UNIT 8 RAPID DETECTION TECHNIQUES FOR FOOD MICRO-ORGANISMS

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8.0 OBJECTIVES

After studying this Unit, we shall be able to:

- appreciate the importance of rapid techniques;
- enlist commercially available biochemical kits;
- specify immunological methods and kits used specially for quick detection of food borne pathogens;
- explain genetic methods employed for detection and enumeration of micro-organisms;
- describe working principles and working of fluorescence, impedance methods e.g. flow cytometry; and
- state the role of biosensors

8.1 INTRODUCTION

Traditional culture techniques are the most common methods of microbiological examination of foods, but these are fraught with a number of disadvantages such as being labour intensive, time consuming and failure to isolate viable but non-culturable organisms. On the other hand, total microscopic count methods are relatively fast, but limitations of these techniques include operator fatigue from prolonged use of microscopes and inability to discriminate between living and dead bacteria. To alleviate problems, associated with culture-based detection systems and direct microscopic methods, various other methods have been developed. These

include colorimetric assay methods based on liberation of dyes from substrates by enzymes, dye reduction tests, and ATP bio-luminescence. The rapid techniques have the futuristic potential to usher in an era of real time microbiology and have wider applications in industry such as food- cosmetic and pharmaceutical industry, control laboratories, biotechnology and environmental testing and research laboratories.

8.2 NEED FOR RAPID DETECTION TECHNIQUES

It is impossible to completely eliminate pathogens from the food supply; some pathogens (e.g., Bacillus cereus) are common in soil and on vegetation, and food handlers often carry others (e.g., *Staphylococcus aureus*), even if they follow standard hygiene practices. Lapses in worker hygiene or sanitation in food processing plants results in a dramatic expansion of the range of bacterial pathogens, because of the broad distribution of pathogenic bacteria and viruses. This ubiquity makes it essential that the food industry have access to efficient diagnostic tools that allow detection and identification of pathogens. These tools are also important for clinicians to help diagnose food borne illnesses, which is usually characterized by gastro-enteritis. Identifying the culprit is important because the effective treatments vary among the pathogens.

Accurate identification of the cause of food-borne illness is also useful to public health officials attempting to identify the source of an outbreak, and for epidemiologists interested in long-term trends, such as the increasing frequency of *E. coli* O157:H7 infections. Public health officials identify sources by testing the stools of affected people for the presence of pathogens. Once a pathogen has been identified, investigators carefully interview victims and attempt to uncover commonalities. For example, all the victims may have eaten at the same restaurant the previous day or may have attended a recent family reunion. In some cases, it is essential to identify all the people affected by an outbreak, so that they can be closely monitored for the eruption of severe symptoms. For example, children under the age of 5 are at risk for developing potentially life-threatening kidney failure after infection by *E. coli* O157:H7; it is essential to identify this pathogen as soon as possible.

Diagnostic speed is also essential in food processing plants. If pathogen contamination can be detected before a product lot leaves the plant, it may be possible to avert an outbreak. However, if enough time elapses before the contamination is detected; it may be too late to prevent an outbreak, depending on the nature of the product and its distribution system. Current methods of microbial identification often require 2 to 3 days, which increases the risk that contamination of food by pathogens will be undetected until it is too late.

Food companies have traditionally relied on end product inspection and testing to ensure that the food that leaves the factory does not contain an excessive load of non-pathogenic and pathogenic microbes. Theoretically, 100% of products can be visually inspected, but human frailties (e.g. distractability, boredom) decrease the efficiency of inspection. Furthermore, many microbial defects cannot be detected by visual inspection. Hence, destructive sampling of end products is often required. In principle, this is a

simple process – a defined proportion (subset) of products is removed at the end of the production line and the level of microbial contamination is assessed. Typically, microbes are put into suspension by grinding each sample in a Stomacher™ or similar apparatus. A dilution series of this suspension is then plated onto an agar medium, and after a suitable period (24 h at 37°C), bacterial of fungal colonies are counted. In some cases, enrichment and selective media are used to detect specific pathogens (*e.g. Salmonella*). This technology can be quite powerful; in some cases, cultural methods can distinguish closely related strains. For example, *E. coli* O157:H7 can be distinguished from non-pathogenic strains through the use of selective and differential media (differential media produce a detectable change in appearance/colour of either the agar medium or the colonies when the target bacterium is present).

Cultural methods such as this are a reliable indicator of microbiological quality in food, but there are many disadvantages of end product microbial analysis. Financial concerns sometimes make it difficult to test an adequate number of samples, because cultural methods are labour and materials intensive. If too few samples are assessed, the risk of missing contaminated product increases. This risk also increases if contamination occurs sporadically, rather than on a regular basis. Also, cultural methods are slow, particularly if the aim is to identify specific pathogens. For example, conventional detection of *Salmonella* requires that a food sample be incubated in three successive media for a total time of 72 h. Additional tests that confirm the presence of *Salmonella* are then required. This confirmation can be **biochemical**; the colonies that are presumed to be *Salmonella* are typically inoculated into a series of media containing a range of different carbon sources (e.g., glucose, sucrose, mannitol, proline, etc.). The bacteria respiring or fermenting (depending on the diagnostic system) these carbon sources produce a 'pattern of utilization' that can be used to identify the bacteria. Unfortunately, the growth period required for these tests further adds to the delay in assessment of contamination. Antibody-based tests, in contrast, can confirm the presence of *Salmonella* immediately. However, if negative, they do not provide any other information that could be used for identification, unlike biochemical tests.

The methods for the rapid detection of food borne micro-organisms, both spoilage and pathogens can be broadly classified into biochemical, immunological and genetic approaches. However, several methods for detection involve the use of more than one approach for identifying micro-organisms. The advantages of these methods are that they are less labour intensive and produce quick results. Most of the rapid methods are available as kits that are very easy to handle and can be carried to the sampling site. However, the main problem faced in the rapid detection of food borne microbes is the presence of inhibitors that interfere with particular detection method. This problem does not occur in the traditional methods because they involve enrichment of the stressed and injured cells from the food sample and this in turn leads to the dilution of inhibitors. Hence in comparison with rapid methods, traditional methods are better if accuracy of detection is to be considered. Food regulatory agencies suggest the use of rapid methods to screen the food samples for the presence of pathogens. In such tests negative, reaction is taken as an absence of pathogen in the food product however, the positive reaction for the presence of a pathogen is considered as presumptive

and the sample is tested by traditional method to confirm the result obtained with a rapid method. Various rapid methods for identification can be categorized as described in following sections.

8.3 BIOCHEMICAL KITS

These kits are designed on the same principles as the conventional methods such as the use of specific or differential media and biochemical tests for the identification of bacteria. However, the format of test is modified. These kits are made up of a disposable device containing an array of different media and substrates used for biochemical identification of bacteria. It will not be wrong to say that these are the miniaturized version of the biochemical tests carried out conventionally for the identification of food borne microbes especially, pathogens. These tests are classified under rapid tests because these kits are commercially available and one does not have to go through the time consuming and cumbersome procedure of preparing the media and test reagents as in the case of conventional methods. However, most of the kits are not truly rapid and require an incubation period of around 24 h to give the results. The accuracy obtained with such kits is around 90-99% as compared to conventional methods. Several such kits are available for the detection of food borne bacteria e.g. API kits developed by bioMerieux for *Enterobacteriaceae, Listeria*, *Staphylococcus, Campylobacter,* non-fermenters and anaerobes. With the advancement of the technology, the automation of such miniaturized kits have been made possible. Such automated systems automatically monitor the biochemical changes and prepare a phenotypic profile of the tested organism which is then matched with the computer database for identification purposes e.g. Vitek automated system developed by bioMerieux for identification of *Enterobacteriaceae*, Gram negatives and Gram positives.

Check Your Progress Exercise 1

Note: a) Use the space below for your answer.
b) Compare your answers with those given at the end of the unit.

1) Enlist the need for rapid detection techniques of micro-organisms in food?

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2) What are biochemical kits?

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8.4 IMMUNOLOGICAL METHODS

Immunological methods of rapid detection are based on the highly specific antigen-antibody reactions and involve the use of monoclonal antibodies for detection of bacteria in the food samples. These methods comprise the largest

group of methods used for detection purposes. The versatility and specificity of these reactions have prompted the production of immunological kits in various formats such as:

8.4.1 Latex Agglutination Assay

This involves the antibodies tagged with the colored latex beads or colloidal gold for the detection purposes e.g. Campyslide, Microscreen for the detection of *Campylobacter*, RIM, Prolex, Ecolex O157, Wellcolex O157 for *E. coli* O157:H7, Microscreen, Listeria Latex for *Listeria*.

8.4.2 Immuno-diffusion Format

This involves the diffusion of antigen through a gel impregnated with antibody. The appearance of precipitation line indicates the presence of specific antigen and is considered a positive test. An example of the kit based on the immunodiffusion is 1-2 test for the detection of *Salmonella*.

8.4.3 Enzyme Linked Immunosorbent Assay (ELISA)

This is the most popular rapid method for the detection of food borne pathogens. This is also based on the specificity of antigen-antibody reaction. In most of the immunological kits, sandwich format for ELISA is used. In this format antibody is coated on the surface of microtitre well. This antibody is known as primary antibody. Bacterial antigen obtained from the food sample is added to the antibody coated wells. Antigen-antibody reaction is allowed to occur followed by washing the wells to remove the unbound antigen. After this, a secondary antibody tagged with an enzyme is added to the microtitre plate. This antibody is also specific for the antigen to be detected but it recognizes a different epitope on the surface of antigen. This step is again followed by washing to remove the unbound antibody, followed by the addition of substrate for the enzyme. This substrate is usually colourless and a coloured product is formed by the action of antibody bound enzyme. The colour development is taken as a positive reaction and even the intensity of the colour can be measured using ELISA reader. This intensity when compared with standard controls can be used for quantification of the antigen or the results may be reported as positive and negative depending on the instructions given with the specific kit. If specific antigen is not present in food then the primary antibody will not bind to the added antigen, which will be removed during washing step. In the absence of specific antigen the secondary antibody will also not bind and will be washed from the well. The absence of enzyme conjugated secondary antibody from the well will effect the formation of coloured product from the substrate, thus giving a negative signal. Several enzymes such as horseradish peroxidase, alkaline phosphatase and p-nitrophenyl phosphatase have been used to tag the secondary antibody. A few examples of some ELISA based kits for the detection of pathogens include EHEC-TEK, Assurance, HECO157, TECRA, Premier O157, *E. coli* Rapitest, Transia Card *E. coli* O157 for *E. coli* O157:H7. Several such kits are also available for other pathogens.

8.4.4 Immuno-precipitation

This format is also a sandwich procedure but it does not make use of enzyme conjugated antibody for the detection purposes, instead it relies on the antibody conjugated with the latex beads or colloidal gold for the detection purposes. These assays are simple and require no washings or manipulations and take very less time to be completed. Examples of the tests based on

immunoprecipitation include PATH-STIK, Reveal, Clearview, for *Salmonella,* choleraSMART, bengalSMART for *Vibrio cholerae*.

8.4.5 Immuno-Magnetic Separation (IMS)

This is basically an enrichment step for the bacteria occurring in food matrix. This test utilizes the antibodies coupled with the magnetic particles. The specific bacteria bind to the antibody and is separated from the food matrix under the influence of magnetic field. It is similar to the enrichment using selective media. This enriched bacterial culture can then be detected by any of the rapid methods being discussed in this chapter.

8.5 GENETIC METHODS

Genetic methods for identification of micro-organisms are based on the detection of the specific gene sequences in the genotype of the organism. These sequences may be selected to detect particular group, genus, species or even strain of the micro-organism e.g. suppose a kit has to be developed for the detection of particular bacterial genus then the DNA sequence occurring only in the members belonging to that genus is to be targeted i.e. the gene sequence should occur in all the species belonging to that genus so that false negative reactions could be avoided, but if a kit for a particular species is to be designed then a DNA sequence occurring only in the members of this species should be targeted. This sequence should not occur in other species belonging to the same genus. The specificity of the detection process totally depends upon the targeted sequence, which is the most important part of designing an assay system. Several genes have been targeted for the detection purposes but rDNA is preferred because it is universally present in all bacteria and also shows required variation for designing the specific assay. Another advantage of targeting this gene is the availability of sequences of rDNA due to the development of databases. Other genes such as those involved in toxin production may also be targeted for this purpose. A genetic method for pathogen detection can be developed in various formats but the two most popular formats are the use of gene probes and polymerase chain reaction (PCR). Both these formats are designed to obtain a signal if the specific gene sequence is present.

8.5.1 Nucleic Acid Probes

For a diagnostic test to achieve widespread use in the food industry, it must also be easy to use. Probe-based tests have achieved this goal through immobilizing probes to inorganic supports (**dipsticks**) that allow the user to easily manipulate the probe (e.g., wash off unhybridized DNA) without damaging or losing it. This is referred to as **solid-phase** hybridization systems (e.g. solution-based) are also possible.

The principle behind the use of DNA probes is quite simple. Short single strands of DNA that are *complementary* to genes present in a pathogenic microbe are synthesized. The food sample must then be treated so that any microbial cells are lysed, releasing their DNA. Microbial DNA is then treated to convert it from double strands to single strands, and the probe is added. Hybridization (annealing of complementary strands) then occurs between the single-stranded DNA probe and single-stranded DNA released from pathogenic microbes present in the food. Probe DNA that has not hybridized must then be removed, usually by washing the sample, and the presence of hybridized DNA probes can then be detected.

Stomach food samples

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Enrichment of Salmonella

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Lyse cells and convert ds DNA to ss DNA

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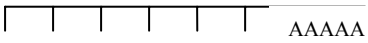
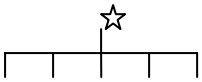
Add capture probe



and detector probe



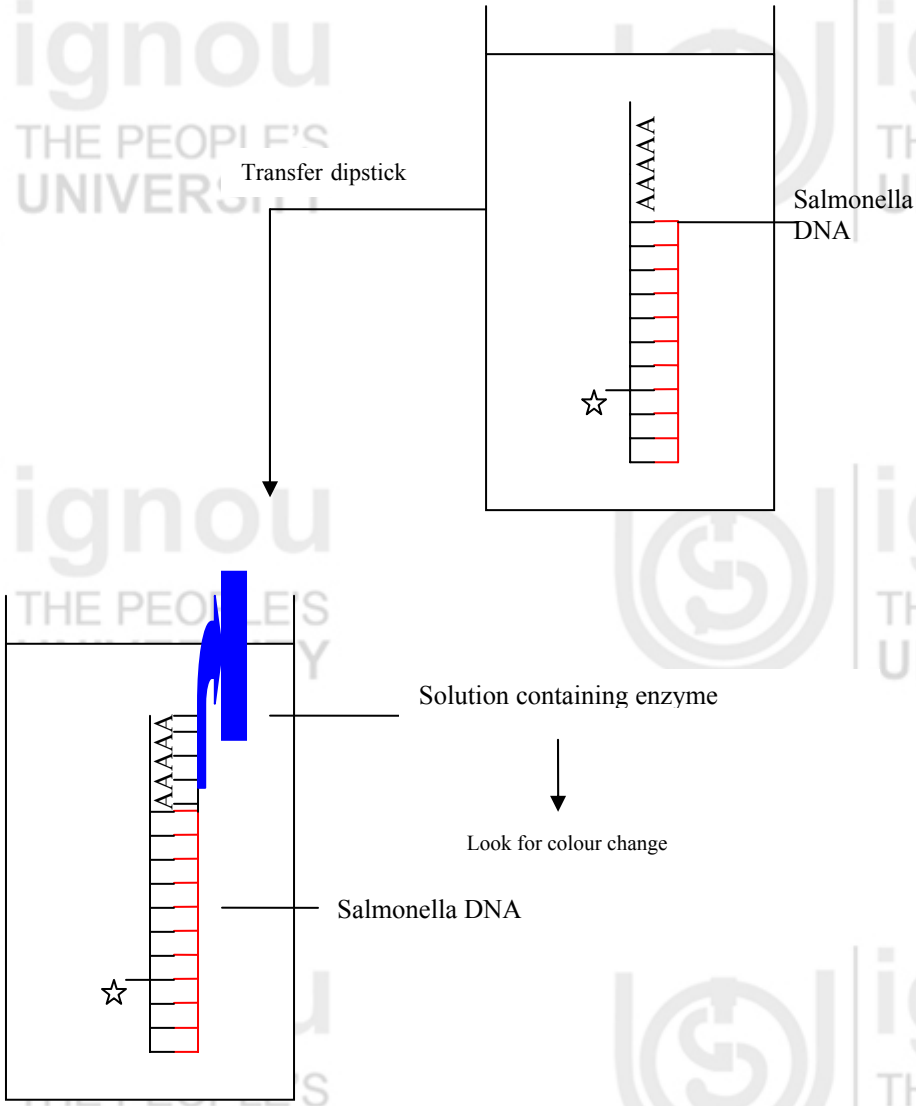


Fig 8.1: The use of a gene probe to detect a pathogen in food. The Gene Trak™ system for the detection of *Salmonella* is used as an example. The capture and detection probes anneal to different regions of the ribosomal DNA gene of *Salmonella*. The dipstick is used to remove the capture probe- *Salmonella* DNA-detection probe complex. The complex is then placed in the appropriate solution that will reveal the presence of the detection probe. In earlier versions of this system, detection was based on fluorescein in the probe binding to anti-fluorescein antibodies, which in turn bind to enzyme-linked antibodies. The enzyme then catalyzes formation of a coloured end product.

The principle advantage of this system is the reduced time required foe positive identification of *Salmonella*. Another advantage is the assay's

specificity; several bacteria found in food (*Citrobacter, Enterobacter, Escherichia, Klebsiella,* and Proteus are very closely related to *Salmonella* and are often able to grow in media that are "selective" for *Salmonella.* Furthermore, some isolates of *Salmonella* are atypical and do not produce the usual colony characteristics of *Salmonella* on differential media. The main problem with the Gene Trak system is that enrichment of *Salmonella* is still required, making "instant" identification of *Salmonella* impossible. However, enrichment is in a sense useful, because it makes it unlikely that deal *Salmonella* will be detected, unless they are present in high numbers. This is important, because dead *Salmonella* do not pose a health hazard, and the detection of deal *Salmonella* constitutes a false positive that may be wasteful of a company's resources. Because it is still possible that dead bacteria could lead to a false positive.

Formats using gene probes are based on the hybridization of the labelled DNA sequence (probe) to its complementary sequence. These probes may be labelled with radioactive or non radioactive reporter molecules. If the desired targeted sequence is present then the signal is obtained due to the hybridization of the labelled probe. If the targeted sequence is absent then probe will not bind and no signal will be obtained. gene probes can be used in dot blot hybridization assays or other formats. Examples of some kits based on gene probes are AccuProbe for *Campylobacter*, GENE-TRAK for *Campylobacter*, *E. coli*, *Listeria*, *Salmonella*, *Staphylococcus aureus* and *Yersinina enterocolitica*.

8.5.2 Polymerase Chain Reaction

This test is also based on the hybridization of the oligonucleotide primer pair followed by amplification of the DNA stretch spanning these primers. First step to carry out PCR is the isolation of DNA from the food matrix or enriched sample. This DNA provides the target for the hybridization of specific oligonucleotide primer and is called the template DNA. In addition to template DNA and primer pair, PCR amplification mix contains four dNTPs (Deoxynucleoside triphosphates, the building blocks from which the DNA polymerases synthesize a new DNA strand), *Taq* enzyme and *Taq* buffer. If a primer is having a complimentary DNA sequence it will hybridize with it. This step is termed as annealing. This DNA stretch between the two primers will be synthesized due to the addition of corresponding dNTPs by the action of *Taq,* which have its optimum activity at 72^{o}C. Once the synthesis is complete the DNA is denatured by heating the mixture to 95^{o}C and cooled again for primers to anneal. The annealing temperature is dependent on the primer sequence. This cycle (**Fig. 8.2)** is repeated several times in a machine called thermocycler to obtain the amplification of DNA. The amplified DNA is visualized by agarose gel electrophoresis, followed by Ethidium bromide (EBr) staining.

Several formats are available for carrying out PCR such as Real time PCR where the amplification of DNA is monitored while the reaction is being carried out, Multiplex PCR where more than one primer pair is used for the simultaneous detection of more than one type of bacteria but all these formats have not been converted to commercially available kits.

Examples of PCR based kits used for pathogen diagnosis are Probelia for *Clostridium botulinum, E. coli* O157:H7, *Salmonella* and BAX *for E. coli* O157:H7, *Listeria* and *Salmonella*.

It is too early to tell if the food industry will embrace PCR-based diagnostics. Considerable expenses are involved in the acquisition of thermal cyclers and the training of personnel in their use. Also, these techniques need to be

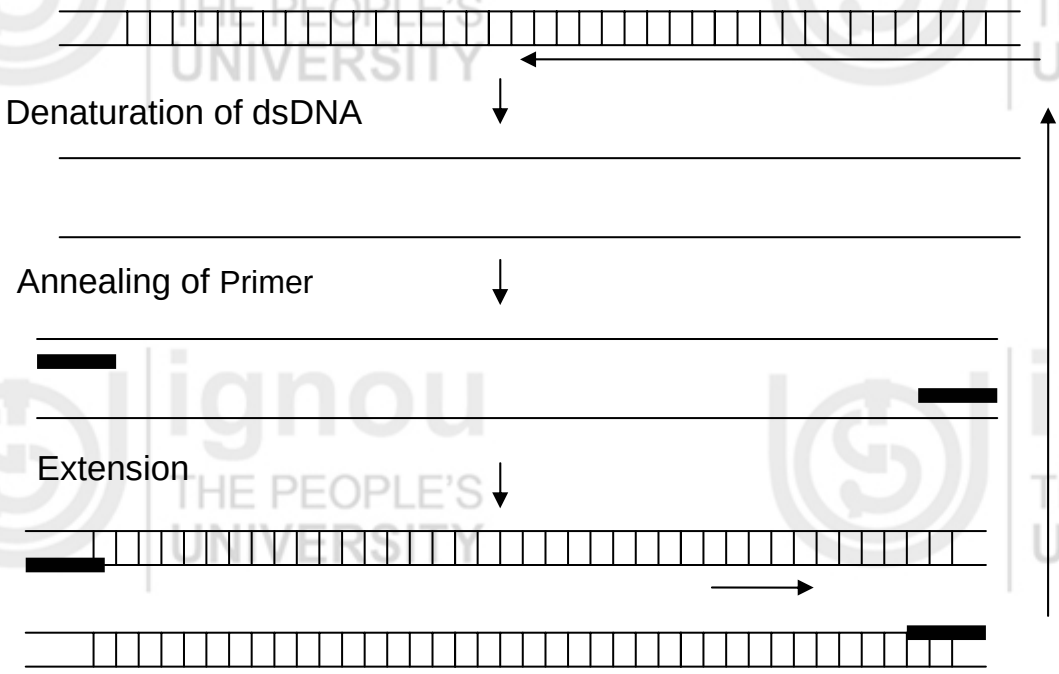


Fig 8.2: Steps in a PCR cycle

extensively validated, through comparison of their ability to detect low levels of contamination in food to those of conventional culture-based methods.

The ability of PCR to detect **dead** organisms is a problem. Tiny amounts of DNA released from pathogens killed by heat treatments, for example, would be amplified by PCR. This is less of a problem if enrichment precedes PCR, but that extends the procedure to at least 1 d. In some situations the presence of **live** *or* **dead** pathogens is an important indicator of food safety. For example, if PCR detects *Clostridium botulinum* in a food sample, it is a cause for concern whether the bacterium is alive or dead, because dead bacteria may have produced botulinum toxin before dying. The toxin could then persist in the food, causing a potential for serious illness.

One final note on PCR: several alternative amplification systems are actively under development. For example, nucleic acid sequence based amplification (NASBA®) uses three viral enzymes to amplify either RNA or DNA targets. The main advantage of this technique is that it is **isothermal** (occurs at a constant temperature), avoiding the need for expensive thermal cyclers. So far, NASBA diagnostics have mainly been aimed at identification of viruses, but applications for the identification of *Campylobacter* and *L. monocytogenes* are also under development.

8.5.3 DNA Chips and Micro-arrays

Few techniques have caused as much excitement among microbiologists as DNA chips and micro-arrays. Both are intrinsically miniaturized extensions of conventional tests of nucleic acid hybridization.

A sample of DNA is placed onto the membrane; labelled probe is then added; and hybridization (if present) is detected. Now consider a slightly different scenario: a number of oligonucleotides (each representing a different gene) are

immobilized onto separate points of a membrane. A bacterial culture is then exposed to a chemical that results in labelled messenger RNA (mRNA) transcripts. The bacteria are lysed and then placed on each oligonucleotide on the membrane. After washing, hybridization between labelled mRNA and immobilized oligonucleotides can be detected. This **micro-array** of oligonucleotide probes allows simultaneous detection of expression of a number of genes.

Now imagine a similar array of immobilized oligonucleotides on a 1 x 1 cm square on a glass microscope slide. Further miniaturization can be achieved with tiny wells etched into a circuit board. Oligonucleotides are then immobilized onto these wells, and the pattern of probe immobilization (i.e , which probes are loaded into which wells) can be controlled electronically. These are often referred to as **DNA chips** or **micro-arrays** (arrays on glass slides are also commonly referred to as micro-arrays). The great advantage of these systems is that they require only small amounts of resources. DNA chips can also be developed into laboratories in a chip, wherein an experimental routine, perhaps involving heating of reagents and mixing of several different chemicals, or even electrophoresis, can occur at a micro scale. If successfully applied to diagnostic testing, micro-arrays embedded into silicon chips could allow efficient testing of large numbers of samples and could even be used to amplify sample DNA using PCR. Theoretically, with one test the investigator could detect the presence of DNA of many different pathogens in a food sample.

Micro-arrays are currently commercially available for assessment of **global gene expression** (the total pattern of gene expression by a cell). Up to 8000 genes can be simultaneously tested for hybridization, allowing investigators to dissect changes in gene expression after different experimental treatments. This type of research has many potential applications in food microbiology (e.g. determining pattern of gene expression in a pathogenic bacterium triggered by exposure to acidic preservatives). Micro arrays also have great potential as diagnostic systems, but this is currently in the research and development phase.

Check Your Progress Exercise 2

Note: a) Use the space below for your answer.
b) Compare your answers with those given at the end of the unit.

1) What are the principles behind immuno-precipitation techniques?

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2) What is a DNA probe?

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3) List the drawbacks of using PCR?

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8.6 FLOW CYTOMETRY

8.6.1 Principle

Flow cytometry uses the principles of light scattering, light excitation, and emission of fluorochrome molecules to generate specific multi-parameter data from particles and cells in the size range of 0.5µm to 40µm diameter. Lasers are most often used as a light source in flow cytometry. One unique feature of flow cytometry is that it measures fluorescence per cell or particle. This contrasts with spectrophotometry in which the percent absorption and transmission of specific wavelengths of light is measured for a bulk volume of sample. Scattered and emitted light from cells and particles are converted to electrical pulses by optical detectors (Fig. 8.3). Collimated (parallel light waveforms) light is picked up by confocal lenses focused at the intersection point of cells and the light source. Light is sent to different detectors by using optical filters. For example, a 525 nm band pass filter placed in the light path prior to the detector will only allow "green" light into the detector. The most common type of detector used in flow cytometry is the photo-multiplier tube (PMT). The electrical pulses originating from light detected by the PMTs are then processed by a series of linear and log amplifiers. Logarithmic amplification is most often used to measure fluorescence in cells. This type of amplification expands the scale for weak signals and compresses the scale for "strong" or specific fluorescence signals.

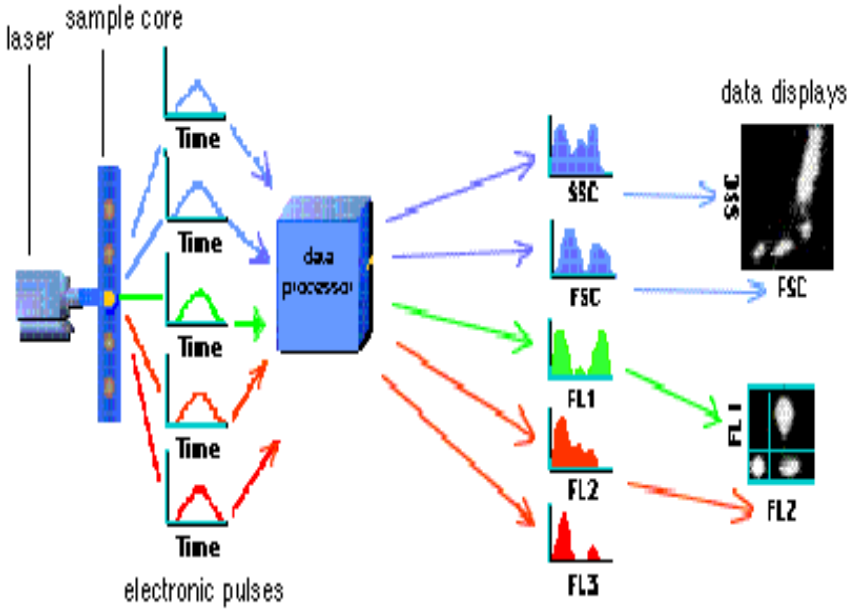

Figure 8.3: Working of a flow cytometer

After the different signals or pulses are amplified they are processed by an Analog to Digital Converter (ADC) which in turn allows for events to be plotted on a graphical scale (One Parameter, Two parameter Histograms).

Flow cytometry data outputs are stored in the form of computer files using the FCS 2.0 or 3.0 standards. Data corresponding to one sample can be stored as a listmode file and/or histogram file.

8.6.2 How does it work?

The tissue sample is broken up into single cells and held in a test tube, which is placed into the flow cytometer. The liquid containing the cells is drawn up from the test tube and pumped into the flow chamber.

1. **Flow chamber** – Cells flow through the flow chamber one at a time very quickly, about 10,000 cells in 20 seconds or 500 cells per second.
2. **Laser** – A small laser beam of very bright light hits the cells as they pass through the flow chamber. The way the light bounces off each cell gives information about the cell's physical characteristics. Light bounced off at small angles is called **forward scatter**. Light bounced off in other directions is called **side scatter**.
3. **Light detector** - The light detector processes the light signals and sends the information to the computer. Forward scatter tells you the size of the cell. Side scatter tells you if the cell contains granules. Each type of cell in the immune system has a unique combination of forward and side scatter measurements, allowing you to count the number of each type of cell.
4. **Filters** – The filters direct the light emitted by the fluorochromes to the colour detectors.
5. **Colour detectors** - As the cells pass through the laser, the fluorochromes attached to the cells absorb light and then emit a specific colour of light depending on the type of fluorochrome. The fluorochromes on the cells act like the bar code on groceries as the cashier passes them over the scanner. Any one cell can have one, both or none of the markers on its surface. The colour detectors collect the different colours of light emitted by the fluorochromes. The fluorochrome data signal is also sent to the computer.
6. **Computer** – The data from the light detector and the colour detectors is sent to a computer and plotted on a graph called a **histogram**.

The possible application of flow cytometry to the analysis of foods for their content of micro-organisms is by staining bacteria with fluorescent dyes, one of which binds preferentially to DNA rich in guanine-cytosine (G-C) while the other binds to adenine-thymine (A-T)-rich DNA. Thus, the rapid identification of bacteria in food based on their specific A-T/G-C ratios is possible. In addition to the simultaneous measurement of protein and DNA in cells and the enumeration of cells based on G-C and A-T content, flow cytometry has been used to distinguish between living and dead cells by dual staining; to determine the ploidy of yeast cells; to differentiate between spores and vegetative cells in *Bacillus* spp.; and to separate pathogenic and non pathogenic amoebae. It has been used to identify *Listeria monocytogenes* present in naturally contaminated milk. Flow cytometry (FCM) is extremely sensitive, avoids the need for culturing or enrichment procedures, and can be both qualitative and quantitative.

Use of fluorescent stains or fluorogenic substrates in combination with FCM allows the detection and discrimination of viable culturable, viable non culturable, and non viable organisms. Furthermore, there is the possibility that numerous (or even rare) microbial cells could be detected against a background of other bacterial or non bacterial particles by combining FCM and specific fluorescently-labeled antibodies or oligonucleotide probes. A flow cytometry method for rapid detection and enumeration of total bacteria in milk has been developed by Gunasekera *et al*. (2000).

8.7 IMPEDANCE

Impedance can be simply defined as the resistance to flow of an alternating current as it passes through a conducting material. To elaborate it further, when two metal electrodes are immersed in a conductive medium the test system behaves as a resistor and capacitor in series. Considering the case where the system is treated as a series combination then application of an alternating sinusoidal potential will produce a resultant current which is dependent on the impedance of the system which in turn is a function of its resistance, capacitance and applied frequency.

When micro-organisms grow in culture media, they metabolize substrates of low conductivity into products of higher conductivity and thereby decrease the impedance of the media. Simple examples include the conversion of glucose from a non ionized substrate to two molecules of lactic acid with a corresponding increase in conductivity. Further metabolism will take the lactic acid and three oxygen molecules to produce carbonic acid resulting in three ion-pairs, including the smaller, more mobile bicarbonate ion which is a more effective electrical conductor than the lactate ion. The impedance of a system is a function of its resistance, capacitance and the applied frequency. It is usually the resistive element which is measured and this is most frequently recorded as changes in conductance. These changes occur in the bulk electrolyte solution (culture medium) due to metabolism of uncharged or weakly charged substrates which are converted to highly charged end products e.g. proteins to amino acids. Capacitance is related to the behaviour of ions at the surface of the electrode and can be monitored separately from conductance. Several instruments which monitor changes in impedance are available and include the Bactometer 123 (Bactomatic Ltd.), Malthus 2000 (Malthus Instruments Ltd) and the Rapid Automated Bacterial Impedance Technique (RABIT) (Don Whitley Scientific Ltd). Detection of bacteria can be by direct conductimetry which is achieved by monitoring changes in the growth medium or by indirect conductimetry which monitors changes due to evolution of CO_2 produced by the metabolism of substrates in the culture medium. When the impedance of broth cultures is measured, the curves are reproducible for species and strains, and mixed cultures can be identified by the use of specific growth inhibitors. The technique has been shown capable of detecting as few as 10 to 100 cells and cell populations of 10^5-10^6/ml can be detected in 3-5 h and 10^4-10^5/ml in 5-7 h. Impedance has been evaluated by a large number of investigators as a means of monitoring the overall microbial quality of various foods.

The redox potential is one of the most complex indicators of the physiological state of microbial cultures and its measurement could be a useful tool for the qualitative and quantitative determination of the microbial contamination. During the bacterial growth, the redox potential of the medium decreases. The shape of the redox potential curve is characteristic of the type of micro-organism, and the rate of the change (dE/dt) is proportional to the living cell concentration. The time required to reach a significant change in redox potential is defined as Time to Detection (TTD). Similarly to the impedimetric measurements, a strict linear correlation could be established between the TTD and the logarithm of the initial concentration of micro-organisms. On the basis of this calibration curve, the determination of living cell concentration could be simplified.

The applications of this technique remains to be fully exploited though it has already been shown to be a powerful tool for working with strains of Aerobic mesophilic micro-organisms, Psychrotrophic micro-organisms, Thermophilic micro-organisms, gram negative bacteria, Enterobacteria, Enterococci, Lactobacilli, Coliforms, *E. coli*, *Salmonella*, *Listeria monocytogenes*, *Staphylococcus aureus*, Beer spoilers, Clostridia, Aerobic spore formers, *Bacillus cereus*, Yeasts and Molds.

8.8 BIOSENSORS

Biosensors have a **biological sensor** that is connected to a **transducer**. A transducer is a device capable of converting signals from the biological sensor into signals (usually electrical) that can be easily recorded and stored. For example, a number of biosensors use the specificity of antibody-antigen binding to detect pathogens in food samples. When the pathogen is present, it binds to the antibody. The key event follows: binding of antigen to antibody produces an electrical signal that can be detected and recorded. Biosensors, then, are an example of what science fiction authors describe as: 'cybernetics'– the fusion of organic matter to electronic circuitry.

8.8.1 Applications

Biosensors have many applications in clinical settings (e.g. diagnosis of food borne pathogens from stool and other samples) and in maintenance of food safety (e.g., assessment of microbial loads or detection of specific pathogens in food). For example, it is theoretically possible to design biosensors that are sensitive (e.g. , able to detect one pathogen in 25 g of food), selective (able to discriminate specific pathogens from a large background of non-pathogenic microbes), fast 9real time), automated, portable, and inexpensive. To date, this potential has not been realized, but research in biosensor development is highly active and steady improvements in design are predicted.

Continuous monitoring is also useful for many food safety or spoilage applications, particularly in the processing of liquids (milk, beer, etc.), where it is desirable to monitor microbial numbers in line in piping systems used to transfer liquids from one vessel to another or to packaging processes). For example, post-pasteurization contamination of beverages and foods is a significant cause of spoilage and has been implicated in outbreaks of food-borne illness.

Contamination of processing equipment is often difficult to eradicate. A continuous monitoring system that detects microbial growth in the lines is much better than monitoring based on examination of discrete samples or equipment swabs. If microbial growth could be immediately detected in transfer lines, the process could be stopped and the contamination eliminated, thus avoiding the production of large amounts of contaminated product.

Conventional diagnostic systems based on enrichment and selective culture are not adaptable for continuous monitoring. Instead, they require the collection of discrete sampling units (**batch samples)**. Each sample is then cultured in the appropriate media. Continuous monitoring using a culture-based system would require an infinite (or at least very large) number of sampling units, whereas biosensors can continuously monitor without the collection of discrete samples.

Biosensors can also be used for batch sampling. One important application of biosensors is to speed up pathogen identification using culture-based methods.

For example, spoilage of fresh meat is an economically important problem and is often linked to the presence of high levels of a variety of spoilage microbes. Conventional monitoring is done through plate counting (total bacterial count) after incubation on non-selective media that allow a wide range of bacteria to grow. However, this requires at least 24 h – not ideal for preventing meat spoilage. Biosensors have been designed that assess levels of microbial contamination after short (1 h or less) incubation of meat samples.

It is also often desirable to continuously monitor physical processes in the food industry. Physical such as temperature can easily be monitored continuously, allowing immediate adjustment if the temperature strays outside a defined range, and also providing a record of temperature changes. A continuous record can be useful if product quality declines; deviations in the temperature of the process could be a causal factor. Biosensors can be used to monitor some physical – chemical processes (e.g., CO_2 production).

8.8.2 Types of Biosensors

Biosensors can be classified according to the type of sensor, the transduction strategy, and the directness of the assay. **Affinity-based** biosensors rely on specific recognition between the sensor and the target. Antibodies are most commonly used, but nucleic acid hybridization, similar to that used in gene probe assays, and receptor-ligand interactions (e.g. insulin binding to insulin receptor molecules) are also used to create affinity-based biosensors. Antibody-based biosensors have been developed for most of the major food-borne pathogens (e.g. *Salmonella, E. coli O 157:H7, and L. monocytogenes*).

Improvements in out ability to miniaturize electronic components and to develop "laboratories in a chip" are also expected to lead to more frequent application of biosensors in the food industry. Applications are currently not common, mostly because of low sensitivity, interference by compounds in food matrices, and difficulties regenerating electrodes. The potential benefits, through, are significant enough to justify continued research and development of these elegant diagnostic systems.

Check Your Progress Exercise 3

Note: a) Use the space below for your answer.
b) Compare your answers with those given at the end of the unit.

1) What is a biosensor?

……

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……

2) Enlist the components of Flow Cytometry?

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……

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3) How can impedance be used to detect microbes?

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……

8.9 OTHER METHODS

These methods include the use of fatty acid profiling e.g. MIS Management Information System for the identification of *Enterobacteriaceae, Listeria, Bacillus, Staphylococcus* and *Campylobacter*, use of specific media with the incorporated flourogenic or chromogenic compounds for the detection of enzymatic activities of bacteria.

Another important rapid method for detection of total bacterial counts in food samples is Bioluminiscence, which can be defined as conversion of chemical energy to light by living organisms. In these assays enzyme luciferase present in fire fly is used, which in presence of ATP and luciferin forms oxy-luciferin that emits light. The fluorescence obtained by this method is measured by luminometer and is proportional to the concentration of ATP which in turn is related to the total bacteria occurring in the sample. The kits for obtaining rapid bacterial counts in the food samples are Enliten, Profile-1, Biotrace and Lightning.

8.10 LIMITATIONS

The main disadvantage of rapid methods is that the results may vary with the variation in the food products. This is because some food products may contain inhibitors for various reactions that form the very backbone of the rapid detection methods. This is observed in case of PCR because it is a very sensitive reaction and presence of very minute quantities of interfering agents may inhibit the reaction. On the other hand some food components may react with the added chemicals and lead to false positive results in the rapid tests.

8.11 LET US SUM UP

Rapid enumeration and detection of food borne micro-organisms both spoilage and pathogens can be accomplished by biochemical, immunological and genetic methods. Broadly, these methods can be categorized in to Biochemical methods, Immunological methods, Genetic methods, Flow Cytometry, Impedance method and miscellaneous methods are commercially available Kits to facilitate the hassle free performance of these methods. The above mentioned methods have been described in this unit along with their working principle, procedure (wherever possible), kits and automated equipment.

8.12 KEY WORDS

API Kits	:	Biochemical kit used for detection of pathogens
ELISA	:	Enzyme Linked Immunosorbent Assay
PCR	:	Polymerase chain reaction
PMT	:	Photomultiplier tube
ADC	:	Analog to Digital Converter
FCM	:	Flow cytometry
RABIT	:	Rapid Automated Bacterial Impedance Technique
TTD	:	Time to Detection
dNTPs	:	Deoxynucleoside triphosphates, the building blocks from which the DNA polymerases synthesize a new DNA strand

8.13 SOME USEFUL REFERENCES

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8.14 TERMINAL QUESTIONS

1. What is ELISA method? How does it work?
2. Describe various steps in a PCR cycle.
3. Describe the principle and working of flow cytometry.
4. How does Impedance technique function? Give names of instruments available for its commercial application as a rapid method for detection of bacteria.
5. Describe the functioning of biosensors.
6. Rapid detection methods have the potential of ushering in a new era of Real Time Microbiology. Give your comments.

8.15 ANSWERS TO CHECK YOUR PROGRESS EXERCISES

Check Your Progress Exercise 1

Your answer should include the following points:

1 Required by: food industry for in-line detection; Clinicians for diagnosis; Public health officials

2. These are the miniaturized version of the biochemical tests carried out conventionally for the identification of food borne microbes.

Check Your Progress Exercise 2

Your answer should include the following points:

1. An antibody is coated on the surface of microtitre well. This antibody is known as primary antibody. Bacterial antigen obtained from the food sample is added to the antibody coated wells. Antigen-antibody reaction is allowed to occur followed by washing the wells to remove the unbound antigen. After this, a secondary antibody tagged with an enzyme is added to the microtitre plate. This antibody is also specific for the antigen to be detected but it recognizes a different epitope on the surface of antigen. This step is again followed by washing to remove the unbound antibody, followed by the addition of substrate for the enzyme. This substrate is usually colourless and a coloured product is formed by the action of antibody bound enzyme. The colour development is taken as a positive reaction and even the intensity of the colour can be measured using ELISA reader.
2. Immobilizing probes having nucleic acid strands specific to the pathogen bound to inorganic supports (**dipsticks**) that allow the user to easily manipulate the probe (e.g., wash off unhybridized DNA) without damaging or losing it.

3. (i) Considerable expenses are involved in the acquisition of thermal cyclers and the training of personnel in their use.

(ii) These techniques need to be extensively validated, through comparison of their ability to detect low levels of contamination in food to those of conventional culture-based methods.

(ii) The ability of PCR to detect **dead** organisms is a problem. In some situations the presence of **live** *or* **dead** pathogens is an important indicator of food safety. For example, if PCR detects *Clostridium botulinum* in a food sample, it is a cause for concern whether the bacterium is alive or dead, because dead bacteria may have produced botulinum toxin before dying. The toxin could then persist in the food, causing a potential for serious illness.

Check Your Progress Exercise 3

Your answer should include the following points:

1) Biosensors have a biological sensor that is connected to a transducer. A transducer is a device capable of converting signals from the biological

sensor into signals (usually electrical) that can be easily recorded and stored.

2) Flow chamber; Laser; Light detector; Filters; Colour detectors; Computer

3) When micro-organisms grow in culture media, they metabolize substrates of low conductivity into products of higher conductivity and thereby decrease the impedance of the media. Simple examples include the conversion of glucose from a non ionized substrate to two molecules of lactic acid with a corresponding increase in conductivity. The impedance of a system is a function of its resistance, capacitance and the applied frequency. It is usually the resistive element which is measured and this is most frequently recorded as changes in conductance. These changes occur in the bulk electrolyte solution (culture medium) due to metabolism of uncharged or weakly charged substrates which are converted to highly charged end products e.g. proteins to amino acids. Capacitance is related to the behaviour of ions at the surface of the electrode and can be monitored separately from conductance.