Demethylase (LSD-type) Activity Assay Kit

Item No. 700400

Customer Service 800.364.9897 * **Technical Support** 888.526.5351
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TABLE OF CONTENTS

GENERAL INFORMATION

Materials Supplied

The kit will arrive as two packages at different shipping temperatures. The LSD Formaldehyde Standard has to be stored ≥4°C or it may polymerize. For best results, remove components and store as stated below.

Item Number	Item	Quantity/Size	Storage
700401	LSD Assay Buffer (10X)	1 vial/5 ml	-20°C
700402	LSD Positive Control	1 vial/100 µl	-80°C
700403	LSD Peptide Substrate	1 vial/1.2 ml	-20°C
700404	LSD Ammonium Acetate	1 vial/5 ml	-20°C
700405	LSD Formaldehyde Detector	1 vial/150 mg	-20°C
700406	LSD DMSO	1 vial/2 ml	Room temperature
700407	LSD Formaldehyde Standard	1 vial/100 µl	Room temperature
400091	Half Volume 96-Well 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) 971-3335. 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.

Formaldehyde is carcinogenic. It is toxic if inhaled, ingested, or if in contact with skin. In case of contact with skin or eyes, rinse immediately with plenty of water for 15 minutes. Keep away from combustible materials.

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

E-Mail: 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

This kit will perform as specified if stored as directed in the **Materials Supplied** section and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A fluorometer with the capacity to measure fluorescence using an excitation wavelength between 365-375 nm and an emission wavelength between 465-475 nm
2. Adjustable pipettes and a repeating pipettor
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable

INTRODUCTION

Background

Histones are evolutionarily conserved proteins that are the building blocks of the nucleoprotein chromatin structure that packages DNA within the eukaryotic nucleus. Chromatin contains individual nucleosomal core particles with eight core histone proteins, two copies each of histones H3, H4, H2B, and H2A, with 146 bp of DNA wrapped around the protein octamer.[1] The histone amino termini extend from the core, where they can be modified post-translationally by acetylation, phosphorylation, ubiquitination, and methylation, affecting their charge and function. These post-translational modifications affect key DNA regulatory processes such as DNA replication, DNA repair, and transcriptional activation and repression.[1] Dysregulation of histone acetylation and methylation can lead to the silencing of tumor suppressor genes and contributes to cancer progression. Inhibitors of enzymes that catalyze the addition and removal of these epigenetic marks thus have therapeutic potential for treating cancer and represent a very active research area in drug development.

Lysine-specific demethylases, LSD1 (KDM1, p110b, BHC110, NPAO) and the recently identified LSD2, belong to the family of flavin adenine dinucleotide (FAD)-dependent amine oxidases that include monamine oxidases (MAOs) and polyamine oxidase (PAO).[2,3] Both enzymes specifically demethylate mono- and dimethylated histone H3 at lysine 4 (H3-K4), resulting in transcriptional repression.[3,4] LSD1 is a component of several histone deacetylase co-repressor complexes, including histone deacetylases 1/2, CtBP, and the neuronal CoREST complexes.[5] LSD1 also controls the tumor suppressor activity of p53 by demethylating lysine 370, which prevents p53 interaction with its co-activator 53BP1 to induce apoptosis.[6-9] As opposed to LSD1, LSD2 does not form a biochemically stable complex with the C-terminal domain of the corepressor protein CoREST and contains a CW-type zinc finger motif with potential zinc-binding sites that are not present in LSD1.[3] While both enzymes may have similar properties, LSD2 is very likely part of a chromatin-remodeling complex that is distinct from LSD1.[3]

About This Assay

Cayman's Demethylase (LSD-type) Activity Assay provides a fluorescence-based method for assaying LSD1 or LSD2 demethylase activity from cell lysates or purified enzyme preparations. The assay is based on a multistep reaction in which LSD first produces formaldehyde during the demethylation of lysine 4 on a peptide corresponding to the first 21 amino acids of the N-terminal tail of histone H3. The detection reaction involves the cyclization between formaldehyde and acetoacetanilide in the presence of ammonia. The resulting fluorescent product is analyzed using an excitation wavelength between 365-375 nm and an emission wavelength between 465-475 nm (see Figure 1).[10]

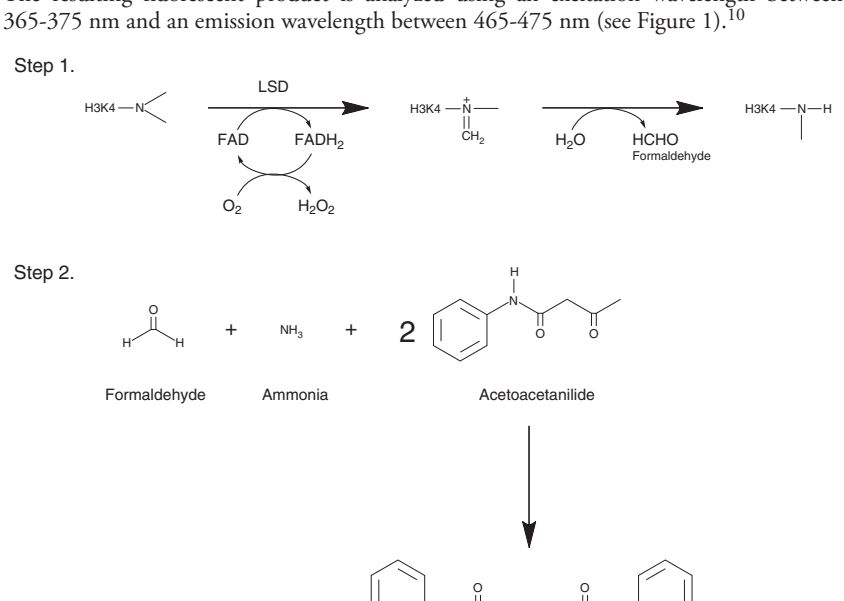

Figure 1. Assay scheme

PRE-ASSAY PREPARATION

Reagent Preparation

1. LSD Assay Buffer (10X) - (Item No. 700401)

The vial contains 5 ml of 500 mM HEPES, pH 7.5. Prior to use, dilute 3 ml of Assay Buffer concentrate with 27 ml of HPLC-grade water. This final Buffer (50 mM HEPES, pH 7.5) will be used in the assay. When stored at 4°C, this diluted buffer is stable for at least six months.

2. LSD Positive Control - (Item No. 700402)

The vial contains 100 µl of human recombinant LSD1. The enzyme is ready to use in the assay. Prior to use, thaw and store the enzyme on ice.

3. LSD Peptide Substrate - (Item No. 700403)

The vial contains 1.2 ml of 1 mM peptide corresponding to the first 21 amino acids of the N-terminal tail of histone H3, with a dimethylated lysine at residue 4 (ARTK(Me2)QTARKSTGGKAPRKQLA). The peptide is ready to use in the assay. The addition of 10 µl to the assay yields a final concentration of 100 µM peptide. If a lower concentration of peptide is desired, dilute with 1X Assay Buffer.

4. LSD Ammonium Acetate - (Item No. 700404)

The vial contains 5 ml of ammonium acetate. The reagent is ready to use in the assay.

5. LSD Formaldehyde Detector - (Item No. 700405)

The vial contains 150 mg of acetoacetanilide. Prior to use, weigh 125 mg into another vial, add 1.2 ml of DMSO, and vortex until dissolved. The reagent is now ready to use in the assay. Do not store on ice; it will freeze. Any unused reconstituted detector can be stored at -20°C for one month. Upon thawing, the detector may appear cloudy. Vortex at room temperature until clear. Additional warming in your hands may be needed to get the detector to go back into solution.

6. LSD DMSO - (Item No. 700406)

The vial contains 2 ml of dimethylsulfoxide (DMSO). It is ready to use to dissolve the detector.

7. LSD Formaldehyde Standard - (Item No. 700407)

The vial contains 100 µl of 5 M formaldehyde. It is ready to use to prepare the standard curve.

Sample Preparation

Cell Lysate

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, do not harvest using proteolytic enzymes; rather use a rubber policeman.
2. Sonicate cell pellet in 0.5-1 ml of cold buffer (*i.e.*, 50 mM HEPES, pH 7.5).
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.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. However, a formaldehyde standard curve and LSD Positive Control in duplicate has to be assayed with the samples. We suggest that each sample be assayed at least in duplicate and to have two wells designated as sample background wells to allow for the correction of non-formaldehyde-generated fluorescence. Record the contents of each well on the template sheet provided on page 23. A typical layout of samples to be measured in duplicate is given below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	S1	S1	S5	S5	S9	S9	S13	S13	S17	S17
B	B	B	B1	B1	B5	B5	B9	B9	B13	B13	B17	B17
C	C	C	S2	S2	S6	S6	S10	S10	S14	S14	S18	S18
D	D	D	B2	B2	B6	B6	B10	B10	B14	B14	B18	B18
E	E	E	S3	S3	S7	S7	S11	S11	S15	S15	S19	S19
F	F	F	B3	B3	B7	B7	B11	B11	B15	B15	B19	B11
G	G	G	S4	S4	S8	S8	S12	S12	S16	S16	S20	S20
H	+	+	B4	B4	B8	B8	B12	B12	B16	B16	B20	B20

A-G = Formaldehyde Standards
+ = LSD Positive Control
S1-S20 = Sample Wells
B1-B20 = Background 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 100 µl in all the wells.
- It is not necessary to use all the wells on the plate at one time.
- We recommend assaying samples in triplicate, but it is the user's discretion.
- The assay is performed at 37°C.
- Monitor the fluorescence with an excitation wavelength between 365-375 nm and emission wavelength between 465-475 nm.

Standard Preparation

Dilute 10 µl of the Formaldehyde Standard with 9.99 ml of HPLC-grade water to yield a concentration of 5 mM. Dilute 200 µl of the 5 mM formaldehyde with 1.8 ml of HPLC-grade water to yield the stock solution of 500 µM. The 500 µM standard will be used to prepare the remaining standards. Take seven clean glass test tubes and mark them A-G. Add the amount of Formaldehyde Standard (500 µM) and HPLC-grade water to each tube as described in Table 1. The diluted standards are stable for four hours at room temperature.

Tube	Formaldehyde 500 µM standard (µl)	HPLC-grade water (µl)	Final Concentration (µM)
A	0	1,000	0
B	10	990	5
C	20	980	10
D	40	960	20
E	100	900	50
F	150	850	75
G	200	800	100

Table 1. Formaldehyde standards to be assayed along with samples

Performing the Assay

1. **Standard Wells** - add 30 µl of diluted Assay Buffer and 10 µl of Standard (tubes A-G) per well in the designated wells on the plate (see **Sample Plate Format**, Figure 2, page 9).
2. **Positive Control Wells** - add 30 µl of diluted Assay Buffer and 10 µl of LSD Positive Control to three wells.
3. **Sample Wells** - add 30 µl of diluted Assay Buffer and 10 µl of sample to three wells.
4. **Sample Background Wells** - add 40 µl of diluted Assay Buffer and 10 µl of sample to three wells.
5. Initiate the reactions by adding 10 µl of LSD Peptide Substrate to only the positive control, standard, and sample wells (**Do Not** add the substrate to the sample background wells).
6. Cover the plate with the plate cover and incubate for 30 minutes at 37°C.
7. Remove the plate cover, add 40 µl of Ammonium Acetate and 10 µl of Detector to all of the wells being used (standard, positive control, sample, and background wells).
8. Cover the plate with the plate cover and incubate for 15 minutes at 25°C.
9. Remove the plate cover and read the fluorescence using an excitation wavelength between 365-375 nm and an emission wavelength between 465-475 nm. It may be necessary to adjust the gain setting on the instrument to allow for the measurement of all the samples.

Steps	Reagent	Std Wells	Positive Control	Sample	Sample Background
1. Add reagents	Assay Buffer	30 µl	30 µl	30 µl	40 µl
	Standard	10 µl	-	-	-
	LSD Positive Control	-	10 µl	-	-
	Sample	-	-	10 µl	10 µl
2. Initiate	LSD Peptide	10 µl	10 µl	10 µl	-
3. Incubate	Incubate 30 minutes at 37°C				
4. Add reagents	Ammonium Acetate	40 µl	40 µl	40 µl	40 µl
	Detector	10 µl	10 µl	10 µl	10 µl
5. Incubate	Incubate 15 minutes at 25°C				
6. Read	Read plate at an excitation wavelength between 365-375 nm and at an emission wavelength between 465-475 nm				

Table 2. Quick Protocol Guide

ANALYSIS

Calculations

1. Determine the average fluorescence of each positive control, sample, and sample background wells.
2. Subtract the fluorescence of the sample background wells from the fluorescence of the sample wells to yield the corrected sample fluorescence (CSF).
3. Determine the average fluorescence of the standards. Subtract the fluorescence value of the standard A from itself and all other standards. This is the corrected fluorescence (CF).
4. Plot the corrected fluorescence values (from step 3 above) of each standard as a function of the final concentration of formaldehyde from Table 1. See Figure 3, on page 15, for a typical standard curve. (Do not use the curve on page 15 for analysis of your samples.)
5. Calculate the demethylase activity using the following equation. One unit is defined as the amount of enzyme that will cause the formation of 1 nmol of formaldehyde per minute at 37°C.

Demethylase Activity (nmol/min/ml) =

$$\left[\frac{\text{CSF - (y-intercept)}}{\text{Slope (CF/µM) x 30 min.}}\right] \text{x Sample dilution}$$

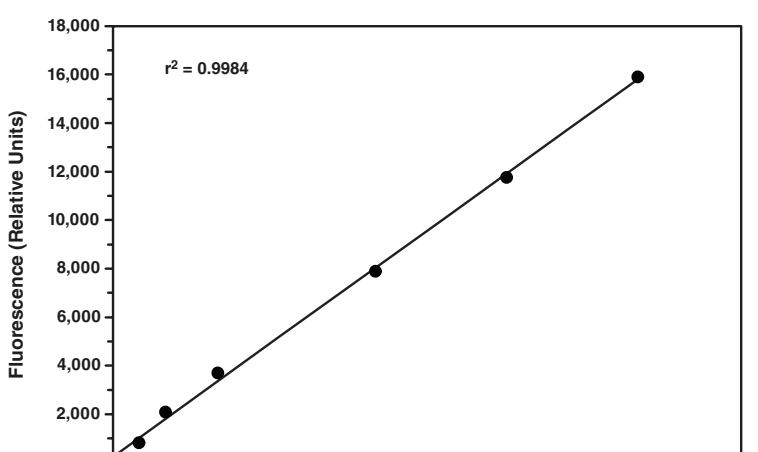

Figure 3. Typical formaldehyde standard curve

Performance Characteristics

Precision:

When a series of 16 LSD1 measurements were performed on the same day, the intra-assay coefficient of variation was 4.6%. When a series of 16 LSD1 measurements were performed on six different days under the same experimental conditions, the inter-assay coefficient of variation was 5.1%.

Assay Range:

Under the standardized conditions of the assay, the dynamic range of the kit is 0-100 µM of formaldehyde or 0-3.4 nmol/min/ml LSD activity.

Inhibition Validation:

Various concentrations of the LSD1 inhibitor, *trans*-2-phenylcyclopropylamine (2-PCPA (hydrochloride), Item No. 10010494) were assayed with the LSD Positive Control to validate that the assay can be used to establish inhibition profiles (see Figure 4, on page 17).[11,12]

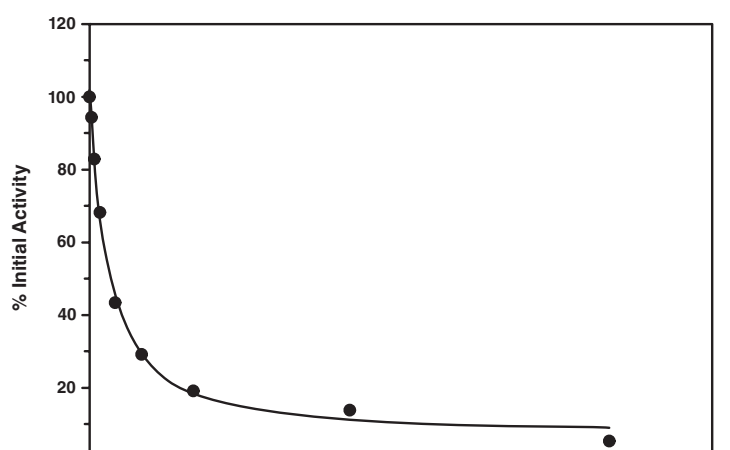

Figure 4. Inhibition of LSD1 by 2-PCPA (hydrochloride) (IC_{50} = 20 µM)

Interferences

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

Reagent		Will Interfere (Yes or No)
Buffers	Tris	No
	HEPES	No
	Phosphate	No
Detergents	SDS	Yes
	Tween 20	Yes
	Triton X-100	Yes
	CHAPS	Yes
Solvents	Acetone (≤1 mM)	No
	Ethanol (5 µl)	Yes
	Methanol (5 µl)	No
	Dimethylsulfoxide (5 µl)	No
Others	Glycerol (10%)	No
	NaCl (≤25 mM)	No
	$CaCl_2$ (≤100 µM)	No
	Iron Sulfate (≤10 µM)	No
	MgCl (≤1 mM)	No
	KCl (≤5 mM)	No
	EDTA (1 mM)	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 fluorescence was detected above background in the sample wells	Sample was too dilute	Re-assay the sample using a lower dilution
The fluorometer exhibited 'MAX' values for the wells	The GAIN setting is too high	Reduce the GAIN and re-read
Sample fluorescence was higher than the last standard	Sample is too concentrated	Dilute sample and re-assay

References

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2. Shi, Y., Lan, F., Matson, C., *et al.* Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* **119**, 941-953 (2004).
3. Karytinos, A., Forneris, F., Profumo, A., *et al.* A novel mammalian flavin-dependent histone demethylase. *J. Biol. Chem.* **284(26)**, 17775-17782 (2009).
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11. Schmidt, D.M.Z. and McCafferty, D.G. *trans*-2-Phenylcyclopropylamine is a mechanism-based inactivator of the histone demethylase LSD1. *Biochemistry* **46**, 4408-4416 (2007).
12. Gooden, D.M., Schmidt, D.M.Z., Pollock, J.A.,*et al.* Facile synthesis of substituted *trans*-2-arylcyclopropylamine inhibitors of the human histone demethylase LSD1 and monoamine oxidases A and B. *Bioorg. Medicinal Chem. Letters* **18**, 3047-3051 (2008).

Related Products

Demethylase (Jumonji-type) Activity Assay Kit - Item No. 700390
HAT Inhibitor Screening Assay Kit - Item No. 10006515
HDAC Fluorometric Activity Assay Kit - Item No. 10011563
HDAC1 Inhibitor Screening Assay Kit - Item No. 10011564
HDAC8 Inhibitor Screening Assay Kit - Item No. 700230
JMJD2A Inhibitor Screening Assay Kit - Item No. 700360
JMJD2D Inhibitor Screening Assay Kit - Item No. 700370
LSD1 Inhibitor Screening Assay Kit - Item No. 700120
Methyltransferase Colorimetric Assay Kit - Item No. 700140
Methyltransferase Fluorometric Assay Kit - Item No. 700150
PAD4 Inhibitor Screening Assay Kit - Item No. 700560
2-PCPA (hydrochloride) - Item No. 10010494
SET7/9 Methyltransferase Inhibitor Screening Assay Kit - Item No. 700270
SET8 Methyltransferase Inhibitor Screening Assay Kit - Item No. 700350
SIRT1 Direct Fluorescent Screening Assay Kit - Item No. 10010401
SIRT1 FRET-Based Screening Assay Kit - Item No. 10010991
SIRT2 Direct Fluorescence Screening Assay Kit - Item No. 700280
SIRT3 Direct Fluorescent Screening Assay Kit - Item No. 10011566
SIRT6 Direct Fluorescent Screening Assay Kit - Item No. 700290

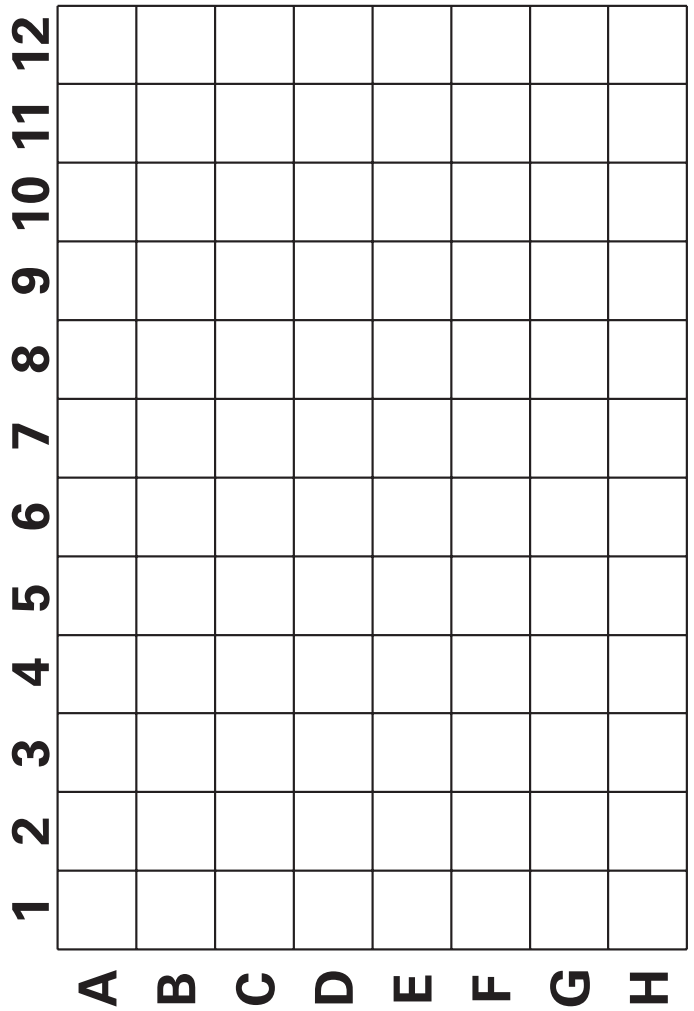
Warranty and Limitation of Remedy

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.



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