Practical activity (5)

Species Identification

(Student version)

I. Background

Adulteration of meat products is a common food fraud committed by food manufacturers. In adulterated meat products, meat from chicken and other low growing-cost species is commonly used to make up the weight of meat of a higher price, such as beef. Apart from giving a false trade description, food adulteration is a public health issue, as adulterated meat products might not be suitable for human consumption. People with allergies to specific proteins may be misled to consume items that contain the allergens. Also, adherents to a specific religion may be deceived into consuming food items that contain animal species which are forbidden by their religion. Therefore, the identification of animal species in meat products is a vital part of promoting food safety and protecting consumers' rights.

Some genes encoding for functional proteins, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and cytochrome b (*Cytb*), are conserved among various species. Thus, they can be used as markers in species identification. GAPDH is a key enzyme in glycolysis, which breaks down glucose for the generation of ATP. *Cytb* is one of the mitochondrial proteins that help electron transfer in cellular respiration for the generation of ATP. Due to their essential functions in cells, the DNA sequences of the genes encoding these two proteins are conserved across different species; however, slight sequential variations do exist. Hence, by comparing the restriction fragments of DNA sequences of these two genes in meat products, the constituting species can be identified.

DNA fingerprinting based on gene amplification by polymerase chain reaction (PCR) and restriction digestion of PCR products are used to determine the constituting species of a meat product. In this experiment, you are going to identify the animal species of an unknown meat product sample and check whether there is any adulteration in it. DNA from reference meats and from the unknown meat product will be extracted for the amplification of the *Cytb* gene. The amplified genes will then be cut into DNA fragments of different sizes by restriction digestion. The DNA fingerprint patterns of agarose gel electrophoresis of these fragments will be analysed.

II. Guiding questions about the design of the experiment

1. What is the purpose of the amplification of the *Cytb* gene?
2. What are the three major events in a polymerase chain reaction (PCR)?
3. What is the purpose of performing gel electrophoresis after PCR of the *Cytb* gene?
4. What is the expected gel electrophoresis result of the restriction digestion products of an adulterated meat product?

III. Objectives of the experiment

1. To amplify the *Cytb* genes of reference meats and the unknown meat sample using PCR;
2. To perform an agarose gel electrophoresis of the PCR products;
3. To perform restriction digestion of the amplified *Cytb* genes;
4. To perform an agarose gel electrophoresis of the restriction digestion products; and
5. To determine the existence of adulteration in the unknown meat sample.

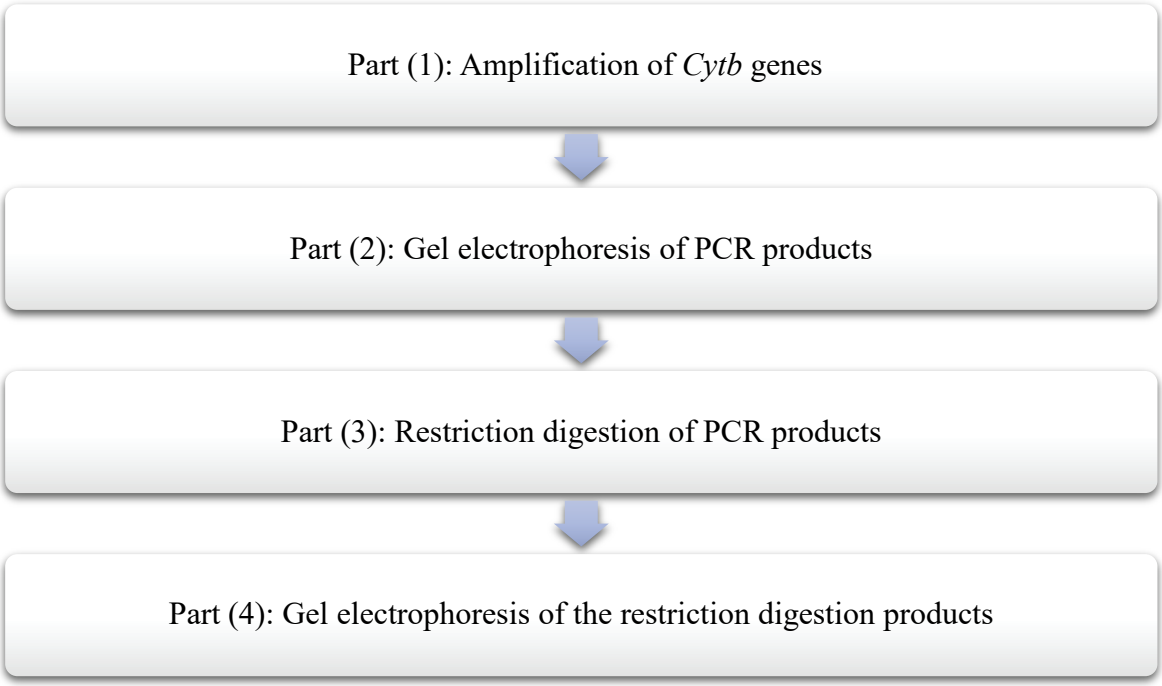
IV. Expected Learning Outcomes

Upon completion of the activities, students should be able to:

1. outline the principle of PCR;
2. recognise the application of PCR in the food industry;
3. outline the principle of DNA fingerprinting; and
4. recognise the application of DNA fingerprinting in identifying food adulteration of meat products.

V. The experiment

A. Overview



B. Part (1) of the experiment: Amplification of *Cytb* genes

a) Equipment and materials (per group)

Equipment

- Thermocycler × 1 (per class)
- Micro-centrifuge × 1
- Micropipettes (P200 and P10) and sterile tips

Materials

- 0.20 ml PCR tube × 5
- PCR-tube rack × 1
- Master mix solution (144 µl) × 1
- Chicken DNA sample (5 µl) × 1
- Pork DNA sample (5 µl) × 1
- Beef DNA sample (5 µl) × 1
- Meat product DNA sample (5 µl) × 1
- Nuclease-free water (5 µl) × 1

- Permanent marker × 1
- 70% ethanol in spray bottle × 1
- Paper towel × 1 box
- Biohazard bag × 1
- Disposal container with 10% chlorine bleach × 1

b) Safety precautions

- Wear a laboratory gown.
- Tie up long hair.
- Wear gloves during the experiment.
- Wash hands to remove all possible contamination before and after the experiment.
- Dispose of or disinfect all materials properly after each experiment.

c) Procedure

1. Label five 0.20 ml PCR tubes as indicated in Table 1, with your group number.

Table 1

Tube	Negative Control	Pure Chicken	Pure Pork	Pure Beef	Meat product
Label	NC	PC	PP	PB	MP

2. Using a P200 micropipette and new sterile tips, transfer 24 µl master mix solution into each of the 0.20 ml PCR tubes (see Fig. 2; the components of the master mix solution are listed in Table 2).

Table 2

Items	Volume (per tube)
Nuclease-free water	17.875 µl
10 × *Taq* buffer	2.5 µl
25 mM $MgCl_2$	1.5 µl
10 mM dNTP	0.5 µl
Forward primer	0.75 µl
Reverse primer	0.75 µl
Taq polymerase (1 U/µl)	0.125 µl
Total	24 µl

3. Using a P10 micropipette and new sterile tips*, add 1 µl DNA sample or nuclease-free water as indicated in Table 3 into the corresponding tubes (See Fig. 2).

*Note: Use a new pipette tip for each sample.

Table 3

Label on the tube	NC	PC	PP	PB	MP
Substance to be added (1 µl)	Nuclease-free water	DNA of pure chicken sample	DNA of pure pork sample	DNA of pure beef sample	DNA of meat product sample

4. Finger-flick the tubes to mix the contents (see Fig. 2).

5. Spin down the solution by the micro-centrifuge (see Fig. 2).

6. Put all tubes into a thermocycler and run the PCR programme as shown in Table 4.

Table 4

Phases in PCR		Temperature	Duration	Number of cycles
(a)		95°C	3 minutes	
(b)	(i)	94°C	40 seconds	(i), (ii) and (iii) × 36 cycles
	(ii)	45°C	30 seconds	
	(iii)	72°C	1 minute	
(c)		72°C	10 minutes	

a. What are the names of Steps (i), (ii), and (iii) in phase (b)?

(i) _____

(ii) _____

(iii) _____

b. What is the purpose of each of the 3 steps in phase (b) of PCR?

_____

_____

_____

_____

_____

c. How much time is needed for the above PCR programme?

_____

7. Store the PCR products at 4°C until the next laboratory session.

Fig. 2 summarises Steps 2–6.

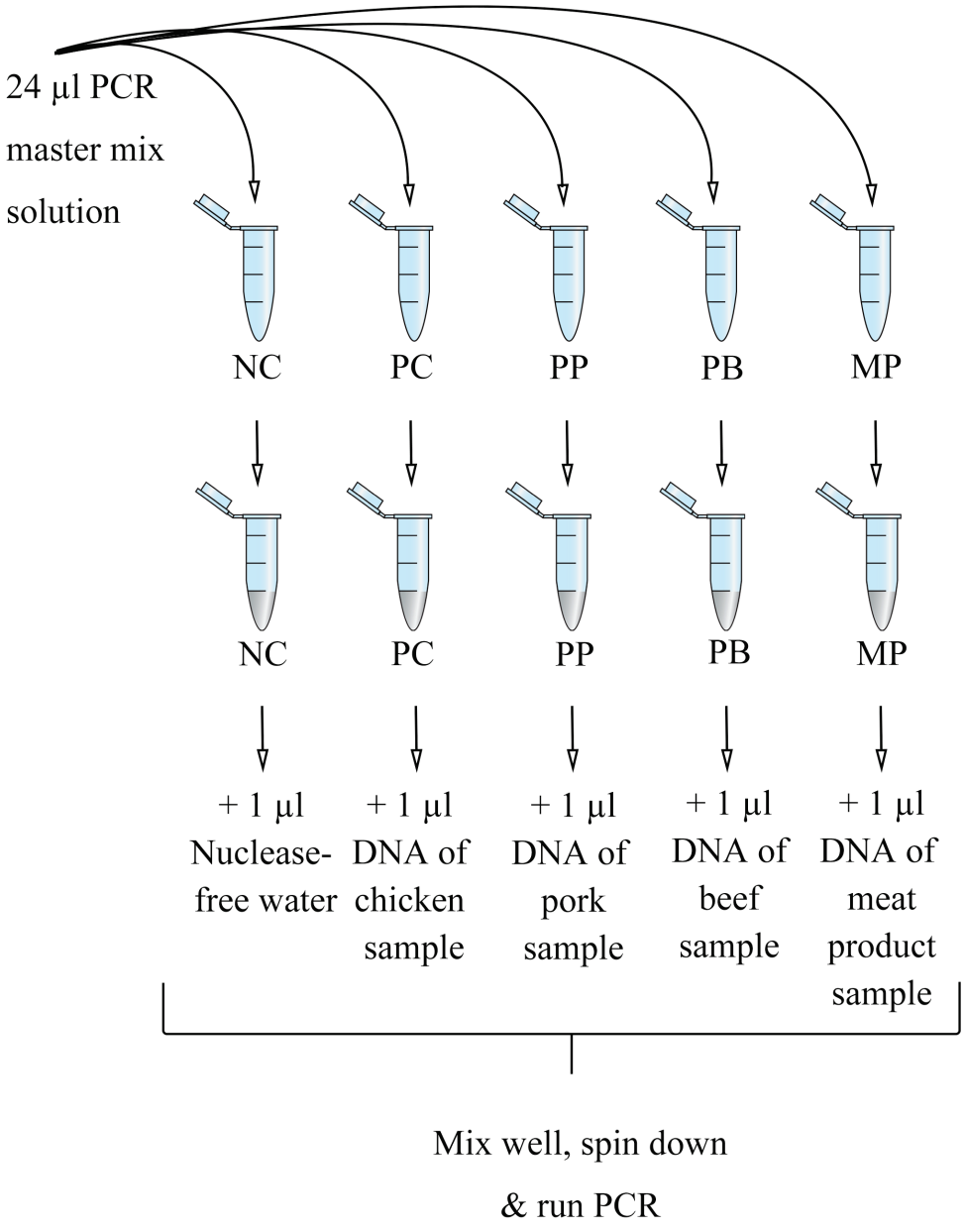


Fig. 2 Flow diagram of Part (1) of the experiment

C. Part (2) of the experiment: Gel electrophoresis of PCR products

a) Equipment and materials (per group)

Equipment

- Electrophoresis tank × 1
- Power supply × 1
- Gel documentation device / UV lamp × 1
- Micropipette (P10) and sterile tips

Materials

- PCR product from Part (1) × 5
- PCR-tube rack × 1
- Agarose gel in casting tray with a comb × 1
- Tris-Borate-EDTA (TBE) buffer (1 L) × 1
- DNA loading dye (10 µl) × 1
- DNA ladder (5 µl) × 1
- Parafilm

- Permanent marker × 1
- 70% ethanol in spray bottle × 1
- Paper towel × 1 box
- Biohazard bag × 1
- Disposal container with 10% chlorine bleach × 1

b) Safety precautions

- Wear a laboratory gown.
- Tie up long hair.
- Wear gloves during the experiment.
- Wash hands to remove all possible contamination before and after the experiment.
- Dispose of or disinfect all materials properly after each experiment.

c) Procedure

Loading of samples into the agarose gel

1. Collect the agarose gel prepared by the teacher/laboratory technician.

2. Pull out the comb carefully from the solidified gel to form wells (Fig. 3). Place the gel in an electrophoresis tank.

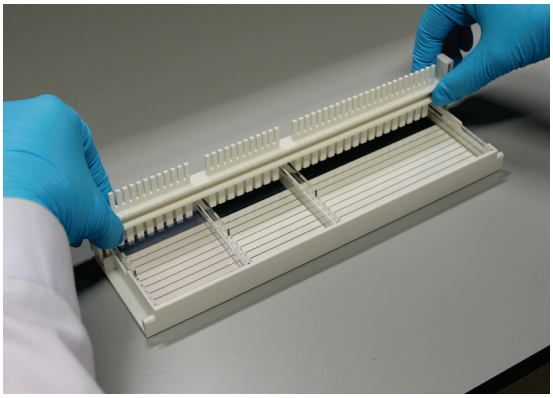


Fig. 3

3. Pour TBE buffer into the tank until the gel is completely covered (Fig. 4).

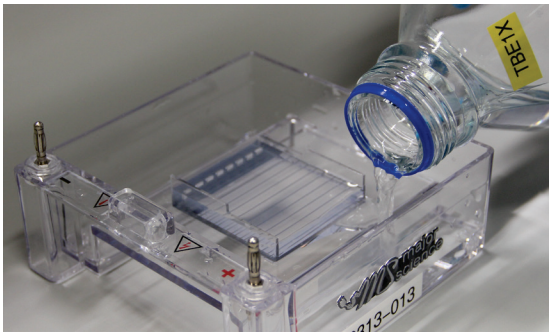


Fig. 4

4. Cut a piece of parafilm. Using a P10 micropipette and a sterile tip, pipette 1 µl of DNA loading dye onto the parafilm (Fig. 5).

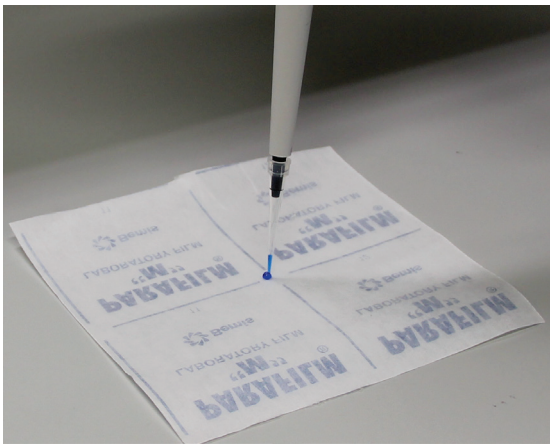


Fig. 5

5. Using a P10 micropipette and a sterile tip, add 4 µl PCR product "NC" onto the drop of the loading dye on the parafilm. Slowly pipette the dye-PCR product mixture up and down until the mixture is uniformly coloured.

6. Using a P10 micropipette and a sterile tip, carefully* load 4 µl dye-PCR product mixture into the well of the gel (Fig. 6) according to the layout shown in Table 5; i.e., load 4 µl dye-NC mixture into Well 2.

*Note: If bubbles are accidentally injected into the well, some DNA sample will be blown out from the well into the electrophoresis tank. This will cause a massive loss of DNA sample and a reduction of the signal.

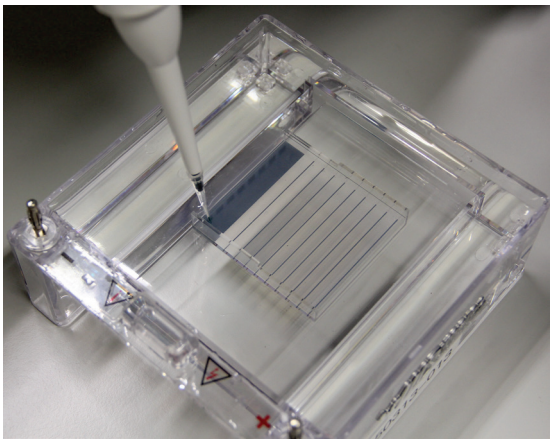


Fig. 6

Table 5 The layout of different samples in the agarose gel

Well position	Well 1	Well 2	Well 3	Well 4	Well 5	Well 6
PCR product	/	Negative control (NC)	Pure chicken DNA (PC)	Pure pork DNA (PP)	Pure beef DNA (PB)	Meat product DNA (MP)
Sample to be loaded	DNA ladder	dye-PCR product NC	dye-PCR product PC	dye-PCR product PP	dye-PCR product PB	dye-PCR product MP
Volume to be loaded	1 µl	4 µl	4 µl	4 µl	4 µl	4 µl

7. Repeat Steps 4–6 for the other PCR products from Part (1), using a new pipette tip for each PCR product.

8. Using a P10 micropipette and a sterile tip, carefully load 1 µl DNA ladder into the well of the gel according to the layout shown in Table 5; i.e., load it into Well 1.

Gel electrophoresis

9. Cover the electrophoresis tank, and connect the electrodes to the power supply.

10. Set the voltage at 80–100V and run the electrophoresis for 25−40 minutes#. Observe for the generation of air bubbles at the two electrodes to confirm a proper connection.

#Note: Running the gel at low voltage for a longer time can increase the separation distance of the bands. Running the gel at a voltage higher than 180V will heat up the buffer and soften the gel. The run time is determined based on the size of the gel, the voltage used, the type of buffer and the length of DNA molecules in the samples. As soon as the dye fronts have migrated to a position at about 80% of the gel length, electrophoresis can be stopped by switching off the power supply.

11. After electrophoresis, take the gel out of the tank, photograph it and record the DNA bands using a gel documentation device or under a UV lamp*.

*Note: Wear nitrile or latex gloves and UV protective eyewear or face shield when working with any UV device.

12. From the printout of the gel electrophoresis result, check the presence of a band of the 5 DNA samples.

13. After observation and analysis, discard the gel in the designated disposal container.

D. Part (3) of the experiment: Restriction digestion of PCR products

a) Equipment and materials (per group)

Equipment

- Micro-centrifuge × 1
- Micropipette (P20) and sterile tips

Materials

- PCR product from Part (1) × 5
- 0.20 ml PCR tube × 8
- PCR-tube rack × 1
- *Bsa*JI master mix solution (75 µl) × 1
- *Rsa*I master mix solution (75 µl) × 1

- Permanent marker × 1
- 70% ethanol in spray bottle × 1
- Paper towel × 1 box
- Biohazard bag × 1
- Disposal container with 10% chlorine bleach × 1

b) Safety Precautions

- Wear a laboratory gown.
- Tie up long hair.
- Wear gloves during the experiment.
- Wash hands to remove all possible contamination before and after the experiment.
- Dispose of or disinfect all materials properly after each experiment.

c) Procedure

1. Label eight 0.20 ml PCR tubes as indicated in Table 6, with your group number.

   Table 6

Restriction enzyme to be used	*Bsa*JI				*Rsa*I			
Sample	Pure Chicken	Pure Pork	Pure Beef	Meat product	Pure Chicken	Pure Pork	Pure Beef	Meat product
Label	BC	BP	BB	BM	RC	RP	RB	RM

2. Using a P20 micropipette and new sterile tips*, transfer 15 µl *Bsa*JI master mix solution into each of the 0.20 ml PCR tubes labelled "BC", "BP", "BB" and "BM" as shown in Table 7.

   *Note: Use a new pipette tip for each sample.

3. Using a P20 micropipette and new sterile tips#, transfer 15 µl *Rsa*I master mix solution into each of the 0.20 ml PCR tubes labelled "RC", "RP", "RB" and "RM" as shown in Table 7.

   #Note: Use a new pipette tip for each sample.

4. Using a P20 micropipette and new sterile tips^, add 5 µl of each PCR product from Part (1) into its corresponding PCR tube as shown in Table 7. In other words, add 5 µl PCR product "PC" into the PCR tube "BC".

   ^Note: Use a new pipette tip for each sample.

   Table 7

PCR Tube	BC	BP	BB	BM	RC	RP	RB	RM
Master mix solution to be added (15 µl)	*Bsa*JI	*Bsa*JI	*Bsa*JI	*Bsa*JI	*Rsa*I	*Rsa*I	*Rsa*I	*Rsa*I
PCR product to be added (5 µl)	PC	PP	PB	PM	PC	PP	PB	PM

5. Finger-flick the tubes to mix the content (see Fig. 7).

6. Spin down the solution by the micro-centrifuge (see Fig. 7).

7. Incubate all tubes at 37°C for 16–24 hours in a thermocycler or water bath (see Fig. 7).

8. After the incubation as stated in Step 7, store the products of restriction digestion at 4°C until the next laboratory session.

The following diagram summarises Steps 2–7.

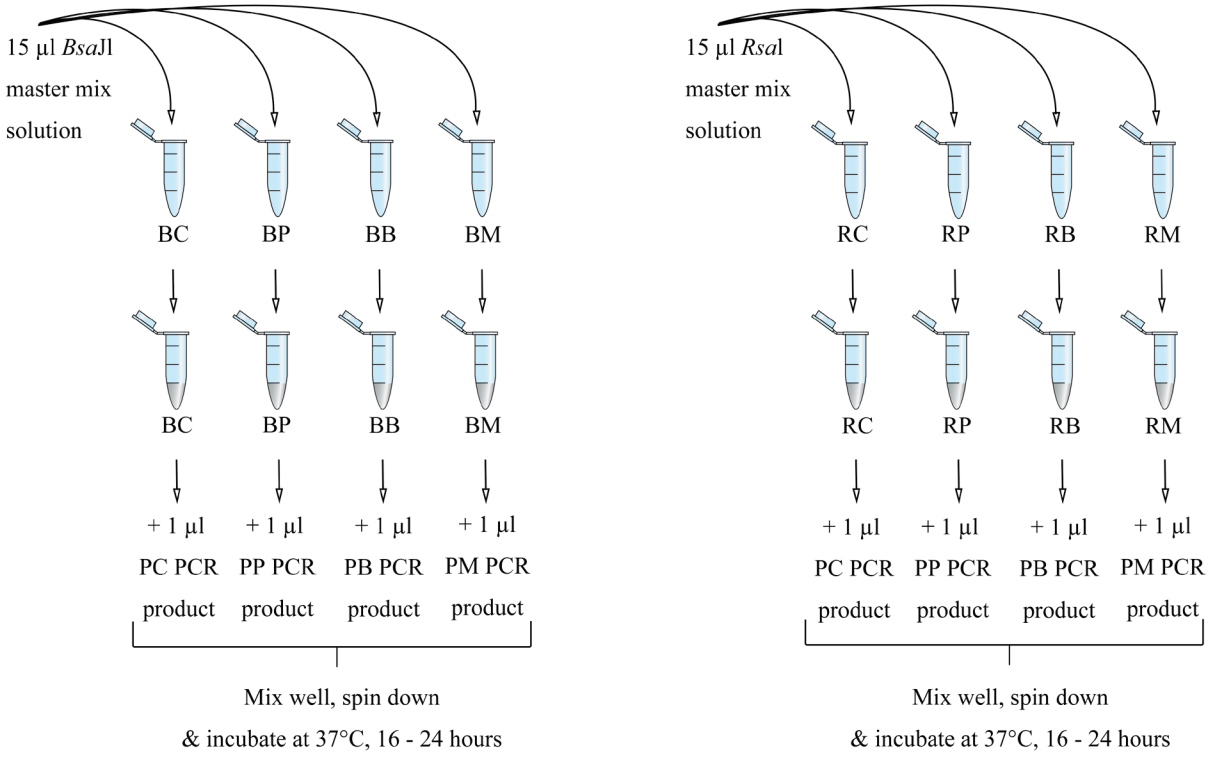


Fig. 7 Flow diagram of Part (3) of the experiment

E. Part (4) of the experiment: Gel electrophoresis of restriction digestion products

a) Equipment and materials (per group)

Equipment

- Electrophoresis tank × 1
- Power supply × 1
- Gel documentation device / UV lamp × 1
- Micropipette (P10) and sterile tips

Materials

- Restriction digestion product from Part (3) × 8
- PCR-tube rack × 1
- Agarose gel in casting tray with a comb × 1
- Tris-Borate-EDTA (TBE) buffer (1 L) × 1
- DNA loading dye (15 µl) × 1
- DNA ladder (5 µ1) × 1
- Parafilm

- Permanent marker × 1
- 70% ethanol in spray bottle × 1
- Paper towel × 1 box
- Biohazard bag × 1
- Disposal container with 10% chlorine bleach × 1

b) Safety precautions

- Wear a laboratory gown.
- Tie up long hair.
- Wear gloves during the experiment.
- Wash hands to remove all possible contamination before and after the experiment.
- Dispose of or disinfect all materials properly after each experiment.

c) Procedure

Loading of samples into the agarose gel

1. Collect the agarose gel prepared by the teacher/laboratory technician.

2. Pull out the comb carefully from the solidified gel, to form wells. Place the gel in an electrophoresis tank (Fig. 8).

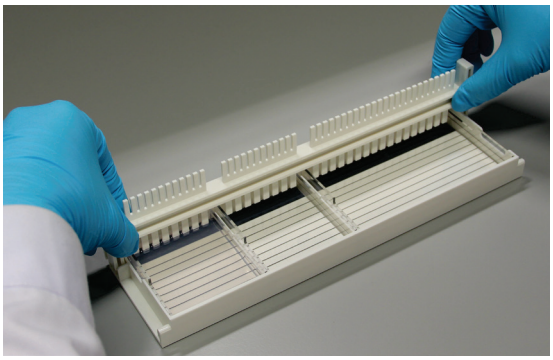


Fig. 8

3. Pour TBE buffer into the tank until the gel is completely covered (Fig. 9).

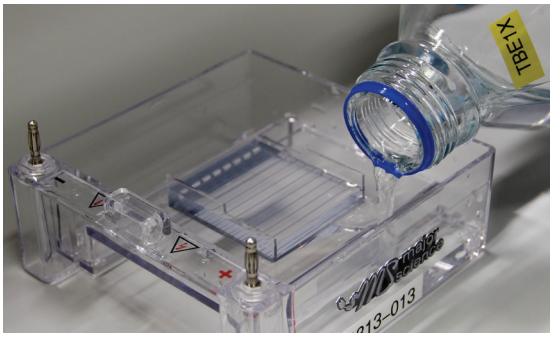


Fig. 9

4. Cut a piece of parafilm. Using a P10 micropipette and a sterile tip, pipette 1 µl DNA loading dye onto the parafilm (Fig. 10).

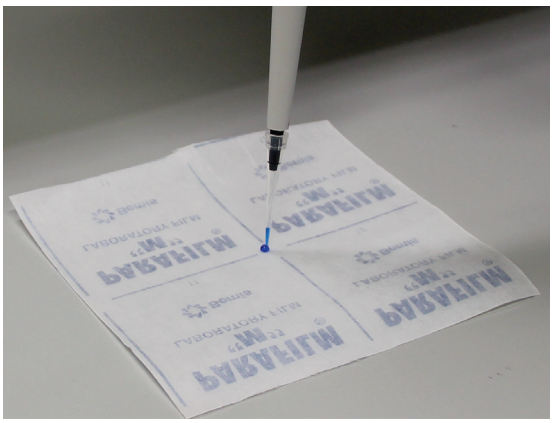


Fig. 10

5. Using a P10 micropipette and a sterile tip, add 4 µl digestion product "BC" onto the drop of the loading dye on the parafilm. Slowly pipette the dye-digestion product mixture up and down until the mixture is uniformly coloured.

6. Using a P10 micropipette and a sterile tip, carefully load 4 µl dye-digestion product mixture into the well of the gel, according to the layout shown in Table 8; i.e. load 4 µl dye-BC mixture into Well 2.

Table 8 The layout of different samples in the agarose gel

Well position	Well 1	Well 2	Well 3	Well 4	Well 5	Well 6	Well 7	Well 8	Well 9
Digestion product	/	BC	BP	BB	BM	RC	RP	RB	RM
Sample to be loaded	DNA ladder	dye-BC	dye-BP	dye-BB	dye-BM	dye-RC	dye-RP	dye-RB	dye-RM
Volume to be loaded	1 µl	4 µl	4 µl	4 µl	4 µl	4 µl	4 µl	4 µl	4 µl

7. Repeat Steps 4–6 for the other *Bsa*JI-digested products, using a new pipette tip for each sample.

8. Repeat Steps 4–6 for the *Rsa*I-digested products, using a new pipette tip for each sample.

9. Using a P10 micropipette and a sterile tip, carefully load 1 µl DNA ladder into the well of the gel, according to the layout shown in Table 8, i.e. load it into Well 1.

Gel electrophoresis

10. Cover the electrophoresis tank and connect the electrodes to the power supply.

11. Set the voltage at 80–100V and run the electrophoresis for 25−40 minutes[*]. Observe the generation of air bubbles at the two electrodes, to confirm the correct connection.

> [*]Note: Running the gel at low voltage for a longer time can increase the separation distance of the bands. Running the gel at a voltage higher than 180V will heat up the buffer and soften the gel. The run time is based on the size of the gel, the voltage used, the type of buffer and the length of DNA molecules in the samples. As soon as the dye fronts have migrated to a position at about 80% of the gel length, electrophoresis can be stopped by switching off the power supply.

12. After electrophoresis, visualise and photograph the DNA bands using a gel documentation device or under a UV lamp[#].

> [#]Note: Wear nitrile or latex gloves and UV protective eyewear or face shield when working with any UV device.

13. From the printout of the gel electrophoresis result, observe the band patterns of the 8 restriction digestion products. Estimate the size of the DNA bands of each sample by comparing them to the position of the bands of the DNA ladder.

14. After observation and analysis, discard the gel in the designated disposal container.

VI. Results

Part (2) of the experiment

1. Paste the image of the gel electrophoresis of PCR products.

2. Is there any band observed from the PCR product "NC"? Suggest the reasons for this.

3. Describe the band patterns of the PCR products on the gel after electrophoresis and what information about the PCR products can you get from the position of the bands?

Part (4) of the experiment

1. Paste the image of the gel electrophoresis of *Bsa*JI-digested products.

2. Paste the image of the gel electrophoresis of *Rsa*I-digested products.

3. Observe the band patterns on the gel and fill in the following table. Count the number of bands for each restriction digestion product. Estimate the size of the DNA sequence at each band by comparing their position with the bands of the DNA ladder.

Restriction enzyme used	*Bsa*JI					*Rsa*I			
Restriction digestion products	Pure chicken	Pure pork	Pure beef	Meat product		Pure chicken	Pure pork	Pure beef	Meat product
Number of DNA bands									
Estimated size of the DNA sequence at each band (bp)									

4. The meat product sample contains _____.

VII. Discussion

1. Describe the band patterns on the gel of the restriction digestion products of pure chicken, pure pork, and pure beef after electrophoresis.

2. Explain the differences in DNA band patterns of the tested meats.

3. Determine and explain whether the unknown meat product sample is adulterated.