SEED STERILISATION PROTOCOL
(This was placed on the electronic Arabidopsis bulletin board; July 1992.)
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* This procedure is for sterilising small numbers of seed (up to about 200) from large numbers of independent lines. We have used it for setting up plate screens of about 4,000 lines from the DuPont T-DNA collection. It may also be useful for others who are interested in plating large numbers of lines or families.

* In brief, seeds are transferred to 96-well ELISA plates, sterilised using bleach, rinsed with sterile water, and resuspended in 0.15% agar for plating. All pipetting steps are done with a Beckman Biomek 1000 Automated Laboratory Workstation. (Alternatively, this process should be adaptable for use with an ELISA plate washer or a hand-held octapette and a support to hold the washer or pipette at the appropriate height above the plate.) Using this procedure, it is possible to transfer 96 lines of seeds from tubes and sterilise them in about 15 minutes of hand work and about 25 minutes of robot work. Seed sterility using this procedure is at least as good as when done by hand. We have observed a low level (~1%) of cross-contamination of seeds. Minor changes in the protocol could probably reduce this cross-contamination, but we have not attempted them since the contamination has been so low that it does not affect our work. More details can be obtained from Tim Caspar. If you would like a copy of the Biomek program code, send him a PC-formatted 3.5 inch floppy disk.

1. OVERVIEW

* Seeds are removed from vials using dampened toothpicks and are washed into the wells of a 96-well ELISA plate in 50% isopropanol. Water is added manually to dilute the isopropanol. The Biomek robot then draws off the dilute isopropanol, adds a bleach/Tween solution, then draws this off and washes the seeds 4 times in sterile water. Finally the seeds are resuspended in dilute, sterile agar for manual plating. A small amount of cross-contamination of the wells should be expected. Some of the seeds (10-40%) are lost during the procedure (the number is roughly proportional to the initial number of seeds in each well).

2. SAFETY PRECAUTION

* The Biomek robot arm is extremely powerful and has no sensors for objects which may block its path. Never place your hands or other objects in the path of the robot arm while the indicator light on the top of the robot arm is illuminated. For an emergency stop of the robot, press the red emergency stop button at the front, middle of the robot unit. For routine stopping, type a "/S" on the computer keyboard then a "/A" to abort the run or a "/C" to continue it.
3. SEED TRANSFER TO 96 WELL PLATES
   (i) Place 150 ul of 50% isopropanol (see note 1) into each well of a 96-well flat bottom ELISA plate (we use Nunc-Micro Well plates, VWR #62409-068).
   (ii) Wet a toothpick in a dish of 50% isopropanol, dip into a seed vial and transfer the seeds which stick to the toothpick to a well by dipping into the 150 ul of isopropanol in the well (see notes 2 and 3).
   (iii) After seeds are distributed to all wells and within 20 minutes, add 100 ul water to each well to dilute the isopropanol. As soon as possible after the seeds are distributed, begin the sterilisation treatment in order to minimize their exposure to the isopropanol.

4. BIOMEK STERILISATION PROTOCOL (see note 4)
   (i) Remove 190 ul from each well and transfer to a waste reservoir (see note 5)
   (ii) Add 200 ul bleach/Tween solution (50% bleach, 0.02% Tween)
   (iii) Add 50 ul bleach/tween solution (see note 6)
   (iv) Remove 200 ul to waste
   (v) Add 150 ul sterile water
   (vi) Remove 200 ul to waste
   (vii) Wash subroutine, repeated 3 times:
      (a) add 200 ul sterile water
      (b) remove 200 ul to waste
   (viii) Rinse pipette tips in water, then agar
   (ix) Add 75 ul sterile 0.15% agar (see note 7)

5. NOTES
   (i) The isopropanol in the 96-well plate is critically important. It wets the seeds so they sink in the sterilising and rinse solutions and also reduces the swelling of the seed mucilage so they clump together less. The seeds are not injured by exposure to isopropanol for the indicated times.
   (ii) During seed transfer to the plates, the seeds will stick to the moistened area of the toothpick. By varying the depth the toothpicks are moistened or the type of toothpick used, the number of seeds transferred can be adjusted.
   (iii) To reduce the chances of cross-contamination during transfer of seeds to the 96-well plates, a Plexiglass mask is used which covers the plate and has a single hole in it which is lined up over the target well. In addition, the plate and the mask are placed on an anti-static mat (available from office supply stores) to reduce the static which causes the seeds to fly around.
   (iv) The entire Biomek unit is placed inside a laminar flow hood. Reservoirs for solutions are sterilised by immersion in 70% ethanol.
   (v) One set of tips is used for the pipetting series for an entire plate. To minimize cross-contamination between wells caused by seeds sticking to the tips, the pipette tips are rinsed with 100% ethanol after each removal of solution from the plate in steps (i), (iv), (vi), and (vii)b. For applications which require less cross-contamination, the tips can be
washed more vigorously or changed more frequently.

(vi) Pipetting steps are determined by the capacity of the octapette (200 ul), the total volume of the wells in the plate, the height above the bottom of the well that the pipette must maintain to minimize loss of the seeds, and the volumes of the reservoirs on the Biomek.

(vii) Resuspension of the seeds in 0.15% agar aids in plating since they remain suspended by the high viscosity of this liquid. For some applications this is not useful and step (ix) can be omitted.