CHAPTER 13

Molecular detection techniques for identification of bacterial pathogens

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DNA ISOLATION

Heat breaks open bacterial cells and releases cell components such as DNA. Once DNA is released from the cells, PCR can be used to amplify any antibiotic resistance genes present in the sample DNA.

Procedure

1. Label the top and side of your clear PCR tube with your sample number.
2. Use the micropipette to add 1ml of sterile DNAase-free water to the tube.
3. Use a sterile pipette tip to 'touch' a series of bacterial colonies on your plate or overnight grown culture from slants. You can sample 10 colonies, but avoid picking up a large amount a bacterial cells. Too much cellular material will inhibit the PCR reaction.
4. Add 0.5 ml of Tris-EDTA buffer to the bacterial culture
5. Close the top of the tube and flick the bottom to mix your bacterial cells.
6. Heat the tube: Place the tube in the heating plate and heat the cells to 95°C for 5 minutes.
7. Immediately transfer the tubes to -20^{0}C.

POLYMERASE CHAIN REACTION

Polymerase chain reaction, or PCR, is an *in-vitro* technique used to amplify particular segments of DNA found in a sample. One of the most commonly used molecular-based method for the detection of foodborne bacterial pathogens is polymerase chain reaction (PCR). PCR was invented about 30 years ago and it allows the detection of a single bacterial pathogen that present in food by detecting a specific target DNA sequence. PCR operates by amplifying a specific target DNA sequence in a cyclic three steps process. Firstly, the target double-stranded DNA is denatured into single-stranded DNA at high temperature. Then, two single-stranded synthetic oligonucleotides or specific primers which are the forward and reverse primer will anneal to the DNA strands. This is followed by the polymerization process whereby the primers complementary to the single-

stranded DNA are extended with the presence of deoxyribonucleotides and a thermostable DNA polymerase. The PCR amplification products are visualized on electrophoresis gel as bands by staining with ethidium bromide. PCR have been used in the detection of numerous foodborne pathogens like *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Staphylococcus aureus*, *Campylobacter jejuni*, *Salmonella* spp. and *Shigella* spp. PCR is used to rapidly produce billions of copies of one or more "target sequences" such as genes or repeating sequences of DNA. Once amplified, the target sequences can be used in a variety of ways. For example, DNA segments copied by PCR can be used for gene detection, gene sequencing, or even be inserted into the DNA of another organism. First, the DNA isolated from a particular sample is placed in a test tube with a "mastermix" containing 1) **Taq polymerase** (a heat-stable DNA polymerase), 2) **primers** specific to the target sequence, 3) **a supply of A, C, T, and G nucleotides**, and 4) **buffers** and **salts** necessary for Taq polymerase to function.

Then, the test tube is placed in a PCR machine, which performs the following steps.

1. **Denaturation**: DNA sample is heated, which breaks the hydrogen bonds and separates the DNA strands.
2. **Annealing**: DNA sample is cooled, which allows primers to hydrogen bond (anneal) with complementary sequences in DNA sample.
3. **Extension**: Once primers anneal to DNA sample, the sample is warmed again so Taq polymerase may add nucleotides to the 3' end of the primer according to base-pairing rules.

The PCR machine repeats these 3 steps for 20 or more cycles to yield over a billion copies of the target sequence ***if it is present*** in your DNA sample.

Procedure

1. Obtain one PCR tube containing the 22 µl PCR mastermix with primers that target the antibiotic reistant gene. Also obtain one PCR tube containing 22 µl PCR mastermix with primers that target the genes.
2. Use the micropipette to transfer 3 µl of template DNA to the mastermix PCR tube.
3. Keep your PCR tubes on ice tray.

4. The PCR tubes will go into the thermocycler, which will control the temperature shifts necessary for the PCR reaction. These shifts will be repeated 20-35 times to yield a large number of copies of the DNA region between the primers (if they are present).
5. After the PCR reactions are completed freeze store your samples for analysis.

GEL ELECTROPHORESIS

Gel electrophoresis is a technique that is used to separate fragments of DNA based on their sizes. DNA samples are loaded onto one end of an agarose gel that is immersed in buffer, and an electric current is used to pull the DNA through the gel. Because DNA is negatively charged, the DNA fragments will move away from the negatively-charged cathode and toward the positively-charged anode. Because the DNA fragments must pass through small pores within the agarose gel, the smaller fragments of DNA are able to move through the gel more easily and quickly. After a sufficient period of time has passed, the electric current will have separated the DNA fragments so that the larger fragments remain closer to the start point, while the smaller fragments have migrated farthest in the gel.

Multiplex PCR (mPCR)

Multiplex PCR offers a more rapid detection as compared to simple PCR through the simultaneous amplification of multiple gene targets. The basic principle of mPCR is similar to conventional PCR. However, several sets of specific primers are used in mPCR assay whereas only one set of specific primers are used in conventional PCR assay. Primer design is very important for the development of mPCR, as the primer sets should have similar annealing temperature in order to produce a successful mPCR assay. Besides, the concentration of primers is also important in mPCR. This is because interaction may occur between the multiple primer sets in mPCR that results in primer dimers, thus, the concentration of primers may need to be adjusted to ensure the production of reliable PCR products. Other important factors for a successful mPCR assay include the PCR buffer concentrations, the balance between magnesium chloride and deoxynucleotide concentrations, the quantities of DNA template, cycling temperatures and Taq DNA polymerase

Real-Time or Quantitative PCR (qPCR)

Real-time PCR or quantitative PCR is different from simple PCR whereby it does not require agarose gel electrophoresis for the detection of PCR products. This method is able to monitor the

PCR products formation continuously in the entire reaction by measuring the fluorescent signal produced by specific dual labelled probes or intercalating dyes. The fluorescence intensity is proportional to the amount of PCR amplicons. Several fluorescent systems have been developed for qPCR and the most commonly used fluorescent systems include SYBR green, TaqMan probes and molecular beacons. SYBR green is a double-stranded DNA (dsDNA)-binding fluorescent dye. This non-sequence-specific intercalating dye emits little fluorescence and the fluorescence signal is enhanced when bound to the minor groove of the DNA double helix. TaqMan probes and molecular beacons are the common alternatives to SYBR green. TaqMan probes, also known as

double-dye probes, are oligonucleotides that contain a fluorophore as the reporter dye at the 5′-end and the quenching dye at the 3′-end. The reporter dye and the quenching dye are close to each other and this prevent the emitted fluorescence of the reporter. TaqMan probe is complementary to a specific nucleotide sequence in one of the strands of amplicon internal to both primers and the system depends on the 5′-3′ exonuclease activity of Taq DNA polymerase that cleaves the probe and separates both dyes in order to generate the fluorophore signal.

Loop-Mediated Isothermal Amplification (LAMP)

LAMP is a novel nucleic acid amplification method developed by Notomi et al. which provides a rapid, sensitivity and specific detection of foodborne pathogens. LAMP is based on auto-cycling strand displacement DNA synthesis carried out by Bst DNA polymerase large fragment under isothermal conditions between 59°C and 65°C for 60 min. In LAMP, four primers comprising two inner primers and two outer primers are used to target six specific regions of target DNA. Cauliflower-like DNA structures bearing multiple loops as well as stem-loop DNAs of different sizes are the final products of LAMP. Large amount of amplicons can be produced by LAMP within 60 min which is usually 103-fold or higher as compared to simple PCR. The LAMP amplicons can be detected by agarose gel electrophoresis or SYBR Green I dye.