Preparation of Metaphase Chromosomes for FISH – (Protocol from the department of Human Genetics)

- ES culture should be sub-confluent and in an exponentially growing phase. Change medium 4 hours prior to start of experiment.
- Incubate ES cells with 100µl of Colcemid (Roche - **10295892001**; toxic-handle and dispose according to safety guidelines) in 5 ml of medium for 3 hours in order to arrest cells in the metaphase stage.
- Wash cells once with PBS and then trypsinise cells as usual, collect in a 15 ml falcon tube and spin down for 5 minutes at 800 rpm
- Remove supernatant carefully
- Snap the tube to loosen the pellet (make sure to completely loosen the pellet). Add 1ml of pre-warmed (37^{o}C) 0.56% KCl dropwise. Snap the pellet gently to get a uniform suspension (no pellet should be visible. If this is the case, pipette gently to get a homogenous suspension). Add an additional 4ml 0.56% pre-warmed KCl and incubate for 10 minutes at 37^{o}C
- Centrifuge the tube at 800 rpm for 5 minutes and remove the supernatant carefully.
- Snap the tube to loosen the pellet. Add 1-1.5 ml of ice-cold fixative (MeOH : Acetic acid ::3:1 – prepared fresh and ice cold) to the above tube. Transfer the contents of the tube to a 1.5ml eppendorf tube. Centrifuge at 6000 rpm for 1 minute and carefully remove the supernatant. Repeat this step two to three times.
- Resuspend in fixative (about 1ml) and this is now ready to be dropped on slides. This preparation can be stored at –20^{o}C for many years in fixative.
- Dip the slides in ice cold 40% methanol and allow to drip on a filter paper. Make sure the methanol coats the entire slide properly after dipping. If uneven film is formed, discard the slide and use a fresh slide repeating the procedure again. With a gilson pipette, drop 25-35µl of cell suspension on the slide from a height of about 10cm. As the fixative gradually evaporates, the surface of the slide becomes grainy. At this moment, place the slide face down into the steam of hot water bath (75^{o}C) for 5-10 seconds and then dry quickly by placing the slide on a metal plate.
- Incubate at 65^{o}C overnight for aeging. The slides can be stored dipped in 100% EtOH at –20^{o}C until use

Labeling of probes by Nick Translation

Labeled probes can be stored for long periods at –20°C without affecting the probe quality. Hence large probe amounts can be labeled at one time.

Reagents:

- Biotin-16-2'deoxy-uridine-5'-triphosphate
  - Concentration : 1mM
  - Store at –20°C
- Digoxigenin-11-2'deoxy-uridine-5'-triphosphate
  - Concentration : 1mM
  - Store at –20°C
- β-Mercaptoethanol (Stock solution)
  - Concentration : 14.44 M/l
  - Store at 4°C

Working Solutions:

- ***DNAse***

  Stock concentration : 1mg/ml

  5mg DNAse
  2.5mg 0.3M NaCl
  2.5mg Glycerine
  Make up the volume to 5ml with aqua bidest.
  Store at –20°C

Working dilution: Dilute 1µl of the stock solution in 1ml of ice-cold water immediately before use. The volume of the working stock used in the nick translation reaction must be tested for each new bath of DNAse I stock solution. Carry out a series of digestions with 2µg of probe DNA, 10µl of 10x buffer, 10µl of b-mercaptoethanol, and 1µl, 2µl, 5 µl and 10 µl of the DNAse I dilution respectively in a 100 µl reaction volume. Incubate for 2 hours at 15°C. Thereafter, takn an aliquot of 5-10 µl and test the size of the digested DNA on a 1% agarose gel using a 1Kb ladder. Choose the volume of DNAse that results in probe fragments of 100-500nt in length for the nick translation reaction. The DNAse stock should be diluted immediately before applying to the nick translation reaction.

- ***dNTP's***
  - Working dilution: Diltute dATP, dCTP, dGTP and dTTP each to 2mM.
  - Add 25µl of dATP, dCTP and dGTP and 5µl of dTTP and make up the volume to 100µl with aq.bidest
  - Store at –20°C

- ***10x Nick translation (NT) buffer***

  0.5M Tris HCl, pH7.5
  50mM Magnesium Chloride
  0.5mg/ml BSA
  Store at –20°C

- ***0.1M β-Mercaptoethanol***

  69µl of 14.4M β-mercaptoethanol and make up the volume to 10ml with aqua. Bidest
  Store at 4°C

- ***Miscellaneous:***

  G50 spin column
  0.5M EDTA
  10% SDS

Procedure:

Combine as follows:

For Biotin labeling:
xµl DNA containing 2 µg DNA
10 µl 10x NT buffer
10 µl 0.1M β-Mercaptoethanol
10 µl dNTP's
2µl Biotin dUTP
x µl DNAse
2 µl DNA polymerase
Make up the volume to 100 µl with sigma water

For Digoxigenin labeling:
xµl DNA containing 2 µg DNA
10 µl 10x NT buffer
10 µl 0.1M β-Mercaptoethanol
10 µl dNTP's
2 µl Digoxigenin dUTP
x µl DNAse (diluted 1:100 fresh)
2 µl DNA polymerase
Make up the volume to 100 µl with sigma water

- Mix the contents by vortexing and briefly spin
- Incubate for 2hours at 15°C
- Place the reaction on ice and keep it on ice until the actual size of the probe molecules is determined
- Take an aliquot of 5-10µl, add gel loading buffer and load it on a standard 1% agarose gel along with a suitable size marker.
- The probe molecules should be visible as a smear. This should contain only fragments smaller than 500nt.and larger than 100nt. If this is the case, proceed with inactivation. If the probe is larger, add more DNAse to the reaction kept on ice and incubate further about 15-30 minutes and repeat the previous step. If part of the DNA is smaller than 100nt, repeat using a higher dilution of DNAse

- The reaction is inactivated by adding 3µl of 0.5M EDTA (15mM final concentration), 1µl of 10% SDS (0.1% final concentration), and heating it for 15mins at 68°C.
- The labeled probe can be separated from the unincorporated nucleotides by gel filtration using a Sephadex G-50 spin column from Amersham. The flow through contains now the labeled probe at a concentration of 20ng/µl. The labeled probe can be stored at –20°C or proceed to the DNA precipitation step

DNA Precipitation

Reagents:

- Mouse-Cot1 DNA
  Store at –20°C. Once thawed, store at 4°C

- 3M Sodium acetate
  40.824 gm Sodium acetate in 100ml water
  Adjust pH to 5.2 with acetic acid
  Store at room temperature or 4°C

Procedure:

- *After the deactivation of the Nick translation reaction, do as follows*

I. Add together for **single copy sequences**

a. 200 ng of biotin labeled DNA (`15µl if yield is good)
b. 200 ng of digoxigenin labeled DNA (`15µl if yield is good)
c. 1 µl Salmon sperm DNA (10µg/ml)
d. 1/10th volume 3M Sodium acetate
e. 2.5 volumes of 100% **chilled** Ethanol (-20°C)

II. Add together for **repetitive sequences**

- 50-200ng labeled DNA (`15µl if yield is good)
- 10µg mouse CotI DNA
- 1/10th volume 3M Sodium acetate
- 2.5 volumes of 100% **chilled** Ethanol (-20°C

- Mix well
- Incubate overnight at –20°C (or for 30 minutes at –80°C)
- Centrifuge at 13,000 rpm for 30 minutes at 4°C
- Discard supernatant
- Wash 3x with 70% Ethanol
- Allow to dry at 37°C

Pretreatment of metaphase chromosomes for FISH

Reagents:

- Pepsin
    - Stock solution is 10% pepsin
    - Store at –20°C
- Pepsin – working dilution
    - 700µl of 1M HCl in 70ml of Aq.bidest water. Warm to 37°C and just before the slides are to be dipped, add 50µl of pepsin and mix well
- 10x PBS
    - 82g NaCl
    - 2g KCl
    - 2g KHPO4
    - 11.5g NaHPO4 x 2H2O
    - Make up the volume to 1L with water
    - Store at room temperature
    - Working dilution : 1x PBS

- PBS-Magnesium Chloride
    - 5ml 1M Magnesium chloride (101.65gm MgCl2 in 500ml water) + 95ml 1x PBS
    - Store at room temperature

- 37% Formaldehyde

- 70%, 90% and 100% Ethanol

- 20x SSC
    - 175.3g NaCl
    - 88.2g Sodium Citrate .2H2O
    - Adjust pH to 7.0
    - Adjust volume to 1L with water
    - Working dilution: 2x SSC

- Denaturation Mix
    - Freshly prepare
    - 100µl 20x SSC
    - 200µl Aq.bidest
    - 7µl 1M HCl
    - 700µl de-ionised formamide

Procedure:

- This procedure can be carried out simultaneously with the precipitated DNA being centrifuged
- Equilibrate the slide by briefly dipping in 2x SSC
- Digest in pre-warmed pepsin solution (remember to add pepsin (50ul) only before incubating the slide!) and incubate at 37°C for 10 minutes in a shaking water bath
- Wash 2x5 mins in 1x PBS at room temperature on a shaker
- Wash for 3 minutes in 1x PBS-Magnesium Chloride at room temperature on a shaker

- *Pre-fixing:* Incubate the slide in 70ml PBS-Magnesium chloride containing 2ml 37% Formaldehyde for 5 minutes at room temperature (no shaking)
- Wash 5 minutes in 1x PBS on a shaker
- Dehydrate the slide by dipping in Ethanol series (3 minutes each in 70%, 90% and 100% Ethanol)
- Allow the slide to air dry
- Place the Ethanol series in –20^{o}C.
- Pre-warm the slides to 60^{o}C
- Once the slide is dry, working quickly, add 100µl of pre-warmed (75^{o}C) denaturation mix on the area of the slide marked with good metaphase chromosomes, place a 24 x 50 mm coverslip over the slide and incubate it in a steel container with lid for **1 minute 45 seconds** at 72^{o}C-75^{o}C or on a heated PCR block (75^{o}C)
- Remove the cover slip immediately and dip the slide briefly in ice-cold 2x SSC
- Dip the slides in **ice cold** Ethanol series (3 minutes each in ice-cold 70%, 90% and 100% Ethanol)
- Allow the slide to air dry
- Slides are ready for hybridization

FISH

Working solutions:

- De-ionized formamide
    - Store at 4^{o}C

- Hybridization buffer
    - Prepare 20x SSC and 50% dextran sulphate solutions. Dissolve dextran sulphate thoroughly, then autoclave it or filter it through nitrocellulose filter. Combine 200µl of 20x SSC and 400 µl 50% dextran sulphate with 400 µl water.
    - Store at 4^{o}C

Procedure:

- To air dried precipitated DNA probe, add 6µl de-ionized formamide and incubate it for about 1 hour at 37^{o}C in a thermoshaker (under shaking conditions)
- Add 6µl of hybridization buffer and mix thoroughly and carefully
- Continue incubation without shaking for an additional 15 minutes
- Denature the DNA by incubating for 5 minutes at 75^{o}C
- Place immediately on ice for about 5 minutes. (If the probe is meant for repetitive sequences, pre-anneal the DNA by incubating the probe at 37^{o}C for 40 minutes)
- Place the probe carefully on the slide area marked with good metaphase chromosomes and place a 18 x 18mm coverslip over the probe, seal around the cover slip with rubber cement and place in a humidified chamber.
- Hybridize overnight at 37^{o}C in a humidified incubator.

DETECTION

Reagents:

- Antifade
    - Mowiol with DABCO
    - Store at 4°C
    - Sensitive to light !!

- Avidin-FITC
    - Fluorescein Avidin DCS
    - Concentration: 2mg/ml
    - Store at -20°C, once thawed, store at 4°C
    - Sensitive to light

- Anti-Avidin
    - Biotinilated Anti-Avidin D
    - Concentration: 0.5mg/ml
    - Store at -20°C, once thawed, store at 4°C

- Anti-DIG
    - Monoclonal Anti-Digoxin (mouse ascites fluid)
    - Store at -20°C, once thawed, store at 4°C

- Anti-Mouse Cy3
    - Anti-Mouse Cy3
    - Store at -20°C, once thawed, store at 4°C
    - Sensitive to light

- DAPI
    - 4'6-Dianidino-2-phenylindol-2HCl
    - Stock concentration: 0.2mg/ml
    - Working dilution: 10µl Stock solution in 100ml distilled water
    - Store in a Coplin jar wrapped in Alu foil at 4°C. Can be re-used many times
    - Sensitive to light!!!

- Tween 20
    - Polyoxyethylensorbitan Monolaurate
    - Store at room temperature

Working dilutions:

- Antibody I
    - Prepare fresh
    - Centrifuge antibody for 5 minutes at 14,000rpm at 4oC
    - Detection solution 1000 µl 200 µl 500 µl

Avidin-FITC	10 µl	2 µl	5 µl

- Antibody 2
  - Prepare fresh
  - Centrifuge antibody for 5 minutes at 13,000rpm at 4oC
  - Detection solution 1000 µl 500 µl 600 µl
    Anti-Avidin 10 µl 5 µl 6 µl
    Anti-Dig 4 µl 2 µl 2.4 µl

- Antibody 3
  - Prepare fresh
  - Centrifuge antibody for 5 minutes at 13,000rpm at 4oC
  - Detection solution 1000 µl 500 µl 600 µl
    Avidin-FITC 10 µl 5 µl 6 µl
    Anti-Mouse-TRITC 20 µl 10 µl 12 µl

- Blocking Solution
  - Prepare fresh
  - 0.09g BSA in 3 ml 4x SSC/Tween 20 solution

- Detection Solution
  - Blocking Solution : 4x SSC/Tween 20 solution ::1:2

- 20x SSC
  - 20 X SSC: siehe Maniatis oder Laborprotokoll

- 4x SSC / Tween 20
  - 140 ml 20x SSC + 560ml Distilled water
  - Place on a magnetic stirrer and add 1.4ml Tween 20
  - Warm to 45°C

- 0.2x SSC
  - 1ml 20x SSC + 99ml Distilled water
  - Warm to 53°C
  - Warm this first before you start your experiment!!

Procedure:

- Remove the rubber cement and cover slip carefully and discard
- Incubate slide for 10mins in 2x SSC at 37°C In a shaking water bath
- Incubate slide 2 x 7 mins in 0.2 x SSC at 53°C in a shaking water bath
- Briefly equilibrate slides in 4x SSC / Tween 20
- Add 150µl of **blocking solution** on the coverslip, place the slide carefully over the coverslip. Place the slide and coverslip in a rack with the coverslip facing down, place the rack in a humidified metal container and incubate at 30°C for 30 minutes

- Carefully remove and discard the coverslip. Wash briefly in 4x SSC / Tween 20 (45°C)
- Incubate with 150µl of **antibody I** as before at 37oC for 30 minutes
- Carefully remove and discard the coverslip. Wash 3 x 5 mins in 4x SSC / Tween 20 (45°C)
- Incubate with 150µl of **antibody II** as before at 37oC for 45 minutes
- Carefully remove and discard the coverslip. Wash 3 x 5 mins in 4x SSC / Tween 20 (45°C)
- Incubate with 150µl of **antibody III** as before at 37oC for 30 minutes
- Carefully remove and discard the coverslip. Wash 3 x 5 mins in 4x SSC / Tween 20 (45°C)
- Incubate for 2-10 minutes in DAPI solution in the dark
- Wash 5x in distilled water. Air dry in dark
- Add two drops of anti-fade on the slide and place a 24 x 50 mm coverslip over it
- Store in dark at 4°C