TABLE OF CONTENTS

1. KIT CONTENTS

Component	Composition	Amount	Storage Temp.	Individual Catalog no.
BACE1 (β-secretase)	50 mM Tris (pH 7.5), 10% Glycerol	2 × 5 U (see note below)	–80°C	P2947
Tb-Anti-Biotin Antibody	Terbium labeled anti-Biotin antibody in HEPES buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM HEPES pH 7.5)	25 µg (see note below)	–20°C	PV4745
Fluorscein-BACE1-Biotin Substrate	Fluorescein-EVNLDAEFK-biotin in TR-FRET dilution buffer	50 nmol	–20°C	PV4747
BACE1 Assay Buffer	50 mM Sodium Acetate (pH 4.5)	20 mL	20–30°C	P2988
BACE1 Assay Stop Buffer	800 mM Tris pH 8.0, 0.01% NP-40	20 mL	20–30°C	PV4744

Note: The volume and concentration of BACE1 enzyme and concentration of Tb-Anti-Biotin-Antibody will vary from lot to lot. The concentration of each reagent will be printed on each bottle.

2. MATERIALS REQUIRED BUT NOT SUPPLIED

The following materials are required but not supplied in the kit:

- A fluorescence plate reader with excitation capabilities at 340 nm and with the appropriate filter sets installed for detecting the fluorescent emission signals of terbium at 495 nm and fluorescein at 520 nm (see Section 4).
- Pipetting devices for 1–1000-µL volumes, suitable repeater pipettors, or multi-channel pipettors.
- Black, 384-well assay plates.
- 100% DMSO

3. INTRODUCTION

BACE1 (β-secretase) is a key enzyme involved in the production of amyloid β-peptides (Aβ) found in extracellular amyloid plaques of Alzheimer's disease (AD). In some cases, early onset familial AD can be attributed to a "Swedish" mutation in the amyloid precursor protein (APP), which dramatically enhances the cleavage of this protein by BACE1. This and other genetic and pathological evidence has led to therapeutic approaches that have focused on the inhibition of BACE1 and other APP-cleaving enzymes, such as gamma-secretase.

Invitrogen's LanthaScreen™ TR-FRET BACE1 components provide a sensitive and robust method for high-throughput screening of potential inhibitors of β-secretase. The kit uses a terbium-labeled anti-biotin antibody and a fluorescein-BACE1-biotin substrate in a homogenous TR-FRET assay format.

3.1 Principle of FRET and TR-FRET

For screening libraries of compounds, TR-FRET is a recognized method for overcoming interference from compound autofluorescence or light scatter from precipitated compounds. The premise of a TR-FRET assay is the same as that of a standard FRET assay: when a suitable pair of fluorophores is brought within close proximity of one another, excitation of the first fluorophore (the donor) can result in energy transfer to the second fluorophore (the acceptor). This energy transfer is detected by an increase in the fluorescence emission of the acceptor and a decrease in the fluorescence emission of the donor. In HTS assays, FRET is often expressed as a ratio of the intensities of the acceptor and donor fluorophores. The ratiometric nature of such a value corrects for differences in assay volumes between wells and corrects for quenching effects due to colored compounds.

In contrast to standard FRET assays, TR-FRET assays use a long-lifetime lanthanide chelate as the donor species. Lanthanide chelates are unique in that their excited-state lifetime (the average time that the molecule spends in the excited state after accepting a photon) can be on the order of a millisecond or longer. This is in sharp contrast to the lifetime of common fluorophores used in standard FRET assays, which are typically in the nanosecond range. Because interference from autofluorescent compounds or scattered light is also on the nanosecond timescale, these factors can negatively impact standard FRET assays. To overcome these interferences, TR-FRET assays are performed by measuring FRET after a suitable delay, typically 50 to 100 microseconds after excitation by a flashlamp excitation source in a microtiter plate reader. This delay not only overcomes interference from background fluorescence or light scatter, but also avoids interference from direct excitation due to the non-instantaneous nature of the flashlamp excitation source.

The most common lanthanides used in TR-FRET assays for HTS are terbium and europium. Terbium offers unique advantages over europium when used as the donor species in a TR-FRET assay. In contrast to europium-based systems that employ APC as the acceptor, terbium-based TR-FRET assays can use common fluorophores such as fluorescein as the acceptor. Because it is straightforward (and inexpensive) to label a molecule such as a peptide with fluorescein, directly labeled molecules may be used in terbium-based TR-FRET assays, rather than biotinylated molecules that must then be indirectly labeled via streptavidin-mediated recruitment of APC. The use of directly labeled molecules in a terbium-based TR-FRET assay reduces costs, improves kinetics, avoids

problems due to steric interactions involving large APC conjugates, and simplifies assay development since there are fewer independent variables requiring optimization in a directly labeled system.

4. INSTRUMENT SETTINGS

General Settings:

Excitation	340 nm filter (30 nm bandwidth)
Emission	520 nm filter (25 nm bandwidth)
Emission	490 or 495 nm filter (10 nm bandwidth)
Delay Time	100 µs
Integration Time	200 µs

The excitation and emission spectra of terbium and fluorescein are shown in Figure 1. As with other TR-FRET systems, the terbium donor is excited using a 340-nm excitation filter with a 30-nm bandwidth. However, the exact specifications of the excitation filter are not critical, and filters with similar specifications will work well. In general, excitation filters that work with europium-based TR-FRET systems will perform well with the LanthaScreen™ terbium chelates.

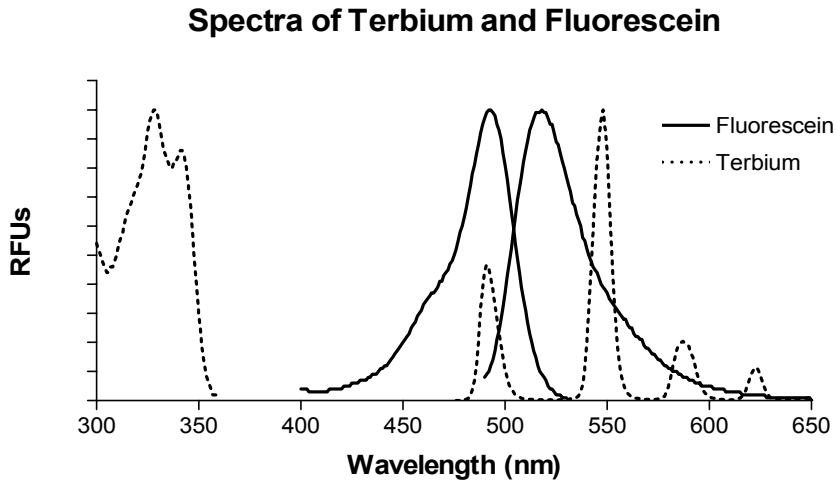


Figure 1: Excitation and Emission spectra of fluorescein and terbium.

As shown in the figure, the terbium emission spectrum is characterized by four sharp emission peaks, with silent regions between each peak. The first terbium emission peak (centered between approximately 485 and 505 nm) overlaps with the maximum excitation peak of fluorescein. Energy transfer to fluorescein is then measured in the silent region between the first two terbium emission peaks. Because it is important to measure energy transfer to fluorescein without interference from terbium, a filter centered at 520 nm with a 25 nm bandwidth is used for this purpose. The specifications of this filter are more critical than those of the excitation filter. In general, standard "fluorescein" filters may not be used, because such filters also pass light associated with the terbium spectra as well. The emission of fluorescein due to FRET is referenced (or "ratioed") to the emission of the first terbium peak, using a filter that isolates this peak. This is typically accomplished with a filter centered at 490 or 495 nm, with a 10 nm bandwidth. In general, a 490 nm filter will reduce the amount of fluorescein emission that "bleeds through" into this measurement, although instrument dichroic mirror choices (such as those on the Tecan Ultra instrument) may necessitate the use of a 495 nm filter. The effect on the quality of the resulting measurements is minimal in either case. Filters suitable for LanthaScreen™ assays are available from Chroma (www.chroma.com) as filter set PV001, or from other vendors. A LanthaScreen™ filter module for the BMG PheraStar is available direct from BMG Instruments.

Aside from filter choices, instrument settings are similar to the settings used with europium-based technologies. In general, the guidelines provided by the instrument manufacturer can be used as a starting point for optimization. A delay time of 100 µs followed by a 200-µs integration time is typical for a LanthaScreen™ assay. The number of flashes or measurements per well is highly instrument dependent and should be set as advised by your instrument manufacturer. In general, LanthaScreen™ assays can be run on any filter-based instrument capable of time-resolved FRET, such as the Tecan Ultra, BMGLabTech PHERAStar, Molecular Devices Analyst, or PerkinElmer Envision. LanthaScreen™ assays have also been performed successfully on the Tecan Safire^2 and Molecular Devices M5 monochromator-based instruments. Contact Invitrogen Technical Services for instrument-specific setup guidelines.

5. BACE1 TITRATION ASSAY

The procedure in this section describes a method for setting up a titration of BACE1 using Tb-Anti-Biotin Antibody. This assay includes a slight excess for dispensing; if your assay method requires a greater dead volume, adjust the volumes accordingly. The final reagent concentrations for the assay and the detection assay are described in the tables below.

Protease Assay: 15 µL

Component	Final Assay Concentration
Fluorescein-BACE1-biotin	200 nM

Detection Assay: 5 µL

Component	Final Assay Concentration
Tb-Anti-Biotin Antibody	5 nM
Tris (pH 8.0)	200 mM

Overall Assay: 20 µL

5.1 Preparing the Assay Components

Prepare 3X BACE1 Enzyme Dilution (30 U/mL)

Note: The BACE1 enzyme should be thawed on ice.

Note: The concentration of BACE1 enzyme varies from lot to lot, and is printed on the bottle supplied in your kit. Use this printed concentration to calculate the following dilution.

1. Calculate the amount of BACE1 needed to make 40 µL of a 30 U/mL solution:

$$\frac{30\ \text{U/mL} \times 40\ \mu\text{L}}{\text{Conc. of BACE1 in U/mL}} = \_\_\_\_\_\ \mu\text{l of stock BACE1 to add}$$

2. Calculate the amount of BACE1 Assay Buffer to add:

   40 µL – _____ µL (from step 1) = _____ µL of BACE1 Assay Buffer to add

3. In a sterile tube, add the volumes of BACE1 from Step 1 and BACE1 Assay Buffer from Step 2 and mix gently by hand or very brief vortexing. Do not overmix.

4. Store the prepared 3X BACE1 on ice until use.

Prepare 3X Solvent Control

Note: The 3X Solvent Control is used as the diluent for the compound of interest. For this BACE1 titration, it will be 3% DMSO.

1. Add 291 µL of BACE1 Assay Buffer to a sterile tube.

2. Add 9.0 µL of 100% DMSO.

3. Mix by vortexing briefly (≤30 seconds).
4. Store the prepared 3X Solvent Control at room temperature.

Prepare 3X BACE1 Substrate Mix (600 nM)

1. Add 296.4 µL of BACE1 Assay Buffer to a sterile tube.
2. Add 3.6 µL of 50 µM Fluorescein-BACE1-Biotin Substrate.
3. Mix the 3X BACE1 Substrate Mix by brief vortexing and store on ice.

Prepare 4X Tb-Anti-Biotin Antibody Stop Mix (20 nM)

Note: The concentration of Tb-Anti-Biotin Antibody varies slightly from lot to lot, and is printed on the bottle. Use this printed concentration to calculate the following dilution.

4. Prepare 10 µL of a 670 nM dilution of Tb-Anti-Biotin Antibody in BACE1 Assay Stop Buffer:

$$\frac{0.670\ \mu M \times 10\ \mu l}{\_\_\_\_\_\ mg/mL\ Tb\text{-}Anti\text{-}Biotin\ Antibody \times 6.7\ \mu M} = \_\_\_\_\_\ \mu l\ of\ stock\ Tb\text{-}Anti\text{-}Biotin\ Antibody$$

10 µl – _____µl of stock = _____µl of BACE1 Assay Stop Buffer to add

5. In a sterile tube, add the volumes of Tb-Anti-Biotin Antibody and BACE1 Assay Stop Buffer specified above and mix by vortexing briefly.
6. Transfer 291 µL of BACE1 Assay Stop Buffer to a tube.
7. Add 9.0 µL of 670 nM Tb-Anti-Biotin Antibody to prepare a 20 nM working stock solution.
8. Mix the 4X Tb-Anti-Biotin Antibody Stop Mix by brief vortexing and store on ice.

5.2 Plate Layout

Prepare a dilution series of BACE1 in triplicate in a black-bottomed 384-well plate as shown below. The BACE1 concentrations listed in columns 1–3 are based on a 15-µL assay (*i.e.,* before the addition of Stop Mix)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	10																							
B	5																							
C	2.5																							
D	1.3																							
E	0.63																							
F	0.31																							
G	0.16																							
H	7.8E-02																							
I	3.9E-02																							
J	2.0E-02																							
K	9.8E-03																							
L	4.9E-03																							
M	2.4E-03																							
N	1.2E-03																							
O	6.1E-04																							
P	0																							

[BACE] (U/mL)

5.3 Running the Assay

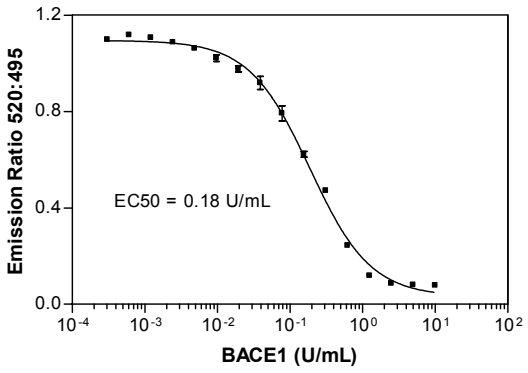
Add the prepared mixtures from Section 5.1 to the plate as follows:

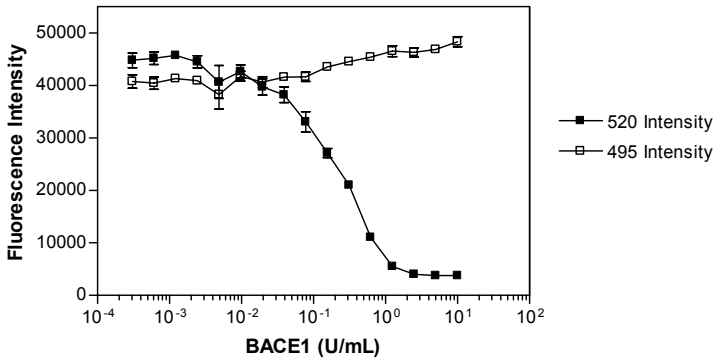
1. Add 10 µL 3X BACE1 to wells A1-A3.
2. Add 5 µL BACE1 Assay Buffer to wells B1-P3.
3. Transfer 5 µL from each well in row A into the corresponding well in row B, 5 µL from B into C, and so on through row O. Discard the extra 5 µL.
4. Add 5 µL 3X Solvent Control to each well in columns 1–3.
5. Add 5 µL 3X BACE1 Substrate Mix to each well in columns 1–3.
6. Mix the plate briefly on a plate shaker, then cover and incubate for 60 minutes at room temperature.
7. Add 5 µL 4X Tb-Anti-Biotin Antibody Stop Mix to each well in columns 1–3.
8. Mix the plate briefly, then cover and incubate for 60 minutes at room temperature.
9. Read the plate using a fluorescence plate reader (see Section 4 for instrument settings).

5.4 Data Analysis

Calculate the TR-FRET ratio by dividing the emission signal at 520 nm by the emission signal at 495 nm. Graph the known inhibitor titration by plotting the emission ratio versus the log [BACE1 enzyme]. To determine the EC_{80} value, fit the data using an equation for a sigmoidal dose response (variable slope), as provided by GraphPad™ Prism® 4.0 or another comparable graphing program.

5.5 Representative Data





6. BACE1 ASSAY-INHIBITOR TITRATION

The procedure in this section describes a method for setting-up an inhibitor titration for BACE1 using Tb-Anti-Biotin Antibody. This assay includes a slight excess for dispensing; if your assay method requires a greater dead volume, adjust the volumes accordingly. The final reagent concentrations for the assay and the detection assay are described in the following tables.

Protease Assay: 15 µL

Component	Final Assay Concentration
BACE1	700 mU/mL
Fluorescein-BACE1-biotin	200 nM

Detection Assay: 5 µL

Component	Final Assay Concentration
Tb-Anti-Biotin Antibody	5 nM
Tris (pH 8.0)	200 mM

Overall Assay: 20 µL

6.1 Preparing the Assay Components

Prepare 3X BACE1 Enzyme Dilution (2.1 U/mL)

Note: The BACE1 enzyme should be thawed on ice.

Note: The concentration of BACE1 enzyme varies from lot to lot, and is printed on the bottle supplied in your kit. Use this printed concentration to calculate the following dilution.

1. Calculate the amount of BACE1 needed to make 40 µL of a 2.1 U/mL solution:

$$\frac{2.1\ \text{U/mL} \times 40\ \mu\text{L}}{\text{Conc. of BACE1 in U/mL}} = \_\_\_\_\_\ \mu\text{l of stock BACE1 to add}$$

2. Calculate the amount of BACE1 Assay Buffer to add:

   40 µL – _____ µL (from step 1) = _____ µL of BACE1 Assay Buffer to add

3. In a sterile tube, add the volumes of BACE1 from Step 1 and BACE1 Assay Buffer from Step 2 and mix gently by hand or very brief vortexing. Do not overmix.

4. Store on ice.

Prepare 3X Test Compound Diluent

Note: This diluent is used to dilute the compound of interest.

1. Add 485 µL of BACE1 Assay Buffer to a sterile tube.
2. Add 15.0 µL of 100% DMSO.
3. Mix by vortexing briefly.
4. Store at room temperature.

Prepare 3X Test Compound Stock

1. In a sterile tube, prepare a 30 µM stock solution of inhibitor test compound by combining the undiluted test compound with 3X Test Compound Diluent (prepared as above).
2. Store at room temperature until needed.

Prepare 3X BACE1-substrate Mix (600 nM)

1. Add 296.4 µL of BACE1 Assay Buffer to a sterile tube.
2. Add 3.6 µL of 50 µM Fluorescein-BACE1-Biotin Substrate.
3. Mix by vortexing briefly and store on ice.

Prepare 4X Tb-Anti-Biotin Antibody Stop Mix (20 nM)

Note: The concentration of Tb-Anti-Biotin Antibody varies slightly from lot to lot, and is printed on the bottle. Use this printed concentration to calculate the following dilution.

1. Prepare a 10 µL of a 670 nM dilution of Tb-Anti-Biotin Antibody in BACE1 Assay Stop Buffer:

$$\frac{0.670\ \mu M \times 10\ \mu l}{\_\_\_\_\_\ mg/mL\ Tb\text{-}Anti\text{-}Biotin\ Antibody \times 6.7\ \mu M} = \_\_\_\_\_\ \mu l\ of\ stock\ Tb\text{-}Anti\text{-}Biotin\ Antibody$$

$$10\ \mu l - \_\_\_\_\_\ \mu l\ of\ stock = \_\_\_\_\_\ \mu l\ of\ BACE1\ Assay\ Stop\ Buffer$$

2. In a sterile tube, add the volumes of Tb-Anti-Biotin Antibody and BACE1 Assay Stop Buffer specified above and mix by vortexing briefly.
3. Transfer 291 µL of BACE1 Assay Stop Buffer to a tube.
4. Add 9.0 µL of 670 nM Tb-Anti-Biotin Antibody to prepare a 20-nM working stock solution.
5. Mix by vortexing briefly and store on ice.

6.2 Plate Layout

Prepare a dilution series of inhibitor test compound in triplicate in a black-bottomed 384-well plate as shown below. The inhibitor concentrations listed in columns 1–3 are based on a 15-µL assay (*i.e.,* before the addition of Stop Mix).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	10000																							
B	5000																							
C	2500																							
D	1250																							
E	625																							
F	312.5																							
G	156.25																							
H	78.125																							
I	39.0625																							
J	19.5313																							
K	9.76563																							
L	4.88281																							
M	2.44141																							
N	1.2207																							
O	0.61035																							
P	0.30518																							

[Inhibitor] (nM)

6.3 Running the Assay

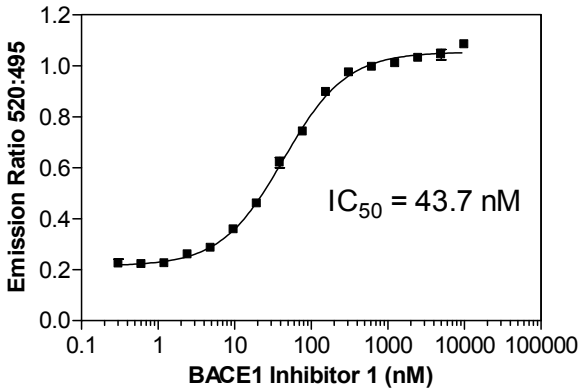
Add the prepared components from Section 6.1 to the plate as follows:

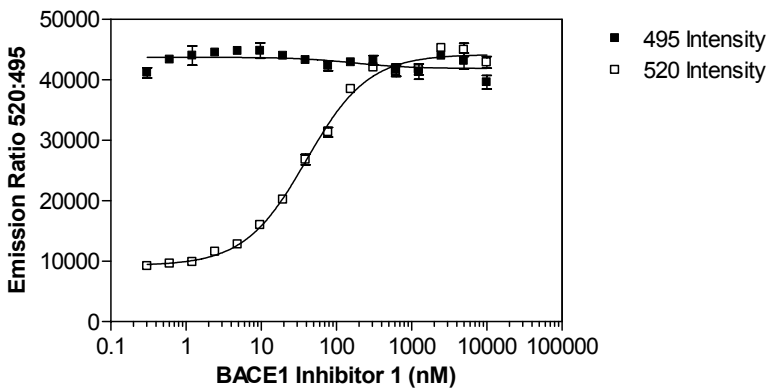
1. Add 10 µL 3X Test Compound Stock to wells A1-A3.
2. Add 5 µL 3X Test Compound Diluent to wells B1-P3.
3. Transfer 5 µL from each well in row A into the corresponding well in row B, 5 µL from B into C and so on through row O. Discard the extra 5 µL.
4. Add 5 µL 3X BACE1 Enzyme Dilution to each well in columns 1–3.
5. Add 5 µL 3X BACE1 Substrate Mix to each well in columns 1–3.
6. Mix the plate briefly on a plate shaker, then cover and incubate for 60 minutes at room temperature.
7. Add 5 µL 4X Tb-Anti-Biotin Antibody Stop Mix to each well in columns 1–3.
8. Mix the plate briefly on a plate shaker, then cover and incubate for 60 minutes at room temperature.
9. Read the plate using a fluorescence plate reader (see Section 4 for instrument settings).

6.4 Data Analysis

Calculate the TR-FRET ratio by dividing the emission signal at 520 nm by the emission signal at 495 nm. Graph the known inhibitor titration by plotting the emission ratio versus the log [BACE1 enzyme]. To determine the IC_{50} value, fit the data using an equation for a sigmoidal dose response (variable slope), as provided by GraphPad™ Prism® 4.0 or another comparable graphing program.

6.5 Representative Data





7. NOTICE TO PURCHASER

Limited Use Label License No. 176: Lanthanide Chelates

This product is the subject of U.S. Patent No. 5,622,821, 5,639,615 and 5,656,433 and foreign equivalents licensed exclusively to Invitrogen Corporation. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) to not transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Invitrogen Corporation will not assert a claim against the buyer of infringement of the above patents based upon the manufacture, use, or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Invitrogen is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Licensing Department, Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, California 92008. Phone (760) 603-7200. Fax (760) 602-6500.

The performance of this product is guaranteed for six months from the date of purchase if stored and handled properly.

Prism® is a registered trademark of GraphPad™ Software, Inc., San Diego, CA.