Myeloperoxidase Peroxidation Fluorometric Assay Kit

Item No. 700160

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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

ASSAY PROTOCOL
11 Plate Set Up
13 Standard Preparation
14 Performing the Assay

ANALYSIS
16 Calculations
21 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	Storage
700164	MPO Assay Buffer (1X)	1 bottle/50 ml	4°C
700023	Resorufin Standard	1 vial/500 µl	-20°C
700001	DMSO Assay Reagent	1 vial/1 ml	RT
700168	MPO Hydrogen Peroxide	1 vial/100 µl	4°C
700166	MPO Enzyme (human)	1 vial/40 µl	-20°C
700167	MPO Inhibitor	1 vial/50 µl	4°C
700002	ADHP Assay Reagent	5 vials	-20°C
400017	96-Well Solid Plate (black)	2 plates	RT
400012	96-Well Cover Sheets	2 covers	RT

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 on 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 fluorescence using an excitation wavelength of 530 nm (530-540 nm) nm and an emission wavelength of 590 nm (570-610 nm)
2. Adjustable pipettes; multichannel or repeating pipettor recommended
3. Materials used for **Sample Preparation** (see page 10)

INTRODUCTION

Background

Myeloperoxidase (MPO) is a heme-containing enzyme and the most abundant protein in polymorphonuclear leukocytes (PMNs).[1] It is composed of two subunits linked by a disulfide bridge with each subunit containing a light and heavy polypeptide chain. MPO is stored in azurophilic granules of PMNs and is released from activated or necrotic PMNs, after which it can bind to and modify acidic serum proteins, as well as recruit additional PMNs.[1] It can oxidize a variety of substrates and catalyzes the formation of highly reactive (pseudo)hypohalous acids and radicals, including hypochlorous acid (HOCl), using hydrogen peroxide (H_2O_2) for chlorination or peroxidation.[1] The use of H_2O_2 by MPO for either its chlorination or peroxidation activities depends on the relative concentrations of chloride and the reducing substrate.[2] MPO also has roles in PMN apoptosis and antimicrobial defense systems, including neutrophil extracellular trap (NET) formation and NETosis.[1,3,4] It enhances neutrophil elastase-induced chromatin decondensation and produces reactive oxygen species (ROS), which trigger NET formation.[5] MPO-derived oxidants and chlorinated products are enriched in LDL and human atherosclerotic lesions.[6-8] In addition, MPO levels in leukocytes and the blood are elevated in patients with coronary artery disease (CAD), and elevated serum levels of MPO in patients with acute coronary syndromes are considered a risk factor for subsequent cardiovascular events.[7-9]

About This Assay

Cayman's MPO Peroxidation Fluorometric Assay provides a convenient fluorescence-based method for detecting MPO peroxidase activity in both crude cell lysates and purified enzyme preparations. The assay utilizes the peroxidase component of MPO, where a single two-electron oxidation of native enzyme (MPO) to compound I (MPO-I) is followed by two successive one-electron reductions back to native enzyme by compound II (MPO-II).[7] The reaction between hydrogen peroxide and ADHP (10-acetyl-3,7-dihydroxyphenoxazine) produces the highly fluorescent compound resorufin (see Figure 1). Resorufin fluorescence can be easily analyzed with an excitation wavelength of 530 nm and emission wavelength of 590 nm. The kit includes an MPO-specific inhibitor for distinguishing MPO activity from MPO-independent fluorescence.

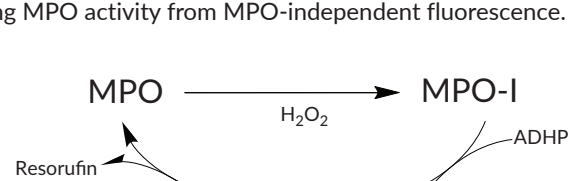


Figure 1. Assay scheme

PRE-ASSAY PREPARATION

Reagent Preparation

1. **MPO Assay Buffer (1X) - (Item No. 700164)**

   This bottle contains 50 ml of 1X Assay Buffer. It is ready to use as supplied.

2. **Resorufin Standard - (Item No. 700023)**

   This vial contains 500 µl of 2 mM resorufin in DMSO. To prepare the 150 µM bulk standard, add 30 µl of Resorufin Standard to 370 µl of MPO Assay Buffer (1X). This bulk standard is ready to use to prepare the standard curve (see page 13). The bulk standard will be stable for two hours at room temperature.

3. **DMSO Assay Reagent - (Item No. 700001)**

   This vial contains 1 ml of DMSO and is ready to use to prepare the MPO Peroxidation Substrate.

4. **MPO Hydrogen Peroxide - (Item No. 700168)**

   This vial contains 100 µl of an 9.8 M solution of hydrogen peroxide. Prior to use in the assay, mix 10 µl with 90 µl of MPO Assay Buffer (1X) to yield a 0.98 M solution. Then, mix 10 µl of the 0.98 M solution with 1.95 ml of MPO Assay Buffer (1X) to prepare a 5 mM solution. The 5 mM solution will be used to prepare the Initiator Solution. The diluted solutions will be stable for one hour at room temperature.

5. **MPO Enzyme (human) - (Item No. 700166)**

   This vial contains a 40 µl suspension of human PMN MPO enzyme. MPO Enzyme should be thawed on ice and mixed by gently pipetting up and down prior to dilution as the enzyme may settle over time. *Do not vortex*. Dilute the MPO Enzyme 1:160 to prepare the MPO enzyme working solution by mixing 5 µl of MPO Enzyme with 795 µl of MPO Assay Buffer (1X). It is recommended that the enzyme be diluted just prior to performing the assay. The diluted enzyme should be kept on ice, where it will be stable for one hour. Any undiluted enzyme can be aliquoted and stored at -20°C, limiting freeze-thaw cycles.

6. **MPO Inhibitor - (Item No. 700167)**

   This vial contains 50 µl of 50 mM 4-aminobenzhydrazide in DMSO. Mix 10 µl of MPO Inhibitor with 490 µl of MPO Assay Buffer (1X) to make a 1 mM solution. This is enough MPO Inhibitor to assay 50 wells. The diluted inhibitor will be stable for four hours.

7. **MPO Peroxidation Substrate**

   Immediately prior to preparing the Initiator Solution, add 120 µl of DMSO Assay Reagent to one vial of ADHP Assay Reagent (Item No. 700002) and vortex until dissolved. Then add 470 µl of MPO Assay Buffer (1X) for a final MPO Peroxidation Substrate concentration of 1 mM. This is enough MPO Peroxidation Substrate to assay 100 wells. Prepare additional vials as needed. The reconstituted MPO Peroxidation Substrate will be stable for 30 minutes.

Sample Preparation

The kit is designed for detection of MPO peroxidation activity in cell lysates and in purified solutions. This assay is not compatible for use with serum samples. Some reagents have been shown to interfere with this assay (see page 22 for a list of compatible reagents). Samples should be processed and assayed immediately after collection; samples that cannot be assayed immediately should be flash frozen in liquid nitrogen and stored at -80°C.

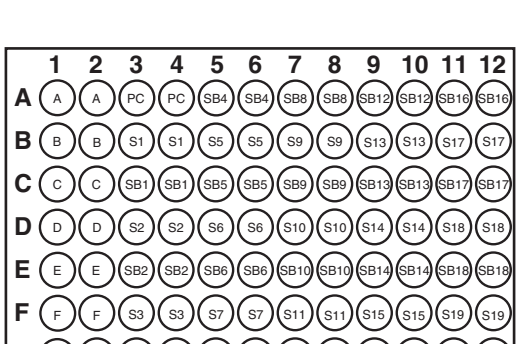
Cell Lysate

1. Collect 2 X 10^6 cells by centrifugation (*i.e.*, 1,000-2,000 x g for 10 minutes at 4°C). For adherent cells, do not harvest using proteolytic enzymes; rather use a rubber policeman.
2. Add 200 µl of cold MPO Assay Buffer (1X) and ~100 mg of glass beads (500-750 µm) to the cell pellet.
3. Vortex for 30 seconds and chill on ice for 30 seconds. Repeat three times.
4. Centrifuge for 15 minutes at 15,000 x g at 4°C.
5. Transfer the supernatant to a new tube and place on ice.
6. Cell lysates should be assayed at multiple dilutions, as antioxidants present in the sample can quench H_2O_2 and interfere with the assay. When sufficiently diluted, the reaction should produce linear slopes upon back calculation. Optimal dilution ranges may vary by cell type.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. However, it is recommended that two wells be designated for the positive control. It is suggested that each standard, sample, and sample background be assayed in duplicate and that the contents of each well be recorded on the template sheet provided on page 25. A typical layout of samples to be measured in duplicate is shown in Figure 2, below.



A-H = Resorufin Standard Wells
PC = MPO Positive Control Wells
S = Sample Wells
SB = Sample Background 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 to achieve 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.

General Information

- The final volume of the assay is 110 µl in all the wells.
- All reagents except the MPO enzyme 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.
- The assay is performed at room temperature.
- Monitor the fluorescence with an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

Standard Preparation

Use the 150 µM bulk standard diluted as described in the **Reagent Preparation** section (see page 8) to prepare the assay standards.

Label eight clean test tubes A-H. Aliquot 990 µl of MPO Assay Buffer (1X) to tube A and 500 µl of MPO Assay Buffer (1X) to tubes B-H. Transfer 10 µl of the 150 µM bulk standard to tube A and mix thoroughly. Serially dilute the standard by transferring 500 µl from tube A to tube B. Mix thoroughly. Next, transfer 500 µl from tube B to tube C. Mix thoroughly. Repeat this process for tubes D-G. Do not add any standard to tube H. The diluted standards will be stable for four hours at room temperature.

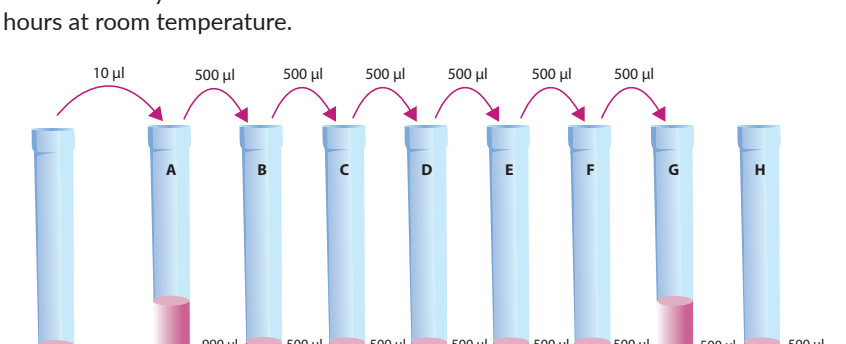


Figure 3. Preparation of resorufin standards

Performing the Assay

NOTE: Due to the rapid reaction kinetics, it is recommended to run no more than 24 wells at a time to avoid missing the linear portion of the reaction.

1. **Standard Wells -** add 110 µl of prepared standards in the designated wells on the plate (see **Sample plate format**, Figure 2, page 11).
2. Read the plate to determine the optimal *gain* setting using an excitation wavelength of 530 nm and an emission wavelength of 590 nm. The optimal *gain* will then be used when assaying the samples.
3. Add the appropriate amount of reagents to the designated wells according to Table 1, below.

Reagent	Positive Control Wells	Sample Wells	Sample Background Wells
MPO Assay Buffer (1X)	50 µl	50 µl	40 µl
Diluted MPO Enzyme (human)	10 µl	--	--
Sample	--	10 µl	10 µl
MPO Inhibitor	--	--	10 µl

Table 1. Pipetting summary

4. Prepare the Initiator Solution according to Table 2, below, and use within 15 minutes.

Reagents	50 wells	100 wells
MPO Assay Buffer (1X)	2.24 ml	4.48 ml
MPO Peroxidation Substrate (1 mM)	250 µl	500 µl
Hydrogen Peroxide (5 mM)	10 µl	20 µl

Table 2. Initiator Solution preparation

5. Initiate the reactions by quickly adding 50 µl of the Initiator Solution to the positive control, sample, and sample background wells. Do not add the Initiator Solution to the standard wells.
6. Read kinetically by measuring fluorescence every 30 seconds for 15 minutes using an excitation wavelength of 530 nm and emission wavelength of 590 nm. *NOTE: Standard well measurements do not have to be read kinetically. Read standard wells as endpoint fluorescence after the 15 minute incubation.*

ANALYSIS

Calculations

Plot the Standard Curve

1. Determine the average fluorescence of the standards. Subtract the fluorescence value of standard H from itself and all other standards. This is the corrected fluorescence.
2. Plot the corrected fluorescence values (from step 1 above) of each standard as a function of the final concentration of resorufin from Figure 3. See Figure 4, on page 17, for a typical standard curve.

Determine MPO Activity

1. Determine the average fluorescence of each sample and sample background.
2. Determine the change in fluorescence (RFU) per minute for the sample and sample background by:
   a. Plotting the fluorescence values as a function of time to obtain the slope (rate) of the linear portion of the curve. An example of human PMN MPO assayed with and without MPO inhibitor over time is shown in Figure 5, on page 18.

   OR

   b. Select two points on the linear portion of the curve and determine the change in fluorescence during that time using the following equation:

$$\text{RFU/min} = \frac{\text{RFU (Time 2)} - \text{RFU (Time 1)}}{\text{Time 2 (min)} - \text{Time 1 (min)}}$$

3. Calculate the MPO activity using the equation below. One unit is defined as the amount of enzyme that will cause the formation of 1 nmol of fluorophore per minute at 25°C.

$$\text{MPO Activity (U/ml)} = \frac{\text{Sample slope (RFU/min)} - \text{Background slope (RFU/min)}}{\text{Resorufin standard curve slope (RFU/µM)}} \times \left(\frac{110\ \mu l}{10\ \mu l}\right) \times \text{Sample dilution}$$

Sample Data:

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

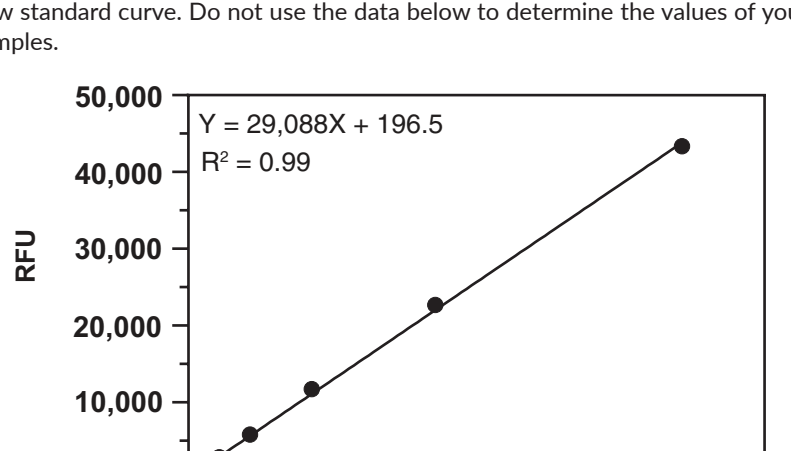


Figure 4. Resorufin standard curve

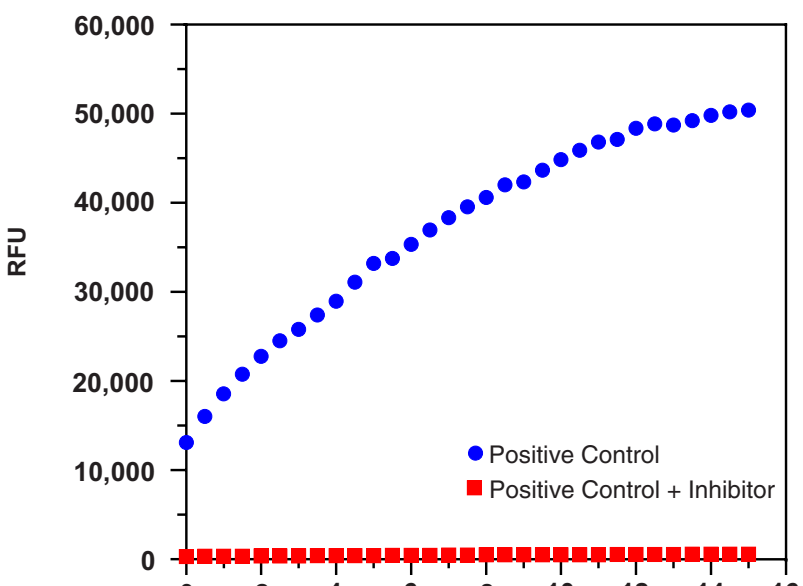


Figure 5. Kinetics of the positive control, MPO Enzyme (human), with and without the MPO inhibitor

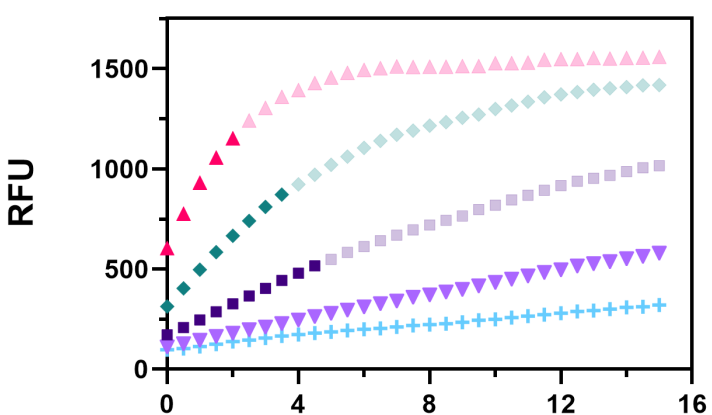


Figure 6. MPO activity measured in HL-60 lysates. 5x10^6 HL-60 cells were lysed in 400 µl of MPO Assay Buffer (1X) according to the protocol outlined on page 10. Cell lysates were diluted 2-, 4-, 8-, 16-, and 32-fold prior to running the assay. The linear range of each dilution (bold) was used to calculate MPO activity.

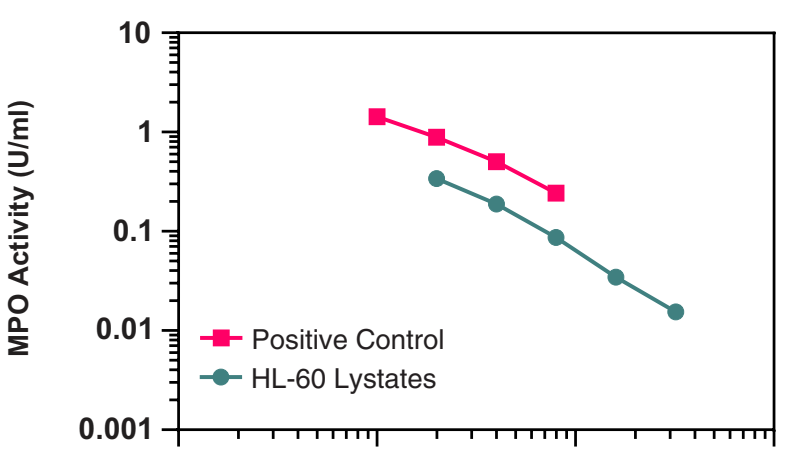


Figure 7. Parallelism of HL-60 cell lysates and MPO Enzyme (human) positive control. The MPO activities measured in HL-60 cell lysates (Figure 6) and the positive control, MPO enzyme, were plotted as a function of sample dilution. Values that fell outside of the linear portion were excluded.

Performance Characteristics

Precision:

When a series of sixteen MPO measurements were performed on the same day, the intra-assay coefficient of variation was 2.8%. When a series of sixteen MPO measurements were performed on five different days under the same experimental conditions, the inter-assay coefficient of variation was 3.1%.

Assay Sensitivity:

Under the standardized conditions of the assay described in this booklet, the sensitivity of this kit is 0.08 U/ml.

RESOURCES

Interferences

The following reagents were tested for interference in the assay:

	Reagent	Will Interfere (Yes or No)
Buffers	Tris	No
	HEPES	Yes
	Phosphate	No
Detergents	Polysorbate 20 (0.1%)	Yes
	Polysorbate 20 (1%)	Yes
	Triton X-100 (0.1%)	Yes
	Triton X-100 (1%)	Yes
Protease Inhibitors/ Chelators/Enzymes	EDTA (1 mM)	No
	EGTA (1 mM)	No
	Trypsin (10 µg/ml)	No
	PMSF (1 mM)	Yes
	Leupeptin (10 µg/ml)	No
	Antipain (10 µg/ml)	No
	Chymostatin (10 µg/ml)	No
Solvents	Ethanol (10 µl)	No
	Methanol (10 µl)	No
	Dimethylsulfoxide (10 µl)	Yes
Others	BSA (0.1%)	Yes
	Glutathione (1 mM)	Yes
	Glycerol (5%)	No

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 signal increase over time observed in samples	A. MPO activity is too low to detect B. There is no MPO in the sample C. If the RFU at t_0 is much higher than the background RFU, the reaction has already reached completion; the enzyme level is too high	A. Concentrate the sample using a concentrator with a MW cut-off of 30 kDa and re-assay B. Verify expression and re-assay C. Dilute the sample and re-assay
No activity detected in samples and positive control	The initiator solution was prepared incorrectly	Prepare the initiator solution and make sure to include all reagents as instructed
No signal change over time in positive control wells	A. Positive control is inactive B. Positive control was not added to the well	A. Prepare a fresh positive control and verify proper storage and handling B. Make sure to add all the components to the wells and re-assay

References

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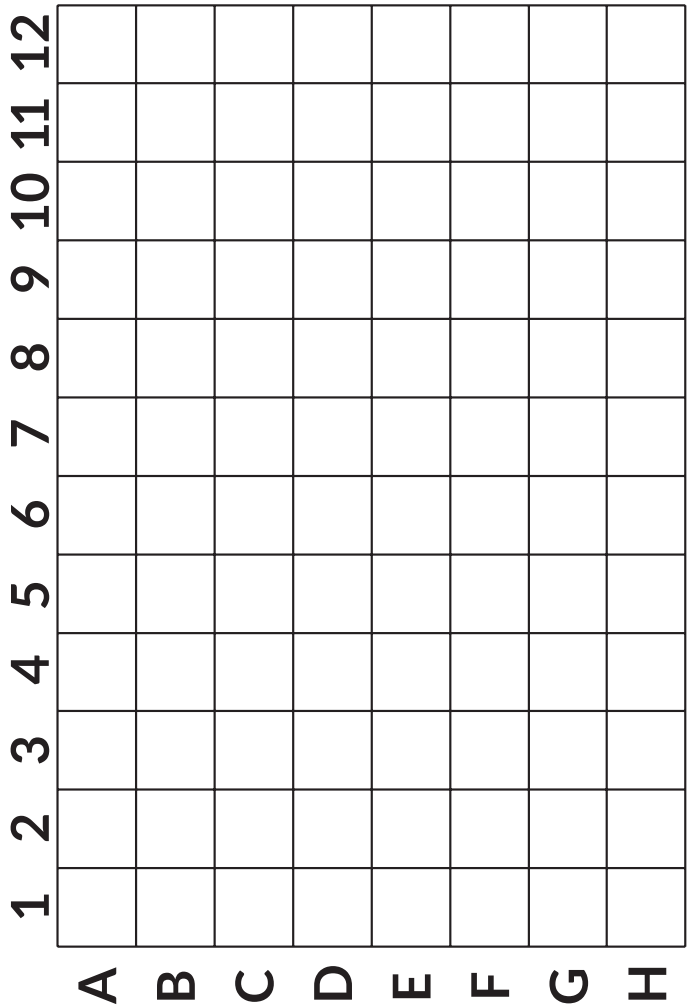

NOTES

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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