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Section: Animal Cell Technology

**Defining viability in mammalian cell cultures**

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## **Abstract**

A large number of assays are available to monitor viability in mammalian cell cultures with most defining loss of viability as a loss of plasma membrane integrity, a characteristic of necrotic cell death. However, the majority of cultured cells die by apoptosis and early apoptotic cells, although non-viable, maintain an intact plasma membrane and are thus ignored. Here we measure the viability of cultures of a number of common mammalian cell lines by assays that measure membrane integrity (a measure of necrotic cell death) and assays that measure apoptotic cells, and show that discrepancies in the measurement of culture viability have a significant impact on the calculation of cell culture parameters and lead to skewed experimental data.

## **Keywords**

Apoptosis · Biopharmaceuticals · Mammalian cell culture · Necrosis · Viability

## **Introduction**

In mammalian cell cultures producing protein therapeutics optimum viability is essential in extending the duration of productive culture and maintaining product quality. Enzymes and incompletely processed product released from lysed cells can degrade protein product, alter glycosylation profiles and affect downstream purification (Gramer and Goochee 1993; Goldman et al. 1997; Hansen et al. 1997). Non-viable cells in culture also have an inhibitory effect on the productivity of the remaining viable population, with a recent study in a hybridoma line showing an inverse correlation between the proportion of apoptotic cells at the beginning of culture and subsequent antibody production (Gregory et al. 2009). Thus, careful monitoring and maintenance of viability in culture is essential.

For the majority of common protocols viability is defined by an intact plasma membrane – measured either through dye exclusion or the release of intracellular enzymes such as lactate dehydrogenase. Other assays using the dyes MTT or Alamar Blue measure metabolic activity. However, these criteria are only relevant to necrotic cell death, whereas the majority of cells in culture die via apoptosis (Singh et al. 1994). Unlike necrosis, which is characterised by a sudden loss of membrane integrity and leakage of intracellular contents, apoptosis is a tightly regulated process of cellular disassembly. Early apoptotic cells, although fully committed to the cell death program, are metabolically active and have an intact plasma membrane, with membrane integrity maintained until the late apoptotic/secondary necrotic phase. However, this fraction of non-viable cells is often overlooked. In this study we compare cell culture data that has been calculated based on the viability values obtained from two different assays – measuring either

necrotic or apoptotic cells – to highlight the effect that different definitions of viability can have on experimental data.

## **Materials and methods**

### Cell lines and culture conditions

GS (glutamine synthetase)-NS06A1 (100)<sup>3</sup> cells were cultured in DMEM/F12 without glutamine containing 10% (v/v) FBS, 2% GS Supplement and 30  $\mu$ M MSX. The NS1 derived murine hybridoma (TB/C3) was maintained in RPMI1640 containing 5% (v/v) FBS. CHOK1 cells were maintained in DMEM/F12 containing 5% (v/v) FBS and 2 mM L-glutamine. The hepatocyte cell line, HepZ, and HEK293 cells were maintained in DMEM/F12 containing 10% (v/v) FBS and 2 mM L-glutamine.

### Measurement of cell number, viability and apoptosis

Viable cell number and viability were measured by Trypan Blue exclusion or by propidium iodide (PI) staining (1  $\mu$ g/ml final concentration).

Apoptotic fractions were determined by the annexin-V-FITC/PI assay. Briefly cells were washed in PBS and resuspended in annexin binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl<sub>2</sub>, pH 7.4). Annexin V-FITC reagent (BD Biosciences) was added and samples incubated

for 20 min in the dark. PI was added to give 1 µg/ml and samples were analysed by flow cytometry.

Measurement of apoptosis by fluorescent microscopy was carried out as previously described (Simpson et al. 1997). Briefly cell suspensions were mixed with Acridine Orange (AO)/PI, each at 10 µg/ml, followed by fluorescent microscopy.

Monoclonal antibody measurement

Recombinant cB72.3 antibody in supernatant samples was measured by ELISA as previously described (Tey and Al Rubeai 2004).

## **Results and discussion**

The apoptotic profile for a typical batch culture of a GS-NS0 myeloma cell line as measured by the annexin-V-FITC/PI assay is shown in Figure 1. This assay is based on binding of annexin-V to phosphatidyl-serine (PS), which is characteristically translocated to the outer plasma membrane of the cell in the early phase of apoptosis, and uptake of the dye PI upon loss of membrane integrity (Vermes et al. 1995). Using these parameters the culture can be divided into fractions of viable (annexin-V negative/PI negative) cells, early apoptotic (annexin-V positive/PI negative) cells that have entered apoptosis but have not yet lost membrane integrity, and late apoptotic/secondary necrotic (annexin-V positive/PI positive) cells where PS is exposed and membrane integrity has been lost. Based on this assay a drop in culture viability relating to the

onset of death phase can be seen from 72 h (Figure 1A), with a corresponding increase in the early apoptotic population (Figure 1B); however, there is no increase in late apoptotic cells, relating to loss of membrane integrity, until 120 h (Figure 1C). Here the early apoptotic population represents a significant proportion of non-viable cells particularly at the early stages of death phase. At 96 and 120 h, early apoptotic cells account for 15% and 34% of the total cells in culture, respectively, representing more than 55% of the dead cell population at both time-points. Based on many of the assays generally used for viability measurements, however, this population of cells would be overlooked and the actual percentage viability of the culture would be significantly overestimated.

To show the extent of this effect we have compared data based on measurement of viable cells by the annexin-V/PI assay to that based on membrane integrity alone through exclusion of PI. The difference in percentage viability as determined by the two assays ranges from just under 20% in the early stages of culture to a peak of 48% at 120 h (Figure 2A). Calculating the viable cell density of the culture based on these different viability measurements leads to a significant difference in the viable growth profile (Figure 2B). The calculation of other cell culture parameters is also affected. Although there is no major impact on the calculation of the doubling time of the culture, there are marked differences in the integral of viable cell concentration (IVCC), which was overestimated by the membrane integrity assay, and of specific production of monoclonal antibody cB72.3, which was actually underestimated based on this assay (Table 1, Figure 2 C&D).

The impact of these differences in determination of viability can be seen to a greater or lesser extent in different cell lines depending on the duration of a particular cell line's transition through the early phase of apoptosis. Figure 3 shows a comparison of cell number and culture viability for a range of common cell lines where viability, as determined by membrane integrity alone, was measured by Trypan Blue exclusion and viability based on the non-apoptotic cell fraction was measured by AO/PI staining and fluorescent microscopy where early apoptotic cells can be identified by visualisation of condensed chromatin stained by AO. Based on these assays the differences in viability measured in early death phase (where the discrepancies between assays is most pronounced) were similar for HEK293, HepZ (a hepatocyte cell line), and TB/C3 (a hybridoma cell line) cell lines at approx. 15%, for CHO-K1 however, a difference of 62% was measured (Figure 3).

**In conclusion,** these results show that an accurate measurement of culture viability can only be established through the use of an assay that takes into account all non-viable fractions of the cell population – early apoptotic, late apoptotic and necrotic. The membrane integrity based assays that are widely used cannot provide a true measure of the level of viability of a culture leading to skewed results and bringing into question the accuracy of experiments. A thorough discussion is required as to what constitutes a “viable” cell and a standardised definition of viability needs to be put in place.

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## Figure Captions

**Figure 1** – Determination of cell fractions in a batch culture of the GS-NS06A1 cell line showing the proportion of (A) viable cells, (B) early apoptotic cells, and (C) late apoptotic/necrotic cells in culture as measured by the annexin-V-FITC/PI assay.  $n = 3$ , bars =  $\pm$  standard deviation (SD). Viable cells are annexin<sup>-</sup>/PI<sup>-</sup> reflecting internal PS and an intact membrane, early apoptotic cells are annexin<sup>+</sup>/PI<sup>-</sup> reflecting PS translocation to the outer membrane but maintenance of membrane integrity, and late apoptotic/necrotic cells are annexin<sup>+</sup>/PI<sup>+</sup> reflecting exposure of PS and loss of membrane integrity

**Figure 2** - (A) Viability of the GS-NS06A1 batch culture as measured by PI exclusion (black circles) or the annexin-V-FITC/PI assay (white circles). (B-D) Comparison of calculated cell culture parameters based on measurement of viability by PI exclusion (black circles) or annexin-V-FITC/PI assay (white circles), (B) viable cell concentration, (C) Integral of Viable Cell Concentration (IVCC), (D) Specific antibody productivity and actual antibody titre (black triangles).  $n = 3$ , bars =  $\pm$  SD.

**Figure 3** – (A) Viable cell concentration and (B) viability as measured by trypan blue exclusion (light grey) vs. AO/PI staining (dark grey) in early death phase of mammalian cell cultures.  $n=3$ , bars =  $\pm$  SD.

**Table 1 – Comparison of cell culture calculations for the NS06A1 cell line based on culture viability as defined by loss of membrane integrity (PI only) or PS exposure (annexin/PI).**

**Results are presented as mean value of 3 replicates  $\pm$  Standard deviation.**

	<b>PI</b>	<b>ANNEXIN/PI</b>	<b>DIFFERENCE</b>
<b>Doubling Time (h)</b>	22.4 $\pm$ 0.92	22.2 $\pm$ 1.19	-0.98%
<b>Maximum Viable Cell Concentration (<math>10^6</math> cells ml<sup>-1</sup>)</b>	1.76 $\pm$ 0.07	1.37 $\pm$ 0.13	-22.16%
<b>Integral of Viable Cell Concentration (<math>10^9</math> cells h<sup>-1</sup> l<sup>-1</sup>)</b>	175.6 $\pm$ 14.75	109.9 $\pm$ 11.94	-37.4%
<b>Specific Mab Productivity (<math>\mu</math>g <math>10^6</math> cells<sup>-1</sup> h<sup>-1</sup>)</b>	2.42 $\pm$ 0.05	3.18 $\pm$ 0.13	+43.8%

Figure 1

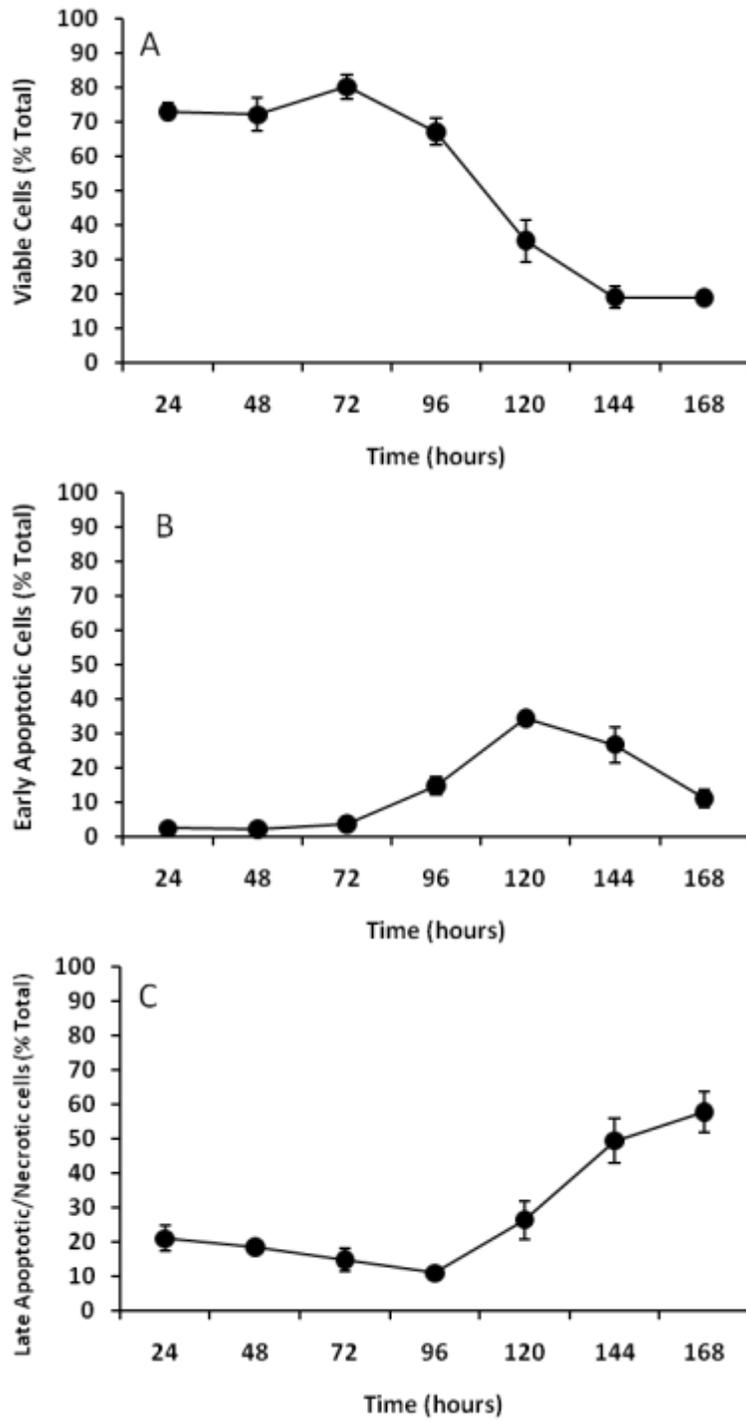


Figure 2

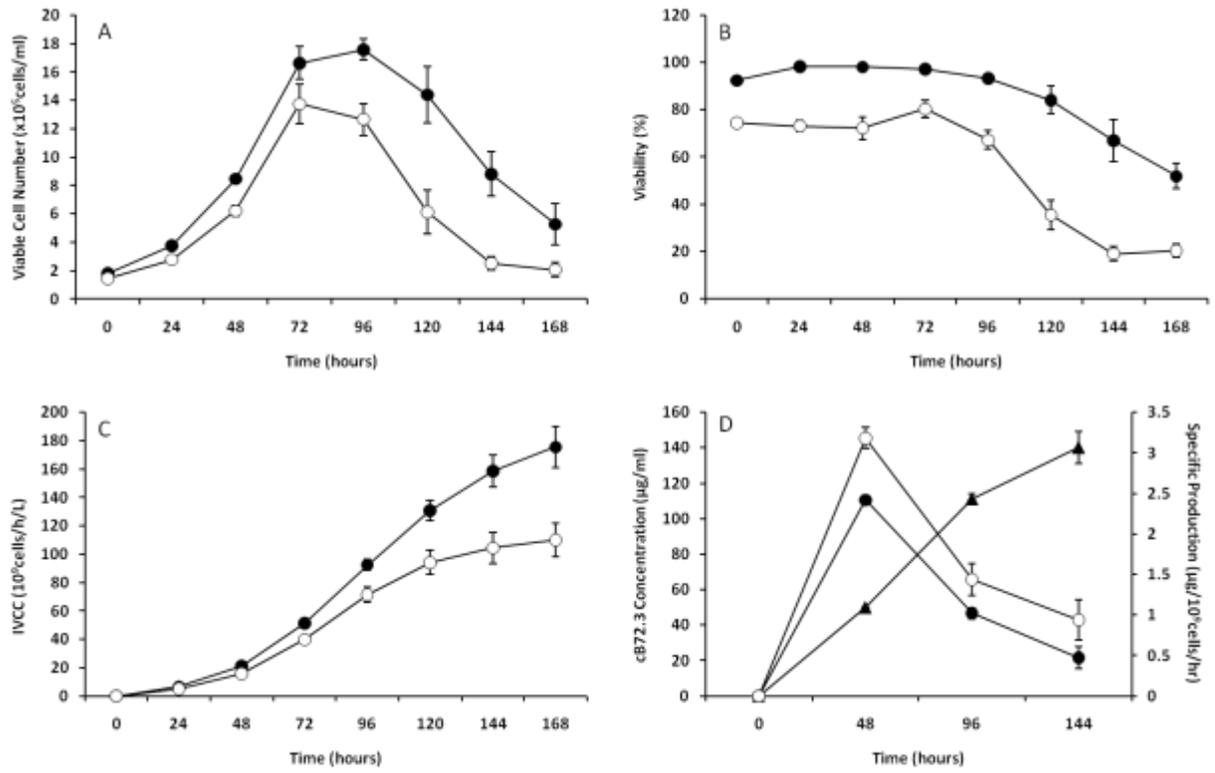


Figure 3

