

# Genome-scale ChIP-chip analysis using 10,000 human cells

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*The technique of chromatin immunoprecipitation (ChIP) is a powerful method for identifying in vivo DNA binding sites of transcription factors and for studying chromatin modifications. Unfortunately, the large number of cells needed for the standard ChIP protocol has hindered the analysis of many biologically interesting cell populations that are difficult to obtain in large numbers. New ChIP methods involving the use of carrier chromatin have been developed that allow the one-gene-at-a-time analysis of very small numbers of cells. However, such methods are not useful if the resultant sample will be applied to genomic microarrays or used in ChIP-sequencing assays. Therefore, we have miniaturized the ChIP protocol such that as few as 10,000 cells (without the addition of carrier reagents) can be used to obtain enough sample material to analyze the entire human genome. We demonstrate the reproducibility of this MicroChIP technique using 2.1 million feature high-density oligonucleotide arrays and antibodies to RNA polymerase II and to histone H3 trimethylated on lysine 27 or lysine 9.*

## INTRODUCTION

We and others (1–5) have developed protocols for studying DNA-protein interactions and histone modifications in living cells or tissues (see also [www.epigenome-noe.net/research-tools/protocols.php](http://www.epigenome-noe.net/research-tools/protocols.php)). In particular, the method of chromatin immunoprecipitation (ChIP) has provided many new insights into gene regulation (6,7). Briefly, ChIP involves crosslinking the transcription factors to their DNA binding sites by treatment of cells with formaldehyde and then preparation of chromatin by sonication of the treated cells. An immunoprecipitation is then performed using the crosslinked chromatin, resulting in the collection of all the binding sites in the genome for the factor of interest. This sample can then be analyzed by PCR to study a particular gene(s) or applied to microarrays for analysis of many genes (8–12). The majority of ChIP studies have used large numbers of cells grown in culture. However, even using the standard amount of 10<sup>7</sup> cells, there is not enough precipitated sample for

hybridization to a single microarray. Investigators have solved this problem by pooling ChIP samples (13,14). However, the problem is exacerbated when considering the number of arrays needed to analyze the entire human genome; pooling samples is simply not practical under these conditions. Therefore, most ChIP-chip protocols incorporate an amplification step that converts the small amount of material present in a ChIP sample into the microgram quantities required for hybridization to an array. Using such amplification techniques, investigators can routinely obtain enough sample material from 10<sup>6</sup> to 10<sup>7</sup> cells for analysis of the human genome.

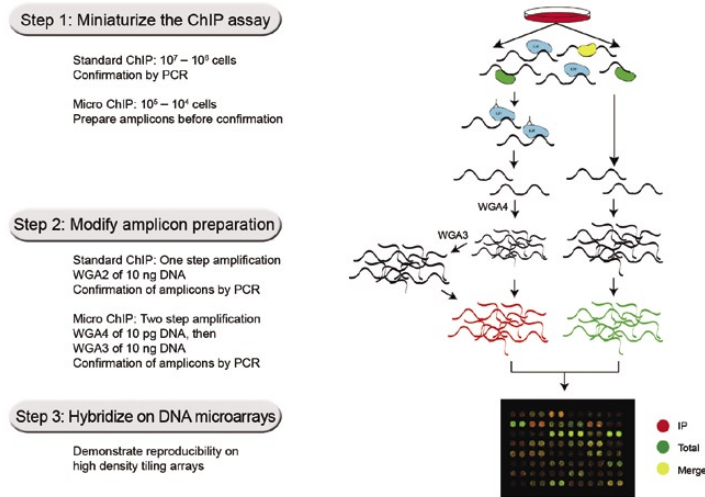
We are interested in applying the ChIP technology to study very small numbers of human cells, such as those obtained from tumor biopsies, fractionation of mixed populations by cell sorting, or differentiation of embryonic stem cells. Although one study has been published in which 1000 cells were used for a ChIP assay, the protocol required mixing a small number of mammalian cells with large numbers of *Drosophila* cells (15).

The success of this protocol was demonstrated by analysis of a few promoters using PCR reactions. Unfortunately, the use of carrier chromatin is not appropriate if the ChIP samples are to be analyzed by sequencing (the carrier DNA would constitute most of the sequenced tags) or by hybridization (due to cross-reaction of the carrier DNA to the oligonucleotides on the array). Therefore, we have taken the approach of altering the ChIP-chip assay such that only 10,000 cells (without carrier DNA) are now required. Importantly, enough sample material can be obtained from 10,000 cells to analyze the entire human genome using microarray technologies.

## MATERIALS AND METHODS

### Cells, Tissues, Antibodies, and PCR

Cells used for the miniaturization optimization are HuH7 human hepatocellular carcinoma cells. Cells were grown at 37°C in a humidified 5% CO<sub>2</sub> incubator in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100 U/mL of penicillin and streptomycin. The cells were crosslinked and chromatin prepared as described in the MicroChIP Protocol (see Supplementary Material available online at [www.BioTechniques.com](http://www.BioTechniques.com)). For the experiments in this study, the chromatin ranged from 0.5 to 3 kb in size, with a median size of ~1 kb. Antibodies to RNA polymerase II (8WG16; Covance, Denver, PA, USA), H3me3K27 (07–449; Upstate, Charlottesville, VA, USA), and H3me3K9 (8898; Abcam, Cambridge, MA, USA) were used in the ChIP assays. Primers used to confirm the binding of factors to the promoter region of the described genes have the following sequence: RNAPII: 5'-agatgaaaccgtgttccaaact-3', 5'-aggttacggcagttgtctctc-3'; SOAT: 5'-ccctactttccaggacaca-3', 5'-acaaatgacatcggaaca-3'; EVX1: 5'-ccgggcttcacacttctaaa-3', 5'-aaaggaaaccgcagcta-3'. and ZNF44: 5'-ggctttccacacaaattcac-3', 5'-aacactcccgcagagtaga-3'; ZNF333: 5'-cacaggagagaagccctacg-3', 5'-tcgcgactcatcagtttc-3'. PCR conditions were 95°C for 3 min, 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s, and then 72°C for 5 min.



**Figure 1. Flowchart diagram of the MicroChIP assay.** We have modified the physical aspects of the ChIP assay (Step 1), incorporated an amplification method that allows linear representation of 10–100 pg of DNA (Step 2), and used high density oligonucleotide arrays to demonstrate that transcription factor binding and histone modifications can be studied on a genome-wide scale using only 10,000 human cells (Step 3).

**MicroChIP Protocol**  
(For  $10^4$ – $10^5$  Cells)

This entire protocol can be performed in ~4 days, assuming that the Staph A cells are prepared in advance (indicated as Day 0). A brief summary of the protocol is provided here; further details, including a step-by-step protocol, catalog numbers for reagents, and composition of each solution is provided in the MicroChIP Protocol (see Supplementary Material).

**Day 0:** Staph A cells, which are used to precipitate the antibody-chromatin complexes, are prepared in large batches, aliquoted, and stored in liquid nitrogen prior to the pre-treatment (blocking) required for the

ChIP assays that are performed on Day 2. Instructions for preparation and storage of the Staph A cells are provided in the MicroChIP Protocol (see Supplementary Material).

**Day 1:** The cells are crosslinked and the chromatin is sonicated and prepared for use in ChIP assays. The crosslinked cells can be used immediately or stored at  $-80^\circ\text{C}$  prior to use in the chromatin preparation step. The chromatin can also be prepared and used immediately or stored at  $-80^\circ\text{C}$  prior to use in the ChIP assay. A portion of the chromatin should be purified by QIAquick column (Qiagen, Valencia, CA, USA) and quantified by NanoDrop (NanoDrop Technologies, Wilmington, DE, USA). Also, Staph A cells, which will be used

to precipitate the antibody-chromatin complexes, are treated overnight with nonspecific protein (BSA) and DNA (herring sperm) to reduce background in the ChIP assay.

**Day 2:** Antibodies are added to the chromatin and allowed to form complexes.

**Day 3:** The antibody-chromatin complexes are purified, the “Input” DNA sample is prepared, and the size of the chromatin is determined. Although one could determine the size of the sonicated chromatin prior to the ChIP assays, this would require using the equivalent amount of chromatin as needed for several ChIP assays. Because this MicroChIP Protocol has been developed for the analysis of limiting amounts of starting material, we recommend that the size of the chromatin be determined using the supernatants from the ChIP samples.

**Day 4:** To obtain enough DNA to analyze by PCR or to hybridize to an array, the ChIP sample must be amplified. A single round of amplification provides enough sample from a MicroChIP assay for many PCR reactions or to probe one or two arrays. To probe a set of arrays that represent the entire genome, a second round of amplification must be performed. PCR reactions are used to confirm positive and negative controls for each set of amplifications.

**ChIP-chip Assays**

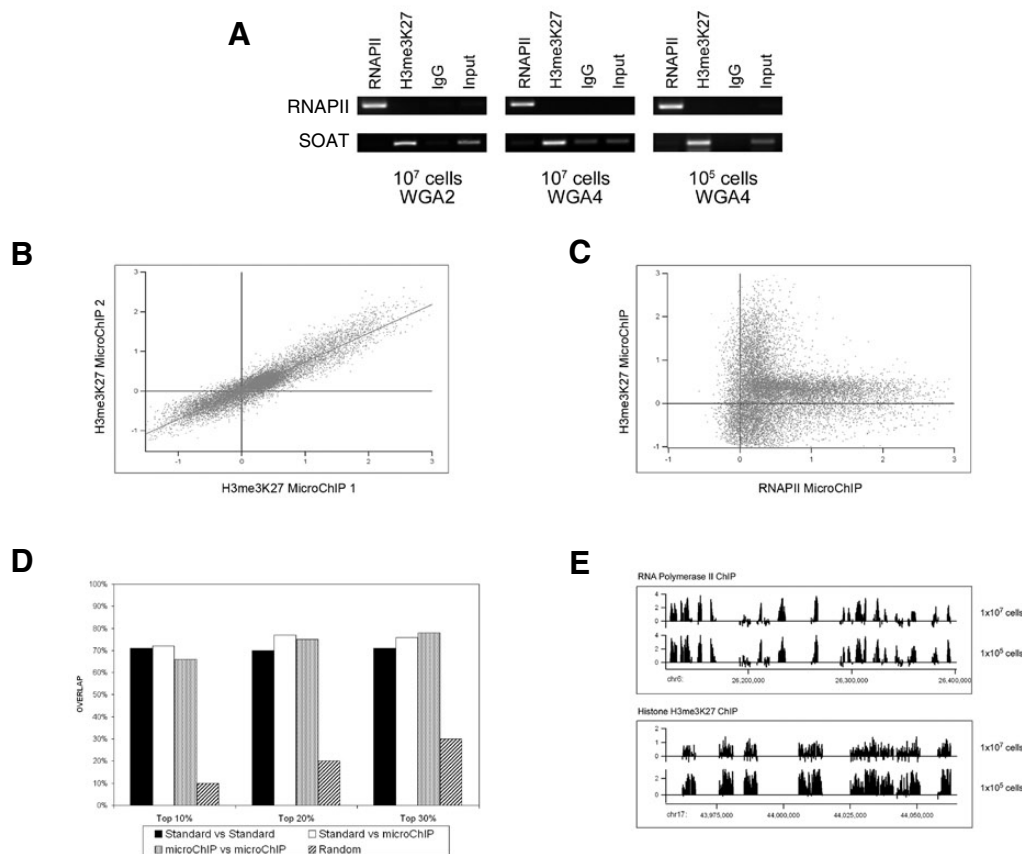
Microarray hybridization was performed by the NimbleGen Service Facility in Iceland or at NimbleGen Systems in Madison, WI, USA, using standard protocols for ChIP-chip arrays, and data analysis was performed using the Maxfour algorithms developed by the Farnham laboratory (9). Briefly, the Maxfour method selects the highest consecutive probes per promoter (4 in a row were used for the RNAPII arrays and 10 in a row were used for the H3me3K27 arrays) and averages them to assign a value to each promoter for ranking analysis. The choice as to the number of consecutive probes to consider for peak calling varies depending on the average peak width of the binding sites for a particular factor. The peaks

**Table 1. Quantitation of ChIP Samples**

Number of cells	Chromatin input	ChIP output
$1 \times 10^7$	200 $\mu\text{g}$	150 ng <sup>a</sup>
$1 \times 10^6$	20 $\mu\text{g}$	10 ng <sup>a</sup>
$5 \times 10^5$	10 $\mu\text{g}$	1.3 ng <sup>a</sup>
$1 \times 10^5$	2 $\mu\text{g}$	300 pg <sup>b</sup>
$1 \times 10^4$	200 ng	30 pg <sup>b</sup>

<sup>a</sup>Output measured using picogreen analysis of a RNAPII ChIP sample.

<sup>b</sup>Output estimated based on larger scale ChIP samples.



**Figure 2. Reproducibility of the MicroChIP assay.** (A) Testing the WGA4 amplification method. ChIP assays using 10<sup>7</sup> cells were performed, then 10 ng of each sample was amplified with WGA2 (left panel), and 50 pg was amplified with WGA4 (middle panel). ChIP assays using 10<sup>5</sup> cells were performed and 50 pg of each sample were amplified with WGA4 (right panel). Primers for the promoters of RNAPII and SOAT were used as the positive controls for RNAPII and H3me3K27 ChIP samples, respectively. IgG ChIP assays were performed as negative controls. (B) Comparison of two H3me3K27 MicroChIP-chip arrays. The Maxfour values from a promoter array for two H3me3K27 ChIP-chip experiments are plotted. (C) Comparison of RNAPII and H3me3K27 data. The Maxfour values from a promoter array for H3me3K27 vs. RNAPII targets are plotted. (D) Comparison of top-ranked RNAPII targets. Two independent standard ChIPs and MicroChIPs were performed, using antibodies to RNAPII. The overlap denotes the common targets in the top-ranked 10%, 20%, and 30% of the promoters on the arrays (which represents ~2000, ~4000, or ~8000 promoters in each set, respectively) bound by each factor in the two independent standard ChIPs, the two independent MicroChIPs, or in the averaged standard vs. MicroChIP experiments. Also shown is the overlap expected by random chance. A similar analysis for H3me3K27 is shown in Supplementary Figure S1. (E) Comparison of binding patterns on standard arrays to those on MicroChIP arrays. Each vertical bar represents the enrichment of a single probe as a log<sub>2</sub> ratio value between the enriched ChIP sample and the Input sample; the chromosomal position is indicated along the bottom axis. The top panel depicts a Histone1 cluster region in chromosome 6, which is enriched for RNAPII binding. The bottom panel shows the HoxB cluster region in chromosome 17, which is enriched for H3me3K27 binding.

for RNAPII binding sites are narrow and thus we analyze 4 probes in a row (~400 bp); the H3me3K27 signals are very broad and thus we analyze 10 probes in a row (~1 kb). Information concerning all of the arrays used in the ChIP-chip experiments is provided in Supplementary Table S1.

## RESULTS AND DISCUSSION

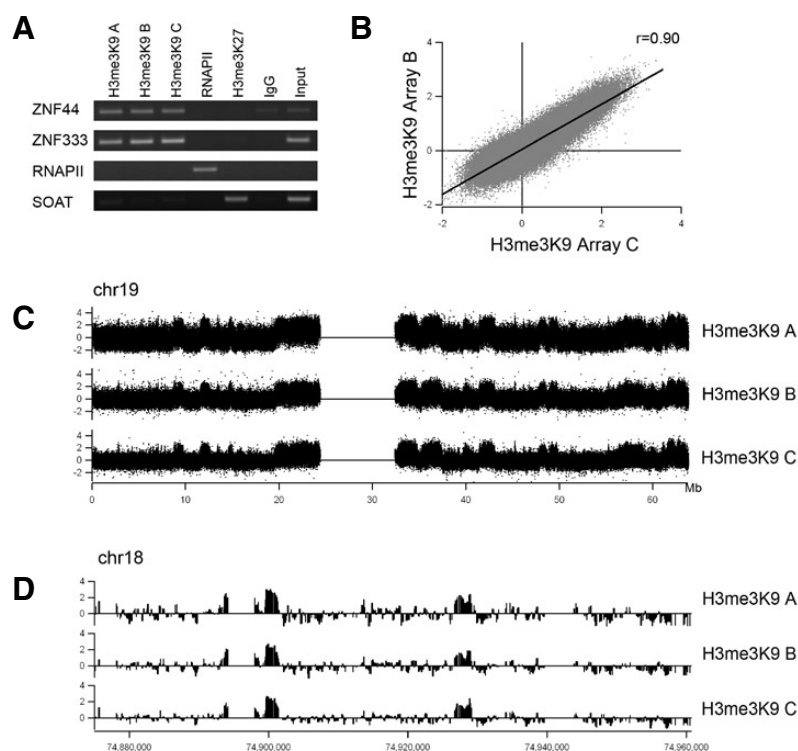
There is widespread interest in the molecular characterization of differences between tumor and normal tissue. Of particular concern is the identification of transcriptionally active genomic regions that have been converted to transcriptionally

silent domains (or vice versa) during neoplastic transformation. These genomic regions can be identified using the ChIP assay and antibodies to RNA polymerase II (to identify active regions) or to histone H3 trimethylated on lysine 27 or lysine 9 (to identify silenced regions). To adapt the ChIP assay such that it is amenable to the analysis of active and silenced genomic regions using the small amount of tissue obtained from tumor biopsies, we have miniaturized the ChIP assay, developed a modified amplification process that allows representative amplification of picograms of ChIP sample, and demonstrated the robustness of this

MicroChIP Protocol by applying the amplicons to high-density oligonucleotide arrays containing 2.1 million features (Figure 1).

## Modification of the Amplicon Preparation Method

Usually the success of a ChIP assay is monitored by PCR analysis of the sample, using primers specific for a genomic region known to be bound by a particular transcription factor or modified histone. Generally 2% or 20% of the enriched material is analyzed in the PCR confirmation step when using 10<sup>7</sup> or 10<sup>6</sup> cells, respectively, in a ChIP assay. However,



**Figure 3. ChIP-chip assays using 10,000 cells and 2.1 million feature arrays.** ChIP assays using 10,000 cells per assay were performed using antibodies to H3me3K9, RNAPII, and H3me3K27. (A) Primers for the promoters of ZNF44 and ZNF333 were used as positive control for the H3me3K9 samples, primers for the RNAPII promoter was used as a positive control for the RNAPII sample, and primers for SOAT were used as a positive control for the H3me3K27 sample. (B) The enrichment values for all of the probes representing chromosome 19 from H3me3K9 sample B and C were compared; this represents ~357,000 probes. See Supplementary Figure S2 for graphs of all three pairwise comparisons for the probes on chromosome 19 and Supplementary Table S2 for all three pairwise comparisons for the probes for the three subarrays on the 2.1 million feature array. (C) The probe enrichment values for the entire chromosome 19 are shown for the three independent H3me3K9 ChIP-chip experiments. (D) The binding patterns (using a 3-oligomer sliding window) for a 80,000-nt region of chromosome 18 are shown for the three independent H3me3K9 ChIP-chip experiments.

when 10,000–100,000 cells are used, the amount of precipitated DNA is quite small (Table 1) and therefore the success of a “miniaturized” ChIP assay is most easily monitored using amplified samples. Therefore, to adapt our ChIP protocol for small numbers of cells, we first had to develop an amplification method appropriate for very small amounts of precipitated DNA. We have recently shown that a whole genome amplification method (16) gives very reliable results using the Whole Genome Amplification 2 kit (WGA2) (Sigma-Aldrich, St. Louis, MO, USA). Unfortunately, the reagents in the WGA2 kit are optimized to give representative amplification of

samples containing 10–100 ng of DNA. We estimate that 10,000 cells (corresponding to ~200 ng of input DNA) would provide only 30 pg of an RNAPII ChIP sample. (Although the amount of precipitated DNA will vary depending on the antibody used and the efficiency of washing the immunoprecipitate, the amount of ChIP sample from 10,000 cells will always be <10 ng.) Therefore, the WGA2 kit is not appropriate for amplification of ChIP samples from such small numbers of cells. Fortunately, a similar amplification method is available (WGA4) that allows representative amplification beginning with 10–100 pg of DNA. Before using this method to amplify

ChIP samples from 10,000 cells, we first optimized the WGA4 amplification protocol using chromatin from a standard ChIP assay that was diluted to the MicroChIP scale. We prepared amplicons from 10 ng (using WGA2) and 50 pg (using WGA4; Sigma-Aldrich) of the same ChIP sample and tested the amplicons in a PCR assay. As shown in Figure 2A, signals from RNAPII and H3me3K27 samples were similar using 10 ng (left panel) or 50 pg (middle panel) to prepare the amplicons. Therefore, we were confident that we could use the WGA4 amplification method to analyze ChIP samples from small numbers of cells.

### Miniaturization of the ChIP Assay

Reduced sample recovery was a major problem that we encountered when using small numbers of cells in the ChIP assay. Therefore, important modifications were incorporated into the ChIP protocol: (i) small volume, low retention siliconized tubes were used at each step; (ii) a bioruptor sonicator was used that allowed a low volume during sonication and no probe contact with the sample; and (iii) a freeze-thaw step was included at the nuclear lysis step. We also found that it is important to quantitate the chromatin using a NanoDrop, to ensure that ~200 ng is used for a MicroChIP experiment. Finally, the procedure used to determine the size of the sonicated DNA in the ChIP assay (which is often very useful as a quality control measure) had to be modified. With the standard assay, investigators usually reserve 10% of the amount used for a ChIP assay, reverse the crosslinks, isolate the DNA, and process this as Input DNA, which can be used to check the size of the chromatin and as a control for PCR assays. However, it is not possible to visualize the DNA on a gel if 10% of a MicroChIP is used. Therefore, we collected all the supernatants from the ChIP precipitation assays (see Supplementary Material: MicroChIP Protocol, Day 3, Step 4), pooled them, reversed the crosslinks, concentrated the sample, cleaned up the DNA, and ran the DNA on a gel to check the size of the chromatin.

Using the modifications described above and in the MicroChIP Protocol (see Supplementary Material), we performed ChIP-chip assays with  $10^5$  cells using antibodies to RNAPII and H3me3K27, to recognize active and inactive chromatin states, respectively. We prepared amplicons from the MicroChIPs using WGA4. We used primers for the promoter of the largest subunit of RNAPII as a positive control for the RNAPII ChIP and primers for the promoter of SOAT as a positive control for the H3me3K27 ChIP. Conversely, the promoter of the largest subunit of RNAPII served as a negative control for the H3me3K27 ChIP, and the promoter of SOAT served as the negative control for the RNAPII ChIP. As can be seen in Figure 2A, right panel, signals from RNAPII and H3me3K27 samples were similar for the MicroChIP/WGA4 samples as for the standard ChIP/WGA2 or standard ChIP/WGA4 (diluted) samples. These controls suggest that our miniaturization of the ChIP protocol was successful. We applied the MicroChIP/WGA4 amplicons to arrays representing 5 kb tiled regions for all known human promoters (~25,000 genes; NimbleGen Systems); hybridization signals from each promoter were ranked using Maxfour (9). As shown in Figure 2B, amplicons from two independent H3me3K27 MicroChIP experiments showed very reproducible results on promoter arrays. In contrast, a comparison of H3me3K27 targets to RNAPII targets showed very little overlap (Figure 2C), as expected when comparing active to inactive marks of chromatin.

We then performed two independent standard ChIP/WGA2 assays using antibodies to RNAPII and H3me3K27 and hybridized these amplicons to the promoter arrays. Again, the promoters were ranked using the Maxfour program and then the top 10%, 20%, and 30% of the promoters were identified. As can be seen in Figure 2D, good reproducibility was obtained between the top targets bound by RNAPII when  $10^7$  cells were used in two independent ChIP-chip experiments (STD ChIP vs. STD ChIP) and when  $10^5$  cells were used in

two independent MicroChIP experiments (MicroChIP vs. MicroChIP). In addition, the comparison between standard and MicroChIP ranked lists showed that the same promoters were identified as top-ranked targets using both methods. Similar comparisons are shown for H3me3K27 in Supplementary Figure S1. For all comparisons of the RNAPII and H3me3K27 ChIP-chip data, the overlaps are much greater than expected by random change (Figure 2D and Supplementary Figure S1). Examples of the raw data from the standard vs. MicroChIP arrays are shown in Figure 2E.

### ChIP-chip Analysis of 10,000 Cells

Having confidence that the miniaturization protocol and new amplification method gave reliable data, we then further reduced the number of cells to 10,000 per sample (corresponding to 200 ng of sample) and performed ChIP assays using antibodies to H3me3K9, RNAPII, and H3me3K27. Our ultimate goal was to identify transcription factor binding sites and chromatin modifications throughout the entire human genome, using small populations of cells. Using the entire ChIP sample in a WGA4 amplification protocol, we could obtain ~5 to 7  $\mu$ g of amplicons from 10,000 cells. However, to probe a 10-array set (representative of the entire human genome) we needed ~40  $\mu$ g of DNA. Therefore, it was clear that we would need to perform a second amplification step, known as a round 2 amplicon. For this, a second round of amplification was performed using the WGA3 kit (which is similar to the WGA2 method, but is used for second round amplification) and 10 ng of the amplified material from the WGA4 round 1. The round 2 amplicons were confirmed in PCR assays using ZNF44 and ZNF333 primers as positive controls for the H3me3K9 samples, RNAPII primers as a positive control for the RNAPII sample, and SOAT primers as a positive control for the H3me3K27 sample (Figure 3A). The amplicons were all clearly highly enriched for the appropriate targets, but not for the negative controls. Also,

none of the promoters were enriched by the IgG sample.

We then probed a high-density genomic array that contains 2.1 million 50-mers probes that represent part of chromosome 16, all of chromosomes 17, 18, and 19, and part of chromosome 20 (NimbleGen HG18\_Tile\_09; HD2\_TILE 25, 26, and 27). These experiments were performed in triplicate, using three independent samples of H3me3K9 amplicons, each prepared from 10,000 cells. As shown in Figure 3B and Supplementary Figure S2, the reproducibility of the MicroChIP-chip protocol is very high when the probe enrichments from any two of the ChIP-chip experiments are compared ( $r = 0.9$ ). Also, the individual probes show very similar patterns when an entire chromosome is viewed (Figure 3C) and when individual peaks are examined (Figure 3D and Supplementary Figure S3).

### Conclusion

ChIP is now a fairly common technique for studying chromatin structure or transcription factor binding patterns. However, researchers typically require large numbers of cells to perform this assay. We have modified the physical aspects of the ChIP assay, incorporated an amplification method that allows linear representation of 10–100 pg of DNA, and used 2.1 million feature arrays to demonstrate that transcription factor binding and histone modifications can be studied on a genome-wide scale using only 10,000 human cells. Several previous studies (15,17,18) have reported modifications to the ChIP assay that allow analysis of a small number of cells. However, it is unclear if the samples produced from the previous protocols could be reproducibly studied using microarrays (especially those samples obtained by the addition of large amounts of cells from other organisms). In contrast, we have demonstrated that our approach can reproducibly identify genes bound by transcription factors (RNAPII) or modified histones using ChIP-chip assays. We have also used this protocol to identify binding sites for KAP1, a co-repressor for site-specific zinc finger-containing transcription factors

(data not shown). We anticipate that the MicroChIP protocol reported here will allow the study of cell populations that are available in limited quantities, such as human tumor biopsies, archival frozen or fixed tissues, or stem cell populations. However, our ultimate goal is to couple the ChIP assay with techniques, such as laser capture microscopy, that involve even smaller numbers of cells. Further modifications to allow the analysis of 1000 cells are in progress.

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## COMPETING INTERESTS STATEMENT

*Several of the authors (A.L.I., H.L.H., X.Z., and R.G.) are employees of NimbleGen Systems Inc., and we have used NimbleGen arrays in our studies.*

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