# TREHALOSE

ASSAY PROTOCOL

## K-TREH
## SKU: 700004348

09/25

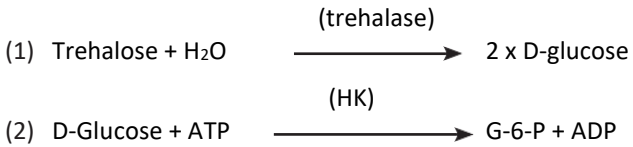
(100 Manual Assays per Kit) or
(1100 Auto-Analyser Assays per Kit) or
(1000 Microplate Assays per Kit)

**INTRODUCTION:**

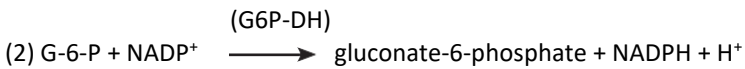
Trehalose is a naturally occurring disaccharide containing two glucose molecules bound in an α,α-1,1 linkage. This structure results in a chemically stable, non-reducing sugar with many important functional characteristics. Trehalose is commonly found in nature, provides a source of energy, and has been shown to be a primary factor in stabilising organisms during times of freezing, drying and heating. Trehalose is consumed as part of a normal diet in mushrooms, honey, lobster, shrimp and foods produced using baker's and brewer's yeast.

**PRINCIPLE:**

Trehalose is hydrolysed to D-glucose by trehalase (1), and the D-glucose released is phosphorylated by the enzyme hexokinase (HK) and adenosine-5'-triphosphate (ATP) to glucose-6-phosphate (G-6-P) with the simultaneous formation of adenosine-5'-diphosphate (ADP) (2).




In the presence of the enzyme glucose-6-phosphate dehydrogenase (G6P-DH), G-6-P is oxidised by nicotinamide-adenine dinucleotide phosphate ($NADP^+$) to gluconate-6-phosphate with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) (2).




The amount of NADPH formed in this reaction is stoichiometric with the amount of D-glucose and thus with half the amount of trehalose. It is the NADPH which is measured by the increase in absorbance at 340 nm.

**SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:**

The assay is specific for trehalose.

The smallest differentiating absorbance for the assay is 0.010 absorbance units. With a sample volume of 0.20 mL, this corresponds to 3.45 mg/L (non-reduced assay procedure) or 18.98 mg/L (reduced assay procedure) of trehalose in the sample solution. The detection limit is 6.9 mg/L (non- reduced assay procedure) or 37.52 mg/L (reduced assay procedure) of trehalose in the sample solution, which is derived from an absorbance difference of 0.020 with a sample volume of 0.2 mL.

The assay is linear over the range of 2 to 40 µg of trehalose 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 0.20 mL, this corresponds to 1.73 to 3.45 mg/L (non-reduced assay procedure) or 9.49 to 18.98 mg/L (reduced assay procedure) of trehalose in the sample solution. If the sample is diluted during sample preparation (as well as that which occurs in the borohydride reduction step), 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 hydrolysis of trehalose and the conversion of D-glucose has been completed within the time specified in the assay (approx. 5 min for each incubation), it can be generally concluded that no interference has occurred. However, this can be further checked by adding trehalose or D-glucose (approx. 40 µg of either in 0.2 mL) to the cuvette on completion of the reaction. A significant increase 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 trehalose 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 from the Megazyme® and Neogen® website.

**KITS:**

Kits suitable for performing 100 assays in manual format (or 1100 assays in auto-analyser format or 1000 assays in microplate format) are available from Neogen. The kit contains:

**Bottle 1:** Buffer (25 mL, pH 7.6) plus magnesium chloride and sodium azide (0.02% w/v) as a preservative.
Store at 4°C. See individual label for expiry.

**Bottle 2:** $NADP^+$ plus ATP.
Store at -10°C. See individual label for expiry.

**Bottle 3:** Hexokinase plus glucose-6-phosphate dehydrogenase suspension, 2.25 mL.
Store at 4°C. See individual label for expiry.

**Bottle 4:** Trehalase suspension (2.25 mL).
Store at 4°C. See individual label for expiry.

**Bottle 5:** D-Glucose standard solution (5 mL, 0.4 mg/mL) in 0.2% (w/v) benzoic acid.
Store at 4°C. See individual label for expiry.

**Bottle 6:** Trehalose dihydrate control (~ 2 g).
Store sealed at room temperature. 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 12 mL of distilled water. **Stable for 4 weeks at 4°C** or stable for years below -10°C (to avoid repetitive freeze/thaw cycles, divide into appropriately sized aliquots and store in polypropylene tubes). This is **$NADP^+$ solution**.

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

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

**6.** Dissolve 200 mg of **bottle 6** (which corresponds to approx. 180.9 mg of trehalose) in 1 L of distilled water. Add 0.2 mL of this solution to assays to check the activity of trehalase enzyme. This is **trehalose control solution**. Stable for 2 years below -10°C.

**NOTE:** The D-glucose standard solution (**bottle 5**) and the trehalose control solution are only assayed when 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 trehalose is determined directly from the extinction coefficient of NADPH (page 6).

**REQUIRED REAGENTS (not included):**

1. **Reagent 1. Sodium hydroxide (50 mM)**
   Dissolve 2.0 g of sodium hydroxide in 900 mL of distilled water. Adjust the volume to 1 L.
2. **Reagent 2. Alkaline borohydride (10 mg/mL sodium borohydride in 50 mM sodium hydroxide)**
   Accurately weigh approx. 50 mg of sodium borohydride (Sigma cat. no. S9125) into polypropylene containers (10 mL volume with screw cap). Record the exact weight on the tubes, seal the tubes and store them in a desiccator with silica gel for future use. When weighing the borohydride, it is suggested that about 10 lots are prepared for convenience.

   Immediately before use, dissolve the sodium borohydride to a concentration of 10 mg/mL in 50 mM sodium hydroxide (Reagent 1). This solution is stable for 4-5 h at room temperature.
3. **Reagent 3. Acetic acid (200 mM)**
   Add 11.6 mL of glacial acetic acid to 600 mL of distilled water and adjust the volume to 1 L.

**EQUIPMENT (RECOMMENDED):**

1. Glass test tubes (round bottomed; 16 x 100 mm).
2. Disposable plastic cuvettes (1 cm light path, 3.0 mL).
3. Volumetric flasks (50 and 100 mL capacity).
4. Micro-pipettors, e.g. Gilson® Pipetman® (20 µL and 100 µL).
5. Positive displacement pipettor, e.g. Eppendorf® Multipette®
   - with 5.0 mL Combitip® (to dispense 0.2 mL aliquots of buffer 1 and 0.1 mL aliquots of $NADP^+$/ATP solution).
   - with 25 mL Combitip® (to dispense 2.0 mL aliquots of distilled water).
6. Analytical balance.
7. Spectrophotometer set at 340 nm.
8. Vortex mixer.
9. Whatman® No. 1 (9 cm) filter paper.

**A. MANUAL ASSAY PROCEDURE:**

**Wavelength:** 340 nm
**Cuvette:** 1 cm light path (glass or plastic)
**Temperature:** ~ 25°C
**Final volume:** 2.54 mL
**Sample solution:** 2-40 µg of trehalose (or 4-80 µg D-glucose) per cuvette (in 0.20 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) | 2.20 mL | 2.00 mL |
| sample solution | - | 0.20 mL |
| **bottle 1** | 0.20 mL | 0.20 mL |
| **$NADP^+$ solution** | 0.10 mL | 0.10 mL |
| **bottle 3** | 0.02 mL | 0.02 mL |
| Mix*, read the absorbances of the solutions ($A_1$) after approx. 5 min and start the reactions by addition of: | | |
| **bottle 4** | 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®.

** if the absorbance continues to increase, this may be due to effects of colour compounds or enzymes in the sample. These interfering substances may be removed during sample preparation.

**CALCULATION:**

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

Determine the absorbance difference ($A_2$-$A_1$) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{trehalose}$. The value of $\Delta A_{trehalose}$ should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of trehalose can be calculated as follows:

$$c = \frac{V \times MW}{\varepsilon \times d \times v2 \times 2} \times \frac{1.1}{0.2} \times \Delta A_{trehalose} \quad [g/L]$$

**where:**

V = final volume [mL]
MW = molecular weight of trehalose [g/mol]
Ɛ = extinction coefficient of NADPH at 340 nm
= 6300 [l x $mol^{-1}$ x $cm^{-1}$]
d = light path [cm]
v = sample volume [mL]
2 = 2 molecules of D-glucose released from each molecule of trehalose hydrolysed
1.1/0.2 = 0.2 mL of the sample extract is treated with borohydride and the final volume after treatment and neutralization is 1.1 mL. This factor is only used if borohydride reduction is used to remove free D-glucose (“B. Removal of Reducing Sugars (borohydride reduction) on pg. 11).

***It follows for trehalose:***

$$c = \frac{2.54 \times 342.3}{6300 \times 1 \times 0.2 \times 2} \times \frac{1.1}{0.2} \times \Delta A_{trehalose} \quad [g/L]$$

$$= 1.8976 \times \Delta A_{trehalose} \quad [g/L]$$

If the sample has been diluted during preparation (other than that involved in the borohydride reduction step), 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 trehalose**

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

When analysing pure trehalose or where the concentration of trehalose is significantly larger than that of free D-glucose, the borohydride reduction step can be omitted. In such cases, the concentration of trehalose can be calculated as follows:

$$c = \frac{2.54 \times 342.3}{6300 \times 1 \times 0.2 \times 2} \times \Delta A_{trehalose} \quad [g/L]$$

$$= 0.3450 \times \Delta A_{trehalose} \quad [g/L]$$

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 trehalose**

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

## B. AUTO-ANALYSER ASSAY PROCEDURE:

**NOTES:**

1. The Auto-Analyser Assay Procedure for trehalose 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 trehalose **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

Reagent preparation is performed as follows:

**Preparation of R1:**

| **Component** | **Volume** |
|---|---|
| distilled water<br>**bottle 1**<br>**$NADP^+$ solution**<br>**bottle 3** | 40.60 mL<br>4.40 mL<br>2.20 mL (after adding 12 mL of $H_2O$ to bottle 2)<br>0.44 mL |
| Total volume | 47.64 mL |

**Preparation of R2:**

| **Component** | **Volume** |
|---|---|
| distilled water<br>**bottle 4** | 5.50 mL<br>0.44 mL |
| Total volume | 5.94 mL |

**EXAMPLE METHOD:**

| | |
|---|---|
| **R1:** | 0.200 mL |
| Sample: | ~ 0.01 mL |
| **R2:** | 0.025 mL |
| | |
| **Reaction time:** | ~ 5 min at 37°C |
| **Wavelength:** | 340 nm |
| **Prepared reagent stability:** | 2 days when refrigerated |
| **Calculation:** | endpoint |
| **Reaction direction:** | increase |
| **Linearity:** | up to 0.37 g/L of trehalose using 0.01 mL sample volume |

## C. MICROPLATE ASSAY PROCEDURE:

**NOTES:**

1. The Microplate Assay Procedure for trehalose 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 trehalose **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:** 0.254 mL
**Linearity:** 0.05-4.0 µg of trehalose (or 0.1-8.0 µg of D-glucose) per well (in 0.02 mL sample volume)

| Pipette into wells | Blank | Sample | Standard |
|---|---|---|---|
| distilled water | 0.220 mL | 0.200 mL | 0.200 mL |
| sample solution | - | 0.020 mL | - |
| **bottle 5** | - | - | 0.020 mL |
| **bottle 1** | 0.020 mL | 0.020 mL | 0.020 mL |
| **$NADP^+$ solution** | 0.010 mL | 0.010 mL | 0.010 mL |
| **bottle 3** | 0.002 mL | 0.002 mL | 0.002 mL |
| Mix*, read the absorbances of the solutions ($A_1$) after approx. 5 min and start the reactions by addition of: | | | |
| **bottle 4** | 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 increase constantly 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).

** if this "creep" rate is greater for the sample than for the blank, extrapolate the sample absorbances back to the time of addition of suspension 4.

**CALCULATION (Microplate Assay Procedure):**

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

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

**SAMPLE PREPARATION:**

**Sample dilution.**

The amount of trehalose present in the cuvette (i.e. in the 0.2 mL of sample being analysed) should range between 2 and 40 µg. The sample solution must therefore be diluted sufficiently to yield a trehalose concentration between 0.01 and 0.2 g/L for sample solutions not requiring borohydride reduction and between 0.05 and 1.0 g/L for sample solutions requiring borohydride reduction to remove free D-glucose.

**Dilution Table 1** (for samples **not requiring** borohydride reduction)

| Estimated concentration of trehalose (g/L) | Dilution with water | Dilution factor (F) |
|---|---|---|
| < 0.2 | No dilution required | 1 |
| 0.2-2.0 | 1 + 9 | 10 |
| 2.0-200 | 1 + 99 | 100 |

**Dilution Table 2** (for samples **requiring** borohydride reduction)

| Estimated concentration of trehalose (g/L) | Dilution with water | Dilution factor (F) |
|---|---|---|
| < 1.0 | No dilution required | 1 |
| 1.0-10.0 | 1 + 9 | 10 |
| > 10.0 | 1 + 99 | 100 |

If the value of $\Delta A_{trehalose}$ is too low (e.g. < 0.100), weigh out more sample or dilute less strongly.

**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.

**1. PREPARATION OF SAMPLE EXTRACTS and REMOVAL OF FREE D-GLUCOSE**

**A. Trehalose Extraction:**

Mill dry samples to pass a 0.5 mm screen; cut solid fatty samples (e.g. chocolate) into fine shavings with a sharp knife; analyse soft food products (e.g. spreads) without further preparation. All samples should be at room temperature before they are weighed.

**Samples containing 0-12% trehalose**

1. Accurately weigh approx. 400 mg of the sample into a dry pyrex beaker (100 mL capacity) and add 40 mL of hot distilled water (~ 80°C). Place the beaker on a magnetic stirrer and stir for 15 min (i.e. until the sample is completely dispersed).

2. Quantitatively transfer the solution to a 50 mL volumetric flask. Adjust the volume to the mark with distilled water, mix the contents thoroughly and allow to cool to room temperature. Re-check the volume and adjust to the mark with distilled water if necessary.

**Samples containing 12-40% trehalose**

1. Accurately weigh approx. 250 mg of the sample into a dry pyrex beaker (100 mL capacity) and add 80 mL of hot distilled water (~ 80°C). Place the beaker on a magnetic stirrer and stir for 15 min (i.e. until the sample is completely dispersed).
2. Quantitatively transfer the solution to a 100 mL volumetric flask. Adjust the volume to the mark with distilled water, mix the contents thoroughly and allow to cool to room temperature. Re-check the volume and adjust to the mark with distilled water if necessary.

**Further treatment of extracts**

3. Filter an aliquot of the solution through a Whatman No. 1 (9 cm) filter circle. *This solution may be slightly turbid, depending on the nature of the sample extracted.* Analyse this solution immediately or store at 4°C until analysed. If a turbidity forms in the solution, filter it again before analysis.

**NOTE:** For samples containing 40-100% trehalose, adjust the volume to 250 mL.

**B. Removal of Reducing Sugars (borohydride reduction):**

1. Accurately dispense a 0.2 mL aliquot of the solution to be analysed (containing approx. 0.05 to 1.0 mg/mL of trehalose) into the bottom of a glass test-tube (16 x 100 mm).
2. Add 0.2 mL of **Reagent 2** (alkaline borohydride solution) to the tube, stir the mixture vigorously and store at 40°C for 30 min to effect complete reduction of reducing-sugars to sugar alcohols.
3. Add 0.5 mL of **Reagent 3** (200 mM acetic acid) to the tube with vigorous stirring on a vortex mixer. **A vigorous effervescence should be observed** (This treatment removes excess borohydride and adjusts the pH to approx. 4.5).
4. After 5 min, add 0.2 mL of solution 1 (kit buffer) to adjust the pH of the solution to approx. 7. This is the **sample solution** and should be analysed as described on page 5.

## 2. PREPARATION OF SAMPLE EXTRACTS NOT REQUIRING REMOVAL OF FREE D-GLUCOSE

**Trehalose Extraction:**

Mill dry samples to pass a 0.5 mm screen; cut solid fatty samples (e.g. chocolate) into fine shavings with a sharp knife; analyse soft food products (e.g. spreads) without further preparation. All samples should be at room temperature before they are weighed.

**Samples containing 0-12% trehalose**

1. Accurately weigh approx. 400 mg of the sample into a dry pyrex beaker (300 mL capacity) and add 200 mL of hot distilled water (~ 80°C). Place the beaker on a magnetic stirrer and stir for 15 min (i.e. until the sample is completely dispersed).
2. Quantitatively transfer the solution to a 250 mL volumetric flask. Adjust the volume to the mark with distilled water and mix the contents thoroughly, and allow to cool to room temperature. Re-check the volume and adjust to the mark with distilled water if necessary.

**Samples containing 12-40% trehalose**

1. Accurately weigh approx. 100 mg of the sample into a dry pyrex beaker (300 mL capacity) and add 200 mL of hot distilled water (~ 80°C). Place the beaker on a magnetic stirrer and stir for 15 min (i.e. until the sample is completely dispersed).
2. Quantitatively transfer the solution to a 250 mL volumetric flask. Adjust the volume to the mark with distilled water, mix the contents thoroughly and allow to cool to room temperature. Re-check the volume and adjust to the mark with distilled water if necessary.

**NOTE:** For samples containing 40-100% trehalose, adjust the volume to 500 mL.

**Further treatment of extracts**

3. Filter an aliquot of the solution through a Whatman No. 1 (9 cm) filter circle. *This solution may be slightly turbid, depending on the nature of the sample extracted.* This is the **sample solution** and should be immediately analysed as described on page 5. Analyse this solution immediately or store at 4°C until analysed. If a turbidity forms in the solution, filter it again before analysis.

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

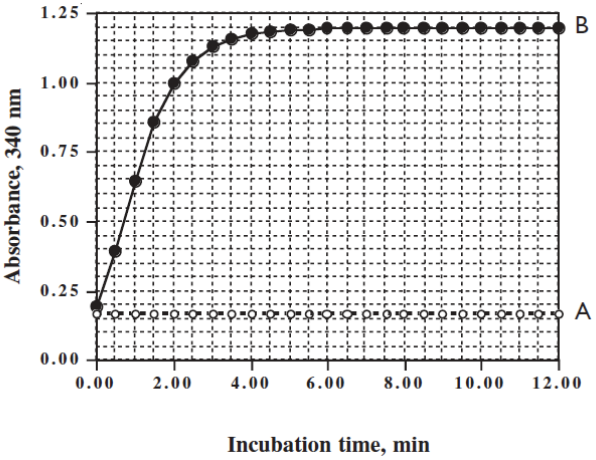



***Figure 1.*** Measurement of trehalose using trehalase enzyme.

A. Incubation mixture with trehalose (100 µg) but no trehalase enzyme.

B. Incubation mixture containing trehalose (100 µg) and trehalase enzyme (9.8 U).

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