Megazyme

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myo-INOSITOL

ASSAY PROCEDURE

K-INOSL 12/18

(*50 Assays per Kit)

**The number of tests per kit can be doubled if all volumes are halved*

INTRODUCTION:

myo-Inositol is a cyclitol present in most eukaryotic cells and exists as the predominant isomer of 1,2,3,4,5,6-cyclohexanehexol. As a key component of eukaryotic cell signalling, *myo*-inositol functions as crucial second messengers in the form of inositol (poly)phosphates and phosphatidylinositides.

The abundance of *myo*-inositol in nature makes it an essential compound for plants and animals, and many microorganisms such as *Bacillus subtilis* are equipped with catabolic pathways to enable the utilisation of *myo*-inositol as a sole carbon source. In plants, *myo*-inositol predominates in the form of phytic acid (*myo*-inositol 1,2,3,4,5,6, hexakisphosphate) where it serves as the major storage of phosphorus in plant seeds contributing ~ 70% of total phosphorus. The abundance of phytic acid in cereal grains is a concern in the foods and animal feeds industries because the phosphorus in this form is unavailable to monogastric animals due to a lack of endogenous phytases.

This method (**K-INOSL**) is suitable for the specific measurement of *myo*-inositol in foods, dairy products and other materials. This kit is not suitable for the determination of *myo*-inositol in baby formula.

Phytic acid content of samples with very low levels of free *myo*-inositol can also be determined using **K-INOSL**. This can be achieved by measuring the amount of *myo*-inositol released after de-phosphorylation of phytic acid with the enzymes phytase and alkaline phosphatase, as used with the Megazyme Phytic Acid/Total Phosphorus Assay Kit (**K-PHYT**).

PRINCIPLE:

myo-Inositol is oxidised by nicotinamide-adenine dinucleotide (NAD^+) in the presence of *myo*-inositol dehydrogenase (IDH), leading to the formation of 2,4,6/3,5-pentahydroxycyclohexanone, reduced nicotinamide-adenine dinucleotide (NADH) and H^+ (1).

(1) *myo*-Inositol + NAD^+ $\xrightarrow{\text{(}myo\text{-Inositol dehydrogenase)}}$ 2,4,6/3,5-pentahydroxycyclohexanone + NADH + H^+

A second reaction catalysed by diaphorase is required, in which NADH reduces iodonitrotetrazolium chloride (INT) to an INT-formazan product, leading to a rapid and quantitative conversion of *myo*-Inositol (2).

(2) INT + NADH + H^+ $\xrightarrow{\text{(Diaphorase)}}$ NAD^+ + INT-formazan

The amount of INT-formazan formed in this reaction is stoichiometric with the amount of *myo*-inositol. It is the INT-formazan which is measured by the increase in absorbance at 492 nm.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

The assay rapidly converts *myo*-inositol and also converts D-glucose and D-xylose, however D-glucose is phosphorylated during the procedure by hexokinase to D-glucose 6-phosphate which does not react.

The smallest differentiating absorbance for the assay is 0.010 absorbance units. This corresponds to 0.41 mg/L of sample solution at the maximum sample volume of 0.5 mL. The detection limit is 0.82 mg/L, which is derived from an absorbance difference of 0.020 with a sample volume of 0.5 mL.

The assay is linear over the range of 2 to 35 µg of for *myo*-inositol 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 0.5 mL, this corresponds to a *myo*-inositol concentration of approx. 0.2 to 0.41 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.

INTERFERENCES:

If the conversion of *myo*-inositol has been completed within the time specified in the assay (approx. 10 min), it can be generally concluded that no interference has occurred. However, this can be further checked by adding *myo*-inositol (approx. 25 µg in 0.1 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 *myo*-inositol to the sample in the initial extraction steps.

High concentrations of L-ascorbic acid, cysteine or sulphite interfere with the assay as they react with INT causing a non-enzymic "creep" rate. These compounds should be removed by treating the sample with H_2O_2 and alkali as follows:

Weigh or pipette sample, diluted if necessary, into a 50 mL volumetric flask. Add water to a volume of approx. 40 mL, and then add 1 mL of 2 M KOH and 0.01 mL of H_2O_2 (30% v/v). Incubate the solution for 10 min at approx. 70°C. Cool to 20-25°C and adjust to pH 8.0 with 1 M H_2SO_4. Fill to the mark with distilled water, mix, filter and use the solution for the assay.

KITS:

Kits suitable for performing 50 assays are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Buffer (5.5 mL, pH 7.4) plus sodium azide (0.02% w/v) as a preservative.
Stable for > 2 years at 4°C.

Bottle 2: ATP.
Freeze dried powder.
Stable for > 5 years below -10°C.

Bottle 3: Hexokinase suspension (1.1 mL).
Stable for > 2 years at 4°C.

Bottle 4: Buffer (53 mL, pH 9.5) plus sodium azide (0.02% w/v) as a preservative.
Stable for > 2 years at 4°C.

Bottle 5: (x2) NAD^+ plus INT.
Freeze dried powder.
Stable for > 5 years below -10°C.

Bottle 6: Diaphorase suspension (1.1 mL).
Stable for > 2 years at 4°C.

Bottle 7: *myo*-Inositol dehydrogenase suspension (1.1 mL).
Stable for > 2 years at 4°C.

Bottle 8: *myo*-Inositol standard solution (5 mL, 0.25 mg/mL) in 0.02% (w/v) sodium azide.
Stable for > 2 years at 4°C.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

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

2. Dissolve the contents of bottle 2 in 5.5 mL of distilled water. Divide into appropriately sized aliquots and store in polypropylene tubes below -10°C between use and keep cool during use. Once dissolved, the reagent is stable for > 2 years below -10°C.

3, 6 & 7. Use the contents of bottles 3, 6 and 7 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 bottles to mix contents before use.
Stable for > 2 years at 4°C.

5. Dissolve the contents of one of bottle 5 in 15 mL of distilled water. Divide into appropriately sized aliquots and store in polypropylene tubes at 20°C between use and keep cool during use if possible. Once dissolved, the reagent is stable for > 2 years below -10°C. **Do not** dissolve the contents of the second bottle until required.

8. Use the contents of bottle 8 as supplied.
Stable for > 2 years at 4°C.

NOTE: The *myo*-inositol 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 *myo*-inositol is determined directly from the coefficient of INT (see page 6).

EQUIPMENT (RECOMMENDED):

1. Disposable plastic cuvettes (1 cm light path, 3 mL).
2. Disposable 1.5 mL polypropylene microfuge tubes.
3. 30 mL polypropylene screw cap tubes.
4. Micro-pipettors, e.g. Gilson Pipetman® (20 µL, 200 µL and 1 mL).
5. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5 mL Combitip® (to dispense 0.1 mL aliquots of Buffer 1 and ATP).
   - with 25 mL Combitip® (to dispense 0.5 mL aliquots NAD^+/INT).
6. Stop clock.
7. Analytical balance.
8. Spectrophotometer set at 492 nm.
9. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
10. Whatman No. 1 (9 cm) filter papers.
11. Microfuge (required speed 13,000 rpm).

PROCEDURE:

Wavelength: 492 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 2.26 mL
Sample solution: 2-35 µg of *myo*-inositol per cuvette (in 0.1-0.5 mL sample volume)

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

Pipette into 3 mL cuvettes:	Blank	Sample
distilled water	0.50 mL	0.40 mL
solution 1 (buffer, pH 7.4)	0.10 mL	0.10 mL
sample	-	0.10 mL
solution 2 (ATP)	0.10 mL	0.10 mL
suspension 3 (Hexokinase)	0.02 mL	0.02 mL
Mix* and incubate at room temperature for 15 min. **Then add:**		
solution 4 (buffer, pH 9.5)	1.00 mL	1.00 mL
solution 5 (NAD^+/INT)	0.50 mL	0.50 mL
suspension 6 (Diaphorase)	0.02 mL	0.02 mL
Mix*, read the absorbances of the solutions (A_1) after 3 min and start the reaction by addition of:		
suspension 7 (IDH)	0.02 mL	0.02 mL
Mix* and read the absorbance of the solutions (A_2) at the end of the reaction (~ 15 min). If the reaction has not stopped after 10 min, continue to read the absorbances at 2 min intervals until the absorbances either plateau or increase constantly over 2 min**.		

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

** if this "creep" rate is greater for the sample than that of the blank, extrapolate the absorbances (sample and blank) back to the time of the addition of suspension 7 (IDH).

NOTE: INT and the reaction system containing INT are sensitive to light. Consequently, reactions must be performed in the dark (e.g. in the spectrophotometer cuvette compartment with the photometer lid closed).

CALCULATION:

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

The value of $\Delta A_{Inositol}$ should generally be at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of *myo*-inositol can be calculated as follows:

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

where:

V = final volume [mL]
MW = molecular weight of *myo*-inositol [g/mol]
ε = extinction coefficient of INT-formazan at 492 nm
= 19900 [L x mol^{-1} x cm^{-1}]
d = light path [cm]
v = sample volume [mL]

It follows for *myo*-inositol:

$$c = \frac{2.26 \times 180.16}{19900 \times 1.0 \times 0.1} \times \Delta A_{Inositol} \quad [g/L]$$

$$= 0.2046 \times \Delta A_{Inositol} \quad [g/L]$$

If the sample has been diluted during the preparation the result must also be multiplied by the additional 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 *myo*-inositol:

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

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

SAMPLE PREPARATION:

1. Sample dilution.

The amount of *myo*-inositol present in the cuvette (i.e. in the 0.1 mL of sample being analysed) should range between 2 and 35 µg. The sample solution must therefore be diluted sufficiently to yield a concentration between 0.02 and 0.4 g/L.

Dilution table

Estimated concentration of *myo*-inositol (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 $\Delta A_{Inositol}$ 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 0.5 mL, making sure that the sum of the sample and distilled water components in the reaction is 0.5 mL and using the new sample volume in the equation.

2. Sample clarification.

a. Solutions:

Carrez I solution. Dissolve 3.60 g of potassium hexacyanoferrate (II) $\{K_4[Fe(CN)_6].3H_2O\}$ (Sigma cat. no. P9387) in 100 mL of distilled water. Store at room temperature.

Carrez II solution. Dissolve 7.20 g of zinc sulphate ($ZnSO_4.7H_2O$) (Sigma cat. no. Z4750) in 100 mL of distilled water. Store at room temperature.

Sodium hydroxide (NaOH, 100 mM). Dissolve 4 g of NaOH in 1 L of distilled water. Store at room temperature.

b. Procedure:

Pipette the liquid sample into a 100 mL volumetric flask which contains approx. 60 mL of distilled water, or weigh sufficient quantity of the sample into a 100 mL volumetric flask and add 60 mL of distilled water. Carefully add 5 mL of Carrez I solution, 5 mL of Carrez II solution and 10 mL of NaOH solution (100 mM). Mix after each addition. Fill the volumetric flask to the mark, mix and filter.

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 red wine or coloured 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 carbon dioxide 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: a sample blank, i.e. sample with no *myo*-inositol dehydrogenase, 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 polyvinylpolypyrollidone (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 distilled 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.

(h) Samples containing protein: deproteinise samples containing protein with Carrez reagents.

SAMPLE PREPARATION EXAMPLES:

(a) Determination of *myo*-inositol in biological samples.

In some cases, it may be possible to test some biological samples directly with an appropriate dilution in distilled water. However, if this is not adequate then deproteinisation may be required. It is assumed that the sample being tested contains *myo*-inositol at a concentration above the limit of detection of the test kit being used after accounting for the dilution during sample preparation.

(b) Deproteinisation with perchloric acid

1 M Perchloric acid
(Sigma cat. no. 244252; MW = 100.46; d = 1.664 (g/mL); 70% assay; 11.59 M)
Add 8.6 mL perchloric acid to 92.4 mL of distilled water and mix thoroughly.

1 M Potassium hydroxide
(Sigma cat. no. 60369; MW = 56.11; 86% assay)
Add 6.5 g of potassium hydroxide pellets to approx. 80 mL of distilled water and stir to dissolve. Make to 100 mL with distilled water.

To the sample add an equal volume of ice-cold 1 M perchloric acid with mixing. Filter or centrifuge at 1,500 *g* for 10 min and adjust the pH of the supernatant to between 7 and 8 with 1 M KOH. Use the supernatant in the assay after appropriate dilution, if required.

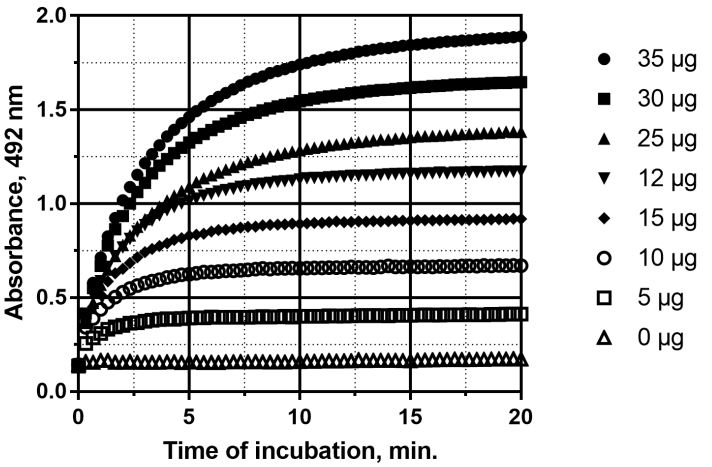

Figure 1. Increase in absorbance at 492 nm on incubation of 0-35 µg of *myo*-Inositol with *myo*-Inositol dehydrogenase and diaphorase in the presence of NAD^+ and INT at 25°C using 1 cm path-length cuvettes.

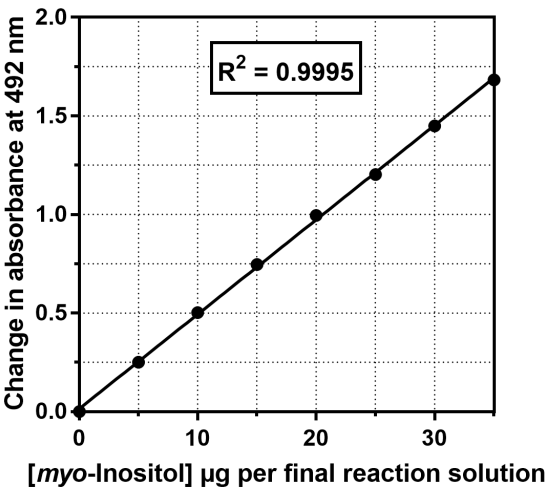

Figure 2. Calibration curve showing the linearity of the described method from 0-35 µg of *myo*-Inositol. The reactions used to generate this calibration curve were performed at 25°C using 1 cm path-length cuvettes.

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