CUT&Tag-IT® Spike-In Control, R-loop

Catalog No. 53173

(Version A1)

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Overview

The Cleavage Under Targets and Tagmentation (CUT&Tag) technique has emerged as a powerful method for investigating the genomic localization of histone modifications of interest. However, the identification of differences between data sets can be challenging when global modification changes occur, such as in the case of studying the effects of chromatin modifying enzyme inhibitors. 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 has now introduced a similar approach for CUT&Tag.

Active Motif's strategy for CUT&Tag normalization is to spike-in cryopreserved *Drosophila* cell nuclei into samples prior to CUT&Tag. Then, during the primary antibody incubation step, a *Drosophila* H2Av antibody is added in addition to the antibody targeting the histone mark of interest. This *Drosophila* H2Av antibody provides a mechanism 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&Tag Spike-in strategy enables normalization of CUT&Tag data independent of the experimental antibody and without bias.

The CUT&Tag-IT® Spike-In Control, R-loop works with the CUT&Tag-IT® R-loop Assay Kit (Catalog No. 53167).

Product	Format	Catalog No.
CUT&Tag-IT® Spike-In Control, R-loop	16 rxns	53174

Kit Components and Storage

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

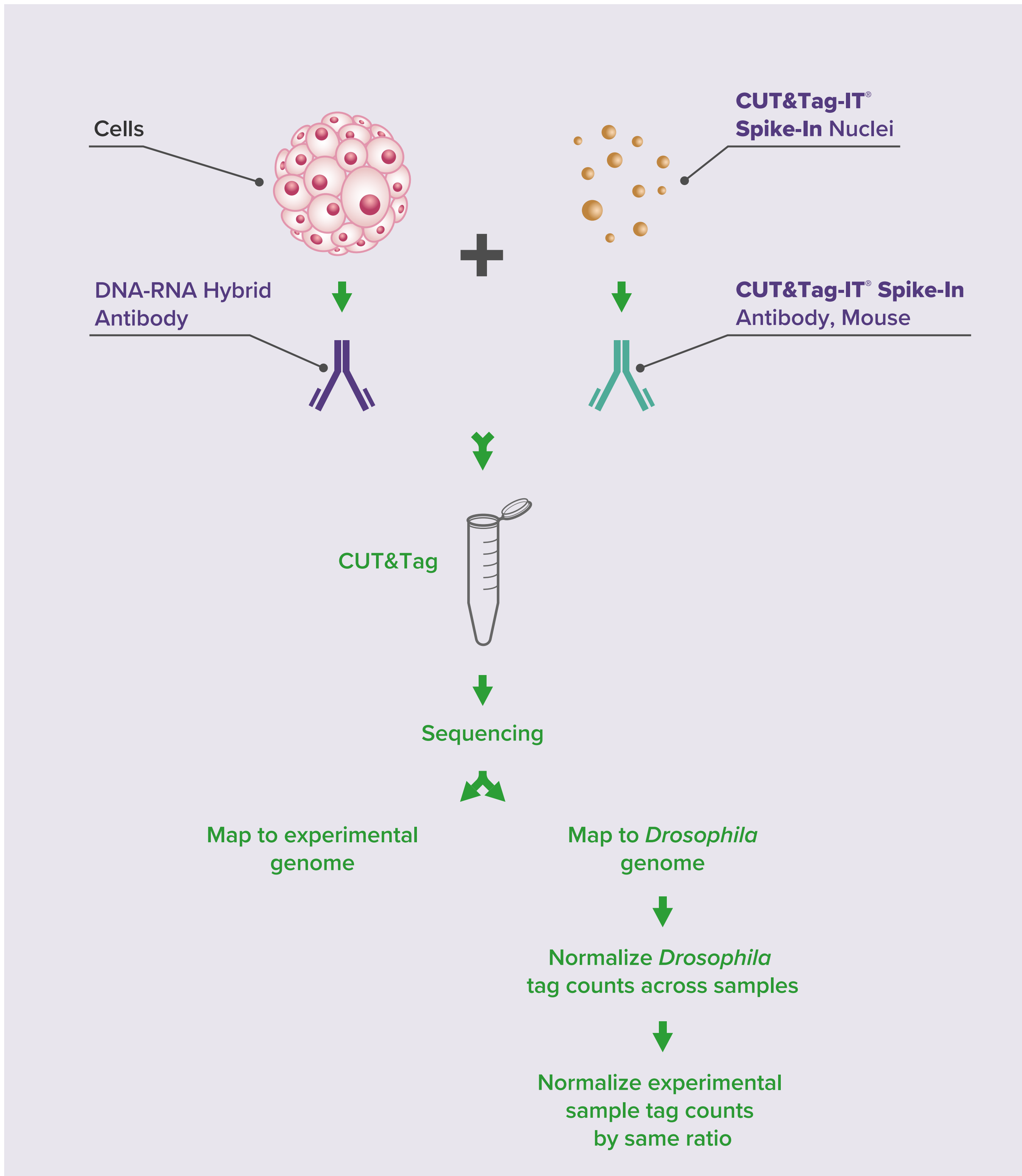
Kit Component	Quantity	Storage
CUT&Tag-IT® Spike-In Antibody, Mouse	16 µL	-20°C
CUT&Tag-IT® Spike-In Nuclei, R-loop	4 x 440 µL	-80°C

CUT&Tag-IT Spike-in Nuclei, R-loop are provided as four 440 µL vials of nuclei at a concentration of 500 nuclei/µL.

We recommend using 50,000 nuclei per 500,000 cells used in CUT&Tag-IT R-loop Assay Kit reaction.

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



CUT&Tag-IT Spike-In Control, R-loop Reaction Guidelines

Active Motif's CUT&Tag normalization strategy[1] may be applied to any mammalian CUT&Tag 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&Tag reaction may need to be optimized with the goal of having *Drosophila* reads make up only 2-10% of the total sequencing reads.

A spike-in:test sample ratio of 1:10 is recommended for CUT&Tag targeting R-loops.

To demonstrate the utility of this approach, DNA-RNA hybrid R-loop level differences were mimicked by setting up CUT&Tag reactions with different amounts of starting cell numbers. Various numbers of cryopreserved human K562 cells (500,000, 375,000, 250,000, and 125,000) were combined with 50,000 of cryopreserved *Drosophila* nuclei for each experiment (Figure 1).

DNA-RNA hybrids were evaluated in the CUT&Tag R-loop Spike-In assay, with biological duplicates included in each experiment. Libraries were quantified and sequenced to a depth of 40-50 million reads per sample. However, sequencing to equal read depth for each sample masks the differences in starting amounts for each sample (Figure 2). 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. After obtaining normalized human read counts, a standard CUT&Tag pipeline was used for peak calling generation of bigWigs. Normalized results correlate with input cell numbers, so that differences between experimental samples can be revealed (Figure 3).

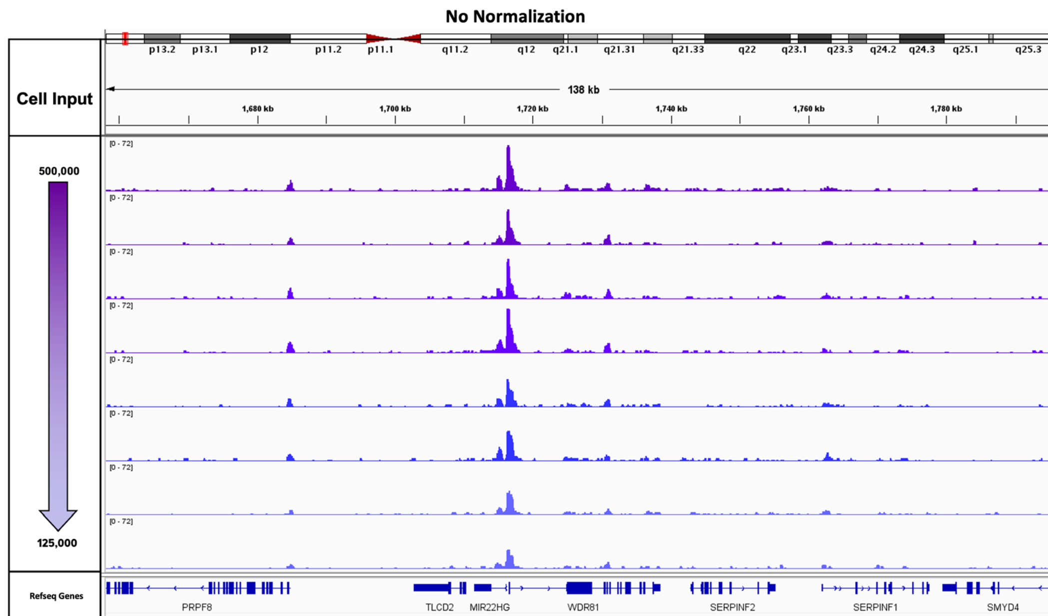


Figure 1: K562 Cell Titration results for CUT&Tag-IT R-loop Assay without CUT&Tag-IT Spike-In Control, R-loop normalization

This experiment used 50,000 *drosophila* nuclei as a spike-in for a mock treatment. The mock treatment was a cell titration performed in duplicate from 500,000 cryopreserved K562 cells down to 125,000 cryopreserved K562 cells. The actual cell numbers are 500,000, 375,000, 250,000, and 125,000 cells. This figure is all of the non-normalized tracks for the titration in duplicates.

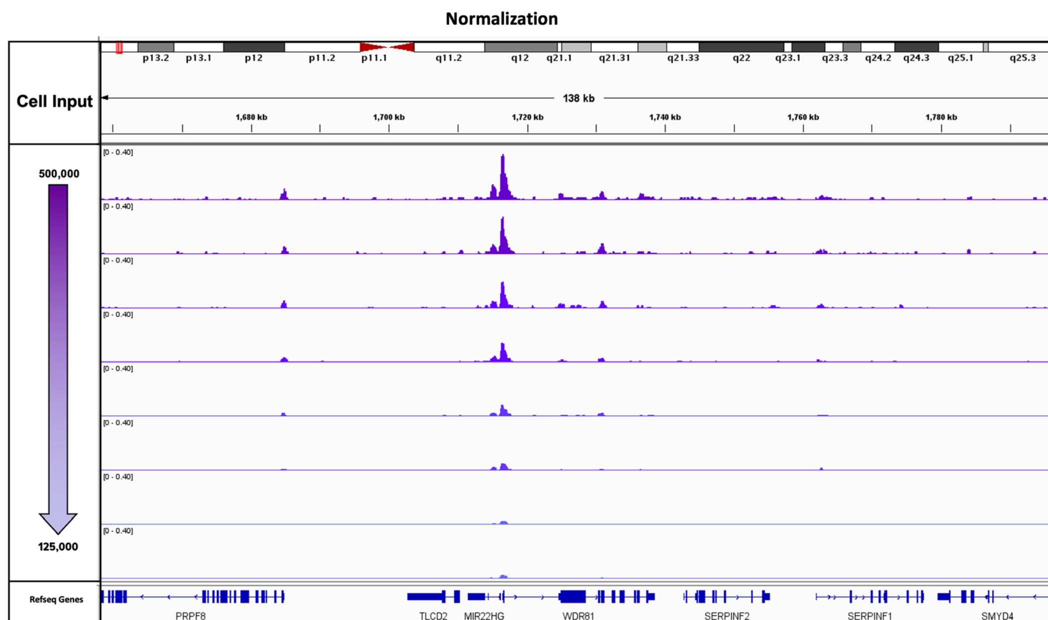


Figure 2: K562 Cell Titration results for CUT&Tag-IT R-loop Assay with CUT&Tag-IT Spike-In Control, R-loop normalization

This experiment used 50,000 *drosophila* nuclei as a spike-in for a mock treatment. The mock treatment was a cell titration performed in duplicate from 500,000 cryopreserved K562 cells down to 125,000 cryopreserved K562 cells. The actual cell numbers are 500,000, 375,000, 250,000, and 125,000 cells. This figure is all of the normalized tracks for the titration in duplicates.

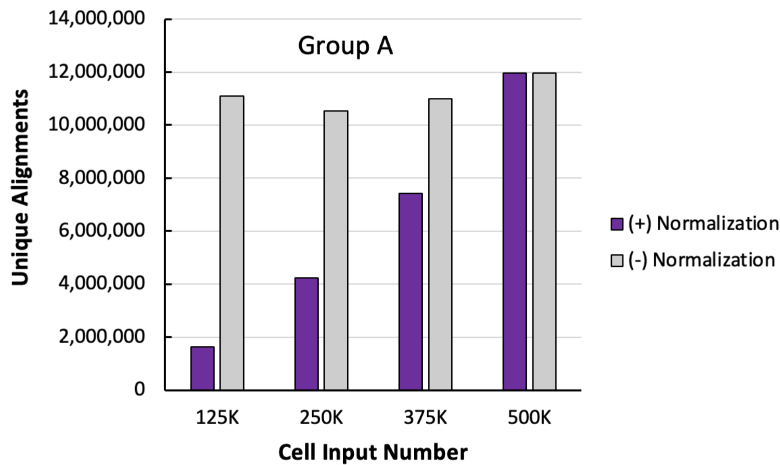


Figure 3: K562 Unique Alignments

This is the unique alignments (to human genome) for one of the duplicates, before (-) and after (+) normalization with the spike in, for each of the 4 cell inputs.

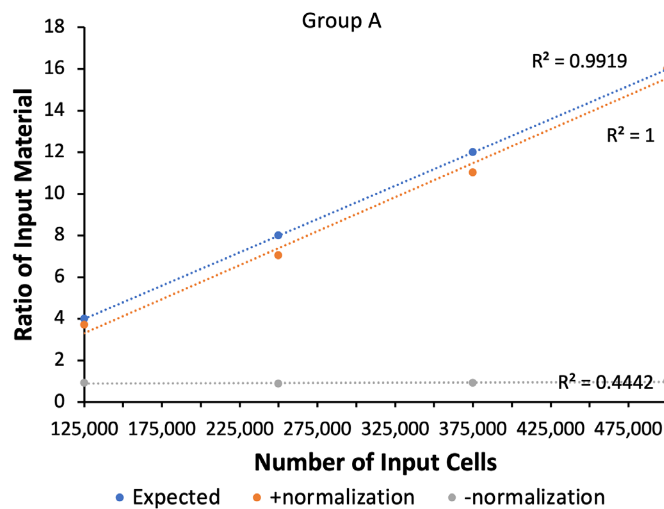


Figure 4: Normalized Data Correlates with Experimental Cell Numbers

This is the ratio of input material. For the sample data (normalized and unnormalized) this was calculated by dividing the number of unique fragments aligning to the human genome for each sample by the number of unique alignments for the max input. For the expected sample – the number of cell input was divided by the max number of cell input.

CUT&Tag-IT Spike-In Control, R-loop Normalization Guidelines

1. Perform CUT&Tag by combining the CUT&Tag-IT® Spike-In Nuclei with the experimental sample cells to be used in the CUT&Tag-IT R-loop Assay. Perform the CUT&Tag-IT R-loop Assay as per the instructions provided in the CUT&Tag-IT® R-loop Assay Kit manual (Catalog No. 53167) and add 1 µL of CUT&Tag-IT® Spike-In Antibody, Mouse in combination with the DNA-RNA Hybrid mAb (Clone S9.6) antibody, during the primary antibody incubation step. Use the guidelines provided for nuclei and antibody quantities based on the antibody target.
2. Sequence the CUT&Tag libraries using an NGS platform.
3. Map the CUT&Tag data to the test reference genome (*e.g.* human, mouse, or other).
4. Map CUT&Tag data to the *Drosophila* 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&Tag 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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