

SCIENCE IMAGING SYSTEMS

Application Note No.17

Northern Hybridization Detection Using Chemifluorescence Method

FLA-2000 / FLA-3000

Foreword

Detection of RNA after Northern hybridization is usually done with a radioisotope. Use of a radioisotope is advantageous from the point of permitting detection of extremely small quantities of RNA but restricts testing to a controlled area. Test methods that can be easily used in an ordinary laboratory are therefore also necessary. One of these is the chemifluorescence method. This method generates a fluorescent substance by reacting a labeled enzyme and a substrate. The substrate AttoPhos™ works with the chemifluorescence method and can be detected at high sensitivity using FLA series imaging systems.

In Note No. 17, Dr. De-Xing HOU of Kagoshima University introduces the detection of Northern hybridization by the chemifluorescence method. Dr. HOU uses this method in his research on effects of functional foods on gene expression and regulation.

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Summary

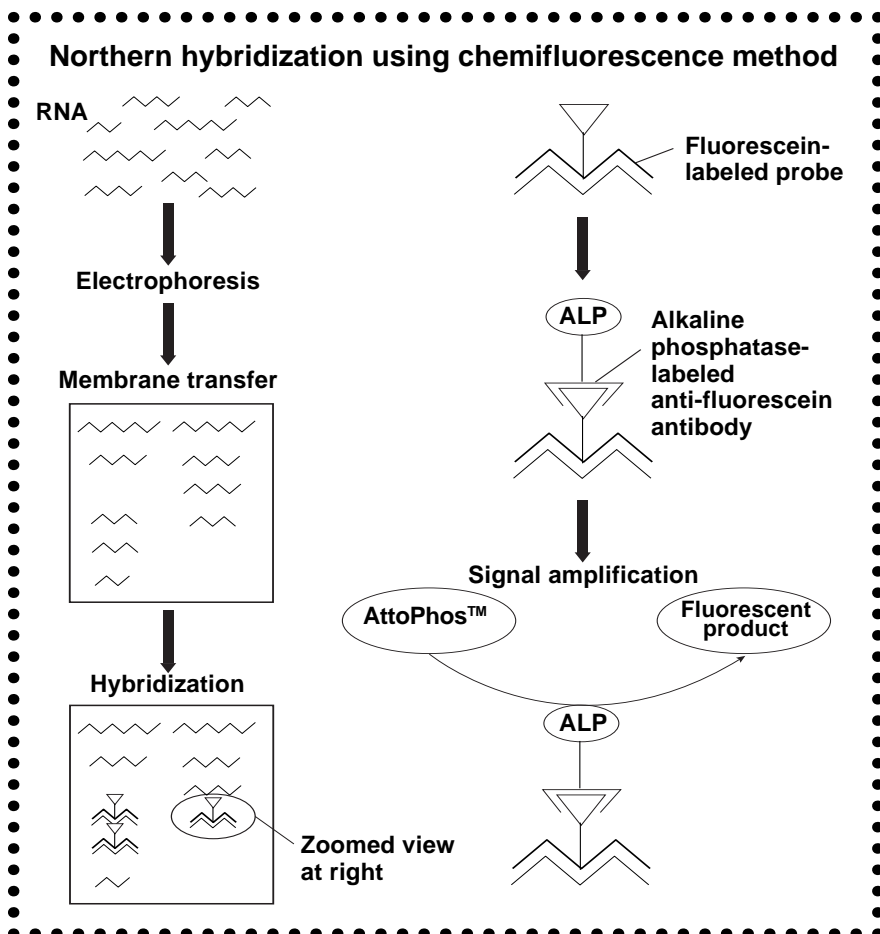
- Northern hybridization detection can be carried out in an ordinary laboratory without using a radioisotope.
- The chemifluorescence method using FLA series imaging systems provides high sensitivity of signal detection and excellent reproducibility.

1 Introduction

In the Northern hybridization method, RNA fixed on a filter is hybridized with a labeled probe. The location and amount of gene expression are then determined from the location and amount of hybridization signal. The method is indispensable for researchers in the field of molecular biology. For many years, the Northern hybridization was conducted using a radioisotope-labeled probe together with X-ray film for detecting the β -rays emitted by ^{32}P . Use of radioisotopes has a number of disadvantages including harm to the human body, troublesome facility maintenance and high cost of waste disposal.

This led to the recent development of non-RI methodologies for mRNA detection like the chemiluminescence method and the fluorescence-labeled probe method. One drawback of chemiluminescence is considerable amount of film waste by difficult control of X-ray film exposure time. Another is that reliable results are frequently hard to obtain because of probe labeling problems, etc.

A need was therefore considered for development of a Northern hybridization methodology that could be implemented without use of either a radioisotope probe or X-ray film. The approach we took at our laboratory was to amplify the signal after Northern hybridization by the chemifluorescence method using alkaline phosphatase-labeled anti-fluorescein antibody and AttoPhos™ as a fluorogenic substrate. The fluorescence signal was detected with a fluorescent image analyzer, FLA-2000. This method, outlined in the following, enabled reliable and stable detection.



2 Preparation of reagents

1. Hybridization buffer

5x SSC
0.1% SDS
5% liquid block
5% dextran sulphate

Storable for three months at -20°C

2. Washing buffers

Washing buffer 1

1x SSC
0.1% SDS

Washing buffer 2

0.5x SSC
0.1% SDS

3. Buffer A

300 mM NaCl
100 mM Tris-HCl, pH 7.5

4. Blocking buffer

Blocking reagent	3	ml
Buffer A	27	ml

5. Signal amplification solution

Anti-fluorescein AP conjugate	4	ml
Bovine serum albumin	0.1	g
Buffer A	20	ml

Use freshly mixed solution

6. 0.3% Tween 20 buffer

Tween 20	0.3	ml
Buffer A	99.7	ml

3 Test protocol

■ RNA sample extraction and RNA filter preparation

The AGPC (Acid Guanidinium-Phenol-Chloroform) method was used for extracting RNA from cultured cells. The RNA filter was prepared by electrophoresing 20 mg of total RNA using denaturing gel and transferring it to a nylon membrane. These methods are described in many laboratory handbooks.

■ Probe preparation and fluorescein labeling

*Probe DNA purification

Digest the plasmid containing interested cDNA fragment with available restriction enzymes. After agarose gel electrophoresis, recover the cDNA fragment and purify it with GENE CLEAN II kit (BIO 101, Inc.).

BIO 101, Inc.

<http://www.bio101.com/index-main.html>

*Probe DNA denaturation

- (1) Seal 150 ng of DNA dissolved in 34ml of distilled water in a microtube and denature it at 96 C for 5 minutes.
- (2) Chill rapidly in ice water for 5 minutes and spin down.

*Fluorescein-labeling of probe DNA

- (1) Add the following reagents to the tube containing the denatured DNA.

Nucleotide mix	10 ml
Primers	5 ml
Enzyme solution	1 ml

- (2) After spinning down, react for 1 hour at 37 C.
- (3) Denature for 5 minutes at 96 C.
- (4) Chill rapidly in ice water for 5 minutes and spin down.

Experimental Tip

As the amount of the probe DNA is extremely small, do not depend on the value obtained by UV absorbance of the sample. Check the amount of DNA by agarose gel electrophoresis comparing with known amount of marker DNA.

■ Northern hybridization

- (1) Place 15 ml of hybridization buffer in a container and preheat to 60 C.
- (2) Wet the membrane with 5x SSC, immerse it in the hybridization buffer avoiding air bubbles, and shake gently for 30 minutes at 60 C.
- (3) Add the probe labeled with fluorescein, mix thoroughly, and shake gently overnight at 60 C.

■ Washing

- (1) Place the membrane in 150 ml of washing buffer 1 preheated to 60 C and shake for 15 minutes at 60 C.
- (2) Transfer the membrane to 150 ml of washing buffer 2 preheated to 60 C and shake for 15 minutes at 60 C.

■ Signal amplification

- (1) Transfer the washed membrane to 100 ml of buffer A and shake for 5 minutes at room temperature.
- (2) Transfer the membrane to 100 ml of blocking buffer and shake for 1 hour at room temperature.
- (3) Transfer the membrane to 20 ml of signal amplification solution and shake for 1 hour at room temperature.
- (4) Transfer the membrane to 100 ml of 0.3% Tween 20 buffer and shake for 10 minutes at room temperature.
- (5) Repeat the procedure of (4) three times.

■ Signal detection and analysis

- (1) Sandwich the membrane between vinyl sheets, add 1 ml of detection reagent, react for 5 minutes, transfer to fresh Saran Wrap®, and carefully seal avoiding air-bubbles.
- (2) Set the membrane wrapped in Saran Wrap® on the FLUOR stage of the FLA-2000.
- (3) Turn on Image Reader software.

Set the FLA-2000 read conditions as shown below and click the Read button.

Gradation:	65536 (16 bit)
Resolution:	50
Sensitivity:	F10 (or F1000)
Sample Mode:	Fluor. 473 nm, Y520 Filter

- (4) When the readout is completed, bring up Image Gauge or MacBAS software. Select "Mode-Quant" and carry out quantitative analysis.

Experimental Tip

The reaction time up to signal detection differs depending on the type of gene probe. At the beginning, a readout should be attempted once every 10 minutes. When no or only a very weak signal can be detected at first, reaction may be done for overnight. However, the results of quantification may be effected when reaction time is too long. We recommend checking the intensity of fluorescent signal frequently after addition of the detection reagent to deal with reaction time.

4 Conclusion

We labeled a DNA probe with fluorescein, carried out Northern hybridization, amplified the fluorescence signal with anti-fluorescein AP conjugate and AttoPhos™, and detected directly with an FLA-2000. Through repeated tests at my laboratory, we found that this detection system has the following features:

- (1) High sensitivity. The results are comparable with those using a radioisotope.
- (2) Reliable results can be obtained with excellent reproducibility.
- (3) Detection can be conducted safely and rapidly because no radioisotope is used and film development is unnecessary.

Still, when working with low-expression genes, it is advisable to do the Northern hybridization after purifying mRNA from the total RNA. For example, we found that it is easy to detect albumin gene expression using total RNA extracted from liver cells, but difficult to detect the expression of liver transcription factor HNF using total RNA.

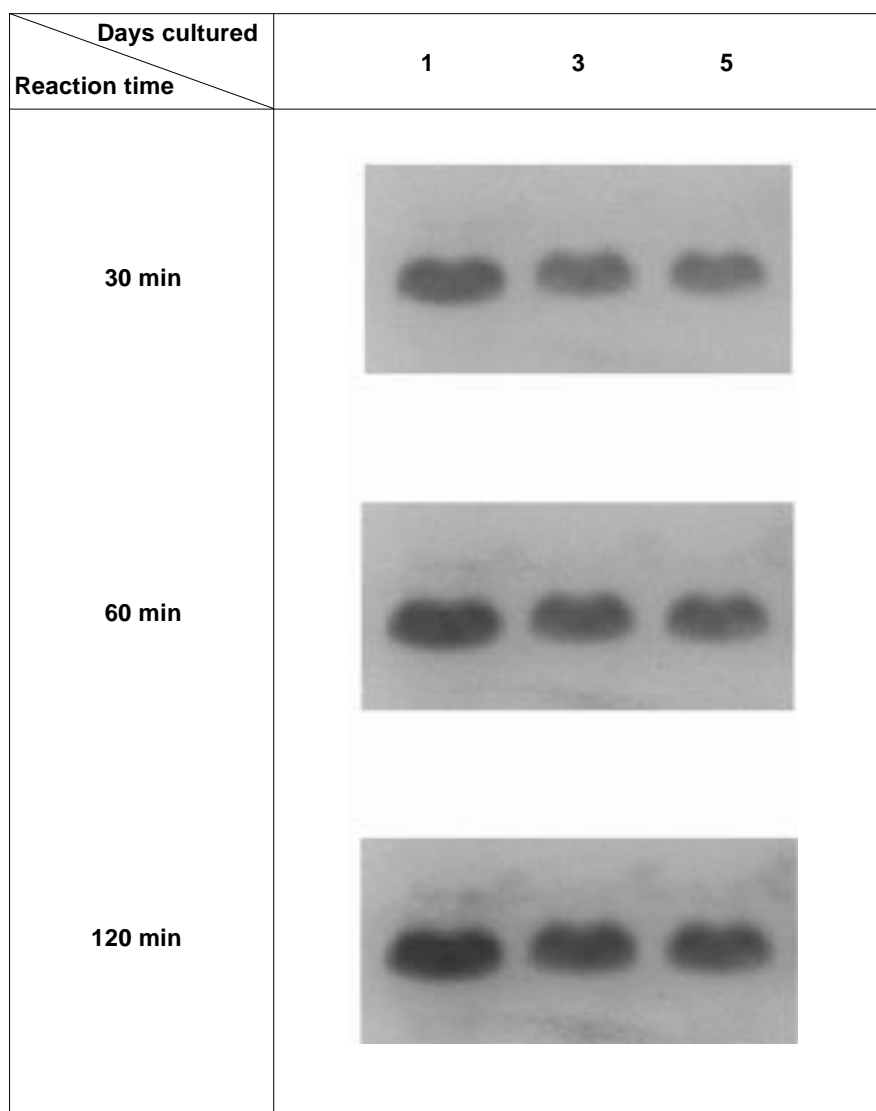


Fig.4.1 Albumin gene expression in primary culture of chicken hepatocytes

Change in hybridization signal with reaction time after substrate addition.

Fig.4-1

HOW TO KEEP THE MEMBRANE FLAT

Plastic plate can be used to ensure sharp readout of membrane samples with FLA series imaging systems.

In the pictures below, a plastic plate of 5 mm thickness was used.

After the membrane has been mounted on the FLUOR stage, the plastic plate was placed on top to keep the membrane surface flat. Effect on background is minimal owing to the use of a low fluorescent material such as poly (methyl methacrylate).



< Procedure >

- (1) Place the membrane face down directly on the FLUOR stage glass.



- (2) Place the plastic plate on the membrane to flatten its surface.



- (3) Set the FLUOR stage in the stage setting section of the FLA series imaging system.
- (4) Conduct readout with Image Reader software.

5 References

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