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# Chop it, ChIP it, check it: the current status of chromatin immunoprecipitation

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## 1. ABSTRACT

Our understanding of the significance of interactions of proteins with DNA in the context of gene expression, cell differentiation or to some extent disease has immensely been enhanced by the advent of chromatin immunoprecipitation (ChIP). ChIP has been widely used to map the localization of post-translationally modified histones or histone variants on the genome or on a specific gene locus, or to map the association of transcription factors or chromatin modifying enzymes to the genome. In spite of its power, ChIP is a cumbersome procedure and typically requires large numbers of cells. This review outlines variations elaborated on the ChIP assay to shorten the procedure, make it suitable for small cell numbers and unravel the multiplicity of histone modifications on a single locus. In addition, the combination of ChIP assays with DNA microrray and high-throughput sequencing technologies has in recent years enabled the profiling of histone modifications and transcription factor occupancy sites throughout the genome and in a high-resolution manner throughout a genomic region of interest. We also review applications of ChIP to the mapping of histone modifications or transcription factor binding at the genome-wide level. Finally, we speculate on future perspectives opened by the combination of emerging ChIP-related technologies.

## 2. INTRODUCTION

Interaction between proteins and DNA is essential for many cellular functions such as DNA replication and DNA repair, maintenance of genomic stability, chromosome segregation at mitosis and regulation of gene expression. Transcription is controlled by the dynamic association of transcription factors (TFs) or chromatin modifiers with target DNA sequences within gene regulatory regions. These associations are modulated by modifications of DNA (methylation of CpG dinucleotides) (1), post-translational modifications of histones (phosphorylation, ubiquitination, sumoylation, acetylation and methylation) (2-4) and incorporation of histone variants (5-9). These alterations are commonly referred to as epigenetic modifications. These modifications constitute an epigenetic code which is read by effector proteins to turn on, turn off, or fine-tune transcription (10,11).

Chromatin immunoprecipitation (ChIP) has become a prominent technique to study protein-DNA interactions inside the cell in the context of embryo development (12,13), cell differentiation (14-16), aging (17) and disease (18,19). In the past decade, ChIP has been used for mapping the localization of post-translationally modified histones and histone variants on the genome, and

for mapping DNA target sites for TFs and other chromosome-associated proteins. Notably, the combination of ChIP with downstream genome-wide high-throughput mapping strategies has enabled the elaboration of a transcriptional regulatory circuitry in embryonic stem (ES) cells (20,21).

A typical ChIP assay is a tedious procedure (Figure 1). DNA and proteins are commonly reversibly cross-linked with formaldehyde (which is heat-reversible) to covalently attach proteins to their target DNA sequences. This ensures that protein-DNA complexes are maintained during the ChIP procedure. Formaldehyde cross-links proteins and DNA molecules within ~2 Å of each other, and thus is suitable for looking at proteins which directly bind DNA. The short cross-linking arm of formaldehyde, however, is not suitable to examining proteins that indirectly associate with DNA, such as those found in larger complexes. To remedy to this limitation, long-range bifunctional cross-linkers such as dimethyl adipimidate, disuccinimidyl suberate, dithiobis[succinimidyl proprionate] or ethylene glycol bis[succinimidyl succinate] have successfully been used in combination with formaldehyde, to detect proteins on their target genes, which with formaldehyde alone are refractory to detection (22). Because the use of long-range cross-linkers is likely to increase the risk of precipitating irrelevant proteins, they should be restricted to analysis of proteins previously established to be in the DNA-binding complex of interest. In contrast to cross-link ChIP, the native ChIP, or NChIP, approach omits cross-linking (23,24). However, this is recommended only for analysis of histones. Regardless of the cross-linking method, the chromatin is then fragmented, either by enzymatic (micrococcal nuclease) digestion after cell lysis, or by sonication of whole cells or nuclei, to fragments of 200-1,000 base pairs in length (and average of 500 base pairs is considered as optimal) encompassing mono- to heptanucleosomes (Figure 1). The chromatin is cleared by sedimentation of the coarse material and protein-DNA complexes are immunoprecipitated from the supernatant using antibodies specific for the protein of interest. Immunoprecipitated complexes are washed under stringent conditions to ensure removal of unspecifically bound chromatin, the cross-link is reversed, proteins are digested and the precipitated ChIP-enriched DNA is purified. DNA sequences associated with the precipitated protein are identified by polymerase chain reaction (PCR), quantitative (q)PCR, labeling and hybridization to genome-wide or tiling DNA microarrays (25-27) or by molecular cloning and sequencing (21,28) (Figure 1).

Despite the power and versatility of ChIP, a major drawback of current protocols is the requirement for large cell numbers (millions per immunoprecipitation) which restricts the application of ChIP to large cell samples. Classical ChIP assays also involve extensive sample handling (23,29), which favors loss of material, creates opportunities for technical errors and hampers consistency of the findings. As a result, alterations have been brought to the ChIP procedure to make it faster and enable the analysis of reduced cell samples or even embryos (24). We review here modifications of conventional ChIP assays that accelerate the procedure and enable examination of smaller cell samples. Analytical tools that may be combined with ChIP to address the landscape of DNA-protein interactions are also addressed.

## 3. TOWARDS A FASTER ChIP ASSAY

Conventional ChIP protocols involve many steps, are time consuming, and as such limit the number of samples that can be analyzed in parallel. To address this issue, Nelson et al. (30,31) have recently introduced two modifications to the ChIP assay, which reduce the length of the protocol. Firstly, incubation of primary antibodies with chromatin substrates in an ultrasonic bath has been shown to substantially increase the rate of antibody-protein binding (32) such that for antibodies tested incubation times were shortened to 15 min (30). Secondly, in a traditional ChIP assay, elution of the ChIP complex, reversal of cross-linking and proteinase K digestion of bound proteins conventionally require ~9 h, and DNA isolation by phenol:chloroform isoamylalcohol extraction and ethanol precipitation takes the best part of day's work. Instead, the Nelson procedure entails a resin (Chelex-100)-based DNA isolation step which reduces the total time for preparation of templates for PCR to ~1 h (30). After washing the ChIP material, Chelex-100 is added to the ChIP pellet, the sample is boiled for 10 min, incubated for 30 min with proteinase K, and proteinase K is then inactivated by boiling for another 10 min. The resulting DNA sample is PCR-ready and can be stored frozen (30) (Figure 2). We have also reported the shortening of cross-linking reversal, proteinase K digestion and SDS elution steps into a single 2-h step without loss of ChIP efficiency or specificity; however our published procedure calls for conventional DNA purification by phenol:chloroform isoamyalcohol extraction (33). Note that it is possible to purify DNA from the ChIP complex with spin columns. Although they can speed up the process, loss of DNA during the procedure may limit the application of such columns to relatively large ChIP assays. We are currently implementing Chelex-100 in our ChIP assay.

As an alternative to Chelex-100 purification of ChIP DNA, using the immunoprecipitate (the ChIP material) directly as template in the PCR has also recently been reported in yeast (34) (Figure 2). The origin recognition complex and the MCM complex, both of which are involved in eukaryotic DNA replication, were selectively immunoprecipitated after overexpression in *Saccharomyces cerevisiae*. Association of Orc1 and Mcm4 with origin sequence ARS1 was determined either by conventionally purifying DNA, or by resuspending the precipitated beads in a Tris-EDTA buffer and using an aliquot directly for PCR. Semi-quantitative comparison of amounts of PCR products obtained by on-bead direct PCR or by PCR using purified DNA showed comparable PCR product (34). The possibility of performing the PCR reaction directly on the immunoprecipitated material indicates that the formaldehyde cross-linking reversion step may be omitted, most likely because the initial PCR heating step suffices to partially reverse the cross-link. Direct PCR, therefore, holds promises for speeding up the analysis of ChIP products.

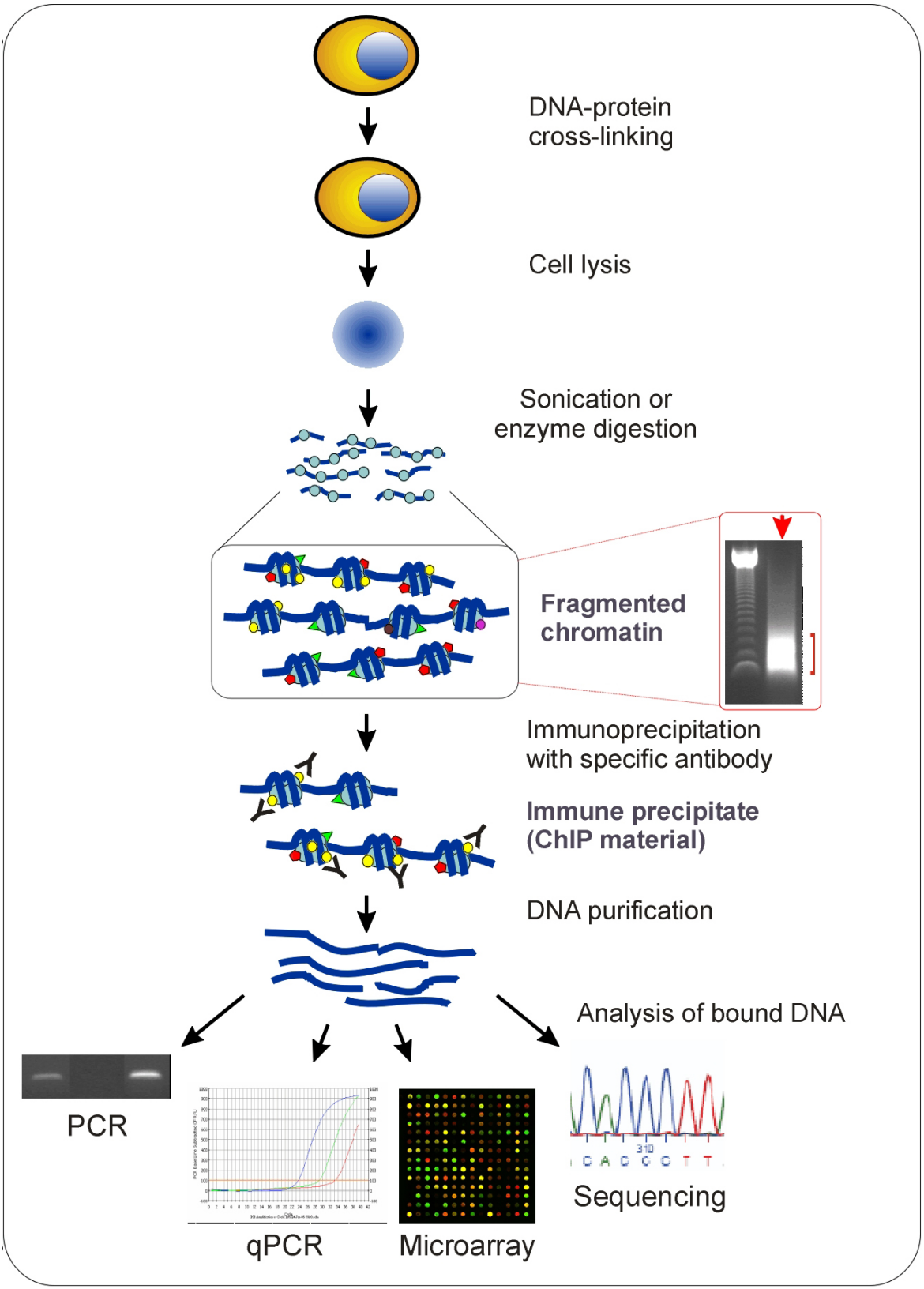


**Figure 1.** The chromatin immunoprecipitation (ChIP) assay.

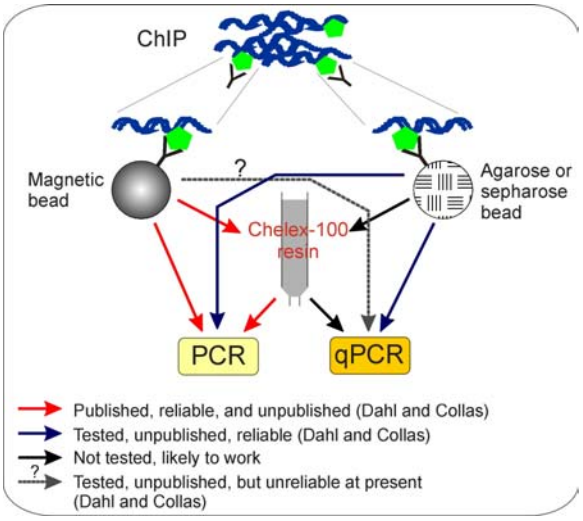


**Figure 2.** Approaches to accelerate analysis of ChIP DNA fragments. ChIP DNA precipitated using magnetic or paramagnetic beads (left) can be directly used as template for PCR (34) or processed through a Chelex-100 DNA purification resin (30,31) prior to PCR. Chelex-100-purifed DNA can also be used in quantitative (q)PCR assays. Use of DNA in the ChIP complex bound to magnetic bead directly as template for qPCR has proven to be unreliable in our hands (unpublished data) due to opacity of the magnetic beads which interferes with SYPBR® Green detection. Alternatively, ChIP complexes are precipitated with agarose or sepharose beads (right). These are compatible with direct PCR and direct pPCR (our unpublished data).

Bearing this in mind, whether end-point or quantitative on-bead PCR can be performed depends at present on the nature of the carrier beads used in the ChIP assay. Direct on-bead PCR has been shown to be successful with magnetic Protein A beads (34) (Dahl and Collas, unpublished data) as well as with agarose-conjugated Protein A/G beads (Dahl and Collas unpublished data). Furthermore, we have shown that ChIP products precipitated by agarose beads can be directly analyzed by qPCR using SYBR® Green (Dahl and Collas, unpublished data). This is in contrast to magnetic beads which due to their opacity, interfere with quantification of the SYBR® Green signal during the real time PCR (Figure 2). Collectively, these observations argue that while direct qPCR is possible with ChIP templates bound to agarose, and probably sepharose, beads, the composition of magnetic beads makes them at present incompatible with qPCR.

## 4. REDUCING CELL NUMBERS FOR ChIP

Until recently, a major drawback of ChIP protocols has been the requirement for large cell numbers. This has been therefore necessary to compensate for the loss of cells upon recovery after cross-linking, loss of material to surfaces throughout the procedure, impaired signal/noise ratio upon reduction of input material, and to some extent for the relative insensitivity of detection of ChIP-enriched DNA. In addition, large cell numbers used for ChIP are presumably the result of the assumption that immunoprecipitation is inefficient. We have recently shown that even cross-linked chromatin can be precipitated with high efficiency (33). The need for elevated cell numbers has limited the application of ChIP to rare or precious cell samples, such as cells from tissue biopsies, rare stem cell populations or cells from embryos. Two recent publications have addressed this issue and report alterations of conventional ChIP protocols to make the technique applicable to smaller numbers of cells.

### 4.1. Carrier ChIP (CChIP)

The rationale behind carrier ChIP, or CChIP, is that the immunoprecipitation of a small amount of chromatin prepared from few mammalian cells (say, 100-1,000) is facilitated by the addition of carrier chromatin from *Drosophila* – or potentially from any species sufficiently evolutionarily distant from the species investigated (24) (Figure 3). We suggest that this is because sample chromatin loss and unspecific background are virtually eliminated by excess *Drosophila* chromatin coating the surface of the reaction vessel. CChIP involves the mixing of cultured *Drosophila* cells with a relatively small number of mammalian cells prior to preparing nuclei and chromatin. Native chromatin fragments are prepared from purified nuclei by partial digestion with micrococcal nuclease and immunoprecipitated using antibodies to modified histones. To compensate for the small amount of DNA precipitated, the ChIP DNA is detected by radioactive PCR using [$\alpha^{32}$P]dCTP, electrophoresis and phosphorimaging (Figure 3). Consequently, specificity of amplification is monitored for each ChIP by determination of the size of the DNA fragment produced (24).

Remarkably, CChIP has been shown to be suitable for the analysis of a few as 100 cells per sample. A limitation, however, is that analysis of multiple histone modifications require multiple aliquots of 100 cells which may or may not be identical depending on how sampling is carried out. Furthermore, in its current published form, CChIP is based on the conventional NChIP procedure (23) and thus is suitable for precipitation of histones but not of TFs. There is, however, no reason to believe that CChIP cannot be applicable to cross-link ChIP and thereby become a more versatile tool. Despite these limitations, the benefit of CChIP for epigenetic analyses of small cell samples is already clear.

Using CChIP, the landmark paper of O'Neill and colleagues (24) reports for the first time an analysis of histone modifications in the two cell types of the mouse blastocyst, namely the trophectoderm (the outer cell layer giving rise to the placenta) and the inner cell mass (ICM) which gives rise to the embryo proper. Analysis of transcriptionally activating (H4K16 acetylation and H3K4 trimethylation) and repressive (H3K9 methylation) histone modifications on the developmentally regulated gene promoters *Nanog*, *Oct4* and *Cdx2* showed that these modifications marked active and silent genes, as anticipated, in the ICM and trophectoderm. However, examination of genes transcriptionally repressed in both

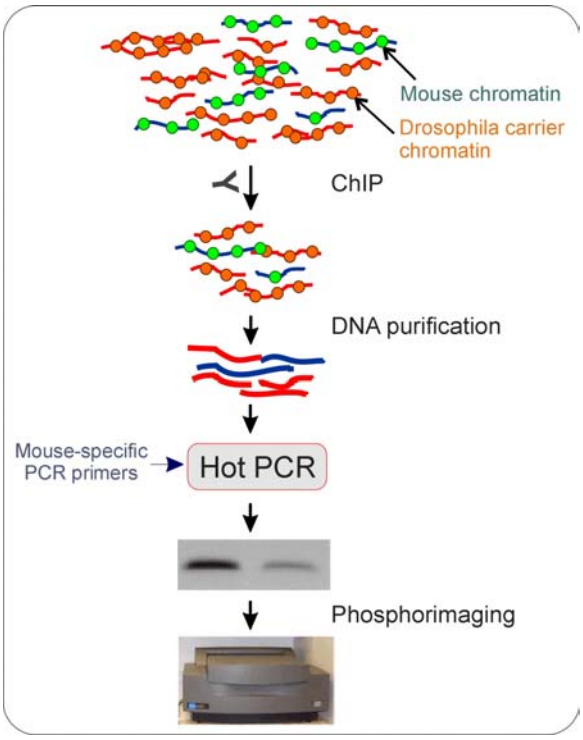


**Figure 3.** Carrier ChIP (CChIP). By mixing a (mammalian) chromatin sample of interest with an excess of *Drosophila* chromatin as a carried, CChIP allows the analysis of histone modifications in small cell numbers (down to 100).

ICM and ES cells (derived from the ICM) indicated that the intensity of silencing histone marks was reduced in ES cells relative to the ICM, suggesting a loosening of chromatin structure at repressed loci upon culture (24). Application of ChIP to embryonic TFs (such as Oct4 and Nanog) in the ICM and ES cells to unravel common and distinct binding sites in the genome should significantly advance our understanding of the molecular basis of pluripotency.

### 4.2. Q$^2$ChIP, a quick and quantitative ChIP assay for small cell numbers

Upon publication of the CChIP assay (24), an alternative ChIP protocol was being developed from conventional ChIP to accommodate relatively small cell samples in a fast and non-radioactive manner (33). We systematically evaluated and modified critical steps in a classical ChIP assay (29) to develop a quick and quantitative (Q$^2$)ChIP protocol suitable for up to 1,000 ChIPs from as few as 100,000 cells (33) (Figure 4A,B). In its current version, Q$^2$ChIP involves a chromatin preparation from a larger number of cells than CChIP, but includes chromatin dilution and aliquoting steps. This enables many ChIPs from the same pool of cells, and therefore the use of chromatin amounts as low as for CChIP (Figure 4A). In addition, Q$^2$ChIP involves a cross-linking step, resulting in samples suitable for immunoprecipitation of any DNA-associated protein (Figure 4C). DNA protein cross-linking in suspension in the presence of the histone deacetylase inhibitor sodium butyrate, elimination of virtually all non-specific background chromatin through a tube-shift step following washes of the ChIP material, and combination of DNA elution, cross-linking reversal and protein digestion into a single 2-h step (see also above) were shown to considerably shorten the ChIP procedure and enhance ChIP specificity and efficiency (33) (Figure 4A,B). Suitability of Q$^2$ChIP to small amounts of chromatin is mainly attributed elimination of background chromatin, to enable signal detection. Furthermore, increased ratio of antibody-to-target epitope in the immunoprecipitation step is critical as this has been shown to enable precipitation of 60-90% of a specific locus (33). The Q$^2$ChIP assay has been validated against the conventional ChIP protocol from which it was derived (29) and used to illustrate changes in histone H3K4 and K9 and K27 acetylation or methylation patterns associated with differentiation of human embryonal carcinoma cells on developmentally regulated gene promoters (33).

Despite its suitability for immunoprecipitation of histones and TFs, Q$^2$ChIP is currently limited by the requirement for 100,000 cells as starting material – although up to 1,000 ChIPs can be performed from a single chromatin preparation. In a second version of the Q$^2$ChIP assay, we have reduced this starting cell number to 1,000, and from this few cells, our procedure enables ~10 different histone ChIPs (Dahl and Collas, unpublished data). Additional refinements in the procedure are expected to make Q$^2$ChIP amenable for several TF immunoprecipitations from 1,000 cells in a single day's work. Furthermore, implementation of an efficient ChIP-enriched DNA amplification step should make Q$^2$ChIP amenable for microarray- or cloning-based genome-wide analyses (see below) from limited cell numbers.

## 5. SEQUENTIAL ChIP: ANALYSIS OF MULTIPLE CO-EXISTING HISTONE MODIFICATIONS ON SINGLE LOCI

The epigenetic profile of mouse ES cells has started to emerge through ChIP-PCR and whole-genome (ChIP-chip; see below) analyses of histone modifications. Notably, trimethylated H3K4 (H3K4m3), a mark typically associated with transcriptionally active chromatin, has been identified on a subset of promoters also bearing H3K27m3, a mark of silent chromatin (35). Interestingly, at the genome-wide level, these so-called "bivalent domains" correspond to TF genes expressed at low levels and to transcriptionally silent developmentally regulated genes (36). These findings led to the proposal that these bivalent histone marks tag silent genes that in undifferentiated ES cells, remain poised for transcriptional activation (35,36). Upon differentiation, H3K27 becomes demethylated, enabling activation of the genes.

A limitation of current ChIP assays, however, is the impossibility to determine whether two distinct histone modifications, such as H3K4m3 and H3K27m3, coexist on the same nucleosome as a bivalent mark, or whether they are found on the same locus but in distinct nucleosomes or in subpopulations of cells in the sample examined. A similar question applies to the coexistence of two

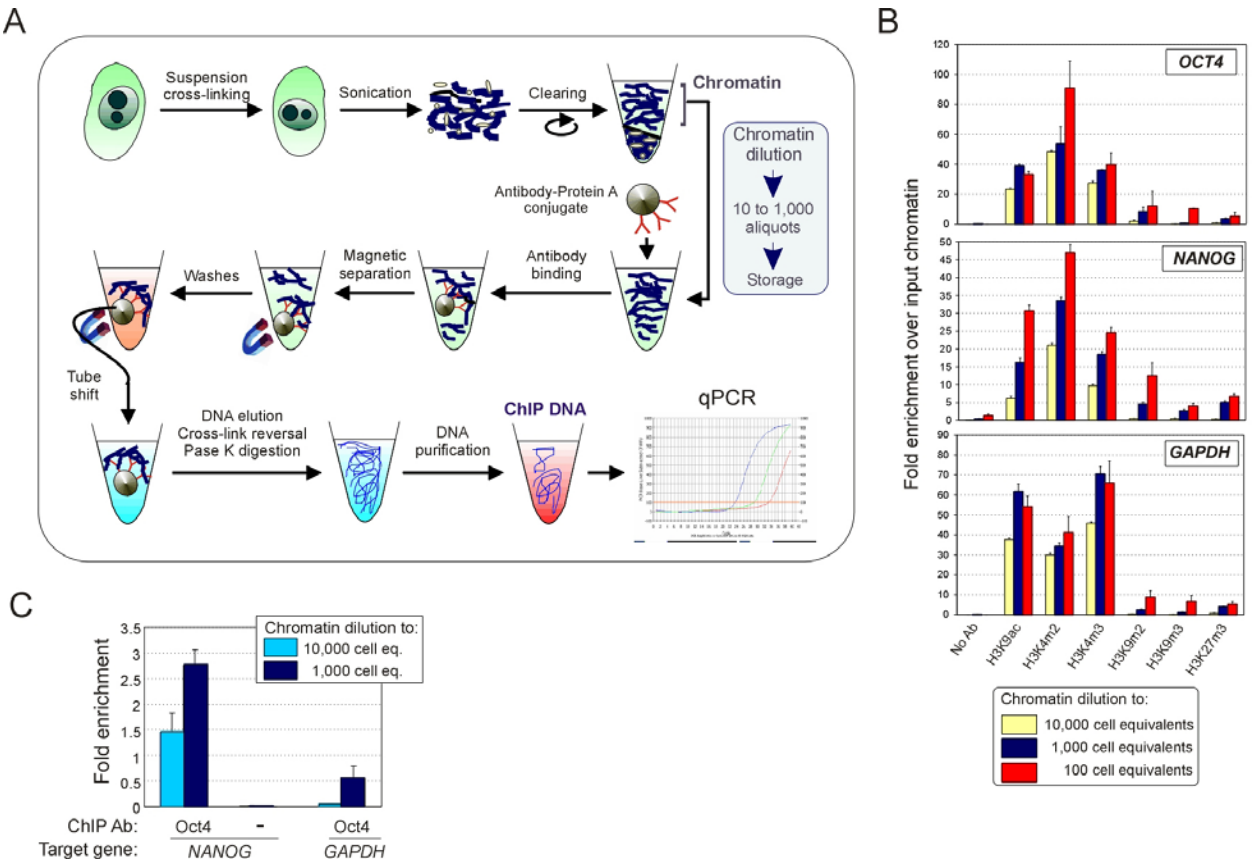


**Figure 4.** Quick and quantitative ($Q^2$)ChIP. (A) The $Q^2$ChIP assay. (B) $Q^2$ChIP analysis of histone modifications on the *OCT4*, *NANOG* and *GAPDH* promoters in embryonal carcinoma cells. Chromatin was prepared from 100,000 cells and diluted 10-, 100- or 1,000-fold prior to ChIP to produce chromatin from 10,000, 1,000 or 100 cell equivalents, respectively. (C) $Q^2$ChIP analysis of Oct4 protein binding to the *NANOG* and *GAPDH* promoters in carcinoma cells. Reproduced and modified with permission from (33).

transcription factors on a given locus. To resolve this issue, Metivier and colleagues (37) have developed a sequential ChIP assay, whereby a first factor is immunoprecipitated from a chromatin sample, and the second factor is subsequently immunoprecipitated from the first ChIP complex. Bernstein et al. (36) have taken advantage of sequential ChIP to demonstrate the existence of bivalent histone marks on specific loci. ES cell chromatin was first immunoprecipitated with antibodies against H3K27m3 and the ChIP chromatin was used for a second immunoprecipitation using anti-H3K4m3 antibodies. Sequential immunoprecipitation, then, retains only chromatin which concomitantly carries both histone marks. This approach showed that the transcription start site of the *Inx2* gene contained both trimethylated H3K4 and H3K27, a result confirmed by reciprocal sequential ChIPs (36). Sequential ChIP has also recently been used to demonstrate that the TFs Oct4 and Sox2 are bound to the same target DNA molecule in mouse ES cells (21). Of note, however, to refine the level of analysis and ensure that TFs or histone modifications examined are really on the same nucleosome on a given locus, it is critical to digest chromatin to mono-nucleosomes, as opposed to poly-nucleosomes.

## 6. ChIP-ON-BEADS: FLOW CYTOMETRY ANALYSIS OF ChIP PRODUCTS

Quantitative determination of the amount of DNA associated with an immunoprecipitated histone modification or TF is routinely carried out by qPCR (33,38,39). The advent of flow cytometry into an increasing number of laboratory protocols has prompted its application to the analysis of ChIP DNA fragments. The method relies on microbead capture of conventional PCR products from a ChIP template and analysis of these products by flow cytometry (40). It is, however, a rather complicated technique. In this assay, standard ChIP is performed, and the ChIP DNA is used as template in an end-point PCR reaction in which primers are tagged in their 5' end with Fam (forward primer) and biotin (reverse primer). The Fam/biotin PCR products are purified and analyzed by flow cytometry after capture onto microbeads. Of note, PCR reactions must be labeled in the linear phase (this is initially determined in parallel qPCRs) to ensure reliable quantification by flow cytometry. The similarity of data obtained by qPCR and by flow cytometry has been shown in a determination of acetylated H4 and methylated H3K4 patterns on the *transglutaminase type-2* (*TGM2*) gene in Jurkat cells (40).

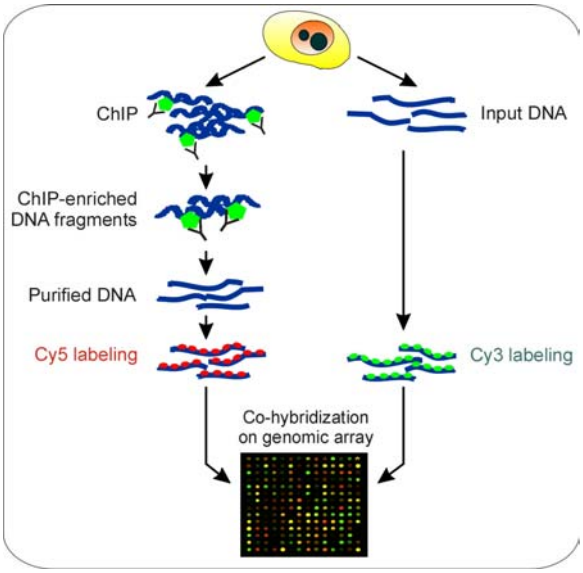


**Figure 5.** The ChIP-chip approach. A protein of interest (green pentagon) is selectively immunoprecipitated by ChIP. The ChIP-enriched DNA is amplified by PCR and fluorescently labeled with, e.g., Cy5. An aliquot of purified input DNA is labeled with another fluorophore, e.g., Cy3. The two samples are mixed and hybridized onto a microarray containing genomic probes covering the whole genome or to a high-resolution tiling array covering a region of interest. Binding of the precipitated protein to a target site is inferred when intensity of the ChIP DNA (red Cy5 labeling) significantly exceeds that of the input DNA (green Cy3 labeling) on the array.

The ChIP-on-beads assay has been proposed to be useful for quantitative assessments ChIP products in a high-throughput manner (40). However, the complexity of the procedure makes it at present difficult to foresee the advantage of ChIP-on-beads over ChIP-qPCR or ChIP-chip approaches (see section 7 below), especially as long as a qPCR analysis of ChIP products remains anyway necessary for evaluation of the linear phase of PCR-mediated labeling step. Nonetheless, considerable simplification of the ChIP DNA fragment labeling procedure would conceivably make the ChIP-on-beads approach amenable for assessing large numbers of samples for a limited number of genes.

## 7. GENOME-WIDE MAPPING OF DNA BINDING PROTEINS

A strong limitation of the ChIP technology has for several years been the restriction of the analysis to pre-determined candidate target sequences analyzed by PCR using specifically chosen primers. Thus, objectivity of the approach has been hampered by a bias towards the sequences of interest. To alleviate this limitation and extend the power of the ChIP assay, several strategies have been developed to enable application of ChIP to the discovery of novel target sites for transcriptional regulators and to map the positioning of post-translationally modified histones throughout the genome. These genome-wide approaches have immensely contributed to characterizing the chromatin landscape in the context of gene expression, "stemness", cell differentiation and disease.

### 7.1. ChIP-chip

The advent of cDNA and oligonucleotides microarrays has in the past decade revolutionized analyses of gene expression and our understanding of transcription profiles. Subsequent development of genomic DNA microarrays (chips) has, when combined with ChIP assays, enabled the mapping of TF binding sites (41,42) and of histone modifications (43,44) on large areas in the genome through an approach known as ChIP-chip (also called ChIP-on-chip). Despite its relatively recent introduction, ChIP-chip has already largely been exploited to, e.g., map c-myc binding sites in the genome (45,46), elaborate Oct4, Nanog and Sox2 transcriptional networks in mouse ES cells (20) and identify polycomb target genes (47,48). Several reviews dedicated to ChIP-chip and variations and limitations thereof have recently been published (25,26,49-52), thus we only provide here a brief account of the technology.

ChIP-chip differs from ChIP-PCR only in the method of analysis of the precipitated DNA (Figure 5). ChIP DNA is eluted after cross-link reversal and the ends repaired with a DNA polymerase to generate blunt ends. A linker is applied to each DNA fragment to enable PCR amplification of all fragments. A fluorescent label (usually Cy5) is incorporated during PCR amplification. Similarly, an aliquot of input DNA is labeled with another fluorophore, usually Cy3. The two samples are mixed and hybridized onto a microarray containing oligonucleotide probes covering the whole genome or portions thereof, or probes tiling a region of interest (such as a given promoter). In this dual-color approach, binding of the immunoprecipitated TF to a specific region is established when intensity of the ChIP DNA significantly exceeds that of the input DNA on the array (Figure 5). Statistical analysis softwares and evaluation by the investigator determine the significance of enrichment of the precipitated factor to a specific motif in the region examined. A detailed procedure for ChIP-chip has recently been published elsewhere (27).

### 7.2. ChIP-display

ChIP-chip approaches are in effect only as informative as the oligonucleotide microarrays onto which the ChIP-enriched DNA is hybridized. This limitation has stimulated the development of methods for unbiased determination of genomic sequences associated with a given histone modification or TF. Novel TF binding sites can be identified by cloning and sequencing DNA from the ChIP material (53,54). However, the overwhelming excess of non-specifically precipitated DNA fragments (such as repeat sequences) makes ChIP-cloning unpractical. To overcome this limitation, a ChIP-display strategy has been designed and applied to the identification of target genes occupied by the TF Runx2 (55) (Figure 6). ChIP-display concentrates DNA fragments containing each target sequence and scatters the remaining, non-specific, DNA. Target sequences are concentrated by restriction digestion, as fragments harboring the same target site acquire the

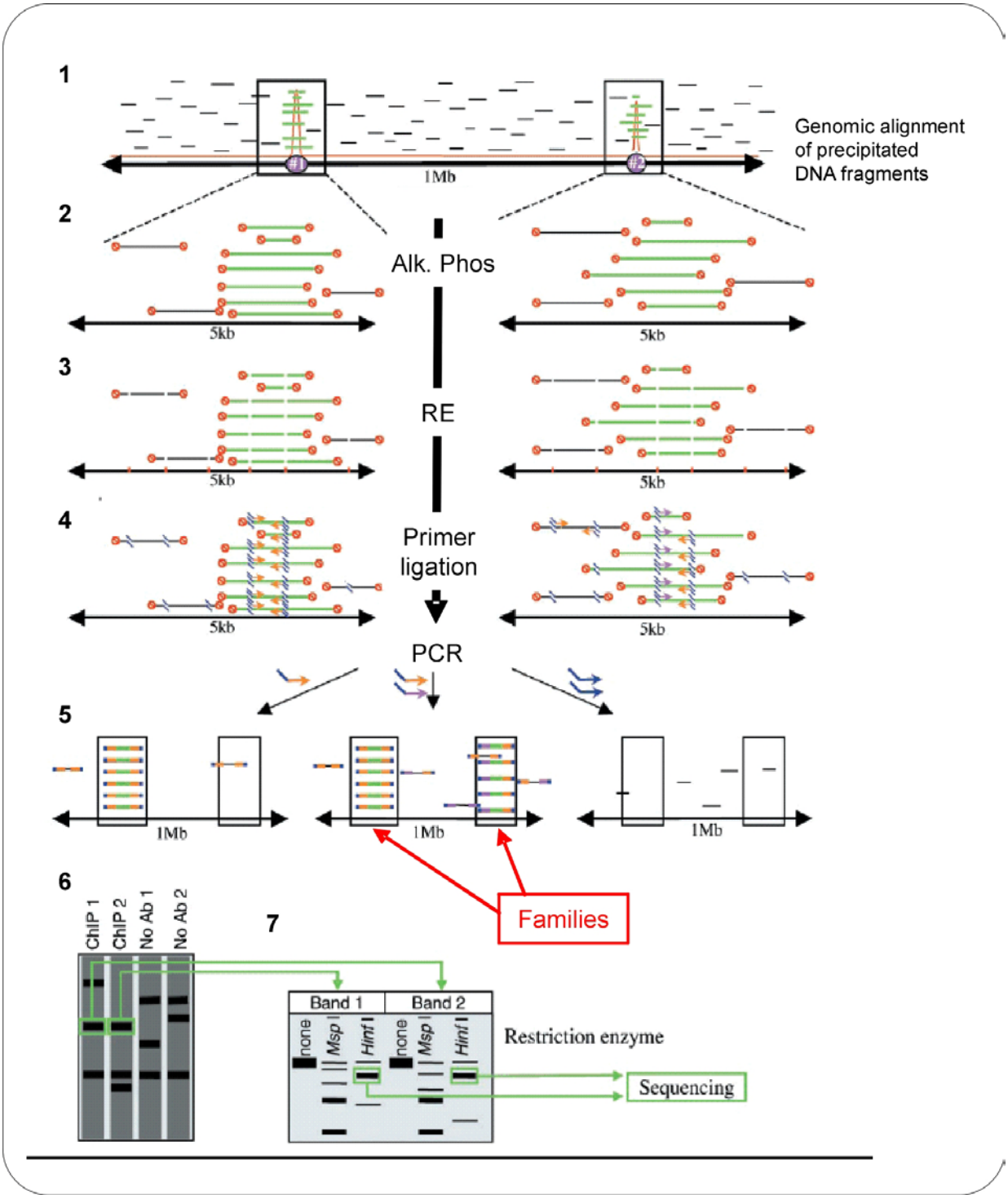


**Figure 6.** ChIP-display. Step 1: non-specific (black bars) and specific (green bars) ChIP DNA fragments are aligned with the genome; two target motifs are presented (#1 and #2). Step 2: DNA fragments are dephosphorylated (red ends) with alkaline phosphatase. Step 3: DNA is digested with a restriction enzyme (RE) and linkers are ligated to the end of the DNA fragments (Step 4). Nested PCR primers that amplify target regions #1 and #2 are shown. Step 5: PCR products amplified in three reactions generating two distinct product families. Step 6: PCR products, here from two ChIPs and two control ChIPs, are separated by PAGE. Step 7: Relevant co-migrating bands from each ChIP are excised, reamplified and analyzed by RE digestion. Strong comigrating bands are sequenced. Reproduced and modified with permission from (55).

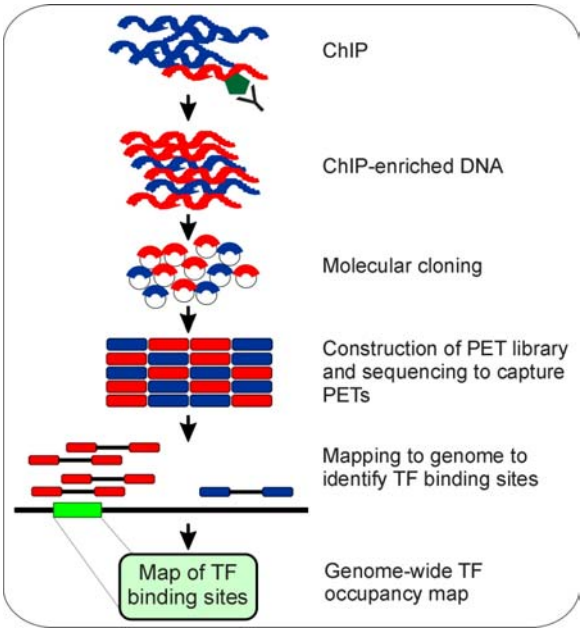


**Figure 7.** ChIP-PET. In the ChIP-PET approach, a TF of interest (green pentagon) is immunoprecipitated by ChIP. The ChIP DNA is cloned into a plasmid-based library, which is restriction-digested to generate a library containing concatenated paired end ditag (PET) sequences. Tags are 18-bp long and contain the 5' and 3' ends of the ChIP DNA fragments cloned into the original library. Location of the sequenced PET sequences is mapped to the genome. Overlap of at least four PET sequences constitutes high-confidence TF binding sites (red tags), whereas random recovery of genomic DNA is manifested in the form of singletons (21). Modified with permission from (21).

same size and therefore can be concentrated by electrophoresis. To scatter non-specific fragments, the total pool of restriction fragments is divided into families on the basis of identity of nucleotides at the ends of these fragments. Because all restriction fragments displaying each given target harbor the same nucleotide ends, they remain in the same family and the family detection signal on gel is not altered. Non-specific background fragments, however, are scattered into many families so that each family detection signal is markedly lower (see Figure 6) (55).

The ChIP-display approach is illustrated in Figure 6 (55). Following ChIP, the precipitated DNA is dephosphorylated by alkaline phosphatase treatment to block ligation of linkers to DNA ends produced by sonication. DNA is digested with a restriction enzyme. Linkers are ligated and multiple combinations of nested primers enable parallel amplification of fragments belonging to one family. DNA fragments amplified from at least two independent ChIPs and two control ChIPs are resolved by polyacrylamide gel electrophoresis (PAGE). Strong bands detected reproducibly among the ChIP products but not in control samples are expected to be target candidates for the TF investigated. These may be excised from the gel, re-amplified, restriction analyzed and products are resolved by PAGE. Co-migrating restriction products are purified from the gel and sequenced.

What are advantages and limitations of ChIP-display? The approach can unravel transcription targets in ChIPs that are enriched for targets by as little as 10- to 20-fold over bulk chromatin (55), and as such shows reasonable sensitivity. Furthermore, as with other ChIP assays, ChIP-display may be used to identify genomic targets for proteins that bind DNA indirectly, depending on the nature of the cross-linker used (see above). The technology appears relatively simple and requires common molecular biology reagents and equipment. Interestingly, gel electrophoresis display of ChIP DNA products allows a direct comparison of patterns (i.e., targets) obtained from different cell types or after different cell treatments (e.g., undifferentiated versus differentiated cells). Indeed, the approach was used to display Runx2 target families from osteoblasts and chondrocytes in parallel (55). ChIP-display is also relatively insensitive to background which characterizes ChIP-PCR or ChIP-chip approaches. Because of the potentially large number of families obtained, however, ChIP-display is not well suited for a comprehensive analysis of target sequences for proteins with a large number of genomic targets, such as SP1, GATA proteins, histone deacetylases, polycomb proteins or RNA polymerase II (55), or for the mapping of histone modifications. It is better suited for TFs with a more limited number of targets, however it lacks the quantification of the relative abundance of a TF associated with a given locus, which is enabled by qPCR.

### 7.3. ChIP-PET

A second strategy developed in response to the limitations of the ChIP-chip assay has been based on the sequencing of portions of the precipitated target DNA. Indeed, with a limited survey of the cloned ChIP DNA fragment library, distinguishing between genuine binding sites and noise without additional molecular validation is challenging. In contrast, with a wide sampling of the ChIP DNA pool, sequencing-based approaches can potentially identify DNA fragments enriched by ChIP. A hindrance to sequencing hundreds of thousands of clones, however, is time and cost involved.

The ChIP-paired end ditag (PET) technology exploits the efficiency of sequencing short tags, rather than entire inserts, to enhance information content and thereby increase accuracy of mapping to the genome (28) (Figure 7). ChIP-PET relies on the recently reported gene identification signature strategy (GIS), in which 5' and 3' signatures of full length cDNAs are extracted into PETs that are concatenated for efficient sequencing (56,57). The sequences are subsequently mapped to the genomic sequences to delineate the transcription boundaries of every gene. As in the GIS strategy, a pair of signature sequences (tags) is extracted from the 5' and 3' ends of each ChIP DNA fragment, concatenated and mapped to the genome (Figure 7).

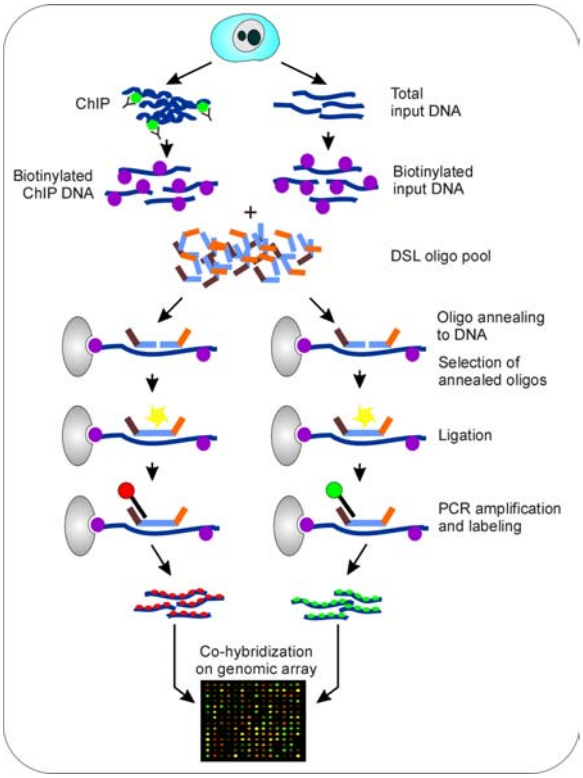


**Figure 8.** ChIP-DSL. A microarray of 40-mer probes is first constructed, onto which the ChIPed and selected DNA fragments will be hybridized. A pair of 20-mer "assay oligos" is synthesized corresponding to each half of each 40-mer. These 20-mer oligos are flanked by a universal primer binding site. Oligos are mixed into a "DSL oligo pool". After conventional ChIP, purified ChIP DNA is biotinylated (purple dots) and annealed to the DSL oligo pool. Annealed fragments are captured on streptavidin-conjugated beads and those paired by a specific DNA target motif are ligated. Thus only correctly targeted oligonucleotides are turned into templates for PCR. One of the PCR primers is fluorescently labeled (red dot) to enable detection after hybridization on the 40-mer probe microarray. The DSL procedure is also carried out for input DNA using PCR primers labeled with a different fluorophore (green dot). Modified with permission from (59).

The PET approach has recently been exploited to characterize ChIP DNA fragments in order to achieve unbiased, genome-wide mapping of TF binding sites (21,28). From a saturated sampling of over 500,000 PET sequences, Wei and colleagues characterized over 65,000 unique p53 ChIP DNA fragments and established overlapping PET clusters to define p53 target sequences with high specificity. The analysis also enabled a refinement of the consensus p53 binding motif and unraveled nearly 100 previously unidentified p53 target genes implicated in p53 function and tumorigenesis (28). In addition, a ChIP-PET analysis of binding sites for Oct4 and Nanog (two ES cell-specific TFs associated with pluripotency) in the mouse ES cell genome has allowed the establishment of a transcription network regulated by Oct4 and Nanog in ES cells, through the identification of over 1,000 and 3,000 high-confidence target sites for the respective factors (21).

### 7.4. How does ChIP-PET compare to ChIP-chip?

ChIP-chip and ChIP-PET are two ChIP strategies that rely on genome-wide or quasi genome-wide methods to determine target sites. In contrast to ChIP-chip, ChIP-PET proves to be a truly genome-wide approach because the ChIP-enriched DNA fragments are cloned and sequenced. ChIP-chip relies on predetermined oligonucleotides printed on an array, and as such is limited by (i) the sequence information encoded in these oligonucleotides, and (ii) quality of the array and of the hybridization. ChIP-PET also enables the prediction of novel DNA motifs that mediate protein-DNA interactions, such as the common Sox2-Oct4 motif and a Nanog binding motif (21). Continuous improvements of genomic arrays, together with increasing probe density throughout the genome, however, largely contribute to making ChIP-chip a reliable method. ChIP-chip is also less demanding than ChIP-PET in terms of time and cost.

How do ChIP-chip and ChIP-PET results compare? A stringent direct technical comparison is at present premature from available information because the species examined to date are different (20,21). Nevertheless, support for each technology in the context of the transcriptional circuitry of ES cells is provided by the findings that Oct4, Sox2 and Nanog occupy both transcriptionally active as well as inactive loci in mouse and human ES cells. In addition, a large fraction of these inactive genes encode transcription factors essential for lineage specification. However, a recent comparison of the Oct4 and Nanog target genes in the Boyer (20) and Loh (21) studies indicates relatively little similarity between the two species (58). Additional detailed analyses will be necessary to distinguish between real species differences and artifacts generated by platform differences.

### 7.5. ChIP-DSL

With the aim of detecting DNA target motifs with higher sensitivity and specificity than through conventional ChIP-chip, a multiplex assay coined as ChIP-DSL was recently introduced. ChIP-DSL combines conventional ChIP with a DNA ligation and selection step (59). The assay involves the pre-determined use, or construction, of a microarray of 40-mer probes onto which the ChIPed and selected DNA fragments are to be hybridized. The reason for this is that a pair of 20-mer "assay oligonucleotides" is synthesized corresponding to each half of each 40-mer. These 20-mer oligonucleotides are flanked by a universal primer binding site. These oligonucleotides are mixed into a "DSL oligo pool" (Figure 8). Following conventional ChIP, the purified ChIP DNA is randomly biotinylated and annealed to the DSL oligo pool. The annealed fragments are captured on streptavidin-conjugated magnetic beads, allowing elimination of the non-annealed fragments (the noise). All selected DNA fragments are immobilized onto the beads and those paired by a specific DNA target motif are ligated. Thus, the correctly targeted oligonucleotides

are specifically turned into templates for PCR amplification. One of the PCR primers is fluorescently labeled to enable detection after hybridization on the 40-mer probe microarray. The DSL procedure is also carried out for input DNA using PCR primers labeled with a different fluorophore (Figure 8).

ChIP-DSL has recently been implemented to identify a large number of novel binding sites for the estrogen receptor alpha in breast cancer-derived MCF7 cells (59). ChIP-DSL has also been used to demonstrate the widespread recruitment of the histone demethylase LSD1 on active promoters, including most estrogen receptor alpha gene targets, and to show that LSD1 is required for gene activation and to antagonize the function of several histone methyltransferases (60).

ChIP-DSL presents several advantages over classical ChIP-chip (59). Firstly, only unique signature motifs are targeted, alleviating potential interference with repetitive and related sequences upon hybridization. Secondly, sensitivity of the assay is increased due to the PCR amplification step. Of note, amplification is claimed to be unbiased because DNA fragments bear the same pair of specific primer binding sites and have the same length. Thirdly, the selection process combined with amplification allows a reduction in starting cell number for ChIP, a significant improvement when considering performing ChIP-chip approaches with limited amounts of material. The counterpart of these advantages, however, is the "homework" required prior to doing the experiment in order to design either the array and/or synthesize the 20-mer paired DSL oligonucleotides.

### 7.6. Comparison of multiple genome-wide ChIP data sets

Inasmuch as with microarray-based gene expression studies (61), robust comparative analysis of genome-wide ChIP data for histone modifications or TFs from different data sets relying on different technology platforms will be necessary to obtain a more uniform and unbiased view of the chromatin landscape in different cell types. A recent comparison of eight independent TF ChIP experiments using three different genome-wide platforms (ChIP-chip with Agilent microarrays, ChIP-chip with Affymetrix microarrays and ChIP-PET) has highlighted key factors to consider when analyzing such data sets (62). In essence, Ji et al. (62) first emphasize that to date's ChIP-chip and ChIP-PET approaches are well suited for non-ambiguous identification of TF target motifs in the mammalian genome because the signal-to-noise ratio is in general sufficient and does not necessarily require independent validation by other means. However, certain sequence patterns may be detected in binding regions of multiple TFs, even after repeated masking, thus these sites should be interpreted with caution. Furthermore, sequence motifs non-specifically enriched in ChIP products may mask the identification of the motif that is really of interest in the various studies, so these should be carefully dissected out. Next, methods for defining threshold values for what is being identified as a target motif and what is not should be carefully chosen. Randomly choosing genomic controls does not appear to be sufficient, because in all eight ChIP experiments analyzed, TF binding regions harbored a higher CG content than the average genome-wide level (62). The increasing number of genome-wide or tiled locus-specific analyses of TF binding sites is expected to provide additional criteria for interpretation of complex data sets. Software and recommendations for analyzing ChIP-chip data based on NimbleGen arrays have recently been published (63). However, no comparative study of genome-wide mapping of histone modifications using different platforms has to our knowledge been reported to date.

## 8. WHICH RELEVANT CONTROL FOR A ChIP EXPERIMENT?

In spite of improvements in the ChIP assays to reduce or eliminate background chromatin (noise) (33), background does exist and needs to be accounted for using appropriate negative controls. A survey of the ChIP literature reveals the use of various controls, the nature of which seems to mainly depend on the investigator. One classical negative control is the use of no antibodies (also often referred to as a "bead-only" control). In effect, bead-only controls for unspecific binding of chromatin fragments to the beads used to precipitate the complex of interest. Although it is useful, this control is not as stringent as using an irrelevant antibody, preferably of the same isotype as the experimental antibody, in a parallel chromatin preparation (27). Enhanced stringency of the control implies the use or an irrelevant antibody against a nuclear protein. A third negative control consists of comparing, in the same ChIP, enrichment of a target sequence relative to enrichment of another, irrelevant, genomic region. This control was, e.g., performed in our laboratory to demonstrate the specificity of occupancy of Oct4 on the *NANOG* promoter in pluripotent carcinoma cells, whereas it is virtually absent from the *GAPDH* promoter (Figure 4C) (33). In ChIP-PCR experiments, the negative control may generate a PCR signal that can be used as reference to express a ChIP-specific enrichment. In addition to a negative control, some investigators use a positive control, such as a high-quality antibody against a well characterized ubiquitous transcription factor (27). Positive control antibodies are particularly important when setting up new methodologies.

## 9. ADDITIONAL DEVELOPMENTS

### 9.1. DamID: A METHYLATION-BASED PROTEIN TAGGING APPROACH

In addition to the techniques reviewed here, several strategies have been designed to investigate other aspects of genome and chromatin function. An alternative to directly immunoprecipitating the protein of interest from chromatin (let it be a histone or a TF) is to label the DNA near the target site of the protein of interest. Labeling may consist of a methylation tag put on by a DNA binding protein (the TF of interest) fused to DNA adenine methyltransferase (DamID approach) (64). Binding of the TF-Dam protein to DNA elicits adenine methylation within GATC sites in the vicinity of the protein target site. As a control, Dam alone is used. Sites methylated by TF-Dam as opposed to Dam alone are detected by digestion with a

methyl-specific restriction enzyme (e.g., *Dpn*I which cuts only at methylated GATC sites). The digestion products are purified or amplified using a methylation-specific PCR assay, labeled, and hybridized onto a microarray. DamID has been successfully used to unravel binding sites for TFs, DNA methyltransferases and heterochromatin proteins in *Drosophila*, *Arabidopsis* and mammals (65-69). DamID has recently been reviewed in the context of gene expression landscaping (70) and as a critical comparison with the ChIP-chip strategy (49).

### 9.2. ChIP-BA: combining ChIP with bisulfite genomic sequencing analysis of DNA methylation

A deeper understanding of the interplay between histone modifications, DNA methylation, TF binding and gene expression will necessitate the combination of multiple analyses from a single chromatin or DNA sample. As mentioned earlier, the CG content of a TF target site, and thus its methylation state, is likely to affect binding (62). In an attempt to relate TF binding to DNA methylation, ChIP has been combined with bisulfite genomic sequencing analysis (ChIP-BA) (71). There, ChIP DNA fragments are processed for both PCR analysis (or potentially for array hybridization) and for bisulfite conversion to determine the CpG methylation pattern. ChIP-BA has been used to in parallel determine the DNA methylation requirements for binding of a methyl-CpG binding protein (71). The method can also potentially be useful to unravel methylation patterns that are compatible, or incompatible, with the targeting of a specific protein to a genomic region (71). A potential problem with the ChIP-BA approach, however, is noise which here is directly turned into a sequence which may be irrelevant. Subtractive strategies may conceivably be utilized provided appropriate controls are performed (see section 8).

### 9.3. Methyl-DNA immunoprecipitation (MeDIP)

A variation of the ChIP assay has been introduced to determine genome-wide profiles of DNA methylation. Methyl-DNA immunoprecipitation, or MeDIP, consists of the immunoprecipitation of methylated DNA fragments using an antibody to 5-methyl cytosine (5mC) (72,73). Here however, the starting material is purified DNA, rather than chromatin, fragmented by sonication or by nuclease digestion. Similarly to ChIP DNA fragments, the MeDIP DNA is eluted, fluorescently labeled and hybridized onto a microarray. MeDIP is being increasingly used to map methylation profiles (the "methylome") in a variety of organisms and cell types, most recently with respect to transcriptional silencing in an evolutionary context (74). Although MeDIP is proving to be a potent method, a constraint of the assay is its limitation to regions with a CpG density of at least 2-3% (72).

## 10. PERSPECTIVES

ChIP has undoubtedly become the technique of choice to investigate DNA protein interactions in the cell, map and identify novel binding sites for TFs and other chromatin-associated proteins, and map the positioning of histone modifications and, most recently, histone variants in the genome (5,6). Collectively, these studies depict an increasingly complex epigenetic landscape in the context of gene expression, definition of gene boundaries, cell differentiation and disease. In addition, the advent of ChIP assays suitable for small cell samples has already moved ChIP into the field of early embryo development. Refinements in "small-scale" ChIP assays, in combination with more robust and unbiased ChIP-enriched DNA amplification procedures, are anticipated to promote genome-wide analysis of histone modifications or TF binding in embryos.

ChIP remains a relatively cumbersome procedure with many steps. Attempts at simplifying the assay, together with possibilities for direct PCR analysis of ChIP samples without DNA purification, are starting to emerge and are expected to lead to robust, fast (one day or less) and highly reliable assays. Companies specialized in the development and commercialization of ChIP assays are already thinking along these lines.

Variations of the ChIP assay, in combination with increasingly varied and sophisticated methods of analysis of ChIP products are inevitably going to lead to novel or improved strategies for high-throughput analyses of the chromatin landscape not only in cultured cells, but also in embryos and small tissue biopsies. In an era which puts forward the concept of personalized medicine in a context where epigenetics is increasingly linked to disease (including cancer), automated whole-genome epigenetic analyses of individual patient material is likely to see the light.

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## 12. REFERENCES

1. F. Antequera: Structure, function and evolution of CpG island promoters. *Cell Mol. Life Sci.* 60, 1647-1658 (2003)
2. T. Jenuwein, C. D. Allis: Translating the histone code. *Science* 293, 1074-1080 (2001)
3. M. Lachner, R. Sengupta, G. Schotta and T. Jenuwein: Trilogies of histone lysine methylation as epigenetic landmarks of the eukaryotic genome. *Cold Spring Harb. Symp. Quant. Biol.* 69, 209-218 (2004)
4. B. D. Strahl, C. D. Allis: The language of covalent histone modifications. *Nature* 403, 41-45 (2000)
5. Y. Mito, J. G. Henikoff and S. Henikoff: Histone replacement marks the boundaries of cis-regulatory domains. *Science.* 315, 1408-1411 (2007)
6. Y. Mito, J. G. Henikoff and S. Henikoff: Genome-scale profiling of histone H3.3 replacement patterns. *Nat. Genet.* 37, 1090-1097 (2005)
7. A. Viens, U. Mechold, F. Brouillard, C. Gilbert, P. Leclerc and V. Ogryzko: Analysis of human histone H2AZ deposition in vivo argues against its direct role in epigenetic templating mechanisms. *Mol. Cell Biol.* 26, 5325-5335 (2006)
8. B. Li, S. G. Pattenden, D. Lee, J. Gutierrez, J. Chen, C. Seidel, J. Gerton and J. L. Workman: Preferential

occupancy of histone variant H2AZ at inactive promoters influences local histone modifications and chromatin remodeling. *Proc. Natl. Acad. Sci. U. S. A.* 102, 18385-18390 (2005)
9. A. Li, J. M. Eirin-Lopez and J. Ausio: H2AX: tailoring histone H2A for chromatin-dependent genomic integrity. *Biochem. Cell Biol.* 83, 505-515 (2005)
10. A. J. Ruthenburg, C. D. Allis and J. Wysocka: Methylation of lysine 4 on histone H3: intricacy of writing and reading a single epigenetic mark. *Mol. Cell* 25, 15-30 (2007)
11. M. S. Cosgrove, C. Wolberger: How does the histone code work? *Biochem. Cell Biol.* 83, 468-476 (2005)
12. K. L. Arney, S. Bao, A. J. Bannister, T. Kouzarides and M. A. Surani: Histone methylation defines epigenetic asymmetry in the mouse zygote. *Int. J. Dev. Biol.* 46, 317-320 (2002)
13. H. D. Morgan, F. Santos, K. Green, W. Dean and W. Reik: Epigenetic reprogramming in mammals. *Hum. Mol. Genet.* 14, R47-R58 (2005)
14. J. H. Lee, S. R. Hart and D. G. Skalnik: Histone deacetylase activity is required for embryonic stem cell differentiation. *Genesis* 38, 32-38 (2004)
15. H. Szutorisz, N. Dillon: The epigenetic basis for embryonic stem cell pluripotency. *BioEssays* 27, 1286-1293 (2005)
16. N. Feldman, A. Gerson, J. Fang, E. Li, Y. Zhang, Y. Shinkai, H. Cedar and Y. Bergman: G9a-mediated irreversible epigenetic inactivation of Oct-3/4 during early embryogenesis. *Nat. Cell Biol.* 8, 188-194 (2006)
17. M. Strunnikova, U. Schagdarsurengin, A. Kehlen, J. C. Garbe, M. R. Stampfer and R. Dammann: Chromatin inactivation precedes de novo DNA methylation during the progressive epigenetic silencing of the RASSF1A promoter. *Mol. Cell Biol.* 25, 3923-3933 (2005)
18. P. W. Laird: Cancer epigenetics. *Hum. Mol. Genet.* 14, R65-R76 (2005)
19. H. Shi, S. H. Wei, Y. W. Leu, F. Rahmatpanah, J. C. Liu, P. S. Yan, K. P. Nephew and T. H. Huang: Triple analysis of the cancer epigenome: an integrated microarray system for assessing gene expression, DNA methylation, and histone acetylation. *Cancer Res.* 63, 2164-2171 (2003)
20. L. A. Boyer, T. I. Lee, M. F. Cole, S. E. Johnstone, S. S. Levine, J. P. Zucker, M. G. Guenther, R. M. Kumar, H. L. Murray, R. G. Jenner, D. K. Gifford, D. A. Melton, R. Jaenisch and R. A. Young: Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell* 122, 947-956 (2005)
21. Y. H. Loh, Q. Wu, J. L. Chew, V. B. Vega, W. Zhang, X. Chen, G. Bourque, J. George, B. Leong, J. Liu, K. Y. Wong, K. W. Sung, C. W. Lee, X. D. Zhao, K. P. Chiu, L. Lipovich, V. A. Kuznetsov, P. Robson, L. W. Stanton, C. L. Wei, Y. Ruan, B. Lim and H. H. Ng: The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nat. Genet.* 38, 431-440 (2006)
22. P. Y. Zeng, C. R. Vakoc, Z. C. Chen, G. A. Blobel and S. L. Berger: In vivo dual cross-linking for identification of indirect DNA-associated proteins by chromatin immunoprecipitation. *Biotechniques* 41, 694, 696, 698 (2006)
23. L. P. O'Neill, B. M. Turner: Immunoprecipitation of chromatin. *Methods Enzymol.* 274, 189-197 (1996)
24. L. P. O'Neill, M. D. Vermilyea and B. M. Turner: Epigenetic characterization of the early embryo with a chromatin immunoprecipitation protocol applicable to small cell populations. *Nat. Genet.* 38, 835-841 (2006)
25. S. E. Hanlon, J. D. Lieb: Progress and challenges in profiling the dynamics of chromatin and transcription factor binding with DNA microarrays. *Curr. Opin. Genet. Dev.* 14, 697-705 (2004)
26. D. Sikder, T. Kodadek: Genomic studies of transcription factor-DNA interactions. *Curr. Opin. Chem. Biol.* 9, 38-45 (2005)
27. T. I. Lee, S. E. Johnstone and R. A. Young: Chromatin immunoprecipitation and microarray-based analysis of protein location. *Nat. Protoc.* 1, 729-748 (2006)
28. C. L. Wei, Q. Wu, V. B. Vega, K. P. Chiu, P. Ng, T. Zhang, A. Shahab, H. C. Yong, Y. Fu, Z. Weng, J. Liu, X. D. Zhao, J. L. Chew, Y. L. Lee, V. A. Kuznetsov, W. K. Sung, L. D. Miller, B. Lim, E. T. Liu, Q. Yu, H. H. Ng and Y. Ruan: A global map of p53 transcription-factor binding sites in the human genome. *Cell* 124, 207-219 (2006)
29. V. A. Spencer, J. M. Sun, L. Li and J. R. Davie: Chromatin immunoprecipitation: a tool for studying histone acetylation and transcription factor binding. *Methods* 31, 67-75 (2003)
30. J. D. Nelson, O. Denisenko and K. Bomsztyk: Protocol for the fast chromatin immunoprecipitation (ChIP) method. *Nat. Protoc.* 1, 179-185 (2006)
31. J. D. Nelson, O. Denisenko, P. Sova and K. Bomsztyk: Fast chromatin immunoprecipitation assay. *Nucleic Acids Res.* 34, e2 (2006)
32. R. Chen, L. Weng, N. C. Sizto, B. Osorio, C. J. Hsu, R. Rodgers and D. J. Litman: Ultrasound-accelerated immunoassay, as exemplified by enzyme immunoassay of choriogonadotropin. *Clin. Chem.* 30, 1446-1451 (1984)
33. J. A. Dahl, P. Collas: $Q^2$ChIP, a quick and quantitative chromatin immunoprecipitation assay unravels epigenetic dynamics of developmentally regulated genes in human carcinoma cells. *Stem Cells* 25, 1037-1046 (2007)
34. H. Kohzaki, Y. Murakami: Faster and easier chromatin immunoprecipitation assay with high sensitivity. *Proteomics* 7, 10-14 (2007)
35. V. Azuara, P. Perry, S. Sauer, M. Spivakov, H. F. Jorgensen, R. M. John, M. Gouti, M. Casanova, G. Warnes, M. Merkenschlager and A. G. Fisher: Chromatin signatures of pluripotent cell lines. *Nat. Cell Biol.* 8, 532-538 (2006)
36. B. E. Bernstein, T. S. Mikkelsen, X. Xie, M. Kamal, D. J. Huebert, J. Cuff, B. Fry, A. Meissner, M. Wernig, K. Plath, R. Jaenisch, A. Wagschal, R. Feil, S. L. Schreiber and E. S. Lander: A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell* 125, 315-326 (2006)
37. R. Metivier, G. Penot, M. R. Hubner, G. Reid, H. Brand, M. Kos and F. Gannon: Estrogen receptor-alpha directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. *Cell* 115, 751-763 (2003)
38. P. M. Das, K. Ramachandran, J. vanWert and R. Singal: Chromatin immunoprecipitation assay. *Biotechniques* 37, 961-969 (2004)

39. M. H. Kuo, C. D. Allis: In vivo cross-linking and immunoprecipitation for studying dynamic Protein:DNA associations in a chromatin environment. *Methods* 19, 425-433 (1999)
40. L. Szekvolgyi, B. L. Balint, L. Imre, K. Goda, M. Szabo, L. Nagy and G. Szabo: Chip-on-beads: flow-cytometric evaluation of chromatin immunoprecipitation. *Cytometry* 69, 1086-1091 (2006)
41. V. R. Iyer, C. E. Horak, C. S. Scafe, D. Botstein, M. Snyder and P. O. Brown: Genomic binding sites of the yeast cell-cycle transcription factors SBF and MBF. *Nature* 409, 533-538 (2001)
42. B. Ren, F. Robert, J. J. Wyrick, O. Aparicio, E. G. Jennings, I. Simon, J. Zeitlinger, J. Schreiber, N. Hannett, E. Kanin, T. L. Volkert, C. J. Wilson, S. P. Bell and R. A. Young: Genome-wide location and function of DNA binding proteins. *Science* 290, 2306-2309 (2000)
43. D. J. Clark, C. H. Shen: Mapping histone modifications by nucleosome immunoprecipitation. *Methods Enzymol.* 410, 416-430 (2006)
44. M. Loden, S. B. van: Whole-genome views of chromatin structure. *Chromosome. Res.* 13, 289-298 (2005)
45. C. V. Dang, K. A. O'Donnell, K. I. Zeller, T. Nguyen, R. C. Osthus and F. Li: The c-Myc target gene network. *Semin. Cancer Biol.* 16, 253-264 (2006)
46. L. A. Lee, C. V. Dang: Myc target transcriptomes. *Curr. Top. Microbiol. Immunol.* 302, 145-167 (2006)
47. L. A. Boyer, K. Plath, J. Zeitlinger, T. Brambrink, L. A. Medeiros, T. I. Lee, S. S. Levine, M. Wernig, A. Tajonar, M. K. Ray, G. W. Bell, A. P. Otte, M. Vidal, D. K. Gifford, R. A. Young and R. Jaenisch: Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature* 441, 349-353 (2006)
48. T. I. Lee, R. G. Jenner, L. A. Boyer, M. G. Guenther, S. S. Levine, R. M. Kumar, B. Chevalier, S. E. Johnstone, M. F. Cole, K. Isono, H. Koseki, T. Fuchikami, K. Abe, H. L. Murray, J. P. Zucker, B. Yuan, G. W. Bell, E. Herbolsheimer, N. M. Hannett, K. Sun, D. T. Odom, A. P. Otte, T. L. Volkert, D. P. Bartel, D. A. Melton, D. K. Gifford, R. Jaenisch and R. A. Young: Control of developmental regulators by Polycomb in human embryonic stem cells. *Cell* 125, 301-313 (2006)
49. M. L. Bulyk: DNA microarray technologies for measuring protein-DNA interactions. *Curr. Opin. Biotechnol.* 17, 422-430 (2006)
50. M. E. Hudson, M. Snyder: High-throughput methods of regulatory element discovery. *Biotechniques.* 41, 673-677 (2006)
51. R. D. Hawkins, B. Ren: Genome-wide location analysis: insights on transcriptional regulation. *Hum. Mol. Genet.* 15 Spec No 1, R1-R7 (2006)
52. L. Elnitski, V. X. Jin, P. J. Farnham and S. J. Jones: Locating mammalian transcription factor binding sites: a survey of computational and experimental techniques. *Genome Res.* 16, 1455-1464 (2006)
53. A. S. Weinmann, S. M. Bartley, T. Zhang, M. Q. Zhang and P. J. Farnham: Use of chromatin immunoprecipitation to clone novel E2F target promoters. *Mol. Cell Biol.* 21, 6820-6832 (2001)
54. B. A. Hug, N. Ahmed, J. A. Robbins and M. A. Lazar: A chromatin immunoprecipitation screen reveals protein kinase Cbeta as a direct RUNX1 target gene. *J. Biol. Chem.* 279, 825-830 (2004)
55. A. Barski, B. Frenkel: ChIP Display: novel method for identification of genomic targets of transcription factors. *Nucleic Acids Res.* 32, e104 (2004)
56. P. Ng, C. L. Wei, W. K. Sung, K. P. Chiu, L. Lipovich, C. C. Ang, S. Gupta, A. Shahab, A. Ridwan, C. H. Wong, E. T. Liu and Y. Ruan: Gene identification signature (GIS) analysis for transcriptome characterization and genome annotation. *Nat. Methods.* 2, 105-111 (2005)
57. P. Ng, J. J. Tan, H. S. Ooi, Y. L. Lee, K. P. Chiu, M. J. Fullwood, K. G. Srinivasan, C. Perbost, L. Du, W. K. Sung, C. L. Wei and Y. Ruan: Multiplex sequencing of paired-end ditags (MS-PET): a strategy for the ultra-high-throughput analysis of transcriptomes and genomes. *Nucleic Acids Res.* 34, e84 (2006)
58. L. A. Boyer, D. Mathur and R. Jaenisch: Molecular control of pluripotency. *Curr. Opin. Genet. Dev.* 16, 455-462 (2006)
59. Y. S. Kwon, I. Garcia-Bassets, K. R. Hutt, C. S. Cheng, M. Jin, D. Liu, C. Benner, D. Wang, Z. Ye, M. Bibikova, J. B. Fan, L. Duan, C. K. Glass, M. G. Rosenfeld and X. D. Fu: Sensitive ChIP-DSL technology reveals an extensive estrogen receptor alpha-binding program on human gene promoters. *Proc. Natl. Acad. Sci. U. S. A.* 104, 4852-4857 (2007)
60. I. Garcia-Bassets, Y. S. Kwon, F. Telese, G. G. Prefontaine, K. R. Hutt, C. S. Cheng, B. G. Ju, K. A. Ohgi, J. Wang, L. Escoubet-Lozach, D. W. Rose, C. K. Glass, X. D. Fu and M. G. Rosenfeld: Histone methylation-dependent mechanisms impose ligand dependency for gene activation by nuclear receptors. *Cell* 128, 505-518 (2007)
61. N. O. Fortunel, H. H. Otu, H. H. Ng, J. Chen, X. Mu, T. Chevassut, X. Li, M. Joseph, C. Bailey, J. A. Hatzfeld, A. Hatzfeld, F. Usta, V. B. Vega, P. M. Long, T. A. Libermann and B. Lim: Comment on " 'Stemness': transcriptional profiling of embryonic and adult stem cells" and "a stem cell molecular signature". *Science* 302, 393 (2003)
62. H. Ji, S. A. Vokes and W. H. Wong: A comparative analysis of genome-wide chromatin immunoprecipitation data for mammalian transcription factors. *Nucleic Acids Res.* 34, e146 (2006)
63. P. C. Scacheri, G. E. Crawford and S. Davis: Statistics for ChIP-chip and DNase hypersensitivity experiments on NimbleGen arrays. *Methods Enzymol.* 411, 270-282 (2006)
64. S. B. van, J. Delrow and S. Henikoff: Chromatin profiling using targeted DNA adenine methyltransferase. *Nat. Genet.* 27, 304-308 (2001)
65. A. Orian, S. B. van, J. Delrow, H. J. Bussemaker, L. Li, T. Sawado, E. Williams, L. W. Loo, S. M. Cowley, C. Yost, S. Pierce, B. A. Edgar, S. M. Parkhurst and R. N. Eisenman: Genomic binding by the Drosophila Myc, Max, Mad/Mnt transcription factor network. *Genes Dev.* 17, 1101-1114 (2003)
66. R. Tompa, C. M. McCallum, J. Delrow, J. G. Henikoff, S. B. van and S. Henikoff: Genome-wide profiling of DNA methylation reveals transposon targets of CHROMOMETHYLASE3. *Curr. Biol.* 12, 65-68 (2002)
67. W. E. de, F. Greil and S. B. van: Genome-wide HP1 binding in Drosophila: developmental plasticity and

genomic targeting signals. *Genome Res.* 15, 1265-1273 (2005)
68. S. B. van: Mapping of genetic and epigenetic regulatory networks using microarrays. *Nat. Genet.* 37 Suppl, S18-S24 (2005)
69. M. J. Vogel, L. Guelen, W. E. de, D. Peric-Hupkes, M. Loden, W. Talhout, M. Feenstra, B. Abbas, A. K. Classen and S. B. van: Human heterochromatin proteins form large domains containing KRAB-ZNF genes. *Genome Res.* 16, 1493-1504 (2006)
70. A. Orian: Chromatin profiling, DamID and the emerging landscape of gene expression. *Curr. Opin. Genet. Dev.* 16, 157-164 (2006)
71. M. R. Matarazzo, F. Lembo, T. Angrisano, E. Ballestar, M. Ferraro, R. Pero, M. L. De Bonis, C. B. Bruni, M. Esteller, M. D'Esposito and L. Chiariotti: In vivo analysis of DNA methylation patterns recognized by specific proteins: coupling CHIP and bisulfite analysis. *Biotechniques* 37, 666-3 (2004)
72. I. Keshet, Y. Schlesinger, S. Farkash, E. Rand, M. Hecht, E. Segal, E. Pikarski, R. A. Young, A. Niveleau, H. Cedar and I. Simon: Evidence for an instructive mechanism of de novo methylation in cancer cells. *Nat. Genet.* 38, 149-153 (2006)
73. M. Weber, J. J. Davies, D. Wittig, E. J. Oakeley, M. Haase, W. L. Lam and D. Schubeler: Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. *Nat. Genet.* 37, 853-862 (2005)
74. M. Weber, I. Hellmann, M. B. Stadler, L. Ramos, S. Paabo, M. Rebhan and D. Schubeler: Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. *Nat. Genet.* 39, 457-466 (2007)

**Abbreviations:** ChIP: chromatin immunoprecipitation; CChIP: carrier ChIP; NChIP: native ChIP; $Q^2$ChIP: quick and quantitative ChIP; Dam: DNA adenine methyltransferase; DSL: DNA ligation and selection; MeDIP: methyl-DNA immunoprecipitation; ES: embryonic stem; ICM: inner cell mass; TF: transcription factor; PCR: polymerase chain reaction; qPCR, quantitative PCR; IP: immunoprecipitation; PAGE: polyacrylamide gel electrophoresis; PET: paired end ditag; BA: bisulfite genomic sequencing analysis

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