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Arraystar R-loop CUT&Tag Library Prep Kit

Cat#: AS-TN-006

Instruction Manual version 1.2

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.

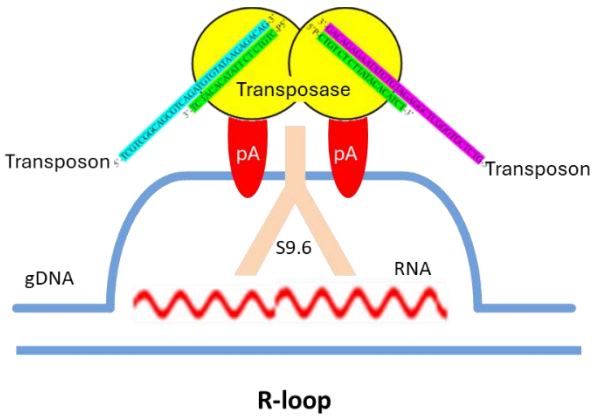

Fig.1. Arraystar R-loop CUT&Tag Library Prep Kit uses transposase/protein A fusion to target and tagment R-loops via S9.6 antibody.

Components

Component	Volume	Storage	
Wash Buffer	1.5 mL	4°C	Box 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	Box 2
Assembled pA-Tn5 Transposome	25 uL	-20°C	
Antibody 2	14 uL		
P5/P7 PCR Primers (20 uM)	7 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 ddH_2O
- 80% ethanol
- BSA dry power
- Vortexer
- Magnetic plate rack
- Centrifuge
- Orbital shaker
- Water bath or incubator
- Thermal cycler
- Analytical balance

Important Notes

• The protocol is lengthy and involves many reagents. We strongly recommend first-time users to read the manual fully before performing the experiment. You may consider a practice run before doing a large scale experiment.

• All reagents must spin down the liquid to the bottom of the tube before opening. Mix gently by finger flicking the tube or pipetting the liquid slowly up and down. For Stop Buffer, it is necessary to dissolve the precipitate back into solution completely and re-mixed. Spin down the reagents again to return the liquid to the tube bottom before use.

• All the reagents have been supplied with amounts more than the stated in "Components" Table. In an unlikely event a reagent is short of the specified amount, please notify us immediately.

• This kit is suitable for cell 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.

• For all steps involving cell resuspension, gently pipette up and down to mix the cells slowly, avoid vigorous agitation or vortex.

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 ddH_2O	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.
Note: It's necessary to gently mix the Antibody 1 with a pipette before use.

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 ddH_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 ddH_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.

Note: *Gently mix the cells once with a pipette at 5 minute time point.*

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.

Note: It's necessary to gently mix the Assembled pA-Tn5 Transposome with a pipette before use.

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.

Note: *gently mix the cells once with a pipette at 5 minute.*

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.

Note: Gently mix the cells once with a pipette at 30-minute time point.

5. Release of DNA

5.1. Add 6 uL Stop Buffer and 1 uL of Neutralize Buffer by gentle pipetting. Incubate at 55°C for 1 hour.

Note: Mix Stop Buffer just before using. Gently mix the cells by vortexing at 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. Wash the beads twice with 350 uL 80% ethanol while keeping the tube on the magnetic rack without disturbing the beads. Air dry at 37°C for 3 min to remove the residual ethanol completely. Resuspend the beads with 20 uL Nuclease-free ddH_2O. Transfer the beads to a new PCR tube.

Note: The complete removal of residual ethanol prior to PCR is crucial, as even trace amounts can inhibit the polymerase reaction.

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. Wash the beads twice with 200 uL 80% ethanol while

keeping the tube on the magnetic rack without disturbing the beads.

7.4. Allow the DNA beads to air dry completely. Add 10-20 uL Nuclease-free ddH_2O to resuspend the beads and elute the DNA.

7.5. 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).

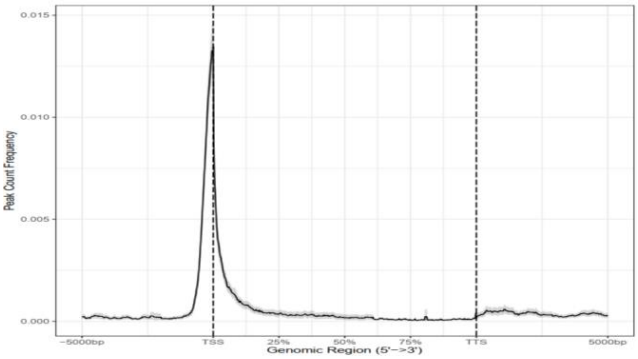

Fig.2. *The distribution of R-loop peaks in the genome, showing characteristic enrichment at TSS.*

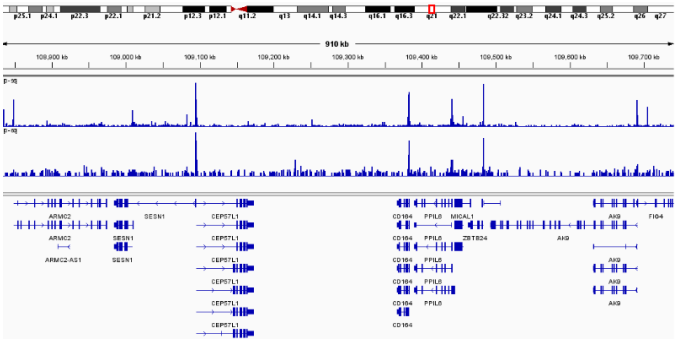

Fig.3. *The IGV view of H3K27ac CUT&Tag (upper) and R-loop CUT&Tag (lower) tracks.*

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. 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. 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
P5, Index 4	AATGATACGGCGACCACCGAGATCTACACAAGGAGTATCGTCGGCAGCGTC
P7, Index 1	CAAGCAGAAGACGGCATACGAGATCTAGTACGGTCTCGTGGGCTCGG
P7, Index 2	CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTCTCGTGGGCTCGG
P7, Index 3	CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTCT
	CGTGGGCTCGG

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'-
AATGATACGGCGACCACCGAGATCTACACIIIIIIIITCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-NNNNNN-
CTGTCTCTTATACACATCTCCGAGCCCACGAGACIIIIIIIIATCTCGTATGCCGTCTTCTGCTTG-3'

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