Absolute Quantification of Architecture (AQuA-HiChIP) Enables Measurement of Differential Chromatin Interactions

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Method Article

Keywords: chromatin architecture, sequencing, epigenetics, topology

Posted Date: November 7th, 2018

DOI: https://doi.org/10.1038/protex.2018.130

Version of Record: A version of this preprint was published on February 12th, 2020. See the published version at https://doi.org/10.1038/s41596-019-0285-9.

Abstract

With emerging methods recently developed to capture protein-anchored 3D epigenome folding we herein report an experimental advance yielding a fundamental and systematic improvement in understanding the 3D genome: integrated normalization of orthologous chromatin for the measurement of absolute changes in the landscape. With Absolute Quantification of chromatin Architecture \(AQuA-HiChIP) global and local changes in the 3D epigenome can be measured, and the absolute differences in protein-anchored folding can be determined. These changes can be defined in a way that couples the relative occupancy of chromatin regulatory factors or histone marks to absolute quantification of 3D chromatin structure. While our method has intrinsic limitations, including restriction by the scope of available ChIP-grade antibodies with mouse/human cross-reactivity, the approach will enable new insights into the topological determinants of transcriptional control and tissue-specific epigenetic memory. We report both experimental and bioinformatic details of AQuA-HiChIP.

Introduction

From the initial 1D sequencing of the human genome[1], it took several years to gain insight into the organization of the 2D epigenome[2-5]. As fundamental insights into the 2D architecture of histone marks and chromatin regulatory factors was revealed in the years following the Human Genome Project \(e.g., ENCODE[6-8]), there was a delay before understanding 3D genome architecture became possible. It was nearly a decade before proximity-ligation coupled to sequencing was developed to illuminate the folding patterns of the human genome, which enabled a contextual understanding for the epigenetic landscape on which histone marks were placed[9]. Measuring genome-wide architectural features mediated by proteins of interest was first achieved with the estrogen receptor α \(ref. 10). In more recent years, significant improvements in chromatin capture methodology have enabled the generation of higher resolution interaction maps[11], and in the case of protein-mediated interactions has also substantially reduced the number of cells required per experiment[12]. This is enabling protein-mediated interactomes to be measured in clinical samples[13], and has also lowered cost per sample by reducing the read depth required to identify structural features \(e.g., enhancer-enhancer and enhancer-promoter interactions). How do the hills \('B-compartments') and valleys \('A-compartments') of the epigenetic landscape[14] regulate \(1) transcriptional control at individual loci, \(2) replication timing, or \(3) tissue-specific memory? These are fundamental questions at the frontier of epigenomics, and lead to critical questions about the etiological roles of architecture in human malignancy: what makes the architecture of a cancer genome different from a healthy cell? On the epigenetic roadmap, what are the roadblocks to understanding connections between architecture and epigenetic deregulation in disease? Currently it is difficult to directly and absolutely compare the epigenome folding of different cell types if relative normalization is carried out based on total interactive PETs. This concept also applies to methods for -omics approaches studying protein localization across the genome \(i.e, epigenomics), where traditional ChIP-seq methods have limiations in revealing global changes to the 2D landscape[15]. ChIP with reference exogenous genome normalization, ChIP-Rx \(ref. 15), where the authors spiked in orthologous species chromatin during the ChIP reaction has facilitated identification of global changes in chromatin state. This has effectively enabled quantitative normalization of the target ChIP in cells where that target is modified globally, because the reference chromatin is identical for each sample. These same intrinsic limitations are placed on the 3D epigenome, especially if global changes occur[11,16,17] rather than locus-specific changes. Thus, we developed and herein report Absolute Quantification of chromatin Architecture \(AQuA-HiChIP) which couples protein-centric 3D epigenome sequencing to internal normalization with orthologous chromatin \(Figure 1). **Figure 1. AQuA-HiChIP uncovers global changes in protein-mediated contact frequency** Induced chromatin interactions anchored with protein contacts will be readily differentiated using AQuA-HiChIP, but with normalizing by total interactive paired-end tags \(PETs), differential interaction frequencies may be more difficult to define. **Applications and outlook** AQuA-HiChIP is most suitable in cases where global gains or losses of chromatin-interactions may occur through perturbation, such as \(1) rapid degradation approaches \(e.g., auxin-inducible degradation[18,19], dTag systems[20]) that reduce the absolute levels of the immunoprecipitation target protein, or \(2) a drug that acutely perturbs the binding interface of chromatin-associated factors across the genome \(e.g., chromatin reader domain inhibitors for proteins such as BRD4, PBRM1), or \(3) a perturbation that globally alters the post-translational modification status of histones \(e.g. lymphocyte activation[21] or HDAC inhibition, Figure 2). These technologies of rapid and global

perturbation are important for facilitating enhanced understanding of direct causal mechanisms in chromatin biology, but results of 3D genome sequencing requiring normalization by read depth or total interacting PETs, in the absence of exogenous spike-in, can lead to masking global changes in the 3D landscape.  **Figure 2. Comparison of HiChIP and AQuA-HiChIP between treated and untreated cells reveals the difference between apparent and absolute contact changes.** Direct comparison of AQuA-HiChIP and non-reference normalized samples indicates apparent chromatin interaction changes anchored in H3-acetylation marks after rapid HDAC inhibition. These changes in architecture are focused near super enhancers \(SEs) proximal to a TSS on chr11. As mentioned previously, analogous concepts in the context of 2D chromatin sequencing have been addressed with global normalization methods and are especially important for rapid small-molecule perturbation of histone marks[15]. As protein-binding to the genome in 3 dimensions can reveal architectural patterns not available from 2D chromatin sequencing, we suggest that this technique will offer a straightforward but meaningful method to facilitate analysis of protein-anchored architecture of the epigenome. We also feel that further applications to non-protein associated 3D genome sequencing with Hi-C \(ref. 9,11; Figure 3) will be interesting for extensions and generalizations of our approach to studying the global chromatin landscape.  **Figure 3. AQuA-HiChIP contact frequency mathematics** a. Valid contacts \(unique: duplicated read pairs removed) were tabulated after separate mapping of the same fastq file to the human \(hg19) and mouse \(mm10) genomes. Trans \(multi-chromosome) and cis \(intra-chromasome) contact frequencies among samples \(DMSO and HDAC inhibitor treated) and across species are presented. b. Direct comparison of mouse to human contacts in DMSO and HDACi treated HiChIP experiments. c. Percentage of mouse and human contacts compared across AQuA-HiChIP and AQuA-input \(HiC, material processed from the same cells, but without a chromatin immunoprecipitation step). d. AQuA Factor for AQuA-HiChIP and AQuA-input \(HiC), both DMSO and HDACi treated. e. Formulas for \(1) calculating matrix of interactions in Contacts Per Million, \(2) calculating the AQuA Factor and \(3) applying it to creating a Reference normalized Contacts Per Million matrix. **Anticipated Results** HiChIP experiments are expected to yield improved signal to noise ratios over HiC, and also contain a higher percentage of information-yielding contacts \(unique paired-end tags, or PETs)[12]. AQuA-HiChIP sequenced at a depth of 100 million paired end reads should contain approximately 70-80 million mapped read pairs to the human genome and 20-30 million to the mouse genome. In a successful experiment, between 20-40% of reads will be duplicates, presumed as artifacts from PCR and discarded. Then, low quality mapping and pairs lacking a predicted digestion site will be discarded. Of the human reads, one should anticipate approximately 25-40 million unique PETs \(valid interaction pairs) to pass all quality filtering steps, and unlike HiC or ChIA-PET, HiChIP contains a high percentage of cis \(not trans) interactions \(Figure 3a). An absolute increase in contacts may be expected to also create a more diverse and higher starting quantity pool of biotinylated DNA fragments, which would result in effectively lowering the duplicate read count and increasing the proportion of valid human reads \(as seen for H3K27ac AQuA-HiChIP under HDAC inhibition conditions, Figure 3b). The relative amount of PETs for human and mouse should trend with the expected total protein-mediated contacts in the treated human sample: for a degron experiment with 80% reduction of the IP target, one would expect an increase from 10% mouse PETs to upwards of 50%; for experiments with an upregulation of a protein target by 2-fold, one would expect a drop in mouse PETs from 10% to 5% \(similar to the decrease seen in Figure 3c). If the same experimental conditions also impact global 3D contact frequencies among all contacts regardless of mediating IP target proteins, one might expect a change in the ratio of human:mouse PETs in the input HiC DNA, although we expect the ratio of human:mouse reads \(and the AQuA Factor) to be less variable than experiments involving a ChIP step \(Figure 3d-e). A successful experiment should produce high quality enrichment and highly informative structural reads, as assessed by eye in a HiC browser such as Juicebox \(ref. 22) \(https://www.aidenlab.org/juicebox/). **Limitations** A primary limitation is that this technique requires the availability of a cross-reactive antibody that binds to both mouse and human targets. For instance, cancer type specific translocation proteins \(ie, PAX3-FOXO1 or EWS-FLI1), or cell-type specific proteins, cannot be assayed for their 3D connectome with spiked-in chromatin, because mouse cells lack the antibody target. **Overview of the Procedure** Here we present a protocol for AQuA-HiChIP, a modified HiChIP \(ref. 12) that uses exogenous species-orthogonal chromatin to accurately detect global increases or decreases in interaction frequencies. This technology can generate high-quality interaction maps with as few as 100 million reads total \(both species, human and mouse herein) per sample. The procedure can be divided into five main components: \(1) multi-species cell preparation, \(2) chromatin contact generation, \(3) sonication and immunoprecipitation, \(4) biotin capture and on-bead library generation, and \(5) cross-species bioinformatics for AQuA-HiChIP normalization.

Reagents

1. NIH-3T3 mouse-derived cells 2. DMSO, molecular biology grade 3. Formaldehyde, molecular biology grade 4. MboI, \(NEB, R0147; New England BioLabs) 5. T4 DNA ligase \(New England BioLabs) 6. DNA Polymerase I, Large \(Klenow) Fragment \(New England BioLabs) 7. Biotin-14-dATP 0.4mM \(ThermoFisher Scientific) 8. dCTP 10 mM \(ThermoFisher Scientific) 9. dGTP 10 mM \(ThermoFisher Scientific) 10. dTTP 10 mM \(ThermoFisher Scientific) 11. dATP 100 mM \(ThermoFisher Scientific) 12. T4 DNA Ligase \(New England BioLabs) 13. Bovine Serum Albumin \(BSA), lyophilized \(Sigma) 14. Proteinase K \(ThermoFisher Scientific) 15. DNA Clean & Concentrator \(Zymo Research) 16. Dynabeads M-280 Streptavidin \(ThermoFisher Scientific) 17. End-It DNA End-Repair Kit \(Lucigen) 18. Klenow Fragment \(3'→5' exo-) \(New England BioLabs) 19. Multiplexing Adapter-Top: 5' Phosphorylation-GATCGGAAGAGCACACGTCT 20. Multiplexing Adapter-Bottom: ACACTCTTTCCCTACACGACGCTCTTCCGATCT 21. Primer M1 \(** denotes Phosphorothioate) CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC**T 22. Primer M2 CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T 23. Primer M3 CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T 24. Primer M4 CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T 25. Primer M5 CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T 26. Primer M6 CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T 27. Primer M7 CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T 28. Primer M8 CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T 29. Primer M9 CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T 30. Primer M10 CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T 31. Primer M11 CAAGCAGAAGACGGCATACGAGATGTAGCCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T 32. Primer M12 CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T 33. Phusion High-Fidelity PCR Master Mix with HF Buffer \(New England BioLabs) 34. Agencourt AMPure XP beads \(Beckman Coulter) 35. E-Gel EX Agarose Gels \(ThermoFisher Scientific) 36. Active Motif cat#39133 anti-H3K27ac 37. Dynabeads Protein A \(ThermoFisher Scientific)

Procedure

Growing control \(untreated) cells, treated cells, and \(control) species-orthogonal cells _Timing: 18-24 hours_ 1. Plate between 10 and 12 million cells \(in our experiments, adherent human cancer cell lines) in a 15 cm dish for each condition, let adhere overnight at 37 °C. According to the biological question being asked, treat one dish of cells with control \(for instance, 0.1% DMSO by volume, 20 µL DMSO in 20 mL of culture media) and a second dish with perturbing agent \(for instance, 1 µM Entinostat, 20 µL of a 1 mM stock solution into 20 mL of culture media) for desired time \(for instance, 6 hours). 2. In parallel, and not necessarily on the same day, grow cells from an orthogonal species. Seed 10 million cells \(in our case, NIH-3T3 mouse cells) and grow until the next day in an incubator set to 37 °C. **Cell harvesting, quantification and formaldehyde fixation** _Timing: 2 hours_ 3. Harvest and count treated and control cells, by aspirating media, washing with PBS gently and applying 4 mL of trypsin \(0.05%) and incubating for 2 minutes until cells are both detached, and clumps are evenly resuspended. Quench trypsin with 9 mL of complete culture media \(10% FBS) and achieve close to single-cell suspension by pipetting cells between 5 and 10 times once transferred into a 15 mL conical tube. 4. Count cells automatically in a Nexcelom Cellometer with at least 4 replicates per condition achieving low standard deviation \(we aim for less than 5%). 5. Calculate the total number of cells in each condition. For the condition with more cells, discard volume to achieve an identical total number of cells per condition, and re-fill to create equivalent volumes \(13 mL each). 6. Add formaldehyde to achieve a final concentration of 1%, and mix gently for 10 minutes at room temperature. For 13 mL of counted cells in culture media from Step 5, add 360 µL of 37% formaldehyde. Quench the formaldehyde with a final concentration of 125 mM glycine at 4°C for 5 minutes. 7. Pellet cells at 1250 x g \(relative centrifugal force) for 3 minutes at 4 °C. Remove and discard supernatant. Resuspend in cold PBS \(5 mL) on ice, and pellet again at 1250 x g, 3 minutes at 4°C. 8. Flash freeze at -80°C or move ahead to Step 10. 9. Prepare exogenous spiked-in cells from an orthogonal species in the same manner, but before freezing, aliquot 2 million cells per 1.5 mL tube \(approximately 6 aliquots for a 15 cm dish of NIH-3T3 cells). **PAUSE POINT** Accurately counted and fixed cell aliquots for paired treated samples and/or exogenous chromatin can be stored at -80°C for up to a year.

Lysis and Restriction Digest \(Steps 10-17, and buffers modified from HiChIP, ref. 12): _Timing: 3.5 hours_ 10. Resuspend ~6 to 10 million crosslinked human cells with 2 million crosslinked mouse cells \(approximately 20% orthologous chromatin, by cell equivalency for AQuA) in 500 µL of ice-cold Hi-C Lysis Buffer and rotate at 4°C for 30 minutes. 11. Centrifuge at 2500 x g for 5 minutes and proceed to next step with pellet. 12. Wash pellet from Step 11 with 500 µL of ice-cold Hi-C Lysis Buffer. 13. Remove the supernatant and resuspend in 100 µL of 0.5% SDS to permeabilize nuclei in preparation for in situ enzymatic digestion steps. 14. Incubate mixture for 10 minutes at 62°C and then add 285 µL of water and 50 µL of 10% Triton X-100 to quench the SDS. 15. Mix by pipetting, spin down sample, and incubate for 15 minutes at 37°C. 16. Add 50 µL of 10X NEB Buffer 2 and 200 U \(8 µL) of MboI restriction enzyme, and digest chromatin for 2 hours at 37°C with rotation. 17. Heat inactivate MboI for 20 minutes at 62°C. **Biotin Incorporation and Proximity Ligation \(Steps 18-22 modified from HiChIP, ref. 12):** _Timing: approximately 5 hours_ 18. After in situ enzymatic digestion with MboI overhangs will be blunted and biotinylated. To the heat inactivated mixture from Step 17, add 52 µL of fill-in master mix: 37.5 µL 0.4 mM biotin-dATP, 1.5 µL 10 mM dCTP, 1.5 µL 10 mM dGTP, 1.5 µL 10 mM dTTP, 10 µL 5U/µL DNA Polymerase I, Large \(Klenow) Fragment \(NEB, M0210). 19. Mix by pipetting and incubate at 37°C for 1 hour with gentle agitation in ThermoMixer. 20. Prepare ligation master mix \(948 µL per sample): 150 µL 10X NEB T4 DNA ligase buffer, 125 µL 10% Triton X-100, 3 µL 50 mg/mL BSA, 10 µL 400 U/µL T4 DNA Ligase, 660 µL Water. Add to samples from Step 19. 21. Incubate at room temperature for 4 hours with gentle agitation in ThermoMixer. 22. Centrifuge reaction mixture at 2500 x g for 5 minutes and remove supernatant. Use pelleted nuclei in subsequent steps. **Sonication:** _Timing: overnight step_ 23. Bring pellet up to 700 µL in Nuclear Lysis Buffer. 24. Use 10 minutes total shearing ‘on’ time with 30s ‘on’ 30s rest with Active Motif Epi-shear probe sonicator at 30% power. 25. Check shearing: take 12.5 µL sonicated lysate and add to 12.5 µL Nuclear Lysis Buffer. Add 1 µL Proteinase K \(Active Motif) and incubate at 65°C overnight after mixing well. Run E-Gel EX with standards to get shearing fragment length approximately ~300bp-1200bp. **Immunoprecipitation, Washes:** _Timing: overnight step_ 26. Spin the sample from Step 25 for 15 minutes at 16000 x g at 4°C \(note: this step may be mostly to homogenize SDS and gently get it back into solution) 27. Add 2X volume of ChIP Dilution Buffer \(split into two tubes of ~400 µL each and add 750 µL Dilution Buffer for 1:3 dilution). Then dilute again 1:1 with ChIP Dilution buffer \(with 1X protease inhibitors). It will be approximately 600 µL per tube in 4 tubes final with approximately 0.16 % SDS \(total 1:6 dilution from 1% SDS). 28. Active Motif #39133 anti-H3K27ac: add 5 µL each per 2 x tubes and incubate at 4°C overnight with overhead rotation. 29. Perform a buffer exchange for Dynabeads Protein A into ChIP Dilution Buffer. Approximately 100 µL Dynabeads Protein A per AQuA-HiChIP sample. 30. Resuspend Protein A beads in Dilution Buffer \(100 µL per AQuA-HiChIP), add to the samples and incubate at 4°C for 2 hours with overhead rotation. 31. Wash beads twice with Low Salt Wash Buffer, High Salt Wash Buffer, and LiCl Wash Buffer. 32. Washing can be performed at room temperature on a magnet by adding 500 µL of a wash buffer, swishing the beads back and forth twice by moving the sample relative to the magnet, and then removing the supernatant. **ChIP DNA reverse crosslinking \(modified from HiChIP, ref. 12):** _Timing: 3 hours_ 33. Resuspend samples on beads in 100 µL of DNA Elution Buffer. 34. Incubate at 23°C for 10 minutes with rotation, followed by 3 minutes at 37°C shaking. 35. For ChIP samples, place on magnet and remove supernatant to a fresh tube. Add another 100 µL of DNA Elution Buffer to ChIP samples and repeat incubations. 36. Remove ChIP eluent again and combine for 200 µL total per sample. 37. Add 10 µL of Proteinase K per sample and incubate at 55°C for 45 minutes with shaking in benchtop ThermoMixer. 38. Adjust to 67°C and incubate for at least 1.5 hours with shaking. 39. Purify the DNA from the samples with DNA Clean & Concentrator \(Zymo Research) and elute in 10 µL per AQuA-HiCHIP immunoprecipitation. **Biotin Capture:** _Timing: 30 minutes_ 40. Prepare for biotin pulldown by washing 5 µL of Dynabeads M-280 Streptavidin beads with Tween Wash Buffer. 41. Resuspend the beads in 10 µL of 2X Biotin Binding Buffer and add to the 10 µL samples. Incubate the mixture at room temperature for 15 minutes with rotation. 42. Let stand on magnet and remove excess buffer. 43. Resuspend beads in 500 µL of Tween Wash Buffer and incubate at 55□C for 2 minutes shaking, and then buffer exchange into 500 µL TE pH 7.4, and leave at room temperature for on-bead library preparation. 44. Proceed to on-bead End-Repair, A-Tail, Adaptor Ligation, Library Amplification \(adapted from ref. 23), but with solid phase purification and washes; buffer exchanges after each step on beads: 1x wash from with high-salt \(Tween Wash Buffer) followed by 1x wash with TE pH 7.4. **End-Repair \(on-bead library preparation deviates from HiChIP, ref. 12):** _Timing: 1 hour_ 45. Resuspend the bead-bound sample from Step 44 with 34 µL TE pH 7.4 and add the following to make End-Repair reaction mix: 5 µL 10X End-Repair Buffer 5 µL dNTPs 5 µL 10 mM ATP 1 µL End-Repair Enzyme mix 50 µL total volume Mix well by pipetting \(do not vortex), and incubate 1 hour at room temperature. 46. Resuspend to wash once with 500 µL Tween Wash Buffer then once with 500 µL TE pH 7.4,

holding beads magnetically. **A-Tailing to 3’ Ends of DNA fragments:** _Timing: 45 min_ 47. Resuspend the bead-bound sample from Step 46 with 32 µL TE pH 7.4 and add the following to make A-Tailing reaction mix: 5 µL 10X Klenow buffer 10 µL 1 mM dATP 3 µl 5 U/ µL Klenow Fragment \(3'→5' exo-) 50 µL total volume Incubate for 45 min at 37 °C in ThermoMixer \(Eppendorf) with gentle rotation. Resuspend to wash once with 500 µL Tween Wash Buffer then once with 500 µL TE pH 7.4, holding beads magnetically **Linker ligation:** _Timing: 1 hour_ 48. Resuspend the bead-bound sample from Step 47 with 22 µL TE pH 7.4 and add the following to make Adaptor ligation reaction mix: 3 µL 10x T4 DNA ligase buffer 2 µL Index PE Adapter Oligo Mix 3 µL T4 DNA ligase \(400 units/µL) 30 µL total volume Incubate for 1 hour min at room temperature with gentle rotation. 49. Resuspend to wash once with 500 µL Tween Wash Buffer then once with 500 µL TE pH 7.4, holding beads magnetically. **Amplify AQuA-HiChIP sequencing libraries with unique indexes:** _Timing: 1-2 hours_ 50. Resuspend beads from Step 49 in 23 µL Water. Add 1 µL Multiplexing PCR FWD Primer \(Box #1), 1 µL REV Primer M1-12, with unique barcode \(Box #1). Mix well with quick vortex, and table-top centrifugation for several seconds. 51. Add 25 µL Phusion High-Fidelity PCR Master Mix with HF Buffer, and pipette to mix. Remove 25 µL of sample and place in 200 µL PCR strip tube to prepare for amplification. 52. Run the following program for on-bead PCR amplification: 30 seconds at 98°C, then cycle: \[10 seconds at 98°C, 30 seconds at 65°C, and 30 seconds at 72°C]. Check library amplicon fragment lengths with aliquots of 2 µL diluted in 10 µL water and run with E-Gel EX. Desired fragment length should be in the range of 300-800 bp. 53. After PCR place libraries on a magnet and elute into new tubes. Save the unused 25 µL PCR reaction mix separately at 4°C to amplify separately after desired cycle number has been defined with the initial on-bead amplification reactions. 54. Add 25 µL qPCR reaction to 25 µL AMPure beads \(1:1 ratio to purify sample from unreacted PCR primers). 55. Mix well and let stand 5 min with beads. Put on magnet for 5 min. Then wash quickly with 80% EtOH x 2 with gentle pipette aspiration. 56. Let stand to dry on magnet for 10-15 min. Add 20 µL ultra pure water. Mix well. incubate 5 min. Put on magnet for 3 min. 57. Remove the 18 µL of purified indexed AQuA-HiChIP DNA. **PAUSE POINT** Indexed AQuA-HiChIP DNA can be stored at -20 °C until ready for sequencing. **Box 1:** "Illumina Sequencing Primers":https://www.nature.com/protocolexchange/system/uploads/7351/original/Box1-Illumina_Sequencing_Primers.pdf?1540660552. **Sequencing and bioinformatic analysis** 58. Quantify the libraries and pool to multiplex according to standard Illumina protocols. 59. Sequence the libraries in 150 bp paired-end mode. A typical run can be performed on an Illumina NextSeq High Output flow cell in paired end mode with 4 samples multiplexed to achieve ~100-120 million reads per sample. 60. Process and demultiplex to create fastq \(R1 and R2) files using standard protocols. 61. Perform dual human and mouse mapping using HiC-pro \(https://github.com/nservant/HiC-Pro). **Attachment: Code and detailed Steps 61-66** For the bioinformatic protocol to process AQuA-HiChIP data, please consult: "Bioinformatic AQuA Protocol":https://www.nature.com/protocolexchange/system/uploads/7349/original/2018_AQuA_Bioinformatic_Protocol.pdf?1540660231. Code for AQuA-HiChIP, with demo data for R scripts, is available "here":https://github.com/GryderArt/AQuA-HiChIP. **In vitro and in silico Fragment Sizes: Bioanalyzer and HiC-pro** 67. Checking fragment size distribution can be done by comparing bioanalyzer results across samples, and also by size distribution from the results of the sequencing as processed by HiC-pro \(Figure 4). These results can be found in the standard subdirectory: HiCpro_OUTPUT/hic_results/pic/Sample_****/ plotHiCFragmentSize_Sample_****.pdf **Figure 4. AQuA-HiChIP size distribution** a. Size of libraries, measured using Agilent Bioanalyzer. b. Size distribution of read-pairs after sequencing, mapping to hg19, and filtering for correct restriction digest at contact junction.

Timing

Overall Timing: Approximately 1 week to grow and treat the cells; 3-4 days from cell harvesting through library preparation. After next-generation sequencing, data analysis can take 1-2 weeks to initially setup, or 2-3 days once the pipeline is established.

Troubleshooting

For troubleshooting consult: "Table 1: Troubleshooting AQuA-HiChIP":https://www.nature.com/protocolexchange/system/uploads/7339/original/Table_1_-_Troubleshooting_AQuA-HiChIP.pdf?1540598454.

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Acknowledgements

We gratefully acknowledge Dr. Y. Song and the NCI Oncogenomics Sequencing Core for assistance. We are grateful to Dr. Tom Misteli and Dr. Justin Demmerle for helpful conversations relating to this work. We thank Dr. Emma Chory for technical advice. We wish to honor the lasting memory of Dr. Joseph P. Calarco. This work was facilitated by Biowulf High Performance Computing Systems and enabled by funding from the Division of Intramural Research from NIH NCI CCR.

Figures

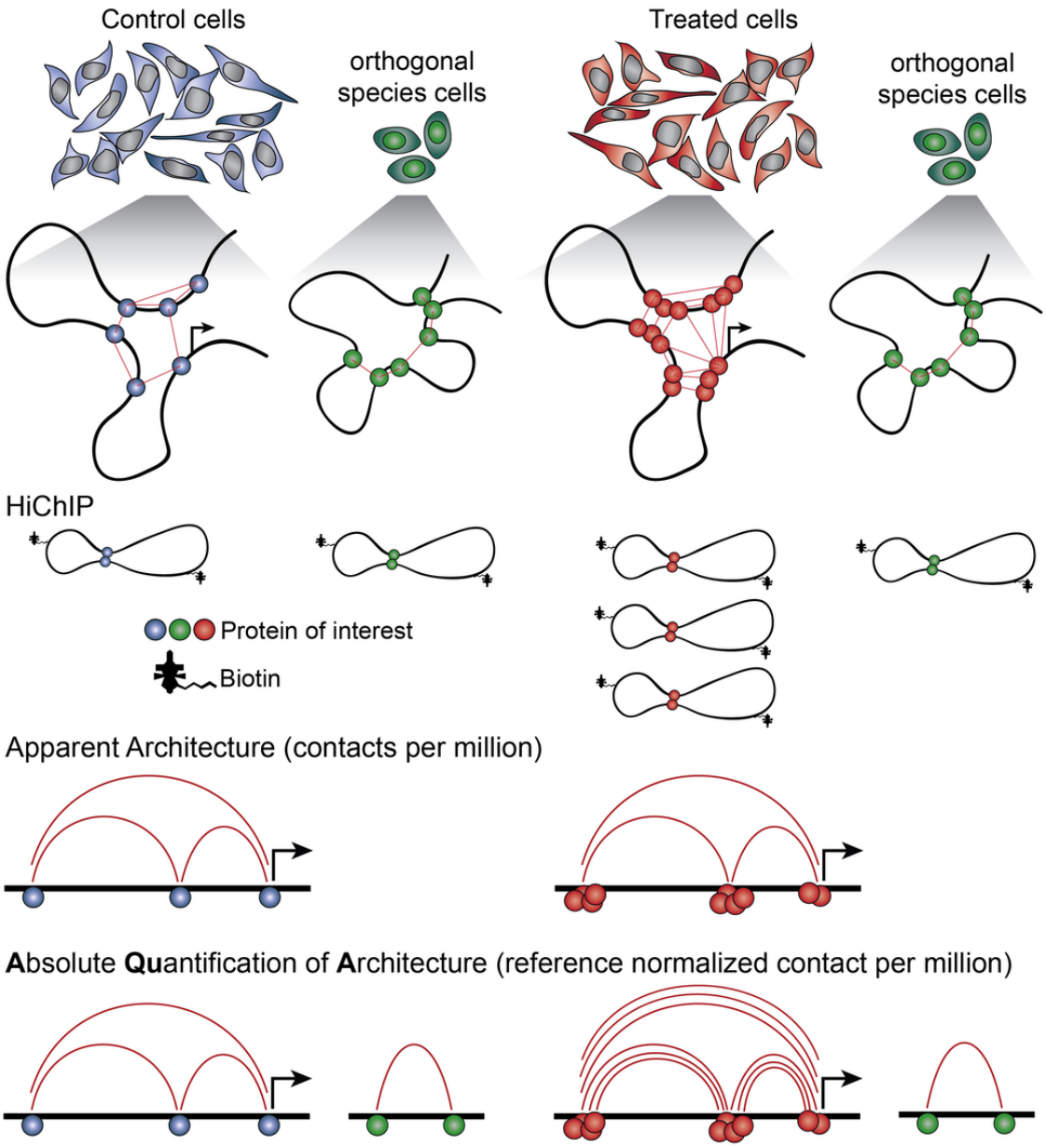

Figure 1

AQuA-HiChIP uncovers global changes in protein-mediated contact frequency Induced chromatin interactions anchored with protein contacts will be readily differentiated using AQuA-HiChIP, but with normalizing by total interactive paired-end tags (PETs), differential interaction frequencies may be more difficult to define.

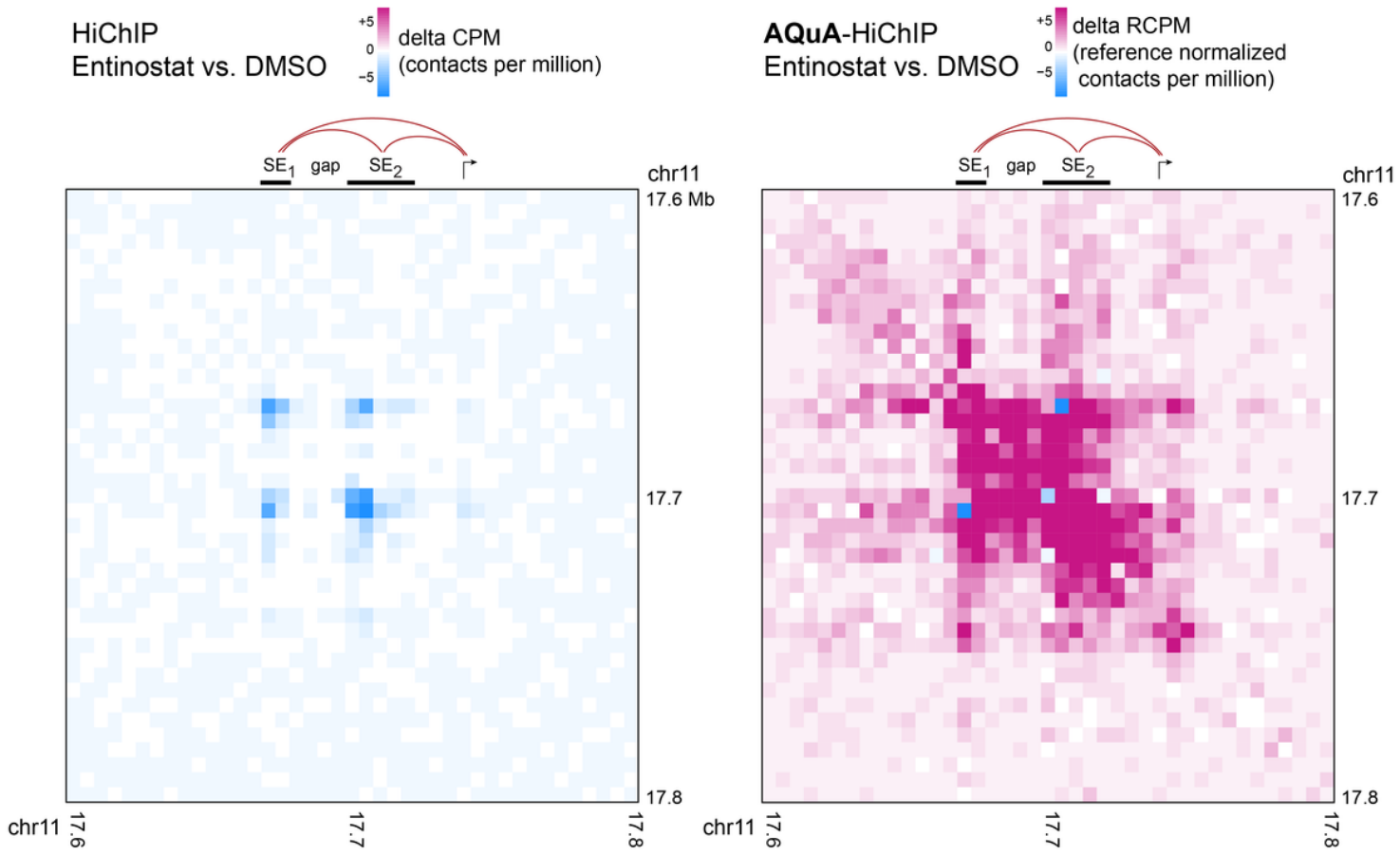

Figure 2

Comparison of HiChIP and AQuA-HiChIP between treated and untreated cells reveals the difference between apparent and absolute contact changes Direct comparison of non-reference normalized HiChIP samples (left) indicates apparent and AQuA-HiChIP (right) indicates absolute chromatin interaction changes anchored in H3-acetylation marks after rapid HDAC inhibition. These changes in architecture are focused near super enhancers (SEs) proximal to a TSS on chr11.

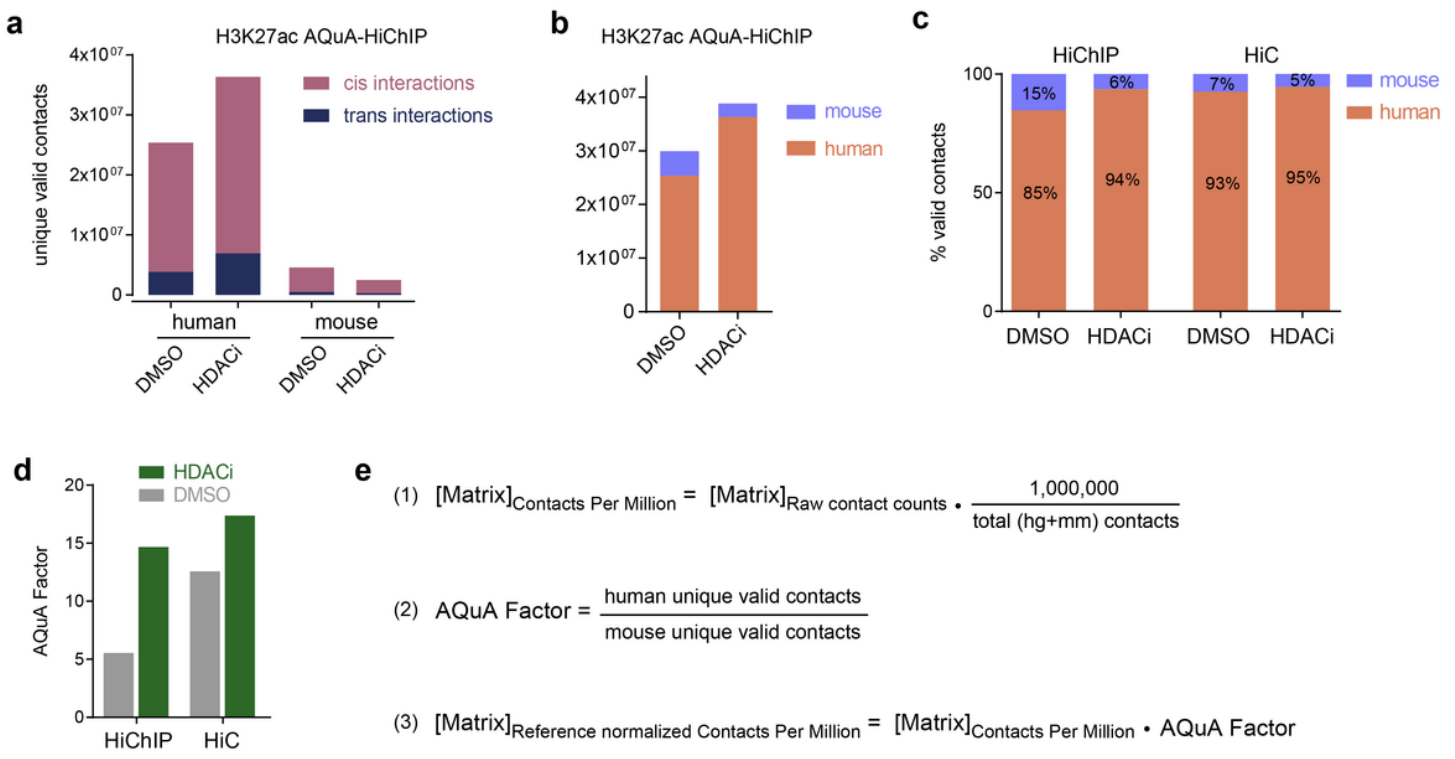

Figure 3

AQuA-HiChIP contact frequency mathematics a. Valid contacts (unique: duplicated read pairs removed) were tabulated after separate mapping of the same fastq file to the human (hg19) and mouse (mm10) genomes. Trans (multi-chromosome) and cis (intra-chromasome) contact frequencies among samples (DMSO and HDAC inhibitor treated) and across species are presented. b. Direct comparison of mouse to human contacts in DMSO and HDACi treated HiChIP experiments. c. Percentage of mouse and human contacts compared across AQuA-HiChIP and AQuA-input (HiC, material processed from the same cells, but without a chromatin immunoprecipitation step). d. AQuA Factor for AQuA-HiChIP and AQuA-input (HiC), both DMSO and HDACi treated. e. Formulas for (1) calculating matrix of interactions in Contacts Per Million, (2) calculating the AQuA Factor and (3) applying it to creating a Reference normalized Contacts Per Million matrix.

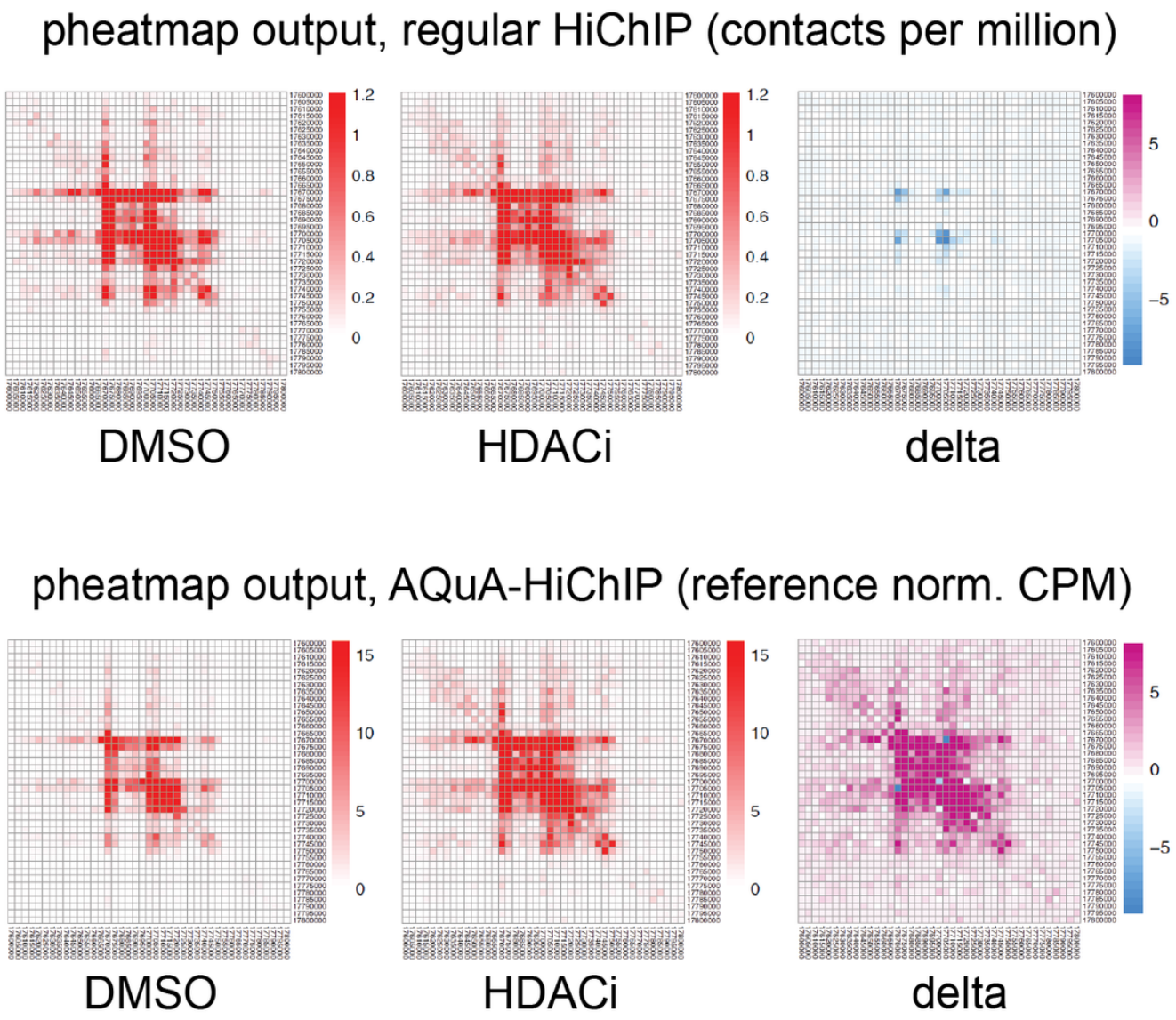

Figure 4

Inline Image 1 Pheatmap output, HiChIP vs. AQuA-HiChIP

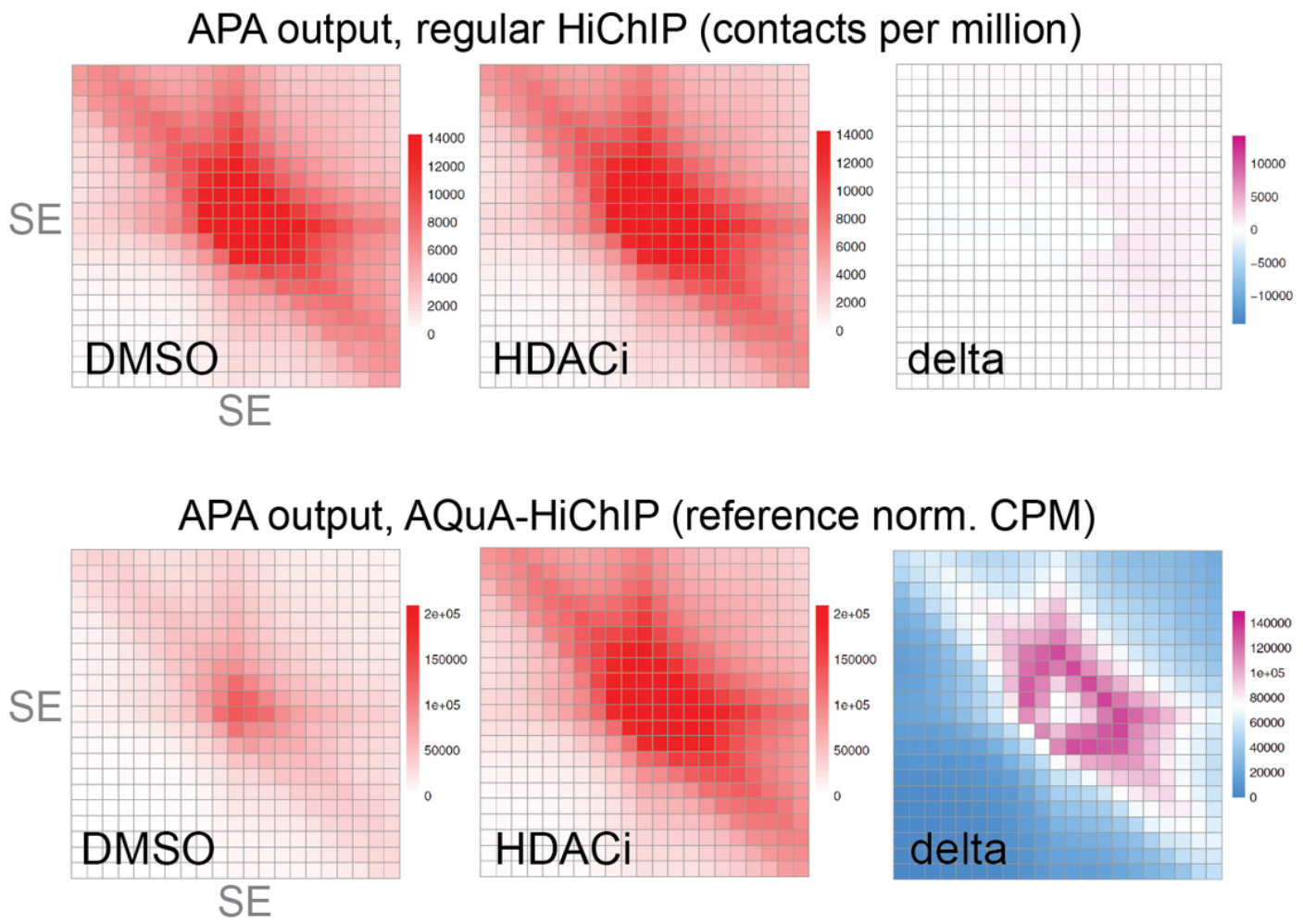

Figure 5

Inline Image 2 APA output, HiChIP vs. AQuA-HiChIP

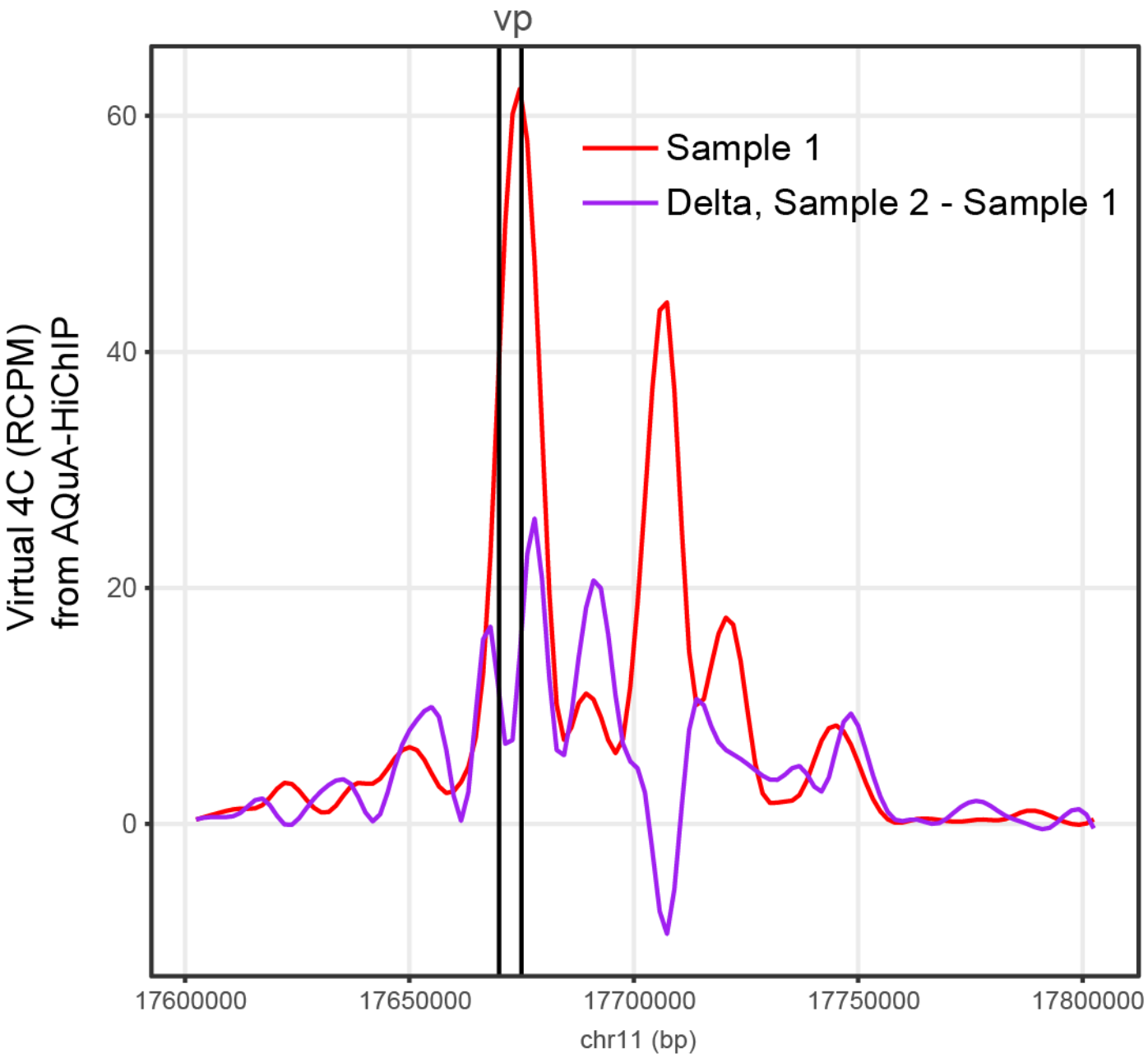

Figure 6

Inline Image 3 Virtual 4C of AQuA-HiChIP

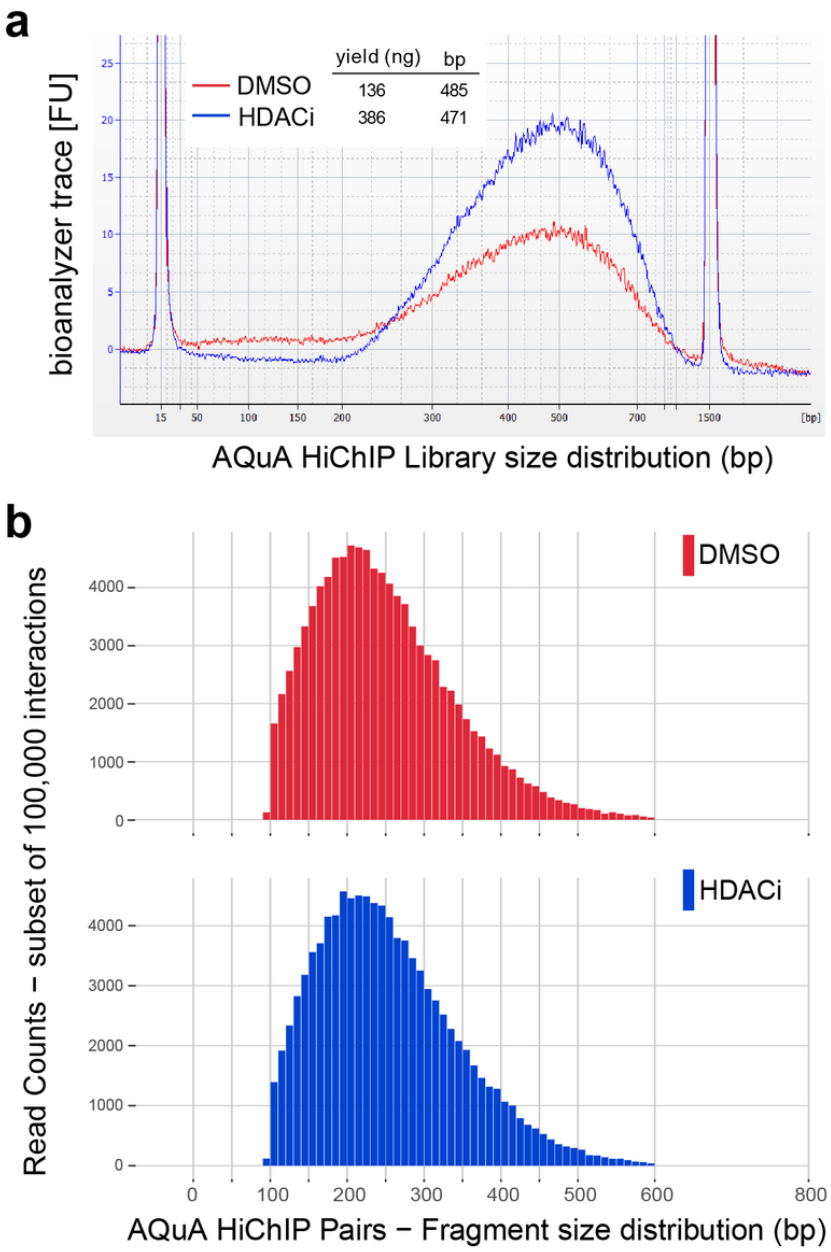

Figure 7

Figure 4 Library Size Distribution

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplement0.pdf
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- supplement0.pdf