Megazyme

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D-GLUCOSE
(MEGAPLEX RED)

ASSAY PROCEDURE

K-MRGLUC 04/21

(1000 Microplate Assays per Kit) or
(100 Manual Assays per Kit) or
(400 Auto-analyser Assays per Kit)

INTRODUCTION:

D-glucose is found in most plant and food products. In plant materials, it occurs as a free sugar or in a range of di-, oligo- and poly-saccharides such as starch, 1,3:1,4-β-D-glucans and cellulose. It is present in significant quantities in honey, wine and beer, and a range of solid foodstuffs such as bread and pastries, chocolate and candies.

Megaplex Red (also known as 10-acetyl-3,7-dihydroxyphenoxazine) is a highly sensitive and stable probe, commonly used to detect hydrogen peroxide (H_2O_2). When this reaction is coupled to glucose oxidase (GOX) it allows for the measurement of D-glucose in a sample (as outlined in the principle section). Integral to this process is the ability of Megaplex Red (in the presence of horseradish peroxidase (HRP) and H_2O_2) to produce the red-fluorescent oxidation product, resorufin. Resorufin has a fluorometric (excitation and emission maxima of 571 and 585 nm respectively) and colourimetric (absorbance maximum at ~ 570 nm) signal allowing for the use of both fluorometric and colourimetric detection methods.

The Megazyme D-glucose Assay Kit (Megaplex Red) (**K-MRGLUC**) is a simple, user friendly test capable of detecting D-glucose in various samples. This kit can be adapted to detect enzymatic activity in reactions where D-glucose is the end product (subject to end user development).

PRINCIPLE:

The D-glucose quantification assay is based on the HRP mediated oxidation of Megaplex Red. Briefly, GOX reacts with D-glucose to form glucono-1,5-lactone and H_2O_2 (1). In the presence of HRP, this H_2O_2 oxidises the colourless Megaplex Red probe in a 1:1 stoichiometry to form a coloured product (resorufin) that can be measured using fluorometer equipped for excitation in the range of 530-560 nm and fluorescence emission detection at ~ 590 nm or a spectrophotometer at 570 nm (2). The concentration of D-glucose in the sample is quantified using a calibration curve in fluouresence mode or a single-point standard of known D-glucose concentration in absorbance mode.

(1) D-glucose + O_2 $\xrightarrow{\text{GOX}}$ glucono-1,5-lactone + H_2O_2

(2) H_2O_2 + Megaplex Red $\xrightarrow{\text{HRP}}$ H_2O + resorufin (coloured)

The assay principle is shown in more detail in Figure 1 (page 12).

LINEARITY:

In fluorescence mode, the detection limit is 0.09 ug/mL. As fluorescence values may vary greatly between instruments, the limit of detection is calculated using the lowest value from the linear range of the assay in fluorescence mode, using the recommended sample volume of 0.01 mL.

The assay is linear over the range of 0.009 to 1.8 µg/mL (0.5 to 10 µM) of D-glucose per assay in fluorescence mode.

In absorbance mode, the detection limit is 0.25 µg/mL which is derived from an absorbance difference of 0.020 with the maximum sample volume of 0.4 mL. The assay is linear over the range of 0.9 to 9.0 µg/mL (5 to 50 µM) of D-glucose per assay in absorbance mode.

Linearity graphs for both detection methods are shown in Figures 2 and 3 (pages 10 and 11).

INTERFERENCE:

Interference caused by the sample matrix can be identified by performing recovery experiments, i.e. by adding a known amount of the D-glucose standard to the sample in the test. Quantitative recovery of this standard would be expected.

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 1000 assays in microplate format (or 100 assays in manual format or 400 assays in auto-analyser format) are available from Megazyme. The kits contain the full assay method plus:

Bottle 1:	Buffer concentrate (20 mL, pH 7.4) plus sodium azide (0.02% w/v) as a preservative. Stable for > 2 years at 4°C.
Bottle 2:	Megaplex Red, lyophilised powder. Stable for > 2 years below -10°C.
Bottle 3:	Dimethyl sulfoxide (DMSO) (0.6 mL). Stable for > 2 years at room temperature.
Bottle 4:	Horseradish peroxidase (HRP) (0.4 mL). Stable for > 2 years below -10°C.
Bottle 5:	Glucose oxidase (GOX) suspension (1.5 mL). Stable for > 2 years at 4°C.
Bottle 6:	D-glucose standard solution (5 mL, 0.09 mg/mL or 500 µM). Stable for > 2 years; store sealed at 4°C.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Visually inspect bottle 1 for crystallisation before use. If the buffer has crystallised, heat to ~ 40°C and mix by inversion until redissolved. Dilute the contents of bottle 1 before use by adding 2 mL of buffer to 18 mL of dH_2O (this is the **Assay Buffer**). This is sufficient for ~ 20 manual assays or ~ 200 microplate assays with a small excess for standard and sample dilution.
   Stable for > 3 months at 4°C.
2. Tap the vial on a solid surface prior to opening to ensure that all powder falls to the bottom of the tube. Dissolve the contents by adding 0.55 mL of bottle 3 (DMSO) into the vial. Recap the tube and mix thoroughly. This is the **Megaplex Red** solution. Split into aliquots of ~ 0.1 mL before storage to avoid repeated freeze thaw cycles.
   Stable for > 2 years below -10°C.
3. Use the contents of bottle 3 as supplied. DMSO has a high freezing point and the contents may freeze if room temperature falls below ~ 20°C. Ensure the DMSO is in liquid form before use by warming gently if necessary.
4. Use the contents of bottle 4 as supplied. Store the bottle in an upright position.
   Stable for > 2 years below -10°C.
5. Use the contents of bottle 5 as supplied. Before opening for the first time, swirl the bottle to remove any protein that may have settled on the rubber stopper. Subsequently, store the bottle in an upright position.
   Stable for > 2 years at 4 °C.
6. Use the contents of bottle 6 as supplied.
   Stable for > 2 years; store sealed at 4°C.

NOTE: The D-glucose standard solution (or user diluted standards in fluoresence mode) should be tested with every set of assays as it is used for the calculation of D-glucose concentration in the sample analysed.

EQUIPMENT (RECOMMENDED):

1. Polypropylene tubes (~ 13 mL capacity).
2. Disposable plastic cuvettes (1 cm light path, 1.5 mL) or microplate suitable for fluorometric detection.
3. Micro-pipettors, e.g. Gilson Pipetman® (200 µL and 1000 µL).
4. Positive displacement pipettor, e.g. Eppendorf Multipette® with 25 mL Combitip® [to dispense 1 mL aliquots of bottle 1, concentrated buffer solution.

5. 20 mL graduated cylinder.
6. Fluorometer equipped for ~ 530-560 nm (excitation) and ~ 590 nm (emission) or Spectrophotometer set at 570 nm temperature controlled to 25°C.
7. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).

PREPARATION OF WORKING SOLUTION:

Pipette the following into a ~ 13 mL polypropylene tube. This volume is sufficient for ~ 200 microplate assays or ~ 20 manual assays.

Component	Volume
Assay Buffer Megaplex red bottle 4 (HRP) bottle 5 (GOX)	9.625 mL 0.100 mL 0.075 mL 0.200 mL
Total volume	10 mL
Mix well. Make fresh on day of use. Do not store.	

The Megaplex Red D-glucose assay procedure may be completed in fluorescence or absorbance mode. In fluorescence mode a 10-fold increased sensitivity is achieved.

A. MICROPLATE ASSAY PROCEDURE (FLUORESCENCE MODE):

NOTES:
For each set of samples analysed a calibration curve must be performed concurrently in fluorescence mode using the same batch of reagents.

PREPARATION OF D-GLUCOSE CALIBRATION CURVE:

Dilute bottle 6 (D-glucose standard) by pipetting 0.5 mL into 4.5 mL of dH_2O in a polypropylene tube. This is the **diluted standard**. Prepare the calibration curve standard D-glucose solutions as described in the table below (standards 1-6, 0.09-1.8 µg/mL of D-glucose in-assay). Aliquot and freeze these solutions for future use. Stable for > 2 years below -10°C.

Pipette into 13 mL polypropylene tubes	**STD 1** 0.09 µg per mL	**STD 2** 0.375 µg per mL	**STD 3** 0.75 µg per mL	**STD 4** 1.125 µg per mL	**STD 5** 1.5 µg per mL	**STD 6** 1.8 µg per mL
assay buffer **diluted standard**	1.44 mL 0.06 mL	1.25 mL 0.25 mL	1.0 mL 0.5 mL	0.75 mL 0.75 mL	0.5 mL 1.0 mL	0.3 mL 1.2 mL
total volume	1.5 mL	1.5 mL	1.5 mL	1.5 mL	1.5 mL	1.5 mL

ASSAY PROCEDURE:

Detection method: Fluorescence
Excitation: 530-560 nm
Emission: ~ 590 nm
Microplate: 96-well (e.g. black, flat-bottomed, polystyrene)
Temperature: ~ 25°C
Final volume: 0.10 mL
Linearity: 0.009-0.18 µg of D-glucose per assay (0.5-10 µM in-assay)

Pipette into wells	Blank	Sample	Standard
Assay buffer	0.050 mL	0.040 mL	0.025 mL
sample	-	0.010 mL	-
standards 0 - 5	-	-	0.025 mL
Mix*, allow the reaction to equilibrate to temperature for 5 min and start the reaction by addition of:			
Working Solution	0.05 mL	0.05 mL	0.05 mL
Mix* and read the absorbance of the solutions (Abs) after exactly 30 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).

CALCULATION:

1. Determine the average of the duplicate readings for the blank, D-glucose standards and samples.
2. Correct for background fluoresence by subtracting the average of the blank values from the D-glucose standard values thereby obtaining $Standard_{cor}$.
3. Plot a calibration curve showing the corrected absorbance values ($Standard_{cor}$) on the X-axis against the concentration of D-glucose [µg/mL in-assay] on the Y-axis using a suitable software such as Excel. A new calibration curve must be completed for each assay.
4. Calculate the linear trendline and the equation of the line based on your calibration curve data.
5. Correct for background fluoresence in the samples of interest by subtracting the average of the blank values from the average sample values thereby obtaining $Sample_{cor}$.
6. Extrapolate the concentration of the corrected sample using the following equation;

$$y = \text{Slope (m)} \times Sample_{cor} + b \quad [\mu g/mL \text{ in-assay}]$$

The concentration of D-glucose in the sample is calculated as follows:

$$\mathbf{c} = \frac{\mathbf{y} \times 0.1 \times F}{0.01} \quad [\mu g/mL]$$

where:

y	=	µg/mL in-assay calculated from the standard curve
0.1	=	total assay volume [mL]
F	=	dilution of sample
0.01	=	sample volume in-assay [mL]

The content of glucose in µM can then be calculated, if required, as follows:

$$\mathbf{c} = \mu g/mL \times 1000 \times \frac{1}{180.16} \quad [\mu M]$$

where:

µg/mL	=	concentration of D-glucose in the sample
1000	=	conversion from mL to L
180.16	=	Molecular weight of D-glucose in g/mol

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).

Example:

The linear trendline for the sample data in Figure 2 (page 12) of this assay protocol gives the following equation:

$$\mathbf{y} = 0.0038x + 0.0081 \text{ (in the form } y = mx + b)$$

If the $Sample_{cor}$ value in this analysis is 300 RFU for an undiluted sample then the concentration of the D-glucose in the assay can be calculated as follows:

$$\mathbf{y} = 0.0038 \times 300 + 0.0081 = 1.15 \text{ µg/mL in-assay}$$

The concentration of D-glucose in the sample is calculated as follows:

$$\mathbf{c} = \frac{1.15 \times 0.1 \times 1}{0.01} = 11.5 \text{ µg/mL}$$

The content of glucose in µM can then be calculated, if required, as follows:

$$\mathbf{c} = 11.5 \times 1000 \times \frac{1}{180.16} = 63.8 \text{ µM}$$

B. MICROPLATE ASSAY PROCEDURE (ABSORBANCE MODE):

In absorbance mode a full calibration curve is not required, a single point standard is sufficient. No dilution of Bottle 6 is required.

Detection method: Absorbance
Wavelength: 570 nm
Microplate: 96-well (e.g. clear flat-bottomed, plastic)
Temperature: ~ 25°C
Final volume: 0.10 mL
Linearity: 0.09-0.90 µg of D-glucose per assay (5-50 µM in-assay)

Pipette into cuvettes	Blank	Sample	Standard
Assay buffer	0.050 mL	0.040 mL	0.045 mL
sample	-	0.010 mL*	-
bottle 6 (standard)	-	-	0.005 mL
Mix**, allow the reaction to equilibrate to temperature for 5 min and start the reaction by addition of:			
Working Solution	0.050 mL	0.050 mL	0.050 mL
Mix** and read the absorbance of the solutions (Abs) after exactly 30 min.			

* the sample volume in the test can be increased to 0.04 mL if an increase in sensitivity is required. If the sample volume is increased, the volume of Assay buffer should be reduced proportionately.
** for example using microplate shaker, shake function on a microplate reader or repeated aspiration (e.g. using a pipette set at 50-100 µL volume).

CALCULATION:

Determine the absorbance difference between the absorbance of the standard and of the blank ($Abs_{standard}$ - Abs_{blank}) after 30 min, thereby obtaining $\Delta Abs_{standard}$.

Calculate **M** as follows:

$$\mathbf{M} = \frac{\text{D-Glucose (µg/mL in-assay)}}{\Delta Abs_{standard}}$$

Determine the absorbance difference between the absorbance of the sample and of the blank (Abs_{sample} - Abs_{blank}) after 30 min, thereby obtaining ΔAbs_{sample}.

The concentration of glucose can then be calculated as follows:

$$\mathbf{c} = \Delta Abs_{sample} \times \mathbf{M} \times \frac{V_1}{V_2} \times F \quad [\mu g/mL]$$

where:

$\Delta Abs_{glucose}$ = Abs_{sample} - Abs_{blank}
M = value of D-glucose standard [$\mu g/\Delta Abs_{standard}$]
V_1 = total assay volume [mL]
F = dilution of sample
V_2 = sample volume in-assay [mL]

The content of D-glucose in µM can then be calculated, if required, as follows:

$$\mathbf{c} = \mu g/mL \times 1000 \times \frac{1}{180.16} \quad [\mu M]$$

where:

µg/mL = concentration of D-glucose in the sample
1000 = conversion from mL to L
180.16 = Molecular weight of D-glucose in g/mol

Example:

Where the kit control is used as decribed in the assay (0.005 mL of bottle 6 containing 0.09 mg/mL of D-glucose in 0.1 mL final volume) the amount of D-glucose in the in the assay is 4.5 µg/mL. If, for example, the $\Delta Abs_{standard}$ measured in the assay is 0.891, then the "M" value can be calculated as follows:

$$\mathbf{M} = \frac{4.5}{0.891} = 5.05$$

If the ΔAbs_{sample} measured in the assay for an undiluted sample is 0.700 then the concentration of glucose is calculated as follows:

$$\mathbf{c} = 0.700 \times 5.05 \times \frac{0.1}{0.01} \times 1 = 35.35 \ \mu g/mL$$

The content of D-glucose in µM can then be calculated, if required, as follows:

$$\mathbf{c} = 35.35 \times 1000 \times \frac{1}{180.16} = 196.2 \ \mu M$$

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).

C. MANUAL ASSAY PROCEDURE (ABSORBANCE MODE):

Detection method: Absorbance
Wavelength: 570 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: 25°C
Final volume: 1.0 mL
Sample solution: 0.9-9.0 µg of D-glucose per assay (5-50 µM in-assay)

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

Pipette into cuvettes	Blank	Sample	Standard
Assay buffer	0.50 mL	0.40 mL	0.45 mL
sample	-	0.10 mL*	-
Bottle 6 (standard)	-	-	0.05 mL
Mix*, allow the reaction to equilibrate to temperature for 5 min and start the reaction by addition of:			
working solution	0.50 mL	0.50 mL	0.50 mL
Mix** and read the absorbance of the solutions (Abs) after exactly 30 min.			

* the sample volume in the test can be increased to 0.4 mL if an increase in sensitivity is required. If the sample volume is increased, the volume of Assay buffer should be reduced proportionately.
** 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 between the absorbance of the standard and of the blank ($Abs_{standard}$ - Abs_{blank}) after 30 min, thereby obtaining $\Delta Abs_{standard}$.

Calculate **M** as follows:

$$\mathbf{M} = \frac{\text{D-Glucose (µg/mL in-assay)}}{\Delta Abs_{standard}}$$

Determine the absorbance difference between the absorbance of the sample and of the blank (Abs_{sample} - Abs_{blank}) after 30 min, thereby obtaining ΔAbs_{sample}.

The concentration of glucose can then be calculated as follows:

$$\mathbf{c} = \Delta Abs_{sample} \times \mathbf{M} \times \frac{V_1}{V_2} \times F \quad [\mu g/mL]$$

where:

$\Delta Abs_{glucose}$ = Abs_{sample} - Abs_{blank}

M = value of D-glucose standard [µg/$\Delta Abs_{standard}$]

V_1 = total assay volume [mL]

F = dilution of sample

V_2 = sample volume in-assay [mL]

The content of D-glucose in µM can then be calculated, if required, as follows:

$$\mathbf{c} = \mu g/mL \times 1000 \times \frac{1}{180.16} \quad [\mu M]$$

where:

µg/mL = concentration of D-glucose in the sample

1000 = conversion from mL to L

180.16 = Molecular weight of D-glucose in g/mol

Example:

Where the kit control is used as decribed in the assay (0.05 mL of bottle 6 containing 0.09 mg/mL of D-glucose in a total assay volume of 1 mL) the amount of D-glucose in the in the assay is 4.5 µg/mL. If, for example, the $\Delta Abs_{standard}$ measured in the assay is 0.891, then the "M" value can be calculated as follows:

$$\mathbf{M} = \frac{4.5}{0.891} = 5.05$$

If the ΔAbs_{sample} measured in the assay for an undiluted sample is 0.700 then the concentration of glucose is calculated as follows:

$$\mathbf{c} = 0.700 \times 5.05 \times \frac{1.0}{0.1} \times 1 = 35.35 \text{ µg/mL}$$

The content of D-glucose in µM can then be calculated, if required, as follows:

$$\mathbf{c} = 35.35 \times 1000 \times \frac{1}{180.16} = 196.2 \text{ µM}$$

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).

D. AUTO-ANALYSER ASSAY PROCEDURE:

NOTE:
For each set of samples tested in in the Auto-Analyser format either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.

Reagent preparation for ~ 100 assays is performed as follows:

R1: Assay buffer, 10 mL (see preparation, page 3)

R2: Working solution, 12 mL (see preparation, page 4)

D-Glucose standard: Before analysis perform a dilution of the kit standard (bottle 6) by pipetting 0.5 mL of the D-glucose kit standard into 0.5 mL of assay buffer.

EXAMPLE METHOD:

R1:	0.1 mL
Sample:	0.025 mL
R2:	0.125 mL
Reaction time:	30 min at 25°C
Wavelength:	570 nm
Prepared reagent stability:	See stability information (pages 3 and 4)
Reaction direction:	Increase
Linearity:	up to 9.0 µg/mL of D-glucose using 0.025 mL sample volume

SAMPLE PREPARATION:

Fluorescence mode:

In fluorescence mode the amount of D-glucose present in the test (i.e. in the 0.01 mL of sample being analysed) should range between 0.009-0.18 µg per assay (0.5-10 µM in-assay concentration). The sample solution must therefore be diluted sufficiently to yield a D-glucose concentration between 0.9-18 µg/mL (5-100 µM) when using the recommended assay volume of 0.01 mL. In order to achieve sufficiently accurate results, the value for the sample in relative fluorescence units (RFU) should not exceed the value achieved for STD 6 (1.8 µg/mL in-assay) in the calibration curve. Recommended dilutions of sample are described in the table below.

D-glucose (µg/mL)	D-glucose (µM)	Dilution with dH_2O	Dilution factor (F)
< 18	< 100	No dilution	1
18-180	100-1,000	1 + 9	10
180-1,800	1,000-10,000	1 + 99	100
> 1,800	> 10,000	1 + 999	1,000

If the RFU value is lower than STD 1 (0.09 µg/mL in-assay) in the calibration curve, weigh out more sample or dilute less strongly.

Absorbance mode:

In absorbance mode (microplate) the amount of D-glucose present in the test (i.e. in the 0.01 mL of sample being analysed) should range between 0.09-0.9 µg per assay (5-50 µM in-assay concentration). In absorbance mode (manual), the amount of D-glucose present in the test (i.e. in the 0.1 mL of sample being analysed) should range between 0.9-9.0 µg per assay (5-50 µM in-assay concentration). The sample solution must therefore be diluted sufficiently to yield a D-glucose concentration between 50-500 µM (9-90 µg/mL) before analysis. In order to achieve sufficiently accurate results, the value of $\Delta Abs_{glucose}$ should as a rule be at least 0.1 absorbance units and not more than 2.0 absorbance units in absorbance mode. Recommended dilutions of sample are described in the table below.

D-glucose (µg/mL)	D-glucose (µM)	Dilution with dH_2O	Dilution factor (F)
< 90	< 0.5	No dilution	1
90-900	0.5-5	1 + 9	10
900-9000	5-50	1 + 99	100
> 9000	> 50	1 + 999	1000

If the value of $Abs_{glucose}$ is too low (e.g. < 0.1), weigh out more sample or dilute less strongly.

APPENDIX:

A. Assay Principle - D-Glucose detection

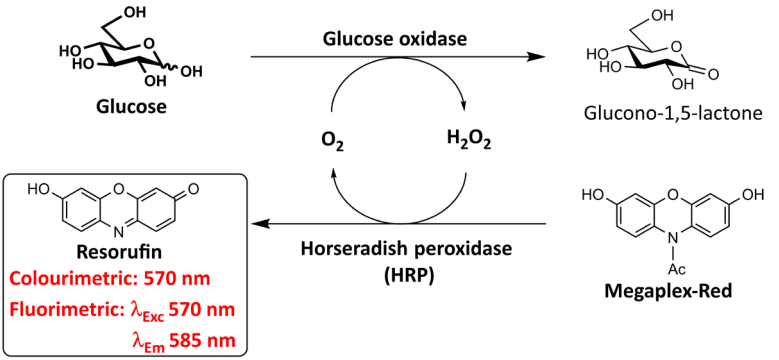

Figure 1. Theoretical basis of the D-glucose detection assay.

B. Linear range of assay

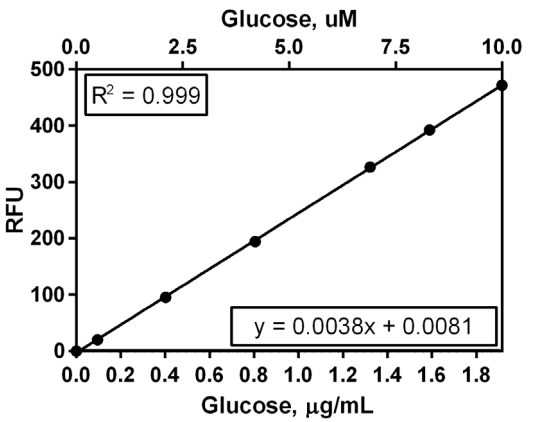

Figure 2. Linearity of the D-glucose assay in fluorescence mode including data points from typical calibration cuve. Fluorescence was measured with a fluorescence microplate reader using excitation at 530 nm and emission at 590 nm wavelengths. A new calibration curve must be completed for each batch of samples, data provided for demonstrative purposes only.

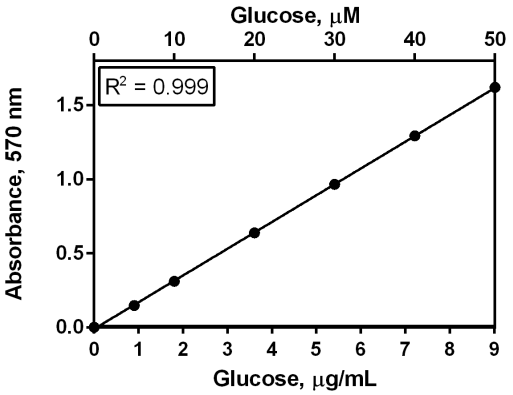

Figure 3. Linearity of the D-glucose assay in absorbance mode.

C. Precision of the assay

Analyte	n	µg D-glucose per test	Mean, RFU D-glucose	Standard Deviation	% CV
D-Glucose	10	0.009	17.37	0.64	3.68
	10	0.018	40.99	2.24	5.47
	10	0.045	115.04	1.73	1.51
	10	0.090	239.09	3.29	1.38
	10	0.180	469.41	20.52	4.37

Figure 4. Intermediate precision values in microplate fluoresence mode obtained using a range of D-glucose standards in the assay kit.

Analyte	n	µg D-glucose per test	Mean, Abs D-glucose	Standard Deviation	% CV
D-Glucose	12	0.9	0.156	0.011	7.1
	12	3.6	0.713	0.031	4.3
	12	9	1.788	0.074	4.14

Figure 5. Intermediate precision values in manual absorbance mode obtained using a range of D-glucose standards in the assay kit.

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