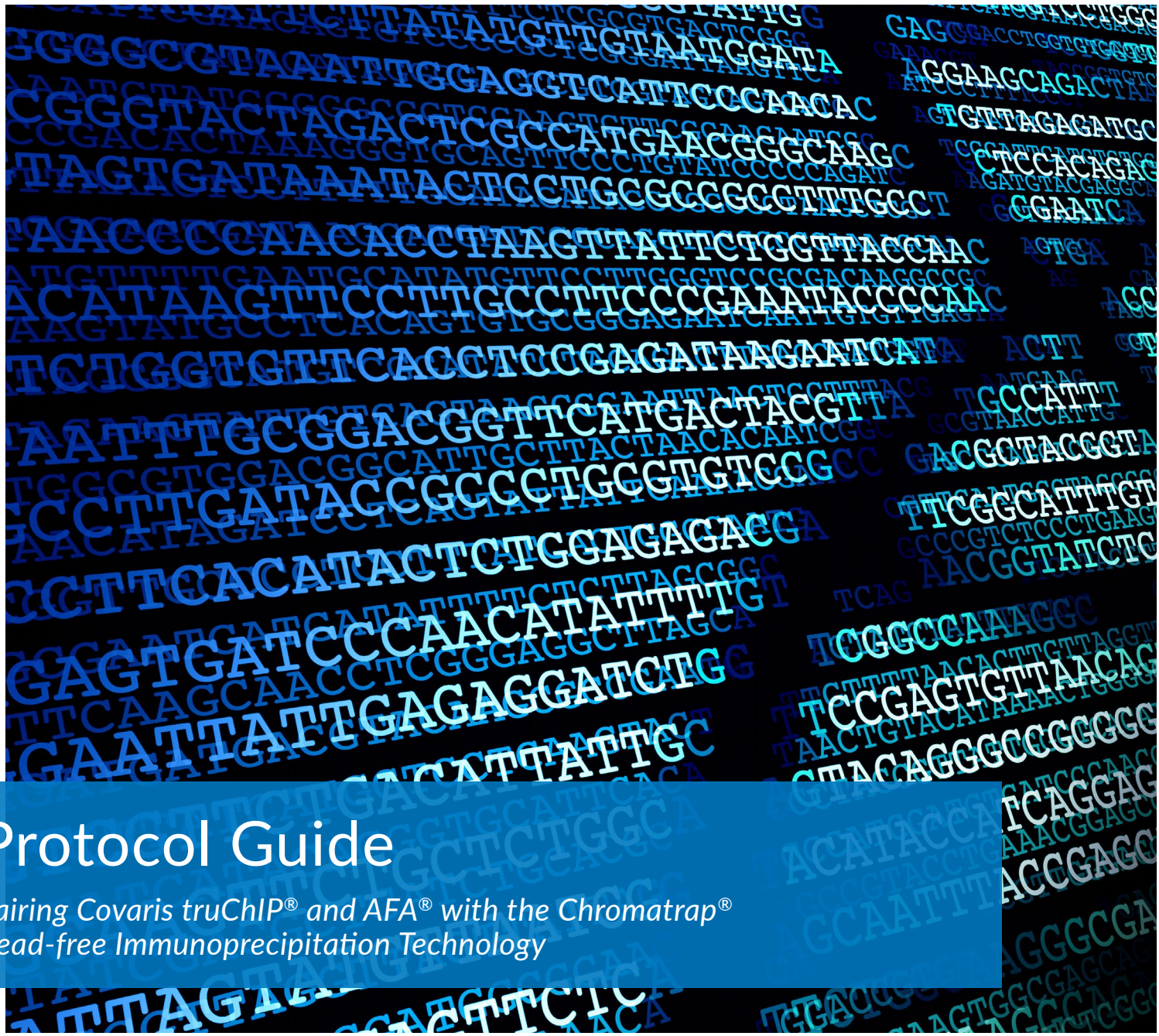


Protocol Guide

Pairing Covaris truChIP® and AFA® with the Chromatrap® Bead-free Immunoprecipitation Technology

Covaris®

Chromatrap

Pairing Covaris truChIP and AFA with the Chromatrap Bead-free Immunoprecipitation Technology

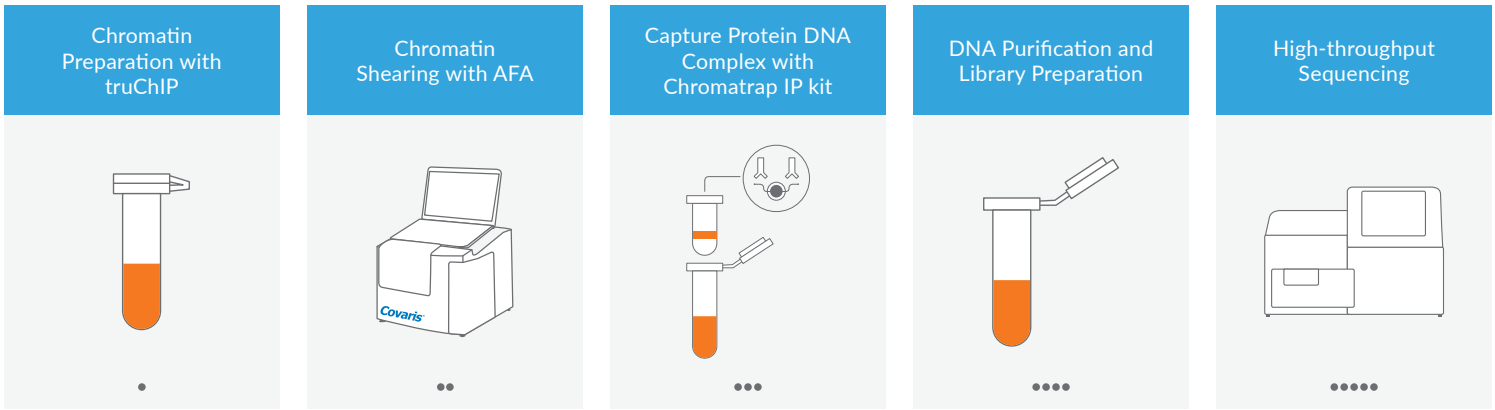
Chromatin immunoprecipitation (ChIP) is a powerful technique used to study the interaction between the genome and proteome. Importantly, this technique is used to interrogate gene expression in biological systems by profiling specific histone modifications, or transcription factor binding events.

Covaris and Chromatrap have partnered to offer a highly scalable and simple ChIP workflow enabling researchers to easily isolate protein-DNA complexes for downstream applications, such as next generation sequencing (NGS), ChIP-qPCR, and Mass Spectrometry (MS). Pairing these two workflows provides unmatched flexibility, speed, broad capability for a wide dynamic range of inputs, and automatable protocols.

This spin column-based workflow eliminates many of the challenges encountered when working with magnetic beads. A typical bead-based workflow takes around 10 hours with over 10 wash steps and incubations. This rapid centrifugation-based workflow requires only three simple washes using individual spin columns or 96-well plates and can be completed in a single day.

Using the Covaris Adaptive Focused Acoustics® (AFA®) technology, researchers can easily isolate and reproducibly shear soluble chromatin without disrupting the protein-DNA interactions for immunoprecipitation (IP) with Chromatrap's bead-free Protein A and G ChIP kits.

Paired Workflow Overview



Step	Process	Time Required (minutes)	Day
1	Crosslink protein-DNA interactions, nuclei preparation, and chromatin shearing	90	1
3	Immunoprecipitation	60	1
4	Crosslink reversal and DNA purification	210	1

Before you begin, ensure that all the materials not provided in the **Covaris truChIP Chromatin Shearing Protocol** and the **Chromatrap ChIP-Seq Protocol** are available.

Part #	Resource	Link
520156 & 520158	Covaris Ultra-low Chromatin Shearing Protocol	Covaris Sample Preparation Protocol
520154 & 520127	Covaris Standard Chromatin Shearing Protocol	Covaris Sample Preparation Protocol
500189, 500190, 500191, & 500192	Chromatrap ChIP-Seq Protocol	Chromatrap ChIP-Seq Protocol

Resources (required, not included)

Reagents and Consumables

- PBS
- Nuclease-free water
- Microcentrifuge tubes (0.5 and 1.5 mL tubes)
- ChIP validated antibodies
- AFA tubes & holders/racks
- Cell scrapers

Equipment

- Covaris Focused-ultrasonicator (M220, ME220, S220, E220 Evolution, E220, and LE220)
- Refrigerated centrifuge having 15,000 x g capability
- Rocking platform for cultured plates/flasks
- Rocker – Nutator® or equivalent
- End-to-end rotator
- Spectrophotometer/fluorometer for DNA quantification

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
- Library preparation kit
- Library quantification kit
- Chromatrap DNA Size Selection kit (Cat. no. 500262)

Optional materials

- Phenol Chloroform
- 3 M Sodium Acetate pH 5.2
- 100% Ethanol
- 70% Ethanol
- Linear Polyacrylamide (LPA)

Procedure Guide

This workflow is compatible with all mammalian cell lines cultured as a suspension or in an adherent monolayer. This protocol has been optimized to process cell inputs in the range of 1,000 to 15 million and/or 500 ng to 50 µg of DNA for immunoprecipitation. Higher and lower inputs can be utilized, however, optimization by the end-user will be required.

Single Sample	Ultra-low Cell	Low Cell	High Cell
Input cell number	<100,000	1 to 3 million (1 to 3 x 10^6) Cells	5 to 30 million (0.5 to 1.5 x 10^7) Cells
Number of samples sheared per kit	50	50	15
AFA tube	microTUBE-130	microTUBE-130	milliTUBE - 1 mL with AFA Fiber
Shearing volume	130 µl	130 µl	1 mL

Purpose

This document is intended to be used as a reference guide, therefore, it is recommended for users to consult the full-length written protocols by Covaris and Chromatrap.

Covaris Workflow

Cell Preparation and Crosslinking

Suspension Cells

1. Based on the estimated cell count, prepare solutions for the appropriate number of samples being processed before starting using the tables provided in the truChIP protocols.
2. Collect cells by centrifugation at 200 x g for 5 minutes at room temperature (RT). Remove media and wash cells once with cold 1X PBS and collect cells again by centrifugation.
3. Re-suspend cells in RT Fixing Buffer A.
4. If processing >100k and up to 30M cells, follow the standard protocol and fix cells by adding freshly prepared 11.1% formaldehyde to a final concentration of 1% and set timer. If processing less than <100k, follow the ultra-low cell protocol and fix cells by adding freshly prepared 5% formaldehyde to a final concentration of 0.25% and set timer.

> ⚠ ***Note 1. The use of fresh methanol-free formaldehyde is required to achieve reproducible results.***
> ***Note 2. Covaris recommends including two fixation times. Typically, 2.5 and 5 minutes for stem and primary cells, & 5 and 10 minutes for all other cell types.***

5. Place cells on a shaking platform at RT for the recommended time.
6. Quench the crosslinking reaction by adding the appropriate volume of Quenching Buffer E to the fixed cells. Keep cells on rocker at RT for an additional 5 minutes.
7. Collect cells by centrifuging at 500 x g for 5 minutes at RT.
8. Aspirate the supernatant and wash twice with cold 1X PBS.
9. Collect cells by centrifugation at 200 x g for 5 minutes, 4C.
10. Proceed to nuclei preparation and chromatin shearing steps.

> ⚠ ***Note. You may flash-freeze the fixed cells in liquid nitrogen at this point and store at -80C for short periods of time (e.g., 2 to 3 days). Longer-term storage is not recommended.***

Adherent Cells

1. Grow the proper amount of cells to conduct a single ChIP assay or the initial time course until they are 80 to 90% confluent.
2. Prepare solutions for the appropriate number of samples being processed fresh before starting.
3. Remove media and wash each plate one time with cold 1X PBS.
4. Remove PBS and add RT Fixing Buffer A to each dish.
5. If processing >100k and up to 30M cells, follow the standard protocol and fix cells by adding freshly prepared 11.1% formaldehyde to a final concentration of 1% and set timer. If processing less than <100k, follow the ultra-low cell protocol and fix cells by adding freshly prepared 5% formaldehyde to a final concentration of 0.25% and set timer.

> ⚠ ***Note 1. The use of fresh methanol-free formaldehyde is required to achieve reproducible results.***
> ***Note 2. Covaris recommends including two fixation times. Typically, 2.5 and 5 minutes for stem and primary cells, & 5 and 10 minutes for all other cell types.***

6. Place cells on a shaking platform at RT for the recommended time.
7. Quench the crosslinking reaction by adding the appropriate volume of Quenching Buffer E to fixed cells. Keep on a shaking platform at RT for an additional 5 minutes.
8. Completely aspirate the solution from the plate.
9. Add cold 1X PBS to each dish and scrape cells from the plate into a proper vessel.
10. Wash the plate with an additional volume of cold 1X PBS to collect any remaining cells.
11. Collect cells at 200 x g for 5 minutes, 4C.
12. Wash cells twice by resuspending in cold 1X PBS, and collecting by centrifugation at 200 x g, 4C.
13. Carefully and completely aspirate the supernatant from the tube(s), and place on ice. Proceed to nuclei preparation and chromatin shearing steps.

> ***Note. You may flash-freeze the fixed cells in liquid nitrogen at this point and store at -80C for short periods of time (e.g., 2 to 3 days). Longer-term storage is not recommended.***

Nuclei Preparation (This section is omitted if following the Covaris Ultra-Low Cell method)

1. Add Lysis Buffer B containing Buffer F inhibitors to cross-linked cells to lyse the plasma membrane. Incubate for 10 minutes on a rocker at 4C. If cells were frozen after formaldehyde fixation, thaw cells on ice first.
2. Collect intact nuclei by centrifugation at 1,700 x g for 5 minutes at 4C. Decant the supernatant without disturbing the nuclei pellet.
3. Gently resuspend pellet in Wash Buffer C containing protease inhibitors and incubate on a rocker for 10 minutes at 4C.
4. Collect nuclei by centrifugation at 1,700 x g for 5 minutes at 4C. Carefully remove and discard the wash solution taking care not to disturb the nuclei pellet.
5. Gently rinse the sides of the tube with Shearing Buffer D3 containing Protease inhibitors. Slowly dispense the buffer down the entire circumference of the upper-inside of the tube taking care not to disturb the nuclei pellet.
6. Collect nuclei by centrifugation at 1,700 x g for 5 minutes at 4C. Decant the supernatant without disturbing the nuclei pellet.
7. Repeat steps 5 and 6 one additional time.
8. Carefully remove and discard the supernatant without disturbing the nuclei pellet.

Chromatin Shearing

1. Resuspend the nuclei in the Covaris Shearing Buffer D3 and transfer to the appropriate AFA tube(s).
2. Shear the chromatin using the settings provided in Appendix A of the truChIP protocol to generate >70% of fragments within the 151 to 700 bp range.
3. Evaluate the chromatin shearing efficiency following the steps outlined in 2c of the Chromatrap ChIP-Seq protocol.
    a. For the most accurate sizing analysis, it is recommended to run the Agilent BioAnalyzer DNA 12 000 chip.
4. Quantify the DNA in the samples using a spectrophotometer at 260 nm. 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 for the Slurry Preparation and Immunoprecipitation.
5. Before beginning the slurry preparation, centrifuge the sheared chromatin at maximum speed for 10 minutes at 4C.

Chromatrap® Workflow

Immunoprecipitation

The Chromatrap Immunoprecipitation technology enables researchers to process 1,000 to 15 million cells or 50 ng to 50 µg of DNA sheared using Covaris AFA. To start, Chromatrap recommends using 10 µg of chromatin per ChIP with 2 to 5 µg antibody. For the positive control for qPCR analysis, prepare the slurry in a 1 mL volume with a 2:1 antibody: chromatin ratio. In addition, prepare negative controls for both antibody validation and the standard assay.

1. Prepare IP slurries in a fresh microcentrifuge tube according to the table below.

Reagent	Immunoprecipitation Slurry (1,000 µl total volume)	Positive Control (1,000 µl total volume)
Chromatin Stock	Up to 100 µl	1 µg
Antibody/IgG	Optimum addition rate	10 µl (2 µg)
Column Conditioning Buffer	Make up to a final volume of 1,000 µl	Make up to a final volume of 1,000 µl

Note 1. Use only the clear supernatant for the immunoprecipitation step.
Note 2. For low concentrated chromatin samples, mix up to 500 µl of sheared chromatin in D3 buffer to a final volume of 1 mL using the provided column conditioning buffer.

   a. For each 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), and label as an input. These will be processed alongside the samples for reverse cross-linking and proteinase K digestion and will be used as controls in the downstream analysis.
   b. Mix well and incubate the IP slurries on an end-to-end rotor for 1 hour at 4C and proceed to steps 2 through 5.
2. Remove the spin column from the collection tube (save for later) and place in an empty 1 mL tip box rack (or alternative holder).
3. Add 600 µl Column Conditioning Buffer to each column and allow to flow through under gravity (around 15 minutes)

Note. Do not close caps when flow is under gravity.

4. Discard the flow through and repeat this conditioning step a second time.
5. Discard the flow through. The columns are now ready for the addition of the IP slurries.
6. Remove slurries from the end-to-end rotator following 1 hour pre-incubation and briefly spin down to remove residual liquid from the caps.
7. Load the entire 1 mL slurry and allow to flow completely through the column at RT (approximately 15 to 20 minutes).
8. 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.
9. 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.
10. 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.
11. Spin dry at top 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).
12. Add 50 µl of ChIP-Seq Elution Buffer to each column, cap, and incubate at RT for 15 minutes.

13. Centrifuge the columns at top speed for 30 seconds to collect the eluted chromatin.
14. Proceed to reverse crosslinking and DNA purification steps.

> ***Note. At this stage, samples can also be analyzed by Mass Spectrometry (MS). Chromatrap recommends pooling Immunoprecipitated samples to achieve sample complexity. For sequencing and qPCR analysis, please proceed directly to reverse crosslinking and DNA purficication***

Protein Crosslink Reversal

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. Input controls, which have not been through the IP process, must be reintroduced at this stage of the protocol.

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.
2. 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.
3. Mix thoroughly and incubate for 2 hours at 65C. If required, the incubation at 65C can be performed overnight.
4. Add 1 µl Proteinase K to each IP and input sample. Vortex briefly and perform a short spin. Incubate for one hour at 37C.
5. Add 2 µl Proteinase K Stop Solution to each IP and input sample. Vortex briefly and perform a short spin.

DNA Purification

Chromatin must be purified before proceeding with qPCR or NGS library preparation. DNA purification columns and reagents are included in all Chromatrap ChIP-Seq kits. 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.

Preparation of DNA Wash Buffer

1. Add 60 mL ethanol (95 to 100%) to the DNA Wash Buffer concentrate before first use and note on label that ethanol has been added.
2. Add 5 volumes of DNA Binding Buffer to 1 volume of sample and mix.

> ***Note. 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 3M Sodium acetate, pH 5, to adjust the pH of the binding mixture, the color of the mixture should turn yellow.***

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

Covaris Products

Product Number	Product	Description
520154	truChIP Chromatin Shearing Kit with Formaldehyde	The truChIP kit contains all the buffers and reagents required to prepare up to 30M mammalian cells for chromatin shearing using a Covaris Focused-ultrasonicator. This kit provides investigators with enough reagents to perform up to 50 reactions.
520127	truChIP Chromatin Shearing Kit	
520156	truChIP Ultra-Low Chromatin Shearing Kit with Formaldehyde	The truChIP Ultra Low Cell Chromatin Shearing Kit with Formaldehyde is designed for efficient chromatin shearing of 100,000 cultured mammalian cells or less using a Covaris Focused-ultrasonicator. This kit provides investigators with enough reagents to perform up to 50 reactions.
520158	truChIP Ultra-Low Chromatin Shearing Kit	The truChIP Ultra Low Cell Chromatin Shearing Kit is designed for efficient chromatin shearing of 100,000 cultured mammalian cells or less using a Covaris Focused-ultrasonicator. This kit provides investigators with enough reagents to perform up to 50 reactions.

Chromatrap Products

Product Number	Product	Description
500189	Chromatrap ChIP-Seq Protein A	Innovative bead-free ChIP reduces laborious manual handling steps while giving you the highest signal-to-noise ratio and sensitivity. Perform up to 24 ChIP assays in a single day. Kit includes purification reagents to elute high quality DNA, ideal for NGS library prepartation.
500190	Chromatrap ChIP-Seq Protein G	
500214	Chromatrap HT ChIP-Seq Protein A	Chromatrap bead-free ChIP technology in plate format. Effortlessly perform up to 96 ChIP assays with unparalleled reproducibility, reliability and sensitivity. Complete kit includes clean-up plate for superior quality DNA purification, ideal for NGS library prepartation.
500215	Chromatrap HT ChIP-Seq Protein G	

Technical Support

If you need assistance at any time, please call Covaris or Chromatrap using the information below.

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