DISCOVER-Seq: MRE11 ChIP Seq

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ABSTRACT

Genome editing using nucleases such as CRISPR-Cas induces programmable DNA damage at a target genomic site, but can also affect off-target sites. We develop a powerful assay for the unbiased identification of off-target sites that we term DISCOVER-seq. This approach takes advantage of the recruitment of endogenous DNA repair factors for genome-wide identification of Cas-induced double strand breaks. One such factor, MRE11, is recruited so precisely to a double stranded break that nuclease cut sites can be determined with single-base resolution. DISCOVER-seq is applicable to multiple types of Cas nucleases and provides an unprecedented molecular picture of events that precede repair of the affected sites. DISCOVER-seq furthermore detects off-targets in cellular models and tissues.

PROTOCOL STATUS

Working

We use this protocol in our group and it is working

STEPS MATERIALS

NAME	CATALOG #	VENDOR
100x Halt protease inhibitor cocktail	78429	Thermo Fisher Scientific
Dynabeads Protein A	10002D	Thermo Fisher Scientific
MRE11 antibody	NB100-142	
RNase A 10mg/ml, DNase and Protease-free	EN0531	Thermo Scientific
Proteinase K	EO0491	Thermo Fisher Scientific
Sodium Acetate 3M, pH 5.2	R1181	Thermo Scientific
MinElute PCR Purification Kit	28004	Qiagen
MinElute Reaction Cleanup Kit	28204	Qiagen

SAFETY WARNINGS

RNP editing of cells

1 Culture your cell line of interest so that you can edit **1x10^7 cells** on the day of nucleofection.

NOTE

It's better to start with lots, if your cell type is tricky or you cannot get that many cells you can start with less. We have successfully done ChIP-Seq with 2x10^6 cells.

NOTE

Best practice is to set up a control experiment of cells edited with a non-targeting gRNA. This sample should be processed the same way as the gRNA of interest. MRE11-ChIP from these cells will serve as a negative control for the analysis later and will help elimate false positives.

2 Harvest cells and spin down in falcon tube.

3 Wash cells with **1ml PBS** and transfer to eppendorf tube. Spin to pellet, discard S/N

4 While cells are spinning: Prepare RNPs.

1. Add **Cas9 Buffer** (20 mM HEPES (pH 7.5), 150 mM KCl, 1 mM MgCl2, 10% glycerol and 1 mM TCEP) such that final volume of all components comes to **15uL**.
2. Add 30 pmol of **Cas9** per one million cells (i.e. for 1x10^7 you need 300pmol Cas9)
3. Add 30 pmol of **sgRNA** per one million cells (i.e. for 1x10^7 you need 300pmol gRNA)
4. Incubate at RT for **5-10 mins** (or longer)

5 Add **60ul of nucleofection buffer** (Lonza) including supplements to your RNPs and pipette up and down. Transfer the whole volume into eppendorf tube with cell pellets and resuspend cells.

NOTE
The type of nucleofection buffer used depends on your cell type. Check Lonza guidelines for the right buffer.

NOTE
The amount of nucleofection buffer added depends on the size of your cell pellet. We found that 1x10^7 pelleted cells have a volume of roughly 40ul. Plus 15ul RNP solution and 60ul nucleofection buffer makes 115ul total which can be nucleofected with 100ul Lonza cuvettes. If you have less cells you can use more nucleofection buffer to make the total volume 100ul. We found that at least 50% of the total solution should be nucleofection buffer. If your cell pellet is too big you can split it up into two nucleofections.

6 Transfect cell suspension into **100ul nucleofection cuvette** and electroporate using the a Lonza 4D X nucleofector.

NOTE
Check with Lonza for the best nucleofection settings for your cell type. We find that FF-120 works great for K562 and DJ-110 works great for B16-F10 cells.

7 After nuclefection, let cells sit at RT in cuvette for 5 mins. Then transfer cells into culture plates with prewarmed complete media and incubate at 37C and 5% CO2.

Crosslinking

8 After **8-24h** of culture, harvest cells into falcon tube. Spin to pellet. Discard media.

IMPORTANT: Before you spin cells down, make sure to take a small split of the cells to keep in culture for a few more days. You will need these cells to later extract gDNA to check on mutation rates at off-target sites. We usually harvest cells for gDNA extraction 4 days post nucleofection.

9 Resuspend cells in **20ml of room temperature media** (You can use IMDM or RPMI) without supplements.
Add 37% Formaldehyde to a final concentration of **1%**. Incubate at RT for **15 min**

NOTE
Make sure you obtain a single cell suspension. For sticky or adherent cell types you can pass cells through a 23G needle with a syringe.

SAFETY INFORMATION

Formaldehyde is toxic and the waste has to be disposed of correctly!

10 Add 2.5 M glycine to quench formaldehyde (final **125mM**). Leave a couple of minutes at RT.

SAFETY INFORMATION

Formaldehyde is toxic and the waste has to be disposed of correctly!

11 Wash cells twice with **10 ml ice cold PBS** (1200 g, 3 min spins – pour off S/N each time). Transfer cells to a 15 mL falcon tube before last spin. Take off S/N completely.

12 Proceed with lysis, or snap freeze cells in liquid nitrogen and **store pellets at −80 °C**.
Crosslinked cell pellets can be stored at -80C for a few months.

Prebinding of antibody to magnetic beads

13

NOTE

This section is optional. We like to pre-incubate the magnetic beads with the antibody and then use bead-antibody complexes for the IP. You can however also add the antibody to lysates directly and then collect Antibody-Antigen complexes with beads afterwards.

Add **100 µl magnetic beads** (Invitrogen, Dynabeads) – protein A to 1.5 ml microfuge tubes. Add **1 ml block solution** (0.5% BSA (w/v) in PBS). If you have multiple IPs you can scale up accordingly to the number of IPs.

e.g.: 3 IPs: use 300ul of beads (still wash with 1ml of Block solution)

Dynabeads Protein A
by Thermo Fisher Scientific
Catalog #: 10002D

14 Collect the beads using magnetic stand. Remove supernatant. Wash beads in **1ml block solution** two more times.

NOTE

With all bead washes (here and subsequent after IP) it is important to touch the beads as little as possible with a pipette tip to avoid losing beads as beads stick to the pipette tip. A better way is to add the wash solution and then turn the tube on the magnetic stand so that beads have to traverse the solution, repeat this twice for each tube and wash.

15 Resuspend beads in **250ul block solution** per IP and add **4ul of MRE11 antibody** (NB100-142).

e.g.: 3 IPs: use 750ul of block solution

MRE11 antibody
Catalog #: NB100-142

16 Incubate for **1-2 hours** at on a rotating platform @ RT.

17 When ready to IP: Wash magnetic beads as described above (3 times in 1 ml block solution). Resuspend in **100 µl block solution** which is to be added to lysate (Step 25).

Cell lysis

18 Resuspend each pellet of crosslinked cells in **10 ml of LB1** (+protease inhibitors) (50 mM Hepes–KOH, pH 7.5; 140 mM NaCl; 1 mM EDTA; 10% Glycerol; 0.5% NP-40 or Igepal CA-630; 0.25% Triton X-100). Rock at **4 °C for 10 min**.

100x Halt protease inhibitor cocktail
by Thermo Fisher Scientific
Catalog #: 78429

19 Spin at 2000 × rcf for 3 min at 4 °C in a tabletop centrifuge. Pour off S/N.

20 Resuspend each pellet in **10 ml of LB2** (+protease inhibitors) (10 mM Tris–HCL, pH8.0; 200 mM NaCl; 1 mM EDTA; 0.5 mM EGTA). Rock gently at **4 °C for 5 min**.

21 Pellet nuclei in tabletop centrifuge by spinning at 2000 × rcf for 3 min at 4 °C. Pour off S/N

22 Resuspend each pellet in each tube in **1.5 ml LB3** (+protease inhibitors) in 2mL tube (10 mM Tris–HCl, pH 8; 100 mM NaCl; 1 mM EDTA; 0.5 mM EGTA; 0.1% Na–Deoxycholate; 0.5% N-lauroylsarcosine).

23 Sonicate lysate **3x for 1 min** on ice. Settings: 0.9 ON 0.1 OFF and 75% amplitude works for our sonicator. Let samples rest on ice inbetween sonications.

NOTE
The right sonication has to be determined experimentally. It varies between cell types, amount of cells used and between cell lines and tissues. it is good to do couple of test runs on unedited cells and run the sonicated products on a gel. The right sonication size is important as it determines the resolution of you ChIP-Seq experiment.

EXPECTED RESULT
You should see smear of DNA between 200-500bp, if you still have unsheared genomic DNA you will see a clear distinct band at high MW and also bigger products (>1000 bp).

24 Spin at **20,000 × rcf for 10 min at 4 °C** to pellet debris. Transfer S/N to new falcon tube. Add **1.5ml of LB3** and **300ul of 10% Triton-X** (final 1%).

NOTE

The amount of LB3 and Triton X-100 is adjusted to the number of ChIPs to be performed. For example to prepare 2 ChIPs from one 1.5 ml sonication you would top up the centrifuged sonication to 6 ml with LB3 and Triton X-100 to 1% final concentration. Mix well and transfer 3mL per IP to 15 ml conical tubes.

25 IMPORTANT: Save 2x 50 µl of cell lysate from each sonication as input DNA in screw cap tubes. Store at −20 °C until the next day.

Now you are ready for IP.

Chromatin IP

26 Add **100 µl** antibody/magnetic bead mix from go to step #17 to cell lysates.

27 Gently mix on rotator or rocker at **4 °C overnight.**

Wash beads and elution

28 Transfer half the volume of an IP to an eppendorf tube.

29 Let tubes sit in magnetic stand to collect the beads. Remove supernatant and add remaining IP. Let tubes sit again in magnetic stand to collect the beads and remove supernatant.

30 Add **1 ml RIPA Buffer** (50 mM Hepes–KOH, pH 7.5; 500 mM LiCl; 1 mM EDTA; 1% NP-40 or Igepal CA-630; 0.7% Na–Deoxycholate) to each tube. Turn tubes to wash beads. Collect beads. Remove supernatant.

31 Repeat this wash **5 more times.**

32 Wash once with **1 ml TBS** (20 mM Tris–HCl, pH 7.6; 150 mM NaCl). Remove S/N.

33 Add **200 µl of elution buffer** (50 mM Tris–HCl, pH 8; 10 mM EDTA; 1% SDS). Transfer to 1.5ml conical screw cap tubes.

34 Elute and perform reverse crosslinking at **65 °C for 6–18 h** rotating or shaking.

35 Thaw 50 µl of the input lysate from go to step #24, add 150 µl of elution buffer and mix. Reverse the formaldehyde crosslinking as in go to step #33 simultaneously with the ChIP samples.

DNA purification

36 Quickspin tubes to collect liquid from lids. Collect beads with magnetic stand. Take 200 µl of supernatant and transfer to a new tube.

37 Add **100 µl of TE** to each tube of IP and input DNA to dilute SDS in elution buffer.

38 Add **8 µl of 1 mg/ml RNaseA** (Ambion Cat # 2271). Mix and incubate at 37 °C for 30 min.

RNase A 10mg/ml, DNase and Protease-free
by Thermo Scientific
Catalog #: EN0531

39 Add **4 µl of 20 mg/ml proteinase K** (Invitrogen, 25530-049). Mix and incubate at 55 °C for 1h.

Proteinase K
by Thermo Fisher Scientific
Catalog #: EO0491

40 Purify DNA using a Qiagen clean up kit.

Make sure to check on pH of your reaction by using the provided pH indicator. Add 5ul 3M Sodium Acetate, pH 5.2 to adjust pH if necessary.

Sodium Acetate 3M, pH 5.2
by Thermo Scientific
Catalog #: R1181

MinElute PCR Purification Kit
by Qiagen
Catalog #: 28004

or

MinElute Reaction Cleanup Kit
by Qiagen
Catalog #: 28204

41 Elute DNA in **41ul of purfied water.**

QC of pulldown

42 Check if ChIP was successful by amplifying a region close to your on-target site (about 200bp from cut site) by quantitative real-time PCR. Also run your input DNA to account for differences in primer efficiencies and amount of starting material.

Compare the Cts obtained in this region to a random region elsewhere in the genome.

For qPCR we dilute input DNA 1 in 49 and IP DNA 1 in 5.

EXPECTED RESULT

If you get good editing of your locus of interest (50-80% indels) you should see a 80-200-fold enrichment of your region of interest in MRE11 ChIP compared to the control region.

Illumina Sequencing

43 If the qPCR was successful proceed to library preparation for Illumina sequencing.

We sequence our samples using 100bp paired-end sequencing on a HiSeq4000 aiming for 12.5 Million reads per sample.

BLENDER bioinformatics pipeline

44 Analyze your sequencing data using BLENDER software which we will make available on github.

Amplicon-NGS for off-targets

45 Don't forget to harvest your cells from go to step #8 for genomic DNA extraction.

Check sites identified by BLENDER for indels using amplicon next-generation sequencing.