J. Tiss. Cult. Meth. 15:37–42, 1993
© 1993 Tissue Culture Association
0271-8057/93 $01.50+0.00

A NEW, RAPID COLORIMETRIC ASSAY FOR QUANTITATIVE DETERMINATION OF CELLULAR PROLIFERATION, GROWTH INHIBITION, AND VIABILITY

Peter Rieck, Dirk Peters, Christian Hartmann, and Yves Courtois

INSERM U 118-CNRS UA 630, Unité de Recherches Gerontologiques, Association Claude Bernard, 29, rue Wilhem, 75016 Paris, France (P. R., D. P., Y. C.); and University Eye Clinic, Joseph-Stelzmann Strasse 9, 5000 Köln (Cologne) 41, Germany (C. H.)

SUMMARY: A sensitive spectrophotometric test has been developed to rapidly quantitate cell growth and viability on monolayer cultures. The method consists of staining fixed cells with the supravital dye Janus green, extraction of the dye from these cells with absolute alcohol, spectrophotometric measurement of the eluant, and referring optical density (OD) readings to a previously established standard curve such that measurements can be expressed directly as number of surviving cells. Staining of cells with the same colorant before fixation allows microscopical or colorimetric quantitation or both of damaged cells still adherent to the substratum. Both steps can be conveniently carried out on the same cell culture plate. More rapid and easier to perform than the usual cell counting or tritiated thymidine assays, this technique may prove to be useful in evaluation of the effect of growth promoting substances, growth inhibitors, or cytotoxic agents on monolayer cultures.

Key words: Janus green; spectrophotometry; tissue culture; cellular viability.

I. INTRODUCTION

The principle of the method described herein is based on the extraction and photometric measurement of the weak basic supravital dye Janus green (JG) from stained cells after termination of experimental protocols, similar to our JG/photometry technique originally designed for isolated corneas (8). JG exhibits the same properties as the widely used trypan blue, i.e. selective staining of damaged cells, with the advantage of being easily extractable with absolute ethanol. More rapid to perform than other colorimetric assays (3,6,10,12) which assess the number of surviving cells after incubation of specific reagents with the culture medium, this test allows a) quantitation of the total cell number per well (fixation of the cells before staining to allow penetration of the dye in all cells), and b) cell viability assessment (direct staining with JG). The procedure is a sensitive and rapid alternative to the usually performed electronic or microscopic cell counting, with subsequent evaluation of the percentage of damaged cells, by counting cells after trypan blue staining. An additional advantage is the possibility to microscopically screen cells for morphologic changes after JG staining. Being of major scientific interest for our research, bovine corneal endothelial cells (BCEC) were used to standardize the technique in 24-well culture plates with photometric readings carried out using a conventional spectrophotometer. Depending on the cell type employed, the method can be adapted to 96-well microtiter plates, and automated photometric measurements can be made with a multiwell scanning spectrophotometer.

II. MATERIAL

A. Equipment
   - Co_2 incubator, model B 50 60 EC CO_2, Heraeus[1]
   - Centrifuge, Centra-3, IEC[2]
   - Microscope, inverted phase, Labovert Wild, Leitz[3]
   - Spectrophotometer Uvicon 930, Kontron[4]
   - Pipet-Aid, Drummond[5]

B. Chemicals
   - Dulbecco's modified Eagle's medium (DMEM), no. 074-01600 N, GIBCO[6]
   - L-Glutamine 200 m*M* (100×), no. 043 05030 H[6]
   - Fetal bovine serum, no. 011 062 90 H[6]
   - Gentamicin, no. 043 05112 D[6]
   - Fungizone 250 $\mu g/\mu l$, no. 043 05209 D[6]
   - EDTA-Versene 1/5000, Eurobio[7]
   - Ethanol, no. 20.821.296, Prolabo[8]

Sodium chloride, no. 27.810.295[8]
Potassium chloride, no. 26.764.298[8]
Calcium chloride, no. 22.319.294[8]
Potassium hydrogen phosphate, no. 26.926.298[8]
Sodium dihydrogen phosphate, no. 28.015.294[8]
Janus green, C.I. 11050, no. 1324, Merck[9]
Isotonic solution of sodium chloride, Meram[10]
Recombinant human basic fibroblast growth factor (bFGF), Farmitalia[11]

C. Supplies

Disposable transfer pipettes, 1, 10 ml sterile, Falcon no. 4-7551-1; 4-7551-3, Becton Dickinson[12]
Petri dishes 100 × 20 mm, sterile, Falcon no. 3005[12]
24-Well cell culture cluster dish, flat bottomed, sterile, no. 3524, Costar[13]
Pasteur pipettes, borosilicate glass 5.75 in., Comptoir de Verrerie[14]
Variable pipettor, 10 to 1000 μl, Gilson[15]
Disposable pipette tips, Polylabo[16]
Filters Sterivex-GS (0,22 μm) no. SVG SO 1015[16]
μ Star LB™ (0,22 μm) no. 81101[16]
Conical plastic centrifugation tubes, 50 ml, sterile, Falcon no. 2095[12]
Microtubes, 1,5 ml, no. 3810, Eppendorf[17]
Hemacytometer, Malassez, Schreck[18]

III. PROCEDURE

A. Preparation of solutions

1. Culture medium
   a. Prepare basal medium according to manufacturer's instruction using double glass distilled water.
   b. For complete medium add fetal bovine serum to 10% by volume.
   c. Add 0.1 ml Gentamycin, 0.1 ml Fungizone, and 1 ml Glutamine per 100 ml medium.
   d. Filter sterilize and store at 4° C in 150-ml aliquots.
2. Phosphate buffered saline
   In 1 liter of double glass distilled water dissolve the chemicals:
   a. 8 g NaCl
   b. 0.2 g KCl
   c. 1.15 g Na_2HPO_4, $2H_2O$
   d. 0.2 g KH_2PO_4
   e. Adjust pH to 7.2.
   f. Sterilize by filtering through a 0.22-μm filter.
3. Trypsin solution
   a. In 250 ml EDTA Versene 1/5000 dissolve 100 ml trypsin 2.5%.
   b. Filter sterilize through μStar LBTM.
   c. Store in 20-ml aliquots at −20° C.
4. Janus green
   a. To 100 ml isotonic sodium chloride solution add 100 mg JG.
   b. Filter and store at room temperature.

B. Plating

Use stock cultures of bovine corneal endothelial cells.
   a. Aspirate medium and rinse with 2 ml trypsin.
   b. Add 2 ml trypsin and transfer to CO_2 incubator for 2 min.
   c. Examine under microscope; ensure that all cells are detached.
   d. Transfer cells into sterile centrifugation tube and add 4 ml of medium.
   e. Pellet cells by centrifugation at 200 $\times g$ for 5 min.
   f. Aspirate supernatant and add 2 ml medium.
   g. Disperse cells carefully and count with hemacytometer.
   h. Seed cells in desired quantity in 500 μl medium/well.

C. Serial plating

1. Standard curve
   a. Seed cells in 24-well tissue culture plates at 3×10^3, 5×10^3, 10^4, 2×10^4, and 3×10^4 cells/well in 500 μl medium/well and add 1 ng of bFGF/ml medium; six replicates of each cell density should be plated every day to observe (Day 2 to Day 6).
   b. Transfer to CO_2 incubator.
   c. Change medium on Day 2 and Day 4.
   d. Count cells in hemacytometer after trypsinizing in 200 μl trypsin and 300 μl of medium.
2. Toxicity test
   a. Seed 2×10^4 cells/well in 500 μl medium/well in a 24-well tissue culture plate and add 1 ng bFGF per well.
   b. Cells are confluent after 72 h in culture.
   c. Change medium and add agents in different concentrations (in general, over 3 to 4 logs) in triplicate, randomly distributed on the culture plate. Preview parallel control cultures (no test agents added) in quadruplicate.
   d. Incubate for 24 h (or other time period desired).
3. Bioassay
   a. Seed 5×10^3 cells/well in 500 μl of medium/well in a 24-well tissue culture plate.
   b. Add different dilutions of agent(s) for 24 h or any desired time interval; include control cultures in quadruplicate randomly located on the plate.

D. Colorimetric assay

1. Quantitation of total cell number per well
   a. Aspirate medium and rinse with 500 μl phosphate buffered saline (PBS).
   b. Add 200 μl of absolute ethanol for 90 s.
   c. Aspirate ethanol; ensure that the remaining alcohol is completely evaporated before adding 200 μl JG for 1 min.
   d. Aspirate the colorant and rinse 3 times with 500 μl PBS.
   e. Transfer culture plate to the microscope and observe homogeneity of staining intensity.
   f. Adding 800 μl of absolute ethanol results in complete extraction of JG from the cells.

g. Transfer the solution from each well to 1.5 ml microtubes and seal carefully to avoid evaporation.
h. Optical density (OD) is measured at 654 nm. The complete procedure: staining with JG, washing, extraction is repeated on two empty wells (containing no cells), the eluant of these wells serve as blanks.

2. Selective staining of damaged cells (optional)
   a. Instead of fixing the cells with absolute ethanol, add the colorant directly after rinsing with PBS.
   b. Aspirate the colorant and rinse 3 times with 500 μl PBS.
   c. Transfer culture plate to the microscope, and screen stained cells for morphologic alterations and staining intensity.
   d. If the staining intensity corresponds to that of cells after ethanol treatment, perform the following steps as described in 1. f–h.
   e. In case of unequal staining intensity, count stained cells in 10 randomly chosen areas representing e.g., 0.03 mm^2 each. Refer counts to total well surface.
3. Interpretation of results
   a. Toxicity test or bioassay: light microscopy
   A scoring system might be established to grade morphologic changes of stained cells. Counted number of damaged cells can be expressed as percentage of total cell number determined by photometry.
   b. Toxicity test: photometry
   Results may be expressed as $JG\text{-}50_R$, i.e. the concentration of a test agent (toxic substance or growth inhibitor) causing 50% *R*eduction in OD.
   c. Bioassay: photometry
   Accordingly, $JG\text{-}50_I$ could represent the concentration of a growth stimulating agent *i*ncreasing the OD by 50%. For both toxicity testing and bioassays, results may also be expressed in cell number by directly referring the OD values to the standard curve. OD readings of elutions from damaged cells can be expressed in percent of the corresponding OD values for total cell number.

IV. DISCUSSION

Biomedical research and toxicology studies increasingly require inexpensive and sensitive in vitro methods for rapid quantitation of the effect of pharmaceuticals or cytotoxic agents on cellular growth and viability. The most widely used electronic cell counting or tritiated thymidine assays, however, do not match this demand because they are time and labor expensive, especially when large numbers of test agents are to be screened. In an attempt to replace the subjective and time-consuming counting of altered or dead cells under a microscope, the JG photometry technique was originally developed to rapidly quantitate cell damage of the endothelium of isolated corneas, in an attempt to reduce animal experimentation (8). The high reliability of this easy test, proven in various experimental protocols (2,7,13,14), has led us to adapt the method to cell culture.

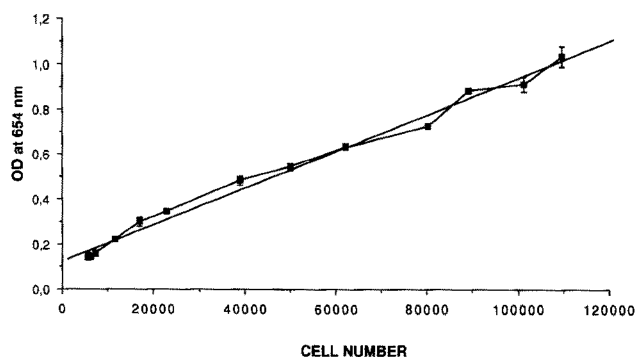

FIG. 1. Janus green standard curve established for BCEC. Cells were seeded in 24-well culture plates at different concentrations ranging from 3 $\times 10^3$ to 3 $\times 10^4$ cells/well, 6 wells/concentration. Cells in each initial concentration range were allowed to grow for 2, 3, 4, 5, and 6 days. At each respective time-point, cells were fixed, stained with 200 μl Janus green, extracted with absolute ethanol, and subsequent spectrophotometry of the eluant was performed as described in Procedure. *Plot symbols* and *error bars* represent mean ± SD. A linear regression curve fit reveals a coefficient of correlation (r^2) = 0.99.

Janus green (molecular weight 511) is a water soluble, weakly cationic dye that selectively stains damaged cells blue. JG gives intensive staining of the nuclei with light staining of the cytoplasm. Morphologic screening is facilitated by the clear outlining of cells stained by JG (Fig. 1). Double staining experiments with trypan blue and JG have shown that JG stains exactly the same cells as does trypan blue (8). The exact mechanism of JG staining is unknown, but it is most likely that altered membrane integrity allows intracellular penetration of the dye, a model that has also been proposed for trypan blue. However, unlike trypan blue, JG is cationic and probably interacts with anionic intracellular organelle membranes, possibly those of the nuclei and mitochondria, through electrostatic and hydrophobic interactions (Hillebrandt D. and Bayer A. G., personal communication). These interactions can easily be disrupted by alcohol, thus allowing the extraction of the dye from tissue or cells in culture.

Beside the rapid quantitative evaluation of total cell number, JG staining before fixation additionally enables visualization and counting of the proportion of damaged cells, a procedure similar to the conventional staining with trypan blue to assess cellular viability. If the staining intensity of stained damaged cells corresponds to that of cells after ethanol treatment, a percentage of damaged cells might also be assessed by colorimetry. Determination of the percentage of damaged cells might not always be necessary, depending on the experimental protocol. However, this step can prove to be useful to screen the overall viability of the cells before incubation with test agents, or to help distinguish between cytotoxic drugs and

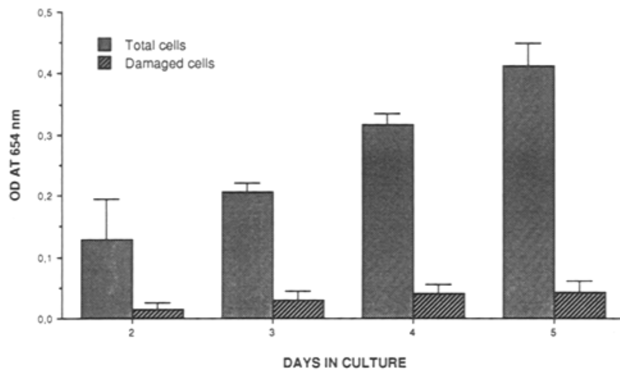

FIG. 2. Growth curves of BCEC as a function of time. Five thousand cells per well were seeded in 24-well culture plates and cells were grown for 2, 4, and 6 days. Total cell number and number of nonviable cells were determined on separate wells, respectively, employing the JG assay as described in Procedure. *Each bar* represents the mean optical density value of three wells ± SE.

growth-inhibiting substances by establishing damaged and surviving cell ratios (Fig. 2).

The major advantage of the technique, however, seems to us to be its simplicity, reliability, and rapidity. Even the nonautomated procedure described above has shown to be considerably quicker than conventional counting of cells in a Coulter counter or hemacytometer. Automatic reading of 96-well microtiter culture plates would further accelerate the screening of large series of experimental studies. The extracted stain does not deteriorate when stored for a long time; thus, measurements can be carried out at any time.

Other colorimetric assays have been introduced as alternatives to cell counting. Different from the JG technique, these tests are based on the determination of viable cells, either by direct staining or by enzymatic conversion of colorimetric reagents. Several of these tests, namely the MTT (12), hexosaminidase (19), and neutral red (3) assays have shown promise in cytotoxicity tests or proliferation assays. However, all methods have potential limitations, e.g., the MTT reagent and neutral red reveal cytotoxic effects after prolonged incubation (4,9). Furthermore, MTT tends to dislodge cells from their substratum (15). Both MTT and hexosaminidase may be induced or inhibited by test agents or simply by fetal bovine serum in the culture medium, leading to over- or underestimated OD readings (1,4). For MTT and neutral red assays, limited sensitivity has sometimes been encountered in certain cell lines (5). The mentioned disadvantages of these tests can be obviated by using the methylene blue uptake assay, another sensitive colorimetric test (6,11). Unfortunately, due to its anionic properties, methylene blue binds firmly to the cells and can be extracted only by prolonged dissolution in, for example, 0.1 *N* HCl. An important contribution to simplicity and rapidity in this context seems to be that the immediate and complete extraction of JG can be done by washing out the dye in absolute alcohol.

Eventual toxic effects of JG to cells represent no technical constraint due to the short incubation time of the dye. Potentiation or suppression of JG staining by test agents is excluded because drugs are not incubated with the colorant. However, some compounds may induce an increase or decrease in the number of mitochondria per cell, thus resulting in increased or reduced staining intensities. Microscopical checks of the stained cultures should be performed routinely to detect this potential source of error.

Beside corneal endothelial cells, the JG assay has been shown to be reproducible on retinal pigment epithelial cells (RPE), epithelial cells, and keratocytes of the cornea, lens epithelial cells, as well as 3T3 cells (Rieck P., Beau I., unpublished observations). Although other cell lines remain to be tested, the JG technique already has proven its usefulness. Further applications may confirm its worth in many other conceivable experimental settings.

V. REFERENCES

1. Alcina, A.; Fresno, M. A colorimetric assay based on cell viability for the indirect detection of intracellular replication and killing of *Trypanosoma cruzi*. J. Immunol. Methods 105:1–5; 1987.
2. Artola, A.; Alio, J. L.; Ruiz, J. M., et al. Corneal endothelial cell protection against free radicals by sodium hyaluronate (Healon). Invest. Opthalmol. & Visual Sci. (Suppl.). 32:1177; 1991.
3. Borenfreund, E.; Puerner, J. A. A simple quantitative procedure using monolayer cultures for cytotoxicity assays (HTD/NR-90). J. Tissue Cult. Methods 9:7–9; 1984.
4. Gerlier, D.; Thomasset, N. Use of MTT colorimetric assay to measure cell activation. J. Immunol. Methods 94:57–60; 1986.
5. Givens, K. T.; Kitada, S.; Chen, A. K., et al. Proliferation of human ocular fibroblasts. An assessment of in vitro colorimetric assays. Invest. Ophthalmol. & Visual Sci. 31:1856–1862; 1990.
6. Goldmann, R.; Bar-Shavit, Z. Dual effect of normal and stimulated macrophages and their conditioned media on target cell proliferation. JNCI 63:1004–1016; 1979.
7. Guthoff, R.; Wendl, U.; Böhnke, M., et al. Zur endothelschützenden Wirkung hochvisköser Substanzen in der Kataraktchirurgie. Fortschr. Ophthalmol. In press: 1992.
8. Hartmann, C.; Rieck, P. A new test for endothelial viability: the Janus green photometry technique. Arch. Ophthalmol. 107:1511–1515; 1989.
9. Isomura, J.; Yoshimatsu, K.; Ikeda, T., et al. A dye uptake method using cultured human cancer cells and its application to a sensitivity test for anti-cancer drugs. Yakagaku Zasshi. 101:227–231; 1981.
10. Landegren, U. Measurement of cell numbers by means of the endogenous enzyme hexosaminidase: applications to detection of lymphokines and cell surface antigens. J. Immunol. Methods 67:379–383; 1984.
11. Oliver, M. H.; Harrison, N. K.; Bishop, J. E., et al. A rapid and convenient assay for counting cells cultured in microwell plates: application for assessment of growth factors. J. Cell. Sci. 92:513–518; 1989.
12. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 67:55–59; 1983.
13. Nuyts, RMMA.; Edelhauser, H. F.; Pels, E., et al. Toxic effects of detergents on the corneal endothelium. Arch. Ophthalmol. 108:1158–1162; 1990.
14. Pharmakakis, N.; Hartmann, C.; Bergmann L. In vitro Hornhautendothelschädigung durch intraokulare Linsen aus PMMA, Silikon und Poly-Hema. Fortschr. Ophthalmol. 86:112–115; 1989.
15. Vaickus, L.; Levy, R. Antiproliferative monoclonal antibodies: detection and initial characterization. J. Immunol. 135:1987–1994; 1985.

[1] Heraeus, Les Ulis, France
[2] International Equipment Company, Dunstable, England
[3] Wild-Leitz GMBH, Wetzlar, Germany
[4] Kontron Instruments, Zürich, Switzerland
[5] Drummond Scientific Co., Broomwall, PA
[6] GIBCO/BRL, DIV of Life Tech., Inc., Gathersburg, MD
[7] Eurobio, Paris, France
[8] Prolabo, Paris, France
[9] Merck, Darmstadt, Germany
[10] Laboratoires Meram, Melun, France
[11] Farmitalia Carlo Erba, Milan, Italy
[12] Becton Dickinson Falcon Labware, Lincoln Park, NJ
[13] Costar Corporation, Cambridge, MA
[14] Comptoir de Verrerie Lyonnais, Villeurbanne, France
[15] Gilson, Villiers de Bel, France
[16] Polylabo, Strasbourg, France
[17] Eppendorf-Netheler-Hinz GMBH, Hamburg, Germany
[18] Schreck GMBH, Hofheim, Germany