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BOVINE SPERM SEXING BY MONOCLONAL ANTIBODY

Surachai Rattanasuk

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy of Biotechnology

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BOVINE SPERM SEXING BY MONOCLONAL ANTIBODY

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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การคัดแยกเพศอสุจิของโคมีความสำคัญต่อวงการอุตสาหกรรมและปศุสัตว์เป็นอย่างมาก เทคโนโลยีการคัดแยกเพศอสุจิของโคนั้นมีหลายวิธี การใช้โมโนโคลนัลแอนติบอดีในการคัดแยกเพศของอสุจิเป็นหนึ่งวิธีที่ได้รับความนิยม เนื่องจากการคัดแยกเพศด้วยวิธีนี้จะไม่ส่งผลกระทบต่อคุณภาพของอสุจิเพศที่ต้องการ การศึกษาครั้งนี้มีจุดประสงค์ที่จะผลิตโมโนโคลนัลแอนติบอดีที่มีความจำเพาะต่ออสุจิเพศผู้ของโค โดยโมโนโคลนัลแอนติบอดีนี้ถูกสร้างขึ้นจากการฉีดกระตุ้นการสร้างแอนติบอดีในหนูขาวเพศเมียสายพันธุ์ BALB/c ด้วยอสุจิเพศผู้ของโค หลัง กระบวนการเชื่อมเซลล์ ได้เซลล์ลูกผสมมากกว่า 2000 โคโลนี จากนั้นนำอาหารเหลวที่ได้จากการเลี้ยงเซลล์ลูกผสมทั้งหมดมาตรวจสอบความจำเพาะต่ออสุจิเพศผู้ของโคด้วยวิธี ELISA และ ตรวจสอบการจับของแอนติบอดีที่ผิวของอสุจิเพศผู้ของโค ซึ่งพบว่า เซลล์ลูกผสม 3 โคโลนี G16G14, G16E7 และ G16E8 สามารถผลิตโมโนโคลนัลแอนติบอดีที่มีความจำเพาะต่ออสุจิเพศผู้ของโคได้ จึงได้นำเซลล์ลูกผสมทั้งสามนี้มาผลิตโมโนโลนัลแอนติบอดีเพื่อใช้ในการคัดแยกเพศของอสุจิโค ในขั้นตอนของการแยกเพศของอสุจิโคนั้น อสุจิเพศผู้ของโคถูกทำลายด้วยกระบวนการทำงานของคอมพลีเม็นต์ของหนูตะเภา จากนั้นอสุจิเพศเมียของโคที่ไม่ถูกทำลายถูกนำไปใช้ในกระบวนการปฏิสนธิในหลอดทดลองเพื่อตรวจสอบความถูกต้องและความแม่นยำของกระบวนแยกเพศอสุจิโคด้วยโมโนโคลนัลแอนติบอดีที่ผลิตขึ้น เพศของตัวอ่อนที่ได้จากการปฏิสนธิในหลอดทดลองถูกตรวจสอบด้วยวิธีพีซีอาร์โดยใช้ไพร์เมอร์ที่มีความจำเพาะต่อโคเพศผู้และต่อโค ผลจากการตรวจสอบเพศของตัวอ่อนที่ได้รับการผสมจากอสุจิที่ผ่านการแยกเพศด้วยโมโนโคลนัลแอนติบอดีที่ผลิตขึ้นพบว่าสามารถเพิ่มค่าเฉลี่ยของตัวอ่อนเพศเมียได้เป็นร้อยละ 78.2, 74.3 และ 79.5

สาขาวิชาเทคโนโลยีชีวภาพ
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ลายมือชื่อนักศึกษา_____
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ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

SURACHAI RATTANASUK : BOVINE SPERM SEXING BY MONOCLONAL ANTIBODY. THESIS ADVISOR : ASSOC. PROF. MARIENA KETUDAT-CAIRNS, Ph.D., 109 PP.

MONOCLONAL ANTIBODY/SPERM SEXING/BOVINE SPERM

Bovine sperm sexing technology is important for both industry and livestock sections. Various methods have been used for bovine sperm sexing. Immunological method is one of the most favorable methods used, because this method does not affect the wanted sperm quality. This study aimed to produce monoclonal antibody (mAb) against bovine Y-sperm. The mAbs were produced by stimulation of female BALB/c mice with bovine Y-sperm injection. More than 2,000 hybridoma colonies were obtained after cells fusion. The cultured supernatants of the hybridoma colonies were used to determine the specificity against bovine Y-sperm by ELISA and sperm surface binding methods. Three hybridoma colonies, G16G14, G16E7 and G16E8 produced mAbs that were specific to bovine Y-sperm. These three hybridoma colonies were used to produce the mAbs to be used for sperm sexing. In the sperm sexing method, bovine Y-sperms were destroyed via cytotoxicity using guinea pig complement. The undestroyed X-sperms were used in *in vitro* fertilization (IVF) to determine the accuracy and precision of the sperm sexing method using the produced mAb. The sex of embryos obtained from IVF was determined by polymerase chain reaction (PCR) method using bovine and bovine Y-specific primers. The embryos sex determination indicates that the three hybridoma colonies can produce mAbs which are specific to

bovine Y-sperm. They can increase the ratio of female embryo to an average of 78.2, 74.3 and 79.5% after sperm sexing and IVF.

School of Biotechnology

Academic Year 2011

Student's Signature _____

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Co-advisor's Signature _____

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Surachai Rattanasuk

CONTENTS

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF ABBREVIATION

×g	=	Force of gravity
µg	=	Microgram
µl	=	Micro liter
bp	=	Base pair
BSA	=	Bovine serum albumin
^{o}C	=	Degree Celsius
CO_2	=	Carbon dioxide
COCs	=	Cumulus-oocyte complexes
cRPMI	=	Complete Roswell Park Memorial Institute medium
$CuSO_4$	=	Copper sulfate
DMSO	=	Dimethyl sulfoxide
dNTPs	=	Deoxynucleotide triphosphate
ELISA	=	Enzyme-linked immunosorbent assay
FBS	=	Fetal bovine serum
FITC	=	Fluorescein isothiocyanate
g	=	Gram
hr	=	Hour
HAT	=	Hypoxanthine-aminopterine-thymidine
HT	=	Hypoxanthine-thymidine
IgG	=	Immunoglobulin G
iv	=	Intravenous
IVM	=	*In vitro* maturation

LIST OF ABBREVIATION (Continued)

IVF	=	*In vitro* fertilization
L	=	Liter
mA	=	Milliampare
mDPBS	=	Modified Dulbecco's phosphate buffered saline
mg	=	Milligram
min	=	Minute
ml	=	Milliliter
mM	=	Micromolar
mm	=	Millimeter
$MgCl_2$	=	Magnesium chloride
mSOF	=	Modified synthetic oviductal fluid
N	=	Normality
NaOH	=	Sodium hydroxide
ng	=	Nanogram
nm	=	Nanometer
N_2	=	Nitrogen
NaCl	=	Sodium chloride
Na_2HPO_4	=	Disodium hydrogen phosphate
NaH_2PO_4	=	Sodium dihydrogen phosphate
NaOH	=	Sodium hydroxide
O_2	=	Oxygen
OD	=	Optical density
PBS	=	Phosphate buffer saline

LIST OF ABBREVIATION (Continued)

PBST	=	Phosphate buffer saline + 0.05% Tween20
PCR	=	Polymerase chain reaction
PEG	=	Polyethylene glycol
pH	=	Potential of hydrogen ion
pNPP	=	para-Nitrophenyl phosphate
RPMI	=	Roswell Park Memorial Institute-1640, serum free RPMI
PVP	=	Polyvinyl pyrolidone
sc	=	Subcutaneous
sec	=	Second
TALP	=	Tyrode albumin lactate pyruvate
U	=	Unit

CHAPTER I

INTRODUCTION

In cattle, the sex ratio is generally 50:50 (male: female). Sperm is the one to determine the sex of the embryo. Spermatogenesis in cattle is the process by which male primary germ cells undergo meiosis. The sperm cells or the male gametes in cattle are heterogametic that contain one of the two types of sex chromosomes. They are either X- or Y-chromosome. In contrast, the egg cells or the female gametes contain only the X-chromosome hence, they are homogametic. Each sperm has an equal chance of carrying the X-chromosome or the Y-chromosome (Pace, 1990). If a sperm cell carrying an X-chromosome fertilizes an egg, the resulting zygote will be female, XX. If a sperm cell containing a Y-chromosome, the resulting zygote will be male, XY (http://biology.about.com/od/basicgenetics/p/chromosgender.htm).

The selection of X- or Y-bearing spermatozoa has been the goal for several methods developed for human and domestic animals. In livestock, various methods have been used to modify mammalian semen to increase the relative percentage of Y- or X-sperm in the semen fraction. In the dairy industry, higher number of cow for milk production is preferred, but higher number of bull is also preferred in beef industry for meat production. Here are some reasons for wanting sexed semen,

1. Production of higher number of bulls for seed stock producers.

2. Production of higher number of bulls for meat production.

3. Production of replacement females from a selected population.

4. Production of females from virgin heifers to reduce calving difficulties.

5. Production of higher number of cows for dairy industry.

Sexed semen has been used to increase the efficiency of the beef and dairy production. It could be used to produce the offspring of the wanted sex. The benefit of using sexed semen is the cost reduction of progeny determination. Sexed semen could also be used to produce specialized, genetically superior replacement heifers from a small proportion of the herd (Hohenboken, 1999).

Various methods including gel filtration, electrophoresis, swimming up, sperm sorting, centrifugation and immunological methods have been used in attempts to fractionate both human and animal sperms into X and Y enriched populations. These methods have been used to separate the spermatozoa into X- and Y-chromosome bearing fractions based on the differences of pH sensitivity (Rothschild, 1960), swimming speed (Ericsson *et al*., 1973; Rohde *et al*., 1973), motility (Sarkar, 1984; Sarkar *et al*., 1984), density (Haevey, 1946; Sumner and Robinson, 1976), surface charge (Kaneko *et al*., 1984; Cartwright *et al*., 1993), sperm surface protein (Prasad *et al*., 2010), size, head and mass (Windsor *et al*.,1993; Gledhill, 1988), adherence to Sephadex (Steeno *et al*., 1975; Adimoelja, 1987), H-Y antigen content (Goldberg *et al*., 1971; Peter *et al*.,1993; Sills *et al*., 1998) and DNA content (Pinkel *et al*., 1982; Johnson *et al*., 1989).

However, no method can give 100% accuracy in the semen sex selection. This problem might come from the large number of spermatozoa produced in each ejaculate and the fact that only one sperm fertilized (Pace, 1990). Semen sexing could also be carried out using gender specific antibodies. This method is based on the differences between antigens on the surface of each sperm gender.

Research objectives

The purpose of this research is to produce specific monoclonal antibody (mAb) against bovine Y-sperms to selectively eliminate the Y-sperms. The left over sperms (majority X-sperms) were used to *in vitro* fertilize (IVF) bovine oocytes. The IVF embryos were checked by PCR to determine the accuracy of the sexed sperm.

The objectives of this research are summarized as below

1. To produce mAb against bovine Y-sperms

    -Select the monoclonal antibodies that are specific to bovine Y-sperms

    -Use the monoclonal antibodies to selectively destroy Y-sperms

    -Determine the quality of sexed sperm by fertilization efficiency

2. To investigate the accuracy and precision of sexed sperms by determine the sex of embryos derived IVF using multiplex PCR

CHAPTER II

LITERATURE REVIEWS

2.1 Antibodies

Antibodies (or immunoglobulin, Ig) are a group of glycoproteins molecules that are synthesized by the host and secreted by B-lymphocytes and plasma cells in the immune system. They are produced in response to the presence of foreign molecules to identify and neutralize invasive foreign molecules in the body.

2.1.1 Antibody structure and classification

The basic structures of each antibody composed of one or more copies of a characteristic unit that can be visualized as a Y forming shape (Figure 2.1). Each Y-shape contains 4 polypeptides including 2 identical heavy (H) chains (~50-70 kDa) and 2 identical light (L) chains (~22-25 kDa) (http://pathmicro.med.sc.edu/mayer /igstruct2000.htm). They are linked by disulfide bonds between heavy and light chains in their constant regions and hydrophobic interaction in both chains.

Antibodies are separated into classes based on physical characteristics including the structure of heavy chains, size, charge, amino acid composition, carbohydrate content and the number of antigen binding sites. Mammalian species produce 5 classes of antibodies, IgG, IgM, IgA, IgE and IgD (Harlow and Lane, 1988) which are distinguished by their heavy chains γ, α, μ, ε and δ, respectively

(Figure 2.2). They are only 2 types of light chains, kappa (κ) and lampda (λ). In mice, 95% are κ light chains and only 5% are λ. Single antibody molecule contains only κ light or λ light chain. Each antibody class differs in valency as a result of different number of Y-shaped units that link to form the complete structure. IgG, IgE and IgD are of the monomeric type which containing 2H2L. IgA is mainly monomeric, but found as a dimer in saliva. IgM is composed of 5 monomeric units and has 10 antigen binding sites. The result from minor differences in the amino acid composition within each class, mouse IgG are divided into 4 subclasses, IgG_1, IgG_{2a}, IgG_{2b} and IgG_3 which have g_1,g_{2a}, g_{2b} and g_3 as heavy chain (Figure 2.3).

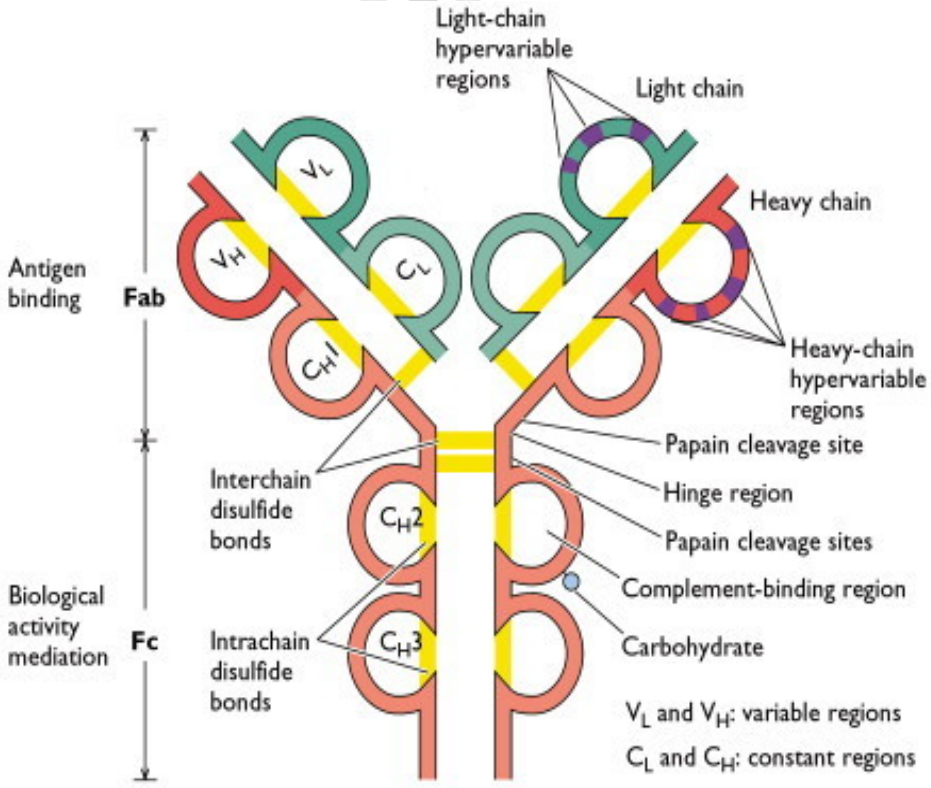

Figure 2.1 Basic Y-shaped structure of antibody consisting 2 heavy chains and 2 light chains. (http://www.virology.ws/2009/07/22/adaptive-immune-defenses-antibodies/)

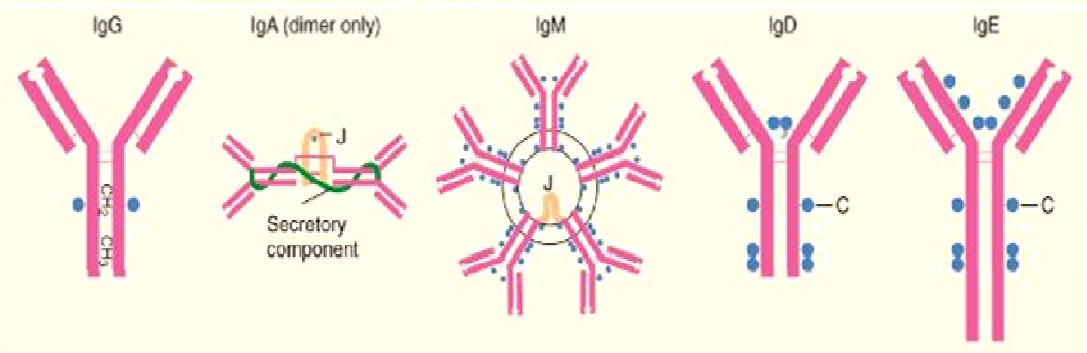

Figure 2.2 Characteristics of the immunoglobulin classes (http://www.pc.maricopa.edu/Biology/rcotter/BIO%20205/LessonBuilders/Chapter%2015%20LB/Ch15LessonBuilder_print.html)

Antibody	Human and Mouse			
	Light Chain	Subtype	Heavy Chain	
IgA	κ or λ κ or λ	IgA_1 IgA_2	α_1 α_2	
IgE	κ or λ	None	ϵ	
IgD	κ or λ	None	δ	
IgM	κ or λ	None	μ	
	Human			Mouse
IgG	Light Chain	Subtype	Heavy Chain	Light Chain	Subtype	Heavy Chain
	κ or λ κ or λ κ or λ κ or λ	IgG_1 IgG_2 IgG_3 IgG_4	γ_1 γ_2 γ_3 γ_4	κ or λ κ or λ κ or λ κ or λ	IgG_1 IgG_{2a} IgG_{2b} IgG_3	γ_1 γ_{2a} γ_{2b} γ_3

Figure 2.3 The overview of antibody classes and subclasses (http://www.rosenthallab.com/gallery/detail.php?fileName=antibody.GIF)

2.1.2 Function

The functions of each antibody class are summarized here (http://biochemis tryquestions.wordpress.com/2009/05/26/immunoglobulins-structure-and-functions/):

IgM antibodies: IgM is the primary antibody that produced first in immune responses. IgM is very effective in the complement activation and bacterial elimination. It compose of pentameric immunoglobulin (2H2L) structure (Janeway *et al*., 2001).

IgG antibodies: IgG is the most important immunoglobulin class which response for long-term protection from disease as a secondary responses. IgG is the dominant antibody found in blood and promotes the uptake of foreign antigens by immune cells.

IgA antibodies: IgA is the secretory antibody found in mucous membrane secretions, tears, colostrums and milk. This is the initial defense in respiratory, genitourinary tracts and gastrointestinal tracts against pathogen and also found in that passively protects the newborn (Woof and Kerr, 2006).

IgE antibodies: IgE is found in blood with very small concentrations. It is also associated with defense against parasite. IgA binds to the receptors on the basophiles and mast cells and plays an important role in the allergic response.

IgD antibodies: IgD is a minor blood component that typically bound to the B-lymphocyte surface.

2.1.3 Polyclonal versus monoclonal antibodies

Polyclonal antibodies (pAb) are a combination of immunoglobulins obtained from different lymphoid organs including spleen, lymph nodes and gut-associated by stimulated plasma cells (B-lymphocytes). In contrast, monoclonal antibodies (mAbs) are the identical immunoglobulin that produced by a single B-lymphocyte which is specific for a single antigen (epitope) (Leenaars *et al*., 1999).

2.2 Monoclonal antibody

In 1975, the mAb production was discovered by Georges J.F. Köhler and César Milstein. They sought the ways to create the long-living cells that could produce large amount of specific antibody. They fused the cancerous form of cells and normal antibody-producing cells together, forming the hybrid cells that could produce a specific antibody and also immortal. From this discovery, in 1984, they got the noble prizes in medicine (http://www.nobelprize.org/nobelprizes/medicine/laureates/1984/speedread.html).

2.3 Myeloma cell

A few mice strains can be induced to produce myeloma cells by injecting mineral oil into the peritoneum (Figure 2.4). In 1972, Potter isolated the myeloma cells from BALB/c mice and the isolated myeloma cells named mineral oil plasmacytoma (MOPC). The derivatives of BALB/c myeloma cells have become the most commonly used in the hybridoma fusion (Table 2.1) (Harlow and Lane, 1988).

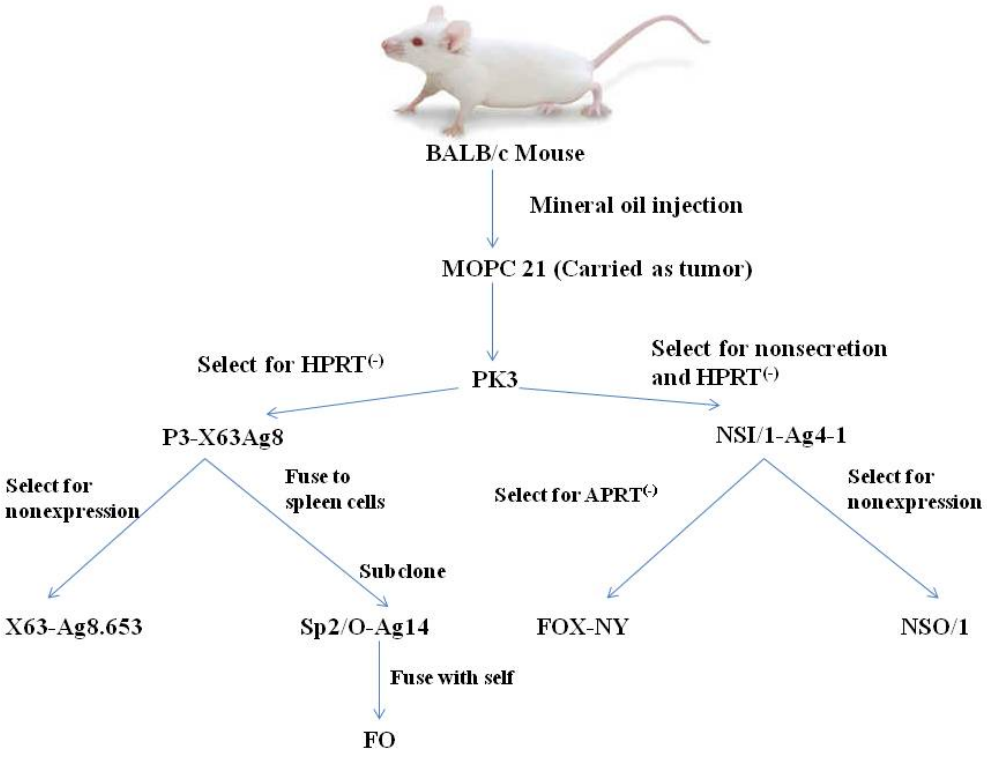

Figure 2.4 Myeloma family tree (adapted from Harlow and Lane, 1988).

Table 2.1 Myeloma cell lines (adapted from Harlow and Lane, 1988)

Cell line	Reference	Derived from	Chains expressed	Secreting	Comments
P3-X63Ag8	Köhler and Milstein (1975)	P3K	γ1, κ	IgG1	Not recommended
X63Ag8.653	Kearney *et al*. (1979)	P3-X63Ag8	None	No	Recommended
Sp2/0-Ag14	Köhler and Milstein (1976) Shulman *et al*. (1978)	P3-X63Ag8 × BALB/c	None	No	Recommended
FO	de St. Groth and Scheidegger (1980)	Sp2/0-Ag14	None	No	Recommended
NSI/1-Ag4-1	Köhler *et al*. (1976)	P3-X63Ag8	Kappa	No	Recommended
NSO/1	Galfre and Milstein (1981)	NSI/1-Ag4-1	None	No	Recommended
FOX-NY	Taggart and Samloff (1984)	NSI/1-Ag4-1	Kappa (?)	No	

2.4 Unfused myelomas drug selection

Hypoxanthine-guanine phosphoribosyltransferase (HPRT) is an enzyme important for purine nucleotide synthesis via purine salvage pathway. HPRT is a transferase that converts hypoxanthine to inosine monophosphate and guanine to guanosine monophosphate by transferring the 5-phosphoribosyl group from 5-phosphoribosyl 1-pyrophosphate to the purine.

In the fusion step, the myeloma cells containg a mutation of HPRT that cannot function in the salvage pathway of purine nucleotide synthesis has been used (Littlefield, 1964). The nucleotide synthesis pathways of both myeloma and normal cells when cultured in media without any drug are shown in Figure 2.5.

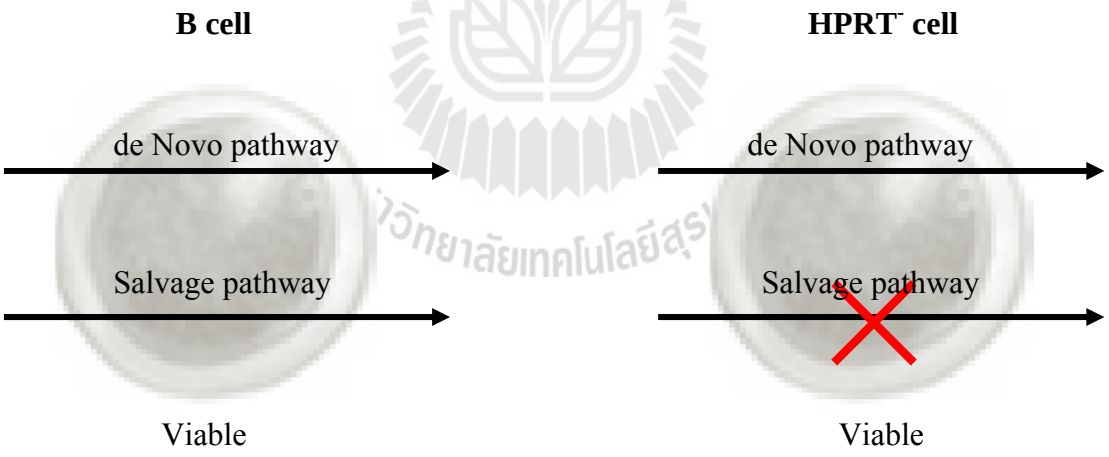

Figure 2.5 Nucleotide synthesis pathways when culture without any drug (Adapted from Harlow and Lane, 1988)

From the above, the myeloma cells can survive and multiply. In contrast, B-cells can survive with normal nucleotide synthesis pathways, but cannot multiply. To force the cells to use only the de novo nucleotide synthesis pathway, drug or

compounds are added into the culture media. Only the B-cells can use the salvage pathway. The drugs commonly use are aminopterin, methotrexate or azaserine. In the mAb production, the selection media used to select the fused or hybrid cells is hypoxanthine-aminopterin-thymidine (HAT) medium. Aminopterin, folic acid antagonist is a synthetic derivative of pterins. A folate analogue works as an enzyme inhibitor by competing for the folate binding site of dihydofolate reductase resulted in the depletion of nucleotide precursors and inhibited the DNA, RNA and protein synthesis (Farber *et al*., 1948).

The selection of fused or hybrid cells using HAT medium, only the fused cells between myeloma cell and B-cell will be able to grow in the media containing HAT as shown in Figure 2.6.

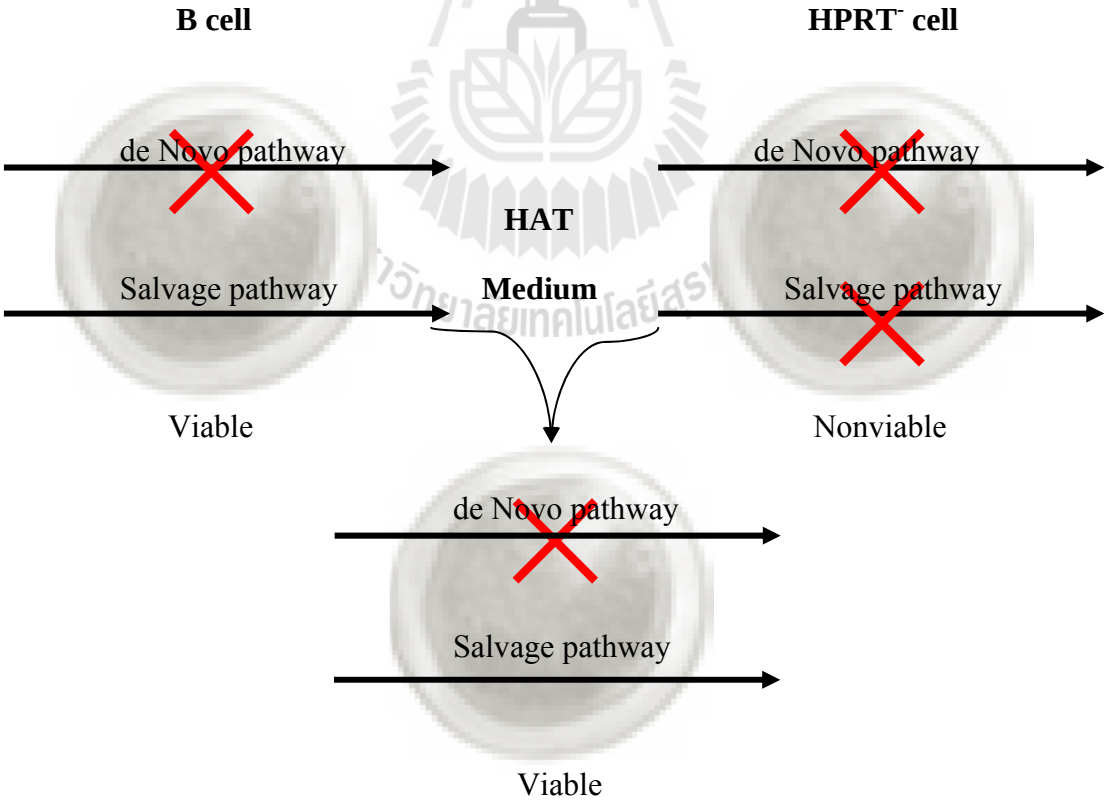

Figure 2.6 Drug selection for viable hybrid cells (Adapted from Harlow and Lane, 1988)

2.5 Immunizations

Immunization is the process of person is made immune or resistant to exposed molecules that are foreign to the body (http://www.who.int/topics/immun ization/en). The ability of a molecule to induce an immune response called immunogenicity. The immunogenicity is determined by chemical structure of the injected molecule and the host recognition of that molecule (Harlow and Lane, 1988).

2.6 Immunizing animals

Immunizing and breeding animals for the production of pAb or use as donors for hybridoma fusions is a technically straightforward and human procedure. The healthy, specific pathogens free and well-cared for animals are essential for obtaining a good immune response. The skill, patience and practical training are required for animal injection to safe and reduce the pain of animal. The choice of animal in Table 2.2 is defined by (Harlow and Lane, 1988):

-How much serum is needed?

-What species is the antigen isolated?

-Are polyclonal or mAbs needed?

-How much antigen is usable?

Table 2.2 Choice of animal to be used for monoclonal and polyclonal antibody production (Harlow and Lane, 1988)

Animal	Maximal amount of Sera (ml)	mAb	Inbred	Comments
Chickens	50	No	No	Good for highly conserved mammalian antigens
Guinea Pigs	30	No	No	Hard to bleed
Hamsters	20	No	Mostly not	Good choice for pAb production when antigen is limiting
Mice	2	Yes	Yes	Often best choice for mAb production, excellent genetics of immune response
Rabbits	500	No	No	Often best choice for pAb production even when antigen limiting
Rats	20	Yes	Yes	Good choice for mAb production

2.7 Adjuvant

Adjuvant is an immunological agent commonly used to enhance or stimulate the immune responses. The action of adjuvants is not fully understood, but incorporated some following components (Chodaczek, 2004; Howard and Kaser, 2006):

i. Act as a reservoir of immunogen forms to protect antigens from rapid catabolism, resulting in the slow release and extent the period of antigen presentation to immune system
ii. Act as an immune stimulating agent that is optimized by focusing a general immunostimulant and antigen in the microenvironment
iii. Cells of the immune system that are nonspecifically activated, so helping those interactions to support the antibody production

Freund's adjuvant

Freund's adjuvant is an adjuvant that most commonly used for research work. Freund's adjuvant is a water-in-oil emulsion prepared with nonmetabolizable oils. They are 2 types of Freund's adjuvant,

i. Complete Freund's adjuvant (CFA): the mixture of water-in-oil emulsion contains killed *Mycobacterium tuberculosis*, a very potent immune-modifier.
ii. Incomplete Freund's adjuvant (IFA): the mixture that identical to CFA except that the *Mycobacterium tuberculosis* is omitted.

It is generally assumed that IFA and CFA act by prolonging the lifetime of injected autoantigen, by stimulating its effective delivery to the immune system and by providing a complex set of signals to the innate compartment of the immune system, resulting in increased leukocyte proliferation and differentiation (Billiau and Matthys, 2001). The disadvantage of Freund's adjuvant is that it is inherently toxic, resulting in granulomas, sterile abscesses and ulcerations after administration (Rizvi and Bashir, 2004). To avoid the side effects of Freund's adjuvant, CFA is recommended to use only in the primary injection, while other boosts should be done with using IFA (http://www.research.uci.edu/ora/acup/freundsadjuvant.htm).

2.8 Dose and form of the antigen

The suitable dose of antigen used to induce a good immune response depends on the host animal and individual antigen. The suitable suggested dose for mice is summarized in Table 2.3.

2.9 Route of injection

The potential routes of introducing an antigen into mice are summarized in Table 2.4.

Table 2.3 Dose of immunogen for mice (Harlow E. and Lane D.1988)

		Primary injections and boosts			Final boosts		
Antigen form	**Examples**	**Possible routes**	**Dose**	**Adjuvant**	**Possible routes**	**Dose**	**Adjuvant**
Soluble proteins	Enzyme Carrier proteins conjugated with peptide Immune complex	ip[a] sc[b]	5-50 µg	+	iv[c]	5-50 µg	-
Particulate proteins	Viruses (killed) Yeasts (killed) Bacteria (killed) Structural proteins	ip sc	5-50 µg	+	iv	5-50 µg	-
Insoluble proteins	Bacterially produced from inclusion bodies Immunopurified proteins bound to beads	ip sc	5-50 µg	+	ip	5-50 µg	-
Live cells	Mammalian cells	ip	10^5-10^7 cells	-	iv	10^6 cells	-
Live Tumorigenic cells	Oncogenic mammalian cells	ip sc	10^4-10^6 cells	-	iv	10^6 cells	-
Carbohydrates	Polysaccharides Glycoproteins	ip sc	10-50 µg	+/-	iv	10-50 µg	-
Nucleic acids	Carrier proteins conjugated with N.A.	ip	10-50 µg	+	iv	10-50 µg	-

[a], Intraperitoneal; [b], Subcutaneous; [c], Intravenous

Table 2.4 Route of mice injection (Harlow and Lane, 1988)

Route	Abbreviation	Uses		Maximum volume (ml)	Adjuvant	Immunogen requirement	Comments
		Primary injection and boosts	Final boost				
Intraperitoneal	ip	Good	Fair	0.5	+/-	Soluble/or insoluble	If used for final boost, wait 5 days before fusion
Subcutaneous	Sc	Good	Poor	0.2	+/-	Soluble/or insoluble	Local response, Serum level slower to increse
Intravenous	iv	Poor	Good	0.2	No Frend's	Soluble, Ionic detergent <0.2% Nonionic detergent <0.5% Salt <0.3M Urea <1M	Poor immunizing
Intramuscular	im	Not recommended for mice					
Intradermal	id	Not recommended for mice					
Lymph node		Special uses		0.1	No Frend's	Soluble/or insoluble	Good applications for experienced workers

2.10 Sperm sexing technology

Sperm sexing technology is a goal in both agricultural industry and livestock sections. This technology used to control the sex of offspring of agriculturally important animals by altering the sex ratio. In livestock, the sex can be altered ratio to increase the milk production for dairy industry and also increase the meat for meat industry. Various methods have been used for sperm sexing including gel filtration, electrophoresis, swimming up, sperm sorting, centrifugation and immunological methods. These methods have been used to separate the spermatozoa into X- and Y-chromosome bearing fractions based on:

I. The differences of pH sensitivity

In 1960, Rothschild proposed that male sperms preferred an alkaline environment and female sperms preferred a more acid environment. Hassan in 2005 also confirmed that the sperm separation efficiency of an alkaline pH 9.0 resulted in the high amount of Y-sperms (60.28%). In contrast, Diasio and Glass in 1971 studied the effect of pH on the migration of X- and Y-sperm. The results indicated that the X- and Y-sperms cannot be distinguished on the basis of the migration through media of varying pH.

II. Motility and swimming speed

Sperm separation using the motility and swimming speed is based on the active sperm migration. In 1973, Rhode *et al*. reported that a small number of Y-sperm from the frontal zone during active migration when using an *in vitro* method for horizontal sperm penetration. In the same time, Ericsson *et al*. (1973) reported that

they could enrich Y-sperm by allowing the sperm to swim into a solution containing high concentration of bovine serum albumin. Sarka *et al*., (1984) also reported the success of enriching X- and Y-sperm to 80% purity using a laminar-flow fractionation method.

Check and Katsoff (1993) found that 88.5% yielded male birth using modified swim-up method for sperm separation and 83.6% of Y-sperms were obtained from stained sperm with quinacrine using the same separation. In contrast, Han *et al*., (1993); De Jonge *et al*., (1997) and De Jonge, (1999) found that this method did not significant change in the ratio of X- and Y-sperm, but increased only 1-2% Y-sperm. In addition, Penfold *et al*., (1998) found that Y-sperm did not swim faster than X-sperm when using simple salt.

III. Density

X-sperm contains DNA content higher than Y-sperm (Simpson and Larson, 2002) which make the Y-sperm lighter and better swimming ability than the X-sperm. From the differences of DNA content, the sperms can be separated on their sedimentation velocity. Cui and Matthews (1993) observed the average size of X-sperm and found that X-sperms are bigger and longer than Y-sperms. They recommended that if the X-sperms head size effects the separation using Percoll gradient, then the X-sperms might sediment faster than the Y-sperms. They found the slight increase of X-sperm when using 95% Percoll (Samura *et al*., 1997).

Bongso *et al*. (1993) reported that the most effective sperm separation method used for frozen sperm is a single Percoll layer. De Maistre *et al*. (1996) also reported that the sperm membrane characteristics were enhanced when separated with

Percoll. But Sharma *et al*. (1997) contradicted that report because they found that the double column was more efficient in separating than single. No difference of the amount of motile sperm was found when using Percoll separation was used by Mushayandebvu *et al*., 1995 and Smith *et al*., 1995. Abnormal sperm samples were separated using Percoll and mini-Percoll columns, the result indicated that the greater motile sperm yields were obtained (Ng *et al*. 1992).

The multiple discontinuous Percoll density gradient centrifugation method has been used for the sperm selection and found that over 77% from many reports were obtained. Watkins *et al*. (1996) reported that the X-sperms obtained from this method have greater longevity in motility and have shorter tails. Andersen and Byskov (1997) found that when seven-step Percoll gradient (100%-40%) underlayered with 100% NycoPrep was used for sperm separation, resulted in the greatly enrichment of X- and Y-sperm.

Mohri *et al*. (1986) reported that the X-sperms sediment faster than the Y-sperms when separated using 12 steps discontinuous Percoll density gradient and 94% purity of X-sperms were obtained. Kobayashi *et al*. (2004) found that the female sex ratio was slightly increased to 52.9% when sperms were separated using discontinuous Percoll gradients method.

However, the sexed-sperms obtained from sperm separation using Percoll gradient were affected from the toxicity of Percoll (Chen and Bongso, 1999). Resende *et al*. (2011) reported the percent increased of female embryos derived IVF using sexed-sperm obtained from Percoll gradient. They found the female sex ratio increased to 62% and considered that sperm selection using Percoll was possible to use for sperm sexing.

Rohde *et al*. (1975) reported that the Y-sperms can be separated by centrifuged at 26,400 ×g in a discontinuous sucrose gradient. Corson *et al.* (1984) and Dmowski *et al*. (1979) also successfully enriched Y-sperms using albumin gradient centrifugation. However, Evan *et al*. (1975) and Ross *et al*. (1975) could not enrich Y-sperm using the same method. Beernink and Ericsson (1982) also observed that 79% male birth ratio from 84 delivered and after that Ericsson and Beernink (1987) reported again that they found 73% male from 454 birth when sperms were separated by albumin gradient. Jaffe *et al.* (1991) confirmed the results of sexed-sperm got from albumin gradient sperm separation increased the Y-sperm ratio after used for IVF. Lobel *et al*. (1993) reported that Y-sperms separation using albumin separation method altered the sex ratio of clinically relevant. However, Zavos (1985) suggested that sedimentation methods using albumin gradients may not be effective for separating X and Y-spermatozoa. Chen *et al.* (1997) also found that Y-sperm separation using albumin did not alter and did not increase the Y-sperm ratio.

IV. Surface charge

The different in DNA content between X- and Y-sperm, might affect the surface proteins of the sperms. Nevo *et al*. (1961) and Bangham (1961) agreed that non motile sperms have negative charge on the outter surface and move to anode. It has been proposed that the electrical charges of each X- and Y-sperm were different. It might be related to the nearaminic acid containing glycoproteins on the X-sperms membrane surface (Prasad *et al.*, 2010). The method used for sperms separation based on the differ of surface charge is the free flow electrophoresis, that used tromethamine (THAM) buffer to separate the sperm into 2 populations. Schroder (1941) reported

that the electrophoresed-sperms used in insemination changed the sex ratio from normally expected. In 1957, Gordon also found the similar results.

Ishijima *et al*. (1991) reported that the sperms can be separated by 2 different methods, using a laser-rotating prism and using an electrophoretic light scattering spectrophotometer. The results found that more than 95% X-sperm and 80% Y-sperm were obtained from using zeta potential at -20 and -15 mV, respectively. Blottner *et al*. (1994) found that X-sperms were enriched in the cathode and Y-sperms were enriched in anode. Manger *et al*. (1997) confirmed that the results from sperm separation using this method were similar to Blottner (1994). However, some research groups found little or no proof to confirm the separation using electrophoresis to increase the sex ratio (Siljander, 1936; Kordts, 1952; Pilz, 1952 and Vesselinovich, 1960) and the motility of the electrophoresed sperms is normally lost (Gledhill, 1988).

V. Sperm surface protein

Histocompatibility Y-chromosome antigen (H-Y) is a male specific antigen found in many male mammalian tissues. H-Y antigen is postmeiotic expressed (Koo *et al*. 1979) and expressed in testis (Müller *et al*, 1978), spleenocytes (Brunner *et al*. 1987) and white blood cells (Brunner *et al*. 1987). From its expression location, H-Y positive sperms could be separated using the antibody against H-Y antigen.

H-Y antigen has been used in sexing method including sperm and embryo sexing by producing antibodies against H-Y antigen (Epstein, 1980; Wachtel, 1988; Avery and Schnidt, 1989; Patthanawong *et al*., 2010). Bennett and Boyse (1973) reported that when sperms were treated with anti H-Y followed by adding the

complement, the X-sperms were higher than Y-sperms 8%. Koo *et al*. (1973) also confirmed that sperms were separated into 2 groups using the H-Y antigen and considered that H-Y antigen was higher expressed in Y-sperm (Koo *et al*. (1979). Zavos (1983) and Ali *et al*. (1990) treated sperms using the anti H-Y antigen. However, they found that H-Y antigen is absorbed on the cell membrane of both X- and Y-sperms during spermatogenesis (Burgoyne *et al*., 1986; Burgoyne, 1987; Hassan, 2005; Prasad *et al*., 2010).

Hendriksen *et al*. (1993) studied about the binding of anti H-Y antigen to separated X- and Y-chromosome bearing porcine and bovine sperm. The results found that their experiments did not demonstrate the H-Y antigen is favored expressed in Y-sperms and found similar in anti H-Y antigen binding between X- and Y-sperms. Howes *et al*. (1997) found that no difference between X- and Y-sperm surface membrane when run on SDS-PAGE, but found the X-specific protein when compare using two-dimensional SDS-PAGE. Scott Sills *et al*. (1998) found the H-Y antigen was higher slightly expressed in human Y-sperms, but it also found the expression in X-sperms. Form their results suggested that H-Y antigen might not be appropriate for human sperms sex selection.

VI. Size and shape

Size and shape are the criteria that often used for sperm separation. X-sperms have been supposed to be bigger than Y-sperms. Shettles (1961) claimed that the sperms can be separated into 2 groups using the different of sperm morphology. In 1972, Roberts present the idea about the different in the size between X- and Y sperms. Mc Evoy (1992) reported that about 3-4% DNA difference in X- and Y-sperm

DNA, but their idea did not have evidences to support and could not be repeated. Cui (1997) found the length, perimeter, sperm head area, the length of sperm neck and tails of X-sperms were significantly larger and longer than Y-sperms. Based on their experimented in 2001, Hossain *et al*. found that size and shape of X- and Y-sperms were similar.

VII. Adherence to Sephadex

Steeno and Adimoelja (1975) reported human sperm separation using filtration though Sephadex gel. Sexed-sperms that bound to Sephadex showed higher percent of Y-sperms (Pearson and Bobrow, 1970). In 1984, Adimoelja reported that sperms that passed though the Sephadex gel filtration are enriched for the X-sperms. Quinlivan *et al*. (1982) have confirmed the results of sperm sex selection using Sephadex method by counting F-body on sperms. Vidal *et al*. (1993) reported that the result of filtrated sperm obtained from Sephadex was slightly increased the Y-sperm to 52.5%. However, other researchers have not been able to repeat this finding (Downing *et al*., 1976; Schilling *et al*., 1978). Beckett *et al*. (1989) also found that the Sephadex treated sperms did not significantly increase the sex ratio of X- and Y-sperms.

VIII. DNA content

Sperm sexing based on DNA content is the most successful method that often used to separate sperms. Flow cytometric cell sorting technique was used to measure the difference of DNA content between X- and Y-sperm (Garner and Seidel, 2008). The difference of DNA content in mammalian X-sperm contain from ranges

2.8-7.5% more DNA than Y-sperm (rabbit 3.0%, pig 3.6%, horse 3.7%, sheep 4.2%, cow and bull 3.8-4.2%) (Renaville *et al*., 2002; Prasad *et al*., 2010). Sperms treated with fluorescence dye and the differences of the fluorescence dye can be used to separate the sperms (http://www.gpo.or.th/rdi/html/Flow-cytometry.html; Garner *et al*. 1983; Van Dilla *et al*., 1985). Johnson *et al*., (1987) and Johnson (1992) separated bull, boars, rams and chinchilla sperm using flow sorting method.

Johnson and Welch (1999) reported the production rated of X- or Y-sperm fraction was obtained at 6×10^6 sexed sperm with 90% or more of one sex sperm and now have been applied in rabbits, swine, cattle or horse (Johnson *et al*., 1989; Rath *et al*., 1999; Cran *et al*., 1995).

The sex ratios of sexed-sperm obtained from flow cytometry increased from 50:50 to 85 to 98% of one sex. The fertility rate of flowed sexed-sperm is about 12 to 25% which is lower than unsexed-sperm but have displayed completely normal morphological appearance and reductive function (Johnson, 1997).Johnson (2000) produced highly enriched X- or Y-sperms using flow cytometry. However, this method is limited by the use of machine, the cost, the low sorting rate, mutagenic effects, very low survived sperm, low fertility, (Hudson *et al*., 2012; Garner and Seidel, 2008; Prasad *et al*., 2010).

2.11 Bovine embryo development

Normal fertilized embryos started mitotic cell division from 1-cell into a multicellular embryo (Figure 2.7). Two-cell embryos stage normally found at day 2 and the embryos continue to multiply and develop to blastocyst and hatched blastocyst at day 7-9 (Wong *et al*., 2010; Leidenfrost *et al*., 2011). The normal bovine embryo

development time is shown in Table 2.5.

Table 2.5 The general bovine embryo development. (Adapted from: http://www.geochembio.com/biology/organisms/cattle/cattle-life-cycle.html)

	Fertilized egg (day)	**2 cell (day)**	**4 cell (day)**	**8 cell (day)**	**16 cell (day)**	**Morula (day)**	**Early Blastocyst (day)**	**Blastocyst (day)**	**Expanded Blastocyst (day)**	**Hatched Blastocyst (day)**	**Expanding Hatched Blastocyst (day)**
Day*	0	1	2	3	4	4-5	6	6-7	6.5-7.5	7-8	8-9

*Day after fertilization

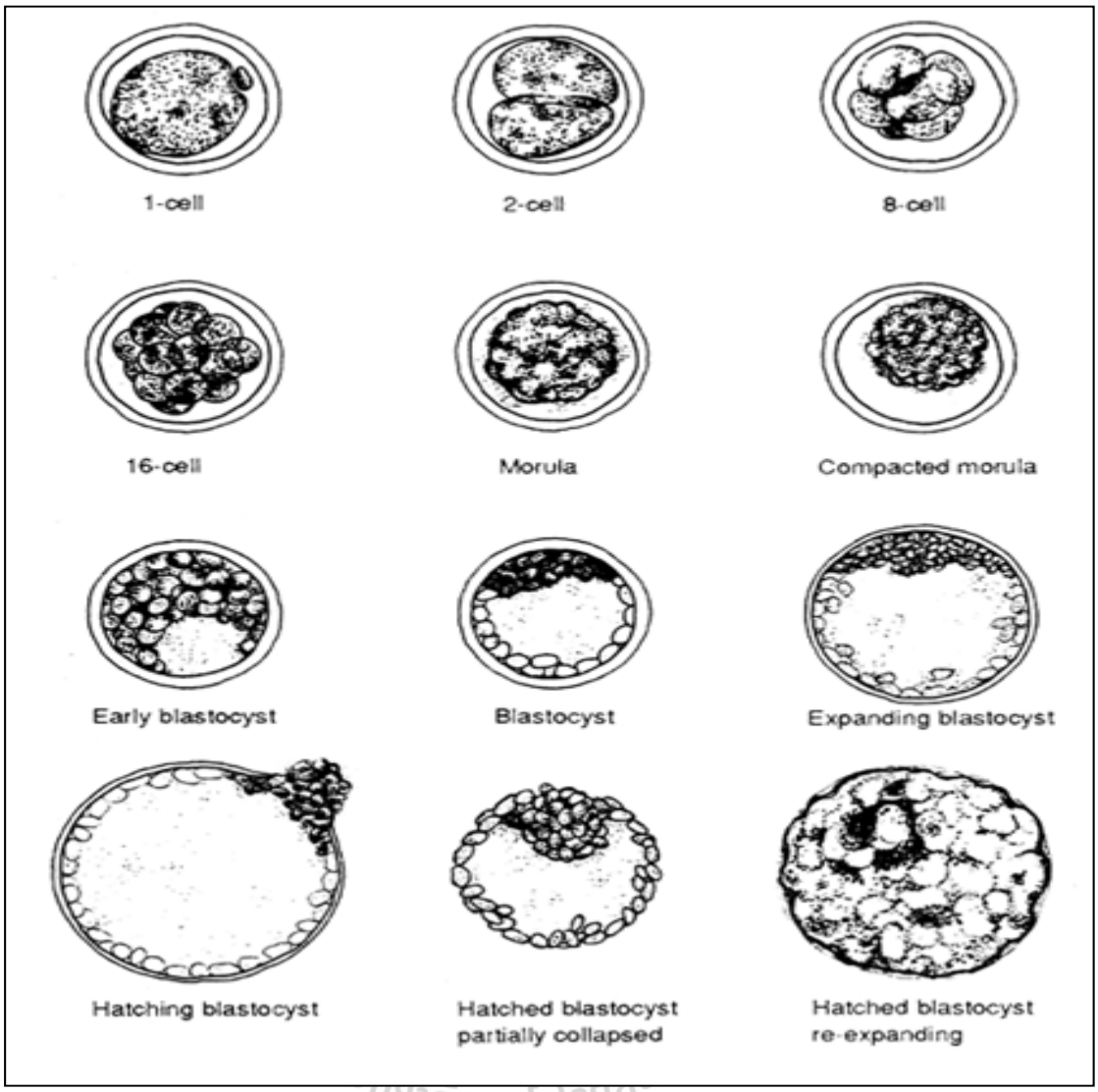

Figure 2.7 Embryo developments in each stage (http://www.fao.org/DOCREP/004/T0120E/T0120E07.htm)

CHAPTER III

MATERIALS AND METHODS

3.1 Monoclonal antibody production

3.1.1 Preparation of antigens for immunization

Frozen bovine Y-sperms purchased from China Mengniu Dairy Company Limited, China, were thawed in 37°C water bath for 30 sec and then 100 µl of Y-sperms were transferred into 2 ml of pre-incubated Tyrode albumin lactate pyruvate medium (TALP). The TALP was pre-incubated at 38.5°C under humidified atmosphere of 5% CO_2 in air for 6 hrs prior to use. The 2 ml TALP containing bovine Y-sperms were incubated at 38.5°C, under humidified atmosphere of 5% CO_2 in air for 45 min. After that, approximately 1,800 ul of the upper layer of supernatant containing motile spermatozoa from each tube were pooled and centrifuged at 540 ×g for 7 min. The pellet was resuspended in 4 ml phosphate buffer saline (PBS), pH 7.2 and centrifuged 2 times. The bovine Y-sperms were then adjusted to 2.5×10^5 sperms/ml for the first injection and the first boost and 2.5×10^6 sperms/ml for the final boost.

3.1.2 *In vivo* immunization

Two 8-weeks old female BALB/c mice purchased from the National Laboratory Animal Center, Mahidol University, were first intraperitoneally (ip) injected with 200 µl mixture of 1:1 bovine Y-sperm and Complete Freund's Adjuvant

(Sigma®). The mice were boosted intravenously (iv) and subcutaneously (sc) 2 times at week 2 and 4 with 200 µl mixture of 1:1 bovine Y-sperm and Incomplete Freund's Adjuvant (Sigma®).

3.1.3 Myeloma cell

X63-Ag8.635 myeloma cell line obtained from Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University was used in the fusion step. Two weeks before the fusion, the cells were rapidly thawed in 37°C water bath until completely melted. Then the thawed myelomas were transferred into 5 ml pre-warmed serum free Roswell Park Memorial Institute-1640 (RPMI) medium and centrifuged at 4°C, 300 ×g for 5 min. The supernatant was discarded and the cell pellet was transferred into a 75 ml culture flasks containing 20 ml pre-warmed Roswell Park Memorial Institute-1640 containing 20% fetal bovine serum (FBS) (cRPMI). The flasks were then incubated at 37°C under humidified atmosphere of 5% CO_2 in air. The cultured media was changed at day 7 and cultured another 7 days until used.

3.1.4 Feeder cells preparation

A 10-weeks old female BALB/c mouse was euthanized by cervical dislocation and the skin was striped from the abdominal wall. Ten ml of cRPMI were injected into the peritoneal cavity. The peritoneal fluid was withdrawn using syringe and kept at 4°C until used. The peritoneal fluid from 1 mouse was brought up to 100 ml by cRPMI.

3.1.5 Spleen cell collection

Two immunized mice (3.1.2) were euthanized by cervical dislocation. The

abdominal area was sprayed with 70% ethanol, and the spleens were removed by sterile operation. After that, both spleens were placed in a sterilized Petri dish containing 70% ethanol for 30 sec and then transferred to sterile Petri dish containing PBS and 3x penicillin-streptomycin (300 unit/L penicillin and 300 μg/L streptomycin) for 5 min to reduce the chance of bacterial contamination. During the soaking, the spleens were trimmed and any contaminating tissues were removed. Then the spleen cells were transferred into sterile Petri dish containing RPMI.

3.1.6 Cell fusion

The spleens were clipped off and minced using 19-gauge needles. The tear was continued until most of the spleen cells have been released. Any cell clumps were disrupted by pipetting. The spleen cells were transferred into a sterile centrifuge tube. The 2 weeks myeloma cells cultured were transferred to another sterile centrifuge tube using pipette. Both spleen cells and myeloma cells were washed with 40 ml prewarmed RPMI 3 times and spin at 300 ×g for 5 min. The cells number of each cell type were counted using hemacytometer chamber. The cells were mixed together at the ratio of 1:6.4 (myeloma cells : spleen cells) in a new sterile centrifuge tube containing prewarmed RPMI and then centrifuged at 37 ^{o}C, 300 ×g for 5 min. The supernatant was discarded and the pellet was left to dry. Prewarmed 1.5 ml polyethylene glycol-4000 (PEG-4000) were gently added and waited 30 sec before slowly added drop wise into 10 ml pre-warmed RPMI over the first 5 min followed by filling up to 50 ml. The cells were collected by centrifugation at 37^{o}C, 300 ×g for 5 min. The supernatant was discarded and the cells were resuspended in pre-warmed hypoxanthine-aminopterin-thymidine (HAT) medium and the cells were adjusted to a concentration of 2-4.5 x 10^{5} spleen cells/ml. The resuspended cells were plated into 24

well culture plates at 1 ml/well and incubated at 37^{o}C under humidified atmosphere of 5% CO_2 in air**.**

At day 4 and 5, the wells were observed under inverted microscope to check for contamination. The contaminated wells were eliminated by adding 200 µl of 20% $CuSO_4$ solution. After 7-10 days, 0.5 ml of the supernatant from each well was discarded and replaced with 0.5 ml HT medium containing feeder cells. Every 3 days, half of the culture medium was replaced.

3.2 Screening assay

When the hybridoma cells were grown to about 2-3 mm, the supernatant was used to screen for antibody specificity against bovine sperms and egg yolk (negative control) using ELISA method.

ELISA

Frozen unsexed bovine sperms were thawed and swamp up as described in 3.1.1. The sperm concentration was adjusted to 2 x 10^5 sperms/ml. Fifty µl of the sperms suspension were added into each well of 96 well ELISA plates. Fifty µl of 0.5% egg yolk in PBS was used as negative control. The plates were incubated at 37^{o}C overnight. The bovine sperm cells were fixed with absolute ethanol for 5 min and washed 3 times with PBS before blocked with 100 µl of 5% skim milk overnight at 4^{o}C. The blocking solution was discarded and the wells were washed 3 times with PBS. Fifty µl of hybridoma cells cultured media used as antibody were added into each wells and incubated at 37^{o}C for 1 hr. The well were washed 3 times with PBS +

0.05% Tween 20 (PBST) and then 50 µl of 1:2,000 alkaline phosphatase-conjugated goat-anti-mouse IgG were added into the wells and incubated at 37°C in for 1 hr. The alkaline phosphatase-conjugated goat-anti-rabbit IgG was discarded followed by washing 3 times with PBST. Fifty µl of *p*-Nitrophenyl phosphate (pNPP) substrate were added to each wells and incubated at room temperature in the dark for 1 hr. Optical density (OD) at 405 nm of each well was measured and recorded.

3.3 Hybridoma cloning (modified limiting dilution method)

The hybridoma cells in the positive wells were diluted by resuspend 5 µl of hybridoma cells into 10 ml cRPMI containing feeder cells. Seven ml of the hybridoma cells suspension were added into the 96 culture plate by dropping 3 drops/well using 10 ml Sero pipette into row A and B, then 2 drops/well into row C and one drop/well into row D. After that 4 ml of cRPMI containing feeder cells were added into the remaining 3 ml of the hybridoma cells suspension. The hybridoma cells suspension were mixed and then dropped 3 drops/well into the row E and F, then 2 drops/well into row G and one drop/well into row H. The plates were then incubated at 37°C under humidified atmosphere of 5% CO_2 in air. At day 5, the single colony was observed and the supernatant was collected for rescreening using ELISA method as described in section 1.3.1.6.

3.4 Monoclonal antibody binding on the bovine Y-sperm surface

To confirm that the selected mAbs were specific to only bovine Y-sperm surface, the bovine Y-sperms were used as positive control while X-sperms were used

as negative control in the binding assays. The sperms were separately smeared on clean glass slides and incubated at 37°C overnight. The coated sperms were fixed with absolute ethanol for 5 min at room temperature and washed 3 times with PBS. The coated sperms were blocked with 5% skim milk for 3 hrs at room temperature. The glass slides were washed 3 times with PBS and 50 µl of cultured supernatant were dropped onto the glass slide. The glass slides were incubated at 37°C for 1 hr and washed 3 times with PBST. One hundred µl of 1:5,000 goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) were dropped onto the glass slide and incubated for 1 hr. The glass slides were washed 3 times with PBST before the binding was observed under fluorescence stereo microscope.

3.5 Hybridoma cells cryopreservation and thawing

The hybridoma cells were harvested by centrifugation at 300 ×g for 5 min. The supernatants were collected and kept at 4°C until used. The cell pellet was mixed with 1 ml of pre-cooled -20°C 10% DMSO (Dimethyl sulfoxide) in cRPMI. The hybridoma cells suspension were rapidly transferred into cryopreservation tubes. After the caps were rapidly closed, the tubes were stored at -80°C overnight and then transferred to aluminum canes in liquid N_2.

The frozen hybridoma cells were thawed by removed the cryopreservation tube from liquid nitrogen (LN_2) and rapidly thawed in 37°C water bath. While tiny pieces of ice still remained in the cell suspension, the cell suspension were diluted with cold 10 ml RPMI drop wise with gentle mixing to reduce the DMSO concentration. The hybridoma cells were harvested by centrifuged at 300 ×g for 5 min at 4°C. The supernatant was discarded. The cell pellet was resuspended in prewarmed 10 ml

cRPMI and transferred to 25 ml culture flasks and cultured at 37^{o}C under humidified atmosphere of 5% CO_2 in air.

3.6 Monoclonal antibody purification

The selected mAb was purified using ammonium sulfate precipitation method. Saturated ammonium sulfate was prepared by adding 761 g of ammonium sulfate to 1 L distilled water. While the mAb is being stirred gently at 4^{o}C, equal volume of saturated ammonium sulfate was slowly added to bring the final concentration to 50% and continue stirring overnight. The precipitant was harvested by centrifuged at 20,000 ×g for 30 min at 4^{o}C. The supernatant was carefully discarded and the precipitant was washed with 50% saturated ammonium sulfate then harvested again. The pellet was resuspended using PBS with the least volume possible. The mAb solution was transferred to dialysis tubing and dialyzed against PBS. The PBS was replaced 3 times before the remaining debris was removed by centrifugation at 20,000 ×g for 15 min at 4^{o}C. The purified mAb was aliquoted to 1.5 sterile tubes and kept at -20 ^{o}C until used.

3.7 Monoclonal antibody characterization

3.7.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of 12% was used to determine the molecular weight of produced mAb compare to standard protein marker (B) according to the Amersham Bioscience's protocol. All reagent used were prepared as shown in the Appendix I.

Polyacrylamide gels were prepared according to standard protocol. Samples were mixed with loading dye and boiled for 5 min before loaded into well. The gels were run at 30 mA in 1x running buffer for 90 min. After that one gel was stained with coomassie for 15 min and then destained with destain solution.

3.7.2 Western blotting

A gel obtained from SDS-PAGE, nitrocellulose membrane and filter papers were soaked in transfer buffer for 5 min before set up. The transfer sandwich was set up by the wet filter papers were firstly placed on the semi-dry transfer chamber and followed by nitrocellulose membrane, gel and filter papers as shown in Figure 3.1. The machine was run at constant 400 mA for 2 hrs. The blotted membrane was blocked by soaking in 5% skim milk in PBS overnight with shaking. The blocking solution was discarded and the membrane was washed 3 times with PBS for 15 min. Diluted of goat anti-mouse Ig (H+L), human adsorbed conjugated with alkaline phosphatase in 10 ml PBS were added into the box. The membrane was incubated at room temperature with shaking for 2 hrs then discarded. The membrane was washed 3 times with PBST for 15 min and 5 ml of 1x of 5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium (BCIP/NBT) was added. The color was developed by incubated the membrane in the dark at room temperature for 1 hr. The reaction of alkaline phosphatase was stopped by washing the membrane with water.

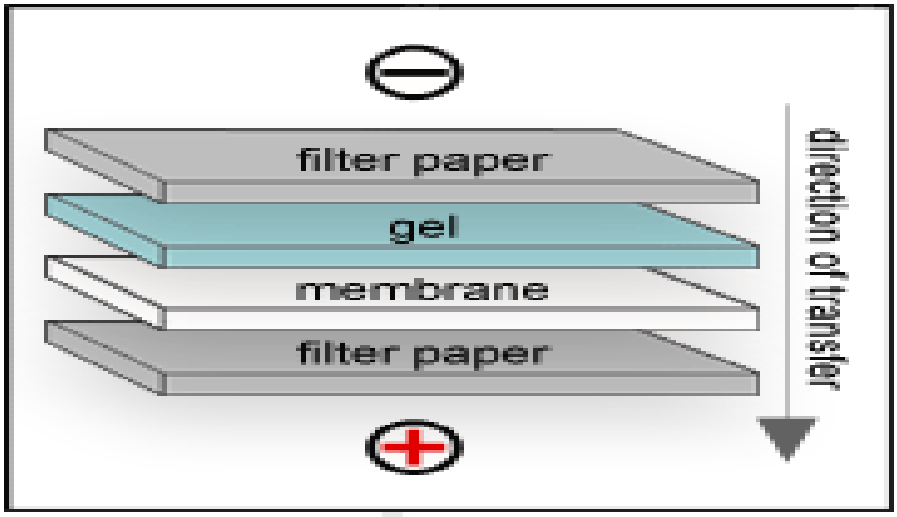

Figure 3.1 The electrophoratic blotting system using semi-dry transfer chamber. (http://www.cardio-research.com/protocols/western-blot)

3.7.3 Monoclonal antibody isotype and subtype identification

The antigen-dependent procedure for isotype determination was used to determine the heavy chain classes and light chain types of the mAbs using ImmunoPure® MAB Isotyping Kit II (PIERCE, Rockford, IL). The procedure was performed followed the manufacturer's recommendations with slight modification. Briefly, 50 µl of the bovine Y-sperm were coated into each of the nine wells and incubated at 37^{o}C for 2 hrs. The sperms were fixed with absolute ethanol for 5 min and washed 3 times with PBS before blocked with 100 µl of 5% skim milk overnight at 4^{o}C. The blocking solutions were discarded and the wells were washed 3 times with PBS. Twenty µl of purified mAbs were added into each wells and incubated at 37 ^{o}C for 1 hr. The wells were washed 3 times with PBST and then 50 µl of normal rabbit serum (negative control) or subclass-specific anti-mouse immunoglobulins were added

to each bovine Y-sperm coated well. The plate was incubated at 37°C for 1 hr and washed 3 times with PBST. Fifty µl of 1:2,000 alkaline phosphatase-conjugated goat-anti-rabbit IgG were added into each wells and incubated at 37°C for 1 hr. The alkaline phosphatase-conjugated goat-anti-rabbit IgG were discarded. The plates were washed 3 times with PBST. Fifty µl of pNPP substrate were added in each wells and incubated at room temperature under dark condition for 1 hr. The Optical density of each well was measured at 405 nm.

3.8 The effects of guinea pig complement concentrations and incubation time on sperm survival

Frozen bovine-sperms were thawed in 37°C water bath for 30 sec and 100 µl of the thawed sperm were transferred into the 2 ml pre-incubated at 38.5°C TALP prior to use. The guinea pig complement was added into each pre-incubated TALP tube to the final concentration of 2, 5 and 10% and then the TALP tubes were incubated at 38.5°C under humidified atmosphere of 5% CO_2 in air with varied incubation time of 15 and 30 min. After the incubation, approximately 1,800 µl of the upper layer of supernatant containing the motile sperm were centrifuged at 540 ×g for 7 min. The sperm pellet was resuspended and the survived sperms were observed and counted under inverted microscope. The final concentration of guinea pig complement with the highest amount of survived sperms was selected. The effect of guinea pig complement incubation time was done as mentioned above excepted only the incubation times were varied to 15, 30 and 45 min. The appropriated final concentrations of guinea pig complement and incubation time were selected for sperm sexing.

3.9 Sperm sexing

Forty µl of each selected mAbs were mixed with 2 ml pre-incubated TALP and incubated at 38.5°C under humidified atmosphere of 5% CO_2 in air. Frozen bovine sperms were thawed in 37°C water bath for 30 sec and 100 µl of the thawed sperm were transferred into the 2 ml pre-incubated TALP containing mAb and then the tubes were incubated at 38.5°C under humidified atmosphere of 5% CO_2 in air for 20 min. The final concentration of guinea pig complement concentration at 2% was mixed into the TALP-sperms containing tube by gently mixing using fine-tip pipette and then incubated at 38.5°C under humidified atmosphere of 5% CO_2 in air. After 45 min of incubation, approximately 1,800 µl of the upper layer of supernatant containing the motile sperm were pooled and centrifuged at 540 ×g for 7 min. The pellet was resuspended in 4 ml pre-incubated TALP and the sexed sperms were harvested by centrifugation at 540 ×g for 7 min again. The sexed-sperms concentration was adjusted to 4×10^6 sperms/ml for *in vitro* fertilization (IVF).

3.10 *In vitro* fertilization and embryos collection

3.10.1 Oocyte collection and preparation

Bovine ovaries were collected from local abattoir and kept in 0.9% NaCl during transported to the laboratory at F1, Suranaree University of Technology. Cumulus-oocyte complexes (COCs) were collected from the follicles of 2-8 mm in diameter using 18-gauge needle attached to a 10 ml syringes (Figure 3.2B). The collected COCs (Figure 3.2C) were washed 5 times with modified Dulbecco's phosphate buffered saline (mDPBS) supplemented with 0.1% polyvinyl pyrolidone

(PVP) and 3 times in *in vitro* maturation (IVM) medium. Twenty to twenty five COCs were then cultured in 100 µl droplets of IVM medium covered with mineral oil under humidified atmosphere of 5% CO_2 in air at 38.5°C for 23 hr.

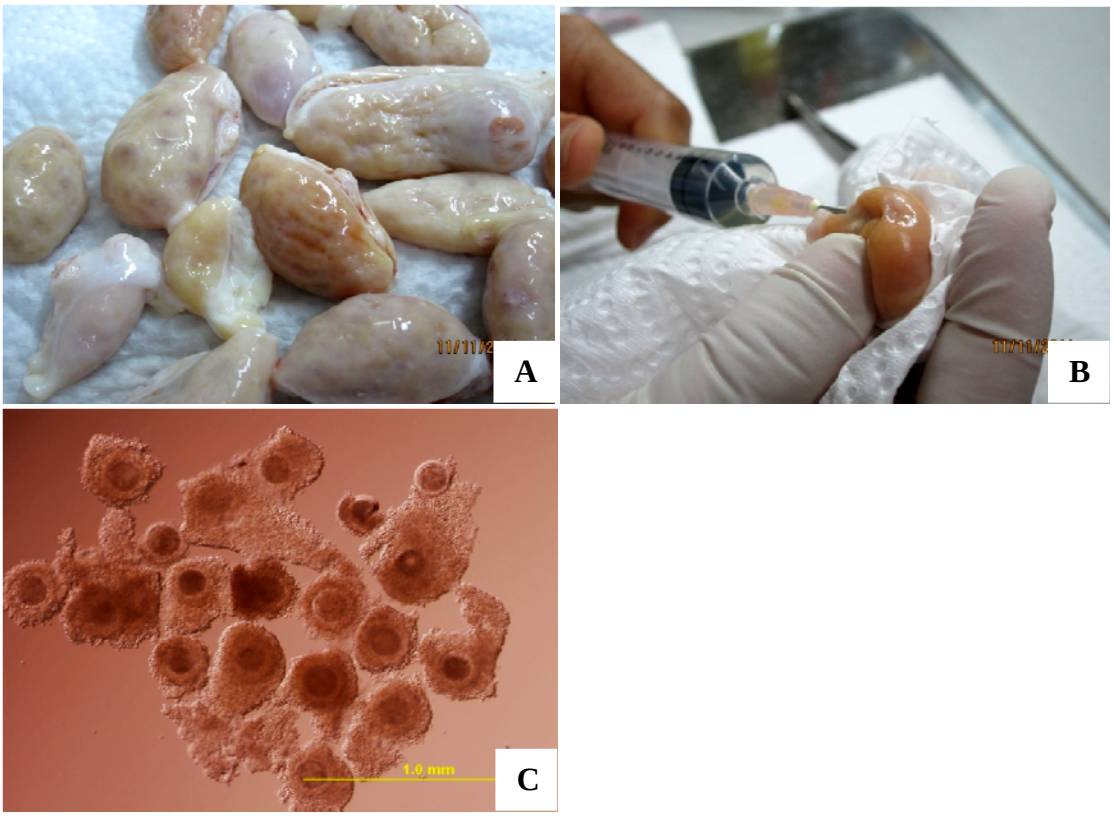

Figure 3.2 Oocyte preparation. Bovine ovaries (A) were collected from local abattoir. COCs were collected from the follicles of 2-8 mm in diameter using 18-gauge needle attached to a 10 ml syringes (B). COCs after aspirated from ovaries (C).

3.10.2 *In vitro* fertilization and embryo culture

After IVM, the cumulus cells were mechanically removed by repeat pipetting in 0.2% hyaluronidase using a fine-tip pipette and were washed 5 times in SYNGRO® Holding medium (BIONICHE ANIMAL HEALTH USA, INC.). The concentration of sexed-sperm was adjusted to 4×10^6 sperms/ml with TALP. Ten to twenty oocytes were grouped and washed 3 times in TALP then placed in fertilization droplets and cultured with the sexed-sperms for 10 hr at 38.5°C under humidified atmosphere of 5% CO_2 in air. The sperms that collected at the same time and same breeder were used as control group. The preparation of sexed-sperms was repeated 4 times for each mAb and the IVF was done with 50 oocytes/treatment. After fertilization, the embryos were subsequently washed and cultured in the mSOF medium supplemented with 3 mg/ml BSA (Laowtammathron *et al*., 2005) and covered with mineral oil. Twenty presumptive zygotes were grouped and placed in each culture drop and then cultured at 38.5°C under humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2 for 2 days. The day of IVF was counted as Day 0. After 2 days, 8-cell-stage embryos were selected and co-cultured with bovine oviduct cells in mSOF medium at 38.5°C under an atmosphere of 5% CO_2 in air for an additional 6 days. The culture medium was changed daily.

3.11 Multiplex polymerase chain reaction (PCR) optimization for embryo sex determination

Genomic DNAs used as positive control in multiplex PCR reaction were extracted from male and female bovine fibroblast using Ultraclean® tissue & cells

DNA isolation Kit (Catalog No. 12334-50, Mo-Bio Laboratories, inc.) followed the manufacturer's protocol. Briefly, male and female bovine fibroblasts were homogenized using bead beating technology to lyse the cells. Lysates were loaded onto a silica spin filter. During a brief centrifuge spin, the DNA selectively bound to the silica membrane while contaminants were passed through. Remaining contaminants and enzyme inhibitors were removed by a washing step. Pure DNA was then eluted by Tris buffer.

Five primers including bovine and Y-chromosome specific primers were combined and compared for the suitability of multiplex PCR to determine the sex of bovine embryos. Primers used in the PCR are shown in Table 3.1. The PCR were performed in a total volume of 50 µl containing 1x Green GoTaq® Flexi buffer (Promega, USA), 2 mM $MgCl_2$ (Promega, USA), 200 mM of dNTPs, 1 U GoTag® DNA polymerase (Promega, USA), 42, 0.42 or 0.0042 ng genomic DNA template and each set of primers. The PCR reactions were done in a DNA Thermal cycler for an initial denaturation at 95^{o}C for 2 min then followed by 45 cycles of 95^{o}C for 20 sec, 52^{o}C (BY/BSP), 33 ^{o}C (ConBV/ConEY) and 55^{o}C (S4BF/S4BR) for 45 sec and 72^{o}C for 50 sec and final extension at 72^{o}C for 10 min. The DNA bands were then visualized on 2% agarose gel under an ultraviolet illuminator.

Table 3.1 Primers used for bovine embryos sex determination

Couple Primer name	Primer sequence	Primer concentration (μM)	Size of amplicon
BSP*	5'-TTTACCTTAGAACAAACCGAGGCAC-3'	0.3	538 bp[B]
	5'-TACGGAAAGGAAAGATGACCTGACC-3'	0.3	
BY*	5'- CTCAGCAAAGCACACCAGAC-3'	0.5	300 bp[F]
	5'-GAACTTTCAAGCAGCTGAGGC-3'	0.5	
ConBV**	5'-TGGAAGCAAAGAACCCCGCT-3'	0.5	216 bp[B]
	5'-TCGTCAGAAACCGCACACTG-3'	0.5	
ConEY**	5'-GATCACTATACATACACCACT-3'	0.5	181 bp[F]
	5'-GCTATGACACAAATTCTG-3'	0.5	
S4BF***	5'-CAAGTGCTGCAGAGGATGTGGAG-3'	0.5	145 bp[B]
S4BR***	5'-GAGTGAGATTTCTGGATCATATGGCTACT-3'	0.5	174 bp[F]

[B] Bovine specific product. [F] Male specific product. * Reed *et al*., 1989; Iwata *et al*., 2002; Iwata *et al*., 2008. ** Pluciennicza*k et al*., 1982; Machaty *et al*., 1993. *** Rivière-Dobigny *et al*., 2009; Kageyama *et al*., 2004.

3.12 Embryos collection

The arrested embryos in each stage and blastocyst stage embryos were collected at each time period. Zona pellucida of the embryos was removed by 0.5% protease and then embryos were washed in PBS. Single embryo was transferred into a microcentrifuge tube, and stored in 10 μl RNAse and DNAse-free distilled water at -20 ^{o}C until used.

3.13 Embryos sex determination

The frozen embryos from IVF using sexed-sperm were lysed by boiling at 100 ^{o}C for 5 min. The lysed embryos were cooled on ice and mixed by vortex then 5µl of boiled embryo were used as template for each 25 µl of multiplex PCR reaction. The Y-chromosome specific primers (BY) and bovine specific primers (BSP) were used to determine the sex of embryos at various stages. The PCR reaction and PCR product bands detection were done as described in the section 3.4 except for the amount of template used.

3.14 Statistical analysis

Data of cleaved embryos and embryo development were analyzed by one-way ANOVA using SAS program. Four replications of each experiment were performed.

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CHAPTER IV

RESULTS

4.1 Monoclonal antibody production

X63-Ag8.635 myeloma cell line (Figure 4.1) was cultured in cRPMI for 2 weeks at 37^{o}C in a humidified atmosphere of 5% CO_2 in air before the fusion step. After 2 BALB/c mice were immunized with bovine Y-sperms, the spleen cells were fused with the myeloma cells at the ratio of 6.4:1 to produce hybridoma cells (Figure 4.2). At day 7-10, small colonies of hybridoma cells can be seen (Figure 4.3). More than 2,000 hybridoma colonies were grown in 24 well tissue culture plates before the screening step. The cultured media were used as an antibody to determine the specificity of secreted antibody on bovine Y-sperms.

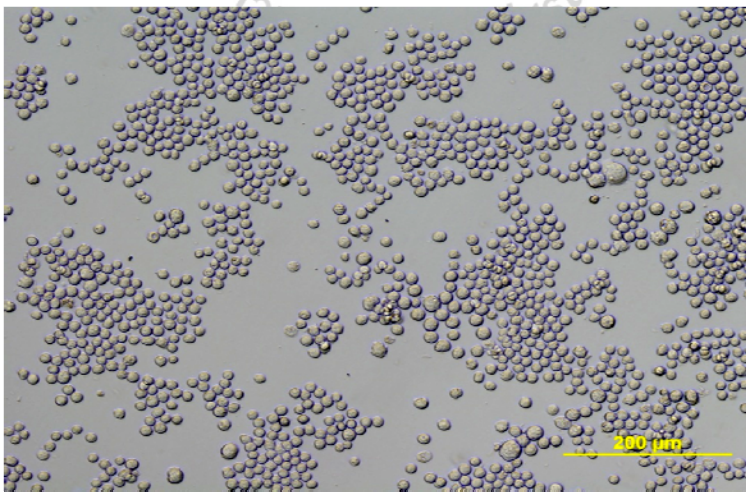

Figure 4.1 X63-Ag8.635 myeloma cell line cultured at day 9

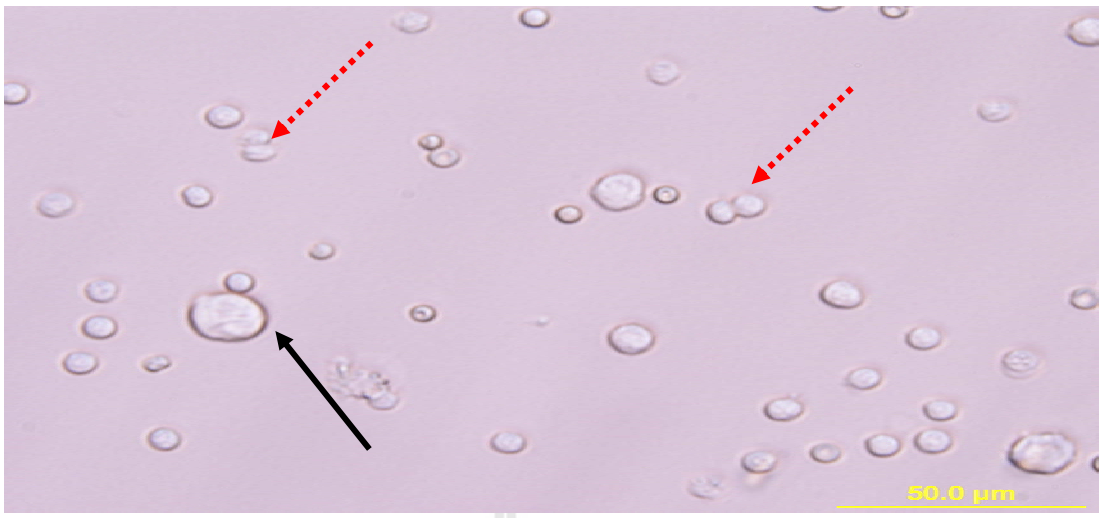

Figure 4.2 Fusion of spleen cells and myeloma cells using PEG-4000. The dotted arrows indicate the fused myeloma-myeloma cell. The black arrow indicate fused myeloma-spleen cell

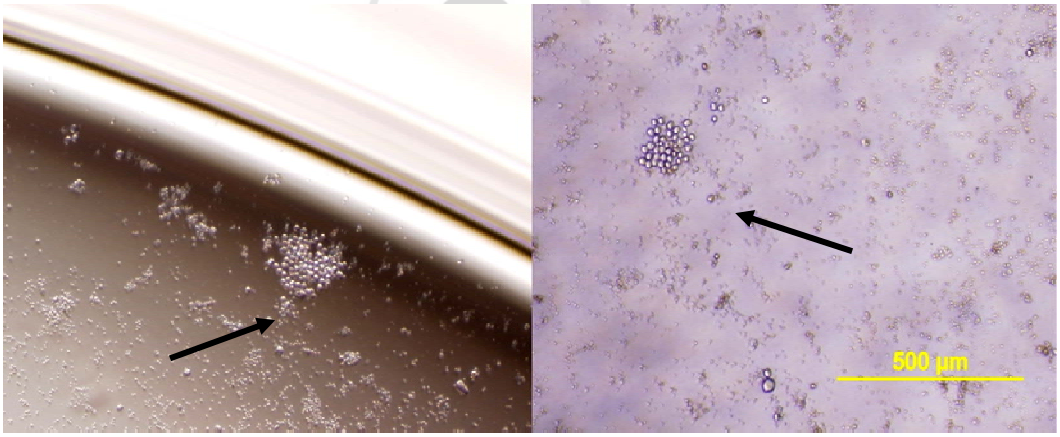

Figure 4.3 Hybridoma colonies grown on plates at day 7-10.

For the 1st screening, unsexed-sperms and egg yolk were used as antigents. The results showed that secreted antibodies from hybridoma cells from 17 wells bound only sperms and not egg yolk and 16 wells bound both sperms and egg-yolk. Limiting dilution method was done using these 33 hybridoma wells. Only 12 hybridoma plates from the 33 plates were grown without any contamination. Five plates from the 12

plates were selected for the 2nd round of ELISA because the antibodies from these 5 plates bound only sperms not egg yolk in the 1st ELISA step. From the 480 wells of the five 96 well tissue culture plates, only 288 monoclones bound only unsexed-sperms and not egg yolk in the 2nd round of ELISA screening. All monoclones were transferred to new culture medium in 24 wells tissue plates and grown for 2 weeks before rescreening with Y- and unsexed-sperms. In the 3rd round of ELISA screening, Y- and unsexed-sperms were used as antigens. Only 96 wells from plate G16 showed higher OD value to Y-sperms than unsexed-sperms. Seventeen wells were randomly selected to rescreen with Y- and X-sperms (7 wells from plate G16 and 10 wells from other plates) in the 4th round of ELISA screening. Only mAb produced from 3 monoclone wells from plate G16 bound specifically to only Y-sperms in the 4th round of ELISA screening. Antibodies from these three wells from plate G16 were used to confirm the specificity to Y-sperms by observed the binding on Y-sperms surface under fluorescence stereo microscopy. The results indicated that the antibody from the 3 wells from the plate G16 bound only the Y-sperms surface (figure 4.4). They were named G16G14, G16E7 and G16E8. From the ELISA screening and the binding of sperms surface results indicated that the three produced mAbs were specific to only Y-sperms without cross-reaction with egg yolk and bovine X-sperms.

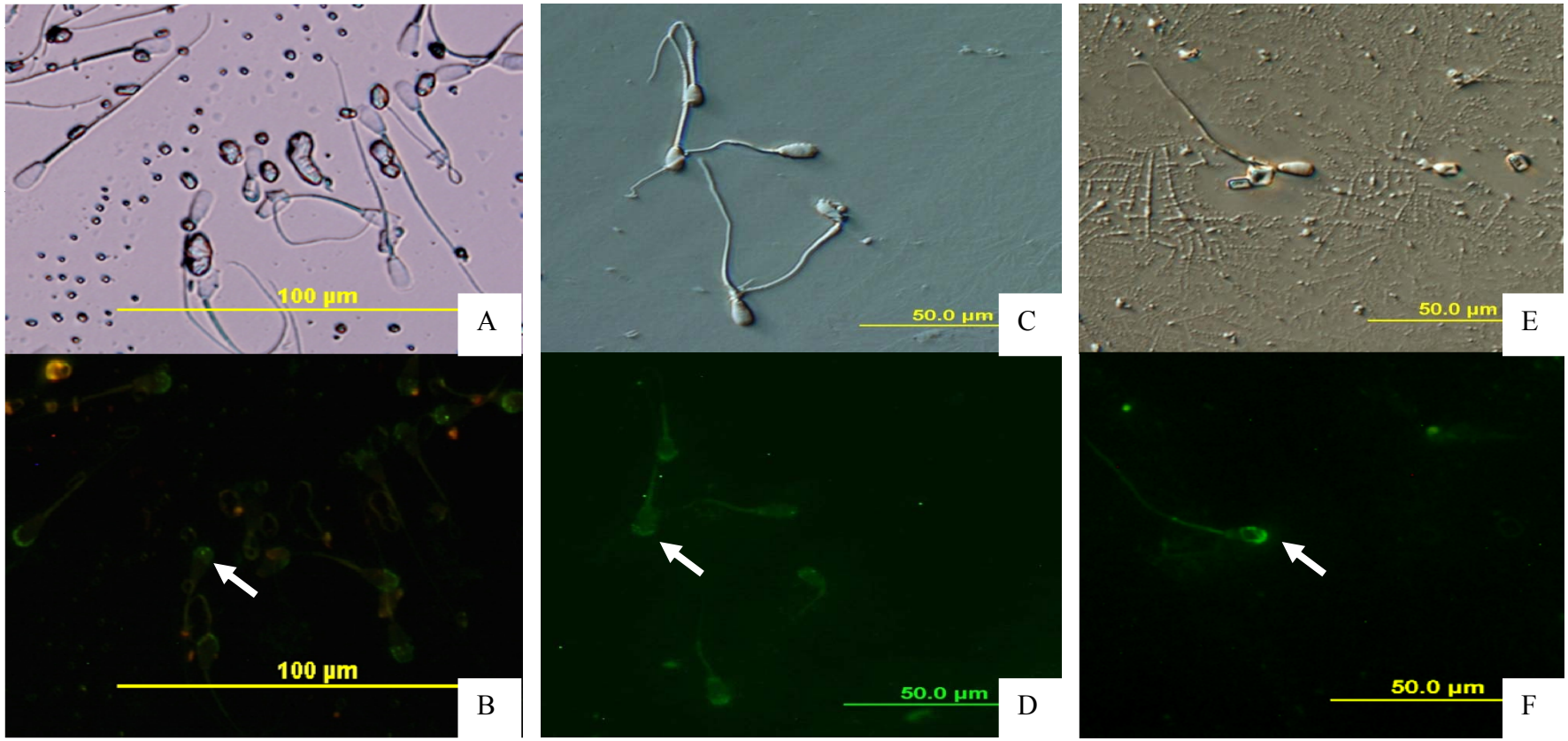

Figure 4.4 mAb binding on the bovine Y-sperm surface. White arrows indicate the position that mAb bound on the bovine Y-sperms surface. Bovine Y-sperms after mAb binding experiments under light microscope (A, C and E). Bovine Y-sperms after mAb binding experiments under fluorescence microscope (B, D and F)

4.2 Monoclonal antibody characterization

4.2.1 Sodium dodecly sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular weight of the three purified mAbs, G16G14, G16E7 and G16E8 were determined by SDS-PAGE. After the electrophoresis and gel staining, very sharp band can be seen at ~66 kDa as (Figure 4.5A).

4.2.2 Western blotting

To determine the molecular weight of 3 purified mAbs, the proteins from unstained gel obtained from SDS-PAGE were transferred to nitrocellulose membrane using semi-dry transfer chamber at constant 400 mA for 2 hrs. The sizes of both heavy and light chains were detected using alkaline phosphatase reaction. The Western blot results indicated that the molecular weight of the heavy and light chain were ~66 and ~24 kDa as shown in Figure 4.5B. The results indicated that the molecular weight of the 3 purified mAb were ~180 kDa (2 heavy chains × 66 kDa) + (2 light chains × 24 kDa).

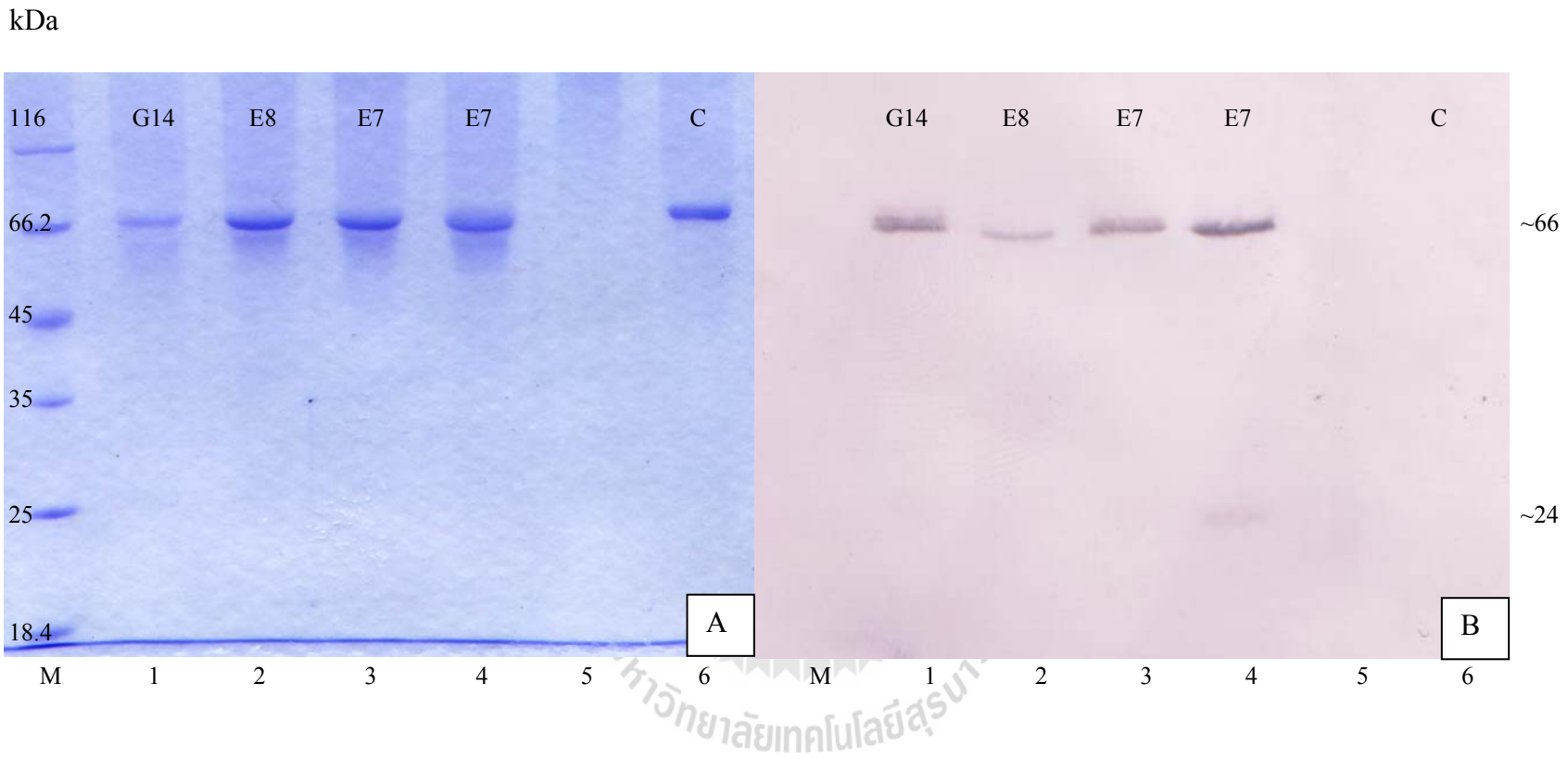

Figure 4.5 SDS-PAGE and Western bolt. A 12 % SDS-PAGE gel of the 3 purified mAbs. The goat anti-mouse Ig was used as control (A). Western blot of 3 purified mAbs and the goat anti-mouse Ig was used as control (B). Lane M; protein marker**,** 1; G16G14, 2; G16E8, 3-4; G16E7 and C; the goat anti-mouse Ig as a control

4.2.3 Monoclonal antibody isotype and subtype identification

The isotype and subtype of produced mAb were identified using ImmunoPure® MAb Isotyping Kit II. The results demonstrated that all 3 clones, G16G14, G16E7 and G16E8 were IgG_{2a} with kappa light chain.

4.3 The effects of guinea pig complement concentrations and incubation time on sperm survival

Three concentrations of guinea pig complement were used to determine the effect on sperm survival. The results showed that guinea pig complement at final concentration of 2% was the most suitable concentration that did not affect the sperm survival (Table 4.1).

Table 4.1 Effect of guinea pig complement on sperm survival

Guinea Pig Complement final concentration at (%)	**Sperm survival**	
	15 min	**30 min**
0	++++++	++++++
2	++++++	++++++
5	++	+
10	-	-

+ = amount of survived sperm

- = no survived sperm

The most suitable time of guinea complement incubation time were determined. Numbers of survived sperms were not different between each incubation time of 15, 30 and 45 min (Table 4.2).

Table 4.2 Effect of guinea pig complement on sperm survival

Guinea Pig Complement (final concentration)	Incubation Time (min)	Sperm survival
2%	15	++++++
	30	++++++
	45	++++++

+ = amount of survived sperm

4.4 Sperm sexing and *In vitro* fertilization

Sperm sexing was done by adding 40 µl of each purified mAb G16G14 (18.1 mg/ml), G16E7 (32.5 mg/ml) and G16E8 (10.9 mg/ml) to 2 ml TALP and 100 µl bovine sperms (25 $\times 10^6$ sperms/ml) were added. The cytotoxicity reaction was done using 2% guinea pig complement incubated at 38.5^{o}C in an atmosphere of 5% CO_2 in air for 45 min. After the cytotoxicity reaction, the concentration of sexed sperm was adjusted to 4 $\times$ 10^6 sperms/ml and was used at 100 µl/20 oocytes in IVF. The sperm sexing of each mAb was done 4 times with 50 oocytes/time. The arrested embryos in each stage and blastocyst stage embryos were collected at each time period. The embryos developments of each sperm sexing treatment are shown in Table 4.3, 4.4, 4.5 and 4.6.

Table 4.3 Development of IVF derived embryos obtained from sexed-sperm using G16G14 mAb at 1:50 dilution

No. of oocytes cultured	No. (%) embryos developed at d 2		No. (%) embryos developed to	
	Cleaved	>4 Cell	Morula	Blastocyst
50	35 (70)	17 (34)	5 (10)	2 (4)
50	29 (58)	15 (30)	5 (10)	1 (2)
50	31 (62)	14 (28)	4 (8)	4 (8)
50	30 (60)	19 (38)	2 (4)	6 (12)

Table 4.4 Development of IVF derived embryos obtained from sexed-sperm using G16E7 mAb at 1:50 dilution

No. of oocytes cultured	No. (%) embryos developed at d 2		No. (%) embryos developed to	
	Cleaved	>4 Cell	Morula	Blastocyst
50	21 (42)	14 (28)	3 (6)	1 (2)
50	11 (22)	7 (14)	4 (8)	1 (2)
50	21 (42)	14 (28)	2 (4)	5 (10)
50	19 (38)	13 (26)	3 (6)	5 (10)

Table 4.5 Development of IVF derived embryos obtained from sexed-sperm using G16E8 mAb at 1:50 dilution

No. of oocytes cultured	No. (%) embryos developed at d 2		No. (%) embryos developed to	
	Cleaved	>4 Cell	Morula	Blastocyst
50	16 (32)	10 (20)	1 (2)	3 (6)
50	16 (32)	14 (28)	0	4 (8)
50	22 (44)	12 (24)	1 (2)	3 (6)
50	30 (60)	19 (38)	6 (12)	3 (6)

Table 4.6 Development of IVF derived embryos obtained from unsexed-sperm (control)

No. of oocytes cultured	No. (%) embryos developed at d 2		No. (%) embryos developed to	
	Cleaved	>4 Cell	Morula	Blastocyst
50	31 (62)	17 (34)	3 (6)	3 (6)

The embryos development between IVF derived embryos obtained from sexed-sperms and unsexed-sperms was not significantly different. The percent of cleaved and blastocyst stage embryos were very low because at the experimental period time, the qualities of oocytes were low.

4.5 Multiplex polymerase chain reaction (PCR) optimization for embryo sex determination (published in Rattanasuk *et al*., 2011)

Five primers including bovine and Y-chromosome specific primers were combined and compared for the suitability of multiplex PCR to determine the sex of bovine embryos. The multiplex PCR conditions for three couple primers were optimized using extracted genomic DNA from male and female bovine fibroblasts as template. The results indicated that only BY/BSP couple primers (Figure 4.6) were able to identify the sex of the bovine DNA without any unexpected bands on gel. Minimum amount of bovine DNA appropriate for amplification using BY/BSP couple primers was 0.42 ng/50 µl reaction (Figure 4.7). To confirm the accuracy of BY/BSP couple primers, this couple primers were used to determine one-cell, two-cell, four-cell and eight-cell stage embryos of known sexed of somatic cell nuclear transfer

(SCNT) derived embryos, the results showed 100% accuracy (Figure 4.8 and 4.9). From the experiment indicated that BY/BSP couple primers were able to identify sex of IVF derived embryos. However, the 300-bp band of BY primer was not as intense due the primer concentration used in this experiment. The couple primers concentration were optimized by varying the BY/BSP at 5:1, 5:2 and 5:3 (Figure 4.10). The results indicated that the most optimal primer concentrations used for multiplex BY/BSP primer were BY primer 0.5 µM and BSP primer 0.3 µM, (5:3).

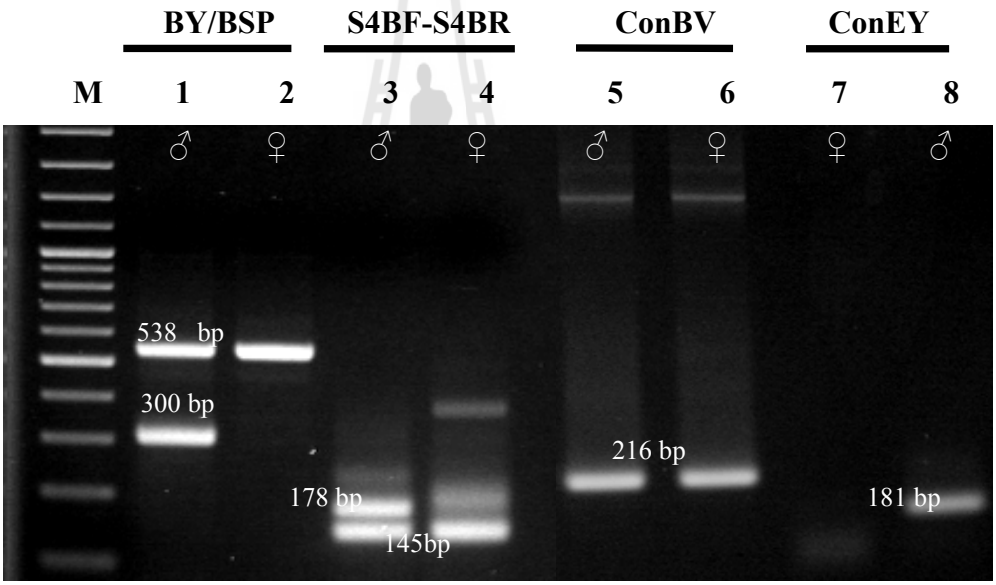

Figure 4.6 PCR results of three different couple primers specific for bovine and male bovine. M: size marker, ♂: male bovine genomic DNA (0.42 ng); ♀: female bovine genomic DNA (0.42 ng). Only BY/BSP couple primers were able to amplify and demonstrate clear results

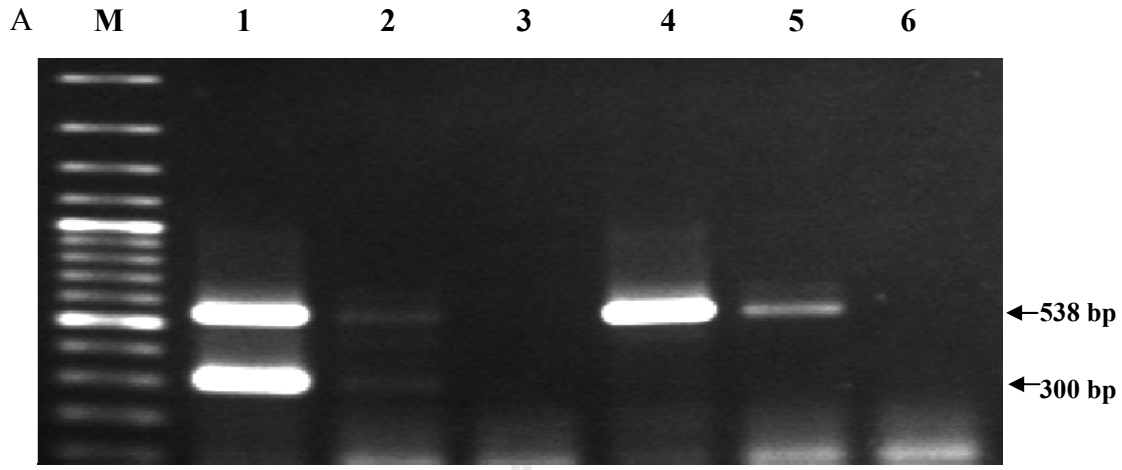

Figure 4.7 PCR sensitivity using BY/BSP primers. M : size marker; Lane 1-3: male bovine genomic DNA (42, 0.42 and 0.0042 ng). land 4-6: female bovine genomic DNA(42, 0.42 and 0.0042 ng)

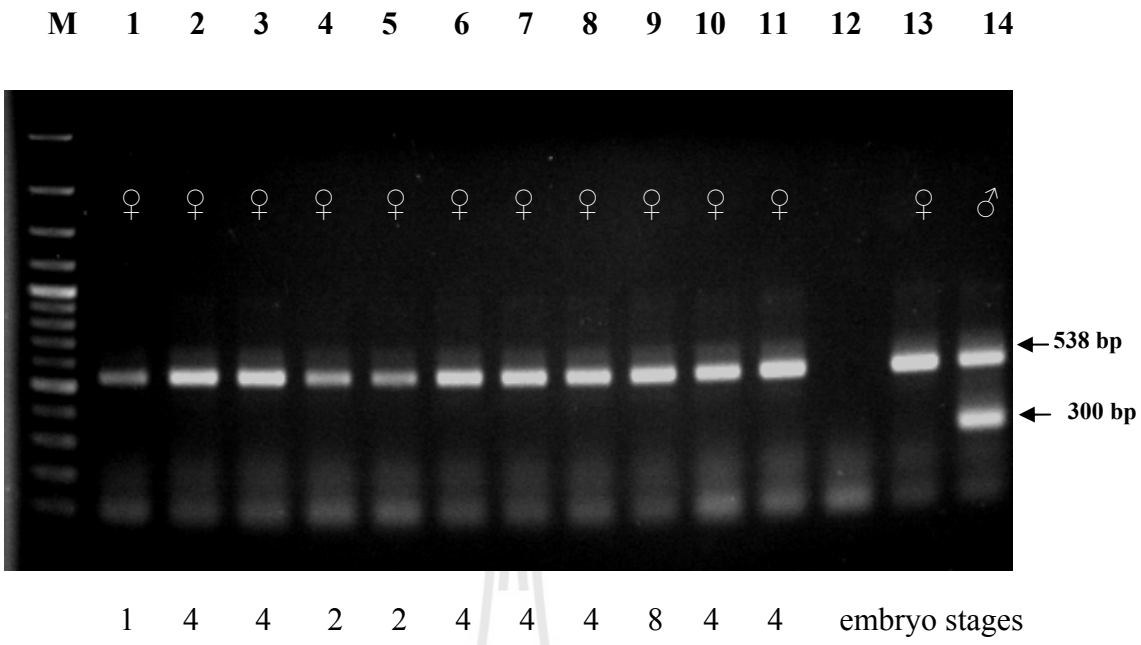

Figure 4.8 Confirmation of BY/BSP couple primers using SCNT female embryos at various stages as templates. M: DNA size marker; lane 1-11: SCNT female embryos; lane 12: (-) control; lane 13: female bovine genomic DNA (0.42 ng) and lane 14: male bovine genomic DNA (0.42 ng), respectively. The stages of embryo are shown by the number under the figure. 1: one-cell, 2: two-cell, 4: four-cell and 8: eight-cell stages

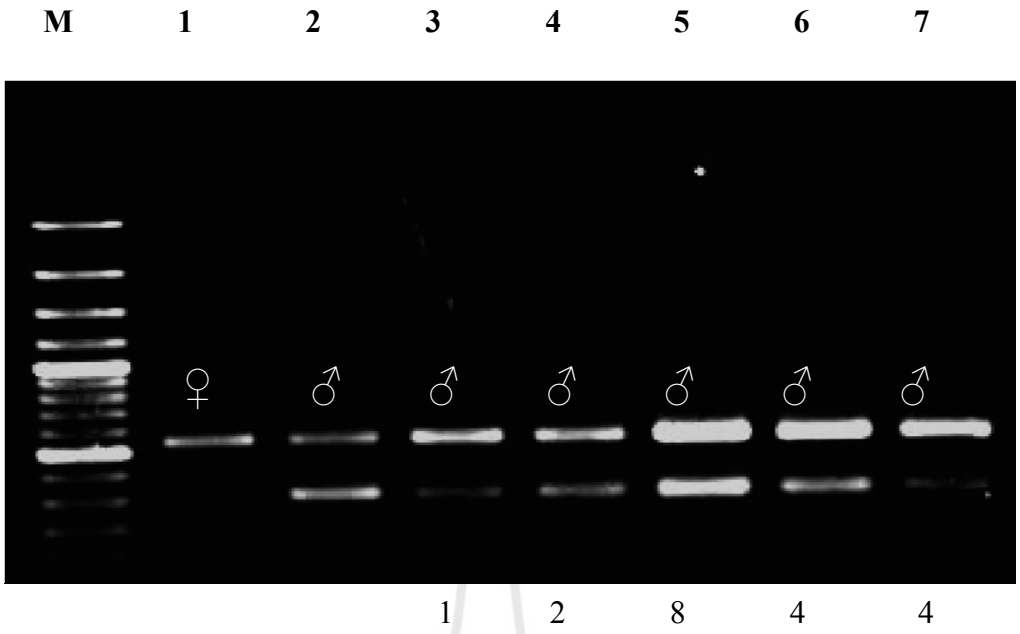

Figure 4.9 Confirmation of BY/BSP couple primers using SCNT male embryos at various stages as templates. M: DNA size marker; lane 1: female bovine genomic DNA (0.42 ng) and lane 2: male bovine genomic DNA (0.42 ng); lane 3-7: SCNT male embryos, respectively. The stages of embryo are shown by the number under the figure. 1: one-cell, 2: two-cell, 4: four-cell and 8: eight-cell stages

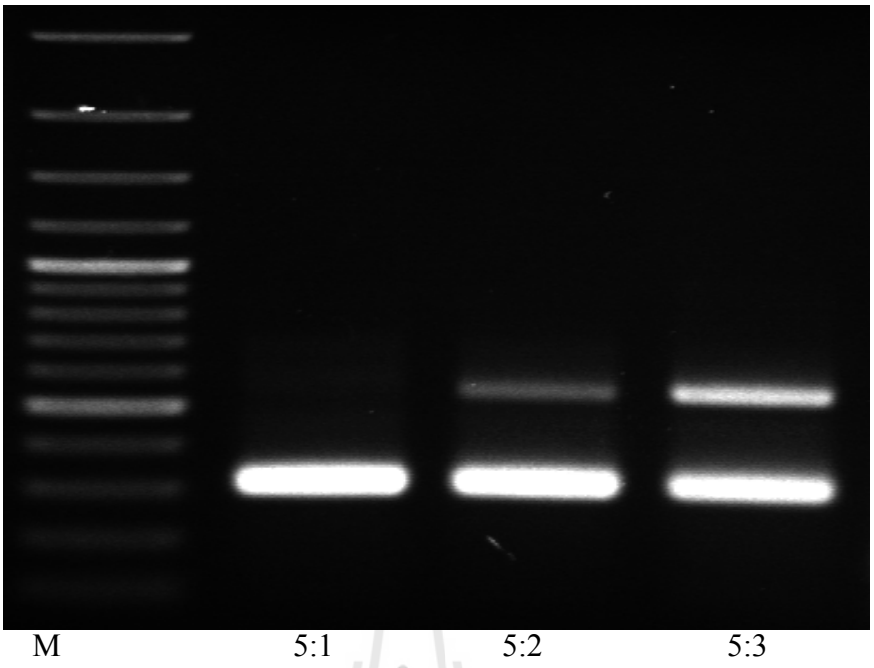

Figure 4.10 Optimal primer concentrations used for multiplex BY/BSP primer. M: DNA size marker; 5:1, 5:2 and 5:3 of BY:BSP primer concentration ratio

4.6 IVF derived embryos sex determination and development

Six hundred IVF derived embryos were used as template in multiplex PCR to test the accuracy and precision of the sexed sperms treated with the produced mAbs. Figure 4.11 showed the sample result of arrested at 2 cell embryos sex determination obtained from IVF using sexed sperms treated with G16E8 mAb. In lane 1; water was used as negative control and lane 2-23; arrested at 2 cell embryos. The results indicated that all arrested at 2-cell embryos were female. Figure 4.12 showed the result of arrested at 8-, 32-cell, morula embryos and blastocyst embryos sex determination. Lane 1-3: blastocyst stage embryos; lane 4-9: morula stage embryos; lane 10-21: 32-cell stage embryos and lane 22-23: 8-cell stage embryos. The results

indicated that female sex ratio of blastocyst stage embryos was 50:50 due to the ambiguous band in lane 1. Sex results from lane 1 were not used to calculate the sex ratio.

The female sex ratios of each stage of IVF derived embryo are shown in Figure 4.13. The results indicated that the 2-cell stage embryos showed higher female sex ratio when compared with all other embryo stages. In contrast, the blastocyst stage embryos contained lowest female sex ratio. The average female sex ratio between G16G14, G16E7 and G16E8 were 78.4±3.9, 74.4±1.5 and 76.0±8.9, respectively which were not significantly different among treatments.

The IVF derived embryos development was observed and the results showed that the percent of cleaved IVF derived embryos was separated into 2 groups, sperms treated with G16G14 mAb and control group were not significantly different but significantly difference from the sperm treated with G16E7 and G16E8 mAb groups (Table 4.7). The factors that might affect embryos development can be sperms health and the fertilization efficiency. However, the percent cleaved of IVF derived embryos of sexed sperm treated with G16G14 mAb was not significantly different to the control. In contrast, the percent of cleaved IVF derived embryos of sexed sperm treated with G16E7 and G16E8 mAb were lower than control. The quality of oocytes used for IVF might also be the reason for low percent cleaved embryo. During the time of their experiments some days the quality of oocyte was low.

The results suggested that G16E7 mAb gave the highest efficient mAb used for sperm sexing. The values of embryo sex from sperm treated the 3 mAb were similar, but G16E7 mAb has the lowest standard deviation. Female sex ratio of the embryos obtained from sperm treated with G16G14 and G16E8 mAb was higher than G16E7

mAb, but the standard deviation were also higher. The G16E7 mAb was used to confirm the effect of sperm sexing using mAb for embryo development (Table 4.8). The results indicated that the sperm sexing using mAb did not show negative effect to the embryo development.

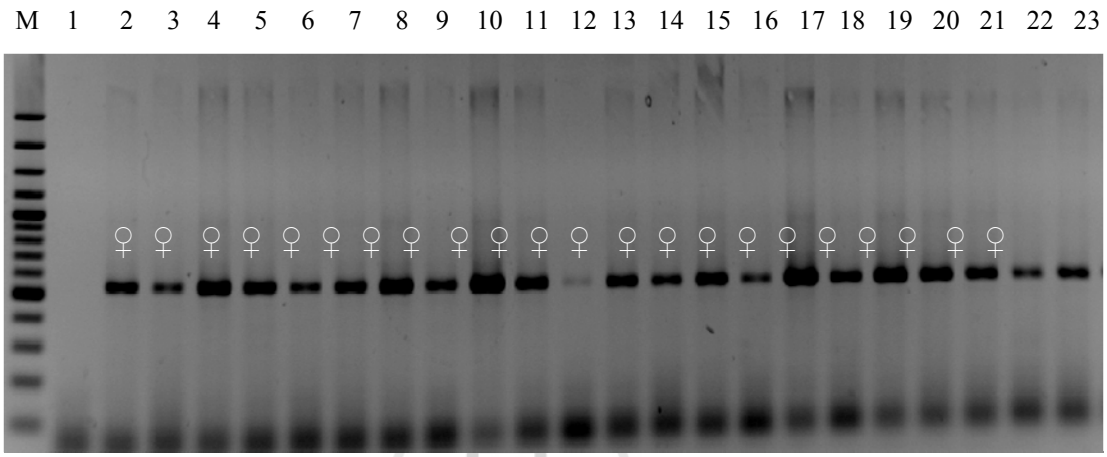

Figure 4.11 IVF derived embryos at 2-cell stage sex determination by the BY/BSP based PCR method. M: 100 bp DNA size marker. Lane 1: negative control; Lane 2-23: 2-cell IVF-derived embryos obtained from sexed sperm treated with G16E8 mAb

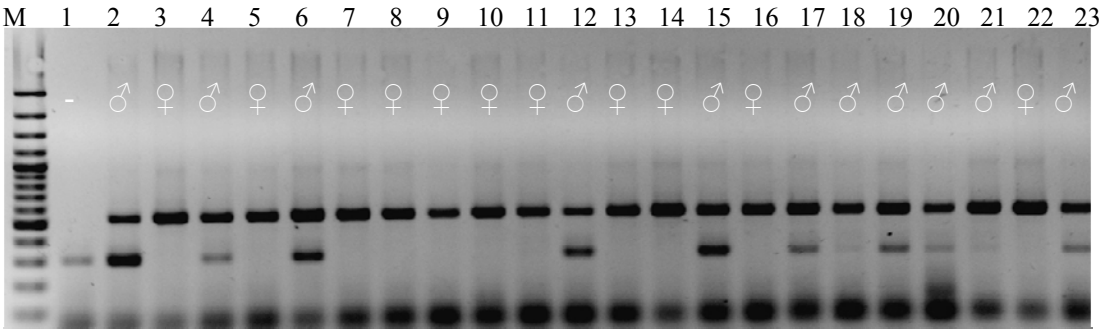

Figure 4.12 IVF derived embryos sex determination by the BY/BSP based PCR method. M: 100 bp DNA size marker. Lane 1-23: day 4 and day 8 IVF-derived embryos obtained from sexed sperm using G16E8 mAb replicated 4

Table 4.7 Embryo development of IVF embryo fertilized with G16G14, G16E7 and G16E8 mAb treated sperms and untreated (control)

Treatments	No. of oocytes culture	No. (%) embryos developed at d 2		No. (%) embryos developed to	
		Cleaved (%)	> 4 cell (%)	Morula (%)	Blastocyst (%)
G16G14	200	125 (62.5)[a]	65 (32.5)	16 (8)	13 (6.5)
G16E7	200	75 (37.5)[b]	48 (24)	12 (6)	12 (6.0)
G16E8	200	84 (42)[b]	55 (27.5)	8 (4)	13 (6.5)
Control	50	31 (62)[a]	17 (34)	3 (6)	3 (6.0)

Values with different superscript within each column are significantly different ($P<0.05$)

Table 4.8 Embryo development of IVF embryo fertilized with G16E7 mAb treated sperms and untreated (control)

Treatment	No. of oocytes culture	Day 2		Day 8	
		Cleaved (%)	> 4 cell (%)	Morula (%)	Blastocyst (%)
G16E7 1	24	22 (91.7)	16 (66.7)	5 (20.8)	5 (20.8)
G16E7 2	28	20 (71.4)	15 (53.6)	7 (25.0)	7 (25.0)
G16E7 3	28	22 (78.6)	17 (60.7)	7 (25.0)	7 (25.0)
G16E7 4	28	22 (78.6)	17 (60.7)	8 (28.6)	8 (28.6)
G16E7 5	28	22 (78.6)	17 (60.7)	9 (32.1)	9 (32.1)
G16E7 Total	**136**	**108 (79.4)**	**82 (60.3)[a]**	**36 (26.5)**	**36 (26.5)**
Control 1	26	22 (84.6)	14 (53.8)	5 (19.2)	5 (19.2)
Control 2	28	21 (75.0)	14 (50.0)	8 (28.6)	7 (25.0)
Control 3	28	21 (75.0)	15 (53.6)	8 (28.6)	8 (28.6)
Control 4	28	20 (71.4)	15 (53.6)	8 (28.6)	8 (28.6)
Control 5	28	20 (71.4)	15 (53.6)	8 (28.6)	8 (28.6)
Control Total	**138**	**106 (76.8)**	**73 (52.9)[b]**	**37 (26.8)**	**36 (26.0)**

Values with different superscript within each column are significantly different (*P<0.05*)

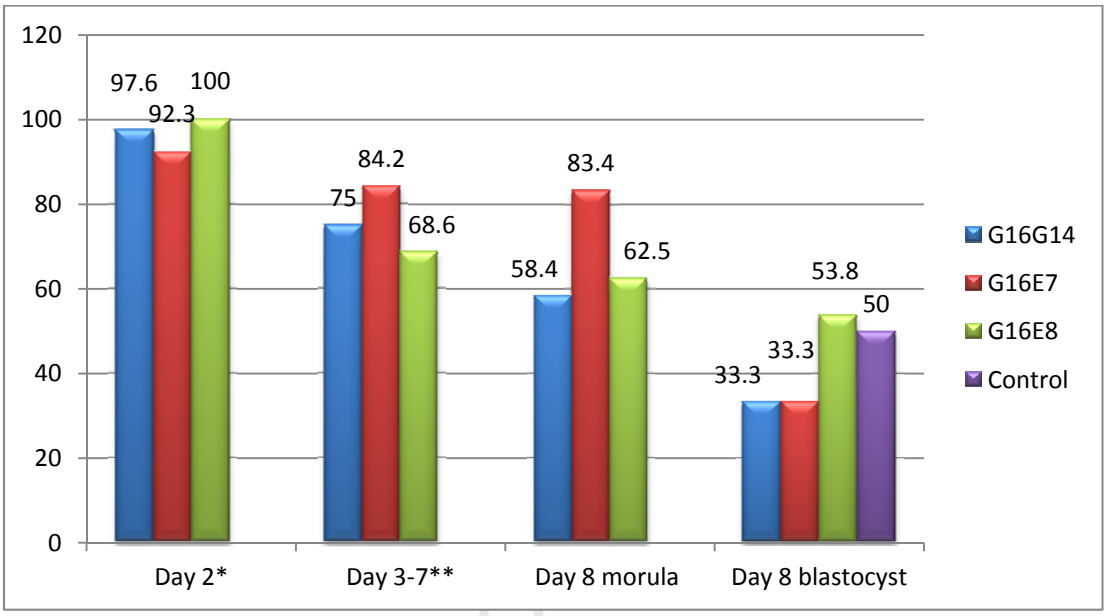

Figure 4.13 Percent of female embryos at different stages of arrested embryos and blastocyst

*; arrested 2 cell stage embryos

**; ≥ arrested 4 cell stage embryos

CHAPTER V

DISCUSSION

The immunological method is the method that does not affect the unbound sperms. The mAb production from this works focused on the surface protein of bovine Y-sperms. In contrast, many researches focused on H-Y antigen (Ali *et al*., 1990: Hendriksen *et al*., 1993; Patthanawong *et al*., 2010; Peter *et al*., 1993; Bradley, 1989; Benjamin *et al*., 2002; Bryant, 1980; Bryant, 1984.). However, few reports on surface protein have also been found (Ambrose *et al*., 1996; Spaulding, 1997). In 1984, Hoppe and Koo (1984) found that they were unable to enrich the sperm sex ratio using antibodies against the H-Y antigen. The failure of this method might be from the mistake of the underlying theory. Garner (1984) found that H-Y antigen was expressed as an integral membrane protein, Y-sperm did not produce H-Y themselves but both X- and Y-sperms adsorbed H-Y antigen to their surface. Hope and Koo (1984) also found the both X- and Y-sperms reacted with the anti H-Y antibody. The experimental technique of each investigator that found that the H-Y is expressed only on Y-sperms may have been a flaw. From these evidences, we decided to produce mAb against surface protein of bovine Y-sperm not the H-Y antigen.

The X63.Ag8.635 myeloma cell line used in this experiment obtained from Department of Immunology, Siriraj Hospital, Mahidol University. This myeloma cell line was recommended for mAb production by Kearney *et al*. (1979) because this cell line does not secrete any antibody by itself. This cell line has been used by many

researches (Millán *et al*., 1982; Carlsson *et al*., 1985; Shiels *et al*., 1986; Treanor *et al.* 1988; Sundström *et al*., 1989; Dungan and Roberson, 1993; Poudrier and Owens 1995; Lin and Hünig, 2003; Fayemi, 2004; Fujii *et al*., 2004; Hifumi *et al*., 2008; Patthanawong *et al*., 2010). Other type of myeloma cell lines such as NS1/1.Ag4 (Stern *et al*., 1978; Dráber *et al*., 1980; Russell *et al*., 1981; Slovin *et al*., 1982; Hosoi *et al*., 1982; De Boer and Wieczorek, 1984; Shone *et al*., 1985; Ambrose *et al*., 1996; Hifumi *et al*., 2008; Ning *et al*., 2012), Sp2/0-Ag14 (Köhler *et al*., 1976; Köhler, 1985; Dungan and Roberson, 1993; Sugaya *et al*., 1997; Mallory *et al*., 2007) have also used in many researches.

In the fusion step, the ratio of immunized mouse spleen and myeloma cells might affect the number of positive clones obtained. Ambrose *et al*. (1996) and Patthanawong *et al*. (2010) fused spleen cells with myeloma cell at the ratio of 1:5. The hybridoma Cloning Kit (Technical manual) also recommended 1:5 spleen cells to myeloma cells (http://www.stemcell.com/~/media/Technical%20Resources/9/3/F/0/1/28411_MAN_2_2_0.ashx). But some protocols recommend 2 spleen cells:1 myeloma cell (people.rit.edu/~gtfsbi/hytc/20103HyTcWeek2.ppt, Fayemi, 2004). The fusion step in this experiment was done using ratio of spleen cells per myeloma cell of 6.4:1. More than 2,000 hybridomas were obtained from this fusion. Only 148 hybridomas were obtained from Patthanawong *et al*. (2010) when 1:5 spleen cells with myeloma cell were used. The amount of hybridomas obtained from each fusion might depend on the ratio and number of spleen and myeloma cell.

The screening step is the most important step for the mAb production. The antigen used in this step affected the specificity of mAb, if unspecific antigen was used in this step, low specificity mAb might be obtained. In this research 3 steps of

screening were done. We found that the errors of ELISA increase when the screening procedure was repeated. In the 1st step, the hybridomas cultured supernatants were screened for the specificity against unsexed bovine sperms using egg yolk as negative control. After that, the positive antibody producing-hybridomas were selected and transferred into new media to increase the amount of selected hybridomas. The 2nd step, the positive hybridoma cultured supernatant were determined its specificity against bovine X- and Y-sperms. In the 3rd step the cultured supernatant of the selected hybridomas were used to check the specificity by sperm surface binding. The produced mAbs from this research believed to be specific to bovine Y-sperms. However, other researches normally screen their mAbs in only one or two steps (Ambrose *et al*., 1996; Patthanawong *et al*., 2010; Chakraborty *et al*., 1985).

The isotype of the 3 mAbs from this research were IgG with κ light chain. That is in agreement with the general corrected antibody isotype in the immune response. For the 1st injection, IgM is a primary antibody response produced and the secondary antibody response, IgG is also produced, but the concentration is lower than IgM (Figure 5.1). For the 2nd and 3rd injection with the same antigen, the IgM will be lower and the IgG is the predominant antibody (Roitt *et al*., 2001).

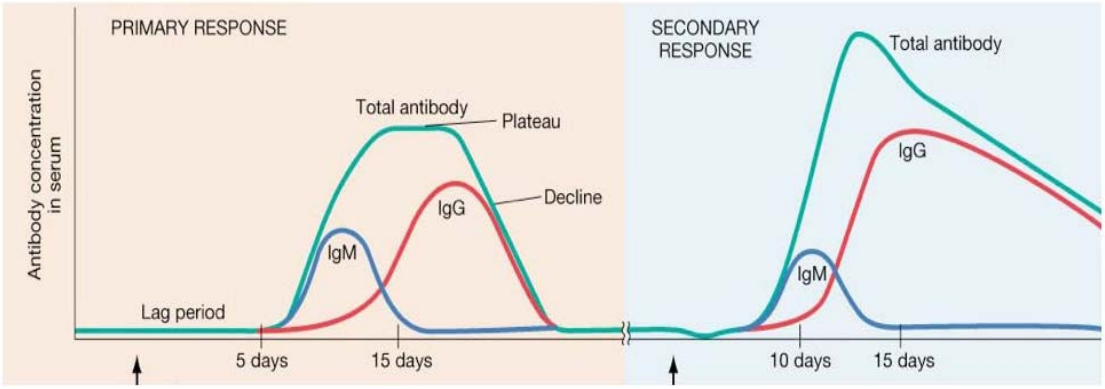

Figure 5.1 Primary and secondary antibody response (http://nfs.unipv.it/nfs/minf /dispense/immunology/lectures/files/somatic_hypermutation.html)

The MW of the three mAbs produced in this research was check using SDS-PAGE and Western blotting. IgG is the monomeric type which containing 2H2L. From the results of SDS-PAGE, a very sharp band can be seen at ~66 kDa (Figure 4.5A). The results from Western blotting showed that MW of the heavy and light chains were ~66 kDa and ~24 kDa. The Figure 4.5B, after finished the Western blot experiment, two bands can be seen but after that the lower band faded away. The results from both methods indicated that the MW of 3 mAbs were ~180 kDa. Cruz et al. (2002) found the IgG obtained from their research was ~180 kDa.

The three produced mAbs against bovine Y-sperms were used for bovine sperm sexing. Y-sperms were destroyed using guinea pig complement via the cytotoxicity reaction. This research determined the effect of guinea pig complement concentration and incubation time used for sperm sexing. The results found that final concentration of 2% guinea pig complement and 45 min for incubation time at 38.5^{o}C under humidified atmosphere of 5% CO_2 in air were the suitable for sperm sexing using the produced mAbs. In 2002, Matta (US Patent No. 20020115055) claimed the method used for sperm sexing by incubating heated guinea pig serum with sperm cells

and mAbs. He claimed that the sperm cells were incubated with mAbs for 45 min at 37^{o}C followed by washing and adding the serum and then incubating for 1 hr at 37^{o}C. Our research here did not inactivate the guinea pig complement. The US Patent No. 20020115055 claimed that inactivated guinea pig complement at 52.2^{o}C for 30 min before used will eliminated the B protein potentially originated from the peritoneal macrophage that will affect the alternative pathway of guinea pig complement (de Resende Matta, 2002). In their experiment no the mAb concentration was reported. Our research, the sperms sexing was combined with the sperm separation using swim up method. The sperms cells were incubated with TALP containing mAb for 20 min before adding guinea complement and then incubated for 45 min at 38.5^{o}C under humidifoed atmosphere of 5% CO_2 in air. The total sexing process time is 65 min. Jamil *et al*. (2007) reported only 30 min in the sperm swim-up procedure, but in our research 65 min was used because the suitable time for guinea pig complement was done followed the product information of 60 min. From de Resende Matta (2002) results showed that 308 IVF derived embryos analyzed, 244 (79.2%) were female, but in our research, found 157 from 200 (78.4%) IVF derived embryos were found to be female.

The development of IVF derived embryos obtained from sexed sperm (Table 4.3, 4.4 and 4.5) and unsexed sperms (Table 4.6) were not significantly different, but the result showed that the percent of cleaved and blastocyst embryos were very low when compared with Sripunya *et al*., 2010. The low number of blastocyst development was due to the oocytes quality obtained during Oct-Dec 2011. The oocytes quality used in the embryo development study between each sexed sperm obtained from 3 mAbs were low. Therefore, the G16E7 mAb was selected to confirm

the development during the season of better oocytes (April 2012). The result indicated that the sperm sexing experiment does not affect to the IVF derived embryo obtained from sexed sperms (Table 4.8) and the percent of blastocyst was higher than previous experiments.

The embryo sex determination is important for manipulating the sex ratio of domestic animals (Handyside *et al*., 1989; Penketh *et al*., 1989). Many approached have been used to determine the sex of bovine embryos but failed to gain much popularity due the lack of either accuracy or speed (Van Vliet *et al*., 1989). To solve the problem, this research combined several PCR primers, one for bovine specific primer and another for male bovine specific primer, to examine as multiplex PCR (Rattanasuk *et al*., 2011). PCR has been used for embryo sexing with various target sequences, including male-specific repetitive sequence (Bredbacka *et al*., 1995; Schroder *et al*., 1990), *ZFY* gene (Aasen and Medrano, 1990; Krikpatrick and Monson, 1993; Kudo *et al*., 1993) and single-copy genes on the Y-chromosome such as amelogenin (Chen *et al*., 1999; Ennis and Gallagher, 1994). The results from the IVF derived embryo obtained from G16G14, G16E7 and G16E8 mAbs treated-sperms were 78.4±3.9, 74.4±1.5 and 76.0±8.9 female, respectively which were not significantly difference among treatments but higher than the 50% in control.

Machaty *et al*., 1993 reported that they used two sets of PCR primer, Y-chromosome specific primers (300 bp) and bovine specific satellite sequence primers (216 bp) and Kitiyanant *et al*., (2000) also used the same primers. Their PCR product sizes were very close and found that some PCR product bands were not sharp and clear. These problems affected the interpretation of the result and if compared with this work, 538 bp and 300 bp, the PCR product bands were separate good enough,

with very sharp and clear bands.

Cong-Ying *et al*. (2004) reported two pairs of nested primers used to determine the bovine pre-implantation embryos, derived from the 5' flanking sequence of the bovine *SRY* gene (male specific primer) and casein protein gene primer (internal control). The nested PCR used in their experiment take longer time than multiplex PCR used in this research.

Lemos *et al*. (2005) and Jinming *et al*. (2007) reported that they designed primer for *TSPY* (male specific primer) and microsatellite DNA (both sex). In their experiment they did not combined the two primers set together so one sample must used 2 reactions, male and microsatellite which will cost higher when compared with multiplex PCR in this research. Some researchers also used Y-specific sequence to screen blood, meat and blastomere sample (Zeleny *et al*., 2002; Alves *et al.,* 2003).

Ekici *et al.* (2006) designed primers used for bovine embryos sex determination using PCR. The bovine Y-chromosome and bovine *G3PDH* gene were selected. The PCR product sizes of these couple primer are 279 and 579 bp. However, the results from their reported found some unspecific bands and smear bands.

Eggtech company reported that the bovine embryo sexing kit, non-electrophoretic sex determination of bovine pre-implantation embryos, is simplest and fastest PCR-based method, convenient for embryo sexing on farm, low cost, no need for electrophoresis, minimal risk of contamination, but only 95% accuracy can be obtained from this kit (http://www.egg tech.co.uk/ resources/Finnzymes%20Ampli-Y.pdf).

Multiplex PCR is a rapid, easy method for large scale embryos sexing. This couple primers (BY/BSP) has been used to determine the sex of somatic nuclear

transfer (SCNT) and IVF derived embryos in various stages. The results showed 100% accuracy (Rattanasuk *et al*., 2011).

In conclusion, this research successfully produced 3 mAbs against bovine Y-sperms that is important for bovine sperm sexing. The sperms treated with these mAbs did not effect to embryo development of the used in IVF. And more that 70% of IVF embryos obtained were shown to be female embryos. The multiplex PCR from this work will also benefit the field of bovine embryo sexing by using multiplex PCR.

CHAPTER VI

CONCLUSION

Two BALB/c mice were immunized with mixed bovine Y-sperms and Complete Freund's Adjuvant for the 1st injection and 2 boosts with mixed Y-sperms and Incomplete Freund's Adjuvant. Three mAb (G16G14, G16E7 and G16E8) against bovine Y-sperm were produced. The specificity of G16G14, G16E7 and G16E8 was determined using ELISA and confirmed by observed the binding on Y-sperms surface. Molecular weight of each mAb was determined by western blot, the molecular weight of each mAb is approximately 180 kDa. The mAb isotype and subtype of the 3 mAbs were IgG_{2a} with κ light chain. The produced mAbs were used to determine the effect on sperm survival by varied the final concentration of guinea pig complement and incubation time. The most suitable condition was used for sperm sexing. The sexed sperms were used in IVF experiment. The result indicated that the 3 mAbs altered the sex ratio by increasing the female sex ratio to 78.4, 74.4 and 76.0%. The G16E7 mAb is the most efficiency mAb used for sperm sexing because of the standard deviation between each group was low. Finally, this work successfully produced mAb against bovine Y-sperm that is important for sperm sexing.

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APPENDICES

APPENDIX I

MEDIA AND REAGENTS

1. 100x L-glutamine (Sigma®)

-L-glutamine	2.92	g
-Water for injection	100	ml

Filter sterilization (0.2 µm)

Aliquot 10 ml/tube and store at -20°C

2. 1000x Penicillin + Streptomycin

-Penicillin G sodium	5×10^6	unit/vial
-Streptomycin	5	g/vial
-Water for injection	50	ml

Filter sterilization (0.2 µm)

Aliquot 1 ml/tube and store at -20°C

3. RPMI-1640 (Gibco®) size 1 bag/1 L

-RPMI	1	bag
-$NaHCO_3$	2	g
-Water for injection	1	L

-10 N HCl

Dissolve RPMI-1640 powder and 2 g $NaHCO_3$ in 900 µl of water for injection

using magnetic stirrer. Adjust the pH to 7.0 by adding 10 N HCl and add water for injection to final volume 1 L. Filter sterilization (0.2 µm) and store at 4°C. Check for contamination by aliquot 2 ml into cell culture disk and store at 37°C in a humidified atmosphere of 5% CO_2 in air overnight.

4. Fetal bovine serum (HyClone FetalClone I, U.S. Origin)

-Heat inactivation at 56 °C for 30 min

-Aliquot 100 ml/tube and store at 4°C

5. Complete RPMI-1640 (cRPMI)

-RPMI	400	ml
-100x L-glutamine	5	ml
-1000x penicillin + streptomycin	0.5	ml
-fetal bovine serum	100	ml

Mix all reagents and store at 4°C

6. Polyethylene glycol (PEG-4000)

-warm the autoclaved PEG-4000 at 65°C until completely melt. Add 15% DMSO in PBS with ratio 5 g PEG-4000/25ml 15% DMSO in PBS. Store at 4°C.

7. 100x Aminopterin (A) stock (Sigma®)

-Aminopterin	1.76	g
-Water for injection	100	ml

-1 N NaOH

Dissolve 1.76 g aminopterin in 90 ml water of injection by add small volume of 1 N NaOH. Adjust the pH using HCl to 7.2 and add water of injection to final volume of 100 ml. Filter sterilization and aliquot 10 ml/tube covered with aluminum foil and store at -20^{o}C.

8. 100x Hypoxanthine and thymidine (HT) stock (Sigma®)

-Hypoxanthine	136.1	mg
-Thymidine	38.75	mg
-Water for injection	100	ml

-1 N NaOH

Dissolve hypoxanthine and thymidine in 90 ml water of injection by add small volume of 1 N NaOH. Adjust the pH using NaOH to 8.1-8.5 and add water of injection to final volume 100 ml. Filter sterilization and aliquot 10 ml/tube. Store at -20^{o}C.

9. HAT working media

-cRPMI	100	ml
-100x HT stock	1	ml
-100x A stock	1	ml

Mix all reagents and store at 4^{o}C. Prepare freshly before each use.

10. HT working media

-cRPMI	100	ml

-100x HT stock	1	ml

Mix all reagents and store at 4^{o}C. Prepare freshly before each use.

11. Phosphate buffer saline (PBS), pH 7.2

-NaCl	8.50	g
-Na_2HPO_4	1.07	g
-NaH_2PO_4	0.30	g
-Deionized H_2O to	1000	ml

Dissolve all reagents and fill deionized water up to 1000 ml. Adjust pH to 7.2.

Filter sterilization with 0.2 µm and store at room temperature.

12. Freezing media

-cRPMI	90	ml
-DMSO	10	ml

Mix all reagents and store at 4^{o}C. (Not allow to filter sterilization)

13. Phosphate buffer saline + 0.05% Tween20 (PBST), pH 7.2

-PBS	100	ml
-0.05% Tween20	50	µl

Mix all reagents and store at room temperature

14. Acrylamide solution (30.8%T 2.7%C_{bis})

-Acrylamide	60	g
-bisacrylamide	1.6	g

-Distilled water to	200	µl

Store up to 3 months at 4^{o}C in the dark

15. 10% Sodium dodecyl sulfate (SDS)

- Sodium dodecyl sulfate	10	g
- Distilled water to	100	ml

Store up to 6 months at room temperature

16. 10% Ammonium persulfate

-Ammonium persulfate	0.1	g
-Distilled water to	1	ml

Prepare just prior to use, do not store

17. 4x Resolving gel buffer

-Tris	36.3	g
-Distilled water	40	ml
-HCl to pH 8.8		
-Distilled water to	200	ml

Store up to 3 months at 4^{o}C

18. 4x Stacking gel buffer

-Tris	3	g
-Distilled water	40	ml
-HCl to pH 6.8		

-Distilled water to	50	ml

Store up to 3 months at 4^{o}C

19. 2x Loading dye

-4x stacking gel buffer	2.5	ml
-10% SDS	4	ml
-Glycerol	2	ml
-Bromophenol Blue	2	mg
-2-Mercaptoethanol	100	µl
-Distilled water to	10	ml

Store in 0.5 ml aliquots at -20^{o}C up to 6 months

20. Tank buffer

-Tris	30.28	g
-Glycine	144.13	g
-SDS	10	g
-Distilled water to	10	L

21. Mixture of separating gel and stacking gel

	12% separating gel	**4% stacking gel**	
-12%Acrylamide solution	3.2	0.83	ml
-Distilled water	2.64	2.77	ml
-4x Separating gel buffer (pH 8.8)	2	-	ml
-4x Stacking gel buffer (pH6.8)	1.26	-	ml

-10% SDS	80	50	µl
-10% Ammonium persulfate	80	50	µl
-TEMED	8	5	µl

22. Coomassie Blue staining solution

-Coomassie Brilliant Blue R250	0.5	g
-Methanol	800	ml
Stir until dissolved, then add		
-Acetic acid	140	ml
-Distilled water to	2	L

Store at room temperature for up to 6 months

23. Destain solution

-Methanol	400	ml
-Acetic acid	70	ml
-Distilled water to	1	L

Store at room temperature

24. Transfer buffer (Western blot)

-Tank buffer	450	ml
-Methanol	50	m

APPENDIX II

PUBLICATION

Rattanasuk, S., Parnpai, R. and Ketudat-Cairns, M. (2011). Multiplex polymerase chain reaction used for bovine embryo sex determination. **Journal of Reproduction and Development** 57(4): 539-542.

BIOGRAPHY

Mr. Surachai Rattanasuk was born on November 5th, 1981 in Nakhon Ratchasima, Thailand. He received his Bachelor's Degree of Science in Biotechnology (2nd Honor) from Maha Sarakham University in Year 2002. In 2005, he had an opportunity to study Doctoral degree in School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima. His thesis is "bovine sperm sexing by monoclonal antibody". He obtained the SUT Excellent Student Scholarship for 3 years before receiving a scholarship from the Commission on Higher Education, Thailand, under the Strategic Scholarships for Frontier Research Network for the Ph.D. Program. He has been a teaching assistant in the subject of Plant Molecular Biology, Genetic Engineering Techniques, and Selected Research in Biotechnology. He is currently a lecturer at the Faculty Arts and Sciences, Roi Et Rajabhat University. Part of his thesis work has been presented at The Annual Meeting and International Conference of the Thai Society for Biotechnology (TSB 2008) at Maha Sarakham and received a poster award. He also presented part of his thesis work at the Commission on Higher Education Congress III: University Staff Development Consortium CHE-USDC Congress III (2010) at Chonburi. Part of his work has been published in Journal of Reproduction and Development (JRD) (Rattanasuk *et al*., 2011).