ACTIVE MOTIF®

Enabling Epigenetics Research

CUT&RUN Spike-In Control

Catalog No. 53183

(Version A2)

Revision	Date	Description of Change
A2	November, 2024	Clarified that nuclei are from *Drosophila S2* strain

Contents

Overview

CUT&RUN (Cleavage Under Targets & Release Using Nuclease) has emerged as a powerful method for investigating the genome-wide distribution of various chromatin-associated proteins and their modifications. However, the identification of differences between data sets can be challenging when global modification changes occur. Additionally inaccurate quantification of starting material or technical variation during processing results in variation across sample data. Currently available bioinformatic-based normalization methods are not applicable in these instances, and the only reliable way to overcome bias and variation is to add a known standard (Spike-In) into all samples. Active Motif offers Spike-In reagents for ChIP-Seq and CUT&Tag, and has now introduced a similar approach for CUT&RUN.

Active Motif's strategy for CUT&RUN normalization is to Spike-In cryopreserved *Drosophila S2* strain cell nuclei into samples prior to CUT&RUN. Then, during the antibody incubation step, a *Drosophila* H2Av antibody is added in addition to the antibody to the target of interest. This *Drosophila* H2Av antibody provides a mechainsm to reliably tag *Drosophila* histones in a consistent way across all samples. A normalization factor is then created based on the *Drosophila* signal and applied to the test genome. This CUT&RUN Spike-In strategy enables normalization of CUT&RUN data independent of the experimental antibody and without bias.

The CUT&RUN Spike-In Control works with the ChIC/CUT&RUN Assay Kit (Catalog No. 53180).

Product	Format	Catalog No.
CUT&RUN Spike-In Control	24 rxns	53183

Kit Components and Storage

All components are guaranteed for 6 months after receipt when stored properly.

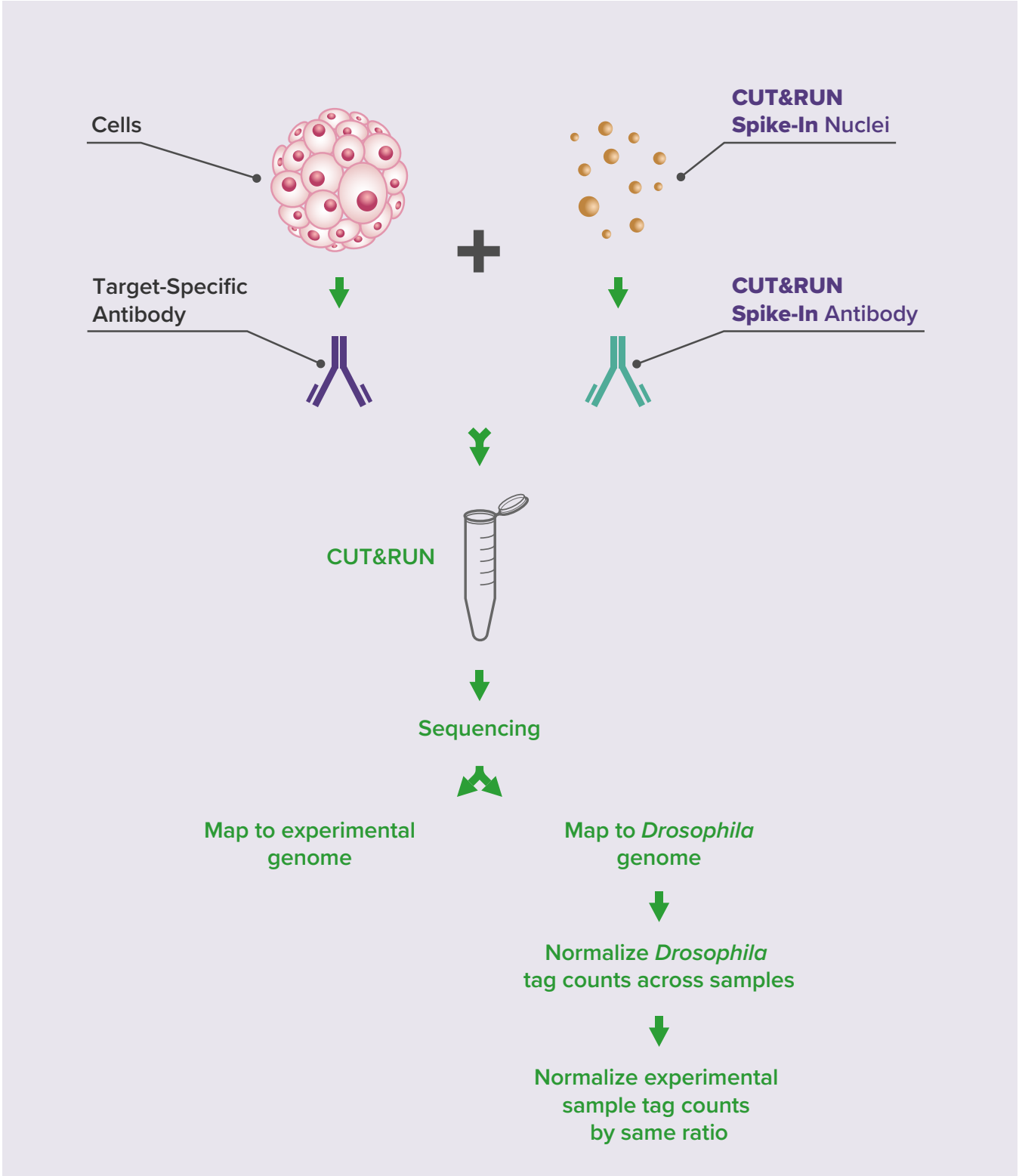
Kit Component	Quantity	Storage
CUT&RUN Spike-In Antibody	24 µL	-20°C
CUT&RUN Spike-In Nuclei	6 x 440 µL	-80°C

CUT&RUN Spike-in Nuclei are provided as six 440 µL vials of nuclei at a concentration of 500 nuclei/µL.

We recommend using a ratio of 1 to 10 nuclei to experimental cells in CUT&RUN reactions.

Note: If more than 1 vial of nuclei are used across an experiment, pool the needed vials of nuclei together to ensure consistency in the amount of nuclei added per reaction for spike-in.

Workflow


Cells
CUT&RUN
Spike-In Nuclei
Target-Specific
Antibody
CUT&RUN
Spike-In Antibody
CUT&RUN
Sequencing
Map to experimental
genome
Map to *Drosophila*
genome
Normalize *Drosophila*
tag counts across samples
Normalize experimental
sample tag counts
by same ratio

CUT&RUN Spike-In Reaction Guidelines

Active Motif's CUT&RUN normalization strategy[1] may be applied to any mammalian CUT&RUN assay reaction due to the lack of cross-reactivity of the Spike-in antibody with mammalian samples. The amount of cryopreserved *Drosophila* nuclei and antibody used per CUT&RUN reaction may need to be optimized with the goal of having *Drosophila* reads make up only 2-10% of the total sequencing reads. However, when using robust antibodies against tightly localized histone modifications, such as H3K4me3, we recommend a spike-in:test sample ratio of 1:20. For antibodies against spreading marks such as H3K27me3, we recommend a spike-in:test sample ratio of 1:10. For antibodies against transcription factors such as YY1, we recommend a spike-in:test sample ratio of 1:100.

Conditions	Human Cells	CUT&RUN Spike-In Nuclei	CUT&RUN Spike-In Nuclei/Human Cells Ratio	%DM/Total
Abundant Histone Mark (H3K27me3)	500K	50K	1:10	5.98%
Medium Abundance Histone Mark (H3K4me3)	500K	20K	1:20	4.57%
Rare Target, Transcription Factor (YY1)	500K	5K	1:100	7.49%

To demonstrate the utility of this approach, histone modification level differences were mimicked by setting up CUT&RUN reactions with different amounts of starting cell numbers. Various numbers of cryopreserved human K562 cells (500,000, 400,000, 300,000, 200,000, 100,000, and 50,000) were combined with 20,000 (for H3K4me3) (Figure 1) or 10,000 (for YY1) (Figure 2) of cryopreserved *Drosophila* nuclei for each experiment.

H3K4me3 and YY1, were evaluated in the CUT&RUN spike-in assay, with biological duplicates included in each experiment. Libraries were quantified and sequenced to a depth of 20-30 million reads per sample. However, sequencing to equal read depth for each sample masks the differences in starting amounts for each sample (Figures 3 and 4). Therefore spike-in normalization is required to reveal the differences in starting material[2]. For normalization, the sample with the lowest number of *Drosophila* reads was used to generate normalization factors across samples, which were then applied to down-sample the human read counts for each sample accordingly. Read counts in CUT&RUN samples were adjusted using a scale factor for peak calling and bigWig files were generated from the normalized read coverage to compensate for sample-to-sample variability. The normalized results correlate with expected results for the ratios of input material (Figures 5 and 6).

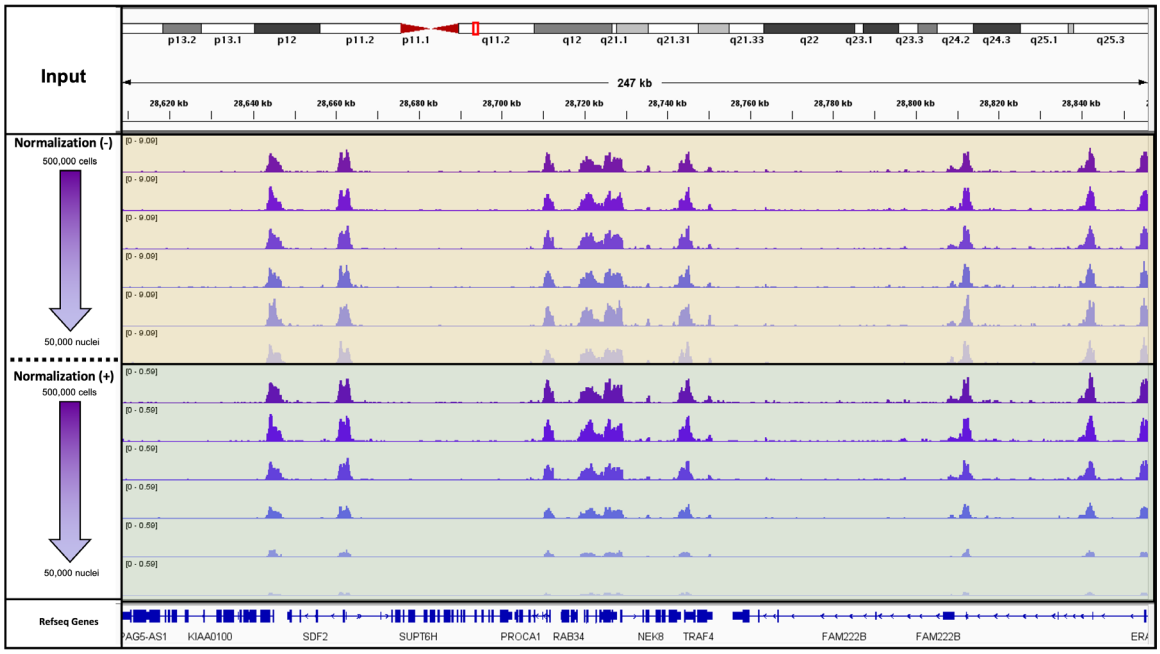


Figure 1: K562 Starting Cell Numbers for CUT&RUN Targeting H3K4me3 with and without Spike-In Normalization

20,000 CUT&RUN Spike-In Nuclei were added to 500,000, 400,000, 300,000, 200,000, 100,000 and 50,000 K562 cells and assayed in CUT&RUN. Results with and without normalization are shown.

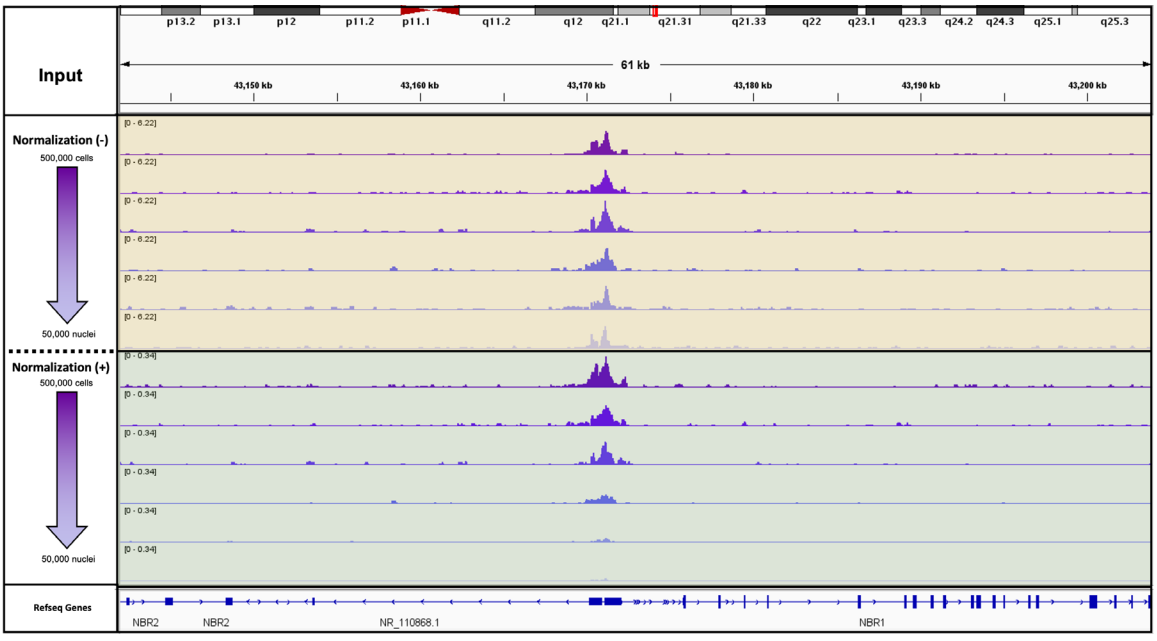


Figure 2: K562 Starting Cell Numbers for CUT&RUN targeting YY1 with and without Spike-In Normalization

5,000 CUT&RUN Spike-In Nuclei were added to 500,000, 400,000, 300,000, 200,000, 100,000 and 50,000 K562 cells and assayed in CUT&RUN. Results with and without normalization are shown.

Fig. 3

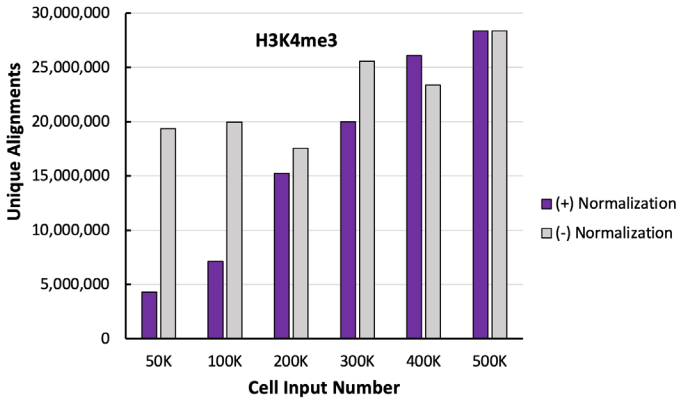


Fig. 4

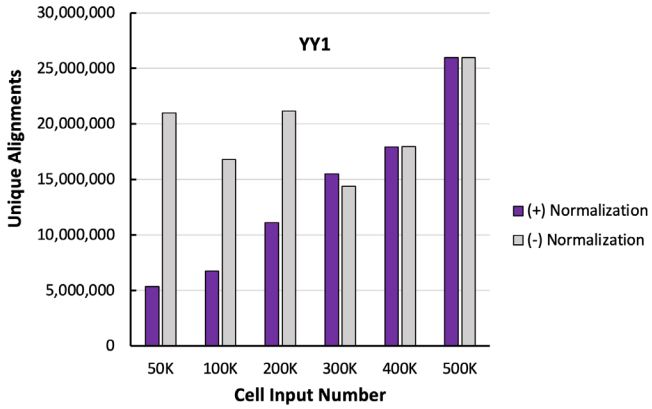


Figure 3: K562 Paired Alignments with and without Normalization Targeting H3K4me3

The number of alignments is not reflective of the number of cells added to the experiment, thus normalization is needed.

Figure 4: K562 Paired Alignments with and without Normalization Targeting YY1
The number of alignments is not reflective of the number of cells added to the experiment, thus normalization is needed.

Fig. 5

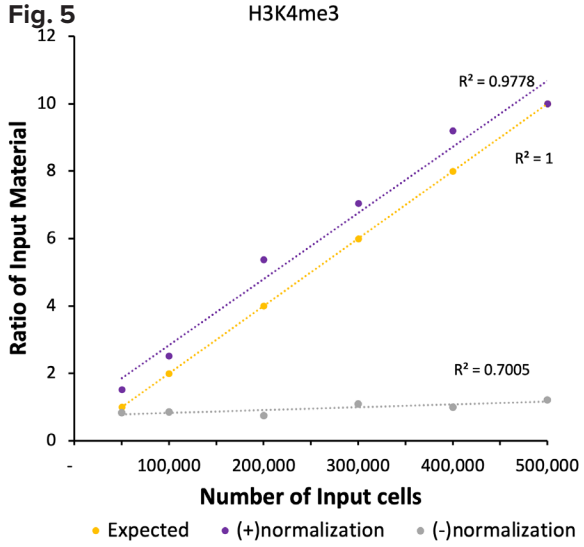


Fig. 6

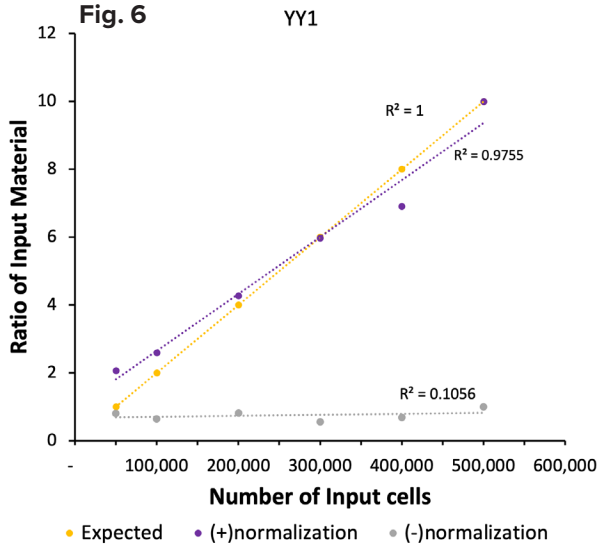


Figure 5: K562 Paired Alignments After Normalization to *Drosophila* Paired Alignments
K562 paired alignments were normalized to the *Drosophila* alignment number from 500,000 K562 cells. Now the expected ratios based on amount of starting cells has been restored in H3K4me3

Figure 6: K562 Paired Alignments After Normalization to *Drosophila* Paired Alignments
K562 paired alignments were normalized to the *Drosophila* alignment number from 500,000 K562 cells. Now the expected ratios based on amount of starting cells has been restored in YY1

CUT&RUN Normalization Guidelines

1. Perform CUT&RUN by combining the CUT&RUN Spike-In Nuclei with the experimental sample cells to be used in CUT&RUN on Step 10 of the ChIC/CUT&RUN Assay Kit (Catalog No. 53180) manual if using cells, or at Step 15 if using nuclei. Perform CUT&RUN as per the instructions provided in Active Motif's ChIC/CUT&RUN Assay Kit (Catalog No. 53180) and add 1 µL of CUT&RUN Spike-In Antibody, in combination with your test antibody, during the primary antibody incubation, Step 29. Use the guidelines provided for nuclei and antibody quantities based on the antibody target.
2. Sequence the CUT&RUN libraries using an NGS platform.
3. Map the CUT&RUN data to the test reference genome (*e.g.* human, mouse, or other).
4. Map CUT&RUN data to the *Drosophila S2* reference genome.
5. Count uniquely aligning *Drosophila* sequence tags and identify the sample containing the least number of tags.
6. Divide the aligned *Drosophila* tag value from the sample with the lowest *Drosophila* tag count by the *Drosophila* tag count value from all other samples to and generate a normalization factor for each sample. (Sample 1 with lowest tag count / Sample 2) = Normalization factor. The sample with the lowest *Drosophila* tag count will have a normalization factor of 1.
7. Generate the normalization factors for all samples using the strategy from step 6.
8. Use the normalization factors to down-sample the read counts for each sample.
9. After obtaining normalized human read counts, use a standard CUT&RUN pipeline starting with the downsampled tag counts for each sample for peak calling and generation of bigWigs.

References

1. Egan, B. *et al.* (2016) *PLoS ONE.* 11(11): e0166438.
2. Taruttis *et al.* (2017) *Biotechniques* 62:53-61

Technical Services

If you need assistance at any time, please call or send an e-mail to Active Motif Technical Service at one of the locations listed below.

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