UV assay for the determination of lactose/D-glucose in foodstuffs and other sample materials
Test combination for 50 determinations

For *in vitro* use only
Store between 2 – 8 °C (36 – 46 °F)

This test was evaluated using selected samples of the following matrices: milk, yogurt, lactose- free milk products, ice cream, whey and skimmed milk powder, chocolate, infant formula, sausage, cheese and soy based products.

Detailed results and information regarding associated validation data are found in the Validation Report.

The test may be used with other foods or sample materials, provided that these are subjected to individual validation by the user.

Note: Low-lactose and lactose-free milk and dairy products are generally produced using β-galactosidase (lactase), a process that results in excess D-glucose and D-galactose from the lactose, which makes it difficult to measure the remaining, small amount of lactose. For this reason, in accordance with §64 01.00-90 of the German Food and Feed Code (LFGB), D-glucose is first removed using glucose oxidase so that the D-glucose hydrolyzed from the remaining lactose can then be measured precisely.

This test Enzytec™ *Liquid* Lactose/D-Glucose, when used in combination with the Enzytec™ *Liquid* D-Glucose test (Art. No. E8140) and the Enzytec™ Glucose Remover (Art. No. E3400), is suitable to confirm that dairy products are lactose-free.

To detect lactose concentrations in the range of the limit value of 0.01 % (10 mg/100 g), the sensitive test procedure (see Chapter 4.2. Sensitive test procedure) should be used.

1. Test principle

In the presence of the enzyme β-galactosidase (β-Gal) and water, lactose is hydrolyzed to D-glucose and D-galactose:

$$\text{Lactose} + H_2O \xrightarrow{\beta\text{-Gal}} \text{D-glucose} + \text{D-galactose}$$

The resulting D-glucose is phosphorylated by a hexokinase (HK) and and adenosine-5'-triphosphate (ATP) to D-glucose-6-phosphate (G-6-P) with the simultaneous formation of adenosine-5'-diphosphate (ADP):

$$\text{D-Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{D-glucose-6-phosphate} + \text{ADP}$$

In the presence of the enzyme glucose-6-phosphate dehydrogenase (G6P-DH), G-6-P is oxidized by nicotinamide adenine dinucleotide (NAD) to D-gluconate-6-phosphate:

$$\text{G-6-P} + NAD^+ \xrightarrow{\text{G6P-DH}} \text{D-gluconate-6- phosphate} + NADH + H^+$$

In this process, NAD is reduced to NADH. The amount of NADH formed is proportional to the converted amount of lactose and free D-glucose and is measured at 340 nm.

2. Reagents

2.1. Content & composition

The test is suitable for manual and automated processing. With manual processing, the reagents are sufficient for 50 determinations. The number of determinations for automated processing is increased by a multiple; however it depends on the device.

- Reagent 1: 2 x 50 mL with buffer, NAD, β-Gal, ATP
- Reagent 2: 2 x 12.5 mL with buffer, HK, G6P-DH

2.2. Reagent preparation

The reagents are ready-to-use and be allowed to reach room temperature (20 – 25 °C / 68 – 77 °F) before use. Do not interchange components between kits of different batches.

2.3. Storage & stability

If stored as directed and between 2 – 8 °C (36 – 46 °F), reagents remain stable until the printed expiration date, even after opening. Reagents must not be frozen.

2.4. Safety & disposal

The test is intended solely for the intended use as described. The provided Instructions for Use must be strictly followed.

Follow standard chemical safety procedures when handling this product. Do not swallow. Avoid contact with skin or mucous membranes.

Detail safety information for individual components is available in the corresponding Safety Data Sheets (SDS).

Dispose of used reagents as laboratory waste in compliance with all relevant regulations. Packaging materials are to be recycled according to local regulations.

3. Sample preparation

3.1. General

- Sample preparation for manual and automated testing is the same.
- Samples solutions should be brought to room temperature before measurement.
- Use liquid, clear and almost neutral sample solutions directly or dilute sufficiently to yield a concentration within the stated measuring range (refer to performance data).
- Neutralize **strongly** acidic samples by adding KOH/NaOH, or alkaline samples by adding HCl, to a pH of approx. 7.
- For turbid test samples: Filter by using fluted paper filter or syringe filter or centrifuge the test solution in a reaction tube (recommended 3000 rpm for at least 5 minutes) until a clear filtrate or supernatant is obtained.
- Degas samples containing carbon dioxide by, for example, stirring them in a beaker or applying a brief ultrasonic pulse (10 s).
- If necessary, decolorize **strongly** colored samples with polyvinyl-polypyrrolidone (PVPP, e.g., 1 g/100 mL sample). Stir or shake for 1 minute and filter or centrifuge at 3000 rpm for at least 5 minutes until a clear supernatant is obtained.
- Crush and homogenize solid and semi-solid samples. Weigh a sufficient quantity of sample in a volumetric flask (considering the measuring range), extract with water; fill up to the mark and filter if necessary (by using fluted paper or syringe filters) or centrifuge in reaction tubes.
  Use Carrez clarification if necessary.
- For fat containing samples, weigh sufficient quantity (considering the measuring range) into a volumetric flask and extract with hot water. Cool to allow the fat to separate, make up the mark, place the volumetric flask in an ice bath for 15 minutes and filter.
- Clarify samples containing proteins or fat alternatively with Carrez reagents: Weigh an appropriate sample quantity accurately into a 100 mL volumetric flask and add approx. 60 mL distilled water. In case of liquid samples, pipette the sample into a 100 mL volumetric flask or beaker pre-filled with 60 mL distilled water. Add 5 mL Carrez I solution (3.60 g potassium hexacyano-ferrate(II)-trihydrate $K_4[Fe(CN)_6] \times 3\ H_2O$/100 mL) and 5 mL Carrez II solution (7.20 g zinc sulfate $ZnSO_4 \times 7\ H_2O$/100 mL). Mix well after each addition. Adjust the pH with 0.1 M NaOH to a value between 7.5 and 8.5.
  Transfer into a 100 mL volumetric flask, fill up to the mark, mix and filter using fluted paper filters or syringe filters.
- Sample preparation in combination with the Enzytec™ Glucose Remover (E3400) is explained in more detail in the corresponding instruction for use.

3.2. Meat products

- Accurately weigh approx. 10 g of the homogenized sample into a resealable 50 mL plastic tube and add 20 mL distilled water.
- Vortex vigorously and fill up to 50 mL with distilled water.
- Heat for 15 minutes at 70 °C (158 °F) in a water bath.
- Add one drop of 98 % sulfuric acid and transfer the suspension quantitatively with water into a 100 mL volumetric flask.

- Allow the solution cool down to room temperature and fill the flask with distilled water up to 100 mL (fat phase above the calibration mark).
- Gently mix by inverting the flask, then filter using a paper or syringe filter.
- Use the filtrate directly for the assay or after dilution.

Important note: If *raw* meat products are analyzed, creep reactions can occur due to interfering effects of enzymes and substrates present in the raw laboratory sample. To prevent these creep reactions, the samples should be heated to approx. 75 °C (167 °F) for 15 minutes before homogenization.

4. Manual test procedure

Wavelength:	340 nm
Temperature (measurement):	20 – 37 °C (68 – 99 °F)
Photometer alignment:	against air (without cuvette)
Measuring range:	45 – 3000 mg/L

	Reagent blank	Samples / controls
Reagent 1	2000 µL	2000 µL
Sample / control	-	100 µL
Dist. water	100 µL	-
Mix, incubate for **20 minutes at 20 – 37 °C (68 – 99 °F)**. **Read absorbance A_1**, then add:		
Reagent 2	500 µL	500 µL
Mix, incubate for **15 minutes at 20 – 37 °C (68 – 99 °F)** and **read absorbance A_2**.		
Note: When performing this enzymatic assay, please ensure that the incubation temperature does not fall below 20 °C, as this will result in reduced enzyme activity and correspondingly lower recovery rates.		

4.1. Important notes for assay procedure

- The reagent blank value (water sample) must be determined **in each series of measurement** and subtracted from **each** sample result.
- Specified incubation times were validated and established at 25 °C (77 °F). The test may generally perform within a range between **20 – 37 °C (68 – 99 °F)**.
- Use separate tips for each sample and the control solutions to avoid cross-contamination; rinse the tip before pipetting.
- A multistepper pipette is recommended for adding reagents. Use a separate tip for each component.
- Stirring spatulas are recommended for mixing each individual cuvette. Remove these from the cuvette immediately before measuring the absorbance
- Always wait for the reaction to end (at least during the first test runs or validation). If the absorbance has not stabilized after the recommended incubation time, continue measuring at 5-minute intervals, for example, until a constant absorbance value is reached.
- If a creep reaction occurs, the reaction will not have finished after stated incubation times and will typically show a constant increase of ΔA. Calculate the analyte-specific ΔA value by plotting the absorbance values against time and performing a linear regression to determine the rate of increase in ΔA per minute related to the creep reaction. Then, extrapolate the absorbance to the time at which reagent 2 is added.
- If the measured absorbance difference of the samples is too small (< 0.020), the sample solution must be prepared again with a higher weight or a lower dilution.
- If the absorbance difference of the samples is very large (e.g., > 1.500), the sample solution must be diluted if necessary.

4.2. Sensitive test procedure

Instructions for sample preparation, test procedure, and result calculation are described in detail in the application *Determination of lactose in low-lactose and lactose-free milk and milk products*, which is available upon request (see also Chapter 7).

5. Calculation of results

5.1. Calculation of sample solutions

5.1.1. Total concentration of lactose

The result of the E8130 test additionally includes the amounts of free D-glucose that might be present in the sample. The sum lactose/ D-glucose is calculated with the molecular weight of lactose (342.3 g/mol) and is referred to as total lactose.

The extinction difference ΔA must be calculated for each sample:

$$\Delta A = (A_2 - df \times A_1)_{\text{sample or control}} - (A_2 - df \times A_1)_{RB}$$

df: Dilution factor
RB: Reagent blank

$$df = \frac{\text{sample volume + R1}}{\text{test volume}} = 0.808$$

The specified df value of **0.808 applies to a base application of 100 µL**. An increase in sample volume is possible (max. 1000 µL; refer to validation report). **While keeping reagent volumes unchanged**, this requires **conversion of the reagent dilution factor (df)** accordingly.

Increasing the sample volume may influence test performance. This must generally be checked depending on the matrix. **The reagent blank value must be adjusted to the changed sample volume.**

The concentration of total lactose is calculated using Lambert-Beer's law:

$$\mathbf{C_{\text{total lactose}}\ [g/L]} = \frac{(V \times MW \times \Delta A)}{(\varepsilon \times d \times v \times 1000)} = \mathbf{1.413 \times \Delta A} \times F$$

If the sample solution was diluted before measurement, this result has to be multiplied with the **sample pre-dilution factor F**.

V:	Test volume basic application [mL]	= 2.600
MW:	Molecular weight lactose [g/mol]	= 342.30
d:	Optical path [cm]	= 1.00
v:	Sample volume [mL]	= 0.100
ε:	Extinction coefficient NADH [L/mmol × cm]	= 6.3 (at 340 nm)

5.1.2. Calculation of the total lactose concentration

To differentiate between lactose and D-glucose, free D-glucose must be determined separately using the Enzytec™ Liquid D-Glucose test (Art. No. E8140). The result is subtracted from the total lactose. In doing so, the ratio of the molecular weights of the two types of sugar must be taken into account:

MW_{lactose} 342.3 g/mol : $MW_{\text{D-glucose}}$ 180.16 g/mol → factor 1.9

$$C_{\text{lactose}}\ [g/L] = C_{\text{total lactose (E8130)}} - 1.9 \times C_{\text{D-glucose (E8140)}}$$

Additional information on how to use Enzytec™ *Liquid* D-Glucose E8140 can be found in the accompanying package insert.

Example: Enzytec™ *Liquid* Multi-Sugar Standard *low* (E8440)

Total lactose (E8130)			= 1.45 g/L
D-Glucose (E8140)			= 0.50 g/L
Lactose	=	1.45 g/L - 1.9 × 0.50 g/L	= **0.50 g/L**

If the D-glucose/lactose ratio is higher than 10:1, the precision of the lactose determination decreases. In this case, the glucose excess must be eliminated with the Enzytec™ Glucose Remover kit E3400.

5.2. Calculation of solid samples

When analyzing solid and semi-solid samples that have to be weighed in for the extraction of the sample, the content is related to the sample weight:

$$\text{Content}_{\text{analyte}}\ [\text{g}/100\ \text{g}] = \frac{C_{\text{analyte}}\ [\text{g/L sample solution}]}{\text{weight}_{\text{sample}}\ \text{in g/L sample solution}} \times 100$$

5.3. Controls & acceptance criteria

Control or reference samples should be included in each run for quality control purposes. Therefore, we recommend Enzytec™ *Liquid* Multi-Sugar Standard *low* (Art. No. E8440; with 0.50 g/L lactose and 0.50 g/L D-glucose).

The theoretical target value for total lactose is calculated as follows:

$$\text{Total lactose} = (0.50\ \text{g/L} \times 1.9)_{\text{D-glucose}} + 0.50\ \text{g/L}_{\text{lactose}} = 1.45\ \text{g/L}$$

The recovery of this multi-standard *low* and other *aqueous* control solutions should be 100 ± 5 %.

As certified reference materials, we recommend, among others:

- NIST 1849a *Infant formula,* lactose monohydrate
- LGC 7016 *Chocolate confectionary,* lactose anhydrous
- QSE Reference *Raw Milk*, F3 # D00M09Y16

The recovery of QSE materials and *extracted* matrices should be within 100 ± 10 %.

6. Performance data

6.1. Specificity

This assay is specific for lactose and D-glucose. However, allolactose (6-*O*-(β-D-galactopyranosyl)-D-glucopyranose) is converted by β-galactosidase to approximately 100 % and thus co-determined.

6.2. Interferences & side activities

The test shows no interferences to different relevant alcohols, acids, sweeteners and most of the sugars. In case of sulfite, there is no interference at or below 0.1 g/L. Oxalic acid and glucosamine did not interfere at or below 1 g/L.

6.3. Linearity, measuring range & sensitivity

Linearity is given up to 3000 mg/L total lactose (sample volume of 100 µL) with a recommended measuring range of 45 – 3000 mg/L.

The limit of detection (LoD) was determined for a sample volume of v = 100 µL according to method DIN 32645:2008-11, using buffered aqueous solutions. This results in an LoD of 10.0 mg/L.

The limit of quantification (LoQ) was determined by precision profile and is 45.0 mg/L.

The smallest absorbance difference that the method can distinguish is ΔA = 0.005. For a sample volume of v = 1000 µL, this results in an LoD of 0.95 mg/L. Based on ΔA = 0.010, an LoQ of 1.9 mg/L was calculated.

6.4. Automation with Pictus 500

6.4.1. Limit of quantification (LoQ)

P500 application	LoQ
High Range	30 mg/L
Basic Range	10 mg/L
Sensitive Range	1.25 mg/L

6.4.2. Measuring ranges

P500 application	Measuring range
High Range	to 15.000 g/L
Basic Range	to 3100 mg/L
Sensitive Range	to 310 mg/L

6.4.3. Precision and accuracy

Data from the measurement of an aqueous solution are shown here.

High Range

Target concentration, mg/L	1450	2000
Mean value, mg/L	1486	2007
SD, mg/L	26.74	22.61
RSD, %	1.80	1.13
Recovery, %	103	100

Basic Range

Target concentration, mg/L	1450	2000
Mean value, mg/L	1478	2028
SD, mg/L	5.11	8.39
RSD, %	0.35	0.41
Recovery, %	102	101

Sensitive Range

Target concentration, mg/L	145	200
Mean value, mg/L	148.0	200.9
SD, mg/L	1.29	1.22
RSD, %	0.87	0.61
Recovery, %	102	100

7. Supporting documents

On request, we offer the following documents:

- Application note for sensitive test procedure: *Determination of lactose in low-lactose and lactose-free milk and milk products*
- Enzytec™ *Liquid* Lactose/D-Glucose Validation Report
- Enzytec™ *Liquid* Lactose/D-Glucose Excel template for results
- Enzytec™ *Liquid* Lactose/D-Glucose Technical information
- Enzytec™ *Liquid* Sample preparation guide
- Enzytec™ *Liquid* Troubleshooting guide

Safety data sheets (SDS) and certificates of analysis (CoA) are available in digital form, quoting the batch number, via the following link:

https://eifu.r-biopharm.com/

8. Limits of this method

Test results may vary depending on the sample matrix, specific test implementation, and laboratory environmental conditions. Detection and quantification limits are dependent on respective sample matrices extraction procedures. Refer toto the current Validation Report for details.

For this test, only the matrices explicitly listed in the documentation were validated, due to the wide variety of food products and other potential sample materials.

When analysing non-validated matrices results should be verified by performing spiking (fortification) experiments. If appropriate or necessary, a suitable sample preparation procedure for the respective matrix must be developed and validated.

The responsibility for validating non-validated matrices and for ensuring the suitability of the assay for its intended use lies solely with the user.

9. Services & technical support

Upon request, we offer the following services, among others:

- Customized troubleshooting
- Workflow analysis
- Data & results analysis
- Customer workshops & webinars
- Automation: application support and technical service

10. Disclaimer

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c. Failure to apply appropriate industry standard practices, including Good Laboratory Practices;
d. Failure to otherwise use, and when necessary validate or verify, suitable controls, samples, matrices, or processing procedures;
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f. Product alterations or modifications;
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