STELA PROTOCOL

Required Solutions

1 M NaPi pH 7.2

(Make in orange cap plastic roller bottle -- this is 2.3 l when filled to the 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
pH to 7.2 with H_3PO_4 (should be close)
fill to 2.3 l with ddH_2O

Church mix

500 ml 1 M NaPi pH 7.2
2 ml 0.5 M EDTA pH 8.0
70 g SDS
10 g BSA
Fill to 1 l with ddH_2O
Filter sterilize through a 0.45 µm filter unit

10x OLB

for 1 ml:

1 M Tris pH 6.8	500 µl
1 M MgOAc	100 µl
1 M DTT	1 µl
10 mg/ml BSA	50 µl
ddH_2O	346 µl

Add 0.6 µl 100 mM (GE Heathcare stocks) of each desired dNTP to 100 µl of 10x OLB
Store at -80°C for up to 1 month.

TNE

10 mM Tris pH 7.4
100 mM NaCl
10 mM EDTA

TNES

10 mM Tris pH 7.4
100 mM NaCl
10 mM EDTA
1% SDS

Day 1

Materials

- Genomic DNA. Make sure the DNA is very clean (smell it for traces of phenol or 2-propanol.
- EcoR1 enzyme and buffer (NEB R0101S)

Restriction Enzyme Cut (for XpYp STELA on human DNA)

To a 1.5 ml tube, add:

- 30 μl genomic DNA (5-10 μg)
- 10 μl 10x EcoR1 buffer
- 5 μl EcoR1 enzyme (20 U/μl)
- 55 μl H_2O

Incubate overnight at 37°C

NOTE: Run 5 μl of the digested DNA on a 0.7% agarose gel to test the efficiency of the digestion. If the DNA is not cut well, it is dirty and needs to be cleaned up.

Day 2

Materials

- Make sure you have all Oligos in stock solutions of 500 μM in TE:
  - Telorette1: TGCTCCGTGCATCTGGCATCCCCTAAC
  - Telorette2: TGCTCCGTGCATCTGGCATCTAACCCT
  - Telorette3: TGCTCCGTGCATCTGGCATCCCTAACC
  - Telorette4: TGCTCCGTGCATCTGGCATCCTAACCC
  - Telorette5: TGCTCCGTGCATCTGGCATCAACCCTA
  - Telorette6: TGCTCCGTGCATCTGGCATCACCCTAA
- Genomic DNA cut with EcoR1 (5-10 μg)
- Hoechst 33258 (to 1 μg/ml) in filter-sterilized TNE (store at 4°C, use at room temperature otherwise the cuvette will fog up).
- T4 DNA ligase and buffer (Amersham E70005Y)

Measure DNA concentration with Hoechst fluorimeter

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. Place 2 ml of the diluted dye in the cuvette.
3. Press <ZERO>.
4. Add 2 μl of ref DNA solution (for example 100 μg/ml). Mix with a P1000 pipette (do not make bubbles).
5. Press <CALIB>, enter the concentration of your standard (e.g. 100) press <ENTER>.
6. Remove sample with suction (p200 tip on Pasteur pipet).
7. Replace with fresh solution and zero again.
8. Add 2 μl of the sample DNA and mix. The concentration of the sample will be displayed

Fluorimeter Notes:

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

Ligation

Make a 10 ng/μl dilution of DNA in H_2O
Set up a ligation reaction. In 10 μl add:
1 μl DNA (10 ng/μl)
1 μl 10x ligase buffer
1.8 μl telorette X (final con. 10^{-2} – 10^{-5} μM)
0.5 μl T4 DNA ligase
5.7 μl H_2O

Incubate overnight at 35°C in a PCR machine.
Controls

- Ligation – no telorette
- Ligation – no ligase
- Telorette3 – used for telomere length analysis

Day 3

Materials

- Make sure you have all oligos in stock solutions of 500 μM in TE:
  - XpYpE2: TTGTCTCAGGGTCCTAGTG
  - Teltail: TGCTCCGTGCATCTGGCATC
- Fail Safe Enzyme Mix (Epicentre FS99100)
- 2X Fail Safe PCR Buffer H (Epicenter FSP995H)

PCR of Ligations

Dilute ligations to 250 pg/μl by adding 40 μl of H_2O to the ligation.
To a PCR tube, in 25 μl add:
1.0 μl DNA (250 pg/ul)
0.5 μl 10 μM XpYpE2
0.5 μl 10 μM teltail
12.5 μl 2x Fail Safe PCR buffer H
0.8 μl Fail Safe Enzyme Mix (2.5 U/μl)
9.7 μl H_2O

Cycles:

94°C 15 sec
25 cycles of:
95°C 15 sec
58°C 20 sec
68°C 10 min
68°C 10 min final extension

Controls: PCR mix, no DNA

PCR Notes:

- The 2x Fail Safe PCR Buffer H tends to stop working after freeze/thawing. After the first thawing, make 500 μl aliquots and store at -20°C.
- For the first time running a particular DNA sample, PCR several different concentrations (i.e. 250 pg, 125 pg and 62.5 pg)

Day 4

Materials

- 50x TAE and 1x TAE
- Agarose
- Orange G loading buffer
- 1x TE
- 10 mg/ml ethidium bromide
- ΦX λ Hind III molecular weight marker
- 20 cm x 20 cm gel mold, 20-well comb
- XpYpB2: TCTGAAAGTGGACCTATCAG (make sure you have all oligos in stock solutions of 500 μM in TE)
- 10 μM XpYpE2 (from STELA PCR)
- 10 ug/μl DNA (EcoR1)
- Takara Taq kit
- QiaQuick gel extraction kit

Southern Blotting Probe

Preparing XpYp Probe (subtelomeric)

To a PCR tube, in 50 μl add:

- 5 μl 10x Takara buffer
- 3 μl Takara $MgCl_2$
- 4 μl 10 mM Takara dNTPs
- 5 μl 10 μM XpYpE2
- 5 μl 10 μM XpYpB2
- 0.5 μl enzyme
- 26.7 μl H_2O
- 1 μl 10 ng/μl DNA (genomic DNA cut with EcoR1 on Day 1)

Cycles:
94°C 15 sec
35 cycles of:
94°C 30 sec
53°C 30 sec
72°C 1 min
72°C 10 min

1. Run 5 μl of PCR on a 1% gel to check that the size of the fragment is ≅ 500 bp
2. Run remain of PCR on a gel and extract the 500 bp band using QiaQuick gel extraction kit.
3. Run extract to check the presence of the band and the approximate concentration.

Note: Do 4 copies of the probe PCR at the same time. This generates enough probe DNA for many STELA blots (100 ng of probe DNA is used per blot).

Pour and run STELA gel

1. Make 300 ml of 0.5% agarose gel in 1x TAE .
2. Add 3 μl of 10 mg/ml ethidium bromide as agarose cools.
3. Pour all 300 ml into a 20 cm x 20 cm gel mold with a 20-well comb.
4. Run 25 μl of PCR product on the gel, with 5 μl Orange G loading buffer.
5. Run 4 μl ΦX λ Hind III MW marker mixed with 1 ng probe DNA.
6. Run gel until the Orange G front is about 1 inch from the bottom of the gel. (overnight at 30 V).

   Gel Controls: ≅1 ng of probe PCR (mixed into the MW marker).

Day 5

Materials

Southern Blot:
Depurination Buffer (0.25 M HCL)

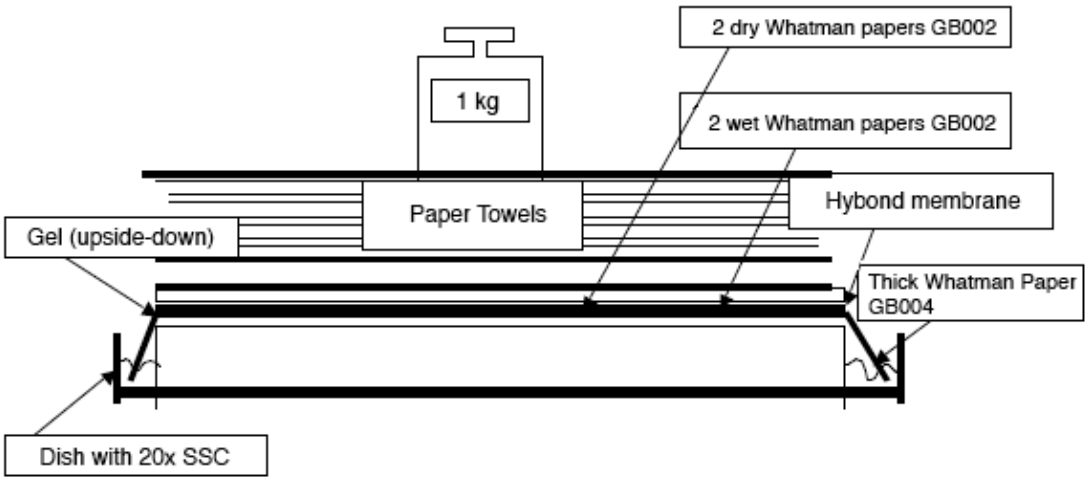
- Denaturation buffer (1. 5 M NaCl, 0.5 M NaOH)
- Neutralization buffer (1 M Tris 7.4, 1.5 M NaCl)
- 20 x SSC
- Hybond N membrane 20 x 20 (Amersham RPN2020N)
- S&S Gel blot paper 20 x 20 (GB002) (VWR 52844-504)
- S&S Gel blot paper 20 x 25 (GB004) (VWR 28153-290)
- paper towels

Probe Preparation:

- Probe PCR (Purified on Day 3)
- ^{32}P -alpha-dCTP (3000 Ci/mmol)
- 10x OLB w/ dTTP, dATP, and dGTP
- 10X=x Random Hexamers (Invitrogen 48190-011)
- Klenow 2 U/μl (Roche 1008-404)

Southern Blot

1. Take a picture of the gel with a ruler next to the markers. (PCR products will NOT be visible).
2. Soak the gel (leave it on its tray, make sure the gel is completely submerged and floating, shake continuously and gently):
    - Depurination 30 minutes 0.25 M HCL
    - Denaturation 2 x 30 minutes 1.5 M NaCl, 0.5 M NaOH
    - Neutralization 2 x 30 minutes 1 M Tris 7.4, 1.5 M NaCl
3. Blot onto Hybond filter. Use clean gloves to handle everything. The blotting buffer is 20x SSC. Pre-wet the filter first by floating on top of ddH_2O and then soak it in 20x SSC.
4. Blot for at least 2 hr or o/n. Set up blotting as follows:



Label Probe

1. Set up a random priming reaction. In a 1.5 ml tube add:
    1 μl probe DNA (100 ng/ul)
    24 μl H_2O
    5 μl 10x random hexamers

2. Boil at 100°C for 5 min.
3. Quickly place on ice for 3 min.
4. Add 5 μl 10x OLB w/ dATP, dTTP, and dGTP.
5. Behind a shield add:
    7 μl ^{32}P-alpha-dCTP (3000 Ci/mmol)
    1 μl Klenow (2 U/μl)
6. Incubate at RT for at least two hours

NOTE: If transferring for 2 hr rather than overnight, the probe should be labeled on the evening of Day 4.

Day 6

Materials

- Saran Wrap
- Seal-a-Meal bag and sealer
- Church Mix
- TNES
- G-50 Sephadex

Continue with Southern Blot

1. Take the filter from the gel, wrap it in Saran Wrap and crosslink it (DNA side up) in Stratalinker (auto-crosslink).
2. Unwrap and rinse the filter in a clean tray with H_2O to rinse off the 20x SSC.
3. Put the filter in a Seal-a-Meal bag, add ~20 ml Church mix and seal without air bubbles.
4. Incubate filter (pre-hybridize) for about 1 hr at 55°C in a shaking water bath.
5. Remove (pre-hyb) Church mix. Open the bag at one corner; remove the pre-hyb church mix completely.
6. Add hybridization mix (see probe preparation below). Using a 10 ml pipet add all the hybridization mix, carefully seal bag with no bubbles.
7. Incubate at 55°C in a shaking water bath overnight. While it is incubating, prepare the probe.

Probe Preparation

1. Prepare a G-50 Sephadex column: put some glass wool into a 3 cc syringe, and stuff it tightly with the plunger, fill the syringe to the TOP with Sephadex G 50 fine (autoclaved in TNE).
2. Equilibrate the G-50 column with 1 ml of TNES.
3. Add 50 µl TNES to the random priming reaction from day 5, mix well and slowly add to the G-50 column,
4. Elute with TNES (void volume = 1000 µl) then collect 800 µl (has the DNA). Don't elute further (you will just get free counts off) When measured with a Geiger counter, the counts from the probe should be ≥ the counts from the column.
5. Heat the probe (800 µl) for 5 min at 100°C, immediately add ~25 ml church mix and filter through 0.2 µm syringe filter (IMPORTANT). This is your hybridization mix.

Day 7

Materials

- Tupperware dish
- Church wash
- Saran Wrap
- phosphoimaging cassette

Southern Blot Continued

1. Remove the filter from the bag and place it in a Tupperware container.
2. Wash with pre-warmed church wash at 55°C, 3 times for 15 min each in a shaking water bath.
3. Wrap the filter in Saran Wrap. Remove all the liquid and tape to Whatman paper

4. Expose the filter o/n (or longer depending on the “hotness” of the filter) using a phosphoimaging cassette at room temperature.

Example of STELA blot: