



**UNIVERSITI PUTRA MALAYSIA**

**THE EFFECTS OF DIFFERENT CRYOPRESERVATION CONDITIONS ON  
FELINE PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCs)  
VIABILITY.**

**SITI AISYAH BINTI AZHAR**

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FPV 2016 34**

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VIABILITY.**

**SITI AISYAH BINTI AZHAR**

A project paper submitted to the  
Faculty of Veterinary Medicine, Universiti Putra Malaysia  
In partial fulfillment for the requirement of the  
DEGREE OF DOCTOR OF VETERINARY MEDICINE

Universiti Putra Malaysia

43400 UPM, Serdang, Selangor Darul Ehsan  
Malaysia.

MARCH 2016

It is hereby certified that we have read this project paper entitled “The Effects Of Different Cryopreservation Conditions On Feline Peripheral Blood Mononuclear Cells (PBMCs) Viability”, by Siti Aisyah binti Azhar and in our opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4999 - Project



---

**DR. FARINA MUSTAFFA KAMAL**  
**DVM (UPM), PhD (UC Davis, USA)**

Senior Lecturer,  
Faculty of Veterinary Medicine, Universiti Putra Malaysia  
(Supervisor)

---

**DR. KHOR KUAN HUA**  
**DVM (UPM), PhD (Queensland, Australia)**

Senior Lecturer,  
Faculty of Veterinary Medicine, Universiti Putra Malaysia  
(Co-Supervisor)

---

**DR. MOHD. HEZMEE MOHD. NOOR**  
**DVM (UPM), PhD (Queensland, Australia)**

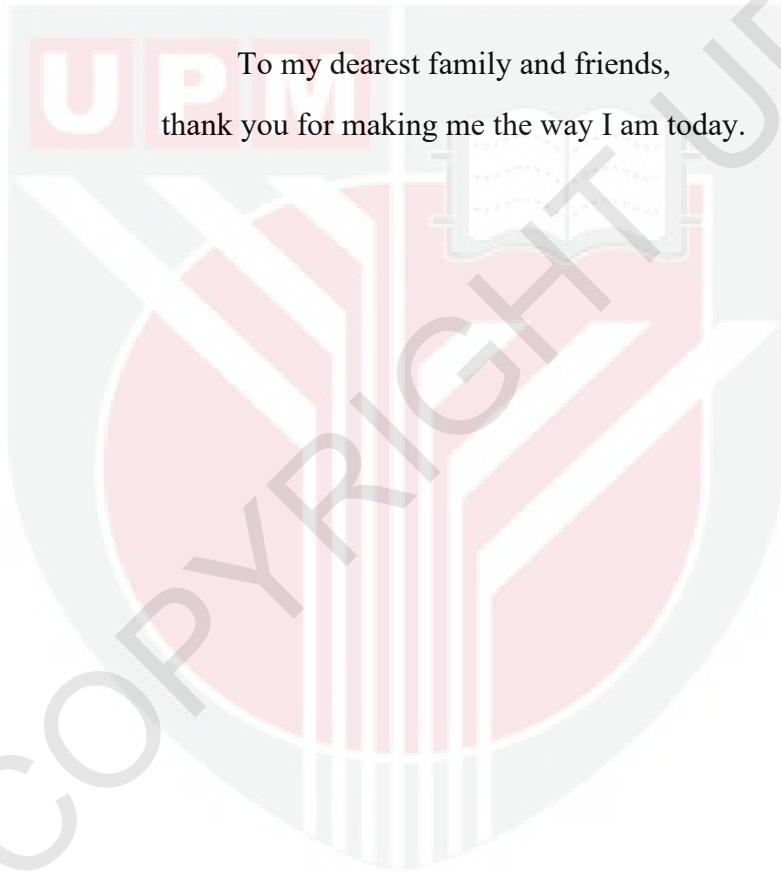
Senior Lecturer,  
Faculty of Veterinary Medicine, Universiti Putra Malaysia  
(Co-Supervisor)

**DEDICATIONS**

“Hakuna matata”

-The Lion King, 1994-

To my dearest family and friends,  
thank you for making me the way I am today.



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## **ACKNOWLEDGEMENT**

In the name of Allah, the Beneficent, the Merciful. I would like to thank the Almighty God for giving me life and granting my wish in completing this project. Million thanks to my parents, Rohayah binti Abdullah and Azhar bin Awi; my two lovely aunts, Rosnine binti Abdullah and Noraini binti Abdullah; and my younger brother, Muhammad Anwar bin Azhar for loving and raising me with all of their heart. My deepest gratitude to my supervisor, Dr. Farina Mustaffa Kamal and co-supervisors, Dr. Khor Kuan Hua and Dr. Mohd. Hezmeem Mohd. Noor, for their guidance throughout the project. I would also like to thank Mr. Mohamad Habid bin Hasan, research assistant who has been sharing his knowledge and willing to help me throughout my final year project. My deepest gratitude to the staffs of University Veterinary Hospital (UVH), especially Dr. Purshyla Manikam, Dr. Hemadevy Manoraj, Dr. Jessie Bay, Mr. Maniam Munusamy, Mr. Mohd. Faizal Hamzah and Mr. Mohd. Azuwan Abu Hassan for being very helpful during my sampling. I would also like to thank Dr. Nor Emeliawati Mohd. Napiah, the private veterinarian for assisting me in my sample collection, and all the cat owners for their willingness in allowing their cats to participate in this research. Million thanks to Dr. Yeap Swee Keong and Dr. Nadia from Faculty of Biotechnology and Biomolecular Science for assisting me in flow cytometry procedure. Thank you to Prof. Mohamed Ariff Omar for assisting me in statistical analysis. Not to forget my dearest friends, Raquel Yong, Nuur Fatin, Nik Fatin, Husna Omar, Husna Atika, Tan Shin Yi, Jong Kwang Yan, Sameerah Hani, and Hakim Narwawi for lending a hand when they are most needed. Last but not least, thank you to all of my classmates, DVM 2016 for being a part of my life.

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**ABBREVIATIONS**

%	percentage
°C	degree Celcius
7-AAD	7-Aminoactinomycin
DMSO	Dimethyl sulfoxide
FBS	Fetal Bovine Serum
FCoV	Feline Coronavirus
FeLV	Feline Leukaemia Virus
FIV	Feline Immunodeficiency Virus
FS	Feline Serum
HBSS	Hank's Balanced Salt Solution
K <sub>2</sub> EDTA	Potassium Ethylenediamine tetraacetic acid
IACUC	Institutional Animal Care and Use Committee
ID	Identification
mL	millilitre
PBMCs	Peripheral Blood Mononuclear Cells
PBL	Peripheral Blood Lymphocyte
PBS	Phosphate Buffered Saline
PI	Propidium iodide
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	Room Temperature
UPM	Universiti Putra Malaysia
UVH	University Veterinary Hospital

**ABSTRAK**

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek.

**IMPAK BERLAINAN KONDISI PENGAWETANKRIO TERHADAP  
KEBOLEHHIDUPAN SEL DARAH MONONUKLEAR KUCING**

Oleh

**SITI AISYAH BINTI AZHAR****2016****Penyelia: Dr. Farina Mustaffa Kamal****Penyelia Bersama: Dr. Khor Kuan Hua****Dr. Mohd. Hezmee Mohd. Noor**

Sel darah mononuklear kucing (PBMCs) mengandungi subset sel imuniti, seperti limfosit dan monosit. Pencirian fungsi imun mereka amat berkait dalam menyediakan maklumat asas mengenai mekanisme dan tindak balas sel. Dalam kajian ini, suhu dan komposisi media pengawetankrio diselidik untuk mengenal pasti kesannya terhadap kebolehhidupan PBMC kucing. Sampel darah telah diperolehi dari 15 ekor kucing sihat dan PBMC kucing diasingkan menggunakan teknik emparan kecerun-tumpatan. PBMC tersebut diawetkrio dalam  $-196^{\circ}\text{C}$  untuk 2 minggu dalam tiga media pengawetankrio berlainan kondisi, iaitu 10% DMSO bersama 90% FBS, 10% DMSO bersama 90% serum kucing (FS), kombinasi 10% DMSO bersama 45% FBS dan 45% FS. Tambahan lagi, dua suhu berlainan ( $4^{\circ}\text{C}$  dan suhu bilik) media

pengawetankrio juga dinilai. Kebolehhidupan sel dinilai menggunakan teknik pengecualian pewarna Trypan Blue dan sitometri aliran. Hasil kajian dari teknik pengecualian pewarna menunjukkan bahawa penambahan FS memberi pemulihan yang sama terhadap PBMC kucing sebaik sahaja dicairkan berbanding penggunaan FBS. Walaubagaimanapun, hasil kajian dari sitometri aliran menunjukkan pemulihan PBMC kucing yang lebih tinggi apabila FS digunakan berbanding FBS. Tambahan pula, suhu media pengawetankrio yang berbeza tidak memberi perbezaan ketara dalam pemulihan PBMC kucing setelah pengawetankrio. Keseluruhannya, penggunaan FS dalam media pengawetankrio memberi keputusan yang sama terhadap kadar pemulihan PBMC kucing sebaik sahaja dicairkan berbanding penggunaan FBS. Tambahan lagi, FS mengekalkan kebolehhidupan PBMC kucing dalam jangka masa yang lebih panjang. Walaubagaimanapun, peranan mereka dalam titik keputusan akhir penilaian klinikal lain tidak boleh dikecualikan.

*Kata kunci: PBMC kucing, pengawetankrio, komposisi media, suhu media, kebolehhidupan sel, sitometri aliran*

**ABSTRACT**

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial requirement for the course VPD 4999 - Project.

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VIABILITY.**

By

**SITI AISYAH BINTI AZHAR**

**2016**

**Supervisor: Dr. Farina Mustaffa Kamal**

**Co-supervisors: Dr. Khor Kuan Hua**

**Dr. Mohd. Hezmee Mohd. Noor**

Peripheral blood mononuclear cells (PBMCs) contain immune cell subsets such as lymphocytes and monocytes. Characterisation of their immune functions are pertinent in providing fundamental information on its mechanism and response. In this study, the temperature and composition of cryopreservation media were examined to determine the effects on feline PBMCs viability. Blood samples were obtained from 15 healthy cats and feline PBMCs were isolated using density gradient centrifugation technique. The PBMCs were stored in  $-196^{\circ}\text{C}$  for 2 weeks in cryopreservation media of three different conditions; namely, 10% DMSO with 90% FBS, 10% DMSO with 90% feline serum (FS) and the combination of 10% DMSO with 45% FBS and 45% FS. In addition, two different temperatures ( $4^{\circ}\text{C}$  and room temperature) of

cryopreservation media were assessed. The cell viability was measured by using trypan blue dye exclusion technique and viability staining using flow cytometry. The result from dye exclusion technique showed that the addition of FS provide a similar recovery of feline PBMCs immediately upon thawing as compared to the utilization of FBS. However, the result from flow cytometric analysis revealed a higher PBMCs viability when using FS as compared to FBS. In addition, different cryopreservation medium temperature did not provide significant recovery of feline PBMCs after cryopreservation. Overall, the use of FS in cryopreservation media has provided comparable feline PBMCs recovery compared to FBS immediately upon thawing. In addition FS may play a role in maintaining feline PBMCs viability. However, their role in the clinical endpoints of other assays could not be excluded.

*Key words: Feline PBMCs, cryopreservation, medium composition, medium temperature, cell viability, flow cytometry*

## 1.0 INTRODUCTION

Peripheral blood mononuclear cells (PBMCs) contain immune cell subsets, namely lymphocytes, monocytes, and macrophages. Characterisation of their immune functions are pertinent in providing fundamental information on its mechanism and response. Feline PBMCs are important in research works, such as isolation of feline parvovirus (Miyazawa *et al.*, 1999), productive infection of feline immunodeficiency virus (FIV) *vif* gene in feline PBMCs and monocyte-derived macrophages (Lockridge *et al.*, 1999), and expression of CXCR4 on feline PBMCs in FIV infection (Willet *et al.*, 2003). Often, analyses could not be performed immediately due to various issues such as logistics, unavailability of materials, or simply time constraint. Therefore, cryopreservation is performed whereby low temperature is used to preserve different types of cells for long term by using liquid nitrogen. Low temperature storage (-196°C) and slow cooling rate (1°C per minute) are generally recommended for optimal cells recovery. In addition, using cryoprotectant such as dimethyl sulfoxide (DMSO) can prevent the formation of ice crystals which can lyse the cells during thawing. The freezing medium is usually supplemented by higher concentration of serum such as fetal bovine serum (FBS). Although certain aspects in cryopreservation have been studied in human PBMCs, none has been done in feline PBMCs. In this study, the temperature and composition of cryopreservation media were examined using dye exclusion technique and cell viability staining by flow cytometry to determine the effects on feline PBMCs viability.

The objectives of this study were:

- 1) To identify the best cryopreservation conditions in order to get the maximum feline PBMCs recovery upon thawing.
- 2) To determine the effect of compositions and temperatures of cryopreservation media on feline PBMCs viability.

The hypotheses for this study were:

- 1) The addition of feline serum in cryopreservation media provides higher feline PBMCs recovery.
- 2) Low temperature (4°C) of cryopreservation media is less toxic to feline PBMCs.

## **2.0 LITERATURE REVIEW**

### **2.1 Feline Peripheral Blood Mononuclear Cells (PBMCs)**

Feline peripheral blood mononuclear cells (PBMCs) consists of cells such as lymphocytes (T cell, B cell, natural killer cell), monocytes, and macrophages. These cells have characteristics of round nucleus and have a lesser density as compared to erythrocytes and polymorphonuclear cells (PMN) such as neutrophils and eosinophils (Ulmer *et al.*, 1984). They play an important role in the immune system to fight infections. To isolate feline PBMCs, buffy coat is extracted from centrifugation of whole, non-coagulated blood and isolated PBMCs can be freshly used or cryopreserved for future use (Makino & Baba, 1997).

PBMCs, mostly from humans have been studied in depth, mostly for purpose of stem cell research, cancer immunotherapy, and biomarker identification (Nazarpour *et al.*, 2012). Research work such as the study of CD4<sup>+</sup> T cell and B cell differentiations have made full use of PBMCs. Among the characteristics of PBMCs which make them valuable in research are the use of PBMCs as potential biomarkers in gene expression profiling and it contains many different cell types (Freshney, 2005). Feline PBMCs are important in research works, such as isolation of feline parvovirus (Miyazawa *et al.*, 1999), productive infection of feline immunodeficiency virus (FIV) *vif* gene in feline PBMCs and monocyte-derived macrophages (Lockridge *et al.*, 1999), and expression of CXCR4 on feline PBMCs in FIV infection (Willet *et al.*, 2003).

## 2.2 Cryopreservation

According to Studdert *et al.* (2012), cryopreservation is defined as maintenance of the viability of cells, excised tissue or organs by utilizing very low temperature storage, while cryoprotectant is defined as a substance added to living biological material that is to be preserved in a viable state by freezing. Ethylene glycol, glycerol, and dimethyl sulfoxide (DMSO) are the examples of cryoprotectants. Cryopreservation can also be referred as cryoconservation. In this process, cells or whole tissues are preserved by cooling to sub-zero temperatures ( $-196^{\circ}\text{C}$ ) by using liquid nitrogen. These cells or tissues are susceptible to damage, which can be triggered by chemical reactivity or time. At a very low temperature, any enzymatic or chemical activity which leads to damage of cells or tissues can be effectively stopped. Besides that, formation of ice during freezing may cause additional damage. Pegg (2015) stated that several theories of freezing injury can be considered such as the piercing or teasing cells apart by the ice crystals, destroying them through direct mechanical action, or damage caused by secondary effects via changes in the composition of the liquid phase. Therefore, cryopreservation methods relied on cryoprotectants; anti-freeze compounds that coat cells. Cryoprotectants reduce the amount of ice formation at any given temperature by increasing the total concentration of solutes in the system. They must be able to penetrate into the cells, with low toxic properties to be biologically acceptable without causing damage to cells. Among the compounds with such properties are glycerol, ethanediol, propanediol, and dimethyl sulfoxide (DMSO).

### **2.3 Dimethyl sulfoxide (DMSO) as Cryoprotectants**

Cryoprotectants are an essential component in cryopreservation as they serve to protect the cell on its journey from room temperature to  $-196^{\circ}\text{C}$ . DMSO is one of the most effective intracellular cryoprotectants with the properties of hydrophilicity, having suitable viscosity and stability (Nazarpour *et al.*, 2012). DMSO works by reducing the formation of intracellular ice crystal without having to endanger cells to water deprivation, consequent to freezing. Simione (2009) described that the addition of DMSO will cause freezing point depression, a process consequent to the act in which adding a solute to a solvent will lead to a decrease in freezing point of the mixture. Cryoprotective agents are said to be most effective when they have the ability to penetrate the cell and delay intracellular freezing. Eventhough DMSO has been traditionally proven to be one of the most effective cryoprotectants (Simione, 2009), it may cause cellular toxicity which is potentiated by warm temperatures (Wewetzer & Dilmaghani, 2001).

### **2.4 The Role of Fetal Bovine Serum in Cryopreservation**

Serum, a component of the blood (without cells and clotting factors) is commonly used as a supplement in medium for cell cultures. The most common type of serum used for this purpose is fetal bovine serum (FBS) due to its high content of embryonic growth promoting factors. The serum is traditionally obtained from foetuses harvested from cows that were slaughtered in abattoirs. These foetuses must be at least 3 months of age in order to qualify for FBS harvest because smaller sized foetuses would not provide sufficient amount of FBS. Blood of foetuses will be extracted under vacuum and kept into a sterile blood collection bag. To separate the

sera, the collected blood is allowed to clot and proceed to refrigerated centrifugation for the sera collection procedure to be completed (Brunner *et al.*, 2010).

Serum provides a wide variety of nutrients, macromolecular proteins, carrier proteins for water-insoluble components, and other compounds required for cells grown *in vitro*. Other than that, serum also ensures the survivability of cells by providing buffering capacity to the medium and binds or neutralizes toxic components in the cell culture. The primary reason of the necessity in using FBS is because cells are programmed to be suicidal and undergo apoptosis when they lack local tissue chemical signals. These signals can be in the form of cell-to-cell contact or secreted factors such as hormones and growth factors (Wang *et al.*, 2006). Without continued stimulus from these signals, cells can sense that they are growing in the ‘wrong place’, thus programming themselves towards apoptosis. The use of FBS, which is rich in the required hormones and growth factors will not only prevent apoptosis, but also has the ability to stimulate replication (Brunner *et al.*, 2010). FBS has a significantly lower amount of antibodies as compared to serum harvested from newborn or adult animals. This particular characteristic of FBS is able to minimize the risk of antibody cross reaction with cells in culture.

## **2.5 The Effects of Cryopreservation towards Cells Detection, Viability, and Response.**

A study has been done in identifying the best cryopreservation condition in human PBMCs. Nazarpour *et al.* (2012) discovered that the survival of PBMCs after cryopreservation is mostly affected by the concentration of DMSO. Meanwhile, the freezing medium temperature seems to have mild effects on the cells viability, and

FBS concentration in freezing medium does not seem to have any significant effect. According to Freshney (2010), 10% DMSO in freezing medium has been suggested to be the minimum concentration and lowering the concentration usually adversely affects the survival of human cells. Nazarpour *et al.* (2012) shown that a higher FBS concentration did not show any improvements in human PBMCs viability compared to a lower FBS concentration. However, in a study conducted on spermatogonial cells freezing medium, an increase in serum concentration from 50 to 70 % has increased the cell viability (Alipoor *et al.*, 2010).

Sattui *et al.* (2012) revealed that cryopreservation imposed a decreased on the flow cytometric detection of surface and intracellular markers of human regulatory T-cells. In another study by Owen *et al.*, (2008), it was found that the response of CD4+ T cells towards Human Immunodeficiency Virus (HIV) peptides is markedly reduced following long term cryopreservation, which is up to one year as compared to less than six months duration.

## **2.6 Methodologies Available in Measuring Cell Viability**

Measuring cell viability is a procedure done to determine the number or percentage of live and dead cells in a cell suspension. Cell viability can be measured through several methods, such as dye exclusion viability assays, metabolic viability assays, flow cytometry and ATP test. Dye exclusion assay involves the use of dye such as trypan blue, propidium iodide, 7-Aminoactinomycin D (7-AAD) and acridine orange. These dyes are able to penetrate damaged cell membrane that has lost its integrity, implying that the cell is dead. However, these dyes are impermeable towards intact cell membrane, thus making it excluded from viable cells (Strober, 2001).

Propidium iodide, 7-AAD and acridine orange can also be used in flow cytometry, as they fluoresce and detected by lasers from the machine. Cells that are stained and fluorescently labelled by the dye will be excited by the laser of flow cytometry to emit light at varying wavelengths. From the light measurements, various information regarding physical and chemical structure of the cells can be collected by the machine. Metabolic viability assay does not work the same as dye exclusion assay. This assay measures the cell's ability in performing specific biochemical reaction that will be measured by the level of absorbance, fluorescence or luminescence. Among the examples of biochemical reactions are function of ion pumps and channels detected by fluorescence, change in pH indicators detected by fluorescence, and cellular and mitochondrial metabolism detected by measuring ATP using luminescence method. According to Riss *et al.* (2004), firefly luciferase is a common reagent used in ATP test in order to estimate the number of viable cells and this assay is the fastest, most sensitive cell viability assay, besides being less prone to artefacts.

### **3.0 MATERIALS AND METHODS**

#### **3.1 Animals**

Fifteen healthy client-owned cats were sampled. The cats were confirmed to be overtly healthy by attending veterinarians. Institutional Animal Care and Use Committee (IACUC) approval was obtained before carrying out this project (FYP.2015/FPV.026). Client consents were obtained from the cat owners prior to blood sampling (Appendix 8.1 and 8.2).

#### **3.2 Sample collection**

Approximately 3 to 5 ml of blood were collected into K<sub>2</sub>EDTA tubes via jugular venipuncture technique. These blood samples were subjected for feline PMBCs Isolation using Ficoll-Paque density gradient technique.

#### **3.3 Inclusion criteria**

Number of viable cells per 10 mL Hank's Balanced Salt Solution (HBSS) was counted for each blood sample using a haemocytometer (Appendix 8.4). Blood samples with a total number of cells of 5 million cells or more were included in the study.

#### **3.4 Preparation of feline serum**

Feline serum was collected from healthy animals, tested negative against FIV, FeLV and FCoV. The sera were then filtered and heat inactivated (Appendix 8.3). The sera had also tested negative for mycoplasma.

### **3.5 Isolation of feline peripheral blood mononuclear cells (PBMCs)**

This procedure was performed according to Ficoll-Paque density gradient technique (Nazarpour *et al.*, 2012). Blood samples were pipetted from K<sub>2</sub>EDTA tubes and transferred into 15 ml conical tubes. The blood was diluted in 1:1 ratio to HBSS. The diluted blood were carefully overlaid on top of an equal volume of Histopaque®-1077 (Sigma-Aldrich®, United States). The tubes were immediately centrifuged at 1500 RPM for 25 minutes. The buffy coat layer was transferred into a new tube and washed with HBSS at 2000 RPM for 10 minutes. The supernatant was poured off and the cells were resuspended in 10 mL HBSS for cell counting.

### **3.6 Determination of Cell Viability before Cryopreservation**

The number of live and dead cells were counted using haemocytometer and the percentage of cell viability was determined. The resuspended cells were diluted in 1:1 ratio with 0.4% Trypan Blue (Gibco®, United States) to determine the cell viability. Live and dead cells were counted from the 4 chambers of the haemocytometer. The figure below shows one of the 4 chambers which consists of 16 smaller squares.

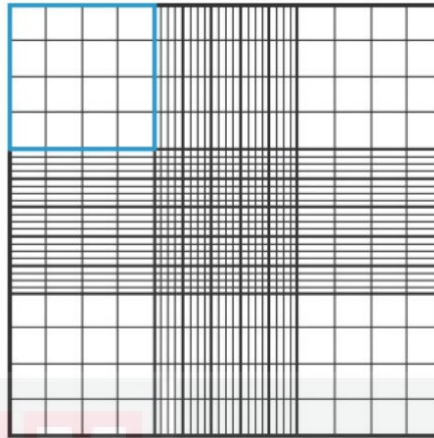


Figure 1: Squares on haemocytometer

The number of cells counted from the four chambers are divided by 4 to get the average number of cells. The following formula was used to obtain total number of cells.

$$\text{Average number of cells} \times \text{Dilution factor} \times 10^4 = \text{Number of cells / ml}$$

For example, for cat number 1, the cell counts for each of the 16 squares were 340, 400, 410 and 330. The total number of cells was:

$$\frac{(340+400+410+330)}{4} \times 2 \times 10^4 = 7\,400\,000 \text{ cells / ml}$$

The percentage of cell viability can be determined from the formula:

$$\text{Total number of cells} = \text{Number of live cells} + \text{Number of dead cells}$$

$$\text{Percentage of viable cells} = \left( \frac{\text{Number of live cells}}{\text{Total number of cells}} \right) \times 100 \%$$

For example, for cat number 1, the number of live cells was 7 400 000, and the number of dead cells was 40 000, the percentage of viable cells would be:

$$\frac{7\,400\,000}{(7\,400\,000+40\,000)} \times 100 \% = 99.5 \%$$

### 3.7 Feline PBMCs Cryopreservation

Different compositions and conditions of cryopreservation media were prepared as shown in the table below:

Condition/ Treatment	Percentage of composition (%)				Media Temperature (°C)
	Dimethyl sulfoxide (DMSO)	Fetal Bovine Serum (FBS)	Feline Serum (FS)	Peripheral Blood Lymphocyte (PBL) media	
1	10	90	0	0	4
2	10	45	45	0	4
3	10	0	90	0	4
4	10	90	0	0	Room temperature
Control	0	0	0	100	4

Table 1: Different Cryopreservation Conditions Based on Media Composition and Temperature.

The cells were divided equally into 5 conical tubes. The conical tubes were centrifuged at 2000 RPM for 5 minutes. The supernatant was discarded and cell pellets were resuspended with the prepared media and transferred into labelled cryopreservation vials. These vials were then stored in -80°C overnight in a polycarbonate freezing container (Nalgene®, United States) which provided the critical, 1°C per minute cooling rate required for successful cryopreservation of cells. Then, the cells were transferred into liquid nitrogen at -196 °C for two weeks.

### 3.8 Recovery of feline PBMCs

After two weeks of cryopreservation, the cells were then thawed in 37°C water bath. The cells were centrifuged at 2000 RPM for 5 minutes and the pellet was resuspended in 1 mL of PBL media. Percentage of viable cells were determined using Trypan Blue as previously described. The percentage of cells viability was compared before and immediately upon thawing by using the following formula:

$$\text{Percentage of decrease in cell viability after cryopreservation} = \frac{(\text{Before Cryopreservation} - \text{After Cryopreservation})}{\text{Before Cryopreservation}} \times 100\%$$

For example, for cat number 1, under treatment 1, the percentage of cell viability before cryopreservation is 99.5%. Meanwhile, the percentage of cell viability after cryopreservation is 84.3%. Therefore, the percentage of decrease in cell viability would be:

$$\frac{(99.5-84.3)}{99.5} \times 100\% = 15.3\%$$

### 3.9 Flow Cytometry Staining

Briefly,  $1 \times 10^6$  cells were washed with phosphate buffered saline (PBS). Cells were then stained with propidium iodide (PI) diluted in Tris staining buffer (100mM Tris, pH 7.4, 150mM NaCl, 1mM CaCl<sub>2</sub>, 0.5mM MgCl<sub>2</sub>, 0.1% Triton X-100) in the dark. The cells were immediately analysed within 15-30 minutes after staining using flow cytometer (BD FACSCalibur™, United States).

### 3.10 Statistical Analysis

All the data was tabulated and descriptive analysis was performed. Non-parametric statistical analyses were performed using Kruskal Wallis and Mann-Whitney statistical test (GraphPad Prism Version 5) because the data were not normally distributed. The percentage of live cells before cryopreservation was determined from each cats. The percentage of live cells upon thawing and the decrease in percentage of cell viability were compared among the five treatments. The association between temperature, composition of cryopreservation media and cells viability was then determined.



## 4.2 Trypan Blue Dye Exclusion Technique

### 4.2.1 Comparison between Different Medium Compositions

#### 4.2.1a Median Percentage of Cell Viability Upon Thawing

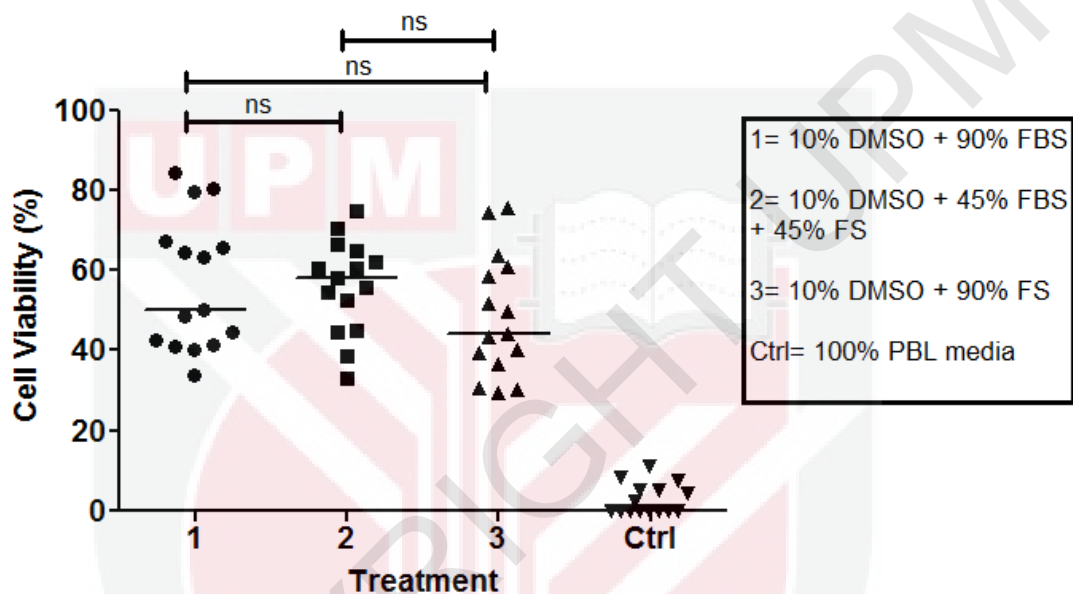


Figure 3: The median percentage of cell viability upon thawing between different medium compositions. Each bar represents the median of each treatment ( $n=15$ ).

Treatment 1, 2 and 3 were comparable with median value of 50%, 58% and 44%, respectively. There was no significant difference in median percentage of cell viability upon thawing between treatment 1, 2, and 3. However, there were significant difference between treatment 1-3 vs. control treatment ( $p < 0.0001$ ). There were no significant difference between treatment 1 vs. 2 ( $p=0.9669$ ), treatment 1 vs. 3 ( $p=0.1585$ ), and treatment 2 vs. 3 ( $p=0.1249$ ).

#### 4.2.1b Median Percentage of Decrease in Cell Viability

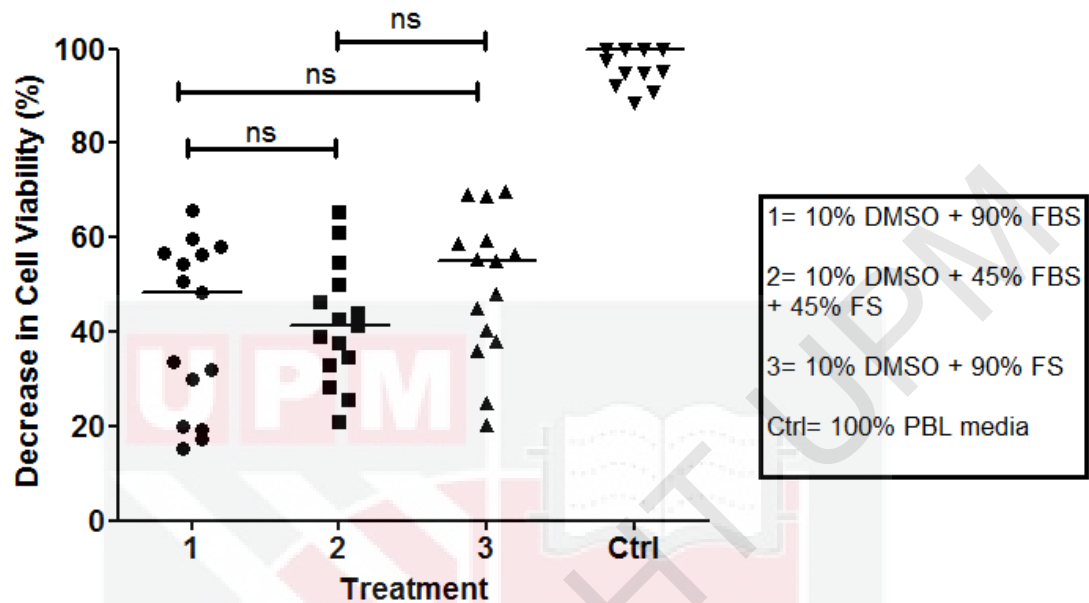


Figure 4: The median percentage of decrease in cell viability between different medium compositions. Each bar represents the median of each treatment ( $n=15$ ).

The median value for treatment 1 was 49%, treatment 2 was 41%, treatment 3 was 55% while control treatment had 100% decrease in cell viability. There was no significant difference in median percentage of decrease in cell viability between treatment 1, 2, and 3. There was a significant difference in the percentage of decrease in cell viability among the treatments ( $p < 0.0001$ ). However, a pairwise comparison showed no significant difference between treatment 1 vs. 2 ( $p=1$ ), treatment 1 vs. 3 ( $p=0.1913$ ), and treatment 2 vs. 3 ( $p=0.1249$ ).

## 4.2.2 Comparison between Different Medium Temperatures

### 4.2.2a Median Percentage of Cell Viability Upon Thawing

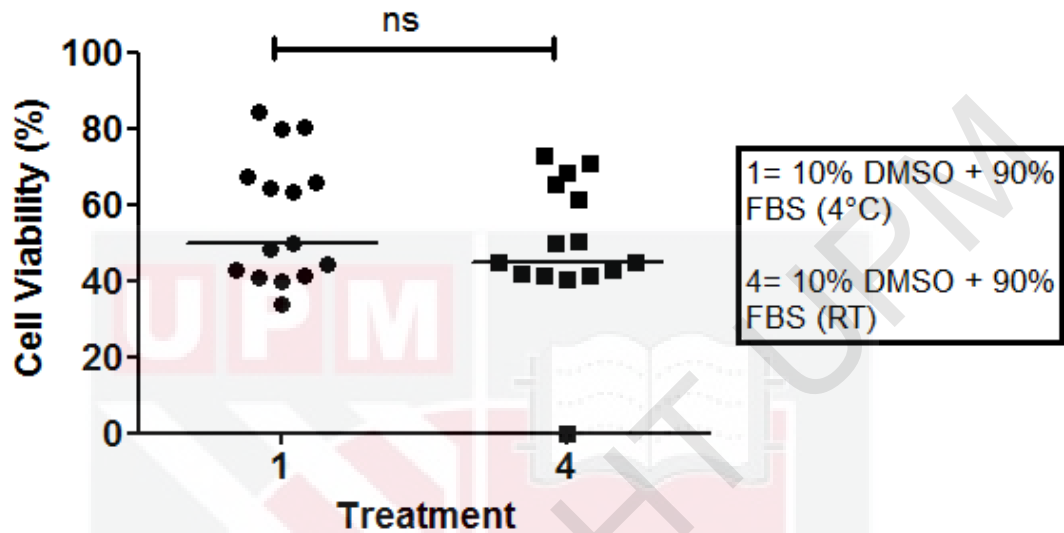


Figure 5: The median percentage of cell viability upon thawing between different medium temperatures. Each bar represents the median of each treatment ( $n=15$ ).

The median value of treatment 1 and treatment 4 were 50% and 45%, respectively. There was no significant difference in median percentage of cell viability upon thawing between treatment 1 and 4 ( $p=0.5613$ ).

#### 4.2.2b Median Percentage of Decrease in Cell Viability

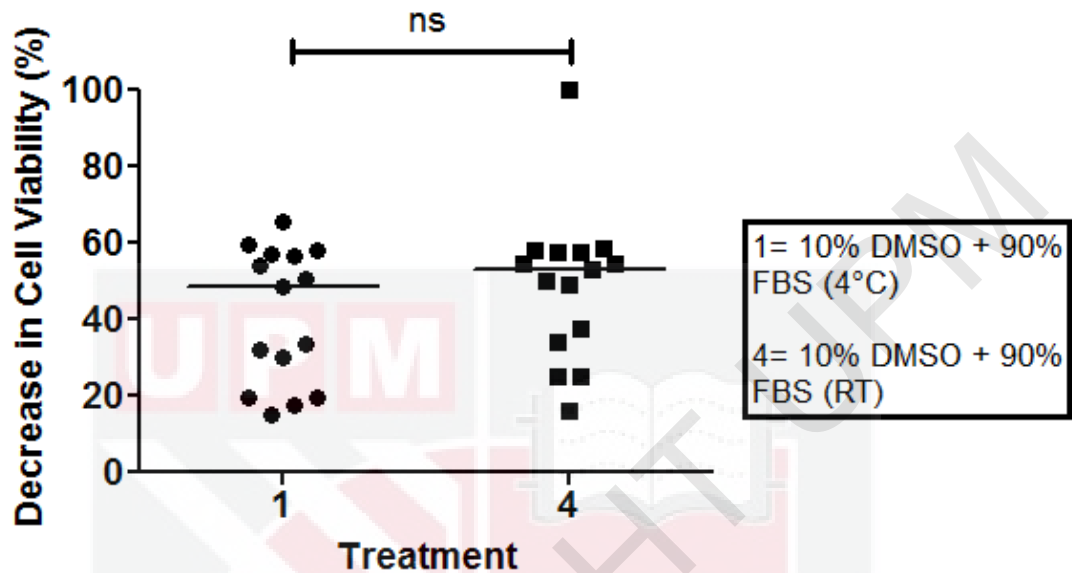


Figure 6: The median percentage of decrease in cell viability between different medium temperatures. Each bar represents the median of each treatment ( $n=15$ ).

The median value for treatment 1 was 49% while treatment 4 was 53%. There was no significant difference in median percentage of decrease in cell viability between treatment 1 and 4 ( $p=0.4185$ ).

### 4.3 Flow Cytometry

#### 4.3.1 Comparison between Different Medium Compositions

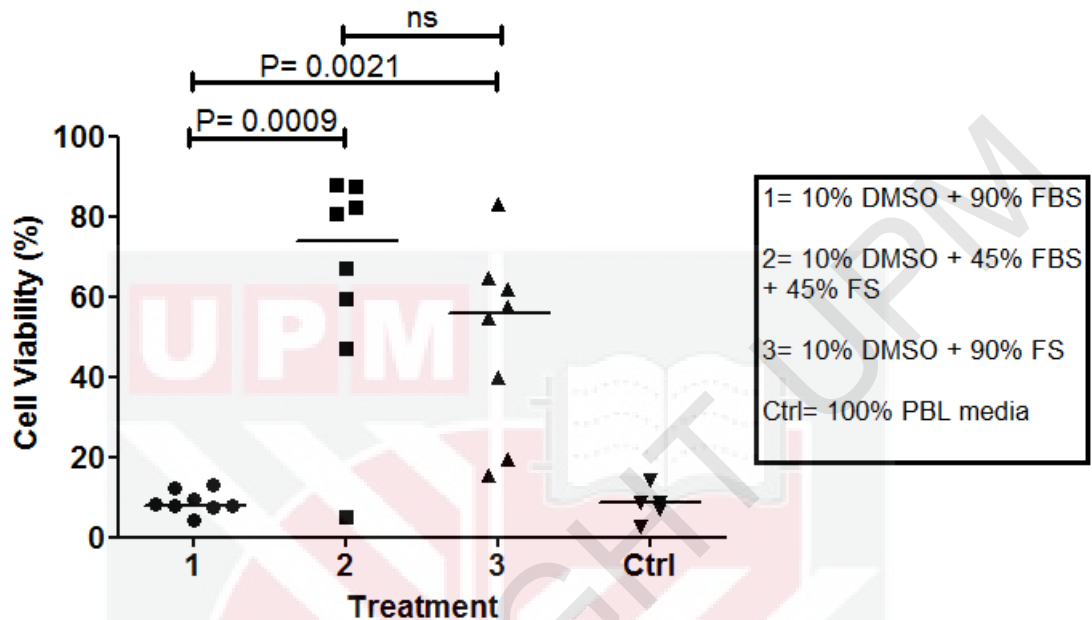


Figure 7: The median percentage of cell viability upon thawing between different medium compositions. Each bar represents the median of each treatment; treatment 1 ( $n=8$ ), treatment 2 ( $n=8$ ), treatment 3 ( $n=8$ ) and control ( $n=5$ ).

The median value of treatment 1, treatment 2, treatment 3 were 8 %, 74% and 56%, respectively. There was a significant difference on percentage of cell viability upon thawing among the four treatments ( $p<0.0001$ ). Treatment 1 showed a significant difference when compared to treatment 2 ( $p=0.0009$ ) as well as treatment 1 vs. 3 ( $p=0.0021$ ). However, treatment 2 was not significantly different from treatment 3 ( $p=0.1665$ ). During staining process in preparation for flow cytometry method, cell loss was experienced. Therefore, the number of samples varied for each treatment.

### 4.3.2 Comparison between Different Medium Temperatures

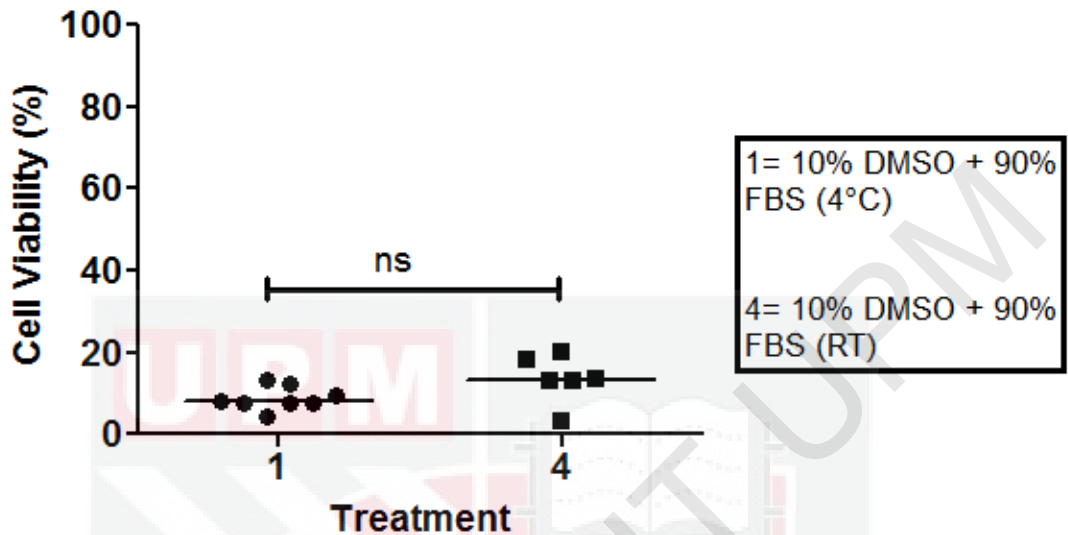


Figure 8: The median percentage of cell viability upon thawing between different medium temperatures. Each bar represents the median of each treatment; treatment 1 ( $n=8$ ), and treatment 4 ( $n=6$ ).

The median value for treatment 1 was 8% while treatment 4 was 13%. There was no significant difference in median percentage of cell viability upon thawing between treatment 1 and 4 ( $p=0.1191$ ).

### 4.3.3 Median Percentage of Cell Viability After Cryopreservation from Flow Cytometry Method

Treatment	Median Percentage of Cell Viability
1	8
2	74
3	56
4	13
5	9

Table 2: Median percentage of cell viability upon thawing from flow cytometry method, with treatment 1 ( $n=8$ ), treatment 2 ( $n=8$ ), treatment 3 ( $n=8$ ), treatment 4 ( $n=6$ ) and control ( $n=5$ ).

Treatment 1 and 4 which consisted of FBS resulted in a lower median percentage of cell viability, 8% and 13%, respectively. Meanwhile, treatment 2 and 3 which contained feline serum as a supplement in the cryopreservation media resulted in a higher median percentage of cell viability, 74% and 56% respectively.

## 5.0 DISCUSSION

According to Brunner *et al.* (2010), appropriate culture conditions with respect to temperature, pH, osmolarity and oxygen supply are necessary for successful growth and maintenance of cells, regardless of the cells' species of origin. The most widely used sera in cell cultures are bovine sera of adult, newborn, or foetal origin. A broad range between 40 to 100% FBS or BSA (Bovine Serum Albumin) has been used as a cryopreservation media supplementation in order to increase survivability of cells (Freshney, 2005). In this study, FBS of 45% and 90% concentration were used, which was a recommended range of FBS in cryopreservation media.

The addition of FS in the cryopreservation media, whether individually or in mixture with FBS provided a comparable feline PBMCs recovery to the use of FBS immediately upon thawing. Interestingly, flow cytometric analysis utilizing PI showed a higher cell recovery when FS were used as cryopreservation media. This may be due to longer duration involved in the preparation and staining of the PBMCs which caused a detrimental effect on the cells viability. Therefore, supplementation with FS allowed longer maintenance of PBMCs, thus higher survivability of cells were observed in comparison to FBS.

According to Freshney (2005), cooling the cryopreservation medium to 4°C before mixing the medium with cell suspension is recommended to decrease the damage caused by toxic DMSO since low temperature will cause a decrease in cell metabolism. However, the act of cooling the medium will prevent DMSO penetration into cells, leading to a greater cryopreservation stress. Kreher *et al.* (2003) stated that adding freezing medium to PBMCs at room temperature will result in a higher cell

recovery instead of keeping the medium cold during cryopreservation. However, in our study, temperature of the cryopreservation medium, whether at 4°C or room temperature did not pose a significant effect on the recovery of feline PBMCs consistent with the findings by Nazarpour *et al.* (2012). In addition, PBL media that served as control resulted in the lowest cell recovery upon thawing. This was due to the absence of DMSO which served as an intracellular cryoprotectant. Cryoprotectant is a very important component in cryopreservation media as it protects the cell on its journey from room temperature to -196°C (Pegg, 2015).

A study done by Sattui *et al.* (2012) found that cryopreservation caused a decrease in proportion of regulatory T cells identified by surface and intracellular markers when comparing freshly stained to frozen thawed cells. However, a study done by Martinello *et al.* (2010) suggested that cryopreservation of multipotent cells isolated from equine peripheral blood does not affect the stem characteristics of the cells after being frozen for 10 to 12 months. Therefore, further evaluation of the advantages in using FS need to be reviewed in order to determine its role in the clinical endpoint of other assays.

## 6.0 CONCLUSION AND RECOMMENDATIONS

As a conclusion, the use of FS in cryopreservation media provided comparable feline PBMCs recovery compared to FBS immediately upon thawing. However, FS may play a vital role in maintaining the survivability of PBMCs. In addition, the temperature of cryopreservation media did not provide any significant difference in the recovery of feline PBMCs, thus played a less significant role in the cells recovery.

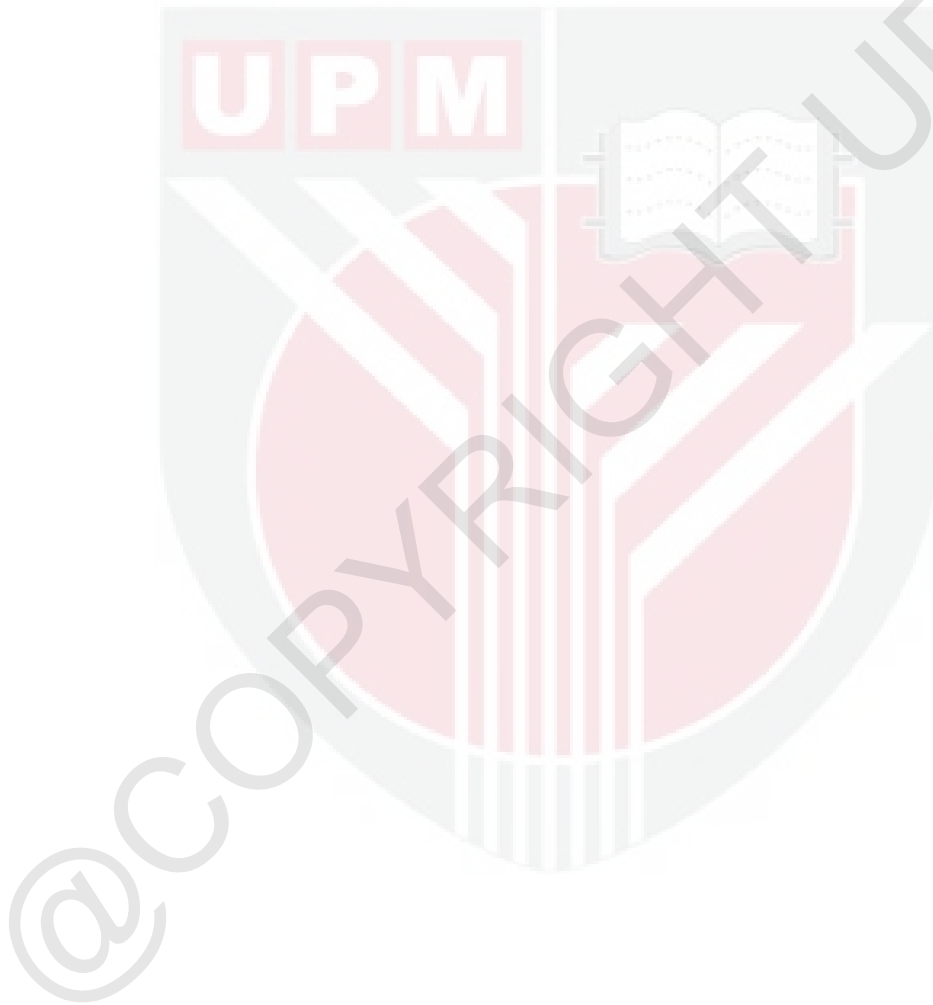
One of the limitations in this study is we could not obtain enough number of cells per cat. Therefore, storing of more than  $10 \times 10^6$  cells per vial is recommended as this is the standard number of cells used for cryopreservation.

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## 8.0 APPENDICES

### 8.1 Client Consent Form (English)



**FACULTY OF VETERINARY MEDICINE  
UNIVERSITI PUTRA MALAYSIA**

**Client Consent Form**

**Research Title: The Effects of Different Cryopreservation Conditions on Feline Peripheral Blood Mononuclear Cells (PBMCs) Viability**

**Period of Study: 9<sup>th</sup> January 2016 - 21<sup>st</sup> February 2016**

I, Siti Aisyah binti Azhar (matric no: 164701), a 5<sup>th</sup> year student of Doctor of Veterinary Medicine (DVM 5), supervised by Dr. Farina Mustaffa Kamal, would like to obtain permission from Mr./Mrs./Miss/Sir to perform **blood collection procedure** on your cat/cats.

This is necessary for me to complete my Final Year Project on 'The Effects of Cryopreservation Conditions on Feline Peripheral Blood Mononuclear Cells (PBMCs) Viability'. From this research study, the best condition to store cat cells for further analysis can be determined.

This procedure does not cause any harm and there will be no clinical conditions or abnormalities expected afterwards. However, this procedure might cause minimal stress to your cat/cats. The participation is voluntary and you may ask to withdraw your cat/cats anytime during the procedure.

Contact number for questions / concerns:

IACUC: 603-89471244

Dr. Farina Mustaffa Kamal (supervisor): 603-86093466

Dr. Khor Kuan Hua (co-supervisor/attending veterinarian): 603-86093926

Investigator,

.....  
(Siti Aisyah binti Azhar)  
Final year student,  
Doctor of Veterinary Medicine.

This application of consent is approved by,

.....  
(Dr. Farina Mustaffa Kamal)  
Supervisor of FYP,  
Department of Veterinary Pathology & Microbiology,  
Faculty of Veterinary Medicine,  
Universiti Putra Malaysia.

Date:

---

I ..... with identification number (I/C)....., the pet owner of a cat/cats named .....hereby is giving permission to Siti Aisyah binti Azhar (matric no: 164701) with the help of UVH personnel to withdraw blood from my cat in order for her to complete her Final Year Project on ‘The Effects of Cryopreservation Conditions on Feline Peripheral Blood Mononuclear Cells (PBMCs) Viability’. I am fully aware of the consequences and the student or any UVH personnel will not held responsible for any impact afterwards.

Sincerely,

Date:

Phone no.:

.....

## 8.2 Borang Persetujuan Pelanggan (Bahasa Malaysia)



**FAKULTI PERUBATAN VETERINAR**

**UNIVERSITI PUTRA MALAYSIA**

**BORANG PERSETUJUAN PELANGGAN**

**Tajuk Kajian: Impak Berlainan Kondisi Pengawetankrio Terhadap Kebolehhidupan Sel Darah Mononuklear Kucing**

**Tempoh kajian: 9 Januari 2016 – 21 Februari 2016**

Saya, Siti Aisyah binti Azhar (no. matrik: 164701), pelajar Tahun 5 bagi Program Doktor Perubatan Veterinar (DVM 5), di bawah pengawasan Dr. Farina Mustaffa Kamal, ingin meminta kebenaran dari pihak Tuan/Puan/Cik/Encik untuk **mengambil sampel darah** dari kucing milik Tuan/Puan/Cik/Encik.

Prosedur ini amat diperlukan bagi saya melengkapkan Projek Tahun Akhir saya yang bertajuk 'Impak Kondisi Pengawetankrio Terhadap Kebolehhidupan Sel Darah Mononuklear Kucing'. Melalui projek ini, kondisi optimum bagi mengawet sel kucing untuk kajian seterusnya dapat dikenal pasti.

Prosedur ini tidak mengakibatkan sebarang kemudaratan atau abnormaliti. Walau bagaimanapun, prosedur ini mungkin akan mengakibatkan stress yang minimum terhadap kucing anda. Penyertaan adalah secara sukarela dan anda boleh membatalkan penyertaan kucing anda bila-bila masa sewaktu prosedur dijalankan.

Nombor telefon bagi soalan atau keraguan:

IACUC: 603-89471244

Dr. Farina Mustaffa Kamal (penyelia): 603-86093466

Dr. Khor Kuan Hua (penyelia bersama/pegawai veterinar terlibat): 603-86093926

Penyelidik,

.....  
(Siti Aisyah binti Azhar)  
Pelajar tahun akhir,  
Doktor Perubatan Veterinar.

Surat permohonan kebenaran ini disahkan oleh,

.....  
(Dr. Farina Mustaffa Kamal)  
Supervisor Projek Tahun Akhir,  
Jabatan Patologi dan Mikrobiologi Veterinar  
Fakulti Perubatan Veterinar  
Universiti Putra Malaysia.

Tarikh:

---

Saya ..... dengan nombor kad pengenalan (I/C) ..... pemilik kepada kucing bernama ..... memberi kebenaran kepada Siti Aisyah binti Azhar (no. matrik: 164701) dengan bantuan staff UVH untuk mengambil darah dari kucing saya untuk melengkapkan Projek Tahun Akhirnya yang bertajuk 'Impak Kondisi Pengawetankrio Terhadap Kebolehhidupan Sel Darah Mononuklear Kucing'. Saya sedar akan kesan dan akibatnya terhadap kucing saya dan pelajar tersebut serta staff UVH tidak akan bertanggungjawab terhadap sebarang impak setelah prosedur dijalankan.

Yang benar,

Tarikh:

No. telefon:

.....

### 8.3 Protocol of Heat Inactivation of Serum



## Heat-Inactivating Serum

#### What is Heat Inactivation?

The practice of heat inactivating serum was originally developed when only serum from adult animals was available for cell culture. Adult serum contains various immune factors, particularly serum complement, which may inhibit or destroy cells under certain conditions. Heating serum is intended to inactivate serum complement. Today, serum is often heat-inactivated without any evidence of beneficial effect, simply because an earlier protocol calls for such action. However, certain applications demand the inactivation of complement; e.g., it is often required to preserve the integrity of immunoassays.

Heat inactivation will increase precipitates and consequent turbidity in the serum.

Gemini will provide heat inactivation for most sera upon request, as a service to our customers. Should you prefer to perform this step in house, we recommend the following protocol:

#### Recommended Protocol

1. Thoroughly thaw serum and swirl to homogenize.
2. Prepare a control bottle containing water. The control bottle should be stored along with the serum bottles to assure identical initial temperatures. This control bottle will be used to monitor the temperature and should be identical to the serum bottle(s) (e.g., PETG with a cap or stopper).
3. Place the bottle(s) of serum and the control bottle into a 56 °C water bath containing sufficient water to immerse the bottle above the serum level. Suspend a thermometer thermocouple in the water bottle. The thermometer should not touch the sides or bottom of the bottle.
4. Swirl the bottles every 3-5 minutes to ensure uniform heating of the serum.
5. Monitor the temperature of the control bottle closely and begin timing when the temperature reaches 56 °C.
6. After 30 minutes (25 minutes for equine/human serum), remove the serum bottles and cool slowly to room temperature. The serum should be left overnight before aliquotting. If the serum is to be filtered to remove precipitate, first it must be cooled to 4 °C overnight, then filtered and aliquotted. Heat-inactivated serum may be re-frozen once, thawed and then used.
7. Heat-inactivated serum should be stored at -20 °C, otherwise the amount of accumulated precipitate will increase as the serum stands in the thawed state, even if left in a refrigerator. The serum should be stored in small aliquots that can be thawed individually as needed. We do not recommend more than one freeze-thaw cycle after heat-inactivation.

## 8.4 Protocol of Counting Cells using a Haemocytometer

### Counting cells using a hemocytometer



Detailed procedure explaining how to obtain a viable cell count from suspension cells using a hemocytometer.

#### Preparing the hemocytometer

1. If using a glass hemocytometer and coverslip, clean with alcohol before use. Moisten the coverslip with water and affix to the hemocytometer. The presence of Newton's refraction rings under the coverslip indicates proper adhesion.
2. If using a disposable hemocytometer (for example, INCYTO DHC-ND1), simply remove from the packet before use.

#### Preparing cell suspension

1. Gently swirl the flask to ensure the cells are evenly distributed.
2. Before the cells have a chance to settle, take out 0.5 mL of cell suspension using a 5 mL sterile pipette and place in an Eppendorf tube.
3. Take 100  $\mu$ L of cells into a new Eppendorf tube and add 400  $\mu$ L 0.4 % Trypan Blue (final concentration 0.08%). Mix gently.

#### Counting

1. Using a pipette, take 100  $\mu$ L of Trypan Blue-treated cell suspension and apply to the hemocytometer. If using a glass hemocytometer, very gently fill both chambers underneath the coverslip, allowing the cell suspension to be drawn out by capillary action. If using a disposable hemocytometer, pipette the cell suspension into the well of the counting chamber, allowing capillary action to draw it inside.
2. Using a microscope, focus on the grid lines of the haemocytometer with a 10X objective.
3. Using a hand tally counter, count the live, unstained cells (live cells do not take up Trypan Blue) in one set of 16 squares (Figure 1). When counting, employ a system whereby cells are only counted when they are within a square or on the right-hand or bottom boundary line. Following the same guidelines, dead cells stained with Trypan Blue can be also be counted for a viability estimate if required.
4. Move the hemocytometer to the next set of 16 corner squares and carry on counting until all four sets of 16 corner squares are counted.

#### Viability

To calculate number of viable cells / mL:

1. Take the average cell count from each of the sets of 16 corner squares.
2. Multiply by 10,000 ( $10^4$ ).
3. Multiply by five to correct for the 1:5 dilution from the Trypan Blue addition.

The final value is the number of viable cells/mL in the original cell suspension.

Example:

If the cell counts for each of the 16 squares were 50, 40, 45, 52, the average cell count would be:

$$(50 + 40 + 45 + 52) \div 4 = 46.75$$

$$46.75 \times 10,000 (10^6) = 467,500$$

$$467,500 \times 5 = 2,337,500 \text{ live cells/mL in original cell suspension}$$

To calculate viability:

If both live and dead cell counts have been recorded for each set of 16 corner squares, an estimate of viability can be calculated.

1. Add together the live and dead cell count to obtain a total cell count.
2. Divide the live cell count by the total cell count to calculate the percentage viability.

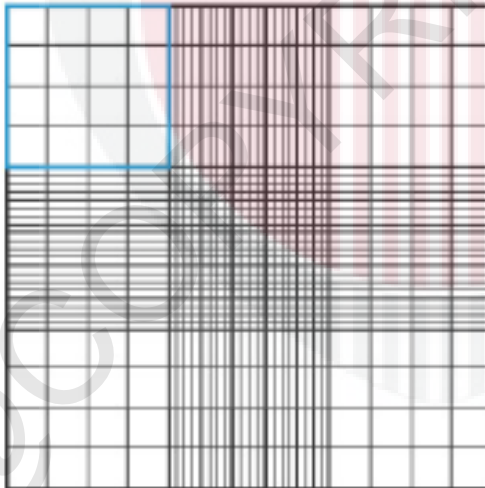
Example:

Live cell count: 2,337,500 cells/mL

Dead cell count: 50,000 cells/mL

$$2,337,500 + 50,000 = 2,387,500 \text{ cells}$$

$$2,337,500 \div 2,387,500 = 97.9\% \text{ viability}$$



**Figure 1. Hemocytometer gridlines**

*Hemocytometer diagram indicating one of the sets of 16 squares that should be used for counting.*

### 8.5 Number of live cells, dead cells and percentage of cell viability before cryopreservation

Date	Cat ID	Condition	No. of live cells	No. of dead cells	Total no. of cells	Percentage of cell viability (%)
15/1/2016	1	1	$7.40 \times 10^6$	$4 \times 10^4$	$7.44 \times 10^6$	99.5
		2	$7.40 \times 10^6$	$4 \times 10^4$	$7.44 \times 10^6$	99.5
		3	$7.40 \times 10^6$	$4 \times 10^4$	$7.44 \times 10^6$	99.5
		4	$7.40 \times 10^6$	$4 \times 10^4$	$7.44 \times 10^6$	99.5
		5	$7.40 \times 10^6$	$4 \times 10^4$	$7.44 \times 10^6$	99.5
15/1/2016	2	1	$7.24 \times 10^6$	$2 \times 10^4$	$7.26 \times 10^6$	99.7
		2	$7.24 \times 10^6$	$2 \times 10^4$	$7.26 \times 10^6$	99.7
		3	$7.24 \times 10^6$	$2 \times 10^4$	$7.26 \times 10^6$	99.7
		4	$7.24 \times 10^6$	$2 \times 10^4$	$7.26 \times 10^6$	99.7
		5	$7.24 \times 10^6$	$2 \times 10^4$	$7.26 \times 10^6$	99.7
15/1/2016	3	1	$5.12 \times 10^6$	$3 \times 10^4$	$5.15 \times 10^6$	99.4
		2	$5.12 \times 10^6$	$3 \times 10^4$	$5.15 \times 10^6$	99.4
		3	$5.12 \times 10^6$	$3 \times 10^4$	$5.15 \times 10^6$	99.4
		4	$5.12 \times 10^6$	$3 \times 10^4$	$5.15 \times 10^6$	99.4
		5	$5.12 \times 10^6$	$3 \times 10^4$	$5.15 \times 10^6$	99.4
18/1/2016	4	1	$2.83 \times 10^6$	$6 \times 10^4$	$2.89 \times 10^6$	97.9
		2	$2.83 \times 10^6$	$6 \times 10^4$	$2.89 \times 10^6$	97.9
		3	$2.83 \times 10^6$	$6 \times 10^4$	$2.89 \times 10^6$	97.9
		4	$2.83 \times 10^6$	$6 \times 10^4$	$2.89 \times 10^6$	97.9
		5	$2.83 \times 10^6$	$6 \times 10^4$	$2.89 \times 10^6$	97.9
18/1/2016	5	1	$4.42 \times 10^6$	$8 \times 10^4$	$4.50 \times 10^6$	98.2
		2	$4.42 \times 10^6$	$8 \times 10^4$	$4.50 \times 10^6$	98.2
		3	$4.42 \times 10^6$	$8 \times 10^4$	$4.50 \times 10^6$	98.2
		4	$4.42 \times 10^6$	$8 \times 10^4$	$4.50 \times 10^6$	98.2
		5	$4.42 \times 10^6$	$8 \times 10^4$	$4.50 \times 10^6$	98.2
18/1/2016	6	1	$2.45 \times 10^6$	$7 \times 10^4$	$2.52 \times 10^6$	97.2
		2	$2.45 \times 10^6$	$7 \times 10^4$	$2.52 \times 10^6$	97.2
		3	$2.45 \times 10^6$	$7 \times 10^4$	$2.52 \times 10^6$	97.2
		4	$2.45 \times 10^6$	$7 \times 10^4$	$2.52 \times 10^6$	97.2
		5	$2.45 \times 10^6$	$7 \times 10^4$	$2.52 \times 10^6$	97.2
18/1/2016	7	1	$2.88 \times 10^6$	$2 \times 10^4$	$2.90 \times 10^6$	99.3
		2	$2.88 \times 10^6$	$2 \times 10^4$	$2.90 \times 10^6$	99.3
		3	$2.88 \times 10^6$	$2 \times 10^4$	$2.90 \times 10^6$	99.3
		4	$2.88 \times 10^6$	$2 \times 10^4$	$2.90 \times 10^6$	99.3
		5	$2.88 \times 10^6$	$2 \times 10^4$	$2.90 \times 10^6$	99.3
20/1/2016	8	1	$4.95 \times 10^6$	$28 \times 10^4$	$5.23 \times 10^6$	94.6
		2	$4.95 \times 10^6$	$28 \times 10^4$	$5.23 \times 10^6$	94.6
		3	$4.95 \times 10^6$	$28 \times 10^4$	$5.23 \times 10^6$	94.6
		4	$4.95 \times 10^6$	$28 \times 10^4$	$5.23 \times 10^6$	94.6
		5	$4.95 \times 10^6$	$28 \times 10^4$	$5.23 \times 10^6$	94.6
20/1/2016	9	1	$3.36 \times 10^6$	$10 \times 10^4$	$3.46 \times 10^6$	97.1
		2	$3.36 \times 10^6$	$10 \times 10^4$	$3.46 \times 10^6$	97.1

		3	$3.36 \times 10^6$	$10 \times 10^4$	$3.46 \times 10^6$	97.1
		4	$3.36 \times 10^6$	$10 \times 10^4$	$3.46 \times 10^6$	97.1
		5	$3.36 \times 10^6$	$10 \times 10^4$	$3.46 \times 10^6$	97.1
20/1/2016	10	1	$3.15 \times 10^6$	$72 \times 10^4$	$3.87 \times 10^6$	81.4
		2	$3.15 \times 10^6$	$72 \times 10^4$	$3.87 \times 10^6$	81.4
		3	$3.15 \times 10^6$	$72 \times 10^4$	$3.87 \times 10^6$	81.4
		4	$3.15 \times 10^6$	$72 \times 10^4$	$3.87 \times 10^6$	81.4
		5	$3.15 \times 10^6$	$72 \times 10^4$	$3.87 \times 10^6$	81.4
20/1/2016	11	1	$5.58 \times 10^6$	$28 \times 10^4$	$5.86 \times 10^6$	95.2
		2	$5.58 \times 10^6$	$28 \times 10^4$	$5.86 \times 10^6$	95.2
		3	$5.58 \times 10^6$	$28 \times 10^4$	$5.86 \times 10^6$	95.2
		4	$5.58 \times 10^6$	$28 \times 10^4$	$5.86 \times 10^6$	95.2
		5	$5.58 \times 10^6$	$28 \times 10^4$	$5.86 \times 10^6$	95.2
21/1/2016	12	1	$4.50 \times 10^6$	$6 \times 10^4$	$4.56 \times 10^6$	98.7
		2	$4.50 \times 10^6$	$6 \times 10^4$	$4.56 \times 10^6$	98.7
		3	$4.50 \times 10^6$	$6 \times 10^4$	$4.56 \times 10^6$	98.7
		4	$4.50 \times 10^6$	$6 \times 10^4$	$4.56 \times 10^6$	98.7
		5	$4.50 \times 10^6$	$6 \times 10^4$	$4.56 \times 10^6$	98.7
21/1/2016	13	1	$3.50 \times 10^6$	$37 \times 10^4$	$3.87 \times 10^6$	90.4
		2	$3.50 \times 10^6$	$37 \times 10^4$	$3.87 \times 10^6$	90.4
		3	$3.50 \times 10^6$	$37 \times 10^4$	$3.87 \times 10^6$	90.4
		4	$3.50 \times 10^6$	$37 \times 10^4$	$3.87 \times 10^6$	90.4
		5	$3.50 \times 10^6$	$37 \times 10^4$	$3.87 \times 10^6$	90.4
20/1/2016	14	1	$3.15 \times 10^6$	$3 \times 10^4$	$3.18 \times 10^6$	99.1
		2	$3.15 \times 10^6$	$3 \times 10^4$	$3.18 \times 10^6$	99.1
		3	$3.15 \times 10^6$	$3 \times 10^4$	$3.18 \times 10^6$	99.1
		4	$3.15 \times 10^6$	$3 \times 10^4$	$3.18 \times 10^6$	99.1
		5	$3.15 \times 10^6$	$3 \times 10^4$	$3.18 \times 10^6$	99.1
20/1/2016	15	1	$4.74 \times 10^6$	$5 \times 10^4$	$4.79 \times 10^6$	99.0
		2	$4.74 \times 10^6$	$5 \times 10^4$	$4.79 \times 10^6$	99.0
		3	$4.74 \times 10^6$	$5 \times 10^4$	$4.79 \times 10^6$	99.0
		4	$4.74 \times 10^6$	$5 \times 10^4$	$4.79 \times 10^6$	99.0
		5	$4.74 \times 10^6$	$5 \times 10^4$	$4.79 \times 10^6$	99.0

### 8.6 Number of live cells, dead cells and percentage of cell viability after cryopreservation

Date	Cat ID	Condition	No. of live cells	No. of dead cells	Total no. of cells	Percentage of cell viability (%)
29/1/2016	1	1	$1.82 \times 10^6$	$3.4 \times 10^5$	$2.16 \times 10^6$	84.3
		2	$1.01 \times 10^6$	$6.2 \times 10^5$	$1.63 \times 10^6$	62
		3	$8.5 \times 10^5$	$2.9 \times 10^5$	$1.14 \times 10^6$	74.6
		4	$2.9 \times 10^5$	$4.1 \times 10^5$	$7 \times 10^5$	41.4
		5	0	$1.6 \times 10^6$	$1.6 \times 10^6$	0
29/1/2016	2	1	$2.54 \times 10^6$	$6.2 \times 10^5$	$3.16 \times 10^6$	80.4
		2	$1.505 \times 10^6$	$8.1 \times 10^5$	$2.315 \times 10^6$	65.0
		3	$1.405 \times 10^6$	$8 \times 10^5$	$2.205 \times 10^6$	63.7
		4	$1.33 \times 10^6$	$7 \times 10^5$	$2.03 \times 10^6$	65.5
		5	0	$6.1 \times 10^5$	$6.1 \times 10^5$	0
29/1/2016	3	1	$1.61 \times 10^6$	$4.1 \times 10^5$	$2.02 \times 10^6$	79.7
		2	$7.8 \times 10^5$	$5.1 \times 10^5$	$1.29 \times 10^6$	60.5
		3	$6.6 \times 10^5$	$6.2 \times 10^5$	$1.28 \times 10^6$	51.6
		4	0	$6.75 \times 10^5$	$6.75 \times 10^5$	0
		5	0	$5 \times 10^5$	$5 \times 10^5$	0
1/2/2016	4	1	$5.9 \times 10^5$	$8.5 \times 10^5$	$1.44 \times 10^6$	41.0
		2	$1.32 \times 10^6$	$1.2 \times 10^6$	$2.52 \times 10^6$	52.4
		3	$1.18 \times 10^6$	$8.4 \times 10^5$	$2.02 \times 10^6$	58.4
		4	$1.17 \times 10^6$	$7.4 \times 10^5$	$1.91 \times 10^6$	61.3
		5	$5 \times 10^4$	$8.6 \times 10^5$	$9.1 \times 10^5$	5
1/2/2016	5	1	$9.8 \times 10^5$	$1.04 \times 10^6$	$2.02 \times 10^6$	48.5
		2	$1.62 \times 10^6$	$6.8 \times 10^5$	$2.3 \times 10^6$	70.4
		3	$1.66 \times 10^6$	$1.06 \times 10^6$	$2.72 \times 10^6$	61.0
		4	$1.16 \times 10^6$	$1.44 \times 10^6$	$2.6 \times 10^6$	44.6
		5	$8 \times 10^4$	$1.47 \times 10^6$	$1.55 \times 10^6$	5
1/2/2016	6	1	$6.7 \times 10^5$	$8.4 \times 10^5$	$1.51 \times 10^6$	44.4
		2	$1.06 \times 10^6$	$8.4 \times 10^5$	$1.9 \times 10^6$	55.8
		3	$1.55 \times 10^5$	$3.6 \times 10^5$	$5.15 \times 10^5$	30.1
		4	$2.9 \times 10^5$	$4.1 \times 10^5$	$7 \times 10^5$	41.4
		5	0	$2.4 \times 10^5$	$2.4 \times 10^5$	0
1/2/2016	7	1	$5.8 \times 10^5$	$8.7 \times 10^5$	$1.45 \times 10^6$	40
		2	$1.03 \times 10^6$	$7.4 \times 10^5$	$1.77 \times 10^6$	58.2
		3	$6.4 \times 10^5$	$8.4 \times 10^5$	$1.48 \times 10^6$	43.2
		4	$6.4 \times 10^5$	$9 \times 10^5$	$1.54 \times 10^6$	41.6
		5	0	$1.4 \times 10^6$	$1.4 \times 10^6$	0
3/2/2016	8	1	$1.48 \times 10^6$	$8.2 \times 10^5$	$2.3 \times 10^6$	64.3
		2	$1.63 \times 10^6$	$5.5 \times 10^5$	$2.18 \times 10^6$	74.8
		3	$1.48 \times 10^6$	$4.8 \times 10^5$	$1.96 \times 10^6$	75.5
		4	$1.76 \times 10^6$	$7.2 \times 10^5$	$2.48 \times 10^6$	71.0
		5	$2 \times 10^4$	$4.2 \times 10^5$	$4.4 \times 10^5$	4.5
3/2/2016	9	1	$2.3 \times 10^5$	$2.3 \times 10^5$	$4.6 \times 10^5$	50
		2	$1.8 \times 10^5$	$1.5 \times 10^5$	$3.3 \times 10^5$	54.5

		3	$2.5 \times 10^5$	$6 \times 10^5$	$8.5 \times 10^5$	29.4
		4	$6.2 \times 10^5$	$2.3 \times 10^5$	$8.5 \times 10^5$	72.9
		5	$3 \times 10^4$	$2.4 \times 10^5$	$2.7 \times 10^5$	11.1
3/2/2016	10	1	$3.9 \times 10^5$	$1.9 \times 10^5$	$5.8 \times 10^5$	67.2
		2	$6 \times 10^5$	$3.9 \times 10^5$	$9.9 \times 10^5$	60.6
		3	$1.5 \times 10^5$	$2.6 \times 10^5$	$4.1 \times 10^5$	36.6
		4	$6.7 \times 10^5$	$3.1 \times 10^5$	$9.8 \times 10^5$	68.4
		5	0	$1.5 \times 10^5$	$1.5 \times 10^5$	0
3/2/2016	11	1	$6.5 \times 10^5$	$9.2 \times 10^5$	$1.57 \times 10^6$	41.4
		2	$6.1 \times 10^5$	$1.24 \times 10^6$	$1.85 \times 10^6$	33.0
		3	$6.5 \times 10^5$	$1.01 \times 10^6$	$1.66 \times 10^6$	39.2
		4	$9.6 \times 10^5$	$1.43 \times 10^6$	$2.39 \times 10^6$	40.2
		5	$2 \times 10^4$	$8.7 \times 10^5$	$8.9 \times 10^5$	2.2
4/2/2016	12	1	$4.8 \times 10^5$	$9.4 \times 10^5$	$1.42 \times 10^6$	33.8
		2	$8.6 \times 10^5$	$1.38 \times 10^6$	$2.24 \times 10^6$	38.4
		3	$4 \times 10^5$	$9 \times 10^5$	$1.3 \times 10^6$	30.8
		4	$6.7 \times 10^5$	$6.6 \times 10^5$	$1.33 \times 10^6$	50.4
		5	0	$4.1 \times 10^5$	$4.1 \times 10^5$	0
4/2/2016	13	1	$1.77 \times 10^6$	$1.03 \times 10^6$	$2.8 \times 10^6$	63.2
		2	$1.16 \times 10^6$	$1.42 \times 10^6$	$2.58 \times 10^6$	45.0
		3	$1.56 \times 10^6$	$1.57 \times 10^6$	$3.13 \times 10^6$	49.8
		4	$1.43 \times 10^6$	$1.93 \times 10^6$	$3.36 \times 10^6$	42.6
		5	$1.4 \times 10^5$	$1.53 \times 10^6$	$1.67 \times 10^6$	8.4
4/2/2016	14	1	$1.75 \times 10^6$	$2.35 \times 10^6$	$4.1 \times 10^6$	42.7
		2	$1.69 \times 10^6$	$8.6 \times 10^5$	$2.55 \times 10^6$	66.3
		3	$9.7 \times 10^5$	$1.23 \times 10^6$	$2.2 \times 10^6$	44.1
		4	$1.16 \times 10^6$	$1.18 \times 10^6$	$2.34 \times 10^6$	49.6
		5	$1 \times 10^5$	$1.22 \times 10^6$	$1.32 \times 10^6$	7.6
4/2/2016	15	1	$9.9 \times 10^5$	$5.2 \times 10^5$	$1.51 \times 10^6$	65.6
		2	$9.1 \times 10^5$	$1.13 \times 10^6$	$2.04 \times 10^6$	44.6
		3	$1.09 \times 10^6$	$1.63 \times 10^6$	$2.72 \times 10^6$	40.1
		4	$7.7 \times 10^5$	$9.4 \times 10^5$	$1.71 \times 10^6$	45.0
		5	0	$4.7 \times 10^5$	$4.7 \times 10^5$	0

### 8.7 Percentage of cell viability before and after cryopreservation, and percentage of decrease in cell viability after cryopreservation

Cat ID	Condition/ Treatment	Percentage of Cell Viability (%)		Percentage of Decrease in Cell Viability (%)
		Before Cryopreservation	After Cryopreservation	
1	1	99.5	84.3	15.3
	2	99.5	62	37.7
	3	99.5	74.6	25.0
	4	99.5	41.4	58.4
	5	99.5	0	100
2	1	99.7	80.4	19.4
	2	99.7	65.0	34.8
	3	99.7	63.7	36.1
	4	99.7	65.5	34.3
	5	99.7	0	100
3	1	99.4	79.7	19.8
	2	99.4	60.5	39.1
	3	99.4	51.6	48.1
	4	99.4	0	100
	5	99.4	0	100
4	1	97.9	41.0	58.1
	2	97.9	52.4	46.5
	3	97.9	58.4	40.3
	4	97.9	61.3	37.4
	5	97.9	5	94.9
5	1	98.2	48.5	50.6
	2	98.2	70.4	28.3
	3	98.2	61.0	37.9
	4	98.2	44.6	54.6
	5	98.2	5	94.9
6	1	97.2	44.4	54.3
	2	97.2	55.8	42.6
	3	97.2	30.1	69.0
	4	97.2	41.4	57.4
	5	97.2	0	100
7	1	99.3	40	59.7
	2	99.3	58.2	41.4
	3	99.3	43.2	56.5
	4	99.3	41.6	58.1
	5	99.3	0	100
8	1	94.6	64.3	32.0
	2	94.6	74.8	20.9
	3	94.6	75.5	20.2
	4	94.6	71.0	24.9
	5	94.6	4.5	95.2

9	1	97.1	50	48.5
	2	97.1	54.5	43.9
	3	97.1	29.4	69.7
	4	97.1	72.9	24.9
	5	97.1	11.1	88.6
10	1	81.4	67.2	17.4
	2	81.4	60.6	25.6
	3	81.4	36.6	55.0
	4	81.4	68.4	16.0
	5	81.4	0	100
11	1	95.2	41.4	56.5
	2	95.2	33.0	65.3
	3	95.2	39.2	58.8
	4	95.2	40.2	57.8
	5	95.2	2.2	97.7
12	1	98.7	33.8	65.8
	2	98.7	38.4	61.1
	3	98.7	30.8	68.8
	4	98.7	50.4	48.9
	5	98.7	0	100
13	1	90.4	63.2	30.1
	2	90.4	45.0	50.2
	3	90.4	49.8	44.9
	4	90.4	42.6	52.9
	5	90.4	8.4	90.7
14	1	99.1	42.7	56.9
	2	99.1	66.3	33.1
	3	99.1	44.1	55.5
	4	99.1	49.6	49.9
	5	99.1	7.6	92.3
15	1	99.0	65.6	33.7
	2	99.0	44.6	54.9
	3	99.0	40.1	59.5
	4	99.0	45.0	54.5
	5	99.0	0	100

**8.8 Summarized table of the number of live cells, percentage of cell viability before & after cryopreservation, percentage of decrease in cell viability after cryopreservation, and result from flow cytometry**

Treatment	Animal	No. of live cells		Percentage of cell viability (%)		Percentage of Decrease in Cell Viability (%)	Flow cytometry (% Gated)		
		Before Cryopreservation	After Cryopreservation	Before Cryopreservation	After Cryopreservation		R1	R2	R3
1 (10% DMSO + 90% FBS at 4°C)	1	7400000	1820000	99.5	84.3	15.3	100	44.63	42.08
	2	7240000	2540000	99.7	80.4	19.4	100	80.32	17.47
	3	5120000	1610000	99.4	79.7	19.8	100	69.9	28.88
	4	2830000	590000	97.9	41	58.1	100	88.15	9.62
	5	4420000	980000	98.2	48.5	50.6	100	90.08	7.42
	6	2450000	670000	97.2	44.4	54.3	100	88.88	7.68
	7	2880000	580000	99.3	40	59.7	undetected		
	8	4950000	1480000	94.6	64.3	32	100	75.42	7.72
	9	3360000	230000	97.1	50	48.5	undetected		
	10	3150000	390000	81.4	67.2	17.4	undetected		
	11	5580000	650000	95.2	41.4	56.5	undetected		
	12	4500000	480000	98.7	33.8	65.8	100	80.02	12.2
	13	3500000	1770000	90.4	63.2	30.1	100	90.83	4.38
	14	3150000	1750000	99.1	42.7	56.9	100	82.27	8.14
	15	4740000	990000	99	65.6	33.7	100	63.85	13.22
2 (10% DMSO + 45% FBS + 45% FS at 4°C)	1	7400000	1010000	99.5	62	37.7	100	47.86	38
	2	7240000	1505000	99.7	65	34.8	100	85.35	12.87
	3	5120000	780000	99.4	60.5	39.1	100	78.85	19.78
	4	2830000	1320000	97.9	52.4	46.5	100	17.25	82.1
	5	4420000	1620000	98.2	70.4	28.3	100	31.31	67.07
	6	2450000	1060000	97.2	55.8	42.6	100	12	87.55

	7	2880000	1030000	99.3	58.2	41.4	100	35.51	59.48
	8	4950000	1630000	94.6	74.8	20.9	100	88.26	5.12
	9	3360000	180000	97.1	54.5	43.9	undetected		
	10	3150000	600000	81.4	60.6	25.6	100	47.57	47.26
	11	5580000	610000	95.2	33	65.3	undetected		
	12	4500000	860000	98.7	38.4	61.1	100	10.51	87.93
	13	3500000	1160000	90.4	45	50.2	100	16.68	80.7
	14	3150000	1690000	99.1	66.3	33.1	undetected		
	15	4740000	910000	99	44.6	54.9	undetected		
3 (10% DMSO + 90% FS at 4°C)	1	7400000	850000	99.5	74.6	25	100	37.69	60.45
	2	7240000	1405000	99.7	63.7	36.1	100	86.54	10.45
	3	5120000	660000	99.4	51.6	48.1	100	64.25	29.34
	4	2830000	1180000	97.9	58.4	40.3	100	33.86	64.85
	5	4420000	1660000	98.2	61	37.9	100	41.09	57.43
	6	2450000	155000	97.2	30.1	69	100	38.25	54.64
	7	2880000	640000	99.3	43.2	56.5	100	74.5	19.55
	8	4950000	1480000	94.6	75.5	20.2	undetected		
	9	3360000	250000	97.1	29.4	69.7	100	66.12	15.65
	10	3150000	150000	81.4	36.6	55	undetected		
	11	5580000	650000	95.2	39.2	58.8	undetected		
	12	4500000	400000	98.7	30.8	68.8	100	12.15	82.99
	13	3500000	1560000	90.4	49.8	44.9	100	55.29	39.81
	14	3150000	970000	99.1	44.1	55.5	100	35.29	62
	15	4740000	1090000	99	40.1	59.5	undetected		

4 (10% DMSO + 90% FBS at RT)	1	740000	290000	99.5	41.4	58.4	100	53.32	41.33
	2	724000	133000	99.7	65.5	34.3	100	97.54	1.68
	3	512000	0	99.4	0	100	100	78.42	19.49
	4	283000	117000	97.9	61.3	37.4	100	84.67	13.17
	5	442000	116000	98.2	44.6	54.6	100	81.15	13.04
	6	245000	29000	97.2	41.4	57.4	100	43.76	18.22
	7	288000	64000	99.3	41.6	58.1	undetected		
	8	495000	176000	94.6	71	24.9	undetected		
	9	336000	62000	97.1	72.9	24.9	100	86.67	3.45
	10	315000	67000	81.4	68.4	16	undetected		
	11	558000	96000	95.2	40.2	57.8	undetected		
	12	450000	67000	98.7	50.4	48.9	100	78.3	13.48
	13	350000	143000	90.4	42.6	52.9	undetected		
	14	315000	116000	99.1	49.6	49.9	100	63.27	20.41
	15	474000	77000	99	45	54.5	undetected		
5 (PBL media at 4°C)	1	740000	0	99.5	0	100	100	26.4	40.1
	2	724000	0	99.7	0	100	100	79.62	7.53
	3	512000	0	99.4	0	100	100	80.88	10.43
	4	283000	5000	97.9	5	94.9	100	81.55	8.81
	5	442000	8000	98.2	5	94.9	100	89.15	8.61
	6	245000	0	97.2	0	100	undetected		
	7	288000	0	99.3	0	100	undetected		
	8	495000	2000	94.6	4.5	95.2	undetected		
	9	336000	3000	97.1	11.1	88.6	100	93.09	2.86

10	3150000	0	81.4	0	100	undetected		
11	5580000	20000	95.2	2.2	97.7	undetected		
12	4500000	0	98.7	0	100	100	74.17	14.08
13	3500000	140000	90.4	8.4	90.7	100	88.62	6.88
14	3150000	100000	99.1	7.6	92.3	undetected		
15	4740000	0	99	0	100	undetected		