COSMO BIO CO., LTD.
Inspiration for Life Science

TOYOBO

PerfectHyb™

Hybridization Solution

Instruction Manual

(Code No.: HYB-101)

Distributor

COSMO BIO CO., LTD.
Inspiration for Life Science

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Warning

・Transportation: This product should be kept at room temperature during transportation.
・Volume and storage methods for this product are as shown below.

Code No.	Volume	Storage
HYB-101	250 ml	Room temperature (shielded from light)
HYB-101S	25 ml	Room temperature (shielded from light)

・When stored at low temperatures, sedimentation may occur. In such cases, the product should be warmed at about 37℃ to dissolve the sediments before use.
・This product is a reagent for use in research. Never use for diagnosis or other clinical purposes. When using this product, follow general laboratory precautions and pay attention to safety.
・This product contains no organic solvents such as formamide.
・When using RI-labeled probes, the following precautions are essential.
  ・Wear protective gloves, clothes, glasses, etc.
  ・Perform experiments involving RI in areas designated for such experiments.
  ・Waste from RI experiments must be disposed of with due care, in accordance with designated procedures.

Introduction

Outline

Characteristics

PerfectHyb™ **Hybridization Solution** is a premixed buffer for hybridization. It has the following characteristics.

- Supports **Northern** and **Southern blotting**
- Hybridization time can be cut
- Allows analysis with both **RI**- and **non-RI-labeled probes**
- Optimum buffer for analysis using a hybridization oven, as **the temperature for hybridization is equal to that for probe cleaning**
- **No need to add salmon sperm DNA, etc.**
- **Low viscosity** makes handling easy

Major uses

- **Northern blotting**

  This product supports analysis using DNA probes, RNA probes and oligonucleotide probes. When used for pre-blotted type membrane* analysis, we recommend use of RI-labeled cDNA or oligonucleotide probes, taking into account background, rehybridization, etc.

  Non-RI-labeled probes sometimes produce background signals depending on detection conditions. Experiments using such probes need to be done carefully, referring to the relevant instruction manuals.

  * Good results have been obtained in analysis involving several manufacturers' pre-blotted membranes.

- **Southern blotting**

  We recommend use of DNA probes and oligonucleotide probes when using this product in Southern blotting. Non-RI-labeled probes sometimes produce background signals depending on detection conditions. Experiments using such probes need to be done carefully, referring to the relevant instruction manuals.

· Hybridization enhancement

This product contains a hybridization enhancer, which allows for shortened hybridization. When used in Northern blotting, this product enhances signals compared to conventional methods.

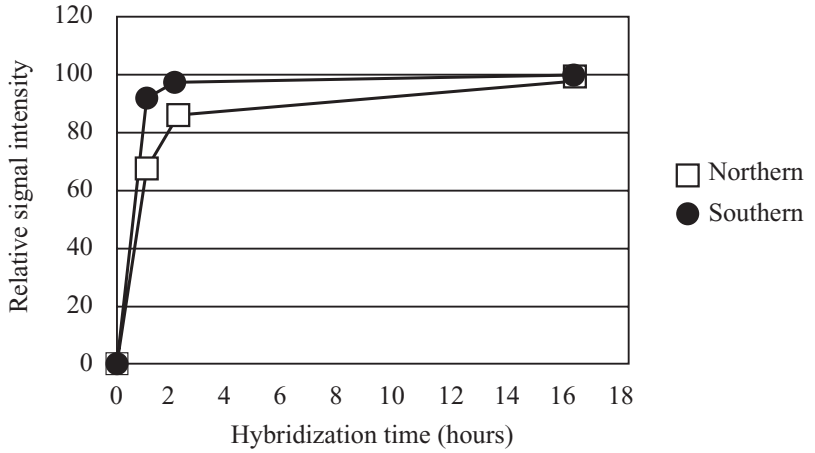

Fig. 1. Hybridization time and relative signal intensity in Northern blot (β-actin cDNA probe) and Southern blot (VNTR probe) tests using non-RI-labeled DNA probes. Amounts used: Total RNA 5 μg, Genomic DNA 1 μg.

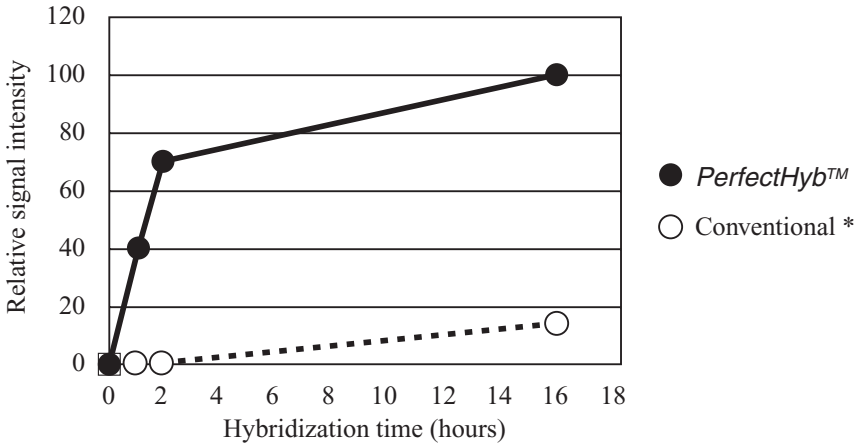

Fig. 2. Detection of low expression gene (transferrin receptor mRNA) with RI-labeled probe. Hybridization time and relative signal intensity. Amounts used: total RNA 5 μg.
* *Molecular cloning* 7.52 (50% formamide)

Additional material required

Introduction
The following are required to perform hybridization experiments using this product.

Reagents
・Cleanser A:
2 × SSC (pH 7.0), 0.1% SDS
・Cleanser B*:
0.1 × SSC (pH 7.0), 0.1% SDS
* Only necessary for Southern blotting or Northern blotting with RNA probes (cf. p.8 for details of reagent preparation)

Other equipment
・Thermostat (preferably with a shaking function) or a hybridization oven
・Heat sealer (unnecessary when an oven is used)
・Hybridization bag (unnecessary when an oven is used)
・X-ray film

Methods

Analysis using DNA and RNA probes

Introduction
This section presents analyses using DNA and RNA probes. Carry out experiments in accordance with protocols, after carefully reading the following instructions about probes and conditions of hybridization. See p.6 for analysis using oligonucleotide probes.

Preparing probes
・Prepare probes in accordance with the instruction manuals given for each labeling kit. After labeling, free nucleotide needs to be removed.
・After purifying RI-labeled probes their relative activity should be measured to confirm they have been labeled efficiently. In cases of non-RI-label probes, perform spot tests in accordance with the instruction manuals for each labeling kit, and calculate probe concentration from labeling efficiency.
・If using a long probe (prepared from 4 kb or longer template) or a probe with repeat sequences, there may be a rise in background signal or an unexpected extra band. Take adequate care when designing probes.

Conditions for hybridization and cleansing

・Perform hybridization and cleansing under the following conditions. The conditions given in the following table should be used when the experiment is performed as in the protocol given below.

	Probe	Label	Hybridization	Primary cleansing	Secondary cleansing
Time	—	—	One hour - overnight	5 min × 2	15 min × 2
Northern	DNA	RI Non-RI	68℃	68℃ (A)	68℃ (A)
	RNA	Non-RI	68℃	68℃ (A)	68℃ (B)
Southern	DNA	RI Non-RI	68℃	68℃ (A)	68℃ (B)

(A): Cleanser A, (B): Cleanser B

・When using a thermostat, the Hybribag is convenient for hybridization. Cleansing should be performed with a tapper, etc. (preferably shaking the container while cleansing).

・In experiments using RI-labeled probes, hybridization ovens are convenient and minimize contamination. When using a hybridization oven, perform hybridization and cleansing within the hybridization bottle.

Probe concentration

Recommended probe concentrations are shown below. Hybridization tends to be promoted as probe concentration increases. Especially when using non-RI-labeled probes concentration should be determined taking into account the possibility that the background signal will rise.

Label	Probe	Probe concentration
RI	DNA	1 - 2 × 10^6 cpm/ml or 1 - 10 ng/ml
Non-RI	DNA	0.2 - 1 ng/ml
	RNA	

Hybridization time

・Hybridization time should be determined with reference to the following table.

	Target gene expression level	Hybridization time
Southern	—	1 hour
Northern	High	1 hour
	Medium	2 hours
	Low	2 hours - overnight
When using a long-chain (≥4 kb) probe		Overnight * (for a non-RI-labeled probe, the concentration should be set lower)

*In experiments using long-chain (≥4 kb) probes that are more likely to produce background signals, overnight hybridization sometimes lowers background signals and increases S/N ratio. When using non-RI-labeled probes, the concentration should be set lower.

・Hybridization tends to proceed more rapidly as probe concentration increases but this also elevates background signals. Hybridization time should be determined on the basis of the target gene expression level and probe concentration.

・For detection of genes with unknown expression level or rare genes, Toyobo recommends overnight hybridization using RI-labeled probes.

Protocol

(1) Add a minimum 5 ml of *PerfectHyb*™ to the 10 × 10 cm (100 cm^2) membrane, followed by pre-hybridization at 68 ℃ for at least 20 minutes.
(2) Boil probe for 5 minutes. *
(3) Combine at least 5 ml (for the 10 × 10 cm (100 cm^2) membrane) of *PerfectHyb*™ preheated at 68 ℃ with the probe. Agitate the mixture.
(4) Discard the pre-hybridization fluid, and add the hybridization fluid prepared in step (3).
(5) Perform hybridization for between one hour and overnight at 68 ℃.
(6) Wash with primary cleanser (preheated at 68 ℃) for 2 sessions (5 minutes/session).
(7) Wash with secondary cleanser (preheated at 68 ℃) for 2 sessions (15 minutes/session).

(8) [If using an RI-labeled probe]
Take the membrane out and dry it on filter paper. Expose it to X-ray film.
[If using a non-RI-labeled probe]
Perform detection in accordance with the protocol for non-RI-labeled probes.

* The probe does not need to be cooled rapidly if used immediately after denaturation.
* Probe denaturation should be performed in a low-salt fluid such as distilled water.
* RNA probes also require denaturation.

Analysis using oligonucleotide probes

Preparing probes

Prepare probes in accordance with the instruction manuals given for each labeling kit. After labeling, free nucleotide needs to be removed. After purifying RI-labeled probes, their relative activity should be measured to confirm that they have been labeled efficiently.

Calculation of a probe's Tm

The probe's Tm is calculated as follows:

・If the oligonucleotide is shorter than 18 b

$Tm = (A + T) \times 2℃ + (G + C) \times 4℃$

・If the oligonucleotide is 18 b or longer

$Tm = 81.5 + 16.6 (\log10 [Na^+]) + 0.41 (\%G + C) – (600/N)$

* A : Number of A in the oligonucleotide (same for T, G and C)
%G + C : Percentage of G + C in the oligonucleotide
N : Number of bases in the oligonucleotide
$[Na^+]$: 0.75 M

Conditions for hybridization and cleansing

・Perform hybridization and cleansing under the following optimal conditions. The conditions given in the following table should be used when the experiment is performed as in the protocol given below.

Label	Hybridization	Primary cleansing	Secondary cleansing
Time	1 - 2 hours	5 min × 2	10 min × 2
RI	Tm-10℃	Tm-10℃ (A)	Tm-10℃ (A)
Non-RI			

(A): Cleanser A

* If the probe's Tm exceeds 70℃, both hybridization and cleansing should be performed at 55-60℃.

・When using a thermostat, the Hybribag is convenient for hybridization. Cleansing should be performed with a tapper, etc. (preferably shaking the container while cleansing).
・In experiments using RI-labeled probes, hybridization ovens are convenient and minimize contamination. When using a hybridization oven, perform hybridization and cleansing within the hybridization bottle.

Probe concentration

Recommended probe concentrations are shown below. Hybridization tends to be promoted as probe concentration increases. Especially when using non-RI-labeled probes concentration should be determined taking into account the possibility that the background signal will rise.

Label	Probe concentration
RI	2.5 - 5 pmoles/ml
Non-RI	0.5 - 1 pmoles/ml

Protocol

(1) Add a minimum 5 ml of *PerfectHyb*™ to the 10 × 10 cm (100 cm^2) membrane, followed by pre-hybridization at the optimal temperature for at least 20 minutes.
(2) Combine at least 5 ml (for the 10 × 10 cm (100 cm^2) membrane) of *PerfectHyb*™ preheated at the optimal temperature with the probe. Agitate the mixture.
(3) Discard the pre-hybridization fluid, and add the hybridization fluid prepared in step (2).
(4) Perform hybridization for 1-2 hours at the optimal temperature.
(5) Wash with primary cleanser (preheated at the optimal temperature) for 2 sessions (5 minutes/session).
(6) Wash with secondary cleanser (preheated at the optimal temperature) for 2 sessions (10 minutes/session).
(7) [If using an RI-labeled probe]
Take the membrane out and dry it on filter paper. Expose it to X-ray film.
[If using a non-RI-labeled probe]
Perform detection in accordance with the protocol for non-RI-labeled probes.

Appendix

Methods for preparing reagents

・20 × SSC
3M NaCl
0.3M sodium citrate
<To prepare one liter>
1. Dissolve NaCl (175 g) and trisodium citrate dihydrate (88 g) in 900 ml of sterile water.
2. Adjust the pH of the solution to 7.0 with 1 N HCl. Increase the volume of the solution to 1 liter by adding sterile water.
3. Store at room temperature.

・10% SDS
<To prepare 500 ml>
1. Dissolve SDS (50 g) in sterile water then increase the volume of the solution to 500 ml by adding sterile water.
2. Store at room temperature.

・Cleanser A
2 × SSC, 0.1% SDS
<To prepare 500 ml>
1. Combine 20 × SSC (50 ml) and 10% SDS (5 ml) with 445 ml of sterile water. Agitate the mixture.
2. Store at room temperature.

・Cleanser B
0.1 × SSC, 0.1% SDS
<To prepare 500 ml>
1. Combine 20 × SSC (2.5 ml) and 10% SDS (5 ml) with 492.5 ml of sterile water. Agitate the mixture.
2. Store at room temperature.

・20 × SSPE
3M NaCl, 173 mM sodium dihydrogenphosphate, 25 mM EDTA
<To prepare one litre>
1. Dissolve NaCl (175 g), sodium dihydrogenphosphate dihydrate (27 g) and EDTA·2Na (7.4 g) in 800 ml of sterile water.
2. Adjust pH to 7.4 with 5N NaOH.
3. Increase the volume of the solution to 1 liter by adding sterile water.
4. Store at room temperature.

Probe stripping

Introduction

Stripped membranes tend to emit less signals, leading to increased background. When re-probing, we recommend UV cross-linking as well as baking after blotting. A typical method for stripping RI-labeled DNA probes is presented below.

- If a pre-blotted membrane is used, perform stripping in accordance with the relevant protocol.
- If a stripping protocol is designated for the membrane, that protocol should be followed.
- Care is needed since the optimal stripping protocol may vary depending on the type of probe used.

Reagents

- Stripping reagent (100 ml)

Formamide	55 ml
20 × SSPE	10 ml
10% SDS	5 ml
Distilled water	30 ml

- Cleanser B

  0.1 × SSC (pH 7.0), 0.1% SDS

Other equipment

- Thermostat (or hybridization oven)

Protocol

(1) Transfer the membrane to the hybridization bag or hybridization bottle.
(2) Apply the stripping reagent (10 ml) to the 10 x 10 cm (100 cm^2) membrane.
(3) Incubate at 68 ℃ for 1-2 hours.
(4) Discard the stripping reagent, and incubate in cleanser B at 68 ℃ for 10 minutes.
(5) Place the membrane between two pieces of wrapping material and measure radioactivity with a survey meter (for reliability, expose the X-ray film overnight to confirm the absence of signals).
(6) If the count is zero, the membrane can be used for the experiment. If the membrane is not used immediately, it should be stored, taking care to avoid drying.

Simplified purification of RI-labeled DNA probes

Introduction

A simplified method for purification of RI-labeled probes, using random priming with Toyobo's MagExtractor® -PCR & Gel clean up- (Code No. NPK-601) is presented below.

Requirements

・MagExtractor® -PCR & Gel clean up- (Code No. NPK-601)
・75% Ethanol
・Magnetic stand (commercially available products such as Toyobo's Mag*ical Trapper* - Code No. MGS-101)

Protocol

(1) Combine labeled DNA probe solution (approx. 50 μl) with an adsorbing agent (200 μl). Agitate the mixture gently.
(2) Add magnetic beads (15 μl) to the mixture. Leave to stand for 2 minutes at room temperature, agitating occasionally with a Vortex mixer.
(3) Separate the beads with the magnetic stand, and collect the supernatant with a pipette. Place in a waste tank.
(4) Add cleanser (300 μl), and agitate the mixture for 10 seconds with a Vortex mixer.
(5) Separate the beads with the magnetic stand, and collect the supernatant with a pipette. Placed in a waste tank.
(6) Add 75% Ethanol (1 ml), and agitate the mixture for 10 seconds with a Vortex mixer.
(7) Separate the beads with the magnetic stand, and collect the supernatant with a pipette. Place in a waste tank.
(8) Centrifuge lightly, and remove ethanol completely with a pipette.
(9) Add distilled water (25-50 μl). Suspend beads completely using a Vortex mixer. Leave the suspension to stand at room temperature for 2 minutes.
(10) Separate the beads with the magnetic stand, and collect the supernatant with a pipette. Place in a new tube.
(11) Repeat steps (9) and (10).

・Manipulate through an acryl screen.
・Contamination can be minimized by the use of a screw cap type 1.5 ml tube.
・Step (11) can be omitted.

Trouble shooting

Trouble	Cause	Countermeasure
Weak signal	Exposure time insufficient	If the background signal is low, extend the exposure time. If background signal is high, take measures referring to the Background section.
	Probe's specific activity is low	If using an RI-labeled probe, measure and check radioactivity count. If using a non-RI-labeled probe, check the labeling efficiency by a spot test.
	Low probe concentration	Elevate probe concentration while taking care to avoid increased background signals.
	Probe is too old	If using an RI-labeled probe, prepare it immediately before hybridization. Avoid storing labeled probes.
	Probe has not been thermally denaturated	If using double-strand DNA probes or RNA probes, denaturate thermally immediately before use.
	Hybridization time insufficient	Overnight hybridization sometimes yields better results when a target gene with low expression level is to be detected by Northern blotting.
	Probing has been repeated	As probing is repeated more often, signals tend to weaken. Extend exposure time.
	Conditions for cleansing inappropriate	If the cleansing time is too long, it should be shortened. Signals may strengthen with elevated concentration of SSC (e.g., from 0.1 x to 0.2 x). However, the SSC concentration should be elevated carefully as this can cause nonspecific bands and background signals.
	Insufficient amount of blotted nucleic acid	Increase the amount of nucleic acid used for blotting.
High background signals	Free nucleotide has not been removed	After preparing probes, remove free nucleotide before use.
	High probe concentration	Particularly when using non-RI-labeled probes, elevating probe concentration can lead to extremely high background levels. The concentration of non-RI-labeled probes recommended for *PerfectHyb*™ is lower than the concentrations set in ordinary protocols.
	Probe designed inappropriately	The background signal level can rise when using long probes prepared from 4 kb or longer templates or probes with repeat sequences. Check the size and sequence of the probe.
	Membrane dried during manipulation	If the membrane has dried during hybridization, cleansing, etc., the background signal can rise. Take care to prevent the membrane from drying.

	Bubbles formed during prehybridization or hybridization	Particularly when using hybridization bags, bubbles can disturb reliable analysis. We recommend removing bubbles completely.
	Amount of cleanser insufficient	Wash with adequate amounts of cleanser.
	The detection system has problems	If using a non-RI-labeled detection system, background signal is likely to increase. Perform detection carefully, with reference to instruction manual.
Extra bands are visible	Probe contains nonspecific sequences	If the probe contained repeat sequences such as Alu, unexpected bands are likely to appear. Take care when designing probes. Extra band formation may be reduced by adding denatured salmon DNA in a concentration of 100 μg/ml during pre-hybridization or hybridization.
	Cleansing is inadequate	Increasing the frequency of cleansing or reducing the SSC concentration of cleanser may eliminate extra bands if the target band is known well.

Related products

Product Names	Package	Storage temperature	Code No.
MagExtractor® -PCR & Gel Clean up-	200 rxns *	Room temperature	NPK-601
Mag*ical Trapper*	1	Room temperature	MGS-101

* This product can be used for 400 purification sessions with RI-labeled probes if 50 μl of probe is purified at a time.

Manufacture

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