

Paraoxonase 1 (PON1) Activity Assay Kit (Fluorometric) (#BN01156)

(Catalog # BN01156; 100 Reactions; Store at -20°C)

I. Introduction:

Paraoxonase 1 (PON1, EC 3.1.8.1) is a 43 kDa enzyme synthesized in the liver and released into the bloodstream, where it associates with high-density lipoprotein (HDL) particles in serum. PON1 is a promiscuous enzyme with broad-spectrum hydrolase activity that was originally named for its ability to hydrolyze highly toxic organophosphorus compounds such as the insecticide paraoxon and various nerve agents used as chemical weapons. The enzyme has subsequently been demonstrated to catalyze hydrolysis of lipid hydroperoxides and lactones. PON1 helps protect serum HDL and LDL particles against lipid peroxidation and inhibits *N*-homocysteinylolation of LDL-associated proteins by hydrolyzing the highly reactive pro-oxidant homocysteine thiolactone. Accumulation of lipid peroxides and homocysteinylated proteins triggers arterial inflammation and eventually leads to atherosclerosis, ischemic stroke and myocardial infarction. PON1 activity is considered to be a clinical biomarker of hepatic and systemic oxidative stress. Measurement of serum PON1 activity has been proposed as a potential test for evaluation of liver function and risk of cardiovascular disease. Assay Genie's Paraoxonase 1 Activity Assay Kit enables rapid measurement of PON1 activity, utilizing a fluorogenic substrate that is converted into a highly fluorescent product (Ex/Em = 368/460 nm). This ensures dramatically greater sensitivity than UV or colorimetric assays and eliminates the need for dangerous toxic substrates. A selective PON1 inhibitor is provided for verification of PON1 specific activity. The assay is simple to perform, high-throughput adaptable and can detect a minimum of 2.0 μ U paraoxonase activity with a sample volume of 5 μ l.



II. Applications:

- Rapid assessment of PON1 activity in biological fluids (serum/plasma) or recombinant PON1 preparations

III. Sample Type:

- Human or animal plasma and serum
- Heterologously expressed recombinant PON1 preparations

IV. Kit Contents:

Components	BN01156	Cap Code	Part Number
Paraoxonase Assay Buffer	50 ml	NM	BN01156-1
Fluorescence Standard	1 vial	Yellow	BN01156-2
PON1 Inhibitor (2-hydroxyquinoline)	1 vial	Clear	BN01156-3
PON1 Substrate	1 vial	Red	BN01156-4
Paraoxonase Positive Control	1 vial	Violet	BN01156-5

V. User Supplied Reagents and Equipment:

- Multiwell fluorescence microplate reader
- Precision multi-channel pipette and reagent reservoir
- Anhydrous (reagent grade) DMSO
- Black 96-well plates with flat bottom

VI. Storage Conditions and Reagent Preparation:

Store kit at -20°C and protect from light. Briefly centrifuge all small vials prior to opening. Allow the Paraoxonase Assay Buffer to warm to room temperature prior to use. Read entire protocol before performing the assay procedure.

- **Fluorescence Standard:** Reconstitute with 55 μ l of DMSO to yield a 5 mM solution. Store at -20°C, stable for 3 freeze/thaw cycles.
- **PON1 Inhibitor (2-hydroxyquinoline):** Reconstitute with 110 μ l of DMSO and vortex to yield a 50 mM stock solution. To prepare a 2 mM working solution (10X final concentration), add 40 μ l of the 50 mM stock solution to 960 μ l of dH₂O. The 2 mM working solution should be stored at -20°C and is stable for 3 freeze/thaw cycles.
- **PON1 Substrate:** Reconstitute with 44 μ l of DMSO to obtain a 250X stock solution. Store at -20°C, stable for 3 freeze/thaw cycles.
- **Paraoxonase Positive Control:** Reconstitute with 110 μ l Paraoxonase Assay Buffer and mix thoroughly. Aliquot and store at -80°C, avoid repeated freeze/thaw cycles.

VII. Paraoxonase 1 (PON1) Activity Assay Protocol:

1. Standard Curve Preparation: Dilute the Fluorescence Standard by adding 10 μ l of the 5 mM stock to 990 μ l Paraoxonase Assay Buffer to obtain a 50 pmol/ μ l Standard solution. Add 0, 2, 4, 6, 8, 12, 16 and 20 μ l of the 50 pmol/ μ l solution into a series of wells in a black 96-well plate and adjust the volume of each well to 100 μ l with Paraoxonase Assay Buffer, yielding 0, 100, 200, 300, 400, 600, 800 and 1000 pmol/well Fluorescence Standard.

2. Sample Preparation:

- Collect plasma or serum samples by standard methods (keep on ice for immediate use or aliquot and store at -80°C for future experiments). For human samples, we recommend adding 5 μ l of undiluted serum/plasma per reaction, although volumes of 2-10 μ l per reaction may be used. The sample volume and/or dilution factor required can vary based upon the nature of the sample. For unknown samples, we suggest doing a pilot experiment by testing several amounts to ensure the readings are within the range of the standard curve.

Note: As PON1 is a strongly Ca^{2+} -dependent enzyme, heparinized plasma samples should be used—plasma specimens collected with EDTA or other Ca^{2+} -chelating anticoagulants may exhibit reduced PON1 activity.

- b. Prepare assay reaction wells according to the table below. In addition to the test sample wells, prepare a background control (no enzyme) well to correct for potential non-enzymatic substrate hydrolysis. For further verification of PON1 specific activity, you may prepare PON1 inhibitor control wells (sample + 200 μM 2-hydroxyquinoline) using the 2-hydroxyquinoline 2 mM working solution (10X final concentration). If desired, you may also prepare positive control (PC) and PC + inhibitor wells using the reconstituted Paraoxonase Assay Buffer. Adjust the volume of all reaction wells to 80 μl /well with Paraoxonase Assay Buffer:

	Test Sample	+ PON1 Inhibitor	Background	Positive Control	PC + Inhibitor
Sample (Serum/Plasma)	2–10 μl	2–10 μl	—	—	—
Paraoxonase Positive Control	—	—	—	10 μl	10 μl
2-hydroxyquinoline Solution (10X)	—	10 μl	—	—	10 μl
Paraoxonase Assay Buffer	to 80 μl	to 80 μl	80 μl	70 μl	60 μl

3. Reaction Mix:

- a. Preincubate the plate for 10 min at 37°C to pre-warm samples and to allow the inhibitor to interact with sample PON1. During the preincubation, prepare a 5X concentrated PON1 Substrate solution by diluting the reconstituted 250X PON1 Substrate stock solution at a 1:50 ratio. Prepare 20 μl of 5X PON1 Substrate solution for each reaction to be performed (for example, for 10 wells, mix 4 μl of 250X PON1 Substrate stock with 196 μl Paraoxonase Assay Buffer).
- b. Start the reaction by adding 20 μl of the 5X PON1 Substrate solution to each reaction well using a multichannel pipette, yielding a final volume of 100 μl /well. *Do not add PON1 Substrate solution to the standard curve wells.*
3. **Measurement:** Immediately (within 1 min) begin measuring the fluorescence at Ex/Em = 368/460 nm in kinetic mode for 60 min at 37°C . We strongly recommend reading in kinetic mode in order to ensure that the measurements recorded are within the linear range of the reaction. Ideal measurement time for the linear range may vary depending upon the sample. The standard curve wells may be read in endpoint mode (Ex/Em = 368/460 nm).
4. **Calculations:** For the fluorescence standard curve, subtract the zero standard (0 pmol/well) reading from all of the standard readings, plot the background-subtracted values and calculate the slope of the standard curve. For the reaction wells (including background control), choose two time points (t_1 and t_2) in the linear phase of the reaction progress curves, obtain the corresponding fluorescence values at those points (RFU_1 and RFU_2) and determine the change in fluorescence over the time interval: $\Delta F = \text{RFU}_2 - \text{RFU}_1$. Calculate specific fluorescence (C_s) by subtracting the background control from each sample: $C_s = \Delta F_s - \Delta F_{\text{bc}}$. Paraoxonase activity is obtained by applying the C_s values to the fluorescence standard curve to get B pmol of substrate metabolized during the reaction time.

$$\text{Sample Paraoxonase 1 (PON1) Activity} = \frac{B}{\Delta T \times V} \times D = \text{pmol/min/ml} = \mu\text{U/ml}$$

Where: **B** is the amount of metabolite produced, calculated from the standard curve (in pmol)
 ΔT is the linear phase reaction time $t_2 - t_1$ (in minutes)
V is the volume of sample added to the well (in ml)
D is the sample dilution factor (if applicable)

Paraoxonase Unit Definition: One unit of paraoxonase activity is the amount of enzyme that generates 1 μmol of fluorescent product per min at 37°C and pH 8.

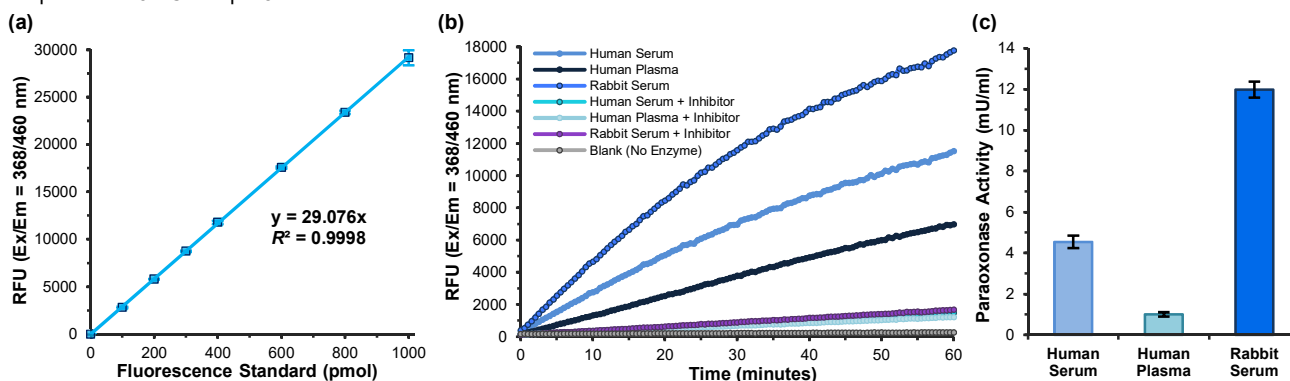


Figure: (a) Standard curve of PON1 Substrate metabolite fluorescence. One mole of fluorescence standard corresponds to the metabolism of one mole of PON1 Substrate. (b) Reaction kinetics of PON1 Substrate metabolism in donor-pooled human serum (5 μl), donor-pooled human plasma (5 μl) and rabbit serum (2.5 μl) in the presence and absence of 200 μM of the selective PON1 inhibitor 2-hydroxyquinoline (no inhibitor conditions contained 0.4% DMSO as a solvent control). (c) Quantification of PON1 activity in serum/plasma samples (mean $\pm$ SEM of four independent replicates). Assays were performed according to the kit protocol.

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