Chromatin Immunoprecipitation of *Drosophila* Blastoderm-stage Embryos:

Shelby Blythe, January 2019, version 6

This is a protocol for performing ChIP on a small number of blastoderm-stage embryos. In general, the protocol is fairly standard as far as the actual ChIP goes, and differs with respect to how the embryos are typically crosslinked. Data generated with this protocol has been published:

Blythe, S. A., and Wieschaus, E. F., 'Zygotic genome activation triggers the DNA replication checkpoint at the midblastula transition.' Cell 2015, Mar 12; 160 (6): 1169-81 (PMC4359640).

Samples produced by this protocol can be analyzed using either PCR or next generation sequencing approaches.

Embryo Crosslinking:

How this is done depends partly on whether embryos will be collected in bulk or if they will be sorted. The ChIP protocol works well enough with ~100 cellular blastoderm embryos per IP or ~200 syncytial blastoderm embryos per IP, if you are sorting. For transcription factors, we have obtained better/more consistent results with 200 cellular blastoderm embryos.

Embryos are crosslinked here for 15 minutes at room temperature. It is critical to start a timer at the moment that the formaldehyde is added to the sample. The stop point of the crosslinking is when PBS/Triton/Glycine is added to the sample to quench the formaldehyde. Therefore, the embryos are incubated in formaldehyde with shaking for 10 minutes, and then in the next 5 minutes, the embryos are centrifuged and collected in a new tube, and at the 15 minute timepoint, the samples are quenched.

Materials:

- Embryos
- Bleach
- PBS/0.5% Triton X-100 (Kept at room temp in a squirt bottle and also a separate stock at 4°C).
- PBS/0.5% Triton X-100/125mM Glycine (that's 235mg Glycine per 25ml)
- Heptane
- 20ml High-Recovery Vials (V-Bottom Tubes) VWR 89042-310
- 20% Paraformaldehyde solution
- Timer
- Tubes that fit with the Pellet Pestle Homogenizers (Kontes)

Procedure:

1) Perfom a timed collection (less important to be precise if sorting embryos by hand).
2) For each sample to be collected, prepare a 2ml PBS/0.5% Triton X-100 + 6ml Heptane solution in a 20ml V-Bottom tube.

3) Dechorionate for ~1 minute in bleach, collect in a basket
4) In the basket, wash embryos extensively in dH_2O. The embryos should float.
5) In the basket, rinse embryos extensively in PBS/0.5% Triton X-100, I typically use enough to fill a 60mm Petri Dish lid. The embryos should sink in this solution.
6) In the basket, blot embryos dry on a paper towel.
7) Transfer embryos to the prepared 20ml V-Bottom tube by inverting the basket in the heptane fraction.
8) Add 180µl 20% Paraformaldehyde solution to the V-Bottom tube, cap the tube, and **start a timer**.
9) Vortex on high for 30 seconds. The solution should turn milky white[1].
10) Mix the samples for 10 minutes on a rotary shaker set to ~120rpm.
11) Collect the embryos at the bottom of the tube and separate the liquid fractions by centrifugation at 500xg for 1 minute. The upper organic phase will have the consistency of shampoo. This is OK.
12) Transfer embryos (now at the bottom of the tube) to a 1.5ml centrifuge tube (you ultimately want the final resting place of the embryos to be in a tube that is compatible with the 'Pellet Pestle' homogenizers. If you plan to collect embryos in bulk, use one of those tubes now. If you plan to sort the embryos, use one of those tubes to collect the sorted embryos). Allow embryos to settle to the bottom. They will probably be medium-clumpy. This is OK.
13) Remove as much supernatant as possible without losing any embryos.
14) **At 15' after the start of fixation** (Step 8), add ~1ml of PBS/0.5% Triton X-100/125mM Glycine to the embryos in order to quench the crosslinking reaction. Vortex for 30 seconds on high. Incubate for at least one minute.
15) Transfer embryos to ice. Wash embryos 3x in ~1ml of ice-cold PBS/0.5% Triton X-100.
16) At this point, if embryos are to be ChIPped in bulk, then they can either be used right away or be stored at -80°C (after removing as much supernatant as possible) until needed. If embryos are to be sorted[2], this must be done before freezing.

[1] This is the downside to including a nonionic detergent in the heptane/PBS/formaldehyde solution. However, I have found that this is essential to getting reproducibly high degrees of crosslinking from batch to batch.

[2] Embryos can easily be sorted on the basis of morphology or for a (bright) fluorescent marker by placing them in a 35mm petri dish lined with agarose (PBS + 1% agarose) containing PBS/0.5% Triton X-100. I prefer to use a hair-loop at this point to push embryos around. Embryos can be kept on ice for an entire day and sorting can be done all at once at the end of the day, if desired.

Chromatin Immunoprecipitation (Day 1):

Once all the samples have been collected, they will be thawed, homogenized, sonicated, and immunoprecipitated. This part of the protocol takes approximately 1h to perform followed by an overnight incubation with the primary antibody. Remember to include a negative control, which should be pre-immune serum or normal IgG derived from the host species for the primary antibod(y/ies) to be used in the experimental IPs. Without any prior empirical determination of how much antiserum to use, 2μl of a ~1μg/μl solution of antibody is as good a starting place as any. Ultimately, controls should be performed to ensure that the IPs are being performed under conditions of antibody excess. With certain targets (such as pan Histone H3), this can be difficult (depending on the particular antiserum). Some folks just use beads (without IgG) as a negative control. While this may yield nice low backgrounds, it is desirable to actually measure the nonspecific binding attributable to IgG, so avoid just doing the 'beads only' control.

Materials:

- RIPA Buffer (Sigma R0278, or can be made up from scratch), stored at 4°C
- Protease Inhibitor Cocktail (Sigma P8340)
- 1M Dithiothreitol (DTT, Sigma 43816)
- Phosphatase Inhibitor Cocktail II (Sigma P5726)
- Phosphatase Inhibitor Cocktail III (Sigma P0044)
- Pellet Pestles (Kontes)
- Sonicator (We have a Branson Digital 450 with a ¼ inch microtip horn)
- Low-retention 1.5 ml microcentrifuge tubes
- Protein G Dynabeads (Invitrogen 100-04D)
- Magnetic Separation Stand
- PBS/ 0.1% Triton X-100/ 3% BSA
- Primary antibodies

Procedure:

1) Everything in this section, unless otherwise stated, is performed on ice.
2) Prepare RIPA buffer: a solution of 1x RIPA, Protease Inhibitor Cocktail (1:100), and DTT (1mM). If the target antigens are phosphoproteins, include 1:100 dilutions of each of the Phosphatase Inhibitor Cocktails.
3) Get each sample to be processed out of the -80°C freezer. Place on ice and immediately add 700μl RIPA. Alternatively, if the embryos for a single sample are spread out in several tubes, subdivide the 700μl volume into however many tubes there are and add it to each tube at the same time.
4) Homogenize the samples using a pellet pestle. The embryos will be reluctant to become homogenized so just do the best that you can.
5) If each sample is spread out in different tubes, collect them all in one tube (you should have about 700μl total at the end).

6) Spin at full speed, 4°C for 5 minutes.
7) Following the spin, the pellet contains crosslinked materials including chromatin. Discard the supernatant and add 700μl fresh RIPA buffer.
8) Resuspend the pellet with a pellet pestle. Try to homogenize the solution as much as possible. It will still have a 'chunky' appearance but should be relatively uniform.
9) Sonicate the sample:
    a. Fill a small plastic beaker with ice.
    b. Place one of your sample tubes in the beaker
    c. Immerse the sonicator horn into the sample
    d. Sonicate for 20 seconds at 20% Output, full duty cycle
    e. Return the sample to the ice bucket, wait at least 30 seconds before repeating
    f. Repeat for a total of 7 rounds of sonication[3].
10) Following sonication, spin the samples for 5 minutes at full speed (4°C).
11) During the spin, for each of the IPs to be performed, block an aliquot of dynabeads: In a low-retention tube, add 30μl of a thoroughly resuspended bead slurry (use a wide-bore pipette tip). Place the tube in the magnetic separation stand, wait ~1minute, remove the storage solution, and replace with 1ml PBS/ 0.1% Triton X-100/ 3% BSA. Incubate on a nutator in the cold room overnight.
12) Following the spin, recover the supernatant, which will now contain the sheared chromatin sample. Note the volume of supernatant recovered. Transfer this to a new tube (on ice). Discard the pellet.
13) Determine the volume of sheared chromatin to be used per IP. Following shearing, there is typically 650μl of solution in the tube. If, for example, three IPs are to be performed, 215μl of sheared chromatin will be used per IP (and 4.3 will be used for the Input control). Remember to account for the Input control.
14) Prepare input samples: To 100μl elution buffer, add a volume of sheared chromatin equivalent to 2% of the volume to be used per IP (calculated above). The tube containing this mixture can be stored at -80°C for now.

[3] The number of sonication rounds is determined empirically. These conditions have been optimized to yield >90% of the total recovered DNA with a size of 1kb or less on our particular sonicator. The average size is ~350bp. Altering the crosslinking time, the concentration of formaldehyde, the sonication power, or the number of rounds of sonication will change this distribution. In addition, using a different sonicator (such as a bath-type sonicator) will likely yield different results. I cannot stress enough the importance of getting this part right. See the appendix for notes on how to check the extent of sonication by agarose gel electrophoresis.

15) Prepare IP samples: in a low retention 1.5ml microcentrifuge tube, transfer the per-IP volume of sheared chromatin and add fresh RIPA buffer to make a final volume of 600μl. Add an appropriate amount of IP antibody to each tube.
16) Incubate IP samples overnight on a nutator at 4°C.

(Day 2):

The antibody complexes that have formed overnight will be immunoprecipitated with blocked Protein G Dynabeads for an hour, followed by several washes, and purified chromatin is eluted from the beads. The purified chromatin will be RNase-treated, proteins digested, and crosslinks reversed.

Materials:

- Blocked Protein G Dynabeads (prepared on Day 1)
- Magnetic Separation Stand
- Wash Buffer I: 20mM Tris-HCl pH8.0, 0.1% SDS, 1% Triton X-100, 2mM EDTA, 150mM NaCl
- Wash Buffer II: Wash Buffer I with 500mM NaCl (final)
- Wash Buffer IV (TE Buffer): 10mM Tris-HCl pH8.0, 1mM EDTA
- Elution Buffer: 50mM Tris-HCl pH8.0, 10mM EDTA, 1% SDS
- RNaseA (25 mg/ml stock)
- 20mg/ml Proteinase K
- Tupperware: capable of holding a rack with 1.5 ml tubes and sealing
- Hybridization Oven capable of doing 65°C

Procedure:

1) Spin the overnight-incubated antibody/chromatin mixtures, full speed 4°C for 5'.
2) In the meantime, collect the tubes with blocked Protein G Dynabeads and load them in the magnetic separation stand. This can be at room temperature.
3) Once the spin is complete, examine the tubes for any evidence of a pellet. Sometimes, some material can precipitate out from the samples. It is probably a good idea to avoid carrying over this material to subsequent steps.
4) Once the magnetic beads have aggregated on the wall of the tube, remove the blocking solution.
5) Transfer the supernatants from the antibody/chromatin mix tubes to the tubes with the magnetic beads (label appropriately).
6) Incubate at 4°C for 1h on a nutator.
7) Following the bead incubation, the samples will be washed a total of five times. Each wash consists of:
    a. Do a quick (~1 sec) spin on a small tabletop centrifuge to get any solution out of the cap (no pellet should form if the spin is quick enough).

b. Place the tubes in the magnetic separation stand and collect beads for ~2 minutes.
c. Discard the supernatant, being careful not to lose any beads.
d. Add 1ml of the appropriate wash buffer.
e. Incubate on a nutator for 3 minutes.

The first two washes use Wash Buffer I, the next two use Wash Buffer II, and the final wash is in Wash Buffer IV (TE) for a total of five washes. More (or less) washes can be used depending on the signal/noise observed.

8) Following the last wash, collect beads on the tube wall using the magnetic separation stand.
9) At this point, thaw the 2% Input sample (made on Day 1), mix it a few times while it is thawing to resuspend any SDS that has come out of solution. Don't do anything to it until after step 15.
10) Discard the supernatant and replace with 100μl of Elution Buffer.
11) Elute purified complexes by incubation in a 65°C water bath for 10 minutes, vortexing the tubes every ~2 minutes to ensure good elution.
12) Spin the tubes at full speed for 1 minute at room temperature.
13) Collect the beads on the tube wall using the magnetic separation stand.
14) Transfer the supernatant to a new 1.5ml centrifuge tube.
15) The rest of the procedure applies to both the IP and the Input samples.
16) Add 3μl RNaseA (25 μg/μl stock) to each tube, mix well, and incubate at 37°C for 30 minutes.
17) Spin tubes briefly at full speed to collect any condensate.
18) Add 5μl of Proteinase K solution to each tube.
19) Place the tubes in a rack in a sealable Tupperware-type vessel. Moisten a paper towel and place it inside. Seal the Tupperware. (This is a makeshift humidified chamber that will minimize sample evaporation during the crosslink reversal / ProK treatment step).
20) Incubate in a 65°C hybridization oven for a minimum of 4h (this can go overnight, if desired).
21) Proceed to DNA Clean up or freeze samples until ready to continue.

DNA Clean Up and routine PCR:

There are several ways to do DNA clean up. Most quickly, samples can be processed directly through a Qiagen PCR Purification kit following the standard protocol with some exceptions: I use 900μl of Buffer PB instead of 500μl for the initial dilution, I wash twice with 750μl Buffer PE, and I elute in 80 μl (for ChIP-PCR) or 50 μl (for ChIP-seq) of Buffer EB after incubating at room temperature for 3 minutes[4].

Alternatively, one can perform a more prolonged DNA extraction, beginning with a standard phenol/chloroform extraction, ethanol precipitation, and finally a pass through the Qiagen kit (standard protocol, without the modifications mentioned above). This is also quite good, but has the drawback of taking several hours and involving phenol. Note that the final step through the Qiagen kit is essential in this case in order to eliminate any residual phenol or ethanol that inevitably survives the initial extraction.

For PCR, I first make two 10-fold dilutions of the Input sample (to yield a set of 2%, 0.2%, and 0.02%). Samples are then analyzed in duplicate by QPCR using SYBR Green detection. I design primers using PrimerBLAST (NCBI) using the default temperature parameters, and limiting product size to be between 50 and 150 base pairs. Each 20μl QPCR reaction contains 2μl of the ChIP product. Primers to single-copy loci typically amplify 2% input between 22 and 26 cycles, the negative control comes up around 32 cycles and the positive results are somewhere in between (or sometimes greater than 2%, in which case: congratulations).

[4] Following the standard PCR clean up protocol results in eluates that will on occasion perform poorly in QPCR. It is suspected that this is due to SDS or guanidine carryover from the buffers. The increased amount of binding buffer and the extra wash were incorporated into the protocol to deal with this and, so far, this seems to be working well. In any case, the problem was worse when the samples were frozen prior to further analysis, so when I prepare sequencing libraries, I usually perform at least the first enzymatic step immediately after elution in order to avoid any potential problems.

Preparation of Illumina Sequencing Libraries:

In the past, I have used the NEBNext ChIP-seq mastermix kit (E6240S (or L)) for preparing libraries for Illumina sequencing. This product is now discontinued by NEB. The replacement is the "NEBNext Ultra II DNA Library Prep Kit for Illumina" (E7645S). For primers, I use the NEBNext Multiplex Oligos for Illumina (E7335S), which come with the adapter and enzyme required to complete the protocol. This new kit is supposed to be more sensitive. Since it is different, I will have to see how well it works compared with my prior experience. One of the obvious differences is that there are no clean up steps in between enzymatic reactions.

The following page contains a useful worksheet for making libraries step-by-step.

Some notes about the making libraries:

1) I make a library out of 75-100% of the ChIP sample, and 0.2% of the input sample (4 μl of the 2% Input sample). This is in the range of 1 ng of input sample. The ChIP samples tend to be in the range of 100-200 pg total, which is a lot less DNA. I am currently testing whether it makes a difference to use a more diluted input sample (0.02 – 0.05 % input) that more closely approximates the amount of DNA in the ChIP samples.

2) Normally, you want to know that the ChIP 'worked' before making the libraries, so unless you are really certain of it, set aside a few microliters of the ChIP sample to perform an initial QPCR verification. Once the experiment becomes routine, I have found it safe to make a library out of the whole ChIP sample. It also remains possible to verify by QPCR that it 'worked' after the library has been made.

3) Multiplexing is good. You don't need 90 million reads per sample unless you have to sequence quite deeply. There's good data in 15-20 million reads. Given that a machine will give ~180 million reads, this means you can multiplex up to 12 samples and get in the range of 15 million reads. I haven't done that many, routinely we multiplex 6-8 samples.

4) You should consult with your sequencing core facility or collaborators about how concentrated the final pooled library samples should be, and whether they require any additional quality control measures. Princeton wants the pooled libraries to be provided in at least a 20 ul volume with a concentration of 3 ng/ul or greater. This protocol is set up to yield under normal circumstances concentrated enough libraries to facilitate pooling 6 samples at a final concentration of 3 ng/ul in 20 ul.

Besides the NEB kit and oligo set, you will need the following additional reagents:

- 10 mM Tris pH 8.0, 10 mM NaCl, sterlie
- Ampure Beads (Beckman Coulter)
- 100% Ethanol
- PCR Strip Tubes
- 100x SYBR Green I
- Additional Q5 Polymerase (purchase an extra vial of the reagent that comes in the kit)
- Real-Time PCR Machine

NEBNext ChIP-seq library preparation worksheet

Version 2, for use with the NEB Ultra II kit

Date: **Description:**

Samples (and IDs):

Volume of Input for Library: Percent of starting sample:

Volume of ChIP Samples for Library:

☐ End Repair

Mix well in a thermocycler tube:

- 50 µl ChIP DNA and TE
- 7 µl Repair Reaction Buffer (green cap)
- 3 µl Repair Enzyme Mix (green cap)

In a thermocycler with a 75°C heated lid:

- 20°C for 30 minutes
- 65°C for 30 minutes
- Hold at 4°C

☐ Adapter Ligation

Dilution of Adapters: 1:25 is good for small number embryo preps

- Make dilutions in 10 mM Tris pH 8.0, 10 mM NaCl

Mix well in the same thermocycler tube from the end-repair step:

- (60 µl End-Repair Reaction product)
- 30 µl Ligation Master Mix (red cap)
- 1 µl Ligation Enhancer (red cap)
- 2.5 µl Diluted Adaptor

In a thermocycler with an unheated lid:

- 20°C for 15 minutes

- + 3 µl USER enzyme

In a thermocycler with a 47°C heated lid:

- 37°C for 15 minutes

Can freeze at -20 overnight at this point

☐ Size Selection (elute in 17µl) || Ampure (volumes adjusted to account for input buffer conditions)

- Add 40 µl Ampure beads to the product of the Adapter ligation reaction.
- 5' room temp incubation
- 5' magnetic separation
- Transfer **supernatant** to a new tube containing 20µl fresh Ampure. Discard old beads.
- 5' room temp incubation
- 5' magnetic separation
- Discard supernatant
- Wash 2x with 200µl fresh 80% EtOH.
- Dry beads until all liquid is gone, but beads are still glossy. Don't over-dry (so that beads are 'cracked').
- Elute by adding 17 µl Qiagen Buffer EB, mix well, collect supernatant.

Can freeze at -20 overnight at this point

Fill in the following table with information from the steps that follow:

Index Primer	Sample ID	*N* additional	Total Cycles

☐ PCR Enrichment (Pre-Amplification):

Mix in a fresh thermocycler tube:

- 15 µl Size-selected sample
- 25 µl NEBNext Ultra II Q5 Master Mix
- 5 µl 10 uM PCR Primer (Universal)
- 5 µl 10 uM PCR Primer (Index)

| mastermix 5.7 µl of each primer, use 10 µl here

Perform PCR with the following Cycling Parameters:

-	1 cycle	30 sec	98° C
-	5 cycles	10 sec	98° C
		75 sec	65° C

Remove tubes from thermocycler and store at 4°C during the next step.

☐ Real-Time PCR Side Reaction:

Mix in a fresh thermocycler tube:

- 5 µl PCR Product from Pre-Amplification Reaction
- 3.66 µl dH2O
- 0.09 µl 100x SYBR Green I
- 0.625 µl 10 uM PCR Primer (Universal)
- 0.625 µl 10 uM PCR Primer (Index)
- 5 µl 2x Q5 Polymerase Mastermix

|| mastermix 73.2 µl of H2O and 1.8 µl of SYBR and use 3.75 µl per reaction.

| and use 1.25 µl of the primer mastermix here

Perform Real Time PCR with the following Cycling Parameters:

-	1 cycle	30 sec	98° C
-	20 cycles	10 sec	98° C
		75 sec	65° C

☐ Complete Library PCR Amplification:

On the basis of the side reaction, determine the number of additional cycles (N) required to complete amplification of each library. Perform N cycles of PCR on each library using the cycling parameters described above. See Appendix for details.

☐ Ampure Clean-Up (0.9x): Repeat if Bioanalyzer reveals significant carryover of self-ligated adapters.

- To the 45 µl of the finished PCR reaction and add 40.5 µl Ampure.
- Mix and incubate for 5 minutes at room temperature
- 5' magnetic separation
- 2x 200µl wash in 80% EtOH
- 10 minutes room temp drying (Don't over-dry.)
- Elute in 20 µl Qiagen Buffer EB.

Post-PCR Analysis of Library Properties

☐ Concentration Measurement: (Qubit)

☐ Bioanalyzer: Make 0.5 – 1.0 ng/ul dilutions of each library in Qiagen Buffer EB

Appendix: Shearing optimization

The first efforts for getting this protocol to work should be focused on producing optimally sheared chromatin. I recommend starting with the fixation conditions described above (15 minutes followed by quenching) using 100 embryo aliquots that are in the ballpark of the stages you want to work with, and trying 3x, 5x, and 7x rounds of sonication for 20 seconds each time. If you want to try a bath type sonicator instead of a horn sonicator, keep in mind that the sonication may take an hour or two (using 20 second rounds followed by 30 seconds rest) since energy transfer is much reduced under these conditions. Under all conditions, care must be taken to ensure that the sample stays as cold as possible during sonication, partly to avoid protein denaturation, but also to preserve the crosslinks. Formaldehyde crosslinks are readily reversed by exposure to high temperatures.

To do this, follow the protocol above from the very beginning through step 10 of "Day 1". Once you collect the supernatants from each of your sheared samples, proceed to step 16 of "Day 2" (ignoring any references to IP or Input samples) and complete the protocol to reverse the crosslinks and extract DNA. You have the choice of either reversing the crosslinks on the whole batch of sheared chromatin, or you could just process 200µl and save the remainder for an actual IP. The only risk in doing that is that the DNA will be too dilute to load, but has the added benefit of letting you do an experiment with the chromatin you prepare. During the DNA extraction, just follow the standard Qiagen PCR purification protocol (i.e. don't worry about the modifications I mention above if you're just going to run a gel).

For running the gel, it is sufficient to load ~500ng of DNA per lane of a 1.5% TAE-Agarose/EtBr Gel. I find that it is useful to use a DNA loading buffer that contains Orange G instead of Bromophenol Blue and Xylene Cyanol. Normally the blue dyes will co-migrate with the most critical parts of the gel, thereby occluding the data. Orange G migrates ahead of anything of interest and is transparent under UV transillumination. An example is provided in Figure 1.

For a semi-quantitative analysis of shearing efficiency (Figure 2), I analyze digital images in ImageJ using it's built in gel analysis functions. By subdividing the lanes according to the molecular weight markers, the fraction of signal within a particular size range (and cumulative distributions) can be calculated easily.

Finally, as an additional verification that this procedure yields samples suitable for ChIP (where binding events can be identified within discrete regions of the genome), I have provided an example of a ChIP for GFP tagged Bcd demonstrating detection of two known binding site clusters near the *hunchback* locus (Figure 3). PCR for regions flanking the known binding sites (plus or minus 1 kb) give signals close to background, which confirms that the distribution of fragment sizes I see by the gel analysis is a close approximation of the degree of shearing in genomic regions of interest.

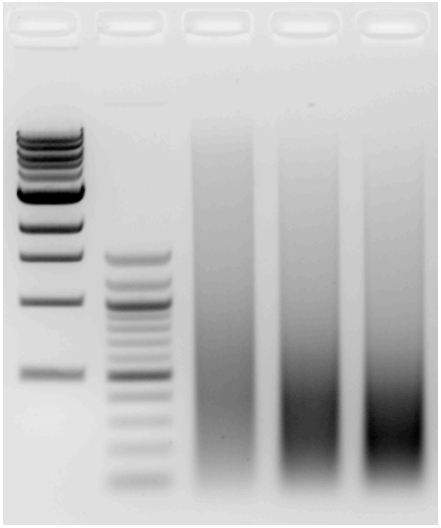

Figure 1: Shearing optimization.
Sheared samples were resolved by 1.5% TAE-Agarose gel electrophoresis. The lanes were loaded with (1) NEB 1kb Ladder; (2) NEB 100bp Ladder; (3) 3x 20 second sonication; (4) 5x 20 second sonication; and (5) 7x 20 second sonication. Approximately 500ng of DNA (as determined by nanodrop following Qiagen Purification) was loaded per lane. For this experiment, the samples were crosslinked for 20 minutes. Ultimately this was determined to be 5 minutes too long (there remains too much high molecular weight material and only ~80% of the sheared DNA in lane 5 is less than 1 kb).

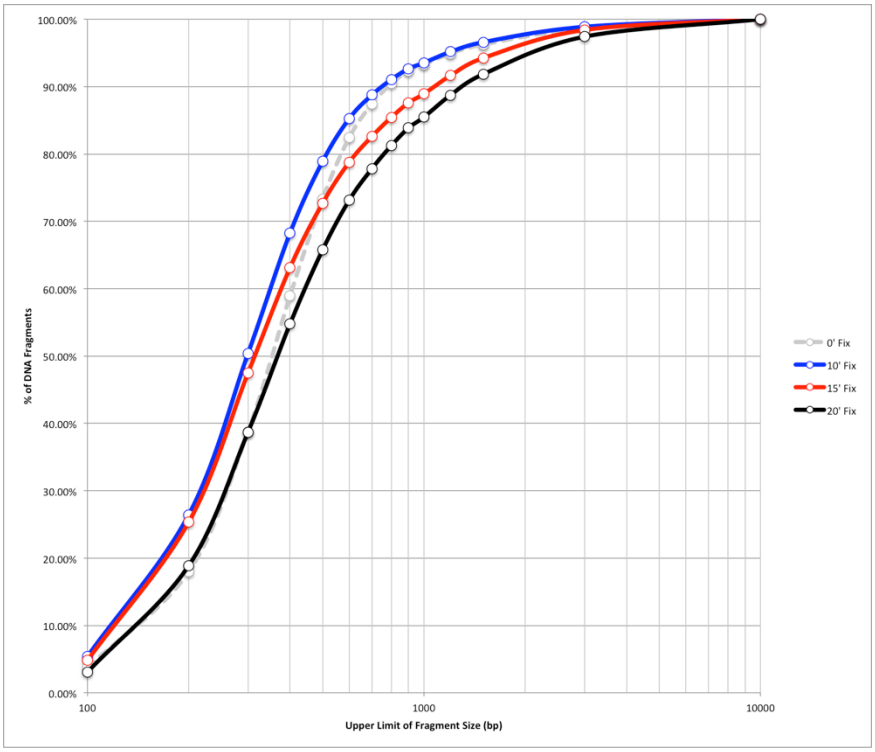

Figure 2: Cumulative Fragment Distributions.
Samples were fixed for 0, 10, 15, and 20 minutes and sheared (7x 20 seconds) before electrophoresis as described for Figure 1. The cumulative distribution of fragment sizes was measured in ImageJ as described in the text and plotted. A 15 minute fixation yields ~90% of fragments with sizes less than or equal to 1 kb, with an average size of approximately 0.3 kb. This distribution is reproducible between multiple independent sample preparations.

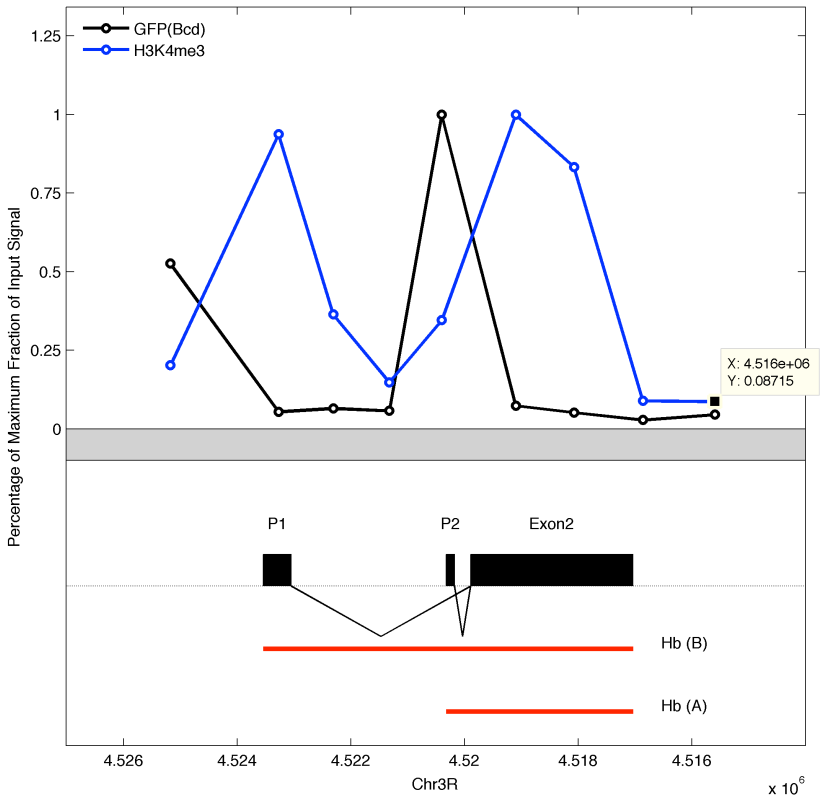

Figure 3: Resolution of discrete transcription factor binding events with at least 1kb resolution
100 Venus-Bcd; *bcd*^{E1} embryos (per IP) were processed according to the protocol and ChIPped for H3K4me3 or GFP(Bcd) (or control IgG) and analyzed by QPCR using primer sets designed at ~1kb intervals along the *Hunchback* locus. The results demonstrate resolution of two known Bcd bound regions by the GFP ChIP (black) and demonstrate enrichment for H3K4me3 within the transcribed regions of the gene. Note that primer pairs flanking the Bcd binding sites (e.g. at P2 exon 1) have little or no positive signal, suggesting that the extent of shearing is sufficient to resolve binding events within (at least)

Appendix: Real Time PCR Side Reaction

This approach is borrowed from the Buenrostro/Greenleaf ATAC-seq sample preparation protocol, and involves doing a small number of pre-amplification PCR steps prior to taking a 5 ul sample for a QPCR “side reaction” to estimate how many total PCR cycles are needed to sufficiently amplify the library. The image below shows the QPCR results following a side reaction. The procedure I have used is, for each curve, to determine the fluorescence level at plateau, divide this number by four, and use this threshold to read off the number of cycles needed to reach that point. Note that the ATAC library procedure divides by three. I adjusted this to prevent over-amplification of the ChIP libraries. For example, the right-most curve plateaus around 70000 units, dividing by four gives 17500, and this would correspond to ~9.7 cycles on the *x*-axis. This value, 9.7, is the *N* number of additional cycles to perform. With respect to rounding: I typically round up only when the *N* value is 0.7 and greater between two integers. From the data below, you can see that these libraries require between 6 and 10 additional cycles of amplification.

In early trials of this protocol, this approach gives from 32 to 126 ng of total DNA in the libraries, which is on the low end of desirable yields. If this is a problem, it is possible to decrease the volume of EB used to resuspend libraries following the final Ampure step (from 20 ul to 15 ul), and this will ensure that libraries will be as concentrated as possible for pooling. Another option would be to divide by three instead of four in the determination of *N*, but keep an eye out for over-amplification artifacts in the bioanalyzer output.