A Universal Spike-In Normalization Strategy for CUT&RUN, CUT&Tag, and ATAC-Seq

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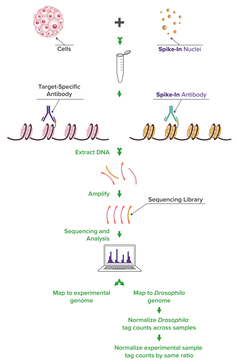
Introduction

The use of Spike-In controls as a means for normalization in NGS based assays has been demonstrated to not only be useful but in some cases necessary to detect differences in gene expression or protein occupancy. The need and usefulness of Spike-Ins have been demonstrated for RNA-Seq[1], ChIP-Seq[2], ATAC-Seq[3], and other sequencing methodologies. Multiple new methods have become commonplace in the field of chromatin biology including CUT&Tag and CUT&RUN, however, normalization has not been demonstrated. Here we applied Spike-In normalization to these techniques using *Drosophila* nuclei. Furthermore, we show that for these methods Spike-In is necessary when using treatments that confer global changes.

Spike-In Workflow



Spike-In Workflow

Spike-In nuclei are mixed with experimental cells prior to assay steps. After mixing, both the target and Spike-In antibody are added to the sample. The assay is carried out and both experimental and Spike-In DNA are isolated and prepared for sequencing. Reads mapping is performed to the experimental and the *Drosophila* genome. For data analysis, first a normalization factor (scale factor) is calculated based on the number of reads aligned to the Spike-In genome. The scale factor is then used to adjust the read depth, peak calling and bigWig files for each sample, compensating for sample-to-sample variability.

Schematic of Experimental Treatment Causing Detectable Global Change

GSK126 Treatment

GSK126 inhibits Enhancer of zeste homolog 2 (EZH2), a methyltransferase subunit in the Polycomb repressive complex 2 (PRC2) that tri-methylates H3K27[4]. **A)** A schematic of a treatment with GSK126 showing lower levels of H3K27me3 detected in treated cells. **B)** A H3K27me3 western blot performed on K562 cells that were treated with either GSK126 (+) or DMSO (-) for 3 days.

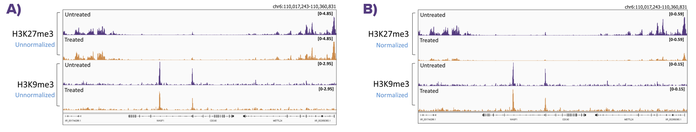
References

1. Risso, D., *et al.*, 2014. Nat. Biotechnol. 31, 896-902.
2. Egan, B., *et al.*, 2016. PLoS ONE. 11, e0166438.
3. Stewart-Morgan, K., *et al.*, 2019. Mol. Cell. 75, 284-297.
4. Zhao, Y., *et al.*, 2019. EMBO. 38, e99599.

Summary

Active Motif's Spike-In control acts as an internal reference that is not sensitive to treatment, therefore the proportion of Spike-In reads in libraries can be used as a normalizing factor. Global enrichment changes are only detectable in CUT&Tag and CUT&RUN when paired with Active Motif Spike-In technology.

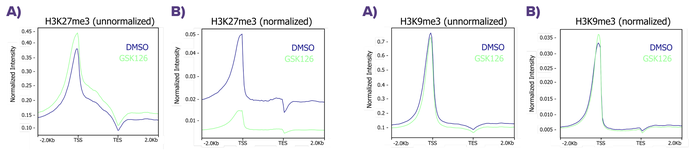
Global Changes Detected Using CUT&Tag with Active Motif Spike-In Technology



Comparison of CUT&Tag with and without Active Motif Spike-In Normalization Technology

Global changes to H3K27me3 induced by GSK126 treatment are only detected when normalized with Spike-In nuclei, while H3K9me3, an unaffected epigenetic mark remains consistent in both data sets. **A)** Genomic browser tracks generated using CUT&Tag data without Active Motif Spike-In normalization. Purple tracks correspond to cells treated with DMSO, gold tracks cell treated with GSK126 for 3 days. **B)** Genomic browser tracks generated using CUT&Tag data with Active Motif Spike-In normalization. Purple tracks correspond to cells treated with DMSO, gold tracks cells treated with GSK126 for 3 days.

Metagene Analysis for H3K27me3 and H3K9me3 (negative control) Enrichment



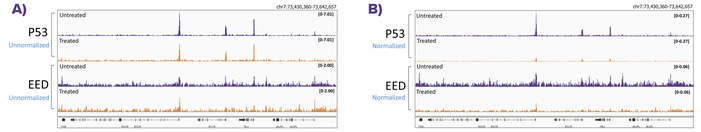
Global reduction of H3K27me3 only detected when using Spike-In normalization

Metagene analysis of CUT&Tag data for H3K27me3 generated **A)** without Spike-In normalization and **B)** with Spike-In normalization.

No detectable change for H3K9me3 when using Spike-In normalization

Metagene analysis of CUT&Tag data for H3K9me3 generated **A)** without Spike-In normalization and **B)** with Spike-In normalization.

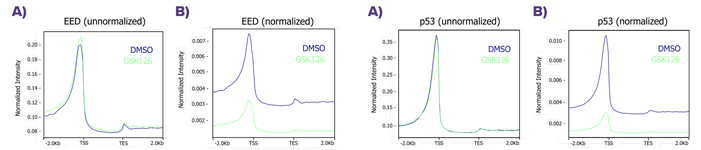
Global Changes Detected Using CUT&RUN with Active Motif Spike-In Technology



Comparison of CUT&RUN with and without Active Motif Spike-In Normalization Technology

Global changes to p53 and EED induced by GSK126 treatment are only detected when normalized with Spike-In nuclei. **A)** Genomic browser tracks generated using CUT&RUN data without Active Motif Spike-In normalization. Purple tracks correspond to cells treated with DMSO, gold tracks cell treated with GSK126 for 3 days. **B)** Genomic browser tracks generated using CUT&RUN data with Active Motif Spike-In normalization. Purple tracks correspond to cells treated with DMSO, gold tracks cell treated with GSK126 for 3 days.

Metagene Analysis for p53 and EED Enrichment



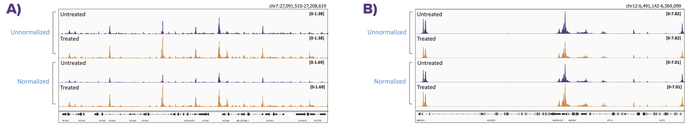
Global reduction of EED only detected when using Spike-In normalization

Metagene analysis of CUT&RUN data for EED generated **A)** without Spike-In normalization and **B)** with Spike-In normalization.

Global reduction of p53 only detected when using Spike-In normalization

Metagene analysis of CUT&RUN data for p53 generated **A)** without Spike-In normalization and **B)** with Spike-In normalization.

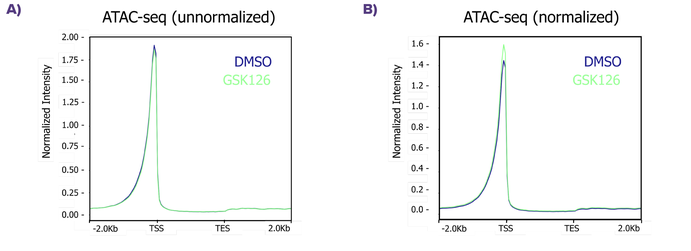
GSK126 Causes Local but Not Global Changes in Chromatin Accessibility



Comparison of ATAC-Seq with and without Active Motif Spike-In Normalization Technology

Local changes in chromatin accessibility are observed at Polycomb protein gene targets but not at house keeping gene loci suggesting GSK126 does not cause global changes. A) Genomic browser tracks generated using ATAC-Seq data at *HOXA* gene cluster with and without Active Motif Spike-In normalization. Purple tracks correspond to cells treated with DMSO, gold tracks cells treated with GSK126 for 3 days. B) Genomic browser tracks generated using ATAC-Seq data at *GAPDH* locus. Purple tracks correspond to cells treated with DMSO, gold tracks cells treated with GSK126 for 3 days.

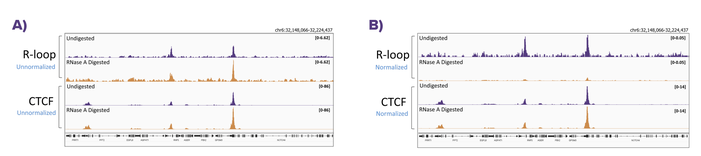
Metagene Analysis for ATAC-Seq Enrichment



No global increase of chromatin accessibility detected for GSK126 treated cells

Metagene analysis of ATAC-Seq data generated **A)** without Spike-In normalization and **B)** with Spike-In normalization. Spike-In normalization gives confidence that changes are not global.

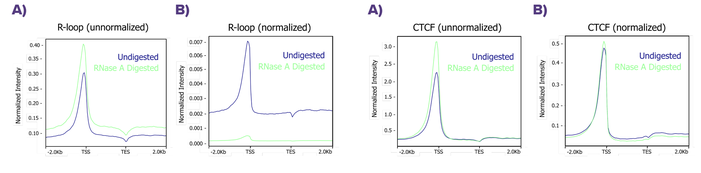
Global R-loop Changes Detected using CUT&Tag with Active Motif Spike-in Technology



Comparison of CUT&Tag data of RNase A digested permeabilized cells with and without Active Motif Spike-In Normalization Technology

Global changes to R-loops induced by RNase A digestion are only detected when normalized with Spike-In nuclei, while CTCF, an unaffected target, remains unchanged in both data sets. A) Genomic browser tracks generated using CUT&Tag data without Active Motif Spike-In normalization. Purple tracks correspond to undigested cells, gold tracks cells digested with RNase A. B) Genomic browser tracks generated using CUT&Tag data with Active Motif Spike-In normalization. Purple tracks correspond to undigested cells, gold tracks cells digested with RNase A.

Metagene Analysis for R-loop and CTCF Enrichment



Global reduction of R-loops by RNase A digestion only detected when using Spike-In normalization

Metagene analysis of R-loop CUT&Tag data generated A) without Spike-In normalization and B) with Spike-In normalization.

No detectable change for CTCF after RNase A digestion when using Spike-In normalization

Metagene analysis of CUT&Tag data for CTCF generated A) without Spike-In normalization and B) with Spike-In normalization.