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Product Information

D-2-Hydroxyglutarate (D2HG) Assay Kit

Catalog Number **MAK320**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description
The level of D-2-Hydroxyglutarate (D2HG) is low in normal cells and tissues, but is significantly elevated in metabolic diseases, such as the rare autosomal disorder D2HG aciduria.[1,2] D2HG is mildly elevated in metabolic disorders resulting from deficiencies of the following enzymes: multiple acyl-CoA dehydrogenase,[3] pyruvate decarboxylase, dihydrolipoyl dehydrogenase, pyruvate carboxylase.[4] D2HG is also mildly elevated in various cancers and neoplasms with mutations in the isocitrate dehydrogenase 1 (*IDH1*) and isocitrate dehydrogenase 2 (*IDH2*) genes.[5,6,7] Detection of elevated D2HG is an important biomarker for early diagnosis, prognosis, monitoring, and development of therapeutic strategies against these diseases.

Developed in partnership with the German Cancer Research Center (DKFZ), the D2HG Assay Kit is a rapid and sensitive enzymatic assay for the detection of D2HG levels in various biological fluids: serum, urine, cell culture supernatants, and cell or tissue lysates. The assay, originally developed by Balss et al.,[8] is based on the oxidation of D2HG to α-ketoglutarate (αKG) by the enzyme (D)-2-hydroxyglutarate dehydrogenase (HGDH) coupled to the reduction of NAD^+ to NADH (see Figure 1). The amount of NADH formed is then quantitated by the diaphorase mediated reduction of resazurin to the fluorescent dye resorufin (λ_{ex} = 540 nm/λ_{em} = 590 nm).

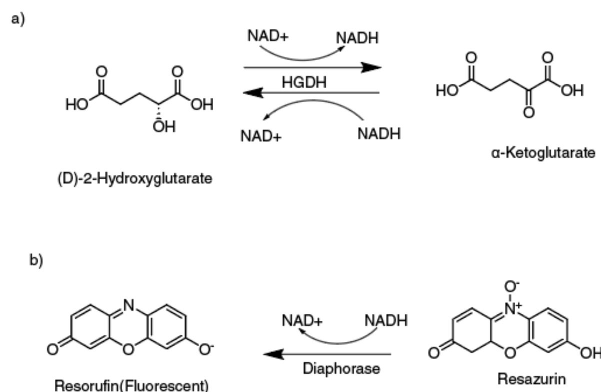
Components
The kit is sufficient for 200 fluorometric reactions

D2HG Standard 10 mM 2-Hydroxyglutarate disodium salt solution Catalog Number MAK320A	100 μl
D2HG Reaction Mixture Reagent mixture, lyophilized Catalog Number MAK320B	2 × 100 rxn
Resazurin Solution 125 μM Resazurin in 50% dimethyl sulfoxide Catalog Number MAK320C	1 ml

Reagents and Equipment Required but Not Provided.
- Extraction buffers
  CelLytic™ M Cell Lysis Reagent, for preparation of cellular extracts, Catalog Number C2978
  CelLytic MT Cell Lysis Reagent, for preparation of mammalian tissue extracts, Catalog Number C3228
- Perchloric acid (PCA), Catalog Number 244252
- Potassium hydroxide (KOH) solution, Catalog Number P4494 or 417661
- Black 96 well plate, Catalog Number P8741
- Multichannel pipette
- Multiwell fluorometer

Precautions and Disclaimer
For R&D use only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Figure 1.
Scheme for detection of D2HG



a) Oxidation of (D)-2-hydroxyglutarate (D2HG) to α-ketoglutarate by (D)-2-Hydroxyglutarate dehydrogenase (HGDH) catalyzes the reduction of NAD^+ to NADH.
b) NADH quantitation by the diaphorase/resazurin system

Preparation Instructions
Use ultrapure water for the preparation of reagents.

D2HG Standard – Dilute 10-fold to a final concentration of 1 mM (1 nmol/μl) using the extraction buffer selected for sample preparation. A total volume of 100 μl is required to generate a complete standard curve in duplicate.

D2HG Reaction Mixture – Reconstitute one vial of lyophilized mixture with 7.1 ml of ultrapure water.

D2HG Complete Reaction Mixture – To the reconstituted D2HG Reaction Mixture add 0.4 ml of Resazurin Solution**.** This amount (7.5 ml) is sufficient for one 96 well plate. It is recommended to prepare enough D2HG Complete Reaction Mixture only for the number of assays to be performed. If a smaller amount is required adjust volumes accordingly.

Storage/Stability
Before opening, store kit protected from light at –20 °C. After reconstitution, the D2HG Reaction Mixture can be stored at 2–8 °C for up to 1 day, or frozen at –20 °C for up to 1 month, protected from light (avoid freeze-thaw cycles).

Procedure
Assay is performed in flat bottom black 96 well plates.

Sample Preparation

- For preparation of cellular extracts it is recommended to use CelLytic M (Catalog Number C2978), while CelLytic MT Cell Lysis Reagent (Catalog Number C3228) is recommended for preparation of tissue extracts.
- Prepare several sample dilutions to ensure the readings are within the range of the standard curve.
- All samples should be deproteinized before analysis, so as to remove interfering proteins from the sample. Perchloric acid (PCA) or Trichloroacetic acid (TCA) precipitation protocols can be used. A protocol for deproteinization by PCA precipitation is given as an example.

Deproteinization by PCA precipitation

1. Prepare sample as recommended in Sample Preparation.
2. Prepare solutions of 4 M PCA and 2 M KOH.
3. Add PCA to each sample to a final concentration of 1 M and vortex briefly to mix well.
4. Incubate samples on ice for 5 minutes.
5. Centrifuge at 13,000 rpm for 2 minutes in a cold centrifuge.
6. Transfer the supernatant to a fresh tube. At this point samples may be stored at –70 °C for up to one month.
7. Neutralize samples by adding a volume of 2 M KOH that equals 34% of the supernatant. Vortex briefly.
   Note: It is important that the pH of the sample at this point is between pH 6.5–8.0. If necessary adjust the pH with 0.1 M KOH or PCA.
8. Centrifuge samples at 13,000 rpm for 15 minutes in a cold centrifuge.
9. Transfer the supernatant to a fresh tube. The samples are ready for the assay.
10. Depending on the expected level of D2HG, either prepare a dilution in the extraction buffer used, or directly transfer 25 μl of sample into designated well. It is recommended to perform assay in duplicates.

Standard Curve Preparation
Note: The standards should preferably be prepared in the same extraction buffer used for Sample Preparation.

1. Place 50 μl of corresponding extraction buffer in wells A2 → A9.
2. Place 100 μl of the prepared 1 mM standard into well A1, and perform 2-fold serial dilutions A1 → A8. Leave well A9 as Reagent blank with buffer only.
3. Place 90 μl of extraction buffer into the wells B1 → B9.
4. Transfer 10 μl from corresponding wells in row A into wells B1 → B9. Mix using a multichannel pipette.
5. Place 25 μl of the serially diluted standard from the row B into rows C and D (work in duplicates). The D2HG amounts in each well are shown in Table 1.

Table 1.
D2HG amounts per well

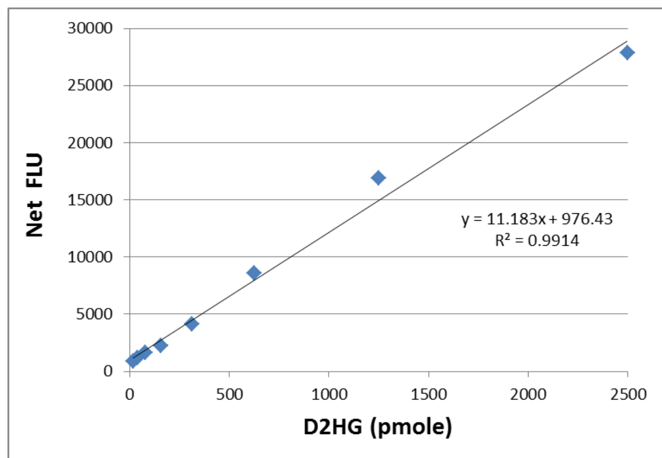
Well number	1	2	3	4	5	6	7	8	9
D2HG (pmol)	2,500	1,250	625	312	156	78	39	19.5	0

D2HG Detection
See Preparation Instructions for reconstitution of D2HG Complete Reaction Mixture.
1. Add 75 μl of the D2HG Complete Reaction Mixture into all wells containing samples and standards. Mix well using a multichannel pipette.
2. Cover the plate with its plastic cover and incubate at 37 °C for 30–60 minutes, protected from light.
3. Read the results in a multiwell fluorometer using the following conditions:
   (λ_{ex} = 540 nm/λ_{em} = 590 nm)

Calculations
1. Subtract the Blank value from all Standard and Samples values.
2. Plot the fluorescence measured for each Standard against the Standard concentration.
3. Determine the linear regression equation and use it to calculate the D2HG concentration of the sample.
4. A supportive Excel calculator can be downloaded from the product page at: https://www.sigmaaldrich.com/catalog/product/sigma/mak320

Results

Figure 2.
Example of D2HG Standard Curve



D2HG dilutions were prepared with ultrapure water, developed with D2HG Complete Reaction Mixture for 60 minutes at 37 °C and fluorescence was measured immediately using a BioTek Synergy™ HT fluorometer, Excitation: 530±25 nm , Emission: 590±35 nm, Sensitivity = 63.

References

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