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BASIC PRACTICAL MICROBIOLOGY

A MANUAL

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Factsheets to support this manual can be downloaded from the www.microbiologyonline.org.uk website.

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BASIC PRACTICAL MICROBIOLOGY

Part 1: THE BASICS

An introduction to microbiology, aseptic technique and safety

As well as causing a familiar range of diseases in animals and plants and problems in food spoilage and deterioration of other materials, microbes are also our "invisible allies". Indeed, life on Earth would not be sustainable without the benefits that many of them provide. The teaching of such an important subject as microbiology cannot be achieved effectively without enhancing the theory with "hands on" experience in the laboratory. The purpose of this manual is to provide teachers and technicians with good techniques in practical microbiology to ensure that investigations proceed safely and achieve the required educational aims successfully.

PREPARATION

Safety guidelines

The small size of microbes and the consequent need to deal with cultures that contain many millions of microbial cells require special procedures for their safe use. Activities involving micro-organisms are controlled by the *Control of Substances Hazardous to Health* (COSHH) *Regulations* and teachers and technicians have a duty under the *Health and Safety at Work Act* to comply with any safety instructions given by their employers. These include using model risk assessments for which it is necessary to refer to appropriate publications such as *Topics in Safety*, 3rd edition (ASE, 2001)*, Microbiology: an HMI Guide* (DES, 1990) and *Safety in Science Education* (DfEE, 1996). The guidelines are straightforward and largely commonsense and, as such, are not an obstacle to conducting interesting microbiological investigations in a school laboratory.

[Factsheet: *Safety Resources*]

Risk assessment

Teachers and technicians may make (but if so must record) sensible adjustments to model risk assessments according to their professional judgement based on the capabilities of the students, themselves and other local circumstances.

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Factors to be considered in risk assessment

Factor	***Relevance***
Level of practical work (Level 1, Level 2, Level 3, *Topics in Safety*, 3rd edition (ASE, 2001), Topic 15) or Factsheet: *Safety Guidelines*	Degree of risk of microbial culture; expertise of teacher; age range of students
Choice of micro-organisms (ACDP Hazard Group 1)	Present minimum risk; refer to list of suitable cultures
Source of cultures	Reputable specialist supplier or approved environmental sample
Type of investigations/activities	Adequate containment of cultures; class practical work *vs.* teacher demonstration
Composition of culture media	Possibility of selecting for growth of pathogens
Volume of cultures	Increased risk with increase in volume of liquid culture
Laboratory facilities	Suitability for level of practical microbiological work
Equipment	Adequate for purpose
Incubation conditions	Possibility of selecting for growth of pathogens
Disposal procedures	Ensures elimination of risk to others
Expertise of technicians and teachers	Competence and level of training in techniques and procedures appropriate to level of practical work
Student age and discipline	Appropriate to level of practical work; confidence in class discipline
Sources of competent advice	ASE*, CLEAPSS*, MISAC, NCBE, SSERC* *members only
Useful check list	*Topics in Safety*, 2nd edition (ASE, 1988), pp 34-37
Essential reference	*Topics in Safety*, 3rd edition (ASE, 2001), Topic 15 or Factsheet: *Safety Guidelines*

Key to abbreviations: Advisory Committee on Dangerous Pathogens (ACDP); Association for Science Education (ASE); Consortium of Local Education Authorities for the Provision of Science Services (CLEAPSS); Microbiology in Schools Advisory Committee (MISAC); National Centre for Biotechnology Education (NCBE); Scottish Schools Equipment Research Centre (SSERC)

[Factsheet: *Safety Guidelines;* Factsheet: *Safe Micro-organisms*]

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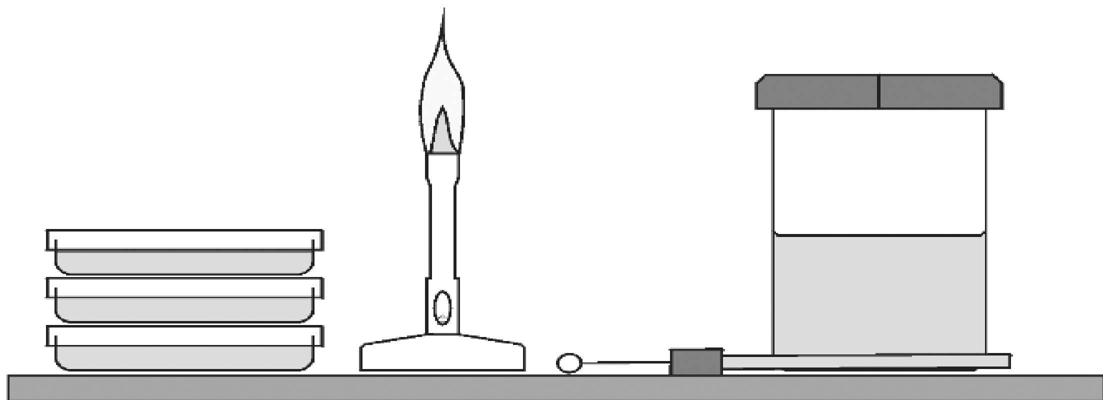
Good microbiological laboratory practice (GMLP)

Training in **GMLP** is aimed at developing proficiency in containing any uncontrolled spread of microbes in order to protect:

- practical investigations from becoming contaminated with microbes from external sources
- the operators (students, teachers and technicians) from the very small possibility of infection.

It is important to arrange the workplace carefully to ensure safe and effective operations.
[Factsheet: *Good Laboratory Practice for All*]



Spillage management

Spills

Spillages of cultures must be reported immediately to the teacher or technician to be dealt with quickly. The keeping of a record of all such incidents is recommended. Spilled cultures and surrounding debris (e.g. glass, cotton wool plugs), if any, **must not** be touched with unprotected hands. Wearing disposable gloves, disinfect the area by covering the spill with several layers of paper towel/cloth soaked in a suitable disinfectant (see " Commonly available disinfectants and their uses" page 7) and leave for 15-30 minutes. Spill debris should then be swept into a dustpan using paper towels. All disposable material should then be transferred to a suitable container, e.g. an autoclave/roasting bag, for autoclaving and disposal. The dustpan must be decontaminated either by autoclaving or by soaking (at least 24 hours) in *Hypochlorite (sodium chlorate I).*

Broken glass

Observe an appropriate disposal procedure for broken glass if present. It should be swept carefully into a suitable container, autoclaved and disposed of in a puncture proof container.

Splashes on clothing and the skin

Contaminated clothing should be soaked in disinfectant. Splashes on the skin should be treated as soon as possible; washing with soap and hot water should be sufficient, but if necessary the skin can be disinfected.

> HINT
> It is useful to have a spillage kit always at hand ready for use.
> Suggested components:
> - beaker for making fresh disinfectant,
> - disposable gloves,
> - dustpan and brush,
> - paper towel/cloth,
> - autoclave/roasting bag

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Aerosols

Spillages also carry a risk of generating aerosols (an invisible "mist" of small droplets of moisture) which may contain microbes and might be inhaled. The risk of spillages occurring is lessened by using cultures grown on agar instead of in liquid media whenever possible. Care should also be taken to avoid generating aerosols during practical work. The risk is minimized by adhering to GMLP with special attention to the correct use of pipettes (see "Inoculation and other aseptic procedures" page 8).

RESOURCES

Equipment

Equipment	***Use***
Loop (wire/plastic)	Routine inoculation of agar slopes/deeps and small volumes of liquid media (up to *ca* 10cm^3); making streak plates
Straight wire	Inoculation from very small colonies; transfer of small inocula from liquid media for nutritional work
Spreader (glass/plastic)	Making lawn/spread plates
Forceps (metal/plastic)	Transfer of sterile paper/antibiotic discs; also plant material, *e.g.* short lengths of root with nodules
Pipette (calibrated/ dropping; glass/ plastic)	Transfer of measured volumes/drops of culture/sterile solutions (dry, non-absorbent cotton wool plug in neck prevents contamination)
Teat	Filling and emptying pipettes safely (never pipette by mouth)
Test tube	Small volumes (*ca* 5-10 cm^3) of liquid media/agar slopes/sterile solutions for inoculation (held in test tube rack; dry non-absorbent cotton wool plug or plastic cap prevents contamination)
Universal bottle (wide neck); McCartney bottle (narrow neck)	Volumes of liquid and agar media/sterile solutions up to *ca* 20 cm^3 for inoculation or for storing sterile media or stock cultures on agar slopes (stay upright on bench; plastic screw cap prevents contamination and reduces evaporation during long storage)
Bijou bottle	Very small volumes (up to *ca* 3 cm^3) of sterile solutions (stay upright on bench; plastic screw cap prevents contamination)
Medical flat	Large volumes of sterile media/solutions for storage; available in range of capacities, 50 - 500 cm^3 (plastic screw cap prevents contamination and reduces evaporation during long storage)
Conical flask	Large volumes of liquid media for inoculation and liquid/media for short-term storage (non-absorbent cotton wool plug prevents contamination but does not reduce evaporation during long storage)
Petri dish (plastic/ glass)	Plastic: pre-sterilised for streak/spread/lawn/ pour plates; Glass: only for materials for sterilization by hot air oven, *e.g.* paper discs
Marker pen	Labelling Petri dishes, test tubes, flasks, bottles and microscope slides
Personal protective equipment (Level 2, Level 3, *Topics in Safety*, 3rd edition (ASE, 2001), Topic 15) or Factsheet: *Safety Guidelines*	Clean laboratory coat/apron: protection of clothing, containment of dust on clothing; Safety spectacles: not considered essential when dealing with suitable cultures and observing GMLP but may be required by local regulations and for dealing with chemicals

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Apparatus

Apparatus	***Use***
Bunsen burner	Sterilization of wire loops and (with alcohol) metal forceps and glass spreaders
Impervious sheet or tray	Provides individual student working area if the bench surface is not appropriately sealed
Autoclave/pressure cooker	Sterilization of media, solutions and equipment before use and contaminated items afterwards; melting solidified agar media for use.
Gas ring/hot plate	Steam generation in autoclave
Autoclavable/roasting bag	Holds contaminated items in autoclave to contain spillages
Hot air oven	Sterilization of glass Petri dishes and pipettes and paper discs (but not essential as autoclaves/ pressure cookers serve virtually all needs)
Discard pots containing disinfectant	Disposal of used pipettes and slides of non-stained microscopical preparations
Microwave oven	Melting solidified agar media for use (but not in vessels with metal caps and not for sterilization)
Incubator	Incubation of cultures (but many cultures will grow at room temperature in the interval between lessons)
Water bath	Suitable temperature for keeping melted agar media molten for use (*ca* 50ºC); accurate temperature control
Thermometer	Checking incubator/water bath temperatures
pH meter	Checking and adjusting pH values of media
Cupboard	Storage of culture media and stock cultures
Refrigerator	Storage of heat-labile materials
Microscope, slides, cover slips, stains, staining rack, immersion oil	Microscopical observations

Materials

Material	***Use***
Culture media ingredients	Stock of a range of culture media in dehydrated form (tablets/powder); available as complete media and as separate ingredients
Disinfectants	Treatment of work surface before and after use and spillages; disposal of used pipettes and microscope slides; in soap form for hand washing
Ethanol (70% industrial methylated spirit)	Sterilization of metal forceps and glass spreaders by ignition
Autoclave indicator tape	Changes colour in response to heat to distinguish those items that have received heat treatment (but is not an indicator of effective sterilization)
Sterilizer control tube/strip	Changes colour when correct temperature has been applied and held for the required length of time to effect sterilization
Non absorbent cotton wool	Plugs for test tubes, flasks and pipettes
Spillage kit	Dealing with spilled cultures

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MEDIA, STERILIZATION AND DISINFECTANTS

MEDIA

Preparation of culture media

Rehydrate tablets or powder according to manufacturer's instructions. Before sterilization, ensure ingredients are completely dissolved, using heat if necessary. Avoid wastage by preparing only sufficient for either immediate use (allowing extra for mistakes) or use in the near future. Normally allow 15-20 cm^3 medium/ Petri dish. Dispense in volumes appropriate for sterilization in the autoclave/pressure cooker.
Agar slopes are prepared in test tubes or Universal/McCartney bottles by allowing sterile molten cooled medium to solidify in a sloped position.
Bottles of complete, sterile media are available from suppliers but are expensive.
[Factsheet: *Suppliers of Cultures and Equipment*]

Pouring a plate

1. Collect one bottle of sterile molten agar from the water bath.
2. Hold the bottle in the left hand; remove the lid with the little finger of the right hand.
3. Flame the neck of the bottle.
4. Lift the lid of the Petri dish slightly with the right hand and pour the sterile molten agar into the Petri dish and replace the lid.
5. Flame the neck of the bottle and replace the lid.
6. Gently rotate the dish to ensure that the medium covers the plate evenly.
7. Allow the plate to solidify.
8. Seal and incubate the plate in an inverted position.

(The base of the plate must be covered, agar must not touch the lid of the plate and the surface must be smooth with no bubbles).

Storage of media

Store stocks of prepared media at room temperature away from direct sunlight; a cupboard is ideal but an open shelf is satisfactory. Media in vessels closed by cotton wool plugs that are stored for future use will be subject to evaporation at room temperature; avoid wastage by using screw cap bottles. Re-melt stored agar media in boiling water bath, pressure cooker or microwave oven. Sterile agar plates can be pre-poured and stored in well-sealed plastic bags (media-containing base uppermost to avoid heavy condensation on lid).

STERILIZATION vs. DISINFECTION

Sterilization means the complete destruction of all the micro-organisms including spores, from an object or environment. It is usually achieved by heat or filtration but chemicals or radiation can be used.

Disinfection is the destruction, inhibition or removal of microbes that may cause disease or other problems e.g. spoilage. It is usually achieved by the use of chemicals.

STERILIZATION

Use of the autoclave/pressure cooker

The principle of sterilization in an autoclave or pressure cooker is that steam under pressure is used to produce a temperature of 121ºC which if held for 15 minutes will kill all micro-organisms including bacterial endospores. For further information on the use of the autoclave/pressure cooker see [Factsheet: *Use of the Autoclave/Pressure Cooker*]

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Sterilization of equipment and materials

Wire loop

Heat to redness in Bunsen burner flame.

Empty glassware and glass (not plastic!) pipettes and Petri dishes

Either, hot air oven, wrapped in either greaseproof paper or aluminium and held at 160ºC for 2 hours, allowing additional time for items to come to temperature (and cool down!).
Or, autoclave/pressure cooker.
Note: plastic Petri dishes are supplied in already sterilised packs; packs of sterile plastic pipettes are also available but cost may be a consideration.

Culture media and solutions

Autoclave/pressure cooker.

Glass spreaders and metal forceps

Flaming in alcohol (70% industrial methylated spirit).

DISINFECTANTS

Choice, preparation and use of disinfectants

Specific disinfectants at specified working strengths are used for specific purposes. The choice is now much more straightforward as the range available from suppliers has decreased.

Commonly available disinfectants and their uses

Disinfectant	*Use*	*Working strength*
VirKon	Work surfaces, discard pots for pipettes and slides, skin disinfection Spillages	1% (v/v) Powder
Hypochlorite (sodium chlorate I)	Discard pots for pipettes and slides	2500 ppm (0.25%, v/v) available chlorine
Ethanol	Skin disinfection	70% (v/v) industrial methylated spirit

When preparing working strength solutions from stock for class use and dealing with powder form, wear eye protection and gloves to avoid irritant or harmful effects.
Disinfectants for use at working strength should be freshly prepared from full strength stock or powder form. Activity of VirKon solution may remain for up to a week (as indicated by retention of pink colour) but less, e.g. 1 day, after use. Use working strength hypochlorite on day of preparation.

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INOCULATION AND OTHER ASEPTIC PROCEDURES

Essential points

There are several essential precautions that must be taken during inoculation procedures to control the opportunities for the contamination of cultures, people or the environment.

- Operations must not be started until all requirements are within immediate reach and must be completed as quickly as possible.
- Vessels must be open for the minimum amount of time possible and while they are open all work must be done close to the Bunsen burner flame where air currents are drawn upwards.
- On being opened, the neck of a test tube or bottle must be immediately warmed by flaming so that any air movement is outwards and the vessel held as near as possible to the horizontal.
- During manipulations involving a Petri dish, exposure of the sterile inner surfaces to contamination from the air must be limited to the absolute minimum.
- The parts of sterile pipettes that will be put into cultures or sterile vessels must not be touched or allowed to come in contact with other non-sterile surfaces, e.g. clothing, the surface of the working area, outside of test tubes/bottles.

Using a wire loop

Wire loops are sterilized using red heat in a Bunsen flame before and after use. They must be heated to red hot to make sure that any contaminating bacterial spores are destroyed. The handle of the wire loop is held close to the top, as you would a pen, at an angle that is almost vertical. This leaves the little finger free to take hold of the cotton wool plug/ screw cap of a test tube/bottle.

Flaming procedure

The flaming procedure is designed to heat the end of the loop gradually because after use it will contain culture, which may "splutter" on rapid heating with the possibility of releasing small particles of culture and aerosol formation.

1. Position the handle end of the wire in the light blue cone of the flame. This is the cool area of the flame.
2. Draw the rest of the wire upwards slowly up into the hottest region of the flame, (immediately above the light blue cone).
3. Hold there until it is red hot.
4. Ensure the full length of the wire receives adequate heating.
5. Allow to cool then use immediately.
6. Do not put the loop down or wave it around.
7. Re-sterilize the loop immediately after use.

> HINT
> If a loop does not hold any liquid the loop has not made a complete circle. To correct the problem, first ensure that the loop has been sterilized and then reshape the loop with forceps. Do not use your fingers because of the possibility of puncturing the skin.

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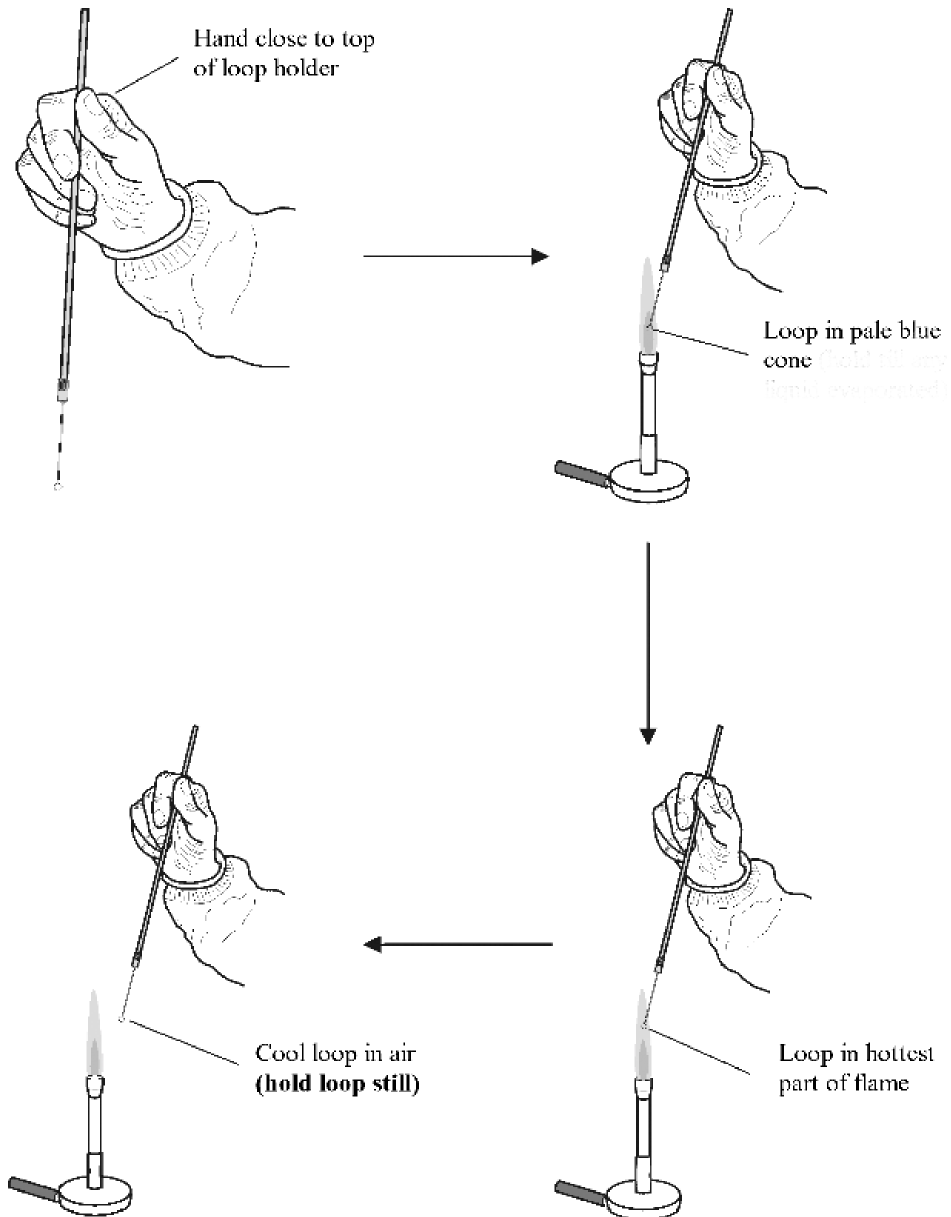
Flaming a loop



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Using a pipette

Sterile graduated or dropping (Pasteur) pipettes are used to transfer cultures, sterile media and sterile solutions.

1. Remove the pipette from its container/ wrapper by the end that contains a cotton wool plug, taking care to touch no more than the amount necessary to take a firm hold.
2. Fit the teat.
3. Hold the pipette barrel as you would a pen but do not grasp the teat.
   The little finger is left free to take hold of the cotton wool plug/lid of a test tube/bottle and the thumb to control the teat.
4. Depress the teat cautiously and take up an amount of fluid that is adequate for the amount required but does not reach and wet the cotton wool plug.
5. Return any excess gently if a measured volume is required.

The pipette tip must remain beneath the liquid surface while taking up liquid to avoid the introduction of air bubbles which may cause "spitting" and, consequently, aerosol formation when liquid is expelled.

6. Immediately put the now contaminated pipette into a nearby discard pot of disinfectant.
   The teat must not be removed until the pipette is within the discard pot otherwise drops of culture will contaminate the working surface.

> HINT
> A leaking pipette is caused by either a faulty or ill-fitting teat or fibres from the cotton wool plug between the teat and pipette.
> A dropping (Pasteur) pipette can be converted to delivering measured volumes by attaching to a non-sterile syringe barrel by rubber tubing.

Flaming the neck of bottles and test tubes

1. Loosen the lid of the bottle so that it can be removed easily.
2. Lift the bottle/test tube with the left hand.
3. Remove the lid of the bottle/cotton wool plug with the little finger of the right hand. (Turn the bottle, not the lid.)
4. Do not put down the lid/cotton wool plug.
5. Flame the neck of the bottle/test tube by passing the neck forwards and back through a hot Bunsen flame.
6. Replace the lid on the bottle/cotton wool plug using the little finger. (Turn the bottle, not the lid.)

> HINT
> Label tubes and bottles in a position that will not rub off during handling. Either marker pens or self-adhesive labels are suitable.
> Occasionally cotton wool plugs accidentally catch fire. Douse the flames by immediately covering with a dry cloth, not by blowing or soaking in water.

Inoculation of media in screw cap bottles and test tubes is usually done by loop.

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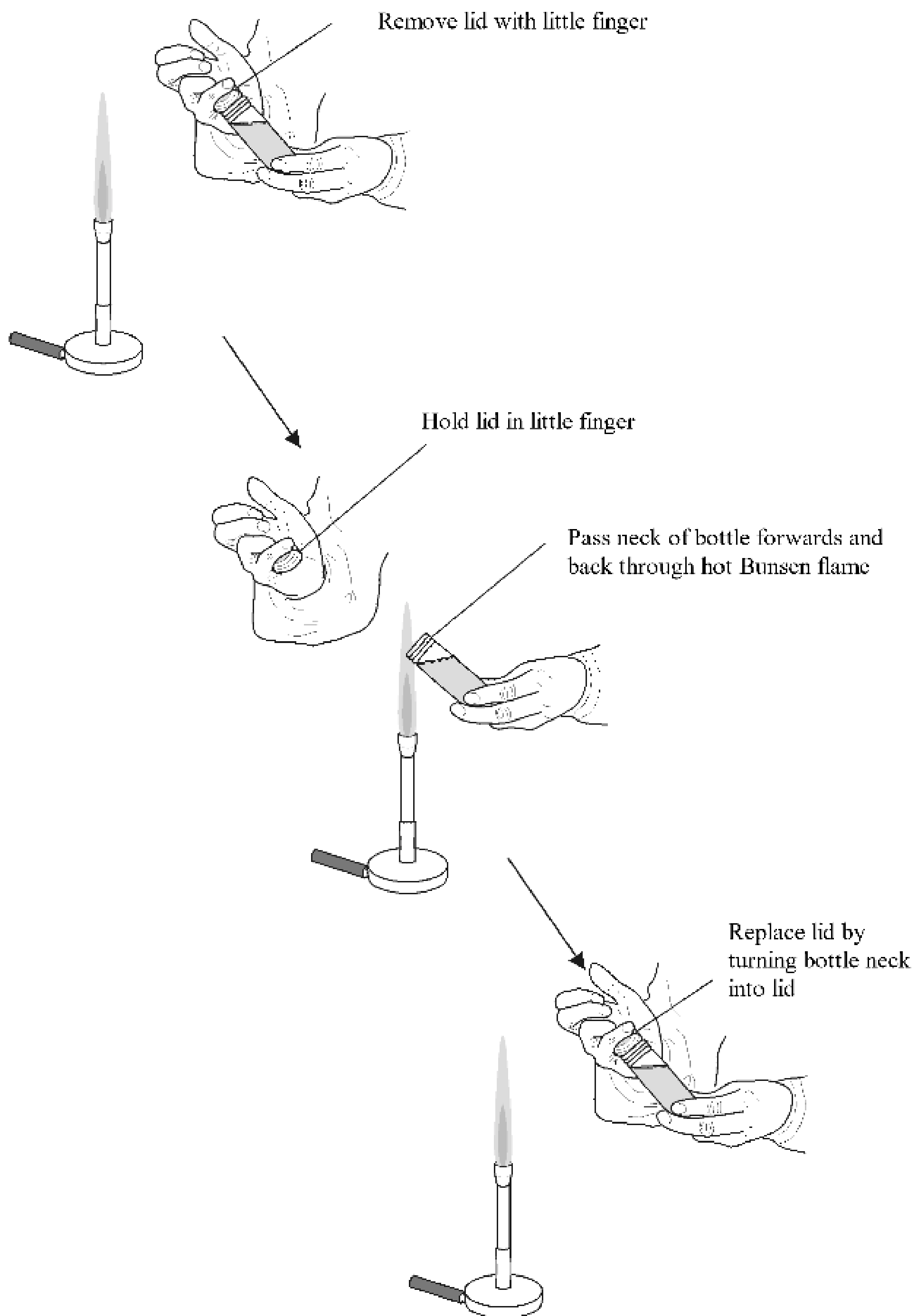
Flaming the neck of bottles



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Streak plate.

The loop is used for preparing a streak plate. This involves the progressive dilution of an inoculum of bacteria or yeast over the surface of solidified agar medium in a Petri dish in such a way that colonies grow well separated from each other.
The aim of the procedure is to obtain single isolated pure colonies.

1. Loosen the top of the bottle containing the inoculum.
2. Hold the loop in the right hand.
3. Flame the loop and allow to cool.
4. Lift the bottle/test tube containing the inoculum with the left hand.
5. Remove the lid/cotton wool plug of the bottle/test tube with the little finger of the left hand.
6. Flame the neck of the bottle/test tube.
7. Insert the loop into the culture broth and withdraw.

At all times, hold the loop as still as possible.

8. Flame neck of the bottle/test tube.
9. Replace the lid/cotton wool plug on the bottle/test tube using the little finger. Place bottle/test tube on bench.
10. Partially lift the lid of the Petri dish containing the solid medium.
11. Hold the charged loop parallel with the surface of the agar; smear the inoculum backwards and forwards across a small area of the medium (see diagram 1 streaked area =A).
12. Remove the loop and close the Petri dish.
13. Flame the loop and allow it to cool. Turn the dish through 90º anticlockwise.
14. With the cooled loop streak the plate from area A across the surface of the agar in three parallel lines (to B see diagram 2). Make sure that a small amount of culture is carried over.
15. Remove the loop and close the Petri dish.
16. Flame the loop and allow to cool. Turn the dish through 90º anticlockwise again and streak from B across the surface of the agar in three parallel lines (to C see diagram 3).
17. Remove the loop and close the Petri dish.
18. Flame the loop and allow to cool. Turn the dish through 90º anticlockwise and streak loop across the surface of the agar from C into the centre of the plate (to D see diagram 3).
19. Remove the loop and close the Petri dish. Flame the loop.
20. Seal and incubate the plate in an inverted position.

HINT

Label the half of the dish that contains medium; use abbreviations and keep them to the edge of the plate so as not to interfere with the later observation of colonies. The same applies to the pour and spread plates described below. Either marker pens or self-adhesive labels are suitable.
There are two approaches to making a streak plate: (1) with the base (containing medium) placed on the working surface, lift the lid vertically (i.e. still covering the base) the least amount that will allow access of the loop; (2) with the lid placed on the working surface, lift out the base, invert it and inoculate the upwards - facing agar surface. The second method is best reserved for older students working in a relatively dust and draught-free laboratory; it is the one used by professional microbiologists.

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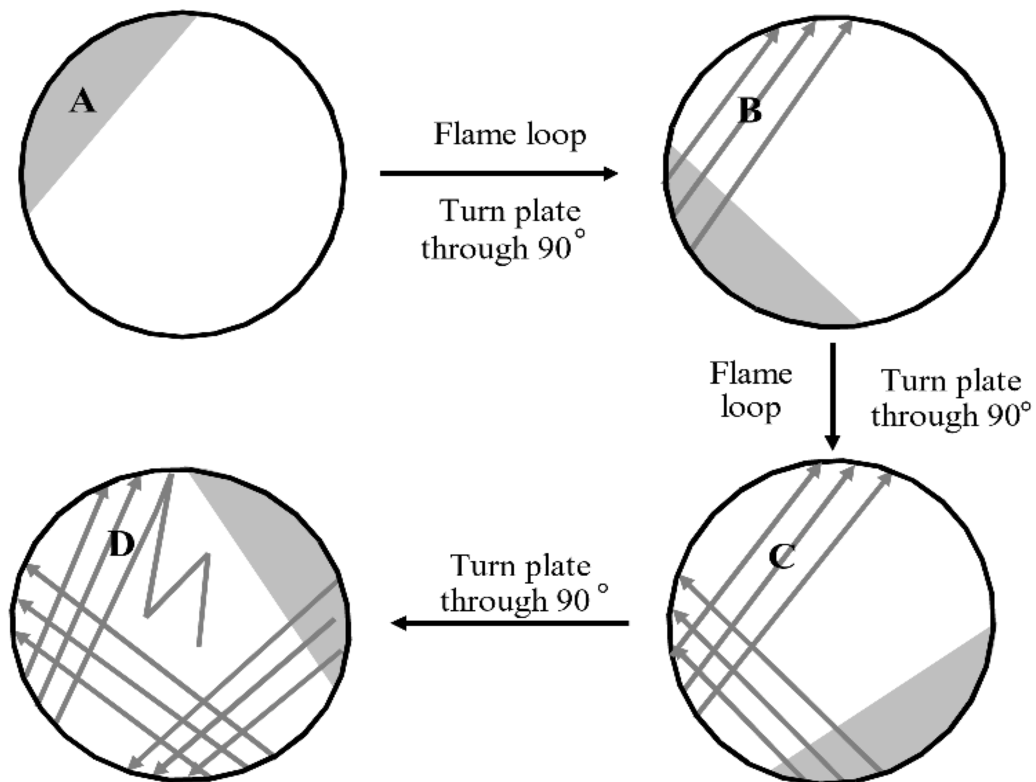
Streak plate



Pour plate

A pour plate is one in which a small amount of inoculum from broth culture is added by pipette to a molten, cooled agar medium in a test tube or bottle, distributed evenly throughout the medium, thoroughly mixed and then poured into a Petri dish to solidify. Pour plates allow micro-organisms to grow both on the surface and within the medium. Most of the colonies grow within the medium and are small in size; the few that grow on the surface are of the same size and appearance as those on a streak plate.
If the dilution and volume of the inoculum, usually 1 cm^3, are known, the viable count of the sample i.e. the number of bacteria or clumps of bacteria, per cm^3 can be determined.

Inoculation using a Pasteur pipette

1. Loosen the top of the bottle containing the inoculum.
2. Remove the sterile Pasteur pipette from its container, attach the bulb and hold in the right hand.
3. Lift the bottle/test tube containing the inoculum with the left hand.
4. Remove the lid/cotton wool plug with the little finger of the right hand.
5. Flame the bottle/test tube neck.
6. Squeeze the teat bulb of the pipette very slightly, put the pipette into the bottle/test tube and draw up a little of the culture. Do not squeeze the teat bulb of the pipette after it is in the broth as this could cause bubbles and possible aerosols.
7. Remove the pipette and flame the neck of the bottle/test tube again, before replacing the lid/cotton wool plug.
8. Place bottle/test tube on bench.

At all times hold the pipette as still as possible.

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Inoculating the cooled molten nutrient agar bottle

1. Pick up the cooled molten nutrient agar bottle.
2. Remove the lid from the molten nutrient agar bottle with the little finger of the right hand which still holds the charged pipette. Do not put down the lid.
3. Flame the neck of the bottle.
4. Insert the pipette into the bottle and gently release the required number of drops of inoculum onto the agar.
5. Flame the neck of the bottle and replace the lid.
6. Put the pipette into a discard pot. Remove the teat while the pipette is pointing into the disinfectant.

Pouring the pour plate

1. Roll the bottle gently between the hands to mix the culture and the medium thoroughly. Avoid making air bubbles.
2. Hold the bottle in the left hand; remove the lid with the little finger of the right hand.
3. Flame the neck of the bottle.
4. Lift the lid of the Petri dish slightly with the right hand and pour the mixture into the Petri dish and replace the lid.
5. Flame the neck of the bottle and replace the lid.
6. Gently rotate the dish to ensure that the medium covers the plate evenly.
7. Allow the plate to solidify.
8. Seal and incubate the plate in an inverted position.

(The base of the plate must be covered, agar must not touch the lid of the plate and the surface must be smooth with no bubbles).

Pouring the inoculated medium

> **HINT**
> If pipettes are not available then a wire loop can be used. Several loopfuls of culture must be added to the cooled molten nutrient agar to ensure that there is enough inoculum present for growth.

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Using a spreader

Sterile spreaders are used to distribute inoculum over the surface of already prepared agar plates.

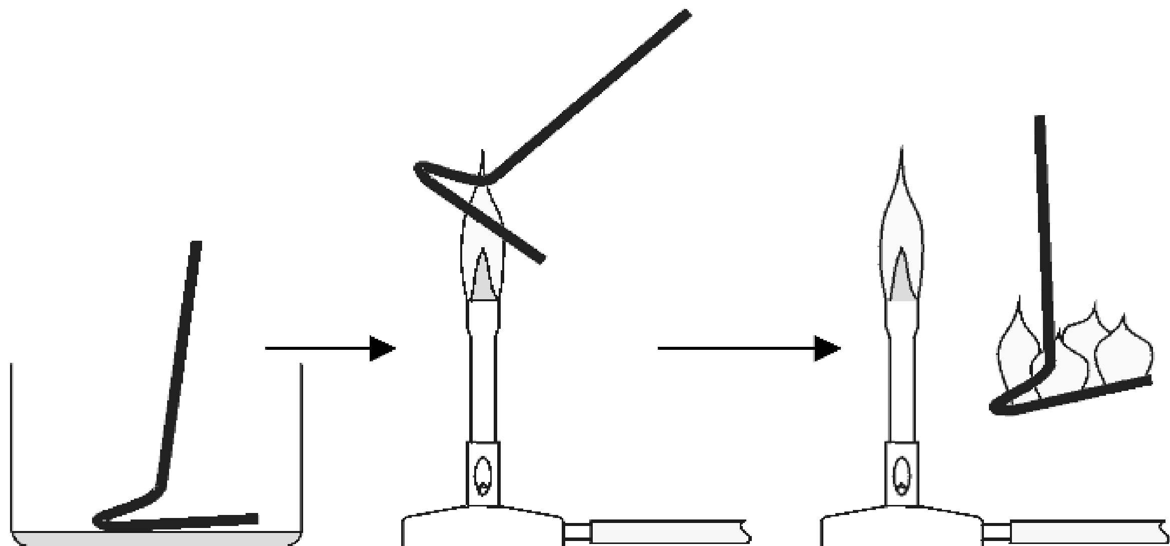
> HINT
> It is advisable to use agar plates that have a well-dried surface so that the inoculum dries quickly. Dry the surface of agar plates by either incubating the plates for several hours, e.g. overnight, beforehand or put them in a hot air oven (*ca* 55-60ºC) for 30-60 minutes with the two halves separated and the inner surfaces directed downwards.

Wrapped glass spreaders may be sterilized in a hot air oven (see "Media, Sterilization and Disinfectants" page 6). They can also be sterilized by flaming with alcohol.

Sterilization using alcohol

1. Dip the lower end of the spreader into a small volume of 70% alcohol contained in a vessel with a lid (either a screw cap or aluminium foil).
2. Pass quickly through a Bunsen burner flame to ignite the alcohol; the alcohol will burn and sterilize the glass.
3. Remove the spreader from the flame and allow the alcohol to burn off.
4. Do not put the spreader down on the bench.

Flaming a glass spreader



> HINT
> Ensure that the spreader is pointing downwards when and after igniting the alcohol to avoid burning yourself.
> Keep the alcohol beaker away from the Bunsen flame.

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Spread plate

Spread plates, also known as lawn plates, should result in a culture spread evenly over the surface of the growth medium. This means that they can be used to test the sensitivity of bacteria to many antimicrobial substances, for example mouthwashes, garlic, disinfectants and antibiotics.
The spread plate can be used for quantitative work (colony counts) if the inoculum is a measured volume, usually 0.1 cm^3, of each of a dilution series, delivered by pipette.

1. Loosen the lid of the bottle containing the broth culture.
2. Hold a sterile pipette in the right hand and the bottle/test tube containing the broth culture in the left.
3. Remove the lid/plug of the bottle/test tube with the little finger of the right hand and flame the neck.
4. With the pipette, remove a small amount of broth.
5. Flame the neck of the bottle/test tube and replace the lid/plug.
6. With the left hand, partially lift the lid of the Petri dish containing the solid nutrient medium.
7. Place a few drops of culture onto the surface about 0.1 cm^3 (ca 5 drops, enough to cover a 5 pence piece).
8. Replace the lid of the Petri dish.
9. Place the pipette in a discard jar.
10. Dip a glass spreader into alcohol, flame and allow the alcohol to burn off.
11. Lift the lid of the Petri dish to allow entry of spreader.
12. Place the spreader on the surface of the inoculated agar and, rotating the dish with the left hand move the spreader in a top-to-bottom or a side-to-side motion to spread the inoculum over the surface of the agar. Make sure the entire agar surface is covered.

This operation must be carried out quickly to minimize the risk of contamination.

13. Replace the lid of the Petri dish.
14. Flame spreader using alcohol.
15. Let the inoculum dry.
16. Seal and incubate the plate in the inverted position.

HINT

Consider the calibrated drop (Miles and Misra) method for colony counts of pure cultures of bacteria and yeast as a more economical method than the pour plate and spread plate. The procedure is as for the spread plate but fewer plates are needed because: (1) the inoculum is delivered as drops from a dropping pipette that is calibrated (by external diameter of the tip) to deliver drops of measured volume e.g. 0.02 cm^3; (2) many drops (six or more) can be put on one plate. The method is not suitable for use with cultures that produce spreading growth including mixed cultures in many natural samples such as soil although yoghurt and cheese are among the exceptions.

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INCUBATION

Note the previous comments on labelling (see "Inoculation and other aseptic procedures" page 8).

For guidance on incubation temperatures see [Factsheet: *Safety Guidelines*].

The lid and base of an agar plate should be taped together with 2-4 **short** strips of adhesive tape as a protection from accidental (or unauthorized!) opening during incubation. (Although tape is the preferred method Parafilm could be used as an alternative for sealing the plates.)

Agar plates must be incubated with the medium-containing half (base) of the Petri dish uppermost otherwise condensation will occur on the lid and drip onto the culture. This might cause colonies to spread into each other and risk the spillage of the contaminated liquid.

The advantages of incubators are that they may be set at a range of temperatures and reduce the possibility of cultures being interfered with or accidentally discarded. However, many cultures suitable for use in schools will grow at room temperature in the interval between lessons and can be incubated satisfactorily in a cupboard. The temperature of an incubator varies from the set temperature, oscillating by several degrees in the course of use.

Water baths are used when accurately controlled temperatures are required, e.g. for enzyme reactions and growth-temperature relationships, when temperature control of incubators is not sufficiently precise. They should be used with distilled or deionised water to prevent corrosion and emptied and dried for storage.

HINT

Overlong incubation of mould cultures will result in massive formation of spores which readily escape, particularly from Petri dishes, and may cause contamination problems in the laboratory and be a health hazard. This can occur in an incubator, at room temperature and even in a refrigerator.

IN CONCLUSION: CLEARING UP

Working surfaces must be cleared after use. If cultures have been used the benches must be swabbed with disinfectant *(VirKon* see "Choice, preparation and use of disinfectant" page 7*)*.

Discarded cultures, empty media tubes and all contaminated material must be placed in the appropriate labelled receptacles. Discard containers must be carefully and securely packed and never overloaded. Plastic Petri dishes must never be stacked above the lip of the discard container.

Cultures and contaminated paper towels, gloves etc. must be autoclaved at 121ºC for 15 minutes before disposal.

Slides, pipettes and Pasteur pipettes must be discarded in the appropriate containers of *Hypochlorite (sodium chlorate 1*) (see "Choice, preparation and use of disinfectant" page 7*)*. They must be soaked for at least 24 hours before disposal.
Never discard sharp or broken items in a way which would endanger anyone (see "Spillage management" page 3).

After sterilization, all materials can be disposed of with normal waste. Care must be taken that glass is adequately packaged to prevent injury.

Before leaving the laboratory, laboratory coats must be removed and hands washed with hot water and soap.

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Part 2: MICROBIOLOGY IN ACTION

Essential methods for maintaining, preparing and using cultures

MAINTENANCE, PREPARATION AND SUB-CULTURING

Obtaining suitable cultures

Micro-organisms on the list approved for use in schools and colleges (see under "Preparation" page 1) present minimum risk given observance of GMLP. The list is not definitive; other organisms may be used if competent advice is taken. Ensure that the current version of the list is consulted because recommendations are altered from time to time with changes in experience and assessment of the risks. Cultures must be obtained from a reputable specialist schools supplier. Isolation of cultures from the environment may be conducted if appropriate to the level of work (i.e. Level 1, Level 2 or Level 3 see Factsheet: *Safety Guidelines*).
[Factsheet: *Safe Micro-organisms*, Factsheet: *Suppliers of Cultures and Equipment*]

Pure cultures

The ability to keep pure cultures from becoming contaminated during inoculation and use is a key feature of GMLP. This skill is crucial for reasons of safety and for maintaining the scientific integrity of an investigation. Clearly, it is also vital skill to recognize when a culture has become contaminated.

Maintaining stock cultures

It may be convenient to maintain a stock of a pure culture instead of re-purchasing it when needed. Most of those considered suitable for use are also relatively easy to maintain by sub-culturing on the medium appropriate for growth but maintenance of stock cultures needs to be well organized with attention to detail. Be prepared to transfer cultures four times a year to maintain viability. Cultures on streak plates are not suitable as stock cultures. Slope cultures in screw cap bottles are preferred because the screw cap reduces evaporation and drying out and cannot be accidentally knocked off (cf. a streak plate culture). Slope cultures are preferred to broth (i.e. liquid medium) cultures because the first sign of contamination is much more readily noticed on an agar surface.
Two stock cultures should be prepared; one is the "working" stock for taking sub-cultures for classes, the other is the "permanent" stock which is opened only once for preparing the next two stock cultures. Incubate at an appropriate temperature until there is good growth.
For growing strict aerobes it may be necessary to slightly loosen the cap for incubation (but close securely before storage) if there is insufficient air in the headspace.
As soon as there is adequate growth, store the cultures at room temperature in either a cupboard or drawer.
Keep on the lookout for contamination.

Checking cultures for contamination

Evidence for a culture being pure or otherwise is given by the appearance of colonies on a streak plates and of cells in a stained microscopical preparation. There should be uniformity of colony form and cell form (and consistency with the appearance of the original culture!). It is sensible to check purity on suspicion of contamination of the working stock culture from time to time and of the permanent stock when preparing new stock cultures.
If a culture becomes contaminated, it is not advisable to try to remedy the situation by taking an inoculum from a single colony from a streak plate of the mixed culture because of the possibility of (1) not being able to distinguish between the colony forms of the contaminant and the original culture, and (2) culturing a variant of the original culture that does not behave as the original culture did. Instead, go back to the working (or permanent) stock cultures; that's what they are for!

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Preventing contamination of cultures and the environment

Cotton wool plugs

Plugs made of **non-absorbent** cotton wool are used in test tubes and pipettes to prevent micro-organisms from passing in or out and contaminating either the culture or the environment. The necessary movements of air in and gaseous products out are not prevented and the gaps between the cotton wool fibres are even wide enough for micro-organisms to pass through. However, this does not happen because micro-organisms (negatively charged) are "filtered" out by being attracted to and adsorbed on the oppositely charged cotton wool. The cotton wool must remain dry because this filtration property is lost if the cotton wool becomes moist – hence the use of **non-absorbent** cotton wool.
For use in test tubes a plug should be properly made to ensure that it can be held comfortably without being dropped and its shape and form are retained while being removed from and returned to a test tube several times. Aseptic technique cannot be maintained with poorly made plugs; working surfaces, floors and cultures may become contaminated and students may become understandably (but avoidably) frustrated and lose interest.

Aseptic transfer of cultures and sterile solutions

Regular practice is necessary to ensure that the manipulations involved in aseptic transfer of cultures and sterile solutions become second nature. Aseptic transfer procedures for dealing with bacteria and yeasts and the appropriate instruments are given in the following table which should be read in conjunction with the following explanatory notes. Absence of an entry in a box indicates that the procedure is very unlikely to have a purpose.

Making a **streak plate** is a basic procedure that tests several skills and serves several purposes. During the inoculation procedure, the agar surface is protected from contamination by micro-organisms that are carried in the air by keeping the time that the Petri dish is open to a minimum. There are two approaches: (1) with the base (containing medium) placed on the working surface, lift the lid vertically (i.e. still covering the base) the least amount that will allow access of the loop; (2) with the lid placed on the working surface, lift out the base, invert it and inoculate the upwards- facing agar surface. The second method is best reserved for older students working in a relatively dust and draught-free laboratory; it is the one used by professional microbiologists.

The choice of **loop or pipette** for transfers between test tubes and screw cap bottles depends on whether they contain agar slopes, liquid media or sterile solutions. Although omitted from the table for simplicity, a straight wire may also be necessary for taking a small inoculum from liquid cultures for nutritional investigations.

The wire loop is usually satisfactory for inoculating a tube or bottle from a separate colony on a plate but a straight wire is occasionally needed for dealing with very small colonies such as occur with pure cultures of some bacteria, e.g. species of *Streptococcus* and *Lactobacillus*, and on plates that are being used for isolating cultures from natural samples.

Appropriate instruments for aseptic transfer procedures

TO / FROM	Test tube	Bottle	Streak plate	Spread plate	Pour plate
Test tube	loop or pipette (see pp 8 & 10)	loop or pipette (see pp 8 & 10)	loop (see p 12)	measuring pipette and spreader (see p 16)	measuring pipette (see p 13)
Bottle	loop or pipette (see pp 8 & 10)	loop or pipette (see pp 8 & 10)	loop (see p 12)	measuring pipette and spreader (see p 16)	measuring pipette (see p 13)
Streak plate	loop (see pp 8 & 10)	loop (see pp 8 & 10)	loop (see pp 8)		
Spread plate	loop (see pp 8 & 10)	loop (see pp 8 & 10)			
Pour plate	loop (see pp 8 & 10)	loop (see pp 8 & 10)			

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Preparing cultures for class use

Microbial cultures for use in a practical class in biology or food studies are the equivalent of, say, solutions of chemicals or electrical circuits in other disciplines. The big difference, however, is that microbial cultures cannot be taken from a shelf and instantly be ready for use. **It is necessary to begin to prepare cultures well in advance** otherwise the outcome might not be as expected and the educational experience will be either diminished or lost.

The key is to transfer cultures several times in advance to ensure that they are growing well and are presented as young, fully active cultures on the day of the practical class. For most cultures of **bacteria and yeasts** this will be after incubation for 1 or 2 days; progress of growth can be followed by observation with the naked eye, looking for growth on an agar surface or turbidity in a broth culture. It is usual to grow **moulds** on the surface of an agar medium, allowing an incubation period of from several days to a week.

The main points to observe are use of an adequate amount of inoculum, an appropriate culture medium and incubation temperature and, if it is necessary to grow a strictly aerobic organism in a single large volume of liquid culture (i.e. more than *ca* 20 cm^3), provision of adequate aeration.

Bacteria and yeast

It will save time in preparing large numbers of cultures of bacteria and yeast for the class if the inoculum is taken by Pasteur pipette from a well-growing (i.e. turbid) broth culture. A line of growth on a slope culture inoculated by wire loop is easy for students to observe but almost the same effect can be achieved with a pipette.

Moulds

It is sometimes appropriate to prepare a mould inoculum as a spore suspension (particular care is necessary to prevent them from escaping into the air) but often the inoculum is a portion of the mycelium taken with a loop or straight wire with the end few millimetres bent at a right angle. When an agar plate with a mould inoculated at the centre is required, it is easy to inoculate accidentally other parts of the plate with tiny pieces of mould, usually spores, that fall off the loop or wire. This can be avoided by placing the Petri dish on the working surface lid down, lifting the base (containing medium) vertically above the lid and introducing the inoculum upwards onto the centre of the downwards-facing agar surface with a bent wire.

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TWO ACTIVITIES

1. Testing sensitivity to antibacterial substances

The agar diffusion method is widely used in industry for testing the sensitivity of micro-organisms to antibiotics, antiseptics, toothpaste, mouthwashes, disinfectants, etc. The method involves preparing a pour or spread plate of a test micro-organism, adding small amount of test substance to either a well cut in the agar medium or (preferably) a paper disc which is then placed on the agar surface. After incubation, an inhibitory effect on the test organism is indicated by a clear zone (no growth) around the test substance; microbial growth is visible to the naked eye in areas of the plate that are unaffected.

This is a straightforward activity that tests several practical skills and is relevant to other aspects of biology and to everyday life. In addition to using laboratory reagents, e.g. stains, and antibiotic discs, many preparations with antimicrobial activity are readily available in pharmacists and supermarkets. There is also opportunity to think of less obvious materials, e.g. plants and their products.

[Factsheet: *Investigating antimicrobial activity for use with students, including notes for teachers and technicians*]

Materials

- Take one of the pour or spread plates prepared earlier in the day.

Sterile:

- Filter paper discs,
- Distilled/demineralised water (control)

Also:

- Samples to be tested, 3 (e.g. mouthwashes, selected for a range of active ingredients)
- Bunsen burner
- Forceps
- *70% (v/v) industrial methylated spirit* in a small beaker covered in foil (CAUTION:flammable, should be kept covered away from flames)
- Incubator at 25-30 °C (if available)

Practical details

Aseptic technique should be used throughout.

1. Mark and label four sections on the base of the Petri dish, for the three different samples and control (sterile water).
2. Using sterile forceps (flamed with alcohol and cooled) remove one filter paper disc. Dip into the first test sample, drain on the side of the container and place firmly onto the appropriate section of the seeded agar plate.
3. Wash the forceps free of the sample.
4. Repeat for the remaining samples and the control (sterile water). Remember to rinse and sterilize the forceps between each sample and to open the plate for the minimum possible time.
5. Seal the lid to the base with tape.

Incubation of the plate.

6. Invert the plate and incubate at 25-30°C or at room temperature for 48 hours.
7. Examine the plate (without opening). Measure and record the size of any zones of inhibition around the filter paper discs. Consider what factors might be affecting the size of the zones of inhibition.

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2. Microscopy

Using the microscope

The setting up of a microscope is a basic skill of microbiology yet it is rarely mastered. Only when it is done properly can the smaller end of the diversity of life be fully appreciated and its many uses in practical microbiology, from aiding in identification to checking for contamination, be successfully accomplished. The amount of magnification of which a microscope is capable is an important feature but it is the resolving power that determines the amount of detail that can be seen. [Factsheet: *Using a Microscope*]

Bacteria and yeast

Yeast can be seen in unstained wet mounts at magnifications x100. Bacteria are much smaller and can be seen unstained at x400 but only if the microscope is properly set up and all that is of interest is whether or not they are motile. A magnification of x1000 and the use of an oil immersion objective lens for observing stained preparations are necessary for seeing their characteristic shapes and arrangements. The information gained, along with descriptions of colonies, is the starting point for identification of genera and species but further work involving physiology, biochemistry and molecular biology is then needed. .

Moulds

Mould mycelium and spores can be observed in unstained wet mounts at magnifications of x100 although direct observations of "mouldy" material through the lid of a Petri dish or specimen jar at lower magnifications with the plate microscope are also informative (but keep the lid on!). Routine identification of moulds is based entirely on the appearance of colonies to the naked eye and of the mycelium and spores in microscopical preparations.

Protozoa and algae

Protozoa and algae are large organisms and therefore are readily visible at a magnification of x10 to x100 in unstained wet mounts. A magnification of x100 is advantageous for observing natural samples that contain a variety of organisms, particularly as many are very motile. Identification of algae and protozoa is based entirely on their microscopical appearance. The common algae are green and non-motile; diatoms have a brown, sculptured outer layer of silica and move slowly. Protozoa are colourless and most are motile. Hay infusions and cloudy vase water are rich in algae and protozoa but clear samples of water are rarely rewarding.
For further information contact Sciento see Factsheet: *Suppliers of Culture and Equipment*

Stained preparations

A "smear" of bacteria or yeast is made on a microscope slide, fixed, stained, dried and, without using a coverslip, examined with the aid of a microsope. Aseptic technique must be observed when taking samples of a culture for making a smear. A culture on agar medium is much preferable to a liquid culture for making a smear. A smear that is thin and even enables the shape and arrangement of cells to be clearly seen and ensures that the staining procedure is applied uniformly. There are two broad types of staining method:

(1) **a simple stain** involves the application of one stain to show cell shape and arrangement and, sometimes, inclusions that do not stain, e.g. bacterial endospores;

(2) **a differential stain** involves a sequence of several stains, sometimes with heating, and includes a stage which differentiates between either different parts of a cell, e.g. areas of fat storage, or different groups, e.g. between Gram-positive and Gram-negative bacteria. The reaction of bacteria to Gram's staining method is a consequence of differences in the chemical structure of the bacterial cell wall and is a key feature in their identification. Yeast cells can be stained by Gram's method but it is of no value in their identification.

The basis of Gram's staining method is the ability or otherwise of a cell stained with crystal violet to retain the colour when treated with a differentiating agent, usually alcohol (although professionals sometimes use acetone). Bacteria that retain the violet/purple colour are called Gram-positive. Those that lose the colour, i.e. called Gram-negative, are stained in the contrasting colour of a counterstain, usually pink/red.

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Making a smear.

1. Clean a plain microscope slide thoroughly using lens tissue.
2. Label a microscope slide with a marker pen to record the culture being used, date and initials; this is also a useful reminder of which side of the slide is being used.
3. Flame a wire loop to ensure that no culture accidentally remains from a previous operation.
4. Transfer one or two loopfuls of tap water on to the centre of the slide.
5. Flame loop and allow to cool.
6. Using aseptic technique, transfer a very small part of a single colony from a plate or slope of agar medium into the tap water.

If the amount of culture on the loop is easily visible you have taken too much!

7. Make a suspension of the culture in the tap water on the slide and thoroughly but gently spread it evenly over an oval area of up to 2 cm length.
8. Flame the loop.

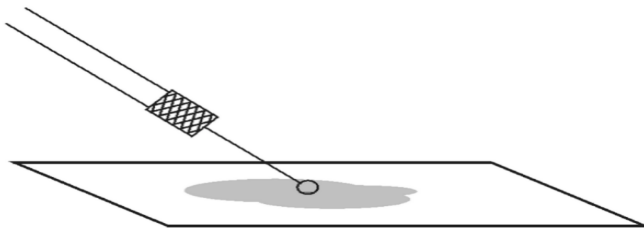
If it is necessary to use a liquid culture or sample, the use of tap water to prepare the smear will probably be unnecessary and may result in a smear with too few cells.

9. Dry the suspension by warming gently over a Bunsen burner flame and then "fix" it by quickly passing it through the flame a few times.

This is called a **heat-fixed smear**; it should be visible to the naked eye as a whitish area. Fixing is necessary to ensure that cells adhere to the slide and to minimise any *post mortem* changes before staining.

The smear is now ready to be stained

Making a smear



Staining solutions (relevant to procedures described below)	
Crystal violet solution:	
A. crystal violet	2.0g
absolute alcohol	100 ml
B. ammonium oxalate	1.0g
distilled/deionised water	100ml
Add 25 ml A to 100 ml B	
Lugol's iodine solution:	
iodine	1.0g
potassium iodide	2.0g
distilled/deionised water	300 ml

A simple stain.

1. Put the slide with the fixed smear uppermost on a staining rack over a sink or staining tray.
2. Thoroughly cover the smear with stain and leave for, usually, 30 seconds.
3. Hold the slide with forceps (optional but avoids stained fingers), at a 45° angle over the sink.
4. Rinse off the stain with tap water.
5. Blot dry the smear with filter/fibre free blotting paper using firm pressure but not sideways movements that might remove the smear.
6. Examine under oil immersion.
7. When finished, dispose of slides into discard jar.

Suitable stains include basic dyes (i.e. salts with the colour-bearing ion, the chromophore, being the cation) such as methylene blue, crystal violet and safranin.

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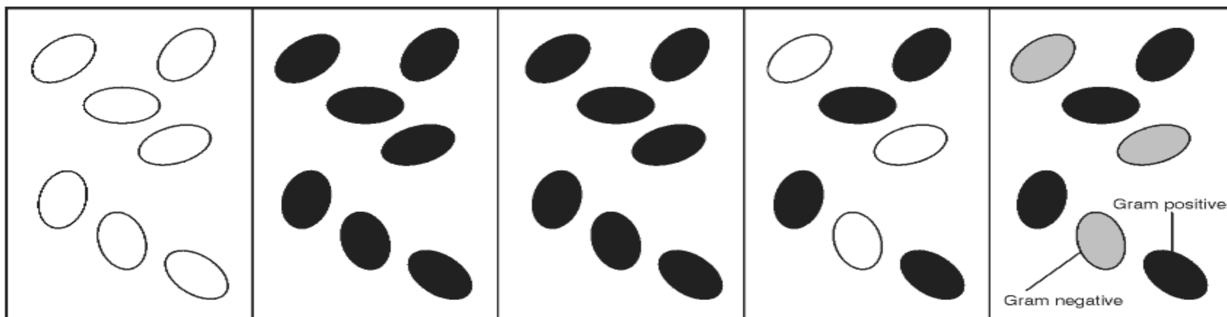
A differential stain: Gram's staining method.

Times of the staining periods depend on the formulation of the staining solutions which are not standard in all laboratories. Therefore, the times given here relate only to the solutions specified here.

1. Put the slide with the fixed smear uppermost on a staining rack over a sink or staining tray.
2. Thoroughly cover the smear with crystal violet solution and leave for 1 minute.
3. Hold the slide with forceps (optional but avoids stained fingers), at a 45° angle over the sink.
4. Pour off the stain, wash off any that remains (and any on the back of the slide) with iodine solution.
5. Put the slide back on staining rack.
6. Cover the smear with iodine solution and leave for 1 minute. Iodine solution acts as a "mordant" (a component of a staining procedure that helps the stain to adhere to the specimen), a crystal violet-iodine complex is formed and the smear looks black.
7. Hold the slide with forceps at a 45° angle over the sink wash off the iodine solution with 95% (v/v) ethanol (not water); continue treating with alcohol until the washings are pale violet.
8. Rinse immediately with tap water.
9. Put the slide back on staining rack.
10. Cover the smear with the counterstain, e.g. safranin solution, 0.5% w/v, for 30 seconds.
11. Rinse off the stain with tap water.
12. Blot dry the smear with filter/fibre free blotting paper using firm pressure but not sideways movements that might remove the smear.
13. Examine under oil immersion.
14. When finished, dispose of slides into discard jar.

[Factsheet*: Using a Microscope*]



(a) before staining all bacteria are colourless
(b) after basic stain *(crystal violet)* all bacteria are stained violet
(c) after mordant *(Lugol's iodine)* stain is fixed more firmly into the cell
(d) after decolouriser (alcohol) some bacteria are colourless (Gram negative) while others are still violet (Gram positive)
(e) after counterstain (safrarin) colourless bacteria (Gram negative) have taken up stain and appear red: Gram positive bacteria remain violet

HINTS:

Always use a young culture because older cultures of Gram-positive bacteria tend to lose the ability to retain the crystal violet-iodine complex and appear to be Gram-negative; but some bacteria are naturally only weakly Gram-positive.

The amount of alcohol treatment (the differential stage) must be judged carefully because over-treatment washes the crystal violet-iodine complex from Gram-positive bacteria and they will appear to be Gram-negative.

Take care to make an even smear otherwise alcohol will continue to wash the violet/purple colour from thick parts of the smear while thin parts are being over-decolorised.

At the end of the procedure, check that the labeling has not been washed off by the alcohol.

Don't despair if the stained smear is not visible to the naked eye; this may happen with a Gram-negative reaction.

Specimen stained slides are available from suppliers.

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Notes

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Notes

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USEFUL ADDRESSES

Association for Science Education (ASE) College Lane, Hatfield, Herts, AL10 9AA www.ase.org.uk
Biotechnology and Biological Sciences Research Council (BBSRC) School's Liaison Office, BBSRC, Polaris House, North Star Avenue, Swindon, SN2 1UH www.bbsrc.ac.uk
CLEAPSS School Science Service Brunel University, Uxbridge, UB8 3PH www.cleapps.org.uk
Microbiology in Schools Advisory Committee (MISAC) c/o SGM Marlborough House, Basingstoke Road, Spencers Wood, Reading, RG7 1AG www.biosci.org.uk/misac
National Centre for Biotechnology Education University of Reading, Whiteknights, PO Box 226, Reading, RG 6 6AP www.ncbe.reading.ac.uk
Society for General Microbiology Marlborough House, Basingstoke Road, Spencers Wood, Reading RG7 1AG www.microbiologyonline.org.uk

USEFUL WEBSITES

American Society for Microbiology www.asmusa.org (go to the section 'For the Public')
Microbial World, University of Edinburgh http//helios.bto.ed.ac.uk/bto/microbes
Schools Science www.schoolscience.co.uk (go to the section 'Microbiology- database of resources')

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