

## ***Dictyostelium* Cultivation, Transfection, Microscopy and Fractionation**

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**[Abstract]** The real time visualisation of fluorescently tagged proteins in live cells using ever more sophisticated microscopes has greatly increased our understanding of the dynamics of key proteins during fundamental physiological processes such as cell locomotion, chemotaxis, cell division and membrane trafficking. In addition the fractionation of cells and isolation of organelles or known compartments can often verify any subcellular localisation and the use of tagged proteins as bait for the immunoprecipitation of material from cell fractions can identify specific binding partners and multiprotein complexes thereby helping assign a function to the tagged protein. We have successfully applied these techniques to the *Dictyostelium discoideum* protein TSPOON that is part of an ancient heterohexameric membrane trafficking complex (Hirst et al., 2013). TSPOON is the product of the *tstD* gene in *Dictyostelium* and is not required for growth or the developmental cycle in this organism. *Dictyostelium* amoebae will exist in a vegetative phase where growth is sustained by the phagocytosis of bacteria. When this food source is spent they enter a developmental phase where the amoebae aggregate, via chemotaxis to extracellular waves of cAMP, into multicellular structures that subsequently form a fruiting body containing viable spores (Muller-Taubenberger et al., 2013). In the laboratory this cycle takes less than 24 h to complete and as a further aid to manipulation the requirement for a bacterial food source has been circumvented by the derivatisation of the wild type and isolation of axenic strains that can also grow in a nutrient rich broth. Axenic strains like Ax2 are the mainstay of laboratory research using *Dictyostelium* (Muller-Taubenberger et al., 2013). A description of *Dictyostelium* cell cultivation, the generation of cell lines that overexpress TSPOON-GFP and TSPOON null cells, and subsequent analysis (Muller-Taubenberger and Ishikawa-Ankerhold, 2013) is detailed below.

### **Materials and Reagents**

1. *Dictyostelium* Ax2 cells can be obtained from the *Dictyostelium* stock centre (<http://dictybase.org/StockCenter/StockCenter.html>)

*Note: Ax2 strain DBS0235521 is recommended due to the relatively few duplications within its genome (Bloomfield et al., 2008).*

2. HL5 with glucose (Formedium, catalog number: HLG0102)
3. LOFLO (Formedium, catalog number: LF1001)
4. Ultra-pure dH<sub>2</sub>O (e.g. Milli-Q system, EMD Millipore)

5.  $\text{KH}_2\text{PO}_4$  (Thermo Fisher Scientific, catalog number: P/4800/53)
6.  $\text{K}_2\text{HPO}_4$  (anhydrous) (Sigma-Aldrich, catalog number: 60353)
7.  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (VWR International, catalog number: 25165.260)
8.  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (VWR International, catalog number: 22317.260)
9. HEPES (Sigma-Aldrich, catalog number: H4034)
10. KCl (Thermo Fisher Scientific, catalog number: P/4280/53)
11. NaCl (VWR International, catalog number: 27810.295)
12.  $\text{NaHCO}_3$  (VWR International, catalog number: 27778.236)
13.  $\text{NaH}_2\text{PO}_4 \cdot 2\text{dH}_2\text{O}$  (Thermo Fisher Scientific, catalog number: 10723621)
14.  $\text{Na}_2\text{HPO}_4$  (anhydrous) (Thermo Fisher Scientific, catalog number: S4520/53)
15. KOH (VWR International, catalog number: 26668.263)
16. Trizma<sup>®</sup> base (Sigma-Aldrich, catalog number: T1503)
17. EDTA disodium salt dihydrate (VWR International, catalog number: 20302.260)
18. HCl (Thermo Fisher Scientific, catalog number: H/1200/PB08)
19. *Klebsiella aerogenes* (available from <http://dictybase.org/StockCenter/StockCenter.html>)
20. 50 mM L-ascorbic acid (Sigma-Aldrich, catalog number: A5960)
21. Ethanol (Sigma-Aldrich, catalog number: 3112)
22. Chloroform (VWR International, catalog number: 22711.290)
23. Plasmids pDT51, pDT61, pDT70 and pJH101 together with gene knockout vectors for *iplA*, *mscS*, *mclN*, *phdA* and *dagA* can be obtained from David Traynor  
*Note: Plasmid pLPBLP can be ordered through dictyBase ([http://www.dictybase.org/db/cgi-bin/dictyBase/SC/plasmid\\_details.pl?id=9](http://www.dictybase.org/db/cgi-bin/dictyBase/SC/plasmid_details.pl?id=9)).*
24. HiSpeed<sup>®</sup> Plasmid Midi Kit (QIAGEN, catalog number: 12643)
25. Hygromycin 100 mg/ml sterile solution (Invivogen HygroGold, catalog number: ant-hg-5)
26. Blastidicin S 100 mg/ml sterile solution (Invivogen, catalog number: ant-bl-1)
27. G418 (20 mg/ml solution) (Life Technologies Geneticin<sup>®</sup>, catalog number: 11811-031)
28. Quick-gDNA MiniPrep kit (Zymo Research, catalog number: D3025)
29. Expand 20 kb<sup>PLUS</sup> PCR system (dNTPack) (F. Hoffmann-La Roche, catalog number: 04743814001)
30. Oligonucleotide primers (custom synthesised by Sigma-Aldrich)
31. Freezing medium [sterilised horse serum containing 7.5% (v/v) DMSO]
32. Horse serum (Sigma-Aldrich, catalog number: H1270)
33. DMSO (Sigma-Aldrich, catalog number: D2650)
34. Master stock of 200 mM cAMP
35. 4x NuPAGE<sup>®</sup> LDS sample buffer (Life Technologies, Novex, catalog number: NP0008)
36. Protein A (PA) sepharose CL-4B beads (GE Healthcare, catalog number: 17-0780-01)
37. Acetone (Thermo Fisher Scientific, catalog number: A/0560/17)
38. Protease inhibitor cocktail (F. Hoffmann-La Roche, catalog number: 11873580001)

39. Precast NuPAGE 4-12% Bis-Tris gels (Life Technologies, Novex)
40. Coomassie G-250 SimplyBlue safe stain (Life Technologies, Invitrogen™, catalog number: LC6060)
41. Pierce™ BCA protein assay (Thermo Fisher Scientific, catalog number: 23225)
42. Phenol solution (Equilibrated with 10 mM Tris HCl, pH 8.0, 1 mM EDTA) (Sigma-Aldrich, catalog number: P4557)
43. Adenosine 3', 5'-cyclic monophosphate (cAMP) (free acid) (Sigma-Aldrich, catalog number: A9501)
44. Triton™ X-100 solution (~10% in H<sub>2</sub>O) (Sigma-Aldrich, catalog number: 93443)
45. Sodium dodecyl sulphate (SDS) solution (10% in H<sub>2</sub>O SDS) (Sigma-Aldrich, catalog number: 71736)
46. SM agar (Formedium, catalog number: SMA0120)
47. SM broth (Formedium, catalog number: SMB0102)
48. Trichloroacetic acid (VWR International, catalog number: 102863H)
49. Q5 (NEB, catalog number: M0491)
50. Pwo (Roche Diagnostics, catalog number: 11644947001)
51. KOD polymerases (Merk Millipore, catalog number: 71086)
52. Antifade mounting medium Fluoromount-G® (Cambridge Bioscience, catalog number: 0100-01) 71086 or Prolong® (Life Technologies, catalog number: P10144)
53. Axenic medium (see Recipes)
54. LOFLO medium (see Recipes)
55. SM agar (see Recipes)
56. SM broth (see Recipes)
57. Trichloroacetic acid (see Recipes)
58. KK<sub>2</sub> buffer (see Recipes)
59. KK<sub>2</sub>C buffer (see Recipes)
60. 1 M MgSO<sub>4</sub> (see Recipes)
61. 1 M CaCl<sub>2</sub> (see Recipes)
62. Electroporation buffer E50 (see Recipes)
63. Dense suspension of *Klebsiella aerogenes* (see Recipes)
64. Tris-HCl buffer (see Recipes)
65. TE buffer (see Recipes)
66. 20 mg/ml G418 (see Recipes)
67. Freezing medium (see Recipes)
68. 50 mM L-Ascorbic (see Recipes)
69. Master stock of cAMP (see Recipes)
70. Phosphate buffered saline (PBS) (see Recipes)
71. PBS-T (see Recipes)
72. Elution buffer (see Recipes)

## **Equipment**

1. Sterile tissue culture dishes [(100 x 20 mm style, Corning, catalog number: 353003 or Nunc, catalog number: 172958) (35 x 10 mm style, Corning, catalog number: 353001)]
2. Sterile 96 flat bottomed well tissue culture dishes (Corning Costar, catalog number: 3799)
3. Sterile 50 ml plastic tubes (SARSTEDT AG, catalog number: 62.547.004 or Corning, catalog number: 352070)
4. 1.8 ml CryoTube vials (Thermo Fisher Scientific, catalog number: 377267)
5. Pipetting reservoir (120 ml capacity) (Thermo Fisher Scientific, catalog number: 10717964)
6. Steribag pouches (140 mm x 50 mm x 330 mm) (Thermo Fisher Scientific, catalog number: 12728995)
7. Microfuge tubes (Sarstedt Micro tube 1.5 ml) (SARSTEDT AG, catalog number: 72.690)
8. Lab-Tek chambered coverglass with cover (Thermo Fisher Scientific, catalog number: 155411 and 155380)
9. Glass bottom microwell 35 mm petri dishes with 20 mm microwell (MatTek, catalog number: P35G-1.5-20-C)
10. Disposable cell spreaders (Biologix, catalog number: 65-100)
11. Cryo preservation module (Agilent StratCooler Lite Preservation module, catalog number: 400006)
12. 21G (0.8 mm x 40 mm) needles (Becton Dickinson, catalog number: 304432)
13. Spatula (150 x 4 mm) (Thermo Fisher Scientific, catalog number: 11503482)
14. Platinum wire inoculating loop and insulated holder (Thermo Fisher Scientific, catalog number: 10669652 and 12882775)
15. Nunc sterile plastic needles and loops (Thermo Fisher Scientific, catalog number: 254399 and 254410)
16. Syringe filters (0.22 µm) for sterilisation of small volumes (Elkay, catalog number: E25-PS22-50S)
17. Sterile disposable syringes for sterilisation of small volumes (Becton Dickinson, Plastipak 50 ml, catalog number: 30086; 20 ml, catalog number: 300613; 10 ml, catalog number: 302188)
18. Swin-Lok™ plastic filter holder 25 mm (GE Healthcare, catalog number: 420200)
19. Prefilters. 25 mm cellulose absorbent pads (Merck Millipore, catalog number: AP1002500)
20. Nucleopore Track-Etch polycarbonate membrane 3.0 µm (GE Healthcare, catalog number: 110612)
21. Micro bio-spin chromatography column (Bio-Rad Laboratories, catalog number: 732-6204)

22. Duran bottles (or equivalent autoclavable media bottles) 1,000 ml, 500 ml and 150 ml (Thermo Fisher Scientific, catalog numbers: FB-800-1000, FB-800-500 and 11699888)
23. Foam stoppers (50 mm x 50 mm and 50 mm x 38 mm) (Thermo Fisher Scientific, catalog number: 11522563 and 11512563)
24. Aluminium foil (300 mm x 75 mm x 0.02 mm) (Prowrap, catalog number: JR307518U)
25. Disposable plastic filter bottle units (150 ml) (Millipore Stericup, SCGPU01RE) or 500 ml (Millipore Stericup, catalog number: SCGPU05RE)
26. Steritop bottle top filter unit (1,000 ml) (EMD Millipore, SCGPT10RE)
27. Corning Filter System (1,000 ml) (Corning, catalog number: 430186)
28. Universal bottle (Thermo Fisher Scientific, catalog number: BTS-402-050M)
29. Temperature controlled shaking incubator (e.g. Infors Multitron standard or Innova 4330)
30. Temperature controlled incubator (e.g. Panasonic, model: MIR-254 PE or MIR-154 PE)
31. Tissue culture hood (e.g. Scanlaf Mars Safety 2)
32. Ultrapure water supply (e.g. Milli-Q<sup>®</sup> Advantage A10 Ultrapure Water Purification System)
33. Autoclave
34. Water bath (e.g. Grant SUB Aqua 12 Plus)
35. pH meter and calibration buffers (e.g. Hanna Instruments Edge Hybrid pH, catalog number: HI-2020-02)
36. Cell counter (e.g. Beckman Coulter Z<sub>1</sub>) or haemocytometer (Weber Scientific)
37. Bench top centrifuge (e.g. Thermo Fisher Scientific, model: IEC CL30 or Eppendorf, model: 5702R)
38. Bench top microfuge (e.g. Eppendorf, model: 5424 or 5424R)
39. Thermocycler (96 x 0.2 ml tube block) (e.g. Biometra TGradeint or Applied Biosystems Veriti)
40. Programmable peristaltic pump (e.g. Watson Marlow, model: 505Di)
41. 8 channel electronic pipette (e.g. eLine 50-1,200 µl) (Sartorius, catalog number: 730391) and 50-1,200 µl tips (SafetySpace<sup>™</sup> filter tips) (Sartorius, catalog number: 79121F)
42. Confocal microscope (e.g. ZEISS, model: LSM710 or LSM780)
43. TIRF microscope (e.g. Nikon Corporation)

*Note: Both the Zeiss confocal and Nikon TIRF microscopes are supplied with the manufacturers image analysis software (Zen and NIS-Elements respectively) but due to the limited time users have on this equipment image analysis is usually performed on a dedicated computer work station running these software packages that are an additional purchase from the manufacturers. Free to use image analysis software (open source) packages are also routinely used. ImageJ and Fiji can be used for the*

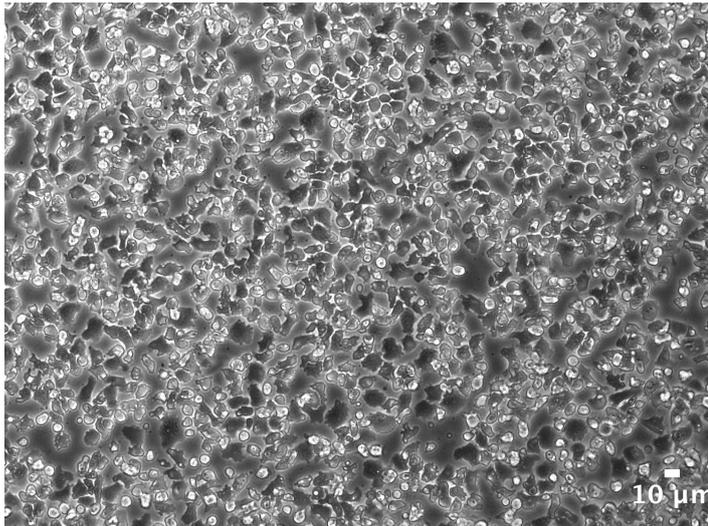
confocal and TIRF images. ImageJ can be downloaded from <http://imagej.nih.gov/ij> and Fiji from <http://fiji.sc/Fiji>.

44. Electroporator (e.g. BIO-RAD Xcell) and 1 mm gap width cuvettes (Cell Projects, catalog number: EP-201)
45. Potter-Elvehjem homogeniser (Wheaton, catalog number: 358049)
46. Ultracentrifuge (Beckman Coulter, model: Optima MAX-XP)
47. Orbitrap Mass Spectrometer (Thermo Fisher Scientific)
48. 0.2 ml PCR strip tube with 12 wells and separate cap (VWR International, catalog number: 53509-306)
49. TLA-110 rotor (Beckman Coulter)
50. XCell SureLock<sup>®</sup> Mini-Cell and XCell II<sup>™</sup> blot module (Life Technologies, Novex, catalog number: EI0002) and power supply (Bio-Rad Laboratories, PowerPac<sup>™</sup>HC High-Current Power Supply, catalog number: 164-5052)

## **Procedure**

### A. Growing *Dictyostelium* cells

1. If possible all work with live *Dictyostelium discoideum* cells should be done in a 22 °C temperature controlled room. The initiation and passaging of cultures either in shaken suspension or in dishes should be done under sterile conditions in a tissue culture hood. It is very important to renew stocks every month from spores or frozen stocks stored at -80 °C or in liquid nitrogen.
2. Ax2 or the TSPOON null cells (HM1725 and HM1727) cells are grown in shaken suspension (22 °C, 180 rpm) in axenic medium in 250 ml or 500 ml conical flasks (seed at  $0.5-2 \times 10^5$  cells/ml) and harvested in mid log phase ( $2-5 \times 10^6$  cells/ml). Doubling time in these conditions is normally 8-11 h.
3. Alternatively, cells can be grown on tissue culture plates and harvested when confluent (each plate should yield  $1-2 \times 10^7$  cells) (Figure 1). This is useful when relatively few are required for confocal or TIRF microscopy.



**Figure 1. A phase contrast light photomicrograph of a near confluent lawn of *Dictyostelium amoebae*.** The cells were grown and photographed in a 10 cm tissue culture dish containing 12 ml of axenic medium. Note that there are few gaps between individual cells. The scale is shown in the bottom right.

- B. Electroporation of *Dictyostelium* cells (based on Pang *et al.*, 1999)
1. Harvest mid log phase cells into 50 ml sterile plastic tubes and centrifuge at 300 x g for 2-3 min to pellet the cells. Aspirate supernatant and add 50 ml of ice cold E50 buffer and resuspend the cell pellet by gently tapping and shaking the tube.
  2. Determine the number of cells/ml using an automated cell counter or a haemocytometer.
  3. Centrifuge at 300 x g for 2-3 min to pellet the cells and then resuspend at  $4 \times 10^7$ /ml in ice cold E50 buffer. Place the cell suspension on ice and incubate for 5 min (if the cells start to settle then gently mix by agitation).
    - a. For overexpression plasmids such as pJH101 (TSPOON-GFP fusion driven by the constitutively active actin15 promoter) or pDT58 and pDT61 (both contain the TSPOON-GFP fusion driven by the TSPOON promoter), transfer 0.1 ml of the cell suspension to a pre-chilled sterile electroporation cuvette (1 mm gap width) and add 10-30 μg of supercoiled plasmid DNA (preferably in  $\leq 15$  μl of Tris-HCl or TE buffer and prepared according to the manufacturer's instructions using the HiSpeed® Plasmid Midi Kit) then mix by gently pipetting up and down avoiding introducing air bubbles to the cell suspension. Return the cuvette to ice (Figure 2).
    - b. The most common and reliable gene disruption in *Dictyostelium* is achieved with *blasticidin S* as the selective agent using a plasmid containing the resistance cassette (such as pLPBLP) flanked on either side with DNA homologous to the targeted gene (Faix *et al.*, 2004; Faix and Kimmel, 2006). This targeting/disruption cassette should be freed from the remainder of the construct by complete digestion with restriction enzymes. To knockout TSPOON, plasmid pDT70 (based

on pLPBLP) was cut with *Apal* and *SacI* (these enzymes generate 3' overhangs in the cut DNA but enzymes that leave 5' overhangs e. g. *BamHI* or blunt cutters like *PvuII* can be used in any combination to liberate the disruption cassette). The enzymes were removed by a single phenol extraction followed by three extractions with chloroform and the cut DNA precipitated with ethanol (Sambrook and Russell, 2001). Resuspend the cut DNA at 2-3 mg/ml in Tris-HCl or TE buffer. Add 15-20 µg of the disruption cassette DNA to each 0.1 ml of cell suspension, again in a chilled sterile electroporation cuvette (1 mm gap width) and mix as in step B4a.



**Figure 2. A photograph of the electroporation apparatus described in this protocol.**

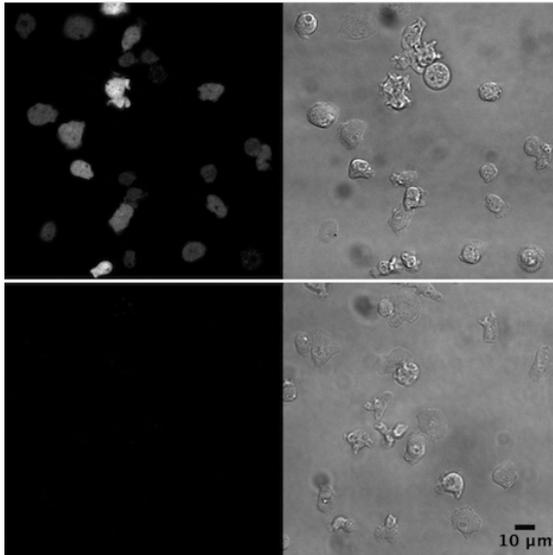
In the background the BioRad Xcell is shown with the pod open and loaded with an electroporation cuvette (1cm gap width). In the ice bucket is two more cuvettes awaiting electroporation. In the foreground a tube (15 ml) containing the axenic medium that will be added (0.5 ml) after each cuvette is pulsed twice to electroporate the cell suspension within. A tissue culture dish with 6 wells each of which contains 3 ml of axenic medium is also shown and after recovery on ice for 5 min, 100 µl of the electroporated cell suspension will be added to each well of this dish.

4. The cell/DNA suspension should be electroporated quickly to minimise cell sedimentation in the cuvette.
  - a. Remove the cuvette from the ice and dry off any excess moisture on the electrodes with a tissue before placing in the electroporation pod.
  - b. The parameters should be set at 0.75-0.85 kV, 25 µF capacitance with resistance set at infinity ( $\infty$ ) for the Bio-Rad Xcell. The same parameters are used on the Bio-Rad GenePulser and GenePulserII however they may need to be optimised with electroporation devices from other manufacturers.
  - c. Deliver two pulses 5 sec apart to each cuvette and then quickly add 0.5 ml of sterile room temperature axenic medium, mix by gentle pipetting up and down and then return the cuvette to ice for 5 min.

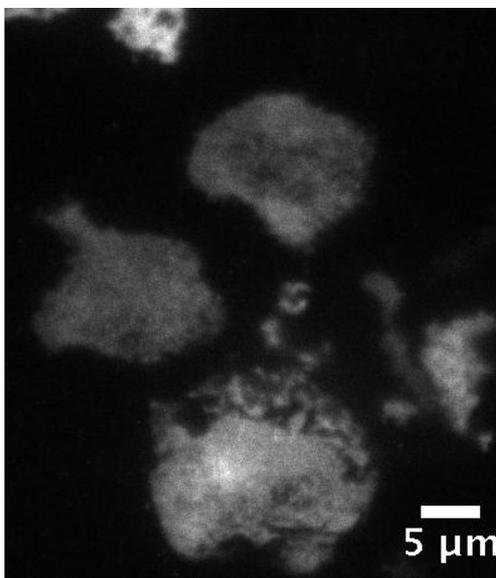
- d. For the selection process and screening of transformants see Faix *et al.* (2004); Kimmel and Faix (2006); Muller-Taubeberger *et al.* (2006).

### C. Overexpression constructs

1. For each cuvette prepare a 6 well tissue culture plate with 3 ml of axenic medium in each well.
2. Remove a cuvette from ice and resuspend the cells by gentle pipetting before adding 0.1 ml to each well of the tissue culture dish.
3. Incubate at 22 °C overnight (normally 16-24 h) before adding, in duplicate wells, 10 µg/ml, 20 µg/ml and 40 µg/ml G418 (for plasmids pJH101 and pDT61).
4. For selection with hygromycin, add 30 µg/ml, 60 µg/ml and 90 µg/ml (for pDT58).
5. Change the selection medium by aspiration to remove dead cells every 2-3 days and the wells should be confluent after 9-14 days. (Hygromycin appears to be less efficient at killing *Dictyostelium* amoebae than G418 and it is therefore important to replace the medium every 2-3 days as directed to ensure the removal of dead and dying cells that will become detached from the tissue culture plate).
6. The wells are screened for optimum expression by dislodging the cells by pipetting up and down and removing 0.3 ml into a single well of a chambered coverslip (8 well).
7. The cells are allowed to settle and attach for 20-30 min before the medium in each well is aspirated and replaced by 0.3 ml of LOFLO medium or KK<sub>2</sub>C.
8. The cells are then examined by confocal or TIRF microscopy for the presence and distribution of the GFP fusion protein (Figures 3 and 4).



**Figure 3. Overexpression of TSPOON-GFP.** The top panel are cells transfected with pDT61 (TSPOON-GFP fusion protein driven by the Tspoon promoter) and selected for 14 days in axenic medium containing 10 $\mu$ g/ml G418. Most cells express the fusion protein but at varying levels. The bottom panel is mock transfected cells. The cells in both panels were transferred to LoFlo medium for 30 minutes before being imaged by confocal microscopy using exactly the same image collection parameters. Note the faint autofluorescence in the mock transfected cells visible as puncta. The scale is indicated in the bottom right of the figure.



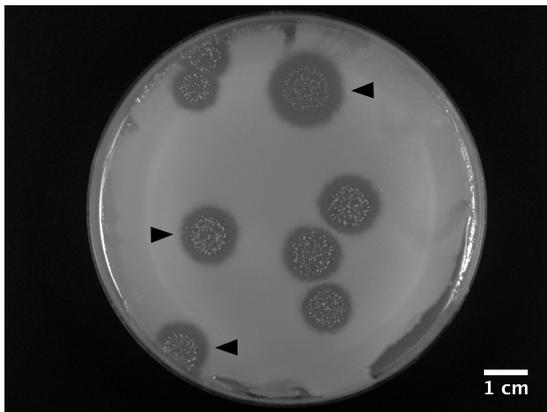
**Figure 4. A TIRF image of TSPOON-GFP.** The amoebae were transfected and selected as in Figure 3. Cells were transferred into KK<sub>2</sub>C (plus 50  $\mu$ g/ml L-ascorbic acid) 3 h prior to image collection. The scale is indicated.

9. To make a permanent stock, the well(s) with the optimum expression are harvested by pipetting the axenic medium up and down to dislodge the cells which are then used to initiate a larger culture (grown with the appropriate levels of G418 or hygromycin) either in shaken suspension or several 10 cm tissue culture dishes each with 12 ml of axenic medium.
10. A minimum of  $8 \times 10^7$  cells should be harvested and pelleted by centrifugation ( $300 \times g$  for 2 min) then resuspended in 1.6 ml of freezing medium and processed as in step C23.
11. To isolate knockout mutants the cell suspension should be diluted to appropriate densities to ensure clonality over a range of cell survival. This is particularly important when the targeted gene affects growth because wild type non-homologous recombinants are likely then to outgrow such a knockout, making the screen and its isolation more difficult.
12. To ensure clonality, the cell suspension from a single cuvette is first added to a total of 15 ml of axenic medium in 50 ml plastic tube so that the cells are approximately  $2.67 \times 10^5$ /ml.
13. This cell suspension is further diluted 10 ( $2.67 \times 10^4$ /ml), 50 ( $5.33 \times 10^3$ /ml), 100 ( $2.67 \times 10^3$ /ml), 200 ( $1.33 \times 10^3$ /ml) and 400 fold ( $0.67 \times 10^3$ /ml). Each dilution is made into a total of 15 ml of axenic medium and 100  $\mu$ l/well is dispensed into a separate 96 well tissue culture plate for each dilution.
14. Normally 4 x 96 well plates are prepared for the 100, 200 and 400 fold dilutions so 50 ml of diluted cells should be prepared for each of these dilutions.
15. Incubate the plates in a moist atmosphere (such as in a large plastic cake storage box lined with wet tissues) at 22 °C overnight (normally 16-24 h) before adding 100  $\mu$ l of axenic medium containing 20  $\mu$ g/ml blasticidin S to each well (10  $\mu$ g/ml final).
16. Change the selection medium to remove dead cells every 2-3 days by forcefully 'throwing' the medium into a large tissue lined (stops splash back into wells) container. Add 200  $\mu$ l of fresh axenic medium containing 10  $\mu$ g/ml of blasticidin S to each well.  
*Note: Plating out the cells into the 96 well plates and replenishing the medium is best achieved with a 8 channel electronic pipette fitted with tips each with a capacity of 1,200  $\mu$ l. The cell suspension or fresh media is dispensed from a sterile plastic reservoir. These reservoirs can be reused after autoclaving in a steribag pouch.*
17. The 96 well plate containing the least diluted cells should have confluent wells after 9 days whereas the plates containing the 100, 200 and 400 fold dilutions may take up to 21 days (times are average based on the use of many different knockout vectors but deletion of a gene that impairs growth in axenic medium is likely to increase these times). Dilutions are usually screened from plates where <30 wells/plate are occupied, as these are likely to be clonal.

18. The cells from each confluent well are harvested by pipetting up and down, then transferred to 1.5 ml microcentrifuge tube containing 0.75 ml of sterile  $\text{KK}_2$  (this is to dilute residual axenic medium after the cells are pelleted).
19. Refill the well with 200  $\mu\text{l}$  of fresh axenic medium containing 10  $\mu\text{g/ml}$  of blasticidin S. The cells are pelleted in a benchtop microfuge at full speed for 20 sec and the supernatant removed by aspiration.
20. Genomic DNA from the cell pellet can then be isolated using a variety of commercial kits but we prefer the Quick-gDNA MiniPrep kit from Zymo Research as it is quick, has few steps and reproducibly yields good quality genomic DNA for screening by PCR using a variety of polymerases [Expand 20 kb plus polymerase (Roche) was found to be optimal for the TSPOON screen but we have also used Q5, Pwo and KOD polymerases].
21. In the case of the TSPOON knockout, isolation and screening took 17 days from the start of selection until confluent wells were observed in the plates containing the 200 and 400 fold dilutions.
22. The PCR screen used TSPOON specific primer (TCP) oligonucleotides TCP15: (5'-GATGAAATTTATCAGATATTGATTTTCATGAATGTTTCACC-3') and TCP16 (5'-CTGCTGATGTTGGTTTATAGGTGGCAAACCATC-3') both of which bind outside the disruption cassette to minimise false positives generated by single crossover recombination events. The gene encoding TSPOON (*tstD*, DDB\_G0350235) is located on chromosome 1 and is flanked by genes DDB\_G0271002 and DDB\_G026990. The exact genomic location of TCP15 is 1:3986383..3986422 and TCP16 is 1:3982321..3982356. All the oligonucleotide primers used in this protocol are located within these coordinates.
  - a. The PCR reactions (in 0.2 ml tubes) consisted of a total of 25  $\mu\text{l}$  containing 1x Expand 20kb<sup>PLUS</sup> buffer, 0.4 nM of each primer, 5  $\mu\text{l}$  of the Quick-gDNA miniprep, 500  $\mu\text{M}$  dNTPs and 0.25  $\mu\text{l}$  (5 units) of Expand 20kb<sup>PLUS</sup> polymerase.
  - b. The cycling parameters were step (1) 92 °C for 1 min 15 sec; (2) 92 °C for 30 sec; (3) 52 °C for 45 sec; (4) 62 °C for 7 min; (5) 62 °C for 5 min. Steps 2 to 4 were repeated a further 36 times before the reactions were held at 4 °C until analysed by gel electrophoresis (Sambrook and Russel, 2001).
  - c. The PCR product from wild-type clones is ~4.1 kb and TSPOON knockout clones ~4.7 kb.
  - d. The insertion of blasticidin resistance cassette and flanking DNA introduces restriction sites into the knockout PCR product that are absent in the wild type. This is a useful further proof of correct targeting as digestion of these products with a restriction enzyme such as SmaI for TSPOON, leaves the wild type product intact but cuts the knockout product into 3 fragments.
  - e. A total of 46 wells were screened by this method, 37 were TSPOON knockouts, 6 were wild type and 3 were not clonal as they contained both PCR products.

Therefore, the targeting frequency for this the *tstD* gene was 80% and though high was in keeping with other genes targeted by this procedure in our laboratory such as 76% for *ipfA*, 67% *mscS*, 57% *mcIN*, 50% *phdA* and 12% *dagA*.

23. Four TSPOON knockouts, isolated from different 96 well plates, were processed further (2 for study and 2 as backup).
  - a. The wells containing these cells were harvested as before, the cells diluted in KK2 to 0.6-1.2 cells/ $\mu$ l (a confluent well contains  $\sim 10^5$  cells).
  - b. SM agar plates were prepared each with a 0.4 ml drop of a dense suspension of *Klebsiella aerogenes* bacteria (Figure 5).



**Figure 5. A SM agar plate covered in a lawn of *Klebsiella aerogenes* with *Dictyostelium* colonies visible.** The translucent periphery of a colony consists of vegetative amoebae while at the centre fruiting bodies and other multicellular structures have formed. Colonies suitable for harvesting and freezing should be  $\sim 1$  to 1.5 cm in diameter and clearly separated from neighbours. Suitable colonies in this example are indicated by the black arrowheads. Approximately 6 amoebae in 10  $\mu$ l of KK<sub>2</sub> were directly added a 400  $\mu$ l drop of a dense suspension of *Klebsiella aerogenes* already on the plate and then evenly spread. Colonies were visible 4 days after plating and this photograph was taken after 6 days. The scale is shown in the bottom right.

- c. Add 10  $\mu$ l of the diluted cell suspension to this drop and spread evenly over the plate with a sterile plastic spreader.
- d. Discrete colonies appear within 4-5 days and one per knockout was harvested when they had reached  $\sim 1.5$  cm in diameter.
- e. The flat end of a sterilised small metal spatula was used to transfer the entire colony directly into 1.6 ml of freezing medium within a CryoTube on ice. The CryoTubes were intermittently vortexed to ensure an even suspension and then transferred to cryo preservation module chilled to 4  $^{\circ}$ C.

- f. The module is then transferred to a -80 °C freezer overnight and then the tubes can remain at -80 °C (cells remain viable for >2 years) or be transferred to a liquid nitrogen storage tank (indefinite viability).

#### D. Microscopy

1. Fixed and live cells were analysed by microscopy at the vegetative or the aggregation competent stage of development.

##### ***For live vegetative cells***

- a. Simply harvest growing cells from axenic culture and transfer an appropriate number of cells so that final density is  $0.5-1.5 \times 10^5/\text{cm}^2$  into a glass bottom dish or chambered coverslip containing LOFLO medium (this has been formulated to minimise background and cellular autofluorescence compared to standard axenic medium and it will keep the cells vegetative for a while) or  $\text{KK}_2\text{C}$  (they will start to develop in this buffer but autofluorescence and photosensitivity will be minimised).
- b. Incubate at 22 °C for 10 min to allow the cells to attach then aspirate the medium (do not allow the cells to dry) and replace with fresh LOFLO or  $\text{KK}_2\text{C}$ . They can then be used for live cell imaging or fixed.

##### ***For developed cells***

- c. Transfer  $0.5-1.5 \times 10^5/\text{cm}^2$  into  $\text{KK}_2\text{C}$  and replace the  $\text{KK}_2\text{C}$  once the cells have attached.
- d. Incubate at 22 °C for 8-10 h to allow the cells to become aggregation competent.
- e. Alternatively transfer  $1 \times 10^6$  cells into a 35 mm tissue culture dish containing 2 ml of  $\text{KK}_2\text{C}$ , allow the cells to attach and then replace the  $\text{KK}_2\text{C}$  and incubate at 22 °C for 1 h.
- f. Transfer the dish to 15 °C overnight (15-17 h) and then return the dish to 22 °C for 1 h before transfer (at  $0.5-1.5 \times 10^5/\text{cm}^2$ ) to a glass bottom dish (the cells should aggregate within 2-3 h at 22 °C).
- g. If larger quantities of developed cells are needed then resuspend vegetative cells at  $2 \times 10^7/\text{ml}$  in  $\text{KK}_2\text{C}$  and transfer them into a conical flask with a volume of at least 5 times that of the cell suspension to ensure optimum aeration. The cells are shaken at 180 rpm at 22 °C for 1 h then a 100  $\mu\text{l}$  droplet of  $\text{KK}_2\text{C}$ , containing enough cAMP (diluted from a master stock) so that the final concentration of the entire suspension is 100 nM, is dispensed into the suspension every six minutes for 3.5-5.0 h via a programmable peristaltic pump. This procedure mimics the pulsatile waves of cAMP that are emitted through a developing population of amoebae and is useful for mutant strains defective in early development and cAMP signalling.



maximises the frame rate (Frigault *et al.*, 2009; Muller-Taubenberger and Ishikawa-Ankerhold, 2011).

- i. For cells expressing the promoter\_TSPOON-GFP construct typical settings used had the 488 nm laser power set at 5% and the pinhole opened up to 2.5 Airy units with a 512 pixels x 300 pixels frame collected every second. For faster temporal resolution switch to a SDCM.
  - j. With fixed cells and ICC photobleaching can be a problem but the use of an antifade mounting medium (typically Fluoromount-G<sup>®</sup>) can minimise this and allows slower scan speeds and averaging to give better images.
3. TIRF microscopy.
- a. The problem of phototoxicity is usually more acute with vegetative *Dictyostelium* cells, in part because although the evanescent field is limited to the initial ~100 nm of the sample from the coverslip, the plasma membrane is strongly illuminated.
  - b. Image cells that have been in LOFLO or KK<sub>2</sub>C for at least one hour. The inclusion of L-Ascorbic acid may be necessary.
  - c. Keep the laser power and the illumination time to a minimum.
  - d. Since there is no scanning the frame rate is higher. A 100x lens (high NA) should be used with an additional 1.5x zoom lens if present as this allows ameobae to be imaged in great detail.
  - e. Filter all buffers (0.22 µm) to remove particulates.
  - f. Typical settings for cells expressing promoter\_TSPOON-GFP construct had the 488 nm laser power set at 7% with an exposure time of 0.08 sec collecting 512 x 512 frames at up to 12 per sec.

#### E. Cell Fractionation

1. Cells expressing A15\_GFP (control) or promoter\_TSPOON-GFP were grown until they reached a density of 2-4 x 10<sup>6</sup>/ml in selective media, and by microscopy >50% of cells were expressing GFP.
2. Starting with a maximum of 8 x 10<sup>8</sup> cells, the cells were washed in KK<sub>2</sub> buffer and then pelleted at 300 x g for 3 min. All subsequent steps were performed at 4 °C.
3. The cells were resuspended in PBS with a protease inhibitor cocktail, lysed by 8 strokes of a motorized Potter–Elvehjem homogenizer (grinding chamber clearance 0.1-0.15 mm) followed by 5 strokes through a 21-g needle to ensure full lysis.
4. Alternatively, the cells can be resuspended at 1-5 x 10<sup>8</sup>/ml and placed in a syringe fitted with a 25 mm filter holder containing a prefilter and a Nucleopore filter (3.0 µm). The cell suspension is then passed through this filter assembly twice.
5. The lysate was then centrifuged at 4,100 x g for 32 min to pellet nuclei and unbroken cells, and the postnuclear supernatant further centrifuged at 50,000 rpm (135,700 x g RCF<sub>max</sub>) for 30 min in a TLA-110 rotor to recover the membrane pellet and cytosolic supernatant.

6. A standard protein assay was used to assess protein recovery in the 2 fractions, and volumes adjusted for equal protein.
7. Alternatively, to equalise volumes, the cytosolic supernatant was concentrated by precipitation with 10% trichloroacetic acid at 4 °C for 30 min and recovered by centrifugation 14,000 x g for 10 min. Samples precipitated with trichloroacetic acid were washed with ice cold acetone (-20 °C), air dried for 2 min and then resuspended in the same volume as the pellet fractions.

#### F. Protein pulldowns

Immunoprecipitations were performed using amoebae stably expressing TSPOON-GFP under a constitutive (A15\_ TSPOON-GFP) and its own promoter (prom\_TSPOON-GFP), and non-transformed cells were used as a control. The amounts given here are recommended for a large scale immunoprecipitation sufficient for proteomic identification of interacting proteins under native conditions. The size of the starting culture, and all subsequent volumes can be reduced accordingly for smaller scale immunoprecipitations, for instance where the identification of proteins are made by Western blotting. The protocol can also be amended for denatured (by heat and/or the presence of SDS but are not detailed here) immunoprecipitations.

1. Cells were grown until they reached a density of 2-4 x 10<sup>6</sup>/ml in selective media, and by microscopy >50% of cells were expressing GFP.
2. Up to 8 x 10<sup>8</sup> cells were pelleted by centrifugation at 300 x g for 2 min, washed twice in 50 ml of KK<sub>2</sub> buffer before being resuspended at 2 x 10<sup>7</sup> cells/ml in KK<sub>2</sub> buffer and starved for 4-6 h at 22 °C whilst shaking at 180 rpm.
3. The cells were then pelleted at 300 x g for 3 min, lysed in 4 ml PBS-T plus protease inhibitor cocktail tablet, extracted for 20 min with rotation at 8 rpm in a 15 ml tube at 4 °C, and then spun 20,000 x g for 15 min to remove debris and insoluble material.
4. By BCA protein assay the resulting lysate contained 10-15 mg total protein.
5. The lysates were precleared by adding 100 µl of PA sepharose bead slurry (50% v/v in PBS) and incubated for 30 min with rotation at 8 rpm, followed by centrifugation at 2,200 x g for 3 min to pellet beads.
6. The supernatant was transferred (~ 5 ml) to a fresh tube, and this is the starting material for the immunoprecipitation (protein concentration should be 2-4 mg/ml).
7. For maximum recovery the lysates were immunoprecipitated using an in-house antibody against GFP overnight with rotation at 4 °C, though it may be sufficient to incubate for as little as 90 min.
8. The appropriate antibody concentration requires individual optimization, though the recommended starting point is generally 2 to 5-fold higher than used for Western blotting.
9. Following incubation with anti-GFP, 50 µl PA sepharose bead slurry (50% v/v in PBS) was added for 90 min at 4 °C with rotation at 8 rpm.

10. An alternative would be to use a commercial source of polyclonal anti-GFP, or an anti-GFP that is already coupled to sepharose, for example GFP-TRAP that has the benefit of the antibody remaining coupled to the PA sepharose when immunoprecipitated proteins are eluted from the beads. In this case the addition of PA sepharose at this step is omitted.
11. The antibody complexes recovered by centrifugation 2,200 x g for 3 min, and then washed twice with 10 ml PBS-T, resuspended in 1 ml of PBS, and transferred into a 1.5 ml microfuge tube.
12. The antibody complexes were washed a further two times with PBS, pelleting the beads at 8,000 x g for 20 sec, and then eluted from the beads with 200 µl elution buffer warmed to 60 °C for 10 min.
13. The beads are then pelleted at 8,000 x g for 20 sec, and the supernatant carefully removed; this can be facilitated by using a fine gel loading tip to remove the last 50 µl as the beads do not enter the tip, or by the use a micro bio-spin chromatography column.
14. Eluted proteins were then precipitated with 1.2 ml acetone overnight (3-20 h) at -20 °C and centrifuged at 10,000 x g for 5 min, 4 °C. An overnight precipitation increases the yield particularly for low protein concentrations but extending it beyond this results in a lower recovery.
15. The supernatant is removed and the pellet air dried for 2 min and resuspended in a buffer of choice (e.g. 1x LDS sample buffer for SDS-PAGE).
16. For Western blotting of samples SDS-PAGE gels were run and transferred according to a standard protocol (Sambrook and Russel, 2001).
17. For proteomics, the samples were run on pre-cast NuPAGE 4-12% Bis-Tris gels, stained with Coomassie G-250 SimplyBlue SafeStain and then cut into 8 gel slices. Each gel slice was processed by filter-aided sample preparation solution digest, and the sample was analyzed by liquid chromatography–tandem mass spectrometry in an Orbitrap mass spectrometer.
18. Proteins that came down in the non-transformed control were eliminated, as were any proteins with less than 5 identified peptides, proteins that did not consistently coimmunoprecipitate in three independent experiments, or proteins of very low abundance compared with the bait (i.e., molar ratios of <0.002). The remaining proteins were considered to be specifically immunoprecipitated.

## **Recipes**

### 1. Axenic medium

35.5 g HL5 with glucose

200 mg dihydrostreptomycin

*Note: Do not use streptomycin as it is inactivated during autoclaving.*

Add ultrapure dH<sub>2</sub>O to 1,000 ml

Dispense into conical flasks

75 ml/250 ml flask stoppered with 50 mm x 38 mm foam bung

150 ml/500 ml flask stoppered with 50 mm x 38 mm foam bung

750 ml/ 2,000 ml flask stoppered with 50 mm x 50 mm foam bung

The top of each flask should be covered over with a small piece (12 cm x 12 cm) of aluminium foil to prevent the top of the stopper being contaminated by dust during storage

Autoclave to sterilise

*Note: 121 °C for no longer than 15 min and remove from the autoclave as soon as possible to avoid caramelisation i.e. the media turning dark brown in colour. With larger more sophisticated autoclaves with extra safety features that extend the cycle time then this may have to be reduced to as low as 7 min.*

Alternatively filter sterilise (Steritop 1,000 ml unit, 0.22 µm) into sterile 1,000 ml Duran bottles or use a 1,000 ml Corning filter unit (0.22 µm)

Stored in the dark at 22 °C

2. LOFLO medium

8.4 g LOFLO medium

100 mg dihydrostreptomycin

Add ultrapure dH<sub>2</sub>O to 500 ml

Filter sterilise (0.22 µm) using a 500 ml Stericup filter unit

Store in the dark at 22 °C (short term) or 4 °C (long term)

3. SM agar

41.7 g SM agar

Add ultrapure dH<sub>2</sub>O to 1,000 ml

Autoclave to sterilise

*Note: 121 °C for no longer than 15 min and remove from the autoclave as soon as possible to avoid caramelisation. With larger more sophisticated autoclaves with extra safety features that extend the cycle time then this may have to be reduced to as low as 7 minutes.*

Cool to 60 °C in a heated water bath prior to pouring 25-30 ml per 10 cm diameter plastic petridish

Once the agar has set and cooled place the dishes in an airtight box or seal up in batches (15-20 plates) in plastic bags (use the bags the dishes were packed in) to minimise desiccation

Stored in the dark at 22 °C (short term <1 week) or 4 °C (long term)

4. SM broth

24.7 g SM broth

Add ultrapure dH<sub>2</sub>O to 1,000 ml

Dispense 10 ml into each 20 ml Universal bottle

Autoclave to sterilise

*Note: 121 °C for no longer than 15 min and remove from the autoclave as soon as possible to avoid caramelisation. With larger more sophisticated autoclaves with extra safety features that extend the cycle time then this may have to be reduced to as low as 7 min.*

Stored in the dark at 22 °C

5.  $\text{K}_2\text{C}$  buffer

2.24 g  $\text{KH}_2\text{PO}_4$

0.52 g  $\text{K}_2\text{HPO}_4$ , anhydrous

Add ultrapure  $\text{dH}_2\text{O}$  to 1,000 ml

Autoclave to sterilise in 1,000 ml Duran bottles (121 °C for 15 min)

pH after autoclaving should be ~6.1

Add 2 ml of sterile 1 M  $\text{MgSO}_4$

Stored at 22 °C

6.  $\text{K}_2\text{C}$  buffer

1,000 ml of sterile  $\text{K}_2\text{C}$

0.1 ml of sterile 1 M  $\text{CaCl}_2$

Stored at 22 °C

7. 1 M  $\text{MgSO}_4$

246.48 g  $\text{MgSO}_4$

Add ultrapure  $\text{dH}_2\text{O}$  to 1,000 ml

Autoclave to sterilise in 150 ml Duran bottles (121 °C for 15 min)

Stored at 22 °C

8. 1 M  $\text{CaCl}_2$

147.02 g  $\text{CaCl}_2$

Add ultrapure  $\text{dH}_2\text{O}$  to 1,000 ml

Autoclave to sterilise in 150 ml Duran bottles (121 °C for 15 min)

Stored at 22 °C

9. Dense suspension of *Klebsiella aerogenes*

Prepare a SM agar plate with *Klebsiella* streaked out on it so that there are individual colonies (stored at 8 °C and renew every 2 months from frozen stocks)

Pick a colony with a flame sterilised platinum loop (or a disposable sterile plastic needle or plastic loop) and use this to seed a universal bottle containing SM broth

Incubate the bottle for 48 h at 22 °C and vortex before using

Stored in the dark at 8 °C

Renew every month

10. Electroporation buffer E50

2.38 g HEPES

1.86 g KCl

0.29 g NaCl

- 0.5 ml of 1 M MgSO<sub>4</sub>  
0.21 g NaHCO<sub>3</sub>  
0.08 g NaH<sub>2</sub>PO<sub>4</sub>·2dH<sub>2</sub>O  
Add ultrapure dH<sub>2</sub>O to 500 ml  
Adjust to pH 7.0 with KOH  
Filter sterilise (0.22 µm) using a 500 ml Stericup filter unit and stored at 4 °C
11. Tris-HCl buffer  
0.12 g Trizma<sup>®</sup> base  
Add ultrapure dH<sub>2</sub>O to 100 ml  
Adjust to pH 8.0 with HCl  
Autoclave to sterilise in a 150 ml Duran bottle  
Stored at 22 °C
12. TE buffer  
0.12 g Trizma<sup>®</sup> base  
0.04 g EDTA  
Add ultrapure dH<sub>2</sub>O to 100 ml  
Adjust to pH 8.0 with HCl  
Autoclave to sterilise in a 150 ml Duran bottle  
Stored at 22 °C
13. 20 mg/ml G418  
552 mg Geneticin<sup>®</sup> G418 sulphate adjusted for a potency of 724 µg/mg  
*Note: Potency varies from batch to batch.*  
Add ultrapure dH<sub>2</sub>O to 20 ml  
Filter sterilise with 20 ml syringe fitted with a 0.22 µm syringe filter  
Stored at -20 °C in 1 ml aliquots in sterile 1.5 ml microfuge tubes the G418 solution retains its potency for >2 years. Working stocks should be stored at 4°C and used within 6 months
14. Freezing medium  
150 ml horse serum  
11.25 ml [7.5% (v/v)] DMSO  
Filter sterilise (0.22 µm) using a 150 ml Stericup filter unit  
Aliquot into sterile 50 ml tubes and stored at -20 °C and should be used within 1 year
15. 50 mM L-Ascorbic  
0.22 g L-Ascorbic  
Add ultrapure dH<sub>2</sub>O to 25 ml  
Filter with 50 ml syringe fitted with a 0.22 µm syringe filter to remove particulates  
Stored in 0.5 ml aliquots at -20 °C
16. Master stock of cAMP  
6.6 g cAMP  
Add ultrapure dH<sub>2</sub>O to 95 ml

Adjust to pH 7.0 very carefully with KOH (*Note: All the cAMP will not dissolve until the pH <5*)

Add ultrapure dH<sub>2</sub>O to final volume of 100 ml

Filter sterilise (0.22 µm) using a 150 ml Stericup filter unit and stored in 20 ml aliquots at -20 °C

17. PBS

8.01 g NaCl

0.20 g KCl

1.42 g Na<sub>2</sub>HPO<sub>4</sub>, anhydrous

0.24 g KH<sub>2</sub>PO<sub>4</sub>

Add ultrapure dH<sub>2</sub>O to 1,000 ml

Autoclave in 500 ml Duran bottles to sterilise (121 °C for 15 min)

Stored at 22 °C

18. PBS-T

PBS adjusted to 1% Triton X-100, using a 10% Triton stock solution

Filter sterilise for long term storage (0.22 µm)

Stored at 22 °C or 4 °C (long term storage)

19. Trichloroacetic acid

100 g trichloroacetic acid

Dissolve in 35 ml ultrapure dH<sub>2</sub>O

Then make up to 100ml with ultrapure dH<sub>2</sub>O

Stored at 22 °C

20. Elution buffer

1.21 g Trizma® base

Add ultrapure dH<sub>2</sub>O to 75 ml

20 ml SDS 2% (w/v) from 10% solution

Adjust to pH 8.0 with HCl

Add ultrapure dH<sub>2</sub>O to a final volume of 100 ml

Filter sterilise (0.22 µm) using a 150 ml Stericup filter unit and stored in 1 ml aliquots at -20 °C

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