Telomere Overhang Assay

(NAR 1999, Vol 27, #20)

This procedure can be done on plugs or on genomic DNA in solution.
We use *MboI* and *AluI* restriction enzymes.

Required Solutions

0.5x TBE Buffer

10x TBE (stock solution)
Dissolve the following in 800 ml ddH_2O:

108 g Tris base
55 g Boric acid
9.3 g EDTA

Adjust pH to 8.3 (the pH should be close to 8.3 even w/o adjusting if correct amounts of ingredients are used)
Adjust to 1 l w/ ddH_2O

0.5x TBE
Dilute the stock solution by 20x in ddH_2O.

TES Buffer

10 mM Tris-HCl pH 7.5
10 mM EDTA pH 8.0
0.1% SDS

TE buffer

10 mM Tris pH 8.0
1 mM EDTA pH 8.0

PMSF

100 mM stock in absolute EtOH stored at -20°C
Add to TE buffer right before use! (The half life of PMSF in aqueous solutions is ~35 min at RT and pH 8.0)

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 (anhydrous)
or 581.71 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)
Fill 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

Mix:
800 ml 1 M sodium phosphate buffer pH 7.2 (see below)
40 ml 0.5 M NaOH-EDTA pH 8
200 g SDS (wear a face mask)
Fill to 20 l with ddH_2O

Church Mix
500 ml 1 M sodium phosphate buffer pH 7.2 (see above)*
2 ml 0.5 M NaOH-EDTA pH 8.0
70 g SDS
10 g BSA
Fill with ddH_20 to 1 l. Heat up to dissolve SDS. Filter through 0.2 µm filter. Store at >20°C.

Denaturing Solution
1.5 M NaCl
0.5 M NaOH

Mix:
1.75 kg NaCl
400 g NaOH
Fill to 20 l with ddH_20.
Mix on stir plate o/n to dissolve.

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

Mix:
3.5 kg NaCl
1.2 kg Tris-base
780 ml HCl (be careful with this much acid; work in hood)
Fill to 20 l with ddH_20 and mix on stir plate o/n to dissolve.

Phenol-Chloroform-Isoamyl Alcohol (Fisher BP1752-400)
Equilibrate with 1/10 vol 100 mM Tris-HCl buffer to pH 7.8
Add hydroxyquinoline (Sigma #H6878) to a final concentration of 0.1% (phenol will turn yellow). Tighten the cap very well and mix vigorously. Let the phenol settle for a few hours at 4°C. Remove excess water layer. Keep separate for genomic preps. Don't pipet with plastic pipets (tips are OK). If phenol turns brownish, it's too old to use. For further information please check the Maniatis handbook.

Exonuclease I (NEB M0293S)
Order a new lot.

Exonuclease I buffer
67 mM Glycine-NaOH, pH 9.5 @25°C
6.7 mM $MgCl_2$
10 mM β-Mercaptoethanol (8 µl/10 ml; add upon usage)

***MboI* and *AluI* restriction enzymes**
MboI from NEB #R0147L
AluI from NEB #R0137L

Proteinase K in digestion buffer
Proteinase K (Roche 03115879001)
100 mM EDTA pH 8.0 (0.5 M stock)
0.2% sodium deoxycholate (10% stock solution)
1% sodium lauryl sarcosine (30% stock solution)
1 mg/ml proteinase K – add right before use!
(10 mg/ml stock solution stored at -20°C in small aliquots; avoid repeated freezing and thawing and use right after thawing out – Proteinase K will eat itself up when in solution)
The buffer can be stored at RT (without the proteinase K)

SeaKem Agarose (Lonza 50004)

Ethidium bromide (BioRad 161-0433)
10 mg/ml

Low Range PFG Marker (NEB N0350S)

Add PMSF stock

Add Orange G loading dye stock

Harvesting the Cells

1. Harvest exponentially growing cells by trypsinization; collect in a 15 ml tube, count cells. Add sufficient media with serum to kill the trypsin (10 ml media / 1 ml trypsin). Keep cells on ice at all times.
   Note: In case of SV40-immortalized MEFs, ~$2x10^6$ cells per 10 cm dish is an optimal density *at the time of harvest*. Plate cells (usually $2.5\text{-}5x10^5$, depending on growth rate) 2-3 days before harvesting to ensure asynchronous growth (if plating 3 days before harvest, exchange media 24 h prior to harvest)
2. Spin 5 min 1000 RPM at 4°C.
3. Remove supernatant and keep cell pellet on ice.
4. Resuspend cells in 0.5-1 ml cold PBS and transfer to an eppendorf tube.
5. Spin 5 min 5,000 RPM in a microcentrifuge at 4°C.
6. Remove supernatant, keep pellet on ice.
7. Continue with DNA prep, plug prep, or flash-freeze cell pellet in liquid N_2 for isolation at a later stage (store pellets at -80°C).

Genomic DNA Isolation

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. Avoid shearing of the DNA, so 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 into a 15 ml tube containing 1 ml TENS/protK (freshly prepared). Mix immediately.
4. Incubate 3 hours or o/n @ 37°C.
5. Transfer to 15 ml pre-spun phase lock gel heavy tube.
6. Add 2 ml phenol-chloroform-isoamyl alcohol, mix for a few minutes at room temperature (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 iso-propanol and 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 with 100 µg/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 @ 37°C.
11. GENTLY resuspend further if needed using a P1000 tip with cut off tip (razorblade).

12. Incubate 2 hrs @ 37°C.
13. Add 0.3 ml TENS/protK mix.
14. Incubate 1 hr @ 37°C.
15. Add 0.6 ml phenol-chloroform-isoamyl alcohol and mix thoroughly by inverting, spin 5 min at full speed.
16. Transfer upper phase to new eppendorf tube with 66 µl 2M NaOAc pH 5.2 (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 buffer.
19. Incubate 30 min @ 37°C, resuspend with P200 tip with cut off end (razorblade) until completely dissolved (will take some time/effort).
20. Store samples at -20°C. Yield: ~200 µg from two 15 cm IMR90 plates.

Exonuclease I Digestion of DNA in Solution (optional)

1. Incubate DNA (4 µg) with 100 U Exo I in Exo I buffer at 37°C o/n in a volume of 200 µl.
2. Transfer to a 2 ml phase lock tube and add 200 µl TE. Add 1 vol (400 µl) phenol-chloroform-isoamyl alcohol.
3. Mix carefully, spin 5 min at full speed.
4. Remove the supernatant. Add 40 µl 3 M NaOAc pH 4.8, and 400 µl isopropanol. Mix thoroughly.
5. Spin full speed for 10 min at 4°C.
6. Remove all isopropanol, wash with 70% EtOH.
7. Resuspend the pellet in 25 µl TE buffer.

Digest genomic DNA with restriction enzymes as needed.

After digestion, carefully measure DNA concentration (triplicate) to ensure equal loading. Best is to use Hoechst.

Agarose Gel Electrophoresis (for separation in the range of 0.2-25 kb)

The gel can be run in duplicate, so one can be hybridized with the G probe, the other with the C probe. Use Exonuclease I to make sure the signal is derived from a 3' ss overhang (see Exonuclease I Digestion of DNA in Solution).

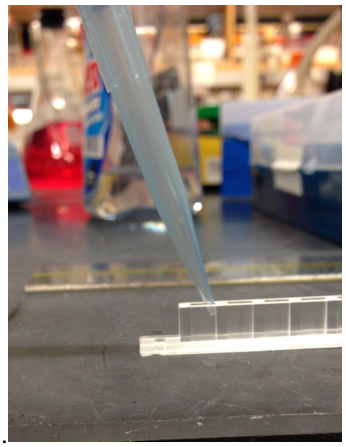
1. Based on Hoechst DNA measurements or cell counts, determine what volume to load to give same amount of DNA per lane (for telomere blots, 1-2 µg/lane, for single copy genes, 12 µg/lane).
2. Adjust the volume of all samples to 30 µl with TE, and add 6 µl of Orange G loading dye.
3. Pre-treat gel boxes, 20x20 cm trays, and combs o/n with 0.25 M HCl to remove plasmid contamination.
4. Load DNA samples on a 20x20 cm 0.7% agarose gel in 0.5x 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 @ 30 V. Run the gel until the Orange G front reaches the bottom of the gel (e.g., o/n @ 45 V). Check the lanes under UV and take a picture. Then continue the run until the 1.3 kb marker is almost off the gel. This takes ~1000-1100 Vhrs total (depending on how much DNA was loaded and the digest, you may run @ 120 V max. to speed things up after the first slow hour). When finished, take a picture with a ruler next to the markers.
6. Cut the upper-right corner off the gel for orientation.
7. Dry gel (on gel dryer) at 45°C on Whatman 3MM paper, until it is completely dry (~2 hrs).
8. Remove Whatman by soaking the gel in water (paper comes off fast).
9. Prehybridize in Church mix and hybridize with hot probe as described below ("Labeling the probe" and "In-gel hybridization")

Preparing Plugs

Notes:

- Many of our conditional shelterin component knockout lines (TPP1$^{F/F}$, TRF1$^{F/F}$, TRF2$^{F/F}$, POT1a/b, shelterin-free etc.) undergo endoreduplication after treatment with Cre and the cell number therefore does not always represent the amount of DNA in the cell pellet. Before making plugs, assess the size of the cell pellets visually and if necessary, adjust the amount of cells so that the pellet sizes are comparable between different samples to ensure equal loading of the gel.
- When making plugs, process one sample at a time, because prolonged incubation of the cell suspension at 50°C will result in cell death. Keep remaining samples on ice.
- For pipetting agarose and the agarose/cell suspension, use a 1 ml pipette w/ a blue tip. Before pipetting, always hold the pipette tip against the inner wall of your agarose bottle (in the 50°C water bath) for a few seconds to warm the tip up. To avoid bubbles in the plug, position the pipette tip to one side of the plug mold when pipetting as depicted below:



1. Boil 2% agarose in PBS, cool to 50°C in water bath.
2. Resuspend the harvested cells in PBS (for one plug mix 1x10^6 cells with 65 µl of PBS) and pre-warm quickly to 50°C (don't let it sit at 50°C).

   Note: If needed, the amount of cells per plug can be scaled down to 3-5x10^5, although longer exposure of the gel to the phospho-screen will be required.

3. Add 65 µl of the melted 2% agarose at 50°C and mix carefully by pipetting up and down. Keep the eppendorf submerged in the 50°C water bath while you mix and pipette all 100 µl into the disposable casting mold.
4. Leave the plug to set 5 minutes at RT and then 15 min at 4°C.
5. Break off the little comb that comes attached to the disposable molds and use it to push the plug into a sterile eppendorf.
6. Add 500 µl of Proteinase K digest buffer (w/ 1 mg/ml Proteinase K) to the plug.
7. Digest at 50°C (in a heating block at 350 RPM) overnight (more than 12 hours). Note: if the plug is opaque after the digestion, the Prot K did not work.
8. Wash the plugs 3 times in TE buffer (1 ml per plug) for 1 hour each at RT on a nutator. Do a 4th wash in TE with 1 mM PMSF (freshly added) to inactivate residual

Prot K activity. (At this point the plugs can be stored at 4°C for up to 3 months.)

9. Perform optional Exonuclease I digestion of DNA in plugs. After Exo I digestion, return to step Wash once again with TE and once more with sterile water for 20 minutes before the digest.
10. Incubate the plug in 0.5 ml of restriction enzyme buffer (**NEB buffer 4** or **CutSmart**) for 30 mins and then digest in new buffer with 60 units of *MboI* (or *AluI* or both) in 0.5 ml overnight at 37°C (in a heating block at 350 RPM).
11. Rinse the plugs with 1 ml TE and equilibrate in 1 ml 0.5x TBE for 1 hour at RT on a nutator.

Exonuclease I Digestion of DNA in Plugs (optional BEFORE MboI/AluI)

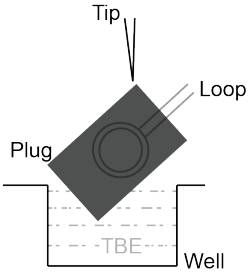
1. Wash plugs with TE buffer (1 ml/plug) for 1 hour on nutator.
2. Wash with ddH_2O for 1 hour on a nutator.
3. Wash 2x with Exo I buffer for 1 hour each (add β-ME to the buffer right before use).
4. Incubate with 1 ml 1x Exo I buffer and 10 µl (100U) Exo I per plug, o/n at 37°C.
5. Next morning, discard old buffer with enzyme and add 1 ml of fresh 1x Exo I buffer with 10 µl Exo I (100U). Incubate for 4 hours at 37°C.
6. Wash 2 times in 1 ml TE buffer per plug for 1 hour each time.

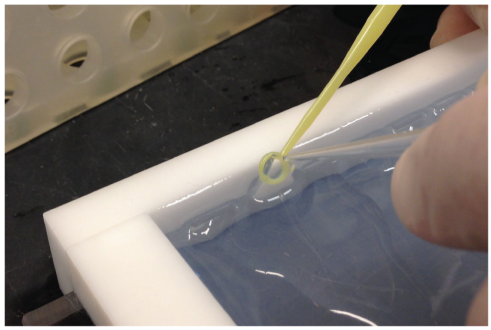
Note: For cells with extremely long overhangs (Shelterin-free 53BP1$^{-/-}$, TPP1$^{-/-}$ 53BP1$^{-/-}$ etc.), the ExoI incubations might have to be prolonged or repeated to ensure complete digestion of the overhang.

PFGE

1. Set gel to cast 1% PFGE grade agarose (Lonza 50004) in 0.5x TBE. Leave some agarose at 50°C for sealing the plugs in step 4.
2. At the same time pre-cool the 3 liters of 0.5x TBE in the PFGE tank with the cooling unit on set at 14°C and the circulation pump on (otherwise the cooling unit freezes and that's bad news).
3. When the gel is set (30 min - 1 hr), fill the wells with 0.5x TBE and load the plugs into the wells alongside a PFG marker (cut a little piece from the stock syringe using a razorblade).

For loading the plugs, use a disposable inoculation loop and a pipette tip as depicted below:





4. Remove excess TBE from the surface of the gel and seal the wells with the remaining agarose and leave to set (a minute or less is sufficient).
5. Run the gel at the following settings:
    - Initial pulse 5s
    - Final pulse 5s
    - Voltage 6V per cm
    - Temp 14°C
    - Time 24 hours
6. Remove the agarose that seals the wells and cut the upper-right corner off the gel for orientation.
7. Stain with EtBr for 30 min gently shaking at RT. Use 20 µl EtBr/300 ml ddH_2O.
8. Wash in ddH_2O for 30 min in shaker at RT.
9. Photograph gel with a ruler alongside the MW markers.
10. Dry gel (on gel-dryer) at 30°C on Whatman 3MM paper, until it is completely dry (~2 hrs).
11. Remove Whatman by soaking the gel in water (paper comes off fast).
12. Prehybridize in Church mix and hybridize with hot probe as described below ("Labeling the probe" and "In-gel hybridization").

Cleaning the PFGE apparatus:

- Drain buffer from tank (buffer can be re-used once).
- Fill tank with H_2O and switch on the pump for at least 10 min. Discard the water and repeat.
- After the procedure is complete, drain water completely (switch on the pump briefly to force the liquid of out the tubing) and wipe the apparatus gently with wet paper towels. Let dry with the lid open and close afterwards to avoid dust accumulation.

Labeling the probe

Prepare oligonucleotide probes for G-overhang assay and, optionally, the C-strand control. The probe needs to be very hot (30,000 cpm per µl). Aliquot the oligonucleotides and do not freeze/thaw it too many times.

TelC oligo = $[CCCTAA]_4$

1. Mix:
   - oligo (50 ng/ µl) 1 µl
   - 10x T4 poly-nucleotide kinase buffer 1 µl
   - ddH_2O 2 µl
   - T4 poly-nucleotide kinase (PNK) 1 µl
   - ^{32}P-γ-ATP 5 µl (6000 Ci/mmol)
2. Incubate 45 min @ 37°C in a waterbath.
3. Add 80 µl TNES buffer.
4. Load on 3 ml G-25 column.
5. Column: put some glass wool into a 3 cc syringe and stuff it down tightly with the plunger. Fill with Sephadex G-25 fine (autoclaved in TE) to the 3 ml mark (bed volume). Equilibrate with 1 ml TNES.
6. Carefully add the probe to the column.
7. Wash column with 700 µl TNES.
8. Elute with 600 µl TNES.
9. Boil probe for 5 min
10. Add to 25 ml Church mix and filter the mixture through a 0.45 µm filter (be very careful during this step, the mixture is very viscous and difficult to pass through the filter. If too much pressure is applied, the filter can be ejected from the syringe resulting in radioactive spillage all over your surroundings).
11. Add to gel as detailed below.
12. Optionally, hybridize with $[TTAGGG]_4$ as a control.

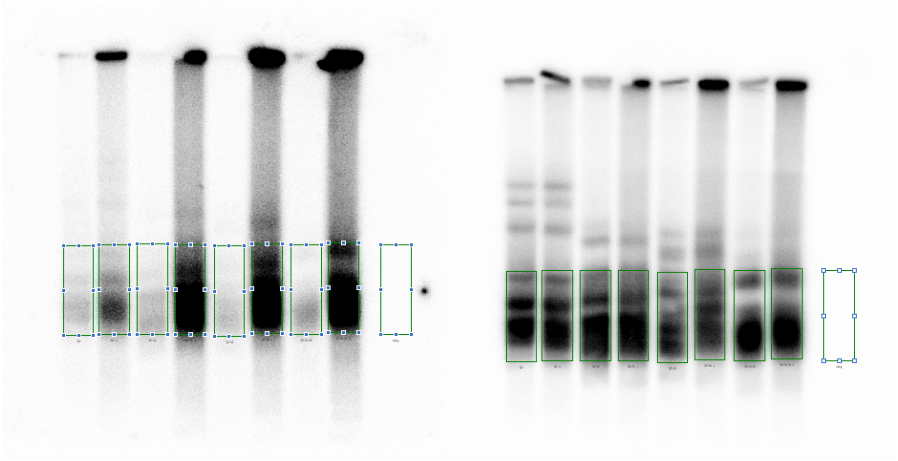
Note: Alternatively, you can use commercial Illustra MicroSpin G-25 columns (GE Healthcare, 27-5325-01) for probe purification. Refer to the manufacturer's manual for instructions. You need to go over column twice. Filter your hybridization mix after adding the probe!

In-gel hybridization

1. Put the gel on a hybridization mesh into a hybridization tube and add 25 ml of Church mix. Pre-hybridize for at least 30 mins at 50°C in a hybridization oven.
2. Discard the pre-hyb Church mix and hybridize the gel at 50°C O/N in ~25 ml Church mix with hot probe.
3. Pour out the probe and save it at RT for hyb after DNA denaturation
4. Wash gel in the tube 3x 30 min in 4x SSC at 55°C in a hybridization oven.
5. Wash 1x 30 min in 4x SSC 0.1%SDS at 55°C.

6. Seal the gel in a plastic bag, cover with Saran wrap and expose to a phosphoimager screen (O/N is usually sufficient). Make sure the screens do not get wet.
7. Denature gel in denaturing solution for 30 minutes at RT in a tray, shaking.
8. Neutralize 2x15 min in neutralizing solution.
9. Rinse in H2O for 3 min.
10. Prehybridize the gel in a tube at 55°C for 1 hour.
11. Hybridize in ~25 ml Church mix with same probe at 55°C o/n.
12. Wash as in steps 3 and 4 above.
13. Expose as to phosphoimager as in step 5 above.
14. Subtract background and normalize native ssDNA signal to denatured dsDNA signal to find relative overhang signal.

Note: Quantify the area of the gel where the bulk telomeres migrate (for MEFs, this usually corresponds to 28-50 kbp) as shown below:



Native gel Denatured gel