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Hybridization Buffers I: Comparison of Formamide vs. Aqueous Hybridization Solutions

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KPL offers the flexibility of two types of hybridization buffers for use in Northern and Southern blot detection, one a formamide-based solution and the second an aqueous buffer. Formamide Hybridization Buffer (Cat. No. 50-86-10) has been designed as part of KPL's Non-Radioactive Detector™ Systems to provide optimal performance in nucleic acid blotting applications. A destabilizer, formamide lowers the melting temperature of hybrids thus increasing the stringency of the probe to target binding. Use of this agent with specified hybridization temperatures results in minimal nonspecific hybridization; less optimization of washes is required by the end user. Formamide Hybridization Buffer is a suitable universal hybridization solution.

For those researchers wishing to minimize hazardous waste, the aqueous Membrane Hybridization Buffer (Cat. No. 50-86-08) is a non-hazardous alternative. It is not recommended for all hybridization applications as will be shown in this paper. However, Membrane Hybridization Buffer does perform very well for hybridizations with plasmid DNA and moderately expressed transcripts.

To demonstrate the utility of the Formamide and Membrane Hybridization Buffers, a series of comparative studies were conducted and summarized in this Application Note. Both buffers were tested for relative performance in Northern and Southern blots, detecting plasmid DNA as well as low and abundantly expressed transcripts.

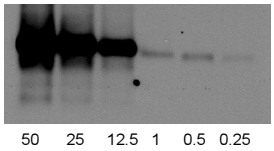
Formamide Hybridization vs. Membrane Hybridization Buffer – Northern Blot Analysis

Using an RNA probe for the detection of a moderate to abundantly expressed message

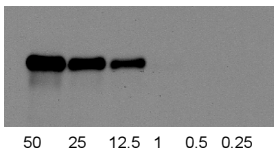
Two-fold serial dilutions starting at 50 ng of total RNA were electrophoresed on a 1% formaldehyde gel. Using alkaline transfer, the RNA was blotted to Biodyne® B membrane. Hybridization and detection were as follows:

1. The membrane was cut in half to represent two duplicate dilution series. One membrane was pre-hybridized for 1 hour at 65°C in Formamide Hybridization Buffer + 100 ug/mL Herring Sperm DNA. The second membrane was pre-hybridized for 30 minutes at 50°C in Membrane Hybridization Buffer + 200 µg/mL Herring Sperm DNA.
2. A biotinylated 18s riboprobe was denatured at 68°C for 10 minutes and immediately placed on ice. (Note: The RNA probes in these studies were generated via *in vitro* transcription using KPL's Detector RNA *in vitro* Transcription Biotinylation Kit, Cat. No. 60-01-02.)
3. The probe was added to each of the blots at a final concentration of 50 ng/mL of hybridization solution.
4. Blots were hybridized overnight at the same temperature as the pre-hybridization step (65°C in Formamide Hybridization Buffer and 50°C in Membrane Hybridization Buffer, respectively).
5. The blot hybridized in the Formamide Hybridization Buffer was washed 2 x 15 minutes at room temperature in 2X SSPE + 0.1% SDS followed by 2 x 30 minute washes at 65°C in 0.2X SSPE + 0.1% SDS and one final wash for 5 minutes in 5X SSPE.
6. The blot hybridized in the Membrane Hybridization Buffer was washed 2 x 10 minutes at room temperature in 0.5X SSPE followed by 2 x 10 minutes at 55°C.
7. Chemiluminescent detection was performed according to the protocol in the Detector™ AP Chemiluminescent Blotting Kit (Cat. No.'s 54-30-01/02) manual. Exposures of the blots to KODAK BIOMAX Light film were performed at 1 and 10 minutes.

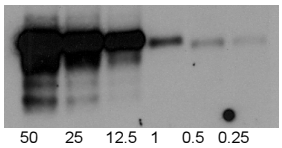
Data and Conclusions



KPL's Formamide Hybridization buffer 1 minute exposure (ng RNA)



KPL's Membrane Hybridization Buffer 1 minute exposure (ng RNA)



KPL's Membrane Hybridization Buffer 10 minute exposure (ng RNA)

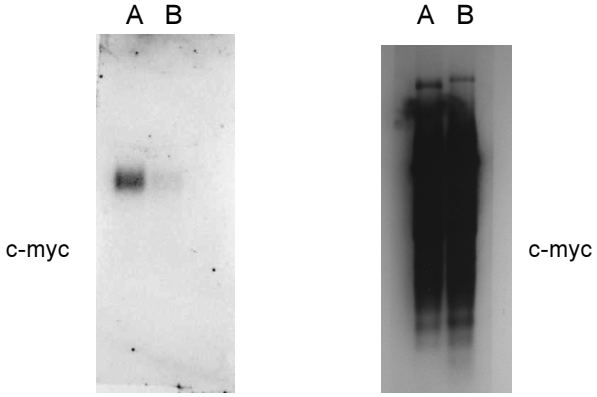
Results of the comparison show that the membranes were sufficiently hybridized such that the riboprobe was able to specifically bind to the target. Additionally, the level of sensitivity achieved is comparable with both hybridization buffers when moderate to abundant messages are probed. The 18s rRNA was detectable to 0.25 ng in both cases. The difference between the blots, however, is observed in the relative sensitivity by exposure time. The Formamide Hybridization Buffer generated stronger signal in a 1-minute exposure to film, whereas equivalent sensitivity was achieved in the blot hybridized with the Membrane Hybridization Buffer after a 10-minute film exposure.

Using an RNA probe for detection of a low expressed transcript

Duplicate lanes of 5 μg of total RNA from WEH1-231 untreated and anti-IgM treated cells were electrophoresed on a 1% formaldehyde gel. RNA was transferred by a 2 hour alkaline method to Biodyne B membrane. Hybridization and detection were as follows:

1. The membrane was cut in half, each blot containing control and treated RNA. One membrane was pre-hybridized for 1 hour in Formamide Hybridization Buffer + 100 μg/mL Herring Sperm DNA at 65°C. The second membrane was pre-hybridized for 30 minutes in Membrane Hybridization Buffer + 200 μg/mL at 55 °C.
2. A biotinylated c-myc riboprobe was denatured at 68°C for 10 minutes and immediately placed on ice.
3. Denatured probe was added to each of the blots to a final concentration of 50 ng/mL of hybridization solution.
4. Blots were hybridized overnight at the same temperature as the pre-hybridization step (68°C in Formamide Hybridization Buffer and 55°C in Membrane Hybridization Buffer, respectively).
5. The blot hybridized in the Formamide Hybridization Buffer was stringently washed 2 x 15 minutes at room temperature in 2X SSPE + 0.5% SDS followed by 2 x 30 minutes at 68°C in 0.2X SSPE + 0.5% SDS and one final wash for 5 minutes in 5X SSPE.
6. The blot hybridized in the Membrane Hybridization Buffer was stringently washed 2 x 10 minutes at room temperature in 0.5X SSPE followed by 2 x 10 minutes at 55°C.
7. Using the Detector™ AP Chemiluminescent Blotting Kit, detection was carried out by standard protocol utilizing 0.5% Detector Block Powder in the block solution. The membrane was exposed to KODAK BIOMAX Light film for 1 minute following a 1 hour incubation.

Data and Conclusions



Formamide **Membrane**

According to the design of this assay, c-myc mRNA should be observed as a single band in the control sample (A) and appear down regulated in the treated sample (B). The blot hybridized with Formamide Hybridization Buffer delivers the expected result. RNA-RNA hybrids are the most stable of the nucleic acid hybrids and thus the most difficult to disassociate even when they are not completely complementary. In this study, Formamide Hybridization Buffer effectively minimized the presence of non-specific hybrids.

However, detection of the transcript with the Membrane Hybridization Buffer could not be achieved.

Significant non-specific binding of the probe to the total RNA was seen when using the Membrane Hybridization Buffer. Multiple attempts were made to increase the stringency of both the hybridization temperature and the washes (data not shown); each blot produced the same pattern. Although the Membrane Hybridization Buffer worked well for the abundantly expressed message, the use of the aqueous Membrane Hybridization Buffer is not recommended in the detection of a low expressed message with a riboprobe. It is also important to note that the stringency of the hybridization temperature and the post-hybridization washes may need to be optimized for each expressed mRNA as detailed in the two experiments shown thus far.

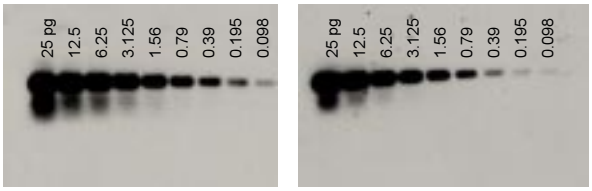
Formamide Hybridization Buffer vs. Membrane Hybridization Buffer – Southern Blot Analysis

Using a random primed DNA probe to detect plasmid DNA

Two-fold serial dilutions of plasmid DNA containing a timp-2 insert were prepared, starting with 25 pg of insert. The dilution series was loaded in duplicate on an agarose gel and subsequently electrophoresed, denatured and neutralized by standard methods. The gel was transferred overnight onto Biodyne® B, a positively charged nylon membrane.

1. The membrane was cut in half to represent two duplicate dilution series. One membrane was pre-hybridized for 1 hour in Formamide Hybridization Buffer + 100 μg/mL Herring Sperm DNA at 42°C. The second membrane was prehybridized for 30 minutes in Membrane Hybridization Buffer + 200 μg/mL at 50°C.
2. A timp-2 random primed biotinylated DNA probe was denatured at 95°C for 10 minutes and immediately placed on ice. (Note: The DNA probe used in this study was biotinylated via KPL's Detector™ Random Primer DNA Biotinylation Kit, Cat. No. 60-01-00.)
3. Denatured probe was added to each of the blots to a final concentration of 50 ng/mL of hybridization solution.
4. Blots were hybridized overnight at the same temperature as the pre-hybridization step (42°C in Formamide Hybridization Buffer and 50°C in Membrane Hybridization Buffer, respecitvely).
5. The blot hybridized with Formamide Hybridization Buffer was stringently washed 2 x 15 minutes at room temperature in 2X SSPE + 0.1% SDS followed by 2 x 30 minutes at 65°C in 0.2X SSPE + 0.1% SDS and one final wash for 5 minutes in 5X SSPE.
6. The blot hybridized in the Membrane Hybridization Buffer was stringently washed 2 x 10 minutes at room temperature in 0.5X SSPE followed by 2 x 10 minutes at 50°C.
7. Detection was carried out by standard protocol using the Detector™ HRP Chemiluminescent Blotting Kit (Cat. No. 54-30-00). Exposure to KODAK BIOMAX Light film was performed for 10 minutes.

Data and Conclusions



Formamide Membrane

Both hybridization buffers worked equally well in the detection of timp-2, each resulting in the detection of as low as 98 fg of plasmid DNA. Relatively, the blot hybridized with the Formamide Hybridization Buffer yielded slightly higher sensitivity with the same exposure time to film. For this type of hybridization where the complexity of the target is not an issue, either buffer is suitable when used under the recommended standard stringent conditions.

However, as the results in the hybridization and detection of the low expressed mRNA show, a similar phenomenon is found in the use of Membrane Hybridization Buffer in single copy genomic Southern blots. As the amount of target in the total sample becomes proportionately smaller, the requirements for stringency becomes greater and the need for further optimization of this hybridization solution also increases. Likewise, greater stringency is required for the hybridization to allow for the specific detection of single copy genes. Therefore, Membrane Hybridization Buffer is not recommended for this application.

Related products:

Description	Size	Catalog No.
Formamide Hybridization Buffer	240 mL	50-86-10
Membrane Hybridization Buffer	240 mL	50-86-08
Detector™ HRP Chemiluminescent Botting Kit	2000 cm^2	54-30-00
Detector™ AP Chemiluminescent Blotting Kit	2000 cm^2 500 cm^2	54-30-01 54-30-02
Detector™ Random Primer DNA Biotinylation Kit	30 reactions	60-01-00
Detector™ PCR DNA Biotinylation Kit	30 reactions	60-01-01
Detector™ RNA *in vitro* Transcription Biotinylation Kit	20 reactions	60-01-02
GeneRuler™ Biotinylated DNA Ladder	20 - 50 lanes	600-0008
Herring Sperm DNA	40 mg	60-00-14
Biodyne® B Nylon Membrane	20 cm x 1 mL roll	60-00-50
Hybridization Bags, 8” x 10”	50/pk	60-00-51

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