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# Arraystar R-loop CUT&Tag Library Prep Kit

Cat#: AS-TN-005

## Instruction Manual version 1.0

**For research use only. Not for diagnostic procedures.**

### Background

R-loops are special RNA:DNA:DNA three-stranded structures that widely exist in the genome. R-loops participate in many biological processes and play important roles in regulating genome stability. Traditional R-loop detection by DRIP-seq is based on immunoprecipitation of ultrasound or enzyme fragmented genomic DNA containing R-loops. Greatly improved upon traditional methods, Arraystar R-loop CUT&Tag Library Prep Kit uses pre-assembled transposomes containing hyperactive Tn5 transposase fusion with protein-A and transposons to target S9.6 antibody bound R-loops and tagment the R-loop sites (CUT&Tag, Cleavage Under Targets and Tagmentation)(Fig.1). This powerful method vastly simplifies the R-loop sequencing library prep, allowing low sample inputs, easy lab procedures, fast and better results.

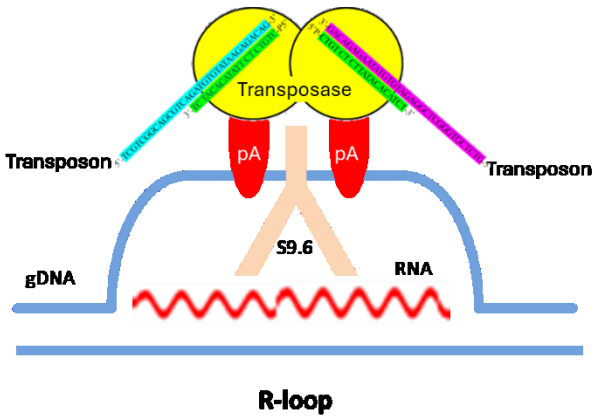


**Fig.1**. Arraystar R-loop CUT&Tag Library Prep Kit uses transposase/protein A fusion to target and tagment R-loops via S9.6 antibody.

### Components

| Component | Volume | Storage | Bag |
|---|---|---|---|
| Wash Buffer | 1.5 mL | 4°C | Bag 1 |
| Antibody Buffer | 1.5 mL | | |
| Dig-150 Buffer (10x) | 2 mL | | |
| Dig-300 Buffer (10x) | 1.5 mL | | |
| Tagmentation Buffer | 20 uL | | |
| Stop Buffer | 80 uL | | |
| Neutralize Buffer | 20 uL | | |
| 60 mM Spermidine | 300 uL | | |
| 1% NP40 | 400 uL | | |
| DNA Beads | 2 mL | | |
| Antibody 1 | 14 uL | -80°C | Bag 2 |
| Assembled pA-Tn5 Transposome | 25 uL | -20°C | |
| Antibody 2 | 14 uL | | |
| P5/P7 PCR Primers (20 uM) | 6 x 12 uL | | |
| PCR Mix (2x) | 280 uL | | |
| 1% Digitonin | 1.8 mL | | |

**Note:** *Store Antibody 1 separately at -80°C upon receipt. Avoid freeze-thaw cycles for 1% Digitonin.*

### Additional Materials

- Nuclease-free dd$H_2O$
- 80% ethanol
- BSA dry powder
- Vortexer
- Magnetic plate rack
- Centrifuge
- Orbital shaker
- Water bath or incubator
- Thermal cycler
- Analytical balance

## Important Notes

• This kit is suitable for cultured cells or cells isolated from tissue samples. The recommended cell viability is > 85%.

• To avoid cell loss during wash processes, leave 3 ~ 5 uL liquid when removing the supernatant. A Horizontal centrifuge is easier for cell collection.

## R-loop CUT&Tag Assay Protocol

The entire protocol takes 3 days to complete.

### DAY 1

Prepare fresh buffers according to the tables below. Keep the buffers on ice.

| 10% BSA | |
|---|---|
| **Component** | **Amount per sample** |
| BSA dry power | 50 mg |
| Nuclease-free ddH2O | 500 uL |

**Note**: *Store the freshly prepared BSA at 4°C. It will be used for buffer prep on Day 1 and Day 2.*

| Complete Wash Buffer | |
|---|---|
| **Component** | **Amount per sample** |
| Wash Buffer | 100 uL |
| 60 mM Spermidine | 0.8 uL |

| Complete Antibody Buffer | |
|---|---|
| **Component** | **Amount per sample** |
| Antibody Buffer | 83 uL |
| 1% Digitonin | 5 uL |
| 1 % NP40 | 1 uL |
| 60 mM Spermidine | 0.8 uL |
| 10% BSA | 10 uL |

### 1. Incubate Antibody 1

1.1. Count cells and check the cell viability (> 85%) by Trypan Blue staining. Aliquot 200,000 cells to a 1.5 mL tube per sample.

1.2. Centrifuge the cells at 600 x *g* at 4°C for 3 minutes. Remove and discard the supernatant.

1.3. Resuspend the cells in 100 uL Complete Wash Buffer. Centrifuge the cells at 600 x *g* at 4°C for 3 minutes. Remove and discard the supernatant.

1.4. Resuspend cells with 99 uL of Complete Antibody Buffer and 1 uL of Antibody 1. Incubate the tube on an orbital shaker at 20 RPM overnight at 4°C.

### DAY 2

Prepare fresh buffers according to the tables below. Keep the buffers on ice.

| Complete Dig-150 Buffer | |
|---|---|
| **Component** | **Amount per sample** |
| Dig-150 Buffer (10x) | 150 uL |
| 1% Digitonin | 75 uL |
| 60 mM Spermidine | 12 uL |
| 10% BSA (from Day 1) | 150 uL |
| 1 % NP40 | 15 uL |
| Nuclease-free dd$H_2O$ | 1098 uL |

| Complete Dig-300 Buffer | |
|---|---|
| **Component** | **Amount per sample** |
| Dig-300 Buffer (10x) | 110 uL |
| 1% Digitonin | 55 uL |
| 60 mM Spermidine | 9.2 uL |
| 1 % NP40 | 11 uL |
| 10% BSA (from Day 1) | 110 uL |
| Nuclease-free dd$H_2O$ | 805 uL |

### 2. Incubate Antibody 2

2.1. Centrifuge the cells at 600 x *g* for 5 minutes at 4°C. Remove and discard the supernatant. Avoid disturbing the cell pellet.

2.2. Resuspend the cells with 200 uL Complete Dig-150 Buffer by gentle pipetting. Incubate for 10 minutes at room temperature, gently mixing the cells once with a pipette at the 5-minute mark.

2.3. Repeat wash steps 2.1 and 2.2 twice (three washes in total).

2.4. Resuspend the cells with 99 uL Complete Dig-150 Buffer and 1 uL Antibody 2 by gentle pipetting. Transfer to a new 1.5 mL tube. Incubate the tube on an orbital shaker at 20 RPM for 1 hour at room temperature.

### 3. Incubate pA-Tn5 transposome

3.1. Repeat wash steps 2.1 - 2.2 three times in total.

3.2. Resuspend the cells with 98 uL Complete Dig-300 Buffer and 2 uL Assembled pA-Tn5 Transposome by gentle pipetting. Transfer to a new 1.5 mL tube. Incubate the tube on an orbital shaker at speed of 20 RPM for 1 hour at room

temperature.

### 4. Tagmentation of target DNA

4.1. Centrifuge cells at 600 x *g* for 5 minutes at 4°C. Remove and discard the supernatant. Avoid disturbing the cell pellet.

4.2. Resuspend the cells with 300 uL Complete Dig-300 Buffer by gentle pipetting. Incubate for 10 minutes at room temperature, gently mixing the cells once with a pipette at the 5-minute mark.

4.3. Repeat wash steps 4.1 - 4.2 twice (three washes in total).

4.4. Resuspend the cells with 49.5 uL Complete Dig-300 Buffer and 0.5 uL Tagmentation Buffer by gentle pipetting. Transfer to a new 1.5 mL tube. Incubate at 37°C for 1 hour, with gentle mixing of the cells once using a pipette at the 30-minute time point.

### 5. Release of DNA

5.1. Mix Stop Buffer just before use. Add 6 uL Stop Buffer and 1 uL of Neutralize Buffer by gentle pipetting. Incubate at 55°C for 1 hour, gently mixing the cells with a pipette at the 20- and 40-minute time points.

5.2. Add 110 uL DNA Beads and mix with a vortexer. Incubate at room temperature for 10 minutes.

5.3. Place the tube on a magnetic rack for 4 minutes to separate the beads. Remove and discard the supernatant.

5.4. Keep the tube on the magnetic rack, add 350 µL of 80% ethanol without disturbing the beads. After 1 minute, remove and discard the supernatant. Repeat this step once for a total of two washes.

5.5. Air dry at 37°C for 3 min to remove the residual ethanol completely. Resuspend the beads with 20 uL Nuclease-free dd$H_2O$. Transfer the beads to a new PCR tube.

### 6. Library amplification

6.1. Add 1 uL P5 PCR Primer (20 uM), 1 uL P7 PCR Primer (20 uM), and 22 uL of PCR Mix (2x) to the PCR tube by gentle pipetting.

6.2. PCR Amplification using the following PCR program:

| Cycle number | Temperature | Time |
|---|---|---|
| 1X (initial heating) | 72°C | 5 minutes |
| 1X (Denaturation) | 98°C | 30 seconds |
| 15 ~ 17X | 98°C | 10 seconds |
| | 60°C | 20 seconds |
| | 72°C | 30 seconds |
| 1X | 72°C | 5 minutes |
| 1X | 4°C | Hold |

### DAY 3

### 7. Enrichment of library fragments

7.1. Resuspend and transfer the DNA beads to a new 1.5 mL tube. Add an extra 27 uL DNA Beads to the PCR product. Mix thoroughly and incubate at room temperature for 10 minutes.

7.2. Place the sample on a magnetic rack for 4 minutes to separate the beads. Transfer the supernatant to a new 1.5 mL tube. Add 17 uL DNA Beads to the supernatant. Mix and incubate at room temperature for 10 minutes.

7.3. Place the tube on a magnetic rack for 4 minutes to separate the beads. Remove and discard the supernatant.

7.4. Keep the tube on the magnetic rack, add 350 µL of 80% ethanol without disturbing the beads. After 1 minute, remove and discard the supernatant. Repeat this step once for a total of two washes.

7.5. Allow the DNA beads to air dry completely. Add 10-20 uL Nuclease-free dd$H_2O$ to resuspend the beads and elute the DNA.

7.6. Use the magnetic rack to separate the beads. Transfer the supernatant (library) into a fresh tube.

### 8. Library quantification

8.1. Take 1 uL library for DNA concentration measurement with a Qubit™ Fluorometer.

- If the total DNA amount is > 20 ng, the library is ready for sequencing.
- If the total DNA amount is < 20 ng, PCR amplify by 2-4 more cycles in step 6.

## Kit performance

200,000 HEK293T cells were used to prepare R-loop CUT&Tag sequencing library using this kit according to the kit protocol. The R-loop profiling results were obtained as below (Fig.2, 3).

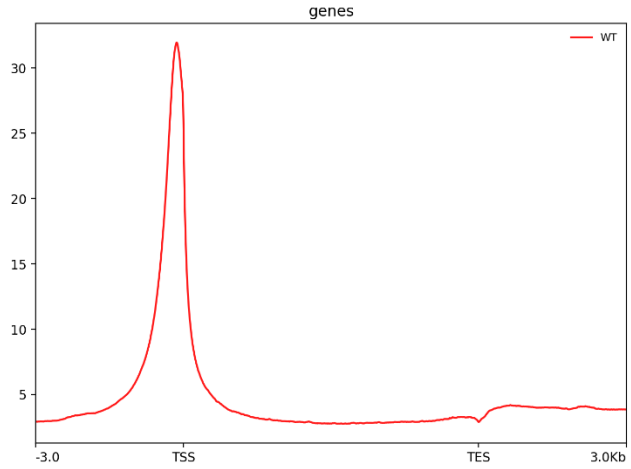


**Fig.2**. *The distribution of R-loop peaks in the genome, showing characteristic enrichment at TSS.*

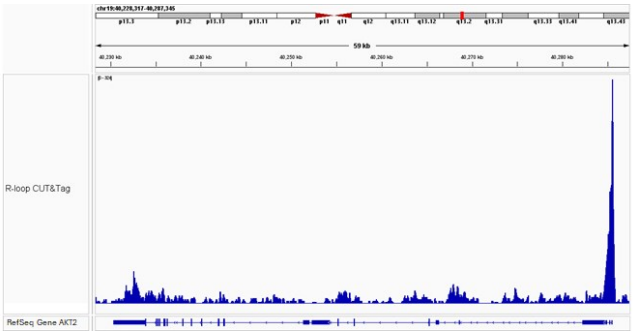


**Fig.3**. *The IGV visualization of R-loop CUT&Tag tracks on the AKT2 gene.*

## Troubleshooting

| Problems | Possible reasons | Solutions |
|---|---|---|
| Low signal-to-background ratios in low-depth sequencing | Often caused by unhealthy cells or otherwise non-ideal input material. | 1. Pre-treat cells with DNase or using flow cytometry to sort viable cells.<br>2. Sequence more deeply. |
| Low library amplification efficiency | Residual ethanol may inhibit the enzyme activity in step 5.3. | 1. Increase the PCR cycle number.<br>2. Ensure ethanol is completely evaporated before DNA elution. |

## Appendixes

### 1. P5 and P7 PCR primer sequences (5'→3')

| | |
|---|---|
| P5, Index 1 | AATGATACGGCGACCACCGAGATCTACACTATCCTCTTCGTCGGCAGCGTC |
| P5, Index 2 | AATGATACGGCGACCACCGAGATCTACACGTAAGGAGTCGTCGGCAGCGTC |
| P5, Index 3 | AATGATACGGCGACCACCGAGATCTACACACTGCATATCGTCGGCAGCGTC |
| P7, Index 1 | CAAGCAGAAGACGGCATACGAGATCTAGTACGGTCTCGTGGGCTCGG |
| P7, Index 2 | CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTCTCGTGGGCTCGG |
| P7, Index 3 | CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTCTCGTGGGCTCGG |

**Note**: *The sample multiplexing indexes are yellow highlighted and marked in green and red. A P5 PCR primer can be paired with a P7 PCR primer in any index combination for sample multiplexing in sequencer.*

### 2. Library fragment schematic sequence:

5'-
AATGATACGGCGACCACCGAGATCTACACIIIIIIIITCGTCGGCAGCGTC
AGATGTGTATAAGAGACAG-NNNNNN-
CTGTCTCTTATACACATCTCCGAGCCCACGAGACIIIIIIIIATCTCGTATG
CCGTCTTCTGCTTG-3'

IIIIIIII: P5, Index, 8 bases
IIIIIIII: P7, Index, 8 bases
NNNNNN: Target DNA sequence