MAF BIOSECURITY NEW ZEALAND

Humulus (Hop)

Post-Entry Quarantine Testing Manual

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BIOSECURITY NEW ZEALAND
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1. SCOPE

The scope of this manual is limited to nursery stock (whole plants and plants in tissue culture), seed for sowing and pollen of *Humulus* species permitted entry into New Zealand as listed in the Ministry of Agriculture and Forestry's (MAF) Plants Biosecurity Index (see: http://www1.maf.govt.nz/cgi-bin/bioindex/bioindex.pl). At the date of publication of this manual, the only species permitted entry into New Zealand is: *Humulus lupulus*.

This manual describes the testing protocols specified in the import health standards for these commodities. The manual also provides an introduction to the crop and guidance on the establishment and maintenance of healthy plants in quarantine.

2. INTRODUCTION

The genus *Humulus* belongs to the family *Cannabinaceae* (Order *Urticales*). The centre of origin of *Humulus* is Asia (probably China) with secondary centres of origin in Europe and North America. *Humulus* is composed of 3 species: *H. lupulus* L., *H. japonicus* Sieb et Zucc. and *H. yunnanensis* Hu. While there are known herbarium specimens of *H. yunnanensis* as well as historical reports of its existence in Yunnan Province, China, no live plants of this reported species are known to exist in any of the major world hop germplasm collections. *H. japonicus* is an annual species that is found throughout Asia. The only species of commercial interest is *H. lupulus*. It is a diploid (2n=2x=20) species which is divided into the following sub-species:
H. lupulus L. var *cordifolius* (Miquel) Maximowicz (origin – Eastern Asia)
H. lupulus L. var *lupuloides* E. Small (origin – North America)
H. lupulus L. var *lupulus* (origin – Europe-Asia)
H. lupulus L. var *neomexicanus* A. Nelson and Cockerell (origin – North America)
H. lupulus L. var *pubescens* E.Small (origin – North America)

The hop (*H. lupulus*) is a day-length sensitive, dioecious, perennial, rhizomatous, wind-pollinated species. The hop has perennial roots and crowns and grows annual shoots forming climbing vines that die back in winter. *H. lupulus* is a short-day species. Hops will flower in mid-summer after a minimum amount of vegetative growth of the vine has taken place. Hops are commercially grown for their unique secondary plant metabolites, which are primarily used in fermented malt beverages (beer) for their flavour and aromatic attributes, but are also now finding increasing uses as nutraceuticals and for their anti-bactericidal properties. The important secondary metabolites are found in resin glands of the 'cone', called lupulin glands, which are mainly found on the bracteoles of the mature female inflorescence called a 'cone' or strobile. There are 3 main groups of compounds found in these glands: hop acids (alpha and beta acids), essential oils, and polyphenols.

Hop acids are the main bittering precursors used in brewing. The alpha acids which are usually found in greater abundance than the beta acids are considered to be more important for brewing. During the brewing process these compounds are converted to iso-alpha acids which are the main bittering component in beer. The bittering value of alpha acids is greater than that of beta acids. There are 3 main analogues each of the alpha and beta acids. The alpha acids are humulone, adhumulone and cohumulone. The beta acids are lupulone, adlupulone and colupulone. Levels of the various alpha acids, in particular low levels of cohumulone (less than 25% of total alpha acids), are considered to be important in brewing. Cohumulone is slightly more soluble in water and this is reflected in higher bittering values, often considered to be harsher to taste. Low levels of cohumulone are associated with good

head retention on beer. Beta acids are also being utilised for their well known anti-bacterial properties in commercial process other than beer production.

The aroma properties of beer are attributed to the oil component of the lupulin gland. Several hundred compounds have been identified from the oil component. In freshly harvested hops, four main terpenoid compounds make up approximately 70-80% of the essential oil profile. The compounds are humulene, farnesene, caryophyllene and myrcene. During the brewing process, many compounds are volatilised or are transformed into a variety of derived compounds that impart a wide range of 'hoppy' aromas and flavour to beer.

Polyphenols are becoming increasingly important in hops due to their health-related properties. In particular the group of compounds called the prenylflavonoids have come under much scrutiny. The main compound, unique to hops, is xanthohumol. Some of the properties of xanthohumol have been attributed to have effects on a broad spectrum of cancer-chemo preventative activities at all stages of carcinogenesis, the inhibition of pre-cancerous lesion formation, inhibition of bone resorption and the inhibition of synthesis of triglycerides. Another prenylflavonoid compound found in hops, 8-prenylnaringenin, is the most powerful phytoestrogen discovered to date.

Hops, found in their native habitat at latitudes 35-55°N, are cultivated under temperate climatic conditions. Traditionally, they have been grown in Germany, Czech Republic, United Kingdom, Slovenia, and North America. The main production areas are Germany and USA, with Czech Republic and China also significant producers. These four countries make up 75-80% of the current world production of approximately 95,000 million tonnes, which is produced from about 50,000 hectares. Today, hops are grown in both hemispheres. Significantly, very few diseases and pests are found in the Southern Hemisphere production areas of Australia, New Zealand and South Africa.

The hop industry in New Zealand has doubled its area of production over the last 25 years. In this time, it has gone from a cottage industry supplying domestic markets, to an export-oriented industry producing unique hops primarily for niche markets overseas. Currently New Zealand produces less than 1% of the world hop production from approximately 400 hectares and the export value of this is about $10 million.

For commercial production, hops are harvested and dried in forced air kilns. Hops are used in a variety of product forms: whole 'cones' or 'leaf' hops; compressed pellets; extracts; and a range of down-stream products. Increasingly, hops are used as the latter category where the hop-derived products, which are now commonly extracted using super-critical CO_2 methods, can be isomerised (to make them light and heat stable) and also split into various fractions. As such, their versatility in the brewing process is further extended.

New Zealand is in the fortunate position of being free of the major diseases and pests that afflict the major hop growing countries. Most hop-producing countries have their own breeding programmes, mainly because of the need to produce cultivars that are adapted to local environmental conditions such as latitude, disease or pest pressure. In New Zealand, the breeding of genetically seedless (triploid) hop cultivars remains the main focus of the long-term research programme and is likely to remain so. There is a need to import germplasm to continue the genetic improvement of hops that are adapted to New Zealand's environmental conditions.

3. IMPORT REQUIREMENTS

The import requirements for nursery stock (whole plants and plants in tissue culture) of *Humulus* are set out in MAF's import health standard "Importation of Nursery Stock" (see: http://www.biosecurity.govt.nz/files/imports/plants/standards/155-02-06.pdf). Imported plants must meet the general requirements (sections 1-3) and the additional specific requirements detailed in the "*Humulus*" schedule. In summary, an import permit is required and a phytosanitary certificate must accompany all consignments certifying that the nursery stock has been inspected and found to be free of any visually detectable regulated pests, and has been treated for regulated insects and mites (whole plants). On arrival in New Zealand, the nursery stock must be grown for a minimum period of 3 months in a Level 3 post-entry quarantine facility where it will be inspected, treated and/or tested for regulated pests. The permit to import will include details of any testing and inspections that will be required in post-entry quarantine.

The import requirements for *Humulus* seed for sowing are set out in MAF's import health standard "Importation of Seed for Sowing" (see: http://www.biosecurity.govt.nz/files/imports/plants/standards/155-02-05.pdf). Imported seed must meet the general requirements (sections 1-2) and the specific requirements detailed in the "*Humulus*" schedule in section 3. In summary, an import permit is required and a phytosanitary certificate must accompany all consignments certifying that the seeds have been inspected and found free of any visually detectable regulated pests. On arrival in New Zealand, the seed must be grown for a minimum period of 1 growing season in a Level 3 post-entry quarantine facility where the seedlings will be inspected, treated and/or tested for regulated pests. The permit to import will include details of any testing and inspections that will be required in post-entry quarantine.

The import requirements for pollen are stated in section 2.2.3 in MAF's import health standard "Importation of Nursery Stock" (see: http://www.biosecurity.govt.nz/files/imports/plants/standards/155-02-06.pdf) and further details can be found in section 7.3 of this manual. The permit to import will include details of any testing and inspections that will be required in post-entry quarantine.

4. PESTS

The following section lists regulated pests of *Humulus* nursery stock, seed for sowing and pollen for which generic or specific measures are required or optional.

4.1 Regulated pests for which specific tests are required

Mite:

Tetranychus kanzawai	[Plants in tissue culture (Nursery stock) only]	

Fungi:

Pseudoperonospora humuli	[Nursery stock & seed for sowing]	Figure 1.3
Verticillium albo-atrum (severe strain)	[Nursery stock & seed for sowing]	Figure 1.4

4.2 Regulated pests for which generic tests are optional

Fungus:

Phacidiopycnis sp.	[Nursery stock only]	Figure 1.1

4.3 Regulated pests for which specific tests are optional

Fungus:		
Podosphaera macularis	[Nursery stock & seed for sowing]	Figure 1.2
Phytoplasma:		
Hop shoot proliferation phytoplasma	[Nursery stock only]	Figure 1.5
Viruses:		
Cherry leaf roll virus (strains not in New Zealand)	[Nursery stock, seed for sowing & pollen]	
Humulus japonicus latent virus	[Nursery stock, seed for sowing & pollen]	
Petunia asteroid mosaic virus	[Nursery stock only]	
Strawberry latent ringspot virus (strains not in New Zealand)	[Nursery stock & seed for sowing]	
Tobacco necrosis virus (strains not in New Zealand)	[Nursery stock only]	
Tobacco ringspot virus (strains not in New Zealand)	[Nursery stock only]	
Viroids:		
Apple fruit crinkle viroid	[Nursery stock only]	Figure 1.6
Hop stunt viroid	[Nursery stock only]	Figure 1.7

5. PROPAGATION, CARE AND MAINTENANCE IN POST-ENTRY QUARANTINE

The imported *Humulus* plants must be maintained under glasshouse conditions that are conducive to symptom expression of the regulated fungal pathogens (listed in sections 4.1, 4.2 and 4.3). Plant growth rate and leaf succulence strongly influence the development of *Podosphaera macularis* (hop powdery mildew) and *Pseudoperonospora humuli* (hop downy mildew). Plants that are not growing vigorously may not become diseased, so maintaining fertilisation and regular irrigation is very important. The optimal day-time temperature for the regulated fungal pathogens is about $20^{o}C$. Suitable night-time temperatures for growing hops are between $15\text{-}18^{o}C$.

Hops are luxuriant feeders and can grow to several metres before flowering. Common nutrient deficiencies of hop are from nitrogen, sulphur, magnesium, zinc and manganese. Photographs of symptoms caused by some of these nutrient deficiencies can be found in Appendix 2. Nitrogen deficiency produces a general yellowing of the foliage. Sulphur and magnesium deficiency shows as inter-veinal yellowing of the older leaves. Zinc deficiency results in small, narrow, stunted leaves, while manganese deficiency shows as an inter-veinal yellowing over the whole plant. Solid fertilisers with a balanced micro-nutrient supplement (e.g. Osmocote® Plus, with N: P: K: S ratios of approximately 15: 5: 11: 3, respectively) should be adequate to maintain good growth.

Hops are classified as "short-day" plants with floral initiation occurring (in New Zealand) from late November to mid January. Hops can be grown successfully in glasshouses from late winter to early autumn in pots using normal potting mixes. Extending the day length to about 16 hours by artificial illumination from incandescent or fluorescent lights assures normal development and vigorous growth during early spring. During winter, it is difficult to

maintain hops in an active growing state even with additional lighting. Therefore, plants are best moved to suitable hardening chambers to experience at least 5-6 weeks of near-freezing temperatures. A common method is to remove the rootstock (i.e. rhizome or “crown”) from pots and store them in plastic bags at 3-5°C.

Because of the dense foliage and luxuriant growth, insect control may be a problem with glasshouse-grown hops and there may be a need to instigate control measures. The most common pests in New Zealand glasshouse-grown hops are two-spotted spider mites (*Tetranychus urticae*) and white fly (*Trialeurodes vaporariorum*).

5.1 Nursery stock

Humulus nursery stock may be imported as whole plants (which includes dormant rhizomes) or as plants in tissue culture.

5.1.1 Whole plants and dormant rhizomes

Rhizomes will more likely be imported than whole plants. Rhizomes develop from shoots that are layered intentionally or from shoots that fail to break the soil surface. The rhizomes may be 5-20 mm in diameter and 20-100 cm long, with several buds at each node. Rhizomes are pruned close to the mother crown and are cut into pieces 10-15 cm in length for planting. In terms of plant importation, this is most likely to be in the form of (1) whole plant dormant “mother” rhizomes (commonly called crowns), or (2) dormant rhizome cuttings (as described above). Whichever form is imported, they should be planted in pots (size PB3-PB5) using a pasteurised peat-based potting media, e.g., 1:1 (v/v) peat:pumice or 1:1 (v:v) peat:perlite. The rhizomes should be covered with potting media so that the dormant buds are about 10 mm below the soil surface. Glasshouse conditions described above should ensure rapid plant development. Once growth is underway, each plant should be trained up either a bamboo stick (about 1.5 m in length) or a string suitably attached to an internal roof structure of the glasshouse.

5.1.2 Plants in tissue culture

Tissue cultured plantlets are the most likely method of importation. They can be successfully rooted by carefully removing plantlets from the container, washing off the agar in running tap water, and planting into seedling potting media with a 1:1 (v/v) peat:perlite mix. Plantlets can be grown in root-trainers or in cell trays. To protect the plantlets and maintain a high humidity atmosphere, they should be kept covered with a plastic bag in shady or low light conditions. The plantlets are then placed on a misting bench for up to 4 weeks and the plastic bag is gradually removed during that time to introduce the rooted plantlet to normal glasshouse conditions. Once acclimatised, plantlets should be moved into PB3-PB5 pots with a suitable temperature (about 20°C during the day and 15-18°C at night) and light (16-hour days) regime. General conditions for growing potted hop plants (described above) can then be implemented. Material imported as tissue cultures will only be tested once they have been deflasked and established in the glasshouse.

5.2 Seed for sowing

The hop seed is a fruit (achene) containing a coiled embryo with 2 cotyledons. Seeds can be stored for a few months at ambient temperatures or for several years in a refrigerator. Hop seeds do not germinate well. To ensure good germination, a cold stratification period is required. Commonly this is achieved by placing surface sterilised hop seeds in a Petri dish

lined with moistened filter paper. They are kept in a refrigerator (3-5°C) for a period of about 4-6 weeks. Periodic checking is required to ensure that the seeds remain moist. Germination of seeds is achieved by sowing the stratified seeds onto seedling propagation trays and covering them with 5 mm of sand. When placed on a misting bench with bottom heat and air temperatures of approximately 20°C, germination should take place within 2 weeks. An 8-hour light period will ensure good germination, followed by spaced planting in a glasshouse.

5.3 Pollen

Humulus pollen needs to be kept in a cool and low moisture environment to maintain and maximise its viability. Pollen should be stored in a container over dry silica gel crystals and can be held in a refrigerator (approximately 4°C) for storage of several months. Regular drying of the silica gel crystals is required to ensure there is no moisture build up, with an associated reduction in pollen viability. For long-term storage, pollen should be enclosed in a container over dry silica gel then frozen at -0°C to -40°C. When removed from the freezer, pollen should be thawed slowly in a refrigerator before use. Pollen containers removed from the refrigerator for use in the field or glasshouse should be kept cool.

Pollen viability can be determined by the fluorochromatic reaction as described by Heslop-Harrison & Heslop-Harrison (1970) or by germination on Petri plates of agar containing dextrose and boric acid. Pollen is germinated for 24 hours at approximately 20°C and then killed by dropping 2 ml of an ethanol-based stain onto each plate. Germination counts are determined with a binocular microscope.

6. INSPECTION

The inspection requirements for the operator of the facility are set out in the "MAF Biosecurity Authority Standard PBC-NZ-TRA-PQCON" (see: http://www.biosecurity.govt.nz/border/transitional-facilities/plants/pbc-nz-tra-pqcon.htm).

Photographs of symptoms caused by significant regulated pests can be found in Appendix 1. Hops in quarantine may also exhibit symptoms associated with abiotic stresses such as water and nutrient deficiencies (see section 5 above).

7. TESTING

Each of the specific tests required in the import health standard (as listed in section 4 and summarised in Tables 1A and 1B) must be performed irrespective of whether plants exhibit symptoms.

Tests are also available for the non-regulated viruses and viroid. Details of these test methods are available from the Investigation and Diagnostic Centre – Tamaki, MAF Biosecurity New Zealand (see the Contact Point, section 8).

Table 1: Summary of the regulated pests for *Humulus* nursery stock (A) or seed for sowing (B) indicating the specific tests that are required (■) or optional (★)

A. Nursery stock

Organism type	**Herbaceous indexing**	**PCR**	**Microscope inspection**
Mites (Plants in tissue culture only)			
Tetranychus kanzawai			■
Fungi			
Podosphaera macularis[1]		★	
Pseudoperonospora humuli		■	
Verticillium albo-atrum		■	
Phytoplasma			
Hop shoot proliferation phytoplasma[1]		★	
Virus			
Cherry leaf roll virus[1,2]	★		
Humulus japonicus latent virus[1]	★	★	
Petunia asteroid mosaic virus[1]	★		
Strawberry latent ringspot virus[1,2]	★		
Tobacco necrosis virus[1,2]	★		
Tobacco ringspot virus[1,2]	★		
Viroids			
Apple fruit crinkle viroid[1]		★	
Hop stunt viroid[1]		★	

[1]These pests are not on the import health standard; therefore testing for these is optional. However, if the test is specified on the permit to import, the test must be completed for the material to be eligible for biosecurity clearance.
[2]Strains not in New Zealand.

B. Seed for sowing

Organism type	**Herbaceous indexing**	**PCR**
Fungi		
Podosphaera macularis[1]		★
Pseudoperonospora humuli		■
Verticillium albo-atrum		■
Virus		
Cherry leaf roll virus[1,2]	★	
Humulus japonicus latent virus[1]	★	★
Strawberry latent ringspot virus[1,2]	★	

[1]These pests are not on the import health standard; therefore testing for these is optional. However, if the test is specified on the permit to import, the test must be completed for the material to be eligible for biosecurity clearance.
[2]Strains not in New Zealand.

PCR testing for powdery mildew and downy mildew is best carried out on the first fully unfurled leaves on relatively young plants; within the first 14-21 days after growth of the plant starts. For PCR analysis of verticillium wilt and phytoplasma, isolate DNA from the vascular tissue from the bottom of the stem (outer layer) or from roots of mature plants.

Herbaceous indexing and PCR tests for viruses and viroids must be carried out using the new flush of spring growth when the stems are 0.5 to 1.0 metre high. Plants must be sampled from at least two positions including a young, fully expanded leaf at the top of the plant and an older leaf from a midway position.

The samples from each plant must be tested as soon as possible after removal from the host. If samples have to be stored before testing, the leaves must be kept whole, all surface water removed and the material stored in a plastic bag at 4°C for no more than 7 days. Samples that become partially decayed or mouldy should not be tested, and further samples must be collected.

7.1 Specific tests for nursery stock

Each plant must be tested separately with the exception that samples from up to 5 plants may be bulked for testing provided that either:

(a) the plants are derived from a single imported dormant rhizome which was split into separate divisions upon arrival in New Zealand, in accordance with the requirements of the permit to import; or
(b) in the case of tissue culture where clonal plants are derived from the same mother plant, and this is confirmed by evidence from the national plant protection organisation in the exporting country.

7.1.1 Herbaceous indexing

It is optional to test each *Humulus* plant by mechanical inoculation onto two indicator plants each of *Chenopodium quinoa* and *Nicotiana clevelandii*.

It is important that the pre- and post-inoculation growing conditions of the herbaceous indicator plants promote their susceptibility. Plants must be grown at 18-25°C before and after inoculation. Select indicator plants at the four- to six-leaf stage of growth with at least two leaves fully expanded.

Recommended method

1. Place indicator plants in the dark for approximately 24 hours prior to inoculation to increase susceptibility.
2. Grind leaf tissue (approximately 1/4; w/v) in 0.1 M sodium phosphate buffer (pH 7.5), containing 5% (w/v) polyvinylpyrrolidone (PVP-40) and 0.12% (w/v) sodium sulphite (Na_2SO_3). A negative (inoculation buffer only) and a positive control must be included on each day that the inoculations are performed. The positive control is a non-regulated virus which is moderately transmissible and produces clear symptoms on the herbaceous indicators, e.g. *Arabis mosaic virus*. Sap expressed from *Humulus* plants contains phenolic compounds that can inhibit virus transmission. If the positive control is not *Humulus* tissue, we therefore recommend mixing healthy *Humulus* leaf material close to the same physiological stage as the test plants with the positive control (approximately 3:1 w/w) prior to carrying out the inoculations. The plants must be inoculated in the following order:
   (a) negative control (inoculation buffer only); then
   (b) test plants; then
   (c) positive control (non-regulated virus).
3. Select two young fully expanded leaves, preferably opposite leaves, to be inoculated on each indicator plant and mark them by piercing holes with a pipette tip (or similar).

4. Lightly dust the leaves with Celite or carborundum powder (wear a mask or perform in a fume hood). Alternatively, a small amount of Celite or carborundum powder may be mixed with the sap extract.
5. Using a gloved finger gently apply the sap to the marked leaves of the indicator plants, stroking from the petiole towards the leaf tip while supporting the leaf below with the other hand.
6. After 3-5 minutes rinse inoculated leaves with water.
7. Maintain inoculated plants for a minimum of 4 weeks. Inspect and record plants twice per week for symptoms of virus infection.

Positive control for herbaceous indexing

The *Arabis mosaic virus* positive control may be obtained from:

1. ATCC Cat. No. PV-192, PV-589, PV-590 (http://www.atcc.org).
2. DSMZ Cat. No. PV-0045, PV-0046, PV-0215, PV-0216, PV-0217, PV-0230, PV-0232 (http://www.dsmz.de).
3. The Investigation and Diagnostic Centre – Tamaki, MAF Biosecurity New Zealand (see the Contact Point, section 8) (available as freeze-dried leaf material). A charge may be imposed to recover costs.

Interpretation of results

The herbaceous indexing results will only be considered valid if:

(a) no symptoms are produced on the indicator hosts with the negative control (inoculation buffer only); and

(b) the correct symptoms are produced on the indicator hosts with the positive control (non-regulated virus). If *Arabis mosaic virus* was used as the positive control, the following symptoms will be produced on the herbaceous indicators:
- *C. quinoa* – local lesions; systemic chlorotic mottling.
- *N. clevelandii* – local lesions, systemic chlorotic spots, rings and lines.

The symptoms produced by each of the regulated viruses on herbaceous indicators are described below.

Cherry leaf roll virus:
- *C. quinoa* – chlorotic or necrotic local lesions; systemic mottle, necrosis and malformation.

Humulus japonicus latent virus:
- *C. quinoa* – necrotic local lesions, systemic chlorosis and necrosis.
- *N. clevelandii* – etched necrotic local lesions, systemic chlorosis and necrosis.

Petunia asteroid mosaic virus:
- *C. quinoa* – necrotic lesions.
- *N. clevelandii* –chlorotic or necrotic local lesions, systemic mottle and necrosis.

Strawberry latent ringspot virus:
- *C. quinoa* – chlorotic or necrotic local lesions, systemic chlorosis and deformation, necrosis or faint chlorotic mottle.

Tobacco necrosis virus:
- *C. quinoa*, *N. clevelandii* – necrotic local lesions, usually no systemic spread.

Tobacco ringspot virus:
- *C. quinoa* – necrotic or chlorotic local lesions.
- *N. clevelandii* – necrotic local lesions; systemic leaf shape malformation.

7.1.2 Molecular assays

PCR **MUST** be carried out for the following fungi:
- *Pseudoperonospora humuli*
- *Verticillium albo-atrum*

PCR is **OPTIONAL** for the following pathogens:
- *Podosphaera macularis*
- Hop shoot proliferation phytoplasma
- *Humulus japonicus latent virus*
- *Apple fruit crinkle viroid*
- *Hop stunt viroid*

7.1.2.1 Polymerase chain reaction (PCR)

PCR primers used to detect fungi, virus, viroids and phytoplasma of *Humulus* are listed in Table 2, along with plant internal control primers for RNA and DNA. The inclusion of an internal control assay is recommended to eliminate the possibility of PCR false negatives due to extraction failure, nucleic acid degradation or the presence of PCR inhibitors. The *Nad5* primers amplify mRNA from plant mitochondria and the Gd1/Berg54 primers amplify the 16S rRNA gene from chloroplasts.

Please note that the primers listed in Table 2 have not been optimised for use in multiplex PCR. We recommend that the pathogen-specific PCR and the internal control PCR are performed simultaneously in separate tubes, unless the PCR has been optimised as a multiplex.

The PCR reagents listed for the methods described in this section have been tested by the Investigation and Diagnostic Centre – Tamaki, MAF Biosecurity New Zealand. Alternative reagents may give similar results but will require validation.

Table 2: PCR primers used for the detection of regulated pests of *Humulus* and plant internal controls

Target organism	Primer name	Sequence (5´-3´)	Tm (°C)	Size (bp)	Reference
Fungi					
Podosphaera macularis	S1	CCCGAACTCATGTAGTTAGTGC	60	282	Patzak, 2005
	S2	GAGCACATCGGTACCGCCACTA			
Pseudoperonospora humuli	P1	CTGAGGGGACGAAAGGCTCTG	60	371	Patzak, 2005
	P2	CTGGTCACATGGACAGCCTTCA			
Verticillium albo-atrum	ITS 1	CCGGTACATCAGTCTCTTTA	60	334	Nazar *et al.*, 1991
	ITS 2	ACTCCGATGCGAGCTGTAAT			
Virus					
Humulus japonicus latent virus	HJLV-F	AACGTGAGAGTACCGCCTGG	60	217	S. Scott, pers. comm.
	HJLV-R	CATCTCCCATGTGAGTGCATGG			
Viroids					
Apple fruit crinkle viroid	AFCVd-C	GACGACGAGTCACCAGG	55	372	Liefting, unpublished
	AFCVd-H	GACGAAGGGTCCTCAGC			
Hop stunt viroid	HSVd-H79	CCGGGGCAACTCTTCTCAGAATCCA	55	300	Hadidi *et al.*, 1992
	HSVd-C60	GGCTCCTTTCTCAGGTAAG			
Phytoplasma					
Universal phytoplasma	P1	AAGAGTTTGATCCTGGCTCAGGATT	53	1,800	Deng & Hiruki, 1991
	P7	CGTCCTTCATCGGCTCTT			Schneider *et al.*, 1995
Universal phytoplasma	R16F2	ACGACTGCTAAGACTGG	50	1,248	Lee *et al.*, 1995
	R16R2	TGACGGGCGGTGTGTACAAACCCCG			
Internal control					
Plant RNA control	*Nad5*-F	GATGCTTCTTGGGGCTTCTTGTT	50-60	181	Menzel *et al.*, 2002
	Nad5-R	CTCCAGTCACCAACATTGGCATAA			
Plant DNA control	Gd1	ACGGAGAGTTTGATCCTG	50-62	1,500	Andersen *et al.*, 1998
	Berg54	AAAGGAGGTGATCCAGCCGCACCTTC			

7.1.3.2.1 Fungal PCR

Recommended method

1. Extract total DNA from leaves for *P. macularis* and *P. humuli* and from vascular tissue from the binds or roots for *V. albo-atrum*. Successful PCR amplification can be achieved using DNA extracted using the DNeasy® Plant Mini Kit (Qiagen Cat. No. 69104). An alternative method may also be used after validation.
2. Optional: Perform a PCR on the purified DNA with the Gd1/Berg54 internal control primers (Table 2) using the components and concentrations listed in Table 3 or Table 4 and cycle under the conditions listed in Table 5.
3. Perform a PCR on the purified DNA with the fungi-specific primers (Table 2) using the components and concentrations listed in Table 3 or Table 4 and cycle under the conditions listed in Table 5. The following controls must be included for each set of PCR reactions:
   (a) positive control: total DNA or a cloned fragment from the appropriate fungus. If the internal control primers are not used, then the fungal DNA must be mixed with healthy *Humulus* DNA to rule out the presence of PCR inhibitors.
   (b) no template control: water is added instead of DNA template.
   When setting up the test initially, it is advised that a negative control (DNA extracted from healthy *Humulus* tissue) is included.
4. Analyse the PCR products by agarose gel electrophoresis.

Fungal positive controls for PCR
DNA of *Podosphaera macularis*, *Pseudoperonospora humuli* and *Verticillium albo-atrum* may be obtained from the Investigation and Diagnostic Centre – Tamaki, MAF Biosecurity New Zealand (see the Contact Point, section 8). A charge may be imposed to recover costs.

Table 3: Generic PCR reaction components for DNA templates using Invitrogen Platinum® *Taq* DNA polymerase

Reagent	Volume per reaction (µl)
Sterile deionised H_2O	11.8
10 × PCR buffer (Invitrogen)	2.0
10 µg/µl Bovine serum albumin (BSA; Sigma A7888)*	1.0
50 mM $MgCl_2$	0.6
10 mM dNTPs	0.4
5 µM Forward primer	1.0
5 µM Reverse primer	1.0
5 U/µl Platinum® *Taq* DNA polymerase (Invitrogen 10966-026)	0.2
DNA template	2.0
Total	**20.0**

*Optional

Table 4: Generic PCR reaction components for DNA templates using Promega GoTaq® Green Master Mix

Reagent	Volume per reaction (µl)
Sterile deionised H_2O	5.0
2 × GoTaq® Green Master Mix (Promega M7122)	10.0
10 µg/µl Bovine serum albumin (BSA; Sigma A7888)*	1.0
5 µM Forward primer	1.0
5 µM Reverse primer	1.0
DNA template	2.0
Total	**20.0**

*Optional

Table 5: Generic PCR cycling conditions

Step	Temperature	Time	No. of cycles
cDNA synthesis (RT-PCR only)	50°C	30 min	1
Initial denaturation	94°C	5 min	1
Denaturation	94°C	30 s	40
Annealing	See Table 3	30 s	
Elongation	72°C	45 s (fungi, virus, viroids) 1 min (phytoplasma)	
Final elongation	72°C	10 min	1

Interpretation of results
The pathogen-specific PCR test will only be considered valid if:
(a) the positive control produces the correct size product as indicated in Table 2; and
(b) no bands are produced in the negative control (if used) and the no template control.
If the Gd1/Berg54 internal control primers are also used, then the negative control (if used), positive control and each of the test samples must produce a 1,500 bp band. Failure of the samples to amplify with the control primers suggests that the DNA extraction has failed, compounds inhibitory to PCR are present in the DNA extract or the DNA has degraded. The

effect of inhibitors may be overcome by passing the DNA through a MicroSpin™ S-300 HR column (GE Healthcare Cat. No. 27-5130-01) or by adding Bovine Serum Albumin (BSA) to the PCR reaction at a final concentration of 0.5 µg/µl.

7.1.3.2.1.1 *Podosphaera macularis*

It is optional to test plants for *Podosphaera macularis* by PCR using the primer pair S1/S2 (Table 2). See section 7.1.3.2.1 for details of test methods and interpretation of results.

7.1.3.2.1.2 *Pseudoperonospora humuli*

Plants must be tested for *Pseudoperonospora humuli* by PCR using the primer pair P1/P2 (Table 2). See section 7.1.3.2.1 for details of test methods and interpretation of results.

7.1.3.2.1.3 *Verticillium albo-atrum* (severe strain)

Plants must be tested for *Verticillium albo-atrum* by PCR using the primer pair ITS 1/ITS 2 (Table 2). See section 7.1.3.2.1 for details of test methods and interpretation of results.

7.1.3.2.2 Phytoplasma PCR

Recommended method

1. Extract total DNA from vascular tissue from the binds or roots. Successful PCR amplification can be achieved using DNA extracted by the following methods:
   (a) DNeasy® Plant Mini Kit (Qiagen Cat. No. 69104); or
   (b) a phytoplasma enrichment procedure as described by Kirkpatrick *et al*. (1987) and modified by Ahrens & Seemüller (1992). See Appendix 3 for details of this extraction method.
   An alternative method may also be used after validation.
2. Optional: Perform a PCR on the purified DNA with the Gd1/Berg54 internal control primers (Table 2) using the components and concentrations listed in Table 3 or Table 4 and cycle under the conditions listed in Table 5.
3. Perform a nested PCR on the purified DNA using the universal phytoplasma primer pair, P1/P7 (Table 2), for first-stage PCR followed by the R16F2/R16R2 primer pair for the second-stage PCR (Table 2).
4. Set up the first-stage and second-stage PCR reactions using the components and concentrations listed in Table 3 or Table 4 and cycle under the conditions listed in Table 5. The first-stage PCR products (1 µl), including the controls, are used directly as the template in the second-stage PCR. The following controls must be included for each set of PCR reactions:
   (a) positive control: DNA from any phytoplasma extracted from any host tissue. A cloned fragment of the phytoplasma may also be used. If the internal control primers are not used, then the phytoplasma DNA must be mixed with healthy *Humulus* DNA to rule out the presence of PCR inhibitors.
   (b) no template control: water is added instead of DNA template. An additional no template control is included in the second-stage PCR.
   When setting up the test initially, it is advised that a negative control (DNA extracted from healthy *Humulus* tissue) is included.
5. Analyse the products from the first and second-stage PCRs by agarose gel electrophoresis.

Phytoplasma positive control for PCR
Phytoplasma DNA may be obtained from the Investigation and Diagnostic Centre – Tamaki, MAF Biosecurity New Zealand (see the Contact Point, section 8). A charge may be imposed to recover costs.

Interpretation of results
See section 7.1.3.2.1 for details of interpretation of results.

7.1.3.2.2.1 Hop shoot proliferation phytoplasma

It is optional to test plants for Hop shoot proliferation phytoplasma by nested-PCR using the universal primers listed in Table 2. See section 7.1.3.2.2 for details of test methods and interpretation of results.

7.1.3.2.3 Virus and viroid reverse transcription-PCR (RT-PCR)

Recommended method
1. Extract total RNA according to a standard protocol. Successful RT-PCR amplification can be achieved using RNA that has been extracted using the following methods:
   (a) RNeasy® Plant Mini Kit (Qiagen Cat. No. 74904); or
   (b) RNeasy® Plant Mini Kit (Qiagen Cat. No. 74904) with a modified lysis buffer as described by MacKenzie *et al*. (1997); or
   (c) silica-based method as described by Menzel *et al*. (2002).
   See Appendix 3 for details of the latter two extraction methods. An alternative method may also be used after validation.
2. Optional: Perform a one-step RT-PCR on the RNA with the *Nad5* internal control primers (Table 2) using the components and concentrations listed in Table 6 and cycle under the conditions listed in Table 5.
3. Perform a one-step RT-PCR on the RNA with the pathogen-specific primers (Table 2) using the components and concentrations listed in Table 6 and cycle under the conditions listed in Table 5. The following controls must be included for each set of RT-PCR reactions:
   (a) positive control: RNA of the appropriate virus or viroid extracted from any host tissue. A cloned fragment of the virus or viroid may also be used. If the internal control primers are not used, then the RNA or cDNA clone must be mixed with healthy *Humulus* RNA to rule out the presence of PCR inhibitors.
   (b) no template control: water is added instead of RNA template.
   When setting up the test initially, it is advised that a negative control (RNA extracted from healthy *Humulus* leaf tissue) is included. Please note that the *Nad5* internal control primers do not reliably amplify a product from RNA extracted from freeze-dried material. We therefore recommend mixing fresh healthy *Humulus* leaf material with freeze-dried positive control material (3:1 w/w) prior to carrying out the extraction.
4. Analyse the PCR products by agarose gel electrophoresis.

Virus and viroid positive controls for PCR
Humulus japonicus latent virus, *Apple fruit crinkle viroid* and *Hop stunt viroid* may be obtained from the Investigation and Diagnostic Centre – Tamaki, MAF Biosecurity New Zealand (see the Contact Point, section 8). A charge may be imposed to recover costs.

Interpretation of results
The RT-PCR or PCR test will only be considered valid if:
   (a) the positive control produces the correct size product as indicated in Table 2; and

(b) no bands are produced in the negative control (if used) and the no template control. If the *Nad5* internal control primers are also used, then the negative control (if used), positive control and each of the test samples must produce a 181 bp band. Failure of the samples to amplify with the internal control primers suggests that the RNA extraction has failed, compounds inhibitory to PCR are present in the RNA extract or the RNA has degraded. The effect of inhibitors may be overcome by adding Bovine Serum Albumin (BSA) to the PCR reaction at a final concentration of 0.5 µg/µl.

Table 6: Generic one-step RT-PCR reaction components using the SuperScript® III One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase

Reagent	Volume per reaction (µl)	
Sterile deionised H_2O	2.1	4.2
2 × Reaction mix (Invitrogen)	5.0	10.0
10 µg/µl Bovine serum albumin (BSA; Sigma A7888)*	0.5	1.0
5 µM Forward primer	0.5	1.0
5 µM Reverse primer	0.5	1.0
SuperScript® III RT / Platinum® *Taq* Mix (Invitrogen 12574-026)	0.4	0.8
RNA template	1.0	2.0
Total	**10.0**	**20.0**

*Optional

7.1.3.2.3.1 *Apple fruit crinkle viroid*

It is optional to test plants for *Apple fruit crinkle viroid* by PCR using the primer pair AFCVd-C/AFCVd-H (Table 2). See section 7.1.3.2.3 for details of test methods and interpretation of results.

7.1.3.2.3.2 *Hop stunt viroid*

It is optional to test plants for *Hop stunt viroid* by PCR using the primer pair HSVd-H79/HSVd-C60 (Table 2). See section 7.1.3.2.3 for details of test methods and interpretation of results.

7.1.3.2.3.3 *Humulus japonicus latent virus*

It is optional to test plants for *Humulus japonicus latent virus* by PCR using the primer pair HJLV-F/HJLV-R (Table 2). See section 7.1.3.2.3 for details of test methods and interpretation of results.

7.2 Specific tests for seed for sowing

Tests are to be carried out on plants germinated from imported seeds. Samples from up to 5 plants may be bulked for testing provided that the plants were derived from the same mother plant and this is confirmed by evidence from the national plant protection organisation in the exporting country.

Seed imported for sowing MUST be tested for the following fungi:

- *Pseudoperonospora humuli*
- *Verticillium albo-atrum* (severe strain)

Testing is **OPTIONAL** for the following pathogens:

- *Podosphaera macularis*
- *Cherry leaf roll virus* (strains not in New Zealand)
- *Humulus japonicus latent virus*
- *Strawberry latent ringspot virus* (strains not in New Zealand)

See Table 1 for the specific tests available for these pathogens and the relevant section for details of the test methods and interpretation of results.

7.3 Specific tests for pollen

Seed resulting from fertilisation of a mother plant with imported pollen must be tested as for seed for sowing (see section 7.2). Tests are to be carried out on plants germinated from the seed. Samples from up to 5 plants may be bulked for testing provided that the plants were derived from the same batch of pollen.

Testing is **OPTIONAL** for the following viruses:

- *Cherry leaf roll virus* (strains not in New Zealand)
- *Humulus japonicus latent virus*

See Table 1 for the specific tests available for this virus and the relevant section for details of the test methods and interpretation of results.

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Appendix 1. Symptoms of significant regulated pests of *Humulus*

1.1 *Phacidiopycnis* sp. (Red crown rot)

Hop roots affected by red crown rot. Roots and crowns of diseased rootstocks are knarled, twisted and covered in loose thick brown bark. The affected core and larger roots are orange to red in colour and the tissue becomes dry and friable. (Top and middle images courtesy P. McGee, right image courtesy D. H. Gent; reproduced, with permission, from the Hop Diseases and Pests Image CD, 2009, American Phytopathological Society, St. Paul, MN, USA)

Above-ground symptoms of red crown rot include uneven thin, weak bines and chlorotic leaves. (Courtesy P. McGee)

1.2 *Podosphaera macularis* (Hop powdery mildew)

The symptoms most commonly associated with hop powdery mildew are powdery and glistening white colonies on tissues that are generally discrete circular areas on leaves or stems (left photo), or non-discrete areas that encompass large areas of tissue or complete organs (right photo). (Left image courtesy W. F. Mahaffee, right image courtesy D. H. Gent; reproduced from the Hop Diseases and Pests Image CD, 2009, American Phytopathological Society, St. Paul, MN, USA)

Hop shoots with *Podosphaera macularis* infection only occurring on the tissue surrounding each node when conditions result in shoot growth that is faster than fungal growth. (Courtesy D. H. Gent; reproduced from the Hop Diseases and Pests Image CD, 2009, American Phytopathological Society, St. Paul, MN, USA)

1.3 *Pseudoperonospora humuli* (Hop downy mildew)

Hop shoot (“spike”) systemically infected with *Pseudoperonospora humuli* showing stunting, shortened internodes and chlorotic to yellow-green leaves that are brittle and down-curled. (Courtesy D. H. Gent; reproduced from the Hop Diseases and Pests Image CD, 2009, American Phytopathological Society, St. Paul, MN, USA)

Secondary basal “spike” infected with *Pseudoperonospora humuli*. (Courtesy D. H. Gent; reproduced from the Hop Diseases and Pests Image CD, 2009, American Phytopathological Society, St. Paul, MN, USA)

Lateral branch systemically infected with *Pseudoperonospora humuli*, producing an aerial "spike". (Courtesy D. H. Gent; reproduced from the Hop Diseases and Pests Image CD, 2009, American Phytopathological Society, St. Paul, MN, USA)

Angular leaf lesions caused by *Pseudoperonospora humuli*; adaxial (left) and abaxial (right). (Courtesy D. H. Gent; reproduced from the Hop Diseases and Pests Image CD, 2009, American Phytopathological Society, St. Paul, MN, USA)

Leaf chlorosis associated with systemic infection by *Pseudoperonospora humuli*. (Courtesy D. H. Gent; reproduced from the Hop Diseases and Pests Image CD, 2009, American Phytopathological Society, St. Paul, MN, USA)

1.4 *Verticillium albo-atrum* (Verticillium wilt)

Bines affected by the lethal form of Verticillium wilt. Bine of a diseased plant early (left photo) and late (right photo) in the season. The symptoms include yellowing and wilting of the leaves. Leaf discolouration begins between the main veins and is followed by marginal and interveinal necrosis. The margins of the leaves turn upwards and the affected leaves fall from the plant easily. (Courtesy S. Radišek; reproduced from the Hop Diseases and Pests Image CD, 2009, American Phytopathological Society, St. Paul, MN, USA)

1.5 Hop shoot proliferation phytoplasma

Shoot proliferation symptoms on a hop plant infected with a phytoplasma (left) compared to a healthy shoot (right). (Courtesy M. Kamińska; reproduced, with permission, from the Hop Diseases and Pests Image CD, 2009, American Phytopathological Society, St. Paul, MN, USA)

Severely stunted growth, shoot proliferation, and leaf chlorosis and malformation caused by phytoplasma infection (photo taken near harvest). (Courtesy E. Solarska)

1.6 *Apple fruit crinkle viroid*

Severe leaf curling in the upper part of bines infected with a hop strain of *Apple fruit crinkle viroid.* (Courtesy T. Sano; reproduced, with permission, from the Hop Diseases and Pests Image CD, 2009, American Phytopathological Society, St. Paul, MN, USA)

1.7 *Hop stunt viroid*

Hop stunt viroid infected plants (left) exhibit reduced lateral branching compared to uninfected plants (right). (Courtesy K.C. Eastwell; reproduced, with permission, from the Hop Diseases and Pests Image CD, 2009, American Phytopathological Society, St. Paul, MN, USA)

Appendix 2. Symptoms of nutrient deficiencies in *Humulus*

2.1 Magnesium deficiency

Hop leaves showing interveinal chlorosis caused by magnesium deficiency. Occasionally scorching of the leaf margin and early defoliation will occur. (Courtesy C. B. Skotland; reproduced from the Hop Diseases and Pests Image CD, 2009, American Phytopathological Society, St. Paul, MN, USA)

2.2 Nitrogen deficiency

Hop leaves showing symptoms of nitrogen deficiency. The leaves develop a pale-yellow colour, are thinner with narrower lobes, red petioles and are shed early. The plant is usually stunted with reduced flowering. (Courtesy C. B. Skotland; reproduced from the Hop Diseases and Pests Image CD, 2009, American Phytopathological Society, St. Paul, MN, USA)

2.3 Zinc deficiency

A hop plant showing symptoms of zinc deficiency. Plants have long shoots with very small yellow leaves that are cupped upward and have deeply cut lobes with sharp marginal serrations. (Courtesy C. B. Skotland; reproduced from the Hop Diseases and Pests Image CD, 2009, American Phytopathological Society, St. Paul, MN, USA)

Appendix 3. Protocols referenced in the manual

3.1 Modified Qiagen RNA extraction method (MacKenzie *et al*., 1997)

1. Grind 0.1 g leaf tissue (1/10; w/v) in extraction buffer (4 M guanidine isothiocyanate, 0.2 M sodium acetate [pH 5.0], 25 mM EDTA, 2.5% [w/v] PVP-40).
2. Transfer the homogenised extract to a microcentrifuge tube containing 100 µl of 20% (w/v) sarkosyl per ml of homogenate.
3. Incubate at 70°C for 10 minutes with intermittent shaking.
4. Then follow steps 4-11 of the protocol for "Isolation of Total RNA from Plant Cells and Tissues and Filamentous Fungi" as described in the RNeasy® Plant Mini Kit (Qiagen Cat. No. 74904) instruction handbook.

3.2 Silica-based RNA extraction method (Menzel *et al*., 2002)

1. Grind 0.2-0.5 g leaf tissue (1/10; w/v) in extraction buffer (6 M guanidine hydrochloride, 0.2 M sodium acetate, 25 mM EDTA, 2.5% [w/v] PVP-40 adjusted to pH 5 with acetic acid).
2. Transfer 500 µl of the homogenised extract to a microcentrifuge tube containing 100 µl of 10% (w/v) SDS.
3. Incubate at 70°C for 10 minutes with intermittent shaking, and then place on ice for 5 minutes.
4. Centrifuge at 13,000 rpm for 10 minutes.
5. Transfer 300 µl supernatant to a new microcentrifuge tube and add 300 µl high salt buffer (6 M sodium iodide, 0.15 M sodium sulphite), 150 µl absolute ethanol and 25 µl silica milk (1 g/ml silicon dioxide, 1-5 µM size particles, suspended in 100 mM glycine, 100 mM NaCl, 100 mM HCl, pH 2).
6. Incubate at room temperature for 10 minutes with intermittent shaking.
7. Centrifuge at 3,000 rpm for 1 minute and discard the supernatant.
8. Resuspend the pellet in 500 µl of wash buffer (10 mM Tris-HCl pH 7.5, 0.05 mM EDTA, 50 mM NaCl, 50% [v/v] absolute ethanol), centrifuge at 3,000 rpm for 1 minute and discard the supernatant. Repeat this wash step.
9. Centrifuge at 3,000 rpm for 1 minute and remove any remaining wash buffer from the pellet.
10. Resuspend the pellet in TE buffer (10 mM Tris-HCl pH 7.5, 0.05 mM EDTA).
11. Incubate at 70°C for 4 minutes then centrifuge at 13,000 rpm for 5 minutes.
12. Transfer 100 µl of the supernatant to a sterile nuclease-free microcentrifuge tube, being careful not to disturb the pellet. Store at -80°C.

3.3 Phytoplasma enrichment extraction method (Kirkpatrick *et al*., 1987 and modified by Ahrens & Seemüller, 1992)

1. Grind 0.3 g leaf petioles and midveins or buds and bark scrapings (1/10; w/v) in ice-cold isolation buffer (0.1 M Na_2HPO_4, 0.03 M NaH_2PO_4, 10% [w/v] sucrose, 2% [w/v] PVP-40, 10 mM EDTA, pH 7.6 to which 0.15% [w/v] bovine serum albumin and 1 mM ascorbic acid were added just before use).
2. Transfer ground sample to a cold 2 ml microcentrifuge tube and centrifuge at 4ºC for 5 min at 4,500 rpm.
3. Transfer supernatant into a new 2 ml microcentrifuge tube and centrifuge at 4ºC for 15 min at 13,000 rpm.
4. Discard the supernatant.

5. Resuspend the pellet in 750 µl hot (55ºC) CTAB buffer (2% [w/v] CTAB, 100 mM Tris-HCl [pH 8.0], 20 mM EDTA [pH 8.0], 1.4 M NaCl, 1% [w/v] PVP-40). It is easier to resuspend the pellet by first adding a small volume of CTAB buffer (e.g. 100 µl) then the remaining volume of CTAB buffer is added once the pellet has been resuspended.
6. Incubate at 55ºC for 30 min with intermittent shaking then cool on ice for 30 seconds.
7. Add 750 µl chloroform:octanol (24:1 v/v), vortex thoroughly and centrifuge at 4ºC or at room temperature for 4 min at 13,000 rpm.
8. Carefully remove upper aqueous layer into a new 1.5 ml microcentrifuge tube.
9. Add 1 volume ice-cold isopropanol, vortex thoroughly and incubate on ice for 4 min. Centrifuge at 4ºC or at room temperature for 10 min at 13,000 rpm. Discard supernatant.
10. Wash DNA pellet with 500 µl ice-cold 70% (v/v) ethanol, centrifuge at 4°C or at room temperature for 10 min at 13,000 rpm.
11. Dry DNA pellet in a DNA concentrator or air-dry.
12. Resuspend in 20 µl sterile distilled water. Incubating the tubes at 55°C for 10 min can aid DNA resuspension.
13. Store DNA at -20ºC for short term storage or -80ºC for long term storage.