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A streamlined method for rapid and sensitive chromatin immunoprecipitation

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Abstract

We report a streamlined procedure to efficiently carry samples from chromatin to qPCR-compatible DNA in as little as 4 hours. We use this streamlined ChIP to quantify histone H3 modifications at active (cad) and repressed (T early alpha) promoters in a Rag1-deficient pro-T cell line after 1–2 hour IP. We further show that the protocol is equally effective when usingreadily quantified histone modifications in chromatin from as few as 10^4 Rag-deficient DN thymocytes. Taken together, these data outline a simple, cost-effective procedure for efficient ChIP analysis.

Keywords

Chromatin immunoprecipitation; histone; epigenetics; cad; TEA; thymocyte; Rag

1. Introduction

The fast-growing fields of epigenetics and epigenomics have necessitated development of assays that probe interactions between DNA, structural histone proteins, and regulatory transcription factors. One of the primary tools used to unravel the so-called “histone code” has been chromatin immunoprecipitation, or ChIP (Dedon et al., 1991; O’Neill and Turner, 1996; Kuo and Allis, 1999). Indeed, ChIP, coupled with realtime PCR (qPCR) has become the gold standard assay for chromatin organization (Jenuwein and Allis, 2001), and is increasingly used to demonstrate differential transcription factor recruitment to various promoters (Morshead et al., 2003; O’Neill et al., 2006). Despite such widespread use, the complexity, lengthiness, and scale of the standard ChIP protocol, which can take up to 3 days and require $\geq 10^6$ cells per reaction, make it extremely sensitive to experimenter-induced variability and to contamination, and limit its utility for scarce cell populations, such as those found in select compartments of the immune system.

A number of groups have now proposed modifications to the standard ChIP protocol (Nelson et al., 2006; O’Neill et al., 2006; Attema et al., 2007; Dahl and Collas, 2007; Dahl and Collas, 2008). The newer protocols have demonstrate that ChIP is amenable to variations in virtually

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every aspect of the assay from the size of chromatin input, the time dedicated to immunoprecipitation, washing, elution, and crosslink reversal, to Proteinase K treatment regiments. By replacing agarose or sepharose beads with Protein A- or Protein G-coupled paramagnetic beads, newer approaches minimize the need to preclear input chromatin of antibody-independent bead binding activities. At the same time, the ability to easily and quantitatively capture magnetic bead complexes eliminates the need for centrifugation, which both reduces the time required for each of the many washes in the ChIP protocol, and reduces the potential for sample loss or contamination during wash aspiration.

We have designed a streamlined protocol that incorporates improvements offered by the Q2-ChIP (Dahl and Collas, 2007), FastChIP (Nelson et al., 2006), ChIP-IT Express (ActiveMotif), and miniChIP (Attema et al., 2007) into a simplified format. We find this streamlined protocol is easily mastered, rapid, and highly reproducible. Demonstrating its adaptability to reduced sample size, our streamlined ChIP readily quantitated histone modifications and even binding of the RAG1 component of V(D)J recombinase in as few as 10^4 CD4/CD8 double negative thymocytes harvested from a Rag-2 deficient mouse. Though we find each of the existing ChIP protocols can be highly effective, we propose our streamlined protocol as a simplified approach for those new to ChIP or for higher throughput ChIP screens.

2. Materials and Methods

2.1 Cells

The RAG1$^{-/-}$, p53$^{-/-}$ pro-T cell line, P5424, has been previously described (Mombaerts et al., 1995). P5424 cells were cultured at 37°C/5% CO_2 in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 0.01% penicillin/streptomycin, and 50 μM β-mercaptoethanol. Thymii were isolated from 4–8 wk old Rag2$^{-/-}$ mice, crushed and filtered to yield a single cell suspension, and red blood cells were removed by hypotonic lysis. The mouse studies described here were reviewed and approved by the institutional animal care and use committee at North Carolina State University.

2.2 Antibodies

Rabbit polyclonal antisera to acetylated H3K9 (06–599) and dimethylated H3K4 (07–030), along with rabbit control IgG (12–370) were purchased from Upstate. Rabbit polyclonal antisera to dimethylated H3K9 (ab1772) and trimethylated H3K4 (ab8580), and mouse monoclonal antibody to RNA polymerase II (ab5408) were purchased from Abcam.

2.3 Chromatin Preparation

Protein:DNA complexes in 4×10^6 P5424 or freshly isolated thymocyte suspension cells were cross-linked using formaldehyde (1% final) in either tissue culture dishes or conical centrifuge tubes with gentle shaking for 10 min. at room temperature. Crosslinking was stopped by drop-wise addition of glycine (125 mM final concentration) and gentle shaking for 5 min. at room temperature. Cells were pelleted, washed in 1X PBS (5 ml). Pelleted cells were resuspended in 500 μl lysis buffer (10 mM Tris-HCl, pH7.5, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP-40) supplemented with 1 mM PMSF and 1X Protease Inhibitor Cocktail (PIC, (Roche), and incubated for 30 min on ice. Nuclei from the lysed cells were pelleted by microcentrifugation (5000 rpm for 10 min. @ 4°C). Nuclei were resuspended in 100 μl MNase reaction buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 4% NP-40) supplemented with 1 mM PMSF and 1X PIC, and chromatin was sheared with the addition of 1–2 U MNase (Sigma) for 10 min at 37°C. Digestion was stopped with the addition of EDTA (10 mM final), and the resultant chromatin was stored @ −80°C.

2.4 Chromatin Immunoprecipitation

For ChIP, paramagnetic Dynabeads (Dynal) separately coupled to Protein A (10 μl/IP) and Protein G (10 μl/IP) were combined in a 1.5 ml microcentrifuge tube, captured by placing the tube against a strong magnet, and the suspension medium was immediately aspirated while the beads were held in place by the magnet. Beads were washed as described (Dahl and Collas, 2007) with the addition of 100 μl/IP 1X RIPA buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% SDS, 0.1% NaDeoxycholate) containing BSA (50 mg/ml) and sheared salmon sperm DNA (0.5 mg/ml), denoted as RBD hereafter. Beads were vortexed (3 sec.), and recaptured. Wash solution was aspirated, and the wash was repeated. Beads were resuspended in RBD (50 μl/IP) containing PMSF and PIC, aliquoted into sterile 200 μl PCR strip tubes containing 1–5 μg antibody, and rotated 1 hr at 4°C. Conjugated antibody:bead complexes were washed 2X in RBD as described above, and resuspended in 75 μl/IP volumes of RBD supplemented with PMSF and PIC.

For each IP, antibody:bead suspensions were mixed with 25 μl undiluted chromatin (10^6 cell equivalents) or chromatin diluted in RBD containing PMSF and PIC as indicated (Fig. 3). Protein-DNA complexes were immunoprecipitated for 2 hours (except Fig. 1) at 4°C with rotation. Bead complexes were captured by placing the PCR strip tubes on a horizontal bar magnet (attached to an empty 200 μl micropipettor tip rack), IP solution was aspirated, and beads were resuspended in 180 μl RBD. To wash the bead complexes, the strip tubes were rapidly moved back and forth across the magnet 5 times (sequentially forcing the beads from one side of the tube to the other). Beads were recaptured and the wash was repeated 3X. Following RBD wash, bead complexes were washed 2X in 180 μl TE, pH 8.0.

To elute protein-DNA complexes, beads were resuspended in 100 mM $NaHCO_3$ (100 μl), strip tubes were resealed with fresh strip caps, and were gently vortexed 15 min @ room temperature. Tubes were returned to the magnet, and protein-DNA eluates were transferred to fresh PCR strip tubes containing 4 μl 5M NaCl. To prepare matched input samples, 10 μl of input chromatin was diluted in 90 μl 100 mM $NaHCO_3$, transferred to a strip tube containing 4 μl 5M NaCl, and carried along with the IP samples for all subsequent steps. Crosslinks were reversed @ 95°C for 15 min., tubes were cooled to room temperature, and then incubated with Proteinase K (10 μg/ml final) 1 hr @ 45°C. Digestion was stopped with addition of PMSF (2 mM final), DNA was purified using Qiaquick nucleotide removal columns (Qiagen) according to the manufacturer's instructions, and eluted in 100 μl Qiagen elution buffer.

2.5 Q-PCR and data analysis

For realtime PCR, bound (3 μl) and input (3 μl of 1:100 dilution) samples were amplified in a MyIQ thermal cycler (Bio-RAD) using 1X SensiMix Plus (Quantace) and primers specific for the cad promoter (forward: 5′-GTCTGCGTGCTTGCCCTGTCTCAGC-3′; reverse: 5′-CGGGCTTGCTTACCCACTTCCCCAGC-3′), or TEA (McMurry and Krangel, 2000; Sikes et al., 2002), or 57prime;PDβ2 promoter (forward: 5′-GTTTCTGAGGCATGTGTCTCTGCG-3′; reverse: 5′-TCCTCTTTGTCACAGTGCCCACC-3′). Cycling parameters for 20 μl reactions were 95°C 10 min., followed by 45 cycles of 95°C, 30 sec.; 57°C (TEA) or 72°C (cad), 30 sec.; 72°C, 30 sec, followed by melt curve analysis. Fold enrichment in the bound fractions relative to input was calculated from as previously described (Ciccone et al., 2004), and the average enrichment for triplicate amplifications was reported.

3. Results

3.1 Synthesis of a streamlined ChIP protocol from existing methods

Although extremely effective, the standard ChIP protocol as described by Kuo and Allis (Kuo and Allis, 1999) remains time-intensive and difficult. By contrast, we recently found that multiple modified ChIP protocols including the Q2ChIP (Dahl and Collas, 2007) and ChIP-IT Express (ActiveMotif) were readily mastered by less experienced lab members. Noting the remarkable differences between each of these effective protocols, we sought to synthesize a single method that would minimize tradeoffs for cost, ease of use, speed, and reproducibility.

For our studies, we used input chromatin prepared essentially as described by Ciccone et al. (Ciccone et al., 2004), in which sonication-based shearing is replaced with micrococcal nuclease digestion. We favored the enzymatic digestion as a means to minimize technique-dependent variability between users and laboratories. Additionally, crosslinking and glycine treatments were performed in conical centrifuge tubes rather than culture dishes. However, this alteration necessitated that we eliminate a PBS wash between the formaldehyde and glycine treatments. Consequently, reactions were stopped by dropwise addition of glycine.

Our antibody:bead conjugation and IP conditions essentially followed the Q2ChIP protocol (Dahl and Collas, 2007). Consequently, preblocking the beads and preclearing chromatin to prevent nonspecific DNA binding proved unnecessary. BSA (50 mg/ml) and sheared salmon sperm DNA (0.5 mg/ml) were added, and antibody:bead conjugates were thoroughly washed to block nonspecific antibody:DNA interactions and reduce ChIP variability. Finally, to increase the uniformity of bead conjugation with different antibody preparations, we incubated 1:1 mixes of Protein A- and Protein G-coupled beads with each antibody for 1 hr, though the manufacturer's instructions and our own tests suggest that conjugation can be reduced to as little as 15 minutes (data not shown).

Conjugates were mixed with varying concentrations of chromatin at 4°C, and immune complexes were allowed to form over a 2 hr window. We adapted the post-IP sample processing procedures used in ActiveMotif's ChIP-IT Express kit, in which the crosslink reversal times are dramatically shortened. We used the Q2ChIP buffers (RIPA and TE) to wash immune complexes 4X and 2X respectively. In each case, washes were performed by repeatedly passing PCR strip tubes containing resuspended immune complexes across an immobilized bar magnet. In this way, all 6 washes for as many as 16 IP reactions could be completed in as little as 15 min. We observed no benefit from incubating complexes in the wash buffers prior to aspiration.

After washing, captured immune complexes were eluted from the beads in 100 mM NaHCO3 by affixing the strip tubes to a benchtop vortexer set at 50% speed for 15 min. Crosslink reversal was similarly limited to 15 min. For this, NaCl (250 mM final) was added, and eluates were immediately recovered from the beads. Crosslinks in the eluates were then reversed in a thermal cycler for 15 min. at 95°C, Proteinase K was added, and eluates were incubated for 1 hr at 45° C. To minimize handling and experimental variability, we stopped protease digestion by adding PMSF, and purified sample DNAs using Qiaquick Nucleotide Removal columns (Qiagen). However, we found that phenol:chloroform extraction and ethanol precipitation were equally effective at yielding Q-PCR-competent DNA.

3.2 Increased IP duration does not enhance specific signal

To determine if the 2 hr IP time could be reduced, we set up ChIP reactions using chromatin from 10^6 $Rag1^{-/-}p53^{-/-}$ P5424 cells (a murine CD4/CD8 double negative pro-T cell line) and antibodies to histone H3 carrying a trimethylation of lysine 4 (H3K4me3). To validate our IP results, we measured Q-PCR signals at 2 distinct promoters, cad, which has previously been shown in P5424 to bear histone modifications consistent with open chromatin and

transcriptional activity (Morshead et al., 2003), and TEA, which exists in a repressive chromatin state in Rag2-deficient thymocytes (McMurry and Krangel, 2000). Two hr IP with anti-H3K4me3 specifically enriched an amplicon within the cad gene promoter >14 fold above untreated input, while IP with nonspecific IgG showed no enrichment (Fig. 1A). Increasing IP duration to 4 or 16 hours did not increase fold enrichment of the anti-H3K4me3 samples relative to input or IgG controls. In fact, enrichment was equally effective after only a single hr IP. In contrast to our cad findings, H3K4me3 levels at TEA were not significantly enriched above control IgG, even after overnight IP (Fig. 1B). We did not determine if IP reactions shorter than 1 hr would be equally effective, though other protocols have limited IP to 45 min. (Nelson et al., 2006).

3.3 Streamlined ChIP effectively distinguishes between accessible and repressed gene targets

To extend our validation, we next screened cad and TEA chromatin with a panel of activation state-specific antibodies (Fig. 2). Two hr IP with antibodies to either dimethylated or trimethylated H3K4 showed equivalent enrichment over input control at the cad promoter (Fig. 2A; 13.25 ± 2.17 and 11.44 ± 1.92, respectively). Enrichment of H3K4me2 was similarly observed at TEA, though H3K4me3 levels were significantly reduced (Fig. 2B; 25.93 ± 0.45 and 2.15 ± 0.70, respectively). We next measured levels of opposing acetylation and dimethylation at H3K9 and recruitment of RNA polymerase II. The cad promoter was enriched for both H3K9 acetylation and RNA Pol II recruitment, both markers of accessible chromatin, but not for the repressive chromatin marker H3K9me2 (Fig. 2A). Conversely, H3K9me2 levels but not H3K9ac levels were elevated at TEA (Fig. 2B), consistent with a repressed chromatin state. When we assessed Pol II binding at TEA, we again saw enrichment equivalent to that at cad. RNA Pol II stalling prior to transcription elongation has been recently shown across the human genome (Muse et al., 2007). Likewise, the differential H3K4 methylation status (me2 +/me3−) of TEA is mirrors that observed for developmentally poised genes (Santos-Rosa et al., 2002;Mikkelsen et al., 2007;Orford et al., 2008). Taken together, our observations suggest that TEA exists in a poised state in P5424 DN cells.

3.4 Streamlined ChIP remains effective with reduced chromatin input

The Q2ChIP is reported to remain effective despite dramatic reduction of input chromatin levels (Dahl and Collas, 2007). To determine if our modified protocol was equally effective in the presence of reduced input, we measured enrichment of H3K4me2 and H3K4me3 at cad after IP of serially diluted P5424 chromatin (Fig. 3). In each case, antibody and bead concentrations were held constant to isolate the impact of chromatin reduction. We found that 10^4 cell equivalency was the minimum amount of chromatin that would still yield reproducible Q-PCR signal for both H3K4 antibodies. AntibodyWhen normalized to input antibody-dependent enrichment when using chromatin from 10^4 cells showed roughly a 2-fold reduction relative to more concentrated input levels. However, PCR signals in control IgG samples failed to cross threshold, suggesting that antibody-specific enrichment was still considerable. Chromatin dilution to 10^3 cell equivalents yielded reproducible Q-PCR signal only for H3K4me2 enrichment, and subsequent dilutions failed to amplify altogether, a marked difference from the Q2-ChIP which showed effective ChIP for $\leq 10^2$ cells (Dahl and Collas, 2007).

Although dilution of chromatin prepared from large-scale P5424 cultures suggested that our ChIP protocol was effective when chromatin was limiting, we wanted to demonstrate its application to analysis of rare cell populations in vivo. Thymii from Rag-deficient mice contain approximately 10^6 thymocytes, all prevented from progressing beyond DN development by their inability to rearrange the TCRβ gene locus (Mombaerts et al., 1992; Shinkai et al., 1992). To test the utility of our streamlined ChIP protocol for limiting cell populations, we

measured histone modifications at TEA in chromatin from 10^4 freshly isolated $Rag2^{-/-}$ thymocytes (Fig. 4). Consistent with our data using 10^6 P5424 cells (Fig. 2B), ChIP of the TEA promoter in Rag2-deficient thymocytes showed elevated H3K9 methylation (9.27 ± 3.79), H3K4me2 (21.65 ± 5.36), and RNA Pol II loading (17.35 ± 8.10), despite moderately increased background levels obtained with control IgG (1.61 ± 0.01). By contrast, enrichments using antibodies to H3K9ac and H3K4me3 (3.01 ± 1.97 and 2.21 ± 0.77, respectively) were similar to IgG. Taken together with our analysis in P5424, these data strongly suggest that TEA exists in a poised chromatin state during DN thymocyte development prior to β selection and the activation of Tcra recombination.

4. Discussion

Although enormously powerful, the conventional methodology for ChIP is a somewhat daunting procedure that requires large amounts of chromatin input and stretches across several days. A series of recent studies have shown that individual steps of the standard ChIP protocol can be extensively modified. We have synthesized the various improvements to the conventional ChIP protocol to develop a simplified method that is significantly shorter, uses a minimum amount of costly reagents and equipment, minimizes potential for sample loss or contamination, and is compatible with reduced chromatin input. Most importantly, IP, post-IP washes, and crosslink reversal times are all dramatically reduced in the streamlined protocol without compromising the ability to quantify gene-specific differences in histone modification and binding site occupancy.

Whereas recent protocols have reported successful ChIP with chomatin prepared from as few as 100 cells (Dahl and Collas, 2007), we found that amplification signals declined when chromatin was IPed from fewer than 10^5 cells (Fig. 3). Despite reduced signal, we had no difficulty quantifying epigenetic alterations in chromatin from 10^4 freshly isolated RAG2-deficient thymocytes (Fig. 4). Though apparently less sensitive than Q2-ChIP, the practical impact of reduced chromatin demand in our protocol allows us to run as many as 100 IP reactions with chromatin isolated from a single RAG knockout animal, a marked difference with standard protocols that would require between 1 and 10 thymii per IP. Quantitation of purified DNA using a NanoDrop spectrophotometer prior to Q-PCR showed that IPs with 10^4 cell equivalent inputs typically yielded between 50 and 100 ng DNA, of which 1 to 3 ng were amplified per reaction. With this amount of input DNA, threshold cycles in both the TEA and cad PCRs ranged from 33 to 36 cycles. Although IP reactions may remain quite effective using even less chromatin, we expect that 10^4 cell equivalents reflects the limit below which our lab cannot effectively amplify available targets.

In developing the streamlined ChIP protocol, we uncovered a previously unreported aspect of thymocyte biology; namely the poised state of TEA in DN cells prior to Tcra recombination. The observed dichotomy between dimethylated and trimethylated H3K4 at TEA has been recently shown to mark poised developmentally regulated promoters at a variety of hematopoietic genes (Orford et al., 2008) as well as at promoters across the genome (Mikkelsen et al., 2007). In addition to selective H3K4 dimethylation, enrichment of RNA pol II at TEA strongly argues that in the DN3 developmental state of P5424 the TEA promoter is repressed but primed for rapid activation in later staged cells.

In summary, the streamlined ChIP protocol outlined here provides a rapid, sensitive, and efficient alternative to the standard protocol. Its ease of use should allow the protocol's ready adoption by programs not currently using ChIP, and its reduced dependence on input chromatin should facilitate extension of current epigenetic studies. Indeed, we are currently using our streamlined ChIP protocol to pursue imposition of a poised state at the TEA promoter during individual stages of T lineage development.

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Abbreviations used in this paper

ChIP
: chomatin immunoprecipitation

H3
: histone 3

Pol II
: RNA polymerase 2

Ab
: antibody

cad
: carbamoylphosphate synthetase/aspartate carbamyltransferase/dihydroorotase gene

TEA
: T early alpha promoter

Rag
: Recombination activating gene

TCR
: T cell receptor

DN
: double negative

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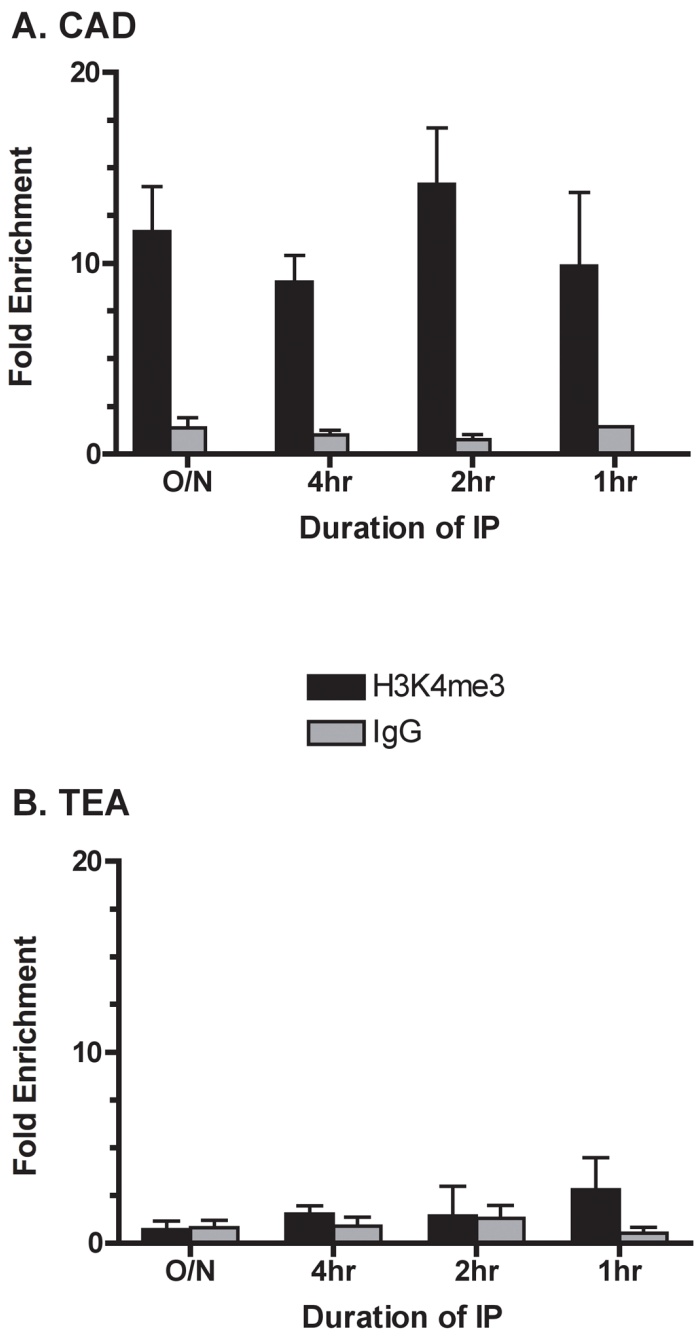

Figure 1.

Sensitivity of ChIP to reduction in the duration of IP incubation. Chromatin from the Rag1$^{-/-}$p53$^{-/-}$ mouse thymocyte cell line, P5424, was immunoprecipitated for the indicated times with paramagnetic beads complexed with either anti-H3K4me3 (black) or control IgG (grey). Fold enrichment of cad (A) or TEA (B) DNA following IP with each antibody is shown relative to an input sample. Bars indicate means (± SD) of triplicate Q-PCRs, and are representative of 2 experiments with independent chromatin preparations.

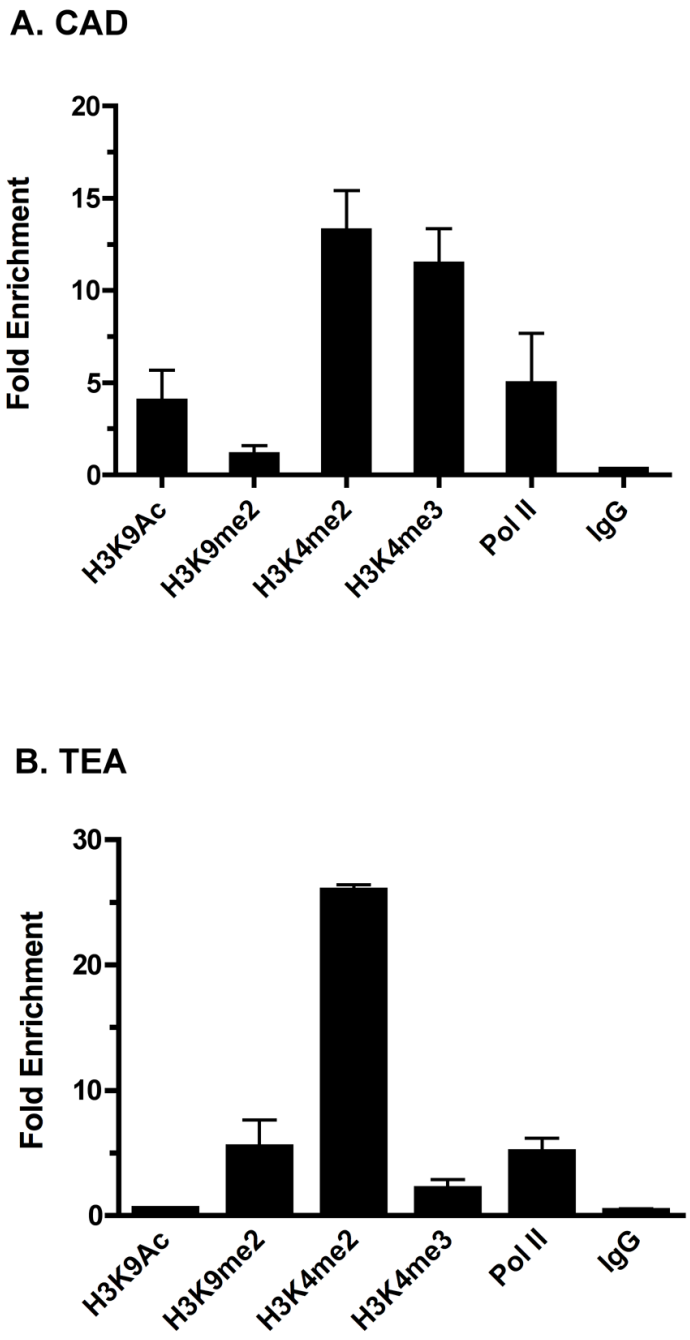

Figure 2.

Analysis of histone H3 modifications at differentially accessible promoters in P5424 chromatin. Bead complexes with each of the indicated antibodies were incubated with P5424 chromatin for 2 hours. Resultant fold enrichments of cad (A) and TEA(B) promoter sequences in the IP samples relative to input are calculated as in Fig. 1.

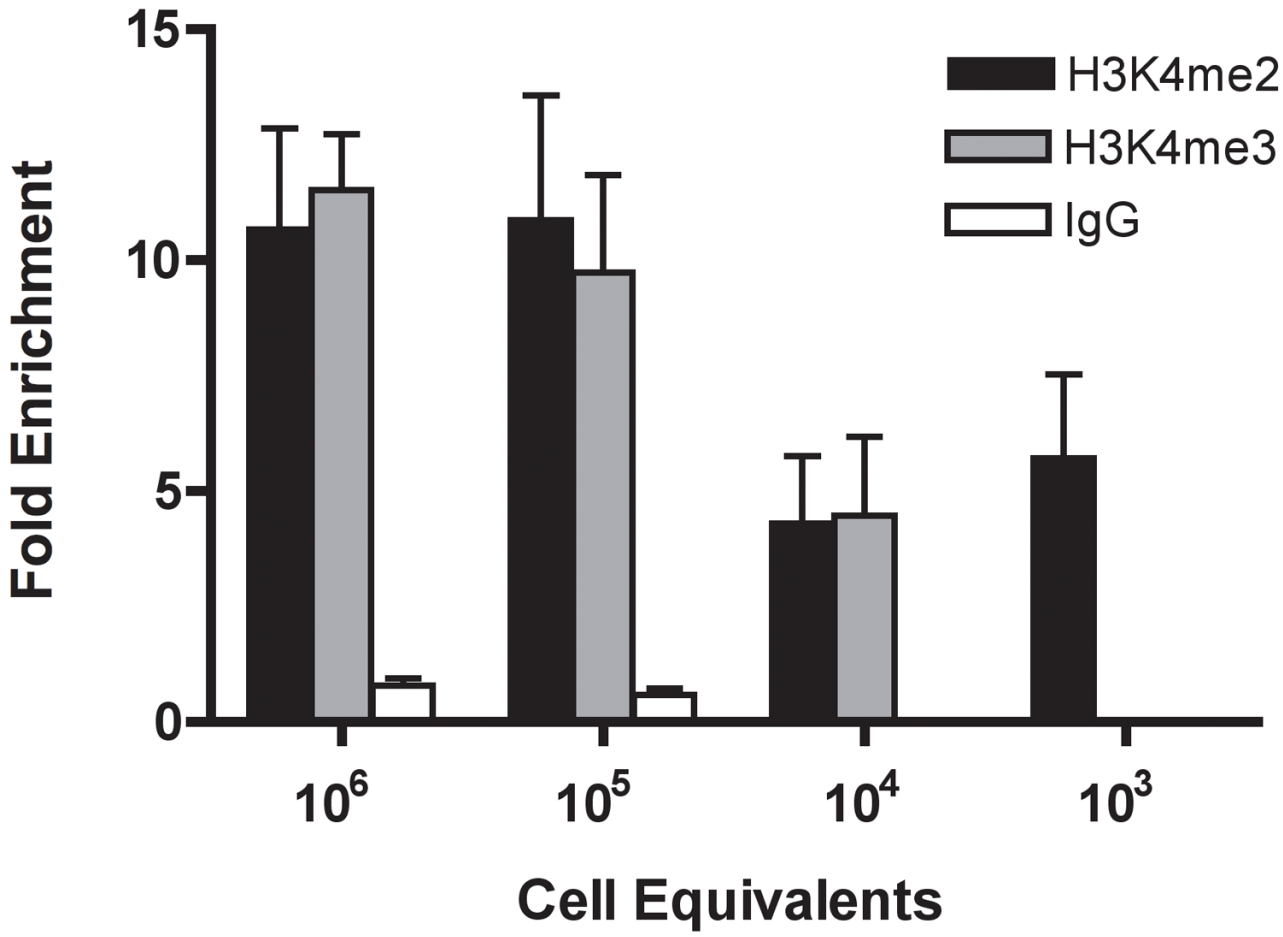

Figure 3.

Sensitivity of ChIP to reduction in the amount of input chromatin. Prior to IP, chromatin was prepared from 10^6 P5424 cells, and serially diluted with RIPA buffer to the indicated cell equivalents. Bead complexes with antibodies to H3K4me2 and H3K4me3, and control IgG were incubated for 2 hours with each chromatin dilution, and fold enrichment of cad promoter DNA in the IP samples relative to input is calculated as in Fig. 1.

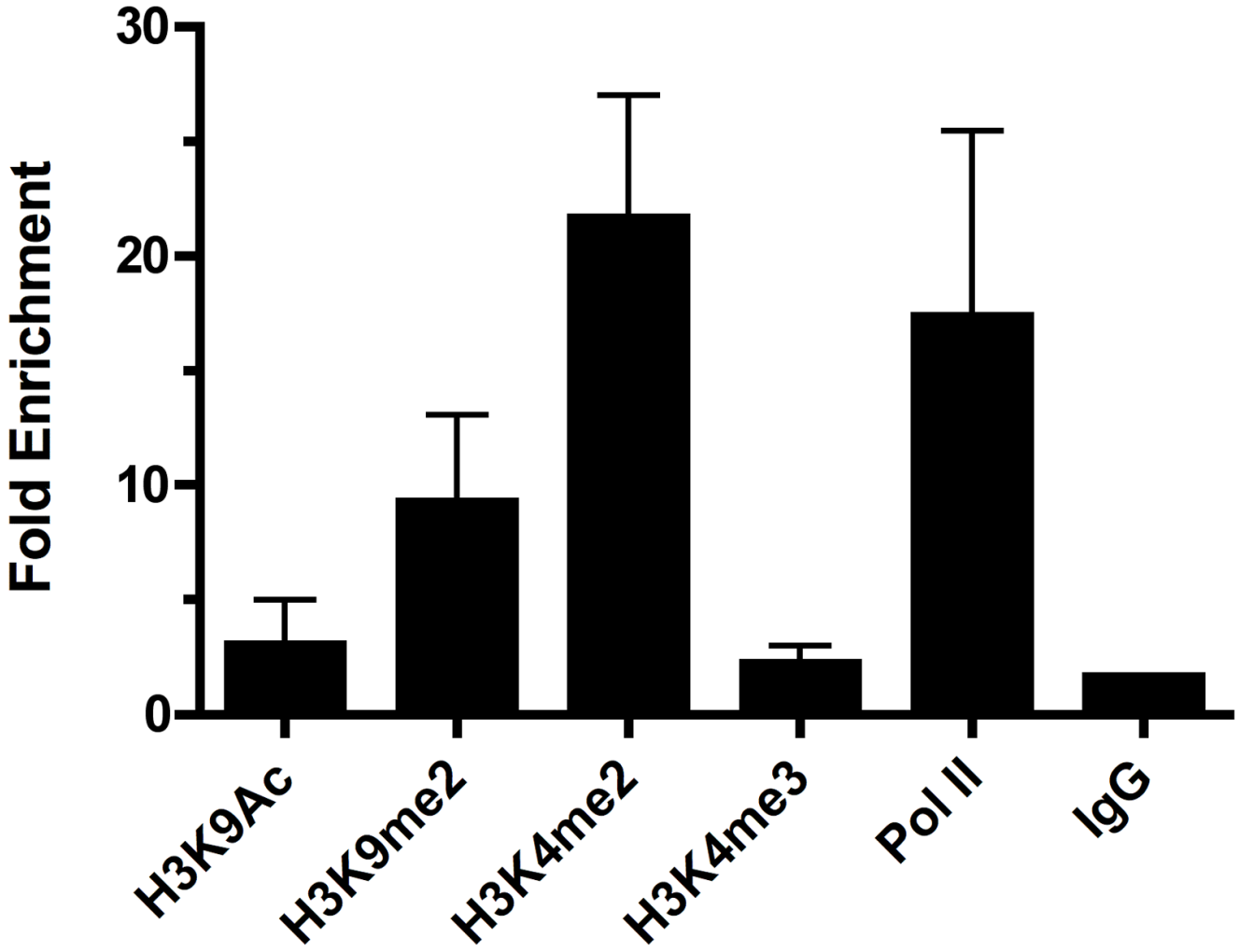

Figure 4.

Chromatin modifications at the TEA promoter of Rag2-deficient thymocytes. Bead complexes with each of the indicated antibodies were incubated for 2 hrs with chromatin prepared from 10^4 freshly isolated Rag2$^{-/-}$ thymocytes. Fold enrichment of TEA in each IP sample relative to input is calculated as in Fig. 1.