Chromatrap

A novel solid-state ChIP platform in high throughput format – Chromatrap® 96 HT

Summary

Epigenetics is increasingly a key focus of research groups in both industry and academia for answers to fundamental transcription regulation questions. Advances in high throughput ChIP coupled with automated liquid handing and next generation sequencing are paving the way for global gene expression analysis and examination of the epigenetic landscape of vast numbers of cell types under varied treatment or disease conditions. In order for this to become a reality, the technology efficiency needs to be advanced, allowing multiple gene loci and signalling mechanisms to be investigated. The novel solid phase technique employed by the Chromatrap® spin column technology has been developed to create a 96 well microplate format for high throughput ChIP. Chromatrap® 96 HT provides a superior platform for large-scale examination of disease and metabolic pathways, biomarkers and biopharmaceutical and small molecule effects on transcriptional regulation, generating a vast array of data from small quantities of starting material.

This technical note demonstrates the tremendous potential of Chromatrap® 96 HT in the field of epigenetics. Sensitive enrichment of gene loci following immunoprecipitation with antibodies directed against transcription factors, alongside indicative histone marks, is clearly shown here to be highly selective, demonstrating excellent reproducibility. Parallel observation of transcription factor recruitment and histone methylation in multiple cell lines is described, demonstrating the capability of the platform to generate vast quantities of high quality, comparable data in a single one-day experiment.

By eliminating the carry-over and unwanted retention often seen with bead-based assays, Chromatrap® 96 HT provides higher purity outputs and with a shorter overall protocol, allowing 96 samples to be processed in one day.

1.0 Introduction

The development of disease and deregulation of cellular signalling pathways involves more than basic genetics and environmental factors. In recent years epigenetic regulation of gene expression has emerged as a significant factor in these processes (Ho and Tang, 2007) and chromatin immunoprecipitation (ChIP) has become a prominent assay for understanding the interaction of regulatory proteins with their target DNA backbone. The majority of ChIP platforms currently available use agarose or magnetic beads. These are associated with a number of difficulties including high background, tedious wash steps which result in sample loss and low binding specificity of beads to antibody. Solid phase immunoprecipitation of chromatin using Chromatrap® spin columns is an exciting new technology advance for ChIP. The Chromatrap® system has been optimized for high sensitivity, low volume analysis of samples to enable targeting of epigenetic regulatory mechanisms alongside low abundant transcription factor binding in model and primary systems (Chromatrap enriches epigenetic marks in primary cells). Chromatrap® solid phase ChIP is rapid (1-day spin column assay) and sensitive with low cell numbers and selective for low abundant transcription factors (targeting the epigenetic landscape).

The limitation of single column analysis is that only 24 samples can be processed at any one time. The advantage of Chromatrap® 96 HT is that it allows up to 96 ChIP reactions to be processed simultaneously. Chromatrap® 96 HT not only offers the technical advantages over magnetic and agarose beads but also means that 96 samples can be processed in one day, with excellent DNA enrichment, and is compatible with automated liquid handling, reducing manual handling error and processing time. The opportunity to perform 96 ChIPs on a microplate means that multiple cell types can be tested in parallel, along with any treatments of interest, with minimal handling time. Further to this, multiple antibody targets can be enriched on the same plate providing a vast array of data and enabling parallel observation of widespread effects in one experiment. The application of

Chromatrap® 96 HT in 1 day high throughput screening of high abundant transcription factors and common epigenetic marks has been demonstrated to be robust and reproducible. The Chromatrap® 96 HT system is also compatible with ChIPseq methods. The major advantage of using Chomatrap® 96 HT in pharmaceutical and industrial settings is the ability to generate large, reliable datasets in a cost-effective and time-efficient manner.

To show the efficiency and sensitivity of Chromatrap® 96 HT an experiment was designed whereby a low abundant transcription factor and associated histone methylation marks were immunoprecipitated from multiple cell lines in parallel. Chromatin was prepared from five human cancer cell lines HepG2 (liver hepatocellular carcinoma cell line), K562 (chronic myelogenous leukemia cell line), HeLa (cervical adenocarcinoma cell line), Ishikawa (endometrial adenocarcinoma cell line) and MCF7 (breast adenocarcinoma cell line) and immunoprecipitated using antibodies directed against the common epigenetic marks H3K4me3 and H3K27me3 alongside the low abundant transcription factor EZH2. The enriched DNA obtained from the Chromatrap® 96 HT precipitation was then subjected to qPCR analysis for a combination of positive and negative gene targets. Using Chromatrap® 96 HT a vast quantity of data was generated, a subset of the results are shown here to demonstrate the robustness and reproducibility of this large scale platform ChIP assay.

2.0 Method

Chromatin Preparation

Chromatin was prepared from five different sources, summarised in Table 1, as per the Chromatrap® protocol using the reagents supplied in the kit. Aliquots of each isolated chromatin stock were used to assess the yield and shearing efficiency, allowing standardisation of chromatin input, therefore facilitating cross-sample signal comparison.

Immunoprecipitation using Chromatrap® 96 HT

Experimental design

The 96 well platform offers enormous loading potential for enrichment of samples and analysis using appropriate controls. To demonstrate the loading capacity of Chromatrap® 96 HT, and the ability to generate vast amounts of data from a single experiment using this platform, 96 well ChIP was carried out to enrich epigenetic marks and transcription factors from chromatin from five different cell lines simultaneously (see Fig.1 for plate layout). Each antibody/chromatin combination was carried out in triplicate to demonstrate the reproducibility of the enrichment using the ChIP matrix.

Antibody and Gene Targets

Details of the target antibodies and the relevant gene loci can be found in Table 2. The epigenetic marks H3K4me3 and H3K27me3 are associated with enhancing and repressing gene transcription respectively (Young et al., 2011). Transcription factor binding and trimethylation of lysine 4 on histone 3 are generally associated with regions of open chromatin (Song et al., 2011), this more relaxed structure enables recruitment of RNA polymerase II (RNA Pol II) and transcription factors (TFs) to the more accessible DNA. RNA Pol II binding alongside H3K4me3 presence at the glyceraldehyde-3-phosphate (GAPDH) gene locus forms an excellent positive target as GAPDH is actively expressed in all cell types (Barber et al., 2005). ß-globin, which is inactivated in non-erythroid tissues (Levings et al., 2002; Goren et al., 2006), is a negative target for H3K4me3 in all included cell lines.

EZH2 encodes the histone-lysine N-methyltransferase, this enzyme forms part of a complex which adds the three methyl groups to lysine 27 on histone 3 and is mainly associated with gene silencing (Cao et al., 2002). Myelin transcription factor 1 (MYT1) is a known positive target of EZH2 (Kirmizis et al., 2004; Booher et al., 1997; Bracken et al.,2006) unlike the

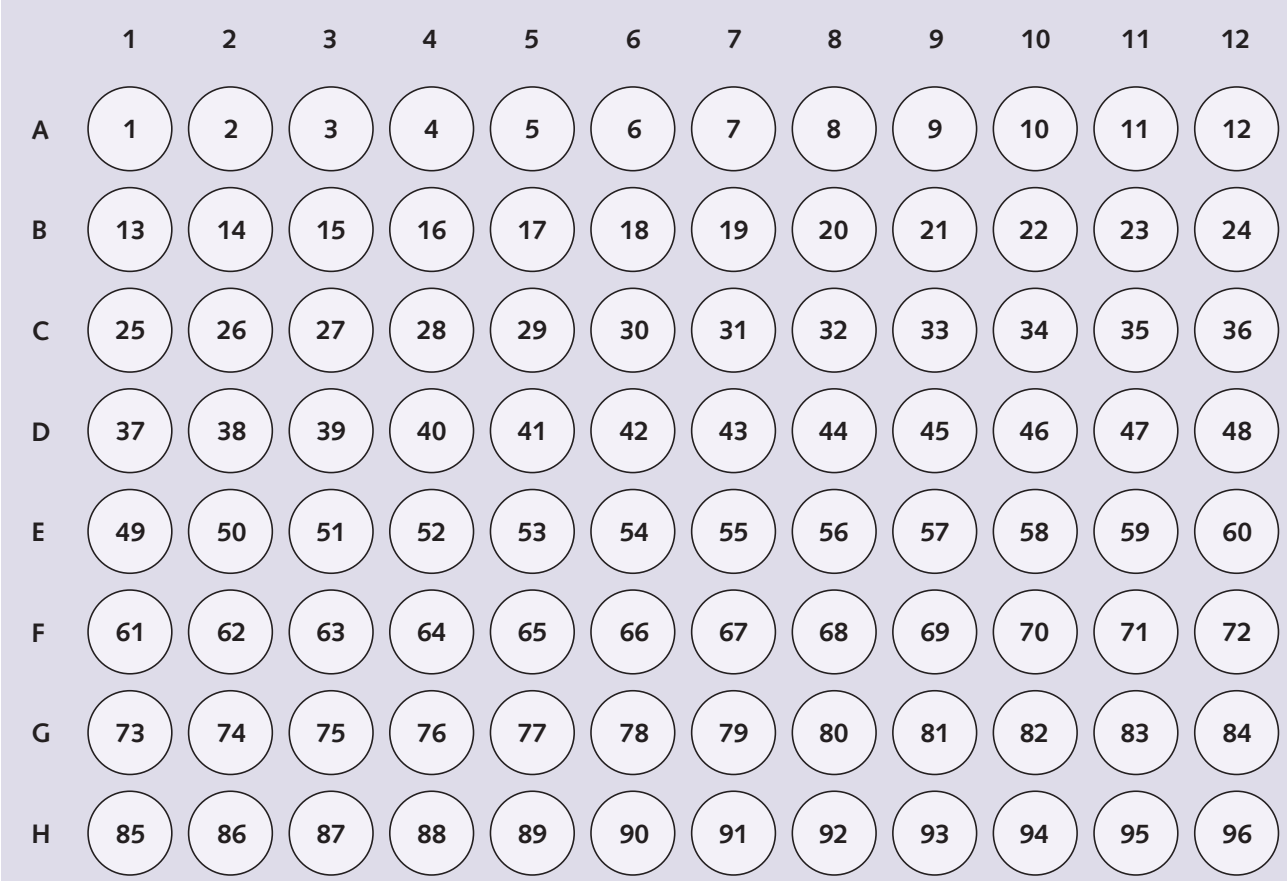
Cell Line	Cell Type	Reference
HepG2	Human liver hepatocellular carcinoma cell line	Knowles et al., 1980
K562	Human chronic myelogenous leukemia cell line	Lozzio & Lozzio, 1975
HeLa	Human cervical adenocarcinoma cell line	Gey et al., 1951
Ishikawa	Human endometrial adenocarcinoma cell line	Terakawa et al., 1987
MCF7	Human breast adenocarcinoma cell line	Soule et al., 1973

Table 1 – *Cell lines included in the study*

negative gene target, ZNF333, a zinc finger binding protein implicated in gene repression (Jing et al., 2004). In addition to negative control gene targets non-specific IgG antibody from the same species as the test sample antibodies was included to demonstrate the low non-specific background obtained using the Chromatrap® technology.

Antibody targets	Positive gene targets	Negative gene targets
RNA Pol II	GAPDH	ß-globin
H3K4me3	GAPDH	ß-globin
H3K27me3	ß-globin, MYT1	GAPDH, ZNF333
EZH2	MYT1	ZNF333

Table 2 – *Antibody targets and gene loci included in this study*



Key:-

Well	Cell Line	Antibody	Well	Cell Line	Antibody	Well	Cell Line	Antibody	Well	Cell Line	Antibody
1-3	HepG2	RNA Pol II	25-27	K562	H3K27me3	49-51	HeLa	HDAC	73-75	Ishikawa	IgG
4-6	HepG2	H3K4me3	28-30	K562	EZH2	52-54	HeLa	IgG	76-78	MCF7	RNA Pol II
7-9	HepG2	H3K27me3	31-33	K562	HDAC	55-57	Ishikawa	RNA Pol II	79-81	MCF7	H3K4me3
10-12	HepG2	EZH2	34-36	K562	IgG	58-60	Ishikawa	H3K4me3	82-84	MCF7	H3K27me3
13-15	HepG2	HDAC	37-39	HeLa	RNA Pol II	61-63	Ishikawa	H3K27me3	85-87	MCF7	EZH2
16-18	HepG2	IgG	40-42	HeLa	H3K4me3	64-66	Ishikawa	EZH2	88-90	MCF7	HDAC
19-21	K562	RNA Pol II	43-45	HeLa	H3K27me3	67-69	Ishikawa	ER-α	91-93	MCF7	ER-α
22-24	K562	H3K4me3	46-48	HeLa	EZH2	70-72	Ishikawa	IgG	94-96	MCF7	IgG

Figure 2 – 96 well ChIP layout

The layout of the Chromatrap® 96 HT well ChIP plate as used in this study. A 40µl slurry containing chromatin from the source detailed in the key along with the antibody described in the key was added in triplicate to the designated wells.

Immunoprecipitation

Chromatin stocks were standardised by preparing 25ng/µl working stocks in sterile distilled water (see Table 3 for example calculations). Addition of 20µl of the relevant chromatin working stock to each of the slurries therefore gives a total chromatin loading of 500ng. Inputs were prepared in parallel containing 500ng chromatin from each cell line in a total volume of 20µl, these samples were used for analyses and not subjected to ChIP enrichment. Each antibody, including the negative control IgG, was added at a 2:1 ratio to the chromatin (1µg antibody:500ng chromatin). Slurries were prepared as a mastermix for each triplicate of replicates for each sample in a microcentrifuge tube (as outlined in Table 4). 40µl of IP slurry was then loaded onto the ChIP, in its corresponding well according to the 96 well plate layout. The plate was incubated for 1 hour at 4°C before washing to remove any unbound chromatin, this is followed by elution of the selectively enriched chromatin specifically precipitated using the appropriate antibody.

500 ng chromatin in 20ul			Chromatin for 25ng/µl working stock		Sterile dH_20 for 25ng/µl working stock	
No. of ChIP's	Chromatin	Starting concentration	1 ChIP	all ChIPS	1 ChIP	all ChIPs
18	HepG2	250ng/ul	2.00µl	36.00µl	18.00µl	324.00µl
18	K562	110ng/ul	4.55µl	81.82µl	15.45µl	278.18µl
18	HeLa	500ng/ul	1.00µl	18.00µl	19.00µl	342.00µl
21	Ishikawa	350ng/ul	1.43µl	30.00µl	18.57µl	390.00µl
21	MCF7	400ng/ul	1.25µl	26.25µl	18.75µl	393.75µl

Table 3 – *Concentrations of chromatin stocks and working stocks used for 96 well ChIP*

Antibodies			ChIP mix (3x)				
Antibody	Stock concentration	Volume for 1µg	Chromatin (25ng/µl)	Wash buffer 1	PIC	dH20	Antibody
RNA Pol II	0.2µg/µl	5µl	60ul	15ul	3ul	27ul	15ul
IgG	0.2µg/µl	5µl	60ul	15ul	3ul	27ul	15ul
EZH2	1µg/µl	1µl	60ul	15ul	3ul	39ul	3ul
ER alpha	0.2µg/µl	5µl	60ul	15ul	3ul	27ul	15ul
H3K4me3	1µg/µl	1µl	60ul	15ul	3ul	39ul	3ul
H3K27me3	1µg/µl	1µl	60ul	15ul	3ul	39ul	3ul
HDAC	0.7µg/µl	1.43µl	60ul	15ul	3ul	37.7ul	4.3ul

Table 4 – *Slurry preparation for single and triplicate sample slurries prepared in 1.5ml microcentrifuge tubes.*

A significant advantage of the Chromatrap® 96 HT solid phase platform over bead-based systems for high throughput ChIP is the suitability of the buffer system for proceeding directly to downstream processing without the need for DNA cleanup. Eluted samples were reverse cross-linked alongside the corresponding inputs and the released protein digested with proteinase K, leaving DNA suitable for use directly in qPCR.

qPCR analysis

qPCR was used to analyse precipitation of the desired gene loci by antibodies directed against proteins of interest known to be present at the given locus. In order to emphasise the selectivity and sensitivity of the ChIP assay, precipitation at a negative gene locus, where the protein of interest should not be present, was also determined. Each precipitation assay was performed using an equivalent loading of IgG antibody as a negative control. The percentage of real signal was calculated as a proportion of the input chromatin, normalised using the signal generated by non-specific binding of unspecific IgG. Error bars represent the standard error of the mean of the triplicate ChIPs.

3.0 Results and discussion

To demonstrate the application of Chromatrap® 96 HT in reliable, rapid and reproducible high throughput ChIP, common epigenetic marks and transcription factors from five human cancer cell lines were selectively enriched using the solid phase platform. The following data represent only a glimpse of the low abundant transcription factor and epigenetic mark target results gained from using Chromatrap® 96 HT, highlighting how much data can be achieved using the high throughput ChIP system.

Using low chromatin loadings (500ng), equivalent to a small number of cells (approx. 80,000), the actively expressed GAPDH gene was precipitated with high specificity using an antibody directed against the epigenetic mark associated with promoter regions being actively transcribed, H3K4me3 (Bartova et al., 2008). A 4.35% real signal was observed for H3K4me3 precipitated at the GAPDH gene locus in HepG2 cells, with around 3.5% for Ishikawa, 3% for MCF7 and around 2% for K562 and HeLa highlighting the ease of observation of variations in signal between cell lines when multiple ChIPs are carried out in parallel. The specificity of this enrichment is demonstrated by the absence of enrichment of the GAPDH promoter region following immunoprecipitation with an antibody directed against modified histone associated with gene repression, H3K27me3 (Portella and Esteller, 2010). A positive enrichment signal for H3K27me3 presence at the GAPDH locus was only observed in chromatin isolated from HeLa cells. A background signal of 0.31% represented a 6.5 fold decrease when compared with 1.97% for the H3K4me3 antibody, leaving no doubt this region of the chromosome is predominantly open in all five cell lines, as expected. Figure 3 demonstrates the value of parallel immunoprecipitation of chromatin from different cell lines or following different treatments using high throughput ChIP. The technique generates data which provides easy visual comparison of variations in gene expression between different cell lines, demonstrating the capacity to monitor multiple cell types or patient response at specific gene loci to the addition of small molecules or biopharmaceuticals, in disease progression or as biomarkers of medical conditions.

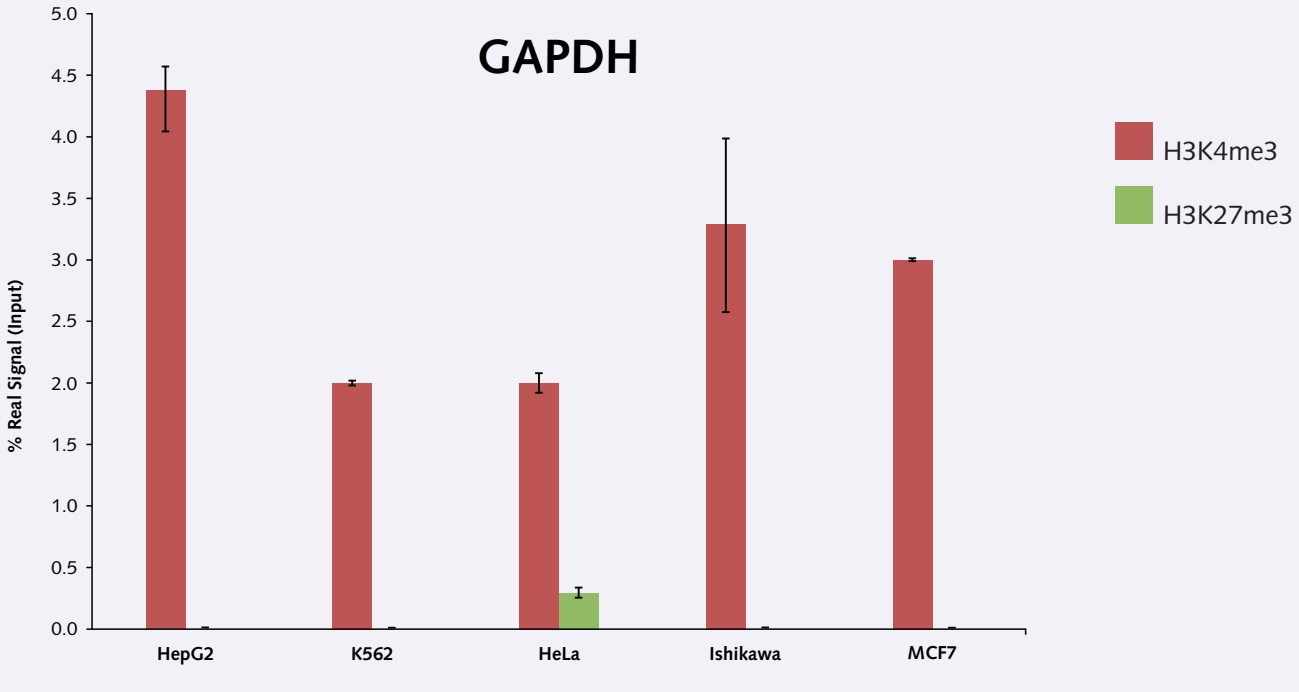


Figure 3 – *Positive and negative GAPDH promoter enrichment from chromatin isolated from five different human cancer cell lines using antibodies directed against H3K4me3 and H3K27me3.*

ß-globin is only expressed in adult erythroid cells (Levings et al., 2002; Goren et al., 2006). Therefore, in the cell lines used in this study the locus would be expected to be in the closed conformation, with absence of RNA Pol II and transcription factor binding. H3K27me3 at the ß-globin locus of the five cell lines demonstrates inactive transcription and repressed gene expression (see Fig. 4). High real signals of 6.31% and 4.54% were observed at the ß-globin locus of HepG2 and HeLa respectively following precipitation with H3K27me3, indicating a tightly closed chromatin conformation and absence of transcription at this site. 3% and 2% real signal for the presence of H3k27me3 were observed in Ishikawa and MCF7 cells at the ß-globin locus, with a comparatively low signal of 0.6% in K562 cells. This low signal however represents a 3 fold increase in signal over background IgG demonstrating the sensitivity of Chromatrap® 96 HT to detect low levels of broad factor epigenetic signal at distinct gene loci. This ability is facilitated by the low level of non-specific binding using solid phase Chromatrap®, resulting in no signal loss with less abundant targets. H3K27me3 signal was low (0.07% in MCF7) or absent in all cell lines confirming the gene locus is not in the open conformation.

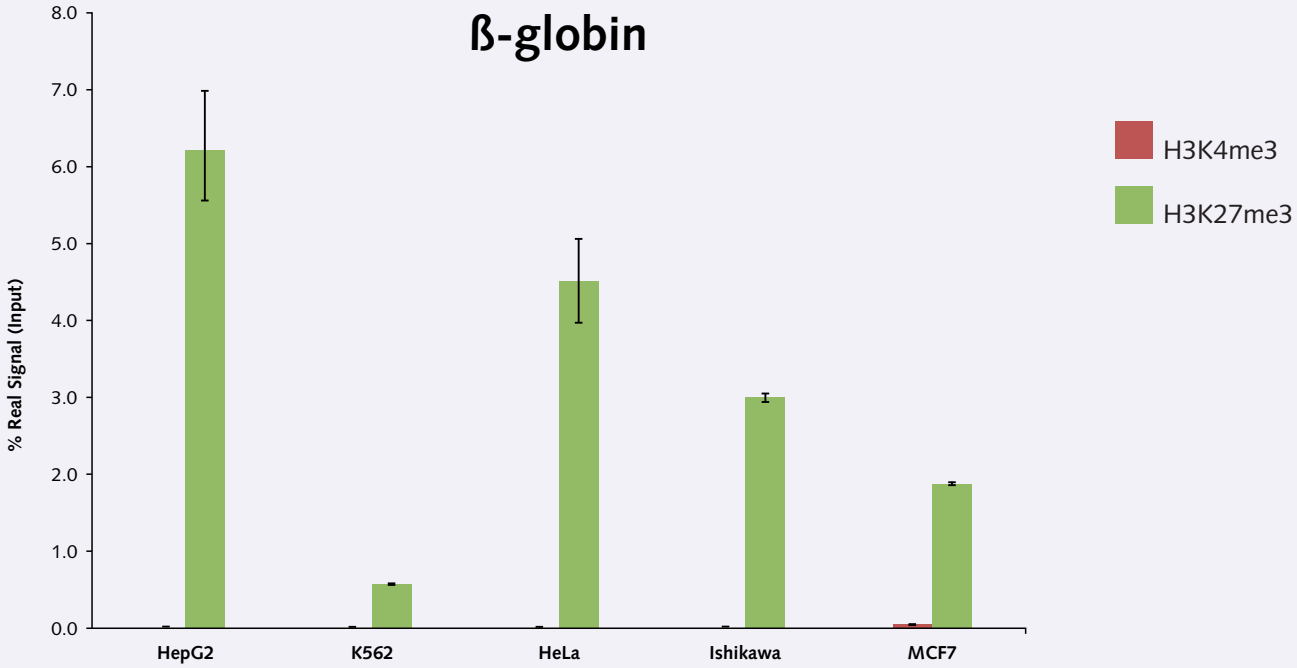


Figure 4 – *Histone methylation signal precipitated at the ß-globin promoter region of five different human cancer cell lines using antibodies directed against H3K4me3 and H3K27me3.*

Precipitation of the relatively low abundant target EZH2 alongside an antibody directed against the epigenetic mark H3K27me3 associated with gene silencing (Young et al., 2011) was chosen here to demonstrate the use of Chromatrap® 96 HT system to monitor a known TF mechanism of gene repression in multiple cell lines. High signal for the neural specific DNA-binding protein MYT1 following immunoprecipitation with both antibodies is indicative of **excellent enrichment of less abundant targets from relatively low chromatin concentration**. Once again, the low non-specific background binding of unspecific IgG using Chromatrap® 96 HT, results in excellent signal to noise ratio even for less abundant targets. **Selectivity** of the assay is demonstrated by the contrast in MYT1 signal with that of the negative target ZNF333. ZNF333 precipitation generated low to no signal in all cell lines with all antibody targets, shown here are the results for chromatin extracted from the diverse cell lines HepG2 and Ishikawa (Figures 5 and 6).

Recruitment of the low abundant transcriptional repressor EZH2 to the MYT1 locus is demonstrated in the liver carcinoma cell line, HepG2, where a 12-fold enrichment was observed over the negative gene target ZNF333. These data are complimented by almost 16% real signal for H3K27me3 at the same locus. Enrichment of H3K27me3 signal at the MYT1 locus was six times that observed using H3K4me3, indicating a closed chromatin conformation and repressed transcription (Figure 5). The same pattern was also observed for the endometrial cell line Ishikawa (Figure 6). Excellent enrichment of MYT1

following immunoprecipitation with EZH2 and H3K27me3 with little variation between replicates and no enrichment of the negative gene target ZNF333 for H3K4me3, H3K27me3 or EZH2 demonstrates the selectivity, sensitivity and reproducibility of the platform.

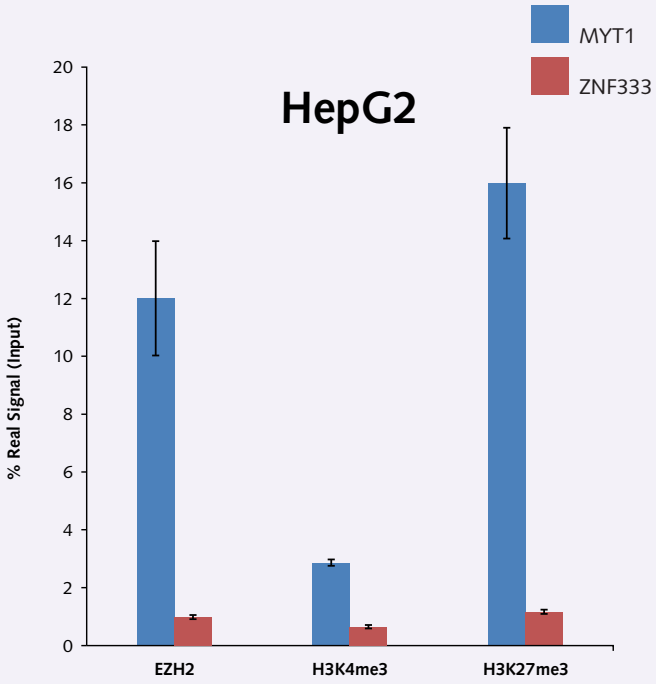


Figure 5 – *EZH2, presence at the MYT1 and ZNF333 loci alongside open and closed chromatin confirmation histone methylation marks reveal insights into the predicted mechanism of repression in the HepG2 cell line.*

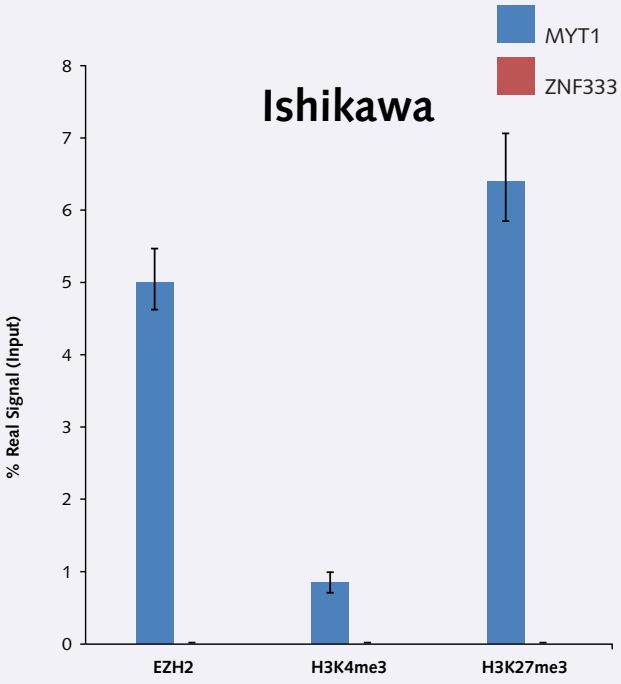


Figure 6 – *EZH2, presence at the MYT1 and ZNF333 loci alongside histone methylation marks indicative of open and closed chromatin confirmation reveal insights into the predicted mechanism of repression in the Ishikawa cell line.*

4.0 Conclusions

Chromatrap® 96 HT provides an excellent platform for highly selective high throughput analysis of gene regulation which is dependent on the interaction of transcription factors and a vast array of epigenetic modifications. This technical note has demonstrated that solid phase ChIP using Chromatrap® 96 HT provides specific and sensitive enrichment of chromatin associated with epigenetic marks and transcription factor binding sites in multiple cell lines with impressive reproducibility. With its suitability for automated high throughput ChIP Chromatrap® 96 HT opens up the opportunity for accurate, reliable analysis of global gene expression and investigation of multiple regulatory pathways following hundreds of different treatments and/or in many different cell lines in parallel.

The format enables investigation of the effects of biopharmaceuticals or small molecules at varying concentrations in different cell lines to be carried out in parallel in a single experiment. Similarly, a wide range of biomarkers or metabolic/disease pathways could be examined along with their regulation/deregulation in different tissues or following different cell/tissue treatment in just one assay, rapidly advancing the knowledge in a single day. Excellent enrichment of high and low abundant targets has been achieved with low concentration chromatin from diverse human cell lines in a robust and reproducible manner demonstrating the potential for this platform to advance epigenetic knowledge in a variety of academic and commercial fields.

5.0 References

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