# Phospholipase D Assay Kit

Item No. 700590

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# GENERAL INFORMATION

## Materials Supplied

Kit will arrive packaged as a -20°C kit. For best results, store kit at -20°C or remove components and store as stated below.

| Item Number | Item | Quantity/Size | Storage |
|---|---|---|---|
| 700591 | PLD Assay Buffer (5X) | 1 vial/5 ml | -20°C |
| 700592 | PLD Enzyme Mixture | 2 vials | -20°C |
| 700593 | PLD Substrate | 1 vial/200 µl | -20°C |
| 700594 | PLD Positive Control | 1 vial/100 µl | -20°C |
| 700112 | Resorufin Standard Assay Reagent | 1 vial/100 µl | -20°C |
| 700001 | DMSO Assay Reagent | 1 vial/1 ml | Room temperature |
| 700002 | ADHP Assay Reagent | 3 vials | -20°C |
| 400091 | Half Volume 96-Well Solid Plate (black) | 1 plate | Room temperature |
| 400012 | 96-Well Cover Sheet | 1 cover | Room temperature |

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 975-3999. We cannot accept any returns without prior authorization.

**WARNING: This product is for laboratory research use only: not for administration to humans. Not for human or veterinary diagnostic or therapeutic use.**

## Precautions

**Please read these instructions carefully before beginning this assay.**

**For research use only. Not for human or diagnostic use.**

## If You Have Problems

### Technical Service Contact Information


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

**Fax:** 734-971-3641

**Email:** techserv@caymanchem.com

**Hours:** M-F 8:00 AM to 5:30 PM EST


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

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

## Materials Needed But Not Supplied

1. A plate reader capable of measuring fluorescence at an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm
2. Adjustable pipettes and a repeat pipettor
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable



# INTRODUCTION

## Background

Phospholipase D (PLD) is a ubiquitous enzyme found in most mammalian cells.[1] There are two human isoforms of phospholipase D: PLD1 and PLD2. The functional differences between the isoforms are not well defined, but the localization of each isoform is unique. PLD1 is localized around the perinuclear region, while PLD2 is located on the plasma membrane.[2] PLD2 is naturally active and regulated by inhibitory mechanisms. PLD1 however has a low native activity and thus requires activation by various proteins, such as Rho and Protein Kinase C.[2,3] Phospholipase D has been linked to the pathogenesis of Alzheimer's disease, Parkinson's disease, alcoholism, and brain aging.[2]

Phospholipase D hydrolyzes phosphatidylcholine to yield choline and phosphatidic acid (PA).[1] Many cellular functions have been associated with PA, including intracellular signaling, mitogenesis in fibroblasts, stimulation of respiratory bursts in neutrophils, increase of cellular $Ca^{2+}$, and activation of specific protein kinases and phospholipases.[3]

PA is further metabolized to 1,2-diacylglycerol (DAG) and lysophosphatidic acid (LPA) by lipid phosphate phosphohydrolases and phospholipase A, respectively.[2,3] The primary cellular function of DAG is to activate protein kinase C. LPA, on the other hand, has a broad range of important functions, including intercellular signaling, calcium mobilization, protein kinase C activation, and growth stimulation.[3]

## About This Assay

Cayman's Phospholipase D Assay provides a convenient fluorescence-based method for determining PLD activity in both crude and purified enzyme preparations. The assay detects choline molecules which are generated during the hydrolysis of phosphatidylcholine. Choline is oxidized by Choline Oxidase to produce betaine and hydrogen peroxide ($H_2O_2$). In the presence of peroxidase, $H_2O_2$ reacts with 10-acetyl-3,7-dihydroxyphenoxazine (ADHP) to yield the highly fluorescent compound resorufin. Resorufin fluorescence can be analyzed using an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm.

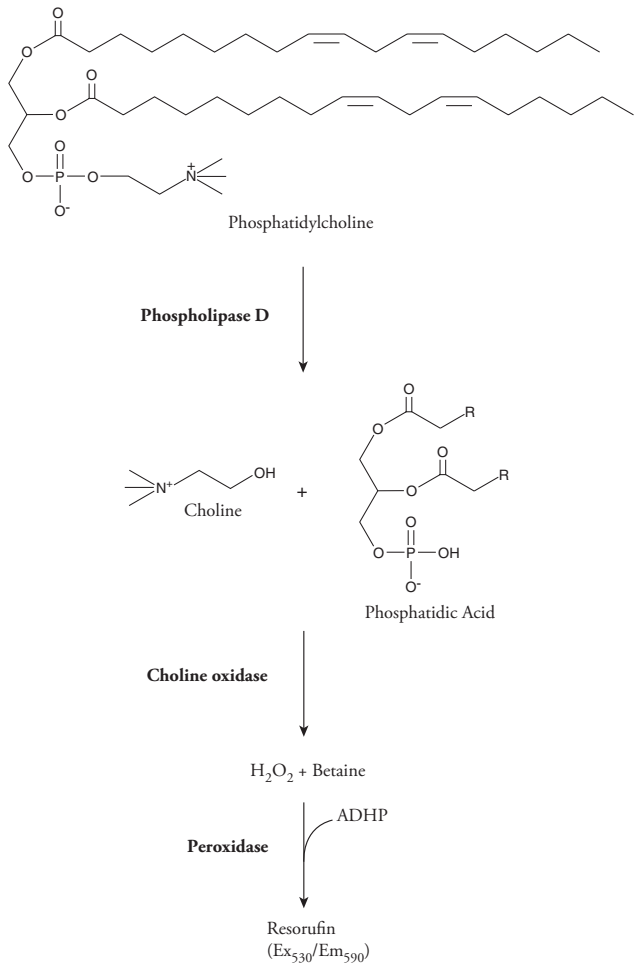



**Figure 1. Assay scheme**



# PRE-ASSAY PREPARATION

## Reagent Preparation

### 1. PLD Assay Buffer (5X) - (Item No. 700591)

The vial contains 5 ml of 250 mM Tris-HCl, pH 8.0, containing 5 mM $CaCl_2$. Dilute the contents of the vial with 20 ml of HPLC-grade water. The diluted Assay Buffer (50 mM Tris-HCl, pH 8.0, containing 5 mM $CaCl_2$) should be used for reconstituting reagents and preparation of the Assay Cocktail (see **Performing the Assay**, pg 13). This diluted buffer is stable for three months when stored at 4°C.

### 2. PLD Enzyme Mixture - (Item No. 700592)

The vials contain a lyophilized powder of choline oxidase and horseradish peroxidase. Reconstitute the contents of one vial with 200 µl of diluted Assay Buffer and store on ice. The reconstituted Enzyme Mixture is used in the preparation of the Assay Cocktail. The reconstituted Enzyme Mixture is stable for two hours when stored on ice or two weeks when frozen at -20°C.

### 3. PLD Substrate - (Item No. 700593)

The vial contains 200 µl of 100 mM phosphatidylcholine in ethanol. It is ready to be used as supplied. The PLD Substrate is used in the preparation of the Assay Cocktail. The substrate is stable for one week at 4°C or six months when stored at -20°C.

### 4. PLD Positive Control - (Item No. 700594)

The vial contains 100 µl of 1,000 U/ml phospholipase D. Dilute 10 µl of PLD with 990 µl of diluted Assay Buffer to yield 10 U/ml PLD. Dilute 50 µl of the 10 U/ml PLD with 450 µl of diluted Assay Buffer to yield a 1 U/ml PLD that will be used in the assay. The diluted Positive Control is stable for two hours when stored at 4°C.

### 5. Resorufin Standard Assay Reagent - (Item No. 700112)

The vial contains 100 µl of 1 mM resorufin in DMSO. It is ready to use to prepare the standard curve.

### 6. DMSO Assay Reagent - (Item No. 700001)

This vial contains 1 ml of dimethylsulfoxide (DMSO). The reagent is used to reconstitute the ADHP Assay Reagent and is ready to use as supplied.

### 7. ADHP Assay Reagent - (Item No. 700002)

This vials contain a lyophilized powder of 10-acetyl-3,7-dihydroxyphenoxazine (ADHP). The ADHP is used in the preparation of the Assay Cocktail, as described on pg 13.

## Sample Preparation

### Plasma

1. Collect blood using an anticoagulant such as heparin or EDTA.
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 until assaying or freeze at -80°C. The plasma sample will be stable for one month. Repeated freeze/thaw cycles should be avoided.
3. Plasma typically does not need to be diluted prior to assaying.

### Serum

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 on the same day, freeze at -80°C. The sample will be stable for one month. Repeated freeze/thaw cycles should be avoided.
4. Serum typically does not need to be diluted prior to assaying.



### Tissue Homogenates

1. Prior to dissection, perfuse or rinse tissue with a solution of phosphate buffered saline (PBS, pH 7.4) to remove any extraneous red blood cells and clots.
2. Homogenize the tissue in 5-10 ml of PBS solution, pH 7.4, per gram weight of tissue.
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant and store on ice. If not assaying on the same day, freeze the sample at -80°C. The sample will be stable for at least one month.
5. Dilution of the sample may be required to obtain results which fall within the standard curve. Samples should be diluted with diluted Assay Buffer.

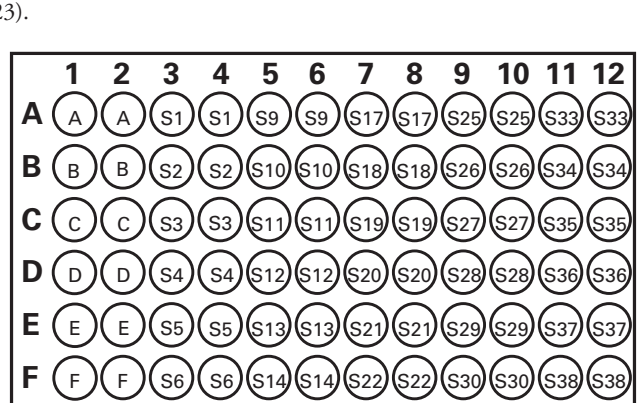
### Cell Lysates

1. Collect cells (~5 x $10^6$) by centrifugation (*i.e.*, 1,000-2,000 x g for 10 minutes at 4°C). For adherent cells, use a rubber policeman to collect cells.
2. Homogenize or sonicate the cell pellet in 0.5-1.0 ml cold buffer (*i.e.*, 100 mM Tris, pH 8.0).
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant and store on ice. If not assaying on the same day, freeze the sample at -80°C. The sample should be stable for at least one month.
5. Dilution of the sample may be required to obtain results which fall within the standard curve. Samples should be diluted with diluted Assay Buffer.

# ASSAY PROTOCOL

## Plate Set Up

There is no specific pattern for using the wells on the plate. A typical layout of resorufin standards, PLD positive control, and samples to be measured in duplicate is given below in Figure 2. We suggest you record the contents of each well on the template sheet provided (see page 23).




A-G = Standards
+ = PLD Positive Control
S1-S40 = Sample Wells

**Figure 2. Sample plate format**

### Pipetting Hints

- It is recommended that a repeating pipettor 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.

### General Information

- The final volume of the assay is 110 µl in all the wells.
- All reagents, except the PLD Positive Control and samples must be equilibrated to room temperature before beginning the assay. Keep the PLD Positive Control and samples on ice until ready to use in the assay.
- 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 at least in duplicate (triplicate is recommended)
- Twenty-six samples can be assayed in triplicate or forty in duplicate.
- The assay should be performed at 37°C.
- Monitor the fluorescence with an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm.

## Standard Preparation

Dilute 40 µl of the Resorufin Standard with 1.96 ml of diluted Assay Buffer to yield a stock concentration of 20 µM. Take seven clean glass test tubes or polystyrene tubes and mark them A-G. Add the amount of resorufin stock (20 µM) and diluted Assay Buffer to each tube as described in Table 1. The diluted Standards are stable for four hours at room temperature.

| Tube | Resorufin stock (µl) | diluted Assay Buffer (µl) | Final Resorufin Concentration (µM) |
|---|---|---|---|
| A | 0 | 400 | 0 |
| B | 20 | 380 | 1 |
| C | 40 | 360 | 2 |
| D | 80 | 320 | 4 |
| E | 120 | 280 | 6 |
| F | 160 | 240 | 8 |
| G | 200 | 200 | 10 |

**Table 1. Preparation of standards**

## Performing the Assay

1. **Standard Wells** - add 10 µl of Resorufin Standard (tubes A-G) and 100 µl of diluted Assay Buffer per well in the designated wells on the plate (see **Plate Set Up** on page 10).
2. Read the plate after five minutes using an excitation wavelength between 530-540 nm and an emission wavelength between 585-595 nm. Reading the standards prior to measuring sample activity allows an appropriate gain to be established for detecting the entire range of standards. This gain must also be used when assaying the samples.

**Preparation of the Assay Cocktail:** *NOTE: This step is performed immediately before running the assay.*

Reconstitute one vial of ADHP Assay Reagent with 100 µl of DMSO Assay Reagent (Item No. 700001) and then add 100 µl of diluted Assay Buffer. The reconstituted ADHP is stable for 60 minutes at room temperature. After 60 minutes, increased background fluorescence will occur.

Combine the following reagents in an appropriately sized vial:

5.80 ml diluted Assay Buffer

120 µl ADHP solution

60 µl Enzyme Mixture solution

30 µl Phosphatidylcholine solution (addition of the PC solution will cause some cloudiness; this is normal)

This is sufficient Assay Cocktail to assay 50 wells. Prepare additional Assay Cocktail if additional wells are to be assayed. Once the reagents have been combined, the Assay Cocktail must be used within 10 minutes or increased background fluorescence will occur.

3. **Positive Control Wells** - add 10 µl of PLD Positive Control to two wells.
4. **Sample Wells** - add 10 µl of sample to at least two wells per sample. To obtain reproducible results, the amount of PLD added to the wells should fall within the range of the assay. When necessary, samples should be diluted with diluted Assay Buffer or concentrated to bring the enzymatic activity to this level
5. Add 100 µl of Assay Cocktail to all sample and Positive Control wells being used. Do not add to the Resorufin Standard wells.
6. Gently shake the plate for 10 seconds.
7. Read fluorescence every minute for 30 minutes at 37°C using an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm.



# ANALYSIS

## Calculations

Determining the Resorufin standard curve:

1. Determine the average fluorescence of each standard. Subtract the fluorescence value of standard A from itself and all other standards. This is the corrected fluorescence.
2. Plot the corrected fluorescence of the standards as a function of the final concentration of resorufin from Table 1. See Figure 3, on page 16, for a typical standard curve.

Determining PLD activity:

1. Determine the change in relative fluorescence (ΔRFU) per minute by:
   a. Plot the fluorescence values as a function of time (in minutes) to obtain the slope (rate) of the linear portion of the curve. An example of the PLD Positive Control over time is shown in Figure 4 on page 17.

   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:

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

2. Using the ΔRFU/min obtained above, the total PLD activity of the sample is calculated with the following equation. One unit is defined as the amount of enzyme that will cause the formulation of 1 nmol of fluorophore per minute at 37°C.

**PLD Activity (nmol/min/ml) =**

$$\left[\frac{\text{ΔRFU/min.}}{\text{Slope from Resorufin standard curve (RFU/µM)}}\right] \text{x Sample dilution}$$

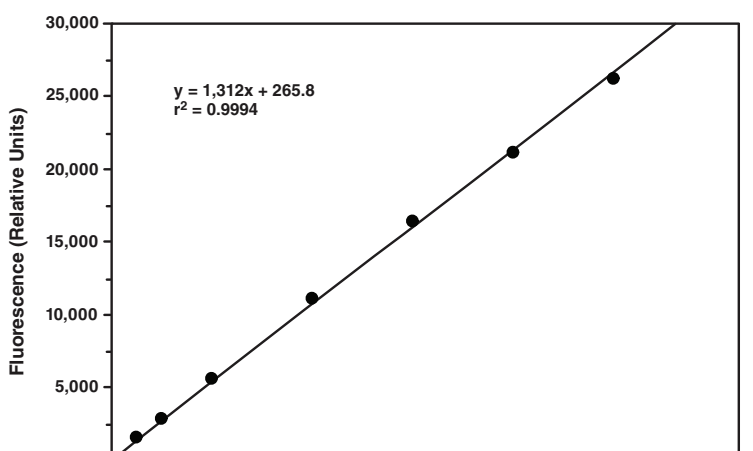



**Figure 3. Resorufin standard curve**

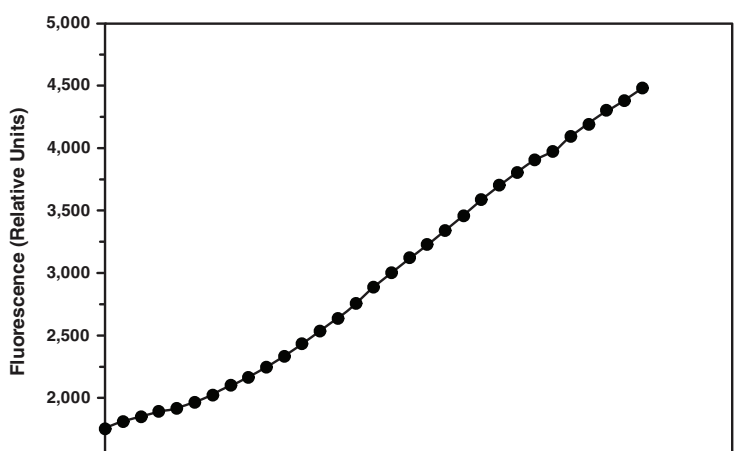



**Figure 4. PLD Positive Control over time**

## Performance Characteristics

### Sensitivity:

This limit of detection for this assay is approximately 0.015 (±0.005) U/ml PLD.

### Precision:

When a series of 80 cell lysate samples were analyzed on the same day under the same experimental conditions, the intra-assay coefficient of variation was 3.4%. When a series of five cell lysate samples were analyzed on five different days under the same experimental conditions, the inter-assay coefficient of variation was 1.6%.

## Interferences

The following reagents were tested in the assay for interference in the assay:

| Reagent | | Will Interfere (Yes or No) |
|---|---|---|
| Buffers | HEPES (100 mM) | Yes (53%) |
| | MES (100 mM) | Yes (62%) |
| | Phosphate (100 mM) | No |
| | Tris (100 mM) | No |
| Detergents | Polysorbate 20 (1%) | Yes (50%) |
| | Triton X-100 (1%) | Yes (54%) |
| Chelators | EDTA (1 mM) | No |
| | EGTA (1 mM) | No |
| Protease Inhibitors/ Enzymes | Antipain (100 µg/ml) | No |
| | Chymostatin (10 µg/ml) | No |
| | Leupeptin (10 µg/ml) | No |
| | PMSF (200 µM) | No |
| | Trypsin (10 µg/ml) | No |
| Solvents | Dimethylsulfoxide (5%) | Yes (57%) |
| | Ethanol (5%) | No |
| | Methanol (5%) | No |
| Others | BSA (1%) | Yes (18%) |
| | Glycerol (5%) | No |
| | Sucrose (250 mM) | No |

## Troubleshooting

| Problem | Possible Causes | Recommended Solutions |
|---|---|---|
| Erratic values; dispersion of duplicates/triplicates | A. Poor pipetting/technique<br>B. Bubble in the well(s) | A. Be careful not to splash the contents of the wells<br>B. Carefully tap the side of the plate with your finger to remove bubbles |
| PLD was not detected in the sample | Sample was too dilute | Re-assay the sample using a lower dilution |
| Fluorescence of samples fell above the standard curve | The concentration of PLD in the sample is too high | Dilute the sample to fall within the range of the standard curve |
| The resorufin standard curve did not work | A. The standards were not diluted properly or the standard has deteriorated<br>B. The gain of the fluorometer is set incorrectly | A. Set up the standards according to Table 1, on page 12, and re-assay<br>B. Increase or decrease the gain as necessary |

## References


1. Krzystanek, M., Trzeciak, H.I., Krzystanek, E. , *et al.* Fluorometric assay of oleate-activated phospholipase D isoenzyme in membranes of rat nervous tissue and human platelets. *Acta. Biochim. Pol.* **57(3)**, 369-372 (2010).
2. Yang, Z., Asico, L.D., Yu, P., *et al.* D5 dopamine receptor regulation of phospholipase D. *Am. J. Physiol. Heart Circ. Physiol.* **288**, H55-H61 (2005).
3. Exton, J.H. Phospholipase D: Enzymology, mechanisms of regulation, and function. *Physiol. Rev.* **77**, 303-320 (1997).


## Related Products

Autotaxin Inhibitor Screening Assay Kit - Item No. 700580
Phosphatidylcholine Assay Kit - Item No. 10009926
$cPLA_2$ Assay Kit - Item No. 765021
$sPLA_2$ Assay Kit - Item No. 765001
$sPLA_2$ (Type V) Inhibitor Screening Assay Kit - Item No. 10004883
Lysophospholipase D Polyclonal Antibody - Item No. 10005375
NAPE-PLD Polyclonal Antibody (aa 6-20) - Item No. 10306
NAPE-PLD Polyclonal Antibody (aa 159-172) - Item No. 10305
Total Phosphatidic Acid Assay Kit - Item No. 700240

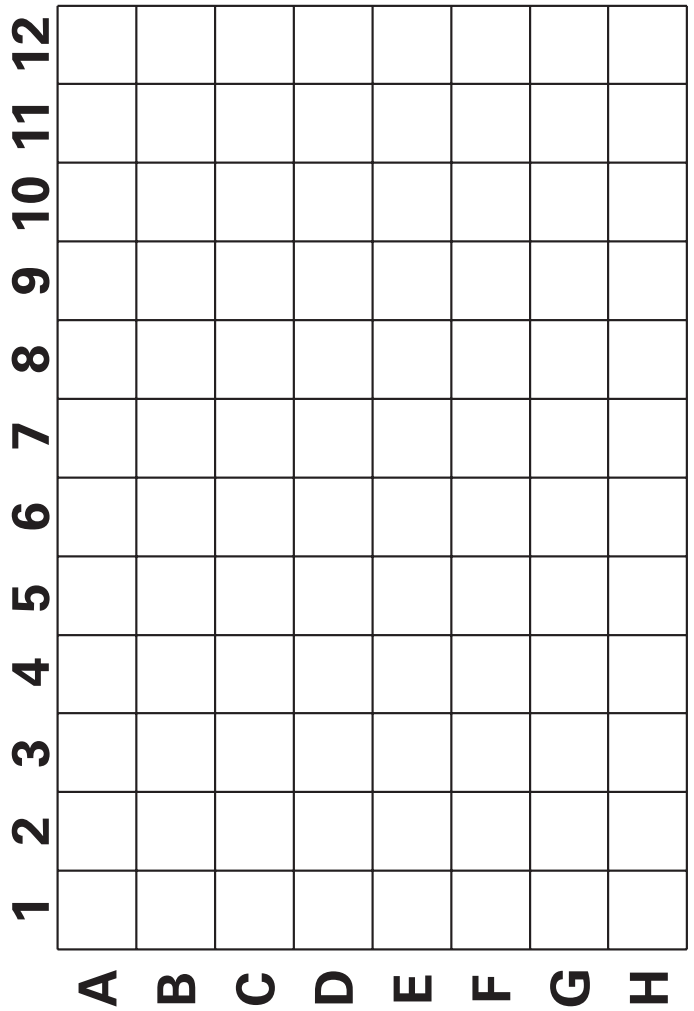
## Warranty and Limitation of Remedy

Cayman Chemical Company makes **no warranty or guarantee** of any kind, whether written or oral, expressed or implied, including without limitation, any warranty of fitness for a particular purpose, suitability and merchantability, which extends beyond the description of the chemicals hereof. Cayman **warrants only** to the original customer that the material will meet our specifications at the time of delivery. Cayman will carry out its delivery obligations with due care and skill. Thus, in no event will Cayman have **any obligation or liability**, whether in tort (including negligence) or in contract, for any direct, indirect, incidental or consequential damages, even if Cayman is informed about their possible existence. This limitation of liability does not apply in the case of intentional acts or negligence of Cayman, its directors or its employees.

Buyer's **exclusive remedy** and Cayman's sole liability hereunder shall be limited to a refund of the purchase price, or at Cayman's option, the replacement, at no cost to Buyer, of all material that does not meet our specifications.

Said refund or replacement is conditioned on Buyer giving written notice to Cayman within thirty (30) days after arrival of the material at its destination. Failure of Buyer to give said notice within thirty (30) days shall constitute a waiver by Buyer of all claims hereunder with respect to said material.

**For further details, please refer to our Warranty and Limitation of Remedy located on our website and in our catalog.**



## NOTES