Cholesterol Fluorometric/Colorimetric Assay Kit

Item No. 10007640

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	96 well Quantity/Size	480 well Quantity/Size
10008052	Cholesterol Assay Buffer (10X)	1 vial/3 ml	1 vial/15 ml
10008053	Cholesterol Assay Standard	1 vial/100 µl	1 vial/500 µl
400610	MaxiProbe	1 vial	5 vials
10008055	Cholesterol Assay Horseradish Peroxidase	1 vial	1 vial
10008056	Cholesterol Assay Oxidase	1 vial	1 vial
10008057	Cholesterol Assay Esterase	1 vial	1 vial
400777	96-Well Black Wall, Clear Bottom, Assay Plate	1 plate	5 plates
400012	96-Well Cover Sheet	1 cover	5 covers

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 specified at -20°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A fluorometric plate reader capable of measuring fluorescence with excitation and emission wavelengths of 530-540 and 585-595 nm, respectively, or a plate reader capable of measuring absorbance at 570 nm
2. Adjustable pipettes and a repeating pipettor
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable
4. Materials used for **Sample Preparation** (see page 10)

INTRODUCTION

Background

Cholesterol is a major sterol produced in mammalian cells that is required for cell viability and proliferation.[1] It is a component of mammalian cell membranes that interacts with membrane phospholipids, sphingolipids, and proteins to influence their behavior. It is also a component of various lipid-based drug delivery (LBDD) systems, including liposomes and lipid nanoparticles (LNPs), where it has a role in membrane stability.[2] Cholesterol is a precursor of steroid hormones, bile acids, and the active form of vitamin D. Impaired cholesterol homeostasis is related to development of various diseases including fatty liver, diabetes, gallstones, dyslipidemia, atherosclerosis, heart attack, and stroke. [3]

About This Assay

Cayman's Cholesterol Fluorometric/Colorimetric Assay Kit provides a simple method for the sensitive quantitation of cholesterol in biological samples, such as plasma, serum, cell extracts, and tissue extracts. The assay is based on an enzyme-coupled reaction that detects both free cholesterol and cholesteryl esters as depicted in Figure 1 below. Cholesteryl esters are hydrolyzed by cholesterol esterase into cholesterol, which is then oxidized by cholesterol oxidase to yield hydrogen peroxide and the corresponding ketone product. By excluding cholesterol esterase, only free cholesterol will be oxidized and therefore can be distinguished from total cholesterol. Hydrogen peroxide produced by cholesterol oxidase activity is then detected using MaxiProbe, a highly sensitive and stable probe for hydrogen peroxide.[4] In the presence of horseradish peroxidase, MaxiProbe reacts with hydrogen peroxide with a 1:1 stoichiometry to produce a compound, the fluorescence of which can be quantified at excitation and emission wavelengths of 530 and 590 nm, respectively. Alternatively, its absorbance can be measured at 570 nm. The fluorometric assay has a range of 0-20 µM with a lower limit of detection of 1 µM. The colorimetric assay has a range of 0-100 µM with a lower limit of detection of 1 µM.

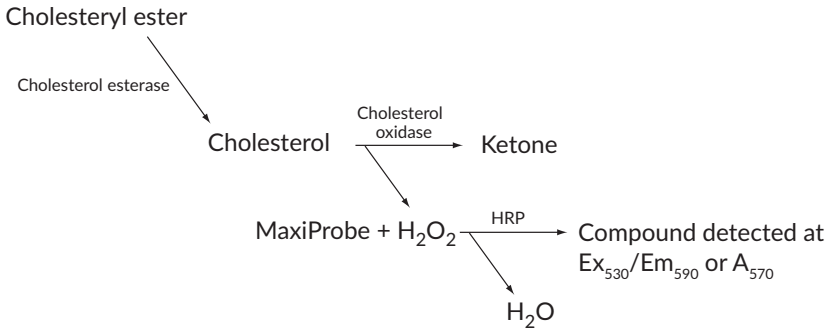


Figure 1. Assay scheme

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Cholesterol Assay Buffer (10X) - (Item No. 10008052)**

   Dilute 3 ml of assay buffer concentrate with 27 ml of pure water to obtain Assay Buffer (1X), which should be used for the preparation of standards and the dilution of samples. This will be stable for at least one week if stored at 4°C.

2. **Cholesterol Assay Standard - (Item No. 10008053)**

   The vial contains 10 mM cholesterol (5-cholesten-3β-ol) in ethanol. The reagent is ready to use for preparation of the diluted cholesterol standards.

3. **MaxiProbe - (Item No. 400610)**

   Each vial contains 250 µl of MaxiProbe in DMSO, which is sufficient to evaluate 100 wells. Maxiprobe will be stable for one hour at room temperature if protected from light. If not using the reagent all at once, prepare aliquots and store at -20°C limiting the number of freeze/thaw cycles to two.

4. **Cholesterol Assay Horseradish Peroxidase (HRP) - (Item No. 10008055)**

   The vial contains a lyophilized powder of HRP. Reconstitute the 1 ea. vial with 200 µl and the 5 ea. vial with 1 ml of pure water. The reconstituted HRP should be stable for at least one week when stored at -20°C. Prepare smaller aliquots before freezing to avoid more than three freeze/thaw cycles.

5. **Cholesterol Assay Oxidase - (Item No. 10008056)**

   The vial contains a lyophilized powder of cholesterol oxidase. Reconstitute the 1 ea. vial with 100 µl and the 5 ea. vial with 500 µl of pure water. The reconstituted reagent should be stable for at least one week when stored at -20°C. Prepare smaller aliquots before freezing to avoid more than three freeze/thaw cycles.

6. **Cholesterol Assay Esterase - (Item No. 10008057)**

   The vial contains a lyophilized powder of cholesterol esterase. Reconstitute the 1 ea. vial with 50 µl and the 5 ea. vial with 250 µl of pure water. The reconstituted reagent should be stable for at least one week when stored at -20°C. Prepare smaller aliquots before freezing to avoid more than three freeze/thaw cycles.

Sample Preparation

Plasma

Typically, cholesterol levels in human plasma are in the range of 2.5-7.5 mM.[5-7]

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 into a clean test tube without disturbing the white buffy layer. Store plasma on ice until assaying or freeze at -80°C. The plasma sample should be stable for at least one month.
3. Typically, a 1:200-1:400 dilution of plasma samples should produce results that fall within the fluorometric standard curve, and a 1:80-1:200 dilution should produce results that fall within the colorimetric standard curve.

Serum

Typically, cholesterol levels in human serum are in the range of 2.5-7.5 mM.[8]

1. Collect blood without using an anticoagulant such as heparin or citrate. Allow blood to clot for 30 minutes at 25°C.
2. Centrifuge the blood at 2,000 x g for 15 minutes at 4°C. Transfer the top yellow serum layer into a clean test tube without disturbing the white buffy layer. Store serum on ice. If not assaying the same day, freeze at -80°C. The sample should be stable for at least one month.
3. Typically, a 1:200-1:400 dilution of serum samples should produce results that fall within the fluorometric standard curve, and a 1:80-1:200 dilution should produce results that fall within the colorimetric standard curve.

Cell Extract

1. Start with $1x10^7$ cells. Wash cells 2-3 times with PBS, centrifuge, and collect the pellet.
2. Add 400 µl chloroform:isopropanol:Igepal™ CA-630 (7:11:0.1) and 200 mg glass beads (500-750 µm).
3. Vortex for 20 seconds and chill on ice for 20 seconds. Repeat five times.
4. Centrifuge for 10 minutes at 15,000 x g at 4°C.
5. Transfer the supernatant to a new tube.
6. Air dry in a ventilated hood overnight to remove organic solvents or evaporate the solvent under a gentle stream of nitrogen to accelerate drying.
7. Add 400 µl of Assay Buffer (1X) to the tube and vortex before analyzing. Optionally, centrifuge for 2-5 minutes at 15,000 x g at 4°C and analyze the supernatant only.
8. To fall within the range of the standard curve, it may be necessary to dilute samples with Assay Buffer (1X) prior to performing the assay.

Tissue Extract

1. Add 10-20 mg tissue to a 2 ml vial containing the Hard Tissue Homogenizing Ceramic Beads (Item No. 10011151). Then, add 400 µl of chloroform:isopropanol:Igepal™ CA-630 (7:11:0.1).
2. Homogenize tissue sufficiently using a homogenizer and following the manufacturer's protocol. For the Precellys® Evolution (available from Cayman Chemical (Item No. 16901)), the recommended cycle is the following:

   Cryolys: OFF

   Speed: 6,800 RPM

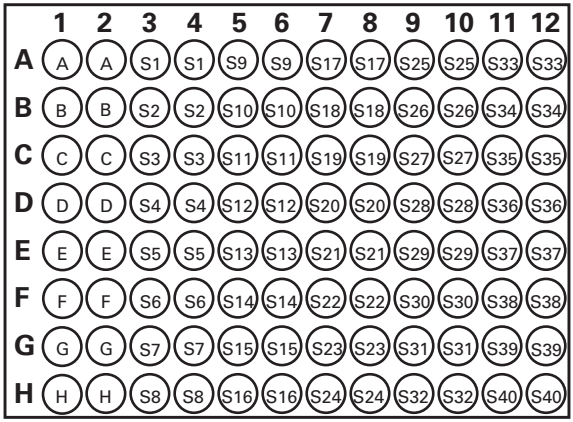
   Cycle: 5 x 20 seconds

   Pause: 30 seconds

   Temperature: 4°C.
3. Centrifuge for 10 minutes at 15,000 x g at 4°C.
4. Transfer the supernatant into a new tube.
5. Air dry in a ventilated hood overnight or under a gentle stream of nitrogen for a few hours until dry.
6. Add 400 µl of Assay Buffer (1X) to the sample and vortex before analyzing. Optionally, centrifuge for 2-5 minutes at 15,000 x g at 4°C and analyze the supernatant only.
7. To fall within the range of the standard curve, it may be necessary to dilute samples with Assay Buffer (1X) prior to performing the assay.

Plate Set Up

There is no specific pattern for using the wells on the plate. It is suggested that each sample and standard be assayed at least in duplicate (triplicate is preferred). A typical layout of standards and samples to be measured in duplicate is shown in Figure 2, below. It is suggested that the contents of each well are recorded on the template sheet provided (see page 26).



A-H = Standards
S1-S40 = Sample wells

Figure 2. Sample plate format

Pipetting Hints

- It is recommended that a multichannel pipette be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- 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(s).

General Information

- The final volume of the assay is 100 µl in all of the wells.
- All reagents except samples must be equilibrated to room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- It is recommended that the samples and standards be assayed at least in duplicate.

Standard Preparation

NOTE: This assay can be read using fluorescence or absorbance. Choose the standard curve preparation that matches the format needed. Both standard curves do not need to be prepared. The same standard curve can be used to determine total cholesterol and/or free cholesterol levels.

Fluorometric Standard Curve Preparation

Dilute 20 µl of Cholesterol Assay Standard (Item No. 10008053) with 980 µl of Assay Buffer (1X). Use this diluted standard (200 µM) to prepare the fluorometric standard curve.

Take eight clean test tubes and mark them A-H. Add the amount of diluted cholesterol standard and Assay Buffer (1X) to each tube as described in Table 1.

Tube	200 µM Cholesterol Standard (µl)	Assay Buffer (1X) (µl)	Final Cholesterol Concentration (µM)
A	0	1,000	0
B	10	990	2
C	20	980	4
D	30	970	6
E	40	960	8
F	60	940	12
G	80	920	16
H	100	900	20

Table 1. Preparation of cholesterol fluorometric assay standard curve

Colorimetric Standard Curve Preparation

Dilute 10 µl of Cholesterol Assay Standard (Item No. 10008053) with 990 µl of diluted assay buffer. Use this diluted standard (100 µM) to prepare the standard curve.

Take eight clean glass test tubes and mark them A-H. Add the amount of cholesterol standard and Assay Buffer (1X) to each tube as described in Table 2.

Tube	100 µM Cholesterol Standard (µl)	Assay Buffer (1X) (µl)	Final Cholesterol Concentration (µM)
A	0	200	0
B	10	190	5
C	20	180	10
D	40	160	20
E	80	120	40
F	120	80	60
G	160	40	80
H	200	0	100

Table 2. Preparation of cholesterol colorimetric assay standard curve

Performing the Assay

1. **Cholesterol Standard Wells** - add 50 µl of cholesterol standard (tubes A-H) per well in the designated wells on the plate (see **Sample Plate Format** on page 13).
2. **Sample Wells** - add 50 µl of sample to two wells. To obtain reproducible results, sample cholesterol levels should fall within the standard curve.
3. Cover the plate with the 96-Well Cover Sheet (Item No. 400012).
4. Prepare the reaction mix(es) for measuring total and/or free cholesterol levels following the chart below. If cholesterol ester levels are to be measured, assay the same sample with both reaction mixes separately and subtract the free cholesterol levels from the total cholesterol levels.

Reaction Mix	Total Cholesterol (96 wells)	Free Cholesterol (96 wells)
Assay Buffer (1X)	4.805 ml	4.81 ml
MaxiProbe	90 µl	90 µl
HRP	50 µl	50 µl
Cholesterol Oxidase	50 µl	50 µl
Cholesterol Esterase	5 µl	--
Final Volume	5 ml	5 ml

NOTE: 5 ml provides enough reaction mix to run the entire 96-well plate. Scale up or down as needed. For best results, use the reaction mix(es) within 10 minutes of preparation.

5. Remove the 96-Well Cover Sheet and initiate the reactions by adding 50 µl of freshly prepared assay reaction mix to all the wells being used.
6. Cover the plate with the 96-Well Cover Sheet and incubate for 30 minutes at 37°C protected from light.
7. Remove the 96-Well Cover Sheet and read fluorescence with excitation and emission wavelengths of 530 and 590 nm, respectively. The background fluorescence intensity will increase over time. If the colorimetric method is used, read absorbance at 570 nm.

Calculations

Calculate total cholesterol and free cholesterol concentrations using the same standard curve, following the steps below. To obtain cholesterol ester levels in a sample, subtract free cholesterol from total cholesterol.

1. Calculate the average fluorescence/absorbance of each standard and sample.
2. Subtract the average fluorescence/absorbance of standard A from itself and all other standards and samples to obtain corrected sample or standard measurements (CSM).
3. Plot the CSM values of the standards (from step 2 above) as a function of the final concentration of cholesterol from Table 1 and Table 2, pages 15 and 16, respectively. See Figure 3 for a typical fluorometric standard curve and Figure 4 for a typical colorimetric standard curve.
4. Calculate the cholesterol concentration of the samples using the equation obtained from the linear regression of the standard curve substituting CSM for each sample.

$$\text{Cholesterol (mM)} = \left[\frac{\text{CSM - (y-intercept)}}{\text{Slope}}\right] \text{x Sample dilution x 0.001 mM/}\mu\text{M}$$

NOTE: To convert the results from mM to mg/dl, divide the cholesterol concentration (mM) by 0.0259.

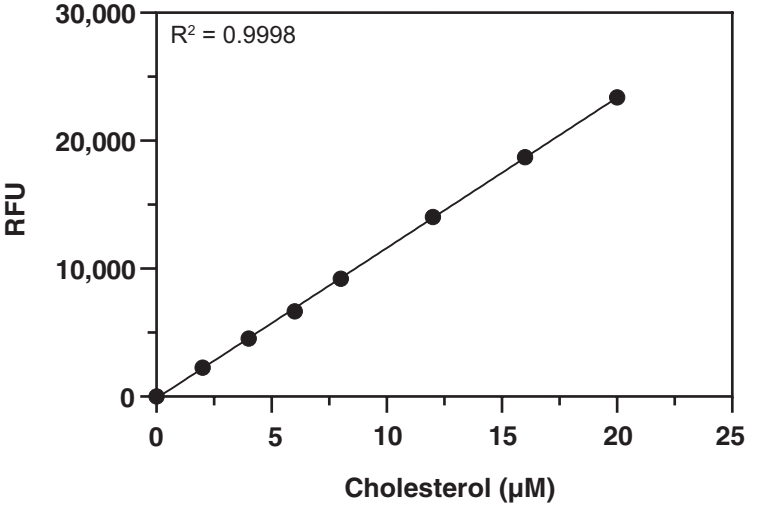


Figure 3. Cholesterol fluorometric standard curve

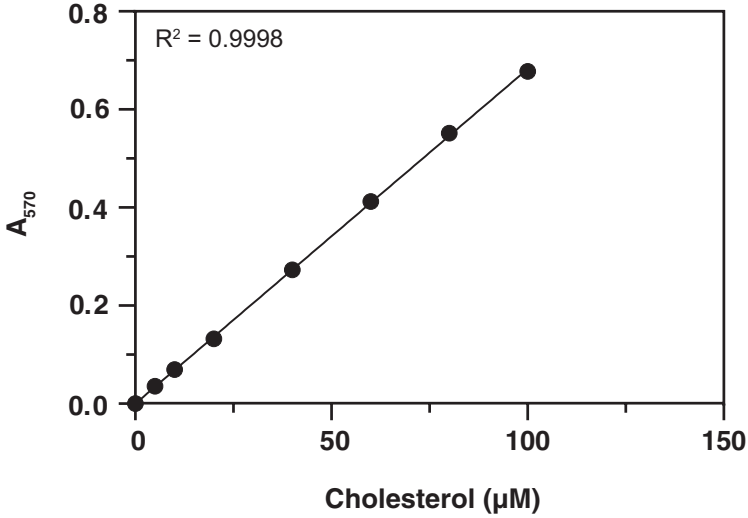


Figure 4. Cholesterol colorimetric standard curve

Performance Characteristics

Sensitivity:

The lower limit of detection (LLOD) for the fluorometric assay is 1 µM and the lower limit of quantification (LLOQ) is 2 µM. The LLOD for the colorimetric assay is 1 µM and the LLOQ is 5 µM.

Precision:

When a series of 65 plasma measurements at a 1:400 dilution were performed using the fluorimetric readout on seven different days under the same experimental conditions, the intra-assay coefficient of variation was 6.4% and the inter-assay coefficient of variation was 3.4%.

Parallelism:

To assess parallelism, various matrices were processed as described in the **Sample Preparation** section (see pages 10-12), serially diluted with Assay Buffer (1X), and evaluated using the fluorometric assay. Measured concentrations were plotted as a function of the sample dilution. The results are shown below.

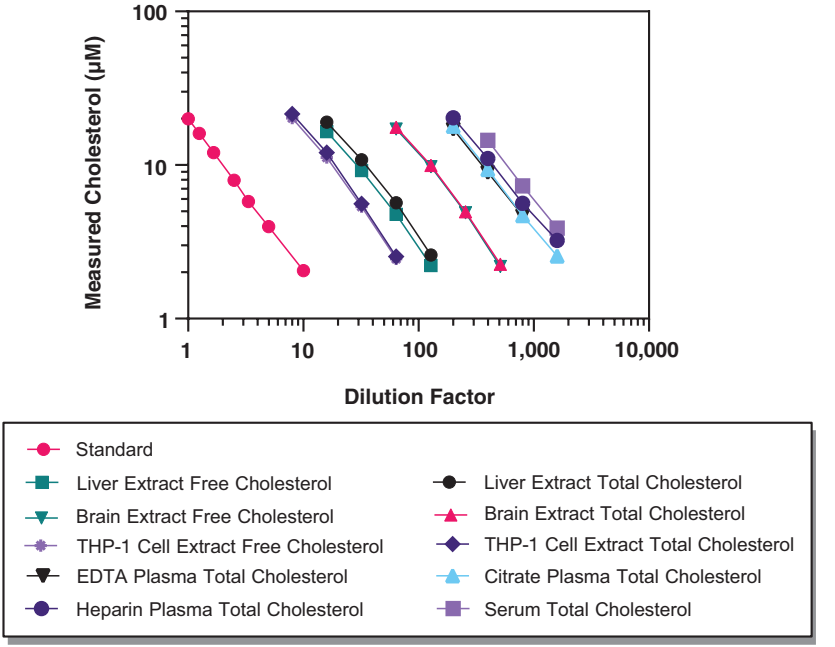


Figure 5. Parallelism of various matrices in the fluorometric assay

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
Cholesterol was not detected in the sample	Sample was too dilute	Re-assay the sample using a lower sample dilution
Signal of sample is higher than most concentrated cholesterol standard	The sample is too concentrated	Dilute your sample with Assay Buffer (1X) and re-assay
The cholesterol standard curve did not work	Either the cholesterol standards were not diluted properly or the cholesterol standard has degraded	Set-up the standards according to Table 1 or 2 and re-assay

Reaction Mix	Total Cholesterol (96 wells)	Free Cholesterol (96 wells)
Assay Buffer (1X)	4.805 ml	4.81 ml
MaxiProbe	90 µl	90 µl
HRP	50 µl	50 µl
Cholesterol Oxidase	50 µl	50 µl
Cholesterol Esterase	5 µl	--
Final Volume	5 ml	5 ml

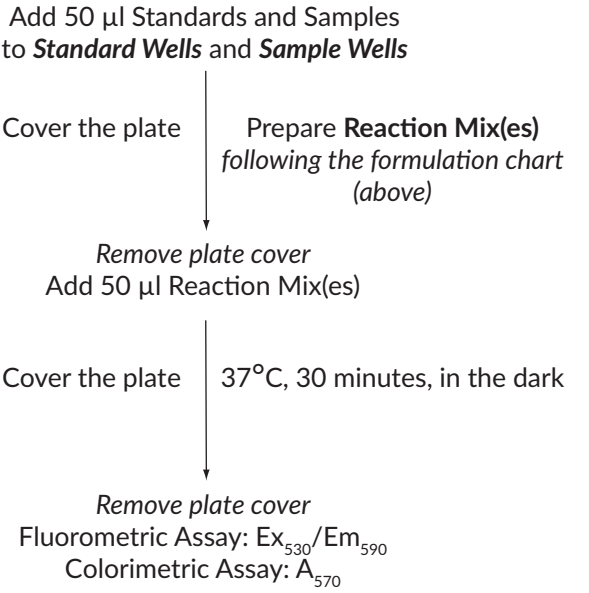


Figure 6. Assay flowchart

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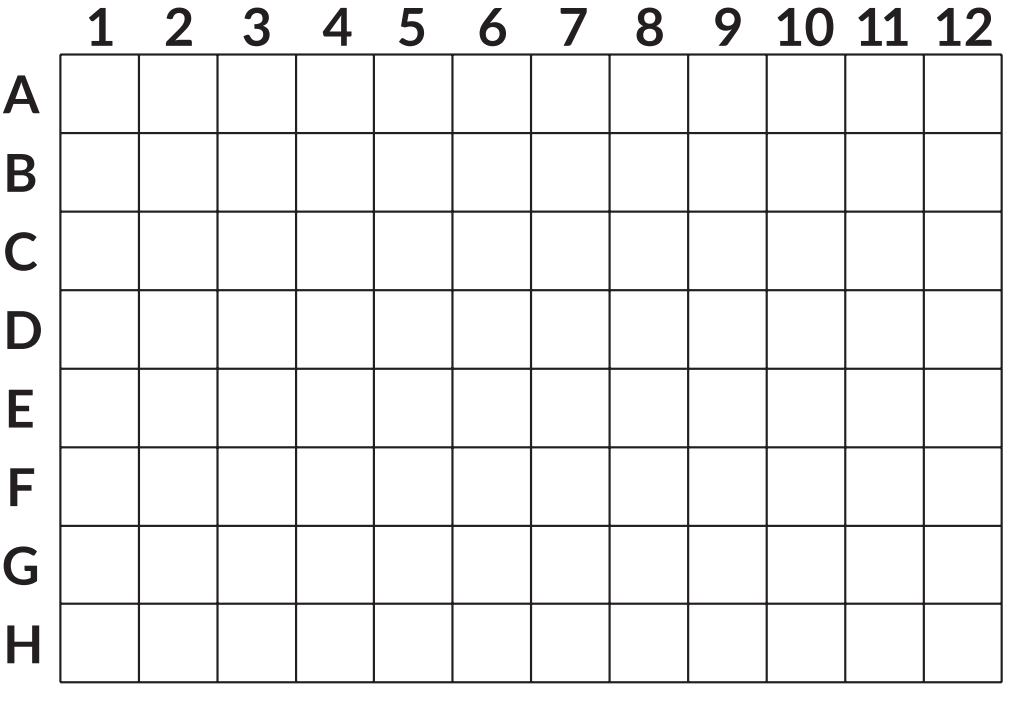
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
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H

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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