Triglyceride Colorimetric Assay Kit

Item No. 10010303

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity/Size
10010509	Triglyceride Standard	1 vial/400 µl
700732	Standard Diluent Assay Reagent (5X)	1 vial/12 ml
700024	NP40 Substitute Assay Reagent (5X)	2 vials/12 ml
10010511	Triglyceride Enzyme Mixture	1 vial
700003	Sodium Phosphate Assay Buffer	1 vial/4 ml
400012	96-Well Cover Sheet	1 cover
400014	96-Well Solid Plate (Colorimetric Assay)	1 plate

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed at -20°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader with the ability to measure absorbance between 530-550 nm
2. Adjustable pipettes and a multichannel or repeating pipette
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable
4. Test tubes
5. 15 ml centrifuge tube
6. Aluminum foil

INTRODUCTION

Background

Triglycerides are water-insoluble lipids consisting of three fatty acids esterified to a glycerol backbone. Triglycerides are transported in the blood as core constituents of all lipoproteins, but are major components of triglyceride-rich chylomicrons and very low-density lipoproteins (VLDL).[1] A major source of triglycerides is dietary fat. Dietary fats are hydrolyzed in the gut into free fatty acids and mono- and diglycerides and then transported through the intestinal villi. After absorption through the gut, they are resynthesized into new triglycerides and assembled into chylomicrons. Triglycerides are rapidly hydrolyzed in the capillary beds by lipoprotein lipase, releasing glycerol and free fatty acids, which are absorbed by adipose tissue for storage. When required, lipases hydrolyze triglycerides from adipose tissue into fatty acids and glycerol, which enter the blood stream. Fatty acids are oxidized in the mitochondria and peroxisomes to produce energy. Triglycerides play an important role in metabolism by containing more than twice as much energy as carbohydrates and proteins.

The measurement of triglyceride levels is useful in the diagnosis of primary and secondary hyperlipoproteinemia, dyslipidemia, and triglyceridemia. Triglyceride concentrations are also useful in the diagnosis and treatment of diabetes mellitus, nephrosis, liver obstruction, and other diseases involving lipid metabolism or various endocrine disorders.[2-4] The most common method to determine triglyceride concentrations is by enzymatic hydrolysis of triglycerides to glycerol and free fatty acids followed by either colorimetric or fluorometric measurement of the glycerol released.[5-8]

About This Assay

Cayman's Triglyceride Colorimetric Assay provides a simple, reproducible, and sensitive tool for assaying triglycerides in plasma, serum, cell lysates, and tissue homogenates. The Triglyceride Colorimetric Assay uses the enzymatic hydrolysis of triglycerides by lipase to produce glycerol and free fatty acids. The glycerol released is subsequently measured by a coupled enzymatic reaction system (Figure 1). The glycerol formed in reaction 1 is phosphorylated to glycerol-3-phosphate in a reaction catalyzed by glycerol kinase (reaction 2). The glycerol-3-phosphate is oxidized by glycerol phosphate oxidase producing dihydroxyacetone phosphate and hydrogen peroxide (reaction 3). Peroxidase catalyzes the redox-coupled reaction of H_2O_2 with 4-aminoantipyrine (4-AAP) and N-Ethyl-N-(3-sulfopropyl)-*m*-anisidine (ESPA), producing a brilliant purple color (reaction 4). The absorbance is measured at 530-550 nm.

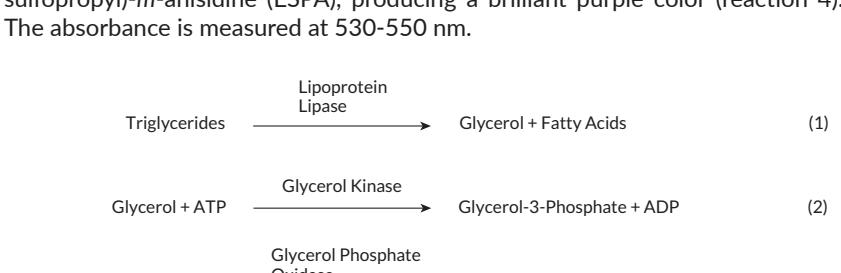

Figure 1. Triglyceride assay scheme

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Triglyceride Standard - (Item No. 10010509)**

   This vial contains 400 µl of a 1,000 mg/dl solution of Triglyceride Standard. It is ready to use as provided to prepare the standard curve. Sufficient Triglyceride Standard is provided to prepare three standard curves.

2. **Standard Diluent Assay Reagent (5X) - (Item No. 700732)**

   This vial contains 12 ml of a 5X salt solution. Prior to use, mix the contents of the vial with 48 ml of HPLC-grade water. This Standard Diluent Assay Reagent (1X) is used to dilute the triglyceride standards, plasma, and serum; as well as preparing the lipid extracts from cells. Standard Diluent Assay Reagent (1X) may be stored for six months at room temperature.

3. **NP40 Substitute Assay Reagent (5X) - (Item No. 700024)**

   This vial contains 12 ml of a 5X surfactant solution. Prior to use, mix the contents of the vial with 48 ml HPLC grade water. This NP40 Substitute Assay Reagent (1X) is used to prepare tissue samples and can be used to prepare the standard curve. NP40 Substitute Assay Reagent (1X) may be stored for six months at room temperature.

4. **Sodium Phosphate Assay Buffer - (Item No. 700003)**

   This vial contains 4 ml of 250 mM sodium phosphate buffer, pH 7.2. Prior to use, mix the contents of the vial with 16 ml of HPLC-grade water. This Sodium Phosphate Assay Buffer (1X) is used to prepare the triglyceride enzyme solution. Sodium Phosphate Assay Buffer (1X) may be stored for six months at room temperature.

5. **Triglyceride Enzyme Mixture - (Item No. 10010511)**

   This vial contains a lyophilized enzyme mixture. Reconstitute the contents of the vial with 1 ml of HPLC-grade water. Transfer the reconstituted solution to a 15 ml centrifuge tube wrapped in aluminum foil. Add 14 ml of the Sodium Phosphate Assay Buffer (1X) to the reconstituted solution and mix by inversion. *NOTE: A portion of the 14 ml should be used to rinse any residual solution from the vial.* This solution is now ready to use in the assay. If the entire solution is not used at one time, the solution should be stored at 4°C. *Do NOT Freeze!* The solution is stable for one month when stored at 4°C; a slight pink discoloration may occur but will have no affect on the assay performance.

Sample Preparation

Plasma

Typically, normal human plasma has triglyceride concentrations in the range of 40-160 mg/dl (male) or 35-135 mg/dl (female).[9]

1. Collect blood using an anticoagulant such as heparin, EDTA, or citrate.
2. Centrifuge the blood at 700-1,000 x g for 10 minutes at 4°C. Pipette off the top yellow plasma layer without disturbing the white buffy layer. Store plasma on ice. If not assaying the same day, freeze at -80°C. The plasma sample will be stable for one month when stored at -80°C.
3. Dilute plasma 1:2 with the Standard Diluent Assay Reagent (1X) prior to the assay.

Serum

Typically, normal human serum has triglyceride concentrations in the range of 40-160 mg/dl (male) or 35-135 mg/dl (female).[9]

1. Collect blood without using an anticoagulant.
2. Allow blood to clot for 30 minutes at 25°C.
3. Centrifuge the blood at 2,000 x g for 15 minutes at 4°C. Pipette off the top yellow serum layer without disturbing the white buffy layer. Store serum on ice. If not assaying the same day, freeze at -80°C. The serum sample will be stable for one month while stored at -80°C.
4. Dilute serum 1:2 with the Standard Diluent Assay Reagent (1X) prior to the assay.

Spike and Recovery

Human plasma was spiked with different amounts of Triglyceride Standard, diluted with the Standard Diluent Assay Reagent (1X) and analyzed using the Triglyceride Colorimetric Assay Kit. The results are shown below. The error bars represent standard deviations obtained from two different dilutions of each sample.

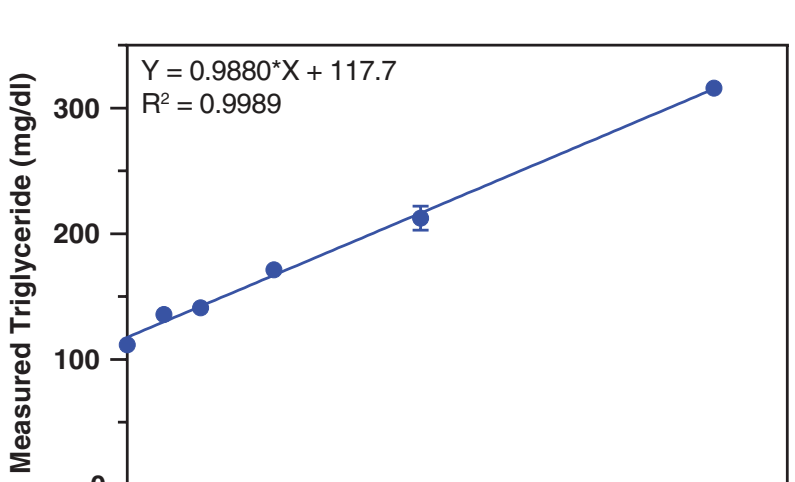

Figure 2. Spike and recovery of triglyceride in human plasma

Extraction of lipids from cells

This protocol may be adjusted to fit the needs of the users' cell cultures or experimental design. Culture cells in a 6-well plate to 90% confluency.

1. Wash cells with 2 ml of PBS buffer two times. Remove all PBS.
2. Add 2 ml of a 3:2 hexane:isopropyl alcohol solution to the well or dish.
3. Allow cells to incubate for 30 minutes with the lid on.
4. Remove the organic solution and save in glass tubes or vials for analysis.
5. Rinse each well or dish with an additional 1 ml of the organic solution. Add the rinse to the saved samples.
6. Allow the organic solution to evaporate completely. This may be done in a fume hood, or under a gentle stream of nitrogen.
7. Reconstitute the lipids in 0.4 ml of Standard Diluent (1X) and test in the assay. Depending on the amount of triglycerides in the sample, they may not require any further dilution.

Tissue Homogenates

1. Weigh tissue and then mince into small pieces.
2. Homogenize 350-400 mg of minced tissue in 2 ml of the diluted NP40 Substitute Assay Reagent containing protease inhibitors of choice (see **Interference** section).
3. Centrifuge at 10,000 x g for 10 minutes at 4°C.
4. Transfer the entire supernatant to another tube. Store the supernatant on ice. If not assaying the same day, freeze at -80°C. The sample will be stable for one month when stored at -80°C. *NOTE: When centrifuged at high speeds some tissue homogenates, such as liver, will leave a layer of insoluble fat at the top of the centrifuge tube. Be sure to include this layer when transferring the supernatant.*
5. Typically, tissue samples require dilutions of at least 1:5 or greater. Dilute the samples using the diluted NP40 Substitute Assay Reagent before assaying.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. A typical layout of triglyceride standards and samples to be measured in duplicate is given below in Figure 3. We suggest you record the contents of each well on the template sheet provided (see page 25).

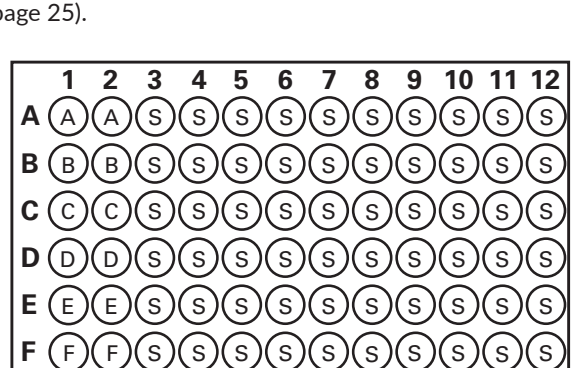

Figure 3. Sample plate format

Pipetting Hints

- It is recommended that a multichannel pipette be used to deliver reagents to the wells.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (i.e., slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- All reagents, except samples, must be equilibrated to room temperature before beginning the assay.
- The final volume of the assay is 160 µl in all wells.
- It is not necessary to use all the wells on the plate at one time.
- It is recommended that the standards and samples be assayed in at least duplicate.
- Measure the absorbance at 530-550 nm using a plate reader.

Standard Preparation

If testing tissue samples it is recommended that the standard curve be prepared in NP40 Substitute Assay Reagent (1X) instead of Standard Diluent Assay Reagent (1X). For all other sample types it is recommended that the standard curve be prepared in Standard Diluent Assay Reagent (1X).

Label eight clean test tubes A-H. Add 200 µl of the Standard Diluent Assay Reagent (1X) or NP40 Substitute Assay Reagent (1X) to tubes B-H. Add 400 µl of Standard Diluent Assay Reagent (1X) (or NP40 Substitute Assay Reagent (1X)) to tube A. Add 100 µl of Triglyceride Standard (Item No. 10010509) to tube A and mix thoroughly. The concentration of tube A is 200 mg/dl (2.26 mmol/L). Serially dilute the standard by removing 200 µl from tube A and adding it to tube B; mix thoroughly. Next, remove 200 µl from tube B and place it into tube C; mix thoroughly. Repeat this process for tubes D-G. Tube H only has Standard Diluent Assay Reagent (1X) or NP40 Substitute Assay Reagent (1X) and is used as the blank. We recommend that you store these diluted standards for no more than one to two hours. See Table 1 below for the triglyceride concentrations of the serial dilutions.

Tube	Triglyceride Concentration (mg/dl)
A	200
B	100
C	50
D	25
E	12.5
F	6.25
G	3.13
H	0

Table 1. Preparation of Triglyceride Standards

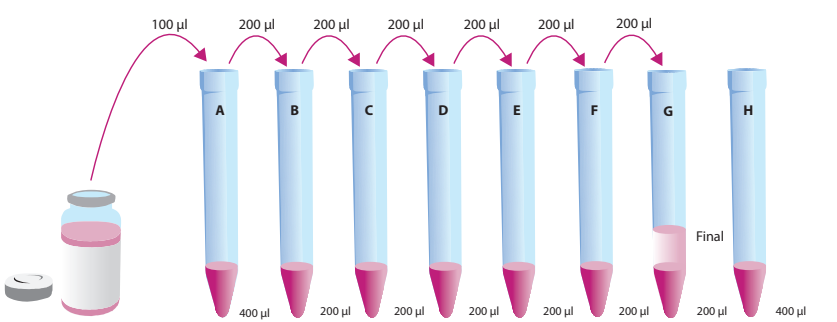

Figure 4. Preparation of Triglyceride Standards
**Standard Diluent Assay Reagent (1X) or NP40 Substitute Assay Reagent (1X)*

Performing the Assay

1. **Triglyceride Standard Wells** - Add 10 µl of standard (tubes A-H) to the designated wells on the plate (see **Sample Plate Format**, Figure 3, page 14).
2. **Sample Wells** - Add 10 µl of sample to two or three wells. *NOTE: The amount of sample added to the well should always be 10 µl.*
3. Initiate the reaction by adding 150 µl of diluted Enzyme Mixture solution to each well.
4. Cover with the plate cover. Carefully shake the plate for a few seconds to mix.
5. Incubate the plate for 60 minutes at room temperature.
6. Measure the absorbance at 530-550 nm using a plate reader.

ANALYSIS

Calculations

1. Calculate the average absorbance of each standard and sample.
2. Subtract the absorbance value of standard H (0 mg/dl) from itself and all other values (both standards and samples). This is the corrected absorbance.
3. Graph the corrected absorbance values (from step 2 above) of each standard as a function of the final triglyceride concentration (mg/dl) (see Table 1, page 16). A typical triglyceride standard curve is shown in Figure 5 on page 21.
4. Calculate the values of triglyceride samples using the equation obtained from the linear regression of the standard curve by substituting the corrected absorbance values for each sample into the equation.

$$\text{Triglycerides (mg/dl)} = \left[\frac{(\text{Corrected absorbance}) - (\text{y-intercept})}{\text{Slope}}\right]$$

Performance Characteristics

Precision:

When a series of sixteen human serum samples were assayed on the same day, the intra-assay coefficient of variation was 1.34%. When a series of sixteen human serum samples were assayed on six different days under the same experimental conditions, the inter-assay coefficient of variation was 3.17%.

Sensitivity:

The lower limit of detection (LLOD) for this assay is approximately 0.5 mg/dl using the Standard Diluent Assay Reagent and 1.6 mg/dl when using the NP40 Substitute Assay Reagent.

Representative Triglyceride Standard Curve

The standard curve, presented below, is an example of the data typically provided with this kit; however, your results will not be identical to these. You must run a new standard curve. Do not use this data to determine the values of your samples.

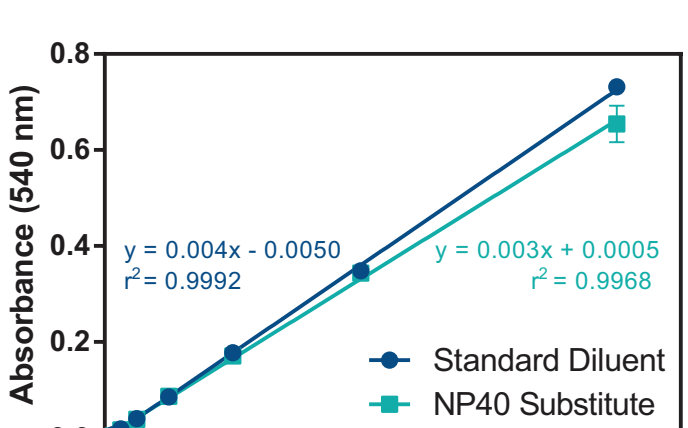

Figure 5. Triglyceride standard curve

RESOURCES

Interferences

The following reagents were tested in the assay for interference:

Reagent		Will Interfere (Yes or No)
Buffers	Tris	No
	Borate	No
	HEPES	No
	Phosphate	No
	MES	No
Detergents	Polysorbate 20 (1%)	No
	Triton X-100 (1%)	No
Protease Inhibitors/ Chelators/ Enzymes	EDTA (1 mM)	No
	EGTA (1 mM)	No
	Trypsin (10 µg/ml)	No
	PMSF (200 µM)	Yes
	Leupeptin (10 µg/ml)	No
	Antipain (100 µg/ml)	No
	Chymostatin (10 µg/ml)	No
	BSA (1%)	Yes
Solvents	Ethanol (5%)	Yes
	Methanol (5%)	No
	Dimethylsulfoxide (5%)	No
Others	Sucrose (250 mM)	No
	Glycerol (5%)	Yes

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
No triglyceride was detected in the sample	Triglyceride concentration was too low or the sample was too dilute	Do *not* dilute samples and re-assay
Sample absorbance values are above highest point in standard curve	Triglyceride concentration was too high in the sample or the sample was too concentrated	Dilute samples with assay buffer and re-assay; *NOTE: Remember to account for the dilution factor when calculating the triglyceride concentration*

References

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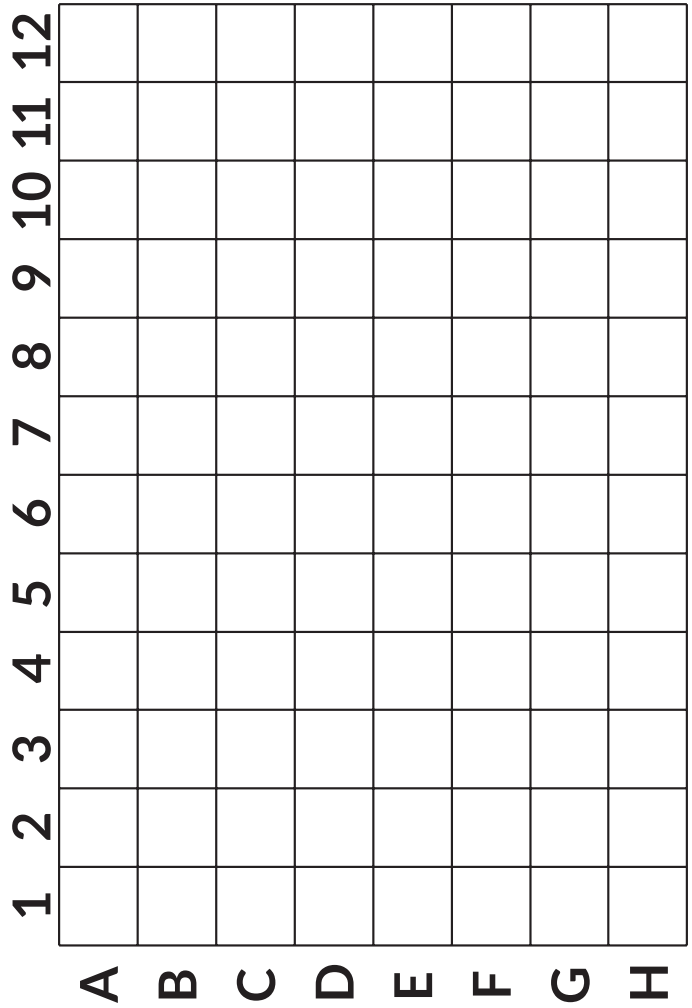

NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.