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# A semiautomated ChIP-Seq procedure for Large-scale Epigenetic Studies

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Cover Letter

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Benjamin Werth
JoVE

To the Editors:

We are submitting a manuscript entitled "*A robust, sensitive and automated ChIP-Seq procedure for Large-scale Epigenetic Studies" by Justin Cayford, Sara Herrera-de la Mata, Benjamin Joachim Schmiedel, Vivek Chandra, Pandurangan Vijayanand, and Grégory Seumois.*

All of the authors agree with this submission, no conflicts of interest.

We present here a semiautomated, microscaled ChIP-Seq protocol that can be readily used for large-scale epigenetic studies. This paper describes the procedure for chromatin immunoprecipitation of DNA regions associated with specific histone modification (H3K27ac) using the IP-Star platform followed by library preparation using tagmentation. The procedure includes quality control assessment for quality and quantity purposes.

This paper describes a full procedure from call to sequencing reads organized into 9 modules. Each section has very critical steps that would benefit from video displays. ChIPseq has always been an challenging procedure because of technical details, this procedure shows that once the steps are well validated, ChIP-seq can be applied to hundreds of samples.

We believe that the approach contained herein, will be of great interest to your readers, especially those who plan to conduct large scale translational epigenetic studies in relation with health or diseases.

Sincerely yours,

Grégory Seumois

**TITLE:**

# A Semiautomated ChIP-Seq Procedure for Large-scale Epigenetic Studies

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**KEYWORDS:**

ChIP-seq, ChIP liquid-handler, tagmentation, H3K27ac, low input, chipmentation, automated

**SUMMARY:**

This paper describes a semiautomated, microscaled ChIP-Seq protocol of DNA regions associated with a specific histone modification (H3K27ac) using a ChIP liquid-handler platform, followed by library preparation using tagmentation. The procedure includes control assessment for quality and quantity purposes and can be adopted to other histone modifications or transcription factors.

**ABSTRACT:**

Chromatin immunoprecipitation followed by sequencing (ChIP-Seq) is a powerful and widely used approach to profile chromatin DNA associated with specific histone modifications, such as H3K27ac, to help identify cis-regulatory DNA elements. The manual process to complete a ChIP-Seq is labor intensive, technically challenging, and often requires large-cell numbers (>100,000 cells). The method described here helps to overcome those challenges. A complete semiautomated, microscaled H3K27ac ChIP-Seq procedure including cell fixation, chromatin shearing, immunoprecipitation, and sequencing library preparation, for batch of 48 samples for cell number inputs less than 100,000 cells is described in detail. The semiautonomous platform reduces technical variability, improves signal-to-noise ratios, and drastically reduces labor. The system can thereby reduce costs by allowing for reduced reaction volumes, limiting the number of expensive reagents such as enzymes, magnetic beads, antibodies, and hands-on time required. These improvements to the ChIP-Seq method suit perfectly for large-scale epigenetic studies of clinical samples with limited cell numbers in a highly reproducible manner.

**INTRODUCTION:**

The wide use of ChIP-Seq assays for determining fragments of DNA associated with specific histone modifications is in part due to its ability to identify cis-regulatory DNA elements, including active enhancers, promoters, silencers, heterochromatin, and others[1-4]. Identification of non-coding regulatory regions across the genome has shown valuable insight to better understand gene regulation in health and diseases[4]. Previous work from the lab has used ChIP-Seq to show that cis-regulatory elements can play important roles in different cell types[5]. Transcription factor (TF) ChIP assays has been utilized to show disease associated risk single-nucleotide polymorphisms[6].

The use of ChIP-Seq with human clinical samples is challenging, mainly due to the limitation of cell numbers or the desired tissue sample. As a result, there has been a concerted effort in the field to improve and microscale these techniques and as a result, several assays have emerged, such as CUT&TAG[5,7-12]. This assay utilizes a transposase to tagment and isolate genomic regions bound by a specific antibody[9]. This technique has been able to reduce the cell numbers down to 1,000s and in some cases to a single-cell, however, the use of this technique in translational research and clinical set-up has shown limitations due to the requirements of using live cells for this method[9,12]. The live cell requirement makes clinical samples logistically difficult to handle and can introduce batch effects if the samples are not processed at the same time. Others have optimized microscaled techniques for formaldehyde-fixed cells, including the development of ChIPmentation[11], which is adapted here in a high-throughput manner. The use of fixed cells allows samples to be stored until collection and subsequent processing of all samples together to minimize batch effects.

Here, a semiautomated microscaled ChIP-Seq assay is described which reduces experimental hands-on time to profile histone modifications[10]. The semiautomated method allows for high-throughput ChIP-Seq assays, allowing for up to 48 samples to be fully processed and ready for sequencing in as little as 5 days, for as few as 10,000 cells per sample using a ChIP liquid-handler. The handler completes the immunoprecipitation (IP) and subsequent washes in an autonomous manner, which helps to reduce variability between samples. The semiautomated method lowers both the hands-on time by over 15 h for 48 samples and the technical variability, enabling large-scale epigenetic studies to be conducted in a reproducible and rapid manner for either primary or cultured cells. The protocol explains the process from start to finish for high quality ChIP-Seq. If the specific machines are not available, the protocol will still be a useful resource to set up and trouble-shoot ChIP-Seq experiments manually.

The assay was performed with three different primary human immune cell types and one cultured cell line (HUT78 – ATCC: TIB-161). For clarity, the protocol has been divided into seven sections: cell fixation, chromatin shearing via sonication, automated chromatin immunoprecipitation, library preparation by DNA fragment tagmentation, library amplification, library purification, followed by DNA quantification. For buffer recipes please refer to **Supplementary Table 1.**

**PROTOCOL:**

The Institutional Review Board (IRB) of the La Jolla Institute for Allergy and Immunology (LJI; IRB protocol no. SGE-121-0714) approved the study. Healthy volunteers were recruited and provided leukapheresis samples after written informed consent.

## 1. Cell fixation

1.1. Bring the cell suspension concentration to 1 to 2 x $10^6$ cells/mL with complete cell culture medium in a 15 mL tube (<10 mL of suspension) or 50 mL tube (10-30 mL of cells). If <1 x $10^6$ cells, use 0.5 mL of the medium in a 1.5 mL tube.

1.2. Gently vortex the cell suspension, add 10x cell fixation buffer dropwise (1:10; vol:vol) and rotate at a low speed for 10 min at room temperature (RT).

1.3. Stop the reaction by gently vortexing and add 2.5 M glycine in the ratio of 1:20 (vol:vol). Invert the tubes a few times and incubate on ice for at least 5 min.

1.4. Perform the remaining steps at 4 °C or on ice. Spin the tubes at 800 *x g* for 5 min at 4 °C and discard the supernatant.

1.5. Resuspend the pellet gently with 5 mL of ice-cold 1x PBS and incubate for 2 min on ice.

1.6. Repeat 1.4 and 1.5 with 1 mL of ice-cold 1x PBS and transfer the sample to a precooled 1.5 mL tube (labeled for long-term storage). If applicable, preparation of aliquots is recommended.

1.7. Spin the tubes at 1,200 *x g* at 4 °C and remove as much of the supernatant as possible without disturbing the cell pellet. Snap freeze the pellet in liquid nitrogen. Store at -80 ˚C.

CAUTION: Take appropriate protection when handling liquid nitrogen.

## 2. Chromatin shearing

NOTE: This protocol is optimized for the chromatin shearing of pellets with 0.3 to 3 x $10^6$ cells in 0.65 mL low binding tubes.

2.1. Remove the samples tube with frozen cell pellets from -80 ˚C and store on dry ice to avoid any thawing of the pellet prior to adding the lysis buffer. This step is critical.

2.2. Add 70 µL of fresh, RT complete lysis buffer to the pellet and keep at RT for 1 min.

2.3. Resuspend the pellet for 1 min without the introduction of any bubbles and then incubate the cell suspension at RT for 1 min before putting the sample on ice.

2.4. Transfer the resuspended pellet into a 0.65 mL low binding tube and keep on ice.

NOTE: To obtain reproducible sonication, pre-warm the sonicator by running it with only blank tubes for 3-6 cycles prior to sonicating the samples.

2.5. Place the samples into the tube holder of the sonicator and fill any gaps with balance tubes filled with 70 µL of water. Leave the samples in the water bath for about 1 min before starting the sonication.

2.6. Perform sonication for *x cycles* (depending on the cell type) with 16 s ON / 32 s OFF per cycle.

NOTE: This step will require validation experiments to determine the optimal number of cycles for efficient sonication.

2.7. After every 3 cycles, remove the samples from the sonicator, gently vortex and pulse spin the tubes before putting them back into the holder. Ensure there are no small droplets on the outside of the tube as that can cause bubble formation.

2.8. After completing the necessary cycles, spin samples at >14,000 *x g* for 15 min at 4 °C. Transfer the supernatant to a new, pre-cooled, low-binding 0.65 mL low binding tube and keep on ice.

2.9. Decrosslink a fraction of the sonicated samples to check for the sonication efficiency.

2.9.1. Transfer 1-7 µL of the supernatant (equivalent to about 250 ng of sheared chromatin) to a 0.2 mL PCR tube and make the volume up to 10 µL with short-term lysis buffer at RT.

2.9.2. Add 1 µL RNase A and incubate for 30 min at 37 °C at 800 rpm, then add 1 µL proteinase K.

2.9.3. Incubate for 2 h at 55 °C with shaking at 1,000 rpm.

2.10. Remove 2 µL of the decrosslinked sample for DNA quantification using fluorescent quantification assay[10] (a spectrophotometer is not recommended as soap and degraded protein can produce bias in the results).

2.11. Run the remaining sample on a 1.2% agarose gel for 1 h at 70 V. Stain with nucleic acid dye (1:20,000) and read the gel using a UV transilluminator.

2.12. Prepare chromatin stock aliquots for storage (dilute the sample to 25 ng/µL in 20 µL with complete lysis buffer). Store all sheared chromatin at -80 ˚C.

## 3. Automated ChIP-Seq for histone modification

NOTE: This protocol is designed to run on a ChIP liquid-handler. Although the system can use customized buffers, all buffers are provided with the ChIP kit. The ChIP strips with 8 tubes used in this section are specific to the ChIP liquid-handler.

3.1. Transfer 16 sample aliquots with 500 ng of sheared chromatin in 20 µL from -80 ˚C and place them on ice to slowly thaw the chromatin. Once thawed fully, vortex briefly and pulse-spin.

3.2. Preparation of chromatin

3.2.1. Pipette 100 µL of tC1 buffer supplemented with 1x protease inhibitor and 20 mM sodium butyrate (complete tC1 buffer) into two ChIP 8-tube strips.

3.2.2. Transfer 20 µL of each chromatin sample into an appropriate tube of the ChIP 8-tube strips containing the 100 µL of complete tC1 buffer. Wash the chromatin tubes by adding 80 µL complete tC1 buffer to the chromatin tubes and then transfer back into the appropriate tube of the ChIP 8-tube strips for a final volume of 200 µL.

3.3. Preparation of the antibody

3.3.1. Calculate the volume of antibody such that 0.5 µg of antibody is in each tube.

$$Volume\ of\ antibody = (number\ of\ samples\ x\ antibody\ per\ reaction) / antibody\ concentration).$$

3.3.2. Add the calculated amount of antibody into 500 µL of tBW1 buffer. Quickly vortex and pulse-spin.

3.3.3. Pipette 70 µL of tBW1 into each of the two ChIP 8-tube strips and add 30 µL of the antibody + tBW1 to each of the tubes. This will bring the total volume in each of the tubes to 100 µL.

3.4. Preparation of the magnetic bead

3.4.1. Vortex the protein A bead solution thoroughly. For 0.5 µg of antibody, pipette 5 µL of beads into a new set of ChIP 8-tube strips and pulse spin.

3.5. Fill the last row of the ChIP liquid-handler with labeled, empty ChIP 8-tube strips.

3.6. Follow the *ChIP-16-IPure-200D* program specifications for the placement of all the strips in the ChIP liquid-handler machine. Add the buffers in the correct position but use tW4 instead of tE1 buffer.

NOTE: Organize the day such that the ChIP liquid-handler will perform the ChIP overnight. The program will run for about 16 h for 16 samples. This marks the end of Day 1.

## 4. Transposase integration of library adaptors for library preparation

4.1. Pre-set a thermomixer to 37 °C and 500 rpm. Cool down a magnet for 0.2 mL tube strips on ice.

4.2. For 16 samples, prepare 440 µL of tagmentation buffer on ice. Pipette 53 µL into a single new 8-tube strip and keep on ice.

4.3. In a new 0.2 mL 8-tube strip, add 220 µL of cold tC1 buffer and keep on ice. The 8 strip tubes can hold this volume and still be capped.

4.4. Remove the "IP samples" strip tube from the ChIP liquid-handler machine (row 12) and cap the tubes prior to pulse-spinning. Capture the beads using the magnet for 8-tube strips for 2 min and carefully remove the supernatant.

4.5. Transfer 25 µL of the tagmentation buffer to the beads with a multi-channel, remove from the magnet, and mix gently until the beads are homogenous (about 5 times up and down with the pipette set to 20 µL).

4.6. Cap the tubes and place into the pre-heated thermomixer and incubate for 3 min. Increasing the time will decrease the efficiency of the library preparation.

4.7. Transfer the tubes to a chilled metal rack and add 100 µL chilled tC1 buffer to each sample. Set a multi-channel pipette to 80 µL and mix the sample until the beads are homogenous, stopping the tagmentation reaction.

4.8. Place the samples back into the ChIP liquid-handler and proceed with the wash procedure *Washing_for_IP-reacts_16_Ipure*. Ensure the washing is performed twice with tC1 buffer and twice with tW4. The elution should be completed as marked by the program layout, with buffer tE1.

4.9. Decrosslinking of the DNA

4.9.1. Remove the ChIP 8-tube strips in the last row of the ChIP liquid-handler and add 2 µL RNase A to each sample.

4.9.2. Cap the tubes, pulse-spin, gently mix the beads with a multichannel pipette until the mixture is homogenous, and re-cap the tubes.

4.9.3. Incubate the samples in a thermomixer for 30 mins at 37 °C and 900 rpm.

4.9.4. Remove the samples from the thermomixer, add 2 µL of Proteinase K. Follow the same procedure as 4.9.2 after the addition.

4.9.5. Incubate the samples in a thermomixer for 4 h at 55 °C and 1,250 rpm, followed by 65 °C at 1,000 rpm overnight.

NOTE: This is the end of Day 2.

## 5. Tagmented DNA fragments purification

5.1. Label sixteen 1.5 mL tubes with the appropriate sample number and add 400 µL of DNA binding buffer from the DNA clean-up kit to each.

5.2. Remove the 8-tube strips from the thermomixer and pulse-spin the strips to ensure any evaporated product is retained. Place strips on an 8-strip magnet to capture the beads.

5.3. Transfer 100 µL of decrosslinked DNA into each of the 1.5 mL tubes. Add 100 µL of the DNA binding buffer to the 8-tube strips to wash the beads and then transfer to the appropriate 1.5 mL tube.

5.4. Vortex for about 10 s and pulse-spin the 1.5 mL tubes.

5.5. Load the columns with the 600 µL containing the DNA binding buffer and ChIP sample.

5.6. Spin samples for 20 s at 10,000 *x g* and reload the column with the flow-through. Spin again with the same conditions and discard the flow-through.

5.7. Wash the columns twice with 200 µL wash buffer (same centrifugation as the previous step) and discard the flow-through.

5.8. Dry the columns by centrifuging for 2 min at 12,000 *x g*.

5.9. Transfer the column to a new 1.5 mL collection tube and add 9 µL warm TE Buffer (pre-heated to 55 °C) directly to the column matrix. Allow the column to incubate for 1 min before centrifugation for 1 min at 10,000 *x g*.

5.10. Transfer the 9 µL of the elute to an appropriate new set of 8-tube strips.

5.11. Complete the elution again with 8 µL TE Buffer as before. Transfer the elute into the appropriate 8-tube strips (final volume 17 µL per sample) and keep on ice.

## 6. Amplification and size-selection of the purified samples

6.1. The following steps use qPCR to determine the number of cycles required for optimal

amplification (CtD – Ct determination)

6.2. Prepare the CtD mix for all samples by multiplying the contents of the CtD Mix Buffer by the number of samples.

6.3. Dispense 3.6 µL of CtD mix into a qPCR plate and add 1.4 µL of tagmented DNA samples (~10 % of the total volume). Perform the following qPCR: 98 °C for 3 min, 72 °C for 5 min, 98 °C for 30 s, 26 cycles of 98 °C for 10 s, 63 °C for 30 s, and 72 °C for 30 s.

6.4. Prepare the Amp Mix for all samples by multiplying the contents of the AMP Mix Buffer by the number of samples. Dispense 14 µL of tagmented DNA into separate wells of a PCR plate, then add 2.5 µL of two sequencing index primers (25 µM) to each sample (final reaction volume is 50 µL).

6.5. Mix the samples by multichannel pipette and perform the amplification program used in the CtD with the appropriate number of cycles.

NOTE: This is a good stopping point as the amplified samples can be stored at -20 ˚C for a few weeks. However, the purification can be completed on the same day. For 48 samples, the steps 3 - 6.5 were completed with two other separate batches and then amplified in one batch as described below.

6.6. Perform post-amplification, size selection, and quantification of tagmented DNA as described below. This can usually be completed with 48 samples (can be completed with fewer samples as desired).

6.6.1. Add 90 µL of paramagnetic beads (1:1.8 ratio) into each well, mix, and allow it to incubate at RT for 2 min.

6.6.2. Capture the beads using a plate magnet and discard the supernatant. Wash the beads 3 times with 200 µL of fresh 80% ethanol without disrupting the bead pellet.

6.6.3. Remove any excess ethanol with 20 µL tips after final wash and leave the beads to dry for 10 min or until cracks appear in the bead pellets.

6.6.4. With the plate still on the magnet, add 40 µL of the pre-warmed water to each well. Seal the plate, vortex thoroughly, and briefly pulse-spin the plate.

6.6.5. Capture the beads by placing the plate back on the magnet and transfer the 40 µL elute to a new “sample” Plate. The samples are now purified, and the next steps enrich fragments ranging from 200-1,000 bp.

6.6.6. Optional QC step: Remove 4 µL from the samples and transfer to a QC plate. Add 4 µL water back to the samples. This determines the percentage of large fragments.

6.6.7. Add 22 µL of paramagnetic beads (1:0.55 ratio) to the samples, carefully mix, and incubate at RT for 2 min.

6.6.8. Place on magnet to capture the beads for 5 min and transfer the supernatants to columns 7-12 of the "sample" plate. Remove the plate from the magnet and add 30 µL of beads (final ratio of 1:1.3). Mix carefully and allow it to sit at RT for 2 min.

6.6.9. Capture the beads for 5 min and then discard the supernatant.

6.6.10. Wash all beads 3 times with 200 µL fresh 80 % ethanol as described previously (step 6.6.2 – 6.6.3).

6.6.11. Once the pellets are dry, elute DNA with 8 µL pre-warmed TE buffer to each well, while still on the magnet.

6.6.12. Remove the plate from the magnet, seal, and vortex thoroughly. Allow the plate to incubate for 2 min at RT, pulse-spin, and place the plate back on the magnet for 2 min. Transfer the supernatant to a new plate (Plate 2).

6.6.13. For maximum recovery, repeat elution with an extra 8 µL of pre-warmed TE buffer. Place the samples into the appropriate wells such that each sample has 16 µL of final library.

NOTE: At the end of this step there should be two plates (one if no QC plate was completed). The QC plate will have the pre-size-selected fragments and the second plate should have 48 wells of final library (16 µL total).

## 7. Quantify the final libraries and QC samples using a fluorescence assay

7.1. Complete DNA quantification using a fluorescence quantifying assay or a similar method.

7.2. If QC quantification was completed, determine the percentage of loss of sample that were < 1,000 bp. There should be no more than about 20% loss – if there was more, there could be an issue with the applied bead ratios.

7.3. Determine the size of the fragments of each sample, preferably using a capillary electrophoresis machine. To calculate the molar concentration use the following equation: [Library concentration (ng/µL) * $10^6$]/[660 * Median fragment size (bp)]).

NOTE: The libraries are ready to be pooled (equimolar amounts) and sequenced following standard next generation sequencing procedures.

## REPRESENTATIVE RESULTS

As proof of concept, ChIP-Seq was completed for six human donors with three sets of immune cell types: naive CD4 T cells (CD4), classical monocytes (MO) and natural killer cells (NK), enriched by FACS sorting as described before[13]. The underlined procedure consists of nine distinct procedures as represented in **Figure 1**.

[Place **Figure 1** here]

After cell isolation by flow cytometry[13], sorted cells were centrifuged and cells fixed and stored as described above. Once all the samples were collected, the samples were lysed and prepared for chromatic shearing in batches of 12 as described above. For each sample, the number of cycles to reach optimal sonication was completed[10]. Quantitative measurement, as well as sheared chromatin fragment size measurements showed great reproducibility of our method on the three sets of immune cells (**Figure 2A**). The different human immune cells were sonicated in separate batches and yielded very consistently with > 70% of the sample between 100 - 500 bp for 14 cycles (16 s ON, 32 s OFF per cycle). At this point, samples with large fragments after sonication (< 70% of the sample between 100 - 500 bp) were considered as failed. These samples could either sonicated for 1-2 additional cycles or were discarded and replaced later with cells from another pellet. Our method showed none of the samples required more sonication or were eliminated, suggesting absolute robustness of the procedure.

[Place **Figure 2** here].

After quantification, the samples were run on a ChIP liquid-handler with H3K27ac antibodies, followed by tagmentation with Tn5 transposase enzyme. To determine the appropriate number of amplification cycles by qPCR, 10% of tagmented samples were used. For the determination of the number of cycles for the amplification of the samples, we find the cycle at which the intensity of the sample is half the average maximum for cycle determination (**Figure 2B)**. Samples with Ct values of more than 18 did not perform well post sequencing and their Ct value was thus indicative of a failed ChIP sample. These samples generally also yielded a lower amount of DNA after amplification. Samples (100,000 cells input) with a Ct value equal or lesser than 15 were ideal and samples between 15 and 18 were acceptable but less consistent post sequencing. For samples with less than 100,000 input cells, the Ct values were usually found between 15 and 18 but did not need more than 18 cycles to yield enough product for sequencing.

After DNA-tagmented amplification, libraries were purified and size-selected to obtain an ideal size distribution, ranging from 200 to 1,000 bp, for the NextGen sequencing. Size distribution assessment on each of the libraries was completed because best sequencing data were obtained when more than 85% of the DNA fragments ranged between 200 to 1,000 bp (**Figure 2C**). Notably, as the same quantity of DNA (measured by fluorescence quantification) was loaded, it was noticed the samples with lower fluorescence intensity generally sequenced poorly (**Figure 2C**).

Post sequencing, standard quality controls based on the ENCODE ChIP-Seq guidelines were applied[5,14,15].

[Place **Figure 3** here]

For visual quality control, H3K27ac enrichment tracks for display in the UCSC genome browser were prepared. For four gene loci, individual tracks for each sample showed high mapping quality and signal-to-noise ratio reflecting the high consistency and robustness of our assay (**Figure 3A**). The two loci to the left harbor well-expressed genes in these cell types, while the genes in the two loci to the right are not expressed and served as background controls[13] (**Figure 3A**). Further, the MEDIPS analysis package was used as post-sequencing variable to assess the correlation index between technical replicates (**Figure 3B**)[5,16,17], establishing the degree of correlation for reads enrichment level for 500 bp bins[16]. For the majority of the pairwise comparisons, Pearson correlations indexes showed more than 90% correlation suggesting high level of consistence between the biological replicates (**Figure 3B**). Replicates with acceptable correlation were merged to increase signal-to-noise ratio. While cell type-specific loci showed high enrichment in the appropriate cells, a house-keeping gene (B2M) showed very consistent histone modification (**Figure 3C**). For the analysis, merging tracks from replicates will increase the enrichment, reinforce the specific signal, including for important cell type-specific enhancers, and reduces the inter-individual variability inherent to human samples[5].

Although 100,000 cells were used for this study, there was high reproducibility for as few as 10,000 cells in a human cultured T-cell line (HUT78). Correlation analysis between ChIP-Seq dataset performed from samples with less than 100,000 cells showed high reproducibility and correlation down to 10,000 cells (**Figure 4A**).

[Place **Figure 4** here].

Pearson correlation analysis showed high correlation index (83% to 92%), suggesting maintenance of signal in low cell number samples. However, there was increased background as the cell numbers were reduced as well as a dropping of the correlation coefficients (**Figure 4B**). To maintain low background signals, technical duplicates were merged, and the correlation was tested between groups (**Figure 4C**).

**FIGURE AND TABLE LEGENDS:**

**Figure 1: General Flowchart for the procedure.** (**A**) A cartoon of the overall procedure (generated in BioRender). (**B**) Flow-chart for all the major steps for the protocol and the estimated hands-on and total time associated with each day. The sequencing could happen at the end of Day 5 or later with multiple rounds. The timeline can also be staggered throughout the week, where sequential Day 3-4 can be completed multiple times in a week to generate 48 ChIP samples.

**Figure 2: Pre-sequencing QC examples.** (**A**) 1.2% agarose gels show reproducibility of sonication. Sonication samples for 6 donors in three cell types: naive CD4 T cells (CD4), Classical monocytes (MO), and Natural killer cells (NK). The samples were sonicated for 14 cycles (16 s ON, 32 s OFF per cycle). For each sample about 200 ng of decrosslinked chromatin were loaded on a 1% agarose gel. Samples were considered good if more than 70 % of the fragments are within 100-

500 bp. (**B**) Top - Analysis qPCR amplification curves to determine the optimal number of cycles for amplification (Ct where there is ½ the max intensity). The ideal samples have a Ct of about 15 and amplification can be completed up to 2 cycle more of the measured Ct. The arrow is an example of a bad example where the Ct is greater than 18. Bottom - An example of a poor set of samples is shown which have a Ct greater than 18. These samples also showed lower fluorescence intensity. (**C**) Left - Fragment analyzer electrophoresis traces showed the distribution of final tagmented libraries after amplification and size-selection. Samples with more than 85 % of fragment library lies within 200-1,000 bp were considered as good samples. Measurement of peak intensity of fluorescence is also considered as an important QC parameter, indeed if signal is low, the sample is unlikely to sequence well. Right - Examples for positive samples in CD4, MO, and NK are shown.

**Figure 3: Reproducibility of the immune-cell samples.** (**A**) H3K27ac tracks (UCSC Genome Browser, maximum intensity, smoothing function of 4, all with equally scaled Y-axis) for 6 donors (100,000 cells per replicate) in each cell type (CD4, MO, and NK). Four exemplary loci are shown, two with (IL2RA locus and PTPRC) and two without enrichment for H3K27ac (CCR4 and MS4A1). (**B**) Pearson correlation between the donors and corresponding correlation plots generated using a 300 bp extension and 500 bp window within the MEDIPS package for each of the cell type replicates[16]. (**C**) Merged donor files for each cell type showing H3K27ac tracks (UCSC Genome Browser maximum intensity, smoothing function of 4) in cell type-specific regions (IL17R for CD4, CCR2 for MO, and KLRC1 for NK) and the house-keeping gene B2M, present in all cell types.

**Figure 4: Reproducibility of low input samples.** (**A**) Examples of the consistency of H3K27ac ChIP-Seq for cells 100,000 down to 10,000 in HUT-78 cells (a T-cell lymphoma cell line). The tracks (UCSC Genome Browser, maximum intensity, smoothing function of 4, all with equally scaled Y-axis) show the IL4 locus. (**B**) Pearson correlations of the replicates using a 300 bp extension and 500 bp window within the MEDIPS package[16]. (**C**) Pearson correlations between the different cell number groups (100,000, 50,000, and 10,000 cells) using the same MEDIPS parameters as in (B)[16].

**Supplementary Table 1: Buffer recipes.**

**Supplementary Table 2: Spearman and Pearson sample correlations for the 6 donors and each cell type.**

**DISCUSSION:**

The method described here expands on the ChIPmentation procedure[11], which implements a tagmentation library preparation protocol prior to DNA purification, by automating and microscaling the protocol. Since the onset of ChIP-Seq, the required cell numbers have been reduced drastically, from about 20 million cells for histones down to hundreds and even single-cells[1,7,10,12,18-21]. These newly developed methods have allowed for a deeper understanding of how *cis*-regulatory mechanisms are working in cells by increasing the sensitivity and allowing for rare clinical cell populations to be tested [5,6,12,17]. For instance, one of the more recent and popular procedure, called CUT&TAG, as robust and sensitive ChIP-Seq alternative[9]. It produces an excellent signal-to-noise ratio as the Tn5 enzyme is covalently bound to protein A and recognizes

the Fc chain of the ChIP antibody with high specificity[9]. Background activity of Tn5-enzyme is reduced as the enzyme is not functional before binding to the target antibody[9]. However, the implementation of this method in a clinical context is limited since it requires non-fixed, live cells. Also, the removal of DNA fragments from the hypotonic nucleus could have negative effects on the chromatin as it is removed from during the assay. The necessary requirement to work with fresh and living cells is a source of issues for rare clinical samples and for large cohorts of samples, since large cohorts can take numerous years to collect[5]. Another type of method, drop-ChIP, elegantly uses a microfluidics device to generate droplet based tagmention prior to processing the ChIP[19]. However, it uses a highly specialized microfluidic device and, while it is possible to complete single-cell ChIP-Seq, it is also limited to the use of live cells[7-9,18,19]. Newer methods relying on ChIP-Seq such as PLAC-Seq or HiChIP, attempt to understanding 3-dimension (3D) interactions between the ChIP-Seq peaks[22,23]. These 3D methods are exciting as they are identifying *cis*-regulatory or TF mediated interactions across the genome and better the understanding of the regulation of gene expression in cell types of interest, in healthy tissues and in the context of disease.

There are a few critical steps to consider for the protocol to be successful such as quality of the sonicated chromatin and quality of the antibody. Shearing efficiency is critical, if the chromatin is not sonicated well, the efficiency of the assay decreases drastically[24]. Sonication is a challenging aspect of ChIP-Seq due to the cell numbers required. On the sonicator used in the protocol, efficiency was drastically reduced under 300,000 cells. This is a challenging aspect in ChIP-Seq as to sonicate under that level would often require enzymatic fragmentation, which is less impartial. As a result, sonication is a major limited factor for true microscaled ChIP-Seq. Other sonication platforms and commercially available kits were tested for sonicating chromatin, but the sonicator used here had the most robust and reproducible results. Another advantage of the sonicator is not having to purchase specialized tubes to run the sonication, which reduces costs when dealing with large number of samples. For optimal sonication, firstly, it is important to pre-warm the sonicator as described above. Second, to lyse the pellet, it is recommended to have the pipette tip touching the bottom of the tube while lysing to break up the cells with more physical constrains. Third, any bubble formation prior to sonication hinders the ability of the sample to be sonicated evenly. If there are any bubbles formed during the lysis, it is important to remove them with a pipette. This can be challenging without removing a lot of sample, but if the tip is lightly pressed against the bubble it can be slowly drawn up without loss of much sample. Lastly, when determining the number of cycles, complete a time-course where every three cycles, sample is removed, purified, and ran on an agarose gel. Avoid over/under sonication of samples as this decreases the ChIP efficiency. If the sample is under sonicated, the large fragments can have a negative effect on the ChIP-Seq quality[24]. On the other hand, if the sample is over sonicated, there is a risk of the target epitope getting lost in the process.

Another essential part of ChIP-Seq is the quality of the antibody. Prior to running any large-scale study, it is necessary to optimize the antibody which will be used. The goal is to obtain a significantly high signal to noise ratio of known regions of the genome and another is the reproducibility. If the antibody is pulling a lot of background signal, it might be recommended to use a larger input or try a different lot/supplier. This will add time before starting a large-scale

experiment, but it is an essential step. To test for the signal-to-noise it is recommended to use qPCR with regions known to be a target of your antibody and another region known to be absent. It has been noticed histone modifications are more robust and easier to optimize than TFs.

The protocol described above provides a robust method for high-throughput histone-modification ChIP-Seq in a semiautonomous, microscaled manner. The method limits the amount of hands-on time and increases the reproducibility over manual ChIP-Seq. Previous studies completed in the lab used manual ChIP on technical replicates and obtained a Spearman correlation average of 0.50[5], however, with the semiautomated system, the Spearman correlation between different donors with an NK cells average of 0.66 (**Supplementary Table 2**). This was also completed with about 40% less hands-on time. The method described here has been optimized for histone-modifications (H3K27ac shown here, but the protocol should not need any modification for others) and would only require minor modifications for to be implemented for TF ChIP-Seq. Despite the quality of the antibody, the main modification would be for the sonication time and potentially the buffers used during the IP. Usually, for TF ChIP assays, the method may work better with slightly longer fragments of chromatin (with a range of around 350-800 bp) as TF:DNA complexes are likely less able to be maintained through rigorous sonication[6]. The buffers might also need to change to a custom mix or other industry available kits, as TFs can behave differently than histone modifications.

Although the automated ChIP liquid-handler was been tested for as few as 10,000 cells, there was a noticeable decrease in reproducibility at lower chromatin concentrations. Due to this, the protocol was not recommended to less than 10,000 cells, with 100,000 cells being the optimal conditions. The protocol was also completed using industry ChIP buffers, which was an added expense but provided higher quality data. The protocol could be modified with regard to the sonication conditions (as long as the sheared chromatin is kept within the same range), buffers could be customized for the immunoprecipitation (IP; optimization may be required), or the ChIP liquid-handler may not be used. A limitation of the protocol is the use of the ChIP liquid-handler, which can be an expensive investment and can only run 16 samples at once. The ChIP liquid-handler is limited to small-scale reactions and cell numbers greater than one million are not recommended. However, the protocol could be completed without it, by completing the IP and wash steps manually. If the IP and washes were completed by hand, the time to complete the assay will increase and the reproducibility may decrease, but this guide will still be useful running a high-quality ChIP-Seq experiment. Of note, other liquid handlers could be adapted to run semiautomated ChIP reactions.

To summarize, the major benefits of this system is the high-throughput nature, since the IP and washing steps are completed autonomously. As such, sequential rounds of ChIP experiments can be completed, allowing up to 48 samples to be fully processed and ready for sequencing in 5 days, with limited hands-on time compared to manual ChIP-Seq experiments. Another benefit is the increased reproducibility since ChIP-Seq can be difficult to obtain highly reproducible results. Other methods either require live cells, complex micro-pipetting systems, or the work to be completed all by hand. This system will have to be optimized for low-input samples (<10,000

cells), ultimately allowing single-cell ChIP reactions. The system is also capable of being adapted for the newer ChIP methods, such as PLAC-Seq and HiChIP[22,23].

**ACKNOWLEDGMENTS:**

We thank the Vijayanand lab members for technical help and constructive discussions and Dr. Sharron Squazzo and Mr. Geoffrey Berguet from Diagenode for technical assistance with the sonicator and ChIP liquid-handler machine and protocols. This work was supported by NIH grants AI108564, R01HL114093, S10RR027366 (BD FACSAria II), and S10OD016262 (Illumina HiSeq 2500).

**DISCLOSURES:**

The authors have nothing to disclose.

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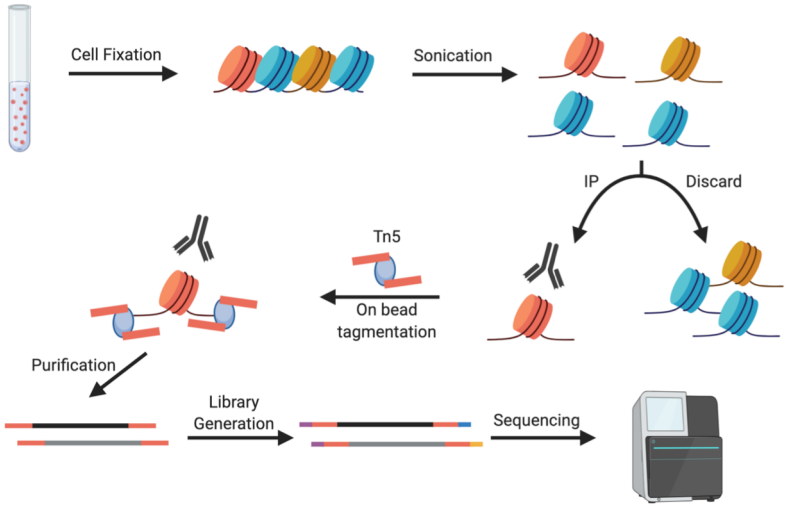
# Figure 1

A.




B.

**Day 1**

**Sample Collection and Processing**

- Preparation and FACS sorting of cells
- Cell fixation

Hands on time: 3 hours
Total time: 5 hours

**Day 2**

**Sonication**

- Cell resuspension and lysis
- Sonication into fragments 150-500 bp
- Sonication QCs and chromatin storage

Hands on time: 3 hours
Total time: 6 hours

**Day 3**

**Automated ChIP on IPStar**

- Machine preparation
- Chromatin, antibody, and bead dilutions
- Start the machine (run time is 16 hours for 16 samples)

Hands on time: 1 hour
Total time: 17 hours

**Day 4**

**ChIP Library Preparation**

- Add Illumina barcode via on bead Tn5 insertion
- Wash the beads on the IP-Star
- Overnight decrosslinking

Hands on time: 1 hours
Total time: 4 hours

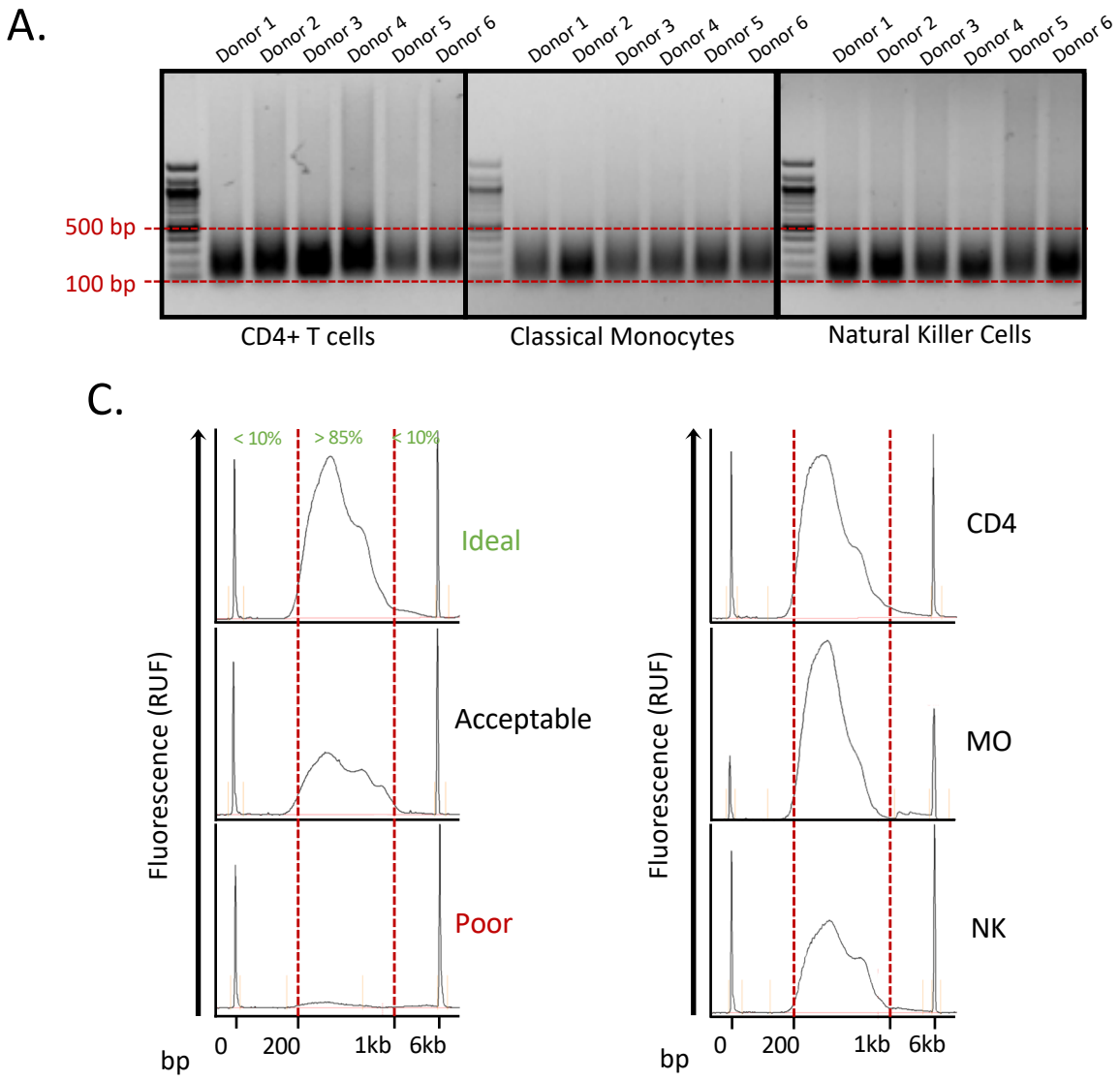
**Day 5**

**ChIP Library Creation**

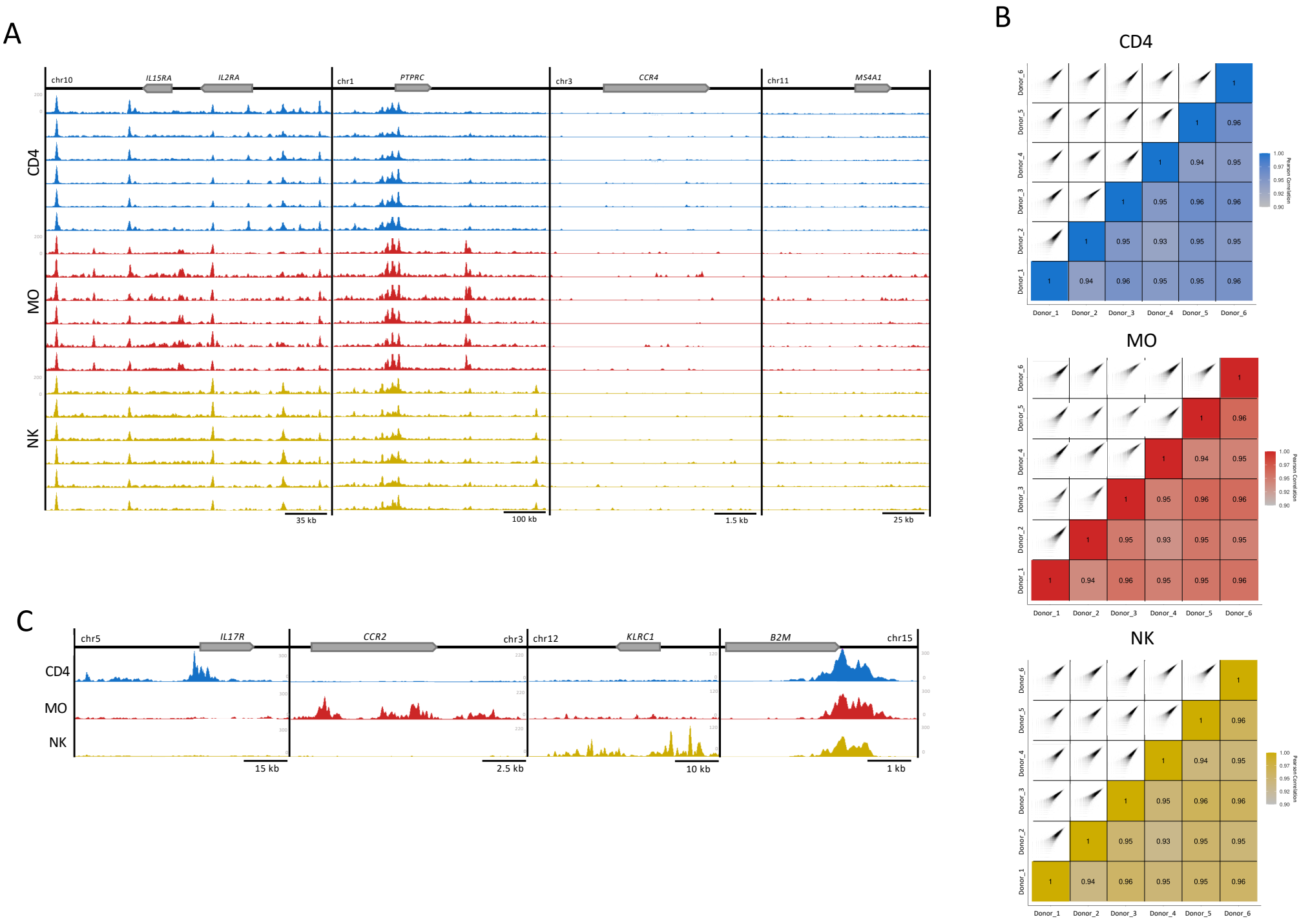
- Purify decrosslinked samples via Zymo columns
- Cycle determination via qPCR
- Amplification for the determined cycles
- Purification of the samples with AMPure Size Selection

Hands on time: 5 hours
Total time: 6 hours

# Figure 2



# Figure 3



# Figure 4

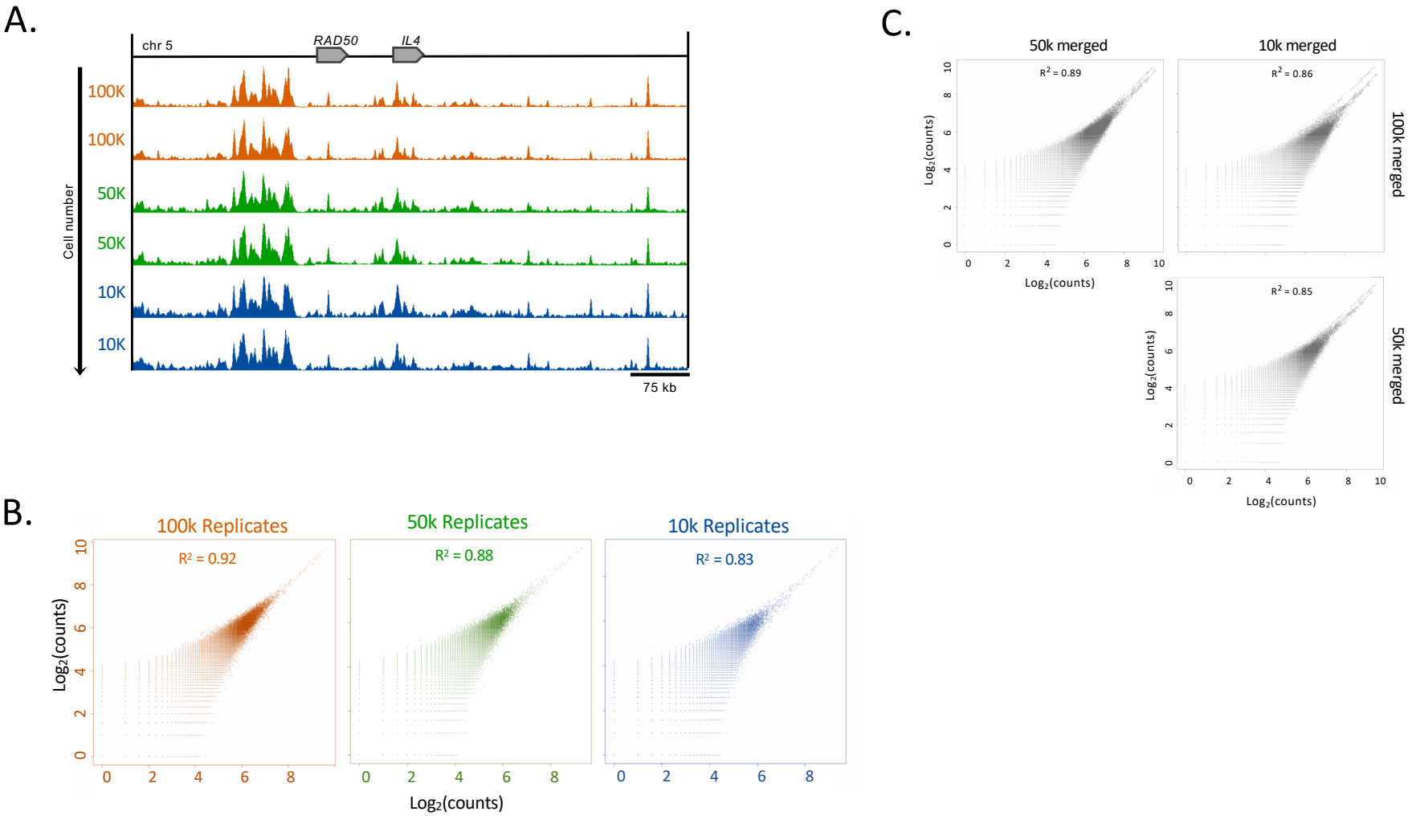


Figure 1 SVG

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**Video or Animated Figure**

Figure 1.svg

Figure 4 SVG

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Figure 4.svg

Figure 3 SVG

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**Video or Animated Figure**

Figure 3.svg

Figure 2 SVG

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**Video or Animated Figure**

Figure 2.svg

Table of Materials

Click here to access/download;Table of Materials;Materials and Reagents.xlsx

| Name of Material/ Equipment | Company | Catalog Number |
|---|---|---|
| 200 µl tube strips (8 tubes/strip) + cap strips | Diagenode | C30020002 |
| AMPure XP for PCR Purification | Beckman Coulter | A63880 |
| Axygen 0.6 mL MaxyClear Snaplock Microcentrifuge Tube | Corning | MCT-060-C |
| Bioruptor Pico Sonicator | Diagenode | B01060010 |
| ChIP DNA Clean & Concentrator (Capped Columns) | Zymo Research | D5205 |
| Dynabeads Protein A for Immunoprecipitation | ThermoFisher | 10001D |
| EDTA (0.5 M), pH 8.0, RNase-free | ThermoFisher | AM9260G |
| EGTA pH 8.0 | Millipore Sigma | E3889-25G |
| Eppendorf ThermoMixer C | Eppendorf | 2231000667 |
| Formaldehyde solution | Millipore Sigma | 252549-1L |
| Glycine | Millipore Sigma | 50046-250G |
| H3K27ac polyclonal antibody - Premium | Diagenode | C15410196 |
| HEPES (1 M) pH 7.5 | ThermoFisher | 15630080 |
| IDT for Illumina Nextera DNA Unique Dual Indexes | Illumina | 20027213 |
| Illumina Tagment DNA Enzyme and Buffer Small Kit | Illumina | 20034197 |
| IP-Star Compact Automated System | Diagenode | B03000002 |
| KAPA HiFi HotStart ReadyMix | Roche | KK2601 |
| Medium reagent container for SX-8G IP-Star Compact | Diagenode | C30020003 |
| MgCl2 (magnesium chloride) (25 mM) | ThermoFisher | R0971 |
| N,N-Dimethylformamide | Millipore Sigma | D4551-250ML |
| NaCl (5 M), RNase-free | ThermoFisher | AM9760G |
| PBS (10X), pH 7.4 | ThermoFisher | 70011044 |
| PCR Flex-free 8-tube stripes, attached individual optical caps | USA Scientific | 1402-4700 |
| Proteinase Inhibitor Cocktail | Millipore Sigma | P8340 |
| Proteinase K Solution (20 mg/mL), RNA grade | ThermoFisher | 25530049 |
| PureLink RNase A (20 mg/mL) | ThermoFisher | 12091021 |
| Quant-iT PicoGreen dsDNA Reagent | ThermoFisher | P7581 |
| QuantStudio 6 Flex Real-Time PCR System | ThermoFisher | 4485699 |
| ROX Reference Dye | ThermoFisher | 12223012 |
| Sodium butyrate | Millipore Sigma | 303410-100G |

| | | |
|---|---|---|
| SYBR Gold Nucleic Acid Gel Stain (10,000X Concentrate in DMSO) | ThermoFisher | S11494 |
| SYBR Green I Nucleic Acid Gel Stain - 10,000X concentrate in DMSO | ThermoFisher | S7563 |
| TE Buffer | ThermoFisher | 12090015 |
| Tips (bulk) | Diagenode | C30040020 |
| True MicroChIP Kit | Diagenode | C01010130 |
| UltraPure 1M Tris-HCl, pH 8.0 | ThermoFisher | 15568025 |
| UltraPure SDS Solution, 10% | ThermoFisher | 24730020 |

| | Comments/Description |
|---|---|
| Strip tubes for use on the IP Star; ChIP 8-tube strip | |
| SPRI beads | |
| 0.65 mL low binding tube | |
| Sonicator used in the lab but others can be used | |
| DNA clean-up kit | |
| Automated system for ChIP-Seq studies; ChIP liquid handler | |
| PCR mix | |
| CAUTION - low flash point | |
| 8 strip tubes, 0.2 mL 8-tube strip | |
| Used in the flourescence quantification | |
| qPCR | |

nucleic acid dye

Tips for the IP Star
Contains all the buffers for the IP; ChIP kit

**Editorial comments:**
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We thank the editor for drawing our attention to this, the manuscript has had any spelling and grammatical errors fixed.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

We apologize for the formatting issue. The protocol was altered such that there is one-line space between every step and the font has also been changed to Calibri 12 points.

3. Please reword the title to directly reflect the protocol being presented. Please tone down the language in the title – remove words like robust, sensitive etc.

We apologize for over extending the language in the title. The title has removed the mentioned words and changed to [**A semiautomated ChIP-Seq procedure for Large-scale Epigenetic Studies**]

4. Please provide an email address for each author.

Justin Cayford: jcayford@lji.org
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Grégory Seumois – gregory@lji.org

5. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: IP-Star, Diagenode Bioruptor Pico (Diagenode, Belgium, SX-8G IP157 Star from Diagenode, Diagenode's True MicroChIP Kit, Protein A Dynabeads, Illumina Library Adaptors, 2X KAPA HiFi HotStart Ready Mix, Nextera index primers, AMPure XP beads , PicoGreen, etc.

We apologize in our use of the commercial language. The commercial products have all been removed and have generic names. The commercial names have been been added to a table of Materials and Reagents.

6. Please remove the commercial term from the keywords and replace it with generic term as well.

We have replaced IP-Star with [**ChIP liquid-handler**] throughout the manuscript.

7. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. Please use generic term for IP-Star throughout.

We apologize the use of IP-Star and have replaced it with a more generic term [**ChIP liquid-handler**]

8. Please avoid the use personal pronouns in the text.

We thank the editor for pointing out this issue and the personal pronouns have been removed from the manuscript with the exception of the acknowledgements.

9. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "This paper describes …"

We thank the editor and made minor changes to the summary to follow the format and reduced the word count to 50.

10. Please ensure the Introduction include all of the following:
a) A clear statement of the overall goal of this method

We have included the goal as described in the third paragraph in lines 107-108 [**a semiautomated microscaled ChIP-Seq assay to profile histone modifications which reduces experimental hands-on time is described**]

b) The rationale behind the development and/or use of this technique

The rationale is discussed in detail in paragraph 2 (lines 93-105), with the need for a method to allow a quicker ChIP-Seq method which uses formaldehyde fixed cells in a highly reproducible manner.

c) The advantages over alternative techniques with applicable references to previous studies

We feel the main advantages are discussed in paragraphs two and three (lines 93-117), with the advantage over single-cell methods and methods such as CUT&TAG being high-throughput ChIP-Seq on fixed, clinical samples.

d) A description of the context of the technique in the wider body of literature

We have included this in paragraphs one, two, and four (lines 37-105, 119-123).

e) Information to help readers to determine whether the method is appropriate for their application

We feel paragraphs three and four help to determine if the method is appropriate (lines 107-123).

11. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly.

We apologize to the editor that the steps were not all in the imperative. As such, the protocol has been changed to reflect a more instructive manner. The notes have also been reduced from 15 to 8 to create a more readable protocol.

12. The Protocol should contain only action items that direct the reader to do something.

We thank the editor for this comment and any steps which were not directing a reader were either altered or removed.

13. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.

We apologize that some of the steps were overly verbose and included many actions. The steps were altered to only have a few actions at most for each. If there were more actions, another step was added.

14. Please ensure you answer the “how” question, i.e., how is the step performed?

We made sure the protocol steps were more clear and thank the editor for this comment.

15. 1: What kind of cells are used in this assay?

We noted a few times in the protocol that either human [**lines 64, 92, 722, 732, 822, and 885**] or cultured cell lines were used **[lines 85, 92, and 885].** We apologize for any confusion without labeling these.

16. Solution recipes can be moved to the a table and uploaded separately as a .xlsx file. Added table of Buffer Recipes

We thank the editor for the suggestion and as a result we added a new table for the **Buffer Recipes** has been included and the recopies have been excluded from the protocol.

17. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We thank the editor and have ensured the protocol is less than 10 pages (7) and the filmable content is less than 2.75 pages (2). The filmable steps have not been altered from the original submission.

18. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

We thank the editor for their concerns but there were not any figures used from a previous publication included.

19. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:

We thank the editor for the concerns expressed in the discussion and have addressed each concern below:

a) Critical steps within the protocol

We included the critical steps are discussed in paragraphs 2-3 (lines 1225-1277).

b) Any modifications and troubleshooting of the technique

We have included the modifications and troubleshooting in paragraphs 4-5 (lines 1279-1303)

c) Any limitations of the technique

We have highlighted the limitations of the paragraph 2 (lines 1225-1268).

d) The significance with respect to existing methods

We have included the significance in detail in paragraph 1 comparing to other microscaled and new ChIP techniques (lines 1200-1223).

e) Any future applications of the technique

We have included any future applications in paragraph 6 (lines 1305-1374), mainly centered along adapting to newer 3D methods (HiChIP) and continually lower cell numbers.

20. Please upload each Figure individually to your Editorial Manager account. Please combine all panels of one figure into a single image file.

We have uploaded each image as a .svg as well as a pdf.

**Reviewers' comments:**
**Reviewer #1:**
Manuscript Summary:
The current ChIP-seq method is labor-intensive and requires large number of cells. The authors describe the semi-automated, micro-scaled ChIP-seq procedure for large-scale epigenetic studies such as the identification of H3K27ac-enriched genomic regions in the cell. This procedure employs tagmentation with less than 100,000 fixed cells combined with the IP-Star platform and ChIPmentation for 48 to 96 ChIP samples. This method can reduce labor and costs with fewer cell numbers of cells, but improve signal-to-noise ratios and reproducibility for large-scale ChIP-seq studies. This procedure is well described in detail, but I have a few comments.

We thank the reviewer for valuing our work and taking the time to review the manuscript. We appreciate their input and have worked to address their concerns, especially the concerns with the verbiage within the protocol.

Major Concerns:
- The IP-Star Platform is important for this semi-automated procedure. Readers may not be familiar with an IP-Star platform. I recommend providing more background of IP-Star in the introduction.

We thank the reviewer for mentioning this and a sentence was added in lines 81-83 **[using a ChIP liquid-handler. The handler completes the immunoprecipitation (IP) and subsequent washes in an autonomous manner. This helped to reduce variability between samples]**. As part of the manuscript. it is not allowed to discuss the specific machine (IP-Star), so subsequent explanation beyond a liquid handle completes the IP and washes were not possible. The IP-Star is listed in the materials spreadsheet, so readers will be able to see what platform was used.

- Figure 1 describes a flowchart for the procedure. If the authors provide the overview figure (cartoon) with sonicated chromatin, Tn5 transposase, library DNA adaptors, etc. as many other method papers provide, it will be helpful to understand this procedure.

We apologize there was not a flowchart included. We altered **Figure 1** to include a new panel **(a)** to show a cartoon of the procedure and the old **Figure 1** is included as panel **(b)**

- Please mention whether the procedure is applicable to both primary cells and cultured cells.

We apologize for not explicitly mentioning this. The ability to use human or cell culture was added in various places in the manuscript: human lines **[115, 119, 798, 808, 961, and 964**] or cultured cell lines were used lines **[115, 120, and 964].**

Minor Concerns:
- This procedure used CD4, MO, and NK cells for the demonstration. It would be good to indicate the source of these cells (for example, transgenic mice, human cultured cell lines, primary cells).

We apologize this was not clear and the additions have been made as seen in the previous concern (lines [**115, 119-120, 798, 808, 961, and 964**]).

- Figure 1 indicates that the IP-Star run time is 16 hours for 16 samples. This manuscript claims 48-96 samples would be applicable to this protocol. For example, step 6.5 indicates that this step is usually performed with 48 samples (Line 278). It would be helpful to mention how to proceed with 48-96 samples instead of 16 samples (e.g., estimated time using IP-Star for 48 samples).

We thank the reviewer for noticing this confusing line. As a result, we removed any mention of 96 samples and instead mentioned in **Note_7** (previously Note_12) in lines 463-455 **[For 48 samples, the Steps 3 - 6.5 were completed with two other separate batches and then amplified in one batch as described below.**

- It is unclear whether the procedure described here is applicable for 48 to 96 samples (Line 26) or up to 48 samples (Line 60).

We apologize for adding this confusing extra information to the manuscript and made this more clear in Note_7 and removed any mention of 96 samples throughout the manuscript are not mentioned. We kept the mention of 48 samples only.

- Line 355: The following sentence is unclear: "we set the intensity of the threshold at half of average maximum intensity."

We altered this phrasing in line 852 to **[find where the intensity of the sample is half the average maximum for cycle determination (Figure 2B).]** to make this more explicit and we thank the reviewer for finding this.

- Many steps in the protocol are unclear. Below are a few examples of them. Please double-check the details of the protocol.

We thank the reviewer for drawing our attention these confusing steps throughout the protocol. We have summarized the changes suggested by the reviewer below:

Line 192 is unclear. It needs to be changed from "about 16 hrs" to "about 16 hrs for 16 samples".

We made this change in line 468 to read **[The program will run for about 16 hrs for 16 samples.]**

Lines 197-200: If 53 ul of tagmentation buffer is added into each sample, more than 440 ul of tagmentation buffer should be prepared for 16 samples.

We agree this was not clear in the protocol and that the 53 ul should be added to a single 8-strip, not two as insinuated. This allows 3 ul extra in each tube after taking 25 ul out for each of the two 8-tube strips in line 487. The correction was made on lines 475 and 549.

Line 198: Please indicate the volume of the tagmentation enzyme instead of mentioning 1:24 (vol:vol).

We added a **Buffer List** which should help to make this more clear as the volume of the tagmentation enzyme correlates directly to the number of samples in the ChIP experiment.

Line 201: 220 ul of cold tC1 buffer is over the limit of 0.2 mL 8-strip tube if we assume 0.2 mL tube can hold up to 0.2 mL.

We have included the tubes in the Materials List can easily hold 250 ul and still be capped, despite being labeled as 0.2 mL. We have included a sentence on lines 478-479 [**The 8-strip tubes can hold this volume and still be capped].**

Line 232: "all 1.5 mL tubes" is unclear. It can be specified like "all 1.5 mL new tubes".

We have altered this to make it clearer. Lines 584-585 have been altered to **[Label 16 1.5 mL tubes with the appropriate sample number and add 400 µL of DNA binding buffer from the DNA clean-up kit to each].**

**Reviewer #2:**

Manuscript Summary:

This manuscript described a comprehensive protocol for an improve method on ChIP-Seq experiments. There are two important features on this protocol: semiautomated and small amount of cell numbers (10k). There is no doubt that it would be useful for this field.

We thank the reviewer for taking the time to review the manuscript and for the comments. Although we were not able to expand the whole paper to be 10k, we hope the reviewer understands the reasoning behind why we use 100k cells in the manuscript and in our studies. We hope to have satisfied the reviewers major concerns.

Major Concerns:

1. The authors emphasized that the traditional ChIP-Seq is 1) labor intensive, 2) technically challenging, and 3) often requires large-cell numbers (>100,000 cells). So, they developed a method to overcome the challenges. However, their protocol was still labor intensive (5 days for 48 samples) and required large-cell numbers (0.3 to 3 million cells). Do not get me wrong, I do understand they have figures to compare the results among different cell numbers (10k, 50k and 100k), but it would be better if they can descript the protocol just according 10k cells since this would be a huge benefit compares to traditional ChIP-Seq method, and there have so many methods for traditional ChIP-Seq already.

We thank the reviewer for the comments. We feel the big difference with the protocol is the amount of hands-on time required for the protocol. The protocol reduced [**the hands-on time by over 15 hours for 48 samples]** and was highlighted in line 113 and later in line 1283 **[completed with about 40% less hands-on time].** The reduction in hours was obtained by

comparing the hands on time for manual and this method. The semiautonomous method requires about 27.5 hours of hands on time to complete 48 samples and about 42 hours. These numbers only account for the hands-on and not the total time. Due to the labor reduction of almost 40% we feel it is much less intensive compared to manual.

We demonstrate the 10k cells in Figure 4, however, we use 100k cells as a standard in the lab as the data quality is higher and more reproducible. We normally have abundant amount of cells to run 100k cells but decided to show the limits of the method for individuals who desire. We apologize to the reviewer for not expanding upon the 10k samples.

Minor Concerns:
1. The title saying that this is "A robust, sensitive and automated ChIP-Seq procedure for Large-scale Epigenetic Studies", but according to the description in the manuscript, instead of "automated", "semiautomated," is more accurate.

We thank the reviewer and altered the title from automated to semiautomated.

2. It looks weird that the authors need to active the hotstart Mix before setting up the PCR. Line 258, 6.1. Activate 2X KAPA HiFi HotStart Ready Mix for 1 minute at 98 ˚C (44 µL for 16 samples).

We apologize for this confusion. This was a potential optimization step that was not removed from the protocol, which did not show significant differences. The activation was changed to be part of the PCR program as noted in line 642 and the pre-activated was removed.

**Reviewer #3:**
Manuscript Summary:
In this manuscript, the authors present a detailed outline of a semi-automated ChIP-seq protocol for histone marks. By using the IP-STAR liquid handler they have managed to reduce the hands-on time and increase the throughput while achieving very good reproducibility. The authors provide representative results at pre- and post-sequencing stages, which would of great use to labs trying to establish the method or even ChIP-seq/ChIPmenation in general. What I find particularly useful is that the authors demonstrate very well which controls need to be performed to ensure the adequate sample quality pre-sequencing and provide examples of "good" and "bad" samples. This is a very useful resource for researchers who would like to

establish ChIP-seq in their laboratories or who struggle with troubleshooting, and has a potential to help in avoiding costs of failed sequencing.

We thank the reviewer for their time in reviewing our manuscript and valuing our work. We thank the reviewer for the comments and agree with some of the fundamental problems with low input ChIP-Seq. We hope to have addressed the reviewers concerns here.

Major Concerns:
Without the access to IP-STAR, it is rather difficult to make out which steps of the classical ChIP/ChIPmentation protocol are being automated. The authors could explain that in more detail, ideally including a diagram/figure or table denoting the automated steps in the method. This would be particularly useful to the newcomers to the field who, together with researchers specifically wanting to use the IP-STAR, would probably be the main target of this publication.

We thank the reviewer for this concern and a sentence was added in lines 110- **[using a ChIP liquid-handler. The handler completes the immunoprecipitation (IP) and subsequent washes in an autonomous manner. This helped to reduce variability between samples]** as well as in lines 1299-1300 [**but instead complete the IP and wash steps manually. If the IP and washes were completed by hand**]. We feel this will give the reader a more complete understanding of what is automated.

Moreover, the authors suggest that their method can be used for as few as 10K cells, however the sonication (which is the limiting step for ChIP) protocol they provide calls for >= 300K cells. The authors should provide some information on how to adapt the protocol for smaller inputs, if they want to make such claims. Similarly, they claim the protocol can be adapted for TFs or other histone marks, however they offer no suggestion or how to do it or information on the robustness of the protocol for marks other than H3K27ac, e.g repressive or sharp-peaked like H3K4me3. This would greatly improve the applicability of the method.

We thank the reviewer for these comments. We agree the sonication has limits for reproducible data and we have found that sonication won't work in a reproducible manner at all >75k cells. So, the only way to do it would be with enzymatic help as noted in line 1227-1231: **[. Sonication is a challenging aspect of ChIP-Seq due to the cell numbers required. On the sonicator used in the protocol, efficiency was drastically reduced under 300,000 cells. This is a challenging aspect in ChIP-Seq as to sonicate under that level often requires enzymatic fragmentation, which less impartial compared to sonication. As a result, sonication is a major limited factor for true microscaled ChIP-Seq].** Due to these issues with sonication, extremely low cell input (~10k cells) is the result of a dilution of a larger batch of chromatin, which will still be useful for the community as it allows for many factors to be tested if there are limits in the cell numbers.

We apologize the alterations for other histones and TFs were note more clear. We have made note that other histone modifications should not have any alterations needed which is noted in lines 107-108 **[Here, a semiautomated microscaled ChIP-Seq assay to profile histone modifications which reduces experimental hands-on time is described]**. Also in lines 1286-1287, it was noted there should not need to be modifications for other histone modification **[histone-modifications (H3K27ac shown here but the protocol should not need any modification for others)].** The number of cycles needed for amplification might be altered with various histone modifications, depending on the abundance, but in Figure 2B we demonstrate the way to determine the number required.

Also, we mentioned there would need to be alterations for TF ChIP, such as sonication conditions in lines 1289-1291 **[Usually, for TF ChIP assays, the method may work better with slightly longer fragment of chromatin (with a range of around 350-800 bp) as TF:DNA complexes are likely less able to be maintained through rigorous sonication[6]. The buffers might also need to change to a custom mix or other industry available kits, as TFs can behave differently than histone modifications]**.

The authors claim that their method reduces variability and reduced signal-to-noise ratio in comparison to standard ChIP-seq. They indeed obtained high reproducibility but since there is no comparison to a typical range of reproducibility that may be expected in e.g. manual ChIP-seq, it is difficult to judge to what extend their claims are substantiated. Again, this could be addressed in discussion. There is a very similar issue with the statement regarding reduced costs (and, to a lesser extent, time), which again has not been backed up by any calculations or comparisons. Especially for the newcomers to the field such information would be essential for the cost/benefits analysis while making the decision whether to adopt this particular approach.

We apologize to the reviewer for not making this more clear. In a previous report we completed a manual ChIP, a Spearman correlation between true sample replicates using the histone mark H3K4me2, a spearman correlation of about 0.50 was obtained. In this study, we had six different human donors which had a Spearman correlation of about 0.66 for NK cells, highlighting that the reproducibility is even greater between donors than pure replicates of manual ChIP. Lines 1281-1285 **[Previous studies completed in the lab used manual ChIP on technical replicates and obtained a Spearman correlation average of 0.50, however, with the semiautomated system, the Spearman correlation between different donors with an NK cells average of 0.66 (supplementary table 1). This was also completed with about 40% less hands-on time**] was added to highlight this. As noted we included a supplementary table which explicitly shows both the spearman and pearson correlations for the 6 donors in this study.

We have also highlighted the hands-on time required in the cost in lines 31-33 as this was one of the largest cost savings: **[The system is also able to reduce costs by allowing for reduced reaction volumes, limiting the number of expensive reagents such as enzymes, magnetic beads, antibodies, and hands-on time required]**.

Minor Concerns:
p. 2
Which tubes are used in the sonication step? "Appropriate to use" sounds ambiguous.

We thank the reviewer for pointing this out. We added the sonication tubes in the Materials List and note that in the protocol in line 203 **[0.65 mL low binding tubes.]**. There is a note in the materials list that this is what is being referenced.

p. 8
How many samples failed/Which percentage are usually expected to fail? (i.e. Ct >18?)
Potential reasons for that?

We apologize we did not make this clear in the study. For this set of samples, there were not any samples which failed the ChIP. We have completed large numbers of H3K27ac ChIP-Seq and the expectation is less than 1% of samples fail prior to sequencing, we have not included this data within the manuscript because there are many variables which can be due to a variety of reasons including fixation, sonication, lower inputs than expected, etc. Overall, the method has been very stable over a large number of samples.

**10X Cell Fixation Buffer**

| Compound | Final Concentration |
|---|---|
| Formaldehyde solution | 11% |
| NaCl | 100 mM |
| EDTA, pH 8.0 | 1 mM |
| EGTA, pH 8.0 | 0.5 mM |
| HEPES, pH 7.5 | 50 mM |

**Complete Lysis Buffer**

| Compound | Final Concentration |
|---|---|
| Tris-HCl, pH 8.0 | 50 mM |
| EDTA, pH 8.0 | 10 mM |
| SDS | 0.25% |
| Sodium Butyrate | 20 mM |
| Protease Inhibitor Cocktail | 1X |

**Short-term Lysis Buffer**

| Compound | Final Concentration |
|---|---|
| Tris-HCl, pH 8.0 | 50 mM |
| EDTA, pH 8.0 | 10 mM |
| SDS | 0.25% |

**Short-term Lysis Buffer**

| Compound | Final Concentration |
|---|---|
| Tris-HCl, pH 8.0 | 10 mM |
| MgCl2 | 5 mM |
| N,N-dimethylformamide | 10% |
| Illumina tagmentation enzyme | 1:24 vol:vol |

**CtD Mix**

| Compound | Per sample (µL) |
|---|---|
| NextEra Index Primer A (25 µM) | 0.275 |
| NextEra Index Primer B (25 µM) | 0.275 |
| 2X KAPA HiFi HotStart Ready Mix | 2.75 |
| 1:1000 SYBR Green dye | 0.11 |
| ROX passive dye | 0.11 |
| Water | Fill to 4 µL |

**AMP Mix**

| Compound | Per sample (µL) |
|---|---|
| 2X KAPA HiFi HotStart Ready Mix | 27.5 |
| Water | Fill to 31 µL |

**CD4 Cells**

| | donor_1 | donor_2 | donor_3 | donor_4 | donor_5 | donor_6 |
|---|---|---|---|---|---|---|
| **donor_1** | 1 | 0.93991217 | 0.95734519 | 0.94740114 | 0.95446144 | 0.9572346 |
| **donor_2** | NA | 1 | 0.95143398 | 0.93374288 | 0.95385862 | 0.94671396 |
| **donor_3** | NA | NA | 1 | 0.94816243 | 0.96480273 | 0.96132311 |
| **donor_4** | NA | NA | NA | 1 | 0.9437577 | 0.94621106 |
| **donor_5** | NA | NA | NA | NA | 1 | 0.96379927 |
| **donor_6** | NA | NA | NA | NA | NA | 1 |

**MO Cells**

| | donor_1 | donor_2 | donor_3 | donor_4 | donor_5 | donor_6 |
|---|---|---|---|---|---|---|
| **donor_1** | 1 | 0.9546523 | 0.94409226 | 0.97107507 | 0.93918596 | 0.95820067 |
| **donor_2** | NA | 1 | 0.93723278 | 0.95750734 | 0.93532734 | 0.94430092 |
| **donor_3** | NA | NA | 1 | 0.94596776 | 0.92728538 | 0.93181063 |
| **donor_4** | NA | NA | NA | 1 | 0.9406978 | 0.95392673 |
| **donor_5** | NA | NA | NA | NA | 1 | 0.92499375 |
| **donor_6** | NA | NA | NA | NA | NA | 1 |

**NK Cells**

| | donor_1 | donor_2 | donor_3 | donor_4 | donor_5 | donor_6 |
|---|---|---|---|---|---|---|
| **donor_1** | 1 | 0.94631894 | 0.95085005 | 0.95119755 | 0.95262655 | 0.95542461 |
| **donor_2** | NA | 1 | 0.93864883 | 0.94255054 | 0.94480115 | 0.94740789 |
| **donor_3** | NA | NA | 1 | 0.94467991 | 0.94639004 | 0.94928552 |
| **donor_4** | NA | NA | NA | 1 | 0.94667872 | 0.95871873 |
| **donor_5** | NA | NA | NA | NA | 1 | 0.95241243 |
| **donor_6** | NA | NA | NA | NA | NA | 1 |

**CD4 Cells**

| | donor_1 | donor_2 | donor_3 | donor_4 | donor_5 | donor_6 |
|---|---|---|---|---|---|---|
| **donor_1** | 1 | 0.60199619 | 0.64140837 | 0.59200316 | 0.60778314 | 0.57652119 |
| **donor_2** | NA | 1 | 0.63649025 | 0.59169885 | 0.6100107 | 0.57199385 |
| **donor_3** | NA | NA | 1 | 0.62592557 | 0.64278774 | 0.60487076 |
| **donor_4** | NA | NA | NA | 1 | 0.59965778 | 0.56121265 |
| **donor_5** | NA | NA | NA | NA | 1 | 0.58167114 |
| **donor_6** | NA | NA | NA | NA | NA | 1 |

**MO Cells**

| | donor_1 | donor_2 | donor_3 | donor_4 | donor_5 | donor_6 |
|---|---|---|---|---|---|---|
| **donor_1** | 1 | 0.47418434 | 0.42834818 | 0.51536417 | 0.48722062 | 0.52058534 |
| **donor_2** | NA | 1 | 0.38472475 | 0.45347787 | 0.42754165 | 0.45715727 |
| **donor_3** | NA | NA | 1 | 0.41258714 | 0.38254706 | 0.41873461 |
| **donor_4** | NA | NA | NA | 1 | 0.45858719 | 0.49896279 |
| **donor_5** | NA | NA | NA | NA | 1 | 0.4583018 |
| **donor_6** | NA | NA | NA | NA | NA | 1 |

**NK Cells**

| | donor_1 | donor_2 | donor_3 | donor_4 | donor_5 | donor_6 |
|---|---|---|---|---|---|---|
| **donor_1** | 1 | 0.66061995 | 0.67028062 | 0.65276731 | 0.64373769 | 0.67553489 |
| **donor_2** | NA | 1 | 0.67238257 | 0.65567443 | 0.64921444 | 0.68079078 |
| **donor_3** | NA | NA | 1 | 0.65910213 | 0.66027609 | 0.68446857 |
| **donor_4** | NA | NA | NA | 1 | 0.63684952 | 0.67205749 |
| **donor_5** | NA | NA | NA | NA | 1 | 0.66179039 |
| **donor_6** | NA | NA | NA | NA | NA | 1 |