

Passage Number Effects In Cell lines

Why they happen and what you can do about it

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Scientific contributions from investigators using continuous cell lines as research tools in biotechnology and medicine are significant.

However, the ability of continuous cell lines to exist almost indefinitely has opened the possibility of questionable subculturing practices and hence, scientific data. A growing body of literature demonstrates that over-subculturing changes cell lines' properties over time.¹⁻⁶ Cell lines at high passage numbers experience alterations in cell morphology, response to stimuli, growth rates, protein expression, transfection and signaling, compared to lower-passage cells. The degree of subculturing a cell line has undergone is often expressed as "passage number," which can generally be thought of as the number of times cells have been subcultured into a new vessel, usually within one lab.

There is a growing awareness within the scientific community that cell line quality is crucial to successful experimentation and that avoiding the use of cell lines that have been in culture too long is an important step to ensure reliable and reproducible results. But while the evidence for passage-number-related effects on cell lines is compelling and awareness of the issue is growing, much less is understood about the mechanisms underlying passage-dependent changes and about actions researchers can take to avoid passage number effects in their experiments.

Compelling data for passage effects

Studies examining passage-dependent effects on cell lines are varied, focusing on a wide range of cell line types and a wide range of behaviors and functions, such as cell morphology, growth rates, protein expression and cell signaling. The following are a few examples from peer-reviewed literature that take different approaches and have different aims, but are centered around how cells change with prolonged subculturing.

A study by Yu et al. (1997) found significant differences in several membrane transport characteristics they evaluated. They also showed cell differentiation for low-passage versus high-passage Caco-2 cells. Alkaline phosphatase activity was reduced significantly in high-passage Caco-2 cells (passage number 93-108) compared to low-passage cells (PN=28-36).

In another study (Esquenet et al. 1997), two samples of LNCaP prostatic adenocarcinoma cells from ATCC, one of low (PN=24) passage number and one of high passage number (PN=80), were examined for their response to the synthetic androgen R1881. The researchers measured ³[H]Thymidine incorporation and PSA secretion after three days of incubation with increasing concentrations of R1881. With this and other data, the authors concluded that low-passage and high-passage LNCaP cells display markedly divergent responses not only to androgens but also to retinoids.

While not the main intent of their research, scientists from Roche Applied Sciences uncovered passage-dependent effects while studying cell line transfection. Using RAW 264.7 cells (ATCC[®] TIB-71™) the Roche data show that high-passage and low-passage cells transfect equally well, but protein expression is significantly reduced in the high-passage samples.⁷

Mechanisms for passage number effects

Documentation of passage-dependent effects on cell cultures is solid, but more fundamental questions remain less addressed: Why and how do these changes occur? What are the mechanisms that underlie passage effects?

Noted cell culturist John Ryan, PhD, of Corning Life Sciences provides a useful framework for answering these questions. Ryan begins with the concept that cells in culture are cells under stress from being in an alien environment. Genetic engineering and cell line transfection place additional stress on cultured cells. Cultured cells should be thought of as microorganisms that adapt to this stress and confusion by evolution — both genotypic and phenotypic.

The stress of culture creates evolutionary pressure and gives rise to a situation conducive to genotypic and phenotypic changes as the cells grow and are subcultured, Ryan explains. This is especially true of transformed cell lines. Changes accumulate with repeated subculturing and over time in vitro. According to Ryan and ATCC scientists, events such as dedifferentiation and loss of tissue-specific function should be considered the norm as passage numbers increase.

Protein expression changes

Many passage-related effects manifest as alterations in cellular protein expression. These changes are complex, as well as cell line- and application-dependent.

Genetic engineering forces a cell to express proteins it does not normally express. Expression of the engineered (possibly non-native) protein at high levels requires energy from the cells. Since cellular energy resources are finite, expression or over-expression of gene products that are forced on the cell artificially takes away from the energy required for cell growth. The result is that the genetically engineered cell line has a slower-than-normal growth rate.

In a situation where a population of clonally isolated cells is expressing a genetically engineered protein, the growth rate will remain slow until a small subpopulation of those cells stops expressing protein (or expresses at lower levels). When that occurs, the nonexpressing group will begin to grow at a more rapid rate because the energy it had used to express foreign protein is now available for growth. Over time, the faster-growing nonexpressing cells will start to outnumber the slower-growing cells, often within several passages. The net effect will be an observed reduction in protein expression at higher passage numbers.

How many passages are too many?

The age of a tree can be determined by counting the rings in a cross-section of the trunk. A similarly straightforward method for determining the passage number of a cell line does not exist. A review of the literature (see reference list) on passage-related effects in cell lines demonstrates that the effects are complex and heavily dependent on a host of factors such as the type of cell line, the tissue and species of origin, the culture conditions and the application for which the cells are used. For example, high-passage Caco-2 cells show an increase in the expression of GFP reporter gene after transfection, while high-passage MCF7 cell lines exhibit a decrease in GFP levels¹³.

Further complicating matters is that a passage level considered “high” for one cell line may not give rise to any significant passage effects in another. But while observed effects appear at different degrees of subculturing for different cell lines and in different applications, the potential consequences of using over-subcultured cell lines remain. Preventing passage-related effects from influencing experimentation becomes a matter of determining the passage number range

under which a set of experiments can be safely performed for a given cell line.

Essential first steps to minimizing cell line passage effects

Researchers have a variety of weapons at their disposal to help avoid passage-dependent effects and ensure valid and reproducible experimental results. In this vein, Corning Life Science's Ryan makes several recommendations that are echoed by ATCC:

- Start with high-quality cells — Cells from well-known biological resource centers (BRCs) are likely to be better characterized and more extensively tested and not be as far removed from the original source material. ATCC, for example, follows a strict seed-stock cell-banking method to ensure distribution of consistent, low-passage cell culture.⁸⁻¹⁰
- Optimize the cells' environment — Carefully select media and sera, control pH with buffers and gases, monitor temperature and pay attention to the growth surface.¹⁰
- Optimize cell culture technique — Focus on harvesting, feeding and storing cells.¹⁰⁻¹²
- Conduct testing on cell lines derived locally or obtained from sources other than BRCs (see section below).

Monitor safe passage number levels

When working with cell lines, it is good cell culture practice to conduct fundamental tests to get an indication of the level of subculturing that maintains consistent cell performance. The following general tests will help researchers obtain valuable information about what passage levels work for their cells and their experiments.

Establish baselines — By routinely monitoring cell lines, researchers can establish baselines and reference points for use in checking for unacceptable differences in experiments or applications. Routine cell line monitoring includes cell morphology checks, identifying markers for genes of interest and correlating expression with passage number, as well as establishing experimental criteria such as growth rates or protein expression levels.

The following includes information about two tests that can be used in monitoring cell lines to establish benchmarks for future comparison with cells of higher passage number.

Morphology check by microscope — Cellular morphology refers to the optical observation of a magnified cell culture. This can be the simplest

and most direct method used to identify the state of cells. Obtaining information morphology from comparative observations both at high and low densities of cultures depends on knowledge of several factors. Morphology can vary between lines depending on the health of the cells and, in some cases, the differentiation state. Morphology can change with plating density as well as with different media and sera combinations. Cell morphology is best monitored through frequent, brief observations. In general, if a culture has an unusual appearance, there is likely a problem. It is recommended that researchers be alert during periodic morphology checks and maintain cell morphology images for comparisons.

Growth curve analysis — Evaluation of cell proliferation can yield valuable information about a culture's response to a stimulus. Variable growth or sudden decreases or increases are a sign that something may be amiss with a cell line. Establishing baselines and quantifying cell culture growth is a crucial element for monitoring the consistency of the culture and determining a number of other factors, such as the best time to subculture, the optimum dilution, and the estimated plating efficiency at various cell densities. Growth curve analysis can also help determine population doubling times and should be performed routinely when enzymatic or functional analysis is imminent. Using cell lines with consistent growth properties should be pursued as a rule.

Conclusion

As there are no tests that directly and absolutely determine cell line passage number, it becomes necessary for investigators to take steps toward ensuring that cell lines used in experiments are giving rise to reliable and reproducible data. If there is any doubt about whether passage levels may affect research results, it is always a good idea to begin with a fresh stock of cells.

References:

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