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Phoenix™ Retrovirus Expression System

Cat. No. RVK-10001

The **PhoenixTM Retrovirus Expression Kit** includes: an Ecotropic, an Amphotropic packaging cell line, and two retroviral expression vectors for high-level protein expression in most mammalian cells.

Contents:
106 PhoenixTM ampho packaging cells
106 PhoenixTM eco packaging cells
10 µg pBMNZ plasmid
10 µg pBMN-GFP plasmid

Storage Conditions:
Store the vectors at –20°C.
Amplify and store aliquots of amplified PhoenixTM cell lines in liquid nitrogen.

The PhoenixTM system is licensed for research use only and not for diagnostic, therapeutic or other commercial applications.
Purchase does not include or carry any right to resell or transfer this product either as a stand-alone product or as a component of another product. Any use of this product other than the permitted use without the express written authorization of Orbigen, Inc. is strictly prohibited.

IMPORTANT NOTE

Before initiating retrovirus experiment, Orbigen recommends that the user follows the NIH guidelines regarding retroviral production and transduction in a Biosafety Level 2 (BL2) facility.
The viral supernatants produced by these methods, depending upon your retroviral insert, may contain potentially hazardous recombinant virus. The user of these systems must exercise due caution in the production, use, and storage of recombinant virions, especially those with amphotropic and polytropic host ranges. This consideration should be applied to all genes expressed as amphotropic and polytropic pseudotyped retroviral vectors. Appropriate NIH and regional guidelines should be followed in the use of these recombinant retrovirus production systems.

PhoenixTM Retroviral Expression System Introduction
The Phoenix™ retroviral system is currently used in over 2500 laboratories worldwide for

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delivery of genes and libraries to cells for biomedical research. An Ecotropic packaging system or an Amphotropic system is currently available for the delivery of genes to most dividing mammalian cell types.

Phoenix™ helper-free retrovirus producer lines:
PhoenixTM retrovirus producer lines, **Phoenix™-Eco and Phoenix™-Ampho (reference)**, for the generation of helper free ecotropic and amphotropic retroviruses are based on the 293T cell line (a human embryonic kidney line transformed with adenovirus E1a and carrying a temperature sensitive T antigen co-selected with neomycin), which is highly transfectable using either calcium phosphate mediated transfection (see section VI) or lipid-transfection protocols.
These retrovirus producer lines were created by placing into 293T cells constructs capable of producing gag-pol and envelope protein for ecotropic and amphotropic viruses. The lines offer many advantages over previous stable systems and these have led to their broad application worldwide.
1. Virus can be produced in just a few days.
2. By introducing an IRES-CD8 surface marker downstream of the reading frame of the gag-pol construct, CD8 expression is a direct reflection of intracellular gag-pol and the stability of the producer cell population's ability to produce gag-pol can be readily monitored by flow cytometry.
3. To prevent potential recombination, different non-Moloney promoters for gag-pol and envelope were used.
4. Both lines are capable of carrying episomes for long-term stable retrovirus production.
5. Helper virus production in these lines have been extensively tested and found to be helper-virus free.

Phoenix™ retrovirus vectors
Encoding either LacZ or GFP, these Moloney murine leukemia virus based vectors were specifically designed to facilitate the optimalization of protein expression in a variety of mammalian cells utilizing X-gal staining or FACS analysis. For comprehensive reviews of retrovirus biology, please refer to listed publications (Section V).

pBMN-GFP
Catalog No: PVL-10014
Quantity: 10 µg in 10 µl of TE (10 mM Tris HCl pH 7.5, 1mM EDTA)
This EBV/retrovirus hybrid plasmid carries gene encoding green fluorescent protein (GFP), internal ribosomal entry site (IRES), and the Epstein Bar virus nuclear antigen (EBNA) gene that allows the establishment of stable episomes at 5-20 copies per cell.

Reference: Heemskerk, M. H. M., Blom, B., Nolan, G., Stegmann, A. P. A., Bakker, A. Q., Weijer, K., Res, P. C. M. & Spits, H. (1997) *J. Exp. Med.* 186, 1597-1602.

pBMN-Z
Catalog No: PVL-10011
Quantity: 10 µg in 10 µl of TE (10 mM Tris HCl pH 7.5, 1mM EDTA)
This parental retroviral vector expresses the gene of interest under the control of viral LTR and carries Ampicillin resistance.

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Protocols

Outline of PhoenixTM retrovirus protocol
Day 0 Seed PhoenixTM cells and grow overnight.
Day 1 Transfect into the appropriate PhoenixTM cells with PhoenixTM vectors encoding gene of interest.
Day 2 Replace medium if necessary.
Day 3 Harvest virus-containing media, which may be stored for future use or utilized immediately to infect target cells for titer determination or for gene expression.
Day 4-5 For transient expression, harvest the cells and assay for protein of interest. For stable expression, initiate drug selection to isolate cells expressing high level of protein of interest.
Day 9 Refresh medium and continue drug selection.
Day 14 Isolate high expression clones.

I. Passaging Phoenix™ cells:
To achieve optimal cell conditions, passage cells at 1:4 or 1:5 at 70-80% confluent every 2-3 days.
Never let cells reach confluence since this will reduce transfection efficiency in the short term.
Passage of Phoenix™ cells every few months in Hygromycin (300 µg/ml) and Diptheria Toxin (1µg/ml) for one week is recommended. Cells can be analyzed and sorted by fluorescent activated cell scan (FACS) for expression of CD8 (a proxy measure of gag-pol in this cell line) and for surface expression of envelope protein with 83A25 antibody.

I-1. Thawing Phoenix™ cells.
It is important to freeze multiple vials of each producer cell line after first receiving and expanding them.
This will ensure a ready supply of backup vials to allow for uniform virus production over several years. If the cells are to be carried in selective media, this should not be applied until after the first passage.
1. Remove the vial containing frozen cells from Liquid Nitrogen or shipping box with dry ice. Thaw rapidly at 37°C by holding the vial gently shaking in the water bath. Take out the vial from the water bath when the frozen cells start to thaw. (about 1-2 minutes, the key point is "Not to thaw the cells completely".)
2. Immediately add 1 ml **Growth Medium (GM:** DME containing 10% heat inactivated fetal bovine serum, 100 U/ml Penicillin, 100 U/ml Streptomycin, 2 mM L-Glutamine) to the freezing vial and gently transfer this solution to a 15 ml sterile conical screw cap tube.
3. Add 2 ml of GM and gently mix the tube to allow for osmotic equilibration.
4. Add 10 ml of GM, close the tube, invert several times and spin cells at 500 x g for five (5) minutes.
5. Remove the supernatant, resuspend cell pellet in GM, and transfer to a 10 cm tissue culture dish.

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I-2. Growth and passage of Phoenix™ cells
PhoenixTM cells derived from 293 cells are carried in GM and grown in a 37°C degree incubator containing 5% CO2. To split and passage the cell lines:
1. Gently rinse x 1 with PBS (without Ca++ or Mg++).

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2. Trypsinize (.05% trypsin/0.53 mM EDTA) until the cells easily detach and can be readily pipetted into a single cell suspension.
3. Trypsinization is quenched with GM prior to subculturing in fresh medium.
It is suggested that the cells not be split at densities more dilute than 1:5 in order to maintain the uniformity of the cells in culture and minimize the outgrowth of clonal variants. The cells should not be allowed to become overconfluent, as this leads to the formation of cell clumps in culture which can cause uneven cell distribution after replating and result in less efficient transfection.

I-3. Freezing Phoenix™ cells:

To assure viability of the cell line, it is recommended that the cells are frozen prior to confluence.
PhoenixTM cells are much less adherent than NIH3T3 cell lines and easily detach from the tissue culture dishes after approximately 30 seconds of treatment with trypsin at room temperature.
1. Wash, trypsinize, and quench cells as described in I.2.
2. Centrifuge the cells at 500 x g for 5 min.
3. Remove the media and add 1 ml of freezing solution (**Freezing Medium:** 90% heat-inactivated fetal calf serum, 10% DMSO) per 106 cells.
4. Transfer to a 2 ml cryogenic vial.
5. Place the freezing vial at -70 °C overnight and transfer to liquid nitrogen on the following day.

II. PhoenixTM retrovirus production

Important Considerations
It is recommended that during initial set up, the user optimize the system by using a retroviral vector expressing an easily detectable marker such as lacZ or a cell surface protein. During optimization, one should check for transfection frequency of the producer clone and test infection rate of target cells. Tests for transfection and infection frequencies using a ßgal or GFP based system can be readily measured by ßgal staining or FACS respectively. Only when one is satisfied with the transfection conditions and infection rates should one proceed to using vectors with no readily detectable marker. It should be possible to scale up the protocols.
The initial plating of the cells may be the most important step in successfully obtaining high retroviral titers. It is extremely important that the cells are not overly clumped and are at the correct density. Unlike NIH3T3-derived cell lines, PhoenixTM cell lines do not readily form well-spread monolayers. Instead, they tend to clump before confluence, and if the clumping is excessive, the cells will never reach confluence during the 48-72 hour period following transfection. In order to prevent clumping, it is essential that the cells are extremely healthy prior to plating. If they are overconfluent, it may be necessary to split them 1:2 or 1:3 for several passages prior to plating for transfection. In addition, the cells are much less adherent than murine fibroblasts and should be handled very gently when washing and changing medium. For consistency, it is important to count the cells rather than estimating the split. The recommended cell number for transfection below is optimized for pBMN-Z. Expression of other inserts may be detrimental to the growth of the cells. This effect may be noted by failure of the packaging cell line to reach confluence by 48-72 hours post-transfection. If this occurs, it may be necessary to plate more cells prior to transfection.

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For example, with constructs expressing either fas or P210bcr/abl, it is necessary to plate 3.0 x 106 cells per 60 mm plate 24 hours prior to transfection. In general, the cells should be plated at a density so that they are 95-100% confluent at 24 hours post-transfection.
The addition of chloroquine to the medium appears to increase retroviral titer by approximately two fold.
This effect is presumably due to the lysosomal neutralizing activity of the chloroquine. It is extremely important that the length of chloroquine treatment does not exceed 12 hours. Longer periods of chloroquine treatment have a toxic effect on the cells causing a decrease in retroviral titers. The range for chloroquine treatment is 7-12 hours with 9-10 hours of treatment giving the best results. For purposes where achieving maximal retroviral titer is not necessary, such as when comparing the relative titers of different constructs, it may be preferable to omit chloroquine treatment. If chloroquine is not used, it is unnecessary to change the medium prior to transfection. On some occasions, we have obtained slightly improved transfection efficiencies by adding the chloroquine to a 1:1 mixture of 293 conditioned media (obtained from any of the 293-based cell lines) and fresh GM.
It is important that the pH of the Hepes be adjusted to 7.05 (within .05 units). Although, we generally add the HBS to the DNA/CaCl2 solution by bubbling, equivalent results can be obtained by adding the HBS to the DNA/CaCl2 solution and immediately inverting or vortexing the tube. The HBS/ DNA/CaCl2 solution should be added to the cells within 1-2 minutes of preparation. It is not only unnecessary to wait for the formation of a visible precipitate, but waiting this long (15-30 minutes) may have a detrimental effect on transfection efficiency and subsequent retroviral titers. In addition, the presence and/or amount of precipitate that one visualizes following transfection is not a reliable indicator of transfection success. We have used DNA prepared by both cesium chloride gradients and several commercial kits and have not found significant differences among titers generated with retroviral plasmids derived from the different preparation methods. It is unnecessary to perform additional phenol or precipitation steps prior to using the DNA. Up to a point, transfection efficiency and retroviral titers increase with increasing amounts of input DNA. The benefit of increasing the amount of input DNA must be weighed against our findings that this appears to have a direct toxic effect to the cell lines. If it is found that the amount of DNA is toxic to the cell line, it may be necessary to decrease the amount of input DNA or increase the number of plated cells.
In some experiments, we have introduced up to 15 µg of DNA to a 60 mm plate during transfection.
Incubator CO2 concentrations, which are outside the range of 4.5%-5.5%, may adversely affect transfection efficiency.

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Day 0: Preparation of Phoenix™ Retrovirus Producer cells for transfection.

At 18-24 hours prior to transfection, seed Phoenix™ cells at 1.5-2 x 106 cells per 60 mm plate in GM. After adding cells gently shake forward and backward, then side to side, 3-4 times to distribute cells evenly about the plate. Set plates in 37ºC incubator. Subconfluent cells (60-70%) are best suited for transfection and potentially generate the highest viral titer. Transfection efficiency of 50-60% should be achieved.

Day 1: Transfection

Transfect subconfluent Phoenix™ cells using either conventional cationic lipids or calcium phosphate protocols (see section VI). Applied about 5-10 µg of plasmid DNA (pBMN-GFP or

pBMN-Z) per 60 mm dish. Including **chloroquine** (25 mM chloroquine stock solution prepared in either PBS or GM and filtered through a 0.2 µM filter and stored at -20°C) at 25 µM during calcium phosphate transfection have been found to yield higher viral titer (2 folds). Transfections of PhoenixTM cells with cationic lipids have been found to work well. After transfection, cells should be incubated at 32 ºC for greater virus stability and thus, higher yield. A lower level of viral production will occur at 37 ºC.

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Day 2: 24 hours post-transfection
Because cells exhibit significant lethality if treated with chloroquine for more than 24 hours, overnight media should be replaced with 3 ml of fresh GM. If transfection was performed with cationic lipid, follow the manufacturer's instruction.

Day 3: Harvest at 48 hours post-transfection
Harvest supernatant from transfected Phoenix™ cells into 15 ml tubes and centrifuge at 2000 x g for 5 minutes to pellet cell debris. The virus stock can be frozen at -80 ºC for future use although the titer drops by one-half for each freeze-thaw cycle and thus, the virus stock should be stored in many small aliquots.

Orbigen do not recommend the use of ultracentrifugation to concentrate the retrovirus stock since hydrodynamic force generated during centrifugation can remove most of the viral glycoprotein and thus, the infectivity of virus particles. Transfection efficiency of transfected Phoenix™ cells should be determined and optimized since this parameter is the primary determinant of high virus yield. If the gene of interest is expressed from Orbigen's retroviral vector pBMN-GFP, the transfection efficiency can be easily monitored under fluorescent microscopy.

III. Determination of virus titer

III-1. Adherent cells

Day 0: Preparation of NIH3T3 cells for PhoenixTM retrovirus infection.
Split NIH 3T3 cells at 5x105 per 35 mm plate.

Day 1: Infecting cells with retroviral supernatant.
Perform a serial dilution of your virus stock (e.g. 10 fold dilution to 10-5) with **standard fibroblast medium** (**SFM**: DME containing 10% heat inactivated donor bovine serum, 100 U/ml Penicillin, 100 U/ml Streptomycin, 2 mM L-Glutamine) supplemented with 15 µg/ml of **polybrene** (*the stock concentration is 4 mg/ml dissolved in PBS and subsequently filtered through a 0.2 µM filter and stored at either 4°C or -20°C*). Infect NIH 3T3 cells on 35 mm plate with 1 ml of diluted stock for 12-24 hours at 37ºC.

Day 2-3: Assay the expression of gene of interest at 24-48 hours post-infection .
The actual reverse transcription and integration take place within 24-36 hours post-infection, depending on cell growth kinetics. Expression can be observed as early as 24 hours and reaching a maximum at 48 hours. Thereafter continued retroviral expression might drop over a period of weeks to months, depending on the cell line, the site of integration, and the relative toxicity of insert.

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III-2. Suspension cells

Day 0: Preparation of Jurkat cells for PhoenixTM retrovirus infection.
Culture Jurkat cells at log phase growth, approximately 5x105 cells/ml.

Day 1: Infecting cells with retroviral supernatant.

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Pellet Jurkat cells by a brief centrifugation at 500xg for 10 min. Resuspend 5x105 cells in 1 ml of fresh media containing diluted retroviral supernatant (>100 folds) and incubate for 12-24 hours at 37ºC.

IV. Infection of target cells.
Efficiency of retrovirus infection is specific to each cell type and can vary greatly.

IV-1. Infection of adherent fibroblasts

Day 0. Preparation of NIH3T3 cells for PhoenixTM retrovirus infection.
Approximately 12-18 hours prior to infection, plate 5 x 105 NIH3T3 cells in SFM (standard fibroblast medium) on a 100 mm plate.

Day 1. Infecting cells with retroviral supernatant.
Prepare a 3 ml infection cocktail consisting of retroviral-containing supernatant, polybrene at a final concentration of 4 µg/ml, and SFM.
Remove the SFM from the NIH3T3 cells and add the 3 ml infection cocktail to the cells and place into incubator for 3 hours.
Remove plate, add 7 ml of SFM to the cells, and continue incubation.

Day 3. Harvest (stain, drug select, etc.) the infected cells at 48 hours post infection.

IV-2A. Infection of non-adherent cells.
The conditions described are for infecting 60 mm plates.

Day 0. Preparation of non-adherent cells for PhoenixTM retrovirus infection.
Prepare an infection cocktail consisting of the medium in which the target cells are grown, retroviral supernatant and Polybrene (2 µg/ml) such that the total volume is 3 ml.

Day 1. Infecting cells with retroviral supernatant.
Centrifuge exponentially growing target cells at 500 x g for 5 minutes. Remove supernatant and resuspend the cells in the infection cocktail at a concentration of 105-106 cells per ml and add to 60 mm plate. Spin infect (spinoculate) cells for 90 min at 30°C at 2500 rpm. Remove virus supernatant and resuspend cells in 3 ml of appropriate media for growth of target cells. Incubate at 37°C.

Day 4. Refresh media.
Allow the cells to grow for 72 hours before drug selection or other assays, such as staining for lacZ activity or flow cytometry for the presence of green fluorescent protein (GFP).

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When working with non-adherent cells, one can add the retroviral supernatant directly to the cells or cocultivating the non-adherent cells with the retroviral producer cells. The advantage of the latter is that there is ongoing retroviral production; however, this must be weighed against the disadvantage of harvesting producer cells together with the target cells. Although we have not tried, it may be possible to minimize this problem by irradiating or mitomycin C treating the producer cells prior to co-cultivation. In general, we have obtained higher infection frequencies by co-cultivation.
For many non-adherent cells, achieving an optimal infection requires growth in the appropriate medium.
Because 293 cells and their derivatives (i.e., Phoenix cells) appear to tolerate many different medium bases and serum types, it is possible to alter the medium at 24 hours post transfection so that the resulting

retroviral supernatant will be harvested in the appropriate growth medium. When infecting with supernatants derived from 293 cells or derivatives, it should be remembered that these cells may provide a different cytokine/growth factor milieu than the NIH3T3-derived producer cells. A careful analysis of factor production by these cells has not been performed.

IV-2B. Infection of non-adherent cells by co-cultivation with retroviral producing cells.
Conditions are described for 60 mm plates.

Day 0. Preparation of PhoenixTM cells for retrovirus infection.
Transfect the PhoenixTM cells as described previously.

Day 1. Infecting cells with retroviral supernatant.
Twenty four hours post-transfection, prepare a 3 ml infection cocktail consisting of (i) Polybrene at a final concentration of 2 µg/ml, (ii) 1 ml of fresh or freshly thawed retroviral supernatant, and (iii) the non-adherent cells at a density of 105-106 cells per ml in the appropriate media for normal growth of the target cells. Remove the medium from the cells and gently add the infection cocktail to the cells (*Add the cocktail to the side of the plate rather than directly to the cells*). Follows spinoculation procedures described above. Return the cells to the incubator.

Day 3. Transfer target cells to fresh medium.
Forty eight hours post-infection, gently remove the medium, which will contain many non-adherent cells, and transfer to a conical tube. Centrifuge for 5 min at 500 x g. Remove the supernatant and gently resuspend the cell pellet in a freshly prepared infection cocktail as described previously. With extreme care to avoid disruption of the adherent cells, add the infection cocktail containing non-adherent cells to the wall of the plate rather than directly to the cells. Return the plate to the incubator.

Day 4. Transfer target cells to growth medium.
Seventy hours post-transfection, remove the non-adherent cells from the dish by gently pipetting. Centrifuge the cells (5 min, 500 x g) and resuspend in the appropriate media for normal growth of the target cells. Allow the cells to grow for an additional 24-48 hours before drug selection or other assays, such as staining for lacZ activity.
At this point, it is important that disturbance of the packaging cells is minimized. Use extreme care when removing the non-adherent cells from the packaging cells and do not wash the

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plate at this step. Also, it is not necessary to remove all of the non-adherent cells at this step since the purpose of this step is to add fresh medium and retroviral supernatant without losing non-adherent cells. Sufficient residual media remains on the plate to maintain the cells during the short centrifugation step described above. With longer centrifugation times, return the culture plates to the incubator.
The plate may be washed at this step; however, extreme care should be used so that the adherent cells do not detach. At this step, one is trying to achieve maximal removal of non-adherent cells. With this procedure, contamination by packaging cells is often less than 10%.

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V. References

Heemskerk, M. H. M., Blom, B., Nolan, G., Stegmann, A. P. A., Bakker, A. Q., Weijer, K., Res, P. C. M. & Spits, H. (1997) *J. Exp. Med.* 186, 1597-1602
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Mulligan, R.C. (1993) The basic science of gene therapy. *Science* 260, 926-932.
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VI. Calcium phosphate transfection protocol

Prepare the DNA in HBS for application to cells.
1. About 5 minutes prior to transfection, replace cell media with 3 ml fresh media supplemented with 25µM chloroquine for each 60 mm plate.
2. For transfection of cells on each 60 mm plate, in a 15 ml tube add the following reagents at room temperature 5-10 µg DNA (*DNA is added in a drop to side of tube*).
438 µ1 dd H2O (*wash the DNA to bottom of tube with water*).
61 µl 2M CaC12 (from Mallinkrodt catalog # 4160) -mix thoroughly with finger tapping.
500 µl total volume.
Scale volume and DNA/reagent amounts if necessary.
3. Quickly add 0.5mL 2xHBS to DNA/CaC12 mixture and combine by bubble vigorously with automatic pipettor (keep eject button depressed) for 3 - 15 sec (actual length of bubbling time depends on each batch of 2xHBS).
4. Immediately transfer 1 ml of HBS/DNA/CaC12 mixture drop wise onto 60 mm dish containing PhoenixTM cells in 3 ml of media and rock plate a few times to distribute DNA/CaPO4 particles evenly.
Observe the cells under a microscope; you should observe evenly distributed VERY small black particles.
5. Put plate(s) in 37°C incubator.

Stock Solutions
2 X HBS: 50mM HEPES (Sigma catalog # H-7006, sodium salt), pH 7.05; 10 mM KCl; 12 mM Dextrose; 280 mM NaCl; 1.5 mM Na2HPO4 (FW 141.96).
1. Make a stock solution of Na2HPO4 dibasic (5.25 g in 500 ml of water).
2. Make 2 x HBS: 8.0 g NaCl, 6.5 g HEPES, 10 ml Na2HPO4 stock solution.

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3. Adjust pH to 7.05 ± 0.05 using NaOH or HCl. Bring volume up to 500 mls. Check pH again.
4. Filter through a 0.2 µM filter, aliquot, and store at -20°C.
The pH is very important and it must be exactly 7.0; therefore, Orbigen recommends that the user make 3 stocks of 2XHBS at pH 6.95, pH 7.00, pH 7.05 and compare the transfection efficiencies generated witheach solution.
Try to avoid multiple freeze/thaw cycles. To thaw, warm to room temperature and invert or vortex the tube to achieve uniform mixing. Although it is unclear why this occurs, the ability of the 2xHBS solution to produce working $CaPO_4$ precipitates deteriorates after 6 months to one year, even when the 2xHBS solution is stored at -20°C.

2 M $CaCl_2$:
Prepare a 2M solution and filter through a 0.2 µM filter, aliquot, and store at -20°C.
All reagents should be at room temperature prior to use.

VII. X-gal Staining Protocol

Stock Solutions:

Fixative
PBS/0.05%
Glutaraldehyde (Glutaraldehyde is 25% stock/500x, from Sigma)

Staining solution
Add 40-50 µl X-Gal (40mg/ml in DMSO--store at –20°C in the dark) to 3 ml ferri/ferrocyanide solution

25X ferricyanide solution:
300mM $[K_3Fe(CN)_6]$ and 130mM $MgCl_2$ in ddH_2O

25X ferrocyanide solution:
300mM $[K_4Fe(CN)_6]$ in ddH_2O.
Keep these solutions away from light and store at 4°C.

Washing solution
PBS
For nonadherent cells add FCS to 1-5%

Procedure:
1. Remove media from adherent cells or spin down nonadherent cells in 15ml conical tube.
2. Add 2 ml fixative to 6cm plate of adherent cells or resuspend non-adherent cells in 1 ml fixative. Leave for 1 min.
3. For adherent cells, remove and wash 3xPBS (first two washes are quick, third is for 3 min).
4. For non-adherent cells, quench fixative by adding 5-lO ml PBS/1-5% FCS to conical tube and spin down again.
5. Layer 3ml staining solution onto adherent cells or resuspend non-adherent cells in 1 ml staining solution and place in well of 24 well plate.

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Optimal staining will occur 24 hours later. If longer, remove staining solution from cells at 24 hours and re-layer/resuspend in ferri/ferrocyanide solution without X-Gal.

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Milder Fixative:

2% Paraformaldehyde/0.2% Glutaraldehyde.
Combine 50 ml 4% paraformaldehyde solution with 49.2 ml 0.1M sodium phosphate pH 7.3 and 0.8 ml 25% glutaraldehyde. Store at 4 °C for up to 1 week.
Should be left on cells for 2 min and washed briefly 3x with PBS.

4% paraformaldehyde stock.
In fume hood, dissolve 8g powder in 150 ml of 0.1M sodium phosphate pH 7.3 (66mM NA2HPO4 or 33mM NAH2PO4), stirring and heating at 60°C.
Add 10N NaOH at rate of 1 drop/min until solution clears. Bring up volume to 200 ml with 0.1 M sodium phosphate pH 7.3. Store at 4°C for up to 1 month.

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