Triglyceride Colorimetric Assay Kit

Item No. 10010303

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TABLE OF CONTENTS

GENERAL INFORMATION
3 Materials Supplied
4 Safety Data
4 Precautions
4 If You Have Problems
5 Storage and Stability
5 Materials Needed but Not Supplied

INTRODUCTION
6 Background
7 About This Assay

PRE-ASSAY PREPARATION
8 Reagent Preparation
10 Sample Preparation
13 Sample Matrix Properties

ASSAY PROTOCOL
15 Plate Set Up
17 Standard Preparation
18 Performing the Assay

ANALYSIS
19 Calculations
20 Performance Characteristics

RESOURCES
22 Interferences
23 Troubleshooting
24 References
25 Plate Template
26 Notes
27 Warranty and Limitation of Remedy

GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity/Size	Storage Temperature
10010509	Triglyceride Standard	1 vial/400 µl	-20°C
700732	Standard Diluent Assay Reagent (5X)	1 vial/12 ml	-20°C
700024	NP40 Substitute Assay Reagent (5X)	2 vials/12 ml	-20°C
10010511	Triglyceride Enzyme Mixture	1 vial	-20°C
700003	Sodium Phosphate Assay Buffer	1 vial/4 ml	-20°C
400012	96-Well Cover Sheet	1 ea	RT
400014	96-Well Solid Plate (Colorimetric Assay)	1 plate	RT
400819	Triglyceride Positive Control	1 vial	-20°C

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 in the **Materials Supplied** section (see page 3) 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 at 540 nm
2. Adjustable pipettes and a multichannel or repeating pipette
3. A source of pure water; glass-distilled water is acceptable. *NOTE: UltraPure water is available for purchase from Cayman (Item No. 400000).*
4. Ethanol
5. Materials used for **Sample Preparation** (see page 10)

INTRODUCTION

Background

Triglycerides, also known as triacylglycerols, are lipids that consist of three fatty acids esterified to a glycerol backbone.[1,2] They are primarily obtained through the diet and subsequently hydrolyzed *via* lipases into free fatty acids and glycerol, monoglycerides, or diglycerides. After intestinal absorption of free fatty acids, triglycerides are resynthesized and combined with cholesterol and apolipoproteins to form chylomicrons in the intestinal epithelium, which are secreted into the lymph and eventually reach the bloodstream.[1] Triglycerides can also be assembled with cholesterol and apolipoproteins to form VLDLs in the liver, which are secreted directly into the bloodstream. Chylomicrons and VLDLs are triglyceride-rich lipoproteins (TRLs) and serve as the main species that transport triglycerides in the blood.[1,2] Fatty acids from triglycerides are released from TRLs through lipoprotein lipase (LPL) and absorbed by adipose or other tissues and reserved for energy storage.[1,3] These free fatty acids can then be oxidized in the mitochondria and peroxisomes *via* β-oxidation to produce acetyl-CoA, which enters the citric acid cycle.[2,4] Triglyceride levels have been used as biomarkers in the diagnosis of atherosclerotic vascular disease, hypertension, type 1 diabetes, dyslipidemia, nephrotic syndrome, and hyperhomocysteinemia, among others.[5]

About This Assay

Cayman's Triglyceride Colorimetric Assay Kit provides a simple, reproducible, and sensitive tool for assaying mono-, di-, and triglycerides in plasma, serum, cell culture extracts, and tissue homogenates. The Triglyceride Colorimetric Assay Kit 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 (H_2O_2) (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 540 nm.

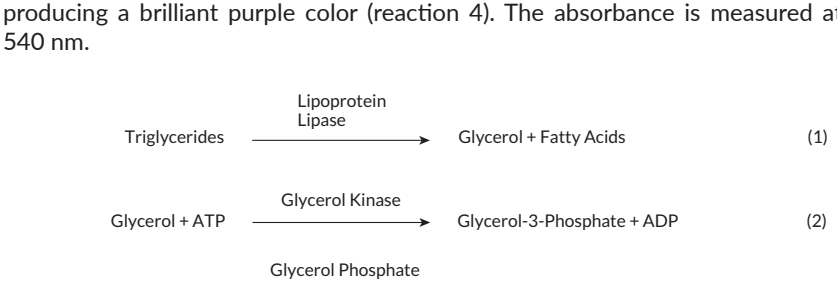

Figure 1. Assay scheme

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.

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 pure 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)**

   Each vial contains 12 ml of a 5X surfactant solution. Prior to use, mix the contents of the vial with 48 ml of pure 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 pure water. This Sodium Phosphate Assay Buffer (1X) is used to reconstitute the Triglyceride Enzyme Mixture (15X). 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 with 1.2 ml of pure water before transferring to a larger container protected from light and diluting with 16.8 ml of Sodium Phosphate Assay Buffer (1X). Use a portion of the buffer to rinse any residual solution from the vial. The solution is now ready for use in the assay. The reconstituted enzyme mixture will be stable for one month at 4°C. *Do NOT Freeze! NOTE: A slight pink discoloration may occur during storage but will not affect assay performance.*

6. **Triglyceride Positive Control - (Item No. 400819)**

   This vial contains a neat oil of 1-palmitoyl-2-oleoyl-3-linoleoyl-*rac*-glycerol. Prior to use, add 1 mL of ethanol and mix well. This solution is stable for one month when stored at -20°C.

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).[6]

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. Transfer the top yellow plasma layer to a separate tube. Do not disturb 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. Prior to the assay, dilute plasma a minimum of 1:2 with Standard Diluent Assay Reagent (1X). For samples with a higher level of triglycerides, it may to necessary to dilute the samples several times with Standard Diluent Assay Reagent (1X).

Serum

Typically, normal human serum has triglyceride concentrations in the range of 40-160 mg/dl (male) or 35-135 mg/dl (female).[6]

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. Transfer the top yellow serum layer to a separate tube. Do not disturb 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. Prior to the assay, dilute serum a minimum of 1:2 with Standard Diluent Assay Reagent (1X). For samples with a higher level of triglycerides, it may to necessary to dilute the samples several times with Standard Diluent Assay Reagent (1X).

Cell Culture Extracts

This protocol may be adjusted to fit the needs of the user's 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 samples in 0.4 ml of Standard Diluent Assay Reagent (1X) and test in the assay. If necessary, dilute with Standard Diluent Assay Reagent (1X).

Tissue Homogenates

1. Add 10-20 mg of tissue to a 2 ml vial containing the Precellys® 2 ml Hard Tissue Homogenizing Ceramic Beads Kit (CK28) (Item No. 10011151). Add 400 µl of chloroform:isopropanol:igepal (CA-630 (7:11:0.1)).
2. Homogenize tissue sufficiently using a homogenizer following the manufacturer's protocol.
3. Centrifuge for 10 minutes at 15,000 x g at 4°C.
4. Transfer the supernatant into a new tube.
5. Dry the supernatant under a gentle stream of nitrogen.
6. Add 400 µl of NP40 Substitute Assay Reagent (1X) to the sample and vortex.
7. To maximize the solubility of the extracted lipid in NP40 Substitute Assay Reagent (1X), it is recommended to heat the sample using a water bath set to 100°C. Incubate the sample in the water bath for 1 minute. Allow the sample to cool completely while vortexing often. Repeat one time.
8. To fall within the the range of the standard curve, it may be necessary to dilute the samples with NP40 Substitute Assay Reagent (1X).

Sample Matrix Properties

Spike and Recovery

Human heparin plasma and serum were spiked with different amounts of triglycerides (Intralipid, Sigma-Aldrich® I141), 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 multiple dilutions of each sample.

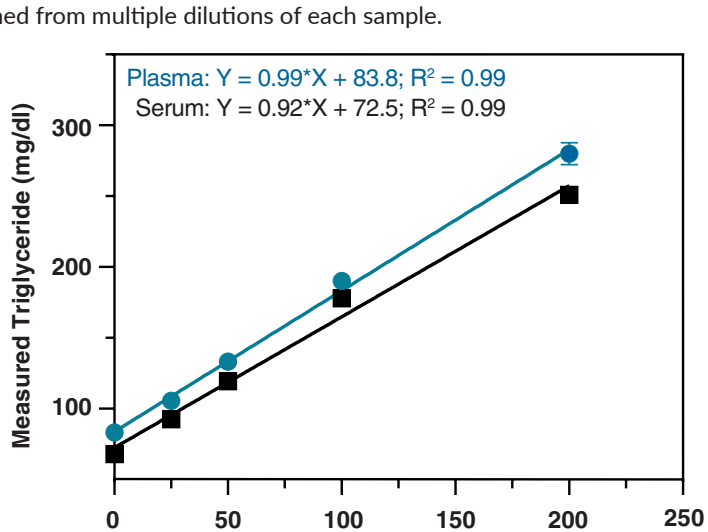

Figure 2. Spike and recovery of triglyceride in human plasma

Parallelism

To assess parallelism, rat visceral fat, mouse liver, HeLa cell culture extracts and Triglyceride Positive Control were assayed at multiple dilutions using the Triglyceride Colorimetric Assay Kit. Tissue homogenates and HeLa cells culture extracts were prepared as in the Sample Preparation (pages 10-12). Concentrations were plotted as a function of sample dilution. The results are shown below.

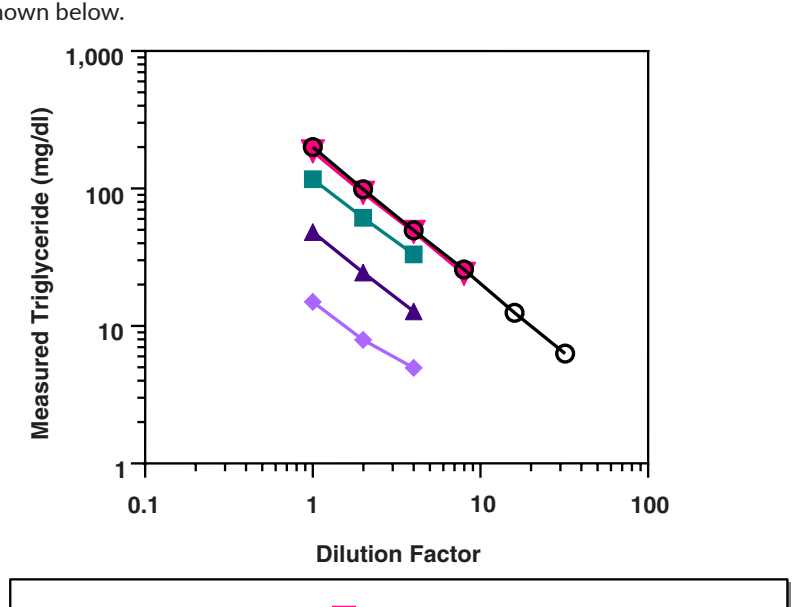

Figure 3. Parallelism of various matrices in the Triglyceride Colorimetric Assay Kit

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. It is recommended that each standard, positive control, and sample be assayed in duplicate and that the contents of each well are recorded on the template sheet provided on page 25. A typical layout of samples to be measured in duplicate is provided below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	TG	TG	8	8	16	16	24	24	32	32
B	B	B	1	1	9	9	17	17	25	25	33	33
C	C	C	2	2	10	10	18	18	26	26	34	34
D	D	D	3	3	11	11	19	19	27	27	35	35
E	E	E	4	4	12	12	20	20	28	28	36	36
F	F	F	5	5	13	13	21	21	29	29	37	37
G	G	G	6	6	14	14	22	22	30	30	38	38
H	H	H	7	7	15	15	23	23	31	31	39	39

A-H = Standard Wells
TG = Triglyceride Positive Control Wells
1-39 = Sample Wells

Figure 4. 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. **Tissue homogenates incubated at high temperatures should be cooled completely prior to 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 540 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 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. The diluted triglyceride standards will be stable for two hours at room temperature.

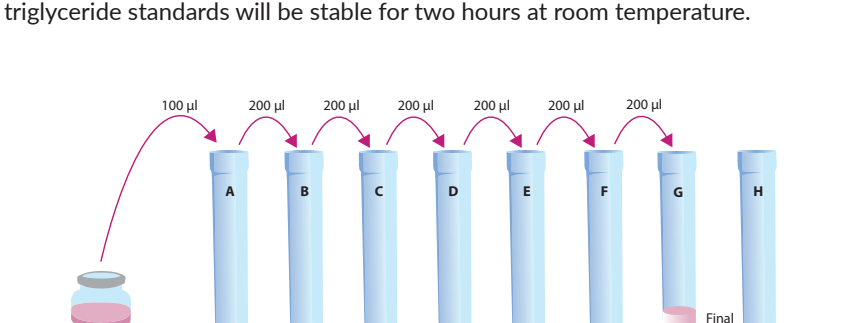

Figure 5. Preparation of the triglyceride standards
**Standard Diluent Assay Reagent (1X) or NP40 Substitute Assay Reagent (1X)*

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 4, page 15).
2. **Sample Wells** - Add 10 µl of sample to the designated wells.
3. **Positive Control Wells** - Add 10 µl of Triglyceride Positive Control (1X) to two wells.
4. Initiate the reaction by adding 150 µl of the reconstituted and diluted Triglyceride Enzyme Mixture to each well.
5. Cover with the 96-Well Cover Sheet (Item No. 400012).
6. Incubate the plate for 60 minutes at room temperature.
7. Remove plate cover and measure the absorbance at 540 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. 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 standard concentration (mg/dl) (see Figure 5 page 17). A typical triglyceride standard curve is shown in Figure 6 on page 21.
4. Calculate triglyceride concentration in 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.

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

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 0.5 mg/dl using the Standard Diluent Assay Reagent (1X) and 1.6 mg/dl when using the NP40 Substitute Assay Reagent (1X). The lower limit of quantification (LLOQ) for this assay is 3.13 mg/dl.

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.

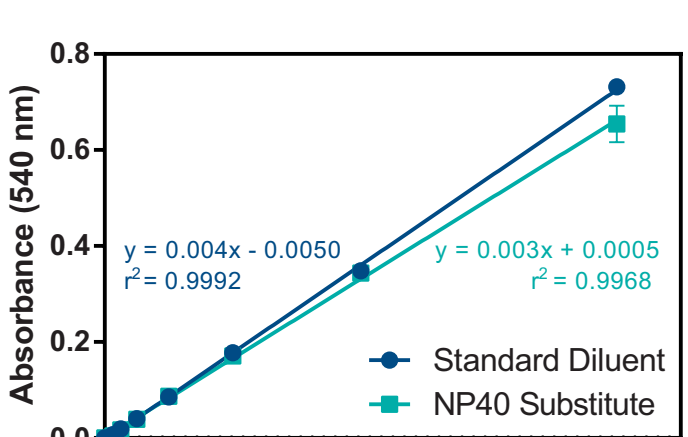

Figure 6. Triglyceride standard curves

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)	No
	Leupeptin (10 µg/ml)	No
	Antipain (100 µg/ml)	No
	Chymostatin (10 µg/ml)	No
	BSA (1%)	Yes
Solvents	Ethanol (5%)	No
	Methanol (5%)	No
	DMSO (5%)	No
Others	Sucrose (250 mM)	No
	Glycerol (5%)	Yes

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of replicates	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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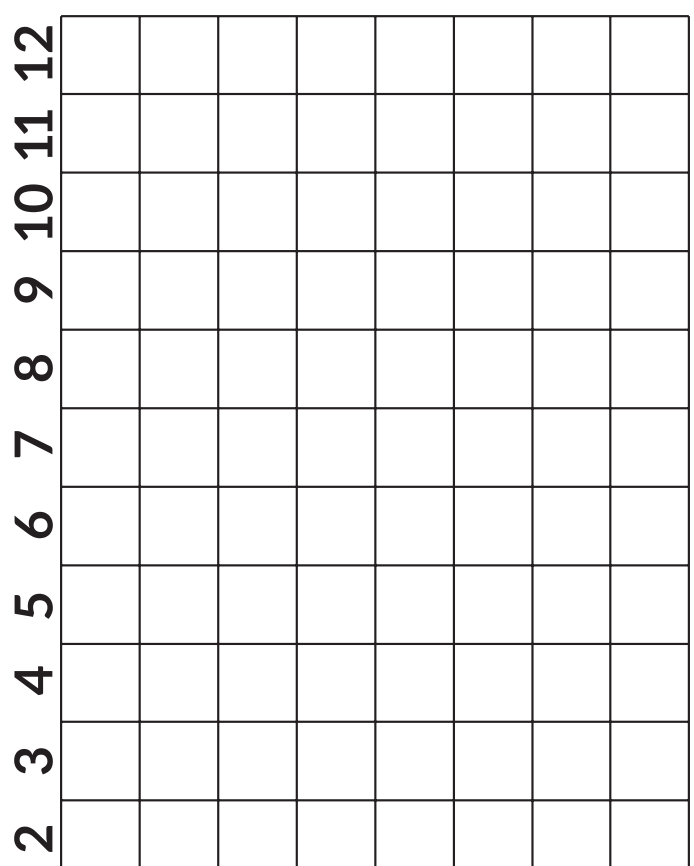

NOTES

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.

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