Megazyme

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D-GLUCURONIC ACID & D-GALACTURONIC ACID

(D-GLUCURONATE & D-GALACTURONATE)

ASSAY PROCEDURE

K-URONIC 08/18

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

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

INTRODUCTION:

D-glucuronic acid and D-galacturonic acid are naturally occurring hexuronic acids present in glycosaminoglucans, glucuronide conjugates in mammals and in plant cell wall polysaccharides.

D-Glucuronic acid exists as a component of glycosaminoglucans such as hyaluronan, heparin and chondroitin sulphate present in mammalian connective tissue such as cartilage. In the synthesis of glucuronide conjugates in mammals glucuronic acid is conjugated to xenobiotic compounds, a biosynthesis reaction known as glucuronidation, catalysed by the enzyme UDP-glucuronyltransferase and considered a detoxifying process.

Both D-glucuronic acid and D-galacturonic acid are major components of plant cell wall polysaccharides. D-glucuronic acid is a component of arabinoxylan, which consists of a β-(1,4)-linked xylan backbone substituted with α-(1-2/3)-linked L-arabinofuranose and α-(1-2)-linked 4-*O*-methylglucuronic acid. D-Galacturonic acid is the major component of pectin comprising the α-(1,4)-linked galacturonan backbone of homogalacturonan and rhamnogalacturonan II, and is present within the repeating disaccharide unit [,4)-α-D-Gal*p*A-(1,2)-α-L-Rhap-(1,] of rhamnogalacturonan I.

This kit (K-URONIC) is suitable for the specific measurement of D-hexuronic acids including D-glucuronic and D-galacturonic acid.

PRINCIPLE:

D-glucuronic acid (1) or D-galacturonic acid (2) are oxidised by the enzyme uronate dehydrogenase in the presence of nicotinamide-adenine dinucleotide (NAD^+) to D-glucarate or D-galactarate, respectively, with the formation of reduced nicotinamide-adenine dinucleotide (NADH).

$$\text{(1)}\quad \text{D-Glucuronic acid} + NAD^+ + H_2O \xrightarrow{\text{(Uronate dehydrogenase; UDH)}} \text{D-glucarate} + NADH + H^+$$

$$\text{(2)}\quad \text{D-Galacturonic acid} + NAD^+ + H_2O \xrightarrow{\text{(Uronate dehydrogenase; UDH)}} \text{D-galactarate} + NADH + H^+$$

The amount of NADH formed in this reaction is stoichiometric with the amount of D-glucuronic acid or D-galacturonic acid. It is the NADH which is measured by the increase in absorbance at 340 nm.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

The assay is specific for D-glucuronic acid (D-glucuronate) and D-galacturonic acid (D-galacturonate).

The smallest differentiating absorbance for the assay is 0.010 absorbance units. This corresponds to a D-glucuronic acid or D-galacturonic acid concentration of ~ 8.7 mg/L of sample solution at a sample volume of 0.1 mL. The detection limit is ~ 17.4 mg/L, which is derived from an absorbance difference of 0.020 with a sample volume of 0.1 mL.

The assay is linear over the range of 5 to 150 µg of D-glucuronic acid or D-galacturonic acid per assay. With a sample volume of 0.1 mL, this corresponds to a D-glucuronic acid or D-galacturonic acid concentration of ~ 0.5 to 1.5 g/L of sample solution. In duplicate determinations using one sample solution, an absorbance difference of 0.005 to 0.010 may occur. With a sample volume of 0.1 mL, this corresponds to a D-glucuronic acid or D-galacturonic acid concentration of ~ 4.4 to ~ 8.7 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 D-glucuronic acid or D-galacturonic acid has been completed within the time specified in the assay (approx. 10 min at 25°C), it can be generally concluded that no interference has occurred. However, this can be further checked by adding D-glucuronic acid or D-galacturonic acid (approx. 50 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 D-glucuronic acid or D-galacturonic acid 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 website.

KITS:

Kits suitable for performing 100 assays in manual format (or 1000 assays in auto-analyser format or 1000 assays in microplate format) are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Buffer (22 mL, pH 8) plus sodium azide (0.02% w/v) as a preservative.
Stable for > 2 years at 4°C.

Bottle 2: NAD^+ freeze dried powder.
Stable for > 5 years below -10°C.

Bottle 3: Uronate dehydrogenase suspension (2.2 mL).
Stable for > 2 years at 4°C.

Bottle 4: D-Glucuronic acid (5 mL; 0.5 mg/mL).
Stable for > 2 years at 4°C.

PREPARATION OF REAGENT SOLUTIONS (SUPPLIED):

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

2. Dissolve the contents of bottle 2 in 22 mL of distilled water. **Stable for > 1 year 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. Use the contents of bottle 3 as supplied. Before opening for the first time, shake the bottle to remove any enzyme that may have settled on the rubber stopper. Subsequently store the bottle in an upright position. **Swirl the bottle to mix contents before use.**
Stable for > 2 years at 4°C.

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

NOTE: The D-glucuronic acid standard solution (bottle 4) 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. To test the assay using the standard solution, add 0.1 mL (50 µg D-glucuronic acid) of standard solution to the assay cuvette in place of the sample. The concentration of D-glucuronic acid is determined directly from the extinction coefficient of NADH (page 5).

EQUIPMENT (RECOMMENDED):

1. Disposable plastic cuvettes (1 cm light path, 3 mL).
2. Micro-pipettors, e.g. Gilson Pipetman® (20 µL, 200 µL and 1 mL).
3. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 25 mL Combitip® [to dispense 0.2 mL aliquots of reagent solutions (solutions 1 & 2)].
4. Stop clock.
5. Analytical balance.
6. Spectrophotometer set at 340 nm.
7. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
8. 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 or 37°C
Final volume: 2.52 mL
Sample solution: 5-150 µg of D-glucuronic acid or D-galacturonic acid per cuvette (in 0.1-2.0 mL sample volume)

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

Pipette into cuvettes	Blank	Sample
distilled water (at ~ 25°C)	2.10 mL	2.00 mL
sample	-	0.10 mL
solution 1 (buffer)	0.20 mL	0.20 mL
solution 2 (NAD^+)	0.20 mL	0.20 mL
Mix*, read absorbances of the solutions (A_1) after approx. 3 min and start the reaction by addition of:		
suspension 3	0.02 mL	0.02 mL
Mix*, read the absorbance of the solutions (A_2) at the end of the reaction (approx. 10 min at 25°C or approx. 5 min at 37°C). If the reaction has not stopped after 10 min, continue to read the absorbances at 2 min intervals until the absorbances remain the same.		

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

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 $\Delta A_{D\text{-hexuronic acid}}$. The value of $\Delta A_{D\text{-hexuronic acid}}$ should be at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of D-glucuronic acid or D-galacturonic acid can be calculated as follows:

$$c = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A_{D\text{-hexuronic acid}} \quad [g/L]$$

where:

V = final volume [mL]
MW = molecular weight of D-glucuronic acid or D-galacturonic acid [g/mol]
ε = extinction coefficient of NADH at 340 nm = 6300 [l x mol^{-1} x cm^{-1}]
d = light path [cm]
v = sample volume [mL]

It follows for D-hexuronic acid (D-glucuronic acid or D-galacturonic acid):

$$c = \frac{2.52 \times 194.14}{6300 \times 1.0 \times 0.1} \times \Delta A_{D\text{-hexuronic acid}} \quad [g/L]$$

$$= 0.7766 \times \Delta A_{D\text{-hexuronic acid}} \quad [g/L]$$

If the sample is 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 D-hexuronic acid (D-glucuronic acid or D-galacturonic acid):

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

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).

B. AUTO-ANALYSER ASSAY PROCEDURE:

NOTES:

1. The Auto-Analyser Assay Procedure for D-glucuronic acid or D-galacturonic acid 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 D-glucuronic acid or D-galacturonic acid **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 solution 1 (buffer) solution 2 (NAD^+)	48.0 mL 5.5 mL 5.5 mL (after adding 22 mL of H_2O to bottle 2)
Total volume	59.0 mL

Preparation of R2:

Component	Volume
distilled water solution 3 (UDH)	6.90 mL 0.55 mL
Total volume	7.45 mL

EXAMPLE METHOD:

R1:	0.200 mL
Sample:	~ 0.01 mL
R2:	0.025 mL
Reaction time:	~ 10 min at 25°C or 5 min at 37°C
Wavelength:	340 nm
Prepared reagent stability:	> 2 days when refrigerated
Calculation:	endpoint
Reaction direction:	increase
Linearity:	up to 1.4 g/L of D-glucuronic acid or D-galacturonic acid using 0.01 mL sample volume

C. MICROPLATE ASSAY PROCEDURE

NOTES:

1. The Microplate Assay Procedure for D-glucuronic acid or D-galacturonic 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 D-glucuronic acid or D-galacturonic **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 or 37°C
Final volume: 0.252 mL
Linearity: 0.1-15.0 µg of D-glucuronic acid or D-galacturonic acid per well (in 0.01-0.20 mL sample volume)

Pipette into wells	Blank	Sample	Standard
distilled water	0.210 mL	0.200 mL	0.200 mL
sample solution	-	0.010 mL	-
standard solution	-	-	0.010 mL
solution 1 (buffer)	0.020 mL	0.020 mL	0.020 mL
solution 2 (NAD^+)	0.020 mL	0.020 mL	0.020 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 2 min and start the reactions by addition of:			
suspension 3 (UDH)	0.002 mL	0.002 mL	0.002 mL
Mix*, read the absorbances of the solutions (A_2) at the end of the reaction (approx. 10 min at 25 or 5 min at 37°C). If the reaction has not stopped after 5-10 min, continue to read the absorbances at 1 min intervals until the absorbances increase constantly over 1 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 3.

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:

1. Sample dilution.

The amount of D-hexuronic acid present in the cuvette (i.e. in the 0.1 mL of sample being analysed) should range between 5 and 150 µg. The sample solution must therefore be diluted sufficiently to yield a concentration between 0.05 and 1.5 g/L.

Dilution table

Estimated concentration of D-uronic acid (g/L)	Dilution with water	Dilution factor (F)
< 1.5	No dilution required	1
1.5-15	1 + 9	10
> 15	1 + 999	100

If the value of $\Delta A_{\text{D-hexuronic acid}}$ 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 to 2.0 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.

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) 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.

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

(f) 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.

(g) Samples containing protein: deproteinise samples containing protein with Carrez reagents, alternatively use ice cold perchloric acid. 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.

SAMPLE PREPARATION EXAMPLES:

(a) Determination of D-glucuronic acid or D-galacturonic acid in plant samples.

Mill plant materials to pass a 0.5 mm screen. Weigh out 1.0 g of sample and extract with 90 mL of water (heated to 80°C). Quantitatively transfer to a volumetric flask and dilute to the mark with distilled water. Mix, filter and use the appropriately diluted, clear solution for the assay. *Dilute appropriately in distilled water and use in the assay.*

(b) Determination of D-glucuronic acid or D-galacturonic acid in fermentation samples and cell culture medium.

Incubate an aliquot (approx. 10 mL) of the solution at approx. 90-95°C for 10 min to inactivate enzyme activity. Centrifuge or

filter and use the supernatant or clear filtrate (diluted according to the dilution table, if necessary) for the assay. Alternatively, deproteinisation can be performed with Carrez reagents. Homogenise gelatinous agar media with water and treat further as described above. *Dilute appropriately in distilled water and use in the assay.*

(c) Determination of D-glucuronic acid or D-galacturonic acid in polysaccharides and fibrous plant material.

Mill plant material or polysaccharide to pass a 0.5 mm screen using a Retsch centrifugal mill, or similar. Accurately weigh approx. 100 mg of material into a Corning screw-cap culture tube (16 x 125 mm). Add 5 mL of 2 M H_2SO_4 to each tube and cap the tubes. Incubate the tubes at 100°C for 6 h. Stir the tubes intermittently during the incubation. Cool the tubes to room temperature, carefully loosen the caps and add 7 mL of 2 M NaOH. Quantitatively transfer the contents of the tube to a 100 mL volumetric flask using distilled water and adjust the volume to 100 mL with distilled water. Mix thoroughly by inversion and filter an aliquot of the solution through Whatman No. 1 filter paper or centrifuge at 1,500 *g* for 10 min. *Dilute appropriately in distilled water and use in the assay.*

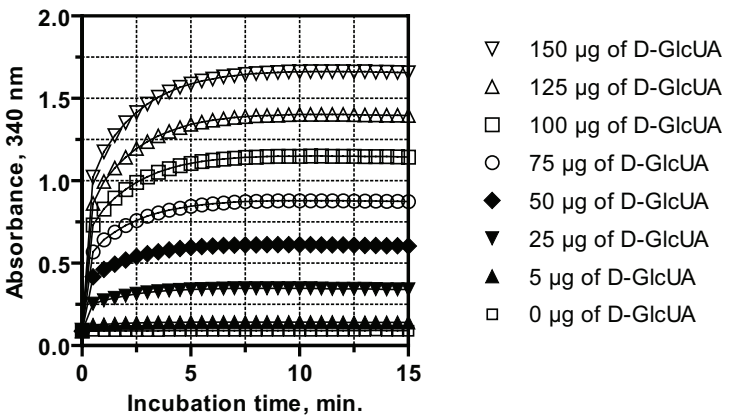

Figure 1. Increase in absorbance at 340 nm on incubation of 0-150 µg of D-glucuronic acid with uronate dehydrogenase in the presence of NAD^+ at 25°C using 1 cm path-length cuvettes (Manual Format; page 4).

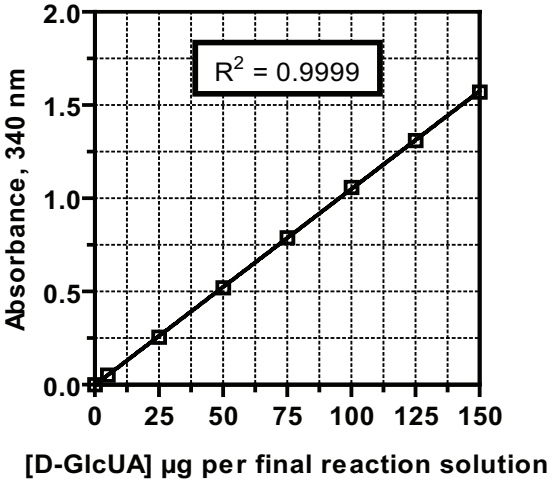

Figure 2. Calibration curve showing the linearity of K-URONIC from 0-150 µg of D-glucuronic acid. The reactions used to generate this calibration curve were performed at 25°C using 1 cm path-length cuvettes (Manual Format; page 4).

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