The Spike-in Normalization Strategy

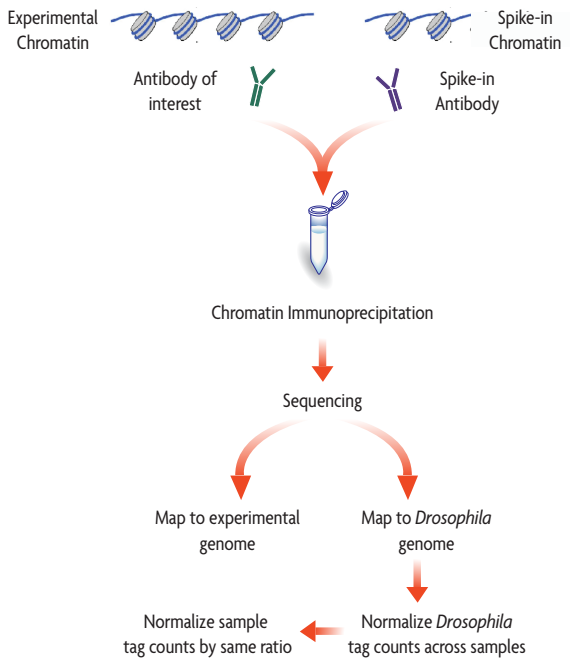
Description

The Spike-in Antibody (Catalog No. 61686) is intended for use with Active Motif's Spike-in Chromatin (Catalog No. 53083) to normalize chromatin immunoprecipitation (ChIP) experiments. ChIP is a multi-step process in which variations caused by sample loss during immunoprecipitation and library preparation, uneven sequencing read depth or user differences can lead to results that are difficult to interpret. To overcome this challenge, Active Motif has developed a spike-in strategy to normalize out technical variation and sample processing bias. Additionally, the normalization strategy can be used to monitor the effects of experimental conditions, such as inhibitory compounds or mutants.

A standard ChIP reaction is set up using experimental chromatin (*e.g.* human) and an antibody of interest. In addition, *Drosophila melanogaster* chromatin (Spike-in Chromatin) is added, or "spiked-in", to each reaction as a minor fraction of the total chromatin. An antibody that recognizes the *Drosophila*-specific histone variant, H2Av, is also spiked-in to the reaction (Spike-in Antibody). This Spike-in antibody provides a mechanism to reliably pull down a small fraction of *Drosophila* chromatin that is consistent across all samples. Since variation introduced during the ChIP procedure will also occur with the spike-in chromatin, a normalization factor can be created based on the *Drosophila* signal and applied to the sample genome. This ChIP Spike-in strategy enables normalization of data across ChIP antibodies and experiments without bias.

Products

- Spike-in Antibody (Catalog No. 61686)
- Spike-in Chromatin (Catalog No. 53083)
- 96 rxns *Drosophila* Positive Control Primer Set Pbgs (Catalog No. 71037)
- 96 rxns *Drosophila* Negative Control Primer Set 1 (Catalog No. 71028)
- ChIP-IT High Sensitivity Kit (Catalog No. 53040)
- ChIP-IT qPCR Analysis Kit (Catalog No. 53029)



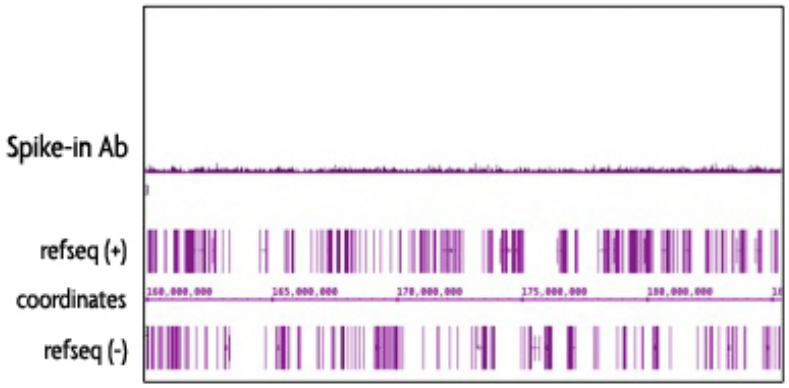
ChIP-Seq Normalization Workflow

Guidelines

Active Motif's normalization strategy may be applied to any mammalian ChIP reaction due to the low cross-reactivity of the Spike-in Antibody with mammalian samples. Researchers may choose to optimize the amount of sample chromatin and antibody used per ChIP reaction. However, we recommend adjusting the amount of Spike-in Chromatin to maintain the same chromatin ratio (sample : spike-in) as recommended on the lot-specific Spike-in Chromatin data sheet that accompanies the purchase of the product.

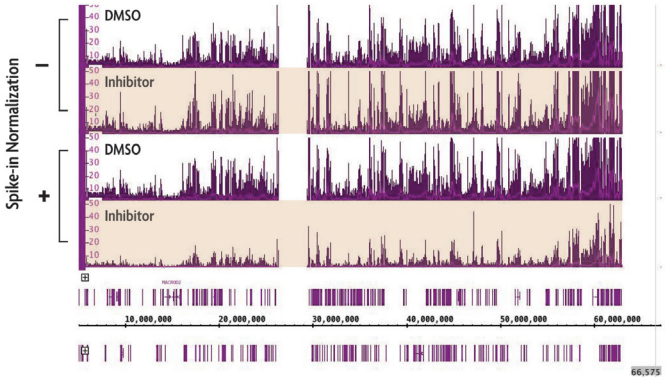
	ChIP Spike-in Reaction Guidelines			
	Sample chromatin	**Spike-in chromatin**	**Antibody of interest**	**Spike-in Antibody**
Robust antibodies against abundant histone modifications	25 µg	Refer to lot-specific data sheet	4 µg	2 µg
Antibodies against transcription factors, histone modifiers or low abundance histone modifications	25 µg	Refer to lot-specific data sheet	4 µg	2 µg

Simply add the Spike-in Chromatin and Spike-in Antibody to your standard ChIP reaction containing sample chromatin and the antibody of interest. Use the recommended ratio based on the antibody target. Perform ChIP as normal. Follow the guidelines to normalize the data for qPCR or ChIP-Seq analysis.



A

The Spike-in Antibody shows minimal cross reactivity with mammalian samples. When the Spike-in Antibody was tested in ChIP-Seq with human chromatin, there is little to no signal detected. This demonstrates the specificity of the Spike-in Antibody for the Spike-in Chromatin ensuring low background during ChIP.



B

ChIP-Seq was performed on untreated cells and cells treated with a small molecule inhibitor of EZH2 methyltransferase. Using standard ChIP-Seq analysis (–) the differences in signal are not detected. Incorporation of the Spike-in Normalization Strategy (+) reveals the expected decrease in H3K27me3 ChIP-Seq signal confirming the value of the normalization strategy for detecting biological changes.

The following guidelines are provided for qPCR normalization:

1. Perform ChIP combining the Spike-in Chromatin, Spike-in Antibody, sample chromatin and antibody of interest into the same tube for immunoprecipitation. Use the guidelines provided for chromatin and antibody quantities based on the antibody target.
2. Following purification of the ChIP DNA, perform qPCR using primers designed to amplify the experimental region of interest. The same ChIP DNA is also used in qPCR with *Drosophila* Positive control Primer Set for the Pbgs gene (Catalog No. 71037) to determine the amount of Spike-in present in each sample.
3. Use data from one of the *Drosophila* positive control IP reactions to determine a normalization factor that equalizes the signal across samples. Always use a positive primer set as the selected data point for normalization as the negative primers should have very low signal.

   (Selected Sample / Selected Sample) = Normalization factor of 1

   (Selected Sample / Sample 2) = Normalization Factor X for IP 2

   (Selected Sample / Sample 3) = Normalization Factor Y for IP 3
4. Normalize the experimental samples by multiplying each qPCR signal by it's corresponding normalization factor. These adjusted values represent the normalized data.

Below is an example of qPCR normalization:

Spike-In Antibody Binding events detected per 1000 cell values	**Example Data for qPCR Normalization**		
	Normal IP	**50% Beads**	**50% Loss**
Drosophila Negative Control Primer Set	23	20	18
Drosophila Positive Control Primer Set Pbgs	**180**	125	110

Antibody of Interest Binding events detected per 1000 cell values	**Example Data for qPCR Normalization**		
	Normal IP	**50% Beads**	**50% Loss**
Human Negative Control Primer Set	0.5	0.4	0.4
Human Positive Control Primer Set	35	24	22

To normalize the data, select a positive control sample from the Spike-in Antibody and use this value to create normalization factors for the rest of the data. In this example select the Normal IP value of 180. Divide the selected value by each spike-in data point to create the normalization factor for each IP reaction.

	Normalization Factor		
	Normal IP	**50% Beads**	**50% Loss**
Drosophila Positive Control Primer Set Pbgs	180 / 180 = 1.0	180 /125 = 1.44	180 / 110 = 1.64

Adjust the antibody of interest data by multiplying each IP reaction by it's corresponding normalization factor.

Antibody of Interest Binding events detected per 1000 cell values	**Normalized Data**		
	Normal IP	**50% Beads**	**50% Loss**
Human Negative Control Primer Set	0.5x 1.0 = **0.5**	0.4 x 1.44 = **0.6**	0.4 x 1.64 = **0.7**
Human Positive Control Primer Set	35 x 1.0 = **35**	24 x 1.44 = **34.6**	22 x 1.64 = **36.1**

The following guidelines are provided for ChIP-Seq normalization:

1. Perform ChIP combining the Spike-in Chromatin, Spike-in Antibody, test chromatin and test antibody into the same tube for immunoprecipitation. We suggest using the guidelines provided for chromatin and antibody quantities based on the antibody target.
2. Follow ChIP with Next-Generation Sequencing.
3. Map ChIP-seq data to the test reference genome (*e.g.* human, mouse or other).
4. Map ChIP-seq data to the *Drosophila* reference genome.
5. Count uniquely aligning *Drosophila* sequence tags and identify the sample containing the least number of tags.
6. Compare *Drosophila* tag counts from other samples to the sample containing the least tags and generate a normalization factor for each comparison.

   (Sample 1 with lowest tag count / Sample 2) = Normalization factor
7. Downsample the tag counts of data sets proportional to the normalization factor determined.

Below is an example of ChIP-seq normalization:

Drosophila **alignments**	**Sample 1**	**Sample 2**	**Sample 3**
Total number of reads	36,024,410	41,210,918	50,337,645
Unique alignments	4,695,354	5,182,606	3,164,431
Final number of tags without duplicate reads	4,039,904	4,136,364	**2,281,096**

Sample genome alignments	**Sample 1**	**Sample 2**	**Sample 3**
Total number of reads	36,024,410	41,210,918	50,337,645
Unique alignments	21,533,256	21,261,810	33,382,716
Final number of tags without duplicate reads	19,221,841	17,600,478	24,839,738

To normalize the data, select the sample with lowest number of *Drosophila* final tags and use this value to create normalization factors for the rest of the data. In this example select 2,281,096. Divide the selected value by the *Drosophila* tag counts from the other samples to create the normalization factor for each IP reaction.

	Normalization Factor		
	Sample 1	**Sample 2**	**Sample 3**
Drosophila final tags without duplicate reads	(2,281,096 / 4,039,904) = 0.5646	(2,281,096 / 4,136,364) = 0.5515	(2,281,096 / 2,281,096) = 1.0

Adjust the sample genome final tag counts by multiplying each sample by it's corresponding normalization factor.

	Normalized Data		
	Sample 1	**Sample 2**	**Sample 3**
Sample final tags without duplicate reads	(19,221,841 x 0.5646) = **10,853,442**	(17,600,478 x 0.5515) = **9,706,201**	(24,839,738 x 1.0) = **24,839,738**

Downsample the tags from each data set to achieve the normalized tag counts.

Technical Services

If you need assistance at any time, please call Active Motif Technical Service at one of the numbers listed below.

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