

# An improved method for staining cell colonies in clonogenic assays

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Received: 20 March 2007 / Accepted: 14 May 2007  
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**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 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

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55 in addition to extracellular constituents such as  
 56 collagen. Despite being a frequently employed  
 57 reagent, there however remain certain disadvantages  
 58 with the MB staining procedure. For example, the  
 59 protocol includes a post-stain washing step with  
 60 water to remove excessive MB from the culture  
 61 plates/cells. Although carefully performed, this step  
 62 may at times wash off cell colonies that are weakly  
 63 adherent to the plastic/substrate matrix, thereby  
 64 creating variability in the assay. More importantly,  
 65 MB is not an effective method for visualizing  
 66 colonies that develop from cell lines that require  
 67 growth on special substrates, such as on collagen  
 68 coated plates, as MB staining of the collagen  
 69 substrate impedes visualization of the colonies. This  
 70 often precludes use of automating counting instru-  
 71 ments, requiring the use of time consuming and error  
 72 prone manual counting of colonies visualized under  
 73 the microscope. In the current study, we describe a  
 74 rapid and improved technique (referred as ETeB) for  
 75 staining cell colonies in clonogenic assays that may  
 76 broadly be applicable to cell lines grown on multiple  
 77 different substrates.

## 78 Materials

79 Ethidium bromide 10 mg/ml (catalog no. 15585-01);  
 80 Invitrogen<sup>1</sup>  
 81 AlphaImager: Alpha Innotech Corporation<sup>2</sup>  
 82 Colon cancer cell lines (Willson et al. 1987;  
 83 Markowitz et al. 1995).

## 84 Procedure

85 Representative colon cancer cell lines (Willson et al.  
 86 1987; Markowitz et al. 1995) were grown either on  
 87 plastic or collagen-coated 6-well culture dishes for  
 88 14 days. Colony staining was performed with either  
 89 MB or ETeB solutions. Cultures grown on plastic  
 90 were incubated in 2% MB (dissolved in 50% ethanol)  
 91 for 3–7 min and washed with running water and air  
 92 dried. Colonies were later visualized and counted

using AlphaImager instrument. For collagen plates, 93  
 0.2% MB was used and colonies incubated for 10 min 94  
 and washed with running water for 5 min and air 95  
 dried. In parallel, an independent set of colonies were 96  
 stained using 0.05% of 10mg/ml ethidium bromide in 97  
 50% ethanol (ETeB). Following the removal of 98  
 culture media, freshly prepared ETeB solution is 99  
 added just enough to cover the colonies within each 100  
 well. After incubating for 10–15 s, the ETeB solution 101  
 is simply removed by aspiration and colonies visu- 102  
 alized immediately after the staining procedure in a 103  
 standard UV illuminator. The colonies were then 104  
 counted and analyzed using AlphaImager. Precau- 105  
 tionary measures similar for DNA/RNA gel electro- 106  
 phoresis should be followed when handling ethidium 107  
 bromide, which is a known mutagen. The stained 108  
 ETeB culture plates should be discarded in appropriate 109  
 biohazard containers. 110

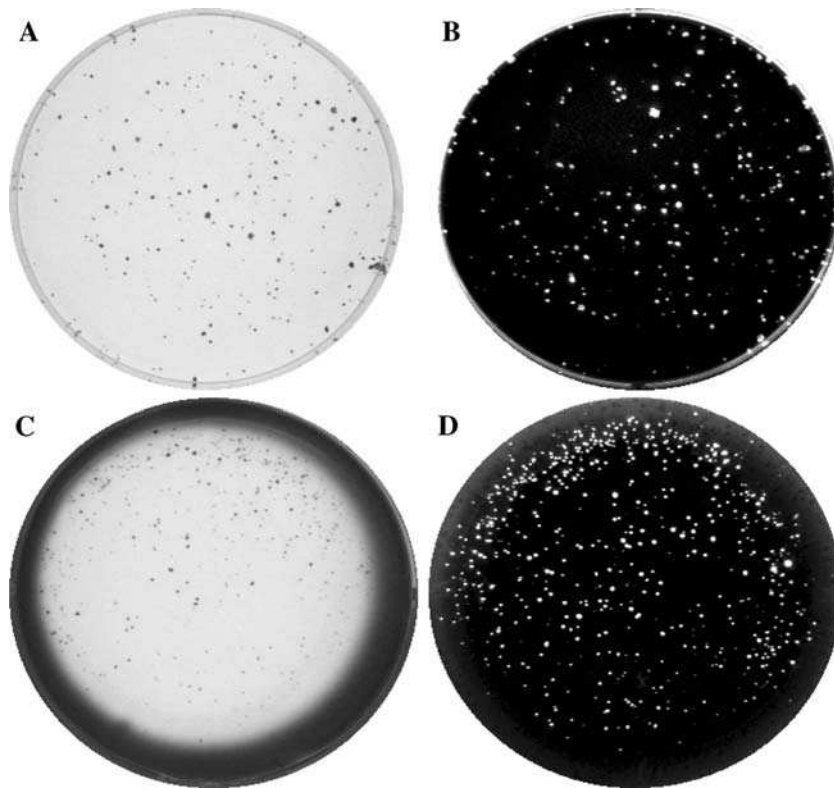
## 111 Results and discussion

112 Figure 1 shows the staining efficiency of MB and 112  
 ETeB solutions in two representative colon cancer 113  
 cell lines, VACO 9M and VACO 576 grown on 114  
 plastic and collagen, respectively. Our results clearly 115  
 demonstrate that the ETeB protocol works with 116  
 similar efficiency as MB on plastic (Fig. 1A, B) 117  
 and is extremely useful for staining colonies on 118  
 collagen coated plates to reduce the background that 119  
 is usually observed with MB (Fig. 1C, D). In order to 120  
 obtain a quantitative estimate of the staining effi- 121  
 ciency, we employed an unpaired t-statistic to 122  
 determine significant differences in colony counts 123  
 between the two stains. We presumed that any 124  
 difference between ETeB and MB staining protocol 125  
 should be reflected in the number of colonies being 126  
 visualized and subsequently counted. While no 127  
 differences in the colony counts between MB and 128  
 ETeB were detected in VACO 9M (Fig. 2A), a 129  
 significantly lower colony number per well (~2-fold, 130  
 $p < 0.0001$ ) was observed in MB stained VACO 576 131  
 cells (Fig. 2B). 132

133 As mentioned above, the ETeB protocol works 133  
 with similar efficiency as MB for colonies grown on 134  
 plastic, thereby resulting in comparable colony 135  
 numbers between the two stains in VACO 9M 136  
 (Fig. 2A). On the other hand, the reason for a lower 137  
 colony number detected with MB when compared to 138  
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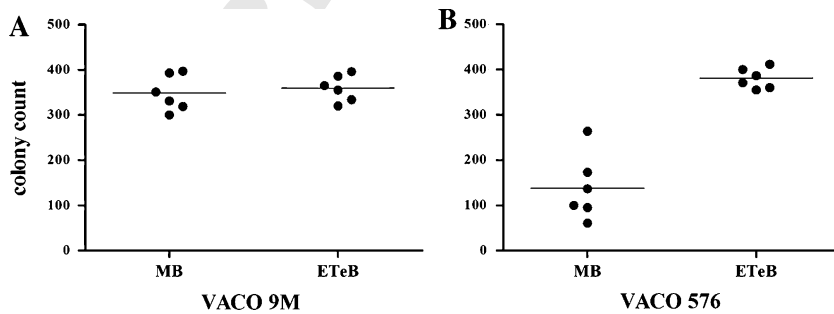
1FL01 <sup>1</sup> Invitrogen Corporation 1600 Faraday Avenue PO Box 6482  
 1FL02 Carlsbad, California 92008.

2FL01 <sup>2</sup> Alpha Innotech Corporation 2401 MercedSt. San Leandro,  
 2FL02 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

139 ETeB in VACO 576 (Fig. 2B) may be attributed to  
140 the inherent disadvantages with MB protocol which  
141 include, (a) VACO 576 cells grow as loosely adherent

colonies on collagen coated matrix. Following MB 142  
staining, the colonies were washed with running water 143  
to remove the excessive stain. Although carefully 144

145 performed, it is possible to lose colonies during this  
 146 procedure. (b) Since MB stains collagen, subsequent  
 147 automated counting leads to additional errors due to  
 148 the non-visibility of some colonies on the collagen  
 149 background (Fig. 1C). This in turn may result in an  
 150 overall reduction in the number of colonies being  
 151 visualized/counted with MB, in addition to a consid-  
 152 erable well to well variation in colony counts  
 153 (Fig. 2A). Our data therefore strongly suggests that  
 154 MB is not suitable for staining colonies grown on  
 155 speciality matrix like collagen.

156 In conclusion, the ETeB protocol described herein  
 157 is an easy, rapid and robust staining technique that  
 158 significantly reduces time together with a minimal  
 159 possibility for poorly adherent colonies to detach  
 160 during the process. This in turn may reduce the  
 161 overall error rate and well to well variation in  
 162 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.

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