IN-Gel Hybridization procedure

Dionne & Wellinger,PNAS 1996, 93,13902

(Our favorite protocol for telomeric single stranded DNA detection)

A) Gel treatments

Gel preparation:

- After running gel (0.5-0.75% in 1xTBE buffer), stain with EtBr and photograph. If this is a telomere blot, you should have digested and loaded at least 3 µg of genomic DNA per lane.

- Immerse the gel in 2x SSC for 30 min at room temperature (RT°).

- Put gel upside down (i.e. the open wells down) on 2 layers of Whatman paper and cover with Saran wrap.

- Dry the gel on the gel dryer at RT° (we use a BIO-RAD 583 dryer) first 12-14 min, stop vacuum by switching valve on pump (but let pump running), flip the gel over and dry for another 12-14 min at RT°, or until the gel is very thin and even.

☞ DO NOT EXCEED 30 min of drying.

- Upon removal, it should stick to the saran wrap, if not just slightly wet the Whatman papers with 2x SSC and then remove the gel.

- If this is for a non-denaturing gel, put the gel now into a sealable plastic hybridization bag and go directly to hybridization!

Hybridization:

- Add ~5 ml of Hybridization solution without probe to verify the integrity of the bag (well sealed).
- Add ~20 ml of Hybridization solution with the hot probe (see below) and seal bag WITHOUT bubbles!
- Hybridize at 37°C, overnight.

Hybridization solution:

25 ml 20x SSC
1 ml 10x P-wash (10x P-wash: 5mM Ppi (inorganic pyrophosphate), 100mM Na_2HPO_4)
10 ml 50x Denhardts (or 5 ml 100x Denhardts)
40 µl 0.1 mM ATP
40 µl denatured salmon sperm DNA (50mg/ml)
Complete to 100 ml with sterile ddH_2O.

B) Rehybridization of gel after denaturation (detection of all DNA)

After appropriate exposures of the non-denaturing gel are obtained, the DNA in the gel can be denatured and rehybridized to detect all DNA species (loading control). To do so:

- Put gel into a glass tray and treat with 500 ml denaturing solution for 25 min at RT°
  Denaturing solution: 150 mM NaCl
  0.5 M NaOH
- Remove the denaturing solution and add 500 ml Neutralizing solution. Gently shake at RT° for 20 min.
  Neutralizing solution: 150 mM NaCl
  0.5 M Tris.HCl pH 8.0

- Remove the gel and put into a sealable plastic bag.
- Add ~5 ml of Hybridization solution without the probe to verify the integrity of the bag.
- Add ~20 ml of Hybridization solution with the hot probe and seal the bag WITHOUT bubbles!
- Hybridize at 37°C, overnight.

C) Probes and washings

Probe - 5'end labelling of oligo:

- Use any gel-purified oligo (we normally use 20-25 mers) at a concentration of 100 ng/µl.

- Radiolabel the 5' end of 200 ng oligo in a final volume of 20 µl. To do so, mix:
  2 µl oligo
  2 µl 10X T4 polynucleotide kinase buffer
  1 µl T4 polynucleotide kinase (9,8U) (USB)
  5 µl γ-ATP^{32}
  10 µl ddH_2O
  - Incubate the reaction at 37°C for 45 min at 37°C.
  - Inactivate the enzyme for 10 min at 68°C
  - Add 30 µl TE and purify on a G-50 Probe Quant column (GE Healthcare)

- 1 µl of radiolabelled primer should give ~ 150 000 cpm

- For a regular gel, you will need 1-2 X 10^6 cpm/gel, for 20 mL hybridization solution.

Washes:

2x 90 min at RT°C (on a horizontal shaking platform) → 0.25X SSC
1x 2h at 30°C → 0.25X SSC
(this step is optional and depends on the signal to noise ratio)

- Expose appropriately (usually requires 1-2 days). If you have excess ssDNA, a few hours to overnight should be OK.

D) Controls

It is very useful to run ssDNA and dsDNA controls in parallel lanes. Usually, you will have a plasmid with the target sequence cloned available. If this is the case, digest some of this plasmid with a restriction enzyme that linearizes the plasmid. For the dsDNA control, load about 1-5ng of this digested plasmid. For the ssDNA control, load the same amount of the linearized plasmid but heat denature the DNA prior to loading (10 min, 95°C).

E) Special Notes:

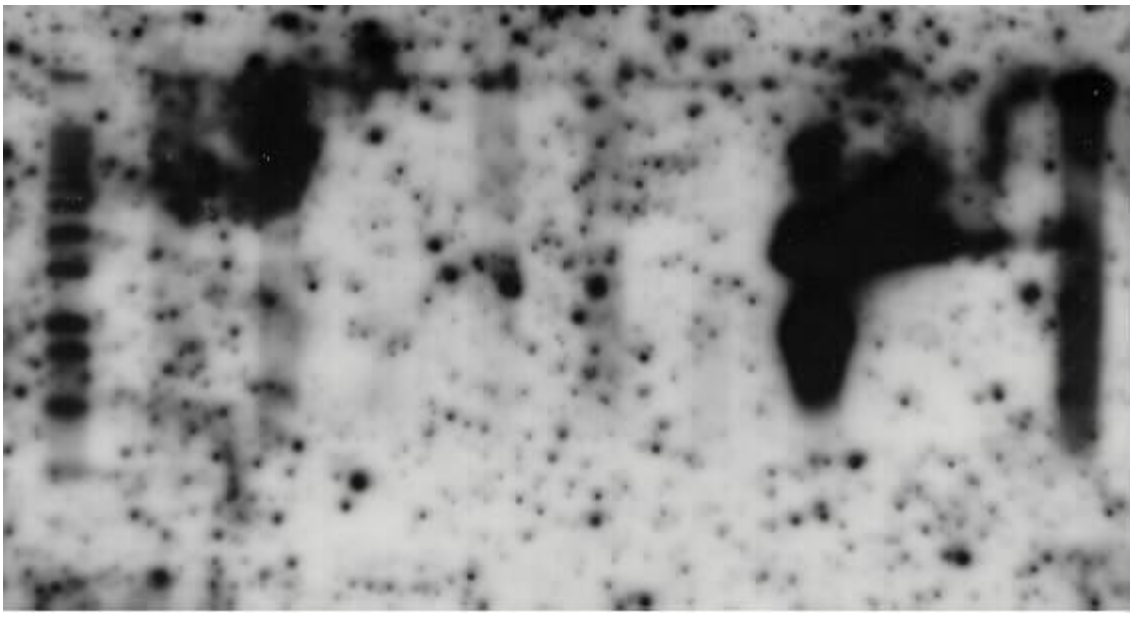
The critical step in this procedure is the gel-drying step. You might have to fiddle around with your gel-dryer setup to get the conditions right. After drying, the gel should be very thin (a bit thicker than 3MM paper; but not as thin as Saran wrap). Should the gel reswell during hybridization, then it was not dried enough. When gels are over dried, DNA fragments smaller than ~1.5 kb tend to be blotted out and end up on your 3MM support instead of staying in the gel. Detection of such DNA in the gel is obviously rather difficult...

With compliments from the Wellinger lab for your excellent choice of methods!!
Good luck!

Troubleshooting

Here are some typical problems that you might encounter when using this technique. As a free service to the community, we also suggest some ways to resolve them.

Case #1: Spotted gel (kindly provided by Julie)

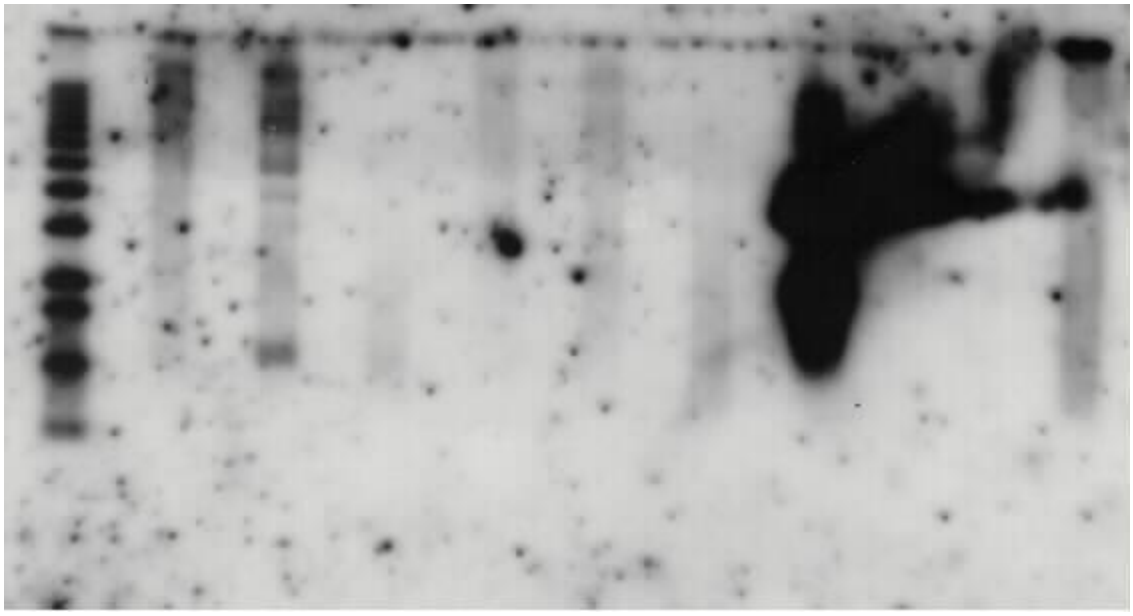


Our interpretation: The spots are caused by aggregation of probe in the gel matrix. Cleaning your probe should resolve the problem for the next gel. It is almost impossible to get rid of such spots and the gel usually has to be declared a total loss! Remember: this is a non-denaturing procedure, you cannot simply heat wash or alkali treat to get rid of the problem.

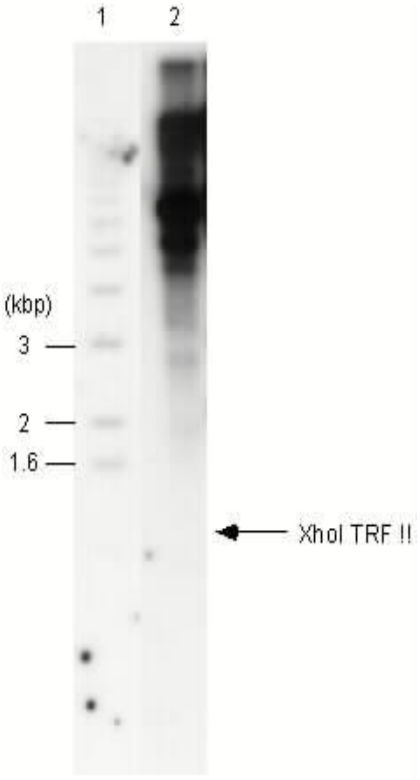
X-File interpretation: Your gel has been infected by an unknown dangerous virus. Run for your life!

Alternatively: It's Michel's fault.

Seriously now, it is almost impossible to get rid completely of such spots. However, rewashing the gel at a slightly higher temperature might greatly improve the result. As an example, the same gel as shown above was rewashed at 30 ^{o}C for 2h30 in 0.25X SSC.



Case #2: Over dried gel (kindly provided by Michel)



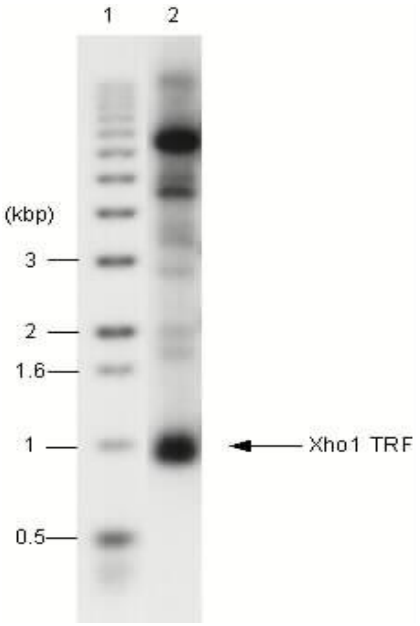
Our interpretation:

When gels are over dried, DNA fragments smaller than ~1.5 kb tend to be blotted out and end up on your 3MM support instead of staying in the gel. This problem is obvious in the ladder lane (lane 1). The 0.5 and 1 kb bands have been blotted out. Consequently, the ~1 kb XhoI TRF (terminal restriction fragment) cannot be detected. (For the connaisseurs of the matter: this DNA was derived from a ku- strain, thus the shorter TRFs).

X-File interpretation:

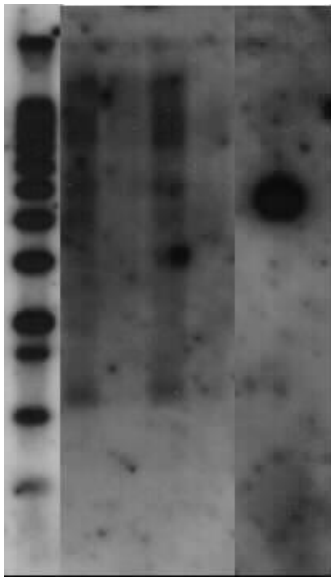
The TRF has been kidnapped by an alien-life form, specialized in making life of telomere researchers miserable.

Alternatively: It's Michel's fault.



Seriously now, you might have to fiddle around with your gel-dryer setup to get the conditions right. After drying, the gel should be very thin (a bit thicker than 3MM paper; but not as thin as Saran wrap). As an example, the same DNA has been analyzed on a gel dried appropriately. Note the 0.5 and 1 kb bands in the ladder lane.

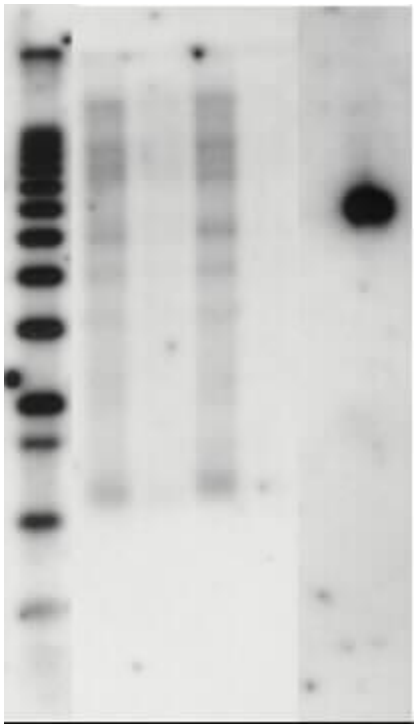
Case #3: Heavy background (kindly provided; sorry, I promised to keep the secret)



Our interpretation: The gel reswelled during hybridization, because it was not dried enough. Consequently there is excess probe all over the gel giving it a hazy (in severe cases, all black) background.

X-File interpretation: Those dammed aliens once again.

Alternatively: It's Michel's fault.



Seriously now, you can try to re-dry and reprobe the gel (but dont forget; do not overdry it!). Alternatively, one or two freeze-thaws of the gel will push the water out. Once you get rid of the excess water a quick wash (1h, 23 ^{o}C, 0.25X SSC) and a re-exposition should do the job. Shown next, the same gel after we did the freeze-thaw procedure to it.