GLYCEROL

PRODUCT INSTRUCTIONS

SKU: 700004292
K-GCROL

01/24

(70 Manual Assays per Kit) or
(700 Microplate Assays per Kit)

Play Training Video

INTRODUCTION:

In the food industry, glycerol is an important moistening agent for baked goods. It is also added to candies and icings to prevent crystallisation and as a solvent for food colours and a carrier for extracts and flavouring agents. As a product of fermentation, glycerol is monitored in the beer and wine industries, where it occurs at concentrations of approx. 1% (v/v) and is an indicator of quality. The smoothness of lotions, creams and toothpaste is due to the incorporation of glycerol. Due to its humectant properties, glycerol is sprayed on pre-processed tobacco to prevent crumbling.

This kit benefits from the presence of NADH, ATP and PEP in the form of highly stable lyophilised powder.

PRINCIPLE:

Glycerol is phosphorylated by adenosine-5'-triphosphate (ATP) to L-glycerol-3-phosphate in the reaction catalysed by glycerokinase (GK) (1).

$$(1)\quad \text{Glycerol} + \text{ATP} \xrightarrow{(GK)} \text{L-glycerol-3-phosphate} + \text{ADP}$$

The adenosine-5'-diphosphate (ADP) formed in the reaction is reconverted by phosphoenolpyruvate (PEP) with the aid of pyruvate kinase (PK) into ATP with the formation of pyruvate (2).

$$(2)\quad \text{ADP} + \text{PEP} \xrightarrow{(PK)} \text{ATP} + \text{pyruvate}$$

In the presence of the enzyme lactate dehydrogenase (LDH) pyruvate is reduced to lactate by reduced nicotinamide-adenine dinucleotide (NADH) with the production NAD^+ (3).

$$(3)\quad \text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{(LDH)} \text{lactate} + NAD^+$$

The amount of NAD^+ formed in the above reaction pathway is stoichiometric with the amount of glycerol. It is NADH consumption which is measured by the decrease in absorbance at 340 nm.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

The assay is specific for glycerol. In the analysis of pure glycerol (free of water), results of approx. 100% can be expected.

The smallest differentiating absorbance for the assay is 0.010 absorbance units. This corresponds to 0.171 mg/L of sample solution at the maximum sample volume of 2.00 mL. The detection limit is 0.342 mg/L, which is derived from an absorbance difference of 0.020 with a sample volume of 2.00 mL.

The assay is linear over the range of 0.8 to 35 µg of glycerol per assay. In duplicate determinations using one sample solution, an absorbance difference of 0.005 to 0.010 may occur. With a sample volume of 2.00 mL, this corresponds to a glycerol concentration of approx. 0.086 to 0.171 mg/L of sample solution. If the sample is diluted during sample preparation, the result is multiplied by the dilution factor, F. If, in sample preparation, the sample is weighed, e.g. 10 g/L, a difference of 0.02 to 0.05 g/100 g can be expected.

INTERFERENCE:

If the conversion of glycerol has been completed within the time specified in the assay (approx. 5 min), it can be generally concluded that no interference has occurred. However, this can be further checked by adding glycerol (approx. 20 µg in 0.1 mL) to the cuvette on completion of the reaction. A significant decrease in the absorbance should be observed.

Interfering substances in the sample being analysed can be identified by including an internal standard. Quantitative recovery of this standard would be expected. Losses in sample handling and extraction are identified by performing recovery experiments, i.e. by adding glycerol to the sample in the initial extraction steps.

SAFETY:

The general safety measures that apply to all chemical substances should be adhered to.

For more information regarding the safe usage and handling of this product please refer to the associated SDS that is available on both neogen.com and megazyme.com.

KITS:

Kits suitable for performing 70 assays in manual format (or 700 assays in microplate format) are available from Neogen. The kits contain the full assay method plus:

Bottle 1: Buffer (20 mL, pH 7.4) plus sodium azide (0.02% w/v) as a preservative.
Store at 4°C. See individual label for expiry.

Bottle 2: NADH plus ATP and PEP.
Store below -10°C. See individual label for expiry.

Bottle 3: Pyruvate kinase plus lactate dehydrogenase suspension, 1.5 mL.
Store at 4°C. See individual label for expiry.

Bottle 4: Glycerokinase suspension (1.5 mL).
Store at 4°C. See individual label for expiry.

Bottle 5: Glycerol standard solution (5 mL, 0.20 mg/mL) in 0.02% (w/v) sodium azide.
Store sealed at 4°C. See individual label for expiry.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Use the contents of **bottle 1** as supplied.

2. Dissolve the contents of **bottle 2** in 7.5 mL of distilled water. This is **NADH solution**. **Stable for ~ 4 weeks at 4°C** or stable for > 2 years below -10°C (to avoid repetitive freeze/thaw cycles, divide into appropriately sized aliquots and store in polypropylene tubes).

3 & 4. Use the contents of **bottles 3** and **4** as supplied. Before opening for the first time, shake the bottles to remove any enzyme that may have settled on the rubber stopper. Subsequently, store the bottles in an upright position.
Swirl the bottle to mix contents before use.

5. Use the contents of **bottle 5** as supplied.

NOTE: The glycerol standard solution is only assayed where there is some doubt about the accuracy of the spectrophotometer being used or where it is suspected that inhibition is being caused by substances in the sample. The concentration of glycerol is determined directly from the extinction coefficient of NADH (page 6).

EQUIPMENT (RECOMMENDED):

1. Volumetric flasks (50 mL, 100 mL and 500 mL).
2. Disposable plastic cuvettes (1 cm light path, 3.0 mL).
3. Micro-pipettors, e.g. Gilson Pipetman® (20 µL and 100 µL).
4. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® (to dispense 0.2 mL aliquots of **NADH solution**).
   - with 25 mL Combitip® (to dispense 1.0 mL aliquots of **bottle 1** and 2.0 mL aliquots of distilled water).
5. Analytical balance.
6. Spectrophotometer set at 340 nm.
7. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
8. Stop clock.
9. Whatman No. 1 (9 cm) filter papers.

A. MANUAL ASSAY PROCEDURE:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 2.34 mL
Sample solution: 0.8-35.0 µg of glycerol per cuvette (in 0.10-2.00 mL sample volume)

Read against air (without a cuvette in the light path) or against water

Pipette into cuvettes	Blank	Sample
distilled water (at ~ 25°C) sample **bottle 1** (buffer) **NADH solution** **bottle 3** (PK/LDH)	2.00 mL - 0.20 mL 0.10 mL 0.02 mL	1.90 mL 0.10 mL 0.20 mL 0.10 mL 0.02 mL
Mix* and read the absorbances of the solutions (A_1) after approx. 4 min (at completion of pre-reaction**). Start the reactions by addition of:		
bottle 4 (GK)	0.02 mL	0.02 mL
Mix* and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 5 min). If the reaction has not stopped after 5 min, continue to read the absorbances at 2 min intervals until the absorbances remain the same over 2 min.		

* for example with a plastic spatula or by gentle inversion after sealing the cuvette with a cuvette cap or Parafilm®.

** it is necessary to wait for completion of the pre-reaction after the addition of **bottle 3** (PK/LDH).

CALCULATION:

> **NOTE:** These calculations can be simplified by using the ***Mega-Calc™***, downloadable from where the product appears on the Megazyme website (www.megazyme.com).

Determine the absorbance difference (A_1-A_2) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{glycerol}$.

The value of $\Delta A_{glycerol}$ should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of glycerol can be calculated as follows:

$$c = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A_{glycerol} \quad [g/L]$$

where:

V = final volume [mL]
MW = molecular weight of glycerol [g/mol]
ε = extinction coefficient of NADH at 340 nm
= 6300 [$l \times mol^{-1} \times cm^{-1}$]
d = light path [cm]
v = sample volume [mL]

It follows for glycerol:

$$c = \frac{2.34 \times 92.1}{6300 \times 1.0 \times 0.10} \times \Delta A_{glycerol} \quad [g/L]$$

$$= 0.3421 \times \Delta A_{glycerol} \quad [g/L]$$

If the sample has been diluted during preparation, the result must be multiplied by the dilution factor, F.

When analysing solid and semi-solid samples which are weighed out for sample preparation, the content (g/100 g) is calculated from the amount weighed as follows:

Content of glycerol

$$= \frac{c_{glycerol} \text{ [g/L sample solution]}}{weight_{sample} \text{ [g/L sample solution]}} \times 100 \quad [g/100\ g]$$

B. MICROPLATE ASSAY PROCEDURE:

NOTES:

1. The Microplate Assay Procedure for glycerol can be performed using either a single point standard or a full calibration curve.
2. For each batch of samples that is applied to the determination of glycerol **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

Wavelength:	340 nm
Microplate:	96-well (e.g. clear flat-bottomed, glass or plastic)
Temperature:	~ 25°C
Final volume:	0.234 mL
Linearity:	0.1-3.5 µg of glycerol per well (in 0.01-0.20 mL sample volume)

Pipette into wells	Blank	Sample	Standard
distilled water	0.200 mL	0.190 mL	0.190 mL
sample solution	-	0.010 mL	-
bottle 5 (glycerol standard)	-	-	0.010 mL
bottle 1 (buffer)	0.020 mL	0.020 mL	0.020 mL
NADH solution	0.010 mL	0.010 mL	0.010 mL
bottle 3 (PK/LDH)	0.002 mL	0.002 mL	0.002 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 4 min and start the reactions by addition of:			
bottle 4 (GK)	0.002 mL	0.002 mL	0.002 mL
Mix* and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 5 min). If the reaction has not stopped after 5 min, continue to read the absorbances at 2 min intervals until the absorbances remain the same over 2 min.			

* for example using microplate shaker, shake function on a microplate reader or repeated aspiration (e.g. using a pipettor set at 50-100 µL volume).

CALCULATION (Microplate Assay Procedure):

$$g/L = \frac{\Delta A_{sample}}{\Delta A_{standard}} \times g/L\ standard \times F$$

If the sample is diluted during preparation, the result must be multiplied by the dilution factor, F.

SAMPLE PREPARATION:

1. Sample dilution.

The amount of glycerol present in the cuvette (i.e. in the 0.1 mL of sample being analysed) should range between 0.8 and 35 µg. The sample solution must therefore be diluted sufficiently to yield a concentration between 0.008 and 0.35 g/L.

Dilution Table

Estimated concentration of glycerol (g/L)	Dilution with water	Dilution factor (F)
< 0.35	No dilution required	1
0.35-3.5	1 + 9	10
3.5-35	1 + 99	100
> 35	1 + 999	1000

If the value of $\Delta A_{glycerol}$ is too low (e.g. < 0.100), weigh out more sample or dilute less strongly. Alternatively, the sample volume to be pipetted into the cuvette can be increased up to 2.00 mL, making sure that the sum of the sample and distilled water components in the reaction is 2.10 mL and using the new sample volume in the equation.

IMPORTANT NOTE: Users should perform in-house matrix validation work prior to routine use. This process will highlight any problematic matrices encountered. The below are suggested sample preparation examples only.

2. Sample clarification.

Samples containing high amounts of protein or fats may cause interference in target analyte determination and require sample clarification prior to analysis using Carrez Clarification reagents. These reagents are available to purchase separately from Neogen in the Carrez Clarification Kit (K-CARREZ). The Carrez Clarification Kit reagents should be diluted prior to use as described in the assay protocol and the procedure for clarification followed as described on page 3 of the assay protocol. The clarified sample solution prepared using the Carrez Clarification Kit can then be analysed for glycerol content as described in this assay procedure.

3. General considerations.

(a) Liquid samples: clear, slightly coloured and approximately neutral, liquid samples can be used directly in the assay.

(b) Acidic samples: if > 0.1 mL of an acidic sample is to be used undiluted (such as wine or fruit juice), the pH of the solution should be increased to approx. 7.4 using 2 M NaOH, and the solution incubated at room temperature for 30 min.

(c) Carbon dioxide: samples containing significant quantities of carbon dioxide, such as beer, should be degassed by increasing the pH to approx. 7.4 with 2 M NaOH and gentle stirring, or by stirring with a glass rod.

(d) Coloured samples: an additional sample blank, i.e. sample with no GK, may be necessary in the case of coloured samples.

(e) Strongly coloured samples: if used undiluted, strongly coloured samples should be treated by the addition of 0.2 g of polyvinylpolypyrrolidone (PVPP)/10 mL of sample. Shake the tube vigorously for 5 min and then filter through Whatman No. 1 filter paper.

(f) Solid samples: homogenise or crush solid samples in distilled water and filter if necessary.

(g) Samples containing fat: extract such samples with hot water at a temperature above the melting point of the fat, e.g. in a 100 mL volumetric flask at 60°C. Adjust to room temperature and fill the volumetric flask to the mark with water. Store on ice or in a refrigerator for 15-30 min and then filter. Discard the first few mL of filtrate and use the clear supernatant (which may be slightly opalescent) for assay. Alternatively, clarify with Carrez reagents (K-CARREZ).

(h) Samples containing protein: deproteinise samples containing protein using K-CARREZ.

SUGGESTED SAMPLE PREPARATION EXAMPLES:

(a) Determination of glycerol in wine.

In general, the glycerol concentration of white and red wine can be determined without any sample treatment (except dilution according to the dilution table). *Typically, a dilution of 1:20 and sample volume of 0.1 mL are satisfactory.*

(b) Determination of glycerol in beer.

After removal of carbon dioxide by stirring with a glass rod, dilute the sample according to the dilution table and analyse. *Typically, a dilution of 1:5 and sample volume of 0.1 mL are satisfactory.*

(c) Determination of glycerol in fruit juice, concentrates and related beverages.
Dilute the sample to yield a glycerol concentration of less than 0.35 g/L (see dilution table). Clear, neutral solutions can generally be determined without any sample treatment (except dilution). Turbid liquids generally only require filtering before the dilution step.

Coloured solutions are usually suitable for analysis after dilution to an appropriate glycerol concentration. However, if coloured solutions require analysis undiluted, they may need decolourising as follows: adjust 25 mL of liquid sample to approx. pH 7.4 with 1 M NaOH and increase the volume to 50 mL with distilled water. Add 0.5 g of PVPP, stir for 5 min and filter through Whatman No. 1 filter paper. Use the clear, slightly coloured filtrate directly in the assay. *Typically, no further dilution is required and a sample volume of 1.0 mL is satisfactory.*

NOTE: If you have questions about these or other matrices, please contact your local sales representative for support.

REFERENCES:

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