Practical Activity (5)

Species Identification

(Teacher's Guide)

I. 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.

II. Expected Learning Outcomes

Upon the 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.

III. Teaching notes:

1. Introduce the task by going through the "Background" with students or assign the students to read through this part and the "Guiding questions about the design of the experiment" as a pre-class activity.
2. Discuss the design of the experiment with the students using the "Guiding questions about the design of the experiment".
3. Plan the laboratory work with reference to the "Time allocation for the experimental activities" in section IV according to the lesson time of your school.
4. Always remind the students about the safety precautions of each part of the experiment.
5. Go through the "Results" and "Discussion" with the students.

IV. Time allocation for the experimental activities:

Experimental activities		Duration	
		Within class	**Out of class** (To be done by the laboratory technicians)
Lab session 1: Part (1) of the experiment			
1	Preparation of PCR master mix		30−45 min
2	Setup of the PCR experiment	30 min	
3	Running of PCR		90 min
4	Storage of PCR products at 4°C		Store until the next lab session
Lab session 2: Part (2) of the experiment			
1	The casting of agarose gel		30−45 min
2	Loading of samples into agarose gel	10 min	
3	Gel electrophoresis of PCR products	40 min	
4	Gel documentation	10 min	
Lab session 3: Part (3) of the experiment			
1	Restriction digestion	40 min	
2	Incubation and storage of restriction digestion products		Incubation at 37°C for 24 hours, followed by storage at 4°C until the next lab session
Lab session 4: Part (4) of the experiment			
1	Loading of samples into agarose gel	10 min	
2	Gel electrophoresis of restriction digestion products	40 min	
3	Gel documentation	10 min	
Total lesson time for the experimental activities		Lab session 1: 30 min Lab session 2: 60 min Lab session 3: 40 min Lab session 4: 60 min	

V. Equipment, materials, and preparatory work for the experiments

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

a) Equipment (per group)

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

b) Materials (per group)

- 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

c) Preparatory work

Extraction of DNA from reference meats **(One day before lab session; to be done by the teacher/laboratory technicians)**

A commercial kit, QIAamp DNA Mini Kit, from Qiagen will be needed for DNA extraction. Some additional equipment or materials (e.g. ethanol and micro-centrifuge tubes) are required.

Local distributor: QIAGEN Hong Kong PTE. LTD.
Phone number: 3652-3652
Website: https://www.qiagen.com/cn/products/top-sellers/qiaamp-dna-mini-kit/#orderinginformation

Equipment:

- Vortex × 1
- Tissue homogeniser × 1

1. Weigh 25 mg of each test sample (chicken, pork, beef, and meat product) in sterile micro-centrifuge tubes.
   Note: Dim sum and other processed meat are suitable to use as the meat product sample in this experiment.
2. Add 180 µl Buffer ATL to the 4 micro-centrifuge tubes.
3. Vortex the tubes for 15 seconds. Spin down the contents in a micro-centrifuge for 15 seconds.
4. Add 20 µl proteinase K to the 4 tubes. Mix the content by turning the tubes upside down 3–4 times. Spin down the tubes briefly.
5. Incubate all tubes in the 56°C water bath for 2–3 hours, with vortexing every 30 minutes, until the meat tissues are completely digested by proteinase K.
6. Homogenise the test samples (chicken, pork, beef, and meat product) in the micro-centrifuge tubes individually, using a tissue homogenizer.
7. Briefly spin down the contents of the tubes.
8. Add 200 µl Buffer AL to the 4 tubes. Mix by vortexing.
9. Incubate all tubes in the 70°C water bath for 10 minutes, with tube inversion every 3 minutes, until the meat tissues are completely dissolved.
10. Briefly spin down the content of the tubes.
11. Add 200 µl ethanol (≥96%) to the 4 tubes. Vortex for 10 seconds at the lowest speed, and briefly spin down the contents of the tubes.
12. According to the manufacturer's instructions, load each sample into one QIAamp mini spin column (included in the DNA extraction kit) with a 2.0 ml collection tube attached.
13. Cap and centrifuge the columns at 6,000 × g (or 8,000 rpm) for 1 minute at room temperature.
14. Transfer each spin column to a new collection tube, and discard the filtrate.
15. Add 500 µl Buffer AW1 to each of the 4 columns.
16. Cap and centrifuge the columns at 6,000 × g (or 8,000 rpm) for 1 minute at room temperature.
17. Transfer each spin column to a new collection tube, and discard the filtrate.
18. Add 500 µl Buffer AW2 to each of the 4 columns.
19. Cap and centrifuge the columns at 20,000 × g (or 14,000 rpm) for 3 minutes at room temperature.
20. Lift up the columns and pour off the filtrate in the collection tubes. Centrifuge the columns at 20,000 × g (or 14,000 rpm) for 1 minute at room temperature.
21. Transfer each spin column to a sterile micro-centrifuge tube, and discard the filtrate.
22. Add 50 µl sterile distilled water to each of the 4 columns. Incubate them at room temperature for 7−8 minutes. Centrifuge them 6,000 × g (or 8,000 rpm) for 1 minute at room temperature.
23. Store the extracted DNA samples at 4°C until use.

Aliquot of experimental materials **(to be done by the teacher/laboratory technicians before lab session)**

1. Aliquot 5 µl chicken DNA sample into 0.20 ml PCR tubes. Prepare 1 tube per group.
2. Aliquot 5 µl pork DNA sample into 0.20 ml PCR tubes. Prepare 1 tube per group.
3. Aliquot 5 µl beef DNA sample into 0.20 ml PCR tubes. Prepare 1 tube per group.
4. Aliquot 5 µl meat product DNA sample into 0.20 ml PCR tubes. Prepare 1 tube per group.
5. Aliquot 5 µl nuclease-free water into 0.20 ml PCR tubes. Prepare 1 tube per group.

Preparation of master mix solution **(to be done by the teacher/laboratory technicians before lab session)**

1. In a 0.20 ml PCR tube, add the following items to a total volume of 144 µl, which is in excess of 5 test samples:

a. Nuclease-free water: 107.25 µl
b. 10 × *Taq* buffer: 15 µl
c. 25 mM $MgCl_2$: 9 µl
d. 10 mM dNTP: 3 µl
e. Forward primer: 4.5 µl
f. Reverse primer: 4.5 µl
g. *Taq* polymerase (1 U/µl): 0.75 µl

2. Prepare 1 tube per group.

   Note: Primers can be custom-ordered from Tech Dragon, Integrated DNA Technologies, BGI BIO-Solutions HK Co. Ltd., or companies that provide custom oligo synthesis services. Other items can be purchased from local biotechnology companies.

   Primer sequences

   Cytb forward primer: CCA TCC AAC ATC TCA GCA TGA TGA AA (26 bp)
   Cytb reverse primer: GCC CCT CAG AAT GAT ATT TGT CCT CA (26 bp)

   [Tip: It is recommended to add the master mix components into the 0.20 ml micro-centrifuge tube in the order in which they are listed in the above table.

   The reasons are

   (i) it is the best practice to add the purest and least expensive component first in order to minimise the loss in case of any mistake made during the preparation; and

   (ii) (ii) it is the best practice to add the component of the largest volume first, as the subsequent components can be dissolved in the solution more easily.]

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

a) Equipment (per group)

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

b) Materials (per group)

- PCR product from Part (1) × 5
- PCR-tube rack × 1
- Agarose gel in casting tray with a comb × 1
- Tris-Borate-EDTA (TBE) buffer (1L) × 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

c) Preparatory work

Casting of agarose gel **(to be done by the teacher/technicians before lab session)**

1. Prepare 1 gel per group.

2. Weigh 0.20 g of agarose powder in a conical flask.

   [Tip: Or weigh the powder on a small piece of weighing paper and then pour the powder into the conical flask after weighing.]

3. Add 20 ml TBE buffer to the conical flask. Heat the mixture in a microwave oven until all agarose powder is dissolved and the agarose solution becomes clear.

   [Important note: Shaking of TBE buffer should be avoided, as the shaking may carry the undissolved agarose aggregates to the sidewall of the conical flask. These aggregates may not get dissolved even after heating.]

   [Tip: Ready-to-use 1X TBE buffer can be purchased from biotechnology companies. Alternatively, 10X TBE buffer can be purchased and diluted to 1X with distilled/deionised water.]

   [Important note: Completely dissolved agarose solution should be transparent and very clear. Sometimes small fibre-like materials may be seen in the transparent and clear agarose solution after heating, when observing at close range. These fibre-like materials are undissolved agarose, and they indicate that further heating is needed.]

4. Cool the agarose solution slightly under running water.

   [Tip: If the agarose solution feels warm, then it is sufficiently cooled. Over-cooling may cause the gel to solidify prematurely.]

5. Add 2 µl of gel red to the solution and mix the solution gently.

   [Important note: Traditional DNA visualising agents are carcinogenic. Modern DNA visualising agents such as gel red and gel green are non-toxic to humans. However, it is still suggested handling gel red with care and avoiding direct skin contact. Adding gel red into a very hot agarose gel should be avoided, as gel red may get vaporised.]

6. Place a 8-well comb into the casting tray and carefully pour in the agarose solution. Remove any bubbles formed with the wide end of a P200 pipette tip.

7. Wait approximately 25−30 minutes for the agarose to get solidified into an opaque gel.

8. Store the gel in TBE buffer at 4°C if it is prepared one day before the lab session.

Aliquot of experimental materials **(to be done by the teacher/laboratory technicians before lab session)**

1. Aliquot 10 µl DNA loading dye into 0.20 ml PCR tubes. Prepare 1 tube per group.

2. Aliquot 5 µl DNA ladder into 0.20 ml PCR tubes. Prepare 1 tube per group.

3. Aliquot 1 L TBE buffer into 1 L bottles. Prepare 1 bottle per group.

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

a) Equipment (per group)

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

b) Materials (per group)

- 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

c) Preparatory work

Preparation of restriction enzyme master mix solutions **(to be done by the teacher/laboratory technicians)**

1. In a 0.20 ml PCR tube, dilute the restriction enzyme (i.e. *Bsa*JI or *Rsa*I) from 10 U/µl to 2 U/µl with TANGO buffer, i.e. add 2.5 µl enzyme solution to 10 µl TANGO buffer.

2. To the tube, add 62.5 µl nuclease-free water to a total volume of 75 µl, which is in excess of 4 test samples.

3. Prepare 1 tube of *Bsa*JI master mix and 1 tube of *Rsa*I master mix per group.
   Note: Restriction enzymes can be purchased from Thermo Fisher Scientific, New England Biolabs, or other biotechnology companies. TANGO buffer is provided as a dilution buffer of the enzyme.

   [Information: The purpose of preparing materials sufficient for 5 samples is to compensate for the pipetting errors made during the aliquot process. As pipetting errors are inevitable, the master mix solution for the last sample will be less than 15 µl if it is prepared only sufficient for 4 samples.]

   [Tip: Vortex is not recommended, as the shearing force may fragment the DNA and denature the restriction digestion enzyme (endonuclease).]

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

a) Equipment (per group)

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

b) Materials (per group)

- 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
- DNA loading dye (15 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

c) Preparatory work

Casting of agarose gel **(to be done by the teacher/ laboratory technicians before lab session)**

1. Prepare 1 gel per group.

2. Weigh 0.40 g of agarose powder in a conical flask.

   [Tip: weigh the powder on a small piece of weighing paper and then pour the powder into the conical flask after weighing.]

3. Add 40 ml TBE buffer to the conical flask. Heat the mixture in a microwave oven until all the agarose powder is dissolved and the solution becomes clear.

   [Important note: Shaking of TBE buffer should be avoided, as the shaking may carry the undissolved agarose aggregates to the sidewall of the conical flask. These aggregates may not get dissolved even after heating.]

   [Tip: Ready-to-use 1X TBE buffer can be purchased from biotechnology companies. Alternatively, 10X TBE buffer can be purchased and diluted to 1X with distilled/deionised water.]

   [Important note: Completely dissolved agarose solution should be transparent and very clear. Sometimes small fibre-like materials may be observed in the transparent and clear agarose solution after heating, when observing at close range. These fibre-like materials are undissolved agarose, and they indicate that further heating is needed.]

4. Cool the agarose solution slightly under running tap water.

   [Tip: If the agarose solution feels warm, it is sufficiently cooled. Over-cooling may cause the gel to solidify prematurely.]

5. Add 4 µl of gel red to the solution and mix the solution gently.

   [Important note: Traditional DNA visualising agents are carcinogenic. Modern DNA visualising agents such as gel red and gel green are non-toxic to humans. However, it is still suggested handling gel red with care and avoiding direct skin contact. Adding gel red into a very hot agarose gel should be avoided, as gel red may get vaporised.]

6. Place a 17-well comb into the casting tray and carefully pour in the agarose solution. Remove any bubbles formed, with the wide end of a P20 pipette tip.

7. Wait approximately 25−30 minutes for the agarose to get solidified into an opaque gel.

8. Store the gel in TBE buffer at 4°C if it is prepared one day before the lab session.

IV. Suggested answers to the "guiding questions and part (1) of the experiment".

1. What is the purpose of the amplification of the *Cytb* gene?

   To increase the copies of the Cytb gene and the signal intensity of the restriction digestion.

2. What are the three major events in a polymerase chain reaction (PCR)?

   - *Denaturation: allows the DNA double helix to separate*
   - *Annealing: allows primer binding*
   - *Elongation / extension: DNA polymerase adds nucleotides and extends the DNA strands in a manner aided by the primers*

3. What is the purpose of performing gel electrophoresis after PCR of the *Cytb* gene?

   To confirm the genes are successfully amplified.

4. What is the expected gel electrophoresis result of the restriction digestion products of an adulterated meat product?

   The gel will show some DNA bands that match the number and size of the DNA bands of restriction digestion products of the meat species not shown on the food label.

Part (1) of the experiment [Step 6]

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

   (i) *Denaturation*
   (ii) *Annealing*
   (iii) *Elongation / extension*

b. What are the purposes of these 3 steps in phase (b) of PCR?

   Denaturation: DNA double helix separates

   Annealing: primers bind to the DNA template through complementary base pairing to form a double strand

   Elongation / extension: DNA polymerase adds nucleotides and extends the DNA strand

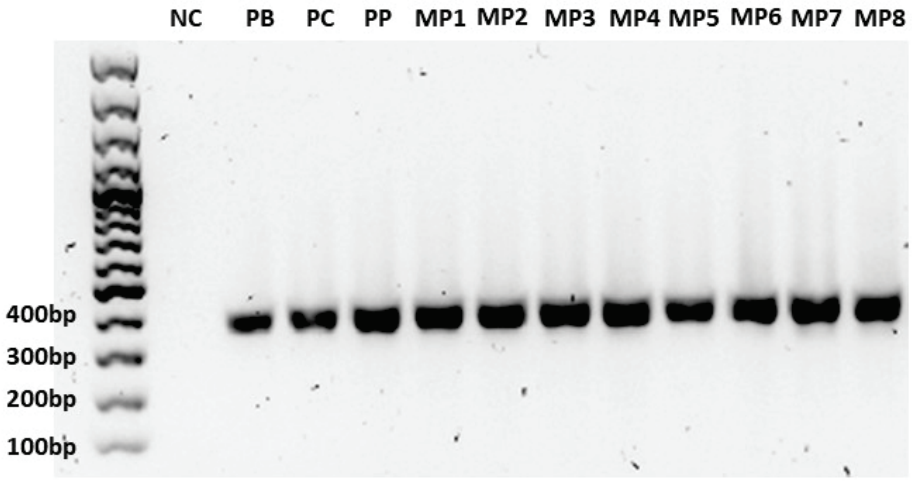
c. How much time is needed for the above PCR programme?

   At least 91 minutes

V. Results

Part 2 of the experiment

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



NC: Negative Control; PB: Pure Beef; PC: Pure Chicken;
PP: Pure Pork; MP1: Beef Ball; MP2: Beef Tendon Ball;
MP3: Pork Ball; MP4: Mushroom Pork Ball; MP5: Sausage;
MP6: Bean Curd Sheet Beef Ball; MP7: Shao mai;
MP8: Chicken Nuggets

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

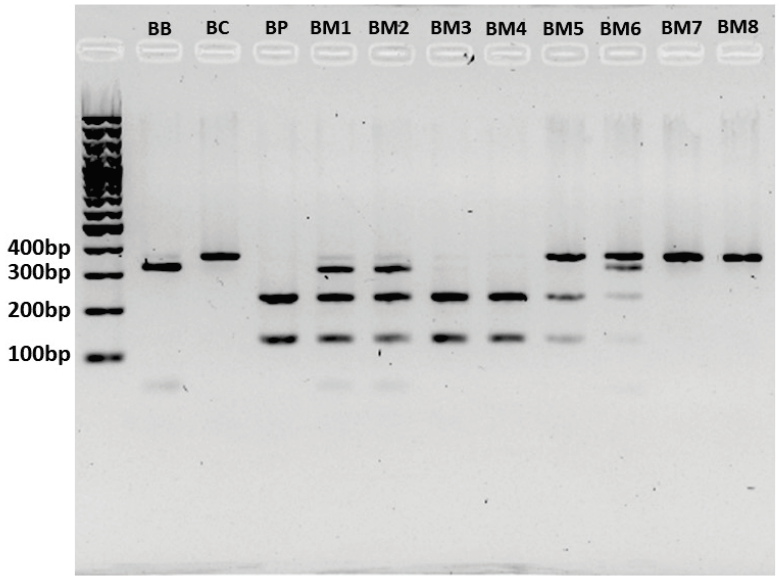
   No band is observed from NC because only water (no DNA sample) is provided in the DNA sample. The absence of a band denotes no contamination of the experimental materials in the samples.

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?

   One band can be observed for each of the PCR products on the gel and the bands are at approximately the same position. This tells us that the size (in approximate no. of bp) of the gene (Cytb) being amplified in the PCR for all the meat samples are of approximately the same size (about 400 bp).

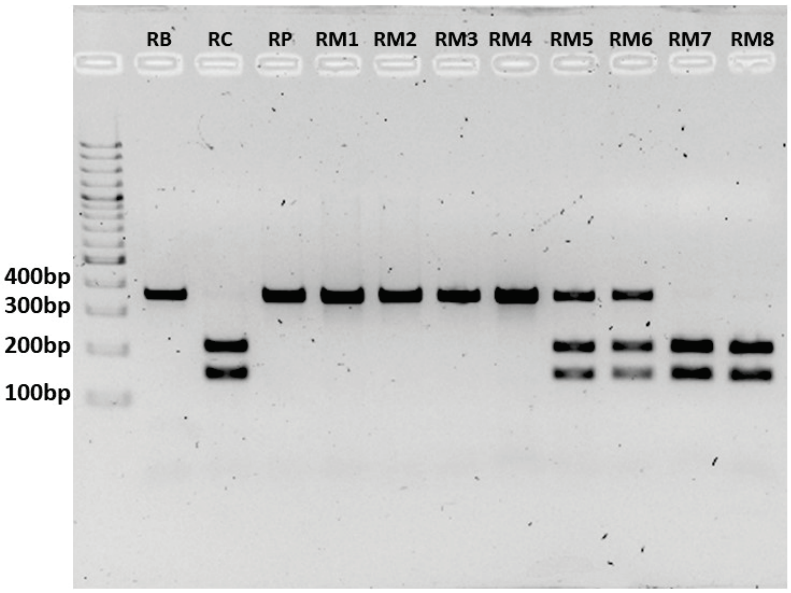
Part 4 of the experiment

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



BB: Beef; BC: Chicken; BP: Pork; BM1: Beef ball; BM2: Beef tendon ball;
BM3: Pork ball; BM4: Mushroom pork ball; BM5: Sausage;
BM6: Bean curd sheet beef ball; BM7: Shao mai; BM8: Chicken nuggets

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



RB: Beef; RC: Chicken; RP: Pork; RM1: Beef ball; RM2: Beef tendon ball;
RM3: Pork ball; RM4: Mushroom pork ball; RM5: Sausage;
RM6: Bean curd sheet beef ball; RM7: Shao mai; RM8: Chicken nuggets.

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	*1*	*2*	*2*			*2*	*1*	*1*	
Estimated size of the DNA sequence at each band (bp)	*380 bp*	*150 bp and 250 bp*	*50 bp and 350 bp*			*150 bp and 200 bp*	*350 bp*	*350 bp*	

4. The meat product sample contains _____.

	Beef Ball	Beef Tendon Ball	Pork Ball	Mushroom Pork Ball	Sausage	Bean Curd Sheet Beef Ball	Shao mai	Chicken Nuggets
Beef	✓	✓				✓		
Chicken					✓	✓	✓	✓
Pork	✓	✓	✓	✓	✓			

[Important: the ingredients of the same type of meat product vary from brand to brand. The results shown here should be taken as a reference only.]

[Tip: Students are recommended to analyse the result of RsaI digestion products before analysing the result of BsaJI digestion products. RsaI digestion of pork and beef DNA samples resulted in a band size of about 350 bp. Upon BsaJI digestion, pork DNAs were cut into fragments of about 150 bp and 250 bp. Beef DNAs were cut into fragments of about 50 bp and 350 bp.]

[Tip: Although all three meat species can be differentiated by using the BsaJI alone, using both BsaJI and RsaI digestions can give us a more conclusive result.

For instance, the difference in the band pattern between BsaJI-digested beef and BsaJI-digested chicken is not distinct, so it is difficult to differentiate the two species. The 50-bp band of BsaJI-digested beef is often pale and may be overlooked or cannot be observed (the smaller the size of the DNA band, the less the gel red molecules can bind, thus less fluorescent signal can be observed).

It is very difficult to differentiate the 350-bp band of the BsaJI-digested beef from the 380-bp band of the BsaJI-digested chicken. As a result, a single band at a position about 350-380 bp is not a conclusive observation for differentiating beef and chicken. The 50 bp band may or may not exist due to its low- signal intensity; thus, it is necessary to use RsaI to differentiate beef and chicken, as it can give a very distinctive band pattern.]

VI. Discussion

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

   - *Pure chicken*
     - *Two bands at about 150 bp and 200 bp in the gel loaded with RsaI-digested products.*
     - *One single band at about 380 bp in the gel loaded with BsaJI-digested products.*
   - *Pure pork*
     - *One single band at about 350 bp in the gel loaded with RsaI-digested products*
     - *Two bands at about 150 bp and 250 bp in the gel loaded with BsaJI-digested products.*
   - *Pure beef*
     - *One single-band at about 350 bp in the gel loaded with RsaI-digested products.*
     - *Two bands at about 50 bp and 350 bp in the gel loaded with BsaJI-digested products.*

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

   - *Each restriction digestion enzyme identifies specific base sequences of DNA and cuts the DNA into fragments;*
   - *There is variation in the base sequences of the Cytb genes of the meat samples being tested. This leads to the differences in the number and position of the restriction digestion sites;*
   - *thus, different numbers and sizes of DNA fragments are generated after the restriction digestion.*

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

   - *[Note: A meat product containing meat from more than one species is not considered adulterated. A meat product is considered adulterated if it contains meat that is not listed on its food label.]*

VII. References

Ong, S. B., Zuraini, M. I., Jurin, W. G. et al. (2007). Meat molecular detection: Sensitivity of polymerase chain reaction-restriction fragment length polymorphism in species differentiation of meat from animal origin. *ASEAN Food Journal*, *14*(1), 51–59.