



Product Information

Dihydrofolate Reductase Assay Kit

Product Code **CS0340**
Storage Temperature $-20\text{ }^{\circ}\text{C}$

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-tetrahydrofolate (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 anti-tumor 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 1-ml tests.

Dihydrofolate Reductase (DHFR) (Product Code D 6566)	0.1 unit
Assay Buffer 10X for DHFR (Product Code A 5603)	30 ml

Dihydrofolic acid (DHFR substrate) (Product Code D 7006)	3 x 10 mg
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Methotrexate [(+)-Amethopterin; MTX] (DHFR inhibitor) (Product Code A 6770)	2 x 10 mg
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NADPH (β -Nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt) (Product Code N 6505)	25 mg
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Equipment required but not provided

- Temperature controlled UV/visible spectrophotometer.
- 1 ml quartz cuvette (Product Code S-10SM).
- Ultrapure water: $17\text{ M}\Omega\cdot\text{cm}$, or equivalent.

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

Note: Use ultrapure water ($17\text{ M}\Omega\cdot\text{cm}$ or equivalent).

1. Dihydrofolic acid (substrate)
Prepare a 10 mM stock solution at pH 7.5 by the addition of 2.2 ml Assay Buffer 10X to the dihydrofolic acid bottle (i.e., add 2.2 ml Assay Buffer 10X to 10 mg powder), and mix well. Aliquot the 10 mM dihydrofolic acid stock solution and store at $-20\text{ }^{\circ}\text{C}$. The solution is stable for 5 days at $-20\text{ }^{\circ}\text{C}$. 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 Assay Buffer 10X to 9.8 ml 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 10X to the bottle. Mix well. Aliquot the 10 mM methotrexate stock solution into working aliquots and store it at -20°C . The solution is stable for at least one month at -20°C .

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

- Dilution to a concentration of $100\ \mu\text{M}$ methotrexate; add $10\ \mu\text{l}$ of the 10 mM stock solution to $990\ \mu\text{l}$ Assay Buffer 10X. Mix well.
- Dilution to a concentration of $10\ \mu\text{M}$ methotrexate; add $100\ \mu\text{l}$ of the $100\ \mu\text{M}$ MTX solution to $900\ \mu\text{l}$ Assay Buffer 10X. Mix well.
- Dilution to a concentration of $1\ \mu\text{M}$ methotrexate; add $100\ \mu\text{l}$ of the $10\ \mu\text{M}$ MTX solution to $900\ \mu\text{l}$ Assay Buffer 10X. Mix well.

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

4. Assay Buffer 1X

Dilute the Assay Buffer 10X for DHFR ten fold in deionized water (i.e. add 5 ml Assay Buffer 10X for DHFR to 45 ml water). Keep at room temperature.

5. Sample

It is recommended to:

- Prepare cell lysates using the CellLytic™ M mammalian cell lysis/extraction reagent (Product Code C 2978). Use extracts at a final concentration of 0.8-2 mg protein /ml reaction mixture.
- Prepare tissue extracts using the CellLytic MT™ Cell Lysis Reagent (Product Code C 3228). Use extracts at a final concentration of 0.5-1 mg protein /ml reaction mixture.

6. DHFR

The amount of DHFR supplied in the kit is approximately 0.1 units (lot specific data is on the component label). 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 label in the following formula:

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

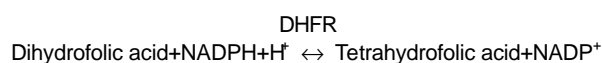
Storage/Stability

The kit is shipped on dry ice. The kit components should be stored at -20°C except for the Assay Buffer 10X for DHFR that can be stored at $2-8^{\circ}\text{C}$.

Procedure

Principle of assay for DHFR activity⁵

The assay is based on the ability of Dihydrofolate reductase to catalyze the reversible NADPH-dependent reduction of dihydrofolic acid to tetrahydrofolic acid.



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 x1	Sample	NADPH	Dihydrofolic acid	Inhibitor
Blank 1	1000 μ l - sample	1.5×10^{-3} units DHFR or $y \mu$ l cell extract	6 μ l	-----	-----
Blank 2	1000 μ l - sample	1.5×10^{-3} units DHFR or $y \mu$ l cell extract	-----	5 μ l	-----
Reaction 1 Activity of the supplied enzyme	1000 μ l – the DHFR volume	1.5×10^{-3} units DHFR	6 μ l	5 μ l	-----
Reaction 2 Inhibition by MTX	1000 μ l – the DHFR volume	1.5×10^{-3} units DHFR	6 μ l	5 μ l	x μ l MTX
Reaction 3 Inhibition by the tested inhibitor	1000 μ l – the DHFR volume	1.5×10^{-3} units DHFR	6 μ l	5 μ l	x μ l Inhibitor tested
Reaction 4 Activity of the sample enzyme	1000- $y \mu$ l	$y \mu$ l sample	6 μ l	5 μ l	-----
Reaction 5 Inhibition of the sample enzyme by MTX	1000-($y+x$) μ l	$y \mu$ l sample	6 μ l	5 μ l	x μ l MTX
Reaction 6 Inhibition of the sample enzyme by the tested inhibitor	1000-($y+x$) μ l	$y \mu$ l sample	6 μ l	5 μ l	x μ l Inhibitor tested

x = amount of tested inhibitor

y = amount of the sample enzyme (not to exceed 100 μ l).

Table 2: MTX concentrations for inhibition

Stock Conc.	Volume to Rx*	Final conc. in Rx
1 μ M	5-15 μ l	5-15 nM
10 μ M	2-10 μ l	20-100 nM
100 μ M	2-10 μ l	200-1000 nM

* Rx=Reaction mixture

Procedure

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

1. Set the spectrophotometer at 340 nm and 22 °C, kinetic program (reading every 15 seconds for 2.5 minutes).
2. Add Assay Buffer 1X to the test microcentrifuge tube according to the reaction scheme and to the test being performed.
3. Add DHFR enzyme or the sample to the appropriate tube, and mix well.

Note: 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.

Inhibition by MTX: For the amounts of MTX to be used in the reaction, see table 2.

5. Transfer the content 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.
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.

Note: 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.

Activity calculation

Measure the decrease in ΔOD obtained during 2.5 min. as $\Delta\text{OD}/\text{min}$. (note that the output of the kinetics program is $\Delta\text{OD}/\text{min}$.) Calculate the specific activity by the formula:

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

where:

DOD/min_{blank}: $\Delta\text{OD}/\text{min}$. for the blank, from the spectrophotometer readings

DOD/min_{sample}: $\Delta\text{OD}/\text{min}$. for the reaction, from the spectrophotometer readings

12.3: extinction coefficient (ϵ , $\text{mM}^{-1}\text{cm}^{-1}$) for the DHFR reaction at 340 nm.⁶

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 $\mu\text{mol}/\text{min}/\text{mg}$ 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 $^{\circ}\text{C}$. (This is equivalent to the conversion of NADPH to NADP)

The equation refers to a reaction volume of 1 ml.

Note: When measuring the activity in cell lysate, take into consideration there is a high background activity. Estimation of the background is performed by 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.

Results

An example for purified DHFR activity calculation:

Sample Type	Sample mg/ml	Sample volume, ml	Dilution factor	OD/min	$\mu\text{mole}/\text{min}/\text{ml}$ sample	$\mu\text{mol}/\text{min}/\text{mg}$ 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 in, can reduce the enzyme activity. It is recommended that 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 that 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 and NP-40 should not exceed 2%, and Tween[®]-20 should not exceed 1%.

References

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5. Mathews, C. K., et al., Dihydrofolate reductase. *Methods Enzymol.*, **6**, 364-368 (1963).
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