BellBrook Labs

interchim

RED FI

TRANSCREENER®

ADP^2 FI Assay

Technical Manual

v060809

Transcreener® ADP² FI Assay

Instructions for Part Numbers 3013-A, 3013-1K and 3013-10K

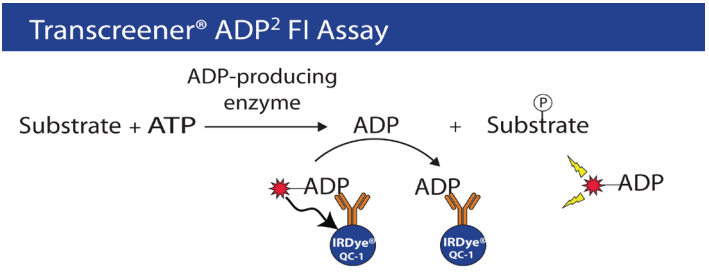
1.0 Introduction p.2
2.0 Assay Components p.3
3.0 EZ Protocol p.4
Determine Antibody Concentration p.4
Instrument Set-up p.5
Enzyme Titration p.6
ADP Detection p.7
4.0 Reagent and Signal Stability p.8
5.0 References p.9
6.0 Appendix p.9

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 fluorescent intensity (FI) Assay extends the Transcreener® platform for ADP detection by utilizing a simple fluorescent intensity output which can be used on both fluorescence readers typically found in academic and therapeutic research labs as well as more complex multimode plate readers more commonly used in core facilities and HTS labs. The Transcreener® ADP² FI Assay is a red, competitive fluorescence intensity (FI) assay based on the detection of ADP and therefore is compatible with any enzyme class that produces ADP, including protein, lipid, and carbohydrate kinases, ATPases, DNA helicases, carboxylases and glutamine synthetase. The Transcreener ADP² Assay is a simple one step homogenous detection assay, and is flexible with regard to ATP concentration (0.1 to 100 µM ATP). The assay provides excellent signal at low substrate conversion, with a Z' ≥ 0.7 at 2.5% ATP conversion using 1 µM ATP.

Figure 1. Transcreener® ADP² FI Assay Principle



The Transcreener ADP² FI Assay was developed to follow the progress of any enzyme that produces ADP. The Transcreener ADP Detection Mixture comprises a quenched ADP Alexa594 Tracer bound to the ADP² monoclonal antibody conjugated to an

IRDye® QC-1 quencher licensed from LI-COR®. The tracer is displaced by ADP, the invariant product generated during an enzyme reaction. The displaced tracer becomes un-quenched in solution leading to a positive increase in fluorescence intensity. Therefore, ADP production is proportional to an increase in fluorescence. The red tracer minimizes interference from fluorescent compounds and light scattering.

2.0 Transcreener® ADP² FI Assay Components

Store reagents at -20°C. Individual reagents tolerate 10 freeze-thaw cycles. Sufficient reagents are provided to complete up to 200 assays (96 well format) with 3013-A, 1,000 assays (384 well format) with 3013-1K and 10,000 assays (384 well format) with 3013-10K; the exact number is dependent on your enzyme reaction conditions.

ADP² Antibody-IRDye® QC-1

A concentrated mouse monoclonal ADP² Antibody-IRDye® QC-1 is provided in 100 mM KH_2PO_4 pH 8.5. Spin the tube briefly after thawing if there is an evident precipitate. The concentration of ADP² Antibody-IRDye® QC-1 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 [ADP² Antibody-IRDye® QC-1] required for ADP detection. Sufficient antibody is included in the kit to complete 200 assays (96 well format) with 3013-A, 1,000 assays (384 well format) with 3013-1K and 10,000 assays (384 well format) with 3013-10K at an ATP concentration up to 100 µM ATP. Please contact BellBrook Labs for custom packaging for enzyme reactions using > 100 µM ATP.

ADP Alexa594 Tracer, 800 nM

The ADP Alexa594 Tracer, 800 nM is provided in 2 mM HEPES, pH 7.5 containing 0.01% Brij-35. The final tracer concentration in the reaction is 4 nM.

Stop & Detect Buffer B, 10X

The Stop & Detect Buffer B, 10X consists of 200 mM HEPES (pH 7.5), 400 mM EDTA, and 0.2% Brij-35. The Stop & Detect Buffer B components will stop Mg^{2+}-requiring enzyme reactions and aid in the detection and stabilization of the FI signal. Stop & Detect Buffer B is at a 0.5X concentration at the time of fluorescent measurement. 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.

5 mM ATP

ATP is common to many laboratories, however, if the ATP stock contains impurities, such as ADP, the assay window will be compromised. ATP supplied in this kit can be used for the enzyme reaction and to create the ATP/ADP standard curve. Contact BellBrook Labs for alternate suppliers and catalog numbers.

5 mM ADP

ADP is not common to all laboratories and therefore is a supplied reagent. 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 Buffer Components
The enzyme buffer components supplied by the end-user include enzyme, enzyme buffer, acceptor substrate, $MgCl_2$ or $MnCl_2$, EGTA, Brij-35, and test compounds. Contact BellBrook Labs Technical Service for supplier recommendations.

Plate Reader
A multidetection microplate reader configured to measure fluorescence intensity of Alexa Fluor®594 is required. The Transcreener ADP² FI Assay has been successfully used on the Tecan Safire²™ and the Molecular Devices Spectramax M2. The assay should be fully compatible with comparable instrumentation. We will update this list as we expand our instrument testing. Contact BellBrook Labs Technical Service for additional information.

Assay Plates
It is important to use assay plates that are entirely black. We recommend Corning® 384 well plates (catalog #3676) and Greiner 96 well, half area plates (catalog# 675076).

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 FI Assay is a universal biochemical assay designed for enzymes that produce ADP. It is designed around your initial ATP concentration and enzyme buffer conditions. The first time you use this protocol there are four steps to complete:

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

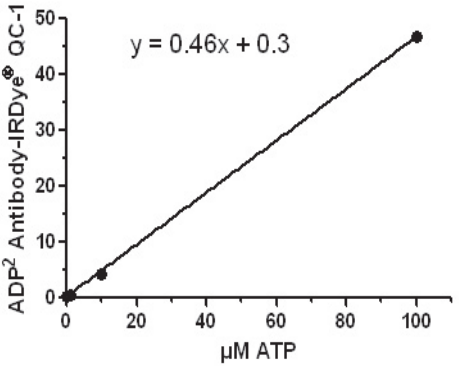
Completing these steps will provide optimal ADP detection results. The enzyme titration need not be repeated unless enzyme reaction conditions change. While instrument set-up is not affected by assay conditions, specific instrument settings may be adjusted for optimal results.

3.1 Determine ADP² Antibody-IRDye® QC-1 Concentration

The Transcreener ADP² FI 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 ADP² Antibody-IRDye® QC-1 determines the total assay window and the ADP detection range, and the amount needed is dependent upon the ATP concentration in the

enzyme reaction.

Figure 2 Linear Relationship between [ATP] and [ADP² Antibody-IRDye® QC-1]



As shown in Figure 2, the relationship between [ATP] and [ADP² Antibody-IRDye® QC-1] is linear. Therefore the quantity of antibody for enzyme reactions that use between 0.1 and 100 µM ATP can be determined using equation of line (y = mx + b; where x = [ATP] in the enzyme reaction, y = [ADP² Antibody-IRDye® QC-1] in the final volume, m (slope) = 0.46 and b (y-intercept) = 1). We recommend a final volume of 20 µL (384 well plate) or 50 µL (96 well plate). For ATP concentrations > 100 µM, use optimization protocol found in the Appendix.

For example, if you are using 3µM ATP in a 10 µl kinase reaction, the optimal antibody concentration in the final 20 µl assay mix (after addition of 10 µl of ADP Detection Mixture) would be [0.46 x 3] + 0.3 = 1.68 µg/ml.

Determining your ADP² Antibody-IRDye® QC-1 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 preparing an ADP² Antibody-IRDye® QC-1 titration in the buffer system ideal for your enzyme target.

3.2 Instrument Set-up

Becoming familiar with ideal instrument settings for fluorescence intensity is essential to the success of the Transcreener ADP² FI Assay.

Verify Instrument Measures Fluorescence Intensity

Becoming familiar with ideal instrument settings for fluorescence intensity is essential to the success of the Transcreener ADP² FI Assay. Make sure your plate reader measures fluorescence intensity. The optimal excitation/emission settings for the ADP Alexa594 Tracer are excitation 590 nM (10 nM bandwidth) and emission 617 nM (10 nM bandwidth). The ADP Alexa594 Tracer has been successfully used at excitations of (580-590) and emissions of (610-620) with bandwidths of 10 nM. Please contact Bellbrook Labs Technical Services for filter recommendations.

Define the Maximum Fluorescence Window for Your Instrument
Measuring low (tracer + antibody) and high (free tracer) relative fluorescence units (RFUs) will define the maximum assay window of your specific instrument.

Low RFU Mixture
Prepare 4 nM ADP Alexa594 Tracer/0.5X Stop & Detect Buffer B with your ADP[2] Antibody-IRDye® QC-1 concentration calculated using the equation of line in Figure 2.

High RFU Mixture
Prepare 4 nM ADP Alexa594 Tracer/0.5X Stop & Detect Buffer B without ADP[2] Antibody-IRDye® QC-1.

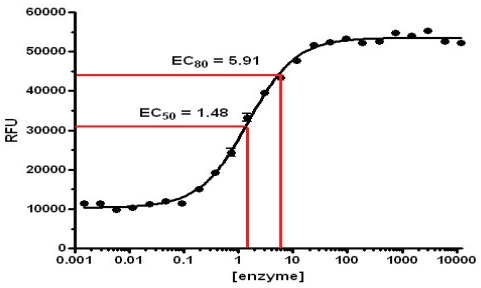
Measure the Fluorescence Intensity
The difference between the low and high RFU values will give you your maximum assay window. The values will differ depending on the units from your plate reader but the ratio of the RFUs from the (High RFU mixture)/(Low RFU mixture) ratio should be >5, if not, please call BellBrook Labs Technical Service.

3.3 Enzyme Titration
Choose enzyme buffer conditions and ADP Detection Mixture that are ideal for your enzyme target. Run your enzymatic reaction at its requisite temperature and optimal duration. Perform an enzyme titration to identify the optimal enzyme concentration for the Transcreener ADP[2] FI Assay. Quality control of the ADP[2] Antibody-IRDye® QC-1 is performed in 50 mM HEPES (pH 7.5), 4 mM $MgCl_2$, 2 mM EGTA, 1% DMSO (test compound solvent), 0.01% Brij-35 and varying ATP concentrations (0.1 to 100 µM).

Enzyme Titration Steps
Perform a serial enzyme titration using a buffer formulation that is ideal for your enzyme target. The ideal enzyme concentration should be determined using a serial titration in the presence of both substrate and ATP. To achieve the most robust assay and a large signal, the quantity of enzyme required to produce a 50%-80% change in fluorescence 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.

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

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 (see p8), the enzyme reaction components (without enzyme), and 100% ATP (0% ADP). This control defines the lower limit of the assay window.

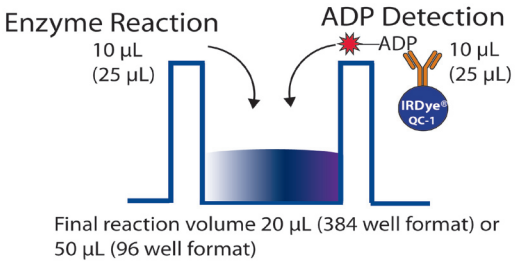
100% ATP Conversion Control
This control consists of the ADP Detection Mixture, the enzyme reaction components (without enzyme) and 100% ADP (0% ATP). This control defines the upper limit of the assay window.

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) and acceptor substrate.

ADP/ATP Standard Curve
Although optional, an ADP/ATP standard curve can be useful to ensure day to day reproducibility 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 prepare 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. Increase each volume to 25 µL if performing the assay in 96 well half-volume plates. The enzyme reaction components (including ATP) and the ADP Detection Mixture are 0.5X in the final 20 µL (384 well plate) or 50 µL (96 well plate) volume.

Figure 4. Protocol



Enzyme Reaction (10 µL or 25 µL)
Add the enzyme reaction mixture to test compounds and mix using a 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 or 25 µL)

The 1X ADP Detection Mixture is prepared by adding ADP^2 Antibody-IRDye® QC-1 (2X the concentration determined using the equation of line in Figure 2) and 8nM ADP Alexa594 Tracer in 1X Stop & Detect Buffer B. Add the 1X ADP Detection Mixture to the enzyme reaction and mix on plate shaker. Incubate at room temperature (20 - 25°C) for 1 hour, and measure fluorescence intensity.

ADP Detection Controls

These controls are used to calibrate the fluorescence intensity reader and are added to wells that do not contain enzyme.

Without Antibody (Free Tracer) Control

This sample contains the ADP Alexa594 Tracer without the ADP^2 Antibody-IRDye® QC-1. This determines the maximum RFU achievable.

Without Tracer Control

This sample contains the ADP^2 Antibody-IRDye® QC-1 without the ADP Alexa594 Tracer and is used as a sample blank for all wells. It is used as a 'buffer blank', but contains the same ADP^2 Antibody-IRDye® QC-1 concentration used in all wells.

Endpoint Assay

The Transcreener ADP^2 FI Assay is designed for endpoint readout. The Stop & Detect Buffer B contains EDTA to stabilize the signal. EDTA stops the enzyme reaction by chelating available $MgCl_2$, which is required for enzyme turnover.

Real-time Assay

The end-user may perform real-time experiments by substituting the Stop & Detect Buffer B, 10X (provided) with a detection buffer that does not contain EDTA. However, the equilibration time for the ADP tracer and ADP^2 Antibody-IRDye® QC-1 is greater than 15 minutes, making it difficult to quantify ADP produced during short term enzyme reactions. Note that the optimal ADP^2 Antibody-IRDye® QC-1 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 RFU assay window at 10% substrate conversion was determined after the addition of the ADP Detection Mixture to the standard samples. The RFU 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, they should first be sealed to prevent evaporation.

ADP Detection Mixture Stability

The ADP Detection Mixture is stable for at least 24 hours at room temperature (20-25°C) before adding to the enzyme reaction (i.e. stored on the liquid handling deck).

Solvent Compatibility

The RFU 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^2 Antibody-IRDye® QC-1 Concentration

The Transcreener ADP^2 FI 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^2 Antibody-IRDye® QC-1 determines the total assay window and the ADP detection range, and the amount needed is dependent upon the ATP concentration in the enzyme reaction. To produce the most sensitive and robust assay signal, it is necessary to perform an ADP^2 Antibody-IRDye® QC-1 titration in the buffer system ideal for your enzyme target.

To determine the optimal antibody concentration, titrate the ADP^2 Antibody-IRDye® QC-1 using the reaction conditions for your enzyme or drug target. The nucleotide substrate concentration in the enzyme reaction generally determines the appropriate concentration of ADP^2 Antibody-IRDye® QC-1. We recommend using the EC_{20} concentration of antibody.

Titrate ADP² Antibody-IRDye® QC-1 in 1X Stop & Detect Buffer B
Prepare 8 nM ADP Alexa594 Tracer in 1X Stop & Detect Buffer B with and without ADP² Antibody IRDye® QC-1 (2 mg/mL). Dispense 20 µL of mixture (with antibody) into wells in column 1. Dispense 10 µL of the mixture (without antibody) 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). If using a 96 well plate, adjust the volumes to 50 µL of mixture (with antibody) into wells in column 1 and 25 µL for the remaining dispensing steps.

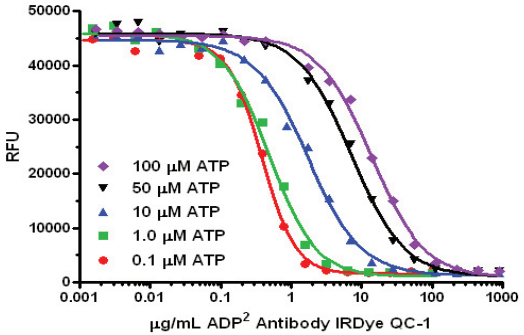
Add Enzyme Reaction Buffer (Containing ATP)
Prepare your enzyme reaction mixture (include substrate and ATP, but omit enzyme) and add 10 µL (25 µL for 96 well plates) to the titrated antibody. Mix the plate, equilibrate at room temperature (1 hour), and measure fluorescence intensity.

Plot RFU vs. log of ADP² Antibody-IRDye® QC-1 Concentration and Calculate the EC_{20}

The antibody concentration at the EC_{20} is often used as a good compromise between sensitivity and maximal assay window. The EC_{20} is determined by inputting the EC_{50} and hillslope values from a sigmoidal dose response curve fit into the equation below.

$$EC_{20} = (20/(100-20))^{(1/hillslope)} * EC_{50}$$

Figure 5. ADP² Antibody-IRDye® QC-1 Titrations at Various ATP Concentrations



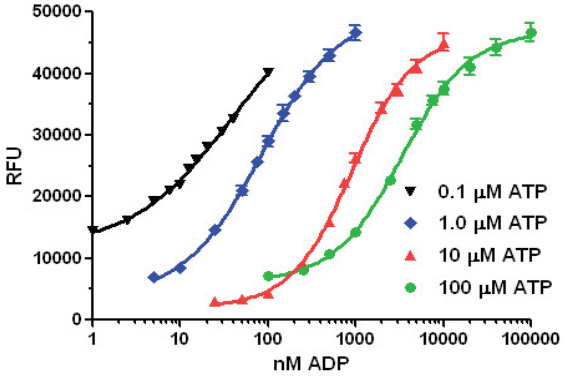
The final 20 µL assay volume consisted of 4 nM ADP Alexa594 Tracer , 0.5X Stop & Detect Buffer B, 0.5X enzyme reaction mixture (50 mM HEPES (pH 7.5), 2 mM $MgCl_2$, 1 mM EGTA, 0.5% DMSO, 0.01% Brij-35, and ATP) and ADP² Antibody-IRDye® QC-1 (n=3).

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). Prepare a twelve-point curve using concentrations of ADP and ATP corresponding to 0%, 2%,

4%, 6%, 8%, 10%, 15%, 20%, 30%, 40%, 60%, and 100% ATP conversion.

Figure 6. ADP/ATP Standard Curves

A)



B)

% ATP Conversion	0.1 µM ATP	1 µM ATP	10 µM ATP	100 µM ATP
0.5	nd	0.4	0.7	0.6
1	nd	0.6	0.8	0.8
2.5	0.3	0.8	0.9	0.9
5	0.7	0.8	0.9	0.9
10	0.7	0.9	0.9	0.9
100	0.9	0.8	0.9	0.9

A) Sample data for 0.1 µM, 1 µM, 10 µM, and 100 µM ADP/ATP standard curves. The nucleotide concentration reflects the amount in the enzyme reaction prior to the addition of the ADP Detection Mixture. 10 µL of reaction mix (50 mM HEPES pH7.5, 4 mM $MgCl_2$, 2 mM EGTA, 1% DMSO, 0.01% Brij, ADP/ATP standards) was added to 10 µL detection mix (ADP[2] Antibody IRDye QC-1, 8 nM ADP Alexa594 tracer, 1X Stop & Detect buffer B using ADP[2] Antibody IRDye QC-1 concentration determined from line in Figure 2 (n=24). The data are plotted as RFU vs log [ADP] using nonlinear regression curve fitting. B) Excellent Z' values are obtained.

$$Z' = 1 - [(3*SD_{(x\% \text{ conversion})}) + 3*SD_{(0\% \text{ conversion})})/|(RFU_{(x\% \text{ conversion})} - RFU_{(0\% \text{ conversion})}]$$

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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