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Product Information

Dihydrofolate Reductase Assay Kit

Catalog Number **CS0340**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

Dihydrofolate reductase (DHFR) is a ubiquitous enzyme present in all eukaryotic and prokaryotic cells, playing a key role in thymidine synthesis. It catalyzes the reduction of 7,8-dihydrofolate (DHF) to 5,6,7,8-tetra-hydrofolate (THF), utilizing NADPH as cofactor. This reaction is an essential step in the biosynthesis of nucleotidic bases of DNA.[1-3] Blockage of the DHFR enzyme causes cell death as a result of DNA synthesis inhibition. For this reason, DHFR is considered an excellent target for antitumor drugs.

The differences between DHFR enzymes from different sources enables the development of species selective DHFR inhibitors.[4] Trimethoprim and methotrexate (MTX) are the two most widely investigated inhibitors of DHFR. Trimethoprim binds more tightly to bacterial DHFR while MTX, an antifolate compound, inhibits both prokaryotic and eukaryotic DHFRs. MTX exhibits antitumor activity.[2]

The Dihydrofolate Reductase Assay Kit is designed for the detection of DHFR activity and for screening DHFR inhibitors. It provides all the reagents required (including a purified enzyme) for the efficient detection of DHFR activity and inhibition in cell lysates, tissue homogenates, or column fractions of purified enzyme.

The kit was tested on recombinant DHFR, A431, NIH 3T3, and CHO cell lines and liver, kidney, brain, and muscle tissue extracts from rat.

Components

The kit is sufficient for 50–100 one ml tests.

Dihydrofolate Reductase (DHFR) 0.1 unit
(Catalog Number D6566)

Assay Buffer 10× for DHFR 30 ml
(Catalog Number A5603)

Dihydrofolic acid (DHFR substrate) 3 × 10 mg
(Catalog Number D7006)

Methotrexate [(+)-Amethopterin, MTX]
(DHFR inhibitor) 2 × 10 mg
(Catalog Number A6770)

NADPH (β-Nicotinamide adenine dinucleotide phosphate, reduced, tetrasodium salt) 25 mg
(Catalog Number N6505)

Reagents and Equipment Required but Not Provided.

- Temperature controlled UV/Vis spectrophotometer
- 1 quartz cuvette capable of holding at least 1 ml such as Catalog Number Z600288
- Ultrapure water (17 MΩ×cm, or equivalent)

Precautions and Disclaimer

This product is 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.

Preparation Instructions

Note: Use ultrapure water (17 MΩ×cm or equivalent).

1. Dihydrofolic acid (substrate)
   Prepare a 10 mM stock solution at pH 7.5 by the addition of 2.2 ml of Assay Buffer 10× to the dihydrofolic acid bottle (i.e., add 2.2 ml of Assay Buffer 10× to 10 mg powder), and mix well. Aliquot the 10 mM dihydrofolic acid stock solution and store at –20 °C. The solution is stable for 5 days at –20 °C. Unused thawed solutions should be discarded the same day.

2. 10 mM NADPH stock solution
   Prepare a 10 ml suspension buffer by adding 0.2 ml of Assay Buffer 10× to 9.8 ml of water. Add 3 ml of the suspension buffer to the NADPH bottle. Mix well and divide this 10 mM NADPH stock solution into working aliquots and store at –20 °C. The solution is stable for at least one month at –20 °C.

3. Methotrexate (inhibitor) stock solution
   Prepare a 10 mM stock solution by adding 2.2 ml of Assay Buffer 10× to the bottle. Mix well. Aliquot the 10 mM methotrexate stock solution into working stable for at least one month at –20 °C.

   Note: For each inhibition experiment using methotrexate, perform a sequential dilution of the methotrexate stock solution:

   a. Dilution to a concentration of 100 μM methotrexate; add 10 μl of the 10 mM stock solution to 990 μl of Assay Buffer 10×. Mix well.
   b. Dilution to a concentration of 10 μM methotrexate; add 100 μl of the 100 μM MTX solution to 900 μl of Assay Buffer 10×. Mix well.
   c. Dilution to a concentration of 1 μM methotrexate; add 100 μl of the 10 μM MTX solution to 900 μl of Assay Buffer 10×. Mix well.

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

4. Assay Buffer 1×
   Dilute the Assay Buffer 10× for DHFR ten-fold in ultrapure water (i.e., add 5 ml of Assay Buffer 10× for DHFR to 45 ml of water). Keep at room temperature.

5. Sample
   It is recommended to:
   - Prepare cell lysates using the CelLytic™ M mammalian cell lysis/extraction reagent (Catalog Number C2978). Use extracts at a final concentration of 0.8–2 mg protein/ml of reaction mixture.
   - Prepare tissue extracts using the CelLytic MT Cell Lysis Reagent (Catalog Number C3228). Use extracts at a final concentration of 0.5–1 mg protein/ml of reaction mixture.

6. DHFR
   The amount of DHFR supplied in the kit is ~0.1 units (lot specific data presented on the certificate of analysis [COA] for the lot). The activity was measured using the substrate supplied with this kit.

   The amount of DHFR in each reaction should be 1.5×10^{-3} units. According to the lot specific data, the volume of enzyme to be used usually varies between 10–30 μl. Since the solution is very viscous, be cautious when sampling low volumes.

   To calculate the volume (in μl) to be used for each reaction, use the information on the COA in the following formula:

$$\text{Volume (}\mu\text{l)} = \frac{1.5 \times 10^{-3} \times 1000}{\text{(units/mg protein)} \times \text{(mg protein/ml)}}$$

Storage/Stability
The kit is shipped on dry ice. The kit components should be stored at –20 °C except for the Assay Buffer 10× for DHFR that can be stored at 2–8 °C.

Procedure
Principle of assay for DHFR activity[5]
The assay is based on the ability of Dihydrofolate reductase to catalyze the reversible NADPH-dependent reduction of dihydrofolic acid to tetrahydrofolic acid.

$$\text{Dihydrofolic acid} + \text{NADPH} + H^+ \xleftrightarrow{\text{DHFR}} \text{Tetrahydrofolic acid} + \text{NADP}^+$$

At pH 7.5, the equilibrium of the reaction lies relatively far to the right, and the reaction goes essentially to completion in the forward direction. The reaction progress is monitored by the decrease in absorbance at 340 nm.

Table 1.
Reaction scheme for DHFR activity detection and DHFR activity inhibition

	Assay Buffer (1×)	Sample	NADPH	Dihydrofolic acid	Inhibitor
Blank 1	1,000 μl – sample	1.5×10^{-3} units DHFR or *y* μl cell extract	6 μl	–	–
Blank 2	1,000 μl – sample	1.5×10^{-3} units DHFR or *y* μl cell extract	–	5 μl	–
Reaction 1 Activity of supplied enzyme	1,000 μl – the DHFR volume	1.5×10^{-3} units DHFR	6 μl	5 μl	–
Reaction 2 Inhibition by MTX	1,000 μl – the DHFR volume	1.5×10^{-3} units DHFR	6 μl	5 μl	*x* μl MTX
Reaction 3 Inhibition by the tested inhibitor	1,000 μl – the DHFR volume	1.5×10^{-3} units DHFR	6 μl	5 μl	*x* μl Inhibitor tested
Reaction 4 Activity of sample enzyme	1,000 – *y* μl	*y* μl cell extract	6 μl	5 μl	–
Reaction 5 Inhibition of the sample enzyme by MTX	1,000 – (*y*+*x*) μl	*y* μl cell extract	6 μl	5 μl	*x* μl MTX
Reaction 6 Inhibition of the sample enzyme by the tested inhibitor	1,000 – (*y*+*x*) μl	*y* μl cell extract	6 μl	5 μl	*x* μl Inhibitor tested

x = amount of tested inhibitor
y = amount of the sample enzyme (not to exceed 100 μl)

Table 2.
MTX concentrations for inhibition

Stock Concentration	Volume added to Reaction mixture*	Final Concentration in Reaction Mixture
1 μM	5–15 μl	5–15 nM
10 μM	2–10 μl	20–100 nM
100 μM	2–10 μl	200–1,000 nM

Assay Procedure
All the reagents should be kept on ice except for the Assay Buffer 1× that should be kept at room temperature.

1. Set kinetic program (reading every 15 seconds for 2.5 minutes) for the spectrophotometer at 340 nm and 22 °C.

2. Add Assay Buffer 1× to the test microcentrifuge tube according to the reaction scheme (see Table 1) and to the test being performed.

3. Add DHFR enzyme or the sample to the appropriate tube, and mix well.

Notes: The DHFR supplied with the kit is in 50% glycerol, which is very viscous. Cut the end from a micropipette tip and remove samples carefully and accurately.

For activity assays, without testing an inhibitor, continue to step 5.

4. For Inhibition assay only, add the inhibitor and mix well.
Note: For Inhibition Reaction with MTX, see Table 2 for amounts of MTX to add to reaction mixture.

5. Transfer the contents of the tube to be tested to a 1 ml quartz cuvette.

6. Add 6 µl of NADPH solution.

7. Cover the cuvette with Parafilm® and mix by inversion.

8. Add 5 µl of dihydrofolic acid just before starting the reaction (dihydrofolic acid is the substrate of the reaction).

9. Cover the cuvette with Parafilm, mix by inversion, and immediately insert the cuvette into the spectrophotometer.

10. Start the kinetics program immediately.
    Notes: The absorbance at 340 nm will decrease (due to decrease in NADPH concentration).
    10-20 µl of the supplied DHFR enzyme will usually give a linear slope during the 2.5 minutes of the detection.

    MTX inhibition occurs within seconds. However, at a low concentration of enzyme and MTX, and a high concentration of dihydrofolic acid, there is a slow development of inhibition,[2] i.e., there may be a need for a pre-incubation period with the inhibitor. MTX at a final concentration of 5–50 nM (in the reaction tube) is recommended for an inhibition of the supplied DHFR. A total inhibition by MTX is achieved at a final concentration of 1 µM MTX in the reaction mixture.

Results

Activity calculation

Measure the decrease in ΔOD obtained during 2.5 minutes as ΔOD/min. Note the output of the kinetics program is ΔOD/min. Calculate the specific activity by the formula:

$$\text{Units/mg P} = \frac{(\Delta\text{OD/min}_{\text{sample}} - (\Delta\text{OD/min}_{\text{blank}}) \times d}{12.3 \times V \times \text{mg P/ml}}$$

where:

$\Delta\text{OD/min}_{\text{blank}}$ = ΔOD/min. for the blank, from the spectrophotometer readings

$\Delta\text{OD/min}_{\text{sample}}$ = ΔOD/min. for the reaction, from the spectrophotometer readings

12.3 = extinction coefficient (ε, $mM^{-1}\ cm^{-1}$) for the DHFR reaction at 340 nm

V = Enzyme volume in ml (the volume of enzyme used in the assay)

d = The dilution factor of the enzyme sample

mg P/ml = enzyme concentration of the original sample before dilution

Units/mg P = Specific activity in µmole/min/mg protein

Unit definition: One unit will convert 1.0 µmole of dihydrofolic acid to tetrahydrofolic acid in 1 minute at pH 7.5 at 22 °C. (This is equivalent to the conversion of NADPH to NADP)

The equation refers to a reaction volume of 1 ml.

Notes: When measuring the activity in a cell lysate, take into consideration the high background activity. Estimation of the background is performed by an inhibition reaction with MTX at a concentration giving maximal inhibition. The recommended starting point is MTX at a concentration 2–4 fold higher than the concentration used for inhibition reactions of the purified enzyme. The residual activity is the background activity, which should be subtracted from the enzyme activity.

An example for purified DHFR activity calculation:

Sample Type	Sample mg/ml	Sample volume, ml	Dilution factor	OD/min	µmole/min/ml sample	µmol/min /mg protein
Blank	–	–	–	0.0008	–	–
Sample	0.032	0.02	1	0.0213	0.0833	2.604

Troubleshooting

Several parameters can affect the enzyme activity and therefore should be taken into consideration:

1. Enzyme – measurement of the activity of a concentrated enzyme can result in a non-linear slope. Perform several dilutions of the enzyme and measure the activity of the diluted enzyme in order to find the linear range.

2. Inhibitors - various solvents, in which certain inhibitors are dissolved, can reduce the enzyme activity. It is recommended the ethanol and methanol concentration in the reaction mixture should not exceed 0.1%. DMSO inhibits DHFR activity at any concentration.

3. Detergents – Cell/tissue extraction buffers other than CelLytic M or MT may contain detergents at concentrations that interfere with the enzyme activity. We recommend performing preliminary tests in order to verify the enzyme buffer is suitable for the detection of the enzyme activity. The final concentration of CHAPS should not exceed 0.1%, Triton™ X-100 should not exceed 2%, and TWEEN® 20 should not exceed 1%.

References

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KH,DS,NDH,PHC,MAM 08/18-1