Fruit Fly ID Australia

Fruit fly molecular diagnostics

REAL-TIME PCR TAQMAN ASSAY FOR THE DETECTION OF B. TRYONI COMPLEX

INTRODUCTION

Genetically similar but morphologically different species have been reported in several fruit fly species (Clarke et al., 2005; Krosch et al., 2012). Such species are considered as belonging to 'species complexes' (Drew, 2004). The commonly called Queensland fruit fly (Qfly) is one such species complex, consisting of four species; *B. tryoni, B. aquilonis, B. neohumeralis* and *B. melas* (Drew, 1989). However, while *B. melas* exists in historic documents, researchers now consider it as a cryptic form of *B. tryoni* (Clarke et al., 2011).

Analysis of microsatellite markers has shown that there is only intraspecies level of differentiation present between *B. tryoni* and *B. aquilonis* (Wang *et al.,* 2003). Those analyses also reflect that *B. neohumeralis* and *B. tryoni* represent separate species (Clarke et al., 2005; Wang et al., 2003). The closely related species *B. curvipennis*, however, is difficult to distinguish from *B. tryoni* complex based upon DNA sequencing of the cytochrome c oxidase subunit 1 (COI) gene (Armstrong and Ball, 2005), although it is morphologically distinct. More comprehensive molecular analysis has similarly indicated that *B. curvipennis* has a paraphyletic relationship with the *B. tryoni* complex and is currently considered very close to, but not within, the *B. tryoni* complex *sensu* (Drew 1989; Blacket et al., 2012; Jiang et al., 2014).

DNA barcoding relies on PCR of predetermined marker genes, DNA sequencing and comparison of those sequences to a database of reference sequences (Armstrong and Ball, 2005). Currently, DNA barcoding of the COI gene is the most commonly used technique for the molecular identification of fruit flies (Armstrong and Ball, 2005; Blacket et al., 2012). This process is still relatively time consuming, with the quickest possible identification requiring a full day. In contrast, real-time PCR, or quantitative PCR (qPCR), drastically reduces the end-to-end time of analysis. This technique is based on the amplification of DNA monitored in real time and therefore without the need of post-PCR processing (e.g. gel electrophoresis), thus it enables identification within a few hours. The premise for this technology is that highly species-specific PCR primers or probes are designed to give positive reactions only with DNA of the target species. A positive identification is determined by the measurably lower number of PCR cycles taken for the amplicon to reach a given concentration, and for fluorescence to be detected (the quantification cycle, Cq), than would occur for a negative or suboptimal amplification, as would be expected from DNA of the wrong species. The TaqMan chemistry has the potential for greater specificity through the incorporation of a third oligonucleotide in the reaction. The method has previously been applied to other fruit fly species on the list in this manual (Burgher-MacLellan et al., 2009; Yu et al., 2004 and 2005).

Here, a real-time PCR assay for the *B. tryoni* complex has been developed, validated and applied by PHEL in the routine diagnostics of intercepted fruit fly material at borders, post borders and in recent New Zealand Qfly responses.

AIM

This assay aims to provide a rapid method for the identification of the *B. tryoni* complex, from any life stage, using the real-time TaqMan techniques.

TARGET

B. tryoni complex (*B. tryoni, B. aquilonis,* and *B. neohumeralis),* and *B. curvipennis.*

PROCEDURE

This document provides all the supporting information for conducting the real-time PCR assay and analysis of the results, including:

1. DNA extraction and subsequent PCR from a range of life stages of the samples including eggs, part of an adult (one leg is commonly used in diagnostics), part of a larva, part of a pupa, empty pupal case;
2. PCR competency test;
3. Procedures for conducting the real-time PCR assays and interpretation of the real-time PCR results;
4. Real-time PCR assay for the *B. tryoni* complex (FAM probe); please note other dyes can be used.
5. Results - analysis of Real-time PCR assays.
6. DNA Extraction protocols

REAL-TIME PCR ASSAY FOR B. TRYONI COMPLEX

Set up the PCR assay in a PCR workstation. The assay is described as run on the CFX1000 ™ real-time PCR system (BioRad), with the data analysed in the CFX manager 3.0 analysis software (BioRad).

Equipment and material

- Primers and Probe (Dhami et al., 2016)
    - Forward primer: Btry2F, 5'-AATTGTAACAGCCCATGC-3'
    - Reverse primer: Btry1R, 5'- GTGGGAATGCTATATCGG-3'
    - Probe: Btry2PL: 6FAM-AG[+C]CA[+G]TTTCC[+G]AA[+A]CC-BBQ (or BHQ1)
- Real-time PCR mastermix
    - SsoAdvanced Universal Probe Supermix (Cat#1725280, Biorad), 2x qPCR mix, containing dNTPs, Sso7d fusion polymerase, $MgCl_2$, stabilisers, ROX normalisation dyes. Note: * other supermix for probe can be used, suggest validation of the assay before applied to diagnostics.
    - Primers/probe, working concentration of 5 µM (primer stock concentration 100 µM, stored at -20°C in dark)

  - BSA (bovine serum albumin, working concentration of 10ng/µl) (Sigma Cat# A788-50g)

- DNA samples of unknown Tephritidae species (see DNA extraction page on Fruit Fly Identification website)
- Internal positive control to test unknown DNA samples for PCR competency to avoid false negative results
  - Conventional PCR with Folmer primers LCO1490 and HCO2198 (Folmer et al., 1994)
  - TaqMan ribosomal RNA control reagents (VIC probe) (Applied Biosystems, cat # 4308329).
- Controls for real-time PCR setup:
  - Positive control to monitor the performance of the real-time PCR
    - DNA samples of *B. tryoni* complex or
    - Plasmid DNA of COI insert of *B. tryoni* complex (available from MPI PHEL, NZ on request)
  - Non-template control
    - Sterile water or PCR-clean water
- PCR work station or DNA/RNA-free area for PCR setup
- DNA spiking area for adding DNA template to the PCR Master Mix
- Thermal cycler: CFX96 or CFX1000 Touch Real-time PCR (BioRad). Other brands of real-time thermal cycler can be used.
- Additional equipment: centrifuge, pipettes, plugged PCR tips, PCR tube, PCR plates, plate seals.

Method

1. PCR competency test: It is recommended prior to any qPCR analyses to test all the DNA extractions for PCR competency, using either conventional PCR or 18S internal control TaqMan assay (see Internal control for PCR competency for details)
2. Set up the real-time PCR Mastermix in a PCR work station or clean area, see below for the compositions. Include enough volume to run the samples in duplicate or triplicate plus positive and non-template controls. It can also be run at 20 µl or 10 µl volume.
   a. For 20 µl volume, aliquot 18 µl of master mix to each PCR tube
   b. For 10 µl volume, aliquot 9 µl of master mix to each PCR tube

TaqMan real-time PCR for B. tryoni complex protocol using SsoAdvanced Universal Probe Supermix (BioRad)

Reagents	1 x reaction (µl)[a]	10x reactions (µl) [b]
Sterile distilled H_2O	2.2	22
2 x Probe Supermix	10.0	100
5 µM Btry2F (400 nM)	1.6	16
5 µM Btry1R (400 nM)	1.6	16
5 µM Btry2PL (250 nM)	1.0	10
BSA (10mg/ml)	1.6	16
DNA template	2.0	

[a] The compositions for 20 µl are listed in this table, halve the volumes for each reagent if using in 10 µl volume.

[b] Using 10x reaction as an example, calculate the volumes of each reagents using the number of reactions you are going to test when conducting the assay; note, one or two extra reaction volumes should be included to account for loss due to retention of liquid in pipette tips.

3. Set up the Master Mix, mix well and spin briefly, aliquot 18 µl of the Master Mix to each labelled PCR tube or well of a plate.
4. In the DNA spiking area, add the appropriate DNA template or control solution to the tubes or the wells of a plate with the aliquoted Master Mix, close the tubes or seal the plate, mix well and spin the tubes or plate briefly
5. Put the tubes/plate into real-time PCR machine and run the program below:

Real-time PCR cycling parameters

1 x cycle	95 °C, 2 min
40 x cycles	95 °C, 15 sec
	65 °C, 60 sec
	Plate read after each cycle

6. Open CFX manager 3.0 program, set up the protocol and run the real-time PCR assay.
7. Name the assay and save the data in a folder.
8. Once the run is finished, the data can be opened in the CFX manager 3.0 and analysed.

ANALYSIS OF REAL-TIME PCR RESULTS

The real-time PCR results are analysed here with the CFX Manager 3.0 (BioRad). If different real-time PCR systems are used, analysis of the amplification curves will need to be carried out according to that manufacturers' manual.

1. Open BioRad CFX Manager 3.0.
2. Click file, open the data file, and choose the real-time PCR data you have saved for the assay.
3. Click on quantification tab, the amplification curve will appear on the screen, see Figure for an example.
4. Report: Click Tools tab and choose reports, an analysis report for the real-time PCR including amplification curves and *Cq* values will be generated. It can be saved as pdf format or print it out.
5. Interpretation of the results – 18S internal control:

    a. If *Cq* values ≤30 cycles, the DNA extraction is PCR competent and the samples can be used for real-time PCR assay against *B. tryoni* complex.

    b. If *Cq* values >30 cycles, this indicates that inhibitors are in the DNA sample or there is insufficient DNA. Re-extraction of the samples will be needed.

6. Interpretation of the results – *B. tryoni* complex assay (use the PCR competent DNA samples only):

    a. For *Cq* values ≤25 cycles, the sample is considered as positive for *B. tryoni* complex.

    b. For *Cq* values between 25-30 cycles, the sample is possible the closely related *B. curvipennis*, further tests are needed to confirm its identity

        i. DNA barcoding needs to be conducted to confirm the species;

        ii. The origin of the specimen might assist in distinguishing *B. tryoni* and *B. curvipennis* (e.g. *B. curvipennis* is endemic to New Caledonia and not present in Australia).

    c. Samples with *Cq* values between 30-35 cycles could be interpreted as either a different species with suboptimal match to the primers or that there is a very low copy number of the target species DNA. These should be considered as questionable and require further investigation by either DNA barcoding or re-extraction of DNA.

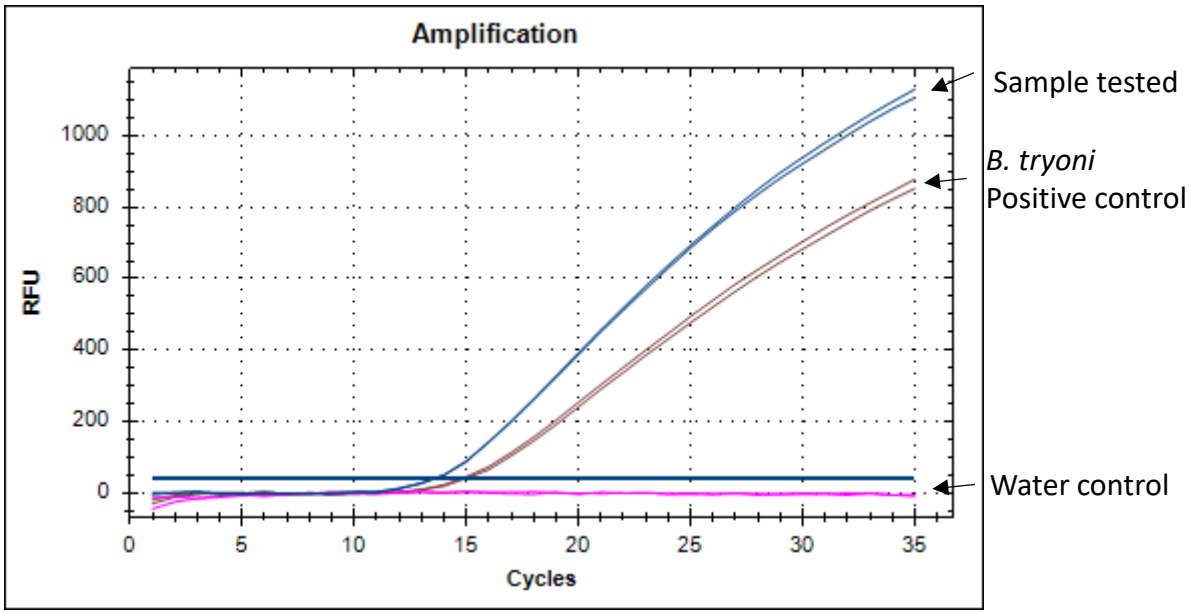
    d. The negative threshold for the assay is *Cq* values >35, at which point the samples should be considered as negative for *B. tryoni* complex, and identification by another method is necessary.

7. Validation of the real-time PCR assay for *B. tryoni* complex used DNA extracted from fresh and aged (up to 2 year old) samples. This included *B. tryoni* from NSW, VIC, QLD (n>80), *B. neohumeralis* from QLD (n>20) and *B. curvipennis* from New Caledonia (n=4). The following results were observed;

a. *Cq* values ≤25 cycles were obtained with the DNA extracted from the *B. tryoni* complex except those with empty pupal cases.

b. *Cq* values between 25-35 cycles were observed for the DNA extracted from *B. curvipennis* samples. DNA barcode sequencing confirmed the identification.

c. Negative results (*Cq* >35) were obtained for all other Tephritidae species tested (see Validation of the real-time PCR assay for B. tryoni).



Amplification curves of real-time PCR assay for *B. tryoni* complex. The sample tested positive for *B. tryoni* complex, DNA sequences has further confirmed the results.

References: see References page on the Fruit Fly Identification Australia website