ab222388
D-2-Hydroxyglutarate (D2HG) Assay kit (Fluorometric)

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

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 (Fluorometric) (ab222388) provides a convenient method to detect low levels of D2HG in plasma, serum and other biological fluids. In this assay, D-2-Hydroxyglutarate is oxidized to α-Ketoglutarate in the presence of D2HG Enzyme Mix and Substrate Mix. The intermediate reduces the PicoProbe to generate a fluorescence signal at Ex/Em = 535/587 nm. The fluorescence signal is directly 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 D-2-Hydroxyglutarate levels less than 0.1 µM in biological samples, where concentration of the metabolism.

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

D2HG

D-2-Hydroxyglutarate dehydrogenase

PicoProbe

Intermediates \[\rightarrow\] Fluorescence Detection

(Ex/Em= 535/587 nm)

In eukaryotic cells, Isocitrate Dehydrogenase (IDH1, IDH2 and IDH3) catalyzes the interconversion of Isocitrate and α-Ketoglutarate. In human cancers, IDH mutations can cause 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) 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 an important biomarker for early diagnosis, prognosis and the development of therapeutic strategies against these maladies.
2. Protocol Summary

Sample preparation

↓

Internal and External Standard preparation

↓

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

↓

Measure fluorescence (Ex/Em= 535/587 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>PicoProbe (in DMSO)</td>
<td>200 µL</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 Developer</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 fluorescence at Ex/Em = 535/587 nm
- Deionized 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 white with clear flat bottom

For serum or plasma:
- 10kD Spin Column (ab93349) – to remove enzymatic activity that can cause assay interference
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 PicoProbe (in DMSO)
Ready to use as supplied. Warm by placing in a 37°C bath for 1 – 5 min to thaw the DMSO solution before use.

\[\text{Note: DMSO tends to be solid when stored at -20°C, even when left at room temperature, so it needs to melt for a few minutes at 37°C.}\]

Aliquot probe so that you have enough volume to perform the desired number of assays. Store at -20°C protected from light. Once the probe is thawed, use within two months.

9.3 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.

9.4 D2HG Developer (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.

9.5 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. Standard Preparation

- Preparation of standard curve dilution for calculation of D2HG concentration in the sample is optional.
- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.

10.1 Prepare 500 µL of 1 mM (1 nmol/µL) D2HG standard by diluting 5 µL of 100 mM D2HG standard in 495 µL of D2HG Assay Buffer.

10.2 Prepare a 10 µM (10 pmol/µL) D2HG standard by diluting the 10 µL of 1 mM D2HG standard in 990 µL D2HG Assay Buffer.

10.3 Using 10 µM D2HG standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

<table>
<thead>
<tr>
<th>Standard #</th>
<th>D2HG Standard (µL)</th>
<th>Assay Buffer (µL)</th>
<th>Final volume standard in well (µL)</th>
<th>End amount D2HG in well (pmol/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>12</td>
<td>138</td>
<td>50</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>126</td>
<td>50</td>
<td>80</td>
</tr>
<tr>
<td>4</td>
<td>36</td>
<td>114</td>
<td>50</td>
<td>120</td>
</tr>
<tr>
<td>5</td>
<td>48</td>
<td>102</td>
<td>50</td>
<td>160</td>
</tr>
<tr>
<td>6</td>
<td>60</td>
<td>90</td>
<td>50</td>
<td>200</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 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.

11.1 Serum or plasma:
11.1.1 Plasma or serum plasma need to be spin filtered using a 10 kD spin column (ab93349) in order to remove enzymatic activity. Use the flow through for the assay.

11.2 Urine:
11.2.1 Urine samples need to be spun down at 10,000 x g for 5 minutes at room temperature.
11.2.2 Collect the supernatant.

Δ Note: We suggest using different volumes of sample to ensure readings are within the External standard range.
12. 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 you do not prepare a standard curve dilution (See Standard Preparation Section), you need to prepare the Internal Standard to calculate the amount of D2HG in your sample.

12.1 Set up reaction wells:

- Reagent Background wells = 50 µL D2HG Assay Buffer.
- Standard wells = 50 µL standard dilutions.
- Sample wells = 0 – 45 µL samples (adjust volume to 50 µL/well with Assay Buffer).
- Internal Standard wells: 0 – 45 µL samples + 5 µL 10 µM D2HG standard (adjust volume to 50 µL/well with Assay Buffer).

⚠️ Note: D2HG concentration in the well is 50 pmol. Internal standard wells are “spiked” sample wells used to correct for sample interference.
- Background Control Sample wells = 0 – 45 µL samples (adjust volume to 50 µL/well with D2HG Assay Buffer).

12.2 D2HG Reaction preparation:

12.2.1 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>47.5</td>
<td>47.7</td>
</tr>
<tr>
<td>D2HG Enzyme</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>D2HG Developer</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>PicoProbe</td>
<td>0.3</td>
<td>0.3</td>
</tr>
</tbody>
</table>
12.2.2 Add 50 µL of Reaction Mix into each standard dilution, internal standard, reagent background and sample wells.
12.2.3 Add 50 µL of Background Reaction Mix into the background control sample wells.

12.3 Plate measurement:
12.3.1 Mix and incubate at 37°C for 60 minutes protected from light.
12.3.2 Measure output on a microplate reader at Ex/Em = 535/587 nm.
13. Calculations

- Standard curve can be used to tool at the linearity of the assay and to ensure all components are working, or to calculate the final concentration of D2HG in the sample.
- Internal standard ("spiked" samples) can be used to calculate the final concentration of D2HG in the sample (ratiometric approach).

13.1 Standard curve calculation:

13.1.1 Subtract the mean fluorescence value of the blank (Standard #1) from all standard readings. This is the corrected fluorescence.

13.1.2 Average the duplicate reading for each standard.

13.1.3 Plot standard curve readings and draw the line of the best fit to construct the standard curve (most plate reader software or Excel can do this step). Calculate the trend line equation based on your standard curve data (use the equation that provides the most accurate fit).

13.2 Measurement of D2HG in the sample:

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

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

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

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

13.2.3 Subtract the Reagent Background Well reading from all sample readings (values from Step 13.2). The resulting value is the “RFU Sample, Corrected (RFUsc)” value for all samples.

13.2.4 Calculate D2HG concentration in the sample using either standard curve dilutions (Step 13.3) or ratiometric approach with Internal standard (Step 13.4).
13.3 D2HG concentration calculation using standard curve:
13.3.1 Apply the corrected sample fluorescence reading to the standard curve to get D2HG (B) amount in the sample wells.
13.3.2 Concentration of D2HG (pmol/µL or µmol/L or µM) in the test samples is calculated as:

\[
\text{D2HG concentration} = \left( \frac{B}{V} \right) \times D
\]

Where:
B = amount of D2HG in the sample well calculated from standard curve (pmol).
V = sample volume added in the sample wells (µL).
D = sample dilution factor if sample is diluted to fit within the standard curve range.

13.4 D2HG concentration calculation using Internal Standard:
13.4.1 Determine the amount of D2HG in the sample wells based on the following equation:

\[
\text{D2HG amount (nmol) "B"} = \\
= \left( \frac{\text{RFU}_{\text{SC}}}{(\text{RFU}_{\text{SS}} - \text{RFU}_{\text{SC}})} \right) \times 50
\]

Where:
OD_{sc} = OD of Samples, Corrected (Step 12.3)
OD_{ss} = OD of Spiked Samples, Corrected (Step 12.5)
50 = Amount spiked in to Internal Standard Wells (50 pmol)

13.4.2 Concentration of D2HG (pmol/µL or µmol/L or µM) in the test samples is calculated as:

\[
\text{D2HG concentration} = \left( \frac{B}{V} \right) \times D
\]
Where:

B = amount of D2HG in the sample well calculated from standard curve (pmol).

V = sample volume added in the sample wells (µL).

D = sample dilution factor if sample is diluted to fit within the standard curve range.

D-2-Hydroxyglutarate MW = 192.08.

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

Typical standard curve – data provided for demonstration purposes only. A new standard curve must be generated for each assay performed.

![Typical D2HG standard calibration curve.](image)

**Figure 1.** Typical D2HG standard calibration curve.
Figure 2. D2HG in human biological fluids. Measurement of D-2-Hydroxyglutarate in Human Serum from pooled healthy people (NS: 10 μL) and human serum from Breast Cancer patient (BCS: 10 μL). Samples were filtered using 10 kD Spin Column (ab93349).
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 reagents and aliquot if necessary; get equipment ready.
- Prepare D2HG standard dilution [40-200 pmol/well]
- Prepare samples.
- Set up plate in duplicate for Internal Standard (50 µL of 50 pmol 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>45.7</td>
<td>47.7</td>
</tr>
<tr>
<td>D2HG Enzyme</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>D2HG Developer</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>PicoProbe</td>
<td>0.3</td>
<td>0.3</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 Ex/Em = 535/587 nm.
## 16. 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</td>
</tr>
<tr>
<td></td>
<td></td>
<td>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>
17. Notes
Technical Support

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