Chapter 1

Plasmids and Their Usefulness in Molecular Cloning

INFORMATION PANELS

PLASMIDS ARE EXTRACHROMOSOMAL MOLECULES OF DNA that vary in size from 1 kb to more than 200 kb. Most of them are double-stranded, covalently closed, circular molecules that can be isolated from bacterial cells in a superhelical form. Plasmids:

- are found in a wide variety of bacterial species; most plasmids have a narrow host range and can be maintained only in a limited set of closely related species.
- are extrachromosomal elements that behave as accessory genetic units that replicate and are inherited independently of the bacterial chromosome.
- have evolved a variety of mechanisms to maintain a stable copy number of the plasmid in their bacterial hosts and to partition plasmid molecules accurately to daughter cells.
- are dependent, to a greater or lesser extent, on the enzymes and proteins encoded by their host for their replication and transcription.

- frequently contain genes coding for enzymes that are advantageous to the bacterial host. These genes specify a remarkably diverse set of traits, many of which are of great medical and commercial significance. Among the phenotypes conferred by plasmids are resistance to and production of antibiotics, degradation of complex organic compounds, and production of colicins, enterotoxins, and restriction and modification enzymes.

The word "plasmid," introduced by Joshua Lederberg in 1952, was defined as an extrachromosomal genetic element. It was supplanted for a while by "episome," a term proposed by Jacob and Wollman (1958) to describe an accessory genetic element that is transmissable from cell to cell and may be propagated either in the cytoplasm or, after insertion, as part of the bacterial chromosome. However, operational difficulties soon arose in deciding whether some extrachromosomal elements were plasmids, because they were never seen to insert into the host chromosome, or episomes that inserted at very low frequency. Hayes (1969) therefore suggested that the term episome "should be thanked for its services and sent into honourable retirement." This has not happened: Both words are now in common use, and the distinction between them has become blurred. However, most of the vectors discussed in this chapter are plasmids as defined by Lederberg and not episomes as defined by Jacob and Wollman. So, for readers who desire firm guidance in this matter, we say that "plasmid" is more correct than "episome" most of the time, but there are of course always exceptions.

THE REPLICONS OF PLASMIDS DEFINE THEIR COPY NUMBER

A replicon is a genetic unit consisting of an origin of DNA replication and its associated control elements. In plasmids, the origin of replication is a defined segment of DNA several hundred base pairs in length: Its set of associated *cis*-acting controlling elements contains sites for diffusible plasmid- and host-encoded factors involved in initiation of DNA synthesis. A plasmid replicon can therefore be defined as the smallest piece of plasmid DNA that is able to replicate autonomously and maintain normal copy number.

The term "replicon" was first used at the 1963 Cold Spring Harbor Symposium (Jacob et al. 1964) in a theoretical paper explaining how circular, extrachromosomal DNA molecules in bacteria might replicate. Since then, many of the predictions of the original prokaryotic model have been validated by biochemical and genetic experiments, and the definition of replicon has expanded to include chromosomal and extrachromosomal replication units in both prokaryotes and eukaryotes.

More than 30 different replicons have been identified in plasmids. However, almost all plasmids used routinely in molecular cloning carry a replicon derived from pMB1, a plasmid originally isolated from a clinical specimen (Hershfield et al. 1974). Plasmids carrying the primeval pMB1 replicon (or its close relative, the colicin E1 [colE1] replicon [Balbas et al. 1986]) maintain between 15 and 20 copies in each bacterial cell. However, over the years, the pMB1/colE1 replicon has been extensively modified to increase the copy number, and hence the yield, of plasmid DNA. High-copy-number plasmid vectors are available in huge variety, are the workhorses of molecular cloning, and are used for almost all routine manipulation of small segments of recombinant DNAs (<15 kb in size). By contrast, low-copy-number vectors, which carry replicons from sources other than pMB1/colE1 (Table 1-1), are required for special purposes. These include (1) cloning of DNA sequences that are unstable and genes that are lethal when propagated in high-copy-number plasmids and (2) constructing bacterial artificial chromosomes (BACs), which are vectors used to propagate large (~100 kb) segments of foreign DNA as plasmids in *Escherichia coli* (please see Chapter 2).

TABLE 1-1 Replicons Carried by Plasmid Vectors

PLASMID	REPLICON	COPY NUMBER	REFERENCES
pBR322	pMB1	15–20	Bolivar et al. (1977b)
pUC	modified form of pMB1	500–700	Vieira and Messing (1982, 1987); Messing (1983); Lin-Chao et al. (1992)
pMOB45	pKN402	15–118	Bittner and Vapnek (1981)
pACYC	p15A	18–22	Chang and Cohen (1978)
pSC101	pSC101	~5	Stoker et al. (1982)
colE1	colE1	15–20	Kahn et al. (1979)

REPLICATION OF PLASMIDS

Stringent and Relaxed Replication

The copy number of a plasmid is defined as the average number of plasmids per bacterial cell or per chromosome under normal growth conditions. Controlled by the plasmid replicon, the copy number can increase or decrease within a narrow range in response to changes in the growth conditions of the bacterial culture. At steady state, the population of plasmid doubles at exactly the same rate as the population of host cells, and the copy number remains constant.

Plasmids, whatever their replicon, maintain harmony between their rate of replication and that of the host by rationing the supply of a molecule that affects the frequency of initiation of plasmid DNA synthesis. In plasmids carrying the pMB1/colE1 replicon, this positive regulatory molecule is an RNA, known as RNAII, which is used to prime initiation of leading-strand DNA synthesis. However, the regulatory molecule of other replicons (e.g., pSC101) is a *cis*-acting protein (RepA) that acts positively on the origin of replication and negatively regulates the transcription of its own gene (Linder et al. 1985; for reviews, please see Nordström 1990; Nordström and Wagner 1994; Helinski et al. 1996). In all cases, the synthesis and activity of positive regulatory RNA and protein molecules are modulated by ancillary *trans*-acting products whose concentration is responsive to plasmid copy number or to alterations in the physiology of the host bacterium.

Plasmids whose positive regulatory molecule is an RNA molecule generally have high copy numbers and do not require any plasmid-encoded proteins for replication. Instead, they rely entirely on long-lived enzymes and proteins supplied by the host, including chaperones, DNA polymerases I and III, DNA-dependent RNA polymerase, ribonuclease H (RNase H), DNA gyrase, and topoisomerase I (for review, please see Helinski et al. 1996). These plasmids, which are said to replicate in a "relaxed" fashion, continue to duplicate when protein synthesis is inhibited by amino acid starvation (Bazaral and Helinski 1968) or by addition of an antibiotic such as chloramphenicol (Clewell and Helinski 1969) (please see the information panel on **CHLORAMPHENICOL**). Because protein synthesis is required for initiation of each round of host DNA synthesis but not for plasmid replication, the content of plasmid DNA in cells exposed to chloramphenicol increases relative to the amount of chromosomal DNA (Clewell 1972). Over the course of several hours of amplification, thousands of copies of a relaxed plasmid may accumulate in the cell; at the end of the process, plasmid DNA may account for 50% or more of the total cellular DNA. By contrast, plasmids such as pSC101 require ongoing synthesis of the RepA protein for replication, and their copy number cannot be amplified, nor their yield increased, by inhibiting cellular protein synthesis. Such plasmids are said to replicate under "stringent" control.

Initiation of DNA Synthesis at colE1 Origins Is Primed by RNAII

Initiation occurs within a 600-nucleotide region that contains all of the *cis*-acting elements required for replication. Synthesis of leading-strand DNA is primed by RNAII (Figure 1-1) (for review, please see Eguchi et al. 1991).

Synthesis of the precursor to RNAII is initiated at a promoter 550 bp upstream of the origin, proceeds through the origin, and terminates at one of a number of closely spaced sites locat-

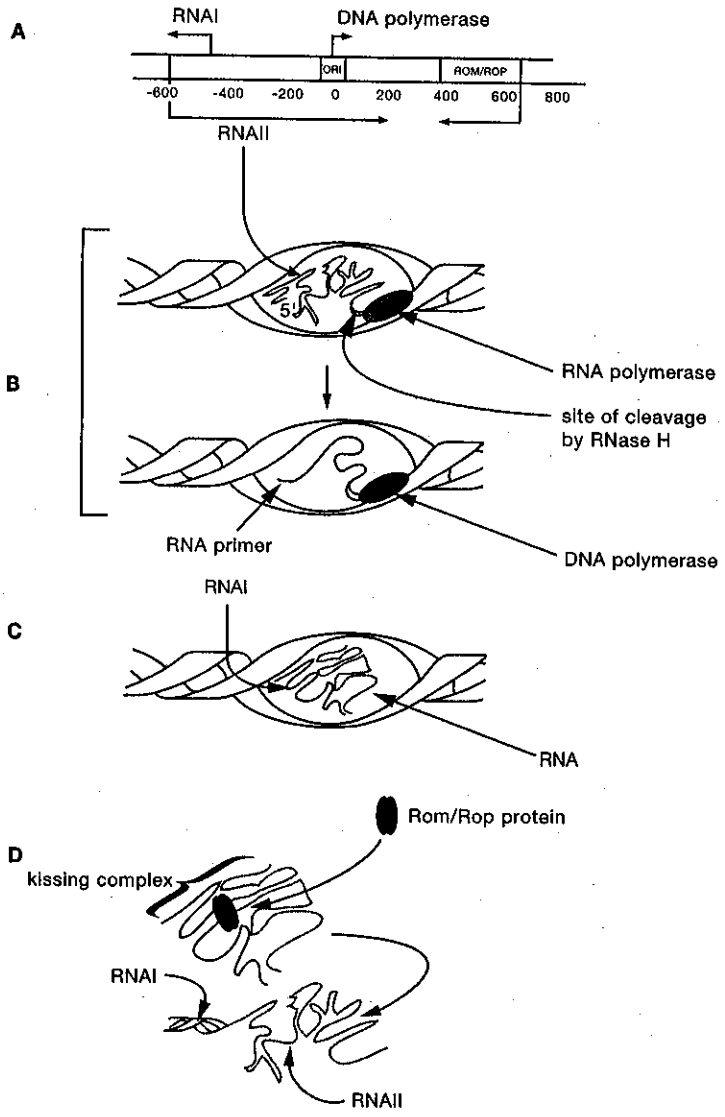

FIGURE 1-1 DNA Synthesis at the colE1 Replicon: Interaction between RNAI and RNAII

(*A*) Genetic map of the colE1 replicon with transcription patterns of this region. (*B*) RNAII serves as the primer for DNA synthesis at the colE1 replicon. During synthesis of RNAII, the 5′ region of the nascent molecule folds into a specific conformation that allows the growing 3′end to form a persistent DNA-RNA hybrid with the DNA template at the origin of replication. The 3′region of RNAII is processed by RNase H to generate a primer that is used for synthesis of the leading strand by DNA polymerase I. (*C*) The "kissing complex" between RNAI and RNAII is stabilized by binding of dimeric Rom/Rop protein. (*D*) Blow up of the "kissing complex" between RNAI and RNAII. The stable complex between RNAI and RNAII prevents DNA synthesis by suppressing the formation of the stable hybrid between RNAII and DNA.

ed ~150 nucleotides downstream. The 5′ end of the ~750-nucleotide primary transcript folds into a complex secondary structure that brings a G-rich loop in RNAII into alignment with a C-rich stretch of plasmid DNA located 20 nucleotides upstream of the origin on the template strand. The RNAII transcript is then processed into mature primer by RNase H, which cleaves the RNA within a sequence of five A residues at the origin. The resulting 555-nucleotide mature RNAII is used as a primer by DNA polymerase I to initiate synthesis of the leading strand (for reviews and references, please see Kües and Stahl 1989; Cesareni et al. 1991; Helinski et al. 1996). Extension of the stable DNA-RNA hybrid exposes sites on the complementary strand of DNA at which discontinuous synthesis of the lagging strand is initiated. Because lagging-strand synthesis is blocked ~20 nucleotides upstream of the origin by the unhybridized segments of RNAII, replication progresses unidirectionally in Cairns or θ structures in plasmids carrying pMB1/colE1 replicons.

RNAI Is a Negative Regulator of Replication

The colE1 replicon is unable to influence the activity of the host enzymes required for plasmid replication and, therefore, is unable to alter the speed or course of events that occur after DNA synthesis has been initiated. Consequently, control of copy number must be exerted at or before initiation of DNA replication. Synthesis of plasmid DNA depends on the formation of a persistent DNA-RNAII hybrid at the origin of replication. Under normal circumstances, initiation is controlled by altering the equilibrium between correctly folded RNAII, which can form the persistent hybrid, and inappropriately folded structures of RNAII, which cannot.

Sway over this equilibrium lies chiefly in the hands of RNAI, a small transcript of 108 bases encoded by the antisense strand of the RNAII gene. RNAI folds into a cloverleaf structure that binds to the nascent RNAII precursor and thereby prevents its folding into the secondary structure required for formation of the persistent hybrid (Lacatena and Cesareni 1981).

The mechanism by which RNAI and RNAII interact has been described in great detail by Tomizawa and his colleagues (e.g., please see Tomizawa 1990a). The picture that emerges is one of dynamic interactions between RNAI and short-lived folding intermediates of RNAII. The folding of RNAII is particularly vulnerable to interference by RNAI when the nascent RNAII transcript is between 80 and 360 nucleotides in length. The initial contacts occur between stem-loops in the two RNA molecules and lead to formation of a segment of double-stranded RNA that involves the 5′ sequences of RNAII and the entire length of RNAI. RNAI therefore controls plasmid copy number by acting as a negative regulator of initiation of plasmid DNA synthesis.

> The copy number of the pUC family of plasmids is much higher than that of other plasmids carrying a pMB1 or colE1 replicon. This is because pUC plasmids carry a point mutation that alters the secondary structure of the positive regulatory molecule (RNAII) in a temperature-dependent fashion. At 37°C or 42°C, RNAII appears to fold into a conformation that is resistant to inhibition by RNAI. Initiation of DNA synthesis is enhanced, resulting in abnormally high copy number. When the bacterial culture is grown at 30°C, the copy number of pUC plasmids is restored to normal (Lin-Chao et al. 1992).

The simplest hypothesis to explain the maintenance of plasmid copy number is that the steady-state concentration of cytoplasmic RNAI is determined by gene dosage (Tomizawa 1987; Chiang and Bremer 1991). Thus, if the copy number of the plasmid increases above normal, the concentration of RNAI will rise and plasmid DNA replication will be inhibited. However, this coupling between plasmid copy number and fluctuations in inhibitor concentration can work only if the half-life of RNAI is short (Pritchard 1984) and if the rate of degradation of RNAI is proportional to the rate of growth of the culture. These two conditions both appear to be fulfilled

under normal conditions of cell growth, where the half-life of RNAI is 1–2 minutes. Kinetic calculations show that this period is sufficiently short for the molecule to act as a real-time sensor of plasmid copy number (Brendel and Perelson 1993).

Degradation of RNAI proceeds in two stages. First, the five 5′-terminal nucleotides are removed by endonucleolytic cleavage by RNase E. The truncated molecule can still bind to RNAII but is now susceptible to degradation by a ribonuclease whose activity is responsive to growth rate (Lin-Chao and Cohen 1991). This regulation provides a mechanism to maintain a constant number of plasmids even when the growth rate of cells is fitful.

The Rom/Rop Protein Empowers the Negative Regulatory Activity of RNAI

The efficiency of binding of RNAI to RNAII is improved by a plasmid-encoded protein known as Rom (RNAI modulator) or Rop (repressor of primer). By improving the efficiency of hybrid formation between RNAI and RNAII, Rom enhances the negative regulatory action of RNAI. Accordingly, deletion of the *rom/rop* gene increases the copy number of colE1 plasmids by at least two orders of magnitude (Twigg and Sherratt 1980). For example, deletion of the *rom/rop* genes raises the copy number of the old war horse pBR322 from 15–20 copies to more than 500 copies per bacterial cell, whereas insertion of a segment of foreign DNA into the *rom/rop* gene causes lethal runaway plasmid DNA replication (Giza and Huang 1989).

Rom is a homodimer of a 63-amino-acid polypeptide encoded by a gene lying 400 nucleotides downstream from the colE1 origin of replication (Twigg and Sherratt 1980; Tomizawa and Som 1984). Each subunit of the dimer consists of two α helices connected by a sharp bend; therefore, the dimer is a tight bundle of four α helices exhibiting twofold symmetry (Banner et al. 1987). Rom binds to RNAI and RNAII with similar affinities (Helmer-Citterich et al. 1988) and drives unstable intermediates formed between the two complementary RNAs into a more stable structure (Lacatena et al. 1984; Tomizawa and Som 1984; Tomizawa 1990b). Most probably, each of the two subunits of Rom recognizes sequence and structural elements in both RNAI and RNAII. Rom binds to a stem on the interacting RNAs, stabilizing the "kissing" complex (Tomizawa 1985) and initiating formation of a perfect RNAI-RNAII hybrid (Eguchi and Tomizawa 1990).

INCOMPATIBILITY OF PLASMIDS

When two plasmids share elements of the same replication machinery, they compete with each other during both replication and the subsequent step of partitioning into daughter cells. Such plasmids are unable to coexist without selection in bacterial cultures. This phenomenon is known as incompatibility (for reviews, please see Davison 1984; Novick 1987).

Plasmids carrying the same replicon belong to the same incompatibility group and are unable to be maintained within the same bacterium. Plasmids carrying replicons whose components are not interchangeable belong to different incompatibility groups and can be maintained in the same bacterium. Examples of plasmids that are compatible with colE1-type plasmids are p15A, R6K, and F.

Plasmids carrying the same replicon are selected at random from the intracellular pool for replication. However, this does not guarantee that the copy numbers of two plasmids will remain constant in a bacterial population. Larger plasmids, for example, require more time to replicate than do smaller plasmids and are at a selective disadvantage in every cell of the bacterial population. Plasmids of similar size may also be incompatible because of imbalances in the efficiency of initiation resulting from stochastic processes within individual bacterial cells. Such turns of

TABLE 1-2 Control Elements That Regulate Replication

Incompatibility Groups	Negative Control Element	Comment
colE1, pMB1	RNAI	controls processing of pre-RNAII into primer
IncFII, pT181	RNA	controls synthesis of RepA protein
P1, F, R6K, pSC101, p15A	iterons	sequesters RepA protein

chance can lead rapidly to drastic differences in the copy number of the two plasmids. In some cells, one plasmid might dominate, whereas in other cells, its incompatible partner might prosper. Over the course of a few generations of bacterial growth in the absence of selection, the minority plasmid may be completely eliminated from some cells of the population. Descendants of the original cell may contain one plasmid or the other, but very rarely both.

The regions of plasmid DNA that confer incompatibility can be identified by introducing segments of the DNA into an unrelated multicopy replicon and determining the ability of a test plasmid to coexist with the hybrid. For example, the incompatibility locus of the stringent, low-copy-number plasmid pSC101 maps to a series of directly repeated ~20-bp sequences known as iterons, located at the origin of replication. The iterons, in conjunction with the nearby *cis*-acting *par* locus, appear to "handcuff" plasmid DNA molecules by sequestering the plasmid-encoded RepA protein so that it can no longer facilitate binding of host-encoded proteins to the origin (for review, please see Nordström 1990). By contrast, in the case of colE1 described above, incompatibility is defined by the inhibitory activity of RNAI (for review, please see Novick 1987). Any two plasmids that are isogenic for RNAI and use it for regulation are incompatible, whether or not they share any other functions (Tomizawa and Itoh 1981).

As discussed earlier, most vectors in current use carry a replicon derived from the plasmid pMB1. These vectors are incompatible with all other plasmids carrying the colE1 replicon but are fully compatible with iteron-binding replicons such as those in pSC101 and its derivatives. Table 1-2 lists several well-known plasmids and negative control elements involved in regulating their replication.

PLASMID VECTORS

Selectable Markers

Plasmid vectors contain genetic markers that confer strong growth advantages upon plasmid-bearing bacteria under selective conditions. In molecular cloning, these markers are used:

- ***To select clones of plasmid-bearing bacteria:*** In the laboratory, plasmid DNA can be introduced into bacteria by the artificial process of transformation. However, even under the best conditions, transformation is generally inefficient, and plasmids become stably established in only a small minority of the bacterial population. Selectable markers carried by the plasmid allow these rare transformants to be selected with ease. These plasmid-encoded markers provide specific resistance to (i.e., the ability to grow in the presence of) antibiotics such as the kanamycins, ampicillin and carbenicillin, and the tetracyclines. The properties and modes of action of these antibiotics are discussed in information panels at the end of this chapter.
- ***To indemnify transformed bacteria against the risks imposed by their burden of plasmid DNA or plasmid-encoded proteins:*** Plasmids present at low copy numbers (<20 copies/cell) do not

appear to unduly handicap their host cells. However, much evidence shows that high-copy-number plasmids and large quantities of recombinant proteins can severely hamper the growth, and even the survival, of transformed cells (Murray and Kelley 1979; Beck and Bremer 1980). To prevent the emergence of bacteria from which the plasmid has been eliminated, it is important to sustain selective pressure by including the appropriate antibiotic in the culture medium at all times.

1973–1978

In the early 1970s, selectable markers, typically *kan*^{r} or *amp*^{r} or *tet*^{r}, were introduced into plasmids carrying the pMB1 (or colE1) replicon (please see the information panels on **KANAMYCINS**, **TETRACYCLINE**, and **AMPICILLIN AND CARBENICILLIN**). The first plasmids used as cloning vectors — pSC101 (Cohen et al. 1973), colE1 (Hershfield et al. 1974), and pCR1 (Covey et al. 1976) — were limited in their versatility: Either they replicated poorly or they carried unsuitable selectable markers, and none of them contained more than two restriction sites that could be used for cloning. The first plasmid to combine all of the then-available desirable features was pBR313 (Bolivar et al. 1977a,b; please see the information panel on **pBR322**). It replicated in a relaxed fashion, contained two selectable markers (*tet*^{r} and *amp*^{r}), and carried a number of useful restriction sites. However, pBR313 was unnecessarily large; more than half of its DNA was not essential for its role as a vector. The first phase of plasmid vector development ended with the construction of pBR322 (Bolivar et al. 1977b), a plasmid of 4.36 kb from which most of these unnecessary sequences had been eliminated. pBR322 was the most widely used cloning vehicle of its day, and many of the plasmid vectors in current use are its distant descendants (for review, please see Balbas et al. 1986).

1978–1983

This period saw the evolution of clunky plasmids such as pBR322 into vectors that were smaller in size, higher in copy number, and able to accept fragments of foreign DNA generated by cleavage with a wider range of restriction enzymes. There is no strict upper limit to the size of DNA fragments that can be cloned in plasmids. However, there are advantages in reducing the size of plasmid vectors to a minimum. Plasmid copy number, stability, and transforming efficiency all increase as the size of their DNA is reduced. Smaller plasmids can accommodate larger segments of foreign DNA before their efficiency begins to deteriorate. In addition, because smaller colE1 plasmids replicate to higher copy numbers, the yield of foreign DNA is increased and hybridization signals are fortified when transformed colonies containing cloned foreign DNA sequences are screened with radiolabeled probes.

In the late 1970s and early 1980s, the problems of unwieldiness and inefficiency were addressed when streamlined derivatives of pBR322 were constructed. These plasmids lacked ancillary sequences involved in the control of copy number and mobilization. Unfortunately, the first-generation high-copy-number plasmids, of which pXf3 (Hanahan 1983) and pAT53 (Twigg and Sherratt 1980) were the best known, suffered from a major defect: Foreign DNA sequences could be inserted only at a limited number of restriction sites located within the "natural" sequences used to construct the plasmid. Within a year or two, these plasmids had been replaced by a revolutionary series of vectors (pUC vectors), in which the number of restriction enzyme cleavage sites was expanded and their distribution within the vector was rationalized (Messing

1983; Norrander et al. 1983; Yanisch-Perron et al. 1985; Vieira and Messing 1987). The pUC vectors were the first plasmids to contain a closely arranged series of synthetic cloning sites, termed polylinkers, multiple cloning sites, or polycloning sites, that consist of banks of sequences recognized by restriction enzymes. In most cases, these restriction sites are unique; i.e., they are not found elsewhere in the plasmid vector. For example, the polycloning site from the vector pUC19 consists of a tandem array of unique cleavage sites for 13 restriction enzymes: *Hin*dIII, *Sph*I, *Pst*I, *Sal*I, *Acc*I, *Hin*cII, *Xba*I, *Bam*HI, *Sma*I, *Xma*I, *Kpn*I, *Sac*I, and *Eco*RI.

Such arrays of recognition sequences provide a vast variety of targets that can be used singly or in combination to clone DNA fragments generated by cleavage with a large number of restriction enzymes. Furthermore, fragments inserted at one restriction site can often be excised by cleavage of the recombinant plasmid with restriction enzymes that cleave at flanking sites. Insertion of a segment of DNA into a polycloning site is therefore equivalent to adding synthetic linkers to its termini. The availability of these flanking sites greatly simplifies the task of mapping the segment of foreign DNA.

A potential disadvantage of drawing together all cloning sites into one location in a plasmid is the inability to use inactivation of a selectable marker to screen for recombinants. This method had been used extensively with first-generation plasmids, such as pBR322, that carry two or more different selectable markers, e.g., *tet*^{r} and *amp*^{r}, each containing a "natural" restriction site. Insertion of foreign DNA sequences into one of these sites inactivated one of the two markers. Bacteria containing recombinant plasmids could therefore be distinguished from those carrying the empty parental vector by virtue of their ability to grow in only one of the two sets of selective conditions (please see the information panel on **AMPICILLIN AND CARBENICILLIN**).

Insertional inactivation is not possible with pUC vectors, which carry only one antibiotic resistance gene (typically *amp*^{r}) and an aggregated set of cloning sites. However, recombinant plasmids can be readily distinguished from parental pUC plasmids by screening the color of bacterial colonies. pUC vectors and many of their derivatives carry a short segment of *E. coli* DNA that contains the regulatory sequences of the *lacZ* gene and the coding information for the amino-terminal 146 amino acids of β-galactosidase. Embedded in the coding information, just downstream from the initiating ATG, is a multiple cloning site. The small amino-terminal fragment of β-galactosidase expressed by pUC vectors in transformed bacteria has no endogenous β-galactosidase activity. However, the amino-terminal fragment, known as the α-fragment, can complement certain mutants of β-galactosidase, which are themselves inactive, producing an enzyme that has abundant catalytic activity. α-complementation occurs when pUC plasmids are introduced into strains of *E. coli* that express an inactive carboxy-terminal fragment (the ω-fragment) of β-galactosidase.

When a segment of foreign DNA is cloned into the multiple cloning site of pUC vectors, the sequence encoding the α-fragment is disrupted, and α-complemention is either greatly suppressed or abolished altogether. Bacterial colonies containing recombinant plasmids are therefore *amp*^{r} and contain little or no β-galactosidase activity. By contrast, bacterial colonies containing empty plasmids are *amp*^{r} and are able to hydrolyze nonfermentable, chromogenic substrates such as 5-bromo-4-chloro-3-indole-β-D-galactoside (X-gal) (Horwitz et al. 1964; Davies and Jacob 1968; please see the information panels on **X-GAL** and on **α-COMPLEMENTATION**). The two types of colonies can therefore be distinguished by a simple, nondestructive histochemical test (Miller 1972). When X-gal is included in the agar medium, colonies carrying parental nonrecombinant plasmids become deep blue, whereas those containing recombinant plasmids either remain an ordinary creamy white or become tinted in pale egg-shell blue (for more details, please see the information panel on **α-COMPLEMENTATION**).

1983–Present

The latest phase of construction of plasmid vectors has involved the incorporation of ancillary sequences that are used for a variety of purposes, including generation of single-stranded DNA templates for DNA sequencing, transcription of foreign DNA sequences in vitro, direct selection of recombinant clones, and expression of large amounts of foreign proteins. These specialized functions are discussed briefly here and in more detail in later chapters.

Plasmid Vectors Carrying Origins of Replication Derived from Single-stranded Bacteriophages

Many plasmid vectors in current use carry the origin of DNA replication from the genome of a single-stranded filamentous bacteriophage such as M13 or f1 (please see Chapter 3). Such vectors, which are sometimes called phagemids, combine the best features of plasmid and single-stranded bacteriophage vectors and have the advantage of two separate modes of replication: as a conventional double-stranded DNA plasmid and as a template to produce single-stranded copies of one of the phagemid strands. A phagemid can therefore be used in the same way as an orthodox plasmid vector, or it can be used to produce filamentous bacteriophage particles that contain single-stranded copies of cloned segments of DNA. Since their introduction in the early 1980s, phagemids have eliminated much of the need to subclone segments of foreign DNA from plasmids into conventional single-stranded bacteriophage vectors.

Production of single-stranded DNA is induced when bacteria carrying a phagemid are infected with a helper bacteriophage that carries the genes required to (1) generate single-stranded DNA from a double-stranded template and (2) package the single-stranded DNA into filamentous virus particles. The defective filamentous virions secreted from a small-scale culture of infected bacteria contain sufficient single-stranded DNA for sequencing (please see Chapter 12; for preparation of radiolabeled single-stranded probes, please see Chapter 9 or for site-directed mutagenesis, please see Chapter 13).

In most cases, pairs of plasmid vectors are available that differ in the orientation of the bacteriophage origin of replication. The orientation of the origin determines which of the two DNA strands will be encapsidated into bacteriophage particles. By convention, a plus sign (+) indicates that the origin in the plasmid and that in the bacteriophage particle are in the same orientation. For more details on the design and use of phagemids, please see Chapter 3.

Plasmid Vectors Carrying Bacteriophage Promoters

Many plasmid vectors carry promoters derived from bacteriophages T3, T7, and/or SP6 adjacent to the multiple cloning site (MCS). Foreign DNAs inserted at restriction sites within the MCS can therefore be transcribed in vitro when the linearized recombinant plasmid DNA is incubated with the appropriate DNA-dependent RNA polymerase and ribonucleotide precursors (please see Chapter 9). These promoters are so specific that RNA polymerase from SP6, for example, will not synthesize RNA from any other bacteriophage promoter located elsewhere in the plasmid.

Many commercial vectors (e.g., vectors of the pGEM series or the Bluescript series) carry two bacteriophage promoters in opposite orientations, located on each side of the multiple cloning site (Short et al. 1988). This organization allows RNA to be synthesized in vitro from either end and either strand of the foreign DNA, depending on the type of RNA polymerase used in the transcription reaction. The RNAs generated in this way can be used as hybridization probes or can be translated in cell-free protein-synthesizing systems. In addition, vectors carrying the T7

promoter can be used to express cloned DNA sequences in bacteria expressing T7 RNA polymerase (please see Chapter 9, Protocol 8) (Tabor and Richardson 1985).

Positive Selection Vectors

Identifying plasmids with DNA inserts can be frustrating and time-consuming. However, a variety of cloning vectors have been developed that allow growth only of bacterial colonies carrying recombinant plasmids (for reviews, please see Burns and Beacham 1984; Hengen 1997). Bacteria containing the empty parental plasmid are unable to form colonies under selective conditions. Typically, the plasmids used in these systems express a gene product that is lethal for certain bacterial hosts; cloning a segment of foreign DNA into the plasmid inactivates the gene and relieves the toxicity. For example, Bochner et al. (1980), Maloy and Nunn (1981), and Craine (1982) describe conditions under which transformed bacteria carrying plasmid vectors coding for *tet*^{r} will die, whereas recombinant plasmids carrying a segment of foreign DNA within the *tet*^{r} gene will grow. Other conditionally lethal genes used in various positive selection vectors include those encoding the bacteriophage λ repressor (Nilsson et al. 1983; Mongolsuk et al. 1994), *Eco*RI methylase (Cheng and Modrich 1983), *Eco*RI endonuclease (Kuhn et al. 1986), galactokinase (Ahmed 1984), colicin E3 (Vernet et al. 1985), transcription factor GATA-1(Trudel et al. 1996), the lysis protein of ϕX174 (Henrich and Plapp 1986), the *ccdB* gene of *E. coli* (Bernard 1995, 1996), and barnase (Yazynin et al. 1996). Ingenious as these positive selection systems may be, few of them have found wide use. In many cases, the number of potential cloning sites is limited, the efficiency of the selection may be variable, special host cells may be required, and the plasmids may be devoid of desirable features (e.g., bacteriophage promoters and bacteriophage M13 origin of DNA replication). In consequence, most investigators prefer to reduce the background of empty plasmids by other means, for example, by optimizing the ratio of vector DNA to insert in the ligation reaction, dephosphorylating the vector, or using directional cloning. Colonies containing the desired recombinant are then identified by hybridization in situ to radiolabeled probes, restriction analysis of small-scale preparations of plasmids, and/or polymerase chain reaction (PCR) amplification of inserts.

Low-copy-number Plasmid Vectors

By contrast to conventional high-copy-number plasmid vectors, which carry souped-up versions of the colE1 replicon, low-copy-number plasmid vectors are built around replicons such as R1 that keep plasmid DNA synthesis under a very tight rein.

The first generation of low-copy vectors — rather bulky and fairly rough-hewn by today's standards — was designed to solve problems of toxicity that arose when particular types of foreign genes and DNA sequences were cloned in plasmid vectors. Many genes coding for membrane and DNA-binding proteins fall into this class, as do certain promoters and regulatory sequences (e.g., please see Fiil et al. 1979; Hansen and von Meyenberg 1979; Little 1979; Murray and Kelley 1979; Beck and Bremer 1980; Spratt et al. 1980; Claverie-Martin et al. 1989). Sometimes, these DNA sequences and gene products are so toxic to the host bacteria that it is simply impossible to isolate transformed strains using high-copy-number vectors. If transformants are obtained, their growth rate is often frustratingly slow, and the cloned foreign DNA sequences are often unstable. To solve these problems, multipurpose low-copy-number vectors have been developed that carry tightly regulated prokaryotic promoters with a low level of basal expression, for example, the pET series of vectors, and prokaryotic transcription terminators to prevent spurious transcription of foreign DNA sequences from upstream plasmid promoters. These low-copy-number vectors now come equipped with multiple cloning sites, origins of replication of single-stranded bacterio-

phages, T3 and T7 promoters, and other useful modular conveniences of proven worth. Most modern low-copy-number vectors also carry *par* loci that promote accurate partitioning of plasmid molecules into daughter cells during cell division. Problems of plasmid instability may also be solved by using an *E. coli* strain that suppresses replication of colE1 plasmids. Most strains of *E. coli* used as hosts for colE1 plasmids carry a wild-type version of a gene known as *pcnB*, which codes for poly(A) polymerase. Wild-type *pcnB* promotes the decay of RNAI, the negative regulator of copy number of colE1 plasmids, by adding adenylate residues to the 3′ terminus of RNAI. In its polyadenylated form, RNAI is highly unstable and is therefore unable to prevent formation of RNAII, the primer for plasmid DNA synthesis. In strains of *E. coli* bearing a mutant *pcnB* allele, RNAI remains unadenylated and its half-life is extended. Processing of RNAII is suppressed and the copy number of colE1 plasmids is thereby reduced by a factor of ~10. Many recombinant colE1 plasmids that are unstable in conventional *E. coli* hosts can be grown successfully in *pcnB* mutant strains (He et al. 1993; Ellis et al. 1995; Podkovryov and Larson 1995; Pierson and Barcak 1999).

Runaway-replication Plasmid Vectors

Runaway vectors replicate in a normal fashion at temperatures up to 34°C. However, their copy number increases as the temperature of the culture is raised until, at 39°C, plasmid replication becomes uncontrolled. Vectors based on the low-copy-number IncFII plasmid R1 have been converted to runaway-replication vectors by artificially increasing the rate of synthesis of *rep*A mRNA, for example, by placing the *repA* gene under the control of the bacteriophage λ p_R or p_L promoter. The activity of this promoter is in turn controlled by the temperature-sensitive λ repressor *c*I857 (for review, please see Nordström and Uhlin 1992). Because runaway amplification occurs in the presence of protein synthesis, the expressed product of a foreign DNA cloned in a runaway plasmid may eventually constitute 50% of the protein in a bacterial cell in which plasmid replication has gone amok (e.g., please see Remaut et al. 1983).

Runaway plasmid replication and associated production of plasmid-encoded proteins place the cell under severe metabolic strain that is reflected in a decreased growth rate and, sometimes, cell death (Uhlin and Nordström 1978; Uhlin et al. 1979; Remaut et al. 1983). For this reason, it is important to ascertain the time of induction required to obtain maximal yields of the intact target protein.

Plasmid Expression Vectors

A large number of plasmid vectors have been constructed that contain powerful promoters capable of generating large amounts of mRNA in vivo from cloned foreign genes. Nowadays, the activity of many of these promoters can be stringently regulated so that there is (1) minimal basal expression of the target gene under repressed conditions and (2) fast and dramatic induction of expression of the cloned gene in response to simple changes in the conditions of culture. For native proteins to be produced in large quantities, the vector must contain an efficient Shine-Dalgarno sequence upstream of the initiating ATG codon. The distance between the Shine-Dalgarno sequence and the ATG codon is crucial (Shine and Dalgarno 1975) if maximal expression of the foreign protein is to be achieved.

In many cases, plasmid expression vectors are designed to express foreign proteins that are not linked to any prokaryotic sequences; more commonly, however, expression vectors generate fusion proteins that are encoded partly by the vector and partly by an open reading frame in the cloned segment of foreign DNA. The foreign protein is therefore synthesized as a fused polypep-

tide containing a tract of amino acids that are not normally part of the native protein. In the early days of cloning, the tract of foreign amino acids was often large enough to produce dramatic changes in the physical and biological properties of the protein under study. Solubility and stability could be altered for better or worse, and there was a good chance that the biological properties of the protein would be compromised, at least to some extent.

During the last few years, the sequences contributed by the vector have shrunk dramatically in size. In most cases, they are less than a dozen residues in length and generally do not affect the function of the protein under study. Frequently, these "tags" are antigenic determinants (epitopes) that are recognized by specific antibodies. Epitope-tagged proteins can be purified with an existing epitope-specific antibody (for reviews, please see Kolodziej and Young 1991; Keesey 1996). The same antibody can be used to detect the epitope tag in a variety of expressed proteins.

The virtues and limitations of expressing proteins in these three forms — as native proteins, as fusion proteins, and as epitope-tagged proteins — are discussed in more detail in Chapter 18.

Finding Plasmid Vectors Appropriate for Specific Tasks

When looking for common or garden-variety plasmids that can be used for a wide range of general purposes, the first port of call should be the catalogs of commercial suppliers. Often, these companies will have something with a suitable combination of markers, modules, cloning sites, and epitopes that can be used without extensive engineering. These off-the-shelf vectors have been tested under a wide variety of conditions in many laboratories. It does not take a rocket scientist to make them work well.

Unfortunately, there is no easy and certain way to search the literature for descriptions of plasmid vectors with unusual properties that are suited to particular purposes. Obviously, the ability to carry out Boolean searches of databases such as Medline, Entrez, and PubMed with the logic operators AND, OR, and NOT is a great advantage. For example, trawling most Medline-based databases with the string (p15A or IncFII)[TW] AND T7[TW] AND low-copy-number should generate a list of references to papers whose title includes "low-copy-number" and whose text contains the words "T7" and either "p15A" or "IncFII." In addition, papers describing specialized or novel plasmid vectors are still published regularly in archival journals such as *Gene* and *BioTechnology*. Once one or two promising papers have been identified — either from the scientific literature or by Boolean searching — they can be used as starting points for an expanded search of databases for additional papers on the same or closely related topics. The PubMed system, which can be accessed via the Internet, is very good at assembling clusters of papers on topics related to a particular keyword. The address of PubMed is http://www.ncbi.nlm.nih.gov/PubMed/.

One never knows whether a vector unearthed from the literature will work as advertised and if it is actually the best currently available. The authors of the paper are usually able to offer sensible advice in this regard. However, beware if they start talking about making improvements to the published vector. This is a sure sign that the original vector did not work as well as advertised; the chances are that the improved version will not be much better. If possible, find out the names of other investigators who have used the vector and who may have found ways to identify and solve the problems.

Choosing an Appropriate Strain of *E. coli*

Most investigators want to use strains of *E. coli* that are easy to transform with plasmid DNA (e.g., DH1 or MM294; for a full list of useful strains, please see Appendix 3). The vast majority of colE1-type plasmids introduced into these strains replicate to high copy number and can be iso-

lated in high yield. However, a significant minority of recombinant plasmids transform strains of *E. coli* such as DH1 and MM294 with low efficiency, generate transformed colonies that are smaller than usual, and produce low yields of plasmid DNA. Most of these "difficult" plasmids can be shown to encode a protein that is toxic to *E. coli* or to contain inverted or repeated DNA sequences.

The problem of toxic proteins can be alleviated by switching to an amplifiable low-copy-number vector or to a high-copy-number vector containing prokaryotic transcription termination signals that suppress readthrough transcription of foreign DNA sequences. Another possibility, however, is to use strains of *E. coli* that suppress the copy number of colE1-based plasmids. Several commercially available strains of *E. coli* (e.g., ABLE C and ABLE K strains from Stratagene) reduce the copy number of colE1 plasmids (and hence the level of plasmid-encoded toxic proteins) by four- to tenfold. The yield of plasmid DNA from such strains, albeit reduced, is sufficient for most purposes in molecular cloning.

If there is reason to suspect that a plasmid may carry repeated DNA sequences that are substrates for the general recombination systems of *E. coli*, consider the possibility of switching to a recombination-deficient strain. For example, strains carrying a *rec*A mutation have almost no recombination capacity (Weinstock 1987) and are the preferred hosts for many targeting vectors used in gene knock-out experiments in mice, such as vectors that contain two copies of a viral thymidine kinase gene. Wayward inserts can also be stabilized in strains carrying *recB* mutations, which inactivate exonuclease V and reduce general recombination to a few percent of normal. Finally, improved yields of certain plasmids have been reported in strains deficient in SOS repair or DNA repair that carry mutations in the *umuC* and *uvrC* genes (Doherty et al. 1993).

Inverted repeat sequences are often lethal to their carrier plasmid. Thus, recombinant plasmids containing perfect or near-perfect inverted repeats longer than ~300 bp fail to transform conventional host strains of *E. coli* or do so with very low efficiency. In many cases, the forced propagation of such clones provokes internal deletions or other rearrangements that remove the center of symmetry of the palindrome (e.g., please see Hagan and Warren 1983). Sequences containing head-to-head palindromes are lethal, perhaps because they inhibit DNA replication by interfering with the passage of replication forks or because they deleteriously affect the state of supercoiling of the plasmid, leaving it open to attack by nucleases (Collins and Hohn 1978; Lilley 1980; Collins 1981; Mizuuchi et al. 1982; Hagan and Warren 1983).

No strain of *E. coli* exists that is guaranteed to propagate all recombinant clones containing palindromic sequences. *E. coli* strains carrying mutations in *recBC* and *sbcBC* genes will support growth of plasmids containing certain palindromic sequences of a variety of sizes and sources. However, plasmids with a colE1 origin are unstable in *recBC, sbcBC* hosts because they form linear multimers (e.g., please see Cohen and Clark 1986), which apparently interfere with replication and partition of chromosomal DNA (Kusano et al. 1989). Multimer formation is dependent on a subset of proteins involved in the RecF recombination pathway function and does not occur in cells that are deficient in RecF, RecJ, RecA, RecO, or RecQ function.

> Only a few strains deficient in *recBC* and *sbcBC* genes also contain mutations that eliminate all known restriction systems. These include PMC128, which is *mcrAΔ(mcrBC-hsd-mrr)recBC sbcBC* (Doherty et al. 1993), and SURE, and SRB (Stratagene), which carry similar mutations.

Finally, if plasmid vectors are used to propagate methylated DNA (e.g., mammalian genomic DNA or DNA synthesized in vitro using methylated analogs of deoxynucleoside triphosphates), then it is essential to use a strain that is deficient in the McrA, McrBC, and Mrr/Mcf restriction systems. The McrA and McrBC systems recognize and restrict certain DNA sequences containing methylated cytosine residues (Raleigh and Wilson 1986), whereas the Mrr/Mcf system recognizes

and restricts certain DNA sequences containing methylated adenine DNA residues (Heitman and Model 1987), as well as additional DNA sequences that contain methylated cytosine residues.

Further information on restriction modification systems can be obtained from REBASE, the Restriction Enzyme Database at http://www.neb.com/rebase. For more details on the properties of useful *E. coli* strains, see Appendix 3.

EXTRACTION AND PURIFICATION OF PLASMID DNA

Many methods have been developed to purify plasmids from bacteria. These methods invariably involve three steps:

- growth of the bacterial culture
- harvesting and lysis of the bacteria
- purification of the plasmid DNA

Growth of the Bacterial Culture

Wherever possible, plasmids should be purified from bacterial cultures that have been inoculated with a single transformed colony picked from an agar plate. Usually, the colony is transferred to a small starter culture, which is grown to late log phase. Aliquots of this culture can be used to prepare small amounts of the plasmid DNA (minipreparation) for analysis and/or as the inoculum for a large-scale culture. The conditions of growth of the large-scale culture depend chiefly on the copy number of the plasmid and whether it replicates in a stringent or relaxed fashion (please see Table 1-3). At all times, the transformed bacteria should be grown in selective conditions, i.e., in the presence of the appropriate antibiotic.

Harvesting and Lysis of the Culture

Bacteria are recovered by centrifugation and lysed by any one of a large number of methods, including treatment with nonionic or ionic detergents, organic solvents, alkali, and heat. The choice among these methods is dictated by three factors: the size of the plasmid, the strain of *E. coli*, and the technique used subsequently to purify the plasmid DNA. Although it is impractical to give precise conditions for all possible combinations of plasmid and host, the following general guidelines can be used to choose a method that will give satisfactory results.

Large Plasmids (>15 kb in Size) Must Be Handled with Care

Plasmids >15 kb in size are susceptible to damage during both cell lysis and subsequent handling. Gentle lysis is best accomplished by suspending the bacteria in an isosmotic solution of sucrose and treating them with lysozyme and EDTA (ethylenediaminetetraacetic acid), which removes much of the cell wall. The resulting spheroplasts are lysed by adding an anionic detergent such as SDS. For methods for tender handling of large DNAs, please see the information panel on **MINIMIZING DAMAGE TO DNA MOLECULES** in Chapter 2.

Smaller Plasmids (<15 kb in Size) Are More Durable

When handling smaller plasmids, more severe methods of lysis can be used, and no special care need be taken to minimize shearing forces. Typically, bacterial suspensions are exposed to deter-

TABLE 1-3 Plasmid Growth and Replication

Replicon (Example)	Copy Number	Stringent or Relaxed	Comments
Modified pMB1 (pUC)	several hundred	relaxed	pUC plasmids contain a modified pMB1 replicon and replicate to a very high copy number. Further amplification of the copy number by addition of chloramphenicol to the growing bacterial culture is unnecessary; instead, the culture should be grown to late log phase with vigorous shaking.
colE1 (pBR322)	15–20	relaxed	The yield of pBR322 and other relaxed plasmids that maintain a low-moderate copy number in transformed cells can be dramatically increased by adding chloramphenicol (final concentration 170 μg/ml) to mid-log phase cultures and continuing incubation for a further 8 hours. Chloramphenicol inhibits host protein synthesis and, as a result, prevents replication of the host chromosome. However, replication of relaxed plasmids continues, and their copy number increases progressively for several hours.
pSC101 (pSC101)	~5	stringent	Stringently replicating, low-copy-number plasmids can be a challenge to grow. Obviously, adding chloramphenicol to the culture is not an option and the only available variable is the culture medium. For example, "Terrific Broth," which has been reported to increase the yield of difficult plasmids (Tartof and Hobbs 1987) might be a better option than standard Luria Broth (LB).

As discussed above, the copy numbers of the current generation of plasmids are now so high that selective amplification in the presence of chloramphenicol is no longer required to achieve high yields of plasmid DNA. However, some investigators continue to use chloramphenicol, not necessarily to increase the yield of plasmid DNA but to reduce the bulk of bacterial cells in large-scale preparations. Handling large quantities of viscous lysates of concentrated suspensions of bacteria is a frustrating and messy business that can be avoided if chloramphenicol is added to the culture at mid-log phase. Because some amplification of copy number — even of such feverishly replicating plasmids as pUC — occurs in the presence of chloramphenicol, equivalent yields of plasmid DNA are obtained from smaller numbers of cells that have been exposed to the drug as from larger number of cells that have not.

gent and lysed by boiling or treatment with alkali. This disrupts base pairing and causes the linear stretches of sheared or disrupted chromosomal DNA of the host to denature. However, the strands of closed circular plasmid DNA are unable to separate from each other because they are topologically intertwined. When conditions are returned to normal, the strands of plasmid DNA fall into perfect register and native superhelical molecules are re-formed.

Prolonged exposure to denaturing conditions causes closed circular DNA to enter an irreversibly denatured state (Vinograd and Lebowitz 1966). The resulting collapsed coil, which cannot be cleaved with restriction enzymes, migrates through agarose gels at about twice the rate of native superhelical closed circular DNA and stains poorly with intercalating dyes such as ethidium bromide. Varying amounts of this collapsed form of DNA can usually be seen in plasmids prepared by alkaline or thermal lysis of bacteria.

Some Strains of E. coli *Should Not Be Lysed by Heat*

Some strains of *E. coli*, particularly those derived from HB101, release relatively large amounts of carbohydrate when they are lysed by detergent and heat. This can be a nuisance when the plasmid DNA is subsequently purified by equilibrium centrifugation in cesium chloride (CsCl)-ethidium bromide gradients. The carbohydrate forms a dense fuzzy band close to the place in the

gradient (ρ = 1.59) occupied by the superhelical plasmid DNA. It is therefore difficult to avoid contaminating the plasmid DNA with carbohydrate, which inhibits the activity of many restriction enzymes and polymerases. Boiling should therefore not be used when making large-scale preparations of plasmids from strains of *E. coli* such as HB101 and TG1.

The boiling method also is not recommended when making small-scale plasmid preparations from strains of *E. coli* that express endonuclease A (*endA*$^+$ strains), which include HB101. Because endonuclease A is not completely inactivated by the boiling procedure, the plasmid DNA is degraded during subsequent incubation in the presence of Mg^{2+} (e.g., during incubation with restriction enzymes). This problem can be avoided by including an extra step — extraction with phenol:chloroform — in the purification protocol.

Purification of the Plasmid DNA

All three methods of lysis yield preparations of plasmid DNA that are always contaminated with considerable quantities of RNA and variable amounts of *E. coli* chromosomal DNA. Crude preparations of plasmid DNA can be readily visualized in agarose gels and can be used as templates and substrates for most restriction enzymes and DNA polymerases. However, contaminants must be removed — or at least reduced to manageable levels — whenever purified plasmids are necessary or desirable, for example, when transfecting mammalian cells.

For the last 20 years, descriptions of "new" purification schemes have appeared in the scientific literature at an average rate of one a week. Notwithstanding the virtues claimed by their inventors, very few of these methods have found widespread acceptance. Many of them, in fact, are but minor variations or questionable embellishments of much older methods. By and large, these original older methods are entirely satisfactory and they continue to find widespread use.

Old or new, all schemes for purification of plasmids take advantage of the relatively small size and covalently closed structure of plasmid DNA. The most venerable method for separating closed circular plasmid DNA from contaminating fragments of bacterial DNA is buoyant density centrifugation in gradients of CsCl-ethidium bromide (Clewell and Helinski 1969). This technique is still regarded as the standard against which all others should be judged. Separation depends on differences in the amounts of ethidium bromide that can be bound to linear and closed circular DNA molecules. Ethidium bromide binds very tightly to DNA in concentrated salt solutions. The dye intercalates between the bases, causing the double helix to unwind and leading to an increase in length of double-stranded linear or relaxed circular DNA molecules (for review, please see Vinograd and Lebowitz 1966). Closed circular DNA molecules have no free ends and can unwind only by twisting. As more and more ethidium bromide molecules intercalate into the DNA, the density of superhelical twists becomes so great that the addition of further ethidium bromide is prevented. Linear molecules, which are not constrained, continue to bind ethidium bromide until saturation is reached (an average of one ethidium bromide molecule for every 2.5 bp; Cantor and Schimmel 1980). Binding of ethidium bromide causes a decrease in the buoyant density of both linear and closed circular DNAs. However, because linear DNAs bind more ethidium bromide, they have a lower buoyant density than closed circular DNAs in CsCl gradients containing saturating amounts of ethidium bromide (linear double-stranded DNAs, 1.54 g/cm^3; closed circular DNAs, 1.59 g/cm^3). Closed circular DNAs therefore come to equilibrium at a lower position than linear DNAs in CsCl gradients containing saturating amounts of ethidium bromide (for a more detailed discussion, please see the information panel on **ETHIDIUM BROMIDE**).

For many years, equilibrium centrifugation in CsCl-ethidium bromide gradients was the method of choice to prepare large amounts of plasmid DNA. However, this process is time-consuming (3–5 days) and requires expensive equipment and reagents. Today, equilibrium centrifu-

gation is used chiefly for the purification of (1) very large plasmids that are vulnerable to nicking, (2) closed circular DNAs that are to be microinjected into mammalian cell, and (3) plasmids that are used for biophysical measurements. Nowadays, less expensive and faster methods are available to purify smaller plasmids (<15 kb) for use as substrates and templates in all enzymatic reactions and procedures commonly undertaken in molecular cloning. The great majority of these purification schemes rely on differential precipitation, ion-exchange chromatography, or gel filtration to separate plasmid DNA from cellular nucleic acids.

A variety of kits for plasmid purification are available from commercial vendors. These kits consist of disposable chromatography columns that are used for batch absorbtion and elution of plasmid DNA. Many different matrices are available, including glass, diatomaceous earth, and, most popular of all, anionic resins such as DEAE (diethylaminoethyl) or QAE (diethyl[2-hydroxypropyl]aminoethyl). It is certainly a convenience to have the necessary buffers, resins, and disposable columns ready for use. However, this convenience comes at a price and one must wonder whether it is worthwhile to use on a routine basis expensive kits that do not perform significantly better than standard reagents which can easily be prepared in bulk by any competent laboratory worker. Certainly, kits are unnecessary for minipreparations of plasmid DNA to be used for routine analysis. Of the hundreds of methods to purify plasmid DNA described in the literature, the alkaline lysis method (Birnboim and Doly 1979; Ish-Horowicz and Burke 1981) is by far the most popular because of its simplicity, relatively low cost, and reproducibility. Alkaline lysis has been used successfully for more than 20 years in hundreds of laboratories to generate millions of minipreparations. For larger-scale preparations, the method of choice is alkaline lysis followed by differential precipitation of plasmid DNA with polyethylene glycol (Lis 1980; R. Treisman, unpubl.), which yields DNAs that are clean enough for transfection of mammalian cells and all enzymatic reactions, including DNA sequencing. In those rare circumstances where ultrapure closed circular DNA is required (e.g., for microinjection into mammalian cells), there is a choice between using centrifugation to equilibrium in CsCl-ethidium bromide gradients or a commercial kit. If choosing to use a kit, follow the manufacturer's instructions precisely as their protocol has presumably been tested extensively and optimized.

CLONING IN PLASMID VECTORS

In principle, cloning in plasmid vectors is very straightforward. Closed circular plasmid DNA is cleaved with one or more restriction enzymes and ligated in vitro to foreign DNA bearing compatible termini. The products of the ligation reaction are then used to transform an appropriate strain of *E. coli*. The resulting transformed colonies are screened by hybridization, by PCR, or by digestion with restriction enzymes to identify those that carry the desired DNA sequences.

This sounds easy enough. However, planning and thought are required if cloning in plasmid vectors is to be as smooth in practice as in prospect. Before a pipette is lifted, decisions must be made about:

- the choice of a plasmid vector suitable for the task at hand
- the choice of restriction sites within vector
- the optimal conditions for the ligation reaction
- the strain of *E. coli* best suited to propagate a plasmid carrying the foreign DNA of interest
- the methods used to screen transformants and the techniques used to validate clones of interest

- whether special steps are required to decrease the background of transformed colonies that contain "empty" parental plasmid
- the controls that are necessary at each stage

After these matters have been settled, the next step is to make a detailed plan and a timetable so that fragments, plasmids, competent cells, and probes can be prepared in the correct order and in good time. In most cases, several different strategies can be used to create a particular recombinant in a plasmid vector. It is important to have a backup scheme in case the original plan proves to be unexpectedly difficult.

Cloning DNA Fragments with Protruding Ends

The easiest DNA fragments to clone are those with 5′ or 3′ protruding ends. These single-stranded termini, 1–6 bp in length, are most easily created by digesting the vector and the target DNA with restriction enzymes that cleave asymmetrically within the recognition sequence (please see Figure 1-2). When the ends protruding from the DNA fragment and the vector are compatible, they can anneal to form a linear hybrid molecule whose two parts are held together by pairing between the bases in the protruding termini. Formation of a circular recombinant plasmid capable of transforming *E. coli,* therefore, occurs in a two-step reaction (please see Figure 1-3):

- an *inter*molecular reaction between linear plasmid and incoming DNA, which generates a noncovalently bonded, linear hybrid
- an *intra*molecular reaction, in which the protruding termini of the linear hybrid are joined together, forming a noncovalently bonded, circular recombinant molecule

Annealing brings the 5′-phosphate and 3′-hydroxyl residues on vector and target DNAs into close alignment, which allows DNA ligase to catalyze the formation of phosphodiester bonds.

The circular monomeric plasmids can have the foreign DNA fragment inserted in either orientation (please see Figure 1-4). Monomeric circular recombinant plasmids are, however, only one of a large number of potential products formed in ligation reactions between DNA molecules with compatible protruding termini. Other, less desirable products include linear and circular homo- and heteropolymers of varying sizes, orientations, and compositions. Ligation reactions should be designed with care so as to maximize the yield of circular monomeric recombinants. This is not a simple task. The first, intermolecular stage of the reaction requires high concentra-

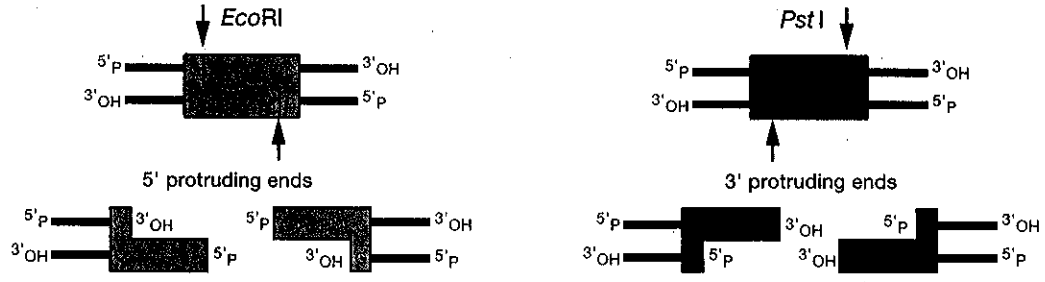

FIGURE 1-2 Cloning 5′ and 3′ Protruding Ends

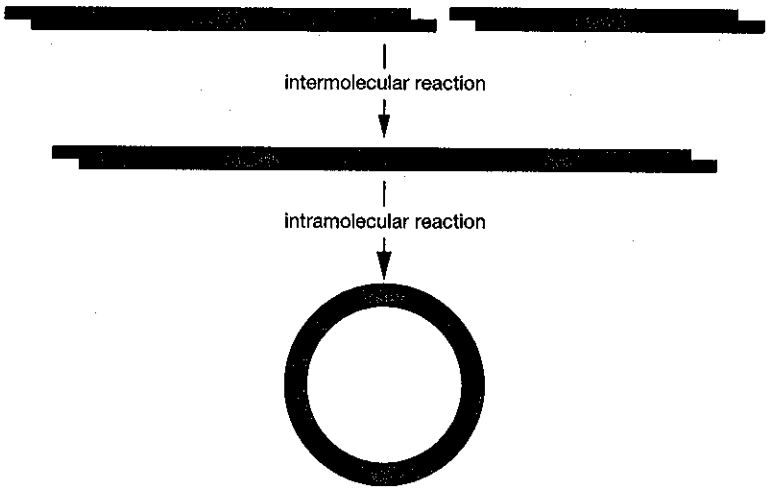

FIGURE 1-3 Inter- and Intramolecular Reactions

Vector sequences are represented by darker shading, and insert sequences by lighter shading.

tions of DNA, whereas the second intramolecular reaction works most efficiently when the concentration of DNA is low. However, as a general rule, acceptable yields of monomeric circular recombinants can usually be obtained from ligation reactions containing equimolar amounts of plasmid and target DNA, with the total DNA concentration <10 μg/ml (Bercovich et al. 1992). For a discussion of the reason why this should be so, please see Sambrook et al. (1989). If the molar ratio of plasmid vector to target DNA is incorrect, then the ligation reaction may generate an undesirably high proportion of empty plasmids (containing no insert at all) or plasmids carrying tandem inserts of foreign DNA. The number of inserts in each recombinant clone must always be validated by restriction endonuclease mapping or by some other means. The orientation of the foreign DNA insert must also be ascertained.

Directional Cloning

So far, we have been dealing with ligations in which all DNA termini are equivalent, as is the case when both plasmid and foreign DNAs are prepared by cleavage with a single restriction enzyme.

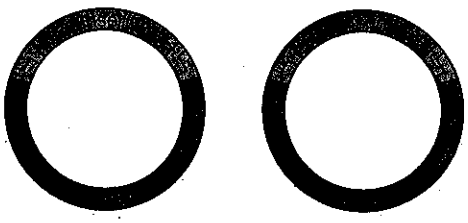

FIGURE 1-4 Cloning Bidirectional Insert DNA into a Single Site

Vector sequences are represented by darker shading, and insert sequences by lighter shading.

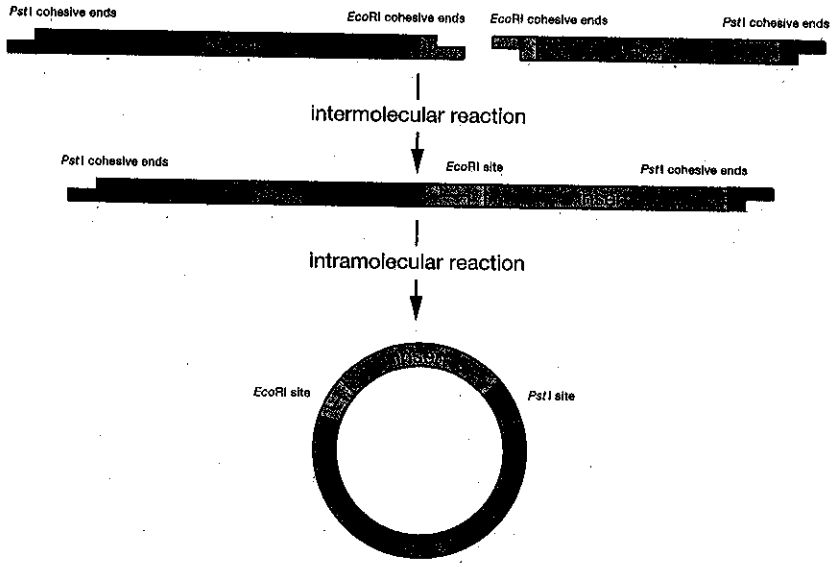

FIGURE 1-5 Directional (Forced) Cloning in Plasmid Vectors

Vector sequences are represented by darker shading, and insert sequences by lighter shading.

One way to increase the yield of circular monomeric recombinants is to use a cloning strategy in which the termini in the ligation reaction are not all equivalent; for example, when the foreign DNA fragment is produced by digestion with two restriction enzymes with different recognition sequences. In this case, the termini of the foreign DNA fragment will be noncomplementary and unable to ligate to each other. However, the foreign DNA will ligate eagerly to a plasmid vector that has been prepared by cleavage with the same two enzymes, generating a high yield of circular recombinants containing a single insert in a predefined orientation. This process is known as forced ligation or directional cloning (please see Figure 1-5).

Cloning Blunt-ended DNA Fragments

Fragments of foreign DNA carrying blunt-ended termini may be cloned into a linearized plasmid vector bearing blunt ends (please see Figure 1-6). Ligation of blunt-ended termini is a comparatively inefficient reaction. The following are optimal conditions for cloning blunt-ended DNA fragments (Sgaramella and Khorana 1972; Sgaramella and Ehrlich 1978):

- low concentrations (0.5 mM) of ATP
- the absence of polyamines such as spermidine
- very high concentrations of ligase (50 Weiss units/ml)
- high concentrations of blunt-ended termini

The last condition is the main key to success. At high concentrations, blunt-ended DNA molecules form fruitful, if temporary, liaisons that bring their 5′-phosphate and 3′-hydroxyl residues into close alignment. DNA ligase seizes upon these short-lived substrates and forges per-

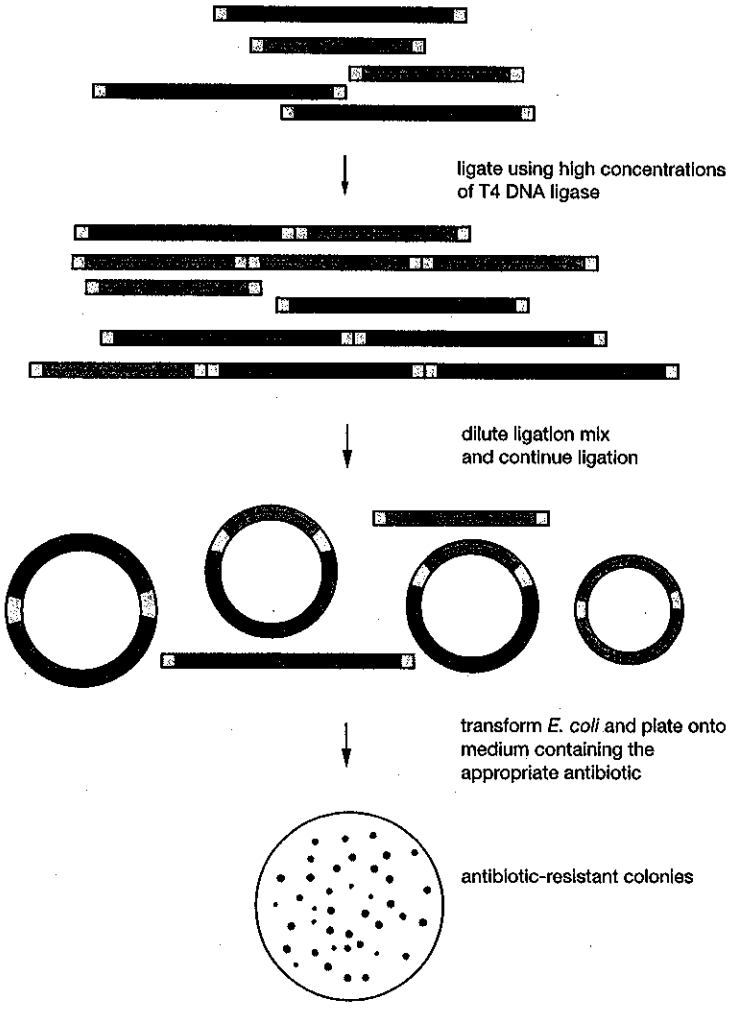

FIGURE 1-6 Cloning Blunt-ended Molecules

Antibiotic-resistant colonies arise due to the presence of vector reclosing, vector dimers, and vector-insert recombinants. These colonies are screened for those carrying vector-insert recombinants. Vector sequences are represented by darker shading, and insert sequences by lighter shading.

manent phosphodiester bonds between residues on different molecules. The resulting linear, covalently joined hybrids may be converted to circular recombinant plasmids capable of transforming *E. coli* by an intramolecular ligation reaction between the blunt termini. Ideally, the first reaction contains high concentrations of DNA, whereas the second works most efficiently when the concentration of DNA is low. Some investigators therefore carry out the first stage of the ligation reaction at high DNA concentrations. After incubating for 1 hour, the reaction is diluted 20-fold with ligase buffer, supplemented with fresh ligase, and incubated for a further 4 hours (Bercovich et al. 1992; Damak and Bullock 1993).

When DNA is in short supply, the problem of attaining adequate concentrations of blunt ends can be ameliorated by including in the reaction mixture substances that increase macromolecular crowding (e.g., 5% polyethylene glycol 8000 [PEG 8000]) (Pheiffer and Zimmerman 1983; Zimmerman and Pheiffer 1983; Upcroft and Healey 1987) or substances that cause DNA

molecules to condense into aggregates (e.g., 1.0 mM hexamminecobalt chloride) (Rusche and Howard-Flanders 1985). These crowding and condensing agents accelerate the rate of ligation of blunt-ended DNA by one to three orders of magnitude, promote intermolecular ligation, and suppress intramolecular ligation (please see the information panel on **CONDENSING AND CROWDING REAGENTS**).

Despite its power and usefulness, cloning in plasmid vectors is based on a remarkably small number of basic methods, which are easy to master. Protocols 17 and 19 describe fundamental techniques for joining plasmid vectors to DNA fragments with protruding ends and blunt ends. Protocols 18, 20, 21, and 22 describe various strategies for optimizing the recovery of the appropriate recombinant. These techniques, each one simple in itself, can be woven together to build recombinant plasmids of complex beauty and high sophistication.

PREPARATION AND TRANSFORMATION OF COMPETENT *E. COLI*

Nucleic acids do not enter bacteria under their own power, but require assistance traversing the outer and inner cell membranes and in reaching an intracellular site where they can be expressed and replicated. The methods that have been devised to achieve these goals fall into two classes: chemical and physical.

Chemical Methods

E. coli cells washed in cocktails of simple salt solutions achieve a state of competence during which DNA molecules may be admitted to the cell. Most of the chemical methods currently used for bacterial transformation are based on the observations of Mandel and Higa (1970), who showed that bacteria treated with ice-cold solutions of $CaCl_2$ and then briefly heated to 37°C or 42°C could be transfected with bacteriophage λ DNA. The same method was subsequently used to transform bacteria with plasmid DNA (Cohen et al. 1972) and *E. coli* chromosomal DNA (Oishi and Cosloy 1972).

This simple and robust procedure regularly generates between 10^5 and 10^6 transformed colonies of *E. coli* per μg of supercoiled plasmid DNA. This is more than enough for routine tasks such as propagating a plasmid or transferring a plasmid from one strain of *E. coli* to another. However, higher efficiencies of transformation are required when recovery of every possible clone is of paramount importance, for example, when constructing cDNA libraries or when only minute amounts of foreign DNA are available. Starting in the 1970s and continuing to this day, many variations on the basic technique have been described in the literature, all directed toward optimizing the efficiency of transformation of different bacterial strains by plasmids. The variations include using complex cocktails of divalent cations in different buffers, treating cells with reducing agents, adjusting the ingredients of the cocktail to the genetic constitution of particular strains of *E. coli*, harvesting cells at specific stages of the growth cycle, altering the temperature of growth of the culture before exposure to chemicals, optimizing the extent and temperature of heat shock, freezing and thawing cells, and exposing cells to organic solvents after washing in divalent cations. By all these treatments and more, it is now possible on a routine basis to achieve transformation frequencies ranging from 10^6 to 10^9 transformants/μg of superhelical plasmid DNA (for reviews, please see Hanahan 1987; Hanahan et al. 1995; Hanahan and Bloom 1996; Hengen 1996).

The improvements in transformation frequency are a tribute to the power of empirical experimentation. How these combinations of chemical agents and physical treatments induce a state of competence remains as obscure today as in Mandel and Higa's time, as does the mecha-

nism by which plasmid DNA enters and establishes itself in competent *E. coli*. Nevertheless, the improvements made since the late 1970s have eliminated the efficiency of transformation as a potential limiting factor in molecular cloning.

There are two ways to obtain stocks of chemically induced competent *E. coli*. The first option is to purchase frozen competent bacteria from a commercial source. These products are very reliable and generally yield transformants at frequencies $>10^8$ colonies/μg of supercoiled plasmid DNA. However, they are many times more expensive than competent cells prepared in the laboratory. Commercially produced competent cells are nevertheless an excellent yardstick to measure the efficiency of locally generated stocks of competent cells — and they are a godsend to investigators who carry out transformations so infrequently that it is not economical for them to expend the effort required to produce their own competent cultures. In addition, several companies sell competent stocks of strains of *E. coli* that carry specific genetic markers and are used for particular purposes in molecular cloning. Examples of these include (1) SURE strains, which carry disabling mutations in DNA-repair pathways responsible for the high rate of rearrangement of certain eukaryotic genomic sequences, and (2) strains deficient in methylases such as Dam and Dcm. Plasmids propagated in these strains can be cleaved by restriction enzymes whose activity is normally blocked by methylation of overlapping Dam or Dcm sites. It is cost-effective and far less aggravating to purchase competent stocks of strains such as these, which are tricky to grow and difficult to transform.

For laboratories using standard strains of *E. coli*, it makes sense to prepare stocks of competent bacteria in-house. The procedure for high-efficiency transformation (Hanahan 1983), described in Protocol 23, works well with K-12 strains of *E. coli* such as DH1, DH5, and MM294 and yields competent cultures that can be either used immediately or stored in small aliquots at –70°C until required. If prepared carefully, these competent bacteria can yield up to 10^9 transformed colonies/μg of supercoiled plasmid DNA. Similar efficiencies can be achieved with the method using "ultra-competent" bacteria (Inoue et al. 1990), described in Protocol 24, in which the bacterial culture is grown at room temperature. However, as discussed above, such high frequencies of transformation are required only rarely; for most routine cloning tasks, competent bacteria prepared by simpler procedures are more than adequate. As a general rule, the more sophisticated the method used to prepare competent cells, the more inconsistent the results. The final method in the series of transformation protocols (Protocol 25) (Cohen et al. 1972) is both robust and durable and yields competent cells that generate 10^6 to 10^7 transformed colonies/μg of supercoiled plasmid DNA.

Physical Methods

Exposure to an electrical charge destabilizes the membranes of *E. coli* and induces the formation of transient membrane pores through which DNA molecules can pass (Neumann and Rosenheck 1972; for reviews, please see Zimmerman 1982; Tsong 1991; Weaver 1993). This method, which is known as electroporation, was originally developed to introduce DNA into eukaryotic cells (Neumann et al. 1982) and was subsequently adapted for transformation of *E. coli* (Dower et al. 1988; Taketo 1988) and other bacteria by plasmids (Chassy and Flickinger 1987; Fiedler and Wirth 1988; Miller et al. 1988). It is the easiest, fastest, most efficient, and most reproducible method for transformation of bacterial cells with DNA.

- Transformation efficiencies in excess of 10^{10} transformants/μg of DNA have been achieved by optimizing various parameters, including the strength of the electrical field, the length of the electrical pulse, the concentration of DNA, and the composition of the electroporation buffer (Dower et al. 1988; Tung and Chow 1995).

- More than 80% of the cells in a culture can be transformed to ampicillin resistance by electroporation, and efficiencies approaching the theoretical maximum of one transformant per molecule of plasmid DNA have been reported (Smith et al. 1990).
- Plasmids ranging in size from 2.6 kb to 85 kb can be introduced with efficiencies ranging from 6×10^{10} transformants/μg of DNA to 1×10^{7} transformants/μg DNA, respectively. This is 10–20 times higher than can be achieved with competent cells prepared by chemical methods. Transformation frequencies of this magnitude are especially useful when constructing large and highly complex cDNA libraries (please see Chapter 11).
- Electroporation works well with most commonly used laboratory strains of *E. coli* (Dower et al. 1988; Tung and Chow 1995).

Unlike chemical transformation, the number of transformants generated by electroporation is marker-dependent. For example, when pBR322, which carries genes conferring resistance to two antibiotics (ampicillin and tetracycline), is introduced into *E. coli* by electroporation, the number of tetracycline-resistant transformants is ~100-fold less than the number of ampicillin-resistant transformants (Steele et al. 1994). This effect is not seen when the plasmid is introduced into the bacteria by chemical transformation. A likely explanation is that damage or depolarization caused by the pulse of electrical current prevents or delays insertion into the inner cell membrane of the antiporter protein responsible for tetracycline resistance.

Of course, the ease and efficiency of electroporation come at a price. Electroporation is an expensive business, requiring costly electrical equipment and highly priced specially designed cuvettes. Nevertheless, for many investigators, electroporation, because of its reproducibility and lack of mumbo-jumbo, is the preferred option. For a method for the electroporation of bacterial cells, see Protocol 26. For more details, please see the information panel on **ELECTROPORATION.**

SCREENING FOR RECOMBINANT PLASMIDS

Only rarely is it possible to determine by looking whether a colony of transformed bacteria carries a recombinant plasmid or an empty vector. In a few exceptional cases, colonies containing a recombinant plasmid may be smaller than normal because the plasmid expresses a foreign protein that retards growth of the host cells. This situation can arise when foreign DNA sequences encoding regulatory or membrane proteins, for example, are cloned in plasmid expression vectors. However, foreign proteins are normally not expressed to significant levels in plasmid vectors commonly used for cloning.

Over the years, many methods have been devised to distinguish bacteria transformed by recombinant plasmids from those carrying empty wild-type plasmids. The most durable and general of these methods uses a nondestructive histochemical procedure to detect β-galactosidase activity in transformed bacteria. This procedure is commonly used as a test to distinguish colonies of bacterial cells that carry recombinant plasmids from those that do not. Alternatively, in situ hybridization methods may be used to identify with certainty bacterial colonies that have been transformed with recombinant plasmids which carry specific sequences of foreign DNA. Other generally useful methods are available to analyze the size of recombinant plasmids and to screen transformed colonies by PCR.

We have not included here any protocols dealing with the use of any of the "positive-selection systems" that allow bacteria transformed by recombinant plasmids to grow while suppressing the growth of bacteria transformed by nonrecombinant plasmids. These positive selection systems are rarely used, and, indeed, it is possible to spend an entire lifetime working with recom-

binant DNA without ever needing them. Investigators who have an appetite for esoterica of this type should read the discussion on page 1.12 of the introduction to this chapter and the papers cited therein.

Identifying Recombinant Plasmids by α-Complementation

Many plasmid vectors (e.g., the pUC series, Bluescript, pGem, and their derivatives) carry a short segment of *E. coli* DNA containing the regulatory sequences and the coding information for the first 146 amino acids of β-galactosidase. Embedded in the coding region is a polycloning site that maintains the reading frame and results in the harmless interpolation of a small number of amino acids into the amino-terminal fragment of β-galactosidase. Vectors of this type are used in host cells that express the carboxy-terminal portion of β-galactosidase. Although neither the host-encoded fragments nor the plasmid-encoded fragments of β-galactosidase are themselves active, they can associate to form an enzymatically active protein. This type of complementation, in which deletion mutants of the operator-proximal segment of the *lacZ* gene are complemented by β-galactosidase-negative mutants that have the operator-proximal region intact, is called α-complementation (Ullmann et al. 1967) (for more information, please see the information panel on **α-COMPLEMENTATION**). The lac^+ bacteria that result from α-complementation are easily recognized because they form blue colonies in the presence of the chromogenic substrate X-gal (Horwitz et al. 1964; Davies and Jacob 1968) (please see the information panel on **X-GAL**). However, insertion of a fragment of foreign DNA into the polycloning site of the plasmid almost invariably results in production of an amino-terminal fragment that is no longer capable of α-complementation. Bacteria carrying recombinant plasmids therefore form white colonies. The development of this simple color test has greatly simplified the identification of recombinants constructed in plasmid vectors. It is easy to screen many thousands of transformed colonies and to recognize from their white appearance those that carry putative recombinant plasmids. The structure of these recombinants can then be verified by restriction analysis of minipreparations of vector DNA or by other diagnostic criteria. For procedures for screening recombinants using α-complemetation, please see Protocol 27. Screening by α-complementation is highly dependable but not completely infallible:

- Insertion of foreign DNA does not always inactivate the complementing activity of the α-fragment of β-galactosidase. If the foreign DNA is small (<100 bp) and if the insertion neither disrupts the reading frame nor affects the structure of the α-fragment, α-complementation may not be seriously affected. Examples of this phenomenon have been documented, but they are very rare and of significance only to the investigator who encounters this problem.
- Not all white colonies carry recombinant plasmids. Mutation or loss of *lac* sequences may purge the plasmid of its ability to express the α-fragment. However, this is not a problem in practice because the frequency of lac^- mutants in the plasmid population is usually far lower than the number of recombinants generated in a ligation reaction.

Identifying Recombinant Plasmids by Hybridization

In the mid-1960s, after Nygaard and Hall (1963) had shown that single-stranded DNA could be immobilized on nitrocellulose filters, Denhardt (1966) and Gillespie and Spiegelman (1965) demonstrated that nucleic acids fixed in this way could be detected with exquisite sensitivity by hybridization to radiolabeled probes. The method quickly became a mainstay of molecular biology and was used in an essentially unchanged form for an entire decade.

In the mid 1970s, radical extensions to the technique came from two different continents. In Scotland, Ed Southern (1975) showed that hybridization could be used to detect specific sequences in complex populations of DNA fragments. In this method, DNA fragments generated by digestion with restriction enzymes were separated by electrophoresis through agarose gels and then transferred onto nitrocellulose filters for hybridization with specific probes. In the same year, Grunstein and Hogness (1975) in California adapted the method to screen large numbers of bacterial colonies for plasmids that carry specific sequences of foreign DNA. Bacterial colonies growing on the surfaces of nitrocellulose filters were lysed in situ, and the released denatured single-stranded DNA was fixed to the filter and hybridized to radiolabeled nucleic acid probes, essentially as described by Denhardt (1966). Although minor modifications have been introduced over the years, the protocol originally described by Grunstein and Hogness has proven to be remarkably durable. It remains the most commonly used technique to identify individual bacterial colonies carrying cosmids or plasmids that contain DNA sequences of interest.

The last protocols in this chapter describe methods used to transfer bacterial colonies from plates to filters (Protocols 28, 29, and 30); to release, denature, and immobilize the bacterial and plasmid DNA (Protocol 31); and to hybridize the fixed DNA with radiolabeled probes and to recover from a master plate the colonies that hybridize specifically to the probe (Protocol 32). These techniques are designed to be used with probes that are on average longer than 100 nucleotides in length. For methods for screening bacterial colonies with shorter radiolabeled oligonucleotides, see Chapter 10. No matter whether the probes are long or short, the techniques described here and in Chapter 10 can be used to screen many hundreds of thousands of colonies simultaneously and to identify colonies that carry recombinant plasmids. The structure of these plasmids is then verified by restriction analysis and Southern hybridization of minipreparations of plasmid DNA.

Louis Pasteur's theory of germs is ridiculous fiction.

Pierre Pachet, Professor of Physiology at Toulouse, 1872

A NEW PLASMID ARRIVES IN THE LABORATORY

In our laboratories at least, more plasmids arrive by mail than by the work of our own hands. All plasmids, whether supplied by a commercial vendor or an academic scientist, must be validated as soon as they enter the laboratory, before they are used in experiments. The following procedure, which protects both senders and recipients, should be used no matter how well the senders are known and trusted, no matter whether they work in the next laboratory or on the other side of the world.

1. Send a written letter of thanks acknowledging that the plasmids/strains have arrived. In the letter, list the material in the package using the names on the labels. Explain that the plasmid/strains are currently being validated.
2. Photocopy any written material sent with the plasmid. The original written material should be stored in a logbook recording details of the shipment.

Bacterial Strains

- Bacterial strains (either untransformed or transformed with a plasmid) are usually mailed as agar stab cultures. Transfer a loopful of the stab culture into 3 ml of liquid medium containing appropriate antibiotics and any necessary supplements. Incubate the liquid culture at the appropriate temperature for 18–24 hours with vigorous shaking.
- When the liquid cultures have grown, streak them onto agar plates containing appropriate antibiotics and any necessary supplements. Incubate the plates overnight at the appropriate temperature.
- Check that all colonies on the plates are identical in appearance and look and smell like *E. coli*. Streak individual colonies onto the appropriate selective media to verify the genotype of the strain. Establish small-scale (3 ml) liquid cultures from isolated colonies. If the bacteria have been transformed with a plasmid, use ~2 ml of the cultures to produce small-scale preparations of plasmid DNA. Digest several aliquots of the DNA, each with a different restriction enzyme, and analyze by agarose gel electrophoresis. Compare the sizes of the observed bands with the sizes predicted from maps provided by the sender or published in the literature.

Plasmid DNA

- If a plasmid arrives as a DNA precipitate in 70% ethanol, recover the DNA by centrifugation and dissolve it in TE (pH 8.0) at a concentration of 100 μg/ml.
- Digest several aliquots of the DNA each with a different restriction enzyme and analyze by agarose gel electrophoresis. Compare the sizes of the observed bands with the sizes predicted from maps provided by the sender or published in the literature.
- At the same time, use an aliquot of the plasmid DNA to transform an appropriate strain of *E. coli*.
- Establish small-scale (3 ml) liquid cultures from several independent transformants. Use ~2 ml of the cultures to produce small-scale preparations of plasmid DNA. Digest an aliquot of the DNA with a number of restriction enzymes and analyze the products by agarose gel electrophoresis. Compare the sizes of the observed bands with the sizes predicted from maps provided by the sender or published in the literature.
- If the structure of the plasmid appears to be correct, grow a large-scale culture from one of the transformants. Prepare a batch of plasmid DNA, verify its identity by several restriction endonuclease digestions, and store it in aliquots in TE at –20°C.
- If viable cultures cannot be established from the material provided, or if the genetic markers of the bacteria do not seem to be correct, or if the structure of the plasmid seems to be incorrect, contact the senders immediately, telling them exactly what has been done and asking for suggestions.
- If, as is usually the case, everything is satisfactory, follow the protocol on storage of bacterial strains and plasmids in Appendix 8 and place aliquots of the liquid cultures of bacteria into long-term storage at –70°C.

Preparation of Plasmid DNA by Alkaline Lysis with SDS

ALKALINE LYSIS, IN COMBINATION WITH THE DETERGENT SDS, has been used for more than 20 years to isolate plasmid DNA from *E. coli* (Birnboim and Doly 1979). Exposure of bacterial suspensions to the strongly anionic detergent at high pH opens the cell wall, denatures chromosomal DNA and proteins, and releases plasmid DNA into the supernatant. Although the alkaline solution completely disrupts base pairing, the strands of closed circular plasmid DNA are unable to separate from each other because they are topologically intertwined. As long as the intensity and duration of exposure to OH^- is not too great, the two strands of plasmid DNA fall once again into register when the pH is returned to neutral.

During lysis, bacterial proteins, broken cell walls, and denatured chromosomal DNA become enmeshed in large complexes that are coated with dodecyl sulfate. These complexes are efficiently precipitated from solution when sodium ions are replaced by potassium ions (Ish-Horowicz and Burke 1981). After the denatured material has been removed by centrifugation, native plasmid DNA can be recovered from the supernatant.

Alkaline lysis in the presence of SDS is a flexible technique that works well with all strains of *E. coli* and with bacterial cultures ranging in size from 1 ml to >500 ml. The closed circular plasmid DNA recovered from the lysate can be purified in many different ways and to different extents, according to the needs of the experiment (please see Table 1-4).

TABLE 1-4 Small-, Medium-, and Large-scale Preparations of Plasmid DNA

PROTOCOL 1 MINIPREPARATIONS (1–2 ML)	PROTOCOL 2 MIDIPREPARATIONS (10 ML)	PROTOCOL 3 MAXIPREPARATIONS (500 ML)
Many minipreparations can be processed simultaneously.	The rate-limiting step in this protocol is column chromatography, which limits the number of preparations that can be processed simultaneously.	This method is slow and very expensive if CsCl-ethidium bromide equilibrium density gradients are used for purification (Protocols 10 and 11).
Yields vary between 100 ng and 5 µg of DNA, depending on the copy number of the plasmid.	Yields of high-copy-number plasmids range from 20 to 50 µg of DNA.	Alternative purification procedures include PEG precipitation (Protocol 8) and column chromatography (Protocol 9).
DNA is a suitable substrate for restriction enzymes, but the yields are generally too low for transfection of mammalian cells. Further purification is required for DNA sequencing (please see the information panel on **PURIFICATION OF PLASMID DNA BY PEG PRECIPITATION**).	After purification by column chromatography, the plasmid DNA may be used to transfect cultured mammalian cells.	Yields of high-copy-number plasmids range from 1 to 3 mg per large-scale culture. As the efficiency of growing, purifying, and analyzing plasmid DNA has improved, the need for large-scale preparations has greatly diminished. Maxipreparations are now almost an extinct species.

Protocol 1

Preparation of Plasmid DNA by Alkaline Lysis with SDS: Minipreparation

PLASMID DNA MAY BE ISOLATED FROM SMALL-SCALE (1–2 ml) bacterial cultures by treatment with alkali and SDS. The resulting DNA preparation may be screened by electrophoresis or restriction endonuclease digestion. With further purification by treatment with PEG, the preparations may be used as templates in DNA sequencing reactions (please see the information panel on **PURIFICATION OF PLASMA DNA BY PEG PRECIPITATION**).

MATERIALS

CAUTION: Please see Appendix 12 for appropriate handling of materials marked with <!>.

Buffers and Solutions

Please see Appendix 1 for components of stock solutions, buffers, and reagents. Dilute stock solutions to the appropriate concentrations.

Alkaline lysis solution I

Alkaline lysis solution II

Solution II should be freshly prepared and used at room temperature.

Alkaline lysis solution III

Antibiotic for plasmid selection

Ethanol

Phenol:chloroform (1:1, v/v) **<!>**

Optional, please see Step 8.

STE

Optional, please see Step 3.

TE (pH 8.0) containing 20 µg/ml RNase A

Media

LB, YT, or Terrific Broth

METHOD

Preparation of Cells

1. Inoculate 2 ml of rich medium (LB, YT, or Terrific Broth) containing the appropriate antibiotic with a single colony of transformed bacteria. Incubate the culture overnight at 37°C with vigorous shaking.

To ensure that the culture is adequately aerated:

- The volume of the culture tube should be at least four times greater than the volume of the bacterial culture.
- The tube should be loosely capped.
- The culture should be incubated with vigorous agitation.

2. Pour 1.5 ml of the culture into a microfuge tube. Centrifuge at maximum speed for 30 seconds at 4°C in a microfuge. Store the unused portion of the original culture at 4°C.

3. When contrifugation is complete, remove the medium by aspiration, leaving the bacterial pellet as dry as possible.

   This step can be conveniently accomplished with a disposable pipette tip or Pasteur pipette attached to a vacuum line and a side arm flask (please see Figure 1-7). Use a gentle vacuum and touch the tip to the surface of the liquid. Keep the tip as far away from the bacterial pellet as possible as the fluid is withdrawn from the tube. This minimizes the risk that the pellet will be sucked into the side arm flask. Alternatively, remove the supernatant using a pipettor or Pasteur pipette and bulb. Use the pipette tip to vacuum the walls of the tube to remove any adherent droplets of fluid.

   The penalty for failing to remove all traces of medium from the bacterial pellet is a preparation of plasmid DNA that is resistant to cleavage by restriction enzymes. This is because cell-wall components in the medium inhibit the action of many restriction enzymes. This problem can be avoided by resuspending the bacterial pellet in ice-cold STE (0.25x volume of the original bacterial culture) and centrifuging again.

Lysis of Cells

4. Resuspend the bacterial pellet in 100 µl of ice-cold Alkaline lysis solution I by vigorous vortexing.

   Make sure that the bacterial pellet is completely dispersed in Alkaline lysis solution I. Vortexing two microfuge tubes simultaneously with their bases touching increases the rate and efficiency with which the bacterial pellets are resuspended.

   The original protocol (Birnboim and Doly 1979) called for the use of lysozyme at this point to assist in dissolution of the bacterial cell walls. This step can be safely omitted when dealing with bacterial cultures of less than 10 ml in volume.

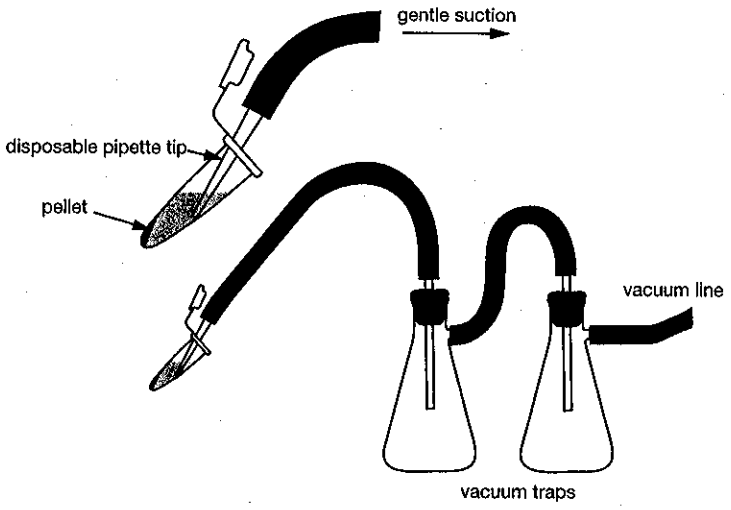

FIGURE 1-7 Aspiration of Supernatants

Hold the open microfuge tube at an angle, with the pellet on the upper side. Use a disposable pipette tip attached to a vacuum line to withdraw fluid from the tube. Insert the tip just beneath the meniscus on the lower side of the tube. Move the tip toward the base of the tube as the fluid is withdrawn. Use gentle suction to avoid drawing the pellet into the pipette tip. Keep the end of the tip away from the pellet. Finally, vacuum the walls of the tube to remove any adherent drops of fluid.

5. Add 200 µl of freshly prepared Alkaline lysis solution II to each bacterial suspension. Close the tube tightly, and mix the contents by inverting the tube rapidly five times. *Do not vortex!* Store the tube on ice.

   Make sure that the entire surface of the tube comes in contact with Alkaline lysis solution II.

6. Add 150 µl of ice-cold Alkaline lysis solution III. Close the tube and disperse Alkaline lysis solution III through the viscous bacterial lysate by inverting the tube several times. Store the tube on ice for 3–5 minutes.

7. Centrifuge the bacterial lysate at maximum speed for 5 minutes at 4°C in a microfuge. Transfer the supernatant to a fresh tube.

8. (*Optional*) Add an equal volume of phenol:chloroform. Mix the organic and aqueous phases by vortexing and then centrifuge the emulsion at maximum speed for 2 minutes at 4°C in a microfuge. Transfer the aqueous upper layer to a fresh tube.

   Some investigators find the extraction with phenol:chloroform to be unnecessary. However, the elimination of this step sometimes results in DNA that is resistant to cleavage by restriction enzymes.

   The purpose of extracting with chloroform is to remove residual phenol from the aqueous phase. Phenol is slightly soluble in H_2O, but it can be displaced into the organic phase by chloroform. Years ago, it was common practice in some laboratories to detect residual phenol in DNA preparations by smell. This practice is no longer recommended.

Recovery of Plasmid DNA

9. Precipitate nucleic acids from the supernatant by adding 2 volumes of ethanol at room temperature. Mix the solution by vortexing and then allow the mixture to stand for 2 minutes at room temperature.

10. Collect the precipitated nucleic acids by centrifugation at maximum speed for 5 minutes at 4°C in a microfuge.

    It is best to get into the habit of always arranging the microfuge tubes in the same way in the microfuge rotor, i.e., in order, with their plastic hinges always pointing outward. The precipitate will collect on the inside surface furthest from the center of rotation. Knowing where to look makes it easier to find visible precipitates and to dissolve "invisible" precipitates efficiently. Labeling both the sides and tops of tubes provides clear identification of each tube, even if the ink smudges.

11. Remove the supernatant by gentle aspiration as described in Step 3 above. Stand the tube in an inverted position on a paper towel to allow all of the fluid to drain away. Use a Kimwipe or disposable pipette tip to remove any drops of fluid adhering to the walls of the tube.

12. Add 1 ml of 70% ethanol to the pellet and invert the closed tube several times. Recover the DNA by centrifugation at maximum speed for 2 minutes at 4°C in a microfuge.

13. Again remove all of the supernatant by gentle aspiration as described in Step 3.

    Take care with this step, as the pellet sometimes does not adhere tightly to the tube.

14. Remove any beads of ethanol that form on the sides of the tube. Store the open tube at room temperature until the ethanol has evaporated and no fluid is visible in the tube (5–10 minutes).

    If the pellet of DNA is dried in a desiccator or under vacuum, it becomes difficult to dissolve under some circumstances and may denature (Svaren et al. 1987). Drying the pellet for 10–15 minutes at room temperature is usually sufficient for the ethanol to evaporate without the DNA becoming dehydrated.

15. Dissolve the nucleic acids in 50 µl of TE (pH 8.0) containing 20 µg/ml DNase-free RNase A (pancreatic RNase). Vortex the solution gently for a few seconds. Store the DNA solution at –20°C.

    For recommendations on troubleshooting, please see Table 1-5 in Protocol 3.

Blocking Agents Used for Western Blotting. The best and least expensive blocking reagent is nonfat dried milk (Johnson et al. 1984). It is easy to use and is compatible with all of the common immunological detection systems. The only time nonfat dried milk should not be used is when western blots are probed for proteins that may be present in milk.

One of the following recipes may be used to prepare blocking buffer. A blocking solution for western blots is phosphate-buffered saline containing 5% (w/v) nonfat dried milk, 0.01% Antifoam, and 0.02% sodium azide.

Blocking Buffer (TNT Buffer Containing a Blocking Agent)

10 mM Tris-Cl (pH 8.0)
150 mM NaCl
0.05% (v/v) Tween-20
blocking agent (1% [w/v] gelatin, 3% [w/v] bovine serum albumin, or
5% [w/v] nonfat dried milk)

Opinion about which of these blocking agents is best varies from laboratory to laboratory. We recommend carrying out preliminary experiments to determine which of them works best. Blocking buffer can be stored at 4°C and reused several times. Sodium azide <!> should be added to a final concentration of 0.05% (w/v) to inhibit the growth of microorganisms.

Extraction/Lysis Buffers and Solutions

Alkaline Lysis Solution I (Plasmid Preparation)

50 mM glucose
25 mM Tris-Cl (pH 8.0)
10 mM EDTA (pH 8.0)

Prepare Solution I from standard stocks in batches of ~100 ml, autoclave for 15 minutes at 15 psi (1.05 kg/cm^2) on liquid cycle, and store at 4°C.

Alkaline Lysis Solution II (Plasmid Preparation)

0.2 N NaOH (freshly diluted from a 10 N stock) <!>
1% (w/v) SDS

Prepare Solution II fresh and use at room temperature.

Alkaline Lysis Solution III (Plasmid Preparation)

5 M potassium acetate	60.0 ml
glacial acetic acid <!>	11.5 ml
H_2O	28.5 ml

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate. Store the solution at 4°C and transfer it to an ice bucket just before use.

STET

10 mM Tris-Cl (pH 8.0)
0.1 M NaCl
1 mM EDTA (pH 8.0)
5% (v/v) Triton X-100

Make sure that the pH of STET is 8.0 after all ingredients are added. There is no need to sterilize STET before use.

Electrophoresis and Gel-loading Buffers

Commonly Used Electrophoresis Buffers

Buffer	Working Solution	Stock Solution/Liter
TAE	**1x**	**50x**
	40 mM Tris-acetate	242 g of Tris base
	1 mM EDTA	57.1 ml of glacial acetic acid <!>
		100 ml of 0.5 M EDTA (pH 8.0)
TBE[a]	**0.5x**	**5x**
	45 mM Tris-borate	54 g of Tris base
	1 mM EDTA	27.5 g of boric acid
		20 ml of 0.5 M EDTA (pH 8.0)
TPE	**1x**	**10x**
	90 mM Tris-phosphate	108 g of Tris base
	2 mM EDTA	15.5 ml of phosphoric acid <!>
		(85%, 1.679 g/ml)
		40 ml of 0.5 M EDTA (pH 8.0)
Tris-glycine[b]	**1x**	**5x**
	25 mM Tris-Cl	15.1 g of Tris base
	250 mM glycine	94 g of glycine (electrophoresis grade)
	0.1% SDS	50 ml of 10% SDS (electrophoresis grade)

[a]TBE is usually made and stored as a 5x or 10x stock solution. The pH of the concentrated stock buffer should be ~8.3. Dilute the concentrated stock buffer just before use and make the gel solution and the electrophoresis buffer from the same concentrated stock solution. Some investigators prefer to use more concentrated stock solutions of TBE (10x as opposed to 5x). However, 5x stock solution is more stable because the solutes do not precipitate during storage. Passing the 5x or 10x buffer stocks through a 0.22-µm filter can prevent or delay formation of precipitates.

[b]Use Tris-glycine buffers for SDS-polyacrylamide gels (see Appendix 8).

Specialized Electrophoresis Buffers

10x Alkaline Agarose Gel Electrophoresis Buffer

500 mM NaOH <!>
10 mM EDTA

Add 50 ml of 10 N NaOH and 20 ml of 0.5 M EDTA (pH 8.0) to 800 ml of H_2O and then adjust the final volume to 1 liter. Dilute the 10x alkaline agarose gel electrophoresis buffer with H_2O to generate a 1x working solution immediately before use. Use the same stock of 10x alkaline agarose gel electrophoresis buffer to prepare the alkaline agarose gel and the 1x working solution of alkaline electrophoresis buffer.

10x BPTE Electrophoresis Buffer

100 mM PIPES
300 mM Bis-Tris
10 mM EDTA

The final pH of the 10x buffer is ~6.5. The 10x buffer can be made by adding 3 g of PIPES (free acid), 6 g of Bis-Tris (free base), and 2 ml of 0.5 M EDTA to 90 ml of distilled H_2O and then treating the solution with diethylpyrocarbonate <!> (final concentration 0.1%; for more details, please see the information panel on **DIETHYLPYROCARBONATE** in Chapter 7).

PREPARATION OF ORGANIC REAGENTS

CAUTION: Please see Appendix 12 for appropriate handling of materials marked with <!>.

Phenol

Most batches of commercial liquefied phenol <!> are clear and colorless and can be used in molecular cloning without redistillation. Occasionally, batches of liquefied phenol are pink or yellow, and these should be rejected and returned to the manufacturer. Crystalline phenol is not recommended because it must be redistilled at 160°C to remove oxidation products, such as quinones, that cause the breakdown of phosphodiester bonds or cause cross-linking of RNA and DNA.

Equilibration of Phenol

Before use, phenol must be equilibrated to a pH of >7.8 because the DNA partitions into the organic phase at acid pH. Wear gloves, full face protection, and a lab coat when carrying out this procedure.

1. Store liquefied phenol at –20°C. As needed, remove the phenol from the freezer, allow it to warm to room temperature, and then melt it at 68°C. Add hydroxyquinoline to a final concentration of 0.1%. This compound is an antioxidant, a partial inhibitor of RNase, and a weak chelator of metal ions (Kirby 1956). In addition, its yellow color provides a convenient way to identify the organic phase.

2. To the melted phenol, add an equal volume of buffer (usually 0.5 M Tris-Cl [pH 8.0] at room temperature). Stir the mixture on a magnetic stirrer for 15 minutes. Turn off the stirrer, and when the two phases have separated, aspirate as much as possible of the upper (aqueous) phase using a glass pipette attached to a vacuum line equipped with appropriate traps (please see Appendix 8, Figure A8-2).

3. Add an equal volume of 0. 1 M Tris-Cl (pH 8.0) to the phenol. Stir the mixture on a magnetic stirrer for 15 minutes. Turn off the stirrer and remove the upper aqueous phase as described in Step 2. Repeat the extractions until the pH of the phenolic phase is >7.8 (as measured with pH paper).

4. After the phenol is equilibrated and the final aqueous phase has been removed, add 0.1 volume of 0.1 M Tris-Cl (pH 8.0) containing 0.2% β-mercaptoethanol <!>. The phenol solution may be stored in this form under 100 mM Tris-Cl (pH 8.0) in a light-tight bottle at 4°C for periods of up to 1 month.

Phenol:Chloroform:Isoamyl Alcohol (25:24:1)

A mixture consisting of equal parts of equilibrated phenol and chloroform:isoamyl alcohol <!> (24:1) is frequently used to remove proteins from preparations of nucleic acids. The chloroform denatures proteins and facilitates the separation of the aqueous and organic phases, and the isoamyl alcohol reduces foaming during extraction. Neither chloroform nor isoamyl alcohol requires treatment before use. The phenol:chloroform:isoamyl alcohol mixture may be stored under 100 mM Tris-Cl (pH 8.0) in a light-tight bottle at 4°C for periods of up to 1 month.