# A Novel TaqMan® assay for *Nosema ceranae* quantification in honey bee, based on the protein coding gene *Hsp70*

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## Abstract

*Nosema ceranae* is now a widespread honey bee pathogen with high incidence in apiculture. Rapid and reliable detection and quantification methods are a matter of concern for research community, nowadays mainly relying on the use of biomolecular techniques such as PCR, RT-PCR or HRMA. The aim of this technical paper is to provide a new qPCR assay, based on the highly-conserved protein coding gene *Hsp70*, to detect and quantify the microsporidian *Nosema ceranae* affecting the western honey bee *Apis mellifera.* The validation steps to assess efficiency, sensitivity, specificity and robustness of the assay are described also.

***Keywords*:** *Apis mellifera*; *Hsp70* gene; *Nosema ceranae*; Nosemosis; qPCR; TaqMan® assay

## Introduction

Nosemosis caused by *Nosema apis* (Zander, 1909) has been the only known microsporidiosis of honey bees (Klee et al. 2007; Paxton et al. 2007) until the first evidence of infection by *Nosema ceranae* (Fries et al. 1996) in *Apis ceranae*. Being detected in *Apis mellifera* (Higes et al. 2006), *N. ceranae* is now another widespread causative agent of nosemosis in the western honey bee (Ansari et al. 2017; Chen et al. 2009; Giersch et al. 2009; Higes et al. 2009b; Jack et al. 2016). The consequences of nosemosis are of increasing concern among both beekeepers and scientists, although the actual impact of this exotic parasite on *A. mellifera* is still debated (Botías et al. 2013). Besides, the microsporidium *N. ceranae* was detected in other Hymenoptera like stingless bee (Meliponini) and the social wasp *Polybia scutellaris* (Vespidae) (Porrini et al. 2017) and it may cause emerging infectious diseases (EIDs) in *Bombus* spp. (Brown 2017; Graystock et al. 2014).

In *A. mellifera*, higher pathogenicity was reported at individual level, leading to increased mortality compared to *N. apis* infections (Higes et al. 2007).

In the colony, correlation was found between *N. ceranae* infections and colony collapses (Higes et al. 2008; Martin-Hernandez et al. 2007). However a metagenomic survey of CCD-affected colonies failed to attribute to this microsporidium the role of primary causative agent (Cox-Foster et al. 2007).

The discrimination between the two *Nosema* parasites affecting the honey bees by light microscopy is hardly achievable and results may be highly biased. Thus, the use of biomolecular techniques has been deeply investigated: several PCR methods have been developed so far, most of them based on the amplification of the microsporidium's

multi-copy gene *16s rRNA* (Chen et al. 2008; Forsgren and Fries 2010; Higes et al. 2006; Klee et al. 2007; Martin-Hernandez et al. 2007).

In principle, multi-copy genes are preferred for molecular diagnostics since they allow to design high sensitivity tests, with obvious advantage when the organism is present in a low number of copies. However, in our case, reliability of quantification may be deeply affected by the fact that the *Nosema* genome contains a highly variable number of *16s rRNA* gene copies (Sagastume et al. 2011).

Moreover, the design of PCR primers on a variable gene may obviously affect their capability to anneal to the template influencing the assay sensitivity. This makes the *16s rRNA* gene a suboptimal candidate for both strain genotyping and designing of PCR diagnostic assays.

An end-point PCR assay has been designed on the gene coding for the DNA-dependent *RNA polymerase II largest subunit* (*RPB1*) (Gisder and Genersch 2013), which provides an alternate molecular tool for nosemosis detection.

However, to assess the effect of the parasite both at individual and colony level and to better understand the effect of multiple infections, quantification is pivotal (Fries et al. 2013). In quantitative diagnostics, probe-based assays are to be preferred over dye-based assays due to better specificity (Newby et al. 2003) and amplicon size ranging from 50-150bp should be used to maximize the reaction efficiency.

The aim of this investigation is to provide a reliable qPCR assay based on a highly-conserved region of the *N. ceranae* genome, namely single copy heat-shock protein coding gene *Hsp70* (Gomez-Moracho et al. 2014; Wang et al. 2017).

## Material and Methods

**qPCR assay design**

An in silico assay design was performed by checking the sequences of 23 clones (Table 1) obtained from homogenates of *A. mellifera* workers, naturally infected with *N. ceranae*, from three colonies: 440 Hungary, 1251 Hawaii and 1324 Hawaii (Gomez-Moracho et al. 2014).

Polymorphisms in the annealing zones were investigated with BioEdit software v7.0.4 (Hall 1999) while potential primers and probe interactions were ruled-out by using AutoDimer software (Vallone and Butler 2004) using Temperature = 56 °C and Total score required = 7 as testing condition.

The primers pair and the TaqMan® MGB Probe were designed with Primer Express® Software v2.0 (Applied Biosystems) using AN: XM_002995382.1 as reference sequence (Table 2). The primers used in the study are located on scaffold NW_003314033.1 of the NcBRL01 genome.

**qPCR optimization**

Regardless the use of TaqMan® chemistry, SYBR® has been chosen in order to spot the presence of non-specific amplification through melting curve analysis.

All the reactions were carried out using an AB7300 thermocycler (Applied Biosystems) under the following cycling conditions: initial activation step 95 °C for 10 min, PCR cycling (40 cycles of 95 °C for 15 sec., 56 °C for 60 sec.) with a final dissociation stage for melting curve analysis.

The reaction was performed using 10 µl of SYBR® Green PCR Master Mix (Applied Biosystems), 2 µl of template (IDT Ultramer®, 1,5 * $10^4$ copies of DNA) and a matrix of all possible combinations between forward and reverse primers at the concentrations

of 1000, 500, 250, 125 and 62,5 nM. Nuclease free water was added to adjust the final volume to 20 µl.

Each primer concentration pair had a corresponding well with no template as a control (NTC) to assess false positives.

**qPCR final setup**

All the reactions in the study were performed according to the following conditions: 4 µl of 5x HOT FIREPol® Probe qPCR Mix Plus (ROX) (Solis Biodyne), 2.5 µl of template, 250 nM of forward primer, reverse primer and probe, and a volume of nuclease free water to adjust to 20 µl.

The thermal cycle followed mix manufacturers' specifications with initial activation step 95 °C for 15 min, PCR cycling (40 cycles of 95 °C for 15 sec., 56 °C for 60 sec.).

**Absolute quantification and Inhibition test**

Synthesized oligonucleotide (IDT Ultramer®), having the sequence of the target fragment has been chosen as standard for absolute quantification.

Serial logarithmic dilutions were performed in order to obtain a reasonable range of copies to build the six-point standard curve ($1.88 * 10^6 – 1.88 * 10^1$ copies).

All the solutions were diluted in IDTE buffer and kept at – 80 °C in single use aliquots, in order to avoid multiple thawing.

Possible bias in sample quantification due to the presence of inhibitors in the honey bee extracts has been investigated through the simultaneous amplification of buffer-eluted standards (as used in qPCR reactions) and standards eluted in DNA extracts from a

single honey bee abdomen (previously tested and resulted negative to *N. ceranae* infection).

Each of the six dilutions were tested in triplicates with the qPCR method proposed in this paper, respectively in IDTE buffer and in three different negative matrixes.

The arithmetic means of the three replicates for each dilution were calculated and then the results of each matrix were compared with One-way ANOVA.

**Performance of the assay**

*Efficiency and $R^2$*

These assay parameters were evaluated by running 10 separate plates arranged with the 6 concentrations of the quantification standards ($1.88 * 10^6 – 1.88 * 10^1$ copies) in triplicate, to evaluate also within run repeatability.

The percentage efficiency for every run examined was calculated with the formula:

E [%] = (10^(-1/Slope)-1)*100

*Linear Dynamic Range (LDR), Limit of Detection (LOD), Limit of Quantification (LOQ)*

The regression line to estimate these parameters has been calculated with the results of a plate arranged with quantification standards ($1.88 * 10^6 – 1.88 * 10^2$ copies) in triplicate plus standards with 24, 12 and 6 copies in 20 replicates each.

*Specificity*

The specificity of the reaction has been investigated with 3 different methods:

1) In silico, searching for nearly exact match on primer set with NCBI-BLAST (Basic Local Alignment Search Tool) (Altschul et al. 1990); the amplicon sequence and both primers were tested with BLASTN algorithm using the "somewhat similar sequence" parameter.

2) Melting profile analysis was performed with AB Prism 7300 SDS software v1.4 and consisted in a gradual increase in temperature (1°C/15 s to 95°C) after the end of the amplification cycles, during which changes in fluorescence were monitored.

3) Experimental evaluation was performed versus the other relevant microsporidia of honey bees, *N. apis*; an end-point PCR assay (Martin-Hernandez et al. 2007) was used to confirm the spores as *N. apis*; two DNA extracts from different stocks of Percoll-purified, microscopy counted (8500 and 12000 spores per microliter respectively), spores have been evaluated in triplicate with SYBR chemistry. The reaction was performed using 10 µl of SYBR® Green PCR Master Mix (Applied Biosystems), 2 µl of template, 250 nM of forward primer and reverse primer and nuclease free water to adjust the volume to 20 µl.

*Repeatability between runs (operator effect)*

Quantification standards and PCR mastermix were prepared from three different operators. The operator involved in PCR mastermix preparation always used a quantification standard set prepared by a different operator.

**Testing the Hsp70 gene-based method on realistic samples**

The reliability of the method was also tested on real honey bee samples (N=100). Ten colonies were randomly selected in summer from apiaries located in the area of

Bologna, Italy. Forager worker bees were sampled from each hive entrance and ten random individuals were chosen from each sample for further analysis.

The honey bees were processed individually. Each abdomen (= gastrum) was dissected and homogenized with Tissue Lyser II (Qiagen, Hilden, Germany) for 3 minutes at 30 Hz. DNA was extracted by ZR Tissue & Insect DNA Microprep© (Zymo Research, USA) following the manufacturer's instructions and the extracts were analysed in duplicate with Real Time PCR as previously described.

## Results

### qPCR assay design

Sequence alignment evidenced a highly-conserved region of 65bp (ranging from 498bp to 562pb of the reference sequence) that was used as target for the assay.

Among the oligos proposed by the Primer Express® Software v2.0, the ones falling on polymorphic sites were discarded whereas the ones in the conserved region were tested for potential interactions.

The resulting following oligo pairs when testing oligos for potential interactions (hairpin, homodimer, and heterodimer structures) satisfied the testing conditions.

### qPCR optimization

The lowest Cq was obtained from the couple Forward 1000 nM / Reverse 500 nM followed by the couple Forward 1000 nM / Reverse 1000 nM and the couple Forward 250 nM / Reverse 250 nM. The NTC amplification failed for all of the primer pairs combination.

The couple chosen for the assay is F250/R250 because, despite the slightly higher Cq

(24.3771 vs 24.2632 of the F1000/R500 couple), it showed a considerably higher melting temperature of the amplicon (77.9 vs 77.4 of the F1000/R500 couple).

**Quantification standards and Inhibition test**

The risk in using synthetic oligos as standards lies in the possible presence of inhibitors in the samples extract, capable of distorting quantifications.

To verify this possibility, a comparison between standards eluted in IDTE buffer and eluted in negative samples has been arranged.

The statistical test (One-way ANOVA: $F_{3,20} = 0.007601$, $p = 0.999$) indicates no significant differences between the matrixes (Figure 1).

Although it is not possible to exclude the presence of inhibitors in every sample without testing them, our data suggest that there are no endogenous inhibitors for the assay in the honey bee abdomen extracts.

**Performance of the assay**

*Efficiency and $R^2$*

The mean value obtained for all the plates with the relative confidence interval is E [%] = 97.5 ± 2.1 %, very close to the ideal value of 100 %.

The mean $R^2$ value was 0.998 ± 0.002.

*LDR, LOD, LOQ*

The LDR covers 6 $\log_{10}$ with a maximum CI of 0.5.

The standard deviation was ≤ 0.250, which is compatible with the possibility to discriminate between a 2-fold dilution in more than 95% of cases for 100% efficient reactions.

The least concentrated standard with a SD ≤0.250 was selected as LOQ (188 copies).

The LOD, intended as the last dilution showing 100% response, was close to 6 copies (Figure 2).

*Specificity*

1) In silico BLAST search returned the following results:

- The whole amplicon sequence returned a BLAST Max score of 118 with 100% query coverage (E-value 8e-24). No other genes showed 100% coverage except for *N. ceranae* Hsp70. Second best match had a max score of 64.4 and 96% coverage (E-value 1e-07).
- The Forward primer best matches against *N. ceranae Hsp70* gene with 100% query coverage (E-value 5e-04); following matches were with organisms not likely to be present in honey bee extract and returned query coverages lower than 76% (E-value lower than 1.9).
- The Reverse primer best matches with *N. ceranae Hsp70* gene with 100% query coverage (E-value 4.8e-02); following matches were with organisms not likely to be present in honey bee extract and (E-value lower than 0.75); the only ubiquitous bacteria (thus possibly present in bee extract) scored was *Bacillus subtilis* (AN: CP015004.1): query coverage 76%; E-value 46.

2) Melting profile analysis confirmed the presence of only one amplicon, melting at 77.7 ± 0.6°C.

3) Experimental evaluation of two different DNA extracts from *N. apis* spores were amplified in triplicates and revealed no amplification.

*Repeatability between runs (operator effect)*

Ct data from 10 runs for each of the three operators involved were analysed. Each of the six dilution points were independently tested and returned non-significant differences for LS-means value (Ct[10^6], P=0.40; Ct[10^5], P=0.35; Ct[10^4], P=0.44; Ct[10^3], P=0.5; Ct[10^2], P=0.17; Ct[10^1], P=0.53).

The complete Ct data set was tested, returning non-significant overall differenced for LS-means value as well (Ct[means], P=0.94). No evidence was attained that the assay results may be significantly affected by the operators.

**Testing the Hsp70 gene-based method on realistic samples**

Amplification of the *Hsp70* gene was achieved in 95 out of 100 honey bee samples (Table 3), with a number of *N. ceranae* copies covering all the orders of magnitude in the range log 10^0 to log 10^6 with respectively 4, 40, 31, 7, 2, 8 and 3 samples.

More precisely, the number of *N. ceranae* copies found with qPCR analysis in the positive foragers ranged from 7 to 1,555,570 and averaged 93,318.7 (± 29,903.0 s.e.) per individual honey bee with a standard deviation of 291,458.7.

## Discussion

The novel qPCR assay exploits the functionality of the TaqMan® probe. It relies on the exonuclease activity of the polymerase, which is best at a lower temperature than the probe's annealing temperature. This also reduces enzyme processivity, hence the need

of small amplicons to maintain high reaction efficiency. The amplicon size of 65bp chosen in our case fits the recommended range of 50-150 bp. Moreover, a shorter amplicon size implies lower sensitivity to sample degradation. It is also convenient to keep the probe short to makes it more likely that a single mismatch with the template prevents annealing and, consequently, a fluorescent signal. However, such a small probe (12bp) needs the use of the MGB moiety attached to the quencher molecule to raise the melting temperature (Kutyavin et al. 2000).

The assay proved to be specific and sensitive, the LOD is very low and the LOQ is far below the identified $ID_{100}$ for the parasite (10^4 spores) (Forsgren and Fries 2010), which allows to discriminate between infected and diseased bees. A reliable quantification of the parasite load is an essential tool to increase knowledge towards this elusive enemy. A recent study (Graystock et al., 2014) showed considerable pathogen spill over between honey bees and bumblebees. It is not surprising that closely related species are susceptible to the same parasite, but only a quantitative approach can disclose the degree of susceptibility (and consequently of damage) of each involved species. Another field that could take advantage of this quantitative molecular approach is the study of genetic tolerance towards *N. ceranae* (Huang et al. 2014) where high accuracy in spore quantification is required.

The analysis of samples collected from colonies in the field confirmed that the method allows to detect *N. ceranae* infections far below the $ID_{50}$ (85 spores) (Forsgren and Fries 2010) that are likely to correspond to subclinical conditions, and corroborated both LOD and LOQ that were calculated in the previous stages of setup.

This novel *Hsp70* gene-based qPCR detection and quantification assay relies on a sequence that is highly conserved (Gomez-Moracho et al. 2014; Wang et al. 2017) and

not reported as subjected to intraspecific genetic variability. It provides reliable and sensitive assay to overt and subclinical *N. ceranae* infections in honey bees. Cross reaction with *Nosema apis* was not detected but, at the moment, this statement cannot be generalised for other *Nosema* species that may come into contact with honey bees. Indeed, sequences for the *Hsp70* gene of *Nosema neumanni*, a new species recently identified only in Ugandan honey bees (Chemurot et al. 2017), and of other closely related species like *Nosema bombi* are not yet available. Further investigation is needed in this respect.

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