Real-time PCR technology for halal authentication

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OUTLINES

- **Halal Authentication**
- **Real-time Polymerase Chain Reaction (PCR)**
- **Preliminary work on qPCR technique for analysis of cosmetic product**

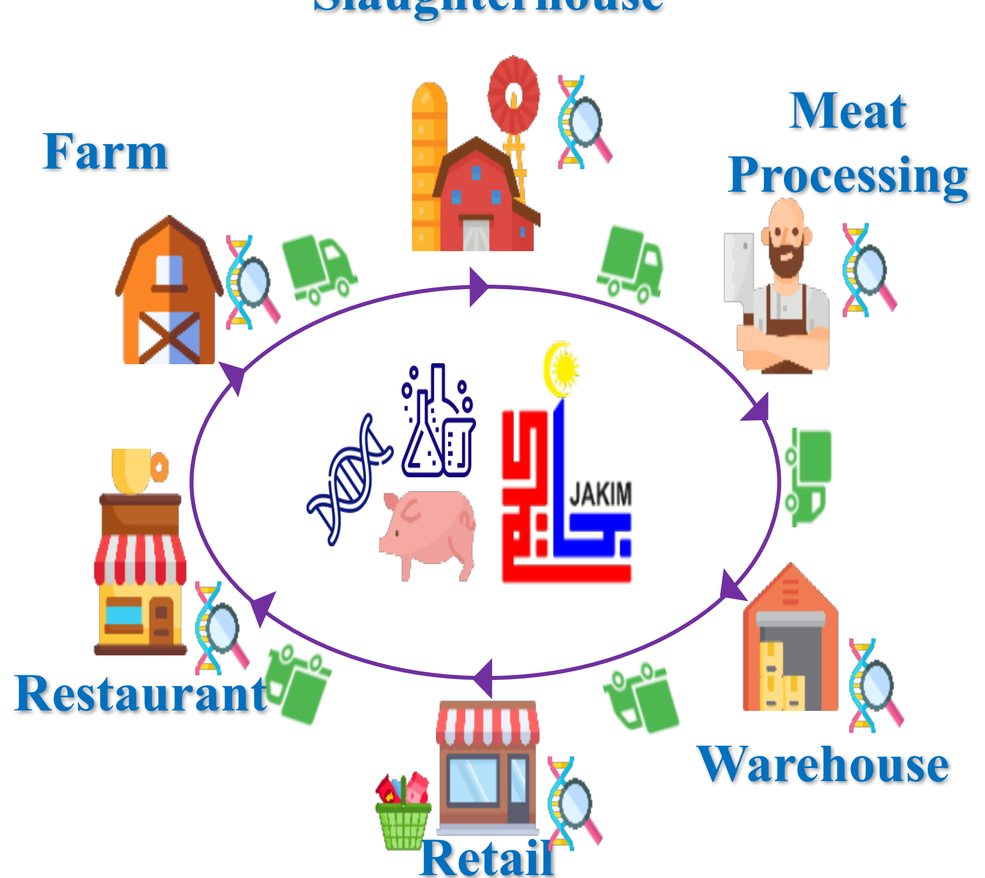
INTRODUCTION

- **Nowadays, the demand on halal products has increased tremendously across the world, both from Muslim and non-Muslim communities.**

- **The trade of halal goods and products is estimated to be worth more than USD 2.1 trillion[1].**

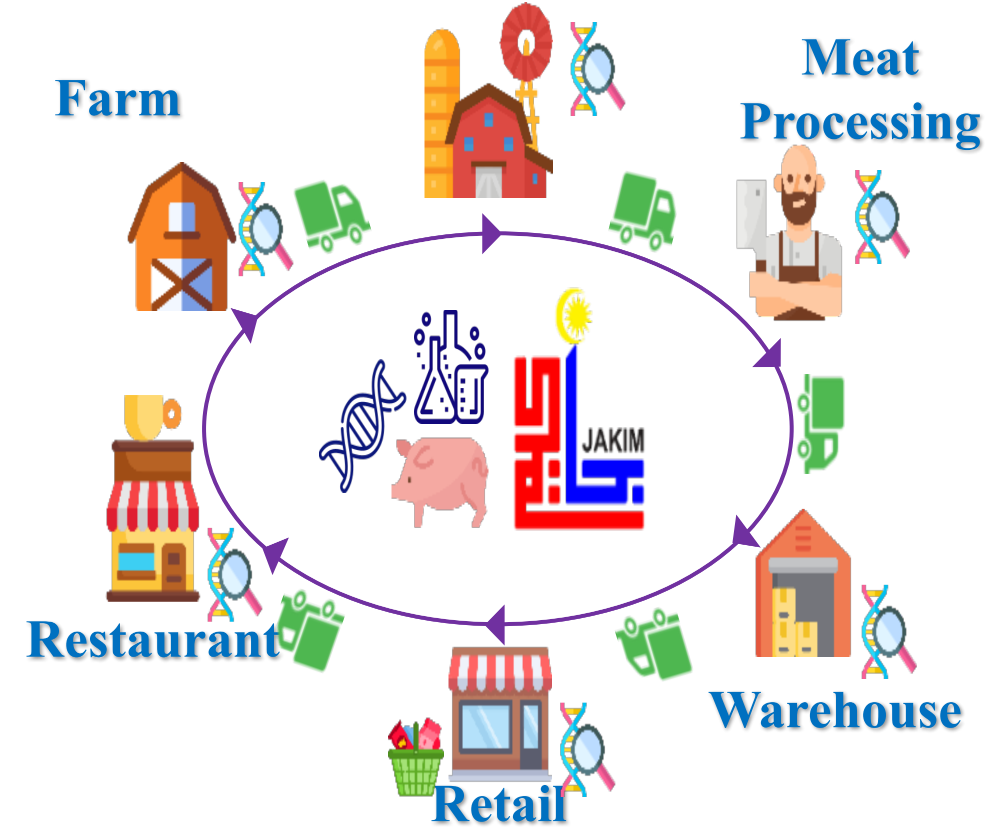
INTRODUCTION

Therefore, both domestic and foreign food manufacturers are putting efforts into making their food products acceptable in current markets.



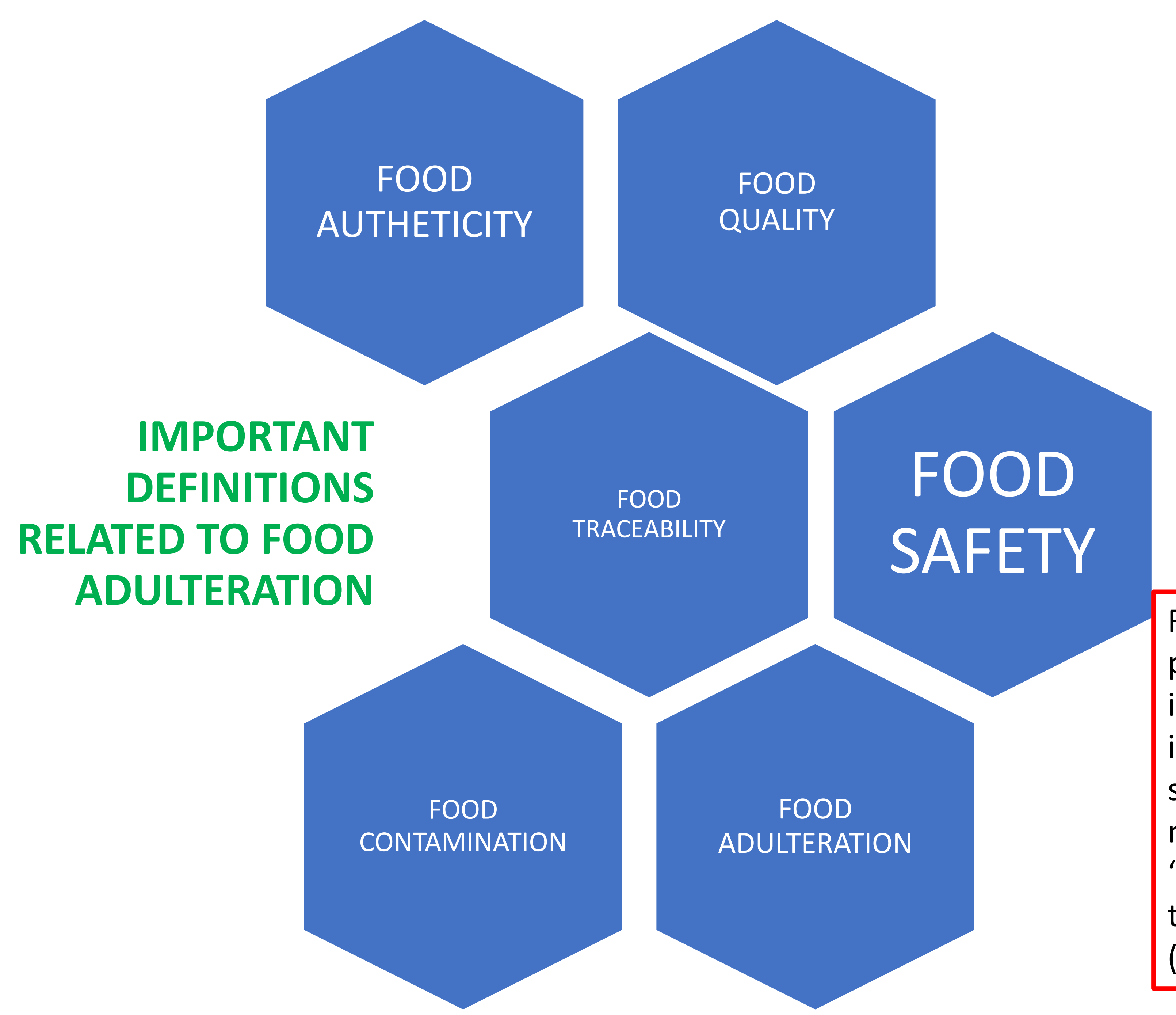
INTRODUCTION

Integrity of halal supply chain management is required to achieve the high level of consumer trust.



Food Adulteration/Fraud

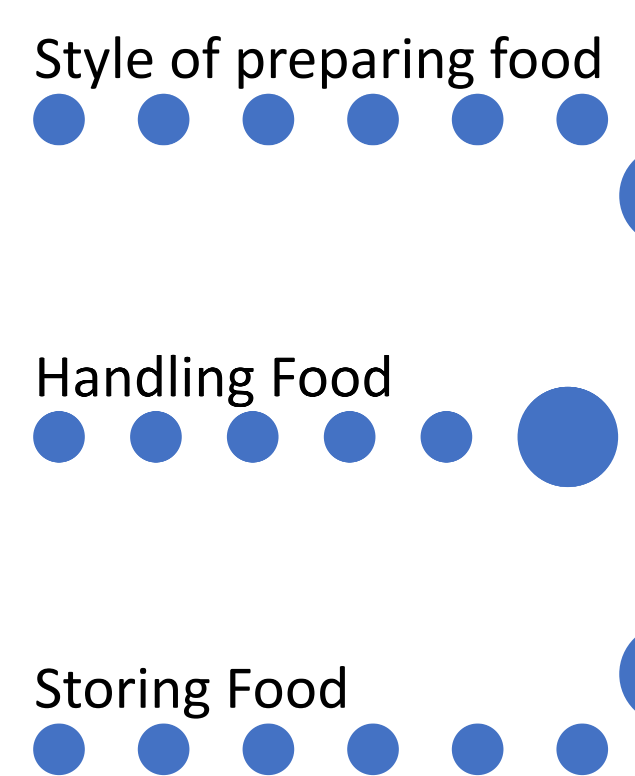
- Is the act of intentionally debasing the quality and the safety of the food offered for sale
- either by the admixture or
- substitution of inferior substance or
- by the removal of some valuable ingredients, tampering, or
- misrepresentation of food, or food packaging;
- or false or misleading statements



Food Authenticity – is defined by **legally recognized description that concerns its characteristics (quality, origin, process, etc)**

Food means any substance whether processed, semi-processed or raw, which is intended for human consumption, and includes drinks, chewing gum and any substances which has been used in the manufacture, preparation or treatment of "food" but does not include cosmetics or tobacco or substances used only as drugs (Codex Alimentarius Commission, 1997)

FOOD SAFETY



Style of preparing food

Handling Food

Storing Food

To prevent infection

To help ensure food retains enough nutrients

Why should we detect adulterated products?

- **Food Adulteration**
  - **Harmful effects on Health**
    - Chronic liver diseases
    - Kidney failure
  - **Economic impact**
    - Lost sales
    - Product recalls
    - Loss of consumer confidences

Health Hazards of Adulterated Foods

Adulterated foods can have a range of harmful effects on health.

For example, the addition of brick powder to red chili powder can cause loss of vision and respiratory diseases (Manasha & Janani, 2016).

Economic cost of adulterated foods

Although some of the food adulteration cases have caused a significant number of hospitalization and fatalities (Crocombe & Miseo, 2017), the economic costs are not widely appreciated.

However, each year, lost sales, product recalls and loss of consumer confidence are estimated to cost legitimate retailers around US$30-40 billion (Oh, 2017)

How to detect adulterated products?

Nowadays, the detection of adulterants is one of the greatest challenges facing not only in food and feed products companies but also cosmetics and pharmaceutical companies.

Therefore, there is a necessity for accurate analytical methods to evaluate Halal authenticity in consumers' products, and a strict regulatory mandate.

Analytical Methods – Porcine Detection

Numerous methods can be used to determine the presence of porcine DNA in mixed samples, processed foods and cosmetic products.

Food Adulteration

Trends in Food Science & Technology
Volume 86, April 2019, Pages 544-552

Commentary

DNAFoil: Novel technology for the rapid detection of food adulteration

Aly Farag El Sheikha [a, b, c]

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Advantages and disadvantages of current approaches utilized for the detection of food adulteration

Detection of Food Adulteration

APPROACH	ADVANTAGES	DISADVANTAGES
Chemicals and biochemical methods • Chromatographic based method (including HPLC and GC)	• High resolution • High sensitivity • Tolerable cost • Wide adulteration (quality control, characterizing food products, detect adulteration)	• Labour intensive • Complex sample preparation • Large consumption of solvent • Expensive equipment
DNA-based methods (including PCR) Ref: Aly Farag El Sheikha et.al (2019) Trends in Food Science & Technology, 86, 544-552.	• High specificity • High-throughput (processing of large numbers of samples in short time) • Highly stable DNA biomarkers • Highly sensitive and can be used with degraded sample • Robust, reproducible and efficient • Adaptable	• Technically challenging • Large consumable requirement • High cost

Why qPCR?

- DNA-based method is used for real-time polymerase chain reaction technique (qPCR).
- Among PCR methods, real-time PCR assays based on target DNA sequences.
- Previous studies revealed that qPCR provides rapid and accurate identification of species of animal origins.

APPLICATION OF THE COMMON COMMERCIAL PCR-BASED KITS

1. Quantitative real-time PCR kits (typically with internal amplification and animal control)

2. Fish species identification

3. Plant species identification

Reference:
Aly Farag El Sheikha et.al (2019) Trends in Food Science & Technology, 86, 544-552.

Molecular techniques: Why DNA-based approaches are more efficient?

1. DNA is unique for every organism in this world.

2. The development of DNA-based techniques, i.e., PCR have the **potential** to complement the physical, chemical and biochemical approaches.

3. As detection tools for food adulteration especially if both adulterant and the original food show high physical similarity.

Reference:
Aly Farag El Sheikha et.al (2019) Trends in Food Science & Technology, 86, 544-552.

How does
PCR work?

How does PCR work?

- PCR is a method where an enzyme amplifies a short specific part of the template DNA (amplicon) in cycles.

- This enzyme (thermostable DNA polymerase) originally isolated in 1960s from bacterium *Thermus aquaticus*, growing in hot lakes of Yellowstone park, USA.

- In every cycle, the number of short specific sections of DNA is doubled, leading to an exponential amplification of targets.

Conventional PCR basics

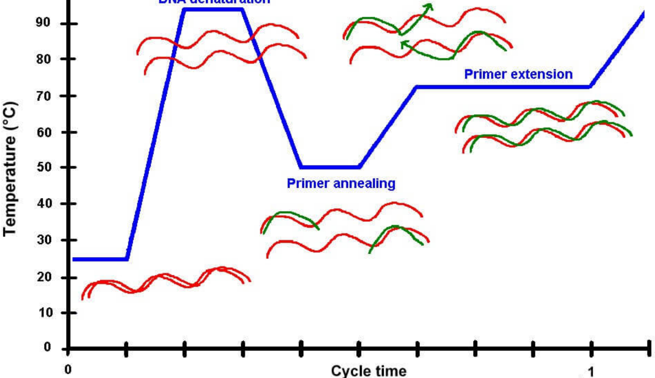
Thermostable DNA polymerase on one side

All these amplification cycles are carried out automatically by a combination of temperature changes and thermostable DNA polymerase which replicates DNA.

In order to prevent the random uncontrolled replication of all or unwanted parts of DNA in the sample, a set of primers are introduced into the reaction mixture: a forward primer that marks the beginning and a reverse primer that marks the end of a section the amplicon.

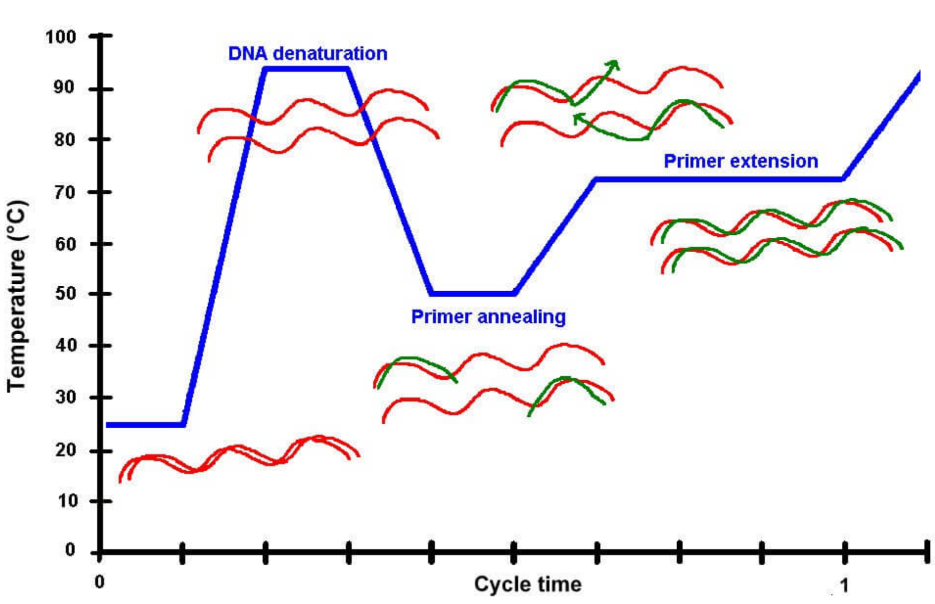
By carefully designing of both primers (known sequence of nucleotides) we can instruct the DNA polymerase exactly which part of the DNA it should amplify.

These short sections of DNA are usually a few hundred base pairs long in conventional PCR and only a few ten base pairs in qPCR.



Temperature cycling on the other

- In the beginning cycles the DNA is heated up to 95°C so it is denatured and single strands of DNA (ssDNA) are obtained from dsDNA.
- In other words, the DNA becomes exposed to the DNA polymerase. But the DNA polymerase requires a double stranded DNA where it can start adding nucleotides to the template DNA strand.
- Here is where a set of primers come in.
- The temperature is now lowered and the primers anneal to the complementary part of the DNA (according to A-T and G-C base pairing).



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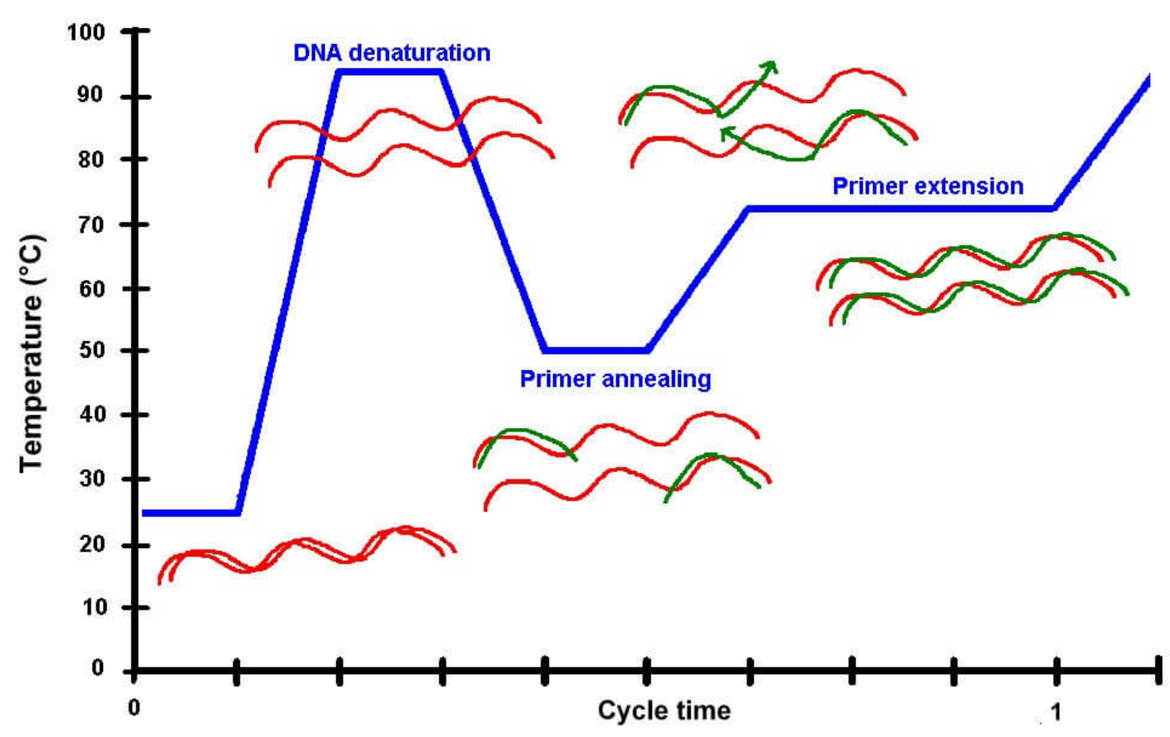
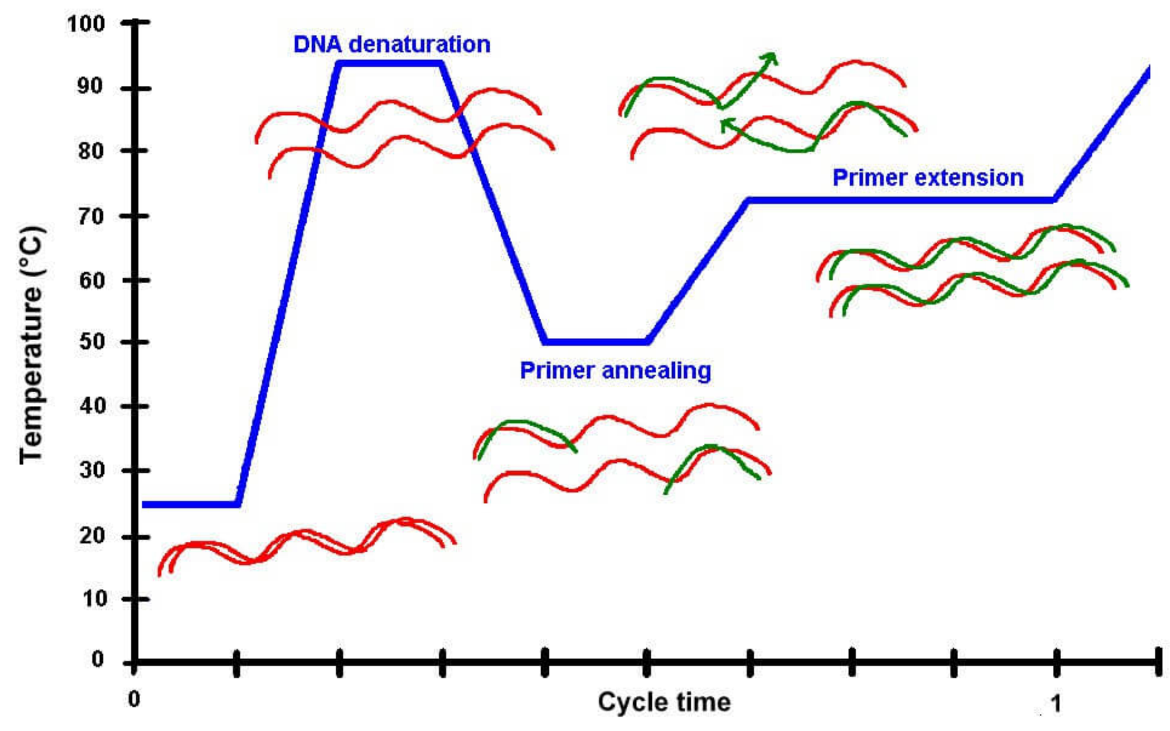


- **…….Temperature cycling on the other**
- Because there is an enormous excess of primers, they anneal to most target sequences on the template DNA even if many copies of it are present.
- Now, the DNA polymerase can start filling in the complementary DNA strand along the template DNA. The DNA polymerase replicates the short section of a DNA until it runs out of the template, filling all the 'gaps'. This is referred to as one cycle of PCR which results in double the amount of target DNA, compared to the beginning of the cycle.



How does qPCR work?

In qPCR, exactly the same procedure happens but with two major differences:

1. The amplified DNA is fluorescently labelled (usually with cyanine based fluorescent dyes)

2. The amount of the fluorescence released during amplification is directly proportional to the amount of amplified DNA.

Fluorescence is monitored during the whole PCR process (along all 30 to 45 cycles). The higher the initial number of DNA molecules in the sample, the faster the fluorescence will increase during the PCR cycles (see Images 1 and 2).

Reference:
https://biosistemika.com/blog/qpcr-technology-basics/

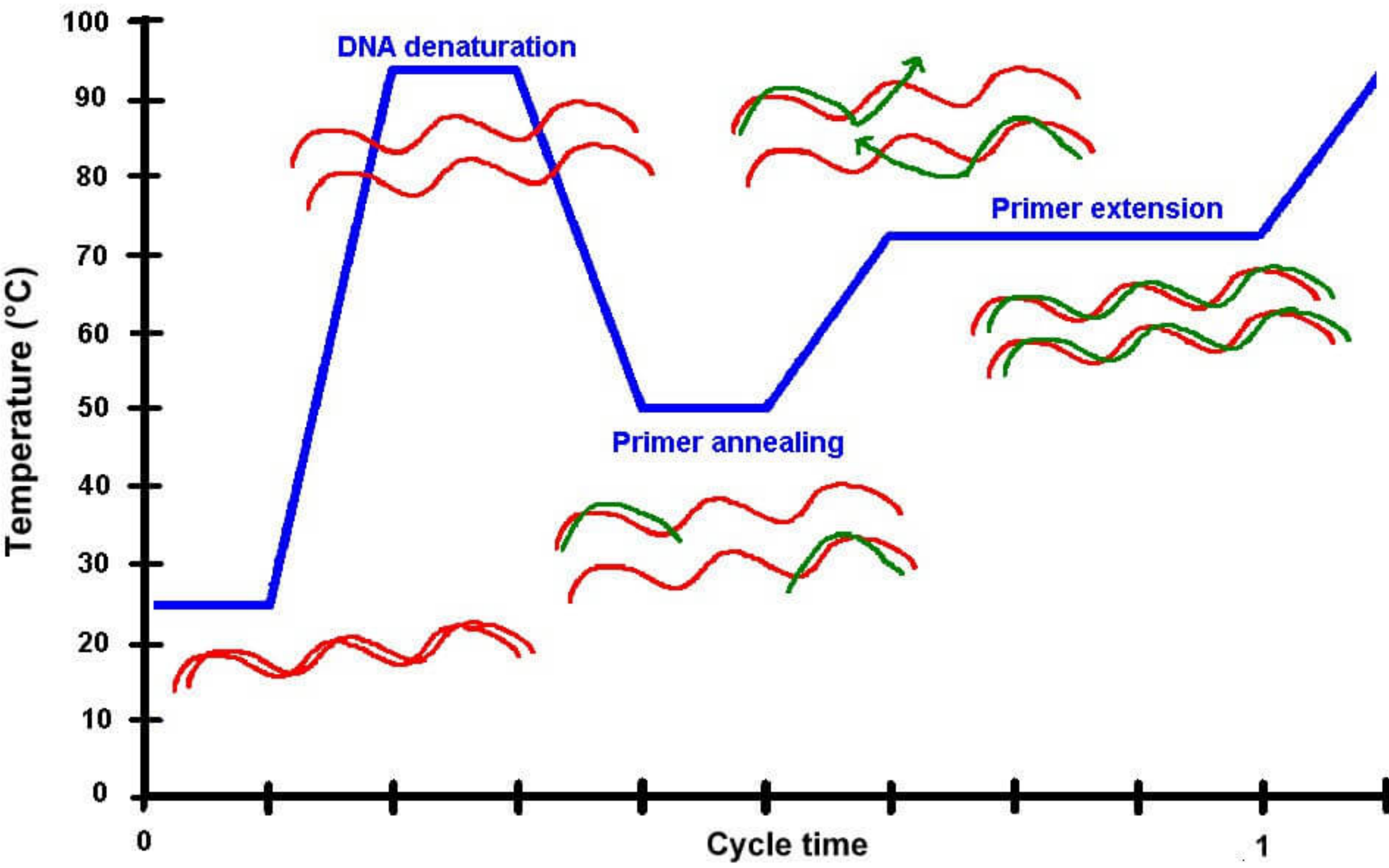


Figure 4 shows the different thermal steps in one polymerase chain reaction (PCR) cycle. Cycling of temperature is the basis of PCR.

A graphical representation of qPCR amplification (the first two cycles) as it happens in the PCR tube. (Source: https://biosistemika.com/blog/qpcr-technology-basics/)

Figure 2 shows amplification plot with five samples (S1 to S5).

- While DNA in each sample is being amplified with every cycle the fluorescence increases.
- Sample S1 contained the highest initial number of target DNA, resulting in the fastest increase of fluorescence.
- Sample S4 contained the lowest initial number of target DNA molecules while S5 did not contain any.

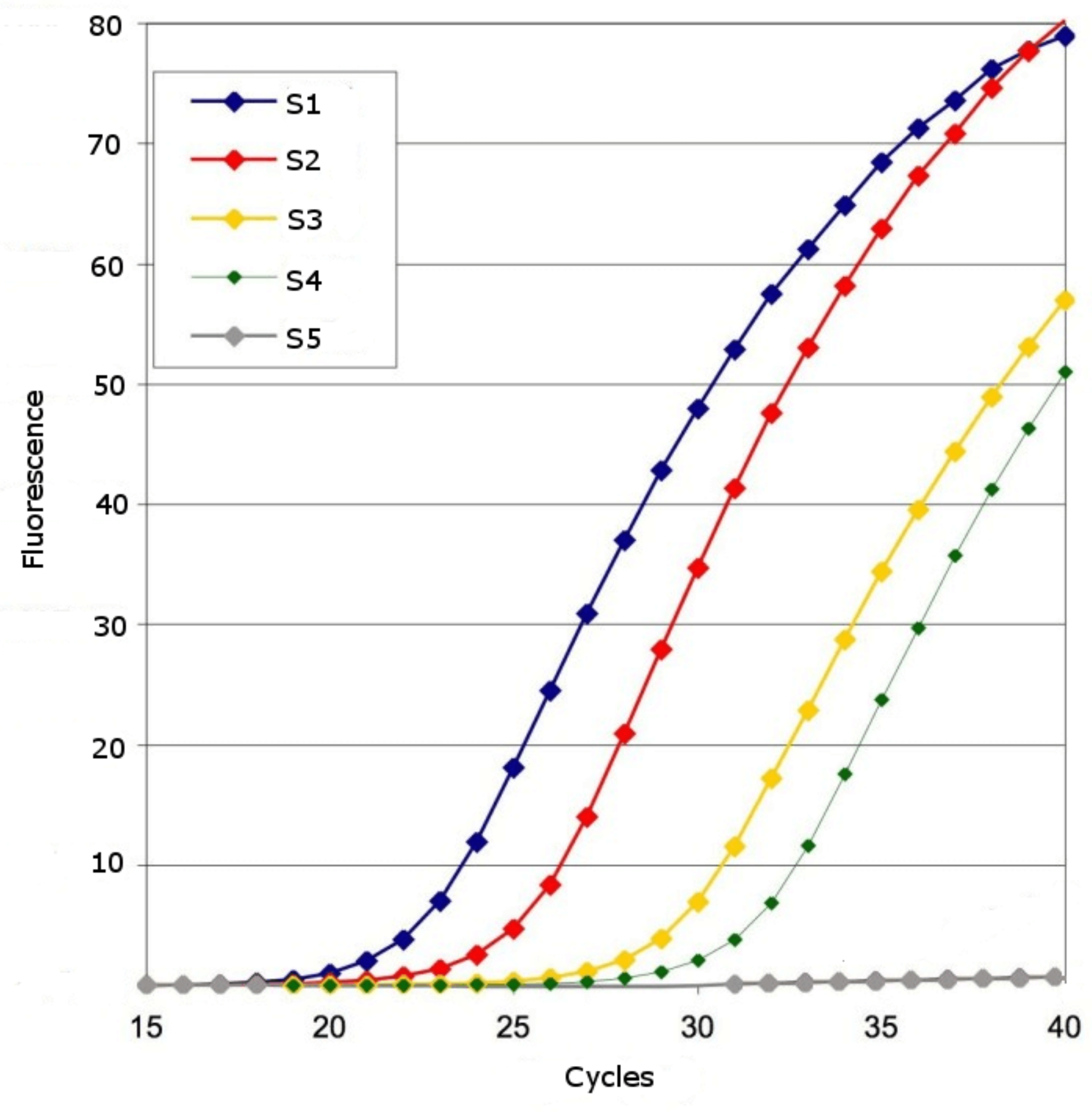
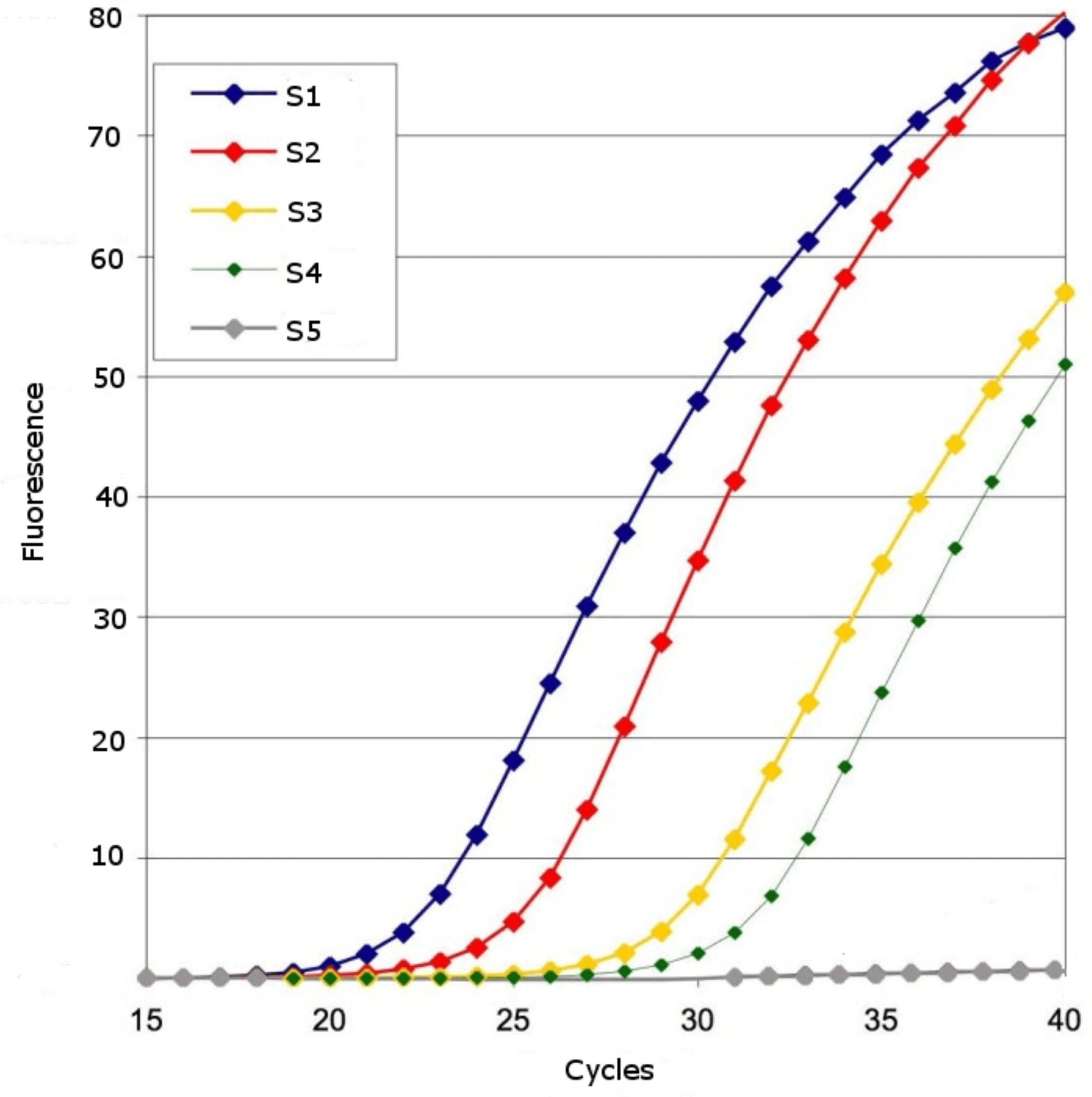


Figure 2 shows amplification plot with five samples (S1 to S5).

The amplified DNA is fluorescently labelled

A fluorescent signal is produced during the PCR reaction, which is quantifiably and directly proportional to the starting amount of DNA.



Advantages of qPCR

Speed: amplified DNA is being detected at the same time as the PCR reaction is taking place, so there is no need for a separate detection after, as is the case in conventional PCR (e.g. on an agarose gel)

Throughput: qPCR is considered a high-throughput method (processing of large numbers of samples in short time), due to its compatibility with liquid handling automation stations for sample preparation (DNA/RNA isolation and loading onto qPCR plates).

Sensitivity: qPCR is able to distinguish two-fold differences in quantity of target DNA molecules, and it can detect down to just a few molecules of initial DNA. When compare to PCR, as little as 1/1000 the amount can be used.

Range of quantification: broad quantification can be performed over several orders of magnitude (up to 107-fold dynamic range).

Reproducibility: generally regarded as highly reproducible.

Disadvantages of qPCR

Cost of equipment: due to the optical components required for sensitive fluorescence detection the qPCR cyclers are five to ten times more expensive than conventional PCR thermal cyclers

Cost of chemicals and consumables: qPCR is a very sensitive method. Therefore, precise composition and high quality of the reaction mixtures is extremely important. This is why ready-to-use reaction mixtures are usually purchased (master mix). Because of the sensitive detection method (fluorescence) a specific set of plastic-ware is required.

Loading times: Loading qPCR samples into plates is usually a much more precise and tedious process when compared to conventional PCR, mainly due to the higher number of reagents and samples being used and the method´s extreme sensitivity.

Disadvantages of qPCR

Inhibition of PCR reaction: due to the complex nature of biological samples, imperfect purification processes during isolation of nucleic acids may leave traces of various substances in isolated samples. PCR reactions are sometimes inhibited by these substances, also called inhibitors of PCR reaction (DNA polymerase is susceptible to certain compounds that inhibit its activity). This can complicate the quantification process.

Sensitivity to errors: qPCR is an extremely sensitive method and as such very prone to errors. This means that even the slightest mistakes can have a significant influence on the final results. The most variable and critical point is the preparation of the samples (DNA extraction and reverse transcription). That is why several control reactions (e.g. no template control, buffer control) need to be included when performing the assay to assure quality control checks in every run.

Data analysis: data analysis and interpretation of results is more complicated than in conventional PCR, granted the results are more informative.

PORCINE DNA EXTRACTION METHOD IN COSMETIC PRODUCTS FOR REAL-TIME PCR AMPLIFICATION

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TYPES OF SAMPLE USED IN THE STUDY

Four types of non-halal skin care were used (Silky balm, clay mask, skin refined cream & moisturizer)

MOISTURIZER

MASK

PRIMER

FOUNDATION

Aim of the study

To exclude PCR inhibitors during the analysis

- To develop porcine DNA extraction method for real-time qPCR amplification

Objectives of the study

1. To extract and optimization method extraction for moisturizer cream samples and spiked samples using modified CTAB (cetrymethylammoniumbromide) method.

2. To measure the quantity and quality DNA sample and spiked samples by reading absorbance at 260 nm and 280 nm using UV spectrophotometer.

3. To develop, optimize and validate method for detection of porcine DNA using Real-Time PCR.

4. To analyse porcine DNA in extracted samples and spiked samples using Real-Time PCR.

Flow Chart of Methodology

1

Collecting of variety moisturizer cream-based product samples

2

Extraction genomic DNA using modified CTAB method

Determination of DNA concentration (quality and quantity) using UV spectrophotometry

Method development of porcine DNA using real-time PCR including limit of detection, selectivity

5

Identifying porcine DNA using real-time PCR

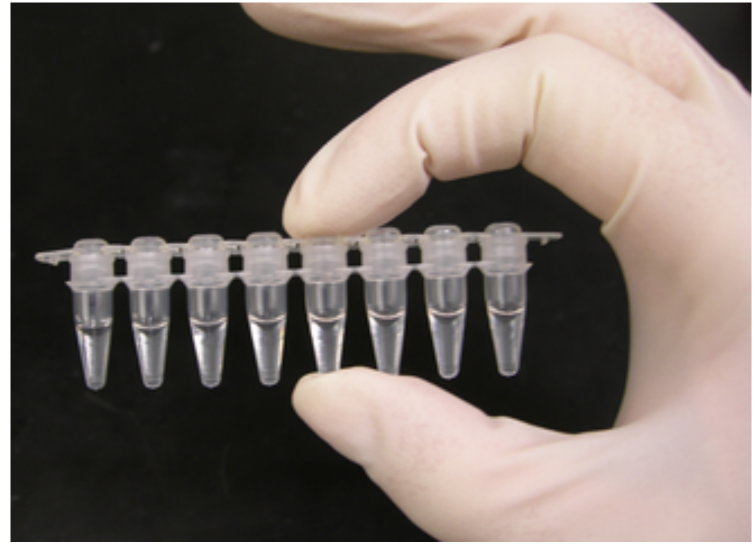
SAMPLE PREPARATION FOR CTAB EXTRACTION

- Weight sample (~0.2 gms) into 2 mL tube. Add 1.6 mL **PBS (Phosphate Buffered Saline).** Vortex sample for few seconds. Incubate sample with 65°C, 1000rpm until sample dissolved.
- Centrifuge sample 13,000 rpm for 10 min. Discard supernatant. Keep pellet only.
- Add 1 mL CTAB buffer, 10 µL RNAse, **5 µL DTT,** 30 µL proteinase K. Incubate at 900 rpm for 60 minutes.
- Transfer 800 µL supernatant to a new tube. Add 800 µL chloroform. Vortex for few second. Centrifuge 8000 rpm for 15 minutes.
- Transfer 300 µL upper layer supernatant to new 1.5 mL tubes. Add 600 µL 100% ethanol and mix well. Centrifuge at 13,000 rpm for 15 minutes.
- Discard ethanol, add 600 µL 80% ethanol. Invert for few times. Centrifuge again at 13,000 rpm for 5 minutes.
- Discard 80% ethanol. Dry the sample using centrivap for 10-15 minutes.
- Add 30 µL TE buffer. Keep at chiller for next step

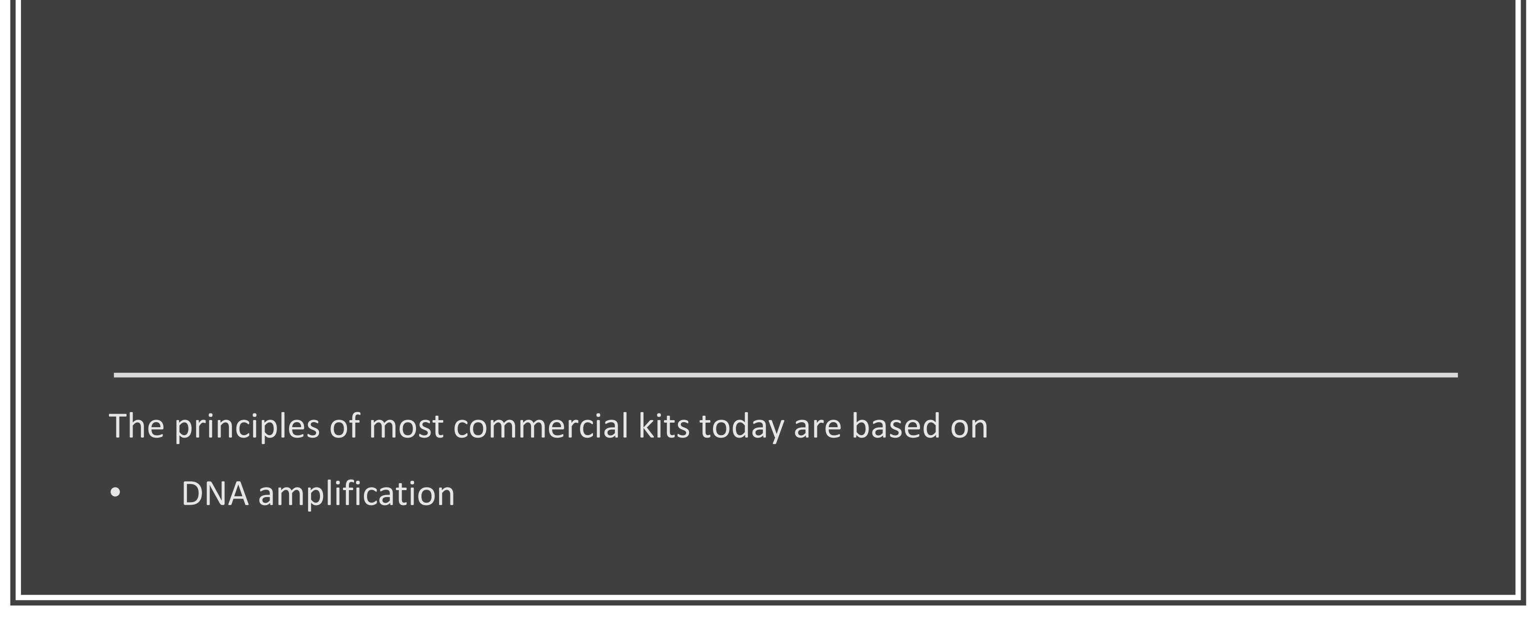
Real-time PCR System

UV/Vis Spectrometer with CCD array detector

Placing a strip of eight PCR tubes into a thermal cycler



A strip of eight PCR tubes, each containing a 100 µl reaction mixture



The principles of most commercial kits today are based on

- DNA amplification

Determination of the Sensitivity and Repeatability of the Assay

- The determination of sensitivity assay was measured in terms of the detection limit of porcine DNA in pure lard extract.
- The replicate of real-time PCR measurements was made of dilution series of (1.0, 0.1,0.01,0.001, 0.0001, 0.00001 ng) porcine gelatin.
- The limit of detection (LoD) was taken as being the lowest amount that could be amplified with a reproducible Ct value.
- The repeatability assay was performed by replication of these dilution series in three replicates.
- (LOD) for porcine DNA was examined and the detection limit was as low as 0.0001 ng

RESULTS

Label	Details	Weight (g)	DNA yields (ng/µL)	CT (Mean)
1	Silky Balm Formulation: Cream Brand: A Purchase: Online Made in: China	0.2346	2.8	UD
1S		0.2470	3.3	UD
2	Clay Mask Formulation: Cream Brand: B Purchase: Online Made in: China	0.2358	9.2	UD
2S		0.2681	10.3	34.56
3	Skin Refined Formulation: Cream Brand: Y Purchase: Online Made in: China	0.2126	5.6	UD
3S		0.2211	8.8	35.44
4	Moisturizer Formulation: Cream Brand: Z Purchase: Online Made in: Korea	0.2388	2.8	29.26
4S		0.2616	13.2	UD
Positive Control	Raw lard extraction	0.2620	24.4	20.19
Negative Control	Blank	0.2	2.5	UD

Table 1: CT values obtained from different brand of cosmetic samples

PowerChek TM Pork Gelatin Real-Time PCR Kit

- To detect the presence of porcine ingredients in commercial non-halal cosmetics, four types of skin care were tested, and the qPCR assay was performed using DNA extracted from each skincare product with the PowerChek TM Pork Gelatin Real-Time PCR Kit.

- To assess the accuracy of the method, the porcine template DNA was extracted from spiked cosmetics samples (Sample 1-4) using the kit and qPCR was used to determine the CT value. Initially, to detect porcine DNA in the cosmetics before spiking, DNA was extracted from sample 1-4 using the Power Prep™ DNA extraction kit. Among 4 samples, the Real-time

PowerChek TM Pork Gelatin Real-Time PCR Kit

- Among 4 samples, the Real-time PCR results showed that porcine DNA was not present in the tested cosmetics except for the moisturizer.
- However, the spike samples of 1, 2 and 3 shown CT value indicating the presence of porcine and showed that we can get the recovery of porcine DNA.

CONCLUSIONS & FUTURE STUDY

1. Using this commercial kit, we can detect as little as 0.0001 ng porcine DNA using raw lard material.

2. Dilution of samples may help to reduce the concentration that suits the kit limitation.

3. By reducing the concentration of the DNA samples as the CT values are high and UD value may due to the high concentration of samples and cannot be detected by the Real-time PCR system.

4. It is crucial to select the appropriate DNA extraction method to exclude these PCR inhibitors

OBJECTIVES OF THE STUDY

1. To screen the presence of porcine DNA in selected non-halal colour cosmetics by real-time PCR using Pork Gelatine DNA extraction kit

2. To determine the sensitivity and repeatability of the kit

RESULTS- QUALITATIVE ANALYSIS OF EXTRACTED DNA

Label	Details	Weight (g)	DNA yields (ng/µL)	CT (Mean)	
1	Silky Balm Formulation: Cream Brand: A Purchase: Online Made in: China	0.2346	2.8	33.42	Positive
1S		0.2470	3.3	UD	
2	Clay Mask Formulation: Cream Brand: B Purchase: Online Made in: China	0.2358	9.2	UD	
2S		0.2681	10.3	34.58	Positive in spike sample
3	Skin Refined Formulation: Cream Brand: Y Purchase: Online Made in: China	0.2126	5.6	UD	
3S		0.2211	8.8	35.44	Positive in spike sample
4	Moisturizer Formulation: Cream Brand: Z Purchase: Online Made in: Korea	0.2388	2.8	29.26	Positive
4S		0.2616	13.2	UD	
Positive Control	Raw lard extraction	0.2620	24.4	20.19	
Negative Control	Blank	0.2	2.5	UD	

The spike samples of 2 and 3 shown CT value indicating the presence of porcine and showed the recovery of porcine DNA.

Dilution of samples may help to reduce the concentration that suits the kit's limitation.

PCR inhibitors may interfere with PCR system

The extraction efficiency depending on the type of samples.

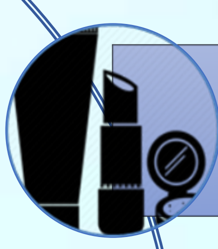
The use of nanocosmeceuticals cause difficulties for lab instrument to detect porcine in cosmetics without spiking.

RESULTS- SENSITIVITY & REPEATABILITY TEST

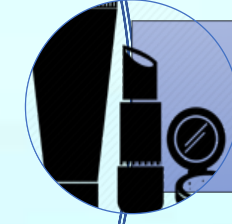
Table 1: LOD determination using raw lard

No.	Concentration (ng)	CT (Mean)
1	1.0	UD
2	0.1	UD
3	0.01	UD
4	0.001	28.16
5	0.0001	36.52
6	0.00001	UD
7	Positive Control (Porcine)	20.19
8	Negative Control (water)	UD

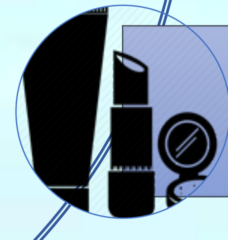
CONCLUSIONS



The Pork Gelatin Real-time PCR kit proved to be a reliable and sensitive tool for detecting porcine DNA fragments present in cosmetic samples silky balm and moisturizer using a 40-cycle Real-time PCR.



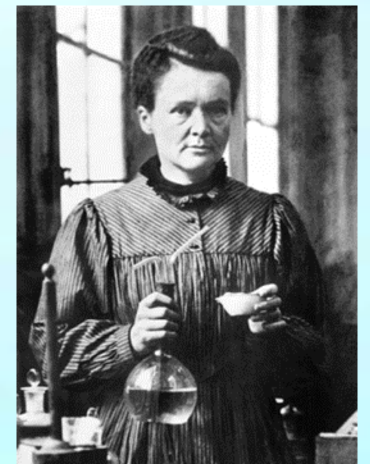
Using this commercial kit, we can detect as little as 0.0001 ng porcine DNA using raw lard material.



This is an easy-to-follow, reliable, and sensitive commercial detection kits, used for monitoring of cosmetic sample products.

Acknowledgements

Ministry of Education Malaysia research grant (grant vote MOO02-2018 awarded to Malaysian Higher Education Consortium of Halal Institutions) and University of Malaya Research Grant Challenge: Grant vote 002B-17HNE.



Thank you

Be less curious about people and more curious about ideas – Marie Curie