1.0 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.

PanVera®'s BACE1 fluorescence resonance energy transfer (FRET) Assay Kit provides a sensitive and efficient method for screening potential BACE1 inhibitors. This kit uses purified baculovirus-expressed BACE1 and a new 'red' FRET peptide substrate based on the "Swedish" mutant. This kit contains enough reagents for 400 high-throughput kinetic or endpoint assays.

2.0 ASSAY THEORY

The BACE1 Assay Kit utilizes fluorescence resonance energy transfer (FRET) technology. Although FRET is commonly used to determine nanometer-scale distances of two molecules in a homogenous solution, it is used in this context to monitor cleavage of a peptide substrate. The principle of the BACE1 FRET assay is as follows: The peptide substrate is synthesized two fluorophores, a fluorescent donor [a rhodamine (Rh) derivative] and a proprietary quenching acceptor. The distance between these two groups has been selected so that upon light excitation, the donor (D) fluorescence energy is significantly quenched by the acceptor (A) through a quantum mechanical phenomenon known as resonance energy transfer (**Figure 1**). Upon cleavage by the protease, the fluorophore is separated from the quenching group, restoring the full fluorescence yield of the donor. Thus, a weakly fluorescent peptide substrate becomes highly fluorescent upon enzymatic cleavage; the increase in fluorescence is linearly related to the rate of proteolysis. FRET methods are widely used because they offer a homogenous and sensitive assay easily adapted for high-throughput screening (HTS).

Figure 1. General Principle of the BACE1 FRET Assay. The FRET protease substrate consists of a fluorescence donor on one end and a quenching acceptor on the other. The intrinsic fluorescence of the intact substrate is dramatically reduced because of intramolecular resonance energy transfer to the quenching group. Upon enzymatic cleavage, the energy transfer is disrupted, and the full quantum yield of the donor is restored. Enzyme activity is linearly related to the increase in fluorescence.

3.0 SAFETY PRECAUTIONS

Normal precautions exercised in handling laboratory reagents should be followed. All reagents in this kit are considered non-hazardous according to 29 CFR 1910.1200. The chemical, physical, and toxicological properties of these products may not, as yet, been thoroughly investigated. We recommend that you use gloves, lab coats, eye protection and a fume hood when working with any chemical reagents.

4.0 KIT COMPONENTS

4.1 Materials provided

Description	Composition	Amount	Part #
BACE1 (β-Secretase) Enzyme	50 mM Tris (pH 7.5), 10% glycerol	5 Units	P2947
BACE1 Substrate, 75 µM	Rh-EVNLDAEFK-Quencher, in 50 mM Ammonium bicarbonate	45 µL	P2986
BACE1 Stop Solution	2.5 M Sodium acetate	5 mL	P2987
BACE1 Assay Buffer	50 mM Sodium acetate (pH 4.5)	20 mL	P2988
BACE1 Product Standard, 25 µM	Rh-EVNL in 50 mM Ammonium bicarbonate	25 µL	P2989

4.2 Materials required but not supplied

- Black microwell plates (96- or 384-wells)
- Multiwell spectrofluorometer instrument capable of 530-545 nm excitation and 570-590 nm emission wavelengths
- Pipetting devices P20, P200, and P1000, suitable repeater pipetters, or multi-channel pipettors

5.0 STORAGE AND STABILITY

The BACE1 (β-Secretase) Enzyme (PanVera® Part No. P2947) should be stored at –80˚C. BACE1 Substrate (PanVera® Part No. P2986), and BACE1 Product Standard (PanVera® Part No. P2989) should be stored at -20˚C. Avoid subjecting these components to repeated freeze-thaw cycles. The BACE1 Assay Buffer (PanVera® Part No. P2988) and BACE1 Stop Solution (PanVera® Part No. P2987) can be thawed upon receipt and stored at room temperature.

6.0 BACE1 FRET ASSAY CONSIDERATIONS

This Assay Kit contains enough reagents to perform 400 assays in 30 µL (kinetic) or 40 µL (endpoint = 30 µL reaction plus 10 µL BACE1 Stop Solution) volumes. The amount of enzyme required per well has been predetermined to give approximately 10% conversion of substrate to product in 60 minutes at room temperature using a spectrofluorometer that can detect 1 nM rhodamine. These conditions result in a Z' >0.6. In constructing BACE1 inhibition curves, a dilution series of test compounds will be added to the reaction. The concentration of a test compound that results in half-maximum change in fluorescence units under these conditions equals the IC_{50} of the test compound.

- ***Enzyme Concentration:*** When using BACE1 from another source, please note that the units of activity may be defined differently. It is recommended that a kinetic assay be performed first with various enzyme dilutions to determine the linear range of the assay.
- ***Controls:*** It is suggested that the following controls be included for a kinetic assay: BACE1 Assay Buffer plus 250 nM BACE1 substrate (baseline); BACE1 Assay Buffer plus 250 nM BACE1 Substrate plus 250 nM BACE1 Product Standard (to represent 100% cleavage of substrate to product). For the endpoint assay include 10 µL BACE1 Stop Solution in all controls (See Appendix A).
- ***Incubation Time:*** If an extended incubation time is preferred (>60 minutes), use less enzyme per well to maintain approximately 10% conversion of substrate to product.
- ***Solvents:*** The BACE1 FRET assay can tolerate up to 10% DMSO and/or 1% MeOH in the standard protocol.
- ***Instrumentation:*** This assay was designed on an instrument that can detect 1 nM rhodamine. If a less sensitive spectrofluorometer is used for the assay, it may be necessary to double the substrate concentration per well to increase the dynamic range of the assay.

7.0 PROCEDURE

7.1 Reagent Preparation

7.1.1 3X BACE1 Enzyme (1.0 Unit/mL)

Dilute the provided BACE1 Enzyme (PanVera® Part No. P2947) with BACE1 Assay Buffer (PanVera® Part No. P2948) to make a 3X working solution (1 Unit/mL). Prepare at least 4.0 mL of this 3X BACE1 enzyme solution. The 3X BACE1 Enzyme solution can be added directly to the wells as described in the assay outline below.

Note: The activity of BACE1 Enzyme varies from lot to lot. This can affect the volume used to prepare the 3X solution.

7.1.2 3X Test Compound (Customer Supplied)

Dilute your compound of interest to a 3X concentration in BACE1 Assay Buffer. The concentration of DMSO at this 3X concentration cannot be greater than 30%, since this will compromise the quality of the assay.

7.1.3 3X BACE1 Substrate (750 nM)

The 75 µM BACE1 Substrate (PanVera® Part No. P2986) is at a 300X concentration for this assay. Dilute this stock into BACE1 Assay Buffer (PanVera® Part No. P2988) to make a 3X stock (750 nM). Add 40 µL of BACE1 Substrate to 3960 µL of BACE1 Assay Buffer. Make and store the 3X solution in an amber container since this is a photolabile molecule. This 3X Substrate is ready to add directly to the wells as described in the assay outline below.

Note: The 3X BACE Substrate solution should be prepared fresh and stored at +4˚C. For best results, use within 24 hours.

7.2 Assay Protocol

7.2.1 Kinetic Assay Steps 1-4, Endpoint Assay Steps 1-6 and Appendix I

1. Add 10 µL 3X BACE1 Substrate to 10 µL 3X Test Compound (or BACE1 Assay Buffer if preparing a control well).
2. Mix gently.
3. To start the reaction, add 10 µL of 3X BACE1 Enzyme.
4. Incubate 60 minutes at room temperature. Follow fluorescence in real-time to track kinetically.
5. Add 10 µL BACE1 Stop Buffer to stop the reaction. Once the stop solution has been added, the signal is stable for > 24 hours.
6. Read the fluorescence.

Note: We have successfully used 545 nm excitation (12 nm bandwidth) and 585 nm emission settings (12nm bandwidth) on a TECAN Safire. Other wavelengths may be optimal on other instruments.

8.0 SAMPLE DATA

8.1 Kinetic BACE1 FRET Assay Performed in the Absence of Inhibitor in a 384-well Format.

The standard assay protocol was designed to convert approximately 10% of substrate to product in one hour at room temperature. To represent 100% conversion, 250 nM peptide product (Rh-EVNL) was used as an internal reference. Substrate was added to the product to account for any fluorescence that was absorbed by the quencher groups.

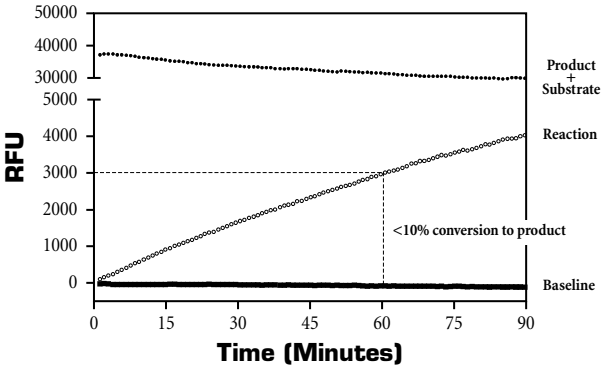

Figure 2. Kinetic BACE1 FRET Assay. The standard enzyme reaction contains 250 nM FRET substrate and 10 milliunits of BACE1 in 50 mM sodium acetate (pH 4.5) (○). Total volume is 30 µL. The reaction was performed at room temperature for 90 minutes on a TECAN Safire spectrofluorometer [545 nm excitation (12 nm bandwidth) and 585 nm emission filter (12 nm bandwidth)]. Complete conversion of the substrate to product is represented by the control reaction containing 250 nM BACE1 Substrate plus 250 nM product in BACE1 Assay Buffer (●). The baseline control contains substrate in the Assay Buffer alone (■). Error bars representing one standard deviation from the mean of three replicates were so small that they were indistinguishable from the data points.

8.2 Inhibition of BACE1 Activity in an Endpoint Assay

To demonstrate inhibition of BACE1 activity in the FRET assay, a Statine-derived inhibitor, Lys-Thr-Glu-Glu-Ile-Ser-Glu-Val-Asn-(Statine)-Val-Ala-Glu-Phe-OH, was serially diluted into the reactions. The IC_{50} was calculated using Prism® Software from GraphPad.

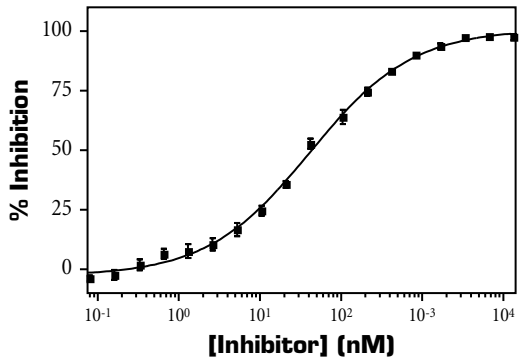

Figure 3. Inhibition of BACE1 by a Statine-derived Inhibitor. STA–200 (Enzyme Systems Products, Cat no. STA-200) (13 µM) was serially diluted 1:2 into a standard reaction mix containing 250 nM BACE1 Substrate and 10 milliunits of BACE1 Enzyme in BACE1 Assay Buffer [50 mM sodium acetate (pH 4.5)]. The reaction was stopped with BACE1 Stop Solution (2.5 M sodium acetate) after 60 minutes incubation at room temperature (total volume = 40 µL). Rhodamine fluorescence was monitored on a TECAN Safire spectrofluorometer ($Ex_{545\ nm}/Em_{585\ nm}$). Error bars represent one standard deviation from the mean of four samples.

9.0 REFERENCES

1. Vassar, R. *et al.* (1999) *Science* **286:**735-41.
2. Yan, R. *et al.* (1999) *Nature* **402:**533-7.
3. Sinha, S. *et al.* (1999) *Nature* **402:**537-40.
4. Hussain, *et al.* (1999) *Mol. Cell. Neurosci.***14:**419-27.
5. Citron, M. (2001) *Expert. Opin. Ther. Targets* **5:**341-3.

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

APPENDIX I

Reactions for the BACE1 Endpoint Assay.				
	Assay Reaction	Controls		
Reagents	BACE1 + Test Compound	Enzyme	No Enzyme	Product + Substrate†
BACE 1 Assay Buffer		10 µL	20 µL	10 µL
3X BACE1 Substrate (750 nM)	10 µL	10 µL	10 µL	10 µL
3X Test Compound	10 µL			
3X BACE1 Enzyme (1.0 U/mL)	10 µL	10 µL		
3X BACE1 Product Standard* (750 nM)				10 µL
BACE1 Stop Solution	10 µL	10 µL	10 µL	10 µL
Total Volume	40 µL	40 µL	40 µL	40 µL

* To prepare 3X BACE1 Product (750 nM), add 10 µL BACE1 Product Standard (25 µM, PanVera® Part No. P2989) to 320 µL BACE1 Assay Buffer (PanVera® Part No. P2988).

† BACE1 Substrate is added to the BACE1 Product Standard to account for fluorescence that is absorbed by the quenching group on intact substrate and cleaved product in the reaction. This is known as the inner filter effect.