Demethylase (Jumonji-type) Activity Assay Kit

Item No. 700390

Customer Service 800.364.9897 * **Technical Support** 888.526.5351
www.caymanchem.com

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GENERAL INFORMATION

Materials Supplied

The kit will arrive as two packages at different shipping temperatures. The JMJ 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
700361	JMJ Assay Buffer	1 vial/10 ml	-20°C
700391	JMJ Positive Control	1 vial/50 µl	-80°C
700392	JMJ Control Peptide	1 vial/200 µl	-20°C
700364	JMJ Ammonium Acetate	1 vial/5 ml	-20°C
700365	JMJ Formaldehyde Detector	1 vial/150 mg	-20°C
700001	DMSO Assay Reagent	1 vial/3 ml	Room temperature
700367	JMJ Cofactor Mixture	2 vials/500 µg	-20°C
700393	JMJ 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

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

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. Appropriate **Jumonji substrate**
3. Adjustable pipettes and a repeating pipettor
4. 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 leads 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.

Histone demethylases catalyze the removal of methyl groups on lysine or arginine residues of histones. Two kinds of histone lysine demethylases have been identified, including lysine specific demethylase 1 and Jumonji C (JmjC) domain family proteins. Five JmjC domain subfamilies that mediate histone demethylation reactions have been identified, including the JHDM1, JHDM2, JMJD2 (JHDM3), JARID1, and UTX/UTY subfamilies.[2] JmjC enzymes are members of the cupin superfamily of 2-oxoglutarate (2OG)-dependent Fe (II) oxygenases and catalyze hydroxylation reactions.[3] In humans, six JMJD2 homologs have been identified, termed JMJD2A through JMJD2F.[4] Currently, researchers are at the early stages of understanding the chemistry of how histone demethylases catalyze specific reactions and exhibit substrate-selective activity. Convenient assay tools are essential in characterizing these enzymes and discovering how these enzymes impact chromosome formation and transcriptional regulation.

About This Assay

Cayman's Demethylase (Jumonji-type) Activity Assay provides a convenient fluorescence-based method for assaying demethylase activity from cell lysates or purified enzyme preparations. The assay is based on the multistep reaction in which the demethylase first produces formaldehyde during the demethylation of a methylated peptide substrate, with the concomitant oxidation/decarboxylation of 2-oxoglutarate. 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.[5] Figure 1 outlines the reaction with JMJD2D (JMJ Positive Control) and histone H3 trimethyl lys9 peptide (JMJ Control Peptide).

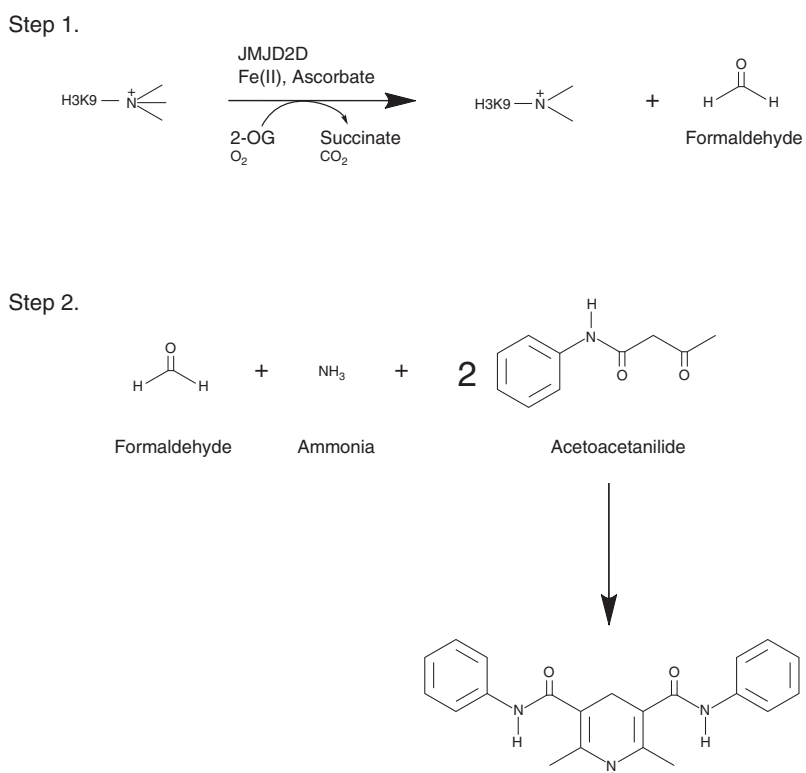


Figure 1. Example of JMJD2D and histone H3 trimethyl lys9 peptide

PRE-ASSAY PREPARATION

Reagent Preparation

1. JMJ Assay Buffer - (Item No. 700361)

The vial contains 10 ml of 20 mM Tris-HCl, pH 7.5, containing 20 mM sodium chloride. It is ready to use in the assay as supplied. Store unused buffer at -20°C.

2. JMJ Positive Control - (Item No. 700391)

The vial contains 50 µl of human recombinant JMJD2D. Thaw the enzyme on ice, add 150 µl of Assay Buffer to the vial, and vortex. The diluted enzyme is stable for four hours on ice.

3. JMJ Control Peptide - (Item No. 700392)

The vial contains 200 µl of 1 mM histone H3 trimethyl lys9 peptide in 20 mM Tris-HCl, pH 7.5. The peptide is ready to use in the assay. The addition of 10 µl to the assay yields a final concentration of 200 µM peptide.

4. JMJ Ammonium Acetate - (Item No. 700364)

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

5. JMJ Formaldehyde Detector - (Item No. 700365)

The vial contains 150 mg of acetoacetanilide. For use in the assay, 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.

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

The vial contains 3 ml of dimethylsulfoxide (DMSO). It is ready to use to dissolve the detector.

7. JMJ Cofactor Mixture - (Item No. 700367)

Each vial contains 500 µg of jumonji cofactors. Reconstitute the contents of the vial with 600 µl of Assay Buffer. This is enough reagent to assay 60 wells. Prepare the additional vial as needed. The reconstituted cofactors are stable for one hour at room temperature.

8. JMJ Formaldehyde Standard - (Item No. 700393)

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 10^8-10^{10}) 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. Pour off media and add 2 ml of PBS (pH 7.2-7.4) to wash cells.
3. Centrifuge at 1,000-2,000 x g for 10 minutes at 4°C.
4. Sonicate cell pellet in 0.5-1 ml of cold buffer (*i.e.*, PBS, pH 7.2-7.4, containing a pan protease inhibitor cocktail; *i.e.*, Roche Item No. 11836170001, Complete Mini, EDTA-free).
5. Centrifuge at 10,000 x g for 15 minutes at 4°C.
6. 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 JMJ 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
+ = JMJ 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 to do so.
- 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.
- **The customer supplies the appropriate peptide for their particular demethylase of interest.**

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 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 HPLC-grade water and 10 µl of Standard (tubes A-G) per well in the designated wells on the plate (see Figure 2, page 10).
2. **Positive Control Wells** - add 20 µl of Assay Buffer, 10 µl of cofactor mixture, and 10 µl of diluted JMJ Positive Control to three wells.
3. **Sample Wells** - add 20 µl of Assay Buffer, 10 µl of cofactor mixture, and 10 µl of sample to three wells.
4. **Sample Background Wells** - add 30 µl of Assay Buffer, 10 µl of cofactor mixture, and 10 µl of sample to three wells.
5. Initiate the reactions by adding 10 µl of JMJ Control Peptide to only the positive control wells and the appropriate jumonji peptide to the standard and sample wells (**Do Not** add the peptide 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 sample 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.

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.
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) =

$$\left[\frac{\text{CSF - (y-intercept)}}{\text{Slope from std curve (CF/µM)} \bullet \text{30 minutes}}\right] \text{x Sample dilution}$$

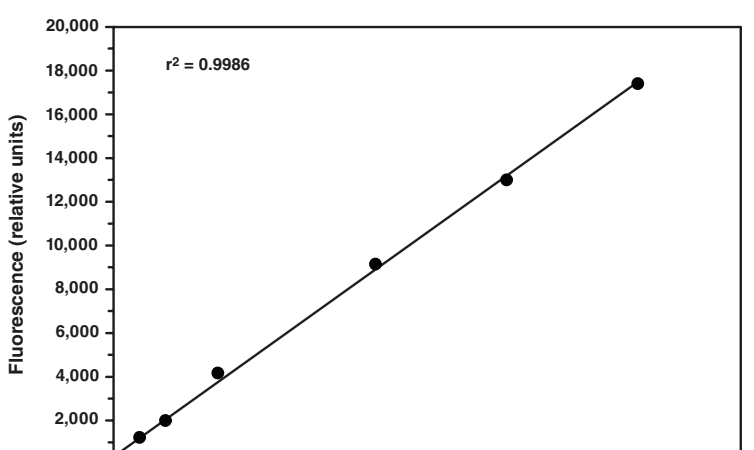


Figure 3. Typical formaldehyde standard curve

Performance Characteristics

Precision:

Under the standardized conditions of the assay, the dynamic range of the kit is 0-100 µM of formaldehyde or 0-3.5 nmol/min/ml demethylase activity.

Assay Range:

When a series of 16 JMJD2D measurements were performed on the same day, the intra-assay coefficient of variation was 4.7%. When a series of 16 JMJD2D measurements were performed on six different days under the same experimental conditions, the inter-assay coefficient of variation was 5.1%.

Inhibition Validation:

Various concentrations of N-Oxalylglycine (NOG), an inhibitor of 2-oxoglutarate-dependent enzymes, was assayed with the JMJ Positive Control to validate that the assay can be used to establish inhibition profiles (see Figure 4, on page 17).[6,7]

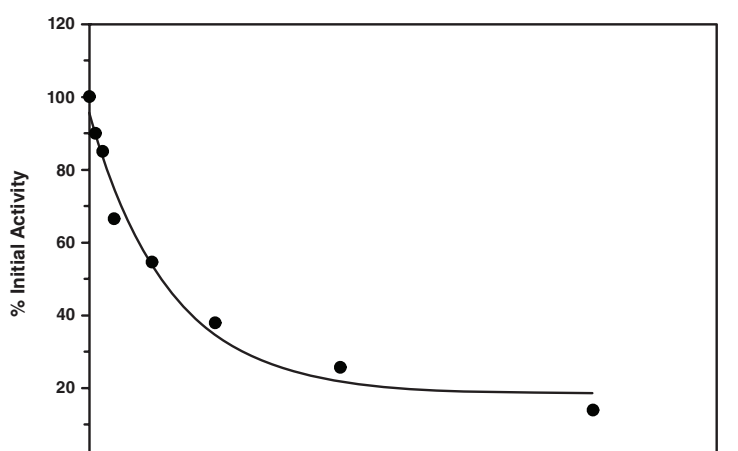


Figure 4. Inhibition of JMJD2D by NOG (IC_{50} = 200 µM)

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

1. Strahl, B.D. and Allis, D. The language of covalent histone modifications. *Nature* **403**, 41-45 (2000).
2. Tian, X. and Fang, J. Current perspectives on histone demethylases. *Acta Biochim. Biophys. Sin.* **39(2)**, 81-88 (2007).
3. Schneider, J. and Shilatifard, A. Histone demethylation by hydroxylation: Chemistry in action. *ACS Chem. Biol.* **1(2)**, 75-81 (2006).
4. Katoh, M. and Katoh, M. Identification and characterization of JMJD2 family genes in silico. *Int. J. Oncol.* **24**, 1623-1628 (2004).
5. Li, Q., Sritharathikhun, P., and Motomizu, S. Development of novel reagent for Hantzsch reaction for the determination of formaldehyde by spectrophotometry and fluorometry. *Anal. Sci.* **23**, 413-417 (2007).
6. Rose, N.R., Ng, S.S., Mecinovic, J., *et al.* Inhibitor scaffolds for 2-oxoglutarate-dependent histone lysine demethylases. *J. Med. Chem.* **51**, 7053-7056 (2008).
7. Rose, N.R., Woon, E.C.Y., Kingham, G.L., *et al.* Selective inhibitors of the JMJD2 histone demethylases: Combined nondenaturing mass spectrometric screening and crystallographic approaches. *J. Med. Chem.* **53**, 1810-1818 (2010).

Related Products

Demethylase (LSD-type) Activity Assay Kit - Item No. 700400
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
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 Fluorescent Screening Assay Kit - Item No. 700280
SIRT3 Direct Fluorescent Screening Assay Kit - Item No. 10011566
SIRT6 Direct Fluorescent Screening Assay Kit - Item No. 700290

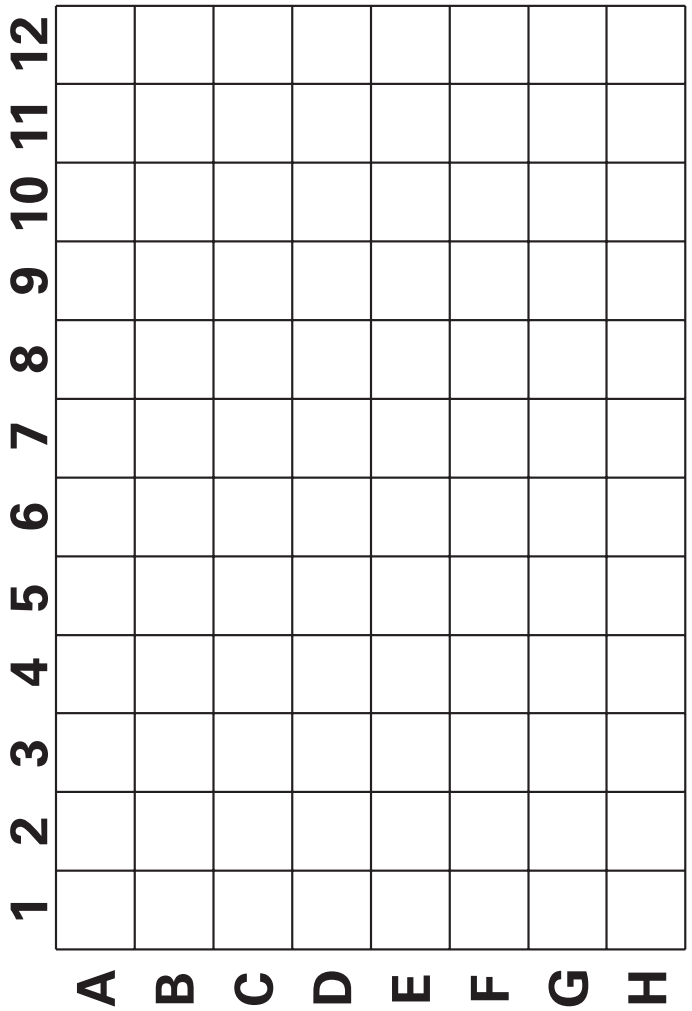
Warranty and Limitation of Remedy

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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