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Genome-wide Chromatin Immunoprecipitation-Sequencing in *Plasmodium*

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Abstract

Chromatin immunoprecipitation (ChIP) studies have been used extensively in recent years to study the functional role of histone marks, variant histones, and other chromatin factors in gene expression in the human malaria parasite, *Plasmodium falciparum*. In this chapter, we present a ChIP-sequencing protocol optimized for blood-stage forms of this parasite. The processing of the immunoprecipitated DNA prior to high-throughput sequencing is performed in a way to minimize amplification biases due to the high genomic AT-content of the parasite.

Keywords

Plasmodium falciparum; Red blood cells; Chromatin immunoprecipitation; ChIP-seq

1 Introduction

The malaria parasite *Plasmodium falciparum* has a complicated life cycle that involves the differentiation and development in distinct host cell environments during the course of an infection. Epigenetic processes are involved in transcriptional control in all life cycle stages. ChIP studies have led to important advances in our understanding of factors contributing to gene regulation in malaria parasites. The technique is used to study the interaction between proteins and DNA in the cell and aims at determining whether specific proteins (transcription factors or other DNA binding proteins) are associated with specific genomic regions. ChIP has been used to establish the specific location of various histone modifications or histone variants in the genome. By combining ChIP with microarray technology (ChIP-on-chip) or next-generation DNA sequencing technology (ChIP-seq) genome-wide studies have produced valuable information and novel insight into epigenetic regulation of virulence genes.

The availability of commercial antibodies to posttranslationally modified histones has been instrumental in the understanding of transcriptional regulation in *P. falciparum*. For instance, using antibodies to trimethylated lysine 9 of the histone H3, it has been shown that this mark is associated with the silencing of several virulence gene families, including the *var* genes and certain invasion-related genes (1–3). In other experiments, it has been found that acetylation of lysine 9 of histone H3 is associated with general transcriptional activity while the trimethylation of lysine 4 of histone H3 appeared to be deposited in a stage-specific manner (4). ChIP assays have also been used to study histone-modifying enzymes, such as the histone acetylase PfGN5 (5), the histone deacetylase PfSir2A (6), and the histone H3 trimethylated lysine 9 binding protein heterochromatin protein 1 (PfHP1) (7,

8) . Similarly, the genomic distribution of PfSip2, a member of the plasmodial AP2 family, has been determined (9) .

Here, we describe a protocol (see Fig. 1) well-suited for mapping the DNA targets of transcription factors or other chromatin-associated proteins that has also been successfully used to determine the distribution of histone marks in *P. falciparum* . The protocol described here uses reversibly cross-linked chromatin as starting material. We use formaldehyde to cross-link the chromatin followed by sonication to generate fragments of 200–600 base pairs (bp) in length. Alternatively, native ChIP (native chromatin sheared by micrococcal nuclease digestion) may be used to improve protein recovery of antibodies but this method is less suitable for non-histone proteins.

Protein-DNA complexes are selectively immunoprecipitated using specific antibodies to the protein of interest, formaldehyde-induced cross-links are reversed and DNA extracted using Proteinase K–phenol–chloroform treatment. To determine the sites of interaction between the protein of interest and the DNA, the DNA associated with the precipitated protein can be identified and quantified by one of several techniques, such as quantitative PCR, dot-blot, DNA microarray or high-throughput sequencing.

While the analysis of immunoprecipitated DNA by quantitative PCR is well established in *P. falciparum* , this approach allows analysis of only a limited number of specific targets (10, 11) . Similarly, dot blot analysis of immunoprecipitated DNA has been used to determine the amount of a specific target sequence. This technique is based on the hybridization of a labeled probe to the immobilized immunoprecipitated DNA and has traditionally been used for the identification and quantification of highly repetitive sequence elements that could not be PCR-amplified (6, 12) . DNA microarrays, just like dot blots, are based on the hybridization of the immunoprecipitated DNA to a specific probe (13) . Microarrays can contain several ten thousand probes and can thus be used for genome-wide analyses of immunoprecipitated DNA. However, all hybridization-based approaches suffer from cross-hybridization artifacts (14) , a limited dynamic range (15) , and the resolution of microarray-based analyses is limited by genomic spacing of the probes used to generate the microarray. Over the past years, high-throughput sequencing of DNA has evolved as an alternative to identify and precisely quantify immunoprecipitated DNA (16) . For ChIP and sequencing (ChIP-seq) analyses, the immunoprecipitated DNA is directly sequenced, the sequenced DNA “tag” is aligned to the genome and for each base pair the number aligned sequenced tags is summed. The number of summed sequence tags corresponds to the relative enrichment of the protein of interest along the genome (13) . Because ChIP-seq analysis does not require previously designed probes, it permits the identification of unanticipated genome rearrangements, small insertions or deletions and allows mapping of protein DNA interaction on a single base-pair resolution. However, just like quantitative PCR and microarray-based analyses, ChIP-seq requires amplification of the immunoprecipitated DNA. PCR-based amplification of highly AT-rich regions, as they are commonly found in *P. falciparum* , can be extremely inefficient and prone to induce significant biases. Several strategies have been proposed to reduce such PCR-induced biases. For example, reducing the PCR extension temperature appears to decrease the introduced bias (17, 18) . Likewise, replacing the DNA polymerase with a T7 RNA polymerase followed by reverse

transcription of the RNA into DNA led to decreased amplification-induced bias (4) . Here, we describe a protocol that incorporates several adjustments that have been found to increase the efficiency of library generation thus permitting fewer cycles of PCR amplification and consequently less bias (19, 20) . To further reduce the AT-induced bias, we used an engineered DNA polymerase that has been demonstrated to exhibit reduced AT-bias compared to conventional high-fidelity DNA polymerases. We find that the protocol described here reduces drastically the amplification bias of extremely AT-rich regions and is a suitable method for the generation of libraries for ChIP seq.

2 Materials

2.1 Formaldehyde Cross-linking and Chromatin Preparation

1. 37% Formaldehyde.
2. 1.25 M glycine.
3. Phosphate-buffered saline (PBS): 135 mM NaCl, 2.7 mM KCl, 8 mM Na₂ HPO₄ · 2H₂ O, 1.5 mM KH₂ PO₄ . Adjust the pH to 7.4 with HCl.
4. Protease inhibitor cocktail (Roche).
5. Saponin.
6. Cold lysis buffer: 10 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM EDTA (pH 8.0), 0.1 mM EGTA (pH 8.0). Store at 4°C and 1 mM DTT and protease inhibitor cocktail prior use.
7. Nonidet-P40 10%.
8. Douncer homogenizer.
9. SDS lysis buffer: 1% SDS, 10 mM EDTA (pH 8.0), and 50 mM Tris–HCl (pH 8.1). Store at 4°C and add protease inhibitor cocktail prior use.
10. IP dilution buffer: 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA (pH 8.0), 16.7 mM Tris–HCl (pH 8.1), and 150 mM NaCl. Store at 4°C and add protease inhibitor cocktail prior use.
11. Bioruptor UCD-200 (Diagenode).
12. Cooled centrifuge for 50 and 1.5-ml tubes.

2.2 Chromatin Immunoprecipitation

1. Salmon Sperm DNA/protein A agarose slurry 50%.
2. Antibodies against proteins of interest.
3. Low salt wash buffer: 0.1% SDS, 1% Triton X-100, 2 mM EDTA (pH 8.0), 20 mM Tris–HCl (pH 8.1), and 150 mM NaCl.
4. High salt wash buffer: 0.1% SDS, 1% Triton X-100, 2 mM EDTA (pH 8.0), 20 mM Tris–HCl (pH 8.1), and 500 mM NaCl.

5. LiCl wash buffer: 0.25 M LiCl, 1% NP-40, 1% Deoxycholate, 1 mM EDTA (pH 8.0), and 10 mM Tris-HCl (pH 8.1).
6. TE Buffer: 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0).
7. Elution buffer: 1% SDS and 0.1 M NaHCO₃.
8. Rotation mixer.

2.3 De-Cross-linking of Chromatin and DNA Purification

1. 20 mg/ml Proteinase K.
2. 0.5 mg/ml RNase.
3. Phenol and Phenol-Chloroform-Isoamylalcohol (25:24:1).
4. 3 M sodium acetate (NaOAc) pH 5.2.
5. 20 mg/ml glycogen.
6. 100% Ethanol.
7. 70% Ethanol.
8. NanoDrop.

2.4 End-Repair of DNA

1. En-It DNA End-Repair Kit (Epicentre, Cat #: ER0720).
2. Ultrapure water.

2.5 Add protruding 3' A Base

1. Low retention 1.5-ml microcentrifuge tube (e.g., from Ambion).
2. 10× NEB Buffer 2 (100 mM Tris-HCl, pH 7.9, 500 mM NaCl, 100 mM MgCl₂, 10 mM DTT, New England Biolabs).
3. 1 mM dATP.
4. Klenow fragment, 3'–5' exo⁻ (New England Biolabs, Cat #: M0212S).
5. Ultrapure water.

2.6 Adapter Ligation

1. DNA adapter mix PE-102-1001 or PE-102-1003 (see Note 1).
2. T4 DNA ligase and 2× ligation buffer (Enzymatics, Cat #: L603-HC-L).
3. Ultrapure water.

2.7 Size-Selection on Agarose Gel

1. Agarose.
2. TBE buffer.

3. DNA loading buffer.
4. Ethidium bromide (EtBr).
5. 100-bp DNA ladder.
6. Clean scalpel.

2.8 Enrich and Amplify Adapter-Containing DNA Fragments

1. PCR primers (Illumina, PE-102-1001 or PE-102-1003 (see Note 1).
2. KAPA HiFi DNA HotStart DNA Polymerase plus buffer and dNTPs (Kapa Biosystems, cat #: KK2501).
3. Thermocycler.

2.9 Cleanup of PCR Product

1. AMPure XP beads (Beckman Coulter, cat #: A63880).
2. Magnetic stand.
3. 80% EtOH.
4. 10 mM Tris-Cl, pH 8.5 (e.g., EB buffer from Qiagen Kits).
5. Agilent Technologies 2100 Bioanalyzer plus DNA Chips.

3 Methods

3.1 Preparation of Cross-linked Chromatin

The step-by-step protocol is described for cultures of erythrocytic asexual stages of *P. falciparum* containing 1×10^9 ring stage parasites or 3.5×10^8 trophozoites or 1×10^8 schizonts. Usually, these amounts of cells allow performing eight immunoprecipitations (IP). Nevertheless, the number of cells required per IP depends on several factors (see Note 2).

1. Add 37% formaldehyde directly to the synchronized parasite culture to get a final concentration of 1%. Mix immediately and incubate at 37°C with agitation for 10 min (see Note 3).
2. To stop cross-linking add the amount of 1.25 M Glycine needed to achieve a final concentration of 0.125 M. Place the flask in ice and agitate for 5 min.
3. Wash sample three times with cold PBS (centrifugations should be carried out at 4°C). The formaldehyde may cause some red blood cell (RBC) lysis.
4. Add saponin so that the final concentration is 0.06%. Incubate for 5–10 min (until complete RBC lysis). If RBC lysis is not complete, add more saponin until the final concentration is 0.15%.
5. Spin the sample ($3250 \times g$, 4°C, 10 min) and wash the pellet with cold PBS until the supernatant becomes clear.

6. Discard the supernatant and proceed to the next step or snap-freeze the pellet and store at -80°C .
7. Prepare nuclei by resuspending the cross-linked parasites in 1 ml of cold lysis buffer.
8. Transfer to a prechilled douncer homogenizer and set on ice for 30 min. Add 10% Nonidet-P40 to reach a final concentration of 0.25%. Lyse the parasite with 200 strokes for rings stage parasites and 100 strokes for trophozoites or schizonts. Check for parasite lysis with the help of a light microscope (phase contrast).
9. Centrifuge the lysate for 10 min at $13500 \times g$ 4°C .
10. Resuspend the pellet in 200 ml of SDS Lysis (see Note 4).
11. Chromatin sonication. Precool the Bioruptor's tank with crushed ice 30 min before starting, to avoid water heating too quickly. Bioruptor settings are high power and 30 s ON, 30 s OFF of cycling parameter.
12. Sonicate for 8 min.
13. Replace the water with cold water and crushed ice.
14. Sonicate for 8 min.
15. Remove debris by centrifuging for 10 min at $12,500 \times g$ at 4°C .
16. Dilute supernatant fraction tenfold in ChIP dilution buffer. Keep a portion of this chromatin solution (20 μl) as DNA input and another portion (80 μl) to check chromatin shearing (shearing check sample). These two portions will be processed with the other immunoprecipitations during DNA purification. The diluted chromatin can be stored at -80°C for months.

3.2 Chromatin Immunoprecipitation

1. To reduce nonspecific background, preclear the 2 ml of chromatin solution with 200 ml of salmon sperm DNA/protein A agarose slurry 50% for 2 h at 4°C with agitation. Pellet agarose by brief centrifugation and transfer the supernatant to a new tube.
2. Make eight aliquots of the chromatin solution (250 μl / aliquot).
3. Add the antibody (the concentration of the antibody should be empirically determined) and incubate overnight at 4°C with rotation (see Note 5). For control IP, use the nonimmune IgG fraction from the same species in which the antibody was produced (see Note 6).
4. To collect immune complexes, incubate the sample with 25 μl per sample of Salmon sperm DNA/protein A agarose slurry 50% for 2 h at 4°C with agitation.
5. Pellet beads by gentle centrifugation ($1,000 \times g$ for 1 min). Carefully remove the supernatant that contains unbound chromatin.

6. Wash the protein A agarose–antibody–chromatin complex for 5 min on a rotating platform with 1 ml of each of the buffers listed below. Discard the wash buffer between steps:
 - Low salt immune complex wash buffer (at 4°C).
 - High salt immune complex wash buffer (at 4°C).
 - LiCl immune complex wash buffer (at 4°C).
 - TE buffer, two washes at RT.
7. Elute immune complexes by adding 100 µl of fresh elution buffer. Vortex briefly to mix and incubate at RT for 15 min with rotation. Spin down beads (1,000 × g for 1 min), carefully transfer the supernatant fraction (eluate) to another tube and repeat elution with 150 µl of fresh elution buffer. Combine eluates.

3.3 DNA Purification

1. Add 230 µl of elution buffer to the input and 170 µl to the shearing check sample. From here, both samples are treated like the IP fractions. To reverse cross-linking, add 5 M NaCl (10 µl) to a final concentration of 200 mM and incubate the sample and the input at 65°C for 6 h.
2. Add 8 mg of RNase to each sample, mix, and incubate at 37°C for 2 h.
3. Add 3 µl of 20 mg/ml Proteinase K and incubate for 2 h at 45°C (see Note 7).
4. Extract with 250 µl of phenol, vortex for 1 min until an emulsion forms and centrifuge for 2 min at 14,000 rpm. Transfer the aqueous phase to a fresh tube.
5. Extract with 250 µl of phenol/chloroform/isoamylalcohol (25/24/1) as described above for extraction with phenol.
6. Add 25 µl of 3 M NaOAc, 1 µl of 20 mg/ml glycogen, and 600 µl of 100% EtOH, and precipitate for 45 min at -80°C.
7. Pellet DNA by centrifugation for 40 min, 4°C, 14,000 rpm.
8. Wash the pellet once with 500 µl of 70% EtOH and centrifuge for 5 min, 4°C, 14,000 rpm.
9. Carefully remove supernatant and store the open tube on the bench at RT until the last traces of fluid evaporated (at least 10 min).
10. Dissolve the DNA pellet in 11 µl of distilled water and rinse the walls of the tube.
11. Use NanoDrop to determine concentration. If concentration is above 10 ng/µl, the adapter concentration used in Subheading 3.6 should be adjusted (see Note 8).

3.4 End-Repair of DNA

1. The ends of the DNA fragments were repaired in a 50- μ l reaction with the End-It DNA end-repair kit (Epicentre) according to the manufacturer's instructions. To a low retention 1.5-ml microcentrifuge tube, add the following: 10 μ l DNA, 5 μ l 10 $\times$ End-Repair buffer (Epicentre), 5 μ l dNTP mix (Epicentre), 5 μ l ATP (Epicentre), 24 μ l H₂O to a total volume of 49 μ l, and 1 μ l End-Repair enzyme mix (Epicentre).
2. Incubate reaction at RT for 45 min.
3. To heat-inactivate enzymes, incubate reaction for 10 min at 70°C.
4. Purify DNA (see Note 9). Final volume should be 32 μ l.

3.5 Add Protruding 3' A Base

1. To a low retention 1.5-ml microcentrifuge tube, add the following: 32 μ l end-repaired DNA, 5 μ l 10 $\times$ NEB Buffer 2 (100 mM Tris-HCl, pH 7.9, 500 mM NaCl, 100 mM MgCl₂, 10 mM DTT, New England Biolabs), 10 μ l 1 mM dATP, 3 μ l (15 U) Klenow fragment, 3'–5' exo⁻ (New England Biolabs), H₂O to a total volume of 50 μ l.
2. Incubate reaction for 30 min at 37°C.
3. Purify DNA (see Note 9). Final volume should be 19 μ l.

3.6 Adapter Ligation

1. Dilute the DNA adapters mix (Illumina) 1:10 with H₂O (see Note 8).
2. To a 200 μ l PCR tube add the following: 19 μ l A-tailed DNA, 1 μ l of diluted adapter mix (Illumina), 25 μ l 2 $\times$ ligation buffer (Enzymatics), 5 μ l of T4 ligase (600 U/ μ l, 3,000 U total, Enzymatics), H₂O to total volume of 50 μ l.
3. Incubate reaction for 15 min at 25°C in thermocycler.
4. Purify DNA (see Note 9). Final volume should be 30 μ l.

3.7 Size-Selection on Agarose Gel

1. Prepare 2.0% agarose gel with 1 $\times$ TBE buffer containing 400 ng/ml EtBr.
2. Load ~500 ng of 100 bp DNA ladder.
3. Mix 30 μ l of purified ligation product with 10 μ l DNA loading buffer.
4. Load the entire sample in one well (leave at least two lanes between ladder and sample, do not load more than one sample per gel to avoid cross-contamination).
5. Run gel at 100 V for 60 min.
6. Using a clean scalpel, excise DNA in the 200 $\pm$ 25 bp range.

7. Use gel extraction kit from Macherey-Nagel or Qiagen to purify DNA. Final volume should be 36 μ l. Important: let agarose solubilize at RT to avoid melting of AT-rich DNA.

3.8 Enrich and Amplify Adapter-Containing DNA Fragments

1. To prepare a PCR mix add the following to a 200- μ l PCR tube:
 - 35.5 μ l of size-selected DNA.
 - 1 μ l of PCR primer 1.1.
 - 1 μ l of PCR primer 2.1.
 - 10 μ l of 5 $\times$ KAPA HiFi reaction buffer.
 - 1.5 μ l of 10 mM dNTP mix.
 - 1 μ l of KAPAHiFi DNA Polymerase (1 U/ μ l).
2. Use the following PCR protocol (see Note 10): 1 min at 98°C, 18–25 cycles (15 s at 98°C; 30 s at 65°C; 1 min at 72°C), and 1 min at 72°C.

3.9 Cleanup of PCR Product

1. Remove the AMPure XP Beads from storage and let them equilibrate to RT for ~30 min.
2. Prior to use, vortex AMPure XP Beads and add 65 μ l (see Note 11) of well-mixed AMPure XP beads to a 1.5-ml low-retention microcentrifuge tube.
3. Add 50 μ l of the PCR reaction mix to tube containing AMPure XP beads and mix thoroughly by pipetting up and down ~10 times.
4. Incubate the reaction for 15 min at RT.
5. Place the reaction on the magnetic stand and wait for solution to clear (~5 min).
6. Remove and discard the supernatant from the tube without disturbing the AMPure XP beads.
7. Keep the tube on the magnetic stand and wash beads (~30 s incubation) with 200 μ l of freshly prepared 80% EtOH without disturbing the beads.
8. Remove and discard the supernatant from the tube without disturbing the AMPure XP Beads.
9. Repeat wash with 80% EtOH.
10. Remove and discard the supernatant from the tube without disturbing the AMPure XP Beads.
11. To collect liquid at bottom of tube, centrifuge for 15 s at $600 \times g$.
12. Place the tube in the magnetic stand, let liquid clear (~1 min), and carefully remove remaining 80% EtOH without disturbing the beads.

13. To dry beads, place tube on 37°C heating block until small cracks can be observed in the dried bead pellet surface (~1 min).
14. To elute DNA, resuspend pellet with 30 µl of 10 mM Tris-Cl, pH 8.5 (e.g., Qiagen EB buffer) and incubate for 2 min at RT.
15. Mix beads and 10 mM Tris-Cl thoroughly by pipetting up and down ~10 times.
16. Place the tube in the magnetic stand, let liquid clear (~1 min) and carefully, without disturbing the beads, transfer eluted DNA (30 µl) to clean 1.5-ml low retention tube.
17. Repeat purification with 39 µl of AMPure XP beads (The ratio of beads to reaction volume should again be 1.3:1).
18. After completion of the second round of purification, elute the DNA with 15 µl 10 mM Tris-Cl, pH 8.5 buffer.
19. To determine the size, purity, and concentration of the sample analyze 1 µl of purified library on an Agilent Technologies 2100 Bioanalyzer (see Note 12).

4 Notes

1. The DNA adapter mix and PCR primers are available as part of a ChIP-seq library preparation Kit from Illumina DNA adapter mix (for 10 reactions, PE-102-1001) or individually (for 100 reactions, PE-102-1003).
2. Amount of recovered DNA depends on the quality of the antibody, binding frequency of protein to DNA, and abundance of the protein. Usually, for each IP reaction 100–150 × 10⁶ ring stage parasites, 30–40 × 10⁶ trophozoites and 10–15 × 10⁶ schizonts are recommended. These numbers of cells are equivalent to 2.5–5 mg of DNA-containing chromatin. However, as little as 500 ng will work for some IP. The amount of cells-chromatin required should be high enough to get the minimum 10 ng of immunoprecipitated DNA necessary for amplification and sequencing.
3. Cross-linking time influences the efficiency of chromatin shearing and the efficiency of precipitating a specific antigen. For some proteins, especially those that do not directly bind DNA, longer cross-linking times or combining the use of formaldehyde with other cross-linking agents improve the formation of covalent links between proteins (21– 23) .
4. 1% SDS improves the efficiency of sonication (next step) but could negatively affect the recovery for some antibodies. Performing the sonication in a 0.1% SDS-containing buffer compromises shearing efficiency; therefore, sonication conditions should be carefully controlled. Another option is to use SDS lysis buffer containing 1% SDS, followed by dialysis against the same buffer with a lower SDS concentration. For histones and other proteins very tightly associated with the DNA/chromatin, native ChIP can be performed (3) . Native ChIP omits cross-linking and chromatin is subsequently fragmented by enzymatic digestion

with micrococcal nuclease (MNase, which digests DNA at the level of the linker, leaving nucleosomes intact).

5. The amount of antibody added should be in excess of the factor being precipitated. Not all antibodies can effectively immunoprecipitate protein-DNA complexes. ChIP assays require highly specific antibodies that must recognize its epitope in free solution and underfixed conditions. For abundant proteins, like histones, 1–2 mg of affinity-purified antibody or 2–4 µl of whole serum per IP is recommended.
6. Common controls used in ChIP experiments are IP with nonimmune IgG antibodies or with no antibody. IP with protein deficient cell line or with cells that do not express the tagged protein of interest (for tag-specific antibodies) are also very useful controls.
7. RNase and Proteinase K treatments can be performed before de-cross-linking as well.
8. If the starting material is larger than 100 ng, the concentration of DNA adapters should be adjusted accordingly.
9. For DNA purifications, we use a NucleoSpin Extract II kit from Machery-Nagel (cat #: 740609.50). Alternatively, kits from Qiagen can be used or DNA can be purified by phenol–chloroform extraction.
10. The final library concentration should be above 1 nM; thus, the number of PCR cycles needed will depend on the amount of immunoprecipitated DNA and the efficiency of the library preparation. The number of PCR should be kept as low as possible to reduce the bias.
11. The ratio of beads to reaction volume is important and influences the size cutoff that enables exclusion of small DNA molecules like primer dimers.
12. The minimal final concentration will depend on the protocol used at the sequencing center; however, concentrations above 1 nM should work without any problems.

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Fig. 1.

Two-step genome-wide ChIP protocol. DNA fragment library preparation is needed for microarray or high-throughput sequencing.