Dihydrofolate Reductase Inhibitor Screening Kit (Colorimetric)

(Catalog #BN00512; 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, playing a key role in folate metabolism. Inhibition of DHFR results in reduction of the intracellular level of tetrahydrofolate, inhibition of RNA and DNA synthesis, and cell death. For this reason, DHFR has been a critically important therapeutic target for anti-tumor drugs. Methotrexate (MTX) is the most widely investigated inhibitor of DHFR, inhibiting both prokaryotic and eukaryotic DHFRs, and has shown antitumor activity. Assay Genie's Dihydrofolate Reductase Inhibitor Screening Kit is designed for screening DHFR inhibitors. MTX is used as a positive control. The DHFR activity is monitored by the reduction in absorbance reading at OD340 nm, while potential inhibitors arrest this decrease. The kit is adapted to a 96-well format and provides a rapid, simple, sensitive, and reliable test for high-throughput screening of DHFR inhibitors.

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

$$\text{Dihydrofolate} + \text{NADPH} + H^+ \xrightarrow[\text{Methotrexate}]{\text{Dihydrofolate Reductase}} \text{Tetrahydrofolate} + NADP^+ \quad \text{(no decrease in OD 340nm)}$$

II. Applications:

- Screening/characterizing Dihydrofolate Reductase inhibitors

III. Kit Contents:

Components	BN00065	Cap Code	Part Number
DHFR Assay Buffer	35 ml	NM	BN00512-1
DHFR Substrate	450 µl	Red	BN00512-2
Dihydrofolate Reductase	30 µl	Green	BN00512-3
NADPH	1 vial	Yellow	BN00512-4
Methotrexate (10 mM)	25 µl	Brown	BN00512-5

IV. User Supplied Reagents and Equipment:

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

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

VI. Dihydrofolate Reductase Inhibitor Screening Protocol:

1. **Methotrexate, Screening Compounds, Inhibitor Control & Enzyme Control preparations:** Dilute Methotrexate 100-fold (i.e. Dilute 2 µl Methotrexate with 198 µl DHFR Assay Buffer). Dissolve test sample to 100X in an appropriate solvent. Add 2 µl of the test sample, DHFR Assay Buffer or Diluted methotrexate into wells assigned as Sample Screening (S), Enzyme Control (EC) or Inhibitor Control (IC), respectively.

   Note:

   a. Do not store the Diluted Methotrexate. Prepare fresh dilutions on the day of the experiment.

   b. High solvent concentration might affect the DHFR enzymatic activity. Prepare parallel well(s) as Solvent Control to test the effect of the solvent on enzyme activity (such as EC in presence of final solvent concentration). In case that Solvent Control is significantly different from EC, use its values in the calculations below.

   c. The absorbance of the sample solution might affect the readings at 340 nm. Prepare parallel well(s) as Sample Background Control to test the effect of the compound on the absorbance (such as S in presence of final test sample). In case that Sample Background Control is significantly different from that of assay buffer alone, use its values in the calculations below.

2. **Dihydrofolate Reductase Enzyme Solution Preparation:** Dilute Dihydrofolate Reductase 400-fold (i.e. Dilute 2 µl Dihydrofolate Reductase with 798 µl DHFR Assay Buffer). Prepare enough enzyme mix for the number of wells to be analyzed. Add 98 µl of Diluted Dihydrofolate Reductase into desired well(s) containing the test samples, Enzyme Control or Inhibitor Control. The partial volume is 100 µl. Add 100 µl DHFR assay buffer to desired well(s) as Background Control.

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 diluted NADPH to each well containing the test samples, Enzyme Control, Inhibitor Control or Background Control. Mix well. Incubate at room temperature for 10-15 min, avoid light.
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 diluted DHFR substrate to each well containing the test samples, Enzyme Control, Inhibitor Control or Background Control. Mix well. The total volume 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 the tube or using an amber-colored 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).
6. **Calculation:** Calculate the slope for all test Inhibitor Samples [S] & Enzyme Control [EC] by dividing the net ΔOD (A_1-A_2) values with the time Δt (t_2-t_1). Subtract the Solvent Control or Inhibitor Background Control readings from its paired sample readings.

$$\% \text{ Relative Inhibition} = \frac{\text{Slope of [EC]} - \text{Slope of [S]}}{\text{Slope of EC}} \text{ X100}$$

$$\% \text{ Relative Activity} = \frac{\text{Slope of [S]}}{\text{Slope of EC}} \text{ X100}$$

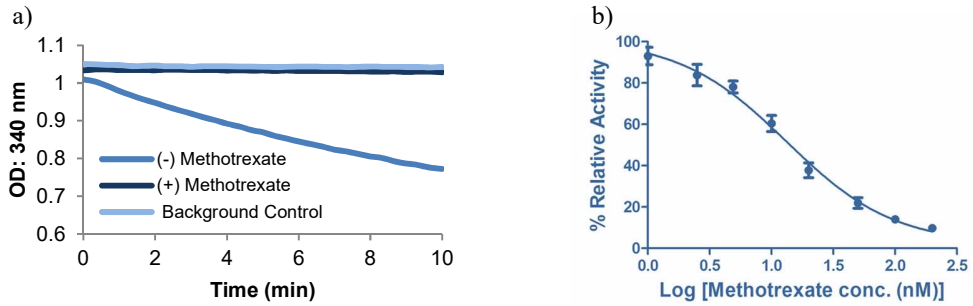

Figure: (a) Dihydrofolate Reductase activity with or without Methotrexate. (b) Inhibition of Dihydrofolate Reductase activity by Methotrexate (final conc.: 1 µM). IC_{50} of Methotrexate was calculated to be 13.25 ±1.18 nM. Assay was carried out following the kit protocol.

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