# Development of detection method as point-of –care using Ultra-rapid PCR and immunochromatography against 11 major pathogen in honeybee

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# Introduction

- **Viral, bacterial and fungal pathogens against honeybee**
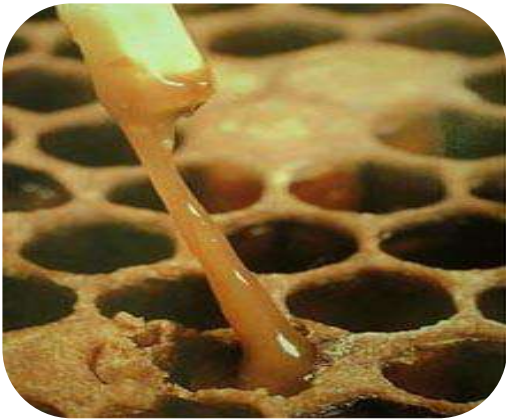
**BQCV**

**DWV**

**SBV**

*Paenibacillus larvae*

*Melissococcus plutonius*

*Ascospaera apis*

**\* Timing of symptom might be too late to control disease.**

# Honeybee pathogens found in Korea:17 species

**Table 1. Honeybee pathogens, parasite and injurious insect in bee hive**

| | Name of pathogens, parasite and injurious insect | Larvae | Adult |
|---|---|:---:|:---:|
| **Bacteria** | *Paenibacillus larvae* (AFB) | O | |
| | *Mellisococcus plutonius* (EFB) | O | |
| **Fungi** | Chalkbrood (*Ascospaera apis*) | O | |
| | Stonebrood (*Aspergillus flavus*) | | O |
| | Nosema disease (*Nosema cerana*) | O | |
| **Virus** | Sacbrood Virus (SBV) | O | |
| | Korean Sacbrood Virus (kSBV) | O | |
| | Acute Paralysis Virus (ABPV) | O | O |
| | Chronic Bee paralysis Virus (CBPV) | | O |
| | Black Queen Cell Virus (BQCV) | | O |
| | Deformed Wing Virus (DWV) | | O |
| | Slow Bee Paralysis Virus (SBPV) | | O |
| | Israelli acute paralysis Virus (IAPV) | | O |
| **Parasite** | *Varroa destructor* | O | O |
| | *Tropilaelaps mercedesae* | O | O |
| | *Acarapis woodi* | | O |
| **Injurious insect** | Small hive beetle (*Aethina tumida*) | O | O |

# Purpose of this study?

\* **Development of <u>sensitive, accurate, rapid and easy</u> detection method**

 - **against all 17  pathogens of honeybee,**
 - **under 30 minutes test,**
 - **on apiary field directly (point-of-care)**
 - **quantitative detection available.**

-> **Ultra-Rapid PCR** **based on molecular detection (UR-PCR)**

# What is Ultra-Rapid PCR ?



**Computer program**

**Rapi:Chip™**
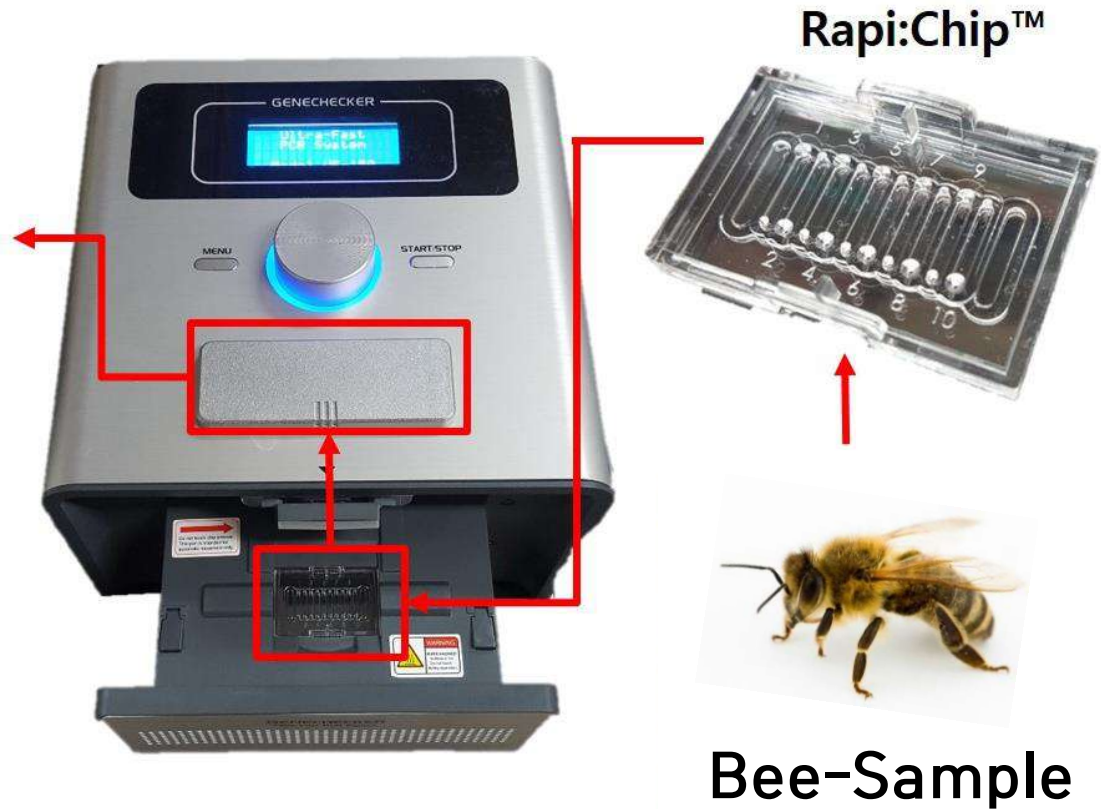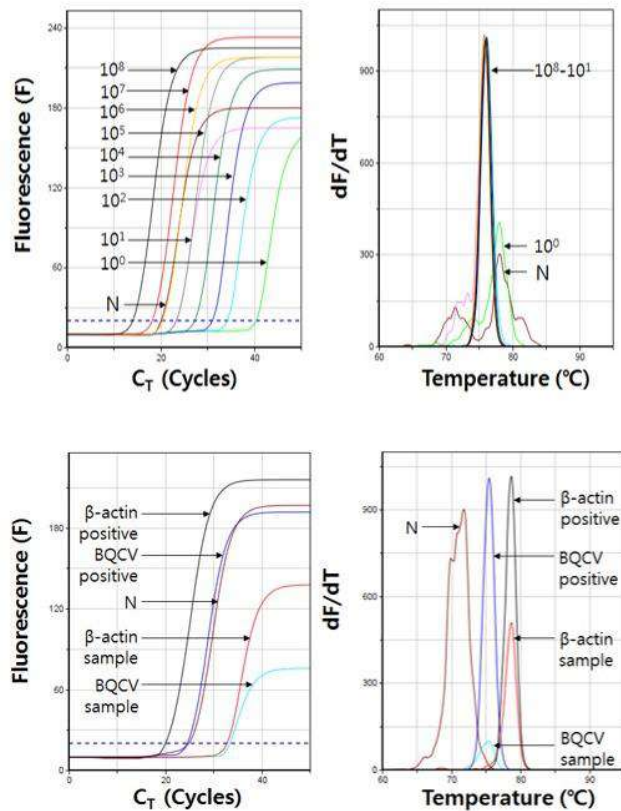
**Bee-Sample**

**Fig. 1. Data analysis, Real-time PCR, PCR-Chip and Bee-sample for Ultra-rapid PCR (UR-PCR) system**

# Materials & Methods

- **Why is Ultra-Rapid PCR so fast?**

**Table 2. PCR condition for pathogen detection.**

| PCR step | Temperature | Time | cycle |
|---|---|---|---|
| Reverse transcription | 50°C | 1 min | 1 |
| Pre-denaturation | 95°C | 20 sec | 1 |
| **Step 1-Denaturation** | 95°C | 1 sec | |
| **Step 2-Annealing** | 45 ~ 68°C | 3 sec | **50** |
| **Step 3-Polymerization** | 72°C | 1 sec | |

UR-PCR: **15 min / 50 cycles**

# Materials & Methods

\* **<u>Ultra-Rapid PCR sensitive and accurated?</u>**
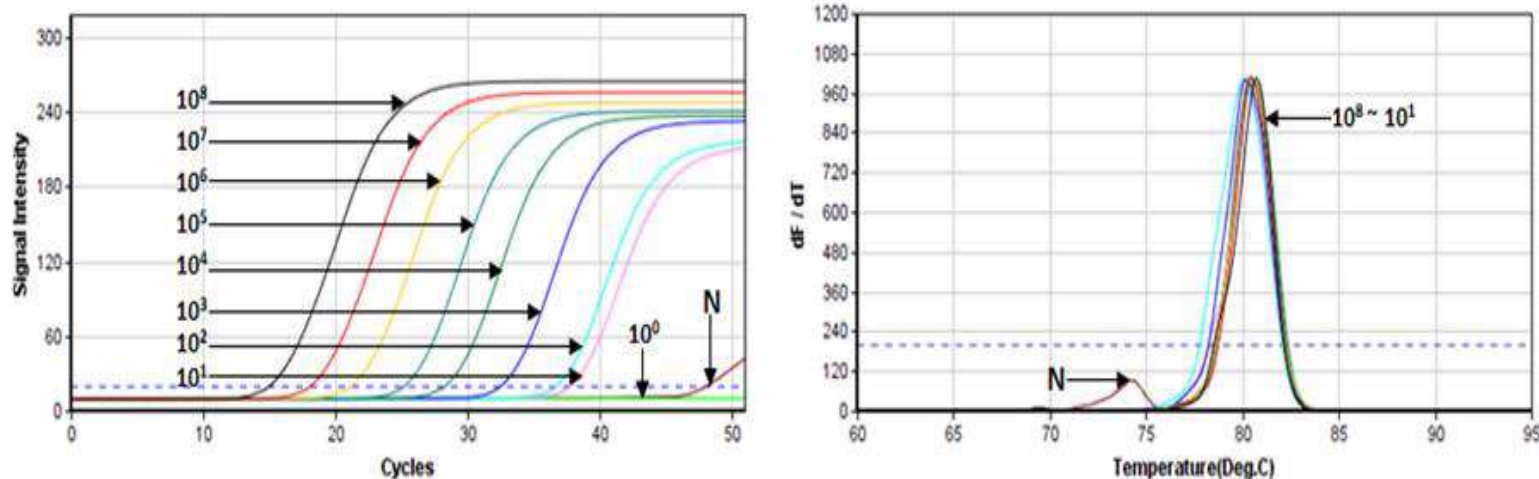


**Fig. 2. Fluorescence curve and melting-point analysis in UR-PCR**
With serially diluted targets ($10^8$ to $10^0$ molecules), UR-PCR performed by 50 cycles. It finished in 14 minutes. <u>Each $10^8$ to $10^1$ molecules of targets was quantitatively measured</u> by $C_T$ (Threshold Cycles), without under 10 molecules or no target. $T_m$ (Temperature of mid-point) of each UR-PCR products were identical, $80.0^o$C.

# Development of 17 UR-PCRs against 17 pathogens

## Table 3. Specific primers for 17 UR-PCRs.

| | Name of primer pairs | Sequence (5'→3') | Expected size (bp) | Reference |
|---|---|---|---|---|
| **Viral pathogens** | | | | |
| **ABPV** | ABPV-RdRp-DF | GCA TTA ATT CGA CAT GTT TT | 208 | Unpublished |
| | ABPV-RdRp-DR | TTA CAT AGC ATT TTG TTC CG | | |
| **BQCV** | BQ-DC-F1 | ACT TTG AAG GTT TCG ACG CTT CGG | 233 | Min *et al.*, 2017 |
| | BQ-DC-R1 | GAC GGA ATT TAT AAT AGC AGT GAG AT | | |
| **CBPV** | CB-DC-F1 | CCGACACATACTTCACTCTCTCAT | 230 | Wang *et al.*, 2016 |
| | CB-DC-R1 | GTACTTGCGGCGAGGTTCTG | | |
| **DWV** | DWV-DP-Heli-F | GTG TGG TGC ATC TGG AAT TGG | 147 | Unpublished |
| | DWV-DP-Heli-R | GGC TGA AAA TCA CAT TGA TCC CA | | |
| **IAPV** | IAPV-F2 | CCA TGG TCT CTT ATG GAG ATG A | 219 | Unpublished |
| | IAPV-R2 | CCC AAA CCT TCC GTT GTT C | | |
| **kSBV** | SBVD51-F | AGA CCA AGA AGA GAA TCA G | 182 | Tai *et al.*, 2018 |
| | SBVD-R | TCC AAA GAT TGR AAA CCC AT | | |
| **SBV** | SBVD-F2 | GGT ACC AAT AGA Y(C,T)AG TTT TCC | 108 | Tai *et al.*, 2018 |
| | SBVD0-R | CCT TAC CCC CAT CGC TAT CT | | |
| **SBPV** | SBPV-F | CGC AGG GTG ATC ATG CTA ATG | 224 | Min *et al.*, 2017 |
| | SBPV-C3G-R | ACA GCA AAC ACC CGG ACT AA | | |
| **Bacterial pathogens** | | | | |
| *Paenibacillus larvae* | P.larvae-DF | ATG ATT CAG ATC CGG CAG CAA G | 233 | Unpublished |
| | P.larvae-DR | TCA TAA GGG GCC AGT GTT ACG G | | |
| *Melissococcus plutonius* | EF-DC-F1 | AAG AGT AAC TGT TTT CCT CG | 208 | Wang *et al.*, 2016 |
| | EF-DC-R1 | TCC TCT TCT GCA CTC AAG TCT TC | | |

# Results:

# Table 3. Primers for 17 UR-PCRs (continued)

| | | Name of primer pairs | Sequence (5'→3') | Expected size (bp) | Reference |
|---|---|---|---|---|---|
| **Fungal pathogens** | *Ascosphaera apis* | AA-ITS294-F | CTG CCG GAG GGG TTA GTT C | **248** | Unpublished |
| | | AA-ITS294-R | GGG ACG ATC GCC CAA CAC | | |
| | *Aspergillus flavus* | A. flavus-18s-199F | GGT GTT TCT ATG ATG ACC CG | **199** | Unpublished |
| | | A. flavus-18s-199R | GAG CTC TCA ATC TGT CAA TC | | |
| | *Nosema ceranae* | NO-DC-F2 | GGT AAT GGC TTA ACA AGG CTG TGA | **346** | Wang *et al.*, 2016 |
| | | NO-DC-R2 | CAG GGT CGT CAC ATT TCA TCT TTC | | This study |
| **Injurious insect** | *Aethina tumida* | SHB-DP-F1 | TGA TTC TTC GGA CAC CCA GA | **205** | Kim *et al.*, 2017 |
| | | SHB-DP-R1 | AGG CTC GAG TAT CAA CGT CT | | |
| **Parasite** | *Varroa distructor* | Varroa-COI-F | GTA TAC AAA GAG GGA AGA AGC A | **293** | Unpublished |
| | | Varroa-COI-R | TAC ACC AGT AAT ACC CCC TAA AG | | |
| | *Acarapis woodi* | AW-COI-F | CTG GTT TAG TTG GTC TAT CAA | **299** | Unpublished |
| | | AW-COI-R | CCC TGT TCC TGA ACC TTT TG | | |
| | *Tropilaelaps sp.* | Tro-COI-F | GGA GCC TCA GTT GAC CTA AGA AT | **203** | Unpublished |
| | | Tro-COI-NR | GTA ATA GCT GCT GCT AGG AC | | |
| **Honey bee** | *Apis mellifera* | β-actin 151 F | ATG CCA ACA CTG TCC TTT CTG G | **151** | Yang and Cox-Foster, 2005 |
| | | β-actin 151 R | GAC CCA CCA ATC CAT ACG GA | | |

# • Quantitative UR-PCRs against DNA pathogen
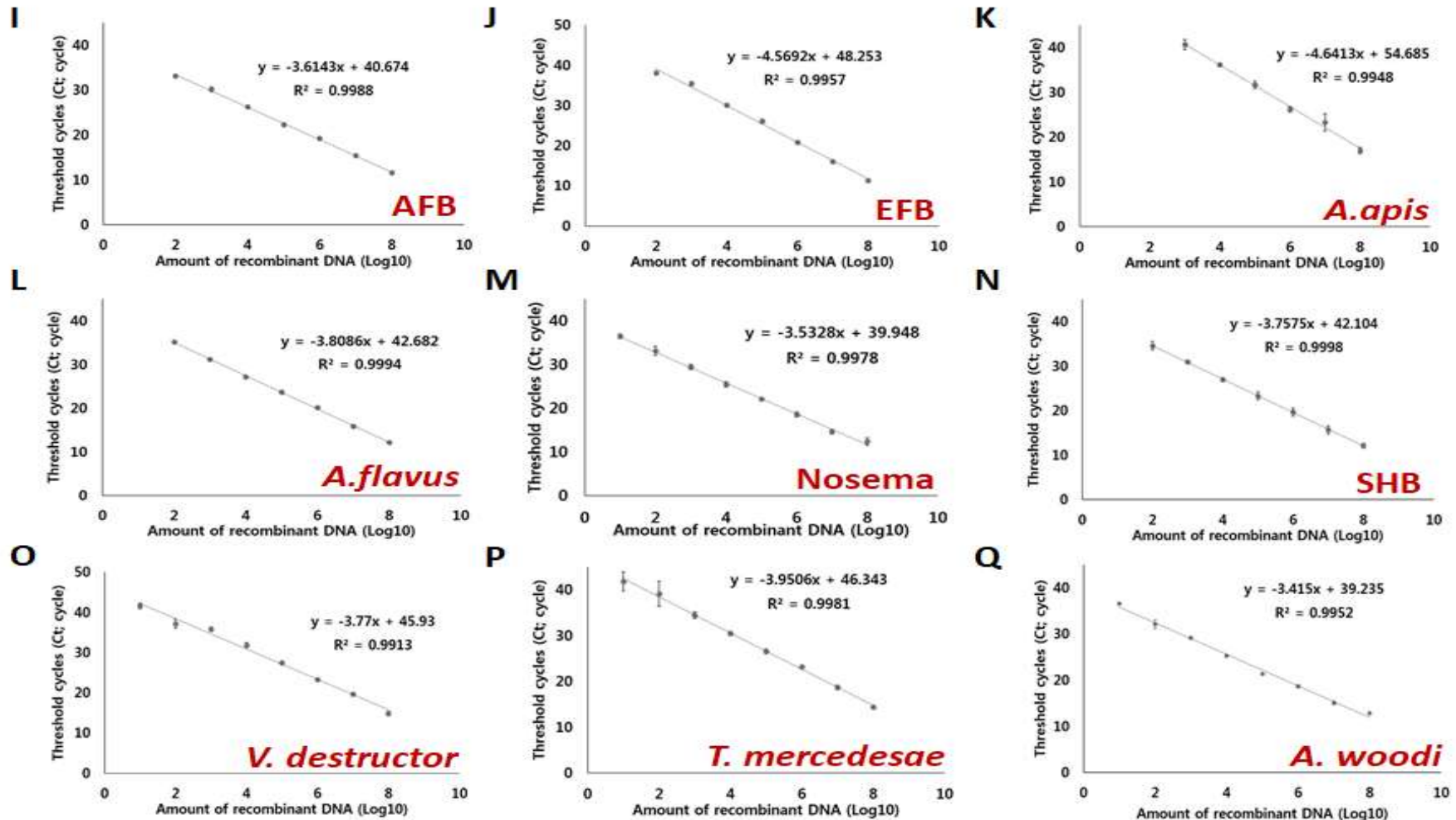


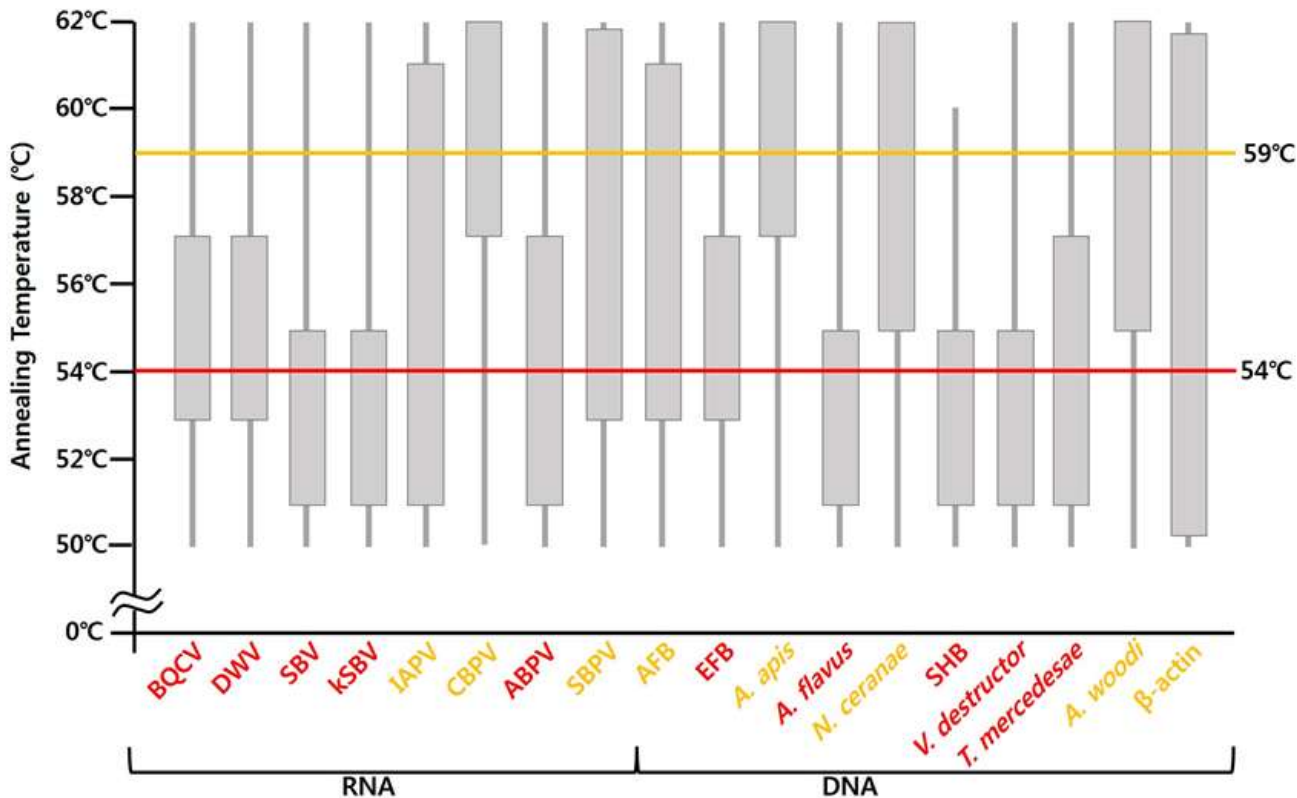**Fig. 3. Regression equation from each UR-PCR against honeybee DNA pathogens.**

DNA pathogens could be also detected from $10^8$ to $10^0$ molecules of PCR-target.

# • **Quantitative UR-PCRs against** **RNA viruses**
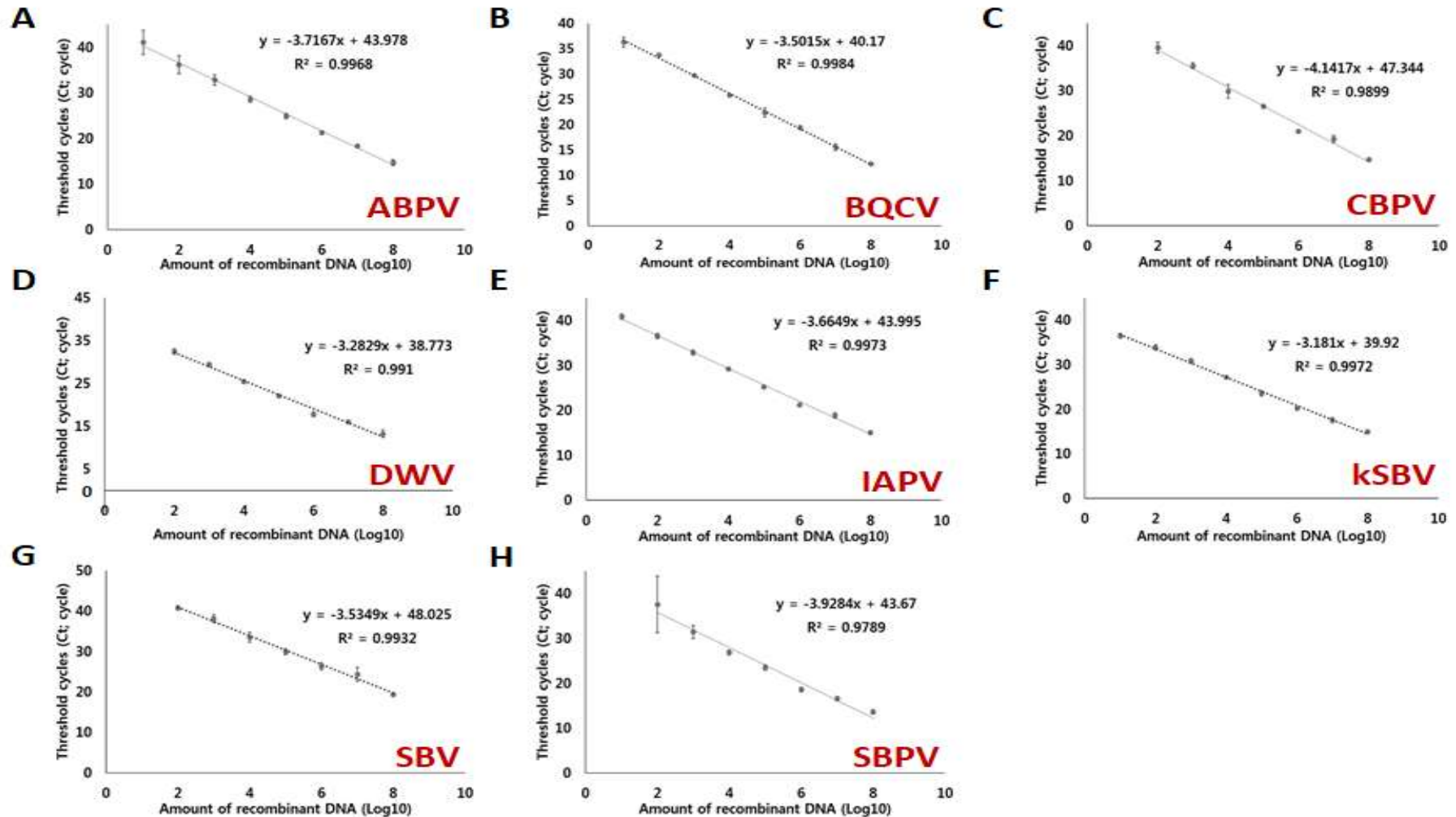


**Fig. 4. Regression equation from each UR-PCR against honeybee RNA pathogens.**

# Results

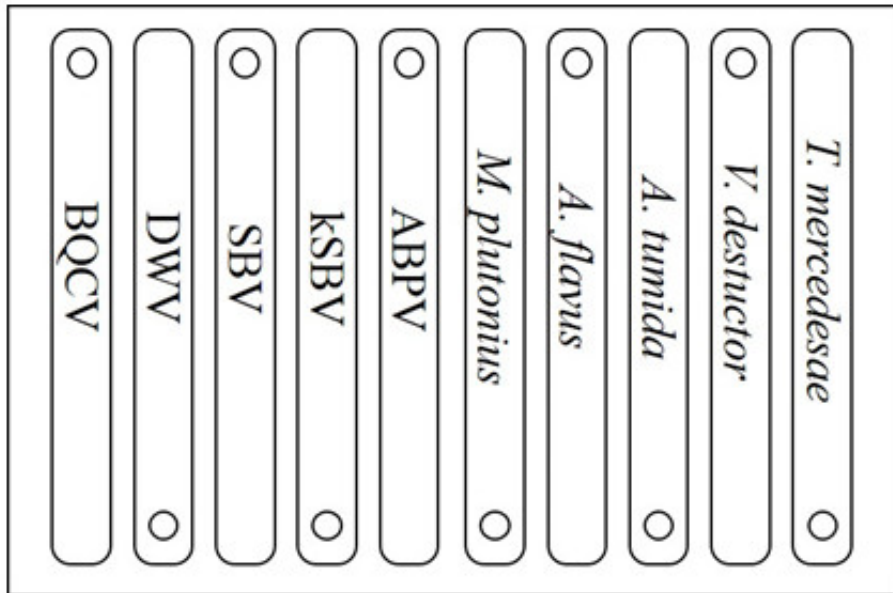## Optimum annealing temperature for each UR- PCR



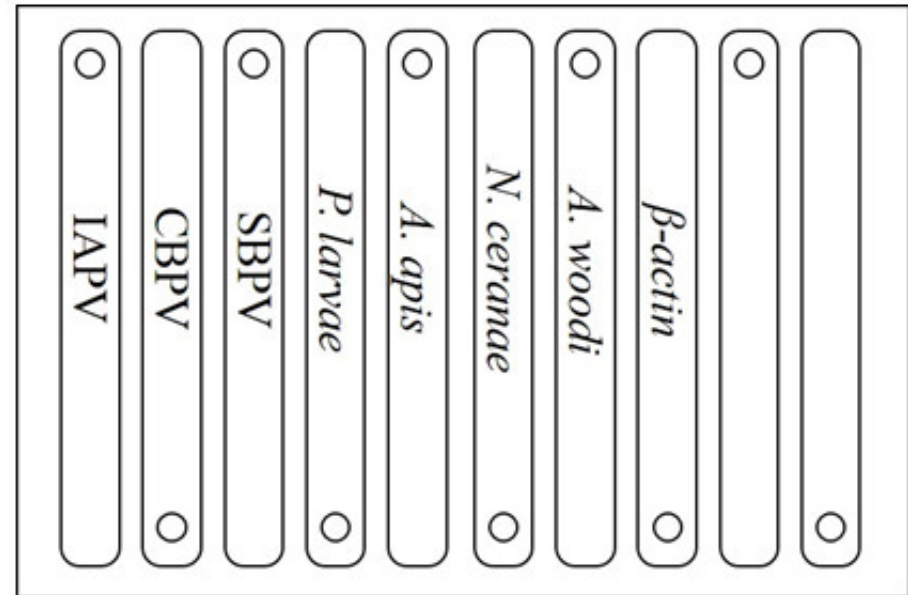**Fig. 5. Optimum annealing temperature in each 17 UR-PCRs.**

Annealing temperature could be integrated to 54°C or 59°C for all 17 detections by UR-PCR.

# Results

## Setting-up of 17 UR-PCRs using PCR chip based on annealing temperatures



Fig. 6. Two DNA-Chips for 17 PCRs against honeybee pathogen

# Summary of quantitative detection by 17 UR-PCRs

**Table 4. Detection limit, Specific Tm value and Regression equation for each pathogen.**

| Target genes | Detection limit (molecules) | Regression coefficient ($R^2$) | Tm value (°C) | Regression equation |
|---|---|---|---|---|
| **ABPV** | $1.01 \times 10^1$ | 0.9968 | 78.66 ± 0.17 | y =-3.7167x+43.978 |
| **BQCV** | $1.01 \times 10^1$ | 0.9984 | 77.15 ± 0.14 | y =-3.5015x+40.17 |
| **CBPV** | $1.01 \times 10^2$ | 0.9899 | 85.55 ± 0.33 | y =-4.1417x+47.344 |
| **DWV** | $1.01 \times 10^2$ | 0.9910 | 79.73 ± 0.26 | y =-3.2829x+38.773 |
| **IAPV** | $1.01 \times 10^1$ | 0.9973 | 77.69 ± 0.16 | y =-3.6649x+43.995 |
| **kSBV** | $1.01 \times 10^1$ | 0.9972 | 79.28 ± 0.31 | y =-3.181x+39.92 |
| **SBV** | $1.01 \times 10^2$ | 0.9932 | 79.16 ± 0.17 | y =-3.5379x+48.025 |
| **SBPV** | $1.01 \times 10^2$ | 0.9789 | 80.34 ± 0.85 | y =-3.9284x+43.67 |
| *P. larvae* | $1.01 \times 10^2$ | 0.9988 | 79.28 ± 0.01 | y =-3.6143x+40.674 |
| *M. plutonius* | $1.01 \times 10^2$ | 0.9957 | 85.92 ± 0.23 | y =-4.5692x+48.253 |
| *A. apis* | $1.01 \times 10^3$ | 0.9948 | 84.48 ± 1.52 | y =-4.6413x+54.685 |
| *A. flavus* | $1.01 \times 10^2$ | 0.9994 | 84.19 ± 0.41 | y = -3.8086x+42.682 |
| *N. Ceranae* | $1.01 \times 10^1$ | 0.9978 | 80.04 ± 0.20 | y =-3.5328x+39.948 |
| *A. tumida* | $1.01 \times 10^2$ | 0.9998 | 74.99 ± 0.16 | y =-3.7575x+42.104 |
| *V. destructor* | $1.01 \times 10^1$ | 0.9913 | 74.80 ± 0.14 | y =-3.77x+45.93 |
| *T. mercedesae* | $1.01 \times 10^1$ | 0.9981 | 74.48 ± 0.15 | y =-3.9506x+46.343 |
| *A. woodi* | $1.01 \times 10^1$ | 0.9952 | 72.84 ± 0.46 | y =-3.415x+39.235 |

# Results:

• **Why nested UR-PCR needed? Different sample !**
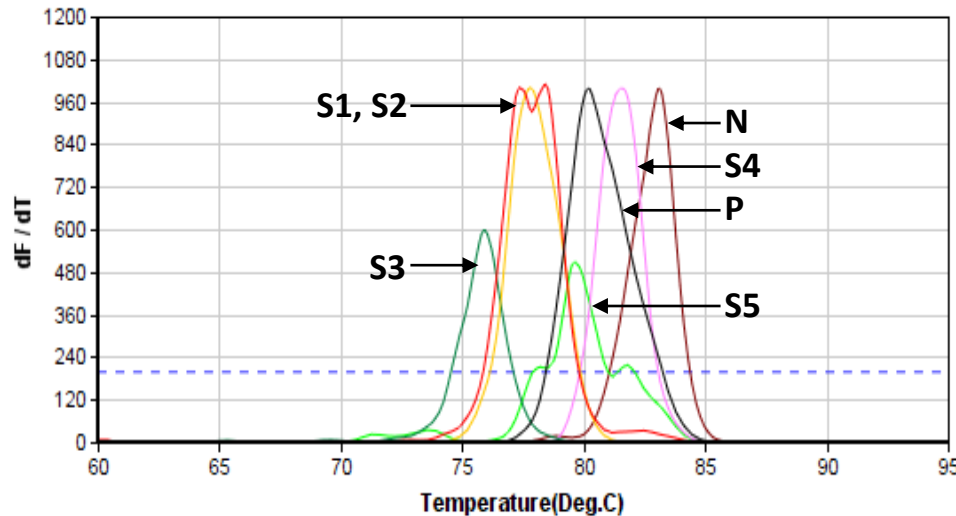


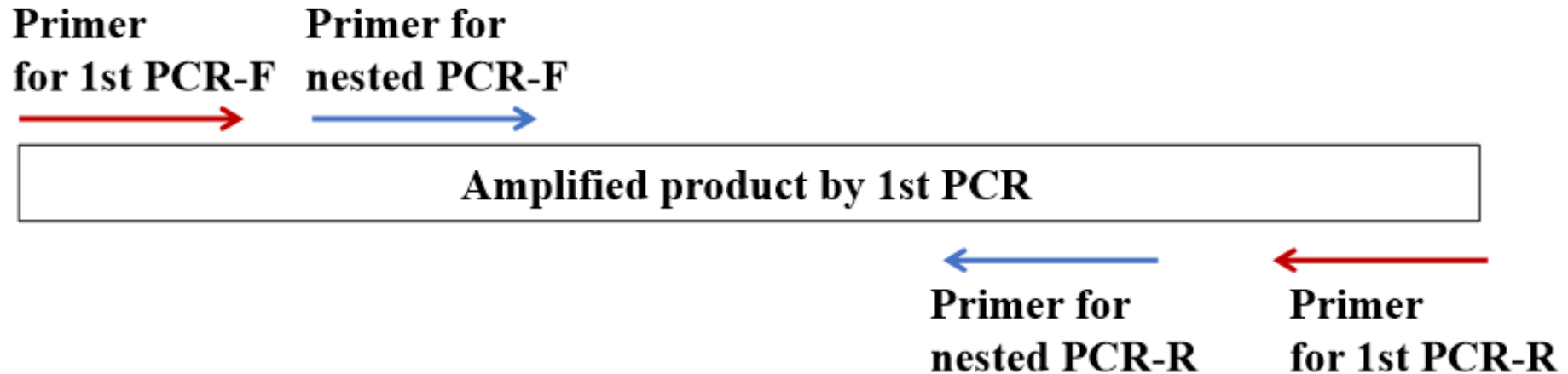**Fig. 7. Detection of SBPV from 5 different samples of honeybee**

Tm of PCR-product was not always identical to positive control because of various PCR samples → SBPV existed or not?

**Table 5. Tm value and results for several samples**

|  | Tm (℃) | Results |
|---|---|---|
| Positive | 80.14 | + |
| S1 | 78.49 | +/- |
| S2 | 77.83 | +/- |
| S3 | 75.85 | +/- |
| S4 | 81.46 | + |
| S5 | 79.48 | + |
| N | 83.11 | - |

# Results:

## **Nested UR-PCR is best re-checking tool.**

Primer for 1st PCR-F     Primer for nested PCR-F

Amplified product by 1st PCR

Primer for nested PCR-R     Primer for 1st PCR-R

**Fig. 8. Schematic diagram of primer positions for nested PCR**
**Advantage of nested PCR : good amplification for very low numbers of target DNA. Disadvantage of nested PCR : double or more time than one round PCR (few hours?)**

**Application of nested UR-PCR against few molecules of target?**
**1. Only 15 minutes (50 cycles) = 15 minutes or more!**
**2. Quantitative analysis available for only few molecules!**

# Results:

## * Nested primers for 17 nested UR-PCR

## Table 6. Specific-primers for 17 UR-nested PCR

| | | Name of primer pairs | Sequence (5'→3') | Expected size (bp) | Reference |
|---|---|---|---|---|---|
| **Viral pathogens** | ABPV | ABPV-NT-F | CTT ATG GAT GTA TAT AAC TCA AC | 166 | Kim et al., 2018 |
| | | ABPV-NT-R | TAA GGG CGG AAT ATT TCT TT | | |
| | BQCV | BQ-DC-NF | ACA GTC TGA TAT ATT GTA TGC TGC | 174 | This study |
| | | BQ-DC-NR | CTA GGA AGA GAC TTA CAC CAC TG | | |
| | CBPV | CBPV-NF | AGT CCG GAT CCC CGA GAC ACT | 174 | Kim et al., 2018 |
| | | CBPV-NR | GCT GAG GAC GCG ATT TCG TGC | | |
| | DWV | DWV-NP-Heli-F | GTT ATA CTT CAA GGA GTA TAT AC | 99 | Unpublished |
| | | DWV-NP-Heli-R | GAT ACC TAT AAT CGG CCG | | |
| | IAPV | IAPV-NF | CAA TGT CAT AAA CTT CAG TGA TG | 123 | This study |
| | | IAPV-NR | GGT ACT TCG CCA TTT ACG C | | |
| | kSBV | kSBV-NF | CAT TTG AGA CTT ACG TGT AT | 207 | This study |
| | | kSBV-NR | GTA TTT TTA GAA CTC CTT CA | | |
| | SBV | SBV-NF | ACC TGA TGG TTA TGA TCC AGT | 80 (semi) | This study |
| | | SBVD0-R | CCT TAC CCC CAT CGC TAT CT | | |
| | SBPV | SBPV-NF | CAT CCA GTT GTT CGT TCT CAG GTA CCT G | 141 | This study |
| | | SBPV-NR | GAG CGC ACT CCC GCA CAT G | | |
| **Bacterial pathogens** | *Paenibacillus larvae* | P.larvae-NF | TGC AGA ACA GGA GAT TGT TGA | 152 | This study |
| | | P.larvae-NR | TGG TTA ACA GGT TCG TTC CA | | |
| | *Melissococcus plutonius* | EF-DC-F1 | AAG AGT AAC TGT TTT CCT CG | 186 (semi) | This study |
| | | EF-NC-R | CAG TTT CCA ATG ACC CTC | | |

# MR,esults:

## • **Nested primers** for 17 nested UR-PCR

## Table 6. Specific-primers for 17 UR-nested PCR (**continue**)

| | Name of primer pairs | Sequence (5'→3') | Expected size (bp) | Reference |
|---|---|---|---|---|
| **Fungal pathogens** | *Ascosphaera apis* | AA-ITS294-F | CTG CCG GAG GGG TTA GTT C | 216 (semi) | Kim et al., 2019 |
| | | AA-ITS216-R | GGG CGC AAT GTG CGT TC | | This study |
| | *Aspergillus flavus* | A. flavus-18s-199F | GGT GTT TCT ATG ATG ACC CG | 179 (semi) | Kim et al., 2019 |
| | | A. flavus-18s-179R | CTT ATT TTG TCT GGA CCT GGT G | | This study |
| | *Nosema ceranae* | NO-DC-F3 | AGG CAG TTA TGG GAA GTA ATA TTA TA | 216 (semi) | Unpublished |
| | | NO-DC-R2 | CAG GGT CGT CAC ATT TCA TCT TTC | | This study |
| **Injurious insect** | *Aethina tumida* | SHB-mt-NF | TGT AGT TAT AGG AAC AGC TTT CC | 334 (semi) | This study |
| | | SHB-mt-DR | GAA TCC TAC AGA ATC CTT TCA TG | | |
| **Parasite** | *Varroa distructor* | Varroa-COI-NF | GCC TTT TGG AAA TTT AGG GAT AA | 214 | This study |
| | | Varroa-COI-NR | CGG GAC ATC TAA TTT AAC TAT AG | | |
| | *Acarapis woodi* | AW-COI-NF | CGA ATA GAA TTA TCA ATT CCA TCC | 217 | Kim et al., 2019 |
| | | AW-COI-NR | TTA GAG AGG ATA ATA AAA GTC AAA ATC | | |
| | *Tropilaelaps sp.* | Tro-COI-NF | GGA TTT TCT TCA ATC CTA GGA GC | 141 | Kim et al., 2019 |
| | | Tro-COI-NR2 | TGG TAA TCT AAG TAA TAA TAA AAT TGC TGT GA | | |

# Results

- **Optimum annealing temperature for nested UR-PCR**

  Table 7. Optimum annealing temperature of each primer pairs for nested PCR.

| | Quantitative range of annealing temperature | Optimum annealing temp. for nested PCR |
|---|---|---|
| ABPV | 40 – 65°C | 48°C |
| BQCV | 53 – 59°C | 53°C |
| CBPV | 61 – 67°C | 65°C |
| DWV | 45 – 59°C | 55°C |
| IAPV | 50 – 59°C | 53°C |
| kSBV | 50 – 59°C | 56°C |
| SBV | 50 – 59°C | 56°C |
| SBPV | 56 – 65°C | 65°C |
| AFB | 53 – 65°C | 65°C |
| EFB | 53 – 62°C | 56°C |
| *A. apis* | 53 – 62°C | 59°C |
| *A. flavus* | 56 – 65°C | 56°C |
| Nosema | 50 – 59°C | 53°C |
| SHB | 45 – 53°C | 50°C |
| *V. destructor* | 53 – 59°C | 56°C |
| *T. mercedesae* | 53 – 59°C | 53°C |
| *A. woodi* | 50 - 54°C | 52°C |

# Results

## · 17 UR-PCR with sample Ulsan (case study)
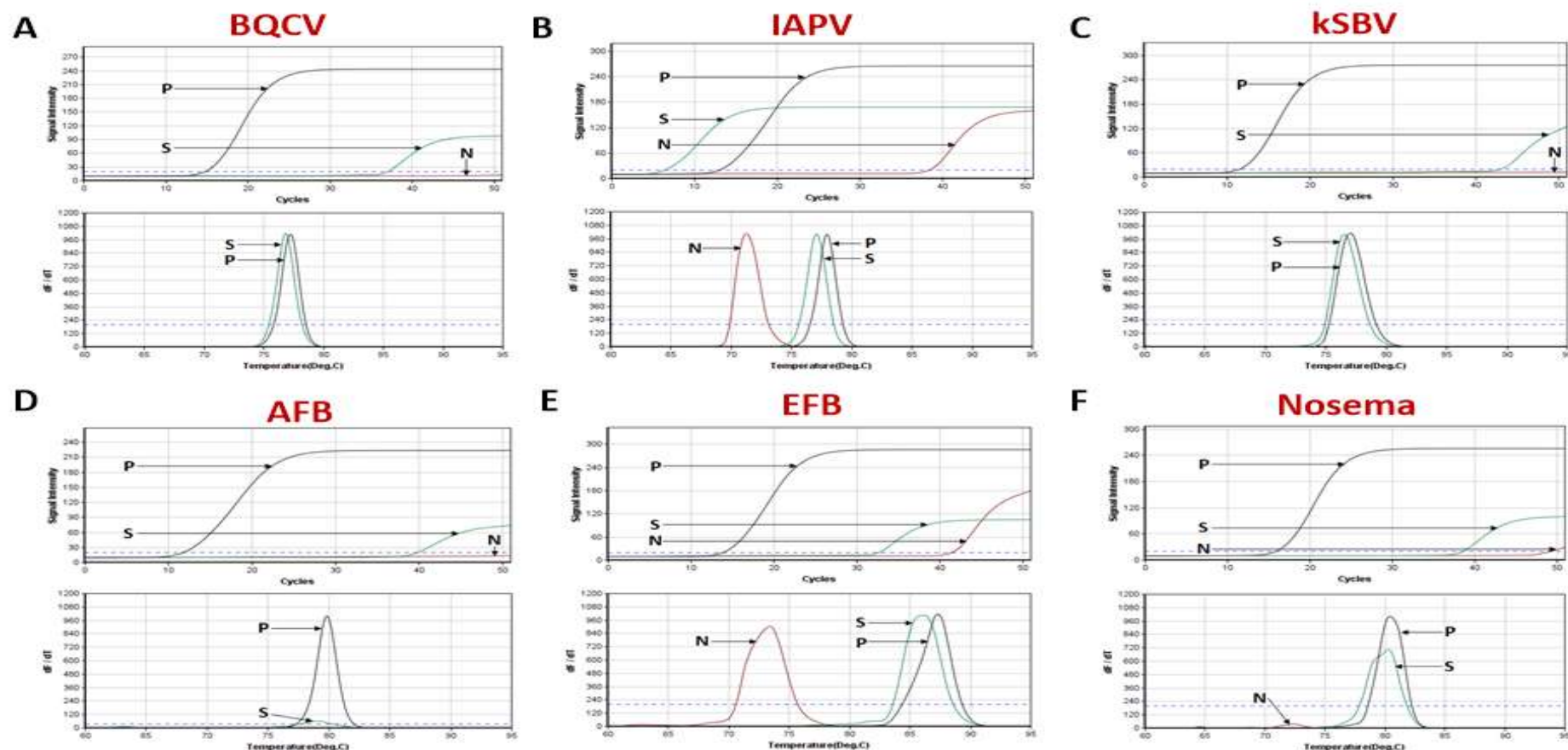


**Fig. 9. 17 UR-PCRs with Ulsan sample**

6 pathogens, BQCV, IAPV, kSBV, AFB, EFB, and Nosema were positively detected from Ulsan honeybee sample. P, Positive; S, Sample.

# Results

- **Re-check by nested UR-PCR** with 1$^{st}$PCR product
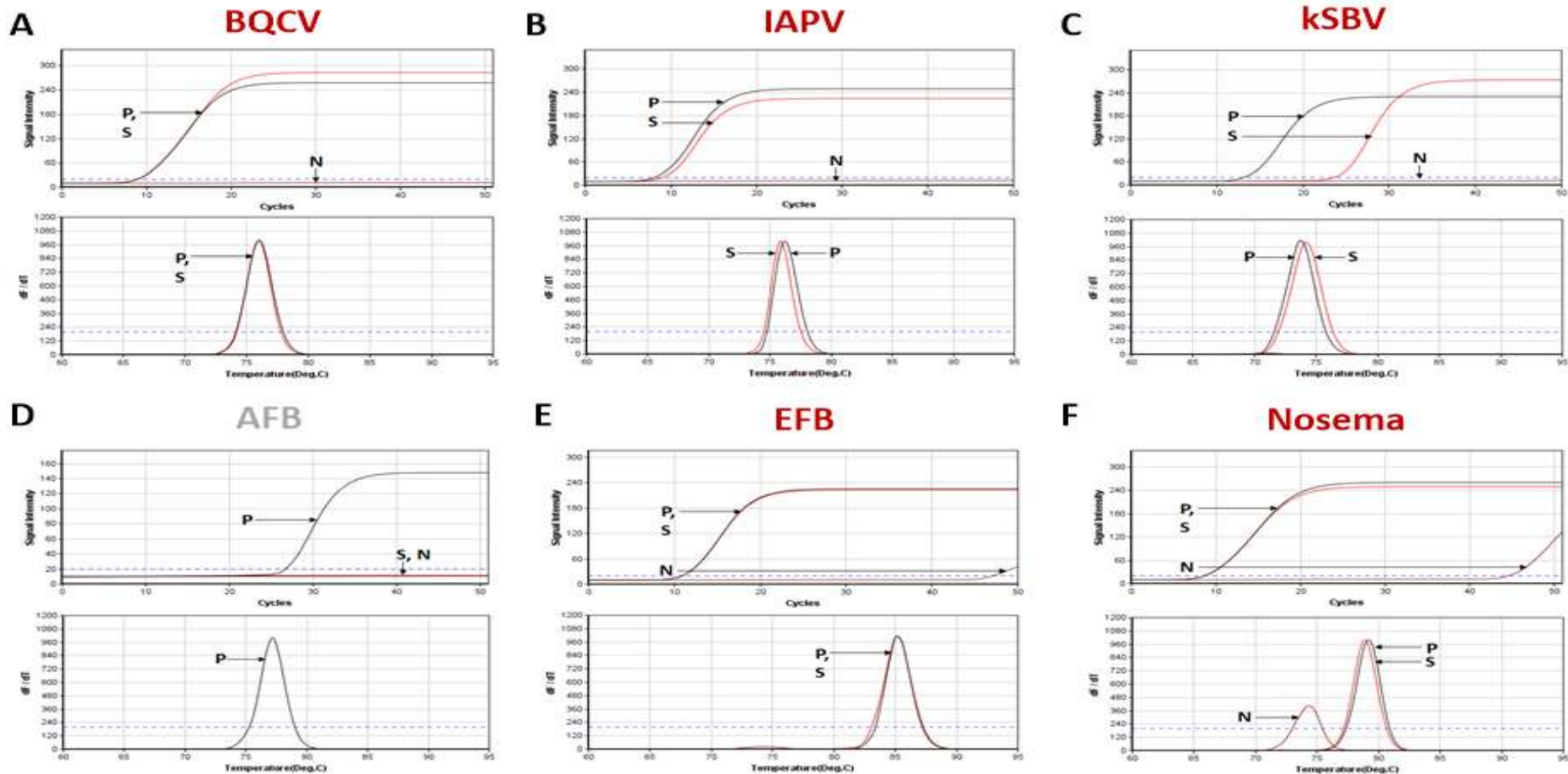


Fig. 10. Nested UR-PCRs with PCR products by 1$^{st}$ UR-PCRs
BQCV, IAPV, kSBV, EFB, and Nosema was detected again quantitatively.
AFB was not amplified by nested UR-PCR (Wrong amplification).

# Results

- ## Quantitative detection against Ulsan bee sample.

**Table 8. Calculations of target molecules from UR-PCR and Nested UR-PCR with Ulsan sample.**

| | BQCV | IAPV | kSBV | EFB | Nosema |
|---|---|---|---|---|---|
| **Ct (cycles)** | 36.54 | 8.40 | 42.23 | 32.31 | 38.10 |
| **Tm (℃)** | 76.68 | 77.01 | 76.64 | 85.97 | 80.14 |
| **Regression equation** | y= -3.5015x + 40.17 | y= -3.6649x + 43.995 | y= -3.181x + 39.92 | y= -4.5692x + 48.253 | y= -3.5328x + 39.948 |
| **Calculated Target molecules** | $1.10 \times 10^1$ $= 11$ | $5.13 \times 10^9$ | $5.37 \times 10^0$ $= 5.37$ | $3.09 \times 10^3$ | $3.31 \times 10^0$ $=3.31$ |

# Results

- **Summary of nested UR-PCRs using 8 samples.**

**Table 9. 17 UR-PCR and nested PCR with 8 different samples.**

| | Sample 1 | | Sample 2 | | Sample 3 | | Sample 4 | | Sample 5 | | Sample 6 | | Sample 7 | | Sample 8 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Area** | Sunchang | | Ulsan | | Yongin | | Suwon | | Suwon | | Suwon | | Suwon | | Suwon | |
| **PCR** | 1st | 2nd | 1st | 2nd | 1st | 2nd | 1st | 2nd | 1st | 2nd | 1st | 2nd | 1st | 2nd | 1st | 2nd |
| **ABPV** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **BQCV** | - | - | + | + | + | + | - | - | - | - | - | - | + | + | - | - |
| **CBPV** | - | - | - | - | - | - | - | - | - | - | - | - | + | + | - | - |
| **DWV** | + | + | - | - | + | + | - | - | - | - | + | + | - | - | - | - |
| **IAPV** | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| **kSBV** | + | + | + | + | - | - | + | + | - | - | + | + | + | + | + | + |
| **SBV** | - | - | + | + | - | - | + | + | - | - | - | - | + | + | + | + |
| **SBPV** | - | - | - | - | + | - | + | + | + | - | + | - | + | + | + | + |
| **AFB** | - | - | + | - | + | - | + | + | - | - | - | - | + | - | - | - |
| **EFB** | - | - | + | + | - | - | - | - | - | - | - | - | - | - | - | - |
| **A. apis** | - | - | - | - | - | - | - | - | - | - | - | - | + | + | - | - |
| **A. flavus** | + | + | - | - | + | - | - | - | - | - | + | + | + | + | + | + |
| **Nosema** | - | - | + | + | + | + | - | - | - | - | + | + | + | + | + | + |
| **SHB** | + | + | - | - | - | - | + | - | - | - | - | - | - | - | + | + |
| **V. destructor** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | + |
| **T. mercedesae** | - | - | - | - | + | - | - | - | - | - | + | + | + | + | + | + |
| **A. woodi** | - | - | - | - | - | - | - | - | - | - | + | + | - | - | - | - |

**\* Among 136 specific amplifications by 1st UR-PCRs, 9 were corrected as wrong amplifications by nested UR-PCRs.**

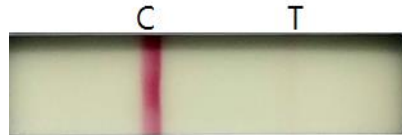# Results : IC-kit against bee-pathogens

- **Immunochromatography against Sacbrood Virus**
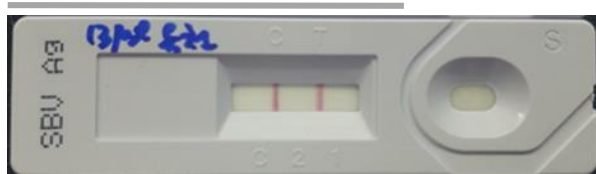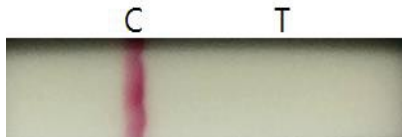


**SBV positive  100%**
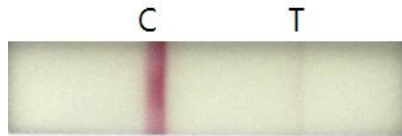
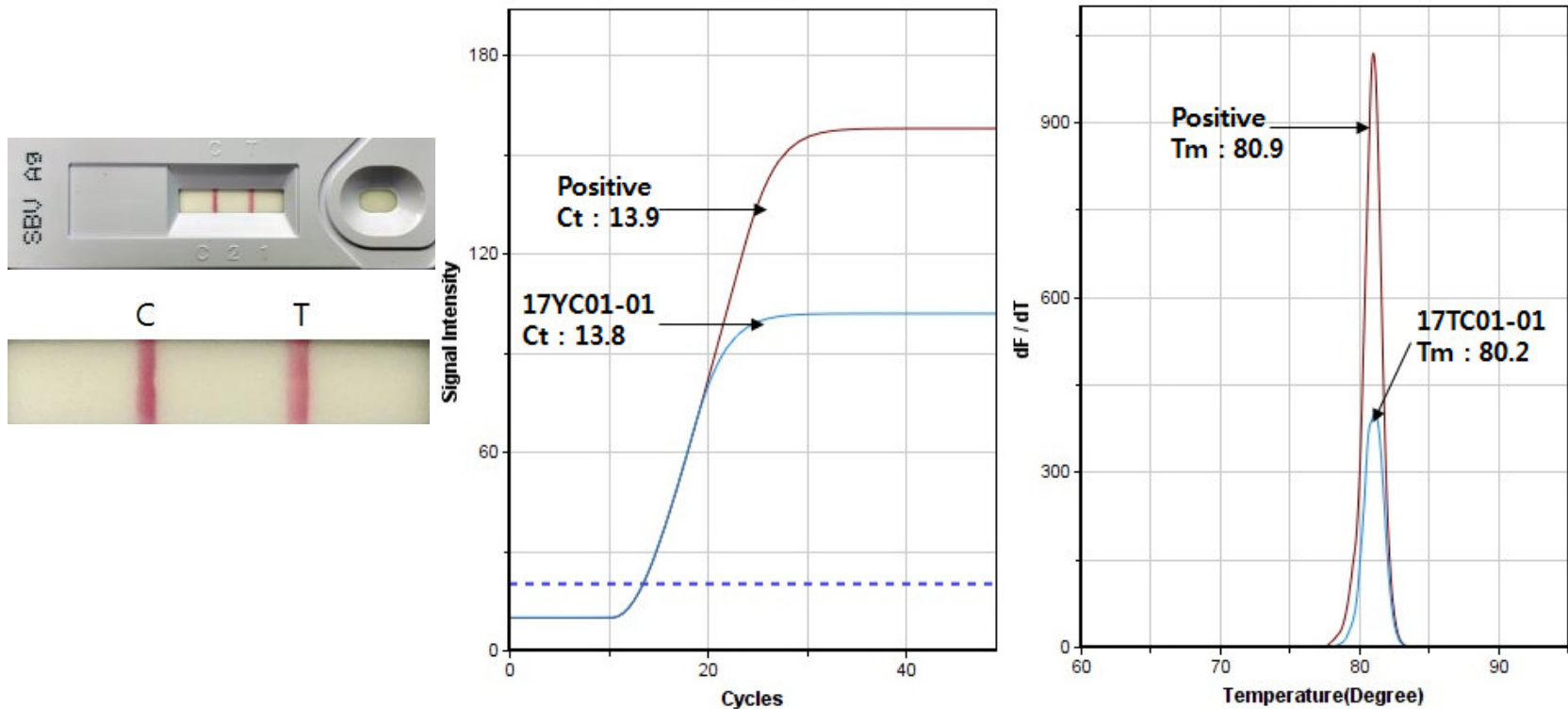**SBV positive      7%**

**SBV positive      1%**

**SBV Negative  100% ?**

**Fig. 11. Positive or negative results using Rapid kit with different samples**
Different larvae samples were used. 1/10 dilution rate, finished on 15 minutes.
No quantitative detection available. No extra apparatus needed.

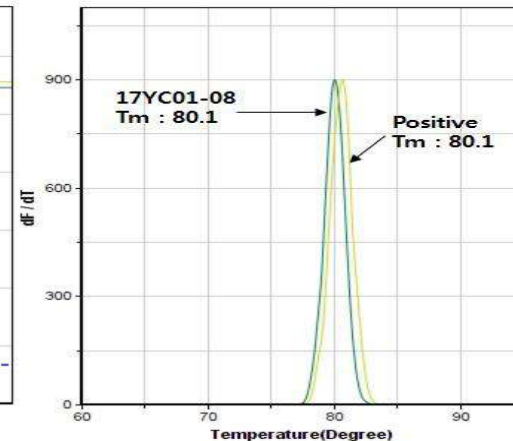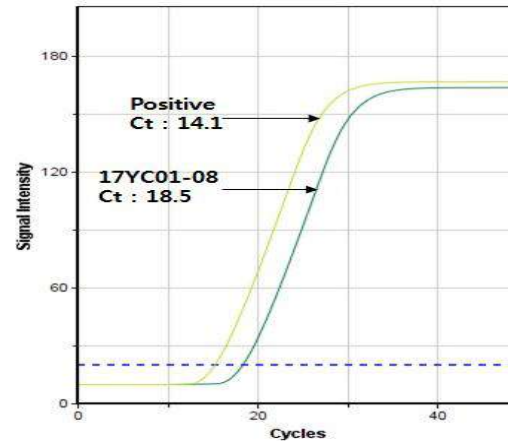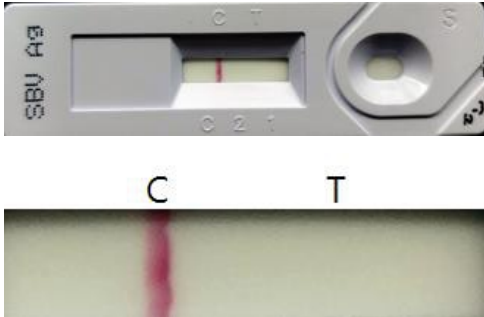# Results

• **Rapid kit** and **Ultra-Rapid PCR** against **Sacbrood Virus**



**Fig. 12. Positive results using Rapid kit and UR-PCR**
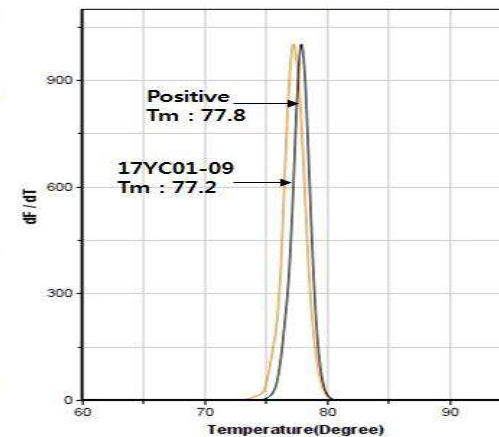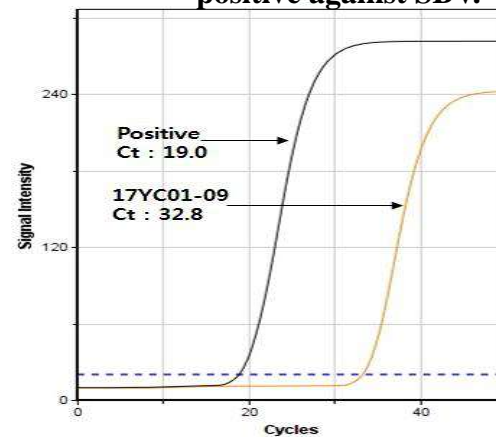Same larvae samples were used for both. Finished in 20 minutes including sample preparation. Both indicate clear positive results against SBV.
Only UR-PCR show quantitative detection by Ct value (13.8 cycles).

# Results

- **Rapid kit** and Ultra-Rapid PCR against **Sacbrood Virus**



**Fig. 13. Positive results using Rapid kit or UR-PCR**

Same larvae samples were used for both. Results of Rapid kit show nearly negative against SBV. However, only results of UR-PCR indicated positive against SBV as quantities (18.5 cycles or 32.8 cycles) and identical Tm (80.1 or 77.2ºC).

# Results

• **Rapid kit** and **Ultra-Rapid PCR** against **Sacbrood Virus**
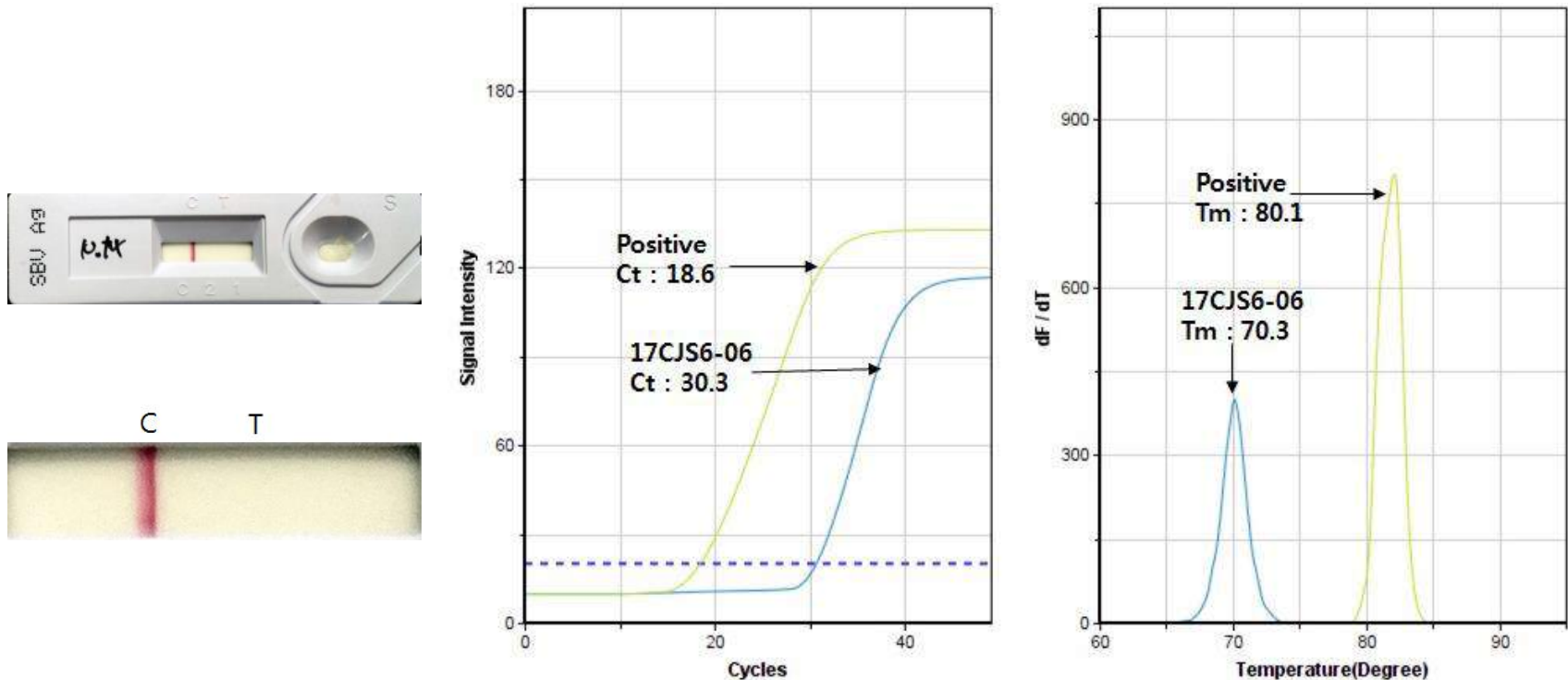


**Fig. 14. Negative result using Rapid kit or UR-PCR**
Same larvae samples were used for both. Results by both indicated clear Negative against SBV. Only UR-PCR show different Tm of PCR-products from Sample or Positive ($70.3^o$C or $80.1^o$C) clearly.

# Summary on development of IC-kit

- **Immunochromatography ( IC-kit or Rapid kit)** against pathogen of honeybee are also **developing now.**

- Especially, against kSBV, SBV, AFB, EFB etc. were already registered or in process in Korea and other lands.

- However, test by IC-kit show **100-1,000 times less sensitive** than UR-PCR now.

# Conclusion :

• **17 Ultra-rapid PCRs (17 UR-PCR) and 17 nested UR-PCR might be one of most sensitive tool to monitor environmental pathogens of honeybee.**

• **Immunochromatography (IC) kit against bee-pathogens are demanded, especially beekeepers, because of easy handling and rapidity.**