BellBrook Labs

FAR RED TR-FRET

TRANSCREENER®

ADP^2 TR-FRET Red Assay

Technical Manual

v073109

Transcreener® ADP² TR-FRET Red Assay

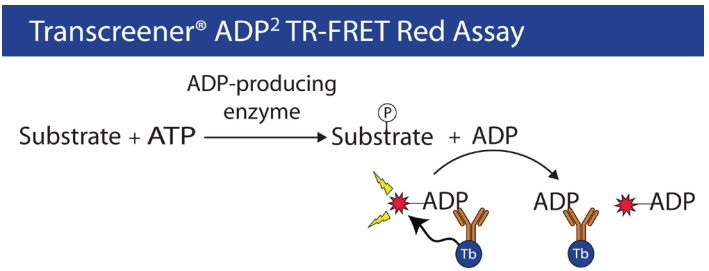
Instructions for Part Numbers 3011-1K and 3011-10K

1.0 Introduction

The Transcreener® HTS Assay platform overcomes the need for time-consuming, one-off assay development for individual members within a group transfer enzyme family by utilizing a single set of assay reagents that detect an invariant product. The generic nature of the Transcreener HTS Assay platform eliminates delays involved in assay development for new HTS targets, and greatly simplifies compound and inhibitor profiling across multiple target families.

The Transcreener® ADP² TR-FRET Red Assay is a competitive immunoassay for ADP with a far-red, time-resolved Förster-resonance-energy-transfer (TR-FRET) readout. It is designed specifically for HTS with a single addition mix-and-read format, reagent stability, and compatibility with commonly used multimode plate readers. Because it is highly selective for ADP, the assay can be used with any enzyme that converts ATP to ADP, including protein, lipid, and carbohydrate kinases, ATPases, DNA helicases, carboxylases and glutamine synthetase, regardless of what other substrates are used. The assay can accommodate ATP concentrations ranging from 0.1 to 1,000 µM and provides excellent data quality ($Z' \geq 0.7$) at low substrate conversion (typically 10% or less).

Figure 1. Transcreener® ADP² TR-FRET Red Assay Principle



The Transcreener ADP Detection Mixture comprises an ADP HiLyte647 Tracer bound to an ADP² Antibody-Tb conjugate. Excitation of the terbium complex in the UV range (ca. 330 nm) results in energy transfer to the tracer and emission at a higher wavelength (665nm) after a time delay. ADP produced by the target enzyme displaces the tracer which causes a decrease in TR-FRET. The time gated nature of the detection method largely eliminates interference that can result from prompt fluorescence

of test compounds. Use of a far red tracer further minimizes interference from fluorescent compounds and light scattering.

2.0 Transcreener® ADP² TR-FRET Red Assay Components

Store reagents at -80°C. Sufficient reagents are provided to complete up to 1,000 assays with 3011-1K and 10,000 assays with 3011-10K; the exact number is dependent on your enzyme reaction conditions.

ADP HiLyte647 Tracer
A 10 µM solution of ADP HiLyte647 Tracer is provided in 2 mM HEPES, pH 7.5 containing 0.01% Brij-35. The concentration of ADP HiLyte647 Tracer needed for an enzyme target is dependent upon the ATP concentration and buffer conditions in the enzyme reaction. In section 3.1, a linear relationship is illustrated between [ATP] in the enzyme reaction and the [ADP HiLyte647 Tracer] required for ADP detection. Sufficient tracer is included in the kit to complete 1,000 assays with 3011-1K and 10,000 assays with 3011-10K at an ATP concentration up to 100 µM ATP. Please contact BellBrook Labs for custom packaging for enzyme reactions using > 100 µM ATP.

ADP² Antibody-Terbium Conjugate, 800 nM
The ADP² Antibody-Tb, 800 nM is provided in HEPES buffered saline. The final antibody concentration in the reaction is 4 nM.

Stop & Detect Buffer C, 10X
The Stop & Detect Buffer C, 10X consists of 500 mM HEPES (pH 7.5), 200 mM EDTA, and 0.2% Brij-35. The Stop & Detect Buffer C components will stop Mg^{2+}-requiring enzyme reactions. To ensure the enzyme reaction is stopped completely, confirm that the EDTA concentration is at least equimolar to the magnesium ion concentration in the enzyme reaction. (Stop & Detect Buffer C is at a 0.5X concentration at the time of FRET measurement.)

5 mM ATP
ATP is a common reagent in many laboratories; however, it is imperative that a highly purified preparation be used for the Transcreener® ADP² Assay. If the ATP stock contains impurities, such as ADP, the assay window will be compromised. The ATP supplied in this kit can be used for the enzyme reaction and to create an ADP/ATP standard curve, if desired. For additional quantities, pricing and alternate supplier information , please contact BellBrook Labs.

5 mM ADP
ADP is used to create the ADP/ATP standard curve.

Materials Required but Not Provided

Ultrapure Water
Some deionized water systems are contaminated with nucleases that can degrade both nucleotide substrates and products, therefore reducing assay performance. Careful handling and use of ultrapure water eliminates this potential problem.

Enzyme
Transcreener® ADP² Assays are designed for use with purified enzyme preparations. Contaminating enzymes such as phosphatases or nucleotidases can produce background signal and reduce the assay window.

Enzyme Buffer Components
The enzyme buffer components supplied by the end-user include enzyme, buffer, acceptor substrate, $MgCl_2$ or $MnCl_2$, EGTA, Brij-35, and test compounds. Contact BellBrook Labs Technical Service for suppliers and catalog numbers.

Plate Reader
A microplate reader configured to measure time-resolved Förster-resonance-energy-transfer (TR-FRET) of the Tb:HiLyte647 donor:acceptor pair is required. This assay has been designed to provide high quality data on any HTS qualified instrument configured to measure TR-FRET using standard Europium or Terbium complexes measuring emissions at 615 nm and 665 nm. Validation was completed using BMG LABTECH's PHERAstar Plus (Ex337/Em620/Em665) and Perkin Elmer's Envision (Ex320/Em615/Em665). Contact BellBrook Labs Technical Service for additional information regarding instrument set-up and TR-FRET measurements.

Assay Plates
It is important to use assay plates that are entirely white with a non-binding surface. We recommend Corning® 384 plates (catalog #3673).

Liquid Handling Devices
Use liquid handling devices that can accurately dispense a minimum of 2.5 µL into 384-well plates.

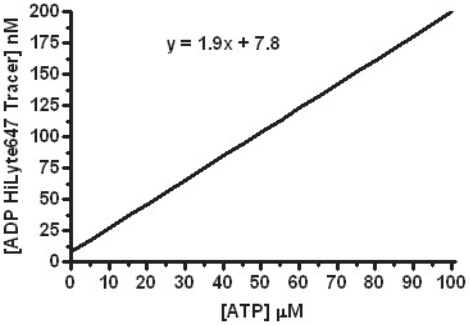
3.0 EZ Protocol
The Transcreener ADP² TR-FRET Red Assay is a universal biochemical assay designed for enzymes that produce ADP. It is designed around your initial ATP concentration and enzyme buffer conditions. There are four steps to complete:

1. Determine antibody concentration
2. Instrument set-up
3. Enzyme titration
4. Detect ADP

Completing these steps will provide optimal ADP detection results.

3.1 Determine the ADP HiLyte647 Tracer Concentration
The Transcreener ADP² TR-FRET Red Assay requires detection of ADP in the presence of excess ATP (assuming initial velocity enzyme reaction conditions) using an antibody with a finite selectivity for the diphosphate vs. the triphosphate. The concentration of ADP HiLyte647 tracer determines the total assay window and the ADP detection range, and the amount needed is primarily dependent upon the ATP concentration in the enzyme reaction.

Figure 2. Linear Relationship between [ATP] and [ADP Tracer]



As shown in Figure 2, the relationship between [ATP] and [ADP HiLyte647 Tracer] is linear. (Though shown for 0.1 µM to 100 µM ATP; the relationship is valid to 1,000 µM ATP.) Therefore the quantity of ADP HiLyte647 Tracer for enzyme reactions that use between 0.1 µM and 1,000 µM ATP can be determined using the equation y = mx + b, where x = [ATP] (µM) in the 10 µL enzyme reaction, y = [ADP HiLyte647 Tracer] (nM) in the 10 µL of 1X ADP Detection Mixture, m (slope) = 1.9, and b (y-intercept) = 7.8. We recommend a final volume of 20 µL.

For example, if you are using 3µM ATP in a 10 µl enzyme reaction, the optimal ADP HiLyte Tracer concentration in the 1X ADP Detection Mixture (assuming 10 µL of ADP Detection Mixture added to each 10 µL enzyme reaction) would be [1.9 x 3] + 7.8 = 13.6 nM.

Determining your ADP HiLyte647 Tracer concentration using this equation will provide excellent results for most assay conditions. If it does not provide the results you require, please refer to the Appendix for instructions on further optimization of the ADP 647HiLyte Tracer concentration in the buffer system ideal for your enzyme target.

3.2. Instrument Set-up

Becoming familiar with ideal instrument settings for fluorescence polarization is essential to the success of the Transcreener ADP^2 TR-FRET Red Assay.

Verify Instrument Measures TR-FRET

Ensure the instrument is capable of measuring TR-FRET (not simply fluorescence intensity) of the terbium:HiLyte647 TR-FRET pair ($Ex_{320}/Em_{615}/Em_{665}$). Please call BellBrook Labs Technical Service if you have questions about settings and filter sets for a specific instrument.

Define the Maximum TR-FRET Window for Your Instrument

Measuring high (0% ATP conversion) and low (100% ATP conversion) FRET will define the maximum assay window of your specific instrument. Prepare High and Low FRET Mixtures below in quantities sufficient to perform at least 6 replicates for each condition.

High FRET Mixture
Prepare a solution containing: 4 nM ADP[2] Antibody-Tb, 0.5X Stop & Detect Buffer C, ADP HiLyte647 Tracer, and ATP. Use ATP at half the concentration present in your enzyme reaction and ADP HiLyte647 Tracer at half the concentration calculated using the equation in Figure 2. This mimics the 2-fold dilution when adding an equal volume of detection mixture to an enzyme reaction.

Low FRET Mixture
Prepare the same mix as used for High FRET Mixture substituting ADP for ATP.

Measure the Time-Resolved Förster-Resonance-Energy-Transfer
Calculate the Z'-Factor using the equation below; values greater than 0.7 are acceptable. Contact BellBrook Labs Technical Service for assistance if your calculated Z'-Factor is less than 0.7.

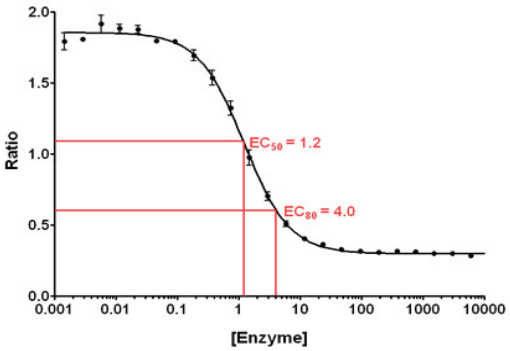
$Z'=1-[(3*SD_{\text{High FRET Mixture}}] + 3*SD_{\text{Low FRET Mixture}})/|[\text{mean of High FRET Mixture ratio 665/615}]- [\text{mean of Low FRET Mixture ratio 665/615}]|$

3.3 Enzyme Titration
An enzyme titration is performed to identify the optimal enzyme concentration for the Transcreener ADP[2] TR-FRET Red Assay. Use enzyme buffer conditions, substrate, and ATP concentrations that are optimal for your target enzyme and ADP HiLyte647 Tracer concentration calculated as described in Figure 2. If a compound screen is planned, you should include the library solvent at its final assay concentration. We routinely use enzyme buffer containing 35 mM HEPES (pH 7.5), 4 mM MgCl2, 2mM EGTA, 1% DMSO (test compound solvent), 0.015% Brij-35 and ATP. Run your enzymatic reaction at its requisite temperature and time period.

Enzyme Titration Steps
To achieve the most robust assay and a large signal, the quantity of enzyme required to produce a 50% - 80% change in polarization signal is ideal (EC_{50} to EC_{80}) for screening of large compound libraries and generating inhibitor dose response curves. To determine the EC_{80} [enzyme] use the equation below.

$$EC_{80} = (80/(100-80))^{(1/\text{hillslope})} * EC_{50}$$

Figure 3. Enzyme Titration Graph



Enzyme Assay Controls
The enzyme reaction controls define the limits of the enzyme assay.

0% ATP Conversion Control
This control consists of the ADP Detection Mixture, the enzyme reaction components (without enzyme), and 100% ATP (0% ADP). This control defines the upper limit of the assay window.

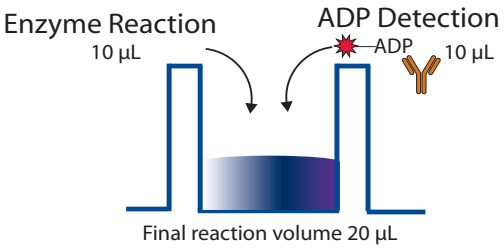
100% ATP Conversion Control
This control consists of the ADP Detection Mixture, the enzyme reaction components (without enzyme) and 100% ADP (0% ATP). This control defines the lower limit of the assay window.

Without Nucleotide Control
To verify that the enzyme does not interfere with the detection module, perform an enzyme titration in the absence of nucleotide (i.e. ATP) or acceptor substrate.

ADP/ATP Standard Curve
Although optional, an ADP/ATP standard curve can be useful to ensure day to day reproducibility and that the assay conditions were performed using initial rates, in addition to being used to calculate inhibitor IC_{50} values. See Appendix for a description of how to run the standard curve.

3.4 ADP Detection
The detection protocol is a single step as shown in Figure 4. 10 µL ADP Detection Mixture is added to the 10 µL enzyme reaction then mixed and incubated for 1 hour. The enzyme reaction components (including ATP) and the ADP Detection Mixture are 0.5X in the final 20 µL.

Figure 4. Detection Protocol



Enzyme Reaction (10 µL)
Add the enzyme reaction mixture to test compounds and mix on plate shaker. Start the reaction by adding ATP and mix. Incubate at temperature and time ideal for enzyme target before addition of the ADP Detection Mixture.

ADP Detection Mixture (10 µL)

The 1X ADP Detection Mixture is prepared by adding ADP^2 Antibody-Tb and ADP HiLyte647 Tracer to Stop & Detect Buffer C. Final concentrations should be 8nM ADP^2 Antibody-Tb Tracer, 1X Stop & Detect Buffer C, and the tracer concentration calculated using the equation in Figure 2. Add an equal volume of 1X ADP Detection Mixture to the enzyme reaction and mix using a plate shaker. Incubate at room temperature (20-25°C) for 1 hour, and measure TR-FRET.

ADP Detection Controls

This control is used to verify background fluorescent levels are negligible and, if required, for subtraction of background signal.

Background Control

This control contains 0.5X enzyme reaction conditions and Stop and Detect Buffer C.

Endpoint Assay

The Transcreener ADP^2 TR-FRET Red Assay is designed for endpoint readout. The Stop & Detect Buffer C contains EDTA to stop Mg^{++} dependent enzyme reactions by chelating available Mg^{++}.

Real-time Assay

The end-user may perform real-time experiments by adding ADP^2 Antibody-Tb and ADP HiLyte647 Tracer directly to an enzyme reaction. ADP detection equilibration time is greater than 30 minutes, making it difficult to quantify ADP produced during short term enzyme reactions, however this method can provide insight into optimal enzyme concentration and incubation time. Note that the optimal ADP HiLyte647 Tracer concentration may change when EDTA is omitted.

4.0 Reagent and Signal Stability

The Transcreener technology provides the end-user with a robust and stable assay method to detect ADP.

Signal Stability

The stability of the TR-FRET ratio assay window at 10% substrate conversion was determined after the addition of the ADP Detection Mixture to the standard samples. The ratio assay window at 10% substrate conversion (10 µM) remained constant (< 10% change) for at least 24 hours at room temperature (20-25°C). If plates are to be read the following day, steps should be taken to prevent evaporation.

ADP Detection Mixture Stability

The ADP Detection Mixture is stable for at least 8 hours at room temperature (20-25°C) before addition to the enzyme reaction (i.e. stored on the liquid handling deck).

Solvent Compatibility

The assay window at 10% substrate conversion (10 µM ATP) remains constant (< 10% change) when up to 10% DMSO, DMF, ethanol, acetonitrile, ethanol, or methanol are used in the enzyme reaction. Contact BellBrook Labs for further reagent compatibility information.

5.0 References

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6.0 Appendix

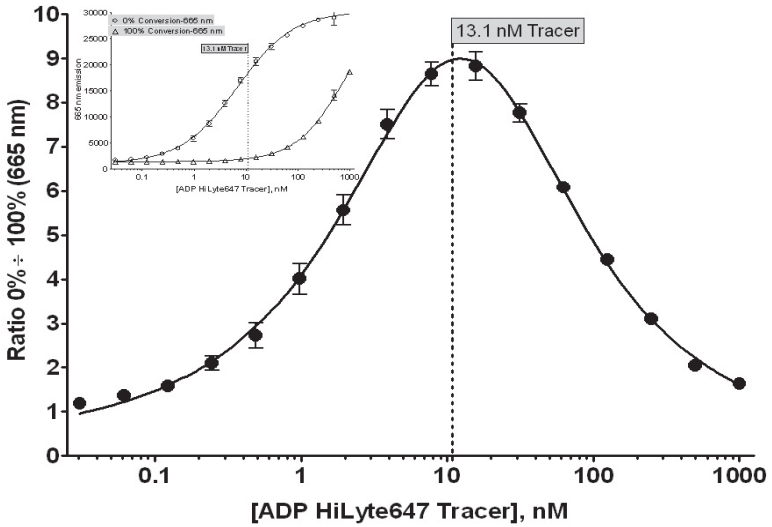
Optimize ADP HiLye647 Tracer Concentration

Using an ADP HiLyte647 Tracer concentration calculated using the equation in Figure 2 will produce excellent results for most users. If it does not produce the results you require, we recommend that you perform an ADP HiLyte647 Tracer titration in the buffer system ideal for your enzyme target in the presence of 0 and 100% converted nucleotide substrate. This will determine the optimal tracer concentration for your assay conditions. The nucleotide substrate concentration in the enzyme reaction generally determines the appropriate concentration of ADP HiLyte647 Tracer. We recommend using the concentration of tracer that produces the greatest 665 nm emission ratio between 0 and 100% converted nucleotide substrate for ATP concentrations up to 100 µM. Please contact BellBrook Labs for tracer optimization recommendations when using ATP at levels greater than 100 µM.

Prepare Reaction Mixes Containing 0 and 100% Converted Nucleotide Substrate
The following is performed in the presence of 0 and 100% converted nucleotide substrate (all ATP or ADP, respectively). Prepare 4 nM ADP[2] Antibody-Tb in a solution containing both 0.5X Enzyme Reaction Conditions and Stop & Detect Buffer C with and without 1 µM ADP HiLyte647 Tracer. Dispense 20 µL of mixture (with tracer) into wells in column 1. Dispense 10 µL of the mixture (without tracer) across a 384-well plate (columns 2-24). Remove 10 µL from column 1 and serially titrate the contents across the plate (to column 24).

Plot the FRET ratio (665/615 nm) vs. log of ADP HiLyte647 Tracer Concentration and Calculate the ratio of signal at 0 and 100% Conversion
The optimal tracer quantity will produce the maximum ratio between FRET ratios at 0 and 100% conversion (Figure 5).

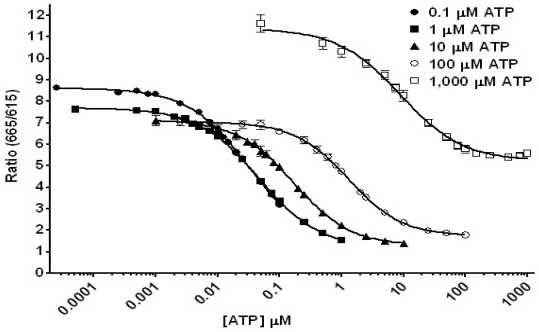
Figure 5. ADP HiLyte647 Tracer Titrations at 10 µM ATP

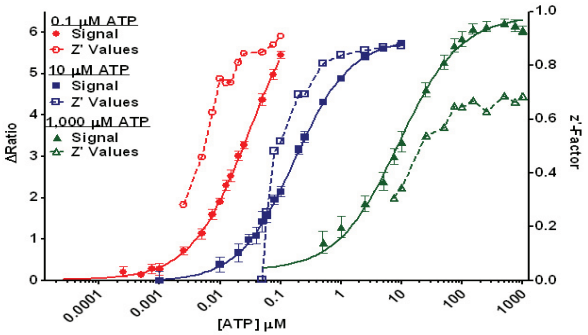


The final 20 µL assay volume consisted of 4 nM ADP[2] Antibody- Tb, 0.5X Stop & Detect Buffer C, and 0.5X enzyme reaction mixture (35 mM HEPES (pH 7.5), 2 mM $MgCl_2$, 1 mM EGTA, 0.5% DMSO, 0.01% Brij-35, and 5 µM ATP) and titrated ADP HiLyte647 Tracer (n=4).

ADP/ATP Standard Curve
The standard curve mimics an enzyme reaction (as ATP concentration decreases, ADP concentration increases); the adenine concentration remains constant. The ADP/ATP standard curve allows calculation of the concentration of ADP produced in the enzyme reaction and therefore the % ATP consumed (% ATP conversion). In this case a 16-point standard curve was prepared using concentrations of ADP and ATP corresponding to 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1.0, 2.0, 2.5, 5.0, 10.0, 25.0, 50.0, and 100% ATP conversion. Commonly 8 to 12 point standard curves are used.

Figure 6. ADP/ATP Standard Curves





A) Sample data for 0.1 µM, 1 µM, 10 µM, 100 µM, and 1000 µM ADP/ATP standard curves. The nucleotide concentration reflects the amount in the enzyme reaction, prior to the addition of the ADP Detection Mixture. Curves are obtained in a final 20 µL assay volume consisting of 35 mM HEPES (pH 7.5), 2 mM $MgCl_2$, 1 mM EGTA, 0.5% DMSO, 0.015% Brij-35, 10 mM EDTA, 4 nM ADP[2] Antibody-Tb, ADP/ATP standards, and ADP HiLyte647 Tracer (Tracer concentration from equation Figure 2) (n=24). The data are plotted as FRET ratio and change in ratio vs. log [ADP] using four-parameter nonlinear regression curve fitting. Alternatively a two phase exponential decay and nonlinear regression can be used to present the data (GraphPad Prism). B) Excellent Z′ values are obtained at < 10% ATP conversion for the range of ATP concentrations. Shown are 0.1 µM, 10 µM, and 1,000 µM ATP standard curves.

$$\Delta ratio = ratio_{initial\ [ATP]} - ratio_{sample}$$

and

$$Z' = 1 - [(3*SD_{initial\ [ATP]} + 3*SD_{sample})/|(ratio_{initial\ [ATP]} - ratio_{sample})|]$$

U.S. Patent 7,332,278, 7,355,010 and 7,378,505 issued. U.S. Patent Application Nos. 11/353,500, 11/958,515 and 11/958,965, U.S. Divisional Application 12/029,932, and International Patent Application Nos. PCT/US07/088111, European Application Nos. 04706975.2 and 05785285.7, Canadian Application 2,514,877, and Japanese Application 2006-503179 applied. 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. BellBrook Labs LLC 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, BellBrook Labs LLC 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, BellBrook Labs LLC, 5500 Nobel Drive, Suite 250, Madison, Wisconsin 53711. Phone (608)443-2400. Fax (608)441-2967.

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