IMMAGINA
• BioTECHNOLOGY •
The Ribosome Company

# CircAID-p-seq for Oxford Nanopore Technologies

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

Shipping: Dry ice

Storage Conditions: store components as indicated on data sheet

Shelf Life: 12 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).

## 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 | 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 |
| **MnCl2** | 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 | 6.5 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*

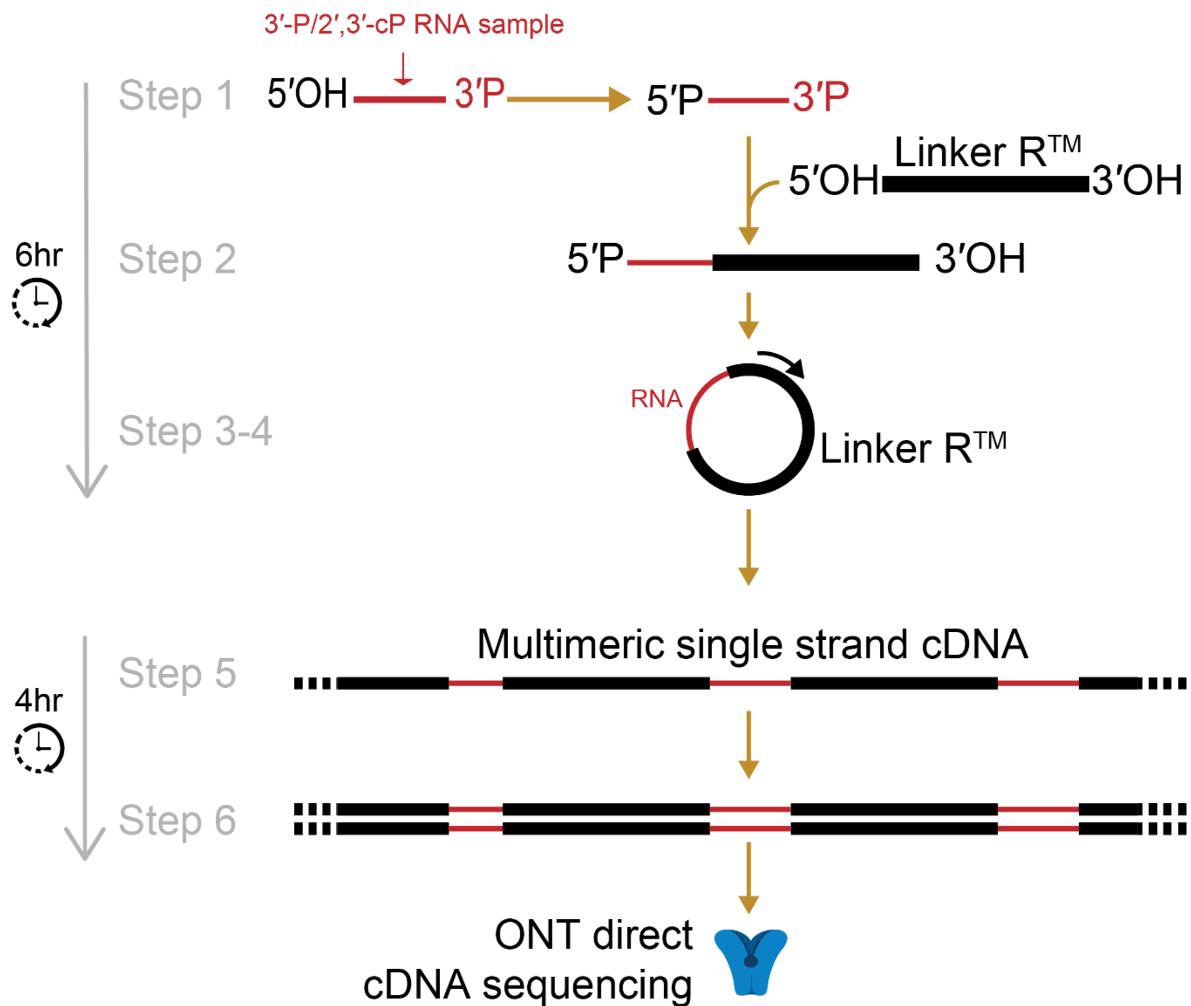
IMMAGINA®
• BioTECHNOLOGY •
The Ribosome Company

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 µM, see sequence at the end of the protocol). For library preparation of the positive control, use 3 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.***

- **<u>Step1:</u>** 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 |
| $H_2O$ | 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, <u>following the protocol for small RNAs</u> and performing the final elution in a volume of 6 µL of nuclease-free water.

Step 2

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

- **<u>Step2:</u>** 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, <u>following the protocol for small RNAs</u> and performing the final elution in a volume of 8 µL of nuclease free water.

Step 3-4

Circularization

- **<u>Step3</u>**: 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.
- **<u>Step 4:</u>** 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.

Add nuclease free water up to 50 µL final volume, then purify the reaction through RNA Clean & Concentrator™-5 kit, <u>following the protocol for small RNAs</u> and performing the final elution in a volume of 10 µL of nuclease free water. **<u>OPTIONAL STOPPING POINT</u>** (*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 µL |
| 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 | 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 6**

**Second strand synthesis**

- **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 | 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 | 30secs |
| | 68°C | 1 min |
| **Hold** | 4°C | |

- Purify the reaction by adding 40µL 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 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 the "**End Prep Step**".

**Appendix 1. Sequences**

**Control RNA**: 5' OH-CUGAGAAAGUAGAGCAAGAAGAAAUAGAGC-3'Phosp

**Linker R**: 5'- ACACAUCGUAUGCGCUGCUAGUAG -3'

**P1**: 5'- CTACTAGCAGCGCATACGATG -3'

**P2**: 5'- CACATCGTATGCGCTGCTAGT -3'