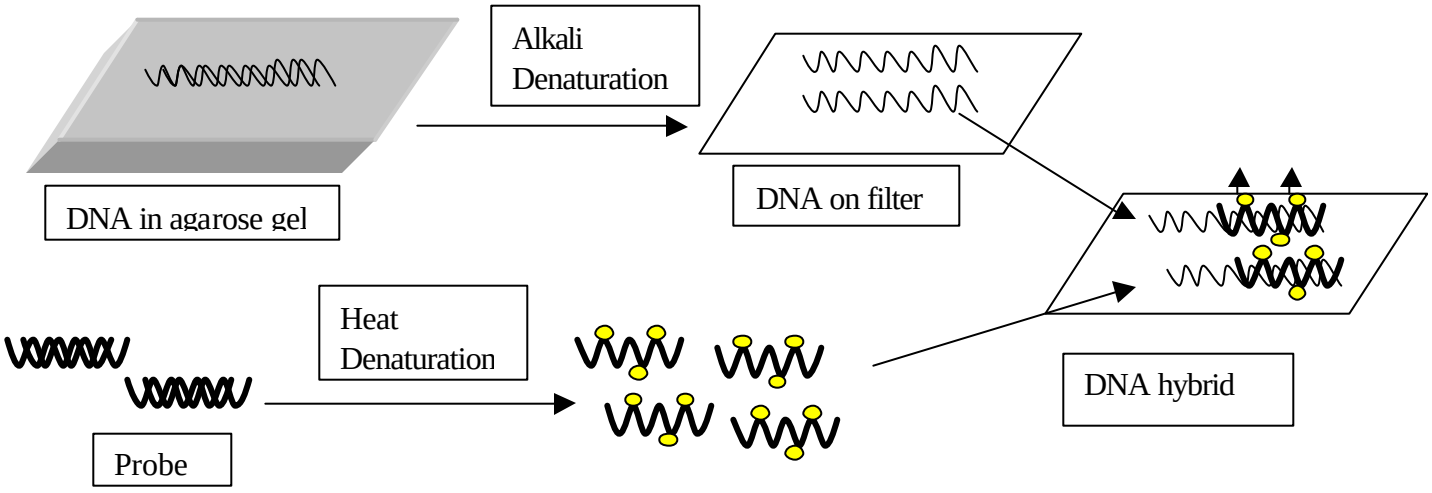
PLB161A Laboratory XII
Genome Mapping

Hybridization

Introduction: Nucleic acid hybridization is the last step towards the detection of RFLPs. The two strands of DNA are held together primarily through complementary base pairing (A to T, C to G). When the duplex molecule is subjected to conditions such as high temperature or alkali treatment, denaturation of the double strands results in a separation into single strand molecules. The DNA transferred to the Nylon membrane was denatured by the alkali treatment and the single strand molecules are immobilized on the membrane. The probe is denatured and the single strand molecules are labeled with ^{32}P-dCTP. Under favorable conditions, in the hybridization bottles, the single strands will reanneal into a duplex state. Some of the labeled single strand molecules of the probe will reanneal with the single strand molecules of the Nylon membrane.

Regions of non-specific attachment of probes to the surface of filters do occur, and to eliminate this effect blocking agents (like PAES) are included in the prehybridization and hybridization steps.

After filter hybridization, the unbound probe is washed off the filter with a series of stringency washes and detection of the DNA:DNA hybrids is done by exposing the membrane directly to an X-ray film.



DNA hybridization and detection

Factors affecting hybridization rate

Nucleic acid hybridization depends on the random collision of two complementary sequences.

Factors affecting **reaction rates**:

- **Concentration** of target DNA in the filter [C_f] and of probe [C_s]. The disappearance of single strand is a function of two competing reactions:

Disappearance of single strands	Hybridisation on the filter		Reassociation in solution
$-d[C_s]/dt =$	$k_1[C_s][C_f]$	+	$k_2[C_s]^2$

At high [C_s] reassociation in solution is favored. During incubation the reaction will change from being in probe excess to being in filter-bond excess.

- **Length of strands**:

	Reannealing of DNA is
In solution	Proportional to the square root of the length of the shorter strand
In filters with low [C_f]	Independent of the length of the probe (nucleation-limited reaction)
In filters with high [C_f]	Inversely proportional to the length of the probe (diffusion limited reaction)

- **G + C content**: small effect on reaction rate
- **Mismatching**: If hybridization is carried at optimum temperature (T_m – 25 °C) the reaction rate is reduced by a factor of two for every 10% mismatch.
- **Ionic strength:** at low ionic strength nucleic acid hybridize very slowly. The effect of higher ionic strength on reassociation rates is more dramatic at low NaCl concentrations.
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SSC	Monovalent cation concentration	Reassociation rate relative to 0.18 M NaCl
0.1	0.015	0.00
0.5	0.075	0.10
1.0	0.150	0.69
2.0	0.300	2.31

2.5	0.375	3.07

High salt concentrations stabilize mismatched duplexes, so to detect heterologous probes, the salt concentration must be kept high.

- **Formamide**
  - Formamide decreases the T_m of nucleic acid hybrids.
  - Concentrations of formamide between 30 – 50 % have no effect on the rate of filter hybridization.
  - By including 30 – 50% formamide in the hybridization solution the incubation temperature can be reduced to 30 – 42 °C.

Factors affecting hybrid stability

The melting temperature of duplex DNA (T_m) is a measure of its thermal stability.

In a perfectly-matched hybrid (homologous probes) T_m is dependent on:

- **Ionic strength**
- **Base Composition**
- **Denaturing agents**

T_m= 81.5 + 16.6(log NaCl M) + 0.41(% G+C) – 0.72(% formamide)

- The T_m of a duplex DNA decreases by 1 °C with every increase of 1% in the mismatched base pairs (depending on the distribution of mismatches this reduction varies between 0.4 to 1.4 °C). At high concentrations of salt mismatched hybrids are more stable.
- Washing should be carried out at T_m -12 °C

%mismatch	0 %		10 %		20 %	
SSC	T_m	Wash	T_m	Wash	T_m	Wash
2X SSC (0.30 M NaCl)	93.3	81.3	83.3	71.3	73.3	61.3
1X SSC (0.15 M NaCl)	88.3	76.3	78.3	66.3	68.3	56.3
0.5X SSC (0.075 M NaCl)	83.3	71.3	73.3	61.3	63.3	51.3

A. Random-Primer labeling of DNA Hybridization Probes.

(Feinberg and Vogelstein (1983) Anal. Biochem. **132**: 6)

Introduction:

This procedure is the preferred method of preparing radiolabeled probes because less amount of template is required, DNA fragments are labeled homogeneously, and probes with high specific activities are produced. Synthesis of radiolabeled DNA probes proceeds using a single-stranded DNA template, one [α-^{32}P] dNTP and three unlabeled dNTPs as precursors to yield probes with specific activities of 5 X 10^8 to 4 X 10^9 cpm/μg. Radiolabeled probes prepared by random priming are usually 400 - 600 nucleotides in length.

The DNA that is going to be used as a probe is denatured by boiling and chilled in ice. The single stranded DNA is mixed with a molar excess of random hexanucleotide primers, and used as a template for the synthesis of radiolabeled DNA fragments. The Klenow fragment of *E. coli* DNA Polymerase I is used in this reaction. This enzyme lacks 5' - 3' exonuclease activity so the radioactive product is synthesized solely by primer extension.

Procedures: for a standard 50-μl reaction

1. Transfer the Plexiglas container of [α-^{32}P] dCTP to the radioactive hood and place it behind the Plexiglas shield at room temperature to defrost.

Caution: Wear the appropriate clothing for dealing with radioactivity

2. Label one 1.5-ml microcentrifuge tube for each probe. Dilute 30 ng of the DNA that will be used as a probe to a total of 31.25 μl with dd$H_2$0.
3. Boil the 1.5-ml tube for 8 minutes and then quench 5 minutes on ice. Spin in microcentrifuge for few seconds.
4. Prepare 13 μl of random-primer mix per sample. Include one extra sample in your calculations to compensate for pipetting errors. Keep all tubes in ice.

   Random primer mix: 5 μl of 10X Klenow buffer + 5 μl of Random Primers pd(N6) + 3 μl of ATG Buffer

   Add 0.75 μl of Klenow per sample just before use. The enzyme is in glycerol and will go to the bottom of the 1.5-ml tube. Mix it slowly by pipetting up and down until the enzyme and the glycerol is uniformly distributed in the solution.

5. Add 13.75 μl of mix to each DNA sample in ice. Behind the Plexiglas shield add 5.0 μl (50 μCi) of [α-^{32}P] dCTP labeled with a specific activity >3000 Ci/mmol, 10 mCi/μl.

Note: Use aerosol tips to prevent contaminating the pipet. All subsequent steps should be performed using aerosol tips and appropriate radiation safety techniques.

6. Incubate in a 37 ^{0}C-water bath for 2-3 hr behind Plexiglas. After 2-3 hr at 37 ^{0}C proceed with the purification of the probe (see "C")

Note: Greater than 70% of the radioactive label, which is the rate-limiting component, should be incorporated into the labeled DNA.

Solutions:

ATG buffer:

Unlabelled dNTPs from Boehringer Mannheim (Cat. N^o dATP: 1051 440, dTTP:1051 482, dGTP:1051 466) are 100 mM.

	Final	1ml
dATP	500 µM	5 µl
dTTP	500 µM	5 µl
dGTP	500 µM	5 µl
3 mM TRIS pH 7; 0.2 mM EDTA in ddH_2O	3 mM TRIS 0.2 mM EDTA	985 µl

10X Klenow buffer (From Current Protocols in Molecular Biology 3.4.2)**:**

	Final concentration	100 ml
1M TRIS, pH 7.5	0.5 M	50 ml
$MgCl_2$ - 6 H_2O	0.1 M	2.033 g
Dithiothreitol (Sigma D-9779)	10 mM	0.154 g
10 mg/ml BSA (New England Biolabs)	0.5 mg/ml BSA	5 ml
ddH_2O		To 100 ml

Usually provided by the Klenow vendor

Random Primers

Prepare under hood, inside the Pharmacia pd(N6) bottle. Dispense in aliquots of 200 µl and freeze (-20 °C). The final concentration is 18 OD_{260} units per ml.

Total	**2800 ml**
Stock Solution A	2.43 ml
2-mercaptoethanol	6.5 µl
10 mg/ml BSA	360 µl
$pd(N)_6$	50 OD_{260} units

Note: $pd(N)_6$ [50 OD_{260} units] available from Pharmacia, catalog # 27-2166-01

Stock Solution A

	Final concentration	For 30 ml
HEPES	670 mM	4.80 g
$MgCl_2$	17 mM	0.10 g
TRIS-HCl	170 mM	0.62 g
ddH_2O		To 30 ml

B. Prehybridization

1. Prepare 20 ml of prehybridization solution per hybridization bottle (PAES, see below). The prehybridization solution may precipitate at room temperature. Pre-warm the solution to dissolve SDS. The prehybridization solution can be stored in the refrigerator.
2. Cover the bench with Saran-Wrap and wet the surface with prehybridization solution. Wet the membrane in the prehybridization solution, make a tight roll and place it into a hybridization bottle. Pour 10 ml of prehybridization solution inside the bottle. Close the bottle and slowly unroll the membrane moving the prehybridization solution up and down along the membrane. This will remove most of the air bubbles.
3. Incubate in the rotary hybridization oven at 65 °C during 30-120 minutes. If this is the first use of the membrane, replace the prehybridization solution by another 10 ml of fresh prehybridization solution before hybridization.

SOLUTIONS:

20X SSC:

	Concentration	Amount for 1 l
Sodium Citrate Dihydrate Fisher S 279-3	375 mM	88.2 g
Sodium chloride (NaCl) Fisher S 271-3	3700 mM	175.3 g
dd H_2O		1 l[1]

[1]Solubilize in 900 ml distilled H_2O. Adjust pH 7.4 with concentrated HCl. Adjust final volume to 1 liter with distilled H_2O

10% SDS:

	Concentration	Amount for 1 l
Sodium Dodecyl Sulfate	10% w/v	100 g
dd H_2O		Adjust to 1000 ml[1]

Heating while stirring will help to dissolve the SDS. No autoclaving is necessary. Weigh under hood or using a mask.

20X SSPE:

	Concentration	Amount for 1 l
Sodium Phosphate Monobasic $NaH_2PO_4 \cdot H_2O$ Fisher S 369-3		27.6 g
Sodium chloride (NaCl)	2.3337 M	174 g
Na_2-EDTA	20 mM	7.4 g (or 40 ml 0.5 M EDTA)
dd H_2O		Adjust to 1 l[1]

[1]Heat in 800 ml of ddH_2O to dissolve, pH to 7.4 with NaOH (~6.5 ml of 10 N solution). Adjust volume with ddH_2O to 1 L and autoclave.

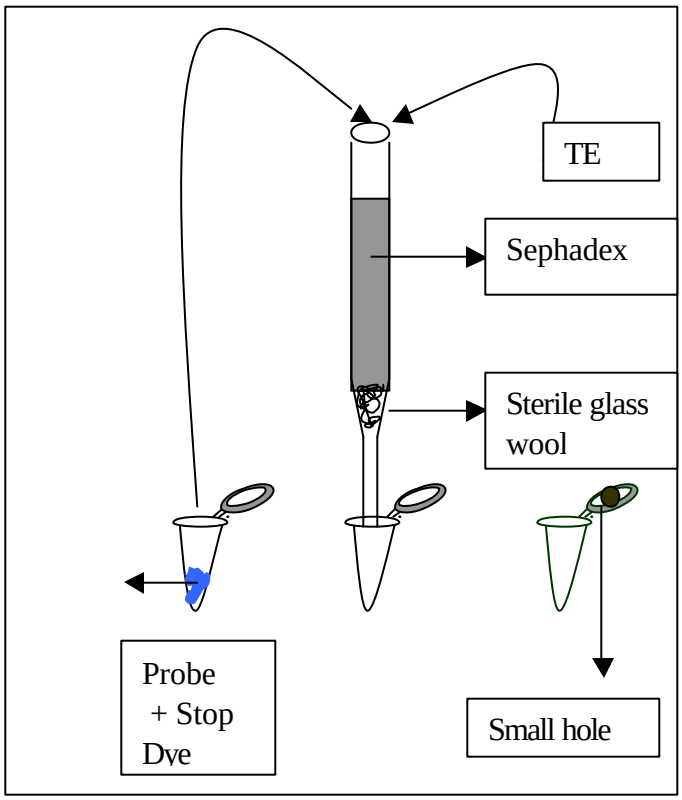
Prehybridization solution:

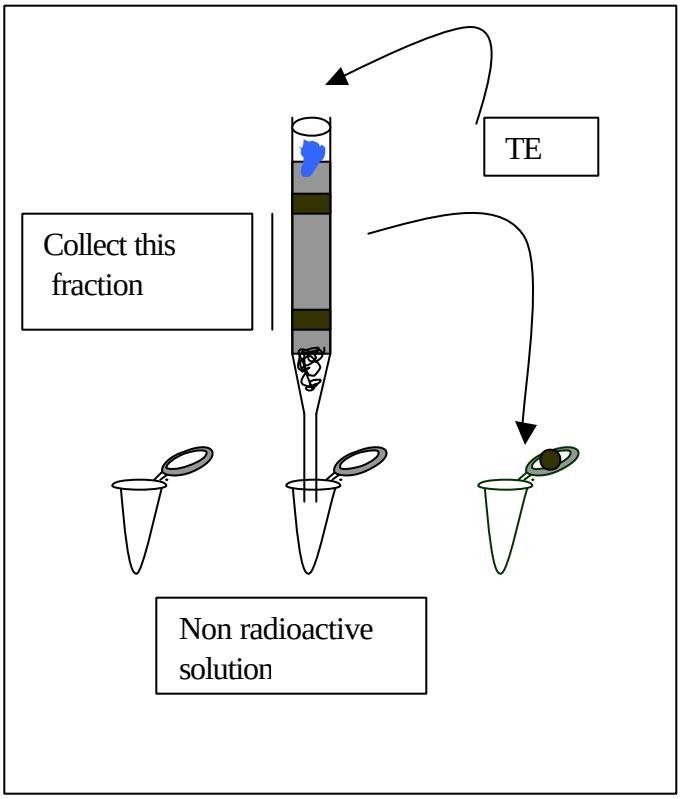
	Final	For 1 liter
20X SSPE	2.5 X	125 ml
10% SDS	1%	100 ml
Sodium Pyrophosphate ($NaP_2O_7 \cdot 10H_2O$) Sigma S-9515	0.01%	0.1 g
PAES: Polyanetolensulfonic Acid	0.1%	1 g
ddH_2O		775 ml

Mix water + SSPE + SDS and heat to 65 °C. Grind the Sodium Pyrophosphate and dissolve it with the PAES in the warm solution.

C. Purification of the probe: (can be also done with commercial columns ProbeQuant® G-50 Amershan Catalogue No. 27-5335-01)

1. All the following work is performed in the hood. Add 50 µl of STOP DYE SOLUTION FOR COLUMNS to each 1.5-ml tube used in part "A". Use a plastic tool to open the 1.5-ml tubes to avoid contamination of the gloves.





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2. Prepare 2 labeled 1.5-ml tubes, one with a little hole in the lid of the tube to collect the purified radioactive probe. Tubes without holes in the cap may explode when boiled producing a radioactive contamination.
3. Remove unincorporated nucleotides from the radiolabeled DNA probe using Sephadex G50 columns. Put the columns in an empty 1.5-ml tube. Add the labeled probe with the Stop Dye solution in the column using 200-µl aerosol tips. Add TE slowly to the top of the column, do not allow the column to dry. When the light blue stain is 1 cm above the bottom of the column, move it to the 1.5-ml tubes with the hole in the cap to collect the labeled insert. Collect until the 1.5-ml tube is almost full or until the second blue dye arrives to the bottom of the column. Put the column in the empty 1.5-ml tube and discard both into the radioactive waste.

Note: Specific activity of the probe should approach 2 X 10^9 cpm/µg. Since probes labeled to this high level of specific activity are subject to damage very quickly by radiochemical decay, radiolabeled probes should be used without delay.

4. Boil the 1.5-ml tube for 10 min, quench immediately on ice, and leave for 5 min.
5. Add the content of the tube to the hybridization bottle using a 1-ml aerosol tip. Check that the bottle does not leak. Place the bottle in the hybridization oven. The direction of the rotor of the oven should be the same as the direction the membrane was rolled when place inside the bottle. If not invert the bottle. Hybridize overnight at 65 ^{0}C.

Solutions

Stop dye solution for columns:

Total	25 ml
Sheared Salmon Sperm 10 mg/ml Sigma Type III sodium salt	5 ml
0.5 M EDTA, pH 8.0	5 ml
2X Stop Dye	15 ml

2X Stop Dye:

1) Dissolve 2.233 g of EDTA and 1.5 g of Blue Dextran in 80 ml of water
2) Dissolve 0.5 g of Bromophenol Blue in 20 ml of glycerol
3) Mix both solutions. Adjust pH to 7.6 with Na(OH)

Preparation of Sephadex G50: Slowly add 30 g of Sephadex G-50 to 500 ml of TE (pH8.0) in a 500-ml beaker or a bottle. Heat at 65 ^{0}C for 1-2 hrs. Allow to cool to room temperature. Decant the supernatant and replace with an equal volume of TE (pH 8.0). Store at 4^{0}C in a screw-capped bottle.

Preparation of the columns: Use disposable 5-ml borosilicate glass pipettes (Pasteur pipette). Plug the pipette with sterile glass wool. Add slowly the Sephadex in TE. Let the Sephadex accumulate in the bottom of the Pasteur pipette by gravity. Keep adding Sephadex until 1cm from the top of the glass pipette, Wash the column with TE. Store at 4^0C in a beaker or bottle with TE.

D. Washing

1. Open the hybridization bottle in the hood. Discard the probe in the liquid radioactive waste. Add 100 ml of 2 X SSC washing solution. Close the bottle and place it in the hybridization oven 30 min at 65 ^{0}C.

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2. Discard the 2 X SSC washing solution in the liquid radioactive waste. Add 100 ml of 1 X SSC washing solution. Close the bottle and place it in the hybridization oven 30 min at 65 ^{0}C.
3. Discard the 1 X SSC washing solution in the liquid radioactive waste. Add 100 ml of 0.5 X SSC washing solution preheated at 65 ^{0}C. Close the bottle and place it in the hybridization oven 15 min at 65 ^{0}C.
4. Discard the 0.5 X SSC washing solution in the liquid radioactive waste. Take out the membrane and place it in a plastic container with 500 ml of cold 0.5 X SSC washing solution. Wash the membranes for a few minutes with gentle agitation and transfer to another container with 500 ml of fresh, cold 0.5 X SSC washing solution.
5. Dry the membrane on paper towels and wrap them in Saran Wrap, without air bubbles.

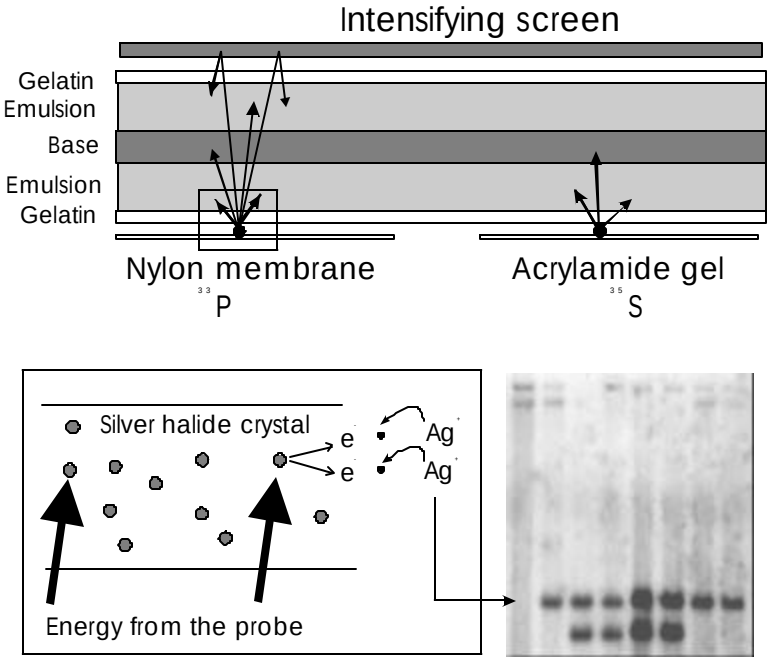
Washing solutions:

Washing solution	100ml		500ml		1000ml	
	20 X SSC	10% SDS	20 X SSC	10% SDS	20 X SSC	10% SDS
2 X	10 ml	5 ml	50 ml	25 ml	100 ml	50 ml
1 X	5 ml	5 ml	25 ml	25 ml	50 ml	50 ml
0.5 X	2.5 ml	5 ml	12.5 ml	25 ml	25 ml	50 ml
0.1 X	0.5 ml	5 ml	2.5 ml	25 ml	5 ml	50 ml

E. Autoradiography

Introduction:

Autoradiography produces permanent images on photographic film of the distribution of radioactive atoms on a two-dimensional surface. In molecular cloning, autoradiography is used for a variety of purposes, including the visualization of bands of radioactive nucleic acids in Southern and northern hybridizations, the identification of bacterial colonies or bacteriophage plaques that hybridize to radioactive probes, and the localization of bands of DNA in gels (e.g., in DNA sequencing). A diagram of the events that occur during exposure of photographic emulsion to radioactivity is shown in the next Figure. The lower rectangle is an amplification of the process that occur within the emulsion of the radiography.



The two isotopes most commonly used for autoradiography are ^{35}S and ^{32}P, both of which emit particles. However, the energies of these particles are very different: ^{35}S emits a particle with a maximum energy of 0.167 MeV that can penetrate film emulsion only to a depth of 0.25 mm. Although this is sufficient to allow the emitted particles to interact productively with silver halide crystals in the emulsion, it is not enough to allow the particles to pass through barriers (e.g., Saran Wrap) that might be placed between the film and the source of the radiation. Thus, when establishing autoradiographs of ^{35}S-labeled material, it is essential that the film and the source of the radiation be directly in contact to one another. Gels should be as thin as possible and should be fixed and dried before autoradiographs are taken.

^{32}P, on the other hand, emits a particle with sufficient energy (1.71 MeV) to penetrate water or plastic to a depth of 6 mm and to pass completely through an X-ray film. Gels and filters therefore need not be completely dried (although the sharpness of the autoradiographic image is much improved if they are) and can be covered with Saran Wrap before they are exposed to the film. To increase the efficiency, with which these strong particles are detected, an intensifying screen may be placed behind the X-ray film. Radioactive particles that pass through the film hit the intensifying screen and cause it to emit photons that are captured by silver halide crystals in the emulsion. This leads to an approximately fivefold enhancement in the intensity of an autoradiographic image when the film is exposed at low temperature (-70 °C). The best intensifying screens are du Pont Cronex Lightening Plus and Fuji Mach 2, both of which are made from calcium tungstate and emit blue light, to which X-ray films are most sensitive.

Procedures:

1. Place the wrapped membranes in a hybridization cassette. In the dark room turn the lights off and place an X-ray film on top of the membrane and an intensifier screen on top of the X-ray film. Close the cassette, and press it between two Plexiglas with four clamps.
2. Place the hybridization cassette at –80 ^{0}C for one week.
3. After one week develop the film in the dark room with the lights off. Turn on the film processor located in the dark room 30 minutes before use. Check that it is working properly with a used film.

F. Stripping

1. After developing the film, remove the radioactive probe from the membrane. Unwrap the membrane and place it 10 seconds in boiling 0.1 X washing solution. Dry it on paper towels and wrap it with Saran Wrap. Store at –20 ^{0}C until next use. Membranes can be reused up to 10 times.

Caution: Wear the appropriate clothing for dealing with radioactivity