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APPLICATION NOTE

Combining the use of the iDeal® CUT&Tag kit and the Tissue Nuclei Isolation Module for chromatin profiling in iPSC-derived cortical brain organoids.

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Background

The iDeal CUT&Tag kit has been designed for efficient chromatin profiling of histone marks and some non-histone proteins. CUT&Tag is a recently developed strategy to profile the location of chromatin components genome-wide (1). The method selectively targets chromatin-bound proteins with specific antibodies in intact nuclei or cells, without fragmentation or immunoprecipitation. It offers many advantages, including a low background and the requirement of low cell inputs and sequencing depth. The team of Gabriel Santpere at Hospital del Mar Research Institute in Barcelona is focused on the experimental determination and analysis of transcription factor (TF) binding sites during neurogenesis and neuronal differentiation (2). In this application note, we detail the procedure and present the results we have obtained from validating the combination of the iDeal CUT&Tag kit and the Tissue Nuclei Extraction Module on cortical organoids derived from induced pluripotent stem cells (iPSC) to test specific TFs. We show that the iDeal CUT&Tag kit for histones together with the Tissue Nuclei Extraction Module can be used to ascertain the genome-wide binding landscape of both histone associated proteins and transcription factors in iPSC-derived cortical brain organoids.

Material

Nuclei Isolation

Tissue Nuclei Extraction module (Diagenode, Cat. No. C01080003)

Cleavage Under Targets and Tagmentation

iDeal CUT&Tag kit for histones (Diagenode, Cat. No. C01070020)
Antibody package for CUT&Tag (Diagenode Cat, No. C01070022)
Primer index for tagmented libraries (Diagenode, Cat. No. C01011033)

Additional supplies

- iPSC-derived cortical organoids of interest
- Cell medium
- Phosphate-buffered saline (PBS) buffer (Gibco, Cat. No. 10010023)
- Ultrapure DNase/RNase-free Distilled water (Thermo Scientific, Cat. No. A57775)
- 100% Ethanol, molecular grade (Thermo Scientific, Cat. No. 445730025)
- Trypan Blue solution 0.4% (Thermo Scientific, Cat. No. 15250061)
- Cell Strainer 40 µm (Thermo Scientific, Cat. No. 08-771-1)
- 10 mM Tris-HCl pH 8 (Thermo Scientific, Cat. No J62745.EQE)
- Antibody of interest (Diagenode)
- Sera-Mag Select reagent (Cytiva, Cat No. 29343045)
- Agilent High Sensitivity DNA Kit for the Bioanalyzer

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- 1.25 M Glycine (Diagenode, Cat. No. C01019019)
- 37% Formaldehyde, molecular grade (Sigma, Cat. No. 252549)
- Antibody CTCF (Diagenode Cat, No. C154110210-50)

Procedure

Tissue Nuclei Extraction module

1. iPSC-derived Organoids lysis

- Take around 2 or 3 organoids per iDeal CUT&Tag reaction and wash them with PBS twice.
- Transfer the organoids to a 1.5 ml tube containing 800 µl of Tissue Lysis buffer.
- Disrupt the organoids by pipetting up and down several times until a homogeneous suspension is obtained.
- Filter the tissue lysis suspension by gravity through a 40-µm cell strainer. Recover the cell suspension in a 50 ml tube.

2. Nuclei extraction

- Transfer 400 µl of the recovered suspension to a 2 ml LoBind tube.
- Follow the manufacturer's instruction to obtain a 3-layer gradient.
- After centrifugation at 3500 x g for 20 minutes at 4°C, the nuclei appear as a thin whitish band between layers 2 and 3. Using a P200 pipette, collect this band (approximately 100-200 µl) and transfer it to a new 1.5 LoBind tube.

3. Nuclei counting and mild fixation

- To a 10 µl aliquot of the nuclei from the previous step, add 10 ul of trypan blue solution, count and evaluate nuclei purity under a wild-field microscope.
- Distribute 300.000 nuclei per CUT&Tag reaction.
- Critical step. Dilute the nuclei solution (100 - 200 µl) from the previous step with 500 µl of PBS and perform a mild fixation by adding 1.4 µl of 37% fresh formaldehyde.
- Gently rotate for 5 minutes at room temperature (RT).
- Quench the fixation by adding 50 µl of 1.25 M glycine, mix by pipetting and rotate for 5 minutes at RT.
- Centrifuge at 500 x g for 5 minutes at RT and gently remove the supernatant.
- Resuspend the nuclei in 250 µl of PBS. Centrifuge at 500 x g for 5 minutes at RT, remove the supernatant and resuspend the nuclei pellet in 250 µl of Complete CT Wash Buffer 1 at RT.
- Centrifuge for 3 minutes at 500 x g at RT and carefully remove the supernatant without disturbing the nuclei pellet.
- Resuspend the nuclei in 100 µl of Complete CT Wash Buffer 1 per reaction (for scaling on a batch basis, adjust the quantities according to the number of reactions). Mix by pipetting up and down several times.

NOTE: *Before the nuclei binding, it is recommended to count and check the nuclei. The nuclei should be clearly visible, intact without any double-membrane left.*

iDeal CUT&Tag kit for histones

STEP 1 - ConA beads binding

- Centrifuge the resuspended nuclei for 3 minutes at 500 x g at RT and carefully remove the supernatant.
- Add washed ConA beads (as indicated in step 1.5 of the iDeal CUT&Tag guide).

- Place the tube on a rotation wheel for 8 minutes at RT.
- Briefly spin the tube to remove the liquid from the cap. Place the tube on a magnetic rack and wait 2 minutes until the solution is clear and remove the supernatant.

STEP 2 - Cell permeabilization and primary antibody binding

- Remove the tube from the magnetic rack and resuspend the pellet in ice-cold Complete CT Antibody Buffer using 50 µl per reaction.
- Add 1 µg of primary antibody to each reaction tube.
- Place the tubes on a rotating wheel at +4ºC and incubate overnight.

NOTE: *1µg of CTCF, IgG and H3K27me3 antibody has been used for this purpose.*

STEP 3 - Secondary antibody binding

NOTE: *Chose the secondary antibody accordingly to the host species of primary antibody.*

STEP 4 - Targeted tagmentation

STEP 5 - DNA purification

STEP 6 - Library amplification

Follow the manufacturer's instructions for the iDealCUT&Tag kit without any further modifications (Diagenode).

STEP 7 - Library clean-up and QC (optional)

NOTE: *After the recommended clean-up from the iDeal Cut&Tag kit protocol, we performed a second clean-up using the Sera-Mag Select reagent to remove fragments above 3000 bp (right side selection) as follows:*

- Add 0.55x Sera-Mag Select reagent to the library DNA samples. Mix thoroughly by pipetting up and down several times and incubate at RT for 5 minutes.
- Place the tubes on a magnetic rack for 2 minutes or until the beads have fully settled.
- Aspirate and RETAIN the supernatant, discard the beads.
- Add 2.5x of Sera-Mag Select reagent to the supernatant from the previous step to trigger maximum DNA binding.
- Vortex the samples and incubate at RT for 5 minutes.
- Place the tubes on a magnetic rack for 2 minutes or until the beads have fully settled.
- Aspirate and discard the supernatant.
- Wash the beads pellet twice with 200 µl 85% ethanol.
- Dry the beads for 5 - 10 minutes at RT to remove the residual ethanol.
- Add 20 µl of TE buffer, vortex for 30 seconds to resuspend the beads and incubate at RT for 5 minutes.
- Return the tube to the magnetic rack for 2 minutes or until the beads have fully settled.
- Carefully aspirate the supernatant containing the size-selected DNA and transfer to a fresh tube.

- Quantify and verify the libraries (in our case we used the Bioanalyzer and the Qubit devices.
- Perform sequencing (in our case we used a NextSeq 500 device.

Result

To test the iDeal CUT&Tag kit for Histones on extracted nuclei, we first desegregated and isolated single nuclei from our iPSC-derived cortical organoids. As shown in **Figure 1**, we successfully used the Tissue Nuclei Extraction Module to obtain good quality single nuclei. In particular, we obtained 2 million nuclei from five 1-month-old cortical organoids. Secondly, we applied the iDeal CUT&Tag kit to profile two chromatin-interacting components with different DNA binding properties: H3K27me3 and CTCF. Each chromatin profiling assay was performed on 300.000 isolated nuclei from iPSC-derived cortical crganoids. An assay only including IgG was performed as a negative control. All experiments were performed twice.

Using the Bioanalyzer (Agilent) we observed library size distributions between 150 and 2000 bp for H3K27me3 and CTCF, whereas the IgG control showed lower abundance indicating the absence of non-specific cuts (**Figure 2**). The fragment size distribution also exhibited mono-, di-, and tri-nucleosomes traces, especially in the H3K27me3 assays, indicating successful protein A-Tn5 digestion. We sequenced the resulting libraries using an Illumina NextSeq 500 sequencer (2x75 cycles Mid Output). Regarding the bioinformatic analyses, we performed all the processing steps, from alignment of reads to peak calling with the Cut&Run tools 2.0 pipeline (3). This pipeline outputs three sets of peaks using different peak calling algorithms: *MACS2* broad, *MACS2* narrow and *SEACR*. For the H3K27me3 CUT&Tag, we used the peaks obtained with *MACS2* broad, and for the *CTCF*, *MACS2 narrow*. To assess the robustness of our peaks, we downloaded public ChIP-seq datasets from the *CistromeDB* database for H3K27me3 and CTCF in brain tissue or neuronal cells (4). We observed a high overlap with our H3K27me3 peaks (**Table 1**) and an even higher overlap between our CTCF peaks and the public CTCF datasets (**Table 2**). To further validate our CTCF peaks, we performed motif enrichment analysis, using two approaches. First, we conducted de novo motif discovery on the peaks using the *MEME* program, which yielded top enriched motifs closely resembling the canonical CTCF binding motif (**Fig. 3A**). We also tested enrichment of known motifs around the summits of the CTCF peaks using *CentriMo*, which also returned the CTCF motif as the most centrally enriched one (**Fig. 3B**).

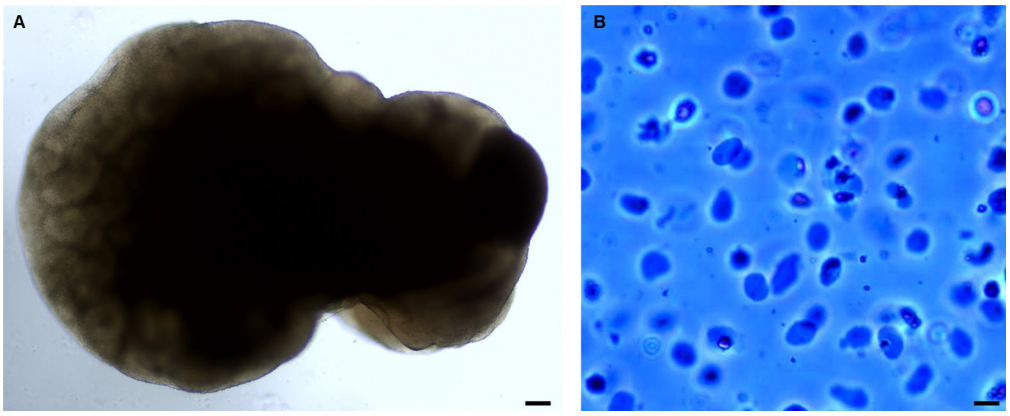

Figure 1. (A) Low-magnification bright-field image of an iPSC-derived cortical organoid. (B) Image of intact isolated nuclei, stained with 0.4% trypan blue. Scale bar 10 µm.

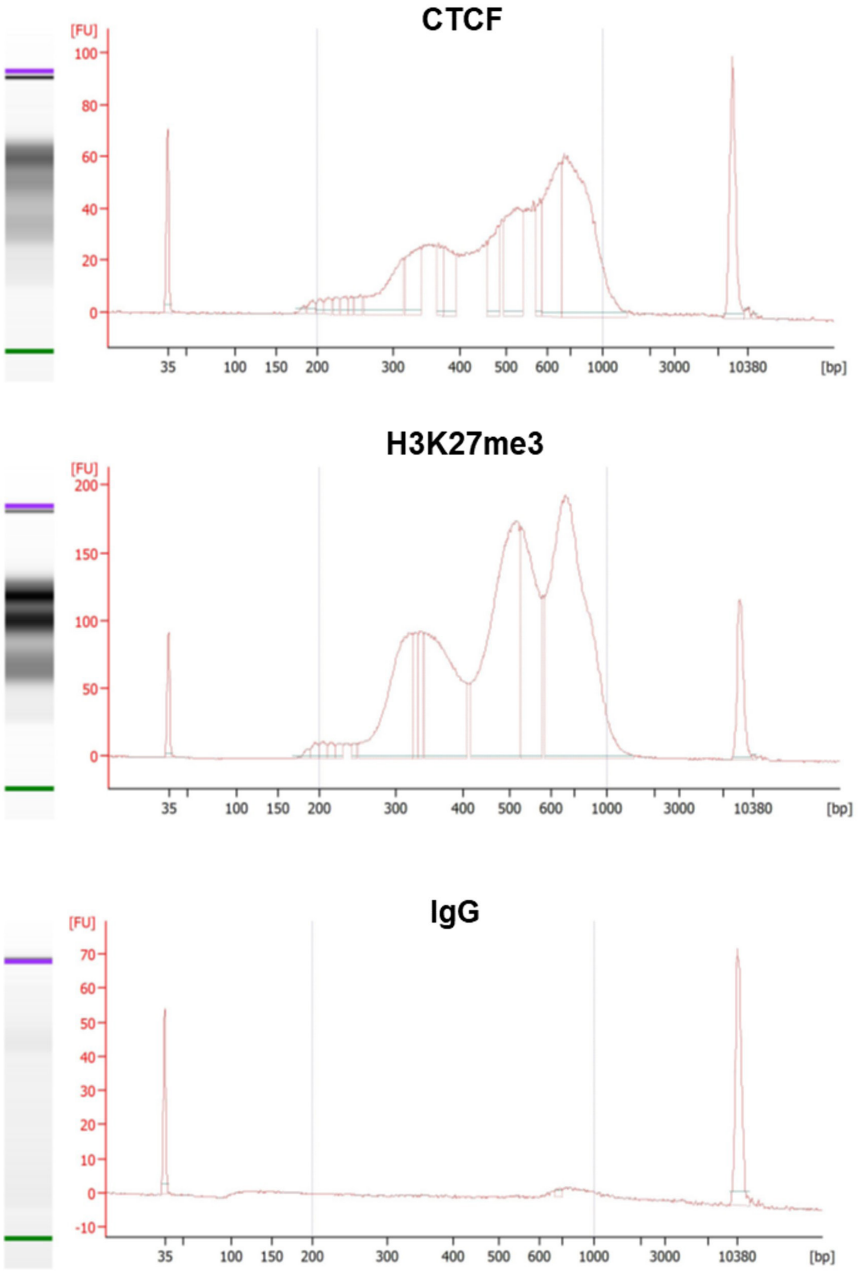

Figure 2. Library size distributions generated by iDeal CUT&Tag protocol using 300.000 isolated nuclei and CTCF (top) and H3K27me3 (middle) primary antibodies and IgG control (bottom).

Public H3K27me3 ChIP-seq datasets				
	Neural progenitors	Fetal brain	Embryonic neurons	Total
H3K27me3 CUT&Tag	3576	6478	15207	33108
Total	5919	42499	57503	

Table 1. Number of intersecting peaks between our H3K27me3 CUT&Tag and several public H3K27me3 ChIP-seq assays, along with the total peaks of each experiment.

Public CTCF ChIP-seq datasets				
	Neuron	Neuroblastoma	Fetal cortex	Total
CTCF CUT&Tag	14914	15856	12915	21621
Total	28652	38706	51159	

Table 2. Number of intersecting peaks between our CTCF CUT&Tag and several public CTCF ChIP-seq datasets.

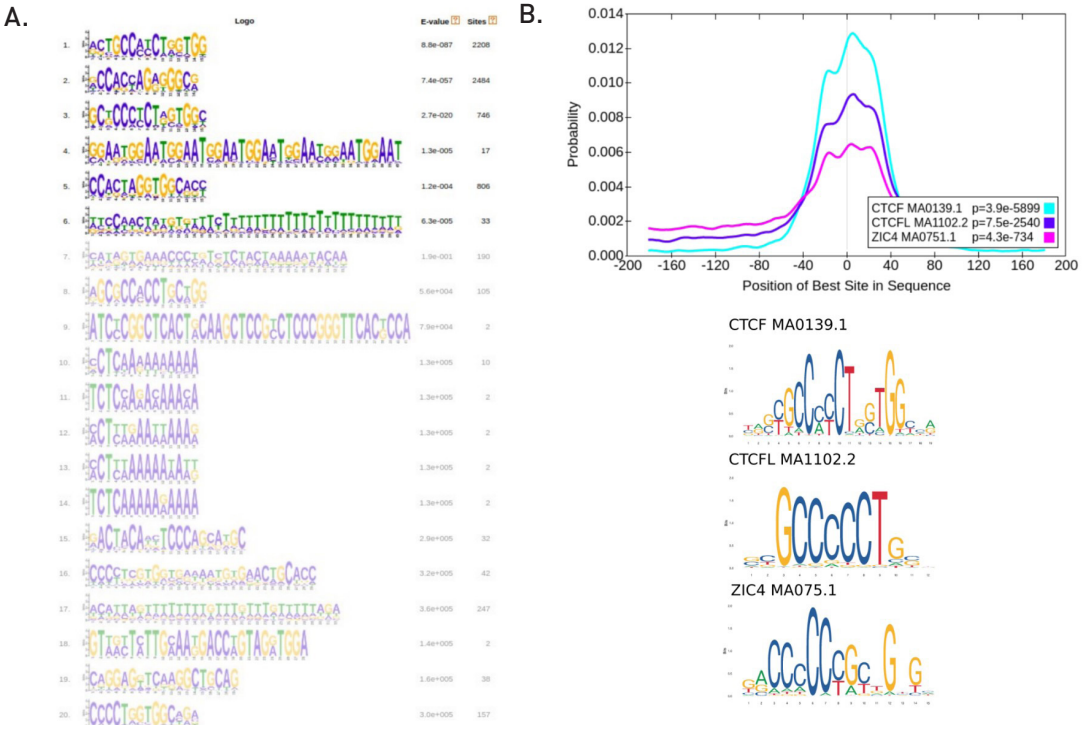

Figure 3. (A) Logos of top 20 most enriched motifs de novo in the CTCF peaks, as found by the MEME program. Faded logos represent motifs that are not significantly enriched. (B) Top 3 motifs from the JASPAR database which are more centrally enriched around the summits of the CTCF peaks. On the top, the distribution of the motifs around the summits of the peaks is displayed, and on the bottom, their sequence logos.

Conclusion

CUT&Tag is a recently developed chromatin profiling method that offers various advantages such as a low starting cell/nuclei number. The method uses the pA-Tn5 enzyme for one-step fragmentation of the DNA and library preparation for sequencing. The method often works with native (or lightly fixed) chromatin from cultured cells. In this application note, we focus on adapting the method to perform CUT&Tag from tissue samples. Our results demonstrate the Tissue Nuclei Extraction Module and the iDeal CUT&Tag kit for Histones can be combined to identify histone modifications and TF binding sites in iPSC-derived cortical brain organoids. This protocol is now established and used routinely in our laboratory, and it could be used as a base for CUT&Tag analysis from tissue samples.

Reference

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(2) X. de Martin, B. Oliva and G. Santpere. Recruitment of homomeric proneuronal factors by conserved CAT-CAT E-Boxes drives major epigenetic reconfiguration in cortical neurogenesis. Biorxiv., (2023)

(3) Yu, F., Sankaran, V. G. & Yuan, G.-C. CUT&RUNTools 2.0: a pipeline for single-cell and bulk-level CUT&RUN and CUT&Tag data analysis. Bioinformatics 38, 252–254 (2021).

(4) Zheng, R. et al. Cistrome Data Browser: expanded datasets and new tools for gene regulatory analysis. Nucleic Acids Res. 47, gky1094- (2018).