Sigma-Aldrich®
Lab & Production Materials

ProClin™ 300 Preservative

An In-depth Technical Analysis

ProClin™ 300 preservative is a highly effective biocide for the control of microorganisms in reagents and products intended for *in vitro* diagnostic use. Due to its broad-spectrum activity, low toxicity at recommended use levels, excellent compatibility, and stability, ProClin™ 300 biocide is the ideal choice as an effective preservative in diagnostic reagents. At low concentrations, it eradicates bacteria, fungi, and yeasts in reagents for prolonged periods, thereby preserving a product's shelf life. This water-soluble preservative permits easy incorporation into reagents. Further, it does not affect the functionality of most enzyme- or antibody-linked reactions and will not interfere with assay indicators.

Features and Benefits

- Broad-spectrum antimicrobial activity
- Compatible with key enzymes
- Does not inhibit antibody binding
- Excellent stability
- Effective at pH ≤ 3.5
- No interfering inert molecules
- No color imparted to reagents
- No health hazards at recommended use levels
- Safe disposal at recommended use levels

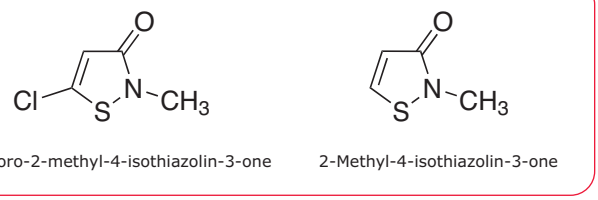
Quick Reference	Page No.

Chemical Identification

Structural Formulas

The active ingredients of ProClin™ 300 preservative are two isothiazolones identified by the IUPAC system of nomenclature as:

5-Chloro-2-methyl-4-isothiazolin-3-one (CMIT) and 2-Methyl-4-isothiazolin-3-one (MIT) see **Figure A**



5-Chloro-2-methyl-4-isothiazolin-3-one 2-Methyl-4-isothiazolin-3-one

Figure A. Active ingredients of ProClin™ 300 Preservative

Chemical Properties

ProClin™ 300 preservative is a precise formulation of active ingredients and an organic stabilizer in a modified glycol (**Table 1**). Typical metals analysis reveals there are no added metals in the formulation (**Table 2**).

Table 1. Properties

Active Ingredients	
5-Chloro-2-methyl-4-isothiazolin-3-one	2.30%
2-Methyl-4-isothiazolin-3-one	0.70%
Inerts	
Modified glycol	93-95%
Alkyl carboxylate	2-3%
Property	**Description**
Appearance	Clear Liquid
Color	Colorless to Pale Yellow
Odor	Mild sweet
Density (lbs/gal)	8.59
Specific gravity (22 °C)	1.03
pH 10% (aqueous solution)	4.1
Viscosity 25 °C	58cPs
Flash Point Seta Closed Cup	224 °F 118 °C
Absorbance Maximum	275nm
Fluorescence	None
Evaporation Rate Bu acetate = 1	<1

Table 2. Typical Metals Analysis

Metal	Volume (mg/L)
Arsenic	<1.0
Selenium	<1.0
Barium	<1.0
Chromium	<1.0
Lead	<1.0
Mercury	<0.01
Antimony	<1.0
Copper	<1.0
Calcium	1.7
Iron	1.0
Magnesium	<1.0
Manganese	<1.0
Nickel	<1.0
Tin	<2.0
Nitrate	<5.0
Nitrite	<5.0
Insoluble Residue	ND (<5.0)

ND = Not Determined (does not constitute specifications)

Solubility

ProClin™ 300 preservative is 100% soluble and completely miscible in water. It is also soluble in a wide range of oxygenated solvents.

Mechanisms of Action

ProClin™ 300 preservative is immediately bacteriostatic upon contact with a microbe (**Figure B**). This is the result of the active ingredients' ability to quickly penetrate cell membranes and inhibit specific enzymes in the cell. ProClin™ 300 preservative attacks the Krebs cycle at four sites: the enzymes pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase, and NADH dehydrogenase (**Figure C**). With the Krebs cycle debilitated, cells rapidly lose the ability to produce energy and subsequently die. ProClin™ 300 preservative is effective against a broad spectrum of microbes because all bacteria and fungi possess at least part of the Krebs cycle. The ability of ProClin™ 300 preservative to act on specific enzymes is reflected in the low levels required to control growth. ProClin™ 300 preservative also targets multiple specific enzymes, reducing the microbes' ability to mutate one target site to achieve any level of resistance. The rapid disruption of cellular metabolism as a result of specific enzyme inhibition severely impairs the ability of the cell to repair damage inflicted upon its components. The accumulation of damage beyond the capacity of the cell for repair results in cell death. Low concentrations of ProClin™ 300 biocide, requires several hours to kill the cell, while higher concentrations exhibit rapid microbiocidal effects (**Figure D**). This process of damage is rate-driven; i.e. higher concentrations of ProClin™ 300 preservative can inflict damage at a greater rate as well as overwhelm the cell's repair functions faster than lower concentrations.

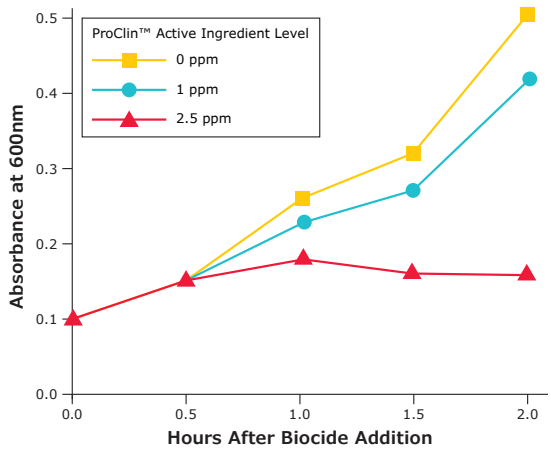


Figure B. Rapid Inhibition of Growth

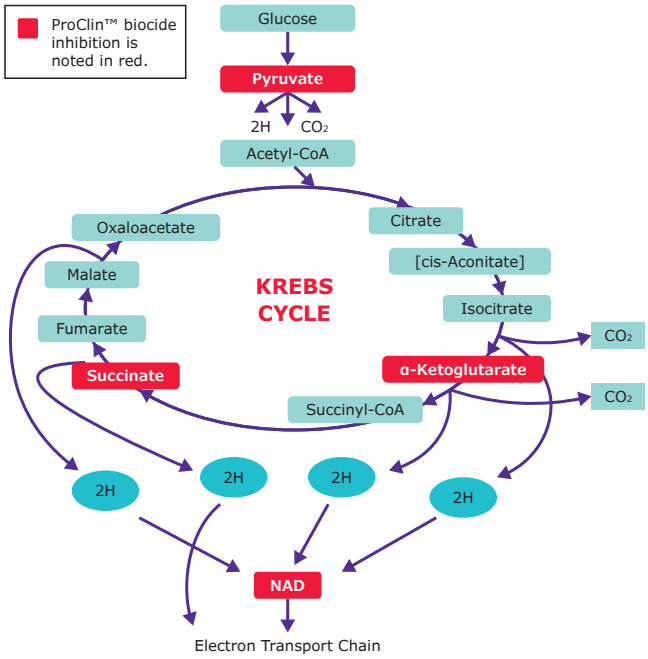


Figure C. ProClin™ biocide inhibits the Krebs Cycle at four key sites.

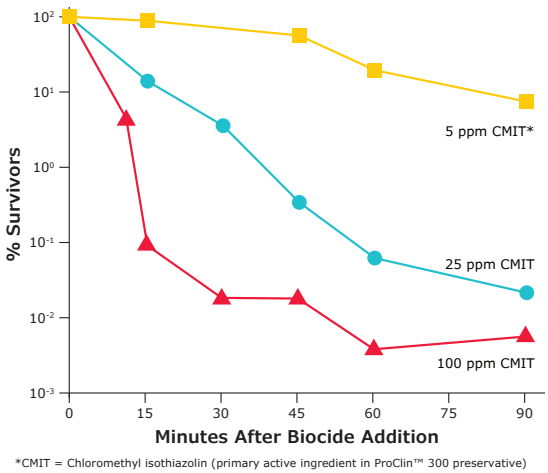


Figure D. Cidal Activity of ProClin™ Preservatives (Cell Death)

Stability

ProClin™ 300 preservative is formulated for a long product shelf life. As determined by our accelerated aging stability study in **Table 3**, after three years of storage at 25 °C, there is no detectable loss of active ingredients. Prolonged storage at high temperatures is not recommended. The level of active ingredients in ProClin™ 300 preservative, as supplied and in formulated products, can be determined by HPLC analysis.

Stability in Reagents

The active ingredients have an established history of successful use as preservatives. However, there are circumstances under which biocide stability should be confirmed prior to use:

Temperature: A rise in temperature may accelerate the rate of degradation of chemicals. Temperatures in excess of 55 °C should be avoided during manufacturing once the biocide has been incorporated into the reagent.

Amines: The presence of amines, particularly secondary amines, has a deleterious effect on the stability of ProClin™ 300 preservative. This can be avoided by reducing the pH of a reagent containing amines below 7, which converts amines to their acid salts.

Reducing Agents: Some reducing agents are detrimental to isothiazolone stability. We have found the use-levels of ProClin™ 300 preservative are stable in the presence of up to ~50 ppm bisulfite (expressed as SO_2).

pH: ProClin™ 300 preservative is stable and effective over a wide pH range. At pH levels below 9, ProClin™ 300 preservative is generally stable over the lifetime of the reagent. The stability of the preservative is reduced when pH exceeds 9.

Serum: The presence of strong nucleophiles such as glutathione and cysteine in blood and serum can have a deleterious effect, but this reaction is dependent on concentration, pH, and other components of the reagent. We recommend that the manufacturer test the performance of ProClin™ 300 preservative in a specific product before its use.

Table 3. Stability of ProClin™ 300 Preservative (as formulated)

Weeks stored at 55°C	Aging Equivalence (when stored at recommended temperature)	Initial % Total Active Ingredient	Final % Total Active Ingredient	% Active Ingredient Remaining
4	1 year	3.3	3.25	>99
8	2 years	3.3	3.25	>99
12	3 years	3.3	3.28	>99
ProClin™ 300 Spec	***3 years***	***3.0 - 3.6***	***2.7 - 3.8***	***Target >95%***

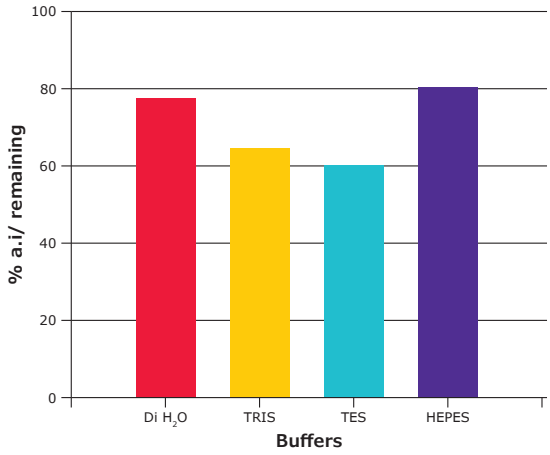


Figure E1. Stability of ProClin™ 300 preservative in the presence of 50 mM physiological buffers after three weeks

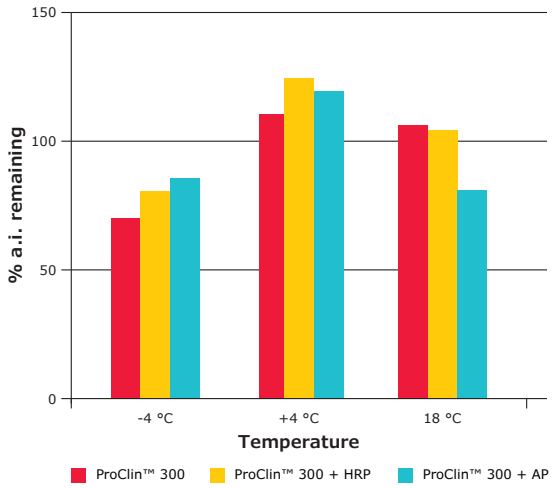


Figure E2. Stability of ProClin™ 300 preservative in the presence of horseradish peroxidase (HRP) and alkaline phosphatase (AP) after six months

Stability in Biological Buffers

Biological buffers (e.g. TRIS, TES, and HEPES) may contain amines that are aggressive above pH 7. The degradation of ProClin™ 300 preservative in these buffers may be minimized by reducing their pH to 7 or lower, which protonates free amines and converts them to a less aggressive acid salt. Short-term stability data is summarized in **Figure E1**.

Stability in Enzyme Conjugates

ProClin™ 300 preservative is unaffected by the presence of enzymes after six months of storage at temperatures ranging from -4 °C to 18 °C. There was virtually no loss of active biocide in horseradish peroxidase (HRP) or alkaline phosphatase (AP) when treated with 250 ppm (**Figure E2**).

Table 4. Minimum Inhibitory Concentration

Organism	ATCC* Number	Active Ingredient (ppm)
Gram-Negative Bacteria		
Achromobacter parvulus	4335	2
Alcaligenes faecalis	8750	2
Azotobacter vinelandii	12837	5
Enterobacter aerogenes	3906	5
Escherichia coli	11229	8
Flavobacterium suaveolens	958	9
Nitrobacter agilis	14123	0.1
Proteus vulgaris	8427	5
Pseudomonas aeruginosa	15442	5
Pseudomonas tolonif	Gibraltar 165	0.75
Pseudomonas fluorescens	13525	2
Pseudomonas oleoverans	8062	5
Salmonella typhosa	6539	5
Shigella sonnei	9292	8
Bacillus cereus mycoides	R&H L5**	2
Bacillus subtilis	R&H B2**	2
Brevibacterium ammoniagenes	6871	2
Cellulomonas sp.	21399	6
Sarcina lutea	9341	5
Staphylococcus aureus	6538	2
Staphylococcus epidermidis	155	2
Streptococcus pyrogenes	624	9
Streptomyces albus	3004	1
Fungi – Yeasts *		
Aspergillus foetidus	16878	9
Aspergillus niger	9642	9
Aspergillus oryzae	10196	5
Aureobasidium pullulans	9348	5
Candida albicans yeast	11651	5
Chaetomium globosum	6205	9
Cladosporium resinae	11274	5
Gliocladium fimbriatum	QM7638	9
Lentinus lepideus	12653	4
Lenzites trabea	11539	6
Mucor rouxii	R&H L5-83**	5
Penicillium funiculosum	9644	5
Penicillium variabile glaucum	USDA**	2
Phoma herbarum pigmentivora	12569	2
Rhizopus tolonifera	10404	5
Rhototorula rubra yeast	9449	2
Saccharomyces cerevisiae yeast	2601	2
Trichophyton mentagrophytes interdigitale	95332	5

* American Type Culture Collection

** Bacteriostatic and fungistatic tests performed by serially diluting test compounds in trypticase soy broth and 1:100 inoculation with 24-hour broth cultures of test bacterium or a fungal spore suspension prepared from 7-to-14 day culture slants washed with 7 mL of deionized water. Minimum inhibitory concentration levels determined visually after 2-4 days' incubation at 30-37 °C for bacteria and 7 days' incubation at 28 °C to 30 °C for fungi.

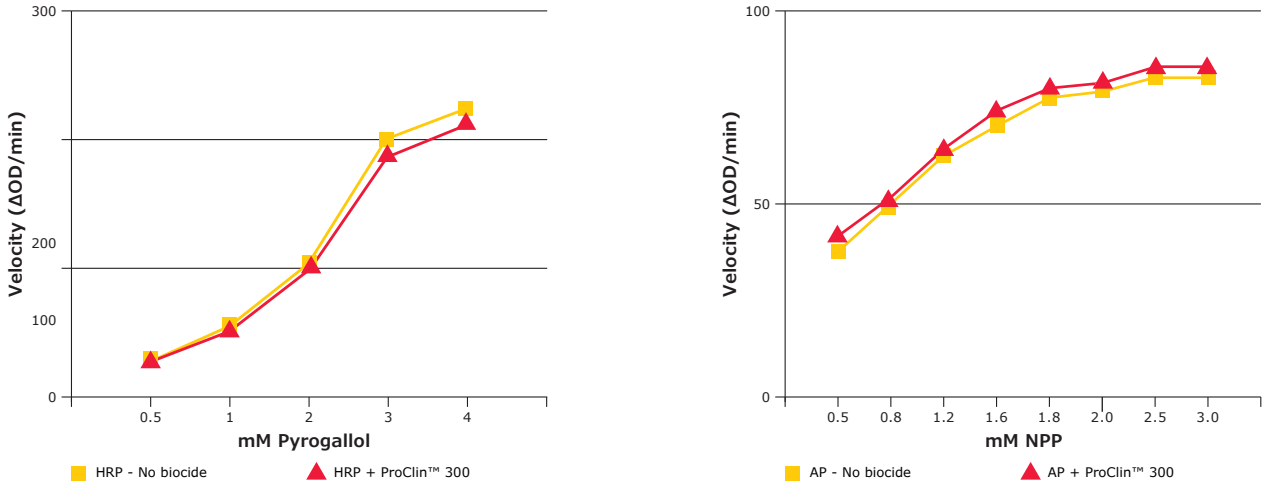


Figure F. Effect of 250 ppm ProClin™ 300 preservative on a substrate vs. velocity plot of HRP and AP

Lyophilization Stability

The use of ProClin™ 300 preservative in lyophilized products is not recommended. Data from several trials indicate the active ingredients are volatilized during the lyophilization process and are not available for preservation once the product is reconstituted.

Degradation Products

As described earlier, ProClin™ 300 preservative may degrade in the presence of high pH, high temperature, reducing agents, and aggressive nucleophiles. The degradative pathway for each of these mechanisms is similar. Degradation products include small organic acids (acetic, formic), carbon dioxide, chloride ion, and elemental sulfur. None of these degradation products have a significant impact on the environment.

Biocidal Efficacy

ProClin™ 300 preservative is an antimicrobial agent with unique biocidal mechanisms. Within minutes after contact with microorganisms, the biocide inhibits growth and respiration. Intracellular energy levels decline rapidly, and macromolecular synthesis is inhibited. Accumulation of damage to cellular constituents results in the death of the cell, possibly induced both by direct covalent modification of protein and due to radical-mediated events. **Table 4** lists the minimum levels of active ingredient that inhibit the growth of various microorganisms. These results were generated under standard laboratory conditions in nutrient-rich growth media and are intended to demonstrate the broad-spectrum activity of ProClin™ 300 preservative.

Actual use concentration will vary according to application.

Efficacy in Enzyme Conjugates

The antimicrobial activity of ProClin™ 300 preservative in HRP is summarized in **Table 5**. Preserved and control solutions were challenged at 0, 2, 4, and 6 weeks with an inoculum consisting of 107 CFU/mL *Pseudomonas aeruginosa* ATCC strain 15442. A concentration of only 15 ppm ProClin™ 300 preservative 0.05% (as supplied) completely eradicated this *Pseudomonas* species in both 1% and 10% HRP solutions after every challenge. This antimicrobial activity was superior to that of sodium azide.

Table 5. Efficacy of ProClin™ 300 Preservative in Enzyme Conjugate

Pseudomonas aeruginosa (CFUs /mL)			
Storage Time (Weeks)	**Control**	**ProClin™ 300 15 (ppm)**	**Sodium Azide (0.1%)**
10% HRP			
0	9.5×10^6	1.1×10^7	1.8×10^7
2	$>1.0 \times 10^9$	<10	<10
4	8.9×10^7	<10	<10
6	4.4×10^8	<10	<10
1% HRP			
0	1.2×10^6	3.5×10^6	2.3×10^6
2	7.2×10^8	<10	1.9×10^3
4	4.4×10^8	<10	<10
6	4.4×10^8	<10	<10

Compatibility with Enzymes

The effect of ProClin™ 300 preservative on both the short-term and long-term activity of HRP and AP was determined using standard assays.

Data from laboratory studies (**Table 6**) indicates that it does not affect the functionality of most key enzymes used for diagnostic testing, including HRP and AP (**Figure F**).

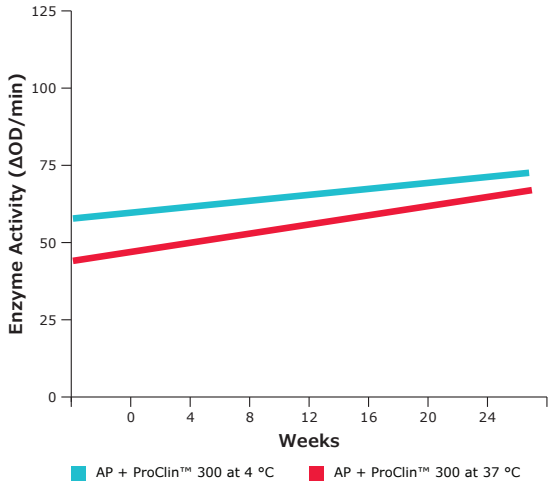


Figure G1. Effect of ProClin™ 300 preservative on the activity of AP at 4 °C and 37 °C

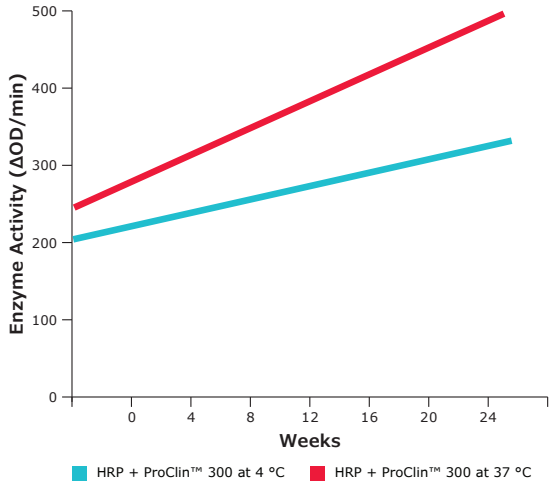


Figure G2. Effect of ProClin™ 300 preservative on the activity of HRP at 4 °C and 37 °C

Table 6. ProClin™ 300 Preservative Short-Term Enzymes Compatibility

No Inhibition	
Acid phosphatase	Malate dehydrogenase
Alkaline phosphatase	Monoamine oxidase
Catalase	NADH dehydrogenase
Chymotrypsin	3-Phosphoglycerate phosphokinase
β-Galactosidase	Pyruvate kinase
Glyceraldehyde-3-phosphate dehydrogenase	Trypsin
Horseradish peroxidase	Tyrosine carboxylase
Inhibition Observed	
α-Ketoglutarate dehydrogenase	Pyruvate dehydrogenase
Lactate dehydrogenase	Succinate dehydrogenase

*30 minute assays using 7500 ppm 25% (as supplied)

Short-Term Effects

ProClin™ 300 preservative has no significant effect on either the maximum velocity (Vmax) or required enzyme concentration (Km) of HRP and AP, even at 15 times the maximum recommended use levels (**Table 7**). A slight decrease in the Km of AP was observed on the presence of 250 ppm active biocide, but the affinity of the enzyme for its substrate was unaffected at the typical use level of 15 ppm.

Long-Term Effects

Enzymes that are compatible in the short term also retain their activity for extended periods. The rate of color development with AP remained constant over 6 months in the presence of 250 ppm active ProClin™ 300 biocide (**Figure G1**). Under the same conditions, HRP activity increased steadily with time (**Figure G2**).

Table 7. Effect of ProClin™ 300 Preservative on the Km and Vmax of HRP and AP

	Km (mM)	Vmax (A/min/mg protein)
Horseradish peroxidase	6.9	904
+15 ppm ProClin™ 300	6.8	861
Horseradish peroxidase	7.9	520
+250 ppm ProClin™ 300	8.3	525
Alkaline phosphatase	0.69	138
+15 ppm ProClin™ 300	0.56	131
Alkaline phosphatase	1.40 *	153
+250 ppm ProClin™ 300	1.20 *	142

*Results for AP were generated separately, using different enzyme preparations in each test.

Effect on Antibody Binding

The effects of ProClin™ 300 preservative on antibody binding were studied using purified mouse IgM as the antigen, and goat anti-mouse IgM linked to HRP as the reporting antibody. Direct effects on binding were determined in the presence of 0-300 ppm active isothiazolones, and long-term inactivation of the reporting antibody was determined by incubating aliquots of the concentrated antibody reagent with 0-50 ppm active isothiazolones for a period of 35 days.

The data in **Table 8** demonstrate that concentrations of ProClin™ 300 preservative up to 300 ppm active 1% (as supplied) had no effect on the extent of antibody-antigen binding. Incubation of HRP-linked antibody reagent with 50 ppm active ProClin™ 300 preservative for 35 days also had no effect on the performance of the reagent when diluted for use in the binding assay. An examination of the effect of 300 ppm active ProClin™ 300 preservative on the rate of color development by HRP-linked antibody indicated that there was no inhibition (**Figure H**).

These results demonstrate that ProClin™ 300 preservative has no effect on the activity of HRP-linked antibodies and is suitable for use with antibody-based diagnostic assays.

Table 8. Effect of ProClin™ 300 Preservative on Short-Term Antibody Binding

ProClin™ 300 Concentration (ppm active)	Percent binding vs. Control*
7.5	101.9
15.0	100.4
22.5	104.9
37.5	102.5
75.0	101.5
300.0	100.7

*values represent the average of three independent experiments with duplicate determinations

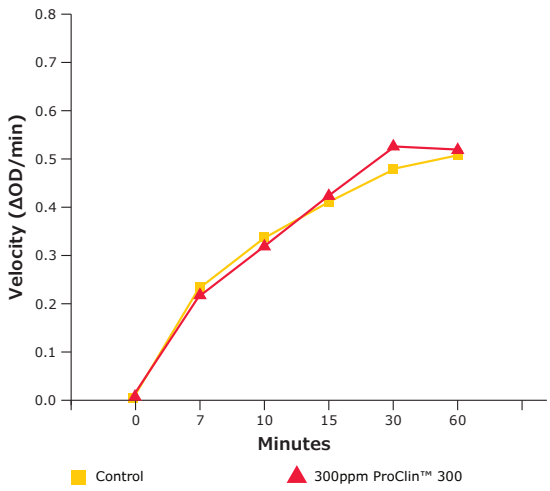


Figure H. Effect of ProClin™ 300 preservative on antibody-bound HRP activity

Toxicological Properties

All toxicological properties have been identified in **Tables 9-11**.

Table 9. Acute Toxicity

Test	Active Ingredient	Results
Oral Toxicity (Rats)		
Male		>500<2000mg/kg
Female		>500<2000mg/kg
Dermal Toxicity (Rabbits)		
Male		>500<2000mg/kg
Female		<2000mg/kg
Inhalation Toxicity (Rats)		
4-hour aerosol (LC50)*		0.7-1mg/L product = 0.2mg/L a.i.
4-hour saturated vapor (LC50)*		>2.31mg/L product = 0.65mg/L a.i.
Primary Skin Irritation (Rabbits)		
Product as supplied	30,000 ppm	Corrosive
Product diluted to 18.6%	5600 ppm	Severe irritant
Product diluted to 9.3	2800 ppm	Moderate irritant
Product diluted to 1.8%	560 ppm	Non-irritant
Primary Eye Irritation (Rabbits)		
Product as supplied	30,000 ppm	Irreversible damage
Product diluted to 18.6%	5600 ppm	Moderate irritant
Product diluted to 9.3	2800 ppm	Slight irritant
Product diluted to 1.8%	560p pm	Non-irritant

*Air saturated with a solution containing ten times the level of active ingredient that is present in ProClin™ 300 preservative.

Table 10. Subchronic Toxicity

Test	Active Ingredient	Response
90-day dietary:rats	30mg/kg/day	No mortalities, no pathological findings
90-day dietary:dogs	27mg/kg/day	No mortalities, no pathological findings
90-day dermal:rabbits	0.4mg/kg/day	No treatment-related pathological effects
90-day drinking water, one generation reproduction:rats	20mg/kg/day	Slight gastric irrita- tion, no adverse effect on reproduction, fetal health or survival
4-week eye irritation: rabbits (8 applications/day, 5 days/week)	8mg/kg/day 56ppm	No effect level Non-irritant
13-week inhalation:rats	0.34mg/m3	No observable effect at that dose (6 hours/ day, 5 days/week)
Teratology:rats	15mg/kg/day	Not teratogenic
Teratology:rabbits	13.3mg/kg/day	Not teratogenic

Table 11. Chronic Toxicity

Test	Active Ingredient	Response
Dermal Oncogenicity, 30-month skin painting: treatment mouse (3 times/week)	400 ppm	Slight skin irritation,no other treatment-related pathology, no increase in tumor frequency

Genetic Toxicity

Based on a battery of genotoxicity tests, as well as the oncogenicity studies, ProClin™ 300 preservative has been determined not to pose any mutagenic or carcinogenic hazards to humans under normal conditions.

Directions for Use

Long-term microbial protection in reagents is accomplished with levels of ProClin™ 300 preservative between 6 and 20 ppm active biocide. Typical use levels fall in the range of 9-15 ppm active 0.03-0.05% (as supplied). It is usually advisable to treat a reagent with a higher level than the minimum effective dose to preserve its shelf life.

Some reagents are sold in concentrated form and require a limited shelf life after opening and dilution by the user. Reagents such as these can incorporate higher levels of ProClin™ 300 preservative, but special labeling may be required to inform the user of sensitization effects (**Table 12**).

Since the components of diagnostic reagents vary considerably and may impact the stability and efficacy of preservatives, we urge each manufacturer to confirm the efficacy of ProClin™ 300 preservative in use.

Table 12. Sensitization Tests

Test	Concentration	Effect
Skin Sensitization		
Guinea Pigs (Buehler Method)	56ppm a.i. in	Sensitizer EC50 induction 90ppm a.i.
Guinea Pigs (Magnusson- Kligman Method)	1, 3, and 10%	EC50 elicitation 429ppm a.i. No sensitization aqueous solution
Photosensitization/Phototoxicity		
Guinea Pigs	0.3% a.i. insult dosage	Not phototoxic; irritant at the higher concentration
	0.3% and 1% a.i. challenges	No photo-sensitization

Safe Handling Information

ProClin™ 300 preservative presents no toxicological problems or health hazards at recommended use levels. However, the following precautions must be observed when handling the undiluted product as it can cause irreversible eye damage and skin burns. These effects may not manifest themselves for several hours after contact. Avoid contact with eyes, skin and clothing.

Personal Protection and First Aid Measures

If ProClin™ 300 preservative makes eye contact, FLUSH IMMEDIATELY with water for at least 15 minutes and get prompt medical attention. If it makes contact with skin, WASH IMMEDIATELY AND THOROUGHLY with soap and water and consult a physician if irritation develops.

Manufacturing personnel handling ProClin™ 300 preservative should wear splash goggles or face shield, rubber gloves, and an impervious apron and boots. Laboratory personnel should also wear a lab coat and safety glasses.

Avoid breathing vapor or mist, and wash hands thoroughly after use to prevent contamination of food and drink. Special care should be taken to avoid contamination of surfaces that may later be handled by unprotected personnel. If inhaled, move person to fresh air immediately. If not breathing, initiate artificial respiration. If breathing is difficult, give oxygen. If swallowed, dilute by giving water to drink. Never give an unconscious person anything to drink.

Note to Physician – Corrosive material. Probable mucosal damage may contraindicate the use of gastric lavage. Measures against circulatory shock, respiratory depression, and convulsions may be needed.

Neutralizing Solution

ProClin™ 300 preservative is quickly neutralized by solutions of sodium bisulfite at acidic pH. To prepare a neutralizing solution, dissolve 1lb. of sodium bisulfite in 1 gallon of water (120g/L). The solution should have a pH of 4.0-5.0 for maximum effectiveness. If a neutralizing solution is permanently maintained, it should be replaced weekly. Keep containers of neutralizing solution covered to reduce the release of sulfur dioxide and maintain solution activity.

Tests indicate that ProClin™ 300 preservative is completely deactivated within 30 minutes when treated with at least 2 volume equivalents of neutralizing solution.

Note: Neutralizing solution should not be used to treat ProClin™ 300 preservative spilled on skin.

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MK_TN6216EN Ver. 0.0
32442
08/2020