

Genetic Transformation Via Microparticle Bombardment

For bombardment in the tissue culture flow hood follow aseptic protocols at all times.

1. Sterilization of Tungsten Microparticles (Sanford et al. 1992):

- Weigh out microparticles (typically 20-40mg) into 1.5ml microcentrifuge tube.
- Add 1ml fresh 70% EtOH, vortex for 5 minutes, then incubate for 15 minutes without shaking.
- Centrifuge max. speed (13,200rpm) for 30s to pellet microparticles.
- Remove EtOH.
- Repeat the following three times:
 - Add 1ml sterile dH₂O.
 - Vortex 1 minute.
 - Incubate without shaking for 1 minute.
 - Centrifuge max. speed for 30s to pellet microparticles. Remove dH₂O.
- Add sterile 50% glycerol to a concentration of 60mg microparticles per ml (assume no loss during sterilization) and resuspend pellet by flicking/vortexing.
- Sterile microparticle stocks can be kept at room temperature for 2 weeks.

2. Preparation for Bombardment:

- Spray gun chamber with 70% EtOH and wipe down.
- Soak the following in 100% EtOH for at least 20 minutes in flow hood, under UV.
 - Loading Stage and cap (in spares box)
 - Firing cylinder (unscrew/remove from gun)
 - Two pairs large/bayonet forceps (Sigma)
 - One pair watchmakers'/needle-nosed forceps

NB/ The stage, firing cylinder and forceps can be sterilised together in a square box of EtOH.

- Metal dies/cartridges. Add EtOH to the storage box.
- Circular wire mesh discs (one per four bombardments). Sterilize with metal dies.
- Large orange plastic macrocarrier discs. Add EtOH to storage box.
- Rupture discs- choice will depend on firing pressure required. **Leave dry in their box inside cabinet, dip each in Propan-2-ol before use as needed.**
- Also place in cabinet before sterilizing:
 - Three 9cm petri dishes with lids (for holding bombardment cartridges etc.)
 - One 3cm petri dish containing propan-2-ol (to sterilize rupture discs).
 - Torque wrench (in spares box).

While this is in progress, coat microparticles with DNA.

3. Coating Microparticles with DNA (modified from Sanford et al. 1992)

- Minimum plasmid concentration of 1ug/ul is required.

- Vortex sterile microparticle stock (see above) 5 minutes to resuspend and break up clumps. **Need to keep microparticles vortexing during this procedure.**

NB/ Can use filter tips in this procedure, but does not appear to be essential.

- Aliquot 50ul microparticle into 1.5ml microcentrifuge tube, one per plasmid and No DNA control. Each aliquot contains sufficient microparticles for 3-5 bombardments (see below). Return tubes to vortex.
- Add the following to each aliquot, in order:
 - 5ul DNA or dH₂O (No DNA control)
 - 50ul filter-sterilized 2.5M CaCl₂
 - 5ul filter-sterilized 0.1M Spermidine
- Continue vortexing tubes for 3 minutes.
- Incubate tubes without shaking for 1 minute. Plasmid-coated microparticles may start to settle out.
- Centrifuge max. speed 30s to pellet microparticles.
- Remove supernatant, taking care not to disturb the pellet.
- Add 140ul 70% EtOH, then remove, taking care not to disturb the pellet.
- Add 140ul 100% EtOH, then remove, taking care not to disturb the pellet.
- Add 50ul 100% EtOH.

Switch UV off and cabinet back on. Remove components in 2. from EtOH and allow to dry completely inside the cabinet.

4. Biolistics gun set-up:

- Attach vacuum hose to outlet at rear of gun.
- Close and tighten vacuum trap.
- Prepare helium source- open main valve on cylinder, and adjust secondary valve to control He2 pressure. Close valve until pressure reading is >1000 psi.
- Remove transformer from flow hood and connect to power socket. Switch gun on (left hand switch on top left of gun front)
- Switch vacuum pump on.

5. Sample prep/loading:

- Using bayonet forceps, insert an orange macrocarrier disc into each cartridge, and press down around the edges until it fits snugly.
- Resuspend microparticles by gently flicking the eppendorf tube. Immediately withdraw a 10ul sample with a pipette and spread evenly across the centre of the orange disc, over the hole in the cartridge. Leave to dry.

NB/ If pellet is clumpy and difficult to resuspend, divide whole aliquot over 3 bombardments. If pellet resuspends quickly and evenly, up to 5 bombardments can be prepared from one aliquot.

- Using watchmakers forceps, soak a rupture disc in propan-2-ol, allow to dry and then place in the firing cylinder, fitting snugly and centrally over the hole.
- Screw firing cylinder back into position inside gun. Tighten using the chuck.
- Place a wire mesh disk over the hole of the firing stage. Ensure that fit is snug and central.

- Place loaded bombardment cartridge above the mesh, flipping over so that the orange disc is on the underside. Screw the stage cap tightly on to hold it in place.
- Place loading stage into the gun chamber above the sample stage, ensuring that it almost abuts the firing cylinder. The loaded microparticles are now on the under-surface of the disk, facing downward.
- Distance to the sample stage can be varied by moving the sample stage to different slots.

6. Particle bombardment:

- Place tissue for bombardment (e.g. agar plate with callus present) on the sample stage, such that the tissue is central, directly beneath the firing position. Close the chamber door.

NB/ Ensure that lid of the plate has been removed.

- Ensure that vacuum pump is on and running. Evacuate chamber (central switch, three positions). Click switch up to create vacuum. Vacuum pressure dial is left of the chamber. When chamber vacuum reaches 28 psi, click switch fully down to the 'hold' position. Gun is now ready to fire.
- Press and hold the rightmost switch up to build helium pressure. Gauge on top of the gun will rise. Keep the pressure rising until the rupture disc bursts (i.e. with an audible pop), then release the switch.
- Move the central switch to the middle 'off' position to release the vacuum.
- When the vacuum is gone, open the chamber, remove the bombarded plate, cover and seal with parafilm. Tissue has now been bombarded.

7. Reloading biolistics gun:

- Remove loading stage from the gun.
- Unscrew the cap and, using forceps, remove the spent cartridge. Orange disc can be discarded, retain cartridge separately. Reseat the wire mesh centrally or remove if bombardment is complete, a new plasmid is to be bombarded or mesh is severely dented (can usually last 4 bombardments).
- If a new plasmid is to be bombarded, wash loading stage in EtOH and allow to dry. Insert new wire mesh.
- Insert fresh cartridge into loading stage and screw on the cap.
- Unscrew the firing cylinder from the gun.
- Using forceps, remove the spent rupture disc and discard. For another bombardment, replace with a fresh rupture disc, making sure that it is seated centrally.
- Screw the firing cylinder into position, tightening with the chuck.
- Replace loading stage in the chamber.

Biolistic gun shutdown:

- Remove loading stage from the cabinet (it doesn't live there), spray with 70% EtOH and wipe clean to remove contaminating microparticles. Store in spares box.
- Turn off power.

- Switch off vacuum pump, loosen the trap and disconnect the hose.
- On the helium cylinder, open the pressure valve and shut the main valve.
- Unplug transformer from the wall socket and return it to the flow hood.
- Clear and switch off flow hood. Empty all used EtOH/propan-2-ol and discard.
- Wash used bombardment cartridges in clean 100% EtOH then wipe each dry individually to remove contaminating microparticles. DO NOT PLACE DIRTY CARTRIDGES IN THE 'CLEAN' STORAGE BOX.
- Used orange macrocarriers, rupture discs and wire meshes are discarded. Return unused ones to storage if clean.