Chapter 1 Administrative

Introduction

Our base funding supports four scientists plus an excellent technical support crew including biological and agricultural science research technicians, program assistant, information technology specialist, and facilities technician. The constant, which has inspired a team effort throughout continuing challenges of low funding cycles, continues to be the golden vision that our staff shares:

PRESERVE DIVERSE PLANT GENETIC RESOURCES FOR ALL PEOPLE FOR ALL TIME.

I thank the following individuals for the latest Revision of the Operations Manual for the USDA-ARS facilities at Corvallis, Oregon, and Palmer, Alaska. I applaud their continuing efforts to enact these procedures towards the collection, preservation, distribution and evaluation of the temperate, arctic and subarctic specialty crops assigned to Corvallis and Palmer genebanks.

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USDA ARS CRIS Project

CRIS 5358-21000-038-00D - National Clonal Germplasm Repository, Corvallis, Oregon

Management of temperate adapted fruit, nut, and specialty crop genetic resources and associated information

Mission

The executive priorities of these management units are 1) FOOD SECURITY, through conservation of plant genetic resources, and 2) GLOBAL CLIMATE CHANGE, through research in plant adaptation to changing environments.

This genebanks was established to collect, maintain, distribute and evaluate germplasm of an array of temperate, arctic, and subartic specialty crops including: *Actinidia, Corylus, Fragaria, Humulus, Mentha, Pyrus, Ribes, Rubus, Vaccinium* and other specialty crops. The research program directly supports agriculture and horticulture in the temperate and region. The research pursued here adheres closely to the practical problems of germplasm storage and maintenance, including in vitro culture and cryopreservation, virus and disease testing, identity verification, and seed and clonal propagation techniques. We are a genebank for invaluable clonal plant resources. We will preserve this diverse living germplasm for all people for all time.

Motto

Plant diversity for a diverse future.

Guiding Principles

Preserve diverse genotypes of assigned genera of temperate, arctic, and subarctic specialty crops.
Distribute plant genetic resources to international researchers.
Operate and act safely with consideration for all employees, collaborators, and the public.
Operate and act without discrimination to race, color, religion, sex, national origin, age or handicap.

Goals

The Corvallis genebank staff has identified major goal areas:

- Collection-preservation: To preserve assigned clonal plant collections in good health and identity.
- Administration-workplace: To effectively manage resources to operate USDA-ARS facilities.
- Information management: To collect, organize, and provide accurate information on plant resources.
- Distribution: To provide plant material to researchers worldwide.
- Pathology: To identify and eliminate germplasm borne pathogens.
- New acquisitions-collections: To locate, collect, and obtain plants to represent world diversity of assigned genera.
- Research: To improve the preservation of the collection, gather information and collaborate with other scientists on crop development.
- Public Relations: To inform and serve the public.

Objectives

1) The NCGR-Corvallis will conserve global genetic resources for hazelnuts, strawberries, hops, mint, currants, gooseberries, blackberries, raspberries, hybrid berries, blueberries, cranberries, lingonberries, kiwifruit, pawpaws, juneberries, mountain ash, quince, elderberries, and graft- or cross- compatible relatives and other edible fruits. Clones will be preserved by keeping living plants in orchards and field collections, in greenhouses and

screenhouses, as tissue cultured plants, and as meristems or other plant parts in cryogenic preservation. Seeds and pollen of species relatives will be stored in freezers and in liquid nitrogen. Pathogen testing- and elimination-procedures will be performed on clonal germplasm. Botanical and horticultural identity of genetic resources will be confirmed through phenotypicl and genotypic approaches.

2) We will develop new or improved short-, medium-, and long-term preservation procedures for the assigned genetic resources. Improvements in micropropagation for selected difficult-to-culture accessions will be developed and improvements in contaminant control will be developed. Medium-term storage techniques and evaluation procedures will be developed to improve the duration of in vitro storage of germplasm collections. Cryopreservation techniques for additional genera will be developed. Genetic stability of cryopreserved meristems will be studied. Cryopreservation technology will be made available to other genetic resources facilities through workshops and manuals.

3) Genetic marker-based approaches for genetic diversity assessment of the genera in the collections will be developed. Clonal fingerprinting techniques will be developed as baselines for clonal horticultural identity recognition. Genetic diversity for other assigned crops will be examined.

4) The NCGR-Corvallis will annually distribute > 5,000 accessions of the genetic resources of specialty crops. Web pages will be developed to transfer information to researchers and the public concerning crop evaluation and unique clonal characters. Order processing and database management will be integrated with the GRIN system. Passport information including pedigree or collection locality, inventory maintenance, and evaluation information will be maintained on the Germplasm Resource Information Network (GRIN) database.

Summary

The major CRIS is assigned to preserve, collect, distribute, and evaluate genetic resources of about 60 temperate and arctic climate genera. These genera fall into the general categories of small fruits, tree fruit, nuts, herbaceous perennials, revegetation, and agronomic crops.

Many countries and every state in the US produces berry crops of those assigned to the Corvallis Repository. Strawberries rank in the top 20 most valuable commercial agricultural crops in the country. Pears, hazelnuts (filberts), and hops, while being crops of regional commercial importance, are also of increasing interest throughout the country. In Alaska, rhubarb has large production. In addition, herbaceous peonies are becoming an expanding economic industry for summer production national and international exports. These high value specialty crops assigned to the Corvallis and Palmer genebanks offer important and healthy variation to American diets, bountiful ornamental crops to beautify our surroundings, and add unique flavors for food products. The fresh and processed value of these crops is significant to domestic markets and international trade. Nursery crop production is also a significant client of genetic resources produced at the genebanks.

The economic importance of these crops is increasing internationally. Many countries are entering in competition to produce these crops as transportation and storage survivability becomes easier. The wild diversity for these crops is great at present, but concern exists as native habitats are encroached by development, and old selections become unavailable through neglect. Native habitats include locations on six continents from sea level to 3000 meters elevation, and from arctic through sub-tropical and tropical zones. Several of the economically important genera include crops with centers of origin in North America. Several of these genera have cultivars which are three to four hundred years old. The mission, objectives, and actions of the repository are essential to the improvement of cultivars and continued or increased use of these high-value specialty crops.

Related practical research is underway concerning taxonomy and cytology of mint, strawberries, blackberries, and raspberries; restriction sites in nuclear and chloroplast genomes of currants and gooseberries; seed germination of raspberries and blackberries; virus detection and elimination in strawberries, hazelnuts, pears, and blueberries and cranberries. Viroid and phytoplasma elimination are now added to health certification of plant genetic resources.

Development of a cooperative plan for in situ preservation of cranberries in the Middle Atlantic States is under development with the US Forest Service, State Heritage Botanists, and other ARS cooperators. Development of efficient methods for germplasm preservation is a high priority throughout the world. Alternative storage and back-up technology is needed to safeguard the traditional preservation methods of orchards, fields, screenhouse and greenhouse-grown plants. These alternative technologies may be labor, space, and cost efficient. In vitro techniques aid in virus elimination when combined with thermotherapy. Virus tested in vitro cultured plants provide clean material for plant distribution, satisfying many quarantine regulations.

The in vitro and cryogenic research laboratory at Corvallis has been a drawing card for international cooperation on clonal research projects. Scientists from Brazil, Egypt, India, Malaysia, Poland, and Russia have spent short terms of study here. The cryogenic techniques under research may provide long term storage for Corylus seed, for which traditional storage life is considered to be less that one year. Major breakthroughs have been noted using differing carbon sources and hormones to improve initiation, growth and rooting of hazelnuts and pears in vitro cultures. The research in bacterial contamination of plant tissue cultures has broad-reaching implications for the in vitro commercial industry, besides the applications in preservation of genetic resources.

How is the Program Unique?

The Corvallis genebank is unique in the world for accumulating the diversity and global representation of wild and cultivated genetic resources of the assigned genera. Fruit genebanks of other countries have tended to specialize in genotypes and species of regional interests. To summarize the program by genus:
Actinidia (hardy kiwifruit): initial representation of cultivated genotypes; beginning representation of world species collections. Some materials from Russia and China.
Corylus (hazelnuts): excellent representation of world genotypes including those from Spain, Italy, Yugoslavia, Romania, France, and New York and Oregon in the United States. Wild species are represented from the US, China, Korea, Republic of Georgia, Azerbaijan, and Europe.
Fragaria (strawberries): excellent representation of the hybrid cultivars from breeding programs in about 20 US states and about 20 countries. Recent collecting trips have brought wild representatives from Chile, the Pacific Northwest, Northern Rocky Mountains, Canada, Alaska, and Japan
Mentha (mint): very good representation of commercial cultivars; excellent representation of world species *Paeonia* (peonies): Beginning representation of cultivated peonies and main species.
Pyrus (pears): excellent representation of European cultivars, very good representation of Asian cultivars; the most comprehensive species collection in the world (some gaps and taxonomic questions from Asia); good representation of intergeneric hybrids.
Ribes (currants and gooseberries): very good representation of cultivated currants and gooseberries; very good representation of North American species (gaps from South America and Asia).
Rheum (Rhubarb): Three species and seventy-five major cultivars are represented.
Rubus (blackberries and raspberries): very good representation of raspberry and blackberry cultivars; very good representation of Chinese species, some South American representatives; good representation of world species.
Vaccinium (blueberries and cranberries): good representation of blueberry and cranberry cultivars; very good representation of lingonberry cultivars; very good representation of North American species, some examples of South American, Japan, Russian Far East, and European species.

Other Specialty Horticultural Genera (Corvallis)

- *Amelanchier* (shadbush): fair representation of cultivars and species
- *Arbutus* (manzanita): poor representation of cultivars or species
- *Asimina triloba* (Pawpaw): variety trial planting started 1995
  (preserved by the satellite repository at Kentucky State University)
- *Cydonia* (quince): good representation of cultivars and species
- *Escallonia* : poor representation of South American shrubby species
- *Gaultheria* (salal): poor representation of species
- *Gaylussacia* (huckleberry): poor representation of species
- *Holodiscus* (ornamental shrub): poor representation of species
- *Lonicera edulus* (edible fruited honeysuckle): wild collections of Asian species

Juglans cinerea (butternut): fair representation of cultivars
Mespilus (mespilus): fair representation of cultivars
Peraphyllum (shrub): poor representation of species
Pycnanthemum (mint relative): very good representation of species
Sambucus (elderberry): poor representation of cultivars and species
Sorbus (mountain ash): fair representation of species

Permits

Quarantine
Title 7 - Agriculture CFR 319.37 and its revisions, describe the plant quarantine programs in place for plant importation. The Repository works with federal quarantine officials to insure that these regulations are followed. We work with USDA-APHIS as closely as possible to coordinate entry of foreign plant materials.
The USDA APHIS now has a website where request for new and renewed permits can be made on-line.

http://www.aphis.usda.gov/plant_health/permits/plantproducts.shtml

We have been granted permits for:

- Post-Entry Quarantine, Permit Number 37-71362. Plant material under this permit is initially inspected in Seattle, USDA-APHIS, and resides on the Corvallis Repository property. The plants are annually inspected by state inspectors (under USDA-APHIS auspices) until released.
- Permit for importation of noxious weeds for importation of *Rubus fruticosus* and *Rubus moluccanus*. This permit was issued in 1992 and in valid for indeterminate amount of time.
- APHIS Departmental Permit No. 61784 for strawberry importation .
- APHIS Departmental Permit No. 61934 for importation of pathogen infected pears in quarantine at Beltsville to assist with heat-therapy and meristem culture
- APHIS Departmental Permit No. 62051 for importation of apple scar skin infected plant materials for research purposes, granted to Joseph D. Postman, November 9, 1995.

Humulus : The presence of races of hop powdery mildew on different continents has caused more stringent requirements for hop importation. Hops require an APHIS Departmental permit from foreign countries. Oregon State has quarantined *Humulus* because of powdery mildew races. A Director's Exemption is required to bring in hops from out of state.

Pyrus is a prohibited genus from every country with about 6 exceptions. Most Pyrus germplasm is susceptible to fireblight. We are helping the national quarantine center all that we can with virus elimination procedures. Some pears requested in 1981, received in 1984 remain in the National Quarantine. These comments apply to *Pyrus* relatives such as *Sorbus, Amelanchier, Mespilus, and Cydonia*.

Ribes is a prohibited genus from all countries except Canada. Plant material is imported through APHIS at Beltsville, Maryland.

Rubus is a restricted genus if certified free of rubus stunt, but prohibited if not. Plant material can move into the US from Canada without US restriction.

Vaccinium: some foreign germplasm to import. *Vaccinium* is a restricted genus and can enter under post-entry quarantine.

Priority Genera

The following genera are assigned to the Corvallis and Palmer Repositories. These genera are ranked according to the current value of the crop to the United States, to the world, and on anticipated value of potential new crops, and intangible values (medicinal or other properties).

Priority 1 - Major genera

GENUS	**CROP**	**US VALUE $ million 2003**	**US Trend**	**2002 World Production (MT)**	**2003 World Production (MT)**	**2007 World Production (MT)**	**2008 World Production (MT)**	**World Trend**
Fragaria	strawberries	1,220	dec	3,237,533	3,165,314	3,998,280	4068454	inc.
Vaccinium	blueberries cranberries	394	inc stable	207,174 303,468	227,570 311,150	279,323 375,890	331,347 440,388	inc. inc.
Pyrus	European pears Asian pears	297	inc	17,115,205	16,988,881	20,612,142	20,998,473	Inc.
Rubus	raspberries blackberries	100	inc inc	414,031	415,836 ------	507,653	458,885	dec. inc.
Mentha	Mint	103	stable	43,730	60,730	73,815	72,285	inc.
Corylus	hazelnuts	19	stable	842,981	830,567	812,645	1,052,001	inc.
Humulus	Hops	16.8	stable	98,140	96,218	119,012	135,254	inc
Actinidia	Kiwifruit	15	stable	996,867	990,306	1,240,447	1,308,424	stable
Ribes	black currants gooseberries	5	inc	653,417 177,694	644,950 117,014	714,560 136,297	602,621 114,217	inc. stable

Priority 2 - Minor Genera

GENUS	**CROP**	**REASON FOR NCGR-CORVALLIS PLACEMENT**
9. Juglans cinerea	butternuts	Davis, CA cannot hold these because of canker quarantine.
10. Asimina	pawpaws	new crop potential, medicinals, natural pesticides, US native
11. Cydonia	quince	dwarfing rootstocks for pear
12. Sorbus	mountain ash	rootstock for pear, ornamentals
13. Intergeneric Crosses		horticultural novelties, rootstocks, genetic studies
14. Amelanchier	shadbush	crop in Northern US, Canada, rootstock for pear
15. Mespilis	medlar	crop in Italy, rootstock for pear, ornamental
16. Sambucus	elderberry	home owner crop in US, juice and wine production Europe
17. Pycnanthemum	mint relative	potential medicinal, US natives
18. Peraphyllum	peraphyllum	rootstock for pear, US native
19. Gaylussacia	huckleberry	blueberry relative, US natives
20. Galtheria	salal, others	blueberry relative, US natives
21. Others	miscellaneous	Arbutus, Ceanothus, Eriobotrya, Escalonia, Holodiscus US natives - ornamentals, in GRIN, grow well in Oregon

State of the Database

Corvallis records are up-to-date locally and on the GRIN database.
We presently enter data to our local database and upload this information periodically to the GRIN system. We have a hardwire link to the internet through Oregon State University. The Repository server is electronically backed up by a tape drive system each week. Tapes are stored in a fireproof cabinet on-site. Each winter we send copies of our database as hardcopies and in magnetic media to NCGRP for archiving and as a data security backup.

Core

Initial core subsets have been designated for the following crops. The core representatives have been flagged on the GRIN accession record.

GENUS	NUMBER ACCESSIONS IN CORE
Actinidia	
Corylus	
Fragaria	
Humulus	
Lonicera caerulea	
Pyrus	
Ribes	
Vaccinium	

Backup Collections

Planned location of primary and back-up collections to be established/maintained during 1998 through 2004.

Crop		Primary Collection		NPGS Back-up preservation		
		Location	Type	Location	Type	Plant Material
Corylus	cultivars species	33447 Peoria Rd	field	NCGR cooler	TC	plantlets (core)
				NCGRP	LN2	embryonic axes
				NCGR dewar	LN2	embryonic axes, pollen
Fragaria	cultivars species	33447 Peoria Rd	screenhouse 8, 9	NCGR cooler	TC	plantlets
				NCGR	LN2	meristems (core)
	species	33447 Peoria Rd	seed freezer	NCGRP	-20	Seed
Humulus	male cultivars	33707 Peoria Rd	field			
	female cultivars	Rt 34 Botany Farm	field	NCGR cooler	TC	plantlets
	species	33447 Peoria Rd	seed freezer	NCGRP	-20	Seed
Mentha	cultivars species	33447 Peoria Rd	Greenhouse 2	NCGR shadehouse	TC	plantlets
				NCGRP	LN2	meristems
Pyrus	cultivars species	33447 Peoria Rd	field	NCGR	TC	plantlets (core)
				NCGR	tubehouse	grafted, potted
	species	33447 Peoria Rd	seed freezer	NCGRP	-20	Seed
				NCGRP	LN2	meristems
Ribes	cultivars species	33447 Peoria Rd	field	NCGR	TC	plantlets (core)

				NCGR	LN2	meristems
	species	33447 Peoria Rd	seed freezer	NCGRP	-20	Seed
				NCGRP	LN2	meristems
Rubus	cultivars species	33447 Peoria Rd	screenhouse 5, 6	NCGR	TC	plantlets (core)
	species	33707 Peoria Rd	field	NCGR dewar	LN2	meristems
Vaccinium	cultivars	33447 Peoria Rd	screenhouse 10	NCGR	TC	plantlets (core)
	species	33447 Peoria Rd	field	NCGR	TC	plantlets (core)
	species	33447 Peoria Rd	seed freezer	NCGRP	-20	Seed
Minor Genera	cultivars species	33707 Peoria Rd	field			

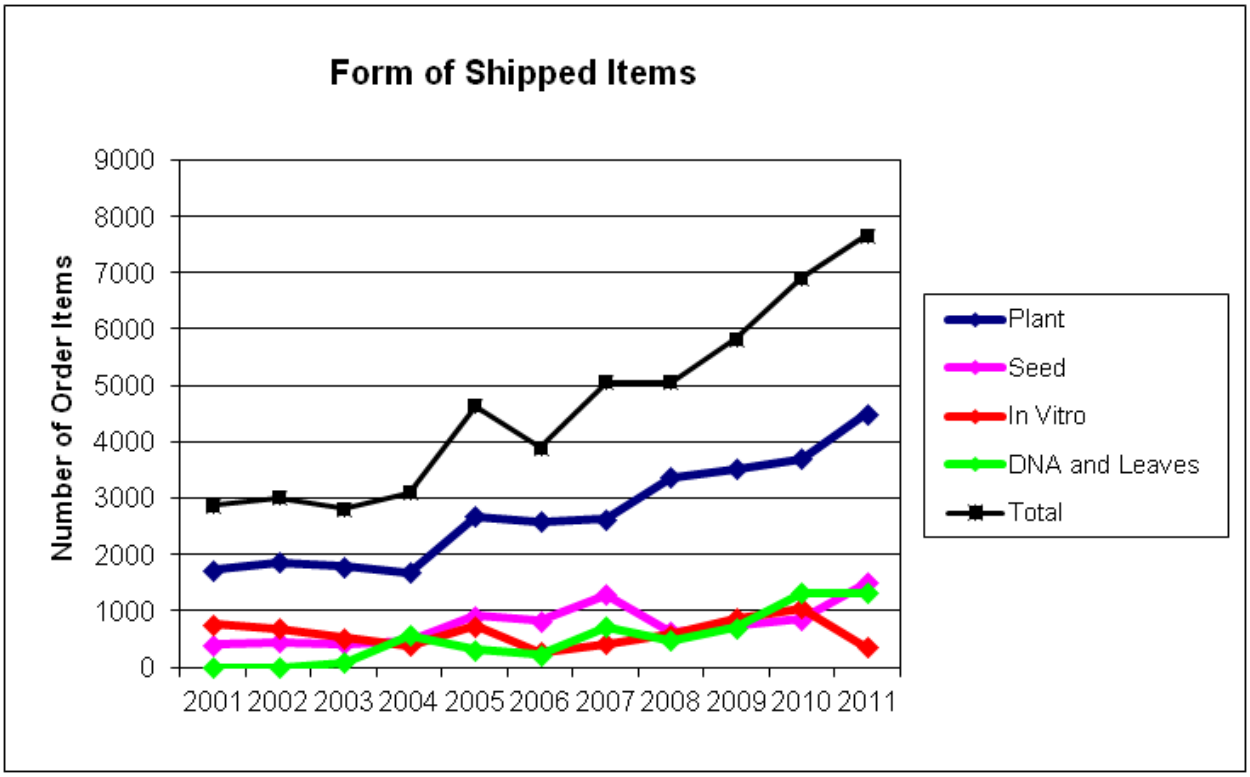
Corvallis Backup as of FY 2010

GENUS	**In Vitro**	**LN2**	**Other Locations**
Corylus	50	50 pollen 2 species embryonic	Oregon State University Department of Horticulture, Corvallis, OR
Fragaria	187	some experimental	CCG, Harrow, Ontario Canada
Humulus	86	25	NCGRP, Ft. Collins, CO
Mentha			Delaware State College
Pyrus	179	39 meristems COR 15 meristems NCGRP some pollen	Oregon State University, Medford, OR USDA-ARS, Kearneysville, MD Seed Saver's Exchange, Decorah, IA
Ribes	50	some experimental	CCG, Harrow, Ontario Canada
Rubus	182	14 meristems COR	CCG, Harrow, Ontario Canada
Vaccinium	120	some experimental	Rutgers University, Chatsworth, NJ
Subtotal	1021		
Minor Genera	0	-----	
TOTAL	1021		

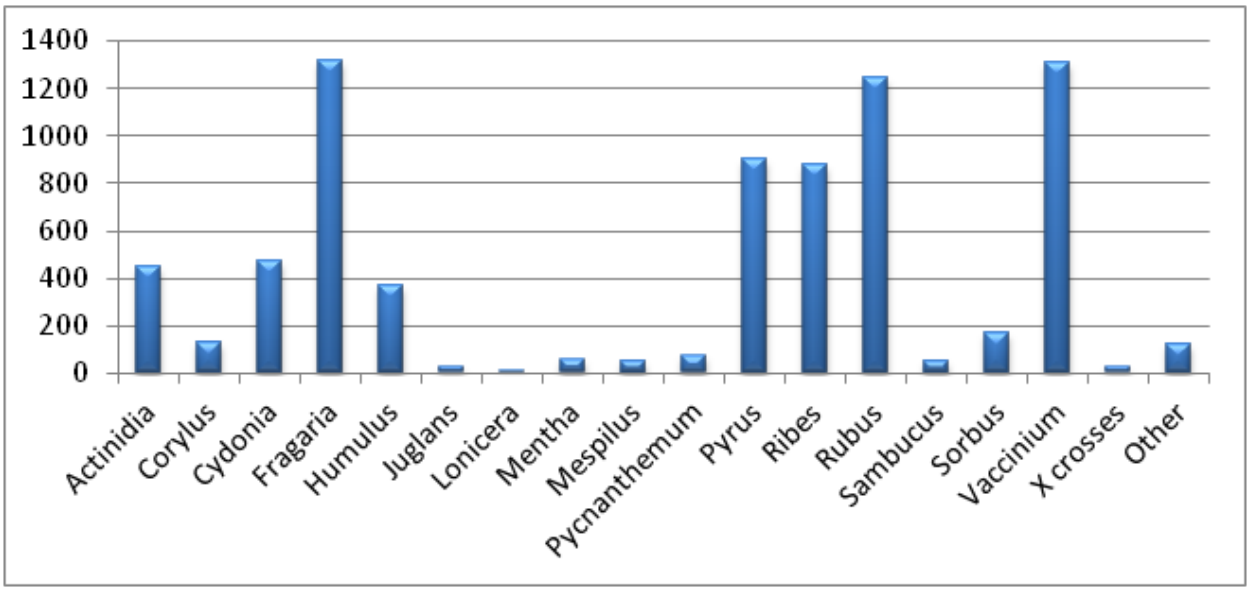
Corvallis Collections Summary, April 2012

Genus	Accessions	Taxa	Countries	Genus	Accessions	Taxa	Countries
Actinidia	107	9	7	*Juglans*	26	3	4
Amelanchier	50	11	7	*Lonicera*	79	8	7
Amelasorbus	1	1	1	*Lycium*	8	4	5
Arbutus	2	1	1	*Malus*	8	4	3
Aronia	8	2	2	*Mentha*	540	36	48
Asimina	48	8	2	*Mespilus*	58	2	11
Castanea	80	5	8	*Peraphyllum*	8	1	2
Ceanothus	40	11	2	*Physocarpus*	1	1	1
Celtis	1	1	1	*Potentilla*	5	3	3
Chaenomeles	13	4	3	*Psammisia*	1	1	1
Cornus	1	1	1	*Pseudocydonia*	2	1	2
Corylus	744	21	39	*Pyracomeles*	1	1	1
Crataegomespilu	3	1	1	*Pyronia*	7	1	4
Crataegosorbus	1	1	1	*Pyrus*	2152	36	59
Crataegus	17	7	5	*Ribes*	1299	110	40
Crataemespilus	1	1	1	*Rubus*	1979	172	55
Cydonia	125	1	17	*Sambucus*	184	20	25
Cynodon	5	1	1	*Schisandra*	11	3	3
Duchesnea	5	1	3	*Sibbaldia*	2	1	2
Empetrum	9	1	5	*Sorbaria*	1	1	1
Epigaea	1	1	1	*Sorbaronia*	7	4	3
Fagus	1	1	1	*Sorbocotoneaste*	3	2	3
Fragaria	1755	44	40	*Sorbopyrus*	11	2	7
Fragotentilla	3	1	1	*Sorbus*	146	48	24
Gaultheria	31	14	4	*Styrax*	1	1	1
Gaylussacia	21	6	2	*Symphysia*	1	1	1
Hippophae	2	1	2	*Vaccinium*	1622	67	33
Holodiscus	4	3	2	*Zoysia*	5	2	1
Humulus	633	8	21	**Total**	**11880**	**704**	**N.A.**

Distribution Summary 2001 to 2011



Distributions by genus in 2011



Budget Estimates for FY 2010

ARMP Code	Function Codes	in $1,000	
1000, 6000	Salary Total (exclude DV)		1,152
2100	Travel		10
2500	Contracts/Services		10
IRC			0
910 and 915	Repair and Maintenance (includes DV salary)		0
	AO support cost		0
	Utilities (includes improvements, supplies, etc.)		0
TG, DV, KH	Total IRC cost	192	192
			0
2554	Research Support (OSU)		10
3100	Equipment		0
2600	Supplies and Materials		0
3200	Land and Structure		0
Subtotal			0
	Total supplies and materials		114
			0
TOTAL			1,488

Purchasing

Those staff members with purchase order training and authorization for visa cards may sign purchases of $2,500 or less up to the total allocated as listed above. The Repository accounting will be kept in financial software provided by the agency.

Non-base and Extramural Funding for the USDA-ARS NCGR

FY 2010

Amount	Purpose	Source
128,000	RosBreed2010	CSREES
12,500	Quince evaluation JP	Winter Pear Commission
15,000	Additional Trusts	Matching for Blueberry SCRI
69,060	Blueberry genetics – NB	SCRI – carryover funding
51,400	Ohelo tc, cryo, id – KH , BR	SCRI – carryover funding
22,000	Tissue culture of pears - BR	OAN- ODA
30,324	Tissue culture of hazelnuts – BR	Oregon Hazelnut Com.
328,284	Total	

FY 2009

Amount	Purpose	Source
10,000	White pine blister rust on Ribes - KH	NW Center Small fruit
12,500	Azores cooperative grant - KH	OIRP
2,200	Summer Student intern – BR	ARS PWA
77,411	Blueberry identification – NB	SCRI – carryover funding
51,734	Ohelo tc, cryo, id – KH BR,and NB	SCRI – carryover funding
19,800	Tissue culture of pears - BR	OAN- ODA
29,156	Tissue culture of hazelnuts – BR	Oregon Hazelnut Com.
29,000	Plant Exploration to Hokkaido – KH and JP	USDA Exploration Grant
231,801	Total	

CHAPTER 9. PATHOGEN DETECTION AND ELIMINATION

Joseph Postman revised October 2012

SECTION 1. OBJECTIVES

The following objectives are consistent with the mission of NCGR-Corvallis to collect, conserve, characterize and distribute healthy plant materials:

- To obtain accessions from “clean” sources free of viruses and other pathogens when possible.
- To maintain plants in a healthy condition and to prevent the vector transmission of pathogens to plants in the repository base collections.
- To detect infected plants by periodic indexing.
- To keep accurate records on virus indexing results for each accession, and to make this information readily available to the NCGR staff and to germplasm users.
- To eliminate viruses from high priority infected clones using appropriate therapy procedures.
- To verify the identities of plants generated following therapy.
- To inform recipients of the known pathogen status of distributed material.
- To distribute pathogen tested plant material to the extent possible.

SECTION 2. INTRODUCTION

2.1 Why Test for Viruses?

Clonally propagated plant materials may accumulate virus and other "germplasm borne" diseases which can be latent or symptomless. Although free of obvious symptoms, plant vigor, productivity, hardiness, graft compatibility and other horticultural characteristics may be affected. Viruses can be detected by bioassay (inoculating sensitive indicator plants) by seroassay (ELISA) when specific antisera are available), and by laboratory procedures such as PCR. ELISA is the most commonly used seroassay. Some viruses produce distinct symptoms and can be detected by simple observation. Each genus differs in the number and identity of important viruses, in the probability of healthy plants becoming infected, and in the tests used to detect latent infections. Pathogen tested plant germplasm tends to be more productive and consistent in growth characters, and better able to satisfy quarantine requirements of other nations, thus promoting reciprocal international exchange of plant genetic resources.

2.2 Priorities

Plants received as clones should be tested for:

a. Viruses which may have quarantine significance in the US

b. Viruses which may have quarantine significance outside the US

c. Viruses for which reliable tests exist.

d. Viruses common in the plant's place of origin.

2.3 Incoming Plant Materials

- Accessions received from foreign sources will be handled according to all state and federal quarantine regulations.
- Arriving packages, especially those from foreign sources, should be opened in a Bio-safety hood to determine if contents are secure and as expected. All packaging should be autoclaved or incinerated.
- The autoclave should be calibrated annually and tested with bacteria ampoules.
- Detailed records will be kept on quantities, locations, and pertinent dates of quarantine and permitted material, and plants will be clearly labeled to indicate their quarantine or permit status.
- Appropriate APHIS and OR Dept. of Agriculture personnel will be notified when plants are received from a restricted or permitted source, or when material is released from quarantine.
- Contact between permitted plant material and its residue, other plants, or contact surfaces should be minimized to prevent the spread of pests. Permitted material should be handled, processed and transported in a dedicated tray which is disinfected after each use.
- Permitted or quarantine plants will be segregated from other closely related plants, using isolation distances and conditions as specified in the permits.

2.4 Isolate Infected Plants

Known pathogen infected plants will be segregated from healthy or untested plants to the extent possible. An intensive vector control program must be maintained in all greenhouses and screenhouses. Flowers should be removed from *Corylus*, *Rubus*, *Fragaria* and

Vaccinium accessions to prevent spread of pollen borne viruses as well as contamination of clones with seedlings. Persons working with protected collections should be certain they are not carrying insects. Persons should not enter screen/greenhouses after visiting field plots. Outer doors should be closed before inner doors are opened. Screen/greenhouses shall be checked periodically to assure they remain insect proof.

Imported accessions will be observed for a period of time in the isolation greenhouse and will not be distributed until they test negative for important virus diseases. Imported accessions which test positive for viruses will undergo therapy, and the infected plants will be destroyed.

SECTION 3. TESTING PROCEDURES

Each genus at the genebank requires specific indexing procedures. Accessions should be evaluated for visible symptoms. Latent infections can be diagnosed by ELISA or other laboratory methods for appropriate viruses, and by inoculating a range of indicator plants. The base collection (primary location used for distribution), or core collection should receive first priority for indexing. If more than one plant represents an accession, samples from each should be pooled for virus tests. Two replicate wells should be used for ELISAs and two replicate indicator plants should be used for graft inoculations. Three to four replicate indicator plants should be used for sap inoculation assays. Healthy and infected checks must be included in each test.

3.1 Bioassays

3.1.1 Mechanical Transmission

Sap inoculation of herbaceous indicators is a good general screen for viruses with a wide host range, and can detect a large number of known and potentially unknown viruses. Symptoms may be local, occurring in the inoculated leaves within 1-5 days, or they may be systemic when the virus infects the indicator plant and produces symptoms in new growth. Systemic symptoms generally occur 1-3 weeks after inoculation. Many herbaceous species are used. *Chenopodium quinoa* and Cucumber are commonly used herbaceous indicators for the fruit crops grown at NCGR-Corvallis.

Procedure For Sap Inoculation

- Inoculum (leaves, young shoots, buds) is collected from new growth in early spring.
- Tissue is ground in a roller press or mortar and pestle with .05 M phosphate buffer (pH 7.0) + 2% PVP (see Martin & Converse 1982; recipe in appendix).

Tissue:Buffer = approx. 1:5 (W:V)

A small amount of celite is added as an abrasive. Alternatively, celite or carborundum (600 mesh) can be dusted on indicator leaves prior to inoculation. 2% nicotine in water is a good alternate extract buffer.

- Young, vigorously growing indicator plants are inoculated by dipping a finger, pipe cleaner or glass rod into sap preparation, and rubbing each leaf 3 - 4 times.
- Indicator plants are lightly rinsed to remove inoculum and are placed on glasshouse bench to await symptom development.

- Indicator plants may react to virus better if they are kept in the dark for 8-24 hrs. prior to inoculation.
- Local symptoms should develop within 2 - 5 days, systemic symptoms within about 2 weeks. Results are recorded and plants are discarded after 2 - 3 weeks.

3.1.2 Graft Transmission

Graft inoculation to plants of the same or a related genus is used to detect viruses that have a narrow host range and which generally cannot be mechanically-transmitted. Chip budding (double budding) is the inoculation method generally used with tree fruits. Leaflet grafting is the inoculation method generally used for small fruits. A bottle graft is an alternative to the leaflet graft, and may be the procedure of choice for graft inoculation of *Ribes* and *Vaccinium* indicator plants. Symptoms may develop within several weeks for greenhouse inoculations, or may take several years for certain field inoculations which produce only fruit or stem symptoms.

Procedure For Double Budding in Glasshouse
(see: Fridlund 1989; Fridlund 1976; ISHS 1985)

- Budding should begin in January or February when indicator wood has received adequate chilling.
- Seedling rootstocks (3/16 to 1/4 inch caliper) are planted in plastic Dee-pots.
- One indicator bud is chip budded, or scion grafted onto each rootstock. Two inoculum bark chips are budded immediately below the indicator graft. For budded indicators, rootstocks are cut back above indicator bud about 1 week after budding.
- Survival has been much better, particularly with small diameter scions such as *Pyronia veitchii*, or *Malus micromalus* if the indicator clone is cleft-grafted using a 2-3 bud scion instead of chip-budded.
- After growing for about 4 weeks, indicator shoots are cut back to about 10 cm. and completely defoliated. Rootstock sprouts should be removed periodically. (Certain indicators produce symptoms in the first flush of growth and may not require defoliation.)
- Plants are grown in a cool (about 22 C), partially shaded glasshouse for best symptom development.
- Indicators are evaluated about 6 weeks after defoliation, or when positive checks are showing distinct symptoms (see rating scale in appendix).
- Two indicator plants are inoculated for each accession being tested. Infected and healthy checks are included in each test.

Procedure For Leaflet Graft
(see: Converse 1987; Frazier 1974)

- Inoculum leaves are collected from plants to be tested and kept between moist paper towels. Inoculum leaves should be young but fully expanded. Lateral leaflets are removed and the terminal leaflet is trimmed to reduce surface area and weight.
- Two leaves on indicator plant are selected for grafting and center leaflets are removed. Lateral leaflets may be trimmed for easier handling and to help locate the grafts later.
- Inoculum leaflets are grafted onto indicator plants. A razor blade or scalpel is used to make a cut down the petiole between the two remaining leaflets of the indicator plant.

Alternatively, a small slice can be made between the leaflets, after which the petiole can be separated by gently pulling apart on the two lateral leaflets. A tapered wedge is cut at the base of the prepared inoculum leaflet, and the leaflet is inserted into the cut petiole and wrapped with parafilm or latex bandage. (See illustrations on page 6-7 of Converse 1987).

- Plants should be periodically sprayed with a mist bottle until they are moved to a mist bench.
- **Important**: All ungrafted leaves (with the exception of new young growth) are removed from indicator plant. This promotes graft survival and virus transmission.
- Indicator plants are placed on mist bench. After 1 week, plants are moved to a glasshouse bench to await symptom development and plants without at least 1 surviving graft are flagged (blue pot label). These flagged plants are not recorded if results are negative, but they are recorded if results are positive.
- At least two indicator plants (total 4 grafts) are inoculated for each accession being tested. Healthy and virus infected checks must be included in each group of tests.
- Indicator plants must be grown in a cool (about 22 C.), partially shaded location and fertilized at least weekly for best symptoms.
- Indicator plants are evaluated 2-3 times per week for symptoms. Plants with questionable symptoms are flagged with a yellow pot label. Plants with definite symptoms are flagged with a red pot label. Most virus symptoms appear about 3 weeks after inoculation. At 6 weeks results are recorded (see rating scale in appendix) and plants are discarded. (some viruses such as Strawberry Pallidosis on UC-10, or Featherleaf on UC-5 may take longer than 6 weeks to produce symptoms).

Procedure for the Bottle Graft

- Inoculum shoots are collected from several branches of the plant to be tested, and are kept moist. Shoots should be about 15cm in length.
- A small test tube is filled with water and tied onto the stem of the indicator plant below the graft location. An inoculum shoot is placed into the test tube, and side-grafted to the indicator plant at a convenient point above the test tube.
- One or two grafts are made per indicator plant, with two indicator plants per sample. Test tubes are kept full of water until graft unions are formed, about 3-4 weeks.
- Inoculated indicator plants may need to be observed for up to 2 years for symptoms.

3.2. ELISA

3.2.1. Antiserum and Testing Sources (updated 10/2012)

ELISA kits, reagent sets, microbial cultures and other various services are available from the following sources. Also, see list of antiserum sources in ISHS (1983).

- AC Diagnostics
  http://www.acdiainc.com/
  phone: (479) 595-0320
  1131 W Cato Springs Road, Fayetteville, AR 72701, USA

- Agdia
  (http://www.agdia.com)
  phone: 574-264-2615
  30380 Country Road 6, Elkhart, Indiana 46514 USA

- American Type Culture Collection
  http://www.atcc.org/
  phone: 703) 365-2700
  ATCC, P.O. Box 1549, Manassas, VA 20108 USA

- Bioreba-AG (Agro-Diagnostics)
  http://www.bioreba.ch/
  Gempenstrasse 8
  CH-4008 BASEL Switzerland
  Telefon 061/35 04 55

- California Seed and Plant Labs
  http://csplabs.com/
  7877 Pleasant Grove Rd
  Elverta, CA 95626 USA
  phone: (916) 655-1581

3.2.2. Antiserum Preparation

If raw antiserum is obtained, IgG must be purified and conjugated to an appropriate enzyme before using in ELISA. (see appendix; and purification and conjugation procedures in: Hampton et. al. 1990, Clark & Adams 1977, or Converse 1984).

3.2.3. ELISA Protocol (Revised 9/3/97)

(see chart in appendix for specific antisera dilutions)

COATING

- Coat ELISA plate with antibody (=IgG) diluted in Coating Buffer according to chart. Prepare 11 ml per plate. Check pH of buffer before using. Use 100 ul octapette to fill plate.
- Incubate overnight (longer OK) in refrigerator.
- Rinse 2 times.
- (Optional) Add Blocking Solution (100 ul per well, dry milk in PBS-tween). Prepare fresh if more than 1 week old. Incubate 15-30 minutes.
- Rinse 1 time.
- Plate can now be stored in the refrigerator for several weeks.

Samples

- Collect samples. Grind in about 3 ml virus buffer. Add 100 ul per well, 2 wells per sample. Can use octapette with 2 tips. Cut off end of tips and rinse several times in two water rinses between samples. (Use special virus buffers for *Vaccinium* samples, or for apple mosaic and prunus ringspot virus - see "Modifications" below).
- Each ELISA plate should contain at least one positive check

- Incubate overnight in refrigerator
- rinse 3 times

Conjugate

- Dilute conjugate in conjugate buffer according to chart. Add 100 ul per well using octapette.
- incubate at room temperature 4 - 6 hours
- Rinse 4 times.

Substrate

- Prepare Substrate. Add 20 mg substrate tablet to 21 ml. substrate buffer or 40 mg. tablet to 42 ml substrate buffer for as needed to allow for about 10 ml per plate. Check pH of buffer before use. All glassware, plasticware, stirbars etc. that contact substrate must be very clean. A dedicated beaker and stirbar can be set aside for substrate only. Add 100 ul per well using octapette.
- Incubate at room temperature
- Record results after 1 hour or when deep yellow color develops in positive controls.
- If color is very faint, incubate longer, or store overnight in refrigerator and read results again next day.
- Read results using plate reader. Enter date etc. and blank recorder on buffer wells (or known healthy wells).

Modifications

- For grinding *Vaccinium* samples .5% nicotine (5ml per liter) must be added to the virus extract buffer (not to the conjugate buffer) to overcome the acidity of the plant tissue, or a higher pH borate buffer can be used instead.
- For ilarviruses (apple mosaic, prunus ringspot, possibly tsv) it may be helpful to add 4.5 g. DIECA (sodium diethyldithiocarbamate trihydrate)per liter of virus extract buffer.

  Volumes for 11 ml per plate
  (@100 ul/well):

  1:500 dilution = 22 ul/11 ml
  1:1000 dilution = 11 ul/11 ml

SECTION 4. Indexing Recommendations for NCGR Genera

The tests listed below are recommended to detect important viruses for NCGR genera. Bolded tests are recommended as high priority for the repository. A list of viruses detected by each indicator is presented in the appendix by genus.

4.1 Corylus

ELISA for:
Apple mosaic virus
PCR for:
Hazelnut stunt phytoplasma

Other:

Possible occurrence of prunus ringspot, tulare apple mosaic virus, and other phytoplasma diseases.

4.2 Fragaria

ELISA for:

Arabis mosaic
Fragaria chiloensis latent
Strawberry mild yellow edge
Strawberry necrotic shock (Tobacco streak)
Tomato ringspot

Graft inoculation of:

UC-5 or UC-4
UC-11 or UC-10
Alpine Seedlings

Sap inoculation of:

Chenopodium quinoa
Cucumber

Viruses Reported to Infect Strawberries (from Martin & Tzanetakis, 2006)

Virus name	Acronym	Transmission	Genus	Laboratory detection
Apple mosaic	ApMV	Pollen, seed	Ilarvirus	ELISA, RT-PCR
Arabis mosaic	ArMV	Nematode, seed	Nepovirus	ELISA, RT-PCR
Beet pseudo-yellows	BPYV	Whitefly	Crinivirus	RT-PCR
Fragaria chiloensis cryptic	FClCV	Unknown	Unknown	RT-PCR
Fragaria chiloensis latent	FClLV	Pollen, seed	Ilarvirus	ELISA, RT-PCR
Raspberry ringspot	RpRSV	Nematode, seed	Nepovirus	ELISA, RT-PCR
Strawberry chlorotic fleck	StCFV	Aphid	Closterovirus	RT-PCR
Strawberry crinkle	SCV	Aphid	Cytorhabdovirus	RT-PCR
Strawberry feather leaf	NA	Unknown	Unknown	NA
Strawberry latent	StLV	Unknown	Cripavirus	RT-PCR
Strawberry latent C	SLCV	Aphid	Nucleorhabdovirus	NA
Strawberry latent ringspot	SLRSV	Nematode, seed	Sadwavirus	ELISA, RT-PCR
Strawberry mild yellow edge	SMYEV	Aphid	Potexvirus	ELISA, RT-PCR
Strawberry mottle	SMoV	Aphid	Sadwavirus	RT-PCR
Strawberry necrotic shock	SNSV	Thrips, pollen, seed	Ilarvirus	ELISA, RT-PCR
Strawberry pallidosis associated	SPaV	Whitefly	Crinivirus	RT-PCR
Strawberry pseudo mild yellow edge	SPMYEV	Aphid	Carlavirus	ELISA
Strawberry vein banding	SVBV	Aphid	Caulimovirus	PCR
Tobacco necrosis	TNV	Oomycete	Necrovirus	ELISA, RT-PCR
Tomato black ring	TBRV	Nematode, seed	Nepovirus	ELISA, RT-PCR
Tomato ringspot	ToRSV	Nematode, seed	Nepovirus	ELISA, RT-PCR

4.3 Humulus

ELISA for:

American hop latent
Apple mosaic
Arabis mosaic
Cucumber mosaic
Hop latent
Hop mosaic
Prunus necrotic ringspot
Tomato ringspot

4.4 Mentha (low priority at NCGR)

Viruses Reported to Infect Mint (from Tzanetakis et al. 2010)

Virus name	Acronym	Genus	Transmission	Detection	Distribution
Alfalfa mosaic	AMV	Alfamovirus	Pollen,aphid	ELISA, RT-PCR	Worldwide
Arabis mosaic	ArMV	Nepovirus	Nematode, seed	ELISA,RT-PCR	Scotland
Cherry raspleaf	CRLV	Cheravirus	Nematode, seed	ELISA, RT-PCR	Worldwide
Cucumber mosaic	CMV	Cucumovirus	Aphid	ELISA, RT-PCR	Europe,China
Impatiens necrotic spot	INSV	Tospovirus	Thrips	ELISA, RT-PCR	USA, Italy
Lychnis ringspot	LRSV-M	Hordeivirus	Seed?	ELISA	Hungary
Mint veinbanding associated	MVBaV	Unassigned	Aphids	RT-PCR	Worldwide
Mint virus-1	MV-1	Closterovirus	Aphid	RT-PCR	USA
Mint virus-2	MV-2	Vitivirus	Aphid	RT-PCR	USA
Mint virus X	MVX	Potexvirus	–	RT-PCR	USA
Peppermint latent	PeLV	Cheravirus	Nematode?	RT-PCR	USA
Peppermint stunt	PmSV	Vitivirus	Unknown	Hybridization	USA
Strawberry latent ringspot	SLRSV		Nematode,seed	ELISA, RT-PCR	Worldwide
Tobacco mosaic	TMV	Tobamovirus	Mechanical	ELISA,RT-PCR	India
Tobacco ringspot	TRSV	Nepovirus	Pollen, nematode	ELISA, RT-PCR	USA
Tomato aspermy	TAV	Cucumovirus	–	–	China
Tomato leafcurl Pakistan	ToLCPKV	Begomovirus	Whitefly	PCR	India
Tomato spotted wilt	TSWV	Tospovirus	Thrips	ELISA, RT-PCR	USA, Italy
Unidentified filiform	–	?	?	?	Bulgaria
Unknown Rhabdovirus	–	Cytorhabdovirus?	?	TEM	Germany

4.5 Pyrus

ELISA for:
Apple chlorotic leafspot
Apple stem grooving
Apple stem pitting

Molecular probes for:
Apple scar skin viroid
Pear blister canker viroid

Graft inoculation of:
Malus micromalus (indicator for ASGV)
***Pyrus communis* Noveau Poiteau**

Virginia Crab (replaced by *M. micromalus*)
Pyronia veitchii
Pyrus communis Bosc (field test for Stony Pit disease)
Pyrus communis Bartlett (field test for bark disorders)

Sap inoculation of:
Chenopodium quinoa

Related genera including *Malus, Cydonia, Mespilus, Crataegus, Sorbus, Amelanchier* may be indexed on the above indicators as well as on Russian apple and Radiant Crab.

4.6 Ribes (low priority, field collection only)

ELISA for:
Alfalfa mosaic (=Lucerne mosaic)
Arabis mosaic
Cucumber mosaic
Raspberry ringspot (European Nepovirus)
Strawberry latent ringspot (European Nepovirus)
Tomato ringspot

Graft inoculation of:
Laxton No. 1
Baldwin
Amos Black (redundant)

Sap inoculation of:
Chenopodium quinoa
Cucumber

4.7 Rubus

ELISA for:
Apple mosaic (uncommon, self indicating)
Arabis mosaic
Cherry leafroll
Cucumber mosaic (uncommon)
Peach rosette mosaic (uncommon)
Raspberry bushy dwarf
Raspberry ringspot (European Nepovirus)
Strawberry latent ringspot (European Nepovirus)
Tobacco ringspot
Tobacco streak (Strawberry necrotic shock)
Tomato blackring (European Nepovirus)
Tomato ringspot

Graft inoculation of:
***Rubus occidentalis* Munger**
Malling Landmark red raspberry
Norfolk Giant red raspberry
Rubus henryi
Alpine strawberry

Sap inoculation of:
Chenopodium quinoa

4.8 Vaccinium

ELISA for:

Blueberry leaf mottle (uncommon)
Blueberry mosaic
Blueberry scorch carlavirus
Blueberry shock ilarvirus
Blueberry shoestring
Peach rosette mosaic (uncommon)
Red ringspot (when antiserum is available)
Tobacco ringspot
Tomato ringspot

Graft inoculation of:

Cabot Blueberry

Sap inoculation of:

Chenopodium quinoa (Detects many sap transmissible viruses including Blueberry leaf mottle, Peach rosette Mosaic, Tobacco ringspot, Tomato ringspot).
Cucumber (Detects Blueberry leaf mottle, Peach rosette Mosaic, Tobacco ringspot, Tomato ringspot.)

SECTION 5. Pathogen Elimination

Viruses can often be eliminated from infected plants by dissecting an apical meristem or shoot-tip from a heat-treated plant, and growing it into a new plant. Many variations of this procedure are used for different plants, but the basic procedure is the same: grow the infected plant at an elevated temperature (about 38°C) for several weeks, and generate a new plant from the apical meristem.

- All accessions indexing positive will be replaced by another plant of the same clone that indexes negative, where possible.
- If a virus free selection is not readily available, the infected clone will undergo thermotherapy and/or meristem tip culture. Resulting plants will be re-indexed. This process will be repeated until a plant is produced that indexes negative.
- Plants produced by thermotherapy or tissue culture must re-index negative after at least one natural cold dormant period, and preferably for 3 years before successful virus elimination is assumed.
- Alternative virus elimination procedures including in vitro heat therapy and in vitro chemotherapy should be considered. In vitro heat therapy can be used for *Fragaria* in particular, where production of runners is difficult using conventional heat therapy.
- Identity of plants should be verified before original plants are discarded.

5.1 Procedure for hot air treatment of trees

- Seedlings potted in clay pots to allow evaporative cooling of root zone. (Lower root temperature favors host survival.)
- Infected scion is grafted onto rootstock, Alternatively, about 5 candidate buds are chip budded onto each rootstock. Take buds from terminal portion of infected plant

to increase possibility of escaping virus. When buds begin to grow, plants can be placed in heat chambers.

- If plants are not treated soon after growth commences, terminal growth can be pinched back to force more lateral shoots.
- Heat treatment same as for small fruits below.

5.2 Procedure for hot air treatment of small fruits

- Well rooted plants are established in clay pots. Shoots can be cut back to encourage lateral growth.
- Plants are placed in heat chambers and temperature is gradually increased over a period of 2 - 4 days.
- Treatment temperatures and times may vary with the ability of the host plant to survive, the method of shoot tip propagation, and the effectiveness of the treatment against the particular virus. If meristems are propagated the treatment times may be reduced. The following temperature regimes have been used successfully:

  A. Constant temperature of 38 degrees C.

  B. Alternating 4 hours 38 C, 4 hours 30 C.

  C. Alternating 8 hours 38 C, 4 hours 25 C.

- Plants should be treated for 4-8 weeks if no meristem culture will occur, however, 3 weeks is adequate for most viruses if followed by apical meristem culture using meristems <0.5 mm in length.
- Heat sensitive clones are more likely to survive alternating temperatures. The 4 hour alternation has worked very well, and has given very satisfactory virus elimination. This is the standard method now used at NCGR-Corvallis. The high temperature may need to be reduced to 35-36 degrees for *Vaccinium* or *Ribes*.
- Meristems of treated plants should be grown in tissue culture where possible. Pyrus shoot tips (about 5.0 mm) can be micrografted onto young seedling rootstock if in vitro methods are not available. Rootstock species different than the scion will aid in differentiating rootsprouts from the graft.

- Plants resulting from heat treatment must reindex negative for 3 years before successful virus elimination is assumed. Virus levels may be reduced to non-detectable levels during the first year or two following thermotherapy.

5.3 Procedure for hot water treatment of dormant propagules

- Hot water treatment will not eliminate viruses, but may be useful for sanitizing mature scionwood or canes. This procedure has not been effective at eliminating virus diseases, however may eliminating most insects, fungi, bacteria, and phytoplasmas.
- Scionwood should be at room temperature prior to heat treatment. (Gradual increase in temperature favors host survival if material has been stored in a cooler).

- Wood is immersed in a large volume of actively circulating water which is maintained at 50 C for 15 minutes.
- Material is then cooled by immersing in cold water for about 5 min.
- Surface moisture is allowed to dry before repackaging for storage.
- Date, source of material, water temperature at start and finish of treatment are recorded in record book. Material is labeled to indicate that it was heat treated.
- Material is now ready to be grafted to clean rootstock, or rooted in a mist bed.

SECTION 6. Records

Indicators are rated for symptoms on a scale of 1 - 9. A rating of 5 or greater is considered positive. Indexing results for each clone are maintained in computer files. A separate directory is maintained for each genus with several key virus indexing files for each genus. "xxx" represents the genus-code or first three letters of the genus.

xxxVIR.dbf = database file with a summary of all bioassay results, ELISA results, notes, and an overall virus 'status' for each accession which is defined below. Virus testing summaries are copied to the inventory files where they are available to the NCGR staff, and to the GRIN database in a field called 'VIRSTATUS'.

The following VIRSTATUS codes are used:

"TESTED"
Clone has been subjected to two or more assays, and no pathogens have been detected, or derived from a meristem from a heat-treated plant.

"UNTESTED"
Clone has not been tested, or very limited testing has been completed, or one or more tests were inconclusive.

"INFECTED"
Clone has tested positive for one or more viruses.

Two additional databases are maintained for each genus containing results of each specific ELISA or bioassay:

xxxLISA.dbf = database with all ELISA results. This data is summarized in the ELISA field of each xxxVIR.dbf and the summary is duplicated in the ELISA field in collection inventories and on GRIN

xxxBIO.dbf = database with **bioassay** results. This data is summarized in the BIOASSAY field of each xxxVIR.dbf and the summary is duplicated in the BIOASSAY field in collection inventories and on GRIN.

SECTION 7. Virus Collection

Positive (virus infected) controls must be included in all bioassays and ELISA tests. A collection of standard isolates of important small fruit, pome fruit, mint, hop and hazelnut viruses are maintained at the repository. With the widespread availability of virus certification programs, and with the increasing use of pathogen-free propagation material by the nursery industry, many viruses are becoming rare. Numerous fruit tree and small fruit viruses can only be stored as infected plants, and these infected plants are an important resource for plant pathologists and other researchers. Virus cultures are needed for developing new detection techniques, comparing with newly characterized pathogens, determining the susceptibility of plants and establishing the effect of these pathogens on growth and production. The NCGR-Corvallis pathologist collects, maintains, evaluates, documents and distributes the pathogen germplasm as an important aspect of the virus indexing program, and as a service to other researchers. A list of the various virus isolate collections is presented in the appendix.

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SECTION 9. Appendix

A. Virus buffer recipes.

B. Antisera inventory and dilutions for ELISA.

C. Procedure for purification and conjugation of antisera.

D. Indicator lists and result codes by genus for:
Fragaria, Pyrus, Ribes, Rubus, Vaccinium

E. Virus collections
E.1 Tree fruit pathogens.
E.2 Small fruit and other pathogens.

Appendix A - Sap Inoculation and ELISA Buffers

PBS 1X (Phosphate Buffered Saline):

	1 liter	3 liters	4 liters
NaCl	8.0 g	24.0 g	32 g
KH_2PO	0.2	0.6	0.8
Na_2HPO_4 - 7 H_2O	2.17	6.51	8.68
KCl	0.2	0.6	0.8
NaN_3	0.2	0.6	0.8

- Adjust pH to 7.4. Store at 5 degrees C.

ELISA Coating Buffer:

H_2O	1.0 liter
Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
NaN_3	0.2 g

- Adjust to pH 9.6 with HCl.
- Can be stored at 5 degrees C
- Reheck pH before use.

ELISA Blocking Solution and Conjugate/Monoclonal Antibody Buffer:
(PBS-Tween + .2% dry milk)

	200 ml	500 ml
PBS	200 ml	500 ml
Tween 20	100 ul	250 ul
Powdered Dry Milk	.4 g	1 g

ELISA Plate Washing Solution:

Use .5 X PBS

ELISA Sample Grinding Buffers:
(Virus Buffer)

PBS	1 liter
Tween 20	.5 ml
Ovalbumin (egg albumin)	2 g
Polyvinylpyrrolidone (PVP mw 10,000)	20 g
Powdered Dry Milk	1 g

- PVP may take a long time to dissolve.
- Store at 5 degrees C and use within 1 month.
- For apple mosaic or prunus ringspot virus add 4.5 g. Dieca/liter (sodium diethyldithiocarbamate trihydrate)
- For extracting *Vaccinium* samples add 5 ml. Nicotine/liter An alternative buffer for *Vaccinium* is .05 M Borate Buffer pH 8.0:

.5 M Boric acid	90 ml
.5 M disodium tetraborate	10 ml
H_2O	900 ml

ELISA Substrate Buffer:

H_2O	1 liter
diethanolamine (liquid)	97 ml
NaN_3	0.2 g

- Adjust to pH 9.8 with HCl.
- Store at 5 degrees C.
- Recheck pH before each use.

Sap Inoculation Buffer:
[.05 M phosphate buffer pH 7.0 + 2% PVP (Martin & Converse 1982)]

H_2O	1	liter
Na_2HPO_4 - 7 H_2O	8.17	g
NaH_2PO_4	2.34	g
Polyvinylpyrrolidone (PVP mw 10,000)	20	g

Serum	Source	IGG Concentration	Conjugate Concentration	Status
American Hop Latent (AHLV)	Hampton 4/89	1:1000	1:1000	
Apple Chlorotic Leafspot (ACLSV)	AGDIA 1988	PRECOATED	-	POOR for pear
Apple Mosaic (=ROSE MOSAIC) (ApMV)	ATCC 254	1:500	1:500	GOOD
Apple Mosaic (ApMV)	ATCC 32	1:500	1:500	GOOD
Apple Mosaic (ApMV)	Hampton 4/89	1:500	1:500	GOOD
Apple Stem Grooving (ASGV)	AGDIA 2/89	PRECOATED		POOR-FAIR
Apple Stem Grooving (ASGV)	East Malling	-	-	POOR
Apple Stem Pitting (Pear Vein Yellows)	Hadidi 89 (from Japan)			GONE
Arabis Mosaic (ArMV)	AGDIA 8/89	1:1000	1:1000	GOOD
Arabis Mosaic (ArMV)	ATCC 192	-	-	GOOD
Arabis Mosaic (ArMV)	Casper 840	-	-	GOOD
Arabis Mosaic-S (ArMV)	East Malling	-	-	GOOD
Blueberry Leaf Mottle (BBLMV)	AGDIA 2/89	PRECOATED		GONE
Blueberry Red Ringspot	Converse 2/90			POOR
Blueberry Scorch Carlavirus	MacDonald 2/89	1:500	1:250	GONE
Blueberry Scorch Carlavirus	Martin 6/92	1:1000	1:1000	V. GOOD
Blueberry Shock Ilarvirus	Martin	1:500	1:250	GOOD
Blueberry Shoestring (BBSSV)	Ramsdell	1:1000	1:800	V.GOOD
Carnation Ringspot (CRSV)	ATCC 21a	-		UNTESTED
Cucumber Mosaic (CMV)	ATCC 242			POOR?
Fra. chiloensis ilarvirus (+ mono)	Martin 94			GOOD
Hop Latent (HLV)	Hampton 4/89	1:1000	1:1000	
Hop Mosaic	Hampton 4/89	-	-	
Mint Veinbanding	Stace-Smith 4/90	1:400	1:500	GOOD
Prunus Ringspot Fulton-G	Hampton 4/89			GOOD
Prunus Ringspot	ATCC 22	1:1000	1:800	GOOD
Raspberry Bushy Dwarf (RBDV)	AGDIA 90		1:1000	GOOD depleted
Raspberry Bushy Dwarf	Martin	1:1000	1:200	V.GOOD
Raspberry Ringspot (RRV)	Casper 979	-	-	UNTESTED
Raspberry Ringspot (RRV)	East Malling	-	-	UNTESTED
Strawberry Latent Ringspot (SLRV)	Casper 339			UNTESTED
Strawberry Latent Ringspot (SLRV)	East Malling			UNTESTED
Strawberry Mild Yellow Edge Potex	Martin 94	1:2000	mc 1:2000	V.GOOD
Tobacco Ringspot (TOBRSV)	ATCC 157	1:1000	1:1600	GOOD

Tobacco Streak (TSV)	AGDIA 2/89	-	-	UNTESTED
Tobacco Streak (TSV)	ATCC 276	1:100	1:400	FAIR-GOOD
Tobacco Streak Virus (+ mono)	Martin 94			
Tomato Black Ring (TomBRV)	Casper 289	-	-	UNTESTED
Tomato Ringspot (TomRSV)	ATCC 174	1:1000	1:400-800	GOOD, Depleted
Tomato Ringspot (TomRSV)	ATCC 239	1:1000	1:500-800	GOOD
Tomato Spotted Wilt - I (TSWV-I)	AGDIA 90	1:1000	1:1000	GOOD
Tomato Spotted Wilt - L (TSWV-L)	AGDIA 90	1:100	1:1000	GOOD

Appendix C - Procedure for purification and conjugation of antisera.

Preperation of immuno gamma globulin (IgG) - Hampton Protocol

1. Combine 1.2 ml whole antiserum with 1.2 ml distilled water.
2. Combine the 2.4 ml. antiserum-water with 2.4 ml of 36% sodium sulfate (Na_2SO_4) (3.6 g sodim sulfate dissolved in 10 ml distilled water) mix and let stand for 10 minutes.
3. Centrifuge, 22C for 15 min. at 12,000 g. Discard supernate - retain white pellet.
4. Add 10 ml of 18% sodium sulfate to pellet. Vortex.
5. Centrifuge as before, retain pellet.
6. Resuspend pellet in 1 ml PBS. Dialyze 3 times against 1 liter PBS at 4 C, one overnight.
7. Examine for visible precipitate; remove any precipitate by centrifugation (15 min.)
8. Estimate IgG concentration.
   (absorbance at 280 nm = 1.4 when IgG = 1mg/ml)

Conjugation of enzyme with IgG - Martin Protocol

Check form of Alkaline Phosphatase. May need to do the following:
Centrifuge the equivalent of 0.1 ml of alkaline phosphatase (0.5mg/ml) in a solution with $(HaH_4)_2SO_4$ for 2 to 3 minutes in a small centrifuge. Discard supernatant.
Dissolve precipitate in 0.2 ml IgG (at 1 mg/ml).

1. Combine 2 mg. alkaline phosphate (Sigma P-6774) with 1 ml. IgG (1 mg/ml).
2. Dialyze overnight against 0.06% glutaraldehyde in PBS.
   (1 ml. 25% glutaraldehyde in 416 ml buffer)
3. Dialyze against PBS to remove excess glutaraldehyde (3 changes, one overnight).
4. Add 5 mg./ ml. bovine serum albumin

optional: Dilute to 1/10 using PBS, and store in siliconized tube.

Appendix E - Virus Collections

E.1 - Tree fruit pathogen collections maintained at NCGR Corvallis.

HOST GENUS	PATHOGEN	ISOLATES
Corylus	Apple Mosaic Ilarvirus	3+
Corylus	Hazelnut Stunt ?Phytoplasma	1
Malus	Apple Blister Bark	1
Malus	Apple Chlorotic Leafspot Trichovirus	2
Malus	Apple Flat Limb	1
Malus	Apple Mosaic Ilarirus	2
Malus	Apple Rubbery Wood	4
Malus	Apple Stem Grooving Capillovirus	4
Malus	Apple Stem Pitting Virus	8
Malus	Apple Swollen Limb	1
Malus	Apple Swollen Stem (twisted twig)	1
Malus	Cherry Rasp Leaf Nepovirus (Flat Apple)	1
Malus	Genetic Variegation (non-chlorophyll mutant)	1
Mespilus	Apple Chlorotic Leafspot Trichovirus	1
Pyrus	Apple Stem Grooving Capillovirus	3+
Pyrus	Bark Disorders	2
Pyrus	Blister Canker	3
Pyrus	Concentric Fruit Ringpattern	1
Pyrus	Hardy Fruit Ring	1
Pyrus	Pear Blister Canker Viroid	2+
Pyrus	Pear Decline Phytoplasma	1
Pyrus	Pear Ringpattern Mosaic Virus (ACLSV)	4+
Pyrus	Pear Vein Yellows Virus	3+
Pyrus	Rough Bark	1
Pyrus	Seedborne Vein Yellowing	3
Pyrus	Stony Pit ?Virus	8
Sorbus	Chlorotic Leafspot ?Virus	2

(+) indicates additional infected plants in clonal germplasm collection

E.2 - Small fruit and miscellaneous other pathogens maintained at NCGR Corvallis.

HOST GENUS	PATHOGEN	ISOLATES
Fragaria	Black Concentric Fruitring (?TSV)	8
Fragaria	Chlorotic Fleck ?Virus	1
Fragaria	Cryptic Virus (Ghost)	1
Fragaria	Fragaria Chiloensis Ilarvirus	2
Fragaria	June Yellows (gentic disorder)	3
Fragaria	Multiplier Disease Phytoplasma	2

Fragaria	Strawberry Crinkle Cytorhabdovirus	2
Fragaria	Strawberry Greenpetal Phytoplasma	1
Fragaria	Strawberry Latent-C ?Rhabdovirus	2
Fragaria	Strawberry Leafroll ?Virus	1
Fragaria	Strawberry Mild Yellow-Edge Luteovirus	3+
Fragaria	Strawberry Mild Yellow-Edge Associated ?Potexvirus	5+
Fragaria	Strawberry Mottle Virus	3
Fragaria	Strawberry Pallidosis ?Virus	5
Fragaria	Strawberry Vein Banding Caulimovirus	3
Fragaria	Strawberry Vein Banding Virus (Western)	1
Fragaria	Tobacco Streak Ilarvirus (Necrotic Shock)	2+
Fragaria	Tomato Ringspot Nepovirus	1
Humulus	American Hop Latent Virus	1+
Humulus	Apple Mosaic Ilarvirus	1+
Humulus	Hop Latent Virus	1+
Humulus	Hop Latent Viroid	1+
Humulus	Hop Latent Carlavirus	1+
Humulus	Hop Mosaic Carlavirus	1+
Kalmia	Rhododendron Necrotic Ringspot ?Potexvirus	1
Mentha	Mint Veinbanding Virus (proposed name)	3
Melissa	Lemon Balm Variegation Virus	1
*Physocarpus**	Tomato Ringspot Nepovirus	1
Ribes	Arabis Mosaic Nepovirus	3
Ribes	Black Currant Yellows	5
Ribes	Tomato Ringspot Nepovirus	2
Ribes	Vein Banding Viruses	5+
Rubus	Alpine mosaic agent (Darrow Sterility)	1
Rubus	Black Raspberry Latent Virus	1
Rubus	Blackberry Calico Carlavirus	4
Rubus	Raspberry Bushy Dwarf Virus	5+
Rubus	Raspberry Mosaic Viruses	4+
Rubus	Rubus Yellow Net Virus	1
Rubus	Strawberry Mild Yellow Edge	1
Rubus	Tobacco Streak Ilarirus	4+
Rubus	Tomato Ringspot Nepovirus	3+
Sambucus	Cherry Leafroll Nepovirus	2
Sambucus	Elderberry Latent ?Carmovirus	2
Sambucus	Elderberry Carlavirus (European)	3
Sambucus	Elderberry Carlavirus (North American)	3
Vaccinium	Blueberry Mosaic ?Virus	1
Vaccinium	Blueberry Mosaic (?genetic variegation)	1
Vaccinium	Blueberry Red Ringspot Caulimovirus	1
Vaccinium	Blueberry Scorch Carlavirus	2
Vaccinium	Blueberry Shock Ilarvirus	1
Vaccinium	Blueberry Shoestring Virus	1
Vaccinium	Cranberry Falseblossom ?Phytoplasma	1

Vaccinium	Cranberry Ringspot	?Caulimovirus	1

* local native plant - common name "Ninebark"

(+) indicates additional infected plants in clonal germplasm collection

CHAPTER 10. Genetics Lab Handbook

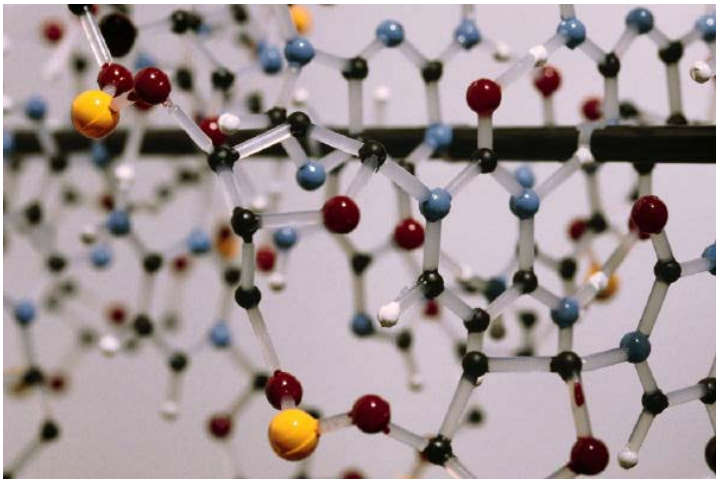
Introduction

The genetics lab at the National Clonal Germplasm Repository (NCGR) in Corvallis, Oregon was established in 2002 with funds coming from the U.S. Department of Agriculture, Agricultural Research Service (USDA-ARS). Our mission is to implement and/or apply molecular tools for the identification and characterization of our diverse germplasm. Presently, Dr. Nahla Bassil is involved in the following projects:

- The development of the first microsatellite markers in *Vaccinium* by her MS student Peter Boches.
- The implementation of PCR-based methods to identify the self-incompatibility genotypes of important European pear cultivars,
- The development of microsatellite markers in hazelnut, pear and strawberry.

Goals

- Research: To improve genetic characterization of the collection, authenticate plant identity using molecular markers, identify duplicates as well as gaps in our present collection and to gather information and collaborate with other scientists on analysis of genetic diversity.
- Information management: To provide accurate and up-to-date information on Genetic analysis of our assigned genera.
- Distribution: To provide plant material and/or DNA to researchers worldwide.
- Pathology: To identify pathogens using PCR-based methods.
- Public relations: To inform and serve the public.



SECTION 1. Safety Guidelines

I. Rules

See appendix 1 and 2.

II. Correct use and management of chemicals and facilities

(See "Chemical Hygiene Plan" in library of NCGR)

III. Fire

A. Pull the nearest alarm pull and call the fire department at 911.
B. Learn the location and operation of fire extinguishers in the work area (see appendix 3). Employees are authorized to use fire extinguishers and have the responsibility to use them in an actual emergency.
C. Fire extinguisher

- Types and use limitations

Classification of fire:

- class A - paper, wood, and rubber.
- class B - oil, gas, grease, and other flammable liquids.
- class C - electrical.

Types of fire extinguishers:

- Tri-class dry-chemical fire extinguisher (Carbon dioxide) can be used for all of the three classes of fires.
- Liquid gas type fire extinguisher is used for class B and C fires only.
- Type of fire extinguisher is clearly labeled on the front.

Operations (PASS, Pull, Aim, Squeeze, Sweep):

- Hold upright and release handle by removing ring pin or tape.
- Stand back eight to 12 feet and aim at the base of fire.
- Press handle-lever and sweep side to side at the base of the fire area.

IV. Bomb threats

- Be aware of any suspicious looking objects or packages - do not touch.
- Notify the research leader and administrative officer immediately.

Bomb threat calls:

- Keep the caller on line as long as possible. (ask him/her to repeat the message and record every word spoken)
- Stay calm.

Questions to ask:

- When is the bomb going to explode?
- Where is it right now?
- What does it look like?
- What kind of bomb is it?
- What will cause it to explode?

- Did you place the bomb?
- What is your address?
- What is your name?

Record description of caller's voice, sex, accent, tone, and any background noises.

Notify research leader and administrative officer immediately.

V. Chemical spills

A. In the case of a non-toxic chemical spill, the spill should be cleaned up immediately and disposed of as directed by the laboratory supervisor.
B. In the case of any spill of a caustic or corrosive chemical, such as a base or acid; or a flammable chemical, contact a member of the safety committee for further action as needed.
C. In any spill of a flammable solvent or a combustible liquid, cover the area immediately with the appropriate chemical spill clean-up kit. Shut off all electrical equipment and immediately contact a member of the safety committee for further action.
D. In any spill of a toxic chemical immediately cover the area with the appropriate chemical spill clean-up kit (if it is safe to do so), and evacuate the area. Call the fire department if needed. Contact a member of the safety committee. The building may need to be evacuated and/or the air exchange system shut off.

VI. Electrical emergency

A. If fire or other emergency warrants, shut off electrical power at the electrical disconnect box. One is on the corridor wall by the TC lab (see attached floor plan for other three).
B. In the event of a power failure, emergency lighting will be activated in the exits. Turn off all electrical equipment. Proceed to main reception area and await instructions.

VII. Safety committee member

- Two staff members are at the location.
- Safety committee - see safety bulletin board.

SECTION 2. General Lab Information

I. Supplies

- When opening the last container of some item or using up commonly used chemical (like agarose), please list the item on the To Order List on the white marker board on the lab door.
- Put everything back on their original place when you are finished.

II. Chemicals

A. MSDS

- MSDS sheets are in a large black binder; one on the shelf in the lab and the other one in Nahla's office.

B. Storage

1. General

- Organic and inorganic chemicals are stored on the shelves of the lab in two cabinets: Miscellaneous and General.
- Any non-hazardous supplies can be stored in storage room no. 3 in breezeway behind TC lab (The key to the storage room is hung on the right side of fume hood with a red tape on it. The keys to the corrosive and flammable cabinets are labeled with blue and yellow tape, respectively).
- Make sure everything is stacked neatly with the labeled side facing the outside door so that people can easily read the label and find what they want efficiently.

2. Corrosive

- Corrosive chemicals, such as acids and bases (nitric acid, phosphoric acid, ammonium hydroxide, potassium hydroxide, and sodium hydroxide), are stored in the blue cabinet in breezeway.

3. Flammable

- Flammable chemicals, such as alcohol and organic solvents (acetone, chloroform, ethanol, isopropanol, etc.), are stored in the yellow cabinet in breezeway. Unopened phenol/chloroform/IAA containers are stored at -20 °C.

III. Cleaning and dish washing

A. Precautions

- Wear gloves.
- Dishwasher detergent is toxic, be careful not to breathe detergent powder.
- Do not put any broken or sharp items or any chemicals that might harm the janitor into the wastebasket.

- Watch for cracks or broken edges of glassware. Discard in plastic bin on shelf to the left of the sink. (Empty that bin into dumpster outside)
- Be patient and cautious.

B. Bleach Treatment

Is used to degrade DNA. Three containers containing 1% bleach are used:

- Following bleach treatment for a minimum of 15 min., mats and beads are thoroughly rinsed with deionized water, then with nanopure water. They are allowed to air-dry. Mats are subsequently autoclaved and re-used for PCR.

Glassware or plastic ware is washed separately with dishwasher soap, rinsed thoroughly with tap water then with three to five rinses with deionized water.

C. Hand dish washing

Never put glassware over to be washed unless it has been rinsed thoroughly.

- Take off any ink (with 95% alcohol in fume hood) and rinse with tap water.
- Soak or wash with tap water containing a couple drops of liquid detergent (Liquinox).
- Use an adequate size of brush to clean up.
- Finally, rinse with tap water followed by three to five rinses with deionized water and put on the spike (above sink) to air dry.

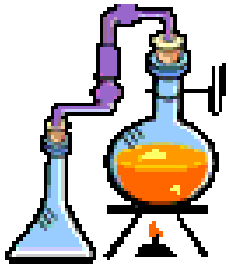
IV. Lab opening and closing

Opening:

- Turn off the thermocycler and place the plate at 4 °C
- Turn on deionized (D.I.) water system in the TC lab (if nobody from the TC lab has done it yet).
- Place clean dishes and electrophoresis accessories in their regular spots.

Closing:

- Turn off the deionized water in TC lab if you are the last person to leave (the red handle pointed towards the wall).
- If a gel was poured, place it in a plastic bag to prevent it from drying out.
- Turn off the lights and close the doors



SECTION 3. Equipment

See operation manual for details and operating directions for each piece of specific equipment.

I. TC lab Equipment

A. Water deionization system in the TC lab

Turn it off at the end of the working day to avoid water leaks when no one is in the building. Two pre-filters and resin filters inside the tank need to be cleaned and changed whenever the orange indicator light on the filter tank turns off. Check with facilities manager**,** Dennis, if problems arise.

- On: red handle pointing to the floor.
- Off: red handle pointing to the wall.

A. Autoclave in TC lab

Sterilization of media and equipment for tissue culture (121 ^{0}C at 1.1 kg.cm^{-2}).

1. Operation
    - Fill four to six quarts of deionized water till just below the indicator level (do not overfill).
    - Check for autoclave tape on items to be autoclaved.
    - Remove all the ink (with 95% alcohol) and parafilm wrap from container before loading autoclave.
    - Close the door tightly.
    - Select exhaust (slow) for media and (fast) for petri dishes, paper towels, water, and instruments (Important: media autoclaved on slow exhaust for 20 minutes. Utensils, glassware etc. can be done for longer time).
    - Set time (dial the time dial clockwise only).
2. Safety: Wear heat resistant gloves for loading and unloading.
3. Drain off the used water from autoclave at the end of the work-week and wastewater should be dumped into sink when it has cooled down.
4. If there are spills in the autoclave, they should be cleaned up immediately.
5. Move contaminated containers to the washing area only after autoclaving.

C. Laminar flow hood

1. Hood pre-filters need to be changed or cleaned every three to four months.
2. Hepa filter should be checked for leaks yearly or whenever hood is moved.
3. Turn off the light and hood power when you are done.
4. If you need a specific hood at a specific time, check with your co-workers about availability.

C. Balance (Mettler PM 460, Delta Range®)

- Check and see if it is level before use by looking at leveling bubble.
- Clean it up after finishing.
- There is a more accurate scale (Mettler AC 100): to use for weighing chemicals between 1.0 g and 10.0 mg (0.01 g). None of our scales will accurately weigh amounts less than 10 mg.

D. pH meter (pH meter 240, CORNING®)

1. Precautions:
   - A 15-minute warm-up time is required to meet the technical specifications of the meter. Connect the pH meter to a power supply outlet and then the meter will perform a self-test routine for five seconds, and the decimal point and the pH indicator will remain lit.
   - Do not use solvents to clean the pH meter.
2. To calibrate in pH mode (calibrate everyday) – see appendix 5
3. To measure pH

   Manual (the method used in the TC lab)
   - Select pH mode (usually it has been set on pH mode).
   - Rinse and put electrode in sample solution (solution must cover over the bottom ball of electrode).
   - Press ***read*** to start; pressing ***read*** again will put it on **standby** and stabilize the reading.
   - Rinse electrode for the next sample or put it back to soak in pH 4.0 buffer (red).

E. Hot plate stirrer (Nuova II, Thermolyne®), hot plate (PC 351, CORNING®)

1. Make sure that you turn it off (indicator light goes off) after you are done.
2. Clean after use with Corning detergent (beside sink) – let it cool some first. .

F. Orbit shakers (Lab-Line Instruments Inc. Junior Orbit Shaker AD-700)

1. Place the 100-ml beakers or 250-ml flasks on the orbit shaker using the clips.
2. Dial the speed button to between the first and the second marker.
3. Keep it clean.
4. Turn it off after you are done.

J. Liquid Nitrogen Tank

1. Turn on the valve on the liquid nitrogen tank in the breezeway outside the TC lab.
2. Place the liquid nitrogen dispenser in the tank to be filled
3. Wear protective goggles and gloves.

4. Turn on the valve on the liquid nitrogen tank in the TC lab Dial and fill up your tank.
5. Turn off the valve, place the lid on your container and
6. Remember to turn off the valve in the breezeway.

II. Genetics Lab Equipment

A. Eppendorf 5417C: (used with 1.7 and 2.0 ml centrifuge tubes)

- Turn centrifuge on (switch in back)
- Press open lid button
- Remove rotor cap by pressing down and turning right
- Load rotor with balanced load
- Do not forget to place the rotor cap back on
- Close lid
- Set spin time and speed
- Press start
- To stop, press ‘Stop’.

B. Thermo Forma High Performance Refrigerated Centrifuge (Two-place deep well plate rotor is used with 96-well plates and Swinging bucket rotor is used with 15 ml and 50 ml centrifuge tubes)

- Turn centrifuge on (switch on left side)
- Press open lid button
- Load rotor with balanced load
- Close lid
- Set speed (not to exceed 4,400 rpm for the two-place deep well plate rotor), enter.
- Set time, enter.
- Set temperature, enter.
- Press start
- To stop, press ‘Stop’.

C. Bio-Rad Power Pac 300 and 3000: The Power Pacs are the high voltage power sources for the electrophoresis equipment. The Power Pac 3000 can produce up to 3,000 volts of electricity and is used for larger power needs like AFLP.

Power Pac 300

- Connect the electrophoresis cell(s) to the power supply
- Turn on the power
- Set the constant parameter to constant voltage (V) with the ‘constant’ key
- Enter the constant value with the scroll keys
- Start the run by pressing the ‘run’ button
- End the run by pressing the ‘run’ button
- Turn off the power before checking the gel
- To program a timed run: before pressing the run button, press the ‘parameter’ button to select ‘Time’ parameter and enter the time with the scroll keys
- To program Power Failure detect, press both scroll keys after programming a run time. This feature will automatically resume the run where it was stopped in the event of a power failure.

Power Pac 3000

- Connect the electrophoresis cell(s) to the power supply.
- Turn on the power
- (Optional) set Power Fail Detect from the main menu with the appropriate soft key
- Select Manual with the appropriate soft key
- Set the constant parameter to constant voltage (V) with the appropriate soft key
- Enter the constant value with the numeric keypad
- Select Time with the appropriate soft key to continue
- Enter a time using the numeric keypad
- Press the 'run' button to start the run
- Press the 'stop' button to stop the run or the 'interrupt' button to pause the run
- You may also create and edit programs with the Power Pac 3000: see the Power Pac 3000 Instruction manual for details

D. Sub Cell GT 96

- Pour a 175 ml gel using the gel Bio-Rad gel caster
- Keep the buffer reservoir filled with clean 1X TBE
- The reservoir holds approximately 1.5 liters
- The reservoir should be filled to the max line with a gel in the reservoir
- Be sure to dry the area around the power leads before connecting to the power supply
- Run time is approximately two and three quarter hours for a 100 bp product in a 1% gel

E. Mini Sub-Cell GT

- Pour a 35-50 ml gel
- Keep the reservoir filled as for the sub cell GT 96 (capacity is approximately .75 liters).
- Run time is approximately 30 minutes for a 100 bp product in a 1% gel

F. Barnstead/Lab-Line Max Q 400 Shaker Incubator

- Turn on the power
- Press and hold the star (*) key and use either the up or down arrows to adjust the set point to the desired temperature
- Allow at least 30 minutes for the chamber temperature to stabilize
- Load the samples into the appropriate racks or clamps
- Press the membrane power switch to turn the shaker on
- Slowly rotate the speed control knob clockwise to increase the shaking speed and counterclockwise to decrease.

- From the 12 o'clock off position, rotate timer knob counterclockwise to the ON position to initiate constant shaking.
- For timed operation, rotate the timer knob clockwise from 1 minute to 60 minutes

G. Lab Line Orbit Shaker

To shake by time:
- Turn the knob on the right until the marker points at the amount of time (1-60 minutes)
- Turn the left knob to adjust the frequency of agitation.

For continuous agitation:
- Turn the right knob to the 'Continuous' position

H. Fisher Scientific Isotemp Digital Dry Bath (Hot Block)

- Push the power switch (located on the left side of the bath) to the on position (up)
- Momentarily pressing the up or down arrow key will display the last temperature setting
- To change the set temperature, hold down the up or down arrow key until the temperature displays the desired set point, then release the key
- Allow approximately 30 minutes for the block temperature to stabilize
- Place tubes containing samples into block wells
- Level of sample in tubes should not extend past the surface of the block

I. Polyscience Microprocessor Digital Water Bath

- Fill water bath with fresh deionized water to the desired level
- Press power switch in the rear to the 'on' position
- Press the on button on the front panel (press hard)
- The display indicates the set temperature for a few seconds, then indicates the actual temperature
- To adjust the set temperature, press the set/menu button
- While the °C light flashes, turn the temperature set knob to the desired temperature
- To change the units from °C to °F, press and hold the set/menu button until the display reads unit, then turn the temperature set knob
- To clean the bath, empty it carefully to avoid spilling fluid onto the control panel. Use only warm soapy water, and do not use steel wool or other strong scouring pads.

J. Thermo Savant SPD111V speedvac concentrator

- Turn the power switch on the right hand side of the UVS 400 to the 'on' position at least 30 minutes before use

- Remove the rubber seal to the vacuum flask on the UVS 400, gently remove the flask, pour off any accumulated liquid, and replace it in the UVS 400
- Place samples in centrifuge tubes into the centrifuge rotor of the SPD 111V with lids open. Be sure to balance the load as for any centrifuge!
- Set the temperature and/or run time using the 'set' and arrow buttons, and begin the run ('manual run' or 'auto run' button if a time has been set).
- Once the rotor has reached full speed, turn the vacuum pump on (front switch on the UVS-400)
- To build the vacuum pressure, the valve on the tube connecting the SPD 111V to the UVS 400 should be turned so the "bleed" arrow points toward the SPD 111V centrifuge
- To apply the vacuum to the samples in the centrifuge, turn the bleed arrow so that it is pointed towards you
- To end the run, slowly turn the bleed arrow to point towards the centrifuge with the rotor still running.
- After the vacuum has dissipated, press the stop button on the SPD 111V and remove the samples
- Turn off all power switches when done

K. Biophotometer

- Vortex for one minute extracted samples and centrifuge for 30 sec at 4400 rpm
- Place 100 µL 1X TE in cuvette
- Turn on Biophotometer (switch is in the back)
- Use extra care when handling quartz cuvettes: they are fragile and cost several hundred dollars
- Carefully place cuvette in hole
- To specify dilution press 'Dilution' and then 'Enter." Enter the amount of desired amount DNA first, then the desired amount of carrier (e.g., TE soln.). For example: for a dilution of 2:100 enter '2' for DNA 'Enter', '2', 'Enter', '98', 'Enter'
- Press 'Blank' (Screen should read 0.00)
- Take cuvette out of hole, pour contents in beaker, and rinse three times with nanopure water
- Gently tap dry on a Kim Wipe or paper napkin
- Pipette 98 µL of TE into cuvette (avoid introducing bubbles), and add 2 uL of DNA sample. Mix DNA into solution by pipetting up and down
- Check to be sure there are no bubbles in the light path, clear bubbles with a pipette tip or by flicking the cuvette
- Press 'Sample'. Annotate reading and the 260/280 ratio

L Mettler Toledo scale (10-1000 mg)

- Turn on scale by pressing left foremost button.

- Lift the lid to place a weigh boat or paper on clean metal plate, tare it off, and add desired amount of powdered chemical on boat/paper by using a spatula. If the spatula is not already in the chemical's container, use a clean one.
- Add desired amount of powdered chemical onto boat/paper. If weigh exceeds the desired amount, return excess to container.
- After weighing your chemicals, make sure the scale plate is clean by lifting it and brushing/wiping with a paper towel. Remember to turn scale off by pressing the arrow (←) and the 'On' together for a couple of seconds.

M. Mettler PC440 scale (> 1 g)

- Turn on scale by pushing the front bar upward.
- If small amounts of chemical are to be weighed, place a weigh boat or paper on clean metal plate, tare it off, and add desired amount of powdered chemical on boat/paper by using a spatula. If the spatula is not already in the chemical's container, use a clean one.
- For large amounts of chemicals (more than 100 g), one may tare off the weigh boat and load chemical by carefully shaking the container until the approximate amount falls in the boat. Then, proceed to add/subtract chemical as needed.
- After weighing your chemicals, make sure the scale plate is clean by lifting it and brushing/wiping with a scale brush. Remember to turn scale off by pushing upward front bar.

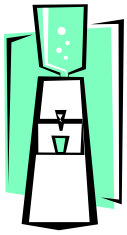
N. Mixer Mill 301

- Place sterile clustered tubes in two 96-tube, labeled racks (type of tissue, date, extraction process, etc.)
- Put a clean bead into each tube
- Add desired tissue to each tube accordingly to protocol specifications
- Add the desired lysing solution to each tube containing tissue
- Cover tubes with lids
- Place rack on white adapter base and cover with black adapter lid making sure the knob is placed at same distance from one long side of rack
- Pull up the clamping bolt so that the grooves are not locking the tightening mechanism and rotate bolt by 90 degrees. The automatic locking is deactivated.
- Turn the hand wheel to maximize the space for rack
- Place the adapter with rack inside in one of the clamps and turn hand wheel until snug
- Rotate clamping bolt back by 90 degrees until it locks back into groove
- Turn the hand wheel again
- One should hear the bolt coming up and down with an audible click as the clamp tightens up, turn the wheel until 12 clicks are heard
- Turn on the Mixer Mill with the switch is on the back.

- Set the grinding time by turning the knob on the left. The time will display on the left dial. Set the frequency by turning knob on the right and dial displays desired frequency. Standard grinding is 1.5 minutes at 30/s
- For even grinding, the sample plates need to be rotated 180° and ground for another 1.5 minutes at 30/s
- To do this, remove the plate from the mixer mill, take off the black cover, rotate the plate 180°, replace the white cover, reinsert the plate into the mixer mill and grind as before

O. Meku Juice Press

- Turn on the juice press water supply with the faucet valve
- Place the intake tube for the peristaltic pump into a beaker of grinding buffer, turn on the pump, and set it to 'prime'
- After the grinding buffer is dripping onto the rollers, switch the peristaltic pump to 'forward' and then set the speed to the desired flow rate
- Turn on the juice press motor switch lever into position 'I'. It is natural for the rollers to squeak when operating dry. Do not operate dry for extended periods
- Insert the first leaf sample into the rollers. The rollers should pick up the tip of the leaf and carry it inwards. Use forceps to grip the leaves if necessary to prevent fingers from being entangled in the rollers
- Allow the first drop or two of juice to spill and catch the appropriate amount of juice in a centrifuge tube
- Press the foot pedal to rinse the rollers with water before inserting the next sample. The rinse time can be adjusted with a knob at the rear of the press.
- When finished juicing, turn off the juice press motor
- Place the intake tube of the peristaltic pump into a beaker of clean water and flush the entire line clean
- Turn off the water supply to the juice press



P. NANOpure Diamond Ultrapure Water Filter System

- Turn the system on by pressing the main power switch to the 'I" position
- When the display reads 'Idle', press the Start/Stop switch
- The system should be left on or in 'Standby' mode during the workday
- Place a container under the dispenser
- Use the dispense knob for manual dispensing
- For volumetric dispensing, press the up or down arrow until the display reads "Auto Dispense Menu?'. Press Enter. Select the volumetric dispense method with the up or down keys and press enter. Enter volume setting with the up or down arrows. Press enter to set the dispense volume. The 'Dispense' button will then automatically dispense this amount.
- At the end of the day, place the system on standby for the night by pressing the STANDBY button

P. Vortex

If a different attachment is needed (depending on shape of container to vortex):

- Turn switch to the off position –control rod is in the central position.
- Remove the installed attachment by bulling straight up.
- Place alternate attachment and rotate until flats align. Press firmly until attachment snaps into place.

For intermittent operation:

- Place control rod in the lowest position.

For continuous operation:

- Pull control rod upward to the highest position. Return the rod to central position to stop vortexing. Speed can be regulated by turning knob as needed.

Q. Matrix Pipettors

- Connect transformer into plug and to the back of the pipettor.
- Press the 'On' switch.
- To fill pipettor press the 'Fill' button and enter the amount to pipette.
- To dispense press the 'Dispense' button and enter the amount to be dispensed. Keep in mind that one can fill pipettor with a multiple amount for repeated amounts to dispense. The pippettor will make a double sound to indicate the full amount has been dispensed and need to be refilled.
- To fill and dispense press the upper button on handle.
- To expel the used tips, press the lower button on handle.

R. Fisher Accumet Basic pH meter

- Keep the pH electrode immersed in pH 7 buffer when not in use
- Always rinse the electrode in deionized water when switching it from one liquid to another for standardization or measurement
- Turn the pH meter on and press and release the mode key until the digital display indicates pH mode
- The pH meter should be standardized before use:
- Standardize the meter using at least two buffers close to the pH you are measuring
- Rinse the electrode and place in a buffer solution
- Press the setup key twice and then the enter key to clear an existing standardization
- Press the std key to access the standardize mode
- Press the std key again to initiate standardization: the meter will automatically recognize the buffer and flash the pH value on the screen
- When the stable icon appears, the buffer value is entered and the meter returns to the measure screen

- Rinse the electrode, place in a new buffer, and repeat standardization by pressing the std button twice

S. Bio-Rad Gel Doc 2000

- Wear gloves at all times
- Turn on the computer, the printer, and the Gel-Doc (the Gel-Doc on switch is in the rear, near the top where the video feed cables enter
- The computer password is 123
- Open the 'Quantity One' software icon and select 'Gel Doc' from the File menu
- Place the gel on the glass illumination tray and press the 'Epi White' button to provide illumination
- You can adjust the zoom, focus, and iris (exposure) of the image using either the buttons on the front of the gel doc or the mouse buttons on the gel doc window in the computer screen
- With the illumination tray locked in place, adjust the position of the gel and the camera zoom so that the gel is centered on the screen at full magnification
- Close and lock the doors (turn handle to right) to the Gel Doc hood and press the 'Trans UV' button to visualize the DNA with UV light. You will need to press the 'Trans UV' button again every time you open the door because the UV light automatically shuts off when the door is opened
- Use the focus and iris buttons to adjust the camera so that you get a clear image. Overexposed areas become orange, underexposed areas will fade to black
- When you are satisfied with the image, click on the 'Video Print' button to send the image to the Mitsubishi printer
- You can also save a copy of the image as a digital file with the save button. If you want to use the software to do image analysis (such as calculating the relative size of bands) you should save a copy
- Wipe the glass tray clean with a Kim Wipe between uses
- To photograph the next gel, click on the 'Live/Focus' button and follow the previous steps to position the gel
- When finished, exit the Quantity one software, shut down the computer, and power off the Gel Doc and printer

NOTE: if the computer enters sleep mode, you may need to restart it in order to access the Gel Doc controller through Quantity One

T. Eppendorf Mastercycler Gradient

- Turn on the thermocycler (large switch in the back)
- Turn the locking button to the 'lid open' icon
- Gently pen the lid to the machine
- Place a PCR plate or tubes with fresh PCR mix into the appropriate wells in the thermocycler chamber

- The screen should begin on the main menu. You can exit to the next highest menu by pressing the ‘Exit’ button
- Use the up/down arrow keys to select an appropriate menu
- First, check the PCR program you plan to use by selecting the Load menu with the arrow keys and pressing enter
- Select the PCR program to view with the arrow keys and pressing enter
- View the program with the arrow keys, press exit when you are satisfied with the program
- Do not save the program unless you have made changes that you wish to keep
- When you are ready to run the PCR program, press ‘Exit’ until you return to the main menu
- Select the ‘Start’ menu with the arrow keys and press enter
- Select the PCR program you want to run from the ‘Start’ menu with the arrow keys and press enter
- The computer will test the program for logical errors and begin cycling

SECTION 4. Solutions

I. Hazelnut Extraction Solutions

A. Grinding Buffer C (1 L)

750 ml ddH_20
119.8 g sucrose
12.1 g Tris
3.7 g KCL
9.3 g EDTA
50.0 g PVP (FW=40,000)
Stir on a hot plate at low heat for 1 to 2.5 hours to dissolve.
Store at 4°C and use cold.

B. Lysing Buffer A (1 L)

(100 mM EDTA, 50 mM Tris, pH 8.0)
200 ml 0.5 M EDTA pH 8.0
50ml 1.0 M Tris-HCL pH 8.0
750 ml ddH_20
Store at 4°C.

C. Lysing Solution D Stock (1 L)

812.5 ml lysing buffer A
110 ml 20% sarkosyl
110 ml 25% Triton X-100
When ready to use, add 293 µl PK to 64 ml lysing solution D

D. Proteinase K (10 ml)

20 mg/mL
Dissolve 0.2 g in 10 ml sterile H_20
Store in 585 µl aliquots at -20°C until needed.

E. RNase A/ 10 ml

40 mg/ 10 ml of sterile water

F. TE (1 L, pH 8.0)

10 ml 1.0 M Tris-HCL, pH 8.0
2 ml 0.5 M EDTA, pH 8.0
988 ml nanopure H_20
Store at 4°C.

G. High Salt TE (1 L)

10 mM Tri-HCL	10 ml of 1 M stock
1 mM EDTA (pH 8.0)	2 ml of 0.5 M stock
2 M NaCl	116.88 g

Adjust the final volume to 1 L.

H. Sodium Acetate (3 M, pH 5.2)

408.24 g Sodium Acetate Trihydrate, FW 136.0
750 ml dH_20
Adjust pH to 5.2 using glacial acetic acid.

I. 95% Ethanol: Sodium Acetate (3 M, pH 5.2)

1000 ml 95% EtOH
50 ml 3 M Sodium Acetate
Store at room temperature.

II. CTAB Extraction Solutions

A. 2 X CTAB Buffer/ 100 ml

	/100 ml
2% Hexadecyltrimethyl ammonium bromide (CTAB)	2 g
1.4 M NaCl	8.18 g OR 35 ml of 4M
20 mM EDTA, pH 8.0	4 ml of 0.5 M
100 mM Tris, pH 8.0	10 ml of 1 M
1% PVP (binds polysaccharides)	1 g
1% Sodium bisulfite (inhibits oxidation of polyphenols)	1 g

Dissolve 2 g CTAB and 1 g PVP in 30 ml by heating or incubating in a 65°C water bath.
Add the remaining ingredients and stir until dissolved.
Adjust the volume to 100 ml.

B. 1 M Tris-HCl, pH 8.0/1 L

121.1 g of Tris base in 800 ml water.
Adjust the pH to 8.0 by adding ~ 42ml HCL.
Allow the Tris to cool before adjusting the pH.
Adjust the pH, then the volume to 1 L.
Dispense into aliquots and autoclave.

C. 0.5 M EDTA, pH 8.0/1 L

186.1 g of EDTA to 800 ml of water.
While stirring, add ~20 g of NaOH pellets (the disodium salt of the EDTA will not go into solution until the pH is close to 8.0).
Adjust the pH to 8.0 and the volume to 1 L.
Can dispense into aliquots and autoclave.

D.TE/1 L

	/ 1 L
10 mM Tris-HCl, pH 8.0	10 ml of 1 M
1 mM EDTA	2 ml of 0.5 M
Water	988 ml

E. 5% CTAB Buffer/ 50 ml

	50 ml
5 % CTAB	2.5 g
0.7 M NaCl	8.75 ml of 4M

F. 13% PEG/ 50 ml

13 g PEG per 50 ml water

G. 4 M NaCl/ 50 ml (MW. 58.44)

11.69 g of NaCl per 50 ml water.

H. 70% Ethanol/ 1 L

750 ml of 95 % ethanol + 250 ml water

I. 10 M Ammonium Acetate/ 100 ml

77 g of ammonium acetate (MW 77.08) in 80 ml water.
Adjust the volume to 100 ml.
Sterilize by filtration.

J. 76% ethanol + 0.01 M Ammonium Acetate/ 100 ml

81 ml of 95 % ethanol
100 µl of 10 M ammonium acetate
Adjust the final volume to 100 ml with nanopure water.

III. SDS-based Extraction Solutions

A. Lysis Buffer/ 100 ml, Accoto et al.

	/100 ml	
100 mM Tris-HCl, pH 8.0	10 ml	1 M Tris-HCl, pH 8.0
50 mM EDTA, pH 8.0	10 ml	0.5 M EDTA, pH 8.0
500 mM NaCl	10 ml	5 M NaCl
1 % SDS	10 ml	10 % SDS
10 mM citric acid	10 ml	0.1 M Citric acid
PVP	2%	

Add 200 mg PVP to 10 ml Lysis Buffer
Incubate at 65°C for 5-10 min until PVP is dissolved
Cool Lysis Buffer to RT.
Store at RT.

B. 5 M Potassium Acetate (MW 98.14)/ 100 ml

49.07 g in 100 ml water (or 24.53 g /50 ml)
Filter sterilize.

C. 10 % SDS/ 100 ml

10 g SDS in 100 ml water (or 5 g /50 ml)

D. 100 mM (0.1 M) Citric Acid/ 100 ml (FW 210.14)

2.1 g citric acid in 100 ml water (or 1.05 g /50 ml)

E. 5 M NaCl/ 50 ml (MW 58.44)

14.61 g in 50 ml water

IV. Agarose Gel Electrophoresis

A. 10 X Tris-borate Buffer (TBE)/ 1000 ml

	/1 L	/2 L
Tris	108 g	216 g
Boric acid	55 g	110 g
500 mM EDTA, pH 8.0	40 ml	80 ml

Adjust final volume.

B. 50 X Tris-acetate Buffer (TAE)/ 1000 ml

	/1 L
Tris	242 g
Glacial acetic acid	57.1 ml
500 mM EDTA, pH 8.0	100 ml

Adjust final volume to 1 L.

C. Loading Dye

	/50 ml
Glycerol (50 %)	25 ml
500 mM EDTA, pH 8.0 (50 mM)	5 ml
Bromophenol Blue (0.05 %)	0.025 g

Adjust the final volume to 50 ml.

D. Ethidium Bromide (10 mg/ml) HIGHLY MUTAGENIC

- After dissolving 50 mg in 5 ml of water, keep in a dark bottle or wrapped in aluminum foil and store at room temperature.
- For the small gels, 8 µl of ethidium bromide stock is freshly added to the ethidium bromide working solution to stain it.
- For the large gels (Sub-Cell GT 96), 12 µl of ethidium bromide stock is freshly added to the ethidium bromide working solution for staining.
- Use an ETHIDIUM BROMIDE REMOVAL SYSTEM 2/PK from ISC to decontaminate the used ethidium bromide solution (ISC, E-3008-2).
- Stained gels are allowed to dry out in the hood before they are placed in garbage bags and discarded in the garbage.

E. Promega's 100 bp Ladder

Add 375 ul Loading dye to the ladder.
Load 5 µl in a narrow gel lane and 8 µl in a wide lane.
Ordered from Fisher: PR-ZG2101 (5 x 250 µl)

F. Fisher's 100 bp Ladder

Add 450 ul Loading dye to the ladder.

Load 5 µl in a narrow gel lane and 8 µl in a wide lane.
Ordered from Fisher: BP2551-50

G. Promega's 1 Kb Ladder

Make up a working ladder solution consisting of equal volume Loading dye and ladder.
Load 5 µl in a narrow gel lane and 8 µl in a wide lane.
Ordered from Fisher: PR-G5711

SECTION 4. DNA Extraction

I. Some Key Points

Genomic DNA: Looks like snot.
Some basics include the following important points:

- DNA is negatively charged.
- DNA has lots of ring structures, therefore quite hydrophobic. AND
- DNA is a very robust molecule, generally, the easiest of the biological macromolecules to work with.

Recommendations:

1. Get it as pure as you can.

- DNA also doesn't like nucleases. Therefore, you want it as pure as you can. Nucleases are everywhere. Be careful handling material. Keep everything cold, since these enzymes are much more active at physiological temperatures. ALWAYS wear gloves.
- If have contamination (by proteins, or whatever) subsequent steps may be hindered. (i.e. restriction digests, PCR, etc).

2. Large pieces of DNA can shear (not actually a big deal for PCR).

- When interested in isolating or dealing with a piece of DNA that is ~100kb in size, grind in liquid nitrogen and do not vortex.

II. Plant Collecting and initial homogenization

A. Meku Juicer

- Fill out a 48 cell master DNA collecting list ordered by location.
- Collect healthy and new growing leaf tissue in spring in 48-compartment boxes the day of the extraction or 24 hours earlier.
- Follow SOP for the juicer. After the buffer starts dripping, feed a leaf between the rollers and collect 500 µl homogenate in a 1.7 ml centrifuge tube.

B. Mixer Mill

- Fill out a 48 cell master DNA collecting list ordered by location.
- Collect healthy and new growing leaf tissue in spring in 48-compartment boxes the day of the extraction or 24 hours earlier.
- Use a paper hole puncher to collect leaf punches. Place 4 – 6 leaf discs in the corresponding cluster tube (arranged in a 96-well plate containing one tungsten bead each)
- Make sure the hole puncher is free of the previous sample's tissue and clean it by punching paper holes and spray or rinse with ethanol occasionally before the next leaf sample is processed.

III. General Procedure

A. Cell Lysis

Lysis step is probably the most varied in literature regarding ways of extracting genomic DNA.
The SDS/proteinase K is a very standard procedure.
The cells are lysed open to make the DNA easily accessible for subsequent steps.

Cell Lysis Components
Tris, a buffering agent, (good @ pH 6-8).
EDTA chelates divalent cations which are necessary cofactors for DNase activity (way of shutting down nucleases).
NaCl at physiological concentration (generally considered to be 100 – 150mM) keeps all molecules happy (particularily proteinase k) and prevents unwanted aggregation.
SDS, is a nasty ionic detergent that is good at breaking membrane. It is also a general denaturant that inhibits enzyme activity. DNA is robust and is not really adversely affected by SDS treatment.
Sarkosyl, is a detergent often used in place of SDS when dealing with plant material. It essentially behaves in a similar manner to SDS.
Proteinase K, is a very effective serine protease (works well at 55 °C) and not particularly susceptible to SDS, and other denaturants such as urea. Proteinase K will chew up protein, which helps lysis in general and frees up the DNA from any protein gunk associated with it. Best used FRESH (*quite an important step). Incubation step generally a minimum of an hour.

B. Purification of DNA

*****Phenol*** is pretty nasty! It will burn.** Do PHENOL addition in fume hood. If you get it on you, do not panic. Quickly rinse off with cold water.
Phenol/chloroform works with any nucleic acid prep. Phenol is usually buffer saturated (i.e. when you buy it, the solution comes in two layers: The top layer is excess buffer and the bottom layer is buffer-saturated phenol).

The pH of the buffer is important: At acidic pH, DNA is soluble in phenol. When purchased from Ambion, the buffer comes in a separate bottle with the phenol/chloroform/IAA. Therefore add the buffer to the phenol solution for DNA extraction (but not for RNA extraction). If not buffer saturated any additional aqueous (i.e. water) solution you add will combine with phenol solution. (This is no good because YOU want the layering effect).

This purification procedure works on the principle of "differential solubility".
Phenol is an organic solvent. Nucleic acids are not soluble at all in it. Therefore, DNA/RNA will stay dissolved in the aqueous phase. Lipids and polysaccharides preferentially go into the phenol phase. Proteins will also selectively go into phenol solution. Phenol also acts as a denaturant. Proteins denature and form aggregates and will collect at the interphase (seen as gunk @ the interphase).
Choloroform, also has same general attributes as phenol (as far as solvent properties) but also stabilizes the rather unstable boundary between aqueous and organic layers. **Isoamyl alcohol** also contributes to interphase stability and also helps prevent frothing.
Phenol/Chloroform/Isoamyl alcohol (at a volume ratio of 24:23:1) is added to the lysate. This step can be done ~2 or 3 times. The more times it is done, the cleaner is the sample (the interphase gets cleaner and cleaner with each step). This procedure is very reliable and does not lose much DNA yield.
Sometimes a final Choloform step is recommended. It is likely done because it evaporates easily. The interphase is a little trickier to handle. However, the tube lid can be left open to make sure ALL the organics are gone from the prep.

C. Precipitation of DNA.

Efficiency of **ethanol** precipitation is dependant on a number of things: Temperature, length of precipitation time and amount of the DNA. It is added at 2 x volume.
Ammonium Acetate (0.5 volume) helps in the precipitation of the DNA in ethanol. **NaCl**, can be used (depending on the concentration of the DNA). Salt will help neutralize negative charge of DNA. It will also sequester the solvent molecules, in this case water.

Isopropanol can also be used (equal volume is added). RNA tends to stay soluble in this solvent. Some people use it for this purpose.

70% ethanol wash is included to get rid of excess salts.

IV. Mixer Mill Gentra DNA Extraction

1. Prepare the extraction buffer by adding 1g polyvinylpyrrolidone-40 to 50 ml Cell Lysis Solution.
   - Incubate at 65ºC for 10 min inverting occasionally until PVP is dissolved.
   - Cool extraction buffer (EB) to RT.

2. Place one tungsten bead per well in a 96 well plate cluster tube rack.
   - Punch leaf discs (20-30 mg) using a paper punch.
   - Place 4 -5 leaf discs per well.
   - Add 500 µl EB to each well.

1. Place in the white mixer mill holder with the A1 well near the mixer mill instrument.
   - Grind for 1.5 min at 30 Hz.
   - Take the rack out, remove the black adaptor lid, rotate so that A1 is towards the outside. Place the black adaptor lid back on.
   - Grind for another 1.5 min at 30 Hz.
   - Spin racks for 35 sec at 3000 rpm to collect the homogenate in bottom.
   - Punch a hole into each cap.

4. Remove rack bottom and incubate at 65ºC for 60 min.
   - After 30 min, place a paper towel on the lid and invert tube 10 times. Repeat inversion after 60 min.
   - Spin again for 35 sec at 3000 rpm.

5. Add 10 µl = 15 µg RNase A solution to the cell lysate using the multi channel pipet (from a master mix prepared from the 10 mg/ml stock).
   - For each 96 well plate, prepare by adding 174 µl RNase A stock solution (10 mg / ml) + 986 µl TE,
   - For 2 x 96 well plates, prepare by adding 348 µl RNase A stock solution (10 mg / ml) + 1972 µl TE,
   - Rinse pipet tips in nanopure water after each addition.
   - Discard the old caps and replace with new caps.
   - Mix the sample by inverting the tube 25 times and incubate at 37ºC for 30 min.

6. Add 150 µl Protein Precipitation Solution to the cell lysate in each well.
   - Vortex vigorously at high speed for 20 sec to mix uniformly.
   - Place the sample at -20 °C for 15 min (necessary to remove polysaccharides)
   - Centrifuge at 4,400 rpm for 15 min at 4 °C. The precipitated proteins should form a tight pellet.

7. To a new 96 well plate, add 400 µl isopropanol using wide pipet tip.

- Transfer 400 µl supernatant to new tubes.
- Close with the same tips.
- Mix by inverting 50 times.
- Place at -20 ºC overnight.

8. Centrifuge at 4,400 rpm for 15 min.
    - Carefully, remove the cap strips one at a time, place on a clean towel in order, pour off liquid and blot off the excess on a paper towel.
    - Add 1 ml 70% ethanol. Invert to wash pellet.
    - Centrifuge at 4,400 rpm for 5 min.
    - Pour off the ethanol again, one strip at a time.

9. Let dry in the hood overnight.
    - Add 500 µl DNA Hydration Solution or TE.
    - Incubate overnight at 4ºC to resuspend.
    - Store at 4ºC.

V. DNeasy 96-well Plate DNA Extraction

1. Add ethanol to Buffers AP3/E and AW.

2. Preheat Buffer AP1 to 65ºC.

3. Add one tungsten carbide bead to each collection microtube.

4. Use a leaf punch to punch leaf discs.
- Weigh out 50 mg equivalent and place in each well.

5. Make Lysis Solution by combining
   90 ml Buffer AP1
   225 µl RNase A, and
   225 µl Reagent DX.

6. Dispense 400 µl of Lysis Solution per well.
- Shake for 1.5 min at 30 Hz. Rotate the plate and shake again for 1.5 min at 30 Hz.
- Centrifuge the collection tubes until the rotor reaches 3000 rpm to collect all the solution in the bottom of the microtubes.
- Remove and discard the caps.
- Add 130 µl Buffer AP2 to each collection tube.
- Close the microtubes with new caps. Add the cluster tube rack cover and shake vigorously for 15 sec.
- Centrifuge the collection tubes again to collect any solution from the caps.
- Incubate the racks at -20ºC for 10 min to precipitate the proteins.

7. Centrifuge for 10 min at maximum speed.

- Remove and discard the caps.
- Transfer 400 µl of each supernatant to new racks of collection microtubes. (Leave the tangsten carbide beads to treat later).

8. Add 1.5 volumes or 600 µl of Buffer AP3/E to each sample.
- Close the tubes with new caps.
- Place the rack cover and shake the racks vigorously for 15 sec.
- Centrifuge the collection tubes again to collect any solution from the caps.

9. Place two DNeasy 96 Plates on top of Square-Well Blocks.
- Remove and discard the caps from the collection tubes.
- Carefully transfer 1 ml of each sample to the DNeasy 96 plates.
- Seal each DNeasy 96 Plate with an Airpore Tape sheet.
- Centrifuge for10 min at 4400 rpm.

10. Remove the tape. Add 800 µl of Buffer AW to each sample.
- Seal with a new AirPore Tape sheet.
- Centrifuge for 20 min at 4400 rpm to dry the DNeasy membranes.
- If a very dark membrane was seen, wash with another 800 µl of 100 % ethanol.
- Empty the flow through from the square-well block before performing the wash step.

11. Remove the tape. To elute the DNA, place the DNeasy 96 Plate on a new rack of elution microtubes RS.
- Add 100 µl of Buffer AE to each sample and seal with a new AirPore tape sheet.
- Incubate for 5 min at RT.
- Centrifuge for 5 min at 4400 rpm.
- Add another 100 µl of Buffer AE to each sample and seal with a new AirPore tape sheet.
- Incubate for 5 min at RT.
- Centrifuge for 5 min at 6000 rpm.
- Use new caps to seal the elution microtubes RS for storage.

VI. Hazelnut DNA Extraction

The DNA extraction protocol is based on that of Davis et al. (1998). The hazelnut tissue used consisted of young leaves in the spring or male catkins in the fall. DNA is extracted over the course of 2-5 days depending on time constraints. Up to 3 sets of 96 samples can be processed every day.

Day 1

- One young leaf is collected from seedlings in the greenhouse or field.
- On the same day/morning of field collection, the samples tissues are ground in the laboratory with a leaf juice press (MEKU, Wennigsen, Germany) using an extraction buffer consisting of 0.35 M sucrose, 100 mM Tris, 50 mM potassium chloride, 25 mM EDTA, and 5% PVP (molecular weight, 40,000).
- About 500 µl of the macerate is collected in a 1.5-ml centrifuge tube and placed on ice.
- Each sample is then centrifuged at 10,000 x g for 5 min. The supernatant is discarded and the pellet is resuspended in 640 µl lysing buffer [78.3 mM EDTA (pH 8.0), 39.16 mM Tris (pH 8.0), 2.12% n-lauroylsarcosine, 2.65% Triton X-100, and 10.7 µg/µl freshly-added Proteinase-K (Fisher Scientific)].
- Protein degradation is carried out by incubating the samples at 37 ºC for 1 h. in a shaker rotating at 160 rpm.
- After centrifugation at 10,000 x g for 5 min, 500 µl of supernatant is transferred to a new tube. An equal volume of isopropanol is added to the sample that is mixed and subsequently incubated at -20 ºC anywhere from 30 min to overnight.

Day 2

- Nucleic acids are precipitated by centrifugation at 10,000 x g for 5 min. The supernatant is discarded, the remaining liquid aspirated and the pellet is resuspended in 220 µl high salt TE buffer (10 mM Tris, 1 mM EDTA (pH 8.0), and 2 M sodium chloride) overnight at 4 ºC.

Day 3

- Samples are extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) by vortexing for 10 sec and then centrifuging for 10 min at 10,000 x g.
- A 150-µl aliquot of the upper aqueous phase is transferred to a new tube and precipitated with 2 volumes (300 µl) of 95% ethanol:3 M sodium acetate, pH 5.2 (20:1) at -20 ºC anywhere from 30 min to overnight.

Day 4

- The tubes are centrifuged, the supernatant poured off and the pellet is washed with 1 ml of 70% ethanol.
- The tubes are again centrifuged for 5 min, the supernatant discarded and the pellet is air-dried overnight.

Day 5

- The dry DNA pellet is resuspended in 500 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

Note:
DNA yield is however variable and depends on leaf age and genotype. The amount of DNA obtained from one leaf weighing 0.5-0.7 g can be as low as 150 ng or as high as 15 µg. Despite such variability, 97% of the DNA samples are amenable to amplification by PCR. The DNA sample is still contaminated with RNA but adequate for PCR. For DNA samples that will be used over a long period of time, further purification by RNase A treatment is done. RNA is degraded by incubating the sample in RNase A-containing TE buffer (10 ng) at 37 ºC for 30 min. RNase A treatment can be performed after isopropanol precipitation or at the end of the DNA extraction protocol. As a last step, RNase treatment has to be followed by another phenol:chloform:isoamylalcohol extraction and ethanol precipitation to respectively eliminate the enzyme and precipitate the DNA.

VII. CTAB DNA Extraction

This procedure is a miniprep method of the original Doyle and Doyle (1987) technique with the addition of a polyethylene glycol precipitation (PEG) step at the end. The description presented here is borrowed from Messinger (1999) and Rowland (1993).

1. Grind .3 g leaf tissue in about 1 ml hot (65 deg C.) CTAB in a 1.5 mL microfuge tubes.

2. Incubate ca. 30 minutes in a 65°C water bath.

3. Let sit at RT for 10 min (for samples to cool)

4. Divide the sample into 2, Tube 1 to follow Method 1 and Tube 2 to follow Method 2.

5. Extract once with equal volume chloroform:isoamyl alchohol 24:1.

6. Spin at about 6,000 RCF for 10 minutes. Most of the debris should be concentrated in the interface. If it is not, try spinning more and faster.

7. Remove the upper, aqueous phase to a clean centrifuge tube

Method 1, CTAB (Doyle and Doyle)

8. To Tube 1, add equal volume chloroform:isoamyl alcohol 24:1.
- Spin at about 6,000 RCF for 10 minutes.
- Most of the debris should be concentrated in the interface.
- Remove the upper, aqueous phase to a clean centrifuge tube

9. Add about equal volume isopropanol, and allow to precipitate at RT overnight.

10. Spin at maximum speed for 5 minutes
- Pour off supernatant

11. Wash with 1 ml ice cold 70% ethanol, vortex, and leave at RT for 20 min.

12. Spin at maximum speed for 5 min,
- Pour off supernatant.
- Spin again for 30 sec, pipet off the remaining liquid.
- Let dry in the hood for 15-20 min.

13. Resuspend in minimal (50 µl) TE at 4 °C overnight.

Method 2, Modified CTAB (Rowland)

1. To Tube 2, add 1/5 volume of 5% CTAB, 0.7 M NaCl solution to the aqueous phase.
- Add about two thirds volume isopropanol,
- Allow to precipitate at room temperature overnight. (As little as 30 minutes may be sufficient).

2. Spin at maximum speed for 30-60 minutes,
- Pour off supernatant
- Dry thoroughly in speedvac with heat off (try 18 minutes).

3. Fill the tube with 76% EtOH/.01M ammonium acetate,
- Let stand for 10-30 minutes,
- Spin briefly,
- Pour off supernatant,
- Dry thoroughly.

4. Resuspend in 0.5 ml TE, add 0.125 ml 4 M NaCl and 0.625 ml 13% PEG.
- Allow to precipitate on ice water for 1 hour.

5. Spin at maximal speed (13,000) for 10 minutes (refrigerate if possible).
- Wash with ice cold 70% ethanol
- Resuspend in minimal (50 µl) TE.

SECTION 5. Polymerase Chain Reaction

I. History

PCR was invented by Kary Mullis, who was awarded the Nobel Prize in Chemistry in October 1993 for this achievement, only seven years after he first published his ideas. Mullis's idea was to develop a process by which DNA could be artificially multiplied through repeated cycles of duplication driven by an enzyme called DNA-Polymerase.

DNA-Polymerase occurs naturally in living organisms, where it functions to duplicate DNA when cells divide. It works by binding to a single DNA strand and creating the complementary strand. In Mullis's original PCR process, the enzyme was used *in vitro* (in a controlled environment outside an organism). The double-stranded DNA was separated into two single strands by heating it to 96°C. At this temperature, however, DNA-Polymerase was destroyed so that the enzyme had to be replenished after the heating stage of each cycle. Mullis's original PCR process was very inefficient since it required a great deal of time, vast amounts of DNA-Polymerase, and continual attention throughout the PCR process.

Later, this original PCR process was improved by the use of DNA-Polymerase taken from *thermophilic* (heat-loving) bacteria that grow in geysers at a temperature of over 110°C. The DNA-Polymerase taken from these organisms is *thermostable* (stable at high temperatures) and, when used in PCR, did not break down when the mixture was heated to separate the DNA strands. Since there was no longer a need to add new DNA-Polymerase for each cycle, the process of copying a given DNA strand could be simplified and automated.

One of the first thermostable DNA-Polymerases was obtained from *Thermus aquaticus* and called *Taq*. Taq polymerase is widely used in current PCR practice (May 2001). A disadvantage of Taq is that it sometimes makes mistakes when copying DNA, leading to *mutations* (errors) in the DNA sequence. Polymerases such as *Pwo* or *Pfu,* obtained from *Archea,* have *proofreading mechanisms* (mechanisms that check for errors) and can significantly reduce the number of mutations that occur in the copied DNA sequence.

II. Key points

PCR is used to amplify a short, well-defined part of a DNA strand.

PCR, as currently practiced, requires several basic components. These components are:

- Open or single stranded target DNA by denaturation of double stranded DNA that is usually accomplished by many enzymes that unwind DNA (such as helicases). With PCR, HEAT is used to open up our DNA.
- Replication also needs a primase enzyme to make primer for the polymerase. BUT, you can make your own! Buy oligo's. Two *primers* determine the beginning and end of the region to be amplified
- *Nucleotides* (can buy these), from which the DNA-Polymerase builds the new DNA
- *Buffer*, which provides a suitable chemical environment for the DNA-Polymerase
- DNA polymerase, which copies the region to be amplified. HOWEVER, this high temperature will basically denature any protein structures, including our polymerase. TO get around this, a heat-stable polymerase isolated from a thermophile (a bug that grows in high temperatures) is used.

The PCR reaction is carried out in a *thermocycler.* This is a machine that heats and cools the reaction tubes within it to the precise temperature required for each step of the reaction. To prevent evaporation of the reaction mixture, a heated lid is placed on top of the reaction tubes or a layer of oil is put on the surface of the reaction mixture.

III. Procedure

The PCR process consists of a series of twenty to thirty cycles. Each cycle consists of three steps (Fig. 1):

- 93 - 95 °C denaturing temperature, 30 - 45 sec
- X °C annealing temperature, 30 - 45 sec
- 72 °C elongation temperature, 30 - 60 sec

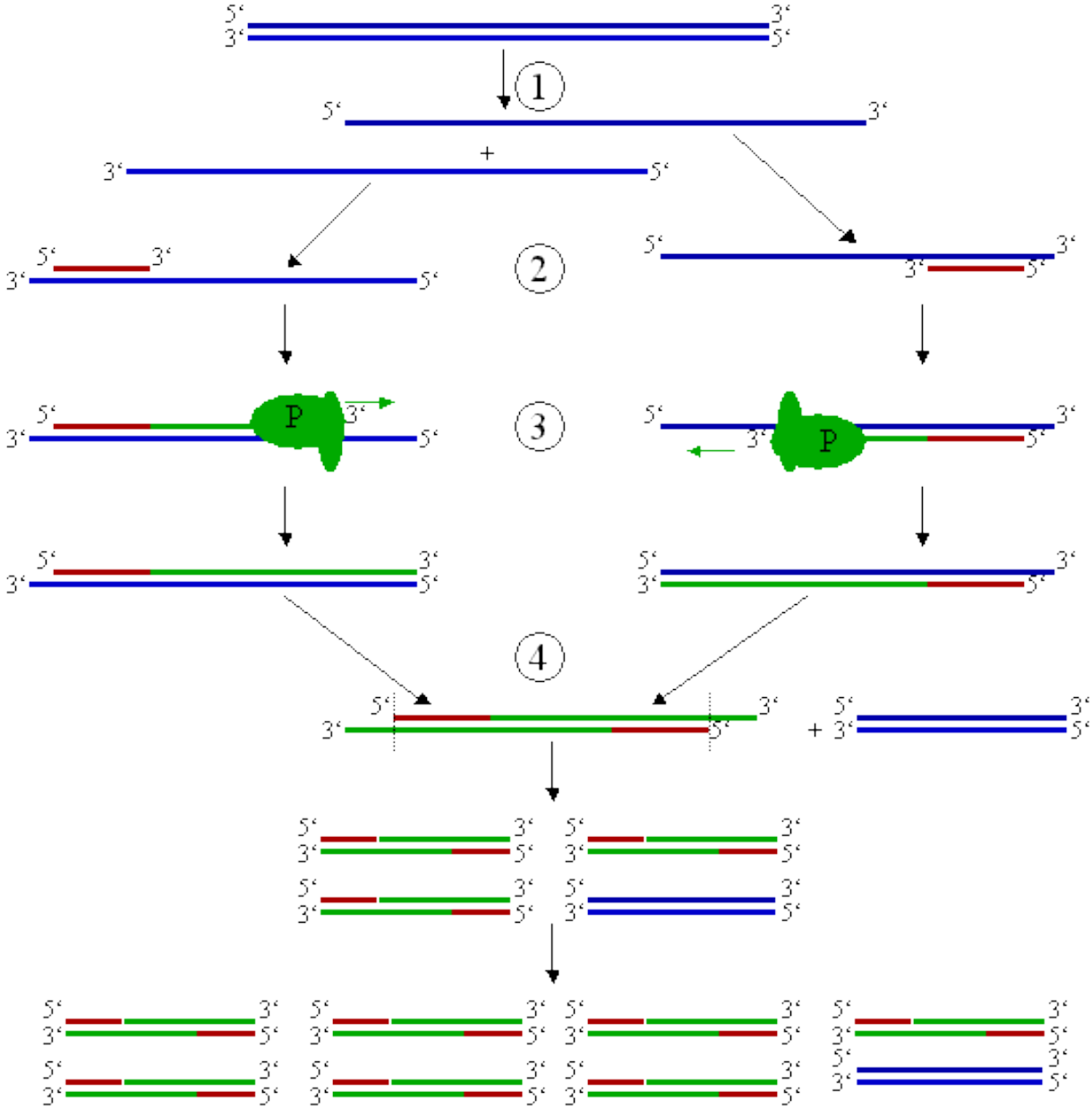


Fig. 1

1. **Initial denaturation or melting** is first performed (before cycling) to completely denature the template double helix, exposing single stranded template DNA for primer annealing and second strand synthesis. The temperature used for this step can actually vary from 90-96°C and depends on both the nature of the template and the enzyme being used. The more complex the template and the more G/C rich a template is, the higher the denaturation temperature required. Some enzymes are more heat labile than others, read the manufacturers product sheet to find the recommended denaturation temperature (or range of denaturation temperatures) for your enzyme.

   Denaturation or melting in each cycle is performed to completely denature the newly synthesized products, and to a lesser extent the initial template, exposing single stranded template DNA for the next round of DNA synthesis, primer annealing and second strand synthesis. As described above, the temperature used for this step can actually vary from 90-96°C, though is usually either 92°C or 94°C. Typically this step is carried out for 30 secs but for labile enzymes and delicate A/T rich-templates, it can be reduced to 10 seconds.

2. **Annealing** is the technical term for two single-stranded DNA molecules combining via hydrogen bonds to complementary bases to form one double stranded molecule. In this context it is normally referring to the binding of the PCR primers to their target site. The temperature at which annealing is allowed to take place during PCR is critical. Normally annealing is performed at 5°C below the lower of the PCR primers melting temperatures (Tms). Any lower and the primers will anneal to non-specific sites and any higher they may not anneal at all. Although this is true in most cases, optimal annealing temperatures can be far from Tm-5°C, and can even be higher than one of the primer Tms. Consequently the annealing temperature may have to be altered in either direction if the conditions above do not result in optimal amplification. As explained, in this style annealing temperature is slowly reduced in the first stage such that amplification occurs first under conditions where specific primer binding occurs.

3. **Extension or elongation** is the stage in which second strand DNA synthesis takes place from the 3' end of the oligonucleotide primer that has annealed to the template. The extension temperature used should be that recommended by the manufacturer, typically 72°C for Taq polymerases or 68°C for **High-Fidelity** and **Long-Range** enzymes. For some applications e.g. amplification of delicate A/T rich templates this temperature may have to be reduced. The manufacturer's product sheet will also state the processivity of the enzyme, i.e. the rate of synthesis. This is normally 1.5-2 kb per minute, though you should allow 1 kb per minute with a minimum of 1 minute.

The cycle (steps 1-3) is repeated 30-40 times.

4. **Final extension** is an optional single cycle step included to allow complete synthesis of any partial products that may have formed in the latter stages of

PCR when the enzyme was less active.

5. **Holding**. To ensure that no degradation of DNA occurs once the cycling is complete, set the temp cycler to hold the reaction at 4°C.

The amount of DNA essentially doubles with every cycle. Therefore after 30 cycles, ~1000000000 molecules of amplified product are obtained. Therein lays the power of PCR. Lots of product is obtained easily from very little material (theoretically one strand is all you need).

III. PCR Reaction Components

PCR buffer: salt (50mM KCl), buffer (10mM Tris-Cl @ pH 9.0), and detergent (0.01% Triton X-100). A new ammonium-based buffer is less sensitive to Mg ion concentration (for eg., the Bioline 10 x buffer).

Target DNA:
For gene amplification, a working stock of 10 ng/ ul is initially used.
For microsatellite analysis, 2.5 ng/ ul is the stock we use.
THE PURER THE BETTER, but PCR is quite forgiving.

Primers: each @ 0.1 - 0.50 μM using a 10 μM stock, about 20 to 30nt in length. The distance between two primers should be < 10kb, but anything > 3kb will affect efficiency. If there are differences, put the differences on the 5' end.
The choice of the length of the primers and their melting temperature depends on a number of considerations. The melting temperature of a primer--not to be confused with the melting temperature of the DNA in the first step of the PCR process--is defined as the temperature below which the primer will anneal to the DNA template and above which the primer will *dissociate* (break apart) from the DNA template. The melting temperature increases with the length of the primer. Primers that are too short would anneal at several positions on a long DNA template, which would result in non-specific copies. On the other hand, the length of a primer is limited by the temperature required to melt it. Melting temperatures that are too high, i.e., above 80°C, can also cause problems since the DNA-Polymerase is less active at such temperatures. The optimum length of a primer is generally from thirty to forty nucleotides with a melting temperature between 60°C and 75°C.

GC content similar to template sequence, avoid polypurines or polypyrimidines.

dNTPs, ~0.2 mM mix (0.02 – 0.2 mM) from a 2.5 mM stock. Enough to make 12.5 μg of product!!!!!

Enzyme usually comes in a stock of about 5 Units/ul.

- **Biolase**: use 0.05 μl per 10 μl PCR reaction
- **Amplitaq Gold** and **Taq Polymerase**: use 0.25 μl per 15 μl reaction

NOTE, that Taq adds an ATP is to the 3' end when done!!!

IV. Troubleshooting:

MgCl2: VERY IMPORTANT probably the most important condition when using Taq polymerase. However, using ammonium-based buffer in the 10 x biolase buffer decreases sensitivity to Mg ion concentration.

Reasons why are a little vague but Mg ions seem to do a number of things.

- They bind DNA: may affect primer/template interactions.
- They bind Taq polymerases and Mg is required as a cofactor
- They influence Taq polymerase's ability to interact with primer/template sequences.
  High Mg ions concentration results in decreased stringency in binding.

GENERALLY, if doing a PCR reaction for the first time using Taq polymerase as an enzyme, do a titration of 0.5 and 2.5 mM final concentration to determine the optimal Mg concentration.

DMSO: denaturant ability. Good at keeping GC rich template/primer strands from forming secondary structures. It doesn't seem to generally affect the reaction. Therefore, most people will include it regardless (use @ 5%).

Glycerol: increases apparent concentration of primer/template mix. Therefore may help in getting good primer/template interactions at high temperatures (use @ 10%).

Annealing temperatures

- If GC < 50%, use 55 °C
- if > 50% use 60 ° C
- OR use the ((A+T) x 2) + ((G+C) x 4) to figure out the Tm.

Extension times:

- Extension at 72 °C: 500 nt, use 30 – 45 sec;
- If greater than 500 nt, use 1 min.

OPTIMIZING the first round of amplification. (IMPORTANT)

Taq polymerase may start replication when the primer is binding at an inappropriate site. This will lead to "pseudo" bands and a diluting out effect of the real template. There are a couple tricks to optimize the first cycle.

- Use a “hot start” polymerase.
- “hot start” the reaction by adding the polymerase after the first denaturation and annealing step.
- Use a Taqstart antibody. It will get denatured upon 95 °C step.

V. PCR Programs

A. Hot Start PCR

"Hot-start" PCR is a method that generally produces cleaner PCR products. Template DNA and primers are mixed together and held at a temperature above the threshold of non-specific binding of primer to template. All the PCR reaction components are added for the extension reaction except one critical reagent (usually the thermostable polymerase).

Just prior to the cycling, the missing component is added to allow the reaction to take place at higher temperature. Due to lack of non-specific hybridization of primers to template, the amplified DNA bands tend to be cleaner; the primers don't have a chance to anneal non-specifically.

This method is difficult to do because the tubes must be kept on a 100 °C heat block as your work surface. There are ways to avoid this however. One way is to quickly cool the tubes on ice while adding the component mix. You can then heat the tubes on the pre-warmed thermocycler just before adding the last component. This may not always be successful due to a thermal ramp that may allow non-specific interactions between primer and template.

Hot starts are also done by creating a physical barrier between the essential components, eg. primers and template. This barrier may be created by putting a half-reaction mixture into the bottom of the tube and melting wax over the mix. The wax used can be "PCR Gems" from Perkin-Elmer/Cetus or any number of home-grown waxes (e.g. paraffin or Paraplast). Cooling solidifies the wax, and the missing components can be placed on top. The mixing of the last component then occurs at high temperature only when the wax melts and the top half-mix is added by convection currents within the tubes. The PCR then proceeds as a normal cycle sequence.

Co-solvents have also been used to eliminate artifacts from PCR reactions. For high fidelity, the specificity of primer to template is desirable. Co-solvents such as glycerol, DMSO, and formamide, work to provide highly stringent reactions by changing the Tm of the primer-template hybridization reaction.

Co-solvents have various effects on the thermostablility of the polymerase enzyme. Glycerol tends to extend the resistance of Taq enzyme to heat destruction, while formamide lowers enzyme resistance.

Hot Start PCR can be achieved by the addition of AmpliTaq Gold® DNA Polymerase, a chemically modified form of AmpliTaq® DNA Polymerase. When the chemical moiety is attached to the enzyme, the enzyme is inactive. During set-up and the first ramp of thermal cycling (when the reaction is going through non-optimal annealing temperatures), the enzyme is inactive. The result is that mis-primed primers are not extended. Once the reaction reaches optimal annealing temperatures, the chemical moiety is cleaved during a heat activation step, releasing active AmpliTaq® DNA enzyme.

The yield of specific product increases because reactants are not wasted in the formation of unintended products. Because AmpliTaq Gold® DNA Polymerase is a chemical hot start enzyme, there is no worry of biological contamination.

AmpliTaq Gold® DNA enzyme's chemical hot-start capability can release active enzyme in a "time-release" manner. With or without a limited up-front heat activation step, active enzyme is released slowly during thermal cycling to match template concentration and increase specificity.

B. Touch Down PCR

This cycling style was developed to simplify the process of finding the optimal annealing temperature.

It involves starting with a very high annealing temperature (10 to 15°C above the expected optimum) and reducing the annealing temperature by 1 to 2°C per cycle over the first 10 cycles, then performing 20 cycles at, or slightly below, the predicted optimum. As a result the first products generated will be those formed from cycles with a high annealing temperature where at some point under these stringent conditions annealing could just occur and then only at 100% homologous sites. In these circumstances (and for the next few cycles) amplification of the desired product only is favored. As a result specific products are generated that will serve as template in the next round of amplification, and specific amplification is obtained without empirical determination of optimal annealing temperature. This cycling style can work well with all types of PCR and can be especially useful for amplification of targets from heterologous templates e.g. PCR of a locus from different genomes using the same set of primers. In this case some genomes will have slightly different sequences at the primer binding sites and at the optimal annealing temperature PCR would fail. But by starting high and slowly dropping to lower annealing temperatures, conditions are found where annealing just occurs and specific products can be synthesized. An example of a "touchdown" program would be:

Initial denaturation[a]	92-94°C	For	2 min	1 cycle
Denaturation[b]	92-94°C	For	10sec	10 cycles
Annealing[c]	Tm(+5°C) minus 1°C/cycle	For	30sec	10 cycles
Extension[d]	72°C	For	1 min per kb*	10 cycles
Denaturation[b]	92-94°C	For	10sec	20 cycles
Annealing[c]	Tm(-5°C)	For	30sec	20 cycles
Extension[d]	72°C	For	1 min/kb	20 cycles
Final extension[e]	72°C	For	7 min	1 cycle
Holding[f]	4°C			

C. Gradient PCR

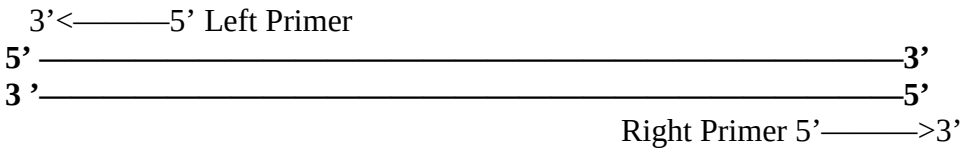
The selection of the annealing temperature is possibly the most critical component for optimising the specificity of a PCR reaction. In most cases, this temperature must be empirically tested. The PCR is normally started at 5ºC below the calculated temperature of the primer melting point (Tm).
However, the possible formation of unspecific secondary bands shows that the optimum temperature is often much higher than the calculated temperature (> 12ºC).The Mastercycler® Gradient enables rapid testing of the optimum temperature conditions on one block and in one experiment. During the PCR, a temperature gradient, which can be programmed between 1ºC and 20ºC, is built up across the thermoblock. This allows the most stringent parameters for every primer set to be calculated with the aid of only one single PCR reaction.

B. Inverse PCR (Bradeen and Simon 1998)

Inverse PCR is a powerful and simple PCR-based technique used to obtain unknown flanking DNA sequences to a known sequence.

Protocol

1. Primers that are looking opposite directions to each other are designed.



2. One microgram of genomic DNA is singly digested with restriction enzymes that don't cut the known sequence, according to the manufacturer's instruction.
3. Digested DNA is desalted by QIAquick® Spin columns and quantified by fluorometry.
4. DNA is diluted to 1ng/µl for ligation reaction containing 500ng/ml DNA and following reaction mixture is prepared and incubated for 16-18 h at 14°C.

Ligation Reaction (/10 µl)

Ligase 10X buffer	1
T4 ligase (3u/µl)	1.7
dATP (1mM)	0.5
DNA (1ng/µl)	5
H_2O	1.8
Total	10 µl

5. Reactions are desalted by spin columns again, dried under vacuum, and resuspended in TE to a final concentration of 1ng/ µl.
6. Inverse PCR reaction is performed according to the following reaction mixture and the temperature cycle is 95°C for 10 min for only 1 cycle, 35 cycles of 95°C for 40s, annealing temperature for 2 minutes, 72°C for 4 min, and for one cycle final polymerization 72°C for 4 min.

iPCR Reaction Mixture (/10 µl)

10 X buffer	5
50 mM $MgCl_2$	2.5
dNTP mix (2.5mM each)	1
Biolase	
0.056	
iPrimer1 (10µM)	0.5
iPrimer2 (10µM)	0.5
Circular DNA (1ng/µl)	
1.25	
H_2O	
39.2	
Total	50

7. iPCR bands from the agarose gel are extracted as following;
    a. iPCR product on loaded on 2% agarose gel and electrophoresed for 2 h at 90V.
    b. The single bands are cut out with sterile scalpel.
    c. The pieces of gels (w/band) are put in 1.7µl

tubes and 50-100µl ddH_2O is added.

d. Tubes freezed (at -70°C) and thawed (2X).
e. 1.2µl of water with DNA is used as template and the following PCR reaction is performed (for 1 sample), 35cycles 94°C 30s, annealing temp. 40s, 72°C 30s.

PCR Reaction (/15 µl)

10 X buffer	1.5
50 mM $MgCl_2$	1
dNTP mix	0.4
Biolase 0.056	
iPrimer1 (10µM) 0.15	
iPrimer2 (10µM) 0.15	
DNA	1.2
H_2O 10.54	
Total µl	15

f. 5µl of PCR product is loaded on agarose gel and the rest is kept for cloning and sequencing.

SECTION 6. Microsatellite Analysis

I. Definition

Microsatellites are tandemly repeated sequences whose unit of repetition is between one and 5 or 6 base pairs.

- Di-nucleotide repeats **GA**GAGA
- Tri-nucleotide repeats **GGA**GGAGGA
- Tetra-nucleotide repeats **CAGA**CAGACAGA
- Hexa-, Penta-

II. Characteristics

- Found in wide variety of eukaryotes (nuclear), in the chloroplast genome of plants, and in mitochondrial genome.
- Classified into three families:
  - Pure CACACACACACACACACACA
  - CompoundCACACACACA**GAGAGAGAGA**
  - InterruptedCACACA**TT**CACACACA**TT**CACA
- Microsatellites are co-dominant and inherited in a Mendelian fashion (based on pedigreee analysis).
- They are neutral.
- Highly polymorphic in natural populations, with average heterozygosity well above 50 % in general.
- Compound and interrupted loci tend to be less polymorphic than pure. Interruptions within the core sequence seem to stabilize arrays of repeats, making interrupted repeats less variable than pure ones.
- The distribution of microsatellites is random when considering the whole genome. High density mapping of vertebrates indicates a clustering of microsatellites as observed in frequent association of different types (di-, tri- and tetra-) in the same cloned insert.
- Mean density of microsatellites varies widely.
- Microsatellites are found within coding and non-coding regions of the genome. However, they are most often found in the non-coding regions, where the nucleotide substitution rate is higher than in coding expressed regions. (Consequently, the strategy of designing 'universal primers' matching conserved sequences, which was very effective for mitochondrial DNA, is more problematic for microsatellites. It is important to note however, that highly conserved flanking regions have been reported in cetaceans, turtles and fish, thus allowing cross-amplification from species that diverged as long as 470 million years ago (Ma).
- Recently, using large scale genomic as well as EST DNA sequencing (transcribed), Morgante et al., estimated microsatellite density in 5 plant species with a 50-fold range in genome size that is mostly attributable to the recent amplification of repetitive DNA: *A. thaliana, Oryza sativa, Glycine max, Zea mays,* and *Triticum aestivum*, which have haploid genome sizes ranging from 125 Mbp to 5600 Mbp. (Much of the
- observed difference in size is attributable to the amount of repetitive DNA , in particular to long-terminal-repeat (LTR) retrotransposon amplification.

Retrotransposon amplification doubled the size of the maize genome over the past 5 million years). They concluded that:

- Among species, the overall frequency of microsatellites was inversely related to genome size and to the proportion of repetitive DNA (i.e., microsatellite frequency was highest in *Arabidopsis*, and increasingly lower as we move towards species with larger genomes such as maize and wheat). Therefore, microsatellite frequency is not a function of genome size but of the relative proportion of single-copy DNA. Consequently, microsatellites are more abundant in single/low-copy DNA than in repetitive DNA. Genes and single-copy regions are the most ancient portion of plant genomes, as their presence predates that of repetitive DNA. Thus, one would expect, and experimental data support, a higher density of microsatellites in species in which the genome expanded less recently, such as conifers.
    - Coding sequences are under negative selection for all microsatellite types except trinucleotide.
    - 5' UTRs are under very strong positive selection.
    - 3' UTRs are under moderate positive selection.
    - Repetitive DNA is under mutational (slippage) pressure that increases microsatellite frequency
    - Nontranscribed DNA is presumably at equilibrium (?????)

A. Disadvantages

- The major drawback is that they need to be isolated de novo from most species being examined for the first time.
- The requirement to determine fragment lengths, which complicates automation (as compared to markers amenable to yes/no tests which can be set up on dense chips, for e.g. single nucleotide polymorphisms)

B. Dinucleotide repeats

- Most frequently used.
- Density varies with species, peaking around one locus per 5 Kb. More characteristically, they occur one every 30 – 40 Kb.
- At each locus, the number of dinucleotide repeats is < 30 in animals, while mean size in plants is 10.
- The animal kingdom is very rich in CA.
- Plants are rich in either TA or GA repeats.
- Over 25 % of identified loci may belong to compound and/or interrupted families.

C. Trinucleotide repeats

- They are often found within exons.
- They are largely studied in connection with human diseases and cancer, where the disease-causing allele may have over 1000 repeats (neurological disorders).
- GTG repeats are preferentially localized subtelomerically on human chromosomes.
- Mean number of repeats and polymorphism is comparable to that of dinucleotide repeats.

D. Tetranucleotide repeats

- Prominent members are GATA/GACA repeats.
- Density varies between species.
- In the human and tomato genomes, they are clustered near centromeres.
- In snakes, they have a sex-chromosome- specific distribution.
- They are often found as compound and/or interrupted stretches.
- They are highly polymorphic, though rarely used.

E. Microsatellite use

- Preferred marker for very-high resolution mapping (dense distribution across the genome)
- Population genetic studies (from identifying relatives to inferring demographic parameters)
- DNA fingerprinting
- Paternity testing
- Conservation/management of biological resources.

III. Analysis Steps

A. Primer Design

Primers flanking the microsatellite are designed using free software Primer 3 @ http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi.
Optimum Primer Size: 22, Range = 20-25
Optimum Primer Tm: 60, Range = 55-63
Optimum GC %: 50, Range = 40-55
The primers are designed to amplify a PCR product that ranges from 100 -350 bp in length.

B. Primer Orders

The forward primers are tagged with the following fluorescent dyes. The size of the PCR determines the dye used (refer to Table 1). All primers except for Ned-labeled primers are ordered from Operon. Ned primers are obtained from ABI. Use the quotes to order primers.

Ned	Hex	Fam	Ned	Hex	Fam
100-150	150-180	180-250	251-280	281-300	>300

Table 1.

C. PCR

1. Reaction

Component	[Stock]	[Final]	Vol/10 ul rxn
Water			5.55
Biolase Buffer 10X	10	1	1
MgCl2 50 mM	50	2	0.4
dNTP 2.5 mM	2.5	0.2	0.8
Primer L 10uM	10	0.3	0.6
Primer R 10 uM	10	0.3	0.6
Biolase 5U/ul	5	0.025	0.05
Template DNA 3 ng/ul	3	0.3	1
Total			10

2. Program

Initial denaturation[a]	94°C	For	3 min	1 cycle
Denaturation[b]	93°C	For	40 sec	35 cycles
Annealing[c]	50-64 °C	For	40 sec	35 cycles
Extension[d]	72°C	For	40 sec	35 cycles
Final extension[e]	72°C	For	30 min	1 cycle
Holding[f]	4°C			

C. Agarose gel Electrophoresis

- Following PCR, 1 µl of each PCR product is transferred to a plate containing 39 µl of nanopure water, i.e. the PCR product is diluted 1:40.
- 3 µl loading dye is added to the remaining 9 µl PCR product and it is loaded on 3 % agarose gel.
- Visualization after ethidium bromide staining confirms amplification before further expensive analysis by capillary electrophoresis at the Central Services Lab (CSL). Unamplified DNA is reamplified.

D. General Steps

1. The primers are designed and ordered.

2. When the primers arrive, sterile TE is added to the primers to a final concentration of 100 µM (i.e., if pmol is 34568, add 345 µl of TE) and left overnight at 4 °C.

3. A 10 µM Primer working stock is prepared from the 100 µM primer stock which is placed in its box at – 20 °C.

4. Optimum annealing temperature of the primers is determined by gradient PCR.

5. PCR is subsequently performed at that optimum annealing temperature.

6. Informative loci are determined and their forward primer is fluorescently tagged.

7. Repeat steps 2 and 3 with the new fluorescent primers.

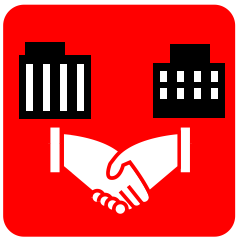
8. Following PCR with the fluorescent primers, 1 µl of each PCR product is transferred to a plate containing 39 µl of nanopure water, i.e. the PCR product is diluted 1:40.
9. 3 µl loading dye is added to the remaining 9 µl PCR product and it is loaded on 3 % agarose gel and amplification is confirmed by visualization after ethidium bromide staining.
10. A dilution series of 1:200 – 1:600 is used and optimum dilution factor for capillary electrophoresis id determined.
11. Once the optimum dilution factor is determined for each PCR product, multiplexing is carried out by pooling the PCR products into one plate. 1 µl is transferrd to an ISC 96-well plate for capillary electrophoresis at the CSL. We are charged for a multiple of 16 samples and the minimum number of samples to submit is 32.
12. Information about Fragment analysis is @ http://www.cgrb.orst.edu/CSL/frag_anal.pdf
13. Submit the job online to the CSL at https://csl-web.cgrb.orst.edu/ordering/index.php3
    - Username is: bassilna
    - Password is: basseem
14. Save the Fragment Analysis 3100-96 well excel sheet on your desktop and fill it in.

Color Key:			
1 = blue 2 = green 3 = yellow 4 = red 5 = orange	column for sample name	column for sample info	column for sample comment

1.0.1

Plate Name=	GS Sample Name	96-Well Color Number	Standard Dye	Dye Set	Color Info	Color Comment
A1		1	4	D		
A1		2	4	D		
A1		3	4	D		
A1		4	4	D		
A1		5	4	D		
B1		1	4	D		
B1		2	4	D		
B1		3	4	D		
B1		4	4	D		
B1		5	4	D		

15. Fill in the Fragment Analysis Form . The job number given at the end is written on the plate and then submitted to the CSL.



SECTION 7. Records Management

Computer records
Any question regarding the manipulation of computer or database in NCGR, ask our computer consultant.

I. FoxPro

- Database software used to record and edit data.
- Login to FoxPro through each terminal in the repository to access up-dated information
- Database information in FoxPro can only be changed by authorized persons. Do not make any changes instead make note of the problem and inform the appropriate person.

II. GRIN (Germplasm Resource Information Network)

- GRIN system is represented on the Internet using World Wide Web or Gopher services.
- It is a network system in the USDA/ARS Maryland allowing us to access the germplasm database of United States.
- We can browse it through web browser such as Netscape or Explorer, by the home page of repository (http://www.ars-grin.gov/ars/PacWest/Corvallis/ncgr/ncgr.html) and link to it, or directly type the URL (Universal Resource Locator): http://www.ars-grin.gov

III. Papyrus

- A bibliographic database of journal articles on file in TC lab and in Nahla's office.
- One instruction book available in Barbara's office and another one in Nahla's office.
- All journal articles used in research is entered in this database and filed in the file cabinet in Nahla's office.

References

Bradeen, J. M. and P. W. Simon. 1998. Conversion of an AFLP fragment linked to the carrot Y2 locus to a simple, codominant, PCR-based marker form. TAG 97 (5/6) p. 960-967.

Hummer, K.E. 1997. Operations manual. USDA-ARS National Clonal Germplasm Repository, Corvallis, OR.

Internet web page: www.ars-grin.gov/cor

Rowland, L.J. and Nguyen, B. 1993. Use of Polyethylene Glycol for Purification of DNA from Leaf Tissue of Woody Plants. BioTechniques 14(5):735-736.

Messinger, Wes. 1999. Master's Thesis: Ribes Phylogeny as indicated by restriction site polymorphisms of PCR amplified chloroplast DNA, (Appendix I). Department of Horticulture, Oregon State University, Corvallis, OR.

APPENDIX 1. Emergency procedures

I. Emergency is defined as a threat to life or property.

II. Emergency response

A. Remain calm.

B. Contact the appropriate party immediately with all related information (address, name, nature of emergency, etc.).

C. Emergency phone numbers.

D. Know how to call the emergency number from a Federal phone (9-911). Stay on the line until the operator has all the information and tells you to hang up.

Fire:	**911**
Police:	**911**
Ambulance/medical:	**911**
Poison control:	**1-800-452-7165**
Research leader: Kim Hummer -	**757-1493 (home)**
Facilities manager: Dennis Vandeveer -	**507-0161 (pager), 928-9866 (home)**
Chemical Hygiene officer: Barbara Reed -	**929-7474 (home)**
Location facilities manager: Tom Garbacik -	**929-6267 (home)**
Administrative officer: Phil Dailey -	**753-6180 (home)**

E. Emergency exit route (see appendix 3)

F. Know the location of at least two exits from the building and be able to find exits in total darkness.

G. Accident and hazard reporting
See "Safety Manual" in the library of NCGR.

H. Health maintenance program
See "Safety Manual" in the library of NCGR.

I. Hazardous waste
See "Safety Manual" in the library of NCGR.

J. Rinse eyewashes weekly

CHAPTER 11. IN VITRO AND CRYOPRESERVATION

1. Staff Requirements:
2. Laboratory Facilities:
3. Transfer Facilities:
4. Growth Room Conditions:
5. Storage Facilities:
6. Storage and Shipment of Germplasm:
7. Initiation, Growth and Rooting of Cultures:
    - General Procedures
    - Detection of Contamination
    - Standard Procedures for each Genus
    - Bacteriology
    - Cryopreservation Protocols

8. References:
9. Standard Equipment and Supplies:

1. Staff Requirements

Educational Background: The level of functioning of an *in vitro* operation is dependent on the personnel involved. Staff with advanced degrees in plant physiology and a major emphasis on micropropagation is needed for continued development of techniques and the optimal level of functioning of the facility. Laboratory technicians should have a good background in plant sciences and micropropagation/tissue culture. Advanced degrees are optional in this position depending on the required level of independence. Laboratory assistants with training in basic botany and/or horticulture and tissue culture techniques are preferred, however in practice, intelligent persons without an extensive educational background can be trained on site.

Training on Site: Because many of the necessary techniques and procedures used will be developed at the facility, on site training is needed even for assistants with plant science and micropropagation backgrounds. The head laboratory technician or lead scientist should be prepared to spend 1 to 2 weeks intensively training each new lab assistant so that procedures and techniques remain standardized within the laboratory. Consistent work protocols will ensure reliable propagules for germplasm storage and reliable research results.

Staffing Levels: The number of personnel involved will depend on the number and diversity of the accessions involved and the results desired. The normal diversity expected of a germplasm collection demands propagation methods that often will be far from routine. The more genera involved and the more complete the collection, the greater number of staff required for optimal functioning. In any facility wishing to have a tissue culture backup or distribution collection, the minimum staff number is one highly qualified technician, loosely supervised by the facility director. However, for active collections with research and development needs, the desired staffing level would include one scientist in charge of planning, research and analysis, one technician in charge of daily operation of the laboratory and two or more laboratory assistants for routine media preparation, transfer of cultures and dishwashing.

2. Laboratory Facilities

Media Preparation: Standard laboratory equipment or household kitchen facilities may be used for defined, reproducible media preparation. Chemical supplies (Appendix 4), refrigerator, balances, pH meter, stir and hot plates, accurate measuring equipment, deionized or distilled water source, media dispensing equipment and autoclave or pressure cooker are the basic items needed. Protective clothing, gloves and safety devices (showers, eyewash, fire extinguishers) are also needed. Arrangement of the equipment and supplies in a logical fashion will allow for the most efficient use of time and should minimize mistakes.

Dish Washing Facilities: Cleanliness of glassware and other items is of prime importance to the health of the *in vitro* collection. A high quality dishwasher with a distilled or deionized water rinse, or very careful workers are needed. Glassware should be dedicated to tissue culture only and not mixed with other laboratory glassware.

Laboratory equipment: The major needs for laboratory equipment are for sterile laminar flow cabinets, growth room and cold room facilities. Transfer of sterile cultures can be accomplished without sterile facilities but it is much more time consuming and would probably have a higher contamination rate. Growth room facilities are critical for optimum survival and growth of cultures. Light fixtures, shelving and temperature control are the most critical elements. The use of an interior room may be helpful in controlling contamination from external sources. The use of lights with ballasts in an external location may be needed to control heat buildup in the culture room.

3. Transfer Facilities

Sterile Transfer Facilities: Several options are available for sterile transfer. The first level would be the laminar flow cabinet. If this is not available, a small room may be designated as a transfer room, fitted with a transfer box to decrease contamination and decontaminated using UV radiation, sterilizing solutions or a combination. The third option is the transfer of cultures on the laboratory bench top using very careful aseptic technique. The latter option is possible only in a room with little airflow or foot traffic.

Instruments: Long lightweight forceps, fine short forceps, scalpels with replaceable blades, alcohol or gas burners and sterile petri dishes for holding sterile tools are needed in most instances. Additional instruments will depend on the culture vessels used and the plant materials cultured.

4. Growth Room Conditions

Light Requirements: Light requirements vary among genera and range from 10 to 1000 $\mu mol \bullet s^{-1} \bullet m^{-2}$. It may be necessary to determine these levels as part of the internal research program. Either warm or cool fluorescent bulbs produce proper spectra for plant growth although there may be variation in light requirements for some genotypes.

Temperature: Temperature requirements also vary among genera and must be determined from the literature or by experimentation. Common growth room temperatures range from 22 to $28^{o}C$. Regulation of the temperature may be accomplished through ventilation systems or air conditioning units, but neither should vent directly onto the culture area. Temperatures will normally stratify from ceiling to floor and these differences may be used to accommodate disparate genera.

5. Storage Facilities

Cold Storage: Storage of cultures at lower than growth room temperatures requires reliable equipment and backup alarm systems. Whether the storage is at 15 or 0°C a malfunction can either overheat or freeze valuable cultures. A fail safe system which shuts off the unit and sounds an alarm if the temperature varies by a set amount is probably ideal. Wiring the alarm into an existing security system is highly recommended.

Warm Storage: In some cases cultures are stored at growth room temperatures. This requires the same monitoring as for the growth room to see that the temperatures remain within preferred limits.

Cryogenic Storage: Storage in liquid nitrogen requires a reliable holding tank and a ready source of liquid nitrogen. This system also requires an alarm to warn of failure of the tank or low nitrogen levels.

6. Storage and Shipment of Germplasm

Condition of Plant Materials: For any *in vitro* culture materials to be stored in cold, warm or cryogenic conditions, they must be in excellent condition and vigorously growing. Small plantlets, clumps or cultures in need of transfer or stressed for other reasons do not store well. All materials should be transferred to storage medium two weeks before actual storage to allow the plant to recover from the transfer and resume growth. Many plants benefit from a cold hardening period before cold storage or cryopreservation. To prepare for cold storage place 10 plantlets in separate cells of two plastic culture bags and heat seal, then place in normal growing conditions. Transfer to cold hardening for 1 week then to cold storage conditions of 4°C in low light or -1°C in the dark. Storage in plastic bags requires a firmer medium than that used for the normal multiplication.

Inventory: It is useful to keep a computerized inventory of *in vitro* plants. These records should include information on explant date, storage date, growth media used, storage media used, and any other pertinent information. This system may also be useful for generating labels for stored plants and producing lists for a variety of uses.

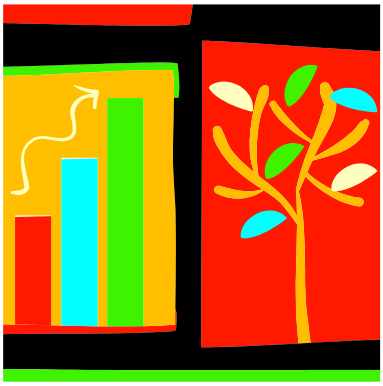
Shipping: Requested plants may be sent directly from the cold storage collection, or if cultures are nearing the usual repropagation interval, they may need to be regrown before being sent out. All plants shipped should be in good condition since they will encounter stress enroute. Do not ship if there is freezing weather at any point along the route. Prolonged heat should also be avoided.

7. Initiation, Growth and Rooting of Cultures

General Procedures, Protocols and Media: Protocols and media recipes for the genera studied follow. These protocols and media are based on standard micropropagation methods, but have been modified to suit the needs of our genera. Careful aseptic technique is required for all work of this type.

Initiation: Cuttings are collected from greenhouse or screenhouse plants if they are available, as they harbor fewer bacterial and fungal contaminants than field material. Dormant field material is usually cut, washed and forced in the greenhouse. Directly collected material is often contaminated, however old leaves may be stripped off, stems washed and new growth forced as with dormant material. Collections from mature trees may be difficult to initiate. It is often easiest to collect dormant budwood, store it for a few months, and graft it onto seedling rootstocks in the greenhouse. The new growth generated from this material will be cleaner and more juvenile than that from the parent tree. This is especially good for hazelnuts.

Standard Procedures for Each Genus: Each genus requires one and sometimes two different media types. Each genus also responds differently to surface sterilization as well as handling procedures. Such individual differences make it difficult to generalize, however standard procedures work for the majority of the members of a genus.



CONTAMINATION DETECTION FOR EXPLANTS AND
MULTIPLYING PLANTLETS AT NCGR

All explants are now initiated in an enriched liquid medium as follows:

a) Initiation/surface sterilization: explants are taken from recently formed runners or shoot tips of plants in the screenhouse collection. Shoots may also be forced from field grown branches, but should be treated for insects (Thrips, mites) with dormant oil or chemical sprays before forcing. Forced shoots should be placed in water with Floralife (florist mix) to inhibit bacterial growth. Plantlets are surface sterilized by placing them in a 10% bleach solution (bleach is 5.25% sodium hypochlorite) with 0.1 ml/l Tween 20 and shaken on a rotary shaker for 10 minutes. Explants are then removed and rinsed twice with sterile water.

b) To detect internal contamination, explants are placed in 1/2 strength liquid MS medium (salts and sucrose only) with 256 mg of peptone and 88 mg of yeast extract and a pH of 6.9. Contamination will look like cloudiness or flocculent growth in the medium. Use standard growing conditions in these steps. If no contamination shows after one week then go on to multiplication. If contamination shows, recollect new tips, sterilize and rinse as before. If a second group are all contaminated, consider antibiotic treatment (Reed et al., 1995; P. Tanprasert, MS Thesis, 1996; Tanprasert and Reed 1997). Some plants do not tolerate submersion in liquid medium so they should be only partially submerged and shaken occasionally to rinse contaminants from the surface into the medium.

c) Multiplying plants suspected of contamination have their bases streaked on petri plates of bacterial detection medium (Viss, et al., 1991. *In Vitro* Cellular and Developmental Biology - Plant 27P:42) containing sucrose 10 g/l, casein hydrolysate 8 g/l, yeast extract 4 g/l, KH_2PO_4 2 g/l, $MgSO_4 \cdot 7H_2O$ 0.15 g/l, and agar 8 g/l. The pH is adjusted to 6.9 before autoclaving. The base of each explant is streaked on the plate before planting in 16 mm tubes containing 5 ml multiplication medium. The plates are incubated for 2-10 days at 24^{o}C. Plantlets showing contamination on the plates are discarded. Some latent bacteria may not be visible for several weeks. Plates should be checked with a dissecting microscope or a hand lens to verify the presence or absence of bacteria.

TISSUE CULTURE PROCEDURES & MEDIA

1. *Corylus*:

a) Medium: NCGR-COR medium (Yu and Reed, Plant Cell Repts., 12:256-259, 1993). DKW salts (Driver, et al. 1984. HortScience 19:507-509) with 200 mg/l Sequestrene 138 Fe (Geigy) for FeEDTA, with (per liter): 30 g glucose, 5 mg (for initiation) or 1.5 mg (for multiplication) BA (N-benzyladenine), 0.01 mg IBA (indole-3-butyric acid), 6.0 g agar (Difco Labs, Detroit) and pH 5.2 before autoclaving.

-Growth conditions: 16 h photoperiod (25 $\mu mol \bullet s^{-1} \bullet m^{-2}$) at 25 ^{o}C.

b) Initiation/surface sterilization: Nodal segments from suckers and branches of greenhouse-grown grafted trees are used as explants. Cold-stored dormant branches could also be used (extra chilling is required). Explants are washed with soapy water and rinsed with tap water, then surface sterilized for 10-15 min in a 10% bleach (bleach is 5.25% sodium hypochlorite) solution with 0.1 ml/l of Tween 20 (on rotary shaker), rinsed 2-3 times in sterile deionized water for 3-5 min each. Explants are recut at the base, planted in 16 mm diameter test tubes with 5 ml of initiation medium and cultured at standard growth conditions for 3-4 weeks. Branches of field-grown trees may also be forced in the greenhouse after chilling, or grafted onto rootstocks and grown in the greenhouse for explanting later. Nodal segments work better than apicals. Most explants contain internal contaminants but initiation could be attempted in liquid medium if desired. Antibiotic treatments for contaminated plants are being studied at this time.

c) Multiplication: Shoot tips and nodal segments from established cultures are planted in Magenta GA7 boxes with 40 ml of multiplication medium and cultured at standard growth conditions and subcultured at 4 week intervals. For cold storage, 10-12 healthy plantlets will be needed.

d) Rooting: Place plantlets (2-3 cm long) on basal medium with or without 1 mg/l IBA at standard growth conditions or root ex-vitro (Yu and Reed 1995 HortScience). Be sure to loosen lids two days before starting the transplant process.

e) Additional information: To prepare for cold storage place 10 plantlets in two plastic culture bags (medium with agar 3.5 g/l, Gelrite 1.45 g/l) and heat seal, then place at standard growth conditions for one week. Transfer to cold-hardening (8 h days at 22^{o}C and 16 h nights at -1^{o}C) for one week then cold storage conditions of 4 ^{o}C in low light.

Revised 9-14-93xl

CORYLUS MEDIUM (NCGR-COR)
(Modified DKW medium HortScience 19:507-509)
In use since 1992 (Yu and Reed 1993. Plant Cell Reports 12:256-259)

Component	Conc./L	Amount Needed
NH_4NO_3	1417 mg	
$Ca(NO_3)_2*4H_2O$	1960 mg	
$CaCl_2*2H_2O$	147 mg	
Zn(NO3)2*6H2O	17 mg	
DKW P.B. Mo	10 ml	
DKW Sulfates	25 ml	
DKW VITAMINS	10 ml	
MS Thiamine	20 ml	
Sequestrene Fe138	200 mg	
Glucose	30 g	

SPS FOR STORAGE ARE 0 HORMONES

Cytokinin: Benzyladenine
(Stock sol. 10 mg/100 ml)

Initiation = 5.0 mg/l	50 ml
Multiplication = 1.5 mg/l	15 ml
Rooting = none	

Auxin : (Stock sol. 10 mg/100 ml)

Initiation = IAA = 0.01 mg/l	0.1 ml
Multiplication = IBA = 0.01 mg/l	0.1 ml
Rooting = IBA = 1 mg/l	10 ml

Bring to final volume

pH	5.2
Boxes and Tubes	
Agar	5 g
Star*packs agar	7 g

2. *Fragaria*:

a) Medium: Tissue culture plantlets of *Fragaria* are grown on a modified MS medium (see below).

-Growth room conditions are 16 h photoperiod (25 $\mu mol \bullet s^{-1} \bullet m^{-2}$) at 25°C.

b) Initiation/surface sterilization: *Fragaria* explants are taken from recently formed runners on plants in the screenhouse collection. Plantlets are surface sterilized by placing them in a 10% bleach solution (bleach is 5.25% sodium hypochlorite) with 0.1 ml/l Tween 20 and shaken on a rotary shaker for 10 minutes. Explants were then removed and rinsed twice with sterile water.

c) To detect internal contamination, explants are placed in 1/2 strength liquid MS medium with 256 mg of peptone and 88 mg of yeast extract and a pH of 6.9. Contamination will look like cloudiness or flocculent growth in the medium. Use standard growing conditions in these steps. If no contamination shows after one week then go on to multiplication. If contamination shows, recollect new tips, sterilize and rinse as before. If a second group are all contaminated, consider antibiotic treatment (Tanprasert MS Thesis, 1996).

Multiplying plants suspected of contamination are streaked on petri plates containing a bacterial detecting medium (Viss, et al., 1991. *In Vitro* 27P:42) containing sucrose 10 g/l, casein hydrolysate 8 g/l, yeast extract 4 g/l, KH_2PO_4 2 g/l, $MgSO_4 \bullet 7H_2O$ 0.15 g/l, and agar 8 g/l. The pH is adjusted to 6.9 before autoclaving. The base of each explant is streaked on the plate before planting in 16 mm tubes containing 5 ml multiplication medium. The plates are incubated for 48 hours at 24°C. Plantlets showing contamination on the plates are discarded.

d) Multiplication: Plantlets are multiplied on base medium as stated above.

e) Rooting: Healthy plants are rooted on regular base medium without hormones.

f) Additional information: Plantlets to be stored are placed on medium without hormones inside plastic tissue culture bags (medium with agar 3.5 g/l, Gelrite 1.45 g/l). After a cold hardening period, the bags are then placed in a storage room at 4°C in low light. Some plants in the screenhouse collection do not produce very many runners. A 500 PPM GA_3 spray combined with long day conditions has been helpful in inducing runnering in these plants.

FRAGARIA MEDIUM (NCGR-FRA)

Revised 12-02-91

Initiation, multiplication and rooting:

M & S Salts and Vitamins (Murashige and Skoog, 1962) with Sucrose (30 g/l)

Growth regulators (mg/l):

Multiplication and initiation:

NaH_2PO_4 (170 mg), Adenine Sulfate (80 mg), Benzyladenine (1 mg = 10 ml stock sol.) (use stock sol. 10 mg/100 ml), IAA (1 mg = 10 ml stock sol.)(Stock sol. 10 mg/100 ml), GA_3 (0.01 mg = 0.1 ml stock sol.) (Stock sol. 10 mg/100 ml)

Rooting and Storage: No growth regulators

pH 5.7

Boxes and Tubes: Agar 3 g and Gelrite 1.25 g (Or agar only 6 g/l)

Star*packs: Agar 3.5 g and Gelrite 1.45 g (Or agar only 7 g)

3. *Humulus*:

a) Medium: MS salts and vitamins with (per liter): 20 g glucose, 0.1 mg BA (N6-benzyladenine), 3.0 g agar (Difco Labs, Detroit), 1.25 g Gelrite at 5.0 pH before autoclaving. This is not an optimized medium.

-Growth room conditions are 16 h photoperiod (25 $\mu mol \bullet s^{-1} \bullet m^{-2}$) at 25^{o}C

b) Initiation/surface sterilization: Collect 7 tips (5-10 mm each) and surface sterilize in a 10% bleach (bleach is 5.25% sodium hypochlorite) solution with 0.1 ml/L of Tween 20 on rotary shaker for 10 min. Rinse twice in sterile water for 5 min each time. Plant in 5 ml enriched 1//2 strength liquid medium in 16 mm tubes. Discard contaminated cultures and transfer to solid medium.

c) Multiplication: Place plantlets in Magenta GA7 boxes with standard medium (40 ml/box) and transfer to new boxes every 3 weeks. For cold storage 12-13 healthy plantlets will be needed.

d) Rooting: Place plantlets on standard medium without hormones in standard growing conditions.

e) Additional information: Storage: Plantlets to be stored are placed on medium without hormones inside plastic tissue culture bags (medium with agar 3 g/l, Gelrite 1.5 g/l). After a cold hardening period, the bags are then placed in a storage room at 4^{o}C in low light. Ideal conditions for storage have not been determined.

-Explants grow vigorously without much contamination.
-Plants in cold storage decline at a faster rate than most other genera.
-It is important to use glucose as a carbon source as sucrose is not satisfactory.

4. *Mentha*:

a) Medium: MS salts and vitamins. Per liter: 30 g sucrose, 0.5 mg BA (N-benzyladenine), 0.1 mg IBA (Indole 3 Butyric acid), 3.0 g agar (Difco Labs, Detroit), 1.25 g Gelrite at pH 5.7 before autoclaving.

-Growth room conditions are 16 h photoperiod (25 $\mu mol \bullet s^{-1} \bullet m^{-2}$) at 25°C

b) Initiation/surface sterilization: Collect 7 tips (5-10 mm each) and surface sterilize in a 10% bleach (bleach is 5.25% sodium hypochlorite) solution with 0.1 ml/L of Tween 20 on rotary shaker for 10 min. Rinse twice in sterile water for 5 min each.

Plant tips are placed in enriched liquid 1/2 MS medium at pH 6.9 to detect contamination . It will look like cloudiness or flocculent growth in the medium. Use standard growing conditions in these steps. If no contamination shows after one week then go on to multiplication. If contamination shows, recollect new tips, sterilize and rinse as before, then place plantlets on liquid medium (pH 6.9) as before with added antibiotics (streptomycin, neomycin, or gentamicin) for 1 week (Reed et al., 1995). If clean, place plantlets in 16 mm tubes with 5 ml standard medium for one week and continue to check for contamination using Viss (523 medium) plates.

c) Multiplication: Place plantlets in Magenta GA7 boxes (40 ml/box) with standard medium and normal growth conditions and transfer to new boxes every 3 weeks. For cold storage 12-13 healthy plantlets will be needed.

d) Rooting: Place plantlets on standard medium without hormones in standard growing conditions or leave on multiplication medium for 6 weeks.

e) Additional information: Plantlets to be stored are placed on medium without hormones inside plastic tissue culture bags (medium with agar 3 g/l, Gelrite 1.5 g/l). After a cold hardening period, the bags are then placed in a storage room at 4°C in low light.

- Expect about 1/3 of the *Mentha* to be contaminated; most will be due to internal contamination. Be careful not to pass contamination.
- Most grow very vigorously. Be sure to check for contamination early because it is difficult to notice in overgrown tubes.
- streak suspected contaminated cultures on Viss (523 medium) plates before storage to determine latent contamination. (Viss, et al., 1991. *In Vitro* 27P:42) containing sucrose 10 g/l, casein hydrolysate 8 g/l, yeast extract 4 g/l, KH_2PO_4 2 g/l, $MgSO_4 \bullet 7H_2O$ 0.15 g/l, and agar 8 g/l. The pH is adjusted to 6.9 before autoclaving. The base of each explant is streaked on the plate to determine bacterial growth.

5. *Pyrus*:

a) Medium: Several variations of modified MS medium were developed for pear. These are MS medium with 1.5 X or 2 X the $CaCl_2.2H_2O$, KH_2PO_4, and $MgSO_4$ and with: a stock solution of Thiamine at 10 mg /100 ml and added to the medium at 2.5 ml/l, inositol 250 mg/l, sucrose 30 g/l, benzyladenine 10 mg/l, agar (Difco Labs, Detroit) 3.0 g/l and Gelrite 1.25 g/l. The medium is brought to a pH of 5.7 before adding the agar/Gelrite, then autoclaved.

b) Growth room conditions are 16 h photoperiod (25 $\mu mol \bullet s^{-1} \bullet m^{-2}$) at 25^{o}C.

c) Initiation/surface sterilization: Force dormant branches in the greenhouse or graft onto seedling rootstocks to obtain lower contamination rates. Collect 7 tips and/or axillary buds (2-3 cm each). Surface sterilize for 10 minutes in a 10% bleach (bleach is 5.25% sodium hypochlorite) solution with 0.1 ml/l of Tween 20 on a rotary shaker for 10 minutes. Rinse twice in sterile water for 5 minutes each time. Explants usually grow well without much contamination.

d) To detect internal contamination, explants are placed in 1/2 strength liquid MS medium with a pH of 6.9. Contamination will look like cloudiness or flocculent growth in the medium. Use standard growing conditions in these steps. If no contamination shows after one week then go on to multiplication. If contamination shows, recollect new tips, sterilize and rinse as before. If a second group is all contaminated, consider antibiotic treatment (Reed et al., In Vitro Plant 31:53-57; 1995 or M. Tanprasert MS Thesis, 1996). Some plants do not tolerate submersion in liquid medium so they should be only partially submerged and shaken occasionally to rinse contaminants from the surface into the medium.

e) Multiplication: Place plantlets in Magenta GA7 boxes with 40 ml of medium / box. Use standard medium with normal growth conditions and transfer to new medium every 3 weeks. Cutting the tops off of plantlets after they have been established in standard medium will encourage growth of new shoots and increase multiplication.

f) Rooting: Place plantlets on standard medium (without BA) with 10 μm indole-3-butyric acid (IBA) for one week then transfer to standard medium without hormones. If plantlets do not root then try standard medium with 10 μm naphthaleneacetic acid (NAA) for one week then transfer to standard medium without hormones. Many are difficult to root (Reed, HortScience 30:1292-1294, 1995)

g) Additional information: Plantlets to be stored are placed on medium without hormones inside plastic tissue culture bags (medium with agar 3 g/l, Gelrite 1.5 g/l). After a cold hardening period, the bags are then placed in a storage room at 4^{o}C in low light. Most store well at 4^{o}C or -1^{o}C.

6) *Ribes*:

a) Medium: Modified MS medium with 30% nitrogen (3 ml MS Nitrates/l) but other salts are MS levels, 50 mg/l of ascorbic acid, 20 g/l of glucose, benzyladenine (BA) 0.1 mg/l, gibberellic acid (GA_3) 0.2 mg/l, agar 3.5 g/l and Gelrite 1.45 g/l, pH 5.7 before autoclaving.

-Growth room conditions are 16 h photoperiod (25 $\mu mol \bullet s^{-1} \bullet m^{-2}$) at 25^{o}C

b) Initiation/surface sterilization: Collect 7 tips (2-3 cm each). Surface sterilize for 10 minutes in a 10% bleach (bleach is 5.25% sodium hypochlorite) solution with 0.1 ml/l of Tween 20 on rotary shaker for 10 minutes. Rinse twice in sterile water for 5 minutes each time. To detect internal contamination, explants are placed in 1/2 strength liquid MS medium with 256 mg of peptone and 88 mg of yeast extract and a pH of 6.9. Contamination will look like cloudiness or flocculent growth in the medium. Use standard growing conditions in these steps. If no contamination shows after one week then go on to multiplication. If contamination shows, recollect new tips, sterilize and rinse as before. If a second group are all contaminated, consider antibiotic treatment (Reed et al., 1995 In Vitro Plant 31:53-57 or M. Tanprasert MS Thesis, 1996). Some plants do not tolerate submersion in liquid medium so they should be only partially submerged and shaken occasionally to rinse contaminants from the surface into the medium.

c) Multiplication: Place plantlets in Magenta GA7 boxes with 40 ml of medium / box. Use standard medium, normal growth conditions and transfer to new boxes every 3 weeks.

d) Rooting: Place plantlets on standard medium without hormones in standard growing conditions. Some are difficult to root.

e) Additional information: Plantlets to be stored are placed on medium without hormones inside plastic tissue culture bags (medium with agar 3.5 g/l, Gelrite 1.75 g/l). After a cold hardening period, the bags are then placed in a storage room at 4^{o}C in low light or -1^{o}C in the dark.

-Explants grow well without much contamination. Ribes seem to exude large amounts of phenolic compounds.
-Many do not elongate and this makes them less likely to survive in cold storage.
-Pass plantlets at three-week intervals or they will decline quickly.

7. *Rubus*:

a) Media: At least two formulations exist for the *in vitro* culture of *Rubus* accessions at NCGR. Most grow well on blackberry medium; however some grow better on Anderson's (Reed, 1990).

Blackberry medium: MS salts and vitamins, with benzyladenine 1 mg/l, indole-butyric acid 0.1 mg/l and GA3 0.1 mg/l (Reed, B.M., Fruit Var. J. 44(3):141-148. l990). For rooting remove BA and increase IBA to 1 or 2 mg/l. pH is 5.7 and agar 3.5 g and Gelrite 1.45 g/l.

Anderson's Raspberry medium (Anderson, l980, Acta Hort. 112:13-20): This medium contains only 1/4 as much nitrates as MS, varying levels of the other salts and includes adenine sulfate at 80 mg/l (see medium sheet).

-Growth room conditions are 16 h photoperiod (25 $\mu mol \bullet s^{-1} \bullet m^{-2}$) at 25^{o}C

b) Initiation/surface sterilization: Explants (3 cm shoots from pot-grown screenhouse plants) are stripped of all but newly forming leaves at the tips and disinfected by immersion for 10 minutes in 10% household bleach (bleach is 5.25% sodium hypochlorite) with 0.1 ml/l Tween 20 on a rotary shaker, followed by two rinses in sterile water. Shoots are trimmed to 1 cm nodal sections and placed in 16 mm tubes with 5 ml medium. To detect internal contamination, explants are placed in 1/2 strength liquid MS medium with 256 mg of peptone and 88 mg of yeast extract and a pH of 6.9 Contamination will look like cloudiness or flocculent growth in the medium. Use standard growing conditions in these steps. If no contamination shows after one week then go on to multiplication. If contamination shows, recollect new tips, sterilize and rinse as before. If a second group is all contaminated, consider antibiotic treatment (Reed et al., 1995 In Vitro Plant 31:53-57 or M. Tanprasert MS Thesis, 1996). Some plants do not tolerate submersion in liquid medium so they should be only partially submerged and shaken occasionally to rinse contaminants from the surface into the medium.

c) Multiplication: Plantlets from the tubes are divided and transferred to fresh multiplication medium in Magenta boxes (40 ml/box) and placed under the same growth conditions.

d) Rooting: Use medium with 1 or 2 mg/l IBA or with 10 μM IBA for one week then to no hormone medium for 3 weeks.

e) Additional information: Plantlets to be stored are placed on medium without hormones inside plastic tissue culture bags (medium with agar 3.5 g/l, Gelrite 1.75 g/l). After a cold hardening period, the bags are then placed in a storage room at 4^{o}C with 8 hr days (10 $\mu mol \bullet s^{-1} \bullet m^{-2}$).

-Start all explants on Blackberry medium. Those which do not multiply but are healthy should have the BA increased to 2 mg/L. Those which do not appear to be healthy and also fail to multiply may do better on Anderson's medium.

Revised 12-15-89

ANDERSON'S RASPBERRY MEDIUM

(Anderson 1980: Acta Hort. 112:13-20)

MULTIPLICATION

Component	Conc./L	Amount Needed
M & S Nitrates	2.5 ml	
M & S Sulfates	10 ml	
ANDERSON'S Halides	10 ml	
ANDERSON'S B,Mo	10 ml	
M & S Iron	20 ml	
M & S Vitamins	10 ml	
NaH_2PO_4	380 mg	
Sucrose	30 g	
Starpacks are 0 Hormone		
Adenine Sulfate	80 mg	
Benzyladenine 1 mg/l (Stock sol. 10 mg/100 ml)	10 ml	
IBA 0.1 mg/l (Stock sol. 10 mg/100 ml)	1 ml	
GA_3 0.1 mg/l (Stock sol. 10 mg/100 ml)	1 ml	

Bring to final volume

pH 5.7

Boxes and Tubes (Or agar only 6g)

Agar	3.0 g
and	
Gelrite	1.25 g

Star*packs (Or agar only 7g)

Agar	3.0 g
and	
Gelrite	1.5 g

8. *Vaccinium*:

a) Medium: Woody Plant Medium (McCown and Lloyd, Comb. Proc. Int. Pl. Prop. Soc. 30:421-427). Zeatin is used for initiation and 2-isopentyl-adenine (2iP) for multiplication (see below). (See media sheet for details)

b) Initiation: Collect tips 2-3 cm long from shoots of healthy plants during the growing season from March to September. Remove all leaves, wash in running tap water for 5 minutes and decontaminate by immersion for 10 min in 10% bleach (bleach is 5.25% sodium hypochlorite) with Tween 20 added at 0.1 ml/L on a rotary shaker. Rinse twice in sterile water, trim to 0.5 to 1.5 cm.

c) To detect internal contamination, explants are placed in 1/2 strength liquid MS medium with 256 mg of peptone and 88 mg of yeast extract and a pH of 6.9. Contamination will look like cloudiness or flocculent growth in the medium. Use standard growing conditions in these steps. If no contamination shows after one week then go on to multiplication. If contamination shows, recollect new tips, sterilize and rinse as before. If a second group is all contaminated, consider antibiotic treatment (Reed et al., 1995 In Vitro Plant 31:53-57 or M. Tanprasert MS Thesis, 1996). Some plants do not tolerate submersion in liquid medium so they should be only partially submerged and shaken occasionally to rinse contaminants from the surface into the medium.

d) Plant clean explants in 16 mm tubes containing 5 ml initiation medium (WPM with 4 mg/l zeatin). Growth conditions: 12 h days, 25^{o}C, and subdued light (3 $\mu M \bullet s^{-1} \bullet m^{-2}$); nights at 22°C.

e) Multiplication: Four weeks after initiation, tips are trimmed to expose fresh basal surfaces and transferred to multiplication medium (WPM with 5 mg/l 2iP). The usual growth period between transfers is four weeks.

f) Rooting: Transfer well-established plantlets to boxes containing WPM without hormones. Many are directly rooted in the greenhouse mist bed.

g) Additional information: Plantlets to be stored are placed on medium without hormones inside plastic tissue culture bags (medium with agar 7 g/l). After a cold hardening period, the bags are then placed in a storage room at 4^{o}C in low light.

The *Vaccinium* collection includes a number of crop types, among them cranberry, highbush, lowbush encourages and rabbiteye blueberries. So far, no single medium has been devised which growth uniformly in all types or even in all accessions of one type.

Besides the variability among plant types, there is reason to believe that the vigor and general health of the parent plant especially influence the success of the *in vitro* cultivation of *Vaccinium*.

Vaccinium plantlets do not grow well with Gelrite substituted for part of the agar.

VACCINIUM MEDIUM
McCown & Lloyd WOODY PLANT MEDIUM (WPM)
(Comb. Proc. Intern. Plant Prop Soc. 30:421-427)

Revised 3-11-97cp

Component	Conc./L	Amount Needed
NH_4NO_3	400 mg	
$Ca(NO_3)_2*4H_2O$	556 mg	
K_2SO_4	990 mg	
$CaCl_2*2H_2O$	96 mg	
M & S Sulfates	10 ml	
M & S P,B,Mo	10 ml	
M & S Iron	10 ml	
Thiamine (Stock 10 mg/100 ml)	6 ml	
M & S Vitamins	10 ml	
Sucrose	30 g	

Starpacks are 0 Hormone

Zeatin	
Initiation = 4 mg/L	40 ml
Multiplication = 2 mg/L	20 ml
Stocks in freezer	
OR	
2-Isopentyladenine 2iP	
5 mg/l	50 ml
(Stock sol. 10 mg/100 ml)	

Bring to final volume

pH	5.2
Boxes and Tubes	
Agar	6 g
Star*packs	
Agar	7 g

BACTERIOLOGY

Contamination: Cultures should be checked at 2 to 4 day intervals and contaminated cultures removed and autoclaved. In the case of rare materials, a second surface sterilization may be attempted to save a culture, or antibiotic treatments may be used. Care should be taken to avoid spreading any contaminants to clean cultures. Transfer tools should be sterilized carefully and alcohol dips changed regularly. The use of 70% ethanol for flaming will decrease the probability of contaminant spread. The use of a 70% dip followed by a 95% dip for flaming may also be used. Instruments should be autoclaved daily and spares should be available for replacement at midday. If bacterial contaminants are common it is recommended to use Gelrite rather than agar to solidify the initiation medium. The clarity of the medium will allow easier detection of contaminants. Specific tests and media recipes follow.

Bacteriology

A. Isolation of bacteria from cultures or Viss plates.
   1. Media choices include
      a. liquid Mentha multiplication medium at pH 6.5-6.9.
      b. the same as a. but at 1/2 concentration to minimize precipitation
      c. 523 (Viss) agar plates
      d. Nutrient broth or Tryptone, glucose, yeast (TGY) medium
   See "Recipes and Formulations for Bacteriology" in this section for specifics.

B. Isolation procedures. Untrimmed stems of suspect contaminated explants may be touched on or stabbed into the surface of a Viss plate. The plate is then incubated at 25 C for several days and examined for evidence of bacterial growth. Usually growth appears at 2-3 days but incubation may be continued for as long as 7 to 10 days. Or, an explant may be immersed in liquid medium and incubated under the same conditions. The development of uniform turbidity or flocs in the clear medium is taken as evidence of bacterial growth. In other situations, from contaminated cultures in Magenta boxes or Starpaks, material may be picked up by using a sterile cotton swab or applicator stick and transferred to a nutrient broth tube and incubated at 25 C until the broth becomes cloudy with growth.

C. Obtaining pure cultures of bacteria. (Chapter 1.4, Isolation of Bacteria, from Methods in Phytobacteriology, ed. Klements, 1990, for more details)

1. Useful media

a. Nutrient agar

b. 523 (Viss) medium or nutrient agar at pH 6.5-6.9, solidified with 1.2% agar or equivalent amount of Gelrite, prepared as poured plates.

c. Other agar media such as King's B, MacConkey's, TGY.

See "Recipes and formulations for Bacteriology" for specifics.

Material from an isolation medium is streaked onto one or more of the above solid media, using a bacteriological loop and standard streaking procedure. Plates are incubated, inverted, at ambient temperature for three or more days and observed to see if only one or more than one colony type is present. Colony morphology, including shape, size, contour, elevation, pigment and consistency is recorded. If well isolated, individual colonies may be picked using a sterilized bacteriological needle and transferred to an appropriate media for further study. A Gram stain is made to determine cell morphology and arrangement. If purity of a culture is uncertain, individual colonies are selected and restreaked on fresh plates until subsequent growth appears uniform.

D. Characterization of bacteria.

1. Colony morphology. Bacterial colonies are described by size, shape, contour, color, elevation, consistency. Descriptive terms include words such as mucoid, dry, umbonate, convex, wrinkled, shiny, round, entire, butyrous and others. A good textbook will define and illustrate these terms; see p. 53 in Klements, 1990.

2. Cell morphology. Bacteria occur as rods and cocci, singly, in pairs, chains and clusters, in chains, or side by side in palisade formation. They are best seen stained and under the oil immersion lens (x100) of a compound microscope. The single most useful bacteriological staining procedure is the Gram stain which uses crystal violet and safranin and results in the differentiation of bacteria into Gram-positive (purple-black) and Gram-negative (red) categories. The categories reflect major differences in the cell wall composition of the two types of organisms and assist materially in their identification and prediction of their responses to antibiotics. Another stain of interest is Leifson's flagella stain, although the availability of the electron microscope and negative staining with heavy metal salts such as sodium phosphotungstate has diminished its popularity. (See Recipes and Formulations for Bacteriology for specifics).

3. Physiological attributes. Bacteria exist as aerobes, anaerobes, and intermediate or facultative organisms. Observation of their growth in broth culture gives good clues to their oxygen requirements; strict aerobes form filmy growth at the surface; facultative organisms tend to cloud the liquid throughout; anaerobes may not grow at all if special conditions are lacking to exclude oxygen, or may grow at the very bottom of tubes containing semi-solid medium. Another factor influencing bacterial growth is pH. Most grow well between pH 6 and pH 8. Whether an organism can grow at pH 4.4 or below provides an added characteristic to aid in its identification. Bacteria

are grouped as psychrophiles, mesophiles, and thermophiles according to their abilities to grow within certain temperature ranges. Most of the microorganisms encountered by plant workers will be mesophiles, growing well between about 10 and 30 C. Some xanthomonads will not grow below 5 C. Other organisms, including *E. coli* and a number of other human-associated bacteria can grow up to 41 C or 43 C. Incubators set up to evaluate maximum and minimum temperature limits for bacteria should have sensitive and precise control mechanisms.

4. Useful diagnostic tests for characterizing bacteria. We have tried the following tests for characterization. The most useful ones are starred and directions for preparation and procedures are given in "Recipes and Formulations for Bacteriology"

a. 3% KOH test
b. oxidase test *
c. oxidation/fermentation test *
d. starch hydrolysis test *
e. motility test *
f. pectolytic enzyme test
g. gelatinase test
h. temperature requirements *
i. pH minimum
j. urease test *
k. arginine hydrolase
l. phosphatase test
m. fluorescence *

GRAM STAINS (REAGENTS AND TECHNIQUE)

1. CRYSTAL VIOLET
   A. Dissolve 2 g. in 20 ml 95% EtOHd
   B. Dissolve 0.8 g. ammonium oxalate in 80 ml of dist.H_2O
      - Mix A & B = STABLE WORKING SOLUTION
      - Stain dry, heat-fixed smears for 1 min.
      - Wash in stream of ultra H_2O from wash bottle

2. LUGOL'S IODINE
-Iodine crystals 1 g
and
- Potassium iodide 2 g Dissolved in 300 ml water
Flood and leave on smears 1 min. Wash and drain slides

3. Decolorize in stream of 95% EtOH for about 10 seconds
(color stops running). Wash and drain

4. SAFRANIN "O"
   - Stock = 2.5% solution in EtOH
   - Working solution = 10 ml stock solution
     + 90 ml distilled water
   - Stain 30 seconds- Wash and air dry.

RECIPES AND FORMULATIONS FOR BACTERIOLOGY

NUTRIENT AGAR: Put the required amount of water into a beaker and add

- Nutrient Broth Powder	8 g/l
- Glucose	10 g/l
- Yeast extract	1 g/l

Adjust pH to 6.9 and transfer to an Erlenmeyer flask before adding the agar. The Erlenmeyer should be able to hold twice the volume to avoid overflow when the autoclave pressure releases.

- Add agar	12 g/l

Bring to a boil, then autoclave for 20 min. Cool the flask contents to 45-50 C in a waterbath before pouring plates to avoid lots of condensation inside the lids.

NUTRIENT BROTH: Put the required amount of water into a beaker and add

- Nutrient Broth Powder	8 g/l
- Glucose	10 g/l
- Yeast extract	1 g/l

Adjust pH to 6.9

Dispense the broth to tubes or other suitable containers. Cap and autoclave for 20 min. under standard autoclave conditions.

LIQUID MS MULTIPLICATION MEDIUM Use the basic formulation for MS medium at 1/2 strength and increase the pH to 6.5 - 6.9 to be more suitable for bacterial growth.

523 (VISS) MEDIUM: To the appropriate amount of deionized water in a beaker add

-Sucrose	10 g/l
-Casein hydrolysate	8 g/l
-Yeast extract	4 g/l
-KH_2PO_4	2 g/l
-$MgSO_4$ $7H_2O$	0.15 g/l

Adjust the pH to 6.9. Transfer to Erlenmeyer flask and add Gelrite 6 g/l

Bring the mixture to a boil and autoclave. Cool to 45-50 C in waterbath and pour into sterile petri dishes. This level of gelling agent gives a soft medium suitable for inoculating with soft plant stems. For streaking plates with bacteria the amount of Gelrite can be increased.

KING'S B MEDIUM FOR DETECTION OF FLUORESCENCE BY PSEUDOMONAS SPECIES
To the required amount of distilled, deionized water add

-Proteose peptone 3 (Difco)	20 g/l
--$KH_2PO_4 \cdot 3H_2O$	2.5 g/l
-$MgSO_4$ $7H_2O$	6.0 g/l
-Glycerol	15 ml/l

Add agar 15 g/l

Boil and autoclave. Cool to 45°C and pour plates. Both *Pseudomonas fluorescens* and *Ps. syringae* produce fluorescent pigments on this medium. A darklight (366 nm) is used to examine colonies growing on this medium.

MOTILITY AGAR: To the required amount of water in a beaker, add

- Peptone 10 g/l
- NaCl 5 g/l

Adjust pH to 6.9, add gelrite 1.7 g/l or agar 5 g/l

Bring to boiling to dissolve gelling agent and dispense into 16 x 100 mm tubes at 5.0 ml per tube. Cap and autoclave for 20 min. under standard conditions.
Note: Gelrite gives a clearer medium and results are easier to read.

GELATINASE: To the required amount of water, add

- Beef extract	3 g/l
- Peptone	5 g/l
Adjust pH to 6.8-6.9	
- add Gelatin	60 g/l

Heat to dissolve the gelatin and dispense the medium in 16 x 100 mm tubes. Autoclave same as for motility medium.

O/F TEST: To the required amount of water, add

- Peptone	2	g/l
- NaCl	5	g/l
- KH_2PO_4	0.3	g/l
- 1% aqueous solution of Bromthymol Blue	3	ml/l
Adjust pH to 7.1, add agar	3	g/l

Heat to boiling. Dispense into tubes at 4.5 ml/tube and autoclave.
While medium is still about 50 C aseptically add 10% glucose solution (filter sterilized); 0.5 ml is added to each tube. Inoculate 2 tubes for each sample. To one of the tubes add a layer of sterile mineral oil one to two cm deep to provide conditions for fermentation.
Note: Sometimes other sugars are used instead of glucose.
If only the tube without oil shows yellowing at the surface, this indicates aerobic oxidative use of the sugar. If yellowing occurs throughout, even in the tube with the oil layer, this indicates a facultative organism. Some bacteria may not give any reaction. Others may cause an alkaline reaction and the medium will become blue. O/F results are recorded as +/-, +/+, -/+, -/-, or alk/-..

STARCH HYDROLYSIS: To the required amount of water add

- Nutrient Broth powder	8	g/l
- Glucose	10	g/l
- Soluble starch	2	g/l
- Yeast extract	1	g/l
Adjust pH to 6.9 and transfer to Erlenmeyer flask		
Add agar	12	g/l

Autoclave and cool to 45-50 C. Pour 20-25 ml into each 15 x 100 cm petri plate.

Plates may be inoculated by sectors or halves, using a single streak of inoculum from a young broth culture to inoculate one sector. After 3-5 days, flood the plate with a 1:2 dilution of Gram's iodine solution. Unused starch gives a dark blue reaction; a clear zone under and around the bacterial growth indicates hydrolysis of the starch.

POTASSIUM HYDROXIDE (KOH) SOLUBILITY:
- 3% KOH
This test is said to corroborate the results of the Gram reaction. When a loopful of bacterial growth is stirred into a drop of 3% KOH on a slide, Gram-negative organisms tend to produce a stringy slime that can be lifted up threadlike from the slide.

OXIDASE REACTION
- 1% aqueous of Tetramethyl-p-phenylenediamine dihydrochloride

Note: This solution should be made fresh on the day it is used and protected from light.

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CRYOPRESERVATION PROTOCOLS

Standard Protocols for Cryopreservation:

Slow freezing: Plants are cold acclimatized for at least one week, meristems removed and placed on medium with 5% DMSO for 48 hr. Meristems are cryoprotected with PGD added gradually on ice over 30 min and chilled for 30 additional min in the refrigerator. Cooling is at 0.1, 0.3,0.5, or 0.8 degrees/min to 40 C followed by a plunge in liquid nitrogen (LN). Rewarming is 1 min in 45C water then 2 min in 25 C water. Meristems are rinsed with MS medium, then pipetted onto sterile dry filter paper to remove excess cryoprotectant and plated in 24 well plates on standard medium for recovery. (See Reed 1990 HortScience). Recovery is seen in 2-3 weeks.

Vitrification: Plants are cold acclimatized for at least one week, meristems removed and placed on medium with 5% DMSO for 48 hr. Meristems are added to 2 drops of MS medium in the cryovial and PVS2 (plant vitrification solution #2: Yamada, 1991) added for 20 min. Vials are capped before the 20 min. are up and at 20 min. are plunged into LN. Rewarming is 1 min in 45C water then 2 min in 25 C water. Meristems are rinsed 2X with MS medium containing 1.2 M sucrose and plated in 24 well plates on standard medium for recovery. Options include a 2 hr. presoak in 2% BSA (bovine serum albumen) prior to PVS2 exposure. This is especially useful for *Ribes* meristems. Recovery is usually seen within 1 week.

Encapsulation-dehydration: Plants are cold acclimatized for at least one week, meristems removed and placed on plates of basal medium until enough are collected for encapsulation. Meristems are encapsulated in alginate beads and dehydrated in sucrose solutions for 18-20 hr followed by drying in the laminar flow hood for 3-4 hr. Moisture content of the beads should reach 20%. Dried beads are placed in cryovials and plunged into LN. Vials are warmed at room temperature and beads are plated on recovery medium. Beads may be soaked for 5 min in MS medium to rehydrate them before plating on 24 cell plates. This allows the plant to break out of the bead more easily. Recovery is usually seen within 1 week.

Protocols for pollen storage are available from the literature.
Dormant buds of cold acclimated plants may also be cryopreserved.

SLOW COOLING
(Reed, 1990)

In vitro plantlets are grown for one week under cold hardening conditions (22°C 8 h days and -1°C 16 h nights) prior to cryopreservation. Meristems (< 1 mm) taken from these plants are grown for two days on a firmer medium with 5% DMSO added before the freezing protocol begins.

Materials to be prepared in advance:

a) small petri dishes with filter paper strips for draining meristems (sterile).
b) small petri dishes (2 per sterile package) for cryoprotectant and sterile medium.
c) sterile liquid medium without hormones (basic MS medium).
d) frozen blue ice.
e) tube container frozen in a block of ice.
f) petri dishes of pretreatment medium (base medium, no hormones, with 5% DMSO and storage levels of agar/gelrite).
g) cell wells with recovery medium.
h) plants cold hardened for 1 week in the incubator.

Dissect meristems 0.8 to 1.0 mm in size and place on pretreatment medium for two days.

1. Just prior to freezing run, mix cryoprotectant solution: 100 ml total
WEAR GLOVES FOR THIS PROCEDURE AND WASH THEM BEFORE TOUCHING OTHER MATERIAL OR TELEPHONE ETC.

a) in a 100 ml graduated cylinder add 40 ml of liquid MS medium (0 Hormones) and small stir bar.
b) add 9.75 ml DMSO.
c) add 10 g glucose (dextrose) stir well.
d) slowly add 10 g polyethylene glycol 10,000 (PGD) while stirring.
e) let stir until dissolved.

2. Filter-sterilize cryoprotectant through filter flask and place in FREEZER until ready for use (at least 30 minutes but not much longer or it will freeze). Wash out the cylinder and stir bar immediately yourself, do not leave it for others. WEAR GLOVES.

3. While the cryoprotectant is cooling you have 30 min to do steps 4 and 5.

4. Label cryovials and place in FROZEN tube container in hood. Unscrew lids but leave them on top of vials. Add 5 drops of liquid medium using a sterile pipette. To keep the pipette sterile, place sterile pipette tip first in a sterile petri dish with the lid covering much of the pipette.

5. Add pretreated meristems to the vials, 5 per vial. Check to be sure they are in the proper vials.

6. Over a 30 min period add the chilled cryoprotectant drop by drop to the meristems. Start with one drop every 2 minutes for 6 minutes then add 2-4 drops every 2 minutes. The vial should be filled to the very top when the 30 minutes are up. WEAR GLOVES
7. Place the tray with the meristems in the REFRIGERATOR for 30 minutes.

8. Start freezer by turning on switch at back (upper left). Set up pen for chart paper and turn on power to chart recorder but not chart drive.

9. Touch [program], touch [1][2][3] [enter] on panel at left to reach the program. Then enter the program number (listed on the top of the freezing chamber). This should bring up the correct program (i.e. 4.1, 5.1 etc.)

10. Remove the tray from the refrigerator, pipette out medium down to the 1 ml mark on the tubes. Close lids, do not over-tighten.

11. Place vials to be frozen (BUT NOT THE CONTROLS) in the freezing unit. Cool chamber to the temperature of the vials by touching the [Cool Plus] button when the chamber temperature (lines down or up) reaches the sample temperature (about 4^{o}C) start programmed freeze by touching Run. Return vial tray to the REFRIGERATOR for the controls to stay cold. Rinse controls and place them on cell wells after the exotherm but before the freezing run ends.

12. When the freezer reaches -9^{o}C after about 25 minutes it will seed the samples by dropping the chamber temperature to -50^{o}C. At this point there should be an exotherm (the sample temperature line should go up by 10-20^{o}C). If this does not occur open the door and shake the sample tray, close the door and see if an exotherm occurs, if not repeat until it does. The sample temperature should not go below -15 at the lowest.

13. Don't forget the controls. Follow directions 6,7,8 and 9 below at the time of the exotherm.

At the end of the programmed freeze:

1. Quickly transfer the vials to a thermos of liquid nitrogen. Cap but do not screw on lid. Let set for 1 h or until ready to thaw. The minimum is about 10 min; the maximum is indefinite as long as there is nitrogen in the thermos. I use 1 h as a standard time.

2. To thaw, fill a 1 liter plastic beaker with hot water (45^{o}C) and one with cold tap water.

3. With long forceps quickly remove 5 vials from the nitrogen and add them to the hot water. Stir with the thermometer for exactly 1 minute then quickly scoop them up with your hand and put into the cold water. Stir.

4. Repeat until all vials are thawed. Vials can be removed from the cold water when they have thawed completely.

5. Place vials in the styrofoam container after shaking off excess water.

6. Place vials in the hood, loosen caps. WEAR GLOVES FOR THE REST OF THIS PROCEDURE.

7. Drain off cryoprotectant down to .25 ml (where the tube tapers) and fill with liquid medium (room temperature).

8. Let vials set for 5 minutes then drain off medium with a pipette, pick up meristems with pipette and place on a small filter paper filled petri dishes. Pick up meristems with a metal pick (be careful not to crush them) and place on growth medium in cell wells.

9. Label cell wells and seal with parafilm. Place on growth room shelf.

10. With gloves on remove filter papers from small petris and discard. Rinse all small petris well and place in rack for washing. Wipe down hood.

11. Check for regrowth weekly for 6 weeks and record callus and plantlet growth.

Basic program for the freezer: Our freezer has been set up with 4 basic programs to fit 4 freezing rates:

Step 1: (ie Program 5.1) initial cooling of sample
Cooling Rate: 0.1, 0.3, 0.5, 0.8 °C/min [enter]
Final Temperature: [sample] -9°C (freezing point of cryoprotectant PGD) [cool plus][enter]

Step 5.2 initiating the exotherm in the cryoprotectant to avoid supercooling
Cooling Rate: 99.9°C/min [enter]
Final Temperature: -50°C [chamber] [cool plus] [enter]

Step 5.3 warming the chamber so the sample doesn't cool too quickly
Heating Rate: 20°C [enter]
Final Temperature: -15°C [chamber] [enter]

Step 5.4 resume cooling the sample
Cooling Rate: 0.1, 0.3, 0.5 °C/min [enter]
Final Temperature: [sample] -40°C [cool plus][enter]

Step 5.5 end

[end] [enter]

VITRIFICATION TECHNIQUE
(Modified from Yamada, et al., 1991)

Prepare in advance:

1. Cold hardened plants for meristems.
2. Petri dishes of pretreatment medium.
3. Liquid medium with 0.4 M sucrose at pH 5.8.
4. Sterile containers for cryoprotectant and liquid media.
5. Liquid medium with 1.2 M sucrose at pH 5.8.
6. Sterile filter paper strips for draining meristems.
7. Recovery medium.
8. Tube holder frozen in a block of ice (or something similar).

Pretreatment Medium: 1.2 M Sorbitol in agar medium or 5% DMSO and increased agar for two days in cold hardening incubator.

Dissect meristems

Cryoprotectant (PVS2)	Glycerol 30%	14.3 ml
	Ethylene Glycol 15%	7.4 ml
	DMSO 15%	7.4 ml

up to 50 ml with liquid medium with 0.4 M sucrose (136.92 g/l) at pH 5.8

Filter sterilize cryoprotectant

Freezing procedure:

1. Fill small dewar with liquid nitrogen.

2. Place 20 meristems in a cryotube with 1 ml PVS2 on ice. START TIMER

3. Hold at 0 C for a total of 20, 25 or 30 minutes from initial contact with PVS2.

4. Submerge in LN2, one vial at a time using forceps. Hold under the surface for 15 seconds before releasing.

Thawing procedure:

1. Transfer from LN2 to 45 C water for 1 min. Then to 25 C water for 1-2 min.

2. Immediately add 1.2 M sucrose liquid medium to the top of the tube.

3. Immediately drain and replace with liquid medium 2 times.

4. Drain onto filter paper and transfer to recovery medium.

ALGINATE BEAD-DEHYDRATION METHOD
(Dereuddre, et al., 1990)

Prepare in advance:

1. Cold hardened plants for meristems.
2. Agar plates for holding meristems temporarily.
3. Liquid MS with 0.75 M sucrose for pretreatment (75 ml in 125 ml flasks).
4. Liquid MS without calcium and with 3% alginate and 0.75 M sucrose (in a flask). This is very difficult to dissolve so heat medium and add alginate slowly, boil to dissolve.
5. Liquid MS with 100 mM calcium chloride for forming beads (in a flask).
6. Sterile 250 ml beakers for forming beads.
7. Sterile pipettes for forming beads.
8. Sterile petri dishes for holding beads during dehydration.

You will need a separate flask of 0.75 M sucrose, 250 ml beaker of calcium chloride medium, 100 ml beaker of alginate solution and at least one sterile petri dish and pipette per treatment (or genotype).

This is a two day procedure once the cold hardening is accomplished.

1. Dissect meristems onto regular plates until enough are collected.
2. Suspend in alginate solution in a small sterile beaker or petri dish.
3. Mix meristems with alginate solution. Using a sterile pipette, drop meristems into a 250 ml beaker of liquid MS with 100 mM calcium chloride medium to make beads.
4. Leave the beads in the solution for at least 20 minutes to firm up.
5. Place beads in 0.75 M sucrose in 125 ml flasks on a shaker for 18 hours (In at 3 PM, out at 9 AM). Drain on sterile filter paper
6. Place beads in sterile petri dishes and dry in the air flow for 3 or 4 hours. They should not touch each other or they will not dry properly. There should be no extra moisture in the dish (absorb with sterile filter paper if necessary).
7. Place beads in cryovials and submerge in liquid nitrogen one at a time, hold under the surface for 30 seconds then release.
8. Hold in LN for 1 hour or more as time permits.
9. Thaw at in 40 ˚C water for 1 min and 25 ˚C water for 2 min, then soak for 5 minutes in liquid MS medium to rehydrate, place on medium to grow (meristem side down).
10. If necessary, remove plantlets from beads after 2 weeks of growth.

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