LACTOSE

ASSAY PROTOCOL

"Sequential / High Sensitivity Method"

K-LOLAC

03/22

A Procedure for the Measurement of Lactose
in Low-Lactose & Lactose-Free Dairy Products
and in Conventional Dairy Products

(65 Lactose Assays per Kit)

Play Training Video

NEOGEN®
NEOGEN.com

Megazyme
Megazyme.com

Introduction:

Lactose (1,4-β-D-galactosyl-D-glucose) is the natural sugar present in milk. It is generally hydrolysed by lactase (β-galactosidase) in the small intestine, with absorption of the released D-galactose and D-glucose. Humans lacking or deficient in this enzyme cannot digest lactose, which therefore passes undigested to the colon where it undergoes microbial fermentation, causing adverse gastrointestinal symptoms associated with lactose intolerance, such as nausea, cramps, gas and diarrhoea.

Lactose intolerance affects approximately 65% of the global human population. The frequency of primary lactose intolerance varies greatly between ethnic and racial populations, with approximately 5% of northern Europeans and greater than 90% of Southeast Asian populations being affected.[1,2]

To address the prevalence of lactose intolerance, dairy manufacturers have introduced low-lactose and lactose-free dairy products, the production of which includes the addition of β-galactosidase to hydrolyse the naturally occuring lactose. The European Food Safety Authority (EFSA) defines “low-lactose” as containing less than 1 g lactose/100 g product while “lactose-free” is between 10-100 mg lactose/100 g product.[3]

It is well known that β-galactosidase catalyses a transglycosylation process in which the released D-galactose is transferred to lactose, pre-formed galacto-oligosaccharides (GOS) and even back to D-glucose and D-galactose. In high concentrations of lactose, this reaction is used industrially to produce GOS. However, this transglycosylation also occurs to some extent during the hydrolysis of lactose (~ 40 mg/mL) in milk in the production of low-lactose and lactose-free milk. In this process, trace quantities of a range of β-D-galactosyl-D-glucose oligosaccharides are formed, of which 1,6-β-D- (allolactose) is the predominant species, but that also include trace amounts of 1,3-β-D- and 1,2-β-D-galactosyl-D-glucose, as well as trace amounts of β-D-galactosyl-D-galactose disaccharides and GOS. While the concentrations of the various β-D-galactosyl-D-glucose disaccharides are very low, they can occur at levels similar to, or greater than, that of lactose in the low-lactose and lactose-free milk products. Allolactose is usually the principal component of the transglycosylation products with concentrations in the range of 0.05-0.5 mg/mL, but in certain samples it can be present in much higher quantities.

Determination of lactose in low-lactose and lactose-free milk products is difficult. HPLC with refractive index determination is insufficiently sensitive, and accurate determination by ion chromatography is variously challenged by the poor chromatographic baseline patterns obtained with real milk samples.

The **K-LACGAR** lactose kit from Megazyme[4] overcomes the problem of high background D-galactose in the sample by performing borohydride reduction to convert all reducing sugars present to their corresponding sugar alcohols. However, this also reduces lactose to lactitol, and hydrolysis of this to D-galactose and D-sorbitol requires the use of *A. niger* β-galactosidase, which is also effective in hydrolysing any lactose analogues or GOS in the sample to D-galactose and D-sorbitol; thus giving overestimation of lactose content. In standard dairy products this is not a problem, but in lactose-free products it is. An alternative existing procedure to measure lactose involves the measurement of D-glucose. Firstly free D-glucose in the sample is removed by the combined action of glucose oxidase and catalase in the presence of hydrogen peroxide.[5] After removal of the bulk of free glucose, samples of the reaction solution are split into 2 aliquots: one is used to analyse remaining D-glucose and the second is used to obtain lactose plus free D-glucose concentration using *E. coli* β-galactosidase (to hydrolyse lactose to D-glucose and D-galactose). Lactose (and lactose analogues) are determined by measuring the difference between the 2 aliquots in an assay procedure that is cumbersome by nature of the fact that two separate incubations need to be performed for each sample.

The lactose test kit (**K-LOLAC**) introduced by Megazyme is unique in offering a sequential assay procedure for measurement of free D-glucose followed by lactose in the same reaction cuvette. The assay format is simple, accurate and sensitive. Lactose in conventional dairy products is also readily assayed with this procedure. The method includes pre-incubation steps to clarify samples and also to remove the high levels of free D-glucose in the samples, allowing accurate measurement of lactose. Both free D-glucose and the D-glucose component of lactose are measured in a single reaction tube in a sequential enzymatic reaction. Free D-glucose is first measured using a HK/G-6PDH/6-phosphogluconate dehydrogenase (6-PGDH) based assay procedure, and then MZ104 β-galactosidase is added to hydrolyse the lactose in the same reaction tube with concurrent measurement of the released D-glucose. The MZ104

β-galactosidase is selective for lactose and the determined lactose values are similar to those obtained by ion chromatography. Adding 6-PGDH to the HK/G-6PDH mixture doubles the sensitivity of the assay. In a further variation of the assay, both diaphorase and iodonitrotetrazolium chloride (INT) can be included, which yields a further 2.5-fold sensitivity increase. Methodology containing INT/diaphorase will be released at a later date. These extensions to the traditional biochemistry employed in lactose measurement greatly improve the limit of detection (LOD) and limit of quantitation (LOQ) of the assay, which are both well within the EFSA recommended levels for low-lactose and lactose free products.

Principle:

Pre-treatment (low-lactose and lactose-free products):

Free D-glucose is efficiently removed from the sample by conversion to D-gluconic acid by the enzymes glucose oxidase and catalase in the presence of oxygen (1) and (2).

$$\text{(1) D-Glucose} + O_2 + H_2O \xrightarrow{\text{(glucose oxidase)}} \text{D-gluconic acid} + H_2O_2$$

$$\text{(2) } 2H_2O_2 \xrightarrow{\text{(catalase)}} O_2 + 2H_2O$$

Assay (measurement of D-glucose/lactose in samples):

Prior to lactose hydrolysis, any remaining free D-glucose is phosphorylated by the enzyme hexokinase (HK), in the presence of adenosine-5'-triphosphate (ATP) to glucose-6-phosphate (G-6-P) with the simultaneous formation of adenosine-5'-diphosphate (ADP) (3).

$$\text{(3) D-Glucose} + \text{ATP} \xrightarrow{\text{(HK)}} \text{G-6-P} + \text{ADP}$$

G-6-P is oxidised by the enzyme glucose-6-phosphate dehydrogenase (G6P-DH) in the presence of nicotinamide-adenine dinucleotide phosphate ($NADP^+$) to gluconate-6-phosphate (gluconate-6-P) with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) (4).

$$\text{(4) G-6-P} + NADP^+ \xrightarrow{\text{(G6P-DH)}} \text{gluconate-6-P} + \text{NADPH} + H^+$$

Gluconate-6-P is immediately converted to D-ribulose-5-phosphate (R-5-P), carbon dioxide (CO_2), and a further molecule of NADPH by the enzyme 6-phosphogluconate dehydrogenase (6-PGDH) (5).

(6-PGDH)

(5) Gluconate-6-P + $NADP^+$ ⟶ R-5-P + CO_2 + NADPH + H^+

Lactose is hydrolysed to D-galactose and D-glucose by β-galactosidase (6). Glucose released from lactose enters the series of reactions catalysed by HK, G6P-DH, and 6-PGDH (3, 4 & 5).

(β-galactosidase)

(6) Lactose + H_2O ⟶ D-galactose + D-glucose

The amount of NADPH formed is stoichiometric to twice the amount of lactose as two molecules of NADPH are produced for each D-glucose molecule originating from the lactose in the sample.

Specificity, Sensitivity, Linearity And Precision:

This kit, which employs MZ104 β-galactosidase, is specific for free D-glucose and selective for D-glucose released from lactose. In contrast, *E. coli* β-galactosidase, which is commonly employed in these types of assays, readily hydrolyses lactose and all lactose analogues (1,3-β-D-, 1,2-β-D and 1,6-β-D-galactosyl-D-glucose), and thus is not selective for lactose. In fact, *E. coli* β-galactosidase can be used in place of MZ104 β-galactosidase to catalyse hydrolysis of lactose and all lactose analogues, allowing measurement of these components as a group.

The detection limit of the assay is 1.62 mg/L, which is derived from an absorbance difference of 0.02 with a maximum sample volume of 0.40 mL. This corresponds to a lactose concentration of 8.75 mg/L in the original low-lactose or lactose-free milk sample, processed using the “Standard Assay Procedure”. The assay is linear over the range of 1 to 50 µg of lactose (or 0.50 to 25 µg of D-glucose). This equates to a lactose concentration range of 0.05 to 2.50 g/L in the original low-lactose or lactose-free milk sample, processed using the “Standard Assay Procedure” and using 0.10 mL of sample. In duplicate determinations using one sample solution, an absorbance difference of 0.005 to 0.010 may occur. With a sample volume of 0.10 mL, this corresponds to a lactose concentration of approx. 8.70 to 17.50 mg/L of sample solution. 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:

Interfering substances in the sample being analysed can be identified by including lactose as 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 lactose to the sample in the initial extraction steps. If the conversion of the D-glucose liberated from lactose has been completed within the time specified in the assay, it can be generally concluded that no interference has occurred. However, this can be further checked by adding lactose (25 µg in 0.10 mL) to the cuvette on completion of the reaction. A significant increase in the absorbance should be observed. MZ104 β-Galactosidase is inhibited at higher levels of free D-galactose, so sample size in the assay is limited to 0.10-0.40 mL (see page 9).

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 website.

Kit Content:

Kits suitable for performing 65 assays are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Buffer A (8 mL, pH 8.0) plus sodium azide (0.02% w/v) as a preservative.
Stable for > 2 years at 4°C.

Bottle 2: Glucose oxidase (GOX) and catalase, lyophilised powder. Stable for > 2 years below -10°C.

Bottle 3: Buffer B (8 mL, pH 7.6) plus sodium azide (0.02% w/v) as a preservative.
Stable for > 2 years at 4°C.

Bottle 4: $NADP^+$ plus ATP.
Stable for > 5 years below -10°C.

Bottle 5: Hexokinase plus glucose-6-phosphate dehydrogenase plus 6-phosphogluconate dehydrogenase suspension (1.4 mL).
Stable for > 2 years at 4°C.

Bottle 6: MZ104 β-galactosidase suspension (1.4 mL).
Stable for > 2 years at 4°C.

Bottle 7: Lactose standard solution (5 mL, 0.25 mg/mL in 0.02% w/v sodium azide)
Stable for > 2 years; store sealed at 4°C.

Preparation of reagent solutions/suspensions:

1. Use the contents of bottle 1 as supplied.
Stable for > 2 years at 4°C.

2. Dissolve the contents of bottle 2 in 14 mL of distilled water. To avoid repetitive freeze/thaw cycles, divide into appropriately sized aliquots and store in polypropylene tubes below -10°C. Stable for > 2 years below -10°C.

3. Use the contents of bottle 3 as supplied.
Stable for > 2 years at 4°C.

4. Dissolve the contents of bottle 4 in 4 mL of distilled water. **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).

5 & 6. Use the contents of bottles 5 and 6 as supplied. Before opening for the first time, shake the bottles to remove any enzyme that may have settled on the stopper. Subsequently store the bottles in an upright position. Swirl the bottle to mix contents before use. Stable for > 2 years at 4°C.

7. Use the contents of bottle 7 as supplied.
Stable for > 2 years; store sealed at 4°C.

NOTE: The lactose standard solution (bottle 7) 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. Interferences in the assay can be tested by adding a known amount of the lactose standard solution to the sample prior to sample preparation and assessing the recovery of this. The concentration of lactose is determined directly from the extinction coefficient of NADPH (page 10).

Preparation of reagents (not supplied):

1. **Concentrated Carrez I solution: 200 mL**

   Dissolve 30 g of potassium hexacyanoferrate (II) trihydrate ($K_4[Fe(CN)_6].3H_2O$) (Sigma cat. no. 60279) in 200 mL of distilled water. Stable for > 3 years room temperature.

2. **Concentrated Carrez II solution: 200 mL**

   Dissolve 60 g of zinc sulphate heptahydrate ($ZnSO_4.7H_2O$) (Sigma cat. no. 31665) in 200 mL of distilled water. Stable for > 3 years room temperature.

3. **Hydrogen peroxide (~ 30% w/w; Sigma cat. no. H1009)**
   Use as supplied. Stable for > 3 years at 4°C.

NOTE: Reagents 1 and 2 for the sample clarification procedure using Carrez are available as a separate kit product K-CARREZ.

Equipment (recommended):

1. Volumetric flasks/beakers.
2. Disposable 1.5 mL polypropylene microfuge tubes, e.g. Sarstedt cat. no. 72.690 (www.sarstedt.com).
3. 13 mL polyproylene tubes, e.g. Sarstedt cat. no. 60.541.685.
4. Analytical balance.
5. Vortex mixer (e.g. Vortex-Genie® 2 mixer).
6. Boiling water bath (set at 100°C).
7. Microfuge (required speed 13000 rpm).
8. Disposable plastic micro-cuvettes (1 cm light path, 1.5 mL), e.g. Plastibrand®, PMMA; Brand cat. no. 759115 (www.brand.de).
9. Micro-pipettors, e.g. Gilson Pipetman® (20 µL, 200 µL and 1 mL).
10. Spectrophotometer set at 340 nm, e.g. MegaQuant™ Wave (Megazyme cat. no. D-MQWAVE).

Standard assay procedure for low-lactose or lactose-free milk:

NOTE: Sample volumes in Section C must not exceed 0.4 mL because at higher levels, free D-galactose in the sample inhibits the β-galactosidase. For samples other than low-lactose or lactose-free milk, refer to "Sample Preparation" sections on pages 14 and 15.

A. Sample preparation:

Pipette into a 1.5 mL polypropylene microfuge tube	Volume
distilled water (at ~ 25°C)	0.90 mL
milk sample	0.50 mL
* concentrated Carrez II solution	0.05 mL
* concentrated Carrez I solution	0.05 mL

Cap the tube, mix by vortex and then centrifuge at 13,000 rpm for 10 min. **Carefully pipette the supernatant for use in section B.**

NOTE: The kit for sample clarification K-CARREZ can be used to carry out the sample preparation step. Refer to the assay protocol for K-CARREZ available on the Megazyme website.

B. Glucose oxidase/catalase pre-treatment

Pipette into a 13 mL polypropylene tube	Volume
distilled water (at ~ 25°C)	0.40 mL
clear supernatant (from section A)	1.00 mL
solution 1 (Buffer A)	0.10 mL
suspension 2 (GOX/catalase mixture)	0.20 mL
* hydrogen peroxide (30% w/w)	0.10 mL

Immediately cap the tubes, mix the contents on a vortex mixer and incubate at room temperature (~ 22°C) for 15 min. After 15 min, slowly loosen the cap to release pressure and re-tighten. Incubate in a 100°C boiling water bath for 5 min. Transfer the solution to a 1.5 mL microfuge tube and centrifuge at 13,000 rpm for 10 min. **Carefully pipette 0.1 mL of the clear supernatant for use as the "sample solution" in the "Enzymatic Determination Reaction" (see section C, page 9).**

* These components should be prepared as described on page 7.

NOTE: If the recommended centrifuge able to reach 13,000 rpm is not available, filtration using Whatman Type I (or equivalent) filter paper or using a syringe filter is also a possibility. Please refer to the relevant FAQ on the Megazyme website (www.megazyme.com).

C. Enzymatic determination reaction:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic; 1.5 mL semi-micro)

Temperature: 25°C
Final volume: 1.17 mL (D-glucose); 1.19 mL (lactose)
Sample solution: 0.5-25 µg of D-glucose or 1-50 µg lactose per cuvette (in 0.1-0.4 mL sample volume)

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

NOTE: Only one "Blank" (Reagent Blank) assay required per batch of samples.

Pipette into cuvettes	Blank	Sample
distilled water (at ~ 25°C)	1.00 mL	0.90 mL
sample solution (see section B, page 7)	-	0.10 mL
solution 3 (Buffer B)	0.10 mL	0.10 mL
solution 4 (NADP+/ATP)	0.05 mL	0.05 mL
Mix*, read absorbances of the solutions (A1) after approx. 3 min and start the reaction by addition of:		
suspension 5 (HK/G-6-PDH/6-PGDH)	0.02 mL	0.02 mL
Mix* and read the absorbance of the solutions (A2) at the end of the reaction (approx. 10 min). **Then add:**		
suspension 6 (β-galactosidase)	0.02 mL	0.02 mL
Mix* and read the absorbance of the solutions (A3) at the end of the reaction (15 min).		

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

NOTE: For low-lactose and lactose-free samples, continue to read the absorbance value exactly at 5 min intervals after the 15 min reaction. Take readings at exactly 20 min and 25 min after the addition of suspension 6. Input the absorbance readings into the "Creep Calculator" tab on the Megazyme ***Mega-Calc***™, downloadable from the Megazyme website. Using this calculator allows more accurate measurement of just lactose and removes some GOS interference.

NOTE: For “low-lactose” and “lactose-free” samples, where a sample has generated absorbance differences (A2-A1) > 0.300 or (A3-A2) > 1.500 refer to “Troubleshooting” on page 14.

Calculation:

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 $\Delta A_{D\text{-glucose}}$.

Determine the absorbance difference (A_3-A_2) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{lactose}$.

Note: The value of $\Delta A_{lactose}$ should, as a rule, be at least ~ 0.05 absorbance units to achieve sufficiently accurate results. While the assay is linear down to a $\Delta A_{lactose}$ of 0.02, the degree of error in the measurement becomes very significant below an absorbance of 0.05.

The concentration of D-glucose and lactose can be calculated as follows:

$$c = \frac{V \times MW}{\varepsilon \times d \times v \times 2} \times F \times \Delta A \quad [g/L]$$

where:

V = final volume [mL]
MW = molecular weight of D-glucose or lactose [g/mol]
β = extinction coefficient of NADPH at 340 nm
= 6300 [1 x mol^{-1} x cm^{-1}]
d = light path [cm]
v = sample volume [mL]
2 = 2 moles of NADPH produced for each mole of D-glucose or lactose
F = dilution factor
= 5.4 [standard preparation for “Liquid Milk”]

It follows for D-glucose:

$$c = \frac{1.17 \times 180.16}{6300 \times 1.0 \times 0.1 \times 2.0} \times F \times \Delta A_{D\text{-glucose}} \quad [g/L]$$

$$= 0.1673 \times F \times \Delta A_{D\text{-glucose}} \quad [g/L]$$

for Lactose:

$$c = \frac{1.19 \times 342.3}{6300 \times 1.0 \times 0.1 \times 2.0} \times F \times \Delta A_{lactose} \quad [g/L]$$

$$= 0.3233 \times F \times \Delta A_{lactose} \quad [g/L]$$

If the sample has been diluted during preparation, the result must be multiplied by the dilution factor (F). Following the standard preparation for "Liquid Milk", the dilution factor (F) is 5.4.

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 D-glucose:

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

Content of Lactose:

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

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

A. Troubleshooting:

Samples with (A_2-A_1) above 0.300 or (A_3-A_2) above 1.500:

Recommendations for re-processing samples that have generated absorbance differences (A_2-A_1) above 0.300 or (A_3-A_2) above 1.500 when processed using the Standard Assay Procedure (section A, B and C, pages 8 and 9) are given in the table below. The suggested recommendations are dependent upon the combination of the two absorbance values generated:

(A_2-A_1)	(A_3-A_2)	Recommendation
> 0.300	< 1.500	Repeat the "Sample Preparation" (section A, page 8) and increase the second incubation of section B (glucose oxidase/catalase) to 30 min. This is a sufficient incubation for the removal of up to 90 mg/mL D-glucose from the original "Milk" sample (section A, page 8). Alternatively, dilute the original sample with distilled water and repeat the "Sample Preparation" (section A, page 8).
> 0.300	> 1.500	Appropriately dilute the original sample with distilled water and repeat the "Sample Preparation" (section A, page 8).
< 0.300	> 1.500	Appropriately dilute the sample after glucose oxidase/catalase pre-treatment (section B, page 8) with distilled water prior to the "Enzymatic Determination Reaction" (section C, page 8) to obtain an absorbance difference (A_3-A_2) between ~ 0.05 to ~ 1.500.

B. Sample preparation:

1. Sample dilution.

Dilution Table

The amount of lactose present in the cuvette should range between 1 and 50 µg (or between 0.5 and 25 µg for D-glucose), i.e. in the 0.1 mL of sample being analysed. The sample solution must therefore be diluted sufficiently to yield a lactose concentration between 0.01 and 0.50 g/L (or between 0.005 and 0.25 g/L for D-glucose).

Estimated concentration of D-Glucose and/or Lactose (g/L)	Dilution with water	Dilution factor (F)
< 0.5	No dilution required	1
0.5-5.0	1 + 9	10
5-50	1 + 99	100
> 50	1 + 999	1000

If the value of $\Delta A_{glucose}$ and/or $\Delta A_{lactose}$ is too low (e.g. < 0.05), prepare a more concentrated sample solution. Alternatively, the sample volume to be pipetted into the cuvette can be increased up to 0.4 mL and the volume of distilled water be reduced accordingly so that the final volume of the assay remains the same.

2. 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, the pH of the solution should be increased to approx. 7.6 using 2 M NaOH, and the solution incubated at room temperature for 30 min.

(c) Carbon dioxide: samples containing a significant amount of carbon dioxide should be degassed by increasing the pH to approx. 7.6 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 β-galactosidase, 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.

Sample preparation examples for low-lactose or lactose -free samples:

(a) Determination of lactose in low-lactose or lactose-free cream or yogurt.

Accurately weigh approx. 10 g of cream or yogurt into a 50 mL volumetric flask. Add approx. 30 mL of distilled water, mix and store at 50-60°C for 15 min with occasional swirling. Add 0.5 mL of concentrated Carrez II solution and mix. Add 0.5 mL of concentrated Carrez I solution and mix. Dilute to volume with distilled water and mix thoroughly. Filter an aliquot of the solution through Whatman No. 1 filter paper. Discard the first few mL of filtrate. Use 1.0 mL of the clear filtrate (sample solution) in the ***"Glucose Oxidase/Catalase Pre-Treatment" step (section B, page 8).*** *Typically, for low-lactose or lactose-free milk, cream and yogurt, no dilution is required and a sample volume of 0.4 mL is used. For calculation purposes a dilution factor (F) of* **1.8** *is required (from the "Glucose Oxidase/Catalase Pre-Treatment" step) and a solid sample concentration of 200 g/L. is used in calculation, i.e. weight*$_{\text{sample}}$ *[g/L sample solution].*

(b) Determination of lactose in low-lactose or lactose-free cheese and chocolate.

Accurately weigh approx. 10 g of grated cheese or grated chocolate to a 50 mL beaker. Add approx. 30 mL of distilled water and a stirrer bar, and mix on a magnetic stirrer at 50-60°C for approx. 15 min. Add 0.5 mL of concentrated Carrez II solution and mix. Add 0.5 mL of concentrated Carrez I solution and mix. Quantitatively transfer the solution to a 50 mL volumetric flask and dilute to volume with distilled water. Mix thoroughly and filter an aliquot of the solution through Whatman No. 1 filter paper. Discard the first few mL of filtrate. Use 1.0 mL of the clear filtrate (sample solution) in the ***"Glucose Oxidase/Catalase Pre-Treatment" step (section B, page 8).*** *Typically, for low-lactose or lactose-free cheese or chocolate, no dilution is required and a sample volume of 0.4 mL is used. For calculation purposes a dilution factor (F) of* ***1.8*** *is required (from the "Glucose Oxidase/Catalase Pre-Treatment" step) and a solid sample concentration of 200 g/L. is used in calculation, i.e. weight*$_{\text{sample}}$ *[g/L sample solution].*

Sample preparation examples for “regular” dairy samples (non low-lactose)

(c) Determination of D-glucose and/or lactose in “regular” milk, cream or yogurt

Accurately weigh approx. 1 g of milk, cream or yogurt into a 250 mL volumetric flask, add approx. 150 mL of distilled water, mix and store at 50-60°C for 15 min with occasional swirling. Add 2 mL of concentrated Carrez II solution and mix. Add 2 mL of concentrated Carrez I solution and mix. Dilute to volume with distilled water and mix thoroughly. Filter an aliquot of the solution through Whatman No. 1 filter paper. Discard the first few mL of filtrate. Use the clear filtrate (sample solution) in the assay. *Typically, for “regular” milk, cream and yogurt, no dilution is required and a sample volume of 0.1 mL is used in the* **“Enzymatic Determination Reaction” step (section C, page 9)**. *For calculation purposes a dilution factor (F) of 1.0 is required and a solid sample concentration of 4 g/L is used in calculation, i.e. weight$_{sample}$ [g/L sample solution].*

(d) Determination of D-glucose and/or lactose in “regular” cheese and chocolate.

Accurately weigh approx. 10 g of grated cheese or 0.5 g of grated chocolate to a 250 mL beaker. Add approx. 150 mL of distilled water and a stirrer bar and mix on a magnetic stirrer at 50-60°C for approx. 15 min. Add 2 mL of concentrated Carrez II solution and mix. Add 2 mL of concentrated Carrez I solution and mix. Quantitatively transfer the solution to a 250 mL volumetric flask and dilute to volume with distilled water. Mix thoroughly and filter an aliquot of the solution through Whatman No. 1 filter paper. Discard the first few mL of filtrate. Use the clear filtrate (sample solution) in the assay. *Typically, for “regular” chocolate and most cheeses no dilution is required and a sample volume of 0.1 mL is used in the* ***“Enzymatic Determination Reaction” step (section C, page 9).*** *For calculation purposes a dilution factor (F) of 1.0 is required and a solid sample concentration of 40 g/L is used for cheese and 2 g/L is used for chocolate in calculation, i.e. weight$_{sample}$ [g/L sample solution].*

References:

1. Azcarate-Peril, M. A., Ritter, A. J., Savaiano, D., Monteagudo-Mera, A., Anderson, C., Magness, S. T. & Klaenhammer, T. R. (2017). Impact of short-chain galactooligosaccharides on the gut microbiome of lactose-intolerant individuals. *Proc. Natl. Acad. Sci. USA*, **114**, 367-375.

2. Levitt, M. 1., Wilt, T. & Shaukat, A. (2013). Clinical implications of lactose malabsorption versus lactose intolerance. *J. Clin. Gastroenterol.,* **47**, 471-480.

3. Scientific Opinion on lactose thresholds in lactose intolerance and galactosaemia. EFSA Panel on Dietetic Products Nutrition and Allergies (NDA). (2010). EFSA Journal, **8(9)**, 1777, 29.

4. McCleary, B. V. & Charnock, S. (2006). Assay for the determination of free D-galactose and/or L-Arabinose. Patent number EP 1 828 407 81.

5. DIN 10486. Milk and milk products - Determination of the lactose content in lactose-reduced milk and lactose-reduced milk products in the presence of glucose - Enzymatic method.

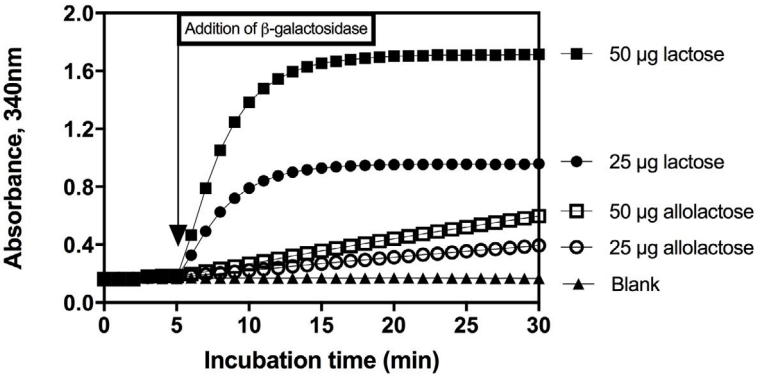

Figure 1. Increase in absorbance at 340 nm on incubation of 25 or 50 µg of lactose and of 25 or 50 µg of allolactose with MZ104 β-galactosidase included in the **K-LOLAC** assay kit (current sequential assay format).

NOTE: The selectivity of the MZ104 β-galactosidase is greater for lactose over allolactose. The linear absorbance increase (i.e. creep) linked to allolactose hydrolysis can be accounted for using the creep calculator. This allows for the removal of any lactose overestimation linked to the presence of allolactose in the sample (see Figure 3).

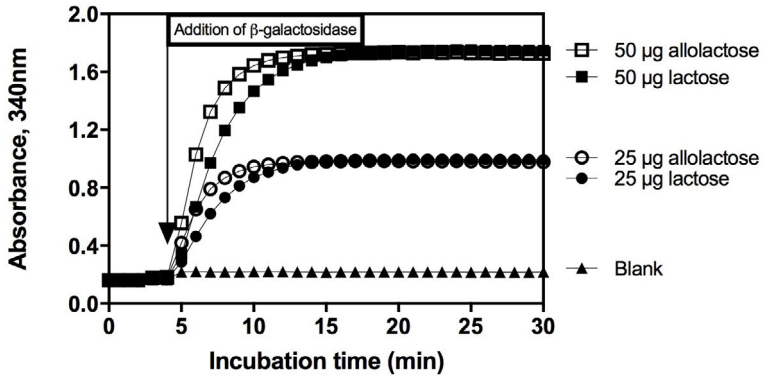

Figure 2. Increase in absorbance at 340 nm on incubation of 25 or 50 µg of lactose and of 25 or 50 µg of allolactose with the enzyme *E. coli* β-galactosidase present in competitor assay kits for lactose measurement (current sequential assay format).

Note that *E. coli* β-galactosidase actually hydrolyses allolactose more rapidly than lactose which is likely to cause lactose over-estimation.

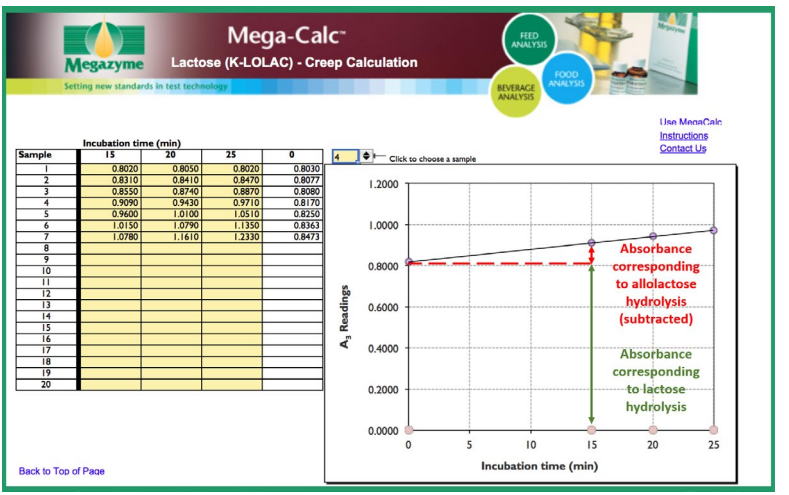

Figure 3. Excel® based "creep calculator" to facilitate calculation of actual lactose content in a sample by identifying the contribution that hydrolysis of allolactose makes to released D-glucose. This sample contains 20 µg lactose and 20 µg allolactose. Hydrolysis of lactose is complete in 15 min. Slow hydrolysis of allolactose continues over the following 30 min. Measuring absorbance at 15, 20 and 25 min and extrapolating back to zero time (time of addition of β-galactosidase to the reaction mixture) allows accurate measurement of the actual lactose content of the sample.

North America
NEOGEN Headquarters

800.234.5333 (USA/Canada)
foodsafety@neogen.com
NEOGEN.com

Europe, Middle East and Africa
NEOGEN Europe

+ 44 (0) 1292 525 600
info_uk@neogeneurope.com
NEOGEN.com

Mexico
NEOGEN Latinoamerica

+52 (55) 5254-8235
informacion@neogenlac.com
NEOGEN.com

Brazil
NEOGEN do Brasil

+55 19 3935.3727
info@neogendobrasil.com.br
NEOGEN.com

China
NEOGEN Bio-Scientific Technology

+86 21 6271 7013
info@neogenchina.com.cn
www.neogenchina.com.cn

India
NEOGEN Food and Animal Security

+91 484 2306598, 2301582
info@neogenindia.com
www.neogenindia.com

NEOGEN

Megazyme Building 1,

Bray Business Park, Bray,

Co. Wicklow,

A98 YV29,

IRELAND.

+353 (1) 286 1220

Megazyme.com

Without guarantee

The information contained in this assay protocol is, to the best of our knowledge, true and accurate, but since the conditions of use are beyond our control, no warranty is given or is implied in respect of any recommendation or suggestions which may be made or that any use will not infringe any patents.