# Evaluation of Anchorage-Independent Proliferation in Tumorigenic Cells Using the Redox Dye alamarBlue™

*BioTechniques 21:780-782 (November 1996)*

Cell proliferation is a key focus of research in cell biology, relating as it does to development, aging, immune activation, tumorigenesis and basic cellular processes such as signal transduction, DNA transcription and DNA replication. A wide variety of techniques for evaluating cell proliferation in culture have been described including direct cell counting by manual or automated means, cell cycle analysis by flow cytometry (8), incorporation of labeled or modified nucleotides into DNA (2,9,12), measurement of total DNA by DNA-binding fluorophores (3,13) and the detection of metabolically reduced dyes by absorbance or fluorescence spectroscopy (10,14). These various methods balance sensitivity, reliability, cost, instrumentation requirements and processibility in varying degrees, but all are directed at evaluating cells growing in suspension or as an attached monolayer.

Proliferation relating to tumorigenesis may be best examined under conditions of anchorage-independent growth, with cells immobilized in soft agar or agarose (6). Evaluation of proliferation under these conditions has been limited to either direct manual counting of clonal colonies or automated image analysis. The former method is time-consuming and not conducive to the analysis of a large number of samples, whereas the latter requires expensive, specialized equipment. Here, we report the use of alamarBlue™ dye (Alamar Biosciences, Sacramento, CA, USA) to evaluate the proliferation of cells immobilized in agarose. AlamarBlue has previously been used to monitor proliferation in cells growing in suspension or as an attached monolayer (2,4,11). When added to culture medium, the dye is reduced by cellular mitochondrial enzymes, yielding a soluble product directly proportional to cell number. The product has a lower peak absorbance value and a substantially enhanced fluorescence (11), which can be measured spectroscopically in cell medium without further processing. Because the procedure does not require processing of either the culture medium or cells for the spectroscopic measurements, it is easily adaptable for use with cells immobilized in agarose or soft agar.

Three tumorigenic cell lines were used in the experiments. EJ cells derived from a human bladder carcinoma (1,5) were maintained at 37°C under 5% $CO_2$ in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/mL), streptomycin (100 µg/mL) and 2 mM glutamine. NCI-H157 cells derived from a non-small cell lung carcinoma (CRL-5802; ATCC, Rockville, MD, USA) and SK-OV3 cells derived from an ovarian adenocarcinoma (HTB 77 ATCC) were similarly maintained in RPMI-1640 medium supplemented as described above.

Experiments were conducted using either 35-mm petri dishes or, in one case, 24-well plates. The petri dishes were prepared with an initial 1-mL underlay of 0.45% agarose in medium. Trypsinized cells were then mixed with a 0.25% agarose/medium solution at 40°C to a concentration of $5–20 \times 10^6$ cells/mL, and 1 mL of this cell mixture was added to each plate on top of the underlay. Following solidification of this cell-agarose layer, an overlay of 0.5 mL medium was placed over the agarose. Microwell plates (24-well) were prepared in a similar manner, except that the volume of the two agarose layers was 250 µL, and the volume of the medium overlay was 200 µL. Cells were grown for 14–21 days in a humidified incubator at 37°C under 5% $CO_2$, with medium overlays replaced every 4 days.

Proliferation was evaluated either by the microscopic counting of individual colonies (>200 µm) or by spectroscopy following addition of alamarBlue dye. For evaluations using the dye, the medium overlay was replaced with alamarBlue diluted in medium (1:2 for use with the 35-mm dishes and 1:3 for use with the 24-well plates), yielding an overall dye concentration for each plate of approximately 10% (vol/vol). The cells were incubated for 18–24 h at 37°C under 5% $CO_2$ until obvious color changes indicated the presence of sufficient amounts of reduced dye for adequate quantitation. For the 35-mm dishes, 200-µL aliquots were removed from each plate at different time points, placed in a 96-well microwell plate and analyzed by light absorbance at 570 nm using 595 nm as the reference wavelength (manufacturer's instructions). For the 24-well plate, fluorescence measurements were taken directly from the plate using a fluorescence plate reader with an excitation wavelength of 560 nm and an emission wavelength of 590 nm (manufacturer's instructions).

In one experiment, EJ cells were plated at varying concentrations, and after 2–3 weeks, they were examined for both colony counts and absorbance following addition of alamarBlue (Figure 1). Both manual colony counts and alamarBlue spectroscopy demonstrated equivalent increases as a function of cell plating concentration, indicating that the dye procedure provides as accurate a measurement of anchorage-independent proliferation as colony counts under these conditions. Similar results were obtained with two other tumorigenic human cell lines (H157 and SK-OV3)

In a second series of experiments, immobilized EJ cells were grown in the presence of varying serum concentrations, a procedure that produces varying proliferation rates (7), and the relia-

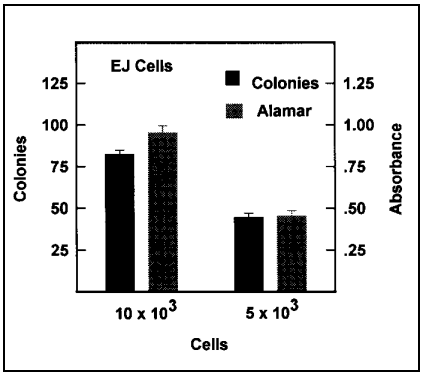


**Figure 1. Anchorage-independent proliferation of EJ cells.** Results represent mean ± SE of 3 plates as evaluated by manual colony counts and light absorbance of the reduced form of alamarBlue in 200-µL aliquots of culture medium. Cells were plated at the denoted concentration in 35-mm plates and grown for 14 days.

bility of alamarBlue spectroscopy to evaluate proliferation under these conditions was examined. One experiment was conducted in 35-mm dishes, with the reduction of alamarBlue measured by absorbance of medium aliquots. A second experiment was performed using 24-well microwell plates, and in this case, the reduction of alamarBlue was evaluated by fluorescence measurements using a fluorescence plate reader (Figure 2). In both experiments, the spectroscopic results reliably reflected the effect of increased serum concentrations. Thus, for example, fluorescence was approximately four times higher for cells grown in 10% serum than for those grown in 2.5% serum.

This study was undertaken to evaluate the use of alamarBlue to measure proliferation in agarose-immobilized, anchorage-independent cells. Simple addition of alamarBlue to the medium bathing the cells, incubation for approximately 24 h and subsequent measurement of the reduced form of the dye by light absorbance or fluorescence spectroscopy provide a reliable and sensitive evaluation of proliferation. alamarBlue is diffusible through the agarose layers in both its oxidized and reduced forms, and as a result, the concentration of the reduced form is directly proportional to cell number. Medium (DMEM or RPMI-1640) containing phenol red indicator and up to 20% serum did not interfere with the spectroscopy results at the cell concentrations used here. Also, alamarBlue spectroscopy did not preclude direct counting of colonies since both procedures could be performed on the same sample at the same time. The dye procedure is therefore a simple, efficient procedure to either supplement or replace the tedious, time-intensive process of manual colony counting.

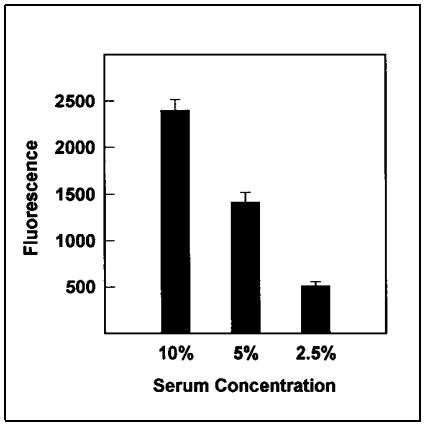


**Figure 2. Anchorage-independent proliferation of EJ cells at varying serum concentrations.** Results represent mean ± SE fluorescence of the reduced form of alamarBlue in 3 wells. Cells were plated at $7 \times 10^3$ cells in a 24-well microwell plate and grown for 14 days in medium containing the denoted serum concentration. Fluorescence was measured directly from the microwell plate using a fluorescence plate reader.

The use of alamarBlue spectroscopy is most useful in experimental situations where anchorage-independent growth involving relatively homogenous cell population is under investigation (e.g., the effect of a particular drug on anchorage-independent growth of a particular tumor cell line). The efficiency of the procedure should allow for the processing of large numbers of samples with minimal time and cost, an advantage especially useful in such research endeavors as drug screening for anti-tumorigenic agents where a number of drugs at various doses need to be assessed.

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*This research was supported by American Cancer Society Grant DHP-105 to E.W. We thank Dr. Christopher Sell for advice on the procedure for plating and growing cells in agarose.* *Address correspondence to Eric Wickstrom, Department of Biochemistry and Molecular Pharmacology, Thomas Jefferson University, Philadelphia, PA 19107, USA. Internet: ewick@lac.jci.tju.edu*

Received 30 October 1995; accepted 27 March 1996.

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