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Longer Life to Cells in Culture with TERT Immortalization Products

Cultivating animal cells in the laboratory is an indispensable technique for cell biologists. However, most normal primary cell lines, while faithfully reproducing the phenotype of their tissue of origin, do not grow indefinitely in culture. After a series of population doublings (the number of which varies by species, cell type, and culture conditions) primary cells enter a state where they no longer divide. This state is called *replicative senescence*.

Replicative senescence is marked by distinct changes in cell morphology, gene expression, and metabolism and can be induced by extrinsic factors, intrinsic factors, or both. Extrinsic factors, irradiation, oxidative stress, or hostile cell culture environments bring about senescence by triggering the activation of various tumor suppressor proteins, including p53, Rb, and P16/INK4A. Intrinsically, the telomeric ends of chromosomes shorten with each mitotic cycle and eventually the short or uncapped ends activate these

same tumor suppressor proteins, inducing senescence. Evidence suggests that this telomere shortening process serves as a counting mechanism to limit the absolute number of cell divisions and in human cells may serve as a tumor suppressor mechanism.

Why is immortalization necessary?

Because 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. To use the same consistent material throughout a research project, researchers need primary cells with an extended replicative capacity, or *immortalized* cells.

Some cells immortalize spontaneously by passing through replicative senescence and thus easily adapt to life in culture.

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Use of Repetitive-Sequence-Based PCR for Genotyping Archaea

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Most methods for characterizing prokaryotes have been developed primarily for bacteria and not archaea. As a result, the techniques tend to be specific for bacteria (fatty acid analysis) or laborious to perform on archaea (ribotyping). With increasing numbers of archaea being described there is a need for rapid, economical identification methods that have phylogenetic breadth. Sequencing of the small subunit ribosomal gene (SSU rDNA) is the standard method for genotypic identification of archaea to the genus and species level; however, SSU rDNA does not often provide reliable strain-level discrimination within a species.

Repetitive-sequence-based PCR (rep-PCR) is a genotyping technique that has been used for over 10 years on a variety of bacteria with much success (1, 2). This method takes advantage of short repetitive oligonucleotide elements that are scattered throughout the genomes of prokaryotes. By using PCR primers specific for these repetitive sequences it is possible to amplify the intervening fragments of DNA. When these DNA fragments are separated electrophoretically they form a fingerprint or barcode that is unique for a given strain. Rep-PCR has become

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Laboratories around the world have sequenced the genomes of a wide range of microbes. ATCC has cultures and genomic DNA from many of these fully sequenced strains. Each of the items below is \$162 with the exception of *Cryptosporidium parvum* (\$180). Order online or call 800-638-6597.

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<i>Bartonella henselae</i> Houston-1	49882™	49882D	<i>Pyrococcus furiosus</i> DSM 3638	43587™	43587D
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<i>Borrelia burgdorferi</i> B31	35210™	35210D	<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Typhi	700931™	700931D
<i>Campylobacter jejuni</i> NCTC 11168	700819™	700819D	<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Typhimurium	700720™	700720D
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<i>Legionella pneumophila</i> subsp. <i>pneumophila</i> Philadelphia 1	33152™	33152D	<i>Xanthomonas campestris</i> pv. <i>campestris</i>	33913™	33913D
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<i>Methanococcus jannaschii</i> DSM2661	43067™	43067D			
<i>Methanococcus maripaludis</i>	43000™	43000D			
<i>Methanosarcina mazei</i> Go1	BAA-159™	BAA-159D			

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Characteristics of the BG01V Human Embryonic Stem Cell Line

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Human embryonic stem (ES) cells have become an increasingly visible and valuable tool in the field of biomedical research, due to their utility as an *in vitro* system for studying developmental biology and drug discovery. More importantly, human embryonic stem cells are a potential source of clinically transplantable cells for treating diseases such as Parkinson's and diabetes.

However, human ES cells pose several technical challenges in the laboratory. They can be difficult to culture, and some ES cell lines fail to remain undifferentiated after serial subculturing.

Most human embryonic stem cell lines can be cultured for many passages without genetic changes (1). However, independent laboratories have observed karyotypic abnormalities in a few human stem cell lines after long-term culture (2).

A karyotypic change in the human ES cell line BG01 derived by BresaGen Inc. (Athens, GA) gave rise to BG01V line (ATCC® SCRC-2002™) with trisomy 12 and 17 and XXY karyotype. These cells are significantly easier to culture than most other human ES stem cells.

We characterized the BG01V cell line for pluripotency, expression of stem cell markers, and differentiation into three germ layers.

Materials and Methods

Immunocytochemistry. Human stem cell markers Oct-3/4 (BD Transduction Laboratories) and SSEA-4, TRA-1-60, and TRA-1-81 (Chemicon) were immunostained on undif-

ferentiated BG01V colonies using fluorescent secondary antibody Alexa Fluor® 488 dye (Invitrogen).

Differentiation. BG01V cells were cultured on mitomycin C-treated mouse embryonic fibroblasts (MEFs, ATCC® SCRC-1040.2) for 3 to 4 days until well-defined colonies were seen. Colonies were dissociated from the culture flask with 200 units/ml collagenase IV (Invitrogen). The suspension of BG01V cell clumps and MEFs was transferred to a flask and incubated for 30 minutes until the majority of the MEFs had attached. The BG01V cell clumps were removed from the flask with a pipette and seeded in low attachment culture dishes. The embryoid bodies were grown for 2 weeks in medium without bFGF.

Presence of germ layers. Two-week-old embryoid bodies were dissociated into single cells with collagenase IV and fixed with 2% paraformaldehyde at room temperature for 20 minutes. The cells were stained for the following markers: stem cell marker Oct-3/4, ectoderm marker GFAP, endoderm marker Troma, and mesoderm marker alpha-actinin. The presence of each marker was evaluated with FACSCalibur™ flow cytometer (BD Biosciences). Undifferentiated BG01V cells were analyzed as a control.

Results

BG01V colonies proliferate significantly faster than normal ES cells. They exhibit normal morphology and are able to expand to relatively large size without spontaneous differentiation. All major stem cell markers are expressed in undifferentiated BG01V colonies (Figure 1).

Furthermore, BG01V cells are able to form embryoid bodies (EB) and express three germ layers. Stem cell marker Oct-3/4 expression was reduced from 72% in undifferentiated sample to virtually no expression in EBs. Ectoderm marker GFAP had a slight increase from 6% to 17%. Endoderm marker Troma had a significant increase in intensity, and mesoderm marker alpha-actinin had a marginal increase.

Because BG01V cells are easy to culture and they retain crucial markers, they are a potentially useful model for studying human embryonic stem cells. Further studies that can be performed to characterize these cells include teratoma formation of undifferentiated BG01V cells (now being tested at BresaGen) and RT-PCR to detect the presence of certain cell types in differentiated EBs.

This study was presented as a poster at the 2004 meeting of the American Society for Cell Biology.

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SCRC-2002™	1 vial	\$600

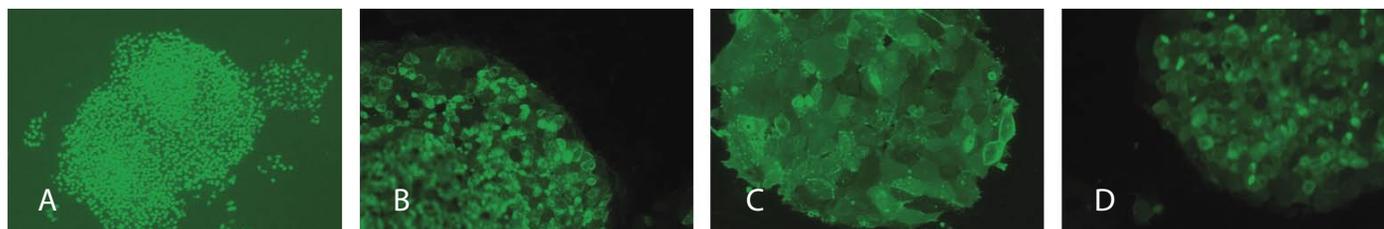


Figure 1. Human embryonic stem cell markers in BG01V cells. A. Oct3/4. B. TRA-1-60. C. SSEA-4. D. TRA-1-81.

Rep-PCR

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a standard method for rapidly genotyping bacterial strains and providing good resolution among multiple strains within a single species.

We evaluated a rep-PCR kit from Spectral Genomics, Inc., that has been optimized for genotyping archaea. The method involves extracting DNA from the source organisms, performing a PCR-based amplification using archaeal primers provided in the kit, and then separating the amplicon based on size using an Agilent Lab-on-a-Chip DNA analyzer. Results were analyzed and compared using software from Spectral Genomics. In this study we evaluated the system for its ability to characterize halophiles and methanogens.

Materials and Methods

Bacterial strains. All strains were obtained from ATCC and propagated on recommended media under described conditions. Media formulations and growth conditions can be found in the catalog description on the ATCC Web site at www.atcc.org.

Genomic DNA extraction. Cells were harvested during late log phase from 1.8 to 10 ml of broth or a few colonies from an agar plate. Genomic DNA was extracted according to procedures described in the Ultra Clean™ microbial DNA isolation kit (Mo Bio Laboratories, Inc.).

Repetitive-sequenced-based PCR. The PCR step was carried out using standard PCR tubes and a GeneAmp® 9700 thermal cycler (Applied Biosystems). The PCR reagents were supplied in the DiversiLab Archaeobacteria Kit, except for AmpliTaq® DNA polymerase

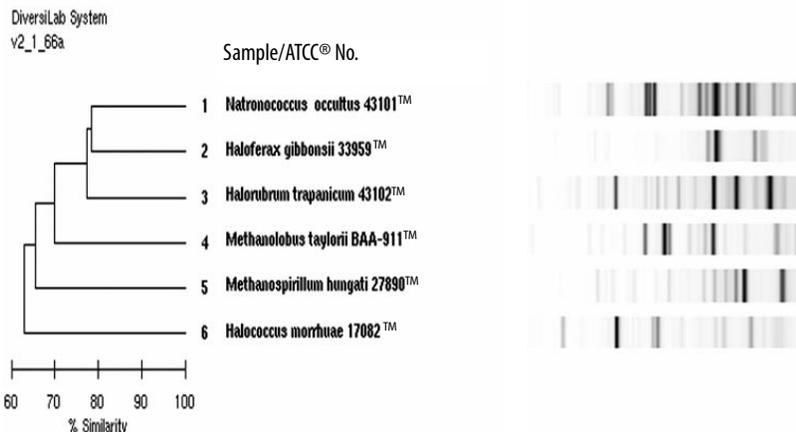


Figure 2. Rep-PCR results for representative strains of archaea.

from Applied Biosystems. Each sample was processed using approximately 100 ng/ μ l genomic DNA and subjected to thermal cycler conditions (35 cycles) according to product instructions.

Separation of amplified DNA. The DiversiLab Fingerprinting Kit includes reagents and LabChip® products (Caliper Life Sciences), which are microfluidic devices for performing electrophoretic separation of DNA fragments based on size. A gel matrix is added to the chip, which has 12 sample wells and one control well. Each sample well received 1 μ l of PCR product and 5 μ l of a standard mix containing high- and low-molecular-weight DNA markers. The control well contained 1 μ l of a DNA ladder mix and 5 μ l of the marker. The LabChip was placed in the Agilent 2100 Bioanalyzer, which includes a laser that can detect the fluorescently labeled DNA to a resolution of one base pair.

DiversiLab analysis. Information from the Bioanalyzer was fed directly into a computer where it was tracked in real time and a sample file created for each chip. After a run was complete, the results were uploaded to the Spectral Genomics Web site for analysis. The software performs statistical quality control on the data from the LabChip, creates libraries, and generates dendrograms based on percent similarity using UPGMA analysis. The entire process from DNA extraction to finished analysis can be completed in 5 hours.

Results

Our study focused on methanogens and extreme halophiles; however, other archaea (including *Thermococcus* and *Sulfolobus*) have been analyzed as well. In total, over 70 different strains representing more than 31 genera of *Crenarchaeota* and *Euryarchaeota* were analyzed (Table 1). Results of the rep-PCR analysis for individual strains have proven to be very reproducible. An example is shown in Figure 1, where five separate cultures of *Methanohalobium evestigatum* were grown up and analyzed at different times. The different batches shared >96% similarity. This reproducibility is essential to ATCC's authentication process, where we typically genotype the original material sent in by the depositor and then compare it to lots prepared at ATCC for seed and distribution.

Sixteen genera of methanogens that included over 24 species were analyzed and all yielded unique genotypes. For halophiles, 27 species in 11 genera and were analyzed and all yielded unique genotypes. The rep-PCR DNA



Figure 1. Reproducibility of different cultures of ATCC® BAA-1072™ *Methanohalobium evestigatum*.

fingerprints for a representative group of halophiles and methanogens is shown in Figure 2.

Rep-PCR was able to differentiate multiple strains of the same species. An example comparing four strains of *Methanosarcina mazei* is shown in Figure 3. Analysis of seven different strains of *Halobacterium salinarium* and four strains of *Methanothermobacter thermautotrophicus* showed that each produced a unique genotype. Analysis of several unknown *Halobacterium* spp. also indicated each was unique, although the clustering of some strains suggests that they are closely related (results not shown).

There was little systematic inference that could be made from dendrograms comparing different strains, species, and genera of archaea based on UPGMA cluster analysis. While some closely related strains did cluster together, intermixing of methanogens and halophiles was common.

Conclusions

The rep-PCR system genotyped a wide variety of archaea, providing a rapid, effective method for authentication. Strains within a species were also reliably distinguished, and fingerprint patterns for the same strain

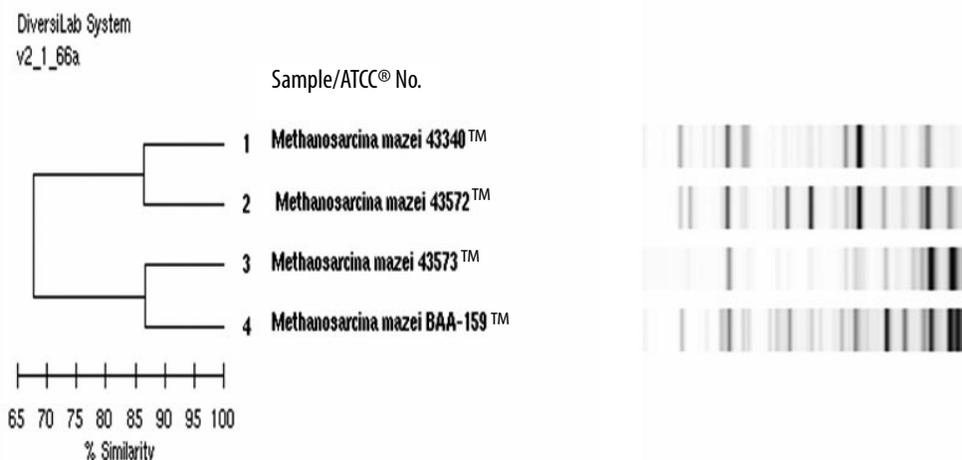


Figure 3. Comparison of four strains of *Methanosarcina mazei*.

were highly reproducible between rep-PCR reactions and multiple LabChips. Based on our results, rep-PCR was a useful tool for the genotyping and strain identification of archaea.

Acknowledgement

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The Polymerase Chain Reaction (PCR) process is covered by patents owned by Roche Molecular Systems, Inc. Use of the process requires a license.

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ATCC and Ardais To Deliver Scalable Biospecimen Management

ATCC and Ardais Corporation have announced a strategic partnership to offer scalable biospecimen management services, addressing the escalating challenges faced by biotechnology, pharmaceutical, and academic institutions in managing biospecimens for drug development.

This partnership will combine ATCC's expertise in biological materials handling with Ardais' expertise in logistics, information technology, and clinical data solutions. "Translational medicine requires large-scale collection, management, and storage of clinically annotated biospecimens," said Donald B. Hawthorne, President and CEO of Ardais Corporation. "With its 80 years of biological

materials handling expertise, ATCC has industrialized biospecimen storage, processing, and distribution. As adoption of the Ardais System accelerates, there will be a greater need for solutions of the scope offered by ATCC."

Biospecimen management is vital for generating and correlating the clinical and molecular data necessary to develop targeted diagnostics, therapeutics, and preventatives. The Ardais System addresses the need for large-scale, systematized, and standardized collection of biospecimens and associated donor- and sample-level clinical and molecular data. The System provides end-to-end support—from donor enrollment and informed consent through sample collection, process-

ing and storage—as well as collection of associated clinical data. It also provides real-time tracking of the chain-of-custody for every biospecimen.

"As the world's leading biological resource center, ATCC offers the scientific community a broad range of products and services for the advancement, validation, and application of scientific knowledge," said Frank Simione, Vice President of Management and Compliance Services at ATCC. "Our partnership with Ardais will add value to our offerings in the form of sophisticated logistics, information technology, and clinical data solutions for biospecimen management."

Immortalization

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However, these spontaneously immortalized cells invariably have unstable genotypes and are host to numerous genetic mutations, rendering them less reliable representatives of their starting tissue's phenotype.

The ideal immortalization protocol, therefore, would produce cells that are not only capable of extended proliferation, but also possess the same genotype and tissue markers of their parental tissue.

How can cells be made immortal?

Several methods exist for immortalizing mammalian cells in culture. Viral genes, including Epstein-Barr virus (EBV), Simian virus 40 (SV40) T antigen, adenovirus E1A and E1B, and human papillomavirus (HPV) E6 and E7 can induce immortalization by a process known as **viral transformation**. Although the process is reliable and relatively simple, these cells may become genetically unstable (aneuploid) and lose the properties of primary cells. For the most part, these viral genes achieve immortalization by inactivating the tumor suppressor genes that put cells into a replicative senescent state.

The preferred method to immortalize cells is through expression of the **telomerase reverse transcriptase** protein (TERT), particularly those cells most affected by telomere length (e.g., human). This protein is inactive in most somatic cells, but when *hTERT* is exogenously expressed the cells are able to maintain telomere lengths sufficient to avoid replicative senescence. Analysis of several telomerase-immortalized cell lines has verified that the cells maintain a stable genotype and retain critical phenotypic markers.

What products are available from ATCC?

A new group of products from ATCC includes TERT-immortalized cell lines and associated molecular tools. A eukaryotic expression plasmid containing the *hTERT* cDNA (catalog number MBA-141) will enable researchers to immortalize their own cells. In addition, two TERT-immortalized cell lines are also available: ATCC®CRL-4000™ *hTERT* RPE-1 (human retinal pigmented epithelium) and ATCC®CRL-4001™ BJ-5ta (human foreskin fibroblast)

These cells are tested for extended proliferative capacity, stable genotype, the presence

of selected phenotypic markers, and the continued expression of the *hTERT* protein. More cell lines and other immortalization tools are planned. Look for the cell immortalization link on our home page at www.atcc.org for details.

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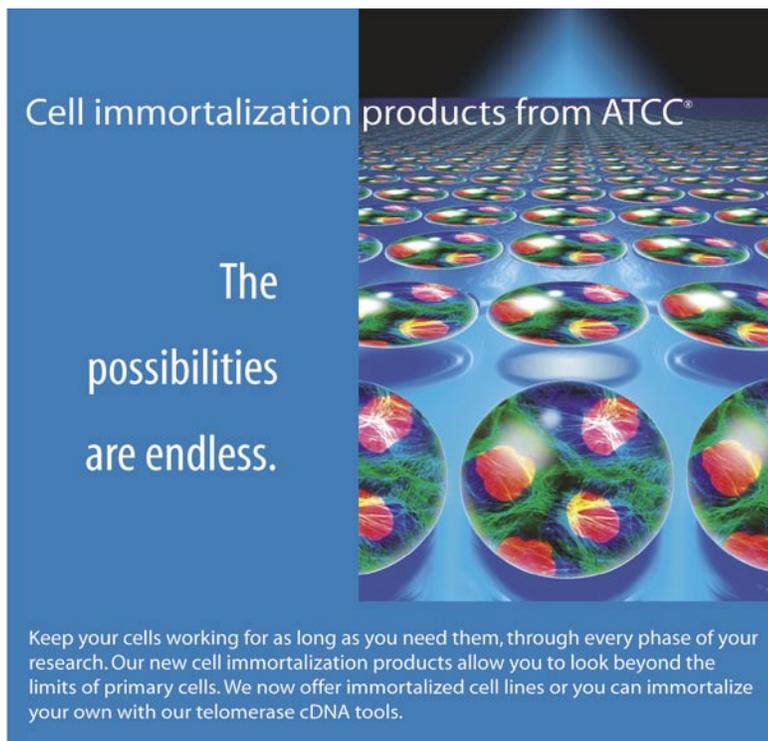
For a more thorough review of cellular senescence and telomerase immortalization we recommend the following references:

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These materials are subject to claims under U.S. Patent Nos. 6,261,836 and 6,337,200, other pending patent applications, and foreign counterparts thereof. They are provided under the ATCC® Material Transfer Agreement as posted on the ATCC Web site (www.atcc.org) and the terms of the Addendum for Commercial and For-Profit Organizations or the Addendum for Noncommercial and Academic Organizations, which are linked to the product's ATCC catalog number on the ATCC Web site.

The TERT-containing plasmid is not available to commercial and for-profit organizations or for work to be conducted under funding from a commercial organization.

Description	ATCC® No.
<i>hTERT</i> RPE-1 cell line	CRL-4000™
BJ-5ta cell line	CRL-4001™
pGRN145 plasmid	MBA-141



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Tech Qs Preparation Is The Key to Culturing New Cells

Many precautions are in place to ensure the safe arrival of a frozen ATCC cell line. The vials are handled carefully and the packaging is specially designed to keep them at a stable temperature for the duration of the trip. How you handle the cell culture *after* it arrives at your lab may be critical to ensuring its viability.

For best results, we recommend that you initiate the culture immediately upon receipt. This means that prior to receiving your cells you should assemble the medium, serum, and reagents required for their growth. Many of these supplies are available from ATCC and can be ordered and shipped along with your cell lines.

Complete instructions detailing the proper handling of an ATCC cell lines are provided in the product information sheet accompanying the cells during shipment. An abbreviated version of this information is also available on our Web site.

If you are unable to grow the cells immediately, place the vial in the vapor phase of a liquid nitrogen unit upon arrival. **Do not store frozen cells in a -80°C mechanical freezer.** This is a common error that may result in poor recovery. Many frozen cell lines are sensitive to damage at -80°C or warmer, and cell viability may decrease over time when stored at this temperature.

For safety reasons do not immerse vials directly in liquid nitrogen. There is the possibility that some liquid will enter the vials, which could explode when the nitrogen converts to gas upon warming. Holding vials in the vapor phase above the liquid safely keeps the contents at sufficiently low temperatures.

Initiating Frozen Cultures

The culture in the cryopreservation vial must be thawed rapidly and then immediately combined with complete culture medium and seeded into the appropriate vessel. For cells grown in monolayers we recommend starting with tissue-culture-grade flasks; cells can be recovered from cryopreservation directly into multiwell plates but the results are not as consistent.

1. Prepare a culture vessel so that it contains the recommended volume of the appropriate culture medium as listed on the product information sheet. Equilibrate for temperature and pH (CO_2) by placing flasks in the incubator for 30 minutes prior to adding cells.
2. Thaw the vial by gentle agitation in a water bath at 37°C or at the normal growth temperature for the cell line. Thawing should be rapid, approximately 2 minutes.
3. Remove the vial from the water bath as soon as the contents are thawed and decontaminate the vial by dipping in

or spraying with 70% ethanol. All of the operations from this point on should be carried out in a laminar flow tissue culture hood under strict aseptic conditions.

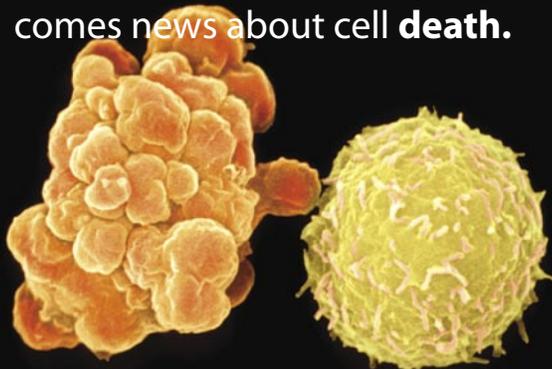
4. Hold the cap of the vial with a sterile square of gauze. Unscrew the cap, transfer the contents to a sterile centrifuge tube and dilute with the recommended medium. Remove the cryoprotectant agent by gentle centrifugation (5 to 10 minutes at $125 \times g$). Discard the supernatant and resuspend the cells in 1 or 2 ml of complete growth medium. Transfer the cell suspension to the prepared culture vessel and mix thoroughly.
5. Examine the cultures after 24 to 48 hours and subculture as needed.

Some cell lines, such as hybridomas, take several days before they fully recover from cryopreservation. Indeed, some hybridomas appear dead the first day in culture and will generate a lot of cellular debris. Viability for most cells declines after freezing and reaches a low point at 24 hours post-thaw. After this point, cells begin to recover and enter exponential growth.

The technical information section of our Web site offers tips on cell culture media and subculturing. Find it by clicking on the tab at the top of our home page at www.atcc.org. You can also contact our technical service department at tech@atcc.org.

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AfCS Constructs from ATCC

The Alliance for Cellular Signaling (AfCS) is a consortium whose goal is to understand how cells interpret signals in a context-dependent manner, including identifying the proteins involved in various signaling systems and assessing how information flows through the systems. AfCS investigators have produced thousands of molecular constructs that are publicly available exclusively through ATCC.

Mouse cDNA Clones

Using Invitrogen Gateway® technology, the AfCS has generated thousands of DNA constructs through different combinations of 1500 cloned, sequence-verified gene sequences and 200 unique parent vectors. cDNAs are available either as entry clones (ready for Gateway-based subcloning to various expression platforms) or as ready-to-use expression vectors with fluorescent protein tags.

Parent Vectors

Over 200 parent vectors are available, including vectors for tagging expressed proteins and lentiviral vectors for expression of short hairpin RNAs (shRNAs).

RNAi Vectors

Two options are available. One set of vectors permits straightforward cloning of shRNAs against specific genes and simple subcloning of shRNA expression cassettes into a variety of expression platforms. There are also over 100 vectors that already contain shRNAs validated in AfCS studies against specific signaling genes.

AfCS and the Nature Publishing Group maintain a comprehensive Web site at www.signaling-gateway.org where you can browse the list of plasmid constructs and perform BLAST or keyword searching. New materials are continually added to the ATCC collection. Visit the molecular genomics section of the ATCC Web site at www.atcc.org and use the keyword search to find AfCS constructs.

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