Chromatrap

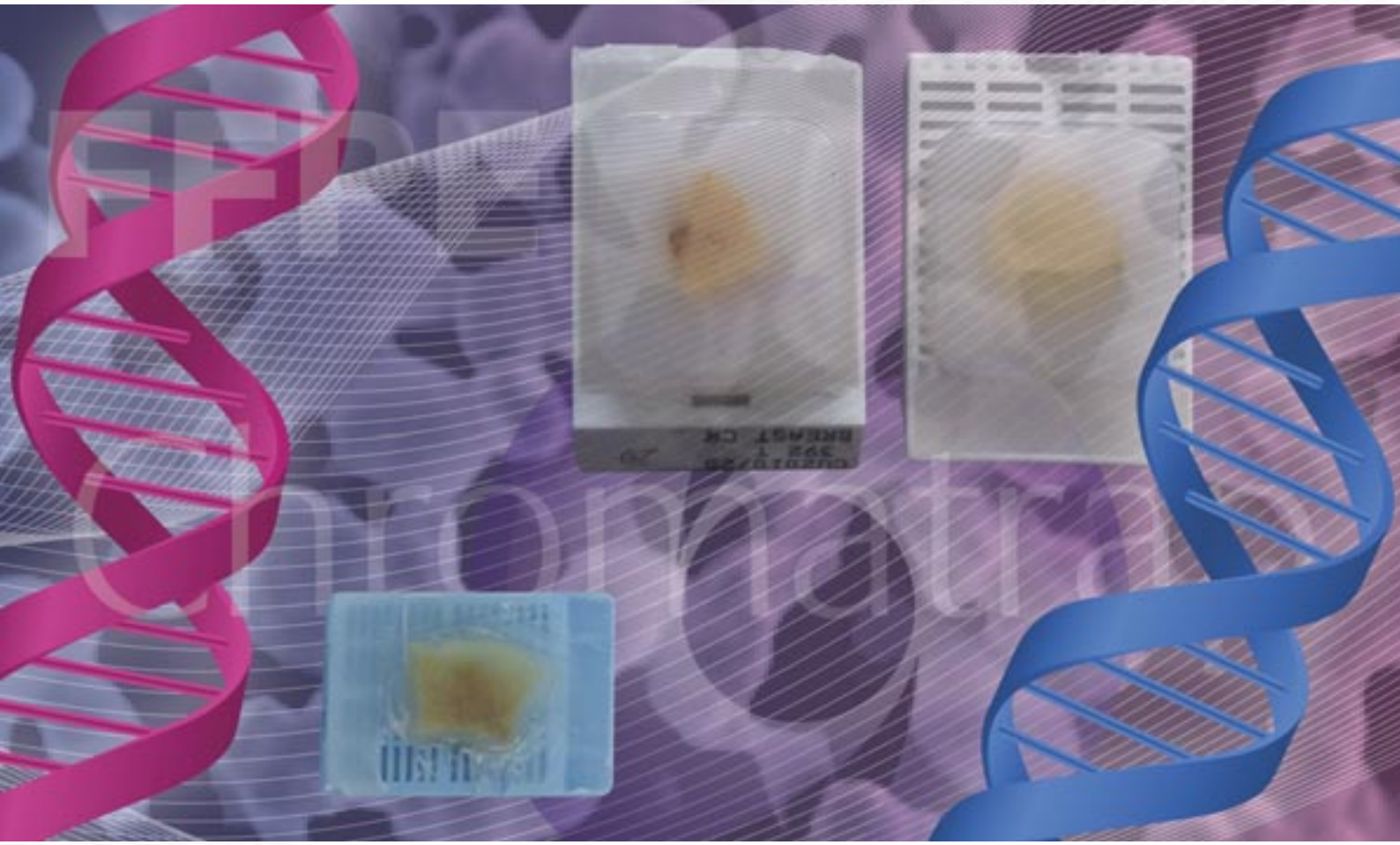
V1.2

Chromatrap® FFPE ChIP kit

A solid phase chromatin immunoprecipitation assay (ChIP) from formalin fixed paraffin embedded (FFPE) tissue

Protocol v1.2

Catalogue no. 500235, 500236



ADVANCEMENTS IN EPIGENETICS

Contents

Introduction

Epigenetics is the study of the molecular mechanisms which control gene expression in a potentially heritable way, which do not involve changes in the underlying DNA sequence. Many disease states involve changes to the epigenetic landscape, therefore pathology tissue can provide an invaluable resource for understanding how these alterations occur in different diseases and tissue types, not only in model systems but also in patient samples.

Chromatin Immunoprecipitation (ChIP) is a technique used to study the association of specific proteins, or their modified isoforms, with defined genomic regions. FFPE-ChIP utilises chromatin extracted from Formalin Fixed Paraffin Embedded (FFPE) tissue. Tissues, such as biopsies, are routinely fixed in high formaldehyde concentration and embedded in paraffin in hospitals under non standardised conditions to allow long term storage without excessive sample degradation. Therefore, a large amount of archived material is available to study. FFPE material has long been used for DNA and RNA analyses but due to difficulties in extracting proteins retaining their antigenicity, ChIP has not been possible until recently (Fanelli et al., 2011).

In a ChIP assay, DNA-protein complexes (chromatin) are selectively immunoprecipitated using matching antibodies and the resulting fractions treated to separate the DNA and protein components. Polymerase Chain Reaction (PCR), Real Time PCR (qPCR), hybridization on microarrays, or direct sequencing are typically used to identify DNA fragments of defined sequence.

An FFPE ChIP assay normally involves five key steps:

- *Preparation of chromatin to be analysed from FFPE tissue*
- *Immunoprecipitation of chromatin using a high quality ChIP-validated antibody specific to the target protein*
- *Isolation of the precipitated chromatin fragments*
- *DNA recovery from the precipitated product*
- *DNA analysis*

Chromatrap® ChIP-seq

Chromatrap®'s unique patented* technology provides a quicker, easier and more efficient way of performing ChIP with unparalleled sensitivity. In this revolutionary system, filter discs of an inert porous plastic, Vyon®, replace magnetic or agarose beads. Protein A or Protein G has been attached in the correct orientation throughout the filter to maximise the capture efficiency of the target chromatin/antibody complex.

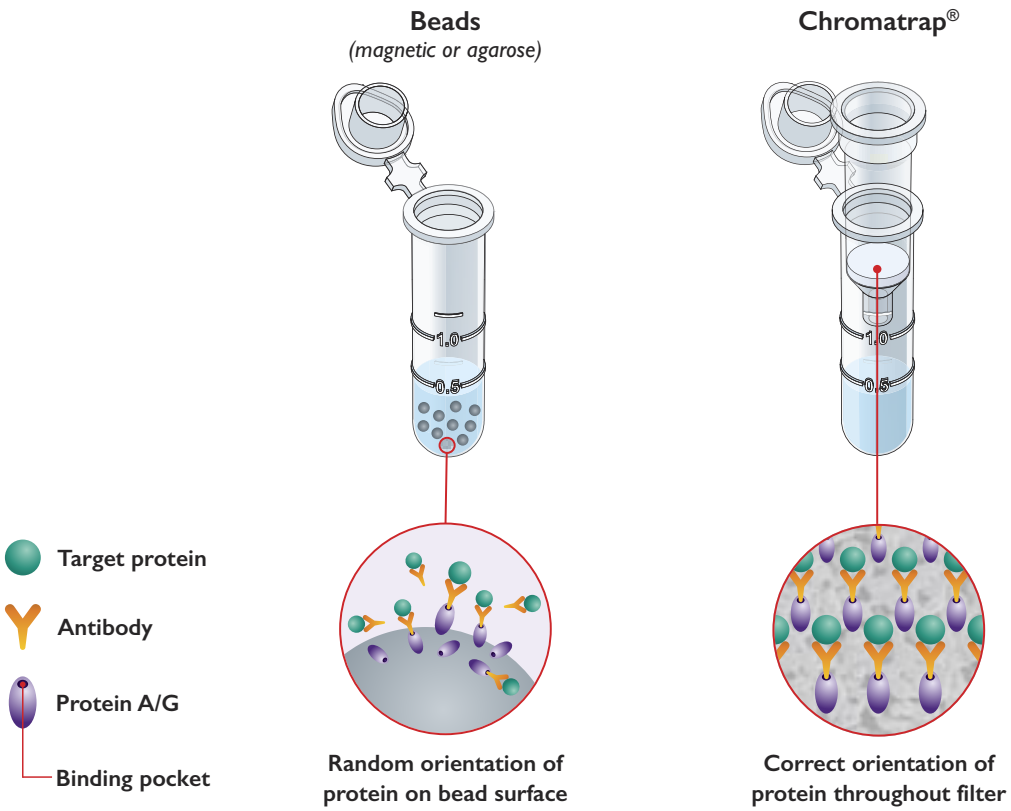


Figure 1: *Chromatrap® ChIP technology*

Advantages of Chromatrap FFPE ChIP

- Maximum chromatin recovery from challenging FFPE tissue
- Highest sensitivity ChIP allows low input material
- Low background due to the inert filter technology
- Fast protocol - no blocking steps or overnight incubations

*UK Patent No. GB2482209, & GB2527623, US Patent No. 9523681 & 10,435,480, Chinese Patent No. ZL 2011 8 0067254.X, Japan Patent No. JP 6088434 and Australian Patent No. AU 2011340263.

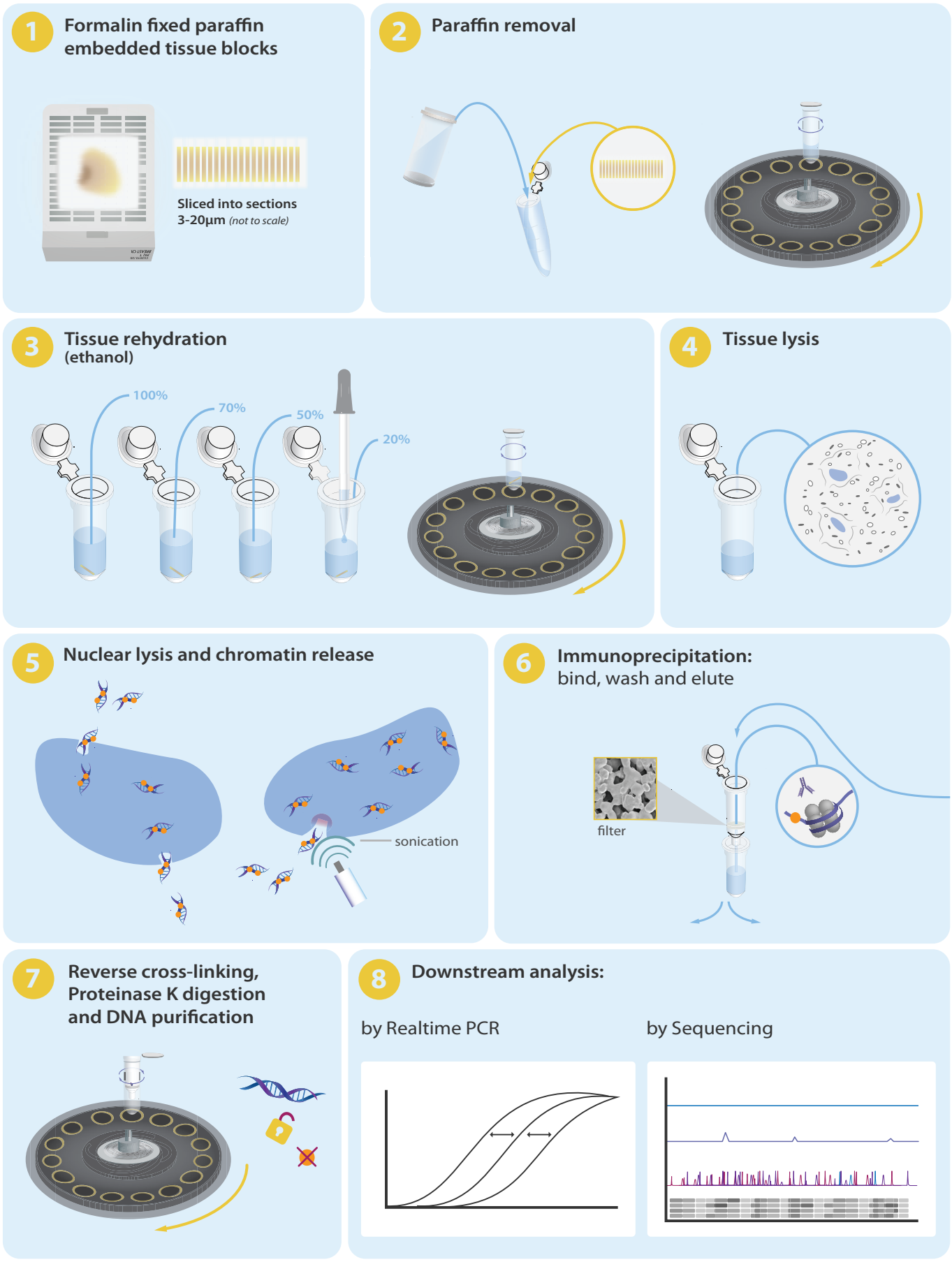


Figure 2: *Overview of the Chromatrap® FFPE ChIP process*

Kit Components

Chromatrap® FFPE ChIPs kits (500235/500236) allow the user to perform up to 24 ChIP ChIP assays from deparaffinisation through to downstream analysis. Upon receipt, please ensure the components are stored at the temperatures listed in Table 1.

Kit Component	Qty	Storage Condition
Chromatrap® ChIP-seq spin columns	24	4°C
Column Conditioning Buffer	60 mls	4°C
Wash Buffer 1	50 mls	4°C
Wash Buffer 2	50 mls	4°C
Wash Buffer 3	50 mls	4°C
ChIP-seq Elution Buffer*	3 mls	4°C
5 M NaCl	500 µl	4°C
1 M $NaHCO_3$	500 µl	4°C
FFPE Lysis Buffer	10 mls	4°C
Protease Inhibitor Cocktail (PIC)	50 µl	-20°C
Proteinase K Stop Solution	400 µl	-20°C
Proteinase K	200 µl	-20°C
1.5 ml Collection tube	50	RT
Paraffin Removal Solution**	4 x 25 ml	RT
Chromatrap® DNA purification columns	24	RT
DNA Binding Buffer	15 mls	RT
DNA Wash Buffer	15 mls	RT
DNA Elution Buffer	2 mls	RT

Table 1: *Chromatrap® FFPE reagents and materials*

*ChIP-seq Elution Buffer must be pre-warmed to 37°C in a waterbath for 30 minutes, with occasional inversion, prior to use to remove any precipitates. Bring the buffer back to room temperature when ready to use.

**Appropriate safety equipment should be used when handling Paraffin Removal Solution which is classified as an irritant (eg. lab coat, goggles and gloves). Do not discard Paraffin Removal Solution down the sink, dispose in a glass container for organic solvents as per your laboratories standard procedure.

The kits are manufactured DNase free and when stored as directed are stable for at least 6 months. Sufficient material is supplied for 24 ChIP assays and up to 10 chromatin sample preparations.

Additional Materials and equipment required

Reagents and Consumables

- *DNase free water*
- *Absolute Ethanol (molecular biology grade)*
- *70% Ethanol*
- *50% Ethanol*
- *20% Ethanol*
- *Microcentrifuge tubes*
- *Pipette tips (filter tips recommended)*
- *ChIP validated antibody directed against protein of interest*
- *qPCR primers for genes of interest*
- *PCR plates*
- *Positive antibody*
- *Negative antibody*
- *Phenol:Chloroform:Isoamyl alcohol (25:24:1)*
- *Chloroform:Isoamyl alcohol (24:1)*
- *3 M Sodium acetate pH 5.0*
- *Tris-HCl pH 8.0*
- *100 bp DNA ladder*

Equipment

- *Rocking platform*
- *Sonicator*
- *Centrifuge (4°C)*
- *37°C waterbath*
- *65°C waterbath*
- *Nanodrop/spectrophotometer/Fluorometer*
- *Fume hood*
- *Agarose gel electrophoresis equipment*

Optional materials

- *Linear Polyacrylamide (LPA)*
- *Low DNA binding microcentrifuge tubes*
- *PCR purification kit*

Experimental Design, Preparation and Planning

It is recommended that you read through the entire protocol before starting.
This protocol details the procedure for extracting chromatin from FFPE tissue blocks. The quality and quantity of chromatin that can be obtained varies greatly between different types of tissue and is dependent on the fixation conditions used. Therefore, for different sample sources, such as unstained slides, the process may need to be optimised by the user.

Consider the following when planning your experiment:

1. Size of the tissue sample
When planning an experiment, it's important to take into account the amount of material available for the experiment. A given amount of tissue will yield a finite amount of ChIP quality chromatin. The Chromatrap® FFPE kit supplies enough reagents for up to 10 chromatin preparations and up to 24 ChIP assays.

2. Chromatin preparation
The success of a ChIP assay is highly dependent on the quality of chromatin prepared. The protocol described here is based on chromatin prepared from up to 20 slices of tissue, up to 20 µm thick. Refer to Step 2b for details on how to check quality of chromatin.

3. Slurry Volume
A key advantage of the Chromatrap® technique compared to conventional bead based assay is the flexibility in chromatin loading. The fundamental requirement for optimal antibody binding is to load sufficient chromatin in a total volume of 1 ml ensuring that the chromatin does not exceed more than 90% (900 µl) of the total 1 ml slurry volume.

4. Quantification
It is critical to ensure sufficient chromatin has been extracted for ChIP. Due to the nature of FFPE tissue low concentrations of chromatin are yielded from extractions. It is strongly recommended chromatin is quantified by a fluorometric method such as the Qubit™ with the High Sensitivity ds DNA quantification kit (Life Technologies) due to difficulties in spectrophotometric quantification with very low concentrations of DNA.

5. Antibodies
The use of ChIP validated antibodies is essential for the success of a ChIP assay. The antibody must recognise and bind to native protein that is bound to DNA. It is essential to include ChIP validated positive and negative antibody controls to ensure chromatin preparation and ChIP methodology are appropriate. Antibodies from other applications do not always work well in ChIP.

6. Quantity of antibody
The user must determine the optimum antibody: chromatin ratio for use in ChIP. Optimal results have been achieved with 4 µg antibody in the Chromatrap® spin columns.

7. Downstream analysis
This protocol has been optimised for q-PCR as a downstream detection method (refer to page 15). Other detection methods will need to be optimised by the user.

8. Quantitative PCR interpretation
The efficiency of immunoprecipitation provides an indicator of the relative success of a ChIP assay and requires the interpretation of qPCR data to determine which DNA fragments have been enriched. This can be expressed as the recovery of the locus calculated as a percentage of input as follows:

$$\% \text{ recovery} = 2^{(Ct_{input} - Ct_{sample})}$$

9. Sequencing
In order to perform NGS following ChIP with FFPE samples it may be necessary to pool replicate samples. Consider the yield from a single IP using chromatin extracted from your tissue and prepare the number of replicate ChIPs that will provide enough DNA for preparation of an NGS library.

Protocol

Wherever this 'pause point' symbol is displayed it signifies that, if required, the sample can be stored under the designated conditions and the protocol continued at a later time.

Step 1 – Paraffin removal and rehydration

The following section describes paraffin removal and rehydration of FFPE tissue slices. A maximum of 10 slices of 20 µM tissue or 20 slices of 10 µM tissue sections should be used per tube to ensure efficient paraffin wax removal. Duplicate tubes of the same tissue sample type can be pooled following homogenisation of tissue.

Step 1a: Paraffin removal

1. Place tissue slices (eg. 5 x 5 µm slices) for each tissue sample in a microcentrifuge tube. If the tissue section is small or a higher concentration of chromatin is desired for ChIP it is recommended to prepare duplicate tubes which can be pooled following homogenisation (Step 2a point 1).
2. Add 1 ml Paraffin Removal Solution to each tube in a fume hood, cap and mix by inversion. Incubate the tubes on a rocking platform for 5 minutes at room temperature.
3. Centrifuge tubes at 18,000 x g for 5 minutes (room temperature). Carefully aspirate and discard the supernatant. Do not disturb the pelletted tissue. Add 1 ml fresh Paraffin Removal Solution to each tube and incubate for 5 minutes at room temperature on a rocking platform.
4. Repeat steps 2 and 3 to a total of 3 washes with Paraffin Removal Solution. If paraffin can still be seen on the tissue in the tube then washing with fresh Paraffin Removal Solution should be repeated to a total of 5 washes.
5. Remove the final Paraffin Removal Solution by careful aspiration with a pipette and proceed immediately to tissue rehydration.

Step 1b: Tissue rehydration

1. Add 1 ml 100% ethanol to each tube and incubate the tubes at room temperature for 5 minutes on a rocking platform. Centrifuge the tubes at 18,000 x g for 5 minutes at 4°C. Carefully aspirate and discard the supernatant.
2. Add 1 ml 70% ethanol to each tube and incubate for 5 minutes at room temperature on a rocking platform. Centrifuge the tubes at 18,000 x g for 5 minutes at 4°C. Carefully aspirate and discard the supernatant.
   N.B. Tissue can be difficult to pellet during rehydration and centrifugation steps may need to be repeated. Extra care should be taken not to disturb or remove small fragments of tissue when aspirating supernatents during rehydration steps.
3. Add 1 ml 50% ethanol to each tube and incubate for 5 minutes at room temperature on a rocking platform. Centrifuge the tubes at 18,000 x g for 5 minutes at 4°C. Carefully aspirate and discard the supernatant.
4. Add 1 ml 20% ethanol to each tube and incubate for 5 minutes at room temperature on a rocking platform. Centrifuge the tubes at 18,000 x g for 5 minutes at 4°C and carefully aspirate and discard the supernatant.
5. Add 1 ml sterile distilled water to each tube and incubate for 5 minutes at room temperature on a rocking platform. Centrifuge tubes at 18,000 x g for 5 minutes at 4°C and carefully aspirate and discard the supernatant.
6. Add 500 µl FFPE Lysis Buffer and 1 µl PIC to each tube, mix by swirling gently and incubate samples in a waterbath at 40°C for 1 hour.
7. Add 12.5 µl Proteinase K, mix well and incubate at 40°C for a further 10 minutes.
8. Add 25 µl Proteinase K Stop Solution to stop the reaction and mix well.

At this point the protocol can be continued or samples can be stored at -80°C overnight and thawed carefully on ice before proceeding

Step 2 – Chromatin Shearing and extraction

Chromatin is sheared and extracted using a unique buffer system and extended sonication. The amount and quality of chromatin that can be extracted from FFPE tissue is highly dependent on the tissue type, the conditions used for fixation and the age of the stored tissue block.

Step 2a: Tissue lysis and chromatin extraction

1. If samples were stored at -80°C defrost carefully on ice before continuing the protocol.
2. Sonicate the samples on ice or in a cooled sonicator to extract the chromatin. The number of sonication cycles and amplitude required will depend on the tissue type, length of fixation and power output of the sonicator used. As a general rule, 40 cycles of 30 seconds on 30 seconds off at 60% amplitude using a probe sonicator, produces sufficient chromatin for ChIP. Alternatively, for processing of multiple samples, chromatin can be extracted using a high power water bath sonicator on 90-100% amplitude for 120 cycles of 30 seconds on, 30 seconds off. It is essential that samples do not become heated during sonication in order to maintain the antigenicity of the proteins in the chromatin.
3. Following sonication centrifuge samples for 10 minutes, 4°C at maximum speed. Transfer the supernatants to fresh microcentrifuge tubes and label these as the soluble fractions. Add 1 µl PIC to each sample.
4. Add 50 µl FFPE Lysis Buffer to each pellet and vortex to resuspend. Add 1 µl PIC to each sample. Label this as the insoluble fraction.

Step 2b: Checking Chromatin Shearing Efficiency

In order to check the quality, quantity and shearing efficiency of the stock chromatin aliquots should be removed, reverse cross-linked and separated on an agarose gel. Prior to IP, 25 µl aliquots of each soluble and insoluble fraction should be used to quantify the chromatin and check that optimal shearing efficiency has been achieved as a standard quality control step at this stage. If the chromatin has already been quantified and sheared proceed to Step 3, 'Slurry preparation and Immunoprecipitation.

1. Take a 25 µl aliquot of each soluble and insoluble chromatin fraction and place in microcentrifuge capped tubes.
2. Add 5 µl of 5 M NaCl and 5 µl 1 M $NaHCO_3$ and make up to a final volume of 50 µl with water, mix thoroughly and incubate the samples at 65°C for 2 hours to reverse the cross-linking (samples can be left overnight if necessary).
3. Remove samples from the waterbath and briefly centrifuge the samples to remove any liquid from the caps.
4. Add 1 µl Proteinase K, mix thoroughly and incubate for 1 hour at 37°C.
5. Return the samples to room temperature and add 2 µl Proteinase K Stop Solution.
6. Purify the samples according to the phenol:chloroform extraction protocol detailed in Step 4b. Resuspend pellets in 25 µl Tris-HCl pH 8.
7. Use the purified samples to quantify the DNA concentration. Preferably, this should be done using a fluorometer as these give a better estimation for low concentration samples. If a fluorometer is not available measure samples on a spectrophotometer or using qPCR method. Spectophotometric methods are highly inaccurate given the low concentration of the obtained DNA.
8. To ensure that 100-500 bp fragments have been obtained during sonication the DNA should be analysed by agarose gel electrophoresis and visualised against a marker of known size DNA fragments (e.g. 100 bp ladder). A smear of DNA sequences 100-500 bp in length is ideal, fragments of smaller or greater length may affect the efficiency of the ChIP reaction.

As a general guide the minimum concentration required for ChIP is 1 ng/µl. If the extraction demonstrated low efficiency (≤10% of the DNA in the insoluble fraction extracted in the soluble fraction) it is possible to add more FFPE Extraction Buffer to the insoluble pellet and sonicate again. The soluble fractions from each sonication series can be pooled. However, it should be noted that increased sonication cycles reduce the number of recoverable epitopes available for ChIP.

Step 3 – Slurry Preparation and Immunoprecipitation

Step 3a: Slurry preparation

The concentration and quality of extracted chromatin varies depending on the fixation conditions used for the tissue along with the storage time and temperature. Therefore, the amount of chromatin required per ChIP must be optimised by the user. The initial recommendation would be to load a minimum of 100 ng chromatin and 4 µg antibody per ChIP. Slurries should be prepared in a total volume of 1ml with the chromatin making up ≤90% of the total slurry volume

1. Thaw chromatin stocks on ice (if necessary).
2. Centrifuge sheared chromatin at max speed for 10 minutes at 4°C, even if previously centrifuged.
   NOTE: Use only the clear supernatant for subsequent steps.
3. Prepare IP slurries in a fresh microcentrifuge tube according to Table 2. For every antibody IP set aside the equivalent amount of chromatin in a microcentrifuge tube and make up to 100 µl with Column Conditioning Buffer (if necessary), label as an input. These will be processed alongside the samples for reverse cross-linking and Proteinase K digestion at Step 4a and will be used as controls in the downstream analysis.
4. Mix well and incubate the IP slurries on an end to end rotator for 3 hours at 4°C.

Reagent	Immunoprecipitation Slurry (1000 µl total volume)
Chromatin stock	Up to 900 µl
Antibody/IgG	Optimum addition rate
PIC	1 µl
Column Conditioning Buffer	Make up to a final volume of 1000 µl

Table 2: *Slurry preparation for FFPE ChIP*

Step 3b: Chromatrap® spin column preparation

The Chromatrap® columns are shipped in a storage solution, prior to use columns must be washed and activated to remove any traces of shipping solution and prepare them for slurry incubation.

1. Remove the spin column from the collection tube (save for later) and place in an empty 1ml tip box rack (or alternative holder).
2. Add 600 µl of Column Conditioning Buffer to each column and allow to flow through under gravity (~30 minutes).
   N.B. Do not close column caps when flow is under gravity.
3. Discard the flow through and repeat this conditioning step a second time.
4. Discard the flow through. The columns are now ready for the addition of the IP slurries, proceed to Step 3c.

Step 3c: Immunoprecipitation

The immunoprecipitation step involves the binding of the antibody of interest to the protein A/G attached to the spin column frit. This allows the selective enrichment of the target protein/DNA complex and allows any non-specific complexes to be washed away. Target chromatin is then eluted using a specially formulated Elution Buffer for optimal target recovery.

N.B. If precipitates have formed in the Elution Buffer then it should be warmed to 40°C in a waterbath for 30 minutes with regular shaking until precipitates have dissolved **before use**.

1. Remove slurries from the end to end rotator following a minumum of 3 hours pre-incubation and briefly spin down to remove residual liquid from the caps.
2. Load the entire 1ml slurry and allow to flow completely through the column at RT (approx 30-40min).
3. Position Chromatrap® spin columns in the collection tubes provided, add 600 µl of Wash Buffer 1 to each column and centrifuge at 4000 x g for 30 seconds at RT. Discard the flow through and repeat.
4. Add 600 µl of Wash Buffer 2 to each column and centrifuge at 4000 x g for 30 seconds at RT. Discard the flow through and repeat.
5. Add 600 µl of Wash Buffer 3 to each column and centrifuge at 4000 x g for 30 seconds at RT. Discard the flow through and repeat.
6. Spin dry at 10,000 x g for 30 seconds at RT to remove any remaining liquid from the spin column. The original collection tubes should be discarded at this point and columns transferred into clean dry 1.5 ml collection tubes (provided).
7. Add 50 µl **ChIP-seq Elution Buffer** to each column, cap and incubate at RT for 15 minutes.
8. Centrifuge the columns at top speed for 30 seconds to collect the eluted chromatin.

Step 4 – Reverse Cross Linking

Chromatin samples must be reverse cross-linked to release the DNA from protein bound complexes. Protein is then degraded by Proteinase K digestion and the DNA purified in Step 4b of the protocol. Input controls which have not been through the IP process (Step 3a.3) must be reintroduced at this stage and treated alongside the samples.

1. To each eluted sample add 5 µl of 1 M $NaHCO_3$, 5 µl of 5 M NaCl and make up to a final volume of 110 µl with water. To each input add 5 µl of 1M $NaHCO_3$ and 5 µl of 5 M NaCl for a final volume of 110 µl. Mix thoroughly and incubate for 2 hours at 65°C. If required, the incubation at 65°C can be carried out overnight. Briefly centrifuge the samples to remove residual liquid from the caps.
2. Add 1 µl Proteinase K to each IP and input sample. Mix thoroughly and incubate for 1 hour at 37°C.
3. Add 2 µl Proteinase K Stop Solution to each IP and input sample. Vortex briefly and perform a short spin to remove residual liquid from the caps.

If required, at this point samples can be stored at -20ºC until purification and downstream processing.

Step 5 – DNA purification

DNA must be purified before proceeding with qPCR or sequencing. **Inputs must be purified by Phenol:chloroform extraction according to section 5a**. It is recommended that an inert carrier such as linear polyacrylamide (LPA) is used to improve DNA recovery during phenol:chloroform extraction. The use of glycogen as a carrier is not recommended due to potential contamination with nucleic acids from a biological source. IP'd samples should be purified using the supplied DNA purification columns according to section 5b.

Step 5a: DNA Purification by Phenol:chloroform extraction and ethanol precipitation

1. If the volume of the sample to be cleaned is less than 100 µl make up to 100 µl by adding sterile distilled water or Tris-HCl pH8.0 and vortex to mix.
2. Add an equal volume of Phenol:Chloroform:Isoamyl Alcohol (25:24:1) to the DNA solution from input or IP'd DNA and mix thoroughly by vortexing.
3. Centrifuge the samples at max speed, 4°C, for 5 minutes to separate the aqueous and organic phases. Carefully aspirate the aqueous phase (top layer) and transfer to a fresh 1.5 ml microcentrifuge tube. If a white precipitate has formed between the two phases following centrifugation, take care not to transfer this precipitate to the fresh tube and repeat Steps 4b.2 and 4b.3.
4. Add an equal volume of Chloroform:Isoamyl Alcohol (24:1) to the transferred aqueous phase and mix thoroughly by vortexing.
5. Centrifuge the samples at max speed, 4°C, for 5 minutes to separate the aqueous and organic phases. Carefully aspirate the aqueous phase (top layer) and transfer to a fresh 1.5 ml microcentrifuge tube.
6. Add 0.1 volumes of 3 M sodium acetate pH5.2 and 9 volumes of 100% ethanol to the transferred aqueous phase. **Optional:** add 25 µg LPA to each sample.
7. Incubate for a minimum of 4 hours at -80°C or overnight at -20°C.
8. Centrifuge at maximum speed for a minimum of 15 minutes at 4°C. Quickly and carefully aspirate and discard the supernatant.
9. Wash the pellet with 500 µl 70% ethanol and centrifuge immediately at maximum speed for a minimum of 15 minutes at 4°C. Quickly aspirate and discard the supernatant, taking care not to disturb the pellet which may be difficult to see at this point.
10. Air dry the pellets at room temperature ensuring all ethanol has evaporated before proceeding. Resuspend the pellet in 50 µl Tris-HCl pH8.0 or sterile distilled water by vortexing and incubate the tubes at room temperature for approximately 20 minutes.

N.B. Take care not to overdry the pellets as this will make resuspension very difficult.

If NGS is the intended downstream process it may be necessary to pool replicate ChIP eluants. This should be done prior to DNA clean up and purification must be carried out according to the Phenol chloroform extraction protocol detailed in 5a for maximum DNA recovery.

Step 5b: DNA purification using Chromatrap® DNA purification columns

Preparation of DNA Wash Buffer
60 ml ethanol (95-100%) must be added to the DNA Wash Buffer concentrate before use and note on label that ethanol has been added.

Some of the components of this product are irritants, refer to MSDS sheet for more information and follow safety guidelines of your research facility.

1. Add 5 volumes of DNA Binding buffer to 1 volume of sample and mix.

DNA binding buffer contains an integrated pH indicator. DNA adsorption requires a pH ≤7.5, and the pH indicator in the buffers will appear yellow in this range. If the pH is >7.5 the binding mixture will turn orange or violet and means that the pH of the sample exceeds the buffering capacity of the DNA binding buffer and DNA adsorption will be inefficient. In these cases add 10 ul 3 M Sodium acetate, pH 5, to adjust the pH of the binding mixture, the colour of the mixture should turn yellow.

2. Place a Chromatrap® purification spin column in collection tube provided and transfer sample onto column.
3. Centrifuge at 16,000 x *g* for 60 seconds. Discard flow through.
4. Add 700 µl DNA Wash Buffer Chromatrap® spin and centrifuge at 16,000 x *g* for 60 seconds. Discard flow through. Centrifuge Chromatrap® purification spin column once more at 16,000 x *g* for 60s to remove residual Wash Buffer.
5. Transfer Chromatrap® purification spin column to a clean 1.5 ml microcentrifuge tube.
6. To elute DNA, add 50 µl DNA Elution Buffer to the centre of the membrane and incubate for 1 minute, centrifuge at 16,000 x *g* for 60 seconds.

Samples are ready for qPCR validation at this stage.

Step 6 – Quantitative PCR analysis

The efficiency of immunoprecipitation provides an indicator of the relative success of a ChIP assay and requires the interpretation of qPCR data to determine which DNA fragments have been enriched. This can be expressed as the recovery of the locus calculated as a percentage of input as follows: % recovery = 2^(Ct input – Ct sample)

qPCR Component	10 µl PCR reaction Volumes required in µl
SsoAdvanced™ SYBR® Green Supermix*	5
Forward Primer	1.25
Reverse Primer	1.25
DNA sample	2.5

Table 3: *Example of a qPCR reaction*

*The following SYBR reagents have all been shown to produce optimal results ; iTaq™ Universal SYBR® Green Supermix, PerfeCTa SYBR Green Supermix, SsoAdvanced™, SYBR® Green Supermix, IQ™ SYBR® Green Supermix.

Instructions for real time PCR instruments should be followed and amplification for each target locus should be optimised. A suggested protocol is as below

95°C 2 minutes 1 cycle
95°C 10s
T_a°C 30s } 40 cycles
72°C 15s
Melt Curve

Troubleshooting guide and FAQs

1. Processing differences of the Chromatrap® spin columns
Chromatrap® spin columns contain protein A or G functionalised inert porous discs which are designed for centrifugation eliminating the need for difficult pipetting stages needed to separate supernatant liquids from retained chromatin complexes. The Chromatrap® technique therefore, significantly reduces the chances of pipetting errors and sample loss during processing. The format supports ease of handling and speed of assay development.

Antibody/chromatin complexes are retained on the column and can be very effectively rinsed to remove any unwanted background. Complexes can then be released at the appropriate time by a specially formulated elution buffer.

2. Should I use Protein A or Protein G Chromatrap® spin columns for my ChIP?
Different antibody sources have different affinities for protein A or G, check the affinity of the host species of your antibody. Protein G has the widest range of antibody affinities.

3. Do the Chromatrap® spin columns require blocking?
There is no requirement to carry out a blocking step to minimise non-specific binding as the composition of the Chromatrap® spin columns and the buffers provided in the kit have been formulated to ensure that non-specific binding is minimised.

4. What controls should I use?
To indicate the efficiency of the immunoprecipitation step it is important to run positive and negative antibody controls. Alternatively, a no antibody control (mock) can be used a negative control.

5. How much chromatin is required per ChIP assay?
Chromatrap® spin columns have much greater sensitivity than competitors' assays, therefore less chromatin per IP is required. Due to variations in chromatin quality and concentration it is recommended that at least 100 ng of chromatin is used per ChIP. While it may be possible to use less chromatin per ChIP this is dependent on the type of tissue, the extent of fixation and the storage conditions of the tissue and will need to be optimised by the user for each of their tissue samples. There is no upper limit to the amount of chromatin that can be loaded per ChIP, providing it can be achieved in the maximum 900 µl sample volume per ChIP.

6. How many ChIPs will I be able to perform from a single chromatin extraction?
The number of ChIPs from an extraction will vary depending on the concentration of the extracted chromatin and the desired chromatin loading per ChIP. The standard protocol lyses and extracts in a 500 µl final volume. After checking the chromatin quality sufficient chromatin remains for one ChIP, with input and negative control if 100 µl chromatin is required per ChIP or up to 3 ChIPs, with input and negative controls, if 50 µl is required per ChIP. Extractions from the same FFPE block can be pooled to increase the number of ChIPs achievable from a given sample, providing the obtained chromatin concentration and quality are similar.

7. Why do I have poor yield of sheared chromatin?
The quality and quantity of chromatin that can be obtained from FFPE tissue varies greatly according to the type of tissue, fixation and storage conditions. If the yield from an extraction is too low it is recommended that more tissue sections are used per extraction. Not all FFPE tissue is suitable from chromatin extraction, in some cases the amount of degradation caused by the fixation process and storage conditions may be too extensive.

8. Why do I have no enrichment with my ChIP antibody?
The use of ChIP-validated antibodies is essential for the success of a ChIP assay. The assay must recognise and bind to native protein that is bound to DNA. It is essential to include ChIP-validated positive and negative controls to ensure chromatin preparation and ChIP methodology are appropriate. Antibodies from other applications do not always work well in ChIP.

The protein epitope may have been destroyed or masked during the cross-linking process, or may be hidden by other proteins in the complex. Not all FFPE tissue is suitable for chromatin extraction and ChIP.

There may have been an incorrect addition of antibody to chromatin sample. Optimal results have been achieved with 4 µg antibody in the Chromatrap® spin columns but this will vary according to the prevalence of the target and the antibody affinity and avidity.

The antibody may be of low affinity and longer incubation of the antibody with the chromatin may be required for target binding. Pre-incubation times (Step 3a.4) can be increased up to overnight to increase the contact time of antibody with chromatin target. High amounts of antibody are added to each ChIP reaction to ensure saturation of the slurry with antibody to maximise binding due to the reduced antigenicity of FFPE chromatin.

9. Why do my qPCR primers have poor efficiency when amplifying my FFPE samples?
The fixation process involved in FFPE often results in highly degraded DNA. The majority of extracted chromatin fragments may be less than 200 bp as a result of this. It is highly recommended that qPCR primers for FFPE ChIP have an amplicon less than 100 bp in length for optimal efficiency. The degradation of the DNA fragments can be checked prior to chromatin extraction by Phenol:chloroform extraction of sections followed by agarose gel electrophoresis. If the majority of the DNA fragments are found to be less than 100 bp in length it is unlikely that the chromatin extracted from the tissue will be suitable for ChIP.

10. What can be the causes of high background in my ChIP data?
The quality and selectivity of the antibody has a major influence on the success of the ChIP assay, ensure the antibody is ChIP validated.

The success of the ChIP assay is highly dependent on the quality of chromatin prepared. High background can be the result of the extensive cross-linking caused by the long fixation and storage times for FFPE tissue. Not all FFPE tissue is suitable for ChIP. Inefficient sonication can result in high background and sonication efficiency can be compromised by excessive sample foaming and poor heat dissipation.

Inefficient washing steps can leave traces of unwanted chromatin alongside that selected by the antibody. The Chromatrap® system is intrinsically better than bead based assays for performing these wash steps. However, if background remains high the number of washes with each buffer can be increased at Step 3c.

An incorrect antibody to target protein can compromise the signal to noise ratio. Optimal results have been obtained with 4 µg antibody per ChIP but this will vary depending on the antibody. It is important for the user to optimise the ratio of antibody to chromatin for their chosen antibody.

11. Do I need to purify my samples before gel analysis?
Due to the nature of the FFPE extraction method it is necessary to purify any chromatin sample by Phenol:Chloroform extraction prior to agarose gel electrophoresis (Step 2b point 9). Purifying samples using a commercial kit is not recommended at this stage due to poor recovery of small fragments of DNA and potential tissue fragment carryover.

12. Why can't I see my bands on a gel?
Different intercalating dyes have varying sensitivities for detecting DNA during agarose gel electrophoresis, the detection limit of DNA bound to ethidium bromide or SYBR® safe is 0.5 to 5.0 ng/band. The concentration of extracted chromatin from FFPE tissue is typically very low. A dye with increased sensitivity, such as SYBR® Gold which can detect as little as 25 pg/band of DNA, may be required in order to assess the degradation of the extracted chromatin. It is recommended that you load 20 µl of your purified reverse cross-linked sample on a gel in order to visualise the DNA. It may not be possible to see chromatin of low concentration on an agarose gel therefore, ensure the quantified DNA is of sufficient concentration to proceed with ChIP.

13. How should I measure the concentration of my chromatin stock?
It is highly recommended that the concentration of chromatin is measured using a fluorimetric method with a high sensitivity kit designed for fragmented double-stranded DNA. Optimal results have been obtained using the Qubit® fluorometer (Life Technologies) with the dsDNA HS assay kit. 5 µl of prepared purified sample should be measured fluourometrically in order to reach the instruments detection limit. Due to the low concentrations of chromatin extracted from FFPE tissue spectrophotometric methods of analysis are very inaccurate and may severely overestimate the concentration of chromatin stocks. Alternatively, DNA can be accurately quantified using a qPCR quantification kit or control DNA of known concentration with qPCR primers which have 100% efficiency.

14. What do I do if my tissue does not pellet properly during rehydration?
Tissue becomes increasingly difficult to pellet during progressive rehydration steps. It may be necessary to increase the speed, length or number of centrifugation steps in order to pellet the tissue sufficiently that the liquid can be aspirated without disturbing the tissue. It is essential that small tissue fragments are avoided during supernatant aspiration to minimise sample loss.

15. Why will my DNA pellet not resuspend/sample concentration cannot be measured?
If the concentration of the chromatin sample cannot be detected using a fluorimetric detection method it may be due to insufficient solubilisation of the DNA pellet after Phenol:Chloroform extraction. A small insoluble pellet may also be seen in the tube. This can be the result of an overdry pellet making resuspension more difficult. Solubility of the pellet can be improved by gently warming the suspension to 37°C, vortexing and briefly spinning down the sample every few minutes. Try to avoid overdrying the samples by adding the resuspension buffer and capping the tubes as soon as the ethanol has dried.

16. Why do I have poor qPCR data for my IP'd DNA?
If the qPCR data for the input is as expected and reflects the concentration of input chromatin this may be due to problems with antibody binding, see point 10. of the troubleshooting guide. The antigenicity of the target proteins may have been irreparably damaged by the fixation or extraction process. Consider using a different tissue sample or reducing the number of sonication cycles. Take care not to heat or emulsify the samples during sonication. If the input also produces poor results this may be due to primer design, see point 11. of the troubleshooting guide.

17. Can chromatin be extracted from FFPE slides?
The protocol is optimised for extraction from sections of FFPE tissue blocks. Tissue sections from other sources, such as pathology slides, may need to be optimised by the user.

18. Can the Chromatrap® FFPE ChIP kit be used for sequencing?
The Chromatrap® FFPE ChIP kit can be used to enrich high quality DNA for next generation sequencing. It is recommended that for sequencing samples should be purified by phenol:chloroform extraction for maximum yield of DNA for library preparation. Due to the relatively low yield of chromatin, and therefore enriched DNA, obtained from FFPE tissue it may be necessary to pool IP'd samples. It is important to pool samples prior to DNA clean up and purification should be carried out according to the protocol in section 5a.

Other products available from Chromatrap®

ChIP products

Product	Quantity	Catalogue no.
Chromatrap® ChIP-seq Pro A	24	500189
Chromatrap® ChIP-seq Pro G	24	500190
Chromatrap® HT ChIP-seq Pro A	1 x 96	500214
Chromatrap® HT ChIP-seq Pro G	1 x 96	500215
Chromatrap® Enzymatic ChIP-seq Pro A	24	500191
Chromatrap® Enzymatic ChIP-seq Pro G	24	500192
Chromatrap® HT Enzymatic ChIP-seq Pro A	1 x 96	500216
Chromatrap® HT Enzymatic ChIP-seq Pro G	1 x 96	500217
Chromatrap® FFPE ChIP-seq Pro A	24	500235
Chromatrap® FFPE ChIP-seq Pro G	24	500236
Chromatrap® Native ChIP-seq Pro A	24	500237
Chromatrap® Native ChIP-seq Pro G	24	500238
Chromatrap® UniqSeq kit Pro A	24	500264
Chromatrap® UniqSeq kit Pro G	24	500265
Chromatrap® UniqSeq Enzymatic Pro A	24	500266
Chromatrap® UniqSeq Enzymatic Pro G	24	500267
Chromatrap® *Drosophila* ChIP-seq kit Pro A	24	500279
Chromatrap® *Drosophila* ChIP-seq kit Pro G	24	500275
Chromatrap® *Drosophila* UniqSeq kit Pro A	24	500276
Chromatrap® *Drosophila* UniqSeq kit Pro G	24	500277
Chromatrap® Sonication Shearing	–	500239
Chromatrap® Enzymatic Shearing	–	500165

DNA products

Product	Quantity	Catalogue no.
Chromatrap® DNA Purification	50	500218
Chromatrap® Gel Purification	50	500219
Chromatrap® HT DNA Purification	2 x 96	500220
Chromatrap® HT DNA Purify and Concentrate	2 x 96	500240
Chromatrap® DNA Extraction	50	500260
Chromatrap® HT DNA Extraction	2 x 96	500261
Chromatrap® Size Selection	50	500262
Chromatrap® HT Size Selection	2 x 96	500263

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