Embrun, 9 & 10 October 2017

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WP3 Activity 3 Health Parameters

Harmonization of methods for measuring viral load and bio-markers of aging

<u>Stage1</u> : Quality check of extracted RNA in Torino

A qPCR on 18S gene showed that the extracted samples also contain genomic DNA.
to remind for primer design on honey bee genes!

<u>Stage 2</u> : Preliminary assays of cDNA synthesis and qPCR with primers targeting the five honey bee viruses

Substitution of one pair of primers (ABPV complex) that showed aspecificity.

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Harmonization of methods for measuring viral load and bio-markers of aging

Stage 3 : Cross-Tests

Bees Origin : INRA France UR406 (17/08/2017).
1 Sample = 40 bees

8 samples (FR-1 to FR-6)

8 samples (FR-10 to FR-17)

For each laboratory

✱ ***RNA Extraction***

4 samples traited with Qiacube Robot and 4 samples traited without Qiacube Robot

➔ **RNA Concentrations and Ratios are good and similar with the two methods in the two laboratories.**

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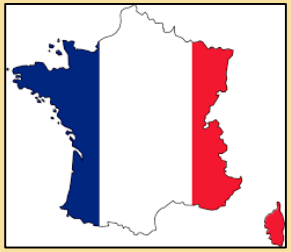
Harmonization of methods for measuring viral load and bio-markers of aging

Stage 3 : Cross-Tests

❋ ***RT-PCR*** : waiting for orders, to follow…

❋ ***qPCR*** : waiting to orders, to follow...



Visit *INRA* to *DISAFA* : 21 and 22 august 2017.

Visit *DIFASA* to *INRA* : planned in october or november 2017.

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Harmonization of methods for measuring viral load and bio-markers of aging

Stage 4 :Production of Positive Controls

Infected samples from Avignon were used as template to clone the virus sequences and to prepare the standard DNA

Selection of 7 positive samples for DWV, BQCV, ABPV, CBPV and SBV
Production of cDNA (RT-PCR)

↓

Production of probes for qPCR by cloning
Ok on the 2 october 2017

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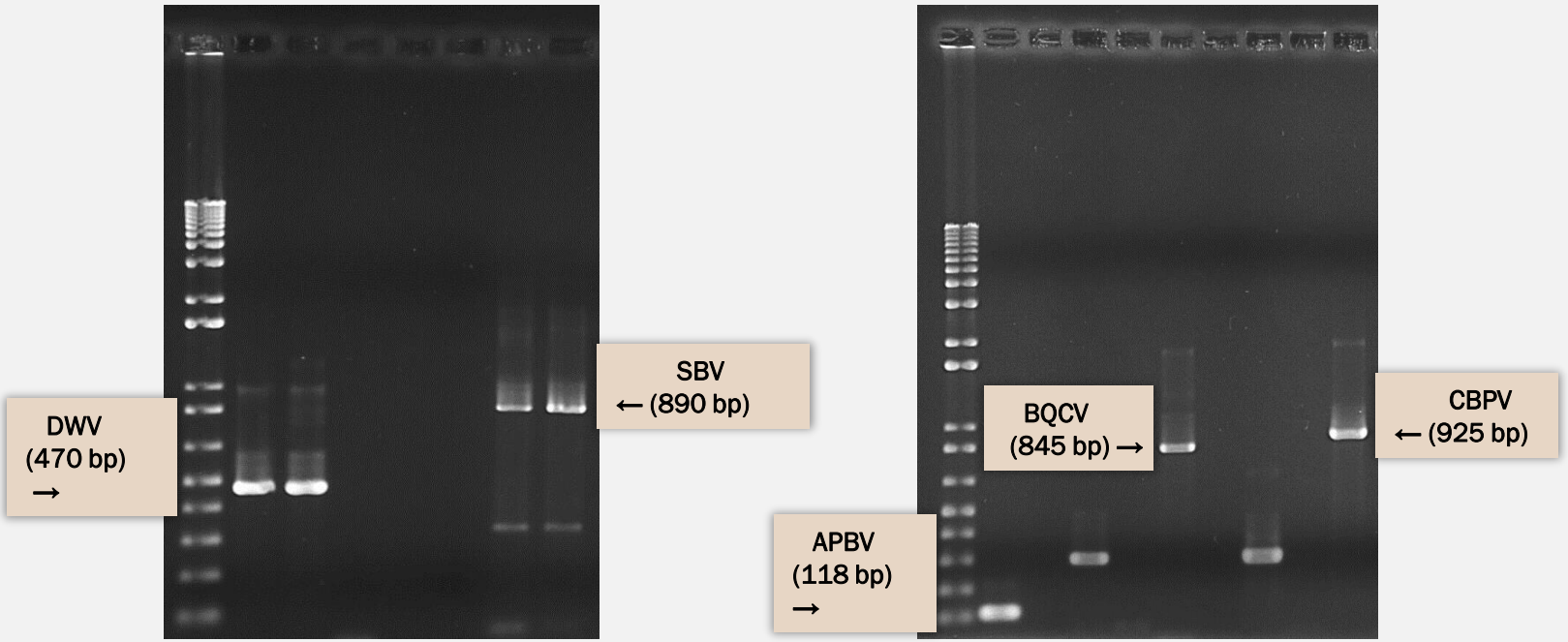
PREPARATION OF STANDARD DNA FOR THE QUANTIFICATION OF VIRUS LOAD IN REALTIME qPCR

1. **Primer design on sequences of virus genomes external to the target regions**
2. **Amplification of cDNA, verification on gel electrophoresis and purification of the PCR products**



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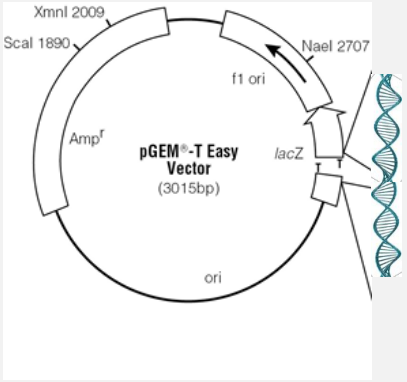
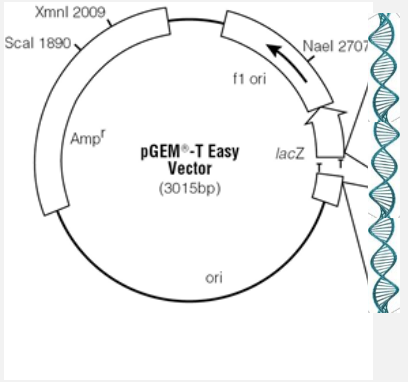
PREPARATION OF STANDARD DNA FOR THE QUANTIFICATION OF VIRUS LOAD IN REALTIME qPCR

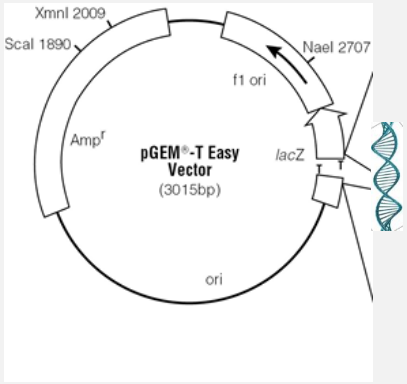
3. Ligation of the inserts (=purified PCR products) into pGEM-T Easy vector by T4 ligase



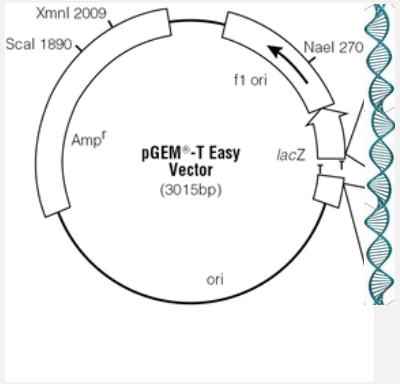
DWV (470 bp)



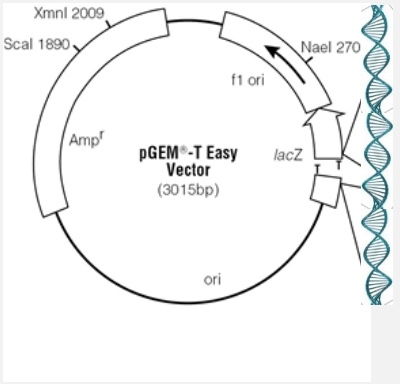
SBV (890 bp)



ABPV (118 bp)



CBPV (925 bp)



BQCV (845 bp)

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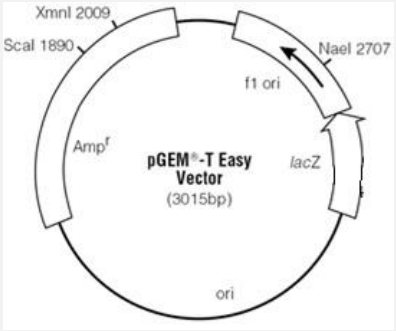
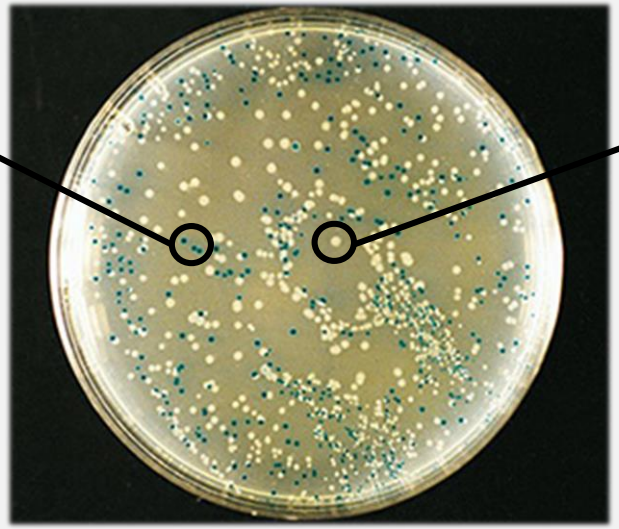
PREPARATION OF STANDARD DNA FOR THE QUANTIFICATION OF VIRUS LOAD IN REALTIME qPCR

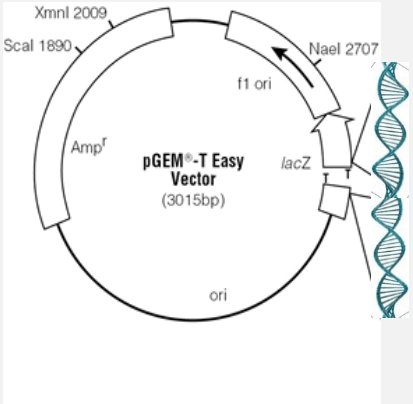
4. **Transformation of *Escherichia coli* DH5α competent cells by heat shock**
5. **Blue/White screening after overnight growth (37 °C) on LB + amp + IPTG + X-Gal**

BLUE = no insert





WHITE = insert interrupting *LacZ* operon



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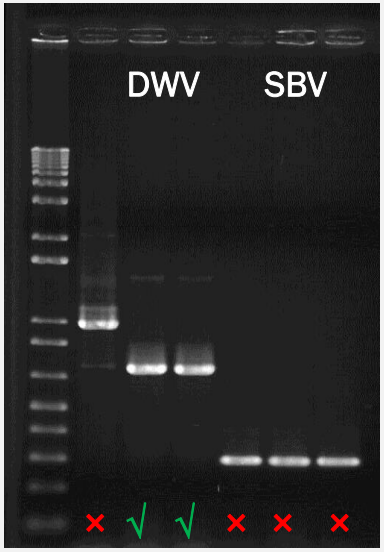
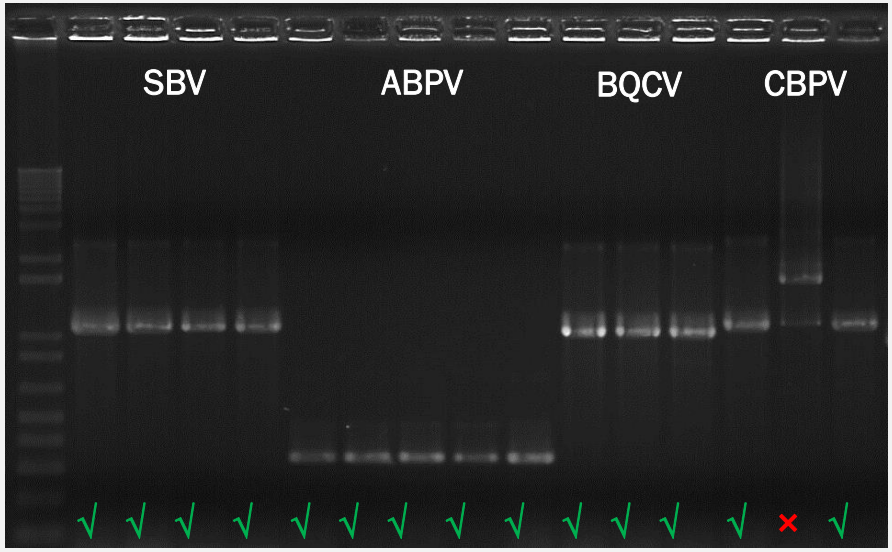
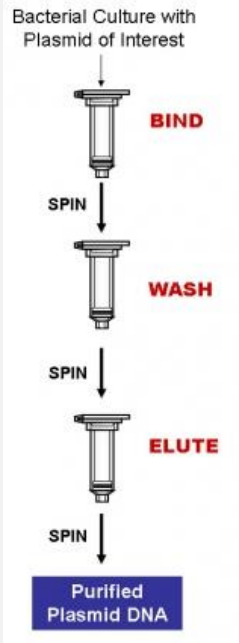
PREPARATION OF STANDARD DNA FOR THE QUANTIFICATION OF VIRUS LOAD IN REALTIME qPCR

6. Colony PCR on white colonies for selection of transformed clones







7. Minipreps of plasmid DNA from the selected colonies

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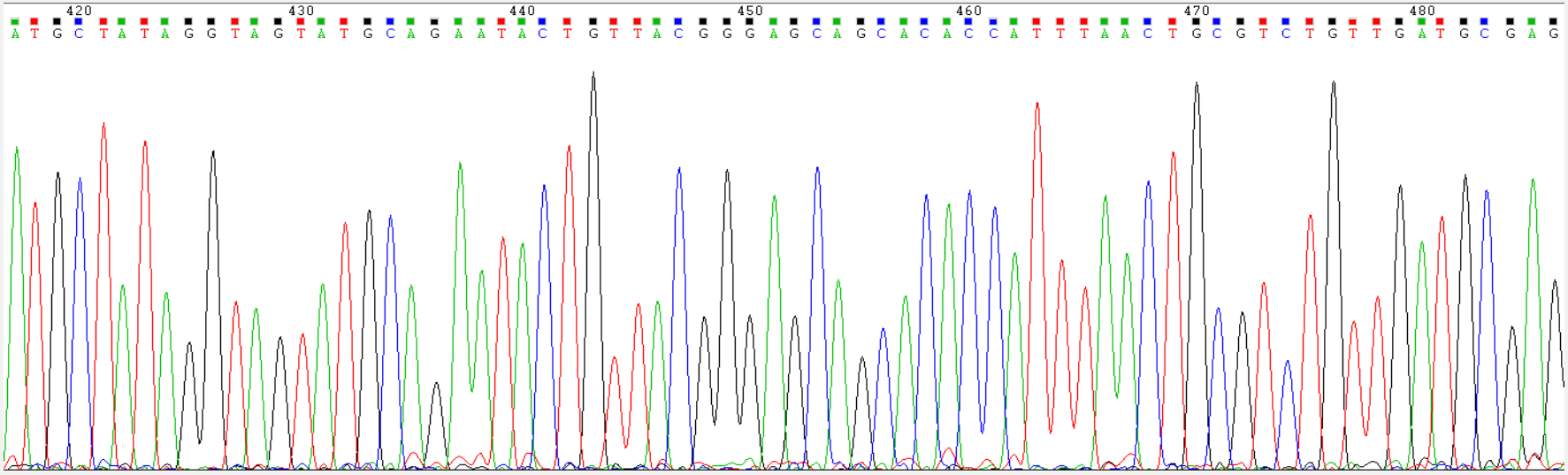
PREPARATION OF STANDARD DNA FOR THE QUANTIFICATION OF VIRUS LOAD IN REALTIME qPCR

8. Sequencing of the inserts



√ Plasmids correctly integrated the five fragments in their entire sequences

× Some mismatches in the target sequences of the primers chosen for the detection of BQCV, CBPV and SBV, however not at the 3' end

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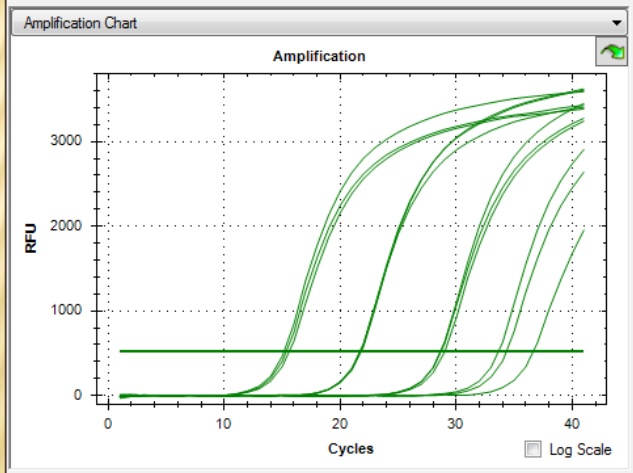
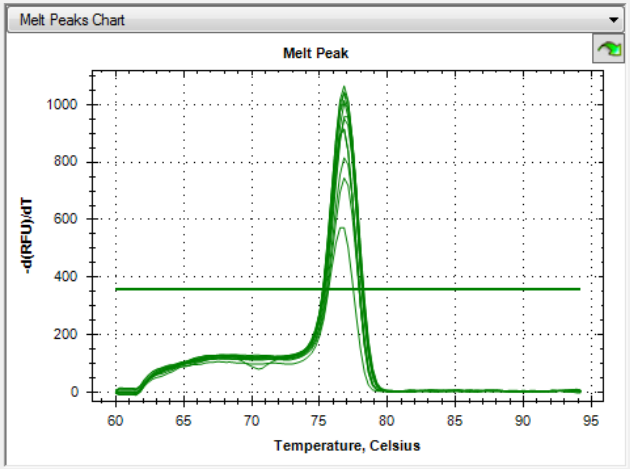
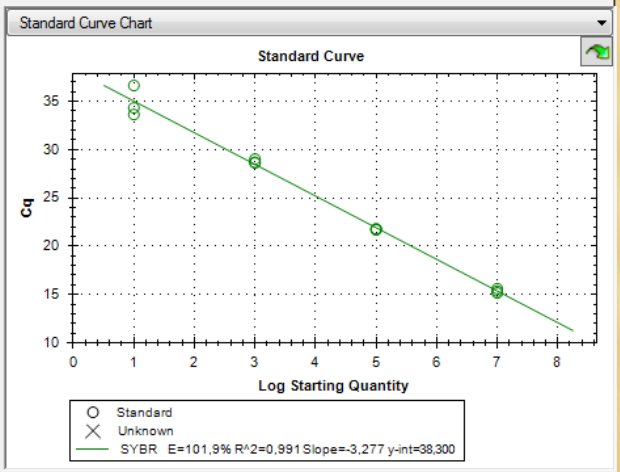
PREPARATION OF STANDARD DNA FOR THE QUANTIFICATION OF VIRUS LOAD IN REALTIME qPCR

9. Realtime qPCR assays with serial dilutions of plasmid DNA: Deformed Wing Virus







Dilution	DNA concentration (ng/µl)	Plasmid copy number	Treshold cycle (avg)
TQ	48,4	1,28E+10	
-1	4,84E+00	1,28E+09	
-2	4,84E-01	1,28E+08	
-3	**4,84E-02**	**1,28E+07**	**15,4**
-4	4,84E-03	1,28E+06	
-5	**4,84E-04**	**1,28E+05**	**21,7**
-6	4,84E-05	1,28E+04	
-7	**4,84E-06**	**1,28E+03**	**28,8**
-8	4,84E-07	1,28E+02	
-9	**4,84E-08**	**1,28E+01**	**34,9**
-10	4,84E-09	1,28E+00	

DWV
(primers: F8688, B8794;
amplicon size = 143)

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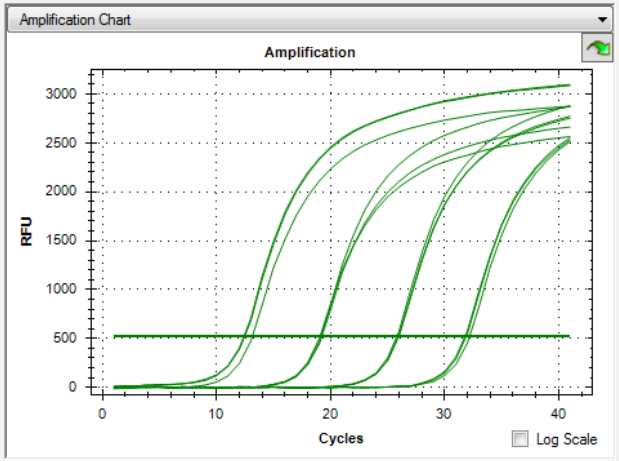
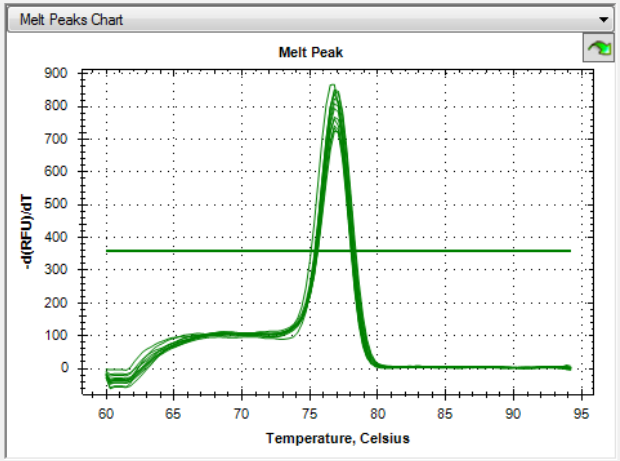
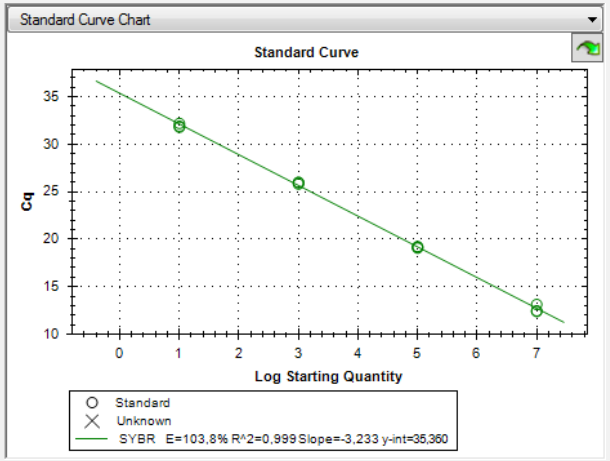
PREPARATION OF STANDARD DNA FOR THE QUANTIFICATION OF VIRUS LOAD IN REALTIME qPCR

9. Realtime qPCR assays with serial dilutions of plasmid DNA: Black Queen Cell Virus







Dilution	DNA concentration (ng/µl)	Plasmid copy number	Treshold cycle (avg)
TQ	37,5	1,11E+10	
-1	3,75E+00	1,11E+09	
-2	3,75E-01	1,11E+08	
-3	**3,75E-02**	**1,11E+07**	**12,7**
-4	3,75E-03	1,11E+06	
-5	**3,75E-04**	**1,11E+05**	**19,2**
-6	3,75E-05	1,11E+04	
-7	**3,75E-06**	**1,11E+03**	**25,9**
-8	3,75E-07	1,11E+02	
-9	**3,75E-08**	**1,11E+01**	**31,7**
-10	3,75E-09	1,11E+00	

ABPV complex (primers: F6099, B6164; amplicon size = 104)

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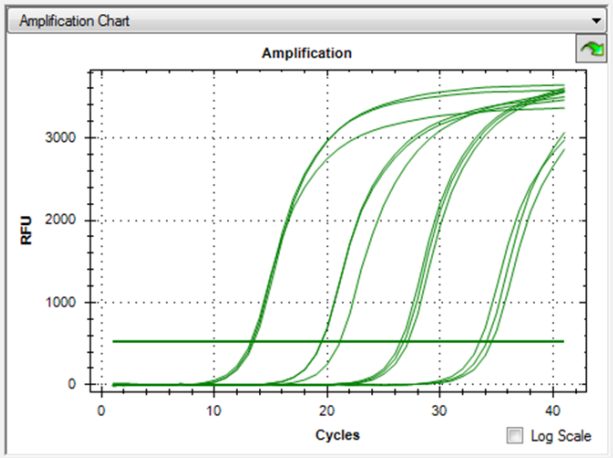
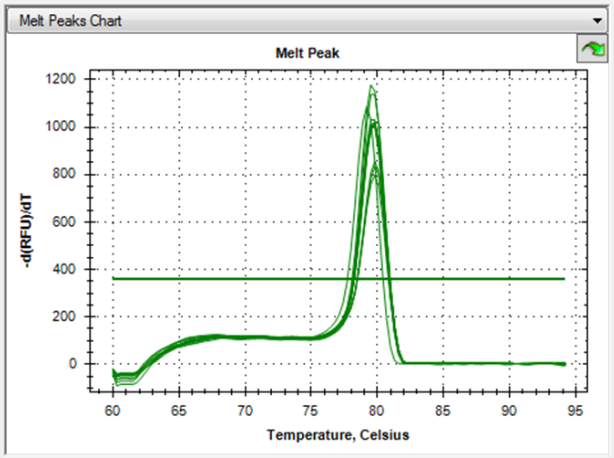
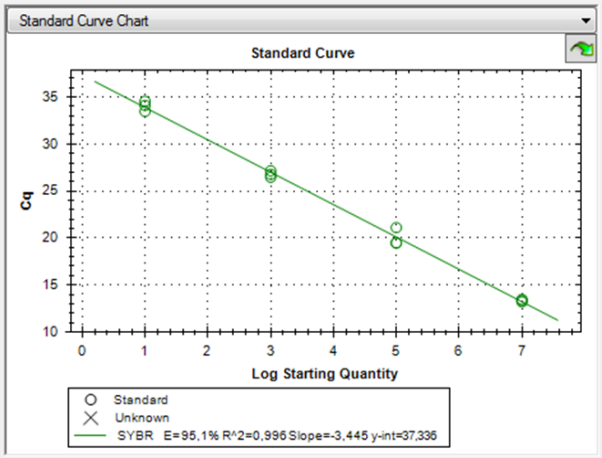
PREPARATION OF STANDARD DNA FOR THE QUANTIFICATION OF VIRUS LOAD IN REALTIME qPCR

9. Realtime qPCR assays with serial dilutions of plasmid DNA: Black Queen Cell Virus







Dilution	DNA concentration (ng/µl)	Plasmid copy number	Treshold cycle (avg)
TQ	25,5	6,14E+09	
-1	2,55E+00	6,14E+08	
-2	2,55E-01	6,14E+07	
-3	**2,55E-02**	**6,14E+06**	**13,3**
-4	2,55E-03	6,14E+05	
-5	**2,55E-04**	**6,14E+04**	**20**
-6	2,55E-05	6,14E+03	
-7	**2,55E-06**	**6,14E+02**	**26,8**
-8	2,55E-07	6,14E+01	
-9	**2,55E-08**	**6,14E+00**	**34,1**
-10	2,55E-09	6,14E-01	

BQCV
(primers: qF7893, qB8150;
amplicon size = 291)

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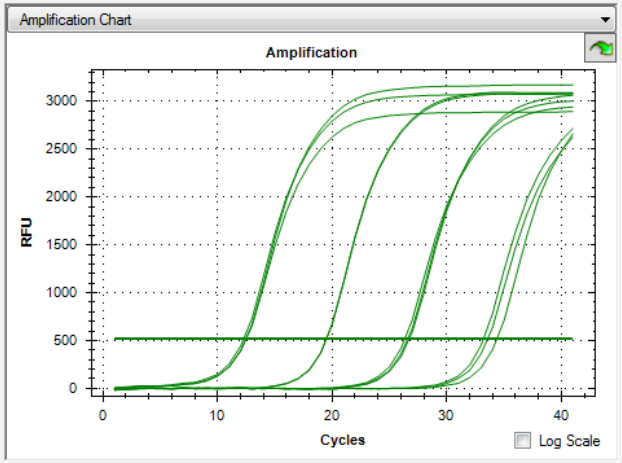
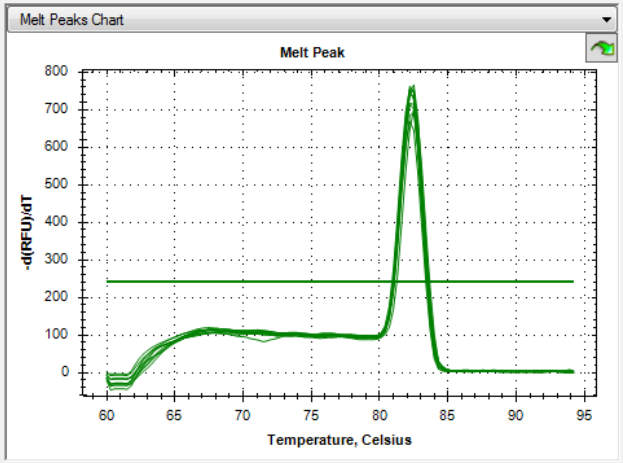
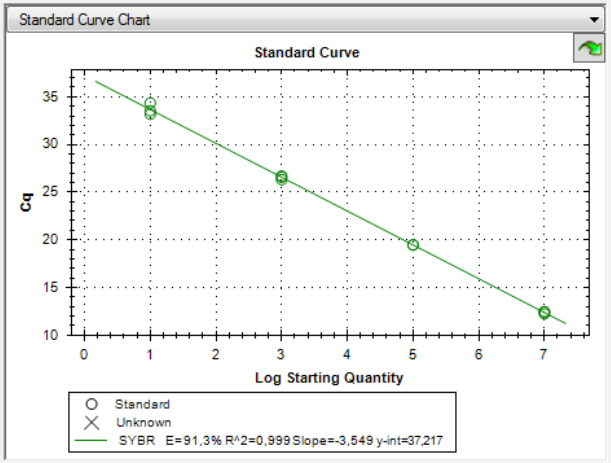
PREPARATION OF STANDARD DNA FOR THE QUANTIFICATION OF VIRUS LOAD IN REALTIME qPCR

9. Realtime qPCR assays with serial dilutions of plasmid DNA: Black Queen Cell Virus







Dilution	DNA concentration (ng/µl)	Plasmid copy number	Treshold cycle (avg)
TQ	43,7	1,03E+10	
-1	4,37E+00	1,03E+09	
-2	4,37E-01	1,03E+08	
-3	**4,37E-02**	**1,03E+07**	**12,4**
-4	4,37E-03	1,03E+06	
-5	**4,37E-04**	**1,03E+05**	**19,6**
-6	4,37E-05	1,03E+04	
-7	**4,37E-06**	**1,03E+03**	**26,6**
-8	4,37E-07	1,03E+02	
-9	**4,37E-08**	**1,03E+01**	**33,7**
-10	4,37E-09	1,03E+00	

CBPV
(primers: qF1818, qB2077;
amplicon size = 296)

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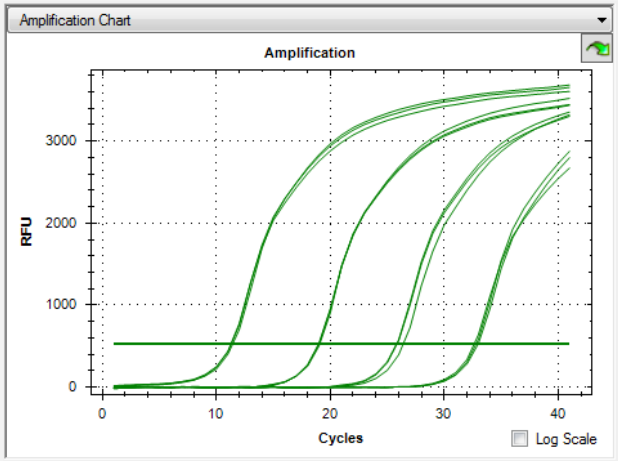
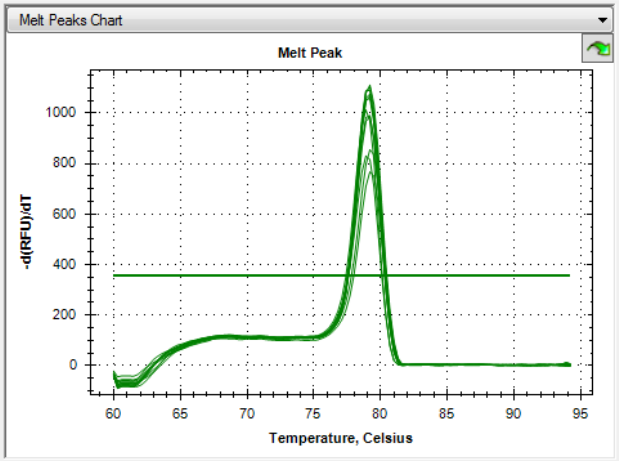
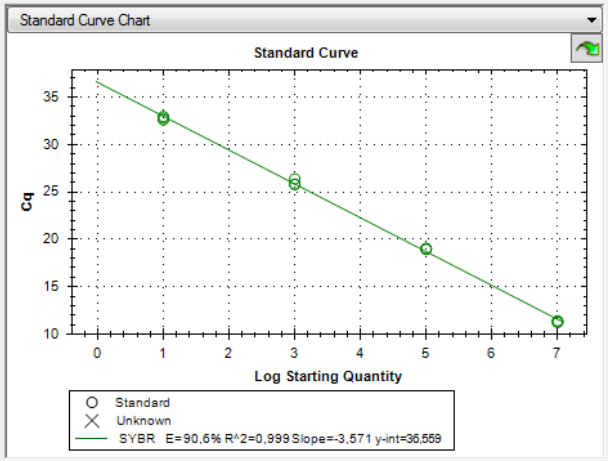
PREPARATION OF STANDARD DNA FOR THE QUANTIFICATION OF VIRUS LOAD IN REALTIME qPCR

9. Realtime qPCR assays with serial dilutions of plasmid DNA: Black Queen Cell Virus







Dilution	DNA concentration (ng/µl)	Plasmid copy number	Treshold cycle (avg)
TQ	48,7	1,16E+10	
-1	4,87E+00	1,16E+09	
-2	4,87E-01	1,16E+08	
-3	**4,87E-02**	**1,16E+07**	**11,3**
-4	4,87E-03	1,16E+06	
-5	**4,87E-04**	**1,16E+05**	**19**
-6	4,87E-05	1,16E+04	
-7	**4,87E-06**	**1,16E+03**	**26**
-8	4,87E-07	1,16E+02	
-9	**4,87E-08**	**1,16E+01**	**32,8**
-10	4,87E-09	1,16E+00	

SBV
(primers: qF3164, B3461;
amplicon size = 335)

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PREPARATION OF STANDARD DNA FOR THE QUANTIFICATION OF VIRUS LOAD IN REALTIME qPCR

To Summarize :

Four dilutions/standard DNA are enough to have good calibration curves :

Target	Efficiency %	Slope	Y-Intercept	R^2
DWV	101,90	-3,277	35,023	0,991
ABPV	103,83	-3,233	35,360	0,999
BQCV	95,09	-3,445	37,336	0,996
CBPV	91,46	-3,545	37,252	0,999
SBV	90,58	-3,571	36,559	0,999

Each dilution tested has been divided in two parts and shared with Avignon lab

Embrun, 9 & 10 October 2017

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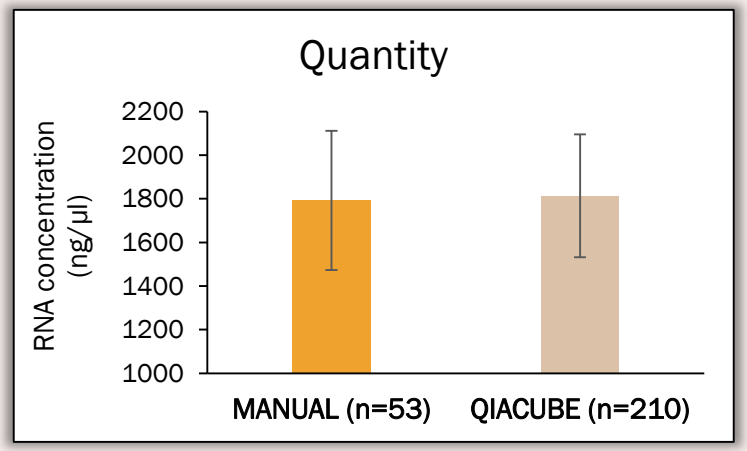
The Samples

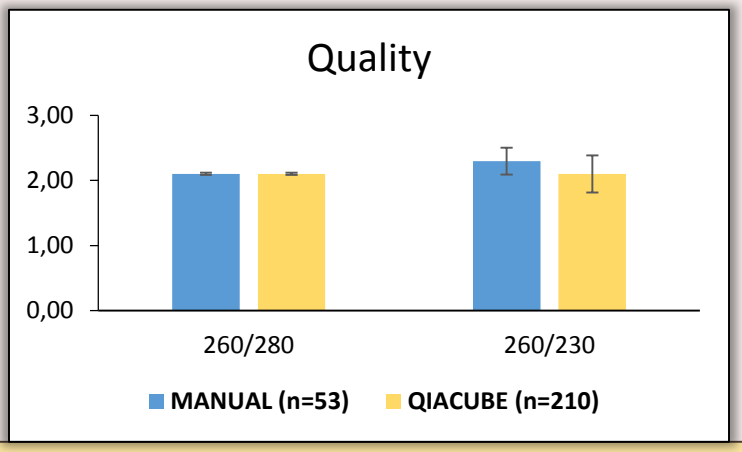
In Italy

2 Biological Replicats for each field sample

	RECEIVED	EXTRACTED
July	148	148
September	140	124

RNA extraction from the samples collected in July and September





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The Samples

In France

2 Biological Replicats for each field sample

	1st Sampling 2017		2nd sampling 2017		
Code Sample	**Sample Date**	**Nb Sample BM**	**Sample Date**	**Nb Sample BM**	**Remarks**
FA17 (1 to 15) 17	08/08/2017	**30**	11/09/2017	30	
FB17 (1 to 15) 17	08/08/2017	**30**	11/09/2017	30	
FC17 (1 to 15) 17	18/08/2017	**30**	26/09/2017	*26* or 28	1 dead colony + *1 dying colony*
FD17 (1 to 15) 17	18/08/2017	30	26/09/2017	22	3 dead colonies + *1 non sampled colony*
FE17 (1 to 7) 17	10/08/2017	14	19/09/2017	14	
FF17 (1 to 7) 17	10/08/2017	14	19/09/2017	14	

90 samples RNA extracts

Questions and Discussion

What are we doing with samples where there are not enough bees ?

- 1 replicat with 40 bees/bag or less than 40 bees/bag ?
- no analyzes of viral load ?

Do the dying colonies and/or not sampled colonies must be sampled to have a value of viral load just before the wasting ?

Embrun, 9 & 10 October 2017

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