MONOCHROMOSOMAL TRANSFER

Materials

Donor cell line (A9 or K1-9, mouse background, carrying specific human chromosome) on four 150mm cell culture plates, in growth medium (DMEM + 10% FCS and 400U/mL Hygromycin B for A9 cells or Ham's F12 + 10%FCS and 500U/mL Hygromycin B for K1-9 cells)

Recipient cell line (fibroblasts (E7 and hTert) or myoblasts (E6/E7 and hTert) on one 100mm plate in growth medium

Bullets for enucleation: custom made by jigsaw from 150mm TC plates to fit reusable centrifuge tubes in pairs.

Centrifuge tubes: Nalgene 50mL, round-bottom, polycarbonate (3117-0500).

Filters: Whatman Cyclopore, 25mm 5µM (70612513), Whatman Nuclepore 25mm 8µM (110614)

Filter holders and Gaskets: Millipore Swinnex 25mm filter holder and gasket (SX0002500, SX0002501)

Hygromycin B (Caibiochem 400051).

Colchicine (Demecolcine, Sigma D1925). 10µg/mL sterile solution. Store 4°C.

Con A (Concanavalin A, Sigma C2631). Powder, store desiccated at 4°C.

WSC (1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metno-p-toluenesulfonate, Sigma C1011). Powder, store desiccated at -20°C.

Cytochalasin B (Sigma C6762). Powder, store protected from light at 4°C. Resuspend at 10 mg/mL in DMSO. Store at -20°C.

Phytohemagglutinin P (PHA-P) (Difco 3110-56-4). Store powder at 4°C. This powder is 50% PHA-P, i.e. 100 mg of powder contains 50 mg of PHA-P. Working solution of 4mg/10mL serum-free medium, filter sterilize. Store aliquoted at -20°C.

Polyethylene glycol (PEG) 1500 (Roche 783641). Store at 4°C.

DMSO (Sigma D2650)

Method

At least a week before transfer

1. Thaw the A9 chromosome specific donor cell line about a 7-10 days before the transfer. For one passage grow without selection, then purge for three passages with 800U/ml hygromycin B (1000U/mL for KI-9 cells). After that the cells are grown continuously in 400U/mL hygromycin B (500U/mL hygromycin B for K1-9). You need a near-confluent 150mm plate three days before the transfer.

2. Thaw the recipient cell line (E7+hTert fibroblasts or E6/E7+hTert myoblasts). You need a near-confluent 100mm plate on the day of the transfer.

Three days before transfer

Split a near-confluent 150mm plate of A9 and/or K1-9 cells into four 150mm plates. You need three plates for the transfer and one plate to continue growin in case the cells are needed for another transfer or for freezing. If to be frozen, remove selection for three passages before freezing.

Two days before transfer

Add 150µL colchicine per 25mL media per plate of donor cells and incubate for 48 hours. Microenucleation can be monitored by observation under phase contrast.

One day before transfer

Bullet preparation (can also be done the same day as the transfer): 5 bullets per plate of donor cells.

1. Sterilization: Sterilize bullets in 70% EtOH overnight, or for a minimum of 2 hours (after transfer store the bullets in 70% EtOH in 50 ml Falcon tubes). Dry them in the hood by resting 5 bullets inclined on the edge of a 150mm plate.

2. Coat bullets with ConA/WSC: Prepare the following reagents just before use:

   Con A: 15mg/mL in 0.9% NaCl. Prepare 20mL for 15 bullets: 300mg ConA ad 20mL 0.9% NaCl. Dissolve in 37°C water bath for 30 minutes (note: it is possible that not all will go into solution). Filter through 0.45µm filter, then 0.2µm filter.

   While ConA is going into solution prepare:

   WSC: 75mg/mL in 0.9% NaCl. Prepare 20mL: 1500mg WSC ad 20mL 0.9% NaCl. Filter sterilize.

3. Lay bullets flat in plates. Pipette WSC solution over the surface of each bullet. Rock back and forth to ensure complete coverage. Aspirate. Repeat with ConA and aspirate. Leave covered in hood for 1-2 hours.

4. Wash the bullets twice with 20mL sterile PBS, rocking dish back and forth to wash. Coated bullets can be stored in PBS overnight at 4°C or used right away.

Prepare media

DMEM (or Ham's F-12) + 10% FCS (about 200 mL)

DMEM (or Ham's F-12) (500 - 700mL).

Enucleation medium: Note: prepare in a sterile glass bottle. DMEM + 10µg/mL cytochalasin B (stock is 10mg/mL so add 300µL to 300mL of serum-free medium). Cytochalasin B can be reused four times, filter sterilize after every use. Store in dark at 4°C.

Autoclave

1. Assemble Millipore Swinnex filters, holders and gaskets. Prepare at least three 5µm and three 8µm filters. The filter is placed shiny side up.
2. Nalgene polycarbonate centrifuge tubes, 1 for every 2 bullets.

Day of transfer

Prewarm centrifuge and Sorvall SS-34 rotor to 28-34°C (definitely not more than 37°C), by spinning at full speed for one hour. Set temperature range from 30-34°C.

Warm in 37°C bath: PBS, Trypsin-EDTA 1x, DMEM (or Ham's F-12) + l0% FCS, DMEM (or Ham's F-12) and myoblast medium if needed.

Microcell preparation

Trypsinize the donor cells and pool the three plates in 30mL medium w/FCS. Pellet the cells in 50mL Falcon tube (1100 rpm x 5-10 minutes).

Resuspend the cells thoroughly in 15mL medium w/FCS.

Aspirate the majority of PBS from the bullets, leaving some on the bullets. Arrange the bullets on the plate so that the edges of adjacent bullets are not touching. Aspirate the PBS from a bullet and overlay it with 1mL resuspended cells.
Let the cells attach for approximately 15 minutes in the hood. Monitor under microscope. Excess cells will not attach.

Flood each dish with 36mL medium w/FCS and incubate until the cells have flattened. Monitor under microscope every 30 minutes. This will take 1.5-3 hours.

When cells have flattened fill a sterilized centrifuge tube with 34mL prewarmed enucleation medium. Place 2 bullets into each tube, back to back. Place the tubes into the EtOH sprayed, prewarmed centrifuge rotor in the hood. Close the rotor and spin at 15000 rpm for 30 minutes at 30-34°C.

After spinning, move bullets back to dish with medium w/FCS and check the enucleation under the microscope. The cells should appear streaked with cytoplasmic remnants. Be careful when removing the bullets so as not to disturb the nuclear pellet in the tube. Place used bullets in a beaker of distilled water for later cleaning. Decant the Cytochalasin B medium into a beaker and filter sterilely into its original bottle if it is to be reused.

Resuspend each pellet thoroughly in 5mL serum free medium. Pool suspensions.

Using a 20mL syringe, filter the suspension gently 2x through an 8µm filter, then 3x through a 5µm filter. Inspect while filtering, paying attention to any leaks from the filter edges. NOTE: The filtration step is extremely important; if mouse cells remain in the filtrate they will end up fusing into recipient cells and form fast growing colonies.

Pellet the filtered microcells in countertop centrifuge at 3500 rpm (2800g) for 10 minutes.

Fusion

Prepare PEG: Add 0.4mL DMSO to the 4ml bottle of PEG. Mix and warm at 37°C.

Prepare PHA: Thaw and prewarm 1.5mL per transfer.

Resuspend the microcells in 1.5mL serum-free medium. Pipette cells up and down vigorously to ensure complete separation of the micronuclei.

Rinse recipient cells 3x with 5mL serum-free medium. Aspirate.

Combine 1.5mL PHA with 1.5mL microcells and mix well. Pour onto recipient cells. Incubate at 37°C for at least 25-30 minutes to allow agglutination of the microcells to the recipient cells. Assess the agglutination under microscope. Usually some cells remain floating.

Fuse one recipient plate at a time. Gently aspirate the PHA/micronuclei mixture being careful not to disturb the attached micronuclei. NOTE: Fusion with PEG is very time dependent. Be ready to time the fusion to be as close as 60 seconds as possible! Add PEG and start timing. Rock plate gently to ensure complete exposure of the cells to the fusogen. Prepare to remove PEG 10 seconds before the endpoint of the exposure. Aspirate the PEG and, as quickly as possible, add 10mL DMEM + 10% DMSO. Aspirate and repeat. Rinse 1x with DMEM. Rinse 1x with DMEM +10% FCS. Avoid spraying the medium directly on the cells. Rock the plate for 1 minute. Change the medium and repeat the washing 2x 1 minute.

Add growth medium (DMEM + 10% FCS for fibroblasts, myoblast medium for myoblasts) + pen/strep or gentamicin, and incubate overnight.

Day 1 post-fusion

Split the recipient plate 1:2, using the original plate and one new 100mm plate.

48-72 hours post-fusion

Change to selection medium: growth medium + G418 400µg/mL and Hygromycin B 100U/mL. (G418 is not absolutely necessary if filtration of micronuclei is complete).

1-4 weeks post-fusion

Usually 7-10 days after the start of selection clones can be recognized and they are ready to be picked within 2-4 weeks. From a typical transfer with 3x 150mm donor plates and 1x 100mm recipient plate expect 1-40 clones.

Picking the clones

Mark the visible clones on the bottom of the plate by looking at them towards a light source. Wash the plate 2x with 15mL PBS. Add 10mL PBS to the plate. Under a dissection microscope focus on a clone. Adjust pipettor to 20µL volume and, while observing under the microscope, trace the outline of the clone, gently lift it as a sheet from the plate and suck it into the pipette. Transfer the clone into a well of a 24-well plate prefilled with selection media. Try to get all the cells of the clone. When all the clones are picked from the plate add growth media to the plate and grow up a plate to be called "pooled". Optionally, the next day the 24-well plate's cell clumps can be redistributed by trypsinization in the wells.