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The 96 well UV plate had been changed to a split module type (Lot.030 ~). Since all wells can be separated by the unit of one well, it can be used without uselessness and economically.
Note: The optical path length is different from the previous plate.
(Refer to VI. Protocols 4. Assay procedure section.)

I. Description:

Biosynthesis of fatty acids, one of the processes of carbon dioxide fixation in the living organisms, takes place by using acetyl-CoA as a direct starting source. Among the synthesized fatty acids, the long-chain fatty acids in particular have surface-activity and cytotoxicity in the free state. Therefore they are not allowed to accumulate in the cells. To avoid this accumulation, the long-chain fatty acids rapidly form esters with glycerol 3-phosphate to generate triacylglycerols(fat) when a large amount of long-chain fatty acids are synthesized in the organisms. In the liver, glycerol 3-phosphate is generated by direct phosphorylation of glycerol, while in the muscles and adiopocytes, it is generated by reducing dihydroxyacetone phosphate, an intermediate product in the glycolytic pathway, with NADH (Fig. 1). Accordingly, lipid synthesis does not occur without glucose in the muscles and adipocytes even if there exist a highest concentration of fatty acids.

Enzyme which catalyzes the reversible conversion between dihydroxyacetone phosphate and glycerol 3-phophate using NAD as a coenzyme is called glycerol 3-phosphate dehydrogenase: GPDH : NAD^+ 2 - oxidoreductase (EC.1.1.1.8) and regarded as a representative marker for adipocyte differentiation from the progenitor cells. In adipocytes and muscles, glycerol 3-phosphate dehydrogenase : NAD^+ 2 - oxidoreductase (EC.1.1.1.8) is found in the cytosol and another glycerol 3-phosphate dehydrogenase : (acceptor) oxidoreductase (EC. 1.1.99.5) using FAD as a coenzyme is responsible for the reverse reaction of glycerol 3-phosphate to dihydroxyacetone phosphate in the mitochondria. These two enzymes exchange the substrates for the reactions to form the glycerol phosphate cycle.

Takara's Glycerol 3-phophate dehydrogenase (GPDH) activity assay kit is designed to determine the activities of GPDH (EC.1.1.1.8), an enzyme that generates glycerol 3-phophate from dihydroxyacetone phosphate using NAD as a coenzyme, in the samples extracted from cells. The GPDH activity is known to rapidly increase on differentiation of adipocyte progenitor cells into adipocytes. In the experimental systems, in which established lines of or primary adipocyte progenitor cells are differentiated, the GPDH activity has been used as a major indicator of the differentiation for screening of the differentiation inhibitors or for elucidating the mechanism of the differentiation. This kit is designed for assays using 96-well plates, which enables determination of a large number of samples at a time. This product allows sample preparation and determination to be completed in a short time, which reduces the risk of enzyme inactivation and ensures acquisition of reproducible data.

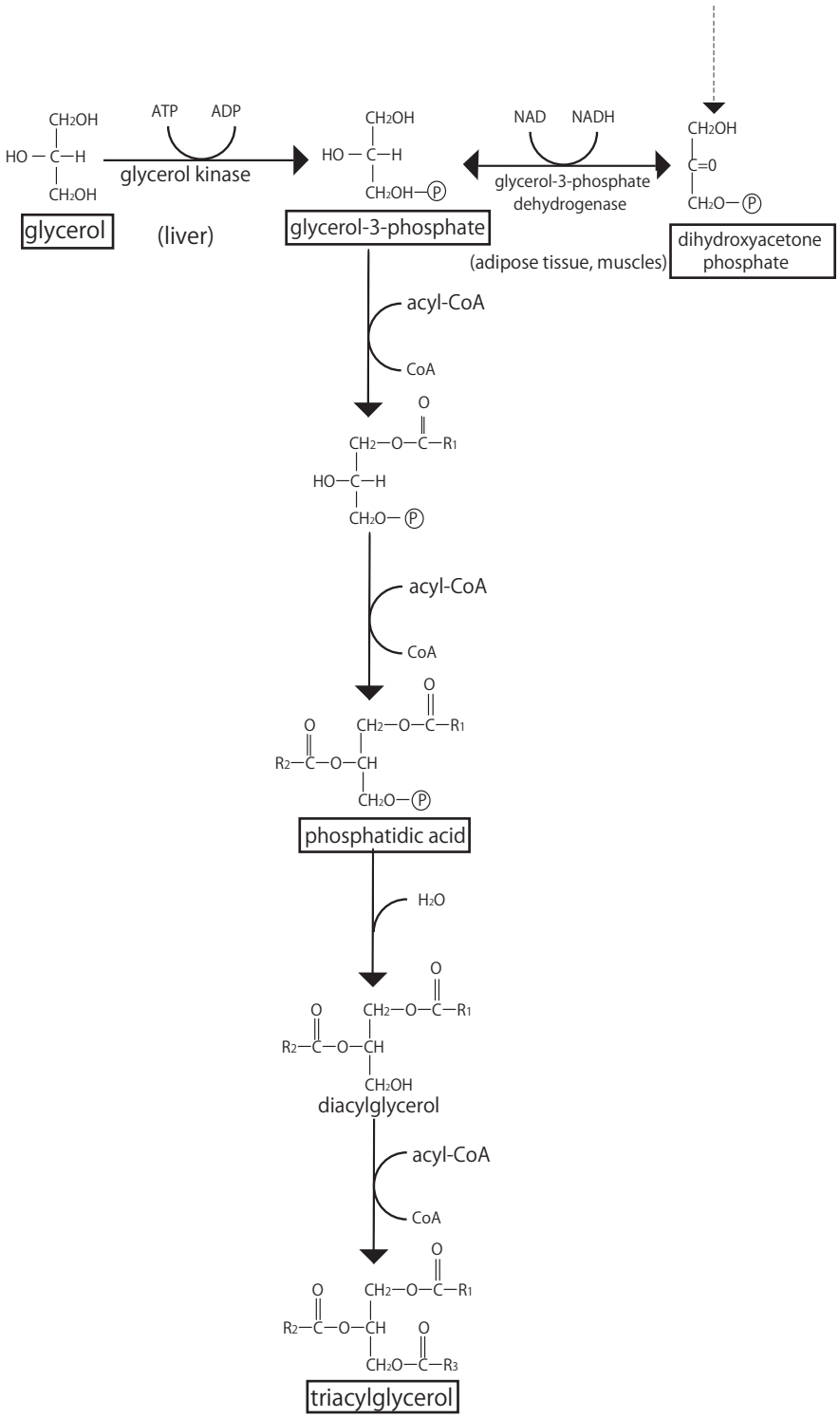


Fig. 1 The pathway for lipid biosynthesis[1)]

Lipid biosynthesis occurs through sequential binding of the fatty acids activated as acyl-CoAs to glycerol-3-phosphate. In the liver, glycerol-3-phosphate is generated from glycerol by the action of glycerol kinase. However, due to the absence of glycerol kinase in the adipose tissue and muscles, glycerol-3-phosphate is generated from glucose by way of dihydroxyacetone phosphate.

II. Features:

- Preparation of reagents is easy.
- The kit contains a 96-well plate exclusively for determination of the UV-transmittancy, and separate purchase of plates is unnecessary.
- The 96 well plate contained in the kit is a split module type that can be separated, so it can be used without uselessness and economically.
- With a 96-well microtiter-plate reader, the activity of a large number of samples can be compared and determined at a time.

III. Principle:

GPDH activity is determined as decrease in NADH by measuring the decrease of absorbance at 340 nm.

Dihydroxyacetone phosphate + NADH + H^+
→ Glycerol-3-phsphate + NAD^+

The GPDH activity (unit/ml) in the test samples can be calculated from the following formula:

$$\text{GPDH activity (unit/ml)} = \frac{\Delta OD_{340} \times A\ (ml) \times \text{Dilution ratio of the test sample}}{6.22 \times B\ (ml) \times C\ (cm)}$$

ΔOD_{340}: Decrease in the absorbance at 340 nm per minute
A (ml): Total reaction volume
B (ml): The volume of enzyme solution (diluted sample) added
C (cm): Optical path length of the cell used*
6.22: Millimolar absorption coefficient of NADH molecules

* For the optical path length of the 96-well plate supplied in this kit, see the protocol on page 7, Note 2. One unit is defined as the amount of enzyme required for consumption of 1 µmol of NADH for one minute at 30℃ .

IV. Kit components: (for assaying the samples in a 96-well plate)

(1) 96 well UV plate: split module type..... one plate
(2) GPDH Substrate (Lyophilized) ..... for 11 ml × 1 bottle
(3) Enzyme Extraction Buffer ..... 11 ml × 1 bottle
(4) Dilution Buffer ..... 11 ml × 2 bottles

V. Storage:

-20℃ or below

VI. Protocols:

This protocol is optimized for assay using the 96 well UV plate (1) supplied in this kit. (Conventional spectrophotometers and quartz cells can be used for the activity assay. If you use your current absorbance spectrometer, you may need to modify the protocol. See Q&A)

1. Required equipment and reagents

- 2-mercaptoethanol (2-ME)
- Micropipettes
- 96-well microtiter-plate for diluting samples
- Distilled water
- Phosphate buffered saline (PBS(-))
  (PBS Tablets: TaKaRa Code T900 is recommended)
- 96-well microtiter-plate reader (with a filter for 340 nm)

For extraction of crude enzyme solutions from test samples, prepare the following equipment, if necessary:

- Incubator
- High speed centrifuge
- Low speed centrifuge

2. Preparation of reagents

[GPDH Substrate (2)]

Dissolve all the product in the bottle in 11 ml of distilled water to use as the substrate solution. This product contains dihydroxyacetone phosphate, NADH and optimal buffer. The solution is stable for two weeks at 4℃ , and for one month at −80℃ after dissolving.

[Enzyme Extraction Buffer (3)]

Thaw at room temperature. The buffer is stable for one year at 4℃ after thawing.

[Dilution Buffer (4)]

Thaw at room temperature. The buffer is stable for one year at 4℃ after thawing. Add 2-ME at the final concentration of 1mM into the necessary volume of this buffer before using. For example, when commercially available 2-ME is used at the concentration of 14.4 M, add 5 µl of 2-ME into 715 µl of distilled water to be 0.1 M, and subsequently dilute 100-fold with this buffer before use. Since 2-ME is not stable, make preparation and addition of this buffer before each use.

3. Preparation of samples

3-1. Preparation from cells cultured in the 96-well plate

1. Remove culture medium and wash twice with PBS (-) (the washing step can be omitted if the cells are easily detached from the plate). Then, add 100-200 µl of "Enzyme Extraction Buffer (3)" to each well. At this step, if necessary, the solution can be sampled for quantitative determination of proteins. Immediately after the extraction, add an equal volume of "Dilution Buffer (4)" supplemented with 2-ME to the sample to prepare two-fold diluent.

2. Determine the GPDH activity in accordance with the assay procedure below after serial dilution to approximately 2^1 - 2^7 with "Dilution Buffer (4)" supplemented with 2-ME.

Note) Centrifuge (at 10,000 rpm for 5 min at 4℃) the sample to separate the aqueous fraction from the fat fraction, if the sample solution is too turbid. Use the aqueous fraction as the sample.

3-2. Preparation from cells cultured in the 90 mm φ plate.

1. Remove culture medium and wash twice with PBS (-). Add 1 ml of "Enzyme Extraction Buffer (3)". Collect the cells with scraper and transfer into a microtube. Vortex the microtube briefly and then centrifuge (at 10,000 rpm for 5 min at 4℃) to separate the aqueous fraction from the fat fraction. The aqueous fraction is used as the sample. At this step, if necessary, the solution can be sampled for quantitative determination of proteins. Immediately after the extraction, add an equal volume of "Dilution Buffer (4)" supplemented with 2-ME to the sample to prepare two-fold diluent.

2. Determine the GPDH activity in accordance with the assay procedure below after serial dilution to approximately 2^1-2^7 with "Dilution Buffer (4)" supplemented with 2-ME.

3-3. Stability of the prepared samples

The samples extracted with Enzyme Extraction Buffer (3) are stable on ice for up to one hour if they are two-fold diluted with Dilution Buffer (4) supplemented with 2-ME.

4. Assay procedure

1. Dispense 100 µl of the substrate solution into each well of a "96 well UV plate split module type (1)" and preincubate the plate at 30℃ .

2. Add 25 µl of a diluted sample and agitate the plate. Determine the decrease in absorbance at 340 nm at 30℃ , using a 96-well microtiter-plate reader, to calculate the change of the absorbance per minute (ΔOD_{340}).
To minimize time differences among the samples till determination of absorbance, it is recommended to dispense 50 µl of the samples previously into each well of another 96-well plate on ice, and take up 25 µl of the sample and add to corresponding wells of the 96-well UV plate split module type using a 8-channel pipette.
When a large number of samples, such as more than 40 samples, should be treated, put the sample in the order of descending ratios of dilution. (The kinetics determination program may be helpful, if it is installed in the plate reader.)

Note 1) In general, the reaction will complete in 2-10 minutes after the sample is added. In a case where the enzyme activity is too high, the reaction will complete in a short time. In such case, appropriate serial dilution of the samples should be used, and ΔOD_{340} is calculated using the measurements obtained from the reactions proceeding linearly. When the kinetics determination program is used, ΔOD_{340} should be calculated while corr. coeff. is in the range of 0.98 - 1.0. Assays according to this method are negative kinetic assays, since reduction of absorbance is monitored.

(Example of the determination program)
measuring time: 15 min., measuring interval: 60 sec. (16 points of measurement), OD limit: -0.5

Note 2) Since this kit is designed for the relative comparison of GPDH activities obtained from a large number of samples at a time, the accurate "optical path length (cm)" of 96 well UV plate split module type (1) is not provided. If users desire to calculate precise values of the activity, it is recommended to use the cells whose optical path length is specified.
When the solutions are added into the well of 96 well UV plate split module type (1) in the amounts specified in the assay procedure on page 6 (125 µl/ 0.263 cm^2), the optical path length is 0.475 cm. Then, GPDH activity is calculated from the following formula:

$$\text{GPDH activity (unit/ml)} = \frac{\Delta OD_{340} \times 0.125 \text{ ml} \times \text{dilution ratio of the sample}}{6.22 \times 0.025 \text{ ml} \times 0.475}$$

Note 3) Even if enzyme reaction appears to proceed linearly, the reaction might have reached a plateau when the enzyme activity is too high. So, use of serial dilutions of samples is recommended. Determination of ΔOD_{340} between 0.02 and 0.01 will give good reproducibility and accurate determination.

Note 4) Note that accurate results cannot be obtained if a reaction solution produces foam or bubbles. They can be removed with the cold blast from a hair dryer. Be sure not to use hot blast, which leads to inactivation of the enzyme.

Note 5) For measurement of supernatants from cultured cells, use unused medium as a blank (control).

The 96 well UV plate: split module type is a product of Greiner-Bio One GmbH (Cat.#705870). Though the well is a frustum form like Figure 2, it is calculated as a cylinder form for convenience sake.

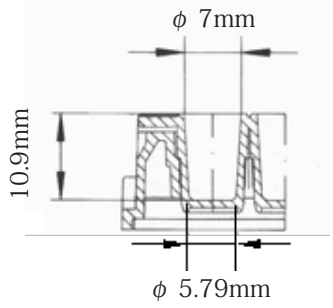
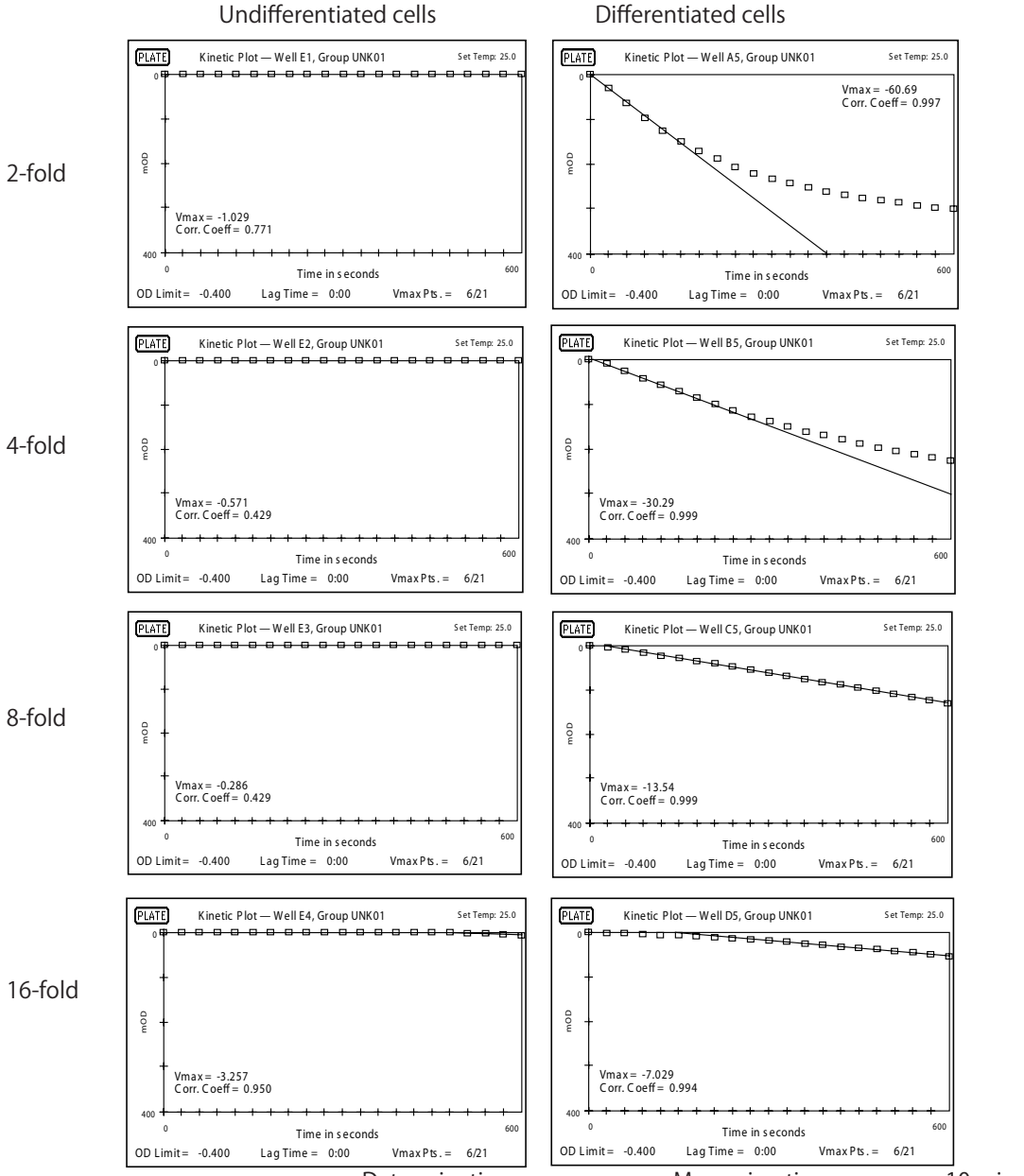


Fig. 2 The specification of 96 well UV plate

VII. Application examples:

1) Time course experiment of the reaction

The rat brown adipocyte progenitor cells (initially 1×10^4 cells/well) were cultured in the collagen-coated 24-well plate, and the cells were allowed to differentiate into adipocytes. The sample for GPDH activity assay was extracted from the cells in each well, using 250 µl of "Enzyme Extraction Buffer (3)". Serial dilutions of the enzyme ranging from 2^1 - 2^4 was prepared with "Dilution Buffer (4)" containing 2-ME. Time course of the GPDH activity was investigated.



Determination program:

Measuring time:	10 min.
Measuring interval:	30 sec.
OD limit:	-0.4

Fig. 3 Time course of the reaction

2) Linearity of dilutions

Linearity of the dilutions using rat brown adipocytes as the sample are shown in the graph.

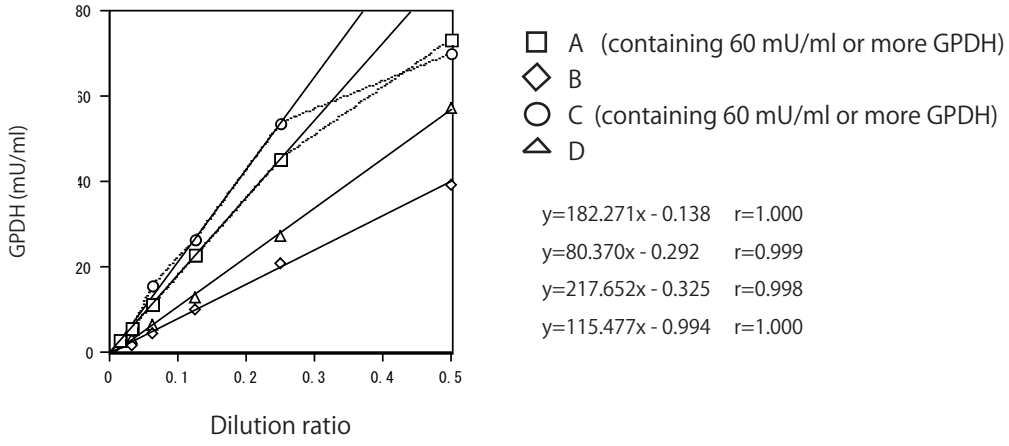


Fig. 4 Linearity of dilutions

3) Application example for investigation of the relation between adipocyte differentiation and EGF

The rat brown adipocyte progenitor cells (initially 1×10^4 cells/well) were cultured in the collagen-coated 24-well plate (three plates). In order to ensure the differentiation of the cells in a short period of time, the cells were treated for two days with 3-isobutyl-1-methylxanthine and dexamethasone as the differentiation-inducing agents. The control group was not induced to differentiate. Subsequently, the medium in each well was replaced with maintenance medium. At this stage, cells were divided into two groups, i.e. they were cultured in the presence or absence of 10 nM EGF (TaKaRa Cat.#T001). For time course determination of the GPDH activity, the sample was prepared by extraction with 150 µl/well of Enzyme Extraction Buffer (3) daily (two wells for each group) and diluted with dilution buffer to prepare serial two-fold dilutions. Daily changes in the GPDH activity were determined.

			induction of differentiation ↓		maintenance medium/presence or absence of EGF ↓				maintenance medium/removal or addition of EGF ↓		
		day 0	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8	day 11
Undifferentiated group	EGF-free	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5.95
Differentiated group	EGF-free				35.07	65.41	62.75	110.00	119.15	118.80	128.46
	EGF-free ↓ EGF-added									129.00	96.65
Differentiated group	EGF-added	0.00	0.00	7.00	30.06	27.83	10.16	0.00	0.00		
	EGF-added ↓ EGF-removed									56.86	60.98

Unit: mU/ml

* EGF showed a differentiation-inhibiting effect during adipocyte differentiation.
* GPDH activity was restored and adipocyte differentiation occured after removal of EGF.
* The effect was observed approximately one day after addition of EGF.
* As shown by the GPDH activity on day 11, the undifferentiated cells differentiated spontaneously.

Fig. 5 Relation between adipocyte differentiation and EGF

VIII. Q&A

Q1: Is it possible to use a spectrophotometer with quartz cells instead of a 96-well microtiter plate reader?

A1: Yes. Mix the substrate solution and sample in 4:1 ratio. The smaller the volume of quartz cells is, the larger number of samples can be measured. Store the samples on ice.

Q2: Is it possible to store the samples extracted from cells in the freezer for later assay? How their stability?

A2: We always determine the activity immediately after the sample preparation because a crude extract of brown adipocytes once has lost its activity due to freeze-thawing. Samples are stable on ice up to one hour. If samples are frozen to store for the experiment, divide the samples in the most concentrated state into small aliquots and store them at -80℃ . Prevent further freeze-thawing.

Q3: The enzyme reaction failed to show linearity.

A3: Excessive concentration of the enzyme is suspected. Serial dilutions should be made using Dilution Buffer containing 2-ME.

IX. Warning

This product is for research use only. It is not for diagnostic or therapeutic use in humans or animals, or for use in food, cosmetics or household articles.

X. References

1) Kozak L. P., *et al* (1974) *J. B. C*. **25**, 7775 - 7781
2) Kuri-Harcuch W., *et al* (1978) *Cell* **14**, 53 - 59.