UNIVERSITI PUTRA MALAYSIA

MOLECULAR PREVALENCE OF FELINE MORBILLIVIRUS IN SHELTER CATS

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MOLECULAR PREVALENCE OF FELINE MORBILLIVIRUS IN SHELTER CATS

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It is hereby certified that we have read this project paper entitled "Molecular Prevalence of Feline Morbillivirus in Shelter Cats", by Nurul Husna Binti Omar and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for course VPD 4999 – Project

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In the name of Allah, The Most Benevolent, The Most Merciful

DEDICATIONS

To my beloved parents,

Omar Bin Hj. Hassan

and

Habsah Binti Semon

For loving and making me who I am today.

To my dearest siblings,

For supporting and cheering me throughout my life.

"A family is a place where principles are hammered

and honed on the anvil of everyday living"

Charles R. Swindoll

ACKNOWLEDGEMENT

In the name of Allah, The Most Benevolent, The Most Merciful

Firstly, I would like to give my sincere thanks to my father, mother and siblings for their support, cheer and love. I would like to thank my best friends Ainul Riza, Aisyah Ridzuan, Husna Atika and ‘Aisyah Aminuddin for their endless help and support. Deepest gratitude to my supervisor, Dr. Farina Mustaffa Kamal for your guidance and continuous help from beginning till the end. This project is a success with your help and assistance. To Dr. Gayathri Thevi Selvarajah, my co-supervisor, million thanks for all the help, especially for my sample collection and always willing to help me on anything. I would also like to acknowledge all the staff of Pusat Perlindungan Kucing Putrajaya, ISPCA and PAWS for their willingness to help and participate in this project. Special thanks to my FYP-mate Aisyah Azhar and also to Mr Maniam, Mr. Kevin and staff of virology laboratory 3 for helping me throughout my project, especially during laboratory works. Thank you to Ms. Hidayah Isa for your guidance, help and knowledge. I would not be able to complete this project without your help. Lastly, thank you to my classmates, DVM 2016 and to all that may have contributed directly or indirectly in this project. Thank you!

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LIST OF ABBREVIATION

%	Percentage
˚C	Degree celcius
cDNA	Complementary DNA
C.I	Confidence interval
DNA	Deoxyribonucleic Acid
FmoPV	Feline morbillivirus
FPV	Fakulti Perubatan Veterinar
IACUC	Institutional Animal Care and Use Committee
ID	Identification
mL	Mililiters
N	Number of cats sampled
RNA	Ribonucleic Acid
PCR	Polymerase Chain Reation
TIN	Tubulointerstitial Nephritis
UPM	Universiti Putra Malaysia

ABSTRAK

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

SARINGAN MOLEKULAR MORBILLIVIRUS FELIN DI PUSAT PERLINDUNGAN KUCING

Oleh,

NURUL HUSNA BINTI OMAR

Penyelia: Dr. Farina Mustaffa Kamal

Penyelia bersama: Dr. Gayathri Thevi Selvarajah

Morbillivirus felin (FmoPV) adalah sejenis virus negatif yang tergolong di dalam keluarga *Paramyxoviridae.* Sebelum ini, tiada virus daripada keluarga morbillivirus yang menyebabkan infeksi didalam kucing domestik. Walau bagaimanapun, terdapat satu kajian di Hong Kong, dimana mereka menjumpai satu virus baru yang kemudiannya dinamakan FmoPV. Ini diikuti oleh penemuan dan pengasingan FmoPV di Jepun dan juga Eropah. Baru-baru ini, satu kajian saringan FmoPV telah dijalankan di Malaysia dan didapati kadar prevalens FmoPV ialah 48.6%. Dalam projek ini, sampel air kencing telah diambil secara rawak daripada 46 ekor kucing daripada tiga buah pusat

perlindungan kucing yang terlibat. Pengesanan FmoPV didalam sampel yang telah dikumpul telah dibuat menggunakan ujian reaksi rantai polimerase (PCR) bersarang untuk membesarkan sebahagian daripada urutan L gen FmoPV untuk produk akhir yang bersaiz 401 bp. Daripada 46 sampel air kencing yang diambil, 19 daripadanya adalah positif untuk FmoPV. Kadar prevalens FmoPV untuk kucing didalam pusat perlindungan kucing adalah 41.3%.

Kata kunci: Morbillivirus felin, kucing, ujian PCR bersarang, L gen, prevalens.

ABSTRACT

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

MOLECULAR SCREENING OF FELINE MORBILLIVIRUS IN SHELTER CATS

By

NURUL HUSNA BINTI OMAR

2016

Supervisor: Dr. Farina Mustaffa Kamal

Co-supervisor: Dr. Gayathri Thevi Selvarajah

Feline morbillivirus (FmoPV) is an enveloped virus with non-segmented negative strands RNA genomes belongs to the genus of *Morbillivirus* under the family of *Paramyxoviridae.* Previously, there were no known morbillivirus infecton in domestic cats. However, a study was done in Hong Kong, in which they discovered a novel virus that was later named as FmoPV. This was followed by discovery and isolation of FmoPV in Japan and Europe. Recently, a screening study of FmoPV was conducted in Malaysia and the prevalence rate was found to be 48.6%. In the present study, urine samples were taken from 46 cats, randomly from three participating shelter organization. The collecting samples were then subjected to nested polymerase chain reaction (PCR) assay amplifying the L gene of FmoPV for final products of 401 bp. Out of 46 samples collected, 19 samples were tested positive for the virus FmoPV. The prevalence rate of FmoPV in shelter cats in Malaysia is 41.3%.

Key words: Feline morbillivirus, shelter cats, nested PCR assay, L gene, prevalence rate

1.0 INTRODUCTION

Morbillivirus belongs to the virus family of *Paramyxoviridae*. It is an enveloped virus with non-segmented negative strand RNA genomes. Other viruses in the genus *Morbillivirus* include measles virus, rinderpest, peste des petits ruminant virus, canine distemper virus, phocine distemper virus and cetacean morbilliviruses (Rory *et al.,* 2015).

A novel virus under the genus of morbillivirus named feline morbillivirus (FmoPV) had been reported to cause infection in domestic cats. It was first isolated in a study done in Hong Kong stray cats (Woo *et al.,* 2012). In addition, studies conducted in Japan and Europe (Italy and Germany) detected similar virus in their pet cats population with >90% sequence similarities with Hong Kong isolate (Furuya *et al.,* 2014; Lorusso *et al.,* 2015; Sieg *et al.,* 2015). To date, feline morbillivirus has been identified only in China, Japan and Europe. The newly discovered FMoPV genomes encode eight non-structural and structural proteins which are N, P/V/C, M, F, H and L proteins. In the study conducted by Woo *et al.* (2012), L protein of the virus was detected and sequenced by using RT-PCR.

Recently, a study was conducted to detect the presence of FmoPV among client-owned cats in Malaysia where the prevalence rate was found to be 48.6% (Manoraj *et al.*, 2015). Due to the fact that pet and shelter cats are exposed to different environment, it would be interesting to investigate the prevalence of FmoPV in shelter settings.

Thus, the objectives of this study were:

1. To detect the presence of feline morbillivirus among shelter cats in Malaysia.
2. To determine the prevalence of feline morbillivirus in shelter cats in Malaysia.

The hypothesis for this project includes:

1. Feline morbillivirus is present in shelter cats.
2. The prevalence of feline morbillivirus in Malaysia is higher than client-owned cats.

2.0 LITERATURE REVIEW

2.1 Paramyxoviruses

Paramyxovirus belongs to the order of Mononegavirales. It is an enveloped, negative-sense, unsegmented, single stranded RNA virus that cause significant disease to both human and animals (Tien Nguyen *et al.,* 2013). The virus was divided into two subfamilies which are *Paramyxovirinae* and *Pneumovirinae*. Paramyxovirinae were then further divided into seven genera which are *Avulavirus, Henipavirus, Morbillivirus, Respirovirus, Ferlavirus, Aquaparamyxovirus* and *Rubulavirus* (Audsley & Moseley, 2013). According to Hubert *et al.* (2014), similar to other viruses in the order of *Mononegavirales*, Paramyxoviruses have six structural and non-structural proteins which are nucleocapsid (N), large polymerase protein (L), phosphoprotein (P), matrix protein (M), haemaagglutinin (H) and fusion protein (F). Two surface proteins, F and H are important for attachment and fusion of the virus with host cell plasma membrane (Barret, 1999).

2.2 Morbillivirus

Morbillivirus is an enveloped, non-segmented negative strand RNA genomes virus that is highly infectious and causes high mortality rates in its natural hosts. It has the ability to cause outbreak in previously unexposed populations (Rory *et al.,* 2015). The virus has been reported to cause a number of significant diseases in human, wildlife, aquatic mammals, and domestic animals. There are six viruses that are classified under

Morbillivirus genus, namely measles virus, phocine distemper virus, cetacean morbillivirus, rinderpest virus, peste des petits ruminants virus and canine distemper virus. Measles virus can affect both humans and also primates while phocine distemper virus and cetacean morbillivirus are known to affect marine mammals (Rory *et al.,* 2015). Rinderpest virus, peste des petits ruminants virus and canine distemper virus, infects susceptible wildlife in the ecosystem as well as the domestic animals. Rinderpest virus and peste des petits ruminants virus cause disease in cattle, small ruminants and even-toed ungulates. Canine distemper virus have been reported to cause disease in animals other than in the family of Canidae, which includes Mustelidae – family of carnivorous mammals including weasel, ferret and badger, and Procyonidae – raccoons and ringtails (Anderson, 1995).

2.3 Diagnosis of Morbillivirus

Polymerase chain reaction (PCR) is one of the reliable diagnostic tools to detect morbillivirus (Saiki *et al.,*1988). According to Barret (1999), Morbillivirus is a single stranded RNA virus, hence, to enable the RNA virus to be run with PCR machines, it has to be first converted into DNA strand by using reverse transcriptase enzyme in reverse transcription-polymerase chain reaction (RT-PCR). Conventional serological techniques and virus isolation can also be used in detection of *morbillivirus* (Barret, 1999).

2.4 Feline Morbillivirus

Feline morbillivirus (FmoPV) is a novel virus in the genus of *Morbillivirus* that has been reported to cause infection in domestic cat (*Felis catus*). Due to the fact that *Paramyxoviruses* infections in cats were previously unexplained and they were known to be reservoirs of many other virus infections, screening study to detect *Paramyxoviruses* in cats was conducted in Hong Kong by Woo *et al.* (2012). The first detection of FmoPV in urine samples, rectal swabs and blood samples was reported in stray cats in Hong Kong (Woo *et al*., 2012). The isolated feline morbillivirus was phylogenetically associated to other *Morbillivirus* (Woo *et al*., 2012). In addition, studies that were done in Japan and Europe detected similar virus to the previously reported FmoPV with more than 90% sequence similarities with Hong Kong isolates (Furuya *et al.,* 2014; Lorusso *et al.,* 2015; Sieg *et al.,* 2015).

Feline morbillivirus with genome size of 16,050 bases is said to be the largest genomes among other viruses of *Morbillivirus* (Woo *et al.,* 2012). Similar to other *Morbillivirus,* it is reported to have six encoding genes which are N, P, M, F, H, and L with N, P and F genes having the highest rates of nucleotide and amino acid polymorphism (Park *et al.,* 2014 ; Woo *et al.,* 2012).

According to the study done by Woo *et al.* (2012), FmoPV have been associated with tubulointerstitial nephritis (TIN) based on the histological examination of various organs in two FmoPV-positive cats. The result revealed inflammatory infiltrates as well as tubular necrosis of the kidney tissue consistent with signs of TIN, supported with a control study in which 12 out of 15 FmoPV cats showed signs of TIN. However, in a study conducted by Sakaguchi *et al.* (2014), it was suggested that no clear relationship was found between FmoPV and TIN. A study conducted in Germany, on the other hand, found a strong association between positive-infected cats with chronic kidney disease in domestic cats (Sieg *et al.,* 2015).

2.5 Detection rate of FmoPV in other studies

Feline morbillivirus infection in Hong Kong revealed that FmoPV was detected in 56 cats out of 457 (12.3%) stray cats by RT-PCR amplifying a 155-bp fragment in L gene of FmoPV in urine, rectal swabs and blood samples (Woo *et al.,* 2012).

In Japan, the detection of FmoPV was done by using nested-set primers in urine, blood samples and formalin-fixed paraffin-embedded (FFPE) kidney tissue of cats that had nephritis. The detection rate was 6.1% (5 out of 82) in urine, 10% (1 out of 10) in blood samples and 40% (4 out of 10) in FFPE tissue (Furuya *et al.,* 2014).

2.6 Current finding of Feline morbillivirus in Malaysia.

In a recent study done in Malaysia, urine and blood samples were collected from 35 client-owned cats. Feline morbillivirus screening was done by using two-steps conventional RT-PCR amplifying N-gene sequence of the virus. The detection rate is 4% (1 out of 25) in blood samples and 63% (17 out of 27) in urine samples. The prevalence rate of FmoPV of client-owned cat in Malaysia is 48.6% (Manoraj *et al.*, 2015). In addition, the study also revealed no association between renal system diseases with the detection of FmoPV consistent with the study done by Sakaguchi *et al.* (2014).

3.0 MATERIALS AND METHODS

3.1 Animals

Forty six cats were randomly selected from three participating shelter associations, PAWS Animal Shelter Society (PAWS), The Ipoh Society for the Prevention of Cruelty to Animals (ISPCA) and Pusat Perlindungan Kucing Putrajaya (PPKP). Institutional Animal Care and Use Committee (IACUC) approval with AUP number (FYP.2015/FPV.027) was obtained before carrying out this project. Letter of authorization and client consent letters were issued for each association to obtain approval and permission to take sample from their place.

3.2 Sample Collection

Three to five ml of urine sample was taken via voided urine, manual compression and cystocentesis. Cystocentesis was done by using 23G needles and 3 mL or 5 mL syringes. The samples that were taken via voided urine and manual compression was kept in urine collection container while samples taken via cystocentesis were kept in the respective syringes used to obtain the urine samples. Fifty grams of kidney samples were taken in available euthanized cases and kept in container filled with RNAlater® solution. All samples were kept on ice prior to processing steps.

3.3 Sample Processing

The urine samples were first filtered by using syringe driven filter 0.45µm size pore into a 15ml centrifuge tube. One ml of RNAlater® was mixed to 5 ml urine and vortex-mixed and aliquoted into 1.5 ml Eppendorf tube. The urine samples were then subjected for viral RNA extraction.

Two ml of phosphate-buffer saline was added to each kidney sample obtained in euthanized cases. The lysate were then homogenized and underwent three times of freeze-thaw process at -20ºC for 15 minutes. The mixture was then centrifuged by using benchtop micro centrifudge at 3000rpm for five minutes at 4ºC. The supernatant obtained from centrifugation was then transferred to 1.5 mL Eppendorf tube and subjected to RNA extraction.

3.4 Viral RNA Extraction

The viral RNA extraction was carried out by using conventional method with TRIzol® Reagent together with other materials (chloroform, isopropyl alcohol, 100% ethanol, RNase-free water) as instructed by the manufacturer (Appendix 8.1). Briefly, 700 µl of TRIzol® Reagent was added to the samples. The mixture need to be shaken gently and incubated in room temperature for 5 minutes. Then, 200 µl of chloroform was added into the mixture; vortex mixed and incubated on ice for 15 minutes. The mixture was then centrifuged at 14000rpm for 20 minutes at 4ºC to get phase separation and 500 µl of the supernatant obtained from the centrifugation was transferred into 1.5 ml Eppendorf tube. In order to precipitate the RNA, 800 µl of isopropyl alcohol was added to the supernatant, gently shaken, incubated at room temperature for 30 minutes and centrifuge at 14000rpm for 15 minutes at 4ºC. After centrifugation, the supernatant was discarded and 1 ml of 100% ethanol was added into the tube to wash the RNA pellet and centrifuge at 14000rpm for 10 minutes at 4ºC. These steps were repeated two times and the RNA pellets were air-dried. The RNA pellets were then dissolved in 30 µl – 50 µl of RNase-free water and stored at -20ºC until subjected for nested reverse-transcriptase polymerase chain reaction (RT-PCR) assay.

3.5 Nested Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

Final product of RNA extraction was subjected to nested RT-PCR to detect the L-gene. In nested RT-PCR, two sets of primer were used to obtain the desired target DNA. The first pair of nested primer were done together with Promega™ GoTaq™ 2-Step RT-qPCR System kit (Promega™ A6010, USA) as instructed by the manufacturer (Appendix 8.2) with primers Nest1F, 5'-GGA ACA TGG CCT CCT GTA GA-3' and Nest1R, 5'-CTC CAT TGG CAA TCA GGT TT-3' following thermal protocol: reverse transcription, 45 minutes at 45°C; initial PCR activation step, 1 minutes at 95°C; 3-step cycling consisting of denaturation, 30 seconds at 94°C; annealing, 30 seconds at 54°C; extension, 2 minute at 72°C (for 34 cycles); final extension 72°C for 10 minutes producing a final product of 487-bp (Furuya *et al.,* 2014).

The second pair of the nested RT-PCR were done with MyTaq™ Red Mix (Bioline, United Kingdom) with primer Nest2F, 5'-CCA AAT CAT GCA TCT GCT GT -3' and Nest2R, 5'- GCG AAC AAT CGA CCT ACC TC -3' as instructed by the manufacturer (Appendix 8.3) following thermal protocol: initial denaturation, 1 minutes at 95°C; 3-step cycling consisting of denaturation, 15 seconds at 95°C; annealing, 30 seconds at 54°C; extension, 30 seconds at 72°C (for 35 cycles) to produce a final product of 401-bp (Furuya *et al.,* 2014). The PCR reactions were performed on a thermal cycler (C1000 Touch™ Thermal Cycler, California).

3.6 Agarose Gel Electrophoresis

The final product of nested RT-PCR was used to run the Agarose Gel Electrophoresis with VP 100-bp Plus DNA ladder and marker. The gel was made using 1.5% agarose powder mixed with Tris-acetate-EDTA (Bio-Rad PowerPac 300, USA). The voltage used to run the electrophoresis was 85V for 35 minutes. The DNA fragment were then visualized using Bio-Rad Gel Doc™ (USA).

3.7 Data Analysis

The data obtained from PCR is recorded and molecular prevalence of FmoPV is calculated. The association between sex and FmoPV infection was analyzed using the Chi-square test, with 95% confidence interval (95% C.I). The statistical analysis was performed using GraphPad Prism® software version 5.0 (GraphPad Software Inc, USA).

4.0 RESULTS

4.1 Number of Samples Taken From Each Association

The number of samples taken for each association was tabulated in Table 1. Out of the 46 samples taken, 16 samples were taken from PPKP, 12 samples were taken from ISPCA and 18 samples were taken from PAWS. In addition, four euthanized cases (kidney sample) were taken from PAWS.

Table 1: Number of Samples Taken From Each Association

Categories	**No. of cats sampled (*n*)**		**Percentage of sample representative (%)/(number of samples/total number of cats in respective shelter)**
	Live cats (*n* = 52)	***Euthanized (*n* = 5)**	
PPKP	16	-	8 (16/200)
ISPCA	12	-	18 (12/64)
PAWS	18	4	11 (22/200)

*only kidney samples was obtained

4.2 Sex

The sex of the cats sampled in this study was tabulated in Table 2. From the 46 samples taken, 14 cats were males while 32 cats were female. Out of 14 male cats, five cats were already castrated and nine cats were intact. In contrast to female cats, there were, 11 spayed cats while 21 cats were intact.

Table 2: Sex categories of the cats sampled

Categories	No of cat sampled	
	Intact	**Neutered**
Male	9	5
Female	21	11

4.3 Detection rate of FmoPV for each shelter organization

4.3.1 Detection rate of FmoPV in urine

The detection of FmoPV for each association were tabulated in Table 3 and illustrated in Figure 1. The detection rate for PPKP was 50%, ISPCA was 58.3% and PAWS was 22%.

Table 3: Detection rate of FmoPV in urine for each shelter organization

Categories	**No. of cats sampled (*n*)**	**FmoPV positive**	**FmoPV negative**	**Detection rate (%)**
PPKP	16	8	8	50
ISPCA	12	7	5	58
PAWS	18	4	14	22

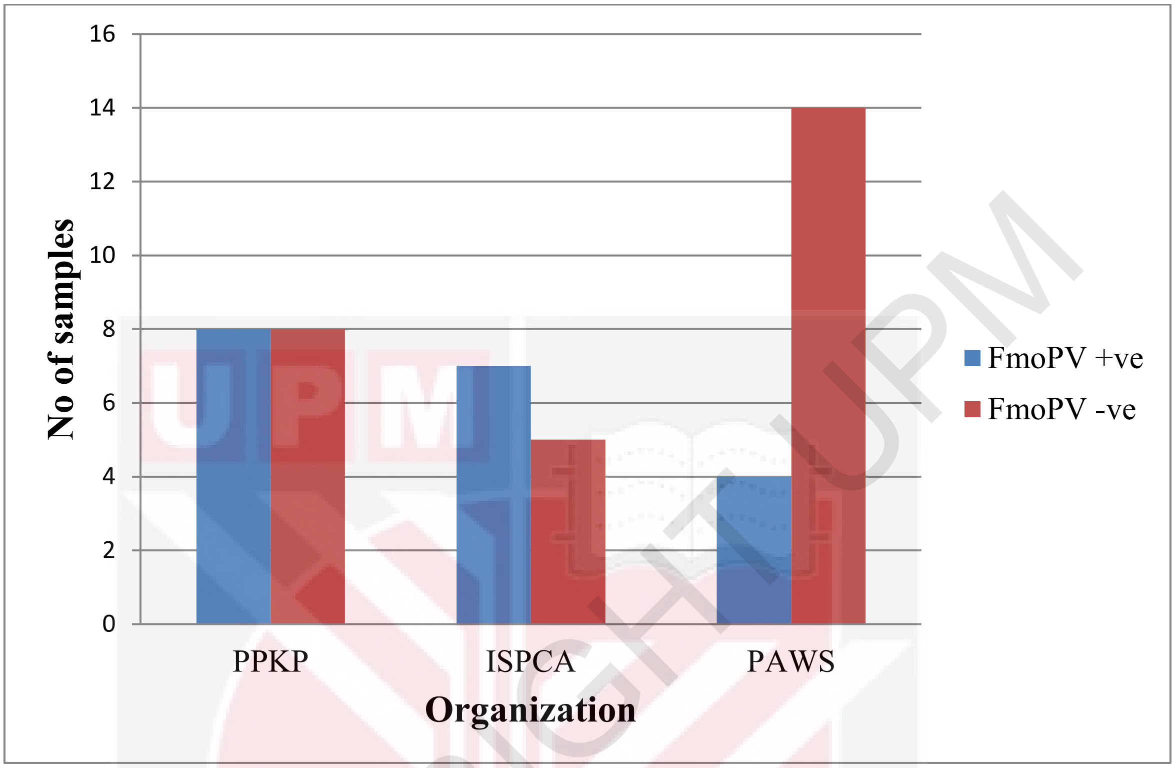

Figure 1: The detection rate of FmoPV in urine for each shelter.

4.3.2 Detection rate of FmoPV in kidney samples

No FmoPV was detected from the kidney samples collected.

4.4 Prevalence of FmoPV

The prevalence rate of FmoPV in this study was 41.3% with 19 out of 46 samples were detected positive for FmoPV (Fig. 2).

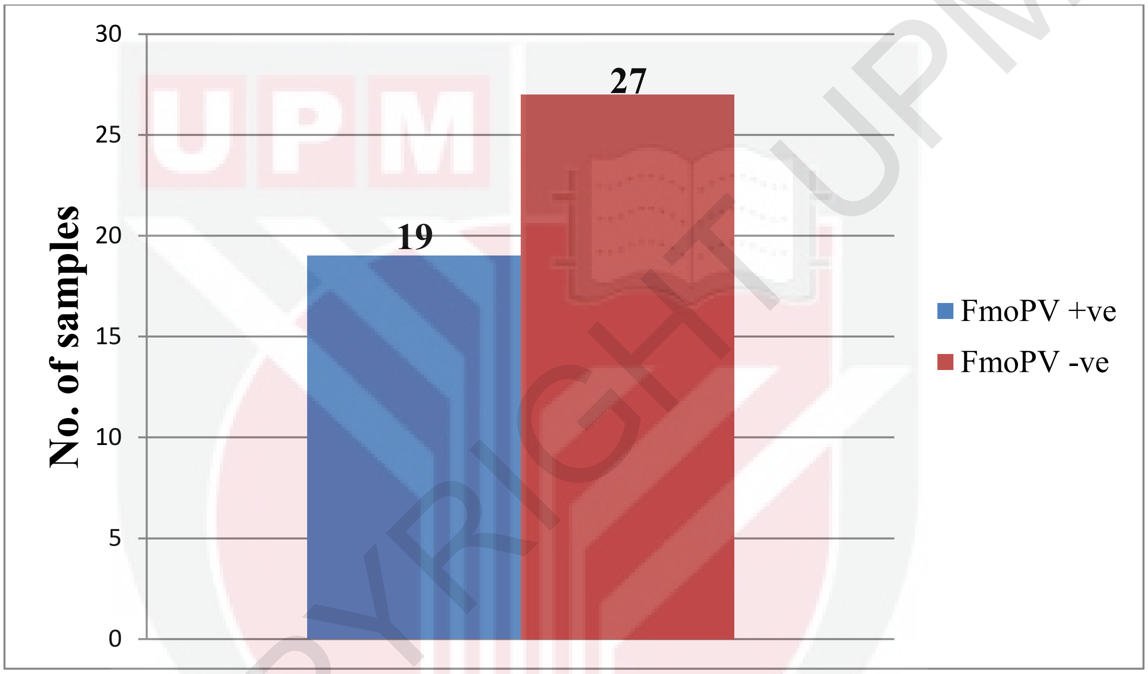

Figure 2: The prevalence rate of FmoPV.

4.5 Association between sex and FmoPV infection

Chi-square analysis was performed and the data was tabulated in Table 4. There was no association (p = 0.10) between sex and FmoPV infection.

Table 4: Data analysis of association between sex and FmoPV infection

<table>
<tr><th rowspan="2">Data Anayzed</th><th rowspan="2">FmoPV positive</th><th rowspan="2">FmoPV negative</th><th rowspan="2">Total</th><th rowspan="2">P value</th><th rowspan="2">Odds Ratio</th><th colspan="2">95% Confidence interval of the differences</th></tr>
<tr><th>Lower</th><th>Upper</th></tr>
<tr><td>Male</td><td>3</td><td>11</td><td>14</td><td rowspan="3">0.10</td><td rowspan="3">0.3091</td><td rowspan="3">0.07225</td><td rowspan="3">1.322</td></tr>
<tr><td>Female</td><td>15</td><td>17</td><td>32</td></tr>
<tr><td>Total</td><td>18</td><td>28</td><td>46</td></tr>
</table>

5.0 DISCUSSIONS

5.1 Detection rate of FmoPV in each organization

The detection rate of FmoPV was found to be 50% for PPKP, 58% for ISPCA and 22% for PAWS. The difference in detection rate between shelters may be due to the different management system between each organization.

In terms of management, the cats in PPKP and ISPCA were allowed to roam freely in an enclosure while cats from PAWS were kept in cage consist of 1 to 4 cats per cage. Woo *et al.* (2012) stated that FmoPV was mostly detected in urine. Therefore, it is possible that this virus may have established in the shelter environment through virus shedding by FmoPV- positive cats. FmoPV can also spread to uninfected cats when they share the same litter box as FmoPV is stable in the environment and can retain it infectivity for 12 days (Koide *et al.,* 2015).

In this study, FmoPV was not detected in the kidney samples. This could be due to the health condition of the cat before euthanasia in which there was no history of renal related diseased.

The lower detection rate in PAWS could be due to the limited cat-to-cat contact in the shelter as the cats were kept in the cage. However, we could only speculate the role of management in the finding in this study as there is no complete establishment of the pathogenicity and epidemiology of FmoPV.

5.2 Prevalence rate of feline morbillivirus

The prevalence of FmoPV in this study is 41.3 % which is higher compared to other studies conducted previously in other countries. The difference in prevalence rate may be due to the limitation of sample region in which this study obtained samples from participating shelters from two states while other studies sampled multiple regions. In addition, the sample size of the studies done in other countries was much larger in comparison to the study done in Malaysia (Sakaguchi *et al.,* 2014).

However, the detection rate of FmoPV in urine detected in this study (41.3%) is lower compared to the study conducted previously in Malaysia by Manoraj *et al.* (2015) which is 63%. This may be due to the background difference of the cats sampled between studies whereby previous study sampled client-owned cats while this study was conducted using samples taken from shelter cats. The difference in the detection rate between pet and stray cats were also observed in previous studies whereby the detection rate was higher in shelter cats. (Furuya *et al.,* 2014; Woo *et al.,* 2012).

Moreover, the difference in detection rate could also happenned due to the different assay and extraction method use between studies. In previous study by Manoraj *et al.* (2015) the RNA extraction was performed using an established commercial extraction kit which was more sensitive compared to the conventional method. Therefore, this factor may also contributed to the higher detection rate.

The sample selection in previous study conducted by Manoraj *et al.* (2015) included cats with renal-related diseases which comprised 37% of the total samples. The inclusion criteria of renal disease in client-owned cats may contributed to the higher detection rate in urine compared to shelter cats, consistent with findings in the study done by Furuya *et al.* (2014). This could be due to the tissue distribution of the virus or a higher FmoPV virus infection in cats with nephritis. This is further supported by a study done by Sieg *et al.* (2015) in Germany where they compared disease and healthy cats and found a strong association between positive-infected cats with chronic kidney disease in domestic cats.

5.3 Association between sex and FmoPV infection

There was no association between sex and FmoPV infection implying that both male and female has equal chance of getting FmoPV infection. The finding of this study is in contrast with a study done by Sieg *et al.* (2015), suggesting that there might be a higher risk for male cats in suffering from FmoPV infections.

6.0 CONCLUSIONS AND RECOMMENDATIONS

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In this study, we concluded that FmoPV is present in shelter cats in Malaysia with the prevalence rate of 41.3%. The detection rate is lower compared to the previous study conducted in Malaysia, 63%. There was no association found between sex and FmoPV infection.

The detection rate of FmoPV is different between shelter organizations. The difference in the detection rate is speculated to be due to the difference in management system. Thus, more studies on the existence or detection of FmoPV in cats in different management should be conducted to determine whether these factors affects the spread of FmoPV in cats. In addition, further studies are needed to investigate and establish the pathogenicity and epidemiology of FmoPV.

Furthermore, sampling collection was done in only three participating shelters in Malaysia. Therefore, it is recommended that sample size and geographical areas should be increased to determine the actual conditions in both shelter and client-owned cats.

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8.0 APPENDICES

8.1 Instruction for RNA extraction using Trizol® Reagent (1/3)

ambion | RNA
by Life technologies

TRIzol® Reagent

Catalog Numbers	Quantity	Store at 2°C to 25°C
15596-026	100 mL	
15596-018	200 mL	

Description

TRIzol® Reagent is a ready-to-use reagent, designed to isolate high quality total RNA (as well as DNA and proteins) from cell and tissue samples of human, animal, plant, yeast, or bacterial origin, within one hour. TRIzol® Reagent is a monophasic solution of phenol, guanidine isothiocyanate, and other proprietary components which facilitate the isolation of a variety of RNA species of large or small molecular size. TRIzol® Reagent maintains the integrity of the RNA due to highly effective inhibition of RNase activity while disrupting cells and dissolving cell components during sample homogenization. TRIzol® Reagent allows for simultaneous processing of a large number of samples, and is an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi (Chomczynski & Sacchi, 1987).

TRIzol® Reagent allows the user to perform sequential precipitation of RNA, DNA, and proteins from a single sample (Chomczynski, 1993). After homogenizing the sample with TRIzol® Reagent, chloroform is added, and the homogenate is allowed to separate into a clear upper aqueous layer (containing RNA), an interphase, and a red lower organic layer (containing the DNA and proteins). RNA is precipitated from the aqueous layer with isopropanol. DNA is precipitated from the interphase/organic layer with ethanol. Protein is precipitated from the phenol-ethanol supernatant by isopropanol precipitation. The precipitated RNA, DNA, or protein is washed to remove impurities, and then resuspended for use in downstream applications.

- Isolated RNA can be used in RT-PCR, Northern Blot analysis, Dot Blot hybridization, poly(A)+ selection, *in vitro* translation, RNase protection assay, and molecular cloning.
- Isolated DNA can be used in PCR, Restriction Enzyme digestion, and Southern Blots.
- Isolated protein can be used for Western Blots, recovery of some enzymatic activity, and some immunoprecipitation.

Caution

TRIzol® Reagent contains phenol (toxic and corrosive) and guanidine isothiocyanate (an irritant), and may be a health hazard if not handled properly. Always work with TRIzol® Reagent in a fume hood, and always wear a lab coat, gloves and safety glasses. Contact your Environmental Heath and Safety (EH&S) department for proper work and disposal guidelines. Avoid direct contact with TRIzol® Reagent, because **contact to skin, eyes, or respiratory tract may cause chemical burns** to the exposed area. **If contact to skin or eyes occurs**, *immediately* wash the exposed area with copious amounts of water for 15 minutes and seek medical attention if necessary. **If you inhale vapors**, move to fresh air and seek medical attention if necessary. For more information, refer to the TRIzol® Reagent SDS (Safety Data Sheet), available from our web site at www.lifetechnologies.com/support.

Contents and Storage

TRIzol® Reagent is supplied in 100 mL (Cat. no. 15596-026) or 200 mL (Cat. no. 15596-018) volumes, and shipped at room temperature. Upon receipt, store TRIzol® Reagent at room temperature. TRIzol® Reagent is stable for 12 months when properly stored.

Intended Use

For research use only. Not intended for human or animal diagnostic or therapeutic uses.

Materials Needed

The following additional materials are needed, but not supplied for the isolation of RNA, DNA or proteins.

RNA Isolation	DNA Isolation	Protein Isolation
• Chloroform • Isopropyl alcohol • 75% ethanol (in DEPC-treated water) • RNase-free water or 0.5% SDS • Centrifuge and rotor capable of reaching up to 12,000 × *g* • Polypropylene microcentrifuge tubes • Water bath or heat block (55–60°C)	• Chloroform • 100% ethanol • 75% ethanol • 0.1 M sodium citrate in 10% ethanol • 8 mM NaOH • Centrifuge and rotor capable of reaching up to 12,000 × *g* • Polypropylene microcentrifuge tubes	• Chloroform • Isopropyl alcohol • 100% ethanol • 0.3 M Guanidine hydrochloride in 95% ethanol • 1% SDS • Centrifuge and rotor capable of reaching up to 12,000 × *g* • Polypropylene microcentrifuge tubes

Part no. 15596026.PPS MAN0001271 Rev. Date: 13 Dec 2012

For support, visit www.lifetechnologies.com/support or email techsupport@lifetech.com. To reorder, visit www.lifetechnologies.com

8.1 Continue (2/3)

Preparing Samples

Homogenizing samples

1. Determine your sample type, and perform homogenization at room temperature according to the table below. The sample volume should not exceed 10% of the volume of TRIzol® Reagent used for homogenization. Be sure to use the indicated amount of TRIzol® Reagent, because an insufficient volume can result in DNA contamination of isolated RNA.

Sample Type	Action
Tissues	1. Add 1 mL TRIzol® Reagent per 50–100 mg of tissue sample. 2. Homogenize sample using a glass-Teflon® or power homogenizer. **Note:** Process or freeze tissue samples *immediately* upon collection.
Adherent Cells (Monolayer)	1. Remove growth media from culture dish. 2. Add 1 mL TRIzol® Reagent directly to the cells in the culture dish per 10 cm^2 of culture dish surface area. **Note:** Add 1 mL TRIzol® Reagent for a 35 mm dish, 3 mL for a 60 mm dish, and 8 mL for a 100 mm dish. 3. Lyse the cells directly in the culture dish by pipetting the cells up and down several times.
Suspension Cells	1. Harvest cells by centrifugation and remove media. 2. Add 0.75 mL of TRIzol® Reagent per 0.25 mL of sample (5–10 × 10^6 cells from animal, plant or yeast origin, or 1 × 10^7 cells of bacterial origin). **Note:** Do not wash cells before addition of TRIzol® Reagent to avoid increased chance of mRNA degradation. 3. Lyse cells in sample by pipetting up and down several times. Disruption of some yeast and bacterial cells may require the use of a homogenizer.

2. *(Optional)* When preparing samples with high content of fat, proteins, polysaccharides, or extracellular material (e.g., muscle, fat tissue, or tuberous plant material), an additional isolation step may be required to remove insoluble material from the samples.

   Note: Do not perform this additional isolation step if you are performing subsequent DNA isolation on your sample.

Sample Type	Notes
Tissue or cells with high content of fat, protein, polysaccharide, or extracellular material	1. Following homogenization, centrifuge your sample at 12,000 × *g* for 10 minutes at 4°C. **Note:** The resulting pellet contains ECM, polysaccharides, and high molecular weight DNA, while the supernatant contains the RNA. In high fat content samples, a layer of fat collects above the supernatant. 2. Remove and discard the fatty layer. 3. Transfer the cleared supernatant to a new tube.

3. Proceed to **Phase separation**, or store the homogenized sample. Homogenized samples can be stored at room temperature for several hours, or at –60 to –70°C for at least one month.

Phase separation

1. Incubate the homogenized sample (see **Homogenizing samples**) for 5 minutes at room temperature to permit complete dissociation of the nucleoprotein complex.
2. Add 0.2 mL of chloroform per 1 mL of TRIzol® Reagent used for homogenization. Cap the tube securely.
3. Shake tube vigorously by hand for 15 seconds.
4. Incubate for 2–3 minutes at room temperature.
5. Centrifuge the sample at 12,000 × *g* for 15 minutes at 4°C.
   Note: The mixture separates into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. *RNA remains exclusively in the aqueous phase.* The upper aqueous phase is ~50% of the total volume.
6. Remove the aqueous phase of the sample by angling the tube at 45° and pipetting the solution out. Avoid drawing any of the interphase or organic layer into the pipette when removing the aqueous phase.
7. Place the aqueous phase into a new tube and proceed to the **RNA Isolation Procedure**.
8. Save the interphase and organic phenol-chloroform phase if isolation of DNA or protein is desired. See **DNA Isolation Procedure** and **Protein Isolation Procedure** for details. The organic phase can be stored at 4°C overnight.

RNA Isolation Procedure

Always use the appropriate precautions to avoid RNase contamination when preparing and handling RNA.

RNA precipitation

1. *(Optional)* When precipitating RNA from small sample quantities (<10^6 cells or <10 mg tissue), add 5–10 µg of RNase-free glycogen as a carrier to the aqueous phase.
   Note: Glycogen is co-precipitated with the RNA, but does not inhibit first-strand synthesis at concentrations ≤4 mg/mL, and does not inhibit PCR.
2. Add 0.5 mL of 100% isopropanol to the aqueous phase, per 1 mL of TRIzol® Reagent used for homogenization.
3. Incubate at room temperature for 10 minutes.
4. Centrifuge at 12,000 × *g* for 10 minutes at 4°C.
   Note: The RNA is often invisible prior to centrifugation, and forms a gel-like pellet on the side and bottom of the tube.
5. Proceed to **RNA wash**.

RNA wash

1. Remove the supernatant from the tube, leaving only the RNA pellet.
2. Wash the pellet, with 1 mL of 75% ethanol per 1 mL of TRIzol® Reagent used in the initial homogenization.
   Note: The RNA can be stored in 75% ethanol at least 1 year at –20°C, or at least 1 week at 4°C.
3. Vortex the sample briefly, then centrifuge the tube at 7500 × *g* for 5 minutes at 4°C. Discard the wash.
4. Vacuum or air dry the RNA pellet for 5–10 minutes. Do not dry the pellet by vacuum centrifuge.
   Note: Do not allow the RNA to dry completely, because the pellet can lose solubility. Partially dissolved RNA samples have an $A_{260/280}$ ratio <1.6.
5. Proceed to **RNA resuspension**.

8.1 Continue (3/3)

RNA resuspension

1. Resuspend the RNA pellet in RNase-free water or 0.5% SDS solution (20–50 µL) by passing the solution up and down several times through a pipette tip.
   Note: Do not dissolve the RNA in 0.5% SDS if it is to be used in subsequent enzymatic reactions.
2. Incubate in a water bath or heat block set at 55–60°C for 10–15 minutes.
3. Proceed to downstream application, or store at –70°C.

DNA Isolation Procedure

DNA is isolated from the interphase and phenol-chloroform layer saved from the **Phase separation** step.

DNA precipitation

1. Remove any remaining aqueous phase overlying the interphase. This is critical for the quality of the isolated DNA.
2. Add 0.3 mL of 100% ethanol per of 1 mL TRIzol® Reagent used for the initial homogenization.
3. Cap the tube and invert the sample several times to mix.
4. Incubate samples for 2–3 minutes at room temperature.
5. Centrifuge the tube at 2000 × *g* for 5 minutes at 4°C to pellet the DNA.
6. Remove the phenol-ethanol supernatant and save it in a new tube if protein isolation is desired. The supernatant can be stored at –70°C for several months.
7. Proceed with the **DNA wash** step using the DNA pellet.

DNA wash

1. Wash the DNA pellet with 1 mL of sodium citrate/ ethanol solution (0.1 M sodium citrate in 10% ethanol, pH 8.5) per 1 mL of TRIzol® Reagent used for the initial homogenization.
2. Incubate for 30 minutes at room temperature. Mix occasionally by gentle inversion.
   Note: The DNA can be stored in sodium citrate/ethanol solution at least 2 hours.
3. Centrifuge at 2000 × *g* for 5 minutes at 4°C. Remove and discard supernatant.
4. Repeat wash (steps 1–3), once.
   Note: Repeat wash twice for large DNA pellets (>200 µg).
5. Add 1.5–2 mL 75% ethanol per 1 mL of TRIzol® Reagent used for the initial homogenization.
   Note: DNA samples may be stored in 75% ethanol at 4°C for several months.
6. Incubate for 10–20 minutes at room temperature. Mix the tube occasionally by gentle inversion.
7. Centrifuge at 2000 × *g* for 5 minutes at 4°C. Remove and discard supernatant.
8. Air or vacuum dry the DNA pellet for 5–10 minutes. Do not allow the pellet to dry out. Do not dry the pellet by vacuum centrifuge.
9. Proceed to the **DNA resuspension** step.

DNA resuspension

Resuspend the DNA in 8mM NaOH at a concentration of 0.2–0.3 µg/µL.

1. Add 0.3–0.6 mL of 8mM NaOH per 50–70 mg of tissue, or per 1 × 10^7 cells.
   Note: Resuspending the DNA is a mild base is highly recommended because isolated DNA does not resuspend well in water or Tris buffer.
2. Remove any insoluble material by centrifuging the sample at 12,000 × *g* for 10 minutes at 4°C.
3. Transfer the supernatant containing the DNA to a new tube. Adjust pH as needed with HEPES and proceed to downstream application of choice. The DNA can be stored overnight at 4°C, but for long-term storage adjust to pH 7–8 with HEPES, and add 1 mM EDTA. Store at 4°C or –20°C.

Determining Yield of RNA and DNA

Use absorbance of RNA and DNA at 260 nm and 280 nm to determine concentration.

Sample	Procedure
RNA	1. Dilute sample in RNase-free water, and measure absorbance at 260 nm and 280 nm. 2. Use the formula A_{260} × dilution × 40 = µg RNA/mL to determine concentration.
DNA	1. Dilute sample in water or buffer (pH >7.5), and measure absorbance at 260 nm and 280 nm. 2. Use the formula A_{260} × dilution × 50 = µg DNA/mL to determine concentration.

Expected yields

The table below presents typical yields of RNA ($A_{260/280}$ of >1.8) and DNA ($A_{260/280}$ of 1.6–1.8) from various starting materials.

Starting Material	Quantity	RNA	DNA
Epithelial Cells	1 × 10^6 cells	8–15 µg	—
New Tobacco Leaf	—	73 µg	—
Fibroblasts	1 × 10^6 cells	5–7 µg	5–7 µg
Cultured cells, mammal	1 × 10^6		5–7 µg
Skeletal muscles and brain Placenta	1 mg 1 mg	1–1.5 µg 1–4 µg	2–3 µg 2–3 µg
Liver Kidney	1 mg	6–10 µg 3–4 µg	3–4 µg 3–4 µg

Protein Isolation Procedure

Proteins are isolated from the phenol-ethanol supernatant layer left over after the **DNA precipitation** step. Isolate the protein using either **Protein precipitation** OR **Protein dialysis**.

Protein precipitation

1. Add 1.5 mL of isopropanol to the phenol-ethanol supernatant per of 1 mL TRIzol® Reagent used for the initial homogenization.
2. Incubate samples for 10 minutes at room temperature.
3. Centrifuge at 12,000 × *g* for 10 minutes at 4°C to pellet the protein. Remove and discard the supernatant.
4. Proceed to the **Protein wash** step with the remaining protein pellet.

8.2 Instruction for Promega™ GoTaq™ 2-Step RT-qPCR System kit

Access RT-PCR System

Quick PROTOCOL

INSTRUCTIONS FOR USE OF PRODUCTS A1250, A1260 AND A1280.

Assemble Reactions

1. Combine the reagents below in a thin-walled 0.5ml reaction tube on ice.

Reagents	Volume	Final Concentration
Nuclease-Free Water (to a final volume of 50µl)	Xµl	
AMV/*Tfl* 5X Reaction Buffer	10µl	1X
dNTP Mix (10mM each dNTP)	1µl	0.2mM
Downstream primer	50pmol	1µM
Upstream primer	50pmol	1µM
25mM $MgSO_4$	2µl	1mM

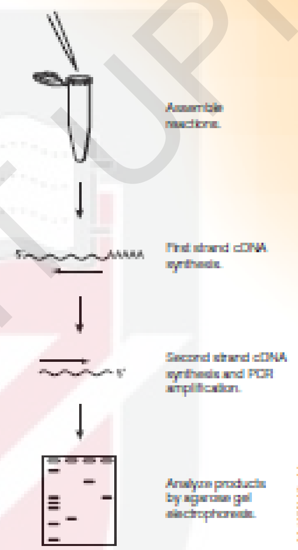
2. Mix by pipetting. Add the remaining components.

AMV Reverse Transcriptase (5u/µl)	1µl	0.1u/µl
Tfl DNA Polymerase (5u/µl)	1µl	0.1u/µl

3. Gently vortex. Initiate the reaction by adding:

RNA template	Yµl	10^3–10^6 copies
	50µl	

4. Overlay the reactions with 1 or 2 drops of mineral oil.



First Strand cDNA Synthesis

These PCR cycling profiles are only a guideline. Cycling conditions should be optimized for each RNA template.

1 cycle	45°C for 45 minutes	reverse transcription
1 cycle	94°C for 2 minutes	AMV RT inactivation and RNA/cDNA/primer denaturation

Second Strand Synthesis and PCR Amplification

40 cycles	94°C for 30 seconds 60°C for 1 minute 68°C for 2 minutes	denaturation annealing extension
1 cycle (optional)	68°C for 7 minutes	final extension
1 cycle	4°C	soak

Analyze 2.5µl of the reaction products by agarose gel electrophoresis. Store the remainder of the reaction at –20°C.

Additional protocol information is available in Technical Bulletin #TB220, available online at: **www.promega.com**

ORDERING / TECHNICAL INFORMATION:
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8.3 Instruction for MyTaq™ Red Mix (1/2)

MyTaq™ Red Mix

Shipping: On Dry / Blue Ice

Catalog numbers

BIO-25043: 200 x 50µl reactions: 4 x 1.25ml

BIO-25044: 1000 x 50µl reactions: 20 x 1.25ml

Batch No.: See vial

Concentration: 2x

Store at –20°C

BIOLINE

A Meridian Life Science® Company

Storage and stability:
MyTaq Red Mix is shipped on dry/blue ice. On arrival store at -20°C for optimum stability. Repeated freeze/thaw cycles should be avoided.

Expiry:
When stored under the recommended conditions and handled correctly, full activity of the kit is retained until the expiry date on the outer box label.

Safety precautions:
Please refer to the material safety data sheet for further information.

Quality control specifications:
Bioline operates under ISO 9001 Management System. MyTaq Red Mix and its components are extensively tested for activity, processivity, efficiency, sensitivity, absence of nuclease contamination and absence of nucleic acid contamination prior to release.

Notes:
Research use only.

Description

MyTaq™ Red Mix is a ready-to-use 2x mix for fast, highly-specific PCR. The advanced formulation of MyTaq Red Mix exhibits more robust amplification than other commonly used polymerases, delivering very high yield over a wide range of PCR templates, and making it the ideal choice for most routine assays. MyTaq Red Mix contain all the reagents (including stabilizers) necessary for trouble-free PCR reaction set up. The product is supplied conveniently all in one tube, reducing the number of pipetting steps and facilitating increased efficiency, throughput and reproducibility.

The specially designed MyTaq Red formulation does not interfere with the PCR and allows users to load samples directly onto a gel after the PCR without the need to add loading buffer.

MyTaq Red Mix only requires the addition of template, primers and water, reducing the risk of pipetting errors and contamination as well as shortening the set-up time.

Components

	200 Reactions	1000 Reactions
MyTaq Red Mix, 2x	4 x 1.25ml	20 x 1.25ml

Standard MyTaq Red Mix Protocol

The following protocol is for a standard 50µl reaction and can be used as a starting point for reaction optimization.

PCR reaction set-up:
All reactions must be set-up on ice.

Template	200ng
Primers (20µM each)	1µl
MyTaq Red Mix, 2x	25µl
Water (ddH_2O)	up to 50µl

PCR cycling conditions:
We suggest these conditions in the first instance:

Step	Temperature	Time	Cycles
Initial denaturation	95°C	1min	1
Denaturation	95°C	15s	25-35
Annealing*	User determined	15s	
Extension*	72°C	10s	

* These parameters may require optimization, please refer to the Important Considerations and PCR Optimization section if needed.

Important Considerations and Optimization

The optimal conditions will vary from reaction to reaction and are dependent on the template/primers used.

Primers: Forward and reverse primers are generally used at the final concentration of 0.2-0.6µM each. As a starting point we recommend using 0.4µM as a final concentration (*i.e.* 20pmol of each primer per 50µl reaction volume). Too high a primer concentration can reduce the specificity of priming, resulting in non-specific products.

When designing primers we recommend using primer-design software such as Primer3 (http://frodo.wi.mit.edu/primer3) or visual OMP™ (http://dnasoftware.com) with monovalent and divalent cation concentrations of 10mM and 3mM respectively. Primers should have a melting temperature (Tm) of approximately 60°C

Template: The amount of template in the reaction depends mainly on the type of DNA used. For templates with low structural complexity, such as plasmid DNA, we recommend using 50pg-10ng DNA per 50µl reaction volume. For eukaryotic genomic DNA, we recommend a starting amount of 200ng DNA per 50µl reaction, this can be varied between 5ng-500ng. It is important to avoid using template resuspended in EDTA-containing solutions (e.g. TE buffer) since EDTA chelates free Mg^{2+}.

Initial denaturation: An initial denaturation step of 1min at 95°C is recommended for non-complex templates such as plasmid DNA or cDNA. For more complex templates such as eukaryotic genomic DNA, longer initial denaturation times of up to 3mins are required in order to facilitate complete melting of the DNA.

Denaturation: Our protocol recommends a 15s cycling denaturation step at 95°C which is also suited to GC-rich templates, however for low GC content (40-45%) templates, the denaturation time can be decreased down to 5s.

PI-50160 V3

Website: www.bioline.com/ email: info@bioline.com

8.3 Continue (2/2)

Annealing temperature and time: The optimal annealing temperature is dependent upon the primer sequences and is usually 2-5°C below the lower Tm of the pair. We recommend running a temperature gradient to determine the optimal annealing temperature, alternatively 55°C can be used as a starting point. Depending on the reaction the annealing time can also be reduced to 5s.

Extension temperature and time: The extension step should be performed at 72°C. The extension time depends on the length of the amplicon and the complexity of the template. For low complexity template such as plasmid DNA, an extension time of 10s is sufficient for amplicons under 1kb or up to 5kb. For amplification of fragments over 1kb from high complexity template, such as eukaryotic genomic DNA, longer extension times are recommended. In order to find the fastest optimal condition, we suggest incrementing the extension time successively up to 30s/kb.

Troubleshooting Guide

Problem	Possible Cause	Recommendation
No PCR product	Missing component	- Check reaction set-up and volumes used
	Defective component	- Check the aspect and the concentrations of all components as well as the storage conditions. If necessary test each component individually in controlled reactions
	Cycling conditions not optimal	- Decrease the annealing temperature - Run a temperature gradient to determine the optimal annealing temperature - Increase the extension time, especially if amplifying a long target - Increase the number of cycles
	Difficult template	- Increase the denaturation time
Smearing or Non-Specific products	Excessive cycling	- Decrease the number of cycles
	Extension time too long	- Decrease the extension time
	Annealing temperature too low	- Increase the annealing temperature
	Primer concentration too high	- Decrease primer concentration
	Extension during set-up	- Make sure all reactions are set-up on ice. Run reaction as quickly as possible
	Contamination	- Replace each component in order to find the possible source of contamination - Setup the PCR and analyze the PCR product in separated areas

Technical Support

If the troubleshooting guide does not solve the difficulty you are experiencing, please contact your local distributor or our Technical Support with details of reaction set-up, cycling conditions and relevant data.

Email: tech@bioline.com

Associated Products

Product Name	Pack Size	Cat No
Agarose	500g	BIO-41025
Agarose tablets	300g	BIO-41027
HyperLadder™ 1kb	200 Lanes	BIO-33025
SureClean Plus	1 x 5ml	BIO-37047

TRADEMARKS

1. MyTaq and HyperLadder are Trademarks of Bioline Reagents Ltd

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8.4 Agarose Gel Representative Image

L	:	Ladder 100-bp
PC	:	Positive control
NC	:	Negative control
P	:	Putrajaya
I	:	ISPCA
W	:	PAWS
Yellow arrow	:	Position of final PCR product – 401-bp

8.5 Samples details

No.	Shelter	Gender	FmoPV urine	FmoPV in kidney
1	Putrajaya	IM	Negative	NA
2	Putrajaya	IF	Positive	NA
3	Putrajaya	IF	Negative	NA
4	Putrajaya	IM	Positive	NA
5	Putrajaya	IM	Positive	NA
6	Putrajaya	IF	Positive	NA
7	Putrajaya	IF	Positive	NA
8	Putrajaya	IF	Positive	NA
9	Putrajaya	IF	Negative	NA
10	Putrajaya	IF	Negative	NA
11	Putrajaya	IF	Negative	NA
12	Putrajaya	IF	Negative	NA
13	Putrajaya	IF	Negative	NA
14	Putrajaya	IF	Positive	NA
15	Putrajaya	IF	Negative	NA
16	Putrajaya	IM	Positive	NA
17	ISPCA	SF	Positive	NA
18	ISPCA	SF	Positive	NA
19	ISPCA	SF	Positive	NA
20	ISPCA	SF	Positive	NA
21	ISPCA	CM	Negative	NA
22	ISPCA	CM	Negative	NA
23	ISPCA	SF	Positive	NA
24	ISPCA	SF	Positive	NA
25	ISPCA	SF	Negative	NA
26	ISPCA	CM	Negative	NA

IM: Intact male

CM: Castrated male

IF: Intact female

SF: Spayed female

- : Unknown

NA: not applicable

27	ISPCA	CM	Negative	NA
28	ISPCA	SF	Positive	NA
29	PAWS	IM	Negative	NA
30	PAWS	IM	Negative	NA
31	PAWS	IF	Positive	NA
32	PAWS	SF	Negative	NA
33	PAWS	IF	Negative	NA
34	PAWS	IM	Negative	NA
35	PAWS	IF	Positive	NA
36	PAWS	IF	Negative	NA
37	PAWS	CM	Negative	NA
38	PAWS	IM	Negative	NA
39	PAWS	IF	Negative	NA
40	PAWS	IF	Positive	NA
41	PAWS	SF	Negative	NA
42	PAWS	IF	Negative	NA
43	PAWS	IF	Negative	NA
44	PAWS	SF	Positive	NA
45	PAWS	IF	Negative	NA
46	PAWS	IM	Negative	NA
47	PAWS	IM	Negative	NA
48	PAWS	-	Negative	NA
49	PAWS	IF	Negative	Negative
50	PAWS	-	Negative	Negative

IM : Intact male

CM : Castrated male

IF : Intact female

SF : Spayed female

- : Unknown

NA: not applicable