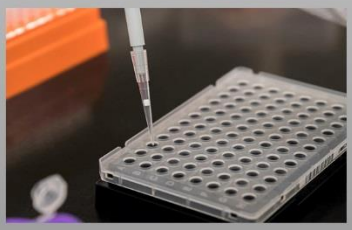

Asian Gypsy Moth (AGM) Taqman Detection Workshop

Part II: *Detection from bulk pheromone trap samples*

Québec, September 18-19, 2018
Laurentian Forestry Centre

1055 rue du PEPS, Québec, G1V 4C7

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DNA Extraction Protocol for Bulk Leg Samples

The DNA extraction is performed using a modified version of the Qiagen DNeasy Blood and Tissue Mini Kit protocol.

1) Place up to 100 moth legs (30-50mg tissue) in a 2ml round bottom Eppendorf safe-lock tube containing a 5mm stainless steel bead.
2) Flash freeze the tube in liquid nitrogen and place at -80^{o}C in a pre-chilled Tissuelyser block.
3) Disrupt the tissue using a Qiagen Tissuelyser machine at a speed of 26 Hz for 60 seconds to produce a fine powder.
4) Add 1080ul of buffer ATL and 120ul of proteinase K solution to the tube and vortex.
5) Incubate samples overnight at 56^{o}C with constant gentle mixing.
6) After incubation, vortex samples and transfer the contents of the tube to a 15ml polypropylene Falcon tube.
7) Add 1.2ml buffer AL to the tube and vortex.
8) Add 1.2ml 100% ethanol and vortex again.
9) For each sample, transfer the lysate (approximately 4ml) to two 2ml Eppendorf safe-lock tubes and centrifuge at 5000 rpm for 1 minute to pellet the debris.
10) Add the cleared lysates to two DNeasy mini spin columns in three steps of 600ul and centrifuge for 1 minute at 8000 rpm after each addition of lysate. Discard the flow-through after each spin.
11) Place the DNeasy spin columns in a new 2ml collection tube, add 500ul of AW1 and centrifuge at 8000 rpm for 1 minute. Discard the flow-through.
12) Place the DNeasy spin columns in a new 2ml collection tube, add 500ul of AW2 and centrifuge at 14,000 rpm for 3 minutes to dry the DNesasy membrane. Discard the flow-through.
13) Place the DNeasy spin columns in clean 1.5 ml microcentrifuge tubes, add 150ul buffer AE directly onto each DNeasy membrane and incubate at room temperature for 1 minute.
14) Centrifuge at 8000 for one minute to elute the DNA.
15) Take the eluate from each tube and pass through the spin column a second time to increase the DNA yield.
16) Combine the eluates from the two tubes to obtain a final volume of 300ul DNA.
17) Quantify DNA on a Nanodrop ND-100 spectrophotometer.

Bulk Extraction DNA Sample List

Table 1: DNA samples used for bulk extraction (mixed sample) Taqman® AGM assay demonstration

Sample Number	Sample Name	Sample ID	Location
1	CFL-	*L. dispar dispar*	Québec
2	CFL+	*L. dispar dispar/L. umbrosa*	Québec/Japan
3	VIC-	*L. dispar dispar*	Québec
4	VIC+	*L. dispar dispar/L. albescens*	Québec/Japan
5	NDL-	*L. dispar dispar*	Québec
6	NDL+	*L. dispar dispar/L. dispar japonica*	Québec/Japan
7	DEN-	*L. dispar dispar*	Ontario
8	DEN+	*L. dispar dispar/L. dispar asiatica*	Ontario/China
9	NTC	water	n/a

L. dispar dispar DNA samples CFL, VIC and NDL were collected in the province of Québec. *L. dispar dispar* DNA sample DEN was collected in the province of Ontario. The bulk samples either consist of 100 *L. dispar dispar* legs (CFL-, VIC-, NDL-, DEN-) or 99 *L. dispar dispar* legs and one leg from either *L. umbrosa, L. albescens, L. dispar asiatica* or *L. dispar japonica* (CFL+, VIC+, NDL+, DEN+). The DNA samples have been normalized to 250,000 copies/ul COI for the purpose of the assay. 2ul of DNA or 500,000 copies of COI are used for the assays.

*Due to time constraints for this workshop, we will not be performing a DNA extraction or SYBR Green quantification reaction for the bulk trap samples. We will be using previously extracted and normalized DNA samples.

SYBR Green Copy Number Quantification – PCR Protocol

Before performing the Taqman® assays to determine if AGM is present in the bulk samples, 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 bulk sample Taqman® assays. In this case, the DNA will be diluted to approximately 250,000 copies of COI/ul DNA or a Ct value of approximately 14.5.

Protocol

We will be amplifying nine samples for this exercise. There will be eight previously extracted DNA bulk samples and a no template control (NTC). There will be two technical replicates for each sample for a total of 18 reactions. We will also add an extra 10% volume to each mastermix component to be sure that there is enough for all reactions.

Table 2: SYBR Green amplification master mix

Reaction Component	Volume per well (µl)	Total volume (µl)
Quantitect SYBR Green 2X master mix	5	100
Primer mix 10X (5 µM)	1	20
Water	2	40
DNA	2	n/a

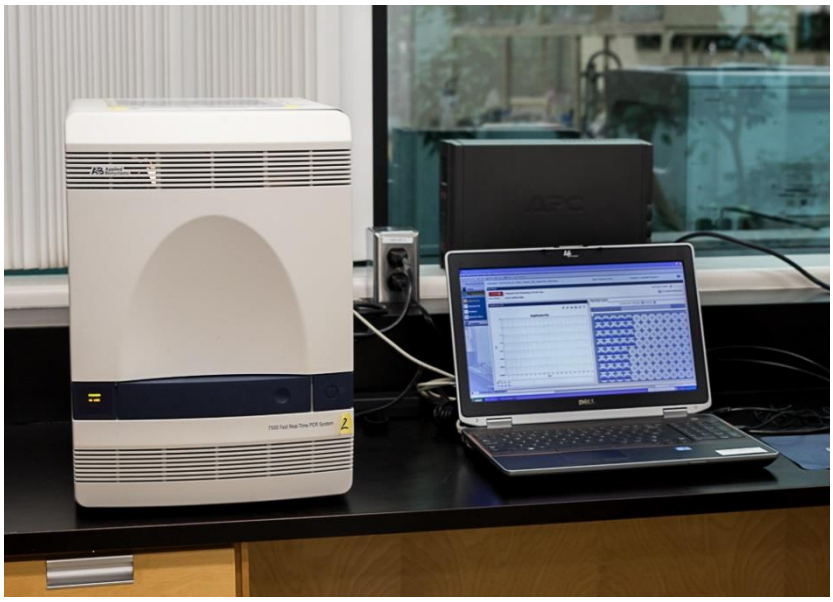
Place a 96 well optical plate in a support base so that it does not come into contact with the benchtop surface. Pipette 8 µl of master mix and 2 µl of DNA into each of the 18 wells of the 96 well PCR plate. Seal the plate with a sheet of optical adhesive film and centrifuge the plate for 30 seconds.

PCR Conditions

Table 3: 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		58°C	30 seconds
Extension		65oC	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 in the AGM detection from egg mass Taqman® workshop guide.

Taqman® AGM Bulk Sample Assay – PCR Protocol

This Taqman® assay consists of a single master mix. It is a triplex reaction, which means that there are three separate assays in the 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 in the AGM detection from egg mass Taqman® workshop guide.

Protocol

We will be amplifying nine samples for the Taqman® assay. These are the same nine 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 18 reactions. We will also add an extra 10% volume to each master mix component to be sure that there is enough for all reactions.

Table 4: Master mix for the bulk sample triplex assay. This assay will detect *L. albescens/L. postalba*, *L. umbrosa, L. dispar asiatica* and *L. dispar japonica* in a background of *L. dispar dispar*.

Reaction Component	Volume per well (µl)	Total volume (µl)	Fluorophore
Quantitect multiplex 2X master mix	5	100	
Primer/probe mix 20X Lumb ND1	0.5	10	HEX
Primer/Probe mix 20X Lalb COI	0.5	10	Cy5
Primer/Probe mix 20X Ldaj cytb	0.5	10	FAM
Water	1.5	30	
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 µl of master mix and 2 µl of DNA into each of the 18 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 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

Analyzing the results of the bulk sample triplex assay is straightforward. There is a single triplex assay to analyze. Figure 1 shows an example of a bulk sample Taqman® assay result.

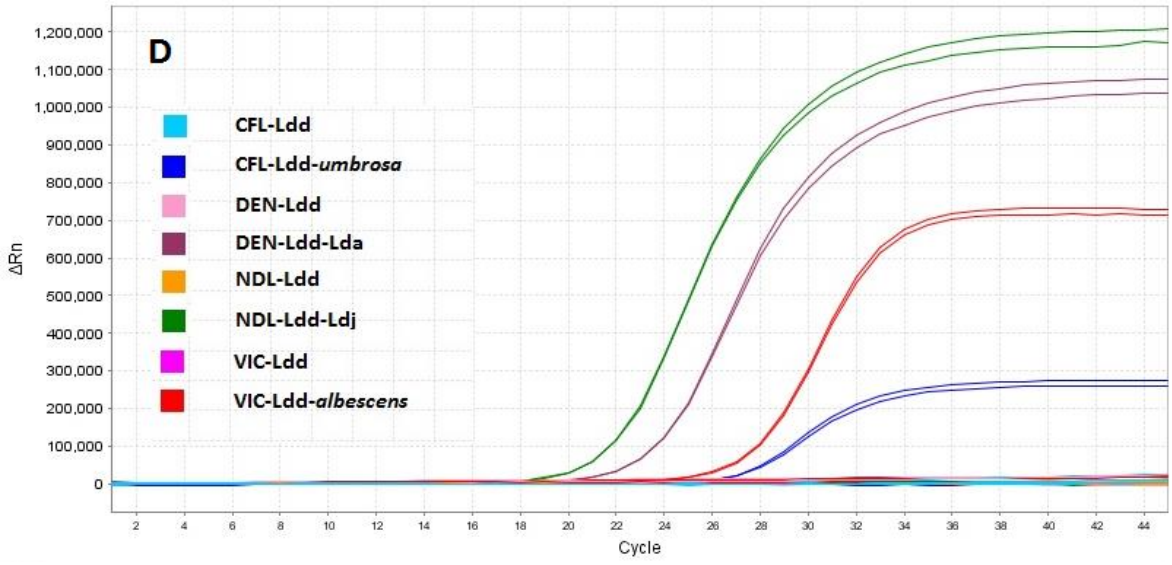


Figure 1: Results from a bulk sample triplex assay run. The blue curves show a positive result for the presence of *L. umbrosa* in a mixed sample, the red curve for *L. albescens/L. postalba* and the burgundy and green curves show a positive result for *L. dispar asiatica*/*L. dispar japonica*.

Appendix A – Taqman® Bulk Sample Assay Primer and Probe List

Table 1: DNA quantification primers for bulk DNA samples and Taqman®® Assay primer sequences. The DNA quantification primers will amplify primarily *L. dispar dispar* DNA but also *L. dispar asiatica, L. dispar japonica* and *L. umbrosa.*

Assay	Assay #	Primer Name	Primer Sequence
DNA quantification	AGM General Primers	Lep COI GEN F306-327	TAGAAAATGGAGCAGGAACAGG
		Lep COI GEN 17W R399-424	TGATGAAATWCCAGCTAAGTGAAGAG
Triplex 1	1A	Lalb COI F497-520	CCTTTATTTGTTTGAAGAGTAGGT
		Lalb COI R562-585	GTTAATAATATTGTAATAGCACCC
	1B	Ldaj cytb 3A/T F120-150	GGATCTTTGTTAGCTTTATGTTTAATTTCC
		Ldaj cytb R339-369	TCCAATTATTCATGTTTGTTTTAAATTAAAA
	1C	Lumb ND1 3C/A F233-262	TATTTTTCTCCTGTATTAGCTTTTGATA
		Lumb ND1 2C/T R375-395	GAATTAGAAGACCATCCTGTC

Assay	Assay #	Probe Name	Probe Sequence	Fluorophore
Triplex 1	1A	Lalb COI T525-552	CAGCTTTCCTTCTACTTTTATCATTACC	Cy5-Tao
	1B	Ldaj cytb T262-273	CT+CT+TC+A+C+G+CT	FAM-LNA
	1C	Lumb ND1 R/C T340-358	AC+A+CTATAAA+C+T+CCAAAAC	HEX-LNA