EnzChek® Paraoxonase Assay Kit

Table 1. Contents and storage information.

Material	Amount	Storage	Stability
Paraoxonase substrate (Component A)	500 µg	• ≤–20°C	When stored as directed, kit components should be stable for at least 6 months.
Fluorescent reference standard (Component B)	250 µg		
20X reaction buffer (Component C)	20 mL		
Stop reagent (Component D)	1 mg		
96-well microplate (Component E)	2 plates		
Adhesive microplate covers (Component F)	2 covers		
Organophosphatase positive control (Component G)	10 units		
DMSO (Component H)	2 mL		
Limit of detection: The limit of detection of this assay is ~50 mU/mL of paraoxonase, where 1 unit of paraoxonase is defined as the amount of enzyme that will liberate 1 nanomole of organophosphate per minute at 37°C.			
Approximate fluorescence excitation/emission maxima: 360/450 nm			

Introduction

Paraoxonase is a mammalian enzyme associated with high-density lipoprotein ("good cholesterol") in serum. Low serum paraoxonase levels are positively correlated with risk of cardiovascular disease,[1] and paraoxonase activity is a better marker than the PON1 genotype for predicting susceptibility to vascular disease.[2] Paraoxonase has multiple activities including organophosphatase, phosphotriesterase, arylesterase, and thiolactonase. The organophosphatase activity confers protection against toxic organophosphates such as insecticides, which are a common source of chemical intolerance, and nerve agents such as sarin and VX. Current methods for measuring paraoxonase in serum, such as the colorimetric paraoxon assay,[3] are relatively insensitive and often toxic themselves, and are compromised by high background and low signal as well.

The EnzChek® Paraoxonase Assay Kit is a highly sensitive, homogeneous fluorometric assay (excitation/emission maxima 360/450 nm) for the organophosphatase activity of paraoxonase, based on the hydrolysis of a fluorogenic organophosphate analog. Under standard conditions the assay requires only 5 µL of serum, yields a signal in as little as 15 minutes, and is linear for up to 60 minutes. The assay is >10-fold more sensitive than the colorimetric paraoxon assay and, unlike the colorimetric assay, can distinguish samples of very similar paraoxonase activity. The assay requires only a single homogeneous reaction, which may be either continuously monitored or terminated using a stop solution. Correlation between the inhibition curves of the fluorogenic paraoxonase assay versus the colorimetric assay is excellent. The Z´ factor (a statistical parameter for evaluating the signal window of an assay)[4] is 0.95 when the assay is performed as described in the 96-well format.

Before You Begin

Before opening a vial, allow it to warm to room temperature.

Preparing the Stock Solution

Allow components to warm to room temperature before preparing the various stock solutions.

1.1 Prepare 20 mL of 1X reaction buffer by adding 1 mL of 20X reaction buffer (Component C) to 19 mL of deionized water. This 1X reaction buffer should be sufficient for approximately 100 assays of 100 µL each, with 10 mL excess for making stock solutions and dilutions.

1.2 Prepare a 110X paraoxonase substrate stock solution (Component A) by adding 127 µL of DMSO to the paraoxonase substrate (Component A) vial. Stored at 2–6°C and protected from light and humidity, this stock solution is stable for up to two weeks.

1.3 If desired, prepare a 10 mM fluorescent reference standard by adding 118 µL of DMSO to the fluorescent reference standard (Component B) vial. Store at 2–6°C and protect from light.

1.4 As needed, dissolve the organophosphatase positive control (Component G) in 1 mL of 1X reaction buffer. This stock solution is sufficient for more than 60 positive control reactions (see step 3.2). For short-term use, store on ice or at 2–6°C overnight. For longer storage, dispense aliquots and freeze at ≤–20°C until required for use. Do not subject the organophosphatase solution to repeated freeze/thaw cycles or vortexing.

1.5 If desired, dissolve the stop reagent (Component D) in 1 mL of DMSO to make a 12X stop solution.

Preparing the Sample

2.1 For each sample that will be assayed (serum samples and organophosphatase positive control, if desired), dispense 245 µL of 1X reaction buffer to a well of microplate 1. Add 5 µL of serum sample, or 10 µL of the organophosphatase positive control stock solution (prepared in step 1.4), in the pattern or replicates desired. Cover the plate with the adhesive microplate cover and store at 2–6°C until needed.

Preparing the Standard Curve

3.1 Prepare the standard curve for the fluorescent reference standard in wells A1, B1, C1, and D1 of microplate 2 as follows: First add 100 µL of 1X reaction buffer to each well. Then add an additional 99 µL of 1X reaction buffer and 1 µL of 10 mM fluorescent reference standard to well A1, and mix thoroughly. Transfer 100 µL from well A1 to well B1, and mix; transfer 100 µL from well B1 to well C1, and mix; transfer 100 µL from well C1 to well D1 and mix. Discard the excess 100 µL from well D1. The concentrations (and amounts) of the fluorescent reference standard in wells A1–D1 will be 50 µM (5 nmol), 25 µM (2.5 nmol), 12.5 µM (1.25 nmol), and 6.25 µM (0.625 nmol), respectively.

3.2 If desired, prepare an organophosphatase positive control dilution series by adding 8 µL, 4 µL, 2 µL, and 1 µL of the organophosphatase positive control stock solution (prepared in step 1.4) to wells E1, F1, G1, and H1, respectively, and bringing the volumes up to 50 µL with 1X reaction buffer. The resulting concentrations of organophosphatase should reflect above-average (E1), average (F1, G1), and below-average (H1) serum paraoxonase activities. Serum samples that fall outside this range should be carefully checked for accuracy.

Experimental Protocol

The following procedure is designed for use with a fluorescence microplate reader, typically in 96-well format.

Note: While the assay is more accurate in the 96-well format, the assay can be scaled down for 384-well plates. Choose the final reaction volume desired, use half that volume of paraoxonase substrate working solution, and make up the remainder of the reaction with sample in 1X reaction buffer. For 384-well black Corning microplates using a total reaction volume of 20 µL per well, a Z´ factor of 0.75 was obtained.

Assaying for Serum Paraoxonase

This protocol describes a paraoxonase assay in a total volume of 110 µL per well. The volumes recommended here are sufficient for ~100 assays.

4.1 To each well of columns 2–12 of microplate 2, add 50 µL of 1X reaction buffer.

4.2 Pipet 10 µL of each serum sample or positive control from microplate 1 (prepared in step 2.1) into the corresponding well in microplate 2. For negative controls, pipet 10 µL of 1X reaction buffer. Each well in columns 2–12 of microplate 2 now contains a total volume of 60 µL. For best results in the subsequent steps, prewarm the plate to 37°C in an incubator at this point.

4.3 Make a paraoxonase substrate working solution by adding 100 µL of the 110X substrate solution in DMSO (prepared in step 1.2) to 5 mL of 1X reaction buffer in a disposable pipetting reservoir. Mix well. This paraoxonase substrate working solution is sufficient for 100 assays and should either be used quickly, or discarded after a few hours if not used up.

4.4 Using a multichannel pipet, add 50 µL of 2X paraoxonase substrate to each well of columns 2–12 of microplate 2, and also to wells E1–H1 containing the organophosphatase positive control dilution series. Mix briefly after each addition. This begins the organophosphate hydrolysis reaction. Proceed promptly to the next step without delay.

4.5 Transfer microplate 2 to a fluorescence microplate reader set to 37°C, and read the plate using excitation at 360 nm and emission at 450 nm. The plate may be read continuously from 15 to 60 minutes. The positive controls should continue to show linear fluorescence increases for up to 60 minutes. Further incubation may result in the control with the highest concentration departing from the linear range of the standard curve.

Note: The reactions may be stopped at any point during the read. Add 10 µL of 12X stop solution (prepared in step 1.5) to any of the sample wells in microplate 2 to stop the reaction in that well.

Analyzing the Data

5.1 Generate a standard curve by plotting the fluorescence of the fluorescent reference standard on the y-axis versus the amount on the x-axis. Figure 1 shows a typical standard curve for this assay.

5.2 Subtract the background fluorescence of the negative controls from all other samples, plot the data, and use the equation of the standard curve to determine the amount of the fluorescent product in each sample well.

5.3 The amount of the fluorescent product formed may be converted to units of paraoxonase (organophosphatase activity) by the following definition:

1 unit (U) of paraoxonase generates 1 nmol of fluorescent product per minute at 37°C.

Example:

Using the equation of the line fit to the standard curve, y = 229.96x + 9.26, a sample with a measured fluorescence of y = 400 gives x = 1.7 nmol of fluorescent product. The fluorescence was measured at 30 minutes after starting the reaction.

1. Using the unit definition, calculate the amount of enzyme in the reaction:

$$\frac{1.7 \text{ nmol}}{30 \text{ min}} \times \frac{1 \text{ U}}{1 \text{ nmol/min}} = 0.057 \text{ U}$$

2. Taking into account that the serum was diluted 50-fold in step 2.1, and that 10 µL of this diluted serum was added to the reaction, calculate the paraoxonase activity of the original serum sample:

$$\frac{0.057 \text{ U}}{10 \text{ µL}} \times 50 = 0.28 \text{ U/µL}$$

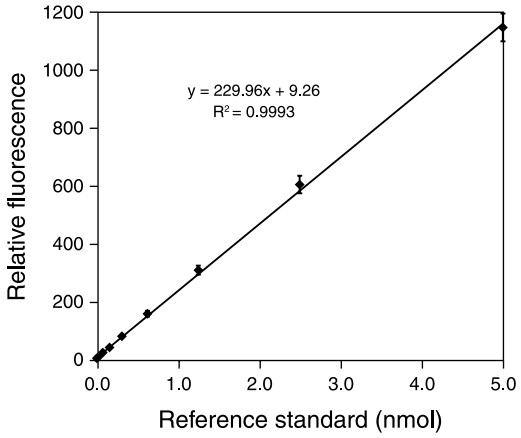

Figure 1. Standard curve for fluorescent reference standard.

References

1. Circulation 107, 2775 (2003); **2.** Arterioscler Thromb Vasc Biol 23, 1465 (2003); **3.** Anal Biochem 180, 242 (1989); **4.** J Biomol Screen 4, 67 (1999).

Product List **Current prices may be obtained from our website or from our Customer Service Department.**

Cat #	Product Name	Unit Size
E33702	EnzChek® Paraoxonase Assay Kit *100 assays*	1 kit

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