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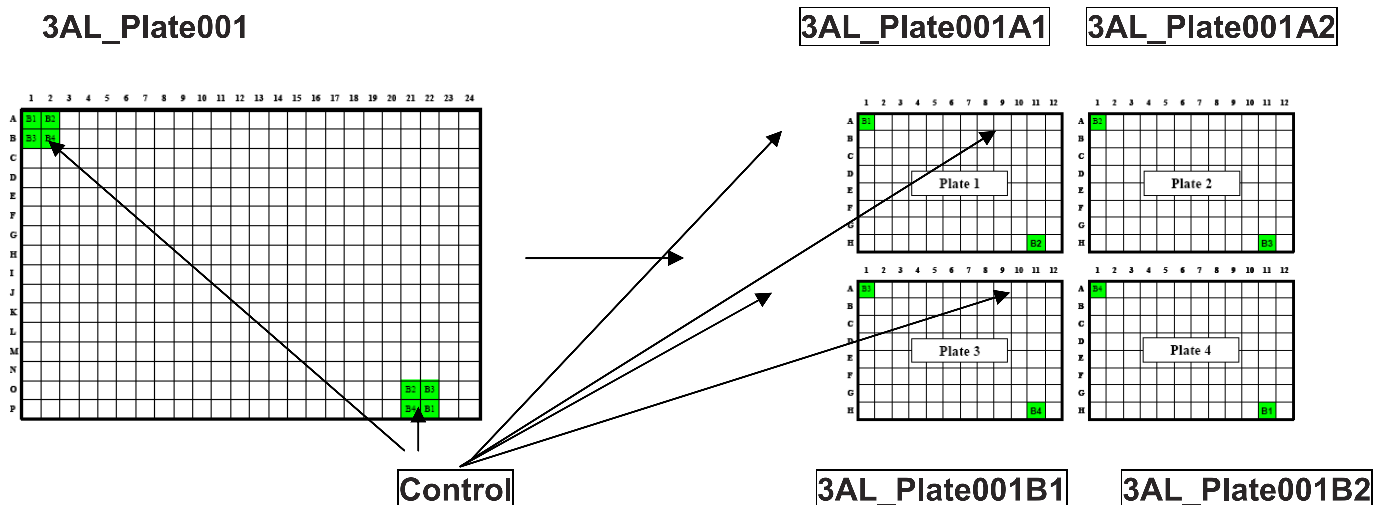
Growing bacterial cultures

1. **Fill each well of a 96 well block with 1.2 ml of 2x YT containing the appropriate selective antibiotic (Chloramphenicol - cM).**
2. **Remove the 384 well plate from the -80C freezer and completely thaw it for inoculation.**
3. **Inoculate the four 96 well blocks with target BAC clones from one 384 well plate.**
   Note:
   a) Flame the bokel twice (ethanol (95%) to sterilize it and then cool it before starting inoculations
   b) Sterilize the bokel between inoculation of each plate by dipping the bokel in ethanol (95%) and flaming and cooling it.
   c) and inculcate twice and shake the bokel in the wells, so that you ensure a proper transfer of inoculums.



4. **Add control clone in each 96 well deep well blocks at positions A1 and H11 (there are 8 control clones for each384 well plates, therefore, two controls clones in each 96 well block.**

5. **Seal the 96 ell deep well blocks with airpore tapes. Aeration of the blocks can be achieved by using AirPore Tape Sheets.**

6. **Fix the blocks in the holder tightly and then incubate the cultures for 18-20 h at 37°C with shaking at 285 rpm.**

Harvest the bacterial cells

1. **Harvest the bacterial cells in the block by centrifugation for 10 min at 2500 rpm in a centrifuge with a rotor for 96-well micro plates. The block should be covered with adhesive tape during centrifugation. Remove medium by inverting the block.**

2. **To remove the medium, peel off the tape and quickly invert the block over a waste container. Tap the inverted block firmly on a paper towel to remove any remaining droplets of medium.**

3. **Freeze the blocks at -20C till further use.**

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R.E.A.L. Prep 96 BAC Protocol

1. Before you start Note: a) Ensure that RNase A has been added to Buffer R1 and stored at 4C always.
b) Buffer R2 should be checked before use for SDS precipitation caused by low storage temperatures.
c) Cool Buffer R3 at 4C before start but store it at room temperature.
d) Use 3 reservoirs and label them as R1 R2 &R3

2. Re-suspend each bacterial pellet in 0.3 ml Buffer R1. Use an 8-channel pipet (fill 1200 ul per channel use stepper to dispense 300ul in 3 columns and dispense the rest back into reservoir). Tape the block and mix by vortexing.
Note: ***The pelleted cells should be resuspended completely, leaving no cell clumps.***

3. Add 0.3 ml Buffer R2 to each well (fill 1200 ul per channel use stepper to dispense 300ul in 3 columns and dispense the rest back into reservoir), seal the block with new tape, *mix gently but thoroughly by inverting 10 times*, and incubate at room temperature for 5 min.
Note: *a) Do not vortex the lysates at this stage, as this will cause shearing of the bacterial genomic DNA.*
b) Do not incubate for more than 5 min. Additional incubation may result in increased levels of open circular plasmid. At the end of the incubation, the lysate should appear viscous and free of bacterial cell clumps.
c) Close the Buffer R2 bottle immediately after use to avoid acidification of Buffer R2 from CO2 in the air.

4. Add 0.3 ml Buffer R3 to each well (in same way as above), seal the block with new tape, mix immediately by inverting 20 times, and incubate on ice for 10 min.
Note: *Thorough mixing ensures uniform precipitation of potassium dodecyl sulfate (KDS) and cellular debris.*

5. Centrifuge at 4500 rpm for 4 mins to settle the debris (Makes the lysate clear).

6. Clearing lysates with QIAfilter 96 : Place a QIAfilter 96 plate (yellow) in the top plate of the QIAvac 96 manifold. Place a new square-well block in the base and reassemble the manifold.
Note: *Ensure that the numbering and orientation of the plate and new square-well block are in accordance with the lysis block, and that the wells of the block are properly aligned with the outlet nozzles of the QIAfilter 96 plate.*

7. Transfer the lysates from step 5 to the wells of the QIAfilter 96 plate.
Note: *Use a multichannel pipet with a sufficiently large fill volume (>1 ml per channel). Try stick the debris with to the walls of the block.*

8. Apply vacuum (–200 to –300 mbar) until the lysates are completely transferred to the square-well block in the QIAvac base.
Note: *Usually 1min of vacuum is enough if you lysate is clear. Do not apply vacuum for very long durations. If 2or 4 wells blocked or slow, use a syringe to manually push the lysate down gently.*

9. Isopropanol precipitation: Take the square-well block containing the cleared lysates from the vacuum manifold. Add 0.7 volumes of room-temperature isopropanol to each well (0.63 ml for 0.9 ml of lysate), tape the block, and mix immediately by inverting 3 times.
Note: *a) Add isoprpanol only when you have your centrifuge ready or keep them on ice wait till centrifuge is available. Isopropanol tends to precipitate salts with time*
b) When preparing multiple sets of 96 samples, add isopropanol to one block, tape, and mix by inversion before proceeding on to the next block. This will minimize separation of the tape from the block before the samples are mixed.

10. Centrifuge the blocks at 6000 x *g* for 35 min at room temperature to pellet the DNA. Remove the supernatants by quickly inverting the block over a waste container, then tapping it firmly, upside down, onto a paper towel.

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11. Wash each DNA pellet with 0.5 ml of 70% ethanol. Let the samples incubate in 70% ethanol for 15 mins before centrifugation. Centrifuge the block (in the same orientation as before) at 6000 x *g* for 15 min to reconcentrate the pellets. Remove the wash solutions by inverting the block, then tapping it firmly, upside down, onto a paper towel.

12. Air dry the pellets for 1hour or until no ethanol remains.
Note: *Ensure that no alcohol droplets are visible after air drying, but do not over dry the DNA pellets as this will make them difficult to dissolve*.