Jan 27, 2020

MojoSort™ Mouse Ig light chain κ Nanobeads Column Protocol

Sam Li[1]

[1]BioLegend

1 Works for me dx.doi.org/10.17504/protocols.io.bbueinte

BioLegend

Sam Li
BioLegend

ABSTRACT

This protocol is about Maintenance and Expansion of induced pluripotent stem cells.

EXTERNAL LINK

https://www.biolegend.com/en-us/protocols/mojosort-mouse-ig-light-chain-k-nanobeads-column-protocol

GUIDELINES

Frequently Asked Questions

Why is the DNA yield so low?

CUT&RUN is performed using low cell numbers and the background signal is considerable lower than e.g. for ChIP. Due to these two factors the amount of recovered DNA is often times too low to be reliably measured based on a fluorometric assay or by capillary electrophoresis. PCR amplification of small CUT&RUN products, i.e. less than 50 bp, can be problematic and is therefore not any option. In order to assess the success of the CUT&RUN method each of the antibodies-online CUT&RUN product sets includes the CUT&RUN Positive Control antibody ABIN6923144•against the abundant H3K27me3 histone modification. DNA fragments prepared using this antibody can be measured by sensitive electrophoresis on a Bioanalyzer or Tapestation or fluorometrically on a Qubit or Nanodrop fluorometer. When using the CUT&RUN Positive Control antibody ABIN6923144•(or any other antibody specific for nucleosomal markers) a ladder corresponding to multiples of the 147 bp long nucleosomes should be visible by capillary electrophoresis.

How can I validate that my primary antibody is working for CUT&RUN?

For a CUT&RUN experiment the validation data could include e.g. a Tapestation or Bionalyzer plot showing the size distribution and qPCR data showing target enrichment.
As mentioned above, the DNA yield of an CUT&RUN experiment appears typically very low compared e.g. to ChIP-seq because of the lower initial sample size and the substantially lower DNA background. In particular for less abundant target protein the concentration is often times too low to be reliably measured using a fluorometric assay or by capillary
electrophoresis. PCR amplification of small CUT&RUN products, i.e. less than 50 bp, can be problematic and is therefore not an option.
Once a sequencing library has been generated and sequenced map sequencing reads and

verify the accumulation of reads at known binding sites.

Why do I need a negative control antibody? Why not just use a no-antibody control?

MNase is an endo- and exonuclease that will unspecifically bind and cleave unprotected DNA in hyper-accessible DNA, e.g. in regions surrounding regulatory elements. Free MNase will preferentially cut DNA within these hyper-accessible regions, thus potentially causing false positives and increased background signal in general.
In order to avoid this undesired effect of untethered MNase, the chromatin is randomly coated with the CUT&RUN Negative Control (ABIN6923140•) prior to the addition of pA/G-MNase is added to the samples. pA/G-MNase is then tethered via its Protein A or Protein G portion to the antbodies' Fc fragment and background DNA fragmentation is dictated by the random antibody binding as opposed to the nuclease digestion of hyper-accessible DNA regions.

Can I replace the antibody negative control using a knock-out (or knock-down) of my protein?

Both controls are useful but address different aspects of the experiment and are therefore not interchangeable.
The CUT&RUN Negative Control (ABIN6923140) antibody is used to establish a reference background for peak calling. This is necessary because of the sparse background signal in CUT&RUN samples compared to ChIP-seq samples. The ko (or kd) control on the other hand gives an impression of unspecific binding of the antibody specific for the prote-
in of interest to other proteins. It is useful to avoid identification of false positive signals.

Do I need to use a secondary antibody? Other CUT&RUN protocols do not use a secondary.

Depending on the host species and isotype of the antibody and the Protein A and/or Protein G MNase fusion protein, a secondary antibody may be necessary for pA/G-MNase binding.
Protein A has good high affinity to all rabbit IgG antibodies but low affinity to rat, goat and sheep IgG isotype antibodies and certain mouse IgG antibody subclasses, in particular IgG1. Protein G on the other hand binds well to the Fc region of mouse, goat, sheep, and most rat IgG. Its affinity to rabbit IgG however is lower than that of Protein A. When using pAG-MNase introduced with the improved CUT&RUN protocol it is therefore generally not necessary to use a secondary antibody. Use of the pA-MNase of the original protocol however might require the use of a secondary antibody raised in rabbit to assure efficient binding of the fusion protein to the antibody.

Should I include heterologous spike-in DNA for quantitation?

Our protocol is largely based on the improved CUT&RUN protocol. Here, the authors show that accurate quantitation is possible using heterologous spike-in DNA or carry-over *E. coli* DNA from the pA/G-MNase purification.

Are there protocols to make this work for tissue, where I cannot necessarily isolate single cells?

The Henikoff lab that published the original CUT&RUN method in 2017 has since developed several variations. One of these modification is AutoCUT&RUN which outlines a workflow to automate CUT&RUN for high-throughput characterization of single cells but also patient samples and xenografts.
In short, the tissue is processed manually or enyzmatically into single, intact cell that are subsequently bound to ConA beads. The improved (and standard) CUT&RUN protocol that serve as basis for the protocol outlined in our CUT&RUN handbook can be adapted accordingly. Presently there is no work published documenting the use of intact tissue.

Is it possible to use the CUT&RUN product sets with plant tissue samples?

The CUT&RUN method can be applied without a problem to plant tissue samples (see e.g.PMID30719569). One essential step in addition to those lined out in the protocol is the generation of spheroblasts so that it becomes possible to permeabilize the plasma membrane for the application of the antibodies and the MNase fusion protein.
The positive control H3K27me3 antibody and the negative control guinea pig anti-rabbit IgG antibody as well as the ConA beads are suitable for use with plant samples. The antibodies that are included in some of the sets, such as the anti-DYKDDDDK antibodies or the secondary antibodies, can also be used with plant samples. Whether they are needed or not depends on your experiment.

Can CUT&RUN be adapted for RIP-seq?

It should be possible to adapt the CUT&RUN protocol for use with RNA as an alternative to RIP-seq. RNA in the cytoplasm will attract the degradation machinery if it is lacking the 5' cap and the 3' poly-A tail. Thus, it is advisable to use isolated nuclei as sample material. Consequently, no digitonin in the different buffers is needed since the nuclear envelope does not contain cholesterol. Isolated nuclei may be immobilized via glycoproteins on the nuclear envelope to the ConA beads like for a CUT&RUN experiment. An antibody against the protein of interest is added and subsequently the pA/G-MNase is tethered to the antibody, thus bringing the MNase into proximity of the RNA of interest. Similar to RIP-seq, isolated RNA is then translated into cDNA and can be sequenced and mapped.

Is it possible to use a cell free extract of a bacterial cell instead of immobilizing and permeabilizing the cell?

It should indeed be possible to use a bacterial extract as starting material instead of immobilized eukaryotic cells. There are a couple of things that should be considered:
One of the factors that makes CUT&RUN so much better than ChIP-seq in a eukaryotic system is that it is done in situ. The intact cells (or isolated nuclei) retain the large, undigested chromatin. Consequently, much fewer off-sites are present in the prepared DNA which is one of the reasons why CUT&RUN data sets have so much less background signal when compared to ChIP-seq data. This enrichment step will be missing when using a cell lysate as starting material. The CUT&RUN result should still be superior to the CHIP-seq result for the same starting material. However, it will be more "noisy" than CUT&RUN sequencing data from intact eukaryotic cells/nuclei.
The buffers described in the CUT&RUN protocol are designed to maintain the cells in the samples intact. The binding buffer contains some divalent cations that are necessary for the binding of the cells to the Concanavalin on the beads that are used for the immobilization. Subsequently, this buffer is changed for an antibody buffer that contains EDTA to chelate the divalent cations and prevent DNA cleavage prior to pA/G-MNase antibody binding. Since you are working with cell lysates as opposed to intact cells there is no need to include digitonin in any of the buffers. You don't need the binding buffer used to immobilize cells on the ConA beads. In order to control premature DNA cleavage by the MNase I would include some EDTA and/or EGTA in the lysis buffer.
Probably, you will have to titrate the amount of pA/G-MNase and try different digestion times to find the best balance between efficient cleavage of the intended sites and too much off-site cleavage.
The original CUT&RUN protocol foresees the use of heterologous spike-in DNA, e.g. from *E. coli.* In its latest iteration it is argued that there is no need to add this DNA since the pA/G-MNase preparation contains sufficient levels of *E. coli* DNA to serve as a standard for quantitation. In a prokaryotic system, spike-in DNA from a eukaryotic organism (e.g. *S. cerevisiae*) is recommended. Also keep in in mind that the *E. coli* DNA carried over with the pA/G-MNase preparation may contain sequences that show homology to the bindings sites of your protein of interest.
Our product sets are optimized for use with eukaryotic cells. The positive control in all sets is a recombinant rabbit anti-H3K27me3 antibody. This control is not suitable for use with prokaryotic samples.

Instead of the proteinase K digestion can I denature the proteins in the CUT&RUN product complexes by heat?
Proteinase K digest followed by a phenol-chloroform extraction has historically been the go-to method to prepare high-molecular weight DNA, primarily from tissues. Subsequently to the diffusion of the CUT&RUN products out of the cells the DNA is present already in a relatively clean form, in particular when compared to a cell lysate like in ChIP-seq. Therefore, it is reasonable to assume that it is possible to forgo the Proteinase K treatment of the cells and instead proceed with the PCI extraction. As a reference, nucleic acid preparation using TRIzol does not include a Proteinase K digest. However, it has also been reported, that a proteinase K digest improves subsequent PCR amplification. Regarding heat inactivation instead of proteinase K digest in step, we recommend againstthis option. Your DNA of interest is at his point present in a complex consisting of the DNA, your antigen, the corresponding antibody, and the pA/G-MNase. If at this stage you boil your sample denature proteins you risk precipitation of your DNA with the protein compounds in this complex upon denaturation. This will also primarily affect the short CUT&RUN products, thus decreasing the signal to noise ratio in your library and potentially also reducing the library's complexity.

What is preferable for DNA extractions prior to library preparation: phenol-chloroform extraction or affinity purification using a column?
In the original publication describing the CUT&Tag method the authors mention the use of AMPure XP beads for the purification of the DNA subsequently to tagmentation and Proteinase K digest. A potential issue is the carry-over of active Proteinase K, which can interfere with the downstream PCR amplification. Therefore, the authors recommend now the phenol-chloroform extraction to assure complete denaturation of Proteinase K.

MATERIALS

NAME	CATALOG #	VENDOR
MojoSort™ Mouse Ig light chain κ Nanobeads	480125	BioLegend

1 Prepare cells from your tissue of interest or blood without lysing erythrocytes.

2 In the final wash of your sample preparation, resuspend the cells in MojoSort™ Buffer by adding up to 4 mL in a 5 mL (12 x 75 mm) polypropylene tube. Note: Keep MojoSort™ Buffer on ice throughout the procedure.

3 Filter the cells with a 70µm cell strainer, centrifuge at 300xg for 5 minutes, and resuspend in an appropriate volume of MojoSort™ Buffer. Count and adjust the cell concentration to 1 x 10^8 cells/mL by adding MojoSort™ Buffer.

4 Aliquot 100µL of cell suspension (10^7 cells) into a new tube.

5 (*Optional*) Add 5µL of Human TruStain FcX™ (Fc Receptor Blocking Solution), mix well and incubate at room temperature for 10 minutes. Scale up the volume accordingly if separating more cells. For example, if the volume of Human TruStain FcX™ for 1x10^7 cells is 5µL, add 50µL for 1 x 10^8 cells. When working with less than 10^7 cells, use indicated volumes for 10^7 cells.

6 Stain 10^7 cells using the mouse Ig light chain κ-containing antibody following manufacturer's instructions for flow cytometric surface staining. Scale up the volume accordingly if staining more than 10^7 cells. Note: For the mouse Ig light chain κ-containing antibodies, we recommend to do a titration to determine the optimal concentration.

7 Wash the cells by adding MojoSort™ Buffer up to 4mL. Centrifuge the cells at 300xg for 5 minutes.

8 Discard the supernatant and resuspend cells in 100uL of MojoSort™ Buffer.

9 Resuspend the beads by vortexing, maximum speed, 5 touches. Add the appropriate volume of pre-diluted Mouse Ig light chain κ Nanobeads to the cell suspension, mix well and incubate on ice for 15 minutes. Scale up the volume accordingly if separating more cells. For example, if the volume of Nanobeads for 1x10^7 cells is 10µL, add 100µL for 1 x 10^8 cells. When working with less than 10^7 cells, use indicated volumes for 10^7 cells.Note: The amount of Nanobeads to use always depends on the frequency of the target, among a few other factors. We recommend doing a titration to determine the optimal concentration.

10 Wash the cells by adding MojoSort™ Buffer up to 4mL. Centrifuge the cells at 300xg for 5 minutes.

11 Discard the supernatant.

12 Add the appropriate amount of MojoSort™ Buffer and proceed to separation. At least 500µL is needed for column separation.Note: There are several types of commercially available columns, depending on your application. Choose the one that fits best your experiment:

	Max. number of labeled cells	Max. number of total cells	Cell suspension volume	Column rinse volume	Cell wash volume	Elution volume
Small Capacity	1 x 10^7	2 x 10^8	500µL for up to 10^8 cells	1ml	1 ml	1 ml
Medium Capacity	1 x 10^8	2 x 10^9	500µL for up to 10^9 cells	3ml	3 ml	5 ml
Large Capacity	1 x 10^9	2 x 10^{10}	500µL for up to 10^{10} cells	20-50ml	30 ml	20 ml

Example of magnetic separation with medium capacity columns:

13 Place the column in a magnetic separator that fits the column.

14 Rinse the column with 3mL of cell separation buffer.

15 Add the labeled cell suspension to the column through a 30µm filter and collect the fraction containing the unlabeled cells.

16 Wash the cells in the column 3 times with 3mL of buffer and collect the fraction containing the unlabeled cells. Combine with the collected fraction from step 3. These cells may be useful as controls, to monitor purity/yield, or other purposes.

17 Take away the column from the magnet and place it on a tube. Then add 5mL of buffer and flush out the magnetically labeled fraction with a plunger or supplied device. These are the positively isolated cells of interest; do not discard. To increase the purity of the magnetically labeled fraction repeat the isolation process with a new, freshly prepared column.