Identification of *Tropilaelaps* spp., using a conventional PCR (in-house method) and sequencing

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Effective date: 21/11/2016

Protocol established according to the ANA-I1.MOA.43_Rev0

For any suspicion of *Tropilaelaps* spp. 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 Honey Bee Health for confirmation.

1 PURPOSE AND SCOPE

This instruction covers the identification by conventional PCR (polymerase chain reaction) of the *Tropilaelaps* spp. in suspect samples of mites. Depending on the condition of the sample, identification is initially attempted by morphological examination of the samples of suspect mites, in accordance with the instruction **ANA-I1.MOA.3500**, and then confirmed by PCR if necessary. If the mites cannot be identified morphologically, the PCR method is applied directly.
This protocol is based on the detection of a sequence of COI gene (cytochrome oxidase I) of mitochondrial DNA. As the primers used are not specific of the *Tropilaelaps* species and that amplification of COI gene from other parasites could be occurred, it is necessary to perform a sequencing when a PCR product of the expected size is obtained.

2 CONTENT

2.1 Principle

The technique used is based on the protocol for conventional PCR described by Anderson and Morgan (2007)[1]. The primer pair used amplifies a 580-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 conventional PCR reagent mix,
4) Preparation of the controls and the samples,
5) PCR reaction and analysis of the PCR results,
6) Sequencing and analysis of the sequence obtained,
7) 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	100 copies / reaction
LOD_{METHOD}	1 specimen	1 specimen
Diagnosis sensitivity	100%	100% (13/13)
Diagnosis specificity	100%	100% (18/18)

NA: not applicable

[1] Anderson & Morgan, (2007) Genetic and morphological variation of bee-parasitic *Tropilaelaps* mites (Acari: Laelapidae): new and re-defined species. Exp Appl Acarol 43, 1–24

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 - Microtube incubator - Conventional PCR system
Consumables	- Sterile 1.5 ml microtubes (RNase-, DNase-free) - Disposable pellet pestles - 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) - **Platinum Taq DNA Polymerase / MgCl2** (Invitrogen, ref 10966-034) - dNTPs (Invitrogen, ref 18427-013) - Specific primers: **COI-TCF1** and **COI-TCR2** (stock solution: 100 µM; working solution: 20 µM in H_2O)

Primers	Sequence
COI-TCF1	5'-CTATCCTCAATTATTGAAATAGGAAC-3'
COI-TCR2	5'-TAGCGGCTGTGAAATAGGCTCG-3'

2.4 Grinding samples from suspect specimens

The protocol used for grinding the samples of suspect insects is described below:
* If possible take 10 individuals
* If the samples have been placed in alcohol, the specimens must be rinsed three times in a large volume of phosphate buffer. The specimens are then transferred to a 1.5 ml microtube. This step is important to avoid inhibition.
* The grinding is done manually using a disposable pellet pestle in the 1.5 ml microtube. The volume of phosphate buffer for 1 to 10 specimens is 200 µl.
It is recommended that samples be stored in a freezer at a temperature ≤ -16°C, and clearly labelled.

[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 Honey Bee Health (see § 2.2.). The EURL for Honey Bee Health can help the laboratory to verify the conformity of its method (contact: eurl.bee@anses.fr).*

2.5 Genomic DNA extraction

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

Notes:

- 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).
- A negative extraction control, to ensure absence of contamination (replacing the sample with ultra-pure water or elution buffer), should be added, regardless of the DNA extraction method used.
- A positive extraction control, to ensure the effectiveness of the extraction, should also be added when available.
- During the PCR, 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).
- 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.

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

The validated method includes an extraction step from **120 µl** of homogenate and the elution volume is **200 µl**. The appropriate "High Pure PCR template preparation" kit from Roche (ref 11796828001) has been used to validate the method.

2.6 Preparation of the conventional PCR reagent mix

➢ Prepare the reagent mix according to the following table:

	Final concentration	Volume for one tube (µl)
Nuclease free H_2O	**/**	**12.4**
Taq DNA pol (5 U/µl)	**0.5U/µl**	**0.2**
Taq DNA pol (10x)	**1x**	**2.25**
MgCl2 (50 mM)	**3.5 mM**	**1.35**
dNTP mix (10 mM)	**450 µM**	**0.9**
COI-TCF1 (20 µM)	**500 nM**	**0.45**
COI-TCR2 (20 µM)	**500 nM**	**0.45**
Mix total volume		**18**

- Amplification is performed in a 20 µl final volume: 18 µl of mix + 2 µl of DNA

- DNA samples are as follows:
  - DNA extracted from samples to be tested
  - DNA extracted from a negative extraction control (buffer used for gridding)
  - *Tropilaelaps* PCR positive control must be added[3].
  - PCR negative control must be added (2 µl of pure distilled water instead of 2 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)
Initial denaturation	1	95	5:00
PCR	35	94	0:30
		58	0:30
		72	0:45
Final extension	1	72	7
Hold		10	∞

2.8 Analysis of the PCR products

Different methods could be used. The EURL has tested two systems: electrophoresis on agarose gel and electrophoresis on a Bioanalyser system.

2.8.1. Electrophoresis on agarose gel

Electrophoresis technique is based on the separation of negatively charged nucleic acids, under the effect of an electric field. This separation is performed into agarose gel matrix: the smallest molecules move faster and migrate farther than the largest ones. Estimation of the size fragment is done by comparison with a DNA-ladder which migrates simultaneously in another well of the same gel.

The staining method used is the revelation with ethidium bromide (EtBr). EtBr is an intercalating agent

[3] The EURL can provide a positive control for this specific PCR (TM11 plasmidic clone) and recommends to use it diluted to a concentration corresponding to the LOD_{PCR} validated by the laboratory multiplied by 10. Use 2 µl of the diluted TM11 clone as template in the tube for positive control.

commonly used in molecular biology laboratories to observe nucleic acids. When exposed to ultraviolet it fluoresces. This fluorescence is 20 times higher when EtBr is linked to DNA molecules.

- **Preparation of the agarose gel**
  - It is recommended to prepare the agarose gel with Tris-acetate-EDTA (TAE) 1X.
  - A gel with 1.2 % (w/v) of agarose must be prepared to observe PCR products (for example weight 1.2 g of agarose for 100 ml of TAE 1X).
  - Heat the TAE-agarose in a microwave until obtaining a transparent liquid.
  - Cool a few minutes before pouring the solution into a gel rack, containing the comb.
  - Wait until the gel has solidified.
  - Remove the comb and place the gel into the electrophoresis tank.
  - Pour TAE 1X to cover the gel.

- **Preparation and loading of the samples**
  - It is recommended to migrate **10 µl** of the PCR products.
  - Loading buffer must be added to the sample before migration.
  - Load the samples into the wells.
  - Load a DNA-ladder.
  - Run the gel.

- **Gel staining with EtBr**

EtBr is a potent mutagen. It is strongly recommended to wear nitrile gloves, a lab coat and protective glasses.

- Put the gel in a 0.5 µg/ml EtBr solution, for at least 20 to 30 minutes. The duration can be increased if the gel contains a high percentage of agarose, if it is too thick or if the EtBr solution is too old.
- Observe the gel with ultraviolet.
- Take a picture of the gel.

2.8.2. Electrophoresis on a Bioanalyser system

PCR products could be analysed on On-chip gel electrophoresis. The chip format dramatically reduces separation time and sample consumption. During chip preparation, the micro-channels are filled with a sieving polymer and fluorescence dye. Once the wells and channels are filled, the chip becomes an integrated electrical circuit. Charged biomolecules like DNA are electrophoretically driven by a voltage gradient-similar to slab gel electrophoresis. As dye molecules are intercalated into DNA, the complexes are detected by laser-induced fluorescence. With the help of a ladder that contains fragments of known sizes and concentrations, a standard curve of migration time versus fragments size is plotted. "Lower" and "upper" markers are internal standards used to align the ladder data with data from the sample wells.

Two different reagents could be used to analyse DNA fragments depending of their size: 7500 DNA kit or 1000 DNA kit.

The present protocol has been validated with a **7500 DNA kit**.

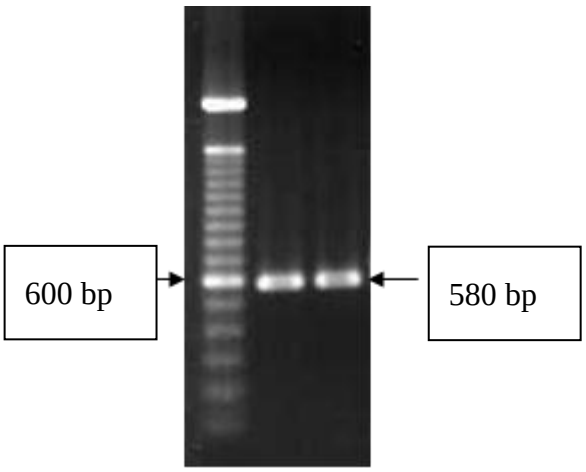
- **Preparation of the chip**
  - Prepare the Gel-Dye Mix as indicated by the supplier: Use the gel-dye within 4 weeks of preparation. Protect the gel-dye mix from light. Store the gel-dye mix at 4 °C.
  - Load the Gel-Dye Mix on the chip (on only well) : pressurize
  - Load the Gel-Dye Mix on two other wells
  - Load the Marker
  - Load the Ladder

- **Load the samples on the chip: 1 µl**
- **Vortex for 60 seconds at 2400 rpm.**
- **Insert the Chip in the Bioanalyzer**
- **Start the Chip Run**

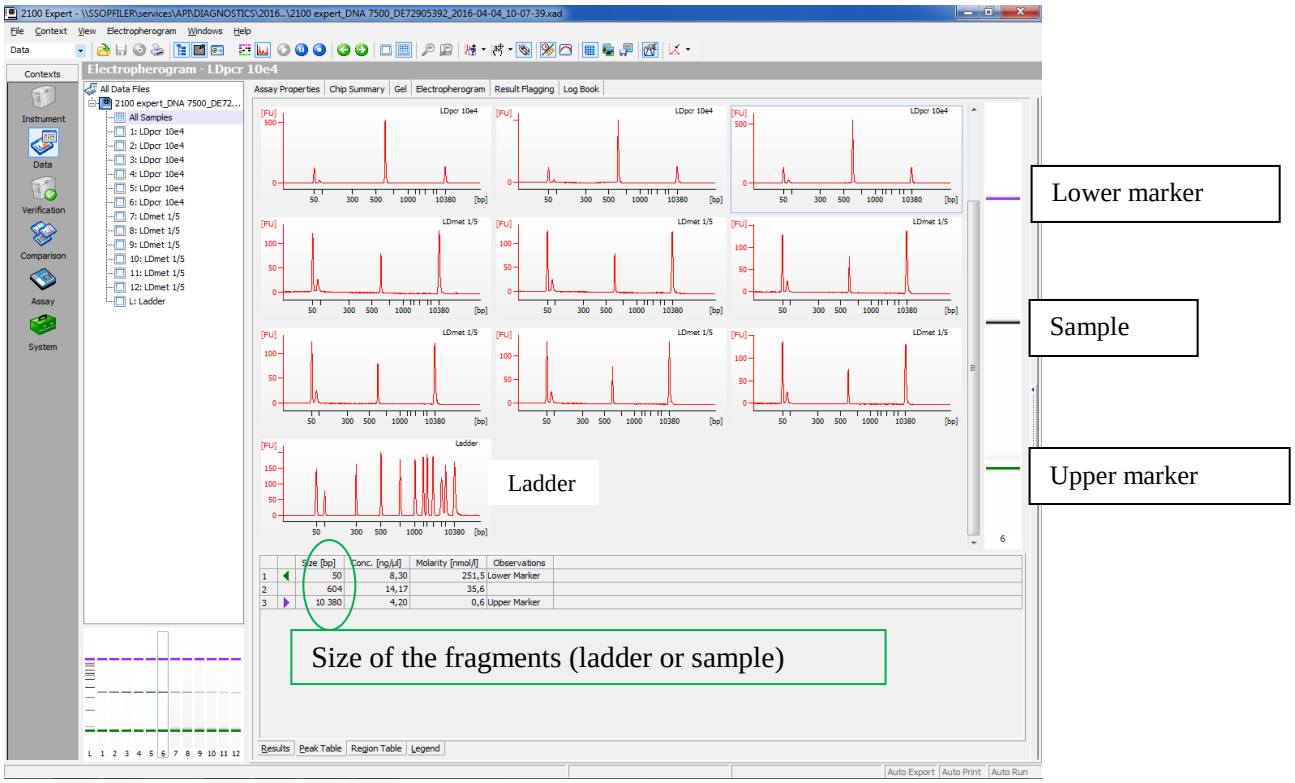
2.8.3. Analysis of the amplification and validation of the PCR

As part of this *Tropilaelaps* PCR, the interpretation of the results is based on the presence or absence of the amplified product: the size of the expected PCR product is 580 bp including the two primers. However, the presence of a PCR product of the right size cannot alone identify the *Tropilaelaps* genus and species and requires to perform a sequencing stage (see § 2.9).

- **After using electrophoresis on agarose gel**



- **After using Bioanalyser system**



2.8.4. Validation of the results

The present conventional PCR is therefore only considered valid if:
- The positive extraction (if available) and PCR controls are positive (presence of the expected band),
- The negative extraction and PCR controls are negative (absence of a specific band).

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

In the event of a negative result for the suspect mite, check for the absence of PCR inhibitor in the extract analysed through the amplification of an external PCR control. Hence PCR inhibitor could lead to a "false negative" result. Inhibition should be overcome by dilution of the sample for example to 1/10.

When the PCR products are analysed with Bioanalyser system, check the electropherogram of the ladder well to ensure the good migration. The expected size of the amplified fragment is **603 ± 5bp**, using a 7500 DNA chip. However, the size could be change with experimental conditions. The size of the fragment is then compared to the size of the positive PCR control.

2.9 Sequencing of the PCR products

In the case of the visualization of a 580 bp band, the PCR product obtained must be sequenced. This step can be subcontracted, and will not be described in this procedure.

Analysis of the sequences obtained could be performed according to the **ANA-P0.PRT.2000** procedure.

A panel of COI sequences available on Genbank must be included in the analysis to construct the phylogenetic three and to identify the species of *Tropilaelaps*. A list of available sequences is provided in Annex 1. This list will be updated as necessary during the analysis of the sequences obtained scientific watch.

2.10 Interpretation of the results

The interpretation of the results of this method (PCR followed by sequencing) is based on the comparison of the sequence obtained on the suspected mite with those described in the literature.

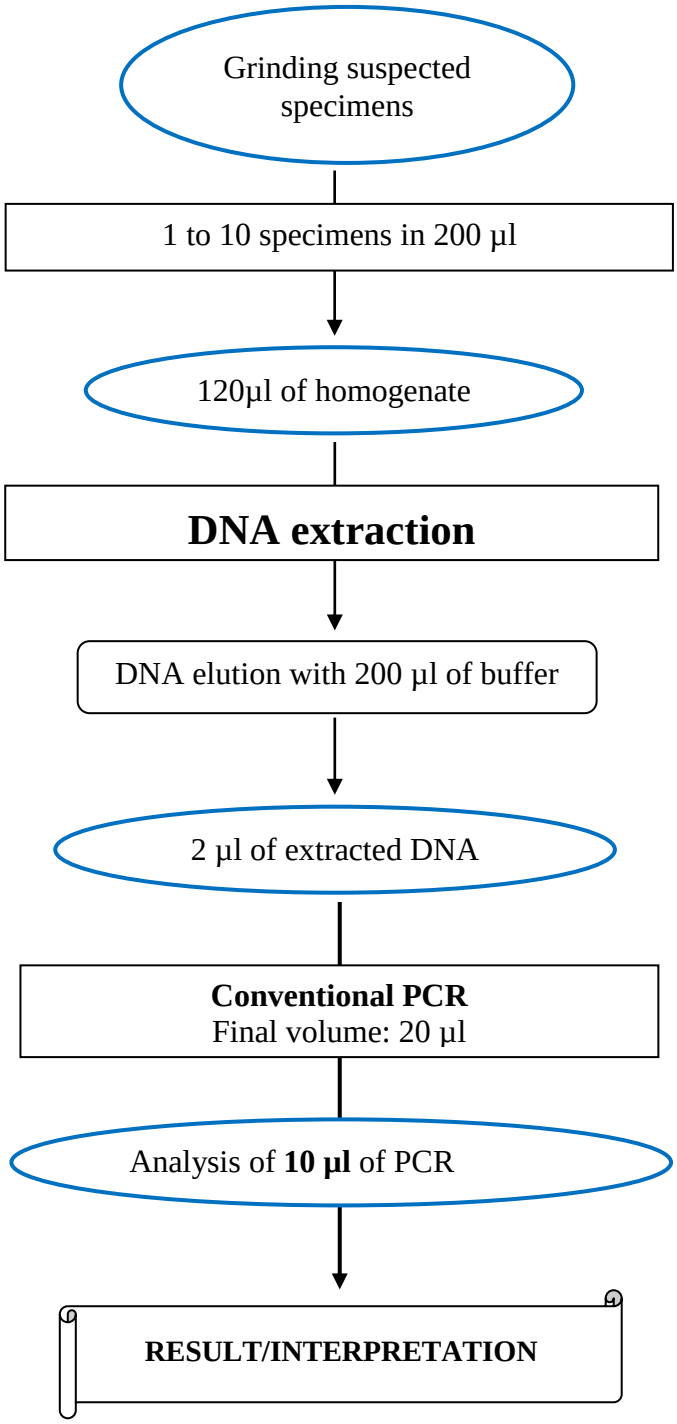
- If the sequence obtained for the suspect specimen matches with the COI gene of *Tropilaelaps* spp., this leads to the conclusion of a **"positive"** identification.
- If the sequence obtained for the suspect specimen does not match with the COI gene of *Tropilaelaps* spp., this leads to the conclusion "**negative**" identification.
- If any PCR inhibition remains, the conclusion is "**uninterpretable**"

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

The samples (homogenate of specimens, DNA extracts) are stored for two months in the freezer at a temperature ≤ -16°C.
Once past these deadlines, the samples are disposed of in an infectious clinical waste container suitable for destruction by incineration, or are preserved for research.

The tubes containing the products of PCR are disposed of using waste containers provided for this purpose.

Synopsis



Annex 1:

- *Tropilaelaps* sequences: GenBank reference

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- Outgroup sequences: GenBank reference

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