DT40 cells transfection for gene targeting

1. Spin down 1 x 10^7 cells

2. Wash once with PBS (sterile PBS)

3. Resuspend cells in 0.450 μL PBS and transfer to transfection cuvette

4. Add 10 μg (around 50μl) of linearised DNA (linearised DNA is purified by potassium acetate precipitation and resuspended in sterile PBS. During the precipitation procedure, the DNA should be well dry before resuspention as presence of leftover ethanol will lower transfection efficiency)

5. Incubate at room temperature for **10 min**

6. Electroporate (550V, 25mFD). (The time constant should be around 0.48-0.56 msec)

7. Incubate at room temperature for **10 min**

8. Transfer cells to 20 mL of fresh media

9. Incubate cells for two doubling times in incubator (approx 16hr)

10. Make up media with required drug for selection and plate cells diluted into selection media into 96 well plates as follows:

Add 5ml of cells into 35ml of media containing drug and mix well. Add 200ul to each well.

Required final concentrations of the selected drug:

G418 (invivogen)	2 mg/mL
Histidinol (invivogen)	1 mg/mL
Hygromycin (Calbiochem)	2 mg/mL
Blasticidin (invivogen)	25 mg/mL
Puromycin (invivogen)	0.5 mg/mL

11. Leave for up to **10 days**.

12. When the clones grow big enough, transfer them to 12 well plates (containing 3ml media in each well, no drug is required at this stage)

13. When clones are confluent, use 1.5ml for freezing down, and 1.5ml for genomic DNA extraction

Genomic DNA extraction

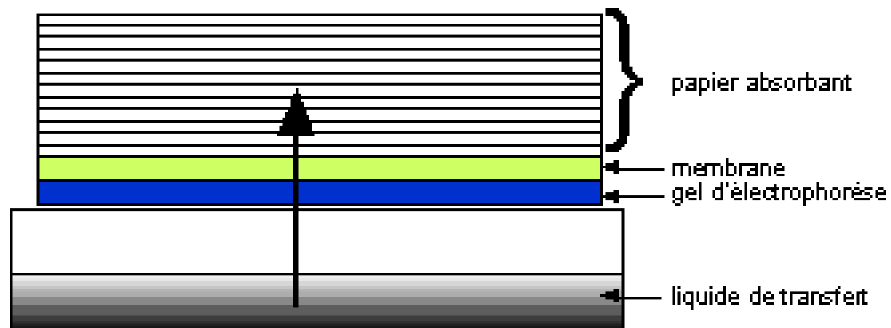
From Xian-Yang Lai (September 2004)

1. Harvest 1.5 x 10^6 cells per eppendorf (or 1.5ml of cells at confluency).
2. Spin down the cells at 1200 rpm for 5 min . Pour off media and remove as much media as possible using tissues.
3. Resuspend each pellet in 500 μL Tail Buffer (50 mM Tris-Hcl, pH 8.8, 100 mM EDTA, 100 mM NaCl, 1% SDS) (Add 3 mL of a 20 mg/mL Proteinase K solution for every 0.5 mL tail buffer used, best to have this added to the buffer before use)
4. Incubate at 55°C for 3 hours, shaking for 5 min each hour in the eppendorf shaker at max speed. You can also incubate this **overnight at 37 ^{O}C**. The rest of the extraction can be performed the subsequent day or the lysed cells can be keeps at -20 ^{O}C until the day of choice.
5. Shake vigorously (in the shaker at 1400 on the 'mix' setting) for 5 min.
6. Add 200 μL saturated NaCl (6M)
7. Shake vigorously in the shaker at 1400 for 5 min.
8. **Spin down for 30 min** at max speed on the desk top centrifuge.
9. Pour off the supernatant into a new eppendorf tube.
10. Add 700 μL of isopropanol, and mix the eppendorf by slowly inverting it. You should be able to see the DNA as you mix, as a 'cloud' in the solution. Look at each sample for this as you will know if the DNA is good or not.
11. Spin down for **10 min** at max speed on desktop centrifuge, look for pellet, will be very clear and spread out.
12. Wash the pellet with 300 μL 70% EtOH.
13. Spin down for **5 min** at max speed on desktop centrifuge afterwards you will see a much more compact pellet. Airdry the pellet before resuspension.
14. Resuspend pellet in 35 μL TE buffer at pH 7.5-8.0). Leave at RT on bench overnight.
15. Digest the totality of gDNA with the appropriate enzyme (1.5μl) in a final volume of 50μl, incubation overnight, and load only half the digested genomic DNA (25μl) on the agarose gel for the Southern.

Southern Blot

Protocol

1. Run genomic DNA digest on 0.8% agarose gel
2. Take photo of gel, include ruler to measure distance from well to band of interest
3. Depurinate gel : Soak in 0.4M HCl for 20 minutes
4. Rinse gel in ddH2O
5. Denature gel : Soak gel in 0.5M NaOH, 1.5M NaCl for 20 minutes (db strand-> single strand)
6. Neutralise gel by rinsing gel in ddH2O for 20 minutes
7. Set up transfer apparatus as show in the diagram



8. Leave to transfer overnight
9. Next day : Mark location of wells on membrane using pencil
10. Dry membrane on filter paper for about 5 minutes
11. Crosslink DNA to membrane with UV (energy = 3000 for 1min)
12. Store membrane at room temperature wrapped in tinfoil

Buffers:

Depurination buffer : Add 22ml concentrated HCL to 1L ddH2O
Denaturation buffer : 0.5M NaOH, 1.5M NaCl
Transfer buffer : 2X SSC (20X SSC stock in the lab, 2x 1L bottles necessary)

Southern hybridation

Hybridization

1. Saturation of the membrane: 20ml of DIG Easy Hyb (bottle 7 preheated at 39°C), **30 min** of incubation at 39°C.

2. Denaturation of the probe: denature the probe at 95°C for 5 min then rapidly cooling in ice for 1 min.

3. Hybridization of the membrane with the probe : Add the denatured probe (500ng DNA) to 15 ml of DIG Easy Hyb pre-heated at 39°C, mix and et pour off to the membrane.

4. Incubate **overnight** with gentle agitation at the optimal temperature corresponding to the following equation.

   Tm= 49.82 + 0.41 (%G+C)- (600/l) (l= length of hybrid in base pair)
   Topt = Tm - 20 to 25°C

5. Stringency wash: 2 wash for **5 min** with 2X SSC, 0.1% SDS at room temperature and then 2 wash for **15 min** at 65° C of 0.5X SSC, 0.1% SDS (100ml for each wash).

Note: DIG Easy Hyb with DIG-labeled probe can be store at -20 °C and reused many time. However, the denaturation of the probe in this buffer has to be performed at 68°C △! for 10min before use. Also, add around 100ng of newly denaturated probe in the reused DIG Easy Hyb/DIG-labeled probe mix.

immunological detection (room temperature)

6. Rinse membrane for **5 min** in washing buffer (≈ 70ml)
7. Incubate for **30 min** in 100ml of blocking solution
8. Incubate for **30 min** in 20 ml of antibody solution
9. Wash **2*15 min** in 100ml washing buffer (≈ 90 ml)
10. Equilibrate **5min** with 20ml of detection buffer (alcaline phosphatase buffer)
11. Place membrane into a plastic film and add 1ml of CSPD ready-to-use.
12. Incubation for **5 min** room temperature.
13. Place membrane into a thin film, put into a cassette with the scale marker
14. Incubate at 37°C for **10 min** to enhance the reaction.
15. Place film.

Maleic Acid buffer
0.1M Maleic acid
0.15M NaCl
NaOH (solid) to adjust to pH 7.5 (20’C)

Washing buffer
250ml Maleic acid buffer
750 µl Tween 20

Blocking solution
12ml blocking buffer
4)
108ml Maleic acid

Antibody solution
2µl Anti-Digoxigenin-AP (vial

20ml blocking solution

Detection buffer
0.1M Tris-HCl
0.1M NaCl
pH 9.5 (20’C)

DIG-Probe synthesis

Probe was originally amplified by PCR from the genomic DNA and cloned into pTOPO vector with the pTOPO kit following the manufacturor’s instructions. DIG labelled probe synthesis is performed using PCR DIG Probe Synthesis Kit from Roche (Cat. No. 11 636 090 910)

Reagent	
pTOPO-Probe (miniprep 1/100)	1µl
Forward primer	1µl
Reverse primer	1µl
Enzyme mix (vial 1)	0.75µl
PCR Buffer (vial 3)	5µl
DIG dNTP Mix (vial2)	5µl
H_2O	36.25µl
Total volume	**50µl**

Step	Cycles	Time	Temperature
Initial denaturation	1	2 min	95°C
Denaturation	25	1 min	95°C
Annealing		1 min	60°C
Elongation		1 min	72°C
Final elongation	1	7 min	72°C

As in the DIG dNTP mix only dUTP is tagged with DIG, a percentage too low of A-T in the probe sequence could be an issue.
In this report, two probe were used:
-Dot1 probe : 53% of A-T
-SUV420h1 probe : 58% of A-T

Immunofluorescence protocol

As DT40 are suspension cells, they must adhere first on polyLysine slides. For this reason, the step 1. And 2. Are specific for DT40. Adherent cells on coverglass can be fixed directly by PFA (step 3).

1. Allow cells to adhere to polylysine slides for **15 min**
2. Aspirate off culture medium (do not wash with PBS or cells will come off!)
3. Fix the cells by adding 4% paraformaldehyde to the slide (PFA is supplied as 16% - dilute in PBS to final concentration of 1X PBS). Leave for **10 min**.
4. Aspirate off PFA and wash slide in 1X PBS, 3 times
5. Permeabilise cells by adding 0.125% triton-X to the slides. Leave for **2 min**.
6. Aspirate off triton X and wash slide in 1X PBS, 3 times
7. Block using 1% BSA (diluted in 1X PBS) for at least **30 min**. (Cover slides with parafilm to prevent evaporation).
8. remove the excess of 1% BSA solution and add add 100ul of primary antibody to the slide at the appropriate dilution in 1% BSA. (Use parafilm to spread the solution over the entire surface of the slide evenly if working on DT40. For Human cells, depose first the primary antibody on parafilm then return the coverglass with cells on the antibody drop and so parafilm)
9. Leave at 37°C for **1 hours** in humid conditions (place a wet tissue in the box containing the slides)
10. Wash slide in 1X PBS, 3 times.
11. Add 100ul of secondary antibody (1/200 dilution in 1% BSA)
12. Leave at 37°C for **45 min to 1h** in humid conditions
13. Wash slide in 1X PBS, 3 times (in dark)
14. Place one drop of Vectashield containing 1X DAPI on the slide. Place a coverslip on the slide (the vectashield spreads across the slide).
15. Allow slide to dry by light pressure of the coverslip on Wattman paper.
16. Seal slides using nail varnish

PI detection by FACS

1. After desired treatment, take about 1 x 10^6 cells (enough in 6w/p) and spin down at 2000 rpm **5min** at 4'C.

2. Resuspend cells in 5ml 1X PBS 0.1% BSA (prechilled at 4'C) and spin down at 2000 rpm **5min** 4'C

3. Resuspend cell in 1ml of PBS at Room temperature and then add 3ml of ice cold ethanol on vortex. Cells can be stored at that stage at 4'C or -20'C

4. Spin down cells at 2000 rpm for 5min. Resuspend in 1X PBS. Leave at room temperature for 30min. Spin down cells at 2000rpm fro 5min.

5. Resuspend cells in 500µL of PBS containing 40µg/mL propidium iodode and 250µg/ml of RNaseA. Incubate the cells for **between 30 min and 1hour** on ice and analyse the samples on the FACS canto.

RnaseA – from Qiagen = 100mg/ml
Propidium Iodide – Stock = 2mg/ml in water

γ-H2AX detection by FACS

1. After desired treatment, take about 1 x 10^6 cells (enough in 6w/p) and spin down at 2000 rpm **5min** at 4'C.

2. Resuspend cells in 0.5ml PBS 0.1% BSA (prechilled at 4'C) and spin down at 2000 rpm **5min** 4'C

3. Resuspend cell in 50μL of PBS 0.1% BSA (4'C) and then add 50μL of 2x PFA-fixation buffer. Mix by pipetting and incubate the cells on ice for **10min**.

4. Add 100μL Block-9 buffer containing 1:500 anti γ-H2AX antibody (Millipore). Mix well and incubate on ice for **1h$^{1/2}$ hour** (mixing periodically, every 30min).

5. Add 1ml of cold PBS 0.1% BSA and spin cell down at 2000 rpm **5min** 4'C

6. Resuspend cells in 50μL Block-9 buffer containing 1:50 FITC-anti-mouse antibody. Mix well and incubate on ice, in the dark for **30min.**

7. Add 1ml of cold PBS 0.1% BSA and spin down cells at 2000 rpm **5min** at 4'C

8. Resuspend cells in 300μL of PBS 0.1% BSA containing 40μg/mL propidium iodode. Incubate the cells for **between 30 min and 1hour** on ice and analyse the samples on the FACS canto.

Reagents:

2x PFA-fixation buffer
50 mM Tris-HCl (ph 7.5)
2% TritonX100
2mM EDTA
0.2g/L BSA
phosphatase inhibitors
0.4% Paraformaldehyde

Block-9 buffer
1X-PBS (to adjust final volume)
1g/L BSA
8% (v/v) Chicken serum
0.1g/L RnaseA
1x phosphate inhibitors
0.25g/L Salmon Sperm DNA
0.1% TritonX100
5mM EDTA
0.05% (w/v) NaN_3 (Na azide)

g-H2AX antibody – Millipore JBW301
(Goat)-anti-Mouse FITC – Jackson immunoresearch lab inc. – 115-096-042
RnaseA – from Qiagen = 100mg/ml
Propidium Iodide – Stock = 2mg/ml in water

Neutral Comet assay (trevigen)

1. Prepare lysis solution and chill at 4'C or on ice for at least 20min
   For 10 slides (2 samples per slides) lysis sol. 40ml
   (DMSO opt. 4ml)

2. Melt LMAgarose in a beaker of boiling water for **5 min** with the cap loosened and then cool in a 37'C wather bath for at least **20min**

3. Combine cell at 1.10^5/ml with molten LMAgarose at a ration 1:10 and immediately pipette 50ul onto cometslide (polylysine slides). Use side of pipette tip to spread agarose/cells over sample area.
   LMA garose 50ul
   Cells in PBS 5ul
   (4.10^5/ml)
4. Place slides at 4'C in the dark for **10 min**, increasing time to **30min** improves adherence of samples
5. Immerse slides in prechilled lysis solution (step1) and leave on ice or 4'C for **1h**
6. Remove slides from lysis buffer, drain the excess and wash by immersing in 50ml of prechilled 1X neutral electrophoresis buffer for **30min** at 4'C
7. Align slides equidistant from electrodes, add 1x neutral electrophoresis buffer not to exceed 0.5cm above slides and apply voltage at 1 volt/cm (measured electrode to electrode: 24volt) for **1h** at 4'C
8. Drain excess neutral Electrophoresis buffer and immerse slides in DNA precipitation solution for **30 min** at room trempterature.
9. Immerse slides in 70% ethanol for **30 min** at room temperature
10. Dry samples at <45'C for **30-45 min** (in 37'C incubator). Drying grings all cells in a single plane to falicitate observation . sample can be stored at Room Temperature.
11. Place 100ul of SYBR green on each sample for **30 min**. Gently tap slide to remove excess of SYBR solution. Allow slide to dry completly at Room Temperature in the dark
12. view slides by microscopy
    (to avoid bleaching effect while taking picture, it is possible to add drops of Vectshield on the slide, cover with a coverglass and seal it with nailvanish).