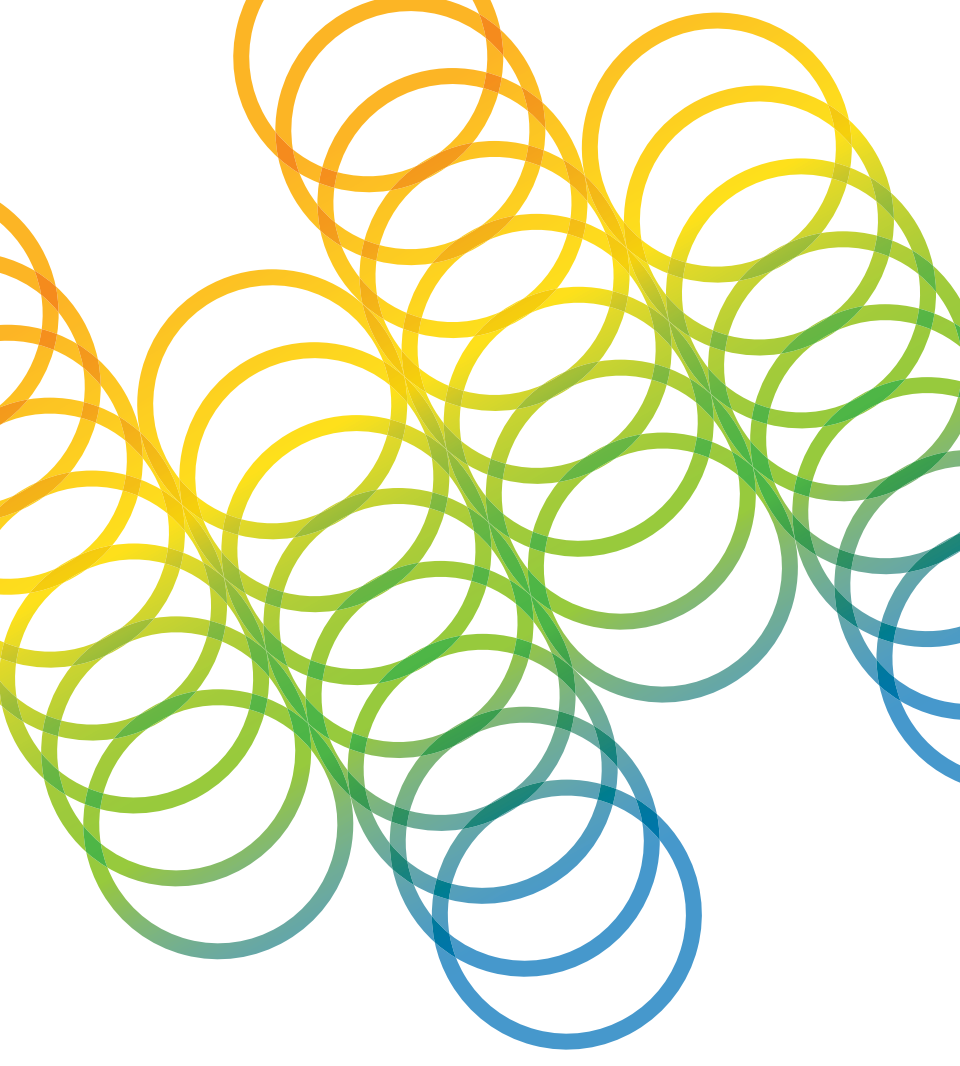

EpiCypher®
Bringing Epigenetics to Life

CUTANA™
ChIC / CUT&RUN Kit
Version 1.0

EpiCypher, Inc.
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Durham, NC 27709

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Kit Version 1.0
User Manual Version 1.0

EpiCypher®

CUTANA™

ChIC / CUT&RUN Kit

Catalog No. 14-1048

48 ChIC / CUT&RUN Samples

Upon receipt, store indicated components at 4°C, -20°C and room temperature (RT)

See p. 8-9 for storage instructions.

Table of Contents

Cleavage **U**nder **T**argets & **R**elease **U**sing **N**uclease (CUT&RUN) is a revolutionary genomic mapping strategy developed by the group of Dr. Steven Henikoff[1]. It builds on Chromatin ImmunoCleavage (ChIC) from Dr. Ulrich Laemmli[2], wherein a fusion of Protein A to Micrococcal Nuclease (pA-MNase) is recruited to selectively cleave antibody-bound chromatin *in situ*[3]. In CUT&RUN, cells or nuclei are immobilized to a solid support, with pAG-MNase cleaved DNA fragments isolated from solution. The workflow is compatible with next-generation sequencing (NGS) to provide high quality genome-wide profiles of histone post-translational modifications (PTMs) and chromatin-associated proteins (e.g. TFs and chromatin remodelers; Figure 1).

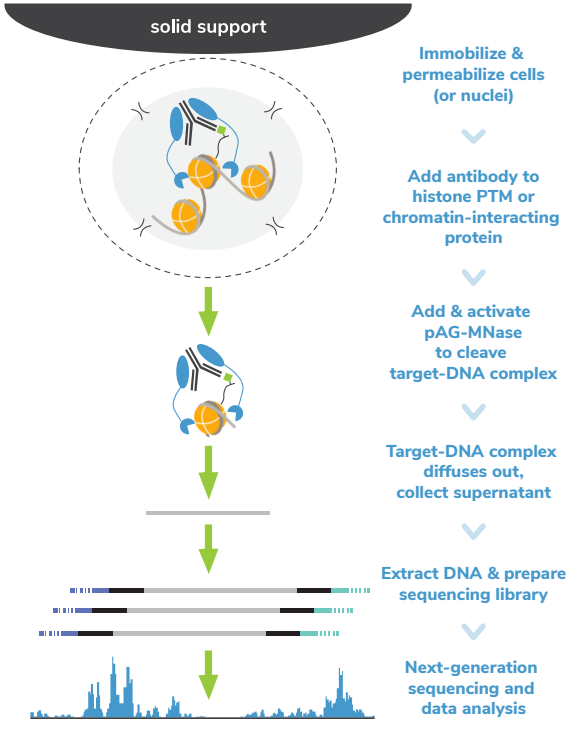

FIGURE 1

Overview of the CUTANA CUT&RUN protocol.

Background and Description

Historically, ChIP-seq is the leading approach for genome-wide mapping of histone PTMs and chromatin-associated proteins. In this approach, bulk chromatin is fragmented by sonication or enzymatic digestion. Target-specific fragments are then immunoprecipitated. Despite extensive optimization and stringent wash conditions, ChIP-seq requires large numbers of cells (typically 10^5 – 10^6 cells) and deep sequencing of both input chromatin and immunoprecipitated material (typically > 30 million reads each) to resolve signal from background.

ChIC and CUT&RUN have revolutionized the study of chromatin regulation by enabling targeted release of genomic fragments into solution. With this innovation, background is dramatically reduced, allowing high resolution genomic mapping for histone PTMs and chromatin-associated proteins using a small number of cells and only 3-8 million sequencing reads per sample (Figure 2). The streamlined workflow and cost savings make ChIC/CUT&RUN amenable to greater experimental throughput, allowing deeper and more rapid investigations to uncover epigenetic biology.

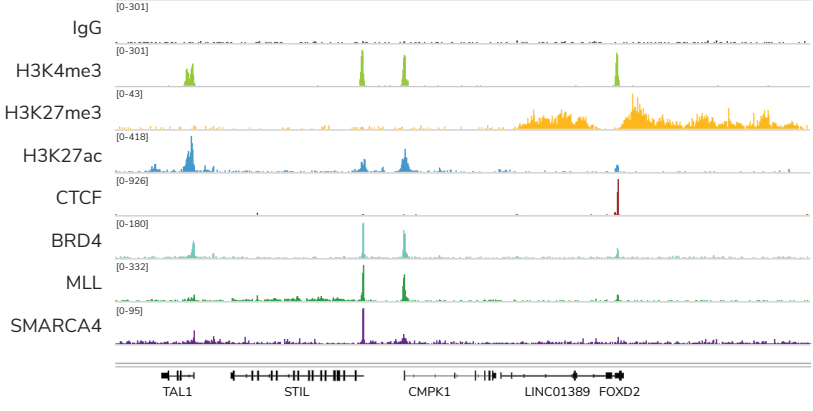

FIGURE 2

Representative genome browser tracks show CUTANA CUT&RUN results using 500,000 K562 cells. Clear peaks with the expected distribution profile are observed using 3-8 million sequencing reads per sample for a variety of epigenetic targets, including histone PTMs (H3K4me3, H3K27me3, H3K27ac), transcription factors (CTCF), epigenetic reader proteins (BRD4), epigenetic writer enzymes (MLL1), and chromatin remodelers (SMARCA4). Rabbit IgG antibody is shown as a negative control (top track).

The CUTANA™ ChIC/CUT&RUN Kit contains sufficient materials for 48 samples and is designed for multi-channel sample pipetting in order to realize the increased experimental throughput advantage of CUT&RUN. The kit includes positive (H3K4me3) and negative (Rabbit IgG) control antibodies. A panel of four designer nucleosomes (H3K4me0, 1, 2, and 3 dNucs™) are spiked-in to control samples to directly monitor experimental success and aid troubleshooting. Additionally, sheared *E. coli* DNA is added to samples after pAG-MNase cleavage to control for library preparation and enable experimental normalization. The kit is compatible with cells and nuclei, including cryopreserved and cross-linked samples (Figure 3 & **Appendix II**). Although it is recommended to start with 500,000 cells, comparable data can be generated using as few as 5,000 cells (Figure 4). The inclusion of rigorous controls as well as compatibility with diverse target types, sample inputs, and cell numbers make the CUTANA kit ideal for a variety of research applications.

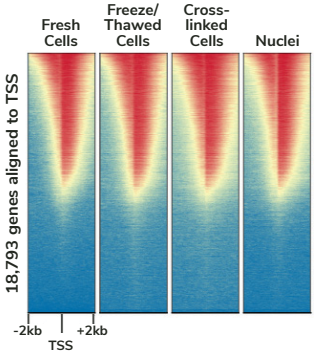

FIGURE 3

Heatmaps showing CUT&RUN signal (red) and background (blue) of H3K4me3-enriched regions flanking annotated transcription start sites (TSS, +/- 2 kb). Gene rows are aligned across the conditions, showing that the genome-wide enrichment pattern is preserved across sample types.

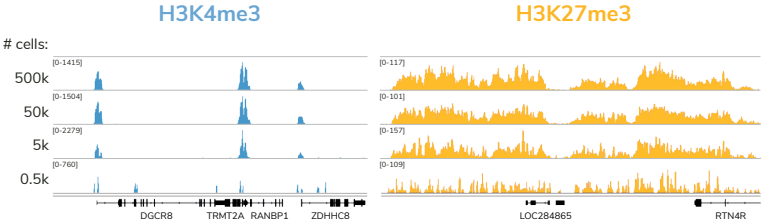

FIGURE 4

Representative genome browser tracks for H3K4me3 (low abundance target) and H3K27me3 (high abundance target) CUT&RUN experiments using decreasing amounts of K562 cells. At 5,000 cells, data quality is largely indistinguishable from standard conditions (500,000 cells).

Included in the Kit

Store at 4°C upon receipt:

Item	Catalog No.	Notes before use
ConA Beads	21-1401	DO NOT FREEZE. Use for immobilizing cells or nuclei. Concanavalin A (ConA) is a lectin, which can cause immune cell activation. For support regarding immune cell studies, contact **techsupport@epicypher.com**.
Bead Activation Buffer	21-1001	Use to prepare ConA beads prior to sample immobilization.
Pre-Wash Buffer	21-1002	Use to prepare Wash, Cell Permeabilization, and Antibody Buffers *FRESH* for each experiment.
Stop Buffer	21-1003	3X concentration: use to terminate pAG-MNase cleavage activity.
SA Beads	21-1402	Streptavidin-conjugated magnetic beads. Use to immobilize CUTANA Spike-in Control dNucs.

Store at -20°C upon receipt:

Item	Catalog No.	Notes before use
Cell Permeabilization Buffer (CP2) Additive	21-1004	Use to prepare Cell Permeabilization Buffer *FRESH* for each experiment. NOTE: Contains 5% digitonin. Final [digitonin] shoud be optimized for each sample type (see **Quality Control Checks**).
pAG-MNase	15-1016	Proteins A and G (pAG) are compatible with a variety of antibody isotypes.
H3K4me3 Positive Control Antibody	13-0041k	0.5 mg/mL. Use as a control in every experiment. SMALL VOLUME: quick spin before use.
Rabbit IgG Negative Control Antibody	13-0042k	0.5 mg/mL. Use as a control in every experiment. SMALL VOLUME: quick spin before use.
E. coli Spike-in DNA	18-1401	100 ng lyophilized *E. coli* DNA for data normalization. Before first use, quick spin and reconstitute in 200 µL DNase-free water (0.5 ng/µL).
CUTANA H3K4 Met-Stat Spike-in Controls	19-1006, 19-1321, 19-1334, 19-1316	Unmodified, H3K4me1, H3K4me2 and H3K4me3 barcoded dNucs (60 nM). Use as a spike-in control with H3K4me3 and IgG control antibodies (see **Quality Control Checks**). SMALL VOLUME: quick spin before use.

Store at room temperature (RT) upon receipt:

Item	Catalog No.	Notes before use
8-strip Tubes	10-0009	Compatible with multi-channel pipettors and Magnetic Separation Rack (EpiCypher Catalog No. 10-0008).
Wash Buffer (W1) Additive	21-1005	10,000X concentration. Use to prepare Wash Buffer *FRESH* for each experiment.
Antibody Buffer (AB3) Additive	21-1006	250X concentration. Use to prepare Antibody Buffer *FRESH* for each experiment.
Chromatin Digest Additive	21-1007	When added to sample, this will activate chromatin tethered pAG-MNase to cleave DNA.
DNA Cleanup Columns	10-0010	Use with the DNA Collection Tubes.
DNA Collection Tubes	10-0011	Use with the DNA Cleanup Columns.
DNA Binding Buffer	21-1008	WARNING: Contains toxic ingredients (see **Appendix IV**).
DNA Wash Buffer	21-1009	Before first use, add 20 mL ≥ 95% ethanol.
DNA Elution Buffer	21-1010	Recover final CUT&RUN DNA in 6 – 20 µL depending on desired final concentration.

Materials Required but Not Supplied

*NOTE: *The kit contains sufficient materials for 48 CUT&RUN samples. Additional materials will need to be procured to fully perform the protocol.*

MATERIALS:

- 1.5, 15 and 50 mL tubes
- Antibody to target of interest (user-dependent)

  *NOTE: *The kit contains H3K4me3 and IgG positive/negative control antibodies to optimize conditions and monitor experimental success. EpiCypher continues to conduct extensive investigations of antibody performance[4]. Contact techsupport@epicypher.com for recommendations.*
- Protease inhibitor (e.g. cOmplete™, EDTA-free Protease Inhibitor Cocktail, Roche #11873580001)
- Trypan blue (e.g. Invitrogen #T10282)
- Qubit™ 1X dsDNA HS Assay Kit (Invitrogen #Q33230)
- Library Preparation Kit (e.g. NEBNext® Ultra™ II DNA Library Prep Kit for Illumina®, New England Biolabs #E7645S)
- AMPure® XP magnetic beads (Beckman Coulter #A63880)

EQUIPMENT:

- 8-strip PCR Tube Magnetic Separation Rack (EpiCypher #10-0008)
- 1.5 mL Tube Magnetic Separation Rack (EpiCypher #10-0012)
- Qubit™ 4 Fluorometer (Invitrogen #Q33226)
- Capillary electrophoresis machine to assess fragment size distribution after library preparation (e.g. Agilent 2100 Bioanalyzer® #G2939A and Agilent High Sensitivity DNA Analysis Kit #5067-4626)
- 8-channel multi-channel pipettor (e.g. VWR #76169-250)
- Multi-channel reagent reservoir (e.g. Thermo Fisher Scientific #14-387-072)
- Tube Nutator for incubation steps (e.g. VWR #82007-202)
- Vortex (e.g. Vortex-Genie®, Scientific Industries #SI-0236)

This kit has been validated for genomic profiling of:

- Histone PTMs (e.g. lysine methylation, lysine acetylation, lysine ubiquitylation, etc.)
- Transcription factors (e.g. CTCF, FOXA1, GATA3)
- Chromatin remodeling ATPases (e.g. SMARCA4-SWI/SNF, SMARCA1/ISWI, INO80, CHD1, CHD3)
- Chromatin writers & readers (e.g. MLL1, BRD4)

1. Always include control conditions (e.g. positive and negative control antibodies with **CUTANA Spike-in Control dNucs**; see **Quality Control Checks**) to confirm experimental success and guide troubleshooting. This is especially critical when evaluating CUT&RUN using previously untested targets and/or antibodies.

2. The **Experimental Protocol** was optimized using 500,000 human K562 cells per sample but is compatible with as few as 5,000 cells.

3. The **Experimental Protocol** describes sample preparation using fresh, non-adherent cells. For adherent cells, **do NOT use trypsin**. For protocol variations, including adaptations for adherent cells, nuclei, cryopreserved samples, and crosslinked material, see **Appendix II**.

4. Take caution throughout the experiment to avoid ConA beads sticking to the sides and caps of tubes. They dry out easily, which can result in sample loss.

5. Although protocols with shortened incubation times have been published[3], such changes can adversely impact yield and reproducibility; therefore they are not recommended.

6. To avoid digitonin precipitation and cell lysis, use the minimal concentration of CP2 Additive that is needed to achieve efficient cell permeabilization. This should be empirically determined for different samples before proceeding with the full CUT&RUN experiment (see **Quality Control Checks**).

7. Paired-end sequencing (mimimum of 50 nucleotides) is recommended to accurately align to the CUTANA Spike-in Control dNucs and enable bioinformatic filtering based on DNA insert fragment size.

8. In CUT&RUN, 3-8 million reads provides adequate coverage for most targets.

*NOTE: *Gather all reagents stored at 4°C and -20°C that are needed for Day 1 (ConA Beads, Pre-Wash Buffer, SA Beads, CP2 Additive, Bead Activation Buffer, H3K4me3 antibody, Rabbit IgG antibody, Spike-in Control dNucs). Place on ice to thaw or equilibrate.*

SECTION I: CUTANA SPIKE-IN CONTROLS & BUFFER PREP (~1 HR)

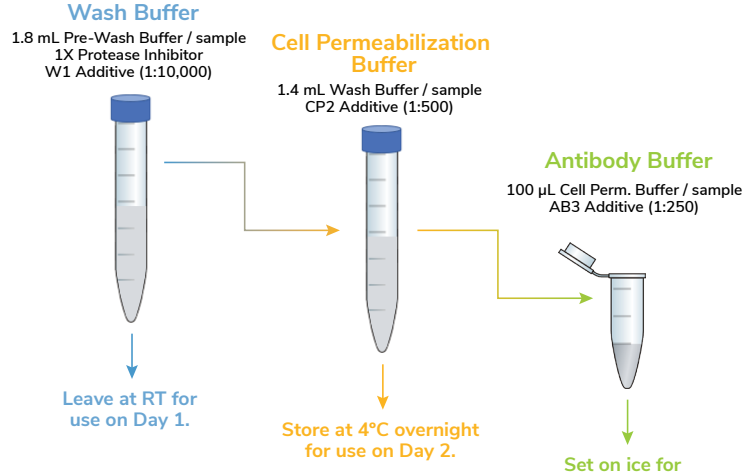

FIGURE 5

Schematic of buffer preparation using the provided Pre-Wash Buffer and additives. Buffers to be prepared fresh the day of use.

A. Prepare immobilized **CUTANA H3K4 MetStat Spike-in Controls** fresh the day of use. Thoroughly mix **SA Beads** by pipetting. Transfer 4 µL into a 1.5 mL tube.

B. Add 150 µL **Pre-Wash Buffer** to beads and pipette mix. Place on magnet and remove supernatant (sup) by pipetting.

C. Remove from magnet and resuspend beads in 210 µL **Pre-Wash Buffer**. Aliquot 50 µL bead slurry into 4 x 1.5 mL tubes.

D. Add 1 µL of a single **CUTANA Spike-in Control dNuc** (H3K4me0, 1, 2, or 3) to each 1.5 mL tube. Pipette to mix and incubate 30 min at RT on nutator.

E. During incubation, make CUT&RUN buffers fresh the day of use (see workflow illustrated in Figure 5 and volume scaling table, right). Add 1.8 mL of **Pre-Wash Buffer** per sample to a single conical tube labeled "**Wash Buffer**".

F. Dissolve 1 protease inhibitor tablet (Roche) in 2 mL water (25X stock). Add 72 µL per sample to the **Wash Buffer**. Store remaining 25X stock for 12 weeks at -20°C.

G. Dilute **W1 Additive** 1:10,000 in the **Wash Buffer**. Store final buffer at RT.

H. Transfer 1.4 mL of **Wash Buffer** per sample into a new conical tube labeled "**Cell Permeabilization Buffer**". Add **CP2 Additive** (1:500 dilution).

*NOTE: *CP2 Additive contains 5% digitonin. A 500X dilution (0.01% final digitonin) is optimal to permeabilize K562, MCF7, and A549 cells. For other cell types, see* ***Quality Control Checks*** *for detailed instructions regarding optimization of permeabilization conditions.*

I. Transfer 100 µL per sample of **Cell Permeabilization Buffer** into a new tube labeled "**Antibody Buffer**". Add **AB3 Additive** (1:250 dilution). Store final buffer on ice.

J. Store the remaining **Cell Permeabilization Buffer** at 4°C overnight (for Day 2 use).

K. Finish preparing the **CUTANA H3K4 MetStat Spike-in Control dNucs**. Quick spin tubes in microfuge to collect samples. Place on magnet and remove sup.

L. Remove tubes from magnet. Add 150 µL **Pre-Wash Buffer** to resuspend beads.

M. Place back on magnet and remove sup. Remove from magnet and resuspend beads in 5 µL **Pre-Wash Buffer**.

N. Combine 4 x 5 µL dNucs into a single tube (20 µL total volume). Place back on magnet and remove sup.

O. Remove from magnet and resuspend in 8 uL **Antibody Buffer**. Place dNucs on ice.

Buffer Sample Scaling Calculations:

NUMBER OF SAMPLES	1X	8X	16X
Wash Buffer - store at RT for use on Day 1			
Pre-Wash Buffer	1.8 mL	14.4 mL	28.8 mL
W1 Additive	0.18 µL	1.44 µL	2.88 µL
25X Protease Inhibitor	72 µL	576 µL	1.15 mL
Cell Permeabilization Buffer - store at 4°C for use on Day 2			
Wash Buffer	1.4 mL	11.2 mL	22.4 mL
CP2 Additive	2.8 µL	22.4 µL	44.8 µL
Antibody Buffer - store on ice for use on Day 1			
Cell Permeabilization Buffer	100 µL	800 µL	1.6 mL
AB3 Additive	0.4 µL	3.2 µL	6.4 µL

SECTION II: BEAD ACTIVATION (~30 MIN)

1. Gently resuspend the **ConA Beads** by pipetting. Transfer 11 µL/sample to a 1.5 mL tube for batch processing.

   *NOTE: *Batch processing at this step is recommended to improve sample handling. If a 1.5 mL tube magnet is not available, the beads can be processed individually (10 µL/sample) in the provided 8-strip PCR tubes using a compatible 8-strip magnet.*

2. Place the tube on a magnet until slurry clears and pipette to remove sup.

   *IMPORTANT: *For all steps involving magnetic racks, take care to avoid disturbing the immobilized beads with pipette tips.*

3. To avoid drying the beads, immediately add 100 µL/sample cold **Bead Activation Buffer**. Pipette gently to mix.
4. Place the tube on a magnet until slurry clears and pipette to remove sup. Repeat previous step for total of two washes.
5. Resuspend beads in 11 µL/sample cold **Bead Activation Buffer**.

   *NOTE: *If not batch processing, use 10 µL/sample at this step. Proceed directly to Section III.*

6. For each experimental condition, aliquot 10 µL/sample of activated bead slurry into separate **8-strip tubes**. Keep on ice until needed.

SECTION III: BINDING CELLS TO ACTIVATED BEADS (~30 MIN)

*NOTE: *For sample inputs other than native, non-adherent cells (e.g. adherent cells, cryopreserved cells, tissue, nuclei, and cross-linked cells) see **Appendix II**.*

7. Harvest 0.5 million cells/sample in 1.5 mL tube. Centrifuge for 3 min at 600 x g at room temperature (RT). Decant or pipette culture media sup.
8. Resuspend cells in 100 µL/sample RT **Wash Buffer**. Pipette to thoroughly resuspend. Centrifuge for 3 min at 600 x g, RT. Decant or pipette sup.
9. Repeat previous step for total of two washes.
10. Resuspend cells in 105 µL/sample in RT **Wash Buffer**. Pipette to thoroughly resuspend. Aliquot 100 µL washed cells to each 8-strip tube containing 10 µL of activated beads. Gently vortex or pipette to mix.

    *NOTE: *Beads are prone to clumping. If your beads are clumped, continue to vortex and/or pipette mix to ensure even resuspension.*

11. Incubate cell-bead slurry on benchtop for 10 min at RT to adsorb cells to beads.

*NOTE: *Count cells by Trypan staining prior to incubation with ConA beads. After incubation with ConA beads, check the sup to ensure most cells have adsorbed to the beads.*

SECTION IV: ANTIBODY BINDING (~30 MIN + OVERNIGHT)

12. If using a multi-channel pipette (recommended) place a multi-channel reagent reservoir on ice.

*IMPORTANT: *If processing > 8 samples (more than 1 x 8-strip tube), for subsequent wash steps remove & replace sups for a single strip before processing the next strip. This avoids bead dry out during wash steps.*

*NOTE: *Multi-channel pipetting is highly recommended through the rest of the experiment. This helps to avoid bead dry out, improves yield, and increases experimental throughput.*

13. Place the 8-strip tubes on an 8-strip tube magnet (high volume setting) until slurry clears. Pipette to remove sup, taking care to avoid disturbing the immobilized beads with pipette tip.
14. Immediately add 50 µL cold **Antibody Buffer** to each sample and gently vortex and/or pipette mix to prevent beads from drying.
15. Add 2 µL **CUTANA H3K4 MetStat Spike-in Control dNucs** to the samples designated for the positive (H3K4me3) and negative (IgG) control antibodies.

*IMPORTANT: *The CUTANA spike-ins provide an essential defined control to ensure assay conditions are optimized (see **Quality Control Checks**). If desired, leftover spike-in controls can also be added to any experimental samples using an H3K4 methylation antibody (H3K4me1, H3K4me2 or H3K4me3 targets).*

16. Add 0.5 µg antibody to each sample and gently vortex.

*IMPORTANT: *Be sure to include the H3K4me3 positive and Rabbit IgG negative control antibodies in every experiment to monitor critical quality controls (see **Quality Control Checks** section). Control antibodies are at 0.5 mg/mL, therefore add 1.0 µL per sample.*

17. Incubate 8-strip tubes on nutator overnight at 4°C.

*IMPORTANT: *To keep beads in solution and avoid drying, slightly elevate the capped end of the 8-strip tubes on the nutator.*

18. Store the **Cell Permeabilization Buffer** at 4°C overnight for use on Day 2.

SECTION IV: ANTIBODY BINDING, CONTINUED (~10 MIN)

19. If using a multi-channel pipette (recommended) place a multi-channel reagent reservoir on ice. Fill with **Cell Permeabilization Buffer.**

    *IMPORTANT: *If processing > 8 samples (more than 1 x 8-strip tube), for subsequent wash steps remove & replace sups for a single strip before processing the next strip. This avoids bead dry out during wash steps.*

20. Place the 8-strip tubes on magnet until slurry clears. Pipette to remove sup.
21. *While beads are on magnet*, add 200 µL cold **Cell Permeabilization Buffer** directly onto beads. Pipette to remove sup.
22. Repeat previous step for total of two washes, without removing 8-strip tubes from the magnet.
23. Add 50 µL cold **Cell Permeabilization Buffer** to each sample. Gently vortex and/or disperse clumps by thorough pipetting.

SECTION V: BINDING OF PAG-MNASE (~30 MIN)

24. Add 2.5 µL **pAG-MNase** (20x stock) to each sample. Gently vortex/pipette mix.

    *NOTE: *To evenly distribute enzyme across cells/nuclei, ensure beads are thoroughly resuspended by gentle pipetting with a P200.*

25. Incubate samples for 10 min at RT. Return 8-strip tube to magnet. Remove sup.
26. *While beads are on magnet*, add 200 µL cold **Cell Permeabilization Buffer** directly onto beads. Pipette to remove sup.
27. Repeat previous step for total of two washes without removing 8-strip tubes from the magnet.
28. Remove 8-strip tubes from the magnet. Add 50 µL cold **Cell Permeabilization Buffer** to each sample. Gently vortex and disperse clumps by pipetting. Cover/put away **Cell Permeabilization Buffer** for later use.

SECTION VI: TARGETED CHROMATIN DIGESTION AND RELEASE (~3 HRS)

29. Place 8-strip tubes on ice. Add 1 µL **Chromatin Digest Additive** to each sample and gently vortex. Ensure efficient digestion by making sure beads are thoroughly resuspended. Gently pipette with a P200 if needed.

30. Incubate 8-strip tubes on nutator for 2 hours at 4°C.
31. Add 33 µL **Stop Buffer** to each sample. Gently vortex to mix.
32. Prior to first use, reconstitute ***E. coli* Spike-in DNA** in 200 µL DNase free water.

*IMPORTANT: *Lyophilized DNA pellet is invisible to the eye. Prior to opening, pellet DNA by quick spin in a benchtop microfuge. After reconstitution, vortex tube on all sides to ensure complete resuspension.*

33. Add 1 µL (0.5 ng) **Spike-in DNA** to each sample. Gently vortex to mix.

*NOTE: *In general, aim for Spike-in DNA to comprise 0.5 – 5% (ideally closest to 1%) of total read counts in the sequencing data. Therefore, while 0.5 ng is a good starting amount for both high (e.g. H3K27me3) and low (e.g. H3K4me3) abundance targets, this may need to be adjusted higher or lower depending on the antibody used, target of interest, total DNA yield, and sequencing results.*

34. Incubate 8-strip tubes for 10 min at 37°C in a thermocycler.
35. Quick spin in benchtop microfuge.
36. Place 8-strip tubes on a magnet stand until slurry clears. Transfer sups containing CUT&RUN enriched DNA to 1.5 mL tubes and discard ConA Beads.

SECTION VII: DNA PURIFICATION (~30 MIN)

*NOTE: *The DNA Cleanup Columns will retain fragments > 50 bp. For specific recommendations regarding smaller fragment size enrichment for TF binding studies, see FAQs, **Appendix III**.*

37. Add 420 µL **DNA Binding Buffer** to each sample. Mix well by vortexing.
38. For every sample, place a **DNA Cleanup Column** into a **DNA Collection Tube**. Load each sample onto a column and label the top.
39. Centrifuge for 30 sec at 16,000 x g, RT. Discard the flow-through. Place the collection tube back on to the column.

*NOTE: *A vacuum manifold can be used in place of centrifugation. For each step, add the indicated buffer, turn the vacuum on, and allow the solution to pass through the column before turning the vacuum off.*

40. Prior to first use, add 20 mL ≥ 95% ethanol to **DNA Wash Buffer**.

SECTION VII: DNA PURIFICATION (~30 MIN), CONTINUED

41. Add 200 µL **DNA Wash Buffer** to each sample column.
42. Centrifuge for 30 sec at 16,000 x g, RT. Discard the flow-through. Place the collection tube back on to the column.
43. Repeat for a total of two washes.
44. Discard the flow-through. Centrifuge one additional time for 30 sec at 16,000 x g to completely dry the column.
45. Transfer column to a clean pre-labeled 1.5 mL microfuge tube, ensuring the column does not come into contact with the flow-through.
46. Elute DNA by adding 12 µL **DNA Elution Buffer**, taking care to ensure the buffer is added to the center of the column rather than the wall. Tap the column + collection tube on the benchtop to ensure all droplets are absorbed onto the resin.

    *NOTE: *12 µL is recommended, however DNA can be eluted in 6 – 20 µL volumes depending on anticipated yield and desired final concentration. Larger elution volumes, longer incubation times, and/or multiple rounds of elution may improve DNA yield. However, sample concentration will be reduced with larger total elution volume.*

47. Let sit 5 minutes, then centrifuge for 1 minute at 16,000 x g, RT.
48. Vortex eluted material and use 1 µL to quantify the CUT&RUN-enriched DNA using the Qubit™ fluorometer as per the manufacturer's instructions. See **Quality Control Checks** section for typical DNA yields.
49. CUT&RUN DNA can be stored at -20°C for future processing.

***IMPORTANT:**

In ChIP protocols, it is common practice to analyze isolated ChIP DNA using agarose gel or capillary electrophoresis to determine fragment size distribution prior to library preparation. However, **electrophoretic analysis of CUT&RUN DNA prior to library preparation is NOT recommended**, since the amount of DNA is likely to be below the sensitivity of detection for these approaches. Instead, proceed directly to library preparation.

SECTION VIII: NGS LIBRARY PREPARATION (~4 HRS)

50. Using 5-10 ng purified CUT&RUN-enriched DNA, prepare sequencing libraries using the NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® (or equivalent approach).

*NOTE: *Although 5-10 ng is recommended, quality CUT&RUN libraries can be prepared when the amount of DNA is undetectable by Qubit™.*

51. Follow the manufacturer's instructions for the following steps:

    a. End repair

    b. Universal adapter ligation

    c. DNA cleanup using 1.0X AMPure® beads to sample volume (this will retain fragments > 150 bp)

*NOTE: *If adapter dimers are observed in the final libraries (~125 bp peak), the ratio of AMPure® beads to CUT&RUN DNA may need to be reduced (e.g. 0.8X beads:sample).*

    d. PCR and primer indexing according to the following cycling parameters:

        i. 45 sec @ 98°C

        ii. 15 sec @ 98°C

        iii. 10 sec @ 60°C

        iv. Repeat steps ii - iii for a total of 14X

        v. 1 min @ 72°C

*NOTE: *The hybrid anneal/extension PCR cycling parameters are intended to enrich for ~100 – 700 bp DNA fragments which are in the sequencing range. Contaminating higher molecular weight species will not be PCR-enriched.*

52. Perform DNA cleanup using a ratio of 1.0X AMPure® beads to sample volume (e.g. 50 µL AMPure® to 50 µL PCR product).

53. Elute DNA in 17 µL 0.1X TE buffer and use 1 µL to quantify the purified PCR product using the Qubit™ fluorometer as per the manufacturer's instructions.

*NOTE: *Typical yield for a purified, PCR amplified sequencing library is ~500 – 750 ng (30 – 50 ng/µL in a final recovered volume of 15 µL TE Buffer)*

54. CUT&RUN libraries can be stored at -20°C for future processing.

SECTION IX: ANALYSIS OF LIBRARY FRAGMENT SIZE (~1 HR)

55. Per sample, prepare 5 µL at 10 ng/µL for loading on the Agilent Bioanalyzer®.

*NOTE: *Record the dilution factor in order to calculate the original sample molarity from the Bioanalyzer reported concentration (reported in nM for the desired DNA size range between 100 – 1,000 bp).*

56. Load 1 µL of 10 ng/µL sequencing libary on Agilent High Sensitivity DNA Chip as per manufacturer's instructions.

*NOTE: *Typical concentration for 15 µL final sequencing library (100 – 1,000 bp region) is 100 – 200 nM.*

57. The final Bioanalyzer® trace should show predominant enrichment of mononucleosomes yielded by the H3K4me3 Positive Control Antibody (~150 bp + sequence adapter length, Figure 6).

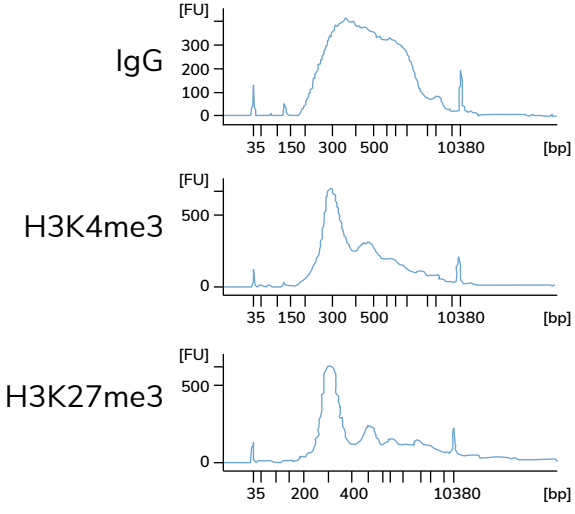

FIGURE 6

Typical CUT&RUN Agilent Bioanalyzer® traces from 5 ng PCR amplified libraries prepared using positive (H3K4me3) and negative (IgG) control antibodies as well as an H3K27me3 antibody (EpiCypher #13-0030). H3K4me3 and H3K27me3 libraries are predominantly mononucleosomes as indicated by the peaks at 275 bp (~150 bp mononucleosome + 125 bp sequence adapters).

SECTION X: ILLUMINA SEQUENCING

58. Based on Agilent Bioanalyzer® molarity calculations, pool libraries at the desired ratios.

*IMPORTANT: *Paired-end sequencing is recommended to enable target footprinting by identifying both ends of MNase cleavage. A minimum of 50 nucleotide paired-end reads are required to read the barcoded DNA on the spike-in CUTANA Spike-in Control nucleosomes.*

*IMPORTANT: *In contrast to ChIP-seq, only 3-8 million paired-end reads are needed for adequate CUT&RUN coverage. For low abundance targets (e.g. H3K4me3), aim for 3-5 million reads per sample. For high abundance targets (e.g. H3K27me3), aim for 5-8 million reads per sample.*

59. To obtain 3-5M PE reads for 8 samples, load 0.8 pM (500 µL) into a MiniSeq™ High Output Reagent Kit, 150-cycles (Illumina #FC- 420-1002).

*NOTE: *Flow cells using the MiniSeq™ High Output Kit typically yield 25 – 40M clusters passing filter (25 - 40M PE reads).*

60. To obtain 3-5M PE reads for > 48-80 samples, load 0.8 pM (1,500 µL) into a NextSeq™ 500/550 High Output Kit v2.5, 150-cycles (Illumina #20024907).

*NOTE: *Flow cells using the NextSeq™ High Output Kit v2.5 typically yield 400M clusters passing filter (400M PE reads).*

SECTION XI: DATA ANALYSIS

61. Align paired-end reads to the appropriate reference genome corresponding to the species used in the experimental samples (e.g. using Bowtie 2).
62. Count the occurrences of the barcoded DNA sequences in the **CUTANA H3K4 MetStat Spike-in Control Panel** using both R1 and R2 paired end reads. A shell script for CUTANA spike-in control alignment is available on the CUT&RUN Kit product page at www.epicypher.com.
63. To determine experimental success, first evaluate performance of the positive and negative (IgG) control samples (see **Quality Control Checks** section).
64. Proceed to further data analysis for experimental samples once the positive controls are confirmed to have produced the expected results. For experimental normalization using the ***E. coli* Spike-in DNA**, see **Appendix I**.

Quality Control Checks

EpiCypher recommends including positive and negative control antibodies and corresponding **CUTANA Spike-in Control dNucs** in every experiment. If the quality control checks for the controls perform as expected, then proceeding to sequencing with all experimental samples is recommended. If sequencing results for control tracks match the expected profile, but the data for experimental cell inputs and/or targets are not satisfactory, further optimization may be necessary (e.g. cell number, sample preparation, digitonin permeabilization, antibody concentration, alternative antibody vendors, etc.).

RECOMMENDED EXPERIMENTAL DESIGN:

- Always include H3K4me3 positive control and IgG negative control antibodies alongside experimental samples.
- Add immobilized **CUTANA Spike-in Control dNucs, H3K4 Panel** into the samples designated for the H3K4me3 positive control and IgG negative control antibodies. This enables a quantitative measure of how much target (H3K4me3) is recovered relative to off-target modifications (H3K4me0, H3K4me1, H3K4me2), providing essential insight for troubleshooting problematic experiments.
- Start with 500,000 cells and optimize conditions before reducing cell number.
- K562 cells can be used as a control cell line since they are known to be permeabilized under standard digitonin concentration and reference data is available for comparison.
- For experimental cells, optimize permeabilization conditions by varying the amount of **Cell Permeabilization Buffer (CP2) Additive**. Use Trypan blue staining to verify that cells have been permeabilized (Figure 7).
- Antibodies that work in well ChIP-seq do not guarantee success in CUT&RUN. For targets of interest, inquire at techsupport@epicypher.com for antibody recommendations, or source multiple antibodies to the same target in order to test for CUT&RUN compatibility.

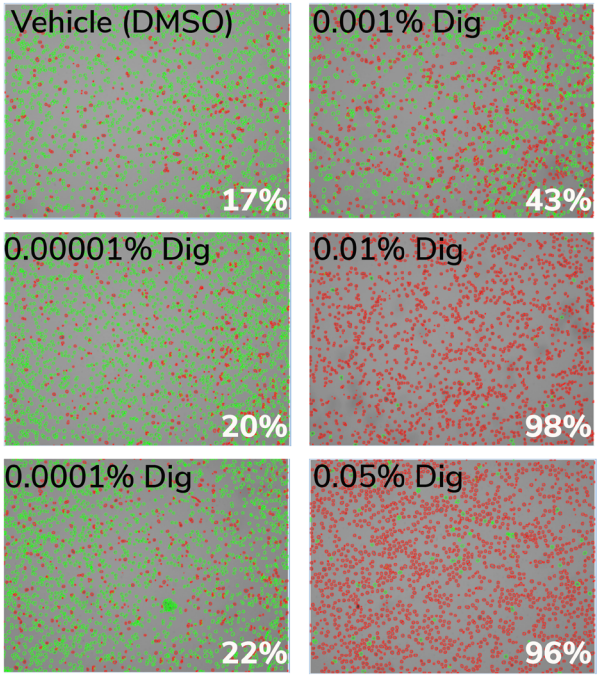

FIGURE 7

Example cell permeabilization optimization experiment. The minimum amount of digitonin needed to permeabilize K562 cells was determined by varying the amount of CP2 Additive (5% digitonin) in the final Cell Permeabilization Buffer. Trypan blue staining was used to evaluate permeability with an automated cell counter (live/dead cell viability analysis). Green cells (Trypan negative) are intact, whereas permeabilized/dead cells (Trypan positive) are indicated in red. The values in the bottom right corner of each panel indicate the percent of dead/permeabilized cells. 0.01% digitonin ("Dig") is the minimum concentration necessary to permeabilize > 95% of total cells.

BEFORE DECISION TO SEQUENCE:

- The yield of CUT&RUN enriched DNA can vary in different experimental settings, starting cell numbers, and for different antibodies (even for antibodies to the same target). Therefore, **DNA yield should not be used as a definitive metric of experimental success.** However, in general the yield from the H3K4me3 positive control antibody should be slightly higher than IgG negative control. Typical results generated using 500,000 K562 cells are shown:

Target	Target Abundance in Cells	Antibody ID	Typical Yield* (ng DNA)
IgG (Negative Control)	None	EpiCypher #13-0042	~2-5
H3K4me3 (Positive Control)	Low	EpiCypher #13-0041	~5-10
H3K27me3	High	EpiCypher #13-0030	~20-50

*NOTE: *Total yields are influenced by a number of considerations, including starting number and type of cells, experimental perturbations, antibody specificity, antibody efficiency, and epitope abundance in each cell type. Yields shown are from 500,000 native K562 cells and are prior to PCR amplification.*

- Yield of library prep and PCR (~750 ng from 5-10 ng library input, see **Section VIII**).
- Fragment size distribution analysis after library preparation (e.g. Agilent Bioanalyzer® or Tapestation® traces) should show enrichment of mononucleosomal fragments (~275 bp = ~150 bp nucleosome + 125 bp adapters, Figure 6).

*NOTE: *Although it may be tempting to analyze fragment size distribution prior to library preparation, remember that CUT&RUN enriched DNA is often below the limit of detection for such approaches. Proceed directly to library preparation after CUT&RUN.*

QUALITY CONTROL CHECKS AFTER SEQUENCING:

- Ensure adequate read depth (3-8 million reads per sample) was achieved.
- Majority of reads (> 80%) should align uniquely to the species genome.
- Sequence duplication levels should be low (< 10% of total reads), but can be filtered out if needed.
- **CUTANA Spike-in Control dNucs** produce the expected results (see next section).
- ***E. coli* Spike-*in* DNA** comprises ~1% of total sequence reads (see **Appendix I**).
- Positive (H3K4me3) and negative (IgG) antibody controls produce the expected distribution and peak structure in the species tested. Results for experimental replicates should be highly reproducible. Representative examples for human K562 cells are shown (Figures 8 and 9).

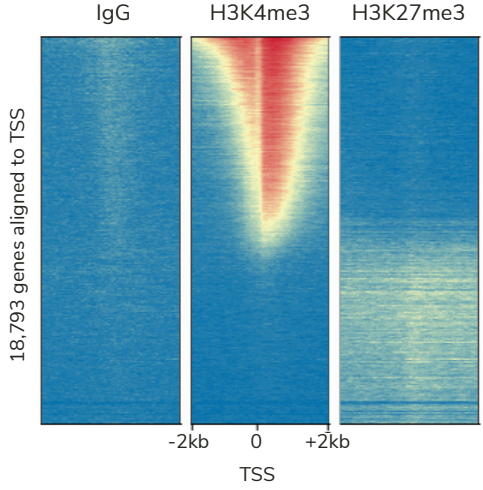

FIGURE 8

Genome-wide analysis of CUT&RUN data presented as a heatmap of signal intensity for Rabbit IgG Negative Control Antibody, H3K4me3 Positive Control Antibody, and H3K27me3 antibody (EpiCypher #13-0030). High (red) and low (blue) signal aligned to the TSS (+/- 2 kb) of 18,793 genes are ranked by H3K4me3 intensity (top to bottom) Gene rows are aligned across the conditions. The heatmaps show that H3K4me3, a mark of active gene transcription, is enriched proximal to the TSS and is anti-correlated with H3K27me3, a mark of transcriptional repression. IgG shows low nonspecific background signal.

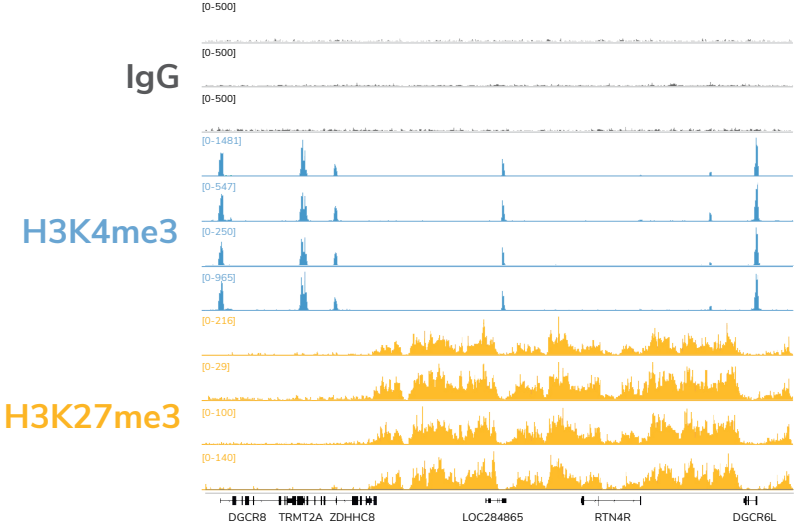

FIGURE 9

Data generated by four independent experimenters are virtually indistinguishable, demonstrating the reproducibility of the CUTANA ChIC/CUT&RUN Kit. Representative genome browser tracks showing CUT&RUN data generated by four independent experimenters using 500,000 K562 cells with Rabbit IgG Negative Control Antibody, H3K4me3 Positive Control Antibody, and H3K27me3 antibody (EpiCypher #13-0030). Total read counts were ~3 million for IgG and H3K4me3 samples, and ~6 million for H3K27me3. H3K4me3 tracks show sharp peaks localized to the TSS, while H3K27me3 tracks show broad peaks spread over transcriptionally repressed genomic regions. IgG shows low nonspecific background enrichment when scaled to the positive control H3K4me3 track.

CUTANA SPIKE-IN CONTROLS

The CUT&RUN Kit includes a spike-in control panel consisting of four DNA-barcoded designer nucleosomes (H3K4me0, H3K4me1, H3K4me2 and H3K4me3) that are to be individually conjugated to streptavidin magnetic beads through biotinylated linker DNA (250x601, Figure 10). Just prior to antibody addition, these beads are spiked into CUT&RUN positive (H3K4me3) and negative (IgG) antibody controls alongside ConA-immobilized cells. pAG-MNase cleavage of the H3K4me3 nucleosome linker DNA in the anti-H3K4me3 positive control sample should specifically release this standard into solution. After sequencing, the relative read count of each spike-in nucleosome provides a quantitative metric of on- vs. off-target PTM recovery (Figure 11), thereby gauging experimental success and guiding troubleshooting efforts for problematic experiments.

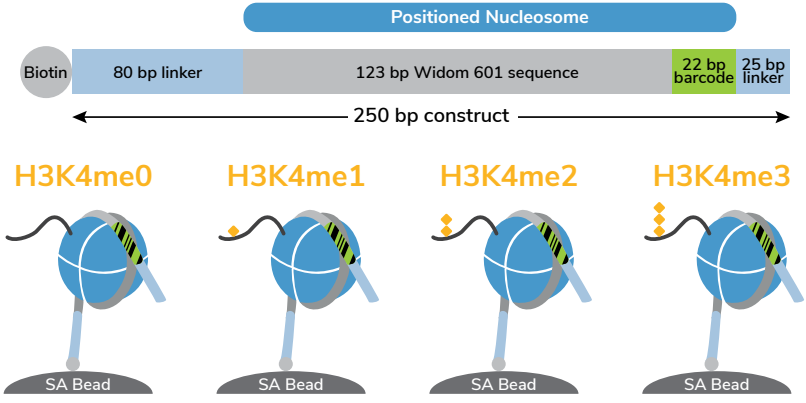

FIGURE 10

Schematic of CUTANA H3K4 MetStat Spike-in Control dNucs, depicting the barcoded designer nucleosomes individually conjugated to magnetic streptavidin beads through biotinylated linker DNA.

Expected results using the CUTANA Spike-in control dNucs (Figure 11):

- Overall read counts for the spike-in panel comprise ~1-8% of total sequencing reads.
- H3K4me3 Positive Control Antibody (EpiCypher 13-0041) shows strong enrichment for H3K4me3 spike-in nucleosomes with less than 5% cross-reactivity to off-target PTMs in the panel, but can be higher in cross-linked samples (~5-15%).
- IgG Negative Control Antibody shows no clear preference for a particular nucleosome in the panel.
- For results that deviate from these conditions: see suggested troubleshooting approaches, next page.

CUTANA Spike-in dNuc	Antibody (Target, Vendor, Cat #): IgG EpiCypher 13-0042	H3K4me1 EpiCypher 13-0040	H3K4me2 EpiCypher 13-0027	H3K4me3 EpiCypher 13-0041	H3K4me3 Shah et al. abMe3-1
H3K4me0	33	2	2	1	5
H3K4me1	23	100	4	2	9
H3K4me2	21	6	100	2	122
H3K4me3	23	2	2	100	100
% on target	23%	91%	93%	96%	42%
% total reads	1.2%	3.9%	0.8%	2.1%	2.2%

FIGURE 11

CUTANA H3K4 MetStat Spike-in Control dNucs were added to CUT&RUN samples alongside the indicated H3K4 methyl state antibodies (top). Sequencing reads were searched for instances of each barcoded nucleosome (left axis) and counts normalized to the on-target PTM (100%, blue). EpiCypher SNAP-ChIP® Certified antibodies to H3K4me1, H3K4me2, and H3K4me3 recovered their respective PTM targets with high specificity. An H3K4me3 antibody previously found to cross-react with H3K4me2 in ChIP (abMe3-1, Shah et al.[4]) has similar cross-reactivity in CUT&RUN. The percentage of reads aligned to the on-target dNuc (relative to the total spike-in reads; % on target) and spike-in read counts (relative to total sequencing reads; % total reads) are shown (bottom).

Problem	Troubleshooting Approach
Using anti-H3K4me3 & IgG Control Antibodies: CUTANA Spike-in Controls produce expected result (**Figure 11**), but genomic signal is poor (e.g. low signal-to-noise, poor peak structure).	• pAG-MNase cleavage and wash conditions are optimized. • Confirm cell viability: low viability will increase noise. • Start with 500k cells & optimize controls before decreasing cell number. • Ideally, start with native cells. If using cross-linked samples, make sure they are only lightly cross-linked (see **Appendix II**). • Ensure cells are not clumped prior to immobilization and beads do not clump throughout protocol. • Ensure cells are adequately permeabilized by optimizing the amount of CP2 Additive (see **Figure 7**). • Optimize conditions in native K562 cells before trying additional sample types.
Using anti-H3K4me3 & IgG Control Antibodies: Both CUTANA Spike-in Controls and samples yield poor/no data.	• Indicates a fundamental failure in the workflow that impaired antibody binding, pAG-MNase cleavage, or adequate DNA recovery. • Always start with 500k cells & optimize controls before decreasing cell number, particularly if this problem is accompanied by low DNA yield. • Ensure Wash, Cell Permeabilization, and Antibody Buffers are prepared fresh the day of use. • Carefully reread the protocol, taking care to pay attention to important notes. • Ensure beads do not clump or stick to side of tube throughout protocol. Gently pipette and vortex to mix as needed. Ensure CP2 Additive is included in both the Cell Permeabilization and Antibody Buffers.
H3K4me3 & IgG Control Antibodies produce the expected results for CUTANA Spike-in Controls (**Figure 11**) and genomic signal (**Figures 8-9**). However, antibodies to additional targets show no clear enrichment.	• The workflow is optimized for controls. • Explore alternate antibodies to the target. Using a "ChIP-grade" antibody does not guarantee success in CUT&RUN. EpiCypher has conducted extensive testing to a variety of histone and non-histone targets in ChIP and CUTANA assays. Contact us for recommendations. • Some targets may benefit from light cross-linking, although yield may be impacted. See **Appendix II**. • Ensure the target of interest is expressed (or localized to chromatin) in the study cells/conditions used. Increase cell number or stimulate target expression if needed.

Appendix I: Experimental Normalization Using *E. coli* Spike-in DNA

1. Aim for *E. coli* Spike-in DNA to comprise ~1% (0.5-5%) of total sequencing reads. In the protocol, 0.5 ng is recommended to start. Based on target abundance, antibody efficiency and experimental conditions, this amount of may need to be adjusted higher or lower to achieve read counts in this range.

2. After sequencing, in addition to the experimental sample reference genome (e.g. human hg19 build), align reads to the *E. coli* K12, MG1655 reference genome. Filter out reads that do NOT align uniquely. https://support.illumina.com/sequencing/sequencing_software/igenome.html

3. For pairwise comparisons, quantify *E. coli* Spike-in DNA reads for treatment and untreated samples and normalize to sequencing depth.

   For example:

   Treatment spike-in = 100,000 Ec reads in 5,000,000 total reads = 2%

   Untreated spike-in = 30,000 Ec reads in 3,000,000 total reads = 1%

4. Calculate normalization factor (see Tay et *al.*[5]) such that after normalization the *E. Coli* spike-in signal is set to be equal across all samples.

   For example:

   Treatment normalization factor = 1 / 2% spike-in bandwidth = 0.5

   Untreated normalization factor = 1 / 1% spike-in bandwidth = 1.0

5. Use single scalar normalization ratio with the --scaleFactor option enabled in deeptools bamCoverage tool (https://deeptools.readthedocs.io/en/develop/content/tools/bamCoverage.html) to generate normalized bigwig files for visualization in IGV.

   For example:

   Treatment sample --scaleFactor = 0.5

   Untreated sample --scaleFactor = 1.0

NOTE: *The effect of normalization on a sample is inversely proportional to the E. coli spike-in bandwidth. In other words, samples with the highest bandwidth will receive the largest reduction in signal after normalization. For further information on experimental normalization using exogenous spike-in controls, see Tay et al.*[5] *and Orlando et al. (2014)*[6].

*NOTE: *The kit is compatible with fresh cells, as well as cryopreserved cells, cross-linked cells, and isolated nuclei (Figure 3). The protocols below provide general guidelines and recommendations for working with these sample preparations.*

SAMPLE PREPARATION: ADHERENT CELLS

Enzymatic treatment (e.g. trypsin) will degrade glycoproteins, thereby impairing cell adsorption to the ConA Beads. Therefore, **do not use trypsin to prepare adherent cells for CUT&RUN**. Instead, we recommend scraping adherent cells to dislodge them from the dish, then pellet by centrifugation for ~3 min at 600 x g, RT. Proceed directly to the CUT&RUN wash steps.

SAMPLE PREPARATION: CRYOPRESERVATION

Cells should be freeze/thawed under conditions that minimize lysis, which can contribute to elevated background in CUT&RUN. Supplement cell culture media with a cryoprotective agent (e.g. 10% DMSO in media) and slow freeze samples (-1°C per minute) in an isopropanol-filled container placed at -80°C (e.g. "Mr. Frosty" or equivalent). When ready to perform CUT&RUN, quickly and completely thaw the samples at 37°C.

SAMPLE PREPARATION: TISSUES

The primary requirement for CUT&RUN is that tissue is processed into a mono-dispersion of cells, typically by mechanical maceration/douncing. EpiCypher has not currently investigated tissue-specific protocols, however a number of groups have reported methods for performing CUT&RUN using human and mouse primary tissues[7-10].

SAMPLE PREPARATION: NUCLEI

Materials Needed	Recipe / Source
Nuclear Extraction Buffer	20 mM HEPES, pH 7.9, 10 mM KCl, 0.10% Triton X-100, 20% glycerol
Protease Inhibitor	Roche #11873580001
Wash Buffer (W1) Additive	EpiCypher CUTANA ChIC / CUT&RUN Kit #21-1005
Phosphate Buffered Saline (PBS)	Any vendor
0.4% Trypan blue	Invitrogen #T10282
Brightfield or phase microscope + hemacytometer slides	Any vendor

1. Prepare 200 µL / sample (+ extra dead volume) Nuclear Extraction Buffer fresh the day of use. Sterile Filter.
2. Add 1:10,000 dilution of Wash Buffer (W1) Additive and 1X Protease Inhibitor to the Nuclear Extraction Buffer. Place final buffer on ice.
3. Harvest 0.5 million cells per sample (+ extra for Trypan blue staining) by centrifugation for 3 min at 600 x g, RT. Remove and discard supernatant (sup).
4. Resuspend cells in 100 µL per sample 1X PBS. Set aside 10 µL cells for future analysis by Trypan blue staining (intact cell control). Centrifuge for 3 min at 600 x g, RT. Remove and discard sup.

   *NOTE: *For all steps, the ratio of buffer volumes : cells scales linearly. For example, use 1 mL buffer for 5 x 10^6 cells.*

5. Resuspend cells in 100 µL per sample cold Nuclear Extraction Buffer.
6. Incubate samples on ice for 10 min.
7. Centrifuge for 3 min at 600 x g, 4°C. Remove and discard sup. The pellet should change in appearance from sticky, pale yellow (cells) to white and fluffy (nuclei).

8. Resuspend nuclei in 100 µL per sample cold Nuclear Extraction Buffer. Set aside 10 uL nuclei for Trypan blue staining.
9. Add 10 uL Trypan blue to the intact cell control (Step 4) and the isolated nuclei (previous step).
10. Load onto hemacytometer slide and examine under brightfield/phase microscope to determine whether nuclei have been efficiently isolated (Figure 12).
11. To cryopreserve nuclei, slowly freeze samples in isopropanol-filled chiller in -80°C freezer.
12. When ready to use samples for CUT&RUN, thaw nuclei quickly by placing on 37°C block.
13. Proceed to CUT&RUN ConA Bead conjugation step (**Experimental Protocol,** Step 10).

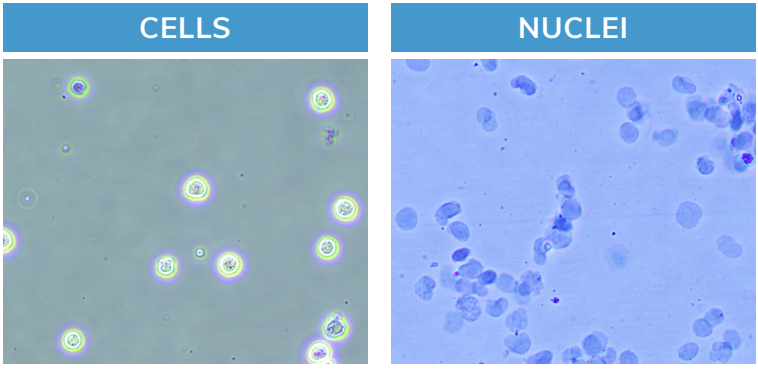

FIGURE 12

Morphology characteristic of intact K562 cells (left) compared to isolated nuclei (right) when visualized under brightfield microscope after Trypan blue staining.

SAMPLE PREPARATION: CROSS-LINKING

It is recommended to first try native samples when possible, as this works well for the majority of targets. However, CUT&RUN signal for labile targets or highly transient chromatin binding proteins may be improved by cross-linking.

In particular, **histone deacetylase activity may contribute to incomplete low resolution genomic profiles in native CUT&RUN**. In these cases, light (e.g. 0.1% formaldehyde, 1 min) to moderate (e.g. 1% formaldehyde, 1 min) cross-linking may improve signal even though total yield may be reduced. However, **heavy cross-linking such as that typically used for ChIP-seq (e.g. 1% formaldehyde, 10 min) has been observed to damage histone acetylation signal in K562 cells** (Figure 13). Therefore, optimal cross-linking conditions for profiling histone acetylation should be empirically determined in the model system of interest. The protocol described provides general guidelines for optimizing fixation conditions.

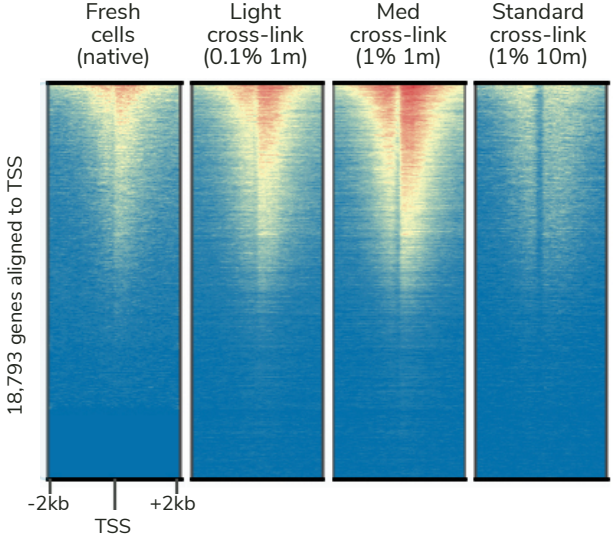

FIGURE 13

H3K27ac CUT&RUN signal is improved by light to moderate fixation (0.1 – 1% formaldehyde for 1 min) compared to native cells. However, heavy (1%, 10 min) cross-linking significantly reduces CUT&RUN DNA yield. CUT&RUN data from 500,000 K562 cells are displayed in a heatmap with each gene row aligned across the conditions.

Materials Needed	Recipe / Source
Pre-Wash Buffer supplemented with detergent	Add 1% Triton X-100 + 0.05% SDS to Pre-Wash Buffer (EpiCypher CUTANA ChIC / CUT&RUN Kit #21-1002)
37% Formaldehyde	Sigma #252549
Glycine	Sigma #50046
10% SDS	Any vendor
20 µg/µL Proteinase K	Ambion #AM2546

1. From suspension tissue culture, transfer 0.5 million cells per sample into a 15 or 1.5 mL tube. For adherent cells, cross-link cells while still attached to plate.
2. Add X µL 37% formaldehyde directly to culture to achieve desired final concentration of formaldehyde (recommended ~0.1% - 1%).

   *NOTE: *In initial experiments, test a range of formaldehyde concentrations to determine the optimal fixation conditions for the cell type and target of interest.*

3. Quickly vortex suspension cells or swirl plate for adherent cells to mix. Incubate for 1 - 10 min at RT.

   *NOTE: *In initial experiments, test a range of times to determine optimal fixation conditions.*

4. Quench fixation reaction by adding glycine to a final concentration of 125 mM. Vortex/swirl to mix. Scrape adherent cells from the plate, transfer to 15 mL tube, and centrifuge for 3 min @ 600 x g, RT.
5. Begin CUT&RUN by starting at the **Experimental Protocol**, Step 7.
   Carry through the protocol with the following modifications:
   a. Use **Wash**, **Cell Permeabilization**, and **Antibody buffers** (Figure 5) supplemented with 1% Triton X-100 + 0.05% SDS.
   b. After 37ºC incubation in the **Experimental Protocol**, Step 34, place 8-strip tubes on magnet stand until slurry clears. Transfer sups containing CUT&RUN enriched DNA to new 8-strip tubes.
   c. Reverse cross-links by adding 0.8 µL 10% SDS and 1 µL of 20 µg/µL Proteinase K to each sample. Mix by vortexing. Incubate overnight at 55ºC using a thermocycler.
   d. After incubation, quick spin samples in benchtop microfuge. Resume protocol at Step 37 and proceed as normal.

Appendix III: Frequently Asked Questions

1. What types of cell inputs are compatible with CUT&RUN?

EpiCypher has confirmed that CUT&RUN is compatible with whole cells and nuclei derived from mammalian suspension, adherent cancer lines, and nuclei derived from mouse primary B-cells. EpiCypher has not yet directly tested other cell types, but a number of groups have successfully performed CUT&RUN on human and mouse primary tissue[7-10], FACS sorted[11] and immune cells[12,13]. Note that lectins (e.g. ConA) play a role in the innate immune system and so immune cell types may be inadvertently stimulated via binding to ConA Beads. To circumvent this potential problem in CUT&RUN, EpiCypher recommends using nuclei[11] or a crosslinking strategy[14].

2. Is CUT&RUN compatible with frozen or cross-linked cells?

Yes. EpiCypher has confirmed that freeze/thawed cells (e.g. slow freeze in 10% DMSO/media, and then quick thaw at 37°C) generate data of indistinguishable quality to fresh material. EpiCypher has also tested previously reported cross-linking conditions and recommended wash buffers[14], and found that while yields may be lower than native cells, the resulting data tracks display similar quality. See **Appendix II** for details.

3. Does EpiCypher's CUT&RUN protocol work on non-PTM targets?

Yes. The current protocol has been used at EpiCypher to generate numerous non-PTM CUT&RUN data, including CTCF, BRD4, and SMARCA4 (BRG1). No protocol modifications were necessary to generate these data since the DNA Cleanup Columns recover > 50 bp fragment sizes.

However, for TFs in particular, which generate sub-nucleosomal size (< 120 bp) DNA fragments, modifications to the library protocol have been reported[9,15] to improve the representation of these smaller fragments during library preparation. Briefly, to enrich for sub-120 bp fragments, the authors reduced the inactivation temperature after end repair from 65°C for 30m to 50°C for 1 hr. Also, after the ligation reaction, the AMPure® bead size-selection ratio was increased to 1.75X volume.

For TF CUT&RUN fragment sizes, both nucleosomal (~150 bp) and sub-nucleosomal (< 120 bp) reflect TF chromatin occupancy locations, however, the sub-nucleosomal fragments provide higher resolution mapping for their locations.

4. What antibodies does EpiCypher recommend for CUT&RUN?

Antibodies that work well in ChIP may not always work in CUT&RUN. Further, using antibodies that cross-react with off target epitopes is likely to compromise biological interpretations[4]. EpiCypher has conducted extensive studies of histone PTM antibodies in ChIP-seq by using defined spike-in controls *in situ* to show that the majority of antibodies are not fit-for-purpose[4 and unpublished results]. To address this unmet need for CUT&RUN, EpiCypher is currently developing full panels of recombinant nucleosome controls that are uniquely designed for this approach, similar to the CUTANA H3K4 MetStat "mini-panel" included in this kit. To provide platform-compatible antibodies while CUTANA spike-ins are still in development, EpiCypher has recently released "CUTANA Compatible Antibodies". These antibodies are verified using SNAP-ChIP® recombinant nucleosome spike-in controls to show that they exhibit best-in-class specificity and target enrichment (EpiCypher "SNAP-ChIP® Certified Antibodies"). Further, they generate highly comparable data in CUT&RUN compared to ChIP-seq, lending confidence for their use in this application. For targets not currently offered, contact us for recommendations (techsupport@epicypher.com).

For more background, see our blog posts:

https://www.epicypher.com/resources/blog/choosing-the-right-chip-antibody-for-your-experiment/

https://www.epicypher.com/resources/blog/epicyphers-h3k4me3-antibody-provides-a-useful-positive-control-for-cutrun/

https://www.epicypher.com/resources/blog/an-h3k27me3-antibody-with-reliable-results-in-chipseq-and-cutrun/

Appendix IV: Safety Datasheet

EpiCypher, Inc.

6 Davis Dr. Ste 755
Durham, NC 27709 USA

Phone: 1-855-374-2461
Fax: 1-855-420-6111
Email: info@epicypher.com

www.epicypher.com

24 Hour Emergency Phone Number:

US & Canada: 1-800-535-5053

International: 1-352-323-3500

Product Identification

Product Name: CUTANA ChIC/CUT&RUN Kit
Synonyms: None.
Molecular Weight: *Not applicable to mixtures.*
Chemical Formula: *Not applicable to mixtures.*
Recommended Use: *This product is for research and development only.*

Component Name	Hazardous Ingredients
Bead Activation Buffer	NA
Pre-Wash Buffer	NA
Stop Buffer	NA
DNA Binding Buffer	Yes
DNA Wash Buffer	NA
DNA Elution Buffer	NA
Wash Buffer (W1) Additive	NA
Cell Permeabilization Buffer (CP2) Additive	NA
Antibody Buffer (AB3) Additive	NA
Chromatin Digest Additive	NA
ConA Beads	NA
pAG-MNase	NA
Rabbit IgG Negative Control Antibody	NA
H3K4me3 Positive Control Antibody	NA
CUTANA dNucs	NA
SA Beads	NA
Spike-in DNA	NA

Hazardous Identification

DNA Binding Buffer

Classification

This chemical is considered hazardous by the 2012 OSHA Hazard Communication Standard (29 CFR 1910.1200)

Label Elements

Acute toxicity – Oral Category 4
Skin corrosion/irritations - Category 2
Serious eye damage/eye irritation - Category 2
Flammable liquids - Category 3

Signal Word

WARNING

Hazard Statements

Harmful if swallowed, causes skin irritation, causes serious eye irritation, flammable liquid and vapor.

Precautionary Statements

Store in a well-ventilated place. Keep container tightly closed.

Prevention

- Wear protective gloves/protective clothing/eye protection/face protection.
- Wash face, hands and any exposed skin thoroughly after handling.
- Do not eat, drink or smoke when using this product.
- Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking.
- Ground/bond container and receiving equipment.
- Use only non-sparking tools.
- Take precautionary measures against static discharge.

Hazardous Identification *continued*

Precautionary Statements

Response

- **IF EXPOSED OR CONCERNED:** Get medical advice/attention.
- **IF IN EYES:** Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
  If eye irritation persists: Get medical advice/attention.
  If skin irritation occurs: Get medical advice/attention.
- **IF ON SKIN (OR HAIR):** Take off immediately all contaminated clothing. Rinse skin with water/shower. Wash contaminated clothing before reuse.
- **IF SWALLOWED:** Call a POISON CENTER or doctor if you feel unwell. Rinse mouth.
- **IN CASE OF FIRE:** Use CO_2, dry chemical, or foam to extinguish.

Composition and Information on Ingredients

DNA Binding Buffer (mixture)

Chemical Name	Kit Volume	CAS Number
Isopropyl Alcohol	< 11 ml	67-63-0
Sodium Perchlorate	< 2 ml	7601-89-0
Guanidine Hydrochloride	< 8 ml	50-01-1

First Aid Measures

General advice: If symptoms persist, call a physician. Do not breathe dust/fume/gas/mist/vapors/spray. Do not get in eyes, on skin, or on clothing.

Inhalation: Remove to fresh air. If breathing is irregular or stopped, administer artificial respiration. Avoid direct contact with skin. Use barrier to give mouth-to-mouth resuscitation. If symptoms persist, call a physician.

Eye contact: Rinse immediately with plenty of water, also under the eyelids, for at least 15 minutes. Keep eye wide open while rinsing. Do not rub affected area. Remove contact lenses, if present and easy to do. Continue rinsing. Get medical attention if irritation develops and persists.

First Aid Measures *continued*

Skin contact: Wash off immediately with soap and plenty of water while removing all contaminated clothes and shoes. Wash contaminated clothing before reuse. If skin irritation persists, call a physician. Immediate medical attention is not required.

Ingestion: Do NOT induce vomiting. Clean mouth with water and drink afterwards plenty of water. Never give anything by mouth to an unconscious person. If symptoms persist, call a physician.

Most important symptoms and effects: May cause redness and tearing of the eyes. Prolonged contact may cause redness and irritation. Vapors may cause drowsiness and dizziness.

Note to Physicians: Treat symptomatically.

Fire Fighting Measures

Suitable Extinguishing Media: Dry chemical. Carbon dioxide (CO_2). Water spray. Alcohol resistant foam.

Specific hazards arising from chemical: Risk of ignition. Keep product and empty container away from heat and sources of ignition. In the event of fire, cool tanks with water spray. Fire residues and contaminated fire extinguishing water must be disposed of in accordance with local regulations.

Special protective equipment for fire-fighters: In the event of a fire, wear full protective clothing and NIOSH-approved self-contained breathing apparatus with full facepiece operated in the pressure demand or other positive pressure mode.

Accidental Release Measures

Spill response: Absorb or cover with dry earth, sand or other non-combustible material and transfer to containers. Soak up condensate or spills with inert absorbent material and collect in ventilated waste container for disposal. Wear lab coat, chemical resistant gloves and safety glasses. Take precautionary measures against static discharges. Wash down the spill site.

Waste disposal method: Dispose of in accordance with all federal, state and local regulations.

Handling and Storage

Safe Handling: Use personal protection equipment. Avoid breathing vapors or mists. Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking. Use grounding and bonding connection when transferring this material to prevent static discharge, fire or explosion. Use with local exhaust ventilation. Use sparkproof tools and explosion-proof equipment. Keep in an area equipped with sprinklers. Avoid contact with skin, eyes or clothing. Take off contaminated clothing and wash before reuse.

Storage Conditions: Keep containers tightly closed in a dry, cool and well-ventilated place. Keep away from heat, sparks, flame and other sources of ignition. Keep in properly labeled containers. Do not store near combustible materials. Keep in an area equipped with sprinklers. Store in accordance with local regulations.

Exposure Controls / PPE

Exposure Limits: The following ingredients are the only ingredients of the product above the cut-off level which have an exposure limit applicable in the region for which this safety data sheet is intended or other recommended limit. At this time, the other relevant constituents have no known exposure limits from the sources listed here.

Exposure Controls / PPE *continued*

Isopropyl Alcohol 67-63-0	STEL: 400 ppm TWA: 200 ppm	TWA: 400 ppm TWA: 980 mg/m3 (vacated) TWA: 400 ppm (vacated) TWA: 980 mg/m3 (vacated) STEL: 500 ppm (vacated) STEL: 1225 mg/m3	IDLH: 2000 ppm TWA: 400 ppm TWA: 980 mg/m3 STEL: 500 ppm STEL: 1225 mg/m3

Engineering Controls: Showers, eyewash stations, ventilation systems.

Personal Protective Equipment

Eye/face protection: Tight sealing safety goggles.

Hand protection: Wear suitable impervious gloves.

Skin and body protection: Wear suitable protective clothing, long sleeved clothing, Chemical resistant apron, and antistatic boots.

Respiratory protection: no protective equipment is needed under normal use conditions. If exposure limits are exceeded or irritation is experienced, ventilation and evacuation may be required.

Physical and Chemical Properties

Appearance:	Colorless Liquid
Odor:	Alcohol
Boiling Point:	90 °C / 194 °F
Melting Point:	No data available
Solubility:	No data available
Flash Point:	27 °C / 80.6 °F
Specific Gravity:	No data available
pH:	5.10-5.30

Stability and Reactivity

Chemical Stability: Stable under ordinary conditions of use and storage.

Hazardous Decomposition Products: None known based on information supplied.

Incompatibilities: Strong oxidizers, strong acids and bases.

Conditions to Avoid: Heat, flame, sparks, incompatibles.

Toxicological Information

Inhalation Specific test data for the substance or mixture is not available. May cause irritation of respiratory tract.

Eye contact Specific test data for the substance or mixture is not available. Irritating to eyes. Causes serious eye irritation.

Skin contact Specific test data for the substance or mixture is not available. Prolonged contact may cause redness and irritation. Repeated exposure may cause skin dryness or cracking.

Ingestion Specific test data for the substance or mixture is not available. Ingestion may cause gastrointestinal irritation, nausea, vomiting and diarrhea. Harmful if swallowed. May cause drowsiness or dizziness.

Symptoms related to the physical, chemical and toxicological characteristics Redness. May cause redness and tearing of the eyes.

Acute toxicity Numerical measures of toxicity. The following values are calculated based on chapter 3.1 of the GHS document: ATEmix (oral) 1,127.90 mg/kg ATEmix (dermal) 4,599.60 mg/kg ATEmix (inhalation-dust/mist) 157.80 mg/

Carcinogenicity. Isopropyl Alcohol IARC Group 3

Target organ effects Respiratory system, Eyes, Skin.

Ecological Information

Chemical Name	Aquatic Plants	Fish	Crustacea
Isopropyl Alcohol 67-63-0	EC50: > 1000 mg/L (72h, Desmodesmus subspicatus) EC50: > 1000 mg/L (96h, Desmodesmus subspicatus)	LC50: =9640 mg/L (96h, Pimephales promelas) LC50: > 1400000 µg/L (96h, Lepomis macrochirus) LC50: =11130mg/L (96h, Pimepha les promelas.	EC50: =13299mg/L (48h, Daphnia magna)
Guanidine Hydrochloride 50-01-1	NA	LC50: =1758mg/L (48h, Leucisc usidus)	NA

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