New Wash Buffer

assay designs
SIMPLIFY YOUR SCIENCE®

*Correlate-EIA*™

# 12(S)-HETE

# Enzyme Immunoassay Kit

## Catalog No. 900-050

## 96 Well Kit

Table of Contents



**FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.**

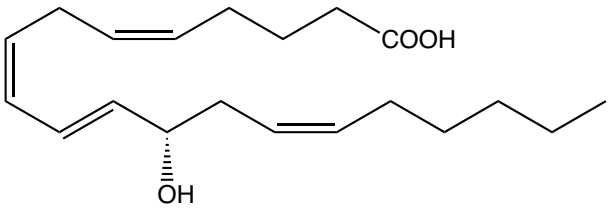
## Description

Assay Designs' Correlate-EIA™ 12(S)-Hydroxyeicosatetraenoic acid [12(S)-HETE] kit is a competitive immunoassay for the quantitative determination of 12(S)-HETE in biological fluids. Please read the complete kit insert before performing this assay. The kit uses a polyclonal antibody to 12(S)-HETE to bind, in a competitive manner, the 12(S)-HETE in the sample or an alkaline phosphatase molecule which has 12(S)-HETE covalently attached to it. After a simultaneous incubation at room temperature the excess reagents are washed away and substrate is added. After another incubation, the enzyme reaction is stopped and the yellow color generated read on a microplate reader at 405 nm. The intensity of the bound yellow color is inversely proportional to the concentration of 12(S)-HETE in either standards or samples. The measured optical density is used to calculate the concentration of 12(S)-HETE. For further explanation of the principles and practice of immunoassays please see the excellent books by Chard[1] or Tijssen[2].

## Introduction

12(S)-HETE is the stereospecific hydroxy product from the reduction of 12(S)-hydroperoxy tetraenoic eicosatetraenoic acid [12(S)-HpETE][3-4], which itself is a 12-lipoxygenase metabolite of arachidonic acid[5]. 12(S)-HETE has been shown to be chemotactic and chemokinetic for polymorphonuclear leukocytes[6] and vascular smooth cells[7]. It also acts as a second messenger in angiotensin-II induced aldosterone production[8]. Evidence also suggests that 12(S)-HETE is involved in suppressing renin production[9], stimulating insulin secretion by pancreatic tissue[10], inducing endothelial cell retraction and tumor cell adhesion[11].

**12(S)-HETE**




## Precautions

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

1. Some kit components contain azide, which may react with lead or copper plumbing. When disposing of reagents always flush with large volumes of water to prevent azide build-up.
2. Stop Solution is a solution of trisodium phosphate. This solution is caustic; care should be taken in use.
3. The activity of the alkaline phosphatase conjugate is dependent on the presence of $Mg^{2+}$ and $Zn^{2+}$ ions. The activity of the conjugate is affected by concentrations of chelators (>10 mM) such as EDTA and EGTA.
4. We test this kit's performance with a variety of samples, however it is possible that high levels of interfering substances may cause variation in assay results.
5. The 12(S)-HETE Standard provided, Catalog No. 80-0607, is supplied in ethanolic buffer at a pH optimized to maintain 12(S)-HETE integrity. Care should be taken handling this material because of the known and unknown effects of eicosanoids.

## Materials Supplied

1. **Goat anti-Rabbit IgG Microtiter Plate, One Plate of 96 Wells, Catalog No. 80-0060**
   A plate using break-apart strips coated with goat antibody specific to rabbit IgG.
2. **12(S)-HETE EIA Conjugate, 5 mL, Catalog No. 80-0610**
   A blue solution of alkaline phosphatase conjugated with 12(S)-HETE.
3. **12(S)-HETE EIA Antibody, 5 mL, Catalog No. 80-0611**
   A yellow solution of a rabbit polyclonal antibody to 12(S)-HETE.
4. **Assay Buffer, 27 mL, Catalog No. 80-1591**
   Tris buffered saline containing proteins and sodium azide as preservative.
5. **Wash Buffer Concentrate, 27 mL, Catalog No. 80-1286**
   Tris buffered saline containing detergents.
6. **12(S)-HETE Standard, 0.5 mL, Catalog No. 80-0607**
   A solution of 500,000 pg/mL 12(S)-HETE.
7. **pNpp Substrate, 20 mL, Catalog No. 80-0075**
   A solution of p-nitrophenyl phosphate in buffer. Ready to use.
8. **Stop Solution, 5 mL, Catalog No. 80-0247**
   A solution of trisodium phosphate in water. Keep tightly capped. Caution: **Caustic.**
9. **12(S)-HETE Assay Layout Sheet, 1 each, Catalog No. 30-0104**
10. **Plate Sealers, 2 each, Catalog No. 30-0012**

## Storage

All components of this kit, **except the conjugate**, are stable at 4 °C until the kit's expiration date. The conjugate **must** be stored frozen at -20 °C.

## Materials Needed but Not Supplied

1. Deionized or distilled water.
2. Precision pipets for volumes between 5 µL and 1,000 µL.
3. Repeater pipets for dispensing 50 µL and 200 µL.
4. Disposable beakers for diluting buffer concentrates.
5. Graduated cylinders.
6. A microplate shaker.
7. Adsorbent paper for blotting.
8. A 37 °C Incubator.
9. Microplate reader capable of reading at 405 nm, preferably with correction between 570 and 590 nm.

## Sample Handling

Assay Designs' Correlate-EIA™ 12(S)-HETE immunoassay is compatible with 12(S)-HETE samples in Assay Buffer. Samples diluted sufficiently into Assay Buffer can be read directly from the standard curve. Please refer to the Sample Recovery recommendations on page 11 for details of suggested dilutions. However, the end user **must verify** that the recommended dilutions are appropriate for their samples. **Samples containing rabbit IgG may interfere with the assay.**

Samples in the majority of tissue culture media, including those containing fetal bovine serum, can also be read in the assay, provided the standards have been diluted into the tissue culture media instead of Assay Buffer. There will be a small change in binding associated with running the standards and samples in media. Users should only use standard curves generated in media or buffer to calculate concentrations of 12(S)-HETE in the appropriate matrix.

Some samples normally have low levels of 12(S)-HETE present and extraction may be necessary for accurate measurement. A suitable extraction procedure is outlined below:

**Materials Needed**

1. 12(S)-HETE Standard to allow extraction efficiency to be accurately determined.
2. 2M hydrochloric acid, deionized water, ethanol, hexane and ethyl acetate.
3. 200 mg $C_{18}$ Reverse Phase Extraction Columns.

**Procedure**

1. Acidify the plasma, serum, urine or tissue homogenate by addition of 2M HCl to pH of 3.5. Approximately 50 µL of HCl will be needed per mL of plasma. Allow to sit at 4 °C for 15 minutes. Centrifuge samples in a microcentrifuge for 2 minutes to remove any precipitate.
2. Prepare the $C_{18}$ reverse phase column by washing with 10 mL of ethanol followed by 10 mL of deionized water.
3. Apply the sample under a slight positive pressure to obtain a flow rate of about 0.5 mL/minute. Wash the column with 10 mL of water, followed by 10 mL of 15% ethanol, and finally 10 mL hexane. Elute the sample from the column by addition of 10 mL ethyl acetate.
4. If analysis is to be carried out immediately, evaporate samples under a stream of nitrogen. Add at least 250 µL of Assay Buffer to dried samples. Vortex well then allow to sit for five minutes at room temperature. Repeat twice more. If analysis is to be delayed, store samples as the eluted ethyl acetate solutions at -80 °C until the immunoassay is to be run. Evaporate the organic solvent under a stream of nitrogen prior to running assay and reconstitute as above.

Please refer to references 12-15 for details of extraction protocols.

## Procedural Notes

1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. Standards can be made up in either glass or plastic tubes.
4. Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.
5. Pipet standards and samples to the bottom of the wells.
6. Add the reagents to the side of the well to avoid contamination.
7. This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4 °C in the sealed bag provided. The wells should be used in the frame provided.
8. **Care must be taken to minimize contamination by endogenous alkaline phosphatase.** Contaminating alkaline phosphatase activity, especially in the substrate solution, may lead to high blanks. Care should be taken not to touch pipet tips and other items that are used in the assay with bare hands.
9. **Prior to addition of substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results.**

## Reagent Preparation

**1. 12(S)-HETE Standard**

Allow the 500,000 pg/mL 12(S)-HETE Standard solution to warm to room temperature. Label five 12 x 75 mm glass tubes #1 through #5. Pipet 1mL of standard diluent (Assay Buffer or Tissue Culture Media) into tube #1. Pipet 750 µL of standard diluent into tubes #2 through #5. Remove 100 µL of diluent from tube #1. Add 100 µL of the 500,000 pg/mL standard to tube #1. Vortex thoroughly. Add 250 µL of tube #1 to tube #2 and vortex thoroughly. Add 250 µL of tube #2 to tube #3 and vortex. Continue this for tubes #4 and #5.

**The concentration of 12(S)-HETE in tubes #1 through #5 will be 50,000, 12,500, 3,125, 781 and 195 pg/mL respectively. See 12(S)-HETE Assay Layout Sheet for dilution details.**

**Diluted standards should be used within 60 minutes of preparation.**

**2. 12(S)-HETE Conjugate**

Allow the conjugate to warm to room temperature. Any unused conjugate should be aliquoted and re-frozen at or below -20 °C. Avoid repeated freeze thaws of the aliquots.

**3. Conjugate 1:10 Dilution for Total Activity Measurement**

Prepare the Conjugate 1:10 Dilution by diluting 50 µL of the supplied Conjugate with 450 µL of Assay Buffer. The dilution should be used within 3 hours of preparation. **This 1:10 dilution is intended for use in the Total Activity wells ONLY**.

**4. Wash Buffer**

Prepare the Wash Buffer by diluting 5 mL of the supplied concentrate with 95 mL of deionized water. This can be stored at room temperature until the kit expiration date, or for 3 months, whichever is earlier.

## Assay Procedure

**Bring all reagents to room temperature for at least 30 minutes prior to opening.**

**All standards and samples should be run in duplicate.**

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the pouch and seal the ziploc. Store unused wells at 4 °C.

2. Pipet 100 µL of standard diluent (Assay Buffer or Tissue Culture Media) into the NSB and the Bo (0 pg/mL Standard) wells.

3. Pipet 100 µL of Standards #1 through #5 into the appropriate wells.

4. Pipet 100 µL of the Samples into the appropriate wells.

5. Pipet 50 µL of Assay Buffer into the NSB wells.

6. Pipet 50 µL of blue Conjugate into each well, except the Total Activity (TA) and Blank wells.

7. Pipet 50 µL of yellow Antibody into each well, except the Blank, TA and NSB wells.

**NOTE:** Every well used should be **Green** in color except the NSB wells which should be **Blue**. The Blank and TA wells are empty at this point and have no color.

8. Incubate the plate at room temperature on a plate shaker for 2 hours at ~500 rpm. The plate may be covered with the plate sealer provided, if so desired.

9. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 2 more times for a total of **3 Washes.**

10. After the final wash, empty or aspirate the wells, and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.

11. Add 5 µL of the blue Conjugate 1:10 Dilution (**see Step 3, Reagent Preparation, on page 5**) to the TA wells.

12. Add 200 µL of the pNpp Substrate solution to every well. Incubate at 37 °C for 3 hours without shaking. The plate should be covered with the provided plate sealer.

13. Add 50 µL of Stop Solution to every well. This stops the reaction and the plate should be read immediately.

14. Blank the plate reader against the Blank wells, read the optical density at 405 nm, preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the Blank wells from all readings.

## Calculation of Results

Several options are available for the calculation of the concentration of 12(S)-HETE in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. If data reduction software is not readily available, the concentration of 12(S)-HETE can be calculated as follows:

1. Calculate the average net Optical Density (OD) bound for each standard and sample by subtracting the average NSB OD from the average OD bound:

   Average Net OD = Average Bound OD - Average NSB OD

2. Calculate the binding of each pair of standard wells as a percentage of the maximum binding wells (Bo), using the following formula:

   $$\text{Percent Bound} = \frac{\text{Net OD}}{\text{Net Bo OD}} \times 100$$

3. Using Logit-Log paper plot Percent Bound versus Concentration of 12(S)-HETE for the standards. Approximate a straight line through the points. The concentration of 12(S)-HETE in the unknowns can be determined by interpolation.

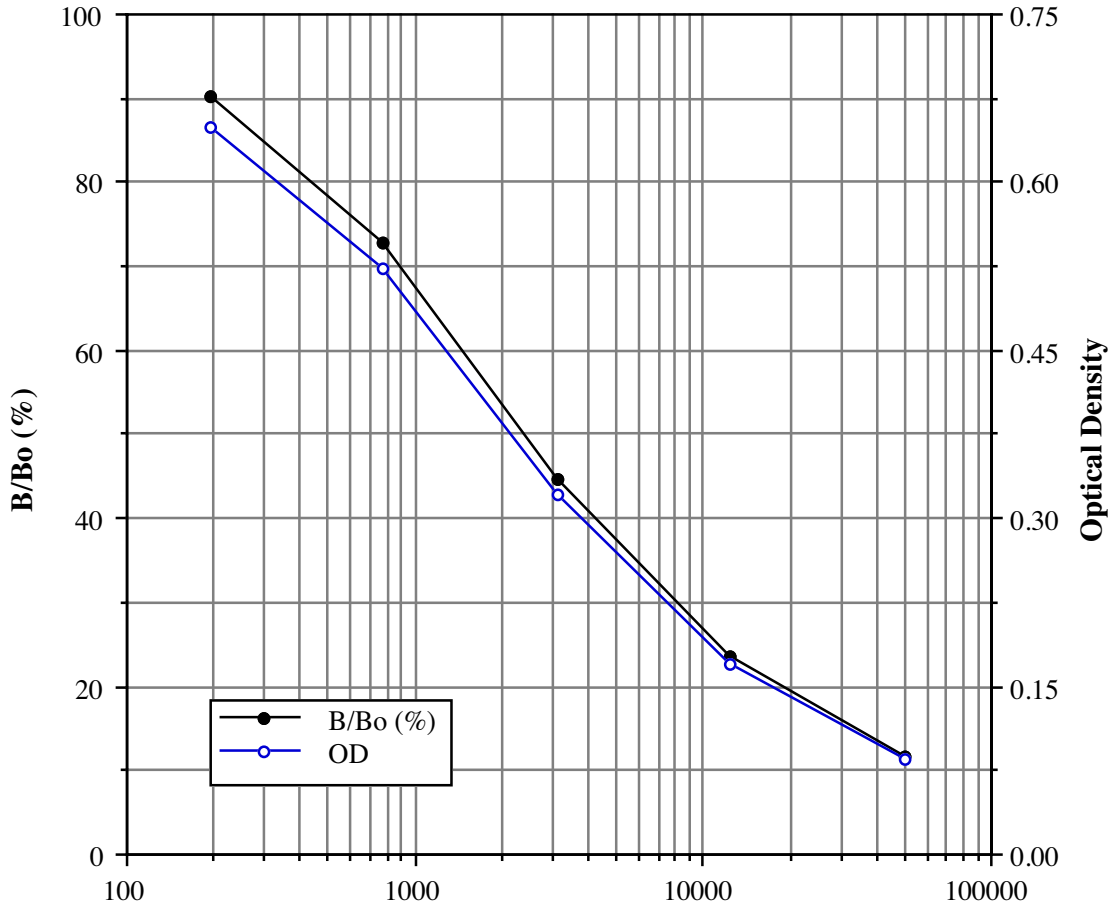
## Typical Results

The results shown below are for illustration only and **should not** be used to calculate results.

| Sample | Mean OD (-Blank) | Average Net OD | Percent Bound | **12(S)-HETE (pg/mL)** |
|---|---|---|---|---|
| Blank OD | (0.094) | | | |
| TA | 0.667 | | | |
| NSB | -0.002 | 0.000 | 0.00% | |
| Bo | 0.718 | 0.720 | 100% | **0** |
| S1 | 0.082 | 0.084 | 11.7% | **50,000** |
| S2 | 0.168 | 0.170 | 23.5% | **12,500** |
| S3 | 0.319 | 0.321 | 44.5% | **3,125** |
| S4 | 0.521 | 0.523 | 72.6% | **781** |
| S5 | 0.648 | 0.650 | 90.3% | **195** |
| Unknown 1 | 0.471 | 0.473 | 65.7% | **1,117** |
| Unknown 2 | 0.145 | 0.147 | 20.5% | **15,608** |

## Typical Standard Curve

A typical standard curve is shown below. This curve **must not** be used to calculate 12(S)-HETE concentrations; each user must run a standard curve for each assay.




## Typical Quality Control Parameters

| | | |
|---|---|---|
| Total Activity Added | = | 0.667 x 10 x 10 = 66.7 |
| %NSB | = | -0.003% |
| %Bo/TA | = | 1.08% |
| Quality of Fit | = | 1.000 (Calculated from 4 parameter logistic curve fit) |
| | | |
| 20% Intercept | = | 16,341 pg/mL |
| 50% Intercept | = | 2,447 pg/mL |
| 80% Intercept | = | 480 pg/mL |

## Performance Characteristics

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols[16].

## Sensitivity

Sensitivity was calculated by determining the average optical density bound for sixteen (16) wells run as Bo, and comparing to the average optical density for sixteen (16) wells run with Standard #5. The detection limit was determined as the concentration of 12(S)-HETE measured at two (2) standard deviations from the zero along the standard curve.

| | |
|---|---|
| Average Optical Density for the Bo = | 0.530 ± 0.021 (4.0%) |
| Average Optical Density for Standard #5= | 0.474 ± 0.019 (4.1%) |
| Delta Optical Density (0-195 pg/mL) = | 0.056 |
| 2 SD's of the Zero Standard = 2 x 0.021 | 0.042 |
| Sensitivity = $\frac{0.042}{0.056}$ x 195 pg/mL = | **146.3 pg/mL** |

## Linearity

A sample containing 9,518 pg/mL 12(S)-HETE was diluted 5 times 1:2 in the kit Assay Buffer and measured in the assay. The data was plotted graphically as actual 12(S)-HETE concentration versus measured 12(S)-HETE concentration.

The line obtained had a slope of 1.025 and a correlation coefficient of 1.000.

## Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of 12(S)-HETE and running these samples multiple times (n=16) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of 12(S)-HETE in multiple assays (n=8).

The precision numbers listed below represent the percent coefficient of variation for the concentrations of 12(S)-HETE determined in these assays as calculated by a 4 parameter logistic curve fitting program.

| | 12(S)-HETE (pg/mL) | Intra-assay %CV | Inter-assay %CV |
|---|---|---|---|
| Low | 342 | 5.2 | |
| Medium | 1,153 | 10.1 | |
| High | 4,762 | 15.5 | |
| Low | 224 | | 4.1 |
| Medium | 1,127 | | 9.1 |
| High | 5,294 | | 20.8 |

## Cross Reactivities

The cross reactivities for a number of related eicosanoid compounds was determined by dissolving the cross reactant (purity checked by N.M.R. and other analytical methods) in Assay Buffer at concentrations from 500,000 to 5 pg/mL. These samples were then measured in the 12(S)-HETE assay, and the measured 12(S)-HETE concentration at 50% B/Bo calculated. The % cross reactivity was calculated by comparison with the actual concentration of cross reactant in the sample and expressed as a percentage.

| Compound | Cross Reactivity |
|---|---|
| 12(S)-HETE | 100% |
| 12(R)-HETE | 2.5% |
| 15-HETE | 0.3% |
| 5(S)-HETE | 0.2% |
| 8,15-diHETE | 0.1% |
| 5,15-diHETE | 0.1% |
| $PGE_2$ | 0.1% |
| $PGF_{2\alpha}$ | 0.1% |
| $PGD_2$ | 0.1% |
| 6-keto-$PGF_{1\alpha}$ | 0.1% |
| Thromboxane $B_2$ | 0.1% |
| Arachidonic Acid | 0.1% |
| Leukotriene $B_4$ | 0.1% |
| Leukotriene $C_4$ | 0.1% |
| Leukotriene $D_4$ | 0.1% |
| Leukotriene $E_4$ | 0.1% |
| 8-HETE | <0.1% |
| 9-HETE | <0.1% |
| 11-HETE | <0.1% |

## Sample Recoveries

Plese refer to pages 4 and 5 for Sample Handling recommendations and Standard preparation

12(S)-HETE concentrations were measured in a variety of different samples including tissue culture media and porcine EDTA plasma. For samples in tissue culture media, ensure that the standards have been diluted into the same media (refer to page 4). 12(S)-HETE was spiked into samples which were diluted with the kit Assay Buffer. The following results were obtained:

| Sample | % Recovery* | Recommended Dilution* |
|---|---|---|
| Porcine EDTA Plasma | 104 | ≥1:10 |
| Tissue Culture Media | 94.4 | 1:2 |

* See Sample Handling instructions on page 4 for details.

## References


1. T. Chard,"An Introduction to Radioimmunoassay & Related Techiques 4th Ed.", (1990) Amsterdam: Elsevier.
2. P. Tijssen,"Practice and Theory of Enzyme Immunoassays", (1985) Amsterdam: Elsevier.
3. A.A. Spector et al., Prog. Lipid Res., (1988) 27(4): 271.
4. H.W-S. Chan,"Autoxidation of Unsaturated Lipids", (1987) Academic Press.
5. M. Hamberg, et al., Proc. Natl. Acad. Sci. USA, (1974) 71: 3400.
6. E.J. Goetzl, et al., J. Clin. Invest., (1977) 59: 179.
7. J. Nakao, et al., Atherosclerosis, (1982) 44: 339.
8. J.L. Nadler, et al., J. Clin. Invest., (1987) 80: 1763.
9. I. Antonipillai, et al., Hypertension, (1987) 10: 61.
10. S.A. Metz, et al., Endocrinology, (1982) 111: 2141.
11. K.V Honn, et al., FASEB J., (1989) 3: 2285.
12. K. Green, et al., Anal. Biochem, (1973) 54: 434.
13. J. Frolich, et al., J. Clin. Invest., (1975) 55: 763.
14. J.E. Shaw & P.W. Ramwell, Meth. Biochem. Anal., (1969) 17: 325.
15. K. Green, et al., Adv. Prostaglandin & Thromboxane Res., (1978) 5: 15.
16. National Committee for Clinical Laboratory Standards Evaluation Protocols, SC1, (1989) Villanova, PA: NCCLS.

## LIMITED WARRANTY

Assay Designs, Inc. warrants that at the time of shipment this product is free from defects in materials and workmanship. This warranty is in lieu of any other warranty expressed or implied, including but not limited to, any implied warranty of merchantability or fitness for a particular purpose.

Assay Designs must be notified of any breach of this warranty within 48 hours of receipt of the product. No claim shall be honored if Assay Designs is not notified within this time period, or if the product has been stored in any way other than outlined in this publication. The sole and exclusive remedy of the customer for any liability based upon this warranty is limited to the replacement of the product, or refund of the invoice price of the goods.

assay designs
SIMPLIFY YOUR SCIENCE®

**For more details concerning the information within this kit insert, or to order any of Assay Designs' products, please call (734) 668-6113 between 8:30 a.m. and 5:30 p.m. EST. Orders or technical questions can also be transmitted by fax or e-mail 24 hours a day.**

**Material Safety Data Sheet (MSDS) available on our website or by fax.**


**Assay Designs, Inc.**
**5777 Hines Drive**
**Ann Arbor, MI 48108**
**U.S.A.**

**Telephone:** **(734) 668-6113**
**(800) 833-8651** **(USA & Canada only)**
**Fax:** **(734) 668-2793**
**e-mail:** **info@assaydesigns.com**
**website:** **www.assaydesigns.com**


***Simplify Your Science®***

KITS FOR CHARITY™


Catalog No. 25-0118

November 2, 2006