**Appendix II: 2,4-dinitrophenylhydrazine a-ketoglutarate detection assay protocol**

**2,4-dinitrophenylhydrazine a-ketoglutarate detection assay for Prolyl Hydroxylase Domain (PHD) proteins**

*Note: this protocol accompanies the following publication:*

S. J. Wong, *et al.*, Development of a colorimetric α-ketoglutarate detection assay for prolyl hydroxylase domain (PHD) proteins. *J. Biol. Chem.* **296**, 100397 (2021).

Assay schematic at a glance (created with BioRender.com)

Diagram

Description automatically generated

Reagent preparation

|  |  |  |  |
| --- | --- | --- | --- |
| **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[[1]](#footnote-1). |
| a-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 |  |

Instructions

In vitro *hydroxylation assay*

1. Prepare 5 Eppendorf tubes containing 50 µl of 10% TCA.
   * Label tubes: 0 min, 1 min, 2 min, 5 min, 15 min.
2. Prepare cofactor solution containing HEPES/MES, catalase, DTT, ascorbic acid, FeSO4, a-ketoglutarate, and peptide in a 150 µl volume in an Eppendorf tube (using the working concentrations).
3. Add 150 µl of 20 µM PHD enzyme into the cofactor solution.
4. Vortex briefly.
5. Place into a 37 oC tabletop shaking incubator and start the timer (counting up).
   * This step equilibrates the temperature of the reaction to 37 oC.
6. 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.
7. 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
8. Briefly vortex the quenched reactions.
9. Keep the quenched reactions at 4 oC until ready for downstream processing.
   * Reactions have been stored up to 3 days with no loss of signal.

*Color development with 2,4-DNPH*

1. Centrifuge the quenched reactions at 13,000 rpm for 15 minutes.
2. Meanwhile, add 10 µl of 0.5 M sodium phosphate to 5 wells of a 96-well plate.
3. Transfer 90 µl of the supernatant of the quenched reaction to a well containing 10 µl of 0.5 M sodium phosphate (VT = 100 µl).
   * Do the same for the other 4 quenched supernatants.
4. Using a multi-channel pipette, add 100 µl of 50 mM 2,4-DNPH to the wells (VT = 200 µl). Pipette up and down gently to mix.
5. Leave at room temperature for 20 minutes.
6. Using a multi-channel pipette, add 50 µl of 6 M NaOH to the wells (VT = 250 µl). Pipette up and down gently to mix.
7. Leave at room temperature for 5 minutes.
8. Read at 425 nm on a spectrophotometer.

*Data handling*

1. Calculate the amount of a-ketoglutarate consumed from a standard curve processed in the same way as the samples.
2. Plot the amount of a-ketoglutarate consumed against time, to obtain a curve that looks like this:

Chart, scatter chart

Description automatically generated

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

1. 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 [↑](#footnote-ref-1)