(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
28 February 2002 (28.02.2002)

PCT

(10) International Publication Number
WO 02/16555 A2

(51) International Patent Classification[7]: C12N 5/00

(21) International Application Number: PCT/GB01/03726

(22) International Filing Date: 17 August 2001 (17.08.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

0020246.5	17 August 2000 (17.08.2000)	GB
60/225,734	17 August 2000 (17.08.2000)	US

(71) Applicant *(for all designated States except US)*: **UNIVERSITY OF WALES COLLEGE OF MEDICINE** [GB/GB]; Heath Park, Cardiff CF4 4XN (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **JONES, Christopher, John** [GB/GB]; 10 Baynton Close, Llandaff, Cardiff CF5 2NZ (GB). **KIPLING, David, Glyn** [GB/GB]; 8 Tydfil Place, Cardiff CF23 5HP (GB). **WILKINSON, Gavin** [GB/GB]; 21 Hampton Crescent West, Cyncoed, Cardiff CF23 6RB (GB). **MCSHARRY, Brian** [GB/GB]; Department of Medicine, University of Wales College of Medicine, Heath Park, Cardiff CF14 4XN (GB). **SKINNER, Julia, Wendy** [GB/GB]; Department of Pathology, University of Wales College of Medicine, Heath Park, Cardiff CF14 4XN (GB).

(74) Agents: **NEWELL, William, Joseph** et al.; Wynne-Jones, Laine & James, Morgan Arcade Chambers, 33 St. Mary Street, Cardiff CF10 1AF (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: CELL LINES, THEIR PREPARATION AND USE

(57) Abstract: An immortalised cell line is disclosed, which is suitable for use in vaccine production, characterised in that the cells thereof are adapted to express hTERT, the catalytic subunit of human telomerase. A suitable cell line comprises human diploid fibroblasts, such as those derivable from MRC-5 or WI38 cell lines, transfected with cDNA of hTERT or infected by a retrovirus carrying cDNA of hTERT, which transfected or infected cells are capable eof supporting antigen production. A method for preparing such cell lines, using recombinant techniques, is also disclosed. The cell lines may be used as a diagnostic test for the presence of a virus, such as HCMV, which assay method includes the step of (a): contacting a sample of the telomerase-immortalised cell line whose genome contains a nucleotide sequence comprising a reporter gene capable of causing a measurable response, which reporter gene is under the control of the virus promoter region whereby expression of the reporter protein corresponds to expression of the virus in the sample. Alternatively, the telomerase-immortalised cell lines may be used to determine the efficacy of anti-viral agents by testing the capability of a modified virus, such as HCMV containing a reporter gene, to infect these cells. The assay method includes the step of (a): contacting a sample of the telomerase-immortalised cells with the modified virus, whereby expression of the reporter protein corresponds to infection of the cells in the sample. Preferably, the methods further include the step of (b): measuring the response of the reporter; and/or (c) comparing the measurement resulting from step (b) with the response from a standard or non-immortalised sample, which has itself undergone assay method steps (a) and (b).

WO 02/16555 A2

CELL LINES, THEIR PREPARATION AND USE

The present invention relates to the use in the replication of a virus of a diploid cell line made immortal by the introduction of the catalytic subunit of telomerase. In particular, the invention relates to a human diploid fibroblast cell line, such as MRC-5, immortalised by ectopic expression of the catalytic subunit of human telomerase. This cell line has the ability, which can be impaired in cell lines immortalised in other ways, of being able to support viral replication. This invention also relates to methods for detecting viral infection and the use of the cell line in the preparation of a vaccine.

Background to the invention

Vaccines provide a way of preparing the immune system to act against entities that are foreign to the body, such as bacteria and viruses. Vaccines are a safe form of the whole or part of the bacterium or virus. The vaccine essentially contains antigens that the immune system processes to provide immunity. Once the body has mounted a response to the vaccine, it is in a better position to react to the presence of these antigens when they appear again, for example, as a result of an infection.

Vaccines can be made in a variety of ways. Modern genetic engineering techniques have allowed the production of individual components of viruses or bacteria that can be incorporated into vaccines. Another way is to introduce an attenuated (*ie* less active) or inactive form of the virus. This requires a method of propagation. The preferred method is to use a cell line, preferably human, that will

produce large quantities of virus but that will be free from any other potentially harmful agents. Currently, a primary (*ie* not derived from a cancer) cell line, MRC-5, is used. This was isolated from the lung of an aborted foetus over 30 years ago. All stocks of MRC-5, world-wide, are taken from this original isolate. Unfortunately, this valuable resource will eventually run out, as the cells have a limited life-span in culture. Problems will occur before then because, as the cell line ages, it becomes less efficient at making viruses for vaccine production.

Although MRC-5 cells have been used as a standard for many years for both fundamental research and vaccine production, they are becoming increasingly difficult to source at low passage number. 'Passage number' relates to how many times the culture has divided and therefore, in MRC-5, it is becoming increasingly difficult to source young stocks of these cells.

Limited growth potential of MRC-5 cells is caused by replicative senescence

Normal human cells have a limited ability to proliferate *in vitro*. They eventually enter a state of viable growth arrest known as cellular or replicative senescence, which arises as a consequence of chromosomal telomere shortening. Senescence becomes apparent when a culture fails to grow in conditions that previously allowed its proliferation. Eventually, after a finite number of population doublings (pd), the culture consists of almost completely permanently growth-arrested cells. The phenotype of senescent cells differs from their 'younger' counterparts in terms of gene activation or repression, and morphologically they appear larger and flatter, with a pronounced cytoskeleton. Senescence should not be confused

with quiescence, terminal differentiation or cell death. Indeed, senescent cells show no change in the rate of apoptosis compared to proliferating cells. Senescent cells can be detected by the presence of a senescence-associated β-galactosidase (SA β-gal) activity that is detectable at low pH (pH 6.0). This assay has shown that senescence is not just an *in vitro* artefact, as SA β-gal-stained cells have been detected *in vivo*. Interestingly, the *in vivo* incidence of SA β-gal staining cells increases with age and tentatively suggests a link between replicative senescence and human ageing.

Cells that have entered a state of replicative senescence usually reside in the G1 phase of the cell cycle and fail to enter the S (DNA replication) phase after the addition of growth factors. Senescence is a dominant state as somatic cell hybrids of old and young cells retain the senescent phenotype. Expression of the cyclin-dependent kinase inhibitor $p21^{WAF1}$ in senescent cells suggests that growth arrest is induced by a p53-mediated damage response. Abrogation of p53 function by SV40 large T antigen prior to senescence confers a limited extension of lifespan in fibroblasts of approximately 20 pd. Micro-injection of anti-p53 antibodies into senescent fibroblasts allows cells to re-enter the cell cycle and resume proliferation.

The senescence of a cell line *in vitro* is an on-going process. At the point where a culture is deemed to be senescent, all the cells will be out of cycle. However, senescent cells will appear very early on in the life of the culture, with the proportion increasing with every passage. Therefore, an MRC-5 culture, for example, will constantly be evolving and, in the case of viral replication, becomes less efficient as time passes.

Therefore, although widely distributed, this cell line is a diminishing resource due to the onset of replicative senescence. Its continued availability is vital to on-going vaccine production. A reliable source of permissive human fibroblasts would be extremely useful to both research and diagnostic laboratories; the currently limited availability of such cells is often a serious impediment.

There are two known routes to overcoming the senescence problem: the first is to find and characterise a new mortal culture able not only to propagate viruses but also meet regulatory requirements. Again, this will be a limited resource as it will ultimately result in senescence problems. Also, regulatory and scientific inertia may prevent its widespread acceptance. The second option is to immortalise MRC-5. Previous attempts to use immortal cells have suffered from the fact that profound changes in cellular physiology can be induced, and the immortalised cells lose the capacity to support the replication of some viruses (*eg* MRC-5VA-SV40 immortalised), which is clearly critical if they are to replace, in practice, the current MRC-5 cell line. Alternative cell lines that have been immortalised deliberately or spontaneously during tumorigenesis are unsuitable, due to the widespread unease about the transmission of oncogenic DNA from these cell lines.

Currently, there is interest in attempting to develop human cytomegalovirus vaccines, and human cytomegalovirus is a potential virus vector. For both vaccine and vector development, it is important to both optimise and standardise conditions. Hence, there is a clear need for a reliable source of permissive cells for use in such work.

Human cytomegalovirus (HCMV)

HCMV is a *herpes* virus that that is ubiquitous in populations world-wide. It is a common virus, with most individuals experiencing infection. In common with other *herpes* viruses, HCMV infection is associated with life-long persistence in its host. In the vast majority of individuals, primary infection with HCMV appears to be asymptomatic or is associated with a mild febrile illness. However, the virus is an important pathogen and, in adults, is a significant cause of infectious mononucleosis. HCMV is a major viral cause of congenital malformation and can be responsible for life-threatening disease in immuno-suppressed or immuno-compromised individuals. Such infections exhibit 'protean clinical manifestations' as a consequence of the virus's capacity for systemic infection and to replicate in a wide variety of tissues.

HCMV is capable of replicating, *in vivo*, in a range of cell types including fibroblasts, smooth muscle cells, endothelial cells, hepatocytes and tissue macrophages. CD34+ haematopoetic progenitors have been implicated as a site of virus latency, with re-activation being detected following differentiation to monocytes and virus release associated with a macrophage population expressing dendritic cell markers.

High titre replication of HCMV in cell culture has previously been possible only in primary human fibroblasts. Productive HCMV replication appears to be restricted almost exclusively to human cells *in vitro*. HCMV is capable of establishing infection and often of producing significant amounts of virus, *in vitro*, in a range of different cell types including endothelial cells, epithelial cells, trophoblasts, and monocyte-derived macrophages. However,

infections tend to be slow, virus yields poor and sometimes the virus has to be specially adapted for the target cell.

As described above, primary human fibroblasts have a limited life span. This is therefore a particular problem for scientists working with HCMV. Experimental variations are caused as different laboratories use different cell sources, and fibroblasts change as they accrue population doublings. Stocks of fibroblasts become exhausted, and adventitious agents (*eg* mycoplasma) may be introduced by using fresh cells (typically human foreskin fibroblasts). It is frequently useful for researchers to be able to provide a complementing gene function or an assayed marker into a permissive cell. However, this requires the cell to be capable of going through a large number of cell doublings (pd).

Disadvantages of previously-available immortalised cells for HMCV production

Most available immortalised human cells (*eg* HeLa cells) are unable to support HCMV replication. Nevertheless, it has been demonstrated that HCMV was able to infect but not replicate in a pluripotent human embryonal carcinoma cell line. However, following the induction of cellular differentiation (by treatment with retinoic acid), virus replication could be detected. This system is therefore a useful model for investigating HCMV re-activation from latency, but it is not suitable for routine virus culture. It has also been demonstrated that human foreskin fibroblasts immortalised using the human papillomavirus type 16 E6 and E7 oncogenes were able to support efficient virus replication. Cell lines generated by this method have proved useful for the complementation

of HCMV deletion mutants. Following immortalisation, however, the cells are atypical; and the endogenous expression of the HPV oncogenes would interfere with assays of gene function, and potentially could be 'picked up' and incorporated into the replicating virus genome.

Therefore, there is a particular need for an immortalised cell line capable of supporting replication of HCMV without exhibiting any of the aforementioned disadvantages.

Immortalisation of cell cultures using human telomerase reverse transcriptase gene (hTERT)

An alternative approach to immortalisation involves using the human telomerase reverse transcriptase gene (hTERT).

Telomeres: form and function

Telomeres are protein-bound DNA structures that define the ends of linear chromosomes. Telomeres are often considered as a cellular clock counting the number of cell divisions, serving as an indicator of the relative age of the culture and as a predictive marker for the remaining lifespan of the population. In human cells, telomeres are characterised by a DNA repeat sequence $(TTAGGG)_n$. The unique nature of the telomeric repeat sequence promotes binding of two proteins TRF1 and TRF2. The amount of $(TTAGGG)_n$ present at chromosome termini greatly exceeds that required simply to supply an end-protective function. Telomeric DNA also offsets a detrimental effect of DNA replication.

Considered in its most basic form, semi-conservative DNA replication requires the activity of an RNA primase

followed by the action of DNA polymerases. Lagging strand synthesis is a discontinuous process involving the synthesis of RNA primers that are, in turn, removed by a ribonuclease (RNase) and replaced by DNA, the polymerase requiring a 3'-OH end to initiate synthesis. However, synthesis at the chromosome terminus is not possible after primer removal and results in the production of an overhang of 50-200 bp. These overhangs are modified, with the result that the chromosome is shortened. Telomere shortening is detectable by Southern blotting. Telomeric DNA, in the form a terminal restriction fragment (TRF) resistant to enzymatic digestion, is detected as a smear representing a distribution of telomeric sequences of all the chromosomes from a population of cells. It is clear from analyses of this nature that TRFs shorten in somatic cells, such as fibroblasts, as the number of pd increases, decreasing to a threshold of about 5 kb (including subtelomeric regions) in senescent cells.

Therefore, in a large number of cell types, telomeres normally show progressive shortening. However, cancer cells, with generally short telomeres, are able to proliferate indefinitely, avoiding replicative senescence. Germ, stem and most cancer cells have reactivated an enzyme, telomerase, which modulates telomere length. It is also apparent that cancer cells with shorter tracts of telomeric DNA have further deregulated their response to shortened telomeres, which is consistent with established models of multistep carcinogenesis. The hypothesis that maintaining telomeres is required to avoid replicative senescence is supported by the observation that the germ line and stem cells have much longer telomeres than somatic cells. Lifespan extension induced by SV40 T is accompanied

by further telomere shortening until the cells enter crisis due, in part, to chromosome fusion events. Therefore, any cell types that have been immortalised by SV40 arise by re-activation of telomerase at crisis. Unfortunately, the culture is much changed from the normal population from which it arose, due to associated genome shredding at crisis and continuing abrogation of p53 function.

Telomerase

The end-replication problem can be overcome by reactivation of the enzyme telomerase. Mammalian telomerase synthesises $(TTAGGG)_n$ repeats *de novo* on to chromosome ends. Reconstitution of telomerase activity requires an RNA template and a catalytic subunit. Telomerase is detected in cell extracts by a telomeric repeat amplification protocol (TRAP assay, see Example 1.6 and Kim, N.W. *et al* (1994) Specific association of human telomerase activity with immortal cells and cancer. *Science*, **266**, 2011-2015). This *in vitro* assay is based on the ability of telomerase to extend an oligonucleotide primer. Extension products are amplified by the polymerase chain reaction (PCR). Extracts with telomerase activity generate a DNA ladder on PAGE gels. Pre-treatment of extracts with RNase A destroys the template and consequently inactivates telomerase.

RNA component of telomerase

The RNA component of telomerase, known as hTR or hTERC (telomerase RNA component) was identified in 1995 through a combination of partial purification of the enzyme, subtractive hybridisation and PCR cyclic selection to enrich for RNAs carrying the complement of the telomeric repeat sequence. hTERC RNA matures to a length of 450 bases

and the template region comprises 11 nucleotides (5'-CUAACCCUAAC). Mutation of this template region produces an equivalent mutation in the telomeric repeat sequence. Expression of antisense hTERC in HeLa cell cultures abolishes telomerase activity and subsequently causes cell death (Feng, J. *et al* (1995) The RNA component of human telomerase. *Science*, **269**, 1236-1241).

Catalytic subunit of telomerase

An expressed sequence tag (EST) generated by the IMAGE consortium (Integrated Molecular Analysis of Genomes Expression Consortium, at NIH, USA) shows considerable homology to the telomerase catalytic subunit of the ciliate *Euplotes aediculatus*. On the basis of this, a full length cDNA encoding the catalytic subunit of human telomerase has been independently identified by a number of groups. The hTERT (telomerase reverse transcriptase, but also known as TCS1, TP2, hTRT, hEST2) gene encodes a protein of calculated M_r 127 kDa, which contains reverse transcriptase domains and a 'T' motif unique to members of the telomerase family (for review see Kipling, D. (1997) Mammalian telomerase: catalytic subunit and knockout mice. *Hum Mol Genet*, **6**, 1999-2004). Introduction of hTERT into normal diploid fibroblasts has a profound effect on replicative lifespan (see below).

Immortalisation of cells by telomerase

The RNA component of telomerase, hTERC, is present in most cell types and ectopic expression of hTERT alone is sufficient to restore telomerase activity in cells (Weinrich, S.L. *et al* (1997) *Nat Genet* **17** 498-502). However, ability to restore telomerase activity is not a

reliable indicator of ability to immortalise. This technique was first reported in human diploid cells by Bodnar *et al* ((1998) *Science* **279** 349-52). Immortalisation of four different cell cultures using this method has been described (Wyllie *et al*, (2000) *Nat Genet* **24** 16-740 & Webley, K.A. *et al*, (2000) *Mol Cell Biol* **20** 2803-8) and the cultures used as controls. These cell cultures were HCA2-normal diploid fibroblasts and three fibroblast cultures (AG1279A, AG05229 and AG3141B) taken from individuals with the progeroid Werner syndrome. Introduction of wild type hTERT into fibroblasts causes telomere lengthening. Fibroblasts expressing telomerase avoid senescence and continue to proliferate (Bodnar, A.G. *et al*, *q.v.*). The cells retain the morphology of younger cells and do not express SA β-gal, unlike vector controls that undergo replicative senescence. Immortalisation of cells by telomerase does not appear to confer any changes associated with malignancy. They have intact cell cycle checkpoints; are karyotypically normal; become quiescent at high density; and, under conditions of serum starvation, fail to grow in soft agar or induce tumours *in vivo*. The cell lines derived from these experiments can be considered truly immortal as they have well exceeded the lifespan of cultures subjected to no intervention or infection with control vectors.

However, no attempt has previously been made to use such telomerase-immortalised cell lines for the replication of viruses. Furthermore, previously-known telomerase-immortalised cell lines are unlikely to be acceptable for use in vaccine production. For example, HCA2 is not likely to be acceptable for use in humans, so it is essential for practical purposes that a cell line be employed that can

acceptably come into contact with and/or be administered to a human patient. Yet further, no attempt has been made to immortalise MRC-5 or other cell lines useful in virus-replication and therefore vaccine production, using the telomerase approach.

Furthermore, there is a marked difference between HCA2 and MRC-5 cells that results in their having differing behaviours and biochemistry. MRC-5 cells are foetal lung pericytes. Although similar in some respects to adult dermal fibroblasts, such as those immortalised with hTERT in Bodnar (*q.v.*) and Wyllie (*q.v.*), there is substantial literature indicating extensive variation within this classification of cells. This is reviewed by Sempowski GD, *et al* in Wound Rep Reg 3 120-131 (1995). Relevant differences include major ones between adult *v.* foetal cells, and between different anatomical sites. Thus, a lung pericyte such as MRC-5 would be expected *a priori* to be a very different cell to an adult dermal fibroblast, such as those that have been immortalised with hTERT.

There is also substantial literature on the ability (or not) of hTERT to immortalise particular cell types. For example, hTERT is sufficient to immortalise human retinal pigmented epithelium and corneal epithelium, but is insufficient for thyroid epithelium and mammary epithelium. Vascular endothelium is immortalised with hTERT, whereas corneal endothelium is not. Because other human fibroblasts (from human breast tissue) have been shown not to become immortalised using hTERT (despite telomerase activity being restored by hTERT therein), as described by O'Hare *et al* in Proc Natl Acad Sci USA 98 646-51 (2001), it might be expected that human fibroblast-derived MRC-5 cells also could not be hTERT-immortalisable.

The Invention

We have now found that primary (non-cancer) MRC-5 cells can be immortalised using hTERT; and, surprisingly (given the failure of the MRC-5 SV40 cell lines to do so), that hTERT-immortalised MRC-5 cells can support the replication of viruses such as HCMV and can therefore be used in the production of vaccines. Furthermore, virus replication occurs at similar rates to those in non-immortalised cells. hTERT-immortalisation does not affect the cell morphology or growth characteristics of MRC-5 cells and hTERT-immortalised MRC-5 cells are substantially free of adventitious agents (as are non-immortalised MRC-5 cells). We have also found that hTERT-immortalised cells can also support the stable maintenance of an Epstein-Barr Virus-based episomal vector.

Accordingly, the present invention provides a telomerase-immortalised cell line capable of allowing human vaccine production.

The invention further provides a telomerase-immortalised cell line for use in the preparation of an antigen, such as a virus, or a component thereof.

By 'component' in this context is meant a single or multiple virus-encoded gene product that may either be incorporated into or excluded from virus particles.

Accordingly, this invention provides an hTERT-immortalised cell line, characterised by being capable of use in vaccine production

Furthermore, there is provided a method of preparing virus, virus-derived agents (such as virus-based episomes) and antigen, or a component thereof, which method comprises

delivering genetic material or infecting with virus a telomerase-immortalised cell line capable of allowing antigen production therein.

The invention therefore provides for the preparation in a telomerase-immortalised cell line of any virus-based therapeutic agent, such as a viral vaccine, a gene therapy agent or expression vector, wherein the gene encoding the antigen or a component of the vector system may be derived from a virus.

Preferably, the telomerase-immortalised cell line is derivable from human diploid fibroblasts. More preferably, the telomerase-immortalised cell line is derivable from MRC-5 or WI38 cell lines, which are the world standard cell lines for vaccine production, especially MRC-5.

Accordingly, the present invention provides a telomerase-immortalised MRC-5 or WI38 cell line, which cell line is capable of protein and antigen production therein, particularly by viral replication. Cells from such cell lines do not produce viruses expressing telomerase.

The invention further provides for the production of a virus, virus-derived agents and antigen, such as that suitable for use in the preparation of a vaccine, preparable or when prepared using a telomerase-immortalised cell line of the invention.

Preferred antigens, virus and virus-derived elements producible in the cell lines according to this invention are those suitable for use in the preparation of a medicine, such as a vaccine. The antigen may comprise any known to those skilled in the art, especially those preparable by use of MRC-5 cells, such as those associated with rubella, hepatitis (such as hepatitis A), polio, varicella zoster, HCMV, and the like. The antigen, or cDNA

encoding therefor, may be incorporated into a suitable plasmid or vector for infecting a cell line or cells therefrom according to the invention.

Still further, the invention provides a vaccine comprising an antigen prepared by or preparable by use of a telomerase-immortalised cell line according to this invention and an antigenically-acceptable carrier therefor.

The telomerase-immortalised cell lines of the present invention may be prepared by any method that enables the delivery of a telomerase gene into a cell, preferably a cell from an MRC-5 cell line, whereby the cell can express hTERT. Any gene delivery system that forces expression of hTERT may be used in such a method.

Preferably, the telomerase-immortalised cell lines of the present invention comprise cells from a clone comprising human diploid fibroblasts transfected with cDNA of hTERT or infected by a retrovirus carrying cDNA of hTERT, whereby cells from the transfected clone are capable of supporting antigen production. More preferably, the cells are derived from the MRC-5 or WI38 cell lines, especially the MRC-5 cell line.

For example, MRC-5 cells may be immortalised with hTERT by plasmid transfection, since dissociating the hTERT-expressing gene from a pro-viral element may be viewed as enhancing safety for pharmacological applications. Also advantageous is when fibroblasts are infected by a retrovirus, since such cells maintain higher expression and longer telomeres when compared with hTERT-transfected fibroblasts. Especially suitable is when the cells remain susceptible to virus infection, especially when the virus is or is derived from human cytomegalovirus (HCMV).

Accordingly, other plasmids or vectors that may be employed in association with a cell line or cells according to the invention include episomal vectors: Suitable plasmids include the pAL288 plasmid, described further below in Examples 2.1 and 2.2. Episomal vectors include those based on the Epstein-Barr virus (EBV) genome, *eg* pAL 105 and pAL357, described further below in Examples 2.3 and 2.4. In these Examples, co-delivery to the immortalised cells of an episomal vector and the replication-deficient adenoviral Rad114 (MOI 30), in which Rad114 is an adenovirus expressing the EBV nuclear antigen EBNA-1, was found to facilitate the generation of stable cell lines containing episomal vectors. Facilitation included both increased frequency with which stable cell lines can be generated and efficiency of transient EGPF expression, where EGPF is an element of a reporter system, described further below.

The use of a replication-deficient adenovirus recombinant encoding EBNA-1 to facilitate the generation of episomal cell lines is novel and, accordingly, the present invention further provides the use of an adenovirus expressing a viral nuclear antigen in the preparation of an episomal cell line.

In order to test the capability of a virus, in particular the HCMV virus, to infect hTERT-immortalised cell lines according to the invention, the invention further provides an assay method, which includes the step of (a): contacting a sample of the telomerase-immortalised cell line (*eg* cells from a clone comprising human diploid fibroblasts transfected with cDNA of hTERT or infected by a retroviral expression vector carrying cDNA of hTERT) with a virus whose genome contains a nucleotide sequence, such as

an inserted DNA fragment, comprising a reporter gene capable of causing a measurable response, which reporter gene is under the control of the virus promoter region, whereby expression of the reporter protein corresponds to expression of the virus in the sample.

A preferred assay method further includes the step of (b): measuring the response of the reporter.

More preferably, the assay method further includes the step of (c): comparing the measurement resulting from step (b) with the response from a standard or non-immortalised sample, which has itself undergone assay method steps (a) and (b).

Alternatively, the cell line of the present invention may be used to determine the efficacy of a candidate or actual anti-viral agent by testing the capability of a modified virus to infect these cells in the presence of the candidate anti-viral agent. In this aspect, the virus is preferably modified so as to allow its presence to be detected, for example, HCMV whose genome is modified by incorporation of a reporter gene. Such an assay method may include the step of (a): contacting a sample of the telomerase-immortalised cell line of the invention in the presence of the candidate anti-viral agent with the modified virus, whereby expression of the reporter protein corresponds to infection of the cells in the sample.

This assay can also be adapted to provide a screening method for identifying antiviral agents capable of inhibiting viral infection and/or replication, which screening method comprises carrying out the above-described assay wherein the contacting step (a) takes place in the presence of a test agent and wherein lack of response at step (b) indicates the test agent has antiviral activity.

The present invention therefore further provides a virus whose genome contains or is associated with cDNA of a reporter gene capable of causing a measurable response, which reporter gene is under the control of the virus promoter, whereby expression of the reporter protein corresponds to expression of the virus.

The present invention also provides an assay adapted to provide a diagnostic method whereby the presence or absence of a virus, such as wild-type virus, in a sample can be detected. Preferably, the diagnostic method includes the step of (a): contacting a telomerase-immortalised cell line (*eg* cells from a clone comprising human diploid fibroblasts transfected with cDNA of hTERT or infected by a retroviral expression vector carrying cDNA of hTERT) incorporating a reporter construct comprising cDNA of a reporter gene capable of causing a measurable response, which reporter gene is under the control of the virus promoter region, with the test sample, whereby expression of the reporter protein corresponds to expression of the virus in the test sample.

Preferred diagnostic methods according to this invention also include step(s) (b) and/or (c), as described above with respect to the screening assay.

Therefore, in the diagnostic assay, the telomerase-immortalised cells are further modified to express a reporter gene when the cells are infected with a virus. The reporter system is chosen such that it will be activated only by viral proteins present as a result of infection. The proteins binding to a virus-specific promoter linked to the gene cause activation of the reporter gene, such as green fluorescent protein, as described below.

Accordingly, the invention further provides a telomerase-immortalised cell line (*ie* cells from a clone comprising human diploid fibroblasts transfected with cDNA of hTERT or infected by a retroviral expression vector carrying cDNA of hTERT) incorporating a reporter construct comprising cDNA of a reporter gene capable of causing a measurable response, which reporter gene is under the control of a virus promoter region.

In the assays according to the invention, the measurable response may comprise a colour change, fluorescence change or emission of light. Accordingly, the reporter construct may be such that the reporter gene expresses an enzyme that causes a measurable response when brought into contact with a suitable substrate, such as a protein. Therefore, in the assay method according the invention, the reporter may be selected from chloramphenicol acetyl transferase (CAT), a luciferase, such as Firefly luciferase and Renilla luciferase, β-galactosidase, alkaline phosphatase and horseradish peroxidase, as well as derivatives of the green fluorescent protein mentioned below.

The present invention therefore further provides an assay method according to the invention comprising, in step (a), the use of a luciferase cDNA driven by a viral promoter; followed by the use of luciferin; and, in step (b), measuring the light output from the luciferinised cells.

Alternatively, the reporter construct may be such that the reporter gene expresses a fluorescent protein. Accordingly, the present invention therefore further provides an assay method according to the invention comprising, in step (a), the use of fluorescent-expressing

cDNA driven by a viral promoter; and, in step (b), measuring the fluorescence output from the fluorescinated cells.

Preferably, when the virus is HCMV, the reporter gene encodes enhanced green fluorescent protein gene (EGFP, available from Clontech). More preferably, the EGFP gene replaces that encoding the β2.7 early gene of HCMV. Still more preferred is when the virus is HMCV strain AD 169-based recombinant virus RCMV288, as described in Example 2.1 hereinbelow, by using a plasmid such as pAL288. More preferably, the reporter construct comprises the plasmid pAL288 having therein the gene encoding EGFP flanked by regions containing both the β2.7 major enhanced promoter and the gene's polyadenylation signal.

In the assay method according to the invention, all reagents used therein may be brought together in one or more steps; and/or two or more of the assay steps may be carried out substantially simultaneously. The assay method may be carried out by manual, partly automated or fully automated means.

The present invention therefore further provides a kit for carrying out an assay according to the invention. For example, a suitable kit may comprise:

(a) a telomerase-immortalised cell line;

(b) a standard sample for the assay (not always necessary);

(c) medium for culturing and/or reconstituting the cells; and

(d) instructions for carrying out the assay; and, optionally,

(e) buffer for lysing the cells; and/or

(f) buffer for the reporter construct; and,

optionally,

(g) means for measuring the response.

For adaptation for use as a screening assay, the kit preferably also comprises a virus whose genome contains a reporter construct comprising cDNA of a reporter gene, such as that for green fluorescent protein, capable of causing a measurable response, such as fluorescence measurable by an inverted fluorescence microscope, which reporter gene is under the control of the virus promoter region, whereby expression of the reporter protein corresponds to expression of the virus.

In practice, the screening methods could be carried out by providing a 96-well tissue culture dish containing telomerase-immortalised MRC-5 cells. To test for agents effective against HCMV, EGFP-tagged HCMV would be added to the wells, together with test agent. If the agent were effective as an anti-HCMV agent, then no fluorescence would be observed.

For adaptation for use as a diagnostic assay, the telomerase-immortalised cells (kit component (a)) are preferably transfected with a reporter construct comprising cDNA of a reporter gene, such as green fluorescent protein, capable of causing a measurable response, such as fluorescence measurable by an inverted fluorescence microscope, which reporter gene is under the control of the virus promoter, whereby expression of the reporter gene corresponds to expression of the virus in the sample.

In practice, the diagnostic methods could be carried out by providing a 96-well tissue culture dish containing telomerase-immortalised MRC-5 cells that have been modified ('indicator cell line') to incorporate a EGFP linked to both the β2.7 major enhanced promoter and the gene's

polyadenylation signal. To test sensitively for presence or absence of HCMV in a patient's sample, the sample would be added to the well. If the sample did not contain HCMV, then no fluorescence would be observed. As well as diagnosing active HCMV infections, the indicator cell line may also be adapted for use in monitoring active HCMV infections and to perform virus titrations. Similar indicator cell lines could be generated, using alternative reporter genes and/or other viral promoters, such as other CMV promoters or those for other viruses.

Other applications for such telomerase-immortalised cell lines such as hTERT-immortalised MRC-5 includes their use in the generation of complementing or 'helper' cell lines, such as for the propagation of HCMV deletion mutants or any other essential gene function. This aspect has applications in vaccine production, for example to limit the replication of certain viruses, such as *herpes simplex*. The use of episomal-based systems in such applications is also contemplated.

The present invention further provides a clone for use in an assay method of the present invention; cells produced by the clone; and cDNA or mRNA for use in preparing such clones and/or cells.

The present invention therefore also may provide:

(a) The use of telomerase-immortalised cell cultures for the detection of viral infection, especially cytomegalovirus.

(b) The use of telomerase-immortalised human cell cultures for the detection of any human infection.

(c) The use of telomerase-immortalised cell cultures for the detection of viral infection by the use of engineered cells containing a reporter construct.

(d) The use of telomerase-immortalised human cell cultures for the detection of viral infection by the use of engineered cells containing a reporter construct.

(e) The use of telomerase-immortalised cell cultures as producer cell lines for the production of modified viral particles for gene therapy.

(f) The use of telomerase-immortalised human cell cultures as producer cell lines for the production of attenuated viral particles for vaccine production. (*eg* viruses having no genetic material).

(g) The use of telomerase-immortalised human cell cultures as producer cell lines for the production of modified viral particles for vaccine production or for viral vector systems.

(h) The use of telomerase-immortalised human cell cultures modified so as to provide new hosts for viruses *eg* expression of a cell surface receptor that makes cells permissive for infection, especially where these modified cells are for use in vaccine production or the production of recombinant agents for gene transfer or gene therapy .

The present invention will now be illustrated by the following non-limiting Examples.

Example 1: Immortalisation of human diploid fibroblasts using telomerase catalytic subunit

A cDNA encoding the catalytic subunit of human telomerase reverse transcriptase has been isolated (Kilian, A. *et al.* (1997). Isolation of a candidate human telomerase catalytic subunit gene, which reveals complex splicing patterns in different cell types. *Hum Mol Genet*, **6**, 2011-9; Meyerson, M*et al.*, (1997) hEST2, the putative human

telomerase catalytic subunit gene, is up- regulated in tumor cells and during immortalization. *Cell*, **90**, 785-95; and Nakamura, T.M. *et al*,. (1997) Telomerase catalytic subunit homologs from fission yeast and human. *Science*, **277**, 955-9).

The cDNA, known as hTERT, encodes a protein of 1132 amino acids with predicted molecular weight 126.9 kDa. It has been demonstrated by a number of laboratories, including ours, that ectopic expression of hTERT alone is sufficient to restore telomerase activity to cells. An RNA molecule, hTERC, which provides the template for synthesis of telomeric repeats at chromosome ends, is constitutively expressed in these cells. It has also been shown that reconstitution of telomerase activity in most human cells is sufficient to confer immortality on these cultures.

The protocols used to immortalise normal human diploid fibroblasts are listed below; all are commonly-used.

Protocols used in Example 1:

1.1 Preparation of pBABE puro plasmid vector containing hTERT cDNA.

1.2 Preparation of ecotropic retrovirus.

1.3 Preparation of amphotropic retrovirus.

1.4 Amphotropic retroviral infection of human diploid fibroblasts with hTERT.

1.5 Selection and expansion of immortal, telomerase expressing fibroblast subclones.

1.6 Detection of telomerase activity.

1.7 Measurement of telomere length.

1.8 Measurement of lifespan extension.

1.1 Preparation of pBABE puro plasmid vector containing hTERT cDNA

A bacterial plasmid vector pGRN121 (7029 bp) was obtained from Geron Corporation, USA. The vector contains the hTERT cDNA (4070 bp, Figure 1, being sequence referenced AF015950 in the EMBL database) cloned into the *Eco*RI site of the polylinker sequence of the plasmid pBluescript II SK+ such that the 5′ end of the cDNA was near to the T7 promoter in the vector. One nanogram of this vector was transformed into the *E. coli* strain DH5α made competent using a calcium chloride based method. Successful transformants were identified by their ability to grow on LB agar plates containing the antibiotic ampicillin (100 μg/ml). One clone was taken and grown in a volume of 500 ml of LB broth containing ampicillin (100 μg/ml) until stationary. Highly purified closed circular plasmid DNA was isolated by alkaline lysis of the cells, neutralisation and ion exchange chromatography (Hybaid recovery quickflow maxi kit).

An aliquot (1 μg) of pGRN121 was then digested with the restriction enzyme *Eco*RI in order to release the hTERT cDNA from the plasmid backbone. The digest was then separated by electrophoresis on a 0.7% agarose gel until 2 DNA species were apparent (Fig. 2a). The DNA fragment containing hTERT was excised from the gel and recovered using mini ion exchange columns (Qiagen Qiaquick kit).

A retroviral expression vector pBABE puro (Fig. 3) was linearised with *Eco*RI. Equimolar amounts of purified hTERT cDNA and linearised pBABE puro were taken and incubated at 16°C for 17 hours in 10 μl of buffer containing 1X OnePhorAll buffer (Amersham Pharmacia Biotech, APB), 1mM ATP, 10 mM DTT and T4 DNA ligase (APB). Ligation products

were transformed into DH5α (see above). Successful recombinants were identified as those that released the hTERT insert DNA following EcoRI digestion. The orientation of the hTERT gene in the vector was determined by digestion with BamHI. hTERT in the sense direction released a fragment of 2.5 kb (Fig. 2b). The resulting plasmid was designated pBABE puro hTERT. The bacterial culture containing pBABE puro hTERT in the sense orientation was re-streaked on LB agar ampicillin plates and incubated overnight at 37°C. One clone was taken and grown in a volume of 500 ml of LB broth containing ampicillin (100 μg/ml) until stationary. Highly purified closed circular plasmid DNA was isolated by alkaline lysis of the cells, neutralisation and ion exchange chromatography (Hybaid, as previously).

1.2 Preparation and use of stable ecotropic retroviral producer lines

All cell cultures were routinely maintained at 37°C under 5% CO_2.

An ecotropic producer cell line ΩE was plated out at a density of 1-2x10^5 cells/60mm dish in DMEM supplemented with 10% foetal calf serum (FCS). ΩE is a high titre ecotropic helper-free packaging cell line derived from NIH 3T3. It is ideal for use with pBABE vectors as the risk of generation of wild type virus is reduced due to extensive changes in the codon usage of the viral proteins (Morgenstern, J.P.and Land, H. (1990) Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res*, **18**, 3587-96).

Four hours prior to transfection, fresh medium (3.6 ml, HEPES-free) was added to the dish. Aliquots of pBABE puro hTERT and pBABE puro (control) plasmid DNA (10 μg) were mixed with 200 μl of 0.25 M $CaCl_2$ and taken up in a syringe with a 21 gauge needle. The needle was exchanged for a 25 gauge needle. This solution was then added dropwise to 200 μl 2X Hepes buffered saline (HBS; 25 mM Hepes, pH 7.05-7.12, 0.015 M Na_2HPO_4, 0.28 M NaOH). Air was passed through the 2X HBS while the DNA solution was added. The solution was left for 20-30 mins for a precipitate to form. The precipitate was gently resuspended and the total volume of 400 μl was added dropwise to the 60 mm dish *via* a 19 gauge needle. The cells were returned to the incubator and left overnight and washed twice with 5 ml warmed phosphate buffered saline (PBS). Fresh medium was then added to the cells, which were allowed to recover for 2 days. The cells were then trypsinised and passed into 100 mm dishes and fresh medium added. The cells were left to attach overnight. The following day fresh medium containing puromycin (2.5 μg/ml) was added.

The aim of puromycin selection was to isolate cells that had taken up and integrated pBABE puro and its hTERT derivative. The purpose of the ecotropic producer line is to provide retroviral particles that will in turn infect an amphotropic producer line. Although it is perfectly acceptable to transfect amphotropic producers directly with pBABE vectors, we have found more stable and higher titres are achieved using the ecotrope/amphotrope approach. The ecotrope producer cell lines are therefore a transitional stage in the production of amphotropic cell lines and hence no effort is made to isolate individual clones. We allowed dishes of colonies to expand and were passed into 75 cm^2 TC

flasks. We harvested ecotropic retrovirus from these pooled populations.

1.3 Preparation and use of stable amphotropic retroviral producer lines

1.3.1 Infection of ΨCRIP

ΩE producer cells were grown to 95% confluency in selective medium. 12-18 hours prior to infection, the medium was removed and the cells washed in pre-warmed HBSS or medium, before adding 10 ml pre-warmed "harvest medium" (appropriate for the target cells) which contained freshly added glutamine but no selective agent. The cell line used for the production of amphotropic retroviral particles was ψ CRIP, derived from NIH3T3 (Danos, O.and Mulligan, R.C. (1988) Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges. *Proc Natl Acad Sci USA*, **85**, 6460-4). ψ CRIP cells were plated 18-24 hours before retroviral infection in 60 mm dishes, in DMEM supplemented with 10% FCS, at a density that would give a subconfluent population 3 days after plating ($2x10^5$ cells). One hour prior to infection, the cells were re-fed with fresh medium containing 8 μg/ml polybrene.

Ecotropic producer supernatant was collected and cell debris removed by centrifugation at 1000 rpm. Supernatant was sterilised by 0.45 μm filters pre-equilibrated with 1 ml medium. Polybrene was then added to a final concentration of 8 μg/ml. Medium was removed from ψCRIP target cells and 3 ml of filtered supernatant added and incubated at 37°C. After 4 hours, 2 ml of normal growth medium was added. Fresh medium was added 24 hours later.

The cells were harvested 48 hours after infection and seeded into 100 mm dishes at 1/2, 1/10, 1/50, 1/250 and 1/500 fold dilutions. After a further 24 hour incubation, puromycin was added at a concentration of 2.5 μg/ml.

1.3.2 Establishing titre of retroviral producer lines

The addition of puromycin killed off cells that had failed to integrate retrovirally transduced genes. After a few days, puromycin-resistant colonies became apparent, and these were isolated by trypsinisation within cloning rings and passage into 12-well dishes. Supernatants were collected from these as before and titred.

Retroviral supernatants were titred on the human cell line A431 (derived from vulva epithelial carcinoma). A431 cells were plated at a density of $1x10^5$ in RPMI supplemented with 10% FCS, in 60mm dishes and infected as before using 1/10 and 1/1000 fold dilutions of retroviral supernatant. Two days after selection, cells were replated at a dilution of 1/100, 1/1000 and 1/10,000 and 24 hours later re-fed with medium containing puromycin at 2.5 μg/ml. The titre of the viral supernatant was estimated after the selective agent had killed the non-infected cells. Colonies were detected by staining in 10% Giemsa in Sorensons buffer following methanol/acetone (3:1) fixation. ψCRIP producers for pBABE puro and pBABE hTERT puro with the highest titres were identified and the population expanded. Retroviral supernatant was collected, cell debris removed by centrifugation at 1000 rpm and snap frozen in liquid nitrogen in 5 ml aliquots and stored at -80°C.

The ψ CRIP cells were frozen for long-term storage, as follows:

Cells were trypsinised, removed in serum-containing medium and collected by centrifugation (5 mins, 1000 rpm). Cells were resuspended in 0.5 ml growth medium-containing serum. An equal volume of a freezing mixture (20% DMSO, 80% foetal calf serum) was added. Cells were then transferred to a freezing vial and placed in a storage box containing isopropyl alcohol at room temperature. This, in turn, was placed in a -80°C freezer subjecting the cells to a cooling rate of 1°C per minute. The following day, the vials were moved to long-term storage in liquid nitrogen. One vial was later recovered to confirm that the cells had been frozen in a viable state.

1.4 Amphotropic retroviral infection of MRC-5 human diploid fibroblasts with hTERT

MRC-5 human diploid fibroblasts were received from ECACC (European Collection of Cell Cultures, Porton Down, UK) at passage 16 with an indication that they would undertake a further 20 passages. Passage number is rather an arbitrary definition and does not take into account what proportion of cells is carried over each time or indeed their viability. A truer reflection of the age of the culture is to calculate the number of population doublings (pd) by counting cells after trypsinisation and determining the percentage of viable cells re-seeded. All references to the age of the cultures are based on quantifying pd in this way.

MRC-5 cells were seeded in 60 mm dishes, in DMEM supplemented with 10% FCS, at a density of $1x10^5$ and infected with retroviral supernatants derived from ΨCRIP producers cells expressing pBABE puro control virus or its pBABE puro hTERT derivative. Cells were passed into new

100 mm dishes 2 days after infection at 1/2, 1/10, 1/50, 1/250 and 1/500 fold dilutions and the following day the selective agent puromycin (1 μg/ml) was added. The cells seeded at high density rapidly formed monolayers and were subcultured into flasks. These populations were designated MRC-5 puro mixed and MRC-5 hTERT mixed. However, these pools contain cells that have integrated the retroviral cassette into different areas of their genomes and also sub-populations that may express the selectable marker but not the hTERT gene. Our long-term aim was to isolate MRC-5 hTERT cell lines cloned from single cells. Obtaining 1 million cells from a single cell requires 20 population doublings assuming exponential growth (i.e. 2^{20} = 1.05 x 10^6).

Therefore, while waiting for the subclones to achieve a density sufficiently high for analysis, the mixed population could still be used to give an indication that our initial hypothesis that telomerase expressing MRC-5 cells would support CMV replication was correct. Therefore, the cultures were used to test whether (i) telomerase activity could be reactivated in these cells (see 1.6) and (ii) that hTERT puro infected MRC-5 cells would support CMV replication (see 2.1).

1.5 Selection and expansion of immortal, telomerase expressing MRC-5 fibroblast subclones

In dishes seeded at low density after infection, colonies became apparent and were isolated by trypsinisation within cloning rings and passage in 12-well dishes as before. One MRC-5 puro (clone 1) and three MRC-5 hTERT clones (clones 2,3,4) were isolated. For the cloned cells it was assumed that 20 pd had passed in order to

arrive at 1 million cells. These individual clones were also tested for telomerase expression, CMV replication and lifespan extension. For routine subculture of these clones, 1/10 of the cells were reseeded into 75cm^2 tissue culture flasks each passage. As a safety measure the MRC5 hTERT subclones were further tested and shown to be free of replication competent retroviruses, indicating that the cells were stable and unable to pass the hTERT gene onto other cell types.

1.6 Detection of telomerase activity

Telomerase is detectable as an enzymatic activity present in whole cell extracts. As mentioned hereinbefore, the telomeric repeat amplification protocol (TRAP assay) is a polymerase chain reaction- based protocol. The version of the TRAP assay described here is based on the method of Kim *et al.* ((1994) Specific association of human telomerase activity with immortal cells and cancer. *Science*, **266**, 2011-2015), with some modifications.

The TRAP assay is a two-stage protocol designed to detect telomerase activity in cell extracts prepared from tissue culture cells or tumour samples. A positive control is required; commonly extracts of the immortalised human embryonic kidney cell line 293. In the first stage a primer is extended by telomerase which adds copies of the repeat sequence TTAGGG. These extension products are undetectable by conventional means and are amplified in the second stage by polymerase chain reaction. PCR products are resolved on acrylamide gels. Telomerase requires the presence of its associated RNA template hTERC and thus the entire protocol is undertaken in RNase free conditions using DEPC-treated consumables and solutions.

1.6.1 *Preparation of cell extracts*

Cells were trypsinised, harvested, washed once in serum containing medium, counted and given a further wash in PBS. These were then resuspended in 1 ml of a wash buffer (10 mM Hepes.KOH, pH 7.5, 1.5 mM $MgCl_2$, 1 mM KCl, 1 mM dithiothreitol) and transferred to 1.5 ml microtubes and centrifuged at 15,000 x g for 2 mins at 4°C. Cells were resuspended in a lysis buffer (10 mM Tris -HCl pH 8.3, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 0.5% CHAPS, 5 mM 2-mercaptoethanol, 1 mM phenyl-methyl-sulphonyl-fluoride (PMSF)). When cell numbers are not limiting lysis buffer is added at a concentration of 185 μl per 10^7 cells. However in the studies described here the cells were resuspended at a density of 5000 cells per μl lysis buffer. After a 30 minute incubation the lysate was centrifuged at 100,000 x g for 30 min at 4°C. The supernatant (extract) was snap frozen on dry ice in 10 μl aliquots. The pellet containing genomic DNA was retained for further analysis.

1.6.2 *Telomeric Repeat Amplification Protocol (TRAP Assay)*

In the first stage of the assay, 1 μl of diluted cell extract (equivalent to 3000 cells) was added to 50 μl of oil-capped buffer containing 20 mM Tris-HCl , pH 8.3, 1.5 mM $MgCl_2$, 63 mM KCl, 0.005% Tween 20, 1 mM EGTA, 50 μM dCTP, 50 μM TTP, 50 μM dGTP, 50 μM dATP, 0.1 mg/ml acetylated BSA, 1 μg T4 gene 32 protein and 100 ng TS primer (5'- AATCCGTCGAGCAGAGTT-3') and incubated for 30 mins at 30°C in a thermal cycler. The temperature was then raised to 94°C to destroy the telomerase activity and maintained, while 2.5 U Taq polymerase, 100 ng CX

primer:(5'-CCCTTACCCTTACCCTTACCCTAA-3') and 0.5 x 10^{-18} g of ITAS (150 bp internal standard) were added. The samples were then subjected to 31 cycles of denaturation (94°C, 30 s), annealing (50°C, 30 s) and extension (72°C, 90 s) and then held at 4°C.

Negative controls were duplicate samples where the extract was heat denatured at 85°C for 10 mins prior to addition to the reaction. The 293 cell line provided the telomerase positive control. Reaction products were separated on 10% non-denaturing polyacrylamide (19:1) 20 cm Protean II gels of 1 mm thickness in 1.5 X TBE at about 300 V for 4 hours. Gels were then washed with a 1:10000 dilution of Sybr Gold for 10 mins followed by de-staining in water and scanned on an APB STORM 860 system using blue fluorescence mode. An extract is said to be telomerase-positive if a DNA ladder of 6 bp periodicity is present and there is no corresponding signal in the heat-treated control. An extract is said to be telomerase-negative if no DNA ladder is present and the internal standard, a single band at 150 bp, indicates that the PCR reaction was not inhibited. An alternative to post-staining of PCR products by fluorescent dyes is to use radiolabelled nucleotides or oligonucleotide primers. The products can be detected on X ray film or phosphor screens within a few hours.

1.6.3 Detection of telomerase activity in MRC-5 cells infected with pBABE puro hTERT

In order to demonstrate that MRC-5 would express telomerase after infection with pBABE puro hTERT, it first had to be established that MRC-5 cells were telomerase negative. TRAP assay analysis of an aliquot of 3000 cell

equivalents (CE) of an extract prepared from MRC-5 showed that this was the case (Fig. 4). It was then shown that pBABE puro hTERT infection of MRC-5 re-activated telomerase in these cells. Figure 5 shows that extracts prepared from the MRC-5 hTERT mixed culture were indeed telomerase-positive. The absence of telomerase activity in the MRC-5 puro mixed culture demonstrates that the act of retroviral infection alone was not responsible for hTERT induction. Individual MRC-5 subclones were also tested for telomerase activity. MRC-5 puro clone 1 assayed at 26 pd after infection remained telomerase negative (Fig. 6). MRC-5 puro hTERT clones 2, 3 and 4, assayed at pd 30, 24 and 24 respectively, remained telomerase positive indicating that expression of hTERT was maintained and not a transient effect.

1.7 Determination of terminal restriction fragment (TRF) length by in-gel hybridisation

This method digests genomic DNA with restriction enzymes and detects the terminal restriction fragment length of chromosomal DNA containing the telomere and undigested sub-telomeric DNA. 1 μg of genomic MRC-5 DNA was digested overnight in a 30 μl volume in Buffer M (Amersham Pharmacia Biotech) containing 15 units of Hinf I and Rsa I. 1 μl of this sample was run out on 0.7% agarose gel to test if DNA was fully digested. Successful digests were separated on a 15 x 15 cm 0.5% agarose gel (~200 ml) in TBE. One lane contained 32-P labelled λ Hind III markers. Samples were run at 100V for 20 min followed by 20 V overnight. When bromophenol blue had run approx 3/4 of the way down the gel, it was denatured with a buffer containing 1.5M NaCl, 0.5M NaOH for 15 mins and neutralized with 1.5M

NaCl, 0.5M Tris, pH8.0, for 10 mins. The gel was dried at room temperature for 1 hour (*ie* under vacuum but with lid of gel drier up) followed by 30 min at 50°C and gently lifted off the paper.

Hybridisation was in bottles in a volume of 25 ml containing 5X SSC, 0.1X P Wash (1X is 5mM sodium pyrophosphate, 100mM Na_2HPO_4), 5X Denhardt's. An oligonucleotide DNA probe 5'-CCCTAACCCTAACCCTAA-3' (500 ng) end-labelled with 32P using γATP and T4 polynucleotide kinase were added and hybridized at 37°C overnight and washed as follows: 2 x 7 mins at 37°C in bottles with 0.1 X SSC, 3 x 7 mins at RT in bottles with 0.1X SSC and 1 x 7 mins in tray with buffer preheated to 37°C. Gel was wrapped in Saranwrap and placed in cassette with film or phosphor screen. Mean terminal restriction fragment (TRF) length was calculated following densitometry using the method of Kruk *et al.*, ((1995) DNA damage and telomeres-relation to aging. *Proc. Natl. Acad. Sci. USA*, **92,** 258-262). It can clearly be seen that in MRC-5 hTERT clones 2,3 and 4 that the telomeres have been substantially extended by the action of telomerase (Fig 7).

1.8 Measurement of life-span extension

The definitive test of whether telomerase expression had an effect on MRC-5 was to determine if the cells had become immortal. This is simply determined by counting the number of pd the hTERT expressing clones would undertake. The MRC-5 bulk culture obtained from ECACC underwent replicative senescence (Fig 8) showing the morphological changes associated with senescent cells (Fig 9).

The kinetics of growth of telomerase-positive MRC-5 subclones 2, 3 and 4 (Fig. 10) and their cellular

morphology (Fig. 11) strongly imply that these cells are immortal. The sub-clones have been in continuous culture for almost one year and have undertaken at least twice as many population doublings as the control cell line, MRC-5 puro clone 1. MRC-5 puro clone 1 appears to have senesced before the bulk population of uninfected MRC-5. The bulk culture is a mix of cells that will arrive at the senescence threshold at different times. The seemingly premature senescence of MRC-5 puro clone 1 reflects that the cell from which this clone was derived was likely to be at the low end of the range of replicative potentials of cells in the bulk culture.

Example 2: Viral replication in hTERT immortalised human diploid fibroblasts

The following protocols describe the evidence indicating that telomerase immortalisation has no obvious effect on the ability of HMCV to infect cells modified in this way. A reporter system for HCMV infection is described, as are two systems demonstrating the cells' ability to maintain episomal vectors.

2.1 Determination of viral titre on human cell lines.

2.2 Time course of virus production on human cell lines.

2.3 Propagation of an episomal vector in hTERT immortalised human foreskin fibroblast cells.

2.4 Propagation of an episomal vector in hTERT immortalised MRC-5 cells.

2.1 Determination of viral titre on human cell lines

An assay was performed to assess the capability of HCMV to infect hTERT-immortalised fibroblasts. A defined RCMV288 virus stock infected nine different cell lines in order to determine the viral titre on each cell type. RCMV288 is based on HCMV strain AD169 but has a copy of GFP (green fluorescent protein) inserted in one copy of the HCMV long repeat under the control of the HCMV ß-2.7 early promoter. This insertion does not incapacitate the virus but provides a convenient reporter system for monitoring infection in live cells.

In RCMV288, deletion of only one of the two copies of the gene encoding β2.7 ensures RCMV288 has the same phenotype as the parental virus due to persistence of the second copy of β2.7. RCMV288 virus was constructed by co-transfecting DNA of the HCMV strain AD169 with the linearized plasmid pAL288. pAL288 was designed to promote homologous recombination into the inverted repeats flanking the unique long segment of the AD169 genome replacing the gene encoding the β2.7 early RNA of HCMV with that encoding EGFP (Clontech). pAL288 contains the gene encoding EGFP flanked by the regions both 5' and 3' to the β2.7 gene which contain the β2.7 major early promoter and the gene's polyadenylation signal respectively. Thus in RCMV288 EGFP expression is controlled by the major early promoter (see Fig. 12).

Cell lines tested using RCMV288 were HCA2; human foreskin fibroblasts (HFF) HCA2 immortalised by hTERT; Ihfie1: HFF cell line immortalised with E6/E7 of human papillomavirus (HPV) expressing IE1 of HCMV; Ihfie2: HFF cell line immortalised with E6/E7 of HPV; HFFF, human foetal foreskin fibroblasts; MRC-5: foetal lung fibroblasts

and MRC-5-hTERT 2,3,4, three individual clones of hTERT immortalised MRC-5.

Confluent 6-well plates (30 mm dishes) of each of the 9 cell lines were infected in triplicate at four different dilutions of virus. All cells were cultured in Eagle's minimum essential medium (MEM) supplemented with Earle's salts containing 10% (v/v) foetal calf serum, 1 x 10^5 IU/L penicillin, 100 mg/L streptomycin and 2 mM glutamine. RCMV288 stock was produced on human foreskin fibroblasts. 1 ml of inoculum was added to each well and was allowed to adsorb in a 37°C rocking incubator for 90 minutes. The inoculum was then removed and the cells washed with PBS and fresh medium added. Ten days after infection green plaques (*ie* expressing GFP) were enumerated using an inverted fluorescence microscope and the virus titre on each cell line was calculated (see Table 1).

Table 1: Viral titres of a series of human cell lines

Cell line	Virus Titre (pfu/ml)
HCA2	3.33×10^7
HCA2-hTERT	1.33×10^7
Ihfie1	1.5×10^7
Ihfie2	1.2×10^6
HFFF p18	9.33×10^7
MRC-5 p26	8.33×10^7
MRC-5-hTERT clone 2	7×10^7
MRC-5-hTERT clone 3	5.67×10^7
MRC-5-hTERT clone 4	6.33×10^7

Key to Abbreviations:
HCA2: Fibroblast cell line
HCA2-hTERT: Immortalised HCA2 cell line
Ihfie1: HFF cell line immortalised with E6/E7 of Human papillomavirus (HPV) expressing IE1 of HCMV
Ihfie2: HFF cell line immortalised with E6/E7 of HPV
HFFF: Human foetal foreskin fibroblasts
MRC-5: Foetal lung fibroblasts

MRC-5-hTERT 2,3 and 4: Three individual clones of hTERT immortalised MRC-5

The viral titres were similar on the three hTERT-immortalised MRC-5 cell lines, MRC-5 and HFFF. A slightly lower titre was obtained in Ihfie1 cell produced by immortalisation with the HPV oncogenes but titre was similar to another fibroblast of this type expressing the HCMV IE1 gene (IHFie1 cells) (Greaves, R.F.and Mocarski, E.S. (1998) Defective growth correlates with reduced accumulation of a viral DNA replication protein after low-multiplicity infection by a human cytomegalovirus ie1 mutant. *J Virol*, **72**, 366-79). This assay demonstrates that immortalisation of fibroblasts with hTERT was compatible with efficient infection with HCMV. Examples of RCMV288 plaques formed in primary and hTERT immortalised MRC-5 cells are shown in Figure 13.

2.2 Time course of virus production on human cell lines

We were interested in determining whether the rate of virus replication was affected in hTERT immortalised fibroblasts. To evaluate this aspect, 25 cm^2 flasks of HFFF, MRC-5 or MRC-5-hTERT clone 3 were infected with 5 $x10^4$ plaque forming units (PFU) of RCMV288 (MOI 0.1). The inoculum was allowed to adsorb for 90 minutes in a 37°C rocking incubator before the virus was removed, the cells washed with PBS and fresh medium added. At 3, 6, 9, 12 and 15 days after infection, medium was removed from the infected cells and stored at -70°C. This medium was then used to determine the virus titre produced by the three cell types at each time point by plaque assay on HFFFs.

It can be seen from Fig 14 that virus harvested from the MRC-5 and MRC-5-hTERT cell lines have very similar growth curves. This indicates that the two cell lines have similar rates of infection and virus production. Virus replication proceeded more rapidly in MRC-5 and MRC-5-hTERT cell lines than in HFFF cells. Infection with EGFP-HCMV induces typical HCMV plaque production and no change in plaqueing efficiency is seen with telomerase-immortalised MRC-5 cells, even at high passage (pd 89). The kinetics of HCMV infection in telomerase-immortalised MRC-5 cells is identical to the non-immortalised cells, with rate of infection and peak rate of virus production not significantly affected. In high passage cells, the rate of infection is slightly slower, but the peak level of virus production is similar. Overall, HCA2 cell lines show lower virus titres than those for MRC-5 cell lines.

2.3 Propagation of an episomal vector in hTERT-immortalised human foreskin fibroblast HCA2 cells

Episomal vectors used are based on the Epstein-Barr virus (EBV) genome which is maintained as an episome in human cells. Such vectors contain the EBV origin of replication (oriP) and nuclear antigen (EBNA-1), which are necessary for high copy episomal replication in human cell lines. Vectors used in these studies also contain the hygromycin selectable marker to allow selection of cells. Episomal vectors have been shown to produce stable and strong transgene expression over time. Prior infection with RAd114, an adenovirus expressing the EBV nuclear antigen EBNA-1, up-regulates the transfection efficiency of EBV episomal vectors.

HCA2-hTERT cells were seeded into a 60mm dish at a density of 2 x 10^5. The following day, these cells were infected with a replication-deficient adenovirus RAd114 (MOI 30). Twenty-four hours after infection, the cells were transfected with an episomal plasmid, pAL105, containing the gene encoding LacZ under the control of the major early promoter of CMV. Transfections were performed using Effectene transfection reagent (QIAGEN) according to the manufacturer's protocol. The cells were allowed to recover for 96 hours after transfection before selection using media containing hygromycin at 30 μg/ml.

A bulk population of hygromycin-resistant cells was allowed to grow. In order to test for LacZ expression the cells were infected with HCMV in order to transactivate the major early promoter. When all cells exhibited a cytopathic effect the cells were fixed with 2% paraformaldehyde and incubated with a 0.02% solution of X-Gal, 3 mM potassium ferrocyanide, 3 mM potassium ferricyanide, 1.3 mM magnesium chloride in PBS for 4 hours at 37°C. Cells that stain blue are expressing β-galactosidase indicating that the episomal vector is present and the LacZ gene is being expressed (see Fig. 15). The episomal vector has been maintained in these cells through at least 15 passages.

hTERT-HCA2s can therefore support stable maintenance of EBV-based episomal vector with efficient transgene expression when driven by the HCMV immediate early promoter. Stable HCA2-hTERT cell lines containing an episome encoding EGFP were readily generated, demonstrating that that these hTERT-immortalised cells were capable of efficiently maintaining an EBV-based episomal vector.

2.4 Propagation of an episomal vector in hTERT-immortalised MRC-5 cells

1×10^5 MRC-5 cells were seeded into a 6-well plate (30 mm dishes). The following day, these cells were infected with a replication deficient adenovirus RAd114 (MOI 30). Twenty-four hours post infection the cells were transfected with an episomal plasmid, pAL357, which expresses GFP under the control of the major immediate early promoter of CMV. Transfections were performed using Superfect reagent (QIAGEN) according to the manufacturer's protocol. The cells were allowed to recover for 96 hours before media containing hygromycin B at 20 μg/ml was used to select out those cells that have been transfected with the episome.

CLAIMS

1. A cell line suitable for use in vaccine production, characterised in that the cells thereof are adapted to express hTERT, the catalytic subunit of human telomerase.

2. An hTERT-immortalised cell line, characterised by being capable of use in vaccine production.

3. A cell line according to claim 1 or claim 2, which is a primary (non-cancer) cell line.

4. A cell line according to any preceding claim for use in human vaccine production.

5. A cell line according to any preceding claim, wherein the cells are substantially free from adventitious agents.

6. A cell line according to any preceding claim, for use in the preparation of an antigen, such as a virus, a virus-derived agent, such as a virus-based episome, or a component thereof.

7. A cell line according to any preceding claim, comprising human diploid fibroblasts transfected with cDNA of hTERT or infected by a retrovirus carrying cDNA of hTERT, which transfected or infected cells are capable of supporting antigen production.

8. A cell line according to any preceding claim, derivable from MRC-5 or WI38 cell lines.

9. A cell line according to any preceding claim, which is an hTERT-immortalised MRC-5 cell line.

10. A cell line according to any preceding claim, transfected or infected with an antigen or cDNA encoding therefor, which antigen is selected from those derivable

from rubella, hepatitis, such as hepatitis A, polio, varicella zoster and human cytomegalovirus (HCMV).

11. A cell line according to any of claims 1 to 9, comprising human diploid fibroblasts transfected with cDNA of hTERT or infected by a retrovirus carrying cDNA of hTERT, further transfected or infected with an episomal vector or cDNA encoding therefor, or a component thereof, such as the EBV-based episomal vector comprising EBNA-1.

12. An assay method suitable for testing the capability of a virus, such as the HCMV virus, to infect an hTERT-immortalised cell line according to any preceding claim, which assay method includes the step of (a): contacting a sample of the telomerase-immortalised cell line with a virus whose genome contains a nucleotide sequence comprising a reporter gene capable of causing a measurable response, which reporter gene is under the control of the virus promoter region, whereby expression of the reporter protein corresponds to expression of the virus in the sample.

13. An assay method according to claim 12, which further includes the step of (b): measuring the response of the reporter.

14. An assay method according to claim 13, which further includes the step of (c): comparing the measurement resulting from step (b) with the response from a standard or non-immortalised sample, which has itself undergone assay method steps (a) and (b).

15. An assay method according to any of claims 13 to 14 adapted to provide a screening method for identifying antiviral agents capable of inhibiting viral infection and/or replication, which screening method comprises carrying out the assay method wherein the

contacting step (a) takes place in the presence of a test agent and wherein lack of response at step (b) indicates that the test agent has antiviral activity.

16. A diagnostic method for detecting the presence or absence of a virus in a sample, which diagnostic method includes the step of (a): contacting a telomerase-immortalised cell line incorporating a reporter construct comprising cDNA of a reporter gene capable of causing a measurable response, which reporter gene is under the control of the virus promoter region, with a test sample, whereby expression of the reporter protein corresponds to expression of the virus, when present, in the test sample.

17. A diagnostic method according to claim 16, which further includes the step of (b): measuring the response of the reporter.

18. A diagnostic method according to claim 17, which further includes the step of (c): comparing the measurement resulting from step (b) with the response from a standard sample not infected with the virus, which has itself undergone assay method steps (a) and (b).

19. A telomerase-immortalised cell line incorporating a reporter construct comprising cDNA of a reporter gene capable of causing a measurable response, which reporter gene is under the control of a virus promoter region.

20. An assay or cell line according to any of claims 12 to 19, wherein the measurable response comprises a colour change, fluorescence change or emission of light.

21. An assay or cell line according to any of claims 12 to 20, wherein the reporter gene expresses an enzyme selected from chloramphenicol acetyl transferase (CAT), a luciferase, such as Firefly luciferase and Renilla luciferase, β-galactosidase, alkaline phosphatase and

horseradish peroxidase, and derivatives of the green fluorescent protein.

22. An assay or cell line according to any of claims 12 to 21, wherein the reporter gene encodes enhanced green fluorescent protein gene (EGFP, available from Clontech).

23. An assay or cell line according to claim 22, wherein the EGFP gene replaces that encoding the β2.7 early gene of HCMV.

24. A kit for carrying out an assay according to any of claims 12 to 18 or 20 to 23, which kit comprises one or more of:

(a) a telomerase-immortalised cell line according to any of claims 1 to 11 or 19;

(b) instructions for carrying out the assay; and, optionally, one or more of:

(c) medium for culturing and/or reconstituting the cells;

(d) a standard sample for the assay;

(e) buffer for lysing the cells;

(f) buffer for the reporter construct; and

(g) means for measuring the response.

25. A kit according to claim 24 for use in a screening assay, the kit further comprising a virus whose genome contains a reporter construct comprising cDNA of a reporter gene.

26. A kit according to claim 24 for use in a diagnostic assay, the kit comprising component (a) in which the telomerase-immortalised cells are transfected with a reporter construct comprising cDNA of a reporter gene, such as green fluorescent protein, capable of causing a measurable response, such as fluorescence measurable by an inverted fluorescence microscope, which reporter gene is

under the control of the virus promoter, whereby expression of the reporter gene corresponds to expression of the virus in the sample.

27. An antigen, such as a virus, a virus-derived agent, such as a virus-based episome, or a component thereof, whenever prepared from a cell line according to any of claims 1 to 11.

28. A vaccine, comprising an antigen according to claim 27 in association with a pharmaceutically acceptable adjuvant therefor.

29. A nucleic acid sequence, such as a cDNA or an mRNA sequence, specific for the preparation of a cell line according to any of claims 1 to 11.

30. A cell line, method, kit, antigen, vaccine or nucleic acid sequence according to any preceding claim, substantially as hereinbefore described, with particular reference to the Examples.

hTRT-cDNA in pGRN121 -> List

DNA sequence 4070 b.p. gaattcgcggcc ... ggccgcgaattc linear

```
        |  10     |  20     |  30     |  40     |  50     |  60
   1 gaattcgcgg ccgcgtcgac GCAGCGCTGC GTCCTGCTGC GCACGTGGGA AGCCCTGGCC 60
  61 CCGGCCACCC CCGCGATGCC GCGCGCTCCC CGCTGCCGAG CCGTGCGCTC CCTGCTGCGC 120
 121 AGCCACTACC GCGAGGTGCT GCCGCTGGCC ACGTTCGTGC GGCGCCTGGG GCCCCAGGGC 180
 181 TGGCGGCTGG TGCAGCGCGG GGACCCGGCG GCTTTCCGCG CGCTGGTGGC CCAGTGCCTG 240
 241 GTGTGCGTGC CCTGGGACGC ACGGCCGCCC CCCGCCGCCC CCTCCTTCCG CCAGGTGTCC 300
 301 TGCCTGAAGG AGCTGGTGGC CCGAGTGCTG CAGAGGCTGT GCGAGCGCGG CGCGAAGAAC 360
 361 GTGCTGGCCT TCGGCTTCGC GCTGCTGGAC GGGGCCCGCG GGGGCCCCCC CGAGGCCTTC 420
 421 ACCACCAGCG TGCGCAGCTA CCTGCCCAAC ACGGTGACCG ACGCACTGCG GGGGAGCGGG 480
 481 GCGTGGGGGC TGCTGCTGCG CCGCGTGGGC GACGACGTGC TGGTTCACCT GCTGGCACGC 540
 541 TGCGCGCTCT TTGTGCTGGT GGCTCCCAGC TGCGCCTACC AGGTGTGCGG GCCGCCGCTG 600
 601 TACCAGCTCG GCGCTGCCAC TCAGGCCCGG CCCCCGCCAC ACGCTAGTGG ACCCCGAAGG 660
 661 CGTCTGGGAT GCGAACGGGC CTGGAACCAT AGCGTCAGGG AGGCCGGGGT CCCCCTGGGC 720
 721 CTGCCAGCCC CGGGTGCGAG GAGGCGCGGG GGCAGTGCCA GCCGAAGTCT GCCGTTGCCC 780
 781 AAGAGGCCCA GGCGTGGCGC TGCCCCTGAG CCGGAGCGGA CGCCCGTTGG GCAGGGGTCC 840
 841 TGGGCCCACC CGGGCAGGAC GCGTGGACCG AGTGACCGTG GTTTCTGTGT GGTGTCACCT 900
 901 GCCAGACCCG CCGAAGAAGC CACCTCTTTG GAGGGTGCGC TCTCTGGCAC GCGCCACTCC 960
 961 CACCCATCCG TGGGCCGCCA GCACCACGCG GGCCCCCCAT CCACATCGCG GCCACCACGT 1020
1021 CCCTGGGACA CGCCTTGTCC CCCGGTGTAC GCCGAGACCA AGCACTTCCT CTACTCCTCA 1080
1081 GGCGACAAGG AGCAGCTGCG GCCCTCCTTC CTACTCAGCT CTCTGAGGCC CAGCCTGACT 1140
1141 GGCGCTCGGA GGCTCGTGGA GACCATCTTT CTGGGTTCCA GGCCCTGGAT GCCAGGGACT 1200
1201 CCCCGCAGGT TGCCCCGCCT GCCCCAGCGC TACTGGCAAA TGCGGCCCCT GTTTCTGGAG 1260
1261 CTGCTTGGGA ACCACGCGCA GTGCCCCTAC GGGGTGCTCC TCAAGACGCA CTGCCCGCTG 1320
1321 CGAGCTGCGG TCACCCCAGC AGCCGGTGTC TGTGCCCGGG AGAAGCCCCA GGGCTCTGTG 1380
1381 GCGGCCCCCG AGGAGGAGGA CACAGACCCC CGTCGCCTGG TGCAGCTGCT CCGCCAGCAC 1440
1441 AGCAGCCCCT GGCAGGTGTA CGGCTTCGTG CGGGCCTGCC TGCGCCGGCT GGTGCCCCCA 1500
1501 GGCCTCTGGG GCTCCAGGCA CAACGAACGC CGCTTCCTCA GGAACACCAA GAAGTTCATC 1560
1561 TCCCTGGGGA AGCATGCCAA GCTCTCGCTG CAGGAGCTGA CGTGGAAGAT GAGCGTGCGG 1620
1621 GACTGCGCTT GGCTGCGCAG GAGCCCAGGG GTTGGCTGTG TTCCGGCCGC AGAGCACCGT 1680
1681 CTGCGTGAGG AGATCCTGGC CAAGTTCCTG CACTGGCTGA TGAGTGTGTA CGTCGTCGAG 1740
1741 CTGCTCAGGT CTTTCTTTTA TGTCACGGAG ACCACGTTTC AAAAGAACAG GCTCTTTTTC 1800
1801 TACCGGAAGA GTGTCTGGAG CAAGTTGCAA AGCATTGGAA TCAGACAGCA CTTGAAGAGG 1860
1861 GTGCAGCTGC GGGAGCTGTC GGAAGCAGAG GTCAGGCAGC ATCGGGAAGC CAGGCCCGCC 1920
1921 CTGCTGACGT CCAGACTCCG CTTCATCCCC AAGCCTGACG GGCTGCGGCC GATTGTGAAC 1980
1981 ATGGACTACG TCGTGGGAGC CAGAACGTTC CGCAGAGAAA AGAGGGCCGA GCGTCTCACC 2040
2041 TCGAGGGTGA AGGCACTGTT CAGCGTGCTC AACTACGAGC GGGCGCGGCG CCCCGGCCTC 2100
2101 CTGGGCGCCT CTGTGCTGGG CCTGGACGAT ATCCACAGGG CCTGGCGCAC CTTCGTGCTG 2160
2161 CGTGTGCGGG CCCAGGACCC GCCGCCTGAG CTGTACTTTG TCAAGGTGGA TGTGACGGGC 2220
2221 GCGTACGACA CCATCCCCCA GGACAGGCTC ACGGAGGTCA TCGCCAGCAT CATCAAACCC 2280
2281 CAGAACACGT ACTGCGTGCG TCGGTATGCC GTGGTCCAGA AGGCCGCCCA TGGGCACGTC 2340
2341 CGCAAGGCCT TCAAGAGCCA CGTCTCTACC TTGACAGACC TCCAGCCGTA CATGCGACAG 2400
2401 TTCGTGGCTC ACCTGCAGGA GACCAGCCCG CTGAGGGATG CCGTCGTCAT CGAGCAGAGC 2460
2461 TCCTCCCTGA ATGAGGCCAG CAGTGGCCTC TTCGACGTCT TCCTACGCTT CATGTGCCAC 2520
2521 CACGCCGTGC GCATCAGGGG CAAGTCCTAC GTCCAGTGCC AGGGGATCCC GCAGGGCTCC 2580
2581 ATCCTCTCCA CGCTGCTCTG CAGCCTGTGC TACGGCGACA TGGAGAACAA GCTGTTTGCG 2640
2641 GGGATTCGGC GGGACGGGCT GCTCCTGCGT TTGGTGGATG ATTTCTTGTT GGTGACACCT 2700
2701 CACCTCACCC ACGCGAAAAC CTTCCTCAGG ACCCTGGTCC GAGGTGTCCC TGAGTATGGC 2760
2761 TGCGTGGTGA ACTTGCGGAA GACAGTGGTG AACTTCCCTG TAGAAGACGA GGCCCTGGGT 2820
2821 GGCACGGCTT TTGTTCAGAT GCCGGCCCAC GGCCTATTCC CCTGGTGCGG CCTGCTGCTG 2880
2881 GATACCCGGA CCCTGGAGGT GCAGAGCGAC TACTCCAGCT ATGCCCGGAC CTCCATCAGA 2940
2941 GCCAGTCTCA CCTTCAACCG CGGCTTCAAG GCTGGGAGGA ACATGCGTCG CAAACTCTTT 3000
3001 GGGGTCTTGC GGCTGAAGTG TCACAGCCTG TTTCTGGATT TGCAGGTGAA CAGCCTCCAG 3060
3061 ACGGTGTGCA CCAACATCTA CAAGATCCTC CTGCTGCAGG CGTACAGGTT TCACGCATGT 3120
3121 GTGCTGCAGC TCCCATTTCA TCAGCAAGTT TGGAAGAACC CCACATTTTT CCTGCGCGTC 3180
3181 ATCTCTGACA CGGCCTCCCT CTGCTACTCC ATCCTGAAAG CCAAGAACGC AGGGATGTCG 3240
3241 CTGGGGGCCA AGGGCGCCGC CGGCCCTCTG CCCTCCGAGG CCGTGCAGTG GCTGTGCCAC 3300
3301 CAAGCATTCC TGCTCAAGCT GACTCGACAC CGTGTCACCT ACGTGCCACT CCTGGGGTCA 3360
3361 CTCAGGACAG CCCAGACGCA GCTGAGTCGG AAGCTCCCGG GGACGACGCT GACTGCCCTG 3420
3421 GAGGCCGCAG CCAACCCGGC ACTGCCCTCA GACTTCAAGA CCATCCTGGA CTGATGGCCA 3480
3481 CCCGCCCACA GCCAGGCCGA GAGCAGACAC CAGCAGCCCT GTCACGCCGG GCTCTACGTC 3540
3541 CCAGGGAGGG AGGGGCGGCC CACACCCAGG CCCGCACCGC TGGGAGTCTG AGGCCTGAGT 3600
3601 GAGTGTTTGG CCGAGGCCTG CATGTCCGGC TGAAGGCTGA GTGTCCGGCT GAGGCCTGAG 3660
3661 CGAGTGTCCA GCCAAGGGCT GAGTGTCCAG CACACCTGCC GTCTTCACTT CCCCACAGGC 3720
3721 TGGCGCTCGG CTCCACCCCA GGGCCAGCTT TTCCTCACCA GGAGCCCGGC TTCCACTCCC 3780
3781 CACATAGGAA TAGTCCATCC CCAGATTCGC CATTGTTCAC CCCTCGCCCT GCCCTCCTTT 3840
3841 GCCTTCCACC CCCACCATCC AGGTGGAGAC CCTGAGAAGG ACCCTGGGAG CTCTGGGAAT 3900
3901 TTGGAGTGAC CAAAGGTGTG CCCTGTACAC AGGCGAGGAC CCTGCACCTG GATGGGGGTC 3960
3961 CCTGTGGGTC AAATTGGGGG GAGGTGCTGT GGGAGTAAAA TACTGAATAT ATGAGTTTTT 4020
4021 CAGTTTTGAA AAAAAgtcga cgcggccgcg gtcgacgcgg ccgcgaattc             4070
        |  10     |  20     |  30     |  40     |  50     |  60
```

Figure 1

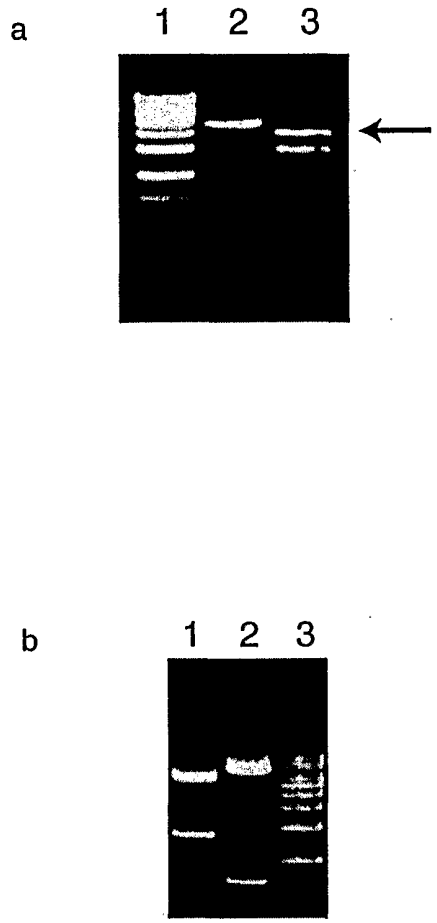


Figure 2 Preparation and verification of pBABE puro hTERT.
a. Lane 1 kb ladder; 2 pBABE puro EcoRI digest; 3 pGRN121 EcoRI digest. Arrow indicates hTERT cDNA.

b. Lanes 1 and 2, BamHI digestion of miniprep DNA. In lane 1 BamHI produces a 2.5 kb fragment indicating hTERT is in the sense orientation, lane 2 antisense. Lane 3, kb ladder

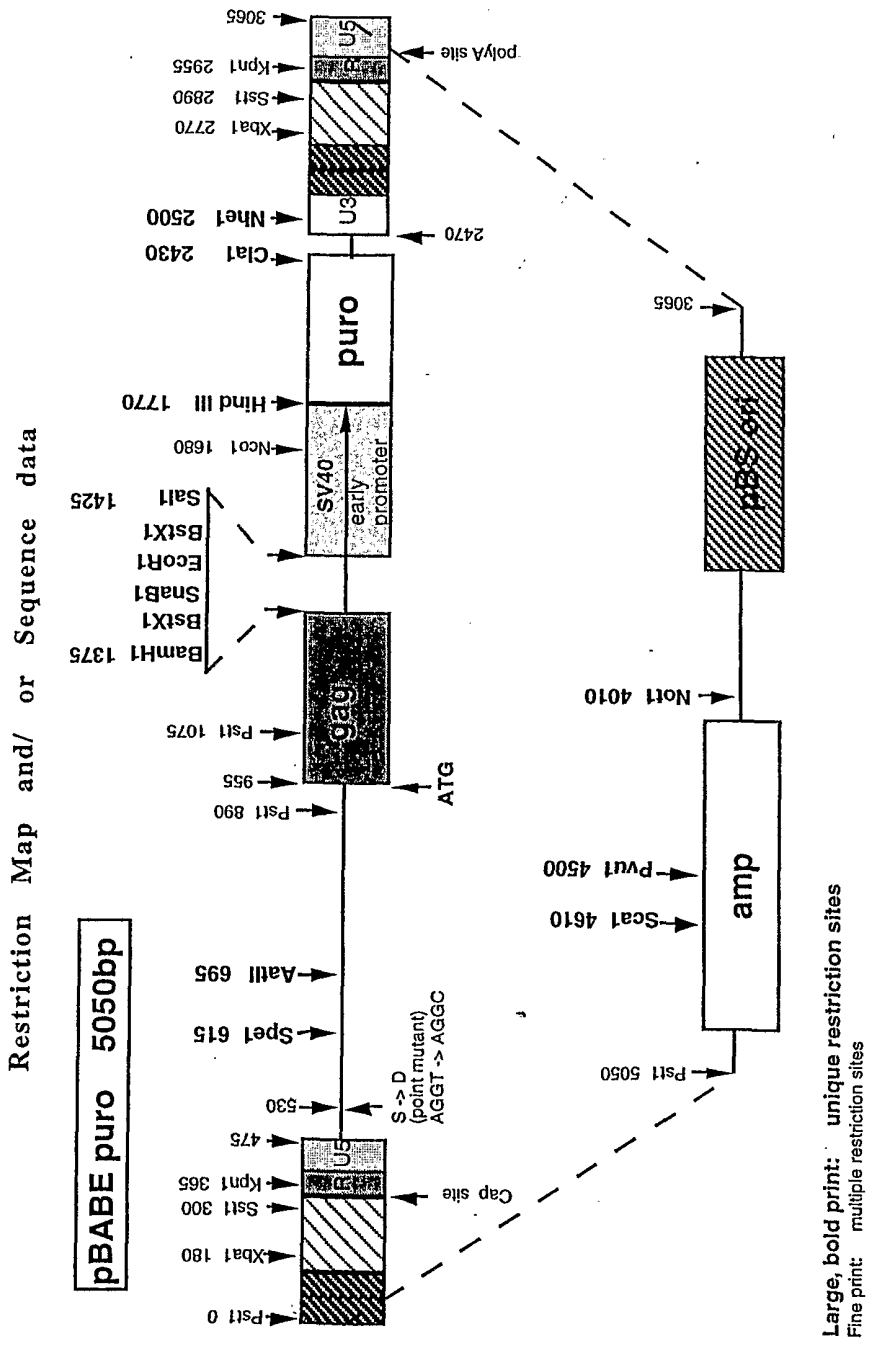

Restriction Map and/ or Sequence data
pBABE puro 5050bp
Large, bold print: unique restriction sites
Fine print: multiple restriction sites
Select for constructs derived from this vector in Ampicillin (100µg/ml)
Select cells infected in culture in 1.0 - 2.5µg/ml puromycin
puro
amp
pBS ori
gag
SV40 early promoter
U3
R
U5
polyA site
Cap site
ATG
S -> D (point mutant) AGGT -> AGGC
PsiI 0
XbaI 180
SstI 300
KpnI 365
475
530
SpeI 615
AatII 695
PsiI 890
955
PsiI 1075
BamHI 1375
BstXI
SnaBI
EcoRI
BstXI
SalI 1425
NcoI 1680
HindIII 1770
ClaI 2430
NheI 2500
2470
XbaI 2770
SstI 2890
KpnI 2955
3065
3065
NotI 4010
ScaI 4610
PvuI 4500
PsiI 5050

Figure 3

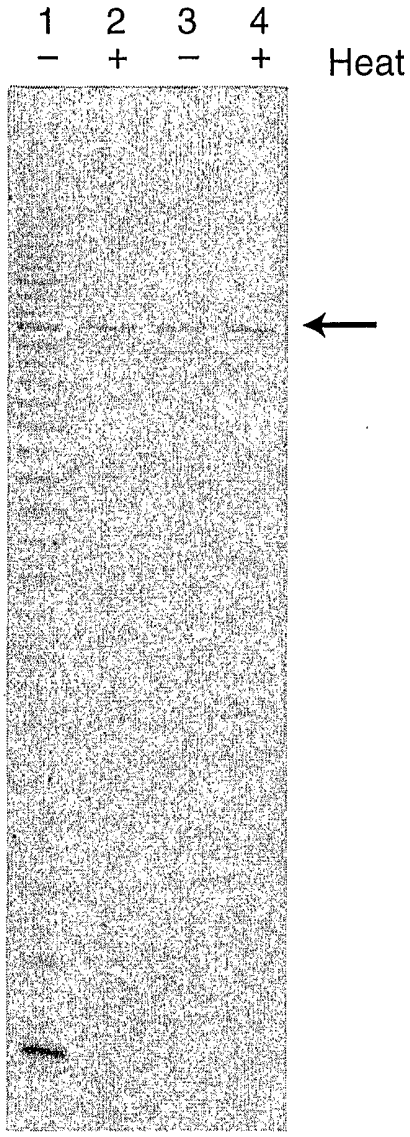


Figure 4. Analysis of telomerase activity in MRC5 cells. Analysed at passage 23. Lane 1,2 293; 3,4 MRC5

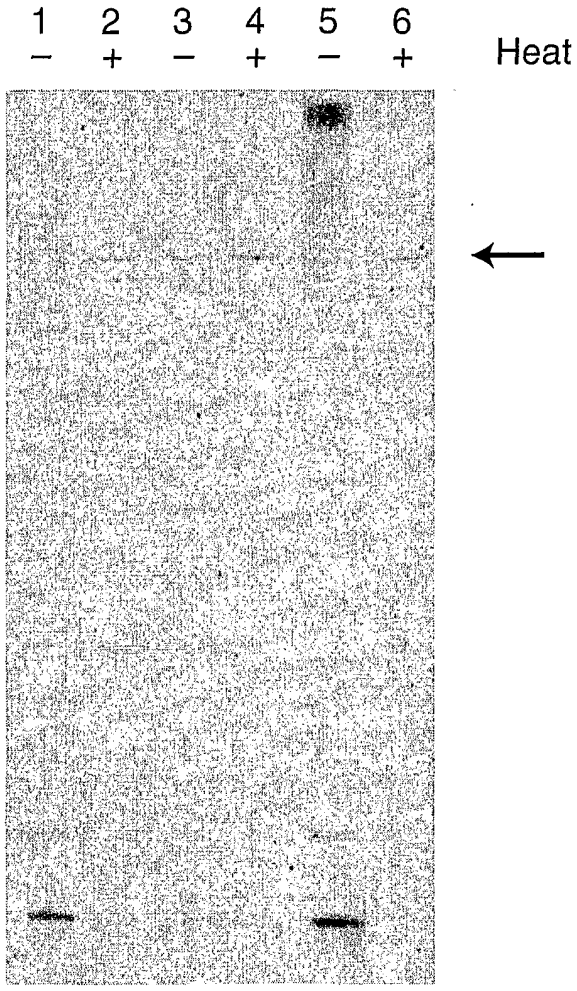


Figure 5 Analysis of telomerase activity in pooled MRC5 cultures.
Lane 1,2 293; 3,4 MRC5 pBABE puro; 5,6 MRC5 puro hTERT.

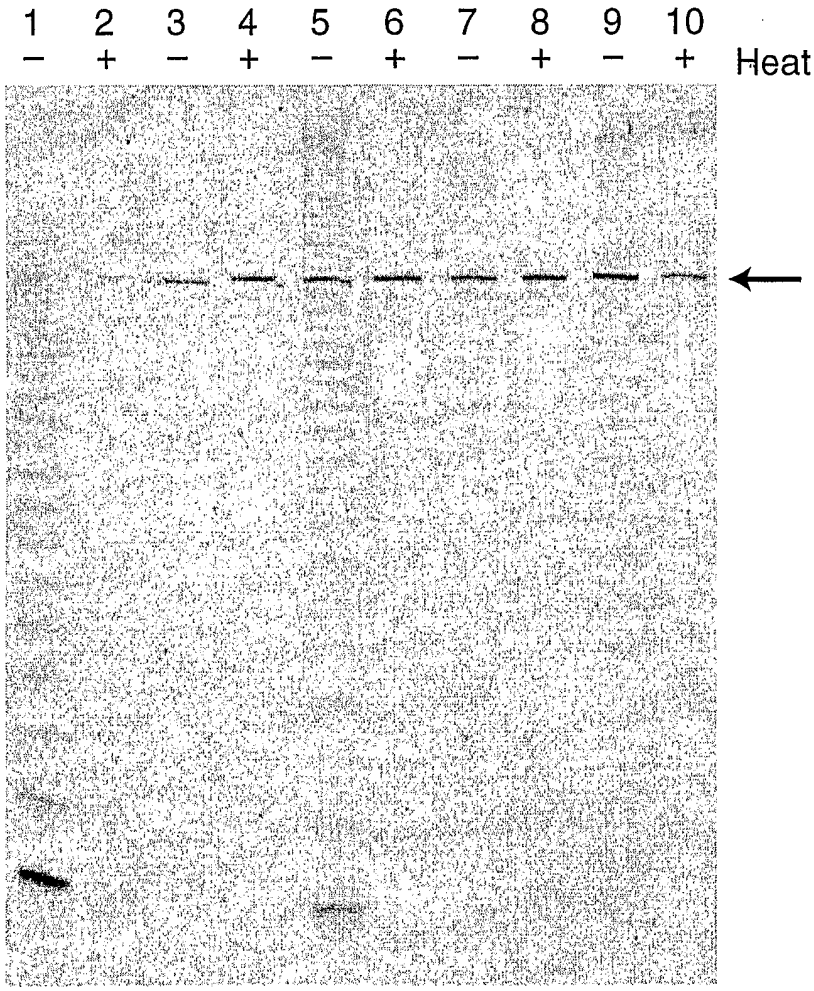


Figure 6 Analysis of telomerase activity in MRC5 subclones. Lane 1,2 293; 3,4 MRC5 pBABE puro clone 1 analysed at 26 pd after infection; 5,6 MRC5 puro hTERT clone 2 (pd 30); 7,8 MRC5 puro hTERT clone 3 (pd 24); 9,10 MRC5 puro hTERT clone 4 (pd 24).

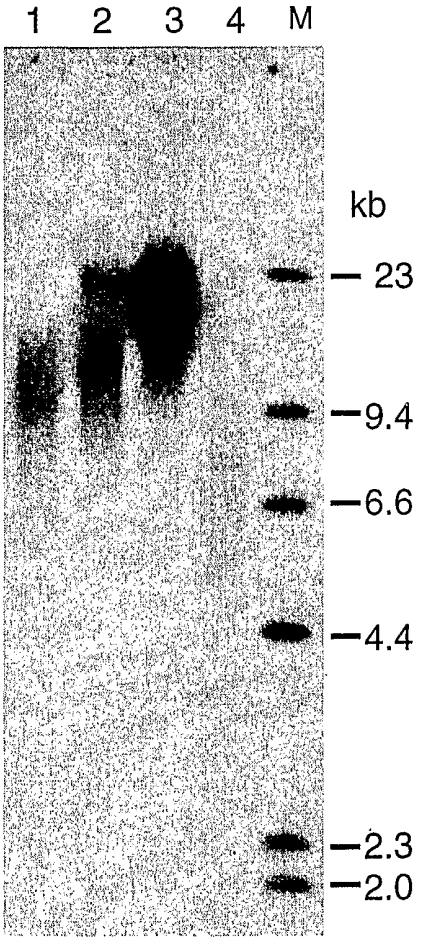


Figure 7. Terminal restriction fragment analysis of hTERT immortalised MRC5 cells. Lanes 1, 2, 3 MRC5 hTERT clones 2, 3, 4 respectively; lane 4 Mrc5 bulk culture pd 18. M is a 32P-labeled λ HindII digest. Note the higher molecular weight smears of telomeric DNA in the hTERT immortalised clones indicating continued activity of telomerase.

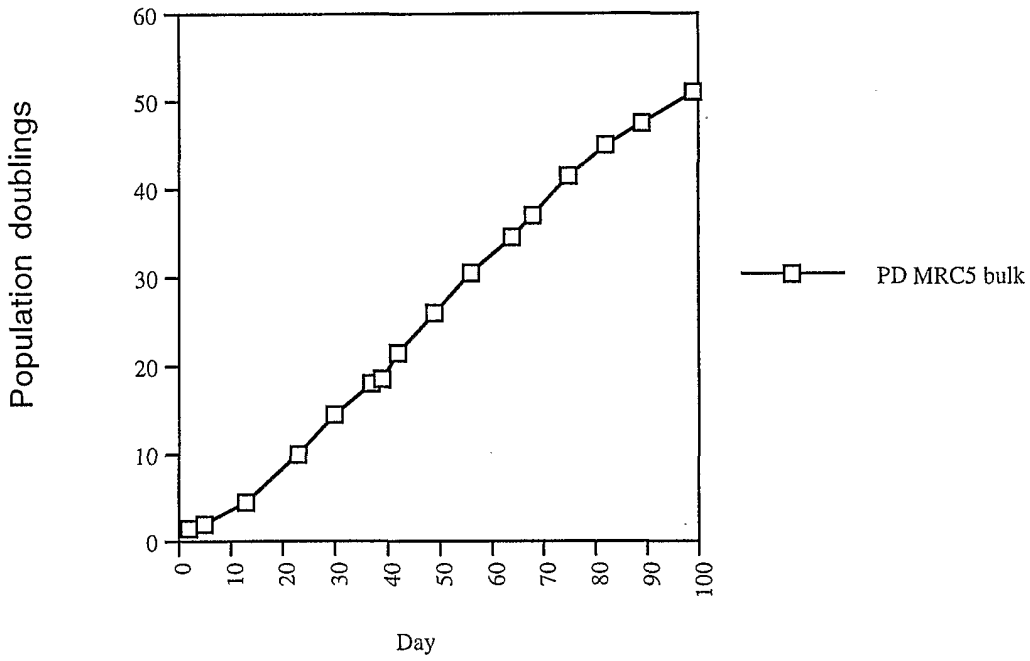


Figure 8. Growth curve of MRC5 culture

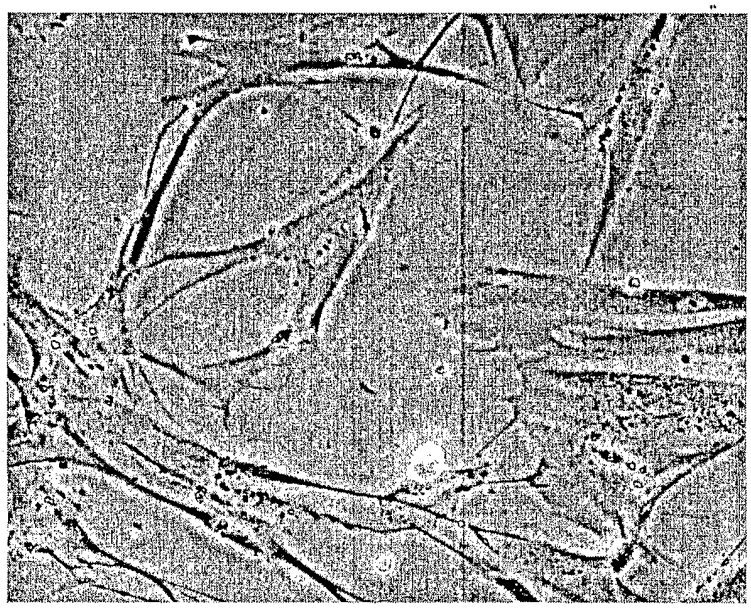


Figure 9. MRC5 cells at 54 pd in continuous culture. Note the larger flattened senescent cells appearing in the culture.

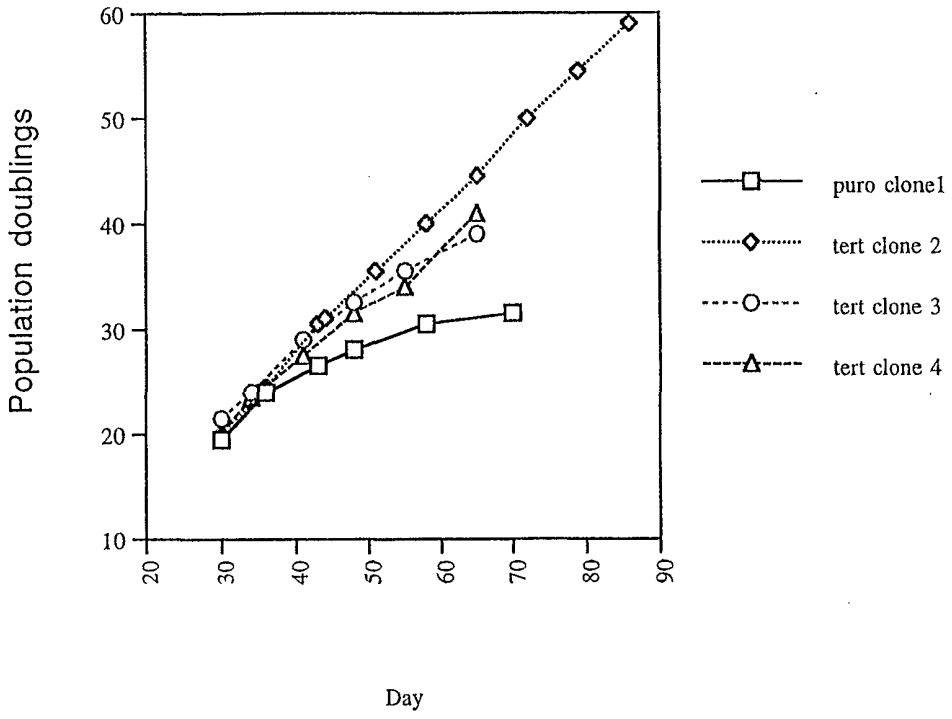


Figure 10. Growth curves of MRC5 puro clone 1 and MRC5 hTERT clones 2, 3, 4.

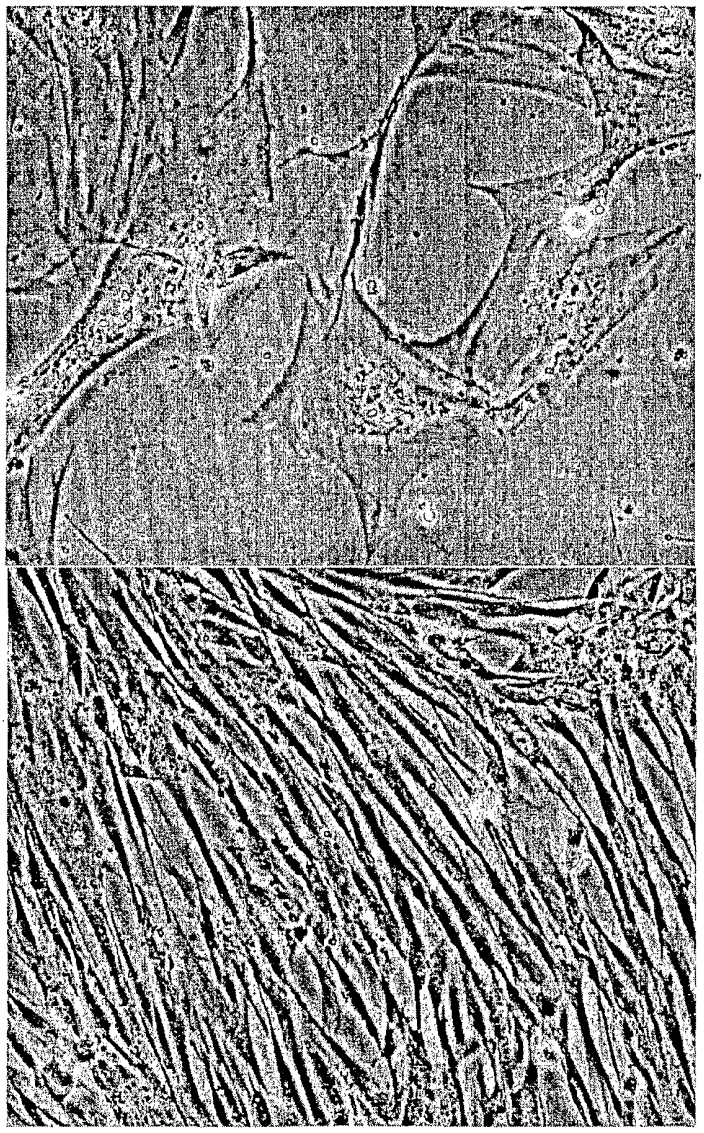


Figure 11. Upper panel MRC5 pBABE puro clone 1 at 31 pd after infection. Lower panel MRC5 pBABE puro hTERT clone 2 at 45 pd after infection. Note the "youthful" morphology of the hTERT expressing cells compared to the larger flattened senescent vector control culture.

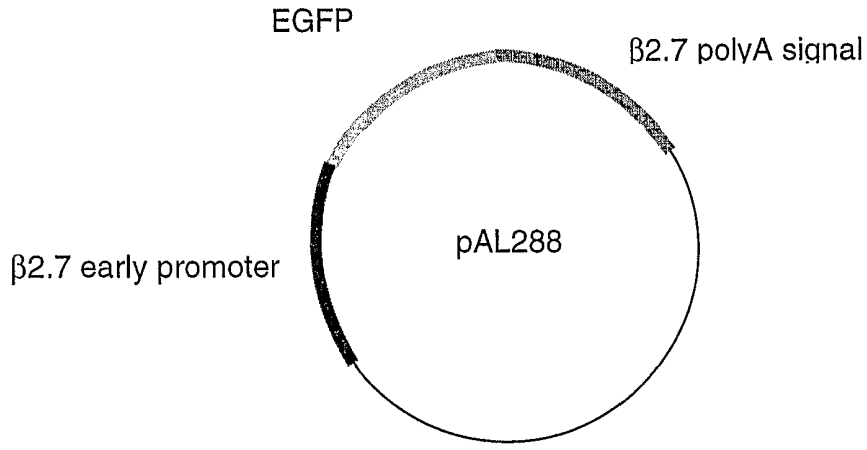
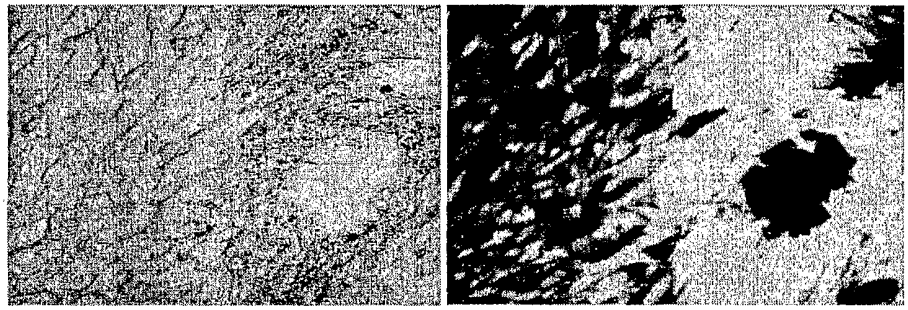


Figure 12. Schematic representation of pAL288

MRC5



MRC5 hTERT Clone 3

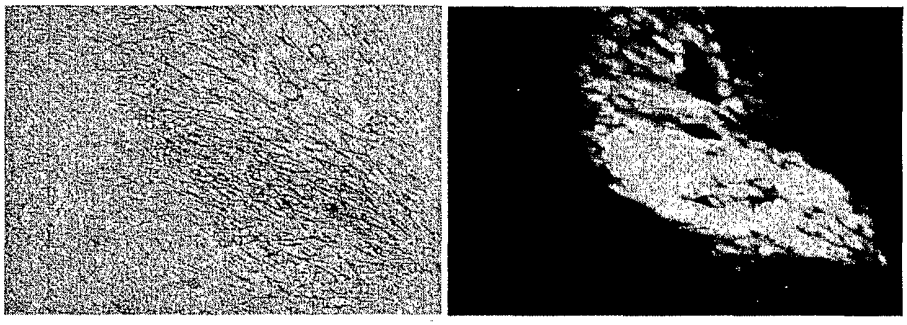


Figure 13. Plaques produced by infection with RCMV288 on primary and hTERT immortalised MRC5 cells. Cells visualised by phase contrast (left panels) and FITC filter (right panels) to detect EGFP expression.

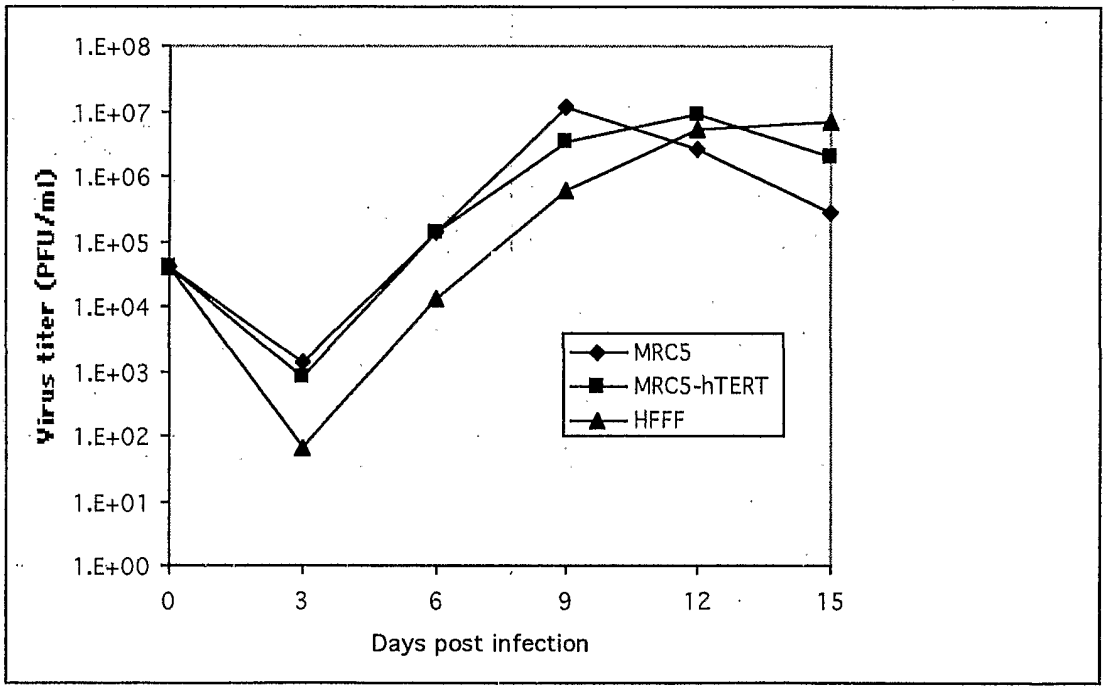


Figure 14 Rate of replication of HMCV in different cell types

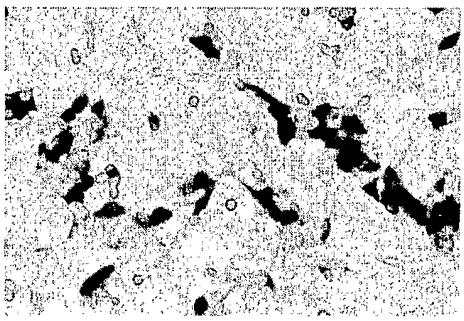


Figure 15. Maintenance of episomal vector in hTERT immortalised HCA2 cells.