



Protocol No.: ECN/PC/001.00

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TITLE: Efficiency Testing for Disinfectant

AUTHOR:

Name /Title/ Department

Signature/Date

CHECKED BY:

Name /Title/ Department

Signature/Date

APPROVED BY:

Name /Title/ Department

Signature/Date

REVISIONS:

No.	Section	Pages	Initials/Date
00	Physicochemical characteristic of Hypochlorous acid and Inhibition of bacterial growth	20	



SUBJECT: Efficiency Testing for Disinfectant - Hypochlorus acid (HOCl) AQUAOX

Purpose :

To ensure that physical and chemical characteristics of disinfectant (Hypochlorus acid (HOCl) AQUAOX)

To ensure that disinfectant used to inhibit the growth of specified microorganisms.

Responsibility :

It is the responsibility of the ECOICONS manager (Physicochemical division and Microbiology division) to follow the protocol.

Definitions :

Disinfectant is specifically defined as Hypochlorus acid (HOCl) AQUAOX that to know the characteristics with respect to sun light and room temperature.

Disinfectant is specifically defined as Hypochlorus acid (HOCl) AQUAOX that inhibit the growth of microbial population in the form of vegetative nature.

Study Frequency as per protocol :

The plate count technique is used for evaluating its effectiveness on microorganism for various contact times.

The characteristics of disinfectant were test for store at various temperature and time intervals.

Equipments for Characteristics assay :

Glass beakers

pH meter

TDS meter



Burette

Conical flask

Pipette

Sodium Thio sulphate ($\text{Na}_2\text{S}_2\text{O}_3$) 0.025 N

Glacial Acetic acid

Potassium iodide

Starch Solution 5% w/v

Equipment for Microbiological assay :

Incubator 25°C to 35°C

Vortex Mixer

Laminar Air Flow unit (LAF Unit)

Bacterial Cultures :

- *Pseudomonas aeruginosa* (NCTC 12924)
- *Escherichia coli* (NCTC 12923)
- *Salmonella abony* (ACM 5080)
- *Staphylococcus aureus* (NCTC 10788)

Sterile test tubes

Sterile water

Sterile normal saline water 0.85% w/v NaCl

Poured, sterile Soybean Casein Digest Agar (SCDA) petriplates

Sterile Nutrient broth test tubes



Sterile pipettes, 10 ml, 5 ml and 1ml sizes

Sterile spreader

Sterile loop

Disinfectant (Hypochlorous acid)

PROCEDURE :

1. Preparation of sample for characteristics of disinfectant for Physio chemical evaluation. :

Collect each 500 ml of disinfectant in a beaker for different temperatures conditions and different time intervals. Keep them in sun light and room temperatures.

Assay at 1hr, 4hrs and 8 hrs intervals, measure 100 ml for each test pH and TDS of various temperature.

Titrimetric method for assay of Free Activated Chlorine :

For assay of Free Activated Chlorine take measured 25 ml of sample in a conical flask then add 5 ml of Glacial acetic acid and 1 g of Potassium iodide and titrate against 0.025N Sodium Thio Sulphate solution note down the initial reading and final reading of burette volume. Repeat the test for 3 time and the values should be vary ± 0.2 . Calculate the chlorine by the formula.

Formula :

$$\text{Free chlorine} = \frac{\text{Volume of Titrate} \times \text{Normality of Titrate} \times \text{Molecular Wt of Chlorine} \times 1000}{\text{Volume of sample}}$$

Based on the calculation the result were expressed in mg/L or PPM



2. Preparation of challenge inocula for Microbiological assay :

A loop full of pure culture from stock preparation and inoculate in 10 ml of sterile Nutrient broth. Incubate at 30 to 35°C for 18 to 24 hours. Do a microbial plate count of suspensions as necessary and dilute with appropriate saline to obtain final working suspensions of 10^{12} CFU/ml.

Plate count technique :

Pipette 2 ml of 10^{12} CFU/ml culture of each challenge organism in sterile test tubes. For each challenge organism pipette 3.0 ml of disinfectant (Hypochlorous acid (HOCl) AQUAOX) mix well. Assay at 5 min, 1 hr and 24 hrs intervals, perform the entire operation in a Laminar Air Flow. At each interval Pipette 0.5 ml of test sample of each challenge organism inoculate in sterile Soybean Casein Digest Agar petriplates and spread uniformly with the help of sterile spreader.

Pipette 0.5 ml of culture, inoculate in sterile Soybean Casein Digest Agar petriplates and spread uniformly with the help of sterile spreader for a positive control, pipette 0.5 ml of sterile saline water in sterile Soybean Casein Digest Agar petriplates use for a negative control.

Incubate all plates at 30 to 35°C for 18 to 24 hours. After incubation examine the sign of growth (24 48 hrs.) of each challenge organism and take a count of colonies of organisms.

Positive control should show the growth of microorganisms, negative control should not show any growth of microorganisms.

Acceptance criteria :

The recommended disinfectant solution must be able to establish inhibit the growth of 10^9 CFU/ml inoculated microorganism with in 5 min, 1hr and 24 hrs.

REASONS FOR REVISION

Effective date :

- First time issued for M/s. Medmokk Enterprises, affiliates and contract manufacturers.



Analytical report for characteristic assay :

Titration table

S.No.	Burette reading		F - I
	Initial volume ml (I)	Final volume ml (F)	
1	0.0	7.2	7.2
2	7.3	14.4	7.1
3	14.4	21.5	7.1
4	21.5	28.6	7.1
5	28.6	35.8	7.2

$$\begin{aligned} \text{Average volume of sodium thio sulphate} &= \frac{\text{Consumed Na}_2\text{S}_2\text{O}_3 \text{ volume of burette}}{\text{No. of times repeated}} \\ &= \frac{35.8}{5} \\ &= 7.16 \text{ ml} \end{aligned}$$

$$\begin{aligned} \text{Free chlorine} &= \frac{\text{Volume of Titrant(ml)} \times \text{Normality of Titrant} \times \text{Molecular Wt of Chlorine} \times 1000}{\text{Volume of sample (ml)}} \\ &= \frac{7.16 \times 0.025 \times 35.45 \times 1000}{25} \\ &= 253.82 \text{ mg/L or PPM} \end{aligned}$$

Day 1:

Time	1hr		4 hr		8 hr	
	pH	FAC ppm	pH	AFC ppm	pH	FAC ppm
Room temperature	6.77	216	6.70	205	6.67	202
Sun light	6.13	187	5.98	109	5.68	81



Day 2 :

Time	1hr		4 hr		8 hr	
	pH	FAC ppm	pH	AFC ppm	pH	FAC ppm
Room temperature	6.89	225	6.69	212	6.62	208
Sun light	6.19	192	5.93	102	5.71	79

Day 3 :

Time	1hr		4 hr		8 hr	
	pH	FAC ppm	pH	AFC ppm	pH	FAC ppm
Room temperature	6.75	219	6.65	206	6.58	201
Sun light	6.15	189	5.91	105	5.68	75

Summary for characteristic assay :

The disinfectant is analyzed for pH, Free Active Chlorine (FAC) with respect to different time intervals (3 Days) at different temperature (i.e. Room Temperature & Sun Light).

Conclusion for characteristic assay :

The pH and Free Active Chlorine (FAC) value is changing with respect to Room Temperature & Sun Light.



Analytical report for Microbiological assay :

Disinfectant establishment :

Disinfectant concentration: 250 ppm

Dilutions	50 PPM	100 PPM	150 PPM	200 PPM	250 PPM
Disinfectant (ml)	4	8	12	16	20
Diluents (ml)	16	12	8	4	0

Inocula establishment

Dilutions	10¹	10²	10³	10⁴	10⁵	10⁶	10⁷	10⁸	10⁹	10¹⁰	10¹¹	10¹²
Normal Saline	9ml	9ml	9ml	9ml	9ml	9ml	9ml	9ml	9ml	9ml	9ml	9ml
Inocula	1ml	1ml of 10 ¹	1ml of 10 ²	1ml of 10 ³	1ml of 10 ⁴	1ml of 10 ⁵	1ml of 10 ⁶	1ml of 10 ⁷	1ml of 10 ⁸	1ml of 10 ⁹	1ml of 10 ¹⁰	1ml of 10 ¹¹

Concentration of inocula: 10¹² CFU/ml

Concentration of disinfectant: 50 PPM, 100 PPM, 150 PPM, 200 PPM, 250 PPM

Protocol studies

Volume of 10¹² CFU/ml : 2.0 ml

Volume of disinfectant: 3.0 ml

$$\frac{10^{12} \text{ CFU}}{1.5} = 10^{11} \text{ CFU} \quad (3/2 = 1.5)$$

If 10¹¹ CFU/ml Inoculums pipette 1 ml and added to plate count became 10¹⁰ CFU

If 10¹¹ CFU/ml Inoculums pipette 0.5 ml and added to plate count became 10⁹ CFU



PLANNING SHEET

Planning for 50 PPM concentration Disinfectant (Hypochlorous acid (HOCl) AQUAOX)

	<i>E.coli</i>	<i>P.aeruginosa</i>	<i>S.abony</i>	<i>S.aureus</i>	
SET - 1					} 5 min
SET - 2					
SET - 1					} 1 hr
SET - 2					
SET - 1					} 24 hr
SET - 2					



Planning for 100 PPM concentration Disinfectant (Hypochlorous acid (HOCl) AQUAOX)

	<i>E.coli</i>	<i>P.aeruginosa</i>	<i>S.abony</i>	<i>S.aureus</i>	
SET - 1					} 5 min
SET - 2					
SET - 1					} 1 hr
SET - 2					
SET - 1					} 24 hr
SET - 2					



Planning for 150 PPM concentration Disinfectant (Hypochlorous acid (HOCl) AQUAOX)

	<i>E.coli</i>	<i>P.aeruginosa</i>	<i>S.abony</i>	<i>S.aureus</i>	
SET - 1					} 5 min
SET - 2					
SET - 1					} 1 hr
SET - 2					
SET - 1					} 24 hr
SET - 2					



Planning for 200 PPM concentration Disinfectant (Hypochlorous acid (HOCl) AQUAOX)

	<i>E.coli</i>	<i>P.aeruginosa</i>	<i>S.abony</i>	<i>S.aureus</i>	
SET - 1					} 5 min
SET - 1					
SET - 2					} 1 hr
SET - 2					
SET - 1					} 24 hr
SET - 2					



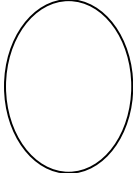
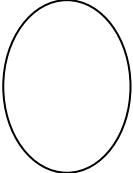
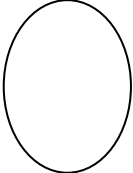
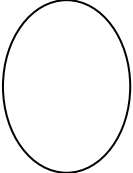
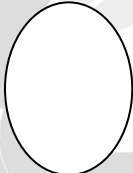
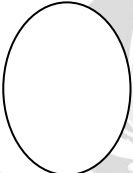
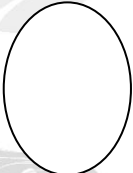
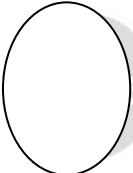
Planning for 250 PPM concentration Disinfectant (Hypochlorous acid (HOCl) AQUAOX)

	<i>E.coli</i>	<i>P.aeruginosa</i>	<i>S.abony</i>	<i>S.aureus</i>	
SET - 1					} 5 min
SET - 1					
SET - 2					} 1 hr min
SET - 2					
SET - 1					} 24 hr
SET - 2					

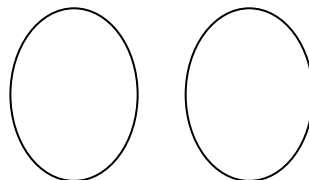


Planning for positive and negative control :

POSITIVE CONTROL

	<i>E.coli</i>	<i>P.aeruginosa</i>	<i>S.abony</i>	<i>S.aureus</i>
SET - 1				
SET - 2				

NEGATIVE CONTROL





RESULT for 5 min's

Concentration of disinfectant: 50 PPM

Volume of sample pipette: 0.5 ml

	<i>E.coli</i>	<i>P.aurgenosa</i>	<i>S.abony</i>	<i>S.aureus</i>
SET – 1	No Growth	No Growth	No Growth	No Growth
SET – 2	No Growth	No Growth	No Growth	No Growth

Concentration of disinfectant: 100 PPM

Volume of sample pipette: 0.5 ml

	<i>E.coli</i>	<i>P.aurgenosa</i>	<i>S.abony</i>	<i>S.aureus</i>
SET – 1	No Growth	No Growth	No Growth	No Growth
SET – 2	No Growth	No Growth	No Growth	No Growth

Concentration of disinfectant: 150 PPM

Volume of sample pipette: 0.5 ml

	<i>E.coli</i>	<i>P.aurgenosa</i>	<i>S.abony</i>	<i>S.aureus</i>
SET – 1	No Growth	No Growth	No Growth	No Growth
SET – 2	No Growth	No Growth	No Growth	No Growth

Concentration of disinfectant: 200 PPM

Volume of sample pipette: 0.5 ml

	<i>E.coli</i>	<i>P.aurgenosa</i>	<i>S.abony</i>	<i>S.aureus</i>
SET – 1	No Growth	No Growth	No Growth	No Growth
SET – 2	No Growth	No Growth	No Growth	No Growth

Concentration of disinfectant: 250 PPM

Volume of sample pipette: 0.5 ml

	<i>E.coli</i>	<i>P.aurgenosa</i>	<i>S.abony</i>	<i>S.aureus</i>
SET – 1	No Growth	No Growth	No Growth	No Growth
SET – 2	No Growth	No Growth	No Growth	No Growth



RESULT for 1 Hr.

Concentration of disinfectant: 50 PPM

Volume of sample pipette: 0.5 ml

	<i>E.coli</i>	<i>P.aurgenosa</i>	<i>S.abony</i>	<i>S.aureus</i>
SET – 1	No Growth	No Growth	No Growth	No Growth
SET – 2	No Growth	No Growth	No Growth	No Growth

Concentration of disinfectant: 100 PPM

Volume of sample pipette: 0.5 ml

	<i>E.coli</i>	<i>P.aurgenosa</i>	<i>S.abony</i>	<i>S.aureus</i>
SET – 1	No Growth	No Growth	No Growth	No Growth
SET – 2	No Growth	No Growth	No Growth	No Growth

Concentration of disinfectant: 150 PPM

Volume of sample pipette: 0.5 ml

	<i>E.coli</i>	<i>P.aurgenosa</i>	<i>S.abony</i>	<i>S.aureus</i>
SET – 1	No Growth	No Growth	No Growth	No Growth
SET – 2	No Growth	No Growth	No Growth	No Growth

Concentration of disinfectant: 200 PPM

Volume of sample pipette: 0.5 ml

	<i>E.coli</i>	<i>P.aurgenosa</i>	<i>S.abony</i>	<i>S.aureus</i>
SET – 1	No Growth	No Growth	No Growth	No Growth
SET – 2	No Growth	No Growth	No Growth	No Growth

Concentration of disinfectant: 250 PPM

Volume of sample pipette: 0.5 ml

	<i>E.coli</i>	<i>P.aurgenosa</i>	<i>S.abony</i>	<i>S.aureus</i>
SET – 1	No Growth	No Growth	No Growth	No Growth
SET – 2	No Growth	No Growth	No Growth	No Growth



RESULT for 24 Hr's.

Concentration of disinfectant: 50 PPM

Volume of sample pipette: 0.5 ml

	<i>E.coli</i>	<i>P.aurgenosa</i>	<i>S.abony</i>	<i>S.aureus</i>
SET – 1	No Growth	No Growth	No Growth	No Growth
SET – 2	No Growth	No Growth	No Growth	No Growth

Concentration of disinfectant: 100 PPM

Volume of sample pipette: 0.5 ml

	<i>E.coli</i>	<i>P.aurgenosa</i>	<i>S.abony</i>	<i>S.aureus</i>
SET – 1	No Growth	No Growth	No Growth	No Growth
SET – 2	No Growth	No Growth	No Growth	No Growth

Concentration of disinfectant: 150 PPM

Volume of sample pipette: 0.5 ml

	<i>E.coli</i>	<i>P.aurgenosa</i>	<i>S.abony</i>	<i>S.aureus</i>
SET – 1	No Growth	No Growth	No Growth	No Growth
SET – 2	No Growth	No Growth	No Growth	No Growth

Concentration of disinfectant: 200 PPM

Volume of sample pipette: 0.5 ml

	<i>E.coli</i>	<i>P.aurgenosa</i>	<i>S.abony</i>	<i>S.aureus</i>
SET – 1	No Growth	No Growth	No Growth	No Growth
SET – 2	No Growth	No Growth	No Growth	No Growth

Concentration of disinfectant: 250 PPM

Volume of sample pipette: 0.5 ml

	<i>E.coli</i>	<i>P.aurgenosa</i>	<i>S.abony</i>	<i>S.aureus</i>
SET – 1	No Growth	No Growth	No Growth	No Growth
SET – 2	No Growth	No Growth	No Growth	No Growth



Positive control

Volume of inocula pipette: 0.5 ml

	<i>E.coli</i>	<i>P.aurgenosa</i>	<i>S.abony</i>	<i>S.aureus</i>
SET – 1	41	37	63	27
SET – 2	45	31	57	24
Total count	86	68	120	51

Average count of bacteria = $\frac{\text{Total colony forming units plates}}{2 \text{ Plates (used as Duplicate)}}$

$$\begin{aligned} E.coli \text{ s} &= 86/2 \\ &= 43 \times 10^9 \text{ CFU/ml} \end{aligned}$$

$$\begin{aligned} P.aurgenosa &= 68/2 \\ &= 34 \times 10^9 \text{ CFU/ml} \end{aligned}$$

$$\begin{aligned} S.abony &= 120/2 \\ &= 60 \times 10^9 \text{ CFU/ml} \end{aligned}$$

$$\begin{aligned} S.aureus &= 51/2 \\ &= 25 \times 10^9 \text{ CFU/ml} \end{aligned}$$

Negative control

SET – 1	No Growth
SET – 2	No Growth



Summary for microbiological assay

1. Microbiological cultures: M/s. BioBall
2. Inoculums establishment of 10^{10} CFU/ 1.0 ml or of 10^9 CFU/ 0.5 ml.
3. Sample of disinfectant of known inoculums mix up 10^{12} CFU/ 1.0 ml.
4. Protocol study of disinfectant exposed to 5 min, 1hr and 24 hrs contact with challenged Microorganism such as *E.coli*, *P.aeruginosa*, *S.abony*, *S.aureus* and inoculated at 30-35°C for 24 to 48 hrs.
5. Compare the individual CFU/ml used microorganism with positive control (10^9 CFU) and negative control Microbiology media as per study protocol.
6. The disinfectant (Hypochlorus acid) used in this study inhibit the challenged microorganisms at the population of 10^9 CFU were exposed between 1 hr to 24 hrs intervals.

Conclusion for microbiological assay

Hence, AQUAOX brand disinfectant containing Hypochlorus acid (HOCl), when exposed to specified microorganism in a given contact time intervals (5 min's, 1 hr, 24 hrs.) as per this study protocol. This study resulted to confirm this disinfectant (Hypochlorus acid- HOCl (AQUAOX) would be in a position to inhibit the challenged population of individual bacteria $> 10^9$ CFU/ml (Billion viable cells/ml) of ≥ 50 ppm.



Abbreviations :

1. CFU - colony forming units.
2. ml - milli Litre.
3. ppm - parts per million.
4. mg - milli gram.
5. L - litre.
6. Min's - minutes.
7. Hr - hour.
8. Hrs - hours.
9. TDS - Total Dissolved Solids.

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