# TiterZyme ®EIA

# human Cytochrome c

# Enzyme Immunometric Assay Kit

## Catalog No. 900-141

## 96 Well Kit

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## Description

Assay Designs' human Cytochrome c TiterZyme® Enzyme Immunometric Assay (EIA) kit is a complete kit for the quantitative determination of Cytochrome c in cell lysates. Please read the complete kit insert before performing this assay. The kit uses a monoclonal antibody to Cytochrome c immobilized on a microtiter plate to bind the Cytochrome c in the standards or sample. A native human Cytochrome c Standard is provided in the kit. After a short incubation, the excess sample or standard is washed out and a biotinylated monoclonal antibody to Cytochrome c is added. This antibody binds to the Cytochrome c captured on the plate. After a short incubation, the excess antibody is washed out and streptavidin conjugated to Alkaline Phosphatase is added, which binds to the biotinylated monoclonal Cytochrome c antibody. Excess conjugate is washed out and substrate is added. After a short incubation, the enzyme reaction is stopped and the color generated is read at 405□nm. The measured optical density is directly proportional to the concentration of Cytochrome c in either standards or samples. For further explanation of the principles and practices of immunoassays please see the excellent books by Chard[1] or Tijssen[2].

## Introduction

Cytochrome c, ~13□kDa and 105 amino acids long, is a nuclear-encoded component of the mitochondrial respiratory chain that is imported as an apoenzyme into mitochondria, where it is converted to the mature form by the addition of a heme group. It catalyzes electron transfer between complexes III and IV of the respiratory chain, moving within the planar surface of the inner mitochondrial membrane[3]. In addition to its role in oxidative phosphorylation, the release of Cytochrome c from the mitochondrial intermembrane space to the cytosol results in nuclear apoptosis[4]. Binding of APAF1 to Cytochrome c allows APAF1 to form a ternary complex with and activate the initiator Procaspase-9 in the presence of dATP. Active Caspase-9 then triggers downstream effector caspases, beginning the death cascade apoptosis[5]. Recent studies have demonstrated that Cytochrome c directly microinjected in the cytoplasms of a variety of cell types is capable of initiating apoptosis on its own, while APAF-1 is needed as a cofactor in cell free extracts thus emphasizing Cytochrome c's crucial role in apoptosis[6]. In contrast, examples of apoptosis without Cytochrome c release indicate that this event may not be necessary in all apoptotic pathways[7].

## Precautions

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1. Stop Solution is a solution of trisodium phosphate. This solution is caustic; care should be taken in use.
2. The activity of the alkaline phosphatase conjugate is dependent on the presence of $Mg^{2+}$ and $Zn^{2+}$ ions. The activity of the conjugate is affected by concentrations of chelators (>10 mM) such as EDTA and EGTA.
3. We test this kit's performance with a variety of buffers; however high levels of interfering substances may cause variation in assay results. **For best results, samples should be prepared in the buffers recommended and included in this kit.**
4. The human Cytochrome c Standard provided, Catalog No. 80-1323, should be handled with care because of the known and unknown effects of Cytochrome c.
5. The Digitonin Cell Permeabilization Buffer should be handled with care; gloves should be worn as protection.

   **CAUTION: The human Cytochrome c standard is derived from human placenta. Treat as a biohazard.**

## Materials Supplied

1. **Cytochrome c Microtiter Plate, One Plate of 96 Wells, Catalog No. 80-1336**
   A plate using break-apart strips coated with a mouse monoclonal antibody specific to Cytochrome c.
2. **Cytochrome c EIA Antibody, 11 mL, Catalog No. 80-1338**
   A yellow solution of biotinylated monoclonal antibody to Cytochrome c.
3. **Assay Buffer 13, 100 mL, Catalog No. 80-1509**
   Tris buffered saline containing proteins, detergents and protease inhibitor.
4. **Cytochrome c EIA Conjugate, 11 mL, Catalog No. 80-1337**
   A blue solution of streptavidin conjugated to Alkaline Phosphatase.
5. **Wash Buffer Concentrate, 100 mL, Catalog No. 80-1287**
   Tris buffered saline containing detergents.
6. **human Cytochrome c Standard, 2 vials, Catalog No. 80-1323**
   Two vials each containing 450 pg of native human Cytochrome c.
7. **pNpp Substrate, 20 mL, Catalog No. 80-0075**
   A solution of p-nitrophenyl phosphate in buffer. Ready to use.
8. **Stop Solution, 5 mL, Catalog No. 80-0247**
   A solution of trisodium phosphate in water. Keep tightly capped. Caution: **Caustic.**
9. **RIPA Cell Lysis Buffer 2, 100 mL, Catalog No. 80-1284**
   50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1□mM EDTA, 1□mM EGTA, 1% Triton X-100, 1% Sodium Deoxycholate and 0.1% SDS**.**
10. **Digitonin Cell Permeabilization Buffer, 100 mL, Catalog No. 80-1187**
    250 mM Sucrose, 137 mM NaCl, 70 mM KCl, 4.3 mM $Na_2HPO_4$, 1.4 mM $K_2HPO_4$, 0.2□mg⁄mL Digitonin and 0.1% Hydorol M.**Wear gloves during use.**
11. **Cytochrome c Assay Layout Sheet, 1 each, Catalog No. 30-0217**
12. **Plate Sealer, 3 each, Catalog No. 30-0012**

## Storage

All components of this kit, **except the standard**, are stable at 4□°C until the kit's expiration date. **The Standard <u>must</u> be stored at or below -20□°C.**

## Materials Needed but Not Supplied

1. Deionized or distilled water.
2. Precision pipets for volumes between 100 µL and 1,000 µL.
3. Repeater pipet for dispensing 100 µL.
4. Disposable beakers for diluting buffer concentrates.
5. Graduated cylinders.
6. A microplate shaker.
7. Adsorbent paper for blotting.
8. Microplate reader capable of reading at 405 nm, preferably with correction between 570 nm and 590 nm.
9. Graph paper for plotting the standard curve.

## Sample Handling

Assay Designs' TiterZyme® EIA is compatible with Cytochrome c samples from cell lysates. Poor recoveries are obtained when serum is used as a sample. In addition, some tissue culture media contain L-glutamine or antibiotics as well as serum, and trace amounts of these materials will interfere with the quantitation of Cytochrome c in this assay. If cell samples are grown in media containing these materials, care should be taken to sufficiently process the samples to remove **all** traces of L-glutamine, antibiotics and serum.

### Cell Lysates

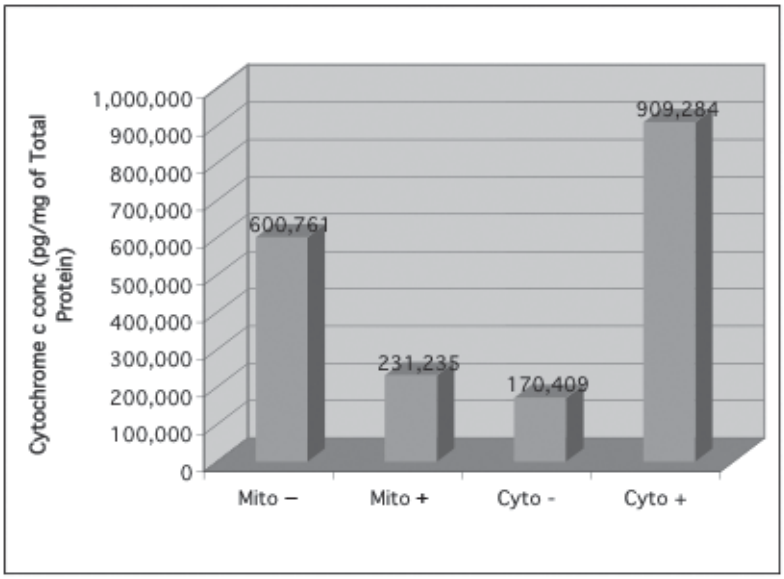
Cytochrome c is found in the mitochondria and released into the cytoplasm shortly after induction by apoptotic stimuli. Cytoplasmic Cytochrome c levels may change after apoptosis is induced. There may be two fractions of interest — the cytoplasmic and mitochondrial fractions. Cell lysate samples diluted sufficiently into Assay Buffer 13 can be read directly from a standard curve. Please refer to the Sample Recovery recommendations on page 11 for details of suggested dilutions. It is recommended that the cytosolic fraction be isolated with the provided Digitonin Cell Permeabilization Buffer and that the remaining mitochondrial fraction be lysed with the provided RIPA Cell Lysis Buffer 2.

There is no matrix interference with the RIPA Cell Lysis and Digitonin Cell Permeabilization buffers. However, due to the high levels of Cytochrome c found in the cell, a dilution may be required to get the levels to fall within the standard range. A 1:300 dilution of the cytosolic fraction isolated with Digitonin Cell Permeabilization yielded ≤ 23,333 cells per mL with a calculated recovery of 112.7%. A 1:2,000 dilution of the subcellular pellet lysed in RIPA Cell Lysis Buffer yielded ≤3,500 cells per mL with a calculated recovery of 102.2%. Note that the above dilutions are based on the lysis of 7 Million HeLa cells per mL.

Do not use buffers or components from other kits to prepare samples. If the end user chooses to use a buffer other than those provided, or a greater number of cells, it is up to the end user to determine the appropriate dilution of cells and assay validation. **Only standard curves generated in Assay Buffer 13 should be used to calculate the concentration of Cytochrome c. Samples must be stored frozen at or below -70°C to avoid loss of bioactive Cytochrome c . Excessive freeze/thaw cycles should be avoided. Prior to running the assay, frozen samples should be brought to 4°C slowly and gently mixed.**

## HeLa Cell Treatment with Actinomycin D

This experiment was adapted from a protocol outlined in reference #7. Seven million HeLa cells per mL were used in this experiment. They were treated with 25µg/mL Actinomycin D or with DMSO (for a negative control) for 24 hours at 37°C. Cells were harvested with trypsin and centrifuged briefly at 800 x g and the supernatant was discarded. The cell pellet was resuspended and washed with PBS. Cells were pelleted at 1,000 x g for 5 minutes and the supernatant was discarded. The cell pellet was resuspended with Digitonin Cell Permeabilization Buffer, vortexed and incubated on ice for 5 minutes. Cells were then centrifuged at 1000 x g for 5 minutes at 4°C. **The supernatants were saved, as they contained the cytosolic fraction of Cytochrome c.** The remaining pellet was then resuspended with RIPA Cell Lysis Buffer 2, vortexed and incubated on ice for 5 minutes. The lysate was vortexed and centrifuged at 10,000 x g for 10 minutes at 4°C. Fractions were run in the assay and the resulting picogram determinations were divided by the protein concentration. The resulting values are expressed as pg/mg of total protein from each fraction.




Mito + is the subcellular fraction containing the Mitochondrial fraction, treated with 25 µg/mL Actinomycin D
Mito - is the subcellular fraction containing the Mitochondrial fraction, treated with DMSO carrier
Cyto + is the Cytosolic fraction, treated with 25 µg/mL Actinomycin D
Cyto - is the Cytosolic fraction, treated with DMSO carrier

## Procedural Notes

1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. Standards must be made up in plastic tubes.
4. Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.
5. Pipet standards and samples to the bottom of the wells.
6. Add the reagents to the side of the well to avoid contamination**.**
7. This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4°C in the sealed bag provided. The wells should be used in the frame provided.
8. **Prior to addition of antibody, conjugate and substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results.**

## Reagent Preparation

1. **Wash Buffer**
   Prepare the Wash Buffer by diluting 50□mL of the supplied concentrate with 950□mL of deionized water. This can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier**.**

2. **human Cytochrome c Standards**
   Allow the lyophilized human Cytochrome c Standard to warm to room temperature. Add 500 µL of Assay Buffer 13 to the lyophilized Cytochrome c vial and vortex. Wait 5 minutes and vortex again prior to use. Label the vial standard #1. Label five 12□x□75□mm polypropylene tubes #2 through #6. Pipet 250□µL of Assay Buffer 13 into tubes #2 through #6. Add 250□µL of reconstituted standard #1 to tube #2 and vortex. Add 250□µL of tube #2 to tube #3 and vortex thoroughly. Continue this for tubes #4 through #6**.**

   **The concentrations of Cytochrome c in tubes #1 through #6 will be 900, 450, 225, 112.5, 56.25 and 28.13 pg/mL respectively. See Cytochrome c Assay Layout Sheet for dilution details.**

   **Reconstituted and diluted standards should be used within 60 minutes of preparation.**

## Assay Procedure

**Bring all reagents to room temperature for at least 30 minutes prior to opening.**

**All standards, controls and samples should be run in duplicate.**

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the pouch and seal the ziploc. Store unused wells at 4□C.
2. Pipet 100□L of Assay Buffer 13 into the S0 (0 pg/mL standard) wells.
3. Pipet 100□L of Standards #1 through #6 into the appropriate wells.
4. Pipet 100□L of the Samples into the appropriate wells.
5. Tap the plate gently to mix the contents.
6. Seal the plate and incubate at room temperature on a plate shaker for 1 hour at ~500 rpm.
7. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 3 more times for a total of 4 washes. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
8. Pipet 100 µL of yellow Antibody into each well, except the Blank.
9. Seal the plate and incubate at room temperature on a plate shaker for 1 hour at ~500 rpm.
10. Empty the contents of the wells and wash by adding 400□L of wash solution to every well. Repeat the wash 3 more times for a total of 4 washes. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
11. Add 100 µL of blue Conjugate to each well, except the Blank.
12. Seal the plate and incubate at room temperature on a plate shaker for 30 minutes at ~500 rpm.
13. Empty the contents of the wells and wash by adding 400□L of wash solution to every well. Repeat the wash 3 more times for a total of 4 washes. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
14. Pipet 100□L of Substrate Solution into each well.
15. Incubate for 45□minutes at room temperature on a plate shaker at ~500 rpm.
16. Pipet 25□L Stop Solution to each well.
17. Blank the plate reader against the Blank wells, read the optical density at 405 nm, preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the Blank from all the readings.

## Calculation of Results

Several options are available for the calculation of the concentration of Cytochrome c in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. If data reduction software is not readily available, the concentration of Cytochrome c can be calculated as follows:

1. Calculate the average net Optical Density (OD) bound for each standard and sample by subtracting the average Blank OD from the average OD for each standard and sample.

   Average Net OD = Average OD - Average Blank OD

2. Using linear graph paper, plot the Average Net OD for each standard versus Cytochrome c concentration in each standard. Approximate a straight line through the points. The concentration of Cytochrome c in the unknowns can be determined by interpolation.

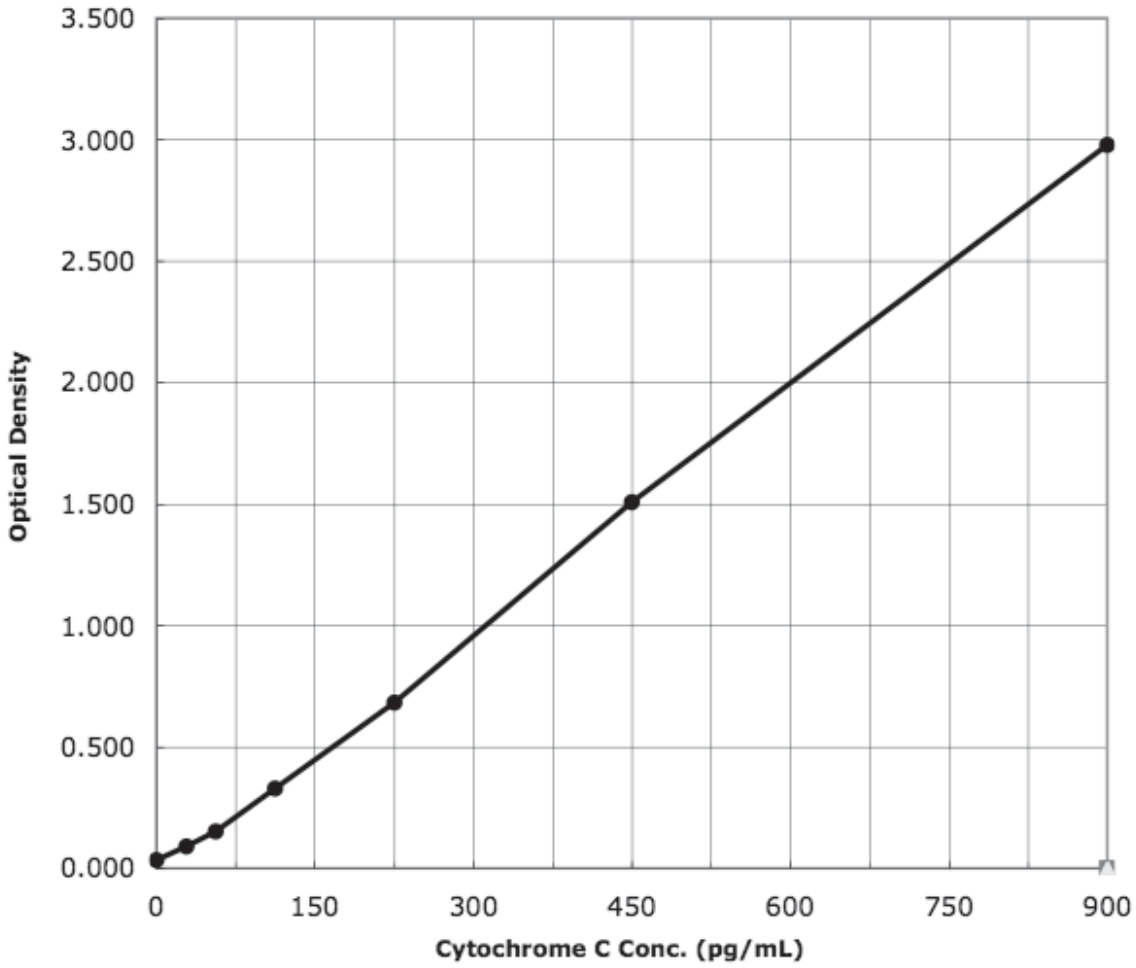
## Typical Results

The results shown below are for illustration only and **should not** be used to calculate results from another assay.

| Sample | Average OD | Net OD | Cytochrome c (pg/mL) |
|---|---|---|---|
| Blank | (0.065) | | |
| S0 | 0.098 | 0.033 | **0** |
| S1 | 3.043 | 2.978 | **900** |
| S2 | 1.572 | 1.507 | **450** |
| S3 | 0.748 | 0.683 | **225** |
| S4 | 0.393 | 0.328 | **112.5** |
| S5 | 0.216 | 0.151 | **56.25** |
| S6 | 0.156 | 0.091 | **28.13** |
| Unknown 1 | 2.189 | 2.124 | **629** |
| Unknown 2 | 0.367 | 0.302 | **108** |

## Typical Standard Curve

A typical standard curve is shown below. This curve must not be used to calculate Cytochrome c concentrations; each user must run a standard curve for each assay.



## Performance Characteristics

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols[9].

## Sensitivity

Sensitivity was calculated by determining the average optical density bound for twenty (20) wells run with 0 pg/mL Standard, and comparing to the average optical density for twenty (20) wells run with Standard #6. The detection limit was determined as the concentration of Cytochrome c measured at two (2) standard deviations from the 0 pg/mL Standard along the standard curve.

| | |
|---|---|
| Mean OD for S0 = | 0.013 ± 0.003 (20.9%) |
| Mean OD for Standard #6 = | 0.041 ± 0.005 (11.1%) |
| Delta Optical Density (28.13 - 0 pg/mL) = | 0.028 |
| 2 SD's of 0 pg/mL Standard = 2 x 0.003 = | 0.006 |
| Sensitivity = $\frac{0.006}{0.028}$ x 28.13 pg/mL = | **6.03 pg/mL** |

## Linearity

A sample containing 782.6 pg/mL Cytochrome c was serially diluted 5 times 1:2 in the Assay Buffer 13 supplied in the kit and measured in the assay. The data was plotted graphically as actual Cytochrome c concentration versus measured Cytochrome c concentration.

The line obtained had a slope of 0.9555 with a correlation coefficient of 0.9995.

## Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of Cytochrome c and running these samples multiple times (n=24) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of Cytochrome c in multiple assays over several days (n=20).

The precision numbers listed below represent the percent coefficient of variation for the concentrations of Cytochrome c determined in these assays as calculated by a 4 parameter logistic curve fitting program.

| | Cytochrome c (pg/mL) | Intra-assay % CV | Inter-assay % CV |
|---|---|---|---|
| Low | 82 | 3.6 | |
| Medium | 257 | 4.2 | |
| High | 466 | 2.7 | |
| | | | |
| Low | 100 | | 10.2 |
| Medium | 251 | | 4.9 |
| High | 486 | | 6.5 |

## Cross Reactivities

The cross reactivities for a number of related compounds were determined by dissolving the cross reactants in the kit assay buffer at a concentration of 10,000 pg/mL. These samples were then measured in the Cytochrome c assay.

| Compound | Cross Reactivity |
|---|---|
| human Cytochrome c | 100% |
| rat Cytochrome c | 6.41% |
| equine Cytochrome c | 0.92% |
| canine Cytochrome c | 0.62% |
| bovine Cytochrome c | 0.61% |
| BAX | < 0.3% |
| Bcl-2 | < 0.3% |
| Bcl-XL | < 0.3% |
| Caspase-9 | < 0.3% |
| cIAP-1 | < 0.3% |
| Survivin | < 0.3% |

## Sample Recoveries

Please refer to pages 4 and 5 for Sample Handling recommendations and Standard preparation.

Cytochrome c concentrations were measured in RIPA Cell Lysis Buffer, Digitonin Cell Permeabilization Buffer and their respective HeLa lysate fractions. For RIPA Cell Lysis Buffer, Digitonin Permeabilization Buffer and their respective HeLa lysate fractions, Cytochrome c was spiked into the undiluted sample which was then diluted with the Assay Buffer 13 and assayed in the kit. The following results were obtained:

| Sample | % Recovery | Recommended Dilution |
|---|---|---|
| RIPA Cell Lysis Buffer 2 | 102.6% | none required |
| Subcellular pellet lysed in RIPA | 102.2% | 1:2,000* |
| Digitonin Cell Permeabilization Buffer | 113.8% | none required |
| Cystolic fraction lysed in Digitonin | 112.7% | 1:300* |

*NOTE: The recommended dilution for the cell lysate fractions is high due to the high concentration of cells (7 million HeLa cells per mL) used. These dilutions were necessary to get the level of Cytochrome c found in the fractions to fall within the standard range.

**WARNING: If the end user chooses to not use the provided buffers, it is up to the end user to determine the appropriate dilution of samples and assay validation for their chosen buffer.**

## References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# NOTES

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## LIMITED WARRANTY

Assay Designs, Inc. warrants that at the time of shipment this product is free from defects in materials and workmanship. This warranty is in lieu of any other warranty expressed or implied, including but not limited to, any implied warranty of merchantability or fitness for a particular purpose.

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**Material Safety Data Sheet (MSDS) available on our website or by fax.**


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