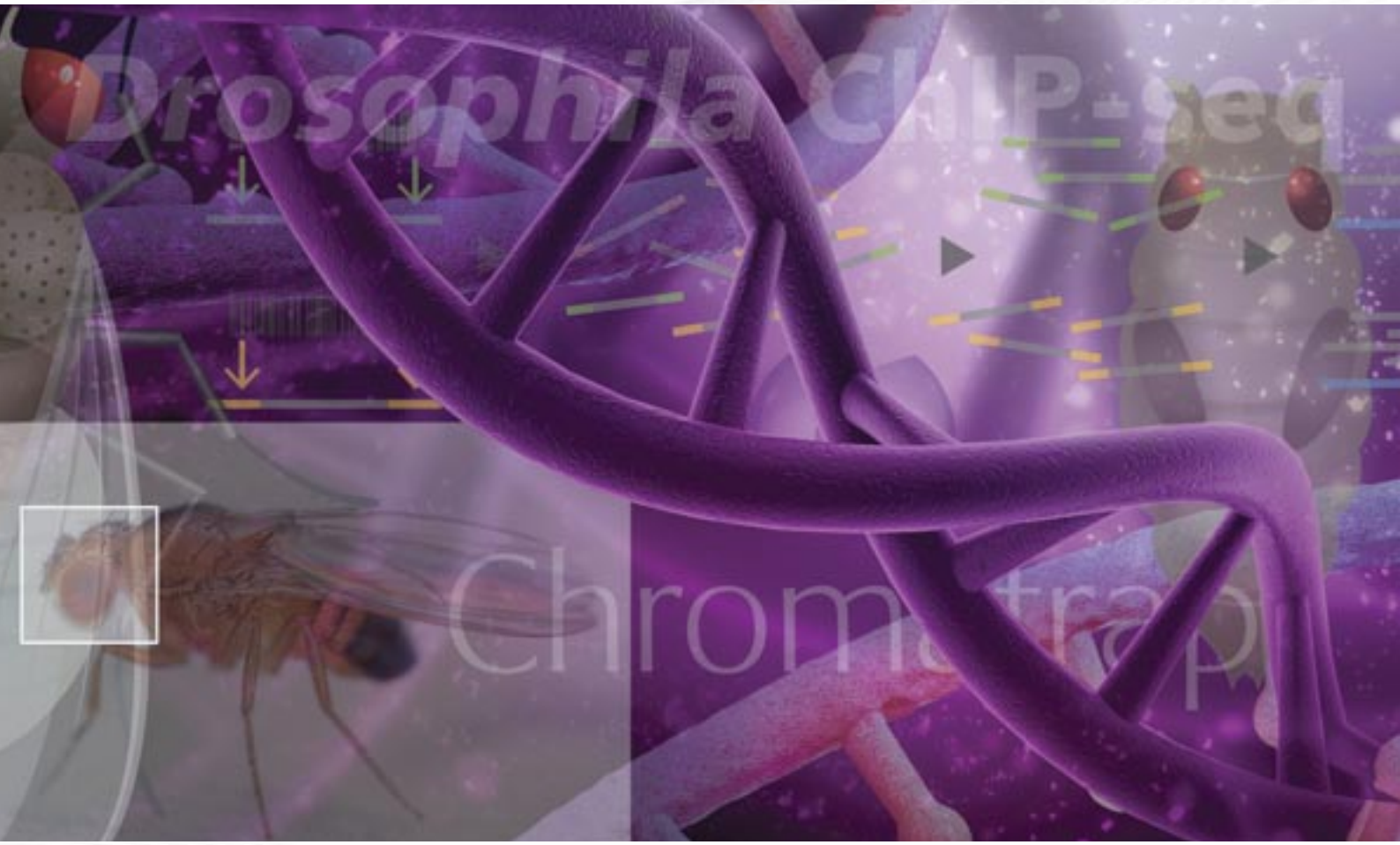
Chromatrap

V1.1

Chromatrap® *Drosophila* ChIP-seq kit

A bead-free assay for chromatin immunoprecipitation from *Drosophila* for qPCR and next generation sequencing

Catalogue no 500274, 500275



ADVANCEMENTS IN EPIGENETICS

Contents

Introduction

Drosophila melanogaster has proven to be a vital model organism in genetic studies for many decades. More recently, the *Drosophila* epigenome is emerging to be a powerful tool in helping to elucidate the role of epigenetics on gene expression regulation in a diverse range of research areas including development, ageing and brain function.

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. Chromatin Immunoprecipitation (ChIP) has proved invaluable in epigenetic research. The technique, used to study the association of specific proteins or their modified isoforms within defined genomic regions, provides a unique snapshot of protein-DNA interactions underlying regulatory mechanisms. In a ChIP assay, protein-DNA complexes (chromatin) are selectively immunoprecipitated using highly specific 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 (Figure 1).

The most widely used tool for examining these Protein-DNA interactions is ChIP followed by massively parallel sequencing (ChIP-seq). Next Generation Sequencing (NGS) of ChIP enriched DNA enables identification of target DNA sites that were in direct physical contact with regulatory mechanisms in vivo. Mapping of these sequenced fragments to whole genome sequence databases allows quick and efficient analysis of the DNA interaction pattern of any transcription factor or epigenetic modification.

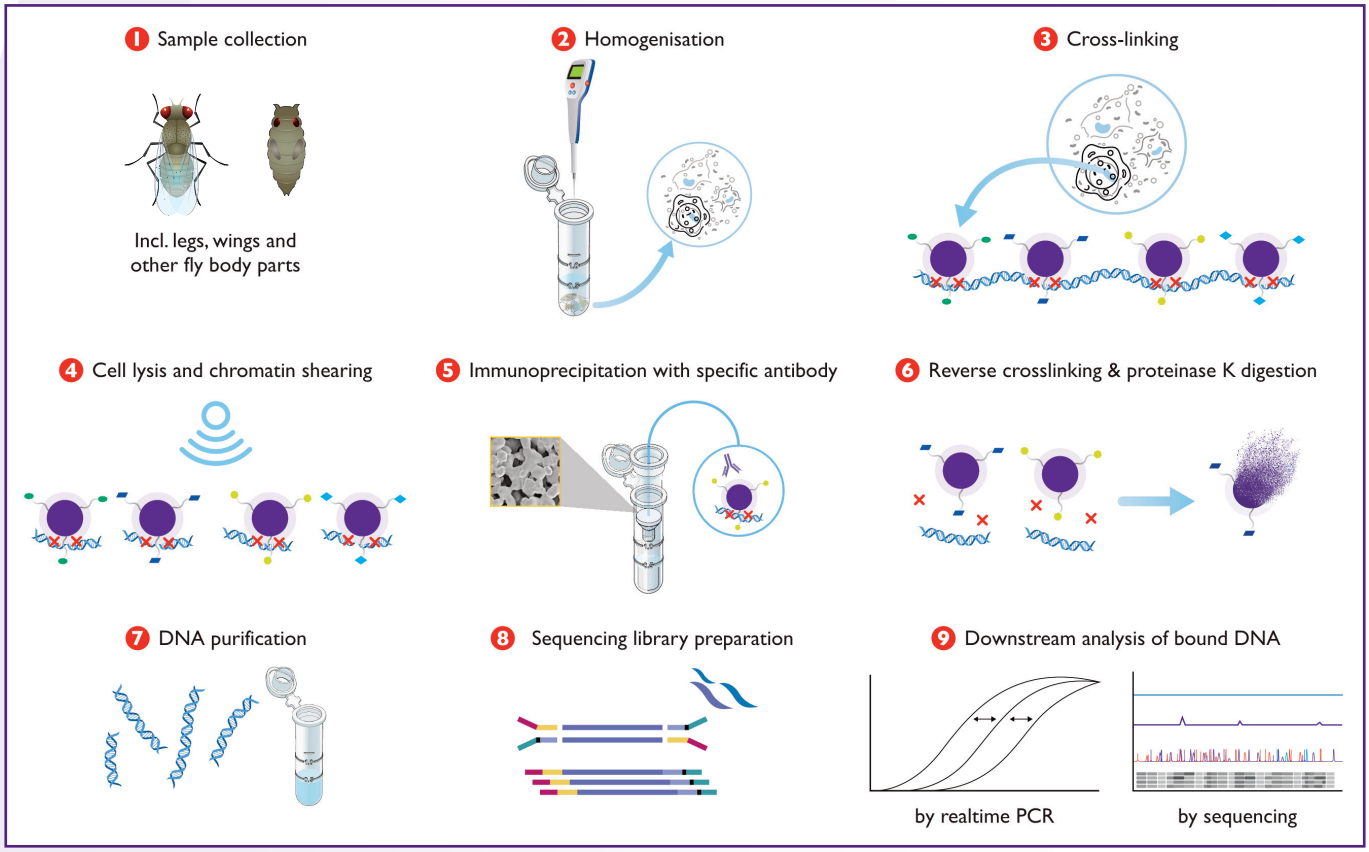


Figure 1: *Overview of the Chromatrap® Drosophila ChIP-seq process.*

Chromatrap® *Drosophila* 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.

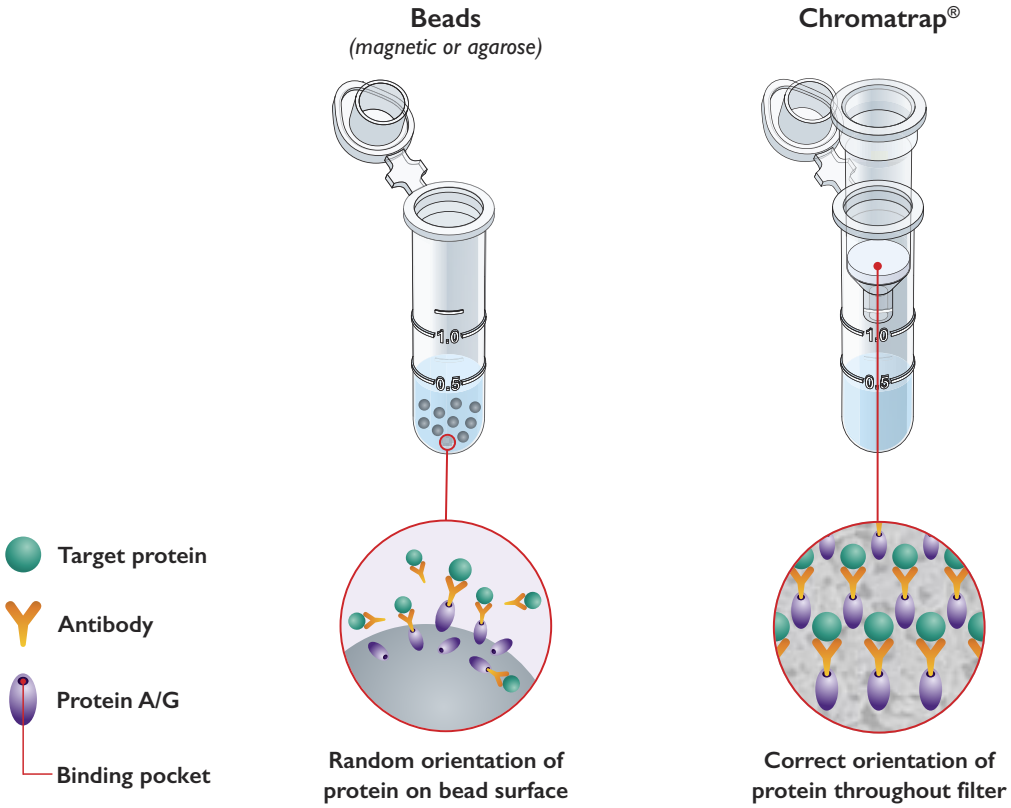


Figure 2: *Chromatrap® ChIP technology*

A Chromatrap® *Drosophila* ChIP-seq assay using whole insects or tissue consists of five key steps:

1. *Preparation of high quality chromatin from Drosophila tissue using the reagents provided*
2. *Immunoprecipitation of chromatin using ChIP-validated antibody of interest specific to the target protein*
3. *Capture of the antibody-chromatin complex using the Chromatrap® spin column technology*
4. *Recovery of the enriched DNA using kit supplied reverse cross-linking, proteinase digestion and DNA clean-up reagents*
5. *DNA analysis*

Chromatrap® utilises this solid state technology in parallel with Next Generation Sequencing to deliver a precise ChIP-seq protocol from whole *Drosophila* insects or insect body parts from various life cycle stages.

Advantages of Chromatrap® *Drosophila* ChIP-seq:

- *Specialised protocol and reagents for maximum recovery of high quality ChIP-ready chromatin from Drosophila tissue*
- *ChIP-seq from as little as 5 Drosophila*
- *Low background due to the inert filter technology*
- *Fast protocol - no blocking steps or overnight incubations*
- *NGS quality DNA from a single IP without the need to pool samples*
- *Compatible with qPCR and sequencing as downstream processes*

**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.*

Kit overview and timetable

The Chromatrap® *Drosphila* ChIP-seq kit allows the user to perform up to 24 ChIP assays from cell collection through to immunoprecipitation, including up to 10 chromatin sample preparations. The kit provides all of the major components required for performing ChIP assays to obtain high quality DNA for qPCR analysis and NGS library preparation.

Step	Process	Time Required	Day
1	Cell fixation and collection	0.5 hours	1
2	Cell lysis and chromatin shearing	0.5 hours	1
3	Immunoprecipitation	1 hour	1
4	Reverse cross-linking and DNA purification	3.5 hours	1
5	Quantitative PCR analysis	1 hour	1
6	*Chromatrap® NGS library synthesis	5 hours	2
7	Library quantification and quality analysis	2 hours	2

Table 1: *Chromatrap® Drosophila ChIP-seq kit protocol overview.*

**When using the Chromatrap® Drosophila UniqSeq library preparation kit (Cat no. 500276/500277). The time for library preparation time with other kits will vary and may take up to 2 days.*

Kit components

The kits are manufactured DNase-free and when stored as directed are stable for up to 6 months. Sufficient material is supplied for 24 ChIP assays and up to 10 chromatin sample preparations. Upon receipt, please ensure the components are stored at the temperatures listed in Table 2.

Kit Component	Quantity	Storage
Chromatrap® ChIP-seq Spin Columns	24	4°C
Column Conditioning Buffer	60 ml	4°C
Wash Buffer 1	50 ml	4°C
Wash Buffer 2	50 ml	4°C
Wash Buffer 3	50 ml	4°C
1.3 M Glycine	500 µl	4°C
Hypotonic Buffer	10 ml	4°C
Lysis Buffer	10 ml	4°C
ChIP-seq Elution Buffer	3 ml	4°C
5 M NaCl	500 µl	4°C
1 M $NaHCO_3$	500 µl	4°C
Protease Inhibitor Cocktail (PIC)	50 µl	-20°C
Proteinase K Stop Solution	100 µl	-20°C
Proteinase K	50 µl	-20°C
1.5 ml Collection Tube	50	RT
Chromatrap® DNA Purification Columns	24	RT
DNA Binding Buffer	15 ml	RT
DNA Wash Buffer	15 ml	RT
DNA Elution Buffer	2 ml	RT

Table 2: *Chromatrap® Drosophila ChIP-seq kit reagents and materials.*

Additional materials required

Reagents and consumables

- PBS
- 37% formaldehyde, molecular biology grade
- Nuclease-free water
- 100 bp ladder
- Microcentrifuge tubes (0.5 ml and 1.5 ml)
- PCR plates
- DNA LoBind tubes (optional)
- Pipettes and tips (filter tips are recommended)
- ChIP validated antibody
- qPCR primer pairs for gene of interest
- 3 M Sodium Acetate pH 5.2

Equipment

- Hand held tissue homogeniser
- Microcentrifuge (4°C)
- Agarose gel electrophoresis equipment
- Fluorometer for DNA quantification
- Sonicator
- End-to-end rotator

Additional materials required for sequencing using Illumina® platforms

- Thermocycler
- Qubit® 2.0 fluorometer with dsDNA high sensitivity kit (or equivalent fluorometric quantification method)
- Agilent Technologies 2100 Bioanalyzer with high sensitivity DNA kit
- Library sample preparation kit (included with Chromatrap® *Drosophila* UniqSeq kit. Cat no. 500276/500277)
- Library quantification kit
- DNA purification kit with size selection, such as the Chromatrap® Size Selection kit (Cat. no. 500262)

Optional materials

- Phenol Chloroform
- 100% Ethanol
- 70% Ethanol
- Linear Polyacrylamide (LPA)

Important considerations for ChIP-seq

Antibody quality

The success and value of any ChIP-seq experiment is dependent on the quality of the antibody used. A highly-specific antibody will increase the relative enrichment of the target compared with the background, making it easier to detect binding events during data analysis. Many commercially available antibodies are listed as ChIP-seq grade and, wherever possible, should be used for your experiments. However, lot-to-lot variations and variability in quality does occur and the antibodies of choice should be validated before use.

Sample quantity

The amount of starting material required to obtain sufficient yields of enriched DNA to prepare a ChIP-seq library will depend on the quality of both chromatin and antibody, the abundance of the target protein and gene and the sensitivity and efficiency of the library preparation kit used. The Chromatrap® *Drosophila* ChIP-seq kit has been optimised for library preparation with the Chomatrap® *Drosophila* UniqSeq kit (Cat no. 500276/500277). High quality ChIP-seq data can be obtained from as little as 5 insects using this workflow. A minimum of 500 pg of DNA is required for library preparation using Chomatrap® *Drosophila* UniqSeq (Cat no. 500276/500277). The input requirements of other library preparation kits will depend on the manufacturers' limits and will need to be optimised by the user. However, if sequence duplication becomes an issue we would recommend increasing the quantity of starting material for library amplification to help minimise duplication levels. Additionally, the number of PCR cycles used during the enrichment step can also be reduced if duplication remains an issue.

Shearing

It is important to optimise shearing to obtain fragments between 100-500 bp. For sequencing the largest proportion of the fragments should be in the 200-300 bp range. Larger fragments not only contribute to high background but can create clustering problems during sequencing.

Control experiment

Peaks identified during sequencing analysis must be compared to the same region in a matched control sample in order to verify their significance. For example, a random region of repetitive sequences may appear enriched due to the number of copies of the region, creating a false-positive result. There are three commonly used controls: input DNA (DNA that has not been immunoprecipitated); mock IP (DNA treated the same but without antibody during the IP); and non-specific IP (IP with an antibody targeting a protein not known to be involved in DNA binding such as IgG). There is no consensus as to which control is most appropriate to use, however, input DNA and IgG controls are commonly used as they account for bias related to the shearing of DNA and amplification. We recommend using input as a control.

Reaction Conditions

ChIP-seq requires careful optimisation of numerous reaction conditions from the amount of *Drosophila* tissue to the number of fragment clusters for optimal sequencing analysis. Chromatrap® *Drosophila* ChIP-seq reduces the number of optimisation steps required and has been tailored for use with Illumina® sequencing platforms. The Chromatrap® *Drosophila* UniqSeq Kit (Cat no. 500276/500277) contains adapters and indices compatible with Illumina sequencing instruments only, such as the MiSeq, HiSeq and NextSeq. For all other sequencing platforms such as Ion Torrent, the SOLiD® system and Roche 454, the appropriate library kit will need to be provided by the user.

Experimental planning

1. Chromatin preparation
This protocol has been optimised for use with *Drosophila* tissue derived from various stages of the life cycle; adult, pupal, larval and embryonic. A guideline number of insects required per extraction can be found in Table 3. The Chromatrap® *Drosophila* ChIP-seq kit provides enough reagents for up to 10 chromatin preparations and up to 24 ChIP assays.

2. Shearing optimisation
The success of a ChIP assay is highly dependent on the quality of chromatin prepared. Guideline sonication conditions for the *Drosophila* life cycle stages are described in Step 2b Chromatin Shearing (page 11). However, these will vary between sonication instruments and may need to be optimised by the user. We recommend checking shearing conditions before progressing with ChIP (see Troubleshooting on page 17 for more information).

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 500 ng - 50 µg chromatin in a total volume of 1 ml, ensuring the chromatin does not exceed more than 10% (100 µl) of the total 1 ml slurry volume.

4. Quantification
For NGS library synthesis, it is critical to determine the concentration of IPd DNA using a high sensitivity fluorescence based quantification method as UV-based spectrophotometers such as the NanoDrop are unreliable for quantification of low quantities of DNA (see Troubleshooting). The concentration of DNA obtained will be influenced by a variety of factors including tissue type, target abundance and antibody affinity.

5. Positive and negative IP controls
In addition to the ChIP validated antibody, we recommend the use of a positive and negative control antibody. We suggest including one negative IgG control antibody corresponding to the host species in which the antibody of interest was raised for each series of ChIP reactions. We recommend using a positive ChIP-seq grade control antibody, such as H3K4me3 (Chromatrap® Cat no; 700010), using 1 µg chromatin and 5 µg antibody to validate successful IP. See Step 3a of the protocol for more information.

6. Quantitative PCR validation
Before beginning library synthesis for sequencing, we recommend analysing the IPd DNA by qPCR using at least one positive and one negative control target of your choice. In order to have sufficient DNA for library preparation, it is recommended that not more than 10% of the total IPd DNA be used for qPCR. If necessary, DNA can be diluted 1:10 to provide an adequate volume for triplicate PCR reactions. Control targets for the antibody of choice should be analysed by the user as appropriate.

7. 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:

% recovery = 2^(Ct input – Ct sample)*dilution*100

Protocol

Wherever this 'pause point' symbol appears, the sample can be stored at -80°C.

The following section describes the fixation and preparation of chromatin from flash-frozen *Drosophila* tissue samples. **N.B.** Depending on the life cycle stage of the *Drosophila* tissue used, different amounts of starting materials may be required (refer to Table 3 for guidelines regarding the amount of starting material).

Life cycle stage	Number of specimens per replicate
Adult	10 females
Pupal	6 (30 if performing sequencing downstream)
Larval	6 (30 if performing sequencing downstream)
Embryonic	~ 1000

Table 3: *Guideline specimens as starting material for chromatin preparation using Drosophila samples at different life cycle stages.*

N.B. Flash-frozen samples can be stored at -80°C for no more than 2 months or at -20°C for no more than a week. Add 1 µl PIC to each sample before storage.

Step 1 – Sample Fixation and collection

1. Thaw tissue samples on ice.

2. Suspend each tissue sample in 500 µl ice cold PBS in a 1.5 ml microcentrifuge tube and vortex at high speed.

3. Crush the tissue on ice using a hand held tissue homogeniser until cuticles are broken-up, no large pieces of tissue remain and the sample is homogenised.

4. Once cuticles are disrupted, add 13.5 µl 37% formaldehyde to the sample (1% final concentration). Further process the sample with a hand held homogeniser for 30 seconds to 1 minute. Incubate samples on ice for 10 minutes, vortexing at low speed every 30 seconds for the duration.

5. Add 28.5 µl 1.3 M glycine solution. Incubate for 5 minutes at RT and gently vortex every 30 seconds during the incubation.

6. Collect cells by centrifugation, 6000 x g for 5 minutes at 4°C. Discard the supernatant.

7. Re-suspend the pellet in 500 µl PBS and centrifuge 6000 x g for 5 minutes at 4°C. Discard the supernatant and proceed to Step 2.

Step 2 – Cell lysis and chromatin shearing

The following section describes the lysis of *Drosophila* tissue and shearing of the chromatin into 100-500 bp fragments. Recommended buffer volumes for the various life stages are detailed in Table 4. Guideline shearing settings recommended in Table 5 have been optimised using a waterbath sonicator on the high amplitude setting and are based on the number of 30 seconds on, 30 seconds off, cycles. Sonication settings will vary between instruments and may need to be optimised by the user.

Step 2a – Cell lysis

N.B. If precipitates have formed in the **Lysis Buffer** then it should be warmed to 37°C in a water bath for 30 minutes with regular shaking until precipitates have dissolved **before use**.

1. Re-suspend the cell pellet in Hypotonic Buffer and incubate the samples at 4°C for 10 minutes (refer to Table 4 for optimal volume for starting material used).

Life cycle stage	Hypotonic Buffer (µl)	Lysis Buffer (µl)
Adult	400	300
Pupal	800	500
Larval	800	500
Embryonic	800	500

Table 4: *Optimum buffer volumes for Drosophila life stage used.*

2. Centrifuge the hypotonic slurry at 5000 x g for 5 minutes at 4°C to collect the nuclei.

3. Discard the supernatant and re-suspend the pellet in **Lysis Buffer (ensure the Lysis Buffer has been pre-warmed prior to use to ensure all precipitates are fully dissolved**, refer to Table 4 for optimal buffer volume). Incubate samples on ice for 10 minutes.

Step 2b – Chromatin shearing

1. Sonicate samples at 4°C until the desired lengths of chromatin fragments are achieved (100-500 bp). Refer to Table 5 for recommended starting sonication cycles for different *Drosophila* life cycle stages using the number of specimens detailed in Table 3.

Life cycle stage	Sonication Cycles
Adult	10
Pupal	10
Larval	10
Embryonic	8

Table 5. *Recommended number of cycles (30 seconds on, 30 seconds off) for Drosophila life cycle stages.*

2. Centrifuge the samples for 10 minutes maximum speed at 4°C and transfer the supernatant to a clean dry microcentrifuge tube.

3. Add 1 µl of PIC to each sample and mix.

4. Chromatin samples are now ready for IP. If samples are not to be used immediately store at -80°C for a maximum of 2 months. It is recommended that the shearing efficiency of each chromatin stock is analysed at this stage.

N.B. Shearing efficiency varies greatly and will need to be optimised and confirmed separately, checking the size of the fragments on an agarose gel as described in step 2c.

Step 2c – Shearing efficiency

In order to verify the quality, quantity and shearing efficiency of the stock chromatin aliquots should be removed, reverse cross-linked and visualised on a 1% agarose gel alongside a DNA ladder of known size. Prior to performing IP the concentration of chromatin should be estimated using a suitable spectrophotometer or fluorometer.

1. Take a 25 µl aliquot of sheared chromatin from each sample and place in a microcentrifuge capped tube.

2. Add 5 µl of 1 M $NaHCO_3$, 5 µl of 5 M NaCl and make up to a final volume of 50 µl with nuclease free water.

3. Mix thoroughly and incubate the samples at 65°C for 2 hours to reverse the cross-linking. If required, samples can be left overnight.

4. Briefly centrifuge the samples to remove any liquid from the caps.

5. Add 1 µl of the Proteinase K solution and mix thoroughly. Incubate for 1 hour at 37°C.

6. Return the samples to room temperature and add 2 µl Proteinase K stop solution.

7. Quantify DNA in the samples using a suitable fluorometer or sensitive spectrophotometer. Multiply the reading by 2 to account for the dilution during the reverse cross-linking. This will be used to determine the volume of chromatin to load in Step 3; Slurry Preparation and Immunoprecipitation.

8. To ensure that 100-500 bp fragments have been obtained during shearing, DNA should be run on an agarose gel and visualised against a marker of known size DNA fragments (e.g. 100 bp ladder). A smear of DNA fragments 100-500 bp in length is ideal, fragments of smaller or greater length may affect the efficiency of the ChIP reaction. If chromatin is over or undersheared please refer to troubleshooting and FAQs.

Step 3 – Slurry preparation and immunoprecipitation

Step 3a: Slurry preparation and column activation

An important consideration when performing ChIP-seq is the amount of chromatin that will need to be loaded onto the column in order to elute sufficient IPd DNA for library synthesis. The DNA yield obtained from ChIP will depend on the quality of the chromatin, the affinity and avidity of the antibody and the abundance of the target. A minimum of 500 pg of DNA is required for preparation of high quality NGS sequencing libraries using the Chromatrap® *Drosophila* UniqSeq Kit (Cat no. 500276/500277). As little as 5 specimens can yield this quantity of DNA from ChIP of an abundant target using a good quality antibody. As a starting point we would recommend using 1 µg chromatin per ChIP with 5 µg antibody.

Remember to prepare negative controls for both antibody validation and the standard assay described in Table 6.

1. Thaw chromatin stocks at 4°C.

2. Centrifuge sheared chromatin at max speed for 10 minutes at 4°C, even if previously centrifuged.
   N.B. Use only the clear supernatant for subsequent steps.

3. Prepare IP slurries in a fresh microcentrifuge tube according to Table 6. 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 rotor for 1 hour at 4°C.

Reagent	Immunoprecipitation Slurry (1000 µl total volume)	Input (100µl total volume)
Chromatin stock	Up to 100 µl	Up to 100 µl
Antibody/IgG	Optimum addition rate	–
PIC	1 µl	–
Column Conditioning Buffer	Make up to final volume of 100 µl	Make up to final volume of 100 µl

Table 6: *Slurry preparation for spin column IP.*

Step 3b: Chromatrap® spin column preparation

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

1. Remove the spin column from the collection tube (save for later) and place in an empty 1 ml tip box rack (or alternative holder).

2. Add 600 µl Column Conditioning Buffer to each column and allow the liquid to flow through under gravity (~ 30 minutes). **N.B. Do not close 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 or 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 ChIP-seq elution buffer for maximal target recovery.

N.B. If precipitates have formed in the **ChIP-seq Elution Buffer** then it should be warmed to 37°C in a water bath for 30 minutes with regular shaking until precipitates have dissolved **before use**.

1. Remove slurries from the end-to end rotator following 1 hour incubation and briefly spin down to remove residual liquid from the caps.

2. Load the entire 1 ml slurry and allow to flow completely through the spin columns under gravity at RT (approximately 30 - 40 minutes).

3. Position Chromatrap® spin columns back into the collection tubes provided and add 600 µl of Wash Buffer 1 to each column. Close the cap 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. Discard final flowthrough and spin dry at maximum speed 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 maximum speed for 30 seconds to collect the eluted chromatin.

Step 4 – Reverse cross-linking

Step 4a: 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 before being 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 as per the IP sample.

1. To each eluted IP 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 DNAse-free water. To each input add 5 µl of 1 M $NaHCO_3$ 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 performed overnight.

2. Add 1 µl Proteinase K to each IP and input sample. Vortex briefly and perform a short spin. 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.

N.B. If validating by qPCR, take a 10 µl aliquot of the input and dilute 1 in 10 or 1 in 100 with water and deduct 3.3/6.6 Cts respectively, with 100% primer efficiency. With the remaining 100 µl input please proceed to Step 4b.

Step 4b – DNA purification

Chromatin must now be purified before proceeding with qPCR or library synthesis. DNA purification columns and reagents are included in the Chromatrap® *Drosophila* ChIP-seq kit to recover ultra-pure DNA from ChIP samples. Alternatively, DNA can be purified by phenol/chloroform extraction using an inert carrier such as linear polyacrylamide (LPA). The use of glycogen as a carrier is not recommended due to potential contamination with nucleic acids from a biological source.

N.B. DNA Wash Buffer must be prepared **before first use**: Add 60 ml ethanol (95-100%) to the DNA Wash Buffer concentrate before first 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 µl 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® DNA purification 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 to each Chromatrap® DNA purification column and centrifuge at 16,000 x g for 60 seconds. Discard flow through. Centrifuge the Chromatrap® DNA purification column once more at 16,000 x g for 60 seconds to remove residual Wash Buffer.

5. Place the Chromatrap® DNA purification column in 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 now ready for qPCR and library preparation.

Step 5 – Quantitative PCR analysis

Prior to sample sequencing, we recommend analysing the IPd DNA by qPCR using at least one positive and one negative control to validate the IP.

Prepare the qPCR reaction mix as follows for a 10 µl reaction volume:

- 5 µl of a 2x SYBR® Green qPCR mix
- 2.5 µl primer mix (combine primers 1:1)
- 2.5 µl IPd or input DNA

Primer concentrations may need to be adjusted but we recommend a final concentration of 1 µM in the reaction mix for each primer.

Program the thermal cycler as follows:

2 minutes at 95°C
10 seconds at 95°C
30 seconds at Ta°C } 40 cycles
15 seconds at 72°C

These conditions may require optimisation depending on the primer, qPCR mix and qPCR system used.

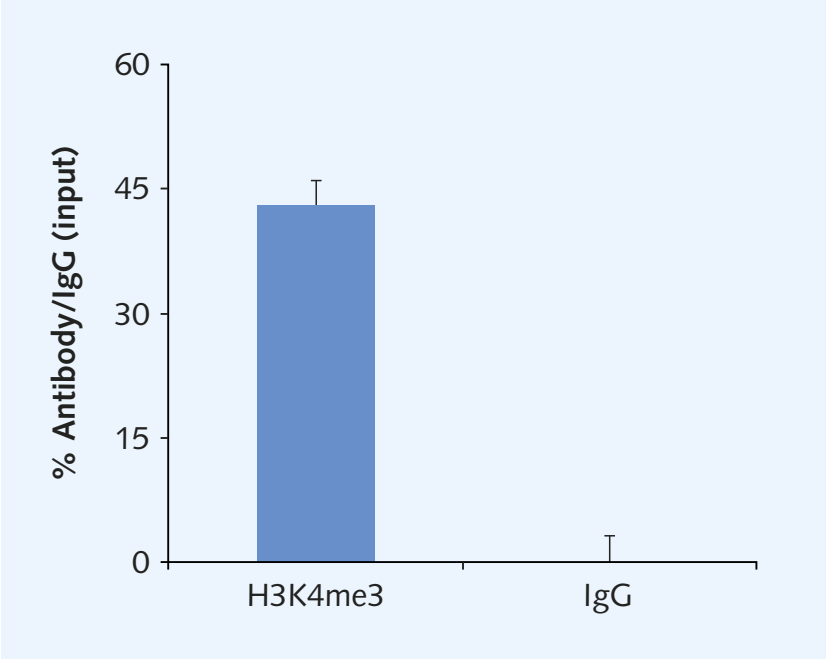


Figure 3: *ChIP was performed using chromatin extracted from pupal tissue using the Chromatrap® Drosophila ChIP-seq kit (Cat no. 500274, 500275). IP was performed with the positive ChIP control antibody H3K4me3 (Chromatrap® Cat no. 700010) using 5 µg antibody and 1 µg chromatin. qPCR was performed with the Drosophila Positive Primer Set (Chromatrap® Cat no. 900037). The data presented as mean % input (the relative amount of IPd DNA compared with input DNA after qPCR analysis).*

Troubleshooting and FAQs

Process	FAQ	Solution
Cross-linking and fixation	How long should the *Drosophila* samples be crosslinked for?	Optimal cross-linking of DNA ensures that the chromatin structure is preserved during the isolation and ChIP procedure. Too little crosslinking will result in DNA loss, elevated background and reduced antigen availability. The optimal time for cross-linking will vary depending on tissue type.
Cell Lysis	How do I ensure cells are completely lysed?	Ensure that adequate Lysis Buffer volume is used for the number individuals of each life cycle stage being processed. During the two lysis steps (Hypotonic and Lysis Buffer) make sure the cells do not settle at the bottom of the tube by flicking the tube every few minutes to ensure efficient cell lysis.
	How do I prevent protein degradation?	Add protease inhibitors to the chromatin at the appropriate step. Proteases can degrade proteins crosslinked to the DNA, resulting in less efficient IP. If protein degradation is a problem, 1 µl PIC can be added to the ice-cold PBS before suspending tissue for chromatin extraction. Ensure that chromatin extraction steps are performed at 4°C and always keep the samples on ice when processing.
	Why is the Lysis and/or ChIP-seq Elution Buffer cloudy?	The Lysis and ChIP-seq Elution Buffers contain detergents which precipitate at 4°C. Warm the buffer to 37°C in a water bath for 30 minutes or until fully redissolved. Return to room temperature before use.
Sample type	What sample types have been validated for use with this protocol?	This protocol has been optimised for *Drosophila* tissue. Careful planning for chromatin collection from different sources needs to be optimised by the user. If using cell lines or non-insect tissue, refer to the Chromatrap® ChIP seq protocol. If using FFPE tissue, refer to Chromatrap® FFPE ChIP protocol. The key requirement of working with tissue samples is to obtain a unicellular starting suspension before proceeding with any sonication steps.

Number of *Drosophila* specimens	How do I determine the optimal number of *Drosophila* specimens for ChIP-seq or qPCR?	An important consideration when performing ChIP-seq is the amount of chromatin that will need to be loaded to the column in order to elute sufficient IPd DNA for library synthesis. The DNA yield obtained from ChIP will depend on the quality of the chromatin, the affinity and avidity of the antibody and the abundance of the target. A minimum of 500 pg of DNA is required for preparation of high quality NGS sequencing libraries using the Chromatrap® *Drosophila* Uniq-seq library preparation kit. The number of *Drosophila* specimens recommended for ChIP seq or qPCR are found in Table 3. As a starting point we would recommend using 1 µg chromatin per ChIP with 5 µg antibody. Depending on the body mass and size of the specimens, the abundance of the target and the quality of the antibody this number may need to be increased.
Chromatin shearing	Why do I have a poor yield of sheared chromatin?	Chromatin may be over cross-linked, making cells resistant to lysis and chromatin resistant to shearing. Ensure specimens are fixed for the appropriate time or reduce incubation with formaldehyde. Make sure that the appropriate buffer volumes have been used.
	What sonication conditions should I use?	We have found that 30 second ON/OFF pulses for the number of cycles indicated in Table 5 at a high power setting produces chromatin fragments of 100-500 bp. Ensure that the sample is kept at 4°C during the OFF phase. Shearing conditions will depend on the sonication instrument used and need to be optimised by the user. Guideline shearing conditions for different *Drosophila* life cycle stages are described in Table 6, however we suggest checking shearing efficiency before starting the immunoprecipitation step of the assay. Short incubations may improve shearing efficiency whilst over-incubation can cause inhibition, hampering the ChIP assay.
	Can I use enzymatic shearing?	Enzymatic shearing is not recommended with this protocol. The reagents provided and the protocol described are only suitable for use with sonication. Enzymatic shearing conditions would need to be optimised by the user.
Shearing efficiency	How much chromatin should I load into the agarose gel?	Adequate chromatin should be loaded into the gel for visualisation against the ladder. Do not over- or under-load as this may hinder visualisation. Typically 15-30 µl of the reverse crosslinked stock is adequate for analysis.
	What percentage of agarose should I use?	Use a 1-2% agarose gel.
	What buffer should I use?	Prepare a 1x TAE or TBE buffer for electrophoresis.
	What electrophoretic conditions should I use?	Run the gel slowly at 100-120 V until the dye-front has migrated at least 2/3 the length of the gel.

Chromatin IP	Do the Chromatrap® spin columns require blocking?	There is no requirement to carry out a blocking step as the spin columns and buffers have been formulated to minimise nonspecific binding.
	How much antibody should be used per ChIP?	This should be determined empirically and is dependent on the amount of chromatin used per IP and the quality of the antibody. We recommend using 1-10 µg antibody per IP taking into account the amount of chromatin used and the quantity of DNA required downstream. Insufficient antibody may result in poor IP whereas excess can cause non-specific binding and lower specificity.
	What is causing high background?	The quality of the ChIP antibody has a major impact on the success of the assay. Use only ChIP validated antibodies. Inefficient wash steps can also leave traces of non-specific chromatin alongside enriched DNA. If background remains high include an additional wash step during the IP protocol.
	Why do I not have any enrichment?	The antibody used must be ChIP validated. It is essential to include ChIP validated positive and negative antibody controls. Antibodies from other applications may not work in ChIP. It is recommended that ChIP is carried out using a positive control antibody such as H3K4me3 (Chromatrap® cat no. 700010), to validate the efficiency of the IP.
Reverse cross-linking	How long should samples be reverse cross-linked?	A minimum of 2 hours at 65°C. Although, samples can be left overnight if necessary. We recommend the use of DNA LoBind tubes to minimise sample loss during heating.
qPCR	What SYBR® reagents can I use?	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.
	What positive and negative controls should I use?	Primers for a gene known to be occupied by the positive control antibody under the conditions used in the experiment should be used. If using H3K4me3 (Chromatrap® cat no. 700010), the Chomatrap® *Drosophila* positive primer set 1 (Chromatrap® cat no. 900037) will provide an excellent positive control. As a negative control, a gene locus not occupied by the target protein can be amplified in qPCR. In addition, it is important to amplify the input with all primer sets for data interpretation.
DNA profiling	Why are the NanoDrop and Qubit readings so dissimilar?	The NanoDrop cannot accurately quantify the typically low concentration of IPd DNA. Use a fluorometer such as the Qubit should be used to accurately quantify DNA before library preparation.
	What concentration of DNA should I use for the Bioanalyzer?	The quantitative range of the Bioanalyzer high sensitivity kit is 5500 pg/µl. IPd DNA may not require diluting; see library synthesis guide for instructions. Load a maximum of 5 ng/µl of sample for analysis using the Bioanalyzer pre-library synthesis.

Library synthesis	How much DNA is required for library synthesis?	The amount of DNA required to prepare an NGS library will depend on the library preparation kit used, check the manufacturers' guidelines. A minimum of 500 pg of DNA is required to prepare a library of sufficient quality and complexity using the Chromatrap® *Drosophila* UniqSeq kit. It is recommended that for optimal library complexity the user prepares libraries using as much ChIP DNA as possible.
	How many PCR cycles should I use for library enrichment?	The number of cycles required to amplify libraries to sufficient concentration depends on the quality and amount of input DNA. It is recommended that the minimum number of PCR cycles that yields sufficient library quantity for sequencing is used to maintain library complexity.
	Can I use the Chromatrap® *Drosophila* UniqSeq kit to prepare libraries for sequencing platforms other than Illumina®?	The adapter sequences supplied with the Chromatrap® *Drosophila* UniqSeq kit are only compatible with Illumina® sequencing instruments. It is possible to prepare libraries for other instruments if appropriate adapters are supplied by the user. It is not necessary to perform the end loop excision step with adapters other than the Chromatrap® Adapter for Illumina®.
	There are bubbles in my reaction mix, will these cause a problem?	A small amount of bubbles in the library preparation reactions will not affect the reaction efficiency. It is very important to mix all the solutions thoroughly before and after addition to the DNA for library preparation.
	Do I need to carry out Loop Restriction Enzyme digestion if I use my own adapters?	Loop Restriction Enzyme is only required for adapters supplied with the Chromatrap® *Drosophila* UniqSeq kit. If other adapters are used the Loop Restriction Enzyme digestion step should be omitted.
Sequencing and data quality	Why do I have adapter dimers in my Bioanalyser analysis and/or sequencing data?	It is essential that libraries are cleaned and size selected both before and after PCR enrichment to ensure unwanted DNA fragments such as unligated primers or primer dimers are not carried over into the sequencing reaction. Ensure the DNA clean up method used removes DNA fragments ≤200 bp in length such as the Chromatrap® size selection kit (Cat no.500262).
	Why do I have high levels of duplication?	ChIP-seq enriches specific fragments of DNA associated with a protein of interest. Therefore high duplication levels in the IP are not unusual. However, if the control sample also has high levels of duplication then we recommend loading more starting material during library preparation to reduce PCR sequence bias introduced during library enrichment. Use the minimum number of PCR cycles necessary to achieve the desired library concentration to minimise PCR bias during enrichment. Loading more DNA generally allows the number of PCR cycles to be reduced and leads to improved duplication rates.

IP sequencing controls	What control should I use?	We recommend using an input as the background control.
Sample storage	How should I store my IPd DNA?	Ideally at -80°C for a maximum of three months. We recommend the use of DNA LoBind tubes to minimise sample loss during storage

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

Worldwide Chromatrap® Technical Support Team
support@chromatrap.com

Worldwide Sales and Customer Support Team
Tel: +44 (0) 1978 666222 sales@chromatrap.com
Clywedog Road South Wrexham Industrial Estate Wrexham LL13 9XS UK

www.chromatrap.com