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A real-time PCR protocol for quantification of *Nosema ceranae* infection in honeybees

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Abstract

- The protocol is dedicated to detecting and quantifying *Nosema ceranae* infection level in individual honeybees *(Apis mellifera)*.
- It relies on real-time PCR (qPCR) absolute quantification of the pathogen's *Hsp70* gene.
- Because *Hsp70* is present in a single copy per spore, the number of *Hsp70* copies can be directly translated into number of spores.
- The qPCR protocol relies on SYBR Green chemistry.
- The target is a 65 base pairs fragment of the *Hsp70* gene.
- For absolute quantification, each reaction plate includes a standard curve, based on serial dilutions of the amplified broader *Hsp70* fragment than the target fragment (824 bp).
- The method's sensitivity is 800 copies per bee.

Guidelines

This protocol allows to analyse up to 23 samples per plate (when analysed in three technical replicates - recommended) or up to 35 samples per plate (in duplicates).

Protocol materials

Zymoclean™ Gel DNA Recovery Kit **Zymo Research Catalog #**D4001

Qubit dsDNA Broad Range assay kit (500 assays) **Invitrogen - Thermo Fisher Catalog #**Q32853

Troubleshooting

Problem

Standard curve not linear; lowest standard concentrations fall below the line

Solution

Frequent reason: Standard degradation Solution: Prepare fresh standard dilutions. If this does not help, prepare fresh standard.

Problem

Additional peaks in the melting curve; especially in samples with low amount of target and in the NTC samples

Solution

Frequent reason: Primer-dimer formation Solutions: - Dilute the template - Dilute the primers - Increase the annealing temperature

Problem

The difference between technical replicates exceeds 1 Cq

Solution

Frequent reason: Pipetting errors Solution: Increase pipetting precision. Mix the DNA extract thoroughly before adding it on a plate.

1. Sample homogenization

1 **Place**: pre-PCR lab

Equipment:

- Homogenizer: **Omni Bead Ruptor Elite** (for 24 samples)
- Centrifuge
- Gas burner
- Metal spoon
- Aluminium foil
- Entomological forceps
- Small scissors
- Permanent marker
- Pipettes (200 µl and 1000 µl)

Materials and reagents (per sample)

- Vials: Screw cap tubes 2.0 ml, Sarstedt, cat. no. 72.694.006 (1)
- dH_2O (800 µl)
- Beads: Omni International 2.8 mm (“big”) ceramic beads (2)
- Omni International 0.5 mm (“small”) ceramic beads (1 metal spoon)
- 200 µl wide-bore pipette tips (1) - **ART™ Wide Bore Filtered Pipette Tips (Catalog number 2069GPK)**
- 1000 µl pipette tip (1 for all samples)
- 1.5 ml Eppendorf tubes (1)

2 **Homogenization steps**

2.1 Label the vials **on the side.** Anything written on the cap will vanish during homogenization due to friction in the homogenizer.

2.2 Sterilize metal instruments in flame. For molecular analyses, it ***does*** need to be repeated between
samples.

2.3 Gently hold the honeybee by the thorax with entomological forceps, then cut off the abdomen with scissors and place it in the vial.

2.4 Add the following materials to each sample vial:

- 800 µl dH_2O
- 2 big beads
- 1 spoon of small beads

2.5 **Tightly** screw the vial caps and place the samples in the homogenizer. Secure the sample holder (not too tight), secure the locker and close the device. The homogenizer accommodates up to 24 samples.

2.6 Turn on the homogenizer. Before starting the program, set the following parameters:

- Volume: 2 ml
- Speed: 5 m/s
- 2 cycles
- Time: 00:30
- Dwell (break between cycles): 00:10

2.7 Remove the samples from the homogenizer. Vortex each homogenate thoroughly before sampling to prevent *Nosema* spores sedimentation. Immediately after vortexing, take 200 µl of homogenate with a wide-bore pipette tip and transfer to a fresh Eppendorf tube to be used for DNA extraction. If the homogenate is not processed further immediately, freeze until further use, if possible perform the digestion step first. This will help to prevent DNA degradation in the homogenate.

2. DNA extraction

3h 19m

3 **Place:** pre-PCR lab
Method: solution-based Promega kit **Wizard® Genomic DNA Purification Kit (promega.com)** + proteinase K
Equipment:

- Thermoblock - holds up to 24 samples.
- Centrifuge. The largest rotor can accommodate up to 30 samples.
- Vortex mixer
- Pipettes (200 µl and 1000 µl)
- Rack for Eppendorf tubes

Materials and reagents (per sample; storage conditions if not at room temperature):

- 1.5 ml Eppendorf centrifuge tubes (2)
- From Promega kit:
  - o **Nuclei Lysis Solution** (600 µl)
  - o **Protein Precipitation Solution** (200 µl)
- **Proteinase K** 600 U/mL (5 µl; freezer)*
- Isopropanol (600 µl)
- 70% ethanol (600 µl)
- 1xTE buffer (100 µl; refrigerator)
- Pipette tips: 1000 µl (3 per batch), 200 µl (1 per batch)

*Proteinase storage conditions may vary between products; follow the manufacturer's guidelines.

4 **DNA extraction steps**

4.1 Add 600 µL **Nuclei Lysis Solution** to each Eppendorf tube containing the homogenate (prepared according to Section 1: Sample homogenization).

4.2 Add 5 µL **Proteinase K**.

4.3 Vortex.

4.4 Incubate at 55 °C for 02:00:00 . If the thermoblock enables mixing, set at least 500 rpm. If mixing is not available, shake manually every 30 min. 2h

4.5 Cool to room temperature.

4.6 Add 200 µL **Protein Precipitation Solution.**

4.7 Vortex or mix by shaking.

4.8 Put into freezer (-20 °C) for 00:10:00 . Place also the alcohols (ethanol and isopropanol) in the freezer, to be cooled for further steps. 10m

4.9 Centrifuge for 00:04:00 at maximum speed **(** 14000 rpm **).** In the meantime, prepare a new tube for each sample, label it accordingly (on the side and on the top of the tube), and add 600 µL **of cooled isopropanol** to each. 4m

4.10 Transfer the supernatant to the tubes with isopropanol (either by pouring or using a pipette). Discard the original tubes containing the pellet.

4.11 Mix by inverting. It can be done one by one or by covering the rack with samples with another empty rack and inverting 3 times. DNA can be visible as white threads. Sometimes the threads may not be visible, especially if the DNA concentration is low.

4.12 Centrifuge for 00:02:30 at 14000 rpm 2m 30s

4.13 Carefully discard the supernatant by decantation.

4.14 Add 600 µL **of cooled ethanol** and mix by inverting the tubes 2-3 times (as in Step 4.11.)

4.15 Centrifuge for 00:02:30 at 14000 rpm 2m 30s

4.16 Carefully discard the supernatant. DNA should be visible as a white or transparent pellet.

4.17 Let the pellets air-dry for 01:00:00 at room temperature by placing inverted tubes on a paper towel (Figure 2.1.). Be careful not to prolong this step to avoid overdrying. Alternatively, remove any remaining liquid by careful aspiration with a pipette tip and leave the tubes open for 5 min. 1h

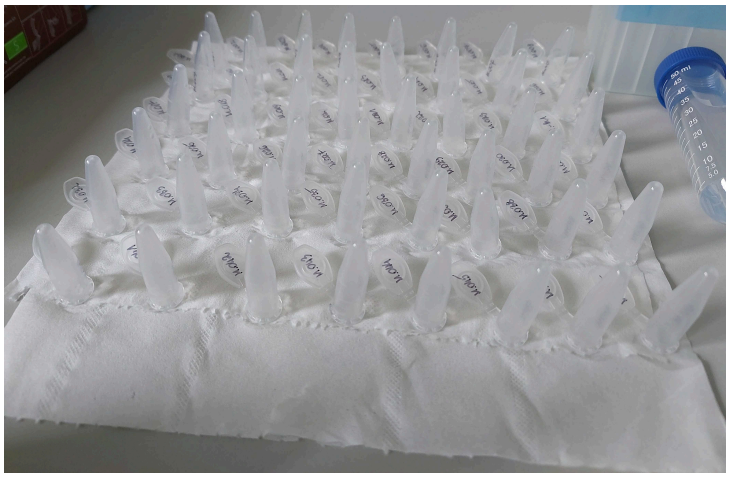


Figure 2.1. Drying the DNA pellets after extraction.

4.18 Add 100 µL **1xTE** and mix with the pellet vigorously, e.g. by dragging the tubes along the rack (to detach the pellet from the tube wall).

4.19 Store in the refrigerator for at least **12 h** (Overnight) before further steps. DNA samples can be kept for weeks to months in the refrigerator, for longer storage aliquot and freeze.

3. NanoDrop

5 **Place:** pre-PCR lab

Equipment:

- NanoDrop
- Pipettes (200 µl and 10 µl)

Materials and reagents:

- DNA extracts
- 200 µl and 10 µl pipette tips
- TE buffer (the same as used to resuspend DNA pellets during extraction)
- ddH_2O

6 **Measurement steps:**

6.1 Clean the NanoDrop pedals with distilled water.

6.2 In the NanoDrop software, select the “Nucleic acid” measurement mode.

6.3 Perform blank measurements with 1 µL ddH_2O and TE buffer as prompted.

6.4 Mix the DNA extracts by pipetting prior to measurement. Measure in 1 µL drops. Record the concentration as well as the 260/280 and 260/230 ratios.

7 **Guidelines**

- Measure samples in duplicates.
- Measurement for concentrations < 10 ng/µl is not accurate with NanoDrop.
- DNA absorption is measured at 260 nm. The 260/280 and 260/230 ratios inform about extract purity:

- The **260/280** ratio informs about protein contamination. Optimal for DNA: **1.7-2.0** (Bio-rad guideline). If the ratio is too low, it indicates high amounts of protein relative to nucleic acid. Protein contamination can inhibit further reactions, e.g. PCR. Solution: increase the amount of proteinase K added in the digestion step, increase the incubation time.

- The **260/230** ratio informs about contamination with salts or other compounds (e.g. EDTA, phenol, guanidine hydrochloride). Optimal value: **>1.5** (Bio-rad guideline). If low, clean the samples using spin columns or re-precipitation, add extra wash steps.

4. Standards preparation for qPCR calibration curve

2m

8 **Purpose:** For absolute quantification in qPCR, serial dilutions of a broader fragment of the *N. ceranae Hsp70* gene than the target fragment are used to generate a standard curve, which is included on each plate alongside the test samples.

Description:

- The reference *Hsp70* sequence used for primer design in **Primer Blast**: was **Nosema ceranae heat shock protein (AAJ76_4900027520), partial mRNA - Nucleotide - NCBI (nih.gov)**, accession no.: XM_024475769.1
- The fragment amplified with the ‘Hsp70-broad’ primers spans positions 216-1039 in the reference sequence (while the target fragment corresponds to positions 498-562 in the reference). Primers were verified *in silico* using **Primer Blast** to exclude off-target amplification in *Nosema ceranae* or *Apis mellifera,* and analyzed in **Multiple Primer Analyzer** for secondary structures and other properties.

- Experimental testing across an annealing temperature gradient of 52–56 °C showed consistent performance, and **56 °C** was selected as the final annealing temperature.

Table 4.1. Primers for 'broad' *Hsp70* fragment (qPCR standard)

	Gene	Primer	Primer sequence (5' - 3')	Tm	Product length (pos. in the reference sequence)
	Hsp70	**Hsp70_broad_F**	TGCGTCTAAGAGATTGCTGGG	56 °C	824 (216-1039)
		Hsp70_broad_R	GCATTCGTGTCATTCCACCC		

9 **1. Stage 1: pre-PCR**

Place: pre-PCR lab

Equipment:
- Thermocycler
- Pipette (200 µl and 10 µl)

Materials and reagents:
- PCR plates/strips
- Pipette tips (200 µl and 10 µl)
- **DreamTaq™ Hot Start PCR Master Mix (thermofisher.com)**
- Primers: Hsp70-broad-F and Hsp70-broad-R (Table 4.1.), [M] 10 micromolar (µM)
- ddH_20
- DNA extract (obtained from bees infected with *Nosema ceranae*)

9.1 **Steps:**

Prepare the PCR reaction in a larger total volume (5×; 125 µL in total) to obtain an excess of product, following the composition in Table 4.2. Place the reactTranion tubes in the thermocycler and run the program specified in Table 4.3.

Table 4.2. PCR mix:

	reagent	1×	5×
	DreamTaq Hot Start PCR Master Mix	12.5 µL	62.5 µl
	Forward primer 10x	1 µl	5 µl
	Reverse primer 10x	1 µl	5 µl
	Template DNA	2 µl	10 µl
	ddH20	8.5 µl	42.5 µl
	Total volume	**25 µL (23 µl mix + 2 µl DNA)**	

Table 4.3. PCR reaction steps:

	step	temperature	step duration	repeats
	initialization	*95 °C*	3 min	
	denaturation	*95 °C*	30 s	40 ×
	annealing	*56 °C (Hsp-broad)*	30 s	
	elongation	*72 °C*	60 s	
	final elongation	*72 °C*	5 min	
	cooling	*8 °C*	∞	

9.2 After the program is finished, transfer the reaction tubes to post-PCR lab.

10 **Stage 2: post-PCR**

Place:
All the remaining standard preparation steps will be performed in the post-PCR lab.
Prepare the following equipment and materials:

Equipment:
- Microwave
- Scale
- Agarose gel electrophoresis system (small)
- Electrophoresis combs (for big wells)
- Gel visualisation system
- Incubator with mixing function
- Pipettes (200 µl and 10 µl)
- Centrifuge
- Qubit fluorometer

Materials and reagents:
- Pipette tips (200 µl and 10 µl)
- Materials for agarose gel electrophoresis: TAE buffer, agarose, MidoriGreen, LoadingDye, Perfect 100-1000 DNA ladder
- Zymoclean™ Gel DNA Recovery Kit **Zymo Research Catalog #**D4001
- 2 ml and 1.5 ml Eppendorf tubes
- Qubit tubes
- Qubit dsDNA Broad Range assay kit (500 assays) **Invitrogen - Thermo Fisher Catalog #**Q32853
- 'Broad' Hsp70 PCR product

11 **Gel electrophoresis of the broad PCR product.**

11.1 Prepare 1.5% agarose gel: buffer: 30 ml, agarose: 0.45 g, MidoriGreen: 1.2 µl

11.2 Distribute the whole PCR volume into two big wells, mixing with concentrated Loading Dye.

11.3 Load 3 µl of DNA ladder to a separate well and run the gel at 70 V for 00:25:00 .

12 **Purifying from gel using columns (ZymoClean Gel DNA Recovery Kit).**
Follow the **manufacturer's protocol** (steps below):

All centrifugation steps should be performed at 10-16,000 g.

12.1 Cut the gel band (Figure 4.1.) and weight it in a 2 ml Eppendorf tube (tare the tube first).

12.2 Add ADB buffer (300 µl per 0.1 g of gel).

12.3 Incubate at 55 °C with mixing for at least 10 min.

12.4 Vortex.

12.5 Transfer the mixture to the columns and centrifuge for 1 min. Discard the flow-through.

12.6 Add 200 µl DNA Wash Buffer.

12.7 Centrifuge for 30 s. Discard the flow-through.

12.8 go to step #12.6 Repeat steps 12.6-12.7.

12.9 Elution: Add water (for sequencing) or ≥6 µl of Elution Buffer (suggested: 15-30 µl). Place the column into a new 1.5 ml Eppendorf tube and centrifuge for 1 min.

If you plan to check the product by Sanger sequencing, use ddH_2O instead of Elution Buffer for the final elution. However, add TE buffer to the remaining stock that will not be used for sequencing, as it improves long-term stability.

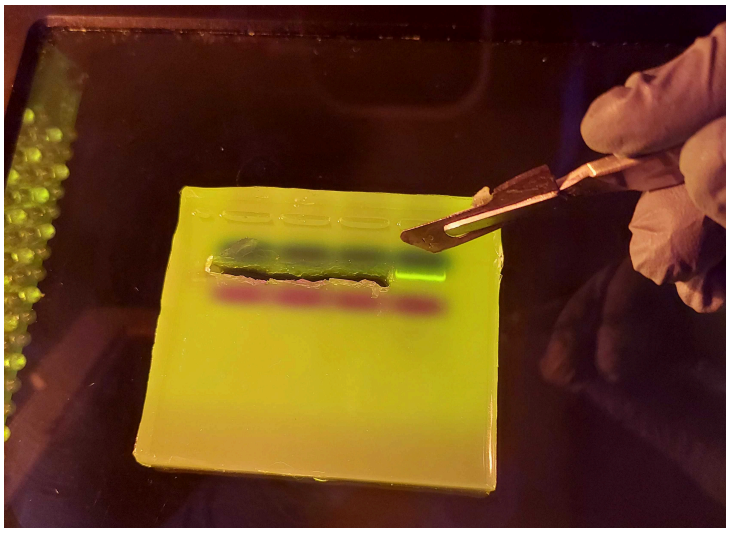


Figure 4.1. PCR product incision from agarose gel.

13 **qPCR standard concentration measurement - Qubit fluorometer**
Follow the manufacturer's **Instruction** for the Qubit BroadRange (BR). Briefly:

13.1 Keep tubes and fthe working mix at room temperature (on the benchtop). Standards should be stored in the refrigerator.

13.2 Allow all the reagents to reach room temperature before use.

13.3 Prepare two standards by adding to the two tubes: 190 µl mix + 10 µl of the appropraite standard solution.

13.4 Prepare the samples by adding to each tube: 198 µl mix + 2 µl sample.

13.5 Vortex and leave for 00:02:00 before measurement. 2m

13.6 Follow the instrument's instructions: measure standards first, then the samples. Set appropriate sample volume and record the DNA concentration in the samples.

13.7 Calculate standard copy number per µl, following **Qiagen** equation:

$$\frac{Xg/\mu l \text{ DNA}}{\text{PCR product length in bp} * 660} * 6.02 * 10^{23} copies/\mu l = Y copies/\mu l$$

14 **qPCR standard dilution series**
Prepare serial dilutions to obtain orders of magnitude from 10^8 to 10^{-1}. The dynamic range of at least 5 concentrations is recommended (Qiagen). **!!!Thorough mixing of the dilutions is essential for the accuracy of the standard curve!!!**

Prepare only the amount of dilutions needed for immediate use (starting with 2 µl of the highest concentration) and keep the dilutions refrigerated. For long-term storage, keep the highest concentration in the freezer.

- 2 µl of 10^{10} + 198 µl ddH_2O → 10^8
- 2 µl of 10^8 + 198 µl ddH_2O → 10^6

- 20 µl of 10^6 + 180 µl ddH_2O → 10^5
- 20 µl of 10^5 + 180 µl ddH_2O → 10^4
- 20 µl of 10^4 + 180 µl ddH_2O → 10^3
- 20 µl of 10^3 + 180 µl ddH_2O → 10^2
- 20 µl of 10^2 + 180 µl ddH_2O → 10^1
- 20 µl of 10^1 + 180 µl ddH_2O → 10^0
- 20 µl of 10^0 + 180 µl ddH_2O → 10^{-1}

5. qPCR

15 **1. Stage 1: pre-PCR**

Place: pre-PCR lab

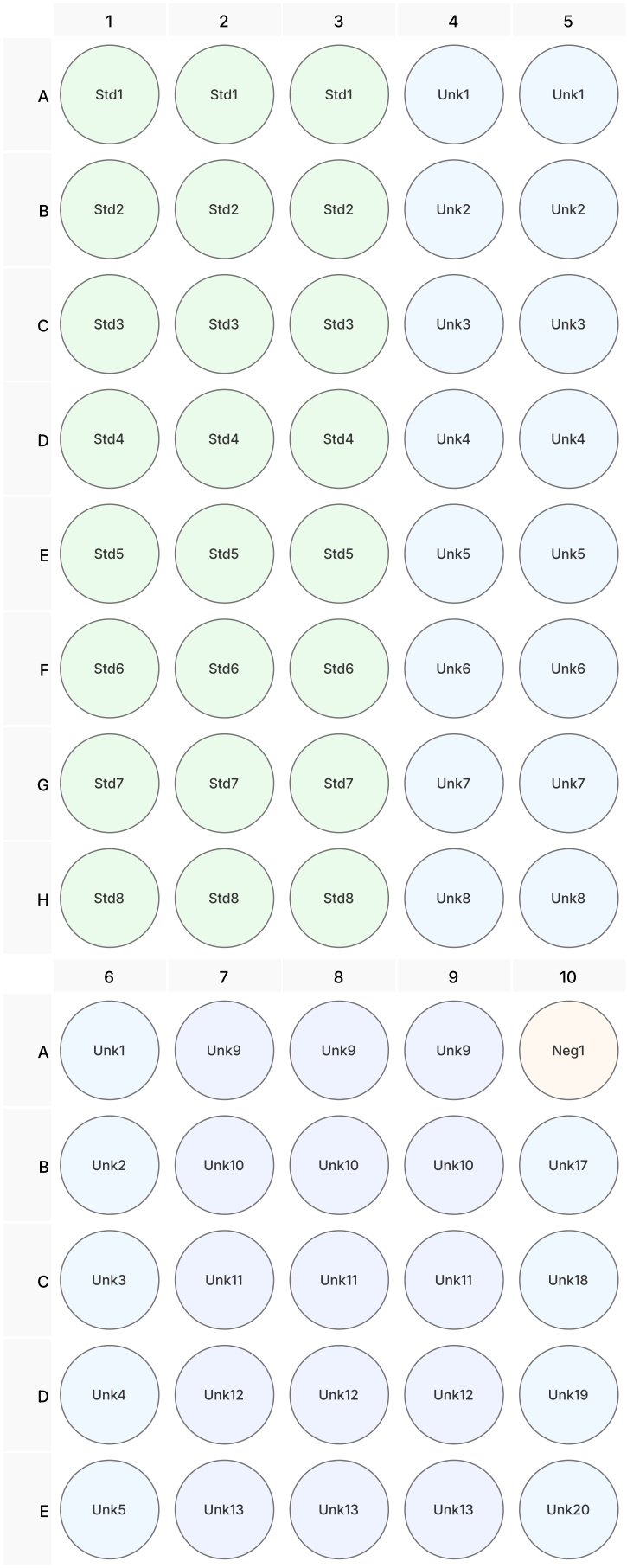
Equipment:
pipettes:
- multi-channel for 20 µl and 5 µl
- 1000 µl
- 100 µl

Materials and reagents:
- qPCR **plates** (BioRad)
- qPCR plate **seal** (BioRad)
- **SYBR Green** (BioRad SsoAdvanced Universal SYBR® Green Supermix)
- Primers: Hsp70_F and Hsp70_R (Table 5.1), 0.2 µM final concentration each (0.8 µl of primers stock diluted 20× to 5 µM)
- ddH_2O
- pipette tips : 1000 µl, 1000 µl, 10 µl
- DNA extracts prepared in Section 2: DNA extraction.
- Box with ice.

Table 5.1 *Hsp70* primer sequences. Reference: Cilia et al. 2018

	Gene	Primer	Primer sequence (5' - 3')	Product length (pos. in the reference sequence)
	Hsp70	Hsp70_F	GGGATTACAAGTGCTTAGAGTGATT	65 bp (498-562)
		Hsp70_R	TGTCAAGCCCATAAGCAAGTG	

15.1 Prepare plate layout, including wells for standards (8 concentrations in 3 replicates each = 24), extraction blank, non-template control (NTC), tested samples in duplicates or triplicates. Example plate layout: Figure 5.1.


1 2 3 4 5
A Std1 Std1 Std1 Unk1 Unk1
B Std2 Std2 Std2 Unk2 Unk2
C Std3 Std3 Std3 Unk3 Unk3
D Std4 Std4 Std4 Unk4 Unk4
E Std5 Std5 Std5 Unk5 Unk5
F Std6 Std6 Std6 Unk6 Unk6
G Std7 Std7 Std7 Unk7 Unk7
H Std8 Std8 Std8 Unk8 Unk8
6 7 8 9 10
A Unk1 Unk9 Unk9 Unk9 Neg1
B Unk2 Unk10 Unk10 Unk10 Unk17
C Unk3 Unk11 Unk11 Unk11 Unk18
D Unk4 Unk12 Unk12 Unk12 Unk19
E Unk5 Unk13 Unk13 Unk13 Unk20

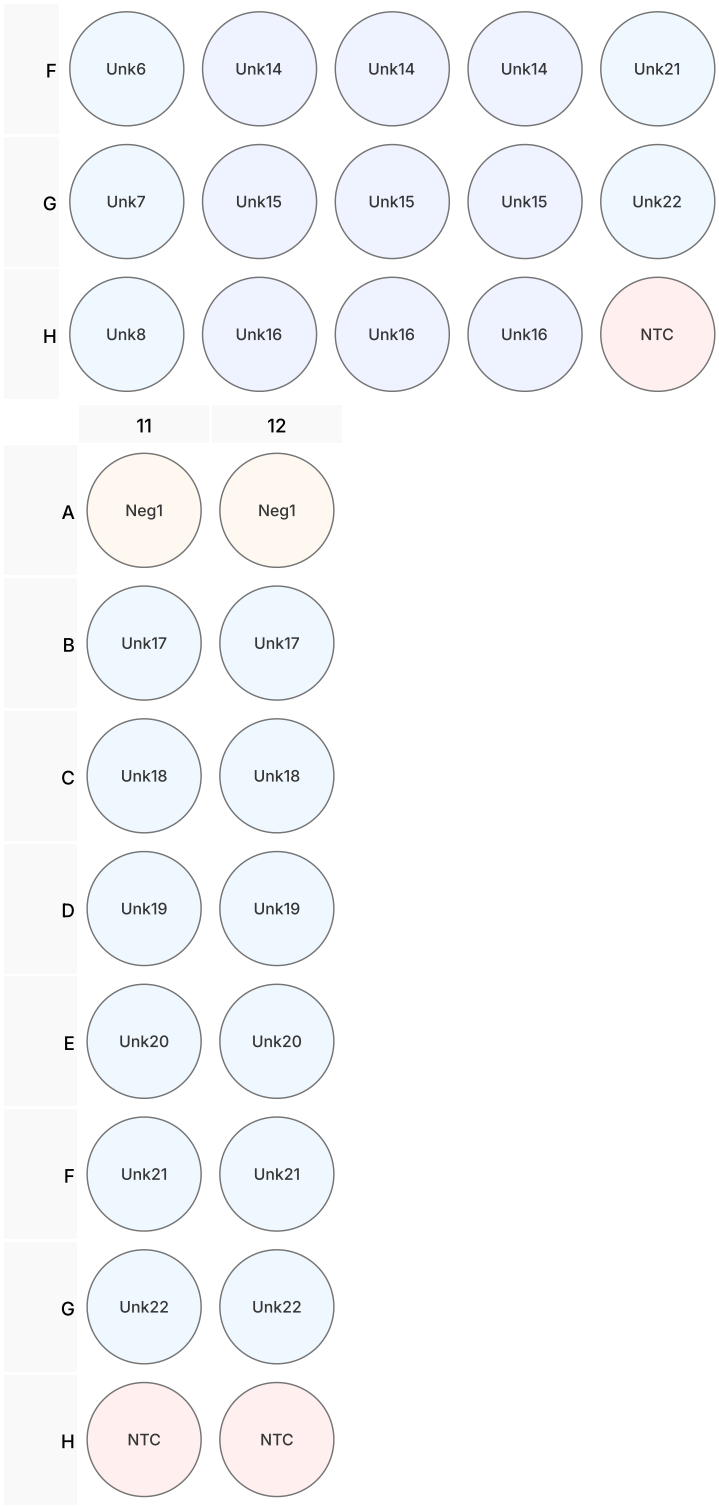


Figure 5.1. Example qPCR plate layout. Std – standards, Unk – tested samples, Neg – extraction blank, NTC – non-template control (qPCR blank)

15.2 Dilute 5 µl of each DNA extract 10× with ddH_2O.

15.3 Calculate the number of wells on the plate that will be used (based on the example in Table 5.2).

Table 5.2. Calculation of the number of wells on the plate and the amount of mix required (example: 7 samples and 7 standards, each in triplicate). The total volume of mix should be prepared with a 15% excess to prevent volume loss due to pipetting.

	Sample category	Number of samples	Number of replicates	Number of samples × number of repliactes	Total numer of well plates	Total + 15%
	Unknown sample	7	3	21	45	52
	Standard	7	3	21		
	Blank	1	3	3		

15.4 Prepare the qPCR mix On ice, according to Table 5.3.

Table 5.3. qPCR mix preparation (for the number of samples calculated in Step 15.3, Table 5.2.).

	Mix (µl)	1×	52×
	SYBR Green	10	520
	Hsp70_F primer 20x	0.8	41.6
	Hsp70_R primer 20x	0.8	41.6
	ddH2O	3.4	176.8

- To each well 15 µl of mix and 5 µl of DNA should be added.

15.5 Distribute 15 µl of reaction mix to each well on the qPCR plate On ice

15.6 Add 5 µl of the diluted DNA extracts to each well in duplicates or triplicates, according to the plate layout.
Mix them well before adding.
Standards will be added later, in post-PCR room.

15.7 Seal the plate (*except for the rows designated for standards*) and move to post-PCR lab.

16 **2. Stage 2 - post-PCR**

Place: post-PCR lab

Equipment:
- Thermocycler: Bio-Rad CFX 96 Touch (CFX96 Touch Real-Time PCR Detection System)
- Computer with BioRad CFX Maestro Software
- Multi-channel pipette for 5 µl
- Centrifuge for PCR plates

Materials and reagents:
- Pipette tips (5 µl)
- Standards (prepared and diluted as in Section 4)
- Reaction plate from previous step.

16.1 Connect the thermocycler to the computer running BioRad CFX Maestro Software. Prepare the plate layout, marking the well categories and replicates. Enter the standards concentrations (in copies/µl, as calculated in Section 4). Program the cycling protocol as shown in Table 5.4.

Table 5.4. qPCR program.

	temperature	duration	repeats
	98 °C	3 min	
	98 °C	15 s	40×
	60 °C	30 s	
	melting curve		
	65-95 °C in 0.5 °C increments	5 s/ step	

16.2 Add standards to the appropriate wells on the qPCR plate, covering the range from 10^6 copies/µl (Std1) to 10^{-1} copies/µl (Std8), in a 10× dilution series, in three replicates.

16.3 Seal the plate and centrifuge shortly.

16.4 Place the plate in the thermocycler and start the program.

16.5 After the protocol is finished, analyse its parameters:

- Melting curve should form a single peak. More peaks indicate possible off-target products of primer-dimers formation.
- The difference between technical replicates should not exceed 1 Cq (Mura et al. 2020).
- PCR efficiency should fall within the range 90-110 (slope -3,58 – (-3.10); **Applied Biosystems Real-Time PCR handbook)**
- Standard curve should be linear (R2 > 0.98; BioRad guidelines).
- Wells with results outside the accepted quality range can be excluded from analysis.
- The desired Cq difference between negative and positive samples should be ≥10.
- No amplification should be detected in the NTC samples.
- If some of the parameters do not meet the desired criteria, refer to the protocol's Troubleshooting section.

16.6 Based on the Mean quantity in the analysed samples, calculated by the software based on a melting curve, calculate the **number of spores per bee:**

N Hsp70 (molecules/µl) × 1 (1 Hsp70 copy per spore) × 100 µl (total DNA extract volume) × 10 (DNA extract dilution) × 4 (as 1/4 of homogenate volume was taken to DNA extraction) = **N Hsp70 (molecules/µl)** × **4000**

Protocol references

Promega extraction kit protocol:
https://pl.promega.com/en/products/nucleic-acid-extraction/genomic-dna/wizard-genomic-dna-purification-kit/?catNum=A1120#protocols

ZymoClean Gel DNA Recovery Kit protocol:
files.zymoresearch.com/protocols/_d4001t_d4001_d4002_d4007_d4008_zymoclean_gel_dna_recovery_kit.pdf

Qubit™ dsDNA BR Assay Kit User Guide:
documents.thermofisher.com/TFS-Assets/LSG/manuals/Qubit_dsDNA_BR_Assay_UG.pdf

AppliedBiosystems Real Time PCR handbook:
ffclrp.usp.br/divulgacao/emu/real_time/manuais/Apostila qPCR-Handbook.pdf#page=6.10

Qiagen: Absolute Quantification qPCR vs Relative Quantification qPCR
Absolute Quantification qPCR | Relative Quantification qPCR

Hsp70 primers source:
Cilia, G. *et al.* (2018) "A novel TaqMan ® assay for Nosema ceranae quantification in honey bee, based on the protein coding gene Hsp70," *European Journal of Protistology*, 63, pp. 44–50. Available at: https://doi.org/10.1016/j.ejop.2018.01.007.

Mura, A. *et al.* (2020) "Propolis Consumption Reduces Nosema ceranae Infection of European Honey Bees (Apis mellifera)," *Insects*, 11(2), p. 124. Available at: https://doi.org/10.3390/insects11020124.

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