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Notes & Tips

An improved cloning strategy for chromatin-immunoprecipitation-derived DNA fragments

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Chromatin immunoprecipitation (ChIP)[1] has become a popular method in regulatory biology for studying the binding of transcription factors to genomic loci. The ChIP technique has been traditionally used for the demonstration of the in vivo binding of a given transcription factor to the genomic regions of interest [1,2]. In recent years, this technique has also become a useful genomic tool for identifying unknown target loci of transcription factors by cloning and analyzing the retrieved DNA fragments that have been bound by the transcription factors [3–5]. Despite the increasing popularity of the latter approach, this ChIP-based cloning approach is still problematic mainly due to the low efficiency of cross-linking and subsequent cloning steps in existing protocols. Here, we describe a simple modification that can dramatically increase the efficiency of existing ChIP-based cloning methods.

Most available ChIP protocols employ a similar overall strategy to retrieve a pool of protein-bound DNAs (Fig. 1). In brief, chromatin is cross-linked by formaldehyde (Step 1), fractionated by sonication (Step 2), and purified by the antibody raised against a given protein (Step 3), and later the bound DNAs are reverse cross-linked by heat treatment and purified from chromatins by proteinase K treatment and phenol/chloroform extraction (Step 5). Since the initial chromatin for ChIP is sheared with sonication, both ends of the purified DNAs are usually modified further by fill-in reactions to generate blunt-end DNA fragments (Step 6). This modified pool of genomic DNAs usually becomes starting DNA material for constructing clone libraries for the identification of unknown target regions for a given DNA-binding protein (Step 7). However, the DNA amounts retrieved from the ChIP and following modifications tend to be very small and DNA fragments with blunt ends need to go through inefficient blunt-end ligation reactions for cloning. The combination of these two inefficient steps has been a major bottleneck for constructing genomic libraries with reasonable clone representation. To circumvent this problem, most existing protocols utilize the PCR amplification (Step 6) for the following cloning step [6], which could easily change the initial representation of the DNA pool and contaminate with unwanted artifact DNAs. We reasoned that the inefficient fill-in and blunt-end ligation reaction (Step 6) could be avoided by the digestion of the antibody-bound chromatin using 4-bp cutters of restriction enzymes before the elution (Step 4). Restriction enzyme digestion of the chromatin that has been immobilized to protein A agarose beads would shorten further the lengths of relevant DNA fragments for the identification of potential binding motifs for a given protein (or transcription factor). The inserts with shorter lengths should make the later-stage sequence inspection more efficient and straightforward.

To test the feasibility of this approach, we performed three trials of ChIP cloning experiments using polyclonal antibodies against mouse DNA-binding proteins, YY1, Suz12, and Peg3 (paternally expressed gene 3). The performance of this approach was compared with the results from parallel ChIP cloning experiments using the traditional approach [4,5]. We prepared chromatin using neonatal mouse brains with the following method. One gram of 10-day-old mouse brain tissues was homogenized in 10 ml PBS buffer containing protease cocktail, cross-linked with 1% formaldehyde for 20 min at 37 °C, washed twice with 10 ml of PBS by centrifugation at 2000 rpm for 10 min to remove cell debris, lysed with 5 ml of the SDS lysis buffer (http://www.upstate.com/img/coa/17-295-32348.pdf) in 10 min on ice, and sonicated with seven strokes of 10-s pulse at the

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[1] *Abbreviations used:* ChIP, chromatin immunoprecipitation; PBS, phosphate-buffered saline.

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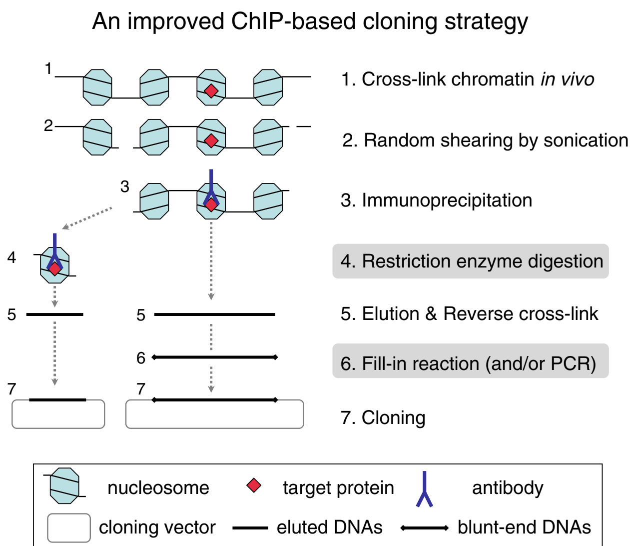

Fig. 1. Schematic diagram of a modified cloning strategy for ChIP-derived DNAs. The traditional approach requires fill-in and blunt-end ligation step for cloning (Steps 1–3, 5–7), whereas the modified approach (shown on the left) adds one restriction enzyme digestion step before the elution step and subsequently skips the fill-in and blunt-end ligation step (Steps 1–5, 7).

30% output of the maximum strength in Sonifier W-350 (Branson Sonic Power Co.). The 5 ml sonicated lysate was aliquoted into 10 fractions of 0.5 ml and stored at an −80 °C freezer. Each ChIP cloning trial used one fraction of these lysates. We followed the ChIP protocol developed by a commercial firm (Upstate Biotechnology Inc., NY) with minimum modifications. In brief, the 0.5 ml lysate was combined with 4.5 ml of the ChIP dilution buffer and incubated with preimmune serum overnight, and the preimmune serum was removed on the following day with 0.1 ml of protein A agarose beads. This precleared chromatin was incubated again with 4 μg of a given antibody at 4 °C overnight. The chromatin was purified using 0.1 ml of protein A agarose beads after sequential washing with 2 ml of each of the following four buffers: LiCl, high salt, low salt immune complex wash, and TE buffer. For the traditional ChIP cloning approach, we eluted the chromatins by incubating the chromatins in 0.5 ml of the elution buffer (0.1 M $NaHCO_3$ and 1% SDS) for 30 min at room temperature. The eluted chromatin was reverse cross-linked by adding 20 μl of 5.0 M NaCl and subsequently incubated at 65 °C overnight. We treated reverse cross-linked chromatin with proteinase K (AMRESCO Inc., OH) for 1 h at 45 °C and extracted DNAs with phenol/chloroform solution. The isolated DNAs were treated with fill-in reactions using Klenow fragments (New England Biolabs, MA). The modified DNAs were cloned into the *Eco*RV-digested pZErO-2 vector or the blunt end TOPO vector (Invitrogen, CA). For the modified approach, we performed restriction enzyme digestion right after the final washing of chromatins with TE (Step 4) by adding 40 units of *Sau*3AI into chromatin

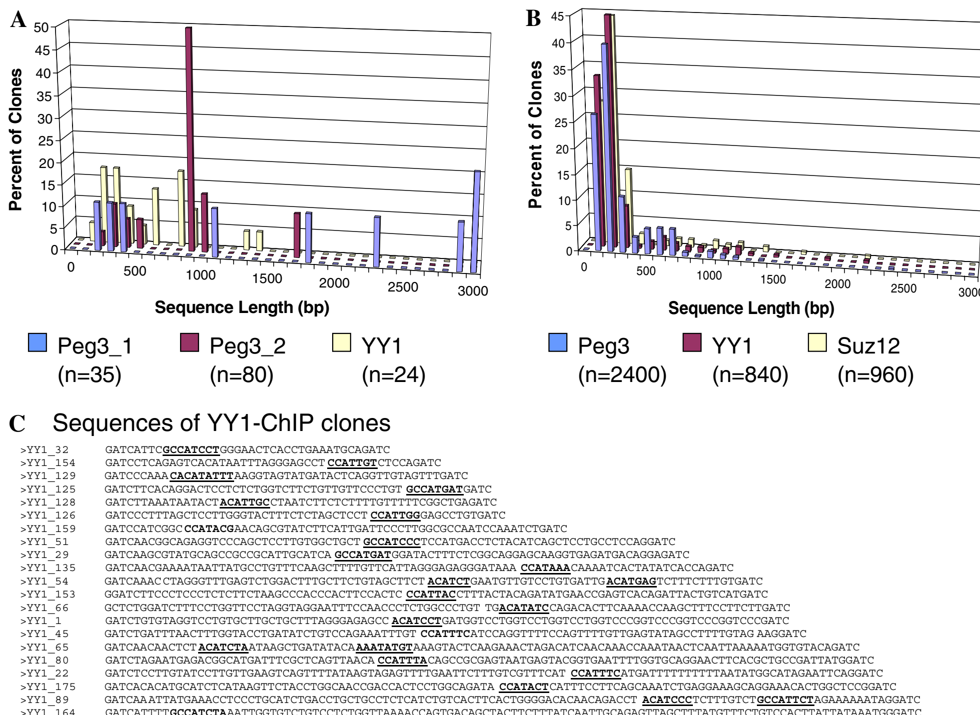

Fig. 2. Comparison of two different cloning approaches for ChIP-derived DNAs. Summary of the total clone number and size distribution of inserts for each ChIP library that has been generated through (A) the traditional and (B) the modified approaches. The total clone number for each library is shown at the bottom. The insert size distribution of each library is presented in a 100-bp increment scale on the *x* axis with the *y* axis representing the percentiles of each category of insert size. (C) A subset of sequences ($n = 21$) derived from the YY1-ChIP library is shown with the potential YY1-binding motif underlined. Two core motifs (ACAT and CCAT) with adjacent nucleotides affecting YY1 binding represent the consensus sequence of YY1 binding sites (CGC/ACATnTT; n means any base) [7].

dissolved in 400 μl of the respective restriction enzyme buffer. After the overnight digestion with gentle rotation at 37 °C, we washed the digested chromatin twice with 500 μl of TE buffer still immobilized to protein A agarose beads. For the remaining steps, the digested chromatin was treated the same as in the traditional approach except for skipping the fill-in reaction. The reverse cross-linked and *Sau*3AI-digested ChIP DNAs were subcloned into the *Bam*HI-digested pZErO-2 vector. Each ligation mixture was transformed into chemically competent TOP10 cells and subsequently plated on LB plates containing 25 μg/ml concentration of Kanamycin.

We assessed the cloning efficiency of the two approaches by comparing the total clone number and the size distribution of clone inserts for each library generated from this study (Fig. 2). The traditional approach generated three libraries with the total clone numbers ranging from 24 to 80, whereas the modified approach generated libraries ranging from 840 to 2400 clones, which represents at least one order of magnitude difference between the two approaches using the same amount of starting chromatin materials. This eliminates one of the main problems in most ChIP cloning protocols, which is the inefficient blunt-end ligation reaction. The median length of clone inserts generated from the traditional approach was 754 bp, while the median length by the modified approach was 137 bp (Figs. 2A and B). This also demonstrated that restriction enzyme digestion before the elution step decreases about six fold the average length of relevant DNA fragments. Our subsequent examination of the 196 sequences derived from the YY1 library further revealed that about 50% of clones have at least one YY1 binding motif within the sequences of their inserts (Fig. 2C), which provides a rough estimate on the feasibility of ChIP cloning approaches for the identification of in vivo binding sites for a given transcription factor. Our strategy using one enzyme, *Sau*3AI, for chromatin digestion, however, needs to be improved further in the future by using different combinations of 4-bp cutters to avoid the potential cloning bias stemming from uneven distribution of restriction enzyme sites in genomes. In conclusion, the modified protocol greatly increased the number of clones and shortened further the size of relevant DNA fragments, which should provide higher resolution to study transcription factor binding sites.

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