*Oxis*Research™

LumiQuick Diagnostics, Inc.

BIOXYTECH® Catalase-520™

Spectrophotometric Assay for Catalase
For Research Use Only. Not For Use In Diagnostic Procedures.
Catalog Number A21042 (AOXRE Catalog Number 21042)

INTRODUCTION

The Analyte

Hydrogen peroxide, a reactive oxygen species (ROS), is a toxic product of both normal aerobic metabolism and pathogenic ROS production. Catalase (EC 1.11.1.6, $2H_2O_2$ oxidoreductase) is a widely distributed enzyme that destroys hydrogen peroxide by dismutation (catalatic activity, Rxn 1) and also demonstrates peroxidatic activity (Rxn 2) (1,2). In the peroxidatic reaction, low molecular weight alcohols can serve as electron donors. The physiological significance of the peroxidatic activity is controversial (2).

Rxn 1	$H_2O_2 + H_2O_2$	$\xrightarrow{\text{Catalase}}$	$O_2 + 2\,H_2O$	Catalatic Activity
Rxn 2	$H_2O_2 + AH_2$	$\xrightarrow{\text{Catalase}}$	$A + 2\,H_2O$	Peroxidatic Activity

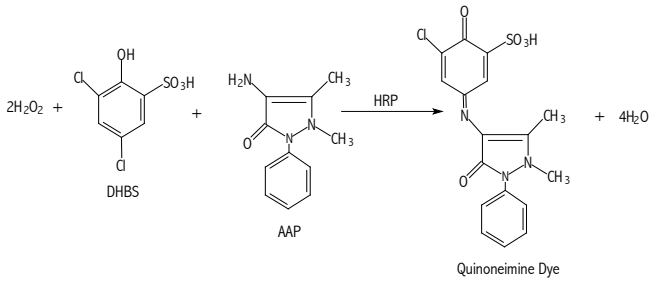
Eukaryotic catalases are heme enzymes but a manganese catalase has been described from prokaryotes (2). In humans, the highest levels of catalase are found in liver, kidney, and erythrocytes, where it is believed to account for the majority of hydrogen peroxide decomposition. Eukaryotic catalases bind NADPH, which stabilizes the enzyme and prevents the formation of Compound II, a form of catalase outside the normal catalytic cycle (3). Acatalasemia is a rare inherited condition in which there is little or no catalase produced (4,5). It is found most commonly in Asian populations, although European and other cases have been reported. Acatalasemic individuals occasionally present with oral ulcers (Takahara's Disease), but the condition is largely asymptomatic. Studies of a mouse model of acatalasemia have demonstrated the importance of catalase in preventing methemoglobin formation in erythrocytes (6). Direct spectrophotometric measurement of catalase activity requires measurement in the ultraviolet region (7), where absorption from protein and other components in the biological sample can interfere. The OXIS Catalase-520 assay utilizes visible light (520 nm), significantly reducing sample interference.

Principle of the Procedure

The Catalase-520™ assay is a two-step procedure. The rate of dismutation of hydrogen peroxide (H_2O_2) to water and molecular oxygen is proportional to the concentration of catalase (reaction 1). Therefore, the sample containing catalase is incubated in the presence of a known concentration of H_2O_2. After incubation for exactly one minute, the reaction is quenched with sodium azide.

Reaction 1

Reaction 2



The amount of H_2O_2 remaining in the reaction mixture is then determined by the oxidative coupling reaction of 4-aminophenazone (4-aminoantipyrene, AAP) and 3,5-dichloro-2-

hydroxybenzenesulfonic acid (DHBS) in the presence of H_2O_2 and catalyzed by horseradish peroxidase (HRP) as shown in reaction 2 (8). The resulting quinoneimine dye is measured at 520 nm (N-(4-antipyrl)-3-chloro-5-sulfonate-p-benzoquinonemonoimine).

REAGENTS

Materials Provided

- Chromogen — 4-aminophenazone, 3,5-dichloro-2-hydroxybenzenesulfonic acid in phosphate buffer, 2 x 110 mL.
- Substrate — 30% hydrogen peroxide, 300 µL.
- HRP — Horseradish peroxidase in phosphate buffer, 400 µL.
- Buffer — Phosphate buffer, 60 mL.
- Sample Diluent — Surfactant in phosphate buffer, 250 mL.
- Substrate Diluent — Phosphate buffer , 2 x 30 mL.
- Stop Reagent — Sodium azide, 2 x 30 mL.
- Standard — Catalase, approximately 160 U/vial, lyophilized, 2 vials.

Items Required But NOT Provided

- Spectrophotometer capable of measuring the absorbance at 520 nm
- Adjustable pipettes, 10 µL to 1 mL with disposable pipette tips
- Spectrophotometric cuvettes with a 1 cm path length, polystyrene cuvettes are recommended
- Polypropylene microtubes or similar tube with cap, 1.5 mL volume
- 12x75 mm Test tubes or similar tube, at least 2.5 mL volume
- Timer

For plasma/serum samples

- Centrifugal ultrafiltration tubes with a 30,000 molecular weight cut-off.
- Centrifuge capable of 10,000*g*, at room temperature.
- Analytical balance

Warnings and Precautions

- **Hydrogen peroxide** is corrosive and is harmful by inhalation or if swallowed. Contact with skin may cause burns. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. May develop pressure. Keep away from combustible materials.
- **Sodium azide** is harmful in contact with skin or if swallowed. Contact with acids liberates a very toxic gas. May react with lead and copper plumbing to form highly explosive azides.
- **4-Aminophenazone** may be harmful if swallowed. May cause eye and skin irritation. May cause respiratory and digestive tract irritation.
- **3,5-Dichloro-2-hydroxybenzenesulfonic acid** causes eye and skin irritation. May cause respiratory and digestive tract irritation.

Reagent Storage and Handling

- It is good practice to transfer the desired volumes of reagents for an experiment to clean glass test tubes or other containers and return reagent bottles to 4°C storage.
- Do not allow the capped reagent bottles to sit at room temperature for long periods of time. When not in use, place the bottles at 4°C.
- Unopened reagents are stable until the indicated expiration date.

PROCEDURE

Reagent Preparation

- HRP/Chromogen: Add 1 volume of HRP to 1000 volumes of Chromogen reagent, e.g. 110 µL in 110 mL. The combined reagent is stable for up to one month if stored sealed at 4°C and protected from light.
- 10 mM Hydrogen Peroxide in buffer: Add 1 volume of 30% H_2O_2 to 1000 volumes of Substrate Diluent, e.g. 30 µL in 30 mL. The working substrate is stable for up to 6 days if stored sealed at 4°C and protected from light.
- All reagents must be brought to room temperature before use.

Standard Preparation

The catalase Standard is provided as a lyophilized powder with approximately 160 U of catalase activity per vial. Prepare the Standard stock by adding sufficient deionized water to the Standard vial to achieve a 160 U/mL catalase activity (see catalase Standard vial label for recommended volume).

Standard dilutions are prepared by combining the indicated volumes of Standard and Sample Diluent specified in the dilution table below. Diluted catalase standards are stable for one week when stored at 4°C.

Standard, µL	0	13.3	28.6	66.7	120	200
Sample Diluent, µL	200	200	200	200	200	200
Final activity, U/mL	0	10	20	40	60	80

Assay

1. Add 30 µL of diluted Standards or Samples into tubes.
2. Add 500 µL of Substrate (10mM H_2O_2) into each tube.
3. Incubate each tube for exactly 1 minute at room temperature.
4. Add 500 µL Stop Reagent into each tube.
5. Cap and mix by inversion (DO NOT VORTEX).
6. Add 20 µL of each reaction mixture into cuvettes or tubes.
7. Add 2 mL HRP/Chromogen reagent into each cuvette or test tube.
8. Mix by inversion (DO NOT VORTEX).
9. Incubate for 10 minutes at room temperature.
10. Read absorbance at 520 nm.

Calculations

1. Using the standard data, perform a second order polynomial regression on A_{520} on U/mL catalase.

$$A_{520} = ax^2 + bx + c$$

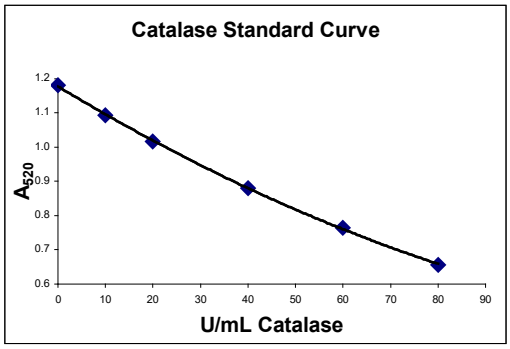
where a, b, and c are the coefficients of the quadratic equation, A_{520} is the absorbance of the sample and *x* is catalase activity in U/mL.

2. Calculate the catalase concentration in the sample using the quadratic formula:

$$\text{Catalase U/mL} = \frac{-b - \sqrt{b^2 - 4a(c - A_{520})}}{2a}$$

Example

Standard Curve. To illustrate the calculation, consider the following experiment: A catalase standard curve was prepared as described in the Assay Procedure above.



U/ml Catalase	A_{520}
0	1.181
10	1.094
20	1.016
40	0.881
60	0.764
80	0.657

The data was then fitted to a second order polynomial:

$$A_{520} = 2.38\text{x}10^{-5}x^2 - 8.39\text{x}10^{-3}x + 1.18$$

The equation for calculating Catalase concentration is thus:

$$\text{Catalase U/mL} = \frac{8.39x10^{-3} - \sqrt{(-8.39x10^{-3})^2 - 4 \cdot 2.38x10^{-5}(1.18 - A_{520})}}{4.76x10^{-5}}$$

Sample. A red blood cell (RBC) lysate was prepared by adding 4 volumes of cold water to a tube containing packed washed RBC. Following a dilution of 1/400 in Sample Diluent, the sample was assayed for catalase as described in the Assay Procedure above. The A_{520} was found to be **0.908**.

1. Use the equation to calculate the Catalase concentration in the diluted sample.

$$\text{Catalase U/mL} = \frac{8.39x10^{-3} - \sqrt{(-8.39x10^{-3})^2 - 4 \cdot 2.38x10^{-5}(1.18 - A_{520})}}{4.76x10^{-5}} = 36.4\text{U/mL}$$

2. Correct for the dilution of the sample:

$$\text{Catalase U/mL(lysate)} = 400 \text{ x } 36.4 = 14{,}560 \text{ U/mL}$$

3. Catalase activity can then be normalized to protein or hemoglobin. In this example, the hemoglobin concentration of the lysate is 60 mg/mL.

$$\text{Catalase} = \frac{14{,}560 \text{ U/mL}}{60.0 \text{ mg/mL}} = 242 \text{ U/mg Hb}$$

PERFORMANCE CHARACTERISTICS

Precision

Three concentrations of catalase were prepared in sample diluent and stored at –70°C in single use aliquots. In addition to the pure catalase samples, a lysate sample was included in each run. The samples were run twice a day for 10 days.

Parameter	Low	Middle	High	Lysate
Mean, U/mL	14.3	33.7	56.9	39.0
Intra-assay %CV	2.38%	1.31%	0.76%	1.28%
Inter-assay %CV	4.03%	3.11%	2.13%	5.55%
Total Precision %CV	4.53%	3.31%	2.23%	5.66%

Sensitivity

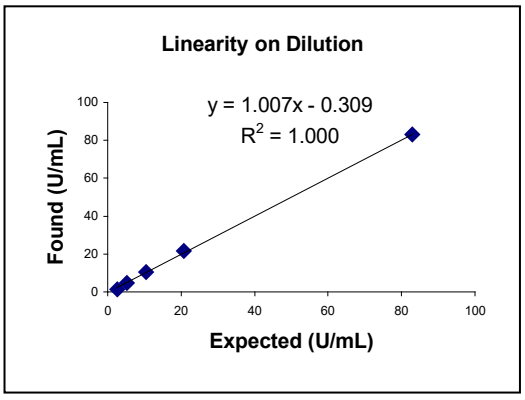
Lower Limit of Detection (LLD)

The LLD is actually a measure of the lowest catalase concentration distinguishable from zero, calculated as 3.29 standard deviations above zero using 20 assays of zero catalase concentration in each precision run. (Read from its individual standard curve)

Number of data points	20
Standard deviation	0.5196
LLD in diluted sample, U/mL	1.71

Linearity on dilution

A RBC lysate sample was diluted in Sample Diluent and the catalase activity was measured in the Catalase-540 assay. The expected values are relative to the undiluted sample. The slope =1.01 and R^2 = 1.00 show linearity upon dilution of lysate.



Assay Range

The range of the catalase assay is defined from the lowest to the highest concentration of catalase that can be added to the reaction and maintain linearity. The range of the assay is between 2-80 U/ml.

Addition Recovery

Whole blood and lysate samples were spiked with 2 different concentrations of measured catalase samples. The recovery of catalase was then calculated by dividing the *Found – Initial* catalase activity by the *Added* catalase.

	Catalase (U/mL)			
	Initial	Added	Found	%Recovery
Whole Blood	20.0	2.6	22.5	97
	19.4	23.7	21.2	90
RBC Lysate	29.5	4.1	33.1	89
	31.0	27.9	56.6	92

Specificity

Possible interferents were tested with and without added catalase. The catalase concentration was determined for each sample. The values are expressed as the percent of catalase activity measured. Ascorbic acid and uric acid show significant interference in the Catalase-520™ assay.

Interferent	Concentration	w/o catalase U/ml	w/ catalase U/ml	Expected catalase u/ml	% Recovery
Ascorbic acid	120µM	-0.242	24.06	42.34	56.84
	60µM	-0.765	28.04	42.34	66.24
	20µM	-0.411	35.44	42.34	83.70
Heparin	143 USP/10mL	0.414	41.75	41.47	100.7
K_3EDTA 15%	17.55mg/10mL	0.46	40.17	41.47	96.9
Hemoglobin	0.18mg/mL	4.0	42.11	41.47	101.6
Cellular GPx	7.5mU/mL	-0.81	42.41	42.05	100.8
Plasma GPx	1.8mg/mL	-0.96	33.4	34.70	96.2
Bilirubin	2.23µM	-0.70	40.58	42.05	96.5
Glucose	0.96mg/mL	-0.95	40.69	42.05	101.8
Citric acid	0.03mg/ml	-0.71	41.6	42.05	98.9
Uric Acid	0.063mg/mL	-1.0	34.48	42.05	82.0
Albumin	50mg/ml	-0.05	23.84	24.41	102.4

Sample Preparation

Whole blood

1. Collect whole blood (WB) in an anticoagulant tube and mix by inversion.
2. Freeze 100 µL of whole blood to lyse.
3. Dilute 1/1000 before use:
    a. 100 µL WB + 900 µL Sample diluent (1/10) then
    b. 10 µL of 1/10 + 990 µL Sample diluent (1/100).
4. Sample should be assayed within one hour after dilution is performed.
5. If whole blood is not to be assayed immediately, it should be stored at –70°C for long-term storage. For short-term storage, it can be stored at 4°C for 10 days, but still needs to be frozen and thawed for lysis to occur.

Erythrocyte Lysate

1. Collect blood in an anticoagulant tube and mix by inversion.
2. Centrifuge at least 500 µL whole blood at 2500g and 4°C for 5 minutes.
3. Discard plasma supernatant, wash 3 times in ice cold 0.9% NaCl.
4. Resuspend erythrocyte pellet in 4 packed-cell volumes of ice-cold deionized water.
5. Keep on ice for 10 minutes.
6. Dilute 1/400 before use:
    a. 100 µL lysate + 900 µL Sample diluent (1/10) then
    b. 25 µL of 1/10 + 975 µL Sample diluent (1/40)
7. Sample should be assayed within one hour after dilution is performed.
8. If lysate is not assayed immediately, it should be stored at –70°C for long-term storage. For short-term storage, it can be stored at 4°C for 5 days.

Blood plasma

1. Collect blood in an anticoagulant tube and mix by inversion.
2. Centrifuge at least 500 µL whole blood at 2500g and 4°C for 5 minutes
3. Collect plasma supernatant.
4. Sample should be used immediately or frozen at –70°C for storage.

5. Weigh filter (without filtrate tube).
6. Add 125 µL plasma to filter tube. Record weight.
7. Add 375 µL assay buffer.
8. Centrifuge for 30 minutes at room temperature, 10,000xg.
9. Discard filtrate.
10. Reconstitute remaining retentate with assay buffer to original weight.
11. Mix well.

Blood serum

1. Collect blood in a tube with no anticoagulant.
2. Allow clotting at room temperature for 30 minutes.
3. Centrifuge at 2500g for 15-20 minutes.
4. Collect serum supernatant.
5. Sample should be used immediately or frozen at –70°C for storage.
6. Follow steps 5-11 for blood plasma samples.

NOTES

Measured Values

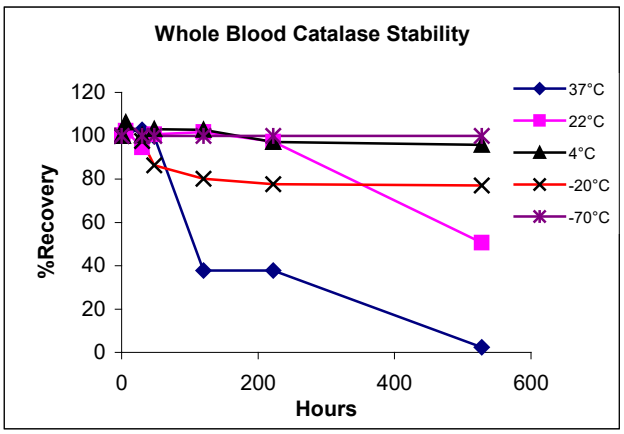
Human whole blood: 20,000-30,000 U/mL (n=4)
Human RBC Lysate: 8000-20,000 U/mL; 140-350 U/mg hemoglobin (n=16)
Human plasma: 6-20 U/mL (n=3)

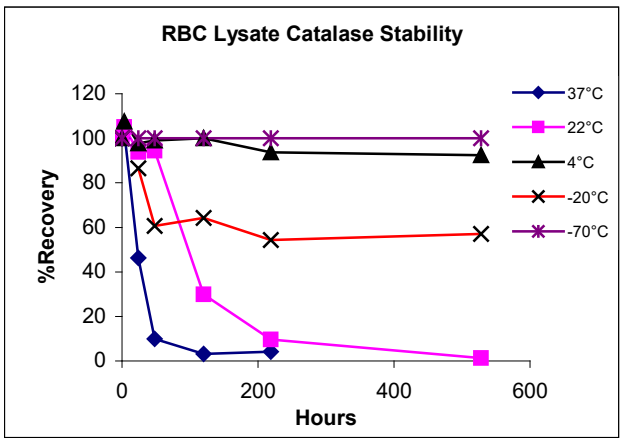
Sample Stability

In general, catalase is very unstable at high dilution and should be kept cold and assayed within 30-60 minutes after dilution.

Blood samples. Whole blood samples may be stored at 4°C for up to two weeks but it is recommended that samples be stored at –70°C for long-term storage. Red blood cell lysates, undiluted, are stable for 5 days at 4°C. Long term storage should be at –70°C.

For both whole blood and RBC lysates, as is shown below, freezing at –20°C results in an initial loss of catalase activity after which activity stabilizes. If –70°C storage is not available, flash freezing in liquid nitrogen or acetone/dry ice and subsequent storage at –20°C (non-self defrosting freezer) may be satisfactory.





Plasma samples

Stability data is not available for serum and plasma samples. It is recommended that the sample be assayed immediately or frozen at –70°C. Contact OXIS Technical Service for additional information.

Tissue homogenates
Stability data is not available for tissue homogenates. It is recommended that the sample be assayed immediately or frozen at –70°C. Contact OXIS Technical Service for additional information

Assay performance

- The quinoneimine dye is stable for at least 30 minutes.
- The working Chromogen/HRP reagent is stable for one month if stored at 4°C, sealed, and protected from light. The development of a slight pink color indicates the reagent is deteriorating and should be replaced.

Interferences
Ascorbic Acid and Uric Acid are interferents in plasma and serum samples but not in whole blood, RBC lysate, or tissue samples. This is due to the large sample dilution prior to assaying results in sufficient dilution of the interferent. The serum/plasma ascorbic acid and uric acid interference can be minimized by using centrifugal a ultrafiltration device (suggest Millipore Ultrafree®-0.5 Centrifugal Filter Device Catalog # UFV5 BTK 25 or equivalent). See suggested procedure in the sample preparation guidelines.

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