Chromatrap

Unlocking the Archive – Chromatrap® ChIP from Formalin Fixed Paraffin Embedded (FFPE) Tissue

Summary

Tissues from biopsies are routinely preserved by formaldehyde fixation and embedding in paraffin wax. These samples provide a vast archive of disease and comparative healthy tissue information. Unlocking the chromatin within this archive would provide the field of epigenetics with a continuing supply of tissue from a multitude of disease states, allowing examination of the in vivo histone modifications and transcription factor occupation of gene promoters from large cohorts. Many of these samples have been preserved for up to 20 years giving the added advantage of the knowledge of patient outcomes of the disease. Extraction of chromatin from FFPE tissue is difficult, time consuming and fraught with problems. The fixation process can result in the damage of many of the protein epitopes, leaving a lower proportion of epitopes available to be recognised by the specific antibodies in ChIP. Chromatrap® FFPE ChIP provides a superior extraction system which results in a much higher yield of chromatin than comparable extraction methods providing more available protein for the ChIP antibody and an efficient immunoprecipitation.

This short technical note demonstrates the success of the Chromatrap® FFPE ChIP kit in the extraction and analysis of chromatin from both human and animal tissue preserved using FFPE. Using chromatin immunoprecipitation (ChIP) targets are selectively and reproducibly enriched using the Chromatrap® spin columns following extraction with Chromatrap® FFPE ChIP kit reagents.

1.0 Introduction

Formalin fixed paraffin embedded (FFPE) tissue is an invaluable source of DNA, RNA and chromatin from clinical and historical samples. Over a billion tissue samples, comprised mainly of FFPE tissue, are estimated to be stored in hospitals, tissue banks and laboratories worldwide (Tang et al, 2009). The vast majority of pathology samples are stored as FFPE blocks for analysis such as immunohistochemistry (IHC). Researchers have already begun unlocking the potential of this tissue using DNA and RNA extraction for genomic and epigenetic analysis. In line with the requirements of the current era in personalised medicine, analysing larger sample cohorts to study numerous biomarkers used for targeted therapies and prognosis, detailed profiling of genomes is becoming increasingly important. Translating the potential of epigenetic profiles for new biomarker discovery and validation requires access to cohorts with associated patient information, diagnosis and treatment outcome. Archived tissue provides an advantage over fresh or frozen tissue in that it remains viable for further analysis over a longer time period, disease outcomes and additional clinical data are often collected long after biopsies are taken. Genotyping technologies have been successfully employed on nucleic acids from FFPE tissue to examine mutations in genes (Beadling et al, 2011, Suz et al, 2011) and gene expression (Fanelli et al, 2011). Utilising the chromatin from these samples has proved more difficult due to extensive cross-linking and damage to

protein epitopes resulting from the fixation process and the destruction of these proteins during typical DNA extraction protocols. Traditional DNA extraction protocols are designed to remove proteins (Fan and Gulley, 2001) and often involve phenol extraction where the protein is separated in the interphase (Pikor et al, 2011).

Sample quality and availability remain limitations for high throughput genetic profiling (Pikor et al, 2011). As a result of the fixation process and the extensive cross-linking which occurs in the preparation of FFPE tissues DNA yield is often low, chemically modified and highly degraded (Bourgen et al, 2014). Formalin fixation leads not only to cross-links between proteins and DNA but also between the strands of DNA themselves (Lin et al, 2009) which causes inhibition of downstream processes such as PCR (Gilbert et al, 2007). Fixation conditions such as extremely low pH cause additional fragmentation of DNA which compounds poor PCR efficiency. In addition to the issues with the DNA component of the genetic material extracting chromatin from FFPE tissue brings its own set of unique challenges.

The Chromatrap® FFPE ChIP kit overcomes these difficulties using an optimised buffer system for extraction which results in a much higher yield of chromatin, leading to more protein epitopes available to ChIP antibodies. Coupled with the increased sensitivity, eliminating the need for high chromatin loading in the Chromatrap® system this makes Chromatrap® FFPE ChIP kit the perfect solution for epigenetic research utilising FFPE tissue.

2.0 Method

FFPE sample preparation

FFPE samples used in this study were rat uterine tissue (fixed for 18 hours in 10% formalin and soaked in 70% ethanol before embedding in paraffin wax, Fig1a) and human breast tissue (Amsbio, Oxford UK), fixed in 10% neutral formalin for 24 hours before embedding in immunohistochemical grade paraffin wax (Fig1b).

Each tissue type was sectioned into 5 µm slices using a microtome (Leica) and the slices placed into a microcentrifuge tube. 20 x 5 µm slices of each tissue type were pooled into a microcentrifuge tube per extraction.

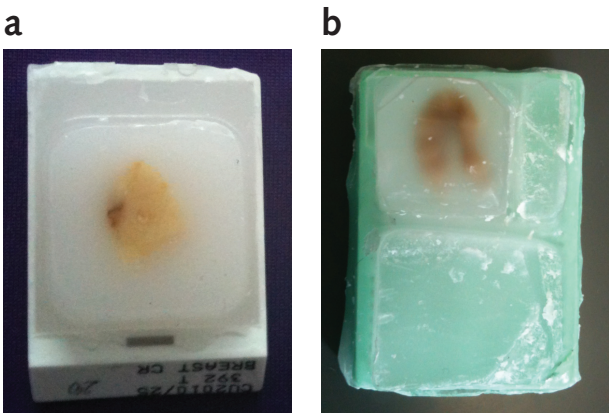
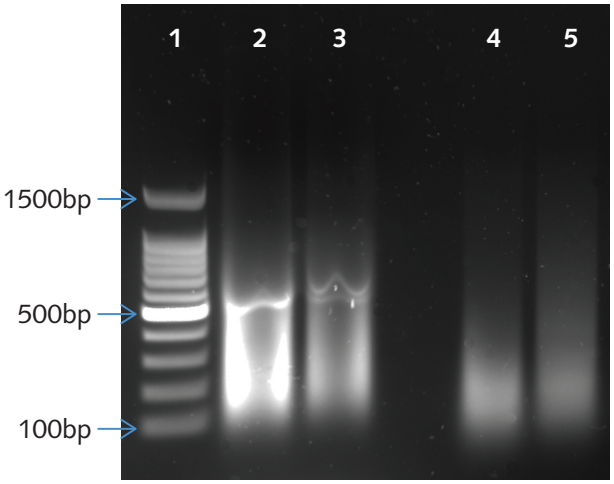


Figure 1 – *rat uterine and human breast tumour FFPE tissue blocks*

Chromatin Extraction

Chromatin was extracted from the FFPE tissue blocks according to the Chromatrap® FFPE ChIP kit protocol. Briefly, paraffin wax was removed from each sample by adding 1 ml Paraffin Removal Solution (PRS) to each tube and incubating samples on a rotating platform for 5 min at room temperature (RT). Tubes were subsequently centrifuged for 5 min at maximum speed at RT and the supernatant carefully aspirated. Fresh PRS was added to each sample and the preceding process repeated to a total of 3 washes in PRS. Following aspiration of the final PRS wash tissue was rehydrated by addition of 1 ml 100% ethanol and tubes were again incubated on a rotating platform for 5 min at RT. Samples were then centrifuged for 5 min at full speed at 4°C before carefully aspirating the supernatant. The washing process was repeated with 70% ethanol, 20% ethanol and finally sterile distilled water. Following aspiration of the final distilled water, the pellet was resuspended in 1 ml FFPE Lysis Buffer and incubated for 30 min at RT on a rotating platform. Samples were centrifuged for 5 min at maximum speed at 4°C, the supernatant was aspirated and the pellet resuspended in 500 µl Digestion Buffer. Samples were homogenised by sonicating for 3 cycles of 30s on 30s off at 42% amplitude before addition of 1 µl Shearing Cocktail. Samples were mixed by pipetting and incubated at 37°C for 5 min before addition of Enzymatic Stop Solution. Pellets were collected by centrifugation, the supernatant carefully aspirated and samples resuspended in 500 µl FFPE Extraction Buffer. Chromatin was extracted by 40 rounds of sonication 30s on 30s off at 42% amplitude. Soluble (supernatant containing chromatin) and insoluble (pellet of tissue debris) fractions were separated by centrifugation. To check the fragmentation and quality of the extracted chromatin 25µl aliquots of each

fraction were reverse cross-linked and proteinase K digested, measured using a Qubit fluorometer (Invitrogen) and analysed by agarose gel electrophoresis. From Figure 2 it can be seen that the chromatin is well sheared (fragments are between 100-500 bp) and of good concentration.



Lane 1 – 100bp DNA ladder
Lane 2 – rat uterine FFPE chromatin, soluble fraction
Lane 3 – rat uterine FFPE chromatin, insoluble fraction
Lane 4 – human breast tumour FFPE chromatin, soluble fraction
Lane 5 – human breast tumour FFPE chromatin, insoluble fraction

FFPE Tissue	Soluble Fraction	Insoluble Fraction
Human breast tumour	6.68 ng/ul	3.44 ng/ul
Rat uterus	48 ng/ul	44 ng/ul

Figure 2 – *Agarose gel electrophoresis and Qubit measurements of chromatin extracted from rat and human FFPE tissue using the Chromatrap® FFPE ChIP kit.*

Chromatin Immunoprecipitation
For the immunoprecipitation slurries were prepared according to the Chromatrap® FFPE ChIP kit protocol. In each 1ml slurry 20 µl of chromatin stock was used with either 4 µg of anti-Histone H3 (Chromatrap® Product Code : 700000) for the positive immunoprecipitation or non-specific mouse IgG for the negative immunoprecipitation. Inputs were prepared in parallel containing 20 µl of the relevant chromatin stock, these were used for subsequent analysis and not subjected to ChIP enrichment. Immunoprecipitation was carried out as per the standard Chromatrap® FFPE ChIP protocol. Briefly, slurries were incubated for 1 hour on an end to end rotator at 4°C prior to loading onto to the relevant Chromatrap® ProA ChIP column, followed by a series of quick and simple centrifugation washes. Chromatin was eluted following a 15 min incubation of the FFPE Elution Buffer on the column. Samples and Inputs were reverse cross-linked for 2 hrs before Proteinase K digestion for 1hr. Finally, samples were cleaned using the supplied Chromatrap® FFPE purification columns and buffers and eluted in 50 µl DNA Elution Buffer.

qPCR analysis
qPCR was carried out using primers which recognise either the human or rat GAPDH locus (Barber et al., 2005). These primer sets generate amplicons <100bp for efficient analysis of highly fragmented FFPE DNA. Subsequent analysis enabled detection of precipitation and therefore, specific enrichment at these gene loci when compared with non-specific IgG. The percentage of real signal was calculated as a factor of the amount of input chromatin to enable relative analysis between samples. Error bars represent the standard error of the mean of triplicate ChIPs.

3.0 Results and Discussion

To demonstrate the application of the Chromatrap® FFPE ChIP kit in the high yield extraction and excellent enrichment of chromatin from FFPE tissue the common epigenetic mark H3 was specifically enriched from chromatin extracted from FFPE rat uterine tissue and human breast tumour tissue.

Excellent signal to noise is demonstrated following enrichment of the GAPDH locus in human (Figure 3) and animal chromatin (Figure 4) from FFPE tissue, using an antibody directed against H3. The sensitivity of the assay is illustrated by high positive antibody signal from very low chromatin concentrations and the superior selectivity by the low non-specific binding. The versatility of the assay allows excellent signal to be obtained from both 100 ng of human breast tumour chromatin or 1 µg rat uterine chromatin.

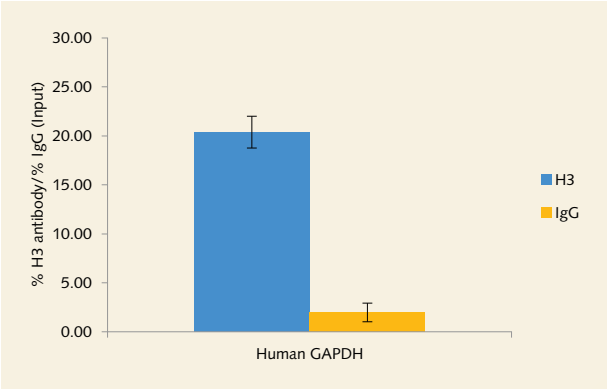


Figure 3 – *Enrichment of the GAPDH locus in human chromatin extracted from FFPE tissue, using anti-histone H3 antibody.*

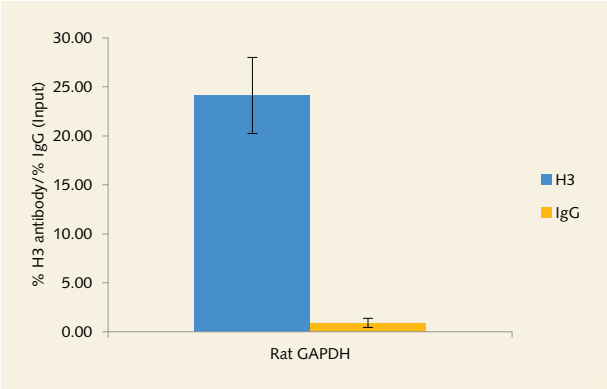


Figure 4 – *Enrichment of the GAPDH locus in rat chromatin extracted from FFPE tissue, using anti-histone H3 antibody.*

4.0 Conclusions

The Chromatrap® FFPE ChIP kit is the perfect assay kit for efficient extraction and immunoprecipitation of chromatin from complex formalin fixed paraffin embedded tissue from human and animal sources. The extraction protocol provides a high yield of chromatin from very difficult sample sources and the superior sensitivity of the unique solid state ChIP columns allows high real signal to be generated from low concentration chromatin. Coupled with shorter protocols and high throughput capability, these advantages make the Chromatrap® FFPE ChIP kit a quick, versatile sensitive and reproducible assay for analysis of patient or research FFPE archives.

5.0 References

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