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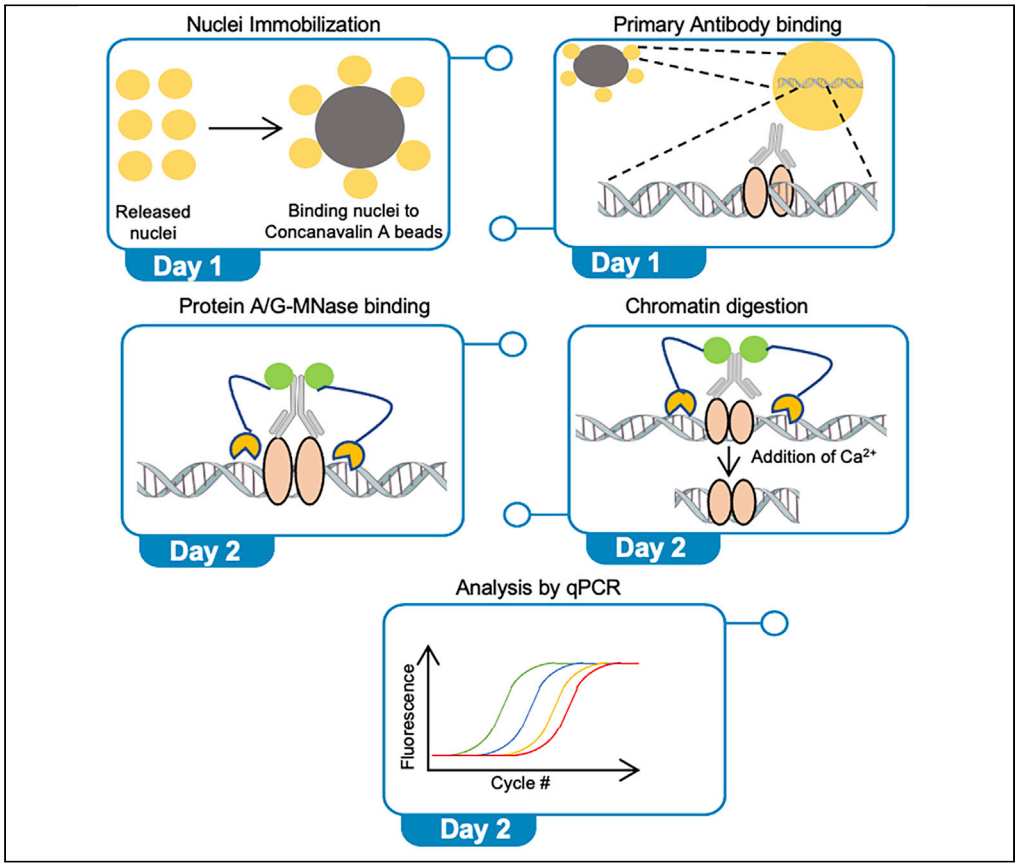
A modified CUT&RUN-seq technique for qPCR analysis of chromatin-protein interactions



Chromatin Immunoprecipitation coupled with quantitative PCR (ChIP-qPCR) even with optimization may give low signal-to-background ratio and spatial resolution. Here, we adapted Cleavage Under Targets and Release Using Nuclease (CUT&RUN) (originally developed by the Henikoff group) to develop CUT&RUN-qPCR. By studying the recruitment of selected proteins (but amenable to other proteins), we find that CUT&RUN-qPCR is more sensitive and gives better spatial resolution than ChIP-qPCR.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

CUT&RUN-seq for classical qPCR analysis of chromatin recruitment of target proteins

Greater sensitivity than ChIP-seq at a site-specific replication fork barrier

Greater spatial resolution than ChIP-seq at a site-specific replication fork barrier

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A modified CUT&RUN-seq technique for qPCR analysis of chromatin-protein interactions

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SUMMARY

Chromatin immunoprecipitation coupled with quantitative PCR (ChIP-qPCR) even with optimization may give low signal-to-background ratio and spatial resolution. Here, we adapted Cleavage Under Targets and Release Using Nuclease (CUT&RUN) (originally developed by the Henikoff group) to develop CUT&RUN-qPCR. By studying the recruitment of selected proteins (but amenable to other proteins), we find that CUT&RUN-qPCR is more sensitive and gives better spatial resolution than ChIP-qPCR.

For complete details on the use and execution of this protocol, please refer to Skene et al. (2018) and Skene and Henikoff (2017).

BEFORE YOU BEGIN

Design and check the quality of primers

Timing: 3 h

1. Design the CUT&RUN-qPCR primers targeting the loci of interest with an annealing temperature around 60°C and an amplicon length of 80–140 bp.
2. Design PCR primer pairs for negative control genomic locus- a locus that is remote from the targeted genomic region or known not to be bound by the protein of interest. We use sequences in the β-actin gene—a locus that is remote from the targeted genomic region. Use the $2^{-\Delta\Delta\ CT}$ method to analyze the Cut&RUN-qPCRdata.
3. To test the specificity of primers, amplify targeted DNA sequence and analyze dissociation curve.

Dissociation curves are carried out at the end of the PCR experiment. Use the instrument software to generate a dissociation curve.

4. A single peak in the dissociation curve indicates a single melting event, and therefore homogeneity of the PCR products and specificity of primers whereas the presence of multiple peaks indicates contamination or non-specific amplification of the input material (Ririe et al., 1997; Zornhagen et al., 2015).

Prepare mouse embryonic stem (mES) cells

Timing: 30 min

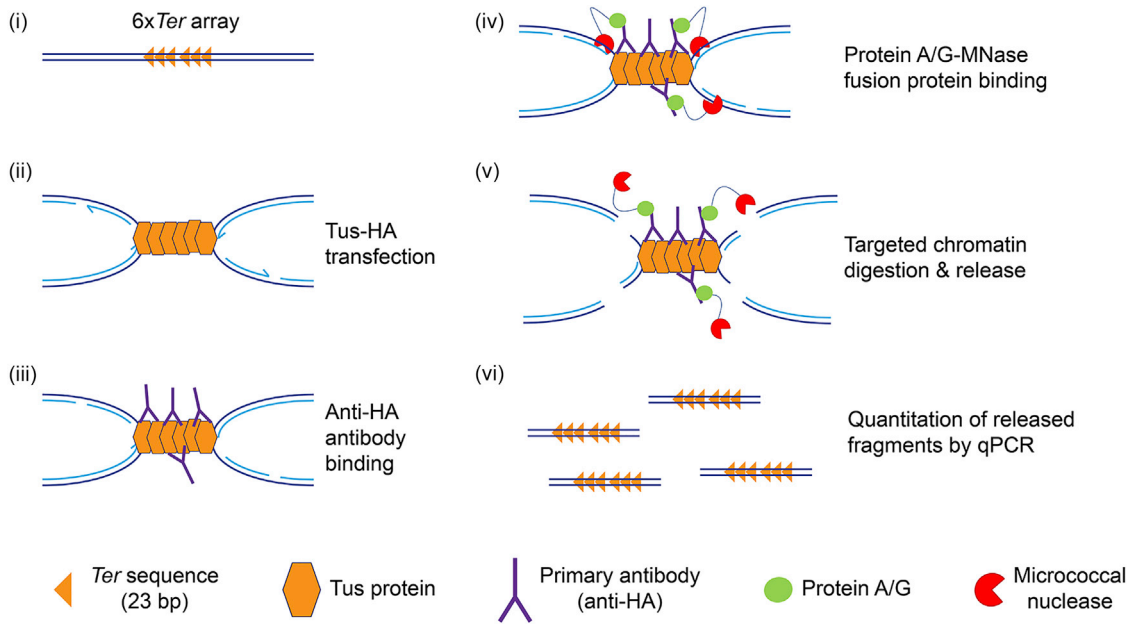

Figure 1. Schematic overview of CUT&RUN-qPCR

(i) We use a Tus/*Ter* reporter system containing 6×*Ter* sequence and targeted it as a single copy to the *Rosa26* locus of mouse chromosome 6 in mES cells. (ii) Transient expression of Tus-HA protein leads to its binding with the 6×*Ter* sequence. (iii) We use Anti-HA antibody as a primary antibody that binds the Tus protein, followed by proteins A/G fused with micrococcal nuclease (iv), to bind the primary antibody. (v) Chromatin is cleaved on either side of the 6×*Ter* sequence and released into solution. (vi) Eluted DNA is quantified and amplified by qPCR using appropriate controls.

5. Culture mES cells under humidified conditions at 37°C 6% CO2 in DMEM supplemented with 15% fetal bovine Serum (FBS), recombinant LIF, 1% penicillin, and streptomycin antibiotics and additional factors as described in the table.

   Note: In this protocol to study the recruitment of protein in response to site-specific stalled fork and site-specific DNA double-strand break (DSB), we used a reporter system. This system uses *Escherichia coli* Tus/*Ter* replication fork barrier (RFB) (Berghuis et al., 2015; Elshenawy et al., 2015) and also contains the I-SceI rare-cutting homing endonuclease cut site that generates site-specific double-strand break and targeted as a single copy to the *Rosa26* locus of mouse chromosome 6 in mES cells (Panday et al., 2021; Willis et al., 2017, 2018). Therefore, this reporter is an ideal system to parallelly study and compare the site-specific recruitment of proteins by ChIP-qPCR and Cut&RUN-qPCR in response to stalled replication fork and DSB (Figure 1)

6. Thaw mES cells into a single well of a six-well plate pre-coated with mouse embryonic fibroblast (MEF) "feeders" cells that have been mitotically inactivated by lethal irradiation. MEFs provide both matrix support for mES cell attachment and secretion of factors that enhance cell survival and pluripotency.
7. Test mES cells regularly for mycoplasma infection by Myco-Alert assay (Panday et al., 2021; Willis et al., 2017, 2018). To perform the Myco-Alert assay, we use MycoAlert Mycoplasma Detection Kit:
   a. Remove and save 500 µL media in a 1.7 mL Eppendorf tube.
   b. Freeze sample(s) until needed.
   c. Spin samples down: 120 × *g* 5 min @RT.
   d. Aliquot 25 µL sample into black flat bottom 96 well suitable for plate reader.
   e. Add 25 µL Reagent buffer, mix by pipetting 5×.
   f. Incubate samples 5 min @RT. Activate plate reader for 5 min warmup.
   g. Read samples by a luminometer.

h. Add 25 μL Substrate buffer, mix by pipetting 5×.
i. Incubate samples 10 min @RT.
j. Read samples using a luminometer.
k. Calculate ratio; Final read: initial read.
   i. Ratio < or = 0.8: sample is myco-free.
   ii. Ratio = 0.9–1.0: sample is borderline and should be retested.
   iii. Ratio is > 1.0 indicates mycoplasma infestation.

Prepare plasmid and buffers

Timing: 5–6 h

Note: We used three different expression plasmids: I-SceI plasmid that expresses rare-cutting homing endonuclease to create site-specific DSB (Puget et al., 2005); Tus plasmid that expresses Tus protein that binds the *Ter* array and stalls replication forks in a site-specific manner (Willis et al., 2014); and empty vector as a control plasmid. I-SceI and Tus protein act on the 6×*Ter*-HR reporter at the *Rosa26* locus of mouse chromosome 6 in mES cells to create a site-specific DSB and to cause bidirectional site-specific replication fork stalling, respectively. The cellular responses to these triggers include the induction of homologous recombination (HR), associated with recruitment of repair proteins (Figure 1) (Panday et al., 2021; Willis et al., 2018). We use parallel transfections of I-SceI to create a site-specific DNA double-strand break (DSB) and empty vector (EV) as a negative control.

8. Transform high-copy mammalian empty vector (EV), Tus, and I-SceI expression plasmids into competent DH5-alpha bacteria. Plate on Luria-Bertani (LB) agar plates supplemented with appropriate selection agent, and incubate overnight at 37°C.
9. Pick 1-day-old colonies directly into 300 mL LB broth containing the appropriate selection agent and incubate the cultures overnight (16–20 h) at 37°C, 200 rpm orbital shaking.
10. Prepare endotoxin-free plasmids using Qiagen Endo-free Maxiprep kits as per manufacturer's instructions.

https://www.qiagen.com/us/resources/download.aspx?id=a48e64ab-27cf-4576-bb93-98bbd0e1229e&lang=en.

11. Prepare binding buffer (store at 4°C up to six months), wash buffer (store at 4°C up to 1 week), and stop buffer (store at 4°C up to 1 week) in advance. Prepare digitonin buffer and antibody buffer freshly.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit Anti-HA tag antibody (3 μL of antibody in 400 μL of antibody buffer)	Abcam	Cat#ab9110, RRID: AB_307019
Rabbit Anti-Rad51 antibody (3 μL of antibody in 400 μL of antibody buffer)	Abcam	Cat#ab176458 RRID: AB_2665405
Bacterial and virus strains		
One Shot Stbl3 Chemically Competent *E. coli*	Thermo Fisher Scientific	Cat#C737303
Chemicals peptides, and recombinant proteins		
500 mL 0.5 M EDTA pH 8.0	Quality Biological	cat# 351-027-101
500 mL Ultra Pure water	Quality Biological	cat# 351-029-101
1 M HEPES buffer	Fisher Scientific	cat# MT25060CI
Gelatin	Sigma Aldrich	cat#G1890-500
0.1 M Sodium Pyruvate	Fisher Scientific	cat#11-360-070
Penicillin-Streptomycin (10,000 U/mL)	Fisher Scientific	cat#15-140-122

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
100× MEM Non-essential amino acids	Fisher Scientific	cat#11-140-050
L-glutamine (100×)	Fisher Scientific	cat#MT25005Cl
Fetal Bovine Serum	Atlanta Biologicals	cat#S11150
ESGRO recombinant mouse LIF Protein	Millipore	Cat#ESG1107
Trypsin-EDTA, Phenol red	Fisher Scientific	Cat#25-200-114
Spermidine trihydrochloride	Sigma-Aldrich	cat# S2501-5G
Glycogen 20 mg/mL	Thermo Scientific	cat# FERR0561
RNAse A 10 mg/mL	Thermo Scientific	cat# FEREN0531
cOmplete EDTA-free tablets	Sigma-Aldrich	cat# 04-693-132-001
Concanavalin A Magnetic Beads	Bangs Laboratories	cat# B P531
Cutana PAG-Mnase	Epicypher	cat# 15-1016
$CaCl_2$ $2H_2O$	Sigma-Aldrich	cat# C3881-500G
$MnCl_2$	Fisher Scientific	cat# M87-100
EGTA	Sigma-Aldrich	cat# E3899
KCl	Fisher Scientific	cat# P217-500
NaCl	Fisher Scientific	cat# s64010
Trypan blue solution	Thermo Fisher Scientific	cat#15250061
Lipofectamine 2000	Thermo Fisher Scientific	cat#11668019
Opti-MEM (1×)	Thermo Fisher Scientific	cat#31985070
Digitonin	Sigma-Aldrich	cat# 300410-1GM
Critical commercial assays		
PCR Purification Kit	QIAGEN	cat# 28106
Endo-free Maxiprep kit	QIAGEN	Cat#12362
2× Power SYBR Green	Applied Biosystems	Cat#4368702
MycoAlert Mycoplasma Detection Kit	Lonza	Cat#LT07-318
Experimental models: Cell lines		
Brca1 exon 11 conditional mouse ES cells	Dr. Chuxia Deng (Xu et al., 1999)	N/A
Oligonucleotides		
Primer name	Forward Primer5′-3′	Reverse Primer5′-3′
109 bp downstream	TCCGGATAGGGATAACAGGGTA	GTCGGCCATGATATAGACGTTG
128 bp upstream	GAGCGCACCATCTTCTTCA	TCCCTACGATGCCCTTCA
443 bp upstream	ACTACCTGAGCACCAGTC	GGGAGGTGTGGGAGGTT
Recombinant DNA		
Plasmid: pcDNA3b-MYC-NLS-Tus-F140A-3×HA	Panday et al. (2021)	N/A
Plasmid: pcDNA3b-MYC-NLS-Tus-F140A-3×FLAG	This study	N/A
Software and algorithms		
Prism 7.0e for Mac	GraphPad Software	https://www.graphpad.com/scientificsoftware/prism/, RRID: SCR_000306
Other		
0.5 mL Eppendorf DNA LoBind tubes	Fisher Scientific	cat# 13-698-790
1.5 mL Eppendorf DNA LoBind tubes	Fisher Scientific	cat# 13-698-791
Dynal MPC-S Magnetic Rack	Fisher Scientific,	cat# 50 114 8229
Nutator mixer	Fisher Scientific	cat# NC0597936
Smartblock (Eppendorf REF 5367000025) 24× 1.5–2.0 mL	Fisher Scientific	cat# 05-412-510
Eppendorf Thermomixer C	Fisher Scientific	cat# 05-412-503

MATERIALS AND EQUIPMENT

mES Cell Media

Reagent	Amount
DMEM, high glucose	500 mL
1 M HEPES pH7.6	10 mL
100× MEM Non-essential amino acids	5 mL

(*Continued on next page*)

Continued	
Reagent	Amount
0.1 M Sodium pyruvate (100×)	5 mL
Beta-mercaptoethanol	4 μL
rLIF 10 million units/mL	25 μL
*Fetal Bovine Serum	75 mL
*L-glutamine (100×)	5 mL
*10,000 U/mL Penicillin/ 10,000 ug/mL Streptomycin (100×)	5 mL

Note: * Add before use. Thaw serum slowly @4°C prior to aliquot for use.

PBS (1×) 1000 mL		
Reagent	Final concentration	Amount
NaCl	13.6 mM	8 g
KCl	0.26 mM	0.2 g
Na_2HPO_4	1 mM	1.44 g
KH_2PO_4	0.17 mM	0.24 g
ddH_2O	n/a	1000 mL
Total	n/a	1000 mL

Note: * Adjust to a final pH of 7.4 with HCl. Autoclave 30 min on liquid cycle. Store @RT.

Binding buffer		
Reagent	Final concentration	Amount
HEPES (1 M)	20 mM	4 mL
KCl (1 M)	20 mM	4 mL
Cacl2 (0.1 M)	1 mM	2 mL
MnCl2 (1 M)	1 mM	200 μL
ddH_2O	n/a	190 mL
Total	n/a	200.2 mL

Note: Binding buffer are filter sterilized through a 0.22 μm filter and stored at 4°C for up to six months.

Wash buffer		
Reagent	Final concentration	Amount
HEPES (1 M)	20 mM	1 mL
NaCl (5 M)	150 mM	1.5 mL
Spermidine trihydrochloride (1 M)	0.5 mM	25 μL
EDTA-free Roche protease inhibitor	n/a	1 tablet
ddH_2O	n/a	47.5 mL
Total	n/a	50 mL

Note: Wash buffers are filter sterilize through a 0.22 μm filter and stored at 4°C for up to 1 week.

5% Digitonin solution		
Reagent	Final concentration	Amount
Digitonin	5% (w/V)	50 mg
Boiled ddH_2O	n/a	1 mL

Note: To make 5% digitonin solution, weigh digitonin in the small boat using high sensitivity scale. Agitate on vortex 5–10 min to dissolve Digitonin. Make fresh to prevent any salt precipitation. DMSO can be used as an alternative to dissolve digitonin.

Caution: Digitonin is toxic and a face mask should be worn when weighing the powder.

Digitonin buffer		
Reagent	Final concentration	Amount
5% Digitonin buffer	0.05%	150 µL
Wash buffer	n/a	15 mL
Total	n/a	15.15 mL

Note: Make digitonin buffer fresh or store at 4°C for no more than 24 h.

Antibody buffer		
Reagent	Final concentration	Amount
Digitonin buffer	n/a	2 mL
EDTA (0.5 M)	2 mM	8 µL
Total	n/a	2.008 mL

Note: Antibody buffers are filter sterilized through a 0.22 µm filter. Make antibody buffer fresh immediately before use and keep chilled for use.

Stop buffer		
Reagent	Final concentration	Amount
ddH_2O	n/a	4.4 mL
NaCl (5 M)	340 mM	340 µL
EDTA (0.5 M)	20 mM	200 µL
EGTA (0.5 M)	4 mM	40 µL
RNAseA (10 mg/mL)	50 µg/mL	25 µL
Glycogen 20 mg/mL	50 µg/mL	12.5 µL

Note: Stop buffer is filter sterilized through a 0.22 µm filter. Store stop buffer at 4°C for up to 1 week.

STEP-BY-STEP METHOD DETAILS

Transfecting the cells with HA-tagged Tus expression plasmid

Timing: 2 days

This step transiently expresses Tus to generate site-specific fork stalling or I-SceI to induce a DSB at the *Rosa26*, to study the recruitment of repair proteins by Cut&RUN-qPCR.

1. Grow 4–5 million cells in a 10 cm dish format. One day prior to transfection, reseed mES cell culture to stimulate proliferation. For ideal cultures displaying 80%–90% confluency, reseed approximately 1/4 of cells. For cultures displaying 40%–60% confluency, reseed approximately 1/3 of cells.

2. On the day of transfection, harvest cells and count viable cells under the microscope using trypan blue exclusion and a hemocytometer.
3. To count the cells, dilute 10 μL of cell suspension into 90 μL trypan blue. Calculate the cell concentration by multiplying the number of cells counted by 10,000 and the dilution factor of 10 (i.e., a count of 34 indicates an original concentration of 3.4 M cells/mL). Resuspend the mES cells in mES cell media to a density of 0.8 million cells/mL.
4. Cover the wells of all the 24-well plates with 0.5 mL of PBS/gelatin (0.2% gelatin in 1× PBS) and incubate the plates for at least 5 min at room temperature to gelatinize.
5. Aspirate the PBS/gelatin and immediately plate 200 μL 0.8 million cells/mL mES cell suspension (160,000 cells) to each well.
6. Prepare plasmid mix by adding 0.5 μg plasmid in 33 μL Opti-MEM per reaction in a 5 mL polystyrene tube. Agitate each tube by gently flicking the tube to mix.
7. Prepare the Lipofectamine mix by mixing 1.2 μL of Lipofectamine 2000 to 33 μL Opti-MEM per reaction in a 5 mL polystyrene tube. Agitate each tube by gently flicking the tube to mix.
8. Incubate the plasmid and Lipofectamine mixes at room temperature for 5 min.
9. Transfer one equal volume of Lipofectamine mix to each plasmid mix to set up the lipofection reactions.
10. Agitate each 5 mL reaction tube by gentle flicking or pipetting using a p1000 pipet. Incubate the lipofection reactions at room temperature for 5–10 min.
11. Transfect target wells by transferring 70 μL of the appropriate lipofection reaction to each designated well. Flick each lipofection mix gently before addition.
12. After the addition of the lipofection reaction, gently agitate the 24-well plate being careful not to swirl the contents of the wells. Place the 24-well transfection plate in the humidified tissue culture incubator at 37°C for 6 h.
13. After 6 h, gently add 1 mL mES cell media to each transfected well using a 25 mL pipet, taking care to avoid touching any individual well's contents with the pipet tip.
14. Return the plate(s) to the tissue culture incubator for incubation overnight.

Cell harvest

Timing: 30 min

These steps involve harvesting transfected cells by detaching them using trypsin from plastic of 24-well plate and make them ready for the first step of Cut&RUN-qPCR.

15. The following day, 24 h after the initiation of transfection, remove mES cell media from each well. Add 500 μL 1×PBS to wash each well of residual media and serum and repeat the decanting process.
16. Using a 5 mL pipet, add two drops (~100 μL) 0.25% trypsin/EDTA to each well and incubate at 37°C for 3 min. Firmly tap to agitate each plate to loosen and dislodge adherent cells from the plastic and to help break up any cell clumps.
17. Using a 5 mL pipet, add two drops (~100 μL) DMEM/EDTA to each well and swirl the wells to mix. Using p1000 tips, pipet each well four-five times and harvest cells as normal and count viable cells using trypan blue exclusion and a hemocytometer.
18. Aliquot 1 million cells per condition (EV, I-SceI, and Tus transfected cells) in mES cell media in 1.7 mL Eppendorf tube.
19. Wash cells twice with sterile 1×PBS, pelleting cells in a microfuge at 600 × *g* for 3 min at room temperature for each wash step.

Note: After the second PBS wash, use a p200 pipet to remove every last drop of the PBS supernatant.

Concanavalin A beads activation

Timing: 15 min

These steps charge the concanavalin A beads and make them ready to bind to cell nuclei.

20. Gently resuspend the Concanavalin A (ConA)-coupled magnetic beads in the stock bottle by slow inversion (5–6×).
21. Calculate volume of beads needed (10 μL per sample) and transfer this volume of beads to a 2.0 mL tube containing chilled 1.5 mL Binding Buffer.

Note: It is very important to use equal amounts of beads in all samples, to avoid sample-to-sample variation in epitope binding and in the release of digested chromatin.

22. Mix tube contents by inversion and place on magnetic separator ~30 s. When liquid clarifies, remove the supernatant with p1000 pipet.
23. Repeat binding buffer wash for a total of two washes. After 2nd wash, resuspend the beads in a volume of binding buffer equal to the initial volume of bead suspension (10 μL per final sample) and place cell-bead suspension on ice.

Cell immobilization- binding cells to concanavalin A activated beads

Timing: 25 min

These steps involve the preparation of cell nuclei and their attachment to a solid support using charged concanavalin A beads.

24. Wash cells twice with 1 mL wash buffer, pelleting cells in a microfuge at 600 × *g* for 3 min at room temperature for each wash step.

Note: Keep wash buffers and steps in this section at room temperature to avoid cell stress.

25. Following second wash, add 1 mL wash Buffer and resuspend cells using p1000.

Note: Perform ConA bead activation steps and cell washing steps in parallel. Adjust the relative timing of these steps in such a way that activated the ConA beads are ready by the time the second wash with wash buffer has been completed (step 21).

26. Add to each sample 10 μL prepared ConA bead slurry. Resuspend cells thoroughly by pipetting to prevent ConA bead clumping.

⚠ CRITICAL: After step 19, the ConA beads will begin to settle. It is critical to resuspend beads before addition to each sample (See troubleshooting).

27. Place samples on Benchmark Nutator Mixer for 15 min at room temperature. Check that the contents of each tube are moving and mixing well. If needed. supplement this rocking by gently agitating samples using a p1000 pipet.

Cell permeabilization and primary antibody binding

Timing: 18 h

These steps make cells permeable to the primary antibody and overnight incubation binds the antibody to target epitopes in the immobilized nuclei.

28. Place the Eppendorf tubes on a magnet stand until the fluid is clear. Remove the liquid carefully.

Note: Use p200 pipet to remove every last drop of liquid.

29. Add 400 µL of chilled freshly prepared Antibody buffer. Resuspend ConA bead/cell conjugate from wall thoroughly by gentle mixing using a p1000 pipet.
30. Transfer entire contents to 0.5 mL low binding tube.
31. Add 3 µL of antibody to each tube. Mix using a p1000 pipet.
32. Incubate samples on the Nutator rocking platform at 4°C overnight.

Note: In this protocol, we used Anti-HA tag antibody and Anti-Rad51 antibody.

⚠ CRITICAL: While rotating samples on the Nutator overnight, beads may tend to become stuck along the wall of the Eppendorf tube or may form clumps within the cell suspension. Therefore, it is important to resuspend the cells attached to ConA beads thoroughly by pipetting before incubating samples overnight.

pA/G-MNase binding

Timing: 45 min

In these steps Micrococcal Nuclease fused to proteins A and G binds the primary antibody.

33. Wash cells twice in chilled digitonin buffer. To do this, flick each tube to consolidate contents and place samples on magnetic separator for ~30 s. Remove supernatant using a p1000 pipet.
34. Add 500 µL chilled Digitonin Buffer and mix beads thoroughly by inversion.

Note: After second wash, use a p200 pipet to remove every last µL of liquid. This step is important, since efficient MNAse activity requires efficient removal of EDTA from the buffer. A dead volume of wash buffer may allow excess EDTA to be carried over.

35. Add 350 µL of chilled digitonin buffer and mix each sample gently using a p1000 pipet.
36. Add 2.5 µL of pAG-MNase and again mix each sample gently using a p1000 pipet.
37. Place each sample on Nutator for 30 min at room temperature to allow pA/G-MNase-antibody chromatin binding.

Targeted chromatin digestion and release

Timing: 5 h

In these steps, exposure to calcium activates MNase to cleave chromatin on either side of the targeted protein and cleaved fragments are released, diffusing out of the immobilized nuclei.

38. Wash cells two times with 400 µL of chilled digitonin buffer by inverting tube 3–4 times.

Note: After second wash, use a p200 pipet to remove every last µL of liquid. This step is important to completely remove non-specific binding of pA/G-MNase.

39. Add 300 µL chilled digitonin buffer and 3 µL chilled 100 mM $CaCl_2$ to activate MNase.
40. Mix samples using a p1000 pipet as fast as possible and rock samples on a Nutator at 4°C for 4 h.

Note: Turn on thermomixer with 1.5–2 mL smart block to preheat to 37°C.

41. Transfer each sample to 1.7 mL Eppendorf tube.
42. Add 100 μL chilled stop buffer to each tube and gently mix using a p1000 pipet.
43. Place samples on thermomixer set to 500 rpm at 37°C for 10 min.
44. Centrifuge samples:16,000 × *g* at 4°C for 10 min.
45. Place the tube on a magnet stand. Once the ConA bead-cell slurry is concentrated at the magnet, transfer the supernatant to a 2 mL tube.
46. Extract the DNA from the supernatant using a Qiagen MinElute PCR purification Kit, following the manufacturer's instructions.
https://www.qiagen.com/cn/resources/download.aspx?id=e0fab087-ea52-4c16-b79f-c224bf760c39&lang=en.
47. Elute the purified DNA in 20 μL of elution buffer.

Note: Purified samples can be stored at −20°C.

Analyzing by qPCR

Timing: 3 h

In this step, qPCR amplifies the eluted DNA fragments and estimates the quantity of initial material.

48. Assess the concentration of the eluted DNA sample using a Nanodrop Spectrophotometer.

Note: The DNA concentration of eluted DNA may be less than 2 ng/uL, which is acceptable.

49. Run qPCR of all the samples in the following system with qPCR machine using 3 technical repeats per experimental repeat.

Note: The Ct values of the triplicates should show minimal variation. Apart from technical triplicates, experimental repeats should show minimal variability, if the samples have been handled appropriately. Variability among experimental repeats may be due to the various reason (see troubleshooting).

A qPCR reaction mix, to be made in triplicate

Sample	Volume
Template DNA/ eluted DNA	3 μL
SYBR Green Master Mix	12.5 μL
Primers (forward 10 μM)	0.5 μL
Primers (Reverse 10 μM)	0.5 μL
ddH20	8.5 μL

qPCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	10 min	1
Denaturation	95°C	15 s	40
Annealing and extension	60°C	1 min	
Melt Curve Stage	95°C	15 s	1
	60°C	30 s	1
	95°C	15 s	1

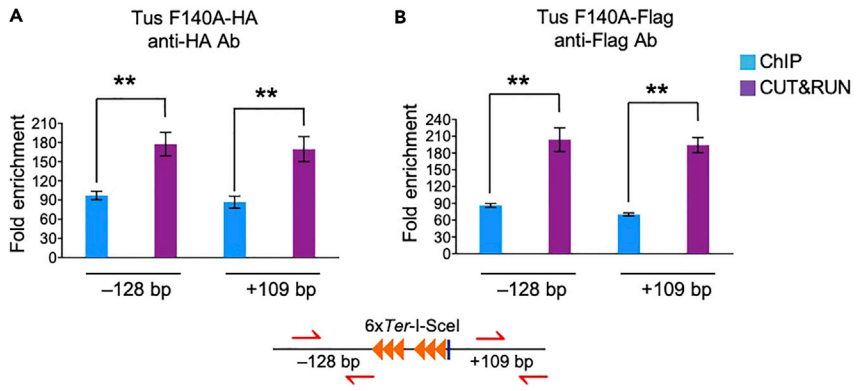

Figure 2. Comparison of Tus enrichment at 6×*Ter* array at *Rosa26* in mES cells using CUT&RUN-qPCR and ChIP-qPCR

(A and B) Data shows signal at Tus/*Ter* RFB for Tus protein C-terminally tagged with HA (panel A) or Flag (panel B). Blue bars: Tus-HA or Tus-Flag enrichment using ChIP-qPCR. Purple bars: Tus-HA or Tus-Flag enrichment using CUT&RUN-qPCR. Numbers indicate distance in base pairs from the outer qPCR primer to the nearest edge of the 6×*Ter* array. Cartoon shows primer positions as red half-arrows. Orange triangles: *Ter* sites. Blue line: I-SceI restriction site. Data in CUT&RUN and ChIP figures show means of $2^{-\Delta\Delta\ CT}$ values, normalized to EV and β-actin control locus. Data show mean ± SD. Statistical analysis by Student's two-tailed unpaired t-test (n = 3), assuming unknown variance. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; ns, not significant. Note more intense enrichment of Tus using CUT&RUN-qPCR than for ChIP-qPCR.

⚠ CRITICAL: To determine the specificity of the PCR reaction, it is important to establish the melting curve properties. It is also important to assess the homogeneity of the PCR products, including screening for the presence of primer–dimers by analyzing the primer pair sequence to avoid complementarity and hybridization between primers. The dissociation curve for a primer should produce a single sharp peak. More than one peak indicates that the PCR reaction is not specific to the target locus.

Analyzing the data

In this step, as $2^{-\Delta\Delta\ CT}$method (Schmittgen and Livak, 2008) used to analyze the CUT&RUN-qPCR data.

We use two sets of negative controls. The first is an untreated control sample of cells, and the second is a control genomic locus, distant from the *Ter* array. For the untreated control sample, we use empty vector-transfected cells in which no replication fork barrier (RFB) is active at the *Ter* array. For the control genomic locus, we use sequences in the β-actin gene—a locus that is remote from the *Ter* array at *Rosa26*—or another locus known not to be bound by the protein of interest. The equation that we use to normalize and analyze the CUT&RUN-qPCR data is the comparative C_T method (Schmittgen and Livak, 2008) (also known as $2^{-\Delta\Delta\ CT}$method).

ΔΔ CT= (C_T at *Ter* locus – C_T at β-actin locus) of sample transfected with Tus – (C_T at *Ter* locus – C_T at β-actin locus) of sample transfected with empty vector.

EXPECTED OUTCOMES

CUT&RUN-qPCR is more sensitive than ChIP-qPCR

We found that the fold enrichment of proteins of interest using CUT&RUN-qPCR is higher than that obtained using ChIP-qPCR. We tested the comparison of Tus enrichment at *Ter* array using the comparative C_T method (Schmittgen and Livak, 2008) (Figure 2). We used expression plasmids encoding C-terminal HA or FLAG epitope-tagged Tus-F140A (pcDNA3b-MYC-NLS-Tus-F140A-3×HA/3×FLAG) derived from the parental Tus expression vector (Willis et al., 2014, 2017). We transfected a mES cell line containing a 6×*Ter* array at *Rosa26* and performed ChIP-qPCR and

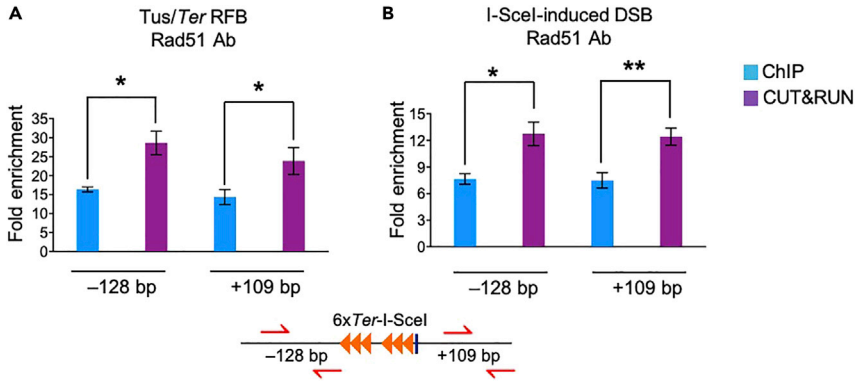

Figure 3. Comparison of Rad51 enrichment at Tus/*Ter* RFB and at I-SceI-induced DSB at *Rosa26* in mES cells, using CUT&RUN-qPCR and ChIP-qPCR

(A and B) Data shows Rad51 signals at Tus/*Ter* RFB (panel A) and I-SceI-induced DSB (panel B), both positioned at the *Rosa26* locus in mES cells.

Blue bar: Rad51 enrichment using ChIP-qPCR. Purple bar: Rad51 enrichment using CUT&RUN-qPCR. Numbers indicate distance in base pairs from the outer qPCR primer to the nearest edge of the 6×*Ter* array. Cartoon features as in Figure 1. Data in CUT&RUN and ChIP figures show means of $2^{-\Delta\Delta CT}$ values, normalized to EV and beta-actin control locus. Data show mean ± SD. Statistical analysis by Student's two-tailed unpaired t-test (n = 3), assuming unknown variance. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; ns, not significant. Note greater sensitivity of CUT&RUN-qPCR Rad51 signal.

CUT&RUN-qPCR in parallel. We found that, irrespective of the epitope tag (i.e., HA or FLAG tag) CUT&RUN-qPCR for the relevant Tus epitope tag gave a higher enrichment of *Ter*-specific signal than ChIP-qPCR performed in parallel in the same experiments (Figure 2).

We next compared the ability of ChIP-qPCR and CUT&RUN-qPCR to detect Rad51 at the Tus/*Ter* RFB or, in parallel, at a site-specific double strand break (DSB) induced at *Rosa26* by the I-SceI rare-cutting homing endonuclease. Rad51 is an early responder at sites of replication fork stalling and also accumulates at double strand breaks undergoing repair by HR. Similar to the results we obtained with the Tus signal at the 6×*Ter* array, we found that the Rad51 signals at both the Tus/*Ter* RFB and at an I-SceI-induced DSB were stronger when assayed by CUT&RUN-qPCR than when assayed by ChIP-qPCR (Figure 3). Thus, CUT&RUN-qPCR detects Rad51 at a Tus/*Ter* RFB and at a DSB with greater sensitivity than ChIP-qPCR.

Next, we assayed the ability of CUT&RUN-qPCR and ChIP-qPCR to detect protein-chromatin signals that are expected to be much less intense than those of Rad51 at an RFB or a DSB. Recruitment of the Bloom's syndrome helicase (BLM) to the Tus/*Ter* RFB is detectable by ChIP-qPCR in wild-type mES cells (Panday et al., 2021). However, in mES cells null for *Fancm*, encoding a stalled fork response DNA translocase and motor protein clones, BLM was not detectable by ChIP-qPCR (Figure 4). Despite the apparent failure of BLM recruitment to Tus/*Ter* in *Fancm*$^{-/-}$ cells, as determined by ChIP-qPCR, we were able to detect residual functions of BLM in Tus/*Ter*-induced repair in *Fancm*$^{-/-}$ cells (Panday et al., 2021). This discrepancy led us to speculate that ChIP-qPCR might lack the sensitivity to detect low levels of residual BLM at the Tus/*Ter* RFB in *Fancm*$^{-/-}$ cells. Notably, when we performed CUT&RUN-qPCR for BLM at Tus/*Ter* at the *Rosa26* locus of wild type mES cells, we were able to detect BLM signals at stronger intensity than those detected by ChIP-qPCR; in addition, CUT&RUN-qPCR revealed low levels of BLM enrichment at Tus/*Ter* in *Fancm*$^{-/-}$ mES cells (Figure 4). Taken together, these data suggest that CUT&RUN-qPCR is more sensitive than ChIP-qPCR. This work identifies CUT&RUN-qPCR as a robust tool to detect low abundance chromatin bound proteins at a defined genomic locus.

CUT&RUN-qPCR yields a spatial resolution superior to that of ChIP-qPCR

In ChIP, the average size of chromatin fragments following sonication is 400–600 bp. This fragment size effectively defines the maximum resolution of ChIP-based methods. However,

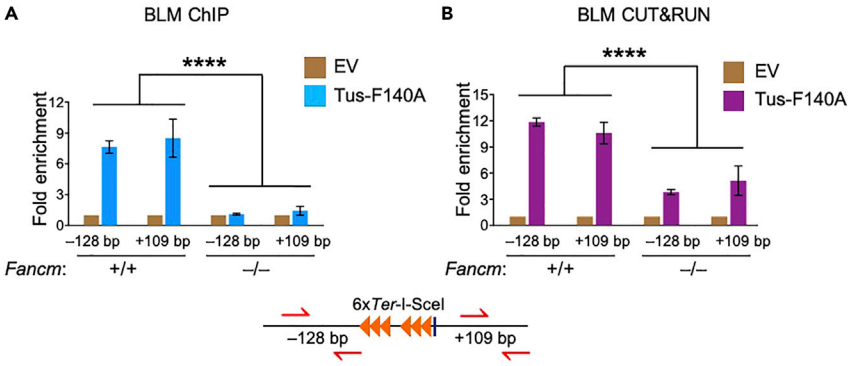

Figure 4. Comparison of BLM enrichment at Tus/*Ter* RFB at *Rosa26* in mES cells, using CUT&RUN-qPCR and ChIP-qPCR

Data shows BLM signal at Tus/*Ter* RFB positioned at the *Rosa26* locus in mES cells, in cells that are wild type (*Fancm*$^{+/+}$) or null (*Fancm*$^{-/-}$) for *Fancm*. Gold bars indicate CUT&RUN-qPCR or ChIP-qPCR signals in cells transfected with empty vector (EV), to show background signal in absence of Tus. Blue bars: BLM signal using ChIP-qPCR. Purple bars: BLM signal using CUT&RUN-qPCR. Numbers indicate distance in base pairs from the outer qPCR primer to the nearest edge of the 6×*Ter* array. Cartoon features as in Figure 1. Data in CUT&RUN and ChIP figures show means of $2^{-\Delta\Delta CT}$ values, normalized to EV and beta-actin control locus. Data show mean ± SD. Statistical analysis by Student's two-tailed unpaired t-test (n = 3), assuming unknown variance. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; ns, not significant. Note greater fold-enrichment of BLM signal in wild type cells using CUT&RUN-qPCR. Note also the ability of CUT&RUN-qPCR to detect a reduced BLM signal at Tus/*Ter* in *Fancm*$^{-/-}$ cells, whereas ChIP-qPCR reveals no BLM signal in this setting.

many chromatin-associated proteins may occupy loci with a span much smaller than 400–600 bp. Therefore, regions of the DNA fragment that is not associated with protein of interest will be enriched together with the physiological binding site, thereby misrepresenting the true distribution of the protein of interest on chromatin. For example, binding of Tus protein is expected to be tightly and specifically localized to the 6×*Ter* array. Nonetheless, ChIP-qPCR for Tus-HA or Tus-FLAG revealed positive signals 443 base pair upstream of the edge of the 6×*Ter* array (Figure 5). We hypothesized that this signal at this exact site is an artifact related to the size of chromatin fragments that are generated during ChIP-qPCR. To test this hypothesis, we performed CUT&RUN-qPCR using the same primer set. Notably, we detected no Tus signal at the remote site 443 bp from the edge of the 6×*Ter* array, while the signal immediately adjacent to the edge of the 6×*Ter* array was robust (Figure 5). These data define the relative spatial resolutions of these two techniques and strongly suggest that CUT&RUN-qPCR yields a spatial resolution superior to that of ChIP-qPCR.

LIMITATIONS

Our data suggest that in terms of sensitivity and spatial resolution CUT&RUN-qPCR out-performs ChIP-qPCR, at least for the proteins analyzed herein. This protocol has been tested with only a limited number of DNA repair proteins and chromatin-bound proteins in mES cells. Thus, for each new application, it will be necessary to optimize the protocol. First, for cell lines other than mES cells, it may be necessary to optimize the cell number per sample empirically. Secondly, for each new protein of interest and each new antibody, it is important to optimize the antibody concentration. It is possible that some antibodies might work well in ChIP-qPCR but not in CUT&RUN-qPCR, and *vice versa*.

TROUBLESHOOTING

Problem 1

Concanavalin beads sediment while sitting on ice.

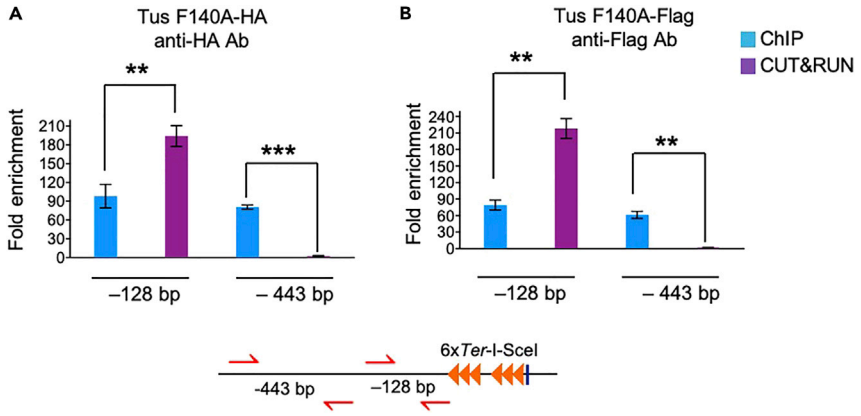

Figure 5. Comparison of resolution of Tus signal at Tus/*Ter* RFB at *Rosa26* in mES cells, using CUT&RUN-qPCR and ChIP-qPCR

(A and B) Data shows signal at Tus/*Ter* RFB for Tus protein C-terminally tagged with HA (panel A) or Flag (panel B). Blue bars: Tus-HA or Tus-Flag enrichment using ChIP-qPCR. Purple bars: Tus-HA or Tus-Flag enrichment using CUT&RUN-qPCR. Numbers indicate distance in base pairs from the outer qPCR primer to the nearest edge of the 6×*Ter* array. qPCR primer positions are shown by red half-arrows. Orange triangles: *Ter* sites. Blue line: I-SceI restriction site. Data in CUT&RUN and ChIP figures show means of $2^{-\Delta\Delta\ CT}$ values, normalized to EV and β-actin control locus. Data show means ± SD. Statistical analysis by Student's two-tailed unpaired t-test (n = 3), assuming unknown variance. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; ns, not significant. Note in CUT&RUN-qPCR data the intense Tus signal position -128 bp (i.e., where the inner PCR primer is immediately adjacent to the 6×*Ter* array), but no signal at position -443 bp (which lacks *Ter* sequences). In contrast, ChIP-qPCR reveals a positive Tus signal at both loci, including at position -443 bp, which lacks *Ter* sequences. This data shows that the spatial resolution of CUT&RUN-qPCR is superior to that of ChIP-qPCR.

Potential solution

Maintenance of beads on ice for more than 5 min will result in sedimentation of the beads. To overcome this problem, resuspend the beads by pipetting every 3–5 min, until the samples are ready for use. Importantly, frequent resuspension by pipetting may lead to the loss of some beads. Therefore, when calculating the total amount of beads required for the complete experiment, it is recommended to prepare extra beads. For example, if there are 10 samples in one experiment, calculate and prepare beads for 12 samples to compensate for bead loss from pipetting.

Problem 2

While rotating samples on the Nutator overnight, beads become stuck to the wall of Eppendorf tube, or form clumps with the cells (Figure 6).

Potential solution

It is important to resuspend the cells attached to ConA beads thoroughly. Use of more than 1 million cells per 10 μL of beads will promote clumping of cells with the ConA beads; therefore, ensure that the cell count is accurate, if necessary by repeated use of the hemocytometer. Further, use of non-adhesive Eppendorf tubes (Catalog no.13-698-790) will help to minimize this problem. If necessary, reducing the digitonin concentration from 2-4% may also help to prevent clumping of the beads.

Problem 3

Variability in fold enrichment between repeats of experiment or no DNA is detected by nanodrop.

Potential solution

There may be various causes of excessive variability of Ct values and thus fold enrichment between experiments. We pointed out those problems and their solutions as follows:

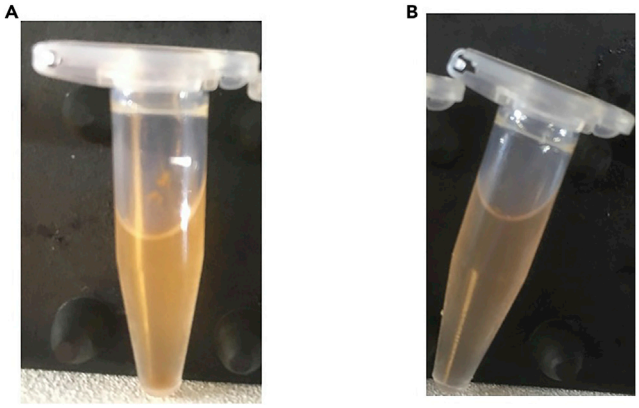

Figure 6. Troubleshooting: avoiding adherence of ConcanvalinA beads to the wall of the Eppendorf tube

(A and B) Use of more than 1 million cells per sample and of regular Eppendorf tube results in beads sticking on the wall of Eppendorf tube (panel A). However, use of 1 million cells and of low-binding Eppendorf tubes with proper resuspension of beads avoids this problem (panel B).

- It is important to test the cell lines for mycoplasma contamination frequently. Using mycoplasma-free cell lines is important for efficient transfection and for all downstream steps involved in CUT&RUN-qPCR.
- Over-trypsinization of cells may lead to cell clumping and reduces the final number of processed cells. After quenching the trypsin activity, use a p1000 pipet to forcibly dislodge the cells off the surface of the plate. Triturate five to ten times using the p1000 pipet to disaggregate all cell clumps.
- This protocol is optimized to 1 million cells. It is important to carefully count the number of cells using a hemocytometer and make sure to have an equal number of cells across various samples.
- With every washing step involving centrifugation and pipetting to remove the supernatant, it is critical to remove the buffer efficiently and equivalently from each sample, without loss of cells. Carefully wash the cells without touching the cell pellet. It is recommendable to use low-binding Eppendorf tubes to avoid any loss of cells.
- Low enrichment of signal or high Ct values may be due to the low abundance of protein of interest at the particular chromatin locus being studied. The signal can be enhanced by increasing the concentration of antibody and by extending the elution time period. Antibody specificity controls could include analysis of cells in which the gene encoding the target protein has been deleted.
- It is important to avoid over-digestion by MNase. Pay careful attention to the MNase digestion time. Chromatin fragmentation can be visualized by Tapestation or bioanalyzer (Figure 7).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Ralph Scully (rscully@bidmc.harvard.edu).

Materials availability

Plasmids and cell lines generated in the study are available upon request from the lead contact.

Data and code availability

This study did not generate any unique datasets or code.

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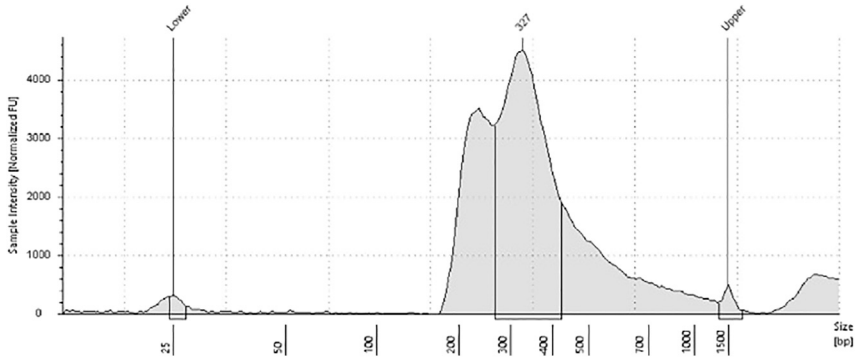

Figure 7. Tapestation analysis

Cell transfected with Tus-HA expression plasmid processed for CUT&RUN using an anti-HA antibody. Tapestation shows cleaved fragments size distribution having a peak of 327 base pairs that match with the Tus binding region (197 base pairs -6× Ter array+126 base pairs- adapter size).

AUTHOR CONTRIBUTIONS

A.P. optimized and conducted the experiments. A.P., R.E., N.A.W., and R.S. designed the experiments. A.P. and R.S. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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