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Genome-wide Mapping of Histone Modifications and Transcription Factor Binding Sites in Neuroendocrine Small Cell Lung Cancer Cell Lines Using CUT&RUN

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Abstract

Chromatin remodeling proteins and transcription factors (TFs) play critical roles in the tumor biology of small cell lung cancer (SCLC). Genome-wide characterization of histone post-translational modifications (PTMs) and TF binding sites is essential for identifying regulatory DNA elements and gene pathways that will lead to a deeper mechanistic understanding of SCLC and nominate targets for therapeutic intervention. Cleavage Under Targets and Release Using Nuclease followed by next generation sequencing (CUT&RUN-seq) is a powerful method for mapping specific histone modifications and determining the DNA-binding profiles of a wide range of proteins *in situ* in the cellular genome. In CUT&RUN, the micrococcal nuclease (MNase) fused to Protein A/G is recruited *via* antibodies to the genomic locations of chromatin-associated proteins, where the underlying DNA fragments are released from bulk chromatin upon MNase activation and cleavage. This localized digestion generates small, locus-specific DNA fragments suitable for sequencing.

Here, we present a detailed protocol for profiling histone modifications H3K4me3 (associated with active or open promoters) and H3K4me1 (associated with active enhancers), as well as the transcription factor E2F7, in SCLC. This protocol has been optimized for neuroendocrine (NE) SCLC cell line models, which are typically characterized by large nuclei, scant cytoplasm, and growth as non-adherent aggregates in suspension.

Introduction

Histone post-translational modifications (PTMs) and transcription factors (TFs) are central regulators of gene expression. Histone PTMs, such as acetylation, methylation, phosphorylation, and ubiquitination, primarily on their N-terminal tails influence chromatin structure[1,2]. In cancer, histone PTMs are often dysregulated, leading to aberrant activation of oncogenes or silencing of tumor suppressor genes[3]. TFs bind specific DNA sequences at promoters or enhancers to activate or repress target genes, often recruiting histone-modifying enzymes that coordinate chromatin remodeling[4]. Enzymes that write, erase, or read histone marks, such as histone acetyltransferases (HATs), histone deacetylases (HDACs), and methyltransferases (HMTs), are frequently mutated or dysregulated in cancer, making them attractive therapeutic targets[5].

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In SCLC, epigenetic changes and lineage-defining transcription factors (LDTFs) are key drivers of tumor biology[6]. Unlike genetic mutations, epigenetic alterations, including DNA methylation and histone modifications, regulate gene expression without changing the underlying DNA sequence. These modifications can silence tumor suppressor genes or activate oncogenes, fueling SCLC's aggressive behavior. For example, the histone methyltransferase KMT2D is frequently mutated with an alteration frequency of 12.9% in a large real-world SCLC patient cohort[7] and is responsible for histone H3 lysine 4 mono-methylation (H3K4me1), a mark associated with active enhancer regions in the genome. SCLC is a molecularly heterogeneous disease. SCLC are classified into molecular subtypes based on differential expression of four LDTFs: achaete-scute homologue 1 (ASCL1), neurogenic differentiation factor 1 (NEUROD1), yes-associated protein 1 (YAP1) and POU class 2 homeobox 3 (POU2F3). The neuroendocrine (NE) subtypes, including ASCL1 and NEUROD1, account for approximately 70-80% of SCLC cases[8]. Therefore, investigating the distribution and profiles of different histone PTMs and TF binding sites in the various SCLC subtypes may elucidate subtype-specific gene programs vulnerable to therapeutic intervention.

The traditional method for identifying histone PTMs and the binding sites of sequence-specific regulatory proteins at the genome-wide level is chromatin immunoprecipitation sequencing (ChIP-seq)[9]. However, ChIP-seq requires a large number of input cells and often yields high background across the genome. As a result, ChIP-seq requires a high level of enrichment of target protein to distinguish true signal from noise and needs deep sequencing for effective data analysis. Additionally, formaldehyde crosslinking used in ChIP-seq can mask epitopes and generate false-positive binding sites[10]. Cleavage Under Targets and Release Using Nuclease followed by next-generation sequencing (CUT&RUN-seq) is a high-resolution alternative for mapping histone PTMs and TF binding sites *in situ* within intact cells. In this method, permeabilized cells are incubated with an antibody targeting the chromatin-associated protein of interest, followed by binding of a Protein A(G)-micrococcal nuclease (MNase) fusion protein. Targeted DNA digestion flanking the protein of interest is then induced under high-calcium/low-salt conditions, releasing specific DNA fragments for purification. The purified DNA fragments are used to construct barcoded sequencing libraries, which can be pooled for high-throughput sequencing[11,12,13,14]. Compared to ChIP-seq, CUT&RUN requires significantly fewer cells as input, produces lower background noise, avoids crosslinking artifacts, and requires fewer sequencing reads - making it particularly suitable for studying dynamic chromatin states in cancer in a cost-efficient manner.

CUT&RUN technology has been developed to investigate the genomic locations of the chromatin-interacting proteins, including histone modifications, TF binding sites, and chromatin-associated complexes, thereby revealing epigenetic regulatory landscapes[11,12]. This technique has enabled detailed analyses of enhancer-promoter architecture, transcriptional network regulation, and chromatin state dynamics during development and disease progression[15]. In cancer research, CUT&RUN allows precise mapping of histone marks[16,17] and oncogenic transcription factor occupancy, helping to identify lineage-specific regulatory programs and potential therapeutic targets[15,16]. Furthermore, CUT&RUN can be performed *in situ* in cells, including CD8+ T cell[17], as well as to tissue samples[18,19].

To detect the genomic location of PTMs and transcription factor E2F7 binding sites in SCLC subtypes, we applied CUT&RUN-seq to four SCLC NE cell line models: H146 and DMS79 (ASCL1 subtype), as well as H446 and H82 (NEUROD1 subtype), with or without KMT2D mutations. We profiled the histone mark H3K4me1, an enhancer-associated modification primarily catalyzed by KMT2D, and E2F7, which has been shown to be a cofactor of ASCL1[20]. The histone mark H3K4me3, which is associated with active promoters, served as positive control, while IgG served as negative control.

Here, we present a detailed CUT&RUN-seq protocol for SCLC NE cell lines, based on the following: a commercially available CUT&RUN kit (see **Table of Materials**), the original work of Skene and Henikoff[11,12,13], and our own laboratory optimizations.

Protocol

NOTE: This protocol (**Figure 1**) provides step-by-step instructions for performing CUT&RUN-seq in SCLC NE cell lines, which typically grow as non-adherent aggregates or floating clusters.

1. Cell Culture

1. Maintain cell lines DMS79 (ASCL1 subtype), H146 (ASCL1 subtype), H82 (NEUROD1 subtype), and H446 (NEUROD1 subtype) in 13 mL of RPMI-1640 media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (see **Table of Materials**) in 75 mL flasks. Cultivate the cells in an incubator at 37 °C with 5% CO_2.

   NOTE: All cells used in this study were purchased from ATCC and have been authenticated with short tandem repeat profiling.

2. Cell permeabilization

NOTE: Proper cell permeabilization is essential for enabling antibodies, pAG-MNase, and digested protein-DNA fragments

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to freely diffuse in and out of the cells. Before starting the CUT&RUN protocol on a new cell line, optimizations should be performed to determine the ideal digitonin concentration for effective permeabilization. The lowest concentration to achieve more than 95% permeabilization (95% of cells "dead") should be used for the CUT&RUN protocol.

1. Place the 5% digitonin stock, protease inhibitor, and spermidine on ice and allow them to thaw completely.
2. Prepare the Wash Buffer according to **Table 1** and keep the buffer on ice .
3. Prepare a 0.05% digitonin working solution by mixing 10 µL of the 5% stock with 990 µL of Wash Buffer. Mix thoroughly by vortexing and keep on ice.
4. Generate a dilution series of digitonin (0.01%, 0.001%, and 0.0001%) by sequentially diluting the previous solution to a final volume of 1 mL of each tube. Vortex each dilution before proceeding to the next. At the same time, prepare a 0.05% Dimethyl Sulfoxide (DMSO) control in Wash Buffer (100 µL total; see **Table 2**).

   NOTE: Digitonin and DMSO are hazardous chemicals. Handle with caution and discard all waste in a properly labeled hazardous-waste container with secure lids.
5. Keep all digitonin dilution solutions and the DMSO control on ice until they are used.
6. Determine the number of cells needed by multiplying the number of permeabilization conditions by the number of cells required per test. Include an additional 20% to compensate for pipetting variation (e.g., for five conditions at 5×10^5 cells each, prepare approximately 5.2 x 5 $x10^5$ cells).
7. Collect the required number of cultured cells by centrifugation at 600 × *g* for 3 min at room temperature (RT). Resuspend the pellet in 600 µL of 1× PBS (100 µL per condition plus 20% extra).
8. Aliquot 100 µL of the PBS-resuspended cells into five 1.5 mL microcentrifuge tubes- one tube for each digitonin concentration and one for the DMSO control.
9. Centrifuge the tubes at 600 × *g* for 3 min at RT.
10. Carefully remove the supernatant and resuspend each pellet in 50 µL of the corresponding digitonin or DMSO control buffer.
11. Incubate the samples at RT for 10 min.
12. Mix 10 µL of the treated cells with 10 µL of trypan blue and load 10 uL of the mixture onto a cell counting slide.
13. Use a cell counter to quantify viable cells (unstained) and non-viable, permeabilized positive cells (stained blue).
14. Identify the lowest digitonin concentration that produces the highest proportion of trypan blue positive cells. Use this concentration for the downstream CUT&RUN experiment. Confirm that the DMSO control maintains viability typical of a normal healthy cell culture.

3. CUT&RUN seq

NOTE: The reagents in this protocol are scaled for 5 reactions, with extra added to account for pipetting loss. For a list of materials and equipment, see **Table of Materials**. At least two independent biological replicates are recommended for each CUT&RUN experiment.

1. Day 1
   1. CUT&RUN buffer prep
      1. Prepare the Wash buffer by combining Pre-Wash Buffer, 25x Protease Inhibitor, and 1 M Spermidine as outlined in **Table 3**.
      2. Prepare the Cell Permeabilization Buffer by adding 5% Digitonin to the Wash Buffer according to **Table 3**.
      3. Prepare the Antibody Buffer by adding 0.4 µL of EDTA to 100 µL of Cell Permeabilization Buffer with the appropriate digitonin concentration, as described in **Table 3**. Place the prepared buffer on ice.
      4. Store the remaining Cell Permeabilization Buffer at 4 °C for use on Day 2.
   2. Concanavalin A (ConA) beads activation
      1. Gently resuspend ConA beads suspension to ensure its even distribution.
      2. Transfer 55 µL of the beads suspension (11 µL per sample) into a 1.5 mL microcentrifuge tube.
      3. Place tube on a 1.5 mL magnetic rack until the beads have fully separated, then pipette to remove supernatant.
      4. Immediately wash the beads twice with 500 µL of ice-cold Bead Activation Buffer (100 µL per sample). Resuspend the beads by gentle pipetting for each wash and use the magnetic rack to discard supernatant between washes.
      5. After the final wash, remove the supernatant and gently resuspend the activated beads in 55 µL (11 µL per sample) of ice-cold Bead Activation Buffer.
      6. Aliquot 10 µL of the beads suspension into each tube of an 8-strip tube set and keep the tubes on ice.

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3. Harvest cells and bind cells to activated beads

NOTE: Cell viability is important for the signal/background ratio. However, the viability of SCLC NE cell lines can be variable (70%-90%) due to inherently high rate of cell turnover and preference to grow as medium-large aggregates in suspension.

1. Remove the cells from the 37 °C incubator and examine them under a microscope to ensure quality.
2. Transfer the cell suspension to a 50 mL conical tube and centrifuge at 1,000-1,200 × *g* for 5 min at RT.
3. Aspirate the medium from the conical tube and rinse the cell pellet with 20 mL of 1× PBS.
4. Centrifuge again, aspirate the PBS, and digest the pellet with Accutase for 3-5 min at 37°C, based on the size of the pellet.
5. Terminate the digestion by adding 5 mL of PBS + 1% FBS. Obtain the single-cell suspension by gently pipetting up and down with a serological pipette.
6. Centrifuge the cells at 1,000-1,200 × *g* for 5 min, discard the supernatant, and resuspend the cell pellet in 5 mL of PBS.
7. Count starting cells, confirm cell viability and integrity.
8. Calculate the total number of cells needed by multiplying the number of reactions by the number of cells per reaction (5×10^5). Add 20% excess to account for pipetting errors (e.g., $5.2 \times 5\times10^5$ for 6 reactions). Centrifuge at 600 x *g*, 3 min at RT.

   NOTE: Prepare at least one additional reaction as a backup.
9. Resuspend the cells in RT Wash Buffer at a density of 5×10^5 cells per reaction (six reactions total), using 100 µL per reaction, gently but thorough pipetting to ensure a uniform suspension. Transfer the cell suspensions to 1.5 mL tubes. Centrifuge at 600 x *g* for 3 min at RT. Pipette to remove supernatant.
10. Repeat the wash step (Step 9) once more.
11. Aliquot 100 µL of washed cells to each tube in the 8-strip set containing 10 µL of activated CoA beads. Gently pipette to mix and quickly spin in a mini centrifuge to collect the slurry (beads should not settle).
12. Incubate bead-cell slurry for 10 min at RT to allow cells to bind to the beads.
13. Place tubes on a 0.2 mL tubes magnet rack, allow the slurry to clear, and pipette to remove supernatant. Save 10 µL of supernatant to confirm cells are bound to beads. Discard the remaining supernatant and move quickly to the next step.
14. Remove tubes from the magnet. Immediately add 50 µL of ice-cold Antibody Buffer to each reaction and gently pipette to resuspend the beads.
15. Mix 10 µL of both the supernatant and 10 µL of the cell/bead suspension (resuspended in Antibody Buffer) with 10 µL of trypan blue and load the mixture onto the cell counter slide.
16. Analyze with a cell counter. Confirm the supernatant contains almost no beads or cells, and the bead/cell mixture contains permeabilized cells surrounded by beads.

    NOTE: It is important to check the Cells CoA beads binding compatibility.

4. Permeabilization and antibody binding

NOTE: The success of CUT&RUN critically depends on the antibody's affinity for its target and its specificity under specific experimental conditions. We recommend the following: 1. Use commercial antibodies that have been validated for CUT&RUN when available. 2. For targets without CUT&RUN-validated antibodies, consider those validated for Immunofluorescence (IF), as IF antibodies recognize epitopes in their native cellular context, similar to in situ binding of antibody to target within permeabilized cells in CUT&RUN. 3. Test 3-5 antibodies in parallel to identify the most effective one for your specific application.

1. Quick spin the K-MetStat Panel and pipette to resuspend. Add 2 µL of K-MetStat Panel to reactions designated for H3K4me3 positive & IgG negative control antibodies. Pipette to mix and quick spin tubes (Optional).
2. Add 0.5 µg each of H3K4me3, H3K4me1, and IgG antibodies, and 0.6 µg of E2F7 polyclonal antibody from different sources to each reaction (**Table 4**). Label the 8-strip tubes.
3. Gently pipette to properly mix each reaction tube. Incubate overnight on a nutator at 4 °C with tube caps elevated. Do not rotate or invert the tubes.

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2. Day 2

    1. Binding of pAG-MNase to antibody

    NOTE: Beads settling overnight is normal and does not affect the results. If the beads become clumpy or sticky after overnight incubation, resuspend the beads by gentle pipetting. To improve consistency and throughput, multi-channel pipetting is recommended. When using 8-strip tubes, remove and replace buffers one strip at a time to prevent ConA beads from drying out.

    1. Put the magnet stand on ice.
    2. Remove the 8-strip tubes from 4 ˚C incubation and quick spin to collect liquid.
    3. Place tubes on the magnet stand until the slurry clears. Carefully pipette and remove the supernatant.
    4. Keep tubes on the magnet stand. Wash tubes twice with 200 µL of cold Cell Permeabilization Buffer. Pipette to remove supernatant.
    5. Remove tubes from the magnet stand and immediately add 50 µL of cold Cell Permeabilization Buffer to each reaction. Gently pipetting to resuspend cells/beads.
    6. Add 2.5 µL of pAG-MNase to each reaction and mix well by gently pipetting.
    7. Incubate reactions for 10 min at RT.
    8. Quick spin the tubes, place them back on the magnet stand, until the slurry clears. Pipette to remove supernatant.
    9. Keeping tubes on magnet stand, wash tubes twice with 200 µL of cold Cell Permeabilization Buffer. Pipette to remove supernatant.
    10. Remove tubes from the magnet stand, gently resuspend in 50 µL of cold Cell Permeabilization Buffer. Place the tubes on ice.

    2. Targeted chromatin digestion and release

    NOTE: Perform Ca^{2+}-dependent digestion at 0 °C to minimize unintended MNase cleavage of accessible DNA[12]. Include spike-in DNA, such as *Escherichia coli (E. coli)*, so that it represents approximately 0.5-5% (ideally ~1%) of total sequencing reads[13]. Spike-in DNA is used to normalize sequencing depth and is particularly important when comparing samples across a series, for example, when evaluating the global histone modification changes after inhibition or genetic deletion of histone modifying enzymes.

    1. Add 1 µL of 100 mM Calcium Chloride to each reaction in the 8-tube strip (from step 3.2.1.10) while on ice. Gently pipette to fully resuspend beads and ensure efficient digestion.
    2. Incubate the tubes on a nutator at 4 °C for 2 h, with the capped ends elevated.
    3. In a new 1.5 mL tube, prepare the Stop Buffer Mix. For each reaction, combine 33 µL of Stop Buffer with 1 µL of *E. coli* spike-in DNA (0.5 ng). Gently vortex to mix.
    4. Add 33 µL of stop buffer to each reaction at the end of the 2 h incubation. Mix by gentle pipetting.
    5. Incubate the reactions in a thermocycler at 37 °C for 10 min.
    6. Quick spin tubes to collect the contents at the bottom, then place them on a magnet rack until the solution becomes clear.
    7. Carefully transfer approximately 85 µL supernatant which contains the CUT&RUN-released DNA into fresh 1.5 mL tubes.

3. DNA purification using the DNA Purification Kit (see **Table of Materials**)

    NOTE: Before first use, add 6.9 mL isopropanol to the DNA Binding Buffer and add 20 mL ≥ 95% ethanol to the DNA Wash Buffer.

    1. Add 420 µL of DNA Binding Buffer to each collected supernatant. Vortex briefly to mix thoroughly.
    2. Place a DNA Cleanup column into its collection tubes.
    3. Load each sample onto the corresponding column.
    4. Centrifuge at 16,000 × *g* for 30 s at RT. Discard the flow-through and return the column to the collection tube.
    5. Add 200 µL of DNA wash buffer to each column and centrifuge at 16,000 x *g* for 30 s at RT. Discard the flow-through and replace the column in the collection tube.
    6. Repeat the washing step once.
    7. Centrifuge again at 16,000 x *g* for 30 s at RT to fully remove residual wash buffer.
    8. Transfer columns to labeled 1.5 mL tubes.

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9. Apply 12 µL of Elution Buffer directly to the membrane at the center of the column.
10. Incubate for 5 min at RT and then centrifuge at 16,000 × *g* for 1 min at RT to elute the DNA.

NOTE: This is a safe stopping point. Store eluted DNA at -20 ˚C for future processing.

4. CUT&RUN DNA quantification and fragment size distribution analysis

NOTE: Quantify DNA using a Fluorometer with the dsDNA High Sensitivity assay Kit, which provides more accurate measurements of double-stranded DNA than absorbance-based methods.

1. Use 1 µL of each sample to determine CUT&RUN DNA concentration using the dsDNA High Sensitivity Assay Kit (see **Table of Materials**).
2. Use an additional 1 µL of DNA to assess the presence and size distribution of cleaved fragments. Perform fragment analysis using the High Sensitivity DNA Kit (see **Table of Materials**) following the manufacturer's guidelines (optional).

NOTE: Highly abundant binding proteins, such as histone post-translational modifications (PTMs), can be readily detected at this step. However, low-abundance transcription factors may be more difficult to detect.

5. Library construction, fragment analysis, and sequencing

NOTE: ≤5 ng total CUT&RUN DNA, as determined by dsDNA High Sensitivity Assay Kit quantification, was used for CUT&RUN next-generation sequencing (NGS) libraries' construction. To minimize adapter dimers, reduce the Adapter from Illumina to 1 µL for the adapter ligation. The number of PCR cycles during library construction can be adjusted based on input amount, library yield, and duplication rate. Different DNA purification Magnetic Beads (see **Table of Materials**) size selection strategies may be used depending on the target-histone PTMs or transcription factors.

1. Library construction was built by using the CUT&RUN library prep kit (see **Table of Materials**) according to the kit manufacturer's instructions, with small modifications. In brief, 5 ng of CUT&RUN DNA was used as the recommended input for library preparation. For DNA less than 5 ng, use as much as possible for library prep. Dual i5 & i7 index primers were used for each sample to ensure successful multiplexed sequencing.

NOTE: This is a safe stopping point. The DNA can be stored at -20˚C for further processing.

2. Measure the concentration of CUT&RUN library with a Fluorometer and the dsDNA High Sensitivity Assay Kit. The size distribution of the CUT&RUN NGS libraries was determined using the High Sensitivity DNA Kit (see **Table of Materials**) based on the High Sensitivity DNA Kit Guide or a comparable capillary electrophoresis instrument TapeStation (see **Table of Materials**).

NOTE: Dilute the CUT&RUN library to bring the concentration within the detection range of the dsDNA High Sensitivity Assay Kit and the High Sensitivity DNA Kit.

3. Pool libraries (40-46) based on equal molecular and unique indexes. After DNA purification Magnetic Beads size selection, Paired-end sequencing (100 bp read length) for the pooled libraries was performed on an Illumina NovaSeq 6000 system for half S-Prime (SP) at a read depth of estimated 10 million reads per library.

6. CUT&RUN data analysis

1. Analyze CUT&RUN data using a custom pipeline (**Figure 5A**). Assess read quality with FastQC. Remove adapter sequences from the start of the reads using Cutadapt. Align paired-end reads to the human genome (hg38) and the *E. coli* reference genome with Bowtie2. Use Samtools to remove unmapped reads. Remove duplicate reads with Picard, and generate bedGraph files using Bedtools. Create bigWig (bw) files with DeepTools. Visualize the data using IGV and DeepTools. Normalize total read counts to the spike-in *E. coli* read counts. Perform peak calling using SEACR (Sparse Enrichment Analysis for CUT&RUN)[19].

Representative Results

Typical or "classic" SCLC NE cell lines are characterized by large nuclei, scant cytoplasm, and growth as non-adherent aggregates or compact spheroids in suspension. ASCL1-high cell lines, such as NCI-H146 and DMS79, and NEUROD1-high cell lines, such as NCI-H82 and H446, exhibit this classic SCLC phenotype: small-to-medium sized cells with scant cytoplasm, finely granular chromatin, and a tendency to grow predominantly as non-

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adherent aggregates or compact spheroids. Although NEUROD1-high models retain NE marker expression, they are enriched for transcriptional programs related to neuronal differentiation, cytoskeletal remodeling, and cell migration. For instance, NCI-H82 grows in large, loosely associated suspension aggregates, while H446 displays mixed morphology-approximately 80% adherent cells and 20% in suspension-highlighting both stem-like features and phenotypic plasticity (**Figure 2**).

Using Agilent chip-based fragment analysis, we successfully detected CUT&RUN DNA for histone marks H3K4me3 and H3K4me1, whereas detection was more challenging for the lower-abundance transcription factor E2F7, whose expression is also likely cell cycle-dependent. CUT&RUN DNA yield and size distribution are summarized in **Figure 3**. For CUT&RUN DNA (**Figure 3A**), the enhancer histone mark H3K4me1 yielded the highest amounts: 175.34 ng in DMS79, 34.70 ng in H146, 61.89 ng in H82, and 125.16 ng in H446. The positive control, H3K4me3, showed moderate yields: 22.18 ng in DMS79, 5.44 ng in H146, 7.54 ng in H82, and 22.82 ng in H446. In contrast, the transcription factor E2F7 yielded low DNA amounts (average 4.13 ng), close to the IgG negative control (average 3.31 ng). Furthermore, CUT&RUN DNA distribution patterns for H3K4me3 and H3K4me1 displayed peak sizes characteristic for mono-nucleosome, di-nucleosome, and genomic DNA (**Figure 3B**). The mono-nucleosome sizes were 167 bp for H3K4me3 and 159 bp for H3K4me1. In contrast, no distinct peaks were detected for E2F7 or the IgG control. Therefore, accurate quantification of CUT&RUN DNA using dsDNA High Sensitivity (HS) Assay Kit is a critical step prior to library preparation.

After quantification of CUT&RUN DNA yield, we proceeded with NGS library construction (**Figure 4**). Despite low DNA yields for E2F7 and IgG (<5 ng), we successfully constructed NGS libraries from these inputs. The average NGS library yields were: 352 ng for H3K4me3, 344 ng for H3K4me1, 327.10 ng for E2F7 (Protein tech antibody), 284.93 ng for E2F7 (Novus Biologicals), and 360 ng for IgG control (**Figure 4A**). Library size distributions are shown in **Figure 4B**: H3K4me3 peaks at ~330 bp, H3K4me1 at ~319 bp, E2F7 at ~244 bp, and IgG at ~240 bp.

CUT&RUN NGS libraries were analyzed using a customized bioinformatics pipeline (**Figure 5A**). Read quality was assessed with FastQC, and adapter sequences were trimmed using Cutadapt. Raw reads were aligned to both the human genome (hg38) and the *E. coli* reference genome using Bowtie2. Samtools was used to filter out unmapped reads, and duplicates were removed with Picard. Bedtools was employed to generate bedGraph files, while DeepTools was used to create bigWig (bw) files, metaplots, and heatmaps. Data visualization was performed using IGV and DeepTools. Spike-in *E. coli* DNA was used for normalization. On average, around 10 million reads were obtained per sample.

The average unique reads of each sample were 7,940,194, ranging from 4,697,841 to 11,570,589. We generated example heatmaps showing the signal intensities of H3K4me3, H3K4me1, E2F7, and IgG across genes aligned to transcription start sites (TSS) in DMS79 (KMT2D wild type, WT) and H146 (KMT2D heterozygous mutant, HET) (**Figure 5B**). As expected, H3K4me3 and E2F7 signals were enriched near the TSS (within ±3 kb), while H3K4me1 showed a broader distribution-both proximal to and beyond 3 kb from the TSS. In KMT2D-HET cell line (H146), the signal intensity of both H3K4me3 and H3K4me1 was reduced compared to KMT2D-WT cell line (DMS79). KMT2D is a key regulator of the enhancer mark H3K4me1 and influences the promoter mark H3K4me3. Consistent with previously reported decreases in KMT2D protein expression[21], loss of a single functional KMT2D allele (DMS79 > H146) resulted in reduced levels of H3K4me1 and to a lesser extent H3K4me3. Additionally, E2F7 binding signal was over twenty-fold lower than that of the histone marks and was close to background levels seen in IgG Controls**.** We attributed this result to the possibility that E2F7 protein expression has been shown to be cell-cycle dependent (e.g., peaking at G_2/M), and that our SCLC cells were not synchronized prior to CUT&RUN-seq.

Finally, we visualized the binding profiles of H3K4me3, H3K4me1, E2F7, and control IgG in a randomly selected genomic region containing multiple genes (Chr20:33,235,071-34,477,922) as well as specifically at the *E2F1* promoter (**Figure 6**). We chose to interrogate binding profiles of various histone PTMs and E2F7 at the *E2F1* promoter because E2F1 expression is high in SCLC and is a well-validated direct E2F7 gene target. **Figure 6A** demonstrated the distribution of unique binding sites (seen as well-defined, sharp peaks) for H3K4me3 (promoter), H3K4me1 (enhancer), and E2F7 throughout the randomly selected chromatin region; importantly, the negative control IgG showed no specific binding, as evidenced by lack of peaks called, throughout the same region. **Figure 6B** shows the specific binding pattern of each antibody at the *E2F1* gene locus. While H3K4me3 and E2F7 binding sites are enriched around the 5' or promoter region, H3K4me1 binding sites are more broadly distributed and detected within the gene body. Finally, no peaks were identified in the negative IgG control sample.

The buffers listed in the tables are prepared according to the recommendations of the commercially available CUT&RUN kit and our own laboratory optimizations.

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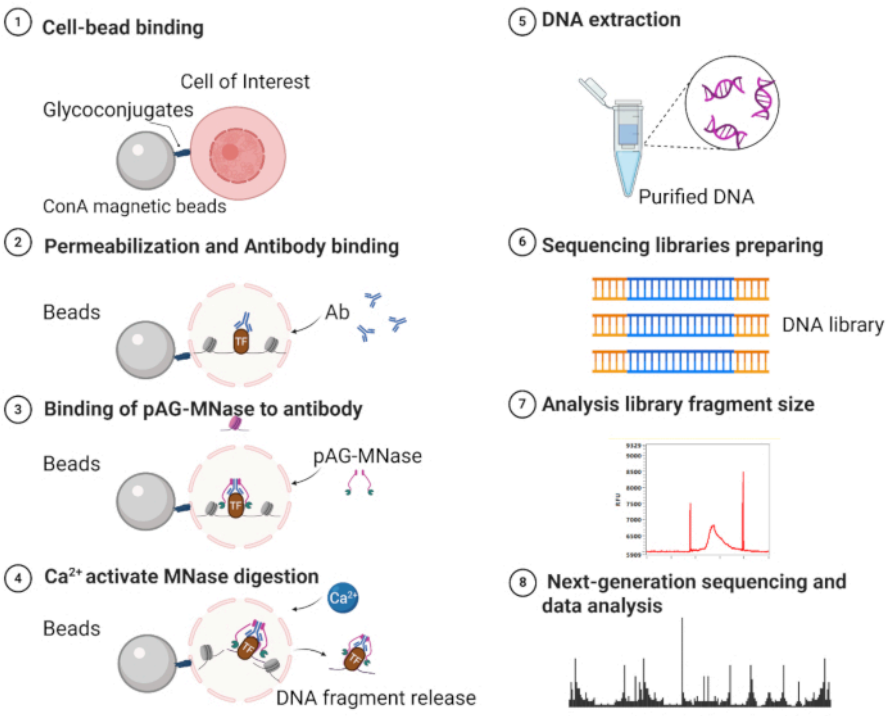

Figure 1: Schematic of the CUT&RUN protocol. Clustered cells are harvested and dissociated into single cells using Accutase. The individual cells are then bound to ConA-coated magnetic beads, permeabilized with an optimized concentration of digitonin, and sequentially incubated with a primary antibody targeting the protein of interest, followed by pA/G-MNase. Upon the addition of calcium, MNase is activated and cleaves the DNA near the bound protein (CUT). The resulting targeted DNA fragments are released into the supernatant[12] and subsequently purified for library preparation. This figure was created with BioRender.com. Please click here to view a larger version of this figure.

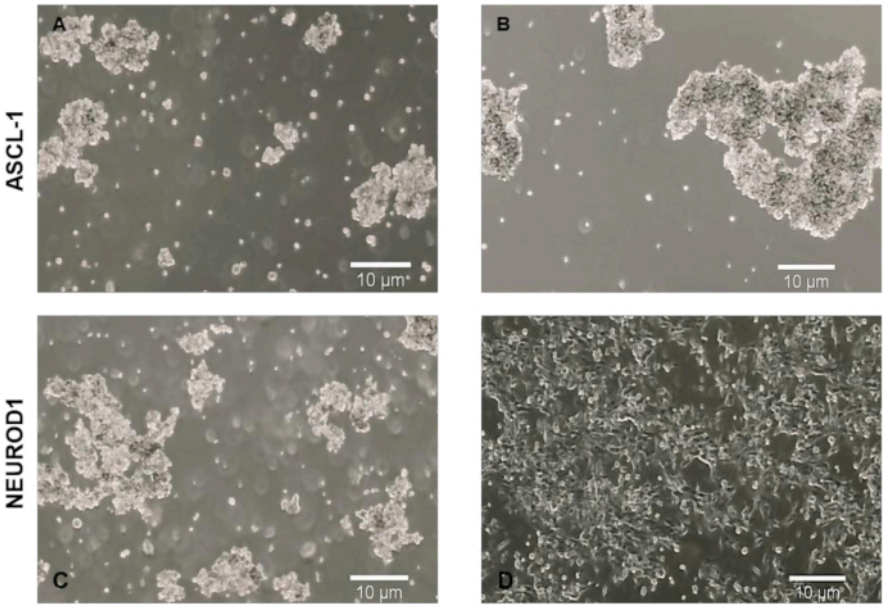

Figure 2: Cell morphology of NE SCLC cell lines representing the ASCL1 and NEUROD1 subtypes. DMS79, H146, and H82 grow as non-adherent aggregates or compact spheroids in suspension (**A**-**C**, respectively). H446 displays mixed morphology, with approximately 80% adherent cells and 20% in suspension (**D**). Please click here to view a larger version of this figure.

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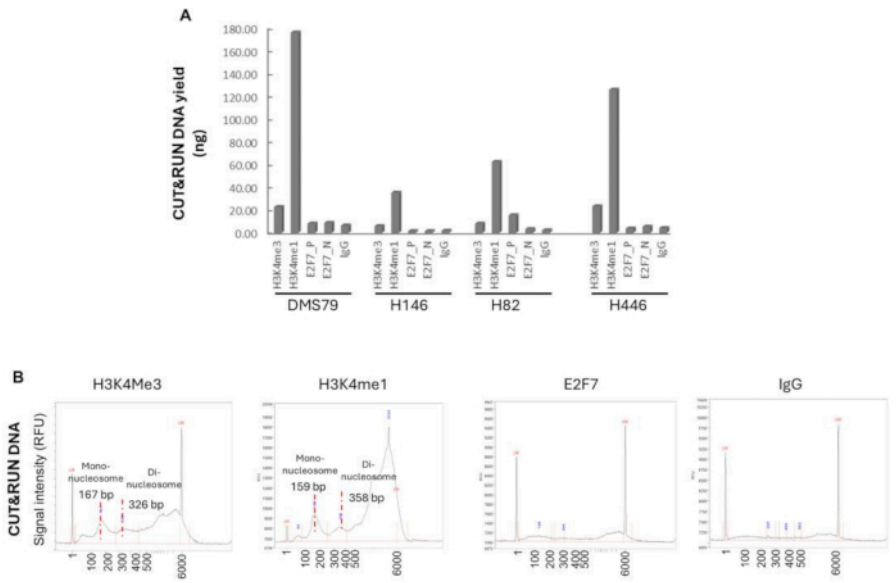

Figure 3: Yield and size distribution of CUT&RUN DNA. (**A**) Yield of CUT&RUN DNA for 0.5 million input cells, showing higher yields for histone marks than for the transcription factor E2F7. The enhancer mark H3K4me1 yielded more DNA than the promoter mark H3K4me3. The DNA yield for E2F7 was slightly higher than the IgG negative control. The yield varied across cell lines. (**B**) Fragment distribution of CUT&RUN DNA showing the typical pattern of mono-nucleosome, di-nucleosome, and genomic DNA fragments. Please click here to view a larger version of this figure.

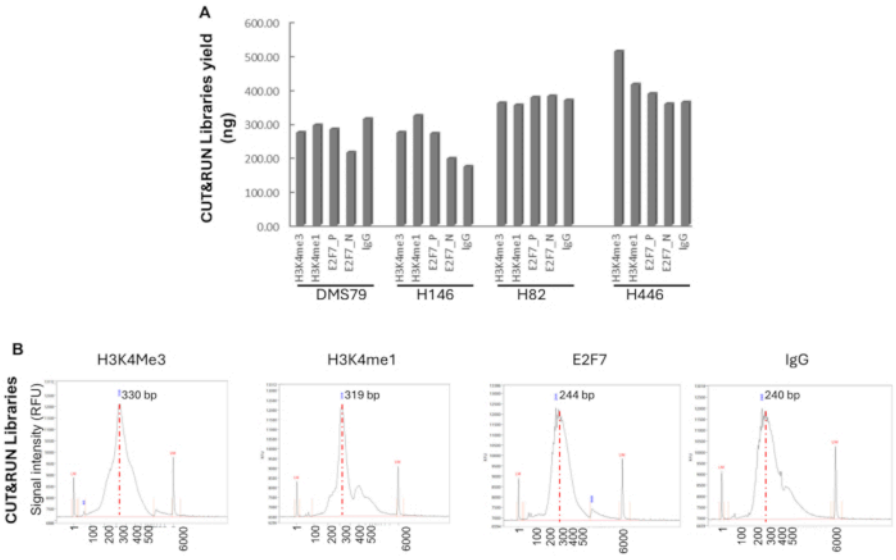

Figure 4: Yield and size distribution of CUT&RUN NGS libraries. (**A**) Yield of CUT&RUN NGS libraries showing consistent library yields across different CUT&RUN targets when using the same amount input DNA (≤5 ng). (**B**) Fragment distribution of CUT&RUN NGS libraries showing a representative sample, where the fragment size corresponds to a single nucleosome plus adaptor sequences. The library size for histone marks (peaks at 330 bp and 319bp) was larger than that for the transcription factor E2F7*(peak at 244 bp)*.Please click here to view a larger version of this figure.

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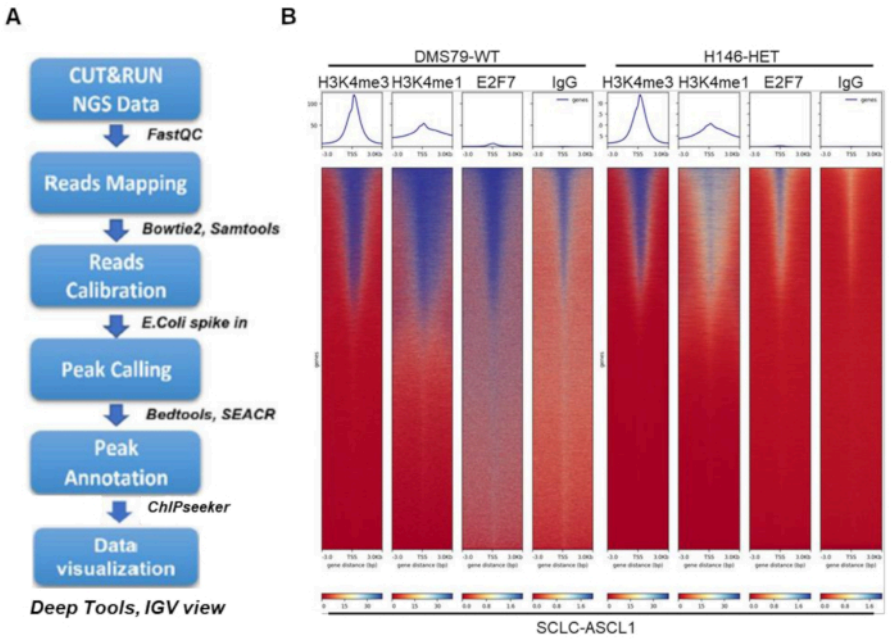

Figure 5: Example of Heatmap and Metaplot visualization of CUT&RUN signal intensity in SCLC cell lines. (**A**) Bioinformatics workflow for data processing and analysis. (**B**) Heatmaps of DMS79 (KMT2D WT) and H146 (KMT2D HET) SCLC cell lines showing enrichment of H3K4me3 and E2F7 near the TSS (within ±3 kb) and a broader distribution for H3K4me1. Signal intensity of H3K4me3 and H3K4me1 was reduced in H146 compared with DMS79. H3K4me3 marks active promoters, H3K4me1 marks enhancers. E2F7 is a transcription factor. IgG served as a negative control. Each row represents a gene, and each column represents a genomic position relative to the TSS. Please click here to view a larger version of this figure.

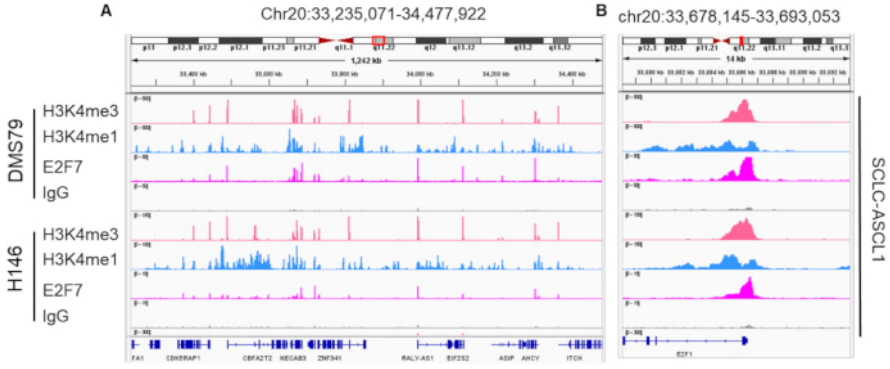

Figure 6: Example of IGV visualization of CUT&RUN-seq peaks in SCLC cell lines. (**A**) Genome browser tracks showing H3K4me3, H3K4me1, E2F7, and IgG binding at Chr20:33,235,071-34,477,922 in DMS79 and H146 cells. Distinct peaks represent specific binding sites for H3K4me3 (promoter), H3K4me1 (enhancer), and E2F7, while IgG shows no enrichment. (**B**) Binding pattern at the *E2F1* promoter illustrate that H3K4me3 and E2F7 peaks are enriched near the 5' promoter region, whereas H3K4me1 signals are broader and extend into the gene body. No peaks were detected in the IgG control. Please click here to view a larger version of this figure.

Wash buffer	Final	Volume
Pre-wash buffer	-	5000 uL
25x Protease inhibitor	1x	200 uL
1M Spermidine	0.5mM	2.5 uL

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Dissolve 1 protease inhibitor tablet (Roche) in 2 mL MQ bi-distillated water (25x)

Table 1: Buffer preparation for serial dilution of 5% digitonin

Final Conc.	Wash Buffer	Digitonin	DMSO
0.05%	990 uL	10 uL	
0.01%	800 uL	200 uL	
0.001%	900 uL	100 uL	
0.0001%	900 uL	100 uL	
0.05%	95 uL		5 uL

Table 2: Serial dilution of 5% digitonin.

Wash Buffer (RT)	**Final**	**Per Reaction**	**5 Reactions**
Pre-Wash Buffer	-	1.8 mL	9.0 mL
25x Protease Inhibitor	1x	72 uL	360 uL
1M Spermidine	0.5mM	0.9 µL	4.5 µL
Cell Permeabilization Buffer 4°C	**Final**	**Per Reaction**	**5 Reactions**
Wash Buffer	-	1.4 mL	7.0 mL
5% Digitonin	0.01%	2.8 µL	14.0 µL
Antibody Buffer (on ice) Prepare fresh	**Final**	**Per Reaction**	**5 Reactions**
Cell Permeabilization Buffer	-	100 µL	500 µL
0.5 M EDTA	2 mM	0.4 uL	2.0 uL

Table 3: Buffer preparation.

Antibody	Company	Recommendation	Volume
H3K4me3	EpiCypher 13-0041	0.5 ug	1 uL

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H3K4me1	EpiCypher 13-0057	0.5 ug	1 uL
Rabbit IgG Negative Control	EpiCypher 13-0042	0.5 ug	1 uL
E2F7 Polyclonal Antibody	Novus biologicals # NBP2-92216	0.6 ug	0.28 uL
E2F7 Polyclonal Antibody	Proteintech catalog # 24489-1-AP	0.6 ug	0.92 uL

Table 4: Antibody specifics.

Discussion

Epigenetic dysregulation and LDTFs are key drivers of SCLC tumor biology[6]. Determining the genomic distribution of specific histone modifications and DNA-binding proteins in SCLC can reveal potential mechanisms of acquired therapeutic resistance and phenotypic plasticity. CUT&RUN-seq is a powerful method for mapping histone and non-histone protein-DNA interactions at high resolution[14] from cells, tissues. This protocol presents an effective and low-cost alternative to traditional ChIP-seq approaches in SCLC NE cell lines to identify new gene pathways suitable for treatment intervention.

CUT&RUN-seq typically produces lower background noise compared to ChIP-seq. However, background signals can still occur and negatively impact peak calling during data analysis. To minimize background reads, it is important to start with cells that have optimal viability. This is especially critical for SCLC NE cell lines, which tend to have more variable viability because of their growth characteristics compared to adherent cell lines. We recommend handling samples gently during the CUT&RUN protocol. Use pipettes, not vertexing, even gently, to resuspend cells. This helps prevent cell shearing, detachment from beads, and antibody denaturation.

Efficient CUT&RUN also requires adequate cell membrane permeabilization to allow antibodies, MNase, and released DNA fragments to move freely across the cell membrane. Digitonin is commonly used for this purpose. Insufficient digitonin results in poor permeabilization, limiting reagent entry and fragment diffusion, whereas excessive digitonin can disrupt membrane integrity and lead to premature cell lysis. Therefore, it is recommended to use the lowest concentration that provides effective permeabilization while maintaining cell viability[14,19].

The success of CUT&RUN largely depends on the affinity and specificity of the antibody for the target protein under the assay conditions. If no CUT&RUN validated antibodies are available for purchase against a protein of interest, we recommend using antibodies that are both suitable for immunofluorescence and demonstrate specificity in immunoblotting. Several target antibodies from different vendors should always be tested in a pilot CUT&RUN experiment to identify which of them can be further used for a larger number of samples. For proteins which are expected to produce low/weak signal (e.g., TFs with oscillating expression throughout the cell cycle), consider increasing the total number of input cells[14], in addition to optimizing conditions for digitonin permeabilization and antibody binding/concentration. In summary, the yield of CUT&RUN DNA (**Figure 3A**) depends on the amount of total (viable) cells used for initial input, intracellular abundance of the target protein at a given point in time, as well as the frequency and durability of target protein-DNA interactions in the cells. As shown in the Results section, the DNA yield of histone marks (H3K4me1 and H3K4me3) is >20-40-fold higher than that of E2F7. This is likely due to the sequence-specific, non-constitutive and/or context-dependent binding of TFs to gene promoters, whereas the histone marks profiled represent modifications that occur genome-wide. Even though we observed a wide range of yields for CUT&RUN DNA, we successfully constructed sequencing libraries from all samples. Although the fragment size distribution of prepared libraries was similar between E2F7 (244 bp) and IgG (240 bp), E2F7 binding occurred and was specific as demonstrated by occupancy on the *E2F1* target gene promoter. Our results suggest that low CUT&RUN DNA yield, even with amounts in the range of a technical negative control (IgG), can/should still proceed with NGS library construction.

As discussed previously, one key challenge of CUT&RUN is the detection of genomic sites bound by TFs, whose expression can vary depending on cell state, with high sensitivity and specificity. In conditions where input materials may be limited or when insights are desired for specific cell populations within a large heterogenous sample, an alternative approach called ultra-low-input CUT&RUN (uliCUT&RUN)[15] may be utilized and has been shown to detect global genome binding of stem cell TFs (e.g. SOX2 and NANOG) at single cell resolution. This method can be applied to multiple human cell and tissue types; alterations to the original protocol[12] were made, including buffer composition, incubation times, quantities of spike-in DNA as well as methods for library preparation and purification[15]. In addition to uliCUT&RUN,

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Cleavage Under Targets and Tagmentation (CUT&TAG) may also be suitable for interrogating chromatin occupancy of non-histone proteins with low input source material[22] . In CUT&TAG, a protein of interest is bound by a specific antibody, which then tethers a fusion transposase, protein A-Tn5. Activation of the transposase generates fragment libraries with high resolution and low background.

To validate the identities of gene targets of TFs from CUT&RUN-seq, one could potentially use the following method of CUT&RUN-qPCR[4,23,24], in which targeted qPCR is performed using gene-specific primers on CUT&RUN DNA. Another traditional validation method is the performance of gene reporter assays, which can further reveal whether a gene is activated or silenced by a specific TF. Finally, genetic controls (if using cell lines), such as knockout or knock-in overexpression of the protein of interest, may aid in the eventual interpretation of data and are important to consider or include when designing CUT&RUN experiments. Regarding the validity of histone marks, the called CUT&RUN peaks can be referenced against published ChIP-seq / ENCODE peaks or other orthogonal datasets for the same cell type/histone mark when available.

In summary, CUT&RUN can be performed using input from cell lines, tissues and frozen cancer tissues[19]. It can be adapted for automated processing in a 96-well format, increasing its scalability and throughput[25]. Additionally, CUT&RUN has been shown to resolve local chromatin architecture, including adjacent nucleosomes and three-dimensional (3D) interactions, through CTCF targeting [11]. Future applications of this technology include combining CUT&RUN with proximity-based ligation techniques (Micro-C)[26] to generate high-resolution, locus-specific maps of 3D nuclear architecture, such as Hi-ChIP[27].

Disclosures

The authors declare no competing interests.

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