

Protocol

Combined 3C-ChIP-Cloning (6C) Assay: A Tool to Unravel Protein-Mediated Genome Architecture

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INTRODUCTION

Progress in technologies to address long-range chromosomal interactions *in vivo* has extensively revised concepts about different aspects of transcriptional regulation. These methods allow probing physical proximities between chromatin elements without specifically identifying the protein components that mediate such interactions. Here we describe a detailed protocol for Combined 3C-ChIP-Cloning (6C) technology, which combines multiple techniques to identify the proteins that bridge distant genomic regions, while simultaneously identifying such physical proximities. This method is also useful for determining if a candidate protein might mediate long-range interactions, both in *cis* and in *trans* in the nucleus. We discuss how the 6C technique can be incorporated with other techniques to discover all the chromatin regions in the nucleus that interact with a given gene or chromatin region of interest in a specific protein-dependent manner. Such information allows complete, cell-type-specific mapping of all the chromatin interactions mediated by specific proteins. The 6C assay advances our understanding of the three-dimensional aspects of the higher-order folding of chromatin and provides an important tool to examine the role of specific proteins in nuclear organization. In addition to providing a detailed protocol of the 6C technique, we discuss how this technology can be used by investigators working in the area of chromatin biology, with special interest in chromatin long-range interactions.

RELATED INFORMATION

The 6C assay combines three different methodologies: chromosome conformation capture (3C) (Dekker et al. 2002), chromatin immunoprecipitation (ChIP), and cloning (Fig. 1). The first step involves conventional 3C methodology: The chromatin is cross-linked, digested with restriction enzymes, and ligated under conditions that favor intramolecular ligation. Immediately after ligation, the chromatin is immunoprecipitated using an antibody against the protein of interest (i.e., the suspected “bridging protein” or the protein whose mediating physical proximities the investigator wishes to map). Thereafter, the cross-links are reversed, and the DNA is purified further. The fragments obtained are then cloned into a vector harboring the same restriction enzyme site overhangs that were generated in the enzyme digestion step of the 3C portion of the protocol. The clones are further screened by digestion with the same restriction enzyme. Ideally, clones showing multiple inserts will result from the intramolecular ligation and should represent physical proximities involving the protein targeted in the immunoprecipitation steps. These clones are chosen for sequencing to reveal the identity of the partners.

For a review of other recent methods developed to examine long-range chromosomal interactions *in vivo*, see Simonis et al. (2007). For additional details on the 6C method presented here, see Tiwari et al. (2008).

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MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <!>, and recipes for reagents marked with <R>.

Reagents

- <!>5-Bromo-4-chloro-3-indoyl- β -D-galactopyranoside (X-gal; 25 mg/mL in DMF [dimethylformamide])
Agarose gel (1%, UltraPure; Invitrogen)
Antibody, specific for the protein of interest
<R>Bead eluting buffer
Bovine serum albumin (BSA; 0.5% in PBS; New England Biolabs)
Cell type of interest, grown under appropriate cell culture conditions with appropriate medium
Cells, bacterial, competent, high-efficiency ($\geq 5 \times 10^9$ cfu/ μ g DNA)
XL10-Gold ultracompetent cells (Stratagene) have produced optimal results.
<R>Cellular lysis solution
<R>ChIP diluent
Cloning vector
The vector used should have enzyme overhangs similar to those generated in the 3C assay. Such vectors can be custom-constructed or obtained commercially. For example, for cloning fragments containing EcoRI ends, use pBluescript II RI Predigested Vector (Stratagene).
<!>DNA gel stain (SYBR Safe, 10,000X concentrate in DMSO [dimethylsulfoxide]; Invitrogen)
DNA ladder, 100-bp and/or 1-kb (New England Biolabs)
DNA polymerase (REDTaq; Sigma D4309) (for polymerase chain reaction [PCR]; see Step 45 and Discussion)
dNTP set (100 mM; Invitrogen) (for PCR; see Step 45 and Discussion)
Ethanol (70% and 100%)
Fetal bovine serum (10% in PBS)
<!>Formaldehyde solution ($\geq 36.5\%$; Sigma-Aldrich 33220)
Glycine (2 M; Fisher BP381)
Glycogen (Roche)
 H_2O , nuclease-free
<R>Immune complex wash buffers (high-salt and low-salt)
Prepare the high-salt and low-salt versions of this buffer separately.
<!>Isopropyl- β -D-thiogalactopyranoside (IPTG; 200 mg/mL)
<R>LB agar
<R>LB-ampicillin agar plates
<R>Alternatively, LB-kanamycin agar plates can be used (see Step 48).
<R>LB (Luria-Bertani) liquid medium
Add the appropriate antibiotic (see Step 51).
<R>Ligation buffer (10X)
Dilute the 10X stock to 1.15X for Step 18.
Magnetic beads, Protein A-conjugated
Magnetic beads, Protein G-conjugated
PCR buffer (10X)
PCR primers, T3 and T7
<!>Phenol:chloroform:isoamyl alcohol, UltraPure (25:24:1 v/v/v; Invitrogen)
Phosphate-buffered saline (PBS; GIBCO 20012)
Protease inhibitor cocktail (Sigma P8340)
<!>Proteinase K (10 mg/mL in TE buffer, pH 8.0; Invitrogen)
PureLink HQ 96 Mini Plasmid DNA Purification Kit (Invitrogen)

This kit is designed to yield high amounts of high-quality DNA from 96 different plasmids in the least amount of time, facilitating the simultaneous growth of several bacterial colonies and large-scale screening of the plasmids. It is particularly useful for screening several plasmids in order to obtain clones having multiple inserts.

Restriction enzyme

The selection of a restriction enzyme is a critical step for 6C methodology that affects both the 3C step and the cloning. It also determines the resolution of interaction maps that can be obtained using the 6C assay in terms of defining the exact chromatin elements that are involved in physical pairing. Enzymes that produce cohesive ends from palindromic recognition sites are preferred in 3C assays, and the six-cutters such as EcoRI, HindIII, and BglII are commonly used. These should cut every 4 kb along the genomic DNA and offer a good starting point. To obtain higher-resolution maps, frequent cutters such as MseI (predicted to cut every 256 bp) can be used. Before beginning a 6C assay, use an aliquot of the restriction enzyme-digested nuclei to test a few regions of the genome for digestion efficiency either by Southern or PCR-based analysis.

Restriction enzyme buffer (10X)

Dilute the 10X stock to 1.14X for Steps 12 and 13.

- <!-->RNase A, DNase-free (10 mg/mL; Sigma R6513)
 - <R><!-->SDS (Sodium dodecyl sulfate; 20% w/v; Fisher BP166)
 - <R>Sodium acetate (3 M, pH 5.2; Fisher BP333)
 - T4 DNA ligase (400 U/μL)
 - <R>TE buffer (pH 8.0)
- Dilute 10X stock to 1X before use.
- <!-->Triton X-100 (20% v/v; VWR)
 - <!-->Trypsin (1X; Invitrogen 25300)

Equipment

- Aluminum foil
- Cell scraper
- Centrifuge, clinical
- Centrifuge, high-speed, refrigerated, equipped with swinging bucket rotor for 14-mL tubes
- Dishes, cell culture
- DNA analysis software (Vector NTI)
- DNA sequencer analysis software (Finch TV)
- Gel electrophoresis tank, horizontal
- Gel imaging system for quantifying PCR products
- Heat block preset to 65°C
- Hemocytometer
- Ice
- Incubator, humidified, equilibrated with 5% CO₂, preset to 37°C
- <!-->Liquid nitrogen (optional; see Step 11)
- Magnetic stand for 1.5-mL tubes (Invitrogen)
- Microcentrifuge, refrigerated
- Microscope, inverted
- Pasteur pipettes
- Pipettors and tips, 5- to 1000-μL
- Refrigerator or cold room preset to 4°C
- Rotating wheel/platform
- Shaking incubator preset to 37°C and 65°C
- Sonicator
- Spectrophotometer
- Thermal cycler, automated
- Timer
- Tubes, microcentrifuge, 1.5-mL
- Tubes, polypropylene, 14-mL (e.g., 17- × 100-mm) for use in high-speed centrifuge
- Tubes, polypropylene, 50-mL
- Tubes, polypropylene, conical, 15-mL (e.g., 17- × 120-mm)
- <!-->UV light
- Vacuum aspirator
- Vortex mixer
- Water bath, variable temperature

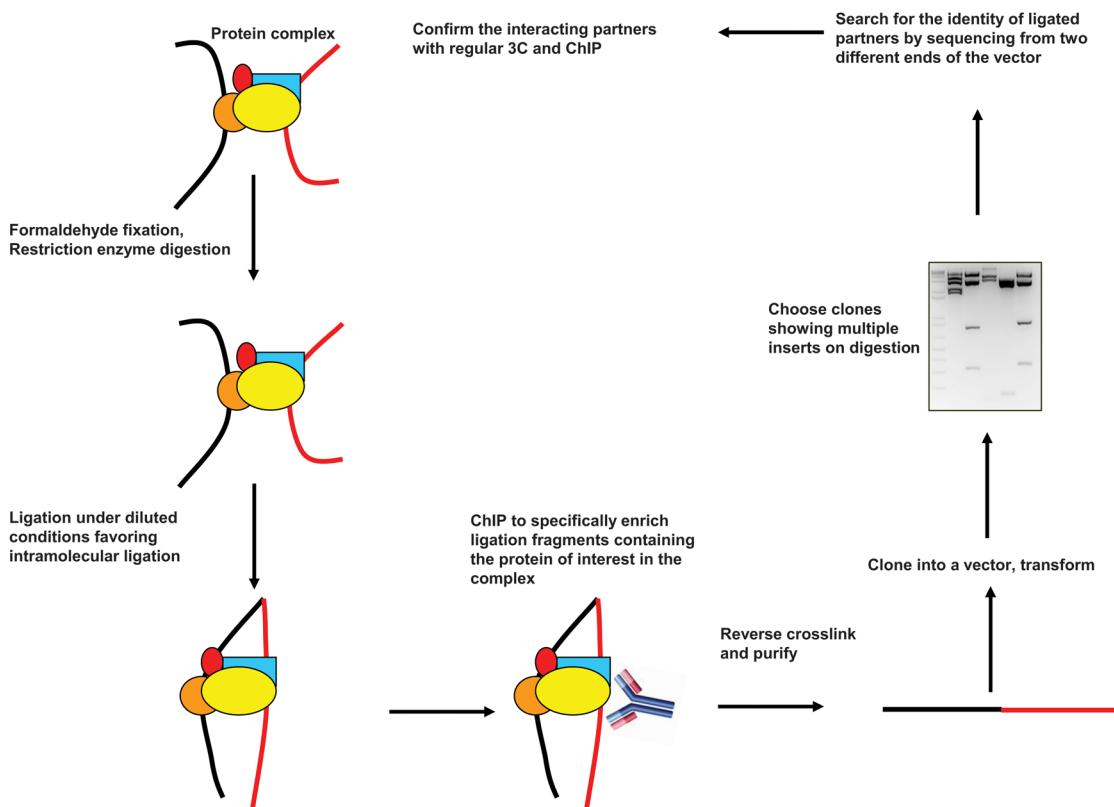


FIGURE 1. Summary of the Combined 3C-ChIP-Cloning (6C) method.

METHOD

Preparing Nuclei

1. Grow 2×10^7 cells of the cell type of interest in an appropriate medium and under suitable conditions until they are 70%-80% confluent.
2. Add 0.86 mL of 37% formaldehyde (to a final concentration of 2%) directly to a cell culture dish containing 15 mL of medium. Swirl gently to mix. Incubate for 10 min at room temperature.
3. Add 1.057 mL of 2 M glycine (to a final concentration of 0.125 M) to the dishes. Swirl gently to mix. Incubate for 5 min at room temperature.
4. Aspirate the medium. Wash the cells with 5 mL of ice-cold PBS containing protease inhibitor cocktail.
5. Prepare 0.2X trypsin diluted in PBS, and add 5 mL of it to the cells. Incubate in 5% CO₂ for 5 min at 37°C.
6. Neutralize the trypsin with 10 mL of 10% fetal bovine serum.
7. Scrape the cells from the plate. Transfer them to 50-mL tubes.
Optionally, scrape a second time with PBS containing protease inhibitor cocktail.
8. Collect the cells by centrifuging at 1300 rpm for 8 min at 4°C.
9. Wash twice with 10 mL of PBS containing protease inhibitor cocktail. Working on ice, pipette gently to make a single-cell suspension.
After resuspending the second time, count the cells before centrifugation.
10. Add 10 mL of cellular lysis solution containing protease inhibitor cocktail to the cells. Mix gently by pipetting. Incubate for 10 min on ice.

Monitor lysis by observing an aliquot under a microscope. These conditions should be sufficient for most cell types. However, certain cell types might need different incubation times and/or different strengths of the buffer for complete lysis.

11. Centrifuge at 1800 rpm for 5 min at 4°C. Discard the supernatant.
At this point, the cell pellet can be snap-frozen in liquid nitrogen and stored at -80°C until use.

Restriction Enzyme Digestion and Intramolecular Ligation

12. Resuspend the pellet in 500 µL of 1.14X restriction enzyme buffer (appropriate for the restriction enzyme to be used in Step 16).
13. Pellet the cells by centrifugation. Resuspend in 500 µL of 1.14X restriction enzyme buffer. Transfer to 1.5-mL tubes.
14. Add 7.5 µL of 20% SDS (to a final concentration of 0.3% SDS). Mix by gentle pipetting. Shake gently for 1 h at 37°C.
15. Add 50 µL of 20% Triton X-100 (to a final concentration of 1.8%). Shake gently for 1 h at 37°C.
16. Add 1200 units of restriction enzyme (i.e., 12 µL of 100 units/µL). Incubate with gentle shaking overnight at 37°C.
17. Add 40 µL of 20% SDS (to a final concentration of 1.6%). Incubate with shaking for 30 min at 65°C.
18. Transfer the sample to a 15-mL tube containing 7 mL of 1.15X ligation buffer.
19. Add 400 µL of 20% Triton X-100 (to a final concentration of 1%). Incubate with occasional gentle shaking for 1 h at 37°C.
20. Add 50 µL of T4 DNA ligase. Incubate for 4 h at 16°C.
21. Incubate for 30 min at room temperature.

Enrichment of Ligation Fragments Containing the Protein of Interest in the Complex

22. Remove 10 µL from the sample (from Step 21). Store at -20°C.
This sample will serve as the "input" sample for subsequent analysis (see Step 37). Prepare one input sample for each treatment group or cell type.
23. Remove an 800-µL aliquot from the sample (from Step 21). Add it to 7.2 mL of ChIP diluent (i.e., a 10-fold dilution).
Multiple ChIP reactions can be performed from one 3C ligation reaction. The same ligation reaction can be split and used for ChIP analyses with different antibodies.
24. Add 4-10 µg of the antibody of choice per immunoprecipitation. Rotate end-over-end overnight at 4°C.
25. Prepare the magnetic beads:
 - i. Mix Protein G-conjugated beads:Protein A-conjugated beads (1:3) in a 1.5-mL tube.
Each immunoprecipitation experiment will need 100 µL of this bead mix. The choice of beads will strictly depend on the type of antibody being used.
 - ii. Add 1 mL of 0.5% BSA in PBS and mix.
 - iii. Place the tube in a magnetic stand for 1 min.
 - iv. Remove the solution by vacuum suction.
Be careful not to aspirate the beads.
 - v. Remove the tube from the stand. Add 1 mL of 0.5% BSA in PBS. Invert the tube to resuspend beads.

- vi. Repeat Steps 25.iii-25.iv.
- vii. Resuspend the beads in 1 mL of 0.5% BSA in PBS. Rotate end-over-end overnight at 4°C.
26. Add 100 µL of the blocked magnetic bead solution to each immunoprecipitate sample (from Step 24). Incubate with rotation for 3 h at 4°C.
27. Prechill one 1.5-mL tube for each immunoprecipitate.
28. Transfer ~1.5 mL of each immunoprecipitate to a separate prechilled tube. Place the tubes in the magnetic stand to collect the beads. Remove the supernatant. Add another aliquot of the remaining immunoprecipitate. Repeat until all the beads have been collected.
Alternatively, pellet the magnetic beads in a centrifuge at 2000 rpm for 5 min. Aspirate some of the supernatant, leaving ~1 mL in the tube. Resuspend the pellet with the remaining supernatant and transfer to a chilled tube. Let the tube sit in the magnetic stand for 1 min to collect the beads. Aspirate the remaining supernatant.
29. Remove the tubes from the magnetic stand. Place on ice.
30. Wash the beads four times with 500 µL of low-salt immune complex wash buffer containing protease inhibitor cocktail. Agitate gently to resuspend the beads. Place the tubes in the magnetic stand to collect the beads. Remove the supernatant.
Use fresh buffer for each wash. If the beads are stuck on the bottom of the tube, place on the magnetic stand momentarily to move the beads to the side and continue to invert.
31. Wash the beads once with 500 µL of high-salt immune complex wash buffer containing protease inhibitor cocktail. Agitate gently to resuspend the beads. Place the tubes in the magnetic stand to collect the beads. Remove the supernatant.
32. Wash the beads once with 1 mL of TE buffer containing protease inhibitor cocktail. Collect on the magnetic stand. Remove the supernatant.
The bead pellet is relatively loose in the TE buffer. Remove the supernatant by pipetting manually instead of vacuum aspiration.
33. Centrifuge at 960g for 3 min at 4°C. Remove any residual TE buffer.
34. Add 210 µL of bead eluting buffer. Incubate for 15 min at 65°C. Vortex briefly every 2 min.
Alternatively, shake gently for 15 min in a shaking incubator pre-set to 65°C.
35. Centrifuge at 16,000g for 1 min at room temperature.
36. Transfer 200 µL of the supernatant to a new 1.5-mL tube.
37. Thaw the inputs (from Step 22). Add 190 µL of bead eluting buffer.
38. Reverse-cross-link samples (from Step 36) and inputs (from Step 37) overnight at 65°C.
Cover the tubes in the heat block with aluminum foil to reduce evaporation.
39. Dilute the SDS by adding 200 µL of TE buffer. Add 8 µL of 10 mg/mL RNase A. Incubate for 2 h at 37°C.
40. Add 8 µL of 10 mg/mL proteinase K. Incubate for 2 h at 50°C.
41. Add an equal volume of phenol:chloroform:isoamyl alcohol to the samples. Shake well. Centrifuge at 10,000 rpm for 15 min. Transfer the upper phase to a fresh tube. Repeat this step with the upper phase.
42. Precipitate the DNA by adding 0.1 volume of 3 M sodium acetate, 1 µL glycogen, and 2 volumes of 100% ethanol.
43. Wash with 0.4 volume of 70% ethanol. Vortex gently after adding the ethanol.
44. Dissolve the pellet in 15 µL of nuclease-free H₂O.
45. Test the success of the ChIP reaction using 1 µL of undiluted immunoprecipitate by PCR with primers specific for a chromatin region known to be occupied by the protein of interest in the cell type under investigation.

Cloning of 3C-Ligated Immunoprecipitated Fragments

46. Using standard cloning protocols, clone the 3C-ChIP products (from Step 44) into a vector that has enzyme overhangs similar to those generated in the 3C assay.
This step typically involves cloning an "IgG/no antibody 3C-ChIP product" and "Plus antibody 3C-ChIP product," in addition to regular cloning controls.
47. Use the ligated vector to transform high-efficiency competent bacterial cells.
48. For experimental samples, spread the surface of LB-agar plates containing the antibiotic of choice (i.e., LB-ampicillin or LB-kanamycin agar plates), with 45 µL of X-gal and 9 µL of IPTG per 100-mm dish before plating the transformed cells.
49. The next day, count the number of blue and white colonies in the bacterial plates from samples immunoprecipitated with the specific antibody as well as the controls (i.e., no antibody or IgG).
White colonies represent bacteria-harboring plasmids with inserts, whereas blue colonies represent bacteria with plasmids without the insert. In an ideal 6C experiment, the number of white colonies should be several-fold higher in samples immunoprecipitated with the specific antibody than in controls. This indicates that the pull-down with a specific antibody enriched specific protein-occupied genomic regions.
50. Pick several white colonies (i.e., containing the insert) from the bacterial plates.
51. Use each such colony to inoculate LB liquid medium containing the appropriate antibiotic. Incubate in a shaking incubator at 220 rpm overnight at 37°C.

Plasmid Purification

52. Purify the plasmids using the PureLink HQ 96 Mini Plasmid DNA Purification Kit according to the manufacturer's instructions.
Alternatively, standard plasmid preparation protocols or kits can also be used.

Screening for Ligated Partners

53. Screen the purified plasmids (from Step 52) by digestion with the same restriction enzyme used for Step 16, using standard restriction digestion protocols.
54. Separate the resulting DNA fragments by electrophoresis in a 1% agarose gel containing SYBR Safe. Use an appropriately sized DNA ladder for reference. Visualize using UV light.
See Troubleshooting.
55. Sequence the plasmids showing more than one insert.
Ideally, multiple inserts mean interacting partners.
56. Sequence the inserts using primers from two different ends of the vector (e.g., T3 and T7 promoter-specific primers for pBluescript II RI Predigested Vector).

TROUBLESHOOTING

Problem: The fragments are too big, and/or there are more than two inserts in the plasmids.

[Step 54]

Solution: Subclone the fragments into a new restriction enzyme-digested vector before proceeding with sequencing.

DISCUSSION

The 6C-captured interactions should be validated by performing independent 3C assays (Tolhuis et al. 2002), in which one performs 3C-PCRs using multiple primer combinations from two different remote sequences. The primers for this purpose are designed exactly as described by Splinter et al. (2004). It also is important to establish, by using separate assays, that the interacting regions captured in the 6C assay are truly occupied by the protein of interest in cells. To this end, perform ChIP

assays (Tiwari et al. 2008) using antibodies specific for the protein of interest. The immunoprecipitated DNA can then be amplified using primers spanning the restriction enzyme site found to be involved in the 3C ligation with other partner(s) in each of the 6C clones.

In early attempts of the 6C assay (Tiwari et al. 2008), screening the clones by restriction digestion identified a very low frequency of clones having multiple inserts (five out of 352). A number of reasons could account for this. First, the rest of the clones that had a single insert probably represented distinct genomic sites that were bound by the specific protein (in this case, EZH2) but were not engaged in any long-range associations. Interestingly, this might also reflect the frequency with which certain protein-dependent long-range associations take place in the nucleus. Second, the number of clones with multiple inserts might also be reduced because of difficulty in cloning (i.e., ligation and transformation) or sequencing of large fragments resulting from the ligation fragments generated by a six-cutter restriction enzyme (e.g., EcoRI). Third, intramolecular ligation after cross-linking and digestion will lead to a fraction of the DNA occurring as circular DNA that cannot be cloned; this might further reduce the number of clones in such an assay. Finally, the study could also have missed some interactions involving partners that bear DNA methylation at the EcoRI site (EcoRI is a DNA methylation-sensitive enzyme). Future work should develop strategies to tackle each of these issues to improve the overall efficiency of the method.

The 6C technique can be combined with other recently published techniques to discover all the chromatin regions in the nucleus that are brought in close physical proximity to the gene (or any other chromatin region of interest) in a specific protein-dependent manner (Fig. 2). Following immunoprecipitation with the antibody of interest, the samples can be subjected to 4C analysis (Zhao et al. 2006) or reverse-cross-linked, purified, digested with a four-cutter enzyme of choice, and processed further for either 3C-chip (Simonis et al. 2006) or the ACT assay (Ling et al. 2006). The 6C procedure can also be used to reveal whether two known chromatin regions are brought into close physical proximity by a specific protein by following the amplification criteria used in the original 3C assay, subsequent to the reversal of cross-linking and purification (Dekker et al. 2002).

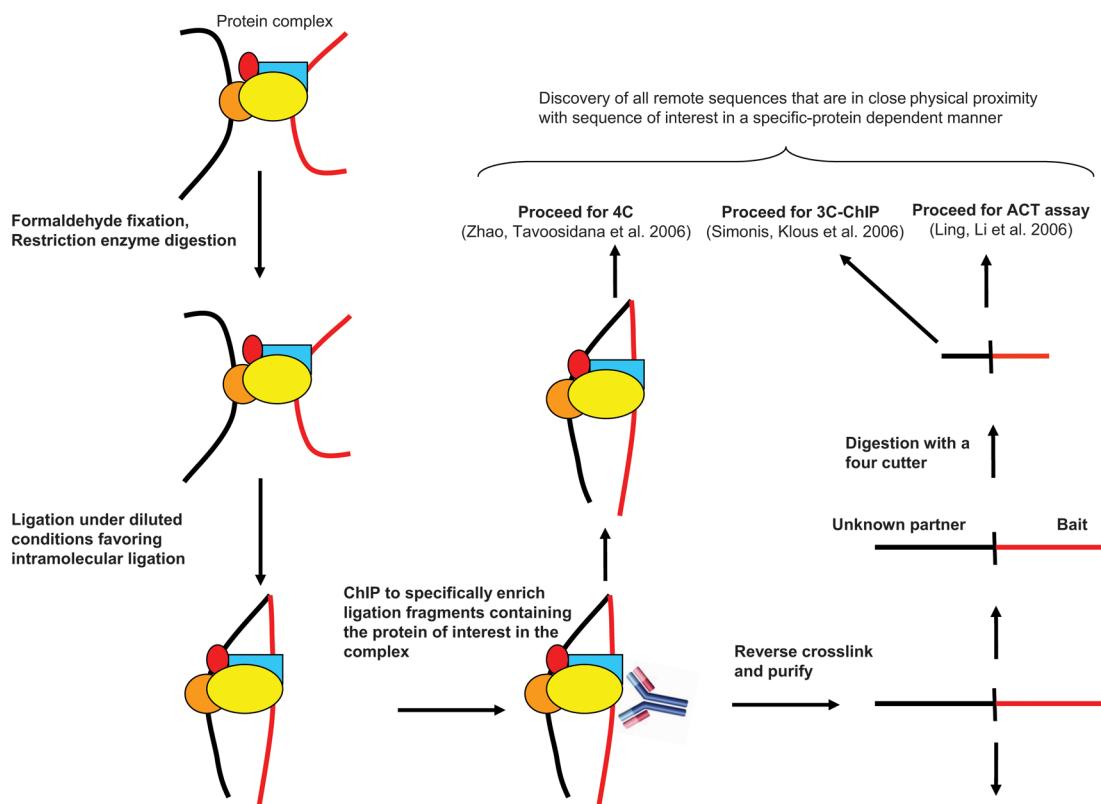


FIGURE 2. Further applications and the future of Combined 3C-ChIP-Cloning (6C) methodology.

REFERENCES

- Dekker, J., Rippe, K., Dekker, M., and Kleckner, N. 2002. Capturing chromosome conformation. *Science* **295**: 1306–1311.
- Ling, J.Q., Li, T., Hu, J.F., Vu, T.H., Chen H.L. Qiu, X.W., Cherry, A.M., and Hoffman, A.R. 2006. CTCF mediates interchromosomal colocalization between *Igf2/H19* and *Wsb1/Nf1*. *Science* **312**: 269–272.
- Simonis, M., Klous, P., Splinter, E., Moshkin, Y., Willemsen, R., de Wit, E., van Steensel, B., and de Laat, W. 2006. Nuclear organization of active and inactive chromatin domains uncovered by chromosome conformation capture-on-chip (4C). *Nat. Genet.* **38**: 1348–1354.
- Simonis, M., Kooren, J., and de Laat, W. 2007. An evaluation of 3C-based methods to capture DNA interactions. *Nat. Methods* **4**: 895–901.
- Splinter, E., Grosveld, F., and de Laat, W. 2004. 3C technology: Analyzing the spatial organization of genomic loci in vivo. *Methods Enzymol.* **375**: 493–507.
- Tiwari, V.K., Cope, L., McGarvey, K.M., Ohm, J.E., and Baylin, S.B. 2008. A novel 6C assay uncovers Polycomb-mediated higher order chromatin conformations. *Genome Res.* **18**: 1171–1179.
- Tolhuis, B., Palstra, R.J., Splinter, E., Grosveld, F., and de Laat, W. 2002. Looping and interaction between hypersensitive sites in the active β-globin locus. *Mol. Cell* **10**: 1453–1465.
- Zhao, Z., Tavoosidana, G., Sjölander, M., Göndör, A., Mariano, P., Wang, S., Kanduri, C., Lezcano, M., Sandhu, K.S., Singh, U., et al. 2006. Circular chromosome conformation capture (4C) uncovers extensive networks of epigenetically regulated intra- and interchromosomal interactions. *Nat. Genet.* **38**: 1341–1347.



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