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Chromosome and genetic testing using ChIP assay

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1. ABSTRACT

Chromatin immunoprecipitation (ChIP) assay can be used to easily visualize information about proteins, DNA, and RNA on chromosomes and is widely used for analysis of genomes, epigenomes, mRNAs, and non-coding RNAs. The ChIP assay can detect, not only DNA-binding proteins of various organisms, but also the temporal and spatial regulating mechanisms of RNA-binding proteins. Because of these features, demand for ChIP assay is expected to grow. Here, by using yeast and *Drosophila* as examples, we describe the superiority of the improved ChIP assay that we have developed.

2. INTRODUCTION

In basic research, the chromatin immunoprecipitation (ChIP) assay can detect the binding sites of transcription factors. It also detects the status of euchromatin or heterochromatin associated with changes in chromatin structure—particularly with changes in histone modification in specific regions on chromosomes (1-13) (Figure 1).

Moreover, the assay can detect the behaviors of molecules that associate or dissociate on chromosomes, depending on the status of the molecules moving on the chromosomes, the stage of the cell cycle, and the stage of development and differentiation (12).

There have recently been an increasing number of reports suggesting that promoter silencing suppresses expression, leading to carcinogenesis. At present, however, testing associated with such findings is being attempted at the basic research level and is not performed in clinical laboratories. Only a few university hospitals in Japan are equipped with chromosomal and genetic testing laboratories, although that number is gradually increasing.

Here, we discuss the effectiveness and potential use in the clinical setting of a highly sensitive simple ChIP assay (11, 13) that we have developed and that can exploit more detailed information than the standard assay.

3. PRESENT STATUS OF CHROMATIN IMMNOPRECIPITATION ASSAYS

The beginning of the ChIP assay dates back to about 20 years ago (8, 9). It was originally used to study transcription in budding yeasts, and it is currently used to study the chromatin structure in, for example, mammalian cells and body tissues. This technique is expected to be developed further in future, with a focus on the association of epigenetic modifications with cancer and with development and differentiation.

However, ChIP assays are currently still at the basic research level and are not used in clinical settings.

4. APPLICATION EXAMPLE IN YEAST

To start with, we used a budding yeast, which is a eukaryote and easy to handle. Although the ChIP assay is very convenient, it consists of several steps that must

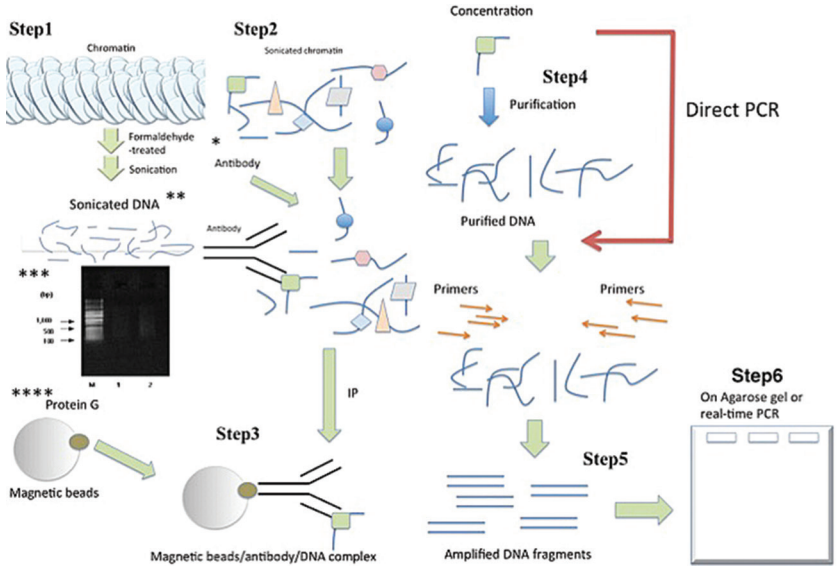

Figure 1. Protocol of the rapid ChIP assay. Steps 1 and 2: Fix cells with formaldehyde and shear chromosomes by sonication. Step 3: React an antibody against the protein of interest and enrich the protein-bound DNA. Step 4: From the recovered complexes, recover DNA by protease K treatment, phenol extraction, phenol-chloroform extraction, chloroform extraction, and RNase treatment. Steps 5 and 6: Determine by PCR whether or not the DNA of interest is included. In our modification of the test, by omitting step 2 the whole assay can be shortened by about 2 days. *Formaldehyde cross-linking: When cells are treated with formaldehyde, formaldehyde enters the cell nuclei, thereby cross-linking the protein. **Sonication: Chromosomes are physically fragmented by sonication (ultrasonic agitation). *** Ethidium-bromide staining of whole-cell extract (WCE) treated with RNase after sonication: Lane one, 4 ml of WCE; lane two, 8 ml of WCE. Suitable DNA size markers are 100, 200, 300, 400, 500, 750, 1000, and 1400 bp. Mean suitable fragment size by sonication is £500 bp. **** Wash magnetic beads several times in bovine serum albumin (BSA) and salmon sperm DNA sonicated in advance, in order to prevent non-specific binding of proteins and DNA. Both BSA and salmon sperm DNA are inexpensive.

be performed correctly (Figure 1). A problematic drawback is that, if the sample is large, 4 or 5 days are needed to yield results. We therefore developed and patented a more efficient, simple ChIP assay. This assay requires no reagents such as protease K and is thus cheaper. Results can be obtained in 2 or 3 days (11), and the detectability is 16 times greater than that of the standard assay. Proteins are detected moving through the cell cycle along the chromosomes, as in the standard assay. If a kit for this simple, rapid assay is developed, it should be immediately usable in clinical settings. Our highly sensitive ChIP assay (11) saves time because one of the conventional steps is omitted, and yet it is extremely sensitive and significantly more sensitive (15 times more sensitive) than the standard assay (11). In the standard assay, the whole-cell extract (WCE) requires various pretreatments. In the rapid ChIP assay, however, we proved that an adequate signal can be obtained with phenol extraction alone. Moreover, the rapid ChIP assay requires at least 16 times less antibody for the same result as in the standard assay (11). The rapid ChIP assay has been successfully patented as a fast and low-cost method. In our investigation of proteins moving along chromosomes, we have examined Mcm4 and confirmed its presence along chromosomes in the G1 to S phases, but not along chromosomes in the M phase (11).

5. APPLICATION EXAMPLE IN *DROSOPHILA*

Drosophila serves as an experimental tool for observing various biological phenomena at the individual level. It is also useful for examining human host defense mechanisms such as immunity (16).

In nature, endoreplication and gene amplification occur in a diverse range of organisms (12, 14-17). In humans, endoreplication occurs in megakaryocytes in the blood (12). In eggs in the ovaries of *Drosophila* females (Figure 2B), endoreplication, followed by gene amplification, occurs specifically as part of development and differentiation. We generated a transgenic *Drosophila* fly model (Figure 2A) (a gift from Dr. Maki Asano of Duke University) carrying Orc1 (which is essential to chromosomal replication) tagged with green fluorescent protein. Fluorescence microscopic observation of the eggs revealed six gene amplifications per follicle cell (18) (Figure 2C); this was also confirmed by immunostaining. These findings suggested that *Drosophila* undergoes gene amplification, which we inferred to be of the onion-skin type (Figure 3D).

We performed a rapid ChIP assay of Orc1 and detected a signal on loci ACE3 and Ori-β, which undergo onion skin-type gene amplification (Figure 2E). Statistical quantification detected from both ACE3 and Ori-β significantly more intense signals than that detected from Act1, which is normally expressed without undergoing gene amplification (N = 8 for each; $P < 0.0.001$).

These findings showed that the rapid ChIP assay effectively detected not only cell rearrangements

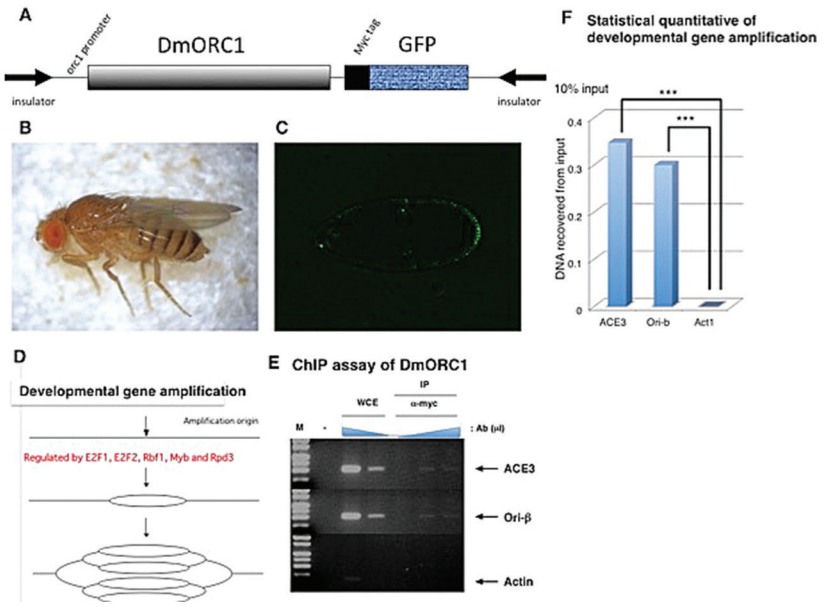

Figure 2. Application of rapid ChIP assay to *Drosophila*. (A) Schematic diagram of the transgene. (B) *Drosophila female* (C) Fluorescence microscopic observation of follicle cells in the ovary (D) Schematic diagram of onion-skin-type gene amplification that occurs in *Drosophila* specifically in development and differentiation. (E) Highly sensitive simple ChIP assay of Orc1-myc-GFP (green fluorescent protein) using *Drosophila* ovary. (F) Data obtained in (E) were quantified and subjected to a *t*-test. ACE3, Ori-β, Act1; N = 8, ***; P < 0.0.001[11].

specific to development and differentiation in various organisms but also development and differentiation–specific behaviors in molecules moving along chromosomes.

6. POSSIBILITY OF USING OF THE CHIP ASSAY IN CLINICAL LABORATORIES

Currently, only a few medical institutions in Japan are equipped with chromosome and genetic testing laboratories, and testing companies are often commissioned to perform such tests. In a questionnaire survey conducted among medical technologists (including our former students), many answered, “If a kit for rapid ChIP assay were developed, I would like to use it.” In fact, chromosome and genetic testing requires considerable capital investment and the number of requests for such tests is still small. In addition, older medical technologists tend to be unfamiliar with chromosome and genetic testing procedures. Nevertheless, the numbers of medical facilities that are installing chromosome and genetic testing laboratories is gradually increasing in Japan. For genetic testing, fully automatic SNP testing is now being done with specific devices (e.g. the i-densy, Arkray, Inc., Kyoto, Japan).

This rapid ChIP assay requires no special, costly instruments, and we have used it on transcription factors (in many cases proto-oncogene products), oncogene products, and molecules moving along chromosomes (11, 12, 13).

ChIP sequencing is commonly used; after a specific DNA fragment is recovered by using the ChIP assay, genome-wide DNA arrays (from genome-wide association studies) can be used to determine the position in which the protein of interest is enriched (19). This approach can thus detect the status of protein modification and the localization of modified proteins on the chromosomes (20). Not only proto-oncogenes, but also histone acetylation and phosphorylation as well as chromosome methylation by DNA methylation antibodies, can be detected. The ChIP assay is thus a promising technology for clinical application.

7. DISCUSSION

The ChIP assay is becoming a basic essential for chromatin study and will continue to be improved, with a focus on convenience in practical use in the clinical setting. ChIP technology is currently being used in basic research to elucidate a variety of biological phenomena. Whereas conventional ChIP assays were time-consuming and expensive, the highly sensitive, simple ChIP assay that we have developed enables detection in a range of organisms in a time-and space-specific way. Nevertheless, the assay is not yet being used in the clinical setting. To overcome this, we have developed a simpler, less expensive, and more convenient method with higher detectability and have proved that this method can detect gene amplification, which is likely involved in cancer. We intend to continue to improve this rapid ChIP system so that it will be usable as a kit in the clinical setting.

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