Ministry for Primary Industries
Manatū Ahu Matua

Multiplex qPCR for detection of *Leptospermum scoparium* DNA from pollen in honey

Molecular Laboratory Method

MPI Technical – Paper No: 2017/31

ISBN No: 978-1-77665-435-9 (online)
ISSN No: 2253-3923 (online)

July 2017

New Zealand Government

Growing and Protecting New Zealand

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1 Introduction

1.1 BACKGROUND

This method was developed and validated by dnature diagnostics & research Ltd and Scion Research as part of the Ministry for Primary Industries Mānuka Honey Science programme. The assay is one of the key tests that is used to verify authenticity of monofloral and multifloral mānuka honey.

The ManKan™ honey test is a multiplex quantitative polymerase chain reaction (qPCR or real time PCR) test designed for the specific detection, differentiation and quantification of mānuka (*L. scoparium*) DNA from other plant species. Following DNA extraction, it can be used to identify DNA from mānuka pollen in honey. Note: the assay is also able to separately detect DNA from kānuka (Kan) pollen but this is not part of the identification criteria for mānuka honey.

To evaluate the specificity of the "Man" primers and probes, a variety of plant species taxonomically identified were included in the assessment. Over 130 plant specimens representing 36 plant species were tested. From the 70+ plant specimens representing 9 different *Leptospermum* species, results show the mānuka component of the assay to be highly specific to *L. scoparium* as it does not detect other species such as *L. polygalifolium* (jellybush), *L. laevigatum, L. grandifolium, L. lanigerum* or *L. petersonii*. *Leptospermum scoparium* samples from a number of different regions in New Zealand were also tested. The assays analytical specificities was also assessed against other plant species (e.g. *Kunzea* species, clover, pohutukawa) associated with honey production in New Zealand (~22 different species tested) with no cross reaction observed. Specimens used for specificity testing are archived at the National Forestry Herbarium hosted by Scion Research.

As part of validation and assay application, this assay has been tested on over 800 honey samples from various floral sources and geographic locations. Samples were sourced from two New Zealand flowering seasons, New Zealand industry archives (up to 5 years) and non-New Zealand sourced honeys (e.g. Australia, China, Africa, USA, Europe).

1.2 SUMMARY OF PROCEDURE

DNA is extracted from pollen present in honey and then tested using a multiplex qPCR assay (ManKan™ honey test) to detect the levels of mānuka (*Leptospermum scoparium*) DNA. Note: qPCR requires facilities that enable separation of key work flow areas to minimise the likelihood of environmental and cross-contamination. Typically this would involve physically separating the laboratory areas used for sample preparation, DNA extraction, preparation of PCR reagents, addition of DNA and DNA amplification. Best practice qPCR guidelines also require that unidirectional workflow is adopted and that there are dedicated equipment in each area plus robust decontamination procedures in place.

1.3 APPLICABILITY

This method is suitable for the quantification of DNA from pollen in honeys of various floral types for the plant species and levels listed in Table 1.

Table 1: DNA from pollen of target plant species

Plant name	**Limit of Reporting[#] (Cq)**	**Linear Range* (Cq)**
Leptospermum scoparium	35.80	35.80 - 11.50

[#]For operational application this was established as being less than or equal to Cq 36. This was determined to be approximately the equivalent of 3 fg/µL following standard curve validation and repeatability testing.

*Note: The linear range validated for this assay exceeds the Cq range found from testing more than 800 honey samples.

2 Equipment

Note: Equivalent equipment may be substituted.

a. Balance top-loading ± 0.01 g
b. Beadbeater (e.g. BioSpec Mini Beadbeater)
c. Drybath/hotblock for 1.5/2 mL tubes (at 65°C) or thermomixer
d. Vortex mixer
e. Centrifuge
f. qPCR instrument capable of detecting the required 2 fluorescent channels
    - Note: if the assay is to be used to also detect kānuka DNA, 3 fluorescent channels are needed.
g. Adjustable calibrated pipettes (2-20 µL, 20-200 µL and 1 mL)

3 Reagents and Consumables

3.1 DNA EXTRACTION

a. 2 mL and 1.5 mL flip top microcentrifuge tubes (e.g. Axygen)
b. 1.5 mL flip top low-binding microcentrifuge tubes (e.g. maxymum recovery (Axygen), Lo-Bind (Eppendorf) or similar)
c. disposable sterile sticks
d. PCR-grade water
e. DNA extraction kit e.g. dnature Genomic DNA Honey Kit
f. > 96% ethanol
g. 0.5 mm zirconia/silica beads
h. 2.3 mm zirconia/silica beads
i. 2 mL screwcap beadbeating tubes (containing O-ring)

3.2 qPCR

a. ManKan™ Honey real time PCR kit (stored at -20°C), which is currently available through dnature diagnostics & research Ltd (www.dnature.co.nz) comprising:

- 5X Mastermix*
- 20X Oligo Mix* (contains primers and probes)
- PCR-grade water

b. 1.5 mL flip top microcentrifuge tubes (e.g. Axygen)
c. qPCR plates/tubes/seals as required for specific brand of qPCR instrument

*Can be stored at 4°C for a period of up to one month without any loss of performance.

Note: Equivalent qPCR reagents may not be substituted

4 Standard(s)

The standard (ManKan™ positive control) is currently available through dnature diagnostics & research Ltd and is supplied with the ManKan™ Honey DNA real time PCR kit in a separate package. The accompanying QC data sheet provides information on performance and shelf life.

Note: Equivalent standards may not be substituted

5 Sample Preparation

5.1 SAMPLE RECEIPT, HOMOGENISATION AND STORAGE

Upon receipt of honey samples in the laboratory, samples should be appropriately labelled. Samples should be brought to room temperature and homogenised using a disposable sterile stick. As pollen can form clumps in the honey, it is very important that the honey is vigorously mixed so that it softens and has a uniform appearance (no granulation or air bubbles). Once sample is thoroughly mixed, sub-sample the required amount.

It is recommended that the remainder of the sample be stored at 4°C until testing is complete and valid results are obtained. If long term storage is needed, -20°C is recommended.

6 Analytical Procedure

6.1 DNA EXTRACTION

The protocol was developed and validated for the extraction of DNA from ~1.4 g (1 mL) of honey using the dnature Genomic DNA Honey Kit. Refer to manufacturers' instructions. Extensive optimisation was performed to maximise DNA recoveries from honey. Other methods (including high throughput and automation) can be used but must undergo appropriate laboratory validation and demonstration of equivalency.

6.2 qPCR ASSAY

The ManKan™ qPCR requires instruments capable of detecting and resolving the following fluorescent channels:

- FAM – mānuka – *L. scoparium*
- ROX/Texas Red/CAL Fluor Red 610 – Internal Control

The protocol has been developed and validated using 10 µL volumes. It is recommended that qPCR reactions are performed with technical replicates whilst establishing laboratory proficiency. Once proficiency is established, appropriate technical replicates should be included within each run.

Note: Instruments able to use these volumes include the Eco qPCR system (Illumina), CFX (Bio-Rad), Mic qPCR system (BioMolecular Systems) and QuantStudio (ThermoFisher). However, machines requiring ROX normalisation should not be used, due to internal control interference.

Other instruments may require larger reaction volumes and 20-25 µL reaction volumes are possible (using 2-5 µL extracted DNA).

Before starting

- Any frozen reagents should be left at room temperature to thaw (protected from light) for up to 1 hour.
- Working reagents (both 20X oligo mix and 5X mastermix) may be stored at 4°C for up to 1 month. Avoid freeze/thawing reagents where possible.
- Briefly vortex and pulse centrifuge both the 20X Oligo mix and the 5X mastermix once fully thawed to ensure even mixing.
- The qPCR instrument should be turned on ahead of time to allow any warmup procedures specific to the instrument to occur. Consult instrument manual.

6.2.1 qPCR set up (prepare in dedicated PCR clean area i.e. PCR cabinet or separate room)

6.2.1.1. Make up a reaction cocktail in a 1.5 mL microcentrifuge tube as follows using the reagents from the ManKan™ Honey DNA real time PCR kit.

Note: Volumes given are for a 10 µL reaction (allow extra volume in reaction cocktail for pipetting error – 10% additional volume is supplied in the kit) and each run should contain a positive control and no template control (NTC):

It is recommended that technical replicates are used for each reaction until laboratory proficiency is established.

To a 1.5 mL tube (per 10 µL reaction) prepare the Mastermix cocktail:

PCR-grade Water	**5.5 µL**
20X Oligo mix	**0.5 µL**
5X Mastermix	**2 µL**

6.2.1.2. Briefly vortex the Mastermix cocktail and pulse centrifuge to collect contents in bottom of tube.

6.2.1.3. Dispense 8 µL working Mastermix per reaction well/tube (a repeating pipette is recommended). Note: If suitable precision can be demonstrated, a multichannel pipette may also be used in conjunction with a low dead volume reagent reservoir.

6.2.2 Addition of DNA (complete in designated DNA area)

6.2.1.4. Briefly vortex DNA prior to addition and pipette 2 µL into duplicate reaction wells/tubes when technical replicates are being used.

6.2.1.5. Add 2 µL positive control DNA and 2 µL PCR grade water to the appropriate wells for positive and no template controls. It is recommended that the positive control DNA is added last.

6.2.1.6. Cap tubes/seal plates as required by instrument manufacturer and centrifuge plates/tubes (if required by instrument instructions).

6.2.3 qPCR cycling protocol and analysis (perform in DNA amplification area)

Illumina Eco/ BMS Mic

Initial denaturation	95°C for 3 min	
Cycling	96°C for 6 sec 62°C for 20 sec	x 40 cycles

Note: For other instruments, times of 10 seconds (96°C) and 30 seconds (62°C) are suggested. A fast cycling mastermix is employed in this test.

Choose the following channels for monitoring:

FAM (mānuka – *L. scoparium*)
ROX/Texas Red/610 (internal control)

6.2.4 Analysis

Due to the difference in fluorescent intensities, it is recommended that each channel is analysed separately (plots will typically autoscale). Whilst recognising that each laboratories' PCR machine, standard curves and reaction efficiencies will vary, to produce comparable results between laboratories the following is recommended. When validating the standard curves, it is important that an appropriate threshold is determined that enables repeatable results to be achieved for known concentrations of DNA, particularly concentrations in the femtogram range. Once determined, the same threshold must be used for each subsequent run to enable comparison between runs. During validation of this assay, the lower limit of reporting (LOR) was determined to be 3 fg/µL which was detected at approximately Cq 36 (see Table 1). In some instances, when this test is performed on different PCR machines, the threshold may need to be set lower on the curve than is usual to achieve a Cq value in the range of 36 for the 3 fg/µL standard. As this is at the lower limit of the detectable range (LOR), some variation in Cq will occur, therefore a result for 3 fg/µL in the range of Cq 36 ± 0.75 is acceptable.

Amplification curves should be verified as being true amplifications, with reactions entering an exponential phase. Reactions that 'creep' upward and generate a Cq result, but are otherwise straight lines, are considered invalid results.

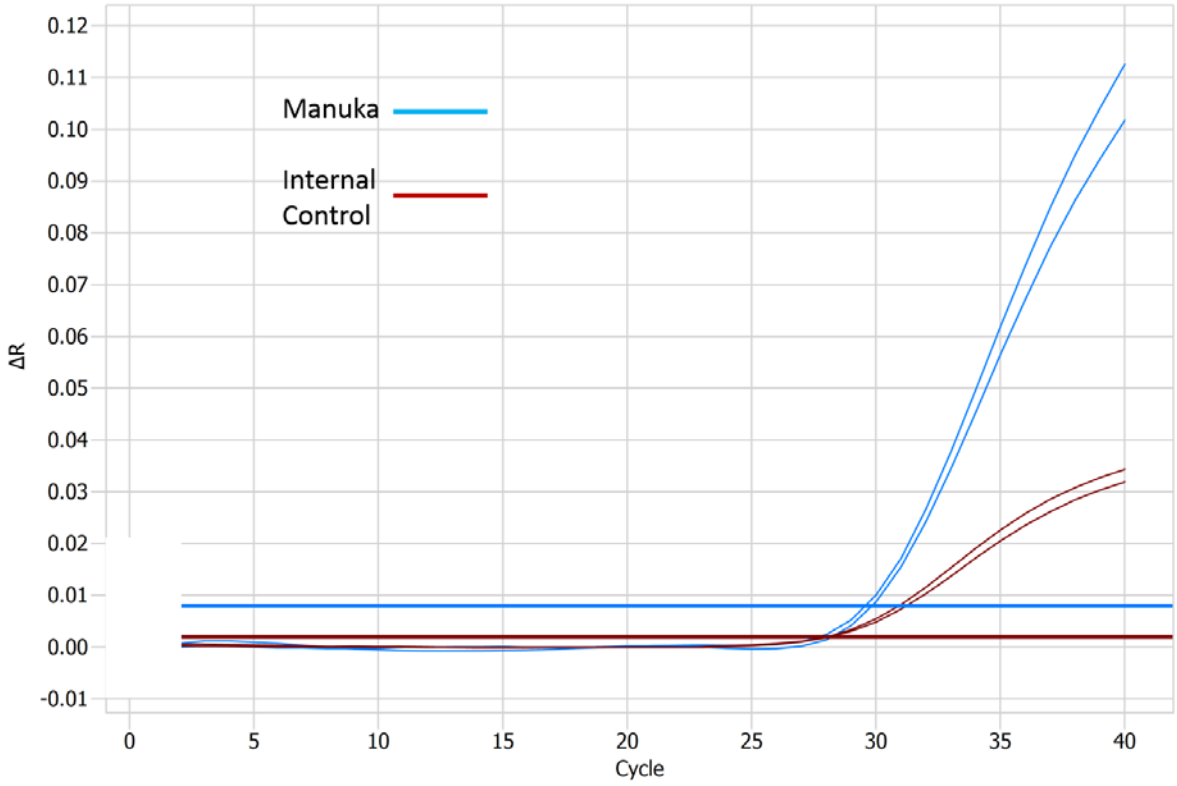

Figure 2: Example of a result from a single honey sample containing DNA from mānuka pollen. Horizontal lines indicate where the thresholds are set.

7 Quality Requirements

7.1 QUALITY CONTROL

Linearity of standards has been checked and found to be good in the range of standards validated for this method. The range is equivalent to DNA concentrations in the range 0.0032 pg/µL -16,200 pg/µL for mānuka DNA. Samples suspected of being above 16,200 pg/µL for mānuka should be diluted to a suitable concentration so that they are within the validated range. However, application of this assay to over 800 honeys samples suggests that concentrations of this magnitude will be uncommon.

At a minimum, a valid assay run requires that the positive control has performed as per the QC data sheet supplied with the ManKan^{TM} Honey DNA real time PCR kit and that the NTC (no template control) is negative.

Note: The NTC should not generate an exponential amplification resulting in a Cq reading in any channel. Amplification curves in such reactions indicate contamination and invalidate the whole qPCR run. The assay must be repeated.

7.2 ACCEPTANCE CRITERIA

A valid test result for an individual sample requires a valid assay run plus:

- #Cq value for the internal control (ROX channel) to be within the validated range of the assay indicating sufficient total plant DNA is present in the test. This was determined to be less than or equal to Cq 36.
- Cq values for 'Man' (FAM channel) to be within the validated range of the assay (for a detected result) of Cq 35.80 for mānuka. For operational application this was established as being less than or equal to Cq 36.
- *Technical replicates when used are to be within the determined relative percent difference (RPD) of <5 %

$$\text{(Value A – Value B)} \,/\, \frac{\text{(A+B)}}{2} \times 100$$

To determine the RPD between replicates, reproducibility data was used from four honey samples extracted in duplicate by two operators. The RPD ranged from 0.24% to 3.65% over 15 mānuka DNA data points. *The number of technical replicates per run should be determined after method proficiency is established.

#Samples that do not generate a valid Cq for the internal control indicate either sub-optimal extraction of DNA, little or no pollen present in the sample possibly from filtration or inhibitors present (although inhibitors are not typically observed with this DNA extraction protocol). In the first instance, the PCR step should be repeated and if yielding similar results, the DNA extraction repeated for the sample and new DNA run in the PCR assay.

8 Reporting

Each target is reported in units of Cq to 2 decimal places and following the limits of reporting. If a sample tests outside the validated range e.g. Cq 38, the result can be reported as a non-detect (ND).

9 Validation data

9.1 CALIBRATION OF STANDARD CURVE AND MEASUREMENT OF UNCERTAINTY

As part of validating this assay for use on honey samples, honey was spiked with specific pollen concentrations followed by DNA extraction and qPCR. Undertaking this work requires access to additional specialist expertise and type plant specimens, it also presented significant technical challenges. The main one being the mechanics associated with recovering significant concentrations of pollen needed for the spiking work.

Therefore it was decided to undertake more extensive validation using purified DNA of the target species against a background of DNA from another plant species as an alternative approach to generating a relevant standard curve.

9.1.1 Mānuka standard curve

The mānuka standard curve was established by diluting purified mānuka DNA (RNA free) against a background of kānuka DNA at a constant concentration (1 pg/µL). Determination of Cq cut-off threshold and linear range was determined using a linear regression model fitted against a 10 point calibration with replicates for each concentration. The model predicted Cq from the natural log analyte concentration and the curve was fitted to data over the range 0.0032-16,200 pg/µL. The relationship became less reliable at the lowest (0.00065 pg/µL) and highest (162,000 pg/µL) concentrations tested.

The regression model is as follows:

$$Cq = 26.60 - 3.660 \times \log(\text{Concentration}),\ R^2=0.998,\ RMSE= 0.347$$

where RMSE is the root mean square error of the regression. Predictions from the regression with 95% confidence intervals for a range of concentrations are shown in Table 2.

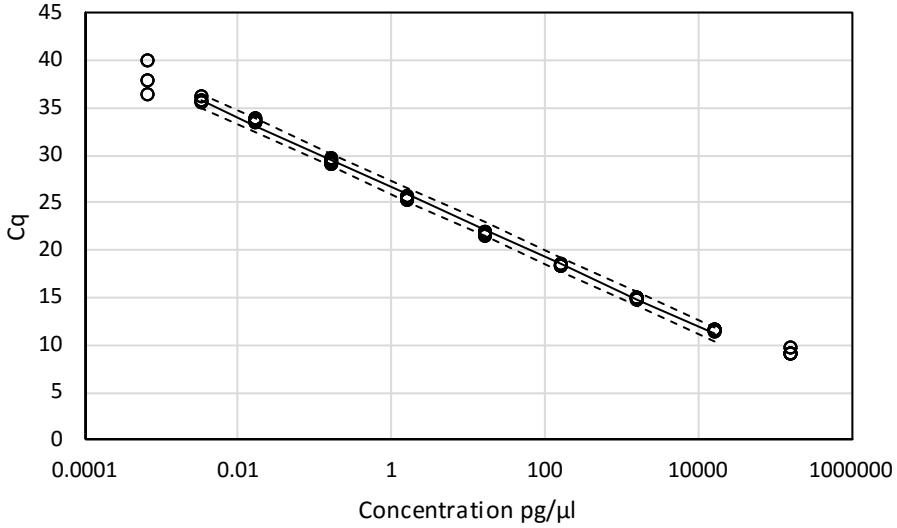

Figure 3: Mānuka standard curve data with linear regression (solid lines) and 95% confidence interval for individual predictions (dashed lines).

Table 2. Predictions of Cq for a range of concentrations with 95% confidence intervals for the mānuka standard curve.

DNA concentration (pg/µL)	Predicted Cq	Measurement uncertainty (95% confidence interval)	MU range
16,200	11.2	±0.75	10.45 -11.95
1,620	14.8	±0.73	14.07 -15.53
162	18.5	±0.73	17.77 -19.23
16.2	22.2	±0.72	21.48 -22.92
1.62	25.8	±0.72	25.08 -26.52
0.162	29.5	±0.72	28.78 -30.22
0.0162	33.1	±0.74	32.36 -33.84
0.00324	35.7	±0.75	34.95 -36.45

Linearity was observed with the internal control within the linearity ranges for the mānuka DNA tested.

It is recommended that the laboratory perform a new standard curve when either a new positive control or mastermix is used. It is also important to note that standard curves must be established for each PCR machine if a laboratory operates more than one type of machine.

9.1.2 Repeatability of samples extracted multiple times from different honeys

Table 3: Repeatability of qPCR performed on two DNA extracts from four honey samples on three separate occasions by a single operator.

	qPCR target	
Sample	IC ± SD	Mānuka ± SD
AHM GT	28.40 ± 0.64	32.86 ± 0.51
AHM 10+	31.53 ± 0.56	33.22 ± 0.25
IH	27.32 ± 1.01	30.12 ± 0.27
PB10+	31.57 ± 1.08	31.32 ± 0.40

9.1.3 The reproducibility of DNA extraction and qPCR assessed using two operators.

Table 4: Reproducibility of DNA extraction and qPCR was assessed using two operators on 4 honey samples, in duplicate, on three occasions. Each cell of the table shows the means of each operator, the absolute difference between the two operators, and the statistical significance of the difference (p-value) in parentheses.

	qPCR target	
Sample	IC	Mānuka
AHM GT	28.6, 29.7, 1.1 (0.0022*)	33.1, 33.2, 0.4 (0.73)
AHM 10+	33.6, 33.7, 0.1 (0.93)	35.5, 34.2, 1.3 (0.10)
IH	27.9, 28.7, 0.8 (0.21)	31.3, 30.3, 1.0 (0.077)
PB10+	32.0, 32.0, 0.5 (0.99)	32.3, 31.2, 1.1 (0.091)

* $p<0.01$

10 Safety Information and Precautions

10.1 REQUIRED PROTECTIVE EQUIPMENT

Personal safety equipment as per laboratory SOPs.

10.2 HAZARDS

Refer to relevant MSDS where applicable.

11 Quality Assurance Plan

To be developed by each laboratory as per accreditation requirements.

11.1 INTER-LABORATORY CHECK SAMPLES

a. System, minimum contents.
   i. Frequency: As available, per analyst when samples tested.
   ii. Records are to be maintained.
b. Acceptability criteria.

Refer to 9.1.

If unacceptable values are obtained, then:

i. Investigate following established procedures.
ii. Take corrective action as warranted.

11.2 CONDITION UPON RECEIPT

c. Room temperature, no evidence of spoilage, leakage or container damage.

12 Approvals and Authorities

12.1 APPROVALS ON FILE

d. Issuing Authority: Manager, Chemical and Microbiological Assurance