(Diagnostic Reagent Grade)

T-227

DIAPHORASE (NADH) [DIII]

from *Bacillus megaterium*
(NADH: acceptor oxidoreductase, EC 1.6.5.2)

$$NADH + H^+ + A \longrightarrow NAD^+ + AH_2$$

A : Hydrogen acceptor

Preparation and Specification

Appearance : Yellow to yellow brownish lyophilized powder
Specific activity : More than 70 U/mg solid

Properties

Substrate specificity	: See Table 1	
Molecular weight	: 50 kDa (SDS-PAGE), 160 kDa (gel filtration)	
Isoelectric point	: pH 4.2	
Michaelis constant	: NADH 5.5 × 10^{-4}M	
Optimum pH	: 8.0–9.0	Figure 1
pH stability	: 6.0–9.0 (37℃, 60 min)	Figure 2
Optimum temperatute	: 40–45℃	Figure 3
Thermal stability	: Stable at 50℃ and below (pH 8.0, 10 min)	Figure 4

Applications for Diagnostic Test

This enzyme is useful for enzymatic determination of **reduced NAD.**

$$NADH + H^+ + NTB \xrightarrow{\text{DIII}} \text{Formazane dye} + NAD^+$$

Table 1. Substrate specificity

Substrate	Relative activity (%)
NADH	100
NADPH	16

Fig.1 pH Optimum

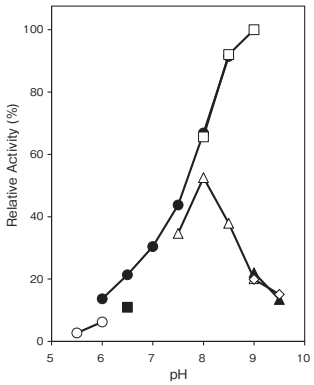


Fig.2 pH Stability

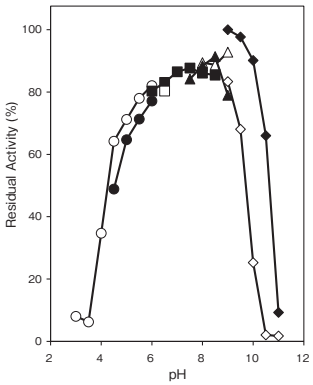


Fig.3 Optimum Temperature

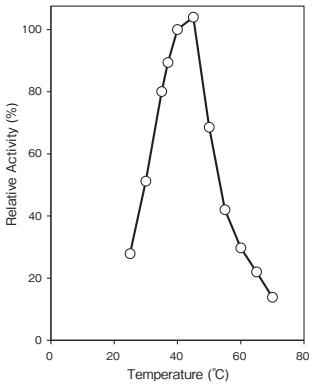
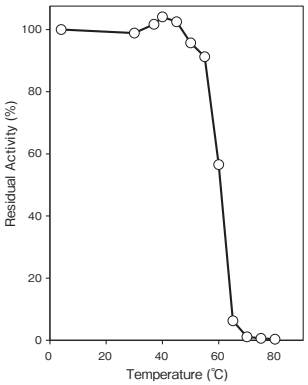


Fig.4 Thermal Stability



D

Assay

■ Principle

The assay is based on the increase in absorbance at 550 nm as the formation of formazane dye ($NTBH_2$) proceeds in the following reaction:

$$NADH + H^+ + NTB \xrightarrow[\text{DI II}]{} NAD^+ + \text{Formazane dye}$$

NADH: Nicotineamido adenine dinucleotide
NTB: Nitrotetrazolium blue

■ Unit definition

One unit is defined as the amount of enzyme which oxidizes 1 μmole of NADH to NAD^+ per minute at 37℃ under the conditions specified in the assay procedure.

■ Reagents

1. Reaction mixture

0.2 M KH_2PO_4–NaOH buffer pH 8.0	0.50 ml
0.25% (W/V) NTB solution	0.10 ml
1% (W/V) BSA solution	0.10 ml
10 mM NADH solution	0.10 ml
Distilled water	0.20 ml

2. Reaction stopper
   0.1 N HCl solution
3. Enzyme dilution buffer
   0.1 M KH_2PO_4–NaOH buffer pH 8.0 containing 0.1% (W/V) BSA
4. Reagents
   NTB: Dojindo Laboratories # 344-02033
   BSA: Millipore Fraction V pH5.2 #81-053
   NADH ($2Na \cdot 3H_2O \cdot$reduced form):
   Kyowa Hakko Co. Ltd.

■ Enzyme solution

Accurately weigh about 20 mg of the sample and add enzyme dilution buffer to make a total of 20 ml. Dilute it with enzyme dilution buffer to adjust the concentration as required.

■ Procedure

1. Pipette accurately 1.0 ml of reaction mixture into a small test tube and preincubate at 37℃.
2. After 5 min, add exactly 100 μl of enzyme solution and mix to start the reaction at 37℃.
   ※ In the case of a test blank, add 100 μl of enzyme dilution buffer in place of enzyme solution.
3. At 10 min after starting the reaction, add 2.0 ml of the reaction stopper to stop the reaction.
4. Measure the absorbance at 550 nm.

Absorbance	sample	: As
	blank	: Ab

$\triangle A = (As - Ab) \leqq 0.370$ Abs

■ Calculation

$$\text{Activity (U/mg of powder)} = \frac{\triangle A/10}{12.4} \times \frac{3.10}{0.10} \times \frac{1}{X}$$

12.4 : millimolar extinction coefficient of Formazane dye at 550 nm (cm^2/ μmole)
10 : reaction time (min)
3.10 : final volume (ml)
0.10 : volume of enzyme solution (ml)
X : concentration of the sample in enzyme solution (mg/ml)

Storage

Storage at − 20℃ in the presence of a desiccant is recommended. Enzyme activity will be retained for at least one year under this condition.

References

1. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem., **193**, 265–275.
2. Gerlo, E. and Charlier, J. (1975) Eur. J. Biochem., **57**, 40–467.
3. Jablonski, E. and DeLuca, M. (1977) Biochemistry, **16,** 2932–2936.
4. Watanabe, H. and Hastings, J. W. (1982) Mol. Cell. Biochem., **44,** 181–187.

D

DI Ⅱ活性測定法 (Japanese)

Ⅰ. 試薬液

1. 反応試薬混合液

0.2M KH_2PO_4–NaOH 緩衝液 pH8.0	0.50 ml
0.25% (W/V) NTB 溶液	0.10 ml
1% (W/V) BSA 溶液	0.10 ml
10mM NADH 溶液	0.10 ml
精製水	0.20 ml

2. 反応停止液
   0.1N HCl 液
3. 酵素溶解希釈用液
   0.1% (W/V) BSA を含む 0.1M KH_2PO_4–NaOH 緩衝液 pH8.0
4. 試薬
   NTB (ニトロテトラゾリウムブルー):
   同仁化学製 #344–02033
   BSA: Millipore 製 Fraction V pH5.2 #81–053
   NADH (2Na･3H_2O･還元型):協和発酵製

Ⅱ. 酵素試料液

検品約 20mg を精密に量り、酵素溶解希釈用液に溶解して全容 20ml とする。
その液を酵素溶解希釈用液で適宜希釈する。

Ⅲ. 測定操作法

1. 小試験管に反応試薬混合液 1.0ml を正確に分注し、37℃で予備加温する。
2. 5 分経過後、酵素試料液 100 μl を正確に加えて混和し、37℃で反応を開始する。
   ※盲検は酵素試料液の代わりに酵素溶解希釈用液 100 μl を加える。
3. 10 分経過後、反応停止液 2.0ml を正確に加えて混和し、反応を停止させる。
4. 550nm における吸光度を測定する。
   求められた吸光度を試料液は As、盲検液は Ab とする。

$$\Delta A = (As - Ab) \leqq 0.370\ Abs$$

Ⅳ. 計算

$$\text{活性 (U/mg)} = \frac{\Delta A/10}{12.4} \times \frac{3.10}{0.10} \times \frac{1}{X}$$

12.4 : $NTBH_2$ の 550nm におけるミリモル分子吸光係数 (cm^2/ μmole)
10 : 反応時間 (min)
3.10 : 反応総液量 (ml)
0.10 : 反応に供した酵素試料液量 (ml)
X : 酵素試料液中の検品濃度 (mg/ml)