[*Plant Bio Techniques Series*(2)]**Genomic DNA Blot Analysis Using Nonradioisotope Probes**

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Hybridization techniques such as Southern blot analysis are fundamental in molecular biology and based on nucleic acid-nucleic acid interactions. To visualize these interactions, people have used radioisotope-labeled probes. However, radioisotopes are toxic to living cells and even carcinogenic due to damage to DNA. Therefore, frequent use of radioisotopes is not recommended as users are forced to take steps to prevent contamination of the environment. In addition, according to safety guidelines, governmental regulation imposes various restrictions. Therefore, the use of radioisotopes is not applicable to all types of research.

Recent development of nonradioactive systems for the labeling and detection of DNA is welcome by all researchers as it guarantees safe and easy hybridization experiments. The principles of the nonradioactive system are relatively simple. First, the hapten such as digoxigenin (DIG)¹⁾, fluorescein (Fl) is bound to uridine-nucleotides (**Fig. 1**) and incorporated enzymatically into nucleic acid probes by the technique of random priming. Then, after hybridization and washing, hapten-labeled

Fig. 1 Digoxigenin-dUTP **Fig. 2** Hapten-mediated nonradioactive detection system

Fig. 3 direct-labeling nonradioactive detection system

probes are detected by a high affinity anti-DIG (or anti-FI)-antibody Fab-fragment conjugated to alkaline phosphatase (AP)²⁾ (or horseradish peroxidase (HRP)). Finally, dephosphorylation of a chemiluminescent substrate³⁾ with AP in the presence of alkaline buffer generates light via an unstable intermediate, which can be documented by a short exposure to X-ray film or detected by a 2-D chemiluminometer (**Fig. 2**). On the other hand, reduction of hydrogen peroxide by HRP generates superoxide and luminol is oxidized by this radical and produces blue light, which is increased and prolonged by the presence of an enhancer⁴⁾ (**Fig. 2**).

Alternatively, nucleic acid probes can be also labeled directly by HRP⁵⁾. This is achieved by completely denaturing the probe so that it is in single-stranded form. HRP, which has been complexed with a positively charged polymer, is added and it forms a loose attachment to the nucleic acid by charge attraction. Addition of glutaraldehyde causes the formation of chemical cross-links so that the probe is covalently labeled with enzyme. After mild hybridization and wash to prevent denaturation of HRP, HRP-labeled probe generates light as above (**Fig. 3**).

The sensitivity of the nonradioactive detection system is comparable to the radioisotope system. According to our experience, it is enough to detect a single copy gene of small-genome plants such as rice (430 Mbp). Highly expressing mRNA can also be detected by Northern blot analysis. Colony and plaque screenings are possible with relatively high backgrounds.

Cost performance will become better when the same DNA fragment is used as a probe repeatedly because hapten-labeled probes are usually stable and hybridization buffer containing probes can be used for several hybridization experiments. In our laboratory, we usually use the same hybridization buffer for five independent hybridization experiments. In addition, hapten-labeled probes can be stored for a long time whereas direct HRP-labeled probes are unstable and recommended to use immediately once labeled.

It is possible to reconstitute the nonradioactive system using commercial reagents, two systems, DIG and ECL, which are ready-to-go kits, can be purchased from Boehringer Mannheim and Amersham, respectively and are recommended. DIG and an AP-conjugated anti-DIG antibody are in the DIG system. On the other hand, FI and both AP- and HRP-conjugated anti-FI antibodies are in the ECL system. In addition, direct nucleic acid labeling and detection systems are also available in the ECL system.

In this paper, we describe two protocols of genomic DNA blot analysis, which are based on the original directions in the kits with slight alterations and work routinely to detect a single-copy gene of rice in our laboratory. Unfortunately, components of some reagents in the kits have not been publically disclosed. Therefore, we use the same nomenclature for these reagents here.

1. *Selection of random prime DIG-labeling or direct HRP-labeling*

Depending on the purpose of experiments, we first select the way of labeling the probes. we routinely use either DIG DNA Labeling Kit (Cat. No. 1175033, Boehringer Mannheim) and DIG Luminescent Detection Kit (Cat. No. 1363514, Boehringer Mannheim) or ECL direct nucleic acid labeling and detection systems (Cat. No. RPN 3005, Amersham).

Points to be considered for the selection are listed below.

Point 1. Compared with the DIG labeling, ECL direct labeling system gives satisfactory results more frequently in our laboratory. This may be because there are fewer steps in this system.

Point 2. ECL direct labeled probes are unstable and users should ensure that temperature of the hybridization mixture never exceeds 42°C.

Point 3. Preparation of nucleic acid fragments for probes is more crucial in the ECL system and high purity of the fragments is required for efficient labeling.

Point 4. Detection sensitivity of the ECL system is significantly lower than that of the DIG system and therefore more genomic DNA should be loaded to one lane.

Point 5. Light output of the ECL system during detection is transient. Therefore, the exposure to a X-ray film becomes a crucial step in the ECL system. On the other hand, AP-producing light in the DIG system lasts for more than three days (Output becomes a peak around 12-24 hrs after the reaction starts). Therefore, many exposure times can be tried to obtain the best results.

Point 6. In the ECL system, users should use freshly-made probes. Therefore, labeling reactions have to be done every time. In the DIG system, once labeled, hybridization buffer containing probes can be stored for a long time and can be used repeatedly after heat denature.

Point 7. Rehybridization with different probes is relatively easy in the ECL system. This may be because of the low background and the ease of inactivation of the light producing enzyme, HRP.

We usually store hybridization buffers containing DIG-labeled probes, which can be used by many lab-members in our laboratory. For individuals, one system is selected according to their familiarity and purpose.

2. *Making genomic DNA-blot membrane*

Rice genomic DNA prepared by conventional methods usually contain polysaccharides which may inhibit digestion by restriction enzymes. Therefore, genomic DNA should be digested at low concentration (1 $\mu\text{g}/200\ \mu\text{l}$) for more than 3 hr. 1 μg of digested rice DNA is loaded to one lane for the DIG system while at least 3 μg of DNA should be for the ECL system. After electrophoresis, the DNA is transferred to a Nylon membrane such as Hybond N⁺ by the conventional way. Transferred DNA is fixed covalently to the membrane by a short UV exposure. Keep it dry at room temperature (RT).

3. *Preparation of DNA fragments for probes*

Purity of DNA fragments to be used as probes is an important factor for labeling efficiency. Although conventional methods, in which a target DNA fragment can be recovered from an agarose gel after electrophoresis, work well, we often prepare the fragment by PCR. This method can greatly reduce contamination of unfavorable DNA fragments such as vector.

Step 1. Design of PCR primers

When a subcloned DNA fragment is used for a probe, primers in the multi cloning site of the vector such as T3, T7 primers can be used for this purpose.

Step 2. PCR reaction

Less than 1 ng plasmid should be used as a PCR template. 20 cycles are usually enough to

amplify the necessary amount of DNA fragment. 25 ng of rice genomic DNA can be used for the same purpose if a single band is amplified.

Step 3. Purification of PCR products

Because residual primers may inhibit proper random priming, PCR products should be ethanol-precipitated after one phenol/CIAA extraction. A column such as Qiagen would also work. DNA precipitate is dissolved in TE buffer to become 100 ng/ μ l.

In the following steps, basic protocols are based on directions with kits. Therefore, we try to describe the modulated points in detail. It is recommended that the original instructions should be read before trying the nonradioactive system.

4. Labeling of probe DNA

For the DIG system,

Step 1. Add reagents to a microfuge tube in the following;

DNA template(denatured)	1 μ l(100 ng)
Hexanucleotide mixture(10 $\times$)	5 μ l
dNTP labeling mixture(10 $\times$)	5 μ l
Water	to 49 μ l
Klenow enzyme	1 μ l(0.1 units)

Step 2. Incubate the reaction tube at 37°C for more than 12 hr. Longer incubation up to 20 hr increases incorporation of DIG-11-dUTP which results in higher specific activity.

Step 3. Precipitate the labeled DNA with 0.1 volume of 2 M sodium acetate and 2 volumes of ethanol. After mixing well, incubate on ice for 15 min. Then centrifuge the reaction tube at 15,000 rpm for 10 min. using a microcentrifuge.

Step 4. Wash the pellet once with 70% ethanol and dry it in the air then resuspend in 50 μ l of TE buffer. The labeled probe can be stored for several months at -20°C.

For the ECL system,

Step 1. Denature 10 μ l of the DNA solution(10 ng/ml) by heating for 5 min. in boiling water.

Step 2. Cool the DNA solution on ice for 5 min.

Step 3. Add 10 μ l of DNA labeling reagent to the cooled DNA.

Step 4. Add 10 μ l of the glutaraldehyde solution.

Step 5. Incubate for 10 min. at 37°C. Use the probe immediately.

5. Hybridization and Wash

For the DIG system,

Step 1. Place the blot in a hybridization bag containing 20 ml prehybridization solution after prewetting the blot in 2 $\times$ SSC and incubate it at 65°C for at least 1 hr.

Prehybridization buffer;

5 $\times$ SSC
1% Blocking Reagent(in the kit)
1% SDS
0.1%(w/v) <i>N</i> -laurolysarcosine

Step 2. Heat the probe solution for 10 min. in boiling water and chill immediately on ice.

Step 3. Add 50 μ l of the probe in the hybridization bag buffer. Incubate the bag with gentle shaking at 65°C for at least 12 hr.

Step 4. Wash the blot for 5 min. in 2 $\times$ SSC 0.1% SDS solution at RT. Wash it for 15 min. in 2 $\times$

SSC 0.1% SDS at 65°C. Finally, wash it for 15 min. in 0.5×SSC 0.1% SDS at 65°C. The hybridization buffer can be stored at -20°C for next hybridizations (The hybridization buffer must be denatured in the boiling water before the next use).

For the ECL system,

Step 1. Place the blot in a hybridization bag containing 20 ml prehybridization solution after prewetting the blot in 2×SSC solution and incubate it at 42°C for 1 hr.

ELC Gold hybridization buffer (in the kit)

Prehybridization buffer; ECL Gold hybridization buffer (in the kit)

0.5 M NaCl

5% (w/v) blocking agent (in the kit)

Step 2. Add 30 μ l of the labeled probe solution to the prehybridization buffer. Incubate it overnight at 42°C.

Step 3. Prepare the primary wash buffer and preheat to 42°C. Wash the blot in the buffer for 20 min. twice ensuring temperature does not exceed 42°C.

Primary wash buffer; 6 M Urea

0.4% SDS

0.5×SSC

Step 4. Wash the blot in the secondary buffer for 5 min. twice at RT.

Secondary wash buffer; 2×SSC

6. Detection

For the DIG system,

Step 1. Equilibrate the membrane in buffer 1 for 1 min.

Step 2. Block the membrane by gently agitating it in buffer 2 for 30 min.

Step 3. Place the blot in the antibody solution (1 μ l of the anti-DIG-AP in 10 ml buffer 2) for 30 min.

Step 4. Wash the blot twice, 15 min. per wash, in 0.3% Tween 20 buffer 1.

Step 5. Equilibrate the blot in buffer 3 for 2 min.

Step 6. Add 150 μ l of LumigenPPD (10 mg/ml) (Cat. No. 532-25651, WAKO) to 15 ml of buffer 3. Incubate the blot in it for 2 min. Drain off excess buffer and wrap the blot by Saran Wrap. The buffer can be used several times and stored at 4°C. LumigenPPD is recently replaced by a new substrate, CSPD (or CDP-Star) in the Boehringer kit. You might use the CSPD in the place of LumigenPPD. According to the direction, light output from the CSPD becomes stronger and reaches a peak earlier than LumigenPPD.

Step 7. Expose the blot to X-ray film for 3 hr. After one overnight, exposure-time can be shortened because of stronger light output.

For the ECL system,

Step 1. Mix 5 ml of detection reagent 1 with 5 ml detection reagent 2.

Step 2. Place the washed blot on a sheet of Saran Wrap, DNA side uppermost. Add the detection reagent directly to the blots.

Step 3. Incubate 1 min. at RT.

Step 4. Drain off excess reagents and cover the blot with Saran Wrap.

Step 5. Place the blot DNA side up in the film cassette and expose X-ray film for 30 min.

Step 6. Remove the film and develop. If required, expose a second film overnight.

7. Troubleshooting

Problem 1. High backgrounds using the DIG system

Recommendation 1. Check and lower the concentration of probe in the hybridization buffer.

Recommendation 2. Use a greater volume of wash buffers.

Recommendation 3. Reduce contamination of extra AP activity. Your hands can be the source of contaminated AP activity.

Problem 2. Little signals using the ECL system

Recommendation 1. Check the purity of the DNA fragment for probe and reprepare it.

Recommendation 2. Ensure the correct temperature during hybridization and wash steps.

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