Analytical method for animal health

REFERENCE: ANSES/SOP/ANA-I1.MOA.3900 - Version 03

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Standard operating procedure: Detection and quantification of the Acute Bee Paralysis Virus (ABPV), Black Queen Cell Virus (BQCV), Deformed Wing Virus (DWV-A & DWV-B), and Sacbrood Virus (SBV) by real-time RT-PCR

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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 the changes	Date	Main changes
V00	Creation	February 27th, 2017	Initial version: protocol established according to the ANA-I1.MOA.39 (version 00)
V01	Major	March 8th , 2018	Description of the real-time PCR for the detection and the quantification of the DWV-B variant.
V02	Major	May 29th, 2018	- Editorial changes (according to ANSES' model) - Use of an alternative real-time PCR thermocycler - Improvement of the real-time PCR for the detection and the quantification of the DWV-B variant
V03	Major	September 24th, 2019	- Sampling extended to 50 honey bees. - Viral loads interpreted taking into account the thresholds between covert and overt infections

Foreword

This method has been optimised by: Unit of Honey Bee Pathology

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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 protocol describes the detection and quantification of the Acute bee paralysis virus (ABPV), Black queen cell virus (BQCV), Deformed wing virus (DWV-A and DWV-B), and Sacbrood virus (SBV) by real-time RT-PCR (RT-qPCR) in honey bee samples.

The ABPV RT-qPCR method is based on the quantification of the capsid protein gene sequence (Jamnikar-Ciglenečki and Toplak, 2012).

The BQCV RT-qPCR method is based on the quantification of the C-terminal part of the polyprotein gene sequence (Chantawannakul *et al*., 2006).

The DWV-A and DWV-B RT-qPCR methods are based on the quantification of the VP3 coding sequence (Schurr *et al*., 2019).

The SBV RT-qPCR method is based on the quantification of the N-terminal part of the polyprotein gene sequence (Blanchard *et al*., 2014).

Note 1: The methods were validated using adult bees. The methods can also be used for quantification of viruses in larvae. However, the methods have not been validated using larvae. Thus, the accuracy of the methods is unknown in larvae.

Note 2: This document describes a new RT-qPCR method targeting the DWV-B variant (also called Varroa destructor virus-1, Martin et al., 2012) or even recombinant viruses between DWV-A and DWV-B (Dalmon et al., 2017). Although the method does not meet the accuracy criteria defined by the EURL for Honey Bee Health, it is implemented to identify the viral aetiology of new bee cases of deformed wings, not associated to DWV-A. This choice is also guided by the increasing frequency of disorders associated with these new variants.

2 Principle of the method

Total RNA is purified from honeybees and used to produce cDNA by reverse transcription. The subsequent real-time PCR technique uses a TaqMan® probe (labelled with a reporter fluorophore at the 5' end and a quencher fluorophore at the 3' end) and a DNA polymerase with an exonuclease activity. During amplification, the 5'-3' exonuclease activity of the polymerase cleaves the probe hybridised to the target sequence. This releases the reporter from the quencher's influence, thereby increasing the fluorescence emitted by the reporter. The amplification cycle number (Ct = threshold cycle) corresponding to a significant increase of emitted fluorescence by the reporter is therefore inversely proportional to the number of copies of the amplified target sequence. The Ct value is compared to a calibration curve obtained from 10-fold serial dilution of standard samples (recombinant plasmid with the target gene sequence) in order to convert the result in virus genome copy number. Then, the quantified viral loads are compared to previously suggested thresholds differentiating covert and overt infections (Schurr et al., 2019). Moreover, this document describes the use of an internal positive control (with its own probe and primers) which is co-amplified during the real-time PCR (duplex amplification reaction). This control checks the absence of PCR inhibitors in the reaction, thus eliminating any false-negative results.

Detection and quantification of viruses by RT-qPCR using bee samples can be divided into six steps (see Supplementary Figure S1):

1. Bee sample
2. Bee homogenisation
3. Total RNA purification from bee homogenate
4. Reverse-transcription of RNAs
5. Real-time PCR reaction
6. Results interpretation

3 Equipment, consumables and reagents

Warning[3]: Trade names or supplier names may be mentioned in the description of the equipment and 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.

Note 3: The final user must verify the conformity of his protocol with the expected criteria defined by the EURL (see Supplementary tables). The EURL can help the laboratory to verify its method conformity (contact: eurl.bee@anses.fr).

Equipment	- Micropipettes (0.5-10 µl, 2-20 µl, 10-100 µl, 100-1000 µl) - Vortex - Ice tray - Manual tissue homogeniser (15 ml Potter), or mechanical tissue lyser (MM301/MM400 lyser Qiagen, or IKA Ultra Turrax Tube Drive), or laboratory mixers (Waring Blendor – two speed) with cutter head (250 ml stainless steel mini-beaker and lid). - Spin micro-centrifuge - 96-well plates centrifuge - micro-tube incubator - Real-time PCR system and a computer coupled to the machine (ABI Prism 7500 Real-Time PCR or Quant Studio 5, Applied Biosystems)
Consumables	- Sterile 2, 1.5, and 50 ml tubes (RNase-, DNase-free) - Sterile IKA Tube DT-20M - Optical 96-well reaction plate (and optical plate sealer film) (Applied Biosystems) - 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 7.0 - TE: Tris 10 mM - EDTA 1 mM, pH 8 - High Pure Viral RNA Kit (Roche Diagnostics)

	- Random Hexamer (pdN6) (Invitrogen) (working solution: 0.3 µg/µl in TE) - Super Script II Reverse Transcriptase Kit (Invitrogen) - RNaseOUT - Recombinant Ribonuclease Inhibitor (Invitrogen) - dNTP 10 mM mix – PCR Grade (Invitrogen) - Real-time PCR Master MIX (Applied Biosystems) - Specific Forward and Reverse primers (stock solution: 100 µM; working solution: 20 µM in H_2O) (Table 1) - Specific Probes (stock and working solution: 50 µM in H_2O) (Table 1). It is recommended to protect the fluorescent probe from the light with a piece of aluminium foil. - Internal Positive Control MIX (if such a control is used) (Applied Biosystems) - Internal Positive Control DNA (if such a control is used) (Applied Biosystems) - Standard samples (Table 2) - External positive control[4]

Note 4: The external positive controls should be produced by the laboratories that would like to implement the RT-qPCR methods described in this document. However, the EURL (contact: eurl.bee@anses.fr) can provide recommendations for the development of such positive controls.

Table 1: Primers and probes

RT-qPCR	Primers and probe	Sequence
ABPV	ABPV1	CAT ATT GGC GAG CCA CTA TG
	ABPVRn	CTA CCA GGT TCA AAG AAA ATT TC
	ABPVnTaq	(6-Fam) ATA GTT AAA ACA GCT TTT CAC ACT GG (Tamra)*
BQCV	BQCV8195F	GGT GCG GGA GAT GAT ATG GA
	BQCV8265R	GCC GTC TGA GAT GCA TGA ATA C
	BQCV8217T	(6-Fam) TTT CCA TCT TTA TCG GTA CGC CGC C (Tamra)*
DWV-A	F-DWV_4250	GCG GCT AAG ATT GTA AAT TG
	R-DWV_4321	GTG ACT AGC ATA ACC ATG ATT A
	Pr-DWV_4293	(6-Fam) CCT TGA CCA GTA GAC ACA GCA TC (Tamra)*
DWV-B	F-VDV1_4218	GGT CTG AAG CGA AAA TAG
	R-VDV1_4290	CTA GCA TAT CCA TGA TTA TAA AC
	Pr-VDV1_4266	(6-Fam) CCT TGT CCA GTA GAT ACA GCA TCA CA (Tamra)*
SBV	SBV-F434	AAC GTC CAC TAC ACC GAA ATG TC
	SBV-R503	ACA CTG CGC GTC TAA CAT TCC
	SBV-P460	(6-Fam) TGA TGA GAG TGG ACG AAG A (MGB)

*: The Tamra labelling at the 3'-end of the probes can be substituted by a non-fluorescent quencher.

Table 2: Standard samples[5]

RT-qPCR	Standard samples	Recommendation
ABPV	**pB2**	Prepared serial dilutions of the clone in TE from 2.0 log_{10} copies/5µl (G2) to 8.0 log_{10} copies/5µl (G8)
BQCV	**pNC1-4**	
DWV-A	**pC1**	
DWV-B	**pFab1**	
SBV	**pD1**	

Note 5: The EURL can provide standard samples (G9 = 9.0 log_{10} copies/5µl) to the laboratories performing the quantitative PCR methods described in this protocol (contact: eurl.bee@anses.fr).

4 Sampling and sample processing

4.1 Bee samples

At least 10 symptomatic or dead bees or larvae must be harvested (minimal prevalence 26% with 95% of confidence), or 50 bees or larvae without clinical signs (minimal prevalence 6% with 95% of confidence). The observed symptoms are reported on a commemorative document. This clinical information is transmitted to the laboratory with the sample. The clinical signs that can be associated with viral diseases are listed in Table 3.

Table 3: Clinical signs of ABVV, BQCV, DWV-A, DWV-B, and SBV infections in honeybees (de Miranda et *al.*, 2013)

ABPV	BQCV	DWV-A and DWV-B	SBV
- Unapparent infection - Dead brood and adult bees from *Varroa destructor* infected colonies	- Cell walls black in patches - Pale yellow larvae - Dead queen larvae and pre-pupae	- Deformed or poorly developed wings *pupae or honeybees* - Brood and adult mortalities from *Varroa destructor* infected colonies	- Sac-like larvae - Pale yellow larvae becoming dark brown from the anterior end - Brood mortalities

4.2 Adult bee or larvae bee homogenisation

- 10 or 50 bees (adult or larvae) are crushed in 10 or 50 ml of ice cooled phosphate buffer 0.01 M pH 7.0 (1 ml per individual), respectively
- Crush manually: at least 20 grinding actions; or homogenize mechanically: 3 min at 30 Hz with MM301 or MM400 lyser, or 3 min power 9 with the Tube Drive lyser; or homogenize for 20 seconds at the lower speed (18,000 rpm) with the Waring Blendor
- Maintained on ice

- Centrifuge 10 min at 8,000 x *g*
- Collect the supernatant
- Centrifuge 10 min at 8,000 x *g*
- Collect the supernatant (final homogenate)

4.3 RNA purification[6]

The extraction of total RNAs is performed using 200 µl of bee homogenate and denaturing solution (guanidine), and the RNAs are purified using spin colon according to the manufacturer's instruction. Used 200 µl of phosphate buffer 0.01 M pH 7.0 (or negative control provide with RNA purification kit) as negative control all along the process. Final RNA recovery is performed with 50 µl elution buffer.

The RNAs are kept in ice until to be quickly analysed.

Note 6: In order to reduce the risk of contamination between samples, the EURL recommends briefly centrifuging the tubes before opening them. In addition, we recommend handling the tubes carefully to prevent aerosol formation and contact with the inside of the tube-caps.

4.4 Reverse transcription (RT)

The cDNA synthesis is performed in two steps:

i. The random hexamer hybridization (Table 4).

Table 4: Hexamer hybridization reaction

Reagent	Volume per reaction (Final: 13.5 µl)
pdN6 (0.3 µg/µl)	1.0 µl
RNA sample	12.5 µl

Incubate 2 min at 70°C and 5 min at 4°C.

ii. The reverse transcription reaction (Table 5).

Table 5: Reverse transcription reaction

Reagent	Final concentration	Volume per reaction (Final: 20 µl)
First Strand Buffer (5 X)	1 X	4.0
dNTP (10 mM)	500 µM	1.0
RNase OUT (40 U/µl)	20 U	0.5
Super Script II RT (200 U/µl)	200 U	1.0
RNA sample/pdN6 (hybridized)		13.5

Incubate 60 min at 42°C and 5 min at 95°C.

4.5 Preparation of the real-time PCR reaction mix[7]

- Prepare the real-time PCR mix (Table 6). Briefly vortex the mixture and protect it with an aluminium foil before use.

Table 6: Real-time PCR reactions

Reagent	ABPV		BQCV or SBV		DWV-A		DWV-B	
	Final	Vol. per reaction (25µl)	Final	Vol. per reaction (25 µl)	Final	Vol. per reaction (25 µl)	Final	Vol. per reaction (25 µl)
H_20	-	2.45	-	3.6	-	3.57	-	4.3
qPCR MIX (2 X)	1X	12.5	1X	12.5	1X	12.5	1X	12.5
Forward Primer (20 µM)	800 nM	1.0	320 nM	0.4	350 nM	0.44	1200 nM	1.5
Reverse Primer (20 µM)	800 nM	1.0	320 nM	0.4	350 nM	0.44	1200 nM	1.5
Probe (50 µM)	100 nM	0.05	200 nM	0.1	100 nM	0.05	400 nM	0.2
Int. Pos. Control Mix (10X)	1X	2.5	1X	2.5	1X	2.5	-	0
Int. Pos. Control DNA (50X)	1X	0.5	1X	0.5	1X	0.5	-	0
cDNA		5.0		5.0		5.0		5.0

Note 7: This protocol uses internal, positive, exogenous, co-amplified control in qPCR reactions targeting ABPV, BQCV, DWV-A and SBV. The low efficiency of the qPCR targeting the DWV-B does not allow to co-amplify this internal control; therefore, it is not added in the reaction mixture for DWV-B qPCR.

4.6 Preparation of plate containing the calibration standards and cDNA samples

- Thaw the calibration curve points (G2 to G8 standard samples – Table 2). Gently vortex the tubes, centrifuge shortly and keep them on ice.
- Place the tubes containing the cDNA samples (from the RT reaction) on ice.
- Add 20 µl of the qPCR reaction mix to each well.
- Add 5 µl of each standard sample (G2 to G8) to the specified wells.
- Add 5 µl of the negative control cDNA in the corresponding wells.
- Add 5 µl of the external positive control cDNA in the corresponding wells.
- Add 5 µl of each sample to be analysed in the corresponding wells.
- Centrifuge the samples rapidly (pulse for a few seconds up to a maximum of 800 × *g*).
- Load the samples in the machine and launch the run.

Incubation conditions:

Uracyl N-glycosylase cleavage:		50°C for 2 min
Polymerase activation:		95°C for 10 min
PCR (40 cycles)	Denaturation:	95°C for 15 sec
	Annealing/Extension:	60°C for 1 min

4.7 Data analysis

4.7.1 Validation of results

- Standard curve:

Report the settings for the analysis and standard curve to a results sheet:

- the threshold value determined by the software (Threshold);
- the slope (Slope) of the standard curve, which must be between -2.92 and -3.92;
- the Y intercept (Y-Inter), which is the expected C_t value for 1 copy;
- the R^2 (correlation coefficient) which must be greater than 0.97.

- External positive control:

This control allows the monitoring of the efficiency of RNA extraction, RT and qPCR:

- report the number of viral genome copies (expressed in $\log_{10}$) found with the positive control in a control chart (ex. Shewhart control charts[8]);
- calculate the measurement uncertainty (*U*) according for example the following equation: $U = 2 \times SD_W$ (SD_W being the within-laboratory standard deviation);
- check if viral load of the external positive control vary in comparison to previous experiments.

If a high variation is observed, an analysis of possible causes and impact on final result should be undertaken.

Note 8: The EURL can provide Shewhart control chart (contact: eurl.bee@anses.fr).

- Negative control:

Check that no viral sequence is detected in the negative process control (Phosphate Buffer or kit negative control).

If viral sequences are detected with a quantity below LOD of the method, verify the samples found positive below the LOD.

If viral sequence quantity found in negative control is over LOD of the method, the complete analysis must be redone after decontamination of work areas and the centrifuge.

- Internal positive control[9]:

This control allows checking that there are no PCR inhibitors in the reaction. Check that the Ct results obtained with this control are comparable in all wells, irrespective of the sample. However, if a sample shows high levels of viral RNA, there will be competition between the two targets to the advantage of the majority target (hence the Ct value will be high or indeterminate). For all the other samples the Ct of the control must not vary.

If the Ct of the control is indeterminate or >35 for an unknown sample (there is a risk of presence of PCR inhibitors), repeat the real-time PCR using a diluted cDNA sample (1/10 or even 1/100)[10]. The C_t value of this diluted sample must be equal to the Ct in the other wells. If this is not the case, repeat the RNA extraction for this sample.

Note 9: For the DWV-B qPCR that does not contain internal positive control, the inhibition interpretation is based on the DWV-A qPCR, both being performed systematically together.

*Note 10: Take into accounts this dilution factor (1/10 or 1/100) for the transformation of raw positive results in viral genome copies per bee (see § 4.7.2). A negative result even from diluted cDNA sample should be interpreted as "**Inhibited sample**".*

4.7.2 Raw data conversion in viral genome copies per bee[11]

Equivalent viral load in the well is determined using the standard curve. The final result corresponding to the viral load present in one honeybee is obtained by adding to the raw results the conversion factor (taking into account the volume proportion used at each step: bee homogenisation, RNA extraction, RT and PCR). For honeybee (adult or larvae), the factor is +1.9 (considering a 100% recovery rate at each step).

Note 11: The EURL recommends converting the results of quantitative PCR into log_{10} (ie. 150,000 copies/bee = 5.2 log_{10} copies/bee). Values are indicated with one digit number.

Example of conversion factor estimation:

- Considering an initial load of **4.0** log_{10} copies per bee;
- 8 bees in 8 ml of homogenate (4.0 log_{10} copies/ml);
- equivalent to 3.3 log_{10} copies in 200 µl of homogenate sample used for RNA purification;
- 3.3 log_{10} copies recovered in 50 µl of total RNA (final elution);
- equivalent to 2.7 log_{10} copies in 12.5 µl of RNA sample used for cDNA synthesis;
- 2.7 log_{10} copies recovered in 20 µl of reverse transcription reaction (final volume);
- equivalent to **2.1** log_{10} copies in 5 µl of cDNA sample use in real-time PCR.

Conversion factor = 4.0 – 2.1 = +1.9 log_{10}

log_{10} copies per PCR reaction +1.9 = log_{10} copies per adult honeybee

4.7.3 Correction of systematic error [12]

ABPV: the systematic bias of virus quantification was estimated to be +0.4 log_{10} genome copies per bee. Consequently, the viral RNA load observed should be corrected as follow:

log_{10} copies/bee (observed) - 0.4 = log_{10} genome copies/bee (final result)

e.g.: 6.5 log_{10} ABPV genome copies/bee - 0.4 = 6.1 log_{10} ABPV genome copies/bee

BQCV: the systematic bias of virus quantification was estimated to be +0.2 log_{10} genome copies per bee. Consequently, the viral RNA load observed should be corrected as follow:

log_{10} copies/bee (observed) -0.2 = log_{10} genome copies/bee (final result)

e.g.: 6.5 log_{10} BQCV genome copies/bee - 0.2 = 6.3 log_{10} BQCV genome copies/bee

DWV-A: no systematic bias was found. Consequently, the viral RNA load observed should not be corrected.

DWV-B: the systematic bias of virus quantification was estimated to be -0.95 log_{10} genome copies per bee. Consequently, the viral RNA load observed should be corrected as follows:

log_{10} copies/bee (observed) + 0.95 = log_{10} genome copies/bee (final result)

e.g.: 6.5 log_{10} DWV-B genome copies/bee + 0.95 = 7.45 log_{10} DWV-B genome copies/bee

SBV: the systematic bias of virus quantification was estimated to be -0.4 log_{10} genome copies per bee. Consequently, the viral RNA load observed should be corrected as follow:

log_{10} copies/bee (observed) + 0.4 = log_{10} genome copies/bee (final result)

e.g.: 6.5 log_{10} SBV genome copies/bee + 0.4 = 6.9 log_{10} SBV genome copies/bee

Note 12: The bias value is intrinsic to each RT-qPCR method. This systematic error is not controllable. Therefore, this bias must be corrected by all laboratories implementing the method. However if any step of the method described in this protocol (from RNA extraction step to qPCR amplification step) is change, the bias should be re-estimated (contact the EURL for support in the bias estimation: eurl.bee@anses.fr)

4.7.4 Expression of results

The results of quantitative PCR are expressed according to the Table 7.

Table 7: Expression of quantitative PCR results

Result (in log_{10} copies per bee)	Result reported	Result expression
Undetermined	<LOD	Viral RNA not detected
≤ LOQ	<LOQ	Viral RNA detected, not quantified
LOQ > x ≥ 10	*Number of viral genome copies per bee* (in log_{10})	Viral RNA detected and quantified
> 10	> 10 log_{10} viral genome copies per bee	Viral RNA detected and quantified

4.7.5 Interpretation criteria [13]

The final interpretation of the viral load must take into account on the one hand, the contextual data, particularly in the case of associated clinical signs (Table 3), and on the other hand, both the threshold separating covert and overt infections (Schurr et al., 2019) and the measurement uncertainty of RT-qPCR methods (*U*; Table 8).

Table 8: Interpretation of viral loads quantified by RT-qPCR

Virus	Threshold* (in log_{10} copies/bee)	Viral load < Threshold - *U*	Threshold - *U* ≤ Viral load ≤ Threshold + *U*	Viral load > Threshold + *U*
ABPV	5	Viral load suggesting a covert infection	Viral load close to the threshold separating covert and overt infections	Viral load suggesting an overt infection
BQCV	8			
DWV-A	6			
DWV-B	7			
SBV	9			

*: The thresholds between overt and covert viral infections have been suggested by Schurr et al. (2019).

Note 13: For the DWV-B RT-qPCR method (which does not meet the validation criteria defined by the EURL), the measurement uncertainty could be higher than 1.0 log_{10} copies/bee.

4.8 Storage and disposal of samples after analysis

Samples storage:

- Honeybee homogenates and cDNAs can be stored in an ultra-low freezer at ≤ -65°C without a predefined time limit;
- Purified RNAs can be stored for one month in an ultra-low freezer at ≤ -65°C.

5 REFERENCES

- AFNOR. 2015. Animal health analysis methods – PCR – Part 2: Requirements and recommendations for the development and the validation of veterinary PCR. NF U47-600-2 (February 7th, 2015), AFNOR ed. France (www.afnor.org).
- Blanchard P, Guillot S, Antùnez K, Köglberger H, Kryger P, de Miranda JR, Franco S, Chauzat MP, Thiéry R, Ribière M. 2014. Development and validation of a real-time two-step RT-qPCR TaqMan(®) assay for quantitation of Sacbrood virus (SBV) and its application to a field survey of symptomatic honey bee colonies. J. Virol. Methods. 197:7-13.
- Chantawannakul P, Ward L, Boonham N, Brown M. 2006. A scientific note on the detection of honeybee viruses using real-time PCR (TaqMan) in Varroa mites collected from a Thai honeybee (*Apis mellifera*) apiary. J. Invertebr Pathol. 91(1):69-73.
- Dalmon A, Desbiez C, Coulon M, Thomasson M, Le Conte Y, Alaux C, Vallon J, Moury B. 2017. Evidence for positive selection and recombination hotspots in Deformed wing virus (DWV). Sci. Rep. 7:41045.
- de Miranda JR, Bailey L, Ball BV, Blanchard P, Budge GE, Chejanovsky N, Chen YP, Gauthier L, Genersch E, de Graaf DC, Ribière M, Ryabov E, De Smet L, and van der Steen JJM. 2013. Standard methods for virus research in *Apis mellifera*. Journal of Apicultural Research 52(4).
- Jamnikar-Ciglenečki U, Toplak I. 2012. Development of a real-time RT-PCR assay with TaqMan probe for specific detection of acute bee paralysis virus. J. Virol. Methods. 184(1-2):63-8.
- Martin SJ, Highfield AC Brettell L,Villalobos EM, Budge GE, Powell M, Nikaido S, Schroeder DC. 2012. Global honey bee viral landscape altered by a parasitic mite. Science 336, 1304-6.
- Schurr F., Tison A., Militano L., Cheviron N., Sircoulomb F., Rivière M.P., Ribière-Chabert M., Thiéry R., Dubois E. 2019. Validation of quantitative real-time RT-PCR assays for the detection of six honeybee viruses. J. Virol. Methods. 270:70-78.

6 Supplementary figure

The Figure S1 shows the successive steps of the complete methods for the detection and quantitation of viruses in bee sample.

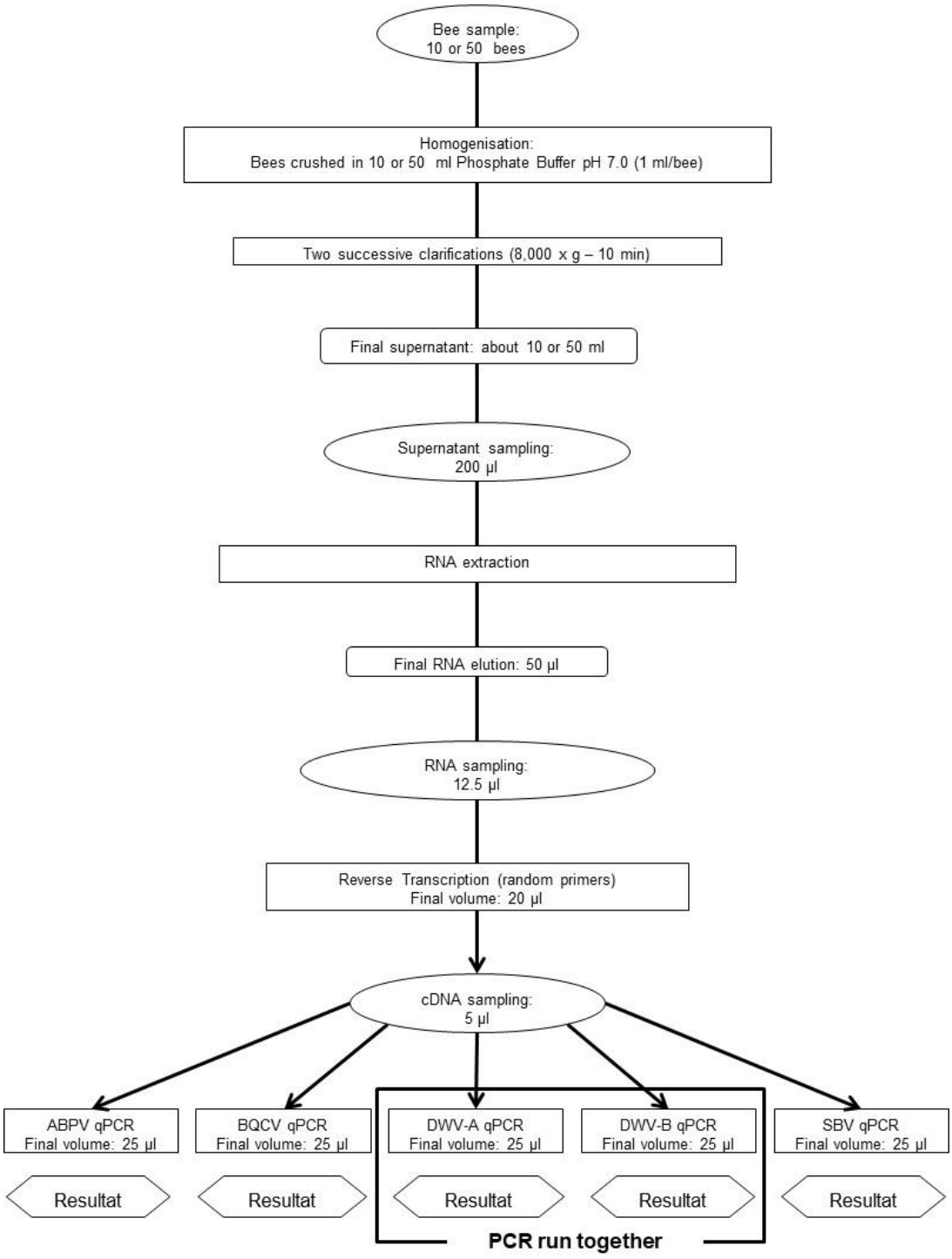

Figure S1: Synoptic of the quantitative PCR methods

7 Supplementary tables

The following tables (Table S1 and Table S2) describe the validation and the performance criteria of the RT-qPCR methods found by the EURL for Honey Bee Health according to the norm NF U47-600 (www.afnor.fr).

Table S1: Validation criteria according to NF U47-600 (AFNOR, 2015)

Criteria	Expected result
Analytical specificity	100%
Limit of detection (LOD) qPCR (minimum: 23 pos. samples/24 tested samples)	≤ 4.0 $\log_{10}$ copies DNA target/5µl
Limit of quantitation (LOQ) qPCR (*Bias* ≤ 0.25 $\log_{10}$)	≤ 4.0 $\log_{10}$ copies DNA target/5µl
Linearity range of standard curve (*Bias* ≤ 0.25 $\log_{10}$)	4.0 to 8.0 $\log_{10}$ copies DNA target/5µl
qPCR Efficiency	80 to 120%
LOD Method (minimum: 8 pos. samples/8 tested samples)	≤ 6.0 $\log_{10}$ genome copies/bee
LOQ Method (uncertainty [*U*] ≤ 1.0 $\log_{10}$)	≤ 8.0 $\log_{10}$ genome copies/bee
Accuracy range (*U* ≤ 1.0 $\log_{10}$)	8.0 to 10.0 $\log_{10}$ genome copies/bee
Method bias (absolute value)	≤ 1.0 $\log_{10}$ genome copies/bee
Diagnosis sensitivity	≥90%
Diagnosis specificity	≥90%

Table S2: Honeybee virus RT-qPCR method performances

Criteria	ABPV	BQCV	DWV-A	DWV-B	SBV
Analytical specificity	a	b	c	d	e
LOD qPCR in $\log_{10}$ genome copies/5µl	2.0	1.7	1.1	2.0	1.7
LOQ qPCR in $\log_{10}$ genome copies/5µl	2.0	2.0	2.0	2.0	2.0
Linearity range in $\log_{10}$ genome copies/5µl	2.0 to 8.0	2.0 to 8.0	2.0 to 8.0	2.0 to 8.0	2.0 to 8.0
qPCR Efficiency	87%	105%	104%	84%	92%
LOD Method in $\log_{10}$ genome copies/bee	4.4	3.0	2.4	5.0	3.4
LOQ Method in $\log_{10}$ genome copies/bee[f]	6.4	3.0	2.4	5.0[g]	3.4
Accuracy range in $\log_{10}$ genome copies/bee[f]	6.4 to 10.4	3.0 to 10.0	2.4 to 10.4	5.0 to 10.0[g]	3.4 to 10.4
Method bias in $\log_{10}$ genome copies/bee	+0.4	+0.2	0	-0.95	-0.4
Diagnosis sensitivity (pos. samples/tested samples)	100% (20/20)	100% (43/43)	100% (37/37)	100% (2/2)	100% (48/48)
Diagnosis specificity (number of bee samples found negative)[h]	12	6	31	11	14

a: Analytical specificity described by Jamnikar-Ciglenečki and Toplak, 2012. Robustness: a nonspecific hybridization of the probe with the CdK4 gene of the honeybee was observed at probe concentration over 100 nM.
b: Analytical specificity described by Chantawannakul *et al*., 2006.
c: The primers and the probe were designed for the detection and the quantification of the DWV-A.
d: The primers and the probe were designed for the detection and the quantification of the DWV-B and several DWV-A and DWV-B recombinants.
e: Analytical specificity described by Blanchard *et al*., 2014.
f: Accuracy range taking into accounts the method bias (systematic error).
g: The average quantification uncertainty of the DWV-B method, in the range of 5.0 to 10.0 $\log_{10}$ copies/bee, is 0.87 $\log_{10}$ copies/bee.
h: The specificity value represents the number of bee samples found negative during the validation process.