**Cell Meter™ Fluorimetric Intracellular Peroxynitrite Assay Kit**  
*Green Fluorescence*

<table>
<thead>
<tr>
<th>Ordering Information</th>
<th>Storage Conditions</th>
<th>Instrument Platform</th>
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<tbody>
<tr>
<td>Product Number: 16315 (100 assays)</td>
<td>Keep in freezer, Protect from light</td>
<td>Fluorescence microplate readers, Fluorescence Microscope</td>
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</table>

**Introduction**

Peroxynitrite (ONOO\(^{-}\)) is a strong oxidizing species and a highly active nitrating agent. Peroxynitrite is formed from the reaction between superoxide radicals and nitric oxide generated in cells. It can cause damages to a wide array of biomolecules including proteins, enzymes, lipids and nucleic acids, eventually contributing to cell death. Meanwhile, peroxynitrite can also have protective activities *in vivo* by contributing to host-defense responses against invading pathogens. Therefore, peroxynitrite is an essential biological oxidant involved in a board range of physiological and pathological processes. Due to its extremely short half-life and low steady-state concentration, it has been challenging to detect and understand the role of peroxynitrite in biological systems. AAT Bioquest’s Cell Meter™ Fluorimetric Intracellular Peroxynitrite (ONOO\(^{-}\)) Assay Kit has been developed to address this unmet need. It provides a sensitive tool to monitor ONOO\(^{-}\) level in living cells. AAT Bioquest’s DAX-J2™ PON Green is developed as an excellent fluorescent probe, which can specifically react with intercellular ONOO\(^{-}\) to generate a bright green fluorescent product. This kit can be used in fluorescence imaging and fluorescence microplate reader.

**Kit Components**

<table>
<thead>
<tr>
<th>Components</th>
<th>Amount</th>
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<tbody>
<tr>
<td>Component A: DAX-J2™ PON Green</td>
<td>1 vial</td>
</tr>
<tr>
<td>Component B: Assay Buffer</td>
<td>1 vial (1 mL/vial)</td>
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<tr>
<td>Component C: DMSO</td>
<td>1 vial (100 μL/vial)</td>
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**Assay Protocol for One 96-Well Plate**

**Brief Summary**

*Prepare cells in growth medium → Co-incubate cells with test compounds and DAX-J2™ PON Green working solution → Monitor fluorescence intensity at Ex/Em = 490/530 nm*

1. Prepare cells:
   1.1. For adherent cells: Plate cells overnight in growth medium at 20,000 to 80,000 cells/well/90 μL for a 96-well plate or 5,000 to 20,000 cells/well/22.5 μL for a 384-well plate.
   1.2. For non-adherent cells: Centrifuge the cells from the culture medium and suspend the cell pellets in culture medium at 80,000-200,000 cells/well/90 μL for a 96-well poly-D lysine plate or 20,000-50,000 cells/well/22.5 μL for a 384-well poly-D lysine plate. Centrifuge the plate at 800 rpm for 2 minutes with brake off prior to your experiment.  
**Note:** Each cell line should be evaluated on an individual basis to determine the optimal cell density.

2. Prepare working solution:
   2.1. Prepare DAX-J2™ PON Green stock solution (500X): Add 20 μL of DMSO (Component C) into the vial of DAX-J2™ PON Green (Component A), and mix them well.  
**Note:** 20 μL of reconstituted DAX-J2™ PON Green stock solution is enough for 1 plate. Unused portion can be aliquoted and stored at ≤-20 °C for more than one month if the tubes are sealed tightly and kept from light. Avoid repeated freeze-thaw cycles.
   2.2. Prepare DAX-J2™ PON Green working solution (10X): Add 10 μL of 500X DMSO reconstituted DAX-J2™ Peroxynitrite Sensor stock solution (from Step 2.1) into 500 μL of Assay Buffer (Component B), and mix them well.  
**Note:** The working solution is not stable; prepare it as needed before use.
3. Run the peroxynitrite assay:
   3.1 Add 10 µL/well (96-well plate), or 2.5 µL/well (384-well plate) of DAX-J2™ PON Green working solution (from Step 2.2) in 90 µL (96-well plate) or 22.5 µL (384-well plate) cell culture per well in the cell plate (from Step 1).
   Note: It is not necessary to wash cells before staining. It’s recommended to stain the cells in full medium.

   3.2 Co-incubate cells with DAX-J2™ PON Green with test compounds in full medium or in your desired buffer at 37°C for desired period of time, protected from light. For control wells (untreated cells), add the corresponding amount of compound buffer.
   Note 1: It’s recommended to stain the cells in full medium. However, if tested compounds are serum sensitive, growth medium and serum factors can be aspirated away before staining. Add 90 µL/well (96-well plate) and 22.5 µL/well (384-well plate) of 1X Hank’s salt solution and 20 mM Hepes buffer (HHBS) or the buffer of your choice after aspiration. Alternatively, cells can be stained in serum-free media.
   Note 2: We co-incubated RAW 264.7 macrophage cells with 50-200 µM SIN-1 and DAX-J2™ PON Green in full medium at 37°C for 1 hour to induce peroxynitrite. See Figure 1 for details.

   3.3 Alternatively, stain cells with DAX-J2™ PON Green at 37°C for 1 hour, protected from light (as in Step 3.1). Remove the working solution, then treat cells with test compounds in full medium or in your desired buffer at 37°C for desired period of time.

   3.4 Monitor the fluorescence increase using microplate reader at Ex/Em = 490/530 nm (cut off = 515 nm) with bottom read mode, or take images using fluorescence microscope with a FITC filter.

Data Analysis

Figure 1. Detection of peroxynitrite in living cells upon SIN-1 treatment using Cell Meter™ Fluorimetric Intracellular Peroxynitrite Assay Kit (Cat#16315). RAW 264.7 cells at 100,000 cells/well/100 µL were seeded overnight in a Costar black wall/clear bottom 96-well plate. Cells were co-incubated with DAX-J2™ PON Green working solution and SIN-1 at the concentration from 50 to 200 µM at 37°C for 1 hour. Cells incubated with DAX-J2™ PON Green without SIN-1 treatment were used as control. The fluorescence signal were monitored at Ex/Em = 490/530 nm (cut off = 515 nm) with bottom read mode using a FlexStation microplate reader (Molecular Devices).

Figure 2. Fluorescence images of intracellular peroxynitrite in RAW 264.7 macrophage cells using Cell Meter™ Fluorimetric Intracellular Peroxynitrite Assay Kit (Cat#16315). Raw 264.7 cells at 100,000 cells/well/100 µL were seeded overnight in a Costar black wall/clear bottom 96-well plate. SIN-1 Treatment: Cells were co-incubated with DAX-J2™ PON Green and 100 µM SIN-1 at 37°C for 1 hour. Untreated control: The RAW 264.7 cells were incubated with DAX-J2™ PON Green without SIN-1 treatment. The fluorescence signals were measured using a fluorescence microscope with a FITC filter.

References