

# *Rubus* (Raspberries, Blackberries & Hybridberries) Post-Entry Quarantine Testing Manual



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# ***Rubus* Post-Entry Quarantine Testing Manual**

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## 1. SCOPE

The scope of this manual is limited to nursery stock (runner tips, stem cuttings and plants in tissue culture), seed for sowing and pollen 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, these species are as follows:

<i>Rubus boysen</i> (= <i>Rubus loganobaccus</i> )	<i>Rubus mollior</i>
<i>Rubus allegheniensis</i>	<i>Rubus mucronulatus</i>
<i>Rubus amplificatus</i>	<i>Rubus nemoralis</i>
<i>Rubus argutus</i>	<i>Rubus nepalensis</i>
<i>Rubus australis</i>	<i>Rubus occidentalis</i>
<i>Rubus biflorus</i>	<i>Rubus ostryifolius</i>
<i>Rubus caesius</i>	<i>Rubus cardiophyllus</i>
<i>Rubus chamaemorus</i>	<i>Rubus palmatus</i>
<i>Rubus cissburiensis</i>	<i>Rubus parviflorus</i>
<i>Rubus cissoides</i>	<i>Rubus parvus</i>
<i>Rubus cockburnianus</i>	<i>Rubus pentalobus</i>
<i>Rubus deliciosus</i>	<i>Rubus phoenicolasius</i>
<i>Rubus echinatus</i>	<i>Rubus polyanthemus</i>
<i>Rubus errabundus</i>	<i>Rubus procerus</i>
<i>Rubus erythropus</i>	<i>Rubus rosifolius</i>
<i>Rubus flagellaris</i>	<i>Rubus rugosus</i>
<i>Rubus fockeanus</i>	<i>Rubus schmidelioides</i>
<i>Rubus fruticosus</i>	<i>Rubus squarrosus</i>
<i>Rubus idaeus</i>	<i>Rubus trivialis</i>
<i>Rubus laciniatus</i>	<i>Rubus tuberculatus</i>
<i>Rubus leptothyrsos</i>	<i>Rubus ulmifolius</i>
<i>Rubus lineatus</i>	<i>Rubus ursinus</i>
<i>Rubus loganobaccus</i>	<i>Rubus</i> × <i>loganobaccus</i>

**Note:** The importation of *Rubus ellipticus* and *Rubus moluccanus* is prohibited.

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

Raspberries, blackberries and hybridberries (including Boysenberries, loganberries and other hybrid types) belong to the genus *Rubus*, in the family Rosaceae. The genus consists of about 750 species (Daubeny, 1996) which have been separated into blackberry (subgenus *Rubus* including *R. armeniacus*, *R. laciniatus* and *Rubus* hybrids) and raspberry (subgenus *idaeobatus* including *Rubus idaeus* and *Rubus occidentalis*) types according to the abscission of the fruit; in raspberry this comes off a woody or fleshy receptacle which remains on the plant, and in blackberry the fruit separates from the plant with the soft, edible receptacle included. The genus is further divided into 12 subgenera in some classifications and 15 in others (Jennings, 1988). Most raspberry species are diploid ( $2x = 14$ ) as are a few

blackberries, but the bulk of blackberry species and all hybridberries are polyploids, ranging from  $3x = 21$  to  $18x = 126$ .

*Rubus* species are spread widely from tropical to temperate and cold temperate regions around the world, excluding areas of desert, extreme altitude and permanent ice cover. From records dating from pre-historic times in the United Kingdom and from early Greek and Roman writers (Roach, 1985a; 1985b), *Rubus* plants and fruits have been used in medicines and as fresh or processed fruit collected from the wild. Several types of blackberries, raspberries and hybridberries have been cultivated since the 19<sup>th</sup> century and in some parts of the world are now grown extensively for machine harvest or for fresh market production, either in open fields or under protected culture.

*Rubus* species are annual, biennial or perennial shrubs and vary in size from small prostrate plants to large shrubs of 5m or more in height. Most species are biennial, growing new canes each year which bear fruit in the following year (floricane fruiting). Blackberries and raspberries have also been selected which bear flowers and fruit on the current years' canes without any chilling requirement or dormancy period (primocane fruiting) (Jennings, 1988; Clark *et al.*, 2007). Selected cultivars from each of these types have also been selected which require little or no chill and they can be grown in sub-tropical to tropical conditions, bearing consecutive crops several times per year without chilling or an extended dormancy period.

*Rubus* flowers vary from small and inconspicuous with white petals to large and bright pink or reddish in colour. Most species have spines which can be small and hair-like or even large and ferocious, similar to thorns in woody plants, and they may be curved or hooked. Some species are smooth-stemmed and there are a few selections of blackberry and raspberry which are spiny only at the base or spines are completely absent. These have been used extensively in breeding and many new cultivars are genetically spineless (Jennings, 1988).

While there are some species barriers to hybridisation within *Rubus* there are also many cases where partial or full fertility is also found, even involving blackberry and raspberry types of different ploidy levels. Natural and artificial use of interspecific hybridisation has given rise to a range of hybridberry types (Lawrence, 1986).

The fruit of many *Rubus* species have limited value for fresh market or processing but some selections have found a place amongst garden plants as ornamentals where attractive flowers, foliage, autumn colour or leafless canes have made these plants popular. Some New Zealand species also have potential for this market.

At the present time, the raspberry crop is the largest of all the cultivated *Rubus*, with over 300,000 tonnes produced annually. Some of this production is harvested by machine but the majority of the processed crop remains harvested by hand, especially in the former USSR and Eastern Europe, in China and South America. The entire fresh market production around the world continues to be harvested by hand.

Annual global production of blackberry and hybridberries is estimated to be 140,292 tonnes (Strik *et al.*, 2007). About 10 % of annual production is collected from the wild, in particular in Chile, Ecuador and Romania. The largest producer of cultivated blackberries and hybridberries is the USA, however, China, Hungary, Mexico and Serbia also produce significant quantities. In the USA, the most important blackberry varieties are 'Marion', 'Thornless Evergreen' and 'Silvan' which are grown and harvested by machine. In Mexico over 4000 tonnes of low chill blackberries is produced, mostly 'Brazos', 'Tupy' and 'Kiowa'.

This area is expanding and is likely to become the major area of world blackberry production, especially for fresh market fruit.

Raspberry production in New Zealand exceeded 2,500 tonnes per annum in the 1980's but in 2002 was estimated to have reduced to 1,500 tonnes (HortResearch, 2005) and production has moved from mostly processing to the fresh market. The principal varieties grown include 'Fairview', 'Heritage', 'Marcy', 'Motueka', 'Skeena', 'Southland', 'Taylor' and 'Waiiau'. Typical yields are around 1.5 tonnes per hectare but may exceed 10 tonnes. Blackberry production is based on cultivars such as 'Navaho', 'Smoothstem' and 'Thornfree' with yields of 10-15 tonnes per hectare and an annual production of approximately 1,000 tonnes. The only hybridberry grown commercially in New Zealand is Boysenberry. In 2002, annual production was estimated to be 1,500 tonnes (HortResearch, 2005) but production in the 2006/07 season had increased to around 2,500 tonnes. The majority of the crop is grown in the Nelson region based on varieties such as 'Mapua', 'Riwaka Choice' and 'Tasman'. Yields are typically 12 tonnes per hectare but may reach 20 tonnes in the most productive fields. Most of the crop is used for processing, either frozen or made into concentrate.

The priorities for importing *Rubus* germplasm into New Zealand include the importation of varieties with sources of pest and disease resistance, adaptation to different climates, high yield and high fruit quality.

### 3. IMPORT REQUIREMENTS

The import requirements for nursery stock (runner tips, stem cuttings and plants in tissue culture) and pollen of *Rubus* are set out in MAF's import health standard "Importation of Nursery Stock" <http://www.biosecurity.govt.nz/files/ih/155-02-06.pdf>. Imported nursery stock must meet the general requirements (sections 1-3) and the additional specific requirements detailed in the "*Rubus*" 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 (runner tips and cuttings only). On arrival in New Zealand, the nursery stock must be grown for a minimum period of 16 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 *Rubus* spp. seed for sowing are set out in MAF's import health standard "Importation of Seed for Sowing" <http://www.biosecurity.govt.nz/files/ih/155-02-05.pdf>. Imported seed must meet the general requirements (sections 1-2) and the specific requirements detailed in the "*Rubus idaeus*" 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 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.

## 4. PESTS

The following section lists regulated pests of *Rubus* nursery stock, seed for sowing and pollen for which generic or specific measures are required. A complete list of regulated and non-regulated pests of *Rubus* can be found in the nursery stock and seed for sowing import health standards (see section 3).

### 4.1 Regulated pests for which generic measures are required

<b>Insects, mites, nematodes, fungi:</b>	Refer to import health standard	
<b>Bacterium:</b>		
<i>Agrobacterium rubi</i>	[Nursery stock only]	
<b>Virus:</b>		
<i>Hawaiian rubus leaf curl virus</i>	[Nursery stock only]	
<b>Diseases of unknown aetiology:</b>		
Alpine mosaic agent	[Nursery stock only]	
Black raspberry streak disease	[Nursery stock only]	Figure 1.1
Raspberry chlorotic net disease	[Nursery stock only]	

### 4.2 Regulated pests for which specific tests are required

<b>Mites:</b>	[Nursery stock: tissue culture only]	
<b>Bacterium:</b>		
<i>Xylella fastidiosa</i>	[Nursery stock only]	
<b>Viruses:</b>		
<i>Black raspberry necrosis virus</i> [Synonym: <i>Black raspberry decline associated virus</i> ] (strains not in New Zealand)	[Nursery stock only]	Figure 1.2
<i>Blackberry calico virus</i>	[Nursery stock only]	Figure 1.3
<i>Blackberry chlorotic ringspot virus</i>	[Nursery stock only]	
<i>Blackberry virus Y</i>	[Nursery stock only]	
<i>Blackberry yellow vein associated virus</i>	[Nursery stock only]	
<i>Cherry rasp leaf virus</i>	[Nursery stock only]	
<i>Raspberry leaf curl virus</i>	[Nursery stock only]	Figure 1.4
<i>Raspberry ringspot virus</i>	[Nursery stock, seed for sowing & pollen]	Figure 1.5
<i>Rubus chlorotic mottle virus</i>	[Nursery stock only]	
<i>Rubus yellow net virus</i>	[Nursery stock only]	
<i>Tobacco necrosis virus</i> (strains not in New Zealand)	[Nursery stock only]	
<i>Tomato black ring virus</i>	[Nursery stock, seed for sowing & pollen]	
<i>Tomato ringspot virus</i> (strains not in New Zealand)	[Nursery stock, seed for sowing & pollen]	Figure 1.6
<i>Wineberry latent virus</i>	[Nursery stock only]	
<b>Phytoplasmas:</b>		
Black raspberry witches' broom phytoplasma	[Nursery stock only]	
Rubus stunt phytoplasma	[Nursery stock only]	

## **Diseases of unknown aetiology:**

Raspberry yellow spot disease

[Nursery stock only]

Note: The nursery stock import health standard requires specific testing for *Bramble yellow mosaic virus* (herbaceous indexing), *Rubus Chinese seedborne virus* (herbaceous indexing), and *Thimbleberry ringspot virus* (graft indexing). However, there are currently no reliable methods to confirm the identity of these viruses as sequence data is not available and no isolates are known to exist in order to obtain sequence data (Dr Yannis Tzanetakis, pers comm.). Should symptoms be observed during herbaceous or graft indexing for other regulated viruses, diagnostic testing will be required to identify the causal organism.

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

Plants must be maintained in a healthy, vigorous state. *Rubus* plants in quarantine may exhibit symptoms associated with abiotic stresses such as water and nutrient deficiencies.

### **5.1 Nursery stock**

*Rubus* nursery stock may be imported as cuttings (runner tips and stem cuttings) and plants in tissue culture.

#### ***Blackberry and hybridberries***

Blackberry and hybridberry cultivars are usually easy to propagate by softwood cuttings, and by tissue culture, although loganberries and other hybridberries with significant raspberry content are often more difficult to root or propagate by tissue culture. The standard propagation method used commercially is tissue culture or rooting hormone-treated (Seredix® #2) softwood cuttings rooted under mist with bottom heat for small numbers of plants.

#### ***Raspberry***

Propagation of raspberries is more difficult than blackberries and hybridberries, and softwood cuttings will rarely root unless given both hormone treatment and fogging. Mist propagation is not suitable for raspberry propagation. Raspberries are not sensitive to downy mildew like blackberries and hybridberries, although they will succumb to this disease if exposed to high inoculation pressure from neighbouring blackberries and hybridberries. Raspberries grown in tissue culture may be rooted in culture but often are rooted under mist and are very sensitive to grey mould (*Botrytis cinerea*) when taken off the mist and grown on in pots. *Botrytis* infection frequently can rot leaves and also girdle the young succulent shoots at the base once growth becomes crowded and dense. This can be combated by spacing plants out when they become larger, by providing significant air movement around the plants and by watering underneath the plants. Control of *Botrytis* can be achieved using a botryticide, e.g. Switch®.

#### ***Glasshouse conditions for effective growth and optimum plant health***

Glasshouse temperatures should be kept within the range of 10-12°C at night to 18-22°C during the daytime. Relative humidity should be around 60% and there should be continuous air movement to limit infection and spread of fungal diseases, especially *Botrytis cinerea*, infecting new plant growth. Irrigation should be consistent and adequate for growth without drought or water logging, and care should be taken to use water that doesn't contain excessive Cl<sup>-</sup> ions as is common in chlorinated water supplies in some parts of New Zealand.

Application of foliar fertiliser sprays may also be used for optimum plant growth and plant health. Use of phosphoric acid as a foliar spray is of particular value as it acts both as a fertiliser and will control downy mildew amongst blackberries, hybridberries and raspberries when they are under high inoculum pressure. Plants should be repotted regularly when they become pot bound or require more root space for growth.

### ***Satisfying chill requirement and longer term growing of Rubus plants in PEQ***

Artificial chilling is essential if plants are required to spend more than a single growing season in a glasshouse before release from quarantine. This may be achieved by placing the plants at 4°C for 6 weeks to 4 months, depending on genotype.

#### **5.1.1 Cuttings (runner tips and stem cuttings)**

Potting mix for growing cuttings should have an air-filled porosity of 16-18% and initial medium fertility to ensure excessive salts are not present so that root formation is promoted and growth is healthy and vigorous. Recommended pH is 5.8-6.5 so that nutrients provided will be available for plant growth. Formulations of slow-release fertiliser can also be added to potting mixes to promote growth further after rooting and initial shoot growth has occurred. Potting mix should not contain too much bark as high tannin levels will also restrict plant growth. Following rooting and shoot development, the rooted cuttings should be side-dressed with fertiliser mix if slow-release fertiliser was not added to the initial mix. Care should be taken not to use chloride-based fertilisers but rather to use nitrate-based formulations. Fertiliser formulations should also include balanced macro nutrients, micro nutrients and trace elements to ensure adequate plant growth and minimise the chance of deficiency symptoms. Plants should be repotted regularly to allow more root space for growth so that they will not become pot bound or stunted and nutrient deficient.

#### **5.1.2 Plants in tissue culture**

Tissue-cultured plantlets offer an easy way to move *Rubus* cultivars and selections, especially from the Northern to the Southern Hemisphere. The condition of material on arrival in New Zealand is very dependent on the tissue culture vessel, the tissue culture medium and the transportation conditions. The use of culture bags for shipment usually allows the plants to arrive in the best possible condition. Tissue-cultured plugs should be sent that have already had the chilling requirement (800-1600 hours between 0 and 7°C) satisfied.

On arrival in New Zealand, the plant material should be inspected and if the medium is broken up the plant material should be sub-cultured onto new media, taking care to remove the medium from the plant tissue in the process. *Rubus* tissue-cultured plantlets to be tested must be deflasked and grown in a Level 3 post-entry quarantine facility, as specified on the permit to import. Wash the plantlets to remove any remaining agar and plant into pots of pasteurised planting media containing 1:1 (v/v) peat:pumice or 1:1 (v/v) peat:perlite with a medium-term, slow-release fertiliser (e.g. Osmocote®). Protect the plantlets from evaporation for approximately 3 weeks by covering with plastic (tub or bag) or misting regularly to keep the planting media just moist and maintain a high relative humidity. If no roots are present, a supply of bottom heat of around 20°C will expedite root formation. Pots should be in bright light but not direct sunlight during this 3 week period. After this period, gradually remove any coverings and move the plants to higher light intensity under normal glasshouse conditions.

Tissue-cultured plants blackberries, hybridberries and particularly Boysenberries, youngberries and derivatives of *R. trivialis* are very susceptible to infection with downy

mildew, *Peronospora rubi*. To combat this disease, sprays at two weekly intervals with phosphoric acid are advised plus the use of Ridomil<sup>®</sup> and/or Aliette<sup>®</sup> or other suitable fungicides. Young plants may also be affected by *Botrytis cinerea* but they are not as susceptible as raspberries (see above).

Some *Rubus* clones may not grow well when deflasked and after several weeks the plants may be stunted and rosetted with little or no apical growth. This can happen with lack of chill on the plant material and usually can be rectified through spraying the young plants with GA<sub>3</sub> (30 ppm solution from Grocel<sup>®</sup> GA<sub>3</sub> tablets) until runoff. If plants are severely checked by lack of chill and back-up tissue culture material is available, it may be worthwhile giving the plants in culture a chilling period of 6-10 weeks at 4°C.

Any remaining tissue-cultured plantlets that are not deflasked remain as a reserve in a post entry quarantine tissue culture laboratory and cannot be released without testing. Tissue-cultured plantlets can be sub-cultured after arrival by excising the growing tips and placing into new tissue culture vessels with fresh nutrient media for growth and rooting. These plantlets must be maintained in a post entry quarantine tissue culture laboratory and do not require testing. Only progeny that is derived from a tissue-cultured plantlet that has undergone testing will be eligible for biosecurity clearance.

## **5.2 Seed for sowing**

Only cleaned seed free of all traces of fruit flesh is permitted entry into New Zealand. Seed is best stored in an airtight container at about 4°C until required for sowing. Seeds must be stratified to break dormancy and enable germination. Scarification by a two-step process can be effective whereby dried seed is treated first by immersion in concentrated sulphuric acid for 5-30 minutes depending on the species, washed and excess carbon rubbed off, prior to being soaked for one week in a 1% solution of Calcium hypochlorite (28.5g/l) and Calcium carbonate (~ 3 tsp per litre). In general, seed from raspberry cultivars require 15 minutes acid scarification (200 seeds in 10 ml of acid) and blackberries require 20 minutes. Seed may require increased or decreased duration of acid treatment according to their seed size and endocarp thickness. Seed should be stratified in sealed bags of moist peat in a coolstore/refrigerator in the dark where temperature is kept at 4± 1°C for 6 weeks to 3 or 4 months (an alternative to this is sowing the seed in flats or pots and then stratifying the pots or flats in plastic bags in a coolstore). Care should be taken to prevent the seed from being frozen as this will kill the seed. For sowing, seed should be mixed with dry sand and sown on the surface of a peat-sand (river sand not crusher dust which sets hard) mixture with added three month Osmocote<sup>®</sup> or given liquid feed after germination. Seed flats should be placed in a warm glasshouse and given supplementary lighting to promote germination. Best results are gained through growing the seedlings from late winter and into the springtime. Excessive heat or light intensity can dry the seed out but otherwise seed germination is promoted by light. Care must be taken to not allow the seed trays to dry out as one dry period will kill the seed and prevent germination. Germination may also be promoted by providing fluctuating day/night temperatures once the seed is sown.

## **5.3 Pollen**

If possible, pollen is collected from plants enclosed in an insect-proof bag or grown in an insect-proof glasshouse or screenhouse. Anthers should be collected from mature but unopened flowers and dried in warm, light conditions. Following this drying period, pollen should be collected into a centrifuge vial or into gel capsules and stored at 4°C in a sealed container in the presence of a strong desiccant such as calcium chloride.

Imported pollen is used to fertilise mother plants of New Zealand origin held in a Level 3 post-entry quarantine facility. The resulting seed is sown as described in section 5.2 and tested for the pathogens of concern described in section 7.3.

## 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/abc-nz-tra-pqcon.htm>).

Photographs of symptoms caused by some of the significant regulated pests can be found in Appendix 1.

## 7. TESTING

Each of the specific tests required in the import health standard (as described in section 4 and summarised in Table 1) must be done irrespective of whether plants exhibit symptoms. This testing is required to detect latent infections.

Country freedom is accepted as an alternative measure for any of the specific tests for nursery stock listed in this manual. Country freedom must be certified by the National Plant Protection Organisation of the exporting country using an additional declaration on the phytosanitary certificate.

Virus testing must be done on new spring growth and phytoplasma and bacteria testing must be done at the end of the summer growth period. At least two fully-expanded leaves must be sampled from different branches of the main stem, one a younger leaf and one an older leaf from a midway position (note: detection of phytoplasmas and *X. fastidiosa* requires testing of leaf petioles and mid-veins). The sampled leaves from each plant must be tested as soon as possible after removal from the host.

If leaf samples have to be stored before testing, the plant material 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.

Graft indexing must be performed using young, vigorous indicator plants and is usually done in summer. Herbaceous indexing for viruses must be carried out in the spring using the new flush of spring growth.

**Table 1: Summary of the regulated pests for *Rubus* indicating the specific tests that are required (■), alternative (□) or optional (★)**

**(a) Nursery stock**

Organism type	Graft indexing	Herbaceous indexing	ELISA	PCR	Binocular microscope inspection
<b>Mites (Tissue culture only)</b>					■
<b>Bacterium</b>					
<i>Xylella fastidiosa</i>				■	
<b>Phytoplasmas</b>					
Black raspberry witches' broom phytoplasma				■	
Rubus stunt phytoplasma				■	
<b>Viruses</b>					
<i>Black raspberry necrosis</i> <sup>1</sup> <i>virus</i>	■			■	
<i>Blackberry calico virus</i> <sup>2</sup>	■				
<i>Blackberry chlorotic ringspot virus</i>		■		■	
<i>Blackberry virus Y</i>				■	
<i>Blackberry yellow vein associated virus</i>				■	
<i>Cherry rasp leaf virus</i>		■	□	□	
<i>Raspberry leaf curl virus</i>	■				
<i>Raspberry ringspot virus</i>		■	□	□	
<i>Rubus chlorotic mottle virus</i>		■		★	
<i>Rubus yellow net virus</i>	■			■	
<i>Tobacco necrosis virus</i> <sup>1</sup>		■	★		
<i>Tomato black ring virus</i>		■		■	
<i>Tomato ringspot virus</i> <sup>1</sup>		■	□	□	
<i>Wineberry latent virus</i>		■			
<b>Disease of unknown aetiology</b>					
Raspberry yellow spot disease	■				

<sup>1</sup>Strains not in New Zealand; <sup>2</sup>The IHS currently states herbaceous indexing, however grafting is recommended as it has been found to be more sensitive.

**(b) Seed for sowing**

Organism type	Herbaceous indexing	ELISA	PCR
<b>Viruses</b>			
<i>Raspberry ringspot virus</i>	★	★	★
<i>Tomato black ring virus</i>	■		■
<i>Tomato ringspot virus</i> <sup>1</sup>	■	□	□

<sup>1</sup>Strains not in New Zealand.

## 7.1 Specific tests for nursery stock

Each tissue-cultured plantlet or cutting must be labelled individually and 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 cutting which was split into separate cuttings upon arrival in New Zealand, in accordance with the requirements of the permit to import; or
- (b) cuttings that are sourced from the same mother plant, and this is confirmed by evidence from the National Plant Protection Organisation in the exporting country. In the case of tissue culture where plants are clonal, and this is confirmed by evidence from the National Plant Protection Organisation in the exporting country.

### 7.1.1 Graft indexing

The bottle graft method, also known as inarch bottle graft, is used to index imported *Rubus* germplasm. This method ensures that the material to be tested is kept alive in a vial of water whilst undergoing grafting onto a woody indicator. Once the graft successfully takes, the indicator plant will develop characteristic symptoms if the imported *Rubus* plant is virus infected.

It is recommended to perform grafting in late spring/early summer when the current season's wood is fairly mature. After approximately 4 weeks when a successful graft union has formed, the indicator plants are given summer growth conditions of 18-22°C with a 16 h photoperiod for 8 weeks, then given a winter chill period of 6 weeks at 4°C. After the chilling period the dormant plants should be brought back into the growth for observation of viral-like symptoms on spring new growth.

To avoid cross-contamination of plants during the grafting process, use a sterile scalpel for each plant to be tested.

#### Recommended method

1. Each imported *Rubus* tissue-cultured plantlet or cutting must be tested by graft inoculation onto at least 2 indicator plants of *Rubus occidentalis* (optional additional indicator plants include *R. henryi*, *R. idaeus* and *R. macraei*<sup>1</sup>). At least two grafts must 'take' for each test plant on each indicator used, for the test to be considered valid.
2. At least 2 indicator plants must be left ungrafted as negative controls.
3. At least 2 indicator plants should be grafted with positive control material (a non-regulated virus), e.g. *Raspberry bushy dwarf virus* (RBDV). It is recommended to use *R. idaeus* 'Autumn Bliss' as the indicator for the positive control as RBDV produces distinctive symptoms on this indicator.
4. Indicator plants must be well-established with stems of at least 5 mm in diameter.
5. Remove lower leaves, side shoots and thorns on 2-3 actively growing stems.
6. For the plants to be tested, select a stem about 5 mm in diameter with firm growth. Remove any soft tip growth, lower leaves and thorns and leave just 2 leaves (this reduces transpiration). If leaves are large reduce them in size by half.
7. Take one stem piece of the test plant and match as closely as possible in stem diameter to the indicator plant. Find a section of stem that will fit together well, e.g. 2 flat sections.

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<sup>1</sup>*R. henryi* and *R. macraei* are new organisms in New Zealand and must be held in a containment facility under approval from the Environmental Risk Management Authority (ERMA).

8. Cut a 5-6 cm strip along the indicator, leaving enough space below the cut for the bottle to fit. Next, cut a matching strip from the stem of the imported cultivar so that the cambium is approximately the same distance apart on both cut surfaces.
9. Place the two cut surfaces together and tie with grafting tape.
10. Place a vial containing charcoal onto the end of the graft. Approximately 6-8 cm of the graft should be placed into the vial. Fill the vial with water.
11. Tie the bottle to the stem with grafting tape and stake the indicator plant to keep the bottle upright.
12. Prune indicators leaving 5-6 leaves above the graft on each indicator stem.
13. Repeat the above steps for other indicator shoots and remove any ungrafted shoots.
14. Fertilise the *Rubus* indicators with a medium-term, slow-release fertiliser (e.g. Osmocote<sup>®</sup>) and place in the glasshouse at 18-22°C for 8 weeks of growth under summer growth conditions with a 16 h photoperiod.
15. Following this period of growth, allow the plants to enter dormancy by altering the growing temperatures and photoperiod to simulate autumn growth conditions of 15-20°C with a 14 h photoperiod.
16. Once indicator plants enter dormancy at the end of the autumn period, the plants will require a cooler winter-chilling period at 4-6°C for 6 weeks.
17. After the chilling period, bring the dormant plants back into growth under spring growth conditions of 15-22°C with a 14 h photoperiod. During the graft period and for at least 3 months, inspect the plants weekly for viral-like symptoms; pay particular attention during the spring flush of growth.

#### Notes:

1. Check water in the vial daily to ensure there is an adequate supply. The graft pieces are totally dependent on this water for a considerable time and water usage increases in hot weather.
2. The graft needs to remain alive for 3-4 weeks for the indexing to be successful.
3. The grafting tape and vial are removed approximately 4 weeks following grafting and once a successful union has formed. Tape should be removed from the indicator plants as the area behind the tapes provides an ideal environment for insects, particularly mealy bugs.
4. Remove all shoots below grafts.
5. Remove all flowers from the indicators.
6. Do not prune back the indicators hard.

#### Interpretation of results

The graft inoculation results will only be considered valid if:

- (a) no symptoms are produced on each of the negative controls (ungrafted plants); and
- (b) symptoms of bright yellow foliage are observed on the grafted *R. idaeus* 'Autumn Bliss' indicators if the non-regulated *Raspberry bushy dwarf virus* was used as the positive control.

#### Positive control for graft indexing

*Raspberry bushy dwarf virus* positive control material 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.

The symptoms produced by each of the regulated pathogens by grafting onto *Rubus* indicators are described below.

*Blackberry calico virus:*

- *R. idaeus* - calico symptoms.

*Black raspberry necrosis virus:*

- *R. occidentalis* and *R. henryi* - shoot tip curls downwards and slowly dies. Leaves beneath the tip wilt and show necrosis. New leaves show chlorotic mottling or mosaic.

*Raspberry leaf curl virus:*

- *R. idaeus* and *R. occidentalis* - leaf curling, proliferation of shoots and stunting.

*Raspberry yellow spot disease*

- *R. occidentalis* – foliar yellow spots of irregular size and shape. Leaf curling, stunting and dwarfing may occur.

*Rubus yellow net virus:*

- *R. idaeus*, *R. macraei* and *R. occidentalis* - chlorotic netting of leaf veins.

*Thimbleberry ringspot virus:*

- *R. henryi* - chlorotic leaf spots.
- *R. idaeus* - faint chlorotic markings.
- *R. occidentalis* - diffuse mottle.

### 7.1.2 Herbaceous indexing

*Rubus* species must be tested by herbaceous indexing. Each imported *Rubus* plant must be tested by mechanical inoculation on two indicator plants each of *Chenopodium quinoa*, *Cucumis sativus* and *Nicotiana clevelandii*. An additional indicator plant, *Chenopodium amaranticolor*, can be included in the herbaceous indexing test, but this is optional.

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 of the leaves being fully expanded. *C. sativus* seedlings should have expanded cotyledons but the first true leaves should be no bigger than 1-2 mm.

#### Recommended method

1. Place indicator plants in dark at 18-25°C for 16-24 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<sub>2</sub>SO<sub>3</sub>). A negative (inoculation buffer only) and a positive control must be included in each batch of inoculations. The positive control is a non-regulated virus which is moderately transmissible and produces clear symptoms on the herbaceous indicators, e.g. a non-regulated isolate of *Arabis mosaic virus*. 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 *Arabidopsis 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 *Arabidopsis mosaic virus* was used as the positive control, the following symptoms will be produced on the herbaceous indicators:
  - *C. amaranticolor* and *C. quinoa* – local lesions, systemic chlorotic mottling.
  - *C. sativus* – local lesions, systemic chlorosis.
  - *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.

*Blackberry chlorotic ringspot virus:*

- *C. quinoa* – mild chlorotic spots.

*Bramble yellow mosaic virus:*

- *C. quinoa* – local lesions, systemic necrotic ringspots or chlorotic mottling.

*Cherry rasp leaf virus:*

- *C. amaranticolor* – systemic mottle about 7 days after inoculation.
- *C. quinoa* – mottle and vein clearing.
- *C. sativus* – chlorotic local lesions, systemic mottle.

*Raspberry ringspot virus:*

- *C. amaranticolor* – chlorotic or necrotic local lesions, not systemic.
- *C. quinoa* – chlorotic or necrotic local lesions, systemic mottling and apical necrosis.
- *N. clevelandii* – chlorotic or necrotic local lesions followed by systemic mosaic (not all isolates).

*Rubus Chinese seedborne virus:*

- *C. quinoa* – chlorotic local lesions, systemic chlorosis, deformation, necrotic flecking.
- *C. sativus* – chlorotic local lesions, systemic chlorotic flecking or chlorosis.

*Rubus chlorotic mottle virus:*

- *C. quinoa* – small yellow lesions.

*Tobacco necrosis virus:*

- *C. amaranticolor* – necrotic local lesions, usually no systemic spread.
- *C. quinoa* – local lesions, usually no systemic spread.
- *N. clevelandii* – necrotic local lesions, usually no systemic spread.

*Tomato black ring virus:*

- *C. amaranticolor* and *C. quinoa* – necrotic local lesions, systemic chlorotic mottling, necrosis.
- *C. sativus* – necrotic local lesions, systemic mottling, necrosis.
- *N. clevelandii* – necrotic local lesions, systemic necrotic streaking, mottle and stunting.

*Tomato ringspot virus:*

- *C. amaranticolor* and *C. quinoa* – chlorotic local lesions, systemic apical necrosis.
- *C. sativus* – necrotic or chlorotic local lesions; systemic mottle.
- *N. clevelandii* – necrotic local lesions; systemic chlorosis, necrosis.

*Wineberry latent virus:*

- *C. amaranticolor* and *C. quinoa* – necrotic local lesions.

### 7.1.3 Serological and molecular assays

ELISA OR PCR MUST be carried out for the following viruses:

- *Cherry rasp leaf virus*
- *Raspberry ringspot virus*
- *Tomato ringspot virus*

ELISA is OPTIONAL for the following virus:

- *Tobacco necrosis virus*

PCR MUST be carried out for the following pathogens:

- *Xylella fastidiosa*
- Black raspberry witches' broom phytoplasma
- Rubus stunt phytoplasma
- *Black raspberry necrosis virus*
- *Blackberry chlorotic ringspot virus*
- *Blackberry virus Y*
- *Blackberry yellow vein associated virus*
- *Rubus yellow net virus*
- *Tomato black ring virus*

PCR is OPTIONAL for the following virus:

- *Rubus chlorotic mottle virus*

### 7.1.3.1 Enzyme-linked immunosorbent assay (ELISA)

#### Recommended method

1. Perform the ELISA according to the manufacturer's instructions. The following controls must be included on each ELISA plate:
  - (a) positive control: infected leaf tissue or equivalent (Table 2); and
  - (b) negative control: *Rubus* leaf tissue that is known to be healthy; and
  - (c) buffer control: extraction buffer only.
2. Add each of the samples and controls to the ELISA plate as duplicate wells. It is not recommended to perform ELISA with plant samples or sap that has been frozen, unless it has been demonstrated that it does not affect the performance of the test.
3. Measure the optical density 60 minutes after addition of the substrate or as recommended in the manufacturer's instructions.

**Table 2: Source of antisera and positive controls for ELISA**

Virus	Antisera <sup>1</sup>	Positive control <sup>2</sup>
<i>Cherry rasp leaf virus</i>	Neogen Cat No. 1303-09 (Identikit96)	Neogen Cat No. 1303-11
<i>Raspberry ringspot virus</i>	Agdia Cat. No. SRA 5940	Agdia Cat. No. LPC 59401
<i>Tomato ringspot virus</i>	Agdia Cat. No. SRA 22000	Agdia Cat. No. LPC 22000
<i>Tobacco necrosis virus</i>	Loewe Cat. No. 07036C	Loewe Cat. No. 07036PC

<sup>1</sup>The catalogue numbers for the complete reagent sets are given; the reagents can also be purchased separately.

<sup>2</sup>The positive control is included if the complete reagent set is purchased.

Further information on the suppliers listed in Table 2 can be found on their websites:

- Agdia Incorporated, USA (<http://www.agdia.com>).
- Loewe Biochemica GmbH, Germany (<http://www.loewe-info.com>).
- Neogen Europe Ltd – Adgen Phytodiagnostics (<http://plant.neogeneurope.com>)

The antisera listed have been tested by the Investigation and Diagnostic Centre – Tamaki, MAF Biosecurity New Zealand. Alternative antisera and positive controls are available from other manufacturers.

#### Interpretation of results

A result is considered positive if the mean absorbance of the two replicate wells is greater than 2 times the mean absorbance of the negative control. The test will only be considered valid if:

- (a) the absorbances for the positive and negative controls are within the acceptable range specified by the manufacturer; and
- (b) the coefficient of variation (standard deviation / mean × 100), between the duplicate wells is less than 20%.

If the test is invalid, it must be repeated with freshly-extracted sample. Samples that are close to the cut-off must be retested or tested using an alternative method recommended in the import health standard (see Table 1).

### 7.1.3.2 Polymerase chain reaction (PCR)

PCR primers used to detect viruses, bacteria and phytoplasmas of *Rubus* are listed in Table 3 along with two different sets of internal control primers for either RNA or 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 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 3: PCR primers used for the detection of regulated pests of *Rubus* and plant internal controls**

Target organism	Primer name	Primer sequence 5'-3'	Tm (°C)	Band (bp)	Reference
<b>Bacterium</b>					
<i>Xylella fastidiosa</i>	RST31	TGGACGTTGTGGTATCGGTG	56	733	Minsavage <i>et al.</i> , 1994
	RST33	TTGAAGTTGACGTGTGGCTG			
	XF-F XF-R XF-P <sup>1</sup>	CACGGCTGGTAACGGAAGA GGGTTGCGTGGTGAAATCAAG FAM-TCGCATCCCCTGGCTCAGCC-NFQ <sup>2</sup>	62	70	Harper <i>et al.</i> , 2010
<b>Phytoplasmas</b>					
Universal phytoplasma	P1	AAGAGTTTGATCCTGGCTCAGGATT	53	1800	Deng & Hiruki, 1991
	P7	CGTCCTTCATCGGCTCTT	50	1248	
	R16F2	ACGACTGCTAAGACTGG			Schneider <i>et al.</i> , 1995; Lee <i>et al.</i> , 1995
	R16F2	TGACGGGCGGTGTGTACAAACCCCG			
Phyto-F Phyto-R Phyto-P <sup>1</sup>	CGTACGCAAGTATGAAACTTAAAGGA TCTCGAATTAACAACATGATCCA FAM-TGACGGGACTCCGCACAAGCG-NFQ <sup>2</sup>	60	75	Christensen <i>et al.</i> , 2004	
<b>Viruses</b>					
<i>Black raspberry necrosis virus</i>	Primer 1-F Primer 1-R	ATGCTGAGCCACTTGTGA CAATGTCTTGGAAGCCAC	55	417	Halgren <i>et al.</i> , 2007
<i>Blackberry chlorotic ringspot virus</i>	BCRV-F BCRV-R	GTTTCCTGTGCTCCTCA GTCACACCGAGGTACT	55	500	Tzanetakakis, <i>et al.</i> , 2007
<i>Blackberry virus Y</i>	BVY312F BVY695R	CTGTGGGGAGATTTGGAGAA TCATTCATGGGTGTGTC	53	383	Susaimuthu <i>et al.</i> , 2008
	BVY F BVY R	GAATTTGATGCAGAGGCCATA TGCTTTAAGTGAGCCTTTCCA	61	200	I. Tzanetakakis (pers comm)
<i>Blackberry yellow vein associated virus</i>	BYVaV CPF BYVaV CPR	AATCAACGGGAGAATGTTAT GGATTGGCAACGTCGG	55	458	Susaimuthu <i>et al.</i> , 2006
<i>Cherry rasp leaf virus</i>	JQ3D3FF JQ3D3FR	GCCAGTTTCTCCAGTGAACC CAGTTGAAACGGATTTAAAC	55	429	James <i>et al.</i> , 2001
<i>Raspberry ringspot virus</i>	RpRSV-F1 RpRSV-R1	TGTGTCTGGCTTTTGATGCT GAGTGCATAGGGGCTGTT	61	385	Ochoa-Corona <i>et al.</i> , 2005
<i>Rubus chlorotic mottle virus</i>	RuCMV 1082 RuCMV 1083	AGTCCTGGGTGTCCATCTTG AAGGCTTCTCAACGGTCTCA	57	325	McGavin & McFarlane, 2009
<i>Rubus yellow net virus</i>	RYN1 F RYN1 R	TCCAAAACCTCCAGACCTAAAAC ATAATCGCAAAGGCAAGCCAC	55	350	Jones <i>et al.</i> , 2002
<i>Tomato black ring virus</i>	TBRV-70F TBRV-70R TBRV-70P	GCTCGTAACAGTTGCGGAGATAT TGTCACACTGTCATGGGA FAM-TGCATAGGCTCACTCCTTGGGA-NFQ <sup>2</sup>	62	72	Harper <i>et al.</i> , 2011
<i>Tomato ringspot virus</i>	U1 D1	TCCGTCCAATCACGGAATA GACGAAGTTATCAATGGCAGC	55	449	Griesbach 1995
<b>Internal controls</b>					
Plant RNA control	Nad5-F Nad5-R	GATGCTTCTTGGGGCTTCTTGTT CTCCAGTCACCAACATTGGCATAA	50-60	181	Menzel <i>et al.</i> , 2002
Plant DNA control & Bacteria	Gd1 Berg54	ACGGAGAGTTTGATCCTG AAAGGAGGTGATCCAGCCGCACCTTC	50-62	1500	Andersen <i>et al.</i> , 1998
Plant DNA control	COX-F COX-R COX- P <sup>1</sup>	CGTCGCATTCCAGATTATCCA CAACTACGGATATATAAGAGCCAAAACCTG FAM-TGCTTACGCTGGATGGAATGCCCT-NFQ <sup>2</sup>	60	74	Weller <i>et al.</i> , 2000

<sup>1</sup>Real-time probe; <sup>2</sup>NFQ= Non-fluorescent quencher.

### 7.1.3.2.1 Virus reverse transcription-PCR (RT-PCR) and PCR

#### Recommended method for RNA viruses: Conventional RT-PCR

1. Extract total RNA according to a standard protocol. Successful RT-PCR amplification can be achieved using the following RNA extraction procedures:

- (a) RNeasy<sup>®</sup> Plant Mini Kit (Qiagen Cat. No. 74904) with a modified lysis buffer as described by MacKenzie *et al.* (1997); or
- (b) silica-based method as described by Menzel *et al.* (2002).

See Appendix 2 for details of these extraction methods. Alternative methods may also be used after validation.

2. Optional: Perform a one-step RT-PCR on the RNA with the *Nad5* internal control primers (Table 3) using the components and concentrations listed in Table 4 and cycle under the conditions listed in Table 6. The *Nad5* primers amplify mRNA from plant mitochondria.

3. Perform a one-step RT-PCR on the RNA with the pathogen-specific primers (Table 3) using the components and concentrations listed in Table 4 and cycle under the conditions listed in Table 6. The following controls must be included for each set of RT-PCR reactions:

- (a) positive control: RNA extracted from virus-infected leaf tissue or equivalent; and
- (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 *Rubus* 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 *Rubus* 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.

#### Recommended method for *Rubus yellow net virus* (DNA virus): Conventional PCR

1. Extract total DNA according to a standard protocol. Successful PCR amplification can be achieved using the Qiagen DNeasy<sup>®</sup> Plant Mini Kit (Qiagen Cat. No. 69104). Alternative methods may also be used after validation.

2. Optional: Perform a PCR on the DNA with the Gd1/Berg54 internal control primers (Table 3) using the components and concentrations listed in Table 5 and cycle under the conditions listed in Table 6. The Gd1/Berg54 primers amplify the 16S rRNA gene from most prokaryotes as well as from chloroplasts.

3. Perform a PCR on the DNA with the pathogen-specific primers (Table 3) using the components and concentrations listed in Table 5 and cycle under the conditions listed in Table 6. The following controls must be included for each set of PCR reactions:

- (a) positive control: DNA extracted from virus-infected leaf tissue or equivalent; and
- (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 *Rubus* leaf tissue) is included.

4. Analyse the PCR products by agarose gel electrophoresis.

## 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 3; and
- (b) no bands are produced in the negative control (if used) and the no template control.

If the *Nad5* or 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 181 bp band (*Nad5*) or a 1500 bp band (Gd1/Berg54). Failure of the samples to amplify with the internal control primers suggests that the RNA/DNA extraction has failed, compounds inhibitory to PCR are present in the RNA/DNA extract or the RNA/DNA has degraded. The effect of inhibitors may be overcome by adding Bovine Serum Albumin (BSA) to a final concentration of 0.5 µg/µl. Alternatively, DNA may be further purified using MicroSpin™ S-300 HR columns (GE Healthcare Cat. No. 27-5130-01).

**Table 4: Generic one-step RT-PCR reaction for RNA templates components using the SuperScript™ III One-step RT-PCR System with Platinum® Taq DNA polymerase**

Reagent	Volume per reaction (µl)
Sterile deionised water	4.2
10 × Reaction mix (Invitrogen)	10.0
5 µM Forward primer	1.0
5 µM Reverse primer	1.0
SuperScript™ III/ RT/ Platinum® Taq Mix (Invitrogen 12574-026)	0.8
10 mg/ml Bovine Serum Albumin (BSA) (Sigma A7888)	1.0
RNA template	2.0
<b>Total</b>	<b>20.0</b>

**Table 5: Generic PCR reaction components for DNA templates using GoTaq® Green Master Mix**

Reagent	Volume per reaction (µl)
Sterile deionised water	5.0
GoTaq® Green Master Mix (Promega M7122)	10.0
5 µM Forward primer	1.0
5 µM Reverse primer	1.0
10 mg/ml Bovine Serum Albumin (BSA) (Sigma A7888)	1.0
DNA template	2.0
<b>Total</b>	<b>20.0</b>

**Table 6: Generic PCR cycling conditions**

Step	Temperature	Time	No. of cycles
cDNA synthesis (RT-PCR only)	50°C	30 min	1
Initial denaturation	94°C	2 min	1
Denaturation	94°C	30 sec	40
Annealing	See Table 3	30 sec	
Elongation	72°C	45 sec (virus, bacteria) 1 min (phytoplasma)	
Final elongation	72°C	7 min	1

### Recommended method for RNA viruses: Real-time RT-PCR

1. Extract total RNA from leaf tissue according to a standard protocol (as described above).
2. Set-up the real-time RT-PCR using pathogen-specific primers (Table 3) and the components and concentrations listed in Table 7 and cycle under the conditions listed in Table 8. Please note that reaction and cycling conditions can be changed depending on the real-time reagents and machine used, but this would require validation.
3. Optional: Perform RT-PCR on the nucleic acid using the COX internal control primers (Table 3), and using the components and concentrations listed in Table 7 and cycle under the conditions listed in Table 8.
4. The following controls must be included for each set of reactions:
  - (a) positive control: RNA extracted from virus-infected leaf tissue or equivalent; and
  - (b) no template control: water is added instead of RNA template.
  - (c) healthy control: RNA extracted from healthy *Rubus* leaf tissue.
5. Analyse real-time amplification data according to the real-time thermocycler manufacturer's instructions.

**Table 7: Generic real-time RT-PCR for RNA templates using Invitrogen Superscript III One-step qRT PCR system**

Reagent	Volume per reaction (µl)
Sterile H <sub>2</sub> O	4.6
2 × Reaction Mix (Invitrogen 11730-017)	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
5 µM Dual-labelled fluorogenic probe	0.4
RNA	2.0
<b>Total</b>	<b>20.0</b>

**Table 8: Generic cycling conditions for real-time RT-PCR**

Step	Temperature	Time	No. of cycles
RT-Step	50°C	30 min	1
Initial denaturation	95°C	2 min	1
Denaturation	95°C	10 sec	40
Annealing	Table 3	45 sec	

### Interpretation of results for real-time RT-PCR

The real-time RT-PCR test will only be considered valid if:

- (a) the positive control produces an amplification curve with the pathogen-specific primers; and
- (b) no amplification curve is seen (i.e. cycle threshold [ $C_T$ ] value is 40) with the negative control (if used) and the no template control.

If the COX internal control primers are also used, then the negative control (if used), positive control and each of the test samples must produce an amplification curve. Failure of the samples to produce an amplification plot with the internal control primers suggests that the nucleic acid extraction has failed or compounds inhibitory to PCR are present in the nucleic acid extract or the nucleic acid has degraded.

### Virus positive controls for PCR

1. The following viruses may be obtained from the American Type Culture Collection (ATCC; <http://www.atcc.org>):
  - (a) *Tomato ringspot virus*; Cat. No. PV-100, PV-174, PV-239.

2. The following viruses may be obtained from the German Resource Centre for Biological Material (DSMZ; <http://www.dsmz.de>):
  - (a) *Raspberry ringspot virus*; Cat. No. PV-0307.
  - (b) *Tomato ringspot virus*; Cat. No. PV-0049, PV-0381, PV-0380.
3. *Cherry rasp leaf virus*, *Raspberry ringspot virus*, *Tomato black ring virus* and *Tomato ringspot virus* may be obtained from the commercial sources listed in Table 2.
4. *Black raspberry necrosis virus*, *Blackberry chlorotic ringspot virus*; *Blackberry virus Y*, *Blackberry yellow vein associated virus*, *Cherry rasp leaf virus*, *Raspberry ringspot virus*, *Rubus chlorotic mottle virus*, *Rubus yellow net virus*, *Tomato black ring virus* and *Tomato ringspot virus* 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.

#### 7.1.3.2.1.1 *Black raspberry necrosis virus*

*Rubus* species must be tested for *Black raspberry necrosis virus* by RT-PCR using the primer pair shown in Table 3. See section 7.1.3.2.1 for details of test methods and interpretation of results.

#### 7.1.3.2.1.2 *Blackberry chlorotic ringspot virus*

*Rubus* species must be tested for *Blackberry chlorotic ringspot virus* by RT-PCR using the primer pair shown in Table 3. See section 7.1.3.2.1 for details of test methods and interpretation of results.

#### 7.1.3.2.1.3 *Blackberry virus Y*

*Rubus* species must be tested for *Blackberry virus Y* by RT-PCR. As the two primer sets shown in Table 3 do not individually pick up all virus strains, plants must be tested with both primer pairs. Note RT-PCR for both BVY primer pairs must be done using the components and concentrations listed in Table 9 and cycling conditions shown in Table 10.

**Table 9: PCR reaction components for BVY one-step RT-PCR using GoTaq® Green Master Mix**

Reagent	Volume per reaction (µl)
Sterile deionised water	1.4
GoTaq® Green Master Mix (Promega M7122)	10.0
5 µM Forward primer	2.0
5 µM Reverse primer	2.0
10 mg/ml Bovine Serum Albumin (BSA) (Sigma A7888)	1.0
200 U/µl SuperScript™ III Reverse Transcriptase (Invitrogen 18080-044)	0.3
0.1 M DTT (Included with SuperScript™ III Reverse Transcriptase)	1.0
40 U/µl RNasin Plus (Promega (Thermofisher Scientific) PMM2611)	0.3
RNA template	2.0
<b>Total</b>	<b>20.0</b>

**Table 10: PCR cycling conditions for BVY one-step RT-PCR**

Step	Temperature	Time	No. of cycles
cDNA synthesis	50°C	30 min	1
Initial denaturation	94°C	2 min	1
Denaturation	94°C	30 sec	40
Annealing	See Table 3	30 sec	
Elongation	68°C	30 sec	
Final elongation	68°C	7 min	1

#### **7.1.3.2.1.4 *Blackberry yellow vein associated virus***

*Rubus* species must be tested for *Blackberry yellow vein associated virus* by RT-PCR using the primer pair shown in Table 3. See section 7.1.3.2.1 for details of test methods and interpretation of results.

#### **7.1.3.2.1.5 *Cherry rasp leaf virus***

*Rubus* species can be tested for *Cherry rasp leaf virus* by RT-PCR using the primer pair shown in Table 3. See section 7.1.3.2.1 for details of test methods and interpretation of results.

#### **7.1.3.2.1.6 *Raspberry ringspot virus***

*Rubus* species can be tested for *Raspberry ringspot virus* by RT-PCR using the primer pair shown in Table 3. See section 7.1.3.2.1 for details of test methods and interpretation of results.

#### **7.1.3.2.1.7 *Rubus chlorotic mottle virus***

It is optional to test *Rubus* species for *Rubus chlorotic mottle* by RT-PCR using the primer pair shown in Table 3. See section 7.1.3.2.1 for details of test methods and interpretation of results.

#### **7.1.3.2.1.8 *Rubus yellow net virus***

*Rubus* species must be tested for *Rubus yellow net virus* by PCR using the primer pair shown in Table 3. See section 7.1.3.2.1 for details of test methods (DNA template) and interpretation of results.

#### **7.1.3.2.1.9 *Tomato black ring virus***

*Rubus* species must be tested for *Tomato black ring virus* by real-time RT-PCR using the primer pair and probe shown in Table 3. See section 7.1.3.2.1 for details of test methods and interpretation of results.

#### **7.1.3.2.1.10 *Tomato ringspot virus***

*Rubus* species can be tested for *Tomato ringspot virus* by RT-PCR using the primer pair shown in Table 3. See section 7.1.3.2.1 for details of test methods and interpretation of results.

### **7.1.3.2.2 Phytoplasma and bacteria PCR**

#### **Recommended method for phytoplasma: Conventional PCR**

1. Extract total DNA from leaf petioles and mid-veins according to a standard protocol. Successful PCR amplification can be achieved using the following DNA extraction procedures:
  - (a) DNeasy<sup>®</sup> 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 2 for details of these extraction methods. Alternative methods may also be used after validation.
2. Optional: Perform a PCR with the Gd1/Berg54 internal control primers (Table 3) using the components and concentrations listed in Table 5 and cycle under the conditions listed in Table 6. The Gd1/Berg54 primers amplify the 16S rRNA gene from most prokaryotes as well as from chloroplasts.

3. Perform a nested PCR on the purified DNA using the universal phytoplasma primer pair, P1/P7 (Table 3), for first-stage PCR followed by the R16F2/R16R2 primer pair for the second-stage PCR (Table 3).
4. Set up the first-stage and second-stage PCR reactions using the components and concentrations listed in Table 5 and cycle under the conditions listed in Table 6. The first-stage PCR products, including the controls, are diluted 1:25 (v/v) in water and 2 µl used as template in the second-stage PCR. The following controls must be included for each set of PCR reactions:
  - (a) positive control: for phytoplasma, total DNA or a cloned fragment from the appropriate organism may be used. If the internal control primers are not used, then the DNA must be mixed with healthy *Rubus* DNA to rule out the presence of PCR inhibitors; and
  - (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 *Rubus* leaf tissue) is included.
5. Analyse the PCR products (second-stage PCR products only) by agarose gel electrophoresis.

#### **Recommended method for bacteria: Conventional PCR**

1. Extract total DNA from leaf petioles and mid-veins. Successful PCR amplification can be achieved using DNA extracted with the DNeasy<sup>®</sup> Plant Mini Kit (Qiagen Cat. No. 69104). An alternative method may also be used after validation.
2. Optional: Perform a PCR with the Gd1/Berg54 internal control primers (Table 3) using the components and concentrations listed in Table 5 and cycle under the conditions listed in Table 6.
3. Perform a PCR with the bacteria-specific primers on the purified DNA using the components and concentrations listed in Table 5 and cycle under the conditions listed in Table 6. The following controls must be included for each set of PCR reactions:
  - (a) positive control: total DNA or a cloned fragment from the appropriate bacteria may be used. If the internal control primers are not used, then the DNA must be mixed with healthy *Rubus* 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 *Rubus* leaf tissue) is included.
4. Analyse the PCR products by agarose gel electrophoresis.

#### **Interpretation of results for conventional PCR**

The pathogen-specific PCR test will only be considered valid if:

- (a) the positive control produces the correct size product as indicated in Table 3; 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 1500 bp band. Failure of the samples to amplify with the control primers suggests that either the DNA extraction has failed or compounds inhibitory to PCR are present in the DNA or the DNA has degraded. The effect of inhibitors may be overcome by adding Bovine Serum Albumin (BSA) to a final concentration of 0.5µg/µl. Alternatively, DNA may be further purified using MicroSpin<sup>™</sup> S-300 HR columns (GE Healthcare Cat. No. 27-5130-01).

### Recommended method for phytoplasma and bacteria: Real-time PCR

6. Extract total DNA from leaf petioles and mid-veins according to a standard protocol (as described above).
7. Set-up the real-time PCR using pathogen-specific primers (Table 3) and the components and concentrations listed in Table 11 for bacteria **or** Table 12 for phytoplasma, and cycle under the conditions listed in Table 13. The reaction and cycling conditions can be changed depending on the real-time reagents and machine used, but this would require validation.
8. Optional: Perform PCR on the nucleic acid using the COX internal control primers (Table 3), and using the components and concentrations listed in Table 11 for bacteria **or** Table 12 for phytoplasma and cycle under the conditions listed in Table 13.
9. The following controls must be included for each set of reactions:
  - (a) Positive control: For phytoplasma and bacteria, total DNA or a cloned fragment from the appropriate organism may be used. If the internal control primers are not used, then the DNA must be mixed with healthy *Rubus* DNA to rule out the presence of PCR inhibitors; and
  - (b) no template control: water is added instead of DNA template
10. When setting up the test initially, it is advised that a negative control (DNA extracted from healthy *Rubus* leaf tissue) is included.
11. Analyse real-time amplification data according to the real-time thermocycler manufacturer's instructions.

### Interpretation of results for real-time PCR

The real-time PCR test will only be considered valid if:

- (a) the positive control produces an amplification curve with the pathogen-specific primers; and
- (b) no amplification curve is seen (i.e. cycle threshold [ $C_T$ ] value is 40) with the negative control (if used) and the no template control.

If the COX internal control primers are also used, then the negative control (if used), positive control and each of the test samples must produce an amplification curve. Failure of the samples to produce an amplification plot with the internal control primers suggests that the DNA extraction has failed or compounds inhibitory to PCR are present in the DNA extract or the DNA has degraded. The effect of inhibitors may be overcome by adding Bovine Serum Albumin (BSA) to a final concentration of 0.5 µg/µl. Alternatively, DNA may be further purified using MicroSpin™ S-300 HR columns (GE Healthcare Cat. No. 27-5130-01).

**Table 11: Generic real-time PCR for bacteria using Invitrogen Platinum® qPCR SuperMix-UDG**

Reagent	Volume per reaction (µl)
Sterile H <sub>2</sub> O	4.6
2 × Reaction Mix (Invitrogen 11730-017)	10.0
10 µg/µl Bovine Serum Albumin (BSA) (Sigma A7888)	1.2
5 µM Forward primer	1.2
5 µM Reverse primer	0.5
5 µM Dual-labelled fluorogenic probe	0.5
DNA	2.0
<b>Total</b>	<b>20.0</b>

**Table 12: Generic real-time PCR for phytoplasma using Roche LightCycler 480 Probes Mastermix**

Reagent	Volume per reaction (µl)
Sterile H <sub>2</sub> O	4.3
2 x Reaction Mix (Roche 04707494001)	10.0
10 µg/µl Bovine Serum Albumin (BSA) (Sigma A7888)	0.8
5 µM Forward primer	1.2
5 µM Reverse primer	1.2
5 µM Dual-labelled fluorogenic probe	0.5
DNA	2.0
<b>Total</b>	<b>20.0</b>

**Table 13: Generic cycling conditions for real-time PCR**

Step	Temperature	Time	No. of cycles
UDG incubation hold (Invitrogen only)	50°C	2 min	1
Initial denaturation	95°C	2 min (Invitrogen) 5 min (Roche)	1
Denaturation	95°C	10-15 sec	40
Annealing	See Table 3	40 sec	

#### Phytoplasma and bacterial positive controls for PCR

1. *Xylella fastidiosa*; ICMP No. 6575, 6576, 8729-8745, 8693, 8694 may be obtained from the Landcare Research International Collection of Micro-organisms from Plants (ICMP; <http://www.landcareresearch.co.nz/research/asp>).
2. Positive control DNA for *Xylella fastidiosa* and phytoplasmas 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.

##### 7.1.3.2.2.1 Black raspberry witches' broom phytoplasma

*Rubus* species must be tested for Black raspberry witches' broom phytoplasma by conventional nested or real-time PCR using the universal primers listed in Table 3. See section 7.1.3.2.2 for details of test methods and interpretation of results.

##### 7.1.3.2.2.2 Rubus stunt phytoplasma

*Rubus* species must be tested for Rubus stunt phytoplasma by conventional nested or real-time PCR using the universal primers listed in Table 3. See section 7.1.3.2.2 for details of test methods and interpretation of results.

##### 7.1.3.2.2.3 Xylella fastidiosa

*Rubus* species must be tested for by *Xylella fastidiosa* by conventional or real-time PCR using the primer pairs listed in Table 3. See section 7.1.3.2.2 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 of *Rubus* spp. 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 viruses:

- *Tomato black ring virus*
- *Tomato ringspot virus* (strains not in New Zealand)

It is optional to test seed for sowing for the following viruses

- *Raspberry ringspot virus*

See Table 1 for the required or alternative tests for these pathogens and the relevant sections 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 in the same way 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.

Tests for the following viruses must be carried out:

- *Tomato black ring virus*
- *Tomato ringspot virus* (strains not in New Zealand)

It is optional to test seed derived from mother plants fertilised with imported pollen for the following viruses:

- *Raspberry ringspot virus*

See Table 1 for the required or alternative tests for these pathogens and the relevant sections for details of the test methods and interpretation of results.

## **8. CONTACT POINT**

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## Appendix 1. Symptoms of significant regulated pests of *Rubus*

### 1.1 *Black raspberry streak virus*



Severe Black raspberry streak on primocane of wild black raspberry. (Courtesy of R.H. Converse; reproduced from the Diseases of Small Fruits CD-ROM, 2000, APS, St Paul, MN, USA).

### 1.2 *Black raspberry necrosis virus*



Black raspberry leaf with symptoms of *Black raspberry necrosis virus*. (Courtesy of USDA-ARS; <http://www.ars-grin.gov/cor/Rubus/ribsymp/ribsymp.html>).

### 1.3 *Blackberry calico virus*



A leaf of ‘Thornless loganberry’ infected with *Blackberry calico virus*. (Courtesy of R.H. Converse; reproduced from the Diseases of Small Fruits CD-ROM, 2000, APS, St Paul, MN, USA).

### 1.4 *Raspberry leaf curl virus*

(a)



(b)



(a) Leaf chlorosis and distortion in ‘New Logan’ black raspberry chronically infected with *Raspberry leaf curl virus*. (b) Dwarfing, chlorosis and leaf distortion in ‘Lloyd George’ red raspberry chronically infected with *Raspberry leaf curl virus* (Courtesy USDA Agriculture Handbook 631; reproduced from the Diseases of Small Fruits CD-ROM, 2000, APS, St Paul, MN, USA).

### 1.5 *Raspberry ringspot virus*



*Raspberry ringspot virus* infection in raspberry 'Malling Jewel', showing chlorotic blotches. (Courtesy of SCRI-Dundee Archive, Scottish Crop Research Institute; <http://www.insectimages.org>).

### 1.6 *Tomato ringspot virus*

(a)



(b)



(a) Crumbly fruit of red raspberry infected with *Tomato ringspot virus*. (b) Foliar ringspot and line patterns on red raspberry infected with *Tomato ringspot virus*. (Courtesy F.D. McElroy; reproduced with permission from the Diseases of Small Fruits CD-ROM, 2000, APS, St Paul, MN, USA).

## Appendix 2. Protocols referenced in the manual

### 2.1 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<sup>®</sup> Plant Mini Kit (Qiagen Cat. No. 74904) instruction handbook.

### 2.2 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 (10mM 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.

### 2.3 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<sub>2</sub>HPO<sub>4</sub>, 0.03 M NaH<sub>2</sub>PO<sub>4</sub>, 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 4°C for 5 min at 4,500 rpm.
3. Transfer supernatant into a new 2 ml microcentrifuge tube and centrifuge at 4°C for 15 min at 13,000 rpm.
4. Discard the supernatant.

5. Resuspend the pellet in 750  $\mu$ l hot (55°C) 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  $\mu$ l) then the remaining volume of CTAB buffer is added once the pellet has been resuspended.
6. Incubate at 55°C for 30 min with intermittent shaking then cool on ice for 30 seconds.
7. Add 750  $\mu$ l chloroform:octanol (24:1 v/v), vortex thoroughly and centrifuge at 4°C 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 4°C or at room temperature for 10 min at 13,000 rpm. Discard supernatant.
10. Wash DNA pellet with 500  $\mu$ l 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  $\mu$ l sterile distilled water. Incubating the tubes at 55°C for 10 min can aid DNA resuspension.
13. Store DNA at -20°C for short term storage or -80°C for long term storage.