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Enabling Epigenetics Research

CUT&Tag-IT™ R-loop Assay Kit

Catalog No. 53167

(Version A3)

Revision	Date	Description of Change
A2	January, 2024	Tagmentation Buffer storage temp changed from -20°C to 4°C or -20°C
A3	February, 2024	Updated table in **Index Primers and Sample Sheet Information** on page 10

Contents

Overview

CUT&Tag (Cleavage Under Targets & Tagmentation) is a genome-wide chromatin profiling technique that utilizes an antibody-based enzyme tethering strategy to target specific histone modifications or proteins to reveal chromatin-binding information that is specific to those sites or proteins of interest. Following antibody binding, NGS libraries are prepared by tagmentation using the protein A-Tn5 (pA-Tn5) transposome enzyme that has been pre-loaded with sequencing adapters.

This CUT&Tag-IT™ R-loop Assay Kit utilizes a CUT&Tag-based approach to profile R-loops genome-wide with a specifically optimized protocol for cell samples and our DNA-RNA hybrid (Clone S9.6) antibody.

R-loops are three-stranded nucleic acid structures that are formed when a single-stranded DNA molecule hybridizes with its complementary RNA molecule, leaving the non-template DNA strand unpaired. These structures form normally during transcription initiation, IgG class switching, and mitochondrial DNA replication. They also appear normally at telomeres[1]. Abnormal R-Loop formation is associated with DNA damage, replication fork stalling, impaired DNA repair, and genomic instability. Cancer cells with aberrantly activated transcriptional programs can have transcription-replication conflicts, enhancing the likelihood of abnormal R-loop formation.

CUT&Tag-IT R-loop Assay Kit Advantages:

- Low background and high signal-to-noise ratio compared with other chromatin profiling techniques
- Faster and more efficient than RNA immunoprecipitation (RIP) and chromatin immunoprecipitation (ChIP)
- Compatible with 200,000 to 500,000 fresh or cryopreserved cells

This CUT&Tag-based approach to studying R-loops provides a powerful and highly sensitive new tool for investigating the formation, regulation, and function of R-loops in various biological contexts, ultimately advancing our understanding of this important molecular process.

Product	Format	Catalog No.
CUT&Tag-IT™ R-loop Assay Kit	16 rxns	53167

Kit Components and Storage

Please store each component at the temperature indicated in the table below.

This kit ships in two boxes so that components that can not freeze, such as SPRI Beads, do not freeze in shipment, and the components that must stay frozen, such as the enzymes, stay frozen. Be sure to store items at their correct storage temperature as indicated in this table below.

All components are guaranteed for 6 months after receipt when stored properly.

Reagents	Quantity	Storage
5% Digitonin	600 µL	-20°C
R-loop RNase A	45 µL	-20°C
Protease Inhibitor Cocktail	1 x 500 µL 2 x 100 µL	-20°C
CUT&Tag-IT™ Assembled pA-Tn5 Transposomes	16 µL	-20°C
10 µg/µL Proteinase K	80 µL	-20°C
DNA-RNA Hybrid mAb (Clone S9.6), 1 mg/mL	50 µL	-20°C
Rabbit Anti-Mouse Antibody	16 µL	-20°C
Tagmentation Buffer	1.5 mL	4°C or -20°C
Q5 Polymerase Master Mix	425 µL	-20°C
i7 Indexed Primer 1	10 µL	-20°C
i7 Indexed Primer 2	10 µL	-20°C
i7 Indexed Primer 3	10 µL	-20°C
i7 Indexed Primer 4	10 µL	-20°C
i5 Indexed Primer 1	10 µL	-20°C
i5 Indexed Primer 2	10 µL	-20°C
i5 Indexed Primer 3	10 µL	-20°C
i5 Indexed Primer 4	10 µL	-20°C
1X Binding Buffer	5.5 mL	4°C
Dig-Wash Buffer	57 mL	4°C
Concanavalin A Beads	320 µL	4°C
SPRI Beads	1 mL	4°C
Dig-300 Buffer	55 mL	4°C

Reagents	Quantity	Storage
Antibody Buffer	800 µL	4°C
1% IGEPAL	220 µL	RT
0.5 M EDTA	250 µL	RT
10% SDS	450 µL	RT
DNA Purification Wash Buffer*	10 mL	RT
DNA Purification Elution Buffer	5 mL	RT
DNA Purification Binding Buffer**	6 mL	RT
3 M Sodium Acetate	500 µL	RT
DNA Purification Columns SF	16	RT

*DNA Purification Wash Buffer must be reconstituted to a final concentration of 80% ethanol prior to use. Add 40 mL of 100% ethanol to the DNA Purification Wash Buffer prior to use.

**DNA Purification Binding Buffer must be reconstituted to a final concentration of 60% isopropyl alcohol prior to use. Add 9 mL of 100% isopropanol to the DNA Purification Binding Buffer bottle prior to use.

Additional Materials Required

- Ethanol, 80%
- Ethanol, 100%
- Isopropanol, 100%
- 1X PBS
- Microcentrifuge
- Vortexer
- Magnetic bar or magnetic plate rack for 0.2 mL tubes
- 0.2 mL PCR tubes
- 1.5 or 2 ml microcentrifuge tubes
- Wet ice and ice bucket
- Nutator or gentle orbital shaker, not an end-over-end rotator
- Nutator or gentle orbital shaker that can be placed at 4°C, not an end-over-end rotator
- 37°C water bath or incubator
- Thermal cycler
- Illumina® Sequencing System

Day 1 Binding Cells to Concanavalin A Beads (approximately 2 hours)

Prepare Buffers

Prepare Complete Antibody Buffer. Protease Inhibitor Cocktail must be added fresh.

Complete Antibody Buffer			
Ingredient	**1 Reaction**	**8 Reactions**	**16 Reactions**
Antibody Buffer	50 µL	400 µL	800 µL
Protease Inhibitor Cocktail	0.5 µL	4 µL	8 µL
5% Digitonin	0.5 µL	4 µL	8 µL
1% IGEPAL	0.5 µL	4 µL	8 µL

Prepare Cells (10 minutes)

Note: When preparing beads, cells or nuclei, the buffers are kept on ice but the samples are handled on the bench at room temperature for convenience.

1. Resuspend the fresh or frozen cell pellet in 1X PBS for cell counting. Aliquot 200,000 to 500,000 cells per CUT&Tag-IT R-loop Assay Kit reaction. Cryopreserved cells can be thawed on the bench or at 37°C water bath or heat block.
2. Prepare Dig-Wash Buffer with Protease Inhibitor Cocktail. 2.1 mL of Dig-Wash Buffer plus 21 µL Protease Inhibitor Cocktail are needed per sample.
3. Centrifuge cells for 3 minutes at 600 x g at 4°C and remove the supernatant.
4. Resuspend cells in 1 mL Dig-Wash (with Protease Inhibitor Cocktail).
5. Centrifuge cells at 600 x g for 3 minutes at room temperature and remove the supernatant.
6. Repeat steps 4-5 for a total of two washes.
7. Resuspend cells with 100 µL of Dig-Wash Buffer (with Protease Inhibitor Cocktail) per each 200,000 to 500,000 cell sample.

Prepare Concanavalin A Beads (20 - 30 minutes)

8. Resuspend the Concanavalin A Beads by vortexing. Aliquot 20 µL per sample into 0.2 mL PCR strip tubes. Resuspend beads as needed during aliquoting.
9. Add 100 µL 1X Binding Buffer per sample.
10. Capture the Concanavalin A Beads on a magnetic plate, remove the supernatant, and resuspend the beads with 100 µL 1X Binding Buffer (mix by pipette).

11. Quickly spin the tubes down, capture the beads on a magnet, and remove the supernatant.
12. Add 10 µL of 1X Binding Buffer to the beads.
13. Add the washed cells from step 7 to the Concanavalin A Beads, resuspend, and incubate using a Nutator or orbital rotator at 20-30 RPM for 10 minutes at room temperature.

DNA-RNA Hybrid (Clone S9.6) Antibody Binding (overnight)

14. Remove tubes from Nutator or orbital rotator and quickly centrifuge. Place the tubes on a magnetic stand or bar to collect beads, and discard the supernatant.
15. Resuspend the beads/cells using 50 µL of ice-cold Complete Antibody Buffer by gentle vortexing. Place tubes with samples on ice.
16. Add 5 µL molecular grade DNase/RNase-free water to samples, or 5 µL of RNase A to negative control sample, and incubate at room temperature for 30 minutes on a Nutator or orbital rotator at 20-30 RPM.
17. Add 2 µL of DNA-RNA Hybrid antibody to each sample with gentle pipetting.
18. Place samples on Nutator or orbital shaker overnight at 4°C at speed of 20-30 RPM. Ensure caps are slightly elevated to avoid beads getting stuck in the lid. Liquid should remain in the bottom and on the side of the tubes while rocking.

Day 2 (approximately 6 hours)

Bind Secondary Antibody

First, Prepare Buffers

Complete Dig-Wash Buffer			
Ingredient	**1 Reaction**	**8 Reactions**	**16 Reactions**
Dig-Wash Buffer	1.3 mL	10.4 mL	20.8 mL
Protease Inhibitor Cocktail	13 µL	104 µL	208 µL
5% Digitonin	13 µL	104 µL	208 µL
1% IGEPAL	13 µL	104 µL	208 µL

Complete Dig-300 Buffer			
Ingredient	**1 Reaction**	**8 Reactions**	**16 Reactions**
Dig-300 Buffer	0.7 mL	5.6 mL	11.2 mL
Protease Inhibitor Cocktail	7 µL	56 µL	112 µL
5% Digitonin	1.4 µL	11.2 µL	22.4 µL

Complete Tagmentation Buffer			
Ingredient	**1 Reaction**	**8 Reactions**	**16 Reactions**
Tagmentation Buffer	100 µL	800 µL	1.6 mL
Protease Inhibitor Cocktail	1 µL	8 µL	16 µL
5% Digitonin	0.2 µL	1.6 µL	3.2 µL

Note: Protease Inhibitor Cocktail must be added fresh.

20. Remove tubes from Nutator and give them a quick spin to collect samples in bottom of tubes. Then place tubes on magnetic stand or bar to pellet beads, and remove and discard the supernatant.

21. Wash the beads by adding 200 µL Complete Dig-Wash Buffer, resuspending the beads, placing the tubes back on the magnetic stand or bar, and removing and discarding the supernatant.

22. Repeat step 21 two times for a total of 3 washes.

23. Dilute the Rabbit Anti-Mouse Antibody 1:100 in Complete Dig-Wash Buffer and add 100 µL per sample. Pipette gently to resuspend pellets.

24. Place the tubes on a Nutator at room temperature for 1 hour.

25. Gently pipette samples to resuspend pellets, and place back on Nutator at room temperature for an additional hour.
26. Remove tubes from Nutator and give the tubes a quick spin.
27. Place the tubes on a magnetic stand to pellet beads, and remove and discard the supernatant.
28. Add 200 µL of Complete Dig-Wash Buffer. Gently pipette to resuspend the beads.
29. Repeat steps 27 - 28 twice for a total of 3 washes, removing and discarding the supernatant after each wash.

Bind CUT&Tag-IT Assembled pA-Tn5 Transposomes (1 hour)

30. Mix CUT&Tag-IT Assembled pA-Tn5 Transposomes in Complete Dig-300 Buffer to a final concentration of 1:100 for 100 µL per sample.
31. After a quick spin, place tubes on a magnetic stand to pellet beads, and remove and discard the supernatant.
32. Add 100 µL of the CUT&Tag-IT Assembled pA-Tn5 Transposomes in Complete Dig-300 Buffer and gently pipette to resuspend the beads.
33. Place the tubes on a Nutator at 20-30 RPM at room temperature for an hour.
34. Remove tubes and quickly spin.
35. Place tubes on magnet or magnetic rack, and remove and discard supernatant.
36. Add 200 µL Complete Dig-300 Buffer to each sample and gently pipette to resuspend the beads.
37. Repeat steps 35 - 36 for a total of 3 washes.

Tagmentation (1 hour)

38. After a quick spin, place the tubes on a magnetic stand to pellet beads, and remove and discard the supernatant.
39. Add 40 µL of Complete Tagmentation Buffer. Gently pipette to resuspend the beads.
40. Incubate at 37°C for 1 hour in a water bath or incubator.

DNA Extraction (1 hour)

41. To stop tagmentation and solubilize DNA fragments, add to each sample:
    2.3 µL of 0.5 M EDTA
    2.8 µL 10% SDS
    0.5 µL 10µg/µL Proteinase K
42. Mix by vortexing at full speed for approximately 2 seconds and quick spin.

Note: It is typical for the beads to form a large clump during this incubation.

43. Incubate the reaction for 1 hour at 55°C in thermal cycler with heated lid set to 65°C.

44. Add 100 µL of water to each sample and mix with pipettor until bead/cell clump is thoroughly homogenized. Set pipettor to 100 µL to avoid pulling up air. Avoid making bubbles as the SDS can cause the sample to become foamy. The samples will be gooey, we suggest using a single channel pipettor and homogenizing one sample at a time.

45. After a quick spin, place the tubes on a magnet tray to clear and transfer supernatant to new microcentrifuge tubes.

Note: Some of the beads will pull off with the DNA, this will not affect the DNA purification.

46. Add 750 µl of DNA Purification Binding Buffer (with 60% isopropanol) to each sample and mix by pipetting up and down. If the color indicator turns violet or orange add 8 µL of 3 M Sodium Acetate.

47. For each sample, place a DNA purification column into a collection tube.

48. The following steps are performed at room temperature. Transfer each sample to its corresponding column, close the cap, and centrifuge at 17,000 x g (approximately 14,000 rpm) for 1 minute.

49. Discard the flow-through and return the collection tube to the column.

Note: 100% Ethanol must be added to the DNA Purification Wash Buffer before the first use, to a final concentration of 80% (40 mL of 100% Ethanol to a new bottle).

50. Add 200 µL of DNA Purification Wash Buffer to the column and cap the column. Centrifuge at 17,000 x g for 1 minute.

51. Discard the flow-through and repeat step 50 for a total of two washes.

52. Discard the flow-through and return the collection tube to the column. Centrifuge the empty tube at 17,000 x g for 2 minutes to remove any remaining DNA purification Wash Buffer.

53. Transfer each column to a new microcentrifuge tube and add 22 µL of DNA Purification Elution Buffer to the center of the column matrix, cap the column and incubate at room temperature for 1 minute.

54. Centrifuge at 17,000 x g for 1 min. Discard the column. DNA purification is complete. The purified DNA can be stored at -20°C or you may proceed directly with the PCR amplification.

PCR Amplification (1 hour)

55. Set up the PCR reactions by adding the components in the order shown below, If libraries are to be multiplexed for sequencing, ensure that a unique i5 - i7 index combination is used for each sample.

Reagent	Volume
Tagmented DNA Sample	20 µL
i5 Indexed Primer	2.5 µL
i7 Indexed Primer	2.5 µL
Q5 Polymerase Master Mix	25 µL
Total Volume	**50 µL**

56. Perform PCR using the following program on a thermal cycler (with a heated lid to 105°C):

72°C for 5 minutes
98°C for 30 seconds

12 cycles of:
98°C for 10 seconds
63°C for 20 seconds

72°C for 1 minute
Hold at 10°C

57. Perform a double-sided SPRI bead clean-up with 25 µL SPRI Bead Solution (0.5X sample volume), pipette to mix and let stand 5 minutes.

58. Place on magnetic stand and move supernatant to new strip tube, and discard the beads.

59. To the supernatant, add 35 µL SPRI Bead Solution (0.7X sample volume, bringing total to 1.2X ratio). Let stand 5 minutes.

60. Place on magnetic stand and remove and discard supernatant. Wash beads twice with 200 µL 80% Ethanol.

61. Allow beads to dry briefly, remove from magnetic stand when the beads turn from shiny to matte in appearance, add 20 µL DNA Purification Elution Buffer, pipette to mix, and incubate for 1 minute at room temperature.

62. Place tubes on magnetic stand to pellet beads. Collect supernatant and transfer to a new tube. Libraries are now ready for quantification and sequencing.

Index Primers and Sample Sheet Information

Index 1 (i7) Primers

CAAGCAGAAGACGGCATACGAGAT[i7]GTCTCGTGGGCTCGG

Index 2 (i5) Primers

AATGATACGGCGACCACCGAGATCTACAC[i5]TCGTCGGCAGCGTC

i7 Index	i7 Sequence	Sample Sheet
N701	TCGCCTTA	TAAGGCGA
N702	CTAGTACG	CGTACTAG
N703	TTCTGCCT	AGGCAGAA
N704	GCTCAGGA	TCCTGAGC

i5 Index	i5 Sequence	Sample Sheet (NovaSeq v1.0 Reagent Kits, MiSeq, HiSeq 2000/2500)
N501	TAGATCGC	TAGATCGC
N502	CTCTCTAT	CTCTCTAT
N503	TATCCTCT	TATCCTCT
N504	AGAGTAGA	AGAGTAGA

i5 Index	i5 Sequence	Sample Sheet (NovaSeq v1.5 Reagent Kits, iSeq, MiniSeq, NextSeq, HiSeq 3000/4000)
N501	TAGATCGC	GCGATCTA
N502	CTCTCTAT	ATAGAGAG
N503	TATCCTCT	AGAGGATA
N504	AGAGTAGA	TCTACTCT

Sequence for Read 1 and Read 2 adapter trimming: CTGTCTCTTATACACATCT.

References

1. Lin R., *et al.* (2021) *Nucleic Acids Research* Vol. 50, D303-D315
2. Wang *et al.* (2021) *Sci. Adv.,* 7:eabe3516

Troubleshooting Guide

Problem/Question	Recommendation
No visible nuclei pellet	The pellet may not be visible, proceed with protocol.
	Be sure to position tubes with caps oriented in centrifuge so that you know where the pellet should be in the tube, and proceed carefully with next steps.
What is the recommended sequencing depth?	25 million reads.
High background	Be sure to start with viable cells.
	Check that cells/nuclei are intact by an automated cell counting method such as the Countess II or by Trypan Blue staining and a hemocytometer.
Will the RNase A negative control produce a library?	Yes. The RNase A treated sample will produce a library. Rnase A will significantly deplete library concentration.
Should I use an IgG control?	No. We found IgG controls to be highly variable and inconsistent in tagmentation-based assays. We recommend a no-DNA-RNA Hybrid-mAb (Clone S9.6)antibody sample as a control.
Why does this kit use RNase A and not RNase H like some publications?	We observed RNase H treatment did not completely abolish CUT&Tag signals, and the extended overnight incubation of RNase H digestion added more time to the protocol without a noticeable benefit.[2]
What percentage of mitochondrial DNA reads are observed?	We observe a range of 36% to 67% of reads mapping to mitochondrial reads. This will vary by cell type and cell treatment.

Technical Services

If you need assistance at any time, please call or send an e-mail to Active Motif Technical Service at one of the locations listed below.

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