Chromatrap

Chromatrap® – targeting the epigenetic landscape alongside low abundant transcription factor recruitment

Application note based on the patented Chromatrap® technology

Chromatin immunoprecipitation (ChIP) is a commonly used IP technique for mapping the DNA-protein interactions in cells which are crucial for correct gene regulation. The molecular mechanisms of epigenetics are being studied to evaluate their effect on chromatin conformation, active or inactive, which control the accessibility of the DNA backbone to transcription factor regulators (TF). Detecting the activity and presence of these mechanisms and their dis-regulation, enables researchers to understand the aberrant epigenetic landscapes which occur in pathology development.

Demonstrated here is the sensitivity, selectivity and reproducibility of solid phase Chromatrap®, as an assay for Chromatin IP from low cell numbers. Using low input chromatin concentration, the utility of Chromatrap® to understand the balance between histone methylation marks and low abundance TF recruitment at positive and negative target gene loci is clearly shown. This exciting new tool enables low volume, fast IP from small samples, eliminating bead loss and high background signals which are prevalent in magnetic bead separation techniques. Handling errors are also reduced, enabling robust analysis of chromatin dynamics and their effect on TF recruitment.

1.0 Introduction

Developed over 20 years ago, ChIP is a technique that allows researchers to take a snapshot of the regulatory mechanisms which interact with the DNA backbone. Formaldehyde-fixed protein/DNA complexes are immunoprecipitated with validated antibodies and analysed, enabling the epigenetic landscape of the cells to be revealed. Multiple platforms are available for carrying out ChIP; however, the novel Chromatrap® platform represents a major advance through using a solid support matrix, which allows the highly sensitive capture of these complexes in low volume and low chromatin concentration samples. By eliminating magnetic bead separation, Chromatrap® speeds up the process whilst providing low background signal, thus enabling difficult low abundant targets to be efficiently detected.

The epigenetic landscape is a complex balance between open and closed chromatin configurations which dictate patterns of active and repressed gene expression. Understanding this balance is crucial, revealing the regulatory molecular mechanisms which underpin normal cell function and pathology development. Detecting these mechanisms, particularly with low abundance targets identified in small cell samples, can be challenging and is a hurdle not easily overcome. Porvair's new Chromatrap®, is an excellent platform for the sensitive and selective capture of high and low abundant transcription factors and histone marks, providing an exciting step change in assay technology.

To demonstrate this, a low volume and high sensitivity ChIP assay was performed on two *in vitro* cell line models, HepG2 and K562. For positive expression indicators the ubiquitously expressed RNA pol II binding onto the Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene loci, which is actively expressed in all cell types, was used[1]. The balance of histone tri-methylation at lysine 4 and 27 in the tails of H3 is used here to suggest the presence of open (high H3k4 and low H3k27) and or closed (low H3K4 and high H3K27) chromatin conformation respectively[5; 6]. The negative gene ß-globin which is activated only in adult erythroid cells and inactivated in non-erythroid tissues[2; 13] was used as a negative gene for RNA pol II and the presence of H3K4me3.

The Enhancer of zeste homologue 2 (EZH2) and histone de-acetylase 1 (HDAC1), were chosen as low abundant TF targets. EZH2 is capable of gene silencing

via the tri-methylation of H3K27[7; 10], and its presence at the positive Myelin transcription factor 1 (MYT1) gene loci is compared to the identified negative zinc finger protein 333 (ZNF333) gene loci[11]. Both Cyclin A and p21 genes are important in regulating cell cycle progression, and HDAC1 recruitment to them has been previously described [8; 14; 15; 16]. Due to their low abundance, aberrant repression of several genes and cell cycle de-regulation associated with cancer development, they are an ideal IP challenge, highlighting the effectiveness of Chromatrap® [7; 8; 9].

2.0 Methodology

2.1 Chromatin preparation

Hep G2, a hepatocellular carcinoma cell line[17], and K562 isolated from chronic myeloid leukaemia[18] were chosen for this study. Cells were grown to confluency and prepared for IP according to the standard Chromatrap® protocol (http://chromatrap.com /downloads/). Briefly cells were fixed in 1% formaldehyde, lysed and the isolated nuclear chromatin fraction was separated by centrifugation. The Chromatin was sheared to obtain fragments of 100 – 500bp in length (Figure 1). The concentration of the chromatin was assessed using a NanoDrop spectrophotometer and aliquots of chromatin were stored to avoid excess freeze thaw cycles.

2.2 Immunoprecipitation and detection

For each antibody slurry preparation 500ng of chromatin, equivalent to approximately 80,000 cells, were precipitated with 1ug of antibody (2:1 antibody: chromatin ratio); buffered with 1µL of PIC and 5µL of Wash buffer 1 (WB1). Each IP reaction was made up to a final volume of 40µl with water. The full list of the antibodies used in each reaction, for both cell lines is given in Table 2. Aliquots of chromatin equivalent to 500ng were set aside as the input for qPCR analysis. Each IP slurry was incubated on the pre-conditioned columns at 4°C for 1 hour. The columns were then washed with appropriate buffers and captured DNA/Protein complexes were eluted as per the protocol. The eluted samples were subjected to reverse cross linking and proteinase K digestion, along with the input sample and then processed directly for qPCR analysis.

2.3 qPCR analysis

Each immunoprecipitation sample was compared with an equivalent loading of the unspecific IgG antibody (Table 1) from the same species, which served as a negative control. Both positive and negative IP were precipitated and analysed at the same time, then compared against the input sample as a percentage signal. Data presented in the results is the % real signal relevant to input ($AB_{\%Signal} - IgG_{\%Signal}$) with the standard error calculated from the minimum of 3 independent repeats to demonstrate reproducibility.

Antibody targets	Positive gene targets	Negative gene targets
H3K27me3	MYT1 ß-globin	ZNF333 GAPDH
H3K4me3	GAPDH	ß-globin
EZH2	MYT1	ZNF333
RNA polymerase II	GAPDH	ß-globin
HDAC1	Cyclin 2A p21	GAPDH

Table 1. *Antibody targets for ChIP and the gene loci at which targets are positive or negative.*

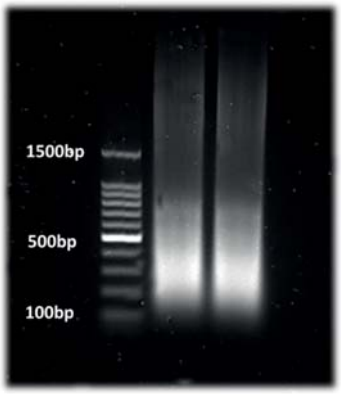

Figure 1. Qualitative analysis of chromatin. *Chromatin was sheared using a Bioruptor for 30 second bursts with 30 second intervals on ice at a power setting of 3 for 15 minutes so that desired fragment lengths between 1-500bp were obtained.*

Antibody target	Type
H3K27me3	pAb / rabbit
H3K4me3	pAb / rabbit
EZH2	mAB / mouse
RNA polymerase II	pAb / rabbit
HDAC1	mAb / rabbit
Unspecific IgG, 1	rabbit serum
Unspecific IgG, 2	mouse serum

Table 2. *Specific and unspecific antibody targets used in ChIP.*

3.0 Results and discussion

Chromatin from both human hepatocellular carcinoma cell line HepG2, and K562 cells isolated from chronic myeloid leukaemia[17; 18], was subjected to IP, using 500ng of input chromatin. RNA pol II presence at the promoter region of GAPDH was screened as a high abundant positive amplification target alongside the negative ß-globin gene locus to ensure selective amplification. The data, from a minimum of three independent repeats is presented as amplified % real signal, where unspecific IgG background signal is subtracted from the positive antibody relative to the input sample (see section 2.3).

3.1 Hep G2 Cells

The presence of RNA pol II at the GAPDH gene was detected alongside the tri-methylation of lysine 4 in the H3 tail, suggestive of open, actively transcribed chromatin (Figure 2). Despite the low loading concentration of input chromatin, a positive 1.7% signal was detected for RNA pol II presence at the GAPDH promoter, 5-fold above the background IgG. Similarly, a strong 5.6% real signal was detected for H3K4me3, a 140-fold increase when compared to the H3K27 tri-methylation signal, supporting an open chromatin conformation in these cells. At the negative ß-globin gene loci, the ratio of H3K4 and H3K27 tri-methylation was reversed, suggestive of a closed chromatin configuration as expected. This was reinforced by the lack of RNA pol II signal at the ß-globin gene (Figure 2). Low RNA pol II recruitment was detected at both Cyclin A2 (0.09%) and p21 (0.16%) gene loci despite the balance of H3k4 and h3K27 tri-methylation presence, being indicative of an open chromatin confirmation. No H3K27me3 signal was detected at the Cyclin A2 gene while 1.6% H3K4me3 signal was observed. The presence of low-level HDAC1 activity (0.1%) suggests interplay between histone methylation and de-acetylase activity at this gene. A 1.5% H3K4me3 signal was detected at the p21 gene loci; however, no HDAC1 activity could be detected at this gene loci in Hep G2 cells.

Low abundant transcription factors HDAC1 and EZH2 are also targeted to highlight the utility and flexibility of the Chromatrap® assay. The lower background IgG signal, afforded by the sensitive Chromatrap® solid phase support, clearly enables these amplification signals. The regulation of the cell cycle is a dynamic process, which can become de-regulated in cancer cells[16]. Cyclin A2 and p21, a cyclin-dependent kinase inhibitor, are important in cell cycle regulation.[5; 16] The expression of these gene transcripts will vary depending on the cell cycle stage at the time of fixation. The low precipitation with RNA pol II on Cyclin A2 and p21 gene loci suggests that they are maintained in a dynamic, open chromatin conformation making them accessible to important transcription factors such as well as known tumour suppressor genes such as p53[19; 20]. The balance of repressive and transcription promoting marks shown in Figure 2 demonstrates, that the Chromatrap® assay is capable of giving an insight into complex epigenetic mechanisms.

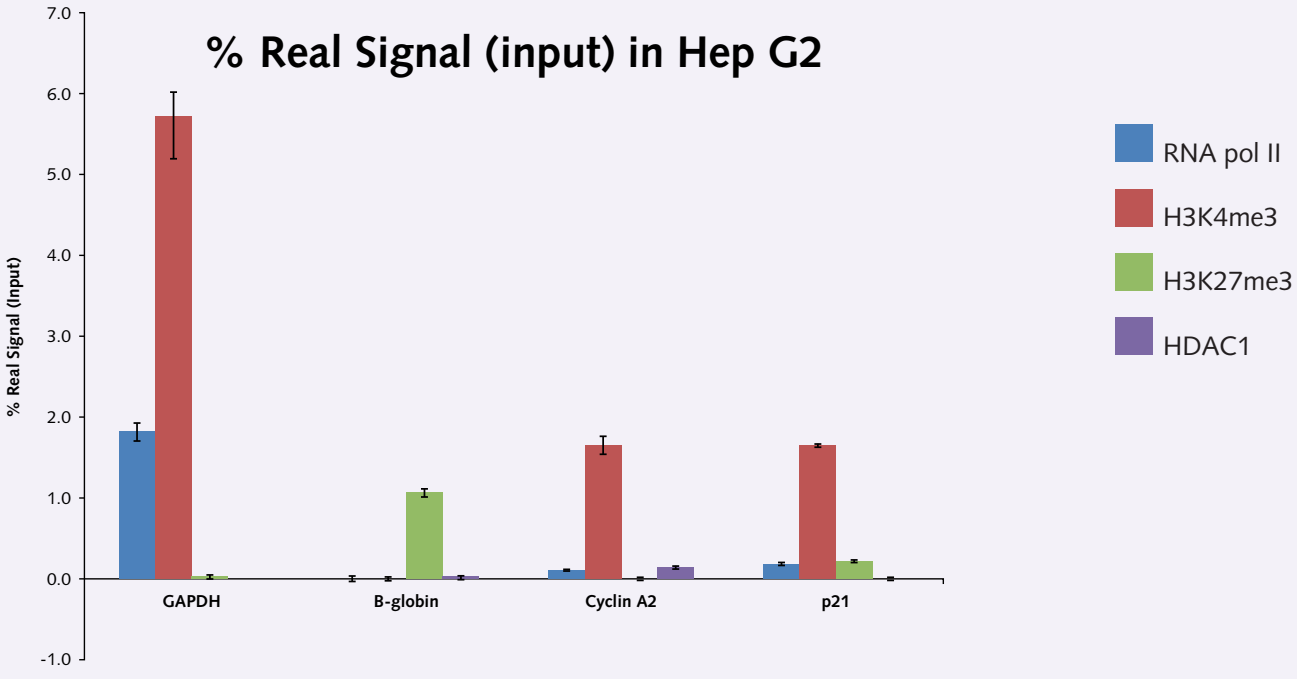

Figure 2. *ChIP results in Hep G2 cell line demonstrating the relationship of methylation marks on potentially transcribed and silenced gene targets.*

MYT1, included here as a positive gene control, showed a high % real signal for the presence of the low abundant transcription factor EZH2 (Figure 3). A 14-fold higher signal (0.7%) was detected at the MYT1 gene loci when compared to the negative control ZNF33 (0.05%). EZH2 is a transcription repressor of MYT1 expression and is thought to be able to recruit the presence of tri-methylation at lysine 27 of histone H3, commonly associated with closed chromatin conformations and gene silencing. This is the case here, where 0.7% signal was amplified at the MYT1 locus, a 12-fold increase when compared to that at the negative ZNF33 loci (0.06%, Figure 3). A very low level, similar to that seen in the negative control gene loci, of H3K4me3 and RNA pol II signal was detected at the MYT1 gene.

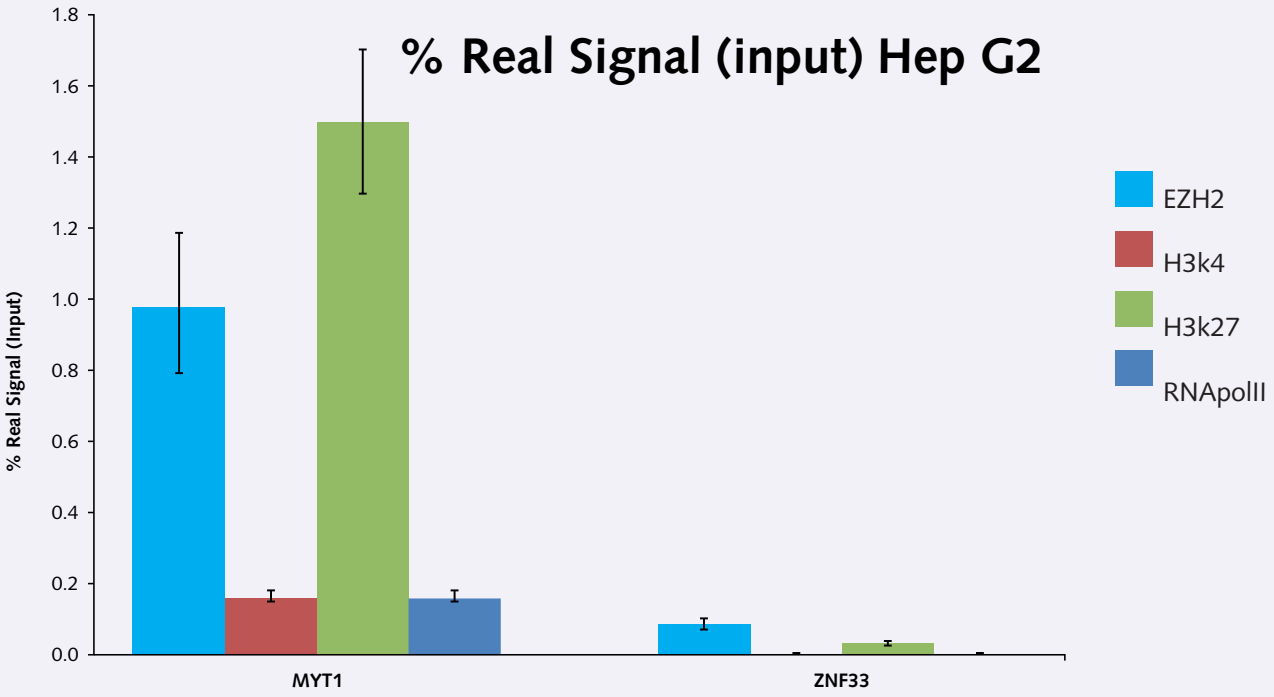

Figure 3. The precipitation with low abundance target EZH2. EZH2 induced presence of H3K27me3 is evident on MYT1 promoter. The % Real Signal (input) values are very low on the negative gene ZNF333 as expected. Highlights sensitivity of the Chromatrap® assay.

3.2 K562 cells

As was the case in the Hep G2 cells, positive amplification of signal was observed relative to background, showing recruitment of RNA pol II onto the GAPDH promoter (1% real signal; Figure 4). Together with a strong 3.7% H3K4me3 real signal, 28-fold higher when compared to the observed H3K27me3 signal, the pattern of epigenetic marks present and recruitment of RNA pol II suggest an open chromatin conformation at the GAPDH gene loci (Figure 4). At the negative ß-globin gene loci, as expected, a reversed H3K4-H3K27 tri-methylation ratio was observed. The H3K27me3 signal was 5-fold higher (0.1 %) than that observed for H3K4me3 (0.02 %), suggesting the ß-globin locus is locked in a closed chromatin conformation. The lack of RNA pol II recruitment at this gene locus agrees with the suggestion that this gene is inactive in these cells at the time of fixation.

A low level of RNA pol II recruitment was observed at the Cyclin A2 loci, alongside a positive 1.2% real signal for H3K4me3. Again, a 25-fold higher signal was observed for H3K4me3 when compared to H3K27me3, suggesting the gene is in the active, open chromatin conformation. As in Hep G2, a low level of HDAC1 precipitation (0.16 %) at Cyclin A2 loci indicates some interaction with histone acetylation. At the p21 gene locus a contrasting profile was observed in K562 cells compared to Hep G2, indicative of the sensitive and selective precipitation capabilities of the Chromatrap assay. No recruitment of RNA pol II was observed at this loci, and H3Kme3 precipitation was 7-fold lower than in Hep G2 (Figures 2 and 4). Together with HDAC1 recruitment at 0.16% and 7-fold higher H3K27me3 signal (1.45%) than H3K4me3 (0.21%), the results suggest that this gene is again, in a closed, inactivated chromatin configuration (Figure 4).

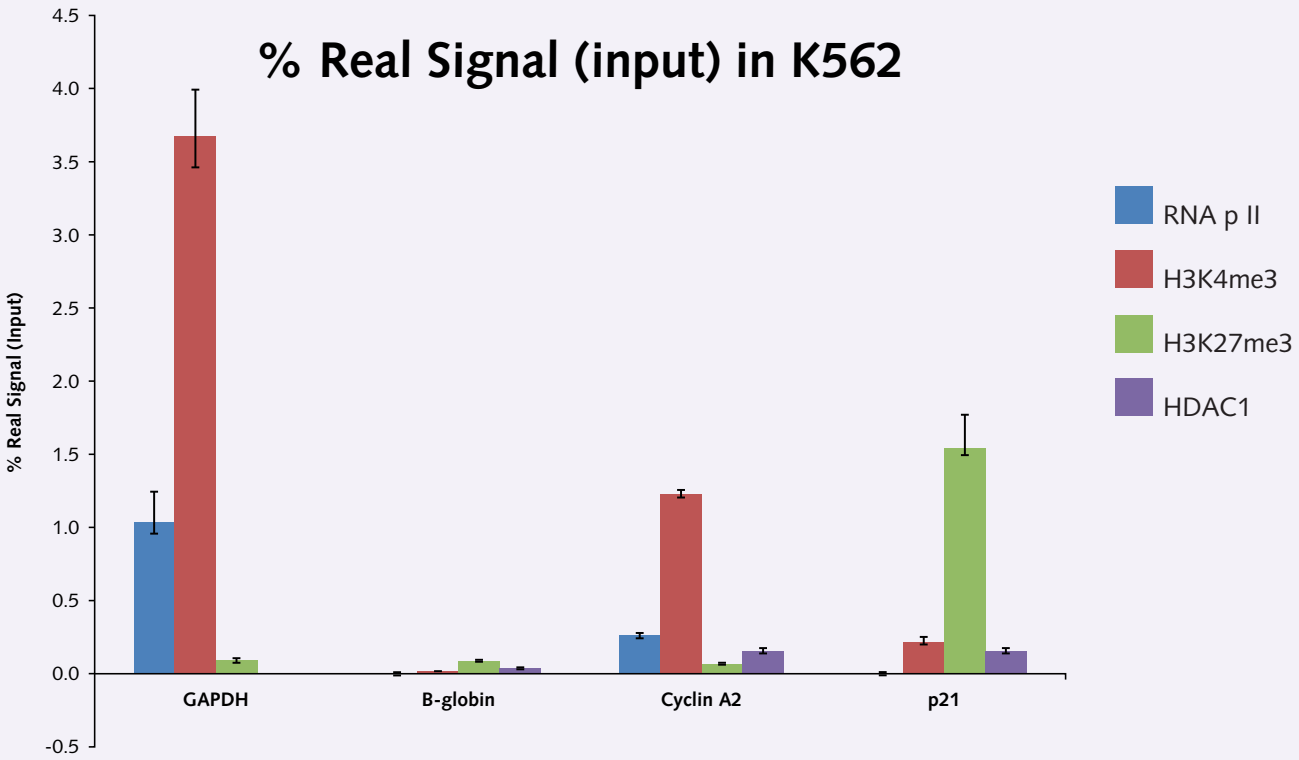

Figure 4. Chromatin Immunoprecipitation of methylation marks and transcription factors at multiple gene loci in K562 cell line.

The K562 cells have some characteristics of erythroid cells, which are capable of expressing embryonic and foetal, but not adult haemoglobin[3; 4]. This is likely to explain the detection of slightly lower H3K27me3 presence at the ß-globin gene loci in K562 compared to HepG2. Although the ChIP signal profile on Cyclin A2 was similar in both cell lines, the p21 gene loci in K562 appears to be in a less accessible conformation than in HepG2, as indicated by reversed H3K27 – H3K4 tri-methylation profile. Positive amplification signal (1%) for the low abundant transcription factor EZH2 recruitment to the MYT1 gene loci was detected using Chromatrap®, a 12-fold increase in signal compared to the negative gene loci (0.08%;ZNF333; Figure 5). Together with the presence of 1.5% H3K27me3 real signal detected at the positive MYT1 locus, 36 fold higher than ZNF333, this data suggests that EZH2 negatively regulates MYT1 gene expression via its recruitment of histone methyl-transferase activity. The RNA pol II and H3K4me3 signals were very low at MYT1 and ZNF333 gene loci as in the Hep G2 cells (Figure 5).

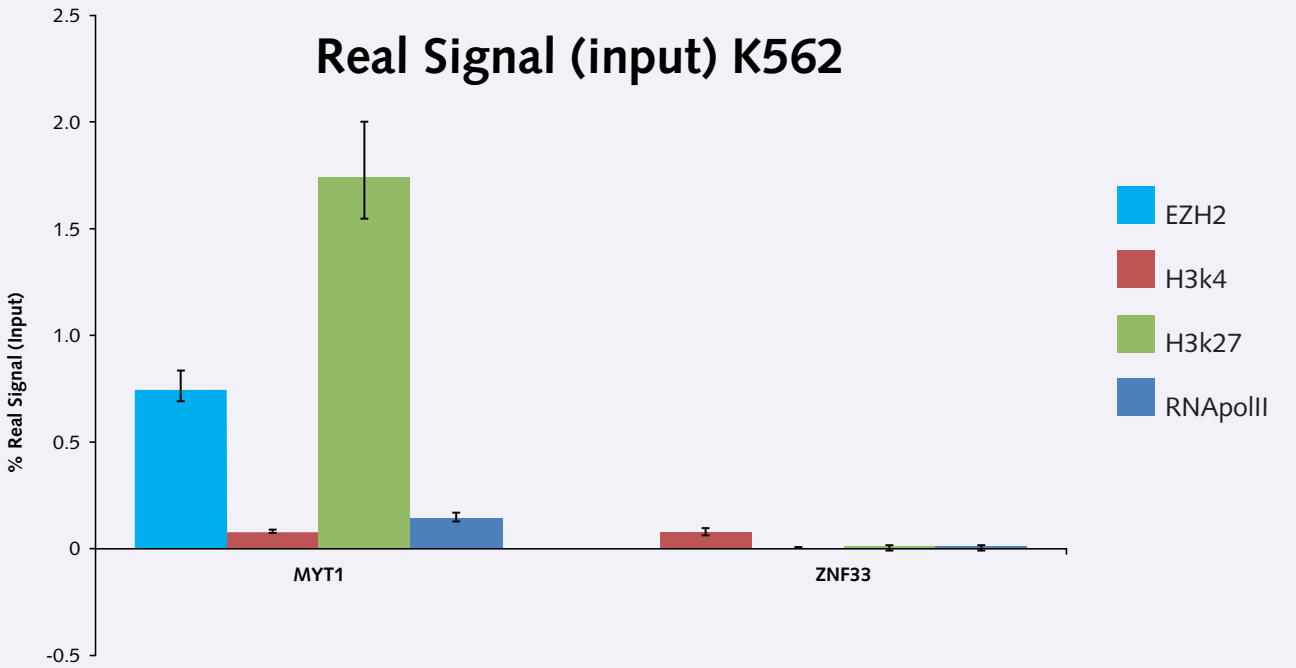

Figure 5. Detected precipitation of low abundant EZH2 at the MYT1 locus.. The % Real Signal (input) values are very low on the negative gene ZNF333 as expected.

4.0 Summary

In order to demonstrate the ease of use and application of Chromatrap® for improved sensitivity and reproducibility of a complex ChIP set up, a matrix of IP assays was designed to simulate a typical gene-specific epigenetic research approach. The balance of histone methylation presence, shown in the literature to be associated with active or inactive transcription, was detected alongside the recruitment of TF's RNA pol II, EZH2 or HDAC1. The balance of these factors is sufficient to allow hypothesis to be drawn regarding the possible chromatin conformation and gene transcription at the time of fixation. The impressive selectivity amplification of signals observed at positive gene loci and absence at the negative genes, clearly demonstrates the utility of this low volume, low input concentration assay. Both the high and low abundance targets were assayed using the same low number of cells (approximately 80,000) showing the flexibility of Chromatrap® assay without the need to increase the amount of starting material and incubation times.

4.1 Conclusion

Chromatrap® from Porvair Sciences is the first solid state chromatin immunoprecipitation assay based on true filtration of antibody-targeted chromatin fragments from unspecific antibody-chromatin interactions. 500ng of chromatin, equivalent to 80,000 cells, subjected to precipitation targeted at high (RNA pol II, H3K4me3, H3K27me3) and low (EZH2, HDAC1) antibody targets is shown here. Up to 140-fold difference between methylation marks is achieved on ubiquitously expressed gene loci, and up to 28-fold on repressed genes, such as MYT1, correlated with binding of a low abundance transcription factor EZH2. Other competitor assays kits are characterised by high concentrations of starting material (chromatin, antibody), or to use prolonged incubation times for increased sensitivity. Chromatrap® overcomes these obstacles without compromising on achievable sensitivity. (http://chromatrap.com/uploads/documents/TL0014__1_Day_Chromatrap_App_Note_1.pdf), clearly demonstrating the value of Chromatrap® in gaining insight into epigenetic gene regulation.

5.0 References

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