Protocol instruction: Test for the presence of the β-actin gene on honeybee samples using PCR (in-house method)

The original is kept at the research unit

Contents

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1 PURPOSE AND SCOPE

This instruction describes the test for the presence of the β-actin gene, specific to the species *Apis mellifera*, in bee samples that have been found to be negative when screening for pathogens. This test is performed by PCR using DNA extracts or cDNA extracts obtained by RT-PCR. It verifies the absence of polymerase inhibitors in the samples tested.

2 CONTENT

2.1 Principle

The technique used is based on the PCR protocol described by Chen *et al*. (2005). The pair of primers used amplifies a PCR product of 181 bp, specific to the species *Apis mellifera* (GenBank accession no. AB023025).

The β-actin gene is detected from DNA extracts prepared for bacterial, fungal or parasitic pathogen detection, or from cDNA extracts prepared when screening for honeybee viruses. Only samples that were found to be negative are tested to verify the positive amplification of the β-actin gene.
The amplification technique is divided into three steps:

1. Sample preparation according to the dedicated protocols for pathogen detection (not described in this instruction)
2. Amplification of target DNA and analysis of PCR products
3. Interpretation and validation of results

2.2 DNA amplification and analysis of PCR products

DNA is amplified by PCR with specific primers. The reaction is based on the succession of temperature cycles, each cycle containing three steps: denaturation, annealing and elongation. Products obtained at the end of each cycle are then matrix for the following cycle. Thus, the amplification is exponential and allows to obtain 2^n copies of DNA (n = number of cycles). Primers and size of PCR products are specific to each sequence, so the primer annealing temperature and the time of annealing and elongation change according to the sequence to be amplified.

2.2.1 Specific points

The specific primers used for the detection of β-actin specific to *Apis mellifera* are as follows:

Primer	Sequence	PCR product size (bp)	Target
A.m-actin-L	5' - AGGAATGGAAGCTTGCGGTA - 3'	181	β-actin, *Apis mellifera*
A.m-actin-R	5' - AATTTTCATGGTGGATGGTGC - 3'		

- The sample volume used is 2.5 µl.
- An Actin PCR positive control must be added (Clone O6 plasmid positive control diluted to the concentration of the PCR limit of detection (LOD_{PCR}) multiplied by 10). Add 2.5 µl of the clone O6 in the corresponding tube.
- A PCR negative control must be added (2.5 µl of pure distilled water instead of 2.5 µl of sample).

2.2.2 Preparation of the reaction mix

Amplification is performed in a 25 µl final volume (22.5 µl of mix + 2.5 µl of DNA).

	Final concentration	For example:	Volume for 1 tube (µl)
H20	/	H20	17.55
Taq polymerase buffer	1X	Taq polymerase buffer (10X)	2.5
MgCl2	1.5 mM	MgCl2 (50 mM)	0.75
dNTP	200 µM	dNTP (10 mM)	0.5
Primer 1	400 nM	Primer 1 (20 µM)	0.5
Primer 2	400 nM	Primer 2 (20 µM)	0.5
Taq Pol	1 unit / 25 µl	Taq Pol (5U/ µl)	0.2

			22.5 µl

Homogenise the mix with a pipette or a vortex and centrifuge quickly in a table-top centrifuge.
Distribute 22.5 µl of mix per tube.
Add 2.5 µl of sample DNA or cDNA, or 2.5 µl of positive control, or 2.5 µl of water in the corresponding PCR tubes.

2.2.3 PCR programme

The specific characteristics of this PCR program have been underlined:

- DNA denaturation — 94°C for 2 minutes
- 30 cycles:
  - Denaturation at 94°C for 30 seconds
  - Primer annealing at 60°C for 30 seconds
  - Elongation at 72°C for 15 seconds
- Final elongation — 72°C for 5 minutes
- Final hold — 10°C

When the run is finished, place the tubes at 5°C +/- 3°C, before the analysis on an agarose gel.

2.3 Analysis of PCR products by 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 comparing 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 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.

2.3.1 Preparation of the agarose gel

- It is recommended to prepare the agarose gel with TAE 1X.
- A gel with 1.2 % (w/v) of agarose can be prepared to observe PCR products. For example weight 1.2 g of agarose for 100 ml of TAE 1X.According to the size of the PCR products agarose percentage can be higher or lower (high concentrations of agarose will be used to observe small PCR products).
- Heat the TAE-agarose in a microwave until obtaining a transparente liquid.
- Cool a few minutes before pouring the solution into a gel rack, containing the comb.
- Wait until the gel has soldified.
- Remove the comb and place the gel into the electrophoresis tank.
- Pour TAE 1X to cover the gel.

2.3.2 Preparation and loading of samples

- It is recommended to migrate 10 µl of 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.

2.3.3 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 0.5µg/ml EtBr solution, for at least 20 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.

2.4 Interpretation and validation of results

When using this PCR to test for the presence of the β-actin gene, interpretation of the results is based on the presence of the amplified product of 181 bp.
A PCR result is only considered valid if:

- The positive extraction and positive PCR controls are both positive,
- The negative extraction and negative PCR controls are both negative.

If the amplification of the β-Actin gene is confirmed, the negative result for the sample tested to screen for the pathogens (carried out previously and tested again during the PCR for β-Actin amplification) is confirmed. Otherwise, the extraction and/or reverse transcription must be repeated for the sample in question.

3 REFERENCES

Chen Y.P., Higgins J.A. and M. F. Feldlaufer (2005) - Quantitative real-time reverse transcription-PCR analysis of deformed wing virus infection in the honeybee (Apis mellifera L.) - Appl. Environ. Microbiol., **71**(1), pp.436-41