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Infinity™ Telomerase-Immortalized Cell Line Culturing Guide

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Catalog #: C4000-1 hTERT-RPE1 Cell Line
C4001-1 hTERT-BJ1 Cell Line

Storage conditions: -150°C

FOR RESEARCH USE ONLY

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I. Introduction

Infinity™ telomerase-immortalized cell lines express exogenous hTERT (human telomerase reverse transcriptase). The introduction of hTERT into these cells produces a stable cell line with an indefinite life span and a normal phenotype. As such, they are an attractive replacement for primary and transformed cell lines and are ideal for studying the long-term biochemical and physiological aspects of cell growth. Furthermore, Infinity cells can be modified by repeated rounds of genetic engineering, enhancing their utility in gene expression studies, cell-based drug screening, and drug toxicity testing. These cells were developed through an alliance between CLONTECH and Geron Corporation. They are the first commercially available immortalized normal human cell lines.

Telomere length controls cellular life span

Telomeres are DNA repeat sequences that stabilize chromosome ends. This stabilization protects chromosomes from degradation by enzymes that repair exposed DNA (Blackburn, 1991; Greider, 1996). The absence of telomeres would lead to chromosome fusion and massive genomic instability. Telomeres also regulate the number of divisions a cell undergoes: as somatic cells divide, their telomeres slowly get shorter (Figure 1). At a critical telomere length, somatic cells enter a nondividing state termed senescence (Bodnar *et al.*, 1998; Harley *et al.*, 1990). In contrast, germline cells have an extended life span and do not undergo senescence. Unlike somatic cells, germline cells actively express telomerase, which maintains their telomere lengths.

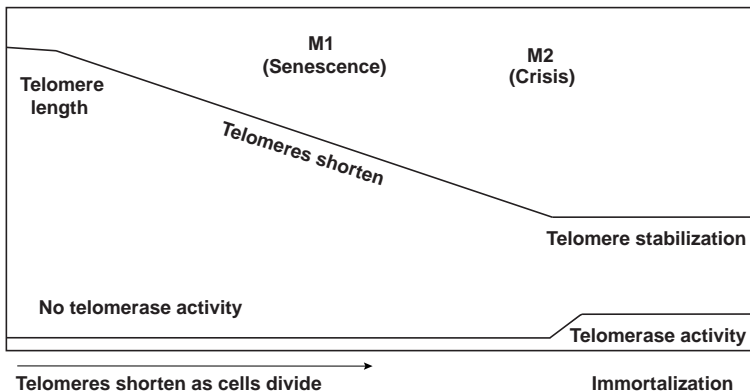


Figure 1. Telomerase activation stabilizes telomere length. The figure shows telomere length relative to the number of cell divisions. The telomeres of normal somatic cells without detectable telomerase activity shorten as the cells divide. This process continues until senescence (M1) occurs. If normal mechanisms of cellular aging are overcome (e.g., by mutation) the cells can continue dividing. However, no telomerase activity is detected. When telomeres become critically short, cells again stop proliferating (M2 or crisis). Cells can only rarely overcome this stoppage of proliferation. When they do, such as in cancer or tumor cells, the cells acquire telomerase activity and become immortal.

I. Introduction *continued*

A series of breakthrough discoveries demonstrated that primary human retinal cells could be immortalized by stable expression of hTERT (Bodnar *et al.*, 1998; Vaziri & Benchimol, 1998; Wang *et al.*, 1998; Yang *et al.*, 1999). Figure 2 compares the life spans of primary cells and hTERT-expressing clones. Primary cells enter into senescence after about 60–80 population doublings, depending on cell type. In contrast, hTERT-expressing clones exceeded the life span of primary cells and show no signs of senescence even after >300 population doublings (Bodnar *et al.*, 1998; our unpublished observations). In fact clones which were selected for long-term studies, continued to divide at the rate of young primary cells. All Infinity cell lines show similar life span characteristics.

To test if the extended life span of Infinity cells correlates with telomerase activity, stable hTERT clones were analyzed for telomerase activity using a telomerase repeat amplification protocol (TRAP; Bodnar *et al.*, 1998). The TRAP assay includes a detergent lysis method that allows uniform extraction of telomerase from a small number of cells. Telomerase activity in a cell extract is determined through its ability to synthesize telomeric repeats onto an oligonucleotide substrate *in vitro*. Figure 3, an example of this assay, shows that primary RPE cells were negative by TRAP for telomerase activity, while hTERT-RPE cells had telomerase activity similar to that of transformed cells. Similar results were obtained for other Infinity cell lines.

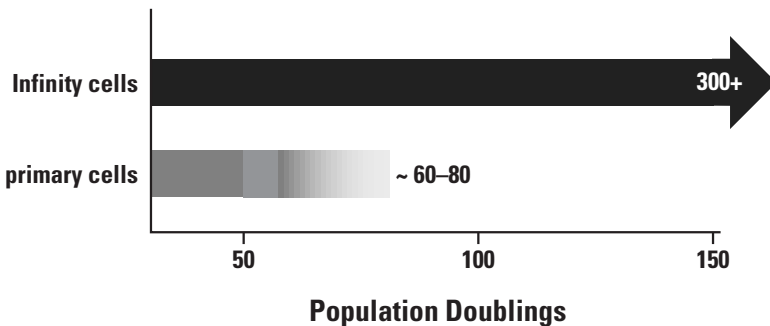


Figure 2. Effect of telomerase expression on cell life span. Primary cells enter senescence after around 60–80 population doublings. Infinity cells continue to divide at a normal rate for more than 300 population doublings.

I. Introduction *continued*

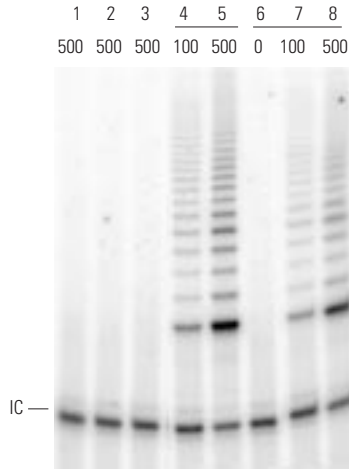


Figure 3. Telomerase activity in hTERT-RPE cells. hTERT-RPE cells were analyzed for telomerase activity by TRAP assay. Lane 1: primary RPE cells. Lanes 2 & 3: primary RPE cells transfected with a control vector (no hTERT insert). Lanes 4 & 5: hTERT-RPE cells. Lanes 6–8: Human lung-cancer cell line H1299. The number of cells assayed is indicated above each lane. IC = internal control. Data provided courtesy of Dr. Choy-Pik Chiu, Geron Corp.

hTERT cells maintain a normal, characteristic phenotype

Although Infinity cells can divide indefinitely, they are functionally and phenotypically normal. Like primary cells that have undergone ~20 population doublings, Infinity cells are arrested by contact and serum deprivation (Table I; Jiang *et al.*, 1999). Furthermore, serum induction stimulates starved clones to resume cell cycling. In contrast, primary cells that have doubled more than 50 times divide slowly under all conditions.

Additional experiments showed that Infinity cells do not grow in soft agar or form tumors in nude mice and respond normally to G1 and G2 phase blockers and spindle inhibitors (Jiang *et al.*, 1999; Morales *et al.*, 1999). Overall, the expression of hTERT in normal cells allows them to grow indefinitely, but does not confer a transformed cell phenotype. Please note, however, that while Infinity cells are phenotypically and functionally normal, karyotype analysis of hTERT-RPE1 cells by G-banding at PD 116 revealed a diploid chromosome number with a partial X:2 translocation. Analysis on later-passage cells at PD177 indicated that this genotype is stable over time and does not appear to be a result of hTERT expression (unpublished observation, Geron Corp.)

I. Introduction *continued*

TABLE I. TELOMERIZED CELLS RESPOND NORMALLY TO CELL DENSITY AND SERUM INDUCTION

	Subconfluence ^b	% of cells in S phase ^a		
		Confluence ^b	0% serum ^c	10% serum ^c
Young primary RPE (21–23 PD)	10.7	3.7	5.3	17.9
Old primary RPE (54–55 PD)	1.9	4.8	2.2	4.0
hTERT-RPE1 (115–125 PD)	20.7	4.2	11.4 (4.8)	18.2

Note: Similar results were obtained for other Infinity cell lines.

PD = Population doublings.

^a The proportion of cells in S phase was determined by flow cytometry. Values represent the average of duplicate cultures analyzed from two separate experiments.

^b Cells were grown in 10% serum at subconfluence or maintained at confluence for 25 days before analysis.

^c Cells were maintained in 0% serum for 3 days (7 days for the number in parentheses) and the cultures were at subconfluence throughout the experimental period. Parallel cultures were then switched to 10% serum and assayed after 24 hr.

Primary and transformed cells vs. telomerase-immortalized cells

Primary and transformed cells are routinely used in gene expression studies, but their usefulness in long-term studies is limited. Primary cell lines are often a heterogeneous collection of cell types, can be hard to obtain, and are difficult to propagate. Even if optimal culturing conditions are established, primary cells only divide 20–100 times before they reach senescence. As a result, they can only be subjected to a limited amount of genetic selection and thus cannot be used in long-term expression studies.

In contrast, transformed cell lines are immortal and do not undergo senescence. However, transformed cells tend to be genetically unstable, as they can become aneuploid by losing or gaining whole or partial chromosomes. In many cases, transformed cell lines exhibit abnormal gene expression profiles that confound the study of biological mechanisms.

Infinity cell lines are derived from single clones, providing you with a relatively uniform cell population that will not undergo senescence. This continuity allows you to compare results from one experiment to the next. Simply freeze reference stocks to ensure an indefinite supply of cells.

II. Additional Materials Required

Note: The following is a list of generally required materials. Materials needed for particular cell lines are listed below.

- **Flasks/culture dishes** (T-75 cell culture flasks)
- **FBS** (hTERT cell lines exhibit optimal growth in CLONTECH's Tet System Approved Fetal Bovine Serum; #8630-1)
- **L-glutamine** (200 mM; VWR #16777-162)
- **Trypan blue solution** (0.4%; Sigma #T8154)
- **DPBS** (VWR #16777-148)
- **DMSO** (Sigma #D2650)
- **Cell freezing medium** (optional; Sigma #C6164)
- **Hemocytometer**
- **Sterile 50-ml conical tubes**
- **Inverted phase contrast microscope**
- **Incubator, humidified, 37°C with 5% CO₂**
- **TeloQuant Kit** (Pharmingen #45227K; for determining telomerase activity or analyzing telomere length.)

hTERT-RPE1 Cells

- **Dulbecco's Modified Eagle's Medium/Hams F-12 Supplement** (DMEM:F-12, VWR #16777-132 or Sigma #D6421)
- **Sodium bicarbonate solution** (7.5%; VWR #16777-160)

hTERT -BJ1 Cells

- **Dulbecco's Modified Eagle's Medium** (DMEM; Sigma #D5671)
- **Medium 199** (Sigma #M4530)
- **Sodium pyruvate** (Sigma #S8636)

III. General Considerations for Infinity Cell Lines

- Culture frozen cells immediately upon receipt. Viability loss may increase after shipping if culturing is delayed. For general information on cell culturing techniques, we recommend Freshney (1993).
- hTERT-RPE1 and hTERT-BJ1 cells were stably selected using hygromycin resistance. If you wish to stably select using your construct, do not use hygromycin as a selection marker.

IV. hTERT-RPE1 Cells

A. Media Preparation

1. Complete Growth Medium: DMEM:F-12 with 10% FBS, 2 mM L-Glutamine, 0.348% sodium bicarbonate.

Note: If you have not purchased DMEM:F-12, prepare a 1:1 mixture of DMEM and Ham's F-12 supplement. Be sure to use low glucose DMEM with no more than 15 mM HEPES for buffering. hTERT-RPE1 cells are particularly sensitive to glucose and HEPES. Add sodium bicarbonate to a final concentration of 0.348%

To prepare medium combine the following:

500 ml	DMEM:F-12 (with 15 mM HEPES and 1.2 g/L sodium bicarbonate)
50 ml	FBS
5 ml	200 mM L-Glutamine
17.3 ml	7.5% sodium bicarbonate solution

Mix well. Store medium at 4°C for up to one month. Replenish L-glutamine after one month of storage. Store medium for an additional month and then discard.

2. Freezing Medium: 70% FBS + 20% DMEM:F-12 + 10% DMSO, or use a commercially available freezing reagent.

B. Seeding Frozen Cultures

1. Thaw cells rapidly in a 37°C water bath with constant agitation. Immediately upon thawing, transfer the contents of the vial to a sterile 50-ml conical tube containing 4 ml of growth medium. Mix gently.
2. Remove 0.1–0.2 ml of the cell suspension, and add an equal volume of 0.4% trypan blue solution. Mix thoroughly; allow to stand for 5–15 min. Use a hemacytometer to count viable cells.

Note: Dilution factor is 2 using this procedure.

3. Calculate the number of viable cells in the total volume of cell suspension remaining in the flask. Record the viable cell number for future reference. Also record the number of population doublings ($PD_{initial}$) noted on the Product Analysis Certificate.
4. Add an additional 10 ml of growth medium to the tube, and transfer contents to a T-75 flask. Place in an incubator at 37°C with 5% CO₂ overnight.
5. The following day, observe the culture. Change growth medium to remove residual cryopreservative. Passage cells if they are greater than 80% confluent.

Note: If viability appears to be significantly less than what you calculated at the time of plating, we suggest trypsinizing the culture, collecting the cells, staining an aliquot with trypan blue, and recounting. This procedure will increase accuracy of the PD number calculation.

IV. hTERT-RPE1 Cells *continued*

C. Passaging Cultures

The following procedure is for trypsinizing cells in a T-75 flask. Adjust volumes accordingly for smaller or larger culture vessels.

1. Aspirate growth medium.
2. Rinse the monolayer briefly with 5–10 ml PBS or 3–5 ml trypsin, and aspirate.
3. Add 1–1.5 ml trypsin to the flask, distribute evenly over the monolayer, and allow cells to detach. hTERT-RPE1 cells are moderately difficult to trypsinize; therefore, trypsinization usually requires 2–5 min in a 37°C incubator.
4. Once cells detach, add 5 ml growth medium to neutralize the trypsin, and disperse cells by pipetting.
5. Repeat the counting procedure (Section B). To determine the PD number of your culture use the following formulas:

$$\text{fold increase in cell number} = n_f \div n_i$$

where: n_f = number of viable cells harvested

n_i = number of viable cells plated

Formula for calculating the total number of population doublings (PD):

$$\text{PD} = \text{PD}_{\text{initial}} + [\ln(\text{fold increase in cell number}) \div \ln 2]$$

6. Expand or split cells as needed. For best results, seed 5×10^5 cells in a T-75 flask or 1×10^6 cells in a T175 flask. Do not allow the cells to overgrow. Passage cultures at <80–90% confluence. The usual passage frequency is 1–2 times per week; generally every 3 or 4 days if seeding at the recommended density.
7. Continue to expand or maintain the culture as needed, recording the number of cells plated and $\text{PD}_{\text{initial}}$ for each passage, and adding the appropriate number of doublings when passaging or harvesting. Because Infinity cells have an indefinite life span, close tracking of the number of PDs may not be essential for some applications; however, we recommend tracking the PD number to use as a point of reference, especially when producing frozen stocks.

D. Freezing Cultures

1. Trypsinize the desired number of flasks or plates.
2. Pool cell suspensions together, count cells, and calculate total viable cell number and the PD number.
3. Pellet cells at 125 x g for 10 min. Aspirate the supernatant.

IV. hTERT-RPE1 Cells *continued*

4. Resuspend the pellet at a density of at least 1×10^6 cells/ml in freezing medium. Freeze cells in the medium described above. Alternatively, use a commercially available freezing medium (Sigma #C6164).
5. Dispense 1-ml aliquots into sterile cryovials.
6. Freeze slowly by reducing temperature by 1°C per minute. If a specialized freezer is not available, we recommend using cryo-containers (Nalgene #5100) at -70°C overnight. Alternatively, place vials in a thick-walled styrofoam container at -20°C for 1–2 hr. Transfer to -70°C overnight. The next day, remove vials from cryo or styrofoam containers, and place in liquid nitrogen storage or an ultra low temperature freezer (-150°C).
7. *Two or more weeks later:* Plate cells to confirm viability of frozen stocks.

V. hTERT-BJ1 Cells

A. Media Preparation

1. Complete Growth Medium: 4:1 ratio of DMEM:Medium 199 with 10% FBS, 1 mM sodium pyruvate, and 4 mM L-glutamine.

To prepare medium combine the following:

400 ml	DMEM
100 ml	Medium 199
50 ml	FBS
10 ml	200 mM L-Glutamine
5 ml	100 mM sodium pyruvate

Mix well. Store medium at 4°C for up to one month. Replenish L-glutamine after one month of storage. Store medium for an additional month and then discard.

2. Freezing Medium: 70% FBS + 20% DMEM + 10% DMSO, or use a commercially available freezing reagent.

B. Seeding Frozen Cultures

1. Thaw cells rapidly in a 37°C water bath with constant agitation. Immediately upon thawing, transfer the contents of the vial to a sterile 50-ml conical tube containing 4 ml of growth medium. Mix gently.
2. Remove 0.1–0.2 ml of the cell suspension, and add an equal volume of 0.4% trypan blue solution. Mix thoroughly; allow to stand for 5–15 min. Use a hemacytometer to count viable cells.

Note: Dilution factor is 2 using this procedure.

3. Calculate the number of viable cells in the total volume of cell suspension remaining in the flask. Record the viable cell number for future reference. Also record the number of population doublings ($PD_{initial}$) noted on the Product Analysis Certificate.
4. Add an additional 10 ml of growth medium to the tube, and transfer contents to a T-75 flask. Place in an incubator at 37°C with 5% CO₂ overnight.
5. The following day, observe the culture. Change growth medium to remove residual cryopreservative. Passage cells if they are greater than 80% confluent.

Note: If viability appears to be significantly less than what you calculated at the time of plating, we suggest trypsinizing the culture, collecting the cells, staining an aliquot with trypan blue, and recounting. This procedure will increase accuracy of the PD number calculation.

V. hTERT-BJ1 Cells *continued*

C. Passaging Cultures

The following procedure is for trypsinizing cells in a T-75 flask. Adjust volumes accordingly for smaller or larger culture vessels.

1. Aspirate growth medium.
2. Rinse the monolayer briefly with 5–10 ml PBS or 3–5 ml trypsin, and aspirate.
3. Add 1–1.5 ml trypsin to the flask, distribute evenly over the monolayer, and allow cells to detach. hTERT-BJ1 cells are moderately difficult to trypsinize; therefore, trypsinization usually requires 2–5 min in a 37°C incubator.
4. Once cells detach, add 5 ml growth medium to neutralize the trypsin, and disperse cells by pipetting.
5. Repeat the counting procedure (Section B). To determine the PD number of your culture use the following formulas:

$$\text{fold increase in cell number} = n_f \div n_i$$

where: n_f = number of viable cells harvested

n_i = number of viable cells plated

Formula for calculating the total number of population doublings (PD):

$$\text{PD} = \text{PD}_{\text{initial}} + [\ln(\text{fold increase in cell number}) \div \ln 2]$$

6. Expand or split cells as needed. For best results, seed 4×10^5 cells in a T-75 flask or 1×10^6 cells in a T175 flask. Do not allow the cells to overgrow. Passage cultures at <80–90% confluence. The usual passage frequency is 1–2 times per week; generally every 3 or 4 days if seeding at the recommended density.
7. Continue to expand or maintain the culture as needed, recording the number of cells plated and $\text{PD}_{\text{initial}}$ for each passage, and adding the appropriate number of doublings when passaging or harvesting. Because Infinity cells have an indefinite life span, close tracking of the number of PDs may not be essential for some applications; however, we recommend tracking the PD number to use as a point of reference, especially when producing frozen stocks.

D. Freezing Cultures

1. Trypsinize the desired number of flasks or plates.
2. Pool cell suspensions together, count cells, and calculate total viable cell number and the PD number.
3. Pellet cells at $125 \times g$ for 5 min. Aspirate the supernatant.

V. hTERT-BJ1 Cells *continued*

4. Resuspend the pellet at a density of at least 1×10^6 cells/ml in freezing medium. Freeze cells in the medium described above. Alternatively, use a commercially available freezing medium (Sigma #C6164).
5. Dispense 1-ml aliquots into sterile cryovials.
6. Freeze slowly by reducing temperature by 1°C per minute. If a specialized freezer is not available, we recommend using cryo-containers (Nalgene #5100) at -70°C overnight. Alternatively, place vials in a thick-walled styrofoam container at -20°C for 1–2 hr. Transfer to -70°C overnight. The next day, remove vials from cryo or styrofoam containers, and place in liquid nitrogen storage or an ultra low temperature freezer (-150°C).
7. *Two or more weeks later:* Plate cells to confirm viability of frozen stocks.

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On the internet:

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Learn about telomerase research at the University of Texas's Southwestern Medical Center at http://www.swmed.edu/home_pages/cellbio/shay-wright/research.html

VII. Related Products

For the latest and most complete listing of all CLONTECH products, please visit www.clontech.com

• hTERT-RPE1 Cell Line	C4000-1
• hTERT-BJ1 Cell Line	C4001-1
• CalPhos™ Mammalian Transfection Kit	K2051-1
• CLONfectin™ Transfection Reagent	8020-1
• Tet System Approved Fetal Bovine Serum	8630-1
• Tet-On™ & Tet-Off™ Inducible Gene Expression Systems	K1621-1, K1620-1
• RevTet-On™ & RevTet-Off™ Gene Expression Systems	K1627-1, K1626-1
• Retro-X™ System	K1060-1
• MSCV Retroviral Expression System	K1062-1
• LRCX Retroviral Vector Set	K1061-1
• Pantropic Retroviral Expression System	K1063-1
• Retroviral Expression Vectors	many
• Living Colors® Vectors	many
• Bicistronic Expression Vectors	many
• pCMV-Myc & pCMV-HA Vector Set	K6003-1
• Hygromycin B	8057-1
• Puromycin	8052-1,-2
• G418	8056-1

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