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**Abstract**

This Cleavage Under Targets and Release Using Nuclease (CUT&RUN) protocol produces genomic occupancy data for a protein of interest in the protozoan parasite *Trypanosoma brucei*. The data produced is analyzed in a similar way as that produced by ChIP-seq. While we describe the protocol for parasites carrying an epitope tag for the protein of interest, antibodies against the native protein could be used for the same purpose.

**Keywords**

*Trypanosoma brucei,* CUT&RUN, genomic occupancy, mapping genomic protein binding sites

**Guidelines**

Pay extra attention to time-sensitive incubation periods, particularly the DNA cleavage step. We find that working through the protocol rather speedily produces the best results, so don’t dawdle.

**Before Start**

We use 50-75 million parasites per sample. Thus, cultures should be prepared in advance so that sufficient numbers of parasites are available for each sample.

The protocol works best when everything is kept cold prior to cutting with the protein A-MNase fusion protein. We recommend keeping buffers chilled on ice and pre-cooling centrifuges to 4-10ºC. Protease inhibitors should be added to the NP-S buffer just before commencing the experiment. The amount of **2X Stop Buffer** required for the experiment should be calculated and yeast spike-in DNA should be added prior to starting (see 2X Stop Buffer recipe below).

**Safety Warnings**

None noted

**Materials**

1. Spermidine trihydrochloride (Sigma-Aldrich, S2501)
2. Saponin (EMD Millipore, 558255, Saponin - CAS 8047-15-2)
3. NaCl (Sigma-Aldrich, S9888)
4. CaCl2 (Sigma-Aldrich, C4901)
5. Tris-HCl pH 7.5 (Millipore Sigma, RES3098T)
6. cOmplete, EDTA-free Protease Inhibitor Cocktail (Sigma-Aldrich, 11873580001)
7. EDTA, 0.5M, pH 8.0 (ThermoFisher Scientific, 15575020)
8. EGTA 0.5M, pH 8.0 (ThermoFisher Scientific, 50-255-957)
9. Mouse anti-rabbit IgG-PE (Santa Cruz, sc-3753)
10. Rabbit anti-HA (Sigma-Aldrich, H6908), primary antibody will vary depending on your system
11. Rabbit anti-H3 (gift)
12. SDS (Sigma-Aldrich, 428018)
13. Proteinase K (ThermoFisher Scientific, EO0491)
14. RNAse A (NEB, T3018)
15. AMPure XP beads (Beckman Coutler, AMPure XP)
16. CUT&RUN pAG-MNase and Spike-In DNA (Cell Signaling Technology #40366)

**Equipment**

1. Microcentrifuge (Eppendorf 5424R)
2. Swinging bucket centrifuge (Eppendorf 5920R)
3. 1.7ml Olympus microtubes (Genesee 22-281)
4. Olympus 50ml Centrifuge Tubes (Genesee 21-108)
5. Roto-Mini Plus Variable Speed Rotator with tube holders, 115V (ThermoFisher Scientific, 1159P34)
6. Novocyte Flow Cytometer 2000

**Citations**

Ashby E, Paddock L, Betts HL, Liao J, Miller G, Porter A, Rollosson LM, Saada C, Tang E, Wade SJ, Hardin J, Schulz D. 2022. Genomic occupancy of the bromodomain protein Bdf3 is dynamic during differentiation of African trypanosomes from bloodstream to procyclic forms. mSphere 7:e00023-22.

Skene PJ, Henikoff S. 2017. An efficient targeted nuclease strategy for high-resolution mapping of DNA binding sites. eLife 6:e21856.

**Additional Notes**

We keep our centrifuges at 10ºC to make sure the samples don’t freeze if the temperature drifts slightly. All spin steps of the protocol can be performed anywhere between 4ºC-10ºC.

**Buffers**

**NP-S Buffer with 0.1% Saponin**

Individual Components

0.5 mM spermidine

0.1% (vol/vol) Saponin

50 mM NaCl

10 mM Tris-Cl (pH 7.5)

Store at 4ºC

Add protease inhibitors just before use

**NP-S Buffer no detergent**

Individual Components

0.5 mM spermidine

50 mM NaCl

10 mM Tris-Cl (pH 7.5)

Store at 4ºC

Add protease inhibitors just before use

**Antibody Buffer**

**NP-S Buffer** with 2mM EDTA

**2X Stop Buffer**

20mM EDTA

40mM EGTA

Add 50pg of yeast spike-in DNA to each reaction, or 50pg/100µl of **2X Stop Buffer**

**Steps**

**Prepare cells**

1. Count parasite cultures with hemocytometer or other preferred counting method.
2. Spin down cells in centrifuge at 2800 x g for 10 minutes. This spin step can be performed at room temperature or at 10ºC. Remove supernatant and resuspend in small amount of remaining media (~100 µL).
3. If needed, combine samples from multiple Eppendorf tubes so that each final tube has 75 million cells, and spin again at 2800 x g for 4 minutes at 10ºC in microcentrifuge. Remove supernatant.

**Permeabilize cells**

1. Wash all samples with 1 mL **NP-S Buffer with 0.1% Saponin**
2. Spin at 4600 x g, 4 min, 10ºC. Remove sup.

**Primary Antibody Binding NO CA**

1. Resuspend each sample in 100µl **NP-S Buffer with 0.1% Saponin**
2. Add EDTA to 2mM final (4µL of 0.05M EDTA/ tube)
3. Add 5 µg α-HA antibody (or other antibody against protein of interest for experimental sample) **OR**

add 5 µg α-H3 antibody (for control sample, might require titration) **OR**

add 5 µg α-IgG antibody (for control sample)

1. Incubate for 45m on tube rotator at room temperature
2. Add 1ml **NP-S Buffer no detergent** to each sample
3. Spin at 4600 x g for 4 minutes at 10ºC. Remove supernatant.
4. Add 1ml **NP-S Buffer no detergent** to each sample
5. Take out aliquot of 1 million cells for flow cytometry analysis (~14µl if starting with 75 million cells). Set aside on ice.
6. Spin the remainder of the sample at 4600 x g for 4 min at 10ºC. Remove supernatant. (total 2 washes)

**pMNAse binding**

1. Resuspend each sample in 100µl of buffer **NP-S Buffer no detergent**
2. Add 1.5µl of pMNase enzyme to each sample.
3. Incubate for 45m at room temperature on rotator

**FACS Sample preparation**

1. Add 1mL **NP-S Buffer no detergent** to 1 million cell aliquot prepared above (we also routinely use HMI-9 media for the flow cytometry antibody incubation and washes)
2. Spin at 4600 x g for 4 min
3. Resuspend in 100uL **NP-S Buffer no detergent**
4. Stain with α-rabbit IgG PE at 1:200 for 15 minutes at room temperature
5. Wash twice in 1mL **NP-S Buffer no detergent** (or HMI-9) at 7,000 rpm for 4 min
6. Resuspend in 300uL **NP-S Buffer no detergent** (or HMI-9)
7. Transfer sample into flow cytometry tube
8. Analyze on flow cytometer

**pMNAse wash** (continued after 45m incubation above)

1. Add 1ml **NP-S Buffer no detergent** to each sample
2. Spin at 4600 x g for 4 minutes at 10ºC. Remove supernatant.
3. Add 1ml **NP-S Buffer no detergent** to each sample
4. Spin at 4600 x g for 4 minutes at 10ºC. Remove supernatant.

**Targeted Digestion Preparation**

1. Make sure to prepare enough **2X Stop** **buffer** with spike in control

This is the section where timing is very important

**Targeted Digestion**

1. Resuspend each sample in 100µl buffer **NP-S Buffer no detergent**
2. Incubate at room temperature for 5 min
3. Add 2µl 100mM CaCl2 to all samples (final concentraiton = 2mM), mix by flicking
4. Incubate all samples at 25ºC for 5 min. We recommend using a heat block as ambient temperature in the lab can vary.
5. Add 100µl **2X Stop** **buffer** to each sample and mix by flicking.

**Chromatin Release**

1. Incubate 10 min at 37ºC to release CUT&RUN fragments from the insoluble nuclear chromatin
2. Spin at 4600 x g for 4 min at 10ºC. Remove supernatant into new tube. SAVE SUPERNATANT

**DNA Extraction**

1. To all samples add 2µl of 10% SDS (final concentration = 0.1%), 3.3µl of 10mg/ml proteinase K (165µg/ml), and 1.33µl of 1mg/ml RNAse A (6.5µg/ml)
2. Mix by gentle flicking and incubate for 10 min at 70ºC
3. Purify using Ampure XP beads at 1.8X or phenol chloroform extraction

Following DNA purification, high-throughput sequencing libraries can be prepared using the preferred method of the research lab.