ab183306
Phospholipase D Activity Assay Kit (Colorimetric)

Instructions for Use

For rapid, sensitive and accurate detection of Phospholipase D Activity.

This product is for research use only and is not intended for diagnostic use.
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1. **BACKGROUND**

Phospholipase D Activity Assay Kit (Colorimetric) (ab183306) provides a simple and sensitive method of measuring Phospholipase D activity using colorimetry (OD 570 nm). In this assay, Phospholipase D cleaves choline from phosphatidylcholine. The free choline is then oxidized by the Phospholipase D enzyme mix to generate an intermediate which reacts with the Phospholipase D Probe to generate color that is detected at OD 570 nm. This high-throughput adaptable assay kit can detect Phospholipase D activity as low as 1.0 mU/mL in a variety of samples.

Phospholipase D is a key player in phospholipid metabolism. Phospholipase D hydrolyzes the phosphodiester bond of the glycerophospholipids, resulting in the production of phosphatidic acid and a free headgroup. Phospholipase D activity regulates the actin cytoskeleton, vesicle trafficking for secretion and endocytosis, and receptor signaling. Abnormalities in Phospholipase D activity and expression have been associated with Alzheimer's disease, stroke, cancer and other brain disorders.
2. **ASSAY SUMMARY**

- Standard Curve Preparation
- Sample Preparation
- Add Reaction Mix
- Measure optical density (OD = 570 nm) in a kinetic mode at 25°C for 30 min*

*For kinetic mode detection, incubation time given in this summary is for guidance only.
3. **PRECAUTIONS**

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. **STORAGE AND STABILITY**

Store kit at -20°C in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature.

5. **LIMITATIONS**

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not use kit or components if it has exceeded the expiration date on the kit labels.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.
6. MATERIALS SUPPLIED

<table>
<thead>
<tr>
<th>Item</th>
<th>Amount</th>
<th>Storage Condition (Before Preparation)</th>
<th>Storage Condition (After Preparation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospholipase D Assay Buffer</td>
<td>25 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Phospholipase D Probe (in DMSO)</td>
<td>200 µL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Phospholipase D Substrate</td>
<td>100 µL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Phospholipase D Enzyme Mix (Lyophilized)</td>
<td>1 Vial</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Choline Standard (Lyophilized)</td>
<td>1 Vial</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Phospholipase D Positive Control (Lyophilized)</td>
<td>1 Vial</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
</tbody>
</table>

7. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully perform this assay:

- MilliQ water or other type of double distilled water (ddH₂O)
- Cold PBS
- Pipettes and pipette tips
- Microcentrifuge
- Colorimetric microplate reader – equipped with filter for OD 570 nm
- 96 well plate with clear flat bottom
- Heat block or water bath
8. **TECHNICAL HINTS**

- This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.

- Keep enzymes, heat labile components and samples on ice during the assay.

- Make sure all buffers and solutions are at room temperature before starting the experiment.

- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.

- Avoid foaming or bubbles when mixing or reconstituting components.

- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.

- Ensure plates are properly sealed or covered during incubation steps.

- Make sure you have the right type of plate for your detection method of choice.

- Make sure the heat block/water bath and microplate reader are switched on.
9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening.

9.1 Phospholipase D Assay Buffer:
Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C. Use within two months.

9.2 Phospholipase D Probe (in DMSO):
Ready to use as supplied. Warm by placing in a 37°C bath for 1 – 5 minutes to thaw the DMSO solution before use. **NOTE: DMSO tends to be solid when stored at -20°C, even when left at room temperature, so it needs to melt for few minutes at 37°C.** Aliquot probe so that you have enough volume to perform the desired number of assays. Store at -20°C protected from light and moisture. Once the probe is thawed, use with two months.

9.3 Phospholipase D Substrate:
Ready to use as supplied. Aliquot substrate so that you have enough volume to perform the desired number of assays. Store at -20°C protected from light. Once the probe is thawed, use within two months.

9.4 Phospholipase D Enzyme Mix (Lyophilized):
Reconstitute in 220 µL Phospholipase D Assay Buffer. Aliquot enzyme so that you have enough volume to perform the desired number of assays. Store at -20°C. Keep on ice while in use. Use within two months.

9.5 Choline Standard (Lyophilized):
Reconstitute the Choline Standard in 100 µL of Phospholipase D Assay Buffer to generate a 50 mM standard stock solution. Aliquot standard so that you have enough volume to perform the desired number of assays. Store at -20°C. Keep on ice while in use.

9.6 Phospholipase D Positive Control (Lyophilized):
Reconstitute in 20 µL PLD Assay Buffer. Store at -20°C. Keep on ice while in use. Use within two months.
10. STANDARD PREPARATION

- Always prepare a fresh set of standards for every use.
- Diluted standard solution is unstable and cannot be stored for future use.

10.1 Prepare a 0.5 mM Choline standard by diluting 10 µL of the reconstituted 50 mM Choline standard with 990 µL of Phospholipase D Assay Buffer.

10.2 Using 0.5 mM Choline standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes.

<table>
<thead>
<tr>
<th>Standard #</th>
<th>Volume of Standard (µL)</th>
<th>Assay Buffer (µL)</th>
<th>Final volume standard in well (µL)</th>
<th>End Conc Choline in well (nmol/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>150</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>144</td>
<td>50</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>138</td>
<td>50</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>132</td>
<td>50</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>126</td>
<td>50</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>30</td>
<td>120</td>
<td>50</td>
<td>5</td>
</tr>
</tbody>
</table>

Each dilution has enough amount of standard to set up duplicate readings (2 x 50 µL).
11. SAMPLE PREPARATION

General Sample information:

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you complete the Sample Preparation step before storing the samples. Alternatively, if that is not possible, we suggest that you snap freeze cells or tissue in liquid nitrogen upon extraction and store the samples immediately at -80°C. When you are ready to test your samples, thaw them on ice. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

11.1 Cell (adherent or suspension) samples:

11.1.1 Harvest the amount of cells necessary for each assay (initial recommendation = 5 x 10^6 cells).

11.1.2 Wash cells with cold PBS.

11.1.3 Resuspend cells in 100 µL of ice cold Phospholipase D Assay Buffer.

11.1.4 Homogenize cells quickly by pipetting up and down a few times.

11.1.5 Incubate cells on ice for 15 – 30 minutes.

11.1.6 Centrifuge sample for 2 – 5 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.

11.1.7 Collect supernatant and transfer to a clean tube.
11.2 **Tissue samples:**

11.2.1 Harvest the amount of tissue necessary for each assay (initial recommendation = 10 mg).

11.2.2 Wash tissue with cold PBS.

11.2.3 Resuspend tissue in 100 µL of ice cold Phospholipase D Assay Buffer.

11.2.4 Homogenize cells quickly by pipetting up and down a few times.

11.2.5 Incubate cells on ice for 15 – 30 minutes.

11.2.6 Centrifuge sample for 2 – 5 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.

11.2.7 Collect supernatant and transfer to a clean tube.

**NOTE:** We suggest using different volumes of sample to ensure readings are within the Standard Curve range.
12. ASSAY PROCEDURE and DETECTION

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- It is recommended to assay all standards, controls and samples in duplicate.
- Prepare all reagents, working standards, and samples as directed in the previous sections.

12.1 Set up Reaction wells:

- Standard wells = 50 µL standard dilutions.
- Sample wells = 1 – 5 µL samples (adjust volume to 50 µL/well with Assay Buffer).
- Background control sample wells= 1 – 5 µL samples (adjust volume to 50 µL/well with Assay Buffer).
- Positive control = 2 µL Positive control (adjust volume to 50 µL/well with Assay Buffer).

12.2 Reaction Mix:

Prepare 50 µL of Reaction Mix for each reaction:

<table>
<thead>
<tr>
<th>Component</th>
<th>Reaction Mix (µL)</th>
<th>Background Control Mix (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLD Assay Buffer</td>
<td>45</td>
<td>46</td>
</tr>
<tr>
<td>PLD Enzyme Mix</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>PLD Probe</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>PLD Substrate</td>
<td>1</td>
<td>-</td>
</tr>
</tbody>
</table>

Mix enough reagents for the number of assays (samples, standards, positive control and background control) to be performed. Prepare a master mix of the Reaction Mix to ensure consistency. We recommend the following calculation:

X µL component x (Number samples + standards +1)

12.3 Add 50 µL of Reaction Mix into each standard, sample and positive control wells. Mix well.
12.4 Add 50 µL of Background Reaction Mix to Background control sample wells.

12.5 Measure output at OD570 nm on a microplate reader in a kinetic mode, every 2 – 3 minutes, for at least 20 – 30 minutes at 25°C protected from light.

**NOTE:** Incubation time depends on the Phospholipase D Activity in the samples. We recommend measuring OD in a kinetic mode, and choosing two time points (T₁ and T₂) in the linear range (OD values A₁ and A₂ respectively) to calculate the Phospholipase D activity of the samples. The Standard Curve can be read in end point mode (i.e. at the end of incubation time).

OD value at T₂ should not exceed the highest OD in the standard curve. For standard curve, do not subtract A₁ from A₂ reading.
13. **CALCULATIONS**

- Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.

- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).

  13.1 Average the duplicate reading for each standard and sample.

  13.2 If the sample background control is significant, then subtract the sample background control from sample reading.

  13.3 Subtract the mean absorbance value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance.

  13.4 Plot the corrected absorbance values for each standard as a function of the final concentration of Choline.

  13.5 Draw the best smooth curve through these points to construct the standard curve. Most plate reader software or Excel can plot these values and curve fit. Calculate the trendline equation based on your standard curve data (use the equation that provides the most accurate fit).

  13.6 Activity of Phospholipase D is calculated as:

\[
\Delta A_{570\text{nm}} = (A_2 - A_{2\text{BG}}) - (A_1 - A_{1\text{BG}})
\]

Where:

- A1 is the sample reading at time T1.
- A1BG is the background control sample at time T1.
- A2 is the sample reading at time T2.
- A2BG is the background control sample at time T2.

  13.7 Use the \(\Delta A_{570\text{nm}}\) to obtain B nmol of choline generated by Phospholipase D during the reaction time \(\Delta T = T_2 - T_1\).
13.8 Activity of Phospholipase D (nmol/min/mL = mU/mL) in the test samples is calculated as:

\[ \text{Sample PLD activity} = \left( \frac{B}{\Delta T \cdot V} \right) \cdot D \]

Where:
- B = Amount of Choline in the sample (nmol).
- \( \Delta T \) = Reaction time (minutes).
- V = Sample volume added into the reaction well (mL).
- D = Sample dilution factor.

**Unit Definition:**

One unit of Phospholipase D activity = amount of enzyme that will generate 1.0 µmol of Choline per min. at pH 7.4 at 25°C.
14. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for demonstration purposes only. A new standard curve must be generated for each assay performed.

Figure 1. Typical Choline Standard calibration curve.

Figure 2. Phospholipase D activity in liver lysate (5 µL) & Positive Control (1 µL).
15. **QUICK ASSAY PROCEDURE**

**NOTE**: This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.

- Prepare standard, probe and prepare enzyme mix (aliquot if necessary); get equipment ready.
- Prepare standard curve.
- Prepare samples in duplicate (find optimal dilutions to fit standard curve readings).
- Set up plate for standard (50 µL), samples (50 µL), and background wells (50 µL).
- Prepare Phospholipase D Reaction Mix (Number samples + standards + 1).

<table>
<thead>
<tr>
<th>Component</th>
<th>Reaction Mix (µL)</th>
<th>Background Reaction Mix (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospholipase Assay Buffer D</td>
<td>45</td>
<td>46</td>
</tr>
<tr>
<td>Phospholipase Enzyme Mix D</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Phospholipase Probe D</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Phospholipase Substrate D</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

- Add 50 µL of Phospholipase D Reaction Mix to the standard, and sample wells.
- Add 50 µL of Background Reaction Mix into the background sample control wells.
- Measure plate in a colorimetric microplate reader at OD=570 nm at 25°C for 20-30 minutes in a kinetic mode.
## Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay not working</td>
<td>Use of ice-cold buffer</td>
<td>Buffers must be at room temperature</td>
</tr>
<tr>
<td></td>
<td>Plate read at incorrect wavelength</td>
<td>Check the wavelength and filter settings of instrument</td>
</tr>
<tr>
<td></td>
<td>Use of a different 96-well plate</td>
<td>Clear plates</td>
</tr>
<tr>
<td>Sample with erratic readings</td>
<td>Samples not deproteinized (if indicated on protocol)</td>
<td>Use PCA precipitation protocol for deproteinization</td>
</tr>
<tr>
<td></td>
<td>Cells/tissue samples not homogenized completely</td>
<td>Use Dounce homogenizer, increase number of strokes</td>
</tr>
<tr>
<td></td>
<td>Samples used after multiple free/thaw cycles</td>
<td>Aliquot and freeze samples if needed to use multiple times</td>
</tr>
<tr>
<td></td>
<td>Use of old or inappropriately stored samples</td>
<td>Use fresh samples or store at -80°C (after snap freeze in liquid nitrogen) until use</td>
</tr>
<tr>
<td></td>
<td>Presence of interfering substance in the sample</td>
<td>Check protocol for interfering substances; deproteinize samples</td>
</tr>
<tr>
<td>Lower/Higher readings in samples and Standards</td>
<td>Improperly thawed components</td>
<td>Thaw all components completely and mix gently before use</td>
</tr>
<tr>
<td></td>
<td>Allowing reagents to sit for extended times on ice</td>
<td>Always thaw and prepare fresh reaction mix before use</td>
</tr>
<tr>
<td></td>
<td>Incorrect incubation times or temperatures</td>
<td>Verify correct incubation times and temperatures in protocol</td>
</tr>
<tr>
<td>Problem</td>
<td>Cause</td>
<td>Solution</td>
</tr>
<tr>
<td>---------</td>
<td>-------</td>
<td>----------</td>
</tr>
<tr>
<td>Standard readings do not follow a linear pattern</td>
<td>Pipetting errors in standard or reaction mix</td>
<td>Avoid pipetting small volumes (&lt; 5 µL) and prepare a master mix whenever possible</td>
</tr>
<tr>
<td></td>
<td>Air bubbles formed in well</td>
<td>Pipette gently against the wall of the tubes</td>
</tr>
<tr>
<td></td>
<td>Standard stock is at incorrect concentration</td>
<td>Always refer to dilutions on protocol</td>
</tr>
<tr>
<td>Unanticipated results</td>
<td>Measured at incorrect wavelength</td>
<td>Check equipment and filter setting</td>
</tr>
<tr>
<td></td>
<td>Samples contain interfering substances</td>
<td>Troubleshoot if it interferes with the kit</td>
</tr>
<tr>
<td></td>
<td>Sample readings above/ below the linear range</td>
<td>Concentrate/ Dilute sample so it is within the linear range</td>
</tr>
</tbody>
</table>
17. FAQ

Q: Do any chemicals or biological materials cause interference in this assay causing compromised results?
A: 1% or more of Triton X-100 will interfere with the assay.
18. NOTES
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