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Second Edition

Diagnostic Microbiology

for DMLT Students





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Foreword
Subir Kumar Dutta

JAYPEE

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2

Chapter

Cleaning, Sterilization and Disinfection

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CLEANING OF GLASSWARE

Most of the containers and measuring devices like flasks, beakers, slides, pipettes, etc. used in a laboratory are made of glass. So, proper cleaning of glasswares are very important factor to maintain the high standard works in laboratory and obviously to avoid the erroneous results.

Cleaning of New Glassware

- Put the new glassware in a plastic bucket containing 1–2% hydrochloric acid.
- Leave them overnight.
- Wash the glassware under *running water* and then with *distilled water*/*deionized water*.
- Dry in air or at 60°C in *hot air oven* → wrap with paper → sterilize by dry heat at 170°C for 60 minutes.

Cleaning of Used Glass Pipette

- As soon as a pipette has been used it must be rinsed *at once* in a stream of *cold water*.
- Take a container 15 inches high and 4 inches diameter (e.g. glass cylinder) filled with 3% Lysol or 0.5–1% *sodium hypochlorite solution*.
- Put the pipette in the container and leave for 30 minutes. If the pipette is *markedly contaminated or blocked; keep it overnight in dichromate sulfuric acid solution* (*See* Chapter 5).
- With gloved hands wash with tap water and then distilled water.
- Draw in *acetone* → keep in air till the internal surface is dry.
- Plug the top end of the pipette with non-absorbent cotton wool → wrap with paper → sterilize by dry heat at 170°C for 60 minutes.

Cleaning of Nondisposable Glassware Used in Bacterial Culture

- All used glassware like Petri plates, tubes with cultured mediums are to be *fully immersed* in a suitable plastic bucket filled with 5% *Lysol*/0.5–1% *freshly prepared sodium hypochlorite solution* for 4 hours.
- Boil the glassware in 5% *soap solution for 1 hour*.
- Remove them from soap solution and scrub the mediums with a brush.
- Rinse the glassware in running water and then with distilled water.
- Dry in air or at 60°C in hot air oven → wrap with paper → sterilize by hot air oven at 170°C for 1 hour.

Cleaning of Used Glass Slides

- If the slides with coverslips, detach them from slides using the tip of a needle and drop them into a beaker filled with 3–5% *Lysol solution*.
- If the slides contain *immersion oil*, remove the oil as much as possible by rubbing them one by one with *newspaper*.
- If the slides have been used for infected specimen they should be immersed in 0.5–1% *sodium hypochlorite solution* for 4 hours.
- Put them in a container filled with *detergent solution* and boil for 1 hour.
- Take the slides one by one out of the boiled solution and rinse the slides under running water.
- Place them *separately* on a *newspaper* and allow to dry.
- After drying, the slides should be *grease-freed* by passing the *both sides* of the slides one by one on the uppermost area of flame for 1–2 times and rubbing with a piece of soft cloth.
- Store the slide in a covered slide-box.

Cleaning of Used Coverslips

- Coverslips are to be *immersed* in 5% Lysol/0.5–1% freshly prepared sodium hypochlorite solution for 4 hours or put them in a container filled with weak bleach-detergent solution (3 mL of detergent + 15 mL of liquid bleach + 200 mL water) for 2–3 hours.
- If Lysol/sodium hypochlorite solution is used → wash the coverslips with soap water.
- Rinse out the beaker with *tap water* four times, shaking gently.
- Rinse finally with distilled water/deionized water.
- Drain the coverslips by tipping them out on a pad of gauze.
- Keep them in a small Petri dish and dry in hot air oven at 60°C.

Cleaning of Glassware Containing Tissue Culture

- *Autoclave* the containers with autoclavable rubber stoppers at 121°C for 20 minutes.
- To remove the cellular materials *scrub* the containers with brush and wash in tap water.
- Place the containers in *mild soap solution* and boil for 15 minutes.
- Wash six times in tap water.
- Keep the glassware in distilled water with 0.05% 1N *hydrochloric acid* for 1 hour.
- Wash three times with distilled/deionized water.
- Dry the glassware → wrap in *aluminum foil* and sterilize in *hot air oven* at 160°C for 3 hours.

Cleaning of Rubber Stoppers

- Wash the *autoclaved* rubber stoppers with hot *tap* water.
- Keep them in 20% $NaHCO_3$ and boil for 20 minutes.
- Wash them with hot tap water → keep them in 0.05% 1N hydrochloric acid for 1 hour.
- Wash in hot tap water.
- After drying place them in aluminum container and autoclave at 121°C for 20 minutes.

Cleaning (Washing) of Hands

As described in Chapter 1.

Cleaning of Specimen Spills

As described in Chapter 1.

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Cleaning of Floor

- About 2–5% *Lysol/cresol* is used for cleaning of floor.
- *Before fumigation* OT floor should be flooded with 2–5% *Lysol/cresol* overnight.

STERILIZATION

Sterilization is the process of freeing of an article, medium or surface from all living microorganisms which include *bacteria* (both vegetative and spore), *fungi* and *viruses*.

Sterilization is extremely necessary to protect the HCW, general public and also to avoid the cross contamination of the articles which are to be reused.

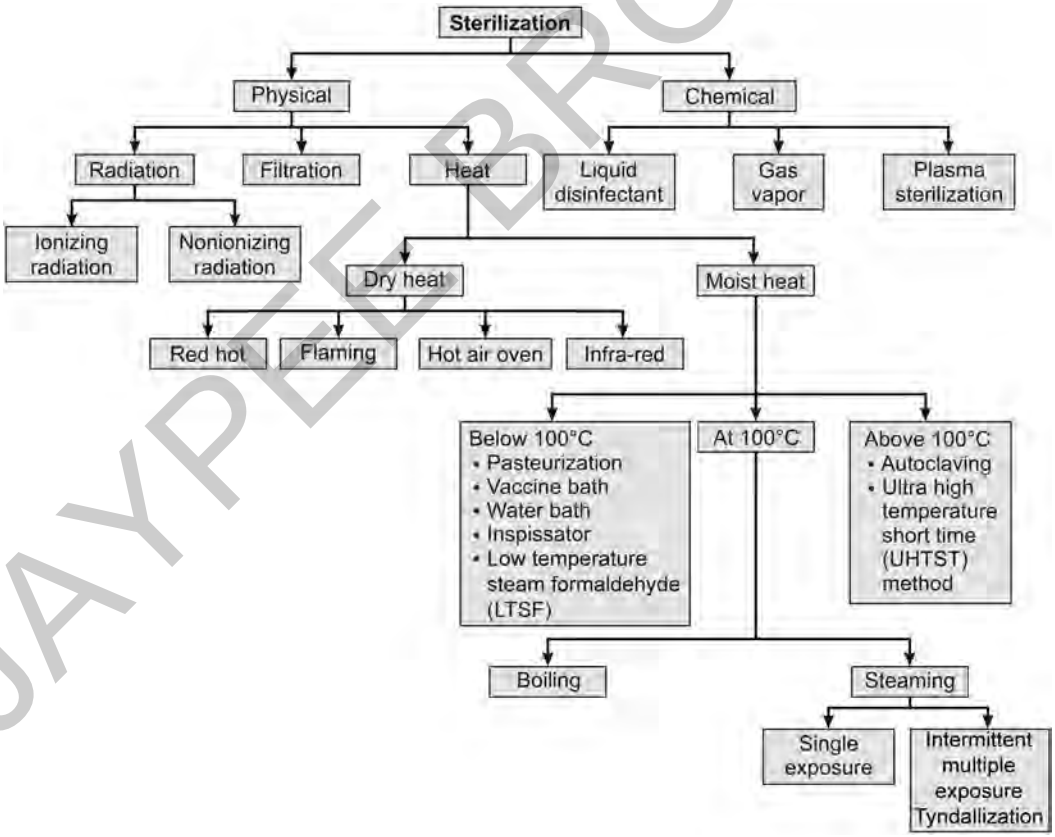
Methods of Sterilization

Flowchart 2.1 shows the methods of sterilization.

Holding Period

Holding period is the total duration of time required for sterilization of the articles at the desired temperature. The time required for the particular temperature to rise is not to be included.

Flowchart 2.1: Methods of sterilization.



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Processes of Sterilization

Physical Sterilization

Processes of sterilization is discusses in Table 2.1.

Table 2.1: Processes of sterilization.

Process	*Holding period/ temperature/time*	*Purpose served*
Radiation		
• Nonionizing radiation		
– Ultraviolet rays	2750Å/240–280 nm wave length	• Can make a bacteria-free zone in *biosafety cabinet* and at the entrance of OT • Cannot penetrate into solid and a few mm into liquid
• Ionizing radiation (cold sterilization) – Cathode rays (by accelerator) – Gamma rays (from cobalt 60) Efficiency checking of this process is done by: Radiochromic chemical/ ATCC # 14884. *Bacillus pumilus* spore strip	2.5 Mard/25 GY	Packed disposable syringes, needle, gloves, catheters can be sterilized. It can be penetrate deep into objects
Heat sterilization		
• Dry heat		
– Red hot	Object should be held in Bunsen-burner flame till red-hot	Inoculating loop wire, tips of needle, forceps, etc. can be sterilized
– Flaming	At 80°C instantaneous (through the flame for few seconds)	To sterilize the mouth of culture tubes and bottles
– Hot air oven	60°C for 30 minutes 100°C for 60 minutes 115°C for 60 minutes 120°C for 16 hours 140°C for 2½ hours 160°C for 2 hours 170°C for 60 minutes 180°C for 30 minutes	To dry the wet glassware To kill the vegetative bacteria To kill the fungal spore • To kill all microorganisms including bacterial spores • For sterilization of predried and packed glassware, e.g. test tubes, Petri dishes, flasks, cylinders, glass syringes with needles, pipettes, cotton wool swabs
Efficiency checking: Use of hot air oven:	Refer to Chapter 3 Refer to Chapter 3 Oils at 160°C for 2 hours Powder at 180°C for 30 minutes	• Powders, fat, petroleum jelly – These should be sterilized in small lots *not exceeding* 10 g and in layers *not exceeding* 0.5 cm depth in a dish
– Infrared radiation		Mainly for sterilization of packed disposable syringes, needles, gloves, catheters
Efficiency checking of this process is done with ATTC # 9372 *B. atrophaeus* (formerly *B. subtilis* var. niger)	Source employed is an electrically heated element to attend 180°C and infra-red rays are directed onto the objects	

Contd...

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Process	*Holding period/ temperature/time*	*Purpose served*
• Moist heat		
– Below 100°C Pasteurization		These two processes can destroy all nonspore forming pathogens that may found in milk except *Coxiella burnetii in* holder method
Holder method	At 63°C for 30 minutes	
Flash method	At 72°C for 15 seconds and instant cooling below 10°C	
Vaccine bath	At 60°C for 60 minutes	To kill most of the nonsporing bacteria but to preserve antigenicity in case of vaccine preparation
Water bath	At 56°C for 1 hour on several successive days	To sterile serum/body fluids containing coagulable protein
Inspissation	Between 80°C and 85°C for 1 hour (1st day) and further at 75°C–80°C for 30 minutes for 2 successive days	The solidifying of protein media and egg in media using heat to coagulate the protein is called *Inspissations* and that can be done by an automatically regulated water jacketed copper box called *Inspissator*
– At 100°C Boiling	Although at 100°C for 10–30 minutes (boiling) most of the vegetative form of bacteria are killed but many spores may withstand boiling for a considerable time. So when better method is not available only then it can be used	Glass syringes, needles, scalpels, forceps, scissors, rubber stoppers can be sterilized
Boiling with increasing pH	Exposure is same as boiling but with addition of 2% sodium carbonate in water	Same as boiling
• Steaming		
– By single exposure	At 100°C in free steam for 90 minutes (in Koch or Arnold steam sterilizer)	• To sterilize the media containing ingredients that would be broken down or inactivated at temperature over 100°C, e.g. Selenite F or Cary-Blair medium • To remelt previously bottled sterile media
– By intermittent multiple exposure		
Tyndallization	At 100°C for 20–45 minutes on each of 3 consecutive days	Where one exposure kill all the vegetative cells → Left overnight at room temperature to encourage the germination of endospores if any. The 2nd and 3rd day the medium is steamed to ensure the destruction of germinated spores
– Above 100°C Autoclaving (Different temperature pressure and holding periods are discussed in Table 2.2)	Complete sterilization can be done by moist heat in this process. After complete air discharge autoclaving is done at the temperature of 121°C (pressure = 15 psi/104 kpa) for 15–20 minutes	For sterilization of most culture media and other laboratory materials wrapped surgical dressings, linen, glassware and biohazard wastes before disposal
– Performance checking and use of autoclave	Refer to Chapter 3	
– UHTST (ultra high temperature short time) method	At 135°C for 2 seconds or at 125°C for 4 seconds and instant cooling	For preparation of ultra-heat treated milk

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Table 2.2: Different pressure, temperature and holding periods of autoclave.

Gauze pressure, i.e. pressure above atmosphere (1 lb/sq inch)	*Temperature (°C) after complete air discharge*	*Sterilizing time by autoclave*
5	109	2½ hours
10	115	50 minutes
15	121	15 minutes
20	126	10 minutes
30	134	3 minutes

Filtration

Many types of filters are used for sterilization. Those are:

- *Chamberland filter*: Made up of unglazed porcelain. Finer grades will allow to pass only certain viruses used to remove the organism from fluid culture in order to obtain bacterial toxin.
- *Berkefeld filter*: Made up of fossil diatomaceous earth.
  Viel type of this filter should not allow to pass a small organism. Used to sterilize fluid culture.
- *Seitz filter*: Made up of asbestos in disk-like shape and of various sizes. Used specially to sterilize serum and it does not pass *S. marcescens* like small organism.
- *Sintered glass filter*: Made up of finely ground glass fused together. Can be cleaned with warm strong sulfuric acid.
- *Cellulose membrane filter*: Made up of cellulose esters named *Millipore filte*r and obtained in size of 13 mm, 25 mm, 47 mm in diameter with 0.22 μm porosity and it is autoclavable. Swinex filter holders (autoclavable) are also available to take membrane filters. It is preferred to others because it filters more rapidly and absorbance is very low. So very small amount of fluid or serum can be filtered by it.
- *HEPA filter*: This filter is generally fitted with containment equipment like Biosafety cabinet, class 2 or Laminar air flow (LAF). It is also used in exhaust-ventilation system of laboratory. HEPA-mask is also available. This filter gives clean and almost sterile air in working area. It removes particles of 0.3 μ or larger (nowadays particles less than 0.1 μ are also arrested).

Chemical Sterilization

By liquid

- *Glutaraldehyde*: It is a good sterilant. A solution of 2% is used.
- *Peracetic acid*: It is an oxidizing agent and nontoxic. A solution of 0.2% is used.

By gas vapor

- *Chlorine dioxide*: It denatures protein by oxidation.
- *Hydrogen peroxide*: It is an effective sterilant and 30% at 55°–60°C is used for sterilization of instruments.
- *Ethylene oxide*: This gaseous disinfectant is colorless liquid; boiling point 10.7°C. Above this temperature it is moderately toxic and explosive if more than 3% is present in air.
  A nonexplosive mixture of 10% ethylene oxide in CO_2 or in halogenated hydrocarbon with a 20–40% relative humidity and 45°C–55°C temperature for 2–5 hours is employed in sterilization process.
  It is generally used to sterile the articles which is liable to damage by heat; like plastics, rubber articles, blankets, pharmaceutical products, surgical machines, disposable syringes and needles.
- *Efficiency checking* of the process: Introduce commercially available

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B. atrophaeus (formerly *B. subtilis* subs niger) ATCC # 9372 strip (available from Hi-media laboratories) at the time of process.

After completion of process put the strip into tryptone soya broth or in *recovery media* incorporated with company pack → incubate at 30°C–35°C aerobically for 7 days.

→ Clearing of broth indicates that the sterilization process has properly done.

- *Formaldehyde*: This irritant water-soluble gas is highly lethal to all kinds of microbes and spores and has a tendency to polymerize to paraformaldehyde.

  Commercial formalin contains 40% (W/V) formaldehyde gas in water to which 10% methanol has been added to prevent polymerization. It is used for disinfection by two ways:

  1. *Spraying*: Spraying of an equal volume mixture of cold formalin-industrial spirit in an atmosphere of 80–90% humidity and a temperature of at least 18°C is useful for disinfection.
  2. *Boiling*: Formaldehyde solution (50 mL) and water (100 mL) can be boiled for disinfection of 100 cubic feet area which gives a vapor concentration of 2 mg gas per liter of air.

  (Without boiling formaldehyde vapor can also be achieved by adding potassium permanganate (25 g) with formalin (50 mL) and water (100 mL) for disinfection of 100 cubic feet area but use of $KMnO_4$ has a risk of explosion if permanganate is at any time present in excess).

- *Plasma gas sterilization*: Plasma describes any gas that consists of electrons, ions or neutral particles. *Hydrogen peroxide vapor or hydrogen peroxide-peracetic acid* mixture is introduced into a chamber under reduced pressure in a dry atmosphere. Vapor diffusion occurs throughout the contents and radio frequency energy excites the H_2O_2 into active radicals and reactive chemical species (plasma). The resulting ultraviolet radiation cause rapid destruction of vegetative organisms including spores, viruses and fungus. On removing the energy source the plasma reverts to water and CO_2. The chamber is then evacuated and vented to atmosphere to dry the load. A high vacuum must be maintained and a low leak-back rate is essential in this process.

  Two commercial plasma sterilizers—*Sterrad 100 S sterilizer and Plazlyte sterilizer* are now being used.

  Efficiency checking of this process is done by using commercially available biological strip containing at least 10^6 spores of *Geobacillus stearothermophilus* (ATCC 7953) or *Bacillus subtilis subsp* niger (ATCC 9372). After culturing no reviveness of spores denotes that the sterilization process is done properly.

DISINFECTION

Disinfection is a process which reduces the number of pathogenic microorganisms but not necessarily bacterial spores from inanimate objects. When this process is used for destroying pathogenic microorganisms on skin/living tissue to a level which is not harmful to health is known as antisepsis.

CHEMICAL DISINFECTION

Although autoclaving or boiling is the method of first choice for sterilization but chemical disinfection can be employed at the last resort especially in case of heat stable articles like medical instruments and reusable equipment where single use is cost effective. Before using of chemical disinfectants one must be cautious about the duration of the action of the disinfectants.

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Influence Factors on the Action Rate of Chemical Disinfectant

Influence factors on the action rate of chemical disinfectant is discussed in Table 2.3.

Chemical Disinfectants

High level disinfectants **(formaldehyde, glutaraldehyde, peracetic acid, hydrogen peroxide)** are also used as sterilizing agent by increasing their concentration and duration of time.

Some Effective Disinfectants and Antiseptics

Aldehydes

Formaldehyde: It is a stable liquid used for disinfection of contaminated surface at the concentration of 5–10%. A 0.04–1.0% solution is used for killing and fixing the bacterial culture or suspension prior to bacterial counting or vaccine preparation. It is also used as sterilizing agent to fumigate the OT room (see gas vapor sterilization).

Table 2.3: Influence factors on the action rate of chemical disinfectant.

Factors	*Influence*
Concentration	In optimum and recommended concentration they work, otherwise may fail to act properly
Temperature	Rise in temperature usually increases the rate of disinfection
pH	Some disinfectants (e.g. glutaraldehyde) function only in alkaline solution whereas some compounds (e.g. phenol) work in acid medium
Formulation	Some disinfectants operate best in aqueous solution (e.g. ethanol acts properly at the dilution of 70%)
Deterioration	Prolong storage, improper dilution and exposure in air may cause of less effectiveness
Inactivation	Chemical disinfectants may be inactivated by certain materials or organic matter, e.g. rubber inactivates phenols and chlorhexidine. Organic matters present in blood may cause of inactivation

Glutaraldehyde: It is available in liquid or powder form. 2% aqueous solution is used to disinfect heat sensitive nondisposable equipment/instrument. Act within 30 minutes. 10 hours immersion is required to destroy spores. To activate add powder/liquid with the solution supplied.

Hydrogen peroxide: It liberates nascent oxygen which oxidizes necrotic matter. It is lethal to bacteria, virus, fungi and spore. Hydrogen peroxide at the concentration of 3–25% is used as high level disinfectant.

Potassium permanganate: It is a water-soluble germicidal which is used for gargling, douching, disinfection of pond and well's water.

Biguanide: *Chlorhexidine:* It is very useful disinfectant for skin preparation especially before bone marrow aspiration, lumbar puncture or any surgery. It is stable and a 0.5% concentration with 70% ethanol is used.

Phenolics: These liquid disinfectants are in liquid form. 2–5% (V/V) solution with water is made and the diluted solution is stable for 24 hours. Useful for cleaning of floor and also for decontamination of reusable articles before autoclaving.

Phenol: It is an antiseptic, relatively weak agent, poor action on spore.

Cresol: It is methyl phenol, more active, used for disinfection of utensils.

Lysol: It is a 50% soapy emulsion of cresol. Used for floor cleaning and decontamination of used articles before disposal or autoclaving.

Quaternary ammonium

Cetrimide: Alone or in combination with chlorhexidine, it is one of the most popular hospital antiseptic and disinfectant for surgical instruments, utensils, bath, etc.

Alcohol

Ethanol/2-propanol: Make 70% with water to use. It is used to disinfect the patient's skin

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surface, to clean laboratory instruments or exterior surface of specimen container but it is only active against bacteria but not viruses or spores.

Borax

Borax-formaldehyde: This liquid disinfectant is made by adding 5 g sodium tetraborate with 4% formaldehyde in 100 ml water. It is stable and used to disinfect the cleaned metallic instrument by overnight immersion in the solution.

Boric acid: It is only bacteriostatic and weak antiseptic.

Metallic salt

Ammoniated mercury: Acts as bacteriostatic. 10% ointment is used for dermatophytosis.

Dyes

Gentian violet: A rosaniline dye active against gram-positive bacteria and fungi only.

Halogens

Iodine and iodophor: Aqueous iodine and **tincture iodine** (iodine 25%, potassium iodide 2.5% and ethanol 70%) are rapid acting, broad spectrum (bacteria, fungi, viruses) bactericidal agent. Used as skin disinfectant. An another good antiseptic, betadine, has chemical complexes with iodine such as polyvinylpyrrolidone (Povidone, PVP) is used in a dilution of 1:2–1:100.

Chlorine compound

Calcium hypochlorite: It is available in powder form. Working solution is made by dissolving 1 g in 1 liter water to get 0.1% available chlorine. Purpose is as sodium hypochlorite.

Chloramine: It is available in powder or tablet form. To get 0.1% available chlorine (20 g is dissolve in 1 liter water) and for higher concentration dissolve 40 g in 1 liter water. It is an all-purpose disinfectant as sodium hypochlorite.

Sodium hypochlorite: It is an excellent universal disinfectant but only effective when it is freshly prepared.

- It is used with a concentration of 0.1% available chlorine (1 g/liter, 1000 ppm) for wiping work surfaces and specimen containers, cleaning of gloves, disinfection of reusable glass syringes and needles.
- To disinfect heavily soiled equipment, blood spills, etc. a stronger solution of 1.0% available chlorine (10 g/liter, 10,000 ppm) is used.
- Household bleach (liquid) generally contains 5% available chlorine.
- Sodium hypochlorite concentration may be expressed in calorimetric degrees (°chlorom). 1° chlorom = 0.3% available chlorine.
- Eau de Javel (15° chlorom) contains approximately 5% available chlorine.
- Chlorinated lime contains approximately 35% available chlorine.

DILUTION OF SODIUM HYPOCHLORITE SOLUTION

Table 2.4 enlist Parts of stock solution.

FUMIGATION AND CLEANING OF OPERATION THEATRE ROOM

For details *See* Chapter 8.

Table 2.4: Parts of stock solution: parts of water.

Required strength	*5% stock solution*	*10% stock solution*	*15% stock solution*
0.1% (1 g/l, 1000 ppm)	1:50	1:100	1:150
1.0% (10 g/l, 10,000 ppm)	1:5	1:10	1:15

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