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CONSTRUCTION OF PLANT BACTERIAL ARTIFICIAL CHROMOSOME (BAC) LIBRARIES: AN ILLUSTRATED GUIDE

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ABSTRACT

Bacterial artificial chromosome (BAC) libraries have become invaluable tools in plant genetic research. However, it is difficult for new practitioners to create plant BAC libraries *de novo* because published protocols are not particularly detailed, and plant cells possess features that make isolation of clean, high molecular weight DNA troublesome. In this document we present an illustrated, step-by-step protocol for constructing plant BAC libraries. This protocol is sufficiently detailed to be of use to both new and experienced investigators. We hope that by reducing the obstacles to BAC cloning in plants, we will foster new and accelerated progress in plant genomics.

Keywords: bacterial artificial chromosome, BAC, genomics, plant, DNA cloning, physical mapping

TABLE OF CONTENTS

CHAPTER 1

Overview

(terms in blue text are defined in the GLOSSARY)

With the creation of yeast artificial chromosomes (YACs) in the late 1980s (Burke et al. 1987), cloning of megabase-sized DNA fragments became possible, and library-based exploration of even the largest genomes appeared practicable. However, YACs have some serious drawbacks as cloning vectors (Anderson 1993). For example, roughly 50% of YAC clones are chimeric or possess insert rearrangements (Burke 1990; Neil et al. 1990; Green et al. 1991; Anderson 1993; Venter et al. 1996; Cai et al. 1998). Such clones are unsuitable for sequencing and mapping research, and a great deal of time is devoted to "weeding out" chimeras and clones with rearranged inserts (Green et al. 1991; Anderson 1993; Venter et al. 1996). Additionally, manipulation and isolation of YAC inserts is difficult and time consuming (O'Conner et al. 1989; Woo et al. 1994).

In the early 1990s, "bacterial artificial chromosomes" (BACs) emerged as an alternative to YACs (Shizuya et al. 1992). Contrary to their name, BACs are not really artificial chromosomes *per se*, but rather are modified bacterial F factors. Though they can carry inserts approaching 500 kb in length, insert sizes between 80 and 200 kb are more typical (*e.g.*, Shizuya et al. 1992; Woo et al. 1994; Cai et al. 1995; Choi et al. 1995; Kim et al. 1996; Zhang et al. 1996; Yang et al. 1997; Tomkins et al. 1999a, Tomkins et al. 1999b). Most BAC vectors possess traditional plasmid selection features such as an antibiotic resistance gene and a polycloning site within a reporter gene (allowing insertional inactivation) (see Choi and Wing 1999 for a review of BAC vectors and FIGURE 1.1 for a diagram of the most common BAC vector, pBeloBAC11). BAC clones have several notable advantages over YACs. In particular, BACs are relatively immune to chimerism and insert rearrangements (Woo et al. 1994; Cai et al. 1995; Kim et al. 1996; Boysen et al. 1997; Venter et al. 1996; Venter et al. 1998). The stability of BAC inserts appears to be due, in part, to F factor genes (*parA* and *parB*) that prevent more than one BAC from simultaneously inhabiting a bacterium (Willetts and Skurray 1987; Shizuya et al. 1992; Cai et al. 1998). An additional advantage of BAC clones is that they are relatively easy to manipulate and propagate compared to viral- or yeast-based clones (O'Conner et al. 1989; Burke and Olsen 1991; Paterson 1996; Marra et al. 1997). Consequently, BACs have supplanted YACs as the dominant vector used in large-scale physical mapping and sequencing (Cai et al. 1998; Kelley et al. 1999)

BAC libraries in which each clone is stored and archived individually (*i.e.*, ordered libraries) are rapidly becoming a central tool in modern genetics research. Such libraries have been made for a host of taxa (*e.g.*, TABLE 1.1; Cai et al. 1995; Choi et al. 1995; Kim et al. 1996; Wang et al. 1996; Frijters et al. 1997; Marec and Shoemaker 1997; Nakamura et al. 1997; Yang et al. 1997; Danesh et al. 1998; Vinatzer et al. 1998; Moullet et al. 1999; Nam et al. 1999; Salimath and Bhattacharyya 1999), and employed in a variety of applications. For example:

(a) The suitability of BACs as DNA sequencing/PCR templates has led to the development of BAC-end sequencing (Venter et al. 1996; Boysen et al. 1997; Rosenblum et al. 1997), fostered advances in STS-based mapping (Venter et al. 1996, Venter et al. 1998), and provided a means to quickly search well-defined genomic regions for phenotypically-

significant genes (Bouck et al. 1998).

(b) The facility of BACs as a large DNA cloning vector (Shizuya et al. 1992) combined with the development of methods for high-throughput DNA fingerprinting (Marra et al. 1997), contig assembly (Gillett et al. 1996; Soderlund et al. 1997; Ding et al. 1999), BAC-end sequencing, and STS-based mapping have helped investigators bridge gaps between DNA markers in physically-large genomes (*i.e.*, physical mapping). Consequently, many interesting and important genes have been isolated (Wang et al. 1996; Nakamura et al. 1997; Yang et al. 1997; Cai et al. 1998; Danesh et al. 1998; Yang et al. 1998; Folkertsma et al. 1999; Moullet et al. 1999; Nam et al. 1999; Patocchi et al. 1999; Salimath and Bhattacharyya 1999; Sanchez et al. 1999). High-throughput physical mapping already has resulted in the construction of BAC contigs encompassing entire chromosomes and/or complete chromosome sets (Mozo et al. 1999).

(c) Many of the DNA probes used to make genetic maps can be localized to specific BACs, providing a means of superimposing genetic maps directly onto BAC-based physical maps (*e.g.*, Yang et al. 1997; Mozo et al. 1999; Draye et al., submitted). This feature also facilitates map-based cloning of genes responsible for specific phenotypes (Danesh et al. 1998; Nam et al. 1999; Patocchi et al. 1999; Sanchez et al. 1999).

(d) BAC-based physical mapping enjoys the fundamental advantage of somatic cell genetics in that it does not require DNA polymorphism (Lin et al. 2000). Therefore it provides an alternative to radiation hybrid mapping in which chromosomes are broken by radiation and propagated in cell cultures (see Goss and Harris 1975; Deloukas et al. 1998). Of particular interest to botanists, this feature has also spawned efficient methods to determine the locus-specificity of individual BACs that correspond to multi-locus DNA probes in a manner that can efficiently be applied on a large scale (Lin et al. 2000).

(e) BAC-based mapping in conjunction with efficient multiplex screening methods (Cai et al. 1998) may open the door to the development of comprehensive "gene maps" (Hudson et al. 1995) for numerous genomes, conferring many of the advantages of complete genome sequencing decades before complete sequences are likely to be available.

(f) BACs have successfully been employed as probes in fluorescence *in situ* hybridization (FISH) (Cai et al. 1995; Hanson et al. 1995; Jiang et al. 1995; Lapitan et al. 1997; Gómez et al. 1997; Morisson et al. 1998; Godard et al. 1999). FISH-based localization of cloned DNA sequences on chromosomes allows molecular and physical maps to be directly superimposed onto the framework of chromosomes, and subsequently provides useful information on the relationship between chromosome structure, DNA sequence, and recombination (Peterson et al. 1999).

(g) Full-scale BAC-based genome sequencing efforts are underway (Venter et al. 1998).

Current published protocols for constructing BAC libraries are not particularly detailed, making it difficult for investigators without previous experience in BAC library construction to create BAC libraries *de novo*. Additionally, creation of plant BAC libraries has been limited because plant cells possess certain natural features that make isolation of "clean", high molecular weight DNA difficult (*e.g.*, cell walls, stored carbohydrates, and volatile secondary compounds). Collectively, we (the authors of this guide) have been involved in the construction of > 20 plant BAC libraries including libraries for species in which secondary compounds, carbohydrates, and/or endogenous nucleases are known to be a problem (TABLE 1.1). Through this document we seek to introduce the new practitioner to efficient BAC cloning of plant DNA, and also to help the experienced investigator streamline the cloning process. We hope that by reducing the

obstacles to BAC cloning in plants, we will foster new and accelerated progress in plant genomics, and contribute to the rapid growth in the plant genomic infrastructure that is opening the door to a new era of botanical discovery.

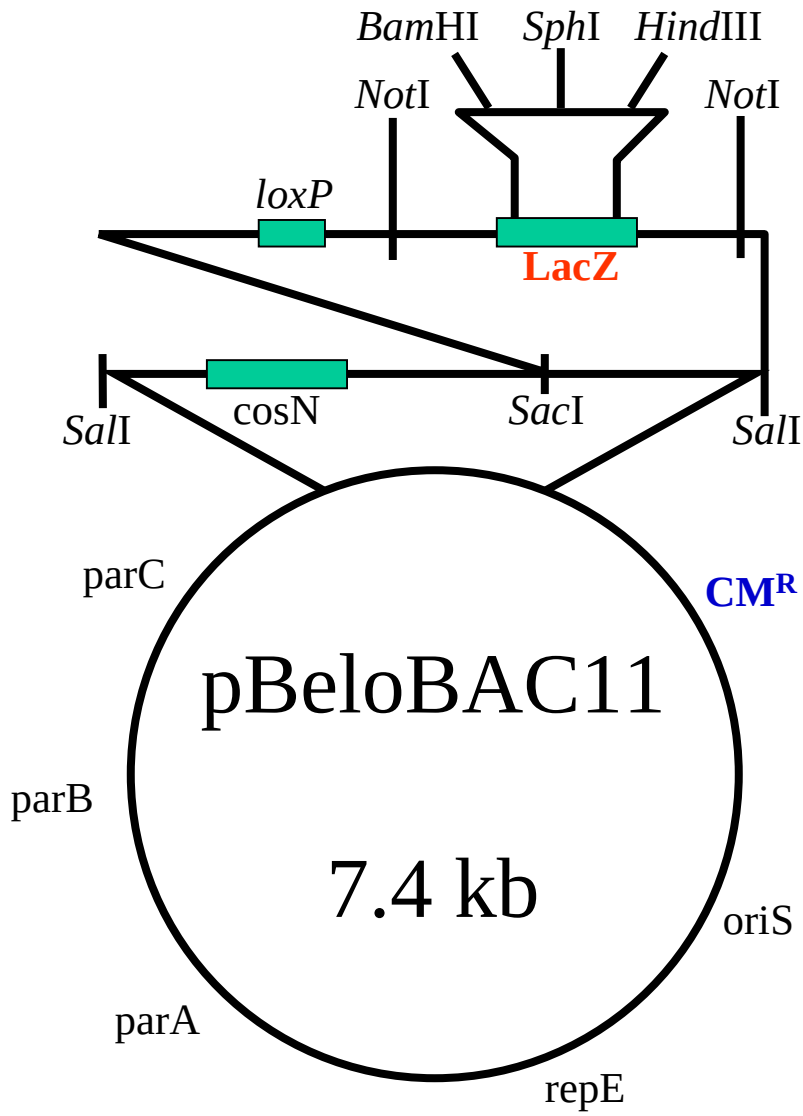


FIGURE 1.1 - pBeloBAC11. The genes *parA, parB, and parC* are required for partitioning. Additionally, *parB* and *parC* are required for incompatibility with other F factors. The *repE* gene encodes a protein essential for replication from the *oriS*. A chloramphenicol resistance gene (CM^R) has been incorporated for antibiotic selection of transformants. pBeloBAC11 has a polycloning site with recognition sequences for three different restriction enzymes (*Hind*III, *Bam*HI and *Sph*I). The polycloning site is located within the *lacZ* gene allowing identification of recombinants by alpha-complementation. The figure above is based on Figure 1 from Choi and Wing (1999).

TABLE 1.1: Plant BAC libraries constructed by the authors and their associates using techniques described in this guide. Except where noted, 1C-genome sizes are from Arumuganathan and Earle (1991).

Species 'cultivar'	Genome size (Mb/1C)	Number of BAC clones	Mean insert size (kb)	Genome coverage	Reference
Arabidopsis thaliana 'Columbia'	100[a]	12,672	100	12.0	Choi et al. 1995
Carica papaya 'Sunup'	372	39,168	132	13.0	Ming et al., in prep.
Poncirus trifoliata 'Rubidoux'	382[b]	45,312	76	9.0	Yang et al. 1999
Glycine max 'P1437654'	1,115	73,728	136	8.7	Tomkins et al. 1999a
Glycine max 'A3244'	1,115	326,000	148	27.0	Tomkins et al. 2000a
Gossypium barbadense 'Pima S6'	2,895[c]	99,840	100	5.0	Abbey et al., in prep.
Gossypium hirsutum 'Maxxa'	2,246[d]	147,456	140	9.2	Tomkins et al., in prep.
Gossypium hirsutum 'Tamcot GCNH'	2,246[d]	51,456	110	2.5	see CUGI website
Gossypium raimondii	1,255[c]	92,160	120	9.0	Peterson et al., in prep.
Hordeum vulgare 'Morex'	5,000	313,344	110	6.3	Yu et al. 1999
Solanum lycopersicum 'Heinz 1706'	954[e]	129,024	115	15.0	Budiman et al. submitted
Oryza sativa japonica 'Nipponbare'	427[e]	36,864	130	10.6	see CUGI website
Oryza sativa japonica 'Azucena'	427[e]	38,784	80	6.8	see CUGI website
Oryza sativa japonica 'Lemont'	427[e]	7,296	150	2.6	Zhang et al. 1996
Oryza sativa indica 'Teqing'	441[e]	14,208	130	4.4	Zhang et al. 1996
Saccharum officinarum 'R570'	3,076[e]	103,296	130	4.5	Tomkins et al. 1999b
Sorghum bicolor 'BTx623'	760[e]	13,440	130	2.6	Woo et al. 1994
Sorghum propinquum	760-835[f]	38,016	126	6.6	Lin et al. 2000
Triticum monococcum	5,751	276,480	115	5.6	Lijavetzky et al., 1999
Vitis vinifera 'Syrah'	483	55,296	135	16.0	Tomkins et al., in prep.
Zea mays 'LH132'	2,504[e]	422,400	120	20.2	Tomkins et al. 2000b
Zea mays 'B73'	2,504[e]	239,616	140	13.5	Tomkins et al. 2000c
Zea mays 'Mo17'	2,504[e]	150,000	120	20.2	Tomkins et al. 2000b

[a] Value from Goodman et al. (1995)
[b] Estimate based on genome size of *Citrus sinensis* (Arumaganathan and Earle 1991), a close relative of *Poncirus trifoliata*
[c] From Bennett et al. (1982)
[d] Mean of two values given for this species in Arumuganathan and Earle (1991)
[e] Median for a range of values given for this species in Arumuganathan and Earle (1991)
[f] K. Arumuganathan, personal communication

CHAPTER 2
Supplies, equipment, and reagents

Names and descriptions of the reagents, supplies, and equipment necessary for BAC library construction are presented below. Additionally, instructions for making and storing solutions and media are discussed in detail.

Manufacturer/distributor names are given for items/components that (to our knowledge) are (a) produced by a single company, (b) known to be well suited for the application at hand, (c) packaged and sold in quantities/forms that make them especially easy to use, and/or (d) rare or difficult to find. While we have used these products in our research, we do not guarantee their availability, quality, *etc*., nor do we wish to imply that these products are inherently superior to those of other manufacturers.

Though each of the following chapters contains a list of reagents, supplies, and equipment necessary for the procedures described in that chapter, consult CHAPTER 2 for details regarding these items. Necessary equipment and supplies common to most cell and molecular biology laboratories are not listed at the beginning of individual chapters. However, these items are listed under the subheadings titled "**Other**" in the sections below.

SUPPLIES (*i.e.*, disposable items)

1. *Cheesecloth*
2. *Miracloth* (Calbiochem, cat. no.475855)
3. *Miracloth squares*: Cut Miracloth into 3 cm^2 pieces and autoclave.
4. *Nitrocellulose filters* (Millipore, cat. no. VSWP 025 00): 0.025 µm pore size
5. *Plug molds*: Plug molds can be purchased commercially from BioRad. We generally purchase the "disposable" plug molds (cat. no. 170-3713) and re-use them numerous times (see CHAPTER 7 for details). BioRad also sells reusable plug molds (cat. no. 170-3622). However, almost any plastic or glass item into which melted agarose can be poured can serve as a plug mold (*e.g.*, microcentrifuge tubes, plastic pillboxes, pipet tips, small syringes, *etc*.). If non-standard objects are used as plug molds, cut the resulting plugs into 2 mm x 5 mm x 10 mm rectangles before placing them in lysis buffer.
6. *Scalpel with #11 blade*
7. *Solution filters* (disposable; Millipore, cat. no. SLGP R25 CS): 0.22 µm pore size; for use in filter sterilization of solutions
8. *384-well or 96-well microtiter plates*: These plates can be purchased from several different companies including Nunc, Genetix, and Genome Systems. Because we use a Genetix QBot for clone picking, library replication, gridding, and arraying, we purchase 384-well plates (Genetix, cat. no. X7001) specifically designed for use with a QBot. The Genetix plates have a relatively low profile (which saves valuable freezer space) and are moderately priced (ca. $2.00 per plate).
9. *Sterile (autoclaved) toothpicks or a hand-held colony picker*: Traditionally, sterile toothpicks have been used in the manual transfer of clones. However, V&P Scientific sells a 12-pin, hand-held colony picker (cat. no. VP 373) that can be used in place of sterile toothpicks. The metal pins of the picker can be flame-sterilized. Use of the hand-held picker reduces the number of movements

between X/I/C trays and microtiter plates.

10. *Library storage boxes*: We store our libraries in cardboard boxes specially designed for holding 36 microtiter plates and/or metal boxes engineered to hold 180 microtiter plates. The cardboard boxes were designed by D. A. Frisch and can be purchased from Southern Container (FIGURE 17.2). The 5-sided metal boxes were designed by A. H. Paterson and can be manufactured by any appropriate metal shop (FIGURE 17.3). The cardboard boxes are less expensive than the metal boxes and have the advantage that stacks of plates are secured on all sides. The metal boxes are sturdier, more space-efficient, and easier to access.
11. *Other*: pipet tips; 15 ml culture tubes with caps (sterile); 50 ml polypropylene centrifuge tubes (sterile); Pasteur pipets; Kimwipes; Parafilm; plastic wrap; sterile 0.65 ml and 1.5 ml microcentrifuge tubes; weighing boats/paper; 50 ml syringes (for use with solution filters in filter sterilization); autoclavable hazardous waste bags; autoclave tape; aluminum foil; marker pens with ink insoluble in water but soluble in ethanol

EQUIPMENT

1. *Standard kitchen blender*
2. *Light microscope*: The microscope should be capable of bright-field and/or phase-contrast illumination and have a total magnification power of at least 200x.
3. *Mortar and pestle*
4. *CHEF gel apparatus*: Instruments suitable for BAC library construction (and general analysis of high molecular weight DNA) are the BioRad CHEF-DR II System (cat. no. 170-3725), the CHEF-DR III Variable Angle System, (cat. no. 170-3700) and the CHEF Mapper™ XA System, (cat. no. 170-3670). All systems are equipped with essentially identical electrophoresis chambers, chiller systems, variable speed pumps, combs, casting stands, *etc*. The three systems differ with respect to the capabilities of their power/control units. Electrophoresis parameters given in the following chapters apply to all three BioRad models.
5. *Large CHEF gel casting stand* (BioRad, cat. no. 170-3704): 21 x 14 cm
6. *45-tooth gel comb* (for use with the large CHEF gel casting stand): BioRad, cat. no. 170-3645.
7. *30-tooth gel comb* (for use with the large CHEF gel casting stand): BioRad, cat. no. 170-3628.
8. *Regular CHEF gel casting stand* (BioRad, cat. no. 170-3689): 14 x 13 cm
9. *15-tooth gel comb* (for use with the regular CHEF gel casting stand): BioRad, cat. no.170-4324.
10. *UV light box equipped with camera or image capture system*: For use in examination and photo-documentation of ethidium bromide-stained agarose gels.

✸ Note 2.1: Always wear appropriate eye and face protection when using a UV light box!

11. *Electroporation system*: Electroporation devices are available from several biotechnology companies. See CHAPTER 5 for a discussion of electroporation and APPENDIX A for specific information regarding use of the Gibco BRL CELL-PORATOR® Electroporation System (cat. no. 11609-013).
12. *Plating rod*: A plating rod is a glass bar or tube bent in such a way that it is well suited for spreading liquid across the surfaces of agar plates (see FIGURE 5.1 and FIGURE 5.2).
13. *Plating trays*: Square, sterile plating trays (500 cm^2) can be purchased from Genetix (Q-Trays, cat. no. X6021). The trays are re-useable. Genetix trays are specifically designed for use with a QBot (a robot designed to pick clones, make BAC grids, *etc*.). However, these trays also are useful for creating "hand-picked" libraries. If trays are being re-used they must be sterilized prior to adding media. To sterilize used trays, wash trays thoroughly with soap and water, allow to air-dry, spray with 80% ethanol, and place the trays in a single layer in a laminar-flow hood equipped with a UV

light for sterilization. Remove the lids from the trays, and stack the lids in the corner of the hood. UV-sterilize the trays for at least 30 minutes. Replace the lids and stack plates on top of each other.

14. *Multi-channel repeat pipettor or automated microtiter plate filler*: Such devices are necessary for filling microtiter plates with media. Multi-channel repeat pipettors suited for filling 96-well plates are available from a variety of biotech companies. To our knowledge, there are no multi-channel repeat pipettors for filling 384-well plates. Automated microtiter plate media dispensers allow rapid filling of both 96-well and 384-well plates. They save time and decrease potential contamination problems associated with hand-held pipettors. The Genetix QFill2 (cat. no. QF21) media dispenser can fill a 384-well plate in less than 20 seconds, has an autoclavable manifold and tubing for rapid and thorough clean up, and can be programmed to accurately dispense volumes between 20 µl and 2.0 ml (see APPENDIX C). BioRobotics and Bioneer also sell a comparable automated media dispenser (the PlateFill). V&P Scientific sells a semi-automated plate filling device (the Multi-Spense).
15. *Hand-held plate replicator (optional)*: Plastic and/or stainless-steel plate replicators are sold by several companies including V&P Scientific, Nunc, Genetix, and Genome Systems.
16. *Electroelution system (optional)*: See CHAPTER 13 for a discussion of electroelution and APPENDIX B for specific information regarding use of the BioRad Model 422 Electro-Eluter, (cat. no. 165-2976).
17. *Automated DNA isolation system (optional)*: BAC minipreps can be prepared using standard plasmid isolation techniques (*e.g.*, CHAPTER 6) and/or a number of commercially available kits. In addition, AutoGen sells robots capable of performing BAC isolations from liquid cultures. The Clemson University Genomics Institute (CUGI) uses an AutoGen 740 for performing BAC minipreps.
18. *High-throughput genomics robot (optional)*: Robots designed specifically for large-scale genomics are available from several companies including Genetix, BioRobotics, GeneMachines, Genomic Solutions, Bioneer, Parallel Biotechnologies, and AutoGen.

    The Genetix "QBot" is specifically designed for high-throughput research and is utilized by the Clemson University Genomics Institute (CUGI) (Rod A. Wing, Director) and the University of Georgia AGTEC Center (AGTEC = Applied Genetic Technology) (A. H. Paterson, Director) as well as many other genomic laboratories around the world. The QBot is designed for high speed, high accuracy colony picking, macroarraying, gridding, replicating, rearraying and microarraying. Its picking head, which consists of 96 separately indexable pins, allows picking rates in excess of 3,500 clones per hour. It has automated plate handling, lid lifting, bar code reading, and pin sterilization.

    High-throughput genomics robots are not yet standard features in all genomics labs. However, there are several companies and non-profit organizations that offer colony picking, library replication, and library gridding services. These include Genomic Solutions, GeneScreen, AGRF, the Clemson University Genomics Institute (CUGI), the University of Georgia AGTEC Center, Genome Systems, and Bioneer.
19. *Other*: laminar-flow hood with UV sterilization lamps and Bunsen burner; water baths (16ºC, 37ºC, 45ºC, 65ºC, 70ºC); incubator shaker (37ºC); incubators (30ºC, 37ºC, 50ºC); assorted digital pipettors; stir/hot plates; pH meter; balances; vortex; microwave oven; small submarine gel apparatus (mini-gel apparatus) with power supply; refrigerated centrifuge(s) with rotors/adapters for 500 ml plastic bottles, 50 ml polypropylene centrifuge tubes (conical), and 15 ml culture tubes; microcentrifuge; refrigerator; -80ºC and -20ºC freezers; 4 L plastic beakers; assorted glassware; stir bars; spatulas; autoclave; sources of distilled and molecular biology-grade water; scissors; a large glass or plastic funnel; water bottles

REAGENTS – Unless otherwise noted, solutions are aqueous.

1. *MBG (molecular biology-grade) water*: MBG water is sterile water that is essentially free of salts, particulates, and dissolved minerals and metals. It may include "double-distilled" (ddH_20) water, deionized water, filtered distilled water, or water purified by other means.

✸ Note 2.2: Unless otherwise noted, all aqueous solutions should be made using MBG water.

2. *Distilled water*: Distilled water is of intermediate quality between tap water and MBG water.
3. *20% SDS (sodium dodecyl sulfate)*: Do not autoclave. Store at room temperature.
4. *5 N NaOH stock solution*
5. *Qiagen Large-Construct Kit* (Qiagen, cat. no. 12462): This kit is specifically designed for isolating highly pure BAC DNA.
6. *Agarose*: Molecular biology grade
7. *HindIII with 10X buffer and 100X BSA* (New England BioLabs, cat. no. 104L): Enzyme concentration is 20,000 units/ml. The 10X buffer and 100X BSA solutions are supplied with the enzyme.
8. *HK phosphatase with 10X phosphatase buffer and 0.1 M $CaCl_2$* (Epicentre Technologies, cat. no. H92050): The enzyme concentration is 1.0 units/µl.
9. *T4 ligase with 10X ligase buffer* (Promega, cat. no. M1804): The enzyme concentration is 3.0 units/µl.
10. *Blue juice (500 ml)*: Mix 350 ml glycerol, 25 ml 10X TBE, 20 ml 0.5 M EDTA (pH 8.0), 5 ml 20% SDS, and 500 mg bromophenol blue. Add MBG water to 500 ml. Store in aliquots at –20ºC.
11. *λH3 DNA* (Gibco BRL, cat. no. 15612013): λH3 DNA is lambda phage DNA digested to completion with *Hind*III. It is supplied at a concentration of 500 ng/µl in a buffer consisting of 10 mM Tris-HCl (pH 7.4), 5 mM NaCl, and 0.1 mM EDTA. λH3 DNA is used as both a size standard and in test ligations.
12. *Ethidium bromide*: 10 mg/ml. Store in a light-proof container at room temperature.

✸ Note 2.3: Ethidium bromide should be handled with great care. It is a mutagen / carcinogen!

13. *5X TBE*: 0.45 M Tris base (THAM), 2.0% v/v 0.5 M EDTA (pH 8.0) stock solution, 0.44 M boric acid. To make 1.0 L, place 54 g of Tris base, 20 ml of 0.5 M EDTA (pH 8.0), and 27.5 g boric acid in a 1 L bottle. Add MBG water to 1.0 L. Adjust the pH to 8.3.
14. *0.5X TBE*: 5X TBE diluted 1:10 with MBG water
15. *PFGE Lambda Ladder* (New England BioLabs, cat. no. 340): The ladder DNA is embedded in agarose loaded in a 1.0 cc syringe. This ladder is suitable for most PFGE applications.
16. *PFGE Midrange ladders I and II* (New England BioLabs, cat. no. 355-1 and 355-2, respectively): Because they have more bands than the PFGE Lambda Ladder, the Midrange ladders are of particular use in accurately determining insert sizes from CHEF gels (see CHAPTER 14).
17. *pBeloBAC11 vector in DH10B (E. coli)*: To obtain pBeloBAC11, write to Dr. H. Shizuya and Dr. M. Simon, Department of Biology, California Institute of Technology, Pasadena, CA 91125, FAX: 808-796-7066 or visit the Caltech Genome Research Laboratory website. Recently BAC vectors derived from pBeloBAC11 have become commercially available (*e.g.*, True-Blue® BAC from Genomics One).

✸ Note 2.4: To prevent confusion, pBeloBAC11 (the most popular BAC vector) will be the only vector discussed in this guide. However, other BAC vectors can be used for cloning, and some vectors are designed for highly specific applications (see Choi and Wing 1999).

18. *T4 ligase with 10X buffer* (New England BioLabs, cat. no. 202L): Enzyme concentration is 3.0 units/µl.

19. *CM (chloramphenicol) stock solution*: 5% w/v dissolved in 100% ethanol. To make 20 ml of CM stock solution, dissolve 1.0 g of chloramphenicol in 20 ml of 100% ethanol. Store in a sterile 50 ml polypropylene centrifuge tube at –20ºC.
20. *Mg^{2+} stock solution*: 1.0 M $MgCl_2 \bullet 6H_20$, 1.0 M $MgS0_4 \bullet 7H_20$. Filter-sterilize and store in sterile container(s) at room temperature.
21. *2.0 M glucose stock solution*: Filter-sterilize and store in a sterile container(s) at room temperature.
22. *1.0 M KCl stock solution*: Filter-sterilize and store in a sterile container(s) at room temperature.
23. *SOC (1.0 L)*: Mix 25 g of granulated LB broth (EM Science, cat. no. 1.10285.5000), 10 ml 1.0 M KCl stock solution, and 980 ml MBG water. Adjust pH to 7.0. and autoclave. Cool to room temperature. Immediately before use add 10 ml of Mg^{2+} stock solution and 10 ml of 2.0 M glucose stock solution.
24. *LB broth (1.0 L)*: Place 25 g of granulated LB broth (EM Science, cat. no. 1.10285.5000) in a 2.0 L autoclavable bottle. Add MBG water to 1.0 L and mix thoroughly. Adjust solution to pH 7.0 and autoclave. Store at room temperature.
25. *LB^{+CM}*: Immediately before use, add 250 µl of CM stock solution to 1 L of LB broth (room temperature) to produce LB^{+CM}.
26. *X-GAL (5-bromo-4-chloro-3-indolyl-beta-D-galactoside) stock solution*: 2% w/v X-GAL dissolved in NN-dimethylformamide. To make 50 ml of X-GAL stock solution, dissolve 1.0 g of X-GAL in 50 ml NN-dimethylformamide. Store in a light proof container at –20ºC.
27. *IPTG (isopropylthiogalactoside) stock solution*: 20% w/v. To make 10 ml of IPTG stock solution, dissolve 2.0 g of IPTG in 10 ml of MBG water. Filter-sterilize and store in 1.0 ml aliquots at -20ºC.
28. *X/I/C (X-GAL, IPTG, CM) agar*: 2.5% w/v granulated LB broth (EM Science, cat. no. 1.10285.5000), 1.5% w/v bacto-agar, 0.45% v/v X-GAL stock solution, 0.045% v/v IPTG stock solution, 0.025% v/v CM stock solution. To make 1.0 L of X/I/C agar, place 25 g of granulated LB broth, 15 g of bacto-agar, and a stir bar in a 2.0 L flask. Add MBG water to 1 L and stir until the LB has gone into solution. Cover the top of the flask with aluminum foil and autoclave. Place the mixture on a stir plate and engage the stir bar. Allow the solution to cool to about 50ºC. Transfer the flask to a laminar-flow hood. Gently lift one corner of the foil and add 4.5 ml X-GAL stock solution, 450 µl of IPTG stock solution, and 250 µl of CM stock solution. Swirl the contents of the flask to facilitate mixing. Use the media to prepare X/I/C Petri plates or X/I/C trays as described below.
29. *X/I/C trays*: Obtain sterile plating trays. If trays are being re-used, wash trays thoroughly with soap and water, dry, and place trays in a single layer in a laminar-flow hood equipped with UV lamps. Remove the lids from the trays and stack the lids in the corner of the hood. UV-sterilize the trays for at least 30 minutes. Replace lids and stack plates. See FIGURE 2.1 for instructions on how to make X/I/C trays using X/I/C agar.
30. *X/I/C Petri plates*: Set up a laminar-flow hood as described in FIGURE 2.1. Remove the lid from a sterile 90 mm Petri plate. Pour approximately 50 ml of 50ºC X/I/C media into the bottom of the plate. Immediately replace the lid and set the plate in the corner of the hood. Repeat this process until all of the X/I/C media has been poured into plates. Allow the plates to sit in the hood for 2 hours. Carefully wipe any condensation from the inside of each Petri plate lid with a Kimwipe. Invert and stack the plates. The plates can be used immediately, wrapped in aluminum foil and stored (upside down) at room temperature for several days, or wrapped in foil and stored (upside down) at 4ºC for several weeks.
31. *ELECTROMAX® DH10B™ competent cells* (Gibco BRL, cat. no. 18290-015): Store at –80ºC. DO NOT ALLOW CELLS TO THAW!

32. *MP-1 (Miniprep solution 1)*: 50 mM glucose, 25 mM Tris HCl (pH 8.0), 10 mM EDTA. Autoclave and store at 4ºC.
33. *MP-2 (Miniprep solution 2)*: 0.2 N NaOH, 1.0% w/v SDS. Make immediately before use from 5N NaOH stock solution and 20% SDS stock solution.
34. *MP-3 (Miniprep solution 3)*: 3.0 M potassium acetate, 28.5% v/v acetic acid. Make immediately before use.
35. *Isopropanol*: Store at –20ºC
36. *NotI with 10X buffer and 100X BSA* (New England BioLabs, cat. no. 189L): The enzyme concentration is 10 units/µl.
37. *MPDB (2-methyl-2,4-pentanediol buffer)* (made day of experiment): 0.5 M 2-methyl-2,4-pentanediol (Aldrich, cat. no. 11,210-0), 10 mM PIPES-KOH, 10 mM $MgCl_2$, 0.5% Triton X-100, 10 mM sodium metabisulfite, 5 mM β-mercaptoethanol, pH 7.0. To make 50 ml of MPD buffer (enough for a typical experiment), place the following into a 100 ml beaker: 2.95 g of 2-methyl-2,4-pentanediol, 0.15 g of PIPES-KOH, 0.10 g of $MgCl_2$, 0.10 g of sodium metabisulfite, 0.25 ml of Triton X-100, and 19.5 µl of β-mercaptoethanol. Bring the total volume to 50 ml with MBG water. Add NaOH until the final pH is 7.0. Place the solution in the refrigerator.
38. *MEB (MPD-based Extraction Buffer)*: 1.0 M 2-methyl-2,4-pentanediol (Aldrich, cat. no. 11,210-0), 10 mM PIPES-KOH, 10 mM $MgCl_2$, 2% polyvinylpyrrolidone (PVP) (Sigma, cat. no. PVP-10), 10 mM sodium metabisulfite, 5 mM β-mercaptoethanol, 0.5% sodium diethyldithiocarbamate (Sigma, cat. no. D-3506; store at -20°C with dessicant), pH 6.0. Prepare MEB on the day of the extraction, and chill the buffer to 4°C before starting the isolation. To make 3 L of MEB (enough for a typical experiment), place the following into a 4 L plastic beaker: 354.54 g of 2-methyl-2,4-pentanediol (Aldrich, cat. no. 11,210-0), 9.07 g of PIPES-KOH, 6.10 g of $MgCl_2 \bullet 6H_20$, 5.70 g of sodium metabisulfite, 15 g of sodium diethydithiocarbamate (Sigma, cat. no. D-3506; store at -20°C with dessicant), 2 L of distilled water, and 1.2 ml of β-mercaptoethanol. Mix using a stir bar. Add 60 g of PVP (Sigma, cat. no. PVP-10) a little at a time to prevent formation of PVP clumps. Add water or ice until the total volume is slightly less than 3 L. Add highly concentrated HCl to the medium until the pH is 6.0. If the pH falls below 6.0, add 1N NaOH until the pH is 6.0. Place the solution in the refrigerator.
39. *TKE (Tris, KCl, EDTA)*: 0.1 M Tris base, 1.0 M KCl, 0.1 M EDTA, pH 9.4-9.5. Autoclave and store at 4°C.
40. *Liquid nitrogen*
41. *SEB (Sucrose-based Extraction Buffer)*: 10% v/v TKE, 500 mM sucrose, 4 mM spermidine, 1 mM spermine tetrahydrochloride, 0.1% w/v ascorbic acid, 2.0% w/v PVP (MW 40,000), and 0.13% w/v sodium diethyldithiocarbamate (Sigma, cat. no. D-3506; store at -20°C with dessicant). To make 1 L, mix 100 ml TKE, 171.2 g of sucrose, 1.0 g spermidine, 0.35 g spermine tetrahydrochloride, 0.45 ml β-mercaptoethanol, 1.0 g ascorbic acid, 20.0 g PVP-40, and 1.3 g sodium diethyldithiocarbamate. Add MBG water to 1 L. Make SEB up fresh the day that it is going to be used.
42. *SEB^{+BME}*: 0.2% v/v β-mercaptoethanol in SEB. Add β-mercaptoethanol immediately before use.
43. *SEB^{+BME}/Triton*: 10% v/v Triton X-100 in SEB^{+BME}
44. *100X TE (Tris-EDTA) stock buffer*: 1.0 M Tris base (also known as THAM), 0.1 M EDTA. To make 2 L, place 242.28 g of Tris-base and 58.44 g of EDTA (or 74.44 g of EDTA disodium salt: dihydrate) in a 4 L beaker. Add distilled water to 1.5 L. Adjust pH with concentrated HCl to yield a solution that is pH 7.0. Add distilled water to a final volume of 2 L. Place the 100X TE in glass bottles.

45. *1X TE slurry (4.0 L)*: Fill a 4-5 L polypropylene beaker to 3.5 L with crushed ice. Add 40 ml of 100X TE stock buffer. Add distilled water to 4 L.
46. *1X TE (non-sterile)*: Dilute the 100X TE stock solution 1:100 with distilled water.
47. *Percoll* (Amersham Pharmacia, cat. no. 17-0891-01): Percoll consists of silica particles (15-30 nm diameter) coated with PVP. Percoll is non-toxic and virtually chemically inert. When spun in a centrifuge, Percoll forms a density gradient within the range of 1.0-1.3 g/ml. Gradients are iso-osmotic throughout.
48. *Proteinase K*: This enzyme can be purchased from a variety of molecular biology companies. When purchasing Proteinase K, make sure that the enzyme you order is suitable for molecular biology applications (*i.e.*, molecular biology grade).
49. *0.5 M EDTA (pH 9.1) stock solution*: Autoclave and store at room temperature.
50. *0.5 M EDTA (pH 8.0) stock solution*: Autoclave and store at room temperature.
51. *1.0 M Tris-HCl (pH 8.0) stock solution*: Autoclave and store at room temperature.
52. *Lysis Buffer*: 1% w/v sodium lauryl sarcosine, 0.1 mg/ml proteinase K, 0.1% w/v ascorbic acid, 2.0% w/v PVP-40, and 0.13% w/v sodium diethyldithiocarbamate (Sigma, cat. no. D-3506; store at -20°C with dessicant) dissolved in 0.5 M EDTA (pH 9.1) stock solution
53. *Diethyl ether* (4°C)
54. *Methylene blue* (1.0% w/v)
55. *LMP (low melting point) agarose* (BMA, SeaPlaque GTG® Agarose, cat. no. 50111)
56. *10X uncut lambda DNA* (Amersham Pharmacia, cat. no. 27-4118-01): The DNA comes frozen in solution. The DNA concentration of the solution is 500 µg/ml.
57. *1X uncut lambda DNA*: 10X uncut lambda DNA diluted 1:10 in sterile 1X TE
58. *WB-A (Wash Buffer-A)*: 0.1% w/v ascorbic acid, 2.0% w/v PVP-40, and 0.13% w/v sodium diethyldithiocarbamate (Sigma, cat. no. D-3506; store at -20°C with dessicant) dissolved in 0.5 M EDTA (pH 9.1).
59. *WB-B (Wash Buffer-B)*: 0.1% w/v ascorbic acid, 2.0% w/v PVP-40, and 0.13% w/v sodium diethyldithiocarbamate (Sigma, cat. no. D-3506; store at -20°C with dessicant) dissolved in 0.05 M EDTA (pH 8.0). Make the 0.05 M EDTA (pH 8.0) by diluting the 0.5 M EDTA (pH 8.0) stock solution.
60. *WB-C (Wash Buffer-C)*: 0.1% w/v ascorbic acid, 2.0% w/v PVP-40, and 0.13% w/v sodium diethyldithiocarbamate (Sigma, cat. no. D-3506; store at -20°C with dessicant) dissolved in 10 mM EDTA, 10 mM Tris-HCl (pH 8.0). Make the 10 mM EDTA (pH 8.0), 10 mM Tris-HCl (pH 8.0) solution by mixing and diluting the 0.5 M EDTA (pH 8.0) and 1 M Tris-HCl stock solutions.
61. *40 mM spermidine*: Dissolve in MBG water, filter sterilize, and store in aliquots at –20°C.
62. *1.0 M DTT (dithiothreitol)*: To make 20 ml of 1.0 M DTT, dissolve 3.09 g of DTT in 20 ml of 0.01 M sodium acetate (pH 5.2). Filter-sterilize, aliquot into 1.0 ml microcentrifuge tubes, and store at –20°C.
63. *H3M (HindIII modified restriction buffer)*: 10% 10X *Hind*III buffer (supplied with enzyme), 1.0 mM DTT, 4 mM spermidine, 1.0% v/v 100X BSA (supplied with enzyme)
64. *0.1 M PMSF (phenylmethylsulfonyl fluoride)*: Dissolve in 100% ethanol and store at -20°C. To make 1.0 ml of 0.1 M PMSF, dissolve 0.017 g of PMSF in 1.0 ml of 100% ethanol. Once in solution PMSF is only effective for 1-2 weeks.
65. *70% (v/v) ethanol and 95% (v/v) ethanol*
66. *50X TAE (Tris, acetic acid, EDTA)*: 2.0 M Tris base (THAM), 5.71% v/v acetic acid, 10% v/v 0.5 M EDTA (pH 8.0) stock solution. To make 1.0 L of 50X TAE, mix 242.2 g of Tris base, 57.1 ml glacial acetic acid, and 100 ml of 0.5 M EDTA (pH 8.0) stock solution in a one liter container. Add

MBG water to 1 liter. Store at room temperature.

67. *1X TAE*: Mix 1 part 50X TAE with 49 parts MBG water.
68. *GELase™ with 50X buffer* (Epicentre Technologies, cat. no. G31200): Enzyme concentration is 0.2 units/µl.
69. *10% (w/v) PEG (polyethylene glycol)*: Use molecular biology-grade PEG.
70. *40 mM $MgSO_4$ stock solution*: Filter-sterilize and store at room temperature.
71. *Freezing media*: 2.5% w/v granulated LB broth (EM Science, cat. no. 1.10285.5000), 13 mM KH_2PO_4, 36 mM K_2HPO_4, 1.7 mM sodium citrate, 6.8 mM $(NH_4)_2SO_4$, and 4.4% v/v glycerol. Autoclave and allow media to cool to < 50ºC. In a laminar-flow hood, add 40 mM $MgSO_4$ stock solution to a final concentration of 0.4 mM ($MgSO_4$ will precipitate if it is autoclaved). Immediately before use, add 250 µl of CM stock solution to each liter of freezing media.
72. *12% v/v bleach*

WEB SITE REFERENCES

AGRF: www.agrf.org.au
AGTEC: www.uga.edu
Aldrich: www.sigma-aldrich.com
Amersham Pharmacia: www.apbiotech.com
AutoGen: www.autogen.com
Bioneer: www.bioneer.com
BioRad: www.bio-rad.com
BioRobotics: www.biorobotics.com.uk
Caltech Genome Research Laboratory: www.tree.caltech.edu
CUGI: www.genome.clemson.edu
EM Science: www.emscience.com
Epicentre Technologies: www.epicentre.com
BMA: www.bioproducts.com
GeneMachines: www.genemachines.com
GeneScreen: www.genescreen.co.uk
Genetix: www.genetix.co.uk
Genome Systems: www.genomesystems.com
Genomic Solutions: www.genomicsolutions.com
Genomics One: www.genomicsone.com
Gibco BRL (Life Technologies): www.lifetech.com
Karlan Research Products: www.karlan.com
Millipore: www.millipore.com
New England BioLabs: www.neb.com
Nunc: www.nalgenunc.com
Parallel Biotechnologies: www.parallelbiotech.com
Promega: www.promega.com
Qiagen: www.qiagen.com
Sigma: www.sigma-aldrich.com
Southern Container: www.southerncontainer.com
V&P Scientific: www.vp-scientific.com

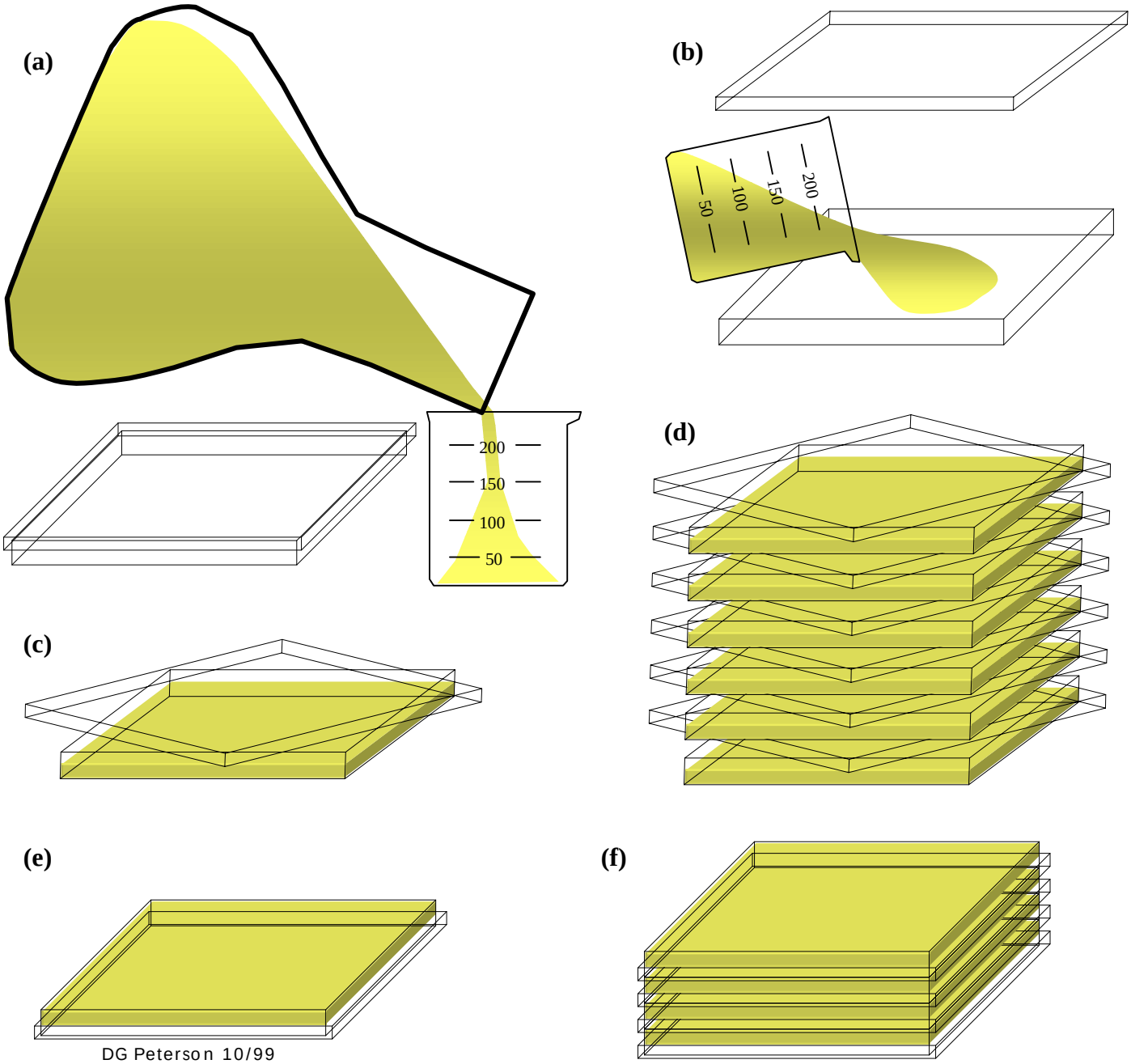


FIGURE 2.1 - Preparing X/I/C trays. The work surface of the laminar-flow hood should be thoroughly wiped clean with 70% ethanol before starting. A Bunsen burner should be set up in the hood. Due to the light-sensitivity of X-GAL, the lights in the laminar-flow hood should be turned off. Room lights also should be dimmed. **(a)** A 250 ml beaker is filled to exactly 200 ml with X/I/C media. Any bubbles in the media are eliminated by quickly passing the flame of the Bunsen burner over the surface of the media. **(b)** The lid is removed from a UV-sterilized 500 cm^2 tray, and the contents of the beaker are poured into the tray. **(c)** The lid is rotated 45° with respect to the tray and placed on top of the tray. **(d)** The first tray is used as a base on which a second tray is prepared, the second tray as a base for preparation of a third tray, *etc*. Ten to twenty plates can be stacked in a column. After all trays have been filled with 200 ml of X/I/C, allow the plates to sit undisturbed in the dark for 45 minutes. During this time the media in the plates should solidify. **(e)** Replace the tray lids, and invert each tray. **(f)** Inverted trays can be stacked as shown. Preferably, X/I/C trays should be plated with transformants the same day they are made. However, trays can be stored in darkness at room temperature for 1-4 days or wrapped in plastic wrap and stored in the dark at 4°C for several weeks. If extra X/I/C agar is left after preparing trays, use the remaining media to make X/I/C Petri plates.

CHAPTER 3
Vector Isolation

PURPOSE

BAC cloning requires successful isolation and preparation of the BAC vector. Poorly prepared vector DNA is one of the primary causes of failure in BAC library construction, and thus vector preparation should be performed with the utmost care.

PREFACE

Vector preparation includes amplification of the BAC vector *via* host cell propagation, isolation of the vector DNA, restriction digest of the vector to produce cohesive termini (sticky ends), and dephosphorylation. Dephosphorylation removes the 5'-phosphate group from the last nucleotide on each sticky end. The absence of 5'-phosphate groups prevents termini from being ligated together but allows ligation between dephosphorylated termini and complementary cohesive termini on DNA molecules that have not been dephosphorylated (*i.e.*, insert DNA) (Sambrook et al. 1989).

Phosphatase, the enzyme used to dephosphorylate the vector, is extremely "aggressive". If the vector DNA is "over-exposed" to the phosphatase enzyme, cohesive termini will not only be dephosphorylated but will be effectively destroyed. Thus exposure of the vector to phosphatase must be limited. One result of limiting vector dephosphorylation is that not all of the vector molecules will be dephosphorylated. In the presence of T4 ligase, vector molecules that have not been dephosphorylated can re-circularize or interact with other phosphorylated and/or dephosphorylated vector molecules to form concatemers. If these recircularized or concatemerized vector molecules are around during transformation, they will result in blue colonies (non-recombinant clones) or false positive colonies, respectively. To get rid of most of the vector molecules that have not been dephosphorylated prior to ligation with insert DNA, the vector DNA can be pretreated with T4 ligase in a "self-ligation" reaction. Vector molecules possessing one or more phosphorylated ends will either recircularize or be ligated to other vector molecules with one or more phosphorylated termini. Concatemers and re-circularized BAC vectors subsequently can be separated from linearized vector molecules by gel electrophoresis. Self-ligation followed by electrophoretic isolation of dephosphorylated vector greatly improves the results of transformation.

EXPERIMENTAL PROCEDURES

SUPPLIES, EQUIPMENT, AND REAGENTS (see CHAPTER 2 *for details)*: Qiagen Large-Construct Kit; pBeloBAC11 vector in DH10B; LB^{+CM}; *Hind*III with 10X buffer and 100X BSA; T4 ligase with 10X ligase buffer; HK phosphatase with 10X phosphatase buffer and 0.1 M $CaCl_2$; 1X TAE; agarose; λH3 DNA; blue juice; ethidium bromide; UV light box equipped with a camera or image-capture system; 1X uncut lambda DNA

METHODS:

1. Isolate BAC vector from DH10B bacterial culture (LB^{+CM}) using a Qiagen Large-Construct Kit. Perform the isolation according to manufacturer's instructions.
2. Set up a restriction digest in a 0.65 ml microcentrifuge tube using 10 µg of the vector as follows:

Restriction Digest
7.5 µl 10X phosphatase buffer
1.5 µl 100X BSA
5.0 µl *Hind*III
10 µg of vector DNA (ca. 50-60 µl)
MBG water to a final volume of 75 µl

✸ Note 3.1: There are three cloning sites (*Hind*III, *Bam*HI, and *Sph*I) in pBeloBAC11 (the most common BAC vector), but only cleavage at the *Hind*III and *Bam*HI sites produce 5' overhangs for easy vector dephosphorylation. In this and following chapters we describe the use of *Hind*III in constructing a BAC library, but *Bam*HI will work also. Additionally, if a vector other than pBeloBAC11 is used, different restriction enzymes may be required (see Choi and Wing 1999).

✸ Note 3.2: Make sure you set up the digestion reaction using the 10X phosphatase buffer supplied with the HK phosphatase (Epicentre Technologies). This allows you to proceed directly to the dephosphorylation step without having to precipitate the DNA.

3. Mix the contents of the microcentrifuge tube by tapping on the tube, and place the reaction at 37ºC for 2 hours.
4. Place the tube in a 65ºC water bath for 20 minutes. This effectively destroys the *Hind*III. Allow the tube to cool at room temperature for 10 minutes and briefly spin the tube in a microcentrifuge (10,000 rpm for 30 sec).
5. To the reaction tube add 5.0 µl HK phosphatase, 5.0 µl of 0.1 M $CaCl_2$, 2.5 µl of 10X phosphatase buffer, and 12.5 µl MBG water.
6. Gently mix the contents of the tube, and place the tube in an incubator at 30ºC for 2 hours or in a water bath at 30ºC for 90 min.
7. Destroy phosphatase activity by placing the reaction at 65ºC for 20 min. Allow the reaction to cool at room temperature for 10 min and briefly spin the tube in a microcentrifuge.
8. To the tube add 12.5 µl 10X ligase buffer, 3.0 µl of T4 ligase, and 9.5 µl of MBG water. Mix gently by tapping on the tube.
9. Place the tube at room temperature for 2 hours or leave it overnight (12 to 16 hours) at 16ºC.
10. Heat-kill the T4 ligase by placing the reaction tube at 65ºC for 20 min.
11. Prepare a small 0.8 % w/v agarose gel in 1X TAE buffer. After the gel has solidified, remove the comb. Using a scalpel, carefully remove the agarose between several of the comb-made wells to produce a slot well (FIGURE 3.1a). Make the slot well large enough that it can accommodate roughly 150 µl of liquid (but not much more).

✸ Note 3.3: We commonly cut agarose gels with a scalpel or razor blade. However, some prefer a coverglass arguing that nucleases may be activated by metal ions from the scalpel / razor blade.

12. Place a thin layer of melted 0.8 % w/v agarose in the bottom of the slot well. Allow the agarose to solidify.
13. Place the gel in an appropriate mini-gel electrophoresis chamber. Add 1X TAE buffer to the electorphoresis chamber until the gel is completely covered with buffer.
14. Add 13 µl of blue juice to the tube containing the dephosphorylated, self-ligated vector solution. Gently mix the tube's contents, and spin the tube in a microcentrifuge at 13,000 rpm for 30 sec.
15. Transfer the contents of the tube into the slot well (FIGURE 3.1a).

16. Place a mixture consisting of 2 µl of λH3 DNA, 5 µl of MBG water, and 2 µl of blue juice in each of the wells flanking the slot well (FIGURE 3.1a).
17. Run the mini-gel for 5 hours at 3.7 v/cm.
18. After electrophoresis, cut the gel with a scalpel as shown in FIGURE 3.1b. Use a plastic ruler as a "straight-edge" to guide the movement of the scalpel through the gel.
19. Gently remove the two flanking gel pieces from the underlying casting tray, and place them in a photographic developing tray. Add enough distilled water that the gel pieces can move freely. Add one drop of ethidium bromide (10 mg/ml) and place the tray on a shaker table. Set the speed of the shaker table so no spilling of ethidium bromide solution occurs. Allow the gel to stain for 20 min. Carefully pour off the ethidium bromide solution (into an appropriate hazardous waste container) and add distilled water to the tray. Place the tray back on the shaker table and allow the gel to "destain" for 20 min.
20. Align the two flanking gel pieces on a UV light box. Turn on the light box.

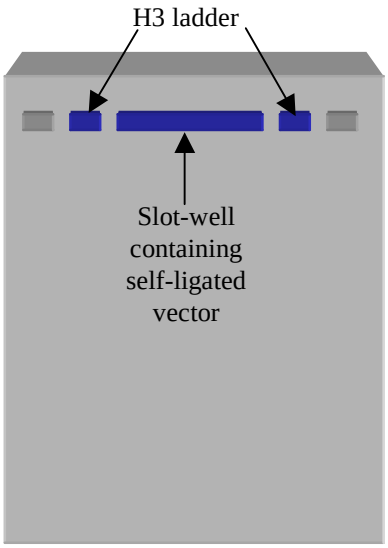
✸ Note 3.4: Always wear eye and face protection when using the UV light box!

21. The dephosphorylated vector should appear at 7.5 kb. Linear concatemeric DNA may be visible at molecular weights > 7.5 kb. Circularized vector and vector concatemers will migrate more slowly than linear vector molecules and thus will be found at molecular weights > 7.5 kb. Using a scalpel, make horizontal incisions in the flanking gel pieces to delimit the 7.5 kb vector band (FIGURE 3.1c).
22. Turn off the UV light box. On a piece of clean plastic wrap on a workbench, assemble the two flanking gel pieces and the unstained central gel piece. Using the incisions on the flanking pieces as guides, cut the 7.5 kb band from the unstained portion of the gel (FIGURE 3.1d). Once the unstained 7.5 kb vector band has been removed, the remaining unstained gel pieces can be stained, and the gel pieces (with the exception of the unstained vector DNA) can be reassembled and photographed for documentation purposes.

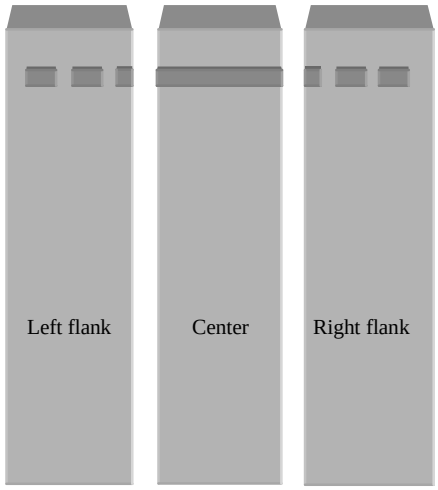
✸ Note 3.5: Never expose the gel piece containing the unstained vector to UV light as this will nick the vector DNA making it unusable in cloning!

23. Cut the gel piece containing 7.5 kb vector DNA into 0.5 g pieces and distribute into 1.5 ml tubes. Freeze the tubes at –80ºC for 1 hour, and then immediately spin the tubes at 13,000 rpm in a microcentrifuge for 20 min at room temperature.
24. Using a pipet, carefully remove liquid (1X TAE containing dephosphorylated vector) from each tube. Place the liquid from all the tubes into a single microcentrifuge tube. It is not necessary to precipitate or desalt vector DNA prepared in this manner.
25. Prepare a 1% agarose submarine mini-gel in 1X TAE. In preparing the gel, use a comb with at least five teeth.
26. Place 0.5, 1.0, 2.0, and 4.0 µl of 1X uncut lambda DNA (*i.e.*, 25 ng, 50 ng, 100 ng, and 200 ng) in separate 0.65 ml microcentrifuge tubes. Add 2.0 µl of blue juice to each.
27. Take a 2.5 µl aliquot from the tube containing the isolated vector and place the solution in a 0.65 ml microcentrifuge tube. Add 2.0 µl of blue juice.
28. Submerge the mini-gel in 1X TAE buffer in an appropriate mini-gel apparatus. Load the gel as shown in FIGURE 3.2. Run the gel at 100 v for 15-20 min. Stain and photograph the gel as described above. Based on comparison of the relative fluorescence in the sample and standard lanes, an estimate of the concentration of the vector DNA can be made.
29. Dilute the vector with sterile water to a final concentration of 10 ng/µl. Store the diluted vector at –20ºC in 25 µl aliquots in 0.5 ml microcentrifuge tubes. By aliquoting the vector DNA, you can avoid freeze/thaw cycles which will damage the vector. We recommend that vector which has been through more than 3 freeze/thaw cycles be discarded.

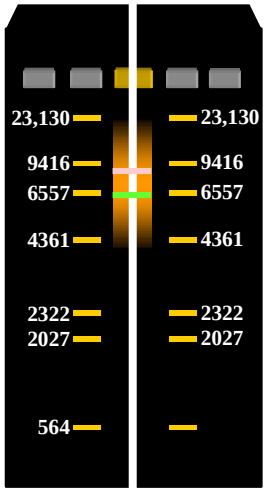
FIGURE 3.1 - Electrophoretic isolation of dephosphorylated vector DNA.



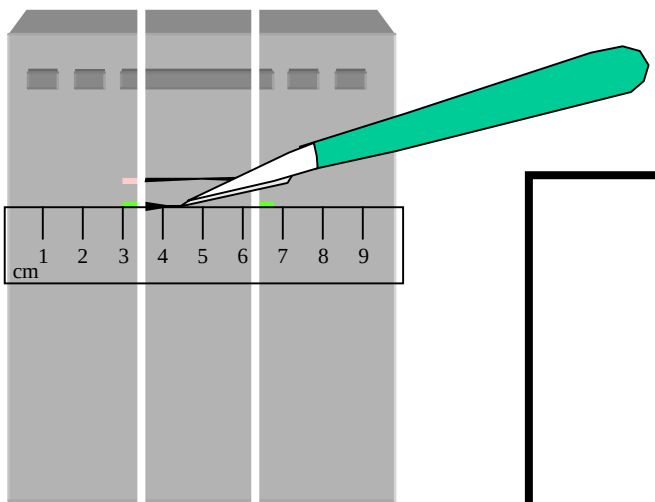
(a) Prepare a "slot-well" by joining several comb-made wells together. Seal the bottom of the slot-well with a thin layer of melted agarose. Place the vector DNA (with blue juice) in the slot-well. Place the H3 ladder in wells flanking the slot well.



(b) After electrophoresis, divide the gel into three pieces as shown. The two flanking pieces should each contain a small part of the slot-well as well as a DNA ladder. Stain the flanking pieces with ethidium bromide. Do not stain the center piece (the gel piece containing most of the vector DNA).



(c) Align the stained flanking gel pieces and examine on a UV light box. There should be a distinct band at approximately 7.5 kb marking the dephosphorylated vector. Using a scalpel, make incisions in the flanking pieces above and below this band (pink and green lines, respectively). Linear concatemers may be visible at (7.5 x 2 =) 15 kb and (7.5 x 3 =) 22.5 kb. Additionally, circular DNA molecules may be appear at molecular weights > 7.5 kb.



(d) Using the incisions in the flanking pieces as guides and a ruler as a "straight edge", cut the 7.5 kb band containing dephosphorylated vector out of the unstained center piece of the gel. DNA is isolated from the gel band containing dephosphorylated vector using a combination of freezing and centrifugation (see **METHODS**).

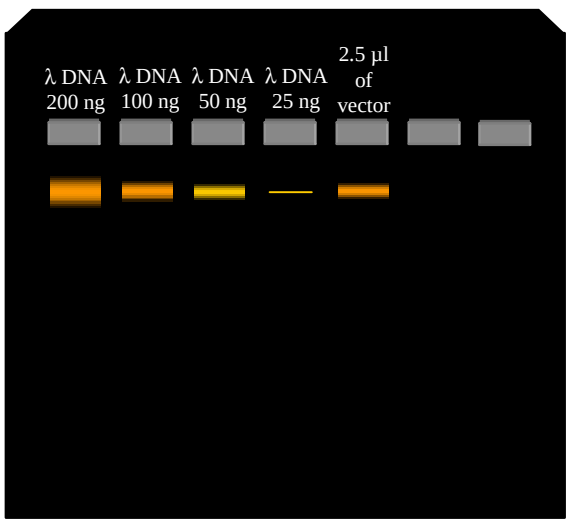


FIGURE 3.2 - Diagram of a mini-gel used to determine the DNA concentration of the dephosphorylated vector DNA. Four different concentrations of uncut lambda DNA are run in the first four lanes while 2.5 µl of the vector is run in lane 5. In this example, 2.5 µl of purified vector stains with an intensity roughly equal to that of the 100 ng of 1X uncut lambda DNA. Because 2.5 µl of vector DNA was placed in lane 5, the concentration of the undiluted DNA sample is about (100 ng/2.5 µl =) 40 ng/µl.

CHAPTER 4
Test ligation

PURPOSE

It is important to see if the vector DNA prepared in CHAPTER 3 is of sufficient quality for use in preparing a BAC library. However, it is generally not a good idea to perform the test ligation with DNA from which you plan to make the library – if the ligation fails, it will not be clear whether the fault is due to the vector or the insert DNA. Rather, the vector can be tested by ligating it with easily obtainable DNA known to be of high quality (*e.g.*, lambda phage restriction fragments).

EXPERIMENTAL PROCEDURES

SUPPLIES, EQUIPMENT, AND REAGENTS (see CHAPTER 2 *for details)*: dephosphorylated pBeloBAC11 vector stock (see CHAPTER 3); λH3 DNA; T4 ligase; 10X T4 ligase buffer; 10% PEG; Millipore nitrocellulose filters

METHODS:

1. Place λH3 DNA, the dephosphorylated pBeloBAC11 vector stock solution, and the T4 ligase 10X buffer on ice. Allow the buffer and the vector stock to thaw. Keep the T4 ligase in the –20ºC freezer until immediately before use.
2. Set up a ligation reaction as described below:

Ligation reaction
20 ng vector DNA
10 µl 10X T4 ligase buffer
2 µl T4 ligase (*i.e.*, 6 units)
200 ng of λH3 DNA
MBG water to give a final reaction volume of 100 µl

3. Gently tap each reaction tube to mix the tube's contents. DO NOT VORTEX OR AGITATE VIOLENTLY AS THIS MAY SHEAR THE INSERT DNA.
4. Incubate the ligation reactions at 16ºC overnight. To create a 16ºC environment, a standard water bath can be placed in a refrigerator and the temperature knob can be adjusted until a stable temperature of 16ºC is attained. Do not incubate ligation reactions for > 20 hrs.
5. Place ligation reaction tubes in a 65ºC water bath for 20-30 min to "heat kill" the enzyme.
6. Ligated DNA must be desalted before it can be used in transformation. Desalting can be performed quite easily using Millipore 0.025 µm filters. Add 30 ml of sterile 10% PEG to a 90 mm Petri plate. Place a Millipore nitrocellulose filter (with its shiny-side facing up) on the PEG surface of the plate. Using a pipettor with a large-orifice pipet tip, transfer the contents of the ligation reaction tube to the center of the Millipore filter (FIGURE 4.1). Place a lid on the Petri plate. Allow the DNA sample(s) to desalt for 90 minutes.

7. Using a pipettor and large-orifice pipet tips, transfer the desalted ligation reaction into a 1.5 ml microcentrifuge tube (*i.e.*, pool the ligation reactions).

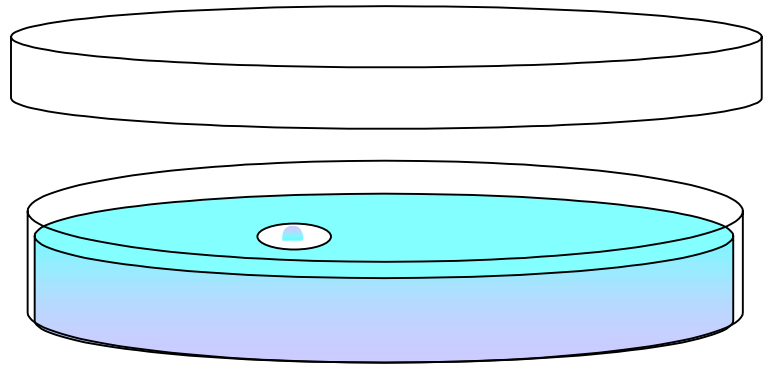
✸ Note 4.1: For each filter, some of the ligation solution will be so closely associated with the filter surface that it will not be recovered.

✸ Note 4.2: Due to osmosis during desalting, the total volume of liquid placed on each filter typically will be one-third to one-half that of the starting volume.

8. Place the tube at 4ºC.

✸ Note 4.3: Ligated DNA is stable at 4°C for at least 5 days.

FIGURE 4.1 - Desalting a DNA solution. A Millipore 0.025 µm filter is floated shiny-side up on 10% PEG in a Petri dish. Using a pipettor with a large-orifice pipet tip, the DNA sample is placed in the center of the filter. Salts in the DNA solution diffuse through the filter into the PEG solution. Additionally, the volume of the DNA solution decreases as water moves by osmosis across the semi-permeable membrane. After about 90 minutes, a pipet is used to remove the now desalted DNA solution from the filter.



CHAPTER 5
Test transformation

PURPOSE

Transformation is the process by which recombinant DNA is taken up by competent bacteria to produce BAC clones. Transformation is an essential step in all DNA cloning protocols.

In order to determine if the vector is suitable for future ligations and transformation, the ligation product produced in CHAPTER 4 is used to transform competent cells. If lambda DNA fragments have been ligated into the polycloning sites of many of the BAC vector molecules, transformation using the test ligation should produce white colonies due to alpha-complementation.

PREFACE

In transformation, competent bacterial cells are mixed with ligate. The resulting mixture(s) is exposed to an electric shock that temporarily creates holes in the plasma membranes of the bacterial cells. This allows BACs to be taken up by the bacteria.

There are currently a variety of commercially available electroporation devices. Because these devices differ in design and operation instructions, researchers should consult manufacturer's instructions to obtain best results. The Gibco BRL CELL-PORATOR™ is a commonly used electroporator. Likewise, it is specifically recommended for use with DH10B cells. Detailed instructions regarding operation of the Gibco BRL CELL-PORATOR are given in APPENDIX A.

EXPERIMENTAL PROCEDURES

SUPPLIES, EQUIPMENT, AND REAGENTS (see CHAPTER 2 *for details)*: 70% ethanol; ELECTROMAX® DH10B™ competent cells; ligated DNA produced in CHAPTER 4; SOC; X/I/C Petri plates; glass plating rod; electroporator with cuvettes

METHODS:

1. Place 2.0 µl of the ligated DNA into four 0.65 ml microcentrifuge tubes. Place the remaining ligated DNA in the refrigerator.
2. Add 20 µl of competent cells to each of the four microcentrifuge tubes. Tap the tubes gently to mix contents. Place the tubes on ice.
3. Using a pipettor equipped with a standard 200 µl tip, transfer the mixture from one of the tubes into an electroporator cuvette. Make sure that there are no bubbles in the ligate/bacterial solution. Load each of the remaining mixtures into its own cuvette.
4. Use an electroporator to apply a shock to the bacteria in each cuvette. Because the lambda DNA fragments are relatively small compared to the fragments that will be used in BAC library construction, a relatively high voltage of 390-400 should be used.

✸ Note 5.1: Be careful not to shock cells in a cuvette more than once.

5. Place the cuvettes on ice.

6. In a laminar-flow hood, place 0.5 ml of sterile SOC into four sterile 15 ml culture tubes (equipped with sterile caps).
7. Using a pipettor, carefully transfer the contents of each cuvette into the media in one of the culture tubes. Label the tubes **I**, **II**, **III**, and **IV**, place a cap on each culture tube, and incubate tubes at 37°C with 250 rpm agitation for exactly one hour.
8. Immediately after placing culture tubes in the incubator, take four X/I/C Petri plates out of the refrigerator. Allow the plates to warm to room temperature, but keep them in a cabinet or wrapped in foil to prevent exposure to light.
9. Remove the "one-hour" culture tubes from the incubator. Transfer culture tubes and X/I/C Petri plates to a laminar flow hood equipped with a Bunsen burner or alcohol lamp.
10. Flame-sterilize a glass-plating rod as shown in FIGURE 5.1. Allow the rod to cool for approximately one minute.
11. Place 50 µl of culture **I** onto the agar of one of the Petri plates. Use the plating rod to spread the culture over the entire agar surface (FIGURE 5.2). Continue moving the rod across the agar until the rod begins to glide with less fluidity, *i.e.*, the friction between the rod and the agar increases. This indicates that the bacterial culture has been absorbed into the agar.
12. Replace the lid and turn the plate upside-down. On the bottom of the plate write "**I**".
13. Prepare a plate for each of the three remaining test transformation reactions (*i.e.*, **II**, **III**, and **IV**) as described in steps 10-12 above.
14. Place the four test plates in a 37ºC incubator overnight. Clones should appear within 15 hours of incubation and should reach a diameter of 1-2 mm by 20 hours.
15. Determine the average number of colonies per plate, the percentage of clones that are white, and the percentage of clones that are blue. Based on the fact that 50 µl of culture was spread on each plate, determine the number of CM-resistant bacteria (colony forming units = cfu) per microliter of liquid culture. For example, if there is an average of 135 colonies per plate, the one-hour cultures possess roughly (135 cfu/plate ÷ 50 µl of one-hour culture/plate =) 2.7 cfu/µl.

INTERPRETING THE RESULTS

- A good transformation is characterized by > 100 cfu/plate (> 2 cfu/µl) and no more than 20% blue colonies. If the titer is < 0.5 cfu/µl, it is possible that the ratio of insert DNA to vector DNA in the ligation reactions was too high. A high percentage of blue colonies suggests problems during ligation and/or dephosphorylation.
- If the titer is > 0.5 cfu/µl and over 50% of the colonies are white, it is generally worth doing minipreps and *Not*I digestions using BACs isolated from white colonies on the test plates (see CHAPTER 6).

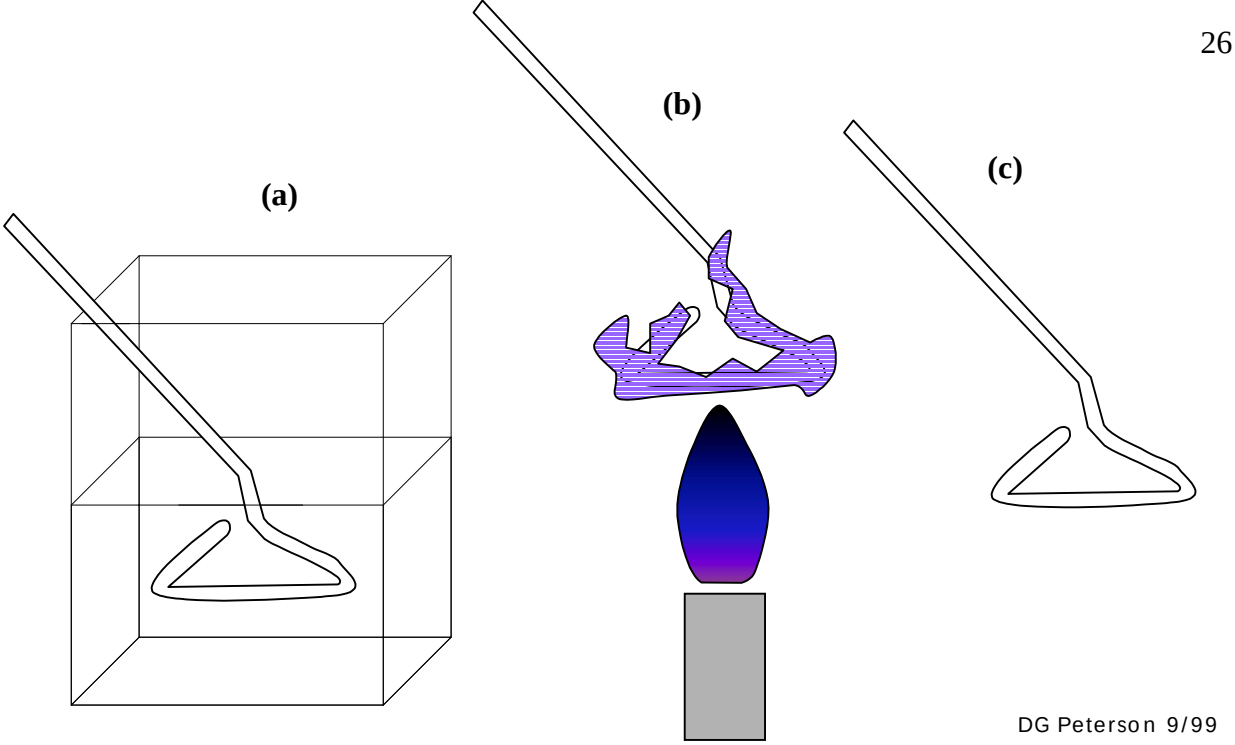


FIGURE 5.1 - Alcohol sterilization of a glass plating rod. **(a)** The "hanger-shaped" end of the rod is inserted into a container in which 70% ethanol has been placed. **(b)** The alcohol drenched rod is ignited by quickly passing it into the flame of a Bunsen burner or alcohol lamp. **(c)** The alcohol on the end of the rod is allowed to burn away. The rod is allowed to cool for several minutes before further use.

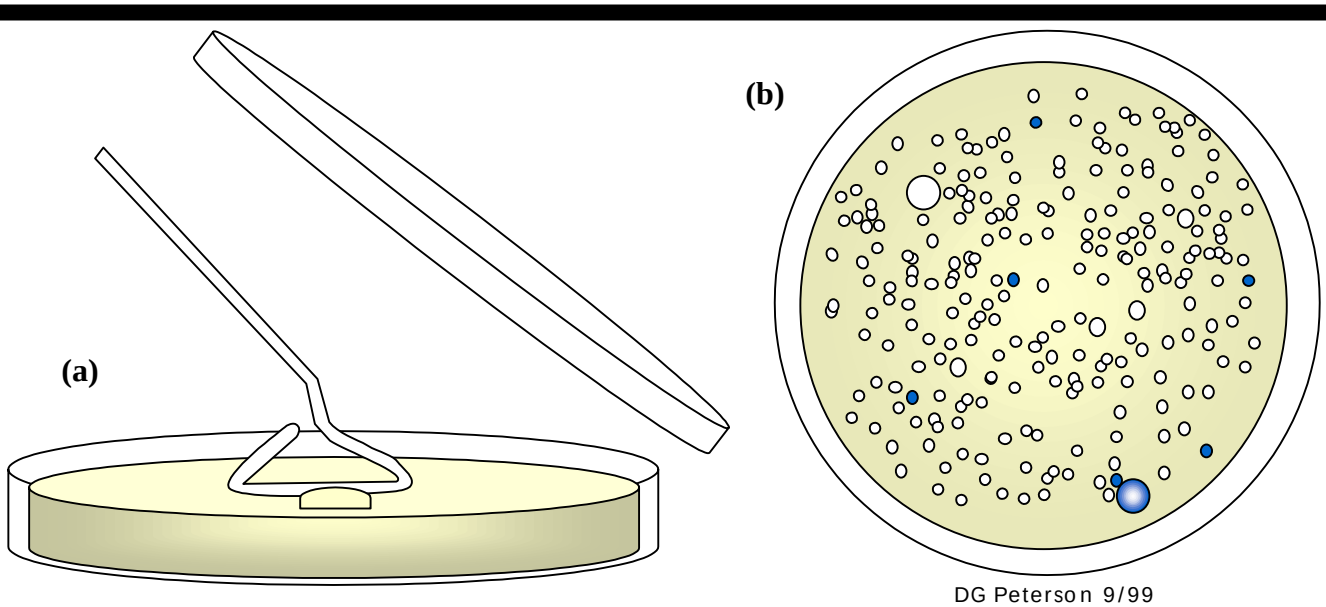


FIGURE 5.2 - Spreading test plates. **(a)** 50 µl of transformation culture is placed in the center of an X/I/C plate. A sterile plating rod is used to distribute the transformation reaction evenly across the surface of the plate. **(b)** Test plates are incubated for 15-24 hours at 37°C. Bacteria without a plasmid lack the resistance gene to chloramphenicol (CM) and thus cannot grow on the X/I/C agar. Blue colonies contain a plasmid, however they are producing β-galactosidase (evident by their ability to convert X-GAL into a blue precipitate) indicating that they do not contain inserts. White colonies represent bacteria that contain a plasmid with an insert (hopefully a large piece of genomic DNA) at the multiple cloning site in the β-galactosidase gene (thus disrupting the gene's activity).

CHAPTER 6

Miniprep & *Not*I digests

PURPOSE

A miniprep is a protocol for isolating plasmids from bacterial cultures. Minipreps from the majority of white clones produced in CHAPTER 5 should result in isolation of BAC vectors containing λH3 DNA inserts. The pBeloBAC11 vector has a *Not*I site on each side of the *Hind*III insertion site. Thus digesting isolated BACs with *Not*I releases inserts from vector DNA. Pulsed-field gel electrophoresis can be used to check whether white clones are "true positives" (whether they actually contain plasmids with a genomic DNA insert). If numerous false positive clones are observed, the vector was most likely damaged during dephosphorylation and/or the T4 ligase is inactive.

EXPERIMENTAL PROCEDURES

SUPPLIES, EQUIPMENT, AND REAGENTS (see CHAPTER 2 *for details)*: test plates with λH3 transformants (see CHAPTER 5); MP-1; MP-2; MP-3; LB^{+CM}; isopropanol (stored at –20ºC); 70% ethanol (-20ºC); 0.5X TBE; PFGE Midrange Ladders or PFGE Lambda Ladder; agarose; *Not*I with 10X buffer and 100X BSA; blue juice; sterile toothpicks; CHEF gel apparatus; large CHEF gel casting stand (21 x 14 cm); 45-tooth gel comb; UV light box equipped with camera or image-capture system

METHODS:

1. For each test plate produced in CHAPTER 5, obtain nine sterile 15 ml culture tubes with caps. Label each set of nine tubes with the Roman numeral designation of one of the test plates (*i.e.*, **I**, **II**, **III**, or **IV**). In a laminar-flow hood, place 3 ml of LB^{+CM} in each tube.
2. Transfer the test plates to the hood. Open a box of sterile toothpicks. Remove the lid from plate **I** and stab an isolated white colony with one of the toothpicks. Drop the toothpick into an appropriately labeled culture tube. Cap the tube. Pull a second sterile toothpick from the container and pick a second white colony off plate **I**. Place the toothpick in a second appropriately labeled tube. Repeat this process until you have transferred bacteria from nine different white colonies on plate **I** to the nine different tubes labeled **I**.
3. Repeat step 2 for each of the other test plates.
4. Place the resulting 36 cultures in a shaker incubator at 37ºC overnight (12-18 hours).

✸ Note 6.1: Minipreps can be performed using the protocol below. However, if desired commercially available minprep kits and/or automated DNA isolation systems can be employed to isolate BACs from liquid cultures (see CHAPTER 2).

5. Place culture tubes in a swinging bucket centrifuge. Centrifuge at 800 x *g* for 20 min. Pour supernatants from the tubes.
6. Resuspend each bacterial pellet in 200 µl of MP-1. Transfer the contents of each tube to an appropriately labeled 1.5 ml microcentrifuge tube. Incubate the microcentrifuge tubes on ice for 5 min.
7. Add 400 µl of MP-2 to each microcentrifuge tube. Mix the contents of each tube by gentle inversion. The mixture in the tube should turn translucent due to lysis of the bacteria. Place

the tubes on ice for 5 min.

8. Add 300 µl of MP-3 to each tube, and mix contents of tubes by gentle inversion. A white precipitate should appear in each tube. This precipitate contains genomic DNA and various proteins.
9. Place tubes on ice for 7 min. Centrifuge the tubes at 13,000 rpm for 25 min.
10. Use a pipettor to carefully transfer the supernatant from each tube (ca. 0.85 ml) to a clean microcentrifuge tube. Label tubes containing supernatants appropriately. Discard the tubes containing the pellets.
11. To precipitate plasmid (BAC) DNA, add 600 µl of ice-cold isopropanol to each tube, and gently mix the contents by inversion. Place the tubes at –80ºC for 20 min.
12. Spin tubes in a microcentrifuge at 13,000 rpm for 30 min. Carefully pour off the supernatants and add 1 ml of ice cold 70% ethanol to each tube. Centrifuge at 13,000 x *g* for 10 min.
13. Gently remove and discard the supernatant from each tube. In the bottom or on the side of each tube, a small translucent pellet should be seen. This is BAC DNA. Place the microcentrifuge tubes upside down on a Kimwipe so that residual liquid can drain from the tubes. Allow the pellets to dry for 1-2 hours in a laminar-flow hood.
14. Add 35 µl of sterile 1X TE to each microcentrifuge tube. Gently tap tubes to mix contents. Make sure that the pellet in each tube is covered in buffer. Place the tubes at 4ºC overnight.
15. Remove tubes from the refrigerator. Gently agitate each miniprep tube by tapping on it. If pellets have not dissolved, agitate the tubes using a vortex and allow tubes to sit at room temperature for a few additional hours. Once pellets have gone into solution, proceed with step 16.
16. Place 6 µl of each miniprep solution into its own 0.65 ml microcentrifuge tube. Label each of the 36 tubes with the designation of its corresponding test plate (**I**, **II**, **III**, or **IV**). The tubes will henceforth be called "*Not*I reaction tubes".
17. Prepare a *Not*I digestion cocktail by placing 58.3 µl of 10X *Not*I buffer, 3.9 µl of 100X BSA, 268.3 µl of MBG water, and 19.4 µl of *Not*I in a 1.5 ml microcentrifuge tube. Mix the contents of the tube using a vortex.
18. Add 9 µl of cocktail to each *Not*I reaction tube. Mix the contents of each reaction tube by tapping.

✸ Note 6.2: Each reaction tube will contain 6 µl of miniprep solution, 1.5 µl 10X *Not*I buffer, 0.1 µl of 100X BSA, 6.9 µl of MBG water, and 0.5 µl of *Not*I.

19. Place the reaction tubes in a microcentrifuge and spin at 10,000 rpm for 30 sec. Transfer the reaction tubes to a microcentrifuge rack. Wrap the rack in aluminum foil and place it at 37ºC for seven hours.

✸ Note 6.3: Reaction tubes can be incubated at 37°C overnight (ca. 12-16 hrs) if necessary.

20. Place 2.5 g of agarose in a 1.0 L flask. Add 0.5X TBE to a final volume of 250 ml. Place the flask on a scale, and tare the scale so the reading is 0.00 g. Remove the flask but do not reset the scale. Heat the mixture in a microwave until all of the agarose has gone into solution (this will require some boiling). Place the flask on the scale. Add MBG water until the scale once again reads 0.00 g. Cover the flask with aluminum foil, and place it in a 45°C water bath.
21. Set up the large CHEF gel casting stand (see FIGURE 6.1). Place the BioRad 45-tooth comb into the comb notches nearest the end of the casting stand. Add agarose to the casting tray until it is near overflowing. Place the remaining melted agarose in a 50 ml polypropylene centrifuge tube. Place the tube in a 65°C water bath for later use. Let the gel solidify at

room temperature for at least one hour.

22. Carefully remove the comb from the gel.
23. Take either the PFGE Lambda Ladder or one of the PFGE Midrange Ladders out of the freezer and remove the red cap from the tip of the syringe. Apply slight pressure to the plunger so that a 1.5 mm long region of agarose-embedded ladder is extruded from the syringe tip. Use a coverglass to slice this agarose from the tip of the syringe. Insert the round agarose slice into the third well of the CHEF gel (FIGURE 6.2). Place a second slice of the ladder in well 42 of the gel.
24. Remove the tube containing the extra agarose from the 65°C water bath. Allow the agarose to cool to about 50°C. Carefully add melted agarose to the wells containing molecular weight ladders and plug pieces (*i.e.,* seal the wells). Allow the melted agarose to solidify

✸ Note 6.4: We commonly cut agarose-embedded DNA with a scalpel or razor blade. However, some prefer a coverglass arguing that nucleases may be activated by metal ions from the scalpel / razor blade.

25. A BioRad CHEF Gel Apparatus is shown in FIGURE 6.3. Place 2.5 L of 0.5X TBE buffer into the electrophoresis chamber. Turn the power and the pump toggle switches on the control/power unit to the "ON" position. Turn on the cooling module, and set the temperature on the cooling module to 14°C. Allow the buffer to cool to this temperature.
26. Remove the gel from the casting stand but leave it on the underlying casting plate. Wipe any agarose off the bottom of the casting plate.
27. Place the plate with the gel on it in the BioRad CHEF apparatus (FIGURE 6.3). The wells should be closest to the back of the apparatus (farthest from the positive electrodes). The gel should be completely submerged in buffer.
28. Unwrap the foil from the microcentrifuge rack containing the *Not*I digests. Add 2.0 µl of blue juice to each tube. Centrifuge the tubes at 10,000 rpm for 30 sec.
29. Place the gel in the electrophoresis chamber of the BioRad CHEF apparatus.
30. Using a pipettor, transfer the contents of the nine *Not*I reaction tubes labeled with the Roman numeral **I** to lanes 5-13, respectively. BE SURE TO CHANGE PIPET TIPS BETWEEN TRANSFER OF EACH SAMPLE.
31. Load lanes 14-22 with the **II** reactions, lanes 23-31 with the **III** reactions, and lanes 32-40 with the **IV** reactions.
32. Run the gel using the following parameters: volts/cm = 6.0, included angle = 120º, run time = 16 hours, initial switch time = 5 sec, final switch time = 15 sec, ramping = linear.
33. Remove the gel (and underlying plate) from the CHEF apparatus. Gently remove the gel from the base plate, and place the gel in a photographic developing tray. Add enough distilled water that the gel can move freely. Add 2 drops of ethidium bromide (10 mg/ml) and place the tray on a shaker table. Set the speed of the shaker table so that the gel is not damaged, and no spilling of ethidium bromide solution occurs. Allow the gel to stain for 45 min. Carefully pour off the ethidium bromide solution (into an appropriate hazardous waste container) and add distilled water to the tray. Place the tray back on the shaker table and allow the gel to "destain" for 45 min. Place the gel on a UV light box and either photograph or digitally capture an image of the gel. A diagram illustrating the appearance of a successful transformation/miniprep/*Not*I digest is shown in FIGURE 6.4. Actual photographs of *Not*I digests of BACs can be seen in FIGURE 14.1.

INTERPRETING THE RESULTS

There are several things to consider when evaluating the results of a BAC *Not*I digest:

- Is ethidium bromide staining visible in the sample lanes? If so, it is likely that the minipreps were successful. If not, the miniprep DNA was most likely lost during a decanting step. The appearance of one or two empty lanes in a gel is fairly common. However, if most of the lanes are empty, it is likely that the isolation of BAC DNA needs to be performed with more care.
- Are distinct bands visible in the sample lanes? If no bands are seen, the digestion has failed (possibly due to an inactive *Not*I enzyme).
- The pBeloBAC11 vector has a length of 7.4 kb. If the digestion was successful, the vector should be visible as a band at 7.4 kb. The vector band should be visible in most (if not all) sample lanes.
- Do the sample lanes contain at least one band in addition to the vector band? Each lane that contains only a vector band represents a false positive. Lanes that contain a vector band and an additional band(s) are "true positives". If more than 10% of the lanes contain false positives, adjustments in the ligation and/or transformation steps are warranted.

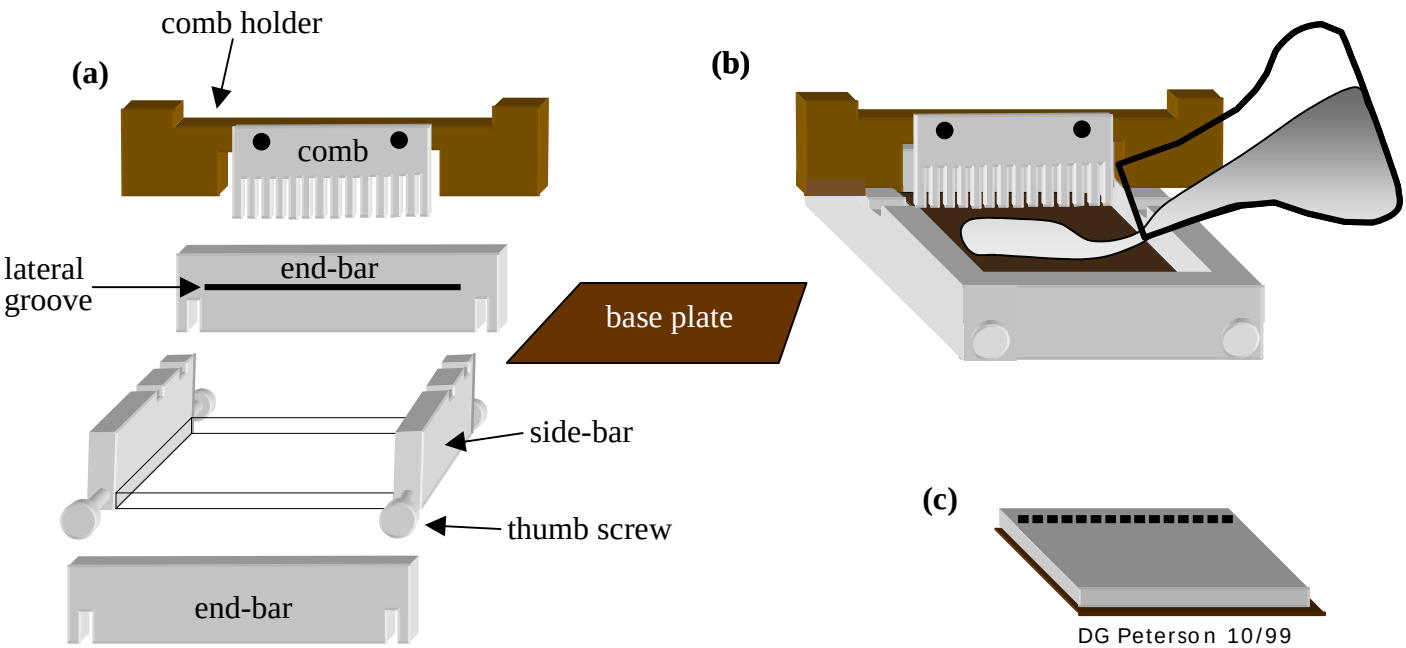


FIGURE 6.1 - Preparing a CHEF gel using the BioRad casting stand. **(a)** The parts of the casting stand. The stand is assembled by placing the large notches on the ventral surface of the end-bars over the thumb screws at the ends of the side-bars of the casting tray. The base plate is set in the casting tray and the upper and lower edges of the base plate are fitted into the lateral grooves of the end-bars. The thumb screws of the side-bars are tightened to form a water-proof seal. The comb holder and attached comb are placed in the distal comb notches on the side bars. **(b)** Approximately 200-250 ml of 1.0% agarose is poured into an assembled casting stand. **(c)** After the gel has solidified, the comb and the side-bars are removed. The gel and its underlying base plate are slid out of the casting tray and placed into the CHEF apparatus (see FIGURE 6.3).

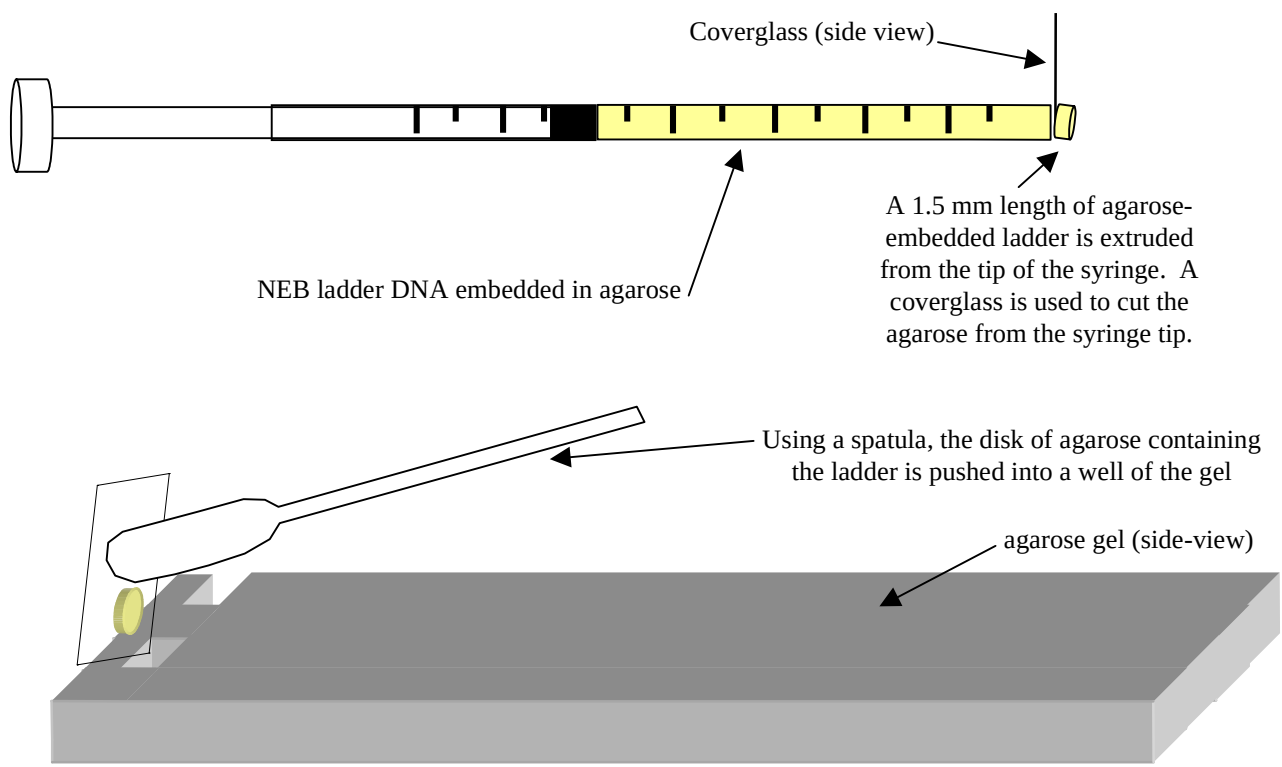


FIGURE 6.2 – Cutting and loading a disk of agarose-embedded ladder for CHEF gel electrophoresis

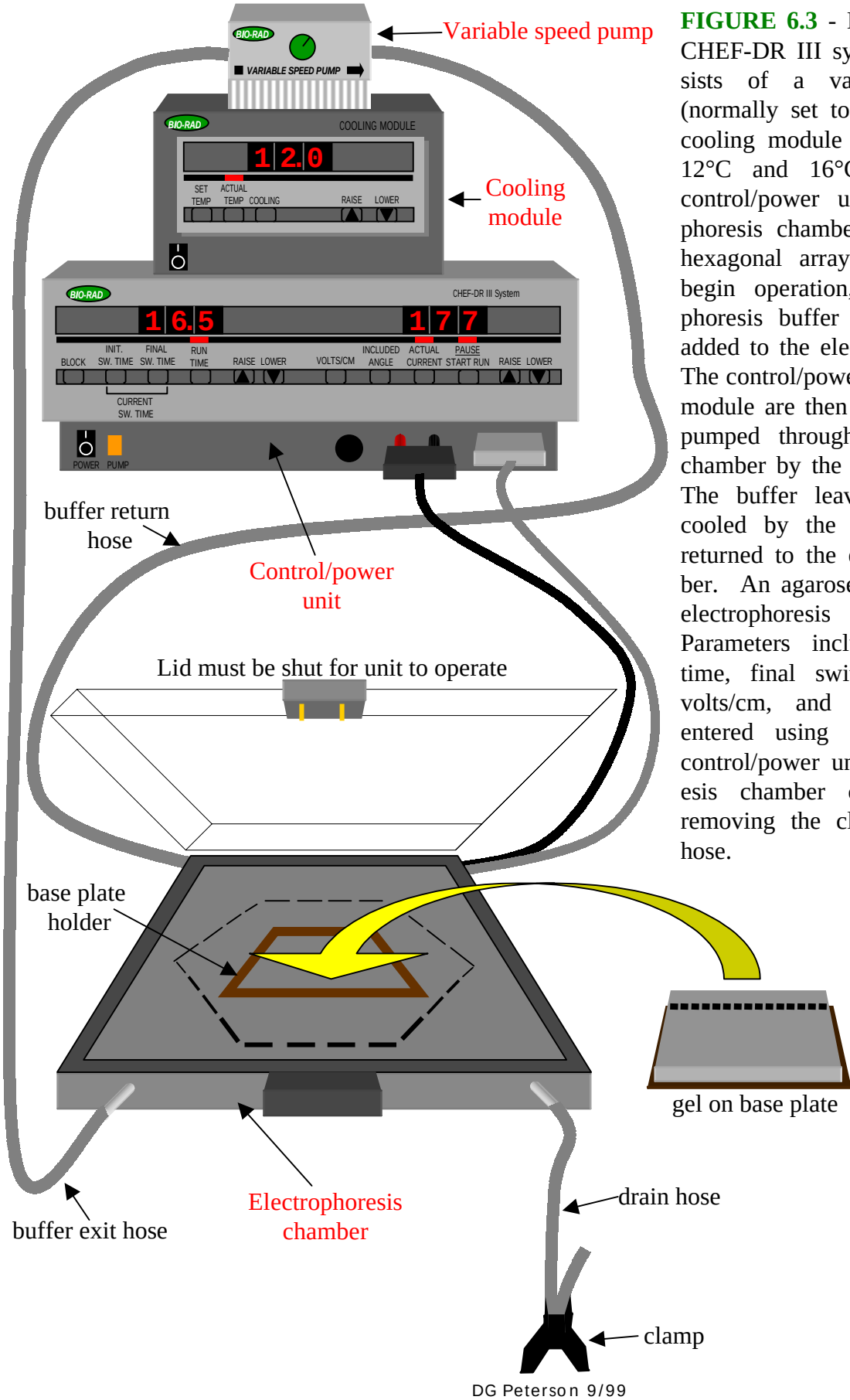


FIGURE 6.3 - Diagram of a BioRad CHEF-DR III system. The unit consists of a variable speed pump (normally set to a speed of "70"), a cooling module (usually set between 12°C and 16°C), a programmable control/power unit, and an electrophoresis chamber characterized by a hexagonal array of electrodes. To begin operation, 2.5 L of electrophoresis buffer (*e.g.*, 0.5X TBE) is added to the electrophoresis chamber. The control/power unit and the cooling module are then turned on. Buffer is pumped through the electrophoresis chamber by the variable speed pump. The buffer leaving the chamber is cooled by the cooling module and returned to the electrophoresis chamber. An agarose gel is placed in the electrophoresis chamber as shown. Parameters including initial switch time, final switch time, run time, volts/cm, and included angle are entered using the keypad on the control/power unit. The electrophoresis chamber can be drained by removing the clamp from the drain hose.

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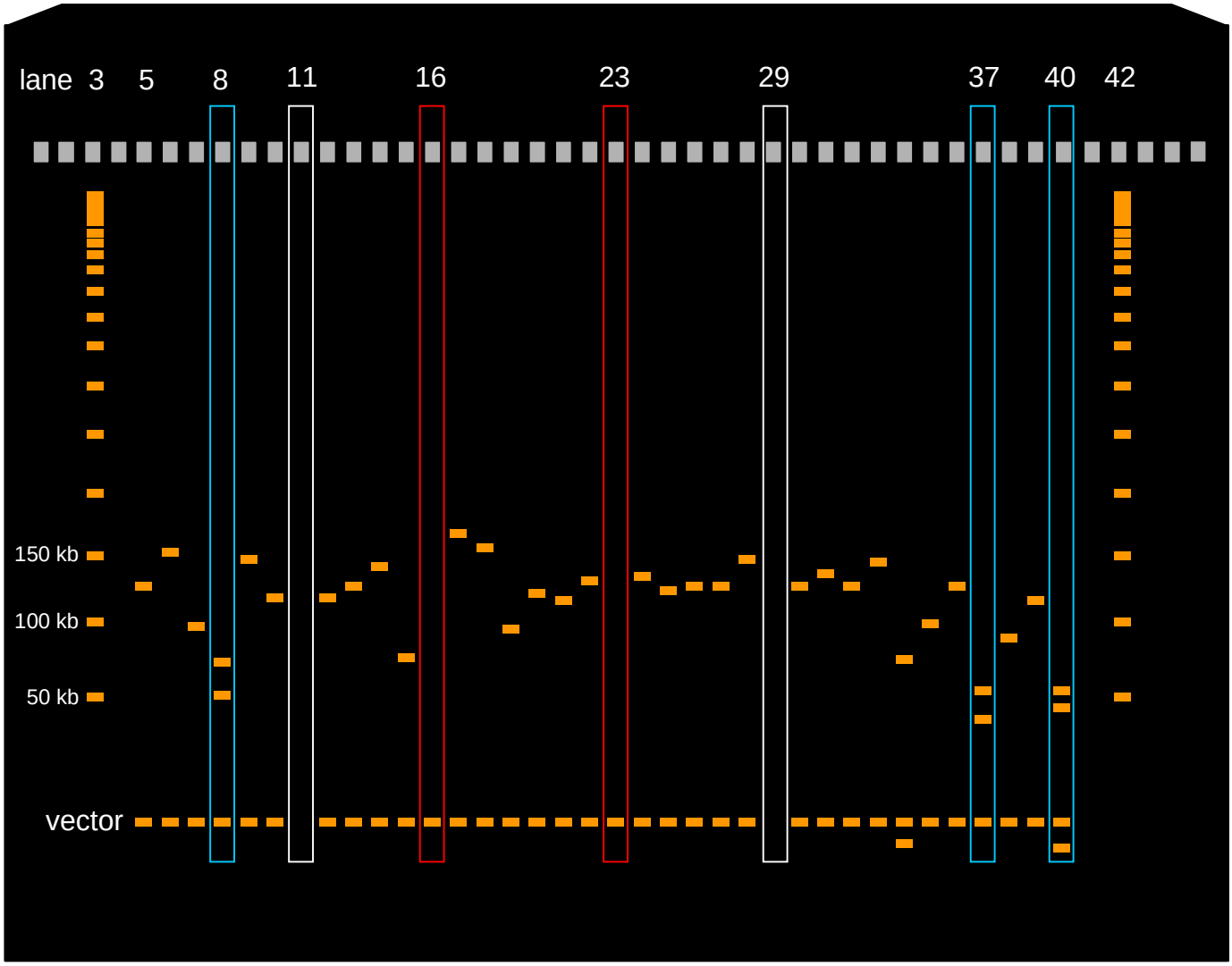


FIGURE 6.4 - Diagram of BACs digested with *Not*I. Lanes 3 and 42 contain the PFGE lambda ladder. All of the sample lanes possess vector DNA except those demarcated by white rectangles. The absence of DNA in these lanes indicates that the BAC DNA was lost during the miniprep protocol. Though most lanes contain one "non-vector" band, some lanes contain several non-vector bands (lanes marked with light blue rectangles). The presence of multiple bands indicates that the insert in question contains one or more *Not*I restriction sites. The insert size for a particular clone is the sum of the base pair lengths of its non-vector bands. For example, in lane 5, the sole insert fragment has a mean length of approximately 130 kb. In lane 8, the insert size is (50 kb + 70 kb =) 120 kb. If the transformation procedure was successful, there should be few (if any) "false positives" (white clones containing a vector but not a piece of exogenous insert DNA. In the figure, examples of false positives are enclosed in red rectangles. Each false positive has a vector band but no insert band(s).

In general, average insert length is best determined using an image capture system with software capable of determining DNA lengths based on a comparison of sample bands to those of a ladder.

CHAPTER 7

Isolation of high molecular weight nuclear DNA

PURPOSE

A BAC vector can carry an insert greater than 100,000 base pairs in length. However, standard DNA isolation methods tend to shear DNA into fragments too small to take advantage of the carrying capacity of BACs. To limit DNA shearing, purified nuclei are embedded in agarose plugs. The agarose provides a physical support for the DNA preventing significant shearing. Plugs are incubated in buffer containing proteinase(s) to digest proteins and detergent to emulsify nuclear lipids, respectively.

PREFACE

Preparing nuclei suitable for BAC library construction can be one of the most difficult steps in making a plant BAC library. The predominant problems involved in trying to isolate plant nuclear DNA are ones that animal researchers do not typically encounter. For example, (a) plant cell walls must be physically broken or enzymatically digested without damaging nuclei, (b) chloroplasts must be separated from nuclei and/or preferentially destroyed, an important process since copies of the chloroplast genome may comprise the majority of DNA within a plant cell, (c) volatile secondary compounds such as polyphenols must be prevented from interacting with the nuclear DNA, and (d) carbohydrate matrices that often form after tissue homogenization must be prevented from trapping nuclei. While it would be ideal if there were a nuclear DNA isolation protocol that worked for all plant species, the biochemical and morphological diversity within the plant kingdom make development of such a protocol unlikely (Loomis 1974, Peterson et al. 1997). Below we present two quite different nuclear DNA isolation protocols that we have used to construct BAC libraries from plants. **OPTION X** is a promising technique that has only recently been used in BAC library construction. **OPTION Y** (or prototypes of this protocol) has been used in the construction of BAC libraries for several years.

OPTION X is an adaptation of a procedure originally designed for isolating milligram quantities of highly pure nuclear DNA from tomato (Peterson et al. 1997, 1998). It has several features that make it well suited for use in BAC library construction as well as other molecular biology applications: (a) Prior to homogenization, tissues are treated with ether to make nuclei more friable. Ether treatment markedly increases the yield of nuclei (Watson and Thompson 1986; our observations). (b) Homogenization is performed using a simple kitchen blender. (c) The nuclear isolation buffer (MEB) is designed to deal with several common problems in plant nuclear DNA extraction. First of all, the buffer contains 2-methyl-2,4-pentanediol (MPD), a compound that helps stabilize nuclei and prevents their premature lysis. Nuclear yield using MEB is > 10 times that obtained using sucrose-based buffers (Peterson et al. 1997 and our observations). The buffer also contains the antioxidants β-mercaptoethanol, sodium diethyldithiocarbamate, and sodium metabisulfite. These compounds limit the oxidation of polyphenols. In their oxidized forms, polyphenols covalently bind to DNA turning it brown and making it useless (Katterman and Shattuck 1983; Guillemaut and Maréchal-Drouard 1992).

Polyvinylpyrrolidone in the buffer adsorbs polyphenolic compounds preventing them from interacting with DNA (Loomis 1974). (d) The low pH of the buffer (pH 6.0) serves to inhibit polyphenol oxidation. (e) After homogenization, addition of Triton X-100 to a concentration of 0.5% results in preferential lysis of chloroplasts and mitochondria. The presence of divalent cations (Mg^{2+}) in the MEB prevents nuclei from being lysed by the Triton X-100 (Watson and Thompson 1986). (e) Nuclei are separated from most debris by centrifugation through a Percoll gradient. Further low-speed centrifugation steps are used to remove some, if not most, of the starch grains that typically pellet with nuclei. (f) Throughout the protocol, nuclear preparations are examined using a light microscope. This allows the investigator to visually assess nuclear concentration and purity.

We have used **OPTION X** to isolate nuclei and nuclear DNA from numerous plant species (*e.g.*, sorghum, sugarcane, grape, cotton, loblolly pine, prickly-pear cactus, fern, peanut, Leyland cypress). The protocol's usefulness in BAC library construction was first demonstrated in August 1999 when it was used to isolate DNA that subsequently was used to construct a 9X library for *Gossypium raimondii* (Peterson et al., in preparation). This success quickly was followed by construction of a 16X library for grape (*Vitis vinifera*) (Tomkins et al., in preparation) and a 9.2X library for *Gossypium hirsutum* (Acala Maxxa) (Tomkins et al., in preparation). Using **OPTION X**, agarose plugs containing nuclear DNA of suitable size and restrictability for BAC library construction have been generated for peanut (*Arachis hypogaea* 'Florunner') and tomato (*Solanum lycopersicum*). The peanut plugs are currently being used in BAC library construction.

OPTION X has produced megabase-sized, restrictable DNA from all dicots in which it has been tested, but whether it will be useful in isolating megabase-sized DNA from monocots is still uncertain. Several attempts at isolating high molecular weight DNA from *Sorghum bicolor* using **OPTION X** yielded fragments too small (< 100 kb) for BAC cloning, presumably due to partial digestion of the DNA by endogenous nucleases. Attempts to limit nuclease activity by removing Mg^{2+} (a DNase cofactor) from the nuclear isolation buffers (either directly or indirectly) resulted in premature nuclear lysis. However, addition of the nuclease inhibitors EGTA (6 mM) and L-lysine-HCl (200 mM) (see Liu and Wu 1999) to the nuclear isolation buffers permitted isolation of sorghum DNA fragments about 800 kb in length without a noticeable decrease in nuclear yield. Further research on the use of **OPTION X** to isolate megabase-sized DNA from monocots is in progress.

Another potential drawback of **OPTION X** is that it is designed for extraction of nuclei from relatively large quantities (ca. 500 g) of fresh tissue. Whether the protocol can be scaled-down to accommodate smaller quantities of tissue and/or used to isolate high molecular weight DNA from frozen tissue has yet to be tested.

OPTION Y is relatively simple and can be used to isolate nuclear DNA from somewhat smaller quantities of fresh or frozen tissues. It has been used to construct DNA libraries from numerous species including rice, sorghum, wheat, sugarcane, cotton, soybean, barley, and *Arabidopsis*. In **OPTION Y**, plant tissue is ground in liquid nitrogen, the resulting homogenate is suspended in a sucrose-based buffer (SEB = sucrose extraction buffer), Triton X-100 is added to destroy chloroplasts and mitochondria, the homogenate is filtered several times to remove most cellular debris, and nuclei are pelleted by centrifugation. The relatively high pH of the SEB (pH 9.1) inhibits the activity of endogenous nucleases. The presence of β-mercaptoethanol and PVP in the SEB counteract some of the negative effects of oxidized polyphenols. **OPTION Y** has been used to prepare megabase-sized DNA from monocots and some dicots. It is particularly well suited for species/tissues that contain little in the way of secondary compounds or carbohydrates. Disadvantages of **OPTION Y** include relatively low nuclear yields (and subsequently production of agarose plugs with lower DNA concentrations), significant contamination of nuclear preps with debris and carbohydrates, and limited control of polyphenols.

For dicots and non-angiosperms, we recommend that investigators start by trying **OPTION X**. At present, we suggest that investigators working on monocots try **OPTION Y** first. If neither **OPTION X** nor **OPTION Y** work for a species of interest, there are a number of other nuclear isolation protocols that can be tried (*e.g.*, see Katterman and Shattuck 1983; Guillemaut and Maréchal-Drouard 1992; Watson and Thompson 1996; Hamilton et al. 1972; Couch and Fritz 1990).

EXPERIMENTAL PROCEDURES
I. ISOLATION OF NUCLEI

A. OPTION X

SUPPLIES, EQUIPMENT, AND REAGENTS (see CHAPTER 2 *for details)*: MEB; MPDB; cheesecloth; 1X TE slurry; 1X TE (non-sterile); Percoll; diethyl ether; 1% methylene blue; kitchen blender; light microscope

METHODS:

1. This nuclear DNA isolation method can be used to isolate DNA from adult plants and seedlings.
   (a) For mature plants, young leaves are gently picked from the plant of interest. After removing a leaf, it is immediately submerged in a 1X TE slurry. Approximately 200-500 g of leaves are collected for one isolation.
   (b) For DNA isolation from seedlings, plant seeds at high density in soil in greenhouse trays, cover seeds with 0.5 cm of soil, water, and place trays in the greenhouse. At the time of harvest, cut the tops from the plants and submerge the tops in a 1X TE slurry. The optimum time of harvest depends on the plant. For monocots, allow seedlings to grow to a height of approximately 20-30 cm before harvesting. For dicots, wait until true leaves appear and flourish before harvesting as cotyledons are senescent tissue and do not yield good quality DNA. Approximately 500 g of seedlings are collected for one isolation.
2. Remove leaves/seedlings from the 1X TE slurry and place them in 1-3 L of ice-cold diethyl ether (*i.e.*, enough to submerge all tissue) for three minutes.

✸ Note 7.1: Perform this step in a fume hood!

   Ether removes waxes and makes cells more friable. Remove leaves/seedlings from the ether and then wash them three times (1 min each wash) in 4 L of 4°C 1X TE (prepared from the 100X TE stock). Place the leaves/seedlings in 3 L of ice cold MEB. Homogenize leaves/seedlings in the MEB using a kitchen blender (highest speed attainable for 30 seconds). Squeeze the homogenate through six layers of cheesecloth, and then filter the resulting filtrate through 32 layers of cheesecloth. If possible, let this second filtration occur by gravity only (*i.e.*, no squeezing).
3. Add Triton X-100 to the filtrate to a final concentration of 0.5% v/v. Fill four 500 ml centrifuge bottles with the mixture and spin at 800 x *g* for 20 min at 4°C. Decant supernatants, place the remaining homogenate in the bottles, and centrifuge as described above (this reduces the number of bottles one has to wash at the end of the experiment).
4. Resuspend each pellet in 4 ml of MPDB, and pool suspensions. Mix a small drop of the suspension with an equal volume of 1.0% methylene blue on a microscope slide. Add a coverglass and examine the slide by phase-contrast and/or bright-field microscopy. If all has gone well, the mixture should contain numerous dark-blue stained nuclei, no intact cells, and

no mitochondria or chloroplasts. Starch grains (visible by phase-contrast microscopy) should stain little, if at all, with methylene blue (see FIGURE 7.1).

5. Place 7.5 ml of Percoll in a 50 ml polypropylene centrifuge tube. Add MPDB to a final volume of 20 ml to produce "37.5% Percoll". Layer the suspension of nuclei onto the 37.5% Percoll bed. Centrifuge the gradient in a swinging bucket rotor at 650 x *g* for 60 minutes (4°C). Nuclei are relatively dense structures and normally form a pellet (along with large starch grains) at the bottom of the centrifuge tube. Less dense materials (*e.g.*, most cell debris, organelles, plastid DNA) remain in the supernatant.

✸ Note 7.2: On occasion, some or all of the nuclei will not pellet. This can occur if the batch of Percoll is more viscous than normal and/or cell debris has formed a semi-solid barrier within the gradient preventing passage of nuclei. Thus it is important not to discard the supernatant unless you are confident that the nuclei are in the pellet. It is prudent to look at a drop of the supernatant under a microscope (see step 4) before discarding it. If the supernatant has several distinct layers, examine a drop from each layer before discarding that layer. If nuclei are found in the supernatant (no matter what the cause), the simplest solution is to add 5-10 ml of MPDB, vortex the mixture, and spin the tube at 650 x *g* for an additional 30 minutes. If the nuclei still have not pelleted, dilute the contents further and spin again (though, in our experience, this has never been necessary).

6. Decant the supernatant, and gently resuspend the pellet in 0.5 ml of MPDB. Examine a drop of the suspension under the microscope as described in step 4 (see FIGURE 7.1). Add 10 ml of MPDB, and transfer the nuclear suspension to a 15 ml polypropylene centrifuge tube.
7. Centrifuge at 300 x *g* for 10 min, and then turn the centrifuge up to a speed equivalent to 650 x *g*. Allow the tube to spin for an additional ten minutes. Discard the supernatant.
8. The resulting pellet may or may not contain two or more distinct layers. If there is a single layer, proceed to step 11. If not, do steps 9 and 10.
9. If more than one layer is present, add two ml of MPDB and gently vortex the tube until the upper-most layer is resuspended. Pour this suspension into its own 15 ml centrifuge tube leaving the rest of the pellet in the first tube.
10. Repeat step 9 until all of the original pellet layers are segregated into different tubes. Prepare a slide from the contents of each tube (see step 4). Based on the microscopy results, pick the tube(s)/layer(s) that contains the most nuclei and the least debris. Discard all other tubes.
11. Add 10 ml of MPDB to the nuclei and mix by gentle inversion. Centrifuge the suspension at 650 x *g* for 10 minutes, and carefully decant the supernatant.

✸ Note 7.3: Highly pure nuclei do not form a very hard pellet and may slide out of the tube if the supernatant is discarded too forcefully.

12. Gently tap the tube so that the nuclei become resuspended in the residual MPDB left in the tube. Place the tube containing the nuclei on ice.
13. Proceed to **II**.

B. OPTION Y

SUPPLIES, EQUIPMENT, AND REAGENTS (see CHAPTER 2 *for details)*: liquid nitrogen; SEB; SEB^{+BME}; SEB^{+BME}/Triton; mortar and pestle; cheesecloth; Miracloth

METHODS:

1. Freeze 15-100 g of fresh plant tissue in liquid nitrogen or take 15-100 g of frozen tissue out of the freezer.
2. Place the frozen tissue in a mortar containing liquid nitrogen. Grind the tissue to a powder with the mortar and pestle.
3. Transfer the powder into ice-cold SEB^{+BME} in an appropriate container(s). For every gram of

tissue, use approximately 10 ml of SEB^{+BME} (*e.g.*, place 20 g of frozen tissue in 200 ml of ice-cold SEB^{+BME}).

4. Place the mixture on ice for 12 minutes. During this time, swirl the contents of the container every two minutes (each swirl time = 20 sec).

✸ Note 7.4: Nuclei in sucrose-based buffers must be handled with extreme care. The absence of divalent cations coupled with the extreme osmotic conditions in the SEB^{+BME} make the nuclei extremely fragile. If roughly agitated, the nuclei will break.

5. Filter the homogenate through 2 layers of cheesecloth and 2 layers of Miracloth into a clean flask(s).
6. Add $^{1}/_{20}$ volume of SEB^{+BME}/Triton to the flask(s), and leave it on ice for 10 min. During this time, swirl the contents of the flask every two minutes.
7. Transfer the mixture into several 50 ml polypropylene centrifuge tubes, and spin the tubes in a centrifuge at 650 x *g* for 15 min (4ºC).
8. Very gently decant the supernatants. Add 10 ml of SEB^{+BME} to each pellet, and gently resuspend nuclei with a small paintbrush. Consolidate the nuclear suspensions into as few 50 ml centrifuge tubes as possible. To each tube add SEB^{+BME} to 50 ml, and centrifuge tubes at 650 x *g* for 15 min (4ºC).
9. Repeat step 8 until all of the nuclear suspensions have been consolidated into a single tube.
10. Decant the supernatant, and resuspend the nuclei in 20 ml of SEB (without β-mercaptoethanol). Centrifuge as described above, and very gently remove all but 1-2 ml of the supernatant with a large pipettor.
11. Gently resuspend the pellet in the residual SEB with a paintbrush. Examine a drop of the suspension by light microscopy (see step 4 in **OPTION X**).
12. Proceed to **II**.

II. PREPARATION OF PLUGS

SUPPLIES, EQUIPMENT, AND REAGENTS (see CHAPTER 2 *for details)*: MPDB or SEB (depending on whether **OPTION X** or **OPTION Y** was used to isolate nuclei); Lysis Buffer; LMP agarose; plug molds

METHODS:

1. In a 50 ml flask, mix 0.15 g of LMP agarose with 10 ml of MPDB (if **OPTION X** was used to isolate nuclei) or 10 ml of SEB (if **OPTION Y** was used to isolate nuclei). Place the flask on a balance. Tare the balance. Transfer the flask into a microwave and heat the LMP agarose/buffer mixture until all of the LMP agarose has gone into solution (this may require some boiling). Place the flask back on the scale. Add MBG water to the flask until the scale reads "0.00 g". Place aluminum foil over the top of the flask, and place the flask in a 45°C water bath.
2. Place an agarose plug mold(s) on ice. If plug molds are being re-used, make sure that they are clean (thoroughly washed and rinsed with ethanol or washed and UV-sterilized) and that fresh tape has been placed on the bottom of the wells.
3. Place the tube containing nuclei in the 45°C water bath for 10 minutes.
4. Mix an equal volume of the LMP agarose solution with the pre-warmed nuclei. Using a plastic pipet with a relatively large-bore tip, place the nuclei/agarose mixture into the wells of the pre-chilled plug mold (FIGURE 7.2). Place the ice bucket containing the plug mold and

plugs in a refrigerator. Allow plugs to solidify for 30 minutes.

5. Push plugs out of plug molds into 50 ml of Lysis Buffer. BioRad plug molds come with a small plastic tab designed for pushing plugs out of the molds (see FIGURE 7.2). However, a clean spatula will suffice. If the nuclear isolation was performed as described above, the plugs should be white to light yellow in color.
6. Incubate the plugs in Lysis Buffer at 50°C for 24 hours. Drain the Lysis Buffer from the plugs, and add 50 ml of fresh Lysis Buffer. Incubate the plugs at 50°C for an additional 24 hours.
7. Transfer plugs into 50 ml of fresh Lysis Buffer and store at 4°C overnight**.**

✸ Note 7.5: The length of time plugs can be stored in Lysis Buffer at 4°C without significant DNA degradation seems to vary from species to species. For optimal results, store plugs in Lysis Buffer for no more than a few days. If a longer storage time is required, transfer plugs (or other agarose pieces containing DNA) to a 50 ml polypropylene tube containing aqueous 70% ethanol (20°C). Allow plugs to equilibrate in the 70% ethanol at room temperature for at least four hours. Place the tube containing the plugs at -20°C. Plugs can be stored in this fashion for at least 6 months (if not considerably longer) without noticeable DNA degradation. Before performing digestions, *etc*., on plugs that have been stored in 70% ethanol, transfer the plugs into 50 ml of an appropriate aqueous buffer at 4°C. Initially, the plugs will float on top of the buffer. However, once the ethanol has diffused out of the plugs they will sink to the bottom of the tube. At this point, the plugs are ready for further use.

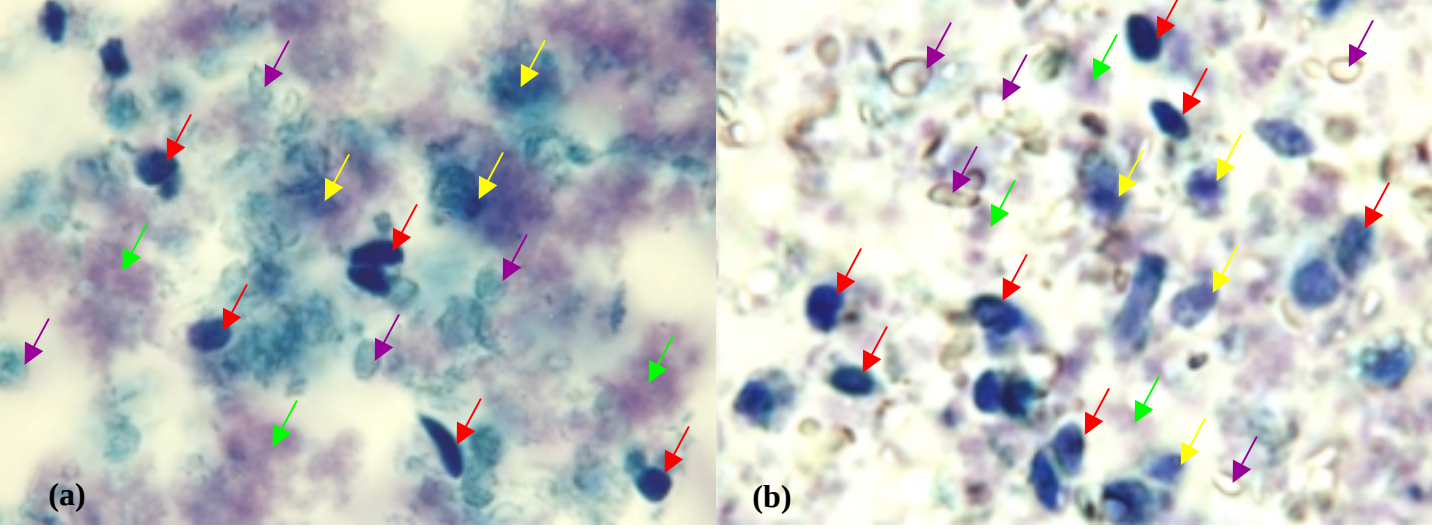
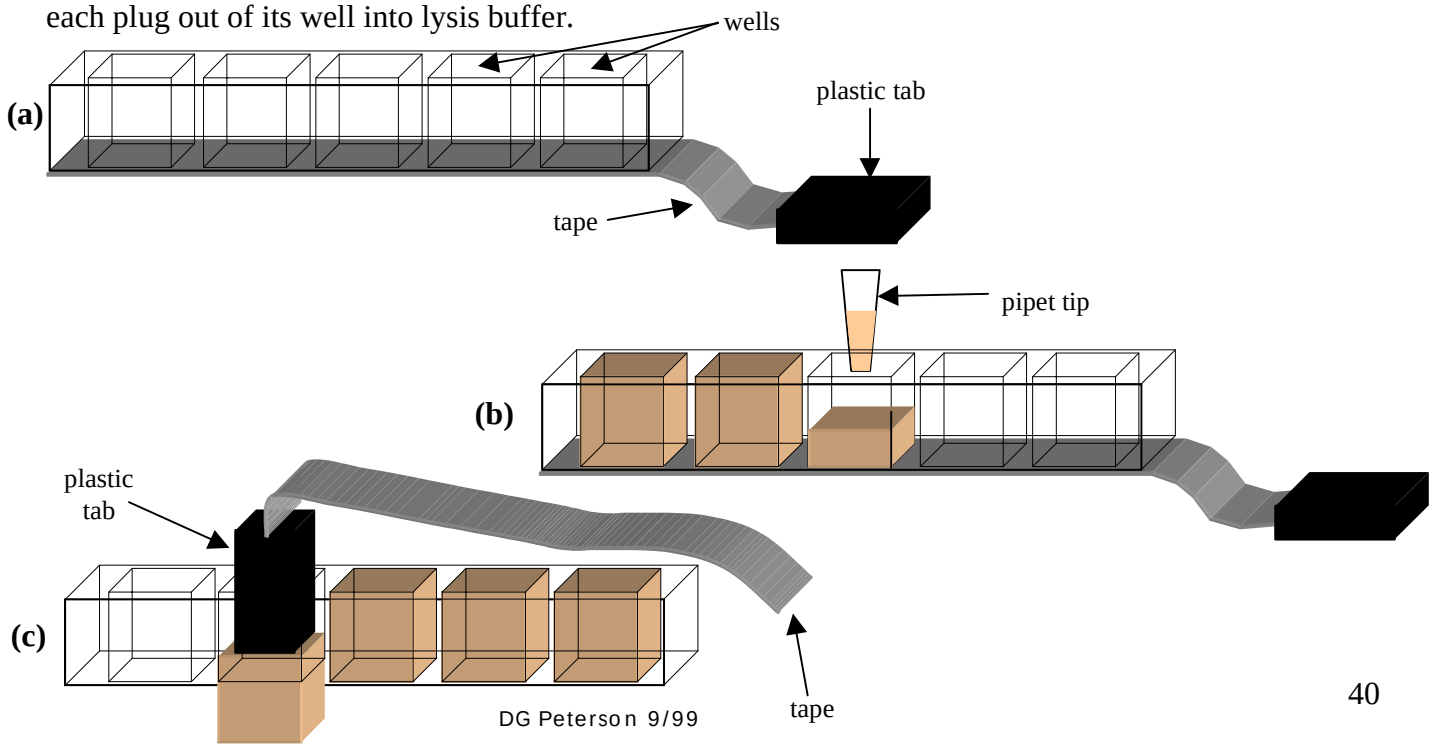


FIGURE 7.1 - Nuclei prepared using **OPTION X** (see CHAPTER 7) from **(a)** *Sorghum bicolor* (grain sorghum) and **(b)** *Solanum lycopersicum* (tomato). The preparations have been stained with methylene blue and photographed using bright-field microscopy. The nuclei stain dark blue and are variable in shape (though most are roughly spherical). Within a picture, much of the observed variation in nucleus size and shape is attributable to the fact that not all of the nuclei lie in the same focal plane. Nuclei within the focal plane (*e.g.*, red arrows) exhibit greater definition than nuclei above or below the focal plane (*e.g.*, yellow arrows). Starch grains (*e.g.*, purple arrows) often pellet with nuclei. However, unlike nuclei, starch grains stain only lightly (if at all) with methylene blue, and tend to have a refractile appearance (especially when viewed by phase-contrast microscopy). Much of the starch can be eliminated from nuclear preps by performing successive low-speed centrifugations as described in the *METHODS*. In most species we examined, some of the debris that pellets with nuclei stains lightly with methylene blue (light purple regions, *e.g.*, green arrows). Magnification = roughly 1000x.

FIGURE 7.2 - Preparation of agarose plugs using a disposable plug mold. **(a)** – An empty BioRad plug mold. **(b)** – The agarose/nuclei mixture is placed into the wells of the plug mold. The plugs are allowed to solidify. **(c)** – The tape strip is removed from the bottom of the plug mold. The plastic tab is used to push each plug out of its well into lysis buffer.



CHAPTER 8
DNA analysis

PURPOSE

Examining DNA from plugs using pulsed-field gel electrophoresis allows one to make sure that the DNA in the plugs is not degraded. If the DNA is not of sufficient length, new plugs will need to be made (possibly using a different nuclear isolation protocol or a different extraction buffer).

Based on relative staining intensity of the samples compared to lambda standards, a mean DNA amount per plug can be estimated.

EXPERIMENTAL PROCEDURES

SUPPLIES, EQUIPMENT, AND REAGENTS (see CHAPTER 2 *for details)*: agarose plugs containing genomic DNA (see CHAPTER 7); 0.5X TBE; agarose; PFGE Lambda Ladder; 10X uncut lambda DNA; blue juice; ethidium bromide; 70% ethanol (in a spray bottle); coverglasses and/or razorblades; CHEF gel apparatus; regular CHEF gel casting apparatus (14 x 13 cm); 15-tooth gel comb; UV light box equipped with camera or image-capture system

METHODS:

1. Make up a 1.0 % agarose/0.5X TBE gel using the regular CHEF gel casting stand and the 15-tooth gel comb. Fill the electrophoresis chamber of the CHEF system with 2.5 L of 0.5X TBE. Set the cooling module so that the buffer is cooled to 12ºC (see CHAPTER 6 for details regarding preparation of gels and operation of the CHEF gel system).
2. Place a slice of the PFGE Lambda Ladder into lanes 3 and 9 of the gel.
3. Place one plug containing the sample DNA onto a clean coverglass (*i.e.*, a coverglass washed with 70% ethanol and dried with a Kimwipe). Using another clean coverglass, cut the plug in half. Take one of the halves and place it into the fifth well of the agarose gel leaving an empty well between the sample and the ladder. Cut the remaining half plug into two pieces (each piece is $^1/_4$ of a plug). Take one of these ¼ plug pieces, and place it in the sixth well of the gel. Take the second ¼ plug piece and divide it into two equal pieces. Take one of the resulting $^1/_8$ plug pieces, and transfer it into the seventh well of the agarose gel. Discard the remaining $^1/_8$ plug.
4. Place 20 µl (10 µg), 10 µl (5 µg), and 5 µl (2.5 µg) of 10X uncut lambda DNA into separate 0.65 ml microcentrifuge tubes. Add MBG water to the second and third tubes so that all tubes contain a total volume of 20 µl. Add 5 µl of blue juice to each tube, mix gently, and spin tubes in a microcentrifuge at 10,000 rpm for 15 sec. Place the complete contents of the tubes containing 10 µg, 5 µg, and 2.5 µg of lambda DNA in lanes 11, 12, and 13, respectively.
5. Run the gel using the following parameters: initial switch time = 1.0 sec, final switch time = 40.0 sec, run time = 18 hours, volts/cm = 6.0, included angle = 120°, ramping = linear.
6. Stain the gel and either photograph or digitally capture its UV fluorescent image. A photograph showing the appearance of a typical gel is presented in FIGURE 8.1. If a photograph is taken, a visual examination of the ethidium bromide fluorescence in the

lambda standard lanes compared with the fluorescence in the sample lanes can be used to estimate the DNA content of a plug. If the image has been digitally captured, imaging software can be used to determine the amount of DNA in a typical plug.

INTERPRETING THE RESULTS

- If the mean DNA length is > 600 kb, the plugs should be further evaluated (CHAPTER 9).
- If the mean DNA length of the test DNA is < 600 kb, it is likely that the DNA in the plugs has degraded too much to be of use in BAC library construction. Consequently, new plugs should be prepared.

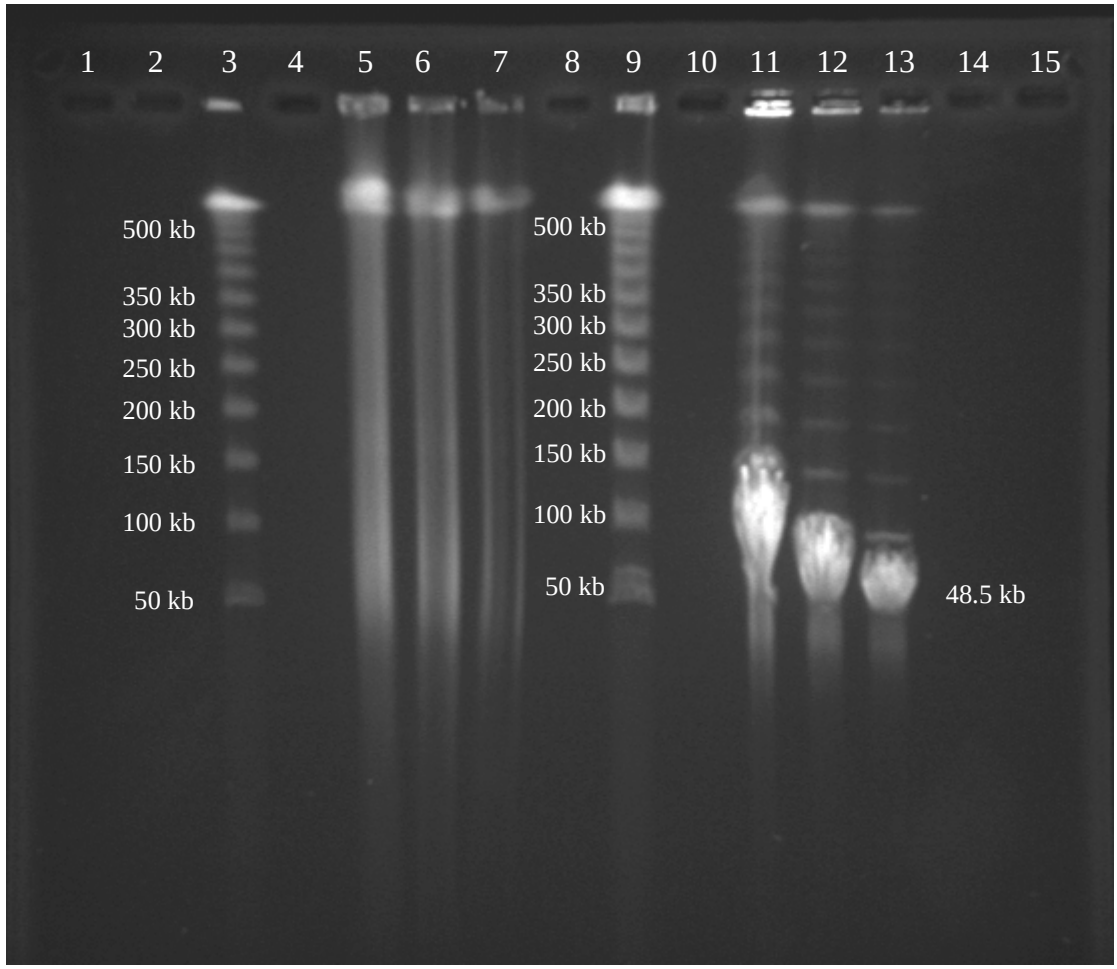


FIGURE 8.1 - CHEF analysis of DNA from plugs of grape (*Vitis vinifera*). Lane 3 = PFGE lambda ladder, Lane 5 = 1/2 plug, Lane 6 = 1/4 plug, Lane 7 = 1/8 plug, Lane 9 = PFGE lambda ladder, Lane 11 = 10 µg uncut lambda DNA, Lane 12 = 5 µg uncut lambda DNA, and Lane 13 = 2.5 µg uncut lambda DNA.

CHAPTER 9
Test restriction digest

PURPOSE

Construction of a BAC library requires generation of relatively high molecular weight restriction fragments. Such restriction fragments will serve as inserts in BAC construction. In general, fragments between 100 kb and 350 kb are desirable. To obtain fragments in this size range, the high molecular weight DNA in the agarose plugs must be partially digested with a restriction enzyme. To determine the conditions that yield a maximum percentage of fragments between 100 and 350 kb, a series of partial restriction digests is performed.

EXPERIMENTAL PROCEDURES

SUPPLIES, EQUIPMENT, AND REAGENTS (see CHAPTER 2 *for details)*: agarose plugs containing genomic DNA (see CHAPTER 7); WB-A; WB-B; WB-C; PMSF; 0.5X TBE; agarose; PFGE Lambda Ladder; aqueous ethidium bromide; H3M; 10X *Hind*III buffer; *Hind*III; 70% ethanol (in a spray bottle); 70% ethanol; microscope slides and coverglasses (slides and coverglasses should be sprayed with 70% ethanol and dried with a Kimwipe prior to use); CHEF gel apparatus; regular CHEF gel casting stand; 15-tooth gel comb; UV light box equipped with a camera or image-capture system

METHODS:

1. Remove the plugs from lysis buffer and place them in a 50 ml tube containing WB-A at 50°C for 1 hour.
2. Decant the supernatant from the tube containing the plugs and add 50 ml of WB-B. Incubate the tube on ice for one hour.
3. Decant the supernatant and incubate the plugs in 50 ml WB-C for 30 min.
4. Remove this buffer and add 50 ml of new WB-C. Add 50 µl of 0.1 M PMSF, mix gently, and place the tube on ice for one hour. The PMSF destroys residual Proteinase K in the plugs.
5. Repeat step 4.
6. Decant the solution and add 50 ml of ice cold WB-C. Incubate the tube on ice. After 30 min remove the WB-C and add 50 ml of ice-cold fresh WB-C.
7. Repeat step 6 two more times (for a total of four post-PMSF washes). These washes remove high concentrations of EDTA from the plugs. EDTA inhibits restriction endonuclease activity. Place the tube in the refrigerator.
8. Make a 1.0% agarose gel in 0.5X TBE using the small BioRad CHEF gel casting stand and the 15-tooth gel comb (see CHAPTER 6 for details regarding gel preparation and loading). Place the PFGE Lambda Ladder in lanes 1 and 13, respectively.
9. Transfer three of the plugs ("test plugs") into 10 ml of H3M. Let the test plugs equilibrate in the H3M for 1 hour.
10. Place eleven 1.5 ml microcentrifuge tubes in a tube rack, and label the tubes with consecutive lower case letters (*i.e.*, label tubes **a**-**k**). Add 250 µl of H3M to each tube.
11. In a 1.5 ml microcentrifuge tube, mix 10 µl of New England BioLabs *Hind*III (20 units/µl)

with 80 µl MBG water and 10 µl 10X *Hind*III buffer to produce a 2.0 unit/µl *Hind*III solution (Dilution 1). Add 10 µl of this solution to 80 µl MBG water and 10 µl 10X *Hind*III buffer to produce a 0.2 units/µl *Hind*III solution (Dilution 2).

✸ Note 9.1: The restriction enzyme used in the partial digest depends upon the sequence of the polycloning site of the vector. There are three cloning sites (*Hind*III, *Bam*HI, and *Sph*I) in pBeloBAC11 (the most common BAC vector), but only cleavage at the *Hind*III and *Bam*HI sites produce 5' overhangs for easy vector dephosphorylation (CHAPTER 3). Consequently, *Hind*III and *Bam*HI are primarily used to construct BAC libraries. In this and following chapters we describe the use of *Hind*III in constructing a BAC library. However, *Bam*HI can be substituted for *Hind*III if desired.

12. Using undiluted New England BioLabs *Hind*III, Dilution 1, and Dilution 2, add a specific quantity of *Hind*III to each of the eleven tubes as directed in TABLE 9.1.
13. Place tubes **a**-**k** on ice.
14. Using a spatula, transfer the three test plugs onto a microscope slide (wiped clean with 70% ethanol). Using a clean coverglass, cut each plug laterally and transversely to produce four pieces of roughly equal size. There should now be twelve ¼ plug pieces.
15. Macerate (thoroughly chop) each ¼ plug piece with a coverglass.

✸ Note 9.2: Chopping the plugs does not result in appreciable DNA shearing (Wang and Schwartz 1993).

16. Using a spatula and a coverglass, place two of the macerated ¼ plug pieces (*i.e.*, ½ a plug) into microcentrifuge tube **a**. Place each of the remaining macerated ¼ plug pieces in one of the remaining ten tubes (**b**-**k**).
17. Gently mix the contents of each tube and incubate tubes on ice for 1 hour. This allows the enzyme to infiltrate the agarose cubes.
18. Gently agitate each tube and place the tubes in a 37°C water bath. Remove the tubes from the water bath after EXACTLY 20 min, and immediately place the tubes on ice.
19. Add 30 µl of 0.5 M EDTA (pH 8.0) to each tube (this inhibits further enzyme activity) and gently agitate tubes to promote contact between the agarose and the EDTA. Keep tubes on ice.
20. Using the pointed end of a spatula, transfer the undigested macerated ½ plug in tube **a** into well 2 of the gel. Likewise, transfer the macerated ¼ plug in tube **b** to well 3, the ¼ plug in tube **c** to well 4, *etc*. Seal wells with leftover melted 1% TBE agarose (50°C) as described in CHAPTER 5. Run the gel using the following parameters: buffer temperature = 12ºC, volts/cm = 6.0, included angle = 120°, initial switch time = 1.0 sec, final switch time = 40.0 sec, ramping = linear, running time = 18 hours.
21. Stain, destain, and examine the gel as previously described in CHAPTER 6. A diagram illustrating how the optimal enzyme concentration is determined from the gel is shown in FIGURE 9.1. A photograph showing the results of an actual test digestion is seen in FIGURE 9.2.

✸ Note 9.3: If the partial digest was successful and you are prepared to do a "large-scale" restriction digest within the next day or so (CHAPTER 10), leave 8-16 plugs in the WB-C and store the remaining plugs in 70% ethanol as described in CHAPTER 7, Note 7.5. If you are not prepared to do a large-scale restriction digest, store all of the plugs in 70% ethanol.

INTERPRETING THE RESULTS

- In reaction **k**, the *Hind*III concentration is extremely high. Consequently, nearly complete digestion of the high MW DNA should be observed for this reaction. If partial to full digestion of the DNA is not observed in this or any of the other lanes, something has gone wrong. There are several possibilities that should be investigated: (a) The *Hind*III has lost its

activity. (b) The DNA in the plugs is not restrictable. (c) One or more of the reagents used in the restriction reactions is inactive/contaminated. (d) EDTA was not sufficiently washed from the test plugs.

- In BAC cloning, it is desirable to have restriction fragments (inserts) between 100 and 350 kb in length. If the DNA is restrictable, one or more of the partial digest variations (reactions **b**-**l**) should produce DNA fragments with a mean length in this size range. The variation that produces the largest percentage of fragments in the 100-350 kb range will be used in performing a mass restriction digest on eight or more plugs (CHAPTER 10).
- The undigested DNA (reaction **a**) should contain DNA with a mean length > 600 kb. If it doesn't, the DNA in the plugs possibly has degraded during storage.

TABLE 9.1 – *Hind*III digests

Tube label	Microliters of enzyme solution added to tube	*Hind*III quantity in tube (units)	Final *Hind*III concentration in tube (units/ml)*
a	0	0	0
b	1 µl of Dilution 2	0.2	0.05
c	2.5 µl of Dilution 2	0.5	0.125
d	5.0 µl of Dilution 2	1.0	0.25
e	7.5 µl of Dilution 2	1.5	0.375
f	1.0 µl of Dilution 1	2.0	0.5
g	1.5 µl of Dilution 1	3.0	0.75
h	2.0 µl of Dilution 1	4.0	1.0
i	2.5 µl of Dilution 1	5.0	1.25
j	5.0 µl of Dilution 1	10.0	2.5
k	2.5 µl of undiluted *Hind*III	50.0	12.5

*Based on a total solution volume of 250 µl per tube.

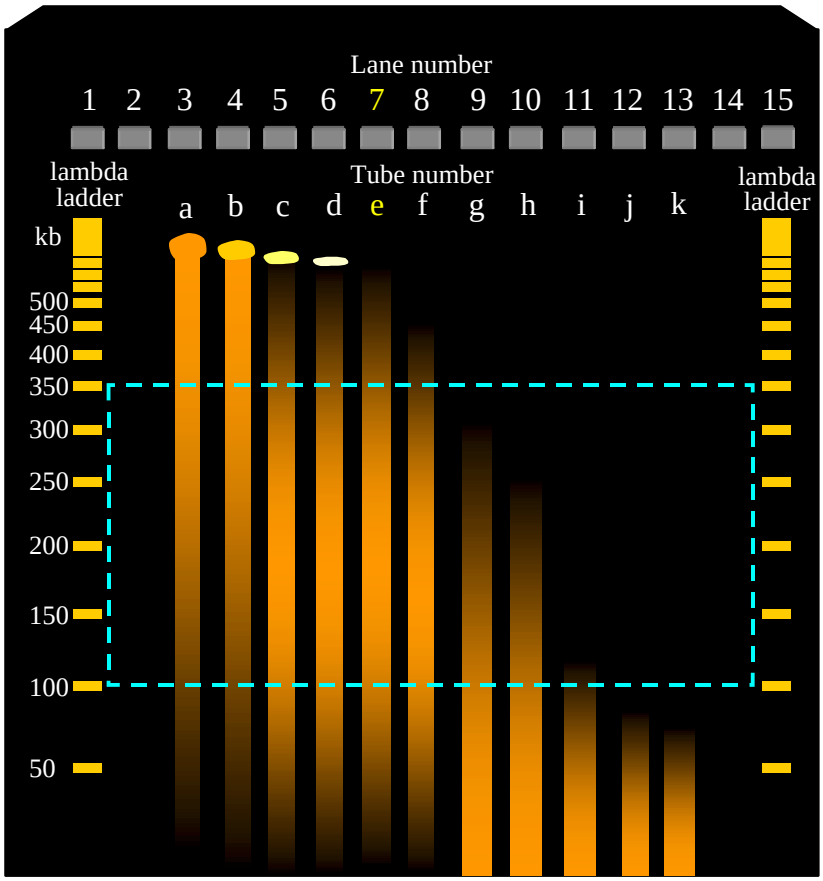


FIGURE 9.1 - Diagram of a test digest of high MW DNA. In this example, comparison of the eleven DNA *Hind*III reactions by gel electrophoresis indicates that the reaction conditions in tube "e" (0.375 units/ml, 20 minute digestion) provide optimal DNA fragments for BAC library construction. The reason for this conclusion is that of all the lanes, lane 7 (the lane in which the contents of tube "e" were placed) contains the largest percentage of fragments within the optimal insert size range of 100-350 kb (*i.e.*, area enclosed by the dotted line). The conditions in tube "d" would also be suitable (and perhaps preferable to those interested in cloning the fragments > 150 kb).

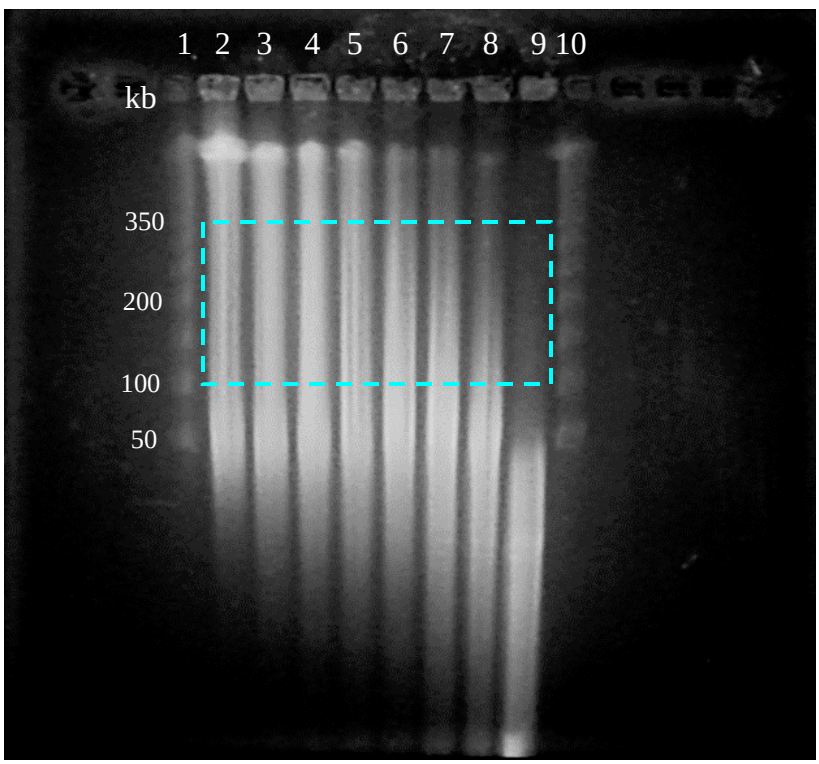


FIGURE 9.2 - A test restriction digest of DNA from cassava (*Manihot duleis*). Lanes 1 and 10 contain the PFGE lambda ladder. Lanes 2-9 contain DNA samples digested with increasingly higher concentrations of restriction enzyme. The conditions used in this test digest differ from those described in CHAPTER 9, however the principle of determining optimal digestion conditions from the gel remains the same (see FIGURE 9.1). A light blue, dotted rectangle has been added to show the optimal fragment size range. In lane 2 the majority of the DNA is > 350 kb, and in lane 5 the majority of the DNA is < 100 kb. However, lanes 3 and 4 appear to contain numerous fragments in the optimal size range. The reaction conditions used in either lane 3 or 4 (or conditions intermediate between the two) should be used in a large-scale restriction digest (see CHAPTER 10).

CHAPTER 10
Restriction digest

PURPOSE

Once the optimal conditions for producing fragments between 100 and 350 kb are determined, a mass digestion using several plugs is performed. The partially digested DNA from these plugs then can be used as insert DNA in construction of a BAC library.

EXPERIMENTAL PROCEDURES

SUPPLIES, EQUIPMENT, AND REAGENTS (see CHAPTER 2 *for details)*: agarose plugs containing genomic DNA (see CHAPTER 7); WB-C; H3M; 10X *Hind*III buffer; *Hind*III; 70% ethanol (in a spray bottle); microscope slides and coverglasses (slides and coverglasses should be sprayed with 70% ethanol and dried with a Kimwipe prior to use)

✸ Note 10.1: Use the same tube of enzyme and same type of buffer used in the test digest!

METHODS:

1. Transfer 8-16 of the PMSF-treated plugs into a 50 ml polypropylene centrifuge tube. If the plugs have been stored in 70% ethanol, allow them to equilibrate in WB-C as described in CHAPTER 7, Note 7.5 before proceeding. If the plugs have been stored in WB-C, proceed directly to step 2.

✸ Note 10.2: For mass restriction digests, we generally use enough plugs to provide 150-250 µg of DNA. The DNA concentration of the plugs was determined in CHAPTER 5.

2. Place the plugs into 20 ml of H3M at 4°C for 1 hour.
3. Place the plugs on 2-3 clean microscope slides. Macerate each plug using clean coverglasses. Place each macerated plug into its own 1.5 ml microcentrifuge tube. Add 1 ml of fresh H3M to each microcentrifuge tube.
4. In CHAPTER 9 an optimal enzyme concentration for producing fragments of 100-350 kb was determined. Add diluted NEB *Hind*III to each tube so that the final enzyme concentration in each reaction tube is the same as the optimal enzyme concentration determined in CHAPTER 9. USE THE SAME TUBE OF ENZYME AND SAME TYPE OF BUFFER USED IN THE TEST DIGEST!

✸ Note 10.3: It is generally best to mix some of the *Hind*III with 1X *Hind*III buffer to produce a 1:10 or a 1:100 enzyme dilution. Aliquots of the dilution can then be added to the reaction tubes (see CHAPTER 9). Diluting the original enzyme eliminates troubles normally associated with pipetting stock enzyme (*e.g.*, contamination, dealing with the viscosity of the stock solution, having to pipet extremely small volumes of solution).

5. Gently mix the contents of each tube, and incubate tubes on ice for exactly 1 hour.
6. Gently tap each tube to mix the contents, and place the tubes in a 37°C water bath. Remove the tubes from the water bath after EXACTLY 20 min and place tubes on ice.
7. Immediately add 150 µl of 0.5 M EDTA (pH 8.0) to each tube (this inhibits further enzyme activity) and gently agitate tubes to promote contact between the agarose and the EDTA. Keep tubes on ice.

✸ Note 10.4: Tubes can be stored at 4°C overnight, but at a significant risk. It is highly recommended that the first size selection (CHAPTER 11) is started before pausing.

CHAPTER 11
First size selection

PURPOSE

Now that the DNA in the plugs has been digested, it is important to check and make sure that the DNA is of an appropriate length for BAC library construction. DNA longer than 350 kb and much of the DNA < 100 kb is removed during this first size selection. Conversely, most of the DNA between 100 and 350 kb is sequestered and taken through a second size selection (CHAPTER 12).

EXPERIMENTAL PROCEDURES

SUPPLIES, EQUIPMENT, AND REAGENTS (see CHAPTER 2 *for details)*: agarose plugs containing partially-digested genomic DNA (see CHAPTER 10); 0.5X TBE; agarose; PFGE Lambda Ladder; aqueous ethidium bromide; 70% ethanol (in a spray bottle); scalpel with #11 blade; CHEF gel apparatus; regular CHEF gel casting stand; 15-tooth gel comb; UV light box equipped with camera or image-capture system

METHODS:

1. Make a 1.0% agarose gel in 0.5X TBE using the regular BioRad CHEF gel casting stand and the 15-tooth gel comb (see CHAPTER 6 for details regarding gel preparation and loading).
2. Using a clean scalpel, cut and remove the agarose between 4-6 of the center wells of the gel to produce one large "slot well". Make sure that the agarose lining the bottom of the wells is removed and that the leading edge of the slot is parallel to the leading edge of the wells (FIGURE 11.1a). The plug pieces will be loaded into the slot well. Based on the volume of agarose plug pieces, extend the slot well in an anterior direction so that all of the plug pieces can fit comfortably into the slot.

✸ Note 11.1: We commonly cut gels with a scalpel or razor blade. However, some prefer a coverglass arguing that nucleases may be activated by metal ions from the scalpel / razor blade.

3. Add a small amount of melted agarose to the slot well so that the bottom of the well is completely sealed. Allow the agarose to solidify.
4. Using the pointed end of a spatula, transfer the macerated, digested plug pieces from all the reaction tubes into the slot well. Press the pieces up against the leading edge of the slot well (FIGURE 11.1b).
5. Place the PFGE Lambda Ladder in lanes flanking the slot well (FIGURE 11.1b).
6. Seal the slot well and the wells containing ladder DNA with melted agarose. Allow the agarose to solidify.
7. Remove the gel from the casting stand, and wipe any agarose off the bottom of the base plate.
8. Place the base plate and overlying gel in the BioRad CHEF electrophoresis chamber. The unit should contain 2.5 L of fresh 0.5X TBE cooled to 12°C.
9. Run the gel using the following parameters: volts/cm = 6.0, included angle = 120°, initial switch time = 1.0 sec, final switch time = 40.0 sec, run time = 18 hours, ramping = linear.

✸ Note 11.2: The gel is generally run overnight.

10. Using a ruler as a "straight-edge", cut the gel with as shown in FIGURE 11.1c.
11. Stain and destain the two peripheral (flanking) pieces of the gel. On a UV light box, align the flanking gel pieces. Turn on the UV light box.

✸ Note 11,3: Always wear eye and face protection when using the UV light box!

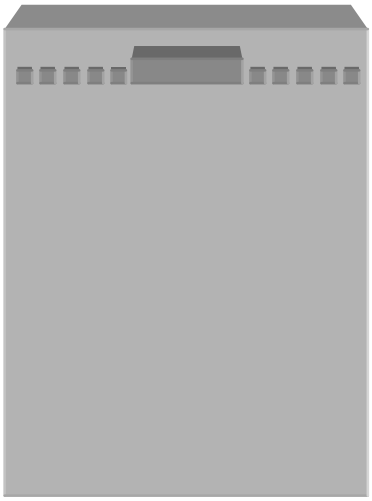
12. Make small incisions in the flanking gel pieces at 100 kb and 350 kb as shown in FIGURE 11.1d.
13. Turn off the light box. On a piece of clean plastic wrap on a workbench, reconstruct the gel by placing the unstained "center piece" between the two flanking gel pieces (FIGURE 11.1e). Using a ruler as a guide, cut the center gel piece as shown in FIGURE 11.1f so that you have three gel blocks; a center gel block containing DNA between 100 and 350 kb and two "end pieces".

✸ Note 11.4: Never expose the center block to UV light as this will break the size-selected DNA!

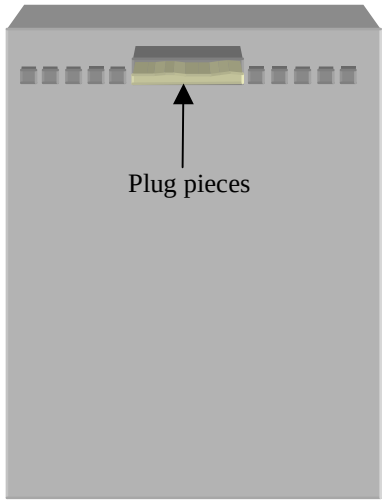
14. Cut the center gel block transversely at two places to yield three blocks (referred to from bottom to top as "*x*", "*y*", and "*z*") of approximately equal size (FIGURE 11.1g). If desired, stain the end pieces with ethidium bromide, partially reconstruct the gel using the various stained pieces, and photograph (*e.g.*, FIGURE 11.2). The unstained pieces should not be included in the reconstruction. Place blocks *x*, *y*, and *z* beneath a piece of plastic wrap to prevent drying.

✸ Note 11.5: Continue with the second size selection (CHAPTER 12) before pausing.

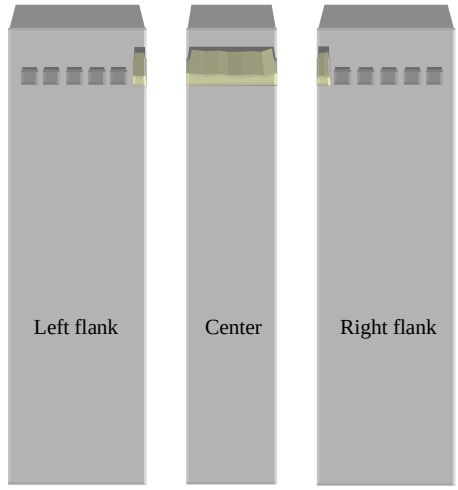
FIGURE 11.1 - Performing the first size selection



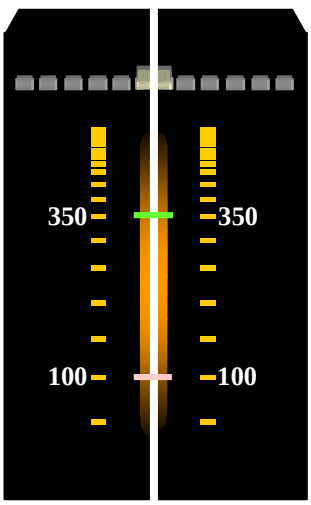
(a) Prepare a "slot well" by joining 4-6 wells together. If necessary, the slot-well can be extended anteriorly to provide more space for the plug pieces. Seal the bottom of the slot-well with a thin layer of melted agarose.



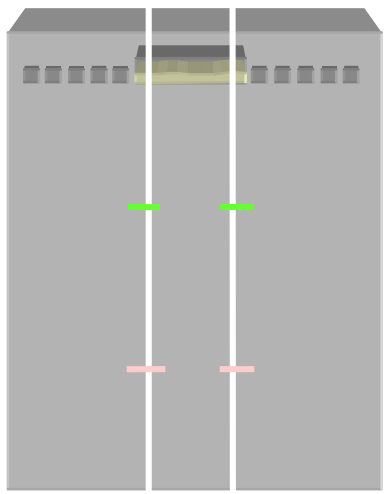
(b) Place plug pieces into the slot-well. Load the PFGE lambda ladder in wells flanking the slot well. Seal all wells with melted agarose.



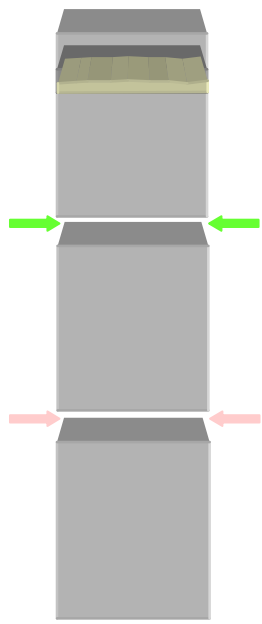
(c) Divide the gel into three pieces as shown. The two flanking pieces should each contain a small part of the slot well as well as a DNA ladder. Stain the flanking pieces with ethidium bromide. Do not stain the center piece (the gel piece containing most of the genomic DNA).



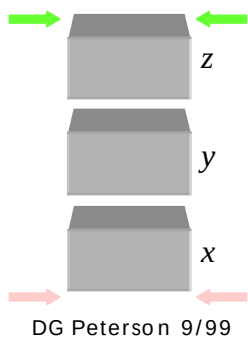
(d) Align the stained flanking gel pieces and examine on a UV light box. If the digestion worked as planned, most of the genomic DNA should lie between 100 and 350 kb. Using a scalpel, make incisions in the flanking pieces marking the 100 kb and 350 kb borders (pink and green lines, respectively).



(e) Reconstruct the gel by placing the unstained center pieces between the two flanking gel pieces. Extend the incisions at 125 kb and 350 kb on the flanking pieces into the center gel piece (green and pink lines).



(f) Cut the center gel piece transversely to connect the two incisions marking 100 kb (pink line) and the two incisions at 350 kb (green line). Stain the resulting upper and lower pieces. The center block will be used in the second size selection.



(g) Cut the center gel block (containing unstained DNA between 100 and 350 kb) transversely at two spots to produce three agarose blocks of roughly equal size. These blocks (from bottom to top) are referred to as *x*, *y*, and *z*, respectively.

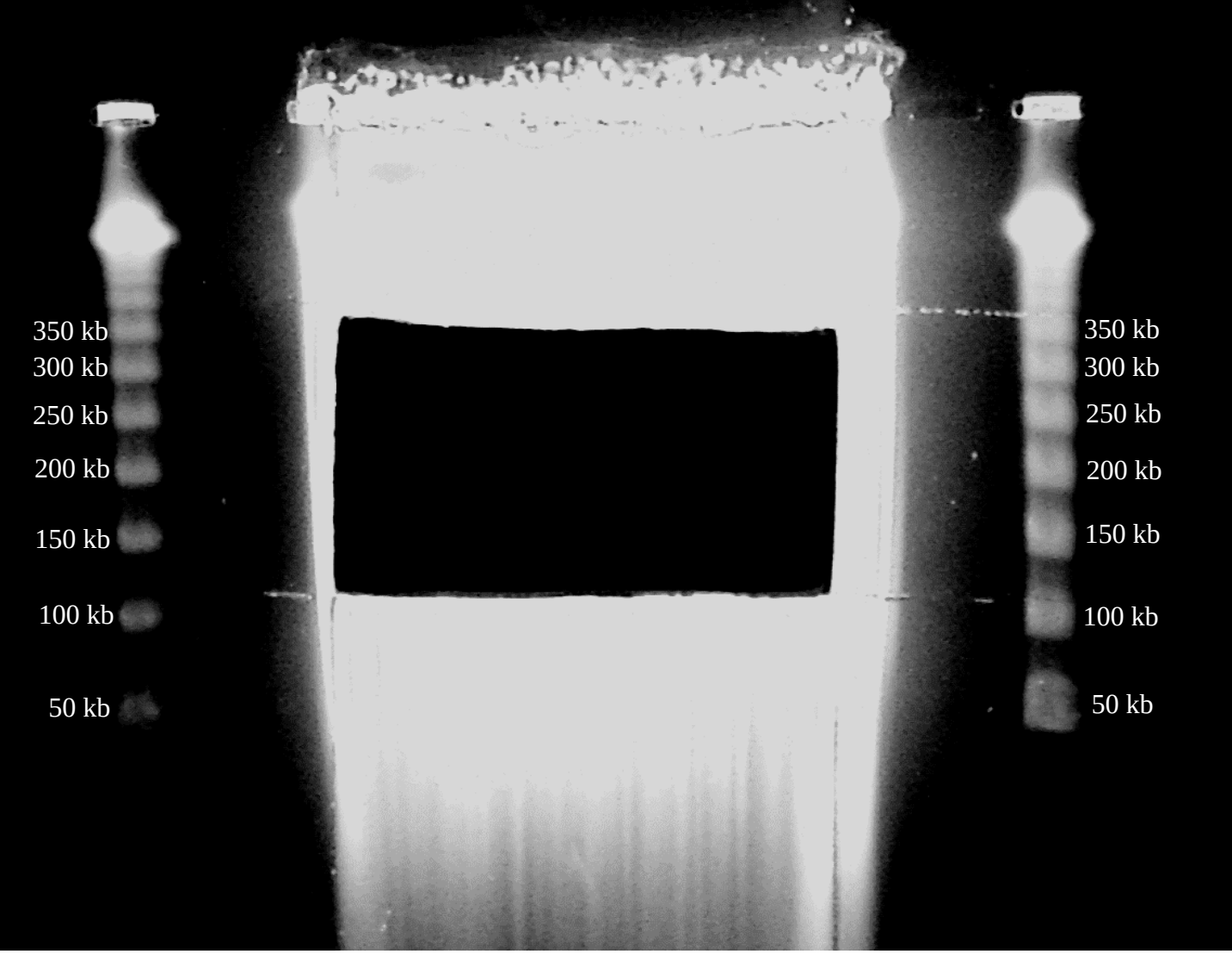


FIGURE 11.2 - Photograph of a partially reconstructed gel. All gel pieces, except for those containing DNA to be used in the second size selection, were stained, placed on a UV light box, reassembled, and photographed. Eight plugs containing *Gossypium raimondii* nuclear DNA were loaded into the slot well. The high density of DNA in the plugs (prepared using **OPTION X**, CHAPTER 7) is reflected in the intense ethidium bromide staining of the *G. raimondii* DNA. The unstained portion of the gel (not shown) containing DNA between 100 and 350 kb was used in a second size selection.

CHAPTER 12
Second size selection

PURPOSE

Though the first size selection gets rid of most of the DNA < 100 kb, some small DNA molecules do get trapped by the longer DNA molecules (this is especially true when the DNA concentration in the plugs is relatively high). The second size selection increases the chance that most of these smaller DNA molecules are eliminated prior to ligation.

EXPERIMENTAL PROCEDURES

SUPPLIES, EQUIPMENT, AND REAGENTS (see CHAPTER 2 *for details)*: agarose cubes *x*, *y*, and *z* containing size-selected DNA (see CHAPTER 11); 0.5X TBE; agarose; LMP agarose; PFGE Lambda Ladder; ethidium bromide; 70% ethanol (in a spray bottle); scalpel with #11 blade; microscope slides; coverglasses; CHEF gel apparatus; large CHEF gel casting stand; 30-tooth gel comb; UV light box equipped with camera or image-capture system

METHODS:

1. Make a 1.0% agarose gel in 0.5X TBE using the large BioRad CHEF gel casting stand and a 30-tooth gel comb. Fill the stand until it is near overflowing. See CHAPTER 6 for additional details regarding gel preparation and loading. Allow the gel to thoroughly solidify.
2. Using a clean scalpel, construct three "slot wells". Each slot well is made by removing the agarose separating four of the comb-made wells (FIGURE 12.1a). Extend each slot well anteriorly to allow enough room for one of the end products of the first size selection (*i.e.*, blocks *x*, *y*, and *z*). Make sure that the agarose lining the bottom of the wells is removed and that the leading edge of each slot well is parallel to the leading edge of the comb-made wells (FIGURE 12.1a).
3. Using a scalpel and a ruler as a "straight-edge", cut a large block of agarose out of the upper-central region of the gel. The cuts should be made exactly as shown in FIGURE 12.1a. Remove all agarose from this region. Completely fill the resulting cavity with 1.0% low melting point (LMP) agarose in 0.5X TBE. Allow the LMP agarose to solidify.
4. Place the PFGE Lambda Ladder in lanes flanking the slot wells.
5. Place blocks *x*, *y*, and *z* in their original top/bottom orientation (see FIGURE 11.1g) in the three slot wells as shown in FIGURE 12.1a. Add LMP agarose to fill any remaining space in each slot well. Gently move each agarose block back and forth to dislodge any bubbles, and position each block so that it touches the leading edge of its respective slot well.
6. Seal the wells containing ladders with melted LMP agarose. Allow the LMP agarose to solidify.
7. Remove the gel from the casting stand, and wipe any agarose off the bottom of the base plate.
8. Place the base plate and overlying gel in the BioRad PFGE chamber. The unit should contain 2.5 L of fresh 0.5X TBE cooled to 12°C.

9. Run the gel using the following parameters: 6.0 v/cm, included angle = 120°, initial switch time = 3.0 sec, final switch time = 5.0 sec, run time = 18 hours, ramping = linear.

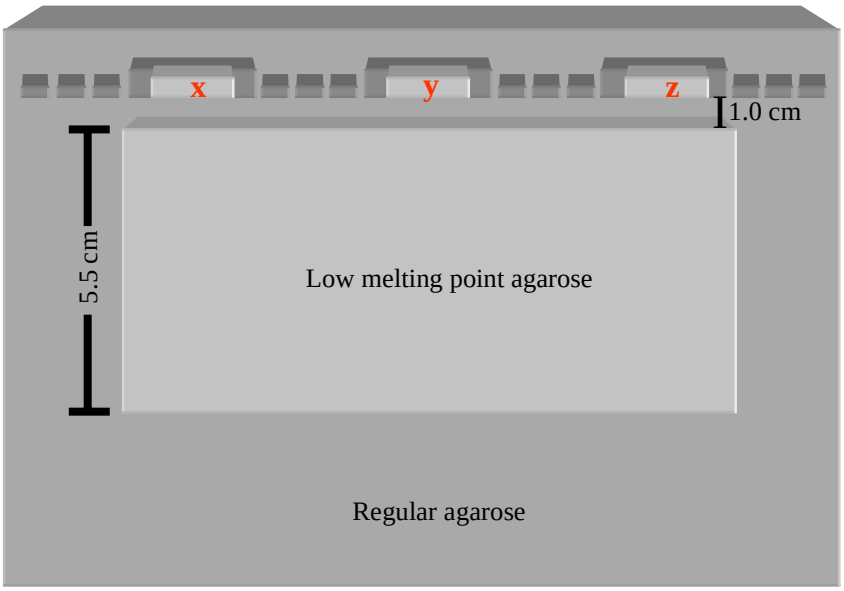
✸ Note 12.1: The gel is generally run overnight.

10. Using a ruler as a "straight-edge", cut the gel with a scalpel as shown in FIGURE 12.1b. There will be seven gel pieces (referred to as 1-7 from left to right). Using a scalpel, place identification notches at the bottom of some of the gel pieces as shown. These notches allow easy reassembly of the gel later in the protocol.
11. Stain and destain the odd-numbered pieces of the gel (*i.e.*, the gel pieces containing the PFGE Lambda Ladder). On a UV light box, realign the stained pieces as shown in FIGURE 12.1c. WEAR EYE AND FACE PROTECTION WHEN USING THE UV LIGHT BOX. While observing the illuminated gel, use the scalpel to make small incisions at 125 kb and 350 kb in each of the stained pieces (FIGURE 12.1c).
12. Turn off the UV light box.

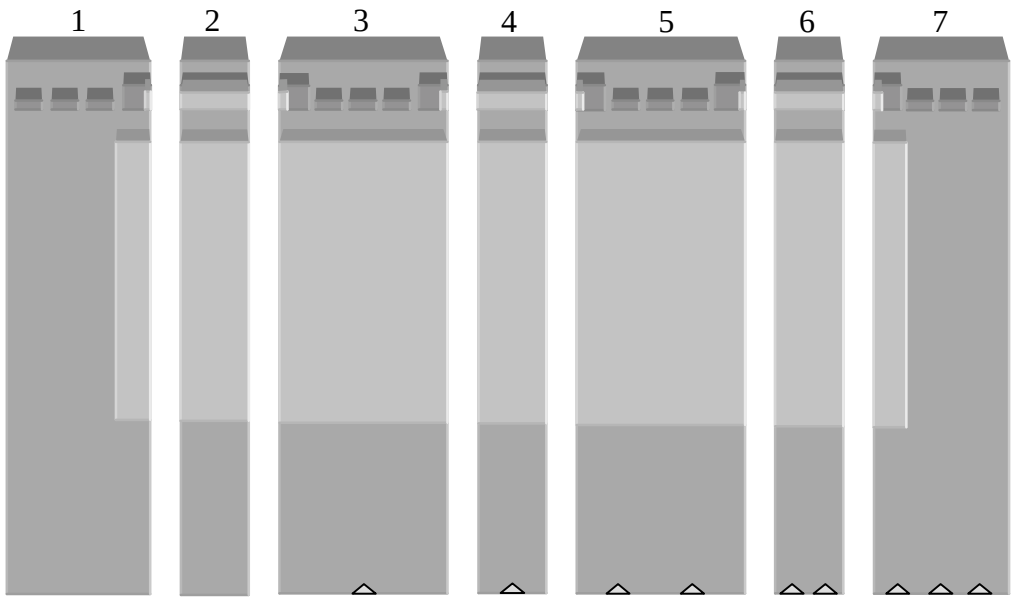
✸ Note 12.2: Never expose the even-numbered gel pieces to UV light as this will break the size-selected DNA and make it unclonable!

13. On a piece of clean plastic wrap on a workbench, reconstruct the gel by placing the unstained even-numbered pieces between their flanking odd-numbered stained pieces (FIGURE 12.1d). Extend the incisions at 125 kb and 350 kb on each stained gel piece into adjacent unstained (even-numbered) gel pieces. Place the stained gel pieces aside.
14. Using a scalpel, coverglass, or razor blade, connect the incisions on each even-numbered gel piece as shown in FIGURE 12.1e. You should now have three unstained gel pieces containing DNA between 125 and 350 kb in length. The DNA from these pieces (referred to from left to right as *q*, *r*, and *s*) will be isolated. Cut piece *q* twice laterally to yield three blocks of roughly equal size. Do the same for pieces *r* and *s* (FIGURE 12.1e). Place the three *q* blocks in a 50 ml polypropylene centrifuge tube. Likewise place the *r* blocks in a new centrifuge tube and the *s* blocks in their own centrifuge tube.
15. If isolation of DNA from the agarose blocks is to be performed within the next two days, 50 ml of sterile 1X TAE should be added to tubes containing gel pieces *q*, *r*, and *s*. Tubes should be stored at 4°C. If DNA isolation is to be performed at a later date, store the agarose pieces in 70% ethanol at –20°C as described in CHAPTER 7, Note 7.5.
16. Stain and destain the remaining gel pieces. Based on the notches made earlier, reconstruct the gel on a UV light box. If desired, photograph the reconstructed gel for documentation purposes (see FIGURE 12.2 for an example of an actual reconstructed gel).

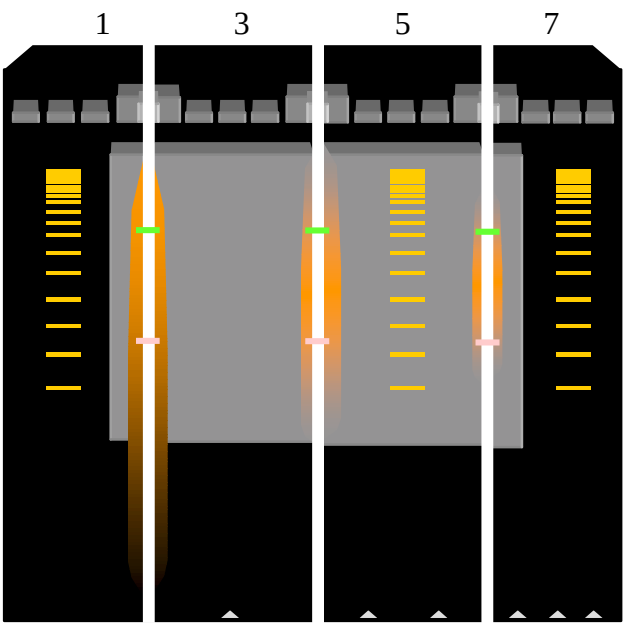
FIGURE 12.1 - The second size selection.



(a) Using a scalpel, join adjacent comb-made wells to form three slot-wells. Place a thin layer of LMP agarose in the bottom of each slot- well and allow the agarose to solidify. Insert blocks *x*, *y*, and *z* into the slot-wells. Place the PFGE lambda ladder into wells flanking the slot-wells as shown. Be sure to push *x*, *y*, and *z* to the leading edge of their respective slot-wells. Seal all wells with LMP agarose. Using a ruler as a guide, use a scalpel to cut out a large block of agarose from the center of the gel (see diagram). Fill the resulting space with 1% LMP agarose, and allow the LMP agarose to solidify. Run the gel using the following parameters: run time = 18 hr, initial switch time = 3 sec, final switch time = 5 sec, included angle = 120°, volts/cm = 6.0, buffer temperature = 12°C.



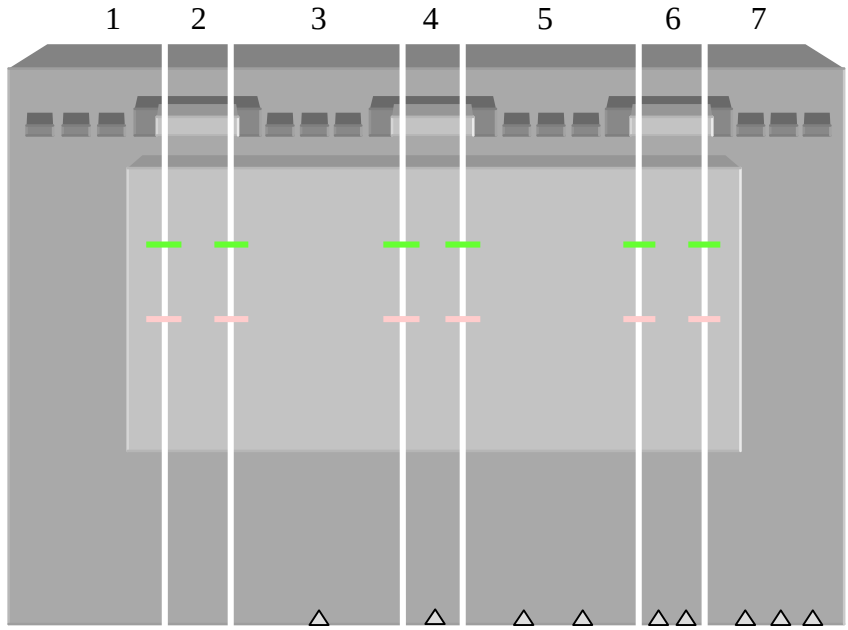
(b) Cutting the gel. After electrophoresis, divide the gel into seven pieces as shown above. The pieces are referred to as 1-7 from left to right. Cut notches into the bottom of pieces 3-7 as shown. Notches facilitate rapid reconstruction of the gel. Stain the odd-numbered pieces with ethidium bromide. Do not stain the even-numbered pieces as these contain genomic DNA which may be used in subsequent ligation reactions.

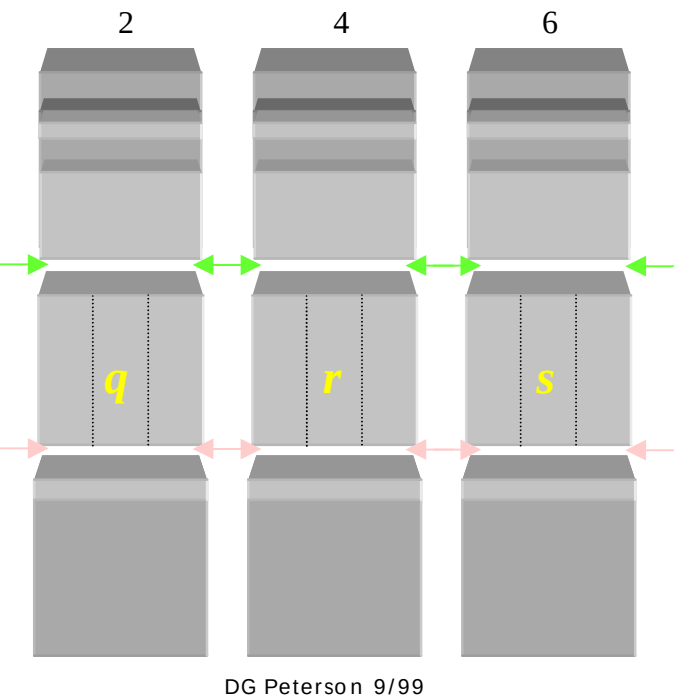


(c) Align the stained (odd-numbered) gel pieces and examine on a UV light box as shown. Using a scalpel, make incisions to mark the 125 kb (pink lines) and 350 kb (green lines) borders.

(d) Reconstruct the gel by placing the unstained even-numbered pieces between the stained odd-numbered pieces. Use the notches at the bottom of pieces 3-7 to facilitate proper assembly of the gel. Extend the incisions at 125 kb and 350 kb on each odd-numbered gel piece into adjacent even-numbered gel pieces (green and pink lines).

✸ Note: The center gel piece should never be exposed to UV light from the light box as this can break the DNA!





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(e) Using a scalpel or razor blade, connect the appropriate incisions on each even-numbered gel piece as shown. The resulting center blocks containing DNA between 125 and 350 kb are referred to as *q*, *r*, and *s*, respectively. Subsequently, cut each of the three center blocks longitudinally to produce three blocks of roughly equal size (dotted lines). Place the three *q* blocks in one centrifuge tube, the three *r* blocks in a second tube, and the three *s* blocks in a third tube. The DNA in these blocks may be suitable for ligation.

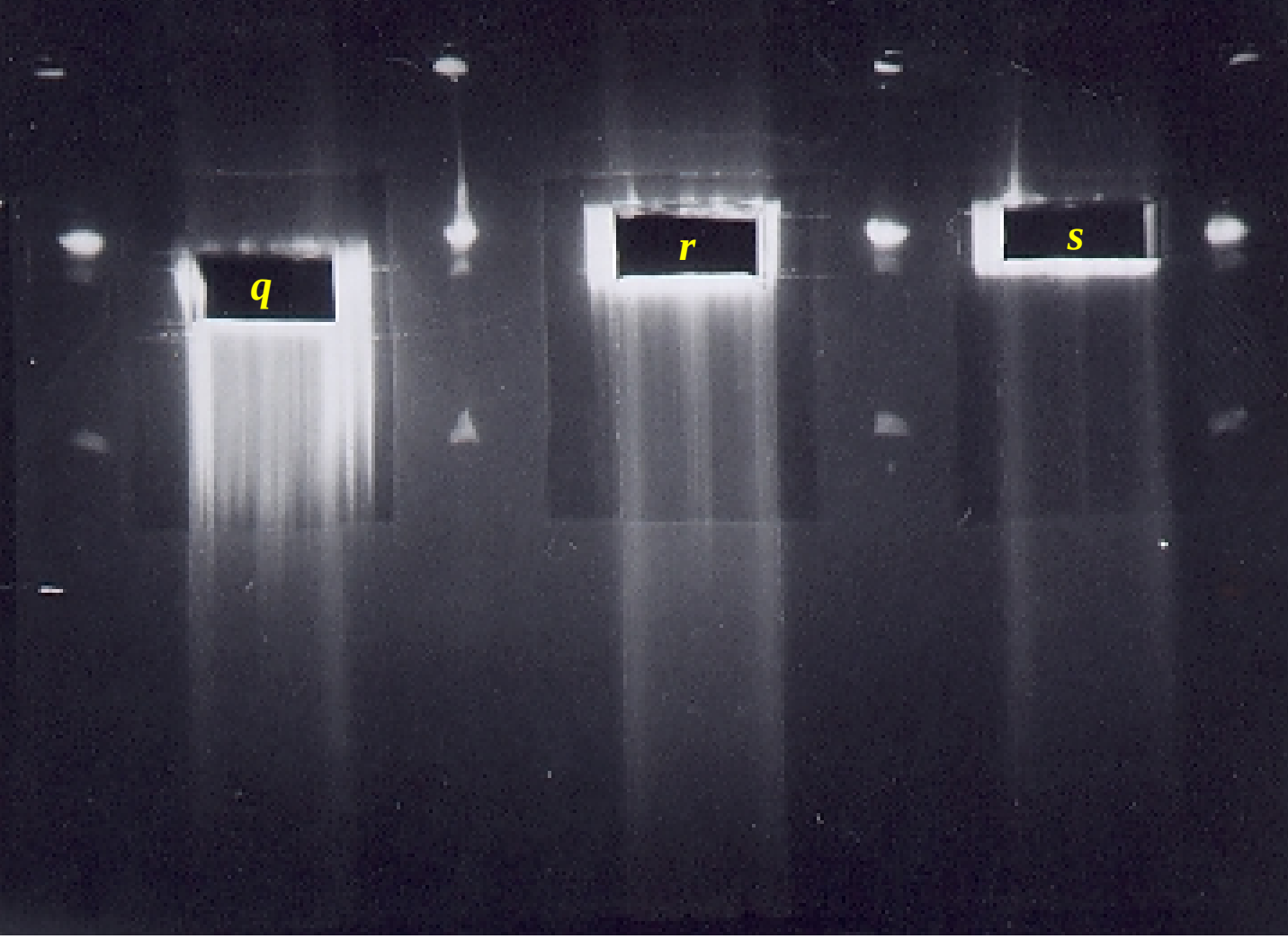


FIGURE 12.2 - Partially-reconstructed gel after a second size selection. This gel shows DNA from *Saccharum officinarum* (sugarcane). Blocks *q*, r, and *s* containing unstained DNA between 125 and 350 kb have been removed from the gel as described in FIGURE 12.1. The spaces where blocks *q*, *r*, and *s* were located have been labeled.

CHAPTER 13
Isolation of size-selected DNA from agarose

PURPOSE

High molecular weight restriction fragments must be removed from surrounding agarose before they can be used in ligation reactions. Presented below are two methods for isolating DNA from agarose plugs.

PREFACE

At present, there are several methods for isolating high molecular weight restriction fragments from agarose. In our experience, both electroelution and GELase digestion work well. Thus each of these methods will be discussed in this chapter. Once size-selected DNA is isolated from LMP agarose blocks it can be ligated into an appropriate vector and used in transformation.

EXPERIMENTAL PROCEDURES

I. DNA ISOLATION

A. ELECTROELUTION

SUPPLIES, EQUIPMENT, AND REAGENTS (see CHAPTER 2 *for details)*: agarose cubes *q*, *r*, and *s* containing size-selected DNA (see CHAPTER 12); electroelution system

METHODS:

In electroelution, a voltage is applied to a gel slice containing DNA. As in standard electrophoresis, the DNA in the agarose moves towards the positive electrode. The DNA exits the gel slice and is collected in a filter cup. DNA solutions removed from filter cups are ready for use in **POST-ISOLATION PROCEDURES** (see **II** below).

There are numerous electroelution devices available. Because these devices differ with regard to design and operation instructions, consult the manufacturer's instructions that come with your electroelution apparatus to get optimal results. The BioRad Electroelution system is commonly used in molecular biology research, and consequently detailed instructions for using this system are given in APPENDIX B.

✸ Note 13.1: Do not use an electroeluter that previously has been used to isolate high-copy plasmid DNA when trying to isolate high molecular weight DNA. This can lead to sample contamination and confusing results.

Electroelution also can be performed using dialysis tubing and a standard horizontal gel apparatus (see Strong et al. 1997 for details).

B. GELase™ DIGESTION

SUPPLIES, EQUIPMENT, AND REAGENTS (see CHAPTER 2 *for details)*: agarose cubes *q*, *r*,

and *s* containing size-selected DNA (see CHAPTER 12); GELase™ (0.2 units/µl) with 50X buffer

METHODS:

1. Place a clean weighing boat on a balance. Tare the balance and place one of the three *q* agarose cubes onto the boat. Record the weight of the agarose cube. Transfer the cube to a sterile 15 ml culture tube.
2. Repeat step 1 using an *r* cube and an *s* cube.
3. Add MBG water to each tube. Place the tubes on ice. After 10 min, decant the water and add fresh MBG water. Repeat this process five times. This step removes electrophoresis buffer from the cubes (which possibly could interfere with ligation).
4. Decant the final wash. For every 50 mg of agarose in a particular tube, add 1 µl of 50X GELase buffer.
5. Place the culture tubes in a 70ºC water bath for 3 min (or until all of the agarose has become liquid). Quickly transfer the tubes to a 45ºC water bath
6. Add GELase to each tube so that there is 1.0 unit of enzyme for every 200 mg of agarose.
7. Gently swirl the contents of each tube. Incubate the tubes at 45ºC for 45 min. The DNA in each tube is now ready for use in **POST-ISOLATION PROCEDURES** (see **II** below).

II. POST-ISOLATION PROCEDURES

SUPPLIES, EQUIPMENT, AND REAGENTS (see CHAPTER 2 *for details)*: DNA isolated from *q*, *r*, and *s* agarose cubes (see above); 1X TAE; agarose; 70% ethanol in a spray bottle, 1X uncut lambda DNA; submarine mini-gel apparatus; UV light box equipped with camera or image-capture system; blue juice; ethidium bromide

METHODS:

1. Place *q*, *r*, and *s* DNA samples isolated using electroelution or GELase digestion ("insert DNA solutions") in their own 1.5 ml microcentrifuge tubes on ice.
2. Prepare a 1% agarose submarine mini-gel in 1X TAE. In preparing the gel, use a comb with at least 7 teeth.
3. Place 0.5, 1.0, 2.0 µl, and 4.0 µl of 1X uncut lambda DNA (*i.e.*, 25 ng, 50 ng, 100 ng, and 200 ng) in separate 0.65 ml microcentrifuge tubes. Add 2.0 µl of blue juice to each.
4. Take a 5 µl aliquot from the tube containing *q* insert DNA and place the solution in a 0.65 ml microcentrifuge tube. Add 2 µl of blue juice. Do the same for the *r* and *s* samples.
5. Submerge the mini-gel in 1X TAE buffer in an appropriate mini-gel apparatus. Load the gel as shown in FIGURE 13.1. Run the gel at 100 v for 15-20 min. Stain and photograph the gel as described in CHAPTER 3. Based on comparison of the relative fluorescence in the sample and standard lanes, an estimate of the concentration of each sample can be made (FIGURE 13.1). Multiplication of a sample's volume by its concentration gives an estimate of the total amount of insert DNA in that sample.
6. Select the insert DNA solution with the greatest concentration of high molecular weight DNA for use in subsequent ligation reactions. In our experience, the *q* solution generally has a DNA concentration of 10-20 ng/µl while the *r* and *s* solutions have concentrations substantially below 10 ng/µl. However, any of the solutions with a DNA concentration > 5.0 ng/µl can be used in ligation.

✸ Note 13.2: If the insert solutions are particularly dilute (< 5.0 ng/µl), they can be

concentrated using Millipore nitrocellulose filters and 10% PEG [see step 6 ("desalting") in CHAPTER 4; also see FIGURE 4.1].
✸ Note 13.3: High MW (insert) DNA samples are quite unstable. Though they can usually be left at 4°C overnight, it is best to perform ligation immediately after checking the DNA concentration.

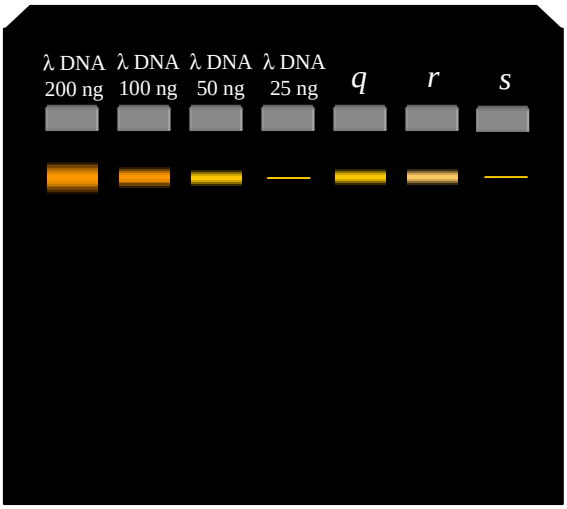


FIGURE 13.1 - Diagram of a mini-gel used to determine the DNA concentrations of DNA samples *q*, *r*, and *s*. Four different concentrations of uncut lambda DNA are run in the first four lanes while the three samples are run in the following lanes. In this example, 5.0 µl of sample *q* stains with an intensity roughly equal to that of the 50 ng of 1X uncut lambda DNA. Because 5 µl of *q* DNA was placed in lane 5, the concentration of the undiluted DNA sample is about (50 ng/5 µl =) 10 ng/µl.

CHAPTER 14

Ligation, test transformation, and *Not*I digest

PURPOSE

During ligation, insert DNA is ligated with vector DNA to form BACs. A test transformation followed by a miniprep/*Not*I analysis provides information on the success of ligation and transformation using plant DNA as the source of inserts.

EXPERIMENTAL PROCEDURES

SUPPLIES, EQUIPMENT, AND REAGENTS (see CHAPTER 2 *for details)*: dephosphorylated pBeloBAC11 vector stock (see CHAPTER 3); insert DNA (see CHAPTER 13); T4 ligase; 10X T4 ligase buffer; 10% PEG; Millipore nitrocellulose filters; 70% ethanol; ELECTROMAX® DH10B™ competent cells; SOC; X/I/C Petri plates; glass plating rod; electroporator with cuvettes; MP-1; MP-2; MP-3; LB^{+CM}; isopropanol (stored at –20ºC); 70% ethanol (-20ºC); 0.5X TBE; PFGE Midrange Ladders or PFGE Lambda Ladder; agarose; *Not*I with 10X buffer and 100X BSA; blue juice; sterile toothpicks; CHEF gel apparatus; large CHEF gel casting stand; 45-tooth gel comb; UV light box equipped with camera or image-capture system

METHODS:

1. Place insert DNA, the dephosphorylated pBeloBAC11 vector stock solution, and the T4 ligase 10X buffer on ice. Allow the buffer and the vector stock to thaw. Keep the T4 ligase in the –20ºC freezer until immediately before use.
2. Set up ligation reactions as described below. The number of reactions that can be prepared depends upon the nanograms of insert DNA available. In general, we make up 150 µl reactions in 1.5 ml microcentrifuge tubes as follows:

Ligation reaction
50 ng vector DNA
15 µl 10X T4 ligase buffer
3 µl T4 ligase (*i.e.*, 9 units)
300 ng of insert DNA
MBG water to give a final reaction volume of 150 µl

✸ Note 14.1: Most BAC libraries have been constructed using a molar ratio of 5-15 parts size-selected DNA to 1 part BAC vector. We typically start off using a 5:1 ratio. If a 5:1 ratio does not produce a satisfactory outcome, changing the ratio of insert to vector can sometimes improve the results.

3. Gently tap each reaction tube to mix the tube's contents. DO NOT VORTEX OR AGITATE VIOLENTLY AS THIS MAY SHEAR THE INSERT DNA.
4. Incubate the ligation reactions at 16ºC overnight as described in CHAPTER 4.
5. Place ligation reaction tubes in a 65ºC water bath for 20-30 min to "heat kill" the enzyme.
6. Desalt the ligated DNA as described in CHAPTER 4. Place no more than 300 µl of ligation

reaction on any particular Millipore nitrocellulose filter.

7. Using a pipettor and large-orifice pipet tips, transfer all of the desalted ligation reactions into a single 1.5 ml microcentrifuge tube (*i.e.*, pool the ligation reactions).

✸ Note 14.2: Due to osmosis during desalting, the total volume of liquid placed on each filter typically will be one-third to one-half that of the starting volume.

8. Place the tube at 4ºC.

✸ Note 14.3: Ligated DNA is stable at 4°C for at least 5 days.

9. Perform a test transformation exactly as described in CHAPTER 5 except expose the contents of each cuvette to 320-330 volts rather than 390-400 volts.
10. After incubating test plates overnight, determine the titer of the transformation reaction and the percentages of white and blue colonies. If more than 60% of the colonies are white, select thirty-six white colonies from the test plates and perform a *Not*I digestion on isolated BACs exactly as described in CHAPTER 6. A diagram illustrating the appearance of a successful transformation/miniprep/*Not*I digest is shown in FIGURE 6.1. Photographs of BAC *Not*I digests from three plant species are shown in FIGURE 14.1.

✸ Note 14.4: Dicots contain few *Not*I restriction sites in their DNA. Thus, *Not*I digests of dicot BACs produce only one or two bands (in addition to the vector band) on agarose gels. In contrast to dicots, *Not*I sites are more frequent in monocot DNA, and consequently more bands are observed in BAC *Not*I digests from monocots (see FIGURE 14.1). Because of the often numerous bands in *Not*I digests of monocot inserts, it is best to use the PFGE Midrange Ladders as size standards rather than the PFGE Lambda Ladder. The PFGE Midrange Ladders possess more fragments covering a wider range of fragment sizes than the PFGE Lambda Ladder, and thus they allow more accurate determinations of insert size in species containing numerous *Not*I sites.

INTERPRETING THE RESULTS

- If the percentage of false positive clones is > 10% it is possible that the vector DNA was damaged during dephosphorylation. An extremely high percentage of blue clones (> 40%) suggests possible problems during vector preparation, ligation, and/or transformation.
- The combined base pair lengths of all non-vector bands in a particular lane constitute the length of the insert (see FIGURE 6.1 and FIGURE 14.1). What is the mean insert length? What is the size of the largest insert? What is the size of the smallest insert? Whether clones contain inserts of adequate size is based upon the needs of those who intend to use the library. The protocol presented in this paper is designed to generate clones with insert sizes between 100 kb and 350 kb. However, smaller insert sizes may be sufficient (or even preferable) for certain applications.

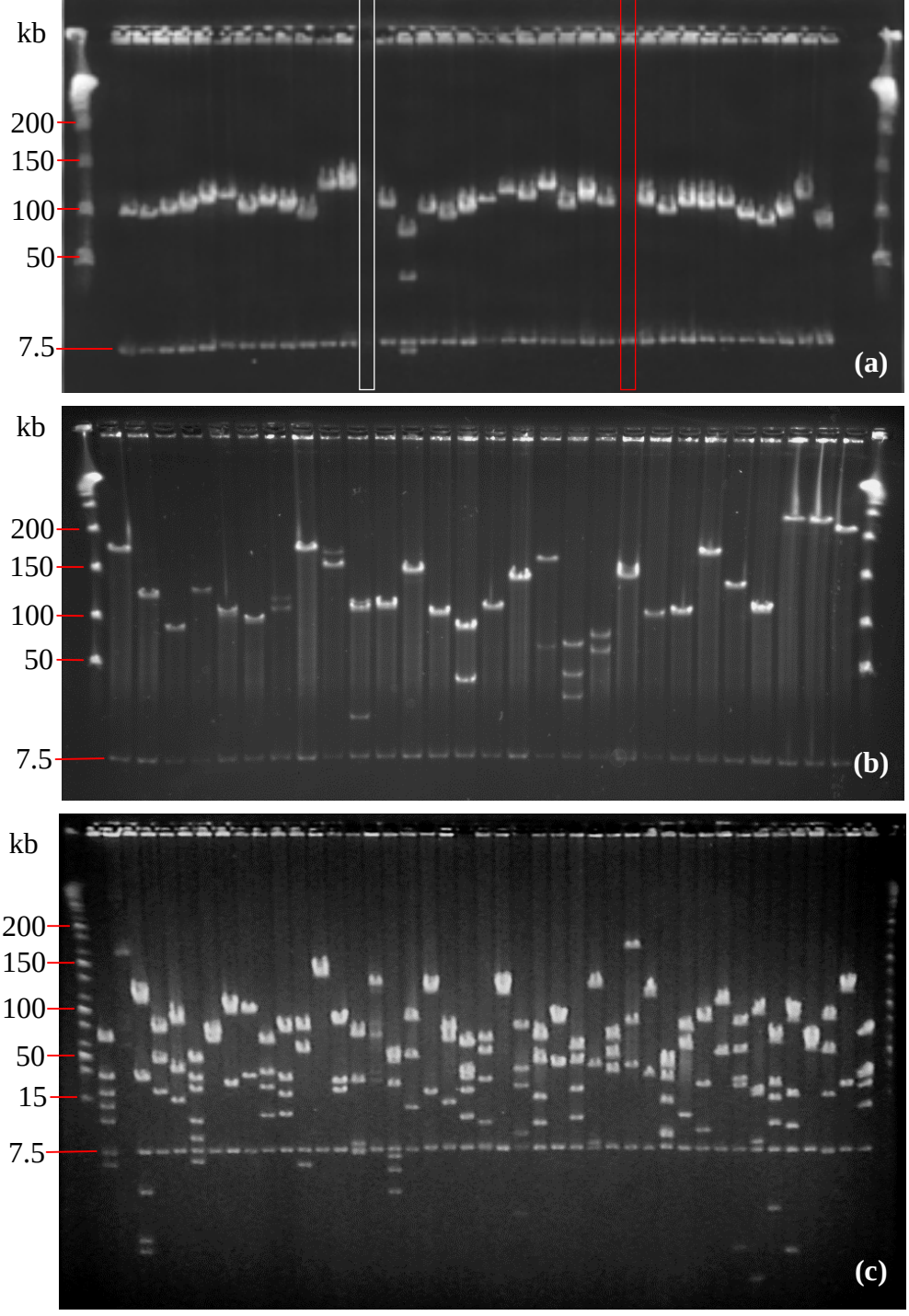


FIGURE 14.1 - *Not*I digests of minipreps from randomly-selected white colonies of **(a)** *Gossypium raimondii*, **(b)** soybean (*Glycine max* 'A3244'), and **(c)** maize (*Zea mays* 'B73') libraries. In **(a)** and **(b)** the PFGE lambda ladder is visible at the far-right and far-left sides of the photograph. In **(c)**, the PFGE Midrange I ladder is in the first and last lanes. In each photo, note the row of vector bands at 7.5 kb. For the dicots *G. raimondii* **(a)** and soybean **(b)**, most lanes contain only one or two non-vector bands indicating relatively few genomic *Not*I sites. As shown in **(c)**, *Not*I restriction sites are considerably more common in monocot DNA. In **(a)**, one of the lanes contains no visible DNA suggesting that the miniprep was lost (white rectangle), and one "false positive" (red rectangle) is visible. No false positives or empty lanes are seen in **(b)** and **(c)**. The average insert sizes for the *G. raimondii*, soybean, and maize libraries are 120, 140, and 140 kb respectively (see TABLE 1.1).

CHAPTER 15
Mass transformation

PURPOSE

If the results of the test transformation are satisfactory, a large-scale transformation is performed. The resulting transformants are plated onto X/I/C trays. White colonies picked from the trays will constitute the BAC library.

EXPERIMENTAL PROCEDURES

SUPPLIES, EQUIPMENT, AND REAGENTS (see CHAPTER 2 *for details)*: 70% ethanol; ELECTROMAX® DH10B™ competent cells; ligated DNA produced in CHAPTER 14; SOC; X/I/C trays; glass plating rod; electroporator with cuvettes (see APPENDIX A for detailed instructions regarding operation of the Gibco BRL CELL-PORATOR)

METHODS:

✸ Note 15.1: Assuming the test transformation yielded satisfactory results, use the same electroporation device and the same instrument settings as were used in the test transformation (CHAPTER 14).

1. Place 2.0 µl aliquots of the ligated DNA into 1.5 ml microcentrifuge tubes. Because ligated DNA has a limited shelf life, it is generally best to go ahead and use all or most of the ligated DNA generated in CHAPTER 14. This means you may have 30 or more microcentrifuge tubes containing 2.0 µl of ligated DNA.
2. Add 20 µl of competent cells to each of the microcentrifuge tubes. Tap the tubes gently to mix contents. Place the tubes on ice.
3. For every six microcentrifuge tubes, place a 15 ml sterile culture tube (equipped with a sterile cap) in a test tube rack. Transfer the rack to a laminar-flow hood. Place 3.0 ml of SOC in each culture tube.
4. Using a pipettor equipped with a standard 200 µl tip, transfer the mixture from one of the microcentrifuge tubes into an electroporator cuvette. Make sure that there are no bubbles in the ligate/bacterial solution. Load as many additional cuvettes as possible.
5. Use an electroporator to apply a shock to the bacteria in each cuvette. Generally, a voltage of 320-330 is adequate.

✸ Note 15.2: Be careful not to shock cells in a cuvette more than once.

6. Place the cuvettes on ice.
7. Once six cuvettes have been shocked, transfer the contents of these cuvettes into one of the culture tubes containing SOC. If need be, new transformation reactions can be loaded from microcentrifuge tubes into these emptied cuvettes.
8. Repeat steps 4-7 until all the transformation reactions have been transferred into culture tubes (six reactions per tube).
9. Place culture tubes in a 37ºC incubator shaker set at 250 rpm for exactly one hour.
10. Prepare a test plate for each “one-hour” culture tube as described in CHAPTER 5. Place the culture tubes in the refrigerator. Incubate the test plates at 37ºC overnight.

11. Based on the mean number of clones on the test plates, calculate a titer for the library (*i.e.*, cfu/µl of "one-hour" culture. Additionally, calculate how many microliters of one-hour culture constitute 2000 cfu. For example, if the average number of colonies on each plate is 150 and each plate was innoculated with 50 µl of one-hour culture, the titer of the one-hour culture would be (150 clones/50 µl =) 3 cfu/µl. 2000 cfu would be present in (2000 cfu ÷ 3 cfu/µl =) 667 µl of overnight culture.

✸ Note 15.3: If the trays are going to be picked by a QBot (see CHAPTER 16), a density of 2,000 clones per plate is desirable. If the clones are to be handpicked, a density of > 3000 clones per plate may be preferable.

12. The number of X/I/C plates one needs to prepare is based on the total volume of one-hour culture and the amount of one-hour culture constituting 2,000 cfu. For example, if you have ten culture tubes each containing 3 ml of culture, you have a total of 30 ml (30,000 µl) of one-hour culture. If 667 µl of one-hour culture contains 2,000 cfu, you should make up (30,000 µl of culture ÷ 667 µl =) 45 X/I/C trays. Prepare X/I/C trays as described in CHAPTER 2. Remember to UV sterilize the trays before pouring.
13. Remove the tubes containing one-hour culture from the refrigerator, and place them in the laminar-flow hood.
14. Flame-sterilize a glass-plating rod as shown in FIGURE 5.1. Allow the rod to cool for approximately one minute.
15. Place 2,000 cfu of one-hour culture onto the agar of one of the X/I/C trays. Use the plating rod to spread the culture over the entire agar surface (FIGURE 15.1). Continue moving the rod across the agar until the rod begins to glide with less fluidity, *i.e.*, the friction between the rod and the agar increases. This indicates that the bacterial culture has been absorbed into the agar.

✸ Note 15.4: In general, 0.5-0.8 ml of culture can be spread on a freshly prepared X/I/C tray without problem. However, smaller volumes may be absorbed into the agar before the bacteria can be thoroughly spread across the plate. This is especially likely if the plates have been stored for awhile before use. Likewise, larger volumes may take an excessively long time to soak into the agar. The former problem can be fixed by diluting the one-hour cultures with MBG water so that 2000 cfu are contained within 0.5-0.8 ml of fluid. The latter situation can be remedied by spinning the one-hour cultures at 650 x *g* for 20 minutes in a centrifuge, decanting the supernatants, and resuspending the pellets in just enough fresh SOC so that 2000 cfu are found in 0.75 ml of the mixture.

16. Replace the lid and turn the plate upside-down. On the side of the plate write information regarding the nature of the library (FIGURE 15.2).
17. Repeat steps 14-16 until all of the one-hour culture has been plated.
18. Place the X/I/C trays in a 37ºC incubator overnight. Clones should appear within 15 hours of incubation and should reach a diameter of 1-2 mm by 20 hours (see FIGURE 15.3).
19. Select a few trays at random and use them to determine an estimate of the average number of colonies per plate, the percentage of clones that are white, and the percentage of clones that are blue.

INTERPRETING THE RESULTS

- The trays should each contain between 2000-3000 colonies, and the vast majority of the clones should be white (*e.g.*, FIGURE 15.3).

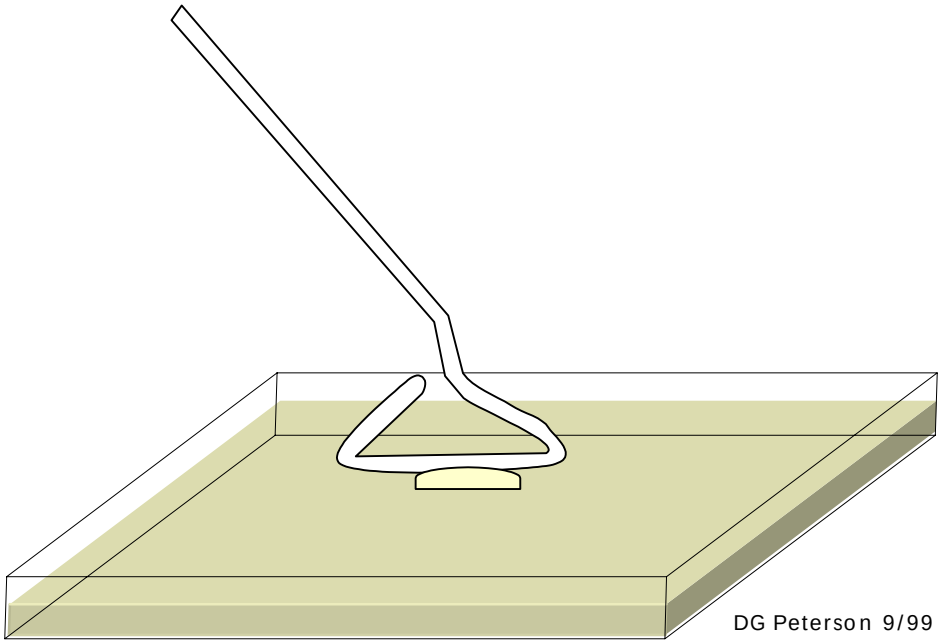


FIGURE 15.1 - A drop of one-hour culture containing 2,000 cfu is spread across the surface of an X/I/C tray.

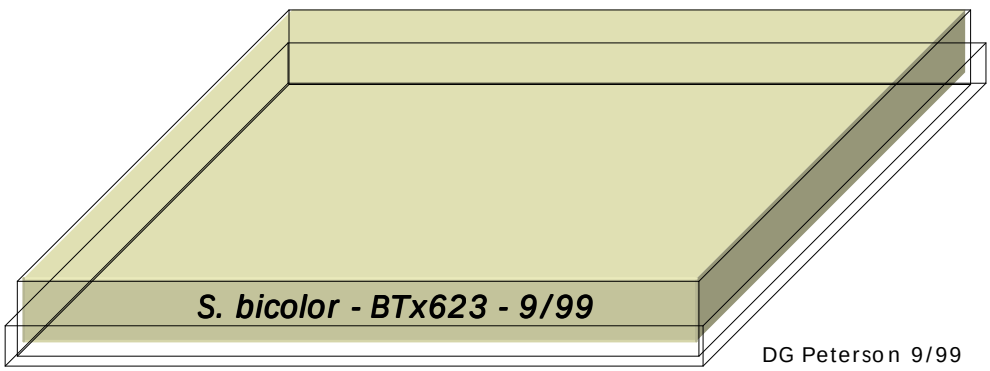


FIGURE 15.2 - Information regarding the nature of the library is written on the side of each X/I/C tray (writing on the bottom of trays can interfere with automated picking) using a marker with ethanol-soluble ink. For example, in the drawing above, the species name (S. bicolor = *Sorghum bicolor*), the cultivar (BTx623), and the date the library was made (September 1999) are written directly on the tray.

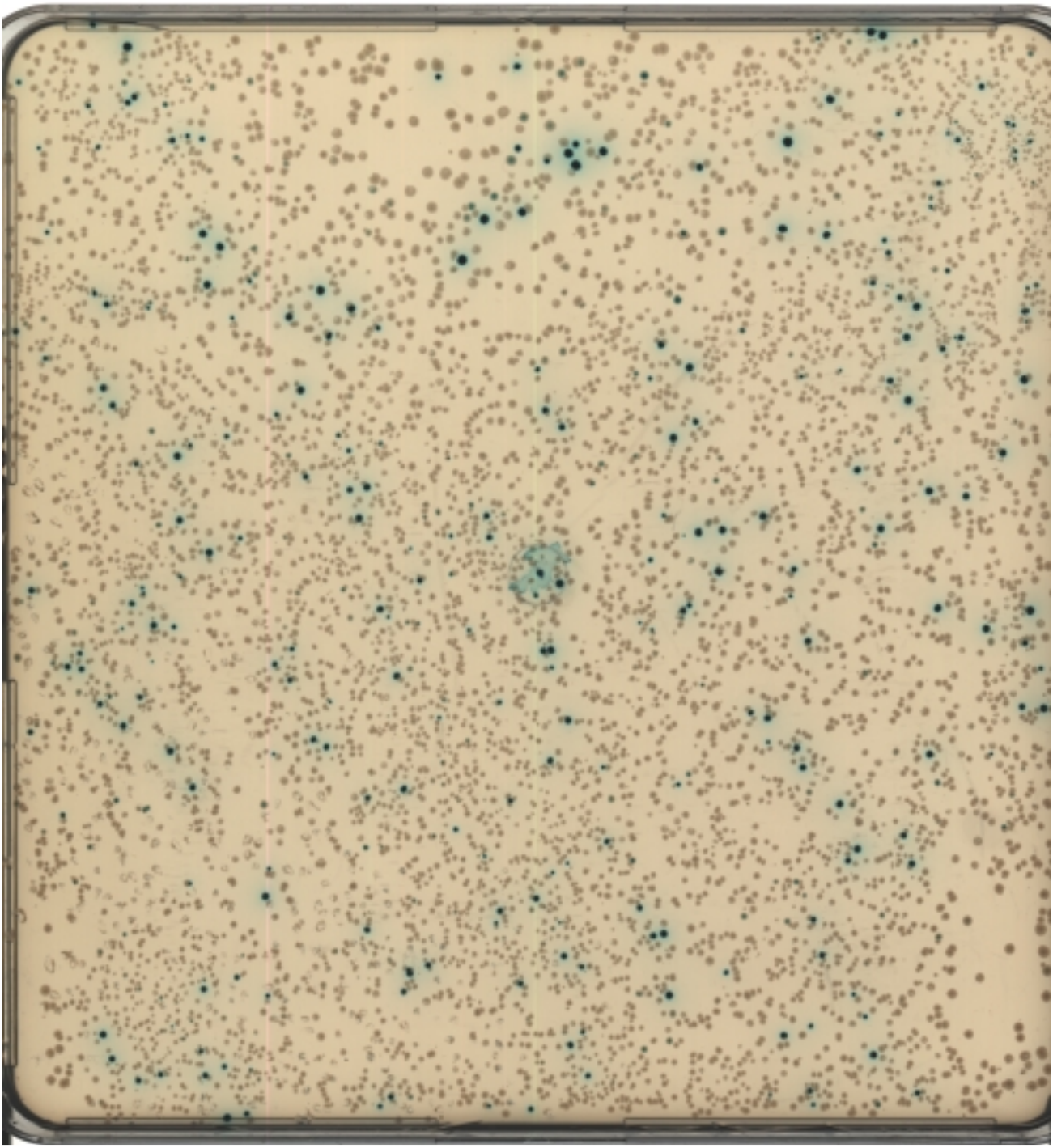


FIGURE 15.3 - Image of an X/I/C tray containing *Gossypium raimondii* BAC clones. The image was obtained by scanning the plate using a desktop scanner. Consequently, there is some color distortion (in particular, white colonies appear brown). The tray contains approximately 3500 clones. About 7% of the clones are blue. The size of the image has been reduced so that it can fit on a single page.

CHAPTER 16
Picking clones

PURPOSE

Once the library (or a portion of the library) has been plated, clones are picked and placed into freezing media in microtiter plates to create an ordered BAC library (see ordered libraries). Each suitable white colony is placed into a single well of a microtiter plate either by hand (using sterile toothpicks) or with an automated picking system.

PREFACE

After clones have been plated and allowed to form colonies, it is important to pick and store clones within the next 14 days. To produce an ordered library, white colonies are transferred from an X/I/C plate into freezing medium in the wells of a microtiter plate. The inoculated microtiter plates are incubated overnight. The 'master copy' of the library is then used as a template for producing replicate copies of the library (see CHAPTER 17).

Clones can be picked by hand using sterile toothpicks or by an automated colony picker. As discussed in CHAPTER 2, there are several companies that currently make colony-picking robots. We use the Genetix QBot for picking as well as other library-related tasks. However, less expensive picking instruments are available, and there are several companies and non-profit organizations that offer picking/replicating/gridding services (see CHAPTER 2). While an in-depth discussion of robotic colony pickers is beyond the scope of this paper, an overview of the principles behind automated picking is presented in the section below.

EXPERIMENTAL PROCEDURES

I. PREPARING MICROTITER PLATES

SUPPLIES, EQUIPMENT, AND REAGENTS (see CHAPTER 2 *for details)*: Freezing medium; sterile 384-well or 96-well microtiter plates; a multi-channel pipettor, a repeat pipettor, or an automated plate filling system (*e.g.*, the Genetix QFill2)

METHODS:

1. Fill sterile 96-well or 384-well plates with freezing medium. If 96-well plates are used, place 150 µl in each well. If 384-well plates are used, place 60 µl of freezing medium in each well. Plates can be filled with hand-held pipettors (preferably multi-channel pipettors or repeat pipettors). However, the easiest and fastest way to fill plates is using an automated plate-filling device such as the Genetix QFill2 (use of the QFill2 is illustrated in APPENDIX C). The number of plates that should be filled will depend upon the desired genome coverage and the number of clones available.
2. Label each plate with regard to organism/genotype, date of library construction, quality designation, and plate number. Plate labels should be placed on the edge of the plate at the "frosted end". An example of a plate-labeling scheme is shown in FIGURE 16.1.
3. Once microtiter plates containing sterile freezing medium have been prepared, picking can

begin. Two methods of picking clones are discussed in section **II** below.

II. CLONE SELECTION

A. PICKING CLONES BY HAND

SUPPLIES, EQUIPMENT, AND REAGENTS (see CHAPTER 2 *for details)*: X/I/C trays with clones (see CHAPTER 15); sterile (autoclaved toothpicks) or a hand-held colony picker; 384-well or 96-well plates filled with freezing medium (see **I** above)

METHODS:

1. Place 10-20 filled and labeled microtiter plates, a box of sterile toothpicks (or a hand-held colony picker; see CHAPTER 2 for details), and one of the X/I/C trays in a sterile laminar flow hood.
2. Wash hands with soap and water. Spray hands with 70% ethanol and wipe dry using a Kimwipe.
3. Open the box of sterile toothpicks, and take the lids off the first microtiter plate and the X/I/C tray.
4. Pick up a sterile toothpick by one of its ends. Carefully stab one of the white colonies on the X/I/C tray with the other end of the toothpick. Make sure that the toothpick only touches one clone.
5. Place the toothpick in one of the wells of the microtiter plate. Leave the toothpick in this well so that there is no doubt that this well has been inoculated (FIGURE 16.2).
6. Repeat steps 4 and 5 until all wells in one row have been inoculated (*i.e.*, all wells contain a toothpick). Carefully remove the toothpicks one at a time from the plate (removal of several toothpicks at once increases the likelihood of cross-contamination between wells). Place the "used" toothpicks in a biohazard bag or autoclave them for re-use.
7. Continue until all of the wells of the plate have been inoculated. Place the lid back on the microtiter plate and set it in the corner of the hood away from the other microtiter plates.
8. Continue the process until you have filled all of the microtiter plates necessary to meet your desired genome coverage and/or you have picked all the useable clones off of your X/I/C plates.

B. AUTOMATED COLONY PICKING

SUPPLIES, EQUIPMENT, AND REAGENTS (see CHAPTER 2 *for details)*: X/I/C trays with clones (see CHAPTER 15); sterile (autoclaved toothpicks); 384-well or 96-well plates filled with freezing medium (see **I** above); a QBot (Genetix) or a similar high-throughput genomics robot

METHODS: Most automated colony picking robots have a similar mode of operation. In general, one or more X/I/C trays are placed in the machine. Lids are removed from the trays. A camera system scans the trays, and the robot's internal computer system uses various algorithms to determine what objects scanned by the camera meet the characteristics of a white colony. The computer's decisions are based upon the relative roundness of an object, its proximity to other objects, and its color relative to the background. The parameters governing the robot's decisions can be adjusted by the user. Once the tray(s) has been scanned and clones have been selected for

picking by the computer, a picking head (composed of a series of pins/needles) is positioned by a robotic arm over the surface of the plate. Each pin is fired into one of the "pre-selected" colonies. After all the pins in the head have been fired, the picking head is moved into a position over an open microtiter plate containing freezing medium. The pins are used to inoculate wells on the plate. The pins are then sterilized and the picking cycle is repeated until the microtiter plate has been completely inoculated. A diagram of the Genetix QBot is shown in APPENDIX D. An overview of how the QBot picks clones is given in APPENDIX E.

III. PLATE INCUBATION

SUPPLIES, EQUIPMENT, AND REAGENTS (see CHAPTER 2 *for details)*: sterile (autoclaved toothpicks); inoculated microtiter plates (see **II** above)

METHODS:

1. Carefully wrap stacks of the inoculated microtiter plates with plastic wrap. These plates constitute the master copy of the library. Place the plates in a 37ºC incubator for 14-16 hours.

✸ Note 16.1: Use of a water-jacketed incubator prevents evaporation from the plates and thus eliminates the need for plastic wrap.

2. Remove the master plates from the incubator. For a given plate, the media in each well should appear turbid due to bacterial growth. If the plates were prepared using an automated picker, it is probable that some of the plates will contain a well or two in which no bacteria have grown (possibly due to a bent or broken pin). If desired, these empty wells can be inoculated by hand and the plates can be placed back into the incubator for an additional 12 hours. Plates without any empty wells should be re-wrapped in plastic and left at room temperature.

✸ Note 16.2: Do not leave plates at room temperature for more than two days. Proceed with replication (CHAPTER 17) as soon as possible.

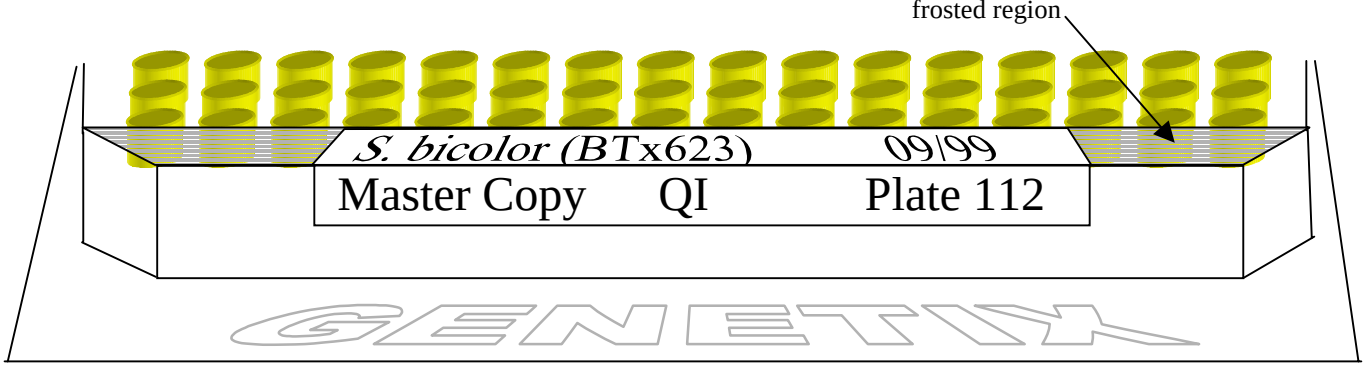


FIGURE 16.1 - Labeling microtiter plates. Each plate should be labeled with information regarding its contents and origin. Traditionally each label is placed on the "frosted" end of the plate. Labels should be uniform in format. In the example above, the species (S. bicolor = *Sorghum bicolor*), the cultivar/genotype (BTx623), and the date that the library was made (09/99 = September 1999) are listed on the horizontal surface of the label. Copy information (Master Copy), a "quality" designation (QI), and the plate number (Plate 112) are given on the vertical surface of the label. The "quality" (Q) of the copy is based upon how far removed it is from the master copy. For example, the master copy itself has the highest quality and is given the designation QI. A library generated using the master copy as a template would be labeled QII (usually Copy 1 and Copy 2 of the library are directly made from the master copy). A copy made from a QII library would be labeled QIII, *etc*.

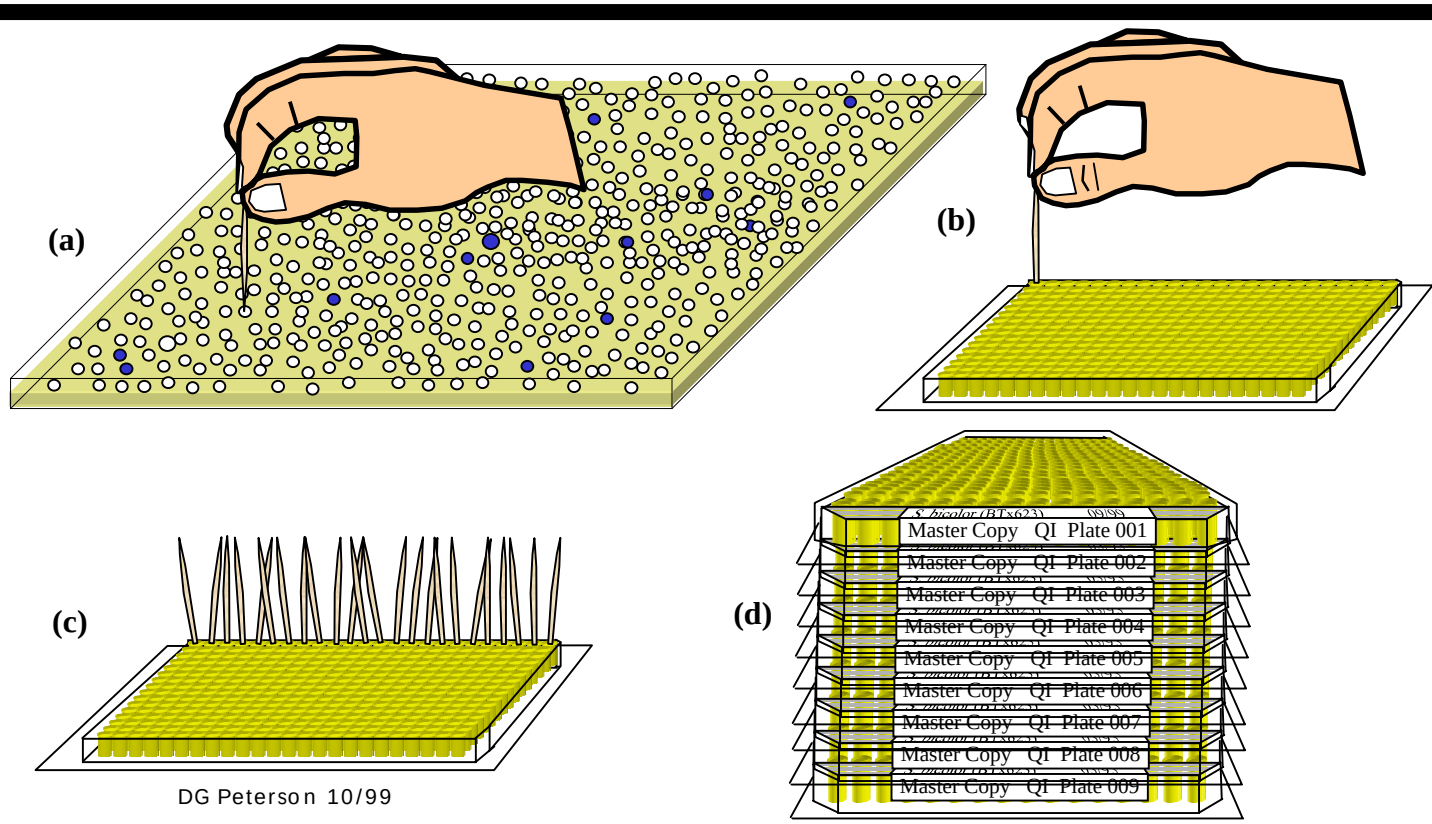


FIGURE 16.2 - Hand-picked libraries. **(a)** A sterile toothpick is used to stab a white colony on an X/I/C plate. **(b)** The end of the toothpick containing the colony is then inserted into the first well of a microtiter plate containing freezing medium. The toothpick is left in the well so that the well is not accidentally inoculated twice. **(c)** Other clones (on toothpicks) are placed in the remaining wells of the first row. Once the first row has been completely inoculated, toothpicks are removed one at a time from the wells. The other rows of the plate are inoculated in this manner until the plate has been completely inoculated. The lid is placed back on the plate, and the plate is set aside. **(d)** Inoculated plates are stacked in columns and incubated at 37°C overnight.

CHAPTER 17
Library replication & storage

PURPOSE

Once the master copy of the library has been made, it is important to make one or two replicates of the master as quickly as possible. Replication can be performed using a hand-held replicator or an automated plate replicator (*e.g.*, a QBot). Once replicates have been made, the master copy can be stored at –80°C indefinitely. The Quality I (QI) replicates can then be used to make Quality II (QII) copies of the library, and QII copies can be used to make QIII working copies of the library (see quality designations for a discussion of the meaning of QI, QII, QIII, *etc*.). Once QIII copies have been made, the QI and QII copies are stored at –80°C as well. QIII plates are used to make BAC grids to which DNA probes of interest can be hybridized.

EXPERIMENTAL PROCEDURES

I. PREPARING MICROTITER PLATES

SUPPLIES, EQUIPMENT, AND REAGENTS (see CHAPTER 2 *for details)*: Freezing medium; sterile 384-well or 96-well microtiter plates; a multi-channel pipettor, a repeat pipettor, or an automated plate filling system (*e.g.*, the Genetix QFill2)

METHODS: For every microtiter plate in the master copy of the library, fill one or two microtiter plates with sterile freezing medium (see CHAPTER 16). Whether you prepare one or two plates depends on whether you want to make one or two QI copies of the library. Label the plates appropriately (see FIGURE 16.1).

II. REPLICATION

A. REPLICATION USING A HAND-HELD REPLICATOR

SUPPLIES, EQUIPMENT, AND REAGENTS (see CHAPTER 2 *for details)*: 384-well or 96-well microtiter plates filled with sterile freezing medium; a hand-held, reuseable plate replicator; the master copy of the library (CHAPTER 16)

METHODS:

✸ Note 17.1: Perform replication in a sterile laminar-flow hood.

1. Sterilize the replicator.
    (a) If a stainless-steel replicator is used, dip the pins of the replicator into a 70% ethanol bath. Remove the replicator from the alcohol bath. Ignite the residual alcohol on the pins by passing the replicator through the flame of a Bunsen burner. Allow the pins to cool for two minutes.
    (b) If a plastic (polypropylene) replicator is used, wash the pins thoroughly in 12% v/v bleach. Rinse the pins thoroughly with MBG water followed by a quick rinse in 95% ethanol. Allow the pins to air dry.

2. Perform replication as shown in FIGURE 17.1.
3. Repeat steps 1-2 until all of the plates in the master copy have been replicated either once or twice.
4. Proceed to **III**.

B. REPLICATION USING AN AUTOMATED REPLICATOR

SUPPLIES, EQUIPMENT, AND REAGENTS (see CHAPTER 2 *for details)*: 384-well or 96-well microtiter plates filled with sterile freezing medium; a QBot (Genetix) or a similar high-throughput genomics robot; the master copy of the library (CHAPTER 16)

METHODS: Plate replication using a high-throughput genomics robot is based on the same principles as manual plate replication (see FIGURE 17.1). However, a robot rather than a human(s) performs plate manipulation and inoculation tasks. An overview of how the Genetix QBot replicates BAC libraries is given in APPENDIX F. Once replication is complete, proceed to **III** below.

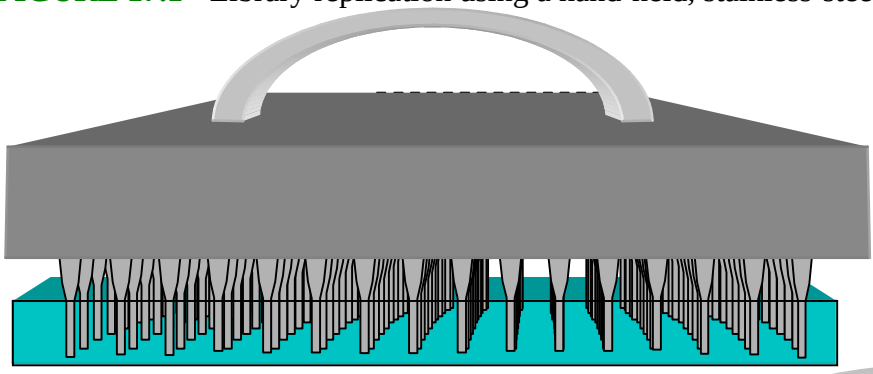
III. LIBRARY STORAGE

SUPPLIES, EQUIPMENT, AND REAGENTS (see CHAPTER 2 *for details)*: the master copy of the library; any other copies of the library that are ready for long-term storage; library storage boxes

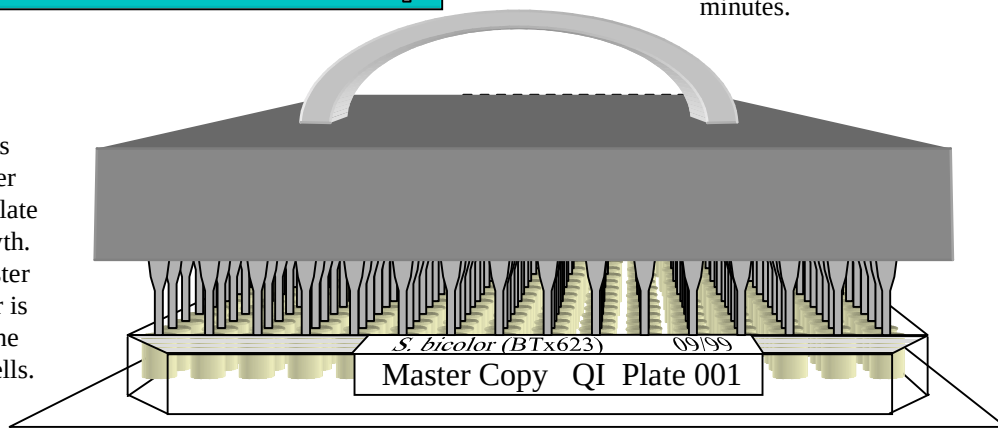
METHODS:

1. Arrange the microtiter plates containing the master copy of the library in stacks of nine plates. For simplicity, place plates 1-9 (from top to bottom) in the first stack, plates 10-18 in the second stack, *etc*. (FIGURE 17.2a).
2. Placing plates in storage boxes:
    (a) If cardboard storage boxes are being used, carefully bind each stack of plates with two rubberbands as shown in FIGURE 17.2a. Be careful not to sharply jar or tilt the plates as this will result in cross-well contamination. Place the first four stacks of plates (*i.e.*, plates 1-36) in a library storage box as shown in FIGURE 17.2b. Close and label the box as shown (FIGURE 17.2c). Place the next four stacks of plates in a second box and label accordingly, *etc*.
    (b) If metal storage boxes are being used, load plates into the metal boxes as shown in FIGURE 17.3.
3. Once the master copy of the library has been placed into storage boxes, place the boxes containing the master copy into a –80°C freezer.
4. Repeat steps 1-3 for all library copies that you want to place in long-term storage.

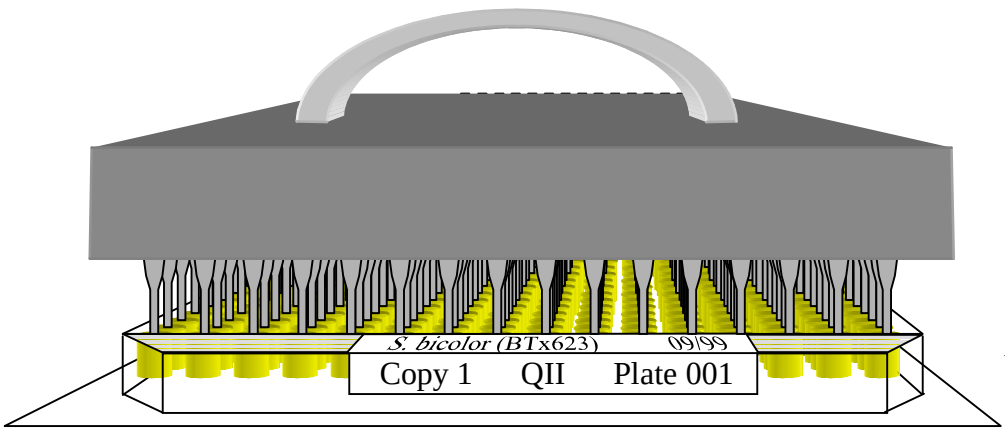
FIGURE 17.1 - Library replication using a hand-held, stainless-steel replicator.



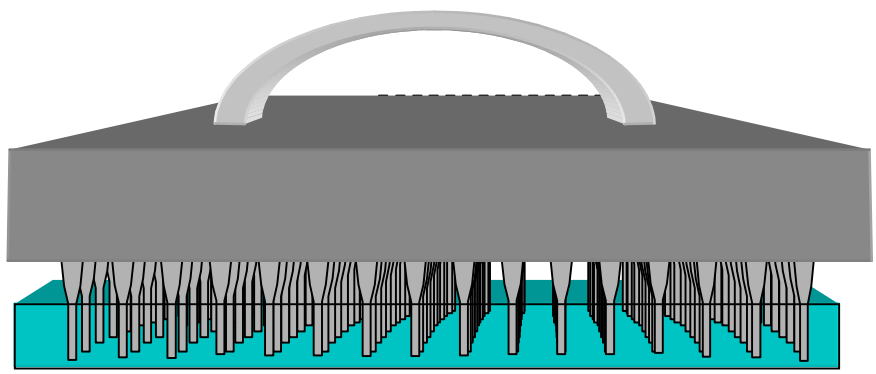
(a) The stainless-steel replicator is placed in a 70% ethanol bath. The replicator is gently moved back and forth through the ethanol to ensure that the pins are thoroughly cleaned. The replicator is removed from the ethanol bath, and the residual ethanol on the pins is ignited by quickly passing the replicator through the flame of a Bunsen burner. Once the alcohol on the pins has burned away, the replicator is allowed to cool for about 2 minutes.



(b) In a laminar-flow hood, the lid is removed from Plate 001 of the master copy. The media in each well of the plate should be turbid due to bacterial growth. The replicator is aligned with the master plate so that each pin of the replicator is directly over an appropriate well. The replicator pins are inserted into the wells.



(c) The replicator is inserted into the wells of a plate containing sterile freezing medium. This newly-inoculated plate should be labeled "Copy 1, Plate 001", and the replicator should be inserted into the plate in exactly the same orientation as it was inserted into the master plate. The lid of Copy 1, Plate 1 is replaced, and the plate is set aside.



(d) The replicator is re-sterilized as described above.

DG Peterson 10/99

(e) The replicator is placed back into Plate 1 of the master copy. A second copy of Plate 1 is generated by using the replicator to inoculate the sterile freezing media in the wells of a second plate designated "Copy 2, Plate 001".

(f) Steps a-e are repeated until each plate of the master copy has been twice replicated (*i.e.*, there are three complete copies of the library).

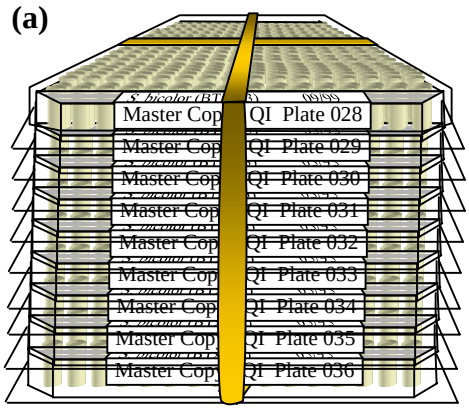
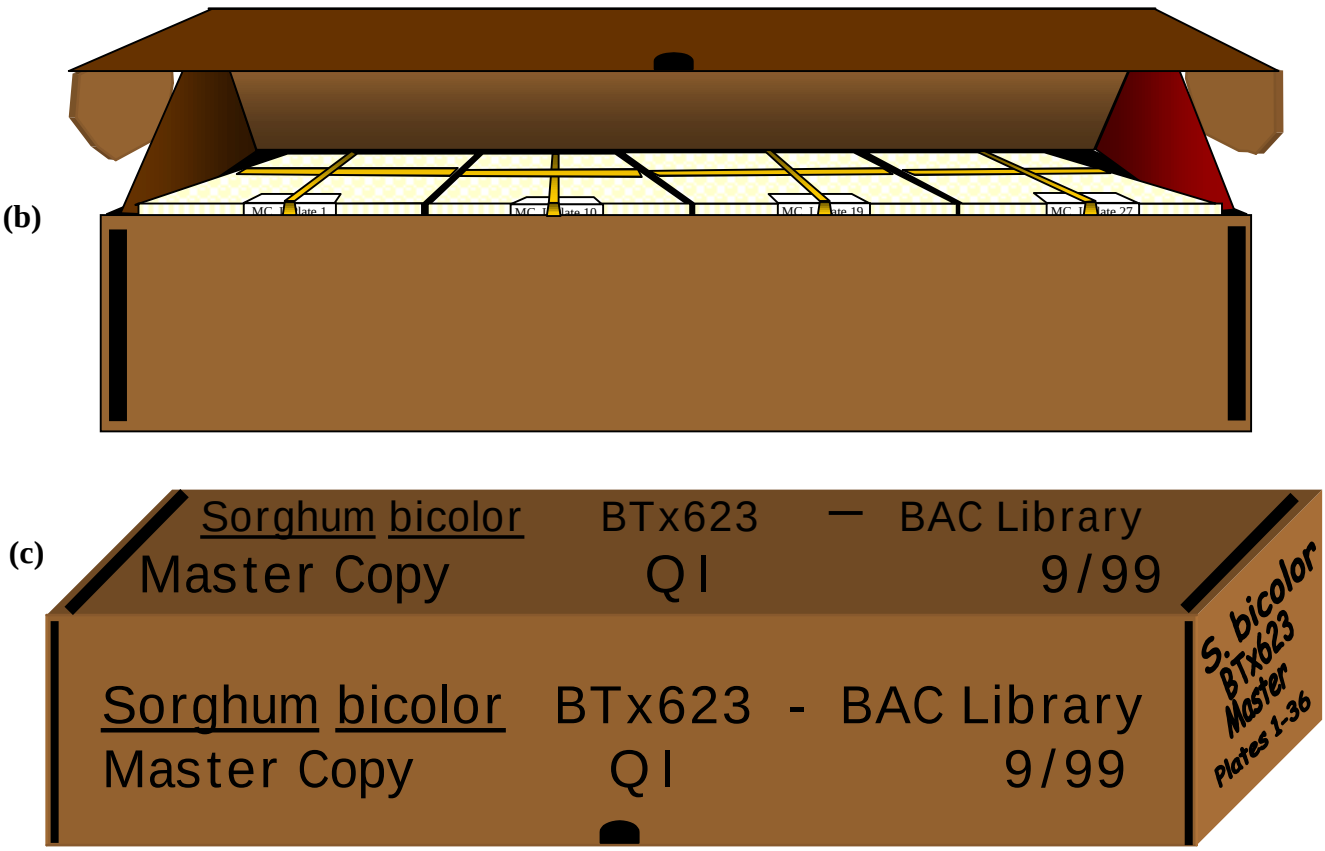
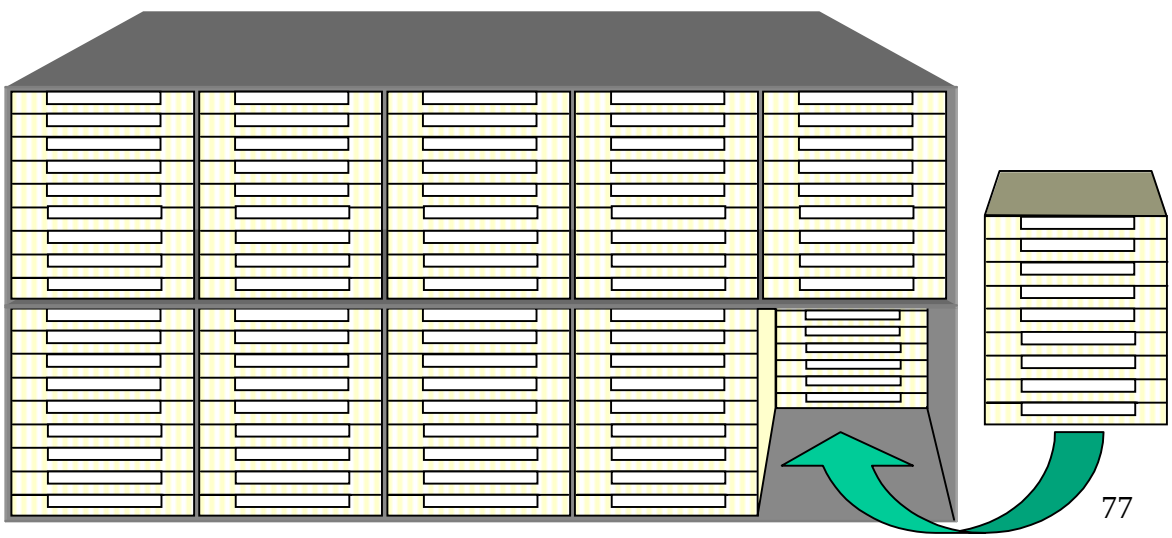


FIGURE 17.2 - Long-term storage of BAC libraries in cardboard boxes. Boxes are 39.5 x 14.7 x 11.6 cm. Once replication of QII plates is finished, the master copy of the library is ready for long-term (and presumably permanent) storage at -80°C. (a) Stack the microtiter plates of the master copy in columns nine plates high. Arrange the plates in a column in ascending numerical order from top to bottom. Secure each column of plates with two rubber bands as shown (BE CAREFUL NOT TO JAR OR SHARPLY TILT PLATES). (b) In the first cardboard storage box, place four columns of plates (in particular, the columns containing plates 1-9, 10-18, 19-27, and 28-36). Close the box. (c) Label the box on top-, front-, and side-panels. Place the next 36 consecutive plates in a second library storage box as described above. Continue the process until all of the library plates are in library storage boxes. Place the labeled library storage boxes containing the master copy of the library in a -80°C freezer.



DG Peterson 10/99

FIGURE 17.3 - Long-term storage of BAC libraries in metal storage boxes. Each box is 56.5 x 22.5 x 13.7 cm and has a shelf that divides it into upper and lower compartments. Each compartment can hold 90 microtiter plates, *i.e.*, a container can hold 180 plates total.



APPENDIX A

Using the Gibco BRL Cell-Porator System

SUPPLIES, EQUIPMENT, AND REAGENTS (see CHAPTER 2 *for details)*: 70% ethanol; DH10B competent cells; vector/insert ligation reaction (see CHAPTER 5, CHAPTER 14, and CHAPTER 15); Gibco BRL CELL-PORATOR® Electroporation System (cat. no. 11609-013); Gibco BRL CELL-PORATOR® Voltage Booster (cat. no. 11612-017); disposable CELL-PORATOR® cuvettes

METHODS:

1. Fill the chamber safe with ice (FIGURE A.1). Turn on the power supply and the voltage booster. Make sure the voltage booster resistance is set on 4000 ohms (4 kΩ). On the power supply, the capacitance should be set to 330 µF, the charge controller should be set to "Charge", the trigger speed should be set to "Fast", and the impedance toggle should be set on "Low Ω".
2. Thaw one tube of DH10B competent cells for every ten microliters of ligation reaction.
3. Make up a transformation reactions by mixing 2.0 µl of ligate with 20 µl of competent cells in a 0.65 ml microcentrifuge tube. Tap tubes gently to mix contents.
4. Using a pipettor equipped with a standard 200 µl tip, transfer the mixture from a tube to an electroporator cuvette. Place the mixture directly between the two electrode knobs of the cuvette (FIGURE A.2). Make sure that there are no bubbles in the ligate/bacterial solution. Close the cuvette.
5. Repeat step 5 until all transformation reactions are loaded into cuvettes.
6. Place the first four loaded cuvettes into the cuvette holder. Place the cuvette holder into the ice-filled chamber safe so that each cuvette is surrounded by ice (FIGURE A.3).
7. Close and lock the lid to the chamber safe. Insert the power-out plug into the plug inlet in the lid of the chamber safe (FIGURE A.4). Turn the cuvette dial on the chamber safe lid so that it is pointing to the number "1". The cuvette lying under the number "1" on the lid is ready for electroporation.
8. With the charge controller set on "CHARGE", push the "UP" button until a voltage of 330 appears in the LED display.
9. Flip the charge controller to the "ARM" position. Allow the voltage to drift down to approximately 325 v. Press the red "TRIGGER" button. The voltage will drop to zero (or thereabouts) as the bacteria in cuvette number one are exposed to a burst of electricity.
10. Flip the charge controller back to the "CHARGE" position. Turn the cuvette dial so that it is pointing to the number "2". Repeat steps 9-10 until all four cuvettes in the chamber safe have been shocked.

✸ Note A.1: Be careful not to shock cells in a cuvette more than once.

11. Remove the power plug from the lid of the chamber safe. Unlock and open the chamber safe. Gently remove the cuvettes and place them on ice.

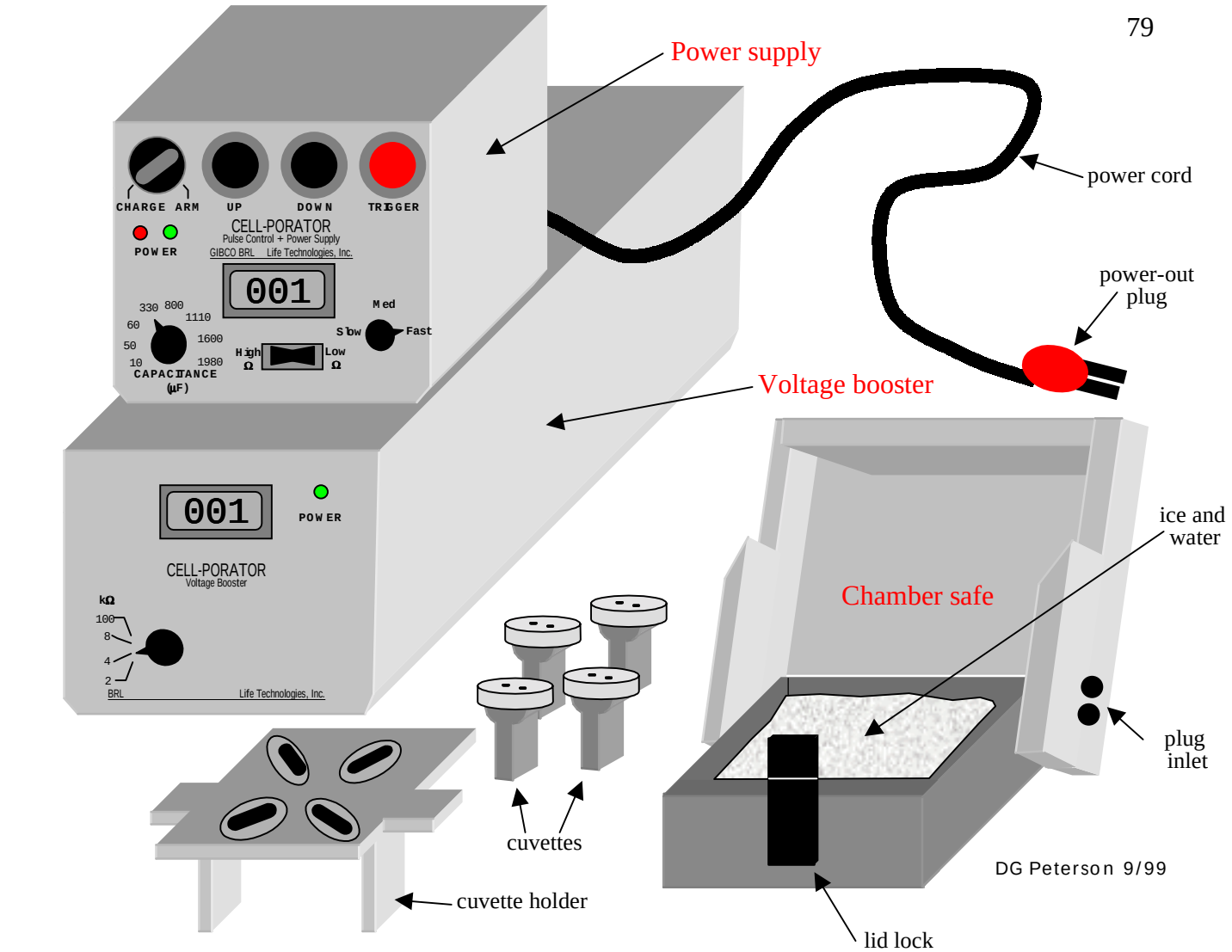


FIGURE A.1 - The components of the Gibco BRL Cell-Porator.

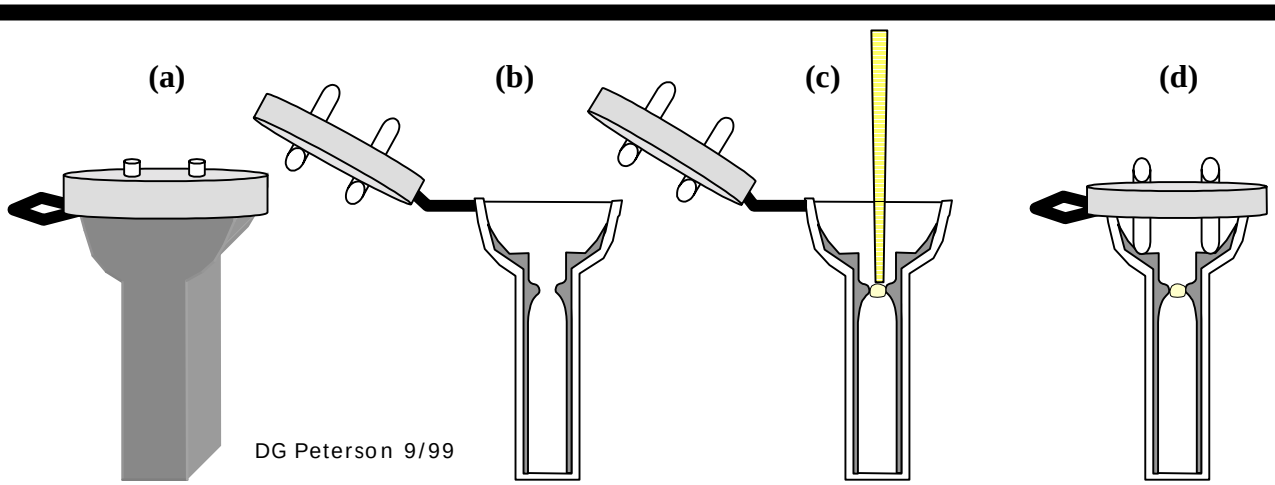


FIGURE A.2 - Loading an electroporator cuvette. **(a)** A cuvette shown from the front. To facilitate understanding of the process of cuvette loading, the front plastic of the cuvette has been made transparent in **b-d**. **(b)** The inner left and right sides of the cuvette are covered by unconnected metal liners. Each liner has a distinctive knob protruding inwards directly opposite the knob on the other liner. **(c)** A combination of competent cells and ligate are placed between the two knobs. **(d)** Closing the lid of the cuvette brings each metal liner into contact with one of the two electrode pins in the lid.

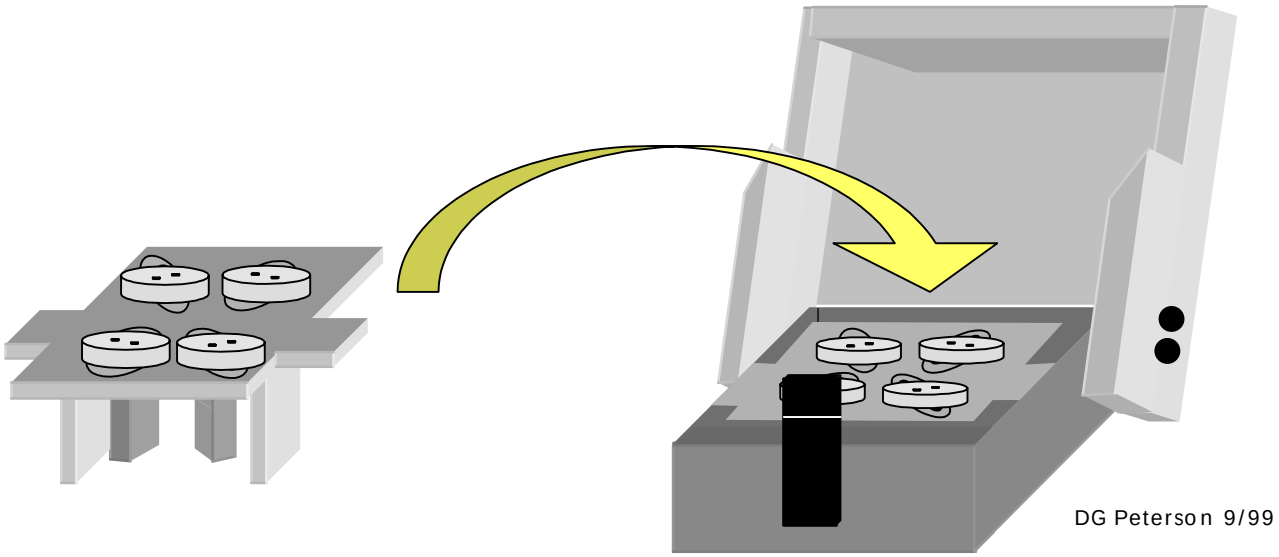


FIGURE A.3 - Loading the chamber safe. Four cuvettes loaded with transformation mixture are placed into the cuvette holder. The filled holder is then gently inserted into the ice-filled chamber safe.

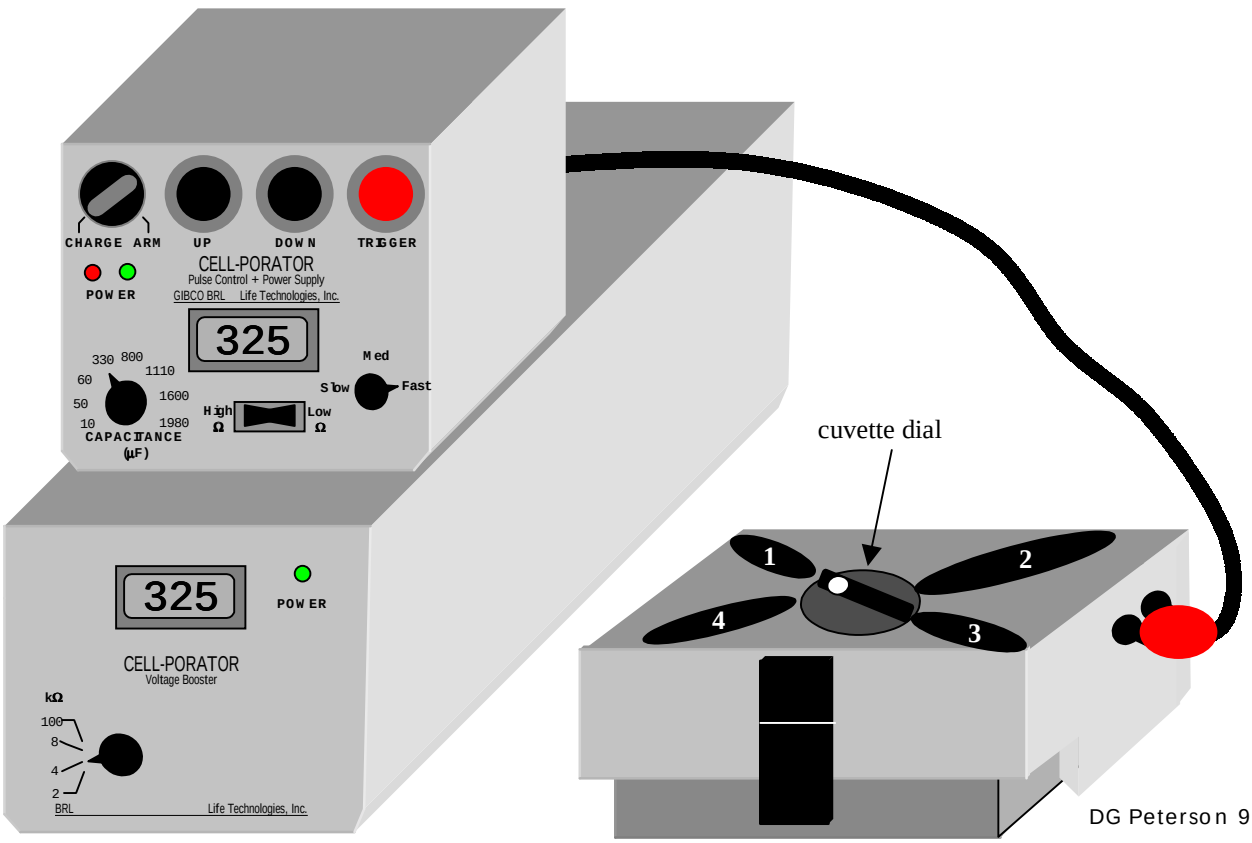


FIGURE A.4 - Electroporation. The lid to the chamber safe is closed and locked, and the power-out plug is inserted into the appropriate holes in the lid of the chamber safe. The cuvette dial on the lid of the chamber safe is turned to "1". A voltage is generated in the power unit. Once a desired voltage is obtained, the "TRIGGER" button is pushed, and the voltage is released into the wires of the power cord, through the power-out plug, into the pins in the lid of cuvette "1", and through the opposed metal liners along the sides of the cuvette. The voltage is eliminated by the passage of current through the path of least resistance (in this case, the transformation mixture). The current applied to the competent bacteria temporarily permeablilizes their cell membranes allowing BACs to enter the cells.

APPENDIX B

Using the Bio-Rad Model 422 Electroelution System

SUPPLIES, EQUIPMENT, AND REAGENTS (see CHAPTER 2 *for details)*: agarose cubes *q*, *r*, and *s* containing size-selected DNA (see CHAPTER 13); 1X TAE; 70% ethanol (in a spray bottle); Miracloth squares; BioRad Model 422 Electro-Eluter (cat. no. 165-2976) electroelution system; BioRad PowerPac 1000 power supply (cat. no. 165-5054)

METHODS:

1. Spray down all plastic and glass components of the electroeluter system with 70% ethanol (FIGURE B.1). Rinse these items with distilled water.
2. Pick out three assembly joints and place them on the Parafilm so that the large orifice of each is pointing up. Place a collection cup under each assembly joint and gently fit the collection cup into the small (dorsal) orifice of the assembly joint (FIGURE B.2).
3. Place a 2" x 10" piece of Parafilm on a clean countertop. Place assembly joints, glass tubes, collection cups, and the cuvette rack on top of the Parafilm. Add 1X TAE to each of the three assembly joint/collection cup combinations until a reverse meniscus is visible at the top of each assembly joint. Gently place a piece of sterile Miracloth onto the reverse meniscus of each assembly joint (FIGURE B.2). Insert a glass tube (frosted side down) into each of the three assembly joints. The three resulting structures are referred to as electroelution cuvettes (see FIGURE B.2).
4. Fill each cuvette with 1X TAE. In the first cuvette place one of the three *q* agarose blocks from CHAPTER 13. Likewise, place an *r* agarose block in the second cuvette and an *s* block in the final cuvette. Let agarose blocks drift through the buffer until they come to lie on the Miracloth stretched across the bottom of the glass tubes. If a block does not naturally drift to the Miracloth boundary, gently use a glass rod to push it to this interface. If the cube is too wide to fit in a cuvette, do not force it through the cuvette. Rather, trim the cube until it will fit in the cuvette.
5. Insert each cuvette into a gasket of the cuvette holder as shown in FIGURE B.3. Place gasket plugs in gaskets that are not being used.
6. Place the cuvette holder into the main buffer tank (FIGURE B.4). Add 1X TAE to the main buffer tank until each assembly joint on the electroelution cuvettes is completely submerged.
7. Fill the upper buffer chamber with 1X TAE. Each cuvette should be full of buffer (FIGURE B.4).
8. Place a stir bar in the bottom of the main buffer tank, and set the apparatus on a stir plate. Activate the stir bar so that the buffer in the main tank is gently agitated.
9. Place the lid on the main buffer tank. Make sure that the red (positive) electrode is next to the red dot on the side of the main buffer tank and that the black electrode is next to the black dot on the main buffer tank (FIGURE B.4).
10. Insert the electrode plug at the end of the electrode wires into an appropriate power supply. Make sure that the red electrode is plugged into the red socket and that the black electrode is

plugged into the black socket.

11. In general, each cuvette requires 10 mA of power. Thus if three cuvettes are used, the power should be set at 30 mA. If the cuvettes are properly loaded, the voltage should start off at about 80-100 v. Higher voltages indicate possible problems with the cuvettes (*e.g.*, trapped air bubbles). Periodically check the voltage. If voltages > 110 are seen upon starting the apparatus, turn off the power supply, remove the main buffer tank lid, and check to see that the agarose cubes are resting on the Miracloth stretched across the bottom of the glass tubes. Dislodge any bubbles trapped below the agarose cubes or otherwise present in the cuvettes. If the voltage drifts to > 130 v over the course of an hour, decrease the amperage until the voltage is between 80-100 v.
12. Run the electroeluter for 2 hours.
13. Recover electroeluted DNA for *q*, *r*, and *s* blocks as shown in FIGURE B.5. TAKE GREAT CARE IN PERFORMING THIS STEP.
14. Place *q*, *r*, and *s* DNA samples in their own 1.5 ml microcentrifuge tubes on ice.

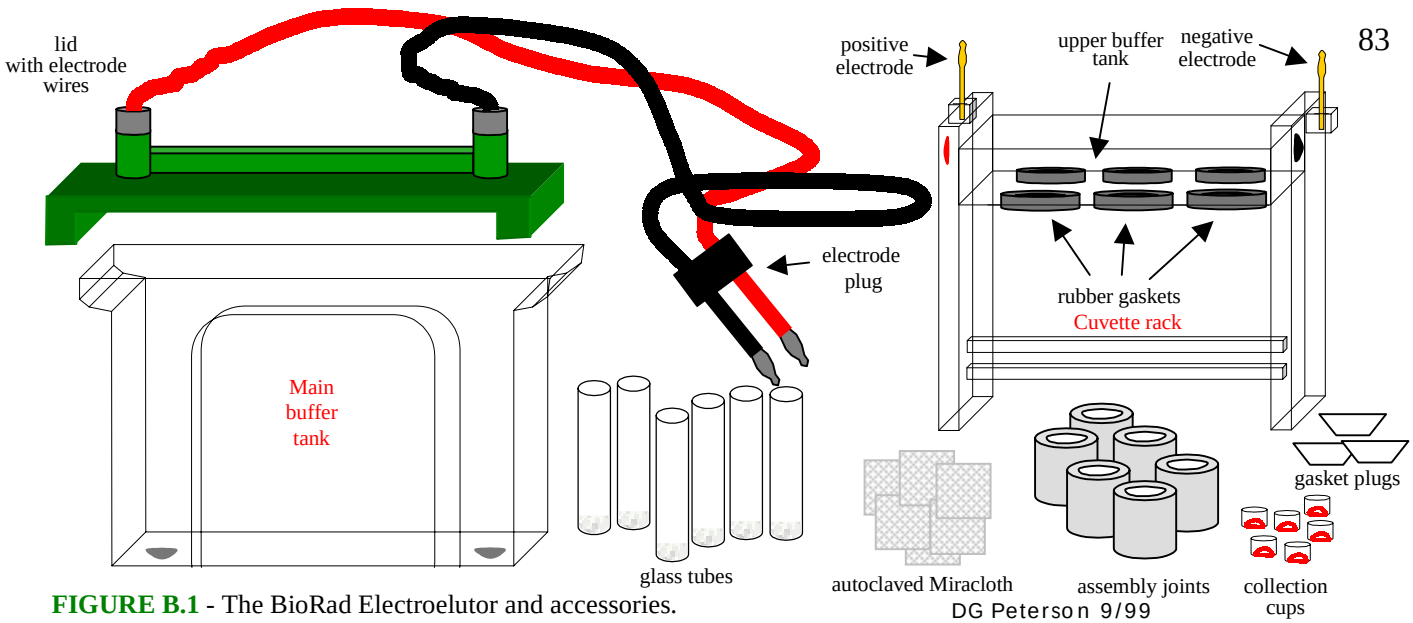


FIGURE B.1 - The BioRad Electroelutor and accessories.

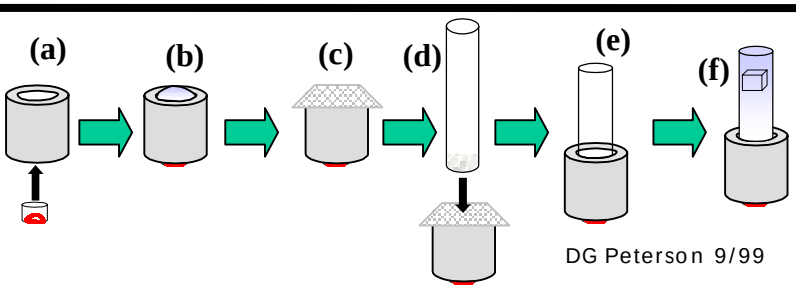
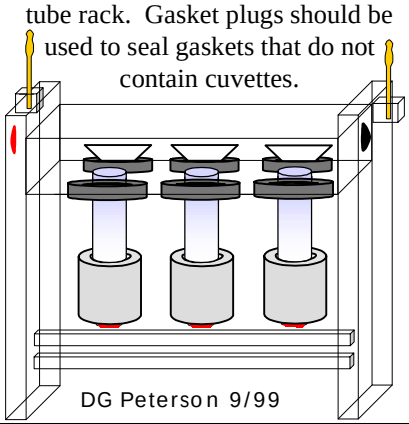


FIGURE B.2 - Assembly and loading of an electro-elution cuvette. **(a)** Insert a collection cup into the small aperture of an assembly joint. **(b)** Add 1X TAE to the cup/joint unit until a reverse meniscus is visible at the top of the joint. **(c)** Place a Miracloth square onto the reverse meniscus. **(d)** Insert the frosted end of a glass tube into the large aperture of the assembly joint. Push the tube in as far as it will go. The Miracloth should be stretched across the bottom of the glass tube. **(e)** The cuvette is now completely assembled and ready for loading. **(f)** Fill the cuvette with sterile 1X TAE buffer. Drop an agarose cube containing size-selected DNA into the cuvette. The cube should drift through the buffer until it rests on the Miracloth stretched across the bottom of the glass tube. Use a glass rod to gently push the cube to the bottom of the tube if it does not drift down by itself.

FIGURE B.3 - Each cuvette is inserted into a rubber gasket on the tube rack. Gasket plugs should be used to seal gaskets that do not contain cuvettes.



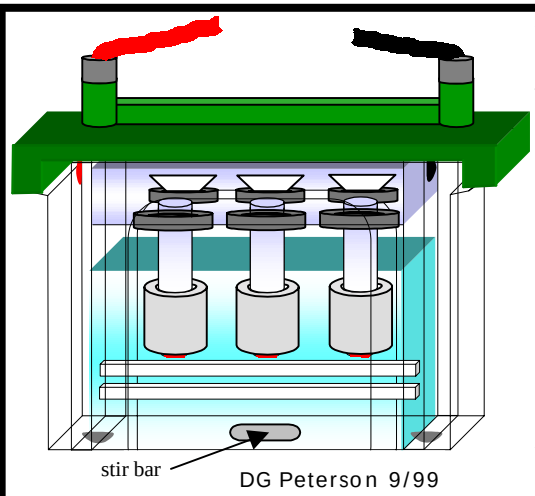
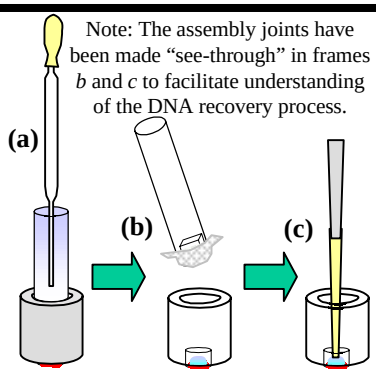


FIGURE B.4 - Electroelution. The cuvette rack is placed into the main buffer tank. 1X TAE is added to the main tank until the assembly joints are covered in buffer. Likewise, 1X TAE is added to the upper buffer tank. Using a stir bar, the buffer in the main tank is gently agitated. The electrode plug is inserted into the BioRad power source (not shown). In general, each cuvette requires 10 mA of power (*e.g.*, if three cuvettes are used, the power should be set at 30 mA). Perform electroelution for two hours (see APPENDIX B for details).

FIGURE B.5 - Recovery of DNA. Once electroelution is complete, lift the cuvette holder from the main buffer tank. Remove buffer in the upper buffer tank using a sterile Pasteur pipet. **(a)** Using the pipet, gently remove all buffer above the surface of the Miracloth at the bottom of each glass tube. PERFORM THIS STEP SLOWLY AND CAREFULLY! IF THE BUFFER IS ASPIRATED TOO RAPIDLY OR IF SUCTION IS APPLIED TO THE MIRACLOTH ITSELF, DNA WILL BE PULLED OUT OF THE COLLECTION CUP! **(b)** Remove a cuvette from the cuvette holder. Gently remove the glass tube, Miracloth, and agarose cube from the assembly joint. A droplet of liquid containing the DNA should be visible at the bottom of the collection cup. **(c)** Using a wide-orifice plastic pipet tip, slowly remove the DNA solution and place it in a sterile 1.5 ml microfuge tube. Approximately 0.4 ml of DNA solution should be recovered from each cuvette.

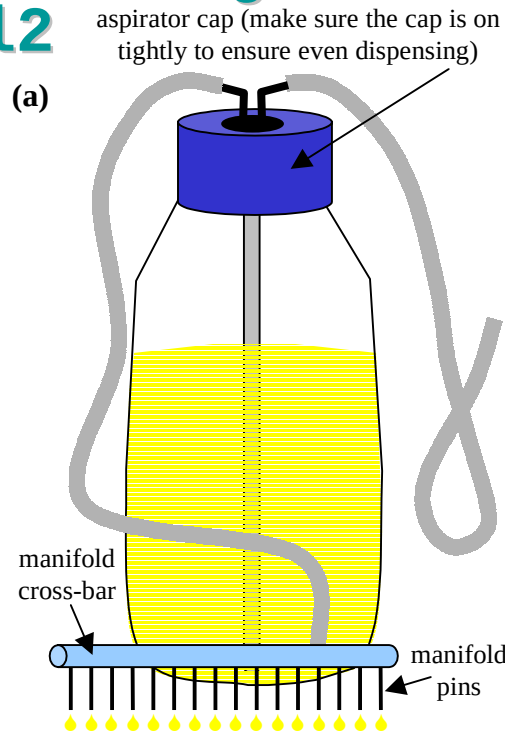


APPENDIX C

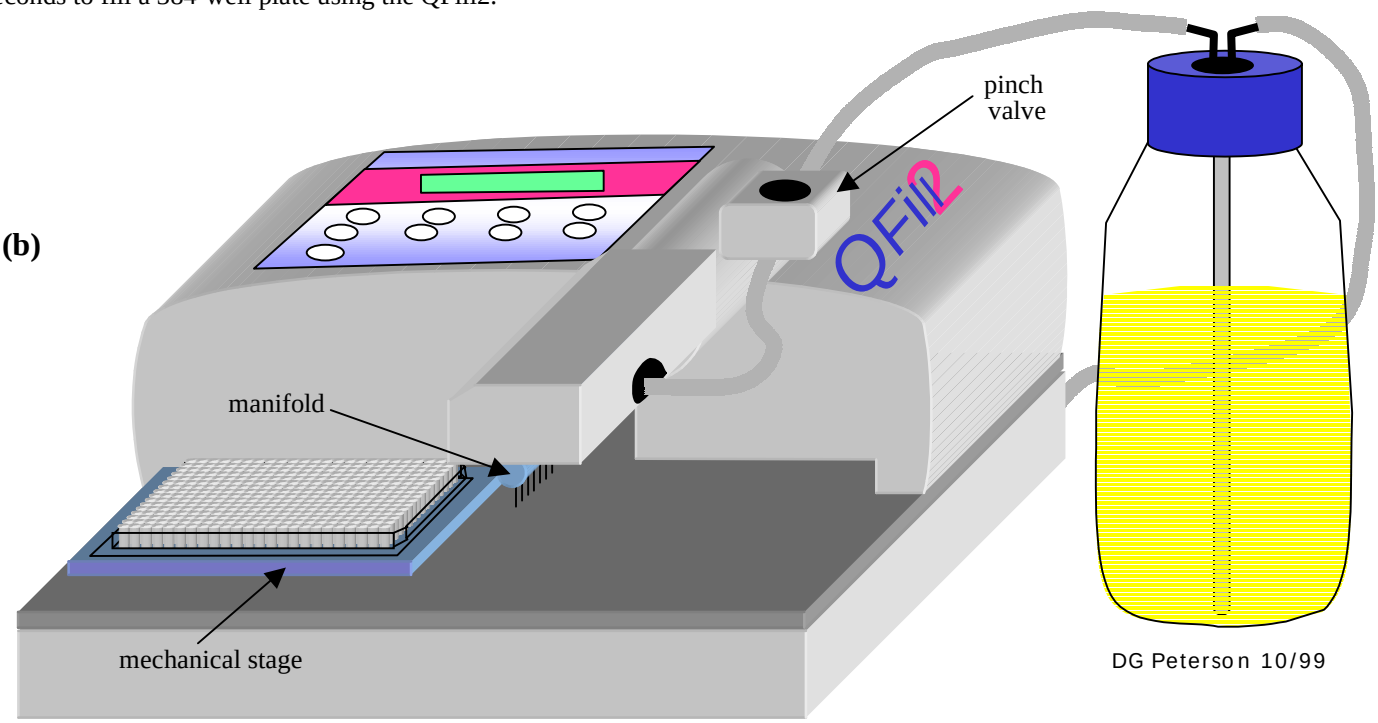
Filling 384-well plates using the Genetix QFill2

(a) A bottle of sterile freezing medium is connected to a 16-pin manifold *via* an aspirator cap and tubing. The pins on the manifold and the manifold cross-bar are hollow. Thus fluid pumped from the bottle into the manifold exits the manifold through the 16 manifold pins. **(b)** The manifold is placed into a holder in the QFill2. The tube attached to the manifold is fitted into a pinch valve located on the upper surface of the QFill2. The other tube is attached to an air-port on the back of the QFill2 creating a pressure in the bottle. A 384-well microtiter plate is placed on the mechanical stage of the QFill2. The plate lid is removed. **(b & c)** Pressing the start button of the QFill2 begins an entirely automated chain of events. The stage moves laterally (to the right) so that the first 16-well row is directly beneath the manifold. A specific volume of media (determined by the user when the unit is first turned on) is dispensed from each manifold pin into the well directly below it. The plate is then moved forward one row, and the second row is filled. This process is continued until all 24 rows of the plate are filled. After the last row of the plate is filled, the mechanical stage moves back to its original position on the left side of the manifold. The operator then can place a lid on the plate, set the filled plate aside, and place a new plate on the mechanical stage. It takes less than 20 seconds to fill a 384-well plate using the QFill2.

(a)

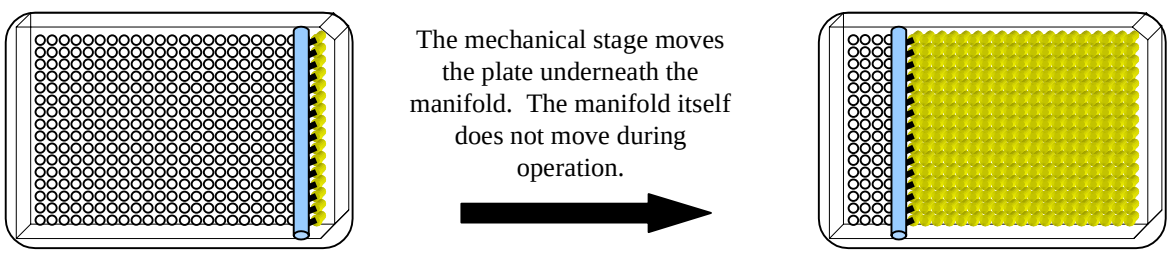


(b)



DG Peterson 10/99

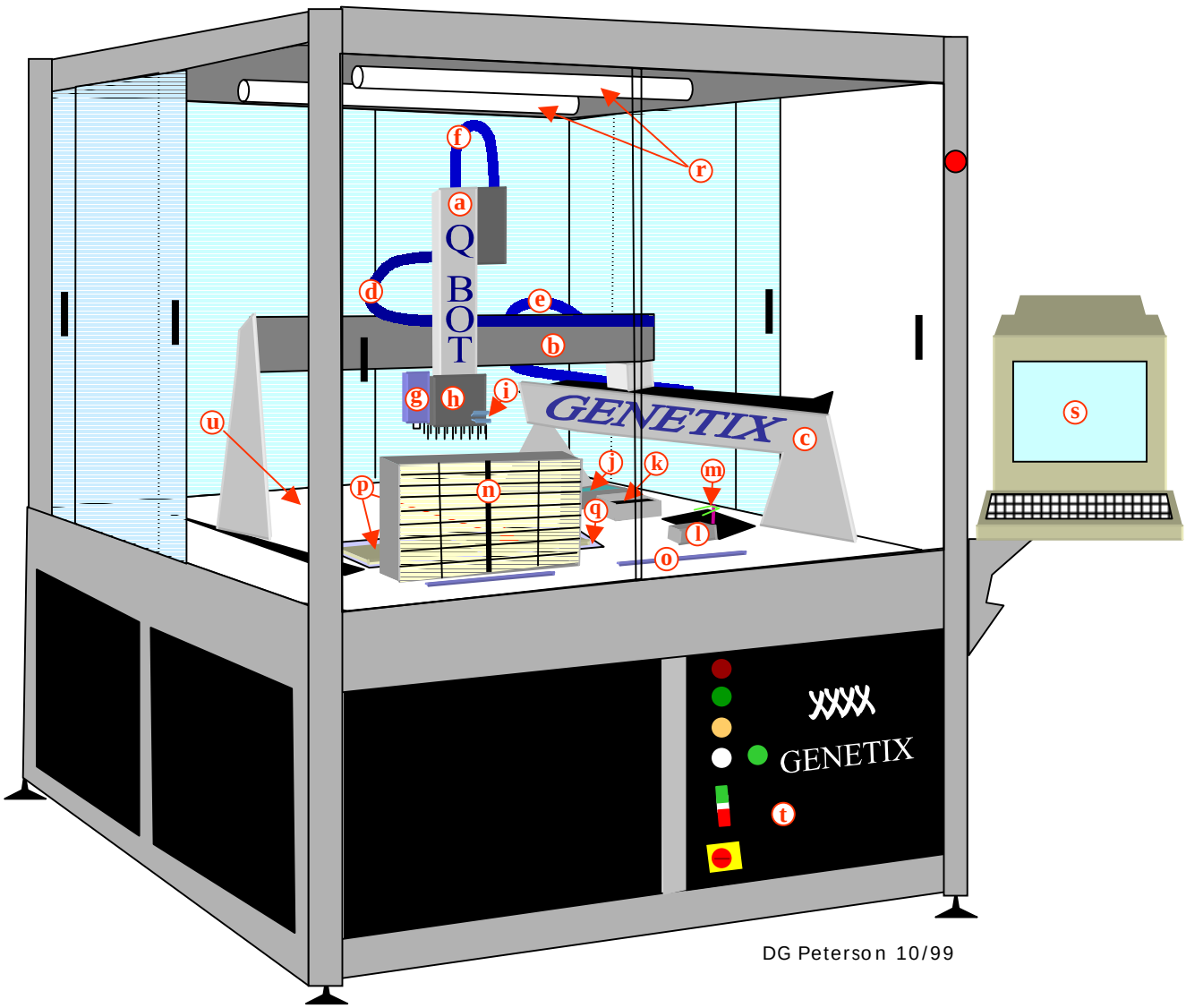
(c)



The mechanical stage moves the plate underneath the manifold. The manifold itself does not move during operation.

APPENDIX D

The Genetix QBot

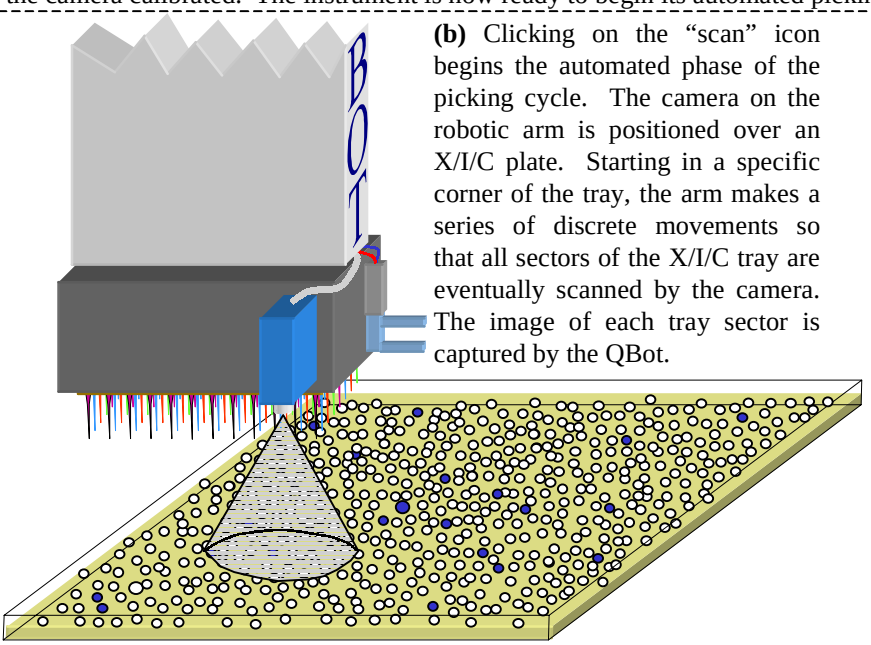


The QBot is roughly cube shaped (length = 2 m, width = 1.6 m, height = 1.97 m) and weighs about 900 kg. It possesses a robotic arm that carries out all of its automated tasks except removing microtiter plate lids from plates placed on the plate holder and shifting the microtiter plates in the plate holder. At the bottom of the arm is a "head" composed of 96 or 384 pins (depending on the application). On the front of the arm is a "plate gripper" which is used to move microtiter plates one at a time between a storage rack (known as a "hotel") and a plate holder. The arm is simultaneously moved along two horizontal perpendicular tracks allowing it to be positioned anywhere in a plane above the work surface. A track running vertically along the back of the QBot arm allows the arm to be moved up and down. In the diagram above, the QBot is set up for picking. In the figure: **a** = robotic arm, **b** = *x* axis track, **c** = *y* axis track, **d** = *x* axis cable guides, **e** = *y* axis cable guides, **f** = *z* axis cable guides, **g** = video camera, **h** = picking head with pins, **i** = plate gripper, **j** = ethanol wash bath, **k** = blow drier (hot air), **l** = plate holder, **m** = microtiter plate lid manipulator, **n** = hotel containing thirty-six 384-well microtiter plates, **o** = site at which a second hotel can be installed, **p** = X/I/C trays with colonies (lids removed), **q** = light table, **r** = UV lamps, **s** = computer monitor and keyboard, **t** = panel with on/off switches and warning lights, and **u** = main bed (used during replication and gridding applications). The QBot's computer is located in the lower half of the machine (hidden from view). When the machine is set up for plate replication, the tray holder (on top of the light table) is removed, and four "microtiter plate holding blocks" (collectively holding 24 microtiter plates) are installed on the main bed of the QBot. During gridding, 15 nylon membranes are placed on 15 "filter holding blocks" on the QBot's main bed.

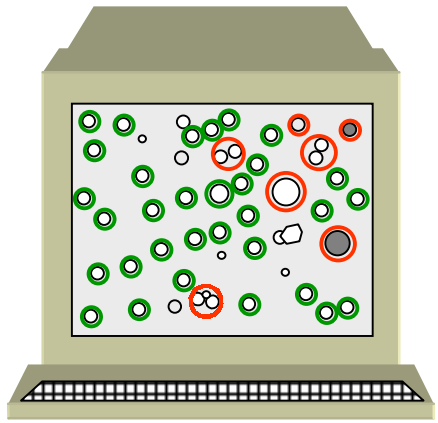
APPENDIX E

Picking clones using the Genetix QBot

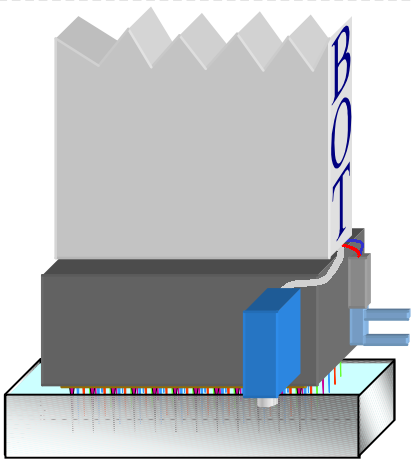
(a) Setting up the QBot for picking: Before picking is initiated, the UV lamps should be used to sterilize the working area in the QBot. After UV-sterilization, the wash bath should be filled with 70-100% ethanol, and one or two X/I/C trays (without lids) placed in appropriate holders on the light table. 384-well microtiter plates containing sterile freezing medium should be placed in the hotel(s), and the hotel(s) should be properly mounted in the QBot. The "Picking" software application should be opened, and the camera calibrated. The instrument is now ready to begin its automated picking tasks.



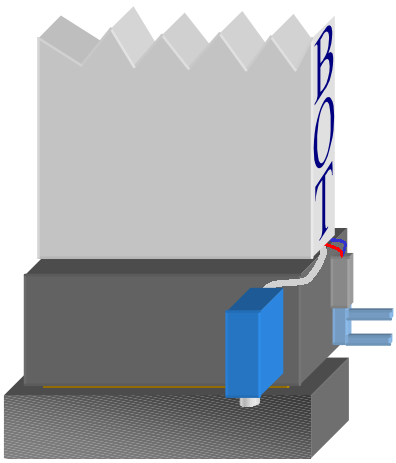
(b) Clicking on the "scan" icon begins the automated phase of the picking cycle. The camera on the robotic arm is positioned over an X/I/C plate. Starting in a specific corner of the tray, the arm makes a series of discrete movements so that all sectors of the X/I/C tray are eventually scanned by the camera. The image of each tray sector is captured by the QBot.



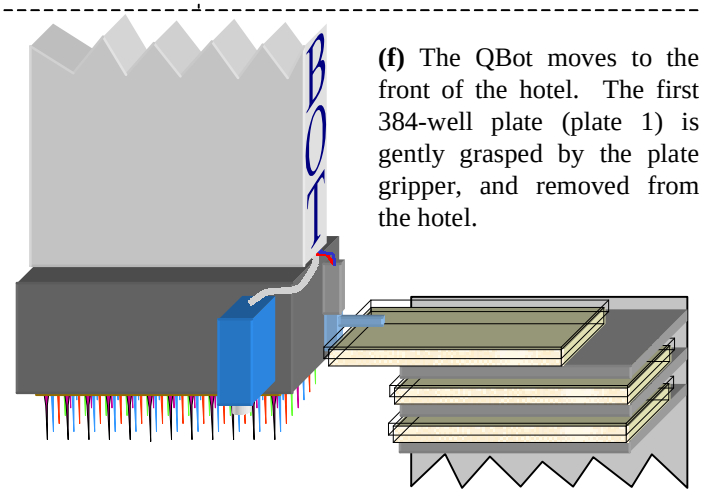
(c) After an image of a particular sector of the tray has been captured, the QBot's picking software analyzes the image and selects objects that meet the characteristics of single, spatially-isolated, white colonies. On the monitor, objects that meet the necessary criteria automatically are circled in green. Objects that meet some, but not all of the criteria are circled in red. Only colonies circled in green will be picked. The user can "fine tune" the selection process for a particular tray by changing the thresholds of various parameters including colony roundness, relative color, and size (this is usually done before automated scanning is actually initiated). After the X/I/C tray(s) has been completely scanned and evaluated by the picking software, the QBot is ready to begin picking.



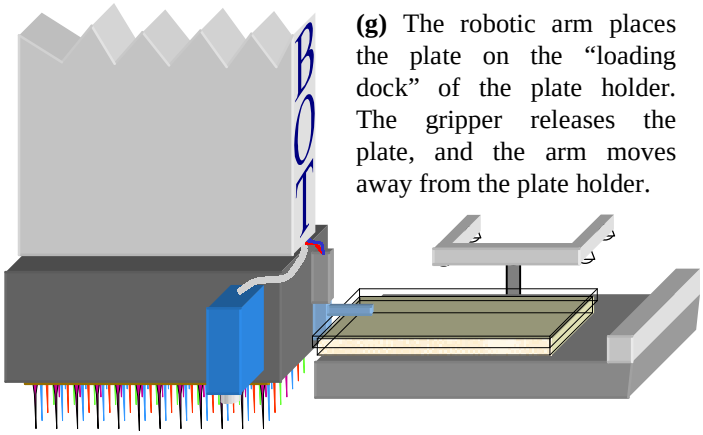
(d) Once the picking cycle is initiated, the QBot's first order of business is to sterilize the picking pins. In brief, the robotic arm places the bottom of the picking head (*i.e.*, the pins) into the ethanol wash bath. The arm makes a series of short, jerky movements effectively moving the pins back and forth through the bath. Groups of clustered bristles lining the bottom of the bath enhance the cleaning process.



(e) Upon completing the wash/sterilization cycle, the arm places the picking head into the blow drier. The warm air released by the blow drier causes evaporation of residual ethanol left on the pins.

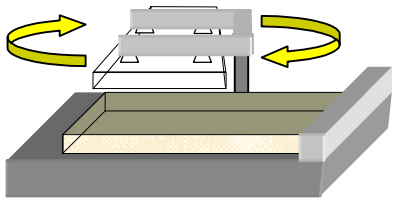


(f) The QBot moves to the front of the hotel. The first 384-well plate (plate 1) is gently grasped by the plate gripper, and removed from the hotel.

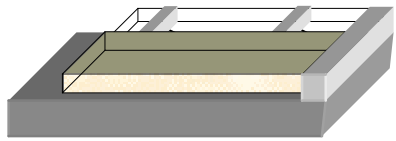


(g) The robotic arm places the plate on the "loading dock" of the plate holder. The gripper releases the plate, and the arm moves away from the plate holder.

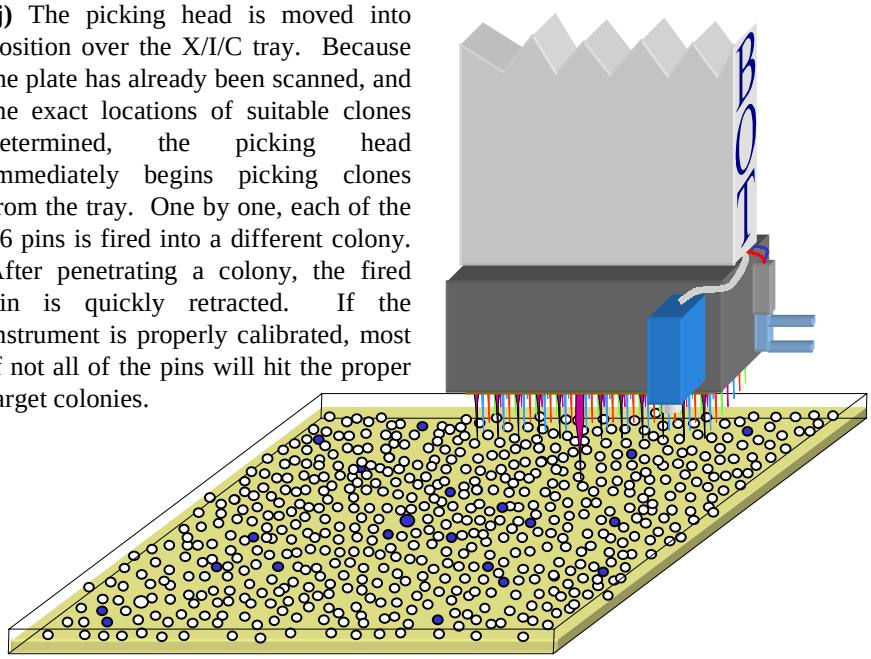
(h) The plate holder moves the plate forward so that it is in "working position". The lid manipulator rotates 180° so that it is directly over the plate. The manipulator drops onto the plate lid. Using four vacuum-controlled suckers, the lid manipulator attaches itself to the lid. The lid is lifted off the plate, and the manipulator rotates back to its original position taking the lid along with it.

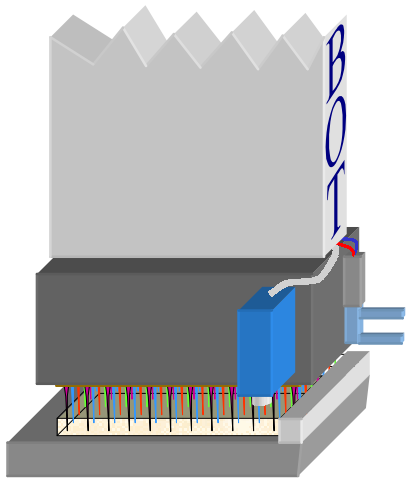


(i) The lid manipulator places the lid on a "low profile" resting pad adjacent to the plate holder. The manipulator remains attached to the lid.

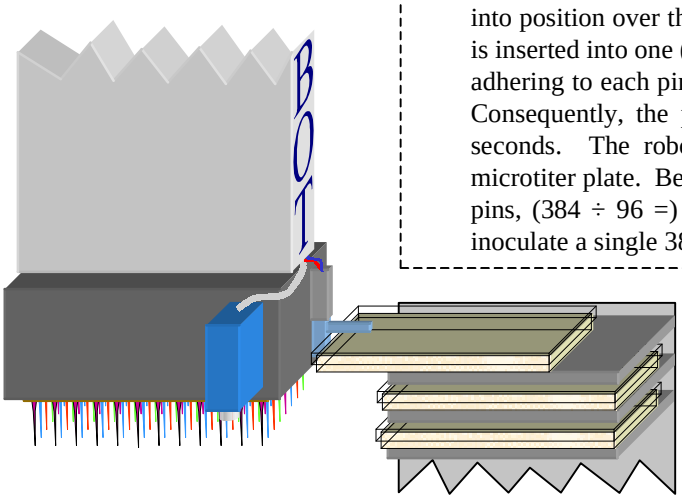


(j) The picking head is moved into position over the X/I/C tray. Because the plate has already been scanned, and the exact locations of suitable clones determined, the picking head immediately begins picking clones from the tray. One by one, each of the 96 pins is fired into a different colony. After penetrating a colony, the fired pin is quickly retracted. If the instrument is properly calibrated, most if not all of the pins will hit the proper target colonies.

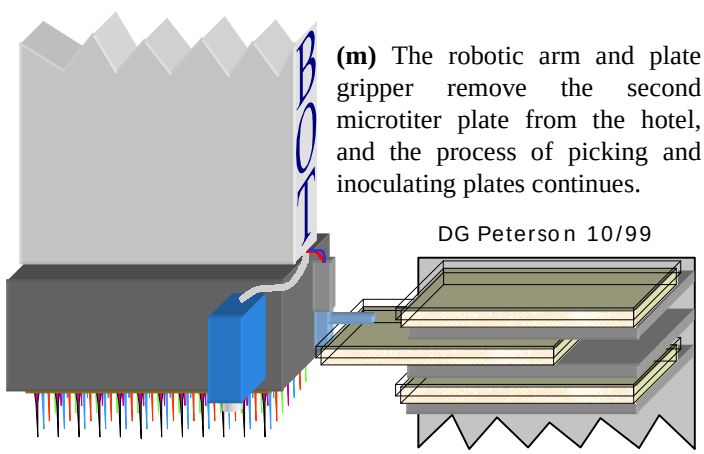




(k) Once all the pins on the picking head have been fired, the picking head is moved into position over the 384-well plate. The head is lowered so that each of the 96 pins is inserted into one (and only one) well of the plate. Presumably, some of the bacteria adhering to each pin are dislodged from that pin when it enters the freezing medium. Consequently, the pins "inoculate" the wells. Inoculation only takes a couple of seconds. The robotic arm then moves the picking head up and away from the microtiter plate. Because there are 384 wells in a microtiter plate and only 96 picking pins, (384 ÷ 96 =) 4 rounds of picking and inoculation are required to completely inoculate a single 384-well microtiter plate.



(l) Once the first plate has been completely inoculated, the lid manipulator puts the lid back on the plate. The plate holder shifts the plate back into "transport position" and the plate is picked up by the plate gripper on the robotic arm. The inoculated plate is placed back into the same slot of the hotel from which it was originally removed.



(m) The robotic arm and plate gripper remove the second microtiter plate from the hotel, and the process of picking and inoculating plates continues.

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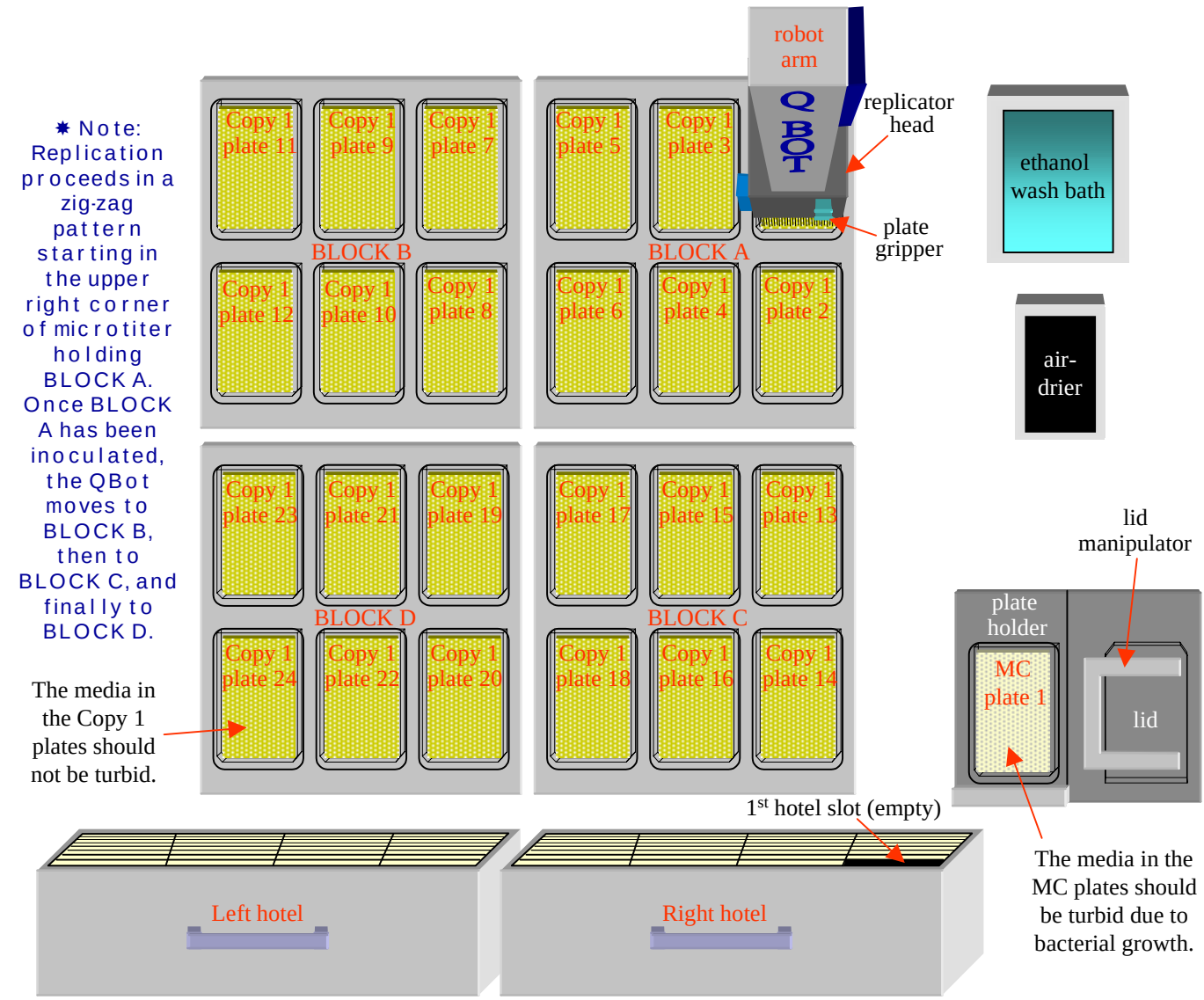
APPENDIX F

Library Replication using the Genetix QBot

(a) Before replication is started, microtiter plate holding blocks are mounted on the main bed of the Q-Bot, and the UV lamps are used to sterilize the working area in the QBot. After UV-sterilization, the wash bath is filled with 70-100% ethanol, and twenty-four 384-well plates containing sterile freezing media are placed on the microtiter plate holding blocks. The plate lids are removed. Hotels containing the first 72 plates of the master copy of the library (36 plates per hotel) are fitted onto the hotel holders. The "Replicating" software application is opened. At this point, the instrument is ready to begin replication. See APPENDIX D for a diagram of the QBot and APPENDIX E for specific details on general operations of the QBot (*e.g.*, pin sterilization, plate manipulation, and inoculation).



(b) At the start of the replication cycle, the replicating head (made up of 384 pins) is sterilized and dried by the ethanol bath and air-drier, respectively. The robotic arm transfers master copy (MC) plate 1 from the first plate slot in the right hotel to the plate holder. The lid is removed from MC plate 1 by the lid manipulator (see APPENDIX E for details on these processes). The replicator head is then positioned over MC plate 1 so that each pin is directly over a corresponding well. The arm is lowered simultaneously inoculating all 384 pins of the replicator head. The robot arm is raised, and quickly moved into position directly over the plate in position 1 of microtiter plate holding BLOCK A. A quick downward movement of the robot arm inoculates the plate (which should be labeled Copy 1, plate 1). The picking head is then re-sterilized as described above. The lid is replaced on MC plate 1, and the plate is returned to the hotel slot from which it was removed. This marks the completion of the first replication cycle. The second replication cycle results in replication of MC plate 2, the third in MC plate 3, *etc.* After 24 complete cycles, all plates on the main bed have been inoculated. At this point, the QBot pauses, and the user can place 24 new microtiter plates on the main bed. Clicking on the "continue" icon on the computer screen will resume the QBot's activities (starting with the replication of MC plate 25).

GLOSSARY

1C is used to designate the nuclear DNA content of a gamete. Basically, an organism's 1C DNA content is the smallest quantity of nuclear DNA that can be used to define that organism. Somatic cells in G1 of interphase have a 2C DNA content, and G2 cells possess a 4C DNA content.

Alpha-complementation is the most common form of insertional inactivation. In α-complementation, the vector molecule contains the regulatory and coding regions for the first 146 amino acids of the β-galactosidase (*lacZ*) gene. A polycloning site has been engineered into the coding sequence without disrupting the activity of the gene product (the amino-terminus of β-galactosidase). During ligation, vector molecules are cut at a particular sequence in the polycloning site and incubated with pieces of insert DNA cut with the same restriction enzyme. In some instances, insert DNA is successfully ligated into a vector molecule causing disruption of the *lacZ* coding sequences on the vector. However, some vectors will not be cut by the restriction endonuclease or will have their ends religated without incorporation of an insert. After ligation, the resulting DNA is used to transform a competent *E. coli* strain in which only the region of the *lacZ* gene coding for the carboxy-terminal portion of β-galactosidase is present. In cells that contain a vector without an insert, the amino-terminal β-galactosidase subunit will interact with the carboxy-terminal β-galactosidase subunit to form an active β-galactosidase enzyme (the inducer IPTG is often necessary for transcription). β-galactosidase catalyzes the transformation of the clear substrate X-GAL into a blue precipitate, giving colonies with an active β-galactosidase protein a distinctive blue color. In contrast, clones containing a polycloning site into which a piece of exogenous DNA has been inserted (*i.e.*, recombinant clones) will not produce a functional amino-terminal subunit, and will consequently appear white in color. It is the recombinant clones that are of interest, and hence they are preferentially selected from agar plates for further analysis (Sambrook et al. 1989).

BAC-end sequencing is a powerful technique used in association with chromosome walking to construct contigs. It is rooted in the principles of Sanger dideoxy DNA sequencing and DNA amplification (Rosenblum et al., 1997). In brief, (a) BAC DNA is isolated from clones using a miniprep procedure, (b) vector-based primers, Taq polymerase, and a nucleotide cocktail containing dye-labeled ddNTPs are added to each miniprep, (c) the mixtures are heat-denatured, cooled to allow primer hybridization, and warmed to a temperature that allows DNA polymerization, (d) each reaction is electrophoretically resolved on an acrylamide sequencing gel, and (e) the gel is analyzed using an automated gel analysis system (see Boysen et al. 1997; Rosenblum et al. 1997; Kelly et al. 1999 for reviews). If the BAC DNA concentration in the minipreps is particularly low, several thermocycle runs can be used to produce more sequencing substrates (Liu and Whittier 1995).

The sequences generated by BAC-end sequencing represent regions of insert DNA adjacent to insert sites ("BAC ends"). Once BAC end sequences for a particular clone are obtained, probes based on that clone's end sequences can be used to screen the BAC library and find clones that overlap the starting clone (*i.e.*, chromosome walking). When applied on a large

scale, probing libraries with BAC end sequences can lead to relatively rapid construction of physical maps.

Chromosome walking: In chromosome walking, the end sequences of a "starter" clone(s), typically associated with an EST or RFLP marker, are used to probe colony blots/grids. DNA fingerprints of positive clones are compared to the fingerprint of the starter clone, and those exhibiting a minimal amount of overlap with the starter are grouped into a contig with that clone.

Contig: A contig is a set of clones containing partially overlapping pieces of insert DNA that collectively represent an uninterrupted stretch of genomic DNA. Contigs are constructed using physical mapping techniques.

Chimeras are clones that possess two or more noncontiguous DNA inserts. Chimeras can result from insertion of more than one noncontiguous DNA fragment into a single vector molecule, recombination between inserts in two different vectors, and/or inclusion of two or more recombinant molecules (vectors with inserts) in a single host cell (Green et al. 1991; Shizuya et al. 1992).

Concatemers are DNA molecules composed of a vector/genome repeated in tandem (*e.g.*, several BAC vector molecules ligated together) (Lewin 1997).

DNA fingerprinting is a means of analyzing the similarity between several DNA samples based upon the presence or absence of specific restriction sites within their sequences. In DNA fingerprinting, two or more DNA samples (*e.g.*, BAC clones) are digested with the same set of restriction enzymes. The digested DNA samples are run on a gel and blotted onto nitrocellulose (Southern blotting). Blots are hybridized with labeled probe sequences, and similarities/differences between the hybridization patterns for the DNA samples are noted.

In BAC-based physical mapping, DNA fingerprints of BAC clones can be compared. Those clones that have considerable overlap in their fingerprint patterns can be grouped together into contigs (Marek and Shoemaker 1997; Marra et al. 1997).

False positive clones do not contain inserts from the experimental organism, yet exhibit the phenotype of recombinants based upon their growth pattern and colony color on selective media (*e.g.*, white color, chloramphenicol-resistance). False positives generally do contain a vector molecule. However, the marker gene has been inactivated by an event other than insertion of a large DNA fragment into the polycloning site.

F factors are naturally occurring episomes (*i.e.*, DNA elements that can exist as circular plasmids or can be integrated into the bacterial chromosome) found in some bacterial strains. In its free plasmid (circular) form, the typical F factor is approximately 100 kb and is maintained at a level of one copy per bacterial genome. When inserted into the bacterial genome, F factors are replicated along with the bacterial genome. Integrated F factors can be present in more than one copy per cell. Bacteria that possess an F factor (F-positive bacteria) can conjugate with strains that do not contain an F factor (F-negative bacteria). During conjugation (which is mediated by F factor gene products), an F-positive bacterium containing a free plasmid transfers a copy of the F plasmid to an F-negative bacterium. If the F factor is integrated into the F-positive bacterium's

genome, the F factor and part or all of the donor's chromosomal DNA may be transferred into the F-negative bacterium (Willetts and Skurry 1987; Lewin 1997).

Although BACs are derived from F factors, only a few of the F factor genes have been preserved in BACs. Genes involved in conjugation and regions involved in insertion of the F factor into the bacterial genome have been eliminated. The endogenous F factor genes left in BACs serve to prevent more than one BAC/F factor from inhabiting a cell and ensure proper replication and segregation of the BAC into daughter cells (see FIGURE 1.1).

Fluorescence *in situ* hybridization (FISH) is a technique in which hapten-labeled DNA probes are hybridized to chromosomes that have been spread on glass microscope slides. Antibodies or other affinity reagents conjugated to fluorochromes are used to detect (directly or indirectly) sites of hybridization (Peterson et al. 1999).

Genome coverage is the combined base pair length of all the inserts in a genomic library divided by the 1C genome content of the organism for which the library was made. The level of genome coverage for a particular library can be determined using the following formula:

$$\mathbf{W = NI/G}$$

Where W = coverage, N = total number of clones in the library, I = mean length in base pairs of DNA inserts, and G = the 1C genome size (in base pairs) of the organism from which the library was made.

For example, suppose that a BAC library was constructed for soybean (*Glycine max*). The 1C DNA content for soybean is approximately 1.115×10^9 base pairs. If the library contained 100,000 clones with an average insert size of 120,000 bp, the library coverage would be

$$W = (100{,}000 \text{ clones} \times 120{,}000 \text{ bp})/1.115 \times 10^9 \text{ bp}$$
$$W = 10.8$$

To put it another way, the library would contain 10.8 genome's worth of soybean DNA or 10.8 times (10.8X) the amount of nuclear DNA in a soybean gamete.

With 3X coverage, the chance of finding a particular genomic sequence in a library is approximately 95%. Increasing coverage to 5X improves the chances that a library is truly representative (includes all of the sequences within the genome of interest) to 99%. Naturally, increasing the genome coverage above 5X affords even higher confidence levels (Paterson 1996).

Insertional inactivation can be used to differentiate recombinant clones from non-recombinants. Most plasmid vectors (including most BACs) contain a reporter gene into which a polycloning site has been engineered. Ligation of a piece of exogenous (insert) DNA into the polycloning site of a vector results in disruption of the reporter gene whereas a vector molecule that either was not cut by the restriction enzyme or that has had its termini ligated back together contains an intact reporter gene. After transformation, bacteria are plated onto nutritive agar. In recombinant clones (clones in which the reporter gene has been disrupted by an insert), a functional version of the reporter gene protein (reporter protein) will not be produced. In clones containing a plasmid without an insert (non-recombinants), the reporter protein will be expressed. The reporter protein is typically an enzyme (or part of an enzyme complex) that catalyzes a colorimetric reaction using a component in the selective media as a substrate. Consequently, recombinant and non-recombinant clones can be differentiated based on colony

color (see alpha-complementation).

Insert rearrangements include deletions, transpositions, and inversions. Insert rearrangements, especially in clones containing tandemly repeated DNA sequences, are relatively frequent in yeast artificial chromosome systems (Neil et al. 1990).

Map-based cloning is the use of physical mapping and molecular mapping to isolate a gene(s) involved in a particular phenotype. Basically, molecular mapping is used to determine where on the molecular map the gene is located. Once the two markers that most closely flank the gene have been determined, physical mapping is used to isolate a contig containing the DNA between the two markers. This contig, which presumably contains the gene(s) of interest, can be further evaluated.

Master copy is a term used with regard to ordered libraries. In brief, the plates produced by transferring bacteria directly from colonies on agar plates into microtiter wells constitute the original or "master" copy of the library. Once the bacteria in the master copy plates have been allowed to propagate overnight, copies of the library can be made using the master copy as a template (see CHAPTER 17).

Polycloning site: A polycloning site is a relatively short region within a vector into which several restriction sites have been engineered for the purpose of DNA cloning. These restriction sites are not found elsewhere on the vector molecule. In many instances, a polycloning site is engineered into a reporter gene allowing insertional inactivation. In order for insertional inactivation to work, addition of the polycloning site must not prevent proper transcription of the reporter gene or significantly alter the activity of the reporter gene product.

Multiplex screening is a colony hybridization strategy for efficiently screening ordered libraries with multiple radioisotope- or fluorochrome-labeled probes. Briefly, probes of interest are labeled and arranged in a series of rows and columns in a microtiter plate. Probes from an entire row are pooled and used to screen a set of library grids. Likewise, probes from one column are pooled and used to probe an identical set of grids. Hybridization patterns on both sets of grids are recorded and compared. If a particular clone is recognized by both the pooled row and pooled column probes, that clone most likely contains a DNA sequence complementary to the probe found at the intersection of the row and column on the microtiter plate. Computer analysis of the hybridization patterns of all pooled column and row combinations allows clones to be assigned to probes using a minimum number of hybridizations (Cai et al. 1998).

Ordered libraries: In an ordered BAC library, bacteria from positive colonies (*i.e.*, colonies presumably containing insert DNA) are picked from agar trays and placed into freezing medium in individual wells of microtiter plates (one clone per well). Letters along the Y-axis and numbers along the X-axis of each plate provide each well with a specific alphanumerical designation (*e.g.*, well G13). Additionally, the microtiter plates in a library are numbered consecutively. Consequently, any particular clone in the library possesses its own unique address (*e.g.*, plate 131, well G13).

Segregation of individual clones into separate wells coupled with automation allows complete libraries to be gridded onto filters in a highly specific manner, *i.e.*, each clone is

gridded onto a filter based on its address in the library. If a probe hybridizes to a specific spot(s) on a grid, the relative location of the spot can be used to determine the exact location of the clone within the library.

Ordered libraries save valuable time and resources by increasing the efficiency and speed of library screening (see Choi and Wing 1999 for review).

Physical mapping: Physical mapping is the grouping of clones into contigs using physical mapping techniques. The goal of most physical mapping projects is to assemble contigs that encompass entire chromosomes/genomes as a prelude to genome sequencing (*e.g.*, Mozo et al. 1999).

Physical mapping techniques are any techniques used in contig construction. The most common BAC physical mapping techniques include chromosome walking, BAC-end sequencing, STS-based mapping, map-based cloning, and DNA fingerprinting.

Quality designations are placed on the labels of microtiter plates as an indicator of how "far removed" a library copy is from the original or master copy. The master copy itself has the "highest quality" denoted on labels by the letter "Q" for "quality" and the Roman numeral "I" (*i.e.*, QI). Libraries prepared using the master copy as a template have the second highest quality designation, *i.e.*, "QII". Copies made from QII plates are designated QIII, *etc.*

Radiation hybrid mapping: In radiation hybrid mapping, cells from a species of interest (donor species) are exposed to radiation of sufficient intensity to cause chromosome fragmentation. The irradiated cells are then fused with cultured cells from a second species (host species). The host cell line often contains a mutation that prevents its growth on selective media. Over time, the hybrid cells lose most of the chromosome fragments from the donor species. However, one or two chromosomal fragments from the donor species may become stably transmitted and expressed in some of the fusion products. If the hybrid cells are placed in selective media, only those cells in which the mutated host gene has been complemented by a DNA fragment from the donor will survive. Viable hybrids can then be tested for the presence of other markers from the donor species. The likelihood that a donor-specific marker linked to the gene complementing the host's mutation will be found in viable hybrid cells is inversely related to the physical distance between the marker and the gene. Consequently, comparison of the transmission of donor-specific markers with the complementing phenotype can be used to determine gene order and estimate distances between markers (Goss and Harris 1975).

Secondary compounds are chemicals not required in the normal metabolic and developmental pathways common to plants (or at least large sub-groups of plants). Examples of secondary compounds are latex, polyphenols (*e.g.*, tannins), and alkaloids (*e.g.*, caffeine, cocaine, nicotine, quinine). Some secondary compounds are apparently involved in plant defense while others have no known function (Salisbury and Ross 1992). Secondary compounds often make isolation of nucleic acids, organelles, and proteins difficult (Loomis 1974; Peterson et al. 1997).

STS-based mapping is a physical mapping technique rooted in the principles of PCR. In general, primer pairs are designed from cDNAs and/or genomic regions known to be single-copy in nature. In the presence of labeled nucleotides, PCR is performed using a particular primer pair(s) and a set of BAC templates (*e.g.*, minipreps from a BAC library). Those BACs that

function as templates for a particular primer pair are grouped into a contig. If different primer pairs produce amplification products from the same BAC template, those primers represent loci that are physically close to one another. The amplification product of a primer pair is called an STS marker. Those amplification products generated using primers derived from cDNAs often are called ESTs (expressed sequence tags) because they presumably correspond to expressed genes.

Yeast artificial chromosomes (YACs) are linear DNA vectors equipped with the essential elements of yeast chromosomes (Hieter et al. 1990; Burke and Olsen 1991). When introduced into living yeast cells, YACs are replicated and segregated along with the standard yeast chromosome complement. During meiosis a YAC will pair and synapse with "homologous" YACs if any are present (Shero et al. 1991).

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REFERENCES

1. Anderson, C. (1993) Genome shortcut leads to problems. *Science*, **259**, 1684-1687.
2. Arumuganathan, K. and Earle, E.D. (1991) Nuclear DNA content of some important plant species. *Plant Mol. Biol. Reptr.*, **9**, 208-219.
3. Bennett, M.D., Smith, J.B. and Heslop-Harrison, J.S. (1982) Nuclear DNA amounts in angiosperms. *Proc. Royal Soc. London B*, **216**, 179-199.
4. Bouck, J., Miller, W., Gorrell, J.H., Muzny, D. and Gibbs, R.A. (1998) Analysis of the quality and utility of random shotgun sequencing at low redundancies. *Genome Res.*, **8**, 1074-1084.
5. Boysen, C., Simon, M.I. and Hood, L. (1997) Fluorescence-based sequencing directly from bacterial and P1-derived artificial chromosomes. *BioTechniques*, **23**, 978-982.
6. Burke, D.T. (1990) YAC cloning: options and problems. *GATA*, **7**, 94-99.
7. Burke, D.T., Carle, G. and Olsen, M.V. (1987) Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors. *Science*, **236**, 806-812.
8. Burke, D.T. and Olsen, M.V. (1991) Preparation of clone libraries in yeast artificial chromosome vectors. *Meth. Enzymol.*, **194**, 251-270.
9. Cai, L., Taylor, J.F., Wing, R.A., Gallagher, D.S., Woo, S.-S. and Davis, S.K. (1995) Construction and characterization of a bovine bacterial artificial chromosome library. *Genomics*, **29**, 413-425.
10. Cai, W.-W., Reneker, J., Chow, C.-W., Vaishnav, M. and Bradley, A. (1998) An anchored framework BAC map of mouse chromosome 11 assembled using multiplex oligonucleotide hybridization. *Genomics*, **54**, 387-397.
11. Choi, S., Creelman, R.A., Mullet, J.E. and Wing, R.A. (1995) Construction and characterization of bacterial artificial chromosome library of *Arabidopsis thaliana*. *Plant Mol. Biol. Reptr.*, **13**, 124-128.
12. Choi, S. and Wing, R.A. (1999) The Construction of bacterial artificial chromosome (BAC) libraries. In Gelvin S. and Schilperoort, R. (eds.), *Plant Molecular Biology Manual*, 2nd Edition, Suppl. IV. Kluwer Academic Publishers, The Netherlands, pp. 1-32.
13. Couch, J.A. and Fritz, P.J. (1990) Isolation of DNA from plants high in polyphenolics. *Plant Mol. Biol. Reptr.*, **8**, 8-12.

14. Danesh, D., Peñula, S., Mudge, J., Denny, R.L., Nordstrom, H., Martinez, J.P. and Young, N.D. (1998) A bacterial artificial chromosome library for soybean and identification of clones near a major cyst nematode resistance gene. *Theor*. *Appl*. *Genet*., **96**, 196-202.
15. Deloukas, P., Schuler, G.D., Gyapay, G., Beasley, E.M., Soderlund, C., Rodriguez-Tome, P., Hui, L., Matise, T.C., McKusick, K.B., Beckmann, J.S., et al. (1998) A physical map of 30,000 human genes. *Science*, **282**, 744-746.
16. Ding, Y., Johnson, M.D., Colayco, R., Chen, Y.L., Melnyk, J., Schmitt, H. and Shizuya, H. (1999) Contig assembly of bacterial artificial chromosome clones through multiplexed fluorescence-labeled fingerprinting. *Genomics*, **56**, 237-246.
17. Folkertsma, R.T., Spassova, M.I., Prins, M., Stevens, M.R., Hille, J. and Goldbach, R.W. (1999) Construction of a bacterial artificial library of *Lycopersicon esculentum* cv. Stevens and its application to physically map the *Sw-5* locus. *Mol*. *Breed*., **5**, 197-207.
18. Frijters, A.C.J., Zhang, Z., van Damme, M., Wang, G.-L., Ronald, P.C. and Michelmore, R.W. (1997) Construction of a bacterial artificial chromosome library containing large *Eco*RI and *Hind*III genomic fragments of lettuce. *Theor*. *Appl*. *Genet*., **94**, 390-399.
19. Gillett, W., Hanks, L., Wong, G.K.-S., Yu, J., Lim, R. and Olson, M.V. (1996) Assembly of high-resolution restriction maps based on multiple complete digests of a redundant set of overlapping clones. *Genomics*, **33**, 389-408.
20. Godard, S., Oustry-Vaiman, A., Cribiu, E.P. and Guérin, G. (1999) FISH mapping assignment of two horse BAC clones containing HMS41 and HTG3 microsatellites. *Anim*. *Genet*., **30**, 233.
21. Gómez, M.I., Islam-Faridi, M.N., Woo, S.-S., Schertz, K.F., Czeschin, Jr., D., Zwick, M.S., Wing, R.A., Stelly, D.M. and Price, H.J. (1997) FISH of a maize *sh2*-selected sorghum BAC to chromosomes of *Sorghum bicolor*. *Genome*, **40**, 475-478.
22. Goodman, H.M., Ecker, J.R. and Dean, C. (1995) The genome of *Arabidopsis thaliana*. *Proc*. *Natl*. *Acad*. *Sci*. *USA*, **92**, 10831-10835.
23. Goss, S.J. and Harris, H. (1975) New method for mapping genes in human chromosomes. *Nature*, **255**, 680-684.
24. Green, E.D., Riethman, H.C., Dutchik, J.E. and Olson, M.V. (1991) Detection and characterization of chimeric yeast artificial-chromosome clones. *Genomics*, **11**, 658 - 669.
25. Guillemaut, P. and Maréchal-Drouard, L. (1992) Isolation of plant DNA: A fast, inexpensive, and reliable method. *Plant Mol*. *Biol*. *Reptr*., **10**, 60-65.
26. Hamilton, R.H., Künsch, U. and Temperli, A. (1972) Simple rapid procedures for isolation of tobacco leaf nuclei. *Anal*. *Biochem*., **49**, 48-57.
27. Hanson, R.E., Zwick, M.S., Choi, S., Islam-Faridi, M.N., McKnight, T.D., Wing, R.A., Price, H.J. and Stelly, D.M. (1995) Fluorescent *in situ* hybridization of a bacterial chromosome. *Genome*, **38**, 646-651.
28. Hieter, P., Connelly, C., Shero, J., McCormick, M.K., Antonarakis, S., Pavan, W. and Reeves, R. (1990) Yeast artificial chromosomes: Promises kept and pending. In Davies, K.E. and Tilghman, S.M. (eds.), *Genetic and Physical Mapping*. Cold Spring Harbor Laboratory Press, Plainview, NY, pp. 83-120.
29. Hudson, T.J., Stein, L.D., Gerety, S.S., Ma, J., Castle, A.B., Silva, J., Slonim, D.K., Baptista, R., Kruglyak, L., Xu, S.-H., et al. (1995) An STS-based map of the human genome. *Science*, **270**, 1945-1954.
30. Jiang, J., Gill, B.S., Wang, G-L., Ronald, P.C. and Ward, D.C. (1995) Metaphase and interphase fluorescence *in situ* hybridization mapping of the rice genome with bacterial

artificial chromosomes. *Proc. Natl. Acad. Sci. USA*, **92**, 4487-4491.
31. Katterman, F.R.H. and Shattuck, V.I. (1983) An effective method of DNA isolation from the mature leaves of *Gossipyium* species that contain large amounts of phenolic terpenoids and tannins. *Preparative Biochem.*, **13**, 347-359.
32. Kelley, J.M., Field, C.E., Craven, M.B., Bocskai, D., Kim, U.-J., Rounsley, S.D. and Adams, M.D. (1999) High throughput direct end sequencing of BAC clones. *Nucleic Acids Res.*, **27**, 1539-1546.
33. Kim, U.-J., Birren, B.W., Slepak, T., Mancino, V., Boysen, C., Kang, H.-L., Simon, M.I. and Shizuya, H. (1996) Construction and characterization of a human bacterial artificial chromosome library. *Genomics*, **34**, 213-218.
34. Lapitan, N.L.V., Brown, S.E., Kennard, W., Stephens, J.L. and Knudson, D.L. (1997) FISH physical mapping with barley BAC clones. *Plant J.*, **11**, 149-156.
35. Laurie, D.A. and Bennett, M.D. (1985) Nuclear DNA content in the genera *Zea* and *Sorghum*. Intergeneric, interspecific and intraspecific variation. *Heredity*, **55**, 307-313.
36. Lewin, B. (1997) *Genes VI*. Oxford University Press, New York.
37. Lijavetzky, D., Muzzi, G., Wing, R.A. and Dubocovsky, J. (1999) Construction and characterization of a bacterial artificial chromosome (BAC) library for the *A* genome of wheat. Abstract P99, *Plant & Anim. Genome Conf. VII*, San Diego, 1999.
38. Lin, Y-r., Draye, X., Qian, X., Ren, S., Zhu, L-h., Tomkins, J., Wing, R.A., Li, Z. and Paterson, A.H. (2000) Locus-specific contig assembly in highly-duplicated genomes using the BAC-RF method. *Nucleic Acids Res.*, in press.
39. Liu, D. and Wu, R. (1999) Protection of megabase-sized chromosomal DNA from breakage by DNase activity in plant nuclei. *BioTechniques*, **26**, 258-261.
40. Liu, Y.G. and Whittier, R.F. (1995) Thermal asymmetric interlaced PCR: automatable amplification and sequencing of insert end fragments from P1 and YAC clones for chromosome walking. *Genomics*, **25**, 674-681.
41. Loomis, W.D. (1974) Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles. *Methods Enzymol.*, **31**, 528-544.
42. Marek, L.F. and Shoemaker, R.C. (1997) BAC contig development by fingerprint analysis in soybean. *Genome*, **40**, 420-427.
43. Marra, M.A., Kucaba, T.A., Dietrich, N.J., Green, E.D., Brownstein, B., Wilson, R.K., McDonald, K.M., Hillier, L.W., McPherson, J.D. and Waterson, R.H. (1997) High throughput fingerprint analysis of large-insert clones. *Genome Res.*, **7**, 1072-1084.
44. Morisson, M., Pitel, F., Fillon, V., Pouzadoux, A., Bergé, R., Vit, J.P., Zoorob, R., Auffray, C., Gellin, J. and Vignal, A. (1998) Integration of chicken cytogenetic and genetic maps: 18 new polymorphic markers isolated from BAC and PAC clones. *Anim. Genet.*, **29**, 348-355.
45. Moullet, O., Zhang, H.-B. and Lagudah, E.S. (1999) Construction and characterisation of a large DNA insert library from the D genome of wheat. *Theor. Appl. Genet.*, **99**, 305-313.
46. Mozo, T., Dewar, K., Dunn, P., Ecker, J.R., Fischer, S., Kloska, S., Lehrach, H., Marra, M., Martienssen, R., Meier-Ewert, S. and Altmann, T. (1999) A complete BAC-based physical map of the *Arabidopsis thaliana* genome. *Nat. Genet.*, **22**, 271-275.
47. Nakamura, S., Asakawa, S., Ohmido, N., Fukui, K., Shimizu, N. and Kawasaki, S. (1997) Construction of an 800-kb contig in the near-centromeric region of the rice blast resistance gene *Pi-ta*^{2} using a highly representative rice BAC library. *Mol. Gen. Genet.*, **254**, 611-620.
48. Nam, Y.-W., Penmesta, R.V., Endre, G., Uribe, P., Kim, D. and Cook, D.R. (1999) Construction of a bacterial artificial chromosome library of *Medicago truncatula* and

identification of clones containing ethylene-response genes. *Theor. Appl. Genet.*, **98**, 638-646.
49. Neil, D.L., Villasante, A., Fisher, R.B., Vetrie, D., Cox, B. and Tyler-Smith, C. (1990) Structural instability of human tandemly repeated DNA sequences cloned in yeast artificial chromosome vectors. *Nucleic Acids Res.*, **18**, 1421-1428.
50. O'Connor, M., Peifer, M. and Bender, W. (1989) Construction of large DNA segments in *Escherichia coli*. *Science*, **244**, 1307-1312.
51. Paterson, A.H. (1996) The DNA revolution. In Paterson, A.H. (ed.), *Genome Mapping in Plants*. Academic Press, San Diego, CA, pp. 7-21.
52. Paterson, A.H., Schertz, K.F., Lin, Y.-R., Liu, S.-C. and Chang, Y.-L. (1995) The weediness of wild plants: Molecular analysis of genes influencing dispersal and persistence of johnsongrass, *Sorghum halepense* (L.) Pers. *Proc. Natl. Acad. Sci. USA*, **92**, 6127-6131.
53. Patocchi, A., Vinatzer, B.A., Gianfranceschi, L., Tartarini, S., Zhang, H.-B., Sansavini, S. and Gessler, C. (1999) Construction of a 550 kb BAC contig spanning the genomic region containing the apple scab resistance gene *Vf*. *Mol. Gen. Genet.*, **262**, 884-891.
54. Peterson, D.G., Boehm, K.S. and Stack, S.M. (1997) Isolation of milligram quantities of DNA from tomato (*Lycopersicon esculentum*), a plant containing high levels of polyphenolic compounds. *Plant Mol. Biol. Reptr.*, **15**, 148-153.
55. Peterson, D.G., Lapitan, N.L.V. and Stack, S.M. (1999) Localization of single- and low-copy sequences on tomato synaptonemal complex spreads using fluorescence *in situ* hybridization (FISH). *Genetics*, **152**, 427-439.
56. Peterson, D.G., Pearson, W.R. and Stack, S.M. (1998) Characterization of the tomato (*Lycopersicon esculentum*) genome using *in vitro* and *in situ* DNA reassociation. *Genome*, **41**, 346-356.
57. Rosenblum, B.B., Lee, L.G., Spurgeon, S.L., Khan, S.H., Menchen, S.M., Heiner,C.R. and Chen, S.M. (1997) New dye-labeled terminators for improved DNA sequencing patterns. *Nucleic Acids Res.*, **25**, 4500-4504.
58. Salimath, S.S. and Bhattacharyya, M.K. (1999) Generation of a soybean BAC library, and identification of DNA sequences tightly linked to the *Rsp1*-k disease resistance gene. *Theor. Appl. Genet.*, **98**, 712-720.
59. Salisbury, F.B. and Ross, C.W. (1992) *Plant Physiology*. Wadsworth Publishing Company, Belmont, CA.
60. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning*: *A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
61. Sanchez, A.C., Ilag, L.L., Yang, D., Brar, D.S., Ausubel, F., Khush, G.S., Yano, M., Sasaki, T., Li, Z. and Huang, N. (1999) Genetic and physical mapping of *xa13*, a recessive bacterial blight resistance gene in rice. *Theor. Appl. Genet.*, **98**, 1022-1028.
62. Shero, J.H., Koval, M., Spencer, F., Palmer, R.E., Hieter, P. and Koshland, D. (1991) Analysis of chromosome segregation in *Saccharomyces cerevisiae*. *Meth. Enzymol.*, **194**, 749-773.
63. Shizuya, H., Birren, B., Kim, U.-J., Mancino, V., Slepak, T., Tachiiri, Y. and Simon, M. (1992) Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using an F-factor-based vector. *Proc. Natl. Acad. Sci. USA*, **89**, 8794-8797.
64. Soderlund, C., Longden, I. and Mott, R. (1997) FPC: A system for building contigs from restriction fingerprinted clones. *CABIOS*, **13**, 523-535.
65. Strong, S.J., Ohta, Y., Litman, G.W. and Amemiya, C.T. (1997) Marked improvement of

PAC and BAC cloning is achieved using electroelution of pulsed-field gel-separated partial digests of genomic DNA. *Nucleic Acids Res*., **25**, 3959-3961.
66. Tomkins, J.P., Byrum, J., Frisch, D.A. and Wing, R.A. (2000a) Construction and characterization of a BAC library for the soybean cultivar A3244. *Soybean Genet. Newsletter*, **27**, www.soygenetics.org.
67. Tomkins, J.P., Frisch, D.A., Byrum, J.R., Jenkins, M.R., Barnett, L.J., Wicker, T., Luo, M. and Wing, R.A. (2000b) Construction and characterization of a maize bacterial artificial chromosome (BAC) library for the inbred line LH132. *Maize Genet. Coop. Newsletter*, 74, www.agron.missouri.edu/mnl/74.
68. Tomkins, J.P., Frisch, D.A., and Wing, R.A. (2000c) Construction and characterization of a maize BAC library for the inbred line B73. *Maize Genet. Coop. Newsletter*, in press.
69. Tomkins, J.P., Mahalingham, R., Miller-Smith, H., Goicoechea, J.L., Knapp, H.T. and Wing, R.A. (1999a). A soybean bacterial artificial chromosome library for PI 437654 and the identification of clones associated with cyst nematode resistance. *Plant Mol. Biol*., **41**, 25-32.
70. Tomkins, J.P., Yu, Y., Miller-Smith, H., Frisch, D.A., Woo, S.S. and Wing, R.A. (1999b) A bacterial artificial chromosome library for sugarcane. *Theor. Appl. Genet*., **99**, 419-424.
71. Venter, J.C., Adams, M.D., Sutton, G.G., Kerlavage, A.R., Smith, H.O. and Hunkapiller, M. (1998) Shotgun sequencing of the human genome. *Science*, **280**, 1540-1542.
72. Venter, J.C., Smith, H.O. and Hood, L. (1996) A new strategy for genome sequencing. *Nature*, **381**, 364-366.
73. Vinatzer, B.A., Zhang, H.-B. and Sansavini, S. (1998) Construction and characterization of a bacterial artificial chromosome library of apple. *Theor. Appl. Genet*., **97**, 1183-1190.
74. Wang, G.-L., Warren, R., Innes, R., Osborne, B., Baker, B. and Ronald, P.C. (1996) Construction of an *Arabidopsis* BAC library and isolation of clones hybridizing with disease-resistance, gene-like sequences. *Plant Mol. Biol. Reptr*., **14**, 107-114.
75. Wang, Y.-K. and Schwartz, D.C. (1993) Chopped insert: a convenient alternative to agarose/DNA inserts or beads. *Nucleic Acids Res*., **21**, 2528.
76. Watson, J.C. and Thompson, W.F. (1986) Purification and restriction endonuclease analysis of plant nuclear DNA. *Methods Enzymol*., **118**, 57-79.
77. Willetts, N. and Skurray, R. (1987) Structure and function of the F factor and mechanism of conjugation. In Neidhardt, F.C. (ed.), *Escherichia coli and Salmonella typhimurium*: *Cellular and Molecular Biology*. American Society for Microbiology, Washington, D.C., Vol. 2, pp. 1110-1133.
78. Woo, S.-S., Jiang, J., Gill, B.S., Paterson, A.H. and Wing, R.A. (1994) Construction and characterization of a bacterial artificial chromosome library of *Sorghum bicolor*. *Nucleic Acids Res*., **22**, 4922-4931.
79. Yang, D., Parco, A., Nandi, S., Subudhi, P., Zhu, Y., Wang, G. and Huang, N. (1997) Construction of a bacterial artificial chromosome (BAC) library and identification of overlapping BAC clones with chromosome 4-specific RFLP markers in rice. *Theor. Appl. Genet*., **95**, 1147-1154.
80. Yang, D., Sanchez, A., Khush, G.S., Zhu, Y. and Huang, N. (1998) Construction of a BAC contig containing the *xa5* locus in rice. *Theor. Appl. Genet*., **97**, 1120-1124.
81. Yang, Z., Choi, S., Frisch, D.A., Fang, D., Moonan, F., Molina, J., Plancarte, J.G., Roose, M.L., Wing, R.A. and Mirkov, T.E. (1999) Towards map-based cloning of the *Ctv* resistance gene from *Poncirus trifoliata*. Abstract P110, *Plant & Anim. Genome Conf. VII*, San Diego, CA.

82. Yu, Y., Tomkins, J., Frisch, D., Waugh, R., Brueggeman, R., Kudrna, D., Kleinhofs, A. and Wing, R. (1999) Construction and characterization of a barley (*Hordeum vulgare*) cv. Morex BAC library. Abstract P102, *Plant & Anim. Genome Conf. VII*, San Diego, CA.
83. Zhang, H.B., Choi, S., Woo, S.-S., Li, Z. and Wing, R.A. (1996) Construction and characterization of two rice bacterial artificial chromosome libraries from the parents of a permanent recombinant inbred mapping population. *Mol. Breeding*, **2**, 11-24.