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Protocol for MC1061/p3 strain and supF plasmids

From: Seed Lab
Additional information can be found at:
http://genetics.mgh.harvard.edu/seedweb/requests/request.htm

The host strain is MC1061/p3. The genotype of MC1061 is: hsdR, araD139, Δara-leu)7697, ΔlacX74, galU, galK, mcrA, mcrB, rpsL (str-r). The p3 plasmid is RP1 amp(am), tet(am), tra. We do not recommend maintaining strains bearing p3 on kan plates, because they tend to become mucoid and/or lose transformation efficiency.

The DNA concentration is 0.1 mg/ml. For the highest yields, we recommend preparing plasmid DNA from freshly transformed bacteria. Following are protocols for (1) high efficiency transformation of *E. coli* MC1061 and related bacteria; (2) propagation of suppressor plasmids; (3) isolation of plasmid DNA; and (4) DEAE Dextran transfection.

(1) High efficiency transformation in *E. coli*

This protocol is a larger scale variant of one due to Mike Scott, Department of Neurology, UCSF. Streak out the desired strain on an LB plate. The next day, innoculate a single colony into 20ml TYM broth (recipes below) in a 250ml flask. Grow the cells to midlog phase (OD_{600} ~0.2-0.8), pour into 2l flask containing 100ml TYM, continue vigorous agitation until cells grow to 0.5-0.9 OD, then dilute again to 500ml in the same vessel. When cells grow to OD_{600} 0.6, put flask in ice-water, and shake gently to assure rapid cooling. When culture is cool, spin 4.2k rpm, (5000xg) 15 minutes (Beckman J6 centrifuge or equivalent). Pour off supernatant, resuspend pellet in ~100ml cold TfB I (below) by gentle shaking on ice. Respin in same bottle 4.2k rpm, 8

minutes (J6). Pour off supernatant, resuspend pellet in 20ml cold TfB II by gentle shaking on ice. Aliquot 0.1 to 0.5 ml aliquots in prechilled microfuge tubes, freeze in liquid nitrogen, and store at -70º. For transformation, remove an aliquot, thaw at room temperature until just melting, place on ice, add DNA, let sit on ice 15-30 minutes, incubate at 37º for 5 minutes (6 minutes for 0.5ml aliquots), dilute 1:10 in LB and grow for 90 minutes before plating or applying antibiotic selection. Alternatively, the heat-pulsed transformation mix can be plated directly on antibiotic plates onto which a thin (4-5ml) layer of antibiotic-free LB agar has been poured just before plating.

Media and Buffers: TYM: 2% Bacto-Tryptone, 0.5% Yeast Extract, 0.1M NaCl, 10mM $MgSO_4$ (can be added before autoclaving). TfB I: 30mM KOAc, 50mM $MnCl_2$ 100mM KCl, 10mM $CaCl_2$, 15% (v/v) glycerol. TfB II: 10mM Na-MOPS, pH 7.0, 75 mM $CaCl_2$, 10mM KCl, 15\% glycerol.

(2) Propagation of Suppressor tRNA Plasmids

SupF plasmids can be selected in nonsuppressing hosts containing a second plasmid, p3, which contains amber mutated ampicillin and tetracycline drug resistance elements (Seed, 1983). The p3 plasmid is derived from RP1, is 57kb in length, and is a stably maintained, single copy episome. The ampicillin resistance of this plasmid reverts at a high rate, so that amp^r plasmids usually cannot be used in p3-containing strains. Selection for tet resistance alone is almost as good as selection for amp+tet resistance. However spontaneous appearance of chromosomal suppressor tRNA mutations presents an unavoidable background (frequency about 10^{-9}) in this system. Colonies arising from spontaneous suppressor mutations are usually bigger than colonies arising from plasmid transformation. We typically select for suppressor plasmids in LB medium

containing amp at 12.5 μg/ml and tet at 7.5 μg/ml. For large plasmid preps we use M9 casamino acids medium containing glycerol (0.8%) as a carbon source, and grow the bacteria to saturation.

(3) Isolation of Plasmid DNA

The following prep is for plasmid from 1 liter of saturated cells. Spin down cells in 1 liter J6 bottles, 4.2k rpm, 25 minutes. Resuspend in 40 ml 10mM EDTA pH 8 (Thump on soft surface). Add 80 ml 0.2M NaOH, 1% SDS, swirl until clearish, viscous. Add 40 ml 5M KOAc, pH4.7 (2.5M KOAc, 2.5M HOAc), shake semivigorously (till lumps are 2-3 mm in size). Spin (same bottle) 4.2k rpm, 5'. Pour supernatant through cheesecloth into 250 ml bottle. Fill bottle with isopropyl alcohol. Spin J6, 4.2k rpm, 5'. Drain bottle, rinse with 70% EtOH (gently--pellet can fragment). Invert bottle, remove traces of EtOH with Kimwipe. Resuspend in 3.5 ml Tris base/EDTA 20mM/10mM. Add 3.75 ml of resuspended pellet to 4.5g CsCl. Add 0.75 ml 10mg/ml ethidium bromide, mix. Fill VTi80 tubes with solution. Run ≥ 2.5 h, 80k rpm. Extract bands by visible light with 1 ml syringe and 20 gauge or lower needle. Cut top off tube with scissors, insert the needle upwards into the tube at an angle of about 30 degrees with respect to the tube, (i.e. as shallowly as possible) at a position about 3mm beneath the band, with the bevel of the needle up. After the band is removed, pour tube contents into bleach). Deposit extracted bands in 13 ml Sarstedt tube. Fill tube to top with n-butanol saturated with 1M NaCl, extract. If truly enormous amount of DNA, reextract. Aspirate butanol into trap containing 5M NaOH (to destroy ethidium). Add about equal volume 1M ammonium acetate to DNA (squirt bottle). Add about 2 volumes 95% ethanol (squirt bottle). Spin 10k rpm, 5' J2-21. Rinse pellet carefully with 70% ethanol. Dry with swab, or lyophilizer.

(4) DEAE Dextran transfection

We typically split the cells to be transfected the night before, to give 25-50% confluence. One simple way to achieve this is to resuspend the trypsinized cells from a confluent 10cm dish in 10ml of medium, and distribute 6 drops from a 10ml pipet into each 6cm dish to be transfected. The higher the degree of confluence, the greater the ability of the cells to withstand the transfection conditions; however expression following transfection at high density is typically inferior to that at lower density.

Cells are transfected in DME or IMDM containing 10% heat-inactivated NuSerum (Collaborative Research). NuSerum allows the cells to withstand the transfection conditions for greater lengths of time, resulting in improved net expression. If Calf or Fetal Bovine Serum is used, a thick precipitate forms in a substantial fraction of transfections, which results in severely eroded cell viability and expression. Presumably the lower protein concentration in the NuSerum obviates this.

If the cells were split into DME or IMDM/NuSerum, the medium need not be removed for the transfection. Usually 1/5 of a miniprep from 1.5ml of culture is used per 6 cm dish in a volume of 1.5-2 ml of medium. Chloroquine phosphate and DEAE Dextran are added to 100 micromolar and 400 microgram/ml final concentrations respectively.

After 4 hours at 37 degrees the DEAE-containing medium is removed by aspiration, and 2 ml of 10% DMSO in PBS added. After two minutes or longer at room temperature (timing is not important), the PBS/DMSO is removed, fresh medium is added, and the cells left to incubate for however long is desired before assay (usually two to three days).

It is generally a good idea to check the cells after about 3 hours of exposure to the DEAE transfection mix, as their health can decline precipitously. This is particularly true of

chloroquine transfections, and it is usually better to shorten the transfection than to try to tough it out when the cells look bad.

Transfection with CsCl-purified DNA can be carried out in the same way (for example for the first round of library screening) but less DNA seems to be required. 20 ng/ml appears to be adequate and higher amounts don't hurt.

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