DEVELOPMENT OF A REAL-TIME PCR ASSAY FOR MOUSE FUR MITES AND EVALUATION OF PILOT STUDY

by

Allison Poore

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Accepted:

_____	_____
Dr. Craig Laufer, Ph.D. Committee Member	Dr. Ann Boyd, Ph.D. Director, Biomedical Science Program
_____	_____
Dr. Ann Boyd, Ph.D. Committee Member	Dr. April Boulton, Ph.D. Dean of the Graduate School
_____	
Dr. Wang-Ting Hsieh, Ph.D. Thesis Adviser	

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DEDICATION

To Scott. And to my Mom and Dad.

I love you.

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Thank you to the National Cancer Institute for funding and for their continued work to improve the lives of patients living with cancer.

Thank you to all the Hood College faculty who have supported me throughout my time as a graduate student. A special thanks to Dr. Ann Boyd, who made every effort to help me finish my degree through every challenge. An additional special thank you to Dr. Rachel Beyer, who also took extra time and provided accommodation to help me finish my classes.

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ABSTRACT

Mouse fur mites *Myobia musculi* and *Myocoptes musculinus* are common parasites of mice and present a unique challenge for exclusion from laboratory animal facilities. In this study, a real-time PCR test was developed to detect both mouse fur mites using distinct dye in a single reaction well. Confirmatory endpoint PCR tests were also designed, to confirm equivocal results. The assays were then tested during a pilot study, where known positive animals were used to evaluate the accuracy of environmental targets for screening. In all cages were mice were positive for the fur mite real-time PCR, environmental targets were also positive within 10 days of placement in a new, clean cage. This study shows that the real-time PCR assay and environmental targets are an effective combination for screening of mouse fur mites in laboratory animal facilities.

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LIST OF ABBREVIATIONS

LASP	Laboratory Animal Sciences Program
OTS	Operations and Technical Support contract
PCR	Polymerase chain reaction
NCI	National Cancer Institute
R&Q	Receiving and Quarantine
MPV-1	Mouse Parvovirus 1
ELISA	Enzyme-Linked Immunosorbent Assay
DNA	Deoxyribonucleic acid
NK cells	Natural Killer cells
Th2	T helper cell 2
Th1	T helper cell 1
Il or IL	Interleukin
IVC	Individually vented cage
mRNA	Messenger RNA
PCR-MAP	PCR-based mouse antibody product test
FNLCR	Frederick National Laboratory for Cancer Research
IACUC	Institutional Animal Care and Use Committee
CV	Coefficient of variation
ADL	Animal Diagnostics Laboratory
Ct	Cycle threshold
NCBI	National Center for Biotechnology Information
BLAST	Basic Local Alignment Search Tool

INTRODUCTION

The monitoring of health in laboratory animals is a significant element in the field of animal research. The field of health monitoring is constantly evolving to meet the dynamic needs of scientific research, to improve animal welfare, and to improve the quality and quantity of scientific data.

The Laboratory Animal Sciences Program (LASP) within Leidos Biomedical Research provides comprehensive animal health services under an Operations and Technical Support (OTS) contract for the National Cancer Institute (NCI). LASP provides a full spectrum of animal health services to hundreds of investigators, with the goal of providing and maintaining animals that are suitable for scientific research. This includes health monitoring of hundreds of thousands of mice, housed in 25 animal facilities over two different campuses (Laboratory Animal Sciences Program 2011). Successful health monitoring is the foundation of all animal science.

The Animal Diagnostics Laboratory within LASP provides molecular diagnostics, polymerase chain reaction (PCR)-based genotyping, bacteriology, parasitology, screening of cell lines for use in animals, and other diagnostic services (Laboratory Animal Sciences Program 2011). The focus of this paper will be on an expansion of the molecular diagnostic assays included in the health monitoring program to address vulnerabilities of traditional detection methods for fur mites. The goal of the project is the development of a PCR test for mouse fur mites *Myobia musculi* and *Myocoptes musculinus.*

Establishment of a health monitoring program is a requirement for any animal facility used for scientific work, both by accreditation bodies and by the federal

government. Each program consists of protocols to determine: which pathogens or parasites to allow or exclude (from the entire facility or from specific projects), the frequency of monitoring, sampling methods, diagnostic testing technology, training of animal and laboratory staff, reporting of significant results, shipping/receiving and quarantine of animals, and outbreak/contamination procedures (Mähler *et al.* 2014a). In addition to these basic requirements, the programs are expected to evolve to meet the growing needs of research, including: evaluation of new technologies, implementation of staff training for developing animal health concerns, and the addition of new pathogens of concern to routine monitoring protocols. Global standard health monitoring recommendations for mice include viruses, bacteria, fungi, and parasites and are published routinely as agents and methods change (Mähler *et al.* 2014a). The health monitoring program at each institution reflects the diversity and complexity of ongoing scientific research as well as the facility size.

LASP within Leidos Biomedical Research is a complex and diverse set of animal facilities, primarily devoted to mouse-model based cancer research. In published global recommendations for health monitoring, this institution has many of the high risk factors listed for risk of introduction of excluded organisms, including: moving animals in and out of a facility (for experimental purposes such as imaging), possible access of feral mice to animal facilities, facilities that house animals involved in different experiments, shared equipment between groups, small work spaces, and research staff that enter animal rooms in additional to animal care staff (Mähler *et al.* 2014a). The institution requires constant adherence to a health monitoring program to prevent introduction of unwanted organisms and to continue to produce valuable scientific research.

Animal research institutions have a dedicated location for Receiving & Quarantine (R&Q), which isolates animals before they are released into any animal facilities. Within LASP, mice enter through R&Q and are housed there until multiple testing events have occurred over a predetermined time period. Within the R&Q facility, there are various levels of animal health statuses, used to separate animals from approved and non-approved vendors. This classification depends on the health monitoring programs of the sending institution or commercial company and is determined by LASP based on available health data. The goal is to separate animals positive for excluded organisms from those negative so that negative animals are not contaminated. If animals pass the required health status screens, they can then be transferred into the desired facility. Animals that are unable to match the required health status of a specific building can undergo rederivation, where embryos are transplanted into a clean mouse for gestation. These progeny can be tested again for the organism of concern, and then upon negative result be released into the facility (Laboratory Animal Sciences Program 2011).

The health status of laboratory mice is an important consideration in research. Animals of identical health status are necessary for reproducible scientific results. Opportunistic organisms play a large role in monitoring, as they can show no clinical signs until a variable is introduced (such as a drug, treatment, or stress). Commensal organisms typically have no effect on a feral animal, but can be devastating to immune-deficient mouse models or those with other genetic modifications.

Another concern is that accepted treatments or prevention methods for an organism are not effective in all mouse models due to their genetic differences. *Corynebacterium bovis* causes dermatitis, skin lesions, and other clinical symptoms in most immune-

deficient mouse strains, including athymic nude mice. Standard antibiotic therapy for this infection was not effective in athymic nude mice (Burr *et al.* 2011). One issue not discussed in this study is the practicality of treatments, including antibiotics, for use during scientific studies. Even if there is an effective treatment for an infection or parasite, administration of the treatment can confound research results and make animals unusable for an experiment.

Animal facilities often house a diverse array of genetically distinct mice, all with their own specific health concerns. Commonly used mouse models are purposefully immunocompromised in order to be used for scientific experiments. This makes them significantly more susceptible to infection and the infection may be more serious. The method of screening plays a role in the accuracy of the results. Each mouse strain may manifest the infection differently, and may not become positive on an assay depending on what it looks for. Mouse Parvovirus 1 (MPV-1) could not be detected reliably in various strains of mice when screened by serological methods such as Enzyme-Linked Immunosorbent Assay (ELISA). Not all mouse strains seroconvert reliably or on an expected timeline due to their genetic manipulations. In the same study, a PCR test for this virus was able to reliably detect infection in all but DBA/2 mice (Besselsen *et al.* 2000). DBA/2 mice have numerous deleterious phenotypes as well as altered natural killer (NK cells). These mice also have no cell surface expression of CD94 due to a known homozygous deletion (The Jackson Laboratory). DBA/2 mice may have a unique response to MPV-1 infection, which prevented detection of viral DNA by PCR. Overall, the detection method and what diagnostic target it is screening for (antibody, nucleic acid,

visual clinical symptoms) matters when interpreting results and there is little room for error when managing over 25 facilities.

Additional considerations include the credibility of research if the organism or its side effects impact results. For example, B6 mice infested with fur mite *M. musculinus* were shown to have an induced Th2 (T helper cell 2)-type immune response from the mite infection. The study evaluated gene expression of cytokines and a transcription factor associated with a Th2 response, including *Il4*, *Il5*, and *Gata3*, over the course of mite infection. Gene expression of these two cytokines and transcription factor increased significantly during mite infection in B6 mice. The authors indicated a direct link between mite infection and initiation of a Th2 immune response (Moats *et al.* 2016). These mice would not be suitable for use in any evaluation of the immune system or in those evaluating treatments that modulate the immune response. The cytokines and transcription factor tested have numerous and diverse effects on cell signaling and transcription. For this reason, there may be many other cellular targets that are modulated by the same fur mite infection. Using these mice for scientific experiments carries the risk of rendering data unusable.

Parasite *Toxoplasma gondii* can infect mice and cause toxoplasmosis infection. *T. gondii* infection proceeds by induction of a Th1 immune response. One study showed that co-infection of *M. musculinus* and *T. gondii*, which would activate both Th1 and Th2 immune response, lead to significantly increased mortality among C57BL/6 mice (Welter et al. 2006). BALB/c mice are resistant to *T. gondii* infection. However, after one month of *M. musculinus* infection, BALB/c mice were susceptible to *T. gondii* infection and

developed toxoplasmosis (Welter et al. 2007). Results of mouse toxoplasmosis studies may be confounded by fur mite infection.

Collaboration among scientific institutions, which includes the transfer of animals and data from one facility to another, is heavily influenced by the health status of animals at each institution. Reproducing scientific results relies on animals of the same, known, health status. Many institutions provide standardized animal health reports for each facility that are publicly available, as recommended by oversight committees in the United States and abroad (Mähler *et al.* 2014b).

Once an organism has contaminated a facility, it can be difficult, time consuming, and prohibitively expensive to eradicate it. Scale is an essential consideration here: in one study of fur mite eradication, the institution had to implement a plan for approximately 30,000 mouse cages (Ricart Arbona et al. 2010). The most effective solution to facility outbreaks continues to be prevention.

Housing and husbandry practices are the foundation of preventing disease spread in animal facilities. The most common type of mouse housing is the individually ventilated cage (IVC), which contains mice in their own closed, filter top environment. This housing may reduce transmission of illnesses from one cage to the next (Mähler *et al.* 2014a) but does not eliminate it . The institution used in this study uses primarily IVC caging and this will be the primary environment for detection of fur mites.

Many organisms are screened for using sentinel mice that undergo exposure to dirty bedding from other mice in their defined area. After a predetermined amount of time, based on institutional health monitoring standards, dirty bedding from mice is transferred

to the associated sentinel mice in that area. For many commonly screened pathogens and parasites, the route of transmission to sentinel and the time required for a positive diagnostic result from a sentinel animal are known (Macy *et al.* 2011). Based on known transmission data, institutional health monitoring programs allow the sentinel mice to undergo adequate exposure time to be infected by any organisms present. The sentinel mice are then euthanized and screened for numerous pathogens. If there are unexpected positive results, further testing is required and is typically urgent. The subsequent testing will usually be pooled samples, which allows mice all in one rack or all in one row to be screened by pooling the diagnostic material. For example, if feces is the sampling material, one fecal pellet from each cage in a row of cages can be pooled and screened. This reduces the overall sample load and helps to quickly identify which row of cages is infected. Once an infected row is identified, individual cages will then be sampled and screened. This is the standard institutional protocol following a significant positive test during sentinel screening. Sentinels are replaced every quarter (Laboratory Animal Sciences Program 2017). In this proposal, transfer by sentinel will be used interchangeably with dirty bedding transfer or sentinel detection. The use of sentinels provides a comparatively inexpensive and quick health evaluation, compared to individual animal testing. Sentinel monitoring programs are the foundation of an animal health program.

However, there are organisms that are not reliably transmitted via sentinel bedding transfer, which presents a unique problem when establishing a screening program. For example, *Cornyebacterium bovis* is an asymptomatic commensal bacteria in immunocompetent mice. This bacteria is not reliably transmitted to an immunocompetent sentinel by infected immunocompromised animals, even with dirty bedding transfer

(Mähler *et al.* 2014a). The immunocompetent sentinel animals typically clear the infection before it can be discovered by screening, giving a false negative result. For organisms with this concern, other methods of screening must be implemented to ensure exclusion.

Murine fur mites *M. musculi* and *M. musculinus*, are not reliably transmitted through sentinel, making them difficult to detect. While some facilities report timely and robust detection of fur mites by sentinel monitoring (Ricart Arbona *et al.* 2010), others report transmission as low as 3% with complete dirty bedding transfer over a 12 week study (Lindstrom *et al.* 2011). Differences in detection rate may be related to unique facility construction and makeup, as well as husbandry practices and staff training.

This is an important consideration for our institution, since we house animals in multiple, distinct, buildings. Any detection method we employ must work in every facility. Based on the wide range of detection probabilities published in the literature, sentinels cannot be used reliably for detection of mouse fur mites. Transfer and subsequent detection of organisms via sentinel animals relies on the efficacy of transferring the organism through dirty bedding. The organism then spreads from the bedding to the naïve sentinel mouse. Without successful dirty bedding transfer, sentinel mice will produce a false negative diagnostic result. Any vulnerabilities in the sentinel detection program may allow for

serious infections to proliferate through the animal facilities, compromising both science and animal welfare (Figure 1).

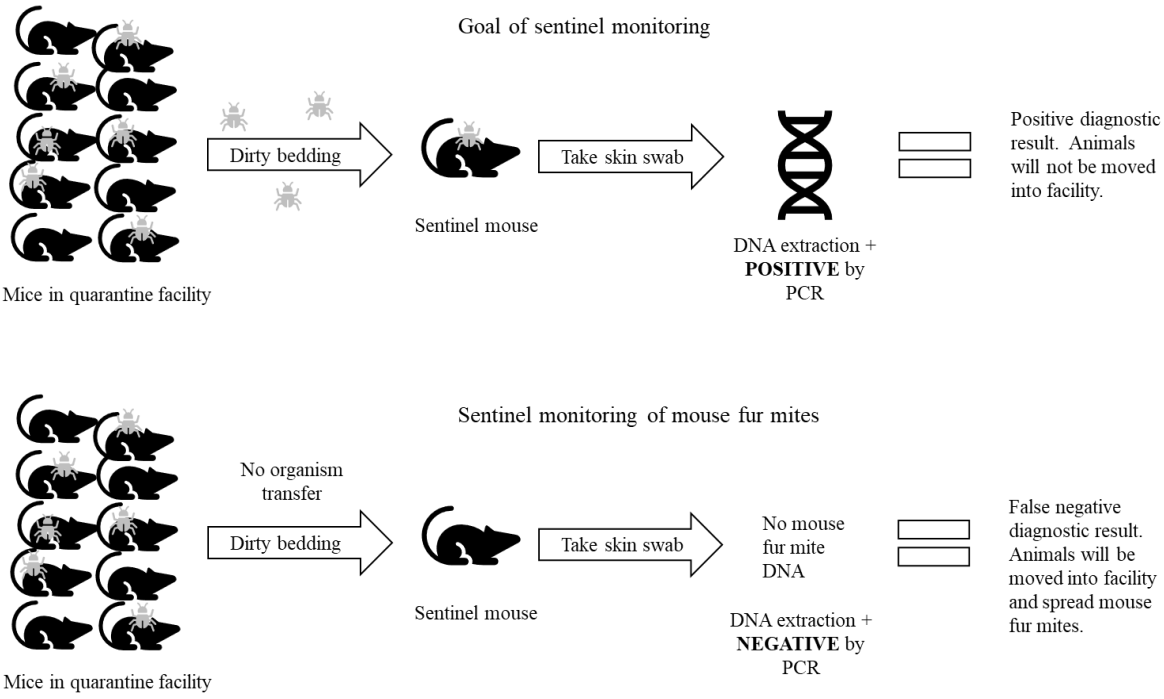


Figure 1. The goal of sentinel monitoring compared with sentinel monitoring of mouse fur mites. Fur mites do not reliably transfer from mice to their dirty bedding, and are therefore not able to infect the sentinel mouse. The sentinel mouse is tested for mouse fur mites, and produces a false negative result. This is the primary source of introduction of mouse fur mites into animal facilities.

Both species of fur mites are known to transfer during close contact with another susceptible host (The Mouse in Biomedical Research 2006) and this provides another explanation for lack of efficacy of transfer to sentinels and subsequent detection. Sentinel transfer does not include close contact of the animals, and the mites may not physically reach bedding to be transferred. The density of the mite population and life cycle stage may also contribute to lack of efficacy for sentinel bedding transfer. New approaches for

detection are needed for organisms, such as fur mites, that do not reliably transmit via sentinel.

This project focuses on two species of fur mites that can infect laboratory mice: *M. musculi* and *M. musculinus*. The ecology and life cycle of the two mites are essential to the discussion of their detection in animal facilities.

M. musculi is a mite in the family *Myobiidae*, with adults measuring less than 0.5mm. Their life cycle includes: egg, larva, multiple nymph stages, and adult, which in total lasts about 23 days. *M. musculi* preferentially live in the head, neck, and shoulder areas of infected mice. Adult mites feed on interstitial fluids from the host mouse. This is the primary route of exposure for immune response in the mouse, as seen clinically. Transmission of mite infection occurs through direct transfer of adult mites, typically during close contact (The Mouse in Biomedical Research 2006).

M. musculinus is a mite in the family *Myocoptidae*, measuring less than 0.38mm. *M. musculinus* often exists in a mixed infection with *M. musculi*, although the two species do typically infest different physical areas of the mouse (Percy and Barthold 2013). However, the two mite species are readily distinguishable based on anatomical differences. The *M. musculinus* life cycle includes: egg, larva, two nymph stages, and adult, which lasts about 14 days. *M. musculinus* preferentially inhabit the inguinal, ventral abdomen, and back regions of the mouse (The Mouse in Biomedical Research 2006). These mites feed on the skin of an infected mouse, this being the primary route of exposure for clinical response in the animal. These mites also transfer directly through close contact with other susceptible hosts (Percy and Barthold 2013).

Symptoms of fur mite infection for the two species are similar, with the most common symptom being skin lesions that vary greatly in severity, from mild dermatitis to ulceration and pyoderma. Each mouse strain varies in the severity of reaction to fur mite infection. Infection can also be discovered clinically by the presence of alopecia, again ranging in severity, as well as secondary infections of the skin irritation. However, mite infections can transition to a systemic reaction and cause severe immune dysfunction (The Mouse in Biomedical Research 2006). Severe infection can also lead to animal welfare concerns, where mice respond to the infection by scratching and severely damaging their skin and these injuries can generate dangerous secondary infections (Bino Sundar *et al.* 2017).

Many of the mice used within LASP facilities for research are immunocompromised. Immunocompromised mice have been shown to have exacerbated symptoms of fur mite infection, associated with significantly higher mite burden and an apparent lesser ability to control the infection than immunocompetent animals. $Rag1^{-/-}$ mice, who lack mature T and B cells and therefore lack adaptive immunity, had higher mite burdens in one study compared to a B6 immunocompetent mouse. The higher mite burden was associated with greater severity of known symptoms, including: weight loss, inability to gain weight, pruritus (skin itching), alopecia (hair loss or patchy fur), a range of dermatitis, and skin lesions. The study measured weight throughout infection and found that even immunocompetent B6 infested mice weighed significantly less than non-infested mice (Figure 2; Moats *et al.* 2016).

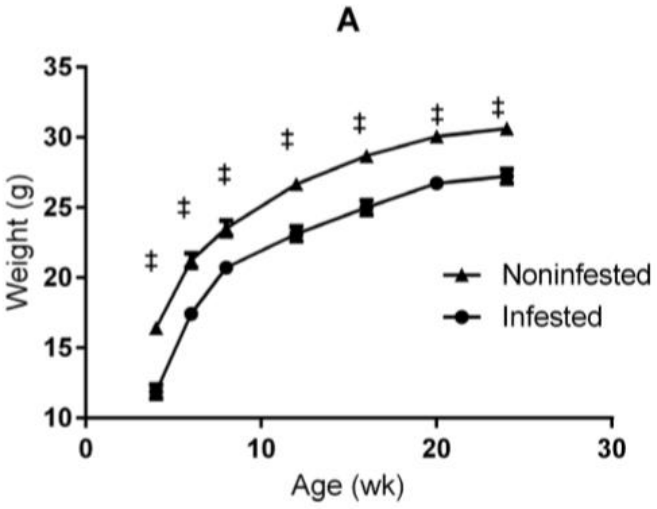


Figure 2. Comparison of weights from infested and non-infested male mice over a 30 week period. Weight of uninfected mice was significantly higher than those with *M. musculinus* infection (Moats et al. 2016).

Rag1$^{-/-}$ and B6 mice are used frequently in LASP facilities and weight is a commonly used parameter for animal studies. If mice are infected with fur mites, and this infection affects their weight, this valuable data may be confounded by the mite infection. Other studies within LASP examine skin changes over time for dermatological experiments. Mite infection would confound this data as well, with a wide range of severe dermatological symptoms.

The same study also explored the effects of fur mite infection in immunocompetent B6 mice on immune system markers, including genes associated with Th1 and Th2 type immune responses. Gene expression of *Il4*, *Il5*, *Gata3*, *Il2*, and *Tbx21* increased throughout the infection (Figure 3; Moats *et al.* 2016). The three cytokines listed as increasing during mite infection, IL4, IL5, and IL2 are used in studies of the immune system by investigators at the LASP animal facilities. Investigators may be monitoring the

exact same parameter: gene expression of these cytokines over time. Any impact on these cytokines caused by mite infection may confound research results. If the infection is serious enough it may even halt research altogether due to animal welfare concerns.

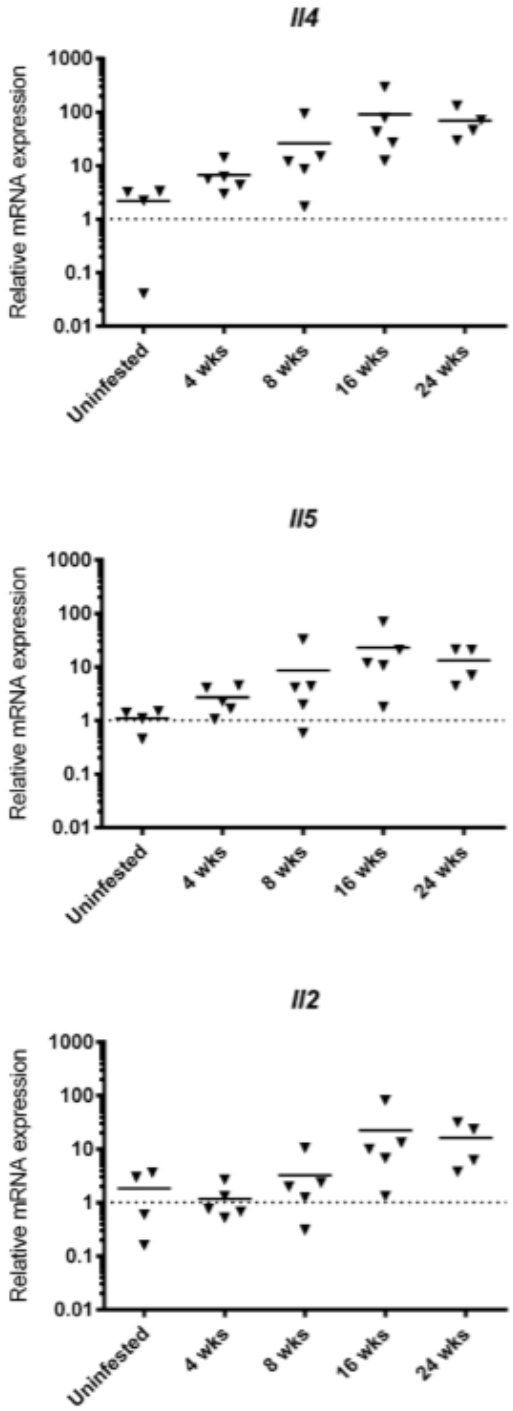


Figure 3. Comparison of relative mRNA expression of cytokines of animals before infection with *M. musculinus* and throughout infection. mRNA was taken from mouse lymph nodes. Gene expression of *Il2*, *Il4*, and *Il5* increased throughout the mite infection (Moats et al. 2016).

Another study done in BALB/c mice that had ulcerative dermatitis due to *M. musculinus* infection shows severe, systemic, immune system dysfunction. The immune system impact included: decreased T and B cells in blood and organs and increases in IgE and IgA. The mice also had the typical clinical symptoms including, weight loss, alopecia, and lymph node swelling (Jungmann *et al.* 1996). Again, many studies within LASP involve the monitoring of T and B cells and immunoglobulins to evaluate immune response to either cancer or a proposed cancer treatment. Mite infection significantly decreases the legitimacy of these results or may completely invalidate them.

Diagnosis of mite infection has historically included tape tests, fur plucks, and direct pelt examination. Tape testing involves affixing a piece of transparent tape to a mouse, and then evaluating the tape under a microscope for mites or their eggs. Fur pluck testing is similar, where multiple plucks of fur are pulled from a mouse and then evaluated under a microscope for eggs or mites. Fur mites can also be diagnosed by direct examination of the entire surface of the mouse, of a euthanized or anesthetized animal (The Mouse in Biomedical Research 2006).

Standard practice of monitoring for fur mites within LASP was a combination of hair plucks and direction examination. Hair was plucked from sentinel mice using forceps from the neck, base of tail and "a third dorsal or lateral location" and placed on a microscope slide to be evaluated by staff for mites or eggs. The sentinel animals were also submitted for complete necropsy, and during necropsy the fur was visually inspected for mites or eggs. In addition, because of known inefficiency of mite transmission to sentinels,

additional fur plucks were instructed to be collected "periodically" for screening from individual animals, not sentinel mice. The animals and appropriate time to complete this spot screening of individual animals was decided by the animal facility's veterinarian when animals with symptoms of mite infection were observed, primarily pruritus or dermatitis (Laboratory Animal Sciences Program 2011).

The institution's standard practice for identifying mouse fur mites left vulnerabilities to outbreak by this organism. Detection rates for fur pluck and visual testing are low and transmission to sentinel is an unreliable detection workflow for mouse fur mites. The periodic screening of animals besides sentinels does not guarantee successful prevention of outbreak. Different strains of mice have varying clinical symptoms and for example, may have alopecia with no associated dermatitis or pruritis. Also, symptoms of mite infection change over time and as mice age, they may be chronically infected with a low mite burden that shows no clinical signs, or a subclinical infection (Moats *et al.* 2016). Mice with subclinical infection would not be identified under this protocol for additional screening. Additionally, this spot testing of mice with symptoms would mean that the animals in question already had a significant infection, because clinical symptoms are shown to be associated with higher mite burden (Moats *et al.* 2016). If these animals were being used for an experimental protocol, this timeline of detection is too late. The experiment would have to account for fur mite infestation in its results. The late detection also means more time and expense wasted if the results are unusable.

Treatment of mice for fur mites is expensive and labor-intensive. Treatment typically consists of acaricides, such as ivermectin, which can be delivered individually to each mouse or in some cases through drinking water or mouse feed. However, many

treatments of this nature are not safe for infant mice and recent mothers, since infant mice are exposed to levels of ivermectin in milk (Arbona *et al.* 2010). Each study of acaricide treatment for toxicity and other side effects often only explores those concerns in one mouse strain. As our facilities have hundreds of distinct mouse strains, there is no guarantee that these treatments will work in all mice or that they will be safe in terms of animal welfare. Additionally, there would be serious considerations about the validity of any research done on animals that have been exposed to acaricides throughout an experiment. This underscores the concern to detect fur mites accurately and fast, so that an outbreak never happens in the first place. Prevention is always the goal of animal health monitoring.

Through technology improvements, fur mites have also been diagnosed by PCR testing. Samples tested by PCR are often skin swabs but can include other sample types (Karlsson *et al.* 2014). DNA is isolated from a sample and then run under conditions of heat cycling, which allows for organism-specific primers to exponentially amplify a piece of fur mite DNA. This result can then be visualized either by viewing the PCR product on an endpoint assay such as a gel or capillary electrophoresis system, or amplification can be viewed throughout the cycling using real-time PCR technology.

At the time of this project, the ADL already had capability to detect numerous murine pathogens and parasites by real-time PCR. These include: *Helicobacter sp.*, *C. bovis*, Pinworms (*Syphacia obvelata* and *Aspiculuris tetraptera)*, Murine Norovirus, Mouse Parvovirus, *Mycoplasma sp.*, and a commonly used virus and bacteria panel called the PCR-MAP test, which is the PCR-based mouse antibody testing that screens cell lines for use in animals. The PCR-MAP test includes a panel of more than 18 different virus

and bacteria species. Assays within the ADL use different sample material based on the organism, which can be feces, lymph node, cell line, or environmental swabs.

There are many techniques used to detect murine fur mites and each can be used in conjunction with sentinel monitoring or individual animal testing. However, these techniques show significant differences in the rate of false negative results, an important indicator of diagnostic accuracy particularly when screening is done during a quarantine period. Non-molecular methods such as tape tests and fur plucks have high false negative rates around 25% in one evaluation. Direct pelt examination of individual animals can be used effectively (Karlsson *et al.* 2014), however to evaluate every animal in a facility is not feasible. Without reliable sentinel transmission, direct pelt examination or individual animals is impractical to implement on a large scale and would be unusable in our facilities. In the same evaluation, PCR had a false negative rate around 2%, indicating that it is an attractive option for the screening of mouse fur mites. However, the PCR results reported in this article give further justification for high-throughput PCR sampling methods. In this article, swabs of the individual animal were used to detect fur mites with high reliability. This sampling method, while accurate for diagnostic evaluation, is not a reasonable solution for large animal facilities (Karlsson *et al.* 2014). It shows the need for environmental targets that can screen many animals at once with a single swab.

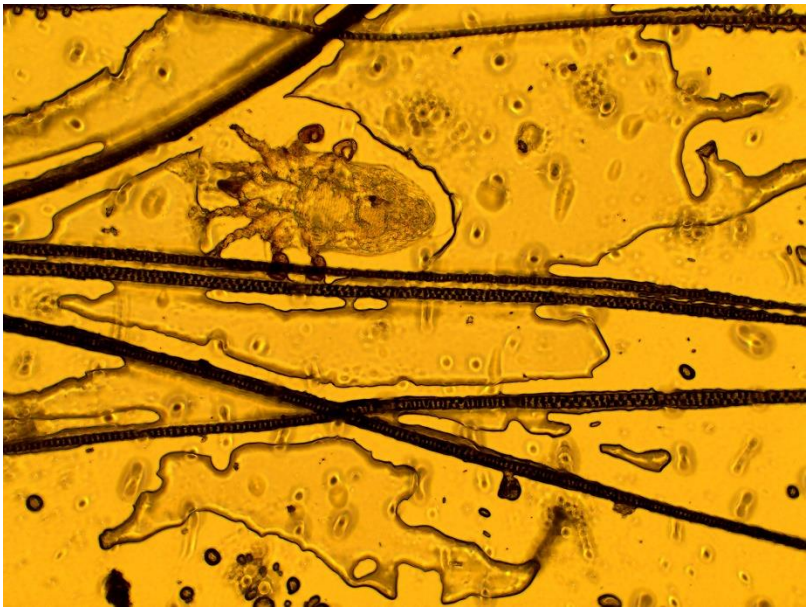
With the unique challenges of monitoring for mouse fur mites, a new approach is clearly needed for more accurate identification of infection. However, a new testing process must still be able to canvas a large number of animals in an area so that testing remains inexpensive and requires little time to collect and process samples. A more recent study showed the use of a commercial PCR test to detect mouse fur mites and also

evaluated the use of the exhaust manifold of the IVC system as an environmental screening target to detect mouse fur mites. Fur mites were accurately detected with a 94% probability on the exhaust manifolds within 4 weeks of placement of known positive animals on the rack (Jensen *et al.* 2013). Using the same commercial PCR testing, another group was able to detect fur mites with a 100% success rate after 30 days by extracting DNA from multiple IVC filters, creating a pooled sample for screening (Gerwin *et al.* 2017). These studies showed the efficacy of an environmental target for the screening of mouse fur mites.

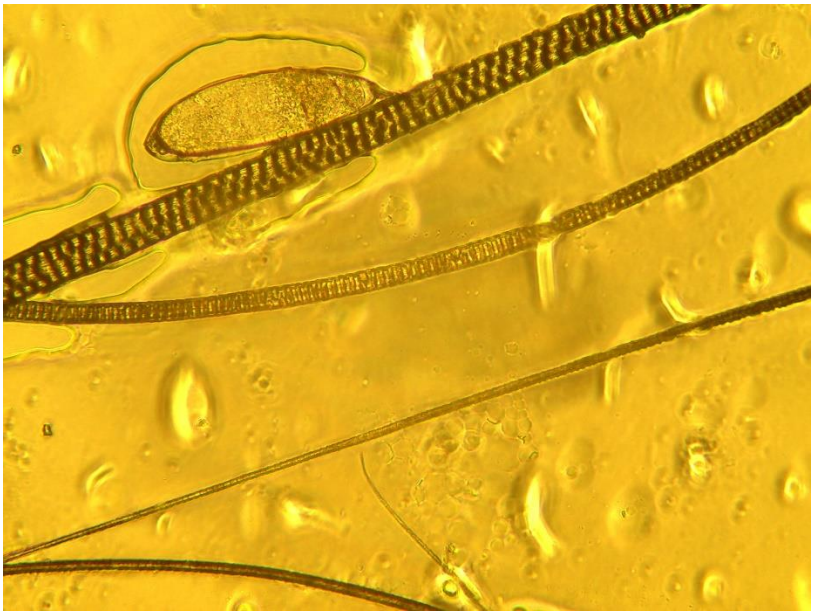
However, these published studies also pose a problem for the internal diagnostics program within LASP. The detection rates are appealing, however the program prefers to have an option to test using in-house diagnostics and these studies rely solely on a commercial vendor for PCR testing. While this is one option, having an in-house test allows more flexibility especially during a potential outbreak. During an outbreak or even a suspected outbreak, the facilities require sample results within 24 hours. Commercial vendors can't always provide this turnaround and if they do, there is typically a large surcharge for this service. This problem is an opportunity for the development of an internal assay that would detect mouse fur mites.

This project will focus on development of a real-time PCR assay to detect both *M. musculi* and *M. musculinus*. It will also include development of endpoint PCR assays for each mite that will be used for confirmation of equivocal results as well as sequencing analysis. Multiple sample types will be evaluated for their use in mite detection, and a pilot study will be conducted to test the detection protocol in a quarantine facility.

Completing the diagnostic testing in-house instead of relying on commercial vendors allows for faster processing, higher control over the testing process, and faster coordination during a potential outbreak. For samples that are not highly positive, we will confirm results using organism-specific endpoint PCR. Additionally, samples positive by real-time PCR can be evaluated using the endpoint PCR assay and subsequent sequencing to confirm the origin of a positive result. Once the testing protocol is established, we will evaluate different sample types, including the rack filters and cage tops to determine if these are reliable diagnostic targets in our quarantine facility for the detection of mouse fur mites. This testing will be done using known mite positive animals on a rack of naïve animals, so that testing is performed under real transmission conditions.



Myocoptes musculinus on a fur pluck slide. Image courtesy of the Animal Diagnostics Laboratory.



Myocoptes musculinus egg on a fur pluck slide. Image courtesy of the Animal Diagnostics Laboratory.

MATERIALS AND METHODS

Recombinant DNA plasmid insert sequence was selected using GenBank JF834895.1 (*Myobia musculi*) and JF834893.1 (*Myocoptes musculinus*) 18S Ribosomal RNA genes. The plasmids were synthesized by Genewiz (Plainfield, New Jersey) using pUC57-Kan vector sequence. DNA quantity was determined using a SPECTROstar Nano spectrophotometer (BMG Labtech).

Primers and probes were designed using Primer Express Software v2.0 (ThermoFisher Scientific). Primers were synthesized at 25nmole by Integrated DNA Technologies (IDT). All probes were TaqMan minor groove binder (MGB) and synthesized by ThermoFisher Scientific (Cat no. 4316034). All primers and probes were given names based on standard laboratory practice.

Real-time PCR thermocyclers used throughout the project include the Applied Biosystems 7500 (ThermoFisher Scientific) and the Stratagene Mx3000P (Agilent).

All mice used in this experiment were housed at the Frederick National Laboratory for Cancer Research (FNLCR) R&Q facility, located in Frederick, Maryland. All work was done following Institutional Animal Care and Use Committee (IACUC) guidelines. Mouse genomic DNA was purified from mouse lymph node punches taken during complete necropsy, using the QIAamp Mini Kit (Qiagen, Cat no. 51306) with an overnight proteinase K digest designed for tissue lysis. The proteinase K used was included in the kit. The concentration of DNA was determined using the SPECTROstar Nano spectrophotometer (BMG Labtech).

Swabs used for animal and environmental testing were Sterile HydraFlock 6 inch Sterile Standard Flock Swab with Polystyrene Handle, 80mm breakpoint (Puritan Medical, Cat no. 25-3306-H). Pilot study swabs labeled "Mice" were brushed over the mouse's abdomen and back. Swabs labeled "Top" were brushed over the IVC cage top, on the side facing the inside of the cage. Swabs labeled "Cage" were brushed on each of the four walls in the inside of the mouse cage. DNA from all experimental swabs was extracted using the QIAamp DNA Investigator Kit (Qiagen, Cat no. 56504) following the manufacturer's protocol for isolation of DNA from swabs. All incubation steps were carried out using a thermomixer heated to the vendor recommended temperature. Samples were incubated for 3 minutes at 56°C after adding the 20µl of final elution buffer, Buffer ATE, which was included in the kit.

All real-time PCR reactions were completed using FastStart Universal Probe Master with Rox reference dye (Sigma-Aldrich, Cat no. 4914058001) and molecular biology grade water. Final reaction volume was 25µl with a 2µl sample volume. Real-time PCR cycling conditions were initial activation at 95°C for 10 minutes followed by 45 cycles of denaturation at 95°C for 15 seconds and annealing and extension at 60°C for 1 minute. Threshold for FAM and NED dyes was the laboratory standard of 0.1. Threshold for VIC was laboratory standard of 0.01. Coefficient of variation was calculated as the ratio of the standard deviation to the average. CV values 3% or below were considered acceptable, indicating that the data points included in the CV calculation have acceptable variation from the mean. For a standard curve spanning at least 6 orders of magnitude, an acceptable R^2 value was 0.99 or higher, indicated a linear relationship between the Ct value and the log of the plasmid copy number.

All endpoint assays were completed on C/S 1000 thermocyclers (Bio-Rad). The endpoint assays used MyTaq Hot-Start DNA polymerase and associated 5X buffer (Bioline, Cat no. BIO-21113). The final volume of all reactions was 40µl with a 2µl sample volume. Cycling conditions were initial activation at 95°C for 3 minutes followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 1 minute. Cycling was followed by a five minute extension at 72°C. All endpoint PCR products were separated by capillary electrophoresis using a LabChipGX and analyzed using LabChipGX software provided by the vendor. The DNA 5K Reagent Kit (Perkin Elmer, Cat no. CLS760675) was used with a DNA 5K/RNA/Charge Variant Assay LabChip (Perkin Elmer, Cat no. 760435).

RESULTS

Recombinant DNA plasmids were generated for use in assay quantitation and the generation of a quantitative standard curve. The plasmid sequence was selected using the 18S ribosomal RNA gene from each organism. The plasmids were quantified and a copy number was calculated (Table 1).

Table 1. Recombinant DNA plasmids were generated for assay quantitation.

Plasmid name	Detection target	Plasmid DNA concentration (ng/µl)	Calculated copy number (copies/µl)
pMbia	*Myobia musculi* 18S ribosomal RNA gene (JF834895.1)	58.4	1.76×10^{10}
pMcop	*Myocoptes musculinus* 18S ribosomal RNA gene (JF834893.1)	60.5	1.82×10^{10}

Primers and fluorescent probes were selected for the developmental of individual assays for each organism. The same detection target was used. Primers are given an identifier that begins with "YXXX" for reference. Both probes were TaqMan minor groove binder and FAM-labeled (Table 2).

Table 2. Primers and probes selected for developmental of individual assays. Standard laboratory nomenclature of reagents indicated in parentheses.

	Myobia musculi	*Myocoptes musculinus*
Forward primer	CCGGCGATGTATCCTTCAA (Y390)	CTTGTGCCGGCGACATATC (Y398)
Reverse primer	GGCGCATAACCTACCATCGA (Y391)	AGGCATATAACCTACCATCGA AAG TT (Y399)
Probe	6FAM-CGTCTGCCTTATCAAC –MGBNFQ (MbiaPb1)	6FAM-TTCGAGTGTCTGCCTTAT –MGBNFQ (McopPb1)

An initial standard curve was generated using the listed primer and probe concentrations. The PCR reaction contained primers at 0.9µM and a probe at 0.3µM (Table 3).

Table 3. Initial assay conditions to detect *Myocoptes musculinus* using plasmid pMcop and *Myobia musculi* using plasmid pMbia.

		PCR concentration (µM)
Primers	Y398	0.9
	Y399	0.9
Probe	McopPb1	0.3
		PCR concentration (µM)
Primers	Y390	0.9
	Y391	0.9
Probe	MbiaPb1	0.3

Standard curve results were generated using the listed assay setup and appropriate DNA plasmids. Linear regression statistical analysis was performed to evaluate the

relationship between the log transformed copy number and assay Ct value (Table 4; Figure 4; Table 5; Figure 5). The assay for *M. musculinus* shows a linear relationship between Ct value and the plasmid copy number, indicated by the R^2 value of 0.99. Each copy number tested shows reproducibility and a coefficient of variation (CV) value under 3%, a laboratory standard for every real-time assay designed. The assay for *M. musculi* had an R^2 of 0.94, indicating that this data does not show a linear regression, which is defined as 0.99 or higher. The 10 copy point has a CV above 3%, which indicates that the Ct value at this copy number varies too much to be reproducible, even within the same testing event. All other copy numbers tested had a CV below 3%.

Table 4. Results of standard curve for pMcop using Y398 Y399 and McopPb1.

Copy number	log (copy number)	Ct value	Average	Standard deviation	Coefficient of variation
1.00E+06	6	19.08			
1.00E+06	6	18.99	19.11	0.11	0.6%
1.00E+06	6	19.25			
1.00E+05	5	22.66			
1.00E+05	5	22.63	22.64	0.02	0.1%
1.00E+05	5	22.62			
1.00E+04	4	26.26			
1.00E+04	4	25.76	25.97	0.21	0.8%
1.00E+04	4	25.88			
1.00E+03	3	29.54			
1.00E+03	3	29.24	29.24	0.24	0.8%
1.00E+03	3	28.95			
100	2	32.47			
100	2	32.08	32.39	0.22	0.7%
100	2	32.61			
50	1.7	34.01			
50	1.7	33.87	33.79	0.22	0.7%
50	1.7	33.48			
10	1	34.26			
10	1	34.05	34.08	0.14	0.4%
10	1	33.92			
	R^2	0.99			
	Slope	-3.15			
	Intercept	38.37			

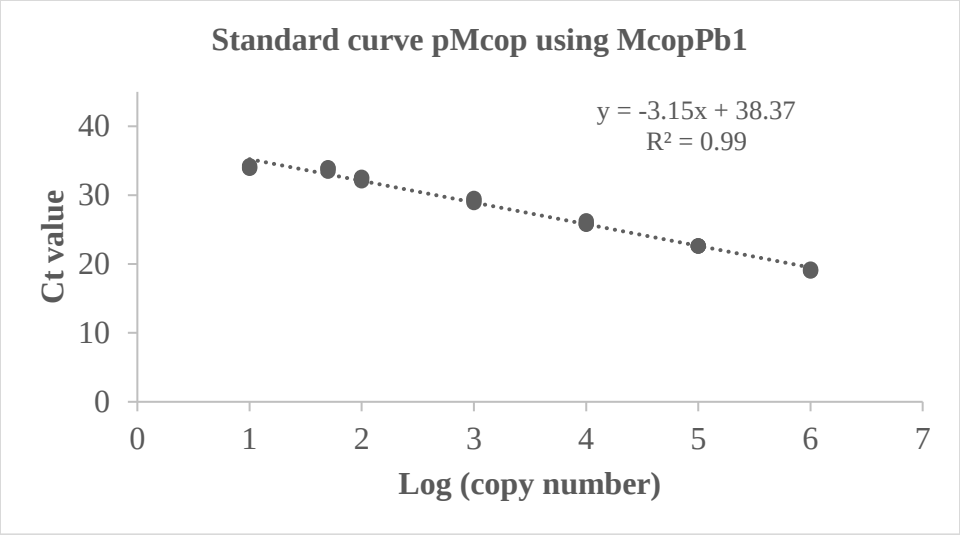


Figure 4. Results of standard curve for pMcop using Y398 Y399 and McopPb1. Linear regression analysis shows a linear relationship between the two variables.

Table 5. Results of an initial standard curve for pMbia using Y390 Y391 and MbiaPb1.

Copy number	log (copy number)	Ct value	Average	Standard deviation	Coefficient of variation
1.00E+06	6	18.43			
1.00E+06	6	18.49	18.50	0.06	0.3%
1.00E+06	6	18.58			
1.00E+05	5	21.97			
1.00E+05	5	21.98	21.94	0.05	0.2%
1.00E+05	5	21.87			
1.00E+04	4	25.09			
1.00E+04	4	25.1	25.14	0.06	0.2%
1.00E+04	4	25.22			
1.00E+03	3	28.53			
1.00E+03	3	28.39	28.41	0.09	0.3%
1.00E+03	3	28.32			
100	2	31.78			
100	2	31.35	31.72	0.28	0.9%
100	2	32.04			
10	1	42.62			
10	1	36.02	37.87	3.39	8.9%
10	1	34.96			
	R^2	0.94			
	Slope	-3.70			
	Intercept	40.21			

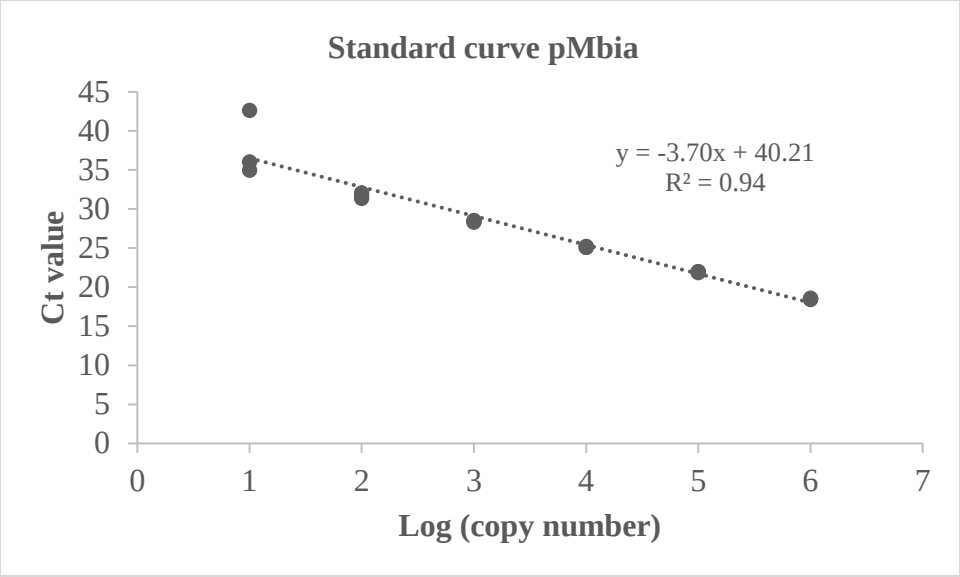


Figure 5. Results of an initial standard curve for pMbia using Y390 Y391 and MbiaPb1. Linear regression analysis shows a linear relationship between the two variables.

A dual detection format allowed both organisms to be detected in a single well. New primers and probes were designed using the 18S ribosomal RNA gene and a conserved probe sequence was determined. The fluorescent probe was FAM-labeled (Table 6). Initial primer and probe concentrations were selected for the generation of a standard curve (Table 7).

Table 6. Dual assay design to detect both fur mites using a conserved probe combined with organism-specific primers.

	Myobia musculi	*Myocoptes musculinus*
Forward primer	CGGAGAGGGAGCCTGAGA (Y453)	CGGAGAGGCAGCCTGAGA (Y51)
Reverse primer	TTATTTTTCGTCACTACCTCCCCGTG (Y454)	TTATTTTTCGTCACTACCTCATCGTT (Y452)
Probe	6FAM-CTACCACATCCAAGGAA –MGBNFQ (FmPb1)	

Table 7. Initial assay to detect *Myocoptes musculinus* and *Myobia musculi* in a dual detection format.

		PCR concentration (µM)
Primers	Y453	0.9
	Y454	0.9
	Y451	0.9
	Y452	0.9
Probe	FmPb1	0.3

Standard curve results were generated using the listed assay conditions and quantified using the plasmid pMcop. Linear regression statistical analysis was performed to evaluate relationship between the log transformed copy number and assay Ct value (Table 8; Figure 6). The assay showed appropriate linear regression, indicated by an R^2 of 1.0. All copy numbers tested show a CV below 3%.

Table 8. Standard curve of dual detection assay using pMcop as the quantitative plasmid.

Copy number	log (copy number)	Ct value	Average	Standard deviation	Coefficient of variation
1.00E+06	6	20.68			
1.00E+06	6	20.36	20.49	0.14	0.7%
1.00E+06	6	20.43			
1.00E+05	5	23.6			
1.00E+05	5	23.78	23.68	0.07	0.3%
1.00E+05	5	23.66			
1.00E+04	4	27.01			
1.00E+04	4	26.87	26.89	0.09	0.3%
1.00E+04	4	26.80			
1.00E+03	3	30.18			
1.00E+03	3	30.09	30.22	0.13	0.4%
1.00E+03	3	30.39			
100	2	33.48			
100	2	33.81	33.65	0.13	0.4%
100	2	33.66			
10	1	36.15			
10	1	36.41	36.21	0.15	0.4%
10	1	36.07			
	R^2	1.00			
	Slope	-3.20			
	Intercept	39.71			

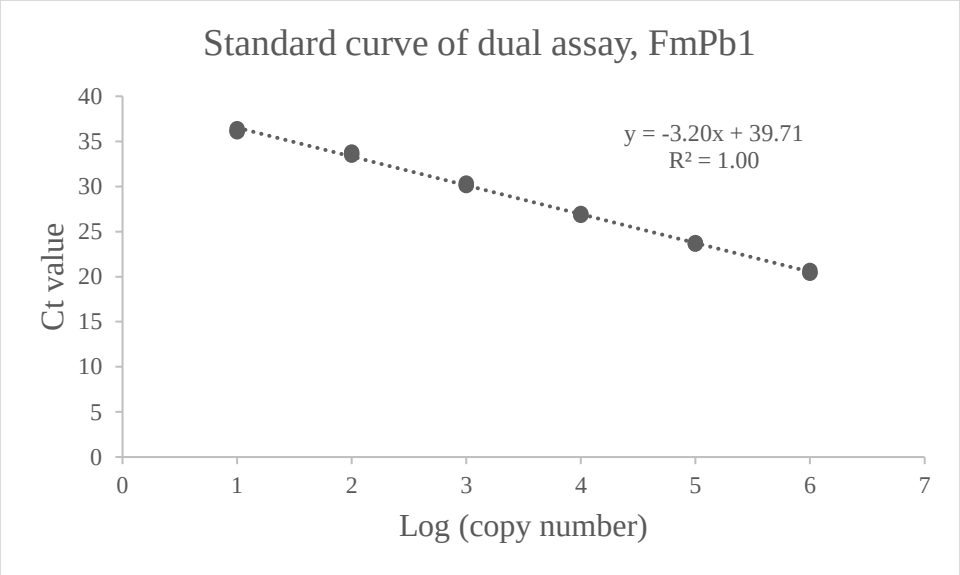


Figure 6. Results of an initial standard curve using pMcop to quantitate the dual detection assay. This assay used primers Y453, Y454, Y451, Y452 and probe FmPb1. Linear regression analysis shows a linear relationship between the two variables.

This assay was evaluated for non-specific amplification with mouse genomic DNA and for inhibition caused by mouse genomic DNA. Mouse genomic DNA is prevalent in the environment at the ADL. If any primers or probes react with mouse genomic DNA in addition to mouse fur mites, the diagnostic results would be compromised. In addition, mouse genomic DNA is in every sample screened for mouse fur mites. It is essential to determine whether the presence of this DNA will inhibit detection of mouse fur mites. Inhibition by genomic DNA would cause a Ct value to be delayed, for example from Ct of 33 to a Ct of 36. This reduces the overall sensitivity of the assay.

Therefore, reactions were evaluated with both plasmid and mouse genomic DNA in the same well. This is completed at copy numbers 1000 and below to screen for reduced sensitivity.

To evaluate, 500ng of purified mouse genomic DNA was added to each reaction and evaluated at copy numbers 1000 and below. The *M. musculinus* primers and probe were evaluated first separately and then the entire dual detection assay was evaluated (Table 9). The *M. musculinus* primers and conserved probe alone showed no difference in Ct value with or without mouse genomic DNA. However, when the *M. musculi* specific primers were added to the reaction to achieve dual organism detection, there is a greater than 10 Ct value difference at the same plasmid copy number. This indicates amplification of non-specific targets. The no template control of this PCR reaction, which just includes assay reagents and molecular biology grade water, also amplified.

Table 9. Comparison of plasmid amplification with and without mouse genomic DNA.

Myocoptes musculinus primers + FmPb1

Copy number	log (copy number)	Ct value	Ct + DNA
1.00E+03	3	30.22	30.13
100	2	33.65	33.77
10	1	36.21	37.39

Dual detection primers (Y451 Y452 Y453 Y454) + FmPb1

Copy number	log (copy number)	Ct value	Ct + DNA
1.00E+03	3	29.93	13.80
100	2	33.17	13.61
10	1	35.40	14.22

Mouse genomic DNA is present in all sample types used in the animal facility to screen for mouse fur mites. This eliminates the primer set Y453 Y454 for *M. musculi* as an option for inclusion into the dual detection assay. New primer sets were designed (Table 10) and evaluated first for amplification and limit of detection (Table 11). Forward primer

Y457 was evaluated with three different reverse primers. These primers were outside of the insert sequence for the original plasmid pMbia. A new recombinant DNA plasmid was generated and named pMyob. All sequence information for both the primers and the plasmid insert sequence was based on the *Myobia musculi* 18S ribosomal RNA gene.

All three primer sets amplified the plasmid, with different limits of detection. However, no primer set was eliminated at this stage as they had yet to be evaluated for non-specific amplification of mouse genomic DNA.

Table 10. Sequences of proposed primers to include in the dual detection format to detect *Myobia musculi*. Y457 is a forward primer and the remaining are reverse primers, generating three unique combinations to evaluate.

Y457	GTTATGCGCCTACCATGGTTGTA
Y458	CGGGATTGGGTAATTTGCGT
Y459	CGTCACTACCTCCCCGTGC
Y460	TTTTGCTCACTACCTCCCCGT

Table 11. Comparison of three *Myobia musculi* primer sets for use in the dual detection assay. These results used pMyob, FmPb1 and then each unique primer pair. No primer pair was eliminated at this stage of testing.

Copy number	log (copy number)	Ct value		
		Y457 Y459	Y457 Y458	Y457 Y460
1.00E+06	6	20.77	20.87	20.45
1.00E+05	5	23.9	23.68	23.77
1.00E+04	4	27.03	27.43	27.17
1.00E+03	3	30.42	30.62	30.31
1.00E+02	2	33.62	34.33	33.41
1.00E+01	1	Undetermined	43.48	37.51

Each primer set was evaluated for non-specific amplification of mouse genomic DNA or inhibition by mouse genomic DNA at copy numbers 1000 and below of pMyob

(Table 12). This was completed using FmPb1, the probe that detects both fur mites. Primer set Y457 Y460 shows inhibition at the lowest copy level of greater than 5 Ct values. Y457 Y459 had the strongest amplification at the 30 copy level, and for this reason was selected to be included in the dual detection assay.

Table 12. Evaluation of three new sets of primers for *Myobia musculi* to be used for the dual detection assay format. Primer set Y457 Y460 shows inhibition at the 30 copy level with mouse genomic DNA included in the reaction. Y457 Y459 shows the strongest amplification at the 30 copy level and was chosen as the primer set to include in the dual assay detection format.

	Y457 Y458		Y457 Y459		Y457 Y460	
Copy number	Ct value	Ct + DNA	Ct value	Ct + DNA	Ct value	Ct + DNA
1.00E+03	30.08	30.09	30.40	30.77	30.13	30.98
1.00E+02	33.18	35.62	34.37	33.44	33.47	33.80
30	38.10	35.89	36.39	35.60	34.51	40.28

Primer set Y457 Y459 to detect *M. musculi* was added to the existing Y451 Y452 to detect *M. musculinus* using the conserved probe FmPb1, which detects both organisms. This dual detection assay combination was evaluated for non-specific amplification of mouse genomic DNA and for inhibition (Table 13). No non-specific amplification was seen. This detection format progressed to further quantification.

Table 13. Dual assay detection with new *Myobia musculi* primers, mouse genomic DNA comparison.

Y451 Y452 Y457 Y459 FmPb1

Copy number	Ct value	Ct value + DNA
1.00E+03	29.19	29.84
1.00E+02	31.48	32.7
30	33	33.46

To establish quantitative standard curves for this new dual assay detection, standard curves were completed using pMcop (Table 14) and pMyob (Table 15). Linear regression was evaluated using statistical analysis (Figure 7). Using the dual detection assay, neither plasmid showed an ideal linear regression relationship, indicated by the R^2 below 0.99. The pMyob standard curve at 30 copies of plasmid had a CV higher than 3%, with all other copy numbers under 3%.

Table 14. Dual assay detection standard curve of pMcop.

Copy number	log (copy number)	Ct value	Average	Standard deviation	Coefficient of variation
1.00E+06	6	19.57			
1.00E+06	6	19.45	19.35	0.23	1.2%
1.00E+06	6	19.04			
1.00E+05	5	22.8			
1.00E+05	5	22.72	22.70	0.09	0.4%
1.00E+05	5	22.58			
1.00E+04	4	23.06			
1.00E+04	4	26.15	24.97	1.36	5.5%
1.00E+04	4	25.69			
1.00E+03	3	29.05			
1.00E+03	3	28.74	29.09	0.30	1.0%
1.00E+03	3	29.48			
100	2	32.73			
100	2	31.52	32.09	0.50	1.5%
100	2	32.03			
30	1.5	33.78			
30	1.5	33.74	34.26	0.71	2.1%
30	1.5	35.27			
	R^2	0.98			
	Slope	-3.267			
	Intercept	38.772			

Table 15. Dual assay detection standard curve of pMyob.

Copy number	log (copy number)	Ct value	Average	Standard deviation	Coefficient of variation
1.00E+06	6	19.96			
1.00E+06	6	20.07	20.22	0.29	1.5%
1.00E+06	6	20.63			
1.00E+05	5	23.04			
1.00E+05	5	23.16	23.25	0.21	0.9%
1.00E+05	5	23.54			
1.00E+04	4	26.21			
1.00E+04	4	27.08	26.66	0.36	1.3%
1.00E+04	4	26.7			
1.00E+03	3	28.92			
1.00E+03	3	29.01	29.19	0.32	1.1%
1.00E+03	3	29.65			
100	2	33.39			
100	2	33.59	33.71	0.32	1.0%
100	2	34.15			
30	1.5	35.00			
30	1.5	34.5	36.00	1.77	4.9%
30	1.5	38.49			
	R^2	0.97			
	Slope	-3.4414			
	Intercept	40.4904			

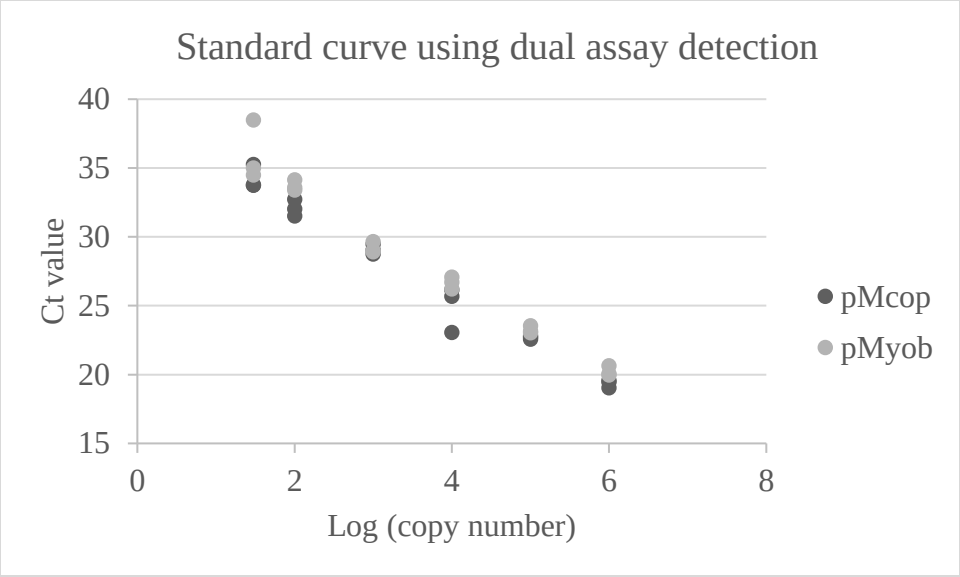


Figure 7. Standard curves using dual assay detection for pMcop and pMyob.

To evaluate the possibility of non-specific amplification with mouse genomic DNA, swabs were taken from laboratory mice that were screened negative for fur mites by PCR from a commercial vendor. Fifty mice were swabbed all over their skin and hair and then DNA was purified from each swab. This was tested using the established dual detection format. The results showed that 58% of swabs had some level of non-specific amplification. This indicates that the dual detection assay, even with re-designed *M. musculi* primers is not suitable for screening mice for fur mites.

The original single detection assay for *M. musculi* was used to screen the first eight swabs that had been tested in the group of 50 animals negative for mouse fur mites. With the dual detection assay, five of these eight swabs had some non-specific amplification. Using the single detection format detecting only *M. musculi*, none of these swabs had non-specific amplification. This result indicated that the primers and probe used initially in the single detection screen for *M. musculi* were more specific than those included in the dual

detection format. The initial goal of the dual detection format was to detect both mites with one FAM-labeled probe. After these results, another dual detection format was sought, where each organism had a distinct probe label. This would still detect both organisms in a single well, with a unique fluorescent probe label for each organism. *M musculinus* was kept FAM-labeled and a standard laboratory inhibition screen is VIC-labeled, which was scheduled to be added to this assay before it is used to do live screening. Therefore, various dyes for probes were evaluated for their ability to multiplex with FAM and VIC, with the ultimate goal of a three dye detection multiplex assay. Dyes NED and TET had emission and excitation values that did not indicate interference with FAM and VIC. All three dyes would also coincide with the light filters already available on existing lab real-time PCR thermocyclers.

Therefore, the initial probe MbiaPb1 was ordered with the same nucleic acid sequence but one probe TET-labeled and one probe NED-labeled. Standard curves using each probe were generated using *Myobia musculi* specific primers and probe (Table 16).

Table 16. Primer and probe concentrations used to generate standard curves for NED and TET labeled pMbiaPb1 probe.

		20X mix concentration (µM)	PCR concentration (µM)
Primers	INTC-F	10	0.5
	INTC-R	10	0.5
Probe	INTC-PV	4	0.2

Both probes amplified the associated plasmid pMbia, however the TET-labeled probe showed some amplification using the VIC dye channel. This would interfere with adding the laboratory standard inhibition screen, which uses a VIC-labeled probe. For this reason, the NED-labeled probe was selected to include in the multiplex assay.

An additional probe would be included in the multiplex assay, which is an internal control probe that detects plasmid pINTC. This is a laboratory standard internal control reaction that evaluates each reaction well for inhibition. It was developed previously and is used in all real-time PCR reactions within the ADL. The appropriate volume of pINTC is added to the reaction master mix so that each well receives 50 copies of plasmid. This plasmid is then detected by specific primers and probes (Table 17). A 20X concentrated mix of the primers and probes is generated for standard use and 1.25µl of the 20X mix is used in a standard 25µl PCR reaction (Table 18).

Table 17. Primer and probe sequences for the internal control assay.

	Internal control
Forward primer	GTC CGG CGT ACC TGT TGA TT (INTC-F)
Reverse primer	GTG ATC TAA ACA ATG GCC GTT TC (INTC-R)
Probe	VIC-AAA TTC TTG GAT TCT CTC TGA C- MGBNFQ (INTC-PV)

Table 18. Concentrations of the internal control assay primers and probe in a standard laboratory reaction.

		PCR concentration (µM)
Primers	INTC-F	0.5
	INTC-R	0.5
Probe	INTC-PV	0.2

A final version of this proposed multiplex assay to detect both fur mites would include a FAM-labeled probe (*M. musculinus*), a NED-labeled probe (*M. musculi*), and a VIC-labeled probe (internal control assay detecting plasmid pINTC). With three differentially labeled probes, dye competition was evaluated in reaction wells including both detection plasmids. This experiment simulates if a sample was positive for both mites and shows if there is competition between the dyes, most importantly at copy numbers 1000 and below (Table 19). Results show no competition between the dyes, indicated by a CV of less than 3% at copy numbers 1000 and below.

Table 19. Evaluation of dye competition in reactions with both plasmids pMbia and pMcop at the listed copy numbers to simulate a sample with dual infection. Results show no competition between the dyes, indicated by a CV of less than 3% at all points 1000 copies and below.

Myobia musculi

Copy number	NED only	NED + FAM	NED/FAM + VIC	CV
1.00E+06	19.98	19.62		
1.00E+05	23.32	22.97		
1.00E+04	26.61	26.3		
1.00E+03	29.87	29.55	28.51	2.0%
100	33.3	32.95	31.73	2.1%
10	36.33	36.61	35.09	1.8%

Myocoptes musculinus

Copy number	FAM only	NED + FAM	NED/FAM + VIC	CV
1.00E+06	18.98	18.78		
1.00E+05	22.35	22.16		
1.00E+04	25.62	25.42		
1.00E+03	28.92	28.66	29.29	0.9%
100	32.4	31.88	32.75	1.1%
10	35.97	35.33	36.17	1.0%

A 20X mix was generated for the FAM/NED fur mite detection assay (Table 20). This concentrated mix that includes all primers and probes necessary for mouse fur mite detection is used in the lab for fast and efficient PCR reaction setup, reducing the number of pipetting steps required.

Table 20. Prepared Fur Mite 20X concentration for use in standard laboratory reactions.

Component	Concentration in 20X mix (µM)	PCR concentration (µM)
Y398	12	0.6
Y399	12	0.6
Y390	12	0.6
Y391	12	0.6
McopPb1 (FAM)	6	0.3
MbiaPb1 (NED)	6	0.3

Non-specific amplification with mouse genomic DNA was evaluated using all three dyes, the FAM/NED 20X mix and the VIC internal control 20X mix. This assay showed no amplification of mouse genomic DNA.

The stability of the fur mite 20X mix was evaluated to determine how long it can be stored at 4°C (Table 21). The CV for each copy number tested was below 3%, except the *M. musculi* assay at 10 copies. Prepared mix is stable for over one month.

Table 21. Evaluation of stability of fur mite 20X mix. Prepared mix is stable for over one month, as seen by a coefficient of variation under 3% for all points 1000 copies and below.

Myobia	initial	+ 6 days	+22 days	+36 days	CV
1.00E+03	29.29	29.67	29.99	29.18	1.1%
100	32.75	32.92	33.27	32.30	1.1%
10	36.17	35.84	36.92	33.33	3.8%

Myocoptes	initial	+ 6 days	+22 days	+36 days	CV
1.00E+03	28.51	28.68	29.26	29.24	1.2%
100	31.73	31.88	32.50	32.26	1.0%
10	35.09	34.69	36.81	35.99	2.3%

Endpoint assays were designed using primers within the pMcop and pMbia plasmid insert sequences (Table 22). Endpoint PCR assays are used for confirmation of equivocal results, which are defined as any Ct value between the positive control and 40 cycles, as determined by laboratory standard. Endpoint assays can also be used to generate a PCR product from a positive sample for sequencing.

Table 22. Sequences of all primers evaluated for fur mite endpoint assays.

Primer name	Sequence
Y435	CCG AAA AGC CTC ATC GTA AG
Y436	ACC CTG ATT CTC CGT TAC CC
Y437	AAG GTG GCA ACA CCT AAT GG
Y438	CTT CCT TGG ATG TGG TAG CC
Y439	GGG GAG GTA GTG ACG AAA AA
Y440	AGA CAA ATC GCT CCA CCA AC
Y441	TCA AGC GTC TGC CTT ATC AA
Y442	GGC CTC AAT AGA GTC CCG TA

Each endpoint assay was designed to detect only one organism. Primer sets Y435 Y436 and Y437 Y438 were evaluated for detection of pMcop for *Myocoptes musculinus* (Figure 8). The primer set Y435 Y436 was selected for further testing. This primer set was then evaluated with additional PCR cycles, still at a 60°C annealing temperature, done twice on different days to show reproducibility (Figure 9). The limit of detection for this assay was determined to be 15 copies.

Figure 8. Two primer sets were evaluated for use in an endpoint assay to detect *Myocoptes musculinus*. This was done using a 60℃ annealing temperature with 35 cycles. The primer set Y435 Y436 was selected for further testing because it amplified stronger at the 100 copy level.

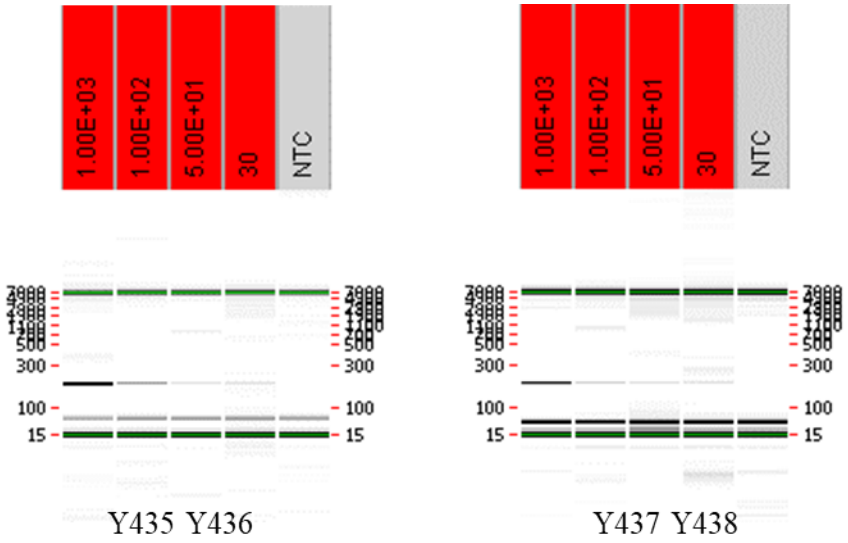
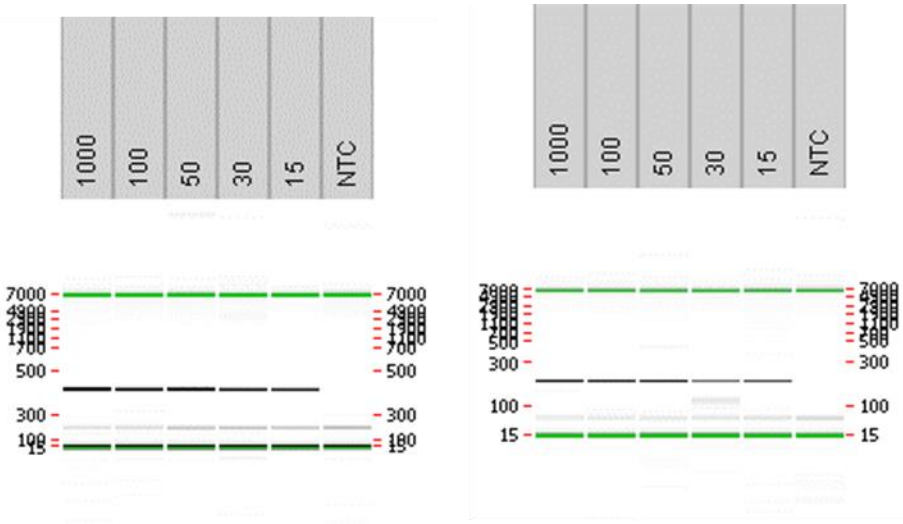
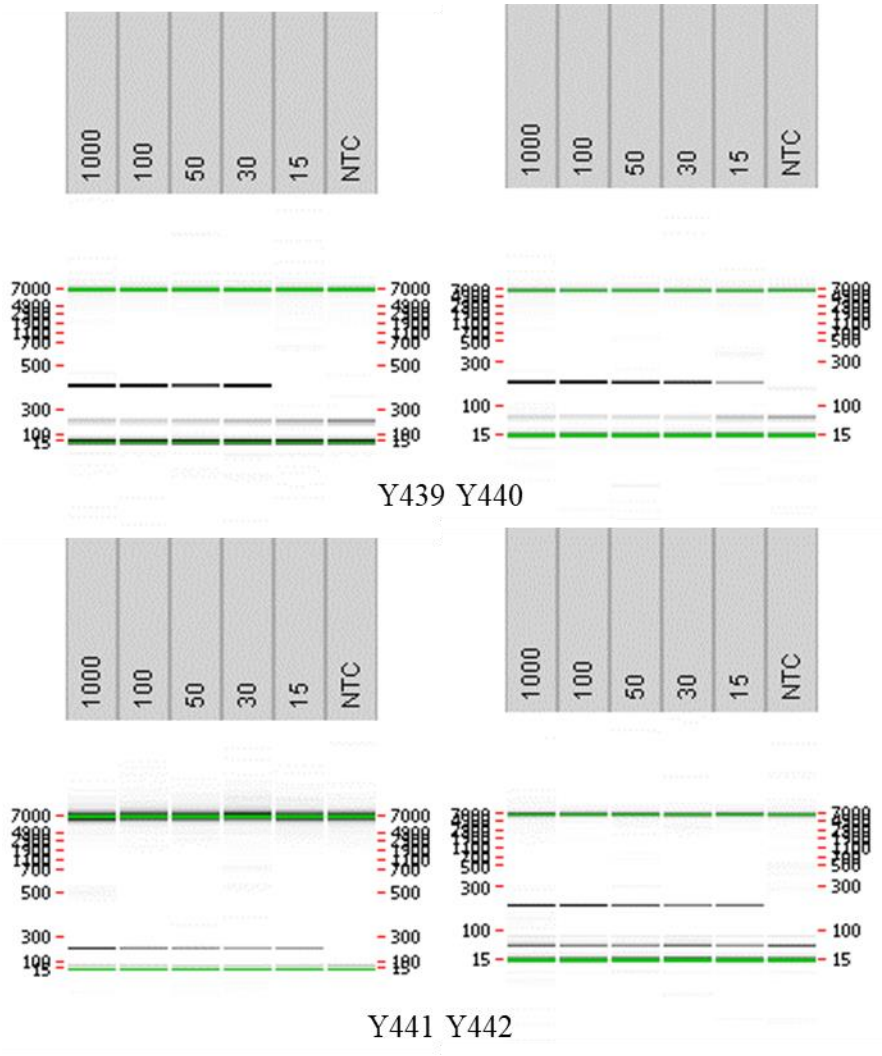


Figure 9. Primers Y435 Y436 were used to amplify pMcop using a 60℃ annealing temperature and increased to 40 cycles of PCR. These two images show two separate experiments, done on different days. This assay has a limit of detection of 15 copies.



Two primers sets were evaluated for *Myobia musculi* using a 60℃ annealing temperature and 40 PCR cycles (Figure 10). Both assays showed a limit of detection of 15 copies.

Figure 10. Primers Y439 Y440 and Y441 Y442 were screened for use in a M*yobia musculi* endpoint assay. Both primer sets amplified down to 15 copies in separate experiments on different days, showing reproducibility.



Animals positive for *Myocoptes musculinus* were imported to the R&Q facility after the completion of development of the fur mite assay, providing an opportunity for a pilot study (Table 23). The identity of the species of fur mite was not known to the author at the initial time of testing, although the tape test and subsequent visual inspection of the animals in R&Q identified the mites as *M. musculinus*. The assay results indicated *M. musculinus*, confirming the species identification from traditional detection methods. Some samples

were also evaluated using the endpoint assay for *M. musculinus*, adding an additional level of specificity. In cages where the direct swabs of mice detected a positive, the assay was able to detect all of them via environmental targets by 10 days post cage change. It detected fur mites in 50% of the environmental targets at four days post cage change.

Table 23. Mice known positive for *Myocoptes musculinus* were evaluated in a pilot study. Animals were moved to clean cages and swabs were taken of the animal's skin, the internal cage walls, and the filter top of the cage at various time points post cage change. A blank space indicates that this sample was not tested. Undet. indicates that the sample was tested but showed no Ct value. Pre-cage change results indicate before dirty bedding was cleaned out and a clean cage and bedding were provided for the mice in the experiment. All samples passed the internal control VIC assay, with Ct values between 32-35, the laboratory standard.

Sample name	Pre-cage change	+ 1 day	+ 2 days	+ 3 days	+ 4 days	+ 10 days
1 Mice	Undet.	38.32	25.88	26.49	34.05	25.94
1 Cage					Undet.	Undet.
1 Top					Undet.	Undet.
1B Mice	32.56	26.85	30.32	27.56	29.41	32.62
1B Cage	33.55	34.60	Undet.	31.65	32.73	Undet.
1B Top	27.97	36.47	36.38	37.24	36.99	31.28
1C Mice	34.34	40.80	35.56	34.16	33.45	36.69
1C Cage	36.52	Undet.	Undet.	35.38	37.50	Undet.
1C Top					Undet.	Undet.
2 Mice					Undet.	36.81
2 Cage					Undet.	Undet.
2 Top	31.78	Undet.	Undet.	Undet.	35.64	40.46
1A Mice					Undet.	27.41
1A Cage					Undet.	35.16
1A Top					Undet.	34.87

DISCUSSION

Mouse fur mites are not detected accurately with non-molecular methods and are not detected reliably via sentinel bedding transfer. This project aimed to design a real-time PCR assay to detect both species *M. musculi* and *M. musculinus*.

Due to non-specific amplification present after the design of an assay with a single, conserved fluorescent probe that could detect both mites, an alternate detection method was sought. The real-time PCR assay consisted of two species-specific probes, each labeled with a unique fluorescent reporter, either FAM or NED. These two individual assays were then evaluated in a multiplex reaction format, where both tests could be added to the same well for parallel detection. In addition, an internal control assay used within the ADL that has a VIC-labeled fluorescent probe was added to complete the reaction mix. All three targets were able to be detected in a single well and showed no competition, which would reduce assay sensitivity. Both organisms can be detected with a limit of detection at 10 copies or below. Another benefit to the final assay design is that any positive diagnostic result can be reported as a species after the real-time PCR test is complete. This is the downside to a conserved probe that detects both species, because the species identity can only be determined by another assay.

Reproducibility of the assays designed was tested by running the same experiment on different days and with different reagents and plasmid dilutions. These tests confirm that the assays designed are reproducible over time.

The ADL is currently able to receive samples for mouse fur mites and detect and identify the species as quickly as the next business day. While the development of this test

was successful, no facility sees zero outbreaks per year. There is no perfect diagnostic method. However, the ability to quickly provide diagnostic results is an essential capability for identifying and quickly stopping a breach from an excluded organism. The lab has overseen the containment of at least two small outbreaks since the generation of this test, and the outbreaks were quickly contained due to efficient diagnostic testing. Positive animals were quickly separated and euthanized based on results, which prevents the infection from further spread. These live scenarios provided additional confidence in the reliability and efficiency of the test itself, as there is no better evaluation than a live outbreak scenario.

The fur mite real-time assay was the first three dye multiplex test completed within the ADL. The evaluation of the combination of FAM/VIC/NED dyes was an important milestone for the diagnostic program overall. This combination of dyes showed no competition or reduced sensitivity when used in the same reaction well, and they work within light filters that are pre-calibrated on all the existing thermocyclers in the lab. It has the added benefit of allowing for species identification using the real-time PCR results. Other diagnostic tests used within the ADL are considered universal, meaning they may detect all viruses of a specific kind. While there are benefits to this detection format, to identify the specific species of a positive result requires a different test to be completed. This requires extra time and resources to be able to report a species to the facility. The fur mite real-time assay serves as a proof of concept for future development that can detect two distinct species within the same reaction well.

The amplification of mouse genomic DNA by the dual detection assay with a single conserved probe proved to be a challenge throughout the development of the fur mite real-

time PCR assay. After the design of these primers and probe and before ordering, all sequences were subjected to the National Center for Biotechnology Information's (NCBI) Basic Local Alignment Search Tool (BLAST) to screen for alignment with any other species, including mouse. However, virtual tools such as BLAST can't replace testing in the lab with real sample material. BLAST will provide sequences with 100% match to a given set of primers or a probe. During a real-time PCR reaction, even a template with one or two mismatches can amplify at a low efficiency and still be seen when evaluating results. There is no replacement for testing primers and probes using true sample material to confirm specificity of the test as early as possible in the developmental process. Challenging the assay for specificity early allows for a redirect with different primer and probe sequences, as was seen in this experiment. Testing for specificity by using mouse genomic DNA and skin swabs of clean mice served as essential developmental tools to test the assays designed in this project.

Endpoint PCR assays were also designed as confirmatory tests for equivocal samples or for generation of a product to sequence. Both endpoint PCR assays have a limit of detection at 15 copies, providing a sensitive screen for mouse fur mites.

Shortly following the development of all the PCR assays to detect mouse fur mites, animals positive for *M. musculinus* were imported into the R&Q facility, providing an opportunity for a pilot study to evaluate what sample types could be used effectively. In cages where direct swabs from the mice were positive, the real-time PCR assay detected *M. musculinus* in 100% of environmental targets by 10 days post cage change. This is an additional milestone, as it shows that environmental targets such as the top of an IVC cage or the cage walls can be used effectively to detect mouse fur mites within 10 days of

receiving the animal into R&Q. Environmental targets are desired because they are faster and less expensive to sample than individual animals. They also provide a method of testing where scale would prohibit individual animal testing.

Future work regarding the detection of mouse fur mites should include a larger pilot study with additional controls. In this pilot study, no negative control mice were included, which weakens the overall conclusions. Additionally, not all data points were tested, which may not give a complete picture of detection capability at every time point. The ability to design a pilot study within the facilities is limited by space to house animals and availability of animal care staff.

Future work for the development of tests within the ADL includes increased focus on multiplex assay development as well as improved environmental detection. For any parasite, virus, or bacteria that the ADL already has a PCR assay to detect, ideas to make them more efficient are of the utmost importance. Multiplex assays are typically cost and time-saving, as they require fewer overall reactions and a single test run. This is a constant goal within the ADL, to provide fast, accurate, and reasonably priced diagnostic services to investigators at the NCI. Pilot studies evaluating the efficacy of environmental targets such as swabs of animal racks, cages, cage tops, filters, walls, and equipment continue to be a focus within the ADL. These are often completed using assays that are already designed, simply to confirm the reliability of detecting environmental targets using existing PCR testing technology.

Future work will also include development of assays for new and emerging organisms of concern. Within laboratory animal science, new organisms can emerge with

significant clinical symptoms, especially from immunocompromised animals. Additionally, mice that are humanized may be susceptible to human pathogens or could potentially carry and spread them to animal staff. These are all essential considerations in the future of the animal diagnostic program.

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