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For *In Vitro* Diagnostic Materials:

Efficacy vs. Thimerosal and Sodium Azide

Background

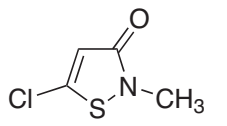
The traditional preservatives used to prevent microbe growth in *in vitro* diagnostic products are not ideal. Thimerosal is expensive and, because it contains mercury, is classified as toxic for disposal. It is banned from use in some countries, including Japan. Sodium azide is primarily biostatic, not biocidal, at levels normally used, thus it might not effectively control microorganisms in a product. As a powder, sodium azide is both hazardous and inconvenient to work with. In acidic solutions, it can release harmful vapors. Antibiotics offer protection against specific groups of microorganisms, rather than broad-spectrum protection. Combinations of antibiotics expand the range of protection, but combinations become complicated and expensive, and protection remains incomplete.

Environmental friendly preservatives

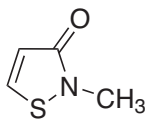
New preservative materials can eliminate the drawbacks associated with traditional preservatives. Two isothiazolones (**Figure 1**), the active ingredient in ProClin™ 150, ProClin™ 200, ProClin™ 300 and ProClin™ 950 preservatives, have a unique mechanism of action that both inhibits bacterial, fungal, and yeast growth and causes cell death. Within minutes after contacting a microorganism, these molecules penetrate the cell wall and inhibit specific enzymes in the Krebs cycle, the central metabolic cycle of the cell, inhibiting growth, macromolecule synthesis, and respiration, and causing intracellular energy levels to fall rapidly.[1] With energy production disrupted, the cell cannot synthesize chemicals for routine operation or repair, and ultimately dies. Because the target enzymes are central to the cell's ability to function, microbes are afforded little chance to develop resistance. Unlike traditional preservatives, there are no disposal restrictions on the isothiazolones when they are used at recommended levels; the ProClin™ 150, ProClin™ 200, ProClin™ 300 and ProClin™ 950 preservatives as supplied can be neutralized and disposed of as nonhazardous waste.

The active ingredients in ProClin™ preservatives are effective at very low concentrations, 6-20 ppm, but the effectiveness of these materials relative to traditional preservatives has not been established. This paper presents the results of an efficacy trial designed to make the comparison. The format of the trial is similar to that of a United States Pharmacopeia (USP) efficacy study.

Figure 1.



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



2-Methyl-4-isothiazolin-3-one

Objective

To compare the antimicrobial efficacy of ProClin™ 150 preservative, ProClin™ 300 preservative, sodium azide, and thimerosal in phosphate buffered saline/TWEEN® 20 (polysorbate 20). The study was conducted by an independent laboratory.

Preservative test solutions

Phosphate buffered saline/TWEEN® 20 (mPBS) was prepared by emptying 4 packets of the reagent (Sigma-Aldrich® Cat. No. P3563) into a sterile graduated cylinder. The volume was brought to 4.0 litres, using sterile dionized water.

1. 0.5 mL ProClin™ 150 (Supelco®, Lot No. LA75145) was added to 250 mL mPBS. The solution was mixed, then 249.5 mL mPBS was added to achieve the final test solution concentration of 15 ppm ProClin™ 150.
2. 0.25 mL ProClin™ 300 (Supelco®, Lot No. LA76183) was added to 250 mL mPBS. The solution was mixed, then 249.75 mL mPBS was added to achieve the final test solution concentration of 15 ppm ProClin™ 300.
3. 1 g sodium azide (Sigma-Aldrich® Lot No. 58H2504) was introduced into a sterile graduated cylinder, then sterile deionized water was added to bring the volume to 10 mL. 5 mL of this 10% sodium azide solution was aded to 495 mL mPBS to achieve the final test solution concentration of 0.1% sodium azide.
4. 0.05 g thimerosal (Sigma-Aldrich®, Lot No. 78H1337) was introduced into a sterile graduated cylinder, then sterile dionized water was added to bring the volume to 10 mL. 5 mL of this 0.5% thimerosal solution was added to 495 mL mPBS to achieve the final test solution concentration of 0.005% thimerosal.

Microbial challenge

Suspensions of the following microorganisms were prepared at a target level of 2.0 x 10^8 - 2.0 x 10^9 CFU/mL:

Pseudomonas aeruginosa (ATCC 9027)
Escherichia coli (ATCC 8739)
Staphylococcus aureus (ATCC 6538)
Burkholderia (formerly *Pseudomonas*) *capacia* (ATCC 25608)
Candida albicans (ATCC 10231)
Aspergillis niger (ATCC 16404)

An inoculum was prepared by combining 1 mL of each suspension in a sterile centrifuge tube. 20 mL aliquots of each test solution were placed into separate sterile centrifuge tubes. Each tube was inoculated with 0.1 mL of the microorganism mixture (target inoculum level 1.0 x 10^6 - 1.0 x 10^7 CFU/mL). Unpreserved mPBS was inoculated as a positive control. To verify the inoculum level, a plate count of the inoculum was performed concurrently with the inoculations, using tryptic soy broth agar (TBSA) for the (aerobic) bacteria and potato dextrose agar (PDA) for the yeast and mold.

Inoculated test solutions were incubated at 20-25 °C for 14 days, then total aerobic bacteria and yeast/mold counts were performed on each solution, using a plate count (all test solutions except sodium azide solution) or membrane filtration procedure (sodium azide solution). Plates for total aerobic bacteria counts were incubated at 30-35 °C for not less than 48 hours before counts were made; plates for yeast/mold counts were incubated at 20-25 °C for not less than 4 days.

Immediately after the plate counts were made for each test solution, the microbial challenge was repeated. Each centrifuge tube was reinoculated, incubated and recounted. This process was repeated until growth was observed on the plates, or until a total of four successive inoculations had been made.

Conclusions

15 ppm ProClin™ 150 preservative, 15 ppm ProClin™ 300 preservative, and 0.005% thimerosal effectively preserved a phosphate buffered saline/TWEEN® 20 solution from a range of microorganisms under the conditions specified. 0.1% sodium azide did not preserve phosphate buffered saline/TWEEN® 20.

This study indicates that ProClin™ 150 and ProClin™ 300 preservatives can be effective replacements for thimerosal, and offer better protection than sodium azide, without the handling and disposal concerns associated with either traditional preservative. Additional information is also available at **sigmaaldrich.com/proclin**.

[1]The enzymes affected are pyruvate dehydrogenase, a-ketoglutartate dehydrogenase, succinate dehydrogenase, NADH dehydrogenase.

Results of microbial challenge

Table 1. ProClin™ Preservatives, Thimerosal Pass Microbial Challenge (All values in CFU/mL).

Test Solution	First Inoculation	14-Day Count	Second Inoculation	14-Day Count	Third Inoculation	14-Day Count	Fourth Inoculation	14-Day Count
Unpreserved mPBS								
TSBA	3.38 x 10^6	4.2 x 10^6	3.30 x 10^6	6.5 x 10^6	a			
PDA	1.93 x 10^6	2.1 x 10^6	3.56 x 10^6	1.31 x 10^7	a			
Sodium Azide								
TSBA	3.38 x 10^6	b	3.30 x 10^6	d	a			
PDA	1.93 x 10^6	c	3.56 x 10^6	e	a			
0.005% Thimerosal								
TSBA	3.38 x 10^6	<10	3.30 x 10^6	<10	1.88 x 10^6	<10	4.50 x 10^6	<10
PDA	1.93 x 10^6	<10	3.56 x 10^6	<10	1.16 x 10^6	<10	2.75 x 10^6	<10
15 ppm ProClin™ 150								
TSBA	3.38 x 10^6	<10	3.30 x 10^6	<10	1.88 x 10^6	<10	4.50 x 10^6	<10
PDA	1.93 x 10^6	<10	3.56 x 10^6	<10	1.16 x 10^6	<10	2.75 x 10^6	<10
15 ppm ProClin™ 300								
TSBA	3.38 x 10^6	<10	3.30 x 10^6	<10	1.88 x 10^6	<10	4.50 x 10^6	<10
PDA	1.93 x 10^6	<10	3.56 x 10^6	<10	1.16 x 10^6	<10	2.75 x 10^6	<10

a Test ended due to growth on plates
b Aerobic bacteria (*C. albicans*) count = 870 (membrane filtration method)
c Yeast/mold (*A. niger*) count = 950 (membrane filtration method)
d Aerobic bacteria (*C. albicans*) count = >3,000 (spread plate method)
e Yeast/mold (*A. niger*) count = 980 (membrane filtration method)

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MS_5935EN Ver. 1.0 31650 05/2020