

The use of a synthetic histone peptide-DNA conjugates as a spike-in reagent to normalize chromatin immunoprecipitation experiments

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Abstract

Chromatin immunoprecipitation (ChIP) is an analytical method used to investigate interaction of proteins with specific genomic DNA regions *in vivo* and provide a better understanding of the mechanisms of gene regulation, DNA replication and DNA repair. Variations in the efficiency of the immunoprecipitation, background signal from carryover in immunoprecipitation, and loss of material during the purification of the ChIP DNA are sources of variability that restrict the use of ChIP as a quantitative tool. We have developed a method to improve the consistency and quantification of ChIP data based on the use of a synthetic peptide-DNA spike in reagent that mimics the behavior of cross-linked chromatin in the immunoprecipitation and recovery reactions. A fixed amount of the synthetic peptide-DNA complex is spiked into the chromatin and co-ChIPed simultaneously with the antibody of interest, and the amount of the recovered synthetic DNA is then used to normalize the results obtained with the antibody of interest. The spike in control can be designed using a peptide specific to the ChIP antibody of interest and acts as an exquisitely sensitive molecular readout for antibody-immunogen affinity. Our data show that use of the spike in control improves the dynamic range of ChIP signal, can strongly reduce the variability between ChIP assay replicates or user to user variation, and is very useful for characterization of antibody specificity. The sequence of the spike-in can also be incorporated into NGS libraries to provide a reference signal and normalize off target effects from antibody dependent ChIP-seq signals.

Material and Methods

Peptides conjugated to 125 base oligonucleotides were manufactured at 21st Century Biochemical as described in US 20130217027 A1 (Mass General Hospital). Double stranded peptide conjugated molecules were diluted in a carrier buffer containing PBS, glycerol and BSA to prevent adherence to the microfuge tubes. Chromatin immunoprecipitations were performed using the EMD Millipore Magna ChIP™ HiSens kit. Briefly, cells were cross-linked with 1% formaldehyde. Nuclei were isolated and sonicated (with and without varying quantities of the spike in reagent) to produce chromatin of ~500 bp. For chromatin samples where spike in was not included during sonication, spike in was added at the indicated concentrations. Antibodies of interest were bound to magnetic protein A/G beads in an Eppendorf® tube at 4° C for 2 hours. ChIP reactions were performed overnight followed by wash with high and low stringency buffers. After protein digestion and crosslink reversal, antibody bound DNA was analyzed by qPCR at positive, negative loci, as well as quantitated for amplification of the spike in DNA sequence without additional clean-up. ChIP efficiency (percent of input) for each locus was then analyzed by comparing ChIP DNA with input sample. ChIP results for each locus were then normalized to the internal control locus. For ChIP-seq experiments, ChIP'd DNA was extracted via Phenol:chloroform following proteinase K digestion, and immunoprecipitations of 1e6 cell equivalents of chromatin was performed with varying amounts of spike in reagent with 20 uL Magna ChIP Protein A/G beads. Following standard Illumina library construction, libraries were size selected with Agencourt bead using a modified cutoff of ~200 bp to allow retention of the adapted spike in sequences (~250 bp). Prior to alignment of FastQ files, tags from the spike in sequence were counted and filtered prior to Bowtie mapping.

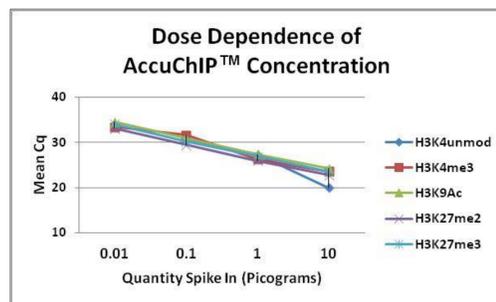


Figure 2. Dynamic range and dose dependence of AccuChIP spike in reagents in ChIP. Various designs of peptide-nucleic acid conjugates were spiked into 1e5 HeLa cell equivalents from 10 fg to 10 pg and the recovery of the synthetic standard in ChIP reactions was monitored via qPCR detection of the spike in nucleic acid. Antibodies were used at optimal ChIP concentration and included 05-1341 (anti-H3K4unmod), 05-745R (anti-H3K4me3), ABE18 (H3K9Ac), 07-452 (K3K27me2), and 07-449 (HeK27me3).

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Results

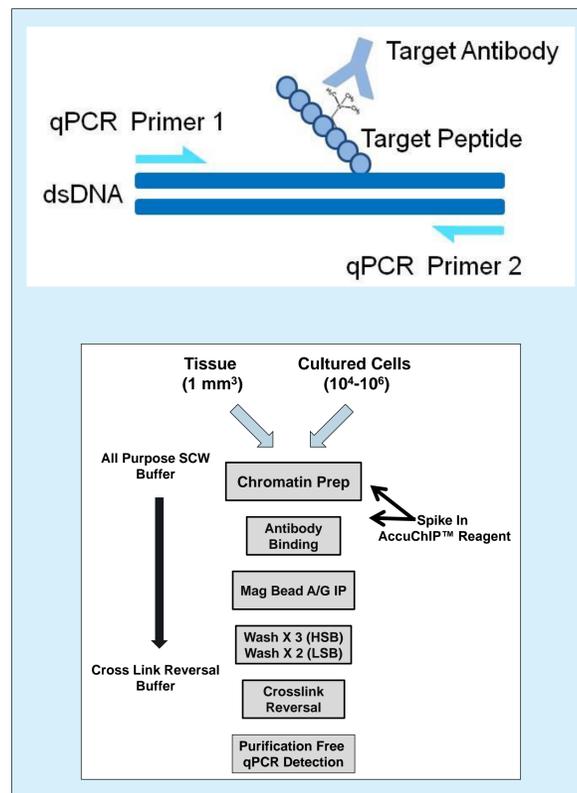


Figure 1. Design of AccuChIP Affinity Reagent and Use in Magna ChIP HiSens Protocol. Spike in reagent is spiked in to chromatin either during chromatin preparation or prior to antibody binding at concentrations that minimally compete or do not interfere with chromatin signal. Magna ChIP HiSens is a simplified ChIP protocol using Magnetic Protein A/G beads and a single buffer for sonication, ChIP, and washes (SCW Buffer). DNA purification is optional with this protocol (recommended for ChIP-seq application) and additional qPCR reactions are programmed to include amplification of the reference Spike in sequence as a quantitative measure of antibody affinity and molecules of recovered antigen. Values for the spike in can then be used to scale ChIP data at various loci, chromatin types or conditions to reduce variability introduced by the user's manipulation of the immunoprecipitation reaction.

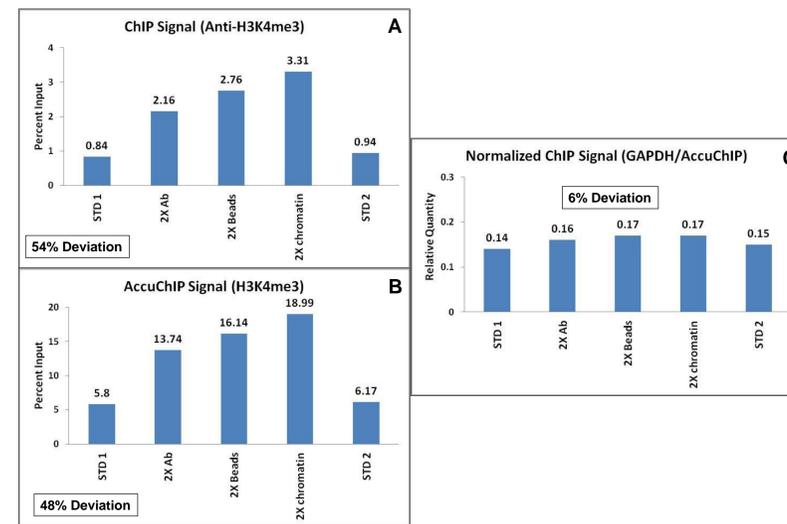


Figure 3. Reduction in ChIP variability using the AccuChIP spike in reagent. The AccuChIP™ reagent H3K4me3 was spiked into HeLa chromatin for various experiments at 10 pg/1e6 cell equivalents and ChIP performed Magna ChIP™ HiSens and 05-745R Anti-H3K4me3. Experiment parameters were varied including a standard reaction, 2X antibody, 2X beads, 2X chromatin compared to the standard, and the positive control ChIP locus (Gapdh promoter, A) was measured by qPCR compared to the same DNA samples where spike in amplification was measured (B). Percent Input values of ChIP Signal were divided by the reference spike in signal to obtain relative normalized ChIP units. IgG was eliminated from the analysis once background levels of the ChIP reaction were measured. Relative values show little fluctuation and a significant reduction in variability in normalized signal as a result of use of the AccuChIP™ spike in.

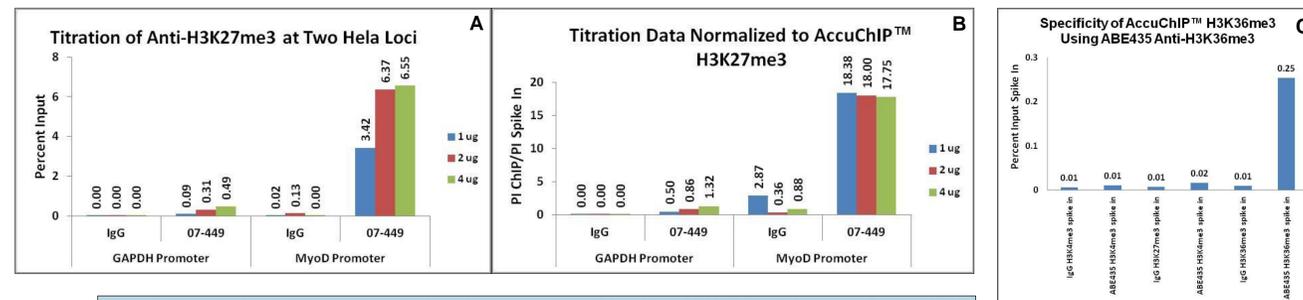


Figure 4. Normalization of differences in antibody performance and specificity of AccuChIP™ Spike In Reagents. The AccuChIP™ reagent H3K27me3 was spiked into 1e5 HeLa chromatin equivalents and Magna ChIP HiSens ChIP performed using 1, 2 and 3 ug of Anti-H3K27me3 (07-449). ChIP'd DNAs were analyzed using qPCR assays for a negative (GAPDH Promoter) and positive (MyoD Promoter) locus and percent input calculated using ddCt analysis (A). Percent input values for the spike in sequence specific to the AccuChIP were used to normalize the locus specific data, resulting in reduction of the impact of varying the amount of antibody used for ChIP (B). To demonstrate specificity of the individual AccuChIP reagents, 1e5 HeLa chromatin equivalents were programmed with 5 pg of H3K4me3, H3K27me3 and H3K36me3 (C). ChIP was performed using the anti-H3K36me3 antibody (ABE436), and presence of each spike in reagent was measured by qPCR.

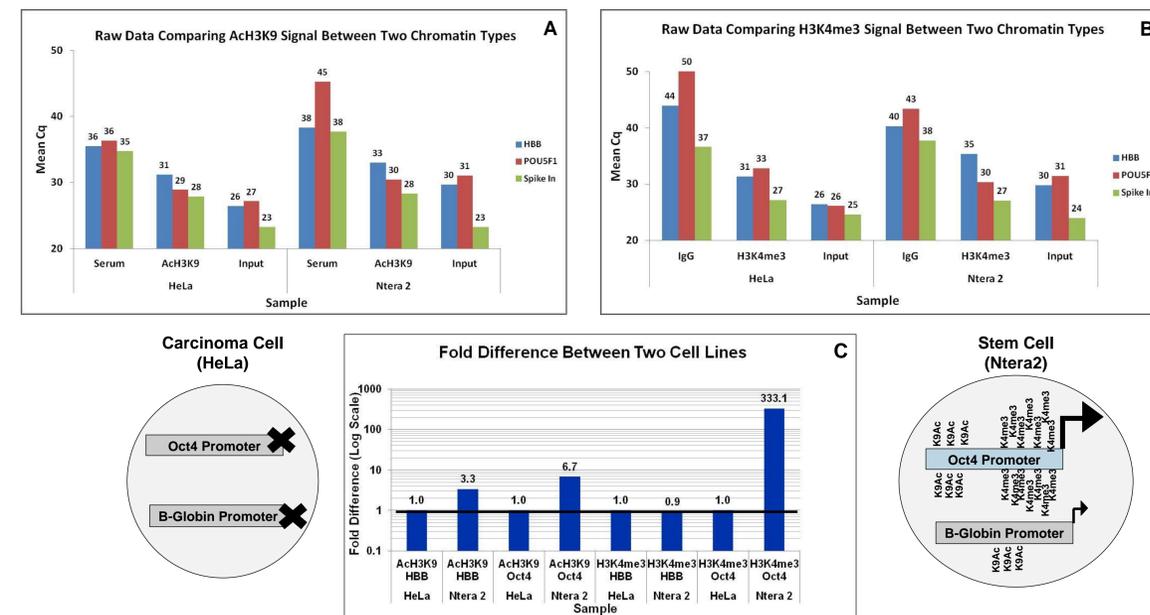


Figure 5. Use of Two AccuChIP spike in reagents to quantitatively assess representation of H3K4me3 and Ach3K9 at two loci in two chromatin types. The AccuChIP™ reagents H3K4me3 and Ach3K9 were spiked into 1e5 chromatin equivalents of HeLa cells or Ntera2 cells at 5 pg spike in each and Magna ChIP HiSens ChIP performed using Anti-Ach3K9 (ABE18) and Anti-H3K4me3 (05-745R). Mean Ct values are shown for Ach3K9 (A) and H3K4me3 (B) at the B-globin promoter or the Oct 4 (POU5F1) promoter. Following determination of Percent Input values for all amplicons, data was normalized to yield relative fold difference of site occupancy between the chromatin types at the two loci by setting HeLa values at each locus to 1 and plotting data on a base 10 log scale (C). Significant enrichment of the H3K4me3 was observed in the stem like cell line Ntera2 at the Oct 4 Promoter, while a modest increase in acetyl H3K9 signal was seen in Ntera2 over HeLa signal.

Summary

- AccuChIP Spike In Reagents are useful for removing variability in ChIP reactions due to user error, and make ChIP reactions more flexible regarding quantity of chromatin, antibody, beads and assay performance.

- These reagents can also be used in mixed panels to determine specificity of antibodies used in ChIP

- As a synthetic spike in that is exquisitely sensitive to antibody affinity and specificity, the spike in can be used to normalize data from ChIP qPCR experiments and add a level of quantitative control to multisample ChIP experiments

- Use in ChIP-seq is possible given appropriate blending of reference spike ins