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S-Gal™: An Autoclavable Dye for Color Selection of Cloned DNA Inserts

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ABSTRACT

Blue/white selection is the standard method for detecting a cloned DNA fragment. In the absence of an insert, uninterrupted expression of the vector-encoded α -complement of β -galactosidase (β -gal), results in the hydrolysis of X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside) and the subsequent blue staining of the host colony or bacteriophage plaque expressing the carboxyterminal portion of the β -gal gene (*lacZ*). A white or clear colony or plaque indicates the presence of an insert. Because of its water insolubility, X-gal is dissolved in hazardous solvents such as dimethylformamide and then added to the medium following autoclaving. X-gal can be spread on previously plated medium, but this may result in an uneven color development. Also, incubation at 4°C is frequently required for the distinction between a positive recombinant (unstained colony or plaque) and a stained negative. S-Gal™ (3,4-cyclohexenoesculetin- β -D-galactopyranoside), a novel β -gal substrate, is autoclavable and microwavable, allowing for dry-blending of the dye directly into the medium. Black S-Gal-stained colonies are visibly distinguishable from unstained colonies at an earlier time than X-gal. In addition, detection of the unstained signal over background is enhanced by 25% using S-Gal-containing medium, compared to medium containing X-gal. These characteristics offer convenience and better suitability for automated colony or plaque analyses.

INTRODUCTION

Blue/white color screening is well established as a means for identifying a ligation product or denoting the presence of a cloned DNA insert. The method is based on the ability of β -galactosidase (β -gal) to hydrolyze X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside) (2), resulting in the characteristic blue staining of a colony or phage plaque. The multiple cloning regions of numerous plasmid and phage vectors are embedded

within an isopropyl- β -D-thiogalactoside (IPTG)-induced amino-terminal fragment of the β -gal gene (*lacZ*) (3). Successful ligation of a DNA fragment into the multiple cloning sites disrupts the expression of this amino-terminal fragment (α -complement), but without insertion, expression is uninterrupted. Thus, when an *E. coli* host, expressing the carboxyterminal portion of *lacZ*, is transformed with a plasmid vector or infected with a phage lacking a DNA insert, a blue colony or plaque results. Transformation or infection of a vector with DNA fragment insertion results in a white or clear colony or plaque.

Color selection was initially introduced as a means of reducing the labor-intensive process of isolating a desired ligation product, or of distinguishing vectors, with or without cloned DNA inserts, during library construction and screening. With the advent of increasingly efficient cloning systems, this methodology has become less critical for routine cloning, although it provides an additional level of certainty. The construction of the genomic sequences for various species and the extensive use of libraries for numerous purposes (e.g., drug discovery and the elucidation of pathways associated with development and disease) continue to depend extensively on color selection as a vital component of the high-throughput process. Moreover, automated colony or plaque analyses (counting and picking) require the timely detection of recombinants from non-recombinants and distinct contrast between the unstained colony or plaque and the background of the medium.

Despite its utility, X-gal has many drawbacks. Blue and white colonies are often difficult to identify, particularly during early colony formation. Slow color development and ambiguous staining often make it necessary to chill plates at 4°C before colonies or plaques can be picked, adding an additional waiting period before clones can be further analyzed. X-gal is light sensitive, requiring stock solutions and stored plates to be protected from light. It is not soluble in water and is often prepared with solvents such as dimethyl formamide, which is very

toxic and possibly carcinogenic. Routinely, X-gal is added to agar medium or "top agar" following the autoclave process—an additional inconvenience. It can be applied directly to previously poured plates, but color development may be uneven.

S-Gal™ (3,4-cyclohexenoescluletin- β -D-galactopyranoside) (Sigma, St. Louis, MO, USA), a newly developed β -gal substrate, directly addresses these concerns. S-Gal, when blended with IPTG and LB agar medium, is autoclavable or microwavable (Figure 1). When hydrolyzed by β -gal, the non-sugar sidegroup chelates iron (Fe^{+3}) from added ferric ammonium citrate to produce an intense black stain. This stain provides enhanced contrast and earlier color detection. It is more soluble in water than X-gal and is not light sensitive. Functionally, S-Gal readily substitutes for X-gal for color selection; darkly stained colonies or plaques indicate the absence of a cloned DNA fragment, while the unstained colony or plaque denotes the presence of a cloned insert. Here, we illustrate the advantages of using S-Gal versus the X-gal dye, emphasizing its potential utility for enhancing automated bacterial colony and plaque analyses.

MATERIALS AND METHODS

The temperature stability of S-Gal during autoclaving or microwaving was evaluated by chemical analysis and direct application testing. For chemical analysis, ferric ammonium citrate, S-Gal, and IPTG were added to 50-mL aliquots LB broth or deionized water at final concentrations of 500, 300, and 30 $\mu\text{g/mL}$, respectively (all from Sigma). S-Gal dye was initially dissolved in N,N-dimethylformamide (DMF) (Sigma) at a concentration of 200 mg/mL and was added to water or medium for analysis at the concentration stated above before autoclaving or microwaving. For this part of the evaluation, a stock solution of S-Gal in DMF was used for convenience and for the consistent addition of S-Gal to media or aqueous solutions. S-Gal, determined empirically to be approximately 70% more soluble than X-gal at room temperature (data not shown), is suitable for the blended-medium product but requires dissolving in DMF for the preparation of more concentrated solutions such as the stock solution described here. IPTG was added to the water or medium from an aqueous stock of 100 mg/mL. Ferric ammonium citrate was prepared as an aqueous stock solution of 200 mg/mL. For microwaving, the medium was heated until boiling, followed by three separate 30-s intervals of further microwaving. The additional intervals of microwaving correspond to the additional heating often required to dissolve the agar component when preparing plate medium. Autoclaved medium was heated for 20 min at 120°C and 15 lb/in² using a model GE6915-AR1 autoclave (Getinge Castle, Rochester, NY, USA) with a PACS 2000™ controller (PACS Industries, Great Neck, NY, USA). Twenty minutes represents the time of sustained chamber temperature and pressure of 122°C and 15 lb/in², respectively. Before drawing samples for analysis, microwaved and autoclaved media were brought to their original volume with deionized water. All samples, including unheated controls containing S-Gal, were then analyzed by reverse phase high-performance liquid chromatography (RP-HPLC) at ambient temperature using a HP1100™ HPLC System (Hewlett Packard, Palo Alto, CA, USA) and a Discovery® C18 column (I.D., 4.6 mm; length, 25 cm) (Sigma). Sample components were separated

using mobile phases A [0.1% trifluoroacetic acid (TFA) in water (Sigma)] and B [0.08% TFA in acetonitrile (Aldrich Chemical, Milwaukee, WI, USA)] in a linear gradient from 5% to 80% B. Samples were detected at 220 nm. The peak corresponding to S-Gal was integrated and tabulated for this study.

S-Gal was evaluated as a component of the S-Gal/IPTG LB Agar blend when testing the stability of prepared medium, and for comparison with X-gal with respect to color development and contrast between stained colonies, unstained colonies, and medium background. In addition to S-Gal, IPTG and ferric ammonium citrate are dry-blended with standard LB agar at the final concentrations stated above. The powdered blend was suspended in deionized water and autoclaved as previously described. For the preparation of microwaved medium, the suspended blend was heated until boiling, followed by swirling to allow the remaining agar component to go into solution. In this case, the antibiotic ampicillin was added after autoclaving (100 mg/mL). Kanamycin is also compatible with S-Gal (data not shown). For comparison with standard color selection, X-gal and IPTG were added to LB agar, following autoclaving at final concentrations of 267 and 67 $\mu\text{g/mL}$, respectively (1).

Next, 50 μL subcloning efficiency DH5 α ™ *E. coli* (Life Technologies, Rockville, MD, USA) were transformed with 500 ng pUC18 DNA (Sigma) or 500 ng pFLAG™-CMV-1-BAP (a component of the Mammalian Transient Expression Kit, FLMA; Sigma) and spread onto plated medium containing S-Gal or X-gal. The pUC18 vector possesses the amino-terminal fragment of the β -gal gene, but pFLAG-CMV-1-BAP does not (i.e., simulating the case in which an insert interrupts the expression of β -gal). Therefore, bacteria transformed with pUC18 form stained colonies (i.e., no cloned insert), while transformation with pFLAG-CMV-1-BAP results in unstained, "positive" colonies. Aliquots of both transformation reactions, 10 μL or 20 μL of pUC18 and 25 μL or 50 μL of pFLAG-CMV-1-BAP, were spread onto test plates. When evaluating the contrast between stained and unstained colonies and plate background, plates were spread with different quantities of these two transformation reactions, resulting in correspondingly different colony densities. This was done to reflect varying degrees of ligation efficiency or the representation of recombinants versus non-recombinants in a library.

Contrast and color development were evaluated and recorded using the Gel-Doc™ 2000 and Quantity One™ 4.0.3 software (Bio-Rad Laboratories, Hercules, CA, USA). Contrast was measured as intensity, with light detection expressed as counts. Darker colonies have lower numerical values; plated media (i.e., background) are represented as higher values. The lighter, unstained colonies are intermediate in numerical value. Plate images were viewed as transmitted light from the UV Transilluminator 2000™ (Bio-Rad Laboratories) with wavelength-shifting filter. The intensities of 10 positive or negative colonies or points of background ($n = 10$) for four plating conditions with varying colony density (see above) and for S-Gal- or X-gal-containing medium were measured and averaged (Table 1A). The absolute difference of intensity for (i) unstained colonies and background and (ii) for stained and unstained colonies was determined for each plating condition and medium type. The contrast comparison between S-Gal- and X-gal-containing medium was represented as the ratio of these differences of intensity, calculated for each plating condition and averaged overall for (i) and (ii) (Table 1B).

Table 1. Contrast between Stained and Unstained Colonies and Background on S-Gal- versus X-gal-Containing Medium

A. Relative Intensities of Colonies or Background for Medium Containing S-Gal or X-gal				
	Condition: Number of Colonies per Plate			
	(1) 2× Stained 2× Unstained	(2) 2× Stained 1× Unstained	(3) 1× Stained 2× Unstained	(4) 1× Stained 1× Unstained
Intensities				
S-Gal Medium				
Unstained	141.4 ± 11.4	134.8 ± 9.3	147.7 ± 12.4	164.0 ± 10.1
Stained	127.1 ± 9.6	112.8 ± 12.4	125.9 ± 18.7	124.9 ± 8.0
Plate Background	212.4 ± 5.9	204.0 ± 7.5	225.6 ± 4.4	227.8 ± 6.4
X-gal Medium				
Unstained	174.6 ± 8.4	173.3 ± 17.0	192.8 ± 10.0	200.4 ± 7.3
Stained	156.8 ± 18.7	158.0 ± 8.3	154.1 ± 10.4	154.5 ± 8.1
Plate Background	238.5 ± 5.4	226.3 ± 6.0	252.0 ± 3.1	250.8 ± 5.6
B. Differences in Colony or Background Intensity, S-Gal versus X-gal				
			<u>Average</u>	<u>SD</u>
(Unstained - Background _{S-Gal} / Unstained - Background _{X-gal}) _{av1-4}			1.249	0.095
(Stained - Unstained _{S-Gal} / Stained - Unstained _{X-gal}) _{av 1-4}			0.914	0.371

E. coli transformed with pUC18 (stained colonies) or pFLAG-CMV-1-BAP (unstained colonies) were plated as indicated (conditions 1–4; 1× is equivalent to approximately 75 colonies/plate) on medium containing S-Gal or X-gal. (A) The average relative intensity and SD (counts) of 10 colonies or background points were calculated for each type of medium and each plating condition. (B) The absolute difference of the averaged intensities for (i) unstained colonies versus background, and for (ii) stained colonies versus unstained colonies was then determined for each plating condition and type of medium. The ratio of absolute differences for each plating condition, S-Gal versus X-gal, was calculated, with the averages for (i) and (ii) shown.

RESULTS

Figure 2 illustrates RP-HPLC analysis of the temperature stability of S-Gal. S-Gal, added with IPTG and ferric ammonium citrate to LB broth or water, is unaffected by either microwaving or autoclaving. The data for each condition shown are an average of three injections from the same sample. These conditions were tested on two separate occasions (*n* =

Figure 1. S-Gal/IPTG LB agar versus standard X-gal/IPTG LB agar. Plated media were spread with *E. coli* transformed with pUC18 and pFLAG-CMV-1-BAP (a non-β-gal-expressing vector) as described in Materials and Methods. S-Gal-containing medium is shown at left, and X-gal-containing medium is shown at right. The photograph was taken 23 h after plating.

2). The SD of these data for S-Gal in LB broth ranged from 0.29% to 17.90%, whereas in water, the SD ranged from 0.22% to 0.62%. The largest discrepancy (17.9%) was seen in the microwaved LB broth sample. The SD of the unheated LB control was 0.63% and 0.29% for the autoclaved LB broth sample. The effect of repeated microwaving of the S-Gal/IPTG LB agar medium was also tested (data not shown). Following an initial autoclaving of 200 mL S-Gal/IPTG LB agar blend, the medium was subjected to six 3.5-min cycles of microwaving with intervals of congealing at 4°C. Application testing for black/white selection on medium plated immediately after autoclaving and for each microwave cycle indicated no observable effect on color development, contrast, or bacterial growth.

We compared the color development and contrast on plated medium containing S-Gal or X-gal. The rate of color development observed was similar for both formulations (Figure 3), with a distinction between stained and unstained colonies observed visually at 16–18 h after plating. However, the black S-Gal colonies began to appear as early as 14 h. This enhancement, due to the black staining versus blue, was evident throughout colony development.

The comparative intensity of colonies and plate background was analyzed at 23 h after plating using the Gel-Doc 2000 as previously described. Four different plating conditions [i.e., plates with differing numbers and ratios of β-gal-expressing (stained) and nonexpressing colonies (unstained)] were tested on medium containing S-Gal or X-gal. Table 1B shows that, across the four conditions, discernment of stained

from unstained colonies using S-Gal-containing medium was equivalent to the medium containing X-gal. However, unstained colonies were approximately 25% more visible on average over background for medium containing S-Gal.

Although shown to be stable under the high temperatures of medium preparation, it was important to demonstrate the stability of S-Gal-containing medium during its use and storage. Furthermore, it was necessary to show that trace contaminants typically eliminated by autoclaving, such as spore-forming microorganisms (e.g., yeast and other fungi), would not inter-

fere with the utility of microwaved plates (data not shown). Application tests with plated S-Gal/IPTG LB agar medium (ampicillin added after autoclaving or microwaving) stored at 4°C for two weeks showed no effect on contrast or color development. Microwaved S-Gal/IPTG LB agar medium (with and without added ampicillin) was incubated at 37°C for 60 h with no indication of microbial growth, indicating the absence of bacterial or fungal spore germination. Additionally, microwaved S-Gal/IPTG LB agar-plated medium (with added ampicillin) stored for two weeks at 4°C showed no sign of contamination when subsequently incubated at 37°C for 48 h.

Finally, S-Gal was shown not to be sensitive to light. Ambient light exposure to S-Gal/IPTG LB agar-plated medium stored at 4°C for up to two weeks showed no significant effect on color development and contrast (data not shown).

DISCUSSION

S-Gal offers several significant advantages over X-gal. The stability of S-Gal at high temperatures is an obvious convenience. Autoclaving and microwaving have no significant effect on the compound (Figure 2). This allows the dye to be blended directly into the medium, eliminating the need for preparation and handling of stock solutions in toxic solvents. Prepared medium is stable at 4°C and, unlike X-gal, S-gal is not sensitive to light. During the evaluation, small 50-mL volumes of medium were observed to be more prone to superheating (heating to temperatures above 100°C). The effect of this phenomenon was the apparent breakdown of nutrients,

Figure 2. Autoclavability/microwavability of S-Gal: RP-HPLC detection. S-Gal (300 µg/mL in water solution or LB broth) was microwaved or autoclaved as described in Materials and Methods. IPTG (30 µg/mL) and ferric ammonium citrate (500 µg/mL) were also present in the solution or medium. This graphic display represents the integrated area of the S-Gal peak. (The control was unheated medium or solution with additives.)

Figure 3. Time course of color development: S-Gal versus X-gal. Plated media were spread with *E. coli* transformed with pUC18 and pFLAG-CMV-1-BAP as described in Materials and Methods. Plates were incubated at 37°C for the times indicated. Arrows refer to specific colony positions on plates for the comparison of color development.

Figure 4. Chemical structure of S-Gal. The O-linkage between the galactoside and the cyclohexenonescutilin side group of S-Gal is hydrolyzed by β -gal. Two S-Gal side groups chelate Fe^{+3} , resulting in black staining of the respective colony or plaque.

resulting in poor or no bacterial growth (data not shown). This effect was not observed when larger volumes were prepared (200 mL or more), even with multiple cycles of microwaving. As reported, multiple cycles of microwaving had no significant effect on color development and contrast. Taking reasonable care when microwaving small volumes (i.e., stopping when boiling is first observed and mixing to allow the agar to go into solution) avoids this problem. Both microwaved and autoclaved media perform consistently without any problems when they are prepared as recommended.

The higher SD observed for the microwaved S-Gal/IPTG LB medium (Figure 2) was most likely caused by the inconsistent injection of the sample. Triplicate injections of each sample were analyzed. For this particular sample, the area count for the third injection was significantly lower (approximately 28% lower) than for the average of the first two injections (i.e., the variation in the area count of the first two injections was less than 5.3%). The S-Gal peak area was not the only peak observed to be lower. All peak areas recorded from the third injection of sample were approximately 30% lower than those observed from the other two injections, indicating a possible instrument error.

The primary advantages of S-Gal over X-gal are found with contrast and color development. Automated colony or plaque counting, or screening and recovery of recombinant clones by colony or plaque picking, requires a definitive contrast between positive and negative signals and the background of the medium. Contrast and early discernment between unstained and stained colonies or plaques are highly desirable for rapid analyses. The rate of color development on plates containing S-Gal or X-gal is similar (Figure 3). In both cases, observation suggests that β -gal hydrolyzes the O-linkage (oxygen-linkage) between the galactoside and the respective side group of both molecules with similar efficiency. For X-gal, the result is a blue color development. In the case of S-Gal, two cleaved cyclohexenonescutilin side groups (Figure 4) chelate Fe^{+3} (provided from added ferric ammonium citrate) to produce a black stain, which is more easily detected at a consistently earlier time.

The density of the bacterial colonies or plaques on plated medium will affect the efficiency of staining by either S-Gal or X-gal. A colony or phage plaque (e.g., M13) will draw dye from the medium in its immediate vicinity. The greater the density of colonies, the less substrate is available per colony and, hence, the less intense the staining of β -gal-expressing colonies. For this reason, four plating conditions were used

for assessing the differences in contrast on S-Gal- and X-gal-containing medium. The difference in intensity between stained and unstained signals at 23 h after plating is comparable for both S-Gal and X-gal (Table 1B). However, unstained colonies on S-Gal-containing medium are consistently more detectable over the medium background than for X-gal-containing medium. This increased sensitivity, a 25% enhancement in signal, and earlier detection of unstained colonies indicate that S-Gal is preferable to X-gal for the analytical methods described above.

It remains to be demonstrated whether this staining method is applicable for other bacterial or eukaryotic systems. Conceivably, glucose or glucuronic acid derivatives could be developed for applications with other species of bacteria and yeast. Cytochemical detection of β -gal expression in eukaryotic cells would be another potential use for S-Gal. Again, it remains to be determined whether ferric ammonium citrate is compatible with other biological systems and methods or whether an alternative iron source will be required.

In conclusion, we have demonstrated that the S-Gal dye is more convenient to use because of its heat and overall stability, and its intense black color that allows for the earlier detection and enhanced contrast of unstained colonies, making it preferable to X-gal for both visual and automated colony or plaque analyses.

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