BASIC PROTOCOL 1: Determining PON1 activity using a non-organophosphate (OP) substrate

The non-OP determination of PON1 activity involves a kinetic measurement of PON1, using the substrate phenyl acetate (arylesterase activity). The assay is conducted using an absorbance microplate reader, with controlled temperature (25°C), and with the enzymatic reactions continuously monitored at 270 nm for 4 min. The activity rates from the microplate reader (mOD/min) are then converted to enzymatic activity units (U/mL or µmol/min/mL) using the Beer-Lambert law. The conversion of activity rates (mOD/min) to enzymatic activity units (U/mL) is necessary to enable comparison of the results with other reports using the same enzymatic assay. All buffers should also be maintained at 25°C.

Materials–Samples: blood samples (plasma or serum).

Dilution buffer (see Reagents and Solutions)

Phenyl acetate (#108723, Sigma)

Arylesterase assay buffer (see Reagents and Solutions)

Equipment– Refrigerated microcentrifuge (or tabletop microcentrifuge placed in a cold room)

Water bath

UV-transparent 96-well microplates

0.5 mL and 1.5 mL Eppendorf tubes or similar

50 mL screw-cap polypropylene tubes

UV/visible microplate spectrophotometer (e.g. SpectraMax Plus Microplate Spectrophotometer, Molecular Devices), pre-set at 25°C

Microplate spectrophotometer acquisition and analysis software (e.g. SoftMax Pro data acquisition and analysis software, version 5.4, if using a Molecular Devices microplate reader).

Protocol Steps

Arylesterase activity measurement

1. Collect mouse blood using at least 4 mL green-top (lithium heparin) blood collection tubes.

   A total of 5 µL of plasma/serum are needed for PON1 status, but we recommend having at least 10 µL available in case the assay needs to be repeated.

2. Centrifuge the blood-containing tubes for 10 min at 2,000 x g, 4°C.

   After centrifugation, cells in plasma will remain at the bottom layer, and plasma/serum (yellow) will be on the top layer.

3. Immediately transfer the plasma/serum (top layer) using a Pasteur-type pipette into 0.5 mL polypropylene tubes.

   In general, it is important to store plasma/serum samples in small aliquots to avoid freeze-thaw cycles because this is detrimental to many serum components. However, freeze-thaw cycles do not seem to affect PON1 activity (Richter RJ., personal communication).

4. Mix plasma/serum samples by inverting the tubes gently, and then centrifuge plasma/serum samples for 5 min at 10,000 x g, at 4°C.

   This step is necessary to avoid pipetting any particulate matter present in the sample.

5. Take 7 µL of each plasma/serum sample and mix with 63 µL of dilution buffer (1:10 dilution) in separate tube. Mix well.

6. Transfer 20 µL of each diluted plasma/serum sample into a well of a **UV-transparent** 96-well plate. Do this in triplicate (i.e. do this three times, in three separate wells, per sample).

7. In each plate, transfer 20 µL of dilution buffer into an empty well, to be used as blank.

8. Prepare the substrate by adding 25 µL of phenyl acetate to 25 mL of arylesterase assay buffer in a 50 mL polypropylene tube (substrate concentration in the final working assay solution will be 6.52 mM). Screw the cap tightly and shake vigorously for 1 min.

   Phenyl acetate is an oily solution. For this reason, vigorous shaking is required to ensure that it goes into solution.

   Freshly prepared substrate should be used within 1 h, to avoid degradation and evaporation.

9. Add 180 µL of the working assay solution to each well in the 96-well plate that contains sample or a blank (final concentration of the substrate in each well will be 5.86 mM).

10. Transfer the 96-well plate rapidly into the plate reader (previously set to 25°C).

11. Use the mixing program in the plate reader (e.g. SoftMax Pro) to mix the samples for 5 s prior to the start of the readings.

    This will ensure an even distribution of the substrate and minimize assay variability.

12. Monitor hydrolysis of phenyl acetate at 270 nm. Set the readings for every 15 s and read for at least 4 min. Use only the initial linear rates of hydrolysis for calculating activity values in mOD/min.

    Plotting the signal obtained (y-axis) versus time (x-axis) yields curves that readily identify the initial linear rates of hydrolysis for each assay. Most microplate spectrophotometer software programs have the capability to provide these plots, making it simple to select the initial linear rates. The activity values in mOD/min are calculated by the software. Calculate the standard deviation of the triplicate values obtained for each sample. If the standard deviation is 10 mOD/min or greater, repeat the assay.

    It is essential that the enzyme reaction conditions operate within the linear portion of the instrument capacity. Use of non-linear rates can lead to false measurements of hydrolysis rates.

13. Once the monitoring is complete, use the plate reader to measure the light pathlength (cm) of each well.

Pathlength values are used to normalize absorbance values measured with a microplate reader to correspond with absorbance values measured in a standard precision quartz cuvette. The liquid pathlength in a microplate is not accurate, compared with standard precision quartz cuvettes (1 cm fixed pathlength). Most software programs in microplate spectrophotometers have the capability to correct for the variability of the light pathlength in each well.

The pathlength correction feature corrects for volume irregularities and pipetting errors between wells, and normalizes absorbance values for each well.

14. Divide the rates of arylesterase hydrolysis (step 13) by the light pathlength values (step 14) obtained in each well. Then, calculate the average of the pathlength-corrected triplicate values for each sample.

REAGENTS AND SOLUTIONS

Use ultra-pure water or equivalent for the preparation of all buffers, unless otherwise specified.

Dilution buffer (1 L): Combine 9 mL of UltraPure 1 M Tris-HCl, pH 8.0 (9 mM final, #T1080, Teknova) with 0.9 mL of 1 M $CaCl_2$ (0.9 mM final, #C0477, Teknova), and add water to a final volume of 1 L. Mix well and store for up to 6 months at room temperature.

Tris-HCl is also known as Tris (hydroxymethyl) aminomethane (THAM) hydrochloride.

You may prefer to purchase Tris-HCl pH 8.0 and $CaCl_2$ as prepared solutions, which are available from vendors including ThermoFisher Scientific (Teknova), MilliporeSigma, and VWR. This is a convenient alternative to preparing these solutions each time they are needed, minimizing inter-assay variability. Both solutions are stable at room temperature and are supplied sterile.

Tris-HCl solutions are generally available at one concentration (1 M) in a variety of pH values, ranging from 6.8 to 10.2.

Arylesterase assay buffer (1 L): Combine 800 mL water containing 20 mL of UltraPure 1 M Tris-HCl pH 8.0 (20 mM final) and 1 mL of 1 M $CaCl_2$ (1 mM final). Mix well and bring volume to 1 L with water. Store for up to 6 months at room temperature.

Critical Parameters

PON1 is a calcium-dependent enzyme. Calcium chloride must be included in all buffers and is a critical component in maintaining the activity of plasma PON1. In this regard, PON1 status cannot be carried out using plasma collected using ethylenediaminetetraacetic acid tubes (EDTA, purple or pink-top tubes), as EDTA is a calcium chelator and it results in irreversible inhibition of human plasma PON1 (Erdos et al., 1959). Sodium heparin or lithium heparin plasma (green-top tube, plasma) or serum are preferred specimens (Ferré et al., 2005). Sodium citrate plasma (blue-top tube) can also be used (Richter et al., 2004).

The substrate used in this non-OP PON1 status analysis, phenyl acetate, is an oily liquid. For this reason, when added to the assay buffer, it does not readily dissolve. When pipetting the substrate, always wash the pipet tip 3-4 times in the assay buffer. Then, mix the tube containing the substrate solution (substrate plus assay buffer) vigorously for at least 30 s. To prevent unnecessary splashes due to a faulty tube or not-well-capped tube, we suggest adding parafilm outside the cap and performing the vigorous shaking with the tube facing the laboratory sink. Since the substrate degrades or volatilizes over time once in an aqueous solution, discard the substrate solution after 1 h.

Plasma/serum PON1 activity is very stable over time and is not highly affected by multiple

freeze-thaw cycles when stored at -80°C for 2 years. We have found PON1 activity to be stable for at least 9 years when plasma samples remain frozen at -80°C (Richter RJ., personal communication). This is particularly relevant for longitudinal studies.

The viscosity of plasma/serum is much different than that of water and other aqueous solutions, thus, careful mixing of plasma/serum samples is important. If users do not take this into account when pipetting plasma/serum samples, it can result in inaccurate sample transfers. Ensure use of standard or wide orifice tips, slow down the dispense speed, and only immerse the pipette tip 1-2 mm below plasma/serum surface to decrease presence of excess droplets on the tip that can lead to loading more plasma/serum sample than needed.

As soon as samples are diluted, they should be analyzed, as diluted plasma PON1 degrades fast over time. Leftover diluted samples should not be stored or re-used in the future. If PON1 status needs to be repeated for any reason, fresh plasma/serum dilutions should be prepared. The use of multichannel pipettes is highly recommended to speed up the process of pipetting both diluted plasma/serum samples and the enzymatic substrate to the 96-well microplate, to minimize the time from the samples mixing with substrates to the time of the determination of rates of hydrolysis in the microplate reader. Our laboratory uses the E1-ClipTip Equalizer pipettes (ThermoFisher Scientific), which are adjustable tip spacing electronic pipettes, allowing volume transfers between any type of tube, rack, or microplate.

It is critical to ensure that the activity rates (mOD/min) obtained from the spectrophotometer that will be used in calculations are derived from only the linear portion of the rate vs. time plots generated during the kinetic analyses. If rates become nonlinear during the 4 min enzymatic assay, it is necessary to select a shorter range of time during which the rate remains linear and adjust for the time change in your calculations.

It is recommended to run the enzymatic assay in triplicate and to use the same brand of 96-well microplates throughout the study. This will help to minimize any plate-to-plate variability.

Troubleshooting

Ensure that the blank sample activity rate (mOD/min) is close to zero before using it to subtract from the activity rates of the plasma/serum samples. The blank absorbance rates should be close to zero in all cases. If the blank sample shows a significant absorbance, we recommend preparing new dilution buffer and substrate buffer and repeating the assay. In addition, check the expiration date of the substrates used. Contamination of either the dilution buffer or the microplate well containing the blank sample with plasma/serum would cause unexpected increase in an activity rate. In the case of the substrate buffer, if the substrate has been in an aqueous solution for a prolonged time, it could be degraded and result in increased absorbance. This would show up as an increased starting absorbance. For this reason, it is always best to prepare the substrate buffer shortly before it is needed and to not use it after 1 h of preparation.

Good techniques in order to avoid non-acceptable/questionable values are: 1) using only the initial linear part of the assay rate curves; 2) assaying samples in triplicate; 3) paying attention to the standard deviation of the obtained hydrolysis rates between; 4) making sure the plasma/serum sample does not include any precipitates or unusual color; and 5) not using samples collected in EDTA-containing blood tubes.

The inter-assay coefficient of variation between triplicate values should be less than 15%. If it is higher, ensure that the enzymatic assays are carried out as soon as plasma/serum samples are diluted and with the substrate assay buffer as freshly prepared as possible. In addition, make sure the pipettes used are calibrated.

The temperature of the laboratory where analyses are conducted should stay at ≤ 25°C when possible, and the plate reader temperature controller should be set at 25°C. Higher temperatures will result in increased activities. In the case that the ambient temperature of the laboratory is higher than 25°C, the buffers should be placed in 25°C water baths.

If the activity assays do not show any rates of hydrolysis, ensure that the 96-well plates used are UV-rated. The absorbance of light in regular 96-well plates (made usually of polystyrene or polypropylene) in the visible range of the electromagnetic spectrum is very low. However, these materials absorb light in the UV range. UV transparent 96-well plates do not absorb light in the UV spectrum, and provide low background and consistent performance for absorbances between 260 and 280 nm.

Understanding Results

The non-OP PON1 activity assay described in this protocol provides easy-to-follow steps for obtaining activity rates (mOD/min) that are then normalized with the pathlength and converted into enzymatic rate activity units (U/mL).

Time Considerations

A total of 30 samples, plus a control sample and a blank sample, can be run in triplicate in a single 96-well microplate. Taking into account that the dilution of the sample for both assays is the same, and the use of multichannel pipettes, the determination of the non-OP PON1 status for 30 individuals can easily be completed in a half-day. If the person carrying out the non-OP PON1 status analysis is experienced with this method, 90 samples (three 96-well microplates) can be assayed in a single day.