User Guide

Catalog No.
CHROP–15 RXN

Imprint® Ultra Chromatin Optimization Kit

sigma-aldrich.com/life-science/epigenetics/chip2.html

Imprint® Ultra Chromatin Optimization Kit

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CHROP Kit Components and Storage

The CHROP kit is shipped at two different temperatures. The wet ice shipment contains Parts 1 and 2. The dry ice shipment contains Part 3. Storage temperatures are indicated below:

Reagent Description	Catalog Number	CHROP-15 Rxn
Part 1, stored at room temperature (RT)		
Cell Lysis Buffer	B5938	3.3 mL
Igepal CA-630, for molecular biology*	I8896	1 mL
Nuclei Lysis Buffer*	B6438	1.65 mL
Water, Molecular Biology Reagent	W4502	5 mL
Sodium chloride solution, 5 M, for molecular biology	S5150	1.5 mL
CHP2 DNA Purification Columns	C6874	15 ea
CHP2 DNA Purification Collection Tubes	T5205	15 ea
CHP2 DNA Binding Buffer	B5563	15 mL
CHP2 DNA Wash Buffer**	B5813	3 mL
CHP2 DNA Elution Buffer	B5688	1.5 mL
Part 2, stored at refrigerator temperature (2-8 ˚C)		
Glycine solution, 1.25 M	G7173	16.5 mL
Part 3, stored at freezer temperature (-20 ˚C)		
4-(2-Aminoethyl)benzenesulfonyl fluoride (AEBSF)	A8456	25 mg
Protease Inhibitor Cocktail (PIC), without metal chelators	P8340	0.05 mL
Ribonuclease A solution, for molecular biology	R4642	10 mg

* These reagents should be warmed up to room temperature for 15 minutes before use in order to dissolve any detergents that may have precipitated during shipping. Warm and mix to obtain a clear solution before use.

** Add 12 mL Ethanol (459844, not provided) to the DNA Wash buffer concentrate (B5813, Part 1, RT) before use.

Product Description

The Imprint® Ultra Chromatin Optimization Kit provides reagents needed to optimize chromatin sonication/shearing conditions before performing ChIP. It is imperative that chromatin is completely sheared into fragments with a peak around 500 bp for ChIP-qPCR assays, and < 500 bp for ChIP-Seq and ChIP-chip, for maximum specificity and resolution, and to ensure low background. For higher resolution applications like genome-wide location analysis by ChIP-chip or ChIP-Seq, sonication of input chromatin to below 500 bp, with a peak around 250 bp, is critical.

Sufficient reagents are provided to perform three sets of chromatin sonication optimization experiments. Each set would include five different conditions for sonication to optimize the parameters. Reagents are provided for a total of 15 reactions including preparation of cell lysates from 10^7 cells/reaction, nuclei lysis, sonication, cross-link reversal and DNA purification.

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Storage and Stability

All components, if stored at the appropriate temperatures as suggested in the reagents table, are stable for up to 12 months.

Care must be taken not to introduce DNases. We recommend the use of DNase-free pipette tips, preferably those having an aerosol barrier. Wear latex gloves and change them frequently. Keep bottles and tubes closed when not in use.

Reagents and Equipment Recommended but Not Provided

- Cells or tissue
- 0.25 % Trypsin-EDTA solution , **T4049**
- Hank's balanced salt solution, HBSS, **H6648**
- Formaldehyde solution, 37% in water, **F8775** or **252549**
- 10X Phosphate Buffered Saline,(PBS), pH 7.4, **P7059**
- Heat block or Oven capable of 65 °C, 100 °C and 37 °C
- Refrigerated Microcentrifuge for 1.5 mL microcentrifuge tubes (capable of 16,000 × g)
- 1.5 mL screw-cap tubes, Corning **430909**
- Microcentrifuge tube holders, **Z708372** ; optional
- Ethanol, **459844**
- Sonicator – Diagenode BioRuptor™ recommended
- Polystyrene tubes for sonication in the BioRuptor, 1.5 mL (Diagenode M-50050) and 15 mL (Falcon 352099)
- Proteinase K, **P4850**
- Direct load wide range DNA size marker, **D7058** , or equivalent

Preparation Instructions

- **Optimize shearing conditions for your cell type:** The equipment and cell type used in preparing the sheared chromatin can have a significant impact on the success of the experiment. The Imprint Ultra ChIP kit (**CHP2**) does not include sufficient reagent volumes to optimize shearing and also to perform all 24 or 48 ChIPs. Thus, we recommend using this Chromatin Sonication Optimization kit (**CHROP**-15Rxn) to standardize the sonication/chromatin shearing conditions before performing the ChIP reaction.
- **Thoroughly mix reagents.** Examine the reagents for precipitation. If any reagent has formed a precipitate, warm at 55 °C until dissolved. Allow to equilibrate to room temperature before use.
- **DNA Wash Buffer, B8313:** Dilute the DNA Wash Buffer Concentrate with 12 mL of ethanol (**459844**) before use. After each use, tightly cap the diluted DNA Wash Buffer to prevent the evaporation of ethanol.

TF abundance	Application	Number of cells
Histone modification or abundant TF	qPCR	0.5 - 2 x 10^6
	Microarray	10^4 - 10^6
	Sequencing	10^5 – 10^7
Medium abundance TF, e.g., RNAP II	qPCR	2-10 x 10^6
	Microarray	10^5 – 10^6
	Sequencing	10^6 – 10^7
Rare TF (Low abundance TF and co-activator/co-repressor that does not bind DNA directly, e.g., EZH2	qPCR	5-10 x 10^6
	Microarray	50 x 10^6 (5 ChIPs)
	Sequencing	100-200 x 10^6 (10-20 ChIPs)

Table 1: Guidelines for number of cells required, depending on the relative abundance of ChIP target and downstream application.

Procedure

Section 1. Preparation of Cross-linked Cells:

Pre-requisites:

- Cells grown in large (245 mm x 245 mm, 500 cm^2) dishes, Corning, **CLS431110**, or flasks
- Warm complete medium for the cells of interest and 0.25 % Trypsin-EDTA solution (**T4049**, not provided) in a 37 °C water bath
- Hank's balanced salt solution, HBSS (H6648, not provided) at RT
- 37% Formaldehyde (**F8775**, not provided), ice-cold 1.25 M Glycine (G7173, Part 2, 4 °C) and ice-cold 1× Phosphate Buffered Saline, PBS (diluted from 10× PBS, pH 7.4, **P7059**, not provided)
- Pre-cool centrifuge and rotor to 4 °C for pelleting cells in 50 mL conical tubes

1. Cell cultures should be healthy and not density arrested prior to cross-linking. Generally, you will need 1 x 10^7 cells per antibody per ChIP. Fewer cells, as few as 2 x 10^6 cells, can be used but may result in lower signal to noise ratio. See **Table 1** for recommended number of cells to use for your intended application. A confluent 500 cm^2 dish will typically yield between 50 -100 x 10^6 cells, depending on the cell type.

   Note: *Volumes below are for 50 -100 x 10^6 cells, harvested from one confluent 500 cm^2 dish.*

2. Remove medium from cells. Wash 50 -100 x 10^6 cells with 35 mL of Hank's Balanced Salt Solution, HBSS (**H6648**), aspirate HBSS completely, and trypsinize the cells with 9 mL of Trypsin-EDTA solution (T4049). Rock side to side to distribute and coat the monolayer and incubate 3-5 minutes at 37 °C. Dislodge the cells by tapping the sides of the dish and rocking side to side to release the cells.

   Note: *Alternately, the cells may be cross-linked directly in the dishes/flasks by adding formaldehyde (1% final concentration) directly to the medium and rocking gently at RT for 10 minutes. However, releasing cells before cross-linking allows more efficient recovery of chromatin and is also helpful for cells that are difficult to swell during chromatin preparation.*

3. Immediately add 31 mL of the warm complete medium to stop trypsinization.

   Note: *Incubation longer than 5 minutes in trypsin may lead to degradation of the cells and dramatically reduced ChIP yields.*

4. Disperse the cells by pipetting, transfer to a 50 mL conical tube, and use an aliquot to count the cells.

5. In fume hood, add formaldehyde to final concentration of 1% (i.e., 1.2 mL of 37 % stock, **F8775** or **252549** to 40 mL of cell suspension). Close the tubes tightly and invert by end-to-end rotation for 10 minutes at RT.

   Note: *Cross-linking for ≥ 15 minutes may cause cells to form aggregates that do not sonicate efficiently.*

   Warning: Formaldehyde liquid and vapors are carcinogenic. Always use proper personal protection, work with open containers in a fume hood, and dispose of waste properly.

6. In fume hood, stop the cross-linking reaction by adding one-tenth volume of 1.25 M Glycine (G7173, Part 2, 4 °C), to give a final Glycine concentration of 0.125 M. For example, add 4.1 mL of Glycine per 41.2 mL of formaldehyde-treated cells. Continue to rock/invert for 5 minutes at RT. The amine groups in Glycine will quench the formaldehyde cross-linking and the color of the solution will become lighter.

7. Centrifuge cells at 200 rcf (≈1000 rpm) for 10 minutes at 4 °C.

8. Discard the spent formaldehyde in an appropriate chemical waste container in the fume hood. Carefully aspirate supernatant so as to not lose cells.

9. Wash the cells by resuspending pellet in 50 mL of ice-cold 1× Phosphate Buffered Saline.

10. Centrifuge cells at 200 rcf (≈1000 rpm) for 10 minutes at 4 °C and aspirate the supernatant.

11. Cross-linked cells can either be used immediately for ChIP assays or snap frozen in liquid nitrogen, and stored in liquid nitrogen or in a –80 °C freezer indefinitely.

Sections 2. Preparation of Cross-linked Chromatin

Pre-requisites:

- Pre-cool a microfuge to 4 °C and cool a glass Dounce homogenizer on ice
- Prepare 10% IGEPAL by diluting the provided 1 mL of IGEPAL (**I8896**, Part 1, RT) with 9 mL of warm water and store at RT
- Reconstitute AEBSF (**A8456**, Part 3, –20 °C): add 1.04 mL water (**W4502**, Part 1, RT) to the 25 mg bottle to generate a 0.1 M AEBSF stock. Mix and vortex until dissolved, and keep on ice. After use, store at –20 °C
- Thaw Protease Inhibitor Cocktail (PIC, **P8340**, Part 3, –20 °C) and keep at RT (it will freeze on ice)
- **Complete Cell Lysis Buffer:** Prepared on ice. Use 1 mL per 50 x 10^6 cells. Add 100 µL of 10 % IGEPAL per 900 µL of Cell Lysis Buffer (B5938, Part 1, RT) for a final concentration of 1% IGEPAL. Just before use, add 10 µL of 0.1 M AEBSF and 10 µL of PIC per mL of Complete Cell Lysis Buffer. Mix and keep on ice. Scale as needed, see table below
- **Complete Nuclei Lysis Buffer:** Prepared at room temperature. Use 0.5 mL per 50 x 10^6 cells. Just before use, add 5 µL each of 0.1 M AEBSF and PIC to 0.5 mL of Nuclei Lysis Buffer (B6438, Part 1, RT). Mix and keep at RT, as it will precipitate on ice. Scale as needed, see table below

Component	Requirement for 5 sonications	Use this column to calculate requirements for your experiment
# Cells: (recommend 10^7 cells/ChIP)	e.g., For 50 x 10^6 cells i.e., 5 sonications @ 10M/reaction	
Volume of Complete Cell Lysis Buffer to prepare:	1 mL	
Cell Lysis Buffer:	0.9 mL	
+ 10 % IGEPAL (10×):	0.1 mL	
+ PIC (100×):	10 µL	
+ 0.1 M AEBSF (100×):	10 µL	
Volume of Complete Nuclei Lysis Buffer to prepare:	0.5 mL	
Nuclei Lysis Buffer:	0.5 mL	
+ PIC (100×):	5 µL	

- Thaw the frozen cross-linked, washed cell pellet from –80 °C rapidly by rubbing between hands or swirling in a 37 °C water bath, and place on ice
- Chill the appropriate size vessel for sonication. For sonication with a Diagenode BioRuptor (recommended), chose the tube depending on total volume of chromatin:
  a. 1.5 mL tube (**Polystyrene** recommended, Diagenode M-50050): 100 – 300 µL
  b. 15 mL tube (**Polystyrene only!** Falcon 352099): 500 µL – 1 mL
- Set up the BioRuptor or other sonicator in a cold room, if possible. If not possible, cool the water chamber (BioRuptor) by filling it with ice before use

Section 2. Preparation of Chromatin from Cross-linked Cells:

12. Resuspend cells (from step 11) with freshly prepared Complete Cell Lysis Buffer, use 1 mL per 50×10^6. Scale volume proportionately if processing more or less than 50×10^6, i.e., 2 mL for 100×10^6).
13. Incubate on ice for 20 minutes and flick the tube occasionally to resuspend the cells.
14. Dounce up to 1 mL of the cell suspension on ice using a 2 mL glass Dounce homogenizer with 15 strokes of the B pestle to release nuclei and disperse cell clumps. If you have more than 1 mL of cell suspension, Dounce only 1 mL at a time or use a larger dounce.
15. Transfer dounced cell lysate to 1.5 mL tubes.
16. Spin at 2,500 rcf for 5 minutes at 4 °C to pellet nuclei, pour off supernatant or aspirate carefully.
17. Resuspend nuclei in freshly prepared Complete Nuclei Lysis Buffer (NLB), use 0.5 mL per 50×10^6 cells. Scale volume proportionately if processing more or less than 50×10^6, i.e., 1 mL for 100×10^6 cells.

    Note: *Resuspend nuclei immediately after adding NLB. If sample (from 100×10^6 cells) was split into two tubes for douncing, use 1 mL of complete NLB to resuspend the first pellet, then transfer quickly to second pellet and resuspend).*

    Transfer immediately after complete resuspension into a pre-chilled polystyrene tubes(s) for sonication. For consistent performance do not sonicate > 1 mL of nuclear lysate per 15 mL polystyrene tube. Samples may be split between multiple tubes to avoid going over maximum volume.
18. Incubate on ice for 10 minutes.

    Sonicate chromatin either in the BioRuptor (Section 3) or microtip based sonicator (Section 4) and process the 10 µL chromatin aliquots as described under cross-link reversal and DNA purification (Section 5).

Section 3. Chromatin Sonication Optimization using BioRuptor:

Note: *Sonication must be optimized for each cell line and instrument. We recommend the Diagenode BioRuptor (water based sonication) set up in a cold room for reproducible sonication without foaming and heating, thus preserving the cross-linked DNA-protein complexes. The procedure below is optimized for this system. User optimization is required for other systems.*

Warning: Wear appropriate ear protection.

Sonicator Preparation: Remove tube holder and some water from the sonication chamber and supplement with 0.5 cm of crushed ice, swirl to mix and check that water/ice level is at blue "water level" mark (remove excess water, if needed). Do not add excess ice so as to impede the circular motion of the tubes in water during sonication. Use appropriate tube holder and accessories that have been rinsed thoroughly with water followed by ethanol between uses.

19. Place tubes in sonicator, balancing as for a centrifuge.
20. Input sonication settings: the pulse duration, intensity and number will vary depending on the extent of cross-linking and cell type. Therefore, you must optimize for your experiment. Ideally the least amount of input energy that gives satisfactory fragmentation should be used. Set LMH dial to High, set interval by adjusting red needle (sonication time, usually 15 sec or 30 sec) and the green needle (rest interval, 30 sec or 1 minute standard to allow cooling). Then set the timer.

    For optimization, we recommend performing a series of five 10 minute sonication treatments (total sonication time of 50 minutes) on the High "H" setting with 30 seconds "ON" and 30 seconds "OFF" cycle. Remove 10 µL aliquots after each 10 minute interval and process as described below. Alternatively, chromatin could be sonicated in 5 separate tubes containing 0.5 ml each on "H" setting for 10, 20, 30, 40 or 50 minutes in 10 minute intervals (with fresh crushed ice added to the chamber and the water level reset after each interval). Remove a 10 µL aliquot of sonicated chromatin from each time point for determining the DNA size range following cross-link reversal and DNA purification as described below.

    Note: *For ChIP-Seq experiments it is recommended that the chromatin be fragmented to < 500 bp. For the cell lines tested, we have found a setting on "H" with 30 sec ON and 30 Sec OFF for 4-5 x 10 minutes gave good results (< 500 bp, **Figure1**).*
21. Rinse used tube holder and accessories thoroughly with water and then with ethanol.
22. Determine optimum sonication conditions after cross-link reversal and DNA purification from the 10 µL sonicated chromatin aliquots, as described below.

23. If using 15 mL tubes, transfer sonicated chromatin to 1.5 mL tubes.
24. Microfuge samples (hinge facing outward) at 14,000 rcf for 10 minute at 4 °C.
25. Transfer supernatant to a new 1.5 mL tube, or to several tubes in conveniently-sized aliquots for storage. Chromatin from 10×10^6 cells (≈100 µL) will be used per ChIP for rare transcription factors. See Table 1 for recommended number of cells for your application. Sonicated chromatin can be stored at –80 °C and used up to a year later.

Section 4. Sonication Conditions for a Microtip Sonicator:

This protocol describes recommendations for the Sonicator 3000 (MISONIX, Part # S3000) with a 1/16" microtip. The sonication conditions need to be optimized for different sonicators and different cell lines.

1. Prepare nuclei lysate as described above (step 18).
2. Place the tube in a wet ice/ ethanol bath or wet ice bath.
3. Insert the probe (1/16" microtip) to a depth of 2-3 mm into the cell lysate.
4. Shear the cell lysate using the following settings: Power: 0.5 W; Time: 2 sec ON / 15 sec OFF; Total time: 16 sec (8 times per round).

   Note: *If sample starts to bubble; STOP the sonication immediately! Centrifuge the sample at 6000 rcf for 5 min at 4 °C to eliminate bubbles. Continue the sonication with previous settings. If bubbling continues, adjust the settings as follows: Power: 2.5 W; Time: 10 sec ON / 30 sec OFF; Total time: 1 to 3 min (6 to 18 times per round)*
5. Mix the cell lysate by pipetting, remove a 10 µL aliquot.
6. Repeat steps 4 and 5 twice, for a total of 3 rounds.
7. Reverse cross-linking, purify DNA, and evaluate fragment size as described below.
8. Pellet the debris from remaining sonicated chromatin by centrifugation at 14,000 rcf for 10 min at 4 °C.
9. Transfer supernatant to a new 1.5 mL tube, or to several tubes in conveniently-sized aliquots for storage. Chromatin from 10×10^6 cells (˜100 µL) will be used per ChIP for a rare transcription factor target. See **Table 1** for recommended number of cells for your application. Sonicated chromatin can be stored at –80 °C and used up to a year later.

Section 5. Cross-link Reversal and DNA Purification:

Pre-requisites:

- Elution Buffer (1% SDS, 50 mM $NaHCO_3$): Made fresh just before use as follows:

Water:	425 µL (W4502, Part 1, RT)
10 % SDS:	50 µL (L4522, Part 1, RT)
1 M NaHCO3:	25 µL (S6326, Part 1, RT)
Total:	500 µL

- Add 12 mL Ethanol (459844, not provided) to the DNA Wash buffer concentrate (B5813, Part 1, RT) before use

26. Use 10 µL sonicated chromatin sample and add 40 µL Elution Buffer.

    Add 1 µL of 10 mg/ml RNase A solution (**R4642**, 10 mg/mL, Part 3, –20 °C) and incubate at 37 °C for 15 minutes.
27. For maximum yields of sonicated chromatin, add 1 µL of Proteinase K solution (**P4850**, not provided) and incubate at 65 °C for 1 hour.
28. Reverse cross-link by adding 6 µL of 5 M NaCl (**S5150**, Part 1, RT) and boil for 15 minutes.
29. Place samples on ice for 5 minutes.

    Note: *Any white precipitate that forms after boiling and chilling does not interfere with the subsequent DNA purification.*
30. Add 250 µL of DNA binding buffer (B5563, Part 1, RT).

31. Assemble a DNA purification column (C6874, Part 1, RT) in a collection tube (T5205, Part 1, RT) and transfer the DNA + binding solution into the column.
32. Centrifuge at > 12,000 rcf for 1 minute at RT.
33. Discard the solution from the collection tube and wash the DNA by adding 200 µL of DNA Wash buffer (to be reconstituted by adding 12 mL Ethanol before use) into the column.
34. Centrifuge at > 12,000 rcf for 1 minute at RT.
35. Repeat the wash (steps 34 and 35 above).
36. Add 10 µL of DNA Elution Buffer (B5688, Part 1, RT) to the column and transfer the column to a clean eppendorf tube (T3566).
37. Elute DNA by centrifugation at > 12,000 rcf for 1 minute at RT.
38. Estimate DNA concentration on a Nanodrop spectrophotometer.
39. Load the remaining 8 µL of sonicated DNA on a 2% agarose gel and determine the size range of the smear by comparing with a DNA size marker (such as Direct load wide range DNA size marker, **D7058**). See **Figure 1** below for an image of a gel showing chromatin that has been sonicated below 500 bp for ChIP-Seq assays. Alternately, the sonicated DNA samples may also be analyzed on a Bioanalyzer DNA chip.
40. The sonication condition that gives a smear of DNA sizes from 200 bp to 1 kb with a peak around 500 bp (2-3 nucleosomes) should be used for ChIP-qPCR reactions. For more demanding (higher resolution) applications like ChIP-Seq, it is recommended to sonicate the chromatin to < 500 bp.

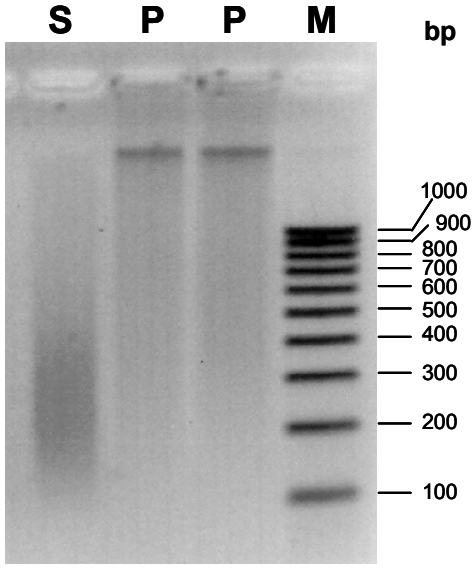

Figure 1. Sonicated chromatin: Following the protocol described above, sonicated (**S**) chromatin was prepared from formaldehyde cross-linked DU145 cells and analyzed on a 2 % agarose gel-stained with ethidium bromide. **M**, size marker - 100 bp ladder (**D3687**), bp, base pairs. Sonication conditions: 30 sec ON, 30 sec OFF, Time: S = 2 x 15 mins (good sonication to below 500 bp), **P** (partial sonication) = 1 x 15 min (most of the genomic DNA is unsheared as indicated by the high MW band).

References:

1. Kuo and Allis, *In vivo* cross-linking and immunoprecipitation for studying dynamic Protein:DNA associations in a chromatin environment. *Methods*, **1999**, 19:425-433
2. O'Geen, H., *et al.,* Comparison of sample preparation methods for ChIP-chip assays. *Biotechniques*, **2006**, 41:577-580
3. Acevedo LG, *et al.,* Genome-scale ChIP-chip analysis using 10,000 human cells. *Biotechniques*, **2007**, 43:791-797
4. O'Geen, H., *et al.,* Genome-Wide Analysis of KAP1 Binding Suggests Autoregulation of KRAB-ZNFs. *PLoS Genet*, **2007**, 3:916-92

Troubleshooting Guide

Observation	Cause	Recommended Solution
High molecular weight genomic DNA	Sub-optimal cross-linking	Titrate the length of time cross-linking is performed from 5-20 min. Excessive cross-linking will hinder sonication.
	Sub-optimal sonication	Optimize sonication conditions by varying number and length of sonication bursts. Avoid foaming and keep sample on ice during sonication. Large volumes and/or too much sample in one tube yields inefficient DNA fragmentation.
	Incomplete disruption of chromatin aggregates	Digesting chromatin with Proteinase K releases aggregates and prevents trapping of DNA during cross-link reversal by boiling
Low yield of sonicated DNA < 500 bp	Insufficient cell number or tissue amounts	Accurate cell count post trypsinization will help in determining the amount of cell lysis and nuclei lysis buffer needed. Optimize sonication from at least 5-10 × 10^6 cells/time point or setting
	Insufficient cell or tissue lysis	Check lysis efficiency by observing a 5 µL aliquot of the lysate under a microscope.
	Harsh sonication conditions	Sonication must be optimized for appropriate shearing conditions. Sonicating in warm conditions, for too long, or at too high a setting may disrupt the protein-DNA complex. Always sonicate on ice, at the lowest setting and the shortest amount of time possible that still yields appropriately fragmented DNA (~200-500 bp)
	Incorrect temperature or insufficient DNA release and cross-link reversal	Longer or shorter times used in cross-linking may require different incubation times for the DNA release and cross-link reversal steps.

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