ab211070
D-2-Hydroxyglutarate Assay Kit (Colorimetric)

For the sensitive and accurate measurement of D-2-Hydroxyglutarate (D2HG) in various biological samples.

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

D-2-Hydroxyglutarate Assay Kit (Colorimetric) (ab211070) provides a convenient method to detect D-2-Hydroxyglutarate (D2HG) in urine, cell lysate and tissue lysate. In this assay, D-2-Hydroxyglutarate is oxidized to α-Ketoglutarate in the presence of D2HG Enzyme and Substrate Mix. The intermediate reduces the probe to a colored product with strong absorbance at OD 450 nm. This absorbance is proportional to the amount of D2HG present in the samples. This assay kit is fast, sensitive, easy to use and high-through adaptable. It can measure as low as 10 μM D-2-Hydroxyglutarate in various samples.

This product is specific for D-2-Hydroxyglutarate and does not detect L-2-Hydroxyglutarate.

\[
\text{D-2-Hydroxyglutarate + NAD} \quad \xrightarrow{\text{D-2-Hydroxyglutarate dehydrogenase}} \quad \text{probe} \quad \xrightarrow{\text{α-Ketoglutarate + NADH}} \quad \text{Colored product (OD 450 nm)}
\]

In eukaryotic cells, Isocitrate Dehydrogenase (IDH1, IDH2 and IDH3) catalyzes the interconversion of Isocitrate and α-Ketoglutarate. In human cancers, an IDH mutation causes a gain-of-function, which reduces its affinity for isocitrate and facilitates the conversion of α-ketoglutarate to D-2-Hydroxyglutarate in the presence of NADP. D-2-Hydroxyglutarate (D2HG, D-2-hydroxyglutaric acid) is present at low level in normal cells and tissues, but is significantly elevated in metabolic diseases and various cancers. Therefore, detection of elevated D2HG is important for early diagnosis, prognosis and the development of therapeutic strategies against these maladies.
2. Protocol Summary

Sample preparation

\[ \downarrow \]

Internal and External Standard preparation

\[ \downarrow \]

Add reaction mix and incubate for 60 minutes at 37°C

\[ \downarrow \]

Measure optimal density (OD450 nm)
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.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipette by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

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.

△ Note: Reconstituted components are stable for 2 months.
5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.

- 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>Quantity</th>
<th>Storage temperature (before preparation)</th>
<th>Storage temperature (after preparation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2HG Assay Buffer</td>
<td>20 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>D2HG Enzyme</td>
<td>1 vial</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>D2HG Substrate Mix</td>
<td>1 vial</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>D2HG Standard</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:

- Microplate reader capable of measuring absorbance at OD 450 nm
- MilliQ water or other type of double distilled water (ddH₂O)
- Cold PBS
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- 96 well plate with clear flat bottom
- Dounce homogenizer (if using tissue)
- (Optional) 0.22 µm filter or 10kD Spin Column (ab93349) – to clarify samples if not clear after preparation
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.
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- 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.
- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.
- Make sure all necessary equipment is switched on and set at the appropriate temperature.
9. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

9.1 D2HG Assay Buffer (20 mL)
Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.

9.2 D2HG Enzyme (lyophilized):
Reconstitute in 220 µL of D2HG Assay Buffer. Pipette up and down to dissolve completely. Aliquot enzyme so that you have enough to perform the desired number of assays. Store at -20°C. Keep on ice while in use. Stable for 2 months.

9.3 D2HG Substrate Mix (lyophilized):
Dissolve in 220 µL of ddH₂O. Pipette up and down to dissolve completely. Aliquot substrate mix so that you have enough to perform the desired number of assays. Store at -20°C. Use within to 2 months.

9.4 D2HG Standard (lyophilized):
Reconstitute with 50 µL ddH₂O to generate 100 mM (100 nmol/µL) D2HG Standard solution. Keep on ice while in use. Aliquot standard so that you have enough to perform the desired number of assays. Store at -20°C. Use within 2 months.
10. 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 snap freeze your samples in liquid nitrogen upon extraction and store them immediately at -80°C. When you are ready to test your samples, thaw them on ice and proceed with the Sample Preparation step. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

10.1 Cell lysates:
10.1.1 Harvest the amount of cells (adherent/suspension) necessary for each assay (initial recommendation: 1 x 10^7 cells).
10.1.2 Wash cells with cold PBS.
10.1.3 Resuspend cells in 100 µL of D2HG Assay Buffer on ice.
10.1.4 Homogenize cells quickly by pipetting up and down a few times.
10.1.5 Incubate cell homogenate for 10 minutes on ice.
10.1.6 Centrifuge 5 minutes at 4°C at 10,000 x g in a cold microcentrifuge to remove any insoluble material.
10.1.7 Collect supernatant and transfer to a new tube.
10.1.8 Keep on ice.

△ Note: if samples are not clear, they need to be clarified through a 0.22 µm filter or spin filtered using a 10kD Spin column (ab93349) in order to remove insoluble components. Use flow through for the assay.

10.2 Tissue lysates:
10.2.1 Harvest the amount of tissue necessary for each assay (initial recommendation: 10 mg).
10.2.2 Wash tissue in cold PBS.
10.2.3 Resuspend tissue in 100 µL of D2HG Assay Buffer.
10.2.4 Homogenize tissue with a Dounce homogenizer or pestle sitting on ice, with 10 – 15 passes.
10.2.5 Incubate tissue homogenate for 10 minutes on ice.
10.2.6 Centrifuge 5 minutes at 4°C at 10,000 x g in a cold microcentrifuge to remove any insoluble material.
10.2.7 Collect supernatant and transfer to a new tube.
10.2.8 Keep on ice.

**△ Note:** if samples are not clear, they need to be clarified through a 0.22 µm filter or spin filtered using a 10kD Spin column (ab93349) in order to remove insoluble components. Use flow through for the assay.

10.3 Urine:
10.3.1 Urine samples need to be spun down at 10,000 x g for 5 minutes at room temperature.
10.3.2 Collect the supernatant.

**△ Note:** Ensure readings are close to the external standard reading, preferably between 10-400 uM.

**△ Note:** We suggest using different volumes of sample to ensure readings are within the External standard range.
11. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all standards, controls and samples in duplicate.
- Prepare all reagents, working standards, and samples as directed in the previous sections.
- If samples OD450 nm is greater than 1.4, we recommend diluting samples further.

**\(\Delta\) Note**: NADH present in cell or tissue extracts will generate background in this assay. Set up Sample Background Controls to correct for background noise in the event that samples exhibit high background signal.

11.1 Standard Preparation:

11.1.1 Prepare a 1 mM D2HG standard (1 nmol/µL) by diluting 10 µL of the 100 mM D2HG standard stock solution (Step 9.4) with 990 µL D2HG Assay Buffer. Mix well.

11.2 Set up reaction wells:

- Reagent Background wells = 50 µL D2HG Assay Buffer.
- External Standard wells: 20 µL D2HG 1 mM standard (Step 11.1.1) + 30 µL D2HG Assay Buffer (20 nmol or 400 µM D2HG).
- Internal Standard wells: 0 – 45 µL samples + 5 µL of 1 mM D2HG standard (5 nmol or 100 µM D2HG) (adjust volume to 50 µL/well with D2HG Assay Buffer). These wells are “spiked” sample wells used to correct for sample interference.
- Sample wells = 0 – 45 µL samples (adjust volume to 50 µL/well with D2HG Assay Buffer).
- Background Control Sample wells = 0 – 45 µL samples (adjust volume to 50 µL/well with D2HG Assay Buffer).
11.3 D2HG Reaction preparation:
11.3.1 Prepare 50 µL of Reaction Mix and Background Mix for each reaction. Mix enough reagents for the number of assays to be performed. Prepare a master mix to ensure consistency.

<table>
<thead>
<tr>
<th>Component</th>
<th>Reaction Mix (µL)</th>
<th>Background Reaction Mix (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2HG Assay Buffer</td>
<td>46</td>
<td>48</td>
</tr>
<tr>
<td>D2HG Enzyme</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>D2HG Substrate Mix</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

11.3.2 Add 50 µL of Reaction Mix into each external and internal standard, reagent background and sample wells.

11.3.3 Add 50 µL of Background Reaction Mix into the background control sample wells.

11.4 Plate measurement:
11.4.1 Mix and incubate at 37°C for 60 minutes protected from light.
11.4.2 Measure output on a microplate reader at OD 450 nm.
12. Calculations

12.1 Average the duplicate readings for each standard, sample and control.

12.2 Correct for any sample interference or background noise from samples, by subtracting the Background Control Sample Well reading from all sample readings.

12.2.1 If Background Control Sample well reading is high (ie, sample wells show high background signal), use Background Control Sample well reading for all corrections.

12.2.2 If Background Control Sample well reading is low (ie, is comparable to Reagent Background Well reading), use Reagent Background well reading for all corrections.

12.3 Subtract the Reagent Background Well reading from all sample readings (values from Step 12.2). The resulting value is the “OD Sample, Corrected (ODsc)” value for all samples.

12.4 Subtract the Reagent Background Well reading from the External Standard reading.

12.5 Determine the amount of D2HG in the sample wells based on the following equation:

\[
\text{D2HG amount (nmol)} \ "B" = \frac{\text{OD}_{\text{SC}}}{(\text{OD}_{\text{SS}}) - (\text{OD}_{\text{S}})} \times 5
\]

Where:
- \(\text{OD}_{\text{SC}}\) = OD of Samples, Corrected (Step 12.3)
- \(\text{OD}_{\text{S}}\) = OD of Samples
- \(\text{OD}_{\text{SS}}\) = OD of Spiked Samples
- 5 = Amount spiked in to Internal Standard Wells (5 nmol)
12.6 The D-2-Hydroxyglutarate concentration (nmol/µL or mmol/L or mM) in the test sample is calculated as:

\[
\text{D2HG concentration} = \frac{B}{V} \times D
\]

Where:
B = amount (in nmol) of D2HG in the sample wells calculated from the equation in Step 12.6, above.
V = original sample volume added in the sample wells (µL).
D = sample dilution factor.

D-2-Hydroxyglutarate MW = 192.08.
Sample D-2-Hydroxyglutarate concentration can also be expressed in nmol/mg or µmol/g of sample.
13. Typical Data

Figure 1. Measurement of D-2-Hydroxyglutarate in different cell lysates: 3T3 (80 µg; mouse), Jurkat (12 µg), HeLa (15 µg), MDA-MB-468 (6 µg), and HepG2 (2 µg).

Figure 2. Measurement of D-2-Hydroxyglutarate in rat liver lysate (120 µg) and rat kidney lysate (240 µg).
14. 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 reagents and aliquot if necessary; get equipment ready.
- Prepare samples.
- Set up plate in duplicate for External standard (50 µL of 20 nmol D2HG), Internal Standard (50 µL of 5 nmol D2HG + sample), sample (50 µL), Sample background (50 µL), and reagent background (50 µL buffer).
- Prepare a master mix for D2HG Reaction Mix and a master mix for Background Reaction Mix:

<table>
<thead>
<tr>
<th>Component</th>
<th>Reaction Mix (µL)</th>
<th>Background Reaction Mix (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2HG Assay Buffer</td>
<td>46</td>
<td>48</td>
</tr>
<tr>
<td>D2HG Enzyme</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>D2HG Substrate Mix</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

- Add 50 µL Reaction Mix to standards, reagent background and sample wells.
- Add 50 µL Background Reaction Mix to Sample Background control wells.
- Incubate plate at 37°C for 60 minutes protected from light.
- Measure plate at OD 450 nm.
## 15. Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Assay not working</strong></td>
<td>Use of ice-cold buffer</td>
<td>Buffers must be at assay 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 microplate</td>
<td>Colorimetric: clear plates Fluorometric: black wells/clear bottom plates</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Luminometric: white wells/clear bottom plates</td>
</tr>
<tr>
<td><strong>Sample with erratic readings</strong></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) till 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><strong>Lower/higher readings in samples and standards</strong></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>Reason</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 described in the 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>
Technical Support
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