CIMMYT

International Maize and
Wheat Improvement Center

LABORATORY PROTOCOLS

CIMMYT Applied Genetic Engineering Laboratory

CIMMYT, Applied Biotechnology Center, 1999

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Transformation of wheat and maize

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These laboratory protocols are part of a UNDP-sponsored project to develop insect resistant tropical maize germplasm through the use of genetic engineering.

CIMMYT (www.cimmyt.mx or www.cimmyt.cgiar.org) is an internationally funded, nonprofit scientific research and training organization. Headquartered in Mexico, the Center works with agricultural research institutions worldwide to improve the productivity, profitability, and sustainability of maize and wheat systems for poor farmers in developing countries. It is one of 16 similar centers supported by the Consultative Group on International Agricultural Research (CGIAR). The CGIAR comprises over 55 partner countries, international and regional organizations, and private foundations. It is co-sponsored by the Food and Agriculture Organization (FAO) of the United Nations, the International Bank for Reconstruction and Development (World Bank), the United Nations Development Programme (UNDP), and the United Nations Environment Programme (UNEP). Financial support for CIMMYT's research agenda also comes from many other sources, including foundations, development banks, and public and private agencies.

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Contents

Summary

This manual provides the theoretical and technical basis required for the successful establishment of maize and wheat somatic cell tissue cultures that are suitable for *in vitro* genetic manipulation through the use of biolistic-based and *Agrobacterium*-mediated gene transfer systems for maize and wheat. Achieving effective transfer, integration, and expression of transgenes using appropriate gene constructs promotes understanding of the process of gene transfer and the nature of transgenic plants.

In addition, the manual introduces readers to the basics of plant genome analysis and its application to the analysis of both maize and wheat transgenic plants. Also covered in this manual are the isolation, digestion, electrophoresis, and transfer of DNA to blots; the non-radioactive hybridization and detection technology in use at CIMMYT, and the application of polymerase chain reaction (PCR) technology to the analysis of transgenic plants. To complete the coverage of the topic, information on phenotypic screening of transgenic plants in the biosafety greenhouses is also presented.

Credits

The chapters on tissue culture and transformation for maize and wheat were prepared by Natasha Bohorva. Sarah Fennell produced the sections on molecular analysis for maize and wheat, based on information from the manual *Molecular Marker Applications to Plant Breeding* (CIMMYT 1995). Alessandro Pellegrineschi prepared the section on transformation vectors, and Scott McLean prepared the section on greenhouse protocols and plant analysis.

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INTRODUCTION

Biotechnology is "any technique that uses living organisms, or substances from those organisms, to make or modify a product, to improve plants or animals, or to develop microorganisms for specific issues." Prominent among the "new" biotechnologies is genetic engineering, which gives us the ability to insert and express a specific gene or genes, from a related or completely unrelated species, into the genome of a target organism. For the first time in the history of plant breeding, we are not limited to the pool of genes available within a particular crop species or those of a closely related species.

Genetic engineering is built on an array of processes and techniques. Indeed, without the huge advances in tissue and cell culture, for example, genetic transformation would not be possible. In similar fashion, it is hoped that genetic engineering will also build on the success of conventional plant breeding and thus help overcome some of the problems that have proven intransigent to prevailing methods. The aim is to produce products that can be effectively incorporated into breeding programs, thus providing elite germplasm with an extended array of desirable characteristics.

As with most new technologies, genetic engineering's great potential is accompanied by new considerations. Because the technology has the potential to introduce genes from nearly any organism into new genetic and physical environments, extensive biosafety and regulatory mechanisms must be in place to ensure the safe testing and possible later deployment of such products into farmers' fields. Furthermore, the extension of patent and patent-like protection to an ever-broader range of products, the oft referred to issue of intellectual property rights (IPR), is a significant component of transgenic germplasm development. These issues will have to be tackled by international research centers and national programs if the benefits of transgenic maize and wheat are to reach developing-country farmers.

In the words of Ismail Serageldin, Head of the Consultative Group on International Agricultural Research and Vice President of the World Bank, "Biotechnology will be a crucial part of expanding agricultural productivity in the 21st century. . . . (and). . ."if safely deployed, could be a tremendous help in meeting the challenge of feeding an additional three billion people, 95 percent of them in poor developing countries, on the same amount of land and water currently available."

PLANT TISSUE CULTURE

LABORATORY PROTOCOLS FOR MAIZE AND WHEAT

CIMMYT Applied Genetic Engineering Laboratory

Overview

These protocols are intended mainly for those with little or no previous experience in maize and wheat tissue culture, with the goal of presenting a guide for successful establishment of maize and bread wheat somatic cell tissue cultures suitable for *in vitro* genetic manipulation.

Recent technical innovations have provided researchers with new insights into fundamental aspects of plant differentiation and development, while paving the way for the identification of strategies for the genetic manipulation of plants. Not long ago, plant regeneration techniques for cereals were quite rudimentary, but technology in embryogenic tissue cultures for maize and wheat have developed rapidly and are now used routinely for producing transgenic plants. Factors influencing the expression of totipotency in cell culture are (1) genotype; (2) size of immature embryos; (3) composition of plant culture medium and growth regulators; and (4) the environment where the initial plants were grown. Various nutritional, hormonal, and environmental conditions have direct bearing on callus formation and subsequent regeneration of complete plants. One of most important factors responsible for the success of maize and wheat tissue culture *in vitro* is the selection of a nutrient medium. The nutrient media and concentration of growth substances presented in this laboratory protocol have worked well for a range of maize and wheat genotypes inbred lines, varieties, and genotypes, as have the techniques herein described.

The first steps towards the production of stable transgenic plants are the selection of target genotypes and the development of a system for inducing high-frequency somatic embryogenesis in immature embryos. The long-term maintenance of embryogenic callus and regeneration of plants comprise a general procedure for working *in vitro* from plant to plant.

The foundation for *in vitro* plant regeneration is that certain somatic plant cells are capable of proliferation and organization into tissues and eventually development into a complete plant or organism. This capability of cells is termed totipotency. It was initially thought that all plant cells were capable of expressing totipotency. Some cells in an explant or within a culture are incapable of cellular proliferation and totipotency. Tissue explants are mixture of cells varying physiologically, biochemically and developmentally and the result of this cellular heterogeneity is that only certain cells in an explant will be capable of or "competent" to respond to the *in vitro* culture conditions. Competence refers to the capability of a cell or group of cells to respond to an inductive stimulus for a developmental process.

Many questions follow from this issue of competence. Why are some cells of explants capable of dedifferentiating and resuming mitotic activity? Why do some explant or cultured cells respond to morphogenic stimuli—shoot or root-inducing media? Why do some explant or cultured cells regenerate following protoplast isolation and culture? Some answers may be found in the basic cells types found in any proliferating culture: meristemic and differentiated cells. Meristematic are characterized by having a small isodiametric shape, dense cytoplasm, little to no vacuolization, many starch grains and large nuclei with prominent nucleoli. Differentiated cells are large variably shaped and highly vacuolated cells with a thin cytoplasm and inconspicuous nuclei. Because we do not yet understand plant hormonal mechanisms or plant developmental processes the best approach for developing an *in vitro* regeneration system is still based on empirically testing variations of the three factors until some combination proves successful. Factors affecting *in vitro* culture are genotype, explant source, and culture conditions. The choice of genotype is the most important factor. Some species or cultivars within a species have a greater capacity to regenerate *in vitro* than others. Poor curtability and regenerability of some species or genotypes can be overcome by

alterations in the culture protocol. Genetic components exert considerable influence on the success of *in vitro* culture and plant regeneration. For all species, generally younger or vigorously growing tissues from healthy plants should be used as the explant source. Immature organs or meristematic and undifferentiated tissue are generally the most responsive and reliable explant sources. For dicot species, these include leaf lieces, leaf petiole segments, cotyledonary petioles and pieces, hypocotyl, segments, root segments, stem segments, embryos, and immature embryos; for monocot species, young tissues such as maize where parenchyma cells *in vivo* quickly lose the ability to dedifferentiate. The reason for this stage-specific response may be genetic, epigenetic, or physiological changes that occur in mature cells: In cereals, establishment of embryogenic callus from immature embryo explants is successful because the explant consists of many predetermined embryogenic cells, or cells that have retained the embryogenic program despite their becoming determined as scutellar cells.

Plantlets regenerate from calli by organogenesis or somatic embryogenesis. High cytokinin-to-auxin ratios produce shoots, low cytokinin-to-auxin ratios produce roots, and more equal concentrations of these phytohormones cause callus proliferation.

Organogenesis is a developmental pathway in which shoots or roots have been induced to differentiate from a cell or group of cells. Direct shoot or root is induced and develops directly from a pre-existing cell/s in the explant without undergoing an initial callus phase. Indirect organogenesis involves an initial phase of callus proliferation and growth, followed by shoot or root induction and development from the proliferated callus tissue. Due to potential problems with somaclonal variation in callus cultures, direct organogenesis is more desirable than indirect.

Embryogenesis is a developmental pathway in which embryos have been induced to form from a somatic cell or group of somatic cells. Embryogenic cells appear very similar to meristematic cells in that they are small and densely cytoplasmic, have large nuclei, and contain many small vacuoles, lipid droplets and starch grains. In immature embryos the optimal stage is when the depositing of starch in the scutellar cells has just begun..

Callus is formed from several of the outermost layers of the scutellar cells in the coleorhizal half of the embryos. Somaclonal variation is a term used to describe genetic variation that is frequently observed as a result of tissue culture, due to accumulation of mutations and epigenetic changes. Different types of variations are involved in tissue cultures: changes in chromosome structure due to chromosome breakage, changes in chromosome number, transactions and deletions, DNA methylation, and single gene mutations.

Before Working in Sterile Conditions

In tissue culture work, particular care must be taken to avoid mineral and material contamination, as well as contamination by microorganisms. Some growth substances are active biologically and many minerals are active even in low concentrations.

Sources of microbial contamination are everywhere—in the air, on hands, on the bench as well as on and sometimes in biological material. Many of these microorganisms grow well on the media used for cell, tissue, and organ culture. Not only will such microorganisms consume part of the medium, they also add their metabolic waste to it, thus defeating one of the advantages of this technology: the ability to grow cultures on a chemically-defined medium.

The following procedures will reduce this type of contamination:

1. Glassware is washed thoroughly with hot detergent and rinsed well (tap water, glass distilled water).
2. The mouths of flasks are covered with paper, prior to rapid drying in a forced-draft oven.
3. Autoclave (15 psi. for 20 min) all flasks, media, etc., to be used.
2. Dry sterilize instruments and petri dishes.
3. Wash hands with a disinfectant.
4. Swab working areas with 75% ethanol.
5. There should not be any air drafts across the work area.

MAIZE TISSUE CULTURE

Plant Materials

1. Harvest ears from greenhouse or field-grown plants 12–15 days (depending on genotype) after pollination. The ears may be refrigerated overnight if necessary, but the embryos do best if isolated immediately. Use only healthy-looking plant materials.
2. Optimal length for callus formation in immature embryos is 1.0–1.5 mm.

Explant Sterilization

1. Autoclave a 2 L beaker with large stir bar, glass petri plates, and three 1 L Erlenmeyer flasks containing 600 ml of deionized water each.
2. Whole ears surface sterilized by immersion in 70% ethanol for 40 seconds (cut the ear longitudinally into two segments).
3. Prepare 20% Clorox solution (3 % NaCl) by adding 200 ml Clorox to a 1 L graduated cylinder, plus 10 drops of Tween 80, and fill to 1000 ml with deionized water. Pour this solution into a 2L beaker with a stir bar.
4. Under transfer hood conditions, place the ears in the Clorox solution (if the ear is big, break it in half and place both halves into the solution) and stir slowly the solution for 30 min.
5. Pour off the Clorox solution and add 600 ml of sterile deionized water to rinse the ears. Place on a stir plate for 5 min.
6. Pour off the water and add 600 ml sterilized, deionized water to rinse the ear (3 times).

Immature Embryo Excision

1. Place a dissecting scope under the hood and one petri dish on the scope.
2. Hold the ear in the petri dish and spear the end of the ear on the silk scar side of the kernel with a dissecting needle to anchor the ear while isolating the embryo.
3. Cut the top of the kernels off with a sharp scalpel blade while the kernels are still on the cobs.
   - Dig the endosperm and embryo out of the kernel cup with a spatula. Sometimes the embryo will come out with the endosperm, other times embryos will stay in the kernel.
   - If the ear is young and the embryos are less than 1 mm long, they may be the same transparent color as the endosperm and therefore difficult to find.
4. Place the embryo on the prepared media (N6C1SN or N6C1) with the scutellar side up and the embryo axis side in contact with the media. Up to 6 embryos can be placed on a petri dish with 10 ml N6C1 (callus initiation) medium.
5. Wrap the plates with parafilm and incubate at 27°C in darkness in for 3 weeks. Plates can be placed in an unlit growth cabinet or in a light-tight box in a growth culture room.

WHEAT TISSUE CULTURE

Explant Sterilization (green seeds)

1. Use only healthy-looking plant material for explantation.
2. Obtain green seeds harvested 15–20 days (depending on genotype) after pollination, in which the endosperm is still relatively liquid (medium-late milk to early dough stage).
3. Green seeds: remove from the seed head and surface-sterilize by immersion in 70% ethanol for 40 seconds.
4. Autoclave a 1 L beaker with large stir bar, glass petri dish, one tea-strainer, and three 1 L Erlenmeyer flasks containing 600 ml of deionized water each.
5. Prepare 20% Clorox solution (3% NaCl). Pour by adding 100 ml Clorox to a 500 ml graduated cylinder plus 10 drops of Tween 80 and fill up to 500 ml with deionized water. Pour this solution into the 1 L beaker with the stir bar.
6. Under a transfer hood place the seeds into the Clorox solution and put the solution on the stir plate for sterilization for 30 min.
7. Pour off the solution through a tea-strainer to save the seeds and rinse them in 600 ml of the sterilized deionized water. Place on the stir plate for a 5 min rinse.
8. Pour off the water and add 600 ml sterilized, deionized water to rinse the seeds (3 times).

Immature Embryo Excision

1. Place a dissecting scope under the hood and place one petri dish on the scope.
2. Hold the seeds in the petri dish to isolate the embryo.
3. Cut the seeds with a scalpel. Dig the endosperm and embryo out of the cup with a spatula.
4. Place the embryo on the prepared media with the scutellar side up and the embryo axis side in contact with the media.
5. Place 6 embryos into 6 cm petri dishes prepared with 10 ml medium.
6. Wrap the plates with parafilm and incubate at 26°C in darkness in a growth culture room for 4–5 weeks.

Callus Formation and Plant Regeneration from Immature Embryos

All normal living cells within a plant body possess the potential capacity to regenerate the entire organism, i.e. to express its totipotency. This potentiality has been exploited through the culture of protoplasts, cells, tissue and organ *in vitro* (T. Thorpe 1994).

Organogenesis is the process by which cells and tissues are forced to undergo changes that lead to the production of a unipolar structure, namely a shoot or root primordium, which is connected to the parent tissue.

Somatic embryogenesis leads to the production of a bipolar structure containing a root/shoot axis, with a closed independent vascular system. Plant regeneration via somatic embryogenesis for many species can be divided into two phases: the selection or induction of cells with embryogenic competence and the development of these cells into embryos. Both of these can occur directly on the explant or indirectly via callus, which is wounded parenchyma tissue.

Organogenesis and embryogenesis begin with changes in a single cell (or group of cells) that then divide to produce a global mass of cells and ultimately a primordium or an embryo.

MAIZE
Tissue culture of maize can produce two different types of embryogenic calli:

Type I, compact and nodular with many scutellum-like bodies, grows at a rapid rate, with short-term plant regeneration.

Type II, friable and creamy yellow in color, contains well-organized somatic embryos at the coleorhizal end of the scutellum, is fast growing, with long-term plant regeneration.

Callus Formation
The primary growth region of the cultured embryos is in the scutellum, near the basal end of the embryo, where cells proliferate in 4 days and grow rapidly.

Within one week, the scutellar surface become irregular, the size of embryo doubles and is transformed into dedifferentiate tissue visible to the naked eye.

In two weeks, callus tissue grows and develops, hastening differentiation of small embryonic tissue. Somatic embryo formation is evaluated two weeks after immature embryo initiation using a stereomicroscope.

These tissues finally develop into embryoids (Type II calli) that can be discerned by the naked eye. Tissue producing more than three somatic embryos is considered embryogenic.

It is important at this stage to separate the embryogenic callus from the non-embryogenic callus and subculture it on fresh medium. Type II embryogenic callus is formed either directly on the scutellum of the cultured embryos or appears later as very small sections during the subcultures of Type I callus. Transfer this callus to fresh media every week and select carefully. Remove the hard tissue and retain the soft tissue with embryoids on it. Sometimes you may need to increase the auxin level in order to eliminate differentiation and to maintain the friable callus.

Once formed, Type II callus can be maintained for long time in darkness by routine subculture every two weeks, with care to exclude non-embryogenic segments at the time of each subculture.

Callus Maintenance
The yellow, irregularly shaped, friable tissue usually obtained on initiation media is transferred to the maintenance medium.

The maintenance of the callus is on the same media as for callus initiation (N6C1SN), but without silver nitrate N6C1.

The cultures are incubated in darkness at 27-28ºC and are subcultured every 2–3 weeks.

Plant Regeneration
Embryogenic calli is successfully maintained on N6C1 media before moving to the budding media under light.

Buds are induced by transferring calli to a petri dish containing the same medium as that used for callus formation, but with a low sucrose concentration and no auxins (N6R).

NOTE: We found that MSR medium is more efficient than N6R medium for plant regeneration, and only MSR is now used routinely in our experiments.

Green shoot formation is evident within a week and plantlet regeneration in 20 days on regeneration media (MSR).

Plants are regenerated from embryogenic calli in petri dishes and transferred to baby food jars or Magenta containing basal MSR medium and kept in a growth chamber at 27ºC with a 16:8 light/dark photoperiod.

The percentage of plant regeneration is calculated based on the number of embryos regenerating out of the total number of embryos planted on the callus initiation medium.

Root Formation
To establish good root systems, transfer plantlets with 3–5 leaves to Magenta, containing 30 ml basal MS medium with 1 mg/l naphthalene acetic acid (NAA)—MSE.

Gently excise the plantlets close to the callus base using a scalpel and transfer them onto the MSE medium.

Roots should form in 1 week and must be white.

Transplant small plantlets with 4–6 leaves and good root systems to Jiffy pots, being careful to remove the agar medium from the plantlets; wash gently with tap water to fully remove the agar from the roots (contamination will appear if the agar is not removed).

Irrigate the pots with tempered water and cover them with plastic covers. Keep pots with small plants in a growth chamber for a 1 week adaptation period and then move them to the greenhouse.

WHEAT
Embryogenic Callus
The primary growth region of the cultured embryos is the scutellum where cells proliferate in 4 days and grow rapidly on MSE3 or MSE5 medium, depending on the genotypes used.

Within 2 weeks, the scutellar surface becomes irregular and transforms into callus tissue.

Somatic embryo formation should be evaluated two weeks after immature embryo initiation using a stereo microscope.

Tissue producing more than three somatic embryos is considered embryogenic.

Callus Maintenance
The yellow, irregularly shaped, friable tissue usually obtained 4–5 weeks after plating on initiation media is transferred to a MSE3B maintenance medium.

Friable embryogenic tissue is subcultured every 3–4 weeks on the maintenance medium MSE3B.

Embryogenic calli are successfully maintained on MSE3B media for 2–3 weeks before moving to the maturation medium MSE3C.

The cultures are incubated in darkness at 27°C.

Plant Regeneration
Two to three weeks later, embryogenic calli are transferred to the petri culture dish on regeneration medium (MSR) containing IAA and 6-BAP in a growth chamber at 27∞C with a 16:8 light/dark photoperiod.

Green shoot formation is evident within a week and plantlet regeneration within 20 days. Transfer the tissue to glass vials containing basal MSR medium and keep them in a growth chamber at 27∞C with a 16:8 light/dark photoperiod.

The percentage of plant regeneration is calculated based on the number of embryos regenerating out of the total number of embryos plated on the callus initiation medium.

Root Formation
Gently cut the small plantlets, excise them gently close to the callus base using a scalpel, separate them from the calli or the compact tissue and then transfer them onto MSE medium.

To establish good root systems, transfer plantlets into Magenta, on 30 ml MSE medium containing basal MS with 1 mg/l naphthalene acetic acid (NAA).

Roots should form in 1 week and they must be white.

Transplant small plantlets with good root systems to Jiffy pots and keep in a growth chamber under light for 1 week.

Transfer plantlets to free-living conditions as follows: Remove plantlets carefully from agar medium without damaging the root systems and rinse roots with tap water.

Pot plantlets in small containers or Jiffy peat and enclose with transparent plastic covers. Water pots and keep them in a growth chamber in light for 1 week. Plants 8-10 cm high, with 3-5 fully expended leaves being moved to the greenhouse.

MAIZE CALLUS INITIATION AND MAINTENANCE MEDIA

N6C1 medium

Based on N6 medium: C.C. Chu, C.C. Wang, C.S. Sun, C. Hsu, K.C. Yin, C.Y. Chu, and F.Y. Bin. 1975 (*Scientia Sinica* 18: 659-668) with modifications.

Components	500 ml	1000 ml	3000 ml	5000 ml
Solution 1 (10x)	50 ml	100 ml	300 ml	500 ml
Solution 2 (100x)	5 ml	10 ml	30 ml	50 ml
Solution 3 (100x)	5 ml	10 ml	30 ml	50 ml
Solution 4 (1000x)	0.5 ml	1 ml	3 ml	5 ml
L-proline	1.15 g	2.3 g	6.9 g	11.5 g
Casein hydrolysate	0.100 g	0.2 g	0.6 g	1 g
Dicamba (mg/ ml)	1 ml	2 ml	6 ml	10 ml
Sucrose	15 g	30 g	90 g	150 g
Agar (Bacto)	4 g	8 g	24 g	40 g

The medium can be stored at room temperature

1. Pour 500 ml deionised water into 2,000 ml beaker.
2. Add and stir 2.3 g L-proline.
3. Add 0.2g casein hydrolysate.
4. Add 4 ml dicamba from the stock solution (10 mg dicamba dissolved in 1 ml Et/OH and add 9 ml deionised water—10 mg/10 ml).
5. Mix solutions #1–4 and stir with a magnetic stirrer.
6. Add 30 g sucrose and stir to dissolve.
7. Pour the solution into 1,000 ml graduated cylinder and fill up to 1,000 ml with deionised water.
8. Adjust pH to 5.7 with KOH.
9. Pour 500 ml N6C1 medium into two 1,000 ml Erlenmeyer flasks, add 4 g bacto-agar to each and cap with foil.
10. Autoclave at 120°C for 15 min, 15 psi.
11. After autoclaving, put flask(s) into a 50°C water bath until cool enough to pour. Each 500 ml of media is enough for 20 (100x15 mm) plates, or 40 (60x15 mm) plates.

N6C1SN medium

Based on N6 medium: C.C. Chu, C.C. Wang, C.S. Sun, C. Hsu, K.C. Yin, C.Y. Chu, and F.Y. Bin. 1975 (*Scientia Sinica* 18: 659-668) with modifications.

Components	500 ml	1000 ml	3000 ml	5000 ml
Solution 1 (10x)	50 ml	100 ml	300 ml	500 ml
Solution 2 (100x)	5 ml	10 ml	30 ml	50 ml
Solution 3 (100x)	5 ml	10 ml	30 ml	50 ml
Solution 4 (1000x)	0.5 ml	1 ml	3 ml	5 ml
L-proline	1.150 g	2.3 g	6.9 g	11.5g
Casein hydrolysate	0.100 g	0.2 g	0.6 g	1 g
Dicamba (1 mg/1 ml)	1 ml	2 ml	6 ml	10 ml
Sucrose	15 g	30 g	90 g	150 g
Silver nitrate	0.0075	0.015 g	0.045	0.075
Agar (Bacto)	4 g	8 g	24 g	40 g

The medium can be stored at room temperature

1. Pour 500 ml deionised water into 2,000 ml beaker.
2. Add and stir 2.3 g L-proline.
3. Add 0.2 g casein hydrolysate.
4. Add 4 ml dicamba from the stock solution (10 mg dicamba dissolved into 1 ml Et/OH and add 9 ml deionised water-1 mg/1 ml).
5. Mix solutions #1– 4 and stir with magnetic stirrer.
6. Add 30 g sucrose and stir to dissolve.
7. Pour the solution into 1 L graduated cylinder and fill up to 1,000 ml with deionised water.
8. Add 0.015 g silver nitrate and stir well.
9. Adjust pH to 5.7 with O.1M NaOH.
10. Pour 500 ml N6C1SN medium into two 1,000 ml Erlenmeyer flasks, add 4 g bacto-agar to each and cap with foil.
11. Autoclave at 121°C for 15 min, 15 psi.
12. After autoclaving, put flask(s) into 50°C water bath until cool enough to pour. Each 500 ml of media is enough for 20 (100 x 15 mm) plates, and 40 (60 x 15 mm) plates.

MEDIUM FOR EMBRYOGENIC WHEAT CALLUS FORMATION

MSE3

Based on MS medium:T. Murashige and F. Skoog. 1962. (*Physiologia Plantarum* 15:473-497)

Components	500 ml	1000 ml	2000 ml	3000 ml
MS macroelements (10x)	100 ml	200 ml	400 m	600 ml
MS microelements(10x)	50 ml	100 ml	200 ml	300 ml
MS vitamins (1000x)	0.5 ml	1 ml	2 ml	3 ml
Thiamine Cl	0.020 mg	0.040 mg	0.080 mg	0.120 mg
L-Asparagine	0.075 g	0.150 g	0.300 g	0.450 g
Sucrose	30 g	60 g	120 g	180 g
2,4-D (mg/ml)	1.75 ml	2.5 ml	5.0 ml	7.5 ml
Agar	4 g	8 g	16 g	24 g

This medium can be stored at room temperature.

1. Pour 500 ml deionised water into 2,000 ml beaker.
2. Add 200 ml MS macroelements, 100 ml MS microelements, 1 ml MS vitamins, 0.040 g thiamin-Cl, 0.150 g asparagine, 60 g sucrose, mix well.
3. Add 2.5 ml 2,4-D from the stock solution—10 mg 2,4-D dissolved completely into 1 ml 1M NaOH, add 9 ml deionized water and stir with magnetic stirrer.
4. Pour the solution into 1 L graduated cylinder and fill up to 1,000 ml with deionised water.
5. Adjust pH to 5.7 with 1M NaON.
6. Dispense 1,000 ml E3 medium into two 1,000 ml flasks (500 ml into each), add 4 g Bacto-agar in each and cap with foil.
7. Autoclave at 121°C for 15 min, 15 psi.
8. After autoclaving, put flask(s) into 50°C water bath until cool enough to pour. Each 500 ml of medium is enough for 20 (100x15 mm) petri dishes or 40 (60x15 mm) petri dishes.

MEDIUM FOR CALLUS MAINTENANCE

MSE3B

Based on MS medium:T. Murashige and F. Skoog. 1962. (*Physiologia Plantarum* 15:473-497)

Components	500 ml	1000 ml	2000 ml	3000 ml
MS macroelements (10x)	50 ml	100 ml	200 ml	300 ml
MS microelements (10x)	50 ml	100 ml	200 ml	300 ml
MS vitamins (1000x)	0.5 ml	1 ml	2 ml	3 ml
Thiamin Cl	0.020 mg	0.040 mg	0.080 mg	0.120 mg
L-Asparagine	0.075 g	0.150 g	0.300 g	0.450 g
Sucrose	15 g	30 g	60 g	90 g
2,4-D (mg/ ml)	1.75 ml	2.5 ml	5 ml	7.5 ml
Agar-Bacto	4 g	8 g	16 g	24 g

This medium can be stored at room temperature.

1. Pour 500 ml deionised water into 2.000 ml beaker.
2. Add 100 ml MS liquid macro elements, 100 ml MS liquid microelements, 1 ml MS vitamins, 0.040 g thiamin Cl, 0.150 g asparagine, 30 g sucrose, and mix well.
3. Add 2.5 ml 2,4-D and stir with magnetic stirrer.
4. Pour the solution into 1,000 ml graduated cylinder and fill up to 1,000 ml with deionised water.
5. Adjust pH to 5.7 with NaON.
6. Dispense 1000 ml MSE3B medium into two 1000 ml Erlenmeyer flasks (500 ml into each), add 4 g Bacto-agar in each and capped with foil.
7. Autoclave at 121°C for 15 min, 15 psi.
8. After autoclaving, put flask(s) into 50°C water bath until cool enough for pouring. Each 500 ml of medium is enough for 20 (100x15 mm) Petri dishes or 40 (60x15 mm) Petri dishes.

MEDIUM FOR EMBRYOGENIC CALLUS FORMATION

MSE3C

Based on MS medium:T. Murashige and F. Skoog. 1962. (*Physiologia Plantarum* 15:473-497)

Components	500 ml	1000 ml	2000 ml	3000 ml
MS macroelements(10x)	50 ml	100 ml	200 ml	300 ml
MS microelements (10x)	50 ml	100 ml	100 ml	300 ml
MS vitamins (1000x)	0.5 ml	1 ml	2 ml	3 ml
Thiamin Cl	0.020 mg	0.040 mg	0.080 mg	0.120 mg
L-Asparagine	0.075 g	0.150 g	0.300 g	0.450 g
Sucrose	10 g	20 g	40 g	60 g
2,4-D (1 mg/ ml)	0.5 ml	1 ml	2 ml	3 ml
Agar (Bacto)	4 g	8 g	16 g	24 g

This medium can be stored at room temperature.

1. Pour 500 ml deionised water into 2,000 ml beaker.
2. Add 100 ml MS macroelements, 100 ml MS microelements, 1 ml MS vitamins, 0.040 g thiamin Cl, 0.150 g asparagine, 20 g sucrose and mix well.
3. Add 1 ml 2,4-D from the stock solution and stir with magnetic stirrer.
4. Pour the solution into 1,000 ml graduated cylinder and fill up to 1,000 ml with deionised water.
5. Adjust pH to 5.7 with 1M NaON.
6. Dispense 1,000 ml MSE3C medium into two 1,000 ml Erlenmeyer flasks (500 ml into each), add 4 g Bacto-agar in each and cap with foil.
7. Autoclave at 121°C for 15 min, 15 psi.
8. After autoclaving, put flask(s) into 50°C water bath until cool enough to pour. Each 500 ml of medium is enough for 20 (100x15 mm) petri dishes, or 40 (60x15 mm) petri dishes.

MEDIUM FOR WHEAT EMBRYOGENIC CALLUS

MSE5

Based on MS medium:T. Murashige and F. Skoog. 1962.(*Phys. Plantarum* 15:473-497497)'

Components	500 ml	1000 ml	2000 ml	3000 ml
MS basal salt mixture	2.21 g	4.43 g	8.86 g	13.29 g
Meo-inositol	0.050 g	0.100 g	0.200 g	0.300 g
Glycine	0.0375 g	0.075 g	0.150 g	0.150 g
L-Glutamine	0.438 g	0.877 g	1.754 g	2.631 g
L-Aspartic acid	0.133 g	0.266 g	0.532 g	0.798 g
L-Arginine	0.114 g	0.228 g	0.456 g	0.684 g
2,4 - D (mg/ ml)	1 ml	2 ml	4 ml	6 ml
Kinetin (mg/ ml)	0.1 ml	0.2 ml	0.4 ml	0.6 ml
Gibberellic acid (mg/ ml)	0.05 ml	0.1 ml	0.2 ml	0.3 ml
Sucrose	15 g	30 g	60 g	90 g
Agar (Bacto)	4 g	8 g	16 g	24 g

This medium can be stored at the room temperature.

1. Pour 500 ml deionised water into 2,000 ml beaker.
2. Add 4.430 g MS basal salts mixture, 100 mg Meo-inositol, 0.2 ml Kinetin, 0.1 ml Gibberellic acid, 2 ml 2,4D and 30 g sucrose. Mix well with magnetic stirrer.
3. Pour the solution into 1,000 ml graduated cylinder and fill up to 900 ml with deionized water.
4. Adjust pH to 5.7 with 1N NaOH.
5. Dispense 900 ml MS medium into two 1,000 ml cylinders, 450 ml medium into each, and add 4 g Bacto-agar into each flask and cap with foil.
6. Autoclave at 121°C for 15 min.
7. After autoclaving, put flasks into 40°C water bath until cool enough to add the amino acids solution.
8. Pour 50 ml deionized water into 200 ml beaker and add 0.075 g glycine, 0.877 g L-glutamine, 0.266 g L-aspartic acid (dissolved completely—0.266 g L-aspartic acid into 1 ml NaOH) and mix well. Pour the solution into 100 ml cylinder and fill up to 100 ml with deionized water, using a 0.2 µm filte (ph=5.7).
10. Add 50 ml from solution #8 into each 450 ml cooled enough medium.
11. Mix well with magnetic stirrer and pour into petri dishes. Each 500 ml of medium is enough for 20 petri dishes, 100x15, or 40 petri dishes, 60x15 mm.

MAIZE AND WHEAT REGENERATION MEDIUM

MSR medium with MS powder salt mixture
Based on MS medium: T. Murashige and F. Skoog. 1962. (*Physiologia Plantarum* 15:473-497)

Chemicals	500 ml	1000 ml	3000 ml	5000 ml
MS salts	2.21 g	4.43 g	13.29 g	22.15 g
Thiamin Cl	0.020 mg	0.040 mg	0.120 mg	0.200 mg
L-asparagine	0.075 g	0.15 g	0.45 g	0.75 g
Sucrose	10 g	20 g	60 g	100 g
IAA(1 mg/ ml)	0.25 ml	0.5 ml	1.5 ml	2.5 ml
BAP(1mg/ ml)	0.5 ml	1 ml	3 ml	5 ml
Agar	4 g	8 g	24 g	40 g

This medium can be stored at room temperature for 1 month.

1. Pour 300 ml deionized water and 4.30 g MS salts mixture, 0.040 mg Thiamin Cl, 0.150 g L-asparagine, 20 g sucrose and 0.5 ml IAA into a 500 ml cylinder and fill to 500 ml with deionized water. Stir until the reagents are completely dissolved (MS salts do not readily dissolve).
2. Adjust the pH to 5.7 with 1M NaOH.
3. Pour 500 ml deionized water into 2,000 ml beaker, add 8 g Bacto agar and steam in micro waves to melt the agar (for 500 ml approximately 8 min).
4. Add solution #1–4 (500 ml liquid medium) to the solution #5 and stir 5 min.
5. Pour off the resulting medium into baby food jars (30 ml per jar) or into Magenta vessels (40 ml per vessel), cover the containers with lids and autoclave for 15 min, 15 psi.
6. After autoclaving, allow the media to solidify in the baby food jars and Magenta vessels.

MSR medium with stock solutions
Based on MS medium: T. Murashige and F. Skoog. 1962. (*Physiologia Plantarum* 15:473-497)

Solutions	500 ml	1000 ml	3000 ml	5000 ml
MS 1 (100X)	5 ml	10 ml	30 ml	50 ml
MS 2 (10X)	50 ml	100 ml	300 ml	500 ml
MS 3 (100X)	5 ml	10 ml	30 ml	50 ml
MS 4 (100X)	5 ml	10 ml	30 ml	50 ml
MS 5 (100X)	5 ml	10 ml	30 ml	50 ml
MS 6 (200X)	2.5 ml	5 ml	15 ml	25 ml
MS vitamins (1000X)	0.5 ml	1 ml	3 ml	5 ml
Myo-Inositol	0.050 g	0.100 g	0.300 g	0.500 g
Sucrose	15 g	30 g	90 g	150 g
IAA	0.25 ml	0.5 ml	1.5 ml	2.5 ml
BAP	0.5 ml	1 ml	3 ml	5 ml
Agar	4 g	8 g	24 g	40 g

This medium can be stored at room temperature for 1 month.

1. Pour 300 ml deionized water into a 2,000 ml beaker.
2. Add solutions MS1, MS2, MS3, MS4, MS5, MS6, MS vitamins, 100 mg Myo-Inositol, 30 g sucrose, 0.5 ml IAA, and 0.5 ml BAP, and stir for 5 min.
3. Pour the solution into 500 ml cylinder and fill to 500 ml with deionized water.
4. Adjust the pH to 5.7 with NaOH.
5. Pour 500 ml deionized water into 2,000 ml beaker, add 8 g Bacto agar and heat in micro wave to melt the agar (500 ml requires 8 min).
6. Add solution #1 to solution #5 and stir 5 min.
7. Pour the resulting medium into baby food jars (30 ml per jar) or Magenta vessels (40 ml per vessel), cover the containers and autoclave for 15 min, 15 psi.
8. After autoclaving, allow the media to solidify in the baby food jars and Magenta vessels.

N6R medium

Based on N6 medium: C.C. CHu, C.C. Wang, C.S. Sun, C. Hsu, K.C. Yin, C.Y. Chu, and F.Y. Bin. 1975 (*Scientia Sinica* 18: 659-668).

Components	500 ml	1000 ml	3000 ml	5000 ml
Solution 1 (10x)	50 ml	100 ml	300 ml	500 ml
Solution 2 (100x)	5 ml	10 ml	30 ml	50 ml
Solution 3 (100x)	5 ml	10 ml	30 ml	50 ml
Solution 4 (1000x)	0.5 ml	1 ml	3 ml	5 ml
L-proline	1.15 g	2.3 g	6.9 g	11.5 g
Casein hydrolysate	0.100 g	0.200 g	0.600 g	1.000 g
Sucrose	10g	20 g	60 g	100 g
Agar (Bacto)	4 g	8 g	24 g	40 g

The medium can be stored at room temperature

1. Pour 300 ml deionised water into 2,000 ml beaker and add 2.30 g L-proline, 200 mg Casein hydrolysate, 20 g sucrose, and stir to completely dissolve the components.
2. Pour the solution into 500 ml graduated cylinder and fill up to 500 ml with deionised water.
3. Adjust the pH to 5.7 with 1M NaOH.
4. Pour 500 ml deionized water into 2,000 ml beaker, add 8 g Bacto agar and heat in microwave to melt the agar (for 500 ml require 8 min).
5. Add solution #1 (500 ml liquid medium) to solution #4 and stir 5 min.
6. Pour the resulting medium into baby food jars (30 ml per jar) or Magenta vessels (40 ml per vessel), cover the containers, and autoclave for 15 min, 15 psi.
7. After autoclaving, allow the media to solidify in the baby food jars or Magenta vessels.

MAIZE AND WHEAT ROOTING MEDIUM

MSE medium

Based on MS medium: T. Murashige and F. Skoog. 1962. (*Physiologia Plantarum* 15: 473-497)

Chemicals	500 ml	1000 ml	3000 ml	5000 ml
MS Macroelements	25 ml	50 ml	150 ml	250 ml
MS Microelements	50 ml	100 ml	300 ml	500 ml
Sucrose	10 g	20 g	60 g	100 g
IAA (1 mg/ 1 ml)	0.5 ml	1 ml	3 ml	5 ml
Agar	4 g	8 g	24 g	40 g

This medium can be stored at the room temperature for 1 month.

1. Pour 300 ml deionized water into a 1,000 ml beaker and add 50 ml liquid macroelements from MS basal salt macronutrient solution, 100 ml MS microelements from MS basal microelements solution, 20 g sucrose and 1 ml IAA.
2. Fill to 500 ml with deionized water and mix the solution well with a magnetic stirrer.
3. Adjust the pH to 5.7 with 1M NaOH.
4. Put 8 g agar into 1,000 ml flask and pour 500 ml of deionized water. Melt the agar for 8 min. in a microwave oven.
5. Mix solution #1 with solution #3 and stir with a magnetic stirrer.
6. Pour the resulting medium into baby food jars (30 ml per jar) or into Magenta vessels (40 ml per vessel), cover the containers, and autoclave for 15 min., 15 psi.
7. After autoclaving, allow the media to solidify at room temperature in the baby food jars or Magenta vessels.

MSE with stock solutions

Based on MS medium: T. Murashige and F. Skoog. 1962. (*Physiologia Plantarum* 15: 473-497)

Solutions	500 ml	1000 ml	3000 ml	5000 ml
MS 1 (100X)	2.5 ml	5 ml	15 ml	25 ml
MS 2 (10X)	25 ml	50 ml	150 ml	250 ml
MS 3 (100X)	2.5 ml	5 ml	15 ml	25 ml
MS 4 (100X)	2.5 ml	5 ml	15 ml	25 ml
MS 5 (100X)	2.5 ml	5 ml	15 ml	25 ml
MS 6 (200X)	1.25 ml	2.5 ml	7.5 ml	12.5 ml
MS vitamins (1000X)	0.5 ml	1 ml	3 ml	5 ml
IAA (1mg/1ml)	0.5 ml	1 ml	3 ml	5 ml
Sucrose	10 g	20 g	60 g	100g
Agar	4 g	8 g	24 g	40 g

This medium can be stored at the room temperature for a month.

1. Pour 300 ml deionized water into 2,000 ml beaker.
2. Add solutions MS1, MS2, MS3, MS4, MS5, MS6, MS vitamins, 100 mg Myo-Inositol, 20 g sucrose, 0.5 ml IAA, and 0.5 ml BAP, and stir for 5 min.
3. Pour the solution into 500 ml cylinder and fill to 500 ml with deionized water.
4. Adjust the pH to 5.7 with 1N M.
5. Pour 500 ml deionized water into 2,000 ml beaker, add 8 g Bacto agar and heat in micro wave to melt the agar (500 ml requires 8 min).
6. Mix solution #1 with solution #5 and stir for 5 min.
7. Pour the resulting medium into baby food jars (30 ml per jar) or into Magenta vessels (40 ml per vessel), cover the containers, and autoclave for 15 min, 15 psi.
8. After autoclaving, allow the media to solidify in the baby food jars or Magenta vessels.

STOCK SOLUTIONS FOR TISSUE CULTURE MEDIA

Stocks for N6C1 and N6C1Sn media

SOLUTION 1 (N6S1–10x)

Components	500 ml	1000 ml	2000 ml	4000 ml
$(NH_4)_2SO_4$	2.315 g	4.630 g	9.260 g	18.520 g
KNO_3	14.165 g	28.330 g	56.660 g	113.320 g
$CaCl_2.2H_2O$	0.830 g	1.660 g	3.320 g	6.640 g
KH_2PO_4	2.000 g	4.000 g	8.000 g	16.000 g
$MgSO_4$	0.452 g	0.930 g	1.860 g	3.720 g

This solution can be stored in the refrigerator

1. Dissolve completely 1.660 g $CaCl_2.2H_20$ with 300 ml deionized water in a 500 ml beaker and stir with a magnetic stirrer.
2. Dissolve completely 0.930 g $MgSO_4$ with 300 ml deionized water in a 500 ml beaker and stir with a magnetic stirrer.
3. Dissolve completely 28.330 g KNO3 with 300 ml deionized water in a 500 ml beaker and stir with a magnetic stirrer. Filter with Whatman #1 filter paper.
4. Mix the solutions #1, 2, and 3 and stir with magnetic stirrer.
5. Add 4.630 g $(NH4)_2SO_4$.
6. Add 4 g KH_2PO_4.
7. Pour the solution into a graduated cylinder and fill up to 1000 ml with deionized water.
8. Filter the solution with Watman #1 filter paper.

SOLUTION 2 (N6S2 - 100x)

Components	500 ml	1000 ml	2000 ml	4000 ml
$MnSO_4.H_2O$	0.298 g	0.597 g	1.194 g	2.388 g
$ZnSO_4.7H_2O$	0.075 g	0.150 g	0.300 g	0.600 g
H_3BO_3	0.080 g	0.160 g	0.320 g	0.640 g
KI	0.040 g	0.080 g	0.160 g	0.320 g

This solution can be stored in the refrigerator.

1. Pour 800 ml deionized water into a 2,000 ml beaker and stir with a magnetic stirrer.
2. Add 160 mg H_3BO_3.
3. Add 150 mg KI.
4. Add 597 mg $MnSO_4.H_2O$.
5. Add 150 mg $ZnSO_4.7H_2O$.
6. Dissolve reagents completely.
7. Pour the solution into a graduated cylinder and fill to 1000 ml with deionized water.
8. Pour into 1,000 ml bottle and store in a refrigerator.

SOLUTION 3 (N6S3–100X)

Components	500 ml	1000 ml	2000 ml	4000 ml
$FeSO_4 7H_2O$ (Iron Sulfate)	1.392 g	2.785 g	5.570 g	11.140 g
Na_2 EDTA (Disodium Salt)	1.862 g	3.725 g	7.450 g	14.900 g

This solution can be stored in the dark and the refrigerator.

1. Pour 400 ml deionized water into a 1,000 ml beaker. Add 2.785 g $FeSO_4.7H_20$ and stir with magnetic stirrer at low heat for 1 hour to dissolve the reagent completely.
2. Pour 400 ml deionized water into a 1,000 ml beaker. Add 3.725 g Na_2EDTA and stir with magnetic stirrer at low heat for 1 h to dissolve reagent completely.
3. Pour solutions #1 and #2 into a 1,000 ml graduated cylinder and fill to 1,000 ml with deionized water.
4. Pour the solution into a 1,000 ml bottle and store in the refrigerator.

SOLUTION 4 (N6S4–1000X)

Components	50 ml	100 ml	200 ml	400 ml
Glicine	0.100 g	0.200 g	0.400 g	0.800 g
Nicotic Acid	0.025 g	0.050 g	0.100 g	0.200 g
Thiamin Cl	0.025 g	0.050 g	0.100 g	0.200 g
Pyridoxine Cl	0.050 g	0.100 g	0.200 g	0.400 g

This solution can be stored in the refrigerator.

1. Pour 25 ml deionized water into 100 ml beaker and stir with a magnetic stirrer.
2. Add 100 mg glicine.
3. Add 25 mg nicotinic acid.
4. Add 25 mg thiamin Cl.
5. Add 50 mg pyridoxine Cl.
6. Dissolve reagents completely, pour into 50 ml graduated cylinder and fill to 50 ml with a deionized water.
7. Pour the solution into 50 ml bottle and store in a refrigerator.

STOCK SOLUTIONS FOR MURASHIGE AND SKOOG MEDIA—MSR, MSE

Solution 1 (MS1–100X)

	1000 ml	2000 ml
$CaCl_2$ (anhydrous) 332.20 mg/L	33.20 g	66.40 g
$CaCl_2$ dehydrate) 431.67 mg/L	43.96 g	87.93 g

1. Mix 33.2 g CaCl2 with 800 ml deionized water and stir with a magnetic stirrer until the reagents completely dissolve .
2. Pour the solution into a graduated cylinder and fill to 1,000 ml with deionized water.
3. Pour into a 1,000 ml bottle and store in a refrigerator.

Solution 2 (MS2–10X)

	1000 ml	2000 ml
NH4NO3, 1650 mg/L	16.5 g	33.0 g
KNO3, 1900 mg/L	19.0 g	38.0 g

1. Completely dissolve 16.5 g NH4NO3 completely with 300 ml deionized water into a 500 ml beaker and stir with a magnetic stirrer.
2. Dissolve completely 19 g KNO3 with 300 ml deionized water in a 500 ml beaker and stir with a magnetic stirrer. Filter with Whatman #1 filter.
3. Mix solutions #1 and #2 and stir with magnetic stirrer.
4. Pour the solution into a graduated cylinder and fill to 1,000 ml with deionized water.
5. Pour the solution into a 1,000 ml bottle and store in a refrigerator.

Solution 3 (MS3–100X)

	1000 ml	2000 ml
KI, 0.830 mg/L	83 mg	166 mg
CoCl2.6H2O, 0.0250 mg/L	2.5 mg	5.0 mg

1. Mix completely 83 mg KI and 2.5 mg CoCl2.6H20 in a 1,000 ml beaker and fill to 1,000 ml with deionized water.
2. Pour the solution into a 1,000 ml bottle and store in a refrigerator.

Solution 4 (MS4–100X)

	1000 ml	2000 ml
KH2PO4, 170.0 mg/L	17 g	34 g
Na2MoO4.2H2O, 0.25 mg/L	25 mg	50 mg
H3BO3, 6.2 mg/L	620 mg	1,240 mg

1. Mix completely 17 g KH2PO4, 25 mg Na2MoO4.2H2O, and 620 mg H3BO3 with 800 ml deionized water in a 1,000 ml beaker and stir with a magnetic stirrer.
2. Pour the solution into a graduated cylinder and fill to 1,000 ml with deionized water.
3. Pour the solution into 1,000 ml bottle and store in a refrigerator.

Solution 5 (MS5–100X)

	1000 ml	2000 ml
MgSO4.7H2O, 370 mg/l	37 g	36 g
MnSO4.4H20, 22.3 mg/l	1.69 g	3.38 g
CuSO4.5H2O, 0.025 mg/l	2.5 mg	5.0 mg
ZnSO4.7H2O, 8.6 mg/l	860 mg	1.72 g

1. Pour 500 ml deionized water into a 2,000 ml beaker and add 37g MgSO4.7H2O, 1.69 g MnSO4.4H2O, 2.5 mg CuSO4.5H2O, and 860 mg ZnSO4.7H2O. Stir with a magnetic stirrer and completely dissolve reagents.
2. Pour the solution into graduated cylinder and fill to 1,000 ml with deionized water.
3. Pour the solution into a 1,000 ml bottle and store in a refrigerator.

Solution 6 (MS6 - 200X)

	1000 ml	2000 ml
FeSO4. 7H2O, 37.26 mg/L	7.45 g	14.9 g
Na2EDTA, 27.85 mg/L	5.56 g	11.12 g

1. Pour 400 ml deionized water into 1,000 ml beaker (Amber). Add 7.45 g FeSO4.7H2O. Stir with magnetic stirrer and completely dissolve reagent..
2. Pour 400 ml deionized water into 1,000 ml beaker. Add 5.56 g Na2EDTA. Stir with magnetic stirrer at low heat for 1 hour to completely dissolve reagent.
3. Pour solutions #1 and #2 into a 1,000 ml graduate cylinder and fill to 1,000 ml. with deionized water.
4. Pour the solution into a 1,000 ml amber bottle and store in a refrigerator.

PREPARATION AND STORAGE OF PHYTOHORMONE STOCK SOLUTIONS

Preparation of phytohormone stock

Compound	Deionized water	Dissolve
Gibberellin	100 mg	100 ml ethanol
IAA	100 mg	100 ml 1M HCl
NAA	100 mg	100 ml 1M HCl
Kinetin	100 mg	100 ml 1M HCl
BA (BAP)	100 mg	100 ml 1M HCl
2,4 D	100 mg	100 ml 1M NaOH
Dicamba	100 mg	100 ml 1M NaOH

1. Dissolve sample in an appropriate amount of solvent as indicate:
   Cytokinins: Dissolve 10 mg into 0.5 ml HCl.
   Auxins: Dissolve 10 mg into 0.5 ml NaOH.
   Gibberellins: Dissolve 1 mg into 1 ml 95% (v/v) ethanol.
2. For auxins or cytokinins, add deionized water to final volume.
3. Stir vigorously and pour into a clean container.
4. Label as follows:
   Compound:
   Concentration:
   Date of preparation:

METHODS OF PREPARATION (1mg/ml stock solution)

To prepare a 1 mg/ml stock solution: Add 100 mg of the plant growth regulator to a 100 ml volumetric flask or other glass container. Add 2 ml of solvent to dissolve the powder. Once completely dissolved, bring to volume with deionized water. Stirring the solution while adding water may be required to keep the material in solution. Add 1 ml of the stock solution to 1 L of medium to obtain a final concentration of 1.0 mg/L of the plant growth regulator in the culture medium.

$$\frac{\text{Desired Hormone Concentration} \times \text{Medium Volume}}{\text{Stock Solution Concentration}} = \text{Volume of stock solution required}$$

STORAGE OF PHYTOHORMONE STOCK SOLUTION

Compound	**Storage time**
Gibberellin	Prepare fresh
IAA	1 week
NAA	1 week
Kinetin	2 months
BA (BAP)	2 months
2,4 D	2 months
Dicamba	2 months

1. Store the stock solutions at 4°C.
2. Vitamin stocks can be stored at 4°C for up to one month.

Tissue Culture and Transformation Laboratory Rules

To safely and efficiently accomplish all of our individual and collective tasks, we need your help in following accepted practices.

LABORATORY ORGANIZATION

There are two areas in the Applied Genetic Engineering Laboratory:

- An area for tissue culture and transformation experiments. Located here are the transfer hoods, the gene gun, stereo microscopes, growth culture cabinets, and media storage.
- An area is for media preparation and material sterilization. Located here are the micro balances, transfer hood, and shelves and cabinets for glassware storage.

LABORATORY WARE AND WASTES

Lab ware that contains any biohazardous material must be separated from other ware before the cleaning process begins. All dirty glassware and plastic ware, except for clear, hard plastic ware and sterile distilled water bottles, must be properly placed for washing.

We have established "clean" and "dirty" areas in the lab, usually near the working area. Each person is responsible for separating dirty and clean glassware in the lab.

There are two types of carts in the lab for accommodating all dirty glassware.

- The cart closest to the transfer hood area is for glassware that only needs to be washed. All dirty glassware/lab ware will be washed by standard procedures that include a final D-I water rinse, drying in an automatic dryer, and returning items to their designated shelf or glass cabinet in the lab. Spatulas are hand-washed in the lab.
- The cart near the door of the growth culture room is for collecting biohazardous material coming from transformation work. These items must be steam sterilized before the glassware is emptied and washed.

Biohazard waste bags for disposing of biological wastes.

- Use big red bags (marked with a biohazard label) for waste material that is biologically contaminated and needs to be autoclaved.
- The waste bags near the transfer hoods are for non-autoclaved waste, not for biological waste material.

PREPARATION FOR STERILE PROCEDURES

- Wash hands with soap before working with sterile media flasks.
- Turn hood on at least 15 minutes before cleaning and use.
- Clean surface of hood and any non-sterile equipment used in the hood with 75% ethanol before placing sterile items in the hood.
- Clean hands with 75% ethanol before adding ingredients to sterile media and/or pouring plates.
- No mouth pipetting.
- Work in an area of the hood with an unimpeded air flow.
- Do not place hands, arms, etc. above or behind an open flask or sterile piece of equipment.
- Use only sterile pipettes, tips, etc.
- Flame the forceps, scalpels, and other items in the transfer hood before work.
- Always add sterile ingredients to sterile media in the laminar flow hood.
- When attaching a sterile filter to a syringe, always protect the sterile opening of the filter. Keep the sterile end in the package while pressing the sides of the filter to attach it to the syringe.

- Flame the neck of a sterile flask after adding ingredients and anytime that contamination of the neck is suspected. Hold the flame behind the flask, between the flask and the grid of the hood.
- Wipe up all spills as they occur and discard all plates that have had media spilled on their exterior.
- Discard or save for non-sterile purposes any plates that have fallen open, are cracked, or for any reason you suspect may have been contaminated.
- Limit conversations and distractions while working in a sterile area. Distractions can cause undetected errors, resulting in contaminated products.

THE GENE GUN (BIOLISTIC PDS-1000/HE PARTICLE DELIVERY SYSTEM)

There are several potential hazards involved with the use of the gene gun including: explosion, handling high pressure gas, implosion, high-speed projectiles, and electric shock. Care should be especially taken when handling Tungsten Metal Powder (see material safety data sheet).

To ensure safe operation of the PDS-1000/He, anyone using it must be aware of all safety practices and take precautions as described in the Biolistic PDS-1000/He Particle Delivery System Instruction Manual, Bio-Rad Laboratories. **No one should use the gene gun without first reading and understanding this manual and without adequate supervision.**

ACCIDENTS

The most likely cause of an accident in the AGE laboratory is a fire in one of the laminar flow hoods due to the close proximity of a flame and alcohol. To avoid this happening, it is advisable to develop good working practices in the cabinet by working in an uncluttered environment and by keeping the flame as far as possible from the alcohol.

- If the flask containing alcohol catches fire, place the lid on the flask to exclude oxygen and the fire will be rapidly extinguished.
- If the fire spreads further in the cabinet, cover the area with a fire blanket or use the fire extinguisher in the lab.

The fire extinguisher and fire blankets are located in the same area as the laminar flow hoods.

When working with chemicals that are irritants, use gloves when handling them and thoroughly clean up any spills.

If an irritant comes into contact with your eyes or skin, wash with copious amounts of water.

There are eye wash stations in both areas of the AGE lab.

Chemicals for Maize and Wheat Tissue Culture Media

(Sigma chemical company)

Name	P. M.	Formula	Cat. No.	Unit size
Abscisic acid	264.3	C15H20O4	A-1049	200 mg
Adenine (6-aminopurine)hydrochloride	171.6	C5H5N5	A-9795	20 g
Adenine(FreeBase)6 aminopurine	135.1	C5H5N5	A-2786	100 g
Agar (Agar-Agar Gum agar)			A-9915	1000 g
Agar Bacto			0140-01	
Agar-Washed			A-8678	500 g
Agarose			A-9045	25 g
L-Alanine				
Ammonium nitrate	80.04	NH4NO3	A-3795	1900g
Ammonium sulfate	132.1	(NH4)xSO4	A-3920	1500 g
L-Arginine	174.2		A-3784	
p-Aminobenzoic acid (Free Acid) (PABA)	138/1	C7H7NO2	A-3659	2.5g
L-ascorbic acid	176.1	C6H8O6	A-2174	500g
L-Asparagine (monohhydrate)	150.1	C4H8N2O3H2O	A-4284	100g
L-Aspartic acid	133.1		A-4534	
BAP-6-benzilaminopurine	225.3	C17H19H5O4	B-5898	45g
d-Biotin (Vitamina H)	224.3	C10H16N2O3	B-3399	50mg
Boric acid	61.83	H3BO3	B-9645 /B-0394	190g
Calcium chloride-dehydrate	0.0147	CaCl2 2H2O	C-2536	3800g
Casein,enzimatic hydrolisate (N-ZOAmine A)			C-7290	200g
Cobalt chloride, hexahydrate	237.9	COCl2 6H2O	C-2911	49g
Choline chloride (aprox 99%)	139.6	C5H14ClNO	C-7527	200g
Cupric sulfate pentahydrate	249.7	CuSO4 5H2O	C-3036	240g
Dicamba (Banvel)			Sandoz Agricola	20g
2-4-D Dichlorophenoxyacetic acid	221	C8H6Cl2O3	D-8407	450g
6-898 Dimethylallylaminopurine (sip. N6-(-2-isopentenyl)adenine)	203.2	2 iP	D-8532	1100mg
Ethylenediamine tetracetic acid (EDTA)	372.2	C10H16N2O8	E-6635	200g
Ethylenediamine tetracetic acid-ferric sodium salt.	367.1	C10H11N2NaFeO8	E-6760	180g
Folic acid (pteroglutamic acid)	441.4	C19H19N7O6	F-8890	95g
Ferrous sulfate	278	FeSO4 7H2O	F-8263	55g
Fumaric acid	116.1	C4H4O4	F-8509	
Gamborg's B-5 basal salt mixture			G-5768	56g
Gamborg's vitamine solution (1000x)			G-1019	149ml
Giberelic acid	346.4	C19H22O6	G-7645	100mg
L-Glutamine	146.1	C5H10N203	G-5763	170g
Glycine	75.07	C2H5NO2	G-6143	450g
IAA(IIndole 3-acetic acid)	175.2	C10H9NO2	I-2886	45g
Kao and Michalyluk Basal salt mixture			K-0878	100g
Kao and Michalyluk vitamine solution(100x)			K-3129	950ml
Kinetin (6-furfuryl amino purine)	215.2	C13H17N5O5	K-3378	24g
L-Lysine	146.1		L-1137	
Magnesium sulfate anhydrous	120.4	MgSO4	M-8150	400g
L-Malic acid	134.1	C4H6O5	M-8405	
Manganese sulfate	151	MnSO4	M-7899	
Mercuric chloride	127.5	HgCl2	M1136	200g
Monooleate (twen 80)polyoxyethilene-sorbitan monooleate			P-1754	500ml
Molybdic acid-sodium salt byhydrate	241.9	Na2MCl4 2H2O	M-1651	195 g
Murashige and Skoog Basal powder medium			M-5519	44g
Murashige and Skoog Macronutr. Sol. (10X)			M-0654	
Murashige and Skoog Micronutr. Sol. (10X)			M-0529	
Murashige and Skoog vitamin mixture(1000x)			M-7150	200ml/93
Myo-Inositol	180.2	C6H12O6	I-3011	140g
a-Naphthaleneacetic	186.2	C12H10O2	N-0640	160g
Nicotinic acid (Niacinpyridin-3-carboxylic acid)	123.1	C6H5NO2	N-0765	195g
D-Pantothenic Acid (Hemicalcium salt)	238.3	C9H16NO5	P-6045	95g
Phytagel			P-8169	

Cont'd...

Chemicals for Maize and Wheat Tissue Culture Media Cont'd...

(Sigma chemical company)

Name	P. M.	Formula	Cat. No.	Unit size
Potassium Chloride	74.55	KCl	P-8041	950g
Potassium Iodide	166	KI	P-8166	195g
Potassiun Nitrate	101.1	KNO3	P-8291	1100g
Potassium Phosphato monobasic (anhydrous)	136.1	KH2PO4	P-8416	95g
L-Proline (Hydroxy-L-Proline free)	115.1	C5H9NO2	P-4655.	
Pyridoxine,hydrochloride (vitamin B6)	205.6	C8H11NO3	P-8666	99g
Riboflavin(vitamin B2)	376.4	C17H20N4O6	R-9881	100 g
L-Serine	105.1		S-5511	25g
Silver Nitrate	169.9	AgNO3	S-6506	20g
Sodium phosphate (monosodium phodphate monobasic anhydrous)	120	NaH2PO4	S-5515	200g
Sodium sulfate anhydrous	142	Na2SO4	S-5640	400g
Sucrose (a-D-Glucopyranosyl-B-D-fructofuranoside,saccharose)	342.3	C12H22O11	S-5390	
Thiamine (aneurine vitamin B1, hydrochloride)	337.3	C12H17CIN4OS-HCl	T-3902	90g
d l-a-tocopherol (vitamin E)	482.9	C29H50O2	T-3251	5g
Vitamin B12 (cyanocabalamin)	1355.4		V-6629	500mg
Zeatin (6-(4-Hydroxy-3-methylbut-2-enylamino)purine).	291.2	C10H13N50	Z-0164	12mg
Zinc sulfate heptahydrate	287.5	ZnSO4-7H2O	Z-4750	120g

Abbreviations

Abbreviation	Name	Synonym	Solubility	M.W.
NAA	l-Naphthyl acetic acid	l-Naphthalene acetic acid	Ethanol	186.21
IAA	3-Indole acetic acid	Indol-3-acetic acid	Ethanol	175.19
2,4 D	2,4-Dichloro-phenoxyacetic acid		Ethanol	221.04
Dicamba	3,6-Dichloro-o-anasic acid			
2,4,5 T	2,4,5,-Trichlorophenoxy acetic acid		Ethanol	255.48
pCPA	p-Chloro phenoxyacetic acid		Ethanol	186.6
pABA	p-Aminobenzoic acid		Ethanol	137.14
IBA	3-Indole butyric acid	Indole-3-butyric acid	Ethanol	203.24
GA3	Gibberellic acid		Ethanol	346.38
ABA	Abscisic acid	3-methyl-5-('-hydroxy-4'-oxo-2',6,6', thrimetyl-2cyclohexen-l-yl)cis, trans, 2,4-pentadienoic acid	Alkali (NaHCO3)	264.3
	Zeatin	6-(4-hydroxy-3-methyl-but-2-enyl)-amino purine.	NaOH	
	Kinetin	6-furfuryl-amino purine	NaOH	
6 BAP	6-benzyl amino purine	6-benzyl adenine	NaOH	
2IP	N^6(Isopentyl) adenine		NaOH	
Vitamin B1	Thiamine HCl	Areurine HCl	Water	
Vitamin B2	Riboflavin		Alkali	
Vitamin B3	Nicotic acid	Niacin	Water	
Vitamin B_5	d-Pantothenic acid (Ca Salt)		Water	
Vitamin B_6	Pyridoxine HCl		Water	
Vitamin C	Ascorbic acid		Water	176.13
Vitamin E	- Tocopherol		Alcohol	417.7
Vitamin H	d- Biotin		Dilute alkali Dilute alkali	244.31
Vitamin M	Folic acid			441.4

Commonly Used Terms in Tissue Culture

Aseptic technique: Procedures used to prevent the introduction of fungi,bacteria, viruses, mycoplasma or other microorganisms into cell, tissue and organ cultures.

Callus: An unorganized, proliferative mass of differentiated plant cells; a wound response.

Cell culture: Term used to denote the maintenance or cultivation of cells *in vitro,* including the culture of single cells. In cell cultures, the cells are no longer organized into tissues.

Cell hybridization: The fusion of two or more dissimilar cells leading to the formation of a synkaryon.

Cell line: A cell line arises from a primary culture at the time of the first successful subculture.

Clonal propagation: Asexual reproduction of plants that are considered to be genetically uniform and originating from a single individual or explant.

Clone: A group of plants propagated only by vegetative and asexual means, all members of which have been derived by repeated propagation from a single individual.

Cryopreservation: Ultra-low temperature storage of cells, tissue, embryos, or seeds. This storage is usually carried out at temperatures below -100° C.

Differentiated: Cells that maintain, in culture, all or much of the specialized structure and function typical of the *in vivo* cell type.

Embryo culture: Aseptic culture of a zygotic embryos. *In vitro* development or maintenance of isolated mature or immature embryos.

Embryogenesis: The process of embryo initiation and development.

Epigenetic event: Any change in a phenotype that does not result from an alteration in DNA sequence. This change may be stable and heritable and includes alteration in DNA methylation, transcriptional activation, translational control, and posttranslational modifications.

Epigenetic: Phenotypic variability that has a nongenetic variation basis.

Explant: Tissue taken from its original site and transferred to an artificial medium for growth or maintenance.

Explant culture: The maintenance or growth of an explant in culture.

Feeder layer: A layer of the cells upon which are cultured a fastidious cell type.

Friability: A term indicating the tendency for plant cells to separate from one another.

Gametoclonal variation: Variation in phenotype, either genetic or epigenetic in origin, expressed by gametoclones.

Gametoclone: Plants regenerated from cell cultures derived from meiospores, gametes, or gametophytes.

Induction: Initiation of a structure, organ, or process *in vitro*.

***In vitro* propagation:** Propagation of plants in a controlled, artificial environment, using plastic or glass culture vessels, aseptic techniques, and a defined growing medium.

***In vitro* transformation:** A heritable change, occurring in cells in culture, either intrinsically or from treatment with chemical carcinogens, oncogenic viruses, irradiation, transfection with oncogenes, etc., and leading to the acquisition of altered morphological, antigenic, neoplastic, proliferative, or other properties.

Meristem culture: *In vitro* culture of a generally shiny, dome-like structure measuring less than 0.1 mm in length when excised, most often excised from the shoot apex.

Micropropagation: *In vitro* clonal propagation of plants from shoot tips or nodal explants, usually with an accelerated proliferation of shoots during subcultures.

Morphogenesis: (a) The evolution of a structure from an undifferentiated to a differentiated state. (b) The process of growth and development of differentiated structures.

Organ culture: The maintenance or growth of organ primordia or the whole or parts of an organ *in vitro* in a way that may allow differentiation and preservation of the architecture and/or function.

Organized: Arranged into definite structures.

Organogenesis: A process of differentiation by which plant organs are formed *de novo* or from preexisting structures.

Passage/Subculture: The transfer or transplantation of cell, with or without dilution, from one culture vessel to another.

Plant tissue culture: The growth or maintenance of plant cell, tissue, organs, or whole plants *in vitro.*

Primary culture: A culture started from cells, tissues, or organs taken directly from organisms.

Protoplast: A cell from which the entire cell wall has been removed.

Regeneration: In plant cultures, a morphogenetic response to a stimulus that results in the production of organs, embryos, or whole plants.

Somaclonal variation: Phenotypic variation, either genetic or epigenetic in origin, displayed among somaclones.

Somaclone: Plants derived from any form of cell culture involving the use of somatic plant cells.

Somatic cell genetics: The study of genetic phenomena of somatic cells.

Somatic embryogenesis: The production of embryo-like structures from somatic cells, which develop and germinate into plantlets through the events that correspond with the zygotic occurrences.

Somatic organogenesis: The formation and outgrowth of shoots from callus or initiation and outgrowth of auxillary bud generated from cultured tips, and their subsequent adventitious rooting.

Suspension culture: A type of culture in which cells, or aggregates of cells, multiply while suspended in liquid medium.

Totipotency: A cell characteristic in which the potential for forming all the cell types in the adult organism is retained.

Transformation: The introduction and stable genomic integration of foreign DNA into a plant cell by any means, resulting in a genetic modification.

Type I callus: A type of adventive embryogenesis found with gramineous monocots, which has been induced on an explant in which the somatic embryos are arrested at the coleptilar or scutellar stage of embryogeny. The embryos are often fused together, especially at the coleorhizal end of the embryo axis. The tissue can be subcultured and maintains this morphology.

Type II callus: A type of adventive embryogenesis found with gramineous monocots, which has been induced on an explant in which the somatic embryos are arrested at the globular stage of embryogeny. The globular embryos often arise individually from a common base. The tissue can be subcultured and maintains this morphology.

Undifferentiated: With plant cells, existing in a state of cell development characterized by isodiametric cell shape, very little or no vacuole, a large nucleus, and exemplified by cells comprising an apical meristem or embryo.

Variant: A culture exhibiting a stable phenotypic change whether genetic or epigenetic in organ.

Vegetative propagation: Reproduction of plants using a non sexual process involving the culture of plant parts such as stem and leaf cuttings.

Virus-free: Free from specified viruses based on tests designed to detect the presence of the organisms in question.

Source: SIGMA Cell Culture, Catalogue and Price List, (1993).

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BIOLISTIC AND AGROBACTERIUM-MEDIATED PLANT TRANSFORMATION

(PROTOCOLS)

CIMMYT Applied Genetic Engineering Laboratory

Introduction

With the rapid development of biotechnology, agriculture has moved from a resource-based to a science-based industry. Plant breeding has been dramatically advanced through the introduction of genetic engineering techniques based on knowledge of gene structure and function. This has ushered agriculture into a new era in which it joins ranks with the most sophisticated of biological sciences in using molecular and cellular approaches to work on age-old challenges. Studies based on transgenic plants have focused on a range of topics including the development of new techniques for transformation, basic research on specific genes, and crop improvement aimed at a specific trait of interest.

The objective of this section of the protocols is threefold:
1. to introduce readers to the overall process of genetic transformation in plants;
2. to provide an overview of the tools used for gene transfer, giving particular attention to biolistic (particle bombardment) and *agrobacterium tumefaciens*-mediated gene transfer; and
3. to provide examples of practical applications of the respective technologies and noteworthy transgenic experiments and research.

Gene transfer (or DNA uptake) refers to the process that moves a specific piece of DNA (usually a foreign gene ligated to a bacterial plasmid) into cells. **Genetic transformation** refers to the stable integration of a foreign gene into the genome of a plant regenerated from DNA-treated protoplasts or intact cells. **Transgenic plants** are plants that carry the stably integrated foreign genes. **T0** plants are those regenerated from DNA-treated cells from gene transfer experiments; they are useful for analysis of tissue-specific or developmental stage-specific gene expression. **T1** plants are derived from seeds produced by T0 plants. Credible proof of T1 transgenic plants requires physical and phenotypic evidence of the inheritance of the foreign gene to the offspring, including data on genetic segregation of the gene.

Genetic transformation can be employed for both basic and applied research. Areas of particular interest in basic research using genetic transformation include the functional analysis of gene regulatory elements; the study of protein function; and the molecular biology of viruses and hormonally regulated genes. The applied use of this technology is for crop improvement. Improved tissue culture methodologies and gene transfer techniques will enhance the effectiveness and efficiency of transformation technologies.

The first step in genetic transformation is gene transfer, for which a number of techniques are available.

Microinjection: Multiple microinjections of cells of zygotic embryos are made into plant cells. The technique is based on the assumption that a few zygotic proembryos contain competent cells for transformation. However, researchers are observing that transgenic chimeras occur with the application of this technique.

Macroinjection: The entirety of the DNA from a donor is injected into the embryos of another (recipient) variety to obtain a large number of plants with morphological characteristics similar to those of the donor plant.

Electropuration: Intact tissue is subjected to short electrical pulses of high field strength. DNA uptake is facilitated by increasing the permeability of the protoplast membranes. Similar techniques that focus on protoplast membranes include gene transfer by sonication (a brief exposure to 20kHz ultrasound) and the use of chemical agents (PEG) to stimulate DNA uptake into the protoplasts.

Silicon carbide fibers (0.6 mm diameter x 10–80 mm long) coated with DNA have recently been used to deliver DNA to cells of maize and tobacco in suspension culture. Evidence that the fibers act as microinjection needles, puncturing the cell wall and delivering DNA into the cytoplasm or nucleus, was presented, but no further studies of rates of stable integration or regeneration to plants have been undertaken.

Access by the pollen tube pathway: This technique is based on cutting off the stigma of a recipient floret and applying a drop of DNA solution to the cut end of the style. The method has proven difficult to reproduce and requires further improvement.

Dry embryo incubation: With this technique, donor DNA is taken up by dry plant tissue through membranes, whose physiochemical characteristics change while natural desiccation occurs.

Agrobacterium tumefaciens: Gene transfer to plant tissue using *Agrobacterium tumefaciens* has the unique advantage of transferring some of the bacterium's DNA into the nuclear DNA of the effected host plant. This activity is attributed to the functions encoded on its "Ti" (tumor-inducing) plasmid.

Biolistic gene transfer is explored in greater depth in the following pages.

Biolistic Gene Transfer

Biolistic gene transfer can be defined as the introduction of substances into intact cells and tissues through the use of high-velocity microprojectiles. The term "biolistic" (biological ballistics) was coined to describe the nature of the delivery of foreign DNA into living cells or tissue through "bombardment" with a biolistic device—a particle gun. The process allows scientists to transform many important crop species that have been difficult to transform using other methods. During the past few years it has been reported that foreign genes have been delivered into, and expressed, in both dicots and monocots, including economically important crop species such as maize and wheat.

The DNA microacceleration technology has three basic components:
1. a particle acceleration device;
2. metal particles associated with precipitated DNA; and
3. target plant tissues that are regeneration competent and accessible to particle penetration.

The biolistic process was first described by John Sanford and co-workers in 1987. The evolution of the technology began with the invention of a gunpowder-driven, dual-chambered device. After the apparatus was developed and successfully tested, Sanford and his collaborators began working with the DuPont corporation to make the Biolistics (TM) PDS 1000/He commercially available. The efficiency of biolistic gene transfer depends on both biological and physical factors.

Other types of devices that work on the same principle include: the **particle inflow gun** (PIG), which accelerates particles directly using a stream of low pressure helium; the **pneumatic particle gun** (PPG), a gene delivery system using a gas-pressure-driven apparatus; and an **air-gun** device that utilizes a chemical explosion from a modified 0.22 caliber rifle.

To date, the delivery efficiency into cells with all these devices is low compared to that obtained through other well-established transformation methods, however the biolistic process is still at an early stage of development and there are many biological and physical factors that require further study and improvement.

Microprojectile bombardment is one of the primary means for breaching cell walls and cell membranes, the principal barriers to DNA delivery. However, the exact mechanism for gene integration using microprojectile bombardment is still unknown. In plants, direct gene transfer leads to non-homologous integration into the chromosome, characterized by multiple copies and some degree of rearrangement. Other disadvantages cited in relation to the biolistic particle gun are the emergence of chimeric plants (which will need to be sorted out and stabilized), and a lack of control over the velocity of the bombardment, which often causes substantial damage to the target cells (thus limiting the number of stable transformants that can be obtained). There are, however, many advantages to using the biolistic particle gun. It's clean and safe; it distributes microcarriers more uniformly over target cells than some other methods; and when correctly calibrated, the gene gun is gentler to target cells, more consistent from bombardment to bombardment, and yields 4-300 fold more transformants in the species tested than other technologies. In addition, direct transformation of totipotent tissues should minimize subsequent genotype-independent somaclonal variation.

Successful transformations are contingent on a number of factors that affect the proportion of cells in the target area that are both regenerable and competent for integrating the introduced DNA at the time of bombardment. The factors include

- parameters for DNA delivery
- plasmids used for transformation
- biolistic target-plant material and explant
- culture media
- expression, integration, and inheritance of inserted gene
- accurate screening of transgenic plants using physical and molecular tools
- leaf bioassay

High-frequency of transfer is related to using the appropriate DNA load for bombardment into regenerable cells. An unsuitable load will cause excess damage and impede cell survival and regeneration. Efficient selection or screening for transformants, together with minimal frequencies of undesired genetic change, contributes to stable integration of foreign DNA. Major variations in gene transfer frequency may be caused by: inappropriate or inconsistent DNA precipitation technique; aggregation or settling of particles prior to DNA precipitation; altered precipitation kinetics attributable to changes in temperature, purity, concentration of DNA, or agent stocks; or aggregation of particles during the precipitation or prior to bombardment. Variations can also be caused when the DNA is stripped from the coated projectile particles by excessive sonication or other treatments intended to disperse projectiles that have clumped together.

PARAMETERS FOR DNA DELIVERY

The parameters and/or settings for the PDS-1000/He device affect the rate of stable transformation by determining both the number of particles that penetrate the cell and the degree of injury to the cell. The optimal helium pressure, distance between the microcarrier and rupture disk, macrocarrier travel distance, and microcarrier flight distance must be empirically tested for each cell type. Additionally, biological factors such as osmotic preconditioning of the cell, post bombardment handling practices, and gene promoters/constructs also significantly influence the rate of stable transformation. Tungsten particles, which are less expensive and more heterogeneous in size and shape than gold, are used as microcarriers for gunpowder-driven units. The disadvantages of tungsten are that it can catalytically degrade DNA over time, and it may be toxic to some cell types. In contrast, gold particles are rounder and more uniform in size than tungsten particles, and they are biologically inert so they do not degrade DNA or harm cells. One disadvantage of gold particles is that they tend to agglomerate irreversibly in aqueous solutions. DNA is precipitated onto microcarriers by the addition of $CaCl_2$ and spermidine.

PLASMIDS USED FOR TRANSFORMATION

Nuclear plant genes consist of distinct regions, each with different functions, involved in transcription and translation of mRNA. Starting with the 5' end there are a promoter region that is involved in the initiation of transcription, together with enhancer/silencer regions that confer regulation of expression; a transcriptional start or cap site; and the so-called CAAT and TATA boxes, which function in RNA polymerase II binding. Within the transcribed region, one or more untranslated or intron regions are present. The end of the translated region is determined by a stop codon and followed by a terminator at the 3' end with a polyadenylation signal. There are no special requirements for the form of DNA to be transferred by particle bombardment.

Transformation frequency increases when plasmid DNA is linearized or super coiled, but there are indications that large plasmids (> 10 kbp) may be more prone to fragmentation during particle

bombardment. For expression in plant cells, foreign genes need to have the appropriate promoter, 5' leader, and 3' terminator sequences to ensure efficient transcription, stability, and translation of mRNA. Plant viruses dependent on plant transcription and translation factors have been used as sources of regulatory elements. The most common of these is the promoter of the 35S RNA gene of the cauliflower mosaic virus (CaMV). This promoter is active in all tissue, but its activity varies between different cell types. A further development is the use of the promoter derived from the maize alcohol dehydrogenase 1 (Adh1) 5' franking sequence, which shows a level of expression in monocot cells equivalent to or higher than the CaMV 35S promoter.

Reporter genes are used in transformation for detecting or quantifying the efficiency of a gene transfer. They include the ß-glucoronidase (Gus) gene; luciferase (Luc), which encodes an organic substrate for light emission and is detected using X-ray film or a luminometer; green fluorescent protein (GFP) from the bioluminescent jellyfish *Aequorea victoria;* chloram phenicol acetyltransferase (Cat); and the anthocyianin gene (C1).

These reporter genes have been used extensively to analyze the function of promoters and other gene regulatory sequences. The availability of these reporter genes, which have sensitive, convenient and reliable enzymatic assays, has greatly increased the usefulness of transient assays. In fact the assay for *gus* activity is so sensitive that it can be used to visualize single cells that express *gus* using a histochemical assay. It has been widely used to determine the frequency, type, and distribution of cells expressing activity after the introduction of DNA by microprojectile bombardment. A disadvantage of this reporter gene for applications related to studies of gene expression is the high stability of the *gus* enzyme, which can persist for days or weeks after transcription.

Selection of transformed cells is a key factor in developing successful methods for genetic transformation. A **selectable marker gene** allows the preferential growth of transformed cells in the presence of the corresponding selective agent. Several factors affect the efficacy of chemicals used for selection. The selection agent must be toxic to plant cells, though not so toxic that products from the dying, non-transformed cells kill adjacent, transformed cells. The most effective toxins are those that either inhibit growth or slowly kill the non-transformed cells. Optimal selection pressure uses the lowest level of toxin needed to kill untransformed tissues.

Common selectable marker genes that test for stable integration with cereal transformations are

- *Antibiotics* that affect translational activities of the cells.
- The Neomycin phosphotransferase gene (nptII) from transposon Tn5, which is detoxified by phosphorylation, neomycin, kanamycin, and G418.
- The Hygromycin phosphotransferase gene (hpt) from *E.coli*., which governs resistance to hygromycin.

These marker genes are unsuitable for some monocotyledonous species whose growth is not significantly inhibited by the antibiotic.

Optimal selection systems using herbicides have been developed for many monocot species. The Phosphinothricin acetyltransferase gene (*bar*) isolated from *Streptomyces hygroscopicus* confers resistance to the herbicide phosphinothricin (PPT)—the active ingredient in bialaphos and Basta‰. Glyphosate is the active ingredient in the broad-range herbicide Roundup‰ (Monsanto), which inhibits the enzyme 5-enol-pyruvylshikimic acid 3-phosphate synthase. Modified versions of EPSPS isolated from *E.coli* or *Salmonella typhimurium* confer resistance to glyphosate among transformed plants.

Careful timing of selection pressure is critical in order to limit the number of non-transformed cells that survive through cross-protection by transformed cells.

BIOLISTIC TARGET

A biolistic target can be defined as any organelle, cell, or tissue into which a substance will be delivered via a high-velocity microprojectile. Rapidly dividing, embryogenic cells have proven to be more suitable for successful transformation than slow-growing, non-embryogenic tissues. All plant cell cultures are a heterogeneous mixture of cell types, not all of which are totipotent. Plant cells differ in their capacity to respond to triggers, a phenomenon termed competence. A very small minority of cells in plant tissue is competent for both transformation and regeneration.

The relative composition of cell populations in tissues are determined by the genotype, the type of organ, the developmental state of the organ, and even the individual history of the experimental plant. The choice of genotypes, however, is the most important factor to consider for transformation.

There are two types of cells in any proliferating culture: meristematic, which are small cells with an isodiametric shape, dense cytoplasm, and large nuclei; and differentiated, which are large, variably shaped, and highly vacuolated cells with thin cytoplasm and inconspicuous nuclei. Immature or mature embryos have an especially high capacity for embryogenesis under controlled culture conditions. This is especially prevalent in the region of the epithelium, where the scutellum tissue is highly meristematic; it proliferates rapidly and forms somatic embryoids that produce large populations of phenotypically normal plants. Cells with large vacuoles may be more prone to damage by microprojectiles due to disruption of cellular compartmentation.

For cereal transformation, two types of tissues are commonly used.

1) Organized: apical meristems, somatic embryos, auxiliary buds, inflorescence meristems microspores, pollen, leaf tissues, and intact seedlings. These explants can be excised and regenerated to plants for all plant species with minimal time in tissue culture, but a high proportion of transformed regenerants are likely to be chimeric.

2) Non-organized: (un/dedifferentiated) callus-type I, type II, scutella, suspensions, and protoplasts. With these tissue types, direct production of uniformly transformed plants is possible, but extended periods in tissue culture under selection pressure leads to increased somaclonal variation and/or sterility.

CULTURE MEDIA FOR PLANT TRANSFORMATION

Components of media are

1. Inorganic macro- and micronutrients
2. A source of low nitrogen
3. An energy or carbon source
4. Vitamins
5. Growth regulators

Growth regulator concentrations and ratios have proven to be the most critical factor for culture initiation and morphogenesis. Other culture conditions include light intensity, light quality, photoperiod, and temperature.

SELECTIVE AGENTS

Selective agents differ in their toxicity to plants, which is influenced by the size and developmental state of the plant cells or tissue. Different species also have different sensitivities to selective agents. The selectable agent is usually applied early in the plant selection system to enhance elimination of the nontransgenic cells or allow better selection and continued culture of transgenic cells. Resistance to the selectable agent may be conferred by overproduction of the protein that is targeted by the selectable agent, production of a target protein that is resistant to the selectable agent, or production of a protein that detoxifies the selectable agent.

Antibiotics are one class of selectable agents. Many monocot species show high levels of endogenous resistance to numerous antibiotics. Kanamycin and hygromycin appear to be effective selective agents in selecting transformed cells within large multicellular clusters.

Herbicides are more toxic to plant tissue than antibiotics because of their specific mode-of-action in plant cells. Phosphinothricine (PPT) inhibits glutamine synthesis, causing a rapid accumulation of ammonia that leads to plant cell death. bialaphos is a tripeptide antibiotic consisting of PPT and two L-alanine residues. The growth of maize callus tissue can be fully inhibited by 5-7 mg/l bialaphos and PPT for tropical maize cells, 10 mg/l bialaphos and PPT for wheat cells.

Various methods for transformation have been developed, and this has allowed many of the world's important crop plants to be transformed. Recent advances in plant biology, particularly the identification of genes that control different traits, have opened up new possibilities for improving plants.

RESISTANCE TO INSECTS IN TRANSGENIC PLANTS

Insects and diseases inflict tremendous crop losses annually. It is estimated that US$3-5 billion annually is spent worldwide on chemical control of insect pests. The first transgenic insect-resistant tobacco and tomato plants were reported in 1987 and 1988. They used a native gene from *Bacillus thuringiensis*. subsp.*kurstaki*, which served as a lepidopteran-specific insect toxin. Field tests of tomato plants revealed a significant reduction in damage to tomato fruit by the tomato fruitworm. However, a problem soon became apparent: expression of *Bt* protein in transgenic plants was low. To solve the problem, scientists created a synthetic *Bt* gene, which resulted in a 500-fold increase in expression of the protein compared to the natural gene

Transgenic potato plants expressing high levels of a synthetic gene from *B. thuringiensis*. subsp. *tenebrionis* exhibited a strong resistance to the Colorado potato beetle (CPB). These potatoes have recently been approved for commercial release and are expected to significantly reduce the use of environmentally undesirable insecticides and the input cost incurred on farmers. The US Department of Agriculture (USDA) estimates that growers spend US$75–100 million annually on controlling CPB on about 480,000 ha of potatoes.

Transgenic cotton plants with an agronomically useful level of resistance to bollworm have been developed through the expression, at high levels, of a synthetic *Bt* gene. The varieties were approved for commercial release in 1995. Insect damage is a serious problem for cotton, with yield losses and insect control costs totaling US$645 million per year.

The European corn borer (ECB) is a major maize pest in North America and Europe, causing yield losses of 3-7% annually. Resistance to ECB has been introduced into corn through the use of a synthetic *Bt* gene.

NOTE: Transgenics will reduce the farmers' exposure to insecticides, and stem the build-up of chemical residues in the environment. The major concern regarding the use of Bt proteins for the control of insects in transgenic crops is the possible development of insect resistance to Bt toxins. Strategies to counter this include

- Use of high-dose expression of *Bt* genes;
- Use of host plants for vulnerable insects as refuges;
- Use of agronomic practices that minimize insect exposure to *Bt* and integrated pest management (IPM) approaches.
- Development of non-Bt proteins for the control of insect pests such as lectin, amylase, and protease inhibitors, which are insecticidal proteins of plant origin.

RESISTANCE TO DISEASES IN TRANSGENIC PLANTS

The first transgenic tobacco plants with increased resistance to tobacco mosaic virus (TMV) resulted from the expression of a coat protein (CP) gene. The first field trials of tomato plants exhibiting CP-MR (mediated resistance) were carried out in the USA in 1987. In 1995, Asgrow Seed Company released Freedom II, a variety of yellow squash that is resistant to zucchini yellow mosaic virus (ZYMV) and watermelon mosaic virus II. These two viruses routinely reduce crop yields by 20-80%. Potato, cucumber, and tomato varieties with CP-MR against a diversity of viruses have been commercialized and widely adopted.

Resistance to fungal pathogens in transgenic plants
The production of fungus-resistant crops with commercially useful levels of resistance has been rather limited. The enzymes that degrade the major constituents of the fungal cell wall (chitin and ß-1, 3-glucan) and ribosome inactivating proteins (RIP) *in vitro* have been extensively studied in plants. It has been demonstrated that the constitutive coexpression of chitinase and glucanase genes in tobacco plants confers higher levels of resistance genes to a fungal pathogen than either gene alone, indicating a synergistic interaction between the two enzymes in plants. The effectiveness of this approach was further demonstrated in tomato, in which the coexpression of the genes encoding these enzymes led to a potentially useful level of resistance to a *Fusarium* wilt disease.

Resistance to root-parasitic nematodes in transgenic plants
Nematodes are responsible for losses exceeding US$100 billion annually to world agriculture. Transgenic plant technology offers a specific response for controlling nematodes by employing promoters that direct a specific expression pattern for gene-encoding effector proteins that disrupt modification of the plant cell by the parasite, or act directly against the nematode

Transgenic plants as bioreactors
Transgenic plants can be used to produce proteins, carbohydrates, lipids, saccharides, and fatty acids of nonplant origin. They have the potential to be an economically important system for producing heterologous proteins.

Transgenic plants as vaccine production systems
The genes responsible for encoding antigens of bacterial and viral pathogens can be expressed in plants in a form that allows them to retain their native immunogenic properties. Transgenic potato tubers that expressed a bacterial antigen stimulated humoral and mucosal immune responses when provided as food.

GENES FOR TRANSFORMATION: RESEARCH AND RESULTS

Transgenic Rice
IRRI, Rice Res. Institute, Iran
"Stability of enhanced resistance to two stem borers in an aromatic rice containing a synthetic CryIA(b) gene."

The cryIA(b) gene was controlled by the promoter of the C4PEP carboxylase gene. Several independent transformants, one of which (#827) produced truncated (67kDa) CryIA(b) protein equivalent to about o.4% of total soluble leaf protein. Line #827 contained 3 copies of the cryIA(b) gene, which segregated as a single dominant Mendelian locus in the second (T1) and third (T2) generations and co-segregated with enhanced resistance to first instar larvae of striped stem borer (Chilo suppressalis) and yellow stem borer (Scirpophaga incertulas).

Transgenic Potato
Department of Plant Sciences, Israel; Max Planck Institute, Koln, Germany
"Multiple insertions of a synthetic cryIC in transgenic potato plants co-suppress expression."

The toxic portion of the *Bacillus thuringiensis* ∂-endotoxin cry IC was chemically synthesized to achieve its optimal transcription and translation in higher plants. All four transgenic plant species displayed 100% resistance to young (1st and 2nd instar) and mature (4th instar) larvae of Spodoptera spp. Transformation of potato plants resulted in a population of plants containing 1-10 copies of the synthetic cry ICS gene. The

CryIC protein produced in transgenic plants was found to be more insecticidal and less susceptible to *Spodoptera* larval midgut proteases than the generally used CryIC toxin of identical size produced in *E.coli.* These data suggest that the CryIC toxin is properly folded in plant cells, yielding high levels of insecticidal activity.

Brassica
University of Helsinki, Institute of Genetics and Selection, Moscow
"Toward insect resistant Brassica plants by expressing insecticidal *Cry*IG (Cry 9a1)- endotoxin against *Pieris brassicae* and *Plutella xylostella*"

CryIG- endotoxin is produced by the soil bacteria *Bacillus thuringiensis* sp. *galleriae* and has insecticidal activity against potato tuber moth. CryIG proteins, native and truncated forms of CryIG against *Pieris brassicae* and *Plutella xylostella*. Resynthesis of CryIG for high-level expression in transgenic brassica plants is ongoing.

Transgenic Rice
CIRAD, France; IRTA, Spain
"Striped stem borer (Chilo suppressalis)-resistant Mediterranean rices through transfer of *Bacillus thuringiensis Cry*IA(c) and *Cry*IB synthetic genes"

A set of 26 To independent transformants, having integrated a plasmid bearing the *cry*IA(c) gene driven by the Emu promoter, were tested. One transformation event was found to exhibit clear toxicity against striped stem borer (SSB). Western blot analyses detected 0.2% of total soluble protein. A second set of 25 independent transformants, having integrated a plasmid consisting of a maize ubiquitin promoter and the *cry*IB gene, after only one event was fully protected against SSB damage. Western blot analyses detected 0.25% of total soluble protein.

Rice Transformation
University of Ottawa, Canada; John Innes Centre, U.K.
The Bt insecticidal gene *Cry* IIa produces a delta endotoxin crystal protein that is active against both *Lepidoptera* and *Dipteran* insects.

Transgenic Cabbage
National Institute of Agricultural Science, Korea.
"Transformation of Chinese Cabbage with *Bacillus thuringiensis* seroval. kurstarki, *cry*IIa gene to control the diamond-back moth (*Plutella xylostella* L.)"

Transgenic Potato
Plant Genetics Engineering Laboratory, U.S.A.
"Insecticidal properties of two proteins derived from a *cry*3A gene modified in domain II to resemble cry3B2"

A Coleoptera-specific *Bacillus thuringiensis* insecticidal crystal protein gene (*cry*3A) was synthesized to achieve high concentrations of insecticidal protein in plants to specifically control Colorado potato beetle (CPB). A closely related protein (*cry*3B2) also appears to be effective against *Coleoptera* with specificity for corn root worms (Diabrotica sp.). Both synthetic genes were inserted into a plant transformation expression vector and cry3AB2 has been expressed in tobacco, potatoes, and *E.coli*.

Transgenic Potato
New Zealand
"Magainin II—an antibacterial peptide isolated from African clawed frog (*Xenopus laevis*)"

The peptide has broad range activity against both bacteria and fungi and has been shown to inhibit the *in vitro* growth of *Erwinia carotovora*, the causative agent of soft rot in potato. The chimeric gene included the CAMV 35S promoter; the 5'leader of AMV; the signal peptide from the tobacco PR-S gene, to target export of the protein to the intercellular space; the magainin II coding region and the 3' polyadenylation signal from a nopaline synthase gene. The construct has been ligated proximal to the right border of the binary vector pBINPLUS for *Agrobacterium*-mediated transformation, and 41 independently selected lines of potato cultivar were regenerated into plants.

Transgenic Wheat
ETH, Switzerland

Wheat (Bobwhite) was transformed with constructs designed for constitutive and coupled expression of a ß-1,3-glucanase and a chitinase from barley, in which these proteins normally are expressed in the seeds. Stable inheritance and expression of chitinase and the selectable markers could so far be followed up to the T_4 generation. Presence of ß-1,3-glucanase was not detectable, even though the gene resided on the same DNA fragment as the chitinase gene. In the best case, chitinase was expressed at the level comparable to that in barley seeds. The respective plants are being tested for elevated resistance against infection by various wheat fungi.

Transgenic Rice
IRRI, Kansas State University
"Expression of chitinase and thaumatin like protein gene in transgenic rice for sheath blight resistance"

Rice sheath disease is caused by the borne fungus, *Rhizoctonia solani.* Different chitinase genes were cloned from different sources—Chi11 (rice), RC7 (rice), serpin, MSc (*Menduca septa*)—and the thaumatin-like protein gene, TLP-D-34 was obtained from rice. The presence of chimeric genes in T0 and T1 transgenic rice plants were detected by Southern blot and the leaf protein from the T0 and T1 for the presence of chitinase and TLP-D34 by western blotting.

Transgenic Strawberry, Sour cherry
Puschino, Russia
"Transgenic strawberry (*Fragaria ananassa*) and Sour cherry (*Cerasus vulgaris*) plants with antifreeze protein from Arctic fish"

One of the most important selection problems is developing cultivars with resistance to different abiotic stresses, such as a tolerance for early spring frosts. The expression of antifreeze protein genes from Arctic fish (AFP) in plants is one possible response. The mechanism under study is one that could delay the growth of ice crystals within tissue. Transgenic plants of strawberry and sour cherry were obtained, and the integration of HPT, NPT, and AFT genes in plant genomes was confirmed by PCR analysis. However, additional analysis failed to detect any phenotypic characteristics of the antifreeze gene.

Transgenic Potato
Molecular Biology Institute, Iran
"Enhanced immunogenecity of transgenic plant-derived hepatitis B middle surface antigenen"

Genetically engineering potato plants have been used for exorcission of several therapeutic proteins and peptides: major (S) and middle (M) HBV surface antigene, rabies G protein, human epidermal growth factor (hEGF), and human calcitonin. The expression level of the introduced genes varied from 20 pg up to 90 ng per mg of soluble protein, depending on the gene construction and plant tissue. This finding may afford new opportunities for development of oral immunization techniques against hepatitis B infection.

Fortifying Rice withIron
ETH, Switzerland

Iron is the most common micronutrient deficiency in the human diet, affecting an estimated 1–2 billion people. Ailments resulting from the deficiency include anemia, impaired learning ability in children, low birthweight babies with increased rate of mortality, increased susceptibility to infection, and reduced work capacity. Scientists produced rice plants with grains containing three times more iron than normal rice using the gene for ferritin, an iron-rich soybean storage protein, under the control of an endosperm-specific promoter or the ferritin gene from beans.

Transgenic Tobacco
Central Research Institute of the Electric Power Industry, Japan
"Introduction of soybean ferritin for adaptation to soils of wide-ranging iron concentration"

Ferritin is known as an iron-containing protein. Iron in ferritin is non-toxic and readily available. If the amount of ferritin in plants can be increased through gene manipulation, plants that are highly adaptable to soils with wide ranges of iron concentration could be bred. The cDNA of soybean ferritin was transferred into tobacco. The gene expression was confirmed in all transformants (T0) by western blot analysis. The highest iron content in a transgenic was as much as 1.3-fold compared to the control. The T1 generation plants grew on the excess-iron medium, where non-transformed failed to grow during several weeks of germination.

Transgenic Chickpea and Tobacco
Center for Plant Molecular Biology, India
"Overproduction of essential aminoacids in chick pea and tobacco"

Chickpea is an important grain legume and one of the most valuable sources of proteins in the tropical countries. It is well known that legumes are deficient in the lysine sulfur-containing amino acids—methionine and cysteine. This study demonstrated increased free amino acids content in the leaves of transformed chickpea and tobacco plants using constitutively expressed genes involved in amino acid biosynthesis such as threonine deaminase (TD). TD was cloned from immature seeds of chickpea. Southern hybridizations further supported stable integration of transgenes. Analysis of free amino acids content revealed a several-fold increase in levels of threonine, methionine, and lysine.

Durum Wheat
University della Tuscia, Italy; USDA, Albany, Georgia, USA
"Gene and amino acid sequences of low molecular weight glutenin subunit associated with quality"

Low molecular-weight glutenin subunits (LMW-GS) are the most abundant components of glutenin polymers, the size distribution of which is strongly correlated with gluten technological properties. The 42K-LMW-GS has been purified from a bread wheat cultivar and transformed in Secora roja.

Transgenic Potato

National. Laboratory of Protein Engineering and Plant Genetics Engineering, China

"Field testing of potato transformed with the 15 KD Zein Gene"

The gene encoding 15KD zein (a sulfur rich protein) was placed under the control of the patatin promoter and transferred into potato by *Agrobacterium* mediated procedures. Two lines showed high levels of zein gene expression, resulting in a 3-5 times increase of sulfur containing amino acids and a 10% increase of essential amino acids content in tubers.

Tropical Plants

Taiwan University, Taiwan

"Towards biopolymer production in tropical plants"

Most of plastics produced and used today are resistant to chemical and physical degradation. This presents disposal problems when their usefulness ceases. Development of transgenic tropical plants for the production of biodegradable plastics should lower the cost of production to an economically competitive level. Transformation efforts on tropical plants with three genes taken from *Acaligenes eutrophis*, a bacterium, are underway.

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Agrobacterium tumefaciens-mediated Transformation

Given adequate resources, particle bombardment represents the optimal method for transforming and engineering specific plant species, according to recent comparative studies. The caveat, in this instance, is that not all laboratories or organizations have the aforementioned "adequate resources." The problem then becomes one of technology transfer between well-funded labs with access to these tools and the large number of labs, particularly in developing countries, which do not have access to such technology.

A strong option for less well-endowed labs is the use of *Agrobacterium tumefaciens* technology. Early gene transfer experiments using *Agrobacterium tumefaciens* demonstrated that it is a very effective vehicle for introducing foreign genes into many important crops. There are significant advantages to using the *Agrobacterium*-mediated method of gene transfer, including:

- relatively high frequencies of transformation;
- transfer of relatively large segments of DNA with little rearrangement;
- integration of low numbers of gene copies into plant chromosomes; and
- lower cost than the biolistic transformation system.

PROCESS AND MECHANISMS

Agrobacterium is a natural genetic engineer—a common lab tool for many plant molecular biologists—because it has the unique capacity to transfer a piece of its own DNA, referred to as **T-DNA**, into the nuclear genome of plant cells. When this bacterium infects a plant, it transfers its Ti plasmid to the host cells, whereupon the T-DNA integrates into the plant DNA, causing the abnormal proliferation of plant cells that gives rise to a crown gall. The resultant transformed plant cells produce novel sugar and amino acid conjugates, termed opines, which can be used by the inciting bacteria. Different *Agrobacterium* strains carry different types of Ti plasmids, which can be categorized by the type of opine produced by the transformed plant cells and metabolized by the bacteria.

There are two important genetic components on a Ti plasmid: T-DNA and the Ti plasmid virulence (*vir*) region. T-DNA is copied and transferred to the plant cell. It is defined and delimited by two 25-bp direct repeats at the ends of the T-DNA borders. Any DNA, and only DNA, between these borders is transferred to the plant cell. The second component, the *vir* region, provides most of the transacting products for T-DNA transit. These genes do not enter the plant cell, but must be expressed in the bacterium for T-DNA transfer to occur. The *vir* region is 30 kbp and is organized into seven complementation groups: *virA, virB, vir C, virD, virE, virG,* and *virH.* The *virA* and *virG* loci encode a positive regulatory system that directs *vir* gene expression. The *virA* product specifies an inner membrane protein that recognizes and responds to the presence of plant phenolic compounds and transduces this information to the product of *virG. VirG* then acts as a transcriptional activator of itself and the other *vir* loci. The products of the *vir* C and *vir* D loci are involved in the generation and processing of the T-DNA copy, and the product of the *virB* and *virE* loci are involved in forming most of the structural components that facilitate T-DNA movement. The *vir* H enzymes may allow the bacteria to survive in the presence of bacteriocidal or bacteriostatic plant compounds during the infection process.

The infection process is a complex series of events. Its temporal sequence is defined by cellular activities of the interacting partners; activities include: bacteria colonization and attachment to cells at or near wound sites; the plant wound response; the mobilization of bacterial DNA into plant cells; the integration of the T-DNA into plant DNA, and its expression. One of the earliest stages in the interaction between *Agrobacterium* and plants is the attachment of the bacteria to the plant cell surface. The initial attachment of single bacterial

cells, often in a polar fashion, is followed by massive aggregation of the bacteria at the plant cell surface. These aggregates result from the formation of cellulose fibrils by the bacteria. *A. tumefaciens* attachment presumably involves the interaction of one or more of its surface molecules with the plant cell wall. Three genetic loci have been defined as having roles in *A. tumefaciens* attachment to plant cells: *chv*A, *chv*B, and *psc*A. They are all encoded by the chromosome and are physically linked. Early experiments with *Agrobacterium* indicated that plant wounding is required for tumor development. Expression of the specific set of bacterial genes that are required for tumor induction is induced by compounds produced in wounded plant tissues in a complex series of metabolic and cellular activities. Further analysis has shown that the molecules responsible for *vir* induction, acetosyringone, and related phenolic compounds, are present at very low levels in uninjured plants, but are significantly more abundant in wounded tissues.

There is evidence that there is a "window of competence," a time during which the cells are susceptible to *Agrobacterium* transformation. This window occurs between 24 and 120 hours after the wounding event. Histological analysis of wound tissues indicates that a burst of cell division is initiated and ceases during this time, unless virulent agrobacteria are present. The correlation of cell division with maximal transformation suggests that cell division and/or DNA synthesis may be important to the process.

There is supporting evidence that T-DNA is excised from Ti plasmids following *vir* region induction. This process involves the action of *vir* gene products and the cis-acting border sequence elements. Soon after *vir* induction, processing of the T-DNA begins and a variety of intermediates are produced. The mechanism responsible for release of the T-strand from the Ti plasmid is not yet known. There is evidence of the formation of linear single-stranded DNA molecules, termed T-strands, which correspond to the bottom strand of the T-DNA. T-strands are formed when the bottom strand of T-DNA is nicked at both the right and left borders, followed by release of the bottom strand. Another explanation is that T-DNA transfer to plants may occur by a mechanism analogous to bacterial conjugation.

The process of T-DNA integration is the least understood aspect of *Agrobacterium*-mediated transformation; very little is known about either its specific sites or the mechanism(s). The primary site of T-DNA insertion is the nuclear genome of plant cells. Many investigators suggest that T-DNA integration is a multiple-step process of recombination accompanied by local replicative and repair reactions. They propose the following general model:

1) The T-strand is transferred to the plant cell as a protein-DNA complex.
2) The right border and accompanying protein interact with a nick in the plant DNA.
3) Local torsional strain on the plant DNA produces the second nick on the opposite strand at varying distances from the first nick.
4) The T-strand is ligated to the plant DNA and the homologous strand replicates.
5) Replication and repair of the staggered nick in the plant target DNA results in both the production of a repeated sequence and additional sequence rearrangements at ends of the inserted T-DNA element. Once the T-DNA is integrated into plant DNA, its expression is required.

The application of the *Agrobacterium*-mediated method of gene transfer has until now been limited to dicotyledonous plants. It has been suggested that no T-DNA transfer occurs in monocots either because the bacteria cannot attach to their cell walls or because of an abnormal auxin-cytokinin balance in monocot cells.

There are two basic reasons why an *Agrobacterium* isolate could fail to incite a tumor on a particular plant. First, the T-DNA might not be transferred to a given host because of poor attachment, lack of *vir* region induction, defective *vir* genes, or inefficient T-DNA transfer, integration, and/or expression of the genes.

Second, there is a rough correlation between the capacity of a strain to attach to plant cells and its ability to initiate infection. In many monocots, the cells around the wound site differentiate into lignified sclerified cells without apparent cell division, which relates to differences in physiological and growth characteristics of dicot and monocot cells. In the other cases, T-DNA might be delivered to monocot cells, but the recipient cells either cannot integrate the T-DNA (owing to the lack of wound divisions), are incapable of proliferating, or the transfer of T-DNA does not result in an expansion of the transformed population of cells.

AGROBACTERIUM-MEDIATED TRANSFORMATION FOR MAIZE AND WHEAT

Transformation of plants in Gramineae using *Agrobacterium*-mediated infection has been recently attempted in several laboratories. The choice of starting materials, tissue culture conditions, bacterial strains, and vectors is essential for efficient gene transfer. Several factors contribute to the competent state of a given plant cell, including

- Production of phenolic compounds inducing excision and processing of the T-DNA-protein complex.
- Targeting of the T-DNA-protein complex into the plant cell nucleus.
- Integration into the plant genome.
- Stable expression of the introduced gene.

It is now widely accepted that the most suitable explants for transformation are those that require the least amount of time in tissue culture before and after the transformation step. A key factor in *Agrobacterium* transformation for monocotyledons is the use of embryonic or meristematic tissues that have the following attributes:

- *vir*-inducting substances are produced by embryonic tissue;
- low production of bacteriotoxic substances;
- favorable endogenous hormone levels;
- availability of receptors for attachment of *Agrobacterium*; and
- actively dividing cells with host DNA synthesis.

The activation of *vir* genes in monocots can be obtained by adding phenolic factors to the culture media, thus activating the expression of the *vir* genes on the Ti plasmid. Monocot plants may also produce these compounds from meristematic, undifferentiated cells. Preferred explants for transformation include immature embryos or seed-derived callus (for most cereals); proliferative shoot cultures; and embryo axes (derived from mature or immature seed) for direct meristem transformation. Transformation methods are not limited to specific genotypes of a plant species.

To achieve effective transfer of genetic material into a host, appropriate genetic constructs need to be made to facilitate integration and expression of the foreign gene(s). A typical genetic construct will include a promoter, a coding sequence (transgene), and a terminating signal. Transgenes may be introduced into plant tissues on separate plasmids or on cointegrative vectors where multiple genes, including selectable or screenable markers, are in the same plasmid.

Commonly used promoters for plant transformation include: the cauliflower mosaic virus (CaMV) 35S promoter, a constitutive promoter suitable for driving the expression of foreign genes in dicotyledons; and the maize ubiquitin promoter, also a constitutive promoter that drives strong expression of transgenes in monocotyledons. Organ/tissue specific promoters are also available to drive expression of transgenes in particular parts of a plant. Specific examples include: the vicilin and phytohemaglutinin promoters, derived

from pea and bean, respectively, suitable for seed-specific expression; the high molecular weight glutetin promoter from wheat, also suitable for seed specific expression; and the alfa-amylase promoter, for driving expression in the aleurone grains. Gene promoters such as Actin, Ubiquitin, and alfa-Amylase may be much more effective than the 35S promoter. Different vectors may be used for *Agrobacterium* transformation: co-integrative vectors, which offer plasmid stability in *Agrobacterium,* and binary vectors, extensively used because of their ease of manipulation. Commonly used screenable markers include the ß-glucoronidase and luciferase genes, and more recently, the green fluorescent protein from jellyfish. The most common selectable marker genes encode proteins that detoxify metabolic inhibitors such as antibiotics and herbicides.

Recently, a number of studies indicate that *Agrobacterium*-mediated transformation may lead to two potential problems, either of which needs to be addressed prior to the release of any genetically engineered plant into the environment. The first problem is that molecular analyses of genomic DNA from engineered plants indicates the presence of vector sequences outside the transferred DNA (T-DNA) borders. The second problem is that *Agrobacterium* was found to persist on the surface and within tissues of soil-grown transformed plants up to 12 months following transformation.

Although transformation technology now exists for many species, it remains labor intensive. In addition, the procedures involved tend to be cumbersome, time-consuming, and inefficient. It is certain that improved gene transfer technologies will continue to emerge. Although such technologies may offer alternatives and advantages over existing transformation methods, it is likely that no single gene transfer method will prove suitable for genetic engineering of all plant species.

HISTORICAL REFERENCES FOR TRANSFORMATION OF MAIZE MEDIATED BY *AGROBACTERIUM*

Gould, et al. 1991. Inoculated shoot apices of maize and obtained a few transgenic plants. *Plant Physiol*. 95: 426-434.

Grimsley, et al. 1987. Studied cDNA of maize streak virus and determined how it was delivered to maize plants and how plants became systematically infected. *Nature* 325: 177-179.

Ishida, et al. 1996. Obtained high efficiency transformation (5% and 30%) of A188 maize inbred. *Nature Biotechnology* 14, June: 745-750.

Shen, et al. 1993. Delivered GUS gene to maize shoots. *Proc. Natl. Acad. Sci.* USA 90: 1488-1492.

Zhao, Z., et al. 1997. Conducted a study comparing *Agrobacterium*-mediated transformation with bombardment transformation in maize.(*Book of Abstracts 5th Intern. Congr. of Plant Molecular Biol*. September, 1997, Singapore. Pp. 21-217.

Zhao, Z., et al. at Pioneer Hi-Bred International compared the data generated from Agrobacterium-mediated transgenic T0 plants with the data generated from transformed T0 plants produced by bombardment in HiII genotype. (Results below)

Bombardment

Efficiency of transformation: 10%

- Multievents index: 1.15
- Low simple/low copy number/insertion pattern: 8%
- One desirable event /100 embryos

***Agrobacterium* -mediated Transformation**

- Efficiency of transformation: 39.8%
- Multievents index: 2.5
- Low simple/low copy number/insertion pattern: 58%
- 62 desirable events /100 embryos

HISTORICAL REFERENCES FOR THE TRANSFORMATION OF WHEAT MEDIATED BY *AGROBACTERIUM*

Ming Cheng et al. (1997) developed a rapid *Agrobacterium tumefacients* transformation system for wheat using freshly isolated immature embryos, precultured immature embryos, and embryogenic calli as explants. The explants were inoculated with a disarmed A tumefaciens strain C58 (ABI) harboring the binary vector pMON18365 containing the beta-glucoronidase gene with an intron, and a selectable marker, the neomycin phosphotransferase II gene. The transfer process can be divided into plant cell and bacterial steps: *Plant Physiol.* 115: 971-980.

Genetically Engineered Major Crop Plants

Species	Transformation method	Field trials
Banana	Bombardment/*Agrobacterium*	
Barley	Bombardment	Virus resistance
Bean	Bombardment	
Canola	Bombardment/*Agrobacterium*	Herbicide tolerance; pollination
Cassava	Bombardment/*Agrobacterium*	
Maize	Bombardment/*Agrobacterium*	Insect resistance; herbicide tolerance
Cotton	Bombardment/*Agrobacterium*	Insect resistance; herbicide tolerance
Papaya	Bombardment/*Agrobacterium*	Virus resistance
Peanut	Bombardment/*Agrobacterium*	Virus resistance
Poplar	Bombardment/*Agrobacterium*	Herbicide tolerance
Potato	*Agrobacterium*	Insect resistance; virus resistance; herbicide tolerance
Rice	Bombardment/*Agrobacterium*	Herbicide tolerance
Soybean	Bombardment/*Agrobacterium*	Herbicide tolerance
Squash	Bombardment/*Agrobacterium*	Virus resistance
Sugarbeet	*Agrobacterium*	
Sugarcane	Bombardment/*Agrobacterium*	
Sunflower	Bombardment/*Agrobacterium*	
Tomato	*Agrobacterium*	Delayed ripening, Virus resistance
Wheat	Bombardment/*Agrobacterium*	

(Table from P.Christou; *Trends in Plant Science*, December 1996, vol. 1, No 12)

(The references used for this section are A.N. Binns. 1988. *Ann. Rev. Microbiol*. 42: 575-606; P. Zambryski. 1992. *Annual. Rev. Plant Physiol. Plant Mol. Biol.* 1992. 43: 465-490; P.Christou. 1996, *Trends in Plant Science* 1 (12): 423-431.

BIOLISTIC TRANSFORMATION: PROTOCOLS

GENE TRANSFER TO IMMATURE EMBRYOS

PLANT MATERIAL FOR MAIZE TRANSFORMATION

Immature embryos of CIMMYT inbred maize lines and the hybrids between these lines were the source of immature embryos used for starting *in vitro* cultures and for microprojectile bombardment experiments.

Immature embryos are excised from seeds harvested 15-20 days after self-pollination. Whole ears are surface-sterilized with 70% ethanol for 1 min., followed by 20% Clorox containing 10 drops/L of polyoxyethylene sorbitan monooleate (Tween-80), for 30 min. and rinsed 3 times with sterile distilled water.

PLANT MATERIAL FOR WHEAT TRANSFORMATION

CIMMYT purified bread wheats are used for *in vitro* culture and transformation experiments. The genotypes are purified by controlled selfing for two generations with selections made for uniform progeny.

Use only healthy looking plant material for explantation.

1. Obtain green seeds harvested 15-20 days (depending on genotype) after pollination in which the endosperm is still relatively liquid (medium-late milk to early dough stage). Remove green seeds from the seed head and surface sterilize by immersion in 70% ethanol for 40 seconds.
2. Prepare 20% Clorox solution (3% NaCl). Add 100 ml Clorox to a 500 ml graduated cylinder plus 10 drops of Tween 80 and fill to 500 ml with deionized water. Pour this solution into a 1 L beaker and stir with a stir bar.
3. Under a transfer hood, place the seeds into the Clorox solution and put the solution on the stir plate for sterilization for 30 min. Pour the solution through a tea-strainer to save the seeds and rinse them in 600 ml of the sterilized deionized water. Place on the stir plate for a 5 min rinse. Drain the water and add 600 ml sterilized, deionized water to rinse the seeds (3 times).

EXCISING IMMATURE EMBRYOS FROM MAIZE

1. Place a dissecting scope under the transfer hood and one petri dish on the scope.
2. Hold the ear in the petri dish and spear the end of the ear on the silk scar side of the kernel with a dissecting needle to anchor the ear while isolating the embryo.
3. Cut off the tops of the kernels with a sharp scalpel blade while the kernels are still on the cobs.
4. Dig the endosperm and embryo out of the kernel cup with a spatula. Sometimes the embryo will come out with the endosperm, other times embryos will stay in the kernel.

NOTE: If the ear is young and the embryos are less than 1 mm long, they may be the same transparent color as the endosperm and therefore difficult to find. Usually in transformation experiments, immature maize embryos 1.0–1.5 mm long are isolated and then cultured with the scutellar side facing upward.

5. Place the embryo on the prepared media (N6C1) with the scutellar side up and the embryo axis side in contact with the media. Up to 20 embryos can be placed on a petri dish with 10 ml N6C1 for callus initiation. Wrap the dishes with parafilm and incubate at 28°C in darkness for 4–7 days. Dishes may be placed in an unlit growth cabinet or in a light-tight box in a growth culture room.

EXCISING IMMATURE EMBRYOS FROM WHEAT

1. Place a dissecting scope under the transfer hood and place one petri dish on the scope.
2. Hold the seeds in the petri dish to isolate the embryo.
3. Cut the seeds with a scalpel. Dig the endosperm and embryo out of the cup with a spatula.
4. Place the embryo on the prepared media with the scutellar side up and the embryo axis side in contact with the media.
5. Place 50 embryos in each of the 12 cm petri dishes prepared with 20 ml MSE3 media.
6. Wrap the dishes with parafilm and incubate at 25°C in darkness in a growth culture room for 4–14 days. The primary growth region of the cultured embryos is the scutellum where cells proliferate in 4 days and grow rapidly on MSE3 or MSE5 medium, depending on the genotypes.

GENE DELIVERY PROCEDURES

For bombardment of 20 petri dishes with rings of about 50 embryos, we use the following procedure:

1. Take the solution of size 1 µm gold particles (40 mg gold particles suspended in 1 ml distilled water using a procedure from Bio-Red instruction manual) stored at 4°C and sonicate well to ensure complete mixing and suspension of particles.
2. Prewashed 50 µ aliquots of gold particles are coated with 5 µ plasmid DNA (1 µg/1µ stored at -20°C) or 2.5 µ DNA from two different plasmids. Keep them on the ice.
3. Mix 20 µ of 0.1M spermidine (stored at 4°C) and 50 µ of 2.5M $CaCl_2$ (stored at 4°C) in a centrifuge tube (1.5 ml) and vortex to speed to maximize mixing.
4. Add this solution to the DNA/particles mixture. Vortex the mixture on the vortex shaker with a multiple-platform head for 3 min. at room temperature. Centrifuge the solution in a microfuge for 1 min. at 13,000 rpm g to spin down particles.
5. Remove and discard supernatant, being careful to leave all particles behind.
6. Wash the DNA/particles by adding 250 µ of 75% ethanol, and vortex to make sure all particles are resuspended and washed.
7. Centrifuge the mixture for 1 min. to spin down particles.
8. Carefully remove and discard the supernatant.
9. Resuspend the pellets in 240 µ absolute ethanol using the vortex and check to be sure that all particles are resuspended.
10. Position the macrocarrier in the macrocarrier holder. The outside edge of the macrocarrier should be securely inserted under the lip of the macrocarrier holder.
11. Using an automatic pipette, remove 5 µ from this suspension and spread 3-5 µ of the aliquot onto the center of the inner circle of each macrocarrier disk.
12. Air-dry for about 2 minutes to allow the ethanol to evaporate. The macrocarriers are now ready for bombardment. To obtain the best results, use the prepared macrocarriers as soon as possible.
13. Bombard target plates (embryos pre-treated on N6C1/E3 media containing 12% maltose) with one shot each using the following gene delivery parameters: position the target material approximately 8 cm below the microprojectile stopping plate (3rd shelf from bottom microcarrier assembly); bombard all target materials twice, using particle densities of 30 µg per shot; use rupture discs with a burst pressure of 1,100 psi.
14. Observe target plates after one day and transfer bombarded materials to the fresh media. The number of transiently expressing cells on the embryos in each ring will show the effect of target tissue placement on gene delivery.

OPERATING PROCEDURE FOR GENE GUN PDS-1000/HE
Loading the rupture disk

1. Unscrew the rupture disk retaining cap from the gas acceleration tube.
2. Place the rupture disk of desired burst pressure in the recess of the rupture disk retaining cap and screw the cap onto the gas acceleration tube using a counter-clockwise motion; hand-tighten the cap. If the retaining cap is not tightened sufficiently, the rupture disk may slip out of place before it ruptures.

Loading the microcarrier launch assembly

1. Remove the macrocarrier launch assembly from the sample chamber.
2. Unscrew the macrocarrier cover lid from the assembly.
3. Place a sterile stopping screen on the stopping screen support**. Never operate the PDS-1000/He without a stopping screen in place**. If you do, your sample will be destroyed.
4. Install the microcarrier holder with macrocarrier on the top rim of the fixed nest. The microcarriers should face down towards the stopping screen.
5. Place the microcarrier cover lid on the assembly and turn clockwise until snug; it doesn't have to be tight.
6. Place the microcarrier launch assembly in the second slot from the top in the sample chamber.

Positioning the sample

1. Put the petri dish containing the sample on the petri dish holder. Place the petri dish holder at the desired level inside the sample chamber. The target material is positioned approximately 8 cm below the microprojectile stopping plate (3rd shelf from bottom microcarrier assembly).
2. Close and latch the sample chamber door.

Firing the PDS-1000/He

1. Turn the PDS-1000/He power to **ON.**
2. Turn the vacuum pump to **ON**. To start, use a vacuum of 26–30 inches of mercury or, in the highlands of Mexico, 16–19 inches of mercury (the altitude affects the pressure).
3. Put the vacuum switch in the HOLD position.
4. Press and hold the FIRE switch to allow pressure to build in the acceleration tube. It should take about 12–15 seconds to fill to burst pressure.
5. Release the FIRE switch immediately after the disk ruptures.
6. Release the vacuum in the sample chamber by setting the vacuum switch to the **VENT** position.
7. After the vacuum is released, open the sample chamber door. Remove the petri dish and treat as appropriate.
8. Remove the microcarrier launch assembly. Discard the microcarrier and stopping screen from the microcarrier launch assembly.
9. Unscrew the rupture disk retaining cap from the gas acceleration tube. Remove the remains of the rupture disk.
10. Clean the gun by spraying with ethanol.

Shutting down the PDS-1000/He

1. If the PDS-1000/He is not going to be used again, close the main valve on the helium cylinder and release the helium pressure. Turn the power OFF.
2. Observe target plates after 1 day and transfer bombarded materials to fresh media. The number of transiently expressing cells on the embryos in each ring will show the effect of target tissue placement on gene delivery.

SELECTION OF TRANSFORMED MAIZE EMBRYOS/CALLI AND RECOVERY OF TRANSGENIC PLANTS

- Selection of transformed cells is achieved using either phosphinothricine (PPT) or bialaphos (B) (Meiji Seika Kaisha, Japan).
- After bombardment immature embryos or calli are transferred individually to the N6C1 medium (see tissue culture protocol), or to the first selection medium containing 1 mg/l PPT/bialaphos, and cultured for 7 days in darkness.
- Calli that show visible signs of growth should be cut into small 2 cm parts and transferred onto the second selection medium—which is the same as the callus induction medium N6C1 containing 5 mg/l PPT/bialaphos—for 3 weeks.
- Calli that fail to demonstrate growth after 10 days should be discarded.
- Calli that grow should be transferred onto N6C1B10 containing 10 mg/l PPT/bialaphos. Keep this material for 3 weeks.
- The duration of the selection process is about 50–75 days on the respective medium with 5 and 10 mg/l PPT/bialaphos, depending of the genotype used.
- Keep all culture at 28∞C under dark conditions in the growth culture chamber.
- Each putative transgenic calli will become a mass of embryogenic Type I callus growing in the presence of the bialaphos selection agent.
- A range of concentrations from 1 to 10 mg/L of PPT or bialaphos should be tested with nontransformed calli to get a more efficient selection system.
- Approximately 20 target plates should be prepared for each experiment. Two plates are controls, one grown on N6C1 to monitor health of the culture and the second grown on the same medium but containing 5-10 mg/L bialaphos to demonstrate response of untransformed cells to selection.

NOTE: Phosphinothricine inhibits glutamine synthesis, causing a rapid accumulation of ammonia that leads to plant cell death. Bialaphos is a tripeptide antibiotic, which consists of PPT and two L-alanine-residues. It is an effective selection agent for transgenic maize calli, however it does not always kill untransformed cells. In order to achieve optimum selection, subculture the bombarded cells at low density, about 10 immature embryos per plate.

Plant regeneration of maize plantlets and Basta™ testing

1. All the PPT/bialaphos-resistant callus tissue uniformly growing on the selection medium should be transferred to the regeneration medium at a temperature of 28°C under a photoperiod of 16 h fluorescent light. Plant regeneration from resistant calli is performed on the same medium used for regeneration of tropical maize (see Tissue Culture Protocol) except that 5 mg/L bialaphos is added to the media.
2. The somatic embryos capable of developing into green shoots within 2-4 weeks are characterized as putative transformants. The selected plantlets are transferred to another medium for root formation (MSE) (see Tissue Culture Protocol) but supplemented with 1 mg/L bialaphos to continue the selection.

The plantlets developed on this medium are transferred to controlled growth chambers and greenhouse conditions and grown for further analyses.

3. Plants are further selected by painting the fifth or sixth leaf near the tip of the youngest fully extended leaf with 2% Basta™ solution containing 0.1% Tween 20.
4. Each regenerated plant is tested for its response to Basta‰. The painted leaves of all control plants (non-transformed) will be completely bleached after one week.
5. One week after the first Basta‰ treatment, plants are painted again with a second concentration of 5% Basta‰ and assessed for damage 1 week after the herbicide application.

SELECTION OF TRANSFORMED WHEAT EMBRYOS/CALLI AND RECOVERY OF TRANSGENIC PLANTS.

1. Selection of transformed cells is achieved using either phosphinothricine (PPT) or bialaphos (B) (Meiji Seika Kaisha, Japan).
2. After bombardment immature embryos are transferred individually to the E3 medium. Maintain for 7–14 days.
3. Embryos are transferred onto MSCB5 medium containing 5 mg/l PPT/bialaphos for the first selection medium and cultured for 2 weeks in darkness.
4. Calli that fail to demonstrate growth after this period should be discarded.
5. Excise with scalpel the clusters of the cells that have proliferated on the MSCB5 medium and subculture and transfer them onto MSCB10 supplemented with 10 mg/L bialaphos/PPT. Maintain the material for 4 weeks.
6. The duration of the selection process is about 50–75 days on the respective medium with 5 and 10 mg/L PPT/bialaphos.
7. Keep all culture at 25∞C under dark conditions in the growth culture chamber.
8. Each putative transgenic calli will become a mass of embryogenic callus growing in the presence of the bialaphos selection agent.
9. A range of concentrations from 1 to 10 mg/L of PPT or bialaphos should tested with nontransformed calli to get a more efficient selection system.
10. Approximately 20 target plates should be prepared for each experiment. Two dishes are control, one grown on N6C1 to monitor the health of the culture and the second grown on the same medium but containing 5-10 mg/L bialaphos to demonstrate response of untransformed cells to selection.

NOTE: Phosphinothricine inhibits glutamine synthesis, causing a rapid accumulation of ammonia that leads to plant cell death. bialaphos is a tripeptide antibiotic that consists of PPT and two L-alanine-residues. Bialaphos is an effective selection agent for selection of transgenic wheat calli, however it does not always kill untransformed cells. In order to achieve optimum selection, subculture the bombarded cells at low density, about 10 immature embryos per dish.

PLANT REGENERATION

- Plant regeneration from resistant calli is performed on the same medium used for regeneration of bread wheat genotypes except that 5 mg/L bialaphos is added to the media.
- The somatic embryos capable of developing into green shoots within 2–4 weeks are characterized as putative transformants. The selected plantlets are transferred to the other medium for root formation (MSE) (see Tissue Culture Protocol) but supplemented with 1 mg/L bialaphos to continue the selection. The plantlets developed on this medium are transferred to controlled growth chambers and greenhouse conditions and grown for further analyses.

HISTOCHEMICAL GUS ACTIVITY ASSAY

- GUS activity can be detected histochemically as described by McCabe (1988). About 5 immature embryos or calli from each transformed plate of each experiment are used for GUS assays.
- GUS-expressing cells are routinely visualized 48 h after microprojectile bombardment by incubating bombarded immature embryos or calli in 400 µ of the following X-Gluc solution: 580 mg of ethylenediaminetetraacetic acid (EDTA); 100 mg of 5-bromo-4-chloro-3-indolyl glucuronide (Sigma); 200 µ of Triton X-100 dimethyl sulfoxide; 2.4 g sodium phosphate (NaH2PO4), 42 mg potassium ferrocyanide (K4Fe(CN)6.3H2O) and adjusting total volume to 200 ml with distilled water and pH to 7.0. Plant tissue is incubated in the above mixture at 37∞ C overnight in darkness. ß-glucuronidase activity is determined microscopically.

SOLUTIONS, MATERIALS, AND THEIR PREPARATION

Plasmid DNA

The DNA for bombardment is adjusted to a concentration of 1 µg/µ in TE buffer (1mM Tris, pH 7.8; 0.1 mM Na_2EDTA) and is stored at -20°C.

Microprojectiles and preparation of gold particles (see the instruction manual, Bio-Rad Laboratories)

1. Store dry tungsten microcarriers in a dry, non-oxidizing environment. Gold particles with a diameter of 1.0 µm (available in Bio-Rad) are used.
2. Place 40 mg (we use this quantity of particles; Bio-Rad proposes 60 mg) of dry microcarriers, tungsten or gold, per 1 ml of 100% ethanol in a microtube. Vortex on high for 1-2 minutes or sonicate using a standard tip at low power for 30 seconds. Repeat **three** times.
3. Centrifuge the microtube at 10,000 rpm for one (1) minute. Remove the supernatant, add 1 ml of sterile distilled water, resuspend, centrifuge, and remove supernatant; repeat the process. Resuspend the macrocarriers in 1 ml of sterile distilled water. Aliquot 50 µ (for 4-8 bombardments) of the final suspension into microtubes, while continually vortexing the suspension. Gold aliquots can be stored at 4°C or room temperature.

Preparation of 2.5M CaCl2 (calcium chloride)

Weigh out 36.75 g calcium chloride and dissolve completely with 80 ml deionized water into 200 ml beaker and bring volume up to 100 ml. Stir the solution well with a magnetic stirrer. Filter sterilize the solution. Pipette into sterile 2 ml tubes. Store this solution at -20°C. Use fresh solution for bombardment.

Preparation of 0.1M Spermidine

- A spermidine C7H10N3 (N-[3-Aminopropyl] -1,4-butanediamine with FW=145.2 is air sensitive and very hydroscopic (a Sigma product).
- Dissolve 1.012 gram of the spermidine in 50 ml distilled water (0.1M). This solution should have pH=11-12. Filter sterilize the solution and pipet out 50 µ into each eppendorf tube. Store at 4°C. Use fresh solution for bombardment.

Required materials and equipment

- Biolistic ® PDS-1000/He Particle Delivery System available from Bio-Rad Laboratory.
- The PDS-1000/He requires a cylinder of high purity compressed helium (99.999% pure.) The pressure of the tank should be 2,000 to 2,600 psi.
- The PDS-1000/He also requires an oil filled, rotary vacuum pump (single or dual chamber) with a capacity of 90-150 lpm (3-5cfm). This pump must be capable of pulling pressure inside the sample chamber to 27 inches Hg within 30 seconds.
- Fifty fresh embryos of maize (1-2.5 mm in length are best), aseptically removed from maize caryopses or embryos previously on callus initiation medium for 4 days (placed on disposable petri dishes (100 mm diameter, 15 mm deep) with 10 ml agar-solidified medium N6C1 (see tissue culture protocol). The embryos should be arranged in a circle about 2 cm in diameter in the center of each plate. For osmotic pre-bombardment treatments the material should be placed onto callus induction medium N6C1 containing 12% maltose and kept on the material for approximately 4 hours.
- Sterilized stainless steel mesh for the PDS-1000/He, available through Bio-Rad Labs. Sterilize by autoclaving.
- Sterilize macrocarrier sheets, rupture disks, and stopping screens by placing them in absolute 100% ethanol in a petri dish and allowing the alcohol to evaporate underneath a transfer hood.

Media and solutions for biolistic transformation of maize and wheat

Media for embryogenic callus initiation

- Cultured maize embryos proliferate on N6C1 medium (see tissue culture protocol)
- Cultured wheat embryos proliferate in 4 days on MSE3 medium (see tissue culture protocol).

Media for selection

- Medium for selection of maize transformation is N6C1 (see Tissue Culture Protocol) supplemented with the concentration of the selective agent used in the experiment.
- Medium for wheat transformation is MSE3 medium for embryogenic callus initiation and MSC medium for somatic embryo formation (see Tissue Culture Protocol), but supplemented with the concentration of selective agent used.

Medium for regeneration

MSRB5C is the medium used for maize and wheat regeneration (see Tissue Culture Protocol), but supplemented with the concentration of the selective agent (bialaphos).

Medium for root formation

MSEB1C is the rooting medium used for maize and wheat (see Tissue Culture Protocol), but supplemented with bialaphos.

Reference

Instruction Manual, Bio-Rad Laboratories.

AGROBACTERIUM-MEDIATED TRANSFORMATION: PROTOCOLS

For monocotyledons, the most efficient *Agrobacterium*-mediated gene transfer system is essentially made up of the following materials, procedures, and factors:

- Plant material and explants
- Tissue culture medium and related conditions
- Bacterial strains and vectors
- Inoculation and co-cultivation
- Selection and regeneration of transgenic plants
- Molecular analyses of transformed materials

Agrobacterium Transformation of Maize

Immature embryos of CIMMYT inbred lines and the hybrids between these lines are the source of immature embryos used for starting *in vitro* cultures and for *Agrobacterium* transformation experiments.

Maize inbred lines, crosses between these lines, and A188 and crosses with CIMMYT inbred lines are used as plant material for *Agrobacterium* transformation.

INITIAL PROCEDURE

- Excise immature embryos from seeds harvested 15-20 days after pollination. Surface sterilize whole ears with 70% ethanol for 1 min, follow with treatment of 20% Clorox containing 10 drops/L of polyoxyethylene sorbitan monooleate (Tween-80) for 30 min, then rinse 3 times with sterile distillate water.
- Aaseptically remove immature embryos, 1.5- 2.0 mm in size, from the kernels and place, scutellum up, on the initiation medium for at 4–11 days. Incubate the cultures in darkness at 28∞C.
- For embryogenic callus initiation, N6C1 media (see Tissue Culture Protocol) consists of modified N6 basal medium (N6), supplemented with 200 mg/L casein hydrolysate, 2.302 mg/L L-proline, 3% sucrose, and 2 mg/L dicamba.
- Intact callus pieces derived from immature embryos (about 50 on a petri dish) are used for inoculation with *A. tumefaciens.*

***A.tumefaciens* STRAINS AND PLASMIDS FOR MAIZE TRANSFORMATION**

The following strains and plasmids are used for *Agrobacterium* transformation:

LBA4404 pKUbi:CryIA(b)
LBA4404 containing pBIN UbiGI/Bar:Act /Ubi CryIA(b)
LBA4404 containing pBIN UbiGI/Bar:Act /Ubi CryIB

EHA105 containing pBIN UbiGI/Bar:Act /Ubi CryIA(b)
EHA105 containing pBIN Ubi GI/Bar:Act/Ubi CryIB

pBIN binary vector derived from pBIN 19; Act:5′ untranslated region of rice Act-1 gene; Ubi:5′ untranslated region of maize Ubi-1 gene; GI-intron-containing GUS gene; 1Ab synthetic cry1Ab gene; 1B synthetic cry1B gene

The *intron-gus* gene expresses *gus* activity in plant cell but not in the cells of *A. tumefaciens.*

(*A. tumefaciens* strains come from Japan Tobacco Inc. Plasmids were developed at the University of Ottawa by Dr. Illimar Altosaar or at CIRAD by Dr. R. Frutos and M. Royer)

INFECTION

Cultures of *A. tumefaciens* are initiated from glycerol stocks and grown for 3 days on YPHK medium supplemented with 50 mg/L kanamycin and 50 mg/L hygromycin for LBA4404 pKUbi. CryIA(b). YPK medium supplemented with 100 mg/L kanamycin are used for the rest of the plasmids.

The bacteria are grown in darkness at 28°C in the incubator.

Agrobacteria are grown as single colonies on the surface of the agarose solid bacterial medium in the petri dishes.

1. For the initiation of bacterial suspension, take 12 loops from the single colonies of bacteria and resuspend them in 5 ml liquid N6CI-inf media in the centrifuge tube.
2. Add 200 µM asetocyringon to the suspension.
3. Vortex for 1 min to thoroughly mix the bacteria with the liquid medium.
4. Observe the OD (optical density) of this suspension (OD600). An OD of 1.0-1.5. should be obtained.
5. Transfer the material from the petri dish to the *Agrobacterium* suspension, vortex for 1 min, incubate for 5 min, and transfer onto the surface of the sterile paper (Kleenex). It is not necessary to completely dry the explants before placing them on the agar dishes, however, try to avoid transferring too much solution.
6. Place the embryos on the co-cultivation medium N6CI-As40 and co-cultivate with *Agrobacterium* for 2-3 days under dark conditions at 25°C in the incubator. During incubation, the embryo axes should be in contact with the medium and the scutella exposed to air.

SELECTION AND REGENERATION OF TRANSFORMED MAIZE TISSUE

1. After co-cultivation, the materials are transferred and cultured on solid N6C1 medium without selection and supplemented with 150 mg/L cefotaxime and timentin or 250 mg/L cefotaxime to stop bacterium growth. Place about 50 embryos in a single petri dish.
2. Keep the material in the incubator for 1 week in darkness at 28°C.
3. Cut the calli into small pieces (approximately 2 mm). Each piece of callus is taken from an immature embryo; each piece of inoculated callus is then divided into several small pieces.
4. Transfer the cut pieces to the selection medium N6C1B5CT supplemented with 5 mg/L bialaphos and 150 mg/L cefotaxime and timentin or N6C1H10C supplemented with 20 mg/L hygromycin and 250 mg/L cefotaxime.
5. Incubate the material in the incubator for 2 weeks in darkness at 28°C.
6. Excise the materials with a scalpel and subculture the clusters of the cells that proliferate and show the characteristics of the Type I calli onto the medium (either N6C1B10CT medium supplemented with 10 mg/L bialaphos and 100 mg/L cefotaxime and 100 mg/L timentin or N6C1H30C supplemented with 30 mg/L hygromycin and 200 mg/L cefotaxime).
7. Incubate the material in the incubator for 3 weeks in darkness at 28°C.

8. Calli propagated from the culture are excised again and cultured on the same selection medium for the other 3 weeks under the same culture conditions.
9. Clusters formed from the last selection are excised again and cultured on MSRB5C supplemented with 5 mg/L bialaphos and 200 mg/L cefotaxim or MSRH30C medium supplemented with 30 mg/L hygromycin and 200 mg/L cefotaxime for elimination of *A. tumefaciens.*
10. Transfer petri dishes with the transformed material to a controlled environment at 28°C, illuminated 16 hours daily (dark for 8 h) for 2 weeks to proliferate shoots.
11. When the green shoots are formed, place the regenerated material into the same medium in baby food jars and grow material for plantlet initiation.
12. Cut small plantlets with 3–5 leaves and transfer them into rooting medium (MSEB1C supplemented with 1 or 3 mg/L bialaphos and 200 mg/L cefotaxime or MSEH10C supplemented with 10 mg/L hygromycin and 200 mg/L cefotaxime) for final selection.
    NOTE: The *Agrobacterium* are still alive at this stage of plant development.
13. Plantlets with good root systems are transferred into pots with soil, grown in a growth culture room, and then transferred to the greenhouse.

Agrobacterium Transformation of Wheat

INITIAL PROCEDURE

- Begin with plant appropriate material (refer to Biolistics Protocols).
- The yellow, irregularly shaped, friable tissue, usually obtained 4–14 days after planting on MSE3 initiation media is used for *Agrobacterium* transformation.
- The intact callus pieces derived from immature embryos (about 50 on a petri dish) are used for inoculation with *A. tumefaciens.*
- The cultures are incubated in darkness at 28°C.

***A. tumefaciens* STRAINS AND PLASMIDS**

The following strains and plasmids are use for *Agrobacterium* transformation:

- LBA4404 containing pBIN UbiGI/Bar; Act/Ubi CryIB
- pBIN binary vector derived from pBIN 19; Act:5′ untraslated region of rice Act-1 gene; Ubi:5′ untraslated region of maize Ubi-1 gene; GI-intron-containing GUS gene; 1Ab synthetic cry1Ab gene; 1B synthetic cry1B gene
- The *intron-gus* gene expresses *gus* activity in plant cells, but not in the cells of *A. tumefaciens*
- *A. tumefaciens* strains come from Japan Tobacco Inc.
- Plasmids were developed at CIRAD by Dr. R. Frutos and M. Royer.

INFECTION

1. Cultures of *A. tumefaciens* are initiated from glycerol stocks and grown for 3 days on YPK medium supplemented with 100 mg/L kanamycin.
2. The bacteria are grown in the incubator in darkness at 28°C.
3. Agrobacteria are grown as single colonies on the surface of the agarose solid bacterial medium in the petri dishes.
4. For the initiation of bacterial suspension, take 12 loops from the bacterial single colonies, and resuspend them in 5 ml liquid N6CI-inf medium in the centrifuge tube. This medium has a high sucrose concentration and a low pH.
5. Add 400 µM asetocyringon to the suspension.
6. Vortex for 1 min to thoroughly mix the bacteria with the liquid medium.
7. Check the OD (optical density) of this suspension (OD600). It should be in the range of 1.0–1.5.
8. Transfer the material from the petri dish to the *Agrobacterium* suspension, vortex for 1 min, incubate for 5 min, then remove the suspension and transfer the material to the surface of the sterile paper (Kleenex). It is not necessary to completely dry the explants before they are placed on the agar plates, however, try to avoid transferring too much solution.
9. Place the embryos on the co-cultivation medium N6CIAS40 and co-cultivate with *Agrobacterium* in the incubator for 2–3 days under dark conditions, at 25°C. During incubation, the embryos should be in contact with the medium and the scutella exposed to air.

SELECTION AND REGENERATION OF TRANSFORMED WHEAT TISSUE

1. After co-cultivation, transfer and culture the materials onto the solid MSE3C medium without selection. The medium is supplemented with 150 mg/L cefotaxime and 150 mg/L timentin to stop bacterium growth.
2. Place about 50 embryos in each petri dish.
3. Keep the material in the incubator for 1 week in darkness at 25°C.

4. Cut the calli into small pieces (approximately 2 mm). Each piece of callus is derived from one immature embryo.
5. Transfer the cut pieces to the selection medium MSE3C-B5CT supplemented with 5 mg/L bialaphos, 100 mg/L cefotaxime, and 100 mg/L timentin.
6. Incubate the material in the incubator for 2 weeks in darkness at 25°C.
7. Excise the materials with a scalpel and subculture the clusters of the cells that proliferated calli on MSE3CB10CT medium supplemented with 10 mg/L bialaphos, 100 mg/L cefotaxime, and 100 mg/L timentin.
8. Incubate the material in the incubator for 3 weeks in darkness at 25°C.
9. Calli propagated from the culture are excised again and cultured on the same selection medium for another 3 weeks under the same culture conditions.
10. Clusters propagated from the last selection are excised again and cultured on MSRB5C supplemented with 5 mg/L bialaphos and 250 mg/L cefotaxime to eliminate *A. tumefaciens* in the petri dishes.
11. Transfer petri dishes with the transformed material for shoot proliferation to a controlled environment for 2 weeks at 28°C under light 16 hours daily (8 hours darkness).
12. When the green shoots are formed, place the regenerated material into baby food jars with the same medium and grow the material for plantlet initiation.
13. Cut small plantlets near the callus from where they originated and transfer the plantlets with 3–5 leaves into rooting medium (MSEB1C supplemented with 1 or 3 mg/L bialaphos and 100 mg/L cefotaxime) for final selection. Small plantlets go into MSEB1C supplemented with 1 mg/L bialaphos, while the more vigorous plantlets are placed into MSEB3C medium containing 3 mg/L bialaphos. Both media are supplemented with 200 mg/L cefotaxime.

NOTE: The *Agrobacterium* are still alive at this stage of plant development.

14. Plantlets with good root systems are transferred into soil in pots, grown in a growth culture room for 1 week, and then transferred into the greenhouse.

HISTOCHEMICAL GUS ACTIVITY ASSAY

GUS activity is detected histochemically as described by McCabe (1988). About 5 immature embryos or calli from each transformed plate of each experiment are used for the GUS assays. The histochemical assay is for both maize and wheat material.

GUS-expressing cells are routinely seen 48 hours immediately after co-cultivation or 3–4 days after co-cultivation by incubating immature embryos or calli in 400 µ of the following X-Gluc solution: 580 mg of ethylenediaminetetraacetic acid (EDTA); 100 mg of 5-bromo-4-chloro-3-indolyl glucuronide (Sigma); 200 µ of Triton X-100 dimethyl sulfoxide; 2.4 g sodium phosphate (NaH2PO4), 42 mg potassium ferrocyanide (K4Fe(CN)6.3H2O). Adjust total volume to 200 ml with distilled water and adjust pH to 7.0. Plant tissue is incubated in the above mixture at 37∞C overnight in darkness. ß-glucuronidase activity is determined microscopically.

Media and Solutions for *Agrobacterium* Transformation of Maize and Wheat

YP medium for bacterial growth

Bacto yeast extract	5 g/L
Bacto peptone	10 g/L
NaCl	5 g/L
Agarose	6 g/L
pH = 6.8	

After autoclaving put flasks with YP medium into 40°C water bath until they cool enough to add the Kanamycin/Hygromycin.

Medium for bacterium suspension N6C1-inf.
This is the same N6C1 liquid media supplemented with sucrose 60 g/L, glucose 40 g/L, with pH = 5.2 (see Tissue Culture Protocols)

Medium for co-cultivation N6C1As40
This is the same N6C1 media supplemented with sucrose 30 g/L, glucose 10 g/L and supplemented with 200 µM acetosyringone, with pH = 5.8 (refer to the Tissue Culture Protocols).

Medium for selection
Medium for selection of maize is N6C1 and for selection of wheat is MSC (see Tissue Culture Protocols) supplemented with the concentration of the selective agent used in the experiment and cefotaxime or timentin.

Medium for regeneration
MSRB5C is the medium used for maize and wheat regeneration (see Tissue Culture Protocols), but supplemented with the selective agent (bialaphos) and cefotaxime.

Medium for root formation
MSEB1C is the rooting medium used for maize and wheat (see Tissue Culture Protocols), but supplemented with bialaphos and cefotaxim.

VECTORS FOR PLANT TRANSFORMATION

Introduction

Recent developments in plant molecular biology have created the need for versatile cassettes for the expression of foreign genes, binary vectors for gene transfer using *Agrobacterium*, marker gene or phenotypic markers for selection or detection of transformants, and reporter genes for assaying gene expression. To effectively use these tools, it is important to be familiar with some frequently used terms of molecular biology, as well as some basic techniques, such as those covered in this section of the CIMMYT AGE Manual. It also important to know that several other protocols and commercially-available kits may be obtained by those in the field.

Terminology

Expression cassettes
Cloning a coding sequence into a polylinker of an expression cassette in the proper orientation will result in the expression of the gene after its transfer to the plant cell. Transcription will occur from the promoter located upstream from the polylinker and the mRNA will be polyadenilated at the site(s) present downstream of the polylinker. The polylinker may contain a translation start codon, in which case the coding sequence must be positioned in frames with the cassette ATG codon for correct translation.

Reporter genes
A reporter gene codes for an enzyme or other proteins that can be quantified using a biochemical assay. The most widely used reporter gene in plant transformation is the *E. coli Gus* gene coding for the b-glucuronidase enzyme. The *Cat* and *Luc* genes coding for bacterial chloramphenicol acetyltransferase and firefly luciferase, respectively, can also be used in plants for accurate assays of gene expression.

Selectable markers
A selectable marker gene enables the recovery of transformants in a population of untransformed cells by giving them the ability to emerge in the presence of a selective agent that is toxic to untransformed cells

Phenotypic markers
In contrast to selectable markers—which require the use of a selective agent to recover a trasformants—phenotypic markers give transformants distinct features that can be easily identified. The thaumatin II gene has been used as a simple marker to confirm the transformed genotype of potato plants. Confirmation of the presence of the gene in the plants was given by an intensely sweet taste, resulting from the presence of thaumatin in transformed tissues. Thaumatin could be detected in as little as 0.1 g of leaf.

Binary vectors
Binary vectors were developed to exploit the ability of *Agrobacterium* strains that have a Ti plasmid depleted of its T-DNA (disarmed) to transfer to plants cells a fragment of DNA comprised between T-DNA borders carried on another plasmid. The plasmid possesses the function for its mobilization from *E. coli* to *Agrobacterium* and for its replication in these two hosts. Selectable marker gene(s) for the selection of bacterial transformants or exconjugants are present in the vector and a selectable marker gene for selection of transformed plant cells can be cloned into unique restriction site(s) close to the selectable marker, between the T-DNA borders.

Protocols

Several protocols for plasmid extraction and manipulation have been published in the recent past. We will describe only those protocols that have proven most effective and useful for plant transformation and cloning for plant transformation vectors. It is important to note that although the techniques are easy, plasmid construction requires a carefully designed cloning strategy to avoid problems in the late stage of cloning (e.g., no matching restriction sites or incorrect orientation of inserts)

NOTE: In all of the following protocols, use autoclaved tubes and solutions whenever possible, and wear gloves to minimize nuclease contamination from fingers.

PLASMID ISOLATION (*E. coli*)

1. Grow 5 ml of culture overnight.
2. Harvest cells by centrifuging entire culture in a 15 ml centrifuge tube for 5 min. at 3,000 g.
3. Discard the supernatant.
4. Add 400 µl of Tris pH 8 1.0 M, EDTA pH 8 0.5 M Glucose 50 mM.
5. Vortex to resuspend the cells.
6. Add 300 µl of NaOH 0.2 M, SDS 1%) and incubate in ice for 10 min.
7. Add 300 of K-Acetate 3 M pH 5.5 and incubate at room temperature for 15 min.
8. Centrifuge for 15 min at 14,000 rpm.
9. Add half volume of isopropanol, mix and centrifuge at 14,000 rpm.
10. Clean the pellet with 70 % alcohol.
11. Treat for RNAase.
12. Add 5 µl of proteinase K and incubate for30 min at 37°C.
13. Extract DNA with 200 µM phenol/chloroform.
14. Centrifuge at 14,000 rpm for 2 min.
15. Add 100 µl of 7.5 M NH_4OAc and 750 µl of absolute ethanol. Incubate in ice for 30 min.
16. Wash pellet with 1 ml 70% ethanol and dry the pellet under vacuum.
17. Resuspend the pellet in sterile dH_2O.

PLASMID ISOLATION (*A. tumefaciens*)

1. Grow 5 ml culture overnight.
2. Transfer 1.5 ml of the bacteria suspension into a 1.5 ml centrifuge tube and centrifuge for 20 seconds. Repeat the steps 2 and 3 until the entire suspension has been centrifuged.
3. Resuspend the bacterial pellet in 150 µl of solution 1 (Glucose 50 mM, Tris Hcl pH 8 25 mM, EDTA 10 mM). Mix by inverting the tube (4–5 times), then incubate for 5 min on ice.
4. Add 300 µl of solution #2 (NaOH o.2 M, SDS 1%) and mix by inverting the tube (4–5 times). Incubate for 5 min on ice.
5. Add 225 µl solution #3 (60 ml K-Acetate 5M, Acetic Acid 11.5 ml, dH_2O 28.5 ml) and vortex the solution. Incubate in ice for 5 min.
6. Centrifuge the suspension and transfer the supernatant (600µl). Clean the supernatant with the same volume of Phenol/chloroform. Centrifuge for 20 seconds
7. Clean the supernatant with chloroform/alcohol isoalmilic. Centrifuge the for 20 seconds.
8. Transfer the supernatant to a clean centrifuge tube.
9. Add half volume of isopropanol to the supernatant. Mix well and centrifuge for 1 min.
10. Eliminate the supernatant.

11. Clean the pellet with 70% ethanol.
12. Remove the supernatant and dry the pellet under vacuum.
13. Resuspend the pellet in 50 µl of TE or sterile dH_2O.

PLASMID DIGESTION

1. Take 1 L of plasmid and add the proper buffer.
2. Add 25–50 units of the chosen enzyme.
3. Digest for a minimum of 2 h.
4. Run a minigel to confirm the digestion.

BACTERIA TRANSFORMATION (E. coli)

1. Add 40 ng of plasmid DNA to 20 ml of thawed competent cells.
2. Place on ice for 15–60 minutes (min/max).
3. Heat shock the bacteria in a water bath at 42∞C for 40 seconds.
4. Add 300 μl of LB broth and incubate for 30 min at 37°C.
5. Plate the bacteria on LB with the proper antibiotic and incubate overnight at 37°C.
6. Isolate one colony and check for the presence of the plasmid.

MOLECULAR TECHNIQUES FOR SCREENING TRANSGENIC PLANTS (PROTOCOLS)

CIMMYT Applied Genetic Engineering Laboratory

Abbreviations/Acronyms

Amp	ampicillin
APS	ammonium persulfate
BME	b-mercaptoethanol
BPB	bromophenol blue
BSA	bovine serum albumine
CSPD	Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′(5′-chloro)tricyclo [3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate
CTAB	mixed alkyltrimethyl-ammonium bromide
dATP	deoxyadenosine 5′-triphosphate
dCTP	deoxycytidine 5′-triphosphate
ddH_2O	double-distilled water
dH_2O	distilled water
dGTP	deoxyguanosine 5′-triphosphate
Dig	digoxigenin
Dig-dUTP	digoxigenin-11-dUTP
DNA	deoxyribose nucleic acid
dNTPs	deoxynucleoside 5′-triphosphates
DTT	dithiothreitol
dUTP	deoxyuridine 5′-triphosphate
EDTA	ethylenediaminetetraacetate
EtBr	ethidium bromide
EtOH	ethanol
g	gram(s)
h	hour(s)
HYB	hybridization
kb	kilobases
KOAc	potassium acetate
LMP	low melting point
mA	milli Amperes
min	minute(s)
ml	milliliter(s)
MSI	Micron Separations Inc.
MW	molecular weight
NaOAc	sodium acetate
ng	nanogram(s) = 10^{-9} gram
nm	nanometer(s) = 10^{-9} meter
OD	optical density
OD_x	optical density at x nm
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
RFLPs	restriction fragment length polymorphisms
RNA	ribonucleic acid
RT	room temperature
RXN	reaction(s)
S&S	Schleicher & Schuell
SDS	sodium dodecyl sulphate
sec	second(s)
SGB	sample gel buffer
SS DNA	salmon sperm DNA
SSC	saline sodium citrate
STE	sodium Tris-EDTA (also TEN)
TAE	Tris-acetate EDTA (buffer)
TBE	Tris-borate EDTA
TE	Tris-EDTA (buffer)
TEMED	N,N,N′,N′-Tetramethylethylenediamine
TNE	Tris Sodium (Na) EDTA (buffer)
Tris	Tris(hydroxymethyl)amino-methane
TTE	Triton Tris-EDTA (buffer)
TTP	thymidine 5′-triphosphate
U	unit(s) of enzyme
UV	ultraviolet
V	volts
XC	xylene cyanole
[FINAL]	FINAL concentration
[Stock]	stock concentration
°C	degree Celsius
µg	microgram(s) = 10^{-6} gram
µl	microliter(s) = 10^{-6} liter

Cloning DNA in Bacterial Plasmids

OVERVIEW

Cloning involves the use of recombinant DNA technology to propagate DNA fragments inside a foreign host. The DNA segment of interest (along with suitable promoters, etc.) is incorporated into a carrier (or vector). Following introduction into suitable host cells, the DNA fragments can then be reproduced along with the host cell DNA. Vectors are DNA molecules originating from viruses, bacteria, and yeast cells. They accommodate various sizes of foreign DNA fragments ranging from 300 bp for bacterial vectors (plasmids and cosmids) to 1 Mb for yeast vectors (yeast artificial chromosomes).

For transformation of plants, the kinds of vehicles used for carrying the genes of interest etc. are plasmid vectors. Bacterial plasmids are double-stranded closed circular DNA molecules that range in size from 1 kb to more than 200 kb. They are found in a variety of bacterial species, in which they behave as accessory genetic units that replicate and are inherited independently of the bacterial chromosome. Nevertheless, they rely on enzymes and proteins encoded by the host for their replication and transcription. Frequently, plasmids contain genes coding for enzymes that under certain circumstances in nature are advantageous to the bacterial host. Among the characteristics found in phenotypes and conferred by plasmids are resistance to antibiotics and the ability to degrade heavy metals. The plasmids found in *E. coli* have been extensively "engineered" to produce cloning vehicles. Among the vectors currently used most widely for use in transformation are pUC19 and pBluescript. Both of these vectors carry the gene conferring resistance to ampicillin as well a short "polycloning site region".

The initial CIMMYT strategy for constructs was to acquire them from other labs or develop them in collaboration with other groups outside CIMMYT. In such cases, we receive constructs that contain transgenes properly linked with a desirable promoter. The gene of interest may be on the same plasmid as that containing the desired reporter gene (eg., GUS or anthocyanin) and/or selectable marker gene, or they may be on separate plasmids in which case a co-transformation (transformation with more than one plasmid) strategy may be chosen. After receiving the plasmid DNA, a transformation into bacteria (*E. coli* or *Agrobacteria*) is performed in our lab. Once the bacteria are transformed and contain plasmid, they can be mixed with glycerol (15%) and stored in a freezer at -80°C for several years. This ensures a permanent stock of each plasmid. Each time more plasmid is required for plant transformation, a stab from the glycerol stock is used to grow an overnight culture and a plasmid mini-prep is then performed. If the plasmid is new to the lab, digestion of the prepared plasmid with the appropriate restriction enzymes is recommended to ensure that the plasmid is what we expect.

Recent developments in plant molecular biology have created the need for versatile cassettes for the expression of foreign genes. With the "new generation" of "flexible" constructs available from many labs, it is possible to "cut" and "paste" more easily . This will allow us to remove certain genes and/or promoters and replace them with others better suited to our final goals. For example, for certain target countries it may be undesirable to deploy a transgenic plant with a herbicide resistance gene and we may want to replace it with an antibiotic selectable marker instead, or to remove all selectable markers. In this case, we would "cut" out the herbicide resistance gene and "paste" in an antibiotic resistance gene in its place, or omit all selectable marker genes. With flexible constructs this is simply achieved by carefully selection of restriction enzymes to cut out the undesirable gene, followed by the use of ligases (enzymes that join pieces of DNA together) to replace it with the desirable gene or promoter.

Cloning a coding sequence into a polylinker of an expression cassette in the proper orientation will result in the expression of the gene after its transfer to the plant cell. Transcription will occur from the promoter

located upstream from the polylinker and the mRNA will be polyadenylated at the site(s) present downstream of the polylinker. The polylinker may contain a translation start codon, in which cases the coding sequence must be positioned in frame with the cassette ATG codon for correct translation.

Protocols for bacterial transformation and plasmid preparations are given below. It should be noted, however, that several other protocols and commercially-available kits exist.

BACTERIAL TRANSFORMATION

Whether you are receiving plasmid DNA from other labs or preparing CIMMYT constructs, it is necessary to transform the DNA into a bacterial vector to ensure a constant and reliable supply of the plasmid for transformation experiments.

Several protocols for plasmid extraction and manipulation have been published in the past years. We will describe only the most effective and useful for plant transformation and cloning for plant transformation vectors. It is important to note that although the techniques are easy, plasmid construction requires a carefully designed cloning strategy to avoid problems in the late stage of cloning (e.g., no matching restriction sites or incorrect orientation of the inserts)

Note: When applying these protocols, use autoclaved tubes and solutions whenever possible and wear gloves to minimize nuclease contamination from fingers.

1. Add 40 ng of plasmid DNA to 20 µl of thawed competent cells.
2. Mix very gently.
3. Place on ice for 20–30 min.
4. Heat shock at 42°C for 40 seconds in a water bath.
5. Place on ice for 10 min.
6. Add 80 µl of LB broth (without antibiotics).
7. Shake for 2-4 h at 225 rpm at 37°C.
8. Plate on LB + proper antibiotic, spreading cells evenly.
9. Grow overnight at 37°C (or until colonies are distinct).

NOTE: Once frozen competent cells are thawed, they should be discarded if not used. Do not return to freezer for future use.

PREPARATION OF FROZEN COMPETENT CELLS

NOTE: This protocol is recommended for the production of large amounts of competent cells of medium efficiency.

1. Grow overnight culture of desired strain in 5 ml of LB broth (without antibiotic).
2. Dilute the overnight culture 1:100 with LB broth (without antibiotic) and shake at 37°C until the OD_{600} reaches 0.3-0.4.
3. Transfer the cells to 250 ml centrifuge bottles and chill on ice for 10 min.
4. Centrifuge the cells for 7 min. at 3500 rpm at 4°C.
5. Carefully discard the supernatant and resuspend the pellet by gently pipetting 5 ml of sterile, ice-cold 10 mM $MgCl_2$. After cells are resuspended, add an additional 120 ml of 10 mM $MgCl_2$.
6. Centrifuge the cells for 7 min. at 3500 rpm at 4°C.

7. Carefully discard the supernatant and resuspend the pellet by gently pipetting 5 ml of sterile, ice-cold 50 mM $CaCl_2$, 20% glycerol. After the cells are resuspended, add an additional 5 ml of 50 mM $CaCl_2$, 20% glycerol.
8. Place on ice for at least 1 h.
9. Transfer 400 µl aliquots of cells to individual, sterile 500 µl microfuge tubes.
10. Quick freeze cells in dry ice/ethanol bath (or in ethanol at -80°C) and store at -80°C until use.

10 mM $MgCl_2$

STOCK	100 ml	200 ml	300 ml	400 ml	500 ml
1.0 M $MgCl_2$	1 ml	2 ml	3 ml	4 ml	5 ml
ddH_2O	99 ml	198 ml	297 ml	396 ml	495 ml

50 mM $CaCl_2$, 20 % Glycerol

STOCK	100 ml	200 ml	300 ml	400 ml	500 ml
1.0 M $CaCl_2$	5 ml	10 ml	15 ml	20 ml	25 ml
Glycerol	20 ml	40 ml	60 ml	80 ml	100 ml
ddH_2O	75 ml	150 ml	225 ml	300 ml	375 ml

PREPARATION OF FRESH COMPETENT CELLS

NOTE: This protocol is recommended for the production of fairly high efficiency competent cells.

1. Grow overnight culture of desired strain in 10 ml of LB broth (without antibiotic), 2 days before the intended use of the cells.
2. Dilute 1.5 ml of the overnight culture into 40 ml of LB broth preheated to 37°C.
3. Shake at 37°C until the OD_{600} reaches 0.4 - 0.6 (about 2.5–3 h).
4. Transfer the cells to a 50 ml centrifuge tube (e.g., Corning) and chill on ice for 20 min.
5. Centrifuge the cell suspension for 15 min at 3,000 rpm at 4°C.
6. Carefully discard the supernatant and resuspend the pellet by gently pipetting 20 ml of sterile, ice-cold 50 mM $CaCl_2$. Use the tip of the pipette to gently resuspend the cells.
7. Chill on ice for 20 min.
8. Centrifuge the cell suspension for 15 min at 3,000 rpm at 4°C.
9. Carefully discard the supernatant and resuspend the pellet by gently pipetting 4 ml of sterile, ice-cold 100 mM $CaCl_2$. Use the tip of the pipette to very gently resuspend the cells.
10. Place on ice and keep in the refrigerator for use next morning.

50 mM $CaCl_2$

STOCK	100 ml	200 ml	300 ml	400 ml	500 ml
1.0 M $CaCl_2$	5 ml	10 ml	15 ml	20 ml	25 ml
ddH_2O	95 ml	190 ml	285 ml	380 ml	475 ml

100 mM $CaCl_2$

STOCK	100 ml	200 ml	300 ml	400 ml	500 ml
1.0 M $CaCl_2$	10 ml	20 ml	30 ml	40 ml	50 ml
ddH_2O	90 ml	180 ml	270 ml	360 ml	450 ml

PLASMID MINIPREPS.

The most convenient method for preparing sufficient plasmid DNA for transformation experiments, is the alkali method of Birnboim and Doly (1979). The cells are lysed by SDS at high pH and then neutralized. The plasmid DNA renatures but the chromosomal DNA remains denatured and precipitates in a protein-DNA-

SDS complex. This procedure can be scaled-up to any volume. However, for culture volumes of more than 10ml, it is best to deproteinize the plasmid with phenol/chloroform prior to ethanol precipitation. For large and high-quality plasmid preparation, the plasmid can be further purified via CsCl equilibrium gradient centrifugation or through HPLC.

Plasmid preps (*E.coli*)

1. Grow 10 ml overnight culture in LB broth with the proper antibiotic.
2. Harvest cells by centrifuging entire culture in a 15 ml centrifuge tube for 5 min at full-speed in a table-top centrifuge (1300-1500 rpm g). Discard supernatant.
3. Resuspend cell pellet thoroughly by vortexing before adding in 200 µlSolution I containing 5 mg/ml lysozyme (add lysozyme within 1 h of use). Vortex thoroughly and leave at room temperature for 5 min. It is easier to resuspend cells if they are vortexed before adding the lysozyme mix.
4. Add 400 µl Solution II, mix gently (no vortex), and incubate 10 min on ice (solution should be clear).
5. Add 300 µl Solution III, mix gently (no vortex), and incubate 15 min on ice.
6. Centrifuge 15 min at full speed in table-top centrifuge, pour off supernatant into 1.5 ml microfuge tube.
7. Add 600 µl ice cold isopropanol, mix and leave at -20°C for one hour or at - 80°C for 30 min, centrifuge 5 min at full speed in microfuge (~12,000 rpm), drain and dry tube.
8. Redissolve pellet in 190 µl dH_2O. Could place on a vortex for 45 min but with gentle vortexing.
9. Add 5 µl of 1 mg/ml RNAse A and 5 µl of 500 U/ml RNAse T1. Incubate at 37°C (or RT) for 15 min.
10. Add 5 µl of 10 mg/ml Proteinase K. Incubate at 37°C (or RT) for 20 min.
11. Extract with 200 µl phenol [or 200 µl phenol/chloroform (1:1)].
12. Centrifuge for 4 min at full-speed in microfuge (~12,000 rpm). Transfer aqueous (upper) phase to new microfuge tube.
13. Add 100 µl 7.5 M NH_4OAc to precipitate the DNA.
14. Add 800 µl ice cold absolute EtOH, mix gently, incubate at -80°C for 30 min, centrifuge 5 min at full-speed in microfuge.
15. Wash pellet with 1 ml 75% EtOH, centrifuge 4 min in microfuge, pour off supernatant and dry tube in vacuum desiccator (for 20–30 min).
16. Dissolve pellet in 50 µl TE-8.0.

Solution I: 25 mM Tris-8.0, 10 mM EDTA, 50 mM glucose

STOCK	10 ml	20 ml	30 ml	40 ml	50 ml
1.0 M Tris-8.0	250 µl	500 µl	750 µl	1000 µl	1250µl
0.5 M EDTA-8.0	200 µl	400 µl	600 µl	800 µl	1000 µl
Glucose	90 mg	180 mg	270 mg	360 mg	450 mg

NOTE: Solution I may be prepared as a 10X stock solution and stored -20°C, in small aliquots for later use. For use: thaw, dilute, and add lysozyme.

Solution II: 0.2 M NaOH, 1.0% SDS

STOCK	100 ml	200 ml	300 ml	400 ml	500 ml
1.0 M NaOH	20 ml	40 ml	60 ml	80 ml	100 ml
20% SDS	5 ml	10 ml	15 ml	20 ml	25 ml

Solution III: 3 M KOAc, pH 5.5

Dissolve 29.5 g potassium acetate in 60 ml dH_2O. Add enough glacial acetic acid to bring pH to 5.5 (approx. 11 ml). Bring final volume to 100 ml.

BINARY VECTORS

Binary vectors were developed to exploit the ability of *Agrobacterium* strains containing a Ti plasmid depleted of its T-DNA (disarmed) to transfer to plants cells a fragment of DNA comprised between T-DNA borders carried on another plasmid. The plasmid possesses the function for its mobilization from *E. coli* to *Agrobacterium* and for its replication in these two hosts. Selectable marker gene(s) for the selection of bacterial transformants or exconjugants are present in the vector and a selectable marker gene for selection of transformed plant cells can be cloned into unique restriction site(s) close to the selectable marker, between the T-DNA borders.

Plasmid isolation (*A. tumefaciens*)

1. Grow 5 ml of culture overnight.
2. Transfer 1.5 ml of the bacterial suspension into a 1.5 ml centrifuge tube.
3. Centrifuge for 20 seconds.
4. Resuspend the bacterial pellet in 150 µl of solution A (Glucose 50 mM, Tris HCl 25 mM pH 8, EDTA 10 mM).
5. Mix by inverting the tube (4–5 times).
6. Incubate for 5 min on ice.
7. Add 300 µl of solution B (NaOH 0.2 M, SDS 1%).
8. Mix by inverting the tube (4-5 times).
9. Incubate for 5 min on ice
10. Add 225 µl solution C (60 ml K-Acetate 5M, Acetic Acid 11.5 ml, dH_2O 28.5 ml) and vortex the solution
11. Incubate in ice for 5 min.
12. Centrifuge the suspension and transfer the supernatant (600µl) to a new tube.
13. Clean the supernatant with the same volume of Phenol/chloroform (1:1).
14. Centrifuge for 20 seconds.
15. Clean the supernatant with chloroform/IAA (24:1).
16. Centrifuge for 20 seconds.
17. Transfer the supernatant in a clean centrifuge tube.
18. Add half volume of isopropanol to the supernatant.
19. Mix well and centrifuge for 1 min.
20. Eliminate the supernatant.
21. Clean the pellet with 70% ethanol.
22. Remove the supernatant and dry the pellet under vacuum.
23. Resuspend the pellet in 50 µl of TE or sterile dH_2O.

UV QUANTIFICATION OF PLASMID DNA

Plasmid DNA is usually quantified using the mini-fluorometer (see later protocol), but a spectrophotometer can also be used as follows:

Add 5 µl of each plasmid sample to 745 µl TE, read OD_{260} and OD_{280} to determine purity. Dilute sample with TE to 1 µg/µl. Store at -20°C. Sample should be usable for up to 6 months. (See Appendices for Beckman Spectrophotometer program.)

LIGATIONS

1. Transfer 0.1 µg of the vector DNA (dephosphorylated) to a 1.5 ml centrifuge tube.
2. Add 0.1 µg of the foreign DNA.
3. Add 7.5 µl of distilled water.
4. Heat at 45 °C for 3 min.

5. Transfer the tube to ice.
6. Add 1 µl (10X) T4 DNA ligase buffer.
7. Add 0.1 unit of T4 ligase.
8. Add 1 µl of 5mM ATP.
9. Incubate for 2-4 h at 16°C.
10. Use 2 µl of the ligation reaction to transform competent *E. coli* cells.

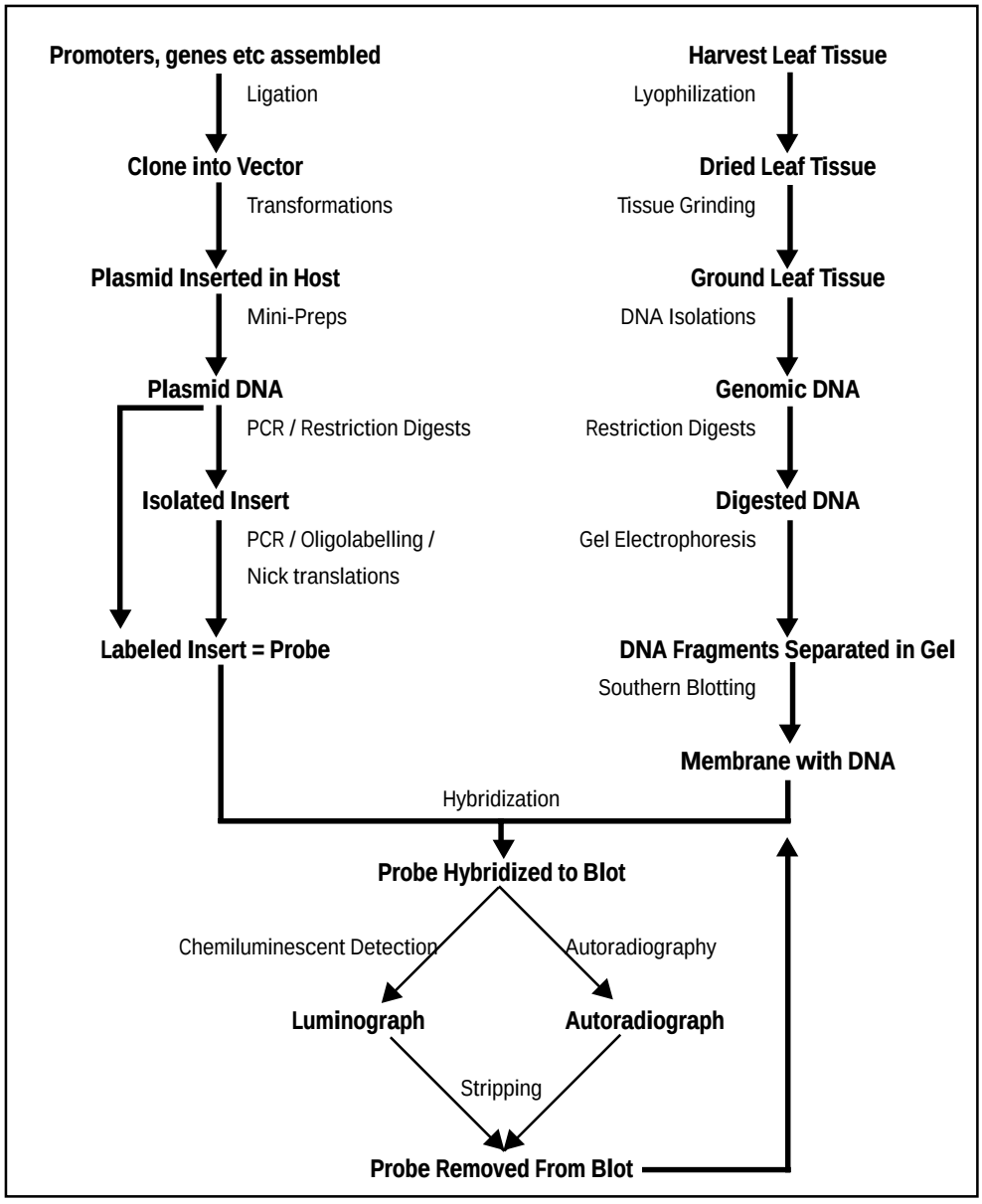
CONFIRMATION OF PLASMID INTEGRITY

1. Prepare bulk digestion mix using the appropriate enzyme and correct enzyme buffer.

STOCK	[FINAL]	Per 30 µl RXN
ddH_2O	—	25.0 µl
10X Buffer	1X	3.00 µl
0.1 M Spermidine	2.5 µM	0.75 µl
Enzyme (10 U/µl)	2.5 U	0.25 µl
Plasmid (1 µg/µl)	1µg	1.00µl

2. Add bulk mix to 500 µl microfuge tube containing plasmid and incubate at 37°C for 2-3 h. A 37°C oven works best because there is minimal condensation on the sides of the tube.
3. Stop reaction by adding 6 µl of 5X SGB.
4. Run the digested plasmid sample on an agarose gel (the % agarose used will depend on the anticipated size of the fragments).
5. Compare the size of the fragment with the information provided on the construct.

Scheme For Analysis Of Transgenic Plants Using Southern Hybridization



DNA Isolation

LYOPHILIZATION

1. Harvest leaves from greenhouse or field grown plants. It is preferable to use young leaves without necrotic areas or lesions, although older leaves that are not senescent may be used.
2. If the midrib is thick and tough, remove it. Cut or fold leaves into 10-15 cm sections and place in a fiberglass screen mesh bag along with the tag identifying the sample (Aluminum foil or paper bags may be substituted if holes are punched to allow good air flow). Place bags in an ice chest or other container with ice to keep samples cool (but do not allow to freeze).
3. Place leaf samples in a styrofoam container or some type of container able to hold liquid nitrogen. Add liquid nitrogen to quick-freeze samples. *Once frozen, do not allow samples to thaw until freeze-dried!*

NOTE: Leaf samples may be stored at -80°C until ready to be lyophilized.

4. Transfer frozen leaf samples to lyophilizer. Make sure that the lyophilizer is down to temperature (the chamber is £ -60°C) and pulling a good vacuum (£ 10 microns Hg) before loading samples. Do not overload lyophilizer—make sure vacuum is always £100 microns and condenser temperature is £ -60°C. Samples should be dry in 72 h. Typically, fresh weight [a] 10X dry weight.
5. Dried leaf samples may be stored in sealed plastic bags at room temperature for a few days or, preferably, at -20°C for several years.
6. Fill out a harvesting record sheet.

GRINDING

1. Grind to a fine powder with a mechanical mill (Tecator Cyclotec Sample Mill, Model 1093), or small coffee grinder if small amounts of tissue are being ground, into a plastic scintillation vial or any other appropriate plastic container that can be closed air tight. The finer the grind, the greater the yield of DNA from a given amount of material.

NOTE: If the plant material weighed less than 4 g fresh weight, grind to a powder with a mortar and pestle in the presence of a pinch of acid washed sand.

2. Store ground samples tightly capped at -20°C. Samples are stable for several years.

GENOMIC DNA EXTRACTION

(Based on method of Saghai-Maroof et al., 1984[*])

Theoretical background

Plants contain three types of DNA: nuclear, mitochondrial, and chloroplast DNA. Although quite elaborate methods exist for the isolation of each type of DNA, most experiments require only the rather simple preparation of total DNA. All DNA preparation methods involve the removal of the envelopes (cell wall and nuclear membrane) around the DNA; the separation of the DNA from all other cell components such as cell wall debris, proteins, lipids or RNA, and the maintenance of the integrity of the DNA during the procedure, i.e. the protection from nucleases and mechanical shearing. In the most common method, which is applicable to a whole range of plant material, cells are opened by grinding in liquid nitrogen. We prefer freezing harvested tissue in liquid nitrogen then lyophilizing it and grinding it to ensure a long-term supply of tissue from the various lines being analyzed. The low temperature prevents nucleases from degrading the DNA.

The problems in isolating DNA, especially from plants, are the presence of DNAase activities, which degrade the DNA, and the presence of other macromolecules, which co-purify with, or polymerize to, the DNA during the isolation procedure. The nuclease problem is reduced by removing cations such $Mg^{++,}$ which are required for nuclease activity. Agents such as EDTA, EGTA, and phenanthroline have been used at a range of concentrations, in different protocols, depending on the plant or animal species being analyzed. In addition

detergent agents such as sodium dodecylsulphate (SDS) are often used to inhibit enzyme activities. Plant researchers often encounter undesirable macromolecules, other than DNA, which create problems in the DNA isolation procedure. The problems created by the presence of phenolic compounds can be reduced by the addition of 1% (or higher) polyvinylpyrollidine (PVP) in the initial isolation buffer. Detergents such as SDS dissolve membranes and dissociate proteins from DNA and make them more accessible to degradation by proteinases used in the DNA isolation. A reagent that is used in several procedures is cetylmethylammonium bromide (CTAB), which binds strongly to DNA displacing protein and preventing degradation. The CTAB itself is removed by chloroform extractions leaving DNA in the aqueous phase to be ethanol precipitated. The aqueous phase contains DNA, RNA, polysaccharides, and some protein, while lipids are found in the organic phase and cell debris and most of the protein aggregate in the interphase. Ethanol precipitation concentrates the DNA.

cetylmethylammonium bromide (CTAB) is	$CH_3(CH_2)_{15}N^+(CH_3)_3Br^-$
sodium dodecyl sulphate (SDS) is	$CH_3(CH_2)_{11}OSO_3^-Na^+$
sarkosyl is	$CH_3(CH_2)_{11}N(CH_3)CH_2COO^-Na^+$

1. Weigh 300-400 mg of ground, lyophilized tissue, into a 15 ml polypropylene centrifuge tube. DNA yields range from 50 to more than 100 µg DNA / 100 mg dry tissue.
   If *higher* amounts are needed, start with 1 g lyophilized tissue into a 50 ml polypropylene centrifuge tube, and triple all the amounts given below. If *lower* amounts are needed, then weigh 100-150 mg lyophilized tissue into a 5 ml polypropylene centrifuge tube, and use 1/3 of the amounts given below.
2. Add 9.0 ml of warm (65°C) CTAB extraction buffer to the 300-400 mg ground, lyophilized tissue. It is best to distribute tissue along the sides of the tube before adding buffer, to avoid clumping of dry tissue in the bottom. Mix several times by gentle inversion.
3. Incubate for 60–90 min, with continuous gentle rocking in a 65°C oven.
4. Remove tubes from oven, wait 4–5 min for tubes to cool down, and then add 4.5 ml chloroform/octanol (24:1). Rock gently to mix for 5–10 min.
5. Spin in a table-top centrifuge for 10 min at 1300-1500 x g[1] at RT.

NOTE: Below 15°C, the CTAB/nucleic acid complex may precipitate; this could ruin the preparation and cause damage to the centrifuge.

6. Pour off top aqueous layer into new 15 ml tubes. Add 4.5 ml chloroform/octanol and rock gently for 5–10 min.
7. Spin in a table-top centrifuge for 10 min at 1300-1500 x g[1] at RT.
8. Pipette off top aqueous layer into new 15 ml tubes containing 30 µl of 10 mg/ml RNAse A (pre-boiled). Mix by gentle inversion and incubate for 30 min at RT.
9. Add 6.0 ml of isopropanol (2-propanol). Mix by very gentle inversion.
10. Remove precipitated DNA with glass hook[2]. Continue with OPTIONS A, B, or C.

OPTION A: PHENOL EXTRACTION, TO OBTAIN DNA OF HIGHER PURITY

NOTE: This option is highly recommended for DNA analyses based on the polymerase chain reaction (PCR). For RFLP analyses, this option is usually not necessary unless DNA does not digest properly. In fact, it is better to perform a phenol extraction only after restriction digestion; this improves DNA band separation and resolution after electrophoresis (see later sections for details).

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* Saghai-Maroof, M.A., K. Soliman, R.A. Jorgensen and R.W. Allard. 1984. Ribosomal DNA spacer-length polymorphisms in barley: mendelian inheritance, chromosomal location , and population dynamics. *PNAS* 81:8014-8018.

1 3000-3200 rpm in a Beckman GP or GPR centrifuge with swinging rotor (holding 56 x 15 ml tubes)

2 Prepare glass hook by first sealing the end of a 23 cm glass transfer pipette by heating in a flame for a few seconds. Then gently heat the tip 1 cm while twirling the pipette. When soft, allow the tip to bend into a hook. Cool before use. Used hooks can be cleaned by washing in dH_2O and EtOH.

11. Place hook with DNA in a 5 ml plastic tube containing 1 ml of TE; gently twirl hook until DNA slides off the hook. Cap tubes and rock gently overnight at room temperature to dissolve DNA.
12. Phenol extract each sample with 1 ml (1x original TE volume) of equilibrated phenol. Centrifuge the sample 10 min at 1300 x g[1] in swinging bucket rotor.
13. Transfer top (aqueous) layer to new 5 ml tube. Extract DNA with a 1 ml (1x original TE volume) of chloroform/octanol. Centrifuge the sample 10 min at 1300 x g[1] in swinging bucket rotor. Transfer top (aqueous) layer to new 5 ml tube. Continue with step **14** of OPTION B.

OPTION B: ETHANOL PRECIPITATION

14. Place hook with DNA in 5 ml plastic tube containing 1 ml of TE; gently twirl hook until DNA slides off the hook. Cap tubes and rock gently overnight at room temperature to dissolve DNA.
15. Precipitate DNA by adding 50 µl of 5 M NaCl and then 2.5 ml absolute EtOH (2.5 original TE volume), mix by gentle inversion.
16. Remove precipitated DNA with glass hook. Continue with step **17** of OPTION C.

OPTION C: DNA WASHES

17. Place hook with DNA in 5 ml plastic tube containing 3-4 ml of WASH 1. Leave DNA on hook in tube for about 20 min.
18. Rinse DNA on hook briefly in 1-2 ml of WASH 2 and transfer DNA to 2 ml microfuge tube (preferably Sarsted with screw-on lids to avoid possible evaporation of the TE) containing 0.3-1.0 ml TE (based on our experience we use 0.3-0.5 ml for maize and 0.5-1.0 ml for wheat); gently twirl hook until DNA slides off the hook. Cap tube and rock gently overnight at room temperature to dissolve DNA. Store samples at 4°C.

CTAB Extraction Buffer[1]

STOCK	[FINAL]	1 RXN 10 ml	5 RXN 50 ml	10 RXN 100 ml	20 RXN 200 ml	50 RXN 500 ml	56 RXN 600 ml
dH_2O		6.5 ml	32.5 ml	65.0 ml	130.0 ml	325.0 ml	390.0 ml
1 M Tris-7.5	100 mM	1.0 ml	5.0 ml	10.0 ml	20.0 ml	50.0 ml	60.0 ml
5 M NaCl	700 mM	1.4 ml	7.0 ml	14.0 ml	28.0 ml	70.0 ml	84.0 ml
0.5 M EDTA-8.0	50 mM	1.0 ml	5.0 ml	10.0 ml	20.0 ml	50.0 ml	60.0 ml
CTAB[2]	1 %	0.1 g	0.5 g	1.0 g	2.0 g	5.0 g	6.0 g
14 M BME[3]	140 mM	0.1 ml	0.5 ml	1.0 ml	2.0 ml	5.0 ml	6.0 ml

[1] Use freshly made; warm buffer to 60-65°C before adding the CTAB and BME.
[2] CTAB = Mixed alkyltrimethyl-ammonium bromide (Sigma M-7635)
[3] Add BME (ß-mercaptoethanol) just prior to use, under a fume hood.

WASH 1: 76% EtOH, 0.2 M NaOAc

STOCK	100 ml	200 ml	300 ml	400 ml	500 ml
Absolute EtOH	76 ml	152 ml	228 ml	304 ml	380 ml
2.5 M NaOAc	8 ml	16 ml	24 ml	32 ml	40 ml
dH_2O	16 ml	32 ml	48 ml	64 ml	80 ml

WASH 2: 76% EtOH, 10 mM NH_4OAc

STOCK	100 ml	200 ml	300 ml	400 ml	500 ml
Absolute EtOH	76 ml	152 ml	228 ml	304 ml	380 ml
1 M NH_4OAc	1 ml	2 ml	3 ml	4 ml	5 ml
dH_2O	23 ml	46 ml	69 ml	92 ml	115 ml

CHLOROFORM: OCTANOL: 24:1

STOCK	100 ml	200 ml	300 ml	400 ml	500 ml
Chloroform	96 ml	192 ml	288 ml	384 ml	480 ml
Octanol	4 ml	8 ml	12 ml	16 ml	20 ml

DNA EXTRACTION USING THE 'SAP EXTRACTOR'

(based on method of Clarke et al. 1989[*])

This method is particularly useful when large numbers of DNA samples are needed for PCR analysis as it is relatively quick and, although yields are low compared to the large–scale CTAB extraction method outlined above, it is sufficient for performing several PCR reactions. Potentially DNA from hundreds of samples can be extracted in a single day.

Setting up and using the sap extractor[1]

1. Make sure that the rollers are completely clean and that the flushing system for cleaning the rollers between samples is connected to a high pressure source of deionized water. If you can only use tap water to flush the rollers, make sure that you finally rinse them thoroughly with deionized or dH_2O between samples. Always wipe the rollers dry using clean, soft tissue paper before initiating the following sample extraction.
2. Position the buffer feeding tip over the upper half of the rollers to ensure that the buffer will mix effectively with the pressed tissue sample. Feed the tissue sample between the rotating rollers at a slight angle to ensure even pressure is applied to a single layer of the tissue (the tissue will wrap around one roller in a spiral).
3. Use 150-250 mg of freshly harvested leaf tissue kept in ice (within a tube) or frozen at -80°C (within a tube). It is critical that as you feed the tissue into the extractor, between the rollers, the buffer should already be at that position in the rollers; so make sure that you synchronize well this operation with the pumping of the buffer, otherwise DNA will be degraded. Pump 1.0 ml of extraction buffer and collect the extract in 2 ml tubes at the tips of the rollers.
4. Incubate the extracts in a water bath or an oven at 65°C for 20-40 min.; mix gently twice or continuously during this incubation. Remove the tubes from the heat and let cool down for 5–10 min.
5. Extract the samples with 1 ml of octanol-chloroform (1:24). Mix by inversion for 5 min., then spin in a table-top centrifuge at 3200 rpm for 10 min.
6. Transfer the aqueous supernatant containing the DNA to 2.0 ml Eppendorf tubes. If DNA has to be quantified precisely at the end of the extraction, add 10-20 µl of RNAse A + T1 (see other protocols) in the tube and incubate for 30 min. at 37ºC, or for one hour at RT.
7. Add 75 µl of 5M NaCl and precipitate DNA with 1 ml of cold absolute ethanol.
8. Spin DNA down, decant ethanol and dry under a weak vacuum for 30 min.
9. Resuspend overnight in the cold room in 200–500 µl TE, pH 8.0.
10. Quantify using a gel method or a TKO fluorometer. With this method, a minimum of 15 µg of DNA can be obtained.

Extraction Buffer[1]

STOCK	[FINAL]	10 ml	50 ml	100 ml	200 ml
dH_2O		1.7 ml	8.5 ml	17.0 ml	34.0 ml
1 M Tris-8.0	100 mM	1.0 ml	5.0 ml	10.0 ml	20.0 ml
5 M NaCl	2100 mM	4.2 ml	21.0 ml	42.0 ml	84.0 ml
0.5 M EDTA-8.0	150 mM	3.0 ml	15.0 ml	30.0 ml	60.0 ml
PVP	2 %	0.2 g	1.0 g	2.0 g	4.0 g
CTAB[2]	2 %	0.2 g	1.0 g	2.0 g	4.0 g
14 M BME [3]	140 mM	0.1 ml	0.5 ml	1.0 ml	2.0 ml

[1] Use freshly made; warm buffer to 60-65°C before adding the CTAB and BME.
[2] CTAB = Mixed alkyltrimethyl-ammonium bromide (Sigma M-7635)
[3] Add BME (ß-mercaptoethanol) just prior to use, under a fume hood.

Optional additional steps

If samples are not clean, repeat the octanol: chloroform step as outlined in steps 4 and 5 before continuing with the RNAse treatment or step 6. It is possible to wash the DNA pellet from step 7 with 75% EtOH.

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* Clarke, B.C., L.B. Moran and R. Appels. 1989. DNA analyses in wheat breeding. *Genome* 32: 334-339.

1 Sap (or juice) extractor: MEKU Erich Pollähne G.m.b.H. - 3015 Wennigsen, Am Weingarten 14, Germany.

Quantification and Quality Control of DNA

We use a semi-automated spectrophotometer equipped with a sipper to determine the quantity and purity of the DNA samples. The optical density of a dilution of each DNA sample is measured at 260 and 280 nm. The program in the spectrophotometer will compute the ratio of these two values (an indication of the purity), the DNA concentration (using the relation that an OD260=1 corresponds to a concentration of 50µg/ml), the total yield, and how much TE to add to each sample to bring them to the same concentration.

The TKO 100 minifluorometer is designed specifically to detect relative fluorescence at 460 nm. To achieve this, the TKO 100 uses a mercury lamp with a filtered detector. The lamp's emission spectrum peaks at 365 nm with a bandwidth of 100 nm and the photodetector is filtered to read light emitted at 460 nm with a 10 nm peak width. These optical characteristics make the TKO 100 ideal for DNA assays based on the binding of a fluorescent dye bis benzimidizole, commonly known as Hoechst 33258. DNA measurements using the minifluorometer are based on the binding of this fluorescent dye to DNA; the fluorescence depending partly on the A-T content of the DNA. For this reason calf thymus DNA (which has approximately 60% A-T and is double stranded and highly polymerized) is used for calibration purposes as it is comparable to most animal and plant DNA.

The agarose gel method allows you to check that the DNA is of high molecular weight, i.e., it has not been degraded. The spot fluorescence method is a relatively simple method for estimating DNA concentrations.

UV QUANTIFICATION OF DNA

1. Add 15 µl of each sample to 735 µl TE, mix well, and read OD_{260} and OD_{280} to determine purity. Refer to the Appendices for instructions on how to use the Beckman DU-65 spectrophotometer and for program listing for automated sample reading.
2. After UV quantification, adjust the concentration of each DNA sample to 0.3 µg/µl or a concentration of your choice and store at 4°C. Sample should be usable for up to 6 months.

$$\text{DNA concentration } (\mu g/\mu l) = \frac{(OD_{260} \times 50 \text{ (dilution factor)} \times 50\ \mu g/ml)}{1000}$$

The ratio OD_{260}/OD_{280} should be determined in order to assess the purity of the sample. If this ratio is 1.8 - 2.0, the absorption is probably due to nucleic acids. A ratio less than 1.8 indicates that there may be proteins and/or other UV absorbers in the sample, in which case it is advisable to reprecipitate the DNA. A ratio higher than 2.0 indicates the sample may be contaminated with chloroform or phenol and should be re-precipitated with ethanol (OPTION B).

A program for the Beckman DU-65 Spectrophotometer is included in the appendices which provides automated sample entry (with sipper) and calculates all appropriate values for each sample.

DNA QUANTIFICATION USING THE TKO 100 MINIFLUOROMETER

1. Turn on the fluorometer and allow it to warm up for at least 15 minutes.
2. Prepare the amount needed of working dye solution and cover with foil.
   CALIBRATION
3. Pipette 2 ml of working dye into the glass cuvette. Wipe the cuvette and insert into the well (always place the cuvette in the same orientation in the fluorometer) and close the cover.

4. With the "SCALE" knob adjusted to around 5 clockwise turns from fully counter-clockwise position, adjust the reading to "000" with the "ZERO" knob.
5. Add 2 µl of the DNA standard to the cuvette, mix well and reinsert into the fluorometer (in same position).

NOTE: DNA standard should be a similar type (linear, circular, GC%) to the samples to be measured. For plant DNAs (genomic and inserts) calf thymus DNA is fine. For low DNA concentrations, it is best to use a standard of 25 or 50 ng/µl. For high DNA concentrations, it is best to use a standard of 500 ng/µl.

6. Adjust the "SCALE" knob so that the reading equals the concentration of the standard.
7. Steps 3–6 can be repeated once or twice until little change in the reading is observed.
   QUANTIFICATION
8. Rinse cuvette with dH_20, dry and pipette 2 ml of working dye into the glass cuvette. Wipe the cuvette, insert into the well (always place the cuvette in the same orientation in the fluorometer) and close the cover.
9. With the "ZERO" knob adjust the reading to "000**". DO NOT ADJUST THE "SCALE" KNOB.**
10. Add 2 µl of the DNA sample to the cuvette, mix well and re-insert into the fluorometer (in same position).
11. Repeat steps 8–10 for each sample.
12. If you have many samples, check the 000 reading every 10–15 samples.

NOTE: When the DNA concentrations are very high, it is necessary to make dilutions before reading the concentration with the fluorometer.

10X TNE Buffer (0.1M Tris-7.4, 10 mM EDTA, 1 M NaCl)

STOCK	500 ml	1000 ml	2000 ml
Tris-base (MW 121.10)	6.05 g	12.1 g	24.2 g
EDTA (MW 372.2)	1.85 g	3.7 g	7.4 g
NaCl (MW 58.44)	29.20 g	58.4 g	116.8 g

Adjust pH to 7.4 with HCl, add ddH_2O to make 1 liter, filter to sterilize and to remove any particulate matter.

1 mg/ml H33258 Dye Stock Solution

STOCK	10 ml
Hoechst 33258	10 mg
Filtered ddH_2O	10 ml

DO NOT FILTER OR AUTOCLAVE DYE SOLUTION.
Wrap tube with aluminum foil and store in refrigerator.

CAUTION: The Hoescht dye is toxic, irritant and carcinogenic — wear gloves and goggles when handling and use extra precaution.

Working Dye Solution

STOCK	50 ml	100 ml
1 mg/ml Dye Stock	10 µl	20 µl
10X TNE	5 ml	10 ml
Filtered ddH_2O	45 ml	90 ml

DNA QUANTIFICATION BY SPOT FLUORESCENCE

1. Place a UV-transparent Plexiglas tray over UV Transilluminator (a gel tray is fine).
2. Spot several 3 µl drops of TE containing 2 mg/ml EtBr on tray.
3. Add 1 µl of a series of known DNA concentration (l DNA at 0, 1, 2.5, 5, 10 & 20 ng/µl) standards to the EtBr drops. Mix by pipetting up and down several times.
4. Add 3 dilutions (1/2, 1/4, 1/8) of each of your unknown DNA samples to the ethidium bromide drops as follows:
   Mix 1 µl of DNA sample and 1 µl TE, pipette 1 µl into a 3 µl EtBr drop and mix.
   Add 1 µl of TE to that sample drop, pipette 1 µl into a 3 µl EtBr drop and mix.
   Add 1 µl of TE to that sample drop, pipette 1 µl into a 3 µl EtBr drop and mix.
5. Photograph the spots using UV illumination so that the lowest concentration standard is barely visible (this is essential for precise quantification).
6. Estimate the concentration (in ng/µl) of the DNA sample by comparing the intensity of the fluorescence in the samples with the standard drops.

NOTE: The DNA standards should be similar in structure to the sample you wish to measure. Work very fast to avoid any evaporation from the drops before the photograph is made.

DNA QUALITY CONTROL

This step is essential for checking that the isolated DNA is of high molecular weight. For adequate resolution of RFLPs, native DNA should migrate as a tight band of molecular weight ≥40 Kb. However, degradation of part of the isolated DNA is inevitable, and the protocol below is designed to run the DNA under optimal conditions for ascertaining the relative amounts of degraded and high molecular weight DNA. The procedure also allows for verifying the UV quantification performed above.

If you have few DNA samples (say less than 25), check all of them. Otherwise, check only 10-20% of the samples, making sure that the selection is totally random.

1. Prepare a 10 ng/µl dilution of the selected samples (e.g., 4 µl DNA at 0.3 µg/µl + 116µl TE).
2. Load 100 ng of each diluted sample (10 µl DNA + 2 µl 5X SGB) in a 0.7% agarose gel. Include at least one lane per comb of uncut Lambda DNA (l) as a molecular weight marker. Load 100 ng (from a 10 ng/µl dilution) of this marker in order to check both quality and quantity of the sample DNAs.
3. Run the gel at 50 mA for about 90 min. See the section on gel electrophoresis for details about gel preparation, running conditions, and DNA visualization.

Restriction Digestion of Genomic DNA

Theoretical background

Although there are three types of restriction endonucleases, those used in molecular biology are type II enzymes, which recognize specific 4–8 bp long sequences and cut DNA of any source within this sequence. For example the restriction endonuclease *Hind*III cleaves after the first A in the sequence AAGCTT. The assay conditions vary from enzyme to enzyme, but generally require $MgCl_2$, a pH between 7 and 8, NaCl between 10 and 150 mM and an incubation temperature, which is 37°C in most cases.

NOTE: The restriction enzyme must be kept on ice at all times and should be kept outside the -20°C freezer for the minimum time possible. Use a fresh yellow tip every time you pipette enzyme from the stock into your sample.

DNA DIGESTIBILITY TEST

This step is essential before setting up large-scale digestion experiments. A small amount of DNA is digested with restriction endonucleases under the conditions described in the next section in order to check the quality of the digest. If you have few DNA samples (say less than 25), check all of them. Otherwise, check only 10-20% of the samples, making sure that the selection is totally random.

1. Put 2 µg of each DNA sample in a 0.5 ml microfuge tube.
2. Prepare a bulk digestion mix based on the recipe given below, and keep it on wet ice. Add 8 µl of this to each of the tubes containing the DNA. Mix thoroughly but gently and spin down the tube contents.

STOCK	[FINAL] or amount	Per 15 µl RXN	Example of bulk digestion mix for 20 samples*
DNA (0.3 µg/µl)	2 µg	7.0 µl	—
ddH_2O	—	5.6 µl	112 µl
10X Buffer	1X	1.5 µl	30 µl
0.1 M Spermidine	2.5 mM	0.4 µl	8 µl
Enzyme (10 U/µl)	2.5 U/µg DNA	0.5 µl	10 µl

* Always prepare bulk mixes for the total number of reactions +1 to allow for pipetting errors.

3. Incubate at 37°C for 1.5 to 3h. Stop the reactions with 3 µl of 5X SGB.
4. Load samples in a 0.7% agarose gel and run the gel at 40 mA for 2-3 h. Use Lambda DNA digested with *Hind*III as a molecular weight marker. See the section on gel electrophoresis for details about gel preparation, running conditions, and DNA visualization.

LAB PROTOCOL FOR RESTRICTION DIGESTION OF GENOMIC DNA

(based on method from T. Helentjaris, NPI)

NOTES

Essentially two situations arise typically when setting up large–scale digestion experiments. On the one hand there may be a few (£10) DNA samples to be digested in large quantities for screening purposes (say 24 to 48 repetitions). On the other, there may be a large number of samples (e.g. a mapping population) to be digested for a specific number of gel separations (say 4 to 10 repetitions). In both cases, the large amount of DNA of each sample is digested all at once with each enzyme, and in a larger volume than the gel loading volume. Thus, after digestion is complete, the DNA is ethanol precipitated, then resuspended in the proper loading volume. The protocols below therefore include a consideration of reaction volumes and corresponding tube sizes for practical purposes.

Phenol extraction after digestion is only necessary when the highest quality of DNA migration and separation in gels are required, as for example in the case of molecular diversity comparisons or fingerprinting work. The tables given in this protocol assume a DNA concentration of 0.3 µg/µl and an enzyme concentration of 10 U/µl. They contain information for the maximum quantities that can be processed for any given reaction tube size.

BULK DIGESTION OF DNA SAMPLES

Calculations

NOTE: We routinely digest 10 µg maize DNA or 15 µg wheat DNA per single-layer gel lane.

1. Determine the total µg and volume of each DNA sample to be digested with an enzyme in a single tube as follows:
   Total µg DNA = (amount of DNA per lane) x (number of lanes of sample)
   Total µl DNA = (Total µg DNA) / (DNA concentration, µg/µl)
2. Determine the units (U) and volume of enzyme necessary to digest each DNA sample. In general, it is best to use 2.5 U/µg DNA to prevent partial digestions.
   Total U Enzyme = (Total µg DNA) x 2.5
   Total µl Enzyme = (Total U enzyme) / (enzyme concentration, U/µl)
3. Based on the DNA and enzyme volumes, determine the total reaction volume and therefore the tube size to use. The maximum reaction and corresponding maximum DNA volumes possible for different tube sizes are given below.

µg DNA (at 0.3 µg/µl)	Range of DNA vol	Tube size	Vol. of RXN	10X buffer	0.1 M spermidine
10 - 90 µg	35 - 300 µl	1.5 ml	400 µl	40 µl	10 µl
90 - 120 µg	300 - 400 µl	2.0 ml	550 µl	55 µl	14 µl
120 - 300 µg	400 - 1000 µl	5.0 ml	1300 µl	130 µl	33 µl
300 - 900 µg	1000 - 3000 µl	15.0 ml	4000 µl	400 µl	100 µl

4. Determine the volume of ddH_2O per tube as follows[1]:
   µl ddH_2O = (total RXN vol.) - (µl buffer + µl spermidine + µl DNA + µl enzyme)
5. Calculate a bulk digestion mix containing the total volume of ddH_2O, buffer, spermidine, and enzyme needed for the total number of different DNA samples to be **digested by the same enzyme**. To allow for pipetting errors prepare extra bulk mix as follows:
   For 1.5 or 2.0 ml tubes, prepare bulk mixture for one or two additional RXN tubes;
   For 5 ml tubes, prepare 1/4 more bulk mixture;
   For 15 ml tubes, prepare 1/10 more bulk mixture.

Digestion reactions

6. Label the tubes for the reactions, and add the proper amount of DNA sample to be digested.
7. Prepare bulk mix on ice, adding enzyme last; mix well.
8. Aliquot bulk mix into reaction tubes. Mix well (do not vortex).
   µl bulk mix / tube = (µl RXN vol./tube) - (µl DNA/tube)
9. Incubate at 37°C for 3–5 h.

[1] **Calculations for maximum DNA digestions per tube size:**

		µg DNA / Tube Size / RXN vol/			
STOCK	[FINAL] or amount	90 µg /1.5 ml / 400 µl	120 µg /2.0 ml/ 550 µl	300 µg /5.0 ml/ 1300 µl	900 µg /15.0 ml/ 4000 µl
ddH_2O	—	27.5 µl	51 µl	62.5 µl	275 µl
10X Buffer	1X	40.0 µl	55 µl	130.0 µl	400 µl
0.1 M Spermidine	2.5 mM	10.0 µl	14 µl	32.5 µl	100 µl
10 U/µl Enzyme	2.5 U/µg	22.5 µl	30 µl	75.0 µl	225 µl
0.3 µg/µl DNA	—	300.0 µl	400 µl	1000.0 µl	3000 µl

Precipitation of digested DNA

10. Stop the reaction by adding 5 M NaCl to a final concentration of 0.25 M NaCl.
11. Add 2.5 volumes of EtOH, mix well, place at -80°C for 30 min or at -20°C overnight. Precipitated DNA can be stored in EtOH at -20°C for an indefinite time.

Tube size	Vol. of RXN	µl 5M NaCl	µl EtOH	Total vol. after EtOH
1.5 ml	400 µl	20 µl	1000 µl	1420 µl
2.0 ml	550 µl	28 µl	1375 µl	1953 µl
5.0 ml	1300 µl	65 µl	3250 µl	4615 µl
15.0 ml	4000 µl	200 µl	10000 µl	14200 µl

12. Centrifuge in microfuge at full-speed for 10-15 min. For larger digestion volumes use siliconized Corex tubes and centrifuge at 10,000 x g for 10 min.
13. Pour off supernatant and invert tubes to drain. Evaporate EtOH from samples by placing tubes upright in a vacuum desiccator for 10-15 min under low vacuum, or overnight on the bench. Take care to remove all EtOH as this makes samples impossible to load into gels. However, avoid overdrying as this makes samples difficult to resuspend.
14. Dissolve pellet in the proper volume of TE for loading into wells of an agarose gel. Typically, 16 µl of TE and 4 µl of 5X SGB per single-layer well is sufficient, while 40 µl TE and 10 µl 5X SGB are needed for a double-layer well. Dissolve DNA in TE first, then add 5X SGB. Generally, pellets are dissolved in 2–3 h.

10X BRL React® 1 (*Dra*I)

STOCK	[FINAL]	1.0 ml	5.0 ml	10.0 ml
1 M Tris-8.0	500 mM	500 µl	2.5 ml	5.0 ml
1 M $MgCl_2$	100 mM	100 µl	0.5 ml	1.0 ml
ddH_2O		400 µl	2.0 ml	4.0 ml

10X BRL React® 2 (*Eco*RV, *Hin*dIII, *Pst*I, *Xba*I)

STOCK	[FINAL]	1.0 ml	5.0 ml	10.0 ml
1 M Tris-8.0	500 mM	500 µl	2.5 ml	5.0 ml
1 M $MgCl_2$	100 mM	100 µl	0.5 ml	1.0 ml
5 M NaCl	500 mM	100 µl	0.5 ml	1.0 ml
ddH_2O		300 µl	1.5 ml	3.0 ml

10X BRL React® 3 (*Eco*RI, *Bam*HI)

STOCK	[FINAL]	1.0 ml	5.0 ml	10.0 ml
1 M Tris 8.0	500 mM	500 µl	2.5 ml	5.0 ml
1 M $MgCl_2$	100 mM	100 µl	0.5 ml	1.0 ml
5 M NaCl	1 M	200 µl	1.0 ml	2.0 ml
ddH_2O		200 µl	1.0 ml	2.0 ml

10X Boehringer SuRE/Cut Buffer B (*Bam*HI, *Eco*RV, *Hin*dIII)

STOCK	[FINAL]
1 M Tris-8.0	100 mM
1 M $MgCl_2$	50 mM
5 M NaCl	1000 mM
BME[1]	10 mM

10X Boehringer SuRE/Cut Buffer H (*Eco*RI, *Pst*I, *Pvu*I, *Xba*I)

STOCK	[FINAL]
1 M Tris-7.5	500 mM
1 M $MgCl_2$	100 mM
5 M NaCl	1000 mM
DTE[2]	10 mM

10X Boehringer SuRE/Cut Buffer M (*Dra*I, *Hae*III)

STOCK	[FINAL]
1 M Tris 7.5	100 mM
1 M $MgCl_2$	100 mM
5 M NaCl	500 mM
DTE[2]	10 mM

[1] BME ß-mercaptoethanol
[2] DTE dithioerythritol

MOLECULAR WEIGHT MARKERS FOR GEL ELECTROPHORESIS

NOTE: Two types of molecular weight (MW) standards are routinely used for subsequent detection using chemiluminescent techniques. The Lambda/*Hin*dIII MW standards provide a useful reference for calculating molecular weights of DNA fragments after electrophoretic separation; the "internal MW standards" provide a means for normalizing fragment migration distances within each lane to facilitate comparisons between lanes on the same or different luminographs in fingerprinting studies.

End-labeled Lambda (λ) DNA as a molecular weight standard for luminographs:

Digestion of λ DNA with *Hin*dIII:

STOCK	[FINAL] or amount	50 µl RXN
ddH_2O	—	30.3 µl
10X Buffer	1X	5.0 µl
0.1 M Spermidine	2.5 mM	1.2 µl
λ DNA (0.4 µg/µl)*	5 µg	12.5 µl
*Hin*dIII (10 U/µl)	2 U/µg DNA	1.0 µl

Check the concentration of commercial λ and adjust quantities accordingly.

1. Allow to digest at 37°C for 1-2 h.
2. Precipitate by adding 5 µl of 2.5 M NaOAc and 125 µl of absolute EtOH, mix well by inversion, and place at -80°C for 30 min.
3. Centrifuge in a microfuge for 10-15 min at full-speed. Pour off supernatant and invert tubes to drain. It is very important to allow the pellet to dry.
4. Resuspend the pellet in 15 µl ddH_2O. Assuming little or no DNA loss during precipitation, the concentration should be about 5 µg/15 µl or 0.33 µg/µl.

End-labeling of λ/*Hin*dIII DNA with digoxigenin-dUTP (dIG-dUTP):

STOCK	[FINAL] or amount	50 µl RXN
[FINAL]ddH_2O	—	25.0 µl
10X Klenow Buffer	1X	5.0 µl
10 mM dATP	100 µM	0.5 µl
10 mM dCTP	100 µM	0.5 µl
10 mM dGTP	100 µM	0.5 µl
1 mM dig-dUTP	40 µM	2.0 µl
λ/*Hin*dIII DNA*	5 µg	15.0 µl
2U/µl Klenow**	3 U	1.5 µl

* Check the concentration of commercial λ and adjust accordingly.
** Purchase from Fisher Scientific (cat. # PR-M2201 Promega-Biotec) or BRL (cat. # 80125B)

5. Incubate at 37°C for 1.5 h.
6. Stop the reaction by placing at 65°C for 15 min.
7. EtOH precipitate as in (2) above.
8. Resuspend in 250 µl TE to bring to a final concentration of 20 ng/µl. This stock can then be diluted to 1 ng/µl with TE.
9. Verify incorporation of dig-dUTP following the protocol "Checking the Activity of Incorporated Digoxigenin-dUTP."
   Use 5 ng/lane of l DNA digested with HindIII and end-labeled with digoxigenin-dUTP.
10. Prepare working solutions from the stocks based on the following proportions:

STOCK	1 ng/µl STOCK	20 ng/µl STOCK
l DNA end labeled	5 µl	0.25 µl
TE	11 µl	15.75 µl
5X SGB	4 µl	4.00 µl

Digestion of ØX DNA with *HAE*III:

STOCK	[FINAL] or amount	150 µl RXN
ddH_2O	—	68.25 µl
10X Buffer	1X	15.00 µl
0.1 M Spermidine	2.5 mM	3.75 µl
ØX DNA (0.25 µg/µl)*	15 µg	60.00 µl
*Hae*III (10 U/µl)	2 U/µg DNA	3.00 µl

Check the concentration of commercial ØX and adjust quantities accordingly.

1. Allow to digest at 37°C for 2-3 h.
2. Check that the digestion is complete by running about 50 ng on a 0.7% agarose gel.
3. Inactivate enzyme by incubating at 65°C for 10 min. Then add 300 µl TE and 150 µl 5X SGB to bring to a concentration of 25 ng/µl. Aliquot (200 µl per 0.5 ml tubes) and keep at 4°C or in the freezer.

DNA ELECTROPHORESIS AND SOUTHERN BLOTTING

Electrophoresis through agarose or polyacrylamide gels is the standard method to separate, identify and purify DNA fragments. Although agarose gels have a lower resolving power than polyacrylamide gels, they have a greater range of separation and are much simpler and easier to handle than the polyacrylamide ones, and have been more widely used in DNA electrophoresis.

Agarose, a purified form of agar isolated from seaweed, is a linear polymer. Various grades and forms of agarose are available commercially. Agarose gels are cast by melting the agarose in the presence of the desired buffer until a clear, transparent solution is achieved. The melted solution is then poured into a mold and allowed to harden. Upon hardening, the agarose forms a matrix, the density of which is determined by the concentration of the agarose. The gel is then submerged in "running buffer" and the DNA samples are loaded into wells that were cast into the gel using a plastic comb at the time of preparation. An electrical field is then applied across the length of the gel and the negatively charged DNA molecules migrate toward the anode of the electrophoretic system. The rate at which linear DNA molecules migrate within the gel depends on their length: larger molecules migrate more slowly than smaller ones because it is more difficult for them to pass through the agarose pores. Thus, the DNA molecules become separated according to their size. At high gel concentrations the resolution of smaller fragments is favored, while at low agarose concentrations that of larger fragments is favored . (see Table 1). DNA is visualized by soaking the gel in a solution of ethidium bromide; this fluorescent dye intercalates between the two strands of DNA and makes them visible under ultraviolet light.

Table 1. Range of separation of DNA fragments in gels containing different amounts of agarose (Sambrook et al. 1989)

Amount of agarose in gel (%w/v)	Efficient range of separation of linear DNA fragments (kb)
0.3	5-60
0.6	1-20
0.7	0.8-10
0.9	0.5-7
1.2	0.4-6
1.5	0.2-3
2.0	0.1-2

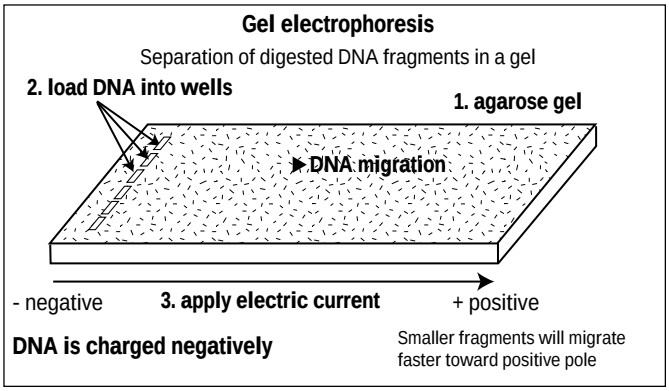


Figure 1. Gel electrophoresis of DNA

NEUTRAL AGAROSE GEL ELECTROPHORESIS

(based on method from T. Helentjaris, NPI)

1. Add agarose to proper amount of 1X TAE Gel Buffer. Typical gel sizes are listed below:

Gel Size	Agarose (0.7%)*	1X Gel Buffer	Sample Volume/Well
6 x 8 cm	0.17 g	25 ml	10 µl
11 x 14 cm	0.52 g	75 ml	20 µl
11 x 20 cm	1.05 g	150 ml	20 µl
20 x 25 cm	2.10 g	300 ml	20 µl

2. Melt agarose in microwave oven, mixing several times during heating. Cool to 55°C (if desired, can place container into cool water to speed cooling) keeping covered to avoid evaporation.
3. Tape the ends of gel tray, pour agarose into tray and insert combs. Allow to solidify (20-30 min).
4. Remove tape and place tray in rig with 1X TAE gel buffer. Pour enough 1X gel buffer into the gel rig to cover the gel by at least 0.5 cm. Remove combs only when ready to load samples.
5. Run samples into gel at 100 mA for 5–10 min, then run at 15-20 mA, constant current, until the bromophenol blue dye has migrated to just above the next set of wells. This will typically take 14–16 hours for a large gel with four combs and a dye migration of about 6 cm. You may run gel at a higher rate, however resolution of the samples may suffer. Resolution can be improved by recirculating the buffer.
6. Remove tray from rig and stain in 1 µg/ml ethidium bromide (100 µl of 10 mg/ml ethidium bromide in 1000 ml dH_2O) for 20 min with gentle shaking.
   CAUTION: *Ethidium bromide is extremely mutagenic—wear double gloves when handling and use extra precaution.*
7. Rinse gel in dH_2O for 20 min, slide gel onto a UV transilluminator and photograph.
   For Fotodyne PCM-10 camera with 20 x 26 cm hood and Type 667 Polaroid film use an f8 or f5.6, 1 second exposure.

* Use higher gel concentrations for separation of small fragments such as plasmids and probe inserts.

10X TAE Gel Buffer: 400 mM Tris, 50 mM NaOAc, 7.7 mM EDTA

STOCK	1 liter	2 liters	3 liters	4 liters	5 liters
Tris Base (MW=121.10)	48.40 g	96.80 g	145.20 g	193.60 g	242.0 g
NaOAc (MW=82.03)	4.10 g	8.20 g	12.30 g	16.40 g	20.5 g
Na_4EDTA (MW=380.20)	2.92 g	5.84 g	8.76 g	11.68 g	14.6 g

pH to 8.0 with glacial acetic acid.

DOUBLE THICK GELS

A "double-thick" gel consists of two layers of agarose poured consecutively into the same mold with the combs in position. After electrophoresis, the two layers are separated and thus yield two separate, duplicate blots. The samples should therefore have the exact volume of the resulting 'double-height' wells; this will ensure that each gel layer contains approximately the same amount of DNA per lane.

There are at least two good reasons for running double-thick gels: the procedure cuts in half the number of potential loading mistakes, and it doubles the output of membranes given a fixed number of double-thick gels. In our laboratory, one person can load, run and blot a maximum of four double-thick gels in one and a half normal working days. This represents a total output of 4 x 2 x 120 = 960 lanes for analysis.

1. Add agarose to total amount of 1X TAE Gel Buffer.

Gel Size	Agarose (0.7%)	Total 1X Gel Buffer	First Layer	Second Layer	Sample Volume
20 x 25 cm	4.62 g	660 ml	280 ml	380 ml	50 µl

2. Melt in microwave oven, mixing several times during heating. Cool to 55°C (if desired, can place container into cool water to speed cooling) keeping covered to avoid evaporation.
3. Tape the ends of gel tray so that the tray will be able to accommodate 2 layers. Pour the indicated first layer amount of agarose measured in a clean, warmed, graduated cylinder into tray and then insert combs. Allow to solidify 20–30 min.
4. Allow second layer of gel solution to cool to 55°C and pour over first layer. Pour the solution slowly, gradually moving back and forth across the bottom end of the gel rig so as to avoid melting a hole in the bottom layer. Allow to solidify 20–30 min.
5. Remove tape and place tray in rig. Pour enough 1X gel buffer into the gel rig to cover the gel, then remove combs and load samples into the wells. Load the wells of the gel to the top of the second layer. It typically takes 50 to 60 µl to fill each well.
6. Run samples into gel at 100 mA for 5-10 min, then run at 25 mA, constant current, until the bromophenol blue dye has migrated to just above the next set of wells. Typically the gel will be done after 14-16 h. Resolution can be improved by recirculating the buffer.
7. Remove tray from rig. Place the double thick gel in a large tray with 1X gel buffer from the run to almost cover the gel. Split the gel layers at the corner of the double gel with a thin spatula. Then, starting at this split, slowly run a 1 ml glass pipette between the two layers at a slight angle. Hold the pipette firmly at both ends with two hands and slide it until the two gel layers come apart. Take care not to break the gel along the wells.
8. Stain each gel in 1 µg/ml ethidium bromide (100 µl of 10 mg/ml ethidium bromide in 1000 ml dH_2O) for 20 min with gentle shaking.
   CAUTION: Ethidium bromide is extremely mutagenic—wear double gloves when handling and use extra precaution.
9. Rinse gel in dH_2O for 20 min, slide gel onto a UV transilluminator and photograph.
   For Fotodyne PCM-10 camera with 20 x 26 cm hood and Type 667 Polaroid film use an f8 or f5.6, 1 second exposure.

10X TAE Gel Buffer - see previous protocol

Southern Blotting

When plant or animal DNA is digested with restriction endonucleases and the DNA fragments are separated by gel electrophoresis, the result is a smear of millions of bands (Fig. 2). If, however, the vast array of fragments are transferred to nitrocellulose or nylon membranes and assayed for *specific* sequences a limited number of size classes of fragments are visualized and these provide a glimpse into DNA structure surrounding the sequence which was used as a probe.

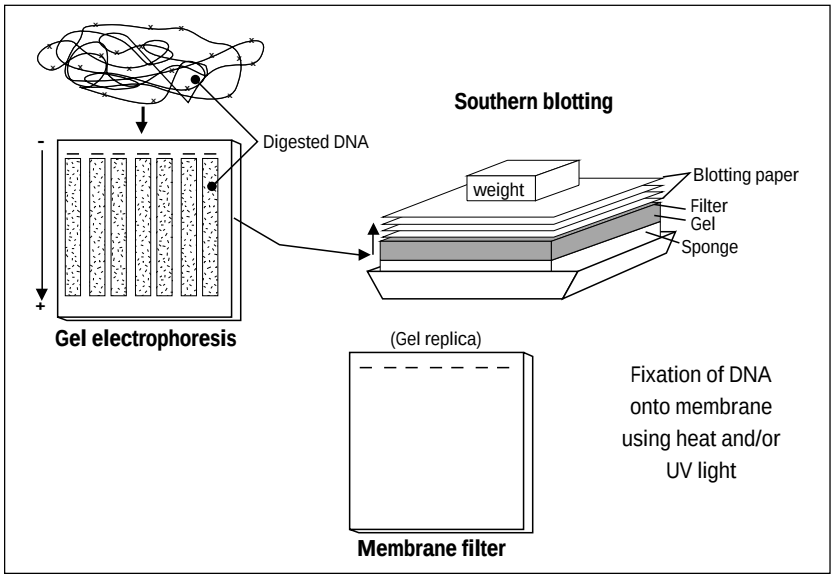


Figure 2. Digested DNA and capillary blotting.

Transferring single-stranded DNA and denatured double-stranded DNA from an agarose gel onto a filter matrix made of nylon or nitrocellulose is achieved by **Southern blotting**. The steps involved are:

- preparation of the agarose gel for blotting,
- blotting, and
- fixation of the DNA to the filter membrane.

There are a number of ways to perform a Southern blot which include capillary blotting, electrophoretic transfer (electroblotting), vacuum blotting and positive pressure blotting. Capillary blotting is by far the most popular method for blotting agarose gels and is illustrated in Fig. 2. Three steps prior to blotting the gel are essential for the further successful use of the resulting blot. These are depurination, denaturation, and neutralization of the DNA in the agarose gel.

In capillary blotting, DNA fragments are transferred out of the gel in a flow of liquid drawn through the gel by simple capillary action. The larger the fragments that leave the gel, the slower the rate at which they transfer. As blotting proceeds, the gel becomes dehydrated and thus more concentrated, up to a point at which DNA molecules will, for practical purposes, not leave the gel any longer. At this point, smaller fragments will have mostly transferred and therefore something may have to be done to increase the efficiency of transfer of larger DNA molecules. This may be done by partial depurination of the DNA in the gel, which involves the removal of some of the purines from the electrophoresed DNA and ultimately its cleavage into smaller fragments. The covalent bond that connects a purine base to a deoxyribose unit in DNA is more sensitive to HCl than the bond that connects this sugar with a pyrimidine base; thus, treatment of the gel with HCl will preferentially remove purines from DNA.

During the next step, that of denaturation of the DNA in the gel, the phosphodiester bonds connecting the backbone of the DNA strand are cleaved at sites of depurination resulting in DNA fragmentation. The single-stranded DNA resulting from denaturation will transfer more efficiently than double-stranded DNA (this treatment alone may be sufficient for effective transfer under certain conditions) and is of course essential for the subsequent hybridization of this template to DNA probes. Finally, in order to avoid some of the hybridization background problems that may arise after alkali treatment of the gel (and ultimately the membrane filter), it is recommended that this is neutralized before blotting. If you do perform an alkali blotting procedure, you will have to neutralize the actual blot, as nucleic acid hybrids are unstable at high pH.

After blotting it is essential to fix the DNA onto the membrane following the instructions of the manufacturer. Fixing can be achieved by baking the membrane at high temperatures (80-95°C) and/or by UV cross-linking of the DNA to the filter matrix; the latter method is one major advantage of nylon membranes over nitrocellulose ones. In this process, covalent bonds are formed between the DNA and chemical groups on the surface of the membrane.

SOUTHERN BLOTS USED FOR ANALYSIS OF TRANSGENIC PLANTS

Southern blot analysis is one of the more powerful tools available for molecular characterization of transgenic plants. Depending on how the restriction digests are designed, information regarding the complexity of transgene insertion(s), the number of transgene copies present, the integrative status and the number of chromosomal sites where the transgene(s) has inserted can be obtained.

Often three treatments are needed for plant DNA digestion: (1) undigested plant DNA; (2) digested by enzymes which excise the entire fragment for which information is desired, most often this will be a plant transcription unit (PTU) (all the vector components that contribute to transgene expression, ie. promoter, coding sequence and any regulatory sequences); and (3) digested by one enzyme which cuts the vector at only one site. It is common to use a transgene coding sequence as the probe used for hybridization. The vector DNA used for transformation should be digested and analyzed alongside sample genomic DNA to provide necessary controls for evaluating rearrangements and copy numbers. Sample bands of a different size from the control band are considered rearranged. For positive controls a known transgenic plant containing the same gene as the "putative" transgenics being evaluated is very useful. If a known transgenic plant is not available the vector DNA can be used instead. For negative controls a non-transgenic plant, of the same genotype as the "putative" transgenic, should be included.

Copy number

Copy number reconstructions can be made by diluting vector DNA to appropriate concentrations in a matrix of DNA from untransformed plants of the same genotype as the transgenic plant.

To determine copy numbers of transgene, the following formula is used:

$$\frac{\text{Size of plasmid (base pairs)}}{\text{Size of plant genome (bp)}} = \frac{\text{DNA equivalent to one copy of transgene in the plant}}{\text{Amount of plant DNA used in the reconstruction}}$$

DNA equivalent to one copy of the transgene in the plant can be calculated using this formula and is then digested with the same restriction enzyme(s) as used for the transgenic plants being screened. The same can be done to reconstruct 5, 10 or any number of copies. Following hybridization with an appropriate labeled probe, signal strengths of these controls can be compared with those in the samples to estimate copy number. However, only rough estimates (+/- 2-3 fold) can be made using this approach. If a more accurate estimate of copy number is required it is possible to probe for an endogenous gene having a known copy number.

INTEGRATION COMPLEXITY

Southern analysis can be used to provide evidence for transgene integration although care should be taken when evaluating results. Analysis is simpler when the material being analyzed was produced using *Agrobacterium*, as the T-DNA right border typically provides a defined junction between vector and plant DNA that can be exploited. In this case, the presence of multiple bands provides evidence for multiple insertion sites, but not proof, as multiple bands can also result from transgene rearrangement.

When plants have been transformed using direct DNA delivery, such as particle gun bombardment, vector-plant DNA junctions are more random and therefore two approaches can be taken. In one, the restriction enzyme cuts only within the plant DNA and not the vector used for transformation. The presence of multiple DNA bands is diagnostic for integration at multiple sites. Because the resulting transgene-containing fragments can be fairly large (particularly if the DNA used for transformation was larger than approximately 5-8 kb), multiple fragments may not be resolved using standard agarose electrophoresis conditions. An alternative is to use a restriction enzyme that cuts once within the vector. This approach tends to produce smaller transgene-containing fragments but multiple bands due to transgene rearrangements and multimerisation at single insertion sites can also occur. To demonstrate unambiguously that a transgene has been integrated into the plant genome it is necessary to observe Mendelian inheritance in subsequent generations.

PROBLEMS WITH ENDOGENOUS SEQUENCES

Southern analysis of transgenes can be particularly problematic when PTU sequences are present in the untransformed host genome. In some cases., this problem can be circumvented by using a probe to a part of the PTU not present in the genome, but this is not always possible. In cases where the probe hybridizes to endogenous, as well as transgene, DNA, several steps can be taken. The first thing is to try to use restriction enzymes that produce as simple a pattern as possible when the untransformed DNA is analyzed. Secondly, these fragment size(s) should be sufficiently different from those of the transgene fragment, such that interpretation of the band pattern in transformed material is not ambiguous. It is also vital that untransformed genomic DNA controls are included on every row of a gel. Another possible way around this problem is to incorporate a molecular "tag" of known sequence into the construct used for transformation. Antibodies against this tag can be used to detect the expression of the known sequence and, so, instead of screening for the presence of the transgene itself it is possible to screen indirectly for the known tag.

SOUTHERN BLOTTING ONTO NON-CHARGED MEMBRANES

(based on method from T. Helentjaris, NPI)

NOTE: The matrix we use is MSI Magnagraph Nylon membrane, non-charged, 0.45 µm pore size, 20 cm x 3 m rolls available from Fisher Scientific or MSI (Cat. # NJ4-HY000-10), and more recently Gibco BRL's Biodyne A nylon non-charged membrane, 20 cm x 10 m rolls (Cat. # 10134-013).

1. The best surface of a gel for regular contact with a membrane filter is that which was formed by the bottom of the gel mold. It is therefore advisable to flip the gel before constructing a blot, and preferably before denaturation. Sandwich the gel between 2 thin acrylic plates, hold firmly at the corners and flip it in one swift movement. Leave one of the plates under the gel to help handling the gel in subsequent operations.
2. Denature gel for 30 min in 0.4 N NaOH, 0.6 M NaCl; treat each gel in about 3 times its volume of solution.
3. Neutralize gel for 30 min in 0.5 M Tris-7.5, 1.5 M NaCl; treat each gel in about 3 times its volume of solution.

Construction of Wet Blot Transfer System

4. Cut nylon membrane to the same dimensions as gel. Label (S&S marker pen) or nick the upper left corner of the membrane for later identification. Place in transfer buffer.
5. Place a plastic grid in a shallow tray to allow transfer buffer (25 mM $NaPO_4$, pH 6.5) access to center of sponge.
6. Place a 6-8 cm thick, clean sponge on the center of the plastic grid, sponge surface should be equal to or greater than the gel to be blotted. Soak sponge thoroughly in transfer buffer.
7. Briefly, dip 1 sheet of blotting paper (extra thick) in transfer buffer and place on top of sponge.

NOTE: Make sure that there are **NO** air bubbles between blotting paper, gel, and membrane. Use transfer buffer between each layer and roll a glass pipette on the exposed surface to avoid bubble problems.

8. Place gel onto blotting paper on sponge, open-side of wells facing down.
9. Place cut piece of matrix on gel, label-side down to identify transfer side of matrix. Use a glass rod to smooth matrix on gel surface.
10. Place 1 sheet of wetted blotting paper on matrix.
11. Carefully place a 10 cm stack of paper towels on top of the blotting paper. An optional light weight can be placed on top, if used with a flat surface, to provide even pressure to blotting surface.
12. Add transfer buffer to tray, so that the buffer level remains high during blotting process.

NOTE: Paper towels do not need to cover entire area of gel. However, if they extend beyond the sides of the blotting paper, a piece of plastic (old X-ray film works great) or Saran wrap should be placed between the two layers of blotting paper, isolating the paper towels from the lower blotting paper and buffer solution. This will avoid short-circuiting the transfer. Instead of paper towels, a second 6-8 cm sponge may be used on top. Wet the sponge with transfer buffer and wring out as much of the buffer as possible. Place on top of the blotting paper and place a light weight on top.

13. Allow to transfer overnight (16–18 h). It is a good idea to carefully remove the bottom wet layer of paper towels after the stack has absorbed 5–8 cm of transfer buffer.

NOTE: If sponge is used, remove and wring out buffer after 4–5 h of transfer.

14. Remove matrix and immediately place in 2X SSC. With a gloved hand, you may gently rub off any agar particles. Wash blot for 15 min, shaking in 2X SSC.
15 Air or drip dry until *moist* but not wet (usually 2–5 min); do not allow to dry.
16. Place membrane on a moist filter paper and UV cross link in Stratagene UV Crosslinker using auto setting (120,000 µjoules/cm^2).
17. Bake at 95°C on or between clean filter paper for 1.5–2 h.
18. Briefly check transfer under UV light. If membrane was not previously labeled, label with a permanent marker pen or pencil on DNA bound side.
19. If blot is not going to be used for a week or more, store in between clean filter paper in a sealed plastic bag in a cool, dry place (can be stored at 4°C).

Denaturation Solution - 0.4 N NaOH, 0.6 M NaCl (1 liter/gel)

STOCK	1 liter	5 liters	10 liters	20 liters	40 liters
NaOH (MW=40.00)	16.0 g	80.0 g	160.0 g	320.0 g	640.0 g
NaCl (MW=58.44)	35.0 g	175.3 g	350.6 g	701.3 g	1402.6 g

Dissolve first the NaCl then the NaOH to avoid precipitate formation

Neutralization Solution - 0.5 M Tris-7.5, 1.5 M NaCl (1 liter/gel)

STOCK	1 liter	5 liters	10 liters	20 liters	40 liters
Tris-HCl (MW=156.60)	63.5 g	317.5 g	630.5 g	1270.0 g	2540.0 g
Tris-base (MW=121.10)	11.8 g	59.0 g	118.0 g	236.0 g	472.0 g
NaCl (MW=58.44)	87.6 g	438.0 g	876.0 g	1752.0 g	3504.0 g
-OR -					
Tris-base (MW=121.10)	60.6 g	302.8 g	605.5 g	1211.0 g	2422.0 g
NaCl (MW=58.44)	87.7 g	438.3 g	876.6 g	1753.2 g	3506.4 g
Conc. HCl	25.0 ml	125.0 ml	250.0 ml	500.0 ml	1000.0 ml

Transfer Buffer: 25 mM $NaPO_4$, pH 6.5 (5 liters/gel)

STOCK	1 liter	5 liters	10 liters	20 liters	40 liters
1 M $NaP0_4$-6.5	25 ml	125 ml	250 ml	500 ml	1000 ml

2X SSC

STOCK	250 ml	500 ml	750 ml	1000 ml	2000 ml
25X SSC	20 ml	40 ml	60 ml	80 ml	160 ml

Preparation of Probes for Nucleic Acid Hybridizations

In preparing probes for hybridization it is necessary to amplify and label the piece of DNA of interest, e.g., part of a gene of interest, marker gene etc. The most common way to do this has been to radioactively label the probe with ^{32}P. Various other molecules have also been used in non-radioactive labeling of DNA fragments. Regardless of which compound is used as the label, three methods are available when labeling cloned DNA fragments: (1) Amplification and labeling by the polymerase chain reaction (PCR); (2) Nick translation; and (3) Random oligonucleotide priming.

PCR labeling

The use of primers on either side of the cloning site of the vector, or primers specific for the gene of interest, or another part of the construct can be used to produce large amounts of the probe for labeling by PCR. Inclusion of ^{32}P or digoxigenin labeled nucleoside triphosphates in the reaction leads to highly labeled probes. The PCR labeling strategy is the one used most often in our lab.

Nick translation

The enzyme **DNA polymerase I** from *E. coli* adds nucleotide residues to the 3'-hydroxyl terminus that is created when one strand of a double-stranded DNA molecule is nicked. The nicking is achieved by including very small amounts of **DNAase I** in the reaction. In addition, the DNA pol I, by virtue of its 5' to 3' exonucleolytic activity, can remove nucleotides from the 5' side of the nick. The simultaneous elimination of nucleotides from the 5' side and the addition of nucleotides to the 3' side results in movement of the nick along the DNA. By including in the reaction all four nucleotides of which at least one is labeled, the newly synthesized molecule will become labeled and ready for use in nucleic acid hybridization.

Random oligonucleotide priming

This method is used to generate probes from denatured closed circular DNA or denatured linear dsDNA. Oligonucleotides serve as primers for initiation of DNA synthesis on such single stranded templates by the *E. coli* **DNA polymerase I Klenow** fragment. This enzyme lacks the 5' to 3' exonucleolytic activity, so that the labeled product is synthesized exclusively by primer extension rather than by nick-translation and is not degraded exonucleotically. Random, heterogeneous primers (usually 6 bases long) are used to form hybrids at many positions along a given sequence of DNA so that every nucleotide of the template gets copied at equal frequency into the labeled product. These primers are available commercially.

PROBE PREPARATION USING PCR FOR INCORPORATION OF DIGOXIGENIN-DUTP

1. Prepare a bulk reaction mix containing all the components listed below except plasmid.

STOCK	[FINAL] or amount	2.5% Dig 100 µl RXN	5.0% Dig 100 µl RXN
dH_2O	—	46.5 µl	46.38 µl
Taq Buffer (10X; Mg-free)	1X	10.0 µl	10.0 µl
$MgCl_2$ (50 mM)[1]	2 mM	4.0 µl	4.0 µl
Glycerol[2]	15 %	15.0 µl	15.0 µl
dNTP Mix-dTTP(10 mM each)	50 µM each	1.5 (0.5 µl each)	1.5 (0.5 µl each)
dTTP (10 mM)	48.75 or 47.5 µM	0.4875 µl	0.475 µl
Dig-dUTP (1 mM)[3]	1.25 or 2.5 µM	0.125 µl	0.250 µl
Taq Enzyme (5U/µl)[1]	2.0 U	0.4 µl	0.4 µl
Primer 1 (2 µM)[4] ,[5]	0.2 µM	10.0 µl	10.0 µl
Primer 2 (2 µM)[4,5]	0.2 µM	10.0 µl	10.0 µl
Plasmid (5 ng/µl)[4]	10 ng	2.0 µl	2.0 µl

2. Add 98 µl of bulk mix into each tube.
3. Add 2 µl of plasmid to each tube. Mix briefly and centrifuge.
4. Overlay each sample with 50 µl of ultrapure mineral oil.
5. Place in PCR machine, making sure there is sufficient oil in each well to provide proper contact with tube.
6. Amplify using following program[6] :

1 Cycle of:	25 Cycles of:	1 Cycle of:
94°C for 1 min	94°C for 1 min 55°C for 2 min 72°C for 2 min *	72°C for 4 min

* Note: You may need to double the extension time for inserts longer than 1.5 Kb.

7. Remove oil by adding 25 µl TE + 50 µl chloroform. Mix and centrifuge. Pipette top aqueous layer into new tube.
8. Quantify yield of insert using one of the three methods described in later sections: gel quantification, fluorometry or spot fluorescence (see other protocols).
9. Gel quantification is a good choice since it also allows one to check the amplification product and the incorporation of Dig-dUTP into this product. Details on these protocols are given in the Gel Quantification section.

1 It may be necessary to determine optimal concentrations of $MgCl_2$ and *Taq* with each new lot of enzyme.
2 This optional ingredient has been found to help amplify large or "difficult" inserts.
3 Digoxigenin-11dUTP, Boehringer Mannheim, Cat. # 1093088 (25 nmoles/25µl)
4 Diluted in DNA dilution buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 10 mM NaCl).
5 Examples of primer sequences.

pUC and M13	**CV72**	5′ - ACGACGTTGTAAAACGACGGCCAGT - 3′
derived vectors	**CV76**	5′ - AAACAGCTATGACCATGATTACGCC - 3′
CryIA(b) 5'		5' - CGACATCTCCTTGTCCTTGACAC -3'
CryIA(b) 3'		5' -ACACCCTGACCTAGTTGAGCAAC -3'

6 Conditions optimized for *ERICOMP TwinBlock*™ System thermocycler.

INCORPORATION OF DIGOXIGENIN-DUTP INTO DNA USING NICK TRANSLATION

(based on BRL Bionick Kit)

1. Prepare a bulk mix of all chemicals except DNA:

STOCK	[FINAL]	(2.5% Dig) 50 µl RXN
10 X NT Buffer	1X	5.0 µl
10 mM dATP	100 µM	0.5 µl
10 mM dCTP	100 µM	0.5 µl
10 mM dGTP	100 µM	0.5 µl
10 mM TTP	97.5 µM	0.4875 µl
1 mM Dig-dUTP	2.5 µM	0.125 µl
10 X Enzyme Mix	2.5 U Pol I 0.0375 U DNase I	5.0 µl
ddH_2O		to make 50.0 µl
DNA	0.5-1 µg	up to 38.0 µl

2. Add desired amount of DNA to 500 µl microfuge tubes.
3. Add proper amount of bulk mix to each tube containing DNA.
4. Incubate at 16°C for 1–2 h.
5. Stop the reaction by adding 2 µl of 0.5 M EDTA-8.0.
6. Unincorporated dig-dUTP can be removed by ethanol precipitation.
7. Check incorporation of dig-dUTP.

10 X NT Buffer (from BRL Bionick Kit)

500 mM Tris-7.8
50 mM $MgCl_2$
100 mM b-mercaptoethanol
100 µg/ml BSA (nuclease-free)

Enzyme Mix (from BRL Bionick Kit)

0.5 U/µl DNA Polymerase I
0.0075 U/µl DNase I
50 mM Tris-HCl, pH 7.5
5 mM MgOAc
1 mM b-mercaptoethanol
0.1 mM phenylmethylsulfonyl fluoride
50 % glycerol
100 µg/ml BSA (nuclease-free)

INCORPORATION OF DIGOXIGENIN-DUTP INTO DNA USING RANDOM PRIMING

1. Prepare a bulk mix of all chemicals except DNA and ddH_2O:

STOCK	[FINAL]	2.5% Dig 50 µl RXN
10 X Klenow Buffer	1X	5.0 µl
10 X Primer/BSA*	1 X	5.0 µl
10 mM dATP	100 µM	0.5 µl
10 mM dCTP	100 µM	0.5 µl
10 mM dGTP	100 µM	0.5 µl
10 mM TTP	97.5 µM	0.4875 µl
1 mM Dig-dUTP	2.5 µM	0.1250 µl
2 U/µl Klenow polymerase**	4 U	2.0 µl
DNA	0.5-1 µg	up to 36.0 µl
ddH_2O		to make 50.0 µl

* BSA can be added separately; note that recommended quantities of other components would change.

** Purchase from Fisher Scientific (cat. # PR-M2201 Promega-Biotec) or BRL (cat # 80125B)

NOTE: DO NOT allow the Klenow fragment of the polymerase to stand at room temperature, remove required amount at freezer and immediately return to freezer, add to bulk mix just prior to use.

2. Add desired amount of DNA and ddH_2O to 500 µl microfuge tubes.
3. Boil tubes containing DNA for 5–10 min and place on ice.
4. Add proper amount of bulk mix to each tube containing DNA.
5. Incubate at 37°C for 1 h or at RT for 2–3 h.
6. Stop the reaction by adding 2 µl of 0.5 M EDTA-8.0.
7. Unincorporated dig-dUTP can be removed by either ethanol precipitation or by Sephadex G-50 spin columns.
8. Check incorporation of dig-dUTP.

10 X Klenow Buffer

100 mM Tris-HCl, pH 8.5

100 mM $MgCl_2$

10 X Primer/BSA

Dissolve 50 A_{260} units of random primers, $p(dN)_6$ from Boehringer Mannheim in 900 µl ddH_2O. Add 100 µl of 1 mg/ml BSA (nuclease-free).

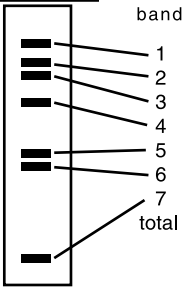
GEL QUANTIFICATION OF PCR-LABELED PROBE

NOTE: After PCR amplification it is essential to check whether the reactions were successful, what their yield has been, and also, when digoxigenin labeling has been performed, whether the incorporated label has the expected activity.

1. Prepare a 1:5 dilution of each amplified insert (at least 2 µl insert into 8 µl TE); this will bring the concentration of the insert to within the range of the molecular markers used as explained below.
2. Load 2 µl of these dilutions with 4 µl of diluted SGB (3:1, TE : 5X SGB) in a medium-sized 1% agarose gel. Load one or two wells per comb with a mixture of molecular weight markers covering the expected range of insert sizes and insert concentrations (see below). A good mixture can be made from Lambda/*Hin*dIII and PhiX174/*Hae*III. Use exactly 60 ng of each of these standards.
3. Run the gel at 40 mA for 2-3 h or until the bromophenol blue has migrated about 4 cm. Stain well with ethidium bromide and de-stain well in water.
4. Take a photograph of the gel with the wells and fragments parallel to the UV lamps of the transilluminator. The exposure has to be calibrated under your conditions so that the strongest band of the molecular standards almost, but does not saturate the film.
5. Estimate the amount of insert in each lane by comparing its intensity to two or three standard bands having *similar molecular weights*. Refer to the table below for these comparisons. **Remember that the concentration of the insert is five times this estimate**.
6. Calculate the size of the amplified inserts based on the molecular weight standards, and compare these sizes with those expected from previous work.

Molecular Weight Markers

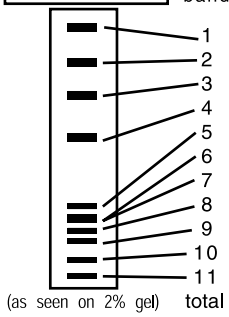
λ / Hind III



band	Lambda/ HindIII	% of total	ng band in 60 ng total	ng band in 100 ng total	ng band in 200 ng total
1	23130	48	29	48	96
2	9416	19	12	19	39
3	6557	14	8	14	27
4	4361	9	5	9	18
5	2322	5	3	5	10
6	2027	4	3	4	8
7	560	1	1	1	2
total	48373 bp		60	100	200

(real size: 48502 bp)

φ X174/ HaeIII



band	Lambda/ HindIII	% of total	ng band in 60 ng total	ng band in 100 ng total	ng band in 200 ng total
1	1353	25	15	25	50
2	1078	20	12	20	40
3	872	16	10	16	32
4	603	11	7	11	22
5	310	6	3	6	12
6	281	5	3	5	10
7	271	5	3	5	10
8	234	4	3	4	9
9	194	4	2	4	7
10	118	2	1	2	4
11	72	1	1	1	3
total	5386 bp		60	100	200

Table 2.

CHECKING THE ACTIVITY OF INCORPORATED DIGOXIGENIN-DUTP

NOTE: This can be achieved by using the quantification gel for PCR-labeled inserts, in which case start at step 2. If other labeling procedures have been used, start with step 1.

1. Load 1-5 µl of each labeled reaction in a 1% medium-sized agarose gel. Run gel at 40 mA for 2-3 h, then stain and de-stain. Remember that a smear, sometimes quite faint, is expected when labeling by nick translation or random priming.

NOTE: Denaturation and neutralization of the gel are not necessary since there is no hybridization step in this procedure.

2. Construct a dry blot transfer in the following way:
   - Lay a piece of Saran wrap on a level, clean bench, larger than the size of the gel.
   - Place two layers of blotting paper (extra thick) soaked wet in transfer buffer, slightly larger than the size of the gel.
   - Place the gel upside down onto the filter paper and lay a piece of blotting membrane on top of it, making sure that there are no bubbles between the layers.
   - Place a thin, dry filter paper of the same size as the matrix, and finally a small stack of dry paper towels cut to the size of the gel. Place a weight on this construction and leave to transfer for 4 h to overnight .
3. Dismantle the blot construction and wash the membrane in 2X SSC for 5 min. Drip dry and either UV cross link in Stratagene UV Crosslinker using auto setting (120,000 µjoules/cm^2), or bake for 1 h at 90°C.
4. Detect the incorporated digoxigenin following the protocols of **Detection of Dig-labeled Probes** except that the duration of each step can be shortened as indicated below:

Solution	Operation
Buffer 1	rinse
Buffer 2	wash 5 min
Anti-Dig	incubate 10 min
Buffer 1	wash 5 min
Buffer 3	rinse
CSPD	incubate 5-10 min

5. Expose the membranes for 45-60 min at 37°C.

NUCLEIC ACID HYBRIDIZATION

The duplex state of DNA strands are held together by H-bonds, primarily through complementary base pairing, A to T and C to G. When the duplex molecule is subjected to conditions such as high temperature or alkali treatment, denaturation of the double strands results in a partial or complete separation into single stranded molecules. Under favorable conditions the reannealing of the single strands into a duplex state can be attained. Renaturation of nucleic acids is not limited to single strands of the same DNA molecule, but can occur between DNA from different populations containing similar sequences. The formation of DNA:RNA hybrids is also possible. The incorporation of radioisotopes such as ^{32}P-dCTP or a non-radioactive nucleotides such as digoxigenin-dUTP, into a purified nucleic acid sequence to make "probes" allows the detection of related nucleic acid sequences in similarly or distantly related organisms. In filter hybridizations the denatured probe is usually in solution while the other population of single stranded DNA molecules are immobilized on a solid support such as nitrocellulose or nylon filters (see Fig. 3)

To ensure maximum detection of nucleic acid hybrids it is essential to optimize the factors affecting the reaction kinetics and maintenance of stable duplexes or hybrids. A useful measure of the stability of DNA duplexes or DNA:RNA hybrids is their melting temperature (T_m), the temperature at which 50% of the

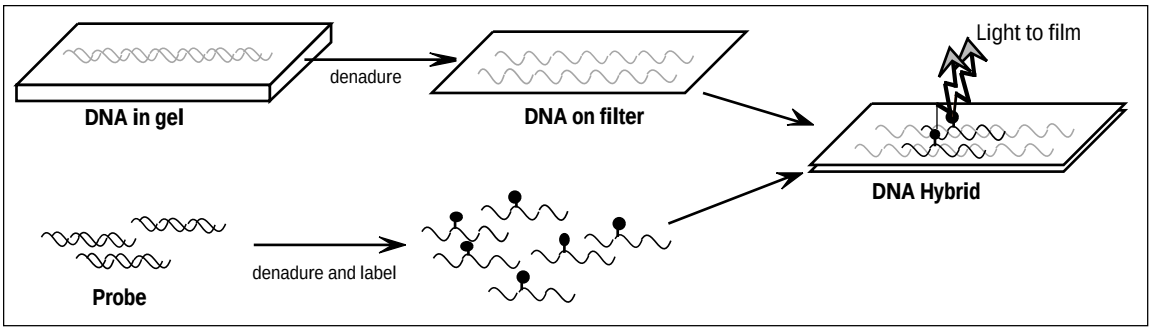


Figure 3. DNA hybridization and detection.

nucleic acids remain dissociated or denatured. Hybridization in aqueous solutions are carried out usually at temperatures of 65-68°C at which the stability of probe to nucleic acid hybrids are maintained at about 20-25°C below its T_m. The addition of compounds such as formamide decreases the T_m of nucleic acid hybrids. Hybridization solutions containing 50% formamide allow for a lower incubation temperature, usually at 42°C which is less harsh on filters and the probes are relatively more stable at lower temperatures. Hybridization of nucleic acids occur slowly at low ionic strengths, and by increasing the ionic concentration to about 1.5M Na^+ the reaction rate is enhanced. The stability of mismatched duplexes, for example in cross hybridization of probes to different species is maintained at high salt concentrations (e.g. 6X SSC).

The inclusion of inert polymers such as polyethylene glycol or dextran sulphate leads to an increased rate of hybridization. About a tenfold increase in the reaction rate is obtained in the presence of 10% dextran sulphate in the hybridization buffer. This effect is thought to be associated with an increase in the effective concentration of the probe solution due to its exclusion from the volume occupied by the polymer. However, the inclusion of these inert polymers increases the viscosity of the hybridization buffer (creates difficulties in handling) and can lead to high backgrounds.

Regions of non-specific attachment of probes to the surface of filters do occur, and to eliminate this effect blocking agents are included in the prehybridization and hybridization steps. The most common blocking agent is Denhardt's reagent (which contains BSA, PVP, Ficoll 400); non-fat dried milk also serves a similar purpose. These agents are often used in combination with sheared or sonicated salmon sperm or calf thymus DNA. When nylon filters are used in hybridization it is recommended that blocking agents be omitted from the hybridization buffer since high concentrations of protein hinder the annealing of the probe to its target. The principles which apply to hybridizing labeled probes to DNA bound to membrane filters also applies to *in situ* hybridization where the DNA is in cytological preparations of chromosomes or nuclei.

After filter hybridization, the unbound probe is washed off the filter with a series of stringency washes and detection of the DNA:DNA hybrids is done by exposing the filter directly to an X-ray film in the case of radioactively labeled probes. For non-radioactive methods, a series of detection steps are essential before exposing the filter to the X-ray.

Non-Radioactive Procedures for Detecting DNA Sequences

Although probes labeled with ^{32}P are at present the most widespread basis for detection of hybridization signals after Southern blotting, they have two major disadvantages. Firstly, the b-particles emitted by ^{32}P are energetic and thus there is a potential health risk for personnel involved in the daily work. This requires careful screening to guard against radioactive contamination. Second, the short half-life of ^{32}P (14 days) means that in almost all cases, probes cannot be stored and reused but must be labeled each time a hybridization is to be performed. This is especially so with single copy probes, which are close to the limit of detection with ^{32}P.

Consequently, there has been considerable work aimed at developing non-radioactive probes and detection systems. They all rely on

- Incorporation of chemically modified nucleoside triphosphates into the probe.
- A system for identifying the chemically modified probe (e.g., an antibody to the chemical).
- A method for visualizing the identified chemical group (e.g., an easily detected enzyme coupled to the antibody).

ELISA technology has provided many of the methods used. An early system involved using biotin-labeled nucleoside triphosphates in the labeling reaction, detecting the biotinylated probe with streptavidin (or anti-biotin antibodies) coupled with peroxidase or alkaline phosphatase, followed by histochemical staining for the enzymes. Systems such as this have been quite successful but have had two major drawbacks. Firstly, they have never been quite sensitive enough for routine use with single copy probes in complex genomes. Secondly, the histochemical detection systems rely on producing an insoluble colored precipitate on the membrane. This is extremely difficult to remove, making it virtually impossible to strip and reprobe the membrane.

The chemiluminescent system used exclusively at CIMMYT overcomes some of the above difficulties. It uses

- Nucleoside triphosphates labeled with digoxigenin (a steroid occurring only in *Digitalis*).
- Detection with an antibody-enzyme conjugate (anti-digoxigenin-alkaline phosphatase).
- Detection by a chemiluminescent substrate CSPD (or Lumi-Phos) (Fig. 4) and exposure to X-ray or polaroid film.

This method is sensitive enough for single copy probes and because detection is via a film and not on the membrane, stripping and reprobing are just as easy as with ^{32}P.

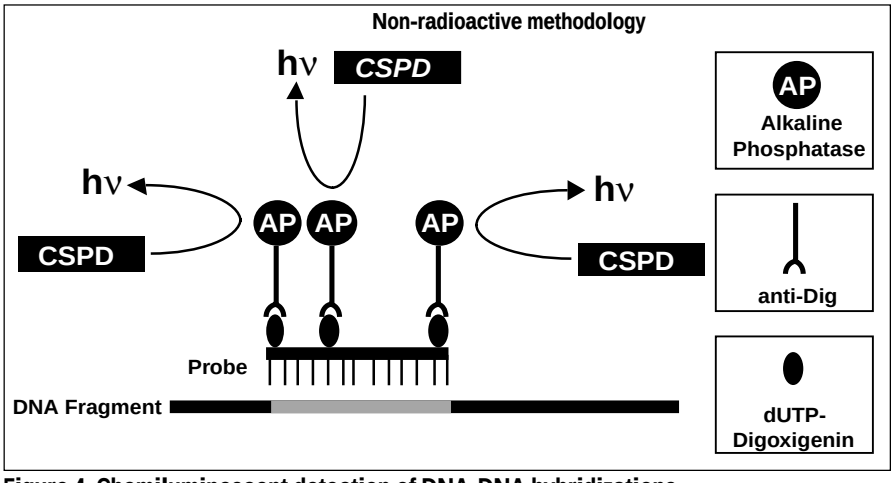


Figure 4. Chemiluminescent detection of DNA-DNA hybridizations.

HYBRIDIZATION AND DETECTION OF DIG-LABELED PROBES

NOTE: These protocols have been optimized for hybridizations in **siliconized** glass bottles (e.g., *Robbins Scientific Corp.* or similar) and in polypropylene Corning tubes; handle membranes with extreme care by their top or bottom edges using clean filter forceps (*Nalgene*) and make sure that they never dry.

1. Prehybridize blots for 1-3 h (at least 2 h the first time) in an oven at 65°C, in a tray with enough HYB solution to cover well all the blots. The HYB solution used for pre-hybridization can be stored frozen or at 4°C and be re-used three to four times or until precipitated material will not go into solution upon heating.
2. Roll wet membranes on a thick glass pipette on top of a flat, clean surface wetted with some of the HYB solution from the tray, and insert them into clean hybridization bottles. Make sure that they do not roll on themselves upon rotation in the oven ("taco" syndrome, check direction of rotation of rotisserie mechanism), and avoid the formation of air bubbles or *any* drying of the membranes. You can place up to five 500 cm^2 membranes in one bottle. Smaller membranes can be placed in 15 or 50 ml Corning polypropylene tubes which can be fitted into sections of common PVC tubing of the right diameter, and long enough to take two tubes each.
3. Add 2-3 ml/100 cm^2 HYB solution for first blot and 1 ml more for each extra 500 cm^2 membrane; in the case of small membranes in tubes, adjust the volume accordingly. The HYB solution should contain 50-100 ng/ml of 2.5-5% Dig-labeled probe [denature probe by heating at 95°C for 10 min and quenching on ice]. If HYB solution containing probe has been previously used and stored frozen, thaw and denature for 20 min at 95°C.

NOTE: After the first use, the intensity of the signal on the membrane will start to decrease; it will thus be necessary to gradually increase the concentration of the probe in the HYB solution and/or increase the concentration of CSPD (see below), with each re-use.

4. Hybridize for 15-18 h (overnight) at 65°C in bottles in hybridization oven.
5. Remove membranes from bottle(s) and wash together in trays of adequate size with shaking as follows:

NOTE: HYB solution containing probe may be saved at -30°C for re-use. Clean hybridization bottles immediately to avoid formation of HYB residues.

2 x 5 min	0.15X SSC, 0.1% SDS	RT	0.5 ml/cm^2
3 x 15 min	0.15X SSC, 0.1% SDS	65°C	0.5 ml/cm^2
OR, for lower stringency,			
3 x 15 min	0.15X SSC, 0.1% SDS	RT	0.5 ml/cm^2
1 x 15 min	0.15X SSC, 0.1% SDS	50°C	0.5 ml/cm^2

It is essential that the wash temperatures be monitored to make sure that the above treatments are respected consistently; undue lowering of the temperature or shorter treatment times may result in higher background noise and less predictable results.

6. Rinse membranes in Buffer 1 at RT*.
7. Incubate membranes in Buffer 2 for 30 min at RT with shaking (5 ml/100 cm^2).
8. Incubate membranes in fresh anti-Dig solution (5 ml/100 cm^2) for 30 min at RT with shaking; this solution may be re-used in the same or the next two days of first use. [Centrifuge anti-Dig immediately prior to use and carefully pipette desired amount].
9. Wash membranes with shaking as follows:

3 x 10 min	Buffer 2	RT	0.5 ml/cm^2
3 x 10 min	Buffer 1	RT	0.5 ml/cm^2
1 x 5 min	Buffer 3	RT	0.5 ml/cm^2 *

* Can leave membranes in this solution for longer periods of time if necessary.

10. Incubate membranes in CSPD solution (5 ml/100 cm^2), for 20 min at RT with shaking, preferably in the dark.

    [Save CSPD solution between uses in refrigerator in a bottle wrapped in aluminum foil]

11. Remove each membrane from CSPD tray slowly, letting solution drip off the membrane; then place, DNA-side down, on top of GladWrap (or equivalent wrapping plastic film). You can do several membranes in a row on a long stretch of film secured on a table with tape. Place another sheet of GladWrap on top (back side of membranes), cut GladWrap between membranes and seal edges on back side of each membrane.
12. Place membranes in cassettes and expose to XAR-5 X-ray film overnight (15-18 h).

NOTE: This long exposure has been sought in the development of this protocol in order to facilitate the simultaneous handling of several dozen large membranes; in addition, it provides a natural overnight break for the worker in charge.

13. Develop X-ray film for 6 min in GBX (*Kodak*) developer, rinse in H_2O for 30 sec, fix in GBX fixer for 3 min, and rinse for 3 min in running H_2O.

NOTE: If signal is weak (you can at least see some faint bands), the membranes can be incubated in higher strength CSPD and re-exposed by starting with either Buffer 2 (Step 7) or Buffer 3 (Step 9) wash above.

14. To ensure longer life of the membranes and a successful stripping of the probe, proceed **immediately** to remove them from their plastic wrapping and immerse them in 0.1X SSC, 0.1% SDS ("Highest Stringency Wash") or in 2X SSC in a tray at RT. **Do not allow the membranes to dry!** You may keep them for a few days in this solution at 4°C or, better still, strip them right away (see next protocol).
15. Score your results on a "Hybridization data sheet" (example given at end of protocol) for signal, lane background, and general background using the following rating scale:

0	1	2	3
none	faint	medium	strong

This will help you keep track of probe quality and/or other problems during these steps.

HYB Solution

STOCK	[FINAL]	25 ml	50 ml	75 ml	100 ml	150 ml
25X SSC	5X SSC	5 ml	10 ml	15 ml	20 ml	30 ml
10% Laurylsarcosine	0.01%	25 µl	50 µl	75 µl	100 µl	150 µl
20% SDS (good)	0.02%	25 µl	50 µl	75 µl	100 µl	150 µl
Blocking reagent[1]	0.2%	50 mg	100 mg	150 mg	200 mg	300 mg
(Boehringer Mannheim)	0.3%	75 mg	150 mg	225 mg	300 mg	450 mg

[1] Add after heating the solution to 65°C and checking that the pH is 7.4. We use 0.2% for maize and 0.3% for wheat.

0.10X SSC, 0.1% SDS: Highest Stringency Wash

STOCK	1000 ml	2000 ml	3000 ml	4000 ml	5000 ml	6000 ml
25X SSC	4.0 ml	8.0 ml	12.0 ml	16.0 ml	20.0 ml	24.0 ml
20% SDS (cheap)	5.0 ml	10.0 ml	15.0 ml	20.0 ml	25.0 ml	30.0 ml

0.15X SSC, 0.1% SDS: Higher Stringency Wash

STOCK	1000 ml	2000 ml	3000 ml	4000 ml	5000 ml	6000 ml
25X SSC	6.0 ml	12.0 ml	18.0 ml	24.0 ml	30.0 ml	36.0 ml
20% SDS(cheap)	5.0 ml	10.0 ml	15.0 ml	20.0 ml	25.0 ml	30.0 ml

0.20X SSC, 0.1% SDS: High Stringency Wash

STOCK	1000 ml	2000 ml	3000 ml	4000 ml	5000 ml	6000 ml
25X SSC	8.0 ml	16.0 ml	24.0 ml	32.0 ml	40.0 ml	48.0 ml
20% SDS (cheap)	5.0 ml	10.0 ml	15.0 ml	20.0 ml	25.0 ml	30.0 ml

Buffer 1

STOCK	[FINAL]	500 ml	1000 ml	2000 ml	4000 ml
1 M Tris-HCl, pH 7.5	0.01 M	5.0 ml	10.0 ml	20.0 ml	40.0 ml
5 M NaCl	0.15 M	15.0 ml	30.0 ml	60.0 ml	120.0 ml

Buffer 2

STOCK		[FINAL]	500 ml	1000 ml	2000 ml	4000 ml
1 M Tris-HCl, pH 7.5		0.01 M	5.0 ml	10.0 ml	20.0 ml	40.0 ml
5 M NaCl		0.15 M	15.0 ml	30.0 ml	60.0 ml	120.0 ml
Blocking reagent (Boehringer Mannheim)	maize	0.1%	500.0 mg	1000.0 mg	2000.0 mg	4000.0 mg
	wheat	0.2%	1000.0 mg	2000.0 mg	4000.0 mg	8000.0 mg

To dissolve the blocking reagent, first heat the solution to 65°C before adding it (Beware: never heat solution already containing blocking reagent in microwave). You may prepare this solution up to a day before use, but make sure to use it at room temperature.

Buffer 3

STOCK	[FINAL]	100 ml	200 ml	400 ml	500 ml
1 M Tris-HCl, pH 9.5	0.01 M	1.0 ml	2.0 ml	4.0 ml	5.0 ml
5 M NaCl	0.10 M	2.0 ml	4.0 ml	8.0 ml	10.0 ml

Autoclave solution before use or use autoclaved stocks and ddH_2O.

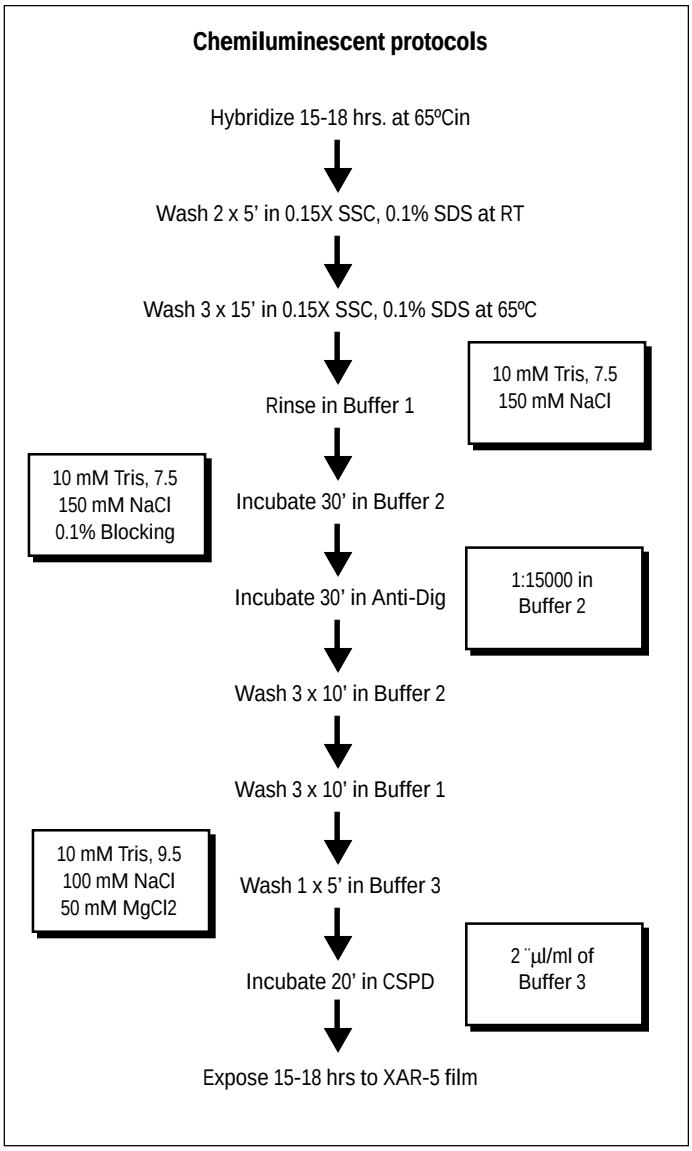
Anti-Dig (1:15000)

Buffer 2 + 1 µl/15 ml anti-Dig (Anti-digoxigenin-AP, *Boehringer Mannheim*, Cat. # 1093274, 150 Units/200 µl)

CSPD Solution (2-15 µl/ml)

Buffer 3 + 2 µl/ml CSPD (*Tropix* , Cat. No. PD250-B, 10 mg/ml)

NOTE: The concentration of CSPD can be increased after few uses and the signal decreases with each re-use of the membrane. Diluted CSPD solution should be stored at 4°C in a bottle wrapped in aluminum foil. The solution can be re-used several (5-10) times and can be filter sterilized every few uses to avoid contamination.



REMOVAL OF PROBE FOR RE-USE OF MEMBRANES

One of the main problems associated with chemiluminescent detection methods as sensitive as those found in these protocols, is that even a very small amount of labeled probe remaining on the blot after stripping can be detected. In many cases this "carry-over" signal will add to the complexity of the resulting banding patterns after re-probing with a different probe and may thus hinder proper data capture and interpretation.

Another problem is that, in an effort to avoid "carry-over", it may be possible to "overstrip" the membrane in a way that eliminates carry-over signal but unfortunately reduces both the overall signal to noise ratio and the life of the membrane.

We only recommend the following procedure if you have followed precisely the preceding protocols for blotting, fixing the DNA, hybridization and detection. It has given us good results for at least 7 re-uses of the membranes with insignificant background noise, and no carry-over signal or only a tolerable, very faint level of such signal. As in the previous protocol, handle membranes with extreme care by the top or bottom edges using clean filter forceps (*Nalgene*), and never let them dry.

The duration and temperature of the wash are the key factors for successful, repeatable stripping.

Strip washes using homemade washing tank

NOTE: In order to scale-up this delicate operation, we constructed a washing tank fitted with a water heater/circulating unit in one corner (e.g., Cole Parmer's *Polystat Immersion Circulator*). It is large enough to loosely fit a flat stack of large blots (say 50) in the space left by the heating unit. The bath is also fitted with a draining outlet to facilitate changes of solution and cleaning.

1. **Immediately** after exposing the membranes to film, transfer them into 2X SSC or TE to avoid over-drying or mold growth if left in the exposure cassettes.
2. Preheat stripping solution (0.1X SSC, 0.1% SDS) to 93°C in the water bath.
3. Wash membranes in tank for **4 to 6 minutes** maximum at **90-93°C**.

NOTE: In order to introduce quickly the membranes into the heated solution, we first lay them as a flat stack in a basket made of plastic mesh (1 cm^2 holes) that we constructed for this purpose. This basket has string handles that are long enough to facilitate the introduction and removal of the basket in and out of the solution. After introduction of the membranes into the solution, use forceps to minimize any sticking between them or rolling, and allow good circulation of the solution in the basket.

4. Rapidly transfer the membranes into a container containing TE or 2X SSC at RT. Either proceed immediately with your next re-hybridization (see step 1 of previous protocol), store at 4°C, or air-dry thoroughly on clean filter paper and store in **sealed** plastic bags at room temperature or in the refrigerator.

Strip washes using plastic containers and oven

1. Place membranes in a plastic box with a tight-fitting lid containing enough 0.1X SSC, 0.1% SDS to ensure that the membranes float freely in the solution (e.g., 2000 ml for 10 large blots). Put box in a 65°C oven until the solution reaches 60–65°C. Meanwhile, heat the stripping solution to 95°C in a microwave oven and place in an oven at 90°C.
2. Drain solution from box and add the stripping solution (0.10X SSC, 0.1% SDS at 90-95°C) for 10 min. Make sure that the membranes are not sticking to each other.

NOTE: Monitor temperature to make sure that it is maintained throughout the treatment. The time required to remove the probe from the membrane at 90°C may vary from lot to lot of membrane. In any case, use the minimal time required to result in no signal following re-detection of the probe.

3. Repeat step 2.
4. Drain solution from box and immediately add cool (RT) TE or 2X SSC. Membranes can be placed immediately in trays for re-hybridization (see step 1 of previous protocol), or can be stored in TE or 2X SSC at 4°C, or air-dried thoroughly on clean filter paper and stored in **sealed** plastic bags at room temperature or in the refrigerator.

0.1X SSC, 0.1% SDS: Strip wash (also Highest Stringency Wash)

STOCK	1000 ml	2000 ml	3000 ml	4000 ml	5000 ml	6000 ml
25X SSC	4.0 ml	8.0 ml	12.0 ml	16.0 ml	20.0 ml	24.0ml
20% SDS	5.0 ml	10.0 ml	15.0 ml	20.0 ml	25.0 ml	30.0ml

The Polymerase Chain Reaction (PCR)

PCR can amplify a desired DNA sequence of any origin (virus, bacteria, plant, or human) hundreds of millions of times in a matter of hours, a task that would have required several days with recombinant technology. The PCR reaction, only described in 1985, is revolutionizing the way molecular biology is being carried out. PCR is especially valuable because the reaction is highly specific, easily automated, and capable of amplifying minute amounts of sample.

PCR is an *in vitro* procedure for the enzymatic synthesis of DNA, using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA. The procedure enables small amounts of specific DNA fragments (which may be mixed with large amounts of contaminating DNA) to be amplified between 10^6 and 10^{12} times (a million to a million million fold).

The PCR amplification is carried out in a Thermal Cycler through three steps:

1. **Denaturation of genomic DNA** (template) by heating the reaction mixture to 92–95°C. High temperatures should be avoided if high fidelity is required because thermal damage of DNA leads to an increased nucleotide misincorporation during PCR.
2. Annealing of primers by cooling the reaction mixture to **Tm**/annealing temperature. The annealing temperature (**Tm**) is determined by the length and base composition of the primer(s), and can be calculated for a particular primer using the following equation:

   Tm = [(number of A+T) x 2°C + [(number of C+G) x 4°C] - 3°C

   The calculated **Tm** only act as a reference point to begin experimentation. The optimum annealing temperature should be determined empirically.
3. Primer extension (synthesis of new DNA strands) by DNA polymerase using four deoxynucleotides (dNTPs). Primer extension is usually performed at about 72°C, which is the optimum temperature for *Taq* DNA polymerase. One minute is usually enough for the extension step.

The amplification is dramatic because the extension products of one cycle serve as templates for the following reactions and thus the number of target copies double at every cycle. For instance, after 20 cycles of PCR a 10^6-fold amplification (2^{20}) is achieved (Fig. 5).

The key factor in the widespread use of PCR was the introduction, in 1986, of the thermostable DNA polymerase (*Taq* polymerase) from *Thermus aquaticus*. The commercial supply of the enzyme (Cetus) meant that the reaction components (template, primers, *Taq* polymerase, nucleoside triphosphates and buffer) could be simply mixed and subjected to temperature cycling. The number of cycles is usually 25–35. The desired product will not be increased after 35 cycles. The final cycle ended with longer extension time (up to 10 min) to ensure that all product molecules are fully extended. The PCR-amplified products are fractionated by agarose gel electrophoresis and visualized under UV light by staining the gel with ethidium bromide.

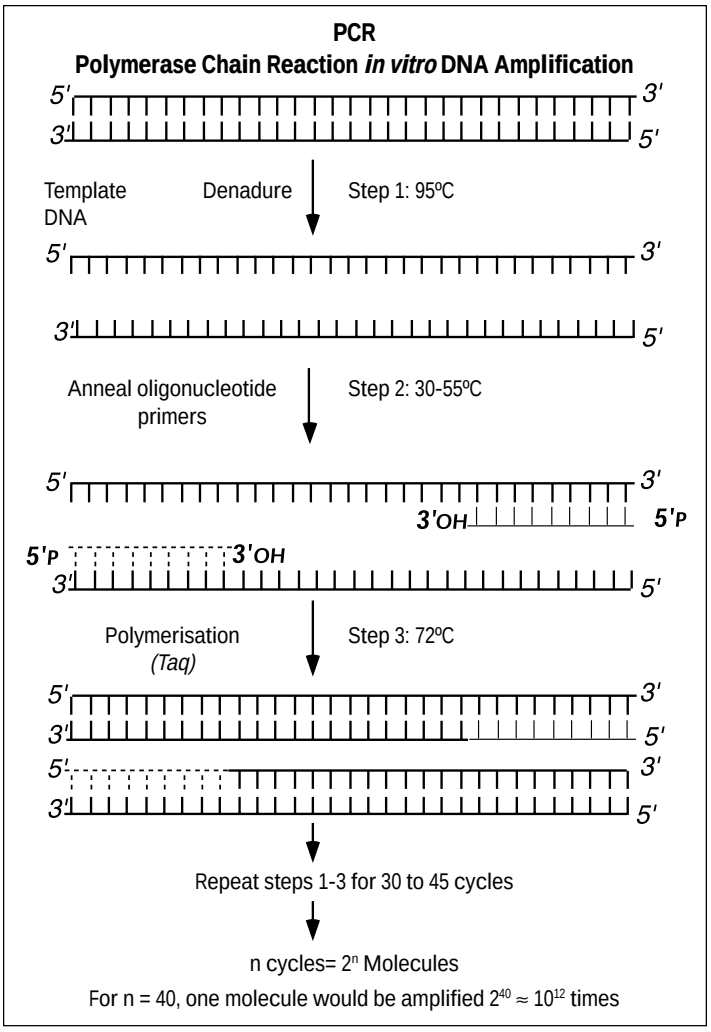


Figure 5. The polymerase chain reaction

Amplification reactions are performed in volumes of about 25–50 µl in a tube containing proper amounts of genomic DNA, specific primers (usually are 20–25-mer primers), dNTPs, thermostable DNA polymerase (such as *Taq*), $MgCl_2$, and reaction buffer. The reaction mixture is overlaid with 1 drop of light mineral oil to prevent evaporation of the samples.

Excess DNA will reduce the amplification efficiency because of annealing of target strands as their concentration increases.

Taq from *Thermus aquaticus* has been the most extensively used thermostable DNA polymerase for PCR assays. DNA polymerase, unlike RNA polymerase, requires a short DNA segment, or primer, to anneal to a complementary sequence and prime synthesis. The amount of enzyme becomes limiting after 25–30 cycles of PCR. The enzyme activity also becomes limiting due to thermal denaturation of the enzyme during the process. The enzyme should be the last component to be added to the reaction mixture, should not be taken out from freezer a long time before use, and should be always kept on ice and put back in the freezer (-20°C) immediately after use.

PCR primers are available commercially and can be purchased from many companies including Operon Biotechnologies (Alameda, Calif.).

The $MgCl_2$ concentration in the final reaction mixture is around 2 mM (the exact concentration for a given lot of enzyme and DNA should be determined empirically). Mg^{2+} ions form a soluble complex with dNTPs that is essential for dNTP incorporation; they also stimulate the polymerase activity and increase the **Tm** of the double-stranded DNA and primer/template interaction.

PCR FOR ANALYSIS OF TRANSGENIC PLANTS

PCR is a very sensitive technique to detect foreign genes in plants. The advantages of using PCR are that it is quick and easy to screen a large number of putative transgenic plants. But we need to be very careful when making conclusions from the presence or absence of a particular band and it is absolutely essential to include a full range of controls. Positive controls should include plasmid DNA (the same as used to transform the plant) and, if possible, transgenic plant DNA. Negative controls should include negative plant DNA (from a non-transgenic plant of the same genotype as the putative transgenic plant being tested) and a non-DNA control that has all components of the reaction mixture, except DNA, to ensure none of the reagents are contaminated. The size of the band produced from the PCR amplification is an important indication that plants contain the specific transgenes. A positive PCR result does not indicate whether the template DNA source is the intended sample material or due to contamination, nor whether the template DNA is integrated into the plant genome. Despite this limitation, PCR is still an excellent "first test" for screening a large number of putative transgenic plants.

PCR, as used for transgenic plant analysis, has different requirements than PCR used for cloning. Robustness is crucial when PCR is used for analysis of transgenics as a 10% failure rate is unacceptable for analytical purposes. To maximize robustness and specificity, several extra steps can be taken—for example, keeping reaction products under less than 1,000 base pairs in length, using relatively long primers (25–30 nucleotides) and using primers with a high Tm (melting temperature) (65–68°C). Ensuring that primers are sufficiently sensitive for reproducible detection of single copy sequences is crucial and primer location affects the utility of the information provided by PCR. Amplification of a known endogenous single copy gene, in addition to the transgene, provides a useful control for monitoring template DNA quality.

PCR PROTOCOL

The protocol below can be taken as a standard "template," but depending on the length (in base pairs) and percentage of G+C etc. of the primers used to detect the particular transgene, it may be necessary to change the annealing temperature. Our "standard" set of conditions is as follows:

1. Prepare a bulk reaction mix containing all the components listed below except DNA.

STOCK	[FINAL] or amount	25 µl RXN	Bulk Mix ____ RXNs
ddH_2O	—	16.05	
Taq Buffer (10X; Mg-free)	1X	2.5 µl	
$MgCl_2$ (50 mM)	2.5 mM	1.25 µl	
dNTP Mix (10mM each)	0.2 mM each	0.5 µl each	
Primer 1 (10 µM)	0.2 µM	0.5 µl	
Primer 2 (10 µM)	0.2 µM	0.5 µl	
Taq Enzyme (5 U/µl)	1 U	0.2 µl	
DNA (100 ng/µl)[4]	100 ng	1.0 µl	
[optional glycerol (100%)	10%	1.0 µl	]

2. Aliquot bulk mix into each labeled tube.
3. Add DNA sample to each tube. Mix briefly.
4. Overlay each sample with 2 drops or 50 µl of ultrapure mineral oil.
5. Place in PCR machine, making sure there is sufficient oil in each well to provide proper contact with tube.

6. Amplify using following program[6]:

1 Cycle of:	35 Cycles of:	1 Cycle of:
94°C for 1 min	94°C for 1 min	72°C for 5 min
	55°C[7] for 1 min	
	72°C for 1 min	

7. *Optional*: remove oil by adding 25 µl TE + 50 µl chloroform. Mix and centrifuge. Pipette top aqueous layer into new tube.
8. Add 5 µl 5X SGB to each tube. Mix well and centrifuge briefly.
9. Load 12 µl of each sample in a 1-2% agarose gel (depending on size of amplification products expected) prepared with 1X TBE gel buffer (this gives resolution equal to novel agarose types).
10. Electrophorese at 35-40 mA, constant current, with buffer recirculation until the blue dye has migrated as required (approximately 3 h for 20 x 25 cm gels).
11. Stain gel in 1 µg/ml ethidium bromide (100 µl of 10 mg/ml ethidium bromide in 1000 ml dH_2O) for 20 min with gentle shaking.

    CAUTION: Ethidium bromide is extremely mutagenic—wear double gloves when handling and use extra precaution.
12. Rinse gel in dH_2O for 30 min, slide gel onto a UV transilluminator and photograph.
    For Fotodyne PCM-10 camera with 20 x 26 cm hood and Type 667 Polaroid film use an f8, 1 second exposure.
    For Fotodyne MP-4 camera and Type 665 Polaroid (negative) film use an f8, 2 min exposure.

5X TBE Gel Buffer: 0.45 M Tris-borate, 10 mM EDTA

STOCK	1 liter	2 liters	3 liters	4 liters	5 liters
Tris Base (MW=121.10)	54.0 g	108.0 g	162.0 g	216.0 g	270.0 g
Boric Acid (MW=61.83)	27.5 g	55.0 g	82.5 g	110.0 g	20.5 g
0.5 M EDTA pH 8.0	20.0 ml	40.0 ml	60.0 ml	80.0 ml	100.0 ml

pH to 8.0 with glacial acetic acid.
A precipitate may form when stored for long periods of time.

NOTE: PCR is capable of amplifying as little as a single molecule of DNA. Precautions should be taken to guard against contamination of the reaction with trace amounts of DNAs that could serve as templates. Disposable gloves should be worn throughout the preparation of the PCR. Always include a control that contains all the components of the PCR except the template DNA.

NOTE: This protocol has been optimized for a specific set of conditions and the following precautions should be noted.

- Always resuspend DNAs in sterile ddH_2O (Sigma's Cell Culture Water, Cat. # W-3500, provides an excellent standard).
- Accurate quantification of DNA amounts is important; fluorometric measurements are usually more accurate than spectrophotometric ones at low concentrations. The concentration of all DNAs should be adjusted to 100 ng/µl, and they should be aliquoted in appropriate amounts and frozen until needed.
- Take note of the lot number of the available *Taq* enzyme and perform the necessary experiments to determine the optimum amounts of enzyme and of $MgCl_2$ for best performance. Repeat these tests whenever you change to a new lot of enzyme.
- Use filtered pipette tips for the preparation of all stocks of reagents included in these protocols, as well as for pipetting DNAs and primers.
- To ensure repeatability and consistent results, it is strongly recommended that all working solutions (primers, buffers, nucleotides) be prepared in advance for all planned experiments using the same

[6] Conditions optimized for *ERICOMP TwinBlock*™ System thermocycler.
[7] Will depend on length and GC content of the primers

reagents and high quality ddH_2O. With this strategy, aliquots of the working stocks may be prepared for each experiment and stored frozen (-20°C); these would then be thawed only once, on the day of a particular experiment.

- All conditions have been optimized for *ERICOMP TwinBlock*™ system thermocyclers.

POLYACRYLAMIDE GEL ELECTROPHORESIS

Nearly all analytical electrophoresis of proteins is carried out in polyacrylamide gels under conditions that ensure dissociation of the proteins into their individual polypeptide subunits and that minimize aggregation. Most commonly, the strong anionic detergent SDS is used in combination with a reducing agent and heat to dissociate the proteins before they are loaded on the gel. The denatured polypeptides bind SDS and become negatively charged. Because the amount of SDS bound is almost always proportional to the molecular weight of the polypeptide and is independent of its sequence, SDS-polypeptide complexes migrate through polyacrylamide gels in accordance with the size of the polypeptide.

In most cases, SDS-polyacrylamide gel electrophoresis is carried out with a discontinuous buffer system in which the buffer in the reservoirs is of a different pH and ionic strength from the buffer used to cast the gel. The sample and the stacking gel contain Tris.Cl (pH 6.8), the upper and lower buffer reservoirs contain Tris-glycine (pH 8.3), and the resolving gel contains Tris. Cl (pH 8.8). The chloride ions in the sample and stacking gel form the leading edge of the moving boundary, and the trailing edge is composed of glycine molecules. After migrating through a stacking gel of high porosity, the SDS-polypeptide complexes are deposited in a very thin zone (or stack) on the surface of the resolving gel.

Between the leading and trailing edges of the moving boundary is a zone of lower conductivity and steeper voltage gradient which sweeps the polypeptides from the sample and deposits them on the surface of the resolving gel. There, the higher pH of the resolving gel favors the ionization of glycine, and the resulting glycine ions migrate through the stacked polypeptides and travel through the resolving gel immediately behind the chloride ions. Freed from the moving boundary, the SDS-polypeptide complexes move through the resolving gel in a zone of uniform voltage and pH and are separated according to size by sieving. The ability of discontinuous buffer systems to concentrate all of the complexes in the sample into a very small volume greatly increases the resolution of SDS-polyacrylamide gels.

Acrylamide is a monomer whose structure is

$$\mathbf{CH_2 = CH\text{-}C{=}ONH_2}$$

In the presence of free radicals, which are usually supplied by ammonium persulphate (APS) and stabilized by TEMED (N, N, N′, N′-tetramethylethylenediamine), a chain reaction is initiated in which monomers of acrylamide are polymerized into long chains. When the bifunctional agent N,N′methylenebisacrylamide is included in the polymerization reaction the chains become cross-linked to form a gel whose porosity is determined by the length of the chains and degree of cross-linking. The size of the pores in the acrylamide gel decreases as the bisacrylamide:acrylamide ratio increases. Table 3 shows the linear range of separation obtained with gels cast with concentrations of acrylamide that range from 5% to 15%.

Table 3. Effective range of separation of SDS-polyacrylamide gels

Acrylamide concentration (%) *	Linear range of separation (kD)
15	12 - 43
10	16 - 68
7.5	36 - 94
5.0	57 - 212

A molar ratio of acrylamide: bisacrylamide is 29:1

Western Blotting

Western blotting is to proteins what Southern blotting is to DNA. In both techniques, electrophoretically separated components are transferred from a gel to a solid support and probed with reagents that are specific for particular sequences of amino acids (western blotting) or nucleotides (Southern hybridization). In the case of proteins, the probes are usually antibodies that react specifically with antigenic epitopes displayed by the target protein attached to the solid support. Western blotting is therefore extremely useful for the identification and quantification of specific proteins in complex mixtures of proteins.

The critical difference between Southern and western blotting lies in the nature of the probes. Whereas nucleic acid probes hybridize with a specificity and rate that can be predicted by simple equations, antibodies behave in a less predictable way. An individual immunoglobulin may preferentially recognize a particular conformation of its target epitope (e.g. denatured or native). Consequently, not all monoclonal antibodies are suitable for use as probes in western blots, where the target proteins are thoroughly denatured. Polyclonal antisera, on the other hand, are undefined mixtures of individual immunoglobulins, whose specificity, affinity and concentration are often unknown. Consequently, it is not possible to predict the efficiency with which a given polyclonal antiserum will detect different epitopes of an immobilized, denatured target protein.

Most problems arising from western blotting can be solved through the design of adequate controls. These include the use of (1) antibodies that should not react with the target protein and (2) control preparations that either contain known amounts of target antigen or lack it altogether.

If there is a choice of immunological reagents available for western blotting, either a high-titer polyclonal antiserum or a mixture of monoclonal antibodies raised against the denatured protein should be used. Reliance on a single monoclonal antibody is hazardous because of the high frequency of spurious cross-reactions with irrelevant proteins.

In western blotting, the samples to be assayed are solubilized with detergents and reducing agents, separated by SDS-polyacrylamide gel electrophoresis, and transferred to a solid support (nitrocellulose or paper derivatized with diazobenzyloxymethyl groups), which may then be stained. The filter is subsequently exposed to unlabeled antibodies specific for the target protein. Finally the bound antibody is detected by a secondary immunological reagent (linked to, for example, horseradish peroxidase). As little as 1–5 ng of an average-sized protein can be detected by western blotting.

PROTEIN EXTRACTION

Extremes of temperature and pH (acidity and alkalinity) cause proteins to lose their natural shapes (denaturation). Plant cells are lysed by a combination of chemical and thermal means. The chemicals break the cell walls and the freeze/thaw action also helps the lysis process. With the addition of reducing agents (e.g., ß-mercaptoethanol) and heat, all inter- and intrachain disulfide bonds break, leaving the denatured protein fully reduced and separated into individual polypeptide subunits.

Preparation of samples for extraction of protein

1. Harvest leaves from greenhouse or field-grown plants (it is preferable to use young leaves); then lyophilize the tissue.
2. Grind the samples to a fine powder with a mill or coffee grinder, and put them in an appropriate container. The samples can be stored at -20°C or -80°C.

3. It is also possible to extract proteins from fresh tissue. In such cases, the extraction would be done using a SAP extractor and lysis buffer (instead of extraction buffer as used for DNA extraction). Then follow the protein extraction process from vortexing in Step 2 through Step 5.

Extraction of proteins

Lyophilized tissue can be processed as follows:

1. Weigh 100 mg of lyophilized plant tissue into an eppendorf tube (1.5 ml).
2. Add 500 µl of lysis buffer and vortex for 3 min.
3. Freeze the contents of the tubes by placing them at -20°C or -80°C for 30 min.
4. Thaw the tubes at room temperature, repeat once from vortex to thawing.
5. Centrifuge the samples at 10,000 rpm for 5 min at 4°C.
6. Take an aliquot for the protein assay and further aliquots for a dot/slot blot or for loading onto a gel if a western blot is being carried out.

NOTE: It is important to keep lysed samples on ice at all times.

QUANTITATIVE ASSAY FOR DETERMINATION OF PROTEIN

Protein standard curve

1. Make a stock solution of albumin of 1mg/ml , aliquot into Eppendorf tubes (0.5ml) and store in the freezer (-20°C)
2. Take 10 µl of buffer containing different quantities (0–40 µl) of soluble protein from the albumin stock to give a range of 0–40 µg protein into a 1.5ml Eppendorf tube. Add water up to a total volume of 110 µl and 1.0 ml of dye (Bio-Rad protein assay).
3. Read the absorbance at 595 nm using a spectrophotometer
4. Construct a protein standard curve

TUBE #	PROTEIN (µg)	H_2O(µl)	LYSIS BUFFER (µl)	DYE (ml)
1	0	100	10	1.0
2	2	98	10	1.0
3	4	96	10	1.0
4	6	94	10	1.0
5	8	92	10	1.0
6	10	90	10	1.0
7	20	80	10	1.0
8	30	70	10	1.0
9	40	60	10	1.0

Assay samples under the same conditions and compare their concentrations to those on the standard curve.

TUBE #	H2O(µl)	SAMPLE IN LYSIS BUFFER (µl)	DYE (ml)
	100	10	1.0

POLYACRYLAMIDE GEL ELECTROPHORESIS

Preparation of sds-page (polyacrylamide) gels

Assemble the glass plates according to the manufacturer's instructions.

1. For Mini-V 8.10 Vertical Gel Electrophoresis System make sure the glass plates, spacers, and combs are clean by washing with soap and water and then 100% ethanol.
2. Place one spacer along each short edge. Next, place the short plate on top of the spacers. Make sure that the sides and bottoms of both plates and the spacers are approximately even. Stand the plates up on a clean flat surface to align the bottom edges.

3. Clamp the assembly together with a spring clip at each short edge over the spacers.
4. Prepare 5 to 10 ml of molten 1% (w/v) agarose in electrophoresis or resolving gel buffer. Allow it to cool to 50° to 60°C.
5. On the surface of a glass support plate or a piece of parafilm, apply a straight line of molten agarose 10 cm long. Immediately place the glass plate assembly upright on the line of agarose. It will form a plug to seal the bottom of the glass assembly. Allow the agarose to solidify for 5 to 10 min. It is also possible to seal the bottom with 5 ml of resolving gel, instead of agarose.

Protein gels (discontinuous denaturing gels)

Use an acrylamide : bis acrylamide stock solution (ratio 39:1) but you may need to optimize this ratio depending on the protein with which you are working.

CAUTION: *Acrylamide and bisacrylamide are potent neurotoxins and are absorbed through the skin. Their effects are cumulative. Wear gloves and a mask when weighing acrylamide and bisacrylamide. Polyacrylamide is considered to be non-toxic, but it should be treated with care because it may contain small quantities of unpolymerized material.*

Resolving gels

6. Prepare the appropriate volume of solution (10ml if using the Mini-v.8.10 system) containing the desired concentration of acrylamide for the resolving gel. Mix the components in the order shown. Polymerization will begin as soon as the TEMED has been added.

	Volume(ml) by gel percentage			
Component	**7.5%**	**10%**	**12.5%**	**15%**
Distilled water	4.8	4.0	3.1	2.3
1.5M Tris.HCl (pH8.8)	2.5	2.5	2.5	2.5
Acrylamide/bis stock (39:1)	2.5	3.3	4.2	5.0
10% (w/v) SDS	0.1	0.1	0.1	0.1
10%(w/v)APS	0.1	0.1	0.1	0.1
TEMED	0.01	0.01	0.01	0.01

7. Carefully pipette the resolving gel acrylamide solution into the gel plate assembly to within 2.5 cm of the top of the plate. Overlay the solution with a little water to keep the gel surface flat. Keep the assembly vertical while the acrylamide polymerizes.

Stacking gels

8. To prepare 10 ml of 4% acrylamide solution (for 2 gels):

Component	**Volume (ml)**
Distilled water	6.0
0.5M Tris.HCl (pH6.8)	2.5
Acrylamide/bis stock (39:1)	1.3
10% (w/v) SDS	0.1
10%(w/v)APS	0.1
TEMED	0.01

9. Add the first four components, then put under a vacuum until bubbles appear in the solution. Wait until all bubbles have dispersed before switching off the vacuum.
10. After the resolving gel has polymerized, pour off the water. Add the APS and TEMED to the partially prepared stacking gel mixture and immediately pour it to cover the resolving gel. Insert the comb fully (avoid bubbles). Allow the acrylamide solution to polymerize completely before removing the comb.
11. Prepare western running (Tris-glycine electrophoresis) buffer.

Denaturing protein samples before electrophoresis

12. The protein samples in lysis buffer are denatured by adding an equal volume of Laemmli Denaturing/ Reducing Sample Buffer (2X) and then heating them to 100°C for 4 min.

13. Place on ice immediately.
14. Centrifuge samples briefly before loading.
15. Before loading the gel, it is important to thoroughly wash out all the wells to remove any unpolymerized acrylamide.
16. Run 10-50 µg of the protein sample in each well using western running (tris/glycine) buffer at 125 or 150 volts until the protein front reaches the bottom of the gel.
17. After electrophoresis, remove the gel plate assembly from the gel frame and lay it on a paper towel. Use a thin spatula to carefully pry the upper glass plate away from the gel.
18. The gel is now available for further processing. Either stain the gel or transfer the protein to a solid support (both options are outlined below).

Staining sds-page gels with coomassie brilliant blue (optional step)

1. Immerse the gel in 12% trichloroacetic acid solution for 5 minutes.
2. Dissolve 0.25g of Coomasie Brilliant Blue R250 in 90 ml of methanol: H_2O (1:1 v/v) and 10 ml of glacial acetic. Filter the (staining) solution.
3. Immerse the gel in at least 5 volumes of staining solution and place on a shaker (slowly) for a minimum of 4 h at room temperature.
4. Destain the gel by soaking it in the methanol/acetic acid solution without dye, on a shaker (slowly) for 4-8 h, change the destaining solution 3 or 4 times.
5. Store the gels in water in a sealed plastic bag or store them in water containing 20% gylcerol (also the gels can be dried).

Transfer of proteins from sds-page gels to solid support (electrophoretic blotting)

Transfer with a tank system (using a Mini-V 8.10 Vertical Gel Electrophoresis System)

1. Prepare 1.5 L of transfer buffer and store it at 4°C.

Preparation of SDS-PAGE gel and the Immobilon-P Membrane

Preparing the gel

2. Immerse the gel in the transfer buffer for 15 min to equilibrate it (notch a corner of the gel to know the orientation of the membrane after the transfer).

Preparing the membrane

3. Immobilon-P transfer Membrane (Millipore) can be used for western blotting.
4. Cut a piece of membrane to the dimensions of the gel. Notch or label one corner of the membrane to correspond to a corner of the gel.
5. Wet the membrane in 100% methanol for a minimum of 15 seconds. Then transfer it to a container with distilled water for 2 min.
6. Equilibrate the membrane for at least 5 min in the transfer buffer.

Assembling the transfer stack (Blot)

7. Cut 2 pieces of heavy filter paper to the same dimensions as the transfer pressure. Saturate both pieces of filter paper and three pads in transfer buffer.
8. Place the blot restrainer in a suitable container, containing 1 to 2 cm of cold transfer buffer.
9. Place one transfer pressure pad (foam pad) on the restrainer (cassette holder). Cover it with one piece of wet filter paper (to ensure an even transfer, remove air bubbles by carefully rolling a pipette over the surface of each layer in the stack).
10. Place the gel (prepared) on top of the filter paper. Then put a the sheet of the Immunobilon-P membrane (prepared) on top of the gel.
11. Place one sheet of filter paper on top of the stack, followed by a transfer pressure pad. The stack should look like a sandwich.

12. Close the tank cassette holder, (slide the assembled blotting stack into the blot support frame).
13. Place the assembled blot module in the buffer tank in either orientation. Be sure the buffer covers the upper electrodes by 3 to 5 mm, but is 8 to 10 mm below the top edge of the tank. (Do not overfill).
14. Place the safety lid on the buffer tank and connect the power cords to a power supply. Cool the buffer during the transfer to prevent overheating by passing cooling water from the tap through the black lead (to the negative electrode). The red lead (from the positive electrode) carries the cooling water to the sink.

Conditions of the transfer.

15. Turn on the system for 1 to 2 hours at 6 to 8 V/cm inter-electrode distance to transfer the proteins to the membrane. For protein blots a starting point is 150V (constant voltage). Typical operating parameters for protein blotting are shown in Table 4.

Table 4.

Apparatus type	Voltage(V)	Current (mA)	Temperature (°C)
Conventional	100	220-400	28-35
Mini-V 8.10	150	85-160	23-35

Drying the blotted membrane

16. After transfer, remove the cassette holder from the blotting apparatus. Remove the foam pad and filter papers with forceps. Remove the blotted membrane. It is essential to dry it before continuing on with immunodetection. You can select one of the following methods for drying the membrane after blotting:
    - Soak the membrane in 100% methanol for 10-20 seconds to drive out water. Then place the blot on a piece of filter paper. Wait for the methanol to evaporate (approx. 15 min).
    - Place the blot in a vacuum chamber (put the blot between two sheets of filter paper), apply a vacuum. This process usually requires 30 min.
    - Incubate the blot at 37°C.
    - Place the blot on a lab bench to let it dry at room temperature (for approx. 2 h).

IMMUNOLOGICAL DETECTION OF IMMOBILIZED PROTEINS

1. After drying the blotted membrane, transfer it to western blocking buffer with 3% non-fat dry milk for at least 1 h.
2. Rinse with western wash buffer + 3% non-fat dry milk.
3. Incubate with antibody (e.g., anti- rabbit cryIA(b)) at 1µg/ml diluted in western wash buffer with 3% non-fat milk, for at least 3 h or overnight at room temperature.
4. Wash 3X in western wash buffer, 5 min each time.
5. Incubate with an appropriate conjugate (eg rabbit anti-goat horseradish peroxidase Bio-Rad 172-1034) at 1:1000 dilution in western wash buffer with 3% non-fat dry milk, leave for at least 1 h at room temperature.
6. Wash 3X in western wash buffer, 5 min per wash.
7. Develop with Sigma fast DAB tablets (3, 3' Diaminobenzidine) (Sigma D-4418).
    - Remove one DAB and one Urea Hydrogen Peroxide tablet from the freezer, allow them to reach room temperature. Open these tablets and drop them into a appropriate container. Do not touch the tablets with your fingers.
    - Add 15 ml of distilled or deionized water. Vortex until dissolved. This solution should be used within 1 h.
8. Cover the membrane treated with DAB solution.

The reaction should be monitored to prevent over development. Stop the reaction by gently washing the membrane in water or PBS (Sigma P-4417).

Laemmli Denaturing/Reducing Sample Buffer (2x)

	10ml solution
0.5M Tris-HCl, pH 6.8	2.5ml
Glycerol 100%	2.0ml
10% (w/v) SDS	4.0ml
0.1% bromophenol blue	0.5ml
ß-mercaptoethanol (14.3M)	0.5ml
deionized water	to 10 ml

Lysis Buffer

50mM NaH_2PO_4
10mM EDTA
0.1% Triton X-100
0.1% Sarkosyl
10mM ß-mercaptoethanol
25 µg/ml PMSF

Adjust pH of the solution to pH 6.8

CAUTION: PMSF is extremely destructive to the mucous membranes of the respiratory tract, the eyes and skin. PMSF may be fatal if inhaled, swallowed or absorbed through the skin. In case of contact, immediately flush eyes or skin with copious amounts of water. Discard contaminated clothing.

Transfer Buffer

25mM	Tris base
192mM	Glycine
10%	Methanol

Western Blocking Buffer (1x)

	[stock]	[final]	1 liter
Tween 20	20%	2%	100 ml
Tris HCl pH 10.2	1M	30 mM	30 ml
NaCl	5M	150mM	30ml
ddH_2O			to 1 liter

Western running buffer

25mM Tris Base
192mM glycine
0.1%(w/v) SDS
pH 8.3

Western Washing Buffer (10X)

	[final]	2 liters
Tween 20 (20%)	0.05%	50 ml
Tris HCl (2M, pH 7.5)	30 mM	300 ml
NaCl (5M)	150mM	60ml
ddH_2O		to 2 liters

General Stock Solutions

am1 M NH_4OAc: 1 M Ammonium acetate
Dissolve 7.71 g ammonium acetate (MW=77.08) in dH_2O to a final volume of 100 ml. Filter sterilize.

am7.5 M NH_4OAc: 7.5 M Ammonium acetate
Dissolve 57.83 g ammonium acetate (MW=77.08) in dH_2O to a final volume of 100 ml. Filter sterilize.

bo50 mg/ml BSA: bovine serum albumin
Purchase DNAse-free BSA from BRL (cat. #5561) or use the 1 mg/ml BSA provided by Promega with restriction endonucleases.

ca1 M $CaCl_2$: 1 M Calcium chloride
Dissolve 11.0 g $CaCl_2$ (anhydrous MW=110.0) in dH_2O to a final volume of 100 ml. Autoclave.

10 mM dATP (Boehringer Mannheim 103 977) MW=589.2
Dissolve 10 mg in 1697 µl of OLB TE-7 (dissolve directly in original bottle). Store in 50 µl aliquots at -20°C. Mark tubes with black tops.

10 mM dCTP (Boehringer Mannheim 104 035) MW=511.1
Dissolve 10 mg in 1956 µl of OLB TE-7 (dissolve directly in original bottle). Store in 50 µl aliquots at -20°C. Mark tubes with blue tops.

10 mM dGTP (Boehringer Mannheim 104 094) MW=551.2
Dissolve 10 mg in 1804 µl of OLB TE-7 (dissolve directly in original bottle). Store in 50 µl aliquots at -20°C. Mark tubes with green tops.

10 mM dNTP (-T)
Mix equal amounts of 10 mM dATP, 10 mM dGTP, and 10 mM dCTP. Store in 10 µl aliquots at -20°C. Mark tubes with blue dot.

DNA dilution buffer: 10 mM Tris-8.0, 1 mM EDTA, 10 mM NaCl

STOCK	[FINAL]	100 ml	200 ml	300 ml	400 ml	500 ml
1 M Tris-8.0	10 mM	1.0 ml	2.0 ml	3.0 ml	4.0 ml	5.0 ml
0.5 M EDTA-8.0	1 mM	0.2 ml	0.4 ml	0.6 ml	0.8 ml	1.0 ml
5M NaCl	10 mM	0.2 ml	0.4 ml	0.6 ml	0.8 ml	1.0ml
ddH2O		98.6 ml	197.2 ml	295.8 ml	394.4 ml	493.0 ml

ed0.5 M EDTA-8.0
Dissolve 186.12 g $Na_2EDTA \bullet 2H_20$ (MW=372.24) in approx. 750 ml of dH_2O. Add NaOH pellets to bring pH to 8.0. After EDTA is in solution, bring to 1000 ml with dH_2O. Autoclave.

la20% Laurylsarcosine
Dissolve 200 g of N-laurylsarcosine (sodium salt, MW=293.4, Sigma #L5125) in dH_2O to a final volume of 1000 ml. Stir for several hours to dissolve completely. Filter sterilize and aliquot in sterile 15 ml tubes (e.g., Corning)

LB Media

Per liter:	10 g	Bacto-tryptone
	5 g	Bacto-Yeast extract
	10 g	NaCl

Adjust pH to 7.5 with 1 M NaOH.

LB + Amp

Autoclave and let cool to 50°C. Add 100-250 mg Ampicillin (sodium salt, Sigma #A9518) per liter sterile LB. Do not autoclave solution containing antibiotics.

LB + Amp for Plates

Add 15 g Bacto-agar per liter of LB. Dissolve agar in microwave, autoclave. Add Amp pour 25 ml per plate.

LB + Amp for Stabs

Add 7 g Bacto-agar per liter of LB. Autoclave. Add Amp, pour stabs.

1 M $MgCl_2$: 1 M Magnesium chloride

Dissolve 20.33 g $MgCl_2 \bullet 6H_2O$ (MW=203.30) in dH_2O to a final volume of 100 ml. Autoclave.

OLB TE-7 : 3 mM Tris-HCl, 0.2 mM EDTA, pH 7.0

Add 300 µl of 1 M Tris-HCl pH 7.5, and 40 µl of 0.5 M EDTA-8.0 to 90 ml of ddH_2O (the purest you can get; we use Sigma/Cell Culture Water, Cat. # W-3500). Check pH by dropping a few µl onto a pH paper, do not contaminate this solution since it is used for PCR reactions! If necessary, bring pH to 7.0 with HCl and make volume up to 100 ml.

1 M $NaPO_4$ - 6.5: Blot Transfer Phosphate Buffer

For approximately 1 liter, start with 660 ml 1 M NaH_2P0_4 and add 1 M Na_2HP0_4 to bring pH to 6.5 (approx. 330 ml).

- or -

STOCK	500 ml	1000 ml	2000 ml	5000 ml
$NaH_2PO_4 \bullet H_2O$ (MW=137.99)	46 g	92 g	184 g	460 g
$Na_2HPO_4 \bullet 7H_2O$ (MW=268.07)	45 g	90 g	180 g	450 g

Adjust pH to 6.5 with NaOH pellets. Autoclave.

Phenol (equilibrated)

Equilibrate melted (at 65°C) ultra-pure, molecular biology grade phenol by adding an equal volume of Tris - 9.5. Shake well and allow to separate, vacuum aspirate off aqueous (top) layer. Repeat equilibration two more times with Tris - 9.5, and twice with TE-8.0. Verify using pH paper that the phenol pH is grater than 7.0. Leave a small layer of TE on the phenol. Aliquot equilibrated phenol into 50 ml tubes with caps, wrap each in foil, and store at 4°C.

10 mg/ml Proteinase K

Dissolve 100 mg of Proteinase K (BRL # 5530UA) in ddH_2O to a final volume of 10 ml. Dispense 200 µl aliquots into 0.5 ml tubes and store at -20°C.

10 mg/ml RNAse A

Dissolve 100 mg of RNAse (Sigma # R4875) in 10 ml of 10 mM Tris - 7.5, 15 mM NaCl. Heat in boiling water for 15 min and allow to cool slowly to room temperature. Dispense into 1 ml aliquots and store at -20°C. Working stock may be stored at 4°C.

500 U/ml RNAse T1
Dilute RNAse T1 (Sigma #R8251) with 10 mM Tris - 7.5, 15 mM NaCl to 500 U/ml. Heat in boiling water for 15 min and allow to cool slowly to room temperature. Dispense into 1 ml aliquots and store at -20°C.

SS DNA: 10 mg/ml Salmon Sperm DNA
Dissolve 100 mg salmon sperm DNA (Sigma #D1626) in TE - 8.0 to a final volume of 10 ml by rotating overnight at 4°C. Shear the DNA by passing through a 22 gauge needle 3-4 times. Denature by placing in boiling water for 10 min followed by cooling on ice. Aliquot and store at 4°C.

20% SDS: 20% Sodium dodecyl sulphate
Dissolve 200 g lauryl dodecyl sulfate, sodium salt (MW=288.40) in 800 ml dH_20. After complete dissolution adjust to final volume of 1000 ml. Can use a low grade (Sigma #L5750) for HYB washes, etc. and a better grade (Sigma #L4390) for HYB solution, plasmid preps, stop solutions, etc.
Prepare the solution in a fume hood and use gloves and goggles.

5X SGB: Sample Gel Buffer

STOCK	[FINAL]	50 ml	100 ml
1 M Tris-8.0	50 mM	2.5 ml	5.0 ml
0.5 M EDTA-8.0	5 mM	0.5 ml	1.0 ml
Sucrose	25%	12.5 g	25.0 g
BPB	2 mg/ml	100.0 mg	200.0 mg
XC (optional)	2 mg/ml	100.0 mg	200.0 mg
ddH_2O		up to 50.0 ml	up to 100.0 ml

BPB = Bromophenol Blue, sodium salt
XC = Xylene cyanole

2.5 M NaOAc: 2.5 M Sodium acetate
Dissolve 20.5 g sodium acetate (anhydrous, MW=82.03) in dH_2O to a final volume of 100 ml. Autoclave.

5 M NaCl: 5 M Sodium chloride
Dissolve 292.2 g NaCl (MW=58.44) in dH_2O to a final volume of 1000 ml. Autoclave.

1 M NaOH: 1 M Sodium hydroxide
Dissolve 40 g NaOH (MW=40.00) in dH_2O to a final volume of 1000 ml. Autoclave. (Best to weigh approx. 40 g of pellets, then determine correct final volume for a 1 N solution.)

1 M Na_2HPO_4: 1 M Sodium phosphate–dibasic
Dissolve 268 g of sodium phosphate, dibasic, heptahydrate (MW=268.07) in dH_2O to a final volume of 1000 mlAutoclave.

1 M NaH_2PO_4: 1 M Sodium phosphate–monobasic
Dissolve 138 g of sodium phosphate, monobasic, monohydrate (MW=137.99) in dH_2O to a final volume of 1000 mlAutoclave.

0.1 M Spermidine
Dissolve 1 g spermidine (MW= 145.2, Sigma # S2626) in ddH_2O to a final volume of 69 ml. Filter sterilize and aliquot into 5 ml tubes. Store at -20°C, working stock may be stored at 4°C.

2X SSC: 3.7 M NaCl, 0.375 M Na-Citrate, pH 7.4

STOCK	10 liter	20 liter
NaCl (MW=58.44)	175.2 g	350.4g
Na-Citrate•$2H_2O$ (MW=294.10)	88.0 g	176.0 g

Adjust pH to 7.4. Autoclave.

25X SSC: 3.7 M NaCl, 0.375 M Na-Citrate, pH 7.4

STOCK	1 liter	2 liter	3 liter	4 liter	5 liter
NaCl (MW=58.44)	219 g	438 g	657 g	876 g	1095 g
Na-Citrate•$2H_2O$ (MW=294.10)	110 g	220 g	330 g	440 g	550 g

Adjust pH to 7.4. Autoclave.

STE: Sodium Tris-EDTA buffer, pH 8.0

STOCK	[FINAL]	100 ml	200 ml	300 ml	400 ml	500 ml
1 M Tris-8.0	10 mM	1.0 ml	2.0 ml	3.0 ml	4.0 ml	5.0 ml
0.5 M EDTA-8.0	1 mM	0.4 ml	0.8 ml	1.2 ml	1.6 ml	2.0 ml
5 M NaCl	100 mM	2.0 ml	4.0 ml	6.0 ml	8.0 ml	10.0 ml

1 M Tris–pH 7.5, 8.0 or 9.5

Dissolve 121 g Tris-Base in approx. 750 ml dH_2O. Add conc. HCl until desired pH is reached (75 ml HCl = pH 7.5, 49 ml HCl = pH 8.0). Bring solution to 1000 ml with dH_2O. Autoclave.

TE-8: 10 mM Tris–8.0, 1 mM EDTA–pH 8.0

STOCK	50 ml	100 ml	500 ml	1000 ml
1 M Tris - 8.0	0.5 ml	1.0 ml	5.0 ml	10.0 ml
0.5 M EDTA - 8.0	0.1	0.2	1.0	2.0
ddH_2O	to volume	to volume	to volume	to volume

10 mM TTP (Boehringer Mannheim 104 264) MW=570.2

Dissolve 10 mg in 1753 µl of OLB TE-7 (dissolve directly in original bottle). Store in 50 µl aliquots at -20°C. Mark tubes with red tops.

Beckmann Du-65 Spectrophotometer DNA Quantification Program for Beckman Du-65 Spectrophotometer

The following are instructions for a program written for a Beckmann DU-65 Spectrophotometer. The program is designed to enable the user to quickly take A_{260} and A_{280} readings of many samples and from these calculate A_{260}/A_{280} ratios, DNA concentrations, total DNA, and the amount of TE needed to bring the samples to a specified concentration.

1. Turn on UV light source for spectrophotometer. It takes approximately 1 minute for the UV light to come on; however, it is best to wait 15 minutes for the lamp to become stable. When the light is on, it will be indicated by the UV letters in the LCD display changing from lower case to upper case. Make sure that the printer is also powered and on-line.
2. Press the PROG button. This will display programs available to the user. Select Program 0: DNA by pressing either STEP or BSTP.
3. When Program 0: DNA is displayed in the LCD display, press R/S.
4. You will be prompted for the following information:
   STORED INFO Y:1 N:0
   Are you recalculating values for previously stored information? Press 1 and ENTER if Yes, or 0 and ENTER if No.
   DILUTION?
   What is the dilution factor for the samples you are going to read? The default is 1:50. If your samples are diluted to something other than 1:50, enter the correct number and press ENTER. To enter the default, simply press ENTER.
   RNA FACTOR?
   The final DNA concentration is divided by this RNA factor to correct for RNA in the sample. The default RNA factor is 1, indicating that RNase was used on the sample and no RNA is present. Otherwise, a factor of 5 is generally used for maize. Enter the desired number and press ENTER. To enter the default, simply press ENTER.
   RESUS. VOLUME?
   At what volume is your final sample from which the aliquots were taken? The default value is 1500 µl. Enter the desired number and press ENTER. To enter the default, simply press ENTER.
   FINAL µg/µl?
   At what concentration would you like your sample, from which this aliquot has been taken, to be diluted to? The default is 0.2 µg/µl. Enter the desired number and press ENTER. To enter the default simply press ENTER.
5. You will be asked to insert a blank. The blank is whatever liquid you have used to dilute your sample aliquot. This will be used to calibrate the instrument. Press R/S. This is very important since all future calculations are dependent upon it.
6. You will then be asked to insert each sample. Press R/S and the spectrophotometer will sip the sample, calculate concentrations, and request the next sample. This will continue indefinitely until PROG is pressed.
7. Once all of your samples have been checked, values for resuspension and so forth can be recalculated. This is done by rerunning the PROG 0: DNA. When prompted at the beginning of the program about STORED INFO Y:1 N:0, enter a 1 for Yes. You will then be prompted as previously for information, however instead of prompting for the samples the spectrophotometer will recalculate values from figures stored from the last run of samples.

PROGRAM LISTING

PROG 0:DNA

```
000:  Strt
001:  disp 5
002:  ABS
003:  1.
004:  STO 006
005:  MSG cSTO
006:  MSG RED
007:  MSG INFO
008:  MSG Y:1
009:  MSG N:0
010:  CALL ENTR
011:  STO 008
012:  50.
013:  STO 000
014:  MSG DILU
015:  MSG TION
016:  MSG ?
017:  CALL COUT
018:  CALL ENTR
019:  STO 000
020:  4.
021:  CALL BLNK
022:  RCL 000
023:  CALL FOUT
024:  CALL CRLF
025:  1.
026:  STO 001
027:  MSG RNA
028:  MSG FACT
029:  MSG OR?
030:  CALL COUT
031:  CALL ENTR
032:  STO 001
033:  5.
034:  CALL BLNK
035:  RCL 001
036:  CALL FOUT
037:  CALL CRLF
038:  1500.
039:  STO 002
040:  MSG RESU
041:  MSG S VO
042:  MSG L?
043:  CALL COUT
044:  CALL ENTR
045:  STO 002
046:  2.
047:  CALL BLNK
048:  RCL 002
049:  CALL FOUT
050:  CALL CRLF
051:  0.2
052:  STO 003
053:  MSG FINA
054:  MSG L uG
055:  MSG :uL?
056:  CALL COUT
057:  CALL ENTR
058:  STO 003
059:  8.
060:  CALL BLNK
061:  RCL 003
062:  CALL FOUT
063:  CALL CRLF
064:  CALL CRLF
065:  1.
066:  RCL 008
067:  x=y
068:  GOTO READ
069:  1.
070:  CALL CHAN
071:  lbl READ
072:  MSG cINS
073:  MSG ERT
074:  MSG BLAN
075:  MSG K
076:  R/S
077:  CALL FILL
078:  280.
079:  LMDA
080:  CALB
081:  260.
082:  LMDA
083:  CALB
084:  1.
085:  CALL CHAN
086:  rtn
```

PROG 1:HEADER

```
000:  Strt
001:  57.
002:  CALL BLNK
003:  MSG cTOT
004:  MSG AL
005:  CALL COUT
006:  8.
007:  CALL BLNK
008:  MSG cTE
009:  CALL COUT
010:  CALL CRLF
011:  35.
012:  CALL ASCI
013:  5.
014:  CALL BLNK
015:  MSG cSAM
016:  MSG PLE
017:  CALL COUT
018:  1.
019:  CALL BLNK
020:  4.
021:  CALL BLNK
022:  MSG cA26
023:  MSG 0
024:  CALL COUT
025:  5.
026:  CALL BLNK
027:  MSG cA28
028:  MSG 0
029:  CALL COUT
030:  5.
031:  CALL BLNK
032:  MSG c260
033:  CALL COUT
034:  47.
035:  CALL ASCI
036:  MSG c280
037:  CALL COUT
038:  5.
039:  CALL BLNK
040:  MSG cuG
041:  CALL COUT
042:  47.
043:  CALL ASCI
044:  MSG cuL
045:  CALL COUT
046:  5.
047:  CALL BLNK
048:  MSG cuG
049:  MSG DNA
050:  CALL COUT
051:  5.
052:  CALL BLNK
053:  MSG cTO
054:  MSG ADD
055:  CALL COUT
056:  5.
057:  CALL BLNK
058:  35.
059:  CALL ASCI
060:  CALL CRLF
061:  CALL LINE
062:  CALL CRLF
063:  2.
064:  CALL CHAN
065:  rtn
```

PROG 2:LOOP

```
000:  Strt
001:  1.
002:  RCL 008
003:  x=y
004:  GOTO LOOP
005:  3.
006:  CALL CHAN
007:  lbl LOOP
008:  disp 3
009:  RCL 006
010:  STO 012
011:  MSG INSE
012:  MSG RT S
013:  MSG AMPL
014:  MSG E
015:  R/S
016:  CALL FILL
017:  260.
018:  LMDA
019:  READ
020:  STO 004
021:  RCL 006
022:  2.
023:  *
024:  STO 009
025:  RCL 004
026:  CALL STOR
027:  280.
028:  LMDA
029:  READ
030:  STO 005
031:  RCL 009
032:  1.
033:  +
034:  RCL 005
035:  CALL STOR
036:  RCL 006
037:  CALL FOUT
038:  disp 6
039:  2.
040:  CALL BLNK
041:  10.
042:  STO 010
043:  lbl LINE
044:  95.
045:  CALL ASCI
046:  dec 010
047:  GOTO LINE
048:  2.
049:  CALL BLNK
050:  RCL 004
051:  CALL FOUT
052:  3.
053:  CALL BLNK
054:  RCL 005
055:  CALL FOUT
056:  3.
057:  CALL BLNK
058:  RCL 004
059:  RCL 005
060:  /
061:  CALL FOUT
062:  5.
063:  CALL BLNK
064:  0.05
065:  RCL 001
066:  /
067:  RCL 004
068:  *
069:  RCL 000
070:  *
071:  STO 007
072:  CALL FOUT
073:  5.
074:  CALL BLNK
075:  disp 6
076:  RCL 007
077:  RCL 002
078:  *
079:  CALL FOUT
080:  5.
081:  CALL BLNK
082:  RCL 002
083:  RCL 007
084:  *
085:  RCL 003
086:  /
087:  RCL 002
088:  -
089:  CALL FOUT
090:  2.
091:  CALL BLNK
092:  disp 3
093:  RCL 006
094:  CALL FOUT
095:  1.
096:  +
097:  STO 006
098:  CALL CRLF
099:  GOTO LOOP
```

PROG 3: REPEAT

```
000:  Strt
001:  lbl READ
002:  disp 3
003:  RCL 006
004:  CALL FOUT
005:  disp 6
006:  2.
007:  CALL BLNK
008:  10.
009:  STO 010
010:  lbl LINE
011:  95.
012:  CALL ASCI
013:  dec 010
014:  GOTO LINE
015:  2.
016:  CALL BLNK
017:  RCL 006
018:  2.
019:  *
020:  STO 009
021:  CALL LOAD
022:  STO 004
023:  0.01
024:  x>y
025:  GOTO OK
026:  60.
027:  CALL ASCI
028:  0.01
029:  CALL FOUT
030:  GOTO LOOP
031:  lbl OK
032:  RCL 004
033:  CALL FOUT
034:  3.
035:  CALL BLNK
036:  RCL 009
037:  1.
038:  +
039:  CALL LOAD
040:  STO 005
041:  CALL FOUT
042:  3.
043:  CALL BLNK
044:  RCL 004
045:  RCL 005
046:  /
047:  CALL FOUT
048:  5.
049:  CALL BLNK
050:  0.05
051:  RCL 001
052:  /
053:  RCL 004
054:  *
055:  RCL 000
056:  *
057:  STO 007
058:  CALL FOUT
059:  5.
060:  CALL BLNK
061:  disp 6
062:  RCL 007
063:  RCL 002
064:  *
065:  CALL FOUT
066:  5.
067:  CALL BLNK
068:  RCL 002
069:  RCL 007
070:  *
071:  RCL 003
072:  /
073:  RCL 002
074:  -
075:  CALL FOUT
076:  2.
077:  CALL BLNK
078:  disp 3
079:  RCL 006
080:  CALL FOUT
081:  lbl LOOP
082:  RCL 006
083:  1.
084:  +
085:  STO 006
086:  CALL CRLF
087:  RCL 006
088:  RCL 012
089:  x<=y
090:  GOTO READ
091:  rtn
```

BIOSAFETY GREENHOUSE AND SCREENHOUSE PROTOCOLS

CIMMYT Applied Genetic Engineering Laboratory

Overview

CIMMYT's biocontainment facilities are relatively new, having been made operational about six years ago. Four existing greenhouses were refurbished and brought up to standards to meet biosafety regulations.

Security measures are critical and all those using the facilities are expected to follow the established rules and procedures regarding their use. To minimize contamination and dissemination of pollen, all four greenhouses are equipped with double doors for entry and egress. The main common entrance to the greenhouses is equipped with a lock as are all of the individual greenhouses. These doors should remain locked at all times except for authorized entrance. Only personnel with specific clearance are allowed access to these greenhouses. All authorized personnel must log in and out of the greenhouse complex. The titles of experiments using genetically modified organisms (GMOs) are posted at the entrance of each greenhouse. Work coats and rubber gloves are highly recommended for all those entering the area. All plant materials, including plant tissue and roots, and even the soil must be sterilized (autoclaved) before being discarded. Plant material may be taken to and from the biosafety laboratories only in authorized containers. All work implements utilized within the greenhouses must be decontaminated (autoclaved) before being removed from the greenhouses. As there are occasional power interuptions in the area, the greenhouses have been equipped with back-up emergency generators to assure a continuous electrical supply.

The greenhouses are constructed of aluminum framing members and glass panes, with concrete floors. All holes and cracks have been sealed with clear silicone. Each greenhouse is equipped with its own water supply, lights, and cooling and heating system. The lights and cooling and heating system are fully automated; in two of the houses, the watering systems are also automated. In the very near future, these greenhouses will be connected to a computer and alarm system to monitor temperature and humidity and activate an alarm if temperatures exceed set limits. Exterior shading of the greenhouses can be manually manipulated. Of the four greenhouses, two are highly self-contained, meaning the air-conditioning system constantly recirculates the same air—no new air is introduced. These two houses are reserved for sexually mature plants in which crosses between and among plants are accomplished. The other two greenhouses are less contained and are reserved for juvenile plant evaluations (maize in these two houses is not allowed to reach sexual maturity). The cooling systems in the less contained greenhouses are what we term "swamp coolers."

Biosafety Internal Guidelines were developed in April 1994 and are now under revision. These guidelines outline the biosafety policies of the International Maize and Wheat Improvement Center (CIMMYT) and were developed with the aid of documentation provided by experts in the field of GMOs and the Ministry of Agriculture of Mexico. These policies and procedures ensure that CIMMYT's research, particularly with GMOs, is conducted safely, and that the products of such experiments will not adversely affect agriculture or the environment. The well-being of CIMMYT employees and the surrounding communities where research may be conducted is also a high priority of these safeguards.

Preliminary Set-Up Procedures for Biosafety Greenhouses and the Screenhouse

SOIL MIX AND POTS

The ideal soil mix for use in the biosafety greenhouses should have the consistency of a sandy loam with a little organic matter in the soil to help retain moisture. For our particular purposes, we have settled on a mixture of 3 parts of alluvial soil to 1 part sand and 1 part peat moss. This mixture is usually made by mixing 3 wheelbarrows of soil to 1 wheelbarrow of sand with 1 bale of sphagnum peat moss. Add 2 kg of (17-17-17) NPK to provide initial essential elements for juvenile plant needs. No additional fertilizer should be applied until after 20 days of growth.

Four different types of durable plastic growing pots are needed. The more flexible pots appear to be the best and longest lasting. Required pots include

- large (12–14 inches in diameter) for plants to be grown to maturity;
- medium (6 inches in diameter) for evaluating plants to be transplanted into large pots;
- small (3 _–4 inches in diameter) for quick evaluations of a large number of genotypes or lines; and
- small transfer pots (1 _–2 inches in diameter for transferring putative plants from growth media to the peat/light soil mixture.

All pots should have sufficient drainage capabilities, so that no water "permanently resides" in the pot.

FERTILIZER TYPES AND APPLICATIONS

Fertilizer should be applied every 20 days. For large pots, 2.5 grams of urea/triple phosphate (2:1) by volume should be added. For medium pots, add 1.25 grams of urea/triple phosphate (2:1) by volume. Once per month, Peters (20-20-20) NPK soluble fertilizer (2 g/L of water) is applied using approximately 500 ml for large pots and 250 ml for medium pots. For new putative transgenic plants that have been brought to the greenhouse and have adjusted to the environment, a small amount of Peters 20-20-20 NPK fertilizer is applied—approximately 250 ml for a tray of plants at the rate of 1 g/L of water. This helps the new putative plants revive from their selection pressure in tissue culture. Before starting selection in the greenhouse, all plants should be as healthy as possible. For plants grown in the screenhouse, apply 2.5 g of urea/triple phosphate (2:1 by volume) to each plant every 20 days, starting at the 8-leaf stage up until flowering. This usually means 3 applications of 2.5 grams of urea/triple phosphate per plant for the life cycle of the plant.

WATERING

Watering systems can be automated for plants that are grown to maturity within biosafety greenhouses. However, one must check daily to make sure that plants are getting enough or not too much water. Smaller pots can be watered more frequently using less water. **A useful rule of thumb is to test each and every pot with your fingers**. The soil mix should be moist to the touch and not dry or too wet. If there is too much organic matter in the soil mix, decomposition may cause the soil to heat up, causing plant leaves to wilt. If this happens, reduce the amount of organic matter in future soil mixtures. Watering of plants should be undertaken in mid-morning each day, in order to decrease incidents of disease problems. **Commonsense is a must in making decisions about watering plants within greenhouses.**

LIGHTING AND TEMPERATURE

Lighting and temperature control are fully automated within CIMMYT's biosafety greenhouses. If you have different crops growing within the greenhouses, they will have different lighting and temperature requirements. For maize, greenhouse temperatures are set at 24–35°C. A temperature of 28°C appears

optimal for maize in our conditions. Temperatures for wheat should be kept at a lower level, probably 15–22°C. If winter type wheat is grown, one must consider vernalization requirements. Supplementary lighting provides extra growth to plants. This is required for growing temperate-type maize germplasm or photosensitive genotypes, or if additional growth is desired for wheat plants (to insure ample growth before the onset of seed production). During the winter months, supplementary light is provided; an additional 2 hours at both sunrise and dusk. During summer months, an additional 1 hour of supplementary light is provided at both sunrise and dusk. Temperatures in the greenhouses are controlled by a combination of air conditioning and steam radiator heating system. The steam heating system does not perform well on very cold days. Recently, a heating and cooling system has been purchased and installed in the designated screenhouse area, thus ensuring good year-round growing conditions.

Obtaining and Screening Putative Transgenic Plants

MAIZE

New putative maize seedlings are obtained from the laboratory nearly every week. These seedlings are transferred in closed containers to Biosafety #4 Greenhouse and placed under shady conditions until seedlings are acclimated to growing conditions within. At the same time, lists with plant number and experiment number are printed and taken to greenhouse staff to record all phenotypic data for each plant. Seedlings are placed in shady conditions for 2–3 days and then transferred to sunlight conditions and grown for another week, after which they are transplanted into larger pots for transgenic evaluations.

All maize seedlings are color coded with stakes, whereby each colored stake undergoes a different screening operation:

- Orange stakes are selected by insects only.
- Blue stakes are selected by herbicide and insects.
- White stakes are selected by herbicide only.

All plants showing a positive phenotypic result for expression of the transgene will be further evaluated using molecular methods. If maize plants show positive responses for both phenotypic and molecular screening methods, they are transferred into the biosafety greenhouse designated for crossing. All of plants showing positive will be crossed with CML 216 to capture the event.

Screening of putative maize transgenic plants can be accomplished in many ways. In cases in which a marker gene (bar gene) has been used in the transformation process, the entire plant is sprayed or painted with the herbicide Basta‰. For biolistic transformation we have been using a 2% concentration of Basta‰ for screening. Maize plants are painted or sprayed three times in order to minimize escapes.. The response of plants to Basta‰ is very clear: susceptible plants show a necrosis of plant tissue, while resistant plants show normal tissue. Responses to the treatment are usually observed within 1–1^{1}/2 weeks.

Another screening approach is to insert insects into putative *cry* gene plants. Insects are used to test successful transformation. For insect infestation, 10-12 first instar larva are placed within the maize plant whorl at the 6–8 leaf stage. Plants must be checked daily to determine if the insects are still alive. Considerable feeding damage will be evident on susceptible (nontransformed) plants, while resistant plants will show only small feeding holes where insects ate and later died as a result of the the *Bt* toxin. Responses to the treatment are usually observed within 1–1^{1}/2 weeks.

WHEAT

For wheat putative plants, procedures are similar to those used for maize, except that only 2 different colored stakes are used.

- Blue stakes signify that the bar gene is present.
- White stakes indicate that only the gene of interest (e.g. antifungal), the transgene, is present.

Phenotypic selection is conducted by spraying plants with 0.3% Basta‰. Molecular methods of evaluation have been used for confirmation.

Pollination, Harvesting, and Inventory of Seed

Pollinations are made on all maize T_0 plants that have been identified phenotypically and or by molecular methods and found to be stable for the expression of the introduced transgene. The new transgenic insect-resistant maize events are crossed, thus producing T_1 seed for further evaluations.

Sometimes, male tassel and female silks are not synchronized to be receptive at the same time for pollination. This can result from the tissue culture process, which can delay synchronization of male and female receptiveness for up to 2 weeks or longer. In such cases, the top end of the ear can be cut to produce silk (however, this practice is not always viewed postively).

When transgenic maize pollinations are made within the biosafety greenhouses, only red pollinating bags are used, thus indicating that the bag contains transgenic seed. Each bag is labeled with the date of pollination and the female parent x male parent. Generally, 2 pollinations are made from the transgenic plant to CML 216 and 1 pollination from CML 216 to the transgenic plant. The goal is to successfully pollinate three ears and produce a good quantity of seed. Following pollination, all tassels are removed to prevent contamination via pollen. Each plant is allowed to dry down until seeds obtain their black layer. Sometimes, husks are opened in the later milk stage to help speed the dry down process. Seed from each ear is shelled by hand and only good viable seed is retained. Each ear's seed is placed into a separate envelope and labeled with its genotype, origin, and the quantity of seed from that particular ear. Inventory of seed is recorded and 16 seeds are planted to test for germination and to evaluate the expression of the transgene.

Because wheat is self-pollinated, glycine pollination bags are placed over each spike of every putative plant and left through maturity. Molecular screening is conducted on each plant and the presence or absence of the transgene is recorded. Seed from each spike is harvested, cleaned by hand, and placed into a small glycine bag. The number of seeds per spike is recorded. Seeds harvested from a plant are placed in their entirety into a larger envelope, which is then labeled with the plant's genotype, origin, and the quantity of seed obtained from that particular wheat plant.

Both wheat and maize transgenic seeds are stored in an authorized locking cabinet. When there is a need for long-term storage, the material may be stored in special locking cabinets in CIMMYT's germplasm banks.

Insect Bioassays for Putative Maize Transgenic Plants

The bioassay described here is designed as a quick method for identifying and comparing the expression of Bt endotoxin in transgenic T_0 plants. The goal is to be able to subsequently focus pollinating resources in the greenhouse on the best expressing T_0 plants, as greenhouse resources are necessarily limited. Therefore, more T_1 seeds of the best plants will be produced for the next generation for use in more detailed studies of gene stability, inheritance, and expression.

PROCEDURE

The bioassays are made in 5 cm petri dishes.

1. Three petri dishes are prepared for each plant/leaf sample to provide replication.
2. Soak a filter paper in distilled water and place in each dish to provide moisture for the sample. Place the leaf sample (approximately 10 cm^2) on the filter paper. (Leaf samples are taken from the largest new leaf (usually third from top) of each new maize plant used in the bioassay.)
3. Ten 1st-instar larvae are placed on the leaf tissue with a small camel hairbrush. As soon as the larvae are placed on the leaf, seal the dish with paraffin.
4. Incubate the bioassays in the insect rearing lab at room temperature for 96 h. Bioassays are repeated 3 times in order to obtain accurate readings.
5. Two variables are collected from each replicate of the bioassays. First, count the number of larvae that are still alive. Second, a rating is made for the amount of feeding damage observed on each leaf sample. Ratings are on a scale of 0–9 and are somewhat subjective. A rating of "0" indicates no feeding marks; a rating of "1" indicates up to 3 small round feeding marks; a rating of "2" indicates more than 3 feeding marks but no elongated feeding marks; and a rating of "9" indicates large elongated areas of feeding and significant growth in the size of the larvae. Resistant plants will show small feeding holes, but no significant damage will occur to the plant sample.

Another successful technique calls for the direct infestation of each progeny plant with 10–12 larvae

Three insect species have been used for bioassays and direct infestations: *Diatraea grandiosella* (Southwestern corn borer), *Diatraea saccharalis* (sugarcane borer), and *Spodoptera frugiperda* (Fall armyworm). Southwestern and sugarcane corn borers are leaf feeders, but later in their life cycles, they will bore into stalks and feed within until pupating. Fall armyworm is generally an aggressive leaf feeder and its migration over the plant tends to exceed that of the Southwestern and sugarcane corn borers.

Embryo Production

MAIZE

Embryos for maize transformation experiments are grown in both the biotech screenhouse and at the Tlaltizapan and El Batan field stations. Several tropical, subtropical, and temperate inbreds have been grown in order to provide embryos to the labs. The number of different maize genotypes grown has been reduced and efforts have concentrated on those genotypes showing the most potential for regeneration; these include CML 72, CML 216, CML 323, CML 327, A188, hybrid (CML 72 x CML 216) and crosses between these combinations. It is thought that single crosses show the most vigorous embryos and that is why crosses are made between these lines.

WHEAT

Embryos for wheat transformation experiments using different genotypes have been planted in the biotech screenhouse. For transformation experiments on bread wheat, Attila, Luan, Kauz, Bobwhite and Baviacora have been used. For the durum types, Minimus-5, Altar, Don Pedro, and Ariza and one triticale, San Gaspar, have been used. Bread wheat types grown to better quality characteristics include the genotypes Star, Opata, Turaco, and Irena. Occasionally, some or all of these genotypes are also grown in the field at the El Batan station to provide an adequate supply of embryos at crucial times.