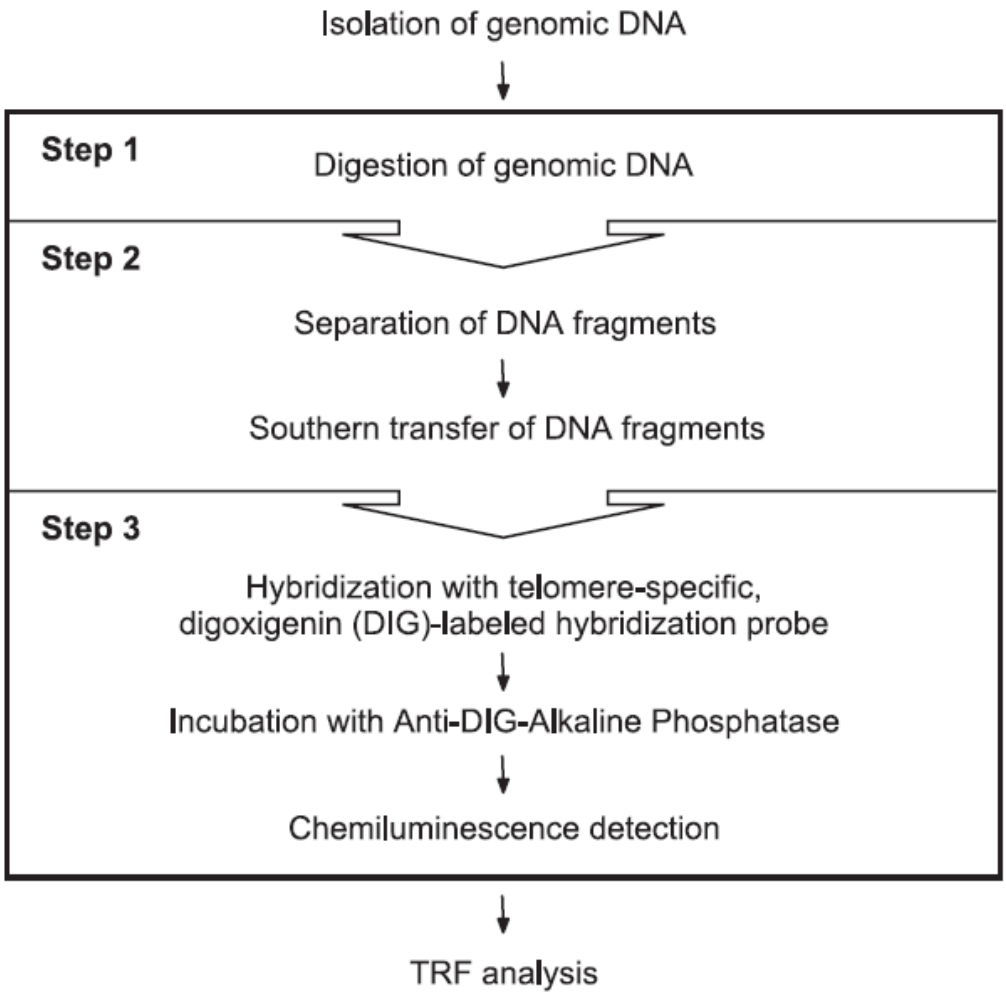
*Telo**TAGGG*** Telomere Length Assay

Test Principle The test principle is shown schematically in the following figure**:**



Note: The volumes used in the following are based on one experiment using a membrane size of 200 cm^2

Storage

Bottle	Content	Temperature
1	Hinf I	-15 to -25°C
2	Rsa I	-15 to -25°C
3	Digestion buffer, 10×	-15 to -25°C
4	Water, nuclease-free	+2 to +8°C
5	Control-DNA	-15 to -25°C

6	DIG Molecular weight marker	-15 to -25°C
7	Loading buffer, 5×	+2 to +8°C
8	DIG Easy Hyb Granules	+15 to +25°C
9	Telomere probe	-15 to-25°C
10	Washing buffer, 10×	+15 to +25°C
11	Maleic acid buffer, 10×	+15 to +25°C
12	Blocking buffer, 10×	+2 to +8°C
13	Anti-DIG-AP	+2 to +8°C
14	Detection buffer, 10×	+15 to +25°C
15	Substrate solution	+2 to +8°C

Step1:Prepare the working solution

1. TAE buffer(1×):

   0.04M Tris-acetate, 0.001M EDTA, PH0.8

   Storage: +15 to +25°C

2. HCI solution:

   0.25M HCI, 250ml

   Storage: +15 to +25°C

3. Denaturation solution:

   0.5M NaOH, 1.5 M NaCl,500ml(=10g NaOH+43.875NaCl+ 500mlH_2O)

   Storage: +15 to +25°C

4. Neutralization solution:

   0.5M Tris-HCl, 3M NaCl, pH7.5, 500ml

   Storage: +15 to +25°C

5. 20×SSC:

   3 M NaCl, 0.3 M Sodium citrate, pH 7.0

   Storage: +15 to +25°C

6. 2×SSC,

1(20×SSC) : 9(autoclaved, redistilled water) ， 1L

Storage: +15 to +25°C

7. DIG Easy Hyb:
   - Reconstitute Bottle 8 with 64ml autoclaved, redistilled water
   - Incubate at 37C until complete reconstitution

   Storage: +15 to +25°C for 3 months

8. Stringent wash bufferⅠ:

   2×SSC,0.1% SDS

   Storage: +15 to +25°C

9. Stringent wash buffer Ⅱ:

   0.2×SSC(=5ml(20×SSC): 995ml(autoclaved, redistilled water), 0.1% SDS, 1L

   Storage: +15 to +25°C

10. Washing buffer 1×: (~300ml per 200cm^2 membrane)

    1(10×Bottle 10) : 9 (autoclaved, redistilled water)=30ml: 270ml

    Storage: +15 to +25°C

11. Blocking solution 1× 100ml

    10ml(10×Bottle 12) : 90ml (maleic acid buffer 1×)

    Note: **Prepare immediately before use**

12. Maleic acid buffer 1×(~200ml per 200cm^2 membrane)

    1(10×Bottle 11) : 9(autoclaved, redistilled water)=20ml:180ml

    Storage: +15 to +25°C

13. Anti-DIG-AP, working solution 50-100 ml

    Dilute an appropriate volume of Anti-DIG-AP (Bottle 13) with blocking solution, 1× (Solution 11) to a final concentration of 75 mU/ml (1:10.000)

    Note: **Prepare immediately before use**

    Spin the vial(Bottle 13) for 5 min at 13000rpm before use

14. Detection buffer 1×(100ml per 200cm^2 membrane)

    1(Bottle 14) : 9(autoclaved, redistilled water)=10ml:90ml

    Storage: +15 to +25°C

Step2: Prepare the genomic DNA

Step3: Genomic DNA digestion(~2h)

Note: Handling of the solutions and pipetting should be done on ice.

1. Prepare a Hinf I/Rsa I enzyme and digestion buffer mixture
   - Prepare a clean sterilized centrifuge tube
   - For 10 DNA samples and 2control DNA, mix 7ul HinfI (Bottle 1), 7ulRsa I (Bottle 2), 28ul digestion buffer, 10×(Bottle 3). (20U/ul for each enzyme)(For each reaction, dispense 3ul mixture. 1 ul of the enzyme mixture and 2ul of digestion buffer are needed for each sample to be tested and for the Control-DNA, respectively.)

   Note: Prepare enough enzyme mixture to digest all the samples to be analyzed in this experiment.
2. Prepare the DNA
   - Prepare a clean sterilized plate
   - Dilute 15ul Control-DNA(Bottle5) with 2ul nuclease-free water(Bottle 4) to produce a final volume of 17ul per reaction vial
   - Dilute 1ug of purified genomic DNA with nuclease-free water(Bottle 4) to a final volume of 17ul per reaction vial.
3. Incubate the reaction mixture for 2h at 37°C.
4. To stop the reaction add 5ul of gel electrophoresis loading buffer, 5×(Bottle 7) and quick-spin the reaction vials.

Step 4: Gel Electrphoresis(2~4h)

1. Prepare a 0.8 % agarose gel (about 15 cm in length)

   Agarose MP 1.6g + 1× TAE buffer 200ml
2. Prepare the molecular weight marker mix
   - Prepare a clean sterilized centrifuge tube
   - Mix 4ul DIG molecular weight marker (Bottle 6), 12ul nuclease-free water (Bottle 4) and 4ul 5× loading buffer (Bottle 7) and quick-spin the vial.
3. Load 1.5ug of each digested DNA sample onto a lane of the gel.

Note: Each sample should contain the same amount of DNA

4. Load one aliquot (10ul) of diluted DIG molecular weight marker to each side of the gel.
5. Run gel at 5 V/cm(75V) in 1× TAE buffer (Solution 1) until the bromophenol blue tracking dye is separated about 10 cm from the starting wells (total run time 2–4 h).
6. To assess the quality of the target DNA, stain the gel briefly in 0.25-0.50ug/ml ethidium bromide. Examine the gel under UV light.

Step 4: Blotting(1h10min+6~12h+20min)

Equipment Needed: Blotting equipment, UV-Crosslinker, shaking water bath, orbital shaker

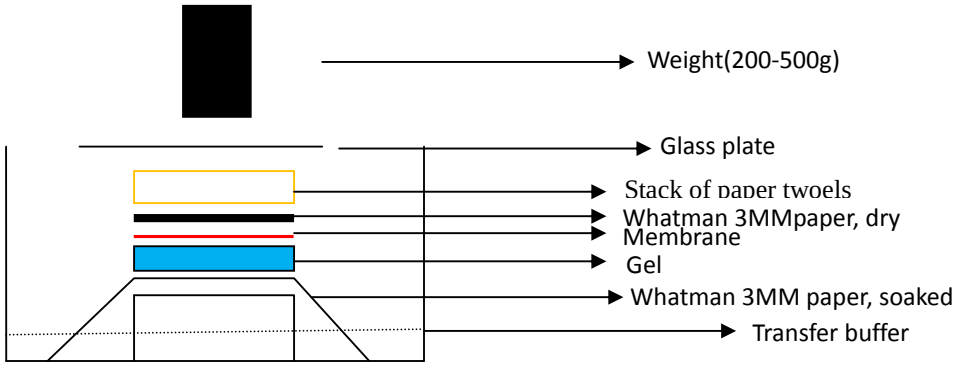
Note: Maximum transfer efficiency and sensitivity are obtained with positively charged nylon membranes using capillary transfer and 20 × SSC transfer buffers.

Note: Steps from 1to 5 should all be done with gentle agitation

1. Submerge the gel in HCl solution (Solution 2) and agitate for 5–10 min at +15 to +25°C, until the bromophenol blue stain changes color to yellow.
2. Rinse the gel 2 times with H2O
3. Submerge the gel in the denaturation solution (Solution 3) for 2 × 15 min at +15 to +25°C.
4. Rinse the gel 2 times with H2O
5. Submerge the gel in the neutralization solution (Solution 4) for 2 × 15 min at +15 to +25°C.

   Note: The following step should be carried out with unpowdered rubber gloves and the membrane should be handled with forceps only at the edges.
6. Set up the blot transfer as follws, avoiding the formation of air bubbles:.
   - Place a piece of Whatman 3MM paper that has been soaked with 20×SSC atop a bridge that rests in a shallow reservoir of 20×SSC
   - Put the gel atop the soaked sheet of Whatman 3MM paper. Roll a sterile pipette over the sandwich to remove all air bubbles that formed between the gel and paper.
   - Cut a piece of positively charged membrane 1mm bigger than the gel. Cut a corner for orientation.
   - Place the dry membrane on the DNA-containing surface of the gel. Use a pipette to eliminate air bubbles as above.
   - Complete the blot assembly by adding a dry sheet of whatman 3MM paper, a stack of paper

towels, a glass plate, and a 200-500g weight. The finished blot transfer sandwich should look like this:.



7. Allow the blot to transfer overnight in Transfer Buffer(20×SSC)
8. While the blot is still damp, fix the transferred DNA on the wet blotting membrane by either of the following methods: (Prewarm approx. 25 ml of DIG Easy Hyb (Solution 7) to +42°C if used immediately for hybridization and chemiluminescence detection step.)
   - UV crosslinking

     -Place the membrane(DNA side facing up)on Whatman 3MM paper that has been soaked in 2× SSC

     -Expose the wet membrane to UV light, according to standard protocols, e.g. 1-3min in a transilluminator

     -Rinse the membrane briefly in sterile, double distilled water.

     -Allow membrane to air dry
   - Baking

     -Wash the membrane briefly in 2× SSC

     -Bake the membrane either: at 120C for a30min, or at 80c for 2h
9. If not used immediately for the hybridization and chemiluminescence detection step, air-dry the blotting membrane and store at +2 to +8°C.

Step5: Hybridization and Chemiluminescence Detection（~6.5h）

Note: The volumes recommended in the following are based on a membrane size of 200 cm^2. The hybridization and stringent wash temperatures should be maintained exactly.

Equipment Needed: hybridization oven, imaging device or X-ray film, orbital shaker, hybridization

tube(?)

1. Prewarm approx. 25 ml of DIG Easy Hyb (Solution 7) to +42°C.
2. For prehybridization, submerge the blot in 18 ml of prewarmed DIG Easy Hyb (Solution 7) and incubate for 30–60 min at +42°C with gentle agitation.(**DNA side in**)
3. Prepare the hybridization solution:

   Add 1.4ul Telomere probe to fresh prewarmed DIG Easy Hyb (Solution 7) and mix.(1ul Telomere probe (bottle 9)per 5 ml fresh prewarmed DIG Easy Hyb. At least 6.5 ml of hybridization solution will be needed per 200 cm^2 blotting membrane.)
4. Discard prehybridization solution completely and immediately add hybridization solution to the membrane.
5. Incubate for 3 h at +42°C with gentle agitation.(Thaw the bottle 12 a half hour before incubation is done)
6. Discard hybridization solution.
7. Wash the membrane twice with stringent wash buffer I (Solution 8) (each wash for 5 min at +15 to +25°C, with gentle agitation).
8. Wash the membrane twice with prewarmed stringent wash buffer II (Solution 9) in a heated water bath (each wash for 15–20 min at +50°C, with gentle agitation).
9. Prepare 100ml 1×blocking solution(Solution 11) as the protocol mentioned above

Note: Clean labware is recommended during the following detection procedure

10. Rinse membrane in at least 100 ml 1 × washing buffer (Solution 10) for 1 – 5 min at +15 to +25°C with gentle agitation.
11. Incubate the membrane in 100 ml freshly prepared 1× blocking solution (Solution 11) for 30 min at +15 to +25°C with gentle agitation. (**Use hybridization tube**)
12. Prepare 50-100 ml Anti-DIG-AP working solution (Solution 13) as the protocol mentioned above. (**Spin the vial for 5 min at 13000rpm before use**)
13. Incubate the membrane in 50-100 ml Anti-DIG-AP working solution (Solution 13), for 30 min at +15 to +25°C with gentle agitation. (**Use hybridization tube**)
14. Wash the membrane twice with 1x washing buffer (Solution 10, 100 ml each time) (2 x 15 min at +15 to +25°C with gentle agitation).

15. Incubate the membrane in 100 ml of 1 × detection buffer (Solution 14) for 2–5 min at +15 to +25°C with gentle agitation.
16. Prepare a glass and parafilm membrane according to the size of membrane.
17. Discard detection buffer (Solution 14) and remove excess liquid from the membrane by placing the membrane, DNA side up, on a sheet of absorbent paper. (**Don´t let the membrane dry. An instant drop on the absorbent paper is recommended**
18. Immediately place the wet membrane, DNA side facing up, on glass and very quickly apply approx. 40 drops (about 3 ml) substrate solution (Bottle 15) to the membrane.
19. Immediately cover the membrane with a sheet of parafilm membrane, being careful to spread the substrate solution homogeneously over the membrane without trapping air bubbles.
20. Incubate the membrane for 5 min at +15 to +25°C.
21. Squeeze out excess substrate solution (bottle 15).
22. Expose to the imaging device or to X-ray film (the membrane should be covered by a piece of plastic membrane)for 20 min at +15 to +25°C.

Step 6: Data Analysis

Equipment: Densitometer

Method: Mean TRF length has been defined according to the following formula:

$$\overline{TRF} = \frac{\Sigma (OD_i)}{\Sigma (OD_i / L_i)}$$

where OD i is the chemiluminescent signal and Li is the length of the TRF at positioni (3). The calculation takes into account the higher signal intensity from larger TRFs due to multiple hybridizations of the telomere-specific hybridization probe.

Calculation

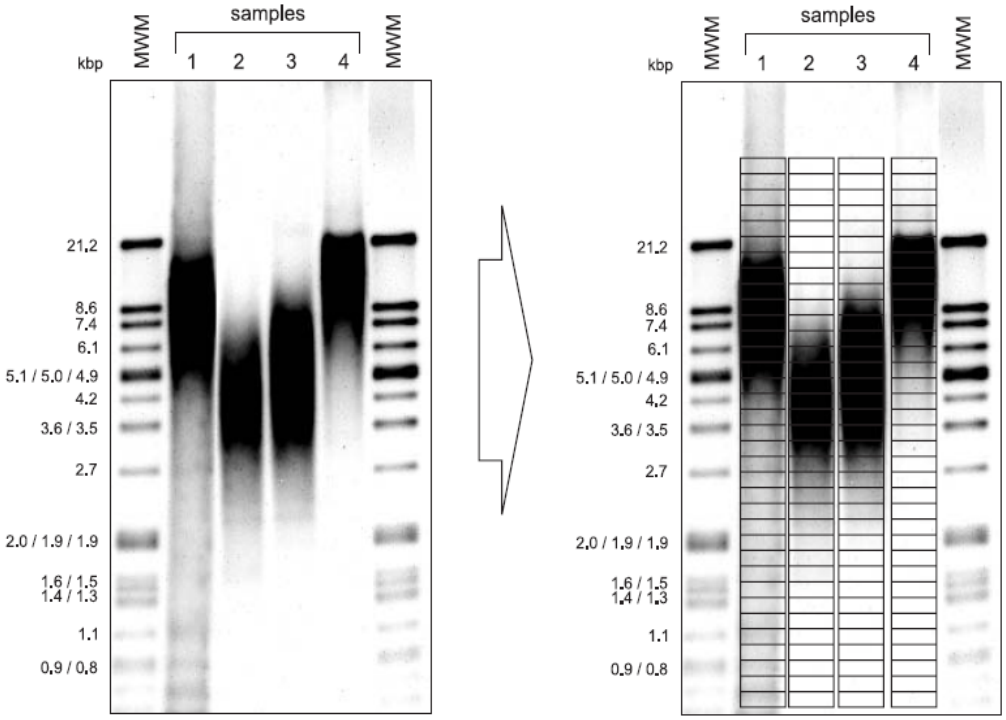
Note: The mean TRF length can be calculated by scanning the exposed X-ray film with a densitometer. To obtain reliable results, the signal strength must be within the linear range of the X-ray film.

1. Scan the exposed X-ray film with a densitometer.

2. Overlay each sample lane of the scanned image with a grid. The height of the individual squares of

the grid determines the resolution of the TRF length calculation. Typically, > 30 squares per lane are recommended.

3. For background subtraction, select several grid squares in each lane where no telomere-specific signal is found and which are representative of the background in that lane. Signals from these squares should be averaged and subtracted from each square that contains DNA.
4. For each square that contains DNA, determine the signal (ODi) and the corresponding length Li where ODi is the total signal intensity within that square and Li is the molecular weight at the mid-point of that square.
5. Calculate the mean TRF length using formula.



Chemiluminescent detection of TRF´s.

Immortalized cell lines (samples 1-4) were analyzed for telomere length using the TeloTAGGG Telomere Length Assay. The data was analyzed as described in Section 4.1 resulting in the following mean TRF length of the various samples:

Sample 1: 7.4 kb; sample 2: 3.9 kb; sample 3: 4.6 kb; sample 4: 10.2 kb.