# Development of a Loop-Mediated Isothermal Amplification Assay as an Early-Warning Tool for Detecting Emerald Ash Borer (Coleoptera: Buprestidae) Incursions

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Research



# Development of a Loop-Mediated Isothermal Amplification Assay as an Early-Warning Tool for Detecting Emerald Ash Borer (Coleoptera: Buprestidae) Incursions

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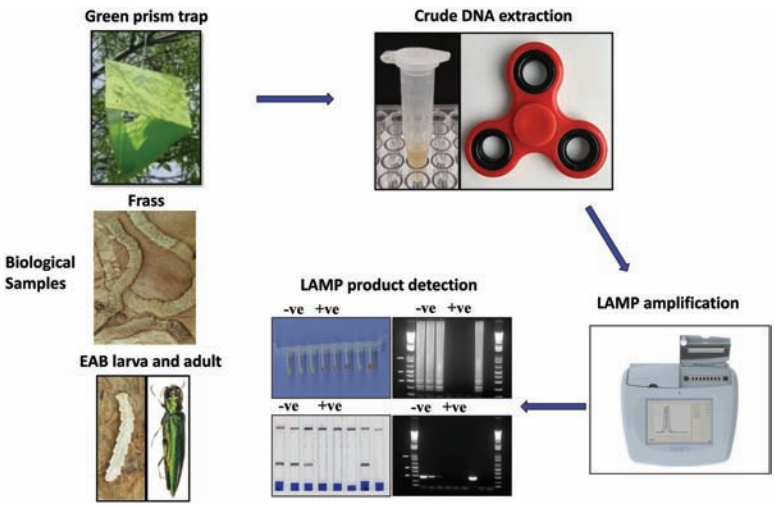
## Abstract

The emerald ash borer (EAB), *Agrilus planipennis* (Fairmaire), is the most destructive invasive insect species of ash (*Fraxinus* spp.) in North America. An accurate method for early detection of this noxious insect pest is indispensable to providing adequate warning of *A. planipennis* infestation. A loop-mediated isothermal amplification (LAMP) assay (EAB-LAMP) was developed based on mitochondrial cytochrome c oxidase subunit I (COI) gene. The EAB-LAMP required only 30 min at 65°C to amplify *A. planipennis* DNA from specimens collected from geographically distinct locations. There was no cross-reactivity with other *Agrilus* and insect species. The developed EAB-LAMP differentially detected traces of *A. planipennis* genome (COI) within frass from various *Fraxinus* species. EAB-LAMP was also able to distinguish among *A. planipennis* DNA and other *Agrilus* species and nontarget insect species in trap captures. By detecting *A. planipennis* DNA in two additional trap captures (in situ), the EAB-LAMP was more sensitive and reliable than visual inspection. We tested the quantitative nature of the assay by evaluating pooled trap samples and demonstrated that the EAB-LAMP was capable of functioning optimally using a pool size of at least five individual trap samples. This potentially circumvents the need to perform large-scale individual analysis for processing trap samples. Considering its performance, specificity, sensitivity, and repeatability, the developed EAB-LAMP could be a valuable tool to support strategy and operation of large-scale surveillance for *A. planipennis* and could profitably be used in routine monitoring programs for effective management of *A. planipennis*.

## Graphical Abstract




**Key words:** *Agrilus planipennis*, COI gene, loop-mediated isothermal amplification, frass, traps

The emerald ash borer (EAB), *Agrilus planipennis* (Fairmaire; Coleoptera: Buprestidae), is a wood-boring beetle native to Asia (China, Japan, Korea, Mongolia, and eastern Russia) that was accidentally introduced to North America. *Agrilus planipennis* was initially discovered in southeast Michigan, United States, in 2002 (Haack et al. 2002). To date, *A. planipennis* has been detected in 35 states in the United States and five provinces in Canada (USDA-ARS, EABInt Maps, 2018 and Canadian Food Inspection Agency 2018); the risk of spreading into new locations is still very high. *Agrilus planipennis* has caused widespread devastation to North American and European ash species (Knight et al. 2013) and it is seriously endangering the existence of North American ash (*Fraxinus*) species. There are 20 species of ash species found in North America; *A. planipennis* attacks and causes mortality to the main ash species thriving in Canada, namely, green ash (*Fraxinus pennsylvanica*), white ash (*F. americana*), black ash (*F. nigra*), and the much less common blue ash (*F. quadrangulata*) and pumpkin ash (*F. profunda*) (ISCC 2019).

Although concerted efforts have been made to contain *A. planipennis* infestations, it is estimated that this wood-boring beetle could damage or kill over 8 billion trees in the next decade (Mercader et al. 2015). It has been estimated that the costs of treating or removing approximately half of the infested ash trees growing on municipal property in urban and suburban communities may exceed $20 billion per year by 2019 (Kovacs et al. 2010). Besides the envisaged economic impact from *A. planipennis* infestation, the loss of certain ash species, especially black ash, could have a devastating impact ecologically, resulting in dramatic shifts in riparian ecosystem structure and function (Telander et al. 2015).

Avoiding incursions of alien species, as well as their establishment and spread, is fundamental to preventing these detrimental effects; hence, accurate and rapid detection and identification of potentially invasive species is extremely important (Rabaglia et al. 2008, Rassati et al. 2015, Brockerhoff et al. 2017). Currently, established EAB surveillance and monitoring techniques include finding larvae developing under the bark of ash trees, and the use of green prism/ green-leaf volatile lure for entomological trapping and branch sampling systems. These established methods and tools are considered to be reliable for detection of incipient *A. planipennis* populations and have been deployed to survey, detect and monitor *A. planipennis* populations by the United States Department of Agriculture (USDA) and Canadian Forest Service (CFS) researchers (Cappaert et al. 2005, McCullough et al. 2015, Ryall 2015). However, monitoring of nascent *A. planipennis* populations can be difficult for obvious reasons; all immature life stages are cryptic and it takes several cycles for signs and symptoms of *A. planipennis* infestations to become apparent (Haack et al. 2002, Cappaert et al. 2005, Poland and McCullough 2006). Additionally, the deployment of established conventional surveillance and monitoring methodologies can be expensive and time-consuming, and more importantly, are not capable of providing real-time information on *A. planipennis* incursions, thereby limiting their practical value to reactive management. Furthermore, in situ trap captures to detect EAB in ash plantations have frequently included other *Agrilus* and insect species (G.K-P., personal observation). In such instances, entomological expertise is required to differentiate among trapped *Agrilus* species. Moreover, the time lapse between trapping specimens, and sorting and identifying them can hamper containment efforts and the ability to effectively manage *A. planipennis* incursions into new locations. Therefore, detection and identification methods that are directly applicable in the speedy processing of field-collected samples would allow more efficient surveillance and monitoring, establishment of quarantine zones and implementation of necessary measures and strategies for *A. planipennis* management.

There are currently no molecular biology-based methods for early detection and identification of incipient *A. planipennis* populations, especially during incursions into new locations. Therefore, to complement the existing traditional surveillance and monitoring methods and tools, our aim was to develop a simple, fast, sensitive, specific, cost-effective, and robust field-deployable molecular assay that is reliable for effective early detection and identification of *A. planipennis*.

Our approach to meet this goal was to take advantage of recent innovations in nucleic acid synthesis and detection using isothermal conditions using loop-mediated isothermal amplification technique (LAMP) (Notomi et al. 2000). LAMP has revolutionized molecular biology and exhibits several significant advantages over conventional polymerase chain reaction (PCR). LAMP assays have a high level of efficiency and specificity and produce large amounts of DNA under isothermal conditions normally ranging from 60 to 65°C for 60 min (Notomi et al. 2000, Nagamine et al. 2002). The amplification does not require sophisticated equipment as the reaction can be performed in a simple water bath or heat block under isothermal conditions (Notomi et al. 2000). Unlike PCR tests, LAMP has shown high tolerance to inhibitors in biological matrices, so DNA purification may not be necessary (Kaneko et al. 2007).

LAMP assay products can be observed through gel electrophoresis and visual inspection after the addition of color-changing reagents (e.g., SYBR Green I; Iwamoto et al. 2003). Additionally, lateral flow devices (LFD) can detect labels incorporated into the products after amplification (Kiatpathomchai et al. 2008) or monitored in real-time via the use of fluorescent dyes with tube scanners (Lucchi et al. 2010, Njiru et al. 2011, Zhang et al. 2014). LAMP amplification procedures and product detection methods are less time consuming than conventional PCR, thus are suitable for large-scale field surveys (Deng et al. 2019). More importantly, LAMP can provide useful real-time information for pest control strategies.

LAMP assays have successfully detected various invasive insect species with high sensitivity and specificity in both sophisticated and basic laboratories (Hsieh et al. 2012; Dickey et al. 2013; Ide et al. 2016a, 2016b, 2018; Sabahi et al. 2018; Suzuki et al. 2018). In addition, they allowed frass/feeding gallery material (frass) to be used as a substrate to detect and identify invasive insects (Ide et al. 2016a, b). In this study, we developed a LAMP assay for *A. planipennis* (EAB-LAMP) and tested it in a series of experiments using *A. planipennis* frass from different ash species as the DNA source. Performance of the EAB-LAMP was compared to conventional PCR assays via sensitivity analysis by employing fresh and aged frass samples. Additionally, to evaluate the EAB-LAMP primer reliability and potential field applicability, we conducted assays using DNA extracted from *A. planipennis* stored for extended periods in trap glue to ensure that the glue does not interfere with amplification of unsorted trap material containing several insect species. Finally, we developed a quantitative form of the assay for use on pooled traps to determine the presence of EAB in new locations.

## Material and Methods

### Biological Materials

*Agrilus planipennis* adults, larvae, and eggs collected from different geographical locations/origins as well as larval frass collected from various infested *Fraxinus* spp., namely, green/red ash (*F. pennsylvanica*), white ash (*F. americana*), black ash (*F. nigra*), blue ash (*F. quadrangulata*), and pumpkin ash (*F. profunda*) (Table 1). All adult and larval *A. planipennis*

**Table 1.** Geographical origin of *Agrilus planipennis* specimens, other insects and frass samples used for EAB-LAMP assay development and evaluation in this study and amplification results from EAB-LAMP assays using the primers shown in Table 2

| Insect species | Origin | Developmental stage | EAB-LAMP[a] |
|---|---|---|---|
| *Agrilus planipennis* | | | |
| APL1 | Changchun, Jilin, China, | Larvae | + |
| APL2 | Chaoyang, Beijing, China | Larvae | + |
| APL3 | Dagong, Tianjin City, China | Larvae | + |
| APL4 | Yushutun, Shengyang, China | Larvae | + |
| APL5 | Zhenshu, Zhenzhuang zi Village, China | Larvae | + |
| APA1 | Davenport, Iowa, United States | Adult | + |
| APA2 | Detroit, Michigan, United States | Adult | + |
| APA3 | Lexington, Kentucky, United States | Adult | + |
| APA4 | Rochester, New York, United States | Adult | + |
| APA5 | Montreal, Quebec, Canada | Adult | + |
| APA6 | Ottawa, Ontario, Canada | Eggs | + |
| APA7 | Sarnia, Ontario, Canada | Adult | + |
| APA8 | Windsor, Ontario, Canada | Eggs | + |
| *A. anxius* | Sudbury, Ontario, Canada | Adult | – |
| *A. bilineatus* | Sudbury, Ontario, Canada | Adult | – |
| *A. subcinctus* | Sarnia, Ontario, Canada | Adult | – |
| *A. sulcicollis* | London, Ontario, Canada | Adult | – |
| *Dendroctonus ponderosae* | Princeton, British Columbia, Canada | Adult | – |
| *D. rufipennis* | Prince George, British Columbia, Canada | Adult | – |
| *Fraxinus* species | Origin | Sample type | |
| *F. pennsylvanica* | Windsor, Ontario, Canada | EAB frass/ phloem tissues | + |
| *F. nigra* | Sarnia, Ontario Canada | EAB frass/ phloem tissues | + |
| *F. americana* | Ottawa, Ontario Canada | EAB frass/ phloem tissues | + |
| *F. quadrangulata* | Detroit, Michigan, United States | EAB frass | + |
| *F. profunda* | Lakeshore, Ontario Canada | EAB frass | + |
| Green/red ash phloem tissues | Petawawa, Ontario, Canada | Non-infested | - |

[a]The positive and negative LAMP by SYBR green, gel electrophoresis, and LFD results are represented by '+' and '–', respectively.

specimens were either stored at –20°C or in absolute ethanol for >1 mo prior to DNA isolation. Although it was not possible to assess the exact length of time that had elapsed since production of the frass, collected samples were preserved at –20°C for less than 1 mo or greater than 3 yr prior to DNA isolation. To ascertain specificity of the assay, adults of four other *Agrilus* species, *A. subcinctus*, *A. bilineatus*, *A. anxius*, and *A. sulcicollis* were used as negative controls. These *Agrilus* species are found throughout the same geographic range as *A. planipennis* in Canada, and were routinely sampled from green prism traps (Synergy Semiochemicals Corp. Burnaby, BC, Canada) during our field trials in Sarnia, ON. In addition, *Dendroctonus rufipennis* and *D. ponderosae* kindly provided by Dr. Katherine Bleiker (Pacific Forestry Centre, Victoria, BC, Canada) were also included as negative controls in the EAB-LAMP primer specificity evaluation (Table 1). Green/red ash phloem tissues from non-infested trees were used as non-insect negative controls to compare with trace amounts of *A. planipennis* DNA that may be present in the larval frass samples. Furthermore, in situ samples extracted from green prism trap devices set up in ash forests at specific locations in the province of Ontario, Canada, where *A. planipennis* had either become established or was non-existent, were used to test the primer sensitivity of our EAB-LAMP assay.

## DNA Extraction

For the LAMP assay development, optimization, and cross-reactivity tests, genomic DNA (gDNA) was isolated from *A. planipennis* adults, larvae and eggs specimen using the Wizard Genomic DNA purification Kit (Promega, Madison, WI), according to the manufacturer's instructions. gDNA from the genus *Agrilus* (*A. subcinctus*, *A. bilineatus*, *A. anxius*, and *A. sulcicollis*), other insect species (*Dendroctonus rufipennis* and *D. ponderosae*), and the green/red ash phloem tissue samples (Table 1), was isolated using the same kit and protocol. The extracted gDNA concentration was quantified using a Nanodrop ND-1000 (Thermo Scientific, Wilmington, DE), then subsequently adjusted with nuclease-free distilled water to ~20 ng/µl and stored at –20°C until analysis. To detect *A. planipennis* genome in frass, total DNA was isolated from frass samples collected from the *A. planipennis*-infested *Fraxinus* spp. named above. Individual frass sample was ground with a sterile mortar and pestle, and five samples of 50 mg each were weighed out and DNA was extracted using NucleoSpin Tissue Kit (Macherey-Nagel, Bethlehem, PA) following the manufacturer's protocol and quantified as above. Each DNA sample was adjusted with nuclease-free distilled water to ~ 20 ng/µl and stored at –20°C until use. To apply the EAB-LAMP assay to detect *A. planipennis* in simulated traps and to ensure that trap glue does not inhibit the LAMP reaction and amplification, we extracted total DNA from *A. planipennis* adults recovered from simulated green prism sticky traps (Material and methods: *A. planipennis* detection in simulated trap samples). Briefly, individual glue-coated *A. planipennis* adults were ground in 500 µl of lysis buffer (40 mM Tris-HCl at pH 6.5, 400 mM NaCl, 0.4% SDS). Homogenates were boiled for 10 min, quickly transferred onto ice for 10 min and incubation at room temperature for another 10 min. Following spinning on a Fidget Gyro Spinner (Jan's Products Inc, Los Angeles, CA), 200 µl of the clarified supernatant were carefully removed and stored at –20°C (crude DNA) until needed or used immediately to conduct the EAB-LAMP assay. To extract gDNA from in situ trap-recovered substrate, samples were ground with a sterile porcelain mortar and pestle, using 500 ml of lysis buffer. The homogenates were either preserved at –80°C or portions used immediately for DNA isolation.

DNA in the lysates (200 ml) were extracted using a Dynabeads DNA DIRECT Universal kit and DynaMag-2 Magnet (Life Technologies, Carlsbad, CA) following the manufacturer's instructions. The DNA was stored at –20°C for future use. A 5-µl aliquot of the DNA was used to conduct the EAB-LAMP assay (Material and Methods: LAMP assay). In addition, homogenates from in situ trap-recovered substrates determined to have varying degrees of positive products or negative product based on *A. planipennis* detection (LAMP amplification) were used to simulate different pooling ratios: 1:4, 1:9, 1:14, 1:19, 1:24, and 1:29 (positive: negative) (Table 4). 500-µl aliquots of the homogenates from individual positive or negative samples in the designated-pooled ratios were combined in new tubes for DNA extraction using the Dynabeads DNA DIRECT Universal kit and DynaMag-2 Magnet (Life Technologies). The DNA was stored at –20°C for future use.

## PCR Primer Design, Amplification, and Sequencing of *A. planipennis mtCOI* Gene

To design EAB-specific LAMP primers that ensure high efficiency and reliability of the assay, DNA samples extracted from individual beetles from various geographical locations (Table 1) were used to sequence a fragment of the mitochondrial cytochrome oxidase subunit I (*mtCOI*) region by employing *COI-5′* (barcode) primers. We designed the primer pair EAB_COIF and EAB_COIR following multiple alignment of *mtCOI* gene sequences of *Agrilus* spp. in the BioEdit software (Hall 1999) to identify diagnostic polymorphism but highly conserved regions common to all *A. planipennis* (Table 2, Supp Table S1 [online only]). For PCR amplification, the 25 µl reaction volume contained 2.0 µl (10 ng/µl) of adult *A. planipennis* DNA, 2.5 µl 10X LA PCR buffer II, 4.0 µl dNTP mixture (2.5 mM each), 0.2 µl TaKaRa LA DNA polymerase Taq (TaKaRa Biotechnology Co. Ltd., Madison, WI), 1.0 µl (10 µM) of each forward and reverse primers, and 16.3 µl of nuclease-free distilled water to the total reaction volume. Amplification was performed in a TouchGene Gradient thermal cycler (Techne Inc., Burlington, NJ). The thermal profile for all PCR reactions comprised an initial denaturation at 94°C for 1 min followed by five cycles of 94°C for 40 s, 45°C for 40 s, and 72°C for 60 s, and 35 complete cycles at 94°C for 45 s, 60°C for 40 s, and 72°C for 60 s. The final extension step was conducted at 72°C for 10 min and the reaction was then held indefinitely at 4°C. PCR products of expected size (~1277 bp) were visualized on a 2% agarose gel pre-stained with GelRed 10,000× fluorescent nucleic acid dye in nuclease-free distilled water (Biotium, Fremont, CA). Successful amplicons were excised and purified using the QIAquick Gel Extraction Kit (Qiagen Inc., Chatsworth, CA) and subsequently cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Five independent clones with inserts were sequenced bi-directionally at the Laval University Hospital Research Center genomic sequencing and genotyping platform, Québec City, PQ, Canada (www.sequences.crchul.ulaval.ca). All generated sequences have been archived and consequently compared to sequences that are currently published in the GenBank database (www.ncbi.nlm.nih.gov) under accession numbers MN548248-MN548260.

## LAMP Primer Design

To guarantee specificity, *A. planipennis mtCOI* gene sequences generated in this study, those archived by other authors and comparative *mtCOI* sequences from other *Agrilus* and insect species available in the GenBank database (Supp Table S1 [online only]) were downloaded and used for primer design. To identify areas/regions with the greatest sequence conservation in all *A. planipennis* samples but with high diagnostic polymorphism of other *Agrilus* and insect species, sequence data

**Table 2.** Primers targeted to insect rDNA and EAB cytochrome b DNA sequences used in this study

| Technique and primer name | Sequence strand (5′→3′) | Gene type | Author/reference |
|---|---|---|---|
| PCR and sequencing | | | |
| EAB_COIF | AGGAATAGTAGGAACAGCCCTTAGA | COI-5′ (barcode) | This study |
| EAB_COIR | TATTTCATCTAAGGTAGGCATCTGG | COI-5′ (barcode) | This study |
| PCR | | | |
| 28SD2F | AGAGAGAGTTCAAGAGTACGTG | 28SrDNA | Belshaw and Quicke 1997 |
| 28SD2F | TTGGTCCGTGTTTCAAGACGGG | 28SrDNA | Campbell et al. 1993 |
| EAB-specific PCR | | | |
| EABFOT | TCAAAGAATGATGTATTTAAGTTTCGATC | COI | This study |
| EABROT | TAGCAATTTTTAGACTTCATTTAGCTGG | COI | This study |
| EABLAMP1 | | | |
| EAB1_F3 | GGCACCTGATATAGCTTTCC | COI | This study |
| EABLAMP1 | | | |
| EAB1_F3 | CTCCCTCCCTCTTTAACATTAC | COI | This study |
| EAB1_B3 | GATCAGACTAGTAGAGGTGT | COI | This study |
| EAB1_FIP[a] (F1c+F2) | ATATTAGCCGCTAATGGTGGGAATAGTCGAAAGAGGAGCAG | COI | This study |
| EAB1_BIP[b] (B1c+B2) | GGCTCTGTTGACTTAGCAAAGGTTATTCCTATTGCTCGC | COI | This study |
| EAB1_LF | ATATACTGTCCAACCAGTCC | COI | This study |
| EAB1_LB | CTGGAATCTCCTCAATTCTAGG | COI | This study |
| EABLAMP2 | | | |
| EAB2_B3 | GGCACCTGATATAGCTTTCC | COI | This study |
| EAB2_B3 | GAGGTGTTTGGTCTAAGG | COI | This study |
| EAB2_FIP (F1c+F2) | ATACTGTCCAACCAGTCCAGATTCTGGCTACTCCCTC | COI | This study |
| EAB2_BIP (B1c+B2) | ATCCACCATTAGCGGCTAGCCCCTAGAATTGAGGAGA | COI | This study |
| EAB2_LF | CTGCTCCTCTTTCGACTA | COI | This study |
| EAB2_LB | GGCTCTGTTGACTTAGCA | COI | This study |

[a]*5′*-Labeled with biotin when used in LAMP-LFD assay.
[b]*5′*-Labeled with 6-FAM when used in LAMP-LFD assay.

were multiply aligned using the BioEdit software. Two sets of six oligonucleotide primers [F3, B3 (external forward and backward), FIP (F1c + F2), BIP (B1c + B2) (forward and backward internal primers), respectively, LF (forward loop) and LB (backward loop)] were designed *de novo* using the online LAMP primer design software package Primer Explorer V5 (http://primerexplorer.jp/e/) with default settings. To avoid cross-reactivity of the designed primers with other insects and organisms, a BLAST search was conducted against all available sequences in the NCBI database (https://www.ncbi.nlm.nih.gov/) to confirm their specificity in silico. Properties of the primers were checked with the software Oligonucleotide properties calculator (Kibbe 2007), NUPACK (www.nupack.org/), and the DINAMelt Web Server (Markham and Zuker 2005). After confirmation, two sets of primers were submitted to Bio Basic Inc. (www.biobasic.com; Markham, ON, Canada) for synthesis. The FIP and BIP primers were purified using HPLC and were either unlabeled or labeled at the 5′-ends with 6-carboxyfluorescein (FIP-6-FAM) or biotin (BIP-biotin) (Bio Basic Inc). The names and sequences of the primers used in this study are in Table 2.

## LAMP Assay and Determination of Optimal Incubation Temperature and Reaction Time

To select a primer set with high specificity and rapid amplification (high efficiency), the LAMP reaction included 15 µl of ISO-001 isothermal master-mix (OptiGene, Ltd, Horsham, UK), 5.0 µl of each specific primer set, EABLAMP1 or EABLAMP2 (10X Primer Mix) mixes which contained final concentrations of 1.6 µM of inner primers FIP and BIP, 0.2 µM of outer primers F3 and B3 and 0.4 µM of loop primers LF and LB. Two µl (10 ng/µl) of gDNA extracts from *A. planipennis* adults, larvae, and eggs were included in the reaction mix and topped up with nuclease-free distilled water to a final reaction volume of 25 µl in order to monitor specific primer performance. Assays were initially performed using a Genie II real-time fluorometer (Optigene Ltd.) (real-time EAB-LAMP assay, EAB-ReaLAMP) which detected the products by fluorescence and reported results as time-to-positive (*Tp*) (hours:minutes:seconds); anneal derivative melting temperature (*Tm*) (°C), indicated when the sample florescence crossed the pre-set threshold of the Genie equipment.

To ascertain the optimal incubation temperature and reaction time for greater efficiency and to select the best primer set concurrently, reactions were performed at different temperatures ranging from 59 to 65°C for 30 min, followed by reducing temperature by increments from 95 to 75°C at a rate of 0.05°C/s. All reactions were repeated three times on different days to assess the repeatability and robustness of the LAMP assay. Fluorescence of the reaction was measured in real-time based on the fastest amplification time (hours:minutes:seconds) and resultant amplification curve. The optimal signal strength was obtained at an incubation temperature and reaction time of 65°C and 30 min, respectively. We subsequently verified the LAMP protocol in a hot water bath or on a Techne PCR machine at the preferred reaction condition of 65°C for 30 min, and similar results were obtained as with the Genie II real-time fluorometer (data not shown).

## Detection of LAMP Amplicons

Three LAMP amplicon monitoring methods or signal readouts were employed in this study, including real-time fluorescence (Optigene Ltd.), SYBR Green I fluorescent DNA-intercalating dye (Lonza, Allendale, NJ), gel electrophoresis (BioRad, Hercules, CA) and lateral flow dipsticks (LFD) (HybriDetect 2T; Milenia Biotec GmbH, Giessen, Germany). Positive amplifications on the Genie II instrument displays a sigmoid shaped amplification curve, amplification time and *Tm* within ± 2°C of the predicted *Tm*. Additionally, direct observation of amplicons was performed with SYBR Green I. Before incubation of the conventional LAMP reaction mix, 1.0 µl of 1/10 diluted original SYBR Green I was added in the tube lids. After completion of the LAMP reaction, tubes were centrifuged briefly to allow mixing of products and SYBR Green I to trigger the color change. Fluorescence was detected either by the naked eye or under UV (254 nm) radiation. A positive LAMP reaction turned green or produced a green fluorescent emission, while negative samples remained pale orange. Three microliters of the SYBR Green I dyed LAMP products were electrophoresed at a constant voltage (80 V) on a 2% (w/v) agarose gel for 80 min and then photographed with a ChemiDoc XRS +System (BioRad, Hercules, CA). Typically, positive LAMP products show a ladder-like pattern following agarose gel electrophoresis. To detect the LAMP products by LFD, the inner primers FIP and BIP were modified at the 5′-end with 6-carboxyfluorescein (6-FAM) and biotin respectively. Post-amplification, the LAMP amplicons were labeled simultaneously with 6-FAM and biotin. The LAMP-LFD detection was conducted by mixing 2 µl of the LAMP amplification product in 98 µl of HybriDetect 2T assay buffer, then immersing the LFD into the mixture at room temperature. Appearance of two crimson bands (test and control) within 5–10 min at the detection regions is an indication of a valid and acceptable positive result. Appearance of a single band at the control detection region indicates negative amplification or non-detectable level of target DNA in the product and confirms the successful test run of the system.

## Evaluation of the EAB-LAMP Assay Specificity for *A. planipennis*

Specificity of the LAMP primers were tested using gDNA extracted from *A. planipennis* adults, larvae, and eggs collected from several geographical regions. To evaluate biological specificity of the EAB-LAMP assay, gDNA extracted from *A. subcinctus*, *A. bilineatus*, *A. anxius* and *A. sulcicollis*, *D. rufipennis*, *D. ponderosae* adults were included as exclusivity panel and controls for nontarget insects. DNA from non-infested green/red ash phloem tissues and nuclease-free distilled water were included as negative and non-template controls, respectively. The LAMP assay components and optimized conditions were as described above (Material and Methods: LAMP assay) and were performed in triplicate on different days using at least two independent gDNA samples from each insect species. Detection of EAB-LAMP amplification products by LFD banding signal was compared to the other two methods (Material and Methods: Detection of LAMP amplicons). The insect diagnostic primer sets, 28SD2F (Belshaw and Quicke 1997) and 28SD2R (Campbell et al. 1993), and the *A. planipennis*-specific primers, EABFOT/EABROT (*A. planipennis* COI gene), designed in this study, were used to verify the quality and amplifiability of all insect gDNA samples. The reaction components, total reaction volume, and thermal profile for the above PCR assays were the same as previously described, except for the annealing temperatures, which were 50 and 60°C for 28SD2F/28SD2R and EABFOT/EABROT, respectively. Amplified products were visualized on a 2% agarose gel pre-stained with GelRed (Biotium, Fremont, CA).

### Comparison of EAB-LAMP and PCR Assay Sensitivity Using Frass DNA

gDNA isolated from frass was used to ascertain reliability and sensitivity of the developed EAB-LAMP assay. Given that undiluted DNA from these frass showed a rather strong inhibitory effect in the conventional PCR and LAMP reactions (data not shown), each DNA sample was subsequently diluted 10 times with nuclease-free distilled water and 2 µl of the diluent was used in the EAB-LAMP reaction (Material and Methods: LAMP assay). The relative detection limit was determined using total DNA extracted from fresh (<1 mo) and stored (>3 yr) frass. The sensitivity assessment was conducted using gDNA extracted from green/red ash frass, which was diluted in serial 10-fold increments from 10 ng to 100 fg in both EAB-LAMP and conventional PCR assays. We used the optimized EAB-LAMP reaction conditions, 65°C for 30 min and conventional PCR components and thermal conditions as above (EABFOT/EABROT, annealing temperature of 60°C) to perform assays. *Agrilus planipennis* adult gDNA was used as a positive control, and nontarget DNA from green/red ash phloem tissues and nuclease-free distilled water were included in the test as negative and non-template controls, respectively. The LAMP and PCR assays were conducted in triplicate on different days using at least two independent DNA extracts from each sample of frass. LAMP amplified products were detected by SYBR Green I color change, ladder-like banding pattern on agarose gel and LFD signal detection.

### *Agrilus planipennis* Detection in Simulated Trap Samples

To simulate experiments that allows transition of the developed EAB-LAMP assay for processing and detecting *A. planipennis* adults (beetles) stored under suboptimal conditions on traps and to ensure that trap glue does not interfere with detection, beetles were stuck on green prism sticky traps and the traps were placed in ash canopies. The traps remained in the ash canopies over a designated period (1 d to 16 wk, Table 3), before recovery for subsequent DNA extraction. Three beetles were recovered from the green prism sticky traps on a bi-weekly basis until 16 wk post-placement. Control *A. planipennis* adults were stuck on another set of glue-coated green prism traps and maintained at room temperature over the same sampling period. For each recovery period, each beetle from the control and field groups underwent a simple crude DNA extraction as described previously (Materials and Method section: DNA extraction). The optimized EAB-LAMP and conventional PCR (EABFOT/EABROT) reaction and amplification conditions indicated above were used in assessing these DNA samples. *Agrilus planipennis* adult gDNA was used as a positive control, and nontarget DNA from green/red ash phloem tissues and nuclease-free distilled water were included in the test as negative and non-template controls, respectively. LAMP amplified products were detected by SYBR Green I color change, ladder-like banding pattern on agarose gel and LFD signal detection.

**Table 3.** Loop-mediated isothermal amplification detection rate of *A. planipennis* stored in trap glue under simulated conditions

| | Preservation conditions | |
|---|---|---|
| | Room temperature | Field |
| Storage time | Positive detection[a] | Positive detection |
| 1 d | 3/3 | 3/3 |
| 1 wk | 3/3 | 3/3 |
| 2 wk | 3/3 | 3/3 |
| 4 wk | 3/3 | 3/3 |
| 6 wk | 3/3 | 3/3 |
| 8 wk | 2/3 | 3/3 |
| 10 wk | 3/3 | 3/3 |
| 12 wk | 3/3 | 2/3 |
| 14 wk | 3/3 | 3/3 |
| 16 wk | 3/3 | 2/3 |

[a]Number of *A. planipennis* detected/total number of *A. planipennis* tested across repeated experiments and at all storage times. For each repeat assay, negative non-template control, *A. planipennis* positive and non-infested green/red ash phloem tissue DNA were included at each storage time tested.

### Investigative Screening for *A. planipennis* in Trap Samples

To demonstrate the potential use of the EAB-LAMP platform as an operational tool, 14 glue-coated green prism traps were set up in the field in Sault Ste. Marie, ON, Canada just before the flight period of *A. planipennis*, followed by weekly inspections post-placement. Most of the insects captured by the traps were identified to the genus/species level. Captured whole insects and appendages with morphological characters suggestive of *Agrilus* species and other insect species were scrapped-off from the 14 green prism sticky traps to perform DNA extraction using the Dynabeads DNA DIRECT Universal kit protocol (Materials and method section: DNA extraction). EAB-LAMP and conventional PCR (EABFOT1/EABROT1) assays were conducted using 2 µl (10-fold dilutions) of isolated DNA as a template. A no-template control was also included to exclude reagent contamination. All reactions were carried out at 65°C for 30 min and signal readout conducted as previously described.

### Exploratory Estimation of Sample Sizes for Pooled Trap Samples

What is more, considering the potential field application of the developed EAB-LAMP assay for large-scale, early detection of new incursions of *A. planipennis* into various geographical regions and monitoring of incipient *A. planipennis* populations, a trial using pooled samples was conducted to decipher cost-efficient processing of trap captures. Individual and pooled trap-captured specimens were employed to estimate potential sample sizes to use in designing pooled sampling schemes for the EAB-LAMP assay. Total DNA samples extracted from captured whole insects and appendages scrape off of each trap were diluted 5, 10, 20, and 50 times and 2 µl of each dilution were used in the EAB-LAMP assay. Preliminary results indicated that the 10X dilution was optimal for the EAB-LAMP assay (data not shown). Subsequently, we estimated sample sizes for pooled unsorted trap samples based on amplification intensity of four positive samples from less intense (weakest) to highly intense (strongest) outcomes (Supp Fig. S3 [online only] and insert). Categorization of these positive samples was based on fluorescence curves/signal strength and amplification time in the EAB-ReaLAMP assay as well as the EAB-ReaLAMP amplicon band intensities on a gel (Supp Fig. S3 [online only] and insert). Then total gDNA extracted from positive and negative traps were used in formulating the different pooling ratios: 1:4, 1:9, 1:14, 1:19, 1:24, and 1:29 (positive:negative), based on categorization of the positive samples above (Table 4). The formulated DNA samples were used in performing the EAB-LAMP assay. Amplification was performed under optimal conditions (65°C for 30 min) and the EAB-LAMP products were detected by all signal readout methods as described prior. Three replications were performed for all reactions on three different days.

**Table 4.** Detection EAB in pooled trap samples by EAB-ReaLAMP using Genie II equipment and gel electrophoresis

| | Pool size and pooling ratio (Pos:Neg) | | | | | |
|---|---|---|---|---|---|---|
| Electrophoresis-band intensity | 5 (1:4) | 10 (1:9) | 15 (1:14) | 20 (1:19) | 25 (1:24) | 30 (1:29) |
| Weakest/low | + | ± | – | – | – | – |
| Medium | + | + | ± | – | – | – |
| Strongest/High 2 | + | + | + | – | – | – |
| Strongest/High 1 | + | + | + | ± | – | – |

Pooled size: Each EAB trap positive sample was pooled with EAB trap negative samples in a range of pooling ratios. Four EAB positive trap samples were selected for pooling with varying number of negative samples. Categorization was based on amplification time in the EAB-ReaLAMP assay and electrophoresis banding (Supp Fig. S3 [online only] and insert): Weakest, Medium, Strongest/High 2 and Strongest/High 1 intensity products: EAB-LAMP assay was conducted using Genie II equipment and products confirmed by gel electrophoresis. Assay was repeated on three different occasions. +: Positive by EAB-ReaLAMP. ±: Indeterminate by EAB-ReaLAMP. –: Negative by EAB-ReaLAMP.

### Sequence Verification of LAMP Amplification Products

To assess the LAMP assay specificity and confirm results obtained with *A. planipennis* frass, DNA from selected trap-captured samples including the two additional traps captures visually verified not to contain *A. planipennis*, LAMP products were electrophoresed on 1.5% agarose gel at 80 V for 80 min. The amplimers just above the 300 bp band on the molecular weight marker were excised and purified with the QIAquick Gel Extraction Kit. To ensure the developed EAB-LAMP assay correctly amplified the target DNA, purified DNA samples were reamplified with the outer primer set, F3/B3 of EABLAMP1, then cloned with the TOPO TA cloning kit as previously described and bi-directionally sequenced at the Laval University Hospital Research Center.

## Results

### DNA Extraction and Competence for PCR and LAMP Assays

To ascertain amplifiability of the extracted gDNA templates, the primer set, EABFOT/EABROT amplified products of ~230 bp in gDNA samples extracted from larvae and adults of *A. planipennis* collected from various geographical regions and *A. planipennis* frass (Figs. 2D and 4D). Fragments of ~460-630-bp were PCR amplified with the primer set, 28SD2F/28SD2R using DNA samples from all *A. planipennis* specimen and frass as well as adults of *A. subcinctus*, *A. bilineatus*, *A. anxius* and *A. sulcicollis*, *D. rufipennis*, and *D. ponderosae* (Fig. 3D). Therefore, any negative EAB-LAMP amplification from nontarget samples was attributable to specificity of the assay and not to lack of DNA quality and amplifiability. In silico tests showed that, despite their widely dispersed geographical range from China to North America (Supp Table S1 [online only]), the partial *mtCOI* sequences (~1277 bp) generated in the current study were 100% identical to other *A. planipennis mtCOI* sequences archived in the GenBank database. The gDNA from adult *A. planipennis* collected from Sarnia, ON, Canada, subsequently was used as our positive control template.

### LAMP Primer Selection, Optimal Incubation Temperature, and Reaction Time Determination

We designed two primer sets, EABLAMP1 and EABLAMP2, to target a portion of the *A. planipennis mtCOI* sequence. The selection of highly specific primer set, optimum temperature, reaction time, was based on several indices, including; high amplification efficacy, fluorescence intensity and *Tm*, and *Tp* on the Genie II equipment and ladder-like bands visualized by agarose gel electrophoresis. Most importantly, we considered the EAB-LAMP primer specificity against other *Agrilus* and insect species commonly found in the ash ecosystem. Both primer sets, EABLAMP1 and EABLAMP2 successfully amplified products from *A. planipennis* gDNA at temperatures and times ranging from 59 to 65°C by 30 min, respectively (Fig. 1). For both primer sets, the minimum positive detection time was less than 10–12 min for 20 ng of input DNA, and reached a maximum at approximately 12–14 min, remaining stable for the remainder of the time course. The same EAB-LAMP products changed color from pale orange to green upon addition of SYBR Green I and exhibited clear ladder-like bands on the gels (Supp Fig. S1A1 and S1A2 [online only]; primer EABLAMP1, and Supp Fig. S1B1 and S1B2 [online only]; EABLAMP2). Although we observed high-intensity ladder-like bands and green coloration on the gels and in tubes, respectively, at temperatures from 59 to 65°C, the reaction at 65°C generated the lowest *Tp* value and the maximum fluorescence intensity (Fig. 1). There was a *Tp* variance of >2 min for EABLAMP1 compared to EABLAMP2. The maximum fluorescence of EABLAMP1 was about 10K relative fluorescence units (RFU) higher than that of EABLAMP2 in the LAMP assays. Based on the visually detectable reaction peak, fluorescence intensity and *Tp* value, EABLAMP1 was undoubtedly a high-efficient primer set choice for subsequent analyses. Consequently, we used the primer set, EABLAMP1, optimal reaction temperature, and time of 65°C and 30 min, respectively, in performing all EAB-LAMP assays.

### Evaluation of the EAB-LAMP Assay Specificity for *A. planipennis*

All *A. planipennis* gDNA samples from geographically distinct locations or containing *A. planipennis* gDNA yielded positive results with the primer set EABLAMP1 as indicated by the SYBR Green I color change from orange to green; the ladder-like pattern on electrophoresis gels and the target banding (test and control bands) simultaneously appearing at the detection region on the LFD (Figs. 2A–C and 3A–C). No gDNA samples from closely-related *Agrilus* species (*A. subcinctus*, *A. bilineatus*, *A. anxius*, and *A. sulcicollis*), the two bark beetles (*D. rufipennis* and *D. ponderosae*), or non-template controls yielded EAB-LAMP products (Fig. 3A–C).

### Comparison of EAB-LAMP and PCR Assay Sensitivity Using Frass DNA

The sensitivity of the EAB-LAMP assay was determined using gDNA from frass obtained from various *A. planipennis*-infested *Fraxinus* species by comparing its performance to the gold standard to the gold standard, conventional PCR. Both diagnostic assays successfully amplified trace amounts of *A. planipennis* gDNA from frass, as well as *A. planipennis* larval and adult gDNA (Fig. 4A–D). There was no cross-reactivity with the non-infested ash phloem tissue

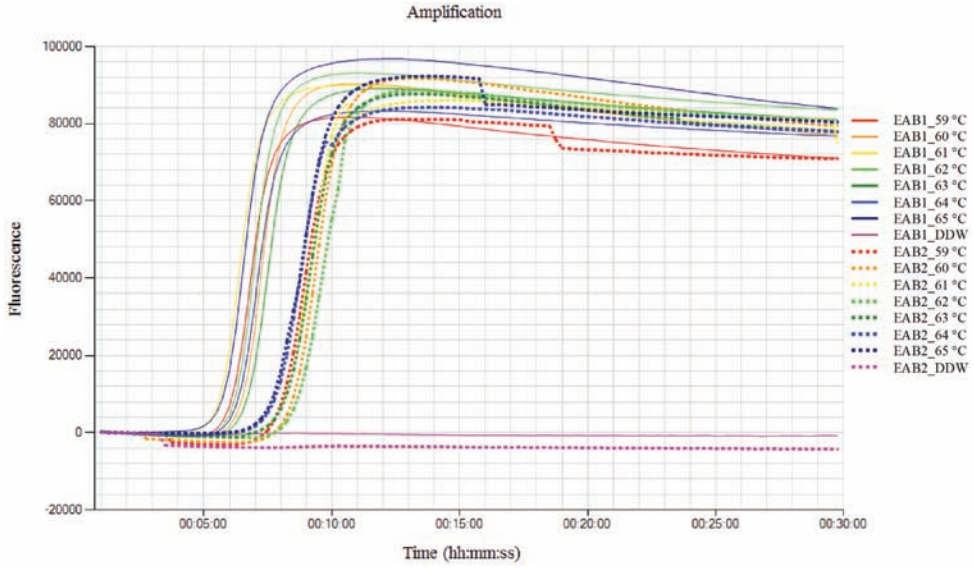



**Fig. 1.** Optimal primer selection for use in the LAMP reaction. Two sets of primers EABLAMP1 (solid line) and EABLAMP2 (broken line), were designed and used in real-time fluorescence LAMP assay (EAB-ReaLAMP) using Genie II equipment. Several indices were used in the optimal primer selection, including the fluorescence intensity and the time-to-positive value (*Tp*) of the real-time fluorescence LAMP and the specificity against other *Agrilus* species. A sample of EAB, 10 ng/µl gDNA was used as a template and the reaction was performed using a temperature range of 59–65°C for 30 min. Nuclease-free distilled water was used as the non-template negative control. At 65°C, the EAB-LAMP assay had the highest amplification efficiency, compared to reactions at other temperatures.

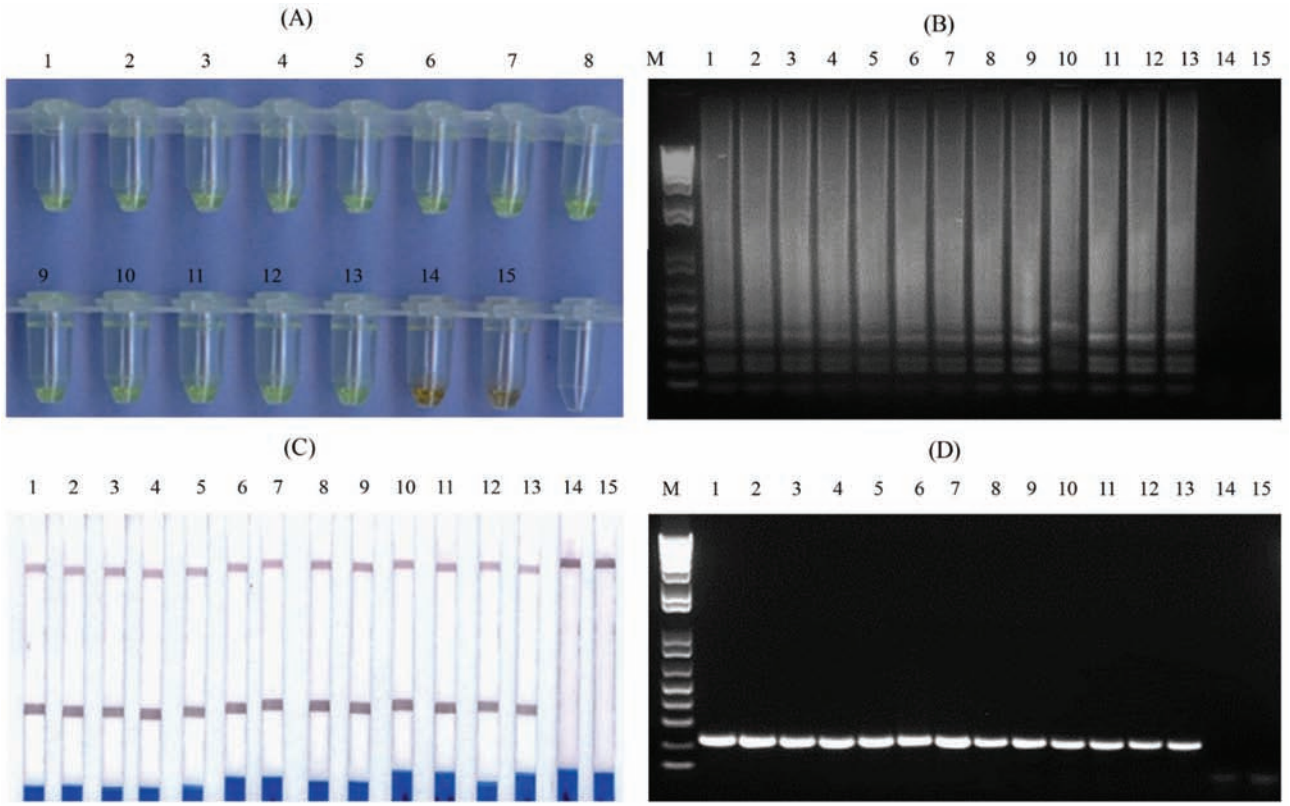



**Fig. 2.** Detection of *Agrilus planipennis* from different geographical regions. Primer set EABLAMP1 was used in the amplification. Assessment of products was based on (A) SYBR Green I color change visualization; (B) gel electrophoresis and (C) Lateral flow dipstick band signal and (D) PCR diagnostic primer set, EABFOT/EABROT amplifying products of ~230 bp in DNA samples containing *A. planipennis* genome. Tubes and lanes 1–13, *A. planipennis* DNA; tubes and lanes 14 and 15, non-infested ash phloem tissues DNA and no-template control (double-distilled water), respectively. The order of *A. planipennis* DNA (from left to right) is the same as in Table 1 (from top to bottom). Lane M, 100 bp DNA ladder size marker.

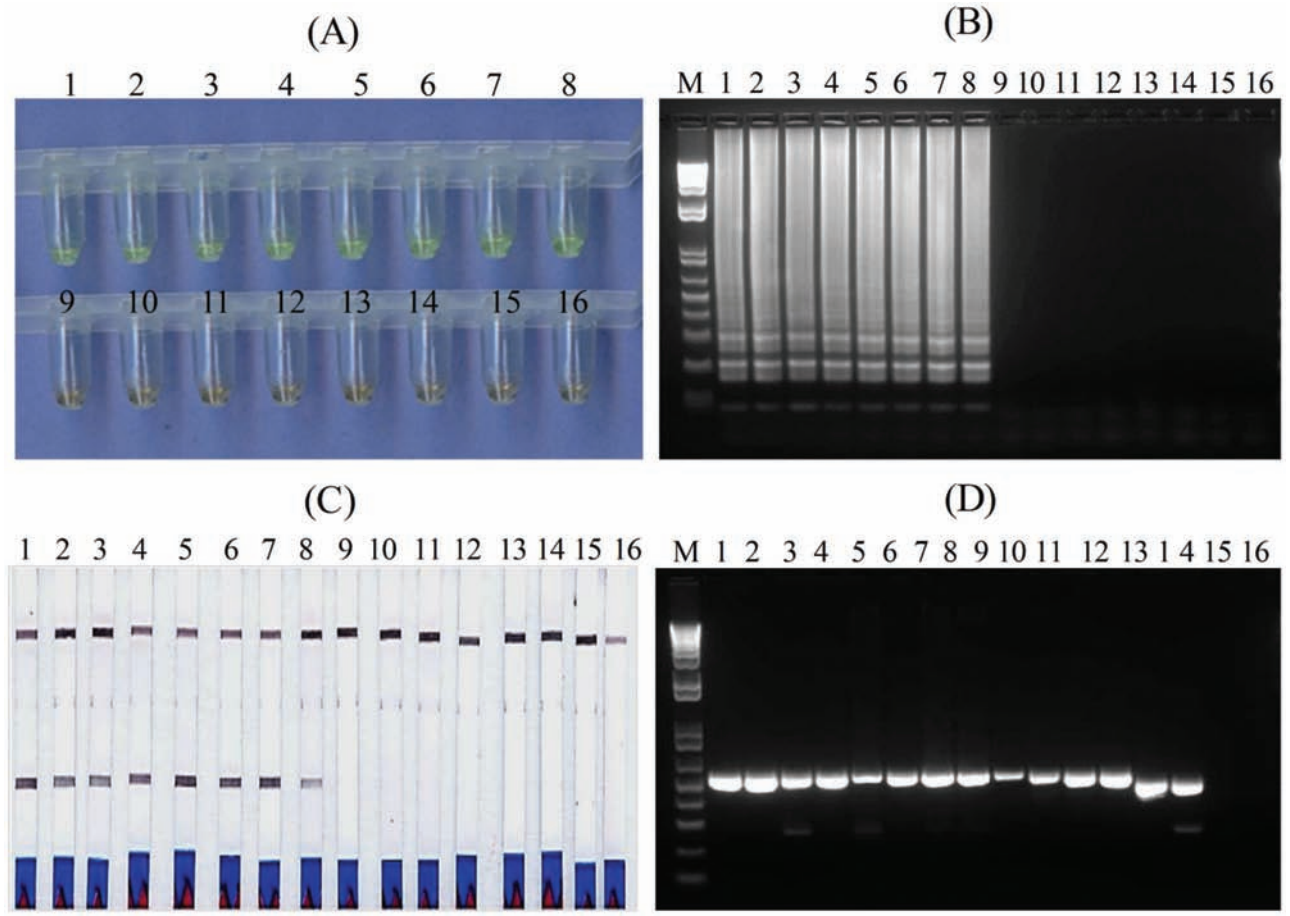



**Fig. 3.** The specificity of the EAB-LAMP detection assay. Assessment of products was based on (A) SYBR Green I color change visualization; (B) gel electrophoresis; (C) lateral flow dipsticks and (D) PCR diagnostic primer set 28SD2F/28SD2R amplifying products of ~460-630-bp from insect DNA. Tubes and lanes 1–3, *A. planipennis* adults; 4–6, *A. planipennis* larvae; 7–8, *A. planipennis* eggs; 9, *Agrilus subcinctus*; 10, *Agrilus subilineatus*; 11, *Agrilus anxius*; 12, *Agrilus sulcicollis*; 13, *Dendroctonus rufipennis*; 14, *Dendroctonus ponderosae*; 15, DNA extracted from non-infested Ash phloem tissues and 16, no-template nuclease-free distilled water. Lane M, 100 bp DNA ladder size marker.

DNA or with the nuclease-free distilled water. We calibrated the detection limits of the EAB-LAMP assay using 10-fold serial dilutions (10 ng to 100 fg/µl) of gDNA from fresh (<1 mo) and stored (>3 yr) green/red ash frass. The frass age affected the detection of target DNA; at the 0.1 ng mark detection limit, there were brighter bands produced for the stored material (Fig. 5B1–B4) than for the fresh material (Fig. 5A1–A4) for both the EAB-LAMP and conventional PCR assays.

## *Agrilus planipennis* Detection in Simulated Trap Samples

As the EAB-LAMP assay performed well, and results characterized with rapidity, high sensitivity and specificity using standard workflow as described prior, we moved forward with refinements that could further aid in processing samples recovered from traps. Trap glue and time of storage did not affect detection of *A. planipennis* recovered from simulated traps (Supp Fig. S2A and S2B [online only]). With *A. planipennis* adults stored in trap glue at room temperature in the laboratory, 3/3 beetles were detected at all recovery time periods tested (Table 3), except at 8 wk post-recovery (2/3). For the *A. planipennis* adults recovered from sticky traps suspended in the ash canopies in the field, 3/3 beetles were detected at all recovery periods except after 14 wk (2/3) and 16 wk (2/3) (Table 3). There was no amplification of DNA from non-infested green/ red ash phloem tissue DNA (negative control) or nuclease-free water without DNA template (data not shown).

## Investigative Screening for *A. planipennis* in Trap Samples

To investigate the applicability and performance of the developed EAB-LAMP assay as a potential surveillance and monitoring tool for *A. planipennis* incursions into new locations, unsorted insects were scrapped-off from 14 green prism sticky traps for DNA extraction. Native supernatants were not amplifiable with the developed EAB-LAMP assay, but 10- to 50-fold dilutions were amplifiable (data not shown). Therefore, with dilutions, the content of non-specific nucleic acids as well as other molecular and macroscopic components present in the crude homogenate did not compromise the ability of the assay to detect *A. planipennis* gDNA. The EAB-LAMP assay detected *A. planipennis* DNA in all seven traps visually verified to have captured *A. planipennis* adults (Fig. 6A–C; tubes and lanes 1–3, 7–9, and 12). The EAB-LAMP assay also detected the presence of *A. planipennis* gDNA in two additional traps visually verified not to contain *A. planipennis* (Fig. 6A–C; tubes and lanes 5 and 11). Mitochondrial cytochrome oxidase subunit I gene sequences generated with DNA from these two traps showed 100% identity compared to *A. planipennis mt*COI gene sequences reported in this study and those archived in the GenBank database (Supp Table S1 [online

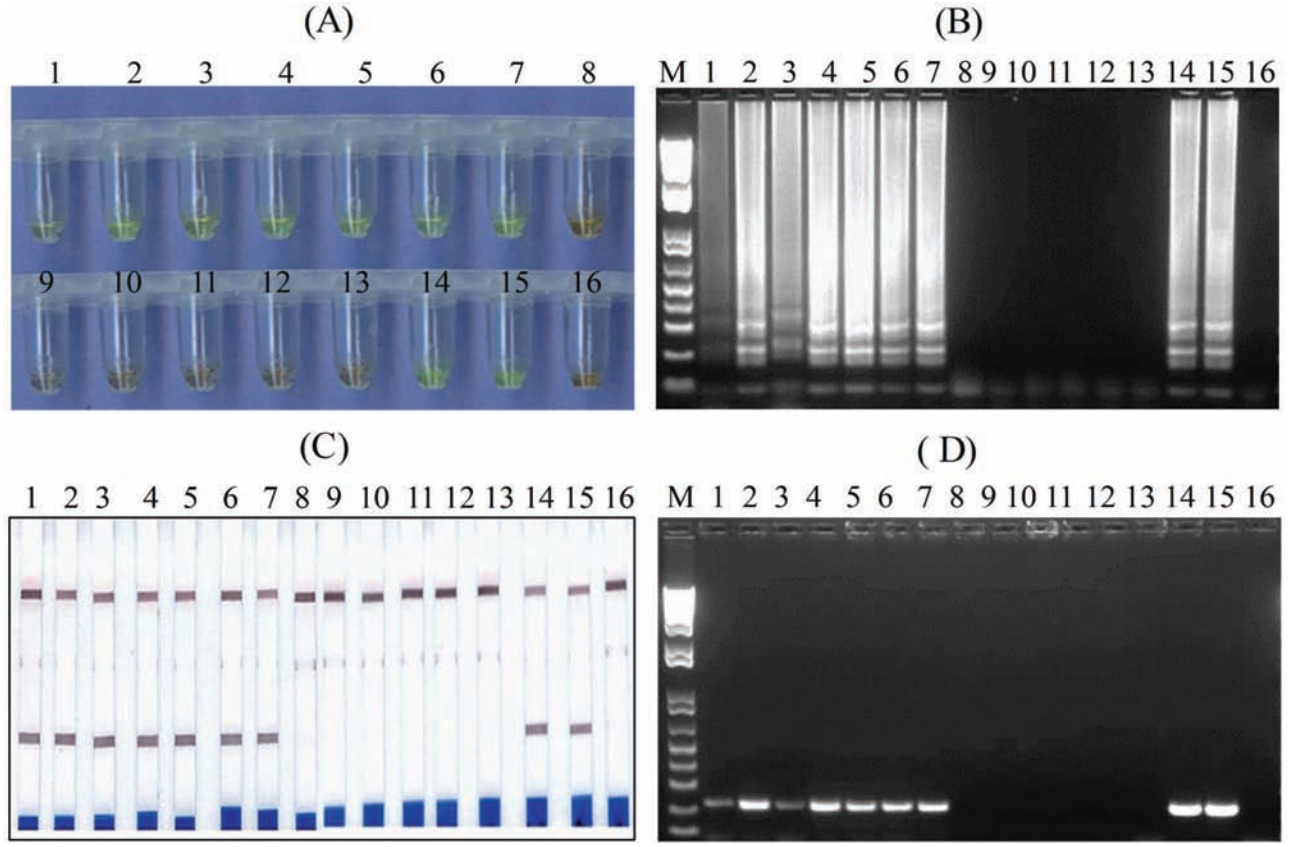



**Fig. 4.** Application of the EAB-LAMP assay using wood samples. Assessment of products was based on (A) SYBR Green I color change visualization; (B) gel electrophoresis; (C) lateral flow dipsticks; and (D) conventional PCR diagnostic primer set, EABFOT/EABROT used to amplify products of ~230 bp in DNA samples containing *A. planipennis* genome. Tubes and lanes 1–5 represent frass DNA obtained from various *A. planipennis*-infested *Fraxinus* species, including green/red ash (*F. pennsylvanica*), white ash (*F. americana*), black ash (*F. nigra*), blue ash (*F. quadrangulata*), and pumpkin ash (*F. profunda*). Tubes and lanes 9–13 are non-infested phloem tissue DNA from respective ash species. Tubes and lanes 6/14 and 7/15 are EAB larval and adult gDNA, respectively (positive control). Tubes and lanes 8 and 16 are no-template nuclease-free distilled water. Lane M, 100 bp DNA ladder size marker.

only]). Based on visual assessment of traps, the accuracy of our developed EAB-LAMP assay for detection of *A. planipennis* from trap samples was 86%, with a detection sensitivity and specificity of 100 and 71.4%, respectively. The results demonstrate that the developed EAB-LAMP assay can detect and identify trap-captured *A. planipennis*.

### Exploratory Estimation of Sample Sizes for Pooled Trap Samples

As a proof of principle experiment, we estimated sample size for pooled traps through experimental simulation of different pooling ratios of *A. planipennis* positive and negative traps. Four *A. planipennis* positive traps were selected from screened positive samples based on amplification signal strength/curves, amplification time in EAB-ReaLAMP with Genie equipment and intensity of the electrophoresis banding of EAB-LAMP products (Supp Fig. S3 [online only] and insert). Positive samples from less intense (weakest) to highly intense (strongest) outcomes from the tests were combined with material scrapped off of *A. planipennis* negative traps at designated ratios (Table 4). Minimum positive detection time for the less intense positive samples occurred at ~36 min (Supp Fig. S3 [online only]; Table 4). The three other positive samples (medium-high intensity banding) can successfully detect *A. planipennis* DNA up to an amplification time of 20–30 min and up to a pool size of 15 trap samples (pool ratio of 1 positive:14 negative). Pooling sizes beyond these ratios showed inconsistent amplification. The three repeats conducted on different days showed similar results, supporting the repeatability of the assay and its potential use for processing field-collected samples to detect *A. planipennis* in traps.

## Discussion

Molecular diagnosis of invasive pests based on DNA amplification has greatly contributed to the monitoring of their presence and subsequent initiation of management schedules (Darling and Blum 2007). Accurate detection and identification is foundational and essential for invasive species surveillance and monitoring purposes and their ecological research. With a trend towards increased *A. planipennis* infestation and with no rapid detection tool, the development of a reliable, rapid, and cost-effective diagnostic tool will contribute to early monitoring, prevention, and effective *A. planipennis* management and control strategies.

The selection of target genes is crucial to development of the LAMP technology and specificity of the assay. COI has been the barcode for genetic distinction (Hebert et al. 2003). COI sequences of many insect species are archived in the GenBank database, allowing them to be employed in the design of species-specific primers (Kitpipit et al. 2014). In this study, we developed a LAMP assay (EAB-LAMP) for the early accurate detection of *A. planipennis*, based on *mt*COI gene sequence which may serve as a potential

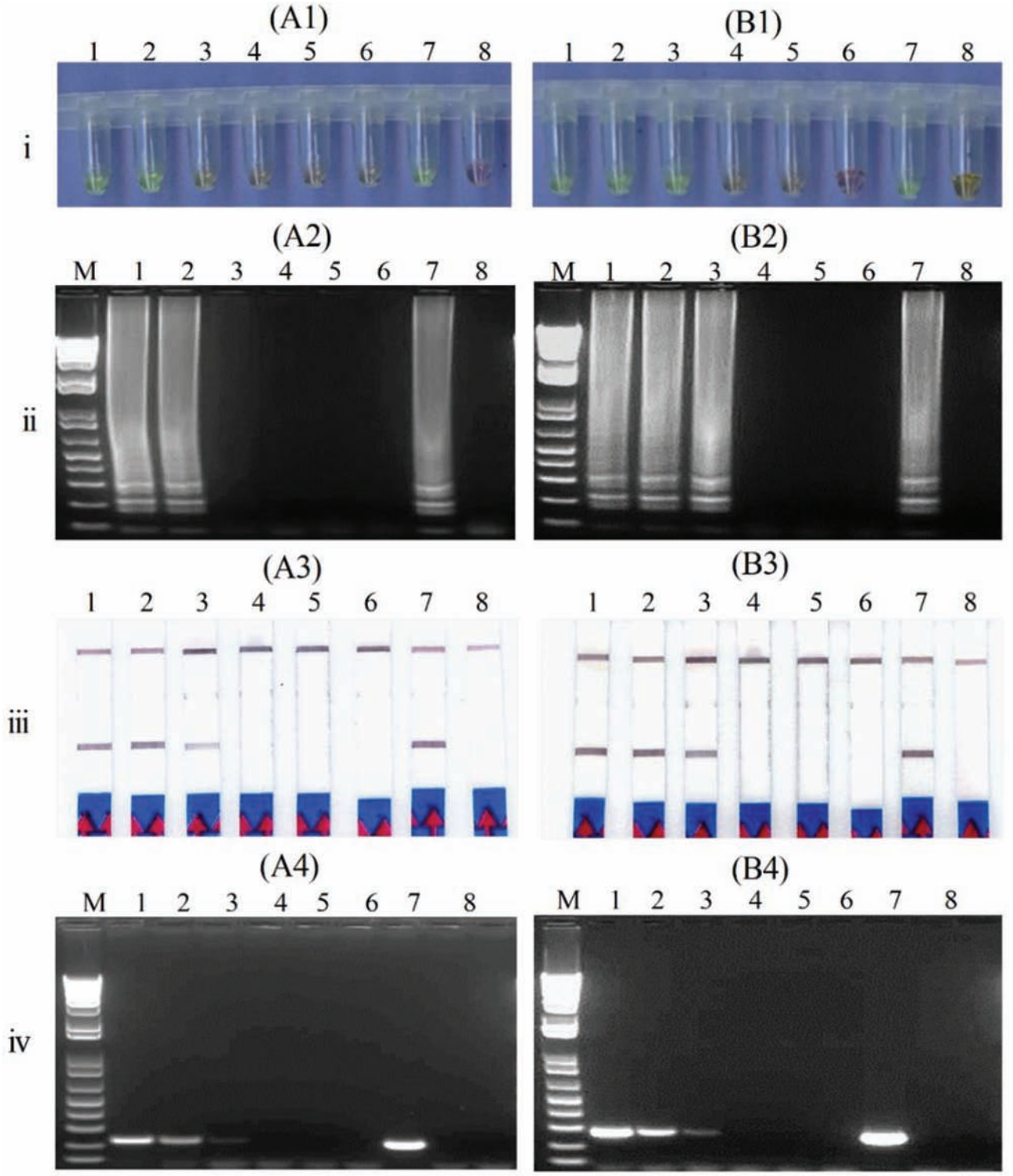



**Fig. 5.** Effect of frass storage on detection of *A. planipennis* genome. (A) Total gDNA from fresh frass (<1 mo) and (B) Total gDNA from dry and old frass (>3 yr). Assessment of products was based on (i) SYBR Green I color change visualization; (ii) gel electrophoresis; (iii) lateral flow dipsticks and PCR diagnostic primer set; and (iv) EABFOT/EABROT PCR amplification products of ~230 bp in DNA samples containing *A. planipennis* genome. Tubes and lanes 1–6 show the detection of gDNA in 10-fold dilutions (10 ng to 100 fg/µl). Tubes and lanes 7 and 8 are gDNA from EAB adult (positive control) and no-template nuclease-free distilled water, respectively. Lane M, 100 bp DNA ladder size marker.

alternative or complement to traditional surveillance and monitoring tools (Cappaert et al. 2005, Ryall et al. 2011, McCullough et al. 2015, Ryall 2015). The ultimate purpose of our study is the application of the developed EAB-LAMP assay to detecting *A. planipennis* in field-collected samples and for conducting future intensive field monitoring and surveillance of *A. planipennis* incursions and associate ecological studies. The developed EAB-LAMP assay successfully detected all *A. planipennis* developmental stages collected from

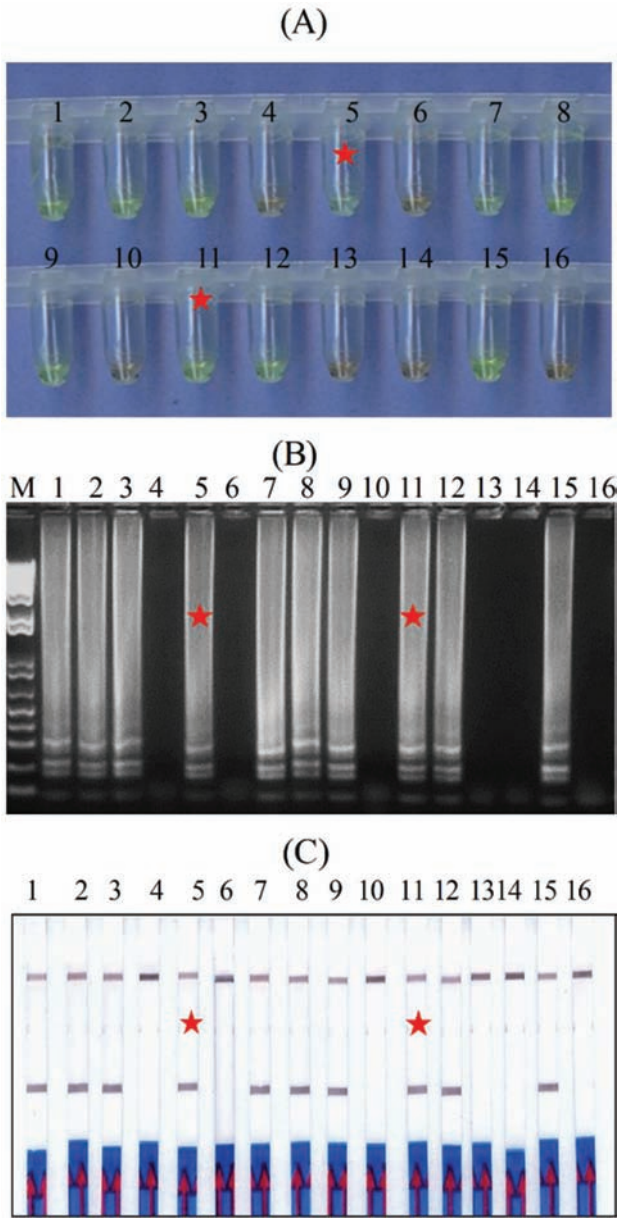



**Fig. 6.** Field validation of EAB-LAMP protocol developed to detect *A. planipennis* caught on glue-coated green prism traps for an early warning system. Product assessment was based on (A) SYBR Green I-visualized color change; (B) gel electrophoresis; and (C) lateral flow dipsticks. Tubes and lanes 1-14, DNA from unsorted green prism trap catches. Tubes and lanes 5 and 11 are the two additional traps visually verified not to contain *A. planipennis* gDNA but detected to contain *A. planipennis* gDNA with the EAB-LAMP assay. Tubes and lanes 15 and 16 are gDNA from EAB adult (positive control) and no template nuclease free distilled water respectively. Lane M, 100 bp DNA ladder size marker.

different geographical regions, from frass and in situ trap samples, confirming its broad detection capabilities, and making it suitable for universal use in surveillance and monitoring of *A. planipennis* worldwide.

Short reaction time and optimal incubation temperature are crucial when developing DNA-based amplification methods for processing large number of field-collected samples (Li and Macdonald 2015). In the current study, the optimal EAB-LAMP reaction conditions were established to be 65°C for 30 min with total detection time of ~2.0 h, which is far shorter and less tedious than required by the traditional branch sampling methods. This rapid turnaround time can improve operational surveillance/monitoring, allow preventative and management measures to be established, and assist in reducing the spread of *A. planipennis* into new locations.

This study also focused on detecting *A. planipennis* genome in frass from different *Fraxinus* species utilize as hosts by *A. planipennis*. In the past, various authors demonstrated and reported the use and capability of different molecular platforms for detecting and identifying target insects using gDNA extracted from frass samples (Kethidi et al. 2003; Fumanal et al. 2005; Scriven et al. 2013; Strangi et al. 2013; Ide et al. 2016a,b, 2018). Although LAMP assays have proven to be useful in detecting many organisms, rarely has the DNA from frass been used in LAMP protocols for detection and identification of alien wood-boring insects. Ide et al. (2016a, b) developed and successfully used LAMP assays to detect traces of insect DNA derived from fecal material, frass substrate, and feeding galleries. Following these authors' footsteps, we effectively developed a robust, rapid, specific, and sensitive LAMP assay that detected trace amounts of *A. planipennis* tissue/epithelial cells DNA in frass collected in the field from various *Fraxinus* species. LAMP has previously been shown to tolerate a range of known inhibitors co-purified during DNA extraction (Kaneko et al. 2007, Tomlinson et al. 2007, François et al. 2011, Kogovšek et al. 2015). It is worth mentioning that due to the low titer of *A. planipennis* DNA in frass, together with high contents of polymerase inhibitory substances in ash phloem tissues, e.g., polysaccharides and polyphenols, the anticipated use of the simple crude DNA extraction method was inappropriate for DNA isolation. Consequently, purified frass DNA was used to perform the EAB-LAMP assay with successful results.

Furthermore, our results also show that long-term storage for at least 3 yr post-collection did not lead to degeneration of the *A. planipennis* DNA in the frass samples. Rather, DNA derived from stored frass (essentially older dehydrated frass) provided increased detection sensitivity of *A. planipennis* DNA in our EAB-LAMP assay. These results suggest that there may be a difference in performance when moisture is either present/absent in frass. Dryness of frass seems conducive to DNA preservation as propounded by other authors (Ide et al. 2016a). These results confirm the suitability of frass as a substrate for use in various molecular assays to detect and identify invasive insect species, especially during the surveillance and monitoring phase.

LAMP-based genetic identification assays have been shown to be useful for quickly identifying insects captured in traps (Chinellato et al. 2013, Kim et al. 2016, Ide et al. 2018). To date, surveillance and monitoring of new *A. planipennis* incursions in the field have been conducted with traps (Crook et al. 2012; Francese et al. 2010 and 2011). However, visual screening of trap captures can miss the presence of *A. planipennis* in new localities, especially when only appendages rather than whole beetles are present. In such cases, morphological identification of *A. planipennis* is extremely difficult, even for experts. To complement the existing screening methodologies for trap-captured samples, we evaluated our EAB-LAMP protocol for accurate detection of *A. planipennis* from traps. The fact that our EAB-LAMP assay picked up *A. planipennis* in two additional traps when visual assessment indicated an absence of the species indicates that there is an accuracy conflict between the two screening approaches. The *mtCOI* sequences generated from the two *A. planipennis* positive traps showed a 100% identity to all *A. planipennis mtCOI* sequences archived in the GenBank database, which confirm results of the EAB-LAMP assay. Thus, the EAB-LAMP

assay had higher specificity and sensitivity, and performed better than visual screening for establishing the presence of *A. planipennis* in traps. Recently Ide et al. (2018) successfully detected *Linepithema humile* (Mayr) in experimental samples containing only an antenna or a hind leg along with 26 whole individuals of six native Japanese ant species using a LAMP assay. The developed EAB-LAMP protocol can serve as a surveillance or monitoring tool to determine the presence of *A. planipennis* appendages in unsorted crude DNA samples; an apparent advantage over traditional surveillance or monitoring methods (Cappaert et al. 2005, Ryall et al. 2011, McCullough et al. 2015, Ryall 2015).

Keeping bulk trap sample processing, operating cost reduction, and on-site applicability of the developed tool in mind, we successfully formulated a proof-of-concept protocol for the detection of *A. planipennis* in pooled trap samples. We tested a semi-quantitative method to estimate the optimal pool size of traps to detect *A. planipennis* (Supp Fig. S3 [online only] and insert). We demonstrated that a pool size of five traps, comprising four traps negative for *A. planipennis*, and one trap positive for *A. planipennis* (providing the least intense positive electrophoresis band from the LAMP products or in the EAB-ReaLAMP assay) can be successfully analyzed in the EAB-LAMP assay. We conclude that testing pooled trap samples with our EAB-LAMP assay will be cost-efficient and will have potential for *A. planipennis* surveillance and monitoring strategies. The estimated expense for DNA extraction, complete EAB-LAMP reaction set up and product detection is approximately $4.50 CAD per sample. Additionally, with the use of the Genie II machine and 15-min crude DNA preparation in a field context, time from start of DNA extraction to detection of *A. planipennis* can be as short as an hour (Tomlinson et al. 2010a). Although our EAB-LAMP assay has many advantages, there are also possible limitations. Due to the high concentration of primers in the reaction, the LAMP technology extremely sensitive, which may, in turn, lead to false-positive results coming from environmental contamination of amplicon aerosols or carryover contamination. To mitigate against such occurrences, stringent spatial separation of reagent preparation, amplification process, and product manipulation are essential to ensure accuracy and reproducibility. Inclusion of wax pellets or adding mineral oil on the top of reaction mixtures to avoid evaporation will reduce contamination (Liang et al. 2013, Lang et al. 2014). Another possibility to eliminate carryover contamination is to limit the amplification time to the absolute minimum, as suggested by Wang et al. (2017). To reduce false-positive results, the amplification time for the current study was limited to 30 min. A further reduction in carryover contamination may be achieved through the addition of Antarctic thermolabile Uracil-DNA Glycosylase (UNG) in the reaction before carrying out the LAMP assay (Wang et al. 2017). Finally, the LAMP assay can be performed on various real-time amplification apparatuses, including the Genie II/III. In this case, target detection is performed using fluorescence, with results appearing in real-time without opening the reaction tubes post-amplification. In such situations, no further DNA product manipulation is required and amplicon aerosols or carryover contamination will be significantly curtailed, which is an advantage for on-site application of the LAMP platform.

Altogether, our results provide an important proof-of-concept that the EAB-LAMP assay can be reliably used in *A. planipennis* detection, identification, surveillance, and environmental monitoring to obtain accurate, sensitive, and rapid genetic results. Results from our EAB-LAMP assay may support the implementation of large-scale active surveillance or monitoring operations toward effective, efficient, and timely management of *A. planipennis* incursions in new locations and incipient populations. The simplicity of the described protocols and features of our developed EAB-LAMP assay should allow field biologists and reliably trained citizen scientists to participate in *A. planipennis* surveillance and monitoring operations, which can be critically important for time-sensitive detection and subsequent management. Furthermore, the EAB-LAMP assay design and development efforts assembled in this study should allow it to be adapted for screening other invasive alien pests. Finally, the developed EAB-LAMP assay has the potential to be developed as an operational tool to complement the existing visual surveys of *A. planipennis* incursions using trapping and branch sampling methods.

## Supplementary Data

Supplementary data are available at *Journal of Economic Entomology* online.

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