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Dihydrofolate Reductase Activity Kit (Colorimetric)

(Catalog # K246-100; 100 assays; Store at -80°C)

I. Introduction:

Dihydrofolate Reductase (DHFR; 5,6,7,8-tetrahydrofolate NADP oxidoreductase; EC 1.5.1.3), is a ubiquitous enzyme that is present in all eukaryotic and prokaryotic cells. It catalyzes the reduction of dihydrofolate (FH2) to tetrahydrofolate (FH4) using NADPH as a cofactor. FH4 is essential for a number of enzymes that are necessary for the *de novo* synthesis of purines, thymidylic acid and some amino acids. Inactivation of the DHFR enzymatic activity causes reduction of the intracellular level of FH4, inhibition of RNA and DNA synthesis, and cell death. For this reason, DHFR has been a critically important enzyme as a molecular target in drug discovery. Biovision's Dihydrofolate Reductase assay kit is based on the ability of DHFR to catalyze the oxidation of NADPH. The reaction progress is followed by monitoring the decrease in absorbance at 340 nm. Our assay has been optimized to be carried out in a 96- well plate. The assay is simple, sensitive and can detect as low as 4 mU/ml in a variety of samples.

$$\text{Dihydrofolate} + \text{NADPH} + H^+ \xrightarrow{\text{Dihydrofolate Reductase}} \text{Tetrahydrofolate} + NADP^+ \text{ (OD 340 nm)}$$

II. Applications:

- Measurement of Dihydrofolate Reductase activity in various tissues/cells
- Analysis of folate metabolism

III. Sample Type:

- Tissue homogenates: liver, spleen, etc.
- Cell culture: adherent or suspension cells
- Purified enzyme preparations

IV. Kit Contents:

Components	K246-100	Cap Code	Part Number
DHFR Assay Buffer	35 ml	NM	K246-100-1
DHFR Substrate	450 µl	Red	K246-100-2
Dihydrofolate Reductase	10 µl	Green	K246-100-3
NADPH	1 vial	Yellow	K246-100-4

V. User Supplied Reagents and Equipment:

- 96-well clear plate with flat bottom
- Multi-well spectrophotometer (ELISA reader)

VI. Storage Conditions and Reagent Preparation:

Upon receiving the kit, store DHFR substrate at -80°C. Store other components at -20°C. Briefly centrifuge small vials prior to opening. Read entire protocol before performing the assay. Upon opening, use within two months.

- **DHFR Assay Buffer:** Warm to room temperature before use. Store at 4ºC or -20ºC.
- **DHFR Substrate:** Aliquot and store at -80°C, protected from light. Avoid repeated freeze/thaw cycles.
- **Dihydrofolate Reductase:** Store at -20°C. Avoid repeated freeze/thaw cycles. Keep on ice while in use.
- **NADPH:** Reconstitute with 200 µl DHFR Assay Buffer to generate 20 mM NADPH Stock Solution. Aliquot and store at –20°C. Keep on ice while in use.

VII. Dihydrofolate Reductase Assay Protocol:

1. **Sample Preparation:** Rapidly homogenize tissue (10-50 mg) or cells (1 x 10^6) with 100 µl ice-cold DHFR Assay Buffer, and keep on ice for 10 min. Centrifuge at 10,000 x g for 10 min at 4 °C to remove cell debris. Transfer the supernatant to a fresh tube. Add 5-50 µl sample per well & adjust the volume to 100 µl with DHFR Assay Buffer. Prepare parallel sample well(s) as sample background control (See Step 4). For the DHFR positive control, prepare a 10-fold dilution of Dihydrofolate Reductase (i.e. Dilute 1 µl of Dihydrofolate Reductase with 9 µl DHFR assay buffer). Add 2-4 µl of diluted Dihydrofolate Reductase into desired well(s) and adjust the final volume to 100 µl with DHFR Assay Buffer. For the DHFR background control, add 100 µl DHFR Assay Buffer into desired well(s).

   Notes:

   For unknown samples, we suggest testing several doses to ensure the readings are within the Standard Curve range.

2. **NADPH Standard Curve:** Dilute 20 µl of the 20 mM NADPH solution with 780 µl DHFR Assay Buffer to generate 0.5 mM NADPH solution. Add 0, 20, 40, 60, 80, 120, 200 µl 0.5 mM NADPH Standards into a series of wells in 96 well clear plate to generate 0, 10, 20, 30, 40, 60, 100 nmol/well of NADPH Standard. Adjust the volume to 200 µl/well with DHFR Assay Buffer.
3. **NADPH Probe preparation:** Prepare a 40-fold dilution of NADPH stock solution (i.e. Dilute 10 µl of NADPH stock solution with 390 µl DHFR Assay Buffer), vortex briefly and keep on ice. Add 40 µl of Prepared NADPH to each well containing the test samples, sample background control, DHFR positive control and DHFR background control. Mix well.

4. **DHFR substrate preparation:** Prepare a 15-fold dilution of DHFR substrate (i.e. Dilute 40 µl of DHFR stock substrate with 560 µl DHFR Assay Buffer), vortex briefly and keep on ice. Add 60 µl of Prepared DHFR substrate to each well containing the test samples, DHFR positive control and DHFR background control. Mix well. For sample background control, add 60 µl DHFR Assay Buffer into well(s) containing sample background control. The total volume in every well (i.e. standards, samples, background controls) should be 200 µl.

   Note:

   a. DHFR substrate is light sensitive and must be protected from light as much as possible during the experiment. We suggest using an aluminum foil to wrap-around the vial or using an amber tube for this purpose.

   b. Do not store the diluted substrate solutions. Prepare fresh dilutions on the day of the experiment.

5. **Measurement:** Measure absorbance immediately at 340 nm in kinetic mode for 10-20 min at room temperature. Choose two time points (t_1 & t_2) in the linear range of the plot and obtain the corresponding values for the absorbance (OD_1 and OD_2). The NADPH Standard Curve can be read in End-point mode.

6. **Calculation:** Subtract 0 Standard reading from all readings. Plot the NADPH Standard Curve. Calculate the Dihydrofolate Reductase activity of the test sample: $\Delta OD = A_1 - A_2$. Apply the ΔOD to the NADPH Standard Curve to get B nmol of NADPH generated during the reaction time ($\Delta t = t_2 - t_1$). Subtract the sample background control reading from its paired sample reading ($B_{\text{test sample}} - B_{\text{sample background control}}$)/$\Delta t$).

$$\textbf{Sample Dihydrofolate Reductase Activity} = \frac{(\textbf{B test sample} - \textbf{B sample control})}{\Delta t \cdot \textbf{M}} = \textbf{nmol/min/mg} = \textbf{mU/mg}$$

Where: **B** = NADPH amount from Standard Curve (nmol)
Δ**t** = reaction time (min.)
M = sample total protein amount added into the reaction well (mg)

Unit Definition: One unit of Dihydrofolate Reductase is the amount of enzyme that oxidizes 1.0 µmol of NADPH per min, at pH 7.5 at room temperature.

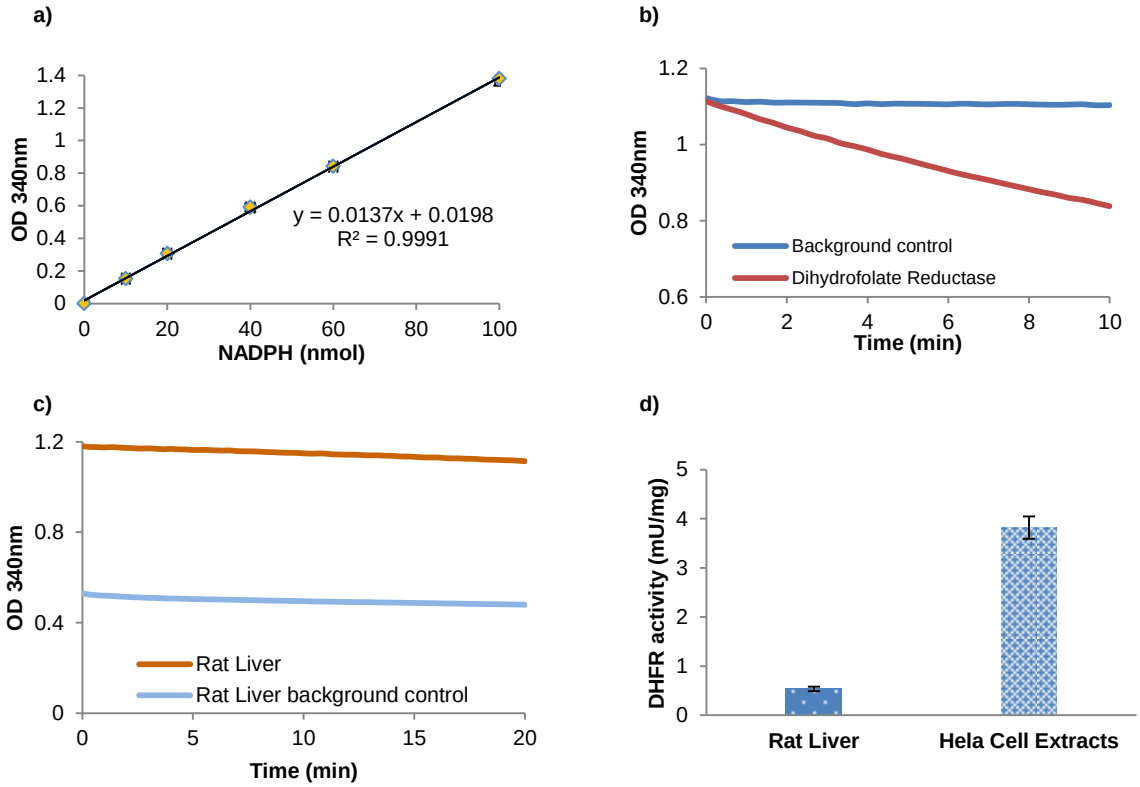

Figure: (a) NADPH standard curve; (b) Purified Dihydrofolate Reductase activity; (c) Dihydrofolate Reductase activity in Rat Liver (100 µg); (d) Dihydrofolate Reductase specific activity was calculated from Rat Liver (100 µg) or Hela cells (80 µg). Assays were performed following the kit protocol.

VIII. RELATED PRODUCTS:

Human Recombinant DHFR (6382-100)
Dihydrofolate Reductase Inhibitor Screening Kit (K247)
Mouse Recombinant DHFR (6383-100)
Dounce Tissue Homogenizer (1998)

FOR RESEARCH USE ONLY! Not to be used on humans.