General Guidelines for Cell Immortalization

SV40 Cell Immortalization System
hTERT Cell Immortalization System
HPV E6/E7 Cell Immortalization System
EBV Cell Immortalization System
MycT58A Cell Immortalization System
RasV12 Cell Immortalization System
p53 Cell Immortalization System

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It has been well-documented that primary cells only undergo a pre-determined and finite number of cell divisions in culture (Stewart SA. 2002). After limited population doublings (the number of which varies by species, cell type, and culture conditions), primary cells enter a state where they can no longer divide. This state is called replicative senescence (Stewart SA. 2002, 2006). Replicative senescence is marked by distinct changes in cell morphology, gene expression, and metabolism. Morphological changes are often associated with increased cell size and the development of multiple nuclei. Activation of tumor suppressor proteins like p53, RB, and p16 are frequently seen gene expression changes. Changes in metabolism are commonly associated with increased lysosomal biogenesis as evidenced by over-expression of endogenous β-galactosidase (Ben-Porath I. 2004, 2005).

The Necessity of Cell Immortalization

As primary cells reach senescence after a limited number of population doublings, researchers frequently need to re-establish fresh cultures from explanted tissue - a tedious process which can also add significant variation from one preparation to another. In order to have consistent material throughout a research project, researchers need primary cells with an extended replicative capacity, or immortalized cells. The ideal immortalized cells are cells that are not only capable of extended proliferation, but also possess similar or identical genotype and phenotype to their parental tissue.

Strategies for Creating Immortal Cells

Several methods exist for immortalizing mammalian cells in culture conditions. One method is to use viral genes, such as the simian virus 40 (SV40) T antigen, to induce immortalization (Jha KK, 1998, Kirchhoff C 2004). SV40 T antigen has been shown to be the simplest and most reliable agent for the immortalization of many different cell types and the mechanism of SV40 T antigen in cell immortalization is relatively well understood (Lundberg AS, 2000). Recent studies have also shown that SV40 T antigen can induce Telomerase activity in the infected cells.

The most recently discovered approach to cell immortalization is through the expression of Telomerase Reverse Transcriptase protein (TERT), particularly for cells that are most affected by telomere length, such as human cells (Lundberg AS. 2005; Fridman AL, 2008). This protein is inactive in most somatic cells, but when hTERT is exogenously expressed, the cells are able to maintain sufficient telomere lengths to avoid replicative senescence. Analysis of several telomerase immortalized cell lines has verified that the cells immortalized by hTERT over expression maintain a stable genotype and retain critical phenotypic markers.

Cell Immortalization Systems

However, over-expression of hTERT in some cell types (especially in epithelial cells) fails to induce cell immortalization and may induce cell death. Recent studies have found that co-expression of hTERT catalytic subunit with either p53 or RB siRNA can immortalize human primary ovarian epithelial cells, providing more authentic and normal cell model with well-defined genetic background (Yang G. Carcinogenesis 2007; Yang G., Oncogene 2007). Likewise, over expression of Ras or Myc T58A mutants have also been found to be able to immortalize some primary cell types (Sears R. 2000). For the most part, viral genes achieve immortalization by inactivating the tumor suppressor genes (p53, Rb, and others) that can induce a replicative senescent state in cells (Lundberg AS. 2000).

With years of experience in cell immortalization, scientists at **abm Inc.** have developed a comprehensive cell immortalization product line that is comprised of retroviral, lentiviral and adenoviral vectors for hTERT, p53, RB, siRNA and SV40 T antigens. All of these tools will make your cell immortalization project simpler and easier than ever before.

General Guidelines for Cell Immortalization

Cell immortalization is a very complicated cellular process and the exact biological mechanisms are still largely not well understood. However, over the years of cell immortalization over a diverse range primary cells, scientists have observed:

1) Based on their *in vitro* culture growth patterns, there are essentially two catagories of primary cells: ones that can be cultured for 20-50 passages before senescence and ones that can only be passed fewer than 10 passages before senescence.

2) Primary cells that have a life span of 20-50 passages under *in vitro* culture conditions include mostly blast cells, such as fibroblasts and retinoblasts. Cells that have a life span of less than 10 passages under *in vitro* culture conditions are mostly epithelial cells, such as breast and ovarian epithelial cells.

3) To immortalize your primary cells, you can use either hTERT or SV40 T antigens for cells that can be cultured for over 10 passages. It is recommended that SV40 T antigens be used for difficult–to–immortalize primary cells such as epithelial cells. Also, a combination expression of Rb or p53 siRNA and hTERT can be used if a more defined genetic background of immortalized cells are required.

General Guidelines for Cell Immortalization

4) It has been shown that the introduction of hTERT may induce apoptosis in primary epithelial cells and other cells that have a life span of less than 10 passages. It is recommended to use SV40 T antigens for these cells. In many epithelial cells, epithelial growth factor (EGF) has been shown to be able to increase their life span to 10-20 passages before senescence (Ahmed N. 2006). Thus, one may try to add some recombinant EGF (10 ng/ml) to expand your cell life span before hTERT gene transduction.

5) For some primary cell types, it has been shown that over-expression of SV40 T antigen or hTERT alone is not sufficient for successful immortalization. However, a combinational expression of SV40 T antigen and hTERT or other genes have been shown to be effective in those cells (Matsumura T. 2004).

6) After transduction, drug selection is generally unnecessary for primary cells that have less than 10 population doublings as the immortalization process will select for the clones capable of growing indefinitely.

General Guidelines about Viral Vectors

Primary cells are known to be resistant to transfection, but receptive to recombinant viral vector transduction, especially adenoviral and lentiviral vectors. To facilitate cell immortalization, scientists at **abm** Inc. have developed a comprehensive range of ready-to-use viral vectors for cell immortalization. The following sections outline their basic features :

Recombinant Adenoviral Vectors

Recombinant adenoviral vectors are proven to be the most efficient viral vector developed to date for delivery of recombinant genes. All types of human cells (except blood cells which lack the adenovirus receptor) can be transduced with adenoviral vectors at 100% efficiency. However, adenoviral vectors will not integrate into target cell genome, giving rise to only transient transgene expression. Vector DNA will eventually be degraded in host cells or diluted with each subsequent cell division. Therefore, primary cells transduced with Adeno-SV40 or Adeno-hTERT are only expected to express SV40 T antigen or hTERT for 1-2 weeks, depending on the rate of cell division.

Recombinant Retroviral Vectors

Retroviral vectors are only capable of transducing actively dividing cells, as they cannot actively transport across the nuclear membrane. During cell division, the nuclear membrane is disintegrated and thus the viral DNA can access the genome. Once the nucleus has been bypassed, retroviruses integrate into the host genome efficiently, giving rise to permanent and stable gene expression. However, the transduction efficiency of target cells using retroviral vectors is low, especially in slowly dividing primary cells.

General Guidelines about Viral Vectors

Recombinant Lentiviral Vectors

Newly developed lentiviral vectors can be used to transduce both dividing and non-dividing cells as lentiviral vectors can actively pass though nuclei membrane. In addition (as in the case of retroviral vectors), lentiviral vectors will integrate into a host cell genome once inside the nucleus. Thus, lentiviral vectors are gaining popularity for both *in vitro* and *in vivo* applications of gene transduction.

One disadvantage associated with lentiviral vectors is the insert size limitation. For most lentiviral vectors developed, the maximum insert size is 5.0 kb. An insert size less than 3.0 kb can be efficiently packaged at a high titer in 293T cells. Viral titers will be significantly decreased by inserts longer than 3.0kb. As the SV40 genome is over 5.0kb, the expected Lenti-SV40 titer is relatively low.

Selection Markers

Vector	Selection Marker
pLenti-MycT58A	Neomycin
iLenti-p53, iLenti-Rb	Neomycin
pLenti-RasV12	Neomycin
pAdeno-SV40	No Selection Marker
pRetro-E2-SV40	No Selection Marker
pLenti-SV40	No Selection Marker
pLenti-SV40-T, pLenti-SV40-Tt	Puromycin
pLenti-HPV-16 E6/E7	Puromycin
pLenti-hTERT,	Puromycin
pLenti-hTERT Antisense	Neomycin
pLenti-EF1a-hTERT-YFP, pLenti-EF1a-hTERT-RFP	Puromycin
pAdeno-hTERT, pAdeno-hTERT Antisense	No Selection Marker
pRetro-E1-hTERT	Puromycin

Note: Antibiotic selection should not be necessary. Non-immortalized cells will die after several passage rounds as they will reach senescence. In these cases, the passaging of the cells can be considered as the selection process. If antibiotics must be used, the optimal concentrations will need to be evaluated by the end user for their cell type.

Lentiviral and Retroviral Protocol

The following procedure outlines how to utilize retroviral and lentiviral vectors to infect target cells with cell immortalizing genes. If you are using an adenoviral vector please continue on to the following section.

1) Thaw the recombinant virus supernatant in a 37°C water bath and remove it from the bath immediately when thawed. After first thaw, we recommend that the remaining supernatant be aliquotted for future use since the viral titer

will decrease significantly with each freeze-thaw.

2) Prepare Polybrene stock to a concentration of 0.8 mg/ml.

3) In the morning, infect the target cells in a 6-well plate with 2 ml/well supernatant in the presence of 2-10 µg/ml Polybrene. Place the remainder of the viral supernatant in the fridge for the second infection in the afternoon. Note: Polybrene is a polycation that neutralizes charge interactions to increase binding between the pseudoviral capsid and the cellular membrane. The optimal concentration of Polybrene depends on cell type and may need to be empirically determined (usually in the range of 2–10 µg/ml). Excessive exposure to Polybrene (>12 hr) can be toxic to some cells.

4) 6-8 hours later, remove the viral supernatant (from first infection) from the wells and re-infect cells with 2 ml/well of fresh supernatant (with polybrene).

5) For Lentiviral vectors, one infection (incubate overnight) works well for most target cells. Dilute Lentiviral vector with fresh complete medium (1:1) if cytotoxicity is a problem.

6) The next day, remove viral supernatant and add the appropriate complete growth medium to the cells and incubate at 37°C.

7) After 72 hours incubation, subculture the cells into 2 x 100mm dishes and add the appropriate selection drug (if applicable) for stable cell-line generation.

8) 10-15 days after selection, pick clones for expansion and screen for positive transgene expression (i.e. Western blotting, RT-PCR).

Adenoviral Protocol

The following procedure outlines how to utilize adenoviral vectors to infect target cells with cell immortalizing genes. If you are using a retroviral or lentiviral vector, please refer to the previous section.

When you place your order for adenovirus, we will ship you seed stock of adenoviral vector at a minumum 250 µl (10^6 pfu/ml). With the seed stock, you can amplify as much adenovirus as you require using the following protocol:

1. When you receive your recombinant adenovirus, make two to three aliquots and use one for amplification in 293 cells. Freeze the remaining aliquots in -70°C as a seed stock for future use.

2. Amplify your adenovirus in HEK 293 cells, plated at 60-70 % confluency. For a 60 mm dish, infect the cells with 70 µl of the adenovirus, for a 100 mm dish, infect the cells with 200 µl of virus.

3. When more than 95% of 293 cells are detached from the dishes (4-5 days later), collect both the cells and medium into a large falcon tube.

4. Freeze (in a -70°C freezer or dry ice / ethanol) and thaw (in a 37°C water bath) the collection three times.

5. Pellet the cell debris by centrifugation at 3,000 rpm at room temperature for 10 minutes.

6. Transfer the supernatant into a fresh tube. Store at 4°C for short-term use (two to three weeks) or add glycerol to a final concentration of 10% and freeze at -70°C (stable for one to two years).

7. Use the supernatant to infect your target cells. Subsequently analyze your transgene expression by Western blot or RT-PCR.

Target cells can be transduced by recombinant adenoviral vectors either by viral supernatant or purified adenovirus. For most *in vitro* applications, target cells can be transduced at 100% efficiency with viral supernatant without further concentration. However, purified high titer of adenoviral preparations are necessary for *in vivo* applications, which require viral preparations free of FBS or other contaminants. Please refer to our Adeno Add-N-Pure products for detailed information on large-scale adenoviral vector purification (Cat. No. A902).

Adenovirus Transduction Protocol

a) Prepare target cells in a 6-well plate or a 10cm dish at 70% confluency at the time of transduction.
b) Aspirate the culture medium and overlay with viral culture supernatant (1ml for 6 well plate and 4-5 ml for 10 cm dishes) to cover the cells for 6-8 hours in a 37°C incubator.
c) After 6-8 hours, remove the medium containing the virus and replace it with fresh, complete medium.
d) Gene transduction can be evaluated 48-72 hrs after transduction via different assays, such as Western blot, qPCR analysis or microscope observation if a fluorescent reporter gene is present.

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