DEVELOPMENT OF ARMS-PCR TECHNIQUE FOR THE DETECTION OF SNPs RELATED TO MEAT QUALITY GENES

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มหาวิทยาลัยเทคโนโลยีสุรนารี

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พัฒนา ARMS-PCR เทคนิค เพื่อการตรวจสอบ SNPs ในยีนที่เกี่ยวข้องกับคุณภาพเนื้อ

นายกิตรติ นาคเกตุ

มหาวิทยาลัยเทคโนโลยีสุรนารี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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DEVELOPMENT OF ARMS-PCR TECHNIQUE FOR THE DETECTION OF SNPs RELATED TO MEAT QUALITY GENES

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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กิตรติ นาคเกตุ: พัฒนา ARMS-PCR เทคนิค เพื่อการตรวจสอบ SNPs ในยีนที่เกี่ยวข้องกับคุณภาพเนื้อ (DEVELOPMENT OF ARMS-PCR TECHNIQUE FOR THE DETECTION OF SNPs RELATED TO MEAT QUALITY GENES) อาจารย์ที่ปรึกษา: รองศาสตราจารย์ ดร.มารินา เกตุทัต-คาร์นส์, 73 หน้า.

คำสำคัญ: ARMS-PCR/ยีนไขมันแทรก/สนิปส์(SNPs)/เกรดไขมันแทรก/โคเนื้อ

โคเนื้อมีความสำคัญต่อระบบเศรษฐกิจในประเทศไทยเป็นอย่างมาก โดยโคที่มีไขมันแทรกสูง (Marbling) จะมีราคาแพงและเป็นที่ต้องการของผู้บริโภค หนึ่งในปัจจัยที่ทำให้โคมีโอกาสไขมันแทรกสูงนอกจากสภาพแวดล้อมและอาหารการเลี้ยงดูคือ กรรมพันธุ์หรือ Genetic โดยยีน Thyroglobulin (*TG5*), Diacylglycerol O-acyltransferase 1 (*DGAT1*) and Fatty acid binding protein (*FABP4*) gene ควบคุม Metabolism ของไขมันที่มีผลต่อไขมันแทรกในโค ซึ่งการศึกษาครั้งนี้ได้ทำการออกแบบไพรเมอร์และหาสภาวะที่เหมาะสมเพื่อตรวจสอบสนิปส์ที่ตำแหน่งต่างๆ ของยีนที่เกี่ยวข้องกับไขมันแทรกในตัวอย่างชิ้นเนื้อที่ทราบเกรดของโคเนื้อวากิวลูกผสมในไทย ด้วยเทคนิค Amplification refractory mutation system PCR (ARMS–PCR) พบว่าความสมดุลของความเข้มข้นไพรเมอร์, $MgCl_2$ และ ชนิดของ *Taq* polymerase มีผลอย่างมากกับเทคนิค ARMS–PCR อีกทั้งในการศึกษาครั้งนี้สามารถให้ผลการตรวจสอบ Genotypes ได้ชัดเจน โดยสนิปส์ตำแหน่ง C422T ของยีน *TG5* ในโคเนื้อวากิวลูกผสมในไทยเป็น T allele มากกว่า C allele ส่วนสนิปส์ตำแหน่ง A10433G และ A10434C ของยีน *DGAT1* เป็นส่วนใหญ่เป็น AA allele และสนิปส์ตำแหน่ง G3691A ของยีน *FABP4* เป็น G allele ซึ่งให้ผล allele frequency เหมือนกับโคเนื้อวากิวของญี่ปุ่นที่มีความสัมพันธ์กับไขมันแทรกในเนื้อโค ดังนั้นสามารถใช้เทคนิค ARMS–PCR ที่พัฒนาขึ้นตรวจสอบ Genotypes ในโคเนื้อวากิวลูกผสมในไทย อีกทั้งยังให้ผลการตรวจสอบที่เร็ว มีความจำเพาะสูง ประหยัดค่าใช้จ่าย เป็นเทคนิคที่เป็นประโยชน์สำหรับในการปรับปรุงพันธุกรรมโคเนื้อในประเทศไทยให้ดีขึ้นและช่วยเกษตรกรลดความเสี่ยงและสามารถตัดสินใจคัดเลือกโคเนื้อก่อนนำเข้าขุนได้อีกด้วย

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2565 ลายมือชื่ออาจารย์ที่ปรึกษา_____

KITRATI NAKKET: DEVELOPMENT OF ARMS-PCR TECHNIQUE FOR THE DETECTION OF SNPs RELATED TO MEAT QUALITY GENES. THESIS ADVISOR: ASSOC. PROF. MARIENA KETUDAT-CAIRNS, Ph.D., 73 PP.

Keyword: ARMS-PCR/Meat quality genes/SNPs/Thai wagyu cattle/Meat quality

Beef cattle is important for the Thai agricultural system. High levels of meat marbling score increases the price. Genetics are the one of the key factors that effect meat marbling in bef. Thyroglobulin (*TG5*), Diacylglycerol O-acyltransferase 1 (*DGAT1*) and Fatty acid binding protein (*FABP4*) genes are important for the determination of metabolism of fat. In the present research, we designed and developed Amplification refractory mutation system PCR (ARMS-PCR) technique to detect single nucleotide polymorphism (SNPs) of marbling genes. The results showed that the balanced concentration of primers and $MgCl_2$, type of *Taq* polymerase are important to the ARMS–PCR and need ed to be optimized. In the Thai crossbreed wagyu in this study, the C422T of TG 5 SNP have higher T allele than C allele. The allele frequency of the A10433G and A10434C of *DGAT1* SNPs were mostly AA allele, whereas the allele frequency for the G3691A SNPs in the *FABP4* gene showed higher G allele than A allele. The allele frequency of meat marbling genes in Thai wagyu crossbreed were similar to Japanese wagyu. This work showed that the ARMS-PCR can be successfully applied to the detection of SNPs in meat marbling genes in Thai wagyu cross breed. The ARMS-PCR technique is more economical, easy to perform, and precise genotyping can be observed. Farmers can use these DNA markers to identify good potential cattle to be used as mother to obtain high quality wagyu cross breed and improve breeding programs.

School of Biotechnology
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Advisor's Signature

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Kitrati Naket

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LIST OF ABBREVIATIONS

M	=	Molar
mM	=	Milli Molar
mg	=	Milligram
µg	=	Microgram
min	=	Minute
mL	=	Milliliter g = Gram
gDNA	=	Genomic DNA
Kg	=	kilogram
M	=	Mole
mg/ml	=	Milligram per milliliter
ml	=	Milliliter
mM	=	Millimolar
mm	=	Millimeter
Ng	=	Nanogram
nt	=	Nucleotide
pmol	=	Picomole
rpm	=	Revolutions per minute
µg/ml	=	Microgram per milliliter
µl	=	Microliter
µm	=	Micrometer
IMF	=	Intramuscular fat
SNPs	=	Single nucleotide polymorphism
FE	=	Feed efficiency
MAS	=	Marker-assisted selection
TG5	=	Thyroglobulin

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LIST OF ABBREVIATIONS (Continued)

DGAT1	=	Diacylglycerol acetyltransferase
FABP4	=	Fatty acid binding protein 4
ARMS–PCR	=	Amplification refractory mutation system PCR
FAO	=	Food and Agriculture Organization
JMGA	=	Japan Meat Grading Association
BMS	=	Beef Marbling Score
LD	=	*Longissimus dorsi*
U.S.A.	=	United States of America
BCS	=	Beef color standard
BTS	=	Beef texture standard
BFS	=	Beef fat standard
RFLP	=	Restriction fragment length polymorphism
MS	=	Marbling score
MC	=	Meat color
FC	=	Fat color
TX	=	Meat texture
MG	=	Meat quality grade
BF	=	Backfat thickness
LDA	=	*Longissimus dorsi* area
CW	=	Carcass weight
MI	=	Meat quantity index
MQ	=	Meat quantity grade
LW	=	Live weight
BFT	=	Backfat thickness
SCD1	=	Stearoyl-CoA desaturase 1
CAST	=	Calpastatin
CAPN1	=	Calpain-1
PUFA	=	Polyunsaturated fatty acids
MUFA	=	Monounsaturated fatty acids
SFA	=	Saturated fatty acids

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LIST OF ABBREVIATIONS (Continued)

PCR	=	Polymerase chain reaction
CYP2E1	=	Cytochrome P450 2E1
h	=	Hour
SF	=	Shear force
dNTPs	=	Deoxyribonucleoside triphosphates

CHAPTER 1
INTRODUCTION

1.1 Introduction

Beef cattle production is important for the agriculture industries. Wagyu cattle is a very distinctive breed because of its high marbling score. It has high visible layers of intramuscular fat. The marbling and tenderness of meat are the key indicators of meat quality and are important issues for beef cattle production due to their major impact on consumer satisfaction (Motoyama et al., 2016).

Marker-assisted selection (MAS) is the method used to select traits of animals to include in breeding program development. Recently, lots of genes associated with fatty acids composition, backfat thickness, meat marbling score and meat tenderness have been particularly of interest (Gotoh et al., 2014). The MAS of meat quality genes could be useful for genetic improvement of economic traits for which phenotypic measurements are difficult or expensive to obtain (Hasanet et al., 2021).

Polymorphisms in the Thyroglobulin (*TG5*), Diacylglycerol acetyltransferase (*DGAT1*) and Fatty acid binding protein 4 (*FABP4*) genes have been shown to affect meat marbling of several breeds of cattle (Winter et al., 2002; Shin et al., 2007; Shin et al., 2012). The single nucleotide polymorphisms (SNPs) for *TG5*, *DGAT1* and *FABP4* genes have been shown to correlate with marbling score in *Bos taurus* and *Bos indicus* breeds. They are important SNPs for beef cattle improvement breeding program (Dadi et al., 2012).

Amplification refractory mutation system PCR (ARMS–PCR) is one of the techniques used to detect SNPs designed by Newton et al. (1989) for the identification of sequence polymorphisms. The ARMS–PCR uses 4 primers which are two outer primers and two inner primers in one PCR reaction (Etlik et al., 2011). The outer primers amplified a common fragment band or reference band of the template gene of interest. Each inner primer combines alleles specific and amplify template of the gene of interest, which are of difference sizes and can simply separated by gel electrophoresis for homozygous and heterozygous. The ARMS–PCR is a fast, easy to

to use, and low-cost protocol for the detection of single nucleotide polymorphisms (SNPs).

The objectives of this study were 1) to develop ARMS-PCR techniques to detect SNPs that have been shown to associated with meat marbling score in the *TG5* (C422T), *DGAT1* (A10433G and A10434C) and *FABP4* genes (G3691A) and 2) to confirm the relation between the genotype of the 3 SNPs mention above with meat quality and carcass composition in Thai Wagyu crossbreeds.

1.2 Research objectives

The objectives of this study were:

1.2.1 To develop ARMS-PCR techniques for the detection of SNPs in the *TG5*, *DGAT1* and *FABP4* genes.

1.2.2 To study the allele frequency of meat quality genes in Thai Wagyu crossbreeds.

1.3 Research hypotheses

1.3.1 The developed ARMS-PCR can be used to detect SNPs in the meat quality genes in Thai Wagyu crossbreeds.

1.3.2 The 3 SNPs are associated with meat marbling score in Thai Wagyu crossbreeds.

1.4 Scope of the study

This research focuses on the development of ARMS-PCR to detect SNPs in the 3 meat quality genes *TG5*, *DGAT1* and *FABP4* correlation of the SNPs in the meat marbling genes and the meat grade of Thai Wagyu crossbreeds.

1.5 Benefits of this research

1.5.1 Easy and simple method (ARMS-PCR) to detect SNPs that are associated with meat marbling score of Thai Wagyu crossbreeds was obtained.

1.5.2 Farmers can use these DNA markers to identify good potential cattle to be used as mother to obtain high quality Wagyu crossbreeds.

CHAPTER 2

LITERATURE REVIEW

2.1 Situation worldwide consumption of meat

Meat is one of the most popular protein sources. In addition, meat have minerals and vitamins excellent for human diet. Protein from meat has great impact worldwide (Salter et al., 2018). The consumption of meat has been rising since the 1960s, particularly between 1980 year to present. While meat products supply has also been increasing. Katare et al. (2020) reported that in 1992 to 2016 the meat consumption increased as high as 500%. Protein from meat products are as high as 58% while from other proteins sources such as grains and wheat. In Europe, one person consumption proteins sources of about 28g/day. Therefore, meat products are the major proteins sources (Bonnet et al., 2020). In 2014-2016 Salter et al. (2018) reported consumption of meat worldwide increased around 60% or 34.1 kg/person.

Australians is one of the top countries indicator for meat consumption. Because increased meat consumption and also has more impacts on human and economy systems. According to Australians data, they have the highest per capita consumption of meat. The United Nations' Food and Agriculture Organization (FAO) reported that the meat consumption (per person) in 1998 to 2018 of Australian is the highest followed by the United States of America and China (Figure 2.1).

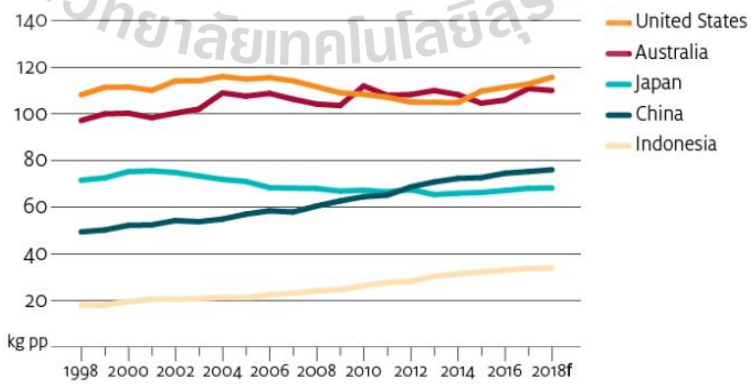

Figure 2.1 Meat consumption (per person) in 1998 to 2018.

Source: Food and Agriculture Organization (FAO) (https://www.agriculture.gov.au/abares/research-topics/agricultural-outlook/meat-consumption).

2.1.1 Situation consumption of meat in Thailand

Meat consumption demand in 2018 to 2022 increased 0.15 %. However, due to the epidemic of an emerging disease, Covid-19 or Corona Viruses and Lumpy Skin, starting in 2019 have damaged the world, resulted in the decrease of meat production to around 251,000 tonne. The lift of lockdown of Covid-19, in 2023 has increased the demand for meat products in restaurants, hotels, and tourism businesses. Moreover, the price is favorable, and the market size continues to increase.

Thailand imported meat from Australia, New Zealand and India. In 2022, Thailand expected to import 25,500 tonne of beef and beef products which is about 7,000 million baht. This was an increase from 22,850 tonne or around 4,697 million baht, or an increase of 11.60% and 49.03%, respectively. And in this year 2023 the expect of import beef and beef products will be increased.

Euromonitor organization reported meat market of Thailand in 2016 to 2020 has been growing around 2% – 10% since 2016 and reported corresponds to " Bangkok post" news Thailand's meat consumption is expected to increase 40 million baht in this year, difference from five years ago which was only 30 million. The consumption of fresh meat increased 60%, this is reason because consumption of local meat and imported meat has increased 10%. Restaurants business have added high quality meat dishes to their menues. Business of meat import plans to prepare more than 1,000 beef items from Australia, Japan, New Zealand, and US to demand enough for population in country.

2.2 Introduction of beef cattle

Beef cattle are high protein sources, The texture softness, good taste normally will come from cattle raised with grassy, grain-fed. However, genetics of the breed are also important, for example Wagyu, Angus, or Australia beef normally have the genetics of meat that will give rise to high marbling and good meat tenderness.

Previous research reported that beef cattle raised with grain fed in a closed system so that the cattle do not have room to exercise will result in high intramuscular fat inserts in muscle or marbling fat. Cattle of grain fed have an approximate fattening period of 100-180 days to increase the amount of marbling fat in muscle. The color of fat for grain fed are more of white without fishy taste and the meat is more juiciness

and tenderness when compared to cattle raised in grassy field (Godde et al., 2021). However, the environment and genetics also contribute to the quality of meat not just the feed.

Cattle of Japan or Wagyu are popular around the world. These breeds are important for the agricultural, history and farming of Japan (Figure 2.2). Japanese Wagyu cattle have been divided into 4 breeds as follow:

1) Japanese Black which are the most studies and investigate for the meat quality and muscle physiology more specifically for beef production.

2) Japanese Brown or Red Wagyu which was certifying in 1944. These cattle are raised primarily in Kumamoto and Kochi regions. They have low-fat content and lean meat.

3) Japanese Shorthorn are raised primarily in Tohoku regions. They were certified as originally breed cattle in 1957 (Takayasu, 1983). These cattle have low-fat content, mild-flavored meat.

4) Japanese Polled which are raised primarily in Yamaguchi regions was breeded by Angus and Japanese Black to improve native breed (Hoashi et al., 2007). These cattle have high lean meat content and high Wagyu flavor.

Figure 2.2 Four Japanese Wagyu breeds.

Source: https://livejapan.com/en/article-a0001254/.

2.2.1 Thai Wagyu crossbreeds

The Beef cattle in Thailand are mostly *B. indicus.* The *B. indicus* are spread across southeast Asia. They have become popular and expanded for farming. However, the number of beef cattle are still not enough for the demand due to the increase of population as well as expansion of economy. Thailand have increased the demand for food consumption in both quantity and quality. As the results motivates the farmer and cattle breeders to improve cattle breeding. Restaurants business are popular in Thailand. They can increase the demand of beef consumption. The beef products from Thai Wagyu crossbreed is one of the option for Thai consumers. To upgrade Thai Wagyu crossbreeds, farmers use pure breed Wagyu semen to artificial inseminate Thai native cattle to produce Thai crossbreeds Wagyu.

Figure 2.3 Wagyu cattle (Breeder) Tajima breed.
Source: https://smartcowboy.blogspot.com/2019/12/Wagyu-cattle.html.

Figure 2.4 Wagyu cattle (Breeder) Tajima breed and Thai Wagyu crossbreed Puparn.
Source: https://www.technologychaoban.com/bullet-news-today/article_150508.

In 1988 Japan Wagyu Association, offered 1 pair of Wagyu cattle Tajima breed (Figure 2.3) to Her Royal Highness Princess Maha Chakri Sirindhorn. The Wagyu cattle were raised in Phupan, Sakhon Nakhon at the Puparn Royal Development Study Centre. In 1990 the bull was used to produce frozen semen (fullblood) to donate to farmers for artificial insemination with local dairy Red Sindhi cattle breed. The young cattle cross breed was used for research on management, raising conditions, feed to improve the breed to be suitable for Thailand.

In 2005 Assoc. Prof. Dr. Rangsun Parnpai from the School of Biotechnology, Institute of Agricultural Technology Suranaree University of Technology (SUT) started up the Korat Wagyu project, by imported Gobori (Full blood Wagyu bull) from Australia. Gobori produced a lot of frozen semen to distributed to the Korat Wagyu corporative farmer to AI with local cattle. The F1 young cattle crossbreed was raise-feed according to formula that was highly researched. At the age of 36 months, they can produce high meat marbling grades 4-6 score (standard of Japan Meat Grading Association 2014 (JMGA 2014)) (Figure 2.5). The Wagyu cattle in this research contain much higher-grade beef than other breeds of cattle produced in Thailand. More frozen semen from full blood Wagyu have been distributed.

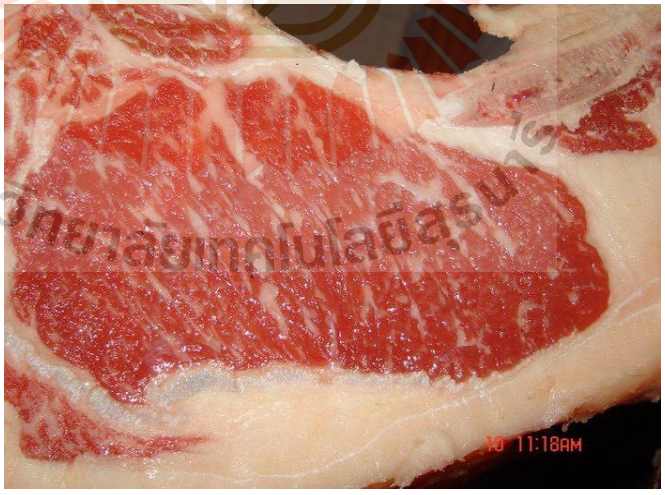

Figure 2.5 Korat Wagyu meat marbling grades 4-6 score.

Source: https://smartcowboy.blogspot.com/2019/12/Wagyu-cattle.html.

2.3 The meat marbling, meat tenderness and grading for beef cattle

The goal of consumption meat requires beef quality because these appearances distribute marbling, juiciness, good flavor, and tenderness effect to of the most influential considered of consumer. Therefore, meat marbling is the most important to consumers and has beneficial to nutrition and health (Gotoh et al., 2015). The meat of Wagyu cattle in Japan (Figure 2.6) has ability to produce meat marbling and high Beef Marbling Score (BMS) on intramuscular muscles due to quality system of raised. The *longissimus dorsi* (LD) muscle can evaluate and identify meat quality grade because in this muscle has attributes of meat marbling (Matsumoto et al., 2012).

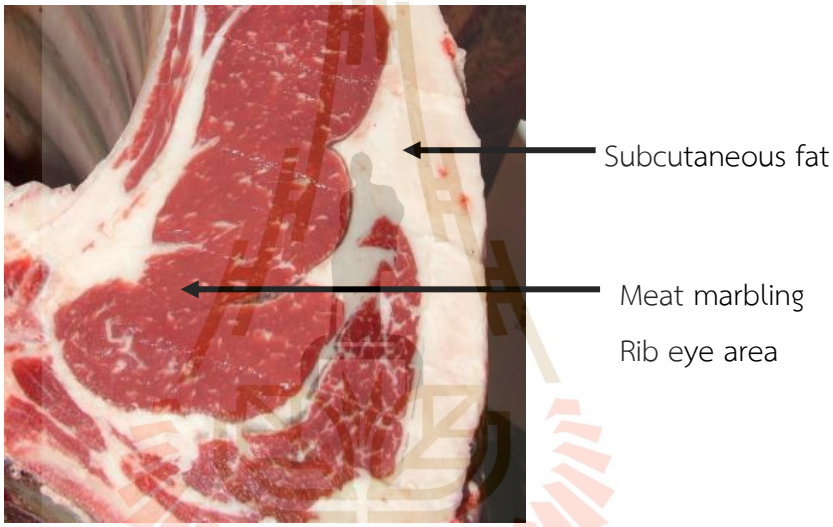

Figure 2.6 Wagyu beef production in *longissimus dorsi* (LD muscle of Japan).
Source: Matsumoto et al., 2012.

The standard determination of meat marbling quality is the United States of America (U.S.A. or USA), Japan and Australia using visual appraisal method evaluation marbling. The U.S.A. standard formulated since 1916 were developed continuities until 1925 used first public to give livestock cattle, slaughterhouse, and agricultural marketing service and in 1997 standard meat grade of U.S.A. were used official standards to identify grade of meat virtually all countries in the U.S.A (United States Standards for Grades of Carcass Beef, 2017). The standards have 10 total number of grades are the very abundant, abundant, moderately abundant, slightly abundant, moderate, modest, small, slight, traces, and practically devoid reference standard by USDA (1997).

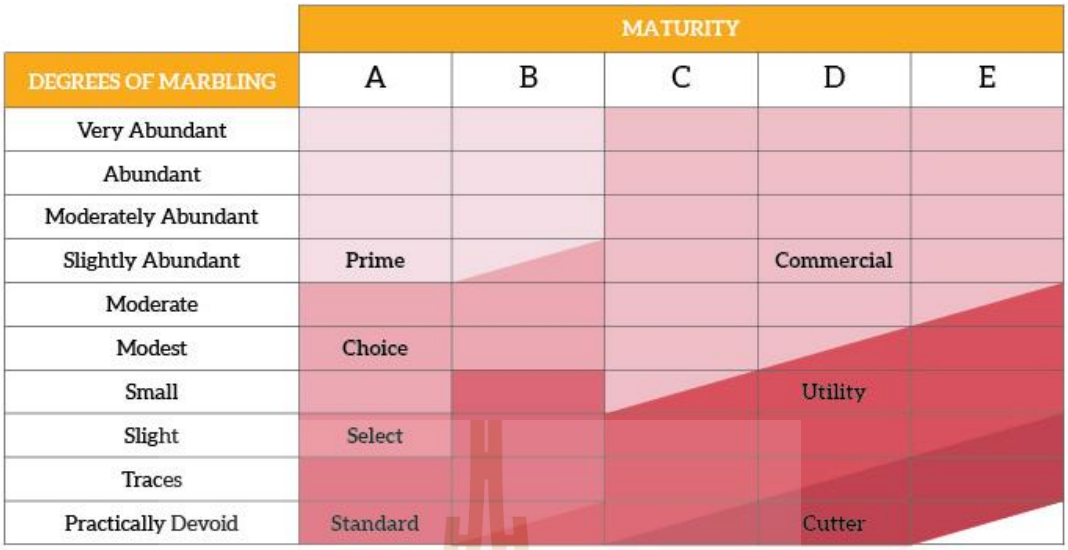

Figure 2.7 Association between carcass and marbling quality grade by USDA (1997).
Source: https://joesbutchershop.com/usda-prime-choice-and-select-what-does-it-really-mean-to-me/.

Figure 2.7 showed association between carcass and marbling quality grade by USDA standard method (1997). In U.S.A., visual evaluation of marbling and meat determinate by the USDA standard (1997), and marbling grade identify in to 10 level (Figure 2.8) three higher level (very abundant, abundant, and moderately abundant) can evaluation with programs (Emerson et al., 2012).

The Japan standard meat marbling grade used Official Japan Meat Grading Association 2014 (JMGA 2014) were established since in 1988 for evaluation other breeds in Japan. The meat marbling grade system in Japan showed yield grade as A, B and C and meat quality grade (1, 2, 3, 4, and 5) (Figure 2.8). These two-evaluator combined show the meat quality grade. As show the highest yield grade and highest meat quality grade because yield grade associated with identify carcass quality of cattle, it is important for evaluation to set and determinate of price. The grading system used reference by Japan Meat Grading Association 2014 (JMGA 2014) are shown in Figure 2.8. The high yield grade and high meat quality grade means have high marbling.

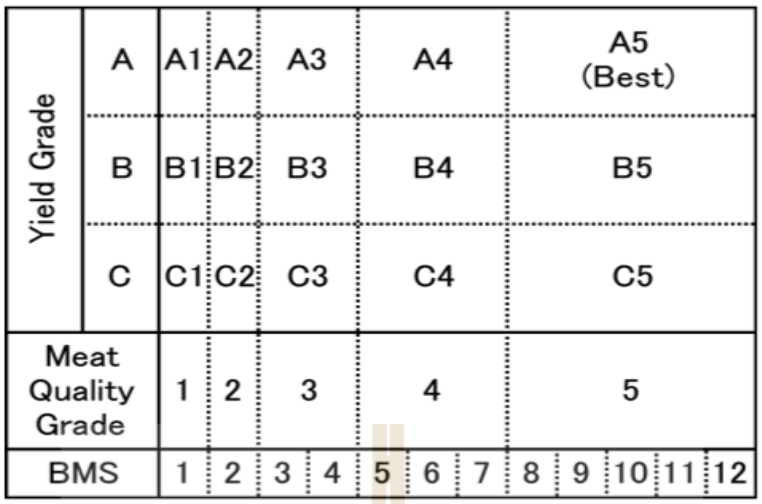

Figure 2.8 The grading guide of Wagyu beef yield grade and meat quality grade comparison of Japan grade to the Beef Marbling Standard (BMS).

Source: Japan Meat Grading Association 2014 (JMGA 2014).

2.3.1 Yield grade (A, B and C)

From Figure 2.9 yield grade that meaning yield score were calculated by rib eye area, level of short ribs, level subcutaneous fat (Figure 2.10) and body weight of carcass. Area and levels were detected by cutting of left carcass between 6th and 7th ribs.

Yield Rate	Meaning	Yield Score
A	Yield is higher than standard	Greater than or equal to 72
B	Carcass has standard yield	Greater than or equal to 69, less than 72
C	Yield is lower than standard	Less than 69

Figure 2.9 Show meaning of yield grade evaluator of cattle compare with standard.

Source: Japan Meat Grading Association 2014 (JMGA 2014).

2.3.2 Conception calculated yield score.

Yield = 67.37 + 0.130 * rib eye area (cm2) + 0.667 * level of short ribs (cm) – 0.025 * body weight of carcass (kg) – 0.869 * level subcutaneous fat (cm).

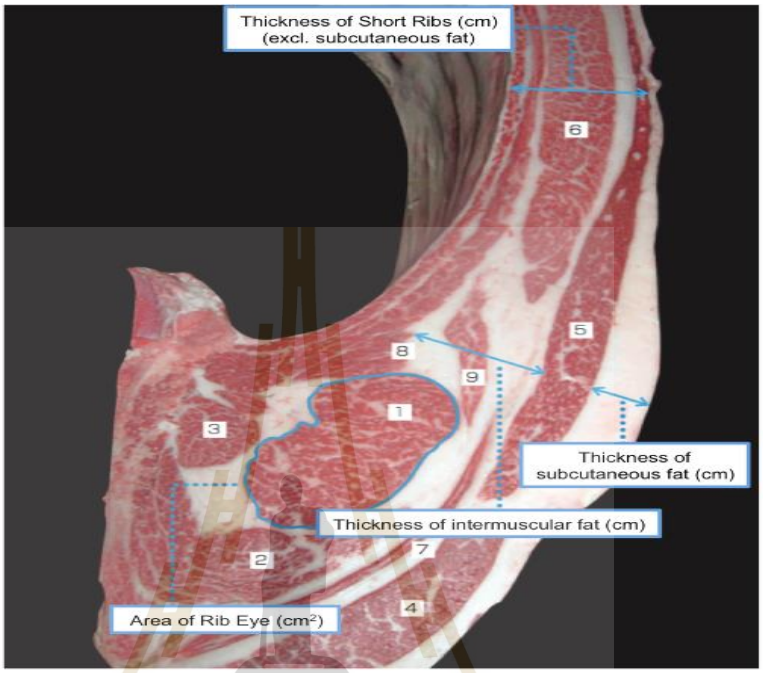

Figure 2.10 Wagyu cattle in rib eye are of left carcass.
Source: Japan Meat Grading Association 2014 (JMGA 2014).

2.3.3 Meat quality grade (1, 2, 3, 4, and 5)

Meat quality grade is the grade determination meat quality such as marbling, color of meat and fat, and meat texture. The grades (1, 2, 3, 4, and 5) would be determine by all five factors, and the highest grade of all five would be the best of meat quality grade of a beef cattle show in Figure 2.8.

2.3.4 Beef marbling standard (BMS)

Marbling is one major factor of meat quality in Wagyu cattle. The Beef Marbling Standard (BMS) to determine meat marbling in Wagyu. In Figure 2.11 and 2.12 shown score of BMS of 1 - 12 determined by analysis marbling in rib eye. BMS of No. 8 to 12 or 5 grade of Meat quality grade has high marbling, and BMS of No. 1 or 1 grade of Meat quality grade that mean cannot found marbling and in addition the BMS more than No. 10 is commonly served on the finest restaurants.

Marbling Grade	1	2	3		4			5				
BMS (No.)	1	2	3	4	5	6	7	8	9	10	11	12

Figure 2.11 Association of Marbling grade and Beef Marbling Standard (BMS).
Source: Japan Meat Grading Association 2014 (JMGA 2014).

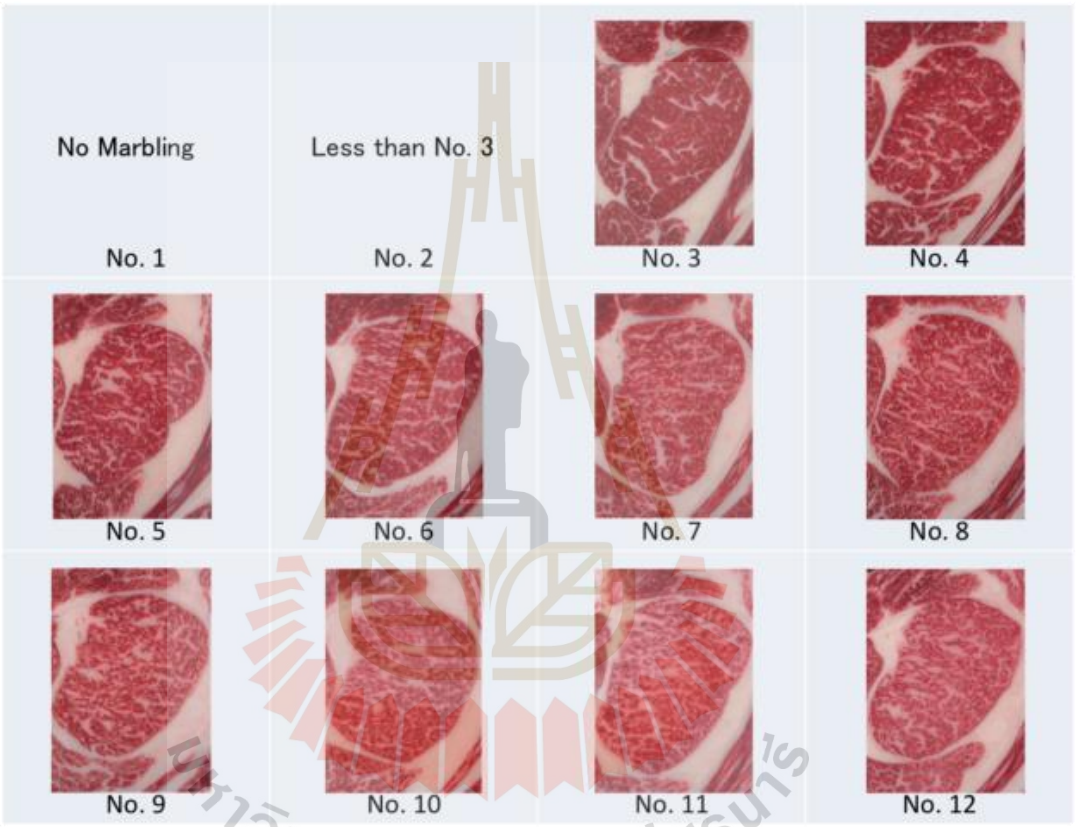

Figure 2.12 Meat marbling grade of Wagyu cattle.
Source: Japan Meat Grading Association 2014 (JMGA 2014).

2.3.5 Beef color standard (BCS)

The grade of Wagyu beef was evaluated by color of meat. The color (BCS) has scored No. 1 to No. 7. (Figure 2.11), color grade based on 1 to 5 have related with color (BCS) and gloss of meat is the meaning for evaluated color grade according shown in the Table 2.3 the medium tones or gloss excellent color (BCS) No.3 to 5 get the best 5 color grade, while the darkest and lightest or gloss close to standard color (BCS) No.1 to 7 get the lower 1 or 2 grades (Figure 2.13 and 2.14).

Figure 2.13 Beef color standard (BCS).

Source: Japan Meat Grading Association 2014 (JMGA 2014).

Color Grade	Color (BCS)	Gloss
5	No.3 to 5	Excellent
4	No.2 to 6	Good
3	No.1 to 6	Standard
2	No.1 to 7	Close to standard
1	Everything else	

Figure 2.14 Association of color grade and beef color standard (BCS).

Source: Japan Meat Grading Association 2014 (JMGA 2014).

2.3.6 Beef texture standard (BTS)

Texture grade standard were evaluated by firmness and texture. The beef texture standard (BTS) has scored No. 1 to No. 5 (Table 2.4), texture grade based on 1 to 5 have related with color firmness and texture of meat is the meaning for evaluated texture grade according shown in Figure 2.15 the firmness excellent or texture excellent get the best 5 texture grade, while the firmness loose and texture loose get the lower 1 grade.

Texture Grade	Firmness	Texture
5	Excellent	Excellent
4	Good	Good
3	Standard	Standard
2	Close to Standard	Close to Standard
1	Loose	Loose

Figure 2.15 Association firmness and texture to effect texture grade.

Source: Japan Meat Grading Association 2014 (JMGA 2014).

2.3.7 Beef fat standard (BFS)

The grade of Wagyu beef is evaluated by color of meat. The color (BFS) has scored of 1 – 7. (Figure 2.12), color grade based on 1 to 5 have related with color (BFS) and gloss and quality of meat are the meaning for evaluated color fat grade according shown in the Table 2.5 the fat of meat gloss and quality has excellent related with color (BFS) No.1 to No.4 get the best 5 color grade, while the gloss and quality has close to standard related with color (BFS) No.1 to No.7 get the lower 1 or 2 grades (Figure 2.16 and 2.17). In Thailand used Japan Meat Grading Association 2014 (JMGA 2014) to determine beef grade for Thai Wagyu crossbreeds.

Figure 2.16 Beef fat standard (BFS).
Source: Japan Meat Grading Association 2014 (JMGA 2014).

Color Grade	Color (BFS)	Gloss and Quality
5	No.1 to 4	Excellent
4	No.1 to 5	Good
3	No.1 to 6	Standard
2	No.1 to 7	Close to Standard
1	Everything else	

Figure 2.17 Association of Color Grade and Beef fat color standard (BFS).
Source: Japan Meat Grading Association 2014 (JMGA 2014).

2.3.8 Meat standard australia (MSA)

Meat Standard Australia (MSA) was improved and developed by Australia meat industry and Meat Livestock Australia (MLA). In Australia consumer meat products, more than dairy products and eggs increasing imports meat product (STATISTICS AUSTRALIA, 2022). Therefore (MSA) important to meat market in Australia. Australian beef cattle or Australian Wagyu used AUS-MEAT determination to be

confirmed graded because popular standard system and were control by Livestock Australia (MLA). AUS-MEAT system is like Japan Meat Grading Association 2014 (JMGA 2014). The AUS-MEAT system has 9 grade is excellent amounts of meat marbling in intramuscular fat while 0 grade is no meat marbling in intramuscular fat (Livestock Australia (MLA)). In Thailand used AUS-MEAT marbling score standard of Australia to determine beef grade for Thai angus cross breeds (Figure 2.18).

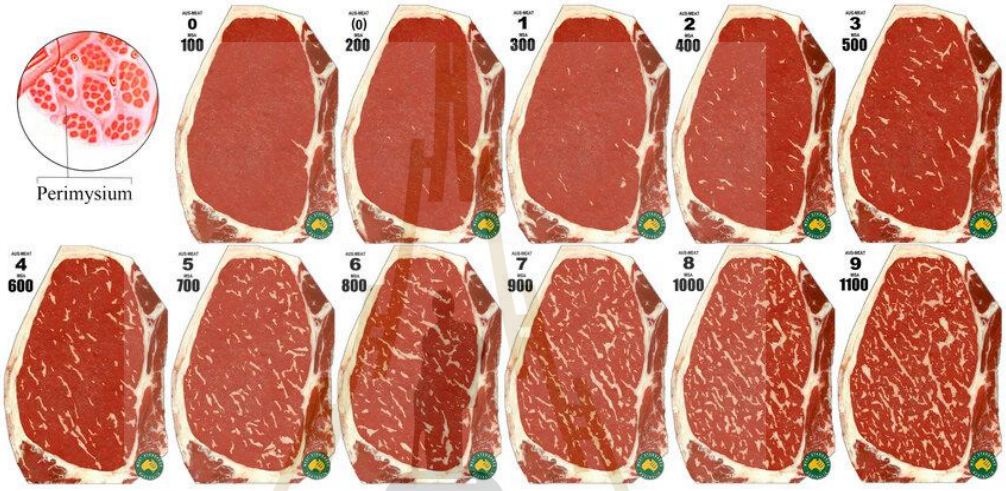

Figure 2.18 AUS-MEAT marbling score standard of Australia.
Source: Livestock Australia (MLA).

2.4 Genetics of meat marbling

2.4.1 Thyroglobulin

Thyroglobulin (*TG*) gene encodes the thyroglobulin protein. This thyroglobulin protein associated with the production of Thyroid hormone (Belew et al., 2003). The Thyroid hormone is important to the metabolism of fat deposition in adipose tissue, and the development of fatty acid content in the muscles.

In 1993 a single nucleotide polymorphism (SNP) in the ‘5 leader sequence of the *TG* gene was reported by Barendse et al. (1993) that it showed significant correlation with meat marbling score (Barendse et al., 1993). The SNP of *TG* gene was used as a marker to be evaluated association of meat marbling after 250-day feed. In 2004 the research reported that this SNP is associated with meat marbling scores ($P<0.05$) in 1750 cattle (Barendse et al., 2004). Therefore, the SNP of *TG* gene has been used for meat quality marker in beef cattle since then.

Shin et al. (2007) studied the correlation between meat quality and single nucleotide polymorphism (SNPs) of *TG* gene in Korean cattle. The *TG* gene association with Thyroid hormones determined fat metabolism to effect intramuscular fat in muscles. The experiment studies 3 SNPs positions: C257T, A335G and C422T of the 5′ promoter region of the *TG* gene (Figure 2.19). These three genotypes were determined by PCR-RFLP method using *MflI* restriction enzyme digestion to identify genotypes (Figure 2.20). The SNP position C422T showed significant association between genotypes and meat marbling score ($P<0.05$). The TT genotype of SNP position C422T showed higher marbling score when compare to CT and CC genotype. The marker of SNP *TG* gene is a useful meat quality gene for selection breeding program (Shin et al., 2007) of Korean cattle.

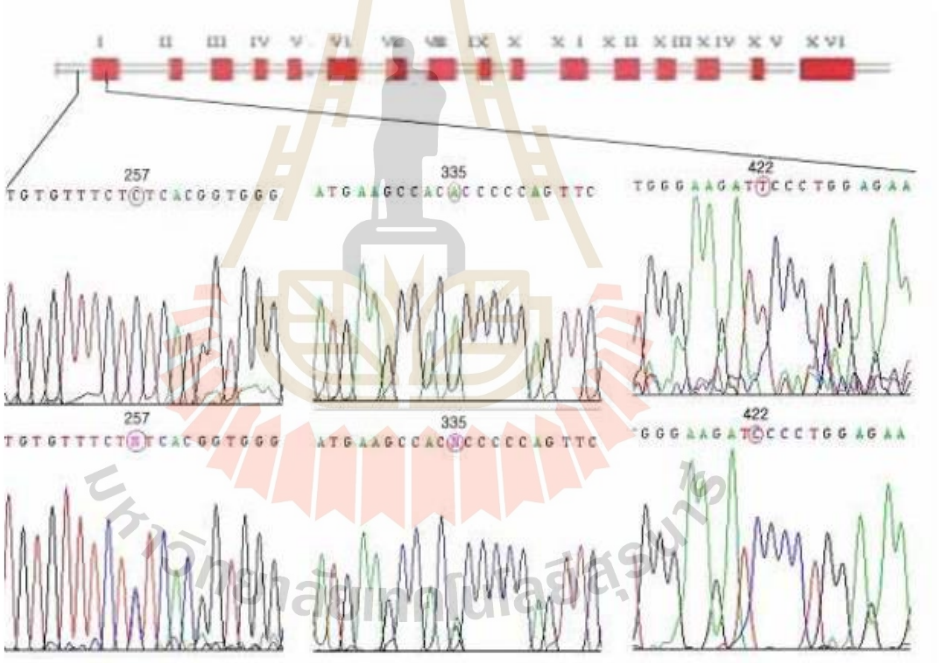

Figure 2.19 Identification SNPs positions C257T, A335G and C422T of 5′ promoter region in the TG gene.

Source: Shin et al., 2007.

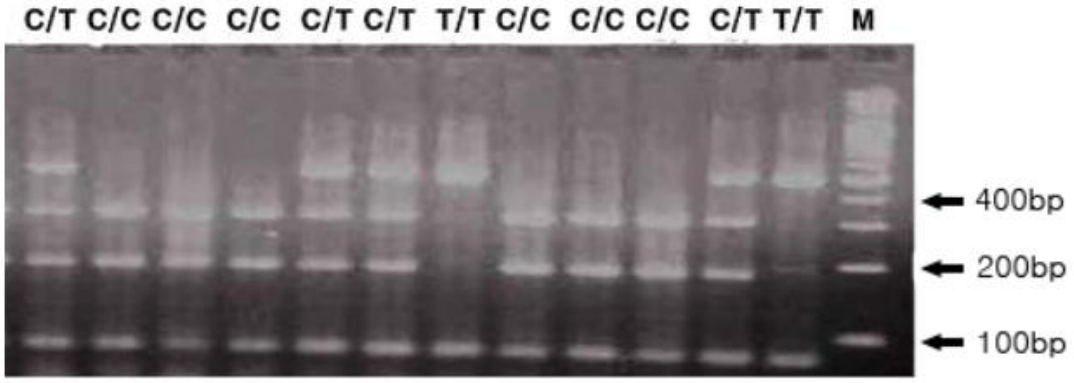

Figure 2.20 PCR-RFLP method using *MflI* restriction enzyme digestion to identify genotypes for SNP C422T of *TG* gene on agarose gel 495 bp, 198 bp and 75 bp indicated homozygous (T/T), those with 300 bp, 198 bp and 75 bp products indicated homozygous (C/C) and the ones with three products of 495 bp, 300pb, 198 bp, and 75 bp are heterozygous (C/T).

Source: Shin et al., 2007.

The positional of *TG* gene was reported to affect fat distribution in muscles of meat cattle, therefore it is candidate gene for identifying meat quality gene (Gan et al., 2008). Previous research (Figure 2.21) showed identification of 6 SNPs of *TG* gene at the position 3' flanking region in Simmental, *Angus*, Hereford, Charolais, Limousin, Qinchuan, Luxi, and Jinnan breed a total of 271 animals. The SNPs positions G133C, G156A, C220T and A506C showed significant relationship with meat marbling score (*P<0.05*). In this research suggests the *TG* gene may be used as the meat marbling marker to identify beef cattle for feeding and crossbreed selection program (Gan et al., 2008).

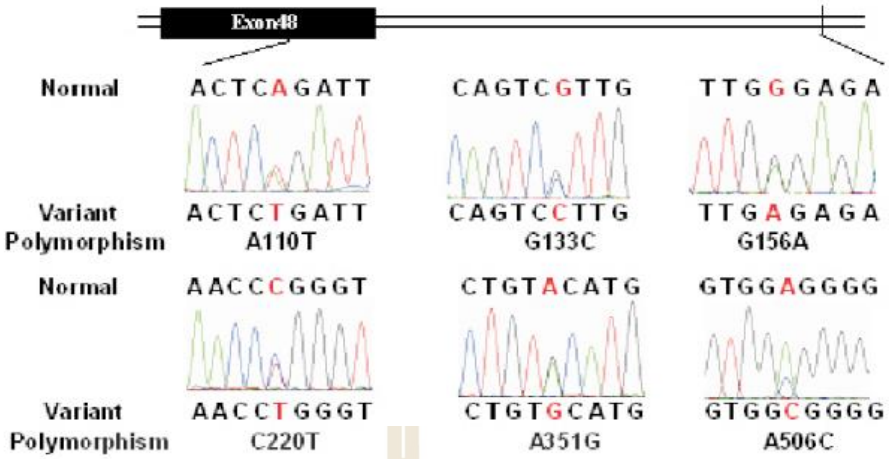

Figure 2.21 Identification of 6 SNPs of TG gene at the position 3' flanking region in cattle.

Source: Gan et al., 2008.

Sedykh et al. (2016) studied the SNPs of *TG5* gene followed Shin et al. (2007). They tested Hereford and Limousine breeds. This study the *TG5* gene was reported the population of cattle have high T allele frequency and significant (*P<0.01*) rates of total carcass body fat, the amount of adipose tissue in the carcass composition and the amount of fat in the longissimus muscles. The *TG5* gene produce protein that is a hormone to produce adipocytes cell and involved of lipid metabolism. Therefore, the results of this research on the SNP of *TG5* gene in population of Hereford and Limousine breeds were association with composition meat quality, they can be used as genetic marker to improve breed of meat cattle (Sedykh et al., 2016).

Moreover, an analysis conducted by Dolmatova et al. (2020) on the SNP position C422T of *TG5* gene according by Shin et al. (2007) to evaluate genotype association with meat quality in dairy cattle (Hereford and Limousine breeds) reported that the TT genotype of C422T SNP shown significant (*P<0.05*) percent of fat yield in the longissimus muscles, percent of fat content in the rib eye and percent of fat content in the general samples of ground beef when compare with the CT and CC genotype (Figure 2.22). Moreover, the TT genotype effected higher yield of milk and fat content in milk of dairy cattle. Therefore, the marker of *TG5* gene can be used to improve good genetic of both dairy cattle and beef characteristics (Dolmatova et al., 2020).

Indicator	Genotype		
	$TG5^{CC}$ (n=20)	$TG5^{CT}$ (n=20)	$TG5^{TT}$ (n=10)
Hereford breed (n=50)			
Bodyweight of newborn calves, kg	33.5±0.28	33.1±0.35	33.0±0.42
Gained bodyweight, kg	576.25±5.74	569.6±5.42	564.7±6.18
Absolute body gain, kg	542.8±5.22	536.5±6.36	531.7±6.33
Average daily gain of bodyweight, g	892.8±8.59	882.4±10.46	874.5±10.97
Before slaughter bodyweight, kg	559.9±5.80	552.4±3.06	549.4±5.20
Carcass yield, %	58.8±0.53	58.7±0.42	58.4±0.60
Internal raw fat weight, kg	19.09±0.51	19.15±0.32	20.09±0.30
Fat yield, %	3.40±0.09	3.40±0.05	3.65±0.07*
Slaughter yield, %	62.20±0.24	62.10±0.27	62.00±0.18
The fat content of the rib eye,%	5.70±0.21	6.05±0.90	6.44±0.15*
Fat content in the general sample of ground beef, %	14.38±0.10	14.54±0.08	14.69±0.06*
Limousine breed (n=50)			
Bodyweight of newborn calves, kg	33.9±0.30	34.2±0.27	34.9±0.23
Gained bodyweight, kg	604.1±3.99	598.1±2.16	581.4±6.60
Absolute bodyweight gain, kg	570.2±4.89	563.9±3.07	546.5±4.47
Average daily bodyweight gain, g	937.8±8.05	927.5±5.05	898.9±7.34
Before slaughter bodyweight, kg	584.0±6.16	577.4±0.44	567.0±4.43
Carcass yield, %	60.0±0.62	59.9±0.96	59.9±0.78
Internal raw fat weight, kg	17.30±0.18	17.80±0.17	18.00±0.33
Fat yield, %	2.95±0.01	3.06±0.14	3.19±0.02*
Slaughter yield, %	62.90±0.11	63.10±0.12	63.10±0.22
The fat content of the rib eye,%	5.60±0.28	5.95±0.28	6.55±0.16*
Fat content in the general sample of ground beef, %	13.16±0.05	13.46±0.03	13.65±0.26*

Figure 2.22 Indicator quality of meat association with genotype of *TG5* gene significance of differences according to Student's t test *$P<0.05$.

Source: Dolmatova et al., 2020.

2.4.2 Fatty acid binding protein 4

The fatty acid-binding protein 4 encoded from the *FABP4* gene involves in lipid hydrolysis and trafficking of intracellular fatty acid in tissues. *FABP4* is essential to produced energy stored in the body. Fatty acids is an important signal molecules for synthesized preadipocyte from in the liver (Chmurzynska et al., 2006; Barton et al., 2015).

Shin et al. (2012) identified 3 SNPs in the *FABP4* gene located in intron 1 C2834G, intron 2 T3533A and in exon 3 G3691A in a total number of 804 Hanwoo breed in Korean. The SNPs locations are shown in Figure 2.23. The genotypes of *FABP4* gene were analyzed by PCR–RFLP method, The GG genotype of SNP G3691A were significantly correlated with marbling score and meat color when compare GA and AA genotype (Figure 2.24). This SNP of *FABP4* gene can be a candidate to identify meat quality. The animal with the G allele can be use in the breeding selection program. The result of this study is similar to the previous research of Michal et al. (2006) which reported that the SNP G3691A of *FABP4* gene associated with marbling scores and subcutaneous fat in Wagyu crossbreed Limousin of F2 generation (Michal et al., 2006). Therefore, the G3691A SNPs in the *FABP4* gene should be useful genetic markers for selection quality of beef cattle.

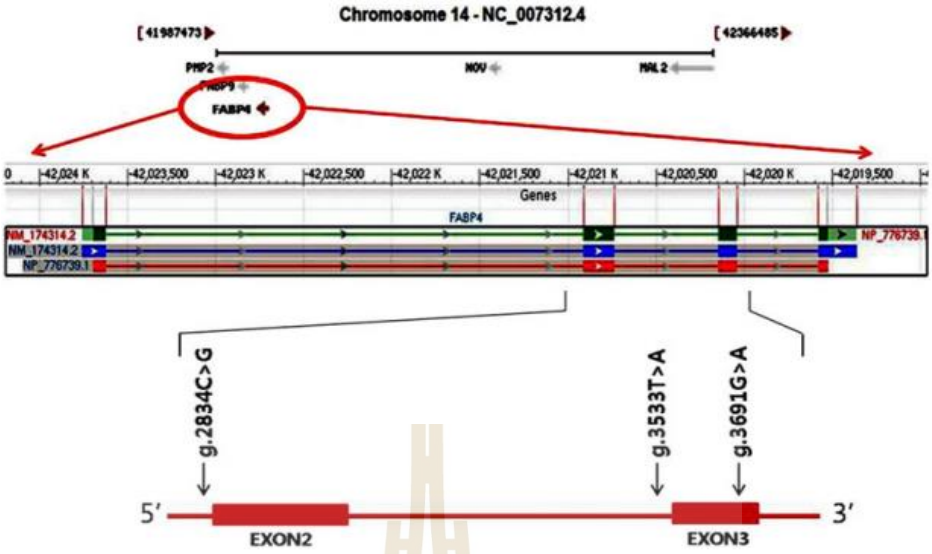

Figure 2.23 SNPs locations C2834G, T3533A and G3691A of *FABP4* gene.

Source: Shin et al., 2012.

Traits	*FABP4* g.3691G>A SNP			*P* value
	GG ($n = 364$)	GA ($n = 203$)	AA ($n = 19$)	
MS	6.131 ± 0.06^{a}	5.767 ± 0.12^{ab}	5.473 ± 0.24^{b}	0.024
MG	2.840 ± 0.03^{a}	2.643 ± 0.06^{ab}	2.526 ± 0.12^{b}	0.019
BF	11.708 ± 0.16	12.452 ± 0.27	12.421 ± 0.89	0.094
EMA	90.123 ± 0.41	89.811 ± 0.67	91.157 ± 1.37	0.819
CW	424.274 ± 2.42	430.094 ± 2.89	443.473 ± 8.49	0.102
MI	65.626 ± 0.15	64.978 ± 0.19	64.853 ± 0.69	0.053

Figure 2.24 Meat quality traits association with genotypes of SNP G3691A in *FABP4* gene MS marbling score, MC meat color, FC fat color, TX meat texture, MA maturity score, MG meat quality grade (The grades were 1–5), BF backfat thickness, EMA, M. Longissimus dorsi area, CW carcass weight, MI meat quantity index, and MQ meat quantity grade. [a, b] Within a row, means with different superscript letter differ (P<0.05) *Effect was significant at P<0.05.

Source: Shin et al., 2012.

In 2017 SNP of *FABP4* gene was study in 400 Holstein breed at the age of 14-21 months. In this study, they reported that GG genotype of SNP position G3691A in exon 3 correlated with significantly (*P<0.05*) highest marbling score and first compressive stress (FCS) of *longissimus dorsi* muscle (Figure 2.25). The animal with G allele has high impact to meat quality more than A allele. A similar result by Shin et al. (2012) showed that the frequency of G allele shown to be associated with meat quality. the results of this study were similar to Shin et al. (2012). They suggested that the G3691A SNP of *FABP4* gene can be use as molecular marker for breeding program (Ardicli et al., 2017).

Genotype	N	LW (kg)	BFT (mm)	MLD (cm^2)	MS (1–9)
3691 G>A					
AA	16	481.0	3.19	94.92	1.59 c
GA	183	498.9	2.98	100.37	2.22 b
GG	201	479.7	2.83	100.68	3.19 a
		NS	NS	NS	P<0.05

Figure 2.25 SNP of *FABP4* gene position G3691A in exon 3 to effect meat quality N: number of animals; LW: live weight; BFT: backfat thickness; MLD: *Musculus longissimus dorsi area*; MS: marbling score; PSE: pooled standard error; NS: non significant. a, b, c - different letters within a column indicate significant difference (P<0.05).

Source: Ardicli et al., 2017.

The corresponding SNP position G3691A of *FABP4* gene research by Yin et al. (2019) studied the variations SNPs of *FABP4* gene that effects the meat quality in Yanbian yellow breed. This research reported that the SNP1 or SNP G3691A was not association with meat marbling, but significantly association with fat content, proteins content and moisture content. These different results when compare to other researches may be due to different background of genetic and difference of size sample and may be the feeding program and environmental factors. However, the SNP1 or SNP G3691A of *FABP4* gene could be a good candidate gene for developed breed of beef cattle (Figure 2.26) (Yin et al., 2019).

Site	Moisture (%)	Fat (%)	Protein (%)	Backfat thickness (cm)	Marbling (grade)
SNP1					
GG	56.67±0.69[a]	13.14±1.65[b]	21.60±1.44[a]	1.30±0.13	3.00±0.71
GA	53.00±2.52[b]	26.00±6.11[a]	15.00±1.15[b]	1.50±0.50	3.00±0.58
AA	53.00±0.58[b]	14.00±2.08[b]	20.67±1.76[a]	1.60±0.40	2.50±0.76

Figure 2.26 Association between SNP1 or SNP G3691A of FABP4 gene and meat traits in Yanbian yellow cattle.

Source: Yin et al., 2019.

2.4.3 Diacylglycerol acetyltransferase

Diacylglycerol acetyltransferase (*DGAT1*) is an important core microsomal enzyme. The function of DGAT1 is to hydrolyze diacylglycerol fatty acid to synthesis of triglyceride fatty acid in the terminal step. It also involved to directly effects fat content in intramuscular fat and milk (Cases et al., 1998; Grisart et al., 2002). In addition, the *DGAT1* gene transcription does not found in only cattle but also in other mammalian species (Humphries et al., 2002). The SNPs located at A10433G and A10434C have been known to associate with meat marbling in *longissimus dorsi* muscle were analyzed by Humphries et al., 2002. The study in Holstein and Charolais breeds of 18 months old showed similar results with Barendse et al. (2009) that the data of fat content in intramuscular muscles correlated with SNPs of *DGAT1* gene. The possibility *DGAT1* gene to be used as candidate gene for meat quality.

Recently, the SNPs located A10433G and A10434C *DGAT1* gene were analyzed in Angus cattle breed in Hungary and after slaughter, carcass physiology characteristics data were recorded (Anton et al., 2012). In this study, they used PCR-RFLP method to check the genotypes. The AA/AA genotype shown to have highest meat marbling score and percent of fat content in *musculus longissimus dorsi (LD)* and *musculus semitendinosus (ST)* when compare AA/GC and GC/GC genotype (Figure 2.27). The cattle with AA allele increase the fat content production in the body more than other allele. The results similar to Thaller et al. (2003) in Holstein and Charolais breeds. The SNPs of *DGAT1* gene can be use as marker assisted selection (MAS) for beef cattle.

Loci	Genotypes	Fat % LD	Fat % ST
Leptin ($n=173$)	CC ($n=97$)	14.43 ± 0.90	8.88 ± 0.51^{a}
	TC ($n=66$)	14.41 ± 0.95	8.62 ± 0.53^{a}
	TT ($n=10$)	15.45 ± 1.25	12.52 ± 0.92^{b}
	Variance (%)#	0	0.1
	Additive effect	0.51	1.82*
DGAT1 ($n=173$)	AA/AA ($n=9$)	18.08 ± 2.16^{a}	12.06 ± 1.2^{a}
	AA/GC ($n=48$)	13.33 ± 1.4^{b}	8.91 ± 0.79^{b}
	GC/GC ($n=116$)	12.87 ± 1.2^{b}	9.04 ± 0.68^{b}
	Variance (%)#	7.1	7.0
	Additive effect	2.61*	1.51*
	Dominance	2.15*	1.64*

Figure 2.27 Percent of fat content in *LD* and *ST* of in Angus bulls.
Source: Anton et al., 2012.

Li et al. (2013) showed the correlation of SNPs in *SCD1*, *CAST*, *leptin*, *CAPN1*, and *DGAT1* genes with carcass physiology characteristics in Angus, Charolais, Hereford, Limousin, and Simmental breeds. The physiology characteristics data were recorded after 7 days of post mortem at 4 °C. The present results SNPs of *DGAT1* and *CAPN1* gene (Figure 2.28) were correlated with intramuscular fat content and to effected high marbling score. For genotypes of *DGAT1* gene AA/GC and AA/AA shown higher of intramuscular fat content when compare with GC/GC genotype. The corresponding Anton et al. (2011) the AA/AA genotype effect to among of fat content in beef cattle because the *DGAT1* gene function catalyzes to synthesis triglyceride fatty acid.

Traits	Markers					Breed
	DGAT1	*LEP*	*SCD1*	*CAPN1*	*CAST*	
Color (n=229)						
Day 0 in air						
L*	0.501	0.176	0.924	0.282	0.553	**0.002**
a*	0.989	0.670	0.103	0.368	0.347	0.258
b*	0.332	0.543	0.190	0.664	0.277	0.752
Chroma	0.654	0.564	0.103	0.558	0.283	0.617
Hue angle	0.441	0.543	0.612	0.085	0.519	**0.012**
DeoxyMb	0.813	0.550	0.396	0.266	0.446	0.900
OxyMb	0.767	0.553	0.084	0.493	0.362	0.297
MetMb	0.385	0.156	0.742	0.090	0.573	0.662
Day 6 in air						
L*	0.213	0.169	0.964	0.693	0.261	0.065
a*	0.751	0.053	**0.003**	0.150	0.606	0.621
b*	0.612	0.085	**0.001**	0.876	0.439	0.973
Chroma	0.991	**0.040**	**0.001**	0.536	0.541	0.860
Hue angle	0.392	0.485	0.334	**0.006**	0.680	0.350
DeoxyMb	0.743	**0.027**	0.498	0.343	0.501	0.225
OxyMb	0.867	0.054	**0.002**	0.357	0.490	0.454
MetMb	0.577	0.154	0.094	0.897	0.760	0.715
Marbling (n=229)						
IMF (%)	**0.017**	0.460	0.942	**0.043**	0.104	**0.039**
Marbling score	**0.021**	0.404	0.561	**0.028**	0.516	0.692
Water holding capacity (n=217)						
Freezing loss (%)	0.099	0.270	0.157	0.594	0.391	0.063
Cooking loss (%)	0.419	0.671	0.972	0.793	0.911	0.073
Total loss (%)	0.734	0.950	0.856	0.169	0.969	**0.015**
pH (n=229)	0.520	0.278	0.479	0.438	0.416	0.565

Figure 2.28 The SNPs of *SCD1*, *CAST*, *leptin*, *CAPN1*, and *DGAT1* and animal physiology characteristics IMF (%) = intramuscular fat content, *P*-values lower than 0.05 are in bold.

Source: Li et al., 2013.

DGAT1 gene has been reported to be associated in cattle, goat, buffalo, and sheep on chromosomes 14, 15, and 9 respectively. Khan et al. (2020) studied the milk yield and meat quality to correlate with *DGAT1* gene SNPs A10433G and A10434C (Figure 2.29) in Cattle, Buffalo, Goat, and Sheep. Presented results SNPs of *DGAT1* to effect on meat quality in cattle and milk yield to effect on cattle, goat, buffalo, and sheep. The future possible can be use genetic marker of *DGAT1* gene to improve good genetics in several animal species (Khan et al., 2020). Similarly, a study reported the *DGAT1* gene in this SNPs correlated with marbling score and intramuscular fat content in Simmental breed of Chinese (Wu et al., 2012).

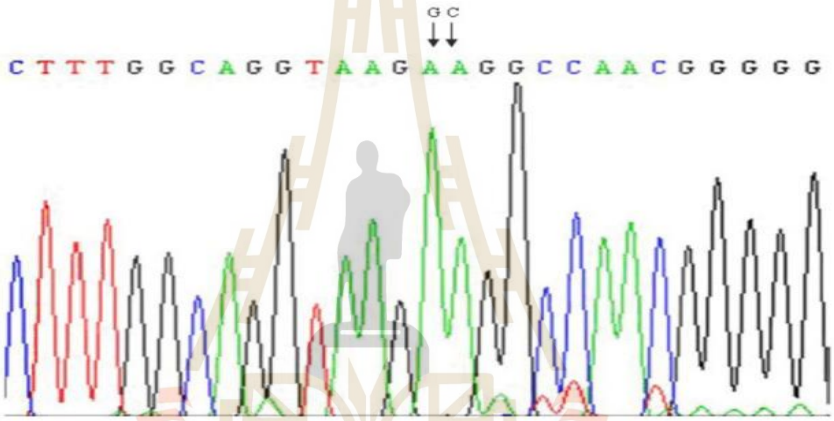

Figure 2.29 The DNA sequencing SNPs positions A10433G and A10434C in exon 8 of *DGAT1* gene in dairy cattle.

Source: Khan et al., 2020.

2.5 Fat compositions in cattle

Meat quality is an important aspect of consumer. The fatty acids composition contributes to adipose tissue and muscle in beef cattle. Fatty acids composition in cattle contains several different types of fatty acids: Polyunsaturated Fatty Acids (PUFA), Monounsaturated Fatty Acid (MUFA) and Saturated fatty acids (SFA). The PUFA are fatty acids that contain more than one double bond structure in the constituent hydrocarbon chain possesses two or more carbon. PUFA can be found many in nuts, fish, seed oils, and meat. The fatty acids of PUFA such as linoleic acid, **α**-linolenic acid (n-3), **γ**-linolenic acid (n-6), arachidonic acid, and so on contain many important compounds such as essential fatty acids (Wu et al., 2017).

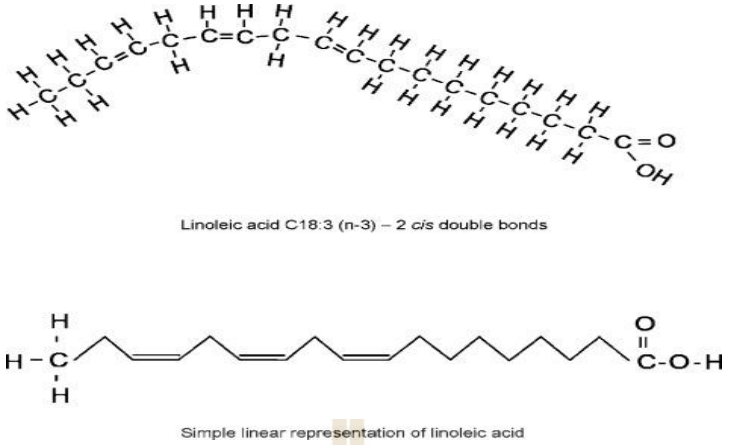

Figure 2.30 Structure of PUFA.
Source: Minihane et al., 2006.

The MUFA structure have a single double bond and have two hydrogen bonds and two carbon bonds in their connected structure by a double bond. The fatty acids of MUFA such as palmitic, oleic, elaidic, vacentic acids and so on contain many oleic acid. MUFA can be found in many sources of oil, canola, peanut, sunflower, corn, soybean and safflower oil, In addition MUFA found that antioxidants content (Nicklas et al., 2004).

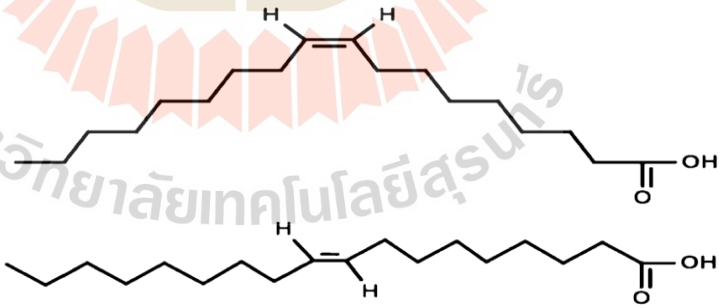

Figure 2.31 Structure of MUFA.
Source: Joris et al., 2016.

The SFA the structure has hydrocarbon chain connected carboxylic head group. The hydrocarbon chain do not contains double bonds. SFA structure can be linear and branched. SFA can be found many in butter, cheese, fat of meat, meat products (e.g., sausages, hamburgers) and raw milk (Eshak et al., 2018).

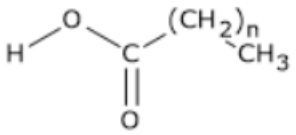

Figure 2.32 Structure of SFA.

Source: https://www.tuscany-diet.net/lipids/fatty-acids/saturated-fatty-acids/.

Garmyn et al. (2011) studied fatty acids compositions fatty acids composition of 1,737 Angus breed. The results showed that in fatty acids composition in Angus breed contained linoleic acid, linolenic acid, arachidonic acid, and PUFA. These fatty acids associated with the strong juiciness with negative correlation ($P < 0.05$). But the MUFA, SFA and PUFA have positive correlation with beef flavors (Garmyn et al., 2011).

The fatty acids in beef is mostly in SFA and MUFA form. The most abundant fatty acids in meat is oleic acid. The fatty acids compositions (Zembayashi et al., 1996). In Wagyu cattle breed the fatty acids compositions fatty acid compositions composed of highest MUFA content when compare with other breeds, similar Hanwoo breed has high fatty acids compositions MUFA (Yang et al., 1999).

Smith et al. (2012) monitored the concentrations of fatty acids in the meat marbling or intramuscular adipose tissue and *longissimus* muscle, in U.S. crossbred cattle and Japanese Black cattle. The results showed that Japanese Black cattle shown higher oleic acid (18:1) when compare with U.S. crossbred cattle (Figure 2.18). The oleic acid (18:1) is the MUFA can be to a lower fat-melting point which helps to up the juiciness and good flavor in meat of Japanese Black cattle.

The content of MUFA can reduce the level concentration of LDL or cholesterol in human (Melton et al., 1982; Smith, 1994). Therefore, fatty acids compositions of beef cattle is important to the beef industry, particularly the meat with high marbling such as Wagyu and Hanwoo breeds.

Zembayashi et al. (1995) have determined the effect and type of cattle and sex on fatty acids compositions fatty acids composition of subcutaneous fat and intramuscular muscle fat of fullblood Japanese black Wagyu, fullblood Japanese brown Wagyu, Holstein and Charolais crossbred. The results showed that the

fullblood Japanese black Wagyu genetics predisposed to increased producing levels of lipids in the body, especially MUFA more than fullblood Japanese brown, Holstein or Charolais crossbred (Zembayashi et al., 1995).

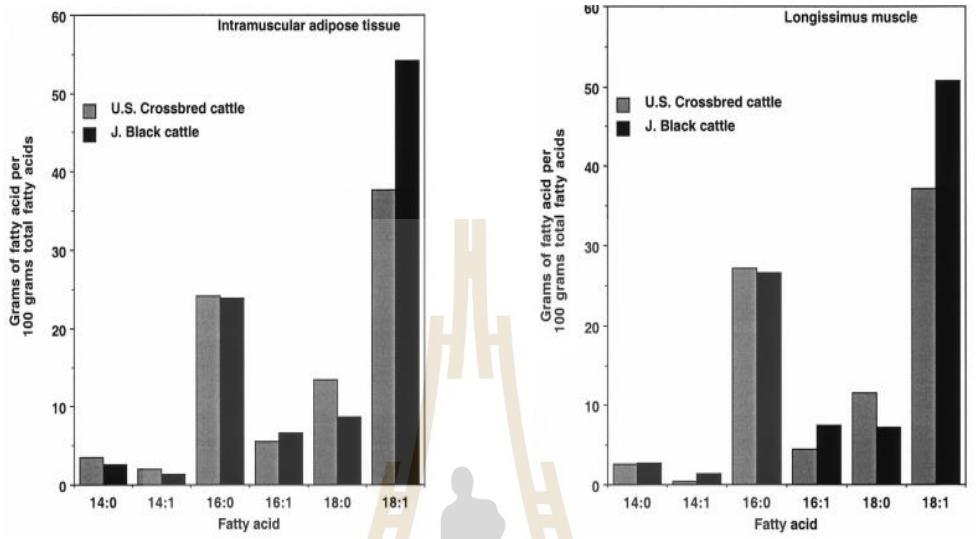

Figure 2.33 Content types of fatty acids in marbling or intramuscular adipose tissue and *longissimus muscle* of U.S. crossbred cattle and Japanese Black cattle.

Source: Smith et al., 2012.

Sturdivant et al. (1992) summarized that fullblood Wagyu cattle fed in Japan is rich in MUFA content. Gotoh et al. (2011) compared fatty acids compositions fatty acid composition content in *longissimus* muscle in Japanese Black cattle and Holstein cattle of 26 month of age feed by using a standard feeding system. The percentage of MUFA such as C16:1, C18:1, and C20:1 content in *longissimus* muscle in Japanese Black cattle breed are higher than the Holstein cattle breed. But the percentage of SFA is C18:0 Holstein cattle breed is higher than in Wagyu. Therefore, the Japanese Black cattle breed has been greater good fatty acids (Gotoh et al., 2014).

Fatty acid	Wagyu (n=6)	Holstein (n=5)	*p*-value[1)]
IMF(%)[2)]	32.066±2.805	17.34±2.864	<0.01
12:0	0.052±0.004	0.037±0.004	<0.05
14:0	2.840±0.172	2.726±0.282	n.s.
14:1	0.848±0.103	0.798±0.121	n.s.
15:0	0.402±0.037	0.351±0.039	n.s.
15:1	0.025±0.002	0.024±0.002	n.s.
16:0	26.144±0.546	28.009±0.661	0.055
16:1	4.069±0.225	3.833±0.162	n.s.
17:0	1.037±0.087	1.004±0.117	n.s.
17:1	0.986±0.082	0.825±0.114	n.s.
18:0	10.484±0.266	12.267±0.516	<0.05
18:1	50.040±0.911	47.465±0.980	n.s.
18:2 n-6	2.116±0.911	1.926±0.129	n.s.
18:3 n-3	0.121±0.036	0.175±0.062	n.s.
CLA 9c, 11t	0.302±0.031	0.260±0.015	n.s.
20:0	0.071±0.003	0.128±0.027	0.051
20:1	0.457±0.046	0.164±0.028	<0.001
ΣSFA[3)]	41.033±0.562	44.524±0.842	<0.01
ΣMUFA[3)]	56.472±0.704	53.112±0.853	<0.05
ΣPUFA[3)]	2.539±0.225	2.363±0.094	n.s.

Figure 2.34 Comparison of fatty acids compositions content in *longissimus* muscle in Japanese Black cattle breed and Holstein cattle breed Values are expressed as mean (%)±S.E. [1)]Student's t-test. [2)]IMF: intramuscular fat. [3)]SFA: saturated fatty acids, MUFA: monosaturated fatty acids, PUFA: polysaturated fatty acids

Source: Gotoh et al., 2011.

Cho et al. (2005) have determined the fatty acids compositions in in *Longissimus muscle* of Hanwoo and Angus breeds. The results shown that significant difference of fatty acids compositions content between both cattle (Table 2.13). The Angus breed has higher n-3 PUFA content while Hanwoo breed had higher n-6 PUFA content in *longissimus muscle* (Cho et al., 2005). The difference types of feeding also effected the fatty acids compositions content in beef cattle because microorganism in rumen hydrogenation (Enser et al., 1998).

Stearoyl-CoA desaturase (SCD) or *SCD* gene was the first gene to be reported that is associated with fatty acids compositions content in beef cattle (Taniguchi et al., 2004). The *SCD* gene catalyzes the enzyme for changing SFA to MUFA in adipocytes tissue of Mammalia (Ntambi et al., 1999).

Fatty acid	Australian Angus	Hanwoo	RSD[a]	Breed F statistic and significance
C14:0	2.56	3.00	0.35	78.24[c]***
C16:0	29.79	28.21	1.85	97.30***
C16:1(n7)	2.70	3.94	1.37	88.19***
C18:0	14.16	9.00	0.89	2180.64***
C18:1(n9)	47.62	52.14	2.26	16.03***
C18:1(n7)	0.24	0.84	1.17	103.40***
C18:2(n6)	1.80	2.11	1.07	260.63***
C18:3(n6)	0.01	0.00	0.06	8.07***
C18:3(n3)	0.21	0.08	0.03	1576.1***
C20:1(n9)	0.24	0.32	0.12	55.49***
C20:2(n6)	0.00	0.01	0.02	29.51***
C20:3(n6)	0.15	0.11	0.11	3.51***
C20:4(n6)	0.37	0.25	0.35	10.57***
C20:5(n3)	0.06	0.00	0.05	461.57***
C22:4(n6)	0.00	0.00	0.05	40.75***
C22:5(n3)	0.08	0.00	0.09	324.92***
SFA[b]	46.51	40.20	2.27	486.32***
USFA[b]	53.49	59.79	2.27	486.14***
MUFA[b]	50.80	57.3	2.32	224.02***
PUFA[b]	2.69	2.56	1.51	102.72***
n3	0.35	0.08	0.15	637.65***
n6	2.34	2.48	1.43	178.91***
n6:n3	7.60	30.79	8.56	1695.1***
MUFA:SFA	1.10	1.44	0.15	321.69***
PUFA:SFA	0.16	0.06	0.04	153.69***

Figure 2.35 Comparison of fatty acids compositions content in *longissimus muscle* in Hanwoo and Angus breeds [a]RSD: residual standard deviation. [b]SFA: saturated fatty acids, USFA: unsaturated fatty acids, MUFA: monosaturated fatty acids, PUFA:polysaturated fatty acids. [c]F-ratio statistic: * if $p<0.05$, ** if $p<0.01$, *** if $p<0.001$.

Source: Cho et al. (2005).

Yang et al. (1999b) reported the relationship of *SCD* gene that effect the catalyzed *SCD* enzyme and fatty acids compositions in adipose tissue of Wagyu cattle. Although the fatty acids synthesis mechanism is very sophisticated, many genes have been specified and verifies to correlate with the functions for synthesis of fatty acids in beef cattle (Gotoh et al., 2014). The content of oleic acid in beef cattle associate with expression of *SCD* gene (Smith et al., 2012). Similar reported with Nogi et al. (2011) the Wagyu cattle have high oleic acid content. The feeding conditions have affected the increased or decreased of oleic acid content. Higher concentration of feeding can also produce higher MUFA content in the *longissimus* muscle of beef cattle.

Barton et al. (2015) have investigated significant the functions of *FABP4* gene to involve metabolism of fatty acids. The SNP 220A>G of *FABP4* gene with palmitic acid and oleic acid in the adipocyte tissue of Japanese black Wagyu cattle crossbreeds. The same SNP position the I/I genotype shown has an increase percentage of C16:1 when compare the I/V genotype and V/V genotype ($P<0.05$) (Chmurzynska et al., 2006). Moreover, Narukami et al. (2011) have investigated the same SNP position in Holstein cattle. The result presented the V/V genotype shown increase percentage of C16:0 when compare the I/V genotype and I/I genotype (Narukami et al., 2011).

Moreover, Yan et al. (2012) have determined that SNP 17924A>G of *FABP4* gene (that changed the amino acid threonine to alanine) in Canadian beef cattle changed the fatty acids compositions content. There are positive correlation SNP of *FABP4* gene with the SFA and MUFA content. Especially, the cattle with AA genotype showed higher level of SFA content but decreased percentage of oleic acid (9c-18:1). Wood et al. (2008) also showed that in Angus breed the SNP 17924A>G of *FABP4* gene, showed that GG genotype contained lower SFA, C14:0 level and C16:0 content. But the AA genotype had higher of C18:1 and MUFA content.

2.6 Polymerase chain reaction (PCR)

The formation of polymerase chain reaction (PCR) radically changed biotechnology and medical science from the first time experienced (Mullis, 1990). The PCR technique allows to determine and produce many DNA templates. The PCR strategically have motivated biggest scientific findings such as Human genome analysis. This technique is popularly used in clinical and research work for DNA. The PCR technique is an important application for disease detection. Moreover, the PCR can be used to identify genetics of animal for breed improvement program. This technique is popular worldwide. The understanding of basic details of PCR is necessary. It can be applied and used to analyze genome and genes of interests.

PCR technique is easily performed. The professor Mullis, this found experiment the PCR technique, recorded it “lets you select the part of DNA of interested and have as many of it as you want” (Mullis, 1990). In PCR technique, the DNA template can be amplified from several sources such as tissues, blood, skin, root hair, spit, and

microorganisms. Low amount of DNA template is needed to generate new DNA copies. For this cause, the PCR is an important, sensitive technique for DNA analysis.

In PCR technique, the components needed are: DNA template, Primers, dNTPs or free nucleotides, buffer reagent, and *Taq DNA polymerase* (Figure 2.36). The *Taq DNA polymerase* is the core enzyme that linked dNTPs or free nucleotides to generate new DNA strand. The dNTPs or free nucleotides comprised of the 4 bases: Adenine=A, Thymine=T, Cytosine=C, and Guanine=G those found in DNA strand. The primers are short DNA fragments with sequence specific for the gene of interest to combined to the complementary and help *Taq DNA polymerase* to extend the new DNA strand. The PCR components or PCR reagent of the PCR technique are mixed and added in PCR tube and in to 96 well plate and added to the thermal block of PCR machine (Figure 2.37).

Figure 2.36 Composition of PCR technique.
Source: https://goldbio.com/goldbios-pcr-overview.

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Figure 2.37 PCR machine and heat block 96 well plate.

Source: Molecular Biology and Protein Engineering F10205 (F10) School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology.

2.6.1 PCR process

First the PCR mixture are heated up to the melting point of double strands of the target DNA to separate the double strand DNA to single strand, this is call the denaturation step. Next decreased the temperature of PCR machine to permit the specific binding of the primers to the target DNA template only. This step is call the annealing step.

Finally heat to increase the temperature again so that the *Taq DNA polymerase* can be actively polymerized the new strand of DNA complementary to the template strand. This step is the extension step. Then the cycle begin again at the denaturation step by the annealing and extension steps. (Figure 2.38).

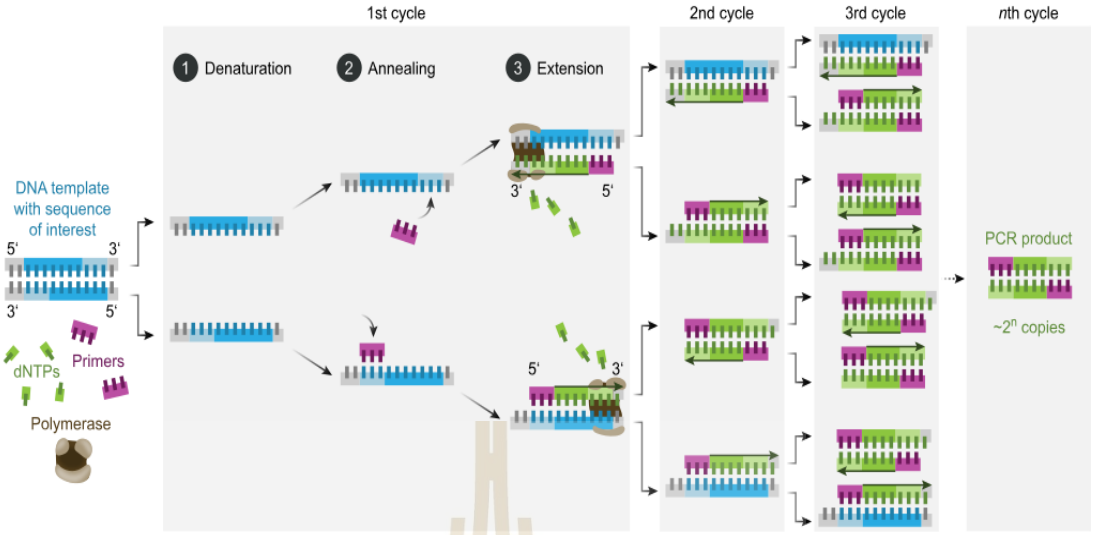

Figure 2.38 Steps of PCR reaction.

Source: https://en.wikipedia.org/wiki/Polymerase_chain_reaction.

2.6.2 Investigation of the PCR product

To detect the PCR products the PCR fragment will be separated in agarose or polyacrylamide gel electrophoresis then stained with dye or if the primers are fluorescently labeled, it can directly be detected. The electrophoresis on agarose gel is the method used worldwide for determining the PCR products, which can separate fragment of PCR products by size and charge of PCR products. In this method is the easily for image and determined the PCR products. A predetermined set of ladder or marker with known size are run simultaneous on the gel as standardized molecular markers to help monitor the fragment of PCR products.

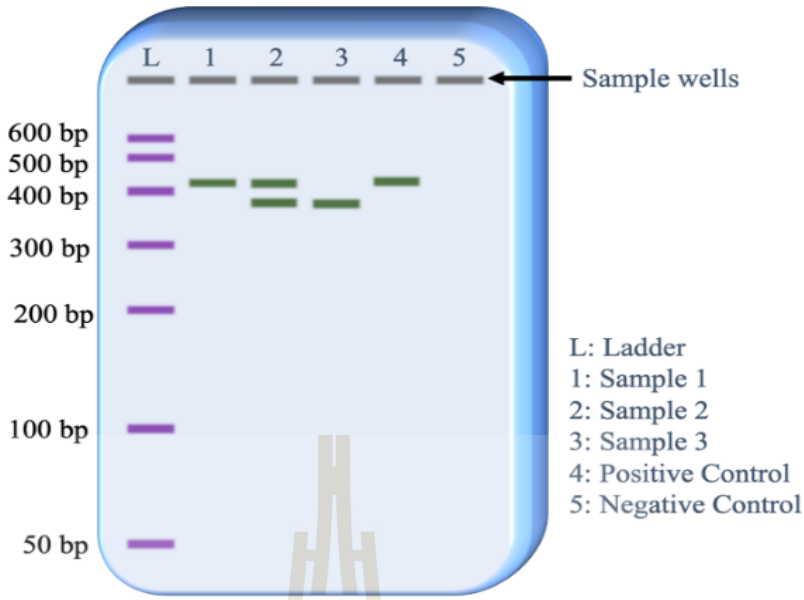

Figure 2.39 Fragment of PCR products on electrophoresis agarose gel method.
Source: https://iastate.pressbooks.pub/genagbiotech/chapter/pcr-and-gel.

2.6.3 The limitations of PCR technique

Although the PCR is a more able technique but have limitation to the experiment. PCR is very sensitive, easily contaminate by any sample which will affect to produce mistake of the results (Smith et al., 2009). In addition, the step for design primers, some previous sequence information is need and primers can be designed that might anneal non-specifically with similar but not exact sequences. Moreover, wrong nucleotides can be added into the fragment by the *Taq DNA polymerase*.

2.6.4 PCR technique for detection SNPs or mutations in meat quality genes

Nucleotide modifications are correlated to meat quality in beef cattle, susceptibility to increase or decrease meat marbling levels, and to the affected meat quality. Nucleotide modifications include nucleotide replacement, insertions, duplications, and fusions. Single-nucleotide replacement, a different nucleotide is replace with any other type of nucleotide. It is called single-nucleotide polymorphisms (SNPs) or base of nucleotides mutations. SNPs can be used as molecular markers for verification of several of cattle genotype such as opportunity has high marble, tenderness, and rate of feeding. The genetic identification can allow to selection good cattle for breeding program to develop and improve breed of beef cattle.

The SNPs mutations in several genes such as *TG5, DGAT1*, *FABP4*, *leptin*, and *SCD1* genes are implicated to be indicators for meat quality of marbling and tenderness and are correlated with predictions breeders of beef cattle. The residence of those mutations direct indicators the presence of opportunity high marble.

The summary PCR technique for detecting SNPs mutations are easy, rapidly to produce results and popular. It can be used to amplify multiple copies of DNA. It can be quantified demonstration how much of specific sequence of meat quality genes of interest. PCR will include several detections and method for produce increase insight into several gene combinations (Botes et al, 2011).

2.7 Amplification refractory mutation system PCR (ARMS-PCR)

Analyzed experiment for several kind of SNPs are real. The experiments have high sensitivity to identify the replaced single base. The amplification of sequence specific has been called amplification refractory mutation system (ARMS).

Newton et al. (1989) proposed the amplification of sequence specific or ARMS-PCR technique to be used to identify SNPs. The details of mismatch positions at the 3'end for primers were design to decrease amplified mistake and amplify only the PCR products that is allele specific only. This technique is mostly essential for analyzed SNPs, and their study has been afterwards referenced by several research purposes to implemented analysis of allele specific amplification or mutations amplification.

In1992, ARMS-PCR technique was reported by Ye et al. (1992) this investigates the known replacement of a single base nucleotide by the fragment of PCR products on gel electrophoresis, which no needed real-time PCR machine (Figure 2.40).

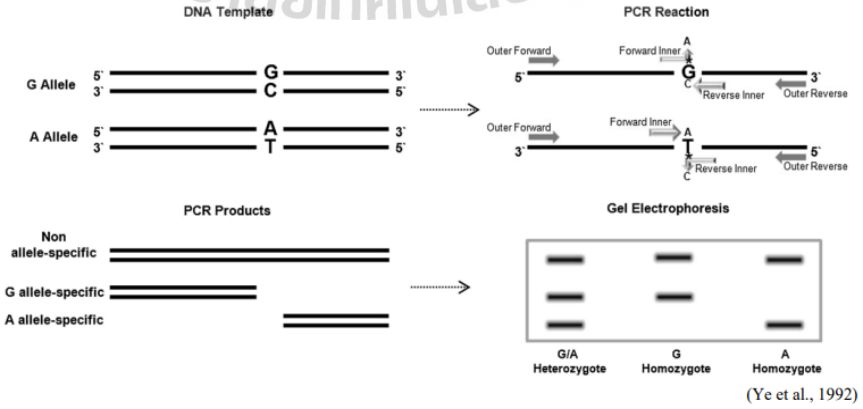

Figure 2.40 The conception of ARMS–PCR reaction for detected SNP.

Source: Ye et al., 1992.

The ARMS-PCR technique is an easy and popular technique used in general laboratories. The technique has been used for analyzing *CAPN1* and *CAST* gene mutation, to identify genetic mutation in beef cattle for breeder selection of good genetic (Rincon et al., 2006). The ARMS-PCR technique is also dependent on DNA quality or suitable of primer concentration and needed preliminary condition of optimization of PCR. For optimization is very hard for SNP of interest with high GC content, and several methods should be attempts in certain situation (Merdrano et al., 2014).

Little et al. (2001) presents the ideal that mismatch options for ARMS-PCR primers can be designed (Figure 2.41). The locations of the suggestion of the consider mismatch can be the terminal sequence or the third base from the 3' end of sequence primers. Much research presented the PCR performance dependents on the locations and type of nucleotide base in each pair of mismatches (Ye et al., 2001; Liu et al., 2012).

	Coding Strand Nucleotide Corresponding to Penultimate Nucleotide in the Primer			
Terminal Mismatch	**A**	**G**	**C**	**T**
AA	A	G	A	G
AG	C	T	A	G
AC	G	A	C	T
TT	C	T	A	G
TG	G	A	T	C or T
TC	C	T	A	G
CC	C	T	A	G
GG	A	G	A	G

Figure 2.41 Mismatch options of ARMS-PCR.
Source: Little et al., 2001.

In 2011, Gábor et al. (2011) improved the ARMS-PCR technique for the analysis of SNP C4685T of *CAPN* gene in a total of 130 cattle population. This *CAPN* gene SNP involve in the enzyme calcium system such as proteinase group which is the core proteolysis structure of myofibrillar proteins between carcass ageing storage. The primers were designed by Tetra-Primer ARMS-PCR program. The sample with C allele is the wild type, the mutation is the T allele. The genotype was investigated by PCR-

RFLP method using the restriction enzyme *BseGI.* The results demonstrated that the ARMS-PCR and PCR RFLP techniques gave similar results (Figure 2.42).

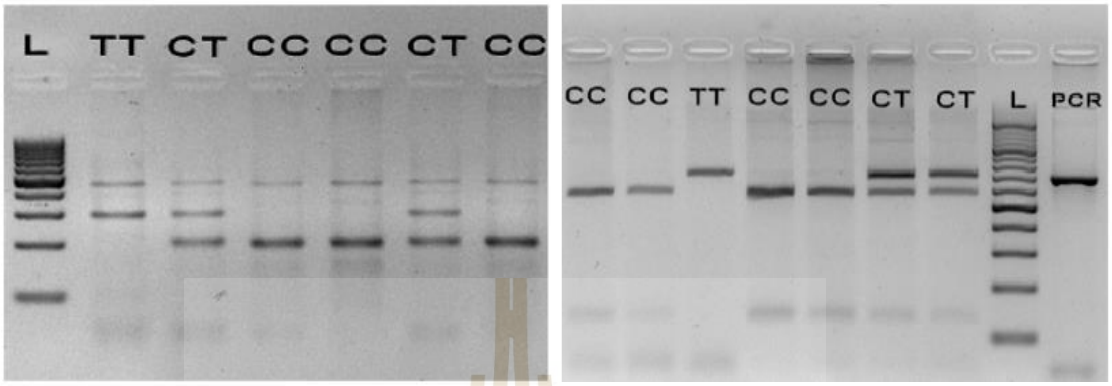

Figure 2.42 A: The genotypes of SNP C4685T of *CAPN* gene detection by ARMS-PCR technique, B: investigate genotypes by PCR-RFLP method was used restriction enzyme *BseGI.*

Source: Gábor et al., 2011.

In 2011, Gábor et al. (2011) improved the ARMS-PCR technique for the analysis of SNP C4685T of *CAPN* gene in a total of 130 cattle population. This *CAPN* gene SNP involve in the enzyme calcium system such as proteinase group which is the core proteolysis structure of myofibrillar proteins between carcass ageing storage. The primers were designed by Tetra-Primer ARMS-PCR program. The sample with C allele is the wild type, the mutation is the T allele. The genotype was investigated by PCR-RFLP method using the restriction enzyme *BseGI.* The results demonstrated that the ARMS-PCR and PCR RFLP techniques gave similar results (Figure 2.42).

Moreover, other analyses have revealed a significant influence of analyzed SNPs by ARMS-PCR. In the study of Rincon et al. (2006) used ARMS-PCR to detect *CAPN1* gene in bovine SNP position C5709G which is call *CAPN316.* This SNPs encode for the amino acid replacement of Gly316 to Ala. And the A4558G which is the *CAPN4751* C6545T encode for the amino acid replacement of Val4751. The *CAPN 316* and *4751* can be found in the GenBank accession number AF252504 and AF248054 respectively. Primer designed based on the published sequences of *B. indicus* and *B. taurus CAPN1* was used in ARMS-PCR. The ARMS-PCR technique was developed to detect SNPs of *CAPN1* gene using outer and inner primer to combing and amplified alleles specific in

one PCR reaction. SNP of *CAPN316* was presented to be correlated with the meat tenderness in beef cattle (*B. taurus*). And SNP of *CAPN4751* showed correlated also with the meat tenderness in beef cattle in both *B. indicus* and *B. taurus* (Rincón et al., 2006).

Ruan et al. (2014) Optimized the ARMS-PCR method. They suggested that the ARMS-PCR method is not easy and very time consuming to develop every single step. Different SNPs cannot use the same condition. The component of ARMS-PCR has to be optimized every time, The purity of DNA templates, temperatures cycling, reagents of PCR reaction, and balancing concentration of $MgCl_2$ and each primers needed to be optimized in ARMS–PCR reaction. All the conditions influenced to produce PCR products by ARMS-PCR technique.

The balance of primers concentration is very important because they are the primers identifying the specific allele. The concentration of the inner primers is importantly to implement which one has the less fragment and promote its fragment by adding its concentration. The detection of ARMS–PCR allows the study of mutation/SNPs in a rapid, dependable, and save cost protocol (Medrano et al., 2014).

Moreover, other method can be used to SNPs in gene of interest. The TaqMan methods (Figure 2.43). This method uses probes with allele specific primers and curve of melting was analyzed in the experiment kits. When used, we needed understanding the basic of the experiment kits, which allowed use to completely. As the TaqMan experiment kits have been improv. We can tests following with standard protocol.

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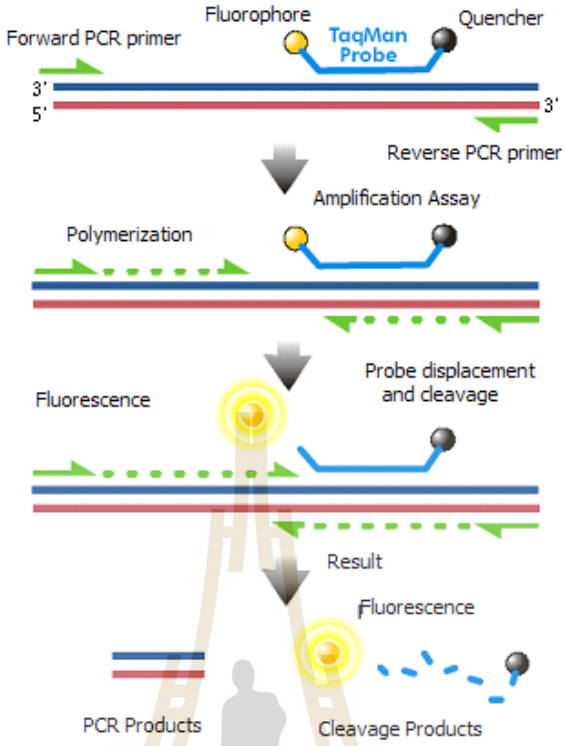

Figure 2.43 TaqMan probes analysis.

Source: https://upload.wikimedia.org/wikipedia/commons/0/07/Taqman.png.

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CHAPTER 3

RESEARCH METHODOLOGY

3.1 Samples

Meat grades from Thai Wagyu and Angus X Thai native crossbreed samples were recorded. Samples were collected from Suranaree University of Technology slaughterhouse, NVK farm, Community enterprise Korat Wagyu cattle, and Salakdai slaughterhouse. The sample from each animal was stored at - 20°C until used. Some of the samples were root hair samples send from farmers around the country.

3.2 Genomic DNA isolation

Genomic DNA (gDNA) were extracted from meat and root hair samples by DNA GF-1 Blood DNA Extraction kit (VIVANTIS, Malaysia). The DNA quality and yield were checked by Nanodrop spectrophotometer (Thermo Scientific, USA). The gDNA from each animal was stored at - 20°C until genotyping determination.

3.3 Determination primers designed of ARMS-PCR primers

Primers were designed using primer1 online program at http://primer1.soton.ac.uk /primer1.html (Ye et al., 2001). The NCBI GenBank accession number of *TG5, DGAT1* and *FABP4* gene used to design the primers are shown in Table 3.1. The GC content of primers were set at 40% to 65% and setup the two alleles of inner primers pair considered by nucleotide base mismatch (Table 4.3) at -3 position from the 3' end to discriminate and combined between the two alleles. After that sequence primers were synthesis by Integrated DNA Technologies Pte. Ltd. company (Singapore). The SNPs of marbling genes were detected by amplification refractory mutation system polymerase chain reaction (ARMS-PCR) with the optimal condition.

Table 3.1 GenBank accession number of marbling genes used for primers design.

Gene name	GenBank accession number	Reference
TG5	X05380	Shin et al., 2007
DGAT1	AJ318490	Winter et al., 2002
FABP4	NC_007312.4	Shin et al., 2012

3.4 Optimization of ARMS-PCR system

The PCR reaction was performed for each gDNA samples in a 25 µl reaction system with 1 × buffer, 1 U Taq polymerase and 50-100 ng of gDNA (of known genotype) Optimization of primers were performed using PCR reaction with vary concentration of outer primers and inner primers (10:1, 5:1, 2:1, and 1:1 µM) (Table 3.2) (Rosman et al., 2022). The concentration for $MgCl_2$ was also varied (1.5, 2.0 and 2.5 mM) (Table 3.3).

Table 3.2 Ratios concentration of outer and inner primers for ARMS-PCR system.

Variation name	Ratios concentration of outer and inner primers (*µM*)
A1	10:1
A2	5:1
A3	2:1
A4	1:1

Table 3.3 Ratios concentration of $MgCl_2$ for ARMS-PCR system.

Variation name	Ratios concentration of $MgCl_2$ (mM)
B1	1.5
B2	2.0
B3	2.5

3.5 Optimization of annealing temperature for ARMS-PCR system

The concept for optimization of the annealing temperature was done following Rincon et al. (2006), briefly touchdown PCR was used with vary annealing temperature set at 65°C for the first cycle and decreased 1°C every two cycles until 61°C for *TG5* gene. For *DGAT1* and *FABP4* genes optimization annealing temperatures were similar to *TG5* but the annealing temperature was set at 60°C for the first cycle and decreased 1°C every two cycles until 56°C.

3.6 Gel electrophoresis for the ARMS-PCR analysis

Amplification products 10 µl were loaded on to 2.5% agarose gels electrophoresis system and ran in 0.5X TAE buffer at 120 V for 25 minutes. The gels were stained with ethidium bromide and detected on gel documentation system (Bio-Rad).

3.7 Grading quality

After 7 days of postmortem aging at 4°C the meat grades were evaluated by authority of slaughterhouse using Marbling score scale (visual evaluation) of Japanese marble score scale standard Japan Meat Grading Association 2014 (JMGA 2014) grade no. 3-no.12 for Thai Wagyu crossbreed.

The evaluation of Thai Angus crossbreed sample was evaluated using USDA grading system (Choice, Prime, Select, etc.), in accordance with the beef carcass grading standards for determining the fat distribution between the muscle fibers reference standards for grades using United States Standards for Grades of Carcass Beef, 2017.

3.8 Evaluation genotype of meat samples with grading quality

After determining the genotypes and the meat grade of 100 Thai Wagyu and Angus X Thai native crossbred samples, the correlation between the genotype and meat grades were evaluated as mentioned in 3.9.

The genotypes of meat samples were used to analysis the allele frequencies. For the study of the relation of SNPs for meat quality genes with meat quality grade.

3.9 Hardy-Weinberg equilibrium calculate (genotype and allele frequencies)

The correlation between allele frequencies and genotype frequencies in Thai Wagyu crossbreeds populations used by principle Hardy-Weinberg Equilibrium and used was as follows:

Allele Frequencies Equation $p + q = 1$ (1)

Genotype Frequencies Equation $P^2 + 2pq + q^2 = 1$ (2)

3.10 Statistical analysis

All measured data were analyzed by IBM SPSS Statistics (version 25) programed for complete design using the Statistical Analysis System (SAS, 2001). Significant differences between genotype of meat samples and grading quality were assessed by Regression model used was as follows:

$$Y = a + bX + e \quad (3)$$

a significant level of $p<0.05$ was used (Steel and Torrie, 1980).

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CHAPTER 4
RESULTS AND DISCUSSION

4.1 DNA extraction

The concentration and purity of genomic DNA (gDNA) is important in molecular research. Therefore, the step of gDNA extraction is very important. In this study, genotypes of marbling genes from meat samples of beef cattle were detect using DNA GF-1 Blood DNA Extraction (VIVANTIS, Malaysia) kit. Our study here showed that the Blood DNA Extraction kit can be used to extract DNA from meat, root hair and frozen semen. As shown in Table 4.1 gDNA from meat samples have high-quality, high purity and high concentration. The concept of gDNA extraction from different samples are similar. DNA extraction from blood used white blood cell and break the cells to release the DNA. The DNA extraction from meat, first step is homogenization of the samples to simply breakdown and break open the cell with some mild chemicals in the lysis buffer. The lysis buffer contained proteinase K to break down the cell membrane. The second step added some buffers to bind the gDNA to the column. Third washing step is to wash all contaminants from the column. Finally, the gDNA is eluted from the column by elution buffer to a collection tube (Rubin et al., 2014).

Therefore, in this study blood DNA Extraction kit was used and shown to work well for our meat, root hair, semen and blood samples. The gDNA has high-quality, high concentration and purity. From this study the gDNA can be used to analyze genotypes of the marbling genes with ARMS-PCR technique.

4.2 Primers designed for ARMS-PCR system

In this study the variants sequence primer marbling genes were designed following Ye et al. (2001). The mismatch for primers design changed at -2 position from the 3′ end of inner primers allele specific and concept changed mismatch followed in Table 4.2. However, the fragment size were control to be around 200-700 bp for every genes. The Table 4.3 showed the sequence of primers, SNPs position, allele specific, and the fragment size for 3 marbling genes.

Table 4.1 Concentration and purity DNA extraction from meat sample analyzed by NanoDrop system.

Sample ID	Nucleic Acid Conc. (ng/µl)	260/280[a]
814	143.5	1.93
รสจรินทร์1	82.5	1.9
อุไรรัตน์1	118.2	1.83
อุไรรัตน์2	152.4	1.94
1465	205.8	1.94
อุกันยา1	148.4	1.9
K.มงคล	162.1	1.91
สำเริง1	137.5	1.92
สุกันยา2	276.1	1.94
สุกันยา3	130.2	1.93
โยชิ3	38.4	1.72
โยชิ4	64.1	1.89
WG-22771	83.9	1.89
WG-23329	114.4	1.89
492	148.9	1.89
25514	152.5	1.95

[a]260/280 = ratio of ~1.8 - ~2.0 is generally accepted as “pure” for DNA.

Table 4.2 Nucleotide base mismatch at -3 position from the 3’ end.

Alleles	Mismatch at -3 position
A/C	G/T, A/C
A/G	G/A, T/C
A/T	C/C, G/G, A/A, T/T
C/G	C/C, G/G, A/A, T/T
C/T	G/A, T/C
G/T	G/T, A/C

The mismatch at -3 positions from the 3´ end G/A, T/C = strong mismatch, A/A, C/C, G/G, and T/T = medium mismatch and C/A, G/T = weak mismatch for opportunity primer amplified complete with SNP of interest.

Table 4.3 Sequences of primers used for ARMS-PCR amplification of marbling genes designed by primer1 online program.

Gene	Primer	Primer Sequences s (5'-3')	SNP position	GenBank accession number	Allele Specific	Fragment size
TG5	Forward inner	GGTTTGATCCCTGGGTTGGGAAGCTT	C422T	X05380	T Allele	197 bp
	Reverse inner	AGTGGGTAGCCATTCCCTTCTCCAGAGG			C Allele	275 bp
	Forward outer	ATTGCTAGGAGGGAAGGAAGGAGCATGG				Common 400 bp
	Reverse outer	AATCTTGTGGAGGCTGTAGGGGAGCAGA				
DGAT1	Forward inner	CGTAGCTTTGGCAGGTAAGGC	A10433G	AJ318490	GC Allele	335 bp
	Reverse inner	TTAGCTCCCCCGTTGGCCTT	A10434C		AA Allele	220 bp
	Forward outer	CACCATCCTCTTCCTCAAGC				Common 516 bp
	Reverse outer	GAAGCAAGCGGACAGTGAG				
FABP4	Forward inner	CCACCATAAAGAGAAAACACG	G3691A	NC_007312.4	G Allele	253 bp
	Reverse inner	CAGCACCATCTTATCATCGAT			A Allele	352 bp
	Forward outer	ACCCCTATGATGCTATTCCACA				Common 602 bp
	Reverse outer	ATACGGTTCACATTGAGAGGGA				

Underlines are nucleotide at the position -3 from the 3' ends of both Forward inner and Reverse inner primers designed to make them more specific for each allele.

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4.3 Optimization of ARMS-PCR by varying primers concentration

The primers were designed using primer1 online program (Ye et al., 2001). The sequence of marbling genes in this study is based on NCBI GenBank accession number database shown in Table 4.2. For optimization concentration ratios of outer and inner primers of the marbling genesis aimed that the appearance of three bands are of reference band and band specific (wild type band and mutation band) can be seen when DNA template of heterozygous genotypes are used as positive control and appearance two band when used DNA template extracted from meat samples of homozygous genotype control (Figure 4.1 and 4.2).

Table 3.2 shows the concept of ration of the primers concentration of outer and inner primers (μM) which were A1 = 10:1, A2 = 5:1, A3 - 2:1, and A4 - 1:1. The 4 conditions were included in this study. PCR products were obtained and resulted were observed under 2.5% agarose gel. The result PCR products of DNA template know genotypes were used to adjust the primers concentration. The outer primers concentration were increased, while the inner primers concentration were decreased to balance the bands between the reference band and the allele specific band in DNA template of known genotypes. For the *TG5* gene the A3 condition was the best condition.

For the *FABP4* gene after optimizing ratios concentration of outer and inner primers 4 conditions included for this study. The result PCR products of DNA template know genotypes, the outer and inner primers equal concentration was suitable for analyzed genotypes completely or the A4 condition to balanced bands between reference band and band specific in DNA template known genotypes.

For *DGAT1* gene used ARMS-PCR technique (Di primer). The result PCR products of DNA template know genotypes, the outer and inner primers equal concentration suitable for analyzed genotypes completely to effected band specific clearly in DNA template know genotypes.

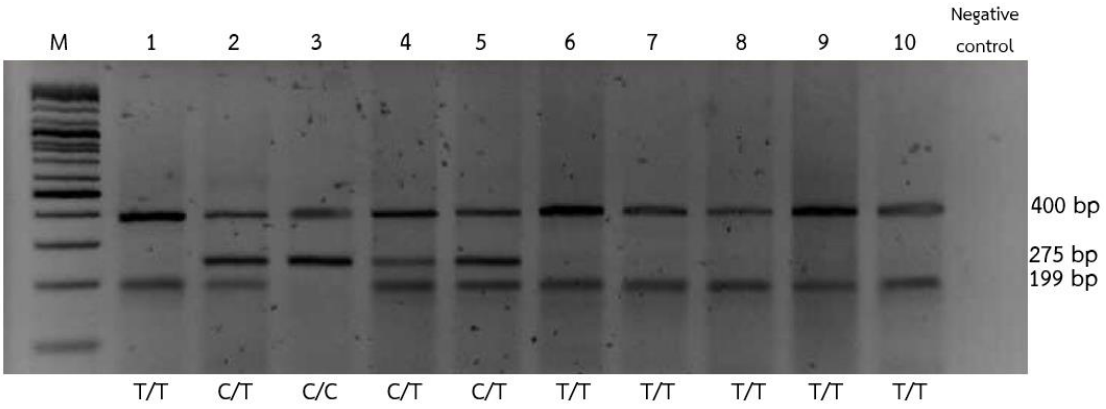

Figure 4.1 Gel electrophoresis of ARMS-PCR technique for SNP C422T of *TG5* gene on 2.5 % agarose gel 400 bp (reference band) and 199 bp (band specific) indicated homozygous (T/T), those with 400 bp and 275 bp (band specific) products indicated homozygous (C/C) and the ones with three products of 400 bp, 275 bp and 199 bp are heterozygous (C/T).

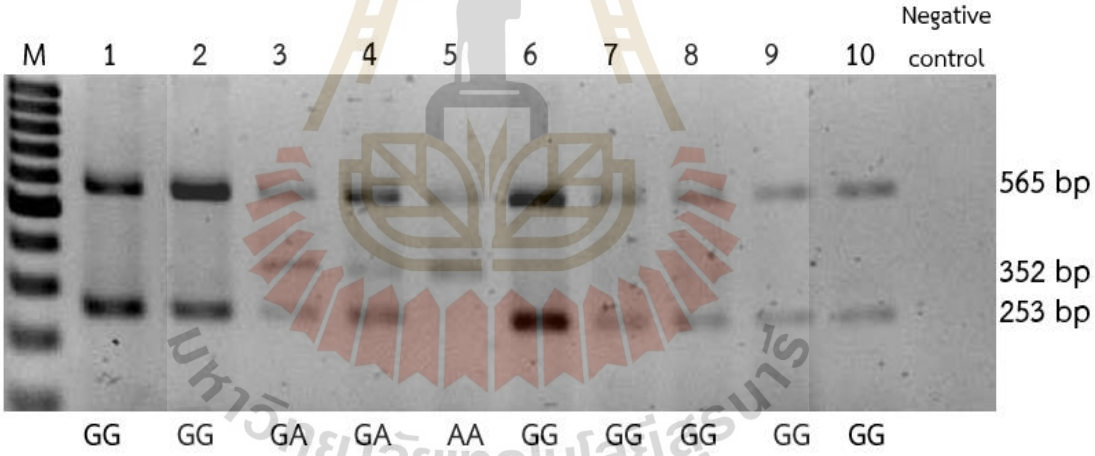

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Figure 4.2 Gel electrophoresis of ARMS-PCR technique for SNP G3691A of *FABP4* gene on 2.5 % agarose gel 565 bp (reference band) and 253 bp (band specific) indicated homozygous (G/G), those with 565 bp and 352 bp (band specific) products indicated homozygous (A/A) and the ones with three products of 565 bp, 352 bp and 253 bp are heterozygous (G/A).

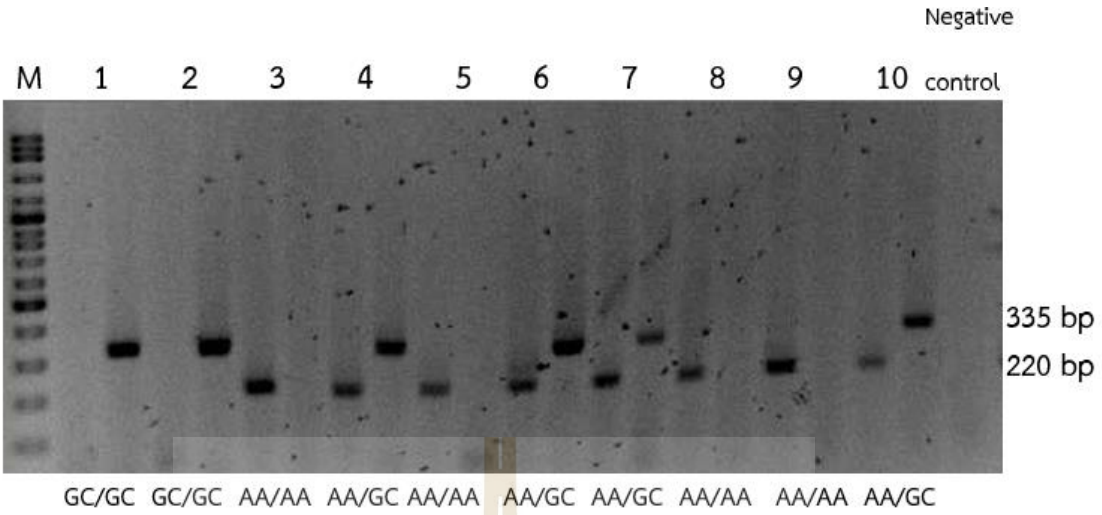

Figure 4.3 Gel electrophoresis of ARMS-PCR technique (Di primer) for SNPs A10433G and A10434C of *DGAT1* gene on 2 % agarose gel 335 bp (band specific) indicated homozygous (GC/GC), those with 220 bp (band specific) products indicated homozygous (AA/AA) and the ones with three products of 335 bp and 220 bp are heterozygous (AA/GC).

4.4 Optimization of ARMS-PCR by varying $MgCl_2$ concentration

Table 3.3 shows the concept of varying the concentration of $MgCl_2$ (mM) which were B1 = 1.5, B2 = 2.0 and B3 = 2.5. The 3 conditions were included in this study. PCR products were obtained on 2.5% agarose gel. The result PCR products of DNA template of know genotypes showed that 1.5 mM $MgCl_2$ decreased the PCR products bands, the concentration of $MgCl_2$ 2 mM was distinct and balanced bands, while the concentration of $MgCl_2$ 2.5 mM was presented bands nonspecific and found smear in background gel of PCR products. Therefore, the concentration of $MgCl_2$ 2 mM was suitable and was used in the ARMS-PCR in this study.

4.5 Optimization of ARMS-PCR by varying annealing temperature

The optimization of annealing temperature was done using touch down PCR technique starting with high temperature and slowly decrease the temperature of the annealing steps until the required terminal of annealing temperature is reached. For the *TG5* gene varies annealing temperature starting from 65°C for the first cycle and

decrease 1°C every two cycles until 61°C and for the *FABP4* gene varies annealing temperature starting at 60°C for the first cycle and decrease 1°C every two cycles until 56°C. The DNA template of positive control of known genotype of heterozygous and homozygous (wild type and mutation) showed correct and clear bands sizes of amplicon without nonspecific bands. After that when repeated with DNA extracted from meat of unknow genotype, in this annealing temperature optimize can amplified bands DNA unknow genotype very clear.

4.6 Analysis genotype and allele frequency

The Genotype and Allele frequency in Thai Wagyu crossbreeds of each genes calculate followed by Hardy-Weinberg Equilibrium model shown in Table 4.4. Present results revealed that the most frequent genotype for *TG5* gene was CT and TT (0.514 and 0.355) and AA/AA and AA/GC for *DGAT1* gene (0.506 and 0.380), for *FABP4* gene was GG and GA (0.536 and 0.464) meat quality marker in Thai Wagyu crossbreeds' bulls, respectively. The T and C allele frequency was 0.613 and 0.387 for *TG5* gene whereas, AA allele and GC allele frequency was 0.696 and 0.304 for *DGAT1* gene; G allele and A allele frequency was 0.741 and 0.259 for *FABP4* gene.

Table 4.4 Genotype and Allele frequency of meat quality genes in Thai Wagyu cross breeds.

Gene	Total No.	Genotype			Allele	
TG5	107	TT	CT	CC	T	C
		(n=38)	(n=55)	(n=14)	(n=131)	(n=83)
		0.355	0.514	0.131	0.613	0.387
DGAT1	79	AA/AA	AA/GC	GC/GC	AA	GC
		(n=40)	(n=30)	(n=9)	(n=110)	(n=48)
		0.506	0.380	0.114	0.696	0.304
FABP4	84	GG	GA	AA	G	A
		(n=45)	(n=39)	(n=0)	(n=129)	(n=45)
		0.536	0.464	0	0.741	0.259

4.7 Optimization ARMS-PCR technique of *TG5* gene

In this research, we have developed ARMS-PCR technique for analyzed genotype of marbling genes in the meat samples of cattle. The developed ARMS-PCR technique follow Tanha et al., 2015.

Shin et al. (2007) indicated that the SNP of *TG5* gene associated with marbling level and meat quality in beef cattle. In this research Shin identified several SNPs in the *TG5* gene, the A335G, C422T and C257T. The SNP of C422T significantly (*p<0.05*) showed relation with marbling level and three different genotypes were observed TT, CT, and CC. The result reported that TT genotype showed higher marbling level in beef cattle when compared to cattle with CT and CC genotype. The studies of Shin 2007 used restriction enzyme in PCR-RFLP method. in the used of PCR RFLP has disadvantage points such as used long time for analyzed genotype and expensive chemical (restriction enzyme).

We have developed ARMS-PCR technique for analyze genotype of the C422T SNP of *TG5* gene. Three different bands were observed at 400 bp (reference band) and 199 bp (band specific) indicated homozygous (T/T), those with 400 bp and 275 bp (band specific) products indicated homozygous (C/C) and the ones with three products of 400 bp, 275 bp and 199 bp are heterozygous (C/T) (Figure 4.1).

The primers of ARMS-PCR technique for analyze genotype of the *TG5* gene SNP C422T were designed by primer1 online program (Table3.1) and changed mismatch at -2 position from the 3′ end of inner primers allele specific and concept changed mismatch followed in Table 4.3 For *TG5* gene SNP C422T allele specific is the C/T therefore designed adding mismatch at -2 position from the 3′ end G/A and T/C strong mismatch followed research by Tanha et al. (2014). The strong mismatch helps amplified template because increased discrimination combined between two alleles or allow specific amplified of sequences *TG5* gene with one base pair changed. And the primers for strong mismatch in this study can make *Taq DNA polymerase* unable to perform extension PCR process (Deekshit et al., 2019).

4.8 Optimization ARMS-PCR technique of *FABP4* gene

FABP4 is fatty acid binding protein 4 found in site the adipocytes cell. The function lipid hydrolysis or breaks down triglyceride fatty acid and intracellular fatty acid trafficking in different tissues (Scifres et al., 2011). Shin et al. (2011) reported genetic

variation SNPs positions of *FABP4* gene found that the position SNP G3691A in exon 3 relation with meat marbling scores in Korean cattle or Hanwoo breed.

Genotype of SNP position G3691A of *FABP4* gene have three different genotypes were observed GG, GA, and AA. Shin et al. (2011) reported effects of GG genotype showed has significantly higher than marbling level and associated meat quality grades when compare GA and AA genotype. Previous studies determined digestion with the restriction enzyme (*Nla III*) using the PCR-RFLP method but in this the method is disadvantage because used long time for analyze genotype and expensive chemical (restriction enzyme).

We have developed ARMS-PCR technique for analyze genotype of the *FABP4* gene SNP G3691A (Figure 4.2) three different band were observed 565 bp (reference band) and 253 bp (band specific) indicated homozygous (GG), those with 565 bp and 352 bp (band specific) products indicated homozygous (AA) and the ones with three products of 565 bp, 352 bp and 253 bp are heterozygous (GA).

The primers of ARMS-PCR technique for analyze genotype of the *FABP4* gene SNP G3691A was designed by primer1 online program (Table 3.1) and changed mismatch at -2 position from the 3′ end of inner primers allele specific. For *FABP4* gene SNP G3691A allele specific is the G/A therefore designed additional mismatch at -2 position from the 3′ end A/G strong mismatch follow research by Tanha et al. (2014). In this study primers were designed able amplified DNA template from meat unknown completely, mismatch designed important step, the inner primers can improve allele specificity of SNPs positions (Ye et al., 2001).

4.9 Optimization concentration of primers in marbling genes

For optimize concentration of outer and inner primers in marbling genes to decrease the inconsistencies fragment bands DNA templates. The outer and inner primers concentration range of ratio 10:1, 5:1, 2:1, and 1:1 or 4 conditions was determined, concept from an experiment by Rosman et al. (Rosman et al., 2022). ARMS-PCR for *TG5* gene and *FABP4* gene required concentration of outer and inner primers difference to avoid amplified fragment nonspecific (Chiapparino et al., 2004). Previous studies was optimization SNP in *CYP2E1* gene, varies primers concentration were useful bands, thus shown the core importance concentration of each primer (Suhda et al., 2016).

The outer and inner primers concentration range of ratio in this study were (10:1= 2 μM and 0.2 μM), (5:1=1 μM and 0.2 μM), (2:1=0.4 μM and 0.2 μM), and (1:1=0.2 μM). Each range of ratio showed difference concentration to amplified good fragment meanwhile decreased products of nonspecific band. For *TG5* gene was amplified genotypes of DNA templates positive control and gDNA of meat samples (unknow genotype) were enhanced by increasing the concentration of outer primer and reducing concentration of inner primer because outer primers generate common band and specific band with inner primers. When we reduce concentration of outer primers disappeared decreased common band and specific band. Therefore, the optimal varies of primers concentration is A3 or 2:1, while *DGAT1* and *FABP4* gene the optimal varies of primers concentration is A4 or 1:1, due to the primers sequence able extension SNP position and can discrimination have combined between two alleles. In this studies DNA templates from meat samples were optimal concentrations and high quality that could be amplified the target sequenced (Forootan et al., 2017; Klymus et al., 2020).

4.10 Annealing temperature ARMS-PCR technique in marbling genes

The optimized annealing temperature in *TG5* gene is a core experiment to developed ARMS-PCR system because it is simple to modify and measure (Markoulatos et al., 2002; Sipos et al., 2007). In the varies annealing temperature using touch down PCR technique set at starting high annealing temperature for the first cycle, decreasing low annealing temperature every two cycles until finish terminal annealing temperature set, rang were 64°C to 60°C, 62°C to 58°C and 60°C to 56°C. When set lower annealing temperature (60°C to 56°C) were appeared smeared and nonspecific band because the primers can't combined with DNA templates completely (Zhang et al., 2019). While set at higher than annealing temperature (64°C to 60°C) appeared fewer smeared and nonspecific band, but decreasing target fragment intensity, due to primers lower combined with DNA templates (Hecker et al., 1996). Therefore, in this study used touch down PCR technique because used single annealing temperature cannot amplified template, For *DGAT1* and *FABP4* gene concept annealing temperature like *TG5* gene but varies lower annealing temperature. And rang were 62°C to 58°C, 60°C to 56°C and 58°C to 54°C. In this study the optimized annealing temperature set at starting 64°C to 60°C suitable for PCR reaction in *TG5* gene, while *DGAT1* and *FABP4* gene set at starting 60°C to 56°C suitable for PCR reaction.

Therefore, we determined optimized temperatures of each primer because each primers have suitable difference annealing temperature and previous optimize annealing temperature is the best were obtaining clear bands and decreased nonspecific bands for marbling gene used ARMS–PCR detection.

In addition, the master mixes sensitive for optimize condition ARMS-PCR technique the concentration $MgCl_2$ in this study 2.0 mM suitable for *TG5* gene, and research related with Medrano et al. (2014). And $MgCl_2$ is the major sensitive to PCR reaction when slightly changed concentration effect to disappear nonspecific bands, but the forward inner primer did not amplify as well (Medrano et al., 2014). And the optimization of ARMS-PCR is too sensitive to variations of annealing temperature and concentration $MgCl_2$.

The dNTPs in this study using concentration 0.4 mM found that suitable for optimized ARMS-PCR technique. In this study which is consistent with the researcher of Kumar et al. (2010) the component dNTPs (deoxyribonucleoside triphosphates) dATP, dTTP, dGTP and dCTP, are to help increase to discrimination combined between two alleles.

4.11 Genotype and Allele frequency in Thai Wagyu crossbreeds

The allele frequency for the SNPs A10433G and A10434C of *DGAT1* gene showed higher AA allele than GC allele. Whereas the allele frequency for the SNP G3691A of *FABP4* gene higher with the G allele than A allele. These 3 marbling genes in Thai Wagyu crossbreed were similar to Japanese Wagyu. Similarly, Nicol et al. (2001) reported the frequencies of T allele than C allele for the *TG5* gene mentioned alleles in the Wagyu cattle. The T allele has been associated with increases in marbling score in both long-fed (> 250 days on feed; Barendse, 1999). For *FABP4* gene in this study higher with the G allele than A allele similarly with Shin et al. (2012) reported the frequencies of G allele was significantly correlated with marbling score and meat color when compare animal A allele. The allele frequency of *DGAT1* gene in this study higher with the AA allele than GC allele similarly with Anton et al. (2012) reported the frequencies of AA allele shown to have highest meat marbling score and percent of fat content in musculus *longissimus dorsi* (LD) and musculus semitendinosus (ST) when compare GC allele and the cattle has AA allele increase the fat content production in

the body more than other allele. The results similar to Thaller et al. (2003) in Holstein and Charolais breeds. Therefore these 3 marbling genes can be use as marker assisted selection (MAS) for beef cattle and candidate gene to identify meat quality in Thai Wagyu crossbreeds.

4.12 Genotype of Meat quality genes in different beef cattle breed

The *TG 5* gene of Wagyu and Thai Wagyu crossbreeds show high TT genotype when compared to *B. taurus* and *B. indicus.* For *FABP 4* gene most cattle have GG genotype. And for the *DGAT 1* gene, the AA/AA was found to be high in Thai Wagyu crossbreeds and Holstein and lower in Red Angus. However, AA/AA was not detected in Charolais (Table 4.5).

Nicol et al. (2001), have shown that TT genotype was associated with high marbling score in Wagyu breed. In this work, high TT genotype was also detected in Thai wagyu cross. In Holstein cattle from Turkey, Ardicli et al., 2017 showed that GG genotype in *FABP 4* gene provides high meat marbling score. In Thai wagyu cross, GG genotype was also high. And in Thai Wagyu crossbreeds high AA/AA genotype was found in the *DGAT 1* gene similar to the Holstein breed which shown higher intramuscular fat content than the cattle with AA/GC or GC/GC genotype (Anton et al., 2011).

This is important because the potential impact of selecting for a genetic marker depends on both the magnitude of its effect and genotype in the population. The meat quality genes in this study would be candidate molecular markers for assisting selection of Thai Wagyu crossbreeds. Further study on evaluation of the association of this Biomarkers with marbling score need to be take in Thai Wagyu crossbreeds. The meat quality genes marker will be one of several SNPs markers for meat quality and meat marbling score and can be used to for markers assisted selection (MAS) in the crossbreed beef cattle breeding program.

Table 4.5 Genotype of Meat quality genes in different beef cattle breed.

Gene	Breed	Genotype (%)				Reference
		TT	CT	CC		
TG 5	Wagyu	38	50	12	Japan	Nicol et al., 2001
	Red Angus	16	42	42	Australia	Nicol et al., 2001
	Black Angus	9	38	53	Australia	Nicol et al., 2001
	Charolais x Angus	5	34	62	United States	Van et al., 2007
	Angus	3	32	64	Australia	Berandse et al., 2004
	Hereford	1	18	81	United States	Van et al., 2007
	Brahman	1	4	95	United States	Casas et al., 2007
	Angus x Thai native	1	40	59	Thailand	Nachai et al., 2007
	Thai Wagyu	36	51	13	Thailand	This study
		GG	GA	AA		
FABP 4	Hanwoo	62	35	3	Korea	Shin et al., 2012
	Holstein	50	46	4	Turkey	Ardicil et al., 2017
	Yanbian yellow	69	19	12	China	Yin et al., 2020
	Aberdeen Angus	46	36	15	Turkey	Ardicil et al., 2021
	Hereford	53	35	12	Turkey	Ardicil et al., 2021
	Thai Wagyu cross	54	46	0	Thailand	This study
		AA/AA	AA/GC	GC/GC		
DGAT 1	Holstein	18	54	28	Germany	Thaller et al., 2003
	Charolais	0	22	78	Germany	Thaller et al., 2003
	Red Angus	5	28	67	Hungary	Anton et al., 2011
	Angus	2	33	65	Sweden	Li et al., 2013
	Charolais	0	21	79	Sweden	Li et al., 2013
	Thai Wagyu cross	51	38	11	Thailand	This study

CHAPTER 5
CONCLUSION

In this study, Amplification Refractory Mutation System-Polymerase Chain Reaction (ARMS-PCR) technique for SNPs of marbling genes *TG5*, *DGAT1* and *FABP4* were developed to study the SNPs in Thai Wagyu crossbreeds. The ARMS-PCR technique in this study has advantages over the traditional technique. It is a fast, easily uses, and save cost technique to detect single nucleotide polymorphisms (SNPs) in genomic DNA of meat sample. The primers designed, conditions of annealing temperature and concentration of primers were the main issues that were optimized and develop. In the future correlation between genotypes of marbling genes analyze by ARMS-PCR technique and grade of meat of Thai Wagyu crossbreeds will be study. Moreover, the developed ARMs-PCR have shown to be accuracy and specific. It can be applied to use with other gene of interest. Farmers can use these DNA markers to identify good potential cattle to be used as mother to obtain high quality Wagyu crossbreeds or improve breeding program, to produce high quality young cattle crossbreeds. And up value the meat products of Thailand.

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APPENDIX

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APPENDIX

Figure A.1 The National Center for Biotechnology Information advances science and health by providing access to biomedical and genomic information.

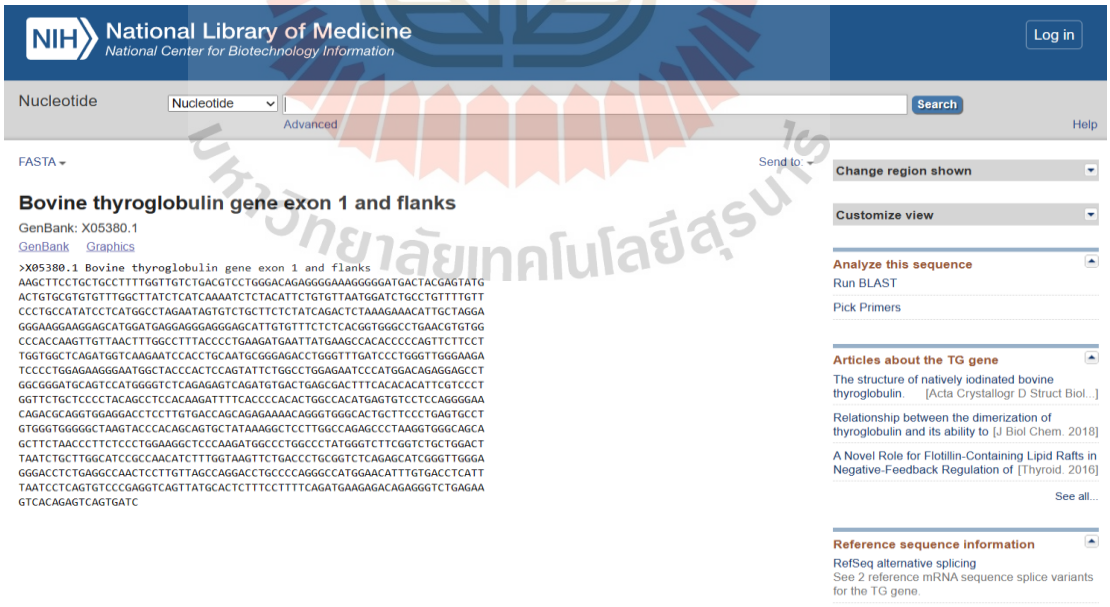

Figure A.2 The National Center for Biotechnology Information advances science and health by providing access to biomedical and genomic information.

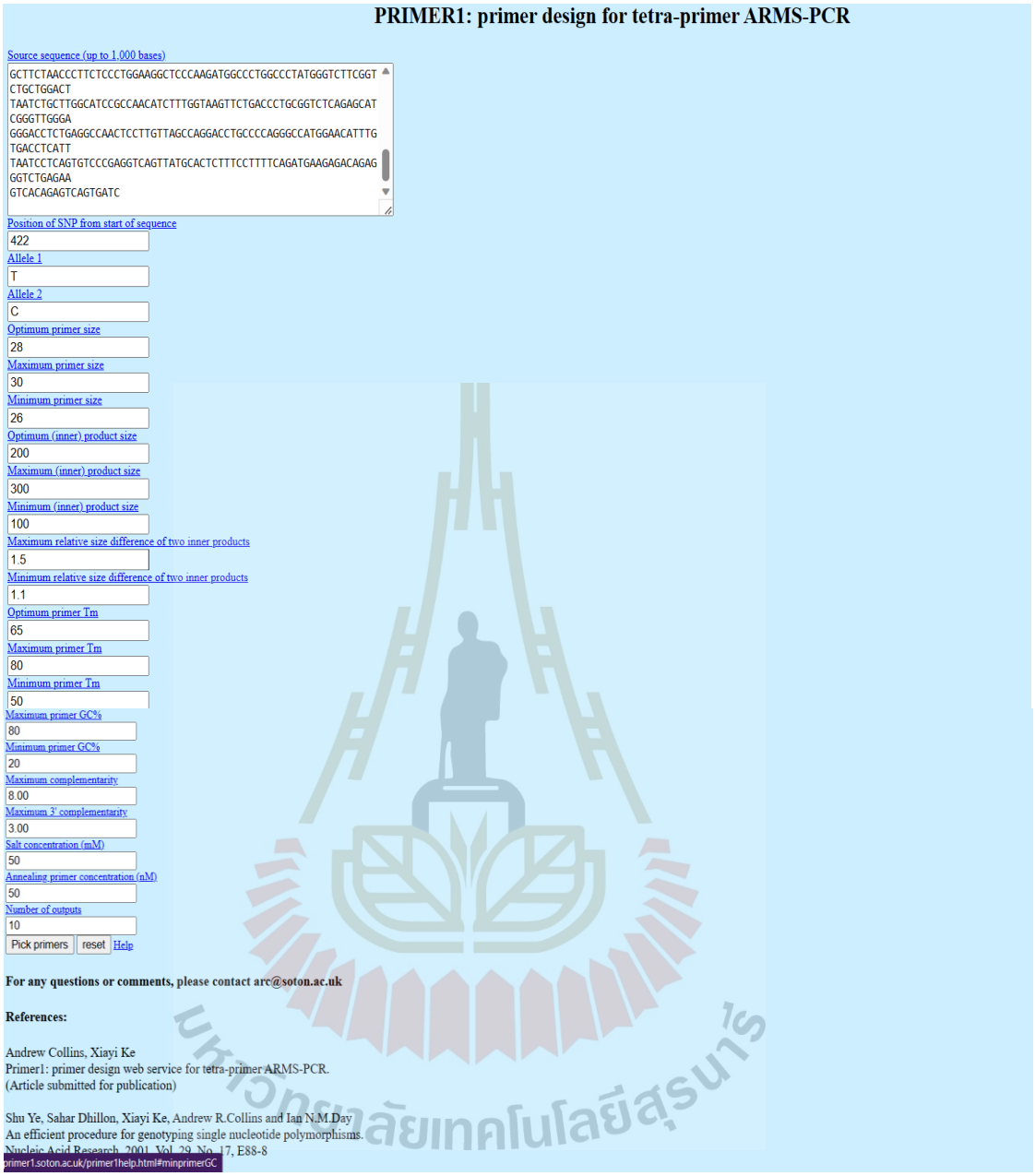

Figure A.3 PRIMER1: primer design for tetra-primer ARMS-PCR.

```
*****************************OUTPUT 1*****************************
Forward inner primer (T allele):                Melting temperature
397 GGTTTGATCCCTGGGTTGGGAAGCTT 422                      72

Reverse inner primer (C allele):
449 AGTGGGTAGCCATTCCCTTCTCCAGTGG 422                    72

Forward outer primer (5' - 3'):
235 AGGGAGGGAGCATTGTGTTTCTCTCACG 262                    72

Reverse outer primer (5' - 3'):
591 AATCTTGTGGAGGCTGTAGGGGAGCAGA 564                    72

Product size for T allele: 196
Product size for C allele: 215
Product size of two outer primers: 357

*****************************OUTPUT 2*****************************
Forward inner primer (T allele):                Melting temperature
397 GGTTTGATCCCTGGGTTGGGAAGCTT 422                      72

Reverse inner primer (C allele):
449 AGTGGGTAGCCATTCCCTTCTCCAGTGG 422                    72

Forward outer primer (5' - 3'):
233 GGAGGGAGGGAGCATTGTGTTTCTCTCA 260                    72

Reverse outer primer (5' - 3'):
591 AATCTTGTGGAGGCTGTAGGGGAGCAGA 564                    72

Product size for T allele: 196
Product size for C allele: 217
Product size of two outer primers: 359

*****************************OUTPUT 3*****************************
Forward inner primer (T allele):              Melting temperature
397 GGTTTGATCCCTGGGTTGGGAAGCTT 422                      72

Reverse inner primer (C allele):
449 AGTGGGTAGCCATTCCCTTCTCCAGTGG 422                    72

Forward outer primer (5' - 3'):
274 CGTGTGGCCCACCAAGTTGTTAACTTTG 301                    72

Reverse outer primer (5' - 3'):
591 AATCTTGTGGAGGCTGTAGGGGAGCAGA 564                    72

Product size for T allele: 196
Product size for C allele: 176
Product size of two outer primers: 318

*****************************OUTPUT 4*****************************
Forward inner primer (T allele):              Melting temperature
397 GGTTTGATCCCTGGGTTGGGAAGCTT 422                      72

Reverse inner primer (C allele):
449 AGTGGGTAGCCATTCCCTTCTCCAGTGG 422                    72

Forward outer primer (5' - 3'):
230 TGAGGAGGGAGGGAGCATTGTGTTTCTC 257                    72

Reverse outer primer (5' - 3'):
591 AATCTTGTGGAGGCTGTAGGGGAGCAGA 564                    72

Product size for T allele: 196
Product size for C allele: 220
Product size of two outer primers: 362
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Figure A.4 Primer design for tetra-primer ARMS-PCR by PRIMER1 program.

```
*****************************OUTPUT 9*****************************
Forward inner primer (T allele):              Melting temperature
397 GGTTTGATCCCTGGGTTGGGAAGCTT 422                  72

Reverse inner primer (C allele):
449 AGTGGGTAGCCATTCCCTTCTCCAGTGG 422                72

Forward outer primer (5' - 3'):
201 ATTGCTAGGAGGGAAGGAAGGAGCATGG 228                72

Reverse outer primer (5' - 3'):
591 AATCTTGTGGAGGCTGTAGGGGAGCAGA 564                72

Product size for T allele: 196
Product size for C allele: 249
Product size of two outer primers: 391

*****************************OUTPUT 10*****************************
Forward inner primer (T allele):              Melting temperature
397 GGTTTGATCCCTGGGTTGGGAAGCTT 422                  72

Reverse inner primer (C allele):
449 AGTGGGTAGCCATTCCCTTCTCCAGTGG 422                72

Forward outer primer (5' - 3'):
273 ACGTGTGGCCCACCAAGTTGTTAACTTTG 301               72

Reverse outer primer (5' - 3'):
591 AATCTTGTGGAGGCTGTAGGGGAGCAGA 564                72

Product size for T allele: 196
Product size for C allele: 177
Product size of two outer primers: 319


Completed
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Figure A.5 Primer design for tetra-primer ARMS-PCR by PRIMER1 program.

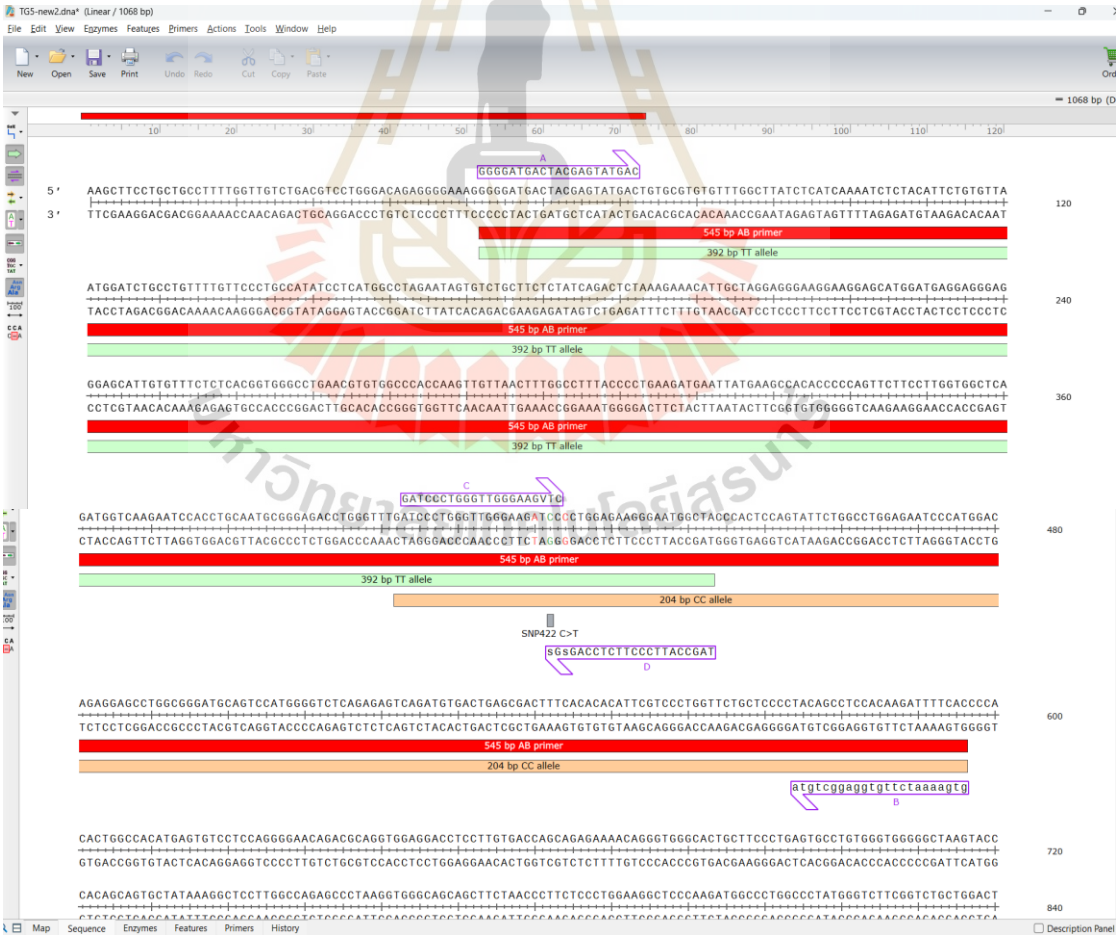

Figure A.6 SnapGene program for PCR primer design.

VITAE

Kitrati Nakket was born on January 2, 1998, in Bangkok, Thailand. In 2016, finished high school from Wangrang Pittayakom school, Nakhon -Ratchasima. In 2020, he graduated with a bachelor's degree from the School of Food Technology at Suranaree University of Technology, Nakhon Ratchasima. Then, he began Master program in the School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, as he received a scholarship from the "SUT - OROG scholarship". As part of before learned Master program, he spent 1 year in the research assistant (RA) of Assoc. Prof. Dr. Mariena Ketudat-Cairns in the School of Biotechnology, learning about PCR technique and methods for DNA extraction.

He presented oral and poster presentation including:

1) Poster presented SUT biotech retreat 2021: Development ARMS-PCR technique to detect SNP of *TG5* gene in Thai Wagyu crossbreeds, Nakhon Ratchasima, Thailand.

2) The 7th SUT International Colloquium on Agricultural Technology, Nakhon Ratchasima, Thailand [2022] Oral presentation on the topic of Development ARMS-PCR technique to detect SNP of *FABP4* gene in Thai Wagyu crossbreeds.

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