Appendix XIV: Deoxyribonucleic Acid (DNA) Identification

DNA analysis is one of the molecular methods for CMM identification, which is less affected by age, physiological conditions, environmental factors, harvest, storage and physical form of CMM. Using polymerase chain reaction (PCR) technique, tiny amount of template DNA can be amplified for typical DNA analysis methods. DNA fingerprinting is one of the DNA analyses to generate a set of DNA fragments which are characteristics of a particular test sample. DNA fingerprints can enable individuals, strains, species, etc. to be distinguished by variations in their genomes. Depending on the existing knowledge on the organism's genome, various techniques can be used for CMM identification, including but not limited to polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), random amplification of polymorphic DNA with sequenced characterized amplified region (RAPD-SCAR), simple sequence repeat (SSR) analysis and amplified fragment length polymorphism (AFLP).

(1) **Apparatus and materials –**

(a) **Incubator or thermal mixer** – A device that controls the incubation temperature for facilitating the lysis of sample in nucleic acid preparation.

(b) **Micro centrifuge** – An equipment that spins samples in small scale (less than 2 mL) at high speeds for pelleting.

(c) **UV-Vis spectrophotometer** – A device that determines DNA concentration and purity.

(d) **Thermal cycler (PCR machine)** – A device that is used to amplify segments of DNA via PCR.

(e) **DNA gel tank** – An apparatus with electrodes that holds an agarose gel used for gel electrophoresis.

(f) **Power supply** – A high voltage power supply that connects to the DNA gel tank setting up an electric field between the two electrodes.

(g) **Ultraviolet (UV) transilluminator** – A device that uses UV radiation to visualize DNA in an agarose gel.

(h) **Gel documentation system** – A system consists of UV transilluminator, image capture device and light source shield that captures and documents the image of nucleic acids in agarose gels.

(i) **Molecular biology grade water** – A type of water that is qualified for use in PCR.

(j) **PCR reagent** – A reagent that contains DNA polymerase, deoxyribonucleotides mix (dNTP) reaction buffer and cofactor of DNA polymerase for PCR.

(k) **Primers** – A short single-stranded DNA, usually 15 to 30 nucleotides in length, serves as a starting point for DNA synthesis in PCR.

(l) **DNA polymerase** – An enzyme that synthesizes DNA molecules. In PCR, DNA polymerase starts replication at the 3'- end of the primer. It is preferable to use high fidelity DNA polymerase with hot-start activity for low error rate during replication.

(m) **DNA ladder** – A set of molecular-weight size standards of double-stranded DNAs that is used to compare against the size of DNA fragment of interest. Mostly, 100 bp DNA ladder, in the range of 100 bp to 1000 bp, is commonly used for the analysis of PCR product size. The molecular size of PCR product should be within the range of DNA ladder.

(n) **DNA dye** – A chemical that stains and visualizes DNA, such as ethidium bromide, SYBR dyes.

(o) **Agarose** – A polysaccharide that can form a matrix for the separation of mixed DNA in different molecular sizes during gel electrophoresis.

(p) **Running buffer** – A buffer that is used in gel electrophoresis. TAE (Tris-acetate-EDTA) and TBE (Tris-borate-EDTA) are common gel electrophoresis buffers.

(q) **Loading buffer** – A buffer that contains density compound and tracking dye to prepare samples for loading on agarose gel.

(2) **Laboratory requirements** – The key advantage of PCR-based test is highly sensitive. However, if care is not taken to avoid contamination in the laboratory environment, false or inaccurate results will be obtained. Good laboratory practices should be adopted and proper precautionary measures should be taken.

(a) Separate areas are designated for different activities as follows:

- pre-PCR area – further divided into different workspaces for sample preparation, sample extraction, reagent preparation and conducting PCR;
- post-PCR area – for PCR product manipulation and analysis.

(b) The movement of materials or nucleic acid samples should be unidirectional (i.e. from pre-PCR area to post-PCR area) to prevent carryover contamination resulting from introducing high concentration products of previous PCR assay into pre-PCR area.

(c) Each area or workspace should have dedicated apparatus and materials. Those apparatus and materials should not allow to be taken to another workspace. Decontamination of apparatus and materials should be done whenever necessary before taking in and out of the area or workspace.

(d) There should be dedicated and separated workspace for storage of reagents and samples.

(3) **Procedure –**

(a) In general, the DNA test process can be divided into sample handling and sample preparation, DNA extraction, DNA amplification, detection of amplified products and results interpretation.

(b) DNA extraction is the most critical step in the DNA test process. Two quality controls, extraction positive control (EPC) and extraction negative control (ENC), should be run in parallel with the samples (details refer to part 4). The yield and purity of DNA extracts should be evaluated by means of spectrophotometric or fluorescence analysis.

(c) DNA amplification –

- The amplification is carried out according to the concentration of PCR components, amount of DNA template, and recommended cycling conditions specified in the monograph concerned.

- EPC and ENC, PCR negative control (PNC) should be run in parallel with the interest samples (details refer to part 4).

- PCR master mix, by combining all components required for PCR except template DNA, should be prepared and then aliquoted to each reaction tube. Add sample, EPC, ENC, water (for PNC) in the corresponding reaction tube accordingly.

(d) Detection of amplified products – The PCR amplified products can be resolved based on their molecular sizes achieved by agarose gel electrophoresis or capillary electrophoresis. The amplified product profile should be recorded by using gel-documentation system.

(4) **Quality control –**

(a) It should analyze every sample in, at least, duplicate for qualitative DNA fingerprinting method.

(b) Controls – At least one, preferably two, of each following control should be run in parallel with each batches of samples (for example every 20 samples), whichever is the less:

- **EPC** – It is the positive control of the DNA extraction process and it would reveal any defect or problem in the reagents used or the performance during the DNA extraction. This control is a reference material of known taxonomic identity or the organisms of interest with taxonomic identity verified by appropriate identification or certification through museums, national authorities, universities or research institution.

- **ENC** – It is the negative control of the DNA extraction process and it should be demonstrated by the absence of sample added to the control tubes. The tubes should always be the last in each batch of DNA extraction.

- **PNC** – It is negative control of PCR amplification reaction and it would reveal any defect and problems in the reagents used or the performance during the PCR reaction. This control should be demonstrated by free from any contaminating nucleic acids in the PCR reagent batches used. Sterile water should be added instead of template DNA.

(5) **Results interpretation** – The test results from the samples and the quality control should be interpreted as below:

(a) The test results from samples and EPC should be interpreted according to the specified monograph concerned.

(b) The sample must give a DNA band(s) of expected size corresponding to those of EPC.

(c) It should be no occurrence band in ENC and PNC.

(d) Every DNA band in DNA ladder should be shown clearly and well separated.