

**A BRIEF INTRODUCTION TO
MICROBIOLOGY AND THE USE OF
3M™ PETRIFILM PLATES™.**

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1. INTRODUCTION.

Microbiology is essentially the study of organisms too small to see with the naked eye (for at least part their life cycle). Sometimes we can see microorganisms such as moulds on cheese or bread with the naked eye but part of the lifecycle of moulds is microscopic so we still consider these organisms to be part of microbiology.

Microorganisms are ubiquitous which means they are found everywhere.

Throughout history microorganisms have been both “friend” and “foe” to mankind.

As a “foe”, organisms such as *Yersinia pestis* (the plague), *Mycobacterium tuberculosis* (tuberculosis), *Mycobacterium leprae* (leprosy), *Vibrio cholerae* (cholera), *Salmonella*, *Campylobacter*, *Staphylococcus* and *Listeria* (various forms of food poisoning), come to mind.

As a “friend”, organisms such as *Lactobacillus* (fermented meats, cheese’s, yoghurt’s), *Saccharomyces cerevisiae* (beer, wine, and bread), *Acetobacter* species (vinegar), *Propionibacterium* species (holes in Swiss cheese), various mould species (antibiotics and other medicinal drugs), come to mind.

Most microorganisms are more or less generally harmless other than spoiling food, or discolouring the bathroom tiles, but we should always understand that life on earth would not be possible without microorganisms.

Whilst we now know a lot about microbiology and microorganisms in general, this knowledge has been historically fairly recent. The microscope, which allows us to see microorganisms directly, was not invented until the Seventeenth Century. Even after it’s invention the full ramifications of microbiology did not become apparent until the Nineteenth Century.

Louis Pasteur (1822-1895) was able to use the microscope to demonstrate that in certain alcoholic fermentations (beer and wine production), the fermentation process was carried out by living microorganisms (yeasts), and that it was a specific type of yeast that produced a good batch whilst other types produced bad batches.

Consequently the concept of aetiology (study of cause and effect) has developed within microbiology. For example, food spoils because microorganisms degrade the food. If you eliminate the microorganisms such as sealing the food in a tin can and heat processing the tin to kill all of the microbes, the food will last for years. So the cause is microbes and the effect is the spoilage.

Robert Koch (1843-1910) was able to demonstrate that certain bacteria caused certain diseases (he showed that the aetiological agent for anthrax was *Bacillus anthracis*), and in so doing developed all of the basic microbiological techniques we still use to this very day.

Since then vaccines and cures have been developed for the great majority of diseases in man and animals and we can test foods for the absence of known pathogenic (harmful) bacteria, test for indicator organisms to see the likelihood of pathogens, and test for spoilage organisms to see if foods will make their use by dates.

This manual describes some basic microbiological techniques along with safety tips when dealing with live microorganisms and has some fun experiments to do in the classroom to understand how microorganisms grow and how to isolate and study them.

You must remember that all experiments deal with live organisms and therefore safety is very important.

2.MICROBIOLOGICAL TECHNIQUES.

Petrifilm plates are a thin film, sample ready, dehydrated, version of the conventional petri dish agar plate. They are ready to use immediately after taking them out of their packets and have several advantages over conventional agar plates, such as built in biochemical confirmation, ease of use and interpretation, no preparation, and smaller volumes of space used in incubation (10 Petrifilm plates takes the same space as one agar dish).

All plates described in this manual require a one ml sample inoculation.

There are four plates described, which are considered to be safe for general educational use. Plates used for the isolation of known pathogens are not described. Petrifilm plates have International recognition such as AOAC and AFNOR, and are widely used in industry in Australia and Internationally.

Aerobic Count Plate (AC).

The AC plate counts nearly all aerobic and facultative anaerobic bacteria in a sample. It contains plate count nutrients, a coloured dye called triphenyl tetrazoliumchloride (TTC) which colours all bacterial colonies red, and a cold water soluble gelling agent. Terms previously used for this count are total viable count (TVC), standard plate count (SPC), and plate count (PC).

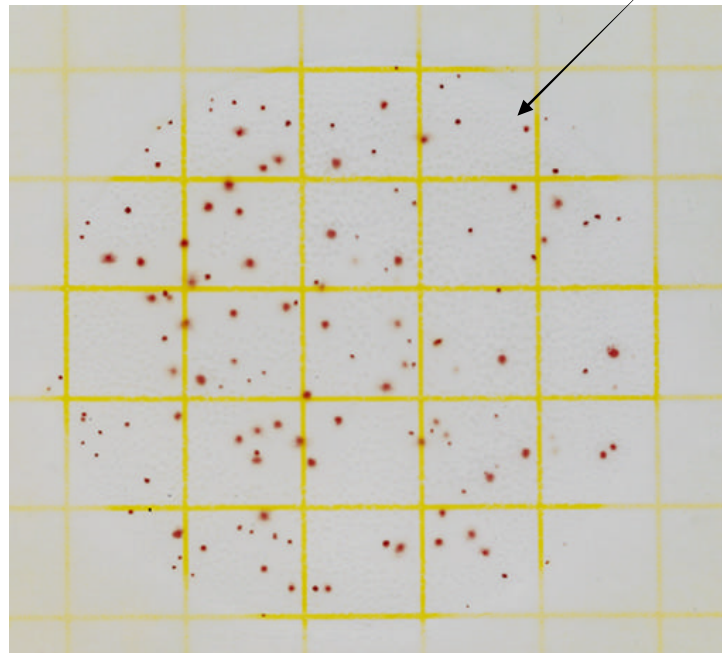
Whilst yeasts and moulds are capable of growing on this plate they generally do not appear within the 2-3 day incubation time and are easily differentiated from bacteria since they do not reduce the TTC to produce a red colour.

Count all red colonies regardless of their size or intensity.

Incubation is at 35 degrees for 2 days.

Count all red colonies.

Aerobic Count Plate showing TTC reaction.



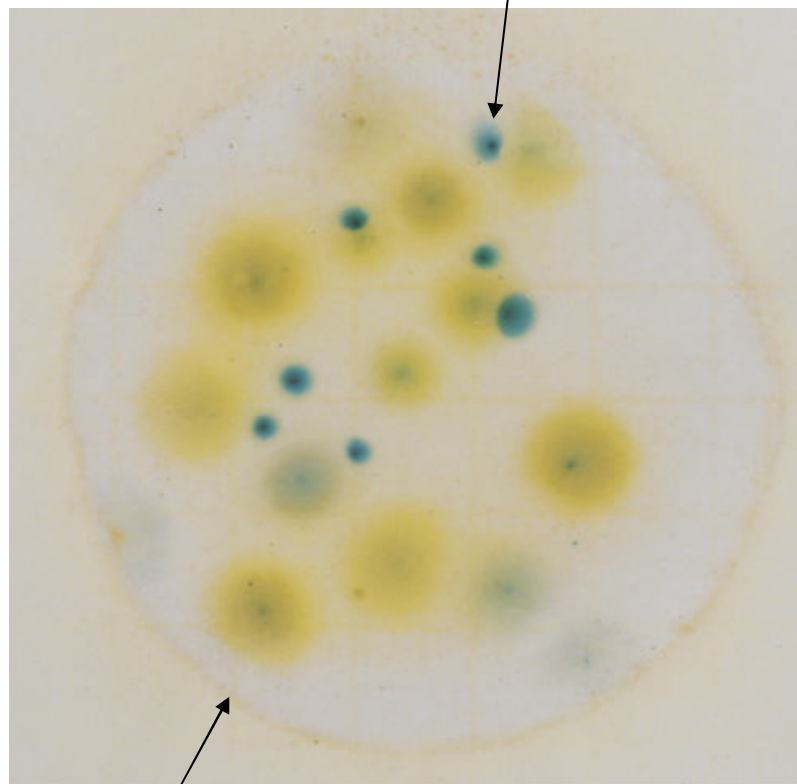
Yeast and Mould Count Plate (YM).

The YM plate counts nearly all common yeast and mould species in a sample. It contains modified Sabroud's dextrose nutrients, two broad spectrum antibiotics to suppress bacterial growth, an alkaline phosphatase indicator which colours all yeasts aqua green, and a cold water soluble gelling agent.

Yeasts appear as small regularly shaped aqua green colonies whilst moulds appear as larger variable coloured colonies with diffuse edges and a central focal point (they appear furry).

Incubation is at 20-25 degrees for 3-5 days. (Ambient temperature will suffice).

Yeasts and
Moulds together
on an YM plate.



Yeast (characterised by aqua green colour).

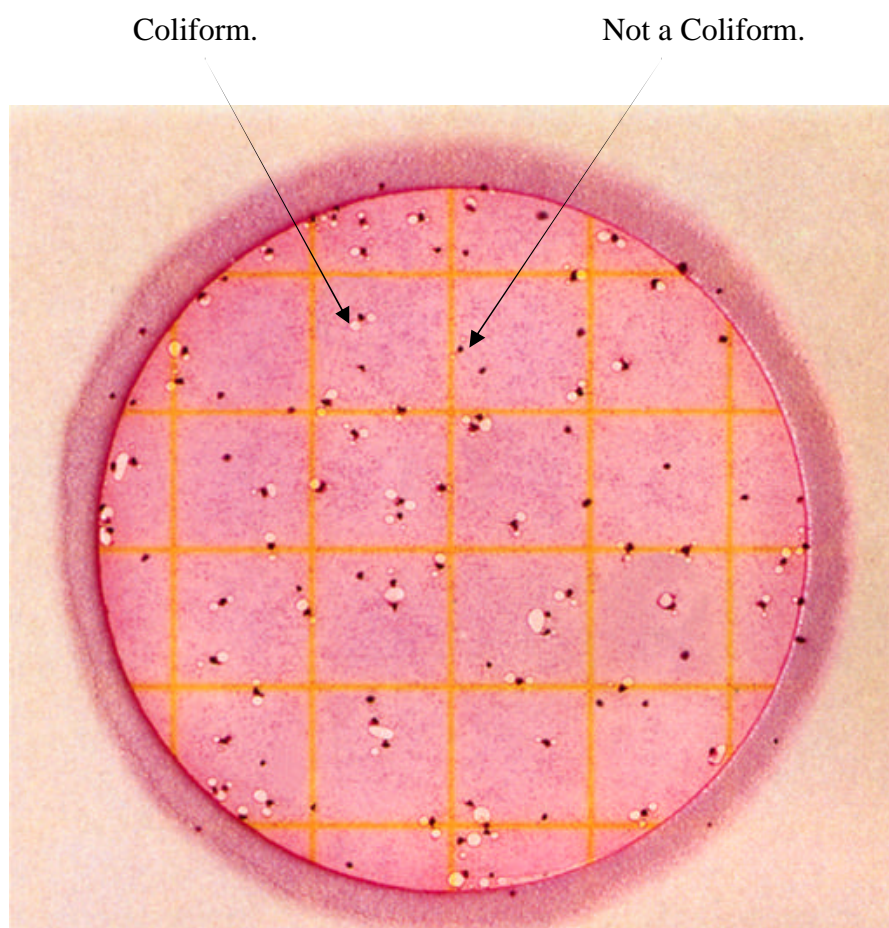
Mould (characterised by central focal point).

Coliform Count Plate (CC).

The CC plate counts all coliforms within a sample without differentiating between the genera. Coliforms by definition are the members of the family Enterobacteriaceae which ferment lactose to produce gas. This count has long been used as a measure of faecal contamination in dairy products and other foods.

The CC plate contains violet red bile lactose nutrients, TTC indicator and a cold water soluble gelling agent. Organisms which ferment lactose to produce gas will have gas bubbles trapped in the gel next to the colonies. The bile salts in the medium select for the family Enterobacteriaceae and the TTC indicator assists in visualising the colony. Count all colonies which appear red and which are associated with gas bubbles as confirmed coliforms.

Incubation is at 35 degrees for 24 hours.



Coliforms and non Coliforms on a CC plate.

E.coli/Coliform Count Plate (EC).

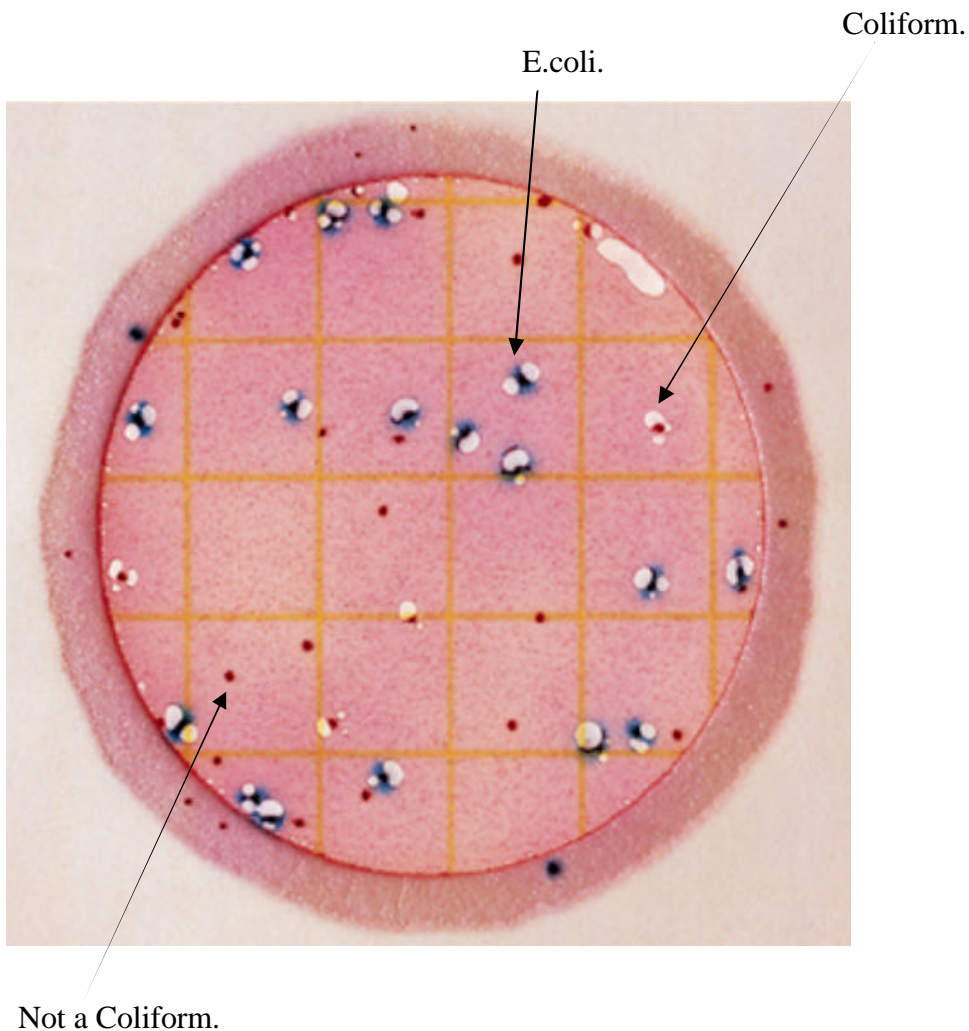
The EC plate counts all coliforms in a sample and differentiates *Escherichia coli* from other coliforms. *E.coli* is differentiated from other coliforms by the BCIG reaction which colours *E.coli* blue. This plate is essentially identical to the CC plate except that it has the BCIG chromogenic (colour producing) indicator.

E.coli has long been an indicator of faecal contamination in meat products and other foods.

Count all blue colonies associated with gas bubbles as *E.coli* and all red colonies associated with gas bubbles as other coliforms.

Incubation is at 35 degrees for 24-48 hours.

E.coli, Coliforms, and non Coliforms on an EC plate.



3. SAFETY IN MICROBIOLOGY.

Since experiments in this manual deal with live microorganisms it is essential that some cautions be exercised.

When plates are inoculated prior to incubation the plates may only contain a few organisms per plate. After incubation each single microbial cell will have multiplied to over 1,000,000 cells and at that level may present a risk.

Plates presented to the class for counting and examination should either be taped shut or placed in zip-lock plastic bags so that they cannot be opened.

Plates with viable colonies must be disposed of in a responsible way such as autoclaving, soaking in an appropriate disinfectant or using a contract collection service, such as is provided by Stericorp.

Adequate antibacterial hand wash and hand rub solutions must be provided with all students washing their hands prior to leaving the class.

4. EXPERIMENTS.

i. Yeasts and Moulds in the Air.

Petrifilm plates can be rehydrated and stored refrigerated for up to 2 weeks prior to use.

Rehydrate as many Petrifilm YM plates (with sterile diluent and a sterile pipette) as are required for the class, and allow to gel for at least 1 hour. The exact procedure is described in the “Environmental Monitoring Procedures”, manual and can be sourced from www.3M.com/microbiology or from Southern Biological.

Peel back the top film without touching the rehydrated culture media and expose the plate to the air for 5 minutes precisely. Placing plates in front of air conditioners or air vents will guarantee a high count.

Plates can be held open by the use of double sided tapes (fold single sided tape over itself to make it double sided). Retain one or two plates as controls. WHY?

Since the YM plate is 30 square centimetres and the top and bottom parts of the plate are exposed, the total exposure area is 60 square centimetres.

The resultant count should therefore be expressed as cfu/square cm/minute.

In this way a meaningful comparative result between samplings of the same site as well as samplings of different sites can be made.

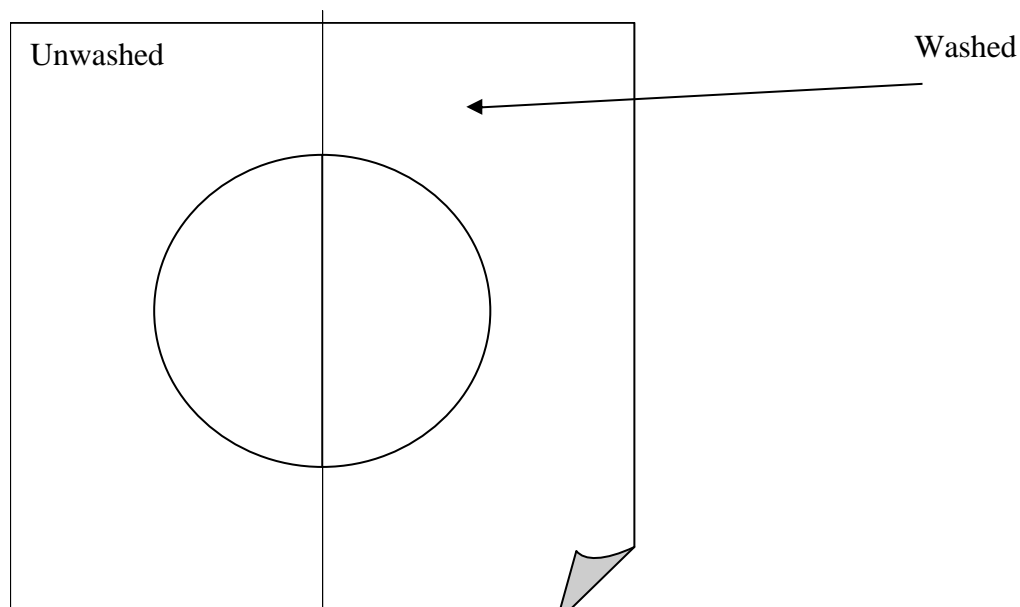
Incubation should be for 3-5 days at 20-25 degrees (Ambient temperature will suffice).

Count colonies as described in the YM plate section.

ii. Bacterial Populations on Fingers. (Use of Topical Antimicrobials).

Petrifilm plates can be rehydrated and stored refrigerated for up to 2 weeks prior to use. This experiment requires the use of a topical antibacterial rub such as Avagard, Chlorhexidine/ Alcohol Rub.

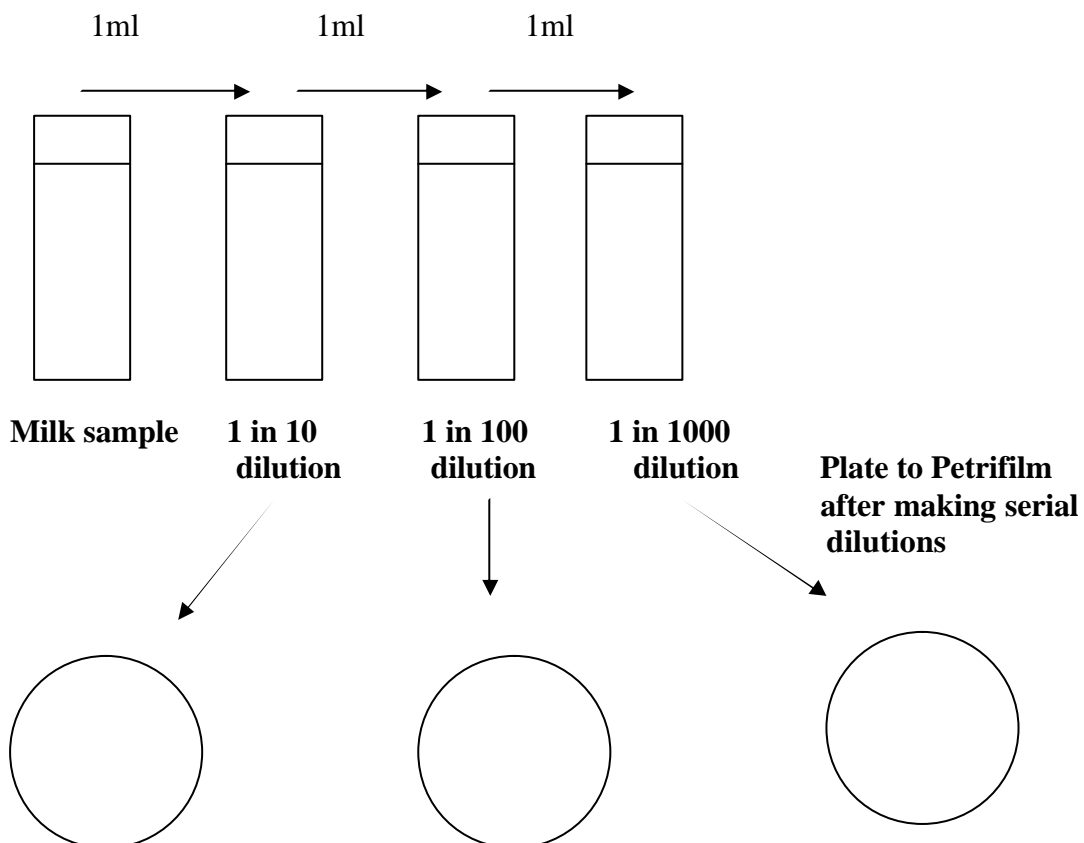
Rehydrate as many Petrifilm AC plates (with sterile diluent and a sterile pipette) as are required for the class, and allow to gel for at least 1 hour. The exact procedure is described in the “Environmental Monitoring Procedures”, manual and can be sourced from www.3M.com/microbiology or from Southern Biological.



Using a marker pen divide the plate in two by marking the top film as in the diagram above as well as marking the left side unwashed and the right side washed. Peel the top film back and touch the inside of the top film (left side only) directly on the gel with the middle three fingers. Sanitise both hands with the chlorhexidine/alcohol rub and allow to air dry. This usually involves allowing contact with the solution for at least 30 seconds whilst paying particular attention to the finger tips. Immediately repeat the procedure with the right side. Incubate the plates at 35 degrees for 2-3 days. All bacterial colonies will appear as red dots on the plates. Plates can be refrigerated for up to 2 weeks and still show typical colonies or frozen almost indefinitely. This comparison demonstrates quite graphically the benefits of washing and sanitising hands.

iii. Total Bacterial Populations in Milk. (Shelf Life Determinations).

This experiment requires the use of 9ml bottles of sterile diluent and sterile pipettes. By law, pasteurised milk must have a bacterial count of less than 50,000 cfu /ml prior to leaving the factory or otherwise it will not meet it's use by date of 2 weeks. By allowing fresh milk to sit at room temperature for 8 to 24 hours the bacterial population will increase substantially thus guaranteeing a reasonable count. This also allows for a second experiment to be performed namely, "Temperature Effects on Bacterial Populations", described in the next experiment but ideally performed in conjunction with this one.



Take 1 ml of the temperature abused milk with a sterile pipette and place it into a 9ml bottle of sterile diluent. Mix well and discard the pipette.

This is a 1 in 10 dilution. By repeating this procedure, using a fresh sterile pipette each time (Why?), we can get to very high dilutions. This is known as serial dilution, which allows us to reduce the bacterial population to a countable range. Since a single plate can count up to about 250 colonies per plate, then we aim for less than this much per plate.

Repeat the procedure as in the above diagram.

Take a fresh sterile pipette and plate 1ml of the highest dilution on to a Petrifilm Aerobic Count plate.

Using the same pipette (Why?), plate the second highest dilution to an AC plate, and repeat using the same pipette with the third highest dilution.

Because we don't know what the bacterial population is, it is necessary to plate out to beyond what the actual population is in order to guarantee that we get a plate which is countable.

Incubate the plates for 48 hours at 35 degrees. Count all red colonies.

The bacterial population (measured in colony forming units, cfu), is calculated as follows.

Number of colonies on a plate \times the dilution on that plate.

For example, if the 1 in 100 dilution plate has 56 colonies on that plate, then the count in the milk is $56 \times 100 = 5,600$ cfu per ml of milk. It is common in microbiology to use standard notation so the result would be expressed as $5.6 \times 10(3)$ cfu per ml.

iv. Temperature Effects on Bacterial Populations.

Heating is one of the principle ways in which microorganisms can be killed.

Place the container of temperature abused milk (after the above experiment has been performed so that the actual population is known) in a beaker of water on a hot plate with a thermometer in the water. Heat slowly, until the temperature is 50 degrees.

Remember that it takes some time for the heat to transfer from the water into the container of milk. Take 1ml of the milk and plate directly on to a Petrifilm AC plate using a sterile pipette.

Repeat this procedure at 60, 70, and 80 degrees. Nearly all bacteria should be killed by 80 degrees.

Incubate the plates for 48 hours at 35 degrees. Count all red colonies.

Compare populations at the different temperatures and you should be able to work out what is an effective temperature for pasteurising milk. Traditionally it is between 60 and 70 degrees (63degrees for 30 minutes or 72degrees for 15 seconds). It is worth noting that a temperature as well as time relationship exists for killing microbes. For example more bacteria will be killed at 60 degrees for 30 minutes than at 60 degrees for 5 minutes.

v. Coliforms and E.coli in Ground Meat.

This experiment requires the use of 90ml bottles of sterile diluent, sterile pipettes, and sterile stomacher bags.

E.coli and coliforms are the principle indicators of faecal contamination in the food industry.

The microbiological limits for raw meat as set by the Meat Standards Committee, prescribe the following limits for E.coli in raw meats.

<u>Meat Quality</u>	<u>E.coli cfu per gram</u>
Excellent	not detected
Good	1-10
Acceptable	10-100
Marginal	100-1000

In the previous experiment we were dealing with a liquid sample; here we are dealing with a solid sample, which means we will have to process it in a different way.

Place approximately 10grams of raw minced meat (any kind) in a sterile stomacher bag with a sterile spoon and add 90mls of sterile diluent. Mix in the bag by mashing the mixture with your hands from the outside of the bag for at least 30 seconds. This effectively washes all the bacteria into the diluent.

Using a sterile pipette place 1ml of diluent on to a Petrifilm Coliform Count plate or E.coli/Coliform Count plate.

Incubate for 24-48 hours at 35 degrees. Count all blue colonies as E.coli and all red colonies with gas bubbles as coliforms. In the case of the Coliform Count plate only count red colonies (since the E.coli indicator is not present in this plate).

The actual count is the count per plate x 10 (since 10 is the dilution).

Determine the microbiological quality of the meat according to the Meat Standards Committee criteria.

vi. Count of Bakers/Brewers Yeast.

This experiment requires the use of 9ml bottles of sterile diluent, and sterile pipettes. The yeast *Saccharomyces cerevisiae* is widely used in industry for such things as brewing alcoholic beverages, baking bread and pizza, and is the main ingredient in Vegemite.

Dehydrated yeast is available at any supermarket in sachet form and is very cheap. Take a sachet of dehydrated yeast and place the smallest pinch you can into a 9ml bottle of sterile diluent.

Perform serial dilutions as in experiment iii until you have a 1 in 1 million dilution using a sterile pipette (changing the pipette at each dilution for a fresh sterile pipette). Plate the last 3 dilutions on to Petrifilm Yeast and Mould plates starting from the most dilute using the same sterile pipette (Why?).

Incubate at 20-25degrees (room temperature) for 3-5 days.

Count all colonies that are aqua green.

Calculate the number of yeasts per pinch as described in experiment iii.
 For example, 30 colonies on the 1 in 1 million plate would be 30 x 1 million yeasts per pinch, or 30,000,000cfu per pinch, or 3.0×10^7 cfu per pinch.
 Commercial yeast makers produce yeast in fermentation vessels that are 10 metres in diameter and 4 stories high.
 Try to imagine how many yeasts would be in such a vessel at the end of a fermentation?

vii. Bacterial Populations on Surfaces.

Since we understand that microorganisms are ubiquitous then we should accept they are to be found on almost all surfaces.
 The CSIRO has guidelines on acceptable limits for work surfaces in the food industry. They are as follows.

<u>Surface Type</u>	<u>Acceptable Microbiological Level</u>
General	less than 6cfu/square cm.
Easy-to-clean	less than 1cfu/square cm.

Take a re-hydrated Petrifilm Aerobic Count plate (or Yeast and Mould Count plate) and peel back the upper film without touching the inside surface. Press the re-hydrated part of the inside surface on to the area to be tested. Ensure that all of the media has touched the area to be tested by smoothing down the outside part of the film with your fingers (gently).

Incubate according to the plate being used.

Calculate the microbiological level by counting the colonies per plate, then dividing by the number of square cm's tested. In the case of the AC plate it is 20 square cm, and in the case of the YM plate it is 30 square cm.

For example on the AC plate if the count was 50 cfu per plate, the level would be 50 cfu divided by 20 square cm, therefore 2.5cfu per square cm.

According to the CSIRO guidelines this would be acceptable for a general surface.

Sometimes surfaces are not flat or even, which means the above technique will not be adequate.

In these circumstances the Quick Swab is the preferred method. Snap the ampoule of a Quick Swab and squeeze the bulb so that all of the diluent is pumped into the barrel. Remove the moistened swab from the barrel and swab the area to be tested. For example a door handle, the flush button on a toilet, a tap handle, etc.

Put the swab back into the barrel and shake the swab vigorously for at least 15 seconds. This will wash all of the microbes captured on the swab into the diluent.

Remove the swab again and discard. Plate the contents on to an appropriate Petrifilm. Incubate as per the Petrifilm plate selected.

Count the plate and work out the cfu per square cm tested. With a swabbing technique sometimes you just have to estimate the area.

Work out your acceptance level.

5. GLOSSARY OF TERMS.

AC. Aerobic Count Plate.

Aseptic. Sterile. To keep microorganisms out.

CC. Coliform Count Plate.

CFU. Colony forming unit.

Chromogenic. Producing a visible coloured chemical reaction.

Diluent. Sterile fluid used to dilute a sample or re-hydrate a plate. Usually 0.1% peptone solution.

EC. E.coli/Coliform Count Plate.

Inoculate. To apply a sample or sterile diluent to the test media.

Microbiology. The study of all forms of microorganisms including bacteria, virus, yeasts and moulds, prions, and parasites.

Microorganism. Organisms not visible to the naked eye for at least part of their life cycle. They include bacteria, fungi (yeasts and moulds), virus, parasites, and prions (eg BSE).

Pathogens. Microorganisms which cause harm to people or animals. There are also plant pathogens.

Serial Dilution. Tenfold dilutions (1 in 10 dilutions) of a sample in diluent. Tenfold dilutions are always preferred as this makes final calculations relatively easy.

Ubiquitous. Found absolutely everywhere.

YM. Yeast and Mould Count Plate.

6. APPENDICES.

Websites that could be of interest.

www.3M.com/microbiology

This site allows the user to access information on Petrifilm products including interpretation guides and specific microbiological applications.

www.southernbiological.com

This site has information on Petrifilm plates as well as microbiological experiments suitable for the classroom.

In addition there are other biological and scientific products and information.