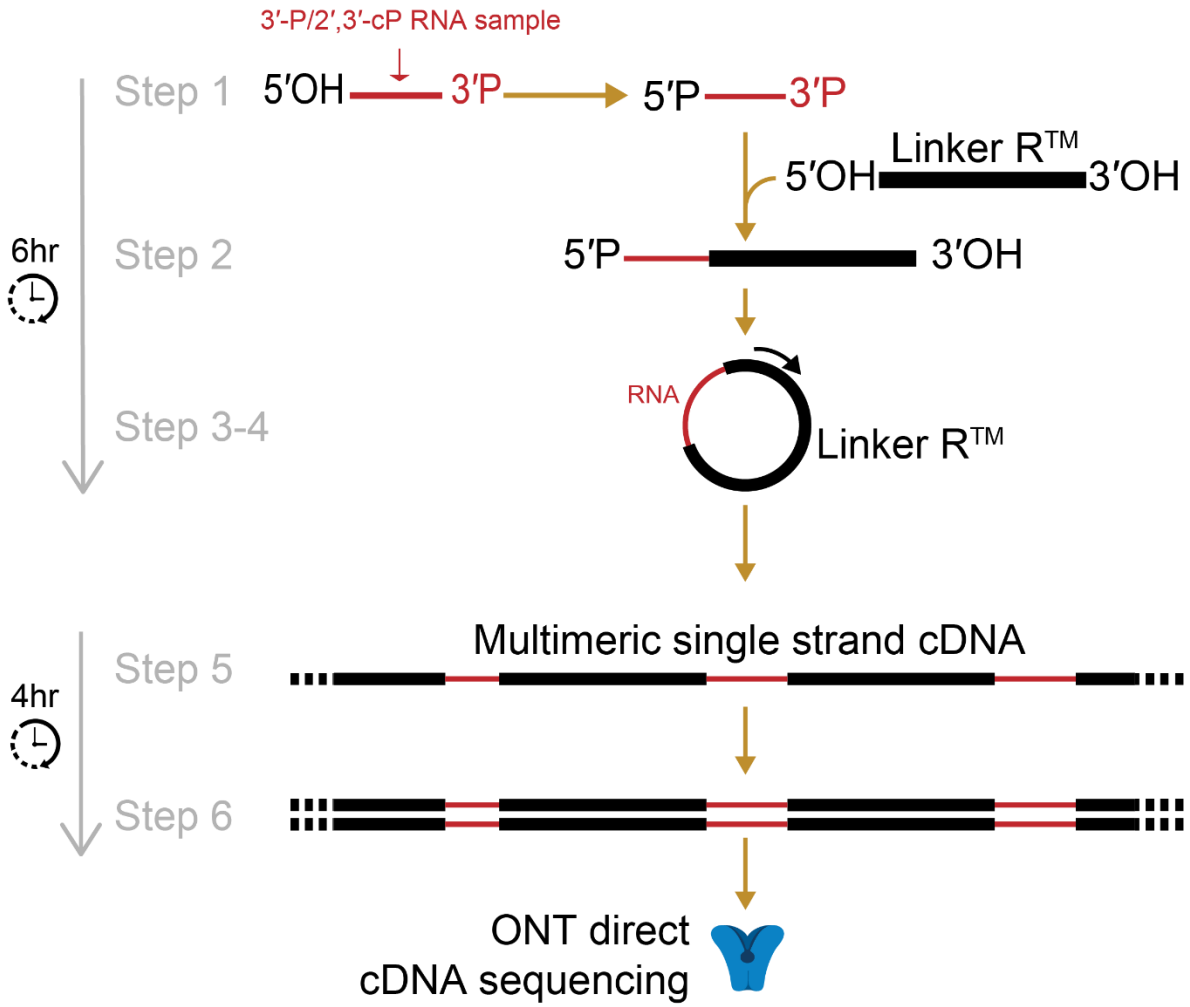
Number of samples (N).....

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
- Qubit™ RNA HS Assay Kit (Thermo Fisher catalog. no. Q32852)
- Direct-cDNA Sequencing kit (SQK-DCS109)

Work always in an RNase-free environment!

Workflow



Sample Recommendations

Input RNA amount: it is recommended to start the protocol with at least 30 pmol of RNA

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

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	30-60 pmol
H_2O	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.

Step 2

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

- **Step2:** Mix the following reagents in a 0.2 mL nuclease-free PCR tube, scaling reagent volumes according to RNA input amounts as reported below:

RNA (from step 1)	**30 pmol**	**45 pmol**	**60 pmol**
Buffer A	1 µL	1.5 µL	2 µL
GTP 1mM	1 µL	1.5 µL	2 µL
$MnCl_2$	1 µL	1.5 µL	2 µL
Enzyme Mix A	1 µL	1.5 µL	2 µL
Linker R™	0.5 µL	1 µL	1.5 µL
H_2O	Up to 10 µL	Up to 15 µL	Up to 20 µL

- Incubate the reaction for 2h 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 8 µL of nuclease free water.

Step 3-4

Circularization

- **Step3**: Prepare 1 mM ATP by diluting the 10 mM stock in nuclease-free water. Assemble the following reaction in a 0.2 mL nuclease-free PCR tube:

Buffer A	2 µL
ATP 1 mM	1 µL
PEG8000	8 µL
Enzyme Mix B	1 µL
RNA (from step 2)	8 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
  - 2 µL Nuclease
  - H_2O up to 25 µL
- Incubate for 1 h at 37 °C in a thermal cycler.

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

- **Recommended:** measure sample concentration using Qubit fluorimeter (Qubit™ RNA HS Assay Kit). Ideal input amounts for the following steps range between 10-100 ng.
- **Step 5:** For the generation of multimeric single-stranded cDNA, combine the following reagents:

dNTPs 10 mM	1 µL
Circular RNA (from step 4)	10-100 ng
P1	2.5 µL
H_2O	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	2 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 6
Second strand synthesis

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

Buffer T	5 µL
dNTPs10mM	1 µL
P2	2.5 µL
cDNA (from step 5)	20 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
	51°C	30secs
	68°C	1 min
Hold	4°C	

- Purify the reaction by adding 45µL 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 25 µl of eluate into a clean nuclease-free 1.5 ml tube.

Step 7
ONT Library preparation

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