dioactive detection of apoB Arg3500 to Gln mutation. The method is quick (results within 2 h after PCR) and reliable.

PCR was performed on genomic DNA prepared from patients previously identified to have the Arg3500 to Gln mutation (4). Oligonucleotides (forward, 5'-TACCAAGTCAAAAACCTC-CTGTCTTCC-3' and reverse, 5'-GCGAATTCCGGCAAGGTCAG-GGAAATC-3') amplified a region of exon 26 of the apoB gene from nucleotide 10489 to 10990. PCR was performed in 50 mM Tris-HCl, pH 9.0, 20 mM (NH4)2SO4 and 1.5 mM MgCl2, using 50 ng of each primer and 350 µM of each dNTP in 50 µL. PCR was performed under the following conditions: denaturation at 96°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 40 s for 33 cycles. For denaturation, 1-µL aliquots of PCR product were mixed with 1 µL of 95% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol and 3 µL of 0.5× TBE (44.5 mM Tris borate, 1 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol). The samples were heated at 98°C for 5 min and snap-chilled on ice. The single-stranded PCR product was then separated on an 8-cm × 9-cm × 0.075-cm mini gel (Hoefer Pharmacia Biotech, San Francisco, CA, USA) using a 10.5% T, 1% C polyacrylamide gel containing 0.5× TBE. The gel was cooled with an external waterbath set at 4°C and run at a constant 3 W for 1.5 h. The gels were stained with ethidium bromide (0.5 µg/mL) for 5 min, and the bands were visualized on a transilluminator. Gels were photographed with a Polaroid® MP4 camera using type 55 film (Sigma Chemical, St. Louis, MO, USA).

Amplification resulted in a 521-bp fragment, which could be separated after denaturation into single-stranded species. Figure 1 shows the results from a control (2 bands) and nine patients containing the Arg3500 to Gln mutation (3 bands). The G→A base change in codon 3500 must have a dramatic effect on the conformation of this fragment as it is generally more difficult to detect mobility shifts in fragments >400 bp. Single-base changes are more likely to have a greater effect on the secondary structure of smaller fragments than larger fragments. However, a C→T change in a 510-bp fragment has also been reported (6). Although it is possible to detect mobility shifts in larger fragments, screening for unknown mutations with smaller fragments would probably provide greater detection sensitivity and accuracy than larger fragments.

In conclusion, this technique is simpler, less time-consuming and less costly than previous methods. Reliable results can be obtained within 2 h after PCR and can be readily used for epidemiological studies of the Arg3500 to Gln mutation.

REFERENCES


Address correspondence to Lori K. Hennessy, Hoefer Pharmacia Biotech, 654 Minnesota Street, P.O. Box 77387, San Francisco, CA 94107, USA. Internet: lori.hennessy@ussfo.pharmacia.se

Received 2 May 1996; accepted 29 July 1996.

Lori K. Hennessy and Clive R. Pullinger
Hoefer Pharmacia Biotech
University of California
San Francisco, CA, USA

Titering Replication-Defective Virus for Use in Gene Transfer

BioTechniques 22:447-450 (March 1997)

To quantitate an infective virus, the parameter that will be used to verify virus entry into target cells must be defined functionally. Some assays measure production of a unique viral product (e.g., reverse transcriptase). Others rely on morphologic changes (i.e., cytopathologic effect) in infected cells. However, most viruses—from bacterio-
phages to herpes viruses—are quantitated by one or another variation of a plaque assay.

**Plaque Assays**

Plaque development reflects the ability of a virus to infect target cells, replicate in them and then lyse them. Infectious progeny spread to adjacent cells where the process continues for a defined period of time. Agar overlays are often used to keep the virus localized after cell lysis. At the end of this time, an effect is detectable (usually visually) centered at the point of the initial infection.

Plaque assays require permissive target cells to support virus replication and a virus that expresses enough of its genome to replicate. However, viruses may infect and express their genes in cells without replicating in them, e.g., in establishing latent or persistent infection (6,12). Some cell types may be infected but either support virus replication poorly (semi-permissive) or not at all (nonpermissive) (3,8). Plaque assays rely on the ability of a virus to produce infectious progeny in at least one cell type.

**Gene Therapy**

Depending on the viral property that is sought, plaque assays may miscalculate the amount of infectious virus. The ability of a virus to infect nonpermissive cells is not necessarily the same as its ability to make plaques in permissive cells. While such discussions may appear arcane, the use of viruses as vectors for therapeutic gene transfer makes this issue important. Specifically, gene therapy applications generally require that viral vectors be replication-deficient in target cells. They may only replicate in a particular packaging cell line or when a helper virus is added. To use infectious titers determined in packaging cell lines as measures of infectivity for other cells and other cell types assumes comparable infectivity for cells derived from different species, taken from different organs, proliferating at different rates, etc.

Thus, a technique that measures the ability of a replication-incompetent virus to enter an array of target cells may be useful for those interested in using virus vectors for therapeutic gene transfer. We have applied in situ polymerase chain reaction (PCR) to measure the ability of a virus to enter target cells, regardless of cell of origin or ability to support virus replication.

**Application of SV40 to Therapeutic Gene Transfer**

This technique was devised in order to measure the infectivity of simian virus 40 (SV40) for a series of target cells. We have developed SV40-derived replication-deficient viruses to be used as gene transfer vectors. SV40 infects cells from many animal species, but it replicates best in monkey cells and generally poorly, if at all, in cells from other animals. We produced replication-defective SV40 derivative viruses by excising an early viral gene, large T antigen (TAg), from the cloned viral genome, and replacing it by the gene (transgene) whose expression is to be transferred (11). COS-7 cells provide TAg, which is necessary for packaging, in *trans*, permitting expression of late genes and production of virus particles containing the viral genome, without TAg, but with the transgene.

**In Situ PCR to Quantitate the Virus in Human Cells**

The use of in situ PCR for measuring SV40-infected cells follows the description of in situ PCR (9). Since the virus constructs used here carried the late SV40 genes, we chose PCR primers from the late region of the viral genome. Our goal was to test the ability of SV40 derivative viruses to infect human T lymphocytes, to assess their

*Figure 1. In situ PCR of TC7 cells.* Utilizing SV40 primer pair for in situ PCR and subsequently using a biotinylated labeled SV40 probe. The arrows in panel A show the TC7 cells infected with the replication-defective SV40 virions. Panel B shows uninfected cells. Both figures are taken from oil-immersion photomicrographs and are presented here, ×2000.
potential as vehicles for gene transfer to such cells.

**In Situ PCR**

The freshly infected cells were transferred to specially-designed, silanated glass slides, containing a 20-mm single well (Erie Scientific, Erie, PA, USA). The cells were air-dried overnight, heat-fixed at 105°C for 10 s and then incubated in 4% paraformaldehyde for 2 h. Paraformaldehyde was removed by incubating slides in 3× phosphate-buffered saline (PBS) for 10 min, and then the slides were washed twice in 1× PBS. The endogenous peroxidase was quenched by incubating slides in 0.3% hydrogen peroxide in PBS overnight. These slides were treated with proteinase K (6 µg/mL; Boehringer Mannheim, Indianapolis, IN, USA) at room temperature for 12 min. Proteinase K was inactivated by incubating slides on a heat block at 95°C for 5 min. The in situ amplification of the SV40 gene was performed with a primer pair (ACTGTGACTGGTGTAGCGCTG and TGGACCCAATGT-CTGGGGTC) that anneals to the SV40 genome at the overlapping VP1 and VP2 genes (2). Subsequently, amplified DNA signals were detected by a biotinylated probe, which anneals at a sequence found within the amplicons CCAGGAATGGCTGTAGATTTGTA-TAGGCCAGATGATTACGA. In situ hybridization was carried out at 37°C using a reaction mixture containing 20 pM probe, 50% deionized formamide, 2× standard saline citrate (SSC), 10× Denhardt’s solution, 1 mg/mL salmon sperm DNA and 0.1% sodium dodecyl sulfate (SDS). Detection was performed using streptavidin-peroxidase and subsequent color development carried out by 3′-amino-9′-ethyl carbazole (AEC; Sigma Chemical, St. Louis, MO, USA) and counterstaining with Gills’ hematoxylin (1). The percentage of SV40-positive cells was determined by at least two independent observers examining at least 10000 cells per slide. All microscopic analyses were performed using coded slides.

We first defined the sensitivity of this assay. Other authors have reported that in situ PCR can detect one cellular or virus gene copy (7,10). The COS-7 cells (ATCC, Rockville, MD, USA) that were used for packaging TAg-SV40 derivative virus were originally produced and analyzed by Gluzman, and they appear to bear one copy of the SV40 genome (5), including the region with the target sequences for in situ PCR amplification and detection. We found that when this in situ PCR approach was applied to COS-7 cells, >95% of the cells were positive (not shown). In parallel, a line of monkey cells, TC7, which does not carry SV40, was negative by this technique. Therefore, the sensitivity of this procedure is approximately one copy of SV40 DNA per cell. This is comparable to the sensitivity reported by others (7).

In our studies, we examined the infectivity of a derivative of SV40, SVluc, in which TAg was replaced by the firefly luciferase reporter gene. Our preparation of SVluc was titered in COS-7 cells using a standard plaque assay (4) at 1.2 ± 0.3 × 10⁸ pfu/mL. Positive reactivity by in situ PCR is shown for SVluc-infected TC7 cells (Figure 1).

The infectivity of the same SVluc preparation was analyzed for two human T lymphoma lines, SupT1 and CEM. The result of in situ PCR assay for SVluc in these cell lines is illustrated in Figure 2. SVluc infectivity for these two cell lines was measured. The infectious titer was approximately 1/10 of that of SVluc for COS-7 cells (i.e., ca. 1.0 × 10⁷ infectious units/mL). In
Benchmarks

contrast, infectious titers for SV1uc measured by in situ PCR using TC7 cells were comparable to those determined in COS-7 cells by conventional plaque assays.

Gene therapy with viral vectors aims to transfer a transgene to a target cell population and to obtain expression of that transgene. In measuring the ability of a viral vector to accomplish these goals, assays of both infectivity and transgene expression are useful. The latter are conceptually more complex, because the desired characteristic is not simply transgene expression, but expression at an effective level. That level will vary according to the individual transgene and its intended effect, and in most situations, that would not be known beforehand. Furthermore, techniques used to detect transcripts or proteins are generally not as sensitive as in situ PCR.

The issue of the infectious titer of a virus preparation is currently of great importance in assessing infective doses of a myriad of replication-defective viruses. These agents are often intended for target cells from species and organs different from those used to package the virus. Our data suggest that the number of plaque-forming units for a packaging cell line does not necessarily describe the infectivity of that virus for other target cells. We further provide an alternative measurement of tissue culture infective dose (i.e., TCID50) that may be tailored to accommodate many different types of viruses and diverse target cells.

REFERENCES


This work could not have been performed without the encouragement and advice of Drs. Roger Pomerantz and Mark Zern. These studies were supported by Grant CA44800 from the U.S.P.H.S. Address correspondence to David S. Strayer, Department of Pathology, Anatomy and Cell Biology, Jefferson Medical College, 1020 Locust Street, Philadelphia, PA 19107, USA. Internet: david.strayer@mail.tju.edu

Received 21 May 1996; accepted 29 July 1996.

D.S. Strayer, L.-X. Duan, I. Ozaki, J. Milano, L.E. Bobraski and O. Bagasra
Jefferson Medical College
Philadelphia, PA, USA