

Cat #	Product Name	Unit Size
A-191	7-amino-4-methylcoumarin *reference standard*	100 mg
C-2110	CellTracker™ Blue CMAC (7-amino-4-chloromethylcoumarin)	5 mg
C-12881	CellTracker™ Blue CMF ₂ HC (4-chloromethyl-6,8-difluoro-7-hydroxycoumarin)	5 mg
C-183	3-cyano-7-hydroxycoumarin	100 mg
D-6566	6,8-difluoro-7-hydroxy-4-methylcoumarin (DiFMU) *reference standard*	10 mg
E-6578	ELF® 97 alcohol *1 mM solution in DMSO*	1 mL
F-1300	fluorescein *reference standard*	1 g
H-6482	7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one) (DDAO) *reference standard*	10 mg
H-189	7-hydroxy-4-methylcoumarin *reference standard*	1 g
P-12925	5-(pentafluorobenzoylamino)fluorescein (PFB-F)	5 mg
R-363	resorufin, sodium salt *reference standard*	100 mg
R-6479	rhodamine 110 (R110) *reference standard*	25 mg

10.2 Detecting Glycosidases

Glycosidase enzymes exhibit very high selectivity for hydrolysis of their preferred sugars. For example, β -galactosidase rapidly hydrolyzes β -D-galactopyranosides but usually does not hydrolyze either the anomeric α -D-galactopyranosides or the isomeric β -D-glucopyranosides. Endogenous glycosidase activity is frequently used to characterize strains of microorganisms^{1–3} and to selectively label organelles of mammalian cells;^{4–6} defects in glycosidase activity are characteristic of several diseases.^{7–9}

In addition, glycosidases are important reporter gene markers. Specifically, *lacZ*, which encodes β -galactosidase, is extensively used as a reporter gene in animals and yeast, whereas the β -glucuronidase (GUS) gene is a popular reporter gene in plants.^{10–13} Glycosidase substrates are also used in conjunction with glycosidase-conjugated secondary detection reagents in immunohistochemical techniques and enzyme-linked immunosorbent assays¹⁴ (ELISAs). Molecular Probes' complete line of fluorogenic and chromogenic glycosidase substrates is listed in Table 10.1.

Some General Fluorogenic β -Galactosidase Substrates

Fluorescein Digalactoside

Probably the most sensitive fluorogenic substrate for detecting β -galactosidase is fluorescein di- β -D-galactopyranoside¹⁵ (FDG, F-1179; Figure 10.10). Nonfluorescent FDG is sequentially hydrolyzed by β -galactosidase, first to fluorescein monogalactoside (FMG) and then to highly fluorescent fluorescein (F-1300, Section 10.1, Figure 1.47). Enzyme-mediated hydrolysis of FDG can be followed by the increase in either absorbance or fluorescence. Although the turnover rates of FDG and its analogs are considerably slower than that of the common spectrophotometric galactosidase substrate, 2-nitrophenyl β -D-galactopyranoside (ONPG),^{16,17} the absorbance of fluorescein is about fivefold greater than that of *o*-nitrophenol. Moreover, fluorescence-based measurements can be several orders of magnitude more sensitive than absorbance-based measurements. Fluorescence-based assays employing FDG are also reported to be 100- to 1000-fold more sensitive than radioisotope-based ELISAs.¹⁸

In addition to its use in ELISAs, the FDG substrate has proven very effective for identifying *lacZ*-positive cells with fluorescence microscopy^{19–22} and flow cytometry.^{23–28} FDG has been employed to identify cells infected with recombinant herpesvirus,²⁹ to detect unique patterns of β -galactosidase expression in live transgenic zebrafish embryos¹⁹ and to monitor β -galactosidase expression in bacteria.^{20,30,31} The purity of FDG and its analogs is very important because a reagent with extremely low fluorescence background is essential for most applications. Our stringent quality control ensures that the fluorescent contamination of FDG is less than 50 ppm.

The FluoReporter *lacZ* Flow Cytometry Kits (50-test kit, F-1930; 250-test kit, F-1931) provide materials and protocols for quantitating β -galactosidase activity with FDG in single cells using flow cytometry. These kits are accompanied by a license to practice patented techniques for loading FDG by hypotonic shock and improving retention of fluorescein in *lacZ*-positive cells.³² In addition to a detailed protocol, each FluoReporter *lacZ* Flow Cytometry Kit contains convenient premixed solutions of:

- FDG
- Phenylethyl β -D-thiogalactopyranoside (PETG; also available separately as a solid, P-1692), a broad-spectrum β -galactosidase inhibitor for stopping the reaction³³
- Chloroquine diphosphate for inhibiting hydrolysis of the substrate in acidic organelles by endogenous galactosidase enzymes
- Propidium iodide for detecting dead cells

A sufficient amount of each reagent is provided in these kits for 50 (in Kit F-1930) or 250 (in Kit F-1931) flow cytometry assays. This assay enables researchers to detect heterogeneous expression patterns and to sort and clone individual cells expressing known quantities of β -galactosidase. Practical reviews on using FDG for flow cytometric analysis and sorting of *lacZ*-positive cells are available.^{34,35}

FDG's fluorescent hydrolysis product, fluorescein (F-1300, Section 10.1, Figure 15.3), rapidly leaks from cells under physiological conditions, making FDG's use problematic for prolonged

studies. Our DetectaGene and ImaGene substrates (see below) have been specifically designed to improve retention of the fluorescent products in cells.

Resorufin Galactoside

Unlike FDG, resorufin β -D-galactopyranoside (R-1159) requires only a single-step hydrolysis reaction to attain full fluorescence.³⁶ This substrate is especially useful for sensitive enzyme measurements in ELISAs.^{14,37} The relatively low pK_a (~6.0) of its hydrolysis product, resorufin (R-363, Section 10.1, Figure 10.5), permits its use for continuous measurement of enzymatic activity at physiological pH. Resorufin galactoside has also been used to quantitate β -galactosidase activity in single yeast cells by flow cytometry³⁸ and to detect immobilized β -galactosidase activity in bioreactors.^{39,40}

DDAO Galactoside

Although 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one) (DDAO)-based substrates are intrinsically fluorescent (excitation/emission ~460/610 nm), β -galactosidase-catalyzed hydrolysis of DDAO galactoside (D-6488) liberates the DDAO fluorophore (Figure 10.11), which absorbs and emits light at much longer wavelengths (excitation/emission ~645/660 nm)⁴¹ (Figure 10.12). Not only can DDAO (H-6482, Section 10.1) be excited without interference from the substrate, but its fluorescence emission is detected at wavelengths that are well beyond the autofluorescence exhibited by most biological samples. The relatively low pK_a of DDAO (~5.5) permits continuous monitoring of β -galactosidase activity at physiological pH.

Methylumbelliferyl Galactoside and Its Difluorinated Analog

The fluorogenic β -galactosidase substrate β -methylumbelliferyl β -D-galactopyranoside (MUG, M-1489) is commonly used to detect β -galactosidase activity in cell extracts,^{42–44} lysosomes⁴⁵ and human blood serum.⁴⁶ However, MUG's hydrolysis product, 7-hydroxy-4-methylcoumarin (β -methylumbelliferone, H-189; Section 10.1; Figure 10.2), has a relatively high pK_a (~7.8), precluding its use for continuous measurement of enzymatic activity. 6,8-Difluoro-4-methylumbelliferyl β -D-galactopyranoside (DiFMUG, D-11945) yields a hydrolysis product — 6,8-difluoro-7-hydroxy-4-methylcoumarin (D-6566, Section 10.1, Figure 10.2) — with a much lower pK_a (4.9),⁴⁷ allowing its detection at physiological pH.^{48,49} Given the very low pK_a of its hydrolysis product, DiFMUG should be especially useful for the continuous *in vitro* assay of β -D-galactosidase activity at any pH > 6. The fluorinated coumarin glycosides are patented by Molecular Probes.

Carboxyumbelliferyl Galactoside and the FluoReporter lacZ/Galactosidase Quantitation Kit

Hydrolysis of 3-carboxyumbelliferyl β -D-galactopyranoside (CUG, C-1488) by β -galactosidase yields 7-hydroxycoumarin-3-carboxylic acid (H-185, Section 10.1). 7-Hydroxycoumarin has a pK_a below the pH at which the turnover rate is optimal, facilitating the use of CUG for continuous measurements of β -galactosidase activity. Unlike most substrates for β -galactosidase, CUG is quite water-soluble and can be used over a wide range of concentrations in enzymatic activity measurements.^{50–52} Our FluoReporter *lacZ*/Galactosidase Quantitation Kit (F-2905) provides a CUG-based method for quantitating β -galactosidase activity in ELISAs or *lacZ*-positive cell extracts. Each kit contains:

- CUG
- 7-Hydroxycoumarin-3-carboxylic acid, a reference standard
- A detailed protocol suitable for use with any fluorescence-based microplate reader

Sufficient reagents are provided for approximately 1000 β -galactosidase assays. We have demonstrated a practical detection limit of ~0.5 pg of β -galactosidase using this kit and a fluorescence microplate reader.

Fluorescent Glycosphingolipids

β -Galactosidase enzymes that act on the lipophilic sphingosyl galactosides, including galactosylceramidase (EC 3.2.1.46) and G_{M1} ganglioside β -galactosidase (EC 3.2.1.23), are particularly important in neurochemistry. The preferred substrates for these enzymes are sphingolipids derived from galactose (Section 13.3). Galactosylceramidase converts

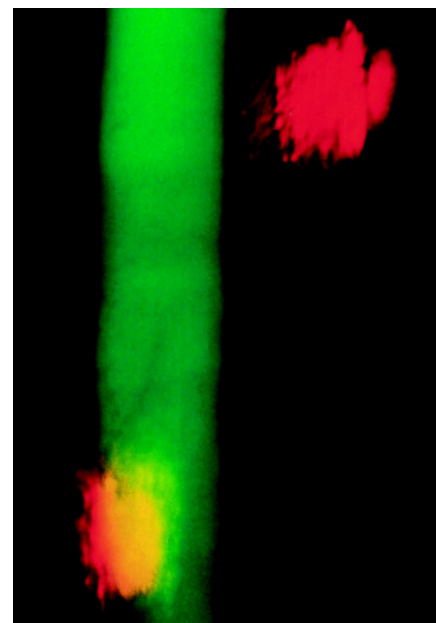


Figure 10.10 Sternomastoid muscle fibers of a living mouse that have been transfected with YOYO-1 dye-stained DNA (red) containing the *lacZ* reporter gene and then stained with the β -galactosidase substrate fluorescein di- β -D-galactopyranoside (FDG, F-1179). DNA stained with YOYO-1 (Y-3601) prior to implantation could still be localized five days after application. Fluorescence signals were visualized *in situ* by epifluorescence microscopy with a low-light level SIT camera and a computer imaging system. Image contributed by Peter van Mier, Department of Anatomy and Neurobiology, Washington University School of Medicine.

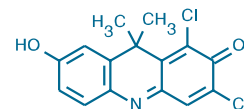


Figure 10.11 H-6482 DDAO.

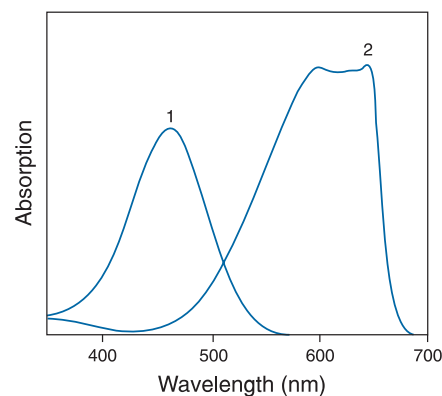


Figure 10.12 Absorption spectra of 1) DDAO galactoside (D-6488) and 2) DDAO (H-6482) at equal concentrations in pH 9 aqueous buffer. These spectra show the large spectral shift accompanying enzymatic cleavage of DDAO-based substrates. DDAO phosphate (D-6487) has very similar spectra.

Molecular Probes' extensive selection of labeled sphingolipids is discussed in Section 13.3 and Section 18.4.

substrates such as our BODIPY FL C₁₂-galactosylceramide (D-7519) back to the ceramide. Purified G_{M1} ganglioside galactosidase removes the terminal galactose residue from lactosylceramides such as our BODIPY FL C₅-lactosylceramide⁵³ (D-13951), yielding the corresponding glucosylceramide.⁵⁴ However, the lack of a spectral shift of the hydrolysis products means that extraction and chromatographic separation of the products is necessary for assessment of the activity.

Table 10.1 Glycosidase enzymes and their fluorogenic and chromogenic substrates.

Carbohydrate (Enzyme)	Notes on Enzyme Activity	Labeled Substrate (Abs/Em of the products) *		Cat #
β-D-Galactopyranoside (β-Galactosidase, E.C. 3.2.1.23)	<ul style="list-style-type: none"> Useful as a reporter gene marker¹⁻⁴ Useful for ELISAs⁵⁻⁸ Useful for enumerating coliforms from the family <i>Enterobacteriaceae</i>⁹⁻¹¹ Useful for classifying mycobacteria¹² 	Blue-fluorescent products	3-Carboxyumbelliferyl (386/448)	C-1488 ^{5,13}
			4-Chloromethylcoumarin (372/470)	D-2921 † ¹⁴
			4-Chloromethyl-6,8-difluoroumbelliferyl (371/464)	C-11946
			6,8-Difluoro-4-methylumbelliferyl (358/452)	D-11945 ¹⁵
			6,8-Difluoro-4-heptadecylumbelliferyl (366/454)	D-11950
			4-Methylumbelliferyl (360/449)	M-1489 ^{16,17}
		Green-fluorescent products	Fluorescein (490/514)	F-1179 ¹⁻³
			5-(Pentafluorobenzoylamino)-fluorescein (490/514)	P-11948
			C ₈ -Fluorescein (490/514)	O-2892 ¹⁸
			C ₁₂ -Fluorescein (490/514)	D-2893, I-2904 ‡ ¹⁹⁻²¹
		Red-fluorescent products	5-Chloromethylfluorescein (490/514)	D-2920 † ²²
			C ₁₂ -Resorufin (571/585)	I-2906 ‡ ^{23,24}
			DDAO (646/659)	D-6488
		Chromogenic substrates	Resorufin (571/585)	R-1159 ²⁵
5-Bromo-4-chloro-3-indoyl (615/NA)	X-Gal; B-1690, B-22015 ²⁶			
β-D-Glucopyranoside (β-Glucosidase, E.C. 3.2.1.31)	<ul style="list-style-type: none"> Deficiency in acid β-glucosidase, which leads to abnormal lysosomal storage, characterizes Gaucher's disease²⁷ Useful as a marker for the endoplasmic reticulum²⁸ 	Green-fluorescent products	Fluorescein (490/514)	F-2881 ^{27,29}
			5-(Pentafluorobenzoylamino)fluorescein (490/514)	P-11947 ³⁰
β-D-Glucuronide (β-Glucuronidase, GUS; E.C. 3.2.1.31)	<ul style="list-style-type: none"> Useful as a reporter gene marker³¹ Useful as a lysosomal marker^{32,33} Useful for detecting <i>E. coli</i>; 94–96% of <i>E. coli</i> contain this enzyme, but it is less common in <i>Shigella</i> (44–58%), <i>Salmonella</i> (20–29%) and <i>Yersinia</i> strains^{9,34-36} 	Blue-fluorescent products	6,8-Difluoro-4-methylumbelliferyl (358/452)	D-11951
			4-Methylumbelliferyl (360/449)	M-1490 ^{16,32,33,37-39}
			4-Trifluoromethylumbelliferyl (385/502)	T-658 ⁴⁰
		Green-fluorescent products	Fluorescein (490/514)	F-2915 ⁴¹
			5-(Pentafluorobenzoylamino)fluorescein (490/514)	P-11949 ⁴¹
			C ₁₂ -Fluorescein (490/514)	I-2908 ‡ ^{42,43}
			ELF 97 (345/530)	E-6587 ^{44,45}
		Chromogenic substrates	5-Bromo-4-chloro-3-indoyl (615/NA)	X-GlcU, B-1691 ^{33,37}
			5-Bromo-6-chloro-3-indoyl (565/NA)	B-8408 ⁴⁶

*Approximate absorption (Abs) and fluorescence emission (Em) maxima of enzymatic hydrolysis product, in nm. † DetectaGene Gene Expression Kit.

‡ ImaGene Gene Expression Kit. NA = Not applicable.

1. Cytometry 17, 216 (1994); 2. Dev Biol 161, 77 (1994); 3. Proc Natl Acad Sci U S A 85, 2603 (1988); 4. Biophys J 74, 11 (1998); 5. Anal Biochem 146, 211 (1985); 6. Exp Parasitol 73, 440 (1991); 7. J Immunol Methods 54, 297 (1982); 8. J Virol Methods 3, 155 (1981); 9. Microbiol Rev 55, 335 (1991); 10. J Appl Bacteriol 64, 65 (1988); 11. Appl Environ Microbiol 35, 136 (1978); 12. Zentralbl Bakteriol 280, 476 (1994); 13. Infect Immun 61, 5231 (1993); 14. Mol Cell Probes 9, 145 (1995); 15. Anal Biochem 273, 41 (1999); 16. Anal Biochem 104, 182 (1980); 17. Anal Biochem 215, 24 (1993); 18. Cytometry 20, 324 (1995); 19. Appl Environ Microbiol 60, 4638 (1994); 20. Proc Natl Acad Sci U S A 89, 10681 (1992); 21. FASEB J 5, 3108 (1991); 22. J Neurosci 15, 1025 (1995); 23. Biotechnol Bioeng 42, 1113 (1993); 24. US Patent No. 5,242,805 (1993); 25. Anal Chim Acta 163, 67 (1984); 26. Biotechniques 7, 576 (1989); 27. Cell Biochem Funct 11, 167 (1993); 28. Nature 369, 113 (1994); 29. Anal Biochem 247, 268 (1997); 30. Blood 89, 3412 (1997); 31. Plant Mol Biol Rep 5, 387 (1988); 32. Cell Signal 3, 625 (1991); 33. J Immunol Methods 100, 211 (1987); 34. Appl Environ Microbiol 59, 3534 (1993); 35. Appl Environ Microbiol 50, 1383 (1985); 36. J Clin Microbiol 13, 483 (1981); 37. J Appl Bacteriol 74, 223 (1993); 38. Arch Biochem Biophys 286, 394 (1991); 39. Plant Sci 78, 73 (1991); 40. Biochem Int 24, 1135 (1991); 41. J Biol Chem 274, 657 (1999); 42. Microbiology 143, 267 (1997); 43. Plant J 10, 745 (1996); 44. Electrophoresis 21, 497 (2000); 45. Tetrahedron 53, 7159 (1997); 46. Biotechniques 19, 352 (1995).

Amplex Red Galactose/Galactose Oxidase Assay Kit

Our Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine, A-12222; A-22177; Section 10.5; Figure 10.49) is an unusually stable peroxidase substrate that we have used in coupled reactions to detect a wide variety of analytes, including both enzymes and their substrates (see Section 10.5 for a list of all of our Amplex Red Kits and reagents). Most of the assays can be performed as continuous assays at neutral or slightly acidic pH and are particularly suitable for automation and high-throughput screening using either an absorption- or fluorescence-based microplate reader.

Rather than requiring an unnatural fluorogenic or chromogenic substrate for β -galactosidase (or α -galactosidase), our Amplex Red reagent-based technology permits the direct quantitation of free galactose, which is produced by a wide variety of enzymes. Even enzymes that act on polysaccharides and glycolipids that cannot be easily assayed with any known chromogenic substrate can, in some cases, be detected and their activity quantitated using the Amplex Red reagent in combination with galactose oxidase and horseradish peroxidase. Unlike glucose oxidase, galactose oxidase can produce H_2O_2 from either free galactose or from polysaccharides — including glycoproteins in solution and on cell surfaces — and from certain glycolipids in which galactose is the terminal residue (Figure 10.13). Because the galactose oxidase-catalyzed reaction does not require prior cleavage of the glycoside to free galactose by a galactosidase, appropriate control reactions must be used to ascertain whether the rate-limiting step is the galactosidase- or galactose oxidase-mediated reaction.

The Amplex Red Galactose/Galactose Oxidase Assay Kit (A-22179) provides an ultrasensitive method for detecting galactose (Figure 10.14) and galactose oxidase (Figure 10.15) activity. This assay utilizes the Amplex Red reagent (Figure 10.49) to detect H_2O_2 generated by galactose oxidase-mediated oxidation of desialated galactose moieties. In the presence of horseradish peroxidase (HRP), the H_2O_2 thus produced reacts with the Amplex Red reagent in a 1:1 stoichiometry to generate the red-fluorescent oxidation product, resorufin.⁵⁵ Resorufin has absorption and fluorescence emission maxima of approximately 563 nm and 587 nm, respectively (Figure 10.5), and because its extinction coefficient is high ($54,000 \text{ cm}^{-1}\text{M}^{-1}$), the assay can be performed either fluorometrically or spectrophotometrically. The Amplex Red Galactose/Galactose Oxidase Assay Kit

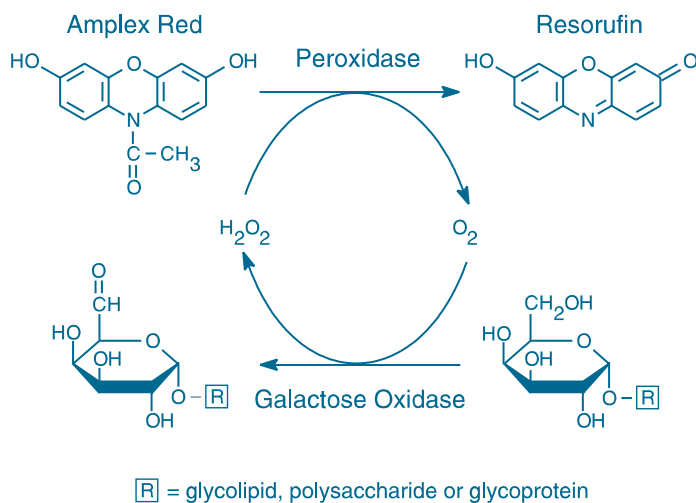


Figure 10.13 Detection scheme utilized in the Amplex Red Galactose/Galactose Oxidase Assay Kit (A-22179). Oxidation of the terminal galactose residue of a glycoprotein, glycolipid or polysaccharide results in the generation of H_2O_2 , which, in the presence of horseradish peroxidase (HRP), reacts stoichiometrically with the Amplex Red reagent to generate the red-fluorescent oxidation product, resorufin.

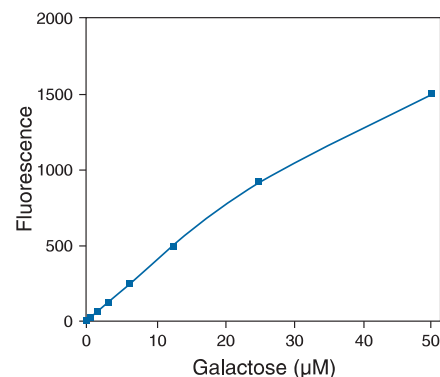


Figure 10.14 Detection of galactose using the Amplex Red Galactose/Galactose Oxidase Assay Kit (A-22179). Each reaction contained $50 \mu\text{M}$ Amplex Red reagent, 0.1 U/mL HRP, 2 U/mL of galactose oxidase and the indicated amount of galactose in $1\times$ reaction buffer. Reactions were incubated at 37°C . After 30 minutes, fluorescence was measured in a fluorescence microplate reader using excitation at $590 \pm 12.5 \text{ nm}$ and fluorescence detection at $590 \pm 17.5 \text{ nm}$. A background fluorescence of 93 units was subtracted from each data point.

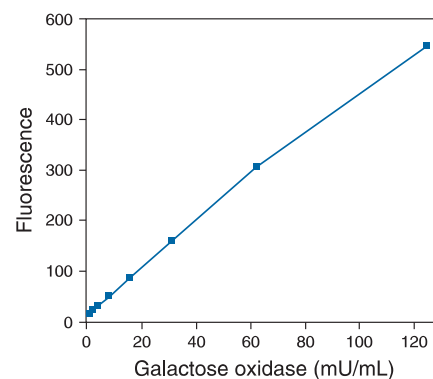


Figure 10.15 Detection of galactose oxidase activity using the Amplex Red Galactose/Galactose Oxidase Assay Kit (A-22179). Each reaction contained $50 \mu\text{M}$ Amplex Red reagent, 0.1 U/mL HRP, $100 \mu\text{M}$ galactose and the indicated amount of galactose oxidase in $1\times$ reaction buffer. Reactions were incubated at 37°C . After 20 minutes, fluorescence was measured in a fluorescence microplate reader using excitation at $530 \pm 12.5 \text{ nm}$ with fluorescence detection at $590 \pm 17.5 \text{ nm}$.

Our Amplex Red technology has additional applications for detection of many other enzymes and analytes; these are described in Section 10.5.

provides all the reagents and a general protocol for the assay of galactose-producing enzymes or for the assay of galactose oxidase, including:

- The Amplex Red reagent
- DMSO
- D-Galactose
- Galactose oxidase from *Dactylium dendroides*
- Horseradish peroxidase (HRP)
- H₂O₂
- A 5× reaction buffer
- A detailed protocol for the assay

Sufficient reagents are provided for approximately 400 assays using either an absorption- or fluorescence-based microplate reader and reaction volumes of 100 μ L per assay. The Amplex Red galactose/galactose oxidase assay accurately measures as low as 4 μ M galactose and 2 mU/mL galactose oxidase activity (Figure 10.14, Figure 10.15). Because of the high absorbance of resorufin, the absorptimetric assay has only slightly lower sensitivity than the fluorometric assay.

Modified Fluorogenic β -Galactosidase Substrates with Improved Cellular Retention

The primary problems associated with detecting *lacZ* expression in live cells using fluorogenic substrates are:

- Difficulty in loading the substrates under physiological conditions
- Leakage of the fluorescent product from live cells
- High levels of endogenous β -galactosidase activity in many cells

Our DetectaGene and ImaGene Kits are designed to improve the sensitivity of β -galactosidase assays by yielding products that are better retained in viable cells and, in the case of the ImaGene

Kits, by providing substrates that can be passively loaded into live cells. The high level of endogenous β -galactosidase activity remains an obstacle when detecting low levels of *lacZ* expression.

DetectaGene *lacZ* Gene Expression Kits

The substrates in our DetectaGene Green and DetectaGene Blue *lacZ* Gene Expression Kits (D-2920, D-2921) — 5-chloromethylfluorescein di- β -D-galactopyranoside (CMFDG) and 4-chloromethylcoumarin-7-yl β -D-galactopyranoside (CMCG), respectively — are galactose derivatives that have been chemically modified to include a mildly thiol-reactive chloromethyl group (Figure 10.16). Once loaded into the cell using the Influx pinocytotic cell-loading reagent (I-14402; included in Kit D-2920 only) or by microinjection, hypotonic shock or another technique (Table 14.1), the DetectaGene substrate undergoes two reactions: 1) its galactose moieties (two per molecule for CMFDG, one for CMCG) are cleaved by intracellular β -galactosidase and 2) either simultaneously or sequentially, its chloromethyl moiety reacts with glutathione and possibly other intracellular thiols to form a membrane-impermeant, peptide-fluorescent dye adduct⁵⁶ (Figure 10.16). Because peptides do not readily cross the plasma membrane, the resulting fluorescent adduct is much better retained than is the free dye, even in cells that have been kept at 37°C. We have found that *lacZ*-positive cells loaded from medium containing 1 mM CMFDG are as fluorescent as those loaded with 40-fold higher concentrations of FDG. Furthermore, unlike the free dye, the peptide-fluorescent dye adducts contain amino groups and can therefore be covalently linked to surrounding biomolecules by fixation with formaldehyde or glutaraldehyde. This property permits long-term storage of the labeled cells or tissue and, in cases where the anti-dye antibody is available (Section 7.4, Table 7.13), amplification of the conjugate by standard immunohistochemical techniques.

The CMFDG substrate in our DetectaGene Green *lacZ* Gene Expression Kit was used to stain *lacZ*-expressing floor plate cells in tissue dissected from a developing mouse embryo,⁵⁷ to identify *lacZ*-enhancer-trapped *Drosophila* neurons in culture and to detect β -galactosidase activity in hippocampal slices.⁵⁸ In the

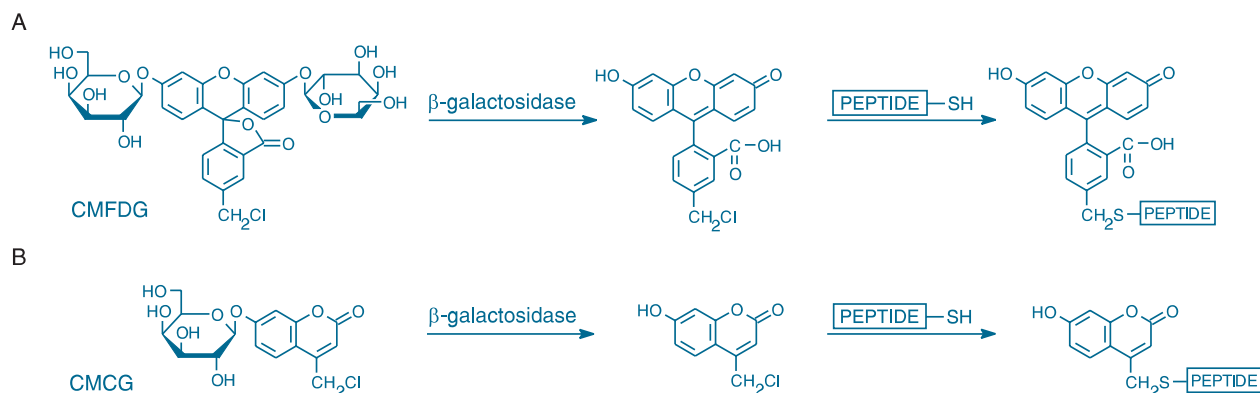


Figure 10.16 Sequential β -galactosidase hydrolysis and peptide conjugate formation of A) CMFDG (D-2920) and B) CMCG (D-2921).

latter study, the fluorescence of the neurons could still be visualized 24 hours after dye loading, and the fluorescent CMFDG-loaded neurons exhibited a normal pattern and time course of axonal outgrowth and branching.⁵⁹ CMFDG also has been microinjected into primary hepatocytes, fibroblasts and glioma cells to detect β -galactosidase activity⁶⁰ and has been incorporated into an electrophysiological recording pipette to confirm the identity of neurons cotransfected with the *lacZ* gene and a second gene encoding Ca^{2+} /calmodulin-dependent protein kinase II (CaM kinase II).⁶¹

The DetectaGene Green CMFDG (D-2920) and DetectaGene Blue CMCG (D-2921) *lacZ* Gene Expression Kits contain:

- The DetectaGene Green CMFDG or DetectaGene Blue CMCG substrate (Figure 10.16)
- Phenylethyl β -D-thiogalactopyranoside (PETG; also available separately as a solid, P-1692), a broad-spectrum β -galactosidase inhibitor for stopping the reaction³³
- Verapamil for inhibiting product efflux^{62,63} (in Kit D-2920 only)
- Chloroquine diphosphate for inhibiting acidic hydrolysis of the substrate
- Propidium iodide for detecting dead cells
- Influx pinocytotic cell-loading reagent for introducing CMFDG into cells (in Kit D-2920 only)
- A detailed protocol for detecting β -galactosidase activity

When used at the recommended dilutions, a sufficient amount of each reagent is provided for approximately 200 flow cytometry tests with the DetectaGene Green CMFDG Kit or 50 flow cytometry tests with the DetectaGene Blue CMCG Kit. Verapamil has been added to the DetectaGene Green CMFDG *lacZ* Gene Expression Kit because we have observed that cell retention of the fluorescent dye-peptide adduct can be considerably improved in many cell types by adding verapamil to the medium.⁶²

PFB Aminofluorescein Digalactoside

Our patented 5-(pentafluorobenzoylamino)fluorescein di- β -D-galactopyranoside (PFB-FDG, P-11948; Figure 10.17) yields the green-fluorescent PFB-F dye (P-12925, Section 10.1), which

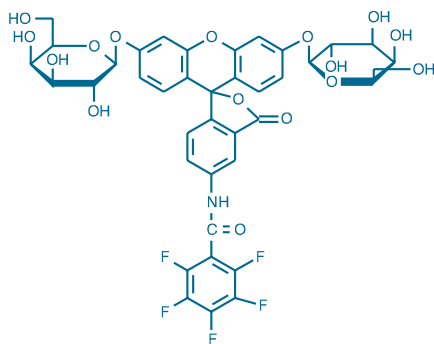


Figure 10.17 P-11948 5-(pentafluorobenzoylamino)fluorescein di- β -D-galactopyranoside (PFB-FDG).

appears to localize to endosomal and lysosomal compartments when loaded into cells by pinocytosis (Figure 10.18), similar to our PFB aminofluorescein diglucoside (PFB-FDGlu, P-11947 see below). Thus, PFB-FDG⁶⁴ is potentially useful for studying lysosomal storage diseases, including Krabbe's disease, G_{M1} gangliosidosis, galactosialidosis and Morquio's syndrome, type B, which are all associated with deficient lysosomal β -galactosidase activity.⁶⁵

ImaGene *lacZ* Reagents and Gene Expression Kits

The fluorescein- and resorufin-based galactosidase substrates in our ImaGene Green and ImaGene Red *lacZ* Gene Expression Kits (I-2904, I-2906) have been covalently modified to include a 12-carbon lipophilic moiety. Unlike FDG or CMFDG (Figure 10.16), these lipophilic fluorescein- and resorufin-based substrates — abbreviated C_{12} FDG (Figure 10.19) and C_{12} RG (Figure 10.20) for the ImaGene Green and ImaGene Red substrates, respectively — can be loaded simply by adding the substrate to

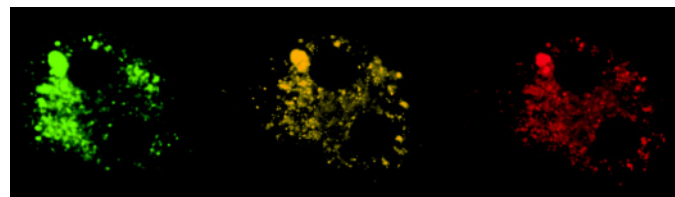


Figure 10.18 Bovine pulmonary artery endothelial cells simultaneously stained with LysoTracker Red DND-99 (L-7528), a cell-permeant, fixable lysosomal stain, and with 5-(pentafluorobenzoylamino)fluorescein di- β -D-galactopyranoside (PFB-FDG, P-11948), a fluorogenic substrate for β -galactosidase. PFB-FDG is nonfluorescent until enzymatically hydrolyzed to green-fluorescent PFB-F. The center image demonstrates colocalization of the LysoTracker Red DND-99 dye and the fluorescent product, PFB-F, to the lysosomes. The left image was acquired with a bandpass filter set appropriate for fluorescein, the right image was acquired with a bandpass filter set appropriate for Texas Red dye, and the center image was acquired with a triple bandpass optical filter set appropriate for DAPI, fluorescein and the Texas Red dye.

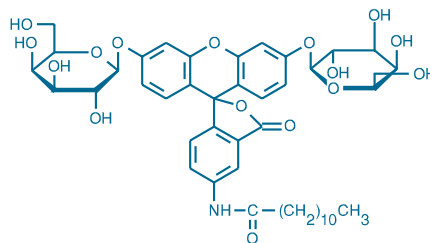


Figure 10.19 5-dodecanoylamino fluorescein di- β -D-galactopyranoside (C_{12} FDG).

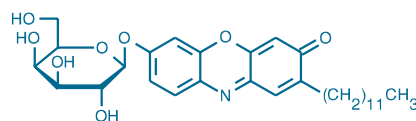


Figure 10.20 2-dodecylresorufin β -D-galactopyranoside (C_{12} RG).

the aqueous medium in which the cells or organisms are growing, either at ambient temperatures or at 37°C. Once inside the cell, the substrates are cleaved by β -galactosidase, producing fluorescent products that are well-retained by the cells, probably by incorporation of their lipophilic tails within the cellular membranes. Mammalian NIH 3T3 *lacZ*-positive cells grown for several days in medium containing 60 μ M C₁₂FDG appear morphologically normal, continue to undergo cell division and remain fluorescent for up to three cell divisions after replacement with substrate-free medium.^{56,66}

The C₁₂FDG substrate in our ImaGene Green *lacZ* Expression Kit (I-2904) is superior to FDG for flow cytometric detection of β -galactosidase activity in live mammalian cells.⁶⁷ Using C₁₂FDG with flow cytometric methods, researchers have:

- Assessed levels of *lacZ* gene expression in recombinant Chinese hamster ovary (CHO) cells throughout the cell cycle, which was monitored with Hoechst 33342⁶⁸ (H-1399; H-3570; FluoroPure Grade, H-21492; Section 8.1)
- Identified endocrine cell precursors in dissociated fetal pancreatic tissue based on their high levels of endogenous acid β -galactosidase⁶⁹
- Measured β -galactosidase activity in single recombinant *E. coli* bacteria⁷⁰
- Detected the activity of β -galactosidase fusion proteins in yeast⁷¹
- Sorted β -galactosidase-expressing mouse sperm cells⁷² and insect cells that harbor recombinant baculovirus^{73,74}

The C₁₂FDG substrate was also useful in a fluorescence microscopy study of zebrafish expressing a *lacZ* reporter gene that was under the control of a mammalian homeobox gene promoter.⁷⁵ In addition, lipophilic β -galactosidase substrates have been employed to diagnose the deficiency in β -galactocerebrosidase activity that typifies Krabbe's disease in human patients.^{8,76} In some cell types, C₁₂FDG produces high levels of background fluorescence that may prohibit its use in assaying low β -galactosidase expression.²¹

Molecular Probes' ImaGene Green C₁₂FDG or ImaGene Red C₁₂RG *lacZ* Gene Expression Kits contain:

- ImaGene Green C₁₂FDG (in Kit I-2904) or ImaGene Red C₁₂RG (in Kit I-2906)
- Phenylethyl β -D-thiogalactopyranoside (PETG; also available separately as a solid, P-1692), a broad-spectrum β -galactosidase inhibitor for stopping the reaction³³

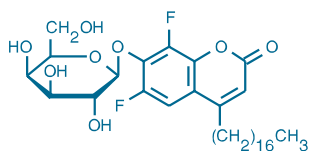


Figure 10.21 D-11950 6,8-difluoro-4-heptadecylumbelliferyl β -D-galactopyranoside (C₁₇DiFUG).

- Chloroquine diphosphate for inhibiting acidic hydrolysis of the substrate
- A detailed protocol for detecting β -galactosidase activity

A sufficient amount of each reagent is provided for 100–200 assays, depending on the volume used for each experiment.

5-Dodecanoylaminofluorescein di- β -D-galactopyranoside (C₁₂FDG) is available separately (D-2893) and we also offer the somewhat less lipophilic 5-octanoylaminofluorescein di- β -D-galactopyranoside (C₈FDG, O-2892). The C₈FDG analog is optimal for investigating the expression of *lacZ* fusion genes in sporulating cultures of *Bacillus subtilis*⁷⁷ and is a better substrate than C₁₂FDG for the detection of β -galactosidase activity in sperm containing the *lacZ* gene.⁷⁸ 5-Acetylaminofluorescein di- β -D-galactopyranoside (C₂FDG, A-22010) is particularly useful for detecting *lacZ* reporter gene expression in slow-growing mycobacteria, including *Mycobacterium tuberculosis*, using a fluorescence plate reader or a flow cytometer.⁷⁹ The ImaGene substrates in these kits are patented by Molecular Probes.⁸⁰

Chloromethyl and Lipophilic Derivatives of DiFMUG

The relatively low pK_a of our 6,8-difluoro-7-hydroxycoumarin derivatives (Figure 1.91) has also allowed us to develop some useful probes for detecting enzymatic activity *in vivo*. Although the β -galactosidase DiFMUG (D-11945) readily enters many live eukaryotic cells, its hydrolysis product (6,8-difluoro-7-hydroxy-4-methylcoumarin, DiFMU; D-6566; Section 10.1; Figure 10.29) is not well retained. To address this limitation, we have developed two modified galactosidase substrates using product-retention strategies that have proven useful for our patented DetectaGene and ImaGene glycosidase substrates.

As with our DetectaGene products, we have replaced the methyl group of DiFMUG with a mildly thiol-reactive chloromethyl group, yielding the β -galactosidase substrate CMDiFUG (C-11946), which is the 6,8-difluorinated analog CMCG (Figure 10.16). We have previously shown that incorporating a chloromethyl group into dyes, as in our CellTracker and MitoTracker probes,^{63,81} considerably improves the retention of fluorescent products in live cells. This enhanced cell retention is at least partially attributable to the formation of dye conjugates with intracellular thiols, including glutathione.⁸² Our results indicate that CMDiFUG discriminates *lacZ*-positive and *lacZ*-negative live cells better than all the other fluorogenic β -galactosidase substrates we have tested, including our ImaGene and DetectaGene substrates.

Similar to our ImaGene products, 6,8-difluoro-4-heptadecylumbelliferyl β -D-galactopyranoside (C₁₇DiFUG, D-11950) contains a lipophilic moiety in place of the methyl group of DiFMUG (Figure 10.21). This modification improves the penetration of this substrate through cell membranes, as well as the retention of the fluorescent product of β -galactosidase activity in live cells. The fluorinated coumarin glycosides are patented by Molecular Probes.

Fluorogenic β -Glucuronidase Substrates

The substrate 4-methylumbelliferyl β -D-glucuronide (MUGlcU, M-1490) is probably the most commonly used fluoro-

genic reagent for identifying *E. coli* contamination and for detecting GUS reporter gene expression in plants and plant extracts.^{83,84} However, β -glucuronidase substrates based on fluorescein may be much more sensitive and yield products that are fluorescent at physiological pH, making them useful for continuous monitoring of enzymatic activity. In addition, we offer a fluorogenic ELF 97 β -D-glucuronidase substrate (E-6587), which produces an intensely green-fluorescent precipitate at the site of enzymatic activity that can be clearly distinguished from most autofluorescence.⁸⁵ This substrate has been used for in-gel zymography to detect β -glucuronidase activity^{86–88} (Figure 9.24, Figure 10.22), immunoassays on protein microarrays⁸⁹ and for the flow cytometric analysis and separation of *E. coli* that had been transfected with *gusA* expression vectors.⁹⁰

Fluorescein Diglucuronide

Fluorescein di- β -D-glucuronide (FDGlcU, F-2915) is colorless and nonfluorescent until it is hydrolyzed to the monoglucuronide and then to highly fluorescent fluorescein (F-1300, Section 10.1). FDGlcU has been used to detect β -glucuronidase activity in plant extracts containing the GUS reporter gene⁸⁴ and may also be useful for assaying lysosomal enzyme release from neutrophils.^{91,92} FDGlcU has also been used in the flow cytometric assay of individual mammalian cells expressing the *E. coli* β -glucuronidase gene.⁹³

PFB Aminofluorescein Diglucuronide

Our patented 5-(pentafluorobenzoylamino)fluorescein di- β -D-glucuronide (PFB-FDGlcU, P-11949) yields the green-fluorescent PFB-F (P-12925, Section 10.1), which appears to localize to endosomal and lysosomal compartments when loaded into cells by pinocytosis, similar to our PFB aminofluorescein diglucoside (PFB-FDGlcU, P-11947). PFB-FDGlcU has been used for the quantitative analysis of β -glucuronidase activity in viable cells and for sorting high-expressing cells by flow cytometry.⁹⁴ Enzyme enrichment has promise as a tool for gene therapy.

Coumarin Glucuronides

4-Methylumbelliferyl β -D-glucuronide (MUGlcU, M-1490) has been used extensively to detect *E. coli* in food,^{95,96} water,⁹⁷ urine⁹⁸ and environmental samples.⁹⁹ MUGlcU is stable to the conditions required for sterilization of media. A fluorogenic bioassay using MUGlcU has been developed to assess the detrimental effects of Li^+ , Al^{3+} , Cr^{6+} and Hg^{2+} on the proliferation of *E. coli*.¹⁰⁰

MUGlcU is also commonly used to identify plant tissue expressing the GUS reporter gene,^{101–103} including nondestructive assays that allow propagation of the transformed plant lines.^{88,104} In addition, MUGlcU has served as a sensitive substrate for lysosomal enzyme release from neutrophils.^{91,92}

Enzyme-mediated hydrolysis of 6,8-difluoro-4-methylumbelliferyl β -D-glucuronide (DiFMUGlcU, D-11951) yields a highly fluorescent product (6,8-difluoro-7-hydroxy-4-methylcoumarin, DiFMU; D-6566; Section 10.1; Figure 10.29) that has a very low pK_a , which should make DiFMUGlcU especially useful for the continuous *in vitro* assay of β -D-glucuronidase activity at a pH greater than or equal to 6. The hydrolysis product of β -trifluoromethylumbelliferyl β -D-glucuronide (T-658) exhibits longer-wavelength excitation and emission spectra than those of either MUGlcU or DiFMUGlcU, which can be advantageous for cells that have high levels of endogenous fluorescence, such as plant cells.

ImaGene Green β -D-Glucuronidase Substrate

Molecular Probes also offers a lipophilic analog of fluorescein di- β -D-glucuronide in our ImaGene Green C₁₂FDGlcU GUS Gene Expression Kit (I-2908). As with our similar ImaGene substrates for β -galactosidase (see above), we have shown that this lipophilic β -glucuronidase substrate freely diffuses across the membranes of viable cultured tobacco leaf cells or protoplasts under physiological conditions. Furthermore, the fluorescent cleavage product is retained in the plant cell for hours to days, facilitating long-term measurements of GUS gene expression. In thin sections of transgenic tomato leaf, the ImaGene Green C₁₂FDGlcU GUS Gene Expression Kit provided a simple and reliable GUS assay that, coupled with confocal laser-scanning microscopy, yielded good cellular resolution.¹⁰⁵ The substrate has also been used to detect β -glucuronidase activity in an *Acremonium* transformant containing the GUS reporter gene.¹⁰⁶

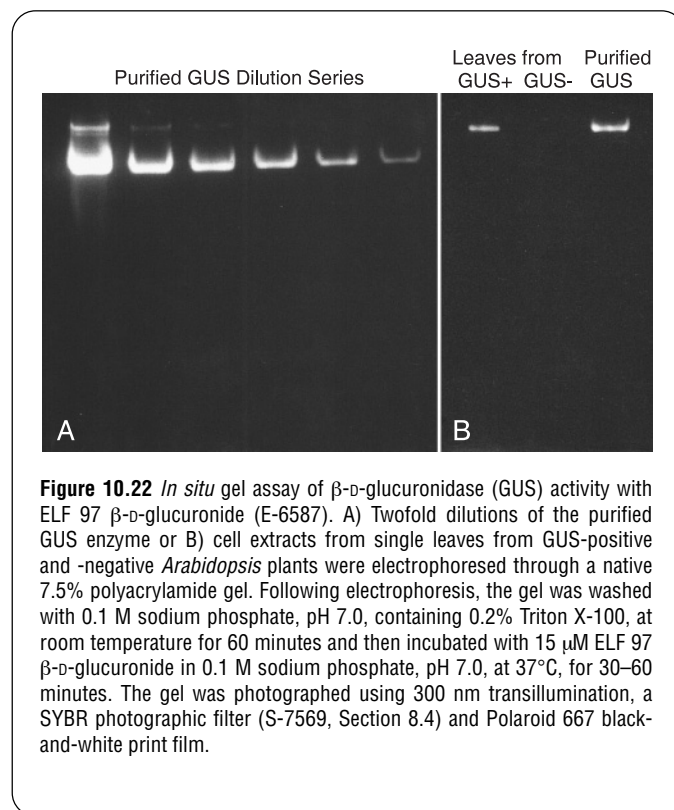
Molecular Probes' ImaGene Green C₁₂FDGlcU GUS Gene Expression Kit contains:

- ImaGene Green C₁₂FDGlcU
- D-Glucaric acid-1,4-lactone, a β -glucuronidase inhibitor for stopping the reaction
- A detailed protocol for detecting β -glucuronidase activity

A sufficient amount of each reagent is provided for approximately 100 tests, depending on the volume used for each experiment.

ELF 97 β -D-Glucuronide

Molecular Probes' ELF 97 β -D-glucuronidase substrate¹⁰⁷ (ELF 97 β -D-glucuronide, E-6587) may be the ideal substrate for analyzing GUS enzyme activity in transgenic plants. Upon hydrolysis, this fluorogenic substrate produces a bright yellow-



green-fluorescent precipitate at the site of enzymatic activity. This fluorescent precipitate has some unique spectral characteristics, including an extremely large Stokes shift (Figure 6.18), that make it easily distinguishable from the endogenous fluorescent components commonly found in plants (see Section 6.3 for a description of our patented ELF technology). We have used this substrate to detect the GUS enzyme in *Arabidopsis* and have found that, after only four hours incubation, signal can be detected in whole-leaf cuttings from GUS-positive plants.⁸⁸ Homogenization of a small portion of a leaf from a GUS-positive *Arabidopsis* plant followed by separation on a nondenaturing gel yields a discrete band corresponding to the glucuronidase enzyme (Figure 10.22). We have also used the ELF 97 glucuronidase substrate for in-gel zymography in one aspect of our Multiplexed Proteomics technology (Section 9.4, Figure 9.24). It is possible to detect as little as 0.5 ng of purified β -glucuronidase in a nondenaturing gel incubated with ELF 97 glucuronide. This substrate may also be useful for detecting GUS fusion proteins in gels, for identifying *E. coli* in agarose-containing medium¹⁰⁸ and for assaying lysosomal enzyme release from neutrophils.^{91,92} The ELF substrates are patented by Molecular Probes.

Fluorogenic β -Glucosidase Substrates

β -Glucosidase, which is a marker for the endoplasmic reticulum (Section 12.4), is present in nearly all species. Its natural substrate is a glucosylceramide (Section 13.3). People with Gaucher's disease have mutations in the acid β -glucosidase gene that result in abnormal lysosomal storage.^{9,109} Enzyme replacement therapy in Gaucher's disease patients¹¹⁰ requires sensitive and selective methods for measuring β -glucosidase activity (Table 10.1). Plant β -glucosidases are implicated in a variety of key metabolic events and growth-related responses.¹¹¹

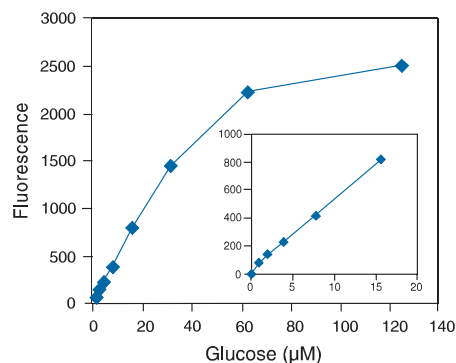


Figure 10.23 Detection of glucose using the Amplex Red Glucose/Glucose Oxidase Assay Kit (A-22189). Reactions containing 50 μ M Amplex Red reagent, 0.1 U/mL HRP, 1 U/mL glucose oxidase and the indicated amount of glucose in 50 mM sodium phosphate buffer, pH 7.4, were incubated for one hour at room temperature. Fluorescence was then measured with a fluorescence-based microplate reader using excitation at 530 ± 12.5 nm and fluorescence detection at 590 ± 17.5 nm. Background fluorescence (arbitrary units), determined for a no-glucose control reaction, has been subtracted from each value. The inset shows the sensitivity and linearity of the assay at low levels of glucose (0–15 μ M).

Fluorescein Diglucoside

As with the other fluorescein diglycosides, Molecular Probes' fluorogenic fluorescein di- β -D-glucopyranoside (FDGlu, F-2881) is likely to yield the greatest sensitivity for detecting β -glucosidase activity in both cells¹⁰⁹ and cell extracts. This substrate has been used to demonstrate the utility of *Saccharomyces cerevisiae* and *Candida albicans* exo-1,3- β -glucanase genes as reporter genes.¹¹² Because these reporter genes encode secreted proteins, assays for reporter gene expression do not require cell permeabilization. FDGlu has been reported to be a selective substrate for the flow cytometric assay of lysosomal glucocerebrosidase activity in a variety of cells.¹¹³ The assay demonstrated the inordinately low glucocerebrosidase activity present in fibroblasts of Gaucher's disease patients.

PFB Aminofluorescein Diglucoside

Through a collaboration with Matthew Lorincz and Leonard A. Herzenberg at Stanford University Medical School,¹¹⁴ our patented PFB aminofluorescein diglucoside (PFB-FDGlu, P-11947) has proven to be an excellent substrate for the flow cytometric discrimination of normal peripheral blood mononuclear cells (PBMC) from the PBMC of patients with Gaucher's disease, a genetic deficiency in lysosomal β -glucocerebrosidase activity.⁶⁴ These researchers loaded the nonfluorescent PFB-FDGlu substrates into cells by pinocytosis, and then observed the green-fluorescent hydrolysis products in endosomal and lysosomal compartments. Under similar loading conditions, we have shown that the hydrolysis products of PFB aminofluorescein digalactoside (PFB-FDG, P-11948; Figure 10.18) and of PFB aminofluorescein diglucuronide (PFB-FDGlcU, P-11949) are similarly localized to endosomes and lysosomes.

Fluorescent Glucocerebrosides

The natural substrates for glucocerebrosidase are sphingosyl β -D-glucopyranosides. Our BODIPY FL analogs of this molecule — BODIPY FL C₅-glucocerebroside^{115,116} (D-7548) and BODIPY FL C₁₂-glucocerebroside (D-7547) — are likely to be substrates for this lysosomal enzyme, which is lacking in Gaucher's disease patients. However, the lack of spectral shift of the hydrolysis products — BODIPY FL C₅-ceramide (D-3521, Section 13.2) and BODIPY FL C₁₂-ceramide — means that extraction and chromatographic separation of the products is necessary for assessment of the activity.

Detection of Glucose and Glucose-Producing Enzymes

Amplex Red Reagent for Glucose

The Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine, A-12222; A-22177; Section 10.5; Figure 10.49) is a colorless, stable and extremely versatile peroxidase substrate.¹¹⁷ In an application similar to our use of the Amplex Red reagent to detect galactose-producing enzymes (see above), we have shown that it is practical to detect free glucose with high specificity at levels as low as 50 ng/mL using the Amplex Red reagent in combination with glucose oxidase (Figure 10.23). Because the peroxidase- and glucose oxidase-mediated reactions can be coupled, it is potentially possible to measure the release of glucose by any glucosidase enzyme — for instance, α -glucosidase, β -glucosidase and glucocerebrosidase — in

either a continuous or discontinuous assay (Figure 10.50). This assay should also be very useful for quantitation of glucose levels in foods, fermentation media and bodily fluids. The long-wavelength spectral properties of resorufin (Figure 10.5) and high sensitivity of the assay result in little interference from colored components in the samples.

Amplex Red Glucose/Glucose Oxidase Assay Kit

Our Amplex Red Glucose/Glucose Oxidase Assay Kit (A-22189) provides all the reagents required for the assay of glucose and enzymes that produce glucose. The kit is also useful for the assay of glucose oxidase activity from cell extracts. We have even shown that the Amplex Red reagent can detect glucose liberated from native dextrans by dextranase¹¹⁸ and from carboxymethylcellulose. The Amplex Red Glucose/Glucose Oxidase Assay Kit contains:

- The Amplex Red reagent
- DMSO and a concentrated reaction buffer
- D-glucose
- Glucose oxidase
- Horseradish peroxidase (HRP)
- H₂O₂ for use as a positive control
- A detailed protocol for the assays

The kit provides a sufficient amount of each reagent for approximately 500 assays using a reaction volume of 100 μ L per assay and can be used with either an absorption- or fluorescence-based microplate reader or a fluorometer.

Amplex Red Neuraminidase/Sialidase Assay Kit

Neuraminidase (NA, also known as sialidase) is a very common enzyme that hydrolyzes terminal sialic acid residues on polysaccharide chains, most often exposing a galactose residue. Although NA is found in mammals, it is predominantly expressed in microorganisms such as bacteria and viruses.¹¹⁹ Anti-influenza drug design has focused on the inhibition of both hemagglutinin and neuraminidase.¹²⁰ Various methods using chemiluminescence, absorption, and fluorescence have been developed to quantitate NA in biological fluids for detection of influenza virus^{121,122} and for screening inhibitors of NA activity in drug development.^{122,123} The ultimate goal has been to develop a rapid, single-step assay that is sensitive and adaptable for a high-throughput screening format. The development of an assay system utilizing the Amplex Red reagent with superior spectral and chemical characteristics meets these needs.

The Amplex Red Neuraminidase (Sialidase) Assay Kit (A-22178) provides an ultrasensitive method for detecting NA activity. This assay then utilizes the Amplex Red reagent to detect H₂O₂ generated by galactose oxidase-mediated oxidation of desialated galactose, the end result of NA action. In the presence of HRP, the H₂O₂ thus produced reacts with a 1:1 stoichiometry with the Amplex Red reagent to generate the red-fluorescent oxidation product, resorufin.⁵⁵ Resorufin has absorption and fluorescence emission maxima of approximately 563 nm and 587 nm, respectively (Figure 10.5), and because the extinction coefficient is high (54,000 cm⁻¹M⁻¹), the assay can be performed either fluorometrically or spectrophotometrically. In a purified system with fetuin as the substrate, NA levels as low as 0.2 mU/mL have been detected with the Amplex Red Neuraminidase (Sialidase) Assay Kit (Figure 10.24). NA activity can also be detected in biological samples such as serum (Figure 10.25). Kit contents include:

- The Amplex Red reagent
- DMSO
- Horseradish peroxidase (HRP)
- H₂O₂
- A 5 \times reaction buffer
- Galactose oxidase from *Dactylium dendroides*
- Fetuin from fetal calf serum
- Neuraminidase from *Clostridium perfringens*
- A detailed protocol

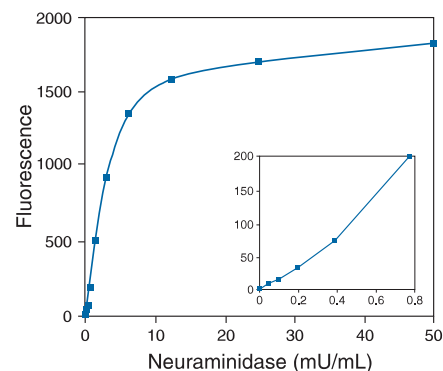


Figure 10.24 Detection of neuraminidase activity using the Amplex Red Neuraminidase (Sialidase) Assay Kit (A-22178). Each reaction contained 50 μ M Amplex Red reagent, 0.1 U/mL HRP, 2 U/mL galactose oxidase, 250 μ g/mL fetuin and the indicated amount of neuraminidase in 1 \times reaction buffer. Reactions were incubated at 37°C. After 30 minutes, fluorescence was measured in a fluorescence-based microplate reader using excitation at 530 \pm 12.5 nm and fluorescence detection at 590 \pm 17.5 nm. A background fluorescence of 70 fluorescence units was subtracted from each data point.

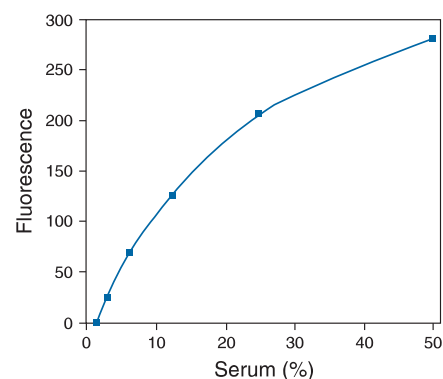


Figure 10.25 Detection of neuraminidase activity in serum using the Amplex Red Neuraminidase (Sialidase) Assay Kit (A-22178). Each reaction contained 50 μ M Amplex Red reagent, 0.1 U/mL HRP, 2 U/mL galactose oxidase, 250 μ g/mL fetuin and the indicated amount of serum in 1 \times reaction buffer. Reactions were incubated at 37°C. After 60 minutes, fluorescence was measured in a fluorescence-based microplate reader using excitation at 530 \pm 12.5 nm and fluorescence detection at 590 \pm 17.5 nm. A background fluorescence of 112 units was subtracted from each data point.

Our Amplex Red Neuraminidase Assay Kit (A-22178) provides the best available technology for assay of this enzyme and uses natural substrates for the enzyme — not synthetic pseudosubstrates.

Each kit provides sufficient reagents for approximately 400 assays using either a fluorescence or absorbance microplate reader and reaction volumes of 100 μL per assay.

Fluorogenic Chitinase/*N*-Acetylglucosaminidase Substrate

Chitin is the second most abundant organic compound in nature¹²⁴ and various chitinases and *N*-acetylglucosaminidases are widely distributed in bacteria, plants and eukaryotic cells. We have utilized our proprietary ELF technology (Section 6.3) to prepare the ELF 97 chitinase/*N*-acetylglucosaminidase substrate (ELF 97 *N*-acetylglucosaminide, ELF 97 NAG; E-22011), which is designed to allow spatially resolved detection of enzyme activity on colony indicator plates and histochemical analysis specimens. Other fluorogenic substrates for these enzymes^{125,126} generate diffusible products and are therefore unsuitable for applications of this type. In addition to the capacity for localized precipitation at sites of enzymatic activity, the ELF 97 alcohol product that is generated upon hydrolysis of ELF 97 NAG is extremely photostable (Figure 6.19) and has widely separated fluorescence excitation and emission peaks ($\sim 360/520$ nm, Figure 6.18). These properties make the signal easy to discriminate from any background fluorescence. ELF 97 NAG has been utilized to differentiate chitinase-active and non-chitinase-active subpopulations of a marine bacterium during chitin degradation.^{127–129}

EnzChek Amylase Assay Kit

α -Amylase is a hydrolytic enzyme that catalyzes the conversion of starch to a mixture of glucose, maltose, maltotriose and dextrans. The levels of α -amylase in various fluids of the human body are of clinical importance,^{130–132} while plant and microbial α -amylases are important enzymes for industry.¹³³

Our EnzChek Amylase Assay Kit (E-11954) provides the speed, high sensitivity and convenience required for measuring

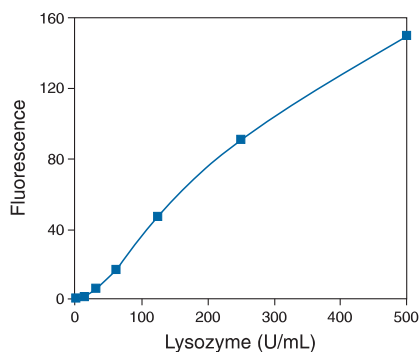


Figure 10.26 Detection of lysozyme activity using the EnzChek Lysozyme Assay Kit (E-22013). Increasing amounts of lysozyme were incubated with the DQ lysozyme substrate for 60 minutes at 37°C. The fluorescence was measured in a fluorescence microplate reader using excitation/emission wavelengths of $\sim 485/530$ nm. Background fluorescence, determined for a no-enzyme control, was subtracted from each value.

α -amylase activity or for screening inhibitors in a high-throughput format. This EnzChek kit contains a starch derivative that is labeled with the BODIPY FL dye to such a degree that the fluorescence is quenched (Figure 10.47). α -Amylase-catalyzed hydrolysis relieves this quenching, yielding brightly fluorescent BODIPY FL dye-labeled fragments. The accompanying increase in fluorescence is proportional to amylase activity and can be monitored with a fluorescence microplate reader, minifluorometer or standard fluorometer. Each EnzChek Amylase Assay Kit includes:

- A heavily labeled BODIPY FL conjugate of starch from corn (DQ starch)
- A 10 \times reaction buffer
- α -Amylase from *Bacillus* sp., for use as a positive control
- A detailed protocol

A sufficient amount of each reagent is supplied for ~ 1000 assays using 200 μL assay volumes and 96-well microplates or ~ 100 assays using 2 mL assay volumes and standard fluorescence cuvettes.

Using 12.5 $\mu\text{g}/\text{mL}$ of the DQ starch substrate and a 60-minute incubation period at room temperature, the assay can detect the activity of this enzyme down to a final concentration of 1×10^{-4} U/mL (~ 0.3 μg protein/mL), where one unit is defined as the amount of enzyme required to liberate 1 mg of maltose from starch in 3 minutes at 20°C, at pH 6.9.

EnzChek Lysozyme Assay Kit

Lysozyme (muramidase) is an important but difficult enzyme to assay. Lysozyme hydrolyzes β -1-4-glycosidic linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues present in the mucopolysaccharide cell wall of a variety of microorganisms. Lysozyme is present in human serum, urine, tears, seminal fluid and milk. Serum and urine lysozyme levels may be elevated in acute myelomonocytic leukemia (FAB-M4), chronic myelomonocytic leukemia (CMML) and chronic myelocytic leukemia (CML).¹³⁴ Increased serum lysozyme activity is also present in tuberculosis,¹³⁵ sarcoidosis, megaloblastic anemias,¹³⁶ acute bacterial infections, ulcerative colitis¹³⁷ and Crohn's disease.^{135,137} Elevated levels of urine and serum lysozyme occur during severe renal insufficiency, renal transplant rejection,¹³⁸ urinary tract infections,¹³⁹ glomerulonephritis and nephrosis.¹⁴⁰

Molecular Probes has developed a simple and sensitive assay that can continuously measure the activity of lysozyme in solution. Our fluorescence-based EnzChek Lysozyme Assay Kit (E-22013) permits the detection of as little as 30 U/mL of lysozyme (Figure 10.26). One unit of lysozyme is the quantity of enzyme that produces a decrease in turbidity of 0.001 optical density units per minute at 450 nm measured at pH 7.0 (25°C) using a 0.3 mg/mL suspension of *Micrococcus lysodeikticus* cells as substrate.¹⁴¹ This assay measures lysozyme activity on *M. lysodeikticus* cell walls that are labeled to such a degree that fluorescence is quenched. Lysozyme action can relieve the quenching, yielding a dramatic increase in fluorescence that is proportional to lysozyme activity. This increase in fluorescence can be measured with any spectrofluorometer, mini-fluorometer or fluorescence microplate reader that can detect fluorescein (excitation/emission maxima $\sim 490/525$ nm).

The EnzChek Lysozyme Assay Kit (E-22013) contains:

- The DQ lysozyme substrate — a fluorescein conjugate of *Micrococcus lysodeikticus* so heavily labeled that its fluorescence is quenched
- Reaction buffer
- Lysozyme from chicken egg white
- A detailed protocol for the assay

Each kit contains sufficient materials for approximately 400 assays of 100 μ L in a fluorescence microplate reader.

Chromogenic Glycosidase Substrates

The widely used β -galactosidase substrate — 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-Gal, B-1690, B-22015) — yields a dark blue precipitate at the site of enzymatic activity. X-Gal is useful for numerous histochemical and molecular biology applications, including detection of *lacZ* activity in cells and tissues. In contrast to β -glucuronidase as a gene marker, β -galactosidase can be fixed in cells and tissues with glutaraldehyde without loss of activity and detected with high resolution with X-Gal.¹⁴² Molecular Probes offers X-Gal at an attractive price, particularly in our 25-gram bulk packaging size (B-22015).

The chromogenic substrate 5-bromo-4-chloro-3-indolyl β -D-glucuronic acid (X-GlcU, B-1691) forms a dark-blue precipitate. X-GlcU is routinely used to detect GUS expression in transformed plant cells and tissues.^{143–149} However, because it is relatively difficult to differentiate the blue color of the product of X-GlcU against the dark-green chloroplasts,¹⁵⁰ we also offer the isomeric 5-bromo-6-chloro-3-indolyl β -D-glucuronide¹⁵¹ (B-8408), which forms a magenta-colored precipitate. X-GlcU can also be used to detect *E. coli* contamination in food and water.^{108,152}

Auxiliary Products for Glycosidase Research

Phenylethyl β -D-Thiogalactopyranoside (PETG)

Phenylethyl β -D-thiogalactopyranoside (PETG, P-1692) is a cell-permeant inhibitor of β -galactosidase activity.^{33,153} We provide PETG in our FluoReporter, DetectaGene and ImaGene *lacZ* Gene Expression Kits for stopping the enzymatic reaction.

β -Galactosidase and Its Streptavidin Conjugate

Molecular Probes also offers the streptavidin conjugate of β -galactosidase (S-931), a reagent used in a variety of ELISAs.¹⁵⁴ Streptavidin- β -D-galactosidase reportedly provided enhanced sensitivity over that obtained with the avidin conjugate of HRP in the detection of a variety of mammalian interleukins and their receptors by ELISA.¹⁵⁵ This reagent has also been used in fluorometric-reverse (IgE-capture)¹⁵⁶ and fluorescence-sandwich¹⁵⁷ ELISAs.

Rabbit Anti- β -Galactosidase Antibody

Molecular Probes offers a polyclonal antibody to the widely used reporter gene product, β -galactosidase. Our rabbit anti- β -galactosidase antibody (A-11132) is raised against *E. coli*-derived β -galactosidase and demonstrates high selectivity for the enzyme. Whether it is being used as a reporter gene or to generate fusion proteins, anti- β -galactosidase provides an easy tool for

detecting the enzyme. The antibody is suited to a variety of techniques, including immunoblotting, ELISA, immunoprecipitation and most immunological methods. β -Galactosidase has been used as a tag for quantitative detection of molecules expressed on a cell surface in unfixed, live cells, using anti- β -galactosidase and a β -galactosidase substrate for detection.¹⁵⁸ This novel “cell-ELISA” technique is reported to be applicable to adherent cells and nonadherent cells and to have utility for large-scale screening for expression of cell-surface molecules and of hybridomas for production of antibodies to cell-surface epitopes.

Rabbit Anti- β -Glucuronidase Antibody

In combination with a fluorophore- or enzyme-labeled anti-rabbit IgG secondary antibody (Section 7.3, Table 7.3), our anti- β -glucuronidase antibody (A-5790) can be used to detect the GUS enzyme in transformed plant tissue^{159,160} and in transfected animal cells¹⁶¹ using Western blotting or immunohistochemical techniques. Furthermore, this antibody, which is raised in rabbits against *E. coli*-type X-A β -glucuronidase, can be immobilized in microplate wells in order to capture the GUS enzyme from cell lysates.¹⁶² The enzymatic activity can subsequently be determined using any of our fluorogenic or chromogenic β -glucuronidase substrates.¹⁶³

ManLev: A Metabolically Active Carbohydrate Analog

N-Levulinoyl-D-mannosamine¹⁶⁴ (ManLev, L-20492; Section 3.2, Figure 3.9) and *N*-levulinoyl-D-mannosamine, tetraacetate (ManLev tetraacetate, L-20493; Section 3.2) are ketone-containing monosaccharides that serve as substrates in the oligosaccharide synthesis pathway, resulting in ketone-tagged cell-surface oligosaccharides.^{165–168} Since ketones are rare in cells, reaction with 2 μ M biotinylated aldehyde-reactive probe (ARP, A-10550; Section 3.2; Figure 3.10) followed by a fluorescent avidin or streptavidin conjugate (Section 7.6) provides a means of identifying and tracing tagged cells by either imaging (Figure 3.11) or flow cytometry.

Related Products for Carbohydrate Research

Molecular Probes offers an extensive assortment of reagents for detection and analysis of carbohydrates that are described in other sections of this *Handbook*. These products include:

- Hydrazine and aromatic amine reagents for derivatization and analysis of carbohydrates by electrophoretic methods (Section 3.2, Table 3.1)
- Lectins and fluorescent lectin conjugates (Section 7.7, Table 7.18)
- Pro-Q Glycoprotein Blot and Gel Stain Kits (Section 9.4)
- Pro-Q Emerald 300 Lipopolysaccharide Gel Stain Kit (P-20495, Section 3.2)
- Fluorescent lipopolysaccharides (Section 13.3, Section 16.1; Table 16.1)
- Fluorescent glycolipids, including phosphatidyl inositol derivatives (Section 13.2, Section 13.3)
- BODIPY FL C₅-ganglioside G_{M1} (B-13950, Section 13.3)
- Fluorescent and biotinylated dextrans (Section 14.5, Table 14.4)
- NBD-glucosamine derivatives for glucose-transport studies (N-13195, N-23106; Section 15.2)
- Fluorescein insulin (I-13269, Section 16.1)
- Fluorescent phosphatidylinositol phosphate derivatives (Table 13.2) and antibodies to phosphatidylinositol phosphates (Section 18.4)

References

1. *J Clin Microbiol* 30, 1402 (1992); 2. *Microbiol Rev* 55, 335 (1991); 3. *Meth Microbiol* 19, 105 (1987); 4. *Nature* 369, 113 (1994); 5. *J Cell Biol* 110, 309 (1990); 6. *Anal Biochem* 148, 50 (1985); 7. *Traffic* 1, 836 (2000); 8. *Clin Chim Acta* 205, 87 (1992); 9. *Crit Rev Biochem Mol Biol* 25, 385 (1990); 10. *Mol Gen Genet* 216, 321 (1989); 11. *Mol Gen Genet* 215, 38 (1988); 12. *Plant Mol Biol* 10, 387 (1988); 13. *Proc Natl Acad Sci U S A* 83, 8447 (1986); 14. *J Immunol Methods* 150, 5 (1992); 15. *Anal Biochem* 257, 234 (1998); 16. *J Immunol Methods* 150, 23 (1992); 17. *J Immunol Methods* 48, 133 (1982); 18. *Exp Parasitol* 73, 440 (1991); 19. *Dev Biol* 161, 77 (1994); 20. *Mol Microbiol* 13, 655 (1994); 21. *J Neurosci* 13, 1418 (1993); 22. *Eur J Immunol* 19, 1619 (1989); 23. *Cytometry* 17, 216 (1994); 24. *Eur J Cell Biol* 62, 324 (1993); 25. *J Biol Chem* 268, 9762 (1993); 26. *Neuron* 9, 1117 (1992); 27. *Science* 251, 81 (1991); 28. *Proc Natl Acad Sci U S A* 85, 2603 (1988); 29. *J Virol Methods* 44, 99 (1993); 30. *Proc Natl Acad Sci U S A* 90, 8194 (1993); 31. *Appl Environ Microbiol* 56, 3861 (1990); 32. Purchase of the FluorReporter lacZ Flow Cytometry Kits is accompanied by a research license under US 5,070,012; 33. *Carbohydr Res* 56, 153 (1977); 34. *Methods* 2, 261 (1991); 35. *Methods* 2, 248 (1991); 36. *Anal Chim Acta* 163, 67 (1984); 37. *Oncogene* 10, 2323 (1995); 38. *Cytometry* 9, 394 (1988); 39. *Ann N Y Acad Sci* 613, 333 (1990); 40. *Anal Chim Acta* 213, 245 (1988); 41. DDAO-based substrates are licensed to Molecular Probes under US 4,810,636 and its foreign equivalents; 42. *Anal Biochem* 215, 24 (1993); 43. *Neuron* 10, 427 (1993); 44. *Proc Natl Acad Sci U S A* 84, 156 (1987); 45. *J Histochem Cytochem* 33, 965 (1985); 46. *Clin Chim Acta* 12, 647 (1965); 47. *Bioorg Med Chem Lett* 8, 3107 (1998); 48. *Anal Lett* 21, 193 (1988); 49. *J Histochem Cytochem* 34, 585 (1986); 50. *Biotechniques* 30, 776 (2001); 51. *Infect Immun* 61, 5231 (1993); 52. *Anal Biochem* 146, 211 (1985); 53. *Proc Natl Acad Sci U S A* 95, 6373 (1998); 54. *J Biochem (Tokyo)* 100, 707 (1986); 55. *J Immunol Methods* 202, 133 (1997); 56. *J Fluorescence* 3, 119 (1993); 57. *Development* 119, 1217 (1993); 58. *Neuron* 14, 685 (1995); 59. *J Neurosci* 15, 1025 (1995); 60. *Exp Cell Res* 219, 372 (1995); 61. *Science* 266, 1881 (1994); 62. *Cytometry* 28, 36 (1997); 63. *J Histochem Cytochem* 44, 1363 (1996); 64. US 5,773,236; 65. *Lysosomes*, Holtzman E pp. 319–361 (1989); 66. *FASEB J* 5, 3108 (1991); 67. *Appl Environ Microbiol* 60, 4638 (1994); 68. *Biotechnol Bioeng* 42, 1113 (1993); 69. *J Clin Endocrinol Metab* 78, 1232 (1994); 70. *Biotechnol Bioeng* 42, 708 (1993); 71. *J Biol Chem* 271, 29312 (1996); 72. *Proc Natl Acad Sci U S A* 89, 10681 (1992); 73. *Methods Cell Biol* 42 Pt B, 563 (1994); 74. *Biotechniques* 14, 274 (1993); 75. *Genes Dev* 6, 591 (1992); 76. *J Lab Clin Med* 110, 740 (1987); 77. *Cytometry* 20, 324 (1995); 78. *Hum Mol Genet* 5, 875 (1996); 79. *FEMS Microbiol Lett* 179, 317 (1999); 80. US 5,208,148 and US 5,242,805; 81. *Cytometry* 12, 184 (1991); 82. *Cytometry* 15, 349 (1994); 83. *Biotechniques* 8, 37 (1990); 84. *Plant Mol Biol Rep* 5, 387 (1988); 85. *Tetrahedron* 53, 7159 (1997); 86. *Electrophoresis* 22, 970 (2001); 87. *Electrophoresis* 21, 497 (2000); 88. *J Biochem Biophys Methods* 33, 197 (1996); 89. *Anal Biochem* 278, 123 (2000); 90. *Biotechniques* 30, 474 (2001); 91. *Cell Signal* 3, 625 (1991); 92. *J Immunol Methods* 100, 211 (1987); 93. *Cytometry* 24, 321 (1996); 94. *J Biol Chem* 274, 657 (1999); 95. *J Assoc Off Anal Chem* 71, 589 (1988); 96. *Appl Environ Microbiol* 50, 1383 (1985); 97. *Can J Microbiol* 39, 1066 (1993); 98. *J Microbiol Methods* 12, 51 (1990); 99. *J Microbiol Methods* 12, 235 (1990); 100. *Biotechniques* 16, 888 (1994); 101. GUS Protocols: Using the GUS Gene as a Reporter of Gene Expression, Gallagher SR, Ed. pp. 61–76 (1992); 102. *Plant Sci* 78, 73 (1991); 103. *Plant Mol Biol* 15, 527 (1990); 104. *Plant Mol Biol Rep* 10, 37 (1992); 105. *Plant J* 10, 745 (1996); 106. *Microbiology* 143, 267 (1997); 107. *Ann N Y Acad Sci* 346, 419 (1980); 108. *J Appl Bacteriol* 74, 223 (1993); 109. *Cell Biochem Funct* 11, 167 (1993); 110. *Neurochem Res* 24, 301 (1999); 111. 533 (1993); 112. *Yeast* 10, 747 (1994); 113. *Anal Biochem* 247, 268 (1997); 114. *Blood* 89, 3412 (1997); 115. *Frontiers in Bioactive Lipids*, Vanderhoeve JV, Ed. pp. 203–213 (1996); 116. *J Cell Biol* 125, 769 (1994); 117. *Anal Biochem* 253, 162 (1997); 118. *Anal Biochem* 260, 257 (1998); 119. *J Biochem Biophys Methods* 22, 23 (1991); 120. *Nat Biotechnol* 18, 835 (2000); 121. *Antiviral Res* 47, 1 (2000); 122. *J Med Chem* 13, 697 (1970); 123. *Anal Biochem* 280, 291 (2000); 124. *Trends Biotechnol* 16, 301 (1998); 125. *Appl Environ Microbiol* 64, 613 (1998); 126. *Anal Biochem* 208, 74 (1993); 127. *Methods Enzymol* 336, 279 (2001); 128. *Appl Environ Microbiol* 66, 3566 (2000); 129. *Appl Environ Microbiol* 66, 3574 (2000); 130. *Medicine (Baltimore)* 55, 269 (1976); 131. *Clin Chem* 21, 57 (1976); 132. *Clin Chem* 30, 387 (1984); 133. *Starch/Stärke* 35, 169 (1983); 134. *Mod Pathol* 7, 771 (1994); 135. *Acta Pathol Jpn* 28, 689 (1978); 136. *N Engl J Med* 277, 10 (1967); 137. *J Clin Pathol* 36, 1312 (1983); 138. *Clin Chem* 32, 1807 (1986); 139. *Toxicology* 28, 347 (1983); 140. *Nephron* 63, 423 (1993); 141. *Biochim Biophys Acta* 8, 302 (1952); 142. *EMBO J* 8, 343 (1989); 143. *Biotechniques* 19, 106 (1995); 144. *Biotechnology (N Y)* 8, 833 (1990); 145. *Plant Cell Physiol* 31, 805 (1990); 146. *Science* 249, 1285 (1990); 147. *Science* 248, 471 (1990); 148. *Nature* 342, 837 (1989); 149. *EMBO J* 6, 3901 (1987); 150. *Biotechniques* 7, 922 (1989); 151. *Biotechniques* 19, 352 (1995); 152. *Lett Appl Microbiol* 13, 212 (1991); 153. *Anal Biochem* 199, 119 (1991); 154. *J Immunol Methods* 125, 279 (1989); 155. *Biochemistry* 26, 4922 (1987); 156. *J Immunol Methods* 116, 181 (1989); 157. *J Immunol Methods* 110, 129 (1988); 158. *J Immunol Methods* 234, P153 (2000); 159. *J Biol Chem* 269, 17635 (1994); 160. *Plant Mol Biol* 15, 821 (1990); 161. *Biotechniques* 27, 896 (1999); 162. *J Clin Microbiol* 32, 1444 (1994); 163. *Appl Environ Microbiol* 53, 1073 (1987); 164. US 6,075,134; 165. *Glycobiology* 11, 11R (2001); 166. *Nat Biotechnol* 19, 553 (2001); 167. *Methods Enzymol* 327, 260 (2000); 168. *Glycobiology* 10, 1049 (2000).

Data Table — 10.2 Detecting Glycosidases

Cat #	MW	Storage	Soluble	Abs	EC	Em	Solvent	Product	Notes
A-12222	257.25	FF,D,A	DMSO	280	6,000	none	pH 8	R-363 *	1
A-22010	713.65	F,D	DMSO	289	5,500	none	MeOH	see Notes	2
B-1690	408.63	F,D	DMSO	290	4,900	none	H ₂ O	see Notes	3
B-1691	521.79	F,D	pH >6	292	4,800	none	MeOH	see Notes	3
B-8408	521.79	F,D	pH >6	294	4,600	none	pH 7	see Notes	4
B-22015	408.63	F,D	DMSO	290	4,900	none	H ₂ O	see Notes	3
C-1488	368.30	F,D	pH >6, DMSO	330	16,000	396	pH 8	H-185 †	5
C-11946	408.74	F,D,L	DMSO, H ₂ O	284	8,000	none	pH 9	C-1288 1*	
D-2893	853.92	F,D	DMSO	289	6,000	none	MeOH	D-109 ‡	
D-2920	705.07	F,L	see Notes	273	4,800	none	MeOH	see Notes	6, 7, 8
D-2921	372.76	F,L	see Notes	321	12,000	394	MeOH	C-2111 §	5, 6, 8
D-6488	470.31	F,D,L	DMSO	465	24,000	608	pH 7	H-6482 *	
D-7519	861.96	FF,D,L	DMSO, EtOH	505	85,000	511	MeOH	see Notes	9, 10
D-7547	861.96	FF,D,L	DMSO, EtOH	505	85,000	511	MeOH	see Notes	9, 10
D-7548	763.77	FF,D,L	DMSO, EtOH	505	85,000	511	MeOH	see Notes	9, 10
D-11945	374.29	F,D	DMSO, H ₂ O	313	6,900	see Notes	pH 9	D-6566 *	5
D-11950	598.72	F,D	DMSO	314	6,400	none	MeCN	see Notes	
D-11951	394.21	F,D	H ₂ O	313	6,200	see Notes	pH 9	D-6566 *	5

Cat #	MW	Storage	Soluble	Abs	EC	Em	Solvent	Product	Notes
D-13951	925.91	FF,D,L	DMSO, EtOH	505	80,000	511	MeOH	see Notes	9, 10
E-6587	483.26	F,D,L	DMSO, H ₂ O	302	14,000	see Notes	MeOH	E-6578 *	5
E-22011	511.34	F,D,L	DMSO	290	11,000	see Notes	DMSO	E-6578 *	5
F-1179	656.60	F,D	DMSO	273	6,400	none	MeOH	F-1300 *	11
F-2881	656.60	F,D	DMSO	272	6,200	none	MeOH	F-1300 *	
F-2905	368.30	F,D,L	pH 7	330	16,000	396	pH 8	H-185 †	5, 8, 12
F-2915	684.56	F,D	pH >6, DMSO	272	5,700	none	MeOH	F-1300 *	
I-2904	853.92	F,D,L	DMSO	289	6,000	none	MeOH	D-109 ‡	8, 12
I-2906	543.66	F,D,L	see Notes	448	20,000	none	MeOH	see Notes	8, 13, 14
I-2908	881.88	F,D	see Notes	290	5,400	none	MeOH	D-109 ‡	6, 8
M-1489	338.31	D	DMSO, H ₂ O	316	14,000	376	pH 9	H-189 *	5
M-1490	352.30	F,D	pH >6	316	12,000	375	pH 9	H-189 *	5
O-2892	797.81	F,D	DMSO	289	5,500	none	MeOH	see Notes	2
P-1692	300.37	F,D	DMSO, H ₂ O	<300		none			
P-11947	865.67	F,D	DMSO	260	26,000	none	MeOH	P-12925 *	
P-11948	865.67	F,D	DMSO	260	25,000	none	MeOH	P-12925 *	
P-11949	893.64	F,D	pH >6, DMSO	260	21,000	none	MeOH	P-12925 *	
R-1159	375.33	F,D,L	DMSO	469	19,000	none	pH 9	R-363 *	
T-658	406.27	F,D	pH >6	325	11,000	410	pH 8	see Notes	5, 15

For definitions of the contents of this data table, see "How to Use This Book" on page viii.

* See Section 10.1. † See Section 1.7. ‡ See Section 13.5. § See Section 14.2.

Notes

1. This substrate is used for peroxidase-coupled detection in our Amplex Red Assay Kits.
2. Enzymatic cleavage of this substrate yields a 5-acylamino fluorescein derivative with spectroscopic properties similar to D-109 (Section 13.5).
3. Enzymatic cleavage of this substrate yields a water-insoluble, blue-colored indigo dye (Abs ~615 nm).
4. Enzymatic cleavage of this substrate yields a water-insoluble, magenta-colored indigo dye (Abs ~565 nm).
5. Fluorescence of the unhydrolyzed substrate is very weak.
6. This product is packaged as a solution in 1:1 (v/v) DMSO/H₂O.
7. Enzymatic cleavage of this substrate yields 5-chloromethylfluorescein, with spectroscopic properties similar to C-1904 (Section 21.2).
8. Data represent the substrate component of this kit.
9. Em for BODIPY FL sphingolipids shifts to ~620 nm when high concentrations of the probe (>5 mol%) are incorporated in lipid mixtures (J Cell Biol 113, 1267 (1991)).
10. Enzymatic cleavage of this product yields a fluorescent ceramide or glycosylceramide with unchanged spectral properties.
11. F-1179 is soluble at 1 mM in water, but it is best to prepare a stock solution in DMSO.
12. This product is supplied as a ready-made solution in the solvent indicated under **Soluble**.
13. This product is packaged as a solution in 7:3 (v/v) DMSO/EtOH.
14. Enzymatic cleavage of this substrate yields 2-dodecylresorufin, Abs = 578 nm (EC = 69,000 cm⁻¹M⁻¹), Em = 597 nm in MeOH.
15. Enzymatic cleavage of this substrate yields 7-hydroxy-4-trifluoromethylcoumarin, Abs = 385 nm (EC = 16,000 cm⁻¹M⁻¹), Em = 502 nm in pH 10 buffer.

Product List — 10.2 Detecting Glycosidases

Cat #	Product Name	Unit Size
A-22010	5-acetylamino fluorescein di-β-D-galactopyranoside (C ₂ FDG)	5 mg
A-22189	Amplex [®] Red Glucose/Glucose Oxidase Assay Kit *500 assays*	1 kit
A-22179	Amplex [®] Red Galactose/Galactose Oxidase Assay Kit *400 assays*	1 kit
A-22178	Amplex [®] Red Neuraminidase (Sialidase) Assay Kit *400 assays*	1 kit
A-12222	Amplex [®] Red reagent (10-acetyl-3,7-dihydroxyphenoxazine)	5 mg
A-11132	anti-β-galactosidase, rabbit IgG fraction *2 mg/mL*	0.5 mL
A-5790	anti-β-glucuronidase, rabbit IgG fraction *2 mg/mL*	0.5 mL
B-1690	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal)	1 g
B-22015	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) *bulk packaging*	25 g
B-1691	5-bromo-4-chloro-3-indolyl β-D-glucuronide, cyclohexylammonium salt (X-GlcU, CHA)	100 mg
B-8408	5-bromo-6-chloro-3-indolyl β-D-glucuronide, cyclohexylammonium salt	25 mg
C-1488	3-carboxyumbelliferyl β-D-galactopyranoside (CUG)	10 mg
C-11946	4-chloromethyl-6,8-difluorumbelliferyl β-D-galactopyranoside (CMDiFUG)	5 mg
D-2921	DetectaGene [™] Blue CMCG <i>lacZ</i> Gene Expression Kit	1 kit
D-2920	DetectaGene [™] Green CMFDG <i>lacZ</i> Gene Expression Kit	1 kit
D-6488	9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) β-D-galactopyranoside (DDAO galactoside)	5 mg
D-7519	<i>N</i> -(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoyl)sphingosyl 1-β-D-galactopyranoside (BODIPY [®] FL C ₁₂ -galactocerebroside)	25 μg
D-7547	<i>N</i> -(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoyl)sphingosyl 1-β-D-glucopyranoside (BODIPY [®] FL C ₁₂ -glucocerebroside)	250 μg
D-7548	<i>N</i> -(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)sphingosyl 1-β-D-glucopyranoside (BODIPY [®] FL C ₅ -glucocerebroside)	250 μg
D-13951	<i>N</i> -(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)sphingosyl 1-β-D-lactoside (BODIPY [®] FL C ₅ -lactosylceramide)	25 μg
D-11950	6,8-difluoro-4-heptadecylumbelliferyl β-D-galactopyranoside (C ₁₇ DiFUG)	5 mg

Product List — 10.2 Detecting Glycosidases — continued

Cat #	Product Name	Unit Size
D-11945	6,8-difluoro-4-methylumbelliferyl β -D-galactopyranoside (DiFMUG)	10 mg
D-11951	6,8-difluoro-4-methylumbelliferyl β -D-glucuronide, lithium salt (DiFMUGlcU)	5 mg
D-2893	5-dodecanoylamino fluorescein di- β -D-galactopyranoside (C ₁₂ FDG)	5 mg
E-22011	ELF [®] 97 chitinase/ <i>N</i> -acetylglucosaminidase substrate (ELF [®] 97 <i>N</i> -acetylglucosaminide; ELF [®] 97 NAG)	5 mg
E-6587	ELF [®] 97 β -D-glucuronidase substrate (ELF [®] 97 β -D-glucuronide)	5 mg
E-11954	EnzChek [®] Amylase Assay Kit *1000 assays*	1 kit
E-22013	EnzChek [®] Lysozyme Assay Kit *400 assays*	1 kit
F-1930	FluoReporter [®] <i>lacZ</i> Flow Cytometry Kit *50 assays*	1 kit
F-1931	FluoReporter [®] <i>lacZ</i> Flow Cytometry Kit *250 assays*	1 kit
F-2905	FluoReporter [®] <i>lacZ</i> /Galactosidase Quantitation Kit *1000 assays*	1 kit
F-1179	fluorescein di- β -D-galactopyranoside (FDG)	5 mg
F-2881	fluorescein di- β -D-glucopyranoside (FDGlu)	5 mg
F-2915	fluorescein di- β -D-glucuronide (FDGlcU)	5 mg
I-2904	ImaGene Green [™] C ₁₂ FDG <i>lacZ</i> Gene Expression Kit	1 kit
I-2908	ImaGene Green [™] C ₁₂ FDGlcU GUS Gene Expression Kit	1 kit
I-2906	ImaGene Red [™] C ₁₂ RG <i>lacZ</i> Gene Expression Kit	1 kit
I-14402	Influx [™] pinocytic cell-loading reagent *makes 10 x 5 mL*	1 set
M-1489	4-methylumbelliferyl β -D-galactopyranoside (MUG)	1 g
M-1490	4-methylumbelliferyl β -D-glucuronide (MUGlcU)	100 mg
O-2892	5-octanoylamino fluorescein di- β -D-galactopyranoside (C ₈ FDG)	5 mg
P-11948	5-(pentafluorobenzoylamino)fluorescein di- β -D-galactopyranoside (PFB-FDG)	5 mg
P-11947	5-(pentafluorobenzoylamino)fluorescein di- β -D-glucopyranoside (PFB-FDGlu)	5 mg
P-11949	5-(pentafluorobenzoylamino)fluorescein di- β -D-glucuronide (PFB-FDGlcU)	5 mg
P-1692	phenylethyl β -D-thiogalactopyranoside (PETG)	10 mg
R-1159	resorufin β -D-galactopyranoside	25 mg
S-931	streptavidin, β -galactosidase conjugate	1 mg
T-658	β -trifluoromethylumbelliferyl β -D-glucuronide	25 mg

10.3 Detecting Enzymes that Metabolize Phosphates and Polyphosphates

Cells utilize a wide variety of phosphate and polyphosphate esters as enzyme substrates, second messengers, membrane structural components and vital energy reservoirs. This section includes an assortment of reagents and methods for detecting the metabolism of phosphate esters. Our diverse array of fluorogenic and chromogenic substrates include substrates for phosphatases, as well as reagents to measure the activity of enzymes such as ATPases, GTPases and DNA and RNA polymerases. In addition, we have several nucleotide analogs and substrates for phosphodiesterases and phospholipases that are described in Section 18.3 and Section 18.4, respectively.

By far the largest group of chromogenic and fluorogenic substrates for phosphate-ester metabolizing enzymes are those for simple phosphatases such as alkaline and acid phosphatase, both of which hydrolyze phosphate monoesters to an alcohol and inorganic phosphate. Conjugates of calf intestinal alkaline phosphatase are extensively used as secondary detection reagents in ELISAs,¹ immunohistochemical techniques² and Northern, Southern and Western blot analyses (Section 8.5, Section 9.4). In addition, phosphatases serve as enzyme markers, allowing researchers to identify primordial germ cells,³ to distinguish subpopulations of bone marrow stromal cells⁴ and to investigate *in vitro* differentiation in carcinoma cell lines.⁵⁻⁷ *P ALP-I*, the gene for human placental alkaline phosphatase, has been used as

a eukaryotic reporter gene that is superior to *lacZ* for lineage studies in murine retina.^{8,9} This gene has also been engineered to produce a secreted alkaline phosphatase (SEAP), allowing quantitation of gene expression without disrupting the cells.¹⁰

Molecular Probes supplies the best phosphatase substrates and assay kits for a wide variety of applications:

- Fluorescein diphosphate (F-2999) — probably the most sensitive substrate available for alkaline phosphatase activity measurements.
- DDAO phosphate, a long-wavelength, dual-purpose phosphatase substrate for both solution-based assays and amplified detection of specific targets on blots (Section 9.4).
- ELF 97 phosphate, an acid and alkaline phosphatase substrate whose hydrolysis immediately yields a green-fluorescent precipitate at the sites of endogenous phosphatase activity in cells. ELF 97 phosphate is also utilized in our Enzyme-Labeled Fluorescence technology for immunostaining and fluorescence *in situ* hybridization applications (Section 6.3).
- DiFMUP, which is available as a standalone reagent (D-6567, D-22065) and as a component of our EnzChek Acid Phosphatase Assay Kit (E-12020). The RediPlate 96 and RediPlate 384 EnzChek Tyrosine Phosphatase Assay Kits (R-22067, R-22068) utilize the DiFMUP substrate for the selective assay