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For any suspicion of *Aethina tumida* infestation in a European apiary, the competent authorities of the country have to be advised.

The suspected samples have to be sent to the European Union Reference Laboratory for Bee Health for confirmation.

1 PURPOSE AND SCOPE

This instruction covers the identification by real time PCR (polymerase chain reaction) of the small hive beetle (SHB) in samples from larvae or adult insects suspected of corresponding to this beetle, collected in bee colonies, or in samples of bees (adults, larvae or eggs) potentially contaminated during the importation of queens. Depending on the condition of the sample, identification is initially attempted by morphological examination of the samples of suspect insects or larvae, in accordance with instruction **ANA-I1.MOA.1500**, then confirmed by PCR if necessary. If the samples of biological material cannot be identified morphologically, the PCR method is applied directly (on larvae,adult insects or eggs).
This protocol is based on the detection of a sequence of the cytochrome oxidase I (COI) gene in mitochondrial DNA that is specific to the SHB.

2 CONTENT

2.1 Principle

The technique used is based on the protocol for real-time PCR described by Ward et al. (2007)[1]. The primer pair used can amplify a 109-bp PCR product. The protocol consists of six steps:

1) Grinding samples from suspect specimens.
2) Extraction of genomic DNA (not described in this protocol).
3) Preparation of the real-time PCR reagent mix.
4) Preparation of the plate containing the controls and the samples.
5) Execution, analysis and interpretation of the results of the real-time PCR.
6) Validation of the results.

2.2 Method performance

The following table describes the performance criteria of the described method validated by the EURL for Honey Bee Health according to the NF U47-600 standard (www.afnor.fr).

Criteria	Expected results	Observed results
Analytical specificity	100%	100%
LOD_{PCR} (Limit of detection)	NA	10^5 copies / reaction
LOD_{METHOD}	1 specimen (adult or larva)	Adult specimen: 1/10 000 Larva specimen: 1/1 000
Diagnosis sensitivity	100%	100% (25/25)
Diagnosis specificity	100%	100% (15/15)

NA: not applicable

[1] Ward et al., (2007) A DNA method for screening hive debris for the presence of small hive beetle (*Aethina tumida*). Apidologie 38, 272–280

2.3 Equipment, consumables and reagents[2]

Equipment	- Micropipettes (0.5-10 µl, 2-20 µl, 10-100 µl, 100-1000 µl) - Vortex - Spin micro-centrifuge - 96-well plate centrifuge - Microtube incubator - Real-time PCR system and a computer coupled to the machine (BIORAD CFX96)
Consumables	- Sterile 50 ml tubes and 1.5 ml microtubes (RNase-, DNase-free) - Disposable pellet pestle - Optical 96-well reaction plate (and optical plate sealer film) (BIORAD) - Sterile filter tips (RNase-, DNase-free) - Powder-free latex gloves
Reagents	- Pure RNase- and DNase-free water (H_2O) - Phosphate buffer 0.01 M pH 8.0 - **High Pure PCR template preparation Kit** (Roche Diagnostics, ref 11796828001) - **SsoAdvanced PCR probe Supermix** (BIORAD, ref 1725231) - Specific SHB primers: SHB207F and SHB315R (stock solution: 100 µM; working solution: 20 µM in H_2O) - Specific SHB probe: SHB245T (working solution: 50 µM in H_2O). It is recommended that the fluorescent probe be protected from the light with a piece of aluminium foil. - **TaqMan® Exogenous Internal Positive Control Reagents (**Applied Biosystems ref 4308323 - VIC™ Probe) - External positive controls (PCR control and extraction control)[3]

Primers and Probe	Sequence
SHB207F	5'- TCT AAA TAC TAC TTT CTT CGA CCC ATC (A/G) -3'
SHB315R	5'- TCC TGG TAG AAT TAA AAT ATA AAC TTC TGG - 3'
SHB245T probe	5'- (6-Fam) ATC CAA TCC TAT ACC AAC ACT TAT TTT GAT TCT TCG GAC (Tamra)-3'

2.4 Grinding samples from suspect specimens

[2] *All commercial references described in this document are given for information. Other equivalent reagents or equipment can be used. The final user must check the conformity of his protocol with the expected criteria defined by the EURL for Bee Health (see § 2.2.). The EURL can help the laboratory to verify the conformity of its method (contact: eurl.bee@anses.fr).*

[3] *The EURL can provide the national reference laboratories, as a priority, with the different controls described in this protocol (contact: eurl.bee@anses.fr).*

The protocol used for grinding the samples from suspect insects is described below. First, if the samples have been placed in alcohol, the specimens (adults, larval Coleoptera or eggs) are rinsed three times in a large volume of phosphate buffer (50 ml tube, for example). The specimen is then transferred to a 1.5 ml microtube. In the case of eggs, this rinsing must be done with great care, due to the small size and the fragility of the eggs. It will not be possible to transfer them to a new tube.This step is important to avoid inhibition.
The grinding of larva or adult insect is done manually using a disposable pellet pestle in the 1.5 ml microtube (or larger tube, if necessary). The volume depends on the size of the sample (example: one adult insect: in 1ml; one larva: in 200 µl).
In the case of eggs, 1 to 10 eggs are taken up with 80µL of phosphate buffer, without grinding.
It is recommended to store the samples in a freezer at a temperature ≤ -16°C, and clearly labelled.

2.5 Genomic DNA extraction

- Extract genomic DNA from suspected specimens with the chosen relevant method ensuring quality of the nucleic acids extracted.

Note: The detection and identification of insect or honeybee pathogens by PCR is wholly dependent upon the availability of 'clean' nucleic acid mixtures to act as a template for the reactions (OIE *Terrestrial Manual*, Guideline 3.2, 2012). This stage is critical because if the target material has not been purified of contaminants in the clinical sample, the assay stage is compromised and may yield false results (OIE *Terrestrial Manual*, Chapter 1.1.5, 2012).
There are a number of specialised methods for particular types of samples and tissues, some of which are now commercially available either as manual or automated systems for robotic workstations.

Note: Regardless of the DNA extraction method used, the EURL for Bee Health recommends adding a positive extraction control, to ensure the effectiveness of the extraction, and a negative extraction control (replacing the sample with ultra-pure water or elution buffer) to ensure absence of contamination; when PCR is used for diagnosis, a great deal of care is required to avoid contamination of the samples because the exquisite sensitivity of the technique can easily lead to false-positive results (OIE *Terrestrial Manual*, Chapter 1.1.5, 2012).

- The positive extraction control can be made from a dilution of SHB homogenate, paying attention to the limit of detection of the method. The EURL recommends estimating the limit of detection of the method in the user's laboratory conditions (following an appropriate validation protocol) to ensure that the method allows to properly detect the targeted insect (OIE *Terrestrial Manual*, Chapter 1.1.5. 2012). The positive extraction control can be aliquoted in a small volume and then stored at a temperature of ≤ -16°C in the freezer. The positive control must be in relation with the matrix extracted: e.g. adult beetle or larva.

Alternatively, positive extraction control homogenate can be provided by the EURL.

- Before DNA extraction, it is recommended to treat the samples with proteinase K, if this step is not included in the DNA extraction method.

The method validated by the EURL includes an extraction step from **80 µl** of homogenate (adult or larval specimen) and the elution volume is **200µl**. The appropriate "High Pure PCR template preparation" kit from Roche (ref 11796828001) is used to validate the method.

2.6 Preparation of the real-time PCR reagent mix

- Prepare the reagent mix according to the following table:

	Final concentration	Volume for one tube (µl)
H_2O	**/**	**4.1**
SsoAdvanced (2x)	**1x**	**12.5**
SHB207F (20 µM)	**320 nM**	**0.4**
SHB315R (20 µM)	**320 nM**	**0.4**
245 probe (50 µM)	**100 nM**	**0.05**
10x IPC MIX	**1x**	**2.5**
50x IPC DNA	**0.1x**	**0.05**
Mix total volume		**20**

IPC: Internal Positive Control

- Amplification is performed in a 25 µl final volume: 20 µl of mix + 5 µl of DNA

- DNA samples are as follows:
  - DNA extracted from samples to be tested
  - DNA extracted from positive extraction control or negative extraction control
  - SHB PCR positive control must be added. The EURL can provide a positive control for this specific PCR (G2 plasmidic clone) and recommends using it diluted to a concentration of the LOD_{PCR} validated by the laboratory multiplied by 10. Use 5 µl of the G2 clone as template in the tube for positive control.
  - PCR negative control must be added (5 µl of pure distilled water instead of 5 µl of sample).

Note: There are a number of reagents for PCR commercially available either separately or in kit form. The protocol recommended here must be adopted by the laboratory as appropriate for their working conditions (reagents, equipment).

2.7 Conditions for amplification

Table showing the amplification cycles:

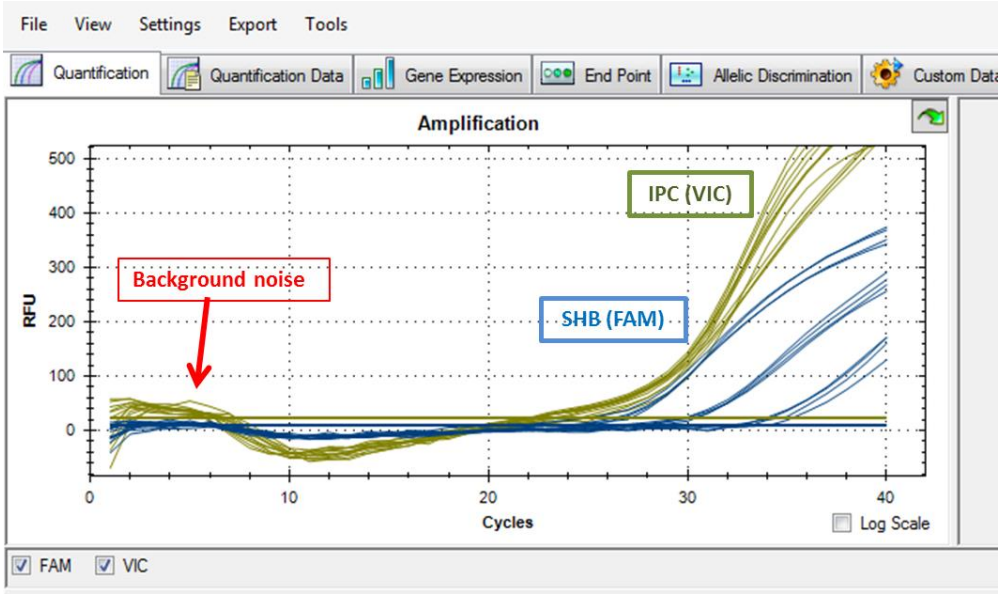
Step	Cycle	Temperature (°C)	Time (min)
Polymerase activation	1	95	3:00
PCR	40	95	0:10
		60	0:30

2.8 Data analysis

2.8.1 Analysis of the amplification

Depending on the amplification system, background noise may appear between cycles 1 and 9. In such cases, analysis is only performed on cycles 10 to 40.

Example:



IPC: Internal positive control
FAM: 6-carboxyfluorescein, fluorophore associated with the probe
VIC®: flurorophore associated with the IPC

2.8.2 Validation of the results

For validation of the results, several criteria are taken into account:

- The threshold cycle (Ct) obtained for the external control (extraction control) must be ≤ 35.
- The Ct obtained for the PCR control must be ≤ 35.
- Confirmation that no amplification has been generated in the negative PCR controls (Ct= N/A).

If one of these controls does not satisfy these criteria, the test is not valid and must be repeated.

A result identifying the SHB by real-time PCR is therefore only considered valid if:
- The positive extraction and PCR controls are positive (Ct ≤ 35),
- The negative extraction and PCR controls are negative (Ct = N/A).

In the event of a negative result for the samples to be tested, check for the absence of PCR inhibitor in the extract analysed through the amplification of the Internal Positive Control (IPC). Hence PCR inhibitor could lead to a "false negative" result. Inhibition should be overcome by dilution of the sample for example to 1/10.

2.8.3 Interpretation of the results

The interpretation of the results of this real-time PCR for the SHB is based on the presence or absence of any amplification, as demonstrated by a Ct.

- A positive result of **identification** by PCR leads to the conclusion "**positive**"
- A negative outcome of **identification** by PCR leads to the conclusion "**negative**"
- If any inhibition remains, the conclusion is "**uninterpretable**"

2.9 Confirmation of the results obtained on the « egg » matrix

When a negative results is obtained on a sample containing eggs, it is necessary to confirm it by the amplification of a part of the COI gene using universal primers LCO1499 (5'-GGTCAACAAATCATAAAGATATTGG-3') / HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (described by Folmer et al. 1994). This PCR is performed on the DNA already extracted following the protocol described above:

Add 2 µl of the DNA template (unknown sample or positive control) or negative control to the reagent mixture to a final volume of 20 µl.

	Final concentration	Volume for one tube (µl)

Nuclease free H_2O	/	12.6
Taq DNA pol (5 U/µl)	0.5 U/µl	0.2
Taq DNA pol buffer (10×)	1×	2.0
$MgCl_2$ (50 mM)	3.5 mM	1.3
dNTP mix (10 mM)	450 µM	0.9
LCO1499 (20 µM)	500 nM	0.5
HCO2198 (20 µM)	500 nM	0.5
Mix total volume		18

An example of thermocycler programme is as follows:

Step	Cycle	Temperature (°C)	Time (minutes)
Initial denaturation	1	95	4:00
PCR	35	94	0:60
		51	0:60
		72	1:30
Final extension	1	72	7
Hold		10	∞

Optimisation of PCR should be carried out according to the Mastermix and PCR machine used, especially testing with different annealing temperatures.

The SHB and negative controls are also included in the test. The amplification products (~660bp) obtained are sequenced using the two primers used during the PCR.
The analysis of the obtained sequences is carried out according to the ANA-P0.PRT.2000 procedure. The obtained sequence is compared to that of the *Aethina tumida* COI sequence and/or a query of the databases by blast is performed to identify the species detected.

2.10 Storage and disposal of samples after analysis, and disposal of waste

Storage and disposal of samples after analysis
The samples (homogenate of specimens, DNA extracts) are stored for 2 months in the freezer at a temperature ≤ -16°C.
Once beyond this deadline, the samples are disposed of in an infectious clinical waste container suitable for destruction by incineration, or are preserved for research.

The 96-well plates containing the products of PCR are disposed of using waste containers provided for this purpose.

Synopsis