Reliable Method of Isolating Transfected Clones from the LNCaP Human Prostatic Cell Line

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The LNCaP human prostatic cell line is the only human prostatic cell line available that is both androgen-sensitive and nonmetastatic when implanted into BALB/c nude mice (4). These properties make it a frequently used model for prostate cancer research. The generation of transfected LNCaP cell lines and the subsequent isolation of transfected clones has been reported by several researchers (3,6,7). Two cloning procedures often used to clone LNCaPs are the cloning ring method (2) and the limit dilution method (5). However, these methods are difficult and tedious and only produce low clone numbers.

Two properties of LNCaP cells make them a particularly difficult cell line to clone. First, LNCaP cells grow in loosely adherent colonies (4) and therefore can easily break away from the main colony. This can make cloning rings unreliable. Second, LNCaP cells have a poor survival rate when plated at very low concentrations in tissue culture flask (unpublished observation), which can affect cloning capacity and efficiency when attempting to produce clonal lines using the limit dilution technique.

To overcome these difficulties in cloning LNCaP cells, we have developed a simple method for isolating clonal LNCaP cells that have been transfected using cationic liposome-mediated transfection (1) with a stable antibiotic resistance marker. We used LNCaP cells (ATCC, Rockville, MD, USA) transfected using LIPOFECTIN® Reagent (Life Technologies, Gaithersburg, MD, USA) with the eukaryotic expression vector pRC/CMV (Invitrogen, San Diego, CA, USA), which contains the neomycin-resistance gene for selection. Transfected cells were then selected in 100 µg/mL of antibiotic G-418 (GENETICIN®; Life Technologies) over 10 days. Because the presence of G-418 was observed to slow down LNCaP cell growth, it was removed, and the heterogeneous population of transfected cells expanded in its absence. These cells have remained stably transfected for at least 90 passages and retained G-418 resistance when reexposed to this antibiotic.

Since G-418 resistance was maintained over the period of study, clones could be established from any passage. Transfected LNCaPs at 60%-70% confluence in a 75-cm² tissue culture flask (Corning Costar, Cambridge, MA, USA) were rinsed with phosphate-buffered saline (PBS). The PBS was removed, and 3 mL 0.2 M EDTA, pH 7.5 were added for 30 s. Following removal of EDTA, cells were incubated in 0.5% trypsin and 5.3 mM EDTA for 15 s, and the excess trypsin was removed. The flask was then placed at 37°C for 5 min; then 3 mL RPMI 1640 medium from the MultiCel™ Kit (with l-glutamine, without sodium bicarbonate) and 10% fetal calf serum (FCS) from the MultiSer™ Kit (both kits from Trace Biosciences, Castle Hill, Australia) were added.

The LNCaPs were then further diluted in RPMI 1640, 10% FCS to a concentration of 25 cells/mL. Forty microliters of this cell suspension were added to each well in a Linbro® 96-well plate (ICN Biomedicals, Costa Mesa, CA, USA) so that, theoretically, each well should only contain one cell. The cells were then left overnight to adhere. The next day, the cell number per well was assessed using a phase-contrast microscope. Two hundred microliters of untransfected LNCaP cells at a concentration of 1 x 10⁶ cells/mL were added to wells that were confirmed to contain one cell. The addition of these untransfected cells acts as a feeder layer and removes the need for addition of any exogenous cytokines or hormones.

Cellular confluence was monitored daily, and cells were passaged when...
they reached 70% confluence to progressively larger plates (24-well, 6-well [both Falcon®; Becton Dickinson Labware, Bedford, MA, USA], 25-cm² flasks [Corning Costar]). Once 60% confluence had been reached in the 25-cm² flask, the appropriate antibiotic used for transfecant selection could be added to eliminate all untransfected cells. In our case, G-418 was added (500 µg/mL) for 12 days to select for resistant cells (Figure 1). After day 12, G-418 was removed, and the surviving transfected clones were allowed to recover in RPMI 1640, 10% FCS. Potential clones were then propagated until they were established in 75-cm² flasks. The complete procedure takes three months. Although it was not possible to determine the exact proportion of G-418-expressing cells in the total population before selection, we observed that after G-418 selection, 15%–20% of wells containing a single transfected cell generated a clonal cell line. Clonal status can then be verified by Southern blot analysis.

This method holds many advantages over other commonly used methods to clone LNCaP cells. For instance, methods relying on a single cell to establish a clonal population can be heavily influenced by long lag phases. In contrast, in our method, the use of untransfected feeder cells of the same cell type reliably promotes the establishment of clones. While it may also be possible to use irradiated, untransfected LNCaP cells as feeder cells, this was not tried because we were concerned that the production of necessary growth factors would remain unchanged. In addition, the use of unirradiated cells kept the procedure simple. Furthermore, unlike the delicate manipulations necessary with cloning rings, the manipulations required for this method are straightforward. This method may be applied to other cell lines, provided that the cells to be cloned contain a stable transfected marker that is toxic to untransfected cells.

REFERENCES
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Rapid Microplate Assay for Substrates and Inhibitors of Proteinase Mixtures

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Evaluation of the hydrolysis of colorimetric substrates by impure proteinase mixtures has in the past been time-consuming and tedious, with the added economic disadvantage of using relatively large amounts of consumables, such as substrates and cuvettes. In the process of characterizing and purifying insect gut proteinases, we have used a microplate reader and a kinetic software program to incorporate efficiency and economy into procedures for rapidly identifying substrates and inhibitors useful in the analysis of proteinases.

We routinely use enzyme substrates conjugated to p-nitroanilide (pNA) to measure proteinase activity. Microplate screening of enzyme activity with substrates conjugated to pNA has been described for porcine pancreatic enzymes (2), but no kinetic data were given. We present a method for an enzyme microplate assay and use the procedure to compare substrate hydrolyses by several mammalian proteinases. In addition, we show how the effects of potential inhibitors on proteinase activity can be measured rapidly and easily, generating inhibitor curves and IC\textsubscript{50} values. This technique will allow researchers to quickly identify inhibitors suitable for use in the isolation and characterization of proteins from crude extracts.

In the substrate assay, different pNA substrates (Sigma Chemical, St. Louis, MO, USA) were added at a concentration of 5 mg/mL in 100 µL assay buffer (0.1 M Tris-HCl, pH 8.1, 0.02 M CaCl\textsubscript{2}) to individual wells in the top row (row A) of a 96-well microplate. All of the remaining wells (rows B–H) contained 50 µL of assay buffer. A multichannel pipettor was used to serially dilute (1:1) the substrates from rows A to G. Row H received no substrate, serving as a negative control. Enzymes (Sigma Chemical) were diluted in assay buffer, and 50 µL were added to each well with a multichannel pipettor. The amounts of enzymes added to each well were 10 µg of trypsin (bovine pancreatic, Type XI, DPCC-treated) or α-chymotrypsin (bovine pancreatic, Type II), or 1.77 U of elastase (porcine pancreatic, Type I).

Samples were immediately incubated at 37°C in a microplate reader (Bio-Tek Instruments, Winooski, VT, USA) for 30 s to allow for temperature equilibration. Absorbance was then measured at 405 nm and monitored at 15-s intervals for 5 min. The mean velocities, in units of mA/min, were calculated by the software program KC3\textsuperscript{®} (Bio-Tek Instruments).

Each mammalian enzyme was analyzed both individually and in combination for the ability to hydrolyze a variety of pNA substrates. From one microplate analysis, we obtained kinetic characterizations of three different pNA substrate hydrolyses by three mammalian enzymes and their mixtures. A representative graph of the data obtained from the hydrolysis of one substrate, N-succinyl-ala-ala-pro-phe ρ-nitroanilide (SAAPFpNA), which is diagnostic for chymotrypsin-like enzymes, is presented in Figure 1. The raw data from the kinetic software program were provided in units of mA/min, which were plotted against increasing substrate concentration. Approximately 90 min were required to set up the assay, measure the absorbances of the progress curves and perform graphical transformation of the data.

Figure 1. Data obtained from a proteinase substrate microplate assay. Rates of hydrolysis of SAAPFpNA by trypsin, chymotrypsin, elastase or a mixture thereof are given as a function of substrate concentration.