

# Asian Gypsy Moth (AGM) Taqman Detection Workshop

**Part I : *Detection from egg mass samples***

Québec, September 18-19, 2018  
Laurentian Forestry Centre

*1055 rue du PEPS, Québec, G1V 4C7*

by  
Don Stewart, Biologist



Natural Resources  
Canada

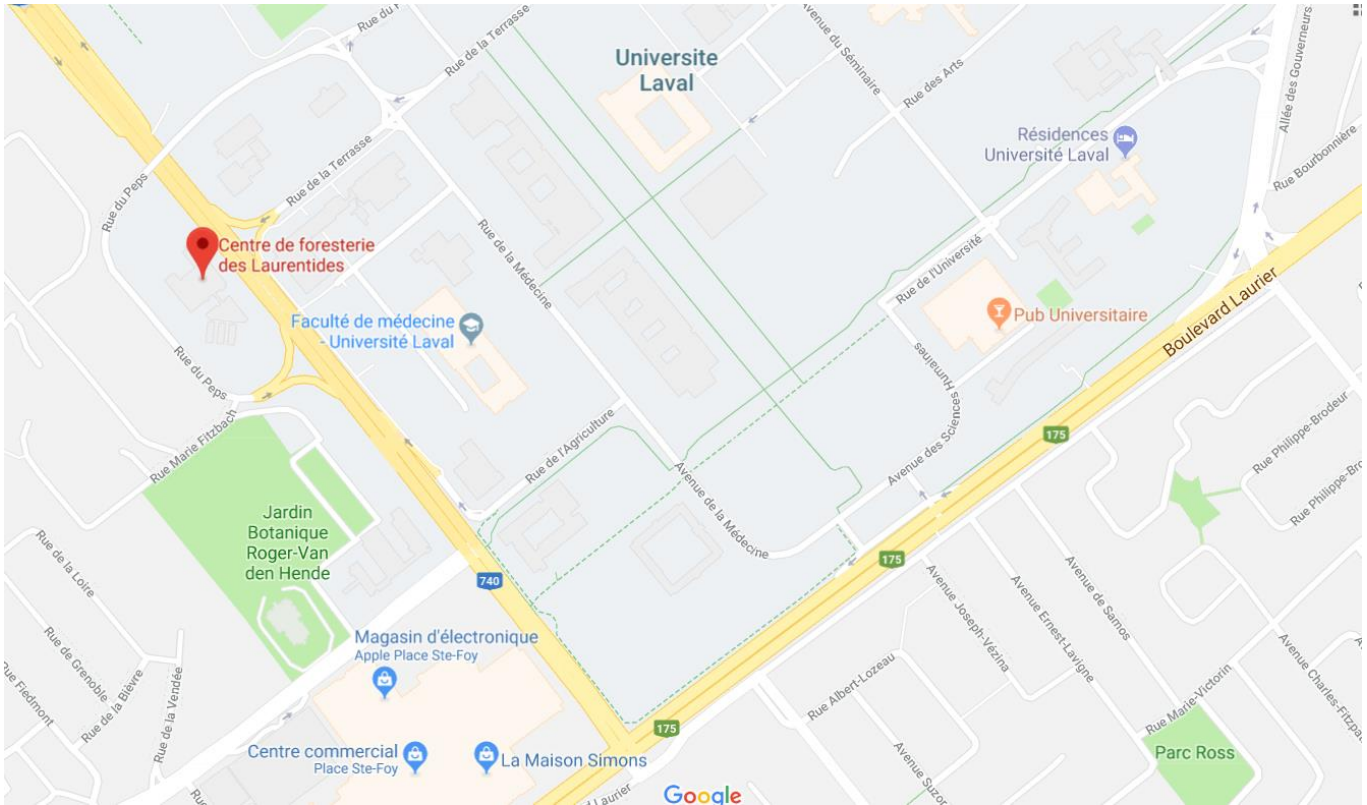
Ressources naturelles  
Canada



Canadian Food  
Inspection Agency

Agence canadienne  
d'inspection des aliments

**Centre de foresterie des Laurentides**  
**1055 rue du PEPS,**  
**Quebec, QC, G1V 4C7**



## **Contact:**

### **Don Stewart**

Biologist, Canadian Forest Service  
Natural Resources Canada / Government of Canada  
[don.stewart@canada.ca](mailto:don.stewart@canada.ca) / Tel: 418-648-4271

### **Catherine Béliveau**

Biologist, Canadian Forest Service  
Natural Resources Canada / Government of Canada  
[catherine.beliveau@canada.ca](mailto:catherine.beliveau@canada.ca) / Tel: 418-648-5806

## Table of Contents

	Page
Agenda .....	1
Procedure flowchart .....	2
DNA extraction protocol .....	3
DNA sample list .....	4
SYBR Green quantification protocol .....	5-6
Taqman <sup>®</sup> assay protocol .....	7-8
Analysis of results.....	9

- Appendix A: PCR and Taqman<sup>®</sup> Assay Theory
- Appendix B: SYBR Green Quantification
- Appendix C: Molecular Key Flowchart
- Appendix D: Species ID Tool
- Appendix E: Primer and Probe Sequences
- Appendix F: Species Discrimination List
- Appendix G: DNA Extraction Protocol for Insect Legs

## Agenda

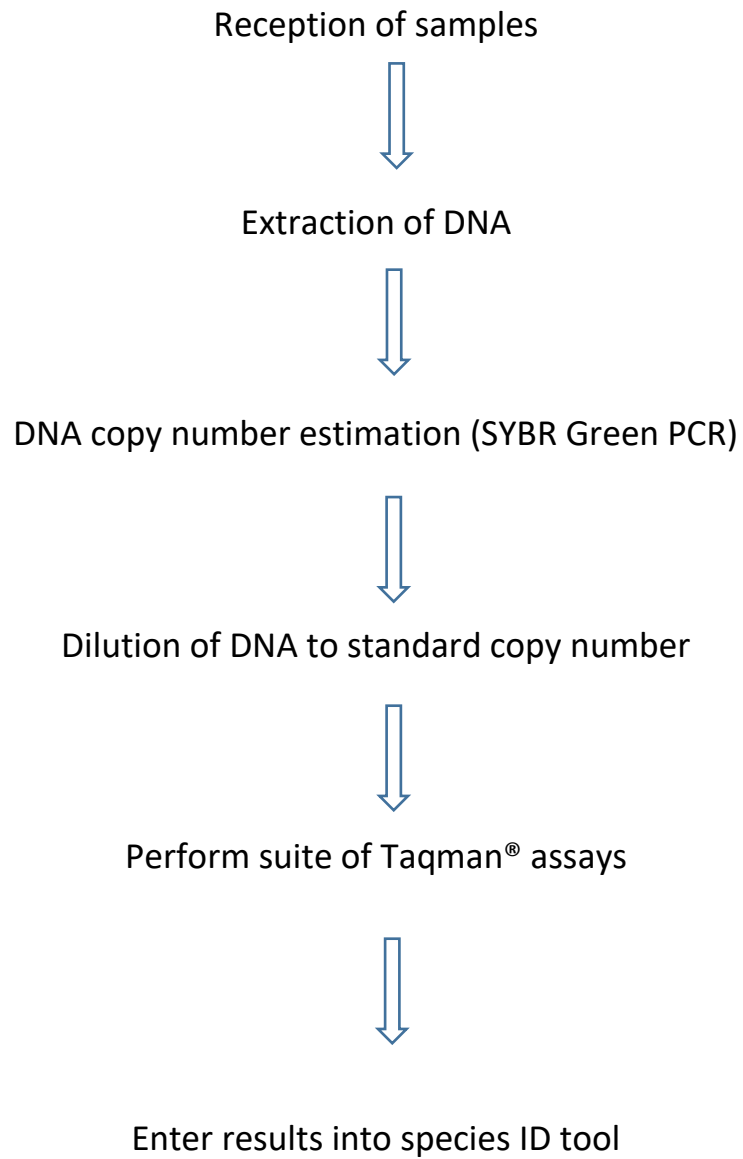
### Day 1 (Tuesday 18<sup>th</sup>)

8:30	Arrival
9:00 -10:00	DNA extraction from egg masses.
10:00 – 10:30	Coffee Break
10:30 – 12:00	SYBR Green PCR for DNA quantification
12:00 – 13:30	Lunch
13:30 – 15:00	Taqman <sup>®</sup> assay preparation (run overnight)
15:00-16:00	Discuss bulk sample Taqman <sup>®</sup> assay (to be run on Day 2)

### Day 2 (Wednesday 19<sup>th</sup>)

8:30 – 9:30	Bulk sample Taqman <sup>®</sup> assay preparation (see separate, “Part 2” protocol)
9:30 –10:30	Analysis of Taqman <sup>®</sup> assay results from Day 1 using species ID tool
10:30 – 11:00	Coffee Break
11:30 – 12:15	Analysis of bulk sample Taqman <sup>®</sup> assay results and workshop wrap up.
12:30	Lunch

## AGM Sample Identification Procedure



## DNA Extraction Protocol for *Lymantria* Eggs

### Edwards Buffer

- 200mM Tris pH 7.5
- 250mM NaCl
- 25mM EDTA
- 0.5% SDS

It may be necessary to heat buffer before use as SDS may precipitate at room temperature.

- 1) Place 2-5 eggs in a 500ul conical PCR tube.
- 2) Add 20ul Edwards buffer.
- 3) Break up eggs with a micropestle
- 4) Add another 20ul of Edwards buffer
- 5) Heat for 10 minutes at 95°C in a PCR thermocycler.
- 6) Transfer buffer and eggs to a 2ml tube containing 960ul of H<sub>2</sub>O to dilute buffer.
- 7) Vortex to mix well.
- 8) Use 2ul in qPCR.



## DNA Sample List

**Table 1:** DNA samples used for Taqman® AGM assay demonstration

Sample Number	Sample Name	Sample ID	Location
1	LYM 02	<i>L. albescens/L. postalba</i>	Japan
2	LYM 04	<i>L. albescens/L. postalba</i>	Japan
3	LYM 06	<i>L. dispar japonica</i>	Japan
4	LYM 15	<i>L. dispar japonica</i>	Japan
5	CFS 04	<i>L. dispar asiatica</i>	Mongolia
6	CFS 10	<i>L. dispar dispar</i>	Lithuania
7	CFS 11	<i>L. dispar dispar</i>	Greece
8	LEP 941	<i>L. dispar dispar</i>	Russia/N. Caucasus
9	egg extraction	<i>L. dispar dispar</i>	Quebec, Canada
10	NTC	water	n/a

**Note:** For the purpose of this training, only Duplex Assays 1-4 will be run. This will allow us to identify all AGM species, as well as *L. dispar dispar*. We will also be able to assess the occurrence of any Asian introgression into *L. dispar dispar*. Duplex 5 (*L. monacha*, *L. fumida*) and Triplex 6 (*L. mathura*, *L. xylina*, *L. lucescens*) will not be included in this training package (see Appendix C for details).

## SYBR Green Copy Number Quantification – PCR Protocol

Before performing the Taqman® assays to identify the unknown egg masses, a SYBR Green amplification step is performed using a pair of *Lymantria* general primers designed in the cytochrome oxidase (COI) gene. This step is included for the following reasons:

- 1) It verifies that the DNA extraction was successful.
- 2) It allows us to dilute the DNA to a copy number that is optimal for the functioning of the AGM Taqman® assays.

### Protocol

We will be amplifying 10 samples for this exercise. There will be eight previously extracted DNA samples, our egg mass DNA extraction and a no template control (NTC). There will be two technical replicates for each sample for a total of 20 reactions. We will also add an extra 10% volume to each mastermix component to be sure that there is enough for all reactions.

**Table 1:** SYBR Green amplification master mix

Reaction Component	Volume per well (µl)	Total volume (µl)
Quantitect SYBR Green 2X master mix	5	120
Primer mix 10X (5 µM)	1	24
Water	2	48
DNA	2	n/a





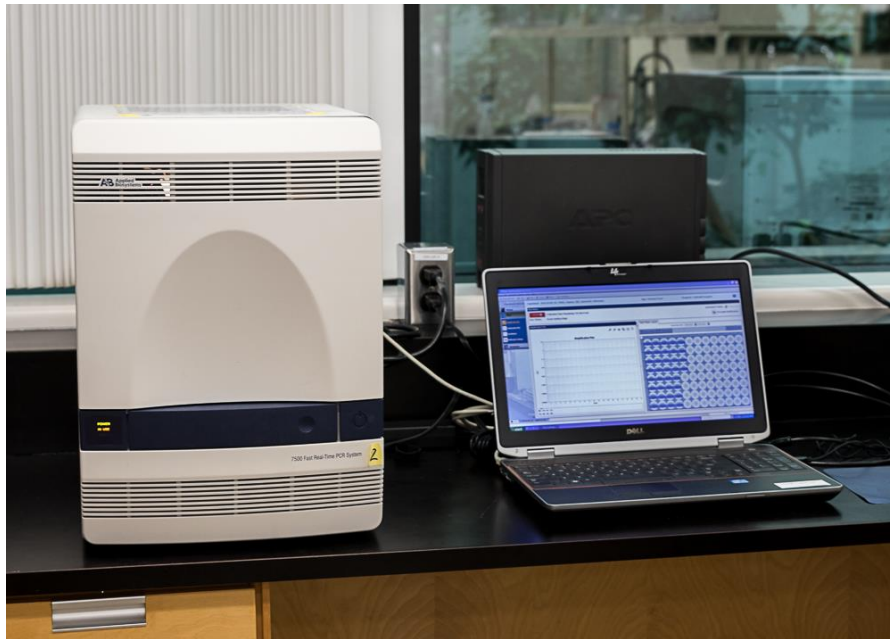
Place a 96 well optical plate in a support base so that it does not come into contact with the benchtop surface. Pipette 8  $\mu$ l of master mix and 2  $\mu$ l of DNA into each of the 20 wells of the 96 well PCR plate. Seal the plate with a sheet of optical adhesive film and centrifuge the plate for 30 seconds.

### PCR Conditions

**Table 2:** PCR conditions for SYBR Green amplification

Stage	Number of Cycles	Temperature	Time
Enzyme activation	1	95°C	15 minutes
Denaturation	40	95°C	10 seconds
Annealing		52°C	30 seconds
Extension		65°C	1 minute

Place the 96 well plate in the PCR machine and begin run.



### Analysis of Results and Dilution of DNA

To calculate Ct value and determine the DNA dilution factor for each DNA sample, refer to Appendix B – SYBR Green Quantification.

## Taqman® Asian Gypsy Moth (AGM) Assay – PCR Protocol

The Taqman® assays consist of four separate master mixes. Each is a duplex reaction which means that there are two assays in each tube. The probe for each assay carries a separate fluorophore which allows the real time PCR machine's detection system to distinguish one from the other. We can combine up to four assays in a single tube. For a more thorough explanation of Taqman® theory, refer to Appendix A – SYBR Green amplification and Taqman® assay theory.

### Protocol

We will be amplifying 10 samples for the Taqman® assay. These are the same 10 samples that were quantified and diluted in the previous SYBR Green amplification step. There will be two technical replicates for each sample for a total of 20 reactions per master mix (remember that we have four master mixes). We will also add an extra 20% volume to each master mix component to be sure that there is enough for all reactions. We are adding 20% instead of 10% this time because we will load the PCR plate with a robotic workstation and a larger volume is required.

**Table 1:** Master mix for Duplex 1 assay. DUP 1A detects *L. albescens/L. postalba* and DUP 1B detects *L. dispar asiatica*, *L. dispar japonica* and *L. umbrosa*.

Reaction Component	Volume per well (µl)	Total volume (µl)	Fluorophore
Quantitect multiplex 2X master mix	5	120	
Primer/probe mix 20X DUP 1A	0.5	12	Cy5
Primer/Probe mix 20X DUP 1B	0.5	12	FAM
Water	2	48	
DNA	2	n/a	

**Table 2:** Master mix for Duplex 2 assay. DUP 2A detects *L. dispar asiatica* and *L. dispar japonica* (negative result = *L. umbrosa*). DUP 2B detects *L. dispar asiatica* (negative result = *L. dispar japonica*).

Reaction Component	Volume per well (µl)	Total volume (µl)	Fluorophore
Quantitect multiplex 2X master mix	5	120	
Primer/probe mix 20X DUP 2A	0.5	12	FAM
Primer/Probe mix 20X DUP 2B	0.5	12	Cy5
Water	2	48	
DNA	2	n/a	

**Table 3:** Master mix for Duplex 3 assay. DUP 3A detects *L. dispar dispar* and “Caucasian” gypsy moth. DUP 3B detects “Caucasian” gypsy moth.

Reaction Component	Volume per well (μl)	Total volume (μl)	Fluorophore
Quantitect multiplex 2X master mix	5	120	
Primer/probe mix 20X DUP 3A	0.5	12	Cy5
Primer/Probe mix 20X DUP 3B	0.5	12	FAM
Water	2	48	
DNA	2	n/a	

**Table 4:** Master mix for Duplex 4 assay. DUP 4A detects the presence of the Asian allele (A) and DUP 4B detects the presence of the North American allele (N). This assay tests for Asian introgression.

Reaction Component	Volume per well (μl)	Total volume (μl)	Volume per well (μl)
Quantitect multiplex 2X master mix	5	120	
Primer/probe mix 20X DUP 4A	0.5	12	FAM
Primer/Probe mix 20X DUP 4B	0.5	12	Cy5
Water	2	48	
DNA	2	n/a	

Place a 96 well optical plate in a support base so that it does not come into contact with the benchtop surface. Proceed to the robotic workstation with the PCR plate, four master mix tubes and DNA sample tubes. The plate will be loaded by the robotic workstation.

### PCR Conditions

**Table 5:** PCR conditions for the Taqman® AGM assay.

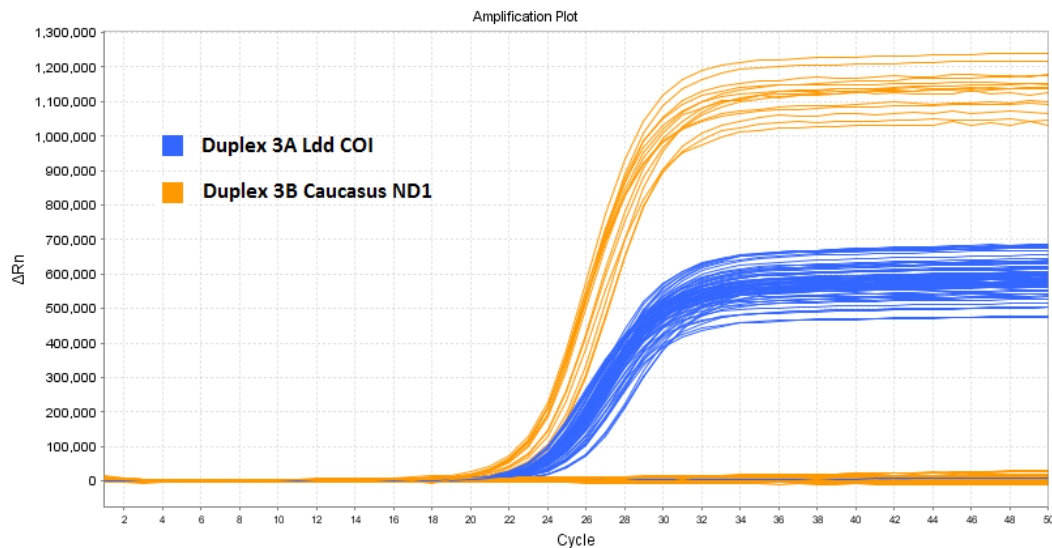
Stage	Number of Cycles	Temperature	Time
Enzyme activation	1	95°C	15 minutes
Denaturation	45	95°C	10 seconds
Annealing/Extension		60°C	60 seconds

Place the 96 well plate in the PCR machine and begin run.

## Analysis of Results

The results can be analyzed and interpreted using the species identification tool, which is an Excel based program that designates a species name to the specimen based on the results of the Taqman® assays. Simply place a yes (Y) or no (N) in the column for each assay for each DNA sample. For a more detailed explanation, refer to Appendix D – Species ID tool.

Figure 1 shows an example of a typical Taqman® assay result.

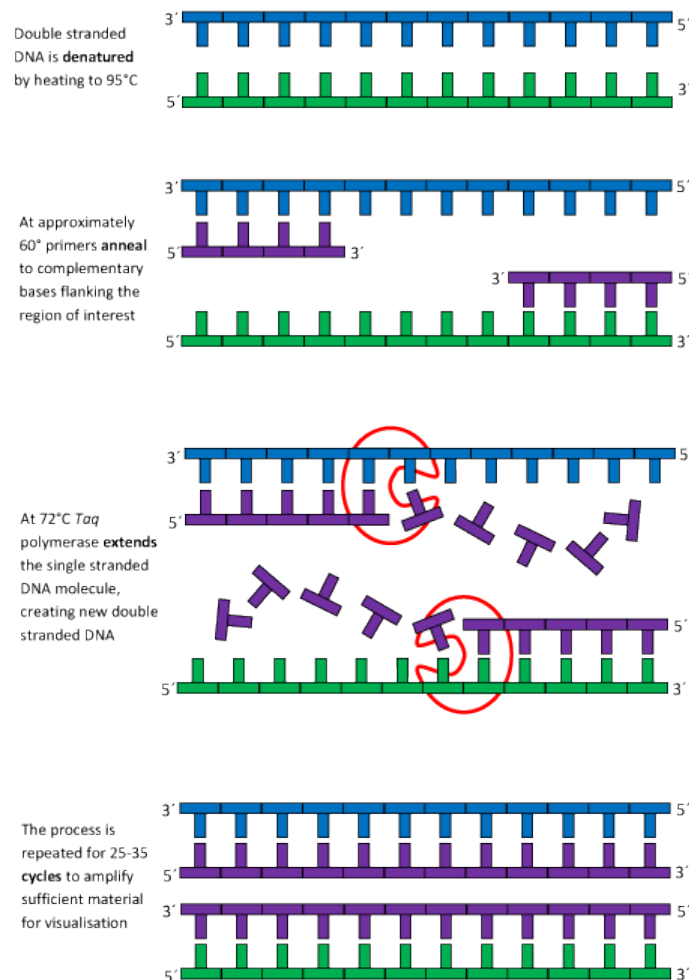


**Figure 1:** Results from a Duplex 3 assay run. The blue curves show a positive result for all *L. dispar dispar* samples and the orange curves show a positive result for a subsample of these that are “Caucasian” gypsy moth. The results are very easy to interpret.

## Appendix A – PCR and Taqman® Assay Theory

### Polymerase Chain Reaction (PCR)

PCR is a technique used to rapidly create multiple copies of a segment of DNA using repeated cycles of DNA synthesis. From trace amounts of DNA used as starting material (template), PCR produces exponentially larger amounts of a specific piece of DNA.

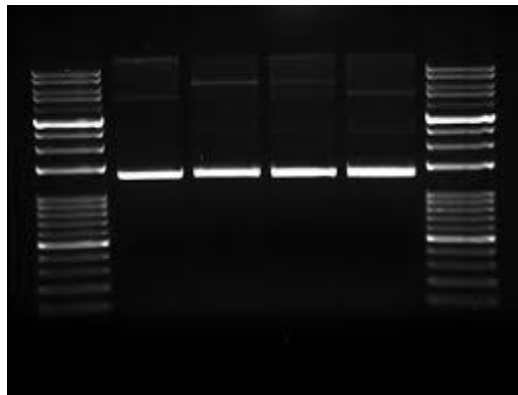


**Figure 1:** An illustration of how PCR works. 1) Double stranded DNA is denatured by heating to 95°C. 2) Primers anneal to the complementary DNA bases flanking the region of interest. 3) Taq polymerase extends the single stranded DNA molecule, creating new double stranded DNA. 4) The process is repeated about 40 times to amplify sufficient material for visualization on an agarose gel.

A few simple things happen quickly in a PCR reaction.

1. Denaturing – at 95°C, the double stranded template DNA is broken apart into two single strands.
2. Annealing – at 50°C to 65°C, primers attach to the homologous DNA sequence that they match. A primer is a short piece of DNA between 15 and 30 base pairs.
3. Extension – Taq polymerase begins adding nucleotides at 65°C and extends the DNA fragment.

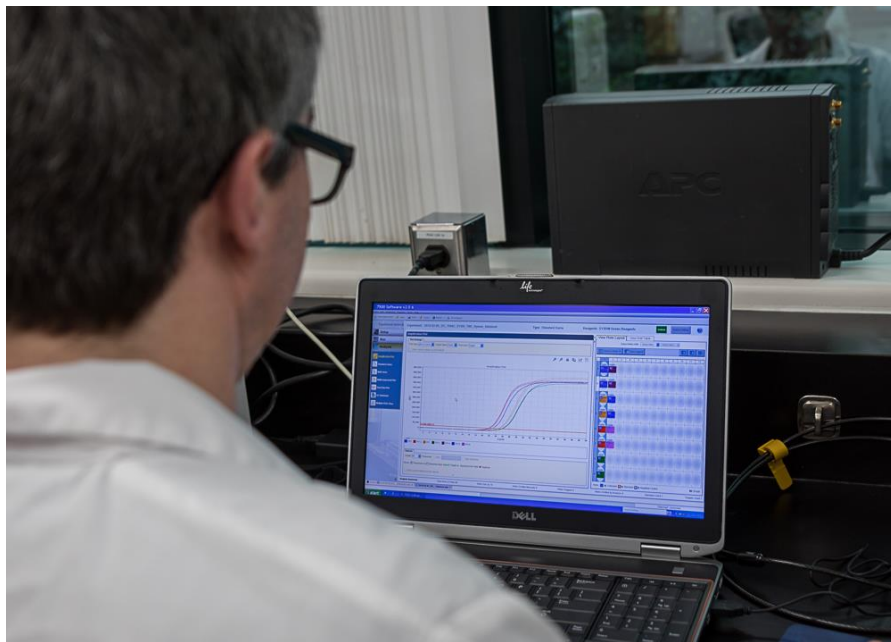
The cycles repeat about 40 times and at the end of each cycle, the DNA doubles 2, 4, 8, 16, 32, 64, 128, 256, 512 copies, etc. At the end of a PCR run, the products can be run on an agarose gel to analyze the DNA bands (Figure 2).



**Figure 2:** Visualization of amplified DNA on an agarose gel.

### Real-Time PCR or Quantitative PCR (qPCR)

The same principle of amplification is employed in qPCR, but instead of looking at bands on a gel at the end of the reaction, the process is monitored in real time. The reaction is placed into a real-time PCR machine that watches the reaction occur with a camera or detector. There are many different techniques used to allow the progress of a PCR reaction to be monitored, but they all have one thing in common. They all link the amplification of DNA to the generation of fluorescence, which can be detected with a camera during each PCR cycle. Therefore, as the number of gene copies increases during the reaction, the fluorescence increases as well.



**Figure 3:** A real-time PCR machine result. The machine monitors the increase of fluorescence in real time.

## Taqman<sup>®</sup> vs SYBR<sup>®</sup> Green

When designing a real-time PCR experiment, a significant decision is choosing the correct detection chemistry for your application. There are two major options to choose from. Most people use either an intercalating dye such as SYBR<sup>®</sup> Green or a hydrolysis probe based detection system such as Taqman<sup>®</sup>. Both technologies are designed to generate fluorescence during the PCR run, which allows the real-time PCR machine to monitor the reaction in real time (Figure 3).

### How SYBR<sup>®</sup> Green dye chemistry works

SYBR<sup>®</sup> Green dye detects polymerase chain reaction (PCR) products by binding to double-stranded DNA formed during PCR (Figure 4).

Denaturation - When the DNA is denatured, the SYBR<sup>®</sup> Green dye is released and the fluorescence is drastically reduced.

Polymerization – During annealing and extension, primers anneal and PCR product is generated.

Polymerization completed – When polymerization is complete, SYBR<sup>®</sup> Green dye binds to the double stranded product, resulting in an increase in fluorescence.

As the PCR progresses, more PCR product is created. SYBR<sup>®</sup> Green dye binds to all double-stranded DNA, so the result is an increase in fluorescence intensity proportional to the amount of PCR product produced.

### How Taqman<sup>®</sup> dye chemistry works (Figure 4)

1. An oligonucleotide probe is constructed containing a fluorescent reporter dye on the 5' end and a quencher dye on the 3' end. While the probe is intact, the proximity of the quencher dye greatly reduces the fluorescence emitted by the reporter dye.
2. If the target sequence is present, the probe anneals downstream from one of the primer sites and is cleaved by the 5' nuclease activity of *Taq* DNA polymerase as this primer is extended.
3. The cleavage of the probe separates the reporter dye from the quencher dye, increasing the reporter dye signal.
4. Additional reporter dye molecules are cleaved from their respective probes with each cycle, resulting in an increase in fluorescence intensity proportional to the amount of amplicon produced.

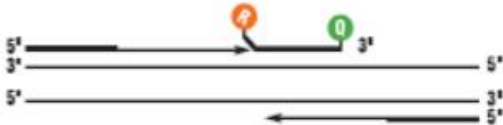


### TAQMAN® PROBE-BASED ASSAY CHEMISTRY

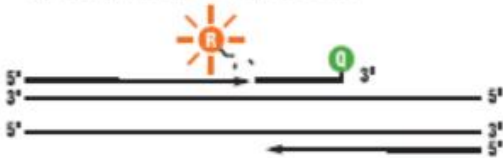
1. **Polymerization:** A fluorescent reporter (R) dye and a quencher (Q) are attached to the 5' and 3' ends of a TaqMan® probe, respectively.



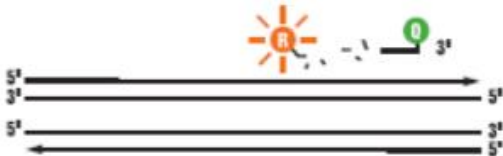
2. **Strand displacement:** When the probe is intact, the reporter dye emission is quenched.



3. **Cleavage:** During each extension cycle, the DNA polymerase cleaves the reporter dye from the probe.



4. **Polymerization completed:** Once separated from the quencher, the reporter dye emits its characteristic fluorescence.



### SYBR® GREEN I DYE ASSAY CHEMISTRY

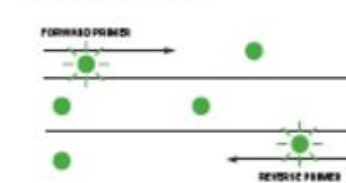
1. **Reaction setup:** The SYBR® Green I Dye fluoresces when bound to double-stranded DNA.



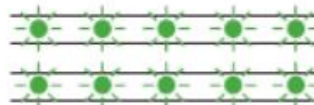
2. **Denaturation:** When the DNA is denatured, the SYBR® Green I Dye is released and the fluorescence is drastically reduced.



3. **Polymerization:** During extension, primers anneal and PCR product is generated.



4. **Polymerization completed:** When polymerization is complete, SYBR® Green I Dye binds to the double-stranded product, resulting in a net increase in fluorescence detected by the 7900HT system.



**Figure 4:** The comparison of SYBR® Green and Taqman® based chemistries.

### **Advantages of SYBR® Green dye**

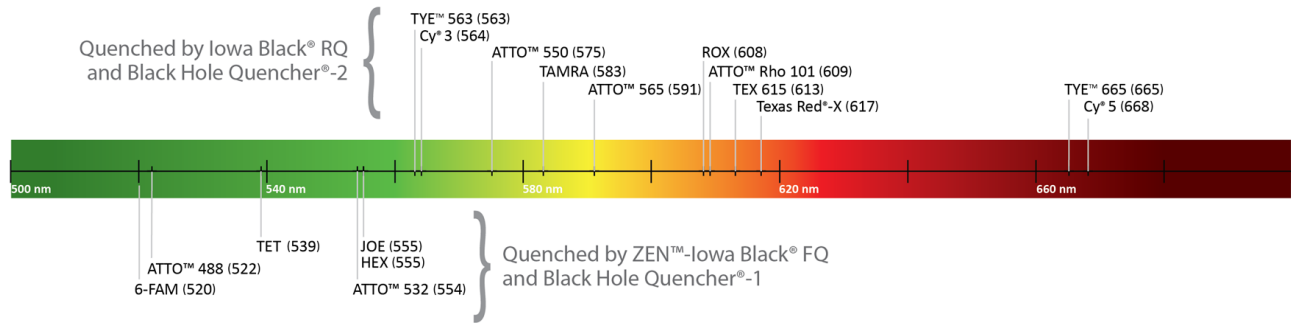
- It can be used to monitor the amplification of any double-stranded DNA sequence.
- No probe is required, which can reduce assay setup and running costs.

### **Disadvantages of SYBR® Green dye**

- The weakness of intercalating dyes such as SYBR® Green is that they are non specific. If your PCR amplifies the wrong target, or even more than one target, you will still get an amplification plot that looks identical to a genuine signal. Intercalating dyes will bind to and report on any double stranded DNA that is formed during the reaction regardless of what it is.
- Because of its inherent lack of specificity, intercalating dyes are less effective when your target of interest is rare. When a target is rare the primers are more likely to form primer dimers or to bind to the wrong target. Even the very best primers will eventually form a primer dimer and give a false signal after enough cycles of PCR if no authentic target of interest is present.

### **Advantages of Taqman®® Chemistry**

- Probes can be labeled with different, distinguishable reporter dyes, which allows amplification and detection of as many as four distinct sequences in one reaction tube (Figure 5).
- Probes are more convenient to work with due to their inherent specificity. When you get a signal from a hydrolysis probe, you can be certain that the signal has come from the amplification of your target sequence. It is only possible to get a signal when the primers and probe bind in the correct place, to the correct target.



**Figure 5:** Emission wavelengths of select dyes and their appropriate quenchers. For multiplexing, it is important to select reporter dyes with the least amount of spectral overlap. Therefore, to perform a 4-plex reaction, a good choice of dyes would be FAM, HEX, Texas Red and Cy5.

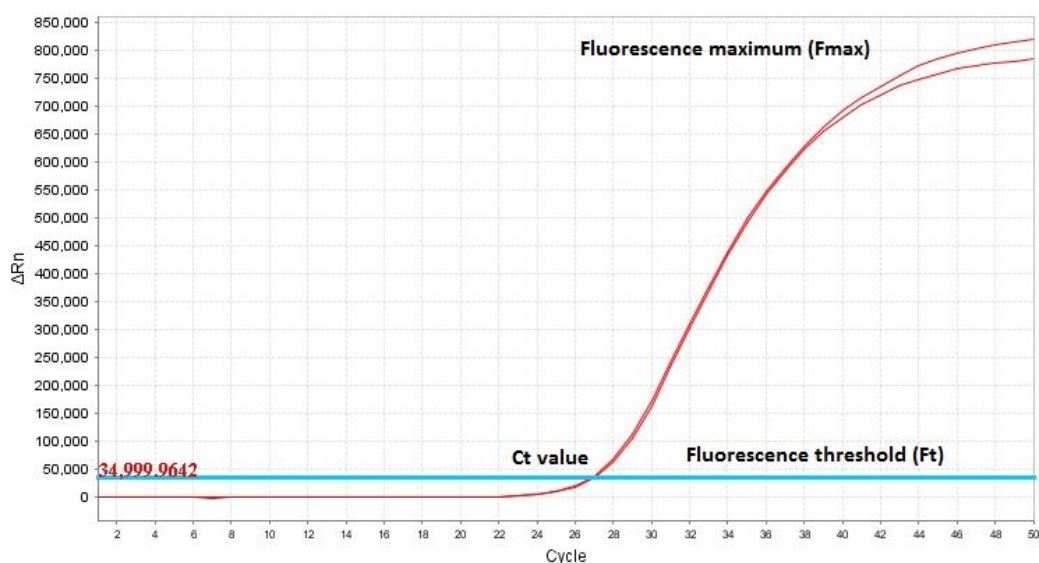
## Appendix B – SYBR Green Quantification

Before performing the Taqman<sup>®</sup> assays to identify the unknown egg masses, a SYBR Green amplification step is performed using a pair of *Lymantria* general primers designed in the cytochrome oxidase (COI) gene. This step is included for the following reasons;

- 1) It verifies that the DNA extraction was successful.
- 2) It allows us to dilute the DNA to a copy number that is optimal for the functioning of the AGM Taqman<sup>®</sup> assays.
- 3) By diluting all DNA samples to the same known Ct value (approximately 22), interpretation of the assay results are simplified. A positive Taqman<sup>®</sup> result is usually 3-5 cycles later than a SYBR Green Ct value. Ct values that are 30 or higher are generally due to cross amplification or cross contamination.

### Calculation of Ct Value

Cycle threshold (or Ct) value is determined by recording the cycle that the amplification curve crosses the fluorescent threshold (Ft) (see Figure 1). For purposes of uniformity, we set the Ft value at 5% of fluorescence maximum (Fmax).



**Figure 1:** Calculation of Ct value. Ct value is the cycle at which the amplification curve (red) crosses the fluorescence threshold (blue line). The fluorescence threshold or Ft is set at 5% of fluorescence maximum (Fmax). In this case, the Fmax value is approximately 800,000 and therefore the Ft value is about 40,000. The Ct value is approximately 27.

## Dilution of DNA

The dilution factor for the DNA is calculated as follows; each decrease of one Ct value represents the doubling of DNA copy number due to the nature of PCR (DNA is doubled during each PCR cycle). Therefore, for a Ct value of 15 the dilution calculation would be as follows;

Initial Ct value 15

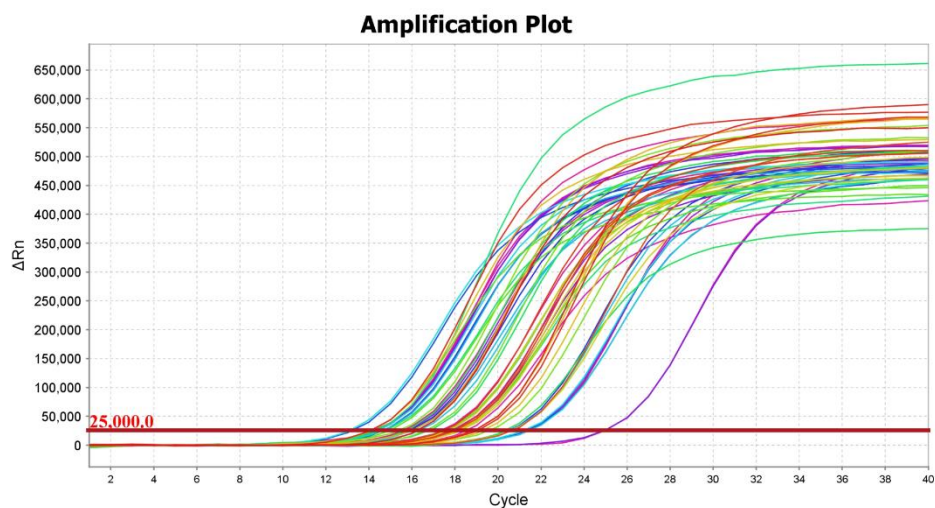
Target Ct value 22

Difference in Ct values  $22 - 15 = 7$  cycles.

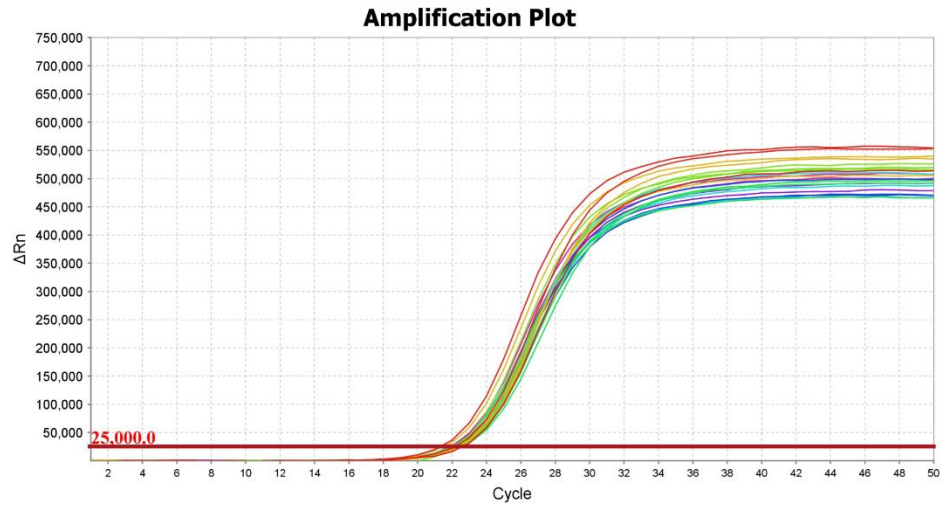
Therefore, to arrive at a Ct value of 22, the dilution factor would be  $2^7$  or  $2 \times 2 \times 2 \times 2 \times 2 \times 2 \times 2 = 128X$

So, for a Ct value of 22, the DNA would need to be diluted 128 times. To dilute the DNA 128X, you simply take  $1\mu\text{l}$  of DNA and dilute it with  $127\mu\text{l}$  of either  $\text{H}_2\text{O}$  or Tris pH 8. Pipette or vortex briefly to be sure that the diluted DNA sample is well mixed.

The two amplification plots below show the result of diluting DNA samples to a common Ct value.



**Figure 2:** A SYBR Green DNA amplification result from extracted moth DNA samples. Copy number varies by as much as 500X (9 cycles) for these extractions.



**Figure 3:** DNA has been diluted to a common Ct value and re-amplified.

## Appendix C – Understanding the “Molecular Key” Flowchart

The architecture of the qPCR assays resembles that of a standard taxonomic key, but one where genomic features substitute for morphological characters. The full assay is partitioned into three taxonomic subgroups (yellow shaded boxes): (i) AGM complex, (ii) EGM and (iii) other threatening lymantriine species (OTLS). Each subgroup comprises several independent assays that are run in duplex or triplex mode, for a total of two assay tubes per subgroup (red boxes) and six tubes for the whole assay when all reactions are run in parallel. For the purpose of this demonstration, we will only consider the first two subgroups, AGM complex and EGM. The flowchart can be viewed below (Figure 1) and a brief description of each assay has been included to facilitate the understanding of the process.

**Duplex Assay 1A:** Is it *L. albescens*/*L. postalba*? This is the first of two assays designed to determine whether the unknown is a member of the AGM complex. It targets SNPs that are unique to the *L. albescens*/*L. postalba* species pair, which are here treated together because of the high degree of sequence identity displayed by their COI-5P regions.

**Duplex Assay 1B:** Is it *L. dispar asiatica*/*L. dispar japonica*/*L. umbrosa*? This assay aims at detecting the presence of any of the three remaining members of the AGM complex in a single qPCR step. If neither assay 1A nor assay 1B produces a positive amplification, then the unknown is not AGM and the molecular key bifurcates to the EGM assay.

**Duplex Assay 2A:** Is it *L. dispar asiatica*/*L. dispar japonica*? This assay provides discrimination between *L. umbrosa* and the two Asian *L. dispar* subspecies: a negative amplification identifies the unknown as *L. umbrosa*, while a positive result detects the presence of either *L. dispar asiatica* or *L. dispar japonica*.

**Duplex Assay 2B:** Is it *L. dispar asiatica*? If the previous assay indicated that the unknown is either *L. dispar asiatica* or *L. dispar japonica*, assay 2B will generate amplification only if the sample is *L. dispar asiatica*; absence of amplification implies that the unknown is *L. dispar japonica*.

**Duplex Assay 3A:** Is it *L. dispar dispar*? When the above-described Duplex assay 1 generates a negative result for the AGM complex, the molecular key then tests the hypothesis that the unknown is *L. dispar dispar*. It must be pointed out here that the design of this assay does not take into account any AGM complex sequences, as this possibility is already eliminated at this intersection of the molecular key.

**Duplex Assay 3B:** Is it “Caucasian” Gypsy moth? There are a population of moths in the Caucasus region that were identified as *L. dispar dispar* with our assay, but are clearly different from *L. dispar dispar*. The addition of this assay will distinguish them from *L. dispar dispar*.

**Duplex Assays 4A and 4B:** Does *L. dispar dispar* show evidence of Asian introgression? Previous studies have reported the existence of gypsy moth populations, primarily from Central Asia, that feature mitochondrial DNA sequences diagnostic of *L. dispar dispar* while displaying biological characteristics that are typical of AGM, including flight capability in females. From a regulatory standpoint, such insects may need to be treated as AGM. To assess the occurrence of Asian introgression into EGM, we made use of the FS1 nuclear marker, for which North American (“N”) and Asian (“A”) alleles have been described. If only Duplex 4B produces a positive result then the *L. dispar dispar* specimen is considered to be homozygous North American (N). If only Duplex 4A produces a positive result then it is homozygous Asian (A) and if both Duplex 4A and 4B are positive then the specimen is heterozygous North American/Asian (N/A).

#### **Other Threatening *Lymantria* Species (Duplex Assays 5A, 5B and Triplex Assays 6A, 6B and 6C)**

In the cases where Duplex assay 3A (Is it *L. dispar dispar*?) produces no amplification, the dichotomic key redirects the identification process to the “other threatening *Lymantria* species” (OTLS) subgroup. The Duplex assay 5 and triplex assay 6 described below may be regarded as forming a single multiplex assay where each individual assay need not be treated in a sequential fashion; separation of these two assays and the sequential presentation of individual assays are done for convenience only.

**Duplex Assay 5A:** Is it *L. monacha*? Duplex 5A will generate a positive result only if the specimen is *L. monacha*.

**Duplex Assay 5B:** Is it *L. fumida*? Duplex 5B will generate a positive result only if the specimen is *L. fumida*.

**Triplex Assay 6A:** Is it *L. mathura*? Triplex 6A will generate a positive result only if the specimen is *L. mathura*.

**Triplex Assay 6B:** Is it *L. xyliana*? Triplex 6B will generate a positive result only if the specimen is *L. xyliana*.

**Triplex Assay 6C:** Is it *L. lucescens*? Triplex 6C will generate a positive result only if the specimen is *L. lucescens*.



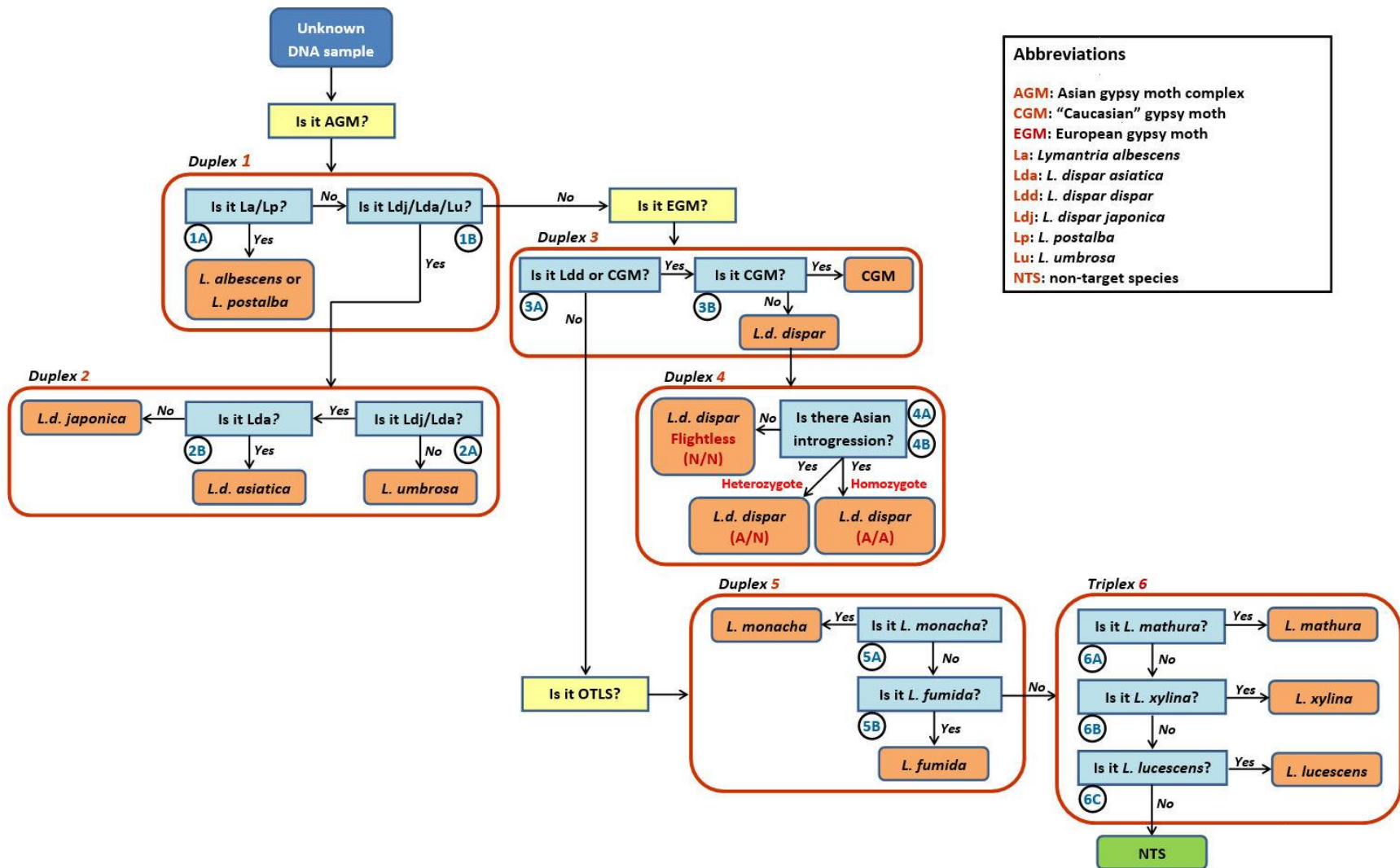
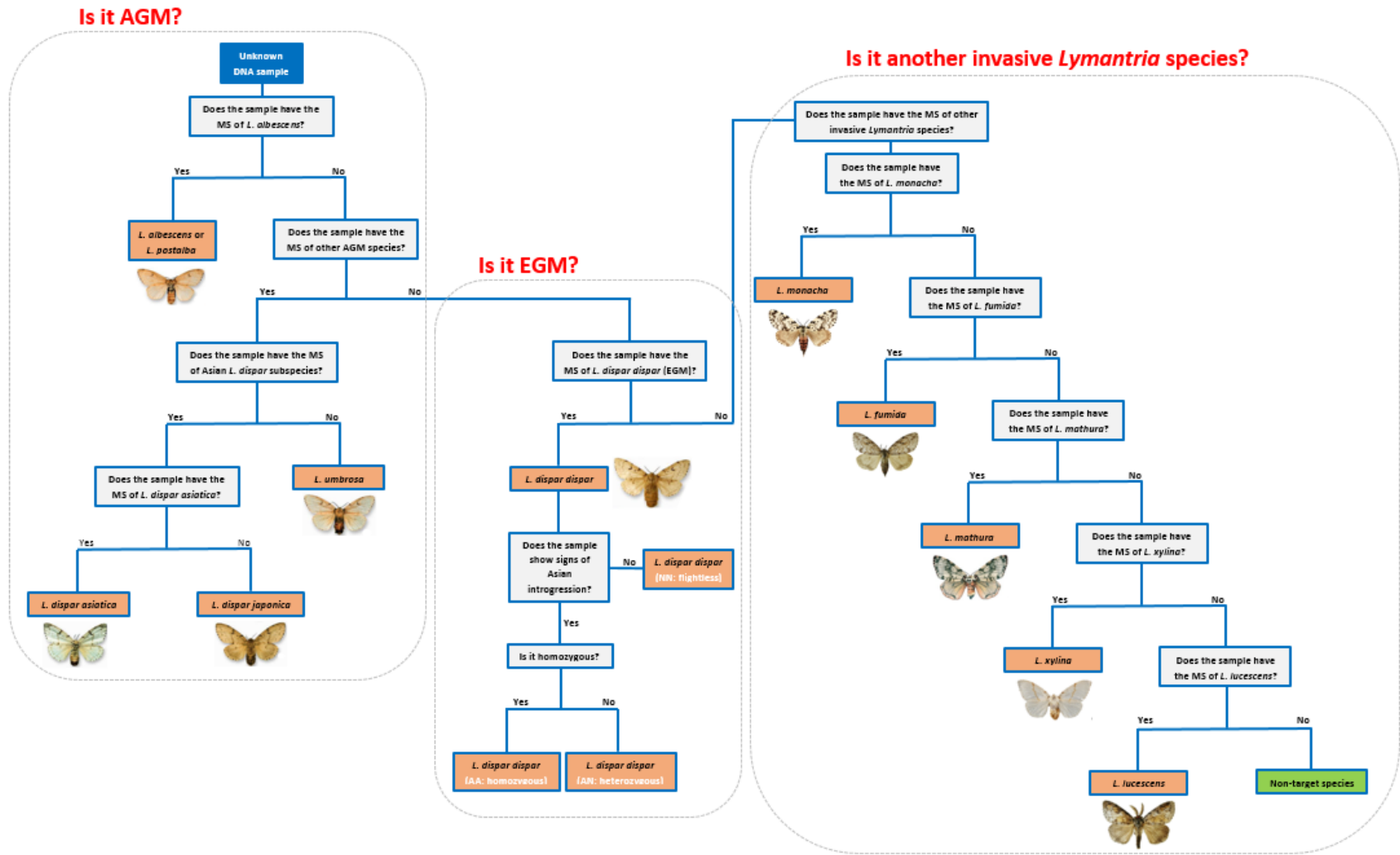


Figure 1: "Molecular key" flowchart for the AGM Taqman® suite of assays.



**Figure 2:** Alternate key for the AGM Taqman<sup>®</sup> suite of assays. MS = molecular signature. The CGM branch of the assay is not present in this representation.

## Appendix D – Species ID Tool

The species ID tool is an Excel based program that uses the Taqman® assay results to identify the unknown samples. You simply enter a y or n (yes or no) in the column for each corresponding assay and it will provide you with a sample species identification. Assays Duplex 1 and Duplex 2 will identify any AGM moths and Duplex 3 will identify any *L. dispar dispar* samples that tested negative in the Duplex 1 and Duplex 2 assays. Duplex 4 will determine whether or not any Asian introgression has occurred in the *L. dispar dispar* specimens

Sample	Duplex 1A	Duplex 1B	Duplex 2A	Duplex 2B	Identification AGM complex
	<i>L. albescens</i> / <i>L. postalba</i> COI	AGM complex COI	Ldaj COI	Lda COI	
1	y	n	n	n	<i>Lymantria albescens/postalba</i>
2	n	y	n	y	<i>Lymantria umbrosa</i>
3	n	y	y	n	<i>Lymantria dispar japonica</i>
4	n	y	y	y	<i>Lymantria dispar asiatica</i>
5	n	n	n	n	Not AGM complex

Sample	Duplex 3A	Duplex 3B	Duplex 4A	Duplex 4B	Identification <i>L. dispar dispar</i>
	Ldd COI	Caucasian ND1	Ldd AGM FS1	Ldd EGM FS1	
1	y	n	n	y	<i>Lymantria dispar dispar homozygous EGM</i>
2	y	y	y	y	<i>Caucasian gypsy moth isolate</i>
3	y	n	y	y	<i>Lymantria dispar dispar heterozygous AGM/EGM</i>
4	y	n	y	n	<i>Lymantria dispar dispar homozygous AGM</i>
5	n	n	n	y	Not <i>Lymantria dispar dispar</i>

**Figure 1:** Species ID tool for the AGM Taqman® assay. By simply entering a y or n for a positive or negative result, this tool will identify the species of your unknown sample. The ID tool for Duplex 5 and Triplex 6 has been left out of this figure.

## Appendix E – Taqman® Assay Primer and Probe List

**Table 1:** DNA quantification primers for Lymantriine species and Taqman® Assay primer sequences.

Assay	Assay #	Primer Name	Primer Sequence
DNA quantif.	Lymantriine general primers	Lep COI GEN F308-327	GAAAATGGAGCIGGWACWGG
		Lep COI GEN R413-435	GCTCCTAAAATWGAIGAAATWCC
Duplex 1	1A	Lalb COI F491-520	CAAATACCTTTATTGTTTGAAGAGTAAGT
		Lalb COI R562-590	GGTCAGTTAATAATATTGTAATAGCACAC
	1B	AGM comp COI F601-622	TACATCCTTTTTGACCCYACR
		AGM comp COI R701-719	TCCTCTTCTTGGGAAATA
Duplex 2	2A	Ldaj COI F325-342	AGGATGAAGTGTATACCC
		Ldaj COI R428-454	GGTAGTAATAAAAATTAATTGACTCTAA
	2B	Lda COI3P F764-783	AGCTATCGGATTATTAGGATT
		Lda COI3P R803-832	GTATCAATATCTATACCYACAGTAAATATG
Duplex 3	3A	Ldd COI F488-513	GATCAAATACCTTTATTTRITTTGAAG
		Ldd COI R574-607	GGATGTATTTAAATTTCCGGTCAGTTAATAATGTT
	3B	Ldd CAUC ND1 F379-405	GGTCTTCTAATTCAAATTATGCT
		Ldd CAUC ND1 R437-463	TAGACAATCTAACTTCATAAGAAATAG
Duplex 4	4A-4B	FS1 F2-16	GATGGTGGGTGTCGT
Duplex 5	5A	FS1 R176-200 (71-95)	GATTCATCTGATCTGATAATTCAT
		Lmon COI F183-205	GATTTGGAAATGATTAGTACCT
	5B	Lmon COI R274-300	TCTTGAAATTAAGGGTTAATGAT
		Lfum COI F445-472	TATTACAACAATTATCAATATACGACTT
Triplex 6	6A	Lfum COI R628-651	TGTTGATATAAAATAGGATCTCCA
		Lmat COI F44-67	ACTTCYCTTAGATTRTTAATTCGC
	6B	Lmat COI R214-230	CTATATCAGGTGCTCCA
		Lxyl COI F335-355	GTTTACCCCTTTATCATTG
	6C	Lxyl COI R421-451	AGTAATAAAATTAATTGCCCTAAAATTGTG
		Lluc COI F455-81	ATCATTAAATGCGATTAACATAATCTT
		Lluc COI R631-652	ATGTTGATAGAGAATTGGATCA

**Table 2:** Taqman® assay probe sequences.

Assay	Assay #	Probe Name	Probe Sequence	Fluorophore
Duplex 1	1A	Lalb COI T 525-552	CAGCTTTCCTTCTACTTTTATCATTACC	Cy5-Tao
	1B	AGM complex COI T 636-649	C+AAT+C+CTTT+A+C+CAA	Fam-LNA
Duplex 2	2A	Ldaj COI T RC 411-424	T+GAT+GAAA+T+A+C+CAG	Fam-LNA
	2B	Lda COI3P T RC 788-797	AG+C+C+CA+AA+CA	Cy5-LNA
Duplex 3	3A	Ldd COI T531-560	TCCTTCTACTTTTATCCTTTACCTGTTTTAG	Cy5-Tao
	3B	Ldd CAUC ND1 R/C T406-417	ACC+TC+C+CAA+C+AA	Fam-LNA
Duplex 4	4A	FS1 AGM T62-86	ACTCAACATAAAGTATGCCAACTCG	Fam-Zen
	4B	FS1 EGM T RC 21-47	AGTACTGCTGTATACATTTTAAACGTC	Cy5-Tao
Duplex 5	5A	Lmon COI T221-234	C+C+A+GATATAG+C+C+TT	Fam-LNA
	5B	Lfum COI T532-560	TCTTTTACTCCTATCTTTACCCGTTTTAG	Cy5-Tao
Triplex 6	6A	Lmat COI T116-139	AATACCATTGTTACAGCTCATGCT	Tex-615
	6B	Lxyl COI T381-412	TAGATTTAGCTATTTTTCCCTTCATTTAGCT	Cy5-Tao
	6C	Lluc COI T532-561	TTTACTCTTCTTCTTACCTGTTTTAGC	Fam-Zen

## Appendix F – Species Discrimination List

Below is a list of Lymantriid species whose egg masses can be discriminated with the AGM Taqman® assay. Species highlighted in yellow can be identified using the Taqman® assays and the other species will be discriminated.

*Lymantria albescens*

*Lymantria postalba*

*Lymantria dispar japonica*

*Lymantria dispar asiatica*

*Lymantria umbrosa*

*Lymantria dispar dispar*

*Lymantria fumida*

*Lymantria lucescens*

*Lymantria mathura*

*Lymantria monacha*

*Lymantria xyliana*

*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*

## **Appendix G - DNA Extraction Protocol for Insect Legs**

### **(Qiagen DNeasy Blood and Tissue Kit)**

- 1) If the samples are in ethanol, take them out at least two hours prior to starting the extraction and let them dry on a piece of paper towel. Place two moth legs in 180  $\mu$ l of Buffer ATL in a 1.5 ml conical tube and homogenize with a microtube pestle.
- 2) Add 20  $\mu$ l Proteinase K stock solution (20mg/ml) and incubate at 55°C for 1-3 hours (2 hours is sufficient). Mix tube occasionally while incubating.
- 3) Add 200  $\mu$ l Buffer AL to the tube. Mix immediately by vortexing for 15 seconds.
- 4) Incubate at 70°C for 10 min.
- 5) Add 200  $\mu$ l of 100% ethanol to the sample, and mix again by vortexing.
- 6) Place a Qiagen spin column in a 2 ml collection tube. Carefully place the contents of the conical tube into the Qiagen spin column, close the cap and centrifuge at 8000 rpm for 1 minute. Discard the collection tube and place the Qiagen spin column in a clean 2 ml collection tube.
- 7) Add 500  $\mu$ l Buffer AW1 to the spin column. Centrifuge at 8000 rpm for 1 minute. Discard the filtrate and place the Qiagen spin column back into the 2 ml collection tube.
- 8) Add 500  $\mu$ l of Buffer AW2 and centrifuge at 8000 rpm for 1 minute.
- 9) Discard the filtrate and place the Qiagen spin column back into the 2 ml collection tube. Centrifuge at 8000 rpm for 3 minutes.
- 10) Place spin column in a new 1.5ml conical tube and discard the collection tube containing the filtrate.
- 11) Elute the DNA with 200  $\mu$ l of Buffer AE (or 50  $\mu$ l for old dry samples) by incubating at room temperature for 1 minute and then centrifuging at 8000 rpm for 1 minute.
- 12) Use 2  $\mu$ l for PCR.