S-Gal™: An Autoclavable, Water-Soluble Dye for Enhanced Color-Selection of Cloned DNA Inserts

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Abstract

S-Gal' (3,4-cyclohexenoesculetin-β-*D*-galactopyranoside) outperforms X-gal as a β-galactosidase substrate for automated and non-automated molecular cloning applications involving color selection. Insertion of a DNA fragment into a vector multiple cloning region embedded in the α-complement of the *lacZ* gene disrupts β-galactosidase activity in the host, resulting in the formation of a colorless or cream-colored colony. Dark black staining of a colony indicates uninterrupted expression of the α-complement (no insertion). Better contrast is observed between the stained and unstained colonies and the background of the plated medium when using S-Gal as opposed to X-gal. As a result, colonies representing recombinants can be distinguished from those containing the parental vector at an earlier timepoint, typically between 19 to 22 hours following plating, using pUC18 and DH5α host strain. Other standard *E. coli* strains (JM109, XL-1Blue, and NovaBlue) have been successfully tested using S-Gal. Additionally, pSTBlue-1 transformants produce black colonies in the presence of kanamycin.

Autoclavable/microwavable S-Gal is blended in LB agar (with IPTG <isopropyl β-*D*-thiogalactoside> and ferric ammonium citrate) at a final plated concentration of 300 micrograms per milliliter. Kanamycin can be added to this blend prior to autoclaving or microwaving for antibiotic selection. Addition of ampicillin to the blend confers selection to microwaved medium. A water-soluble S-Gal sodium salt derivative has been developed which allows for customized adjustment of dye concentration and addition of S-Gal to alternative medium formulations. As in case of the free base form found in the blended agar preparation, the sodium salt has been shown to be heat-stable. Both are light-stable (X-gal is not) and have been tested to be stable in prepared medium containing 100 micrograms per milliliter of ampicillin for up to one month, with no effect on color development or antibiotic selection.

Introduction

Color screening is commonly used for selection of recombinant clones. Typically, β-galactosidase (β-gal) expressed in a bacterial host hydrolyzes X-gal (5-bromo-4-chloro-3-indoyl-β-*D*-galactoside, added to the growth medium), to produce a non-toxic, distinctly colored precipitate that denotes expression of the enzyme in the organism. For most molecular genetic applications, a vector-encoded α-complement (N-terminus) of β-galactosidase, complexes with the C-terminus of the same enzyme expressed by the host, to provide reconstituted enzymatic activity. Interruption of expression of the β-gal α-complement, as result, for example, of insertion of a PCR product into a vector multiple cloning region (embedded in the α-complement coding sequence) results in the absence of staining of the colony or plaque. Staining thus indicates uninterrupted expression, the absence of an insertion.

X-gal has several less than desirable characteristics:

- X-gal is routinely added to medium following autoclaving, after allowing the medium to cool.
- X-gal is light-sensitive.
- X-gal is practically insoluble in water. Solution in solvents such as dimethyl sulfoxide or dimethyl formamide is required.
- Due to ambiguous staining long development time is often required, including over-night storage at 4 °C.
- Even with extended color development, the incidence of false-positives is high, due to poor contrast between the stain and unstained colonies or plaques, and the medium. **This proves to be costly in high throughput applications.**

S-Gal™, 3,4-cyclohexenoesculetin-β-*D*-galactopyranoside, possesses similar utility for *in vivo* detection of β-galactosidase activity. However, its unique properties, shown here, address the negatives that arise with the use of X-gal. To allow for ease of handling, a water-soluble sodium salt derivative has been synthesized, possessing the same advantageous characteristics of the free base.

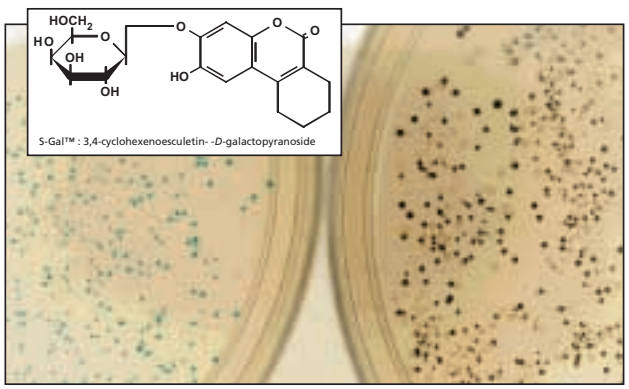

Figure 1. X-gal vs. S-Gal. *E. coli* was transformed with either pUC18 or a plasmid vector not encoding *lacZ*. Aliquots of both transformation reactions were plated on LB agar containing either X-gal (267 µg/ml, left), added following autoclaving; or S-Gal™ (300 µg/ml, right), added prior to autoclaving. (In both cases, ampicillin was added following autoclaving to a concentration of 100 µg/ml. X-gal plates contained 67 µg/ml IPTG <isopropyl-β-*D*-thiogalactopyranoside>, also added post-autoclaving; S-Gal plate contained 30 µg/ml IPTG and 500 µg/ml ferric ammonium citrate, added prior to autoclaving.) Cultures were incubated at 37 °C for 23 hours, post-plating. Stained colonies denote β-galactosidase activity. **The insert illustrates the chemical structure of S-Gal™** (3,4-cyclohexenoesculetin-β-*D*-galactopyranoside). S-Gal hydrolyzes the O-linkage between the galactoside and cyclohexenoesculitin sidegroup. Two sidegroups chelate Fe^{3+}, forming a black precipitate.

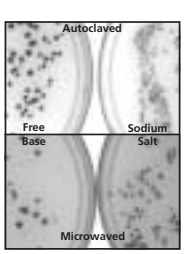

Figure 2. S-Gal free base and sodium salt derivative are equivalent in performance. Transformants were prepared as described in Figure 1 and cultured on plates containing S-Gal free base blended (dry) in the medium prior to addition of water and autoclaving (left), or plates on prepared in the same manner with S-Gal sodium salt (right). (Plates also contained IPTG and ampicillin, as described in Figure 1). Autoclave conditions were heating at 121 °C, 15 lbs/in², for 20 minutes. Microwaved media were heated to boiling, mixed to allow dissolution of the agar component, and heated for 30-second intervals with intermittent mixing until fully dissolved.

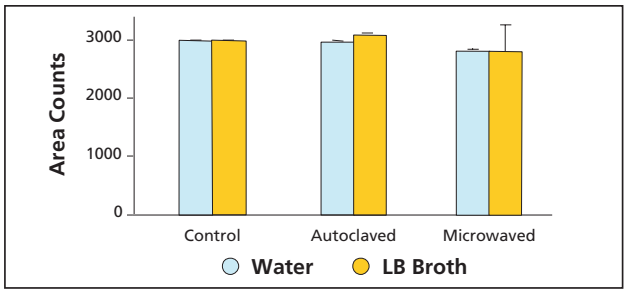

Figure 3. S-Gal is autoclavable and microwavable. Aliquots of LB or water containing S-Gal free base at a concentration of 300 µg/ml (added from a stock solution dissolved in N,N-dimethyl formamide), were autoclaved at 120 °C at 15 lbs/ in² for 20 minutes, or microwaved until boiling followed by three 30-second intervals of further microwaving. (Samples also contained IPTG at 30 µg/ml, and ferric ammonium citrate at 500 µg/ml.) Before analysis, samples were brought to original volume with deionized water. Heated and control samples were analyzed at ambient temperature by reverse phase high performance liquid chromatography, using an HP1100™ HPLC System (Hewlett Packard, Palo Alto) and a Discovery® C18 Column (Sigma).

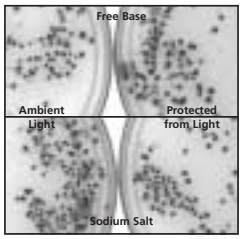

Figure 4. S-Gal is not sensitive to light. Transformants were prepared and cultured as described in Figure 2. Plates were stored at 4 °C for two weeks. Plates in the upper frame were prepared using S-Gal free base; plates in the lower frame were prepared with S-Gal sodium salt. Plates on the right were protected from light; plates on the left were unprotected. Note the accumulation of satellite colonies on plates containing the sodium salt, not seen on plates prepared with the free base. This may be due to the effect of alkaline property of the sodium salt on the ampicillin over the two-week incubation. This can be addressed by adjusting pH of the final medium formulation or providing adequate buffering.

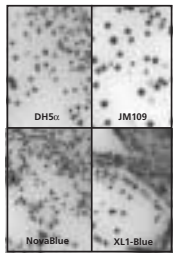

Figure 5. S-Gal is suitable for *E. coli* strains commonly used for color screening. Transformants were prepared and cultured, using the blended S-Gal free base, as described in Figure 2.

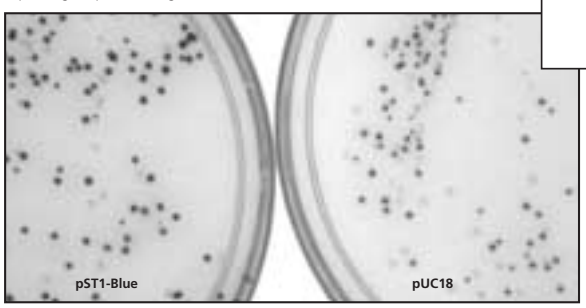

Figure 6. S-gal is suitable for vectors expressing *lacZ*. In addition to pUC18, the utility of S-Gal was tested using pST1-Blue. Transformants were prepared and cultured, using the blended S-Gal free base, as described in Figure 2. (Medium contains ampicillin at a concentration of 100 µg/ml.)

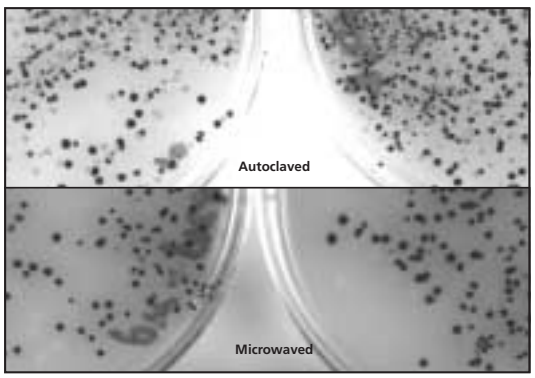

Figure 7. Kanamycin can be added to medium with S-Gal prior to autoclaving or microwaving. *E. coli* were transformed with pST1-Blue or a vector not encoding *lacZ*. Aliquots of both transformation reactions were cultured on medium containing S-Gal (250 µg/ml), IPTG (50 µg/ml), and ferric ammonium citrate (500 µg/ml) (all added prior to autoclaving). Kanamycin was added as follows: 25 µg/ml of kanamycin, following heating (left plates); and 35 µg/ml, prior to heating (right plates).

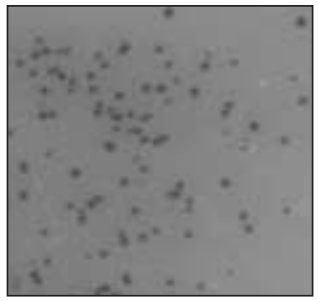

Figure 8. Ampicillin can be added to medium with S-Gal prior to microwaving. Transformants were prepared and cultured as described in Figure 2, using S-Gal free base. Ampicillin was added to the medium at a concentration of 100 µg/ml prior to microwaving.

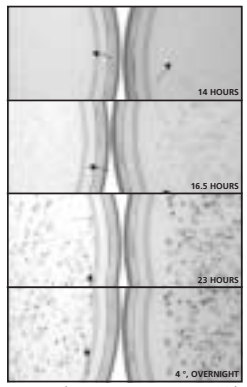

Figure 9. Differentiation between stain and unstained colonies is evident at an earlier time-point for S-Gal versus X-gal. Transformants were prepared and cultured as described in Figure 2. Plates were incubated at 37 °C for the times indicated. (Arrows indicate specific colonies for plate orientation.) S-Gal-stained colonies are first discernable at 13 to 14 hours following plating; positive colonies can be picked as early as 18 to 20 hours.

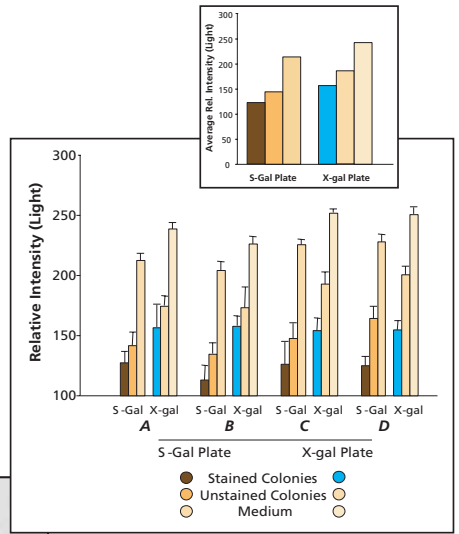

Figure 10. Contrast between stained and unstained signal, and background (medium). Transformants were prepared and cultured on S-Gal or X-gal medium as described in Figure 1. Differing ratios and densities of stained and unstained colonies on a given plate were analyzed: (A) ~250 stained and ~250 unstained colonies, (B) ~250 stained and ~125 unstained colonies, (C) ~125 stained and ~250 unstained colonies, and (D) ~125 stained and ~125 unstained colonies. The average light intensities and standard deviation of ten randomly selected stained or unstained colonies, and ten randomly selected regions of the medium (background) were determined using a Bio-Rad Gel Doc™ 2000 with Quantity One™ 4.03 software (the lower the relative intensity, the darker the signal). Averaged values are shown in the insert.

Analysis of contrast data: S-Gal outperforms X-gal. (from Figure 10 above)

- S-Gal stained signal over background is 11.7 % higher than respective X-gal signal.
- S-Gal unstained signal over background is 25.0 % higher than respective X-gal signal.

∴ Contrast data corroborates visual observations of greater ease of differentiation between stained and unstained colonies when plated on S-Gal-containing medium.

Conclusions

- S-Gal is autoclavable and microwavable.
- S-Gal is not sensitive to light and prepared medium can be stored without protection.
- The S-Gal free base form has similar solubility in water as X-gal. Aqueous stock solutions of at least 50 milligrams per milliliter of the S-Gal sodium salt derivative have been prepared (the limits of solubility have not been tested.)
- With S-Gal, stained and unstained colonies are easily distinguished at 19-22 hours. Overnight incubation at 4 °C is unnecessary.
- Optical contrast for stained and unstained colonies grown on S-Gal-containing medium is measurably better than that observed for X-gal. **This reduces the likelihood of processing a false positive.**

Acknowledgements

We would like to acknowledge Jennifer Cosgrove and Tom Hassell for their assistance with RP-HPLC analyses, and Keming Song and Marketing for their help in this work.

References

1. Greenstein, D. and C. Besmond. 1994. Preparing and Using M13-Derived Vectors, pp. 1.15.1 - 1.15.8. *In* Ausubel, et al. (Eds.), Current Protocols in Molecular Biology. John Wiley and Sons, New York.
2. Horwitz, J.P., J. Chua, R.J. Curby, A.J. Tomson, M.A. DaRooge, B.E. Fisher, J. Mauricio, and I. Klundt. 1964. Substrates for cytochemical demonstration of enzyme activity. I. Some substituted 3-indoyl-β-glycopyranosides. J. Med. Chem. **7:**574.
3. Ullman, A., F. Jacob, and J. Monod. Characterization by *in vitro* complementation of a peptide corresponding to an operator-proximal segment of the β-galactosidase structural gene of *Escherichia coli*. 1967. J. Mol. Biol. **24:**339-343.

Sigma Products

As a result of these product development efforts, the following Sigma products are now available:

Catalog #	Product
S 9811	S-Gal™ (free base)
S 7313	S-Gal™ sodium salt
C 4478	S-Gal™/LB Agar Blend*
S 9938	S-Gal™/LB Agar Blend, without IPTG*
S 1813	S-Gal™/LB Agar/Kanamycin Blend*

*S-Gal medium blends are prepared using the free base.