How to Improve the Reliability of qPCR Detection with Target Gene-optimized Internal Standards

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Achieving robust qPCR results requires both sensitive and accurate detection of target genes. This is especially challenging when working with field samples because the absence of a detection signal does not definitively indicate the absence of the target. Inhibitors present in the sample can mask the signal, leading to false-negative results (i.e., no signal even when the target is present).

A common strategy to mitigate inhibitor effects is to perform qPCR with diluted samples. The expectation is that the inhibitor's impact will decrease disproportionately compared to the target DNA. However, the optimal dilution is often unknown, and multiple trials may be necessary. This approach can be problematic for target genes already present at low levels, as excessive dilution might render them undetectable.

An alternative strategy to assess the impact of potential inhibitors involves using internal standards. These standards are either added at the beginning of sample preparation or are endogenous genes already present in the sample. By monitoring the internal control signal alongside the target gene, it is possible to distinguish between a sample with no target and one with a low-abundance target in the presence of inhibitors. An absence of the internal control signal suggests potential issues with the sample or analysis, rendering the reaction inconclusive. In such cases, the sample should be re-examined to ensure accurate quantification and to minimize the risk of misleading results.

However, the effectiveness of internal controls hinges on the assumption that inhibitors will impact the amplification reactions of both the target and internal standard to the same extent. This assumption has been challenged by research conducted by Huggett et al. [Huggett JF, et al., 2008], which demonstrated that inhibitors can differentially affect the amplification of different genes. This finding undermines the reliability of internal standards as a universal solution for mitigating inhibitor effects in qPCR.

A possible solution to this challenge is adding an internal standard that closely resembles the target gene to each sample. These target-specific internal standards match the target in length, nucleotide composition, and melting temperature. To ensure comparable primer annealing, DNA polymerase binding, and incorporation of the initial nucleotides, the primer regions of the target region are used for the standard. To prevent the standard from outcompeting low-abundance targets, the amount of added internal standard must be carefully calibrated. It should be low enough to avoid interfering with target detection but high enough to ensure its consistent detection.

In the following protocol, we showcase the use of a target-gene optimized internal standard. The goal of the protocol is to use the internal standard to minimize the number of false negatives. In our case, the HLB-infected trees would be misclassified as healthy by qPCR. To discern between a true qPCR-negative,

i.e., a healthy plant, from a false qPCR-negative, i.e., a sick plant, we designed the following synthetic DNA :

TCGAGCGCGTATGCGAATACGAGCGATAAGTTGTGGATCAAGAAGTACGAATCTACCTTTTTCTACGGGATAACGC

This synthetic DNA has the same length and similar composition as the CLas 16S rDNA amplicon used for the detection of CLas. The synthetic DNA is composed of a central region containing a portion of the coat protein gene from citrus leaf blotch virus (underlined), that is flanked by segments of the 16S rDNA from CLas. This design enables the synthetic DNA to be amplified during qPCR with the same primers used for CLas 16S rDNA detection (highlighted in yellow and cyan). However, the unique coat protein sequence within the chimera's amplicon allows for it to be distinguished from genuine CLas amplicon through a sequence-specific hydrolysis probe (highlighted in red).

Since the detection of the internal standard competes with the CLas 16S rDNA amplicon, we aim to add as little as possible of the standard to each sample, yet enough to ensure that only one in a billion reactions will fail to detect the standard by chance. This means we aim to add on average 21 molecules of internal standard to each qPCR reaction[1,2].

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[1] Assuming that adding the internal standard follows a Poisson process, then the probability (P) for k molecules of internal standard per qPCR reaction is:

$$P\{X = k\} = \frac{\lambda^k}{k!}e^{-\lambda}$$

with λ being the average number of molecules of internal standard per qPCR reaction. Therefore, the probability for a qPCR reaction having no internal standard, i.e., k=0, equals:

$P\{k = 0\} = \frac{\lambda^0}{0!}e^{-\lambda}$ which simplifies to $P\{k = 0\} = e^{-\lambda}$

Therefore, the average number of molecules of internal standard present in each qPCR is:

$$\lambda = -\ln\left(P\{k = 0\}\right)$$

For a probability, one negative signal in a billion reactions is:

$$\lambda = -\ln(10^{-9}) = 20.7$$

[2] Table of the probability of a negative signal ($P\{k = 0\}$) for a given average number of molecules of the internal standard ($\bar{x}$)

$P\{k = 0\}$	$\bar{x}$
1 in 10^9	20.7
1 in 10^8	18.4
1 in 10^7	16.1
1 in 10^6	13.8
1 in 10^5	11.5
1 in 10^4	9.2
1 in 10^3	6.9
1 in 10^2	4.6
1 in 10	2.3

The main challenge is to prepare a stock solution that allows the reliable addition of 21 molecules per reaction. In our qPCR detection for HLB, the maximum volume of internal standard that can be added is 3.8 µL per 20 µL qPCR reaction (see "qPCR detection assay for HLB" protocol below). This means we need a working stock solution of 5.5 molecules/µL. To generate a stock of reliable concentration, we prepared an intermediate stock of ~550 molecules/µL and determined its actual concentration using digital droplet PCR (ddPCR). Based on the determined concentration, the final working stock solution of internal standard is prepared.

Determining the concentration of intermediate stock of internal standard by ddPCR

For converting your qPCR assays into ddPCR assays see Maar D. et al. [Maar D., et al., 2020].

Materials:

TE buffer, i.e., 10 mM Tris-HCl with 1 mM EDTA at pH 8.0

Bio-Rad plate heat seal foil (Bio-Rad 1814040)

Bio-Rad 96-well plates (Bio-Rad 12001925)

Bio-Rad PX1 PCR plate sealer

Bio-Rad QX200 digital droplet generator

Bio-Rad C1000 thermal cycler with 96 deep well reaction module

Bio-Rad QX200 digital droplet reader

Reagents:

Bio-Rad droplet generation oil for probes (10 vials of 7 mL each; Bio-Rad Cat# 1863005)

ddPCR buffer control kit for probes (2 vials of 4.5 mL each; Bio-Rad Cat# 1863052)

ddPCR Supermix for probes (no dUTP; 200 rxns; Bio-Rad Cat# 1863023)

Primers:

HLBas2 FWD: TCGAGCGCGTATGCAATACG
HLB RVR: CTACCTTTTTCTACGGGATAACGC

Hydrolysis probe labeled 5' with FAM fluorophore and 3' with MGBNFQ:

AAGTTGTGGATCAAGAAG (Thermo Fisher Scientific # 4316034)

Nuclease-free water

Methods:

Preparation of intermediate stock

The intermediate stock of internal standard is 100x more concentrated than the final working stock solution. On average, our intermediate stock has 550 molecules of internal standard/µL or $550x10^{6}$ molecules/L. For our chimera this works out to be 9.2×10^{-16} moles/liter.

First, the lyophilized stock provided by the manufacturer (IDT, San Diego, CA), is resuspended with TE buffer to an approximate concentration of 25 nM. This stock is diluted with TE buffer to 9.2 x 10^{-16} M.

ddPCR of intermediate stock

Allow all reagents to come to room temperature.

Prepare ddPCR master mix by adding:

Component	**Volume per rxn / [µL]**	**Final concentration**
2x ddPCR supermix for probes (No dUTP)	11 µL	1x
Primer/probe	1.1 µL	900 nM (for primers and probe)
Sample	5.5 µL	250 nM
Nuclease-free water	4.4 µL	-
Total volume	**22 µL**	**-**

Mix ddPCR master mix thoroughly, by vortexing for 20 seconds.

Generate droplets with Bio-Rad QX200 digital droplet generator following the manufacturer's instructions.

Transfer the droplets to the 96 well plate within 5 minutes of generation. Transfer the droplets into the 96 well plate. Aspirate and dispense the droplets slowly, each step should last at least 7 seconds.

Heat seal the 96 well plate with pierceable heat seal foil.

Within 40 minutes, load plate into thermal cycler and start thermal cycling program.

For the Bio Rad system, we used a C1000 thermocycler. Any deep well thermocycler will work.

At the end of the cycling program, remove plate from cycler. Store plate at 4°C for up to 3 days.

Let plate come to room temperature before reading using the Bio-Rad QX200 digital droplet reader. Each column takes 11 minutes to read.

After all the columns of the 96-well plate have been read, the results for each well can be viewed under "Data table". The table includes the concentration, proportion of positive/total droplets, as well as the standard error (see table below).

Molarity	Conc (copies/µL)	upper theo. Conc.	lower theo. Conc.	Poisson Conf Max	Poisson Conf Min
9.13E-20	0			0.246	0
9.13E-18	0.239	0.106	0.0732	0.633	0.0567
9.13E-16	8.97	8.61872	8.653877	10.6	7.32
2.5E-14	227	277.1978	265.6593	236	219
9.13E-14	988			1009	967
2.5E-11	1000000	oversaturated	oversaturated	N/A	N/A

Preparation of the final working stock of internal standard

Materials:

TE buffer, i.e., 10 mM Tris-HCl with 1 mM EDTA at pH 8.0

Calibrated intermediate stock of internal standard
(For calibration see above, "Determining the concentration of intermediate stock of internal standard by ddPCR" protocol.)

Certified volumetric pipettes and flasks

Method:

Following ISO 4787.2010 and ASTM E969-02 norms [Anonymous 2010; Anonymous 2012], the intermediate stock of internal standard was diluted to 5.5 molecules/µL. Then, single use aliquots of the internal standard were made in PCR tubes and stored at -20C.

qPCR detection assay for HLB

To reduce the variability of pipetting, a large volume of sample should be added to each qPCR reaction. Here, we diluted the sample by 5x and added 5 µL of this diluted sample instead adding 1 µL of concentrated sample to each reaction.

Material:

iTaq Universal Probes Supermix (Bio-Rad cat# 1725130)

Primers:

HLBas2 FWD: CGAGCGCGTATGCAATACG
HLB RVR: CTACCTTTTTCTACGGGATAACGC

Hydrolysis probe labeled 5' with VIC fluorophore and 3' with MGBNFQ:

AGACGGGTGAGTAACG (Thermo Fisher Scientific # 4316034)

Both the primers and the hydrolysis probe are stored as 100 µM stocks and titrated to 10 µM working stock concentrations using water as the solvent.

Prepare the master mix for qPCR reaction:

Reagents	1x rxn	___ x rxn
Water / internal standard (5.5 molec./µL)	3.8 µL	
RVR primer	0.48 µL	
FWD primer	0.48 µL	
Hydrolysis probe	0.24 µL	
2x MM iTaq Universal Probes Supermix	10 µL	
5x diluted sample	5 µL	--
Total volume	**20 µL**	

Fill each well of the PCR plate with reaction mix with 16.2 uL of qPCR reaction and 3.8 uL of the internal standard. Avoid introducing air bubbles into solution.

Add 5 µL of 5x diluted sample to each well except for non-template control (NTC). For NTC add 5 µL water.

Seal plate and spin all solutions down to the bottom of well.

Load qPCR machine and run the following thermal cycle program:

95°C	5 minutes	1 cycle
95°C	10 seconds	Repeat for
58°C	30 seconds	39-49 cycles

Reference:

Anonymous (2010) ISO 4787.2010 Laboratory Glassware – Volumetric Instruments – Methods for Testing of Capacity and for Use

Anonymous (2012) ASTM E969-02: Standard Specification for Glass Volumetric (Transfer) Pipets

Anonymous (2022) ASTM E542-22: Standard Practice for Gravimetric Calibration of Laboratory Volumetric Instruments

Maar D., et al. (2020) Transitioning Your Assay from Quantitative PCR to Droplet Digital PCR. Bio-Rad bulletin 7320.

Huggett J.F., et al.(2008) Differential susceptibility of PCR reactions to inhibitors: an important and unrecognised phenomenon. BMC Res Notes, 1, 70. PMID: 18755023. doi: 10.1186/1756-0500-1-70.