

● TOYOBO ENZYMES ●  
(Diagnostic Reagent Grade)

# DIAPHORASE

*from Clostridium sp.*

NAD(P)H:(acceptor)oxidoreductase (EC 1.6.99. —)



## PREPARATION and SPECIFICATION

Appearance	: Yellowish amorphous powder, lyophilized
Activity	: Grade III 30U/mg-solid or more (containing approx. 15% of stabilizers)
Contaminants	: Myokinase $\leq 5.0 \times 10^{-1}\%$ NAD(P)H oxidase $\leq 5.0 \times 10^{-1}\%$
Stabilizers	: FMN, NAD(P)H

## PROPERTIES

Stability	: Stable at $-20^{\circ}\text{C}$ for at least one year	(Fig.1)
Molecular weight	: 24,000 <sup>1)</sup>	
Michaelis constants	: $2.0 \times 10^{-6}\text{M}$ (NADH), $6.0 \times 10^{-6}\text{M}$ (NADPH)	
Structure	: One mol of FMN per mol of enzyme <sup>1)</sup>	
Inhibitor	: N-Ethylmaleimide	
Optimum pH	: 8.5	(Fig.2)
Optimum temperature	: $50^{\circ}\text{C}$	(Fig.3)
pH Stability	: pH 7.5 ( $30^{\circ}\text{C}$ , 3hr)	(Fig.4)
Thermal stability	: below $30^{\circ}\text{C}$ (pH 7.5, 30min)	(Fig.5)
Substrate specificity	: Either NADH or NADPH can be used as a reductant. The catalytic ratio (NADPH/NADH) is 0.6 in the assay method. Neither oxygen nor cytochrome C can be utilized as a hydrogen acceptor.	

## APPLICATIONS

This enzyme is useful for colorimetric determination of NAD(P)H and many dehydrogenases when coupled with various dyes which act as hydrogen acceptors from NAD(P)H.

## ASSAY

### Principle:



The reduction of DCPIP (2,6-dichlorophenol-indophenol) is measured at 600nm by spectrophotometry.

### Unit definition:

One unit causes the decrease of one unit absorbance (1.0) of DCPIP per minute under the conditions described below.

### Method:

#### Reagents

- A. Buffer solution : 0.2M Tris-HCl, pH 7.5  
 B. NADH solution : 6.0mM (Prepare freshly and store on ice)  
 C. DCPIP solution : 1.2mM [3.9mg DCPIP · 2H<sub>2</sub>O/10ml of H<sub>2</sub>O] (Should be prepared fresh)  
 D. Enzyme diluent : Buffer solution(A) containing 0.1% of bovine serum albumin.

#### Procedure

- Prepare the following reaction mixture in a cuvette (d=1.0cm) and equilibrate at 25°C for about 5 minutes.
 

2.4ml	H <sub>2</sub> O	
0.3ml	Buffer solution	(A)
0.1ml	NADH solution	(B)

Concentration in assay mixture	
Tris buffer	27 mM
NADH	0.20mM
DCPIP	40 μM
BSA	ca.33 μg/ml

- Add 0.1ml each of the enzyme solution\* and DCPIP solution (C) in this order and mix by rapid inversion.
- Record the decrease of optical density at 600nm against water for 2 to 3 minutes in a spectrophotometer thermostated at 25°C, and calculate the ΔOD per minute from the initial linear portion of the curve (ΔOD test).

At the same time, measure the blank rate (ΔOD blank) by using the same method as the test except that the enzyme diluent is added instead of the enzyme solution.

- \* Dissolve the enzyme preparation in ice-cold buffer solution (A) (ca.1.0% solution), dilute to 0.4–0.8U/ml with ice-cold enzyme diluent (D) and store on ice.

#### Calculation

Activity can be calculated by using the following formula :

$$\text{Volume activity (U/ml)} = \frac{\Delta \text{OD}/\text{min} (\Delta \text{OD test} - \Delta \text{OD blank}) \times \text{df}}{1.0 \times V_s} = \Delta \text{OD}/\text{min} \times 10 \times \text{df}$$

$$\text{Weight activity (U/mg)} = (\text{U/ml}) \times 1/C$$

V<sub>s</sub> : Sample volume (0.1ml)

1.0 : Unit absorbance at 600nm due to unit definition

df : Dilution factor

C : Enzyme concentration in dissolution (c mg/ml)

## REFERENCES

- F.Kaplan, P.Setlow and N.O.Kaplan; *Arch,Biochem.Biophys.*, 132, 91 (1969).

Table 1. Effect of Various Chemicals on Diaphorase

[The enzyme dissolved in 0.2M Tris-HCl buffer, pH 7.5 (40U/ml) was incubated with each chemical at 25°C for 1hr.]

Chemical	Concn.(mM)	Residual activity	Chemical	Concn.(mM)	Residual activity
None	—	100%	NaF	2.0	102
Metal salt	2.0		NaN <sub>3</sub>	2.0	100
MgCl <sub>2</sub>		99	EDTA	5.0	99
CaCl <sub>2</sub>		102	O-Phenanthroline	2.0	99
Ba(OAc) <sub>2</sub>		100	$\alpha, \alpha'$ -Dipyridyl	1.0	101
FeCl <sub>2</sub>		90	Borate	5.0	100
CoCl <sub>2</sub>		101	IAA	2.0	99
MnCl <sub>2</sub>		96	NEM	2.0	100
ZnCl <sub>2</sub>		100	Hydroxylamine	2.0	101
Cd(OAc) <sub>2</sub>		100	Triton X-100	0.10%	106
NiCl <sub>2</sub>		99	Brij 35	0.10%	104
CuSO <sub>4</sub>		87	Tween 20	0.10%	107
Pb(OAc) <sub>2</sub>		88	Span 20	0.10%	101
AgNO <sub>3</sub>		103	Na-choleate	0.10%	99
HgCl <sub>2</sub>		103	SDS	0.05%	32
PCMB	2.0	90	DAC	0.05%	32
MIA	1.0	100			

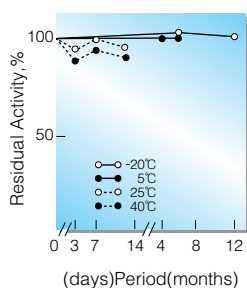


Fig. 1. Stability (Powder form)  
[kept under dry conditions]

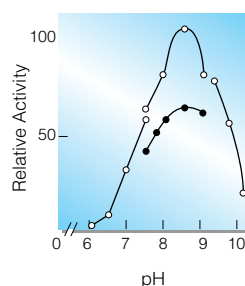


Fig. 2. pH-Activity

[The enzyme reaction was carried out with NADH(○)or NADPH(●).buffer used: pH6.0-7.5, 10mM Veronal-acetate; pH7.5-9.0, 33mM Tris-HCl; pH9.2-10.2, 33mM NH<sub>4</sub>OH-NH<sub>4</sub>OH

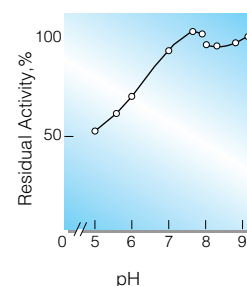


Fig. 4. pH-Stability

[30°C,3hr-treatment with the following buffer solution: pH6.0-7.5, 10mM Veronal-acetate; pH7.5-9.0, 33mM Tris-HCl

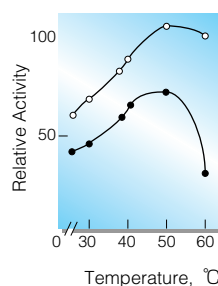


Fig. 3. Temperature activity

[The enzyme reaction was carried out with NADH(○)or NADPH(●). 20mM Tris-HCl buffer, pH8.5

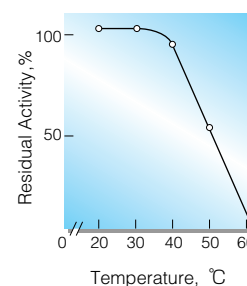


Fig. 5. Thermal stability

[30min-treatment with 0.2M Tris-HCl buffer, pH7.5 enzyme concentration:40U/ml

## 活性測定法 (Japanese)

### 1.原理

$\text{NADH} + \text{H}^+ + \text{DCPIP} \xrightarrow{\text{diaphorase}} \text{NAD}^+ + \text{Leucodye}$   
 DCPIP(2,6-dichlorophenol-indophenol)の還元量を  
 600nmの吸光度の変化で測定する。

### 2.定義

下記条件下で1分間に600nmの吸光度を1.0減少させる  
 酵素量を1単位(U)とする。

### 3.試薬

- A. 0.2M Tris-HCl緩衝液, pH7.5
- B. 6.0mM NADH水溶液(用時調製, 氷冷保存)
- C. 1.2mM DCPIP水溶液〔3.9mgのDCPIP・2H<sub>2</sub>O  
(MW=326.11)を10mlの蒸留水で溶解する〕(用時  
調製)

酵素溶液：酵素標品を予め氷冷した試薬Aに溶解し(約  
 1.0%溶液), 予め氷冷した0.1%牛血清アル  
 ブミン(BSA)を含む試薬Aで0.4~0.8U/mlに  
 希釈し, 氷冷保存する。

### 4.手順

- ①下記反応混液をキュベット(d=1.0cm)に採り, 25°Cで  
 約5分間予備加温する。
 

2.4ml	蒸留水	(A)
0.3ml	Tris-HCl	(B)
0.1ml	NADH水溶液	(C)
- ②酵素溶液, DCPIP水溶液(C)各0.1mlをこの順序で添  
 加し, ゆるやかにかつ速やかに混和後, 水を対照に25°C  
 に制御された分光光度計で600nmの吸光度変化を2  
 ~3分間記録し, その初期直線部分より1分間当りの  
 吸光度変化を求める(ΔODtest)。
- ③盲検は反応混液①に酵素希釈液(0.1%BSAを含む試  
 薬A), DCPIP水溶液(C)各0.1mlを加え, 上記同様に操  
 作を行って1分間当りの吸光度変化を求める(Δ  
 ODblank)。

### 5.計算式

$$\text{U/ml} = \frac{\Delta \text{OD}/\text{min} (\Delta \text{OD test} - \Delta \text{OD blank}) \times \text{希釈倍率}}{1.0 \times 0.1(\text{ml})}$$

$$= \Delta \text{OD}/\text{min} \times 10 \times \text{希釈倍率}$$

$$\text{U/mg} = \text{U/ml} \times 1 / \text{C}$$

1.0 : 活性定義に基づいて定められた600nmにお  
 ける単位吸光度

C : 溶解時の酵素濃度(c mg/ml)