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## Triplex PCR for halal authentication of processed food: Development and Characterization

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## Triplex PCR for halal authentication of processed food: Development and Characterization

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**Abstract.** Awareness of consumers towards the safety of food and labelling statement due to food adulterations is increasing. Simultaneous detection made the developed methods are rapid and economic. In this study we developed triplex PCR for beef and pork detection by using 18SsRNA as the internal control, then further characterized the methods. Primer formulation for triplex PCR being used are 0,8µM porcine, 0,04 µM for each beef and 18sRNA, that all together working at 45°C annealing temperature in one single tube. The amplicon sizes for pork, beef and 18SrRNA are 300, 120 and 99bp respectively. The sensitivity of the method is 0,851ng. This developed method shown as robust method that can detect DNA target from different source of matrices [five products contain pork (meatball, sausage, ham, pasta, cornet), 3 beef processed food (dendeng, rendang and satay) and three products contain pork include beef (sauce curry from three different products)] which could contain some types of PCR inhibitor. Furthermore, the method shows specific detection towards the target that had no cross contamination.

### 1. Introduction

Food safety and quality are important criterion for food authenticity, that support to food certification protected from fraud and adulteration. Especially in developing country, meat adulteration from processed food with the cheap meat such as pork are often happened. Pork was chosen, not only because of the price but also based on the colour and its texture that shown similar with the beef [1,2]. With all of those reasons made detection and identification of meat in processed food are important for protection purpose.

The polymerase chain reaction (PCR) is commonly and popularly used for meat and its derivatives detection and identification in processed food. Simultaneous detection made the developed methods are rapid and economic. DNA based detection have been proven as sensitive rapid and reliable for food authentication. DNA also shown high stability molecule compared with protein which allows analysis from processed and heated treated food source. Several DNA based detection system type already reported are species specific PCR [3], Restriction fragment length polymorphism (PCR RFLP) [4], random amplified polymorphism DNA (RAPD) fingerprinting [5], and PCR sequencing [6]. Carefully designed multiple PCR under optimized condition, allows to avoid from restriction digestion



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and or PCR product sequencing that reducing for both cost and time. Although some of multiplex PCR have been developed for identification of some animal, multiplex PCR design with this activity used primer that has not yet been reported [7-11]. With this activity result could be considered as alternative or complement of others reported developed multiplex PCR. Here in this activity we developed triplex PCR that can simultaneously detected pork, beef and 18S rRNA as specific, rapid, simple and cost effective applying for food fraud and adulteration detection application especially in processed food.

## 2. Methods

### 2.1. Meat sampel

Raw meat of six samples (pork-*Sus sucrofa*, beef-*Bos indicus*, chicken-*Gallus gallus*, Fish- *Oreochormis niloticus*, goat-*Capra aegagrus hircus*, duck-*Cairina moschata*), that commonly found in the market were collected. Raw meat of pork and beef were used as the target for triplex PCR target development also as the target for PCR primer specificity confirmation with other raw meat. Processed food used in this activity are pasta, ham, meatball, cornet, sausage which already known contain pork in the ingredient, then we used satay, dendeng, rendang (Indonesian traditional food), that are known made only by beef. Further we used three different curry sauce that already mentioned in ingredient contain beef and pork.

### 2.2. DNA extraction

DNA total from raw material were extracted used *PureLink Genomic DNA mini kit* from Thermo-scientific, while DNA total from processed food were extracted with *DNeasy maricon Food kit* from Qiagen. DNA extraction methods were done following the instruction prepared from each manufacture kits. DNA concentrations were estimated by UV absorption spectrophotometer at 260nm wavelength.

### 2.3. Single primer test specificity and Triplex PCR assay development

Table 1 are shown the primer used in this activity that specifically targeted each to pork, beef and 18SrRNA

Table 1. Primer specific target

Sekuen 5' → 3'	Amplicon (bp)	Refference
Pork-Fwd: AAGAATATCCACCACACGAA	300	[12]
Pork-Rev: GGTTCAAGTACCCATACAT		
Beef-Fwd: CGGCACAAATTTAGTCGAAT	120	[4]
Beef-Rev: TGGACTATGGCAATTGCTATG		
18S-Fwd: AGGATCCATTGGAGGGCAAGT	99	[13]
18S-Rev: CCAACTACGAGCTTTTAACTGCA		

Before we developed triplex PCR assay, we confirmed specificity of each primer to all of the raw materials. After all primers were confirmed specific only to the target, triplex PCR was optimized with HotStarTaq Master Mix Kit (Qiagen, Germany) with following condition: 95°C denaturation for 30sec, annealing optimizations at 45°C, 47°C, 50°C and 55°C for 30 sec, elongation 72°C for 1min, all cycles were repeated for 35times and continue with elongation 72°C for 10min. After we get the annealing optimum temperature, we optimize the primer formulation with primer specific to pork 0,9µM and 0,8 µM while other primer specific to beef and 18SrRNA were 0,04 µM for each. All PCR amplified products were confirmed in 4% agarose. 18SrRNA detection purpose as internal control for confirming the integrity and the success of DNA extraction result. [14]

### 2.4. Sensitivity (Limit detection of Triplex PCR)

We get DNA extraction from pork with concentration was 143,6ng, while beef DNA concentration was 85,1ng. Limit detection of triplex PCR were confirmed with serial dilution of beef and pork: 85,1ng, 8,51ng, 0,851ng, 0,0851ng. Further we also check for binary DNA mixture. Pork DNA was spiked with beef DNA, mixed together and diluted serially as following in below table. This activity aimed also to demonstrate the PCR sensitivity to detect pork DNA in mixture with beef DNA.

Table 2. Binary DNA mixture of pork and beef.

Diluted	Concentration (ng)	
	Pork	Beef
1	143,6	85,1
2	14,36	85,1
3	1,436	85,1
4	85,1	85,1

### 2.5. Triplex PCR robustness assay with food different matrices

To test the triplex PCR robustness assay, eleven meat products were applied, those were: five products contain pork (meatball, sausage, ham, pasta, cornet) 3 beef processed food (dendeng, rendang and satay) and three products contain pork include beef (sauce curry from three different products). This experiment was duplicated.

## 3. Result and Discussions

### 3.1. DNA extraction

DNA extraction result considered satisfactory-with purity average around 1,8-2 (purity = A260/A280 and 260/230 ratio), and DNA extraction concentration around 9,1-268ng/ $\mu$ l (Table 3).

Table 3. DNA concentration and purity used in this activity

No	Sample	DNA concentration (ng/ $\mu$ l)	Purity (A260/A280)
<b>Raw meat</b>			
	Pork	143,6	1,738
	Beef	85,1	1,795
	Chicken	71	1,972
	Duck	80	1,860
	Goat	85	1,889
	Fish	54	1,929
<b>Processed food</b>			
<b><u>Pork Positive</u></b>			
	Meatball	125,7	1,654
	Sausage	195,7	1,395
	Ham	125	1,866
	Cornet	66,9	1,890
	Pasta	268	1,887
<b><u>Pork Negative</u></b>			
	Meatball	139	1,904
	Cornet	271	1,831
	Abon	40	2,105
	Rolade	266	3,057
<b><u>Beef Positive</u></b>			
	Dendeng	38,4	1,901
	Rendang	96,8	1,851
	Satay	238,9	1,198
<b><u>Pork and Beef Positive</u></b>			
	Carry Sauce 1	9,1	1,896
	Carry Sauce 2	30,8	1,683

Carry Sauce 3

21

1,795

Pork sausage and satay shows have lowest DNA purity while rolade shows have the highest DNA purity. With all of those data shows us quite successful removing PCR inhibitors. Existence of PCR inhibitor will interfere to PCR reaction. Commonly in processed food, oil and fat reported as food ingredients that inhibit polymerases [15]

### 3.2. Single primer specificity test

We confirmed all the primers, each targeted to pork or beef or 18SrRNA, to all DNA extracted from some commonly meat found in the market such as pork-*Sus scrofa*, beef-*Bos indicus*, chicken-*Gallus gallus*, Fish-*Oreochromis niloticus*, goat-*Capra aegagrus hircus*, duck-*Cairina moschata*. These confirmations were done that each primer was detecting only to the target, and targeted PCR does not exist in non-template control (NTC), which means the primer work specifically (Figure 1).

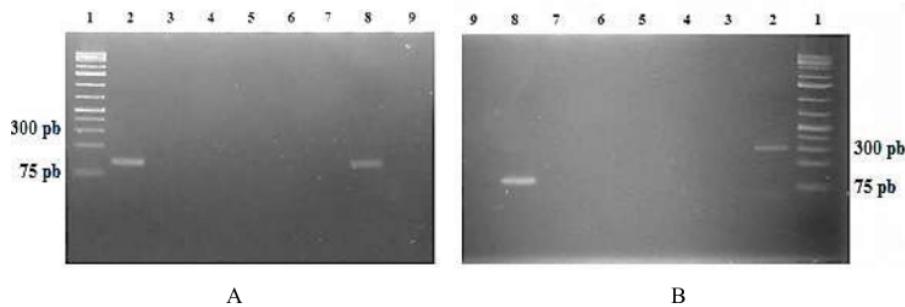


Figure 1. Primer specificity confirmation for A. beef, B. pork.

(A) 1. 1kb DNA ladder plus Thermo scientific, 2. Beef, 3. Pork, 4. Fish, 5. Goat, 6. Chicken, 7. Duck, 8. 18S rRNA, 9. NTC. (B). 1. 1kb DNA ladder plus Thermo scientific, 2. Pork, 3. Beef, 4. Fish, 5. Goat, 6. Chicken, 7. Duck, 8. 18S rRNA, 9. NTC

After confirming the specificity of each primer, we developed pork and beef detection simultaneously. As the strategy for successfully detecting of this method development, avoiding from doubtful thing, we developed simultaneous detection include 18SrRNA detection, that further recognized as triplex PCR. The primer specificity is very important for multiplex PCR development. The selective primer will anneal with the target not with any non-target species under single set PCR including reaction volume, cycling and annealing. [5]

### 3.3. Annealing temperature and primer proportion optimization.

In this triplex PCR development, we used HotStartaq Master Mix (Qiagen, Germany). This Hotstartaq polymerase was activated at 95°C for 15 minutes in the beginning of PCR reaction. This activation effected for avoiding of unspecific product include primer dimer. From four different annealing temperature 45°C, 47°C, 50°C and 55°C, temperature 45°C shows as the optimum annealing temperature for the triplex (Figure 2). This repeated twice, and shown consistently as the beginning result. In this annealing temperature optimization, we used primer with the proportion for each are 0,8 µM for pork while two primers specific to beef and 18SrRNA were 0,04 µM.

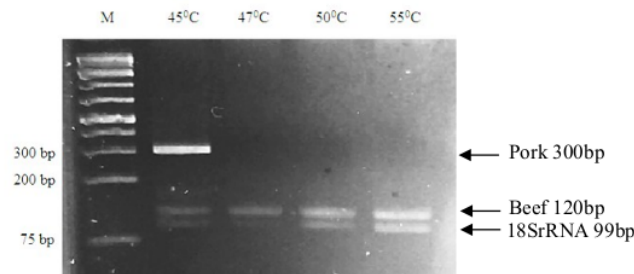


Figure 2. Annealing temperature optimization of Triplex PCR detection. At 45°C, 47°C, 50°C and 55°C. M: 1Kb plus DNA marker.

We also optimize the primer proportion for this triplex PCR. Especially we concern with the pork primer proportions, while other primer proportions were same as the previous proportion. We compared two primer concentration of pork (0,8  $\mu\text{M}$  and 0,9  $\mu\text{M}$ ). As the result, pork primer with concentration 0,8  $\mu\text{M}$  has shown better result than 0,9  $\mu\text{M}$  pork concentration primer. With 0,8  $\mu\text{M}$  primer concentration for pork, all of the target PCR were existed while 0,9  $\mu\text{M}$  are not shown. The activity also repeated two times for result confirmation, and shows consistent data (data was not shown).

#### 3.4. Triplex PCR sensitivity assay

The sensitivity assay for triplex PCR were determined with serial dilution genomic DNA starting from 85,1ng, 8,51ng, 0,851ng, 0,0851ng. The sample sources were from pork and beef meat. Based on this activity assay, the triplex PCR can recognize the sample until 0,851 ng for both DNA target. (Figure 3 only shown sensitivity result for beef). The limit detection of reported multiplex was varied from species to species. Matsunaga et al 1999 reported 0,25ng sensitivity of their multiplex development [16], further Di Pinto et al (2013) also got 0,25ng sensitivity of duplex PCR detection of pork in meat [17], while Ali et al 2015 reported around 0,01-0,02ng sensitivity of multiplex detection of five meat species forbidden in Islamic foods [8].

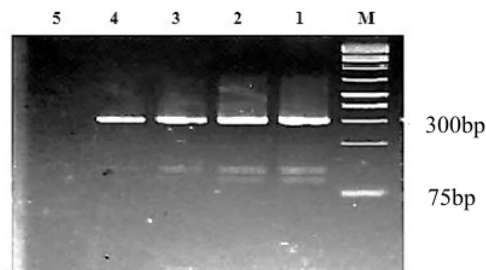


Figure 3. limit of detection developed triplex PCR at serial dilution beef DNA genome. M: 1kb DNA plus, 1. 85,1ng, 2. 8,51ng, 3. 0,851ng, 4. 0,0851ng, 5. NTC (non-template control)

In this activity we also confirmed the sensitivity with binary DNA mixture. All of the targeted DNA were shown in all binary DNA

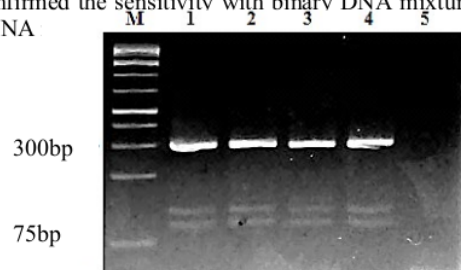


Figure 4. Sensitivity of triplex PCR confirming with Binary DNA mixture. M: 1kb DNA, 1. Pork 143,6ng: beef 85,1ng, 2. Pork 14,36ng: beef 85,1ng, 3. Pork 1,436ng: beef 85,1ng, 4. Pork 85,1ng: beef 85,1ng, 5. NTC (non-template control)

These sensitivity result shows that sensitivity of developed triplex PCR are good, since it can detect low concentration with different proportion of DNA target in wide range DNA proportion and concentration.

3.5. *Triplex PCR robustness assay with food different matrices*

As already mentioned above, triplex PCR were developed to detect for three different target DNA fragments that were 300bp for pork, 120bp for beef and 99bp for 18SrRNA. We test the robustness of developed method with different matrices. We used: five products contain pork (meatball, sausage, ham, pasta, cornet), 3 beef processed food (dendeng, rendang and satay) and three products contain pork include beef (sauce curry from three different products). Based on the PCR result shows that all of the target DNA can detected appropriate as the ingredient of processed food (Figure 5). Existence of PCR inhibitor will interfere to PCR reaction. Commonly in processed food, oil and fat reported as food ingredients that inhibit polymerases [15].

Since this developed method could detect DNA target from different source of matrices which could contain some type of PCR inhibitor, these shows us the robustness of the method. And further this method shows us as high specificity to the target because of no any non-specific amplification fragment.

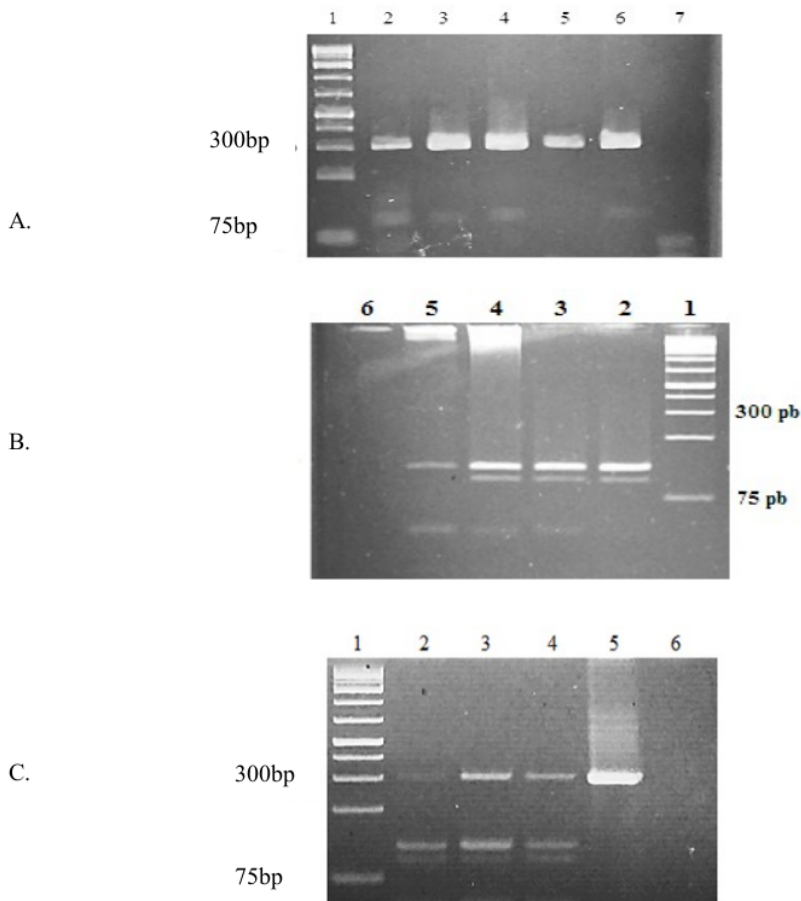




Figure 5. Robustness assay for triples PCR. A. Food process pork contain only. 1. M: 1kb Plus Marker DNA ladder 2. Pasta, 3. Ham., 4. Meatball., 5. Cornet., 6. Sausage 7. NTC; B. Food process beef contain only. 1. M. 1Kb Plus marker, 2. Satay., 3. Dendeng, 4. Rendang, 5. Positive control for beef., 6. NTC. C. Food process contain pork and beef. 1. M 1kb Plus Marker DNA ladder, 2-4 Carry sauce with different commercial brand, 5. Positive control for pork, 6. NTC.

#### 4. Conclusion

Triplex PCR for pork, beef and 18SsRNA have been developed and characterized well. Primer formulation for triplex PCR are 0,8 $\mu$ M primase, 0,04  $\mu$ M for each beef and 18sRNA, that all together working at 45°C annealing temperature. The amplicon size for pork, beef and 18SrRNA are 300, 120 and 99bp respectively. The methods can detect the target with DNA content as low as 0,851ng. Since this developed method could detected DNA target from different source of matrices (five products contain pork (meatball, sausage, ham, pasta, cornet), 3 beef processed food (dendeng, rendang and satay) and three product contain pork include beef (sauce curry from three different products) which could contain some type of PCR inhibitor, these shows us the robustness of the method. And further shows us high specificity to the target.

#### 5. Acknowledgement

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