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Restriction Endonucleases

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BACKGROUND INFORMATION

Molecular biologists routinely use restriction enzymes as key reagents for a variety of applications including genomic mapping, restriction fragment length polymorphism (RFLP) analysis, DNA sequencing, and a host of recombinant DNA methodologies. Few would argue that these enzymes are not indispensable tools for the variety of techniques used in the manipulation of DNA, but like many common tools that are easy to use, they are not always applied as efficiently and effectively as possible. This chapter focuses on the biochemical attributes and requirements of restriction enzymes and delivers strategies to optimize their use in simple and complex reactions.

Which Restriction Enzymes Are Commercially Available?

While as many as six to eight types of restriction endonucleases have been described in the literature, Class II restriction endonucleases are the best known, commercially available and the most useful. These enzymes recognize specific DNA sequences and cleave each DNA strand to generate termini with 5′ phosphate and 3′ hydroxyl groups. For the vast majority of enzymes characterized to date within this class, the recognition sequence is normally four to eight base pairs in length and palindromic. The point of cleavage is within the recognition sequence. A variation on this theme appears in the case of Class IIS restriction endonucleases.

These recognize nonpalindromic sequences, typically four to seven base pairs in length, and the point of cleavage may vary from within the recognition sequence up to 20 base pairs away (Szybalski et al., 1991).

To date, nearly 250 unique restriction specificities have been discovered (Roberts and Macelis, 2001). New prototype activities are continually being discovered. The REBASE database (*http://rebase.neb.com*) provides monthly updates detailing new recognition specificities as well as commercial availability.

These enzymes naturally occur in thousands of bacterial strains and presumably function as the cell's defense against bacteriophage DNA. Nomenclature for restriction enzymes is based on a convention using the first letter of the genus and the first two letters of the species name of the bacteria of origin. For example, *Sac*I and *Sac*II are derived from *Streptomyces achromogenes*. Of the bacterial strains screened for these enzymes to date, well over two thousand restriction endonucleases have been identified—each recognizing a sequence specificity defined by one of the prototype activities. Restriction enzymes isolated from distinct bacterial strains having the same recognition specificity are known as isoschizomers (e.g., *Sac*I and *Sst*I). Isoschizomers that cleave the same DNA sequence at a different position are known as neoschizomers (e.g., *Sma*I and *Xma*I).

Why Are Some Enzymes More Expensive Than Others?

The distribution of list prices for any given restriction enzyme can vary among commercial suppliers. This is due to many factors including the cost of production, quality assurance, packaging, import duties, and freight. For many commonly available enzymes produced from native overexpressors or recombinant sources, the cost of production is relatively low and is generally a minor factor in the final price. Recombinant enzymes (typically overexpressed in a well-characterized *E. coli* host strain) are often less expensive than their nonrecombinant counterparts due to high yields and the resulting efficiencies in production and purification. In contrast, those enzyme preparations resulting in very low yields are often difficult to purify, and they have significantly higher production costs. In general, these enzymes tend to be dramatically more expensive (per unit of activity) than those isolated from the more robust sources. As these enzymes may not be available at the same unit activity levels of the more common enzymes, they can be less forgiving in nonoptimal reaction conditions,

and can be more problematic with initial use. The important point is that the relative price of a given restriction enzyme (or isoschizomer) may not be the best barometer of its performance in a specific application or procedure. The enzyme with the highest price does not necessarily guarantee optimal performance; nor does the one with the lowest price consistently translate into the best value.

Most commercial suppliers maintain a set of quality assurance standards that each product must pass in order to be approved for release. These standards are typically described in the supplier's product catalogs and detailed in the Certificate of Analysis. When planning to use an enzyme for the first time, it is important to review the corresponding quality control specifications and any usage notes regarding recommended conditions and applications.

What Can You Do to Reduce the Cost of Working with Restriction Enzymes?

Most common restriction enzymes are relatively inexpensive and often maintain full activity past the designated expiration date. Restriction enzymes of high purity are often stable for many years when stored at –20°C. In order to maximize the shelf life of less stable enzymes, many laboratories utilize insulated storage containers to mitigate the effects of freezer temperature fluctuations. Periodic summary titration of outdated enzymes for activity is another way to reduce costs for these reagents. For most applications, 1 μl is used to digest 250 ng to 1 μg of DNA. Enzymes supplied in higher concentrations may be diluted prior to the reaction in the appropriate storage buffer. A final dilution range of 2000 to 5000 Umits/ml is recommended. However, reducing the amount of enzyme added to the reaction may increase the risk of incomplete digestion with insignificant savings in cost. Dilution is a more practical option when using very expensive enzymes, when sample DNA concentration is below 250 ng per reaction, or when partial digestion is required. When planning for partial digestion, serial dilution (discussed below) is recommended. Most diluted enzymes should be stable for long periods of time when stored at –20°C. As a rule it is wise to estimate the amount of diluted enzyme required over the next week and prepare the dilution in the appropriate storage buffer, accordingly. For immediate use, most restriction enzymes can be diluted in the reaction buffer, kept on ice, and used for the day. Extending the reaction time to greater than one hour can often be used to save enzyme or ensure complete digestion.

If You Could Select among Several Restriction Enzymes for Your Application, What Criteria Should You Consider to Make the Most Appropriate Choice?

Each restriction endonuclease is a unique enzyme with individual characteristics, which are usually listed in suppliers' catalogs and package inserts. When using an unfamiliar enzyme, these data should be carefully reviewed. In addition some enzymes provide additional activities that may impact the immediate or downstream application.

Ease of Use

For many applications it is desirable and convenient to use 1 μl per reaction. Most suppliers offer standard enzyme concentrations ranging from 2000 to 20,000 units/ml (2–20 units/μl). In addition many suppliers also offer these enzymes in high concentration (often up to 100,000 units/ml), either as a standard product, or through special order. Enzymes sold at 10 to 20 units/μl are common and usually lend themselves for use in a wider variety of applications. When planning to use enzymes available only in lower concentrations (near 2000 units/ml), be sure to take the final glycerol concentration and reaction volume into account. By following the recommended conditions and maintaining the final glycerol concentration below 5%, you can easily avoid star activity.

Star Activity

When subjected to reaction conditions at the extremes of their operating range, restriction endonucleases are capable of cleaving sequences that are similar, but not identical, to their canonical recognition sequences. This altered specificity has been termed "star activity." Star sites are related to the recognition site, usually differing by one or more bases. The propensity for exhibiting star activity varies considerably among restriction endonucleases. For a given enzyme, star activity will be exhibited at the same relative level in each lot produced, whether isolated from a recombinant or a nonrecombinant source.

Star activity was first reported for *Eco*RI incubated in a low ionic strength high pH buffer (Polisky et al., 1975). Under these conditions, while this enzyme would cleave at its canonical site (G/AATTC), it also recognized and cleaved at N/AATTC. This reduced specificity should be a consideration when planning to use a restriction endonuclease in a nonoptimal buffer. It was also found that substituting Mn^{2+} for Mg^{2+} can result in star activity

(Hsu and Berg, 1978). Prolonged incubation time and high enzyme concentration as well as elevated levels of glycerol and other organic solvents tend to generate star activity (Malyguine, Vannier, and Yot, 1980). Maintaining the glycerol concentration to 5% or less is recommended. Since the enzyme is supplied in 50% glycerol, the enzyme added to a reaction should be no more than 10% of the final reaction volume.

When extra DNA fragments are observed, especially when working with an enzyme for the first time, star activity must be differentiated from partial digestion or contaminating specific endonucleases. First, check to make sure that the reaction conditions are well within the optimal range for the enzyme. Then, repeat the digest in parallel reactions, one with twice the activity and one with half the activity of the initial digest. Partial digestion is indicated as the cause when the number of bands is reduced to that expected after repeating the digestion with additional enzyme (or extending incubation time). If extra bands are still evident, contact the supplier's technical support resource for advice. Generally speaking, star activity and contaminating activities are more difficult to differentiate. Mapping and sequencing the respective cleavage sites is the best method to distinguish star activity from a partial digest or contaminant activity.

Site Preference

The rate of cleavage at each site within a given DNA substrate can vary (Thomas and Davis, 1975). Fragments containing a subset of sites that are cleaved more slowly than others can result in partial digests containing lighter bands visualized on an ethidium stained agarose gel. Certain enzymes such as *Eco*RII require an activator site to allow cleavage (Kruger et al., 1988). Substrates lacking the additional site will be cleaved very slowly. For certain enzymes (*Nae*I), adding oligonucleotides containing the site or adding another substrate containing multiple sites can improve cutting. In the case of *PaeR*7I, it has been shown that the surrounding sequence can have a profound effect on the cleavage rate (Gingeras and Brooks, 1983). In most cases this rate difference is taken in to account because the unit is defined at a point of complete digestion on a standard substrate DNA (e.g., lambda DNA) that contains multiple sites. Problems can arise when certain sites are far more resistant than others, or when highly resistant sites are encountered on substrates other than the standard substrate DNA. If a highly resistant site is present in a common cloning vector, then a warning should be noted on the data card or in the catalog.

Methylation

Methylation sensitivity can interfere with digestion and cloning steps. Many of the *E. coli* cloning strains express the genes for *EcoK*I methylase, dam methylase, or dcm methylase. The dam methylase recognizes GATC and methylates at the N6 position of adenine. *Mbo*I recognizes GATC (the same four base-pair sequence as dam methylase) and will only cleave DNA purified from *E. coli* strains lacking the dam methylase. *Dpn*I is one of only a few enzymes known to cleave methylated DNA preferentially, and it will only cleave DNA from dam^+ strains (Lacks and Greenberg, 1977). Another *E. coli* methylase, termed dcm, was found to block *Aat*I and *Stu*I (Song, Rueter, and Geiger, 1988). The dcm methylase recognizes CC(A/T)GG and methylates the second C at the C5 position.

The restriction enzyme recognition site doesn't have to span the entire methylation site to be blocked. Overlapping methylation sites can cause a problem. An example is the *Xba*I recognition site 5′ TCTAGA 3′. Although it lacks the GATC dam methylase target, if the preceding 5′ two bases are GA giving **GA**TCTAGA or the following 3′ bases are TC giving TCTAGA**TC**, then the dam methylase blocks *Xba*I from cutting. *E. coli* strains with deleted dam and dcm, like GM2163, are commercially available and should be used if the restriction site of interest is blocked by methylation. The first time a methylated plasmid is transformed into GM2163 the number of colonies will be low due to the important role played by dam during replication.

Methylation problems can also arise when working with mammalian or plant DNA. DNA from mammalian sources contain C5 methylation at CG sequences. Plant DNA often contains C5 methylation at CG and CNG sequences. Bacterial species contain a wide range of methylation contributed by their restriction modification systems (Nelson, Raschke, and McClelland, 1993). Information regarding known sensitivities to methylation can be found on data cards in catalog tables, by searching REBASE, and in the preceding review by Nelson.

Cloning problems can arise when working with DNA methylated at the C5 position. Most *E. coli* strains have an mcr restriction system that cleaves methylated DNA (Raleigh et al., 1988). A strain deficient in this system must be used when cloning DNA from mammalian and plant sources.

Substrate Effects

More on this discussion appears in the question below, *How Can a Substrate Affect the Restriction Digest?*

WHAT ARE THE GENERAL PROPERTIES OF RESTRICTION ENDONUCLEASES?

In general, commercial preparations of restriction endonucleases are purified and stored under conditions that ensure optimal reactivity and stability over time; namely –20°C. They are commonly supplied in a solution containing 50% glycerol, Tris buffer, EDTA, salt, and reducing agent. This solution will conveniently remain in liquid form at –20°C but will freeze at temperatures below –30°C. Those enzymes shipped on dry ice, or stored at –70°C, will have a white crystalline appearance; they revert to a clear solution as the temperature approaches –20°C. As a rule repeated freeze-thaw cycles are not recommended for enzyme solutions because of the possible adverse effects of shearing (more on the question, *How Stable are Restriction Enzymes?* appears below).

As a group (and by definition), Class II restriction endonucleases require magnesium (Mg^{2+}) as a cofactor in order to cleave DNA at their respective recognition sites. Most restriction enzymes are incubated at 37°C, but many require higher or lower (i.e., *Sma*I requires incubation at 25°C) temperatures. Percent activity tables of thermophilic enzymes incubated at 37°C can be found in some suppliers' catalogs. For most reactions, the pH optima is between 7 and 8 and the NaCl concentration between 50 and 100 mM. Concentrated reaction buffers for each enzyme are provided by suppliers. Typically each enzyme is profiled for optimal activity as a function of reaction temperature, pH (buffering systems), and salt concentration. Some enzymes are also evaluated in reactions containing additional components (BSA, detergents). Generally, these characteristics are documented in the published literature and referenced by suppliers.

Interestingly, a number of commonly used enzymes can display a broad range of stability and performance characteristics under fairly common reaction conditions. They may vary considerably in activity and may exhibit sensitivity to particular components. In an effort to minimize these undesirable effects, suppliers often adjust enzyme buffer components and concentrations to ensure optimal performance for the most common applications.

There is a wealth of information about the properties of these enzymes in most suppliers' catalogs, as well as on their Web sites. The documentation supplied with the restriction endonuclease should contain detailed information about the enzyme's properties and functional purity. It is important to read the Certificate of Analysis when using a restriction enzyme for the first

time, as it may provide important information concerning particular substrate DNAs or alternative reaction conditions for a specific application.

What Insight Is Provided by a Restriction Enzyme's Quality Control Data?

Restriction enzymes are isolated from bacterial strains that contain a variety of other enzyme activities required for normal cell function. These additional activities include other nucleases, phosphatases, and polymerases as well as other DNA binding proteins that may inhibit restriction enzyme activity. In preparations where trace amounts of these activities remain, the end-structure of the resulting DNA fragments may be degraded, thus inhibiting subsequent ligation. Likewise plasmid substrates may be nicked, thus reducing transformation efficiencies.

Ideally the restriction enzyme preparation should be purified to homogeneity and free of any detectable activities that might interfere with digestion or inhibit subsequent reactions planned for the resulting DNA fragments. In order to provide researchers with a practical means to conveniently evaluate the suitability of a given restriction enzyme preparation, suppliers include a Certificate of Analysis with each product, detailing the preparation's performance in a defined set of Quality Control Assays. In order to establish a standard reference for the amount of enzyme and substrate used in these assays, each supplier must first define the unit substrate and reaction conditions for each product.

Unit Definition

A unit of restriction endonuclease is defined as the amount of enzyme required to completely cleave 1 μg of substrate DNA suspended in 50 μl of the recommended reaction buffer in one hour at the recommended assay buffer and temperature. The DNA most often used is bacteriophage Lambda or another well-characterized substrate. Note that the unit definition is not based on classic enzyme kinetics. The enzyme molar concentration is in excess. A complete digest is determined by the visualized pattern of cleaved DNA fragments resolved by electrophoresis on an ethidium bromide-stained gel. Some restriction enzymes will behave differently when used outside the parameters of the unit definition. The number of sites (site density) or the particular type of DNA substrate may have an effect on "unit activity," but it is not always proportional (Fuchs and Blakesley, 1983).

Quality Control Assays—Maximum Units per Reaction

When using procedures requiring larger quantities of enzyme and/or extended reaction times, an appreciation of the quality control data can help determine a safe amount of enzyme for your application.

Overnight Assay

Increasing amounts of restriction endonuclease are incubated overnight (typically for 16 hours) in their recommended buffer with 1 μg of substrate DNA in a volume of 50 μl. The characteristic limit digest banding pattern produced by the enzyme in one hour is compared to the pattern produced from an excess of enzyme incubated overnight. A sharp, unaltered pattern under these conditions is an indication that the enzyme preparation is free of detectable levels of nonspecific endonucleases. The maximum number of units yielding an unaltered pattern is reported. Enzymes listing 100 units or more, a 1600-fold over digestion (100 units $\times$ 16 h), will not degrade DNA up to megabase size in mapping experiments and can be assumed to be virtually free of nonspecific endonuclease (Davis, T. and Robinson, D., unpublished observations).

Nicking Assay

Another sensitive test for contaminating endonucleases is a four hour incubation with a supercoiled plasmid that lacks a site for the enzyme being tested. The supercoil is very sensitive to nonspecific nicking by a single-stranded endonuclease, cleavage by a double-stranded endonuclease, or topoisomerase activity. If a single-stranded nick occurs, the supercoiled molecule, RFI, unwinds and assumes the circular form, RFII. If a double-stranded cleavage occurs, the circle will become linear. High levels of single-stranded nicking leads to linear DNA. All three forms of DNA have distinct electrophoretic mobilities on agarose gels. Enzymes converting 5% or less of the plasmid to relaxed form using 100 units of enzyme for four hours can be considered virtually free of nicking activity. High-salt buffers, especially at elevated temperature, can cause some conversion to relaxed form. A control reaction, including buffer and DNA but lacking enzyme, is incubated and run on the agarose gel for comparison.

Exonuclease Assay

Suppliers use a variety of assays to check for exonuclease activity. A general assay mixture contains a restriction endonuclease

with 1 μg of a mixture of single- and double-stranded, ^{3}H-labeled *E. coli* DNA (200,000 cpm/μg) in a 50 μl reaction volume with the supplied buffer. Incubations (along with a background control containing no enzyme) are at the recommended temperature for four hours. Exonuclease contamination is indicated by the percent of the total labeled DNA in the reaction that has been rendered TCA-soluble. The limit of detectability of this assay is approximately 0.05%. Enzymes showing background levels of degradation with 100 units incubated for four hours can be considered virtually free of exonuclease.

Ligation/Recut Assay

Ligation and recutting is a direct determination of the integrity of the DNA fragment termini upon treatment with the restriction enzyme preparation. Ligation and recut of greater than 90% with a 10- to 20-fold excess of enzyme creating ends with overhangs or 80% for blunt ends indicate an enzyme virtually free of exonuclease or phosphatase specific for the overhang being tested. Alternative assays (i.e., end-labeling) are used to evaluate Type IIS restriction enzymes (e.g., *Fok*I, *Mbo*II). Since these enzymes cleave outside of their recognition sequence, the standard ligation assay would not determine a loss of terminal nucleotides due to exonuclease. The resulting ends could still ligate, and since their recognition sites remain intact, the enzyme would still be able to recut.

Blue-White Screening Assay

The β-galactosidase blue-white selection system is also applied to determine the integrity of the DNA ends produced after digestion with an excess of enzyme to test ligation efficiency. An intact gene gives rise to a blue colony; while an interrupted gene, which contains a deletion due to degraded DNA termini, gives rise to a white colony. Restriction enzymes tested using this assay should produce fewer than 3% white colonies.

The values given for the number of units added giving "virtually contaminant-free" preparations are somewhat arbitrary. They are useful, however, for determining maximum levels of enzyme to use in a reaction for most common applications. Enzymes with quality control results significantly below these values can still be used with confidence under simple assay conditions. As discussed later for complex restriction digestions, caution should be considered when extending reaction times and adding more than 1 to 2 μl of enzyme to 1 μg DNA in 50 μl.

How Stable Are Restriction Enzymes?

As a class, most restriction enzymes are stable proteins. Even during purification periods lasting two weeks, many enzymes lose no appreciable activity at 4°C. At the final stage of purification, the enzyme preparation is typically dialyzed into a 50% glycerol storage buffer and subsequently stored at –20°C. At this temperature the glycerol solution does not freeze. Most enzymes are stable for well over a 12-month period when properly stored. In one stability test of 170 restriction enzymes, activity was assessed after storage for 16 hours at room temperature. Of the enzymes tested, 122 (or 72%) exhibited no loss in activity (McMahon, M., and Krotee, S., unpublished observation). This point is important to note in case of freezer malfunction.

Even under optimal storage conditions, however, some enzymes may begin to lose noticeable activity within a six-month period. The supplier's expiration date, Certificate of Analysis, or catalog will provide more specific information regarding these enzymes. It is best to use these enzymes within a reasonable amount of time after they have been received. Some users employ a freezer box designed to maintain a constant temperature (for short periods at the bench) to store enzymes within the freezer. Alternatively, most enzymes can be stored at –70°C for extended periods. Repeated freeze–thaw cycles from –70°C to 0°C is not recommended. Each time the enzyme preparation solution is frozen, the buffer comes out of solution prior to freezing. As a result some enzymes may lose significant activity each time a freeze–thaw cycle is repeated. Often the extent of an enzyme's stability during storage at –20°C is buffer-related. Identical enzyme preparations obtained from two suppliers, when maintained in their respective storage buffers, may have significantly different shelf lives.

How Stable Are Diluted Restriction Enzymes?

For a discussion, refer above to the question *What Can You Do to Reduce the Cost of Working with Restriction Enzymes*.

SIMPLE DIGESTS

How Should You Set up a Simple Restriction Digest?

Reaction Conditions

Most restriction digests are designed either to linearize a cloning vector or to generate DNA fragments by cutting a given target DNA to completion at each of the corresponding restriction sites. To ensure success in any subsequent manipulations (i.e.,

ligation), the enzyme treatment must leave each of the resulting DNA termini elements intact.

To 1 μg of purified DNA in 50 μl of 1× reaction buffer, 1 μl of enzyme is added and the reaction is incubated for one hour at the recommended reaction temperature. In most instances the amount of DNA can be safely varied from about 250 ng to several micrograms and the volume can be varied between 20 μl and 100 μl. Suitable reaction times may be as little as 15 minutes or as long as 16 hours. Common DNA purification protocols, as well as commercially available kits, yield DNA that is suitable for most digestions. Most commonly used restriction enzymes are of high purity, inexpensive, and provided at concentrations of 5 to 20 units/μl. Using 1 to 2 μl will overcome any expected variability in DNA source, quantity, and purity. The length of incubation time may be decreased to save time or increased to ensure complete digestion of the last few tenths of a percent of substrate, as the reaction asymptotically approaches completion.

Control Reactions

Aside from the mere discipline of maintaining "good laboratory practice," the ultimate savings realized in time and effort by running a simple control reaction is often underestimated. Control reactions can often reveal the cause of a failed digest or point to the step within a series of reactions responsible for generating an unexpected result. For every experimental restriction enzyme reaction set performed, a control reaction (containing sample DNA, reaction buffer, and no restriction enzyme) should also be included and analyzed on the agarose gel. Degradation of DNA in the control reaction may indicate nuclease contamination in the DNA preparation or in the buffer. The control reaction products run alongside the sample reaction products on the agarose gel enables for a more accurate assessment of whether the reaction went to completion. Running the appropriate size markers is also recommended.

Is It Wise to Modify the Suggested Reaction Conditions?

Suppliers devote considerable effort in formulating specific enzyme preparations and the corresponding reaction buffers in order to ensure sufficient enzyme activity for most common applications. In addition suppliers often provide data (Activity Table) indicating the relative activity of each enzyme when incubated under standard reaction conditions for a variety of reaction buffers provided. This is a useful guide when planning multiple

restriction enzyme digests. For enzymes with low activity in these standard buffers, specialized buffers are typically supplied. Restriction enzymes also have a broad range of activity in nonchloride salt buffers. Some suppliers also offer a potassium-acetate or potassium-glutamate single-buffer system that is formulated to be compatible with a significant subset of their enzymes. (McClelland et al., 1988; O Farrell, Kutter, and Nakanishe, 1980). The reaction buffers themselves are typically supplied as concentrated solutions, ranging from 2× to 10×, and should be properly mixed upon thawing prior to final dilution.

It is important to note that the reaction buffer supplied with a given enzyme is the same buffer in which all quality assurance assays are performed, and documented in the Certificate of Analysis provided. Consequently certain modifications to the recommended reaction conditions (i.e., adding components or changing reaction volume, temperature, or time of incubation) may produce unexpected results. Restriction enzymes can vary considerably in sensitivity to particular changes in their reaction parameters. While salt concentration may have a significant effect on activity, salt type (i.e., NaCl vs. KCl) is usually not critical. One exception would be in the case of *Sma*I, which has a strong preference for KCl. For most sensitive enzymes the Certificate of Analysis will detail any reaction modifications not recommended as well as any suggestions for alternative reaction conditions. In order to determine whether a given enzyme may be sensitive to an intended variation in reaction conditions, the Activity Table is also a useful reference. As a rule the most robust enzymes exhibit high relative activity across the range of buffers listed (*Pvu*II). Conversely, those enzymes showing a narrow range for high activity may require additional consideration prior to any change in reaction conditions (*Sal*I) and the technical resources provided by the supplier should be consulted.

All restriction enzymes, as do most other nucleases, require Mg^{2+} as a cofactor for the DNA cleavage reaction; most buffers for restriction enzymes contain 10 mM Mg^{2+}. To protect DNA preparations in storage buffer from any trace nucleases, EDTA (a Mg^{2+} chelator) is used, often stocked as a disodium salt solution. This is commonly used in various stop-dye solutions as well as electrophoresis buffer. DNA preparations with excessive concentrations of EDTA may inhibit restriction endonuclease cleavage, especially if the DNA solution represents a high proportion of the final reaction volume. Addition of Mg^{2+} will alleviate the inhibition.

A reducing agent, like dithiothreitol or β-mercaptoethanol, is a frequent buffer component even though it is not required for enzyme activity. However, as reaction buffers are typically diluted to their final reaction volume with distilled water, oxidation (i.e., from dissolved oxygen) could significantly reduce enzyme activity in the absence of sufficient reducing agent. BSA is frequently added as a stabilizing component to restriction enzyme preparations (Scopes, 1982). BSA increases the overall protein concentration and, by coating the hydrophobic surfaces of plastic vials, prevents possible denaturation. The activity level of many restriction enzymes in a reaction may be significantly enhanced if the final BSA concentration is around 100 μg/ml. Sometimes non-ionic detergents, like Triton ×-100 or Tween 20, are added as stabilizers for particular enzymes (*EcoR*I, *Not*I). A few restriction endonucleases, like *Bsg*I, have their activity significantly increased by the addition of *S*-adenosylmethionine (REBASE).

As most restriction enzymes are isolated from mesophilic bacteria, the vast majority exhibit excellent activity at 37°C in a near-neutral pH buffer. An increasing number of enzymes are being isolated from thermophilic bacteria, which display optimal activity within the range of 50°C to 75°C. As it happens, a good number of these enzymes also retain adequate activity at 37°C, and while this temperature may not be optimal for a particular enzyme, a supplier may list it as such for convenience in double-digest applications.

COMPLEX RESTRICTION DIGESTIONS

Complex reactions include double digests, reactions using nonoptimal buffers, reactions with DNA containing sites close to the ends, reactions with PCR products, and reactions involving multiple steps. In addition these include reactions with DNA concentrations that are significantly higher or lower than the recommended 1 μg/50 μl as well as simple reactions that simply didn't work the first time.

How Can a Substrate Affect the Restriction Digest?

PCR Products

Restriction endonucleases can often be used directly on PCR products in the PCR reaction mix. Suppliers often provide data indicating relative enzyme activity under these reaction conditions. Restriction endonuclease activity is influenced by the buffer used for PCR as well as the enzyme's ability to cleave in the pres-

ence of primers. The excess primers present in PCR reactions have been shown to inhibit *Sma*I and *Nde*I (Abrol and Chaudhary, 1993), but many restriction endonucleases can cleave in the presence of a 100-fold molar excess of primers. If your PCR products were not digested satisfactorily, eliminate the primers by gel purification, desalting column chromatography, membrane filtration or glass (Bhagwat, 1992).

Ends of Linear Fragments

Restriction endonucleases differ in their ability to cleave at recognition sites close to the end of a DNA fragment. Cleavage close to the end of a fragment is important when two restriction sites are close together in the cloning region of a plasmid and when cleaving near the ends of PCR products. Many restriction enzymes can cleave near a DNA end having one base pair in addition to a 1 to 4 single-base overhang produced by an initial cleavage; others require at least 3 base pairs in addition to an overhang (Moreira and Noren, 1995). When designing PCR primers containing restriction sites, adding eight random bases 5′ of the restriction site is recommended for complete digestion of the restriction sites.

Plasmids

Supercoiled plasmids often require more restriction endonuclease to achieve complete digestion than linear DNA. Manufacturers' catalogs often contain tables listing the number of units of restriction enzyme required to completely cleave 1 μg of commonly used supercoiled plasmids.

Inhibitors

Contaminants in the DNA preparation can inhibit restriction endonuclease activity. Residual SDS from alkaline lysis procedures can inhibit restriction endonucleases. High concentrations of NaCl, CsCl, other salts, or EDTA can inhibit restriction enzymes. Salt is concentrated when the DNA is alcohol precipitated. Washes containing 70% alcohol following the initial precipitation will solubilize some salt, but dialysis is preferred.

Protein contaminants in the DNA preparation can influence the restriction digests. Double strand specific exonucleases can co-purify with plasmid DNA when using column purification procedures (Robinson, D., and Kelley, K., unpublished observation). Phenol chloroform extraction followed by ethanol precipitation is an efficient method of removing proteins from DNA samples. The phenol and chloroform as well as the alcohol must

be thoroughly removed to ensure restriction enzyme activity. Residual phenol and chloroform are removed by the alcohol precipitation and 70% alcohol wash steps. Alcohol is removed by desiccation. Dialysis can be used to remove residual alcohol that may be present from a DNA sample that was resuspended before the alcohol was completely removed. Alcohol can be introduced as a wash before elution when using diatomaceous earth as a resin for DNA purification. The resin must be thoroughly dried before DNA elution to remove the alcohol.

Core histones present on eukaryotic chromosomes can be difficult if not impossible to remove. Proteinase K followed by phenol chloroform extraction is often used in these preparations. Proteinase K is also used when preparing intact chromosomal DNA embedded in agarose for megabase mapping by pulse field gel electrophoresis (PFGE). Proteinase K must be inactivated using phenol chloroform or PMSF. Since the inhibition of proteinase K by a proteinase inhibitor such as PMSF is reversible, agarose blocks containing proteinase K should be extensively washed by changing the buffer multiple times. Most restriction enzymes are active in solutions containing PMSF.

Should You Alter the Reaction Volume and DNA Concentration?

Reaction Volume

A standard reaction volume to cleave 1 to 2 μg of DNA is 50 μl. Caution must be used when decreasing the reaction volume. Star activity tends to increase with decreasing reaction volume. The increase is most likely due to the higher glycerol concentration in the smaller volumes. Using 2 μl of *BamH*I containing 50% glycerol in a 10 μl reaction gives a final glycerol concentration of 10%. Increasing the reaction volume is not common unless more than 1 μg of DNA is being digested. Increasing the volume should be less problematic than decreasing the volume.

DNA Concentration

Varying the DNA concentration significantly from the standard (1 μg in 50 μl) can cause problems. Decreasing the amount of DNA or increasing the amount of overdigestion can increase star activity. An additional fourfold overdigestion occurs when 250 ngs are digested compared to 1 μg when using the same number of units of restriction enzyme. Low DNA concentrations near the K_m of a restriction enzyme could inhibit cleavage. The K_m for lambda DNA is 1000-fold less than 1 μg/50 μl (Fuchs &

Blakesley, 1983). Increasing the amount of DNA in 50 μl in most cases will not have a negative impact on the reaction. *Hind*III has been reported to work more efficiently on higher concentration DNA (Fuchs & Blakesley, 1983). Increasing the number of units or length of reaction will make up for the excess DNA. Care must be taken with the addition of extra enzyme, to keep the glycerol concentration to less than 5%. When digesting large quantities of DNA, using a concentrated enzyme is desirable. Inhibition may become a problem if the DNA has contaminants that influence enzyme activity. Salt and other contaminants in the DNA solution are more likely to be problematic if the DNA solution represents a large percentage of the final reaction mix.

Reaction Time

Extended digestion times can be used to increase the performance of a restriction enzyme, but the stability of the restriction enzyme in reaction should be checked by consulting the manufacturer's "survival in reaction" tables. BSA added to 100 μg/ml can increase survival. One should also consider that any trace contaminants in the preparation may continue to be active during an extended reaction. Often lower reaction temperatures can be used with unstable enzymes to increase performance when used for extended periods. One Unit of *Pme*I will digest 1 μg of DNA in two hours at 37°C but can digest 2 μg lambda in two hours at 25°C (Robinson, D., unpublished observation). When using *Pme*I for digesting agarose–embedded DNA, an incubation at 4°C overnight followed by one to two hours at 37°C is suggested.

Double Digests: Simultaneous or Sequential?

Simultaneous

The most convenient way to produce two different ends is to cut both at the same time in one reaction mix. Often the conditions for one enzyme or the other is not ideal. Manufacturers' buffer charts give the percent activity in buffers other than the one in which the enzyme is titered. If there is a buffer that indicates at least 50% activity for each enzyme, a coordinated double digest can be performed. Inexpensive, highly pure enzymes with no notes warning against star activity can be used in excess with confidence. A 10- to 20-fold excess of enzyme is recommended to increase the chances of success. Two microliters of a 10 unit/μl stock will give a 10-fold overdigest when used for one hour on 1 μg in a buffer giving 50% activity. If the enzyme is stable in reaction, then incubating for longer periods will increase

the amount of overdigestion. Consult the manufacturer's stability information.

If the reaction produces extra fragments, possibly caused by star activity, reduce the reaction time or the amount of enzyme. If the reaction is incomplete, individually test each enzyme to determine it's ability to linearize the plasmid. A lack of cutting may indicate an inactive enzyme, absence of the expected site, or inhibitors in the template preparation. Test the enzyme on a second target as a control. If both enzymes are active, and the restriction sites are within several bases of each other, there may be a problem cutting close to the end of the fragment.

Sequential

Enzyme sets that are not compatible for double digests require sequential digestion. Always perform the first digest with the enzyme requiring the lower salt buffer. Either salt (or the corresponding 10× reaction buffer) may then be added to the reaction and the second enzyme can be used directly. To prevent the first enzyme from exhibiting star activity in the second buffer, it is wise to heat inactivate prior to addition of the second enzyme. Addition of BSA, reducing agents, or detergents has no adverse effects on restriction enzymes and may be safely added as required to the reaction.

If the pH requirements between the two enzymes differ by more than 0.5 pH units or the difference in salt requirement is critical (NaCl vs. KCl), alcohol precipitation between enzyme treatments is commonly performed. Alternatively, drop dialysis (see procedure D at the end of this chapter) is an option. A strategy that can often save a dialysis step would be to perform the first reaction in a 20 μl volume and then add 80 μl containing 10 μl of the higher salt buffer and enzyme to the initial reaction. The second reaction approximates the standard conditions for that enzyme.

Expensive enzymes should be optimized and used first in sequential reactions. When planning to use enzymes from different suppliers, first consider their optimal activity by looking at the NaCl or KCl requirements. Compare the buffer charts of both suppliers to determine if the enzyme is used in a standard or optimized buffer. Enzymes that are sold with optimized buffers should be used in those buffers when possible. If the same enzyme is sold by both suppliers, compare the two reaction buffers. Remember, the enzyme is titered in the buffer that is supplied. One supplier may choose to improve titer using a detergent and BSA, while the

other may be using a different salt, pH, or enzyme concentration. In some cases a supplier may be categorizing an enzyme into a core buffer system by increasing the molar concentration of the enzyme. If used in an optimized buffer, this enzyme would titer at higher activity. If an enzyme from another supplier is used in this suboptimal core buffer, poor activity may result.

GENOMIC DIGESTS

When Preparing Genomic DNA for Southern Blotting, How Can You Determine if Complete Digestion Has Been Obtained?

Southern blotting involves the digestion of genomic DNA, gel electophoresis, blotting onto a membrane, and probing with a labeled oligonucleotide. The restriction pattern after gel electrophoresis is usually a smear, which may contain some distinguishable bands when visualized by ethidium bromide staining. It is often difficult to judge if the restriction digest has gone to completion or if degradation from star activity or nonspecific nuclease contamination is occurring. A twofold serial digest of genomic DNA enables a stable pattern, representing complete digestion, to be distinguished from an incomplete or degraded pattern.

Complete digestion is indicated when a similar smear of DNA appears in consecutive tubes of decreasing enzyme concentration within the serial digest. If the tubes with high enzyme concentration show smears that contain fragments smaller than those seen in tubes containing lesser enzyme, then it is likely that degradation is occurring. If the tube containing the most enzyme is the only sample demonstrating a complete digest, then the subsequent tubes (containing less enzyme) will demonstrate progressively larger fragments. A uniformly banded pattern will not occur in serial tubes unless the samples are all completely cut or completely uncut (Figure 9.1).

If the size of the smear does not change even at the greatest enzyme concentration, the digest may appear to have failed. A second possibility is that the fragments are too large to be resolved by standard agarose gel electrophoresis. Rare cutting enzymes may produce fragments greater than 50 kb, may not cleave a subset of sites due to methylation, or their recognition sequence might be underrepresented in the genome being studied. Pulse field gel electrophoresis must be used to resolve these fragments. Tables listing the average size expected from digestion of different species' DNA may be found in select suppliers' catalogs.

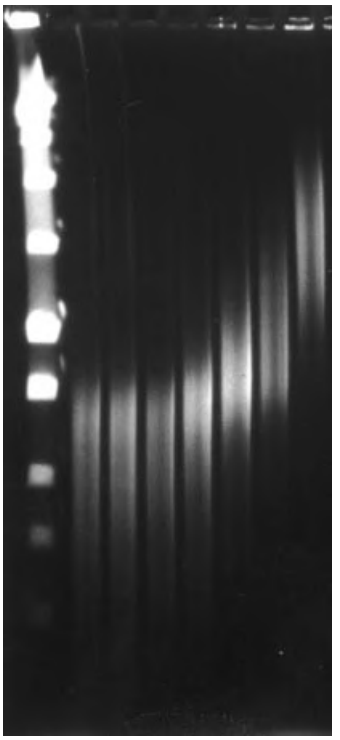

Figure 9.1 Testing for complete digestion of genomic DNA. Twofold serial digest using New England Biolabs *Avr*II of Promega genomic human DNA (cat. no. G304), 0.5 μg DNA in 50 μl NEB Buffer 2 for 1 hour at 37°C. *Avr*II added at 20 units and diluted to 10 units, etc., with reaction mix. The marker NEB Low Range PFG Marker (cat. no. N03050S). Complete digestion is indicated by lanes 2–4. Photo provided by Vesselin Miloushev and Suzanne Sweeney New England Biolabs. Reprinted by permission of New England Biolabs.

How Should You Prepare Genomic Digests for Pulsed Field Electrophoresis?

Pulse field electrophoresis techniques including CHEF, TAFE, and FIGE have made possible the resolution of DNA molecules up to several million base pairs in length (Birren et al., 1989; Carle, Frank, and Olson, 1986; Carle and Olson, 1984; Chu, Vollrath, and Davis, 1986; Lai et al., 1989; Stewart, Furst, and Avdalovic, 1988). The DNA used for pulsed field electrophoresis is trapped in agarose plugs in order to avoid double-stranded breaks due to shear forces. Protocol A has been used at New England Biolabs, Inc. for the preparation and subsequent restriction endonuclease digestion of *E. coli* and *S. aureus* DNA (Gardiner, Laas, and Patterson, 1986; Smith et al., 1986). This protocol may be modified as required for the cell type used.

Protocol A: Preparation of E. coli and S. aureus DNA

Cell Culture

1. Cells are grown under the appropriate conditions in 100 ml of media to an OD_{590} equal to 0.8 to 1.0. The chromosomes are then

aligned by adding 180 mg/ml chloramphenicol and incubating an additional hour.

2. The cells are spun down at 8000 rpm at 4°C for 15 minutes.
3. The cell pellet is resuspended in 6 ml of buffer A at 4°C. Alternatively 1.5 g of frozen cell paste may be slowly thawed in 20 ml of buffer A. Lysed cells from the thawing process are allowed to settle and the intact cells suspended in the supernatant are decanted and pelleted by centrifugation and washed once with 20 ml of buffer A. The pelleted cells are resuspended in 20 ml of buffer A.

DNA Preparation and Extraction

1. The suspended cells are warmed to 42°C and mixed with an equal volume of 1% low-melt agarose* in 1× TE at 42°C. For *S. aureus* cells, lysostaphin is added to a final concentration of 1.5 mg/ml. The agarose solution may be poured into insert molds. Alternatively, the agarose may be drawn up into the appropriate number of 1 ml disposable syringes that have the tips cut off.
2. The molds or syringes are allowed to cool at 4°C for 10 minutes. The agarose inserts are removed from the molds or extruded from the 1 ml syringes.
3. A 12 ml volume of the agarose inserts is suspended in 25 ml of buffer B (for *E. coli*), or 25 ml of buffer C (for *S. aureus*). Lysozyme (for *E. coli*) or Lysostaphin (for *S. aureus*) is added to a final concentration of 2 mg/ml. The solution is incubated for two hours at 37°C with gentle shaking. These solutions may also contain 20 μg/ml RNase I (DNase-free).
4. The agarose inserts are equilibrated with 25 ml buffer D for 15 minutes with gentle shaking. Replace with fresh buffer and repeat. Replace with 25 ml of buffer D containing 2 mg/ml proteinase K. This solution is incubated for 18 to 20 hours at 37°C with gentle shaking.
5. The inserts are again subjected to 15 minutes gentle shaking with 25 ml of buffer E. Replace with fresh buffer and repeat. Then incubate for 1 hour in buffer E, with 1 mM Phenylmethylsulfonyl fluoride (PMSF) to inactivate Proteinase K. As before, wash twice more with buffer E.
6. The inserts are washed twice with 25 ml of buffer F. The inserts are stored in buffer F at 4°C.

**Pulse field grade agarose should be used. The efficiency of the restriction enzyme digestion may vary with different lots of other low-temperature gelling agaroses.*

Digestion of Embedded DNA

Most restriction enzymes can be used to cleave DNA embedded in agarose, but the amount of time and enzyme required for complete digestion varies. Many enzymes have been tested for their ability to cleave embedded DNA (Robinson et al., 1991).

1. Agarose slices containing DNA (20 μl) are equilibrated in 1.0 ml of restriction enzyme buffer. The cylinders of agarose may be drawn back up into the 1 ml syringes in order to accurately dispense 20 μl of the agarose. The solution is gently shaken at room temperature for 15 minutes.
2. The 1 ml wash is decanted or aspirated from the agarose slice. The insert slice is submerged in 50 μl of restriction enzyme buffer. The appropriate number of units of the restriction enzyme with or without BSA is added to the reaction mixture and digested for a specific time and temperature as outlined by Robinson et al. (1991).
3. Following the enzyme digestion, the inserts may be treated to remove proteins using Proteinase K following the steps outlined above. Alternatively, the slices may be loaded directly onto the pulse field gel. Long-term storage of the endonuclease digested inserts is accomplished by aspirating the endonuclease reaction buffer out of the tube and submerging the insert in 100 ml of buffer E at 4°C. Insert slices that have been incubated at 50°C during the endonuclease digestion should be placed on ice for 5 minutes before handling the sample for loading or aspirating the buffer.

List of Buffers

Buffer A Cell suspension buffer: 10 mM Tris-HCl pH 7.2 and 100 mM EDTA.

Buffer B Lysozyme buffer: 10 mM Tris-HCl pH 7.2, 1 M NaCl, 100 mM EDTA, 0.2% sodium deoxycholate, and 0.5% *N*-lauryl-sarcosine, sodium salt.

Buffer C Lysostaphin buffer: 50 mM Tris-HCl, 100 mM NaCl, and 100 mM EDTA.

Buffer D Proteinase K buffer: 100 mM EDTA pH 8.0, 1% *N*-lauryl-sarcosine, sodium salt, and 0.2% sodium deoxycholate.

Buffer E Wash buffer: 20 mM Tris-HCl pH 8.0 and 200 mM EDTA.

Buffer G Storage buffer: 1 mM Tris-HCl pH 8.0 and 5 mM EDTA.

What Are Your Options If You Must Create Additional Rare or Unique Restriction Sites?

Cleavage at a single site in a genome may occur by chance using restriction endonucleases or intron endonucleases, but the

number of enzymes with recongition sequences rare enough to generate megabase DNA fragments is relatively small. When no natural recognition site occurs in the genome, an appropriate sequence can be introduced genetically or in vitro via different multiple step reactions.

Genetic Introduction

Recognition sites have been introduced into *Salmonella typhimurium* and *Saccharomyces cerevisiae* genomes by site specific recombination or transposition (Hanish and McClelland, 1991; Thierry and Dujon, 1992; Wong and McClelland, 1992). Endogenous intron endonuclease recognition sites are found in many organisms. In cases where restriction enzymes and intron endonucleases cleave too frequently, it may be possible to use lambda terminase. The 100 bp lambda terminase recognition site does not occur naturally in eukaryotes. Single-site cleavage has been demonstrated using lambda terminase recognition sites introduced into the *E. coli* and *S. cerevisiae* genomes (Wang and Wu, 1993).

Multiple-Step Reactions

The remainder of this discussion reviews multiple-step procedures that have been used to generate megabase DNA fragments. Our intention is to provide a clear explanation of each procedure and highlight some of the complexities involved. Providing detailed protocols for each is beyond the scope of this chapter but can be found in the references cited.

Increasing the complexity of multiple-step reactions decreases the chances of success. Conditions needed for one step may not be compatible with the next. All of the steps must function well using agarose-embedded DNA as a substrate.

Altering Restriction Enzyme Specificity by DNA Methylation

DNA methylases can block restriction endonuclease cleavage at overlapping recognition sites, decreasing the number of cleavable restriction sites and increasing the average fragment size (Backman, 1980; Dobrista and Dobrista, 1980). Unique cleavage specificities can be created by using different methylase/restriction endonuclease combinations (Nelson, Christ, and Schildkraut, 1984; Nelson and Schildkraut, 1987). The following well-characterized, two-step reaction involves the restriction endonuclease *Not*I and a methylase (Gaido, Prostko, and Strobl, 1988; Qiang et al., 1990; Shukla et al., 1991).

The *Not*I recognition site

$$5' \ldots GC_{\wedge}GGCCGC \ldots 3'$$

$$3' \ldots CGCCGG_{\wedge}CG \ldots 5'$$

will not cleave when methylation at the following cytosine occurs in the *Not*I recognition site:

$$5' \ldots GCGGC^{m}CGC \ldots 3'$$

$$3' \ldots CGCCGGCG \ldots 5'$$

or

$$5' \ldots GCGGCCGC \ldots 3'$$

$$3' \ldots CG^{m}CCGGCG \ldots 5'$$

*Not*I sites that overlap the recognition site of the methylases M. *Fnu*DII, M. *Bep*I, or M. *Bsu*I can be modified as shown above. These methylases recognize the following sequence:

$$5' \ldots CGCG \ldots 3'$$

$$3' \ldots GCGC \ldots 5'$$

They methylate the first cytosine in the 5′ to 3′ direction:

$$5' \ldots {}^{m}CGCG \ldots 3'$$

$$3' \ldots GCG^{m}C \ldots 5'$$

Now the subset of *Not*I sites that are preceded by a C or followed by a G will be resistant to subsequent cleavage by *Not*I.

Resistant sites

$$5' \ldots \mathbf{C}GCGGCCGC \ldots 3'$$

$$3' \ldots \mathbf{G}CG^{m}CCGGCG \ldots 5'$$

or

$$5' \ldots GCGGC^{m}CGC\mathbf{G} \ldots 3'$$

$$3' \ldots CGCCGGCG\mathbf{C} \ldots 5'$$

which are sites flanked by any of the following combinations, will be cleaved by *Not*I:

$$5' \ldots \{A, G, T\}\, GC_{\wedge}GGCCGC\, \{A, C, T\} \ldots 3'$$

$$3' \ldots \{T, C, A\}\, CGCCGG_{\wedge}CG\, \{T, G, A\} \ldots 5'$$

This methylation reaction followed by *Not*I digestion statistically reduces the number of *Not*I sites by nearly half. The larger

fragments produced may be more easily mapped using PFGE. A table of other potentially useful cross-protections for megabase mapping can be found in Nelson and McClelland (1992) and Qiang et al. (1990). A potential problem is that certain methylation sites may react slowly allowing partial cleavage events (Qiang et al., 1990).

DNA Adenine Methylase Generation of 8 to 12 Base-Pair Recognition Sites Recognized by DpnI

*Dpn*I is a unique restriction enzyme that recognizes and cleaves DNA that is methylated on both strands at the adenine in its recognition site (Lacks and Greenberg, 1975, 1977; Vovis, 1977).

*Dpn*I recognizes the following site:

5′ . . . G ^{m}A T C . . . 3′

3′ . . . C T ^{m}A G . . . 5′

The adenine methylases M. *Taq*I (McClelland, Kessler, and Bittner, 1984; McClelland, 1987), M. *Cla*I (McClelland, Kessler, and Bittner, 1984; McClelland, 1987; Weil and McClelland, 1989), M. *Mbo*II (McClelland, Nelson, and Cantor, 1985), and M. *Xba*I (Patel et al., 1990) have been used to generate a *Dpn*I recognition site with the apparent cleavage frequency of a 8 to 12 base-pair recognition sequence (Nelson and McClelland, 1992). The M. *Taq*I/*Dpn*I reaction is detailed below.

The M. *Taq*I recognition site

5′ . . . TCGA . . . 3′

3′ . . . AGCT . . . 5′

methylates the adenine on both strands of the above sequence to produce

5′ . . . T C G ^{m}A . . . 3′

3′.^{m}A G C T . . . 5′

Hemimethylated *Dpn*I sites (in bold below) will be generated when the sequence surrounding the site above is as follows:

5′ . . . T C **G ^{m}ATC** . . . 3′

3′ . . . ^{m}A G **C TAG** . . . 5′

or

5′ . . . **G A T C** G^{m}A . . . 3′

3′ . . . **C T^{m}A G** C T . . . 5′

The hemimethylated *Dpn*I site is cleaved at a rate 60× slower than the fully methylated site (Davis, Morgan, and Robinson, 1990). M. *Taq*I generates a fully methylated *Dpn*I site when two M. *Taq*I recognition sequences occur next to each other. The fully methylated *Dpn*I site is shown in bold below:

5′. . . . TC**G ^{m}A T C** G^{m}A . . . 3′

3′ . . . ^{m}AG**C T ^{m}A G** C T . . . 5′

The apparent recognition site of the M. *Taq*I/*Dpn*I reaction can be simply represented by the eight base pairs 5′ . . . TCGATCGA . . . 3′. The 10 base pair recognition site of the M. *Cla*I/*Dpn*I reaction can be represented by the sequence 5′ . . . ATCGATCGAT . . . . 3′. Notice that M. *Cla*I creates a *Dpn*I site by a slightly different overlap than demonstrated by the M. *Taq*I reaction. The M. *Cla*I/*Dpn*I reaction has been demonstrated on a bacterial and yeast genome (Waterbury et al., 1989; Weil and McClelland, 1989). The M. *Xba*I/*Dpn*I reaction can be represented by the 12 base-pair sequence 5′..TCTAGATCTAGA..3′. This reaction has been demonstrated on a bacterial genome (Hanish and McClelland, 1990).

We performed an extensive study of the M. *Taq*I/*Dpn*I reaction. The goal was to provide a mixture of the two enzymes that could be used in a single-step reaction cleaving the eight base-pairs 5′ . . . TCGATCGA . . . 3′. Several potential problems concerning M. *Taq*I were overcome. M. *Taq*I, a thermophile with a recommended assay temperature of 65°C, maintains greater than 50% of its activity at 50°C. This is the maximum working temperature for low-melt agarose. M. *Taq*I works well on DNA embedded in agarose. Trace *E. coli* Dam methylase contamination was removed from the recombinant M. *Taq*I by heat treatment at 65°C for 20 minutes. This is important because Dam methylase recognizes 5′ . . . GATC . . . 3′ and methylates the adenine creating *Dpn*I sites (Geier and Modrich, 1979). Two properties of the *Dpn*I make the reaction problematic. *Dpn*I does not function well on DNA embedded in agarose and hemimethylated sites are cleaved slowly (Davis, Morgan, and Robinson, 1990; Nelson and McClelland, 1992). A hemimethylated site generated at position 1129 on pBR322 could be completely cleaved with 60 units of *Dpn*I in one hour using the manufacturer's recommended conditions. Partial digestion products were observed with greater than 5 units of *Dpn*I.

As an alternative to agarose plugs, agarose microbeads (Koob and Szybalski, 1992) should be prepared and the DNA embedded

as described. The reduced diffusion distance offered by the agarose microbead matrix provides the enzyme with more effective access to the embedded DNA substrate. *Dpn*I should be diffused into the microbeads by keeping the reaction mix on ice for at least four hours prior to the 37°C incubation. To ensure complete digestion, we suggest a range of *Dpn*I concentrations from 1 to 10 units. Incubation time should not exceed two hours with DpnI concentrations over 5 units.

Reducing the Number of Cleavable Sites via Blocking Agents Coupled with a Methylase Reaction—Achilles's Heel Cleavage

Three classes of blocking reactions have been developed. All three classes rely on the ability of a methylase to protect all but one or more selected DNA sites from digestion by a restriction endonuclease. We can summarize the methodology as follows:

- A restriction endonuclease/methylase recognition site is occupied by a blocking agent.
- The DNA is methylated, blocking subsequent cleavage at all unoccupied sites.
- The blocking agent and methylase are removed.
- Restriction enzyme is added. Cleavage occurs only at previously blocked sites.

1. Achilles' Heel Cleavage–DNA Binding Protein. A blocking reaction using DNA binding proteins followed by restriction enzyme cleavage is termed "Achilles' heel cleavage" (AC) (Koob, Grimes, and Szybalski, 1988a). Unwanted cleavage can occur if the blocking agent interacts with sites other than the one of interest, so blocking conditions should be optimized to minimize nonspecific interactions. These conditions must also allow the methylase to function properly. If the blocking agent doesn't stay bound to the site for the duration of the methylation reaction, the blocking site will be methylated, reducing the yield of the desired product. Finally, all steps must work well on DNA substrates embedded in agarose. The lac and lambda repressors were the first blocking reagents used in this type of reaction (Koob, Grimes, and Szybalski, 1988b); phage 434 repressor (Grimes, Koob, and Szybalski, 1990), and integration host factor (IHF) (Kur et al., 1992) have also been used. Single-site cleavage has been attained using the lac repressor site introduced into yeast and *Escherichia coli* genomes (Koob and Szybalski, 1990).

   Limitations to this strategy include the absence of natural binding protein sites and the low frequency of restriction/methy-

lation sites. Binding protein sites have been engineered into the target DNA, and degenerate sites containing the required restriction/methylation sites have also been added (Grimes, Koob, and Szybalski, 1990). However, modifications in the recognition sequence of the binding protein can decrease the complex's half-life, allowing unwanted methylation at the AC site.

2. Achilles' Heel Cleavage–Triple Helix Formation. The second Achilles' cleavage reaction uses oligonucleotide-directed triple-helix formation as a sequence specific DNA binding protein blocking agent (Hanvey, Schimizu, and Wells, 1990; Maher, Wold, and Dervan, 1989). Pyrimidine oligonucleotides bind to homopurine sites in duplex DNA to form a stable triple-helix structure. The blocking reaction is followed by methylation, removal of the pyrimidine oligonucleotide and methylase, and cleavage by the restriction endonuclease. Single-site cleavage has been demonstrated on yeast chromosomes by blocking with a 24 bp pyrimidine oligo, (Strobel and Dervan, 1991a, 1992) and on human chromosome 4 using a 16 bp oligo (Strobel et al., 1991b). An advantage of this method over the DNA binding protein AC is the increase in frequency of sites. Insertion of the AC site into the genome is not required. Relatively short purine tracts can be targeted using sequence data. Degenerate probes can be used to screen for overlapping methylation/restriction endonuclease sites when suitable sequence data are not available (Strobel et al., 1991b).

   Reaction conditions for successful pyrimidine oligonucleotide AC are complex (Strobel and Dervan, 1992). Triple helix formation using spermine can inhibit certain methylases, or precipitate DNA in the low-salt reaction conditions required by some methylases. The narrow pH range for the protection reaction may not be compatible with conditions required for efficient methylation. Neutral or slightly acidic conditions promote highly stable triple helices but reduce sensitivity to single base mismatches (Moser and Dervan, 1987). Oligonucleotides that bind and protect mismatched sites allow nontarget restriction sites to remain unmethylated and subsequently cleaved. Increasing the pH from 7.2 to 7.8 can decrease the binding to similar sites (Strobel and Dervan, 1990). In higher pH reactions, the oligo does not stringently bind to the intended target, allowing some methylation to occur at the target site. The unwanted methylation reduces cleavage at the Achilles' site, lowering the yield of the desired DNA fragment.

3. Achilles' Heel Cleavage–RecA-Assisted Restriction Endonuclease. RecA-assisted restriction endonuclease (RARE) cleavage is the most versatile of Achilles' cleavage reaction discovered to date

(Ferrin and Camerini-Otero, 1991; Koob and Szybalski, 1992). In vitro studies indicate that in the presence of ATP, recA protein promotes the strand exchange of single-stranded DNA fragments with homologous duplex DNA. The three distinct steps in the reaction are (1) recA protein binds to the single-strand DNA, (2) the nucleoprotein filament binds the duplex DNA and searches for a homologous region, and (3) the strands are exchanged (Cox and Lehman, 1987; Radding, 1991). Stable triple-helix structures, termed "synaptic complexes," can be formed if the nonhydrolysable analog Adenosine 5′-(γ-Thio) triphosphate (ATPγS) is substituted for ATP (Honigberg et al., 1985). The nucleoprotein filament protects against methylation at a chosen site and is easily removed exposing the AC site. Any duplex DNA stretch containing a restriction endonuclease/methylase recognition site, 15 nucleotides (nt) or longer in length, can be targeted (Ferrin and Camerini-Otero, 1991). RARE cleavage has been used to generate single cuts in the *E. coli* genome by single-stranded oligonucleotides in the 30 nt range and on HeLa cell DNA with oligos in 60 nt range (Ferrin and Camerini-Otero, 1991). RecA-mediated Achilles' cleavage of yeast chromosomes using a 36-mer and 70-mer has been demonstrated (Koob and Szybalski, 1992). YACs (yeast artificial chromosomes) have been cleaved using nucleoprotein filaments in the 50 nt range (Gnirke et al., 1993).

Synaptic complex formation can also block cutting by a restriction endonuclease (Ferrin, 1995). Combined with the fact that many restriction enzymes are active in the buffer used to form these complexes, RARE can be applied to eliminate one of a pair of identical restriction sites in a cloning vector. Partial digestion has been applied to achieve a similar result, but this can fail if the desired site is cut at a comparatively slow rate.

The complexities of the recA-mediated Achilles' cleavage reaction include:

- A titration is required to find the exact ratio of recA to oligonucleotide (Ferrin and Camerini-Otero, 1991; Koob and Szybalski, 1992).
- Excess recA inhibits the methylation reaction.
- Complete hybridization of the oligonucleotide is required for stable triplex formation.
- The nucleoprotein complex diffuses slowly into agarose; microbeading is recommended when using this procedure.
- Nucleoprotein filaments produced with oligonucleotides less than 40 nt may not be stable for the length of time required

for diffusion into agarose microbeads (Koob and Szybalski, 1992).

- RecA DNA-binding requires Mg^{2+}.
- The methylases used must be free of contaminating nucleases.

TROUBLESHOOTING

What Can Cause a Simple Restriction Digestion to Fail?

Faulty Enzyme or Problem Template Preparation?

If the suspect enzyme fails to digest a second or control target, the titer of the enzyme activity should be measured by either a twofold serial or a volumetric titration as described below (procedures A and B).

If the titer assay indicates an active enzyme, and the enzyme cleaves a control template but not the experimental DNA, then an additional control digestion (procedure C) should be performed to test for an inhibitor in the template preparation. Often trans-acting inhibitors may be removed by the drop dialysis protocol (procedure D) detailed below. Spin columns may also be used to remove contaminants including primers, linkers, and nucleotides (Bhagwat, 1992). A linearized plasmid containing a single site may be used if cut and uncut samples are available as markers.

As a matter of course, restriction enzyme activity should be assayed by twofold serial titration if an enzyme has been stored for a period longer than a year, an enzyme shipment was delayed, or even if an enzyme was left on the bench overnight. This simple assay may be used to test enzymes under non-optimal conditions as well. Suppliers offer buffer charts that give an indication of an enzyme's expected activity in nonoptimal buffers, and this information may be useful when the sample DNA is in an alternative buffer due to a previous step or adapting digests so that the DNA samples will be optimized for subsequent steps.

Procedure A—Simple Twofold Serial Titer

Ideally the DNA should be the substrate on which the enzyme was titered by the supplier. Lambda phage DNA or adenovirus Type-2 DNA are common substrates used for enzyme titer. Any DNA that contains several sites that produce a distinguishable pattern may be applied.

1. For the following experiment, make a total of 200 μl of reaction mix. The reaction mix contains 1× reaction buffer, 1 μg DNA/50 μl reaction volume and BSA, if required. For this example, the enzyme is supplied with a vial of 10× reaction buffer and 10 mg/ml BSA. The final reaction mix requires 1× reaction buffer and 100 μg/ml BSA. Lambda DNA (commercially available at 500 μg/ml) is the substrate used to titer the enzyme.
   Add, in order:
   a. 170 μl of distilled water
   b. 20 μl of 10× buffer
   c. 2 μl of 10 mg/ml BSA
   d. 8 μl of 500 μg/ml Lambda DNA
2. Label six 1.5 ml microcentrifuge tubes (numbers 1–6). Pipette 50 μl of reaction mix into tube 1 and 25 μl of mix into the remaining tubes.
3. Add 1 μl of restriction endonuclease to the first tube containing 50 μl of reaction mix. With the pipette set at 25 μl, mix by gently pipetting several times.
4. From the 50μl reaction mix/enzyme, transfer 25 μl to the second tube. This dilutes the enzyme concentration in half for each subsequent tube.
5. Repeat step 4 until the final tube is reached. The final tube has the most dilute enzyme, but indicates the highest titer. If the final tube, in the following series, shows a complete digestion, then the titer is at least 32,000 units/ml.
6. Cover each tube and incubate at the appropriate reaction temperature for one hour.
7. The reaction is stopped by adding at least 10 μl stop dye/50 μl reaction volume (50% 0.1 M EDTA, 50% glycerol, 0.05% bromophenol blue). The DNA fragments are resolved by agarose gel electrophoresis, stained with ethidium bromide, and visualized using ultraviolet light.
8. The titer is determined as follows:
   Tube 1 complete: titer ≥1000 units/ml
   Tube 2 complete: titer ≥2000 units/ml
   Tube 3 complete: titer ≥4000 units/ml
   Tube 4 complete: titer ≥8000 units/ml
   Tube 5 complete: titer ≥16,000 units/ml
   Tube 6 complete: titer ≥32,000 units/ml

The titer is based on the unit definition: 1 unit of restriction enzyme digests 1 μg DNA to completion in 1 hour. If the digestion pattern from tube 1 is complete, then 1 μl of the enzyme

added contains at least 1 unit of activity. The concentration 1 unit/μl is the same as 1000 units/ml. With a dilution factor of 2, a complete digestion pattern from tube 2 indicates that the enzyme concentration is at least 2×1000 units/ml = 2000 units/ml. If tube 4 results in a complete digestion, and tube 5 results in a partial banding pattern, the final titer of the enzyme may be conservatively estimated as 8000 units/ml. Similarly a more precise serial dilution may be designed to evaluate the titer value between 8000 and 16,000 units/ml.

Procedure B—Volumetric Titration

The exact method will vary among enzyme manufacturers. You should contact your supplier for the exact method if this information is not found in their catalog.

While not as convenient as serial titration for most benchtop applications, most suppliers use volumetric titration to assay the activity of the restriction endonucleases. This method may yield more consistent results, especially when the enzyme stock is in high concentration. Most volumetric titers require initial dilution of the enzyme (often in 50% glycerol storage buffer) and the use of substantial amounts of substrate DNA/reaction mix. This method maintains constant enzyme addition to increasing amounts of reaction mix volume, while keeping the concentration of DNA substrate constant. The protocol may differ depending on the concentration and dilution of the enzyme. This method is recommended when evaluating an enzyme sample to be ordered in bulk amounts or for diagnostic applications where internal QC evaluation is required.

Procedure C—Testing for Inhibitors

In a single vial with 1× reaction buffer, add 1 μg each of the control and the experimental DNA. Add the restriction enzyme and incubate at the recommended temperature and time. If there is an inhibitor (often salt or EDTA), the mixed control substrate will not cut.

Procedure D—Drop Dialysis (Silhavy, Berman, and Enquist, 1984)

Many enzymes are adversely affected by a variety of contaminating materials in typical DNA preparations (minipreps, genomic and $CsCl_2$ preparations, etc.). The following drop dialysis method has been successfully used to remove inhibitory substances (e.g., SDS, EDTA, or excess salt) from substrates intended for subsequent DNA manipulations. It is particularly effective for assuring

complete cleavage of DNA by sensitive restriction endonucleases, increasing the efficiency of ligation and preparation of templates for DNA sequencing.

1a. For purification of genomic DNA, miniprep DNA, or DNA used as a standard template for DNA sequencing: Phenol extract, chloroform extract, and then alcohol precipitate the DNA. Pellet the DNA in a microcentrifuge, pour off the supernatant, and rinse the pellet with 70% ethanol. Dry the pellet and resuspend it in 50 μl H_20. (Proceed to step 2.)

1b. For purification of templates for DNA sequencing of PCR products: Phenol extract and then chloroform extract the aqueous layer of the PCR reaction. Follow this with an alcohol precipitation. Pellet the DNA by microcentrifugation, pour off the supernatant, and rinse the pellet with 70% ethanol. Dry the pellet and resuspend it in 50 μl H_2O. Alternatively, purify the PCR product through an appropriate spin column, precipitate, and recover the DNA as described above. PCR products that are not a single band on an agarose gel should be gel-purified in low-melt agarose and then treated with β-agarase I or a purification column technology. When using β-agarase, treatment should be followed by extraction, precipitation, and recovery, as described above. When using a purification column, consult the manufacturer's recommendations for the particular column employed.

2. Pour 30 to 100 ml of dialysis buffer, usually double-distilled water or 1× TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), into a petri plate or beaker.

3. Float a 25 mm diameter, Type VS Millipore membrane (cat. no. VSWP 02500, MF type, VS filter, mean pore size = 0.025 mm, Millipore, Inc.) shiny side up on the dialysis buffer. Allow the floating filter to wet completely (about 5 minutes) before proceeding. Make sure there are no air bubbles trapped under the filter.

4. Pipette a few microliters of the DNA droplet carefully onto the center of the filter. If the sample has too much phenol or chloroform, the drop will not remain in the center of the membrane, and the dialysis should be discontinued until the organics are further removed. In most cases this is performed by alcohol precipitation of the sample. If the test sample remains in the center of the membrane, pipette the remainder onto the membrane.

5. Cover the petri plate or beaker. Dialyze at room temperature. Be careful not to move the dish or beaker. Dialyze for at least one hour and no more than four hours.
6. Carefully retrieve the DNA droplet with a micropipette.

Note that step 4 may be tricky for those with shaky hands or poor hand-eye coordination. The filter has a tendency to move briskly around the surface as you touch it with the pipette tip. Practice with buffer droplets to master the technique before you try using a valuable sample.

Dialysis against distilled water is also recommended, especially if one is proceeding to another step where EDTA might be a problem.

The Volume of Enzyme in the Vial Appears Very Low. Did Leakage Occur during Shipment?

Some enzymes (some offered at high concentration) may be supplied in a very low volume and the vial may appear empty. During shipment, the enzyme may be dispersed over most of the interior surface of the vial or trapped just under the cap. Follow the steps below to ensure that the enzyme volume is correct. (Since the volume is very low, it is important to keep the entire vial under ice or as cold as possible by working quickly.)

1. Carefully check the exterior of the enzyme vial, noting any signs of glycerol leakage.
2. Add the enzyme's expected volume as water to an identical vial (for a counterbalance).
3. Briefly spin the enzyme vial in a microcentrifuge along with the counterbalance.
4. With both vials on ice, estimate the volume of the enzyme by comparison to that of the counterbalance.

The Enzyme Shipment Sat on the Shipping Dock for Two Days. Is It Still Active?

Restriction enzymes are shipped on dry ice or gel ice packs, depending on the supplier. When enzyme shipments arrive, there should still be a good amount of dry ice left; or if shipped with ice packs, these should still be cold, solid and not soft. For overnight shipments, most suppliers include sufficient thermal mass to maintain proper shipping temperature for at least 36 hours. If the shipment was delayed en route, misplaced, or left in receiving for one or more days, you should:

- Examine the contents, noting the integrity of the container.
- If contents are still cold (but questionable in terms of actual temperature), place a thermometer in the container, re-seal the lid, and note the temperature after 10 minutes.
- After collecting details regarding the shipment's ordering information, contact the supplier. Customer service should provide detailed information regarding the specific products in question and, if warranted, shipping details for a replacement order.

Generally, if the enzyme package is still cold to the touch, most enzymes should be completely active, even if the 10× buffers have recently thawed. Due to their salt content, the concentrated buffers would be liquid even at 0°C. If the enzyme is required for use immediately and no alternative source is available, the enzyme may be tested for activity by serial titration, as described above. Also bear in mind that many enzymes retain their activity after a 16 hour incubation at room temperature (McMahon, M., and Krotee, S., unpublished observation).

Analyzing Transformation Failures and Other Multiple-Step Procedures Involving Restriction Enzymes

A restriction digest is rarely the ultimate step of a research procedure, but instead an early (and essential) reaction within a multiple-step process, as in the case of a cloning experiment. Therefore, when troubleshooting restriction enzymes, and more so than other reagents, it is essential to objectively list *all* the feasible explanations for failure as noted in step 2 of the troubleshooting strategy discussed in Chapter 2, "Getting What You Need From A Supplier." The following discussion illustrates the importance of identifying and investigating all the possible causes of what appears to be a restriction enzyme failure.

If background levels are high after transformation, the enzyme activity should be checked. Alternatively, the vector may have ligated to itself. If the vector had symmetric ends, were the 5′ phosphates removed by dephosphorylation? Was the effectiveness of the dephosphorylation proved? Incomplete vector digestion might be caused by contaminants in the DNA preparation, incompatible buffer, insufficient restriction enzyme, or sites that are located adjacent to each other. If the vector had two different termini, was the success of both digestions verified by recircularization experiments?

Exonuclease contamination in the restriction enzyme or DNA preparation can prevent insert ligation, but ligation might

proceed if the ends are blunted by the exonuclease. In this scenario the restriction site would be lost and the reading frame shifted. Phenol chloroform extraction followed by ethanol precipitation will remove exonuclease from DNA preparations. Check the restriction enzyme quality control data for exonuclease, ligation, and blue-white selection. Do not extend the digestion time if an exonuclease problem is suspected.

DNA preparations can contain contaminants that inhibit ligation as well as restriction endonuclease digestion, and the use of very dilute DNA solutions can amplify inhibition. Higher stock vector and insert concentrations are preferable because less of the final reaction volume comes from the DNA solution. If the DNA is stored in Tris-EDTA, the EDTA may inhibit the ligation or restriction digest. Using dilute DNA solutions gives less flexibility when choosing the molar ratio of insert to vector and final DNA concentration of the reaction; both parameters directly affect the quantity of desirable products produced in the ligation reaction.

Failed ligation can occur if the molar ratio of insert to vector is not sufficient. A molar ratio of 3:1 insert to vector should be used for asymmetric ligations and symmetric ligations with small inserts. Symmetric ligations with inserts greater than 800 bp should use 8 μg/ml insert to 1 μg/ml vector (Revie, Smith, and Yee, 1988). In general, the vector concentration should be kept at 1 μg/ml. Total DNA concentration should be kept to 6 μg/ml or less (Bercovich, Grinstein, and Zorzopulos, 1992). Blunt ends are treated as symmetric, and overnight ligation at 16°C is recommended. The addition of 7% PEG 8000 can also stimulate ligation. Single-base overhangs are more difficult to ligate than blunt ends; overnight ligation at 16°C using concentrated ligase is also suggested here. Even so, less than 20% ligation is seen for *Tth111*I under these conditions. Filling in the 5′ single-base overhang with Klenow resulting in a blunt end will increase ligation to about 40% (Robinson, D., unpublished observation).

Transformants containing only deletions indicate problems with ligation or dephosphorylation. Blunt end ligation of a PCR product made with unphosphorylated primers into a dephosphorylated vector will result in a failed ligation, although competent cells will take up some linear molecules. Cells can scavenge the antibiotic resistance gene used for selection, and the scavenged gene is normally found on a vector containing a deletion. The miniprep DNA from the transformants will often run smaller than the control linearized vector.

Faulty DNA ligase, a reaction buffer lacking ATP, and the addi-

tion of too much ligation mix to the competent cells can result in low colony count. An antibiotic in the plate that doesn't match the resistance gene within the vector or leaky expression of a toxic protein can kill competent cells, which could mimic a restriction enzyme failure. Cells can be tested by transformation using uncut vector. In addition, as restriction enzymes are excellent DNA binding proteins, they can remain bound to DNA termini and inhibit ligation. Active restriction enzyme can recleave ligated DNA. Often, after incubation, this effect may be minimized by either heating the reaction to 65°C or proceeding with an alternative purification step.

Failure at any one of the many steps of a cloning experiment can give the impression of a restriction enzyme failure. The same principle holds true for the many other applications that involve restriction enzymes.

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10

Nucleotides, Oligonucleotides, and Polynucleotides

Alan S. Gerstein

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NUCLEOTIDES

Nomenclature: De facto and Du jour

Lehninger (1975) provides a thorough discussion of proper nucleotide nomenclature and abbreviations. Unfortunately,

commercial catalogs and occasionally the research literature introduce different notations. Some consider "NTP" a general term for deoxynucleotides, but the absence of the letter "d" indicates a ribonucleotide to others. Commercial literature also describes ribonucleotides as "RTP's." If the letter "d" is present, the name describes a deoxynucleotide. If "d" is absent, check the literature piece closely to avoid a common purchasing error. Dideoxynucleotides are generically referred to as "ddNTP's."

What Makes a Nucleotide Pure?

Using dATP as an example, what categories of impurities could be present? One potential contaminant is a nucleotide other than dATP, such as dCTP. A second class of impurity could be the mono-, di-, or tetraphosphate form of the deoxyadenosine nucleotide. Since most if not all commercial nucleotides are chemically synthesized from highly analyzed precursors, contamination with a nucleotide not based on deoxyadenosine is very unlikely. A third class of impurities is the non-UV-absorbing organic and inorganic salts accumulated during the synthesis and purification procedures.

While essentially all commercial nucleotides are chemically synthesized, the final products are not necessarily identical. Manufacturing processes vary; raw materials and intermediates of the nucleotide synthesis reactions are subjected to different purification strategies and processes. It is these intermediate steps, and the scrutiny of the products' final specifications, that allow manufacturers to legitimately claim that nucleotides are extremely pure.

A formal definition of *extremely pure* does not exist, but commercial preparations of such products typically contain greater than 99% of the desired nucleotide in the triphosphate form. Contaminating nucleotides are rarely detected in commercial preparations, even using exceedingly stringent high-performance chromatography procedures, but some contaminants escape HPLC detection. Freedom from non-UV-absorbing materials is typically judged by comparison of a measured molar extinction (A_m) coefficient to published extinction coefficients (ε)values. Nuclear magnetic resonance (NMR) may also be used to monitor for contaminants such as pyrophosphate.

Are Solution Nucleotides Always More Pure Than Lyophilized Nucleotides?

Nucleotides were first made commercially available as solvent-precipitated powders. The lyophilized and extremely pure solution

forms appeared in the early 1980s. Some lyophilized preparations approach 98% purity or more but rarely match the >99% achieved by extremely pure solutions. Generally, solution nucleotides are purer than the lyophilized version, but unless supporting quality control data are provided, it should not be concluded that a solution nucleotide is extremely pure or even more pure than a lyophilized preparation.

Are Solution Nucleotides More Stable Than Lyophilized Nucleotides?

Peparations of deoxynucleoside triphosphates decompose into nucleoside di- and tetraphosphates via a disproportionation reaction. This reaction is concentration and temperature dependent. At temperatures above 4°C, lyophilized preparations of deoxynucleotides undergo disproportionation faster than nucleotides in solution. In contrast, the rate of degradation for both forms is less than 1% per year at –20°C and below (Table 10.1). Solutions of dideoxynucleotides and ribonucleotides are similarly stable for many months at temperatures of –20°C and below. Most, but not all, dideoxy- and ribonucleotides are stable for many months at 4°C.

Table 10.1 Storage Stability of Nucleotides

		% Triphosphate Form			
	Months	–70°C	–20°C	4°C	21°C
Powder					
dATP	54	99.44	99.14	97.47	93.93 (48 mo) 97.78 (3 mo)
dCTP	54	98.46	95.46	39.3 (33 mo)	39.45 (2.75)
dGTP	54	96.95	95.37	25.74 (27 mo)	34.4 (1.75)
dTTP	54	97.29	94.28	27.4 (30 mo)	39.45 (2.75 mo)
dUTP	N.A.	N.A.	N.A.	N.A.	N.A.
Solution (100 mM)					
dATP	54	99.2	98.75	95.3	91.8 (2 mo) 37.07 (39 mo)
dCTP	54	99.38	99.15	96.98	95.2 (2 mo) 21.25 (42 mo)

Table 10.1 (*Continued*)

	Months	% Triphosphate Form			
		–70°C	–20°C	4°C	21°C
Powder					
dGTP	54	99.63	98.83	95.47	90.5 (2 mo) 19.7 (42 mo)
dTTP	54	99.44	98.87	93.54	95.6 (2 mo) 0.07 (42)
dUTP	54	99.23	98.02	71.55	90.1 (1.2 mo) 40.13 (6 mo)
Solution (10 mM)					
dATP	15		99.68	99.59 (12 mo)	88.6 98.5 (2 mo)
dCTP	15		98.2	99.56 (12 mo)	86.11 98.85 (2 mo)
dGTP	15		98.6	99.51 (12 mo)	89.47 98.35 (2 mo)
dTTP	15		93.57	99.29 (12 mo)	81.05 98.86 (2 mo)
dUTP	15		93.8	99.45 (12 mo)	84.95 98.5 (2 mo)
Solution ddNTP (10 mM)					
ddATP	3		99.69	99.49	94.52
ddCTP	3		100	98.51	97.38
ddGTP	3		98.4	98.08	94.23
ddTTP	3		99.36	99.13	87.06
Solution ddNTP (5 mM)					
ddATP	3		99.77	98.12	68.56
	4		99.63	96.31	2
ddCTP	3		98.77	100	98.4
	4		99.27	99.46	93.72
ddGTP	3		95.61	98	96.67
	4		98.25	97.9	93.68
ddTTP	3		93.1	55.09	49.03
	4		94.25	63.23	3.6
RTP Solutions (100 mM)					
ATP	3		98.57	98.18	95.39
CTP	3		99.25	99.43	98.43
GTP	3		98.46	98.44	96.82
UTP	3		99.71	99.69	97.99

Source: Data based on chromatographic separation of nucleotide species via high performance chromatography on an Amersham Pharmacia Biotech FPLC® System.
Notes: Each sample, 0.2 μmoles (0.2 ml of a 1 mM solution) was injected onto a Mono Q® Ion Exchange column. Using the following buffers:

Buffer A, 5mM sodium phosphate, pH 7.0.
Buffer B, 5mM sodium phosphate, 1M NaCl, pH 7.0.

purification was achieved via a gradient of 5–35% NaCl over 15 minutes using a flow rate of 1 ml/min. Nucleotide peaks were detected at of 254 nm. (Data from Amersham Pharmacia Biotech, 1993a.)

Does Your Application Require Extremely Pure Nucleotides?

Only you can answer this question. Most applications have supporters and detractors for the use of extremely pure nucleotides.

How Can You Monitor Nucleotide Purity and Degradation?

Nucleotides produce very specific spectroscopic absorbance data. Absorbance ratios not within predicted ranges (Table 10.2) indicate a contaminated deoxy- or ribonucleotide, such as if dATP and dCTP were accidentally mixed together. This technique is adequate to quickly determine if a large contamination problem exists, but a high-performance liquid chromatography approach is required to detect minor levels of impurities.

The absorbance ratio will not indicate when the triphosphate form of a nucleotide breaks down into the di- and tetraphosphate forms. This form of degradation can be monitored most effectively

Table 10.2 Nucleotide Absorbtion Maxima

Nucleotide	Lambda Maximum (pH 7.0)	A_m (pH 7.0) molar extinction coefficient
2′-dATP	259 nm	15.2×10^{3d}
2′-dCTP	280 nm[a]	$13.1 \times 10^{3a,e}$
2′-dGTP	253 nm	13.7×10^{3f}
2′-dITP	249 nm	$12.2 \times 10^{3b,h}$
2′-dTTP	267 nm[b]	9.6×10^{3g}
2′-dUTP	262 nm	10.2×10^{3i}
c7-2′-ATP	270 nm	12.3×10^{3j}
c7-2′-dGTP	257 nm	10.5×10^{3c}
2′,3′-ddATP	259 nm	15.2×10^{3d}
2′,3′-ddCTP	280 nm[a]	$13.1 \times 10^{3a,e}$
2′,3′-ddGTP	253 nm	13.7×10^{3f}
2′,3′-ddTTP	267 nm	9.6×10^{3g}
ATP	259 nm	15.4×10^{3}
CTP	280 nm[a]	13.0×10^{3a}
GTP	252 nm	13.7×10^{3}
UTP	262 nm	10.2×10^{3}

Note: The spectral terms and definitions used are those recommended by the National Bureau of Standards Circular LCD 857, May 19, 1947.
[a] Spectral analysis done at pH 2.0.
[b] Spectral analysis done at pH 6.0.
[c] Value determined at Amersham Pharmacia Biotech.
[d] 2′-dAMP NRC reference spectral constants employed.
[e] 2′-dCMP NRC reference spectral constants employed.
[f] 2′-dGMP NRC reference spectral constants employed.
[g] 2′-dTMP NRC reference spectral constants employed.
[h] 2′-dIMP NRC reference spectral constants employed.
[i] 2′-dU NRC reference spectral constants employed.
[j] Leela and Kehne (1983).

by high-performance chromatography, but when such equipment is unavailable, thin layer chromatography can provide qualitative data (Table 10.3).

How Should You Prepare, Quantitate, and Adjust the pH of Small and Large Volumes of Nucleotides?

The following procedure can be used to prepare solutions of deoxynucleotides, ribonucleotides, and dideoxynucleotides provided that the different formula weights are taken into account.

A 100 mM solution of a solid nucleotide triphosphate is prepared by dissolving about 60 mg per ml in purified H_2O. The exact weight will depend on the formula weight, which will vary by nucleotide, supplier, and salt form. As solid nucleotide triphosphates are very unstable at room temperature, they should be stored frozen until immediately before preparing a solution.

Quantitation

Spectroscopy

The most accurate method of quantifying a solution is to measure the absorbance by UV spectrophotometry. A dilution should be made to obtain a sample within the linear range of the spectrophotometer. The sample should be analyzed at the specific λ_{max} for the nucleotide being used. The concentration can then be obtained by multiplying the UV absorbance reading by the dilution factor, and dividing by the characteristic A_m for that nucleotide. These data are provided in Table 10.2.

Table 10.3 TLC Conditions to Monitor dNTP Degradation

dNTP	R_f, Principal	R_f, Trace	Solvent System
dATP	0.25	0.35 (dADP)	A
dCTP	0.15	0.21 (dCDP)	A
dGTP	0.27	0.34 (dGDP)	B
dTTP	0.14	0.21 (dTDP)	A

Note: Solvent System A: Isobutyric acid/concentrated NH_4OH/water, 66/1/33; pH 3.7. Add 10 ml of concentrated NH_4OH to 329 ml of water and mix with 661 ml of isobutyric acid.
Solvent System B: Isobutyric acid/concentrated NH_4OH/water, 57/4/39; pH 4.3. Add 38 ml of concentrated NH_4OH to 385 ml of water and mix with 577 ml of isobutyric acid.
TLC Plates: Eastman chromagram sheets (#13181 silica gel and #13254 cellulose).

Weighing

One would think that the mass of an extremely pure nucleotide could be reliably determined on a laboratory balance. Not so, because during the manufacturing process, nucleotide preparations typically accumulate molecules of water (via hydration) and counter-ions (lithium or sodium, depending on the manufacturer), which signficantly contribute to the total molecular weight of the nucleotide preparation. Unless you consider the salt form and the presence of hydrates, you're adding less nucleotide to the solution than you think. The presence of salts and water also contribute to the molecular weights of oligo- and polynucleotides, which are also most reliably quantitated by spectroscopy.

pH Adjustment

The pH of a solution prepared by dissolving a nucleotide in water will vary, depending on the pH at which the nucleotide triphosphate was dried. An aqueous solution of nucleotide triphosphate prepared at Amersham Pharmacia Biotech will have a pH of approximately pH 4.5. The pH may be raised by addition of NaOH (0.1 N NaOH for small volumes, up to 5 N NaOH for larger volumes). Approximately 0.002 mmol NaOH per mg nucleotide triphosphate is required to raise the pH from 4.5 to neutral pH. If the pH needs to be lowered, addition of a H^+ cation exchanger to the nucleotide solution will lower the pH without adding a counter-ion. The amount of cation-exchanger resin per volume of 100 mM nucleotide solution varies greatly depending on the starting and ending pH. For very small volumes (<5 ml) of nucleotide solutions, a 50% slurry of SP Sephadex can be added dropwise. For larger volumes (>5 ml), solid cation exchanger can be added directly in approximately 0.2 cm^3 increments. The cation exchanger can be removed by filtration when the desired pH is obtained.

The triphosphate group gives the solution considerable buffering capacity. If an additional buffer is added, the pH should be checked to ensure that the buffer is adequate. The pH should be adjusted when the solution is at or near the final concentration. A significant change in the concentration will change the pH. An increase in concentration will lower the pH, and dilution will raise the pH, if no other buffer is present.

Similar results will be obtained for all of the nucleotide triphosphates. Monitor the pH of the solutions as a precaution; purines are particularly unstable under pH 4.5, and all will degrade at acid pH.

Example

To prepare a 10 mM solution from a 250 mg package of dGTP, the dGTP may be dissolved in about 40 ml of purified H_2O. The pH may then be adjusted from a pH of about 4.5 to the desired pH with 1 N NaOH, carefully added dropwise with stirring. About 0.5 ml of 1 N NaOH will be needed for this example. A dilution of 1:200 will give a reading in the linear range of most spectrophotometers. Spectroscopy should be performed at the nucleotide's absorbance maximum, which is 253 nm for dGTP. In this example an absorbance of about 0.700 is expected. The formula for determining the concentration is:

$$\frac{\text{Absorbance at } \lambda_{max} \times \text{ dilution factor}}{A_m} = \text{molar concentration}$$

Using the A_m for dGTP of 13,700, the concentration in this example is found to be

$$\frac{0.700 \times 200}{13,700} = 0.0102\,\text{M}, \quad \text{or} \quad 10.2\,\text{mM dGTP}$$

What Is the Effect of Thermocycling on Nucleotide Stability?

Properly stored, lyophilized and solution nucleotides are stable for years. The data in Table 10.4 (Amersham Pharmacia Biotech, 1993b) describe the destruction of nucleotides under common thermocycling conditions. Fortunately, due to the excess presence of nucleotides, thermal degradation does not typically impede a PCR reaction.

Is There a Difference between Absorbance, A_{260}, and Optical Density?

Readers are strongly urged to review Efiok (1993) for a thorough and clearly written discussion on the spectrophotometric quantitation of nucleotides and nucleic acids.

*Absorbance (*A*)*

Absorbance (A), also referred to as optical density (OD), is a unitless measure of the amount of light a solution traps, as measured on a spectrophotometer. The Beer-Lambert equation (Efiok, 1993) defines absorbance in terms of the concentration of the solution in moles per liter (C), the path length the light travels through the solution in centimeters (l), and the extinction coefficient in liter per moles times centimeters (E):

Table 10.4 Breakdown of Nucleotides under Thermocycling Conditions

		% Purity of Triphosphate	
	Nucleotides	0 PCR Cycles	25 PCR Cycles
Experiment 1	dATP	99.31	92.41
	dCTP	99.47	93.64
	dGTP	99.14	92.43
	dTTP	99.06	93.38
Experiment 2	dATP	99.56	94.17
	dCTP	99.80	95.36
	dGTP	99.78	94.02
	dTTP	99.60	94.17
Experiment 3	dATP	99.40	92.02
	dCTP	99.66	93.84
	dGTP	99.39	92.68
	dTTP	99.15	93.69
Experiment 4	dATP	99.44	92.77
	dCTP	99.59	93.89
	dGTP	99.43	92.88
	dTTP	99.19	93.65

Source: Data from Amerhsam Pharmacia Biotech (1993b).
Note: Each nucleotide was mixed with 10× PCR buffer from the GeneAmp® PCR Reagent Kit (Perking Elmer catalogue number N801-0055)to give a final nucleotide concentration of 0.2 mM in 1× PCR buffer. Noncycled control samples (0 cycles) were immediately assayed. Test samples were cycled for 25 rounds in a Perkin Elmer GeneAmp® PC System 9600 using the cycling program of 94°C for 10 seconds, 55°C for 10 seconds, and 72°C for 10 seconds. After cycling, the samples were stored on ice until assayed.

For analysis, samples were diluted to give a nucleotide concentration of 0.133 mM. The diluted samples were then assayed on FPLC® System using a MonoQ® column. The assay time for a sample was 10 minutes using a sodium chloride gradient (50–400 mM) in 20 mM Tris-HCl at pH 9.0. Nucleotide peaks were detect using a wavelength of 254 nm.

$$A = ClE$$

Since the units of C, l, and E all cancel, A is unitless.

Absorbance Unit

Also referred to as an optical density (OD) unit, an absorbance unit (AU) is the concentration of a material that gives an absorbance of one and therefore is also a unitless measure. Typically, when working with nucleic acids, we express the extinction coefficient in ml per mg times cm:

$$E = \frac{\text{ml}}{\text{mg} \times \text{cm}}$$

Using an extinction coefficient expressed in these terms, one A_{260} unit of double-stranded DNA has a concentration of DNA of 50 μg/ml.

For practical reasons, suppliers typically define the total volume of material to be one milliliter when selling their nucleic acids.

Note that from a supplier's perspective, an A_{260} unit specifies an amount of material and not a concentration. It is the amount of material in one milliliter that gives an absorbance of one. The A_{260} unit value provided by a supplier cannot be substituted into the Beer-Lambert equation to calculate concentration. If this substitution is done, the concentration will be off by a factor of 1000.

*Extinction Coefficient (*E*)*

Also known as absorption coefficient, absorptivity, and absorbency index, the proportionality constant E is a constant value inherent to a pure compound. E will not vary between different lots of a chemical. The units of E are typically ml/mg-cm or L/g-cm. It is experimentally measured by utilizing a method that is not affected by the presence of a contaminant. For example, the extinction coefficient of a nucleotide can be determined by measuring the amount of phosphorous present.

As in the Beer-Lambert equation, the concentration (C) of a solution in mg/ml or g/L = A/El.

Molar Extinction Coefficient (ε) versus A_m

The molar extinction coefficient (also referred to as molar absorbtivity) describes the absorbance of 1 ml of a 1 molar solution measured in a cuvette with a 1 cm path length. For practical reasons a manufacturer may measure a molar coefficient by weighing an amount of the solid material, mixing into a solution and measuring the absorbance of that solution. This way, a molar coefficient is calculated that is not a true molar extinction coefficient because it is affected by the presence of contaminants. To set this measured coefficient apart from a true molar extinction coefficient, companies use the symbol A_m. The A_m for a given chemical will vary from preparation to preparation depending on the presence of contaminants. Using nucleotides as an example, the number of sodium and water molecules present in the finished product can vary from lot to lot, causing the A_m values to also vary slightly between lots. The units of A_m are L/mol-cm.

*Suppose that you have 100 μl of a 5 mM solution of a nucleotide with a molar extinction coefficient of 10.4×10^3, how many A_{260} units do you have? Using the Beer-Lambert equation, the undi-

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luted 5 mM solution of this nucleotide will have an absorbance of 52. $A = 10.4 \times 10^3\,\text{L/(mol} \times \text{cm)} \times 0.005\,\text{M} \times 1\,\text{cm} = 52$. This measure of absorbance is a unitless measure of the opacity of the solution and is independent of the volume of the solution.

To calculate the A_{260} units present as a supplier would define an A_{260} unit, the volume of the solution must be taken into account. This is simply done by multiplying the volume of the solution in milliliters by the absorbance measurement. For the 100 μl of a solution with an absorbance of 52, the number of A_{260} units present is 5.2 units (i.e., 52×0.1 ml = 5.2 units).

Why Do A_{260} Unit Values for Single-Stranded DNA and Oligonucleotides Vary in the Research Literature?

The A_{260} unit values are generated by rearranging the Beer-Lambert equation as per Efiok (1993):

$$OD = ECL$$

$$\frac{C}{OD} = \frac{1}{E} = \frac{1}{\text{AU}}$$

Substituting the value of $E_{1\text{cm}}^{1\text{mg/ml}}$ in Table 10.5 generates the conversion factors to A_{260} data into mg/ml of nucleic acid.

Manufacturer technical bulletins (Amersham Pharmacia Biotech, 2000) and protocol books (Ausubel et al., 1995; Sambrook, Fritsch, and Maniatis, 1989) frequently cite different values for single-stranded DNA and oligonucleotides. Since nucleotide sequence and length alter the value of an extinction coefficient, the variability amongst A_{260} conversion factors is likely caused by the use of different nucleic acid samples to calculate the extinction coefficient. In practice, this means that it probably does not matter which value you use for your work as long as you consistently use the same value for the same type of nucleic acid. However, consider the existence and impact of different conversion factors when attempting to reproduce the work of another researcher.

Table 10.5 Nucleic Acid

	$E_{1\text{cm}}^{1\text{mg/ml}}$ 1	A_{260} (μg/ml)
Double-stranded DNA	20	50
Single-stranded DNA or RNA (>100 nucleotides)	25	40
Single-stranded oligos (60–100 nucleotides)	30	33
Single-stranded oligos (<40 nucleotides)	40	25

Source: From Effiok (1993).

OLIGONUCLEOTIDES

How Pure an Oligonucleotide Is Required for Your Application?

During standard solid phase oligonucleotide (oligo) synthesis, nucleotides are coupled one at a time to a growing chain attached at its 3′ end to a solid support (unlike enzymatic DNA synthesis, chemical DNA synthesis occurs in the 3′ to 5′ direction). To prepare an oligonucleotide where the majority of the product is full length, a coupling efficiency of ≥98% at each nucleotide addition is required. At lower coupling efficiencies, the synthesis will yield a significant amount of oligos that are not full length (failure sequence).

Oligonucleotide impurities may consist of various forms of the desired sequence as well as impurities from the reagents used in synthesis. The ammonium hydroxide that detaches the oligonucleotide from the solid support of a DNA synthesizer and buffer salts carried over from a purificaton process can also be troublesome. Ammonium ions are inhibitory to T4 Polynucleotide kinase, so if the the oligo isn't properly de-salted, subsequent end-labeling reactions will fail.

Your application dictates the level of acceptable purity. The ammonium ions carried over from detaching the oligo from the solid support can completely inhibit end labeling but not other reactions. An oligo preparation that contains less than 50% full-length product will produce miserable sequencing results, but might function as a PCR primer. If your oligo functions reproducibly and verifiably generates data, it's sufficiently pure.

What Are the Options for Quantitating Oligonucleotides?

The concentration of oligonucleotides is most commonly approximated by applying the Beer-Lambert law and a conversion factor ranging from from 25 to 37 μg per A_{260} unit. This approach is inexact, but it is reliable for common molecular biology techniques as long as its limitations are considered. Computer software that predicts an extinction coefficient based on nucleotide sequence and nearest-neighbor analysis is also available. Such predictive software should be employed with caution, since it does not take into account a number of factors, such as the degree of base stacking and the presence of alternate structures commonly found among nucleic acids, that significantly influence the magnitude of the extinction coefficient.

If an exact extinction coefficient is required, a method that directly calculates the quantity of the nucleic acid is required. The

phosphate analysis method of Griswold et al. (1951) is described below.

The method of Griswold et al. (1951) is based on a colorimetric assay (A_{820}) employing ANS (aminonaphtosulfonic acid) dissolved in a sulfite/bisulfite solution. The reaction requires the presence of molybdate prepared in 10 N sulfuric acid. A carefully prepared phosphate solution is utilized to obtain a standard curve by serial dilution (10–100 μM phosphate). DNA test solutions of known absorbance at 260 nm are digested with nuclease P1 and alkaline phosphatase. The phosphate released from the digestion is quantified by monitoring the blue color development at 820 nm following reaction with ANS solution in the presence of molybdate in acidic solution and incubation at 95°C for 10 minutes. The extinction coefficient is determined in accordance with the following equation:

$$E_{260} = \frac{A_{260\,\mathrm{nm}}}{\text{phosphate}\,(\mu\mathrm{M}) \times (n-1)}$$

where A_{260nm} is the original absorbance of the DNA solution, phosphate (μM) represents the value obtained in triplicate of the digested DNA solution extrapolated from the standard phosphate curve, and n is the number of bases comprising the oligonucleotide.

As with nucleotides, determining the amount of an oligo is best done by measuring the absorbance. If you prefer to measure the mass on a very accurate analytical balance, take into account the presence of contaminating salts and water.

What Is the Storage Stability of Oligonucleotides?

The fundamentals of safe DNA storage are discussed in Chapter 7, "DNA Purification," and RNA storage is discussed in Chapter 8, "RNA Purification." Lyophilized oligonucleotides are stable for months or years stored at –20°C and colder in frost-free or non-frost-free freezers. Solutions of DNA oligonucleotides are best stored at –20°C and below at neutral pH. Non-frost-free freezers are preferred to eliminate potential nicking due to freeze–thawing.

In one instance, which was not further investigated, approximately 10% of the phosphate groups were lost from the 5′ ends of phosphorylated oligo dT (approximately 15 nucleotides in length) after 12 months of storage at –20°C (Amersham Pharmacia Biotech, unpublished observations).

Your Vial of Oligonucleotide Is Empty, or Is It?

Lyophilization does not always produce a neat pellet at the bottom of the vial. The material might be dispersed throughout the inner walls of the vial in a very thin layer that is difficult to see. The best method to confirm the absence of the material is to dissolve the vial's contents by thoroughly pipetting the solvent on the vial's inner walls and measuring the absorbance at 260 nm.

SYNTHETIC POLYNUCLEOTIDES

Is a Polynucleotide Identical to an Oligonucleotide?

Manufacturers typically define polynucleotides as single- or double-stranded nucleic acid polymers whose length exceeds 100 nucleotides. Double-stranded polymers can be comprised solely of DNA or RNA, or DNA : RNA hybrids. As illustrated in Figure 10.1, a single preparation of a synthetic polynucleotide contains a highly disperse population of sizes. In comparison, oligonucleotides are almost always single-stranded molecules (RNA or DNA) shorter than 100 nucleotides and typically comprised of a nearly homogeneous population in length and sequence.

Polymer nomenclature is not universally accepted, but the major suppliers apply the following strategy:

- Poly dA—single-stranded DNA homopolymer containing deoxyadenosine monophosphate.
- Poly A—single-stranded RNA homopolymer comprise of adenosine monophosphate.
- Poly A · oligo $dT_{12\text{-}18}$—Double-stranded molecule, with one strand comprised of an RNA homopolymer of adenosine

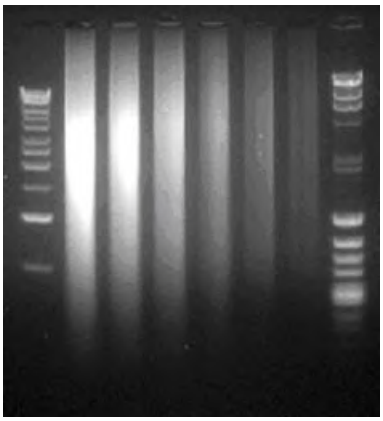

Figure 10.1 Lane 1–1 kb ladder; lane 2–7 poly (dI-dC) · (dI-dC); lane 2–2.0 μg; lane 3–1.5 μg; lane 4–1.0 μg; lane 5–0.5 μg; lane 6–0.25 μg; lane 7–0.125 μg; lane 8–Lambda HindIII/phi X174 Hinc II marker.

monophosphate; a mixture of DNA oligonucleotides 12 to 18 deoxythymidine monophosphates in length and randomly bound throughout the poly A strand.

- Poly dA-dT single-stranded DNA polymer comprised of alternating deoxyadenosine and deoxythymidine monophosphates.
- Poly dA·dT double-stranded DNA polymer containing deoxyadenosine monophosphate in one strand, and deoxythymidine monophosphate in the complementary strand.
- Poly (dA-dT)·(dA-dT) double-stranded DNA polymer comprised of alternating deoxyadenosine and deoxythymidine monophosphates in each strand.

Do double-stranded polynucleotides possess blunt or sticky ends? Yes to both, as explained below.

How Are Polynucleotides Manufactured and How Might This Affect Your Research?

The length of commercially produced polynucleotides varies from lot to lot. Polynucleotides are synthesized by polymerase replication of templates or by the addition of nucleotides to the 3′ ends of oligonucleotide primers by terminal transferase or poly A polymerase. These enzymatic reactions are difficult to regulate, so polymer size significantly varies between manufacturing runs. A second factor that affects the size of double-stranded polynucleotides is that these polymers are affected by annealing conditions. Double-stranded polymers may be produced by synthesizing each strand indpendently and then annealing the two independent strands. In reality, the annealing reaction consists of annealing two populations of strands, each with its own distribution of sizes. Depending on the actual composition of these two populations and the exact annealing conditions, the resulting population of the annealed double-stranded polymer may vary widely (see the discussion about structural uncertainty below for a related case).

Manufacturers apply analytical ultracentrifugation, gel electrophoresis, or chromatography to analyze polymer length. Commercial suppliers provide an average size of the polymer population, but they usually don't indicate the proportion of the different size polymers within a preparation. For example, two lots might have an average size of 500 bp; lot 1 might have a larger proportion of 800 bp polymers and lot 2 a larger proportion of polymers 300 bp in length. Will this affect your experiments? This question can be answered conclusively only at the lab bench, so it

is a good idea to consider performing control experiments when using a new lot of polymer for the first time.

Structural Uncertainty

What is the basic structure of a double-stranded polymer? Is it blunt ended? Will it have overhangs? How long are the overhangs? There is no single answer to these questions due to the heterogeneous nature of the product and the impact of the exact conditions used for dissolving the polymer. The buffer composition, temperature of dissolution, and volume of buffer used will all affect the final structure of the dissolved polymer.

Heterogeneous Nature

If you add equimolar amounts of a disperse mixture of poly dA and a disperse mixture of poly dT, what are the odds that two strands bind perfectly complementary to form a blunt-ended molecule? What's the likelihood of generating the same overhang within the entire population of double-stranded molecules? Does one strand of poly dA always bind to one strand of poly dT, or do multiple strands interact to form concatamers? See Figure 10.2 for examples. Considering the heterogeneous population of the starting material, one should assume that a highly heterogeneous population of double-stranded polymers forms.

Buffer Composition

Double-stranded polynucleotides are usually supplied as lyophilized powders that may or may not contain buffer salts. The pH, salt concentration, and temperature of the final suspension affect the structure of the dissolved polymer. For example, at any specific temperature, the strands of poly dA·dT resuspended in water dissociate much more frequently than the same polymer dissolved in 100 mM sodium chloride. Heating a polymer solution to 85°C for 10 minutes followed by quick chilling on ice produces a different population of polymers compared to poly dA·dT dissolved in the same buffer at room temperature.

Consider these solution variations when attempting to reproduce your experiments and those cited in the literature.

Figure 10.2 Variable products when annealing synthetic polynucleotides.

Would the World Be a Better Place If Polymer Length Never Varied?

Poly (dI-dC)·(dI-dC) is commonly applied to reduce nonspecific binding of proteins to DNA in band shift (gel retardation) experiments. The polymer's average size varies from hundreds of base pairs to several kilobase pairs. Two researchers from one laboratory used the same lot of poly (dI-dC)·(dI-dC) in experiments with different protein extracts. This one lot of poly (dI-dC)·(dI-dC) produced wonderful band shift results for the first scientist's protein extract, and miserable results for the second researcher's extract. Is this Nature's mystique or a lack of optimized band shift conditions?

Oligonucleotides Don't Suffer from Batch to Batch Size Variation. Why Not?

Oligonucleotides are almost always chemically synthesized on computer-controlled instruments, minimizing variation between batches. Different batches of the same oligonucleotide are identical in sequence and length provided that they are purified to homogeneity.

How Many Micrograms of Polynucleotide Are in Your Vial?

At least one manufacturer of polymers reports the absorbance units/mg specification for each lot of polymer. The data from three lots of poly (dI-dC)·(dI-dC) are listed below:

	Absorbance units/mg	μg/absorbance unit
Lot *A*	9.0	111
Lot *B*	13.7	73
Lot *C*	10.4	96

Why is there so much μg/unit variation among the three lots? How should you calculate the mass of material in different lots of this polymer? Should you use 50 μg/unit as you would for double-stranded DNA, or the μg/unit calculated above?

In the tradition of answering one question with another, ponder this. Why do manufacturers quantitate most of their polymer products in terms of absorbance units rather than micrograms? What are the possible explanations?

- It's easier to quantitate polymers on a spectrophotometer than to weigh them on a scale.

- DNA isn't the only material present in the polymer preparation.
- 100 units sounds more generous than 5 mg.

Despite multiple purification procedures that include extensive dialysis, other materials such as water and salts can accumulate in polynucleotide preparations. Since polynucleotides absorb light at 260 nm and the common contaminants do not, manufacturers package polymers based on absorbance units to guarantee that researchers get a consistent amount of nucleic acid.

So, if you choose to define experimental conditions using mass of polymer, use spectrophotometry and a conversion factor. Common conversion factors are 50 μg/absorbance unit (260 nm) for double-stranded DNA polynucleotides, 37 or 33 μg/absorbance unit for single-stranded DNA, and 40 μg/absorbance unit for single-stranded RNA. A conversion factor for synthetic RNA:DNA hybrids has not been defined. Some researchers apply 45 μg/absorbance unit, a compromise between the RNA (40 μg) and DNA (50 μg) values.

Be careful about weighing out an amount of polymer for use in an experiment, or quantitating polymers based on the absorbance units/mg reported within the package insert of a commercial product. Both approaches assume that the polymer is 100% pure and are likely to give higher variation in experimental conditions when changing lots of polymer from the same manufacturer or switching between manufacturers of a polymer.

Is It Possible to Determine the Molecular Weight of a Polynucleotide?

Once the average length of the polymer is known, a theoretical average molecular weight can be calculated based on the molecular weight of each strand or the molecular weight of nucleotide base pairs. Just remember that these calculations are based on the average lengths of disperse populations of polymers.

What Are the Strategies for Preparing Polymer Solutions of Known Concentration?

Suppose that your task was to prepare a 10 μM solution of poly dT. Theoretically you could prepare a solution that was 10 μM relative to the poly dT polymer (molarity calculations would be based on the average molecular weight reported on the manufacturer's certificate of analysis), or 10 μM relative to the deoxythymidine monophosphate (dT) nucleotide that comprises the polymer.

The preferred approach for preparing a polymer solution of a particular molar concentration is to express all concentrations in a concentration of bases or base pairs. The reason for this is that the best way to determine the amount of polymer present is by measuring absorbance. In addition, since the population of polymer molecules is so disperse, approximating the concentration of polymer based on strands of polymer may be misleading. Finally, this approach will maximize the reproducibility of your experiments between different lots of polymer and for those who try to reproduce your work.

10 μM of the dT Nucleotide

As described above, polymer solutions are best quantitated via a spectrophotometer. Before you go to the lab, grab some paper and perform a couple of quick calculations. First, using the molar extinction coefficient, calculate the absorbance of a 10 μM solution. The molar absorbtivity of poly dT is 8.5×10^3 L/mol-cm-base at 264 nm and pH 7.0. This means one mole of dT monomers in one liter will give an absorbance of 8500. Therefore a 10 μM solution (i.e., 0.000010 M) will have an absorbance of 0.085 (i.e., 8500×0.000010).

Next calculate the dilution required of 50 absorbance units to give the absorbance of a 10 μM solution (i.e., 0.085). If you have a vial with 50 absorbance units of polymer and you dissolved the entire 50 absorbance units in 1 ml of buffer, the spectrophotometer would hypothetically measure an absorbance close to 50. To obtain an absorbance of 0.085, the total dilution of the 50 absorbance units would be 588-fold (i.e., 50/0.085 = 588).

In the lab you would never dissolve the entire 50 absorbance units in 588 ml. First, this would limit you to using the polymer at concentrations of 10 μM or less. Second, the dilution may not work as you theoretically calculated. And finally, if the dilution did work as you expected, the solution would have an absorbance of less than 0.100 and therefore not be reliably measured by a spectrophotometer. In practice, you would prepare a stock solution of approximately 10 times the final desired concentration and then dilute to a range that can be measured by a spectrophotometer.

Your Cuvette Has a 10 mm Path Length. What Absorbance Values Would Be Observed for the Same Solution If Your Cuvette Had a 5 mm Path Length?

Half the path length, half the absorbance.

Why Not Weigh out a Portion of the Polymer Instead of Dissolving the Entire Contents of the Vial?

As discussed earlier, would you be weighing out DNA polymer or DNA polymer and salt? Also DNA polymers are very stable in solution when stored at –20°C or colder. (If you have a choice, store unopened vials of polymer at –20°C or colder; see below.) Aliquot your polymer stocks to avoid freeze–thaw nicking and contamination problems.

Is a Phosphate Group Present at the 5′ End of a Synthetic Nucleic Acid Polymer?

Synthetic DNA and RNA polymers are produced by adding nucleotides to the 3′ end of an oligonucleotide primer or by replicating a template by a nucleic acid polymerase. If the primer is phosphorylated, and if the mechanism of the DNA polymerase produces 5′ phosphorylated product, one could conclude that the polymer contains a 5′ phosphate. If your purpose is to end-label a polymer via T4 polynucleotide kinase, it's safest to assume that a phosphate is present, and either dephosphorylate the polymer or perform the kinase exchange reaction (Ausubel et al., 1995).

What Are the Options for Preparing and Storing Solutions of Nucleic Acid Polymers?

Synthetic polymers comprised of RNA and DNA are most stable (years) when stored as lyophilized powders at –20°C or –70°C. Polymer solutions are stable for several months or longer when prepared and stored as described below.

Double-Stranded Polymers

Concentrated Stock Solutions

To maintain principally the double-stranded form of synthetic DNA and DNA–RNA hybrids requires a minimum of 0.1 M NaCl, or lower concentrations of bivalent salts present in the solution (Amersham Pharmacia Biotech, unpublished observations). In the absence of salt, the two strands within a polymer can separate (breathe) throughout the length of the molecule. While its presence won't harm polymers during storage, salt could hypothetically interfere with future experiments. If this is a concern, polymers destined for use in double-stranded form can also be safely stored for months or years in neutral aqueous buffers (i.e., 50 mM Tris, 1 mM EDTA) at –20°C or –70°C, even though they will likely be in principally single-stranded form when heated to room temperature and above.

Preparing Solutions for Immediate Use

DNA alternating co-polymers such as poly (dI-dC)·poly (dI-dC) can be prepared in the salt buffers described above, heated to 60°–65°C, and slowly cooled (no ice) to room temperature to reanneal the strands. Duplexes of poly (dA)·poly (dT) require the salt buffers above, and should be heated to 40°C for 5 minutes, and slowly cooled to room temperature. Duplexes of poly (dI)·poly (dC) and RNA·DNA hybrids require salt buffers and heating to 50°C for 5 minutes, followed by slow cooling. Poly (dG)·poly (dC) can be difficult to dissolve. Even after heating to 100°C and intermittent vortexing, some polymer would not go into solution (A. Letai and J. Fresco, Princeton University, 1986, personal communication).

Single-Stranded Polymers

Single-stranded DNA and RNA polymers are stable in neutral aqueous buffers. Depurination will occur if DNA or RNA polymers are exposed to solutions at pH 4 or lower. In addition, for RNA polymers, pH of 8.5 or greater may cause cleavage of the polymer. Carefully choose your buffer strategy for RNA work, since the pH of some buffers (i.e., Tris) will increase with decreasing temperature.

If a single-stranded DNA polymer is difficult to dissolve in water or salt, heat the solution to 50°C. If heating interferes with your application, make the polymer solution alkaline, and after the polymer dissolves, carefully neutralize the solution (Amersham Pharmacia Biotech, unpublished observations).

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