An improved method for staining cell colonies in clonogenic assays

Kishore Guda · Leanna Natale · Sanford D. Markowitz

Received: 20 March 2007 / Accepted: 14 May 2007 / Published online: 12 June 2007
© Springer Science+Business Media B.V. 2007

Abstract Clonogenic assay is a widely used experimental approach to test for the effects of drugs/genes on the growth and proliferative characteristics of cells in vitro. Accurate quantitation of treatment effects in clonogenic assays depends on the ability to visualize and count cell colonies precisely. We report a novel method (referred as ETeB) for staining cell colonies grown on plastic and specially coated substrates like collagen. Using colon cancer cell lines grown on plastic and collagen, we compared the colony staining efficiencies of the widely used methylene blue, and Ethidium bromide (ETeB) stains. Results show that the ETeB protocol works well on plastic and is extremely effective for staining colonies on collagen when compared to methylene blue. The key features and advantages of ETeB technique are; (a) reduction in background for colonies grown on collagen and possibly other substrates, (b) the whole procedure

takes less than a minute, (c) no post-stain washing step is required which eliminates colony losses for cell lines that are loosely adherent, (d) colony visualization and counting can be done immediately following the staining procedure using a standard UV illuminator and software, and (e) the method works across a wide variety of cell lines. The simplicity and robustness of this procedure should warrant its usage in both small and large-scale clonogenic experiments.

Keywords Clonogenic assay · Colony count · Stain · Methylene blue · Collagen · Ethidium bromide · Cell culture · In vitro

Introduction

Clonogenic assay is a widely used experimental approach in pharmaceutical, clinical and basic research for testing the effects of drugs/genes on the growth and proliferative characteristics of cells in vitro. The technique assesses the difference in colony forming ability of cultures grown under control and test conditions for a given cell line. In order to obtain an accurate quantitation of treatment effects in clonogenic assays, a robust and precise method for visualizing and counting cell colonies is required. A commonly used stain for visualizing adherent cell colonies is methylene blue (MB), a basic aniline dye that stains intracellular components in addition to extracellular constituents such as

K. Guda (✉) · L. Natale · S. D. Markowitz
Department of Medicine, Case Western Reserve
University, University Hospitals of Cleveland, 2103
Cornell road, Wolstein Research Building- 3rd Floor,
Mailstop 3101, Cleveland, OH 44106, USA
e-mail: gkishore@yahoo.com

S. D. Markowitz
Howard Hughes Medical Institute, Cleveland, OH 44106,
USA

K. Guda · L. Natale · S. D. Markowitz
Ireland Cancer Center, Case Western Reserve University,
University Hospitals of Cleveland, Cleveland, OH, USA

collagen. Despite being a frequently employed reagent, there however remain certain disadvantages with the MB staining procedure. For example, the protocol includes a post-stain washing step with water to remove excessive MB from the culture plates/cells. Although carefully performed, this step may at times wash off cell colonies that are weakly adherent to the plastic/substrate matrix, thereby creating variability in the assay. More importantly, MB is not an effective method for visualizing colonies that develop from cell lines that require growth on special substrates, such as on collagen coated plates, as MB staining of the collagen substrate impedes visualization of the colonies. This often precludes use of automated counting instruments, requiring the use of time consuming and error prone manual counting of colonies visualized under the microscope. In the current study, we describe a rapid and improved technique (referred as ETeB) for staining cell colonies in clonogenic assays that may broadly be applicable to cell lines grown on multiple different substrates.

Materials

Ethidium bromide (ETeB) 10 mg/ml (catalog no. 15585-01): Invitrogen¹

AlphaImager: Alpha Innotech Corporation²

Colon cancer cell lines (Willson et al. 1987; Markowitz et al. 1995).

Procedure

Representative colon cancer cell lines (Willson et al. 1987; Markowitz et al. 1995) were grown either on plastic or collagen-coated 6-well culture dishes for 14 days. Colony staining was performed with either MB or ETeB solutions. Cultures grown on plastic were incubated in 2% MB (dissolved in 50% ethanol) for 3–7 min and washed with running water and air dried. Colonies were later visualized and counted using AlphaImager instrument. For collagen plates,

0.2% MB was used and colonies incubated for 10 min and washed with running water for 5 min and air dried. In parallel, an independent set of colonies were stained using 0.05% of 10mg/ml ethidium bromide in 50% ethanol. Following the removal of culture media, freshly prepared ETeB solution is added just enough to cover the colonies within each well. After incubating for 10–15 s, the ETeB solution is simply removed by aspiration and colonies visualized immediately after the staining procedure in a standard UV illuminator. The colonies were then counted and analyzed using AlphaImager. Precautionary measures similar for DNA/RNA gel electrophoresis should be followed when handling ethidium bromide, which is a known mutagen. The stained ETeB culture plates should be discarded in appropriate biohazard containers.

Results and discussion

Figure 1 shows the staining efficiency of MB and ETeB solutions in two representative colon cancer cell lines, VACO 9M and VACO 576 grown on plastic and collagen, respectively. Our results clearly demonstrate that the ETeB protocol works with similar efficiency as MB on plastic (Fig. 1A, B) and is extremely useful for staining colonies on collagen coated plates to reduce the background that is usually observed with MB (Fig. 1C, D). In order to obtain a quantitative estimate of the staining efficiency, we employed an unpaired t-statistic to determine significant differences in colony counts between the two stains. We presumed that any difference between ETeB and MB staining protocol should be reflected in the number of colonies being visualized and subsequently counted. While no differences in the colony counts between MB and ETeB were detected in VACO 9M (Fig. 2A), a significantly lower colony number per well (~ 2 -fold, $p < 0.0001$) was observed in MB stained VACO 576 cells (Fig. 2B).

As mentioned above, the ETeB protocol works with similar efficiency as MB for colonies grown on plastic, thereby resulting in comparable colony numbers between the two stains in VACO 9M (Fig. 2A). On the other hand, the reason for a lower colony number detected with MB when compared to ETeB in VACO 576 (Fig. 2B) may be attributed to

¹ Invitrogen Corporation 1600 Faraday Avenue PO Box 6482 Carlsbad, California 92008.

² Alpha Innotech Corporation 2401 MercedSt. San Leandro, CA 94577.

Fig. 1 Colony staining efficiency of MB and ETeB Panels (A) and (B) represent a colon cancer cell line (VACO 9M) grown on plastic and stained with MB and ETeB, respectively. Panels (C) and (D) represent the colon cancer line VACO 576, grown on collagen and stained with MB and ETeB, respectively. Non-culture control plates with and without collagen stained with ETeB showed no background staining (data not shown).

Images were captured using a UV illuminator or white light (Alpha Imager). For VACO 576, the number of cells for an average size colony is ~ 20 and for VACO 9M, ~ 30 cells. Of note, ETeB stained colony plates maintained at room temperature in the dark wrapped in aluminium foil for more than 4 weeks can still be visualized under UV with no apparent reduction in the staining intensity (data not shown)

Fig. 2 Quantitative analysis of stain-specific colony counts Panels (A) and (B) represent the colony counts for VACO 9M and VACO 576 respectively, stained with MB and ETeB. Each black sphere indicates the number of colonies per well of a 6-well culture plate. The horizontal lines within the graph show

the mean colony counts. An unpaired t-test was used to determine significant stain-specific differences in colony numbers. VACO 576 cells stained with MB show a significantly ($p < 0.0001$) lower count and a sizeable well to well variation when compared to ETeB

the inherent disadvantages with MB protocol which include, (a) VACO 576 cells grow as loosely adherent colonies on collagen coated matrix. Follow-

ing MB staining, the colonies were washed with running water to remove the excessive stain. Although carefully performed, it is possible to lose

colonies during this procedure. (b) Since MB stains collagen, subsequent automated counting leads to additional errors due to the non-visibility of some colonies on the collagen background (Fig. 1C). This in turn may result in an overall reduction in the number of colonies being visualized/counted with MB, in addition to a considerable well to well variation in colony counts (Fig. 2B). Our data therefore strongly suggests that MB is not suitable for staining colonies grown on speciality matrix like collagen.

In conclusion, the ETcB protocol described herein is an easy, rapid and robust staining technique that significantly reduces time together with a minimal possibility for poorly adherent colonies to detach during the process. This in turn may reduce the overall error rate and well to well variation in clonogenic assays.

Acknowledgments We thank Lydia Beard for her technical assistance. Supported by a gift from the National Colon Cancer Research Alliance (S.D.M.) and by a grant from the State of Ohio Biomedical Research and Technology Transfer Commission (BRTT) (S.D.M.). K.G is supported by an NCI Research Oncology Training Grant (T32 CA059366) through the Case Western Reserve University Comprehensive Cancer Center. S.D.M. is an investigator of the Howard Hughes Medical Institute.

References

- Willson JK, Bittner GN, Oberley TD, Meisner LF, Weese JL (1987) Cell culture of human colon adenomas and carcinomas. *Cancer Res* 47:2704–2713
- Markowitz S, Wang J, Myeroff L et al (1995) Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. *Science* 268:1336–1338