2,4-dinitrophenylhydrazine alpha-ketoglutarate detection assay for Prolyl Hydroxylase Domain (PHD) proteins V.1

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The 2,4-dinitrophenylhydrazine (2,4-DNPH) alpha-ketoglutarate detection assay was developed to support the study of prolyl hydroxylase domain (PHD) proteins in a substrate-independent manner. This protocol was extensively optimized for the PHD protein reaction, and is applicable to the study of enzyme kinetics or to high-throughput screening.

2021-01-23 2,4-DNPH assay protocol for deposition to protocols.io.docx

https://www.sciencedirect.com/science/article/pii/S0021925821001691

sjwong 2021. 2,4-dinitrophenylhydrazine alpha-ketoglutarate detection assay for Prolyl Hydroxylase Domain (PHD) proteins. **protocols.io** https://protocols.io/view/2-4-dinitrophenylhydrazine-alpha-ketoglutarate-det-b2fnqbme

protocol

Samantha J. Wong, Alison E. Ringel, William Yuan, Joao A. Paulo, Haejin Yoon, Mark A. Currie, Marcia C. Haigis, Development of a colorimetric α-ketoglutarate detection assay for prolyl hydroxylase domain (PHD) proteins, Journal of Biological Chemistry, Volume 296, 2021, 100397, ISSN 0021-9258, https://doi.org/10.1016/j.jbc.2021.100397.

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The following materials must be prepared fresh:

- 1 mM iron (II) sulfate: Prepare 500 mM in 20 mM HCl, then dilute to 1 mM in water just before use (Hewitson, K. S., Schofield, C. J., & Ratcliffe, P. J. (2007). Hypoxia-inducible factor prolyl-hydroxylase: purification and assays of PHD2. *Methods in enzymology*, *435*, 25–42. https://doi.org/10.1016/S0076-6879(07)35002-7)
- PHD enzyme source: frozen stocks may be use, but fresh enzyme is best, especially for PHD3 due to its lability.
- 2,4-dinitrophenylhydrazine (2,4-DNPH): Dissolve in 0.5 M phosphoric acid, let stand for 30-60 mins, then add water to intended concentration. Filter through 0.45 µM filter to remove precipitate.

General lab items for assay

1. Eppendorf tubes
2. Tabletop vortex
3. Tabletop shaking incubator with temperature control
4. 96-well plate
5. Multichannel pipette
6. Microcentrifuge
7. Plate reader

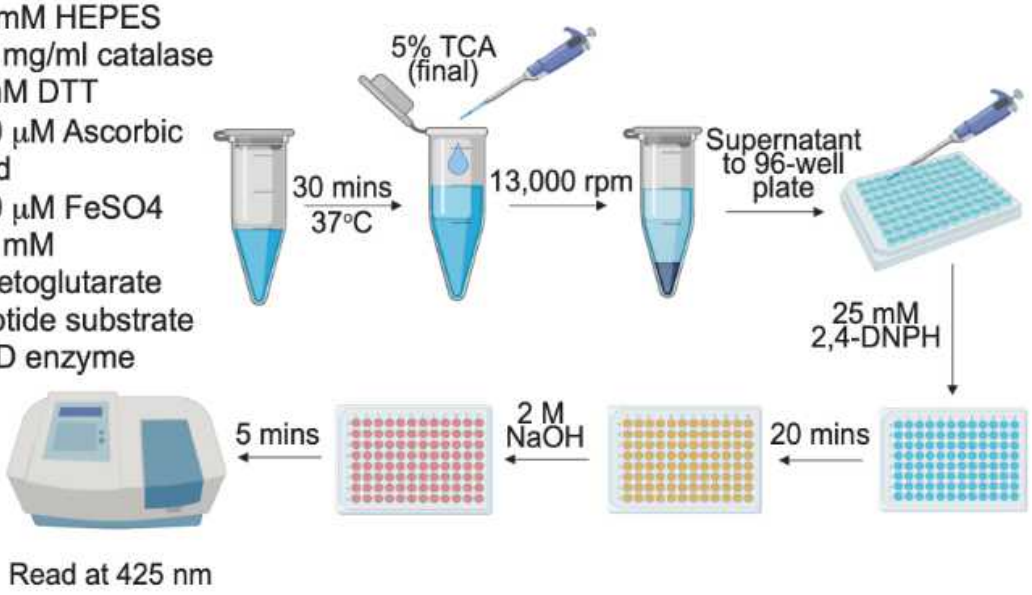
***In vitro* hydroxylation assay materials**

A	B	C	D
Reagent stock	**Stock concentration**	**Working concentration**	**Preparation notes**
HEPES pH 7.0 / MES pH 6.0	0.5 M	50 mM	
Bovine liver catalase	21 mg/ml	0.6 mg/ml	
DTT	10 mM	1 mM	Prepare fresh
Ascorbic acid	40 mM	500 µM	Prepare fresh
FeSO4	1 mM	50 µM	Prepare 500 mM in 20 mM HCl, then dilute to 1 mM in water just before use .
α-ketoglutarate	20 mM	0.5 mM	
Peptide	20 mM	100 µM	Dissolved in DMSO
PHD enzyme source	20 µM	10 µM	Prepare fresh
Trichloroacetic acid (TCA)	10%	5%	
Sodium phosphate pH 7.2	0.5 M	50 mM	
2,4-DNPH	50 mM	25 mM	Prepare fresh. Dissolve in 0.5 M phosphoric acid, let stand for 30-60 mins, then add water to intended concentration. Filter through 0.45 µM filter to remove precipitate.
NaOH	6 M	2 M	

Materials needed to perform the in vitro hydroxylation assay

Overview of assay schematic

1 50 mM HEPES
0.6 mg/ml catalase
1 mM DTT
500 µM Ascorbic Acid
150 µM FeSO4
0.5 mM α-ketoglutarate
Peptide substrate
PHD enzyme



In vitro hydroxylation assay

2 Prepare 5 Eppendorf tubes containing 50 µl of 10% TCA.
- Label tubes: 0 min, 1 min, 2 min, 5 min, 15 min.

3 Prepare cofactor solution containing HEPES/MES, catalase, DTT, ascorbic acid, $FeSO_4$, a-ketoglutarate, and peptide in a 150 µl volume in an Eppendorf tube (using the working concentrations).

4 Add 150 µl of 20 µM PHD enzyme into the cofactor solution.

5 Vortex briefly.

6 Place into a 37 ^{O}C tabletop shaking incubator and start the timer (counting up).
- This step equilibrates the temperature of the reaction to 37 ^{O}C

7 At T = 1 min on the timer, withdraw 50 µl of the reaction solution and quench in the "0 min" tube containing 10% TCA, and replace the reaction tube in the incubator.

8 Repeat this for the other time points.
- At T = 2 min, withdraw 50 µl of the reaction solution and quench in the “1 min” tube
- At T = 3 min, withdraw 50 µl of the reaction solution and quench in the “2 min” tube
- At T = 6 min, withdraw 50 µl of the reaction solution and quench in the “5 min” tube
- At T = 16 min, withdraw 50 µl of the reaction solution and quench in the “15 min” tube

9 Briefly vortex the quenched reactions.

10 Keep the quenched reactions at 4 ^{o}C until ready for downstream processing.
- Reactions have been stored up to 3 days with no loss of signal.

Color development with 2,4-DNPH

11 Centrifuge the quenched reactions at 13,000 rpm for 15 minutes.

12 Meanwhile, add 10 µl of 0.5 M sodium phosphate to 5 wells of a 96-well plate.

13 Transfer 90 µl of the supernatant of the quenched reaction to a well containing 10 µl of 0.5 M sodium phosphate (V_T = 100 µl).
- Do the same for the other 4 quenched supernatants.

14 Using a multi-channel pipette, add 100 µl of 50 mM 2,4-DNPH to the wells (V_T = 200 µl). Pipette up and down gently to mix.

15 Leave at room temperature for 20 minutes.

16 Using a multi-channel pipette, add 50 µl of 6 M NaOH to the wells (V_T = 250 µl). Pipette up and down gently to mix.

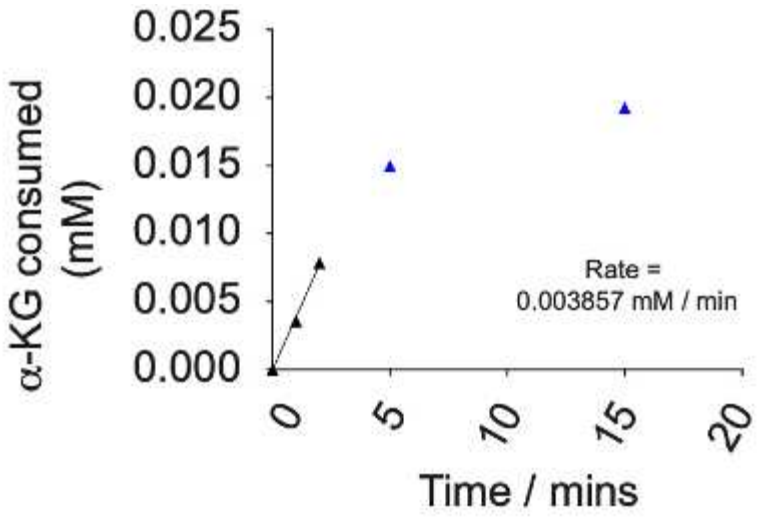
17 Leave at room temperature for 5 minutes.

18 Read at 425 nm on a spectrophotometer.

Data handling

19 Calculate the amount of a-ketoglutarate consumed from a corresponding standard curve processed in *exactly* the same way as the samples.

20 Plot the amount of a-ketoglutarate consumed against time, to obtain a curve that looks like this:



21 The initial rate should be taken as the linear portion of the curve. In this case, from T = 0 to 2 mins.