

## SensiFlex™ ELISA Development Kit with Fluorocillin™ Green Reagent

S33853 includes the goat anti-mouse IgG  $\beta$ -lactamase TEM-1 conjugate

S33854 includes the goat anti-rabbit IgG  $\beta$ -lactamase TEM-1 conjugate

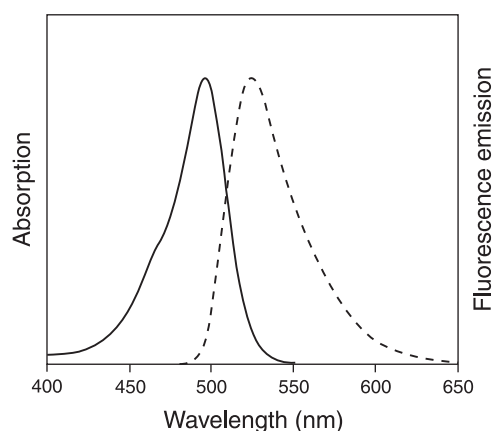
### Quick Facts

#### Storage upon receipt:

- $\leq -20^{\circ}\text{C}$
- Desiccate
- Protect from light

**Ex/Em:** 495/525

**Number of assays:** 500, based on 100 mL reaction volumes



**Figure 1.** Normalized absorption (—) and fluorescence emission (---) spectra of the Fluorocillin™ Green substrate after reaction with  $\beta$ -lactamase.

### Introduction

Molecular Probes SensiFlex™ ELISA Development Kits offer an extremely sensitive fluorometric detection method for  $\beta$ -lactamase-amplified enzyme-linked immunosorbent assays (ELISAs). The Fluorocillin™ Green reagent provided in the kits is a novel substrate, and, following cleavage by  $\beta$ -lactamase, emits a green-fluorescent signal (emission maxima at  $\sim 525$  nm, Figure 1). Fluorocillin™ Green reagent has a broad dynamic range of fluorescence signal (Figure 2), is more sensitive than common colorimetric substrates (Figure 3), and displays only modest hydrolysis when incubated for extended periods from pH 5.5 to 8.0 (Figure 4). Additionally, Fluorocillin™ Green reagent effectively reports  $\beta$ -lactamase activity in the presence of EDTA, many detergents, salts, and sodium azide. Developing ELISA protocols that maximize both dynamic range and limit of detection involves optimizing the concentrations of both the enzyme and substrate. The robust enzyme kinetics of the reaction between  $\beta$ -lactamase and Fluorocillin™ Green reagent are consistent, even in the face of changes in enzyme and substrate concentration. This makes assay development using  $\beta$ -lactamase and Fluorocillin™ Green reagent easier than for assays that employ an enzyme with faster kinetics (e.g., horseradish peroxidase).

The SensiFlex™ ELISA Development Kits contain all that is needed for ELISA detection in assays where goat anti-mouse- or anti-rabbit-IgG can be used as the secondary detection agent. The kits include a  $\beta$ -lactamase TEM-1 goat anti-mouse- or anti-rabbit-IgG conjugate, also available from Molecular Probes as a standalone product (G31567 and G31568). Sufficient reagent quantities are provided in the kit for performing approximately 500 assays using 100  $\mu\text{L}$  volumes per assay.

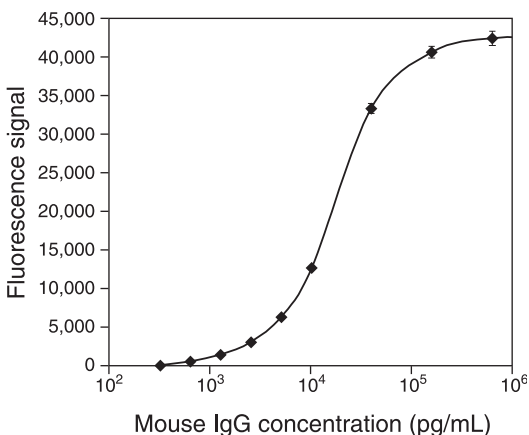
### Product Information

#### Components

- **Fluorocillin™ Green reagent** (MW  $\approx 1100$ , Component A), five vials, each containing 100  $\mu\text{g}$  of reagent
- **Dimethylsulfoxide (DMSO), anhydrous** (Component B), 500  $\mu\text{L}$
- **10X Phosphate buffered saline, (PBS) pH 7.2** (Component C), 200 mL
- **Goat anti-mouse or anti-rabbit IgG (H+L),  $\beta$ -lactamase TEM-1 conjugate** (Component D), 0.5 mg
- **Fluorocillin™ stop reagent** (Component E), five vials, each containing 1.2 mg of reagent
- **0.1 M sodium bicarbonate buffer, pH  $\sim 9.3$**  (Component F), 50 mL
- **Bovine serum albumin (BSA)** (Component G), 1.0 g
- **Tween® 20** (Component H), 1.5 mL
- **Nunc-Immuno™ MaxiSorp™ U96 plate** (Component I), 5 each

#### Storage and Handling

Upon receipt, the SensiFlex™ ELISA Development Kit should be stored at  $\leq -20^{\circ}\text{C}$ , desiccated, and protected from light. Stored properly, the kit components should remain stable for at least six months. Allow the reagents to warm to room temperature before opening the vials. After the 10X PBS (Component C) is thawed for the first time, it can be stored at  $4^{\circ}\text{C}$  and should be



**Figure 2.** Dynamic range of Fluorocillin™ Green substrate. The wells of a microplate were coated with a saturating amount of streptavidin. After incubation and washes, biotinylated IgG was applied to the wells at the stated concentrations (100  $\mu$ L/well). Detection was achieved using 100  $\mu$ L of a 10  $\mu$ g/mL of a  $\beta$ -lactamase-conjugated goat anti-mouse IgG secondary antibody in the presence of 1  $\mu$ g of Fluorocillin™ Green substrate (with appropriate washes between steps). Fluorescence was read at 60 minutes without the addition of the Fluorocillin™ stop reagent. IgG was reliably detected in the range of 320 pg/mL to 640 ng/mL (over three orders of magnitude). Across the range of detection, the accuracy of the data obtained can be dramatically improved if curve fitting (e.g., Hill plot) is employed.

used only as long as it remains free of microbial growth. Component D should be reconstituted and stored according to the instructions in *Reagent Preparation*. The rest of the components of the kits should be returned to storage at  $\leq -20^{\circ}\text{C}$  after each use.

The Fluorocillin™ Green reagent is moisture and light sensitive. Once a vial of the Fluorocillin™ Green reagent is opened and the contents dissolved in DMSO, the reagent should be divided into aliquots promptly and frozen if not used immediately. **PROTECT THE Fluorocillin™ Green REAGENT FROM MOISTURE AND LIGHT.** Each kit provides sufficient reagents for approximately 500 ELISAs using a fluorescence microplate reader and reaction volumes of 100  $\mu$ L per assay.

## Before You Begin

### Materials required but not provided

- Single-channel and multi-channel pipettes (1  $\mu$ L to 1 mL range)
- Fluorescence microplate reader equipped with filters for  $\sim 495$  nm excitation and  $\sim 525$  nm emission (i.e., filters for fluorescein/FITC/Oregon Green dye)
- Deionized water ( $\text{dH}_2\text{O}$ )

### Reagent Preparation

Allow components to warm to room temperature before preparing the various stock solutions.

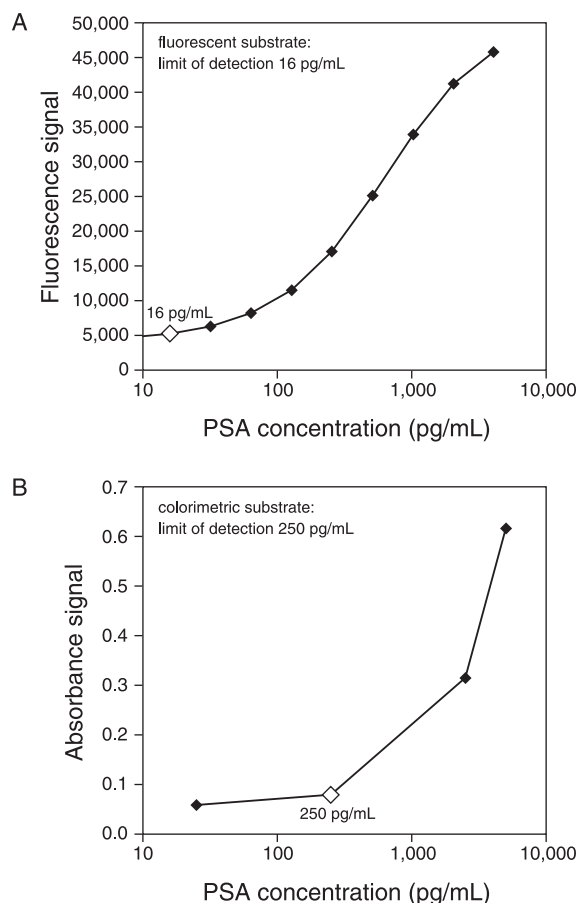
**1.1** Prepare 50 mL of 50 mM sodium bicarbonate buffer by adding 25 mL of 100 mM sodium bicarbonate (Component F) to 25 mL of  $\text{dH}_2\text{O}$ . This stock is sufficient for 500 assays.

**1.2** Prepare 500 mL of 1X PBS by adding 50 mL of 10X PBS (Component C) to 450 mL of  $\text{dH}_2\text{O}$ . This stock of 1X PBS will be used in the preparation of other buffers, as well as in the final Fluorocillin™ reaction.

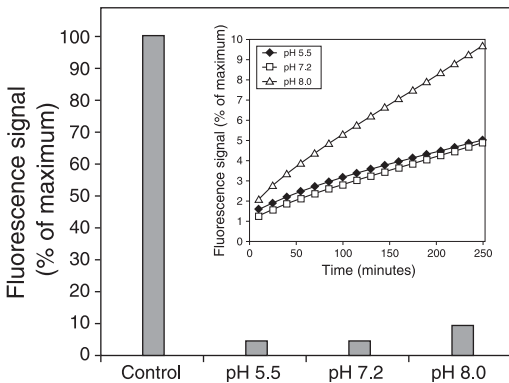
**1.3** Prepare 300 mL of 1X PBS-Tween by adding 300  $\mu$ L of Tween® 20 (Component H) to 300 mL of 1X PBS. Shake well to mix. This solution is sufficient for 100 assays using the protocol provided. Refrigeration is not required, but will not harm this solution.

**1.4** Prepare 100 mL of 1X PBS-BSA by adding 1.0 g of BSA (Component G) to 100 mL of 1X PBS. Dissolve completely. Refrigerate when not in use.

**1.5** Reconstitute the  $\beta$ -lactamase conjugate (Component D) by adding 0.5 mL of  $\text{dH}_2\text{O}$  that includes 2 mM sodium azide directly



**Figure 3.** Sensitivity of Fluorocillin™ Green fluorogenic assay vs. colorimetric assay. For the fluorogenic assay, 100  $\mu$ L/well of a 10  $\mu$ g/mL solution of anti-prostate specific antigen (PSA) antibody was applied to microplate wells. After incubation and washes, a dilution series of purified PSA was applied, and detection was achieved using 100  $\mu$ L of a 10  $\mu$ g/mL of an anti-PSA primary antibody and 100  $\mu$ L of a 10  $\mu$ g/mL of a  $\beta$ -lactamase-conjugated secondary antibody in the presence of 1  $\mu$ g Fluorocillin™ Green substrate (with appropriate washes between steps). The Z-statistic<sup>1</sup> gives a lower limit of detection of PSA in this assay of 16 pg/mL. For the colorimetric assay, a commercially available kit for the detection of PSA in human serum or plasma was used according to the manufacturer's instructions. The assay was performed using the PSA standards provided in the kit. The capture antibody was supplied preimmobilized in the wells, and detection was achieved using an HRP-labeled detection antibody and tetramethyl benzidine (TMB) substrate. The reaction was stopped after 15 minutes using the stop solution provided in the kit and the absorbance was read at 450 nm. The Z-statistic gives a lower limit of detection of PSA in this assay of 250 pg/mL.



**Figure 4.** Background of Fluorocillin™ Green substrate over time as a percentage of maximum signal. Fluorocillin™ Green substrate was prepared at a concentration of 1 µg/mL in three different buffers: MES at pH 5.5, PBS at pH 7.2, and Tris at pH 8.0. The substrate solutions were applied to the wells of a microplate, the plate was protected from light, and fluorescence values were gathered at time points from 10–250 minutes. The bar graph shows the percentage of maximum signal given by each substrate solution after 250 minutes. The line graph (inset) shows the background signal given by each substrate solution as a function of time. Maximum signal was determined by the fluorescence value obtained when 10 µL of a 100 µg/mL solution of β-lactamase was added to control wells. Each point is the mean of 12 replicates.

to the vial, to create a 1 mg/mL stock solution; this should be stored at 4°C. Alternatively reconstitute β-lactamase conjugate by adding 0.5 mL of a solution that is 50% dH<sub>2</sub>O, 50% glycerol (v/v) and contains 2 mM sodium azide; the 50% glycerol stock should be stored at ≤-20°C.

**1.6** Shortly before use, dilute a sufficient amount of the β-lactamase goat anti-mouse- or anti-rabbit-IgG stock solution to 10 µg/mL in 1X PBS-BSA. For example, 100 µL of the 1 mg/mL stock solution diluted into 10 mL of PBS-BSA is sufficient for approximately 100 assays. Note that this concentration of β-lactamase goat anti-mouse- or anti-rabbit-IgG is recommended as an initial concentration to try; the optimal concentration for each particular application may need to be determined empirically.

## Protocol for Sandwich ELISA

The following protocol describes a typical sandwich ELISA (see Figure 5 for schematic diagram of this method). Skip to the next section if you wish to do a direct ELISA. A sandwich ELISA is more sensitive than a direct ELISA; when the detection antibody is polyclonal, the sensitivity is greatest. However, a sandwich ELISA requires both a capture and detection antibody, and there are more steps involved in the procedure. This protocol is written as a convenience for the user and may be replaced with any standard sandwich ELISA protocol at the user's discretion. However, steps 2.10 – 2.12 of this protocol are specific to the β-lactamase enzyme/substrate system.

**2.1** Using 50 mM sodium bicarbonate (prepared in step 1.1), prepare 10 mL of a 10 µg/mL solution of the desired capture antibody and aliquot 100 µL of this solution into each well of the microplate (Component I). The capture antibody should preferably be a monoclonal in order to prevent epitope blocking. Incubate for at least four hours at room temperature, or overnight at 4°C (preferred) (note A).

**2.2** Drain plate and wash wells three times with 200 µL/well of 1X PBS-Tween (prepared in step 1.3), draining after the final wash.

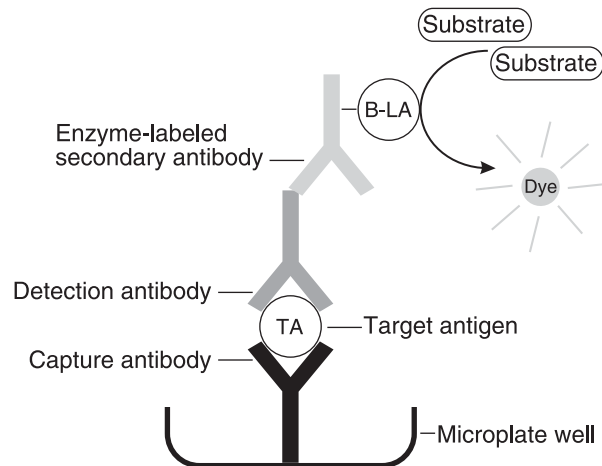
**2.3** Add 200 µL/well of 1X PBS-BSA (prepared in step 1.4). Block the plate by incubating for at least four hours at room temperature, or overnight at 4°C (preferred).

**2.4** Drain plate and wash wells three times with 200 µL/well of 1X PBS-Tween, draining after the final wash.

**2.5** Prepare serial dilutions of the antigen standard in 0.1X PBS-BSA (prepared by diluting the 1X PBS-BSA ten-fold in 1X PBS). Add 100 µL/well of each dilution across a row or column of the microplate, leaving at least one well as a no-antigen control. To the other wells of the microplate, add 100 µL/well of samples with unknown antigen concentrations. Incubate the plate for one hour at room temperature. **UNKNOWN ANTIGEN CONCENTRATIONS CAN ONLY BE QUANTIFIED IF THEY ARE WITHIN THE RANGE OF THE STANDARD CURVE. DO NOT EXTRAPOLATE OUTSIDE OF THE STANDARD CURVE.**

**2.6** Drain plate and wash wells three times with 200 µL/well of 1X PBS-Tween, draining after the final wash.

**2.7** Add 100 µL of diluted detection antibody (anti-antigen) to each well. This antibody needs to be different than the capture antibody used in step 2.1 and also able to react with the β-lactamase conjugate provided in this kit.



**Figure 5.** Schematic diagram of the sandwich ELISA method. The microplate is coated with a capture antibody that has reactivity to the target antigen but has no reactivity to the detection antibody or the enzyme-labeled secondary antibody. A solution containing the target antigen is applied to the plate. A portion of the target antigen is retained by the capture antibody, and nonbinding components of the solution are washed away. A solution containing the detection antibody is applied to the plate. The detection antibody is specific for the target antigen, and any detection antibody not bound to the target antigen is washed away. The β-lactamase-labeled secondary antibody binds to the detection antibody and cleaves the substrate, Fluorocillin™ Green reagent, producing a fluorescent signal. The target antigen described in this assay is “sandwiched” between two antibodies, hence the name “sandwich ELISA.” This is in contrast to direct-capture assays where the plate is coated with the target antigen. Sandwich ELISAs are generally more sensitive than direct-capture ELISAs because of the amplification of signal that arises from the presence of the capture antibody.

**2.8** Incubate at room temperature for 30 minutes, protected from light.

**2.9** Drain plate and wash wells three times with 200  $\mu\text{L}$ /well of 1X PBS-Tween, draining after the final wash.

**2.10** Add 100  $\mu\text{L}$  of the diluted  $\beta$ -lactamase conjugate (prepared in step 1.6) to each well.

**2.11** Incubate at room temperature for 30 minutes, protected from light.

**2.12** Drain plate and wash wells three times with 200  $\mu\text{L}$ /well of 1X PBS-Tween (note **B**), draining after the final wash.

**2.13** Proceed to  *$\beta$ -Lactamase Assay*, below.

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## **Protocol for Direct-Capture ELISA**

The following protocol describes a typical direct ELISA, i.e., an ELISA without the capture antibody. The previous section describes the protocol for a sandwich ELISA. A direct ELISA is less sensitive than a sandwich ELISA, but the procedure requires fewer steps. This protocol is written as a convenience for the user and may be replaced with any standard direct ELISA protocol at the user's discretion. However, steps 3.8 – 3.10 of this protocol are specific to the  $\beta$ -lactamase enzyme/substrate system.

**3.1** Using 50 mM sodium bicarbonate (prepared in step 1.1), prepare serial dilutions of the antigen standard. Add 100  $\mu\text{L}$ /well of each dilution across a row or column of the microplate (Component I), leaving at least one well as a no-antigen control. To the other wells of the microplate, add 100  $\mu\text{L}$ /well of samples with unknown antigen concentrations. In a sandwich ELISA, the antigen is often diluted into a buffer containing some protein (such as BSA) as a stabilizer, but for the direct ELISA use only the bicarbonate buffer. Incubate for at least four hours at room temperature, or overnight at 4°C (preferred). UNKNOWN ANTIGEN CONCENTRATIONS CAN ONLY BE QUANTIFIED IF THEY ARE WITHIN THE RANGE OF THE STANDARD CURVE. DO NOT EXTRAPOLATE OUTSIDE OF THE STANDARD CURVE.

**3.2** Drain plate and wash wells three times with 200  $\mu\text{L}$ /well of 1X PBS-Tween (prepared in step 1.3), draining after the final wash.

**3.3** Add 200  $\mu\text{L}$ /well of 1X PBS-BSA (prepared in step 1.4). Block the plate by incubating for at least four hours at room temperature, or overnight at 4°C (preferred).

**3.4** Drain plate and wash wells three times with 200  $\mu\text{L}$ /well of 1X PBS-Tween, draining after the final wash.

**3.5** Add 100  $\mu\text{L}$  of diluted detection antibody (anti-antigen) to each well. This antibody needs to have reactivity to the  $\beta$ -lactamase conjugate provided in this kit.

**3.6** Incubate at room temperature for 30 minutes, protected from light.

**3.7** Drain plate and wash wells three times with 200  $\mu\text{L}$ /well of 1X PBS-Tween, draining after the final wash.

**3.8** Add 100  $\mu\text{L}$  of the diluted  $\beta$ -lactamase conjugate (prepared in step 1.6) to each well.

**3.9** Incubate at room temperature for 30 minutes, protected from light.

**3.10** Drain plate and wash wells three times with 200  $\mu\text{L}$ /well of 1X PBS-Tween (note **B**), draining after the final wash.

**3.11** Proceed to  *$\beta$ -Lactamase Assay*, below.

### ***$\beta$ -Lactamase Assay***

**4.1** Prepare a 0.90 mM stock solution of the Fluorocillin™ Green reagent: Allow one vial of the Fluorocillin™ Green reagent (Component A) and DMSO (Component B) to warm to room temperature. Just prior to use, dissolve the contents of one vial of Fluorocillin™ Green reagent (100  $\mu\text{g}$ ) in 100  $\mu\text{L}$  DMSO. Each vial of the Fluorocillin™ Green reagent is sufficient for approximately 100 assays, with a final reaction volume of 100  $\mu\text{L}$  per assay. Once prepared, this stock solution should be stored frozen at  $\leq -20^\circ\text{C}$ , protected from light.

**4.2** Optional: If using the Fluorocillin™ stop reagent, prepare a 2X working solution by dissolving one vial of Fluorocillin™ stop reagent (Component E) in 5 mL  $\text{dH}_2\text{O}$ . This 5 mL volume of 2X stop reagent is sufficient for approximately 100 assays. If you are planning on stopping the reaction by the addition of Fluorocillin™ stop reagent, it will be necessary to apply Fluorocillin™ Green reagent to each well at a 2X concentration.

**4.3** Prepare a working solution of 9.0  $\mu\text{M}$  Fluorocillin™ Green reagent by adding 100  $\mu\text{L}$  of the 0.90 mM Fluorocillin™ Green reagent stock solution (prepared in step 4.1) to 9.9 mL of 1X PBS (prepared in step 1.2). This 10 mL volume is sufficient for ~100 assays. Optional: If using the Fluorocillin™ stop reagent, prepare a 2X working solution of 18.0  $\mu\text{M}$  Fluorocillin™ Green reagent by adding 100  $\mu\text{L}$  of the Fluorocillin™ Green reagent stock solution to 4.9 mL of 1X PBS instead of 9.9 mL.

**4.4** Initiate the reactions by adding 100  $\mu\text{L}$  of the Fluorocillin™ Green reagent working solution to each drained microplate well containing the samples and controls (from step 2.12 or step 3.10). Optional: If you will be using the Fluorocillin™ stop reagent to stop the reaction, add 50  $\mu\text{L}$  of the 2X Fluorocillin™ Green reagent working solution to each microplate well.

**4.5** Incubate the reactions for 30 minutes or longer at room temperature, protected from light. Except when using the Fluorocillin™ stop solution, the reactions can be incubated up to 12 hours (note **C**).

**Note:** Addition of the Fluorocillin™ stop reagent abolishes  $\beta$ -lactamase enzyme activity but has no effect on the background hydrolysis of the substrate (see Figure 4).

**4.6** Optional: Stop the reaction by adding 50 µL of the 2X Fluorocillin™ stop reagent (prepared in step 4.2) to each microplate well, and read the plate within an hour. Keep the plate protected from light until it is read.

**4.7** β-lactamase activity can be detected fluorometrically using a fluorescence microplate reader set for excitation in the range of 490–500 nm and emission detection at ~525 nm (see Figure 1).

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## Notes

[A] Overnight deposition of capture antibody provides optimal detection. If desired, 8 hours of room temperature deposition can

be followed by overnight blocking of the plate with 1X PBS-BSA at 4°C.

[B] The stringency of the assay may be adjusted by washing more or fewer times with PBS-Tween, or by incubating or agitating PBS-Tween in the wells for a time during the wash steps.

[C] We recommend reading the plate at 30 minutes as a starting point for the reaction. This incubation time may require optimization for your assay. Reading earlier may provide better differentiation of data points at the high end of the assay; allowing a longer incubation time may increase the sensitivity obtained at the low end of the assay.

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## Reference

1. J Biomol Screen 4, 67–73 (1999).

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## Product List *Current prices may be obtained from our Web site or from our Customer Service Department.*

Cat #	Product Name	Unit Size
F33952	Fluorocillin™ Green 495/525 β-lactamase substrate *soluble product*	5 X 100 µg
G31567	goat anti-mouse IgG (H+L), β-lactamase TEM-1 conjugate *0.5 mg net protein*	0.5 mg
G31568	goat anti-rabbit IgG (H+L), β-lactamase TEM-1 conjugate *0.5 mg net protein*	0.5 mg
S33853	SensiFlex™ ELISA Development Kit for Mouse IgG *with Fluorocillin™ Green 495/525* *500 assays*	1 kit
S33854	SensiFlex™ ELISA Development Kit for Rabbit IgG *with Fluorocillin™ Green 495/525* *500 assays*	1 kit

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## Contact Information

Further information on Molecular Probes products, including product bibliographies, is available from your local distributor or directly from Molecular Probes. Customers in Europe, Africa and the Middle East should contact our office in Paisley, United Kingdom. All others should contact our Technical Service Department in Eugene, Oregon.

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### Molecular Probes, Inc.

29851 Willow Creek Road, Eugene, OR 97402  
Phone: (541) 465-8300 • Fax: (541) 335-0504

### Customer Service: 6:00 am to 4:30 pm (Pacific Time)

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Fax: (541) 335-0238 • [probestech@invitrogen.com](mailto:probestech@invitrogen.com)

### Invitrogen European Headquarters

Invitrogen, Ltd.  
3 Fountain Drive  
Inchinnan Business Park  
Paisley PA4 9RF, UK  
Phone: +44 (0) 141 814 6100 • Fax: +44 (0) 141 814 6260  
Email: [euroinfo@invitrogen.com](mailto:euroinfo@invitrogen.com)  
Technical Services: [eurotech@invitrogen.com](mailto:eurotech@invitrogen.com)

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