Telomere Blots with Genomic DNA

This protocol is for one 15 cm dish of ~80% confluent monolayer cells (= about $1x10^7$ cells, this works for transformed cells). IMR90 and other primary fibroblasts don't pack in tightly and therefore two 15 cm plates should be used. If you're dealing with more cells, scale everything up accordingly.

Required Solutions:

TNE
10 mM Tris pH 7.4
100 mM NaCl
10 mM EDTA
(autoclave)

TNES/protK
10 mM Tris pH 7.4
100 mM NaCl
10 mM EDTA
1% SDS
100 µg/ml proteinase K
(autoclave TNES, store at RT. Add proteinase K right before use.)

TBE: see Maniatis
45 mM Tris base
45 mM boric acid
1 mM EDTA pH 8.0

Phenol-Chloroform-Isoamyl Alcohol (Fisher #BP1752-400)
Equilibrate with Tris buffer to pH 7.8
Add hydroxyquinoline (Sigma #H6878) to a final concentration of 0.1%.
Tighten the cap very well and mix vigorously.
Let the phenol settle for a few hours at 4°C. Keep separate for genomic preps.
For further information please check the Maniatis handbook.

Hoechst Dye (#33258)
Dilute to 1 µg/ml in filter-sterilized TNE. Can be stored at 4ºC.

Gel Loading Buffer (Orange G)
50% Glycerol
0.5% Orange G
(autoclave)

Blotting Buffer (20x SSC)
3 M NaCl
0.3 M Sodium Citrate

3.5 kg NaCl
1.76 kg Sodium Citrate
fill to 20 l with H_2O
Mix on stir plate o/n to dissolve

Depurination Solution
0.25 M HCL

Denaturing Solution
1.5M NaCl
0.5M NaOH

Mix:
1.75kg NaCl
400g NaOH
fill to 20 l with dH_20
mix on stir plate o/n to dissolve

Neutralizing Solution
3M NaCl
0.5M Tris-HCL pH 7.0

Mix:
3.5 kg NaCl
1.2 kg Tris
783 ml HC (careful with this much acid)
fill to 20 l with dH_20 and mix on stir plate o/n to dissolve.

Ethidium Bromide
10 mg/ml
make 10 ml stock, wrap in aluminum foil, store at RT
1 µl/100 ml gel volume

1 M Sodium Phosphate Buffer pH 7.2
(make in orange cap plastic roller; this is 2.3 l when bottle is filled to top bend)
308 g $Na_2HPO_4*7H_2O$
9.2 ml H_3PO_4 -- fill almost completely with ddH_2O leaving room to adjust pH
H_3PO_4 to pH 7.2 (should be close)
filled to 2.3 l with ddH_2O

Church Wash
40 mM sodium phosphate buffer pH 7.2
1 mM NaOH-EDTA pH 8.0
1% w/v SDS

800 ml 1 M NaPi pH 7.2
40 ml 0.5 M EDTA
200 g SDS (wear a face mask)
fill to 20 l with H_2O

Church Mix
500 ml 1 M sodium phosphate buffer pH 7.2
2 ml 0.5 M EDTA pH 8.0
70 g SDS
10 g BSA
Fill with ddH_20 to 1 l. Filter sterilize with a 0.45 µm filter unit.

Harvesting Cells

1. Harvest cells by trypsinization and collect in 15 ml tube. Add sufficient media with serum to kill the trypsin.
2. Spin 3 min at 1000 rpm.
3. Remove the supernatant and resuspend cells in 1 ml PBS, transfer to eppendorf tube.
4. Spin 2 min 4,000 g and remove the supernatant.
5. Continue with DNA prep or freeze cell pellet at -70°C for isolation at a later stage.

Isolation of Genomic DNA

Notes:

- *Make sure that all solutions are absolutely free of traces of plasmids or probes.*
- *Buffers/solutions used for genomic DNA preps should not be used for plasmid preps.*
- *Use a new box of pipette tips, etc. Clean your pipetman thoroughly before you start to remove plasmid droplets from the receptor end.*
- *NEVER use a vortex to suspend your DNA. To avoid shearing the DNA, treat it gently throughout the procedure. Resuspend the DNA with tips that have been cut off so that there is a large opening. Use a sterile razorblade to cut pipette tips.*

1. Thaw out cell pellet quickly at RT.
2. Resuspend cells in 1 ml TNE.
3. Transfer cell suspension to a 15 ml tube containing 1 ml TNES/protK (freshly prepared). Mix immediately.
4. Incubate 3 hours or o/n at 37°C.
5. Transfer a to 15 ml pre-spun phase lock gel heavy tube.
6. Add 2 ml phenol-chloroform-isoamyl alcohol and mix for a few minutes at RT (the phases should mix completely).
7. Spin 5 min at 1500 g at RT.
8. Pour off the water phase into a 15 ml tube containing 0.22 ml 2 M NaOAc (pH 5.5). Mix and add 2 ml isopropanol. Mix gently by inverting several times.
9. Fish out the bundle of DNA with a P200 tip (gently let the isopropanol drip off but don't let the DNA dry out—it will stick to the tip) and transfer to pre-spun 2 ml heavy phase lock tube containing 0.3 ml TNE+100 mg/ml RNase A (DNase free). Help DNA into solution right away by gently pipetting with a P1000 tip with end cut off.
10. Incubate for 30 min at 37°C.
11. GENTLY resuspend further if needed using a P1000 tip with cut off tip.
12. Incubate 2 hrs at 37°C.
13. Add 0.3 ml TNES/protK mix.
14. Incubate 1 hr at 37°C.
15. Add 0.6 ml phenol-chloroform-isoamyl alcohol and mix thoroughly by inverting. Spin 5 min at full speed.
16. Transfer the upper phase to new eppendorf tube with 66 µl 2M NaOAc (carry over as little liquid as possible—it contains SDS).
17. Add 0.7 ml isopropanol, mix completely by inverting.

18. Fish out DNA bundle immediately with a P200 tip, dip it in the isopropanol, let the isopropanol drip off, transfer to new tube with 100 µl TE.
19. Incubate 30 min at 37°C, resuspend with P200 tip with cut off end until completely dissolved, (this will take some time/effort).
20. . Store samples at -20°C. Yield: ~200 µg from two 15 cm IMR90 plates.

Restriction Endonuclease Cleavage

1. Digest approximate amount of genomic DNA samples o/n with frequently cutting enzymes. Make sure the DNA is dissolved.

DNA (from 200 µl prep of 2-4 million cells)		15 µl
H_20		65 µl
10 x NEB buffer 2		10 µl
AluI		5 µl (50U)
MboI		5 µl (50U)
RNAse A: 10mg/ml (DNAse free)		0.02 µl
	Total	100 µl

2. Mix carefully and thoroughly.
3. Digest at least 5 hours or o/n at 37°C in incubator (not water bath).
4. Perform a Hoechst fluorimetric assay to determine DNA concentrations after digest to determine what volume to load per lane.

Measuring DNA Concentrations with Hoechst Fluorimetry

NOTES:

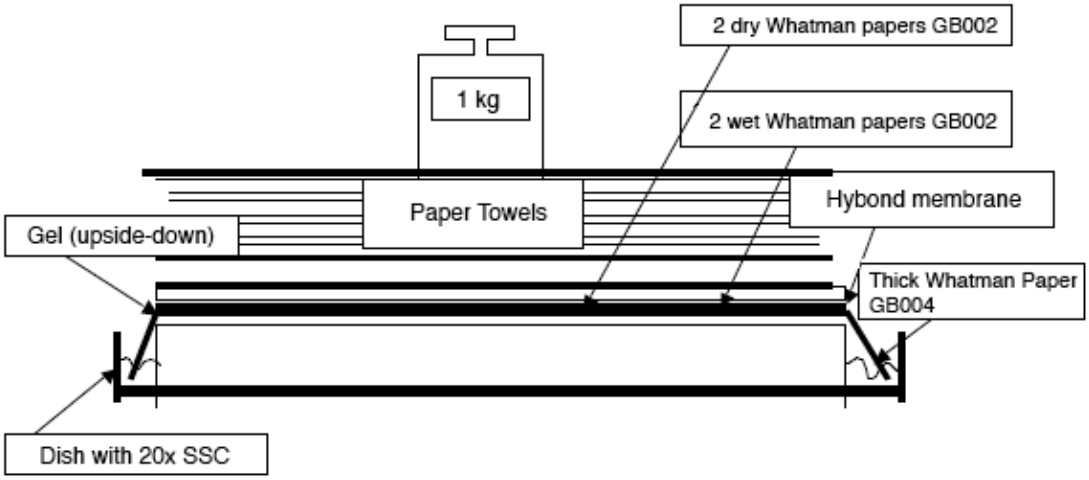
- *It is best to measure [DNA] after digestion (enzymes/NEB buffers don't interfere with Hoechst fluorimetry).*
- *Measure [DNA] in duplicate or triplicate.*
- *Do not forget to zero after new solution is added.*
- *Keep cuvette always in the same orientation.*
- *Do not spill liquid outside of the cuvette.*
- *Avoid dust.*

1. Let the fluorimeter warm up for at least 15 min (keep away from heavy machinery—measurements are sensitive to magnetic fields and very dust sensitive.
2. Dilute Hoechst 33258 (to 1 µg /ml) in filter-sterilized TNE (once prepared can be stored at 4°C, but all solutions should be at room temperature when using them because the cuvette will fog up).
3. Place 2 ml of the diluted dye in a cuvette.
4. Press <ZERO>.
5. Add 2 ml of ref DNA solution (for example 100 µg/ml). Mix with a 1000P pipette—do not make bubbles.
6. Press <CALIB>, enter the concentration of your standard (100) and press <ENTER>.
7. Remove sample with suction (P200 tip on Pasteur, don't scratch the cuvette).
8. Replace with fresh solution and zero again.

9. Add 2 ml of the sample DNA and mix. The concentration of the sample will be displayed.

Southern Blotting and Detection of Telomere Fragments

1. Based on Hoechst measurements, determine what volume to load to have the same amount of DNA per lane (for telomere blots, 1-2 µg/lane, for single copy genes, 12 µg/lane).
2. Adjust the volume of all samples to 30 µl with TE, and add 6 µl of Orange G loading dye.
3. Pre-treat gel boxes, 20 x 20 cm trays, and combs o/n with 0.25 M HCl.
4. Load DNA samples on a 20 x 20 cm 0.7% agarose gel in 0.5 x TBE with ethidium bromide (gel volume ~300 ml; 3 µl ethidium bromide stock). Load 4 µl MW markers in lanes 1 and 24.
5. Run for 1 hr at 30 V. Run the gel until the Orange G front reaches the bottom of the gel (e.g., o/n at 45V). Check under UV the loading of the lanes, take a picture. Then continue the run until the 1.3 kb marker almost runs off. This takes ~1000-1100 Vhrs total (after the first hour you may run at 120V max. to speed things up). When finished, take a picture with a ruler next to the markers.
6. Soak the gel in the following washes (make sure the gel is completely submerged, and released from the tray). Shaking continuously and gently, perform the following washes:
   - Depurination solution 30 minutes
   - Denaturation solution 2 x 30 minutes
   - Neutralization solution 2 x 30 minutes
7. Blot onto Hybond filter. Use CLEAN gloves to handle the Hybond and everything else.
8. The blotting buffer is 20x SSC. Pre-wet the Hybond by first floating it on top of ddH_2O before soaking it in 20x SSC. Mark the position of the slots on the filter with ballpoint pen. Mark the date on the side of the filter that contacts the gel. Blot for at least 2 hrs or o/n. Label probe while blotting (see below).



9. Take the filter from the gel, wrap it up in Saran Wrap, crosslink (DNA side up) in the Stratalinker (auto-crosslink).
10. Unwrap, rinse the filter in a clean tray with dH_2O to rinse off the 20x SSC.
11. Put the filter in a Seal-a-Meal bag or a Hybaid bottle and add ~50 ml Church mix and seal without air bubbles.
12. Incubate (pre-hybridize) for about 1 hr at 65°C in a water bath.

Labeling the Probe

1. Prepare the probe with the following (for two 20 x 20 cm filters):
   - 10 µl 800 bp [TTAGGG]n insert, (~20 ng/µl) (Isolate fragment from pSP73.Sty11x with EcoRI on gel, melt at 65°C).
   - 5 µl [CCCTAA]3 oligo (1 ng/µl)
   - 24 µl ddH_2O
2. Mix, boil 5 min, spin for 5 sec to get condensation down, cool on ice, add:
   - 10xOLB with dATP, dGTP, dTTP 5 µl
   - 32P-alpha-dCTP (3000 Ci/mmol) 5 µl
   - Klenow polymerase 1 µl
3. Mix, incubate 90 min at RT (or longer) add 50 µl TNES, heat to 65 °C for 10 min and immediately load on a 3 ml G-50 column:
   - Put some glass wool into a 3 cc syringe and stuff it tightly with the plunger.
   - Fill the syringe to the top with settled Sephadex G 50 fine (autoclaved in TNE).
4. Equilibrate with 1 ml of TNES (without Prot K).
5. Slowly add the probe.
6. Elute with TNE: void volume = 1000 µl, then collect 800 µl (has all DNA) don't elute further. This should be off-scale when monitored with a Geiger counter.

Note: This protocol will only work for highly abundant sequences such as telomeric DNA

7. Heat the entire 800 µl for 5 min at 100°C. Immediately add ~25 ml Church mix and filter through 0.2 µm syringe filter.

Hybridization

1. Open up the pre-hyb bag at one corner, remove the pre-hyb Church mix completely.
2. Using a pasteur pipette, add the hot probe mix to the filter(s). Remove all air bubbles carefully, seal the bag again and incubate in water bath at 65°C o/n.
3. Remove the filter(s) from the bag into a large tray. Wash with pre-warmed (65°C) Church wash 3 times for 15 min each, shaking.
4. Monitor with a Geiger-counter. The telomeric smear should be detectable in the middle between slot and bottom.
5. Wrap the filter up in Saran Wrap, expose o/n using intensifying screens at -80°C or in a phosphoimager cassette at RT.