Comparison of Diagenode's LowCell# ChIP kit with commercially available ChIP kits

Introduction

Determining the genomic targets of DNA-binding proteins is crucial in understanding transcriptional control and epigenetic silencing. In addition, understanding the association of proteins with specific genomic regions in the context of intact cells helps uncover the mechanisms of gene regulation pathways and cellular proliferation. Chromatin immunoprecipitation (ChIP) allows analysis of this association of proteins with specific genomic regions in vivo and is a powerful method used to study changes in epigenetic signatures, chromatin remodeling and transcription regulator recruitment. To describe ChIP in brief, cells are first fixed with a reversible cross-linking agent followed by shearing of the cross-linked chromatin (the protein-bound DNA). Next, the DNA fragments associated with the protein of interest are immunoprecipitated (IP'd) using specific antibodies. Finally, the immunoprecipitated DNA is analyzed by qPCR, ChIP-chip, or sequencing for enrichment of specific sequences. This enrichment indicates that the sequences are associated with the protein of interest in vivo[1,2].

In recent years, several vendors have developed ChIP kits to overcome both the tedious optimization ChIP typically requires as well as the various challenges the assay presents such as working with limited cell numbers. We have developed the Diagenode LowCell# ChIP Kit which allows researchers to work with fewer amounts of cells than traditionally recommended, requiring only 1000 cell equivalents per ChIP reaction. In this study, we evaluated and compared four commercially available ChIP kits with the Diagenode LowCell# ChIP Kit.

Initially, all kits were evaluated using manufacturer-provided protocols and with ChIP-grade antibodies (Diagenode) directed against histone modifications (H3K9me3 and H3K27me3). Next, kits were tested using a common strategy to purify the IP'd DNA by using the DNA Isolation Buffer provided in the LowCell# ChIP Kit. The results of these experiments revealed poor data when following only manufacturer-recommended protocols. However, DNA purification after ChIP following the Low Cell ChIP kit instructions improved kit performance. In addition, ChIP results with the LowCell# ChIP Kit revealed a higher specificity of results.

Methods

Kits

Four kits were purchased from commercial manufacturers and were compared to the Diagenode LowCell# ChIP Kit.

These kits included:

1. ChIP-IT Express kit (Active Motif)
2. Magna ChIP A kit (Millipore)
3. Imprint Chromatin Immunoprecipitation kit (Sigma)
4. MAGnify Chromatin Immunoprecipitation System kit (Invitrogen)

Antibodies

All ChIP assays were performed with ChIP-grade antibodies from Diagenode. These antibodies are directed against histone modifications. They have previously shown good results in ChIP experiments.

1. H3K9me3. Rabbit polyclonal antibody (Cat. No. pAb-056-050)
2. H3K27me3. Rabbit polyclonal antibody (Cat. No. pAb-069-050)

Cells

Human HeLa cells were used in all the experiments for chromatin preparation. Chromatin was prepared based on manufacturer recommendations for each kit and sheared using the Diagenode Bioruptor®. For sheared chromatin preparation we sought to standardize protocols across all commercial kits to allow a more accurate comparison of ChIP efficiency but were unable to, due to different buffers used in the different kits. Thus we standardized and optimized the quality of sheared chromatin before IP, verified by agarose gel analysis. We found that the most optimal shearing protocol for the different chromatin preparations was shearing for 30 cycles of: [30 seconds "ON", 30 seconds "OFF"] with homogenization and centrifugation of the samples every 10 cycles. To prepare chromatin using the LowCell# ChIP Kit protocol, cells were fixed with 1% formaldehyde for 8 minutes at room temperature and quenched with glycine for 5 minutes. Then, cells were washed twice in ice-cold PBS and resuspended in Buffer B. After 5 minutes of incubation on ice, chromatin was sheared using the Diagenode Bioruptor®

for 25 cycles of: [30 seconds "ON", 30 seconds "OFF"] with homogenization and centrifugation of the samples every 5 cycles. An aliquot of the sheared chromatin obtained from each kit's protocol was reserved for shearing efficiency analysis.

ChIP assays

Most kits gave a range of recommended cell numbers per ChIP reaction to address the amount of chromatin required. The Active Motif kit, however, recommended a range of chromatin quantity expressed as µg per ChIP reaction. To compare kits more accurately, the same number of cell equivalents (100 000 cell equivalents) was used for each ChIP reaction with kits from Millipore, Sigma, Invitrogen, and the Diagenode LowCell# ChIP Kit. This amount was fixed based on recommendations provided for each kit. For ChIP using the Active Motif kit, ChIP experiments were performed with 15 µg of chromatin as recommended in the manufacturer's protocol.

For experiments with a common IP'd DNA purification method, immunoprecipitation and washes were performed according to manufacturer's instructions and the IP'd DNA was eluted in DNA Isolation Buffer (Diagenode) as described in the LowCell# ChIP Kit's protocol. Briefly, after the last wash of the immunoprecipitated material, the beads-IP'd DNA complexes were resuspended in DNA Isolation Buffer with proteinase K. The complexes were incubated for 15 minutes at 55°C followed by an incubation of 15 minutes at 95°C to inactivate proteinase K. The supernatant containing the IP'd DNA was stored and analyzed by qPCR.

qPCR and data presentation

After ChIP, the IP'd DNA was analyzed by qPCR with primers specific for known positive and negative loci. As a negative locus, a region of the c-fos promoter was chosen for both antibodies since these antibodies are directed against histone modifications associated with inactive gene transcription. We selected positive loci based on previous ChIP-sequencing results we obtained with H3K9me3 and H3K27me3 antibodies. Specifically, we selected a region of Zinc Finger 510 as a positive locus for the H3K9me3 antibody and a region in Myt1 as a positive locus for the H3K27me3 antibody. In addition, DNA IP'd with these antibodies was analyzed with primers specific for TSH2B, a locus associated with a repressed expression[3] and previously validated with various ChIP experiments at Diagenode. The qPCR experiments were performed in duplicate for each ChIP reaction. Each qPCR reaction contained 12,5 µl of SYBR Green PCR Master mix (GMO-UN-A100), 1 µl of primer pair (reverse and forward: 5 µM each), 5 µl of IP'd DNA or input, and water to a final volume of 25 µl. A control without DNA was also run during qPCR reactions to check the absence of contamination or primer dimerization.

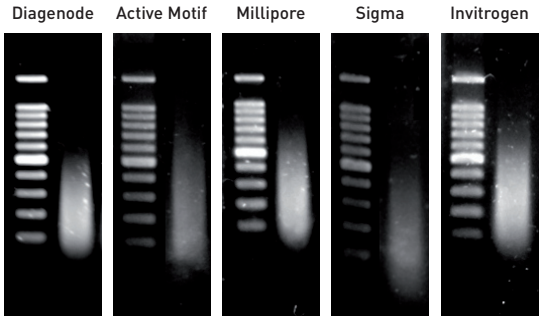
The efficiency of chromatin immunoprecipitation of a particular genomic locus can be calculated from qPCR data and reported as a percentage of starting material: % (ChIP/Total input) =2^[(Ct(x%input)-log(x%)/log2)- Ct(ChIP)]x100. Ct (ChIP) and Ct (Input) are threshold values obtained from exponential phase of qPCR for the IP'd DNA sample and input sample respectively; the compensatory factor (logx%/log2) is used to take into account the dilution 1/x of the input.

The qPCR data also allow us to determine the relative occupancy or specificity, calculated as a ratio of specific signal over the background: Occupancy (Specificity) = % input (specific loci)/ % input (background loci)

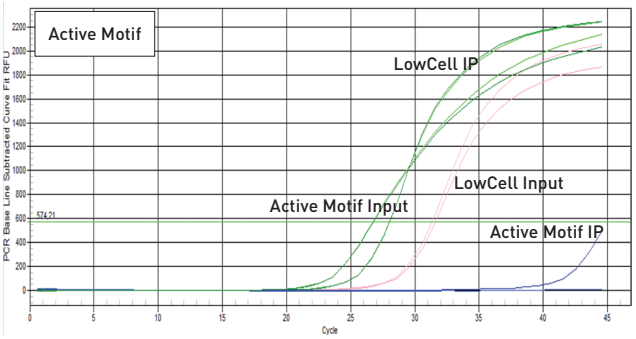
Results

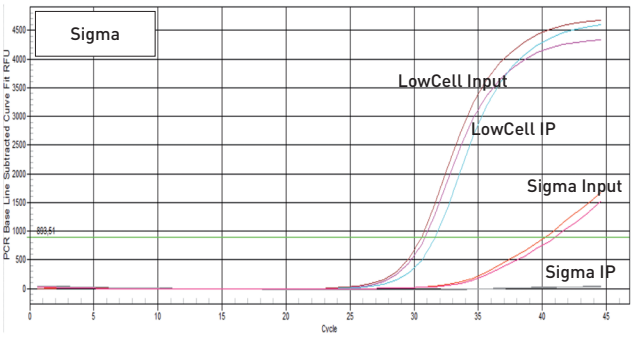
1. ChIP efficiency using each ChIP kit's protocol for all steps

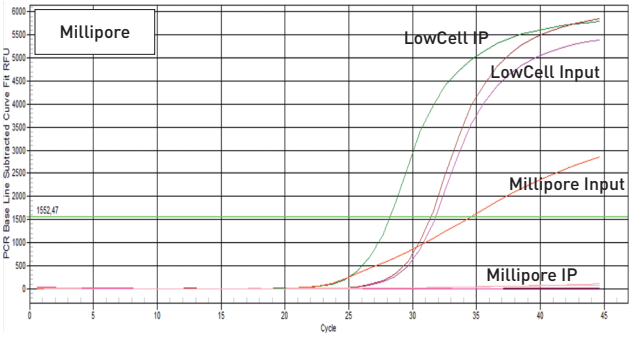
Initially, we evaluated the ChIP kits according to manufacturer recommended protocols. We used a validated ChIP-grade antibody against the H3K9me3 histone modification which had previously given good results in our experiments. Chromatin used in the different assays was prepared according to each kit's protocol. Shearing efficiency was analyzed for each chromatin preparation before ChIP to ensure that kit comparisons were performed on equivalent starting material. As shown in Figure 1, all kits provided chromatin with similar shearing efficiencies (200 to 1000 bp fragments). Thus, all chromatin preparations were suitable for ChIP and kit comparison.

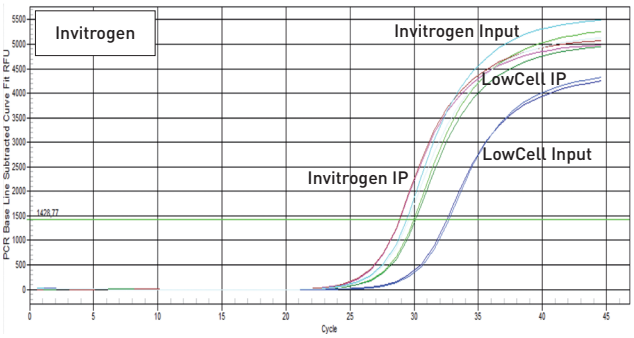


We observed poor qPCR results from most of the commercial kits. Typical qPCR amplification curves are sigmoid with 3 segments: a background phase, an exponential phase, and a plateau phase. However, qPCR curves after ChIP with the Active Motif, Millipore, or Sigma protocols were not sigmoid whereas the Diagenode and Invitrogen ChIP kits displayed sigmoid qPCR curves. Analysis of ChIP efficiency for those two kits revealed a similar percentage of input with both kits for the positive locus Zinc Finger 510. We obtained similar results when the IP'd DNA was analyzed with primers specific for the validated positive locus TSH2B. However, the results obtained using the Diagenode kit indicated higher specificity as shown by the higher specific signal/background ratio. To test whether these observations could be applicable to other antibodies, additional ChIP experiments were performed with another ChIP-grade validated antibody, H3K27me3. As shown in Figure 1, ChIP with the H3K27me3 antibody revealed no significant differences in the percentage of input for Myt1 and TSH2B positive loci when the experiment was performed using the LowCell# ChIP Kit's protocol or the Invitrogen kit's protocol. However, in this case, IP'd DNA analysis for the validated positive locus TSH2B revealed a clearly higher ChIP specificity with the Diagenode kit as shown by the specific signal/background ratio analysis.









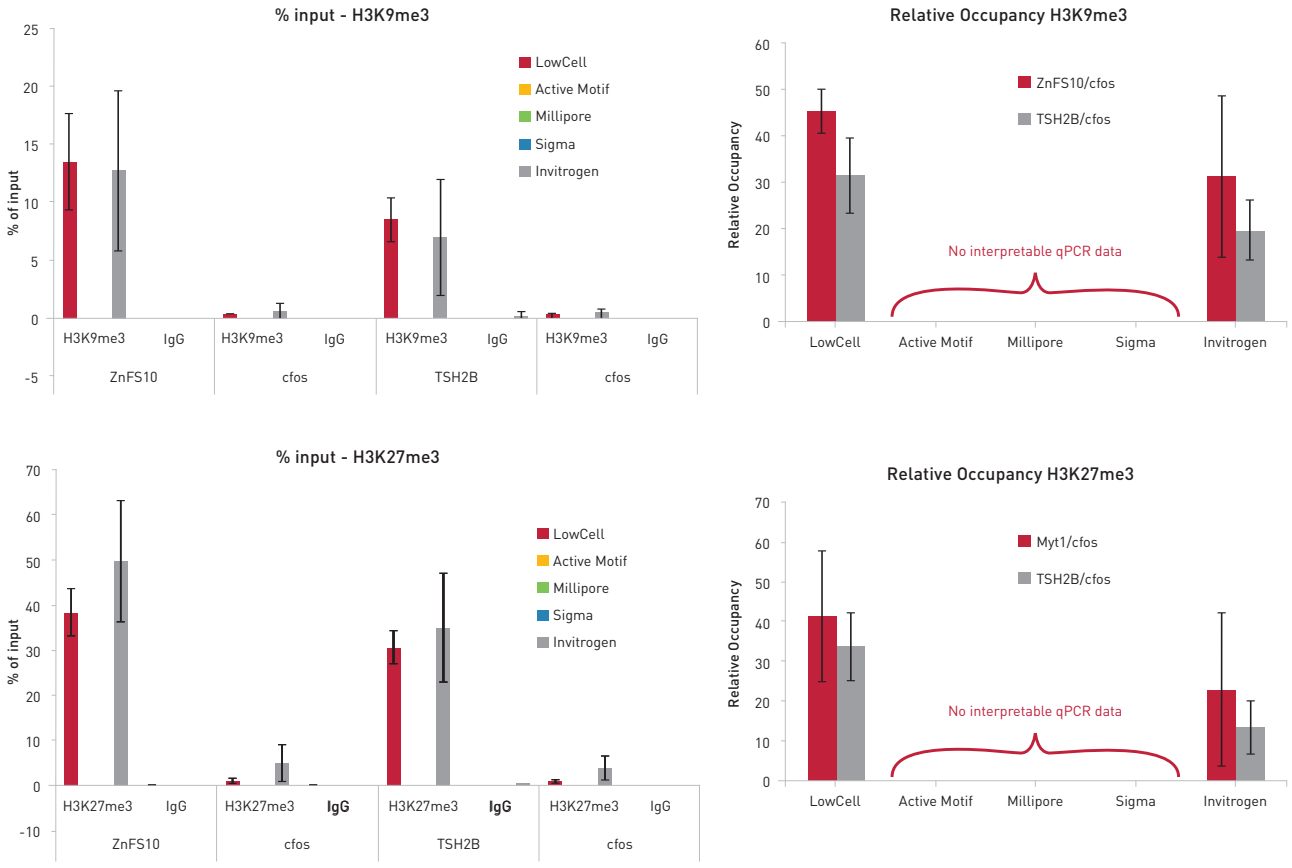
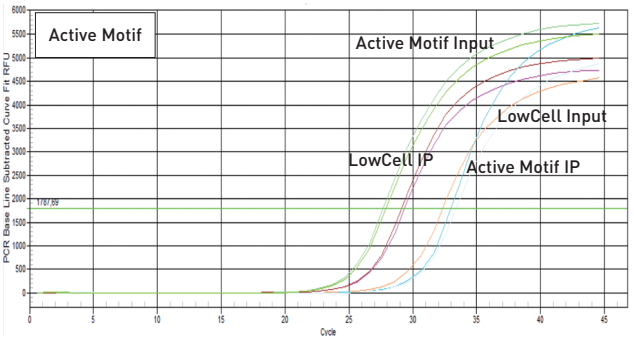


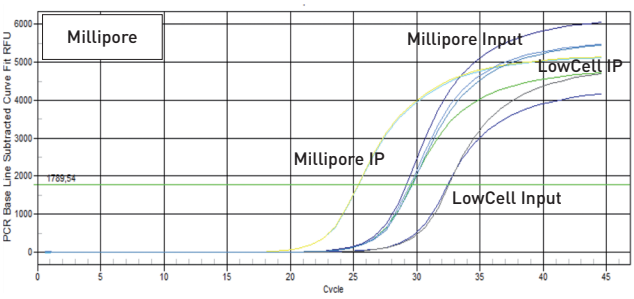
Figure 1
Kit comparisons using each kit's procedure for all steps

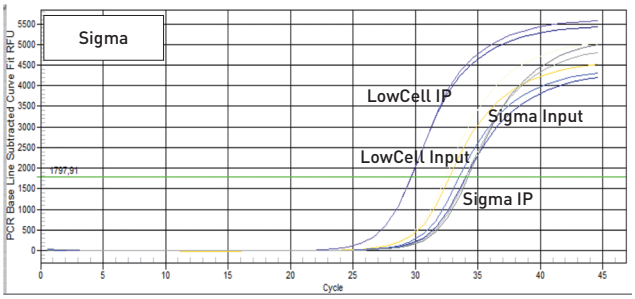
Upper Panel: Analysis of the DNA isolated from all chromatin batches prepared according to each kit's instructions and sheared using the Diagenode Bioruptor. ChIP assays were performed using the LowCell# ChIP Kit from Diagenode or the protocols from the different kits, the chromatin shown in the upper panel, Diagenode antibody against H3K9me3 and against H3K27me3, and optimized primers for qPCR. Middle panel. PCR curves obtained after ChIP with the H3K9me3 antibody (3µg per reaction) using primers specific for TSH2B gene are shown. Lower panels. ChIP results obtained with antibodies against H3K9me3 (3 µg/reaction) and H3K27me3 (2 µg/reaction) according to the different ChIP kit's protocol. IgG was used as negative IP control.

2. ChIP efficiency using the Low Cell Kit's elution method

We observed poor qPCR results from most of the commercial kits, possibly due to inefficiency in the purification methods of the IP'd DNA. In contrast, the Diagenode LowCell# ChIP Kit's purification method using the DNA Isolation Buffer gave efficient and specific results. Therefore, we performed additional ChIP experiments with the different kits that have shown poor qPCR results according to manufacturer recommendations but substituted the Diagenode method for IP'd DNA purification. The use of the Diagenode DNA Isolation Buffer resulted in qPCR curves with a sigmoid shape for all kits, again demonstrating the high performance of the Diagenode DNA elution technique. Analysis of ChIP efficiency using the H3K9me3 antibody showed a similar percentage of input for the positive locus Zinc Finger 510 with the Diagenode and Sigma kits but specificity is higher for the Diagenode kit. A very high percentage of input for this locus was observed after ChIP with the Milipore kit, but this high ChIP efficiency was associated with weak specificity as revealed by specific signal/background ratio analysis. Surprisingly, no enrichment of the positive locus Zinc Finger 510 was shown after ChIP with the Active Motif kit's protocol. These observations were confirmed when the IP'd DNA was analyzed for the presence of the Diagenode validated locus TSH2B. ChIP experiments performed with another antibody H3K27me3 also resulted in a similar percentage of input for all commercial kits except for the Active Motif ChIP kit. Our results clearly showed higher specificity was obtained with the Diagenode kit using the DNA Isolation Buffer.







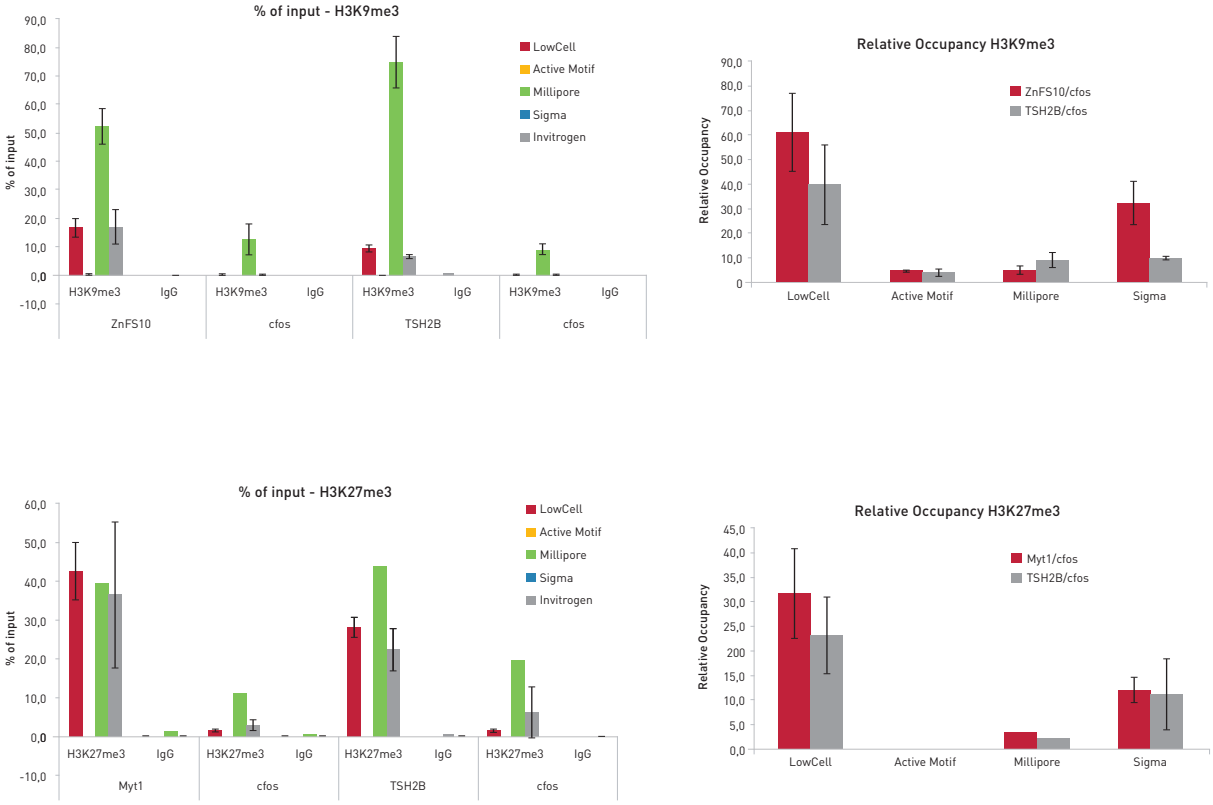


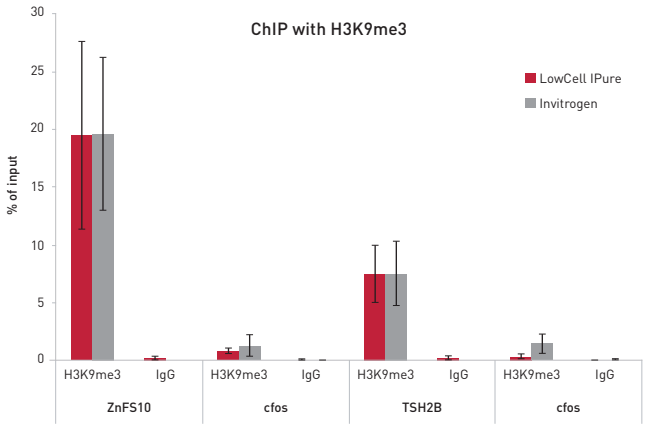
Figure 2
Comparison of ChIP kit efficiency using the Diagenode LowCell# ChIP Kit for IP'd DNA purification.

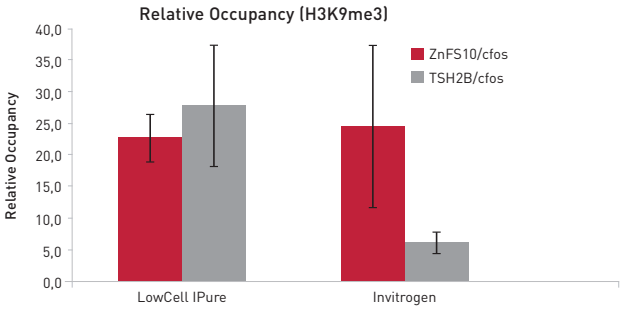
ChIP experiments were performed with 3 µg of H3K9me3 antibody (A, B) or 2 µg of H3K27me3 antibody (C, D) according to each kit's instructions and then the IP'd DNA was purified with the DNA isolation buffer as described in the Diagenode LowCell# ChIP Kit protocol. The qPCR was performed with primers for Zinc Finger 510, TSH2B and Myt1 genes as positive loci and for the c-fos promoter as negative loci. PCR curves obtained after ChIP with H3K9me3 antibody (3 µg/IP) with primers specific for TSH2B are shown in the upper panel. Lower panels: Figures A and C show the recovery, expressed as a percent of input (the relative amount of immunoprecipitated DNA compared to input DNA after qPCR analysis). The relative occupancy (calculated as a ratio of specific signal over background) is depicted in Figures B and D.

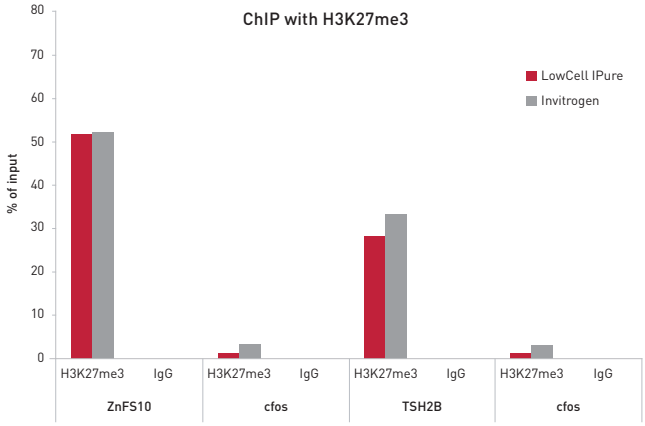
3. ChIP efficiency after IP'd DNA isolation with high purity

The LowCell# ChIP Kit contains DNA Isolation Buffer for efficient purification of IP'd DNA, which provides DNA suitable for qPCR analysis as demonstrated previously. Other downstream applications of ChIP, however, require DNA of higher purity, for which we recommend Diagenode's IPure kit (Cat. No. AL-100-0100) for purification instead of DNA Isolation Buffer. We compared the ChIP efficiency of the LowCell# ChIP Kit in conjunction with the IPure purification method with the ChIP efficiency of the Invitrogen kit (as this kit had given positive data previously, suitable for applications such as next generation sequencing).

Results of this comparison are depicted in Figure 3. ChIP experiments were performed with the H3K9me3 and H3K27me3 antibodies. Analysis of ChIP efficiency revealed a similar percentage of input for the positive loci Zinc Finger 510 and TSH2B using the Diagenode or Invitrogen kit with H3K9me3 antibody. By contrast, higher ChIP efficiency resulted with the Diagenode kit when IP'd DNA (using the H3K27me3 antibody) was analyzed for Myt1 positive locus. However, for both antibodies, specificity of ChIP results seemed better when ChIP was performed using Diagenode's protocol as demonstrated by the ratio of specific signal/background. Overall, the Diagenode ChIP protocol is the more specific method to immunoprecipitate DNA suitable for applications which require high DNA purity.







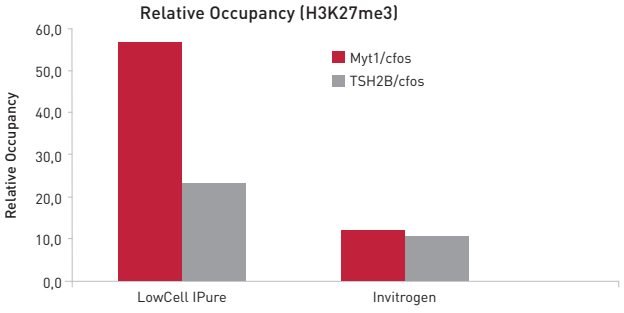


Figure 3

Comparison of ChIP efficiency following the LowCell# ChIP Kit protocol with IP'd DNA elution with the Ipure kit's protocol and the Invitrogen kit's protocol.

ChIP experiments were performed with the Diagenode LowCell# ChIP Kit and Invitrogen kit following instructions provided by each kit using the Diagenode antibodies against H3K9me3 and against H3K27me3 and optimized primers for qPCR. For ChIP with the LowCell# ChIP Kit, the IP'd DNA was eluted following the Ipure kit's protocol. The qPCR was performed with primers for Zinc Finger 510, Myt1 and TSH2B genes ad positive loci and for the c-fos promoter as negative locus. Figure A, C show the recovery, expressed as a percent of input (the relative amount of immunoprecipitated DNA compared to input DNA after qPCR analysis). The relative occupancy (calculated as a ratio of specific signal over background) is depicted in Figure B, D.

4. ChIP efficiency using the LowCell# ChIP kit on few amount of cells

To try to really challenge our LowCell# ChIP kit, we decided to perform ChIP experiments with the Diagenode ChIP kit using decreasing amount of cells. Serial dilutions of a common stock chromatin preparation were used. ChIP reactions were performed with as few as 200 cell equivalent. For these assays, the ChIP-grade antibody directed against H3K27me3 was used.

Results of the down-titration of cell with the Diagenode ChIP kit protocol are depicted in figure 4. Analysis of ChIP efficiency revealed increasing ChIP efficiency when decreasing the amount of cell equivalent from 100 000 to 1000. In addition, an enrichment of the positive locus TSH2B was also obtained with only 200 cells per ChIP reaction. Moreover, these results were associated with high specificity as revealed by specific signal/background ratio analysis. Overall, these results clearly demonstrated that the Diagenode ChIP kit allow efficient and specific ChIP reactions when working with as low cell number as 200 cell equivalent.

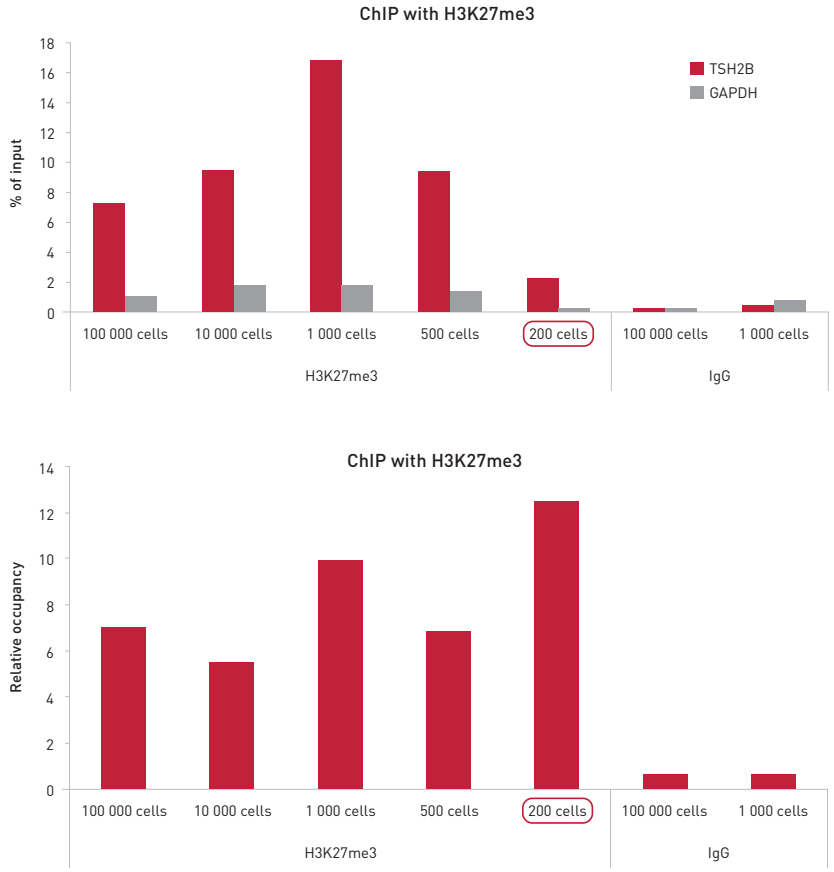


Figure 4
ChIP with the LowCell# ChIP kit on decreasing amount of cells.

ChIP assays were performed using the Diagenode LowCell# ChIP kit, Hela cells, the Diagenode antibody directed against H3K27me3 (Cat No. pAb-069-050) and the optimized primers for qRT-PCR using the SX-8G IP-Star Automated System. Chromatin was sheared from 1 million cells and serial dilutions of this chromatin (from 100 000 to 200 cell equivalent) were used per ChIP reaction. One µg of antibody and 10 µl of beads were used per ChIP experiment performed on 100 000 cell equivalent. ChIP on 10 000 and 1000 cell equivalent were performed using 0.5 µg of antibody and 10 µl of beads and 0.25 µg of antibody and 5 µl of beads were used for ChIP reaction with 500 and 200 cell equivalent. A negative control antibody was included in the ChIP assay (1 µg/IP with 100 000 cell equivalent and 0.5 µg/IP with 1000 cell equivalent). Figure A shows the recovery, expressed as a % of input (the relative amount of immunoprecipitated DNA compared to input DNA after qPCR analysis). The relative occupancy (calculated as a ratio of specific signal over background) is shown in Figure B.

Conclusions

We compared the performance of four commercially available ChIP kits with the Diagenode LowCell# ChIP Kit using two different ChIP-grade antibodies. First, kit performance was evaluated by analyzing ChIP efficiency and specificity following each manufacturer's protocol from start to finish. To allow accurate kit comparison with minimal bias, sheared chromatin of equivalent quality was used as starting material for each experiment. Additionally, to minimize variations in experimental conditions between the different kits, we standardized ChIP conditions by using the same antibody to immunoprecipitate DNA and by using identical qPCR conditions for IP'd DNA analysis. Surprisingly, our qPCR data analysis revealed that only the Diagenode and Invitrogen kits provided interpretable results. Though the Diagenode and Invitrogen kits provided similar ChIP efficiencies, the Diagenode LowCell# ChIP Kit gave results with higher specificity.

Next, to further standardize the experimental conditions for the ChIP kit comparison of the different kits that have shown poor qPCR results, we used the unique Diagenode DNA Isolation Buffer for IP'd DNA purification, as it had previously provided successful results. After using this purification method, we obtained interpretable qPCR data for all commercial kits, highlighting the high performance of the DNA Isolation Buffer. Kit comparison with this common DNA elution method revealed that 1) the Millipore kit gave the highest ChIP efficiency but with low specificity and high background, 2) the Active Motif kit failed to provide enrichment of the positive locus, and 3) the Diagenode kit showed the highest specificity in ChIP results. The increased performance obtained with the Diagenode purification technique suggests defects in purification methods of the other kits. Further, inefficient IP'd DNA purification may explain the poor downstream qPCR results derived from the kits.

Subsequently, we demonstrated the effects on ChIP efficiency when using an IP'd DNA sample of higher purity, achieved by purifying with the IPure kit (Cat. No. AL-100-0100) instead of the DNA Isolation Buffer. We compared ChIP efficiency of the Diagenode kit to the Invitrogen kit since this kit was the only one that provided interpretable results when following manufacturer's instructions. Once more we observed that a higher specificity is obtained when ChIP is performed with the LowCell# ChIP Kit from Diagenode.

Lastly, using decreasing amount of cell equivalent per ChIP reaction, we demonstrated the potential of the Diagenode LowCell# ChIP kit to work with as low cell number as 200 cell equivalent for efficient and specific ChIP results.

In summary, among the different ChIP kits tested using manufacturer protocols, the ChIP kits from Diagenode and Invitrogen were the only ones that yielded qPCR results, with increased specificity from the Diagenode kit. When IP'd DNA purification was performed in DNA Isolation Buffer as described in the LowCell# ChIP Kit, qPCR results improved across all the kits. However, the kit from Diagenode gave higher specificity of results. Finally, assays performed with the IPure kit's (Cat. No. AL-100-0100) protocol revealed a higher specificity for ChIP performed with the Diagenode protocol when a high DNA purity is required. Overall, the Diagenode ChIP Kit is the more specific method to immunoprecipitate DNA.

References

1. Kim and Bing, Genome-wide analysis of protein-DNA interactions. Ann. Rev. Genomics Hum. Gen. (2006) 7:81-102

2. Zecchini and Mills, Putting Chromatin Immunoprecipitation into Context. Journal of cellular biochemistry (2009) 107:19-29

3. Weber et al., Distribution, silencing potential and evolutionary impact of promoter DNA methylation in human genome. Nat. Genet. (2007) 39(4):457-66

WP-LChIP-V1_13_07_11