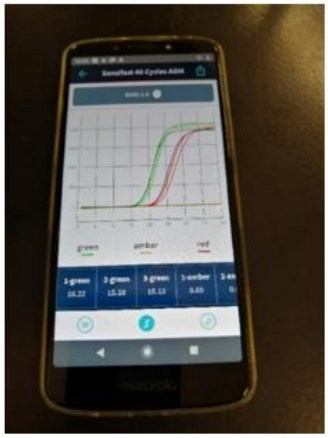

Asian Spongy Moth (ASM) TaqMan® Detection Manual

On-site detection from intercepted egg mass samples

May 2022

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Asian Spongy Moth (ASM) Portable Assay

Introduction

We have developed a portable assay for the detection of Asian spongy moth (ASM) from egg mass samples intercepted on at-risk marine vessels. This is a robust TaqMan® assay system that has been extensively validated. The assay system was developed in collaboration with Precision Biomonitoring (now NatureMetrics), a Guelph, Ontario-based company that specialises in point-of-use DNA detection technologies.

Assays developed earlier and reported in previous publications (1, 3) were adapted for use on the Biomeme Franklin™ three9 portable real-time PCR thermocycler, which is capable of multiplex real-time detection of up to three targets (triplex reaction) for as many as nine samples in a single run.

Our portable assay system consists of a single triplex reaction ("Triplex 1") targeting three different mitochondrial marker genes: 1-*cytochrome c oxidase* (COI) in its barcode region (5P COI), 2- *cytochrome b* (cytb) and 3- *NADH-ubiquinone oxidoreductase chain 1* (ND1). It will identify moths from the ASM complex (*L. dispar asiatica, L. dispar japonica* and *L. umbrosa*).

The cytb assay will also determine whether the unknown is a recently discovered cryptic Chinese ASM moth that features an ESM-like COI sequence.

Abbreviations: ASM - Asian spongy moth, ESM - European spongy moth (*Lymantria dispar dispar*), Lumb - *Lymantria umbrosa*, Ldaj - *Lymantria dispar asiatica* or *Lymantria dispar japonica*.

Note: Asian spongy moth (ASM) was previously referred to as Asian gypsy moth (AGM) and European spongy moth (ESM) was previously referred to as European gypsy moth.

Materials and methods

The portable assay procedure consists of two steps: 1) the collection of an egg mass, followed by rapid, crude DNA extraction, and 2) a single triplex TaqMan® reaction to detect the presence of Asian spongy moth. The entire process can be completed in approximately two hours.

TaqMan® assays

PCR is performed with the Biomeme Franklin™ three9 portable real-time PCR thermocycler. The Biomeme turns your smartphone into a thermocycler computer for real-time PCR analysis. It is battery-operated for maximum portability, allowing users to do a full day's work in the field on a single charge. The thermocycler has three separate channels, allowing for simultaneous detection of three targets in a single tube. PCR results may be analyzed on site by a field inspector or sent to the diagnostics laboratory by uploading the run results to the Biomeme website cloud.

Figure 1: The Biomeme Franklin™ three9 portable real-time PCR thermocycler.

The ASM portable assay (Triplex 1) consists of three TaqMan® subassays grouped together into a single triplex reaction. The TaqMan® subassays are designed to discriminate all Lymantriinae egg mass samples that are difficult to distinguish from ASM eggs (see Appendix D for list). A flowchart describing the assays can be found in Appendix C. All sequence alignments on the basis of which the assay was designed can be found in Supplementary file 1. The assay is organized as follows:

Triplex 1 (ASM)

1. ASM complex COI assay (1A) – This assay will detect *L. dispar asiatica, L. dispar japonica* and *L. umbrosa*.
2. Ldaj cytb assay (1B) – This assay will confirm the ASM complex result (1A). A positive result will give the designation of *L. dispar asiatica/japonica* and a negative result *L. umbrosa*. This assay will also detect a *Chinese ASM variant undetectable by the ASM complex COI assay (or the classic NB restriction enzyme system).
3. Lumb ND1 assay (1C) – This assay will confirm the ASM complex result (1A). A positive result will give the designation of *L. umbrosa* and a negative result *L. dispar asiatica/japonica*.

*Chinese ASM variant with ESM-like COI sequence: any sample that tests negative for the ASM complex COI subassay (1A) but produces a positive result for the Ldaj cytb TaqMan® subassay (1B), will be given the designation of Chinese ASM variant.

Positive controls

Double stranded gBlocks™ gene fragments are used as positive controls for the TaqMan® assays in the triplex reaction. (Integrated DNA Technologies, Coralville, IA, USA). gBlocks™ gene fragments are used at 50 fg per reaction. For Triplex 1A (ASM COI) and 1B (Ldaj cytb), an *L. dispar asiatica* COI-cytb gene fragment is used and for Triplex 1C (Lumb ND1), an *L. umbrosa* COI-ND1 gene fragment is used. *L. dispar asiatica* and *L. umbrosa* gBlocks™ contain concatemerized gene fragments for simplicity's sake. For more detailed information on the gBlocks™ sequences, see Appendix A.

DNA extraction

Eggs from intercepted egg masses are homogenized using a micro tube homogenizer system (Fisher Scientific, cat. no. 03-421-227). The system consists of a 1.5 mL tube and micro pestle. It is important to have a tube/micro pestle combination that fits well to ensure proper homogenization of the sample. DNA is extracted using the ARCIS DNA sample prep kit (ARCIS Biotechnology, cat. no. UFL002). 2-3 eggs from an intercepted egg mass are transferred to a 1.5 mL tube with 100 µL of Reagent 1. The sample is ground with a micro pestle to crush the eggs and release the nucleic acids. The sample is incubated at room temperature for approximately one minute, and then 20 µL is transferred to a separate 1.5 mL tube containing 20 µL of Reagent 2 (1:1 ratio mixture of the two reagents). The tube is flicked to mix the sample and then the mixture diluted 20x with 760 µL H_2O. 20 µL is used for PCR with a lyophilized master mix in the Biomeme Franklin™ three9 portable thermocycler.

Note: DNA may also be extracted from moth legs or antennae using the above protocol. The user may pool egg mass or leg samples if desired (up to 10 per tube).

Figure 2: Left - field inspectors collect a suspect egg mass discovered on an at-risk marine vessel. Middle – magnified view of an intercepted egg mass. Right - micro tube homogenizer system.

PCR conditions

The PCR conditions for the Biomeme Franklin™ can be seen in Table 1. Pre-prepared strip tubes contain lyophilized master mix, primers and probe (see Appendix B for detailed information - Preparation and lyophilization of qPCR mastermix). 20 µL of DNA from the DNA extraction step is added to each tube along with a drop of mineral oil to prevent evaporation of the reaction mix during PCR. The number of technical replicates per egg mass sample can be adjusted from 1-3 depending on the number of samples to test. Two wells should be reserved for positive (gBlocks™ gene fragments) and negative controls.

Table 1: PCR conditions for the TaqMan® ASM assay.

Stage	Number of Cycles	Temperature	Time
Enzyme activation	1	95°C	5 minutes
Denaturation	40	95°C	10 seconds
Annealing/Extension		60°C	45 seconds

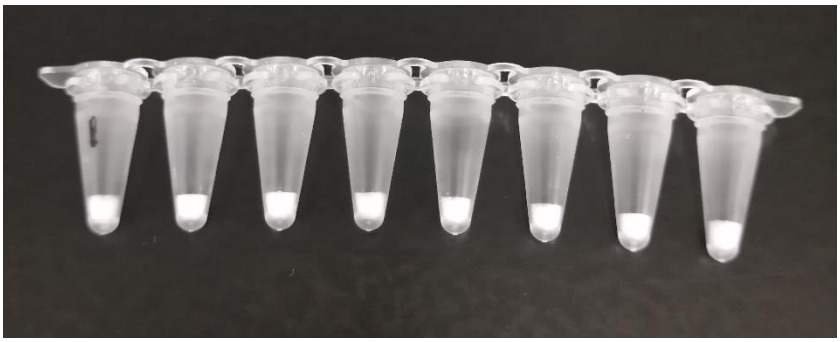


Figure 3: Ready-to-use lyophilized mastermix in Applied Biosystems MicroAmp fast reaction tubes (8 tubes/strip) with optical 8-cap strip.

Results

The output of a Biomeme Franklin™ three9 run can be seen in Figure 4. Results are easy to interpret. In this case, the green curve is the outcome of a positive ASM complex COI TaqMan® assay and the red curve, a positive Lumb ND1 TaqMan® assay.

A more in depth analysis of results can be performed by uploading the qPCR run to the Biomeme website cloud (Figure 5). For example, threshold values can be adjusted for Ct values.

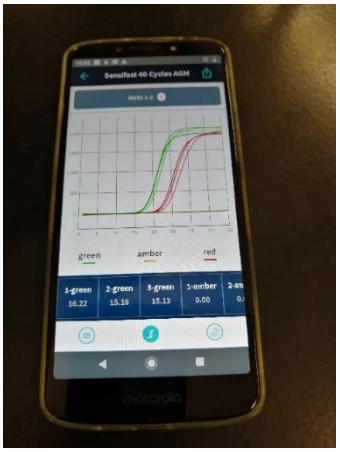


Figure 4: A qPCR result from the Biomeme Franklin™ three9. The thermocycler sends the results in real time to a smartphone via a Bluetooth® connection.

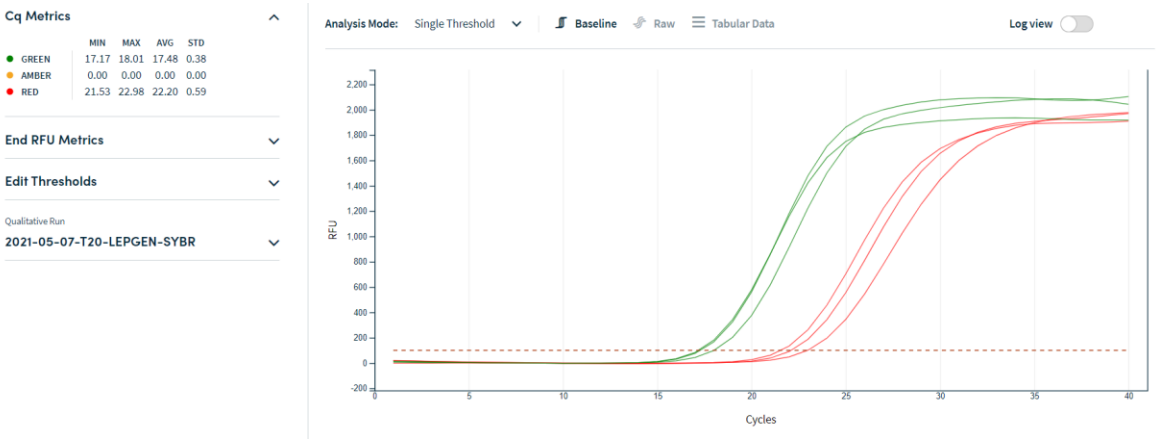


Figure 5: Results from Figure 4 following transmission to the Biomeme website cloud. The fluorescent threshold was set to approximately 10% of Fmax to determine Ct values.

Summary

The procedure described here is an easy-to-use, robust TaqMan® assay, generating results that are easy to interpret.

Acknowledgements

We thank Y. Wu (USDA-APHIS), A. Naum (NatureMetrics) and A. Capron (University of British Columbia) for their roles in the development of the ASM portable assay.

References

1. Stewart D, Zahiri R, Djoumad A, Freschi L, Lamarche J, Holden D, Cervantes S, Ojeda DI, Potvin A, Nisole A, Béliveau C, Capron A, Kimoto T, Day B, Yueh H, Duff C, Levesque RC, Hamelin RC, Cusson M (2016) A Multi-Species TaqMan® PCR Assay for the Identification of Asian spongy moths (Lymantria spp.) and Other Invasive Lymantriines of Biosecurity Concern to North America. *PLoS One* **11(8):** e0160878. https://doi.org/10.1371/journal.pone.0160878.

2. Djoumad A, Nisole A, Zahiri R, Freschi L, Picq S, Gundersen-Rindal D, Sparks M, Dewar K, Stewart D, Maaroufi H, Levesque RC, Hamelin RC, Cusson M (2017) Comparative analysis of mitochondrial genomes of geographic variants of the gypsy moth, *Lymantria dispar*, reveals a previously undescribed genotypic entity. Scientific Reports, **7**: 14245. https://doi.org/10.1038/s41598-017-14530-6

3. Stewart D, Nisole A, Djoumad A, Zahiri R , Lamarche J, Levesque RC, Hamelin RC, Cusson M (2019) A needle in a haystack: a multigene TaqMan® assay for the detection of Asian spongy moths in bulk pheromone trap samples. *Biological Invasions* **21:** 1843-1856. https://doi.org/10.1007/s10530-019-01943-9.

4. Capron A, Stewart D**,** Hrywkiw K, Allen K, Feau N, Bilodeau G, Tanguay P, Cusson M, Hamelin RC In Situ Processing and Efficient Environmental Detection (iSPEED) of tree pests and pathogens using point-of-use real-time PCR. Plos One (2020). https://doi.org/10.1371/journal.pone.0226863

Appendix A – Primer, probe and gBlocks™ gene fragment sequences

Table 1: Primer sequences for the ASM portable TaqMan® Triplex 1 ASM subassays.

Assay	Assay Number	Primer Name	Amplicon Size	Primer Sequence
Triplex 1 ASM	1A	ASM comp COI F3G/A 601-622	142bp	TACATCCTTTTTTGACCCYACR
		ASM comp COI R724-742		TCCTCTTTCTTGGGAAATA
	1B	Ldaj cytb R F120-150	249bp	GGATCTTTRTTAGCTTTATGTTTAATTACC
		Ldaj cytb R339-369		TCCAATTATTCATGTTTGTTTTAAATTAAAA
	1C	Lumb ND1 3C/A F233-262	163bp	TATTTTTCTCCTGTATTAGCTTTTGATA
		Lumb ND1 2C/T R375-395		GAATTAGAAGACCATCCTGTC

Table 2: Probe sequences for the ASM portable TaqMan® Triplex 1 ASM subassays.

Assay	Assay Number	Probe Name	Fluorophore	Probe Sequence
Triplex 1 ASM	1A	ASM comp COI T 636-649	FAM-LNA	C+AAT+C+CTTT+A+C+CAA
	1B	Ldaj cytb T262-273	TEX 615-LNA	CT+CT+TC+A+C+G+CT
	1C	Lumb ND1 T340-358 RC	Cy5-LNA	AC+A+CTATAAA+C+T+CCAAAAC

Table 3: gBlock positive control information. For Triplex 1A and 1B, 50 fg of *L. dispar asiatica* gBlock is used, while for triplex 1C, 50 fg of *L. umbrosa* gBlock is used.

gBlock	Genes	Assay	Positive
L. dispar asiatica	COI-cytb	Triplex 1 ASM	ASM COI - Ldaj cytb
L. umbrosa	COI-ND1	Triplex 1 ASM	Lumb ND1

gBlocks™ sequences

gBlock sequences are shown below. Assay primers and probes are highlighted to show where they are positioned on the gBlock fragments. For Triplex 1A and 1B, gBlock *L. dispar asiatica* COI-cytb is used, while for triplex 1C, gBlock *L. umbrosa* COI-ND1 is used.

ASM comp COI Ldaj cytb Lumb ND1

L. dispar asiatica COI-cytb gBlock (Triplex 1A and 1B positive control)

CCCCTGATATAGCTTTCCCCCGTATAAATAATATAAGATTTTGATTATTACCCCCCTCATTAACCCTTTTAATTTCAAG
AAGAATTGTAGAAAATGGAGCAGGAACAGGATGAACTGTTTACCCTCCTCTATCTTCTAATATTGCTCATGGAGGT
AGATCTGTTGATTTAGCTATTTTTTCTCTTCACTTAGCTGGTATTTCATCAATTTTAGGAGCAATTAATTTTATTACTA
CCATTATTAATATACGATTAAGAAATTTATCGTTTGATCAAATACCTTTATTTGTTTGAAGAGTTGGAATTACAGCTT
TCCTTCTACTTTTATCTTTACCTGTTTTAGCAGGTGCTATTACAATATTATTAACTGACCGAAATTTAAATACATCCTT
TTTTGACCCTGCAGGAGGAGGGGATCCAATCCTTTACCAACATTTATTTTGATTTTTCGGACATCCTGAAGTTTATA
TTTTAATTTTACCAGGATTTGGAATAATTTCCCATATTATTTCCCAAGAAAGAGGAAAAAAGGAAACATTTGGATGT
TTAGGAATAATTTATGCAATATTAGCTATTCTTTAATTGATCTTCCCTCCCCATCAAATATCTCCTATTGATGAAATTT
TGGATCTTTGTTAGCTTTATGTTTAATTACCCAAATCATTACAGGATTATTTCTAACTATATATTATACTGCTAATATT
GAATTAGCTTTTTATAGAGTTAATTATATTTGCCGAAATGTAAATTATGGTTGATTAATTCGAACTCTTCACGCTAAT
GGGGCATCACTCTTTTTTATTTGTATTTATTTACATATTGGACGAGGAATTTATTATGAATCTTTTAATTTAAAACAA
ACATGAATAATTGGAGTAATAATTTTTTTCTTTTAATAGGAACTGCTTTCATAGGATATGTACTACCATGGGGACA
AATATCTTTTTGAGGAGCT

Figure 1: *L. dispar asiatica* COI-cytb gBlock. This concatemerized COI-cytb gene fragment contains the sequence for amplification with the ASM COI subassay (highlighted in light blue) as well as the sequence for Ldaj cytb subassay (highlighted in yellow).

L. umbrosa COI-ND1 gBlock (Triplex 1C positive control)

ACCTGTTTTAGCAGGTGCTATTACAATATTATTAACTGACCGAAATTTAAATACATCCTTTTTTGACCCCACAGGAG
GAGGGGACCCAATCCTTTACCAACATTTATTTTGATTTTTCGGACATCCTGAAGTTTATATTTTAATTTTACCAGGAT
TTGGAATAATTTCCCATATTATTTCCCAAGAAAGAGGAAAAAAGGAAACATTTGGATGTTTAGGAATAATTTATGC
AATATTAGCTATCGGATTATTTGATTTATCCTTATTATTCTTTTATTTTTCTCCTGTATTAGCTTTTGATATATCTTTAT
TAATTTGATTTGTAATTCCTTATTATTTTAATTTAGTTAGATTTAATTTAGGTTTATTATTTATTTTAAGATGTTTAAGT
TTTGGAGTTTATAGTGTTATAGTGACAGGATGGTCTTCTAATTCAAATTATGCTTTGTTGGGGGGTCTTCGGGCTGT
AGCTCAAACTATTTCTTAT

Figure 2: *L. umbrosa* COI-ND1 gBlock. This concatemerized COI-ND1 gene fragment contains the sequence for amplification with the Lumb ND1 subassay (highlighted in green).

Appendix B – Preparation and Lyophilization of qPCR mastermix

There are two options for preparing a lyophilized mastermix. The first is to purchase a lyo-ready mastermix that is glycerol-free and contains lyo-excipients. When lyophilized, this mix will produce a compact white pellet. However, this mastermix is expensive at $1240.00 for 5 ml (500 20 µL reactions). It can be purchased from Froggabio (Lyo-ready qPCR mix, catalogue no. MDX021-5).

The second option is to use a standard TaqMan® mix that contains glycerol and add your own lyo-excipients (we add 30% trehalose to a final concentration of 5% (calculated for a 20 µL final volume). When lyophilized, this mix will produce a loose, clear pellet. This option is very inexpensive at $1800.00 for 50 mL (equivalent to $180.00 for 5 mL). It can also be purchased from Froggabio (SensiFAST™ Probe No-Rox Kit - cat. no. BIO-86005).

Primer/probe mixes

When received, primers and probes are hydrated to a concentration of 100 µM in 10 mM Tris, pH 8. Final primer and probe concentrations for the TaqMan® reactions will be 500 nM primers and 100 nM probe. 20X primer probe mastermixes are prepared, which contain 10 µM of each primer and 2µM of probe.

Each reaction will contain 10 µL of commercial 2X mastermix plus 1 µL of each primer/probe mastermix. The final volume per reaction for the triplex will be 13 µL (we do not bring the volume up to 20 µL with H_2O for mixes that will be lyophilized). The user should calculate the volumes needed for the number of strip tubes to be prepared and add an extra 10% volume to be sure that there will be enough for all tubes. The strip tubes that are used are the Applied Biosystems MicroAmp fast reaction tubes (8 tubes/strip), catalogue number 4358293) and the optical cap strips are the Applied Biosystems MicroAmp optical 8-cap strip, catalogue number 4323032).

Table 1: Mastermix components for the Triplex 1 TaqMan® ASM assay. Final volume per well is 13 µl.

Reaction component	Volume per well (µL)	Stock concentration (µM)	Fluorophore
Commercial mastermix	10		
Primer/probe mix 20X triplex 1A	1	10µM primers /2µM probe	FAM
Primer/probe mix 20X triplex 1B	1	10µM primers /2µM probe	TEX 615
Primer/probe mix 20X triplex 1C	1	10µM primers /2µM probe	Cy5
Total volume	13		

Lyophilization

Place the fast reaction tube strips in a 96 well base, pipette the desired volume (13 µL) into each tube, place the optical cap strips on the fast reaction tubes and place in the freezer at -20°C.

When the user is ready to lyophilize the samples, prechill a 750 mL freeze flask at -20°C. Turn on the freeze dryer about 20 minutes before you are ready to begin your lyophilization reaction as it takes time to get to the correct temperature.

Remove the 96 well base and samples from the freezer and place on ice. Heat the end of a paper clip under a bunsen burner and poke a hole in the middle of the cap of each tube. Return the strips to the freezer until you are ready to lyopholize the mastermix.

Figure 1: 96 well base with fast reaction tubes, cap strip and paperclip used to poke hole in caps.

The tube strips were lyophilized using a Labconco Free-Zone 2.5 L freeze dryer using a collector temperature of -55°C and a vacuum of 0.3 mbar or less. The vacuum is set for 0.002 mbar but does not fall below 0.28 mbar. Samples are lyophilized for 90 minutes under these conditions.

Once the samples have been lyophilized, remove the optical cap strips that have holes in them and replace with new cap strips. The strips should be stored in the dark at -20°C until ready for use. They may also be stored at room temperature in a foil envelope containing a dessi cation packet for up to six months. Be sure to to identify the strips and note the lyophilization date.

Appendix C - TaqMan® ASM portable assay flowchart

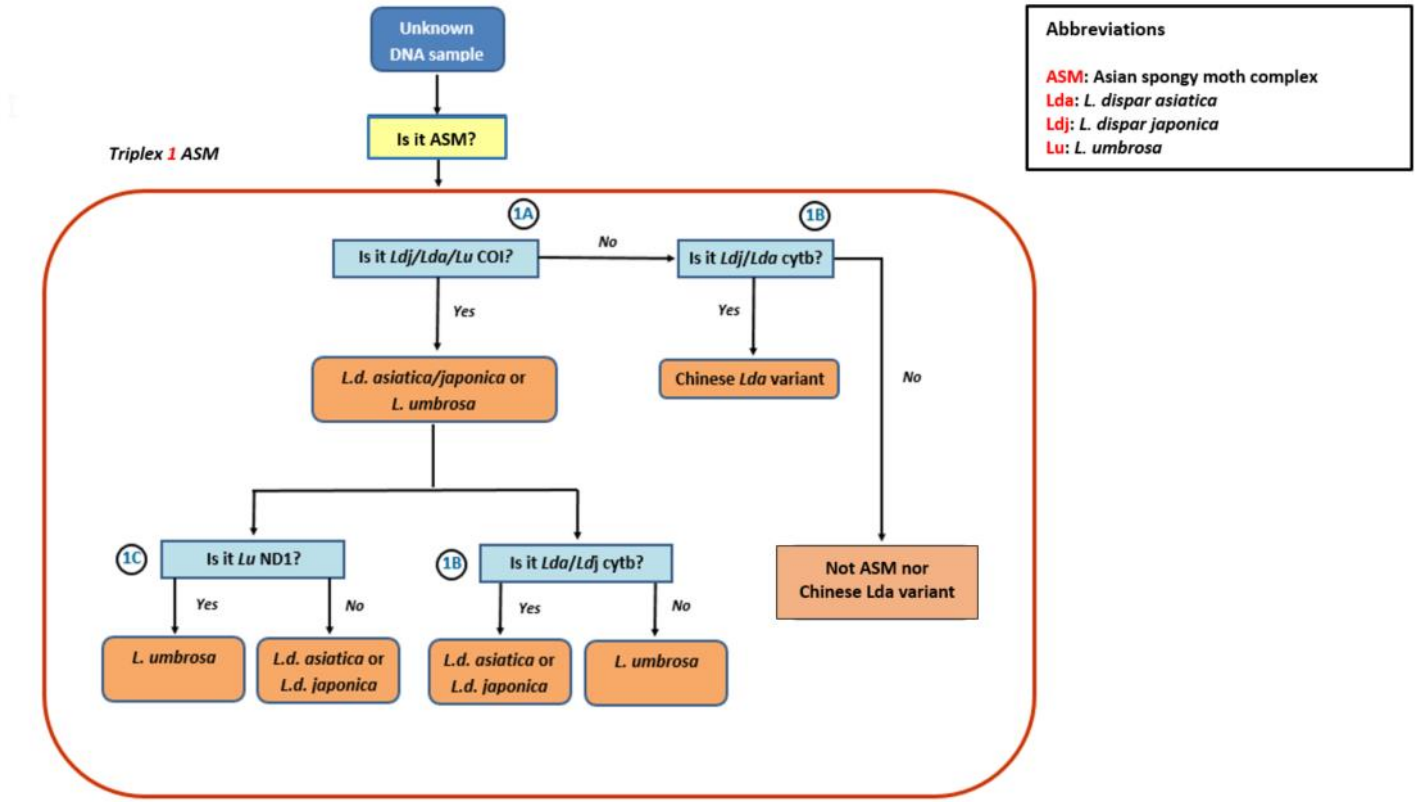


Figure 1: ASM portable TaqMan® assay flowchart. Triplex subassay 1A (ASM COI) will confirm the absence or presence of ASM while subassays IB and IC (Ldaj cytb and Lumb ND1) will provide further species/subspecies resolution. The Triplex 1B subassay is illustrated in two separate places. After a *positive* result for subassay 1A, subassays 1B and 1C will both enable discrimination between *L. umbrosa* and *L. dispar asiatica/L. dispar japonica*, but on the basis of different markers, thus providing confirmatory results. After a *negative* outcome for subassay 1A, subassay 1B will determine whether the unknown is the Chinese *L. dispar asiatica* variant that features an ESM-like COI sequence.

Appendix D - Species discrimination list

Below is a list of Lymantriinae species whose egg masses can be discriminated with the portable ASM TaqMan® assay. Species highlighted in yellow can be identified using the TaqMan® assays while the other species will be discriminated.

Lymantria dispar asiatica
Lymantria dispar japonica
Lymantria umbrosa
Lymantria albescens
Lymantria postalba
Lymantria dispar dispar
Lymantria fumida
Lymantria lucescens
Lymantria mathura
Lymantria monacha
Lymantria xylina
Lymantria atemeles
Lymantria bantaizana
Lymantria concolor
Lymantria minomonis
Lymantria obfuscata
Arctornis l-nigurm
Calliteara abietis
Calliteara pseudabietus
Calliteara pudibunda
Cifuna locuples
Euproctis chrysorrhoea
Euproctis similis
Euproctis subflava
Hylesia nigricans
Leucoma candida
Leucoma salicis
Orgyia anartoides
Orgyia thyellina

ASM TaqMan® Detection Manual - Supplementary Material

1A-ASM COI 601-742

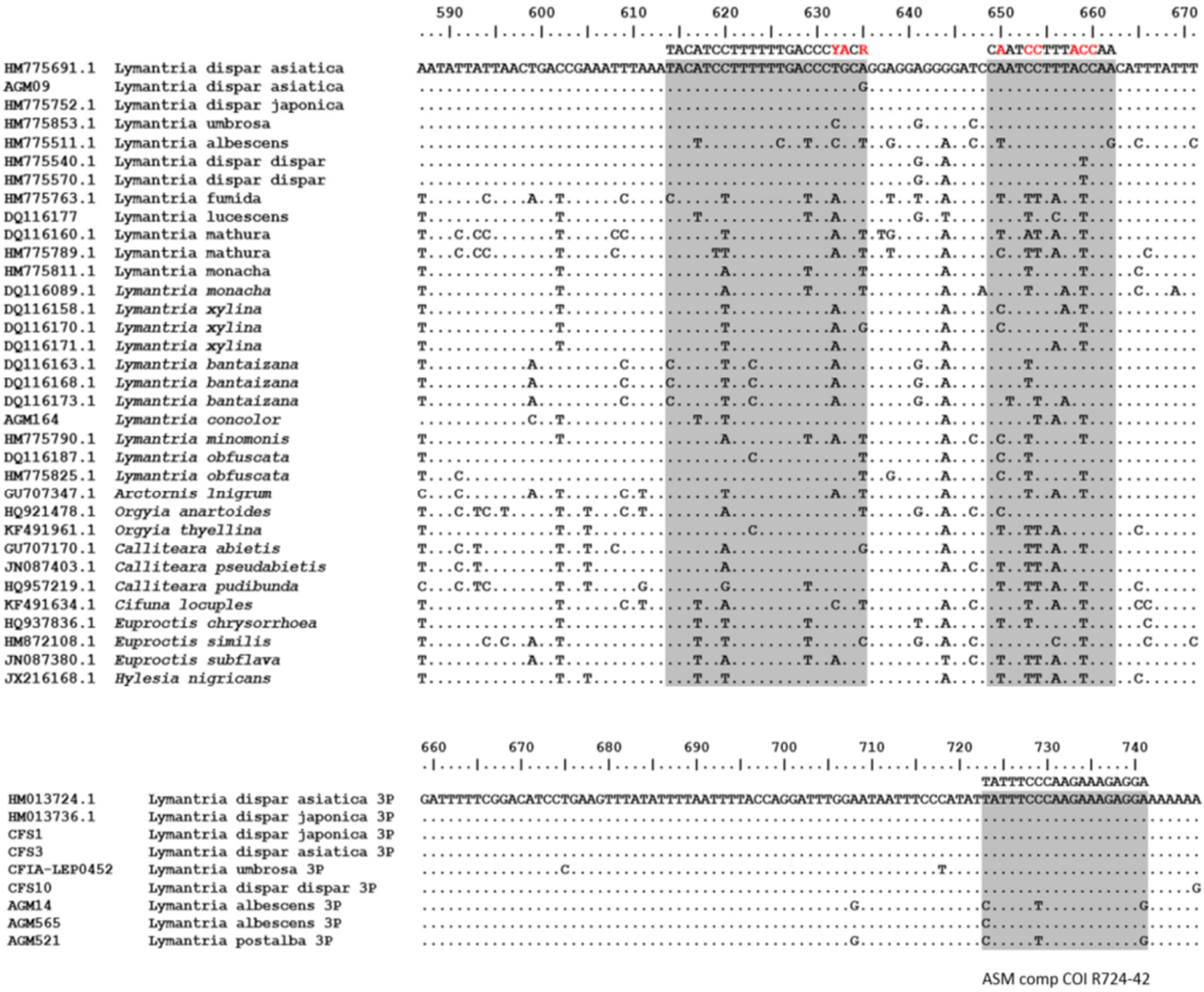


Figure 1: Primer and probe alignment for the ASM complex COI TaqMan® assay. An ARMs base was added to the forward primer to increase discrimination. The majority of the non-target species will be discriminated with the LNA probe (red letters designate LNA bases). The C/T SNP present at position 646 in the probe is sufficient to discriminate L. dispar dispar (the calculated Tm of the probe for L. dispar dispar is 40.5oC). The reverse primer is in the 3P region of the COI gene and was designed to help discriminate L. albescens. Degeneracy was introduced at two sites within the forward primer to enable amplification of L. d. asiatica and L. umbrosa samples.

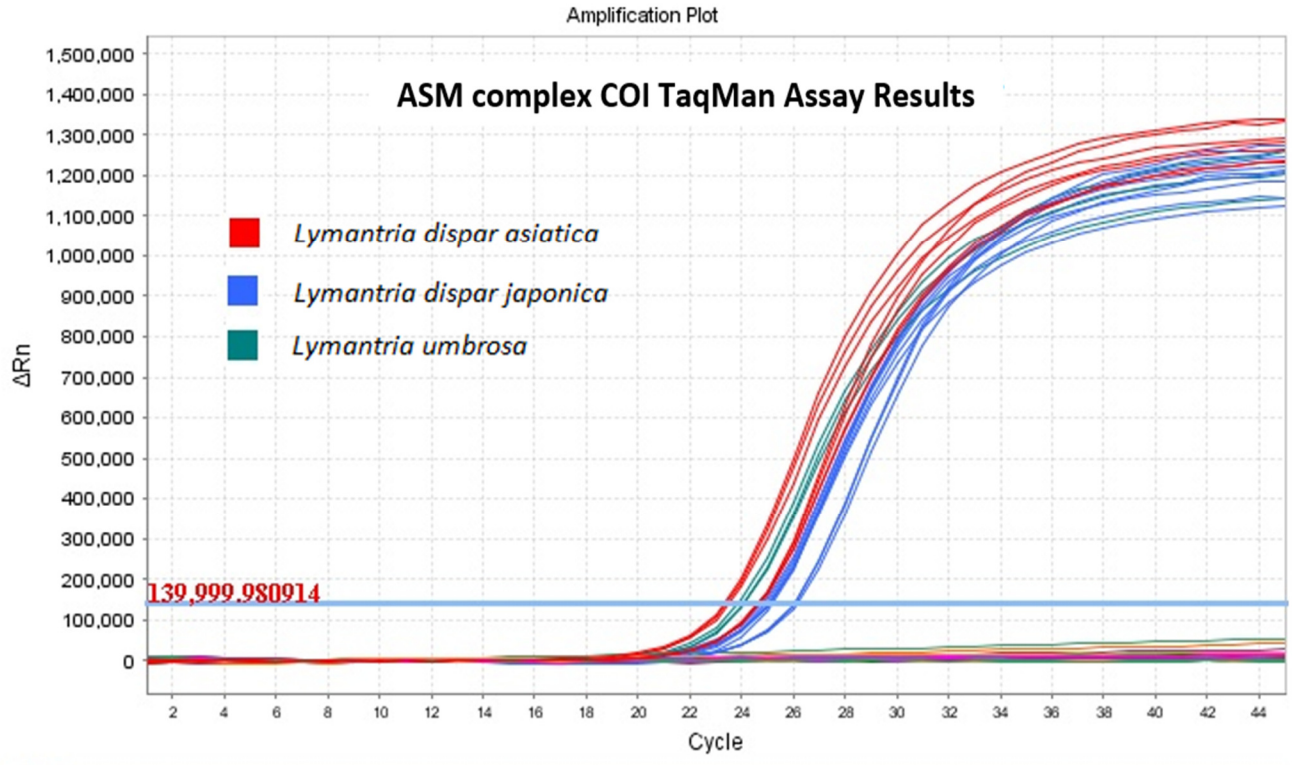
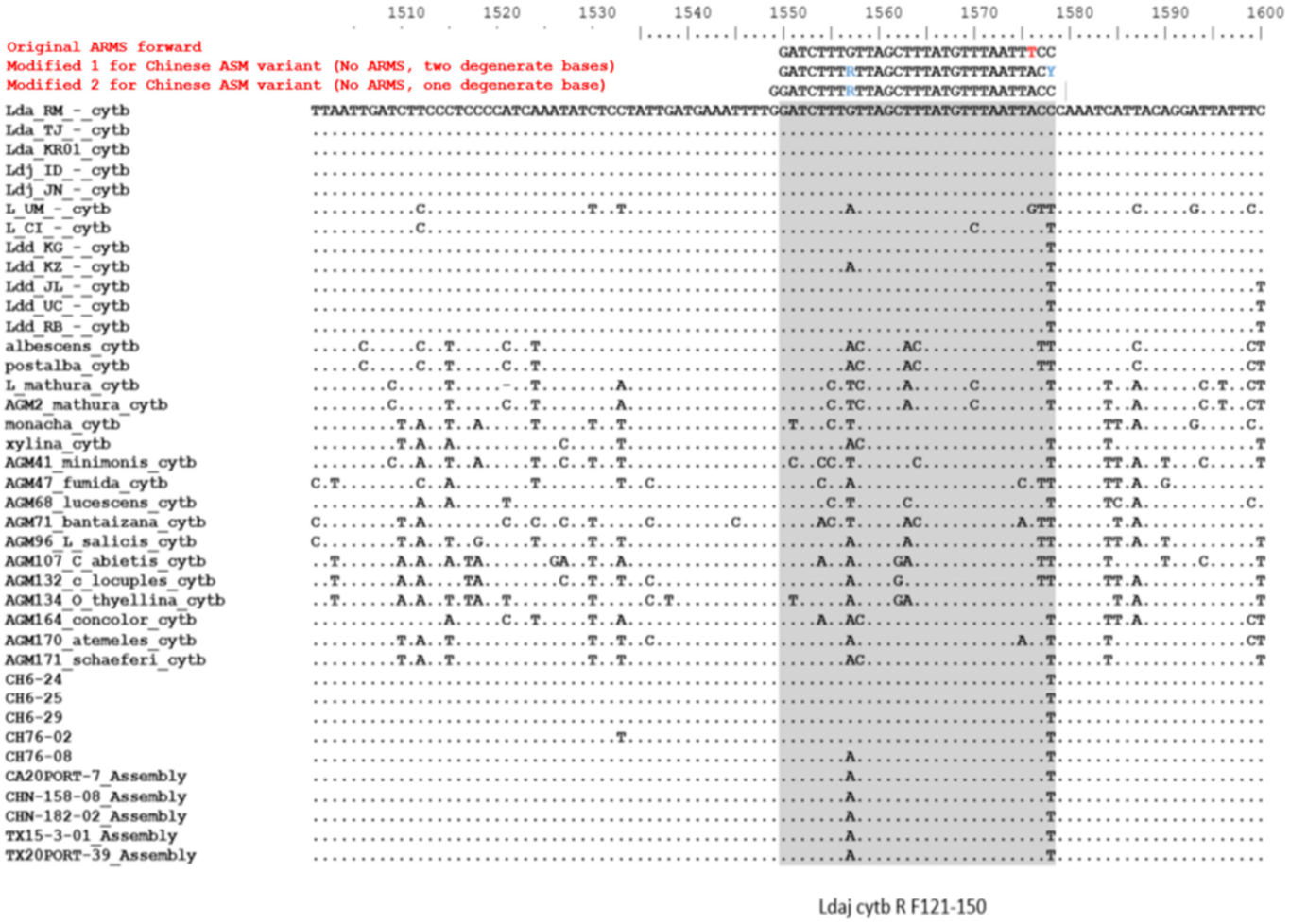


Figure 2: ASM complex COI Taqman® discrimination assay results (Applied Biosystems 7500 Fast Real-Time PCR system). This assay amplifies L. dispar asiatica, L. dispar japonica and L. umbrosa while discriminating all other species on the priority list. Forty-five cycles of qPCR were run and the Ft was set at 10% of Fmax.

1b-Ldaj cytb 120-369



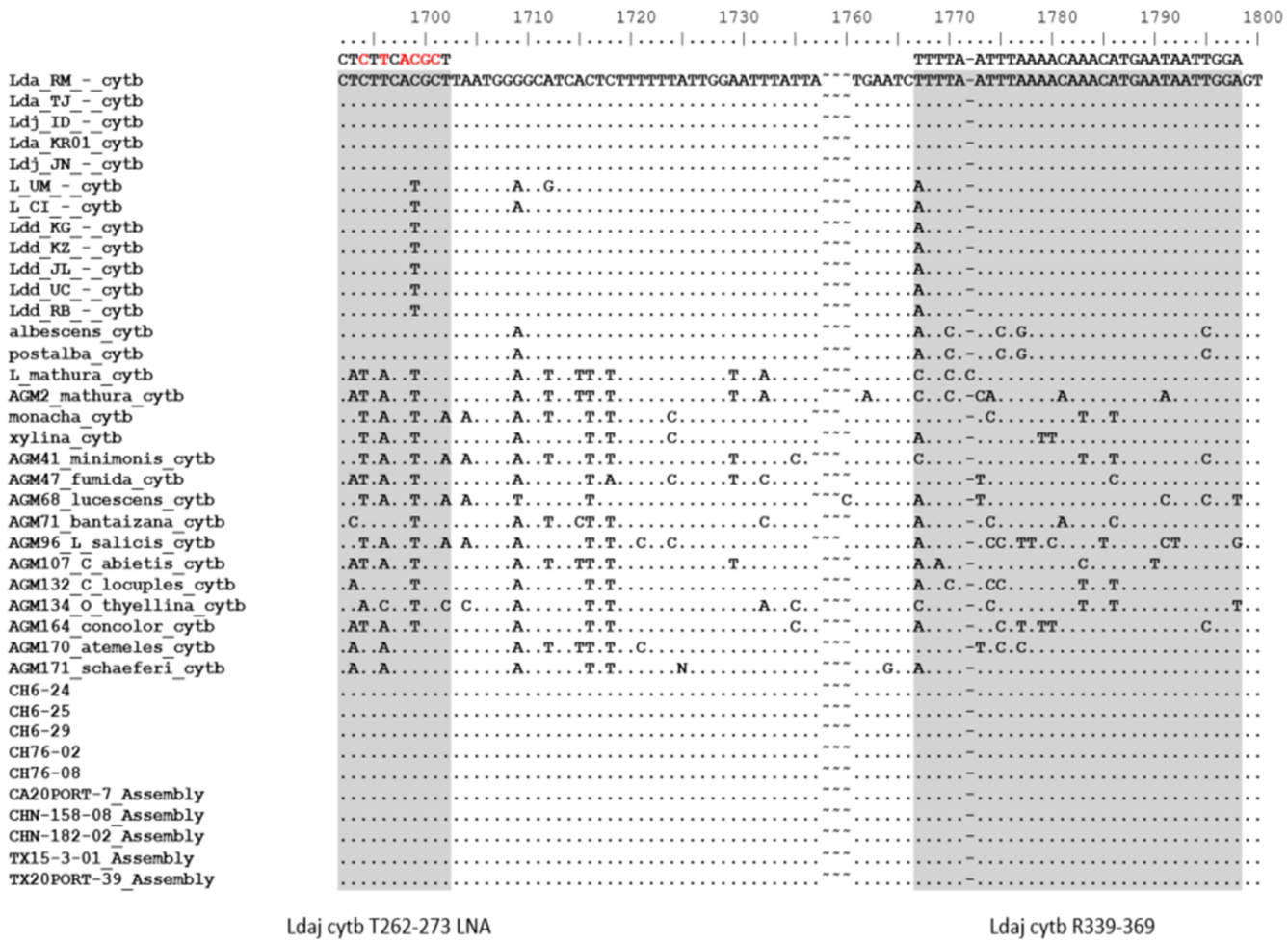


Figure 1. Alignment of the cytb region targeted by the Ldaj cytb assay. This assay was designed to detect L. dispar asiatica and L. dispar japonica while discriminating L. dispar dispar and the other Lymantria species within the ASM complex (L. umbrosa, L. albescens, L. postalba) as well as other non-target species. It will also detect a previously undetected Chinese ASM variant. The original forward ARMS primer was problematic for the detection of the Chinese AGM variant so two different modifications were tested, modified 1 and modified 2. Modified 1 removes the ARMS base and adds an R near the 5P end of the primer and a Y at the 3P end. Modified 2 is the same as modified 1 but is 1bp longer and keeps the 3P C-T SNP intact. Both primers work well for the detection of the Chinese ASM variant. (Figure 3). The 3P C-T SNP by itself only changes the Ct value by one cycle for the Chinese ASM variant but will add discrimination for the L. albescens/L. postalba specimens.

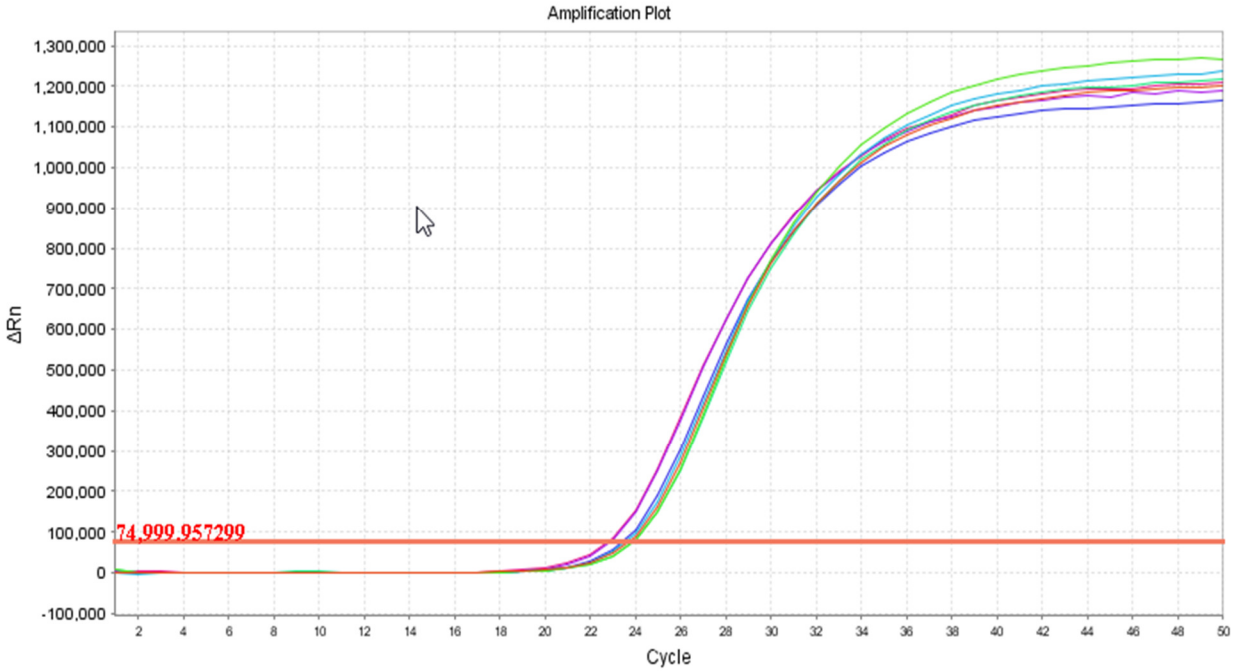


Figure 2: A graphic result for the Ldaj cytb Taqman® assay. The run was performed on an Applied Biosystems 7500 Fast Real-Time PCR system.

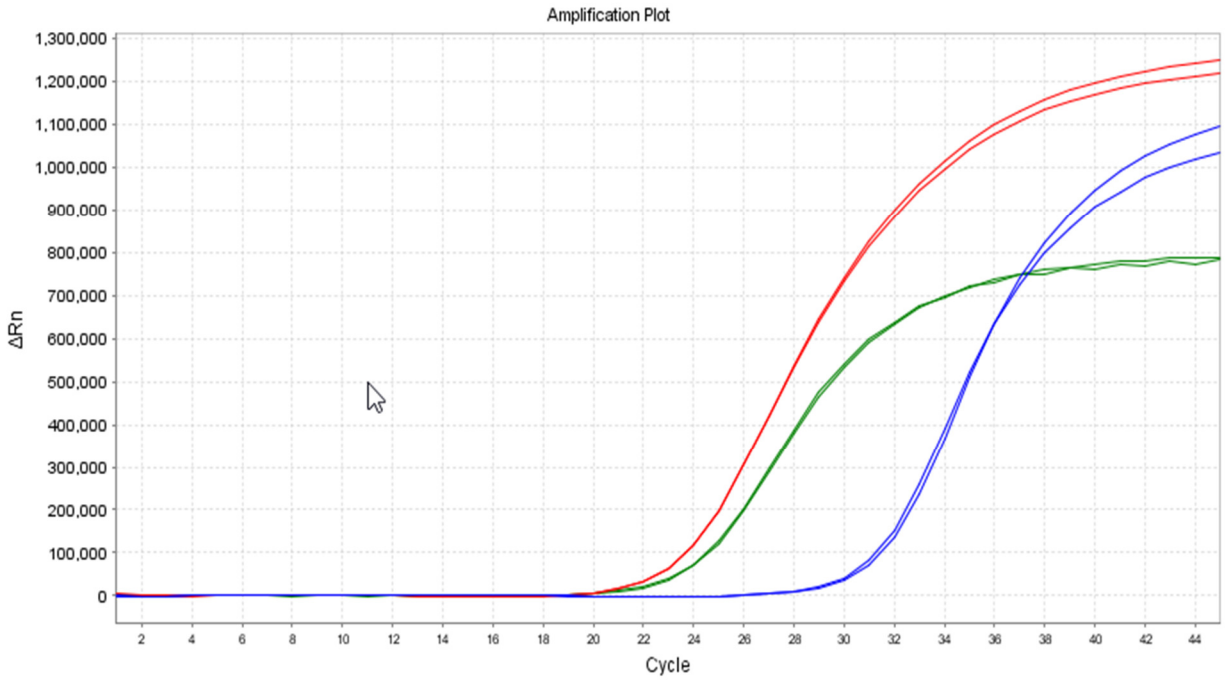


Figure 3: Comparison of three different forward primers for the Ldaj cytb assay with the Chinese ASM variant (20fg cytb gblock DNA). The blue curve is the original ARMS forward primer, the red curve is the modified 1 primer that matches the Chinese ASM variant sequence 100%

and the green curve is the modified 2 primer that keeps the 3P C-T SNP in place to provide some discrimination in the forward primer for L. albescens/L. postalba. This is a weak SNP and produces a Ct value about one cycle later than the primer with a 3P degenerate base. The user can make a decision on which primer to use. When tested against L. albescens/L. postalba, both primers discriminated the L. albescens/L. postalba samples.

1C-Lumb ND1 233-395

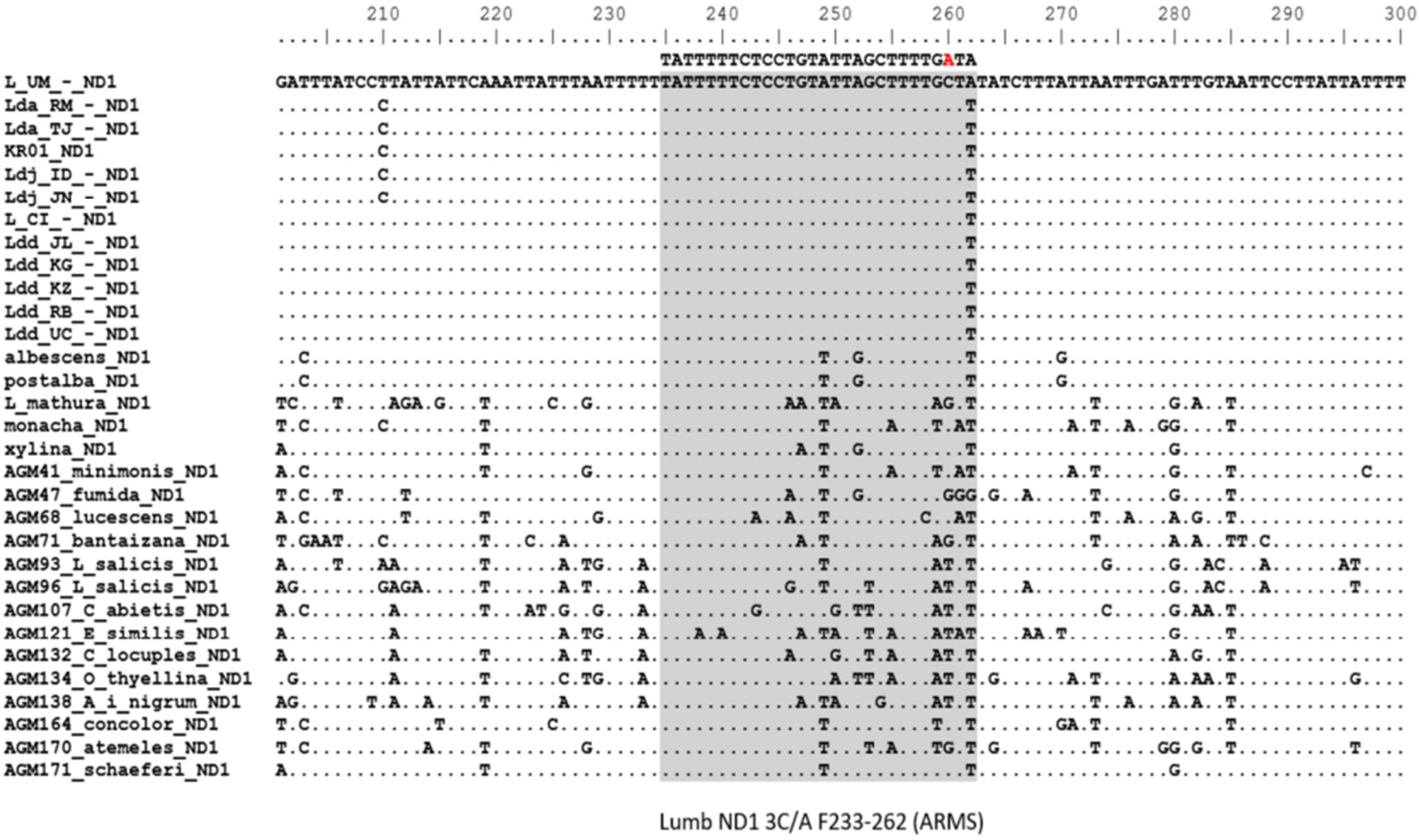
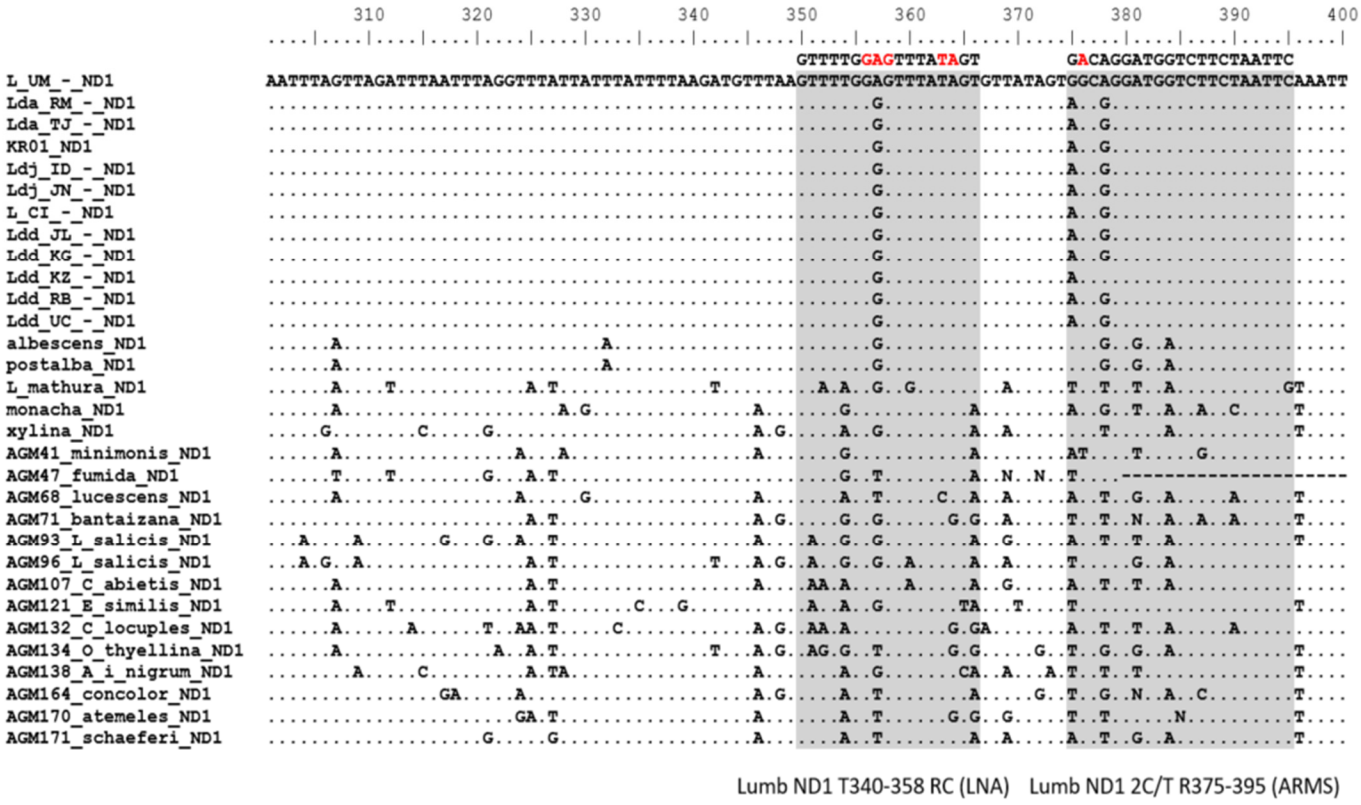


Figure 1. Alignment of the ND1 region targeted by the Lumb ND1 assay. This assay was designed to detect *L. umbrosa.* This assay will discriminate against the other Lymantria species within the ASM complex (*L. dispar asiatica, L. dispar japonica, L. albescens* and *L. postalba*) as well as *Lymantria dispar dispar* and other non-target species.



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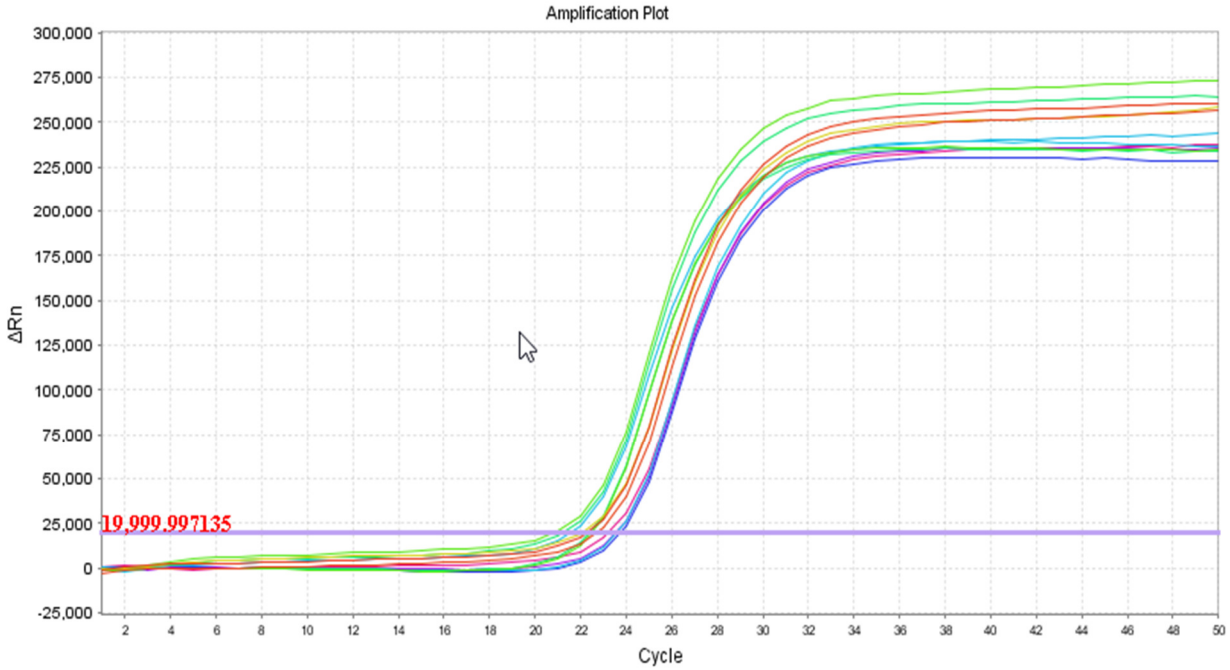

Figure 2: A graphic result for the Lumb ND1 Taqman® assay. The run was performed on an Applied Biosystems 7500 Fast Real-Time PCR system.