Animal health analytical method

REFERENCE: ANSES/SOP/ANA-I1.MOA.2400 - Version 02

April 2020

Standard Operating Procedure

Detection of Paenibacillus larvae, *agent of American foulbrood, and* Melissococcus plutonius, *agent of European foulbrood by qPCR*

Sophia Antipolis Laboratory

National Reference Laboratory – Honey Bee Health

European Union Reference Laboratory – Bee Health

History of the method

A method can be updated in order to take changes into account.

A change is considered major when it involves the analytic process, the scope or critical points of the analysis method, the application of which may modify the performance characteristics of the method and/or the results. A major change requires major adaptations and either total or partial revalidation.

A change is considered minor if it provides useful or practical clarifications, reformulates the text to make it clearer or more accurate, or corrects minor errors. A minor change in the method does not alter its performance characteristics and does not require revalidation.

The table below summarises the version history of this method and provides qualifications for the changes.

Version	Nature of changes (Major / Minor)	Date	Main changes
V 01	Creation	April 27th, 2020	Initial version: Standard Operating Procedure (SOP) established according to the ANA-I1.MOA.24_rev01
V02	Minor Update	July 31st, 2025	Logos updated, removal of appendix 1 related to genomic DNA extraction Synoptic

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Foreword

This method was developed by:

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Contents

Warnings and safety precautions

The user of this method should be closely familiar with standard laboratory practices. It is the responsibility of the user to establish suitable health and safety practices and ensure compliance with the current regulations.

All actions taken in accordance with this method must be performed by employees who have attended relevant training.

1. Purpose and scope

This instruction describes the detection of the pathogenic bacteria ***Paenibacillus larvae*** and ***Melissococcus plutonius***, respectively main agent of American foulbrood and European foulbrood by real-time PCR (qPCR) in crushed bee larvae.

The method is based on the amplification of an ITS (Intergenic Transcribed Spacer) sequence of ***P. larvae*** or a sequence of the gene coding for the 16S rRNA of ***M. plutonius***.

2. Reference documents

[1] ANA-P1.PRT.27 Dossier de validation de la PCR-TR : Détection de *Paenibacillus larvae* par PCR en temps réel (méthode interne)

[2] ANA-P1.PRT.28 Dossier de validation de la PCR-TR : Détection de *Melissococcus plutonius* par PCR en temps réel (méthode interne)

[3] ANA-I1.MOA.01 Recherche de la loque européenne du couvain d'abeilles

[4] ANA-I1.MOA.03 Recherche de la loque américaine du couvain d'abeilles

[5] ANA-I1.MOA.1900 Protocol Instruction PCR AFB PCR (d'après le ANA-I1.MOA.19_Rev02)

[6] ANA-I1.MOA.2100 Protocol Instruction PCR EFB (d'après le ANA-I1.MOA.21_Rev01)

[7] NF U47-600-2 "Méthodes d'analyse en santé animale – PCR (réaction de polymérisation en chaîne) – Partie 2 : Exigences et recommandations

[8] ISO/IEC 17025:2017 General requirements for the competence of testing and calibration laboratories

3. Terms, abbreviations and definitions

Quantification cycle (C_q) or threshold cycle (C_t)

The cycle at which fluorescence from amplification exceeds the background fluorescence has been referred to as threshold cycle (C_t), crossing point (Cp), and take-off point (TOF) by different instrument manufacturers, but is now standardized by the MIQE guidelines as the quantification cycle. A lower C_q correlates with higher target expression in a sample.

Real-time PCR and quantitative PCR (qPCR)

Real-Time PCR refers to the fact that measurements are made during the amplification as opposed to at the end of PCR. qPCR introduces the idea that the data provides quantification of the target. The terms are often used interchangeably. Quantitative real-time PCR is often abbreviated as qPCR. Real-time PCR should not be confused with RT-PCR, which refers to reverse transcription PCR, a technique for reverse transcribing RNA into complementary DNA, which is then amplified.

In this SOP, the qualitative Real-Time PCR is referred to as qPCR.

MIQE

Minimum information for publication of quantitative real-time PCR experiments. These guidelines provide a framework to conduct qPCR experiments and the information and terminology required for submission of qPCR data to any journal. (Bustin SA, Benes V, et al. (2009) The MIQE Guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem, 55(4): 611−622).

4. Principle of the method

The qPCR method is based on TaqMan™ probe hydrolysis technology (Applied Biosystems®), using a probe (oligonucleotide with both a 5' reporter fluorescent dye and a 3' quencher dye attached) and a DNA polymerase with exonuclease activity.

During amplification, the 5'-3 'exonuclease activity of the polymerase cleaves the probe attached to the target sequence, causing the reporter to move away from the quencher, increasing the reporter fluorescence emission.

The amount of fluorescence released by the reporter is therefore directly proportional to the number of copies of the amplified gene.

The process of detecting ***P. larvae*** or ***M. plutonius*** by qPCR from bee larvae samples follows 6 steps:

1. Preparation of crushed bee larvae
2. Extraction of genomic DNA from crushed larvae
3. Preparation of the qPCR reagent mix
4. Preparation of the plate containing the controls and the samples
5. Execution and analysis of the qPCR results
6. Interpretation and validation of the results

5. Reagents

Warning: ***Trade names or supplier names may be mentioned in the description of the products required to implement this method. This information is provided for users of the method and does not mean that ANSES recommends the exclusive use of these products. Similar products may be used if it has been demonstrated that they achieve the same results.***

The final user must verify the conformity of the implemented SOP with the expected criteria defined by the EURL for Bee Health. The EURL for Bee Health can help the laboratory to verify its method conformity (contact: eurl.bee@anses.fr).

The reagents necessary for the detection of ***P. larvae*** and ***M. plutonius*** by qPCR are described in the table above.

SOP processing steps	Storage conditions
1. Preparation of samples for analysis	
Ultra-pure water	5°C ± 3°C
2. Genomic DNA extraction	
Molecular biology grade water (BM) DNase/RNase free	5°C ± 3°C
Absolute Ethanol	Room temperature
Extraction kit : QIAamp® DNA mini kit (Qiagen®)	Room temperature
Lysozyme 20 mg/mL (20 mM Tris-HCl pH 8.0 ; 2 mM EDTA ; 1,2% Triton)	Aliquote at ≤ -16°C
3. Preparation of the qPCR reagent mix	
Molecular biology grade water (BM) DNase/RNase free	5°C ± 3°C
SsoAdvanced™ Universal Probes Supermix 2X (Bio-Rad)	≤-16°C
TaqMan® Exogenous IPC Reagents (Applied Biosystems™) : • Mix IPC 10X (includes probe and specific primers) • ADN IPC 50X	≤-16°C
TE pH 8.0 (10 mM Tris-HCl pH 8.0 ; 1 mM EDTA) DNase/RNase free biffer	5°C ± 3°C
Specific primers to the agent* at 20 µM in TE buffer	≤ -16°C
Specific probe to the agent* at 50 µM in TE buffer	≤ -16°C
4. Preparation of the PCR plate	
Molecular biology grade water (BM) DNase/RNase free	5°C ± 3°C
POSITIVE PCR CONTROL: STANDARD RANGE	**≤-16°C**

The primers used for the amplification of ***P. larvae*** and ***M. plutonius*** target DNA by qPCR are described in the table above.

Primers and probe sequences for the detection of *P. larvae

Primers/probe ID	Oligonucleotide Sequence
PI ITS fwA	5'-CACCTTACGTGTGATTGA-3'
PI ITS revA	5'-TGCTCTTACCAGCTTAAC-3'
PI ITS ProbeA	5'-(6-Fam) CAGCGAAAGCCCTTCAAAGAAA (Tamra)-3'

Primers and probe sequences for the detection of *M. plutonius

Primers/probe ID	Oligonucleotide Sequence
EFB-For	5'-TGTTGTTAGAGAAGAATAGGGGAA-3'
EFB-Rev2	5'-CGTGGCTTTCTGGTTAGA-3'
EFB-PROBE	5'-(6-FAM) AGAGTAACTGTTTTCCTCGTGACGGT (TAMRA)-3'

6. Equipment and materials

Warning: ***Trade names or supplier names may be mentioned in the description of the equipment and materials required to implement this method. This information is provided for users of the method and does not mean that ANSES recommends the exclusive use of these materials. Similar materials may be used if it has been demonstrated that they achieve the same results.***

The equipment and materials used for the amplification of ***P. larvae*** and ***M. plutonius*** target DNA by qPCR are described in the table above.

1. Preparation of samples for analysis	2. Genomic DNA extraction	3. Preparation of the qPCR reagent mix	4. Preparation of the PCR plate
Micropipettes	Dry heating bath	Air-flow cabinet	Real-time PCR system and a computer coupled to the machine
Vortex	Centrifuge	Micro-centrifuge	Air-flow cabinet
	DNA containment chambers	Micropipettes	Micro-centrifuge
	Micropipettes	Vortex	96-well plate Centrifuge
	Thermometer		Micropipettes
	Vortex		Vortex

7. Consumables

The consumables necessary for the amplification of ***P. larvae*** and ***M. plutonius*** target DNA by qPCR are described in the table above.

Consumables for	1. Preparation of samples for analysis	2. Genomic DNA extraction	3. Preparation of the qPCR reagent mix	4. Preparation of the PCR plate
Powder-free latex or nitrile gloves	X	X	X	X
Sterile filter tips (RNase-, DNase-free)	X	X	X	X
Sterile 1.5 ml or 2 mL microtubes (RNase-, DNase-free)	X	X	X	--
Sterile disposable pestle	X	--	--	--
Aluminium foil	--	--	X	--
Adhesive film Microseal B adhesive seals (Bio-Rad)	--	--	--	X
Hard-shell® 96-well pcr plates, thin well, white/clear (bio-rad)	--	--	--	X

8. Samples

8.1. Type of samples

Requested analyses for	Macroscopic and Microscopic examination*	Isolation and culture	PCR*
AFB	Brood* Larvae*	Brood Larvae, Honey, Wax	Brood* Larvae* Bacterial Culture
EFB		Brood Larvae	

(*) The analyses with an asterisk are accredited by the French accreditation body, Cofrac (accreditation number: 1-2229). Scope available on www.cofrac.fr.

8.2. Acceptance conditions for samples and sample storage before analysis

Type of sample	Acceptance requirements	storage
Brood	10x10 cm piece of brood containing at least 10 larvae Entire frame	≤ -16°C
Honey	> 40 g	4°C
Wax	> 5g	4°C
Larvae	2 larvae homogeneised in 500 µl of ultrapure water	≤ -16°C
Bacterial culture	ND	4°C - ≤ -16°C

8.3. Storage of samples or residual materials after analysis

The DNA samples and the ground larvae are kept in dedicated freezers at ≤ -16°C.

They are destroyed four months after sending the results except in special cases:

1. possible request for a second opinion,
2. special request from the client
3. storage for the sample library
4. storage for further research activities.

8.4. Waste disposal

The waste (microtubes, columns, collecting tubes, etc.) is thrown into the benchtop bins in the respective rooms.

The 96-well plates containing the PCR products are thrown into a bin provided for this purpose.

The samples and all the benchtop bins are disposed of in specific yellow containers destined for incineration.

9. Standard operating mode

9.1. Preparation of samples for analysis

1. From a diseased brood, take two larvae in sealed cells that show characteristic clinical signs
2. Put the larvae in a 1.5 ml microcentrifuge tube containing 500 µl of ultra pure water.
3. Crush the larvae with a disposable pestle and homogenise the sample by vortexing.

The obtained homogenates are used for bacterioscopic analyses in order to identify ***P. larvae*** spores as well as for DNA extraction.

Store the tubes containing larval suspensions in the freezer at a temperature of ≤ -16°C.

Note: for this step you can refer to the ANA-I1-MOA.1900

9.2. Genomic DNA extraction

9.2.1. Introduction

The extraction of bacterial DNA is carried out in a DNA/RNA **extraction dedicated room** using the QIAamp® DNA mini kit (Qiagen®).

Briefly, the lysis of the samples is carried out in the presence of lysozyme and proteinase K. Then nucleic acids selectively bind to the silica membrane of the column. After two successive washes to remove potential PCR inhibitors, the nucleic acids are eluted with molecular biology grade water.

9.2.2. Important points

- All the centrifugation steps are carried out at room temperature (20-25 ° C).
- Avoid repeated freezing / thawing of samples to limit DNA degradation.
- Bring the samples and distilled water to room temperature.
- Check the wash buffers AW1 and AW2 have been prepared according to the instructions on the bottles.
- If a precipitate has formed in the AL lysis buffer, dissolve it by incubating at approximately 56 ° C.
- Prepare, just before use, the necessary volume of reagents, according to the number of samples to be treated including BM grade water and ethanol, to avoid possible contamination of the entire extraction kit.

9.2.3. Genomic DNA extraction

- Start a UV decontamination cycle for the DNA containment cabinet.
- Identify each 1.5 mL microtube.
- Add the following controls:
  - **Negative extraction control** (by replacing the sample with BM grade water used for the elution of the DNA),
  - **Positive extraction control** corresponding to the pathogen of interest (see Appendix 2, §6)

- In each 1.5 mL microtube:
  - add 80 µL of sample (or control) and add 180 µL of lysozyme.
    5. Vortex then incubate at least 30 min at 37 ° C with low shaking (approximately 300 rpm).
    6. Centrifuge for a few seconds then
  - add 20 µL of proteinase K and 200 µL of AL buffer.
    7. Vortex then incubate with low shaking (approximately 300 rpm)
    8. 30 min at 56 ° C, then
    9. 15 min at 70 ° C.
    10. Centrifuge for a few seconds then
  - add 200 µL of pure ethanol.
    11. Vortex a few seconds then centrifuge for a few seconds.
- Prepare and identify QIAamp® Mini spin extraction columns.
  - Transfer the entire sample on the corresponding column.
    12. Centrifuge at 6000 g for 1 min.
    13. Discard the collection tube containing the filtrate, and
    14. Place the QIAamp® Mini spin column in a clean 2 ml collection tube,
  - Add 500 µL of buffer AW1 and centrifuge for 1 min at 6,000 g.
    15. Discard the collection tube containing the filtrate, and
    16. Place the QIAamp® Mini spin column in a clean 2 ml collection tube,
  - Add 500 µL of AW2 buffer and centrifuge for 3 min at 20,000 g.
    17. Discard the collection tube containing the filtrate, and
    18. Place the QIAamp® Mini spin column in a clean 2 ml collection tube,
    19. Centrifuge for 1 min at 20,000 g to dry the column and remove the remaining AW2 buffer.
    20. Discard the collecting tube and
    21. Place the column in a sterile 1.5 mL microtube identified,
  - Add 200 µL of BM grade water,
    22. Incubate for 1 min at room temperature and
    23. centrifuge for 1 min at 6000 g for the elution.
- Clean the DNA containment cabinet.

9.3. Preparation of the real-time PCR reagent mix

The qPCR mix is prepared in a **dedicated room** under an Air-flow cabinet.

***Warning**: A non-target internal control, TaqMan® Exogenous Internal Positive Control (IPC) Reagents (Applied Biosystems® - VIC ™ Probe), is analysed in parallel in the same PCR reaction.*

The IPC ensures that there are no PCR inhibitors in the reaction, eliminating false negative reactions.

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

- Start a UV decontamination cycle for the Air-flow cabinet
- Thaw the primers, the probe and the reagents at room temperature.
- Prepare the reagent mix according to the following table:

Component	Final concentration	Volume for one tube (µl)
SsoAdvanced Probe Supermix 2X	1X	12.5
Primer 1 (20 µM stock)	296 nM	0.37
Primer 2 (20 µM stock)	296 nM	0.37
Probe (50 µM stock)	200 nM	0.1
Exo IPC Mix (VIC) 10X	1X	2.5
Exo IPC ADN 50X*	1X	0.5
H_2O	**-**	**3.66**

(*) this component is added to the mix in step 9.4 below

- Protect the Mix tube with aluminum foil.
- Briefly vortex the mixture.

9.4. Preparation of the PCR plate and start of Run.

These steps are carried out in **a dedicated room** under an Air-flow cabinet.

- Start a UV decontamination cycle for the Air-flow cabinet
  - Add the IPC 50X DNA to the Mix according to the amount calculated above
    24. Centrifuge then briefly vortex the tubes.
  - Add 20 µL of Reaction Mix per well.
  - Add 5 µL of the samples to be tested , including:
    25. 5 µL of the **negative extraction control**,
    26. 5 µL of the **positive extraction control** (prepared as described in Annex 2),
    27. 5 µL of BM quality water as **a qPCR negative** control,
    28. 5 µL of **positive qPCR** controls (points of the standard curve),
    29. 5 µL of each **unknown sample** to be tested.-
  - Seal the plate with the transparent adhesive film.
  - Centrifuge the plate quickly (pulse for a few seconds up to a maximum of 800 g).
  - Place the plate in the Real-time PCR machine.
- Start the run with this amplification cycles:

Step	Temperature (°C)	Time (min)	Number of cycles
1	95	3:00	1
2	95	0:10	40
3	55	0:30	

10. Results analysis and interpretation

10.1 Review of the validity of results

***Warning*: For positive amplification signals, make sure that amplification curves have a characteristic appearance (sigmoidal curves, not flattened).**

The validation of the results takes into account several criteria which are reported, for each "run", on a result's sheet.

- Check the following parameters (for the PCR efficiency and the different controls):
  - The standard curve parameters.
    30. Manually set the detection threshold (Cq).

        In general, the Cq is positioned at the middle of the log-linear phase of the amplification curves (when the display is in logarithmic mode).
    31. The **efficiency** of the qPCR must be between **80 and 120%**
    32. The correlation coefficient R^2 must be greater than **0.97**.
  - Negative extraction and qPCR controls: check that no amplification has been generated.

    If amplification is detected in:
    33. the negative qPCR control: repeat the PCR on all the PCR positive samples.
    34. the negative extraction control:
        - the obtained amplification is **<** to the Positive extraction control: repeat the DNA extraction for the positive samples.
        - the obtained amplification is **≥** to the Positive extraction control:
          - proceed with a full decontamination of the working areas and the centrifuge
          - repeat the DNA extraction for all the samples.
  - Positive qPCR controls are positive and conform.
  - Positive extraction control is positive and conform.
    35. Report the Ct value of the Positive Extraction Control on a control chart to monitor assay consistency and detect any potential shifts or deviations in extraction or amplification efficiency over time.
  - IPC control:
    36. The control is validated or not on the basis of qualitative interpretation criteria or acceptable Ct value ranges.

10.2 Interpretation of the results

To be able to interpret the results obtained, it is necessary to have validated the criteria described above.

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

Amplification		Experimental Interpretation	Result reported	Diagnosis interpretation
Sample	IPC			
+	+	Positive qPCR	Positive	Pathogen Detected
-	+	Negative qPCR	Negative	Pathogen Not Detected
-	-	Inhibition Of qPCR	Uninterpretable	Uninterpretable
Strongly positive	Ct ≥ 35	Positive qpcr	Positive	Pathogen Detected

11. APPENDICES

11.1 APPENDIX 1: Preparation of the Positive Extraction Control

The validation of the results of the analysis requires the use of different controls (see §9.2.3 & 9.5). Among these, the Positive Extraction Control is made:

1. From positive and negative, for American or European foulbrood, ground larvae previously characterized using at least one of the following accredited methods:

	Bacterioscopy	Conventional PCR
P. larvae	ANA-I1.MOA.03	ANA-I1.MOA.1900
M. plutonius	ANA-I1.MOA.01	ANA-I1.MOA.2100

2. Following the instructions of the "Standard NF U47-600-2" for the preparation of reference material (methodology 1 of §7.3.1),

   Briefly, the negative ground material of larvae, previously characterised, is spiked with ground positive material containing either ***P. larvae*** / ***M. plutonius***

   2.1. Preparation of uninfected larvae Sampling of larvae

   Using sterile fine forceps 20, asymptomatic for American and European foulbrood, larvae are delicately collected from different colonies belonging to the laboratory apiary:

   - 10 uncapped (just before sealing) cells,
   - 10 sealed (between the sealing and the nymphal stage) cells.

   These will constitute a negative matrix similar in nature to the samples used for the detection of ***P. larvae*** / ***M. plutonius***

   2.1.2. Larvae homogenisation

   In the laboratory, for each colony sampled:

   - Transfer the uninfected larvae from each collection pot into a sterile ULTRA-TURRAX® DT20 tube.
   - Rinse the sampling pot with 2.5 mL of ultra-pure water.
   - Transfer the 2.5 mL into the DT20 tube.
   - Repeat the operation. In total, at the rate of 2 larvae / 500 µl (thus 5 mL of ultra-pure water are added to the 20 larvae.
   - Switch on the ULTRA-TURRAX® Tube Drive and Place the DT20 on the grinder and lock.
   - Grind 50 sec at speed 9.
   - Remove the tube from the device and tap the DT20 on the bench to remove the larvae from the walls.
   - Grind again 30 sec at speed 9.
   - Pipette the crushed material into pre-identified sterile Falcon tubes.

- Store the ground material at a temperature ≤ at -16 °

At least one of the accredited methods (see the table above) is used to analyze the batches from the different colonies separately.

The negative lots for ***P. larvae*** and ***M. plutonius*** are pooled and used for the preparation of the positive extraction controls

2.2. Production of the positive extraction controls

The obtained negative matrix is then spiked with the corresponding Positive ground larvae to obtain a concentration between 10 and 100X the LD_{method} (the values for each species are determined in their respective validation files).

The prepared controls are aliquoted by 100 µl in sterile 1.5 ml microtubes and stored at a temperature ≤ at -16 °.

2.2.1. Homogeneity test

- After a minimum of 24 hours at ≤ -16 ° C,
- 08 aliquots are taken at random and are tested by qPCR according to this SOP
- The samples are considered to be homogeneous: If all of the Cq/Ct values obtained, for these samples, are within a maximum amplitude of 1.5 Ct between the smallest and the largest Ct.
- The results are reported on a control chard (see 0).
- The batch of validated controls is recorded as Reference Material (RM).

Note: if a new batch of RM is prepared, a homogeneity test is carried out and it is checked by comparison with the old batch in use, in order to test and validate its use via the control card. The MR sheets are then updated.