Streamlined Ultra Low Sample Input and Processing Volume Chromatin Shearing Protocols for Fly Embryos and Mammalian Cell Lines

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INTRODUCTION

Rapidly gaining popularity since its inception, Chromatin immunoprecipitation (ChIP) has become the primary method of interrogating the interaction between DNA and protein[1].

Traditional ChIP requires culturing and utilizating large quantities of cells to obtain reliable data. Therefore, investigating the biology of rare cells (e.g. progenitors, early developmental stages) has remained technically challenging despite the very recent adoption of micro-fluidic protocol adaptations. An important characteristic of the ChIP experimental protocol is its modularity. Scientists have begun to modify different aspects of the protocol, which had led to an increase in ChIP sensitivity and a reduction of input material required to apply. [2–5]

In this case study, we present protocols for groups aiming to reduce cell quantities and sample volumes for their ChIP experiments. These protocols have been optimized for both mammalian cell lines and fly embryos. For the chromatin shearing step, the protocols leverage the low sample volume and the high efficiency processing of Covaris E220 Focused-ultrasonicator and microTUBE consumables, which when used together reliably fragment chromatin in low volumes (from 20 to 50 µl) from down to 10,000 mammalian cells and 5 stage-17 Drosophila embryos. Low cell numbers, shearing power, and short shearing time, as compared to typical ChIP protocols, were tested to determine the best method for consistent DNA fragmentation using optimized vessels. Chromatin fragmentation was assessed by loading decrosslinked and RNAse-treated DNA onto an Agilent High Sensitivity DNA chip or a Fragment Analyzer to enrich for DNA fragments ranging in size from 150 to 500 base pairs (bp) for subsequent analysis by next generation sequencing.

This chromatin shearing protocol is scalable and extremely streamlined, enabling batch processing of up to 96 samples for high throughput applications.

MATERIALS AND METHODS:

Instrument & microTUBE:

- Covaris E220 Focused-ultrasonicator
- microTUBE-15 AFA Beads Screw-Cap (PN 520145)
- 8 microTUBE-15 AFA Beads Strip (PN 520159)
- microTUBE-50 AFA Fiber Screw-Cap (PN 520166)
- 8 microTUBE-50 AFA Fiber Strip (PN 520174)
- Rack 24 Place microTUBE Screw-Cap (PN 500308)
- Rack 12 Place 8 microTUBE Strip V2 (PN 500444)

Reagents:

- **Buffer A:**
  - 15 mM HEPES, pH 7.9
  - 60 mM KCl
  - 15 mM NaCl
  - 4 mM $MgCl_2$
- **Buffer A-TX:**
  - Buffer A supplemented with Triton x-100 to 0.1% final
- **Two-phase fixing solution: (Prepare fresh before use, prepare in glass tube)**
  - Methanol free formaldehyde 16% to 1% final in Buffer A
  - Add equal volume of heptane on top
- **Lysis Buffer: (Prepare fresh before use)**
  - 15 mM HEPES, pH 7.9
  - 140 mM NaCl
  - 1 mM EDTA, pH 8
  - 0.5 mM EGTA
  - 1 % Triton x-100
  - 0.5 mM DTT
  - 10 mM sodium butyrate
  - 0.1 % sodium deoxycholate
  - 1 x Protease inhibitor cocktail
- **Fixing Buffer:**
  - 50 mM Hepes-NaOH, pH 7.5
  - 100 mM NaCl
  - 1 mM EDTA, pH 8.0
  - 0.5 mM EGTA, pH 8.0
  - Fixing Buffer with 1% Formaldehyde:
  - 7.5 ml Fixing Buffer 500 µl of 16% formaldehyde
  - note: this solution must be prepared fresh and used immediately
- **2.5 M glycine**
- **Shearing Buffer:**
  - 12 mM Tris-HCl pH 8.0
  - 6 mM EDTA
  - 0.1X PBS
  - 1X Protease inhibitor cocktail (EDTA free)
- **PBS**
- **10% SDS**

MAMALIAN CELL PROTOCOL

Mammalian Cell fixation:

1. Grow 10 cm plate of Human Embryonic Kidney (HEK) 293 T ~ 80% confluency
2. Aspirate media and wash with 10 ml of PBS
3. Add 8 ml of Fixing Buffer supplemented with 1% methanol free formaldehyde
4. Incubate for 5 minutes at room temperature
5. Add 0.5 ml of 2.5 M glycine to each plate for a final concentration 0.125 M
6. Incubate for 5 minutes at room temperature
7. Aspirate media and wash the plate twice with PBS
8. Harvest cells with 5 ml of Shearing buffer using a cell scraper and place into a 15 ml tube kept on ice.
9. Rinse plates with an additional 5 ml of Shearing buffer to get any remaining cells and transfer to the same 15 ml tube
10. After counting and/ or sorting, transfer into a PCR tube and resuspend desired number of cells into Shearing Buffer (9 µl for microTUBE-15 protocol or 22.5 µl for microTUBE-50)
11. Freeze at -80° C or proceed to next step of protocol.

~ Checkpoint: frozen fixed cells

Mammalian chromatin shearing:

12. Thaw cell aliquots in 0.5 ml tubes or PCR strips on ice.
13. Add 10% SDS (1 µl for microTUBE-15 or 2.5 µl for microTUBE-50) to the 0.5 ml tubes to achieve a final concentration of 1 % SDS
14. Re-suspend cells thoroughly with a pipette and incubate for 15 minutes at room temperature.
15. Transfer samples to a precooled microTUBE on ice.
16. Add appropriate volume (10 µl for microTUBE-15 or 25 µl for microTUBE-50) of shearing buffer to a sample's 0.5 ml tube to harvest any remaining sample. Pipette up and down repeatedly followed by transfer to the corresponding microTUBE. (Adding the buffer in multiple stages allows optimal lyses of cells in 1 % SDS while still bringing down the concentration of SDS to acceptable levels for treatment with Covaris Focused-ultrasonicator.
17. Briefly spin down samples to ensure any liquid on the walls of the tube or any bubbles are removed.
18. Treat samples on a Covaris E220 Focused-ultrasonicator with the following settings:
    - microTUBE-15:
        i. Peak Incident Power (PIP): 18W
        ii. Duty factor (DF): 20%
        iii. Cycles/burst (cpb): 50
        iv. Time: 5 minutes
    - microTUBE-50:
        v. PIP: 75W
        vi. DF: 10%
        vii. cpb: 1000
        viii. Time: 7 minutes
19. Transfer resulting supernatant into PCR strips labeled as chromatin extract.

DNA isolation:

20. Quality control: Whole samples are processed to assess DNA size profile (NOTE: after protocol optimization this step should be skipped).
    - Use an equal volume of MilliQ water to wash the microTUBE, and then transfer the water to the corresponding sample's tube. Equilibrate resulting samples with MilliQ water to a final volume of 100 µl.
    - Incubate samples were with RNAse A to a 50 µg/ml final concentration at 37° C while shaking at 300 rpm on a thermomixer for 30 minutes.
    - Incubate samples overnight with proteinase K to a final concentration of 200 µg/ml at 65°C, while shaking at 300 rpm on a thermomixer.
    - Clean samples up with a phenol/chloroform DNA extraction protocol or a PCR cleanup kit from QIAGEN. Final elution with 10 µl of MilliQ water.
    - Determine sample concentration using the High Sensitivity ds DNA Qubit™ reagents according to manufacturer instructions.
    - Run samples on a High Sensitivity DNA Agilent Bio analyzer chip or a Fragment Analyzer (for samples requiring higher sensitivity) and assess DNA fragments size distribution. If longer fragments are desired, a shorter treatment time should be used from chromatin shearing. If shorter fragments are desired, a longer treatment time should be used.
21. Continue to standard immunoprecipitation protocol.

DROSOPHILA EMBRYO PROTOCOL

This protocol is adapted from Löser et al [6]

Embryo collection:

1. Collect stage 17 embryo (~100,000 cells) by incubating male and female flies in egg laying cages for 2 days at 25° C and 70% humidity. After the flies are habituated, change the apple juice plates supplemented with a pea sized amount of yeast in the center. Remove the plate after 30 minutes and incubate for 16 hours at 25° C.

Embryo fixation and dechorionation:

2. Prepare 25 ml of Buffer A-Tx.
3. Prepare fresh 8 ml two-phase cross-linking solution in a screw-cap glass tube (less adhesive to embryos than plastic tubes).
4. Remove any dead flies and excess yeast from the plates. Add 50% bleach directly onto the plate and observe the embryos under a stereomicroscope until you see dechorionation (approximately 2 minutes). Immediately stop dechorionation by rinsing plate with water.
5. Collect the dechorionated embryos on a sieve while actively rinsing the plate and the embryos with water. Wash the embryos extensively in the sieve to remove any remaining bleach. Blot the sieve briefly on a paper towel.
6. Immediately transfer the embryos from the sieve to the heptane

phase of the cross-linking solution with a soft brush soaked in heptane. The embryos should be at the interphase of the two-phase solution. Cap the tube and shake vigorously by hand and place horizontally (with the aid of tape) on a platform shaker for 15 minutes at 250 to 300 rpm at room temperature (make sure liquid is mixing vigorously).

7. Stop the cross-linking by adding glycine to the solution at a final concentration of 0.25 M to quench the cross-linking of formaldehyde. Incubate for 5 minutes while rotating at room temperature.
8. Remove the two-phase cross-linking solution using a glass Pasteur pipette starting with the lower aqueous phase while removing any sinking embryos, followed by the heptane phase (ideally most embryos will be at the interphase, these are the embryos with the optimally fixed chromatin).
9. Wash embryos with 10 ml of buffer A-Tx to the embryos. Incubate for 5 minutes while rotating at low speed to avoid foaming. After 5 minutes, place the tubes upright, the embryos will settle to the bottom of the tube making removing the buffer easier.
10. Repeat wash with 10 ml Buffer A-Tx.
11. Remove supernatant and collect embryos in a precooled Eppendorf tube.
12. Count desired number of embryos to place in individual 0.5 ml tubes under a stereo microscope, while removing unfertilized or undesired stages of embryos.
13. Tubes with desired number of fixed dechorinated embryos can be stored at –80° C until needed or continue to the next step of the protocol.

~ Checkpoint: fixed frozen embryos

14. Prepare 1 ml Lysis buffer basic and place on ice.
15. Resuspend embryos in (20 µl for 15 microTUBE or 50 µl for 50 microTUBE) Lysis buffer basic.

Rupturing the Drosophila embryos:

16. Samples are then treated on a Covaris E220 Focused-ultrasonicator with the following settings to remove the vitelline membrane:
    - microTUBE-15:
        i. PIP: 36 W
        ii. DF: 10 %
        iii. cpb: 50
        iv. Time: Needs to be actively monitored, as it varied depending on number of embryos being sheared.
            - 1 Embryo needed 1 minute to lyse
            - 5 Embryos 3 minutes
            - 10 embryos 6 minutes
    - microTUBE-50:
        v. PIP: 75 W
        vi. DF: 10 %
        vii. cpb: 1000
        viii. Time: 1 minute
    - Important to note that while the vitelline membrane has been broken releasing all the cells into the buffer. The debris is still in the tube and needs to be removed for later steps in immunoprecipitation processing.
17. Lyse the cells by supplementing each tube with SDS and N-Laurylsarcosine to a final concentration of 0.5 %, and incubating samples on ice for 30 to 60 minutes.

Drosophila embryo chromatin shearing:

18. Samples treated on Covaris E220 Focused-ultrasonicator according to the following program:
    - microTUBE-15:
        i. PIP: 18 W
        ii. DF: 20 %
        iii. cpb: 50
        iv. Time: 5 minutes
    - microTUBE-50:
        v. PIP: 75 W
        vi. DF: 10 %
        vii. cpb: 1000
        viii. Time: 8 minutes
19. To remove any remaining debris, samples were centrifuged for 10 minutes at 10,000g at 4° C. Transfer the supernatant to a new tube labeled as chromatin extract.
20. Chromatin can be stored at -80° C until needed, preferably not for periods longer than 1-2 weeks.

~Checkpoint: frozen chromatin extracts (CE)

21. Quality control of chromatin done as described previously for HEK 293T cells.

RESULTS AND DISCUSSION

Results:

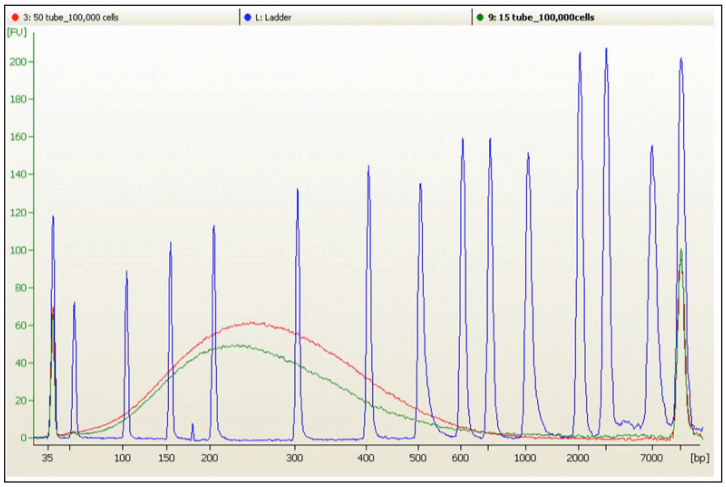

FIGURE1: microTUBE-15 (green) and microTUBE-50 (red) chromatin shearing validation with mammalian cells.

Equal numbers of cells were lysed and the chromatin sheared under identical fixation conditions with microTUBE-50 AFA Fiber and microTUBE-15 AFA Beads. A similar shearing profile was observed with both types of microTUBEs with the majority of chromatin sheared to a range of 200 to 250 bp. The blue peaks correspond to the Agilent High Sensitivity DNA ladder.

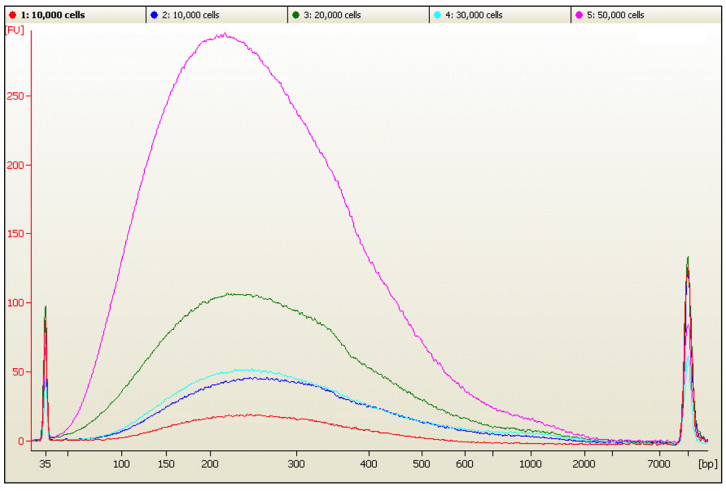

FIGURE2: Leveraging the low volume capacity of microTUBE-15 to reliably shear chromatin for a range of cell quantities.

We observed a proportional stepwise reduction in the area under the curve with the number of cells in the samples. We did observe variability in the concentration of DNA extracted from one of the two samples with 10,000 cells. Nevertheless, the DNA size profile was consistent with all samples, irrespective of the number of cells processed, indicating consistent shearing across samples.

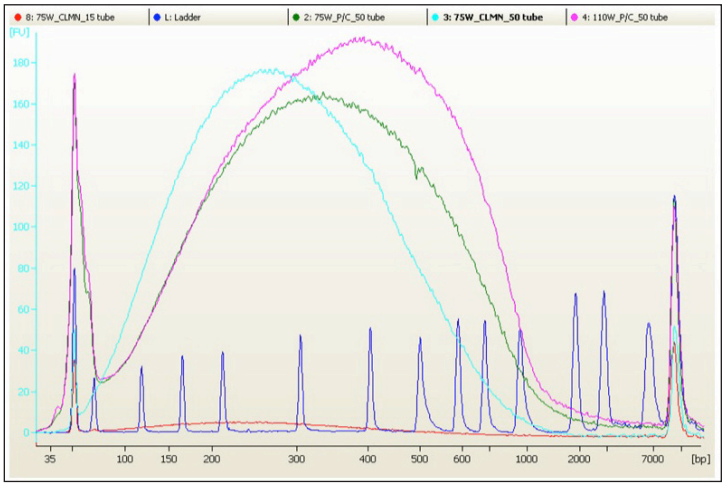

FIGURE3: Validation of Drosophila embryo rupturing, chromatin shearing of ten embryos, and purification method with microTUBE-15 (red) and microTUBE-50 (green, purple and light blue).

microTUBE-15 (red) and microTUBE-50 (light blue) were used to rupture the embryos and shear the chromatin, resulting in similar DNA size profiles. However, significant differences were observed in the concentration of extracted DNA as indicated by the areas under the curves. The data indicates that the microTUBE-50 AFA Fiber is more efficient in embryo rupturing leading to a higher number of released cells. Confirmation of efficient embryo rupture was obtained by visualizing samples after embryo rupture with DAPI under a confocal microscope.

DNA extraction using both Phenol/Chloroform (P/C) or QIAGEN minElute® columns (CLMN) resulted in consistent chromatin profiles. Extracting DNA after de-crosslinking the chromatin with either standard phenol/chloroform (green) or with the QIAGEN PCR purification kit (light blue) showed no significant change in chromatin size profile. However, the concentration of DNA obtained was different, likely due to the columns' cutoff below 70 bp and above 10,000 bp.

Different embryo rupturing protocols utilizing either 75W (green) and 110W (purple) Peak Incidence Power (PIP) resulted in similar sheared chromatin profiles, indicating that chromatin shearing is reproducible regardless of embryo rupturing. The blue peaks correspond to the Agilent High Sensitivity DNA ladder.

Altogether, the results show reliable chromatin shearing regardless of DNA extraction method, embryo rupturing program, and purified DNA concentration.

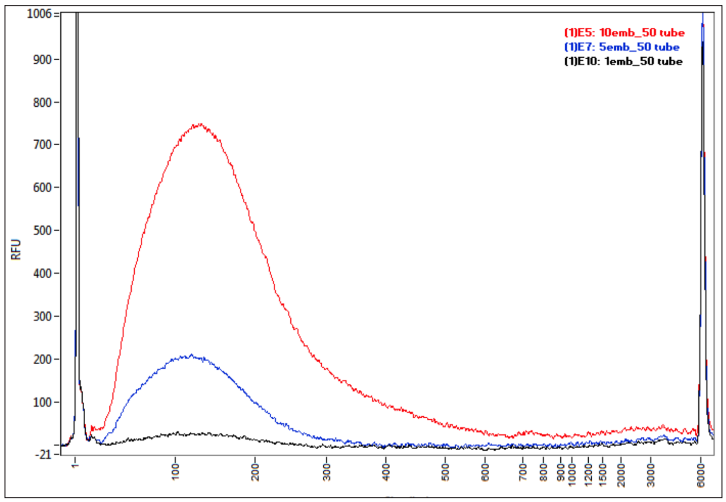

FIGURE4: Consistent chromatin shearing of 1, 5, and 10 embryos using the microTUBE-50.

Samples were run on a Fragment Analyzer, as a higher sensitivity assay was required to detect the DNA profiles. We observe significant enrichment of sheared chromatin fragments larger than 100 bp with as low as 5 embryos. An appropriately sized curve was also visible in the 1 embryo sample, considering the low amount of DNA and high sample to sample variability at this level of biological input. We advise caution and additional optimization if planning experiments with such low sample quantities.

DISCUSSION:

Covaris Focused-ultrasonicators used in conjunction with Covaris microTUBE-15 and microTUBE-50 consumables provide a reliable tool for the fragmentation of chromatin using very low cell numbers. We utilized the HEK cell line and stage 17 Drosophila embryos as our model systems and optimized two separate protocols. Concentration of fixative and fixation time were kept consistent across all samples within each model system, as it can influence the sheared chromatin profile.

We applied this protocol on what can be considered very low cell and embryo quantities. We show ChIP-seq quality DNA fragment profiles of sheared samples down to 10,000 mammalian cells and 5 stage-17 Drosophila embryos, and have seen positive results below these numbers (albeit with higher sample to sample variability). Being able to perform the entire protocol within a single vessel reduced the potential loss of material due to sample transfer between steps, enabling even further reduction in sample input.

In summary, we have shown consistent fragmentation of chromatin in the mammalian system, in both microTUBE-15 and microTUBE-50. Due to the insufficient rupture of the vitelline membrane of embryos in the microTUBE-15, we would recommend the use of microTUBE-50 for the most reliable fly embryo ChIP preparation. That being said, we are optimistic that with sufficient effort, a reliable protocol in the microTUBE-15 is achievable.

The protocols provided in this applications note, are a good starting point for other labs aiming to develop and optimize reduced cell and volume chromatin shearing protocols for their ChIP experiments.

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Note from Covaris: The Covaris E220 Focused-ultrasonicator provides a high throughput automated method to sequentially treat up to 96 samples. Due to the focused ultrasonic energy delivered to the sample, treatment are identical for all samples, ensuring highly reproducible results across a complete 96 samples plate.

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