Megazyme

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CATALASE

ASSAY PROCEDURE

K-CATAL 07/19

(100/200 Manual Assays per Kit)

INTRODUCTION:

Catalase (EC 1.11.1.6) is an important antioxidant enzyme responsible for the degradation of the reactive oxygen species, hydrogen peroxide. It is widely expressed in both mammalian and non-mammalian cells containing the cytochrome system. Catalase catalyses the decomposition of hydrogen peroxide into water and oxygen.

Hydrogen peroxide (H_2O_2) is a by-product of various oxidase and superoxide dismutase reactions which occur within the cell, and is a normal product of cell metabolism. The accumulation of H_2O_2 is extremely harmful to the cell and may lead to oxidation of cellular proteins, lipids and DNA.[1-2] This oxidative damage can ultimately lead to DNA mutagenesis and cell death.[3] In order to prevent this damage and survive, the cell relies on various antioxidants including catalase.

Commercially, H_2O_2 may be used as a germicide to inhibit microbial growth, for example in the dairy industry. It also finds use in the textile industry for the bleaching of fabrics, etc. In these industries (and others), residual H_2O_2 may be removed by catalase treatment to prevent contamination of the product.

This assay kit provides a simple colourimetric method to detect catalase activity. The method is delivered in a fast and reliable format and may be used to detect catalase activity in various samples, including food, biological and bacterial samples. Traditionally, catalase activity is measured by observing the reduction in H_2O_2 absorbance at 240 nm.[4] Within this ultraviolet light region, interference of proteins and other biological components can be observed. The current assay offers the significant advantage of utilising visible light (520 nm), which reduces possible sample interference.

As H_2O_2 is known to inactivate catalase enzymes at concentrations above 100 mM, this assay employs an in-assay substrate concentration of ~ 65 mM with the exact H_2O_2 concentration being factored into the activity calculation in order to maximise the accuracy and reproducibility of the assay.

PRINCIPLE:

The catalase activity in a sample is determined by measuring the decrease in H_2O_2 concentration observed following an incubation of the analyte sample with an H_2O_2 standard solution.

In order to determine catalase activity using the Megazyme Catalase Assay Kit, two separate reactions must be completed.

In reaction A, the catalase sample of interest is incubated with a known concentration (~ 65 mM in assay) of H_2O_2. The reaction

is stopped by the addition of 15 mM sodium azide which strongly inhibits catalase.

Reaction A:

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

In reaction B, the exact concentration of H_2O_2 remaining is measured using an enzyme-linked colourimetric detection method employing 3,5-dichloro-2-hydroxy-benzenesulfonic acid (DHBS), 4-aminoantipyrine (AAP) and peroxidase. The resulting quinoneimine dye is measured at 520 nm.

Reaction B:

$$2H_2O_2 + \text{DHBS} + \text{AAP} \xrightarrow{\text{Peroxidase}} \text{quinoneimine dye} + 4H_2O$$

A 'blank' reaction is performed in parallel to provide the colourimetric response for the initial H_2O_2 concentration. The rate of H_2O_2 decomposition (i.e. catalase activity) can then be determined using either the ***Mega-Calc***™ software provided or the calculation discussed on page 6 of the booklet.

ACCURACY:

Standard errors of less than 3% are achieved routinely (See Appendix C, page 9).

INTERFERENCES:

Depending on the source of the sample, interferences may occur. Normally, as dilutions are required, this is not a problem. However, in samples containing very low catalase activity interferences may become significant. Substances which can interfere with the assay are listed in Table 2, Appendix D (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 these products please refer to the associated SDS that is available from the Megazyme website.

KITS:

An assay kit suitable for performing 100 (3 mL cuvette scale) or 200 (1.5 mL cuvette scale) assays are available from Megazyme. The kit contains the full assay method plus:

Bottle 1: **1.5 M Potassium phosphate buffer (30 mL, pH 7.0)** plus sodium azide (0.02% w/v) as a preservative.
Stable for > 4 years at 4°C.

Bottle 2: **Peroxidase plus 4-aminoantipyrine** and stabilisers. Shipped as a lyophilised powder.
Stable for > 4 years below -10°C.

Bottle 3: **3,5-Dichloro-2-hydroxybenzenesulfonate (DHBS) solution (20 mL)** plus sodium azide (0.02% w/v) as a preservative.
Stable for > 4 years at 4°C.

Bottle 4: **Hydrogen peroxide (H_2O_2) standard solution (10 mL, ~ 1.3 M)** Protect from light.
Stable for > 2 years at 4°C.

Bottle 5: **Catalase Standard (4 g)**
From *Aspergillus niger* (~ 1.6 U/mg). Actual U/mg stated on vial. Store below -10°C. Minimise freeze/thaw cylces. Stable for > 2 years below -10°C.

NOTE:

1. **Please ensure to prepare the reagents in the exact order described below. Failure to follow this procedure will result in the IRREVERSIBLE precipitation of DHBS.**
2. Bottle 1 and 3 contain 0.02% (w/v) sodium azide. This is a poisonous chemical and should be treated accordingly.

PREPARATION OF REAGENT SOLUTIONS (SUPPLIED):

1. Dilute the contents of bottle 1 to approx. 250 mL with distilled water.

2. Use 10 mL of the solution prepared in step 1 to resuspend the contents of bottle 2. Transfer the resulting solution back into the remaining ~ 240 mL solution prepared in step 1. This is the **Peroxidase Solution**.

3. Transfer the contents of bottle 3 into the **Peroxidase Solution** prepared in step 2. Adjust the pH if necessary to pH 7.0 using 1 M NaOH or HCl. Bring the final volume to 300 mL using distilled water. This is the **Colourimetric Reagent Solution**. Aliquot into suitable volumes and store below -10°C. Cover to protect from light. Do not freeze/thaw more than once. The reagent may also be stored

protected from light at 4°C for up to 1 week. The reagent will take on a pink colour upon storage, this is normal. Stable for > 1 year below -10°C.

4. Store bottle 4 at 4°C. Stable for > 2 years. It is necessary to freshly prepare a 130 mM H_2O_2 solution prior to each set of assays. To do this, dilute 0.1 mL of the contents of bottle 4 to 1 mL with **Assay Buffer** (preparation described below). Label this as the **H_2O_2 Substrate Solution**.

5. It is recommended that the contents of bottle 5 are divided into a suitable number of batches and then stored below -10°C. This avoids repeated freeze/thaw cycles and prolongs the life of the catalase control standard. Stable for > 2 years. It is recommended to assay this standard alongside each set of assays. It is necessary to freshly prepare the control solution immediately prior to use. To do this, dilute 100 mg of the contents of bottle 5 in 20 mL **Assay Buffer**. Label this as the **Catalase Control Standard**.

PREPARATION OF ADDITIONAL BUFFERS (not supplied):

(A) Assay Buffer (150 mM Potassium phosphate, pH 7.0)

Dissolve 13.06 g of potassium phosphate dibasic (K_2HPO_4; MW 174.18 g/mol; cat. no. P8281-500G Sigma-Aldrich) in 300 mL of distilled water. Adjust the pH to 7.0 with 1 M KOH or 1 M HCl. Adjust the volume to 500 mL with distilled water. Stable for > 1 month at 4°C.

NOTE:

The assay buffer must be prepared fresh and stored in a well-sealed container at 4°C. The buffer can be preserved by overlaying with a few drops of toluene. Sodium azide must not be used as a preservative as this is a potent inhibitor of catalase.

(B) 15 mM Sodium Azide

Carefully weigh 97.5 mg of sodium azide (NaN_3; MW 65.009 g/mol; cat. no. S2002 Sigma-Aldrich) powder using all suitable safety measures. Add 100 mL of distilled water and allow the solution to dissolve completely. Stable for > 2 years at 4°C..

CAUTION

Sodium azide is a poisonous chemical and should be treated accordingly. Follow all recommended safety precautions as outlined in the SDS.

EQUIPMENT (RECOMMENDED):

1. Disposable plastic cuvettes (1 cm light path, 1.5 mL or 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 50 mL Combitip® (to dispense 3 mL aliquots of reagent solution).
4. Stop clock.
5. Analytical balance.
6. Spectrophotometer set at 520 nm.
7. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
8. Waterbath (25 ± 0.1°C).

ASSAY PROCEDURE:

NOTE:
The number of assays which can be performed per kit can be doubled by halving the volumes of all the reagents used and by employing 1.5 mL cuvettes.

(A) Incubation of catalase sample with H_2O_2 Substrate Solution

Prepare the reaction and blank samples as outlined below. Duplicate reaction blanks are required for each set of assays performed. Duplicate (or triplicate) reactions are also recommended for each sample assayed. Perform in glass test tubes or plastic micro tubes as preferred.

Pipette into tubes	**Reaction Blank**	**Reaction Sample**
H_2O_2 substrate solution (~ 130 mM)	0.050 mL	0.050 mL
assay buffer	0.050 mL	-
catalase sample	-	0.050 mL
Mix and incubate for 5 min at 25°C. **Then add:**		
15 mM sodium azide	0.900 mL	0.900 mL
Mix well and immediately remove an aliquot of 40 µL from the blank and sample reactions. These aliquots are transferred to the colourimetric cuvette assay (Reaction B) described below.		

(B) Determination of remaining H_2O_2 concentration

Perform in cuvettes with 1 cm light path (glass or plastic). Zero the spectrophotometer against air (without cuvette in the light path).

Pipette into cuvettes	Reaction Blank
aliquots from the blank and sample reactions in the catalase/H_2O_2 incubation (Reaction A) above	0.040 mL
colourimetric reagent	3.000 mL
Mix and incubate for 15 min at 25°C.	
Read the blank and sample reaction absorbances at 520 nm and calculate the catalase activity by inputting the values into the K-CATAL ***Mega-Calc***™, available on the Megazyme website (www.megazyme.com). Alternatively, calculate the activity as outlined below.	

CALCULATION OF CATALASE ACTIVITY:

One unit of catalase activity is defined as the amount of enzyme required to decompose 1 micromole of H_2O_2 per minute at pH 7.0 and 25°C at a substrate concentration of 65 mM H_2O_2.

Catalase Units/mL =

$$\frac{\Delta A_{520}}{\text{Incubation Time}} \times \frac{\text{Total Volume in Cell}}{\text{Aliquot Assayed}} \times \frac{1}{\varepsilon_{mM}} \times \frac{A_{520}(65\text{ mM } H_2O_2)}{A_{520}(\text{Blank})} \times \frac{\text{Rxn A Assay Vol.}}{\text{Sample Volume}} \times \text{Dilution}$$

Where:

ΔA_{520} = Abs_{520} (Blank) - Abs_{520} (Reaction)

Incubation Time = 5 min

Total Volume in Cell = 3.04 mL

Aliquot Assayed = 0.04 mL

ε_{mM} = The apparent ε_{mM} for H_2O_2 using the enzyme linked colourimetric determination has been determined experimentally as **21.18**

A_{520}(65 mM H_2O_2) = The absorbance arising from 65 mM H_2O_2 under the conditions specified which has been experimentally determined as **2.01**

A_{520}(blank) = The absorbance obtained for the blank reaction (which allows for any deviation of the H_2O_2 substrate concentration from 65 mM)

Rxn A Assay Volume = 1 mL

Sample Volume = 0.05 mL (volume of catalase sample)

Dilution = Dilution of the catalase sample (if required)

Thus:

Catalase Units/mL:

$$= \frac{\Delta A_{520}}{5} \times \frac{3.04}{0.04} \times \frac{1}{21.18} \times \frac{2.01}{A_{520}(\text{Blank})} \times \frac{1}{0.05} \times \text{Dilution}$$

$$= \frac{\Delta A_{520}}{A_{520}(\text{Blank})} \times 28.85 \times \text{Dilution}$$

NOTE:

1) A suitable dilution is required for each catalase sample to be assayed. It is recommended that, initially, a broad range of dilutions should be prepared and tested. The most suitable dilution is defined as that which generates a ΔA_{520} within the range of 0.2-0.8.
2) The recommended 1:10 dilution required to prepare the 65 mM H_2O_2 substrate solution should result in an A_{520}(Blank) measurement of ~ 1.8-2.2. If necessary, adjust the concentration of the H_2O_2 substrate solution to achieve an A_{520}(Blank) within this range.
3) It has been experimentally shown (see Appendix E, page 10) that the activity of catalase is highly dependent on the concentration of H_2O_2 employed in its assay. The commonly used assay described by Bergmeyer[4] based on the direct measurement of H_2O_2 consumption recommends a 10 mM H_2O_2 concentration and as such will generate activity values approximately one fifth of that measured with this colourimetric assay.

APPENDIX:

A. H_2O_2 colourimetric detection standard curve

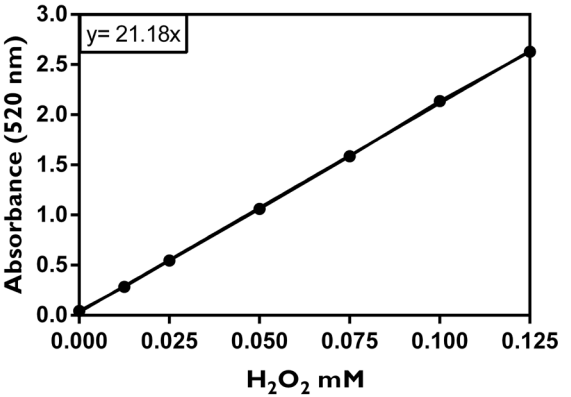

Figure 1. Standard curve relating the absorbance observed at 520 nm for the quinoneimine dye (formed using H_2O_2, AAP and peroxidase) versus a known concentration of H_2O_2. The apparent extinction coefficent (ε_{mM}) is calculated from the equation shown (= 21.18).

B. Catalase assay linearity

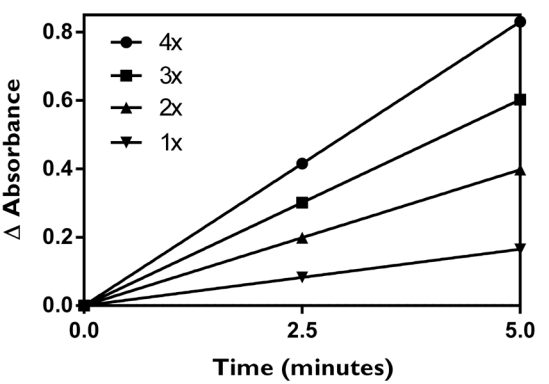

Figure 2. Linearity of the catalase assay over a 5 min reaction time at four concentrations of the *Aspergillus niger* catalase standard.

C. Repeatability and reproducibility

The repeatability and reproducibility of the assay was determined by having two analysts perform a series of assays on three different samples ranging in activity from 17.1-4.3 U/mL over two consecutive days. The results are outlined below.

Table 1.

Catalase Assay	17.1 U/mL ΔAbs (520 nm)	10.7 U/mL ΔAbs (520 nm)	4.3 U/mL ΔAbs (520 nm)
Day 1[A]	1.107	0.771	0.347
Day 2[A]	1.076	0.757	0.343
Day 1[B]	1.106	0.784	0.337
Day 2[B]	1.092	0.749	0.359
Standard Dev. (σ)	0.014	0.015	0.009
%CV	1.3	2.0	2.7

Note: A = Analyst 1, B = Analyst 2

Note that the Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated as 3 x σ of the blank sample solution absorbance and 10 x σ of the blank sample solution absorbance, respectively, using absorbance values from 10 replicates.

LOD = 0.5 U/mL (corresponding to an absorbance of 0.038)
LOQ = 1.8 U/mL (corresponding to an absorbance of 0.126)

D. Known interferences and assay compatibility

Table 2 describes the compatibility of the assay with various concentrations of chemical compounds.

Table 2.

Chemical	Compatibility
Albumin (Bovine)	Up to 50 mg/mL
Ascorbic acid	Up to 20 μM
Glucose	Up to 5 mM
Haemoglobin	Up to 0.75 mg/mL
Heparin	Up to 14 U/mL
Sodium citrate	Up to 20 mM
Tripotassium EDTA	Up to 4 mM
Triton X	Up to 0.5% v/v

E. Effect of H_2O_2 concentration on the activity of the *Aspergillus niger* catalase

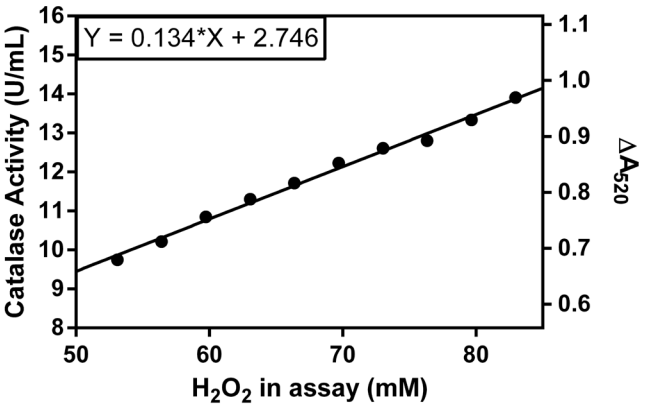

Figure 3. Investigation into the effect of H_2O_2 substrate concentration on the measured activity of the *Aspergillus niger* catalase control standard. The ΔA_{520} obatined in these assays is also shown on the right Y-axis. Any deviation in the H_2O_2 substrate concentration used is accounted for in the activity calculation.

F. Calculation of catalase activity using change in micromole H_2O_2 (alternative method of calculation)

It is recommended that the analyst uses the ***Mega-Calc***™ software provided on the product webpage for ease of calculation of catalase activity. Alternatively, the manual calculation method based on the apparent ε_{mM} of H_2O_2 described on page 6 can be used. The calculation method described below is supplemental and shown only as a similiar method has been reported by other manufacturers.[5]

The micromoles of H_2O_2 present in the blank and the catalase reaction sample can be calculated directly from the A_{520}(Blank) and A_{520}(Reaction) respectively using the standard curve shown in Figure 4. Note that this standard curve is only valid under the recommended assay conditions described here.

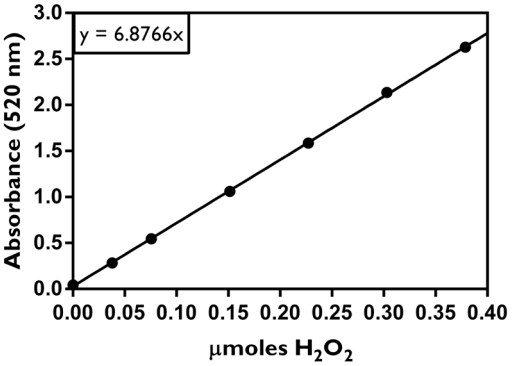

Figure 4. Standard curve relating the number of micromoles of H_2O_2 present to the absorbance observed at 520 nm in the colourimetric reaction cuvette assay.

From the equation shown in Figure 4:

$$\mu\text{moles } H_2O_2 = \frac{A_{520}}{6.877}$$

One unit of catalase activity is defined as the amount of enzyme required to decompose 1 micromole of H_2O_2 per minute at pH 7.0 and 25°C at a substrate concentration of 65 mM H_2O_2.

Catalase Units/mL:

$$= \frac{\Delta\mu\text{moles } H_2O_2}{\text{Incubation Time}} \times \frac{1}{\text{Aliquot Assayed}} \times \frac{1}{\text{Sample Volume}} \times \frac{A_{520}(65\text{ mM } H_2O_2)}{A_{520}(\text{Blank})} \times \text{Dilution}$$

Where:

$\Delta\mu$moles H_2O_2	= µmoles H_2O_2(Blank) - µmoles H_2O_2(Reaction)
Incubation Time	= 5 min
$\frac{1}{\text{Aliquot Assayed}}$	= Aliquot assayed in colourimetric assay
$\frac{1}{\text{Sample Volume}}$	= Sample volume in catalase enzymatic reaction
$\frac{A_{520}(65\text{ mM } H_2O_2)}{A_{520}(\text{Blank})}$	= Factor to allow for any deviation in H_2O_2 substrate concentration from 65 mM
Dilution	= Dilution of the sample (if required)

Thus:

Catalase Units/mL:

$$= \frac{\Delta\mu\text{moles } H_2O_2}{5} \times \frac{1}{0.04} \times \frac{1}{0.05} \times \frac{2.01}{A_{520}(\text{Blank})} \times \text{Dilution}$$

$$= \frac{\Delta\mu\text{moles } H_2O_2}{A_{520}(\text{Blank})} \times 201 \times \text{Dilution}$$

REFERENCES:

1. Gutteridge, J. M. C. & Halliwell, B. (1990). The measurement and mechanism of lipid peroxidation in biological systems. *Trends in Biochemical Sciences*, 15, 129-135.
2. Mello Filho, A. C., Hoffmann, M. E. & Meneghini, R. (1984). Cell killing and DNA damage by hydrogen peroxide are mediated by intracellular iron. *Biochemical Journal*, 218(1), 273-275.
3. Kranner, I. & Birtic, S. (2005). A modulating role for the antioxidants in desiccation tolerance. *Integrative and Comparative Biology*, 45, 734-740.
4. Bergmeyer, H. U. (1983). Isolation and identification of algicidal compound from *Streptomyces* and algicidal mechanism to Microcystis aeruginosa. *Methods of Enzymatic Analysis*, Vol. 3, 273-286.
5. Sigma-Aldrich® Catalase Assay Kit. #CAT100.

NOTES:

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