Molecular Biology Problem Solver: A Laboratory Guide. Edited by Alan S. Gerstein

ISBNs: 0-471-37972-7 (Paper); 0-471-22390-5 (Electronic)

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PCR

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INTRODUCTION

The principle of the polymerase chain reaction (PCR) was first reported in 1971 (Kleppe et al., 1971), but it was only after the discovery of the thermostable Taq DNA polymerase (Saiki et al., 1988; Lawyer et al., 1989) that this technology became easy to use. Initially the thermal cycling was handled manually by transferring samples to be amplified from one water bath to another with the addition of fresh enzyme per cycle after the denaturation step (Saiki et al., 1986; Mullis et al., 1986). Today, 30 years later, we are fortunate to have thermal cyclers, along with enzymes and other reagents dedicated for various PCR applications. The advances in PCR technology and the number of annual publications using PCR in some area of the research has grown tremendously from a single-digit number to 1.6×10^4 in 1999 (Medline search). The popularity of the PCR method lies in its simplicity, which permits even a lay person without a molecular biology degree to run a reaction with minimum training.

However, this easy "entry" can also act as a "trap" to encounter common problems with this technology. The purpose of this chapter is to help you select and optimize the most appropriate PCR strategy, to avoid problems, and to help you think your way out of problems that do arise. While your research topic may be unique, the solutions to most PCR problems are less so. Employing one or a combination of methods mentioned in this chapter could solve problems. I encourage readers to spend time in setting up the lab, choosing the appropriate protocol, optimizing the con-

ditions and analysis method *before* running the first PCR reaction. In the long run, you will save time and resources.

This chapter provides practical guidelines and references to in-depth information. Other useful information is added in the Appendix to help you navigate through various tools available in today's market.

DEVELOPING A PCR STRATEGY: THE PROJECT STAGE

Assess Your Needs

First ask yourself what outcome you need to achieve to feel successful with your experiment (Table 11.1). What kind of information do you need to get? Is it qualitative or quantitative? Are you setting up a routine analysis to run for the next two years, or is this for the manuscript you need to send to the editor in a hurry in order for your paper to get accepted? Your priorities will help you choose the method that best fits your needs.

Table 11.2a shows an example of a list for a researcher who needs to develop a PCR method where approximately 48 genes will be studied for relative gene expression in response to various drug treatments to be given over a three-year period. In contrast, Table 11.2b shows a list of a scientist who wishes to clone a gene with two different mRNA forms generated by alternative splicing

Table 11.1 Priority Check List

Objectives	High/Medium/Low
Quantitative	
Sensitivity	
Fidelity	
High-throughput	
Reproducibility	
Cost-sensitive	
Long PCR product	
Limited available starting material	
Short template size	
Gel based	
Simple method	
Nonradioactivity involved	
Automated	
Long-term project	
DNA PCR	
RNA PCR	
Multiple samples	
Multiplex	

Table 11.2a Priority List: Researcher 1

Objectives	High/Medium/Low
Quantitative	H
Sensitivity	H
Fidelity	M
High-throughput	M
Reproducibility	H
Cost-sensitive	M
Long PCR product	L
Limited available starting material	M
Short template size	H
Gel based	L
Simple method	H
Nonradioactivity involved	H
Automated	H
Long-term project	H
DNA PCR	L
RNA PCR	H
Multiple samples	H
Multiplex	H

Table 11.2b Priority List: Researcher 2

Objectives	High/Medium/Low
Quantitative	M
Sensitivity	M
Fidelity	H
High-throughput	L
Reproducibility	H
Cost-sensitive	H
Long PCR product	L
Limited available starting material	M
Short template size	M
Gel based	H
Simple method	L
Nonradioactivity involved	L
Automated	L
Long-term project	L
DNA PCR	L
RNA PCR	H
Multiple samples	L
Multiplex	L

mechanisms. The purpose of Researcher 2 is to amplify the cDNA and to demonstrate the size difference by separating the two forms by gel electrophoresis. The data are needed for a manuscript due in two months. You can see the differences between the priorities and needs of the two researchers.

After setting clear objectives of what your PCR reaction must accomplish, check that you have the adequate resources. This includes not only budget but also head count, skill level, time, equipment, sequence information, sample supply, and other issues. If time is most critical, then you may require a colleague's assistance or a new instrument to do the project as quickly as possible. In a similar token, if the sample is difficult to obtain in abundance, the choice of PCR that minimizes the sample requirement becomes more important.

Selecting one PCR strategy that optimally satisfies every research need is unlikely. At this early planning stage, a compromise between competing needs will likely be required. Remember that after all the planning is complete, the final PCR strategy still has to evolve at the lab bench.

Identify Any Weak Links in Your PCR Strategy

There are many parameters that affect the outcome of a PCR reaction. Some examples are as follows:

- PCR reaction chemistry (enzyme, nucleotide, sample, primer, buffer, additives).
- PCR instrument type (ramp time, well-to-well homogeneity, capacity to handle many samples).
- Thermal cycling conditions (two-step, three-step, cycle segment length—i.e., denaturation, annealing, and extension—ramp time, etc.).
- Sample collection, preparation, and storage (DNA, RNA, microdissected tissue, cells, and archived material).
- PCR primer design.
- Detection method (simultaneous detection, post PCR detection).
- Analysis method (statistical analysis).

Like the weakest link in a chain, your final result will be limited by the parameter that is least optimum. For example, suppose that you're studying the tissue-specific regulation of two mRNA forms. Regardless of the time spent optimizing the PCR reaction, instrument type, and everything to near-perfection, the use of agarose gel electrophoresis may not allow you to reach the conclusion that there are two different mRNA forms if their molecular weights are similar. You might require a separation technique with greater resolving power.

Suppose that your objective requires quantitative PCR. RNA from 30 samples is collected and RT-PCR is performed. The PCR reaction is run in duplicate and repeated twice on two different

days. One-step RT-PCR is done using the same RNA samples, and PCR products are analyzed by polyacrylamide gel electrophoresis (PAGE). For some unknown reason, the second experiment shows different quantitative data. Which data are correct? Without a sufficient number of samples to calculate standard deviation, one cannot make any quantitative analysis. For quantitative PCR, the sample size has to be large enough and the standard curve must show that PCR was linear within the range one is examining. To do this, serial dilution of a positive control must be run simultaneously, and the test samples have to fall within this range of amplification. Minimums of three to four samples are required for reliable statistical analysis of the data. It is also a good idea to generate enough cDNA to run multiple experiments to reduce error due to differences in the cDNA synthesis step. The positive control must also be properly stored so that loss or damage of DNA does not generate false negative results.

High-tech, automated PCR synthesis and detection systems are useless if the sample preparation destroys the mRNA, co-purifies PCR inhibitors, or the PCR primer design amplifies genomic DNA. Your results will only be as good as the weakest parameter in your PCR strategy.

Manipulate the Reaction to Meet Your Needs

Table 11.3 describes positive and negative effectors of the PCR reaction. These data can help you plan your experiment or modify your strategy if your results aren't satisfactory.

DEVELOPING A PCR STRATEGY: THE EXPERIMENTAL STAGE

What Are the Practical Criteria for Evaluating a DNA Polymerase for Use in PCR?

An appreciation of what your research objective requires from a PCR product should be central to your selection of a thermostable DNA polymerase. Were you planning to identify a rare mutation in a heterogeneous population as in allelic polymorphisms (Frohman, Dush, and Martin, 1988)? As the copy number gets smaller (less than 10), the need for high-fidelity enzyme or enzyme mixes increases, as discussed below. In contrast, if you're screening a batch of transgenic mice for the presence or absence of a marker gene via Southern hybridization, enzyme fidelity might not be as crucial. Most applications do not require high

Table 11.3 Positive and Negative Effectors of a PCR Reaction

To Enhance This Parameter	Manipulate One or More of These Components	
Fidelity and specificity	Enzyme	Select an enzyme with potent 3′–5′ Exonuclase activity.
	Primer design	Include mismatches at 3′ end, which can help discriminate against homologous sequences such as pseudogenes. Enzyme selection can enhance this effect. With Taq polymerase, relative amplification efficiencies with 3′-terminal mismatches is greater for A:G and C:C than for other nucleotide pairs (Kwok et al., 1995). Use longer primers (refer to section "What Are the Steps to Good Primer Design?". Primers less than 15 nucleotides do not give enough specificity from a statistical point of view.
	PCR cycling condition	Increase annealing temperature. Reduce cycle segment time (denaturation, annealing, etc.). Lower cycling number.
	Reaction chemistry	Decrease $[Mg^{2+}]$. Apply a hot start strategy (Erlich, Gelfand, and Sninsky, 1991). Check that concentration and pH of dNTP solution(s) is correct. Decrease primer concentration.
	Template	Confrim that template is intact, not nicked, and free of contaminants and inhibitors. Confirm the presence of sufficient starting copy number.
	Method of analysis	Minimize contamination and handling errors; use an automated analysis system. Use sufficient sample number to enable reliable statistical analysis. Check for erroneous manipulation (pipetting errors, etc.).
	Clean lab practice	Use a positive displacement pipette. Use a separate room to set up experiments. Wear gloves. Use UNG and dUTP (Longo, Berninger, and Hartley, 1990).
	Cycler	Check that the temperature profile is consistent at every position in the heating block. Decrease ramp time. Check for tight fit between reaction vessels and heating block.
Efficiency of doubling/cycle	Reaction	Increase concentration of dNTPs and enzymes. Use minimal concentrations of DMSO, DMF, formamide, SDS, gelatin, glycerol (see Table 11.7).

Table 11.3 (*Continued*)

To Enhance This Parameter	Manipulate One or More of These Components	
	Template	Confirm that template is unnicked, free of contaminants and inhibitors. Use a smaller size template DNA (get more molecules per pg of input template, and less complexity for primer annealing). For example, PCR product vs. genomic DNA. Decrease amplicon size.
	Enzymes	Taq > Pfu, >>Stoffel fragment.
	Cycling	Decrease cycling time or use a shuttle profile (Cha et al., 1992). Decrease the size of the reaction tube. Check for tight fit between reaction vessels and heating block.
	Cycler	Decrease ramp time.
	Primer design	Use forward and reverse primers that have similar length and GC content. Confirm that primers do not form primer-dimer or hairpin structure.
Reproducibility	Sample	Ensure that template is clean and intact. Confirm presence of sufficient starting template and sufficient sample number for statistical analysis.
	Reagents	Use the same lots of primer and buffers between experiments. Store enzyme in small aliquots. Investigate for presence of contaminating template and inhibitors to PCR reaction.
	Controls	Include positive and negative controls with all experiments.
	Cycling	Use a hot-start strategy (Kellogg et al., 1994). Use the same cycler between experiments.
Quantitative	Template	Confirm the quantity of the template. Confirm template preparation is clean. Investigate for presence of contaminating template and inhibitors to PCR reaction.
	Experimental design	Include triplicate or quadruplicate samples. Use a statistically sufficient number of samples. Prepare a standard curve to demonstrate the range over which PCR product yield provides a reliable measure of the template input. Robust: Confirm that chemistry, primer design, tubes, thermal cycler, and other factors are optimized.
	Analysis	Confirm the analytical method's accuracy/resoluton. Is it accurate during the exponential phase of PCR?

Table 11.3 (*Continued*)

To Enhance This Parameter	Manipulate One or More of These Components	
		Use appropriate controls. Repeat experiments when data are outside of standard deviation limits. Minimize the manipulations from start to finish.
	Cycler	Check that the temperature profile is consistent at every position in the heating block.
	Control	Confirm that controls have similar sequence profile and amplification efficiency. Confirm that PCR was linear by producing a standard curve.
	Analysis	Use an automated system to reduce handling steps.
	Detection	Check the detection strategy's senitivity and ability to measure yield in the exponential phase of PCR. Confirm that the technique has high sensitivity and magnitude over a wide dynamic range.
High-throughput	Instrument	Select a system that handles microtiter plates and multiple sample simultaneously.
	Reaction	Use a hot-start PCR strategy (D'Aquilla et al., 1991; Chous et al., 1992; Kellogg et al., 1994). Use a master PCR reagent mix. Use aliquots taken from the same lot of material; don't mix aliquots from different lots.
	Sample preparation	Use of robotics. Storage of sample as cDNA or ethanol precipitate, rather than RNA in solution.
	Cycling	Use one cycling strategy for all samples. Decrease the cycling time.
	Analysis	Use an automated system.
	Detection	Use an automated detection system to monitor the exponential phase.
Sensitivity	Detection	Monitor specific PCR product formation by hybridization via nucleic acid probe. Use fluorescent intercalating dye (Wittwer et al., 1997).
	Reaction	Use a nested PCR strategy (Simmonds et al., 1990). Note: Sensitivity is gained at the expense of quantitation. Use a hot-start PCR strategy. Use UNG and dUTP to prevent carryover.
	Analysis	Use a real time PCR strategy that detects low levels of amplicon missed by gel

Table 11.3 (*Continued*)

To Enhance This Parameter	Manipulate One or More of These Components
	electrophoresis. When hybridization probes are used, primer-dimer formation will not mask the authentic product, even after 40 cycles. This is not true for SYBR® Green or Amplifluor.
	Nested PCR or extra manipulation may be needed for other non-real-time PCR based techniques. "Hot" nested PCR is one such example that elegantly combines the qualities of nested PCR with the high resolution of PAGE (Jackson, Hayden, and Quirke, 1991).
Control	Include positive and negative controls; when the target is not detected, one can conclude that target was below 100 copies, etc., which makes the data more meaningful than just saying it was not detected.
Lab setup	Clean lab.
	No contamination.
Experimental design	Check primer design. If amplifying related genes is a concern, design the primer to create mismatches at the 3′ end using the most heterogeneous sequence region.

fidelity, but one needs to be aware when high fidelity has to be considered. During planning, one should also consider the many ways a PCR reaction can be manipulated to achieve a given end, as discussed throughout this chapter.

The data in Table 11.4 are provided to highlight the biochemical properties of common PCR-related enzymes and help you develop a selection strategy. For a comprehensive comparison of thermostable DNA polymerases, see Perler, Kumar, and Kong (1996), Innis et al. (1999), and Hogrefe (2000). However, biochemical data and logic can't always predict the most appropriate enzyme for PCR; experimentation might still be required to determine which enzyme works best. Abu Al-Soud and Radstrom (1998) demonstrate that contaminants inhibitory to PCR vary with the sample source, and that experimentation is required to determine which thermostable DNA polymerase will produce successful PCR. A second illustration of the difficulty in predicting success based on enzymatic properties are the Archae DNA polymerases, which have not become premiere PCR enzymes despite their extreme thermostability and good proofreading activity.

Table 11.4 Selected Properties of Common Thermostable DNA Polymerases

Enzyme	Proofreading (3′–5′ exonuclease)	5′–3′ Exonuclease	Heat Stability (min before 50% activity remains)	Processivity (dNTP/ binding)	Extension Rate (dNTP/ s/mol)
Taq DNA polymerase	Absent	Present	9 at 97.5°C (40–60 at 95°C. depending on protein concentration[a,b]	50–60	60–150
Stoffel fragment	Absent	Absent	21 at 97.5°C	5–10	130
Tth DNA polymerase	Absent	Present			25
rTth XL	Trace	Present		30–40	
AmpliTaq CS	Absent	Absent		50–60	
UlTma DNA polymerase	Present (low)	Absent	50 at 97.5°C		
Pfu DNA polymerase (native and recombinant)	Present	Absent	1140 at 95°C[a]	10[a]	60
Pfu DNA polymerase (exo-form)	Absent	Absent	1140 at 95°C[a]	11[a]	
(*Pyrococcus species* GB-D)	Present	Absent	1380 at 95°C[c]		>80
(aka Deep Vent®)			480 at 100°C		
Tli Pol (aka Vent®)	Present	Absent	402 at 95°C[c]	7	67
			108 at 100°C		
Herculase enhanced DNA polymerase	Present	Present[a]			
Tbr DNA polymerase (Dynazyme™)[e]	Absent	Present	150 at 96°C		
Platinum *Pfx*[f]	Present	Absent	720 at 95°C 180 at 100°C	100–200	100–300
Platinum *Taq*[f]	Absent	Present	96 at 95°C	50–60	60–150
Advantaq Polymerase[g]	Absent	Absent	40 at 95°C		40
Tac Pol	Present	Absent	30 at 75°C		
Mth Pol	Present	Absent[d]	12 at 75°C		
ThermalAce™ *Pyolobus fumarius*[h]	Present	Absent		5-fold greater than *Taq* DNA Polymerase	
Hot *Tub (T. flaius)*[i]	Present	Absent	Similar to Taq		

Source: Unless otherwise noted, all data from Perler, Kumar, and Kong (1996).
[a] Data provided by H. Hogrefe, Stratagene, Inc.
[b] New England Biolabs Catalog, 2000.
[c] Z. Kelman (JBC 274:28751); present according to Perler.
[d] Data provided by D. Titus, MJ Resesarch, Inc.
[e] Data provide by D. Hoekzema, Life Technologies Inc.
[f] Data provided by J. Ambroziak, Clonetech Laboratories Inc.
[g] Data provided by Invitrogen, Inc.
[h] Lawyer et al. (1993). PCR Methods & application pp. 275–286.
[i] Data provided by Amersham Pharmacia Biotech, Inc.

Fidelity

Fidelity could be defined as an enzyme's ability to insert the proper nucleotide and eliminate those entered in error. As thoroughly reviewed by Kunkel (1992), fidelity is not a simple matter; there are several steps during the polymerization of DNA where mistakes can be made and corrected. Still most practical discussions of fidelity focus on the proofreading function provided by an enzyme's 3′–5′ exonuclease activity. Cline, Braman, and Hogrefe (1996) compared the fidelity of several thermostable DNA polymerases side by side, taking care to optimize the conditions for each enzyme. They observed the following fidelity rates (mutation frequency/bp/duplication), in order: *Pfu* (1.3×10^{-6}) > *Deep Vent* (2.7×10^{-6}) > *Vent* (2.8×10^{-6}) > *Taq* (8.0×10^{-6}) > exo$^-$ *Pfu* and *UlTma* (~5×10^{-5}). These and similar data should be viewed in relative rather than absolute terms, because assay methods affect the absolute number of detected misincorporations (André et al., 1997), and sample source can affect the performance of enzymes differentially and unpredictably (Abu Al-Soud and Radstrom, 1998).

Proofreading activity can also reduce PCR yield, especially in reactions that generate long PCR products. The greater time required to extend the fragment increases the chance of primer degradation by the 3′–5′ exonuclease activity (de Noronha and Mullins, 1992 and Skerra, 1992). The problem of reduced yield can be corrected by including an enzyme with strong proofreading activity into a PCR reaction with a polymerase that lacks a strong proofreading activity (Barnes, 1994; Cline, Braman, and Hogrefe, 1996; MJ Research Inc. Application Bulletin, 2000).

Heat Stability

Is a higher reaction temperature always helpful and necessary? No. For most DNA-based PCR, the consensus is that hot-start PCR increases both sensitivity and yield by preventing nonspecific PCR product formation (Faloona et al., 1990). Higher temperatures can melt secondary structures, but there are limitations to the use of heat. Very high denaturation temperatures can also damage DNA, through depurination and subsequent fragmentation, especially during long PCR reactions (Cheng et al., 1994). It can also increase hydrolysis of RNA in one step RT-PCR in the presence of magnesium ions (Brown, 1974). In order to reduce heat-induced damage, incorporation of additives such as DMSO is used (see later section on additives).

Choosing an enzyme with specialized activities will not produce

the desired results unless the appropriate conditions are applied. For example, UlTma™ DNA polymerase has a pH optimum for polymerase activity of 8.3 and exonuclease activity at pH 9.3 (Bost et al., 1994). Likewise, presence of metal ions can favor one activity over the other for many polymerases.

Long PCR

Additives such as single-stranded binding protein (Rapley, 1994), T4 gene 32 protein (Schwarz, Hansen-Hagge, and Bartram, 1990), and proprietary commercial products may increase the production efficiency of long PCR. However, fidelity was also shown to be crucial to the replication of large products via PCR (Barnes, 1994). By supplementing PCR reactions containing *Taq* DNA polymerase (which lacks proofreading activity) with proofreading-rich *Pfu* DNA polymerase, Barnes generated fragments up to 35 kb. Bear in mind that proofreading activity can potentially reduce yield, especially with large PCR products. As discussed above, this problem can be avoided by utilizing a combination of polymerases that possess and lack strong proofreading activity.

The availability of specialized, designer enzymes are an attractive strategy that shouldn't be ignored. However, selecting the right enzyme(s) is one step among many, and can't guarantee the desired result. One near-term example is the importance of enzyme concentration. The concentration of polymerase applied to a PCR reaction ranges from one to four units per 100 μL. Greater concentrations can increase formation of nonspecific PCR products. The importance of optimizing other parameters, such as buffer component, primer design, and cycling conditions is shown in Table 11.3 and Table 11.5.

How Can Nucleotides and Primers Affect a PCR Reaction?

Nucleotide Concentration

The standard concentration of each nucleotide in the final reaction is approximately 200 μM, which is sufficient to synthesize 12.5 μg of DNA when half of the dNTPs are incorporated. Adding more nucleotide is unnecessary and detrimental. Too much nucleotide reduces specificity by increasing the error rate of the polymerase and also chelates magnesium, changing the effective optimal magnesium concentration (Gelfand, 1989; Coen, 1995).

Primer Concentration

The standard primer concentration is 100 to 900 nM; too much primer can increase the formation of nonspecific products. It is

Table 11.5 Optimizing $MgCl_2$ Concentration for PCR

Component	Final Concentration	Per Reaction	1 mM	2 mM	3 mM	4 mM	6 mM	8 mM	10 mM
10× PCR buffer	1×	10 μl	40.0	40.0	40.0	40.0	40.0	40.0	40.0
50 μM forward primer	0.5 μM	1 μl	4.0	4.0	4.0	4.0	4.0	4.0	4.0
50 μM reverse primer	0.5 μM	1 μl	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Template DNA	Optimum	10 μl	40.0	40.0	40.0	40.0	40.0	40.0	40.0
100 mM $MgCl_2$	Various	Various	4.0	8.0	12.0	16.0	24.0	32.0	40
25 mM dNTP mix	0.2 mM	0.8 μl	3.2	3.2	3.2	3.2	3.2	3.2	3.2
Taq polymerase 5 U/μl	2.5 U	0.5 μl	2.0	2.0	2.0	2.0	2.0	2.0	2.0
H_2O		To 100 ul	To 400 μl	To 400 μl	To 400 μl	To 400 μl	To 400 μl	To 400 μl	To 400 μl

especially important to adjust the primer concentration when the target sequence is rare or the template amount is low. Less primer is needed in these cases; too much primer will generate primer-dimers or smearing of the product visualized by agarose gel electrophoresis. For most applications it is practical to apply the standard concentrations cited above and to focus effort on optimizing other critical parameters. For real-time PCR multiplex applications, it is recommended that a primer matrix study be performed (Table 11.6a,b) to ensure the limiting primer concentration for an endogenous control. This way the target gene amplification is not compromised by competition for reagents in the same reaction tube (well). This recommendation applies to all housekeeping genes regardless of the abundance level (i.e., needed not only for rRNA but also for less abundant genes, e.g., glyceraldehyde 3-phosphate dehydrogenase, cyclophilin, and hypoxanthine-guanine phosphoribosyl-transferase).

The range of final concentration for forward and reverse primers is 100 to 900 nM in the matrix below. Perform an initial series of experiments to find the rough range of an optimum primer concentration. Follow with a second series of experiments to fine-tune the primer concentration range. In the following example, the final results suggest a forward primer concentration

Table 11.6a Primer Matrix Study

Reverse Primer: Primer concentration (nM)	Forward Primer 100	300	600	900
100	++	+	−	−−−
300	+	+	−	−−−
600	−	−−	−−	−−−
900	−−	−−	−−−	−−−

Table 11.6b Primer Matrix Study: Final Primer Optimization Matrix

Reverse Primer: Primer concentration (nM)	Forward Primer 100	120	140	160	180	200
100	+	+	+	++	++	++
120	+	+	+	++	++	++
140	+	+	++	++	++	+++
160	+	+	+	+	+	++
180	−	−	−	−	−	−
200	−−−	−−	−	−	−	−

of 200 nM and reverse primer at 140 nM. Both the specificity and the yield can be scored for excellent (+++), good (++), fair (+), and similarly for poor (−), very bad (−−−) based on no signal, smear, and low yield.

Nucleotide Quality

The benefits of using extremely pure solution nucleotides as compared to standard lyophilized nucleotides include proper pH and absence of nuclease. A nucleotide solution at too low or high a pH can shift the overall pH of the reaction buffer and decrease yield, as can unequal quantities of the four nucleotides. The proper quantitation and pH adjustment of nucleotide solutions is discussed in Chapter 10, "Nucleotides, Oligonucleotides, and Polynucleotides."

How do the Components of a Typical PCR Reaction Buffer Affect the Reaction?

The buffer impacts the amplification by maintaining pH range, minimizing effect of inhibitors, protecting enzymes from premature loss of activity, stabilizing template, and more. Because polymerases have a narrow optimum pH range, a slight shift of pH, as little as 0.5 to 1 can reduce the yield of the PCR products. Because

Tris buffer changes its pH with temperature, it is not an ideal buffer for Taq polymerase. Table 11.7 summarizes the effects of several common additives on Taq polymerase. Their impact and optimum concentrations might differ for other enzymes, but the data regarding Taq polymerase is a starting point. Consult the manufacturers of other enzymes for more details.

Magnesium

The concentration of $MgCl_2$ affects enzyme specificity and reaction yield. In general, lower concentrations of Mg^{2+} leads to specific amplification and the higher concentration encourages nonspecific amplification. The effective concentration of Mg^{2+} is dependent on the dNTP concentration as well as the template DNA concentration and primer concentration. The strategy illustrated in Table 11.5 can be used to optimize Mg^{2+} concentration as well as other additives described below.

Additives and Contaminants

Detergent, gelatin, and other components are often included to reduce the negative effect of contaminants (Gelfand, 1992) (Table 11.7). Tween eliminates the effects of SDS, which can be carried over from sample preparation. Detergent can also stabilize the activity of some enzymes, such as Taq polymerase. When the amount of template is very small, nuclease can degrade the precious DNA, but the presence of "carrier" DNA can prevent this. Gelatin helps prevent the template DNA from getting adsorbed to the surface of the reaction tube and also stabilizes polymerase activity.

The mechanisms behind the effects of some additives and contaminants are unclear. Less than 1% DMSO may affect the T_m of primers, the thermal activity of Taq polymerase and/or the degree of product strand separation. Higher DMSO concentration (10–20%) inhibits Taq polymerase activity from 50% to 90%. Ethanol does not affect activity up to concentrations of 10%.

How Can You Minimize the Frequency of Template Contamination?

Since the power of amplification is so great, the fear of getting a false positive is common (Dieffenbach and Dveksler, 1995). Here is a list of general PCR practices to minimize cross-contamination.

- Wear a clean lab coat and gloves when preparing samples for PCR.
- Have separate areas for sample preparation, PCR reaction setup, PCR amplification, and analysis of PCR products.

Table 11.7 Effects of Additives on *Taq* DNA Polymerase

Chemicals	Mode of Action	Amount for Enhancement or Inhibition
Ethanol		Slight enhancement at 10%
Urea	Lower target T_m for annealing.	Slight enhancement at 1–1.5 M, but inhibition at greater than 2 M
DMSO	Lower target T_m for annealing.	Enhancement at 1–10% (v/v) (*www.alkami.com*) 12–15% (v/v) (Baskaran et al., 1996)[a]
DMF	Lower target T_m for annealing.	Inhibition at 10% or greater
Formamide	Lower target T_m for annealing. Increase specificity and yield by changing T_m of primer-template hybridization and lower heat destruction of enzyme.	Enhancement at 1.25–10% (v/v); Inhibition at 15% or greater
SDS	Prevent aggregation of enzyme.	Inhibition at 0.01% or greater
Glycerol	Enhance specificity by changing T_m. Extends Taq polymerase resistance to heat damage.	Enhancement at 5–20% (v/v) (*www.alkami.com*)
Perfect match polymerase enhancer (PMPE) (Stratagene Inc.)		Approximately 1%
Ficoll 400 (Wittwer and Garling, 1991)		The optimal amount must be empirically determined.
Gelatin		100 μg/ml or 0.01–0.1% (w/v).
Tween 20/NP40		0.1–0.05% (v/v) Tween 20 0.05% (v/v) NP40
T4 Gene 32 protein (Schwarz et al., 1990)	Increase specificity and yield by changing T_m of primer-template hybridization.	0.05–0.1 nmole/amplification reaction (note: original publication incorrectly states 0.5 –1.0 nmole)
Triton X-100	Prevents enzyme from aggregating.	0.01 % (v/v)
Bovine Serum Albumin (BSA)	Neutralizes many factors found in tissue samples which can inhibit PCR.	10–100 μg/ml
Betaine		0.5–2.0 M (Roche Molecular Biochemicals Web site) (1.8–2.5 M) (Baskaran et al., 1996)[a]
Tetramethyl ammonium chloride (TMAC)		10–100 μm
PEG 6000		5–15% (w/v)
Spermidine	Reduces nonspecific reaction between polymerase and template DNA.	

Other references: For Taq DNA polymerase, Gelfand (1992, pp. 6–16); for the polymerase chain reaction, Coen (1995).

[a] Baskaran et al. (1996) claims that combination of DMSO (5–10%) and betaine (1.1–1.4 M) produces best results.

- Open PCR tube containing amplification products carefully, preferably in a room other than where the PCR reactions take place. Spin tubes briefly before opening a lid.
- Use screw cap microfuge tubes for templates and positive controls to control microaerosolization when opening tubes.
- Use a positive-displacement pipette or aerosol-resistant pipette tips.
- Discard pipette tips in a sealed container to prevent airborne contamination.
- Periodically clean lab benches and equipment with 10% bleach solution.
- Prevent contamination by using uracil-N-glycosylase (UNG) which acts on single- and double-stranded dU-containing DNA and destroys the PCR products (Longo, Berninger, and Hartley, 1990).
- Aliquot reagents, sterile water, primers, and other material into tubes to reduce the risk of contamination.
- When possible, avoid using plasmid DNA as a control. The DNA can contaminate the lab like a virus if not handled carefully. A safer control is a sample containing the target at high or low levels. Another method involves a synthetic oligonucleotide template that contains the sequence complimentary to primer binding region plus part of the sequence being amplified by the forward and reverse primers designed just for the initial testing of primers. They have major internal sequence deletions; thus they only serve to validate the primers. They are not amplified simultaneously with the test samples. If you must use plasmid DNA as a control, refer to the Appendix A for preparation of a plasmid DNA control solution that can be stored over a long period of time.

What Makes for Good Positive and Negative Amplification Controls?

The inclusion of reliable positive and negative controls in all your experiments will save time and eliminate headaches. Examples follow:

- Positive controls: Samples containing the target sequence at high copy number.
- Negative controls: One primer only, no Mg^{2+}, no enzyme, sample known to lack the target sequence, no RT step for RT-PCR.

Unfortunately, the above controls can also fail. Most often the failure originates in the preparation of the positive and negative

controls. Plasmid DNA is unstable at low concentrations during storage, especially in plain water or TE (10 Tris, 1 mM EDTA, pH 7). At dilute concentration, DNA can be lost by adsorption to the inner wall of a tube or be degraded by nuclease activity. A good way to store plasmid DNA (or control cDNA or genomic DNA) is in TE with 20 μg/ml glycogen (molecular biology grade, nuclease free) in small aliquots in a –20°C freezer. Repeated freeze–thawing of control DNA should be avoided. The water used for any aspect of a PCR reaction should also be nuclease free, and stored in small volumes. Don't use a bottle of water that's been sitting in the lab for months. Microorganisms are too easily introduced.

What Makes for a Reliable Control for Gene Expression?

Good endogenous controls are constituitively expressed and change minimally while the target gene expression may vary greatly. Poor controls change their expression levels during the treatment, thus masking the target gene expression fluctuation. Bonini and Hofmann (1991) and Spanakis (1993) provide examples where inappropriate controls prevented the detection of biologically significant changes in gene expression. Some popular endogenous controls such as β-actin and glyceraldehyde dehydrogenase (GAPDH) are well known for having pseudogenes, and related genes, adding complexity to interpretation of results (Multimer et al., 1998; Raff et al., 1997). rRNA (28S, 18S, 5.8S, etc.) seems to be more constant in its level than other mRNA type housekeeping genes such as β-actin. Without a housekeeping gene that stays relatively constant (nothing really stays absolutely constant), a subtle change in gene expression will go undetected in the noise, and incorrect conclusions will result. The true level of a control should be monitored rather than taken for granted.

How Do the Different Cycling Parameters Affect a PCR Reaction?

The objective of the information in Table 11.8 is to provide guidelines to help you fine-tune a reaction based on your experimental observations. The data refer to Taq polymerase, but the trends hold true for most thermostable DNA polymerases.

Instrumentation: By What Criteria Could You Evaluate a Thermocycler?

Since the discovery of thermostable Taq DNA polymerase, numerous instrument companies have developed PCR cyclers, not

Table 11.8 Effect of Cycling Parameters on PCR

Stages of PCR	Standard Segment Time and Temperature	Below Optimum Duration	Above Optimum Duration
Initial denaturation	1–3 min 94°C (95°C for higher (55–60%) GC content)	Lower yield or no products Some genomic DNA needs more time, while PCR products or plasmid DNA need less time	Lower yield from premature loss of enzyme activity
Denaturation during cycling	5–20 s	Lower yield	Lower yield
Primer annealing	5–20 s 45–60°C Higher temperatures for more specific annealing	Lower yield	Nonspecific product formation
Primer extension	10–20 s 70–75°C	Lower yield	Lower yield Increased error rate
Cycle number	25–40	Lower yield	Nonspecific product formation
Final extension	1–2 min 70–75°C	Incomplete double-stranded DNA	Nonspecific product formation

only for amplification but for detection and analysis as well. A review of your current and anticipated needs will help you select the most appropriate machine within your budget.

Temperature Regulation

Consistent, predictable ramp times (the time required to transition from one temperature to the next) are crucial to achieve the desired PCR results. The time required to reach the 55°C annealing temperature from the 94°C denaturation temperature can vary one minute or more, depending on the cycler design. The consistency of the heating or cooling profile of samples can also vary with the instrument and introduce errors. If your goal is to run both tubes and plates, make sure that the tube fits the well snuggly, as ill-fit tubes do not transfer heat well.

Programming Capability

If you run different cycling parameters, the capacity to link preexisting programs rather than repeatedly installing old programs will save significant time. The ability to store many programs is also useful if you run many programs routinely or share a cycler with multiple users.

Minimum Manipulations

If your objective requires high-throughput analysis, it is recommended to use a cycler that combines amplification and analysis without further manipulation, such as gel electrophoresis or blotting. These postamplification processes require pipetting, opening and closing of reaction tubes, and so forth, which greatly increase the chance of contamination of other samples throughout the lab as the product contains enormous copies of the target sequence.

Reaction Vessels

Will your planned and unforeseen research require reactions in 0.2 ml, 0.5 ml tubes, or multiwell dishes? The ability to accommodate multiple sample formats usually pays off in the long run.

How Can Sample Preparation Affect Your Results?

Sample preparation can make the difference between good yield and no amplification. The purpose of sample preparation is to eliminate PCR inhibitors as well as to provide the DNA sequence available for PCR reaction. Compounds that inhibit PCR may co-purify with the DNA template and make PCR impossible (Reiss et al., 1995; Yedidag et al., 1996). Inhibitors do not have to be diffusible. Sometimes crosslinking of protein to DNA via carbohydrate groups can cause inhibition (Poinar et al., 1998). Addition of adjuncts such as bovine serum albumin (BSA) or T4 gene 32 protein can sometimes reverse the inhibition (Kreader, 1996). However, it is easiest to remove these inhibitors during the sample preparation than to figure how to reduce the degree of inhibition later. The qualities of good sample preparation follow:

- *Intact*: Undegraded and unnicked. DNA might appear intact immediately after isolation, but repeated use can result in nuclease-mediated degradation. This may result from incomplete removal of nucleases during the initial sample preparation or contamination of the sample during repeated usage; RNA requires a storage pH below 8.0 and special care to avoid RNase contamination.
- *Fixed*: DNA isolated from paraffin-embedded tissue sections and archived fixed tissues may pose problems due to nicking of DNA during tissue preparation. (Note: Human genome haploid equivalent is approximately 3 billion base pairs. Given that the distance between base pair is about 3.4A°, each human cell contains about 2 meters of DNA! A typical DNA isolation method shears genomic DNA in the process.)

- *Inhibitor-free*: Heparin, porpholin, SDS (<0.01%), sarkosyl, heme (Alkane et al., 1994), EDTA, sodium citrate, humic acid (Zhou et al., 1996), phenol, chloroform, xylene cyanol (Alkami PCR manual), and some heavy metals can inhibit PCR.
- *Clean*: $A_{260:280}$ ratio of 1.8 to 2.0; Free of protein and carbohydrate. (See Chapter 4, "How To Properly Use and Maintain Laboratory Equipment," for situations where $A_{260:280}$ ratios prove unreliable.)
- *RNA*: Free of DNA.

How Can You Distinguish between an Inhibitor Carried over with the Template and Modification of the DNA Template?

If it is diffusable inhibition of a thermostable DNA polymerase, adding the sample in smaller quantity lessens the effect whereas the effect worsens with more sample. If the problem is caused by template modification, dilution will have no effect. Compounds such as *N*-phenacylthiazolium bromide (PTB) may eliminate inhibition (Poinar et al., 1998) caused by agents crosslinking to the template. PCR inhibitors can be detected by performing reactions in the presence of commercially available exogenous internal positive controls, which can be added to your PCR reaction without hampering the amplification of your target.

What Are the Steps to Good Primer Design?

Step 1. Consider the Objectives

What must the PCR accomplish? What pressures does this put on the primers?

- Must you identify few or many targets? The identification of several targets requires numerous primers, increasing the difficulty of avoiding 3′ overlaps.
- Must you clone the full-length coding region of a gene? For long PCR, you may use the nearest-neighbor algorithm for selection of T_m (Rychlik et al., 1990).
- Must you generate quantitative data? PCR efficiency becomes more critical, as does avoiding primer-dimers.
- Must you design primers without knowing the exact sequence of the specific species based on information from another species (i.e., design primers for the rat gene X using mouse or human gene sequence for gene X)? If so, aligning as many sequences of gene X from as many organisms as you can collect in order to select the most conserved region for primer design increases the likelihood of success.

- Must you avoid amplifying pseudogenes? What is known about pseudogenes to your target? A preliminary review of the research literature can save you time and headaches. Unfortunately, there are more pseudogenes than are reported. One quick way to search for pseudogene amplification with your selected primer pairs is to do a BLAST search (see Appendix C). However, the only sure way to avoid pseudogenes is to design primers across exon–exon junctions and test for them at the bench by amplifying genomic DNA. Processed pseudogenes do not have introns, so they can be amplified when the PCR primer extend over the two exon junctions.
- Are you searching for a single nucleotide polymorphism (SNP)? SNP primer design requires specialized strategies (Kwok et al., 1995; Wu et al., 1991).
- Must you design a small amplicon to increase detection of the gene in samples where the chance of amplifying a long sequence is unlikely (i.e., paraffin embedded sections, forensic samples, and partially degraded samples)?

Step 2. Apply the Sequence Analysis Programs to Develop Candidate Primers

These programs are described in Appendix B.

Step 3. Apply Good Primer Design

Refer to the generally accepted elements of good primer design (Dieffenbach and Dveksler, 1995). The new nearest-neighbor model based on DNA thermodynamics data for PCR primer design is also recommended (SantaLucia, 1998).

- The optimum length of primers for use with Taq DNA polymerase is between 18 and 28 bases for specificity (This number may vary with enzymes with greater heat stability.) The longer primer gives more specificity but tends to anneal with lower efficiency and results in a significant decrease in yield. A good pair of primers has melting temperature (T_m) 55°C to 60°C. Shorter primers (less than 15 nucleotide long) anneal very efficiently, but they may not give sufficient specificity. Longer primers may be useful when distinguishing multiple gene forms sharing a high degree of sequence homology. The probability of finding a match using a set of 20 nucleotide long primers is $(\frac{1}{4})^{(20+20)} = 9 \times 10^{-26}$ (Cha and Thilly, 1995). It is likely that this set of primers will amplify another gene in the mammalian genome (3×10^9 bp per haploid genome).

- GC content should be between 40% and 60%. The T_m of both primers should be similar to each other and similar to the primer-binding sites at the ends of the fragment to be amplified to achieve an optimal annealing temperature and amplification.
- 3′-end complementarity between primers and self-complementarity within primers must be avoided because it may increase primer-dimer formation and reduce PCR efficiency. This is more problematic when you have a low number of target gene copies.
- Avoid runs of G/C, especially guanidine.
- When performing RT-PCR, design primers to go across exon–exon junctions to avoid amplifying genomic DNA. Since the use of DNase has a negative effect on RNA, it is better to avoid genomic DNA amplification by primer design (Huang, Fasco, and Kaminsky, 1996).
- Include controls lacking RT unless you have shown that this set of primers does not amplify genomic DNA.
- After designing the primers, search for specificity using BLAST (Basic Local Alignment Search Tool), a set of similarity search programs designed to explore all of the available sequence databases regardless of whether the query is protein or DNA (Appendix C). This is especially important for those genes with many pseudogenes and related genes. If you are executing RT-PCR, this is essential. Even a trace amount of genomic DNA left in the RNA sample preparation can give sufficient amplification of those genes. Often these PCR products are indistinguishable by gel electrophoresis and make data interpretation difficult.
- You may add exogenous sequence to the 5′ end of primers for cloning and other purposes.
- When sequence information is ambiguous, substitute deoxyinosine for the unknown nucleotide, and place the ambiguous sequence on the 5′ end. Design and test different primers to determine which works best. Inosine is naturally found in some tRNA. It base pairs with A, C, and U in the translation process (Martin et al., 1985; Kwok et al., 1995).
- Before testing the primers with your test sample, measure the quantity of your primers, and then test with the positive controls. You cannot assume that all primers have the correct sequence. Inefficient desalting, incorrect labeling, and other quality control problems can ruin a primer's performance.

Step 4. Develop and Apply a Primer Testing Strategy

If your goal is to study many genes, then you may want to consider setting a standard thermal cycling condition to run all your PCR reactions, even though they won't produce the optimal PCR results. If your goal is to study a few genes, then the design is more straightforward.

This discussion about primer design is relevant to basic PCR. The bibliography provides references for primer design relevant to multiplex or nested PCR applications.

Which Detection and Analysis Strategy Best Meets Your Needs?

It is crucial that your strategy be consistent with your purpose. If you require quantitative data, a hybridization-based strategy is not ideal. Probe labeling, membrane transfer, and hybridization conditions can introduce variability. If resolution is crucial to your study, PAGE rather than agarose electrophoresis might be required. Because the potential for variability usually increases with the number of manipulations required to generate the data, real-time PCR usually provides greater reproducibility along with time savings.

Table 11.9 compares commonly applied detection methods.

TROUBLESHOOTING

Even the most thorough, insightful planning cannot guarantee success, and PCR can generate results indicative of complete failure or a reaction in need of optimization. The troubleshooting section is organized to reflect the fact that any given PCR problem can have several underlying explanations. The optimization of cycling conditions, primer concentration, and other parameters discussed throughout the chapter can also help resolve a problem.

No Product

Template

Is the target sequence absent?

Amplify housekeeping gene or some gene you know is present as a control; perform standard curve assay with plasmid or amplicon to estimate the dynamic range of detection. This range also indicates the lower limit of detection.

Table 11.9 Comparison of Different Detection Strategies

	Method	Indicates Size	Quantitative	High-throughput	Specificity	Reproducibility
Post PCR	Agarose gel electrophoresis					
	Intact PCR product	Yes	Poor	Poor	Poor	Poor
	Restriction enzyme analysis	Yes	Fair	Poor	Fair	Fair, if the PCR reaction falls in the exponentional phase
	Blots/hybridization	Yes	Poor	Poor	Fair	Poor
	Scanning/densitometer	Yes	Fair	Poor	Fair	Poor; too many variables
	Nested PCR	Yes	Fair	Poor	Good	Fair
	PAGE electrophoresis (sequencing gel)	Yes	NA	Fair	Excellent	Poor
Real time	Automated detection and analysis systems	No	Excellent	Excellent	Excellent	Excellent

Enzyme

Is the enzyme inactive?

Did positive controls work?

Primer

Is the primer poorly designed?

Utilize several different amplicon locations to design the primers to increase your chance of success.

Cycling Parameters

Was there insufficient amplification?

Take a portion of the PCR products and amplify further or repeat with a larger quantity of starting material or test with nested PCR.

Lower or raise annealing temperature (See Tables 11.3 and 11.8).

Buffer

Were one or more buffer components faulty?

Include a positive control such as a commercially tested endogenous control, or a pretested set of reagents

Mg^{2+} concentration is not optimum?

Raise or lower the concentration as per Table 11.5.

Other

Was the detection method sufficiently sensitive?

Prepare a standard curve with a positive control to determine the detection limit.

Smear on the Gel

Template

Was the template copy number too large?
Was the template degraded?

Enzyme

Was too much enzyme and/or too much template included?

Primer

Is the primer design following the design guideline? Is the concentration of primer too low or too high? Do primers lack specificity?

Cycling Parameters

Too many amplification cycles?
Is the annealing temperature too low?

Buffer

Is the Mg^{2+} concentration optimal?

Lower the concentration as per Table 11.5.

Other

Was the appropriate electrophoresis buffer and/or gel concentration used?

Wrong Product

Template

Is the template copy number too large or is the template DNA degraded?

Test a negative control sample to determine if data represent an artifact.

Enzyme

Use a hot-start strategy (Ehrlich, Gelfand, and Sninsky, 1991) to increase specificity.

Primer

Inappropriate primer design?

Apply nested PCR, sequencing, restriction analysis or hybridization to troubleshoot.

Perform a BLAST search to assess possibility of amplifying a different gene (Appendix C).

Cycling Parameters

Too many amplification cycles?
Annealing temperature too low?

Buffer

Is the Mg^{2+} concentration optimal?

Lower the concentration as per Table 11.5.

Other

Was the appropriate electrophoresis buffer and/or gel concentration used?

Faint Band of the Correct Size/Low Yield

Template

Poor quality DNA or RNA?

Check the sample for degradation, inhibitor, or contamination.

Enzyme

Use a hot-start strategy to increase specificity.

Primer

Examine primer design for unmatched T_m of the forward and reverse primers, runs of pyrimidine and purine, or other unfavorable sequence; if a primer-dimer band (lower molecular weight) is visible, a hot-start strategy might increase the yield of the desired product.

Cycling Parameters

Insufficient amplification cycles?

Continue amplification with fresh reagents

Annealing temperature not optimum?

Increase/decrease for more yields.

Buffer

Nonoptimal Mg^{2+} concentration?

Increase concentration as per Table 11.5.

Positive Control Generated Product, but Your Sample Did Not

Template

The sample did not contain the target sequence at detectable level.

Pipetting problem? DNA sample never added to the reaction? It is always a good idea to do two to four reactions to exclude such a possibility.

Enzyme

Low specificity and yield?

Use modified form of Taq DNA polymerase such as TaqGold™ to increase both specificity and yield. This enzyme is inactive until thermal activation to provide a hot-start for increased specificity. At the same time this enzyme is time-released, providing more enzyme in the later cycles when more enzyme increases yield. Decreased mispriming also increases the amount of the desired PCR products (Abramson, 1999).

Primer

Primer design not optimal if primer-dimer is formed?

Redesign.

Cycling Parameters

Insufficient amplification cycles?

Continue amplification with fresh reagents.

If the yield of the positive control is also low, optimize annealing and denaturation temperature and duration of each hold time to increase yield.

Buffer

Mg^{2+} concentration is not optimal?

Increase concentration as per Table 11.5.

Other

Presence of PCR inhibitors?

Test an exogenous IPC (internal positive control) for troubleshooting, or do mixing experiment to test if addition of your sample inhibits the positive control.

Is an inhibitor crosslinked to the DNA template?

Try adding adjunct such as PTB

The troubleshooting discussion above further illustrates how appropriate controls can simplify or eliminate much of the troubleshooting effort. Prevention is the key.

Misincorporations of Nucleotides

Template

Too much single-stranded DNA sample due to insufficient extension time or not having enough quantity of one of the primers?

Enzyme

Too many units of DNA polymerase present?

Primer

Nonoptimal T_m causes pre-PCR annealing to secondary, unintended sites?

Check sequence for hot spots for mispriming.

Cycling parameters

Ramp time too long?
Annealing temperature too low?

Buffer

Mg^{2+} concentration too low?

Check dNTP and template concentration.
Adjust as per Table 11.5.

RT-PCR

Despite the increased interest in RT-PCR, this technique can be more challenging than DNA PCR in many ways. Here are some parameters to keep in mind:

- Isolation and purification of RNA requires greater care.
- Design of primers spanning a large intron may be necessary to avoid amplifying contaminating genomic DNA.
- DNase treatment of RNA preparation may affect different genes differentially for the subsequent PCR (Huang, Fasco, and Kaminsky, 1996); thus use it only as the last resort. Residual DNase I can reduce the yield of PCR products.
- The most frequently used reverse transcriptases are MuLV, *rTth* DNA polymerase, and SuperScript™ (Life Technologies). For RNA with excessive secondary structure or high GC content, apply *rTth* DNA polymerase. Its greater heat stability allows for higher reaction temperatures using a gene-specific reverse primer,

which increases specificity of the RT-PCR reaction. However, these conditions may increase hydrolysis of RNA.

- The choice of primers for the cDNA synthesis includes random primers (nonamers and hexamers), oligo dT and gene-specific primers. For cloning full-length gene, use oligo dT. Use random hexamers for multiplex or when the test sample may not be of good quality (i.e., clinical samples), where full-length mRNA is difficult to obtain (i.e., paraffin-embedded tissue), and where the position of the amplicon is distant from the poly (A) tail. The latter case is especially important when RNA secondary structure prevents full-length synthesis of the first-strand cDNA via the relatively low temperature (37–42°C) RT reaction. Therefore your choice of primers for RT depends on the relative distance between the priming site, the amplicon location and the gene structure. You may want to avoid oligo dT if the following conditions apply to your gene:

  Presence of long 3′-untranslated region (UTR) (>1 Kb) or the length of it is unknown.

  The amplicon site is at the 5′ end of a long transcript.

  The amplicon site is at the 5′ end of a GC-rich gene.

SUMMARY

This chapter has discussed basic PCR technology issues. The complexity of more advanced techniques such as allele-specific amplification, long PCR, RACE, DICE, competitive RT-PCR, touchdown, multiplex PCR, nested PCR, QPCR, and in situ PCR could not be covered in this review.

The intellectual and biochemical strategies discussed within this chapter were not designed to answer every question related to PCR, but to provide a foundation to help you, better ask and answer questions that you will encounter. Combined with the resources provided within this chapter, the author hopes this chapter provides you with new insight to evaluate and meet your PCR needs.

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APPENDIX A

PREPARATION OF PLASMID DNA FOR USE AS PCR CONTROLS IN MULTIPLE EXPERIMENTS

Have you ever failed to amplify a section of a plasmid that previously produced the desired PCR product? Your problem is not unique. Often plasmid DNA at low concentration of DNA is degraded by nuclease or adsorbs to the wall of the plastic tube during storage and handling. This protocol produces plasmid DNA that is stable for months and years if stored at –20°C and generates reproducible standard curves.

The addition of glycogen (20 μg/ml final concentration) in 10 mM Tris, 1 mM EDT (pH8.0) buffer can protect DNA from degradation by nuclease as well as loss from adsorption to the tube. After making serial ten-fold dilutions (100 μl of DNA in 900 μl of TE), aliquot the solution in 100 μl or less volume and store at –20°C. For preparation of TE buffer, use fresh nuclease-free water. "Sterile" water sitting on the lab bench for one week or more may contain contaminants as well as nucleases.

APPENDIX B

COMPUTER SOFTWARE FOR SELECTING PRIMERS*

Primer v. 1.4 (DOS)
PINCERS (Macintosh)
Oligonucleotide Selection Program (Macintosh, DOS, Digital VAX/VMS, SUN SPARC-based workstations)
Right Primer: Primer Design Utility
Gene Runner 3.0
Oligo 5.0 (DOS)
Oligo 4.0
DNASIS 2.0 (Windows)
MacDNASIS (Macintosh)
GeneWorks (Macintosh)
Lasergene (DOS, Windows, Macintosh)
Eugene™ (DOS)
GeneJockey (Macintosh)
Wisconsin Sequence Analysis Package (Digital VAX/VMS, IBM RS6000, Sun SPARC-based workstations, Silicon Graphics Workstation)
MacVector (Macintosh)
PRIMER PRIMER (Macintosh, DOS, PowerMac)
DesignerPCR (Macintosh)
Vector NT1 (Windows, Macintosh)
Primer Designer (Macintosh, DOS)
Primer Express™
HYTHER (PC-Windows, UNIX and Web-based platforms) available for license at *http://jsll.chem.wayne.edu/Hyther/hythermenu.html*.

**Data from Dieffenbach and Dveksler (1995).*

APPENDIX C

BLAST SEARCHES

There are many genes that share local sequence homology with a primer 18 to 30 nucleotide long. For example, the beta-actin primer shares 100% homology with pseudogenes, gamma-actin, and related genes. It is therefore misleading to use this primer to estimate the level of beta-actin gene expression. Some of the PCR products will be derived from these genes, but you have no way to tell how much came from the true beta-actin gene. Pseudogenes are not translated into protein and have no biological significance, so your RT-PCR result may not relate to immunological data or biochemical assays. In some cases it will amplify other genes not even related to the one you are investigating. For this reason it helps to know the BLAST search information before ordering primers.

The BLAST programs (*http://www.ncbi.nlm.nih.gov/BLAST*) have been designed for speed, with a minimal sacrifice of sensitivity to distant sequence relationships. The scores assigned in a BLAST search have a well-defined statistical interpretation, making real matches easier to distinguish from random background hits. BLAST uses a heuristic algorithm that seeks local as opposed to global alignments, and it is therefore able to detect relationships among sequences that share only isolated regions of similarity (Altschul et al., 1997). For a better understanding of BLAST, refer to the BLAST instructional course, which explains the basics of the BLAST algorithm.

APPENDIX D

USEFUL WEB SITES

Topics	Content and URL
Basic PCR information	Weizmann Institute of Science Genome and Bioinformatics site *http://bioinformatics.weizmann.ac.il/mb/bioguide/pcr/contents.html*
Collection of PCR protocols	Standard PCR protocols *http://www.protocol-online.net/molbio/PCR/standard_pcr.htm*
Optimization of PCR	Primer design and reaction optimisation. E. Rybicki, Department of Microbiology, University of Cape Town. In *Molecular Biology Techniques Manual: Third Molecular Biology Techniques Manual*, V. E. Coyne et al., eds. *http://www.uct.ac.za/microbiology/pcroptim.htm*
Standard PCR guideline	PCR Primer: Strategies to improve results provided by G. Afseth of Perkin Elmer at Northwestern University (1997) *http://www.biotechlab.nwu.edu/pe/index.html*
Molecular biology methods	Current Protocols in Molecular Biology *http://www.wiley.com/cp/cpmb/mb0317.htm*
	Elsevier Trends Journals Technical Tips online *http://research.bmn.com/tto*
	Molecular biology reagents and procedures. Dartmouth University *http://www.dartmouth.edu/artsci/bio/ambros/protocols*

APPENDIX D (*Continued*)

PCR protocols and online manual	Alkami Quick Guide™ for PCR. A laboratory reference for the polymerase chain reaction. 1999 *http://www.alkami.com/qguide/idxguide.htm*
PCR protocol	Roche Molecular Biochemicals PCR protocol *http://206.53.227.20/prod_inf/manuals/pcr_man/index.htm*
Links to many sources of basic PCR and other molecular biology information	ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (SIB) Molecular Biology Server *http://www.expasy.ch/*
Multiplex PCR	Multiplex PCR: Critical parameters and step-by step protocol. O. Hehegariu et al., *Biotech.* 23(1997):504–511 *http://info.med.yale.edu/genetics/ward/tavi/bt/BT(23)504.pdf*
Various PCR topics, including multiplex PCR	Tavi's PCR site (Octavian Henegariu) on variety of topics *http://info.med.yale.edu/genetics/ward/tavi/PCR.html*
Primers	Primers! Web site *http://www.alkami.com/cntprmr.cgi?url=http://www.wil liamstone.com/primers/javascript/* Hyther *http://jsll.chem.wayne.edu/Hyther/hythermenu.html*
PCR chat room	Protocol online (discussion) *http://www.protocol-online.net/discussion/index.htm*
Real-time PCR	References for TaqMan real-time assay *http://www.appliedbiosystems.com/ab/about/pcr/sds/taqrefs.html*
Gene quantitation	References on absolute and relative gene quantitation by PE Biosystmes *http://www.appliedbiosystems.com/ab/about/pcr/sds/taqrefs.html#rev*
Gene search and validation BLAST	BLAST (National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) family of programs *http://www.ncbi.nlm.nih.gov/blast/blast.cgi?Jform=0*

Caution: The dynamic nature of the Web allows us to provide more up-to-date information. However, there are major challenges associated with information available on the Web. Some of the major challenges are as follows: (1) Since it is easier for anyone to publish on the Web, its content may not be evaluated nor accurate. (2) The URL address as well as its content may change or even disappear without notice, thus quickly invalidating any list of "useful" sites. All of the Web sites given in this section were selected to give the reader sources of information only and by no means recommended as "valid" source. It is up to the users to determine what is useful. The author highly recommends that readers use their own judgment before adapting any information given.

Molecular Biology Problem Solver: A Laboratory Guide. Edited by Alan S. Gerstein

ISBNs: 0-471-37972-7 (Paper); 0-471-22390-5 (Electronic)

12

Electrophoresis

Martha L. Booz

I am grateful to Bruce Goodrich for the figure on degassing acrylamide, to Fiona Leung for the data regarding the molecular weight vs. relative mobility curve, and to Lee Olech and Dave Garfin for fruitful discussions about many of the questions in this chapter.

Dangerously high voltage and acrylamide, a neurotoxin and suspected carcinogen, are inescapable elements of electrophoresis. Proper personal protection and good laboratory practice will minimize the risk of harming yourself or your colleagues.

CHEMICAL SAFETY

What Is the Safest Approach to Working with Acrylamide?

Unpolymerized, monomeric acrylamide is a neurotoxin in any form. *Bis*-acrylamide is equally dangerous. Protect yourself by wearing gloves, a lab coat, and safety glasses, and never pipet acrylamide solutions by mouth.

Acrylamide powders should be weighed and solutions prepared in a ventilated hood. Acrylamide can be detected in the air above a beaker of acrylamide solution and throughout the laboratory. Values in the single-digit ppm range are detected above a 10% solution at room temperature (Figure 12.1). The detection method involves passing air samples through an acrylamide-binding column, and analyzing the eluant via HPLC (Dow Chemical Company, 1988). The MSDS for acrylamide gives the OSHA permissible exposure limit for acrylamide as 0.3 mg/m^3 for personal exposure in an industrial setting.

The use of pre-cast gels and pre-mixed acrylamide solutions can reduce exposure to acrylamide and *bis*-acrylamide. Even after polymerization, a small fraction of the acrylamide remains in the neurotoxic monomeric form. Wear gloves when handling a polymerized gel.

If you need to cast your own gels, we suggest you use pre-mixed acrylamide solutions, which are also available from many vendors. The pre-mixed solutions avoid the weighing and mixing steps, and generally have a long storage life.

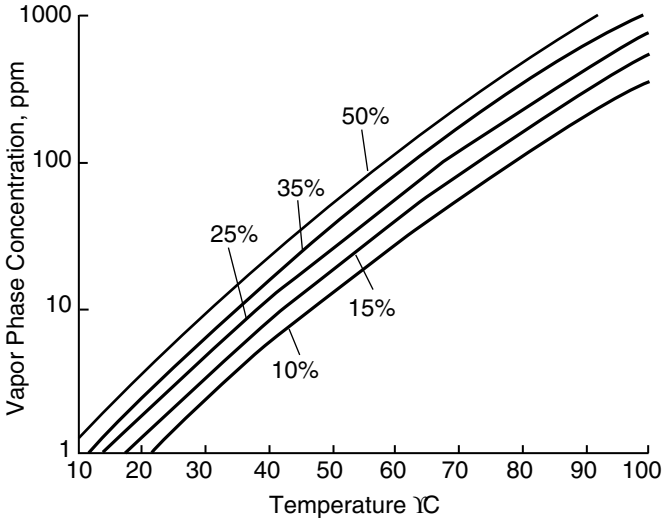

Figure 12.1 Vapor phase concentrations of acrylamide-water solutions (10–50% acrylamide). Cytec Industries Inc., 1995. Reprinted by permission of Cytec Inc.

What Are the Symptoms of Acrylamide Poisoning?

The initial symptoms of acrylamide poisoning on the skin are peeling of the skin at the point of contact, followed by tingling and numbness in the exposed area. If exposure by any means (touch, ingestion, inhalation) continues, muscular weakness, difficulty maintaining balance, and clumsiness in walking and in the use of the hands may develop. A large, acute exposure can produce confusion, disorientation, slurred speech and ataxia (severe loss of balance). Muscular weakness and numbness in the extremities may also follow. Anyone exposed to any form of acrylamide should be immediately examined by a medical doctor (Bio-Rad Laboratories, MSDS, 2000).

What Is the Medical Response to Accidental Acrylamide Exposure?

On your skin: Wash the affected skin several times with soap for at least 15 minutes under running water.

In your mouth: Rinse your mouth immediately with water and seek medical attention immediately.

Swallowed or inhaled: If swallowed, do not induce vomiting. Seek medical attention immediately. If breathed in, get to fresh air, and seek medical attention immediately (Bio-Rad Laboratories, MSDS, 2000).

How Can You Dispose of Excess, Unusable Acrylamide?

Check with your institutional or local county environmental regulators for the disposal requirements in your area. The safest

way to dispose of a small amount of liquid acrylamide is to polymerize it in the hood in a closed plastic bag set into a beaker surrounded by a very large, tightly fastened plastic bag, to prevent spattering as the acrylamide polymerizes.

If you have more than 100 ml to dispose of, contact your local environmental safety officers to determine your recommended procedure. Acrylamide solutions emit significant heat during polymerization, and polymerization of large volumes of acrylamide can be explosive due to rapid heat buildup (Dow Chemical Company, 1988; Cytec Industries, 1995; Bio-Rad Laboratories, 2000).

Acrylamide and *bis*-acrylamide powders must be disposed of as solid hazardous waste. Consult your local environmental safety office.

What Is the Shelf Life of Acrylamide and Acrylamide Solutions?

Commercially prepared acrylamide solutions are stable for as long as one year, unopened, and for six months after opening. The high purity of the solution components and careful monitoring throughout the manufacturing process provides extended shelf life. The lifetime of homemade solutions similarly depends on the purity of the acrylamide and *bis*-acrylamide, the cleanliness of the laboratory dishes, and the purity of the water used to make the solutions.

Solid acrylamide breaks down with time due to oxidation and UV light, producing acrylic acid and ammonia. Acrylic acid in a gel can cause fuzzy bands, or fuzzy spots in the case of 2-D gels, streaking and smearing, and poor resolution (Allen and Budowle, 1994). Acrylamide decomposition occurs more quickly in solution, and it can be accelerated by any impurities within the water (Allen and Budowle, 1994). Thus acrylamide powder should be stored airtight at room temperature, and acrylamide solutions should be stored at 4°C, both in the dark.

Production facilities must establish standards and measures to determine the effective lifetime of unpolymerized acrylamide solutions.

ELECTRICAL SAFETY

What Are the Requirements for a Safe Work Area?

The voltages used in electrophoresis can be dangerous, and fires have occurred due to problems with electrophoresis cells. The

following precautions should be observed to prevent accidents and fires.

- There should be no puddles of liquid on the horizontal surfaces of the electrophoresis cell.
- The area around the power supply and cell should be dry.
- The area for at least 6 inches around the power supply and cell should be bare of clutter and other equipment. Clear space means any fire or accident can be more easily controlled.

What Are the Requirements for Safe Equipment in Good Working Order?

The wires connecting the cell to the power supply must be in good condition, not worn or cracked, and the banana plugs and jacks must be in good condition, not corroded or worn. Broken or worn wires can cause rapid changes in resistance, slow electrophoresis or a halt in the run. All cables and connectors must be inspected regularly for breaks and wear.

The banana plugs on the ends of the wires should be removed from the power supply at the end of the run by pulling them straight out. Grasp the plug, not the wire. If pulled at an angle, the solder joint attaching the banana plugs to the wires can loosen and cause the loss of the electrical circuit. On the cell core, electrode banana posts with flattened baskets do not make good contact with the banana jack in the cell lid, and should be replaced. The banana jacks (female part) in the cell lid should be inspected regularly to make sure there is no corrosion.

Before starting an electrophoresis run, dry any liquid on the horizontal surfaces of the cell, especially near the banana plugs and jacks. Any liquid on the horizontal surfaces of the cell can arc during the run, damaging the cell and stopping the electrophoresis.

POLYACRYLAMIDE (PAGE) GELS—BEFORE SELECTING A GEL: GETTING THE BEST RESULTS FOR YOUR PURPOSE

Before choosing which gel to use, it is important to consider several questions, all of which can help you choose the gel that will give you the best results for your purpose. The next paragraphs provide information on how to select a gel percentage or pore size, when to use SDS-PAGE and when native PAGE, what buffer system to use, which crosslinker to use, and degree of resolution needed.

What Is the Mechanism of Acrylamide Polymerization?

Most protocols use acrylamide and the crosslinker *bis*-acrylamide (bis) for the gel matrix. TEMED (N,N,N′,N′-tetramethylethylenediamine) and ammonium persulfate are used to catalyze the polymerization of the acrylamide and bis. TEMED, a base, interacts with ammonium persulfate at neutral to basic pH to produce free radicals. The free radical form of ammonium persulfate initiates the polymerization reaction via the addition of a vinyl group (Figure 12.2). At an acidic pH, other catalysts must be used, as described in Andrews (1986), Hames and Rickwood (1981), and Caglio and Righetti (1993).

What Other Crosslinkers Are Available, and When Should They Be Used?

Bis-acrylamide is the only crosslinker in common use today. There are others available, for specialty applications. DHEBA (N,N′-dihydroxyethylene-bis-acrylamide) and DATD (N,N′-diallyltartardiamide) were both used historically with tube gels and radioactive samples (before slab gels came into common use). The tube gels were cut into thin discs, the disks were dissolved with periodic acid, and the radioactivity in the disks was counted in a scintillation counter. Of course the periodic acid destroyed some amino acids, so these crosslinkers are not useful for Edman sequencing or mass spectrometry.

Another crosslinker, BAC (*bis*-acrylylcystamine) can be dissolved by beta-mercaptoethanol. It is useful for nucleic acid electrophoresis (Hansen, 1981). However, proteins containing disulfide bonds do not separate on a BAC gel. The subunits with the sulfhydryl moiety bind to the gel matrix close to the origin of the gel, and separation does not occur, so BAC is not recommended for preparative protein electrophoresis, though it is useful for proteins which do not contain any sulfhydryl bonds.

One other crosslinker, piperazine diacrylamide (PDA), can replace *bis*-acrylamide in isoelectric focusing (classical tube gel or flatbed gel) experiments. PDA imparts greater mechanical strength to a polyacrylamide gel, and this is desired at the low acrylamide concentrations used in isoelectric focusing (IEF gels). Some proteomics researchers use PDA to crosslink the 2nd dimension SDS-PAGE slab gels as well, because of the increased mechanical strength, and because the background of a silver stained gel is much better when PDA is used (Hochstrasser, 1988). For further information on these crosslinkers, see Allen and Budowle, 1994.

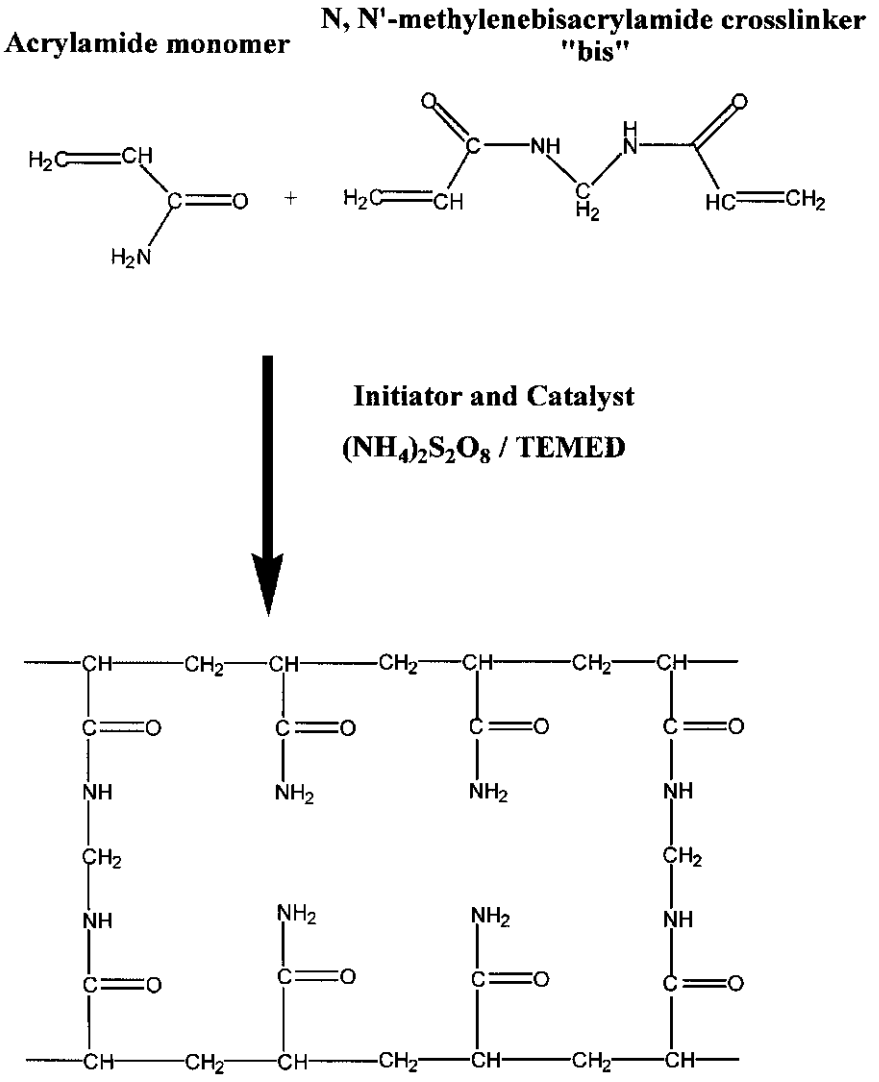

Figure 12.2 Polymerization of acrylamide. Reproduced with permission from Bio-Rad Laboratories.

How Do You Control Pore Size?

Pore size is most efficiently and predictably regulated by manipulating the concentration of acrylamide in the gel. Pore size will change with the amount of crosslinker, but the effect is minimal and less predictable (Figure 12.3). Note the greater impact of acrylamide concentration on pore size, especially at the levels of crosslinker usually present in gels (2.7–5%).

Practical experience with various ratios of acrylamide : bis have shown that it is best to change pore size by changing the acry-

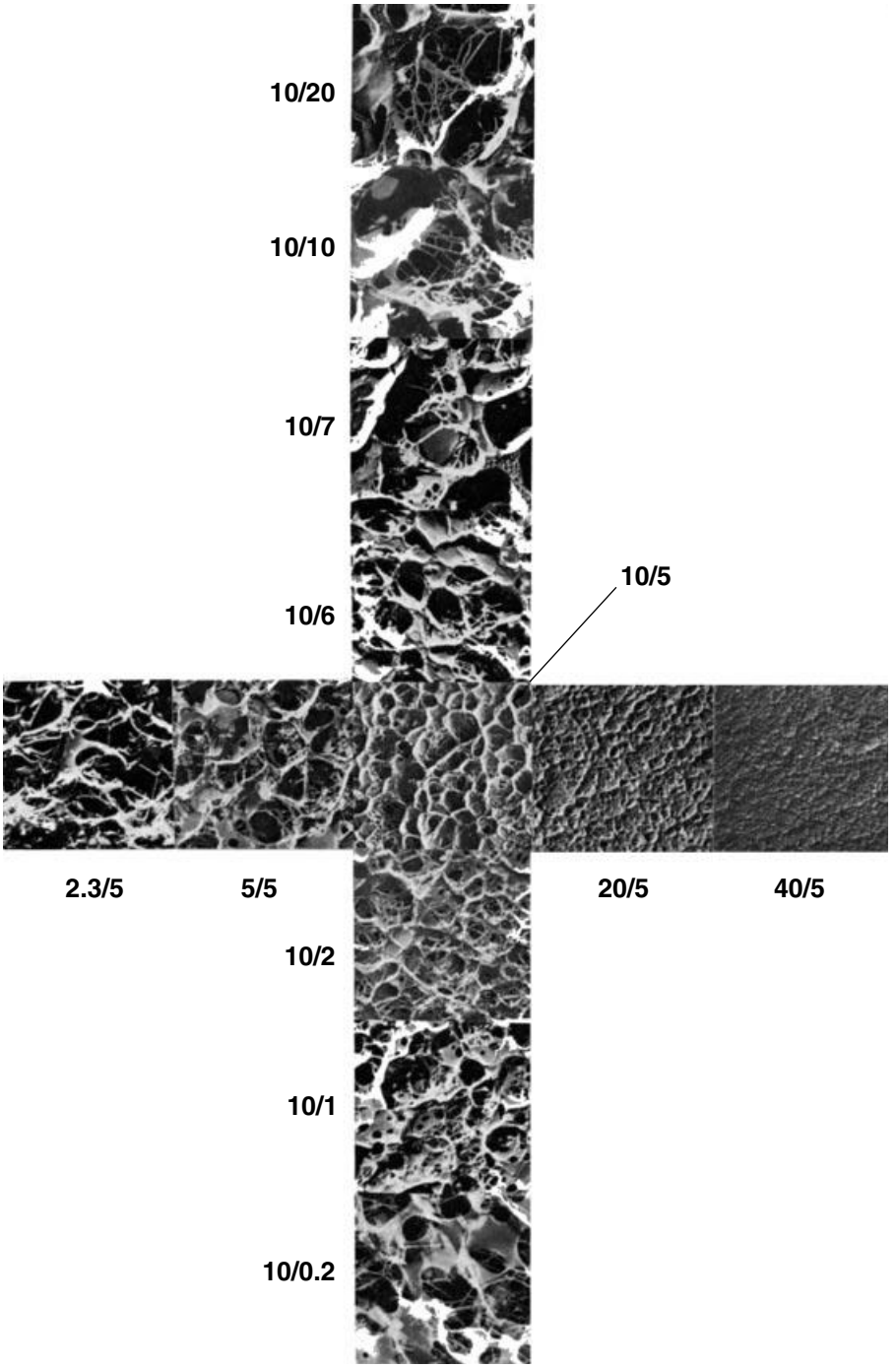

Figure 12.3 Electron micrograph of polyacrylamide gels of various %*T*, showing the change in pore size with the change in %*T* and %*C*. *From* Rüechel, Steere, and Erbe (1978, Fig. 3, p. 569). Reprinted from Journal of Chromatography, volume 166, Ruechel, R., Steere, R., and Erbe, E. Transmission-electron Microscopic Observations of Freeze-etched Polyacrylamide gels. pp. 563–575. 1978. With permission from Elsevier Science.

lamide concentration. A 19:1 ratio of acrylamide to bis (5% *C*; see below for calculation of *C*) is used in low concentration gels, such as IEF gels, and sequencing gels, to impart greater mechanical strength to the gel. A 29:1 ratio (3.4% *C*) is used for concentrations of acrylamide from 8% to 12%, and a 37.5:1 ratio (2.67% *C*) is used for concentrations of acrylamide above 12% to provide flexibility to the gel. SDS-PAGE and native gels are usually run at 10% to 12%. For comparison, a 12% acrylamide gel with a 5% crosslinker concentration will be brittle and will tear easily.

How Do You Calculate %*T* and %*C*?

Percent *T* is $\%T$ = (g acrylamide + g *bis*-acrylamide)/100 ml water × 100.

Percent *C* is $\%C$ = (g *bis*-acrylamide)/(g acrylamide + g *bis*-acrylamide) × 100.

Note that $\%C$ is not the grams *bis*-acrylamide/100 ml water, but rather the percentage of crosslinker as a function of the total weight of acrylamide and *bis*-acrylamide used.

Why Should You Overlay the Gel? What Should You Use for an Overlay?

An overlay is essential for adequate resolution. If you do not overlay, the bands will have the shape of a meniscus. Two closely spaced bands will overlap; the middle of the top band will extend down between the front and back of the bottom band. Overlaying the gel during polymerization will prevent this problem.

Common overlays are best quality water, the buffer used in the gel at a 1× dilution, and water-saturated t-butanol. The choice is a matter of personal preference. Many researchers prefer the alcohol overlay because it will not mix with the gel solution. However, alcohol will turn acrylic plastic (Perspex) from clear to white, and it is difficult to pipet without spills.

Regarding Reproducible Polymerization, What Practices Will Ensure That Your Bands Run the Same Way Every Time?

Reproducible polymerization is one of the most important ways to ensure that your samples migrate as sharp, thin bands to the same location in the gel every time. Attention to polymerization will also help keep the background of your stained gels low. Acrylamide polymerization is affected by the amount of oxygen gas dissolved in the solution, the concentrations and condition of the

catalysts, the temperatures and pH of the stock solutions, and the purity of the gel components. The following paragraphs discuss how to ensure reproducible polymerization and therefore reproducible, excellent gels.

Eliminate Dissolved Oxygen

Oxygen quenches the free radicals generated by TEMED and APS, thus inhibiting the polymerization reaction. Dissolved oxygen must be eliminated via degassing with a bench vacuum or better (20–23 inches of mercury or better) for at least 15 to 30 minutes with stirring (see Appendix A). To achieve reproducible polymerization and consistent pore size, allow the gel solutions, which should be stored in the cold to inhibit breakdown, to come to room temperature before casting a gel. Note that cold gel solutions contain more dissolved oxygen, and low temperature directly inhibits the polymerization reaction. If the temperature during polymerization is not controlled, the pore size will vary from day to day.

Symptoms of Problems with Catalyst Potency

The best indicator of a problem catalyst is poor polymerization of the gel. If you're confident that you have good quality chemicals and water, and have degassed your solutions to remove oxygen, and still the sides of the wells do not polymerize around the teeth of the comb, a degraded catalyst is the likely explanation.

Separation of the gel from the spacers also indicates poor polymerization; the dye front will migrate in the shape of a frown. A third symptom of poor polymerization is *schlieren* in the body of the gel. *Schlieren* are swirls, changes in the refractive index of the gel, where polymerization has been very slow or has not occurred. The gel has no structure at the location of the *schlieren*. It breaks apart in pieces at the *schlieren* lines, when removed from the cassette. *Schlieren* can also be caused by inadequate mixing of the gel solution before pouring it into the gel cassette.

It is difficult to predict the potency of TEMED unless you know its history of use. TEMED is very hygroscopic and will degrade within six months of purchase if it becomes contaminated with water. Therefore store TEMED in a desiccator at room temperature if you use it frequently, or at 4°C if you use it less than once a week. Cold TEMED must be warmed to room temperature before the bottle is opened to prevent condensation from contaminating the TEMED liquid.

Determine the potency of APS by watching it dissolve, or by listening to it. Weigh out 0.1 g of APS in a small weigh boat, and then place the weigh boat with the APS onto a dark surface. Add 1 ml of highest purity water directly to the weigh boat, to make a 10% solution. If the APS is potent, you will see tiny bubbles fizzing off the surface of the APS crystals. No fizzing is observed with deteriorated APS. Or put 0.1 g of APS in a 1.5 ml Eppendorf tube, and add 1 ml of water. Cap it and listen for the fizzing. If you do not hear little crackling noises, like fizzing, it has lost its potency and should be replaced.

Stored solutions of TEMED and APS may polymerize gels, but if you want to minimize the chance of failure and maximize reproducibility, especially with protein gels, prepare APS fresh every day, store TEMED dry at room temperature in a desiccator, and degas your solutions before polymerization.

Temperature

The temperature of polymerization should be 20 to 22°C. If your lab is below 20°C, or if the temperature varies more than five degrees from day to day, reproducibility problems may arise. Note that cold delays polymerization, heat speeds it, and the reaction itself is exothermic.

What Catalyst Concentration Should You Use?

The appropriate catalyst concentration depends on what gel % you are polymerizing. Please refer to Table 12.1.

Note that these catalyst concentrations are for protein PAGE gels only. Sequencing gels are polymerized differently. The final concentrations of catalysts for a 6 %*T* sequencing gel, which allow the solution to be introduced into the gel sandwich before polymerization starts, are TEMED, 0.1% (v/v), and APS, 0.025% (w/v).

What Is the Importance of Reagent Purity on Protein Electrophoresis and Staining?

Reagent purity is extremely important for reproducible results. If the reagents and water you use are very pure, then the polymerization and electrophoresis will be controllable and reproducible from day to day. Any problems you have can be ascribed to the sample and its preparation. The following discussion goes into various reagent purity problems and their resolution.

Table 12.1 Gel Percentage vs. Catalyst Concentration

Gel % Concentration	APS Concentration (w/v)	TEMED (v/v)
4–7%	0.05%	0.1%
8–14%	0.05%	0.05%
⩾15%	0.05%	0.025%

Water

The common contaminants of water are metal ions, especially sodium and calcium, the halide ions, especially chloride, and various organic impurities (Chapter 3 discusses water impurities in greater depth.) Each kind of impurity has a different effect; we will not attempt to enumerate all these effects here. Copper ions inhibit acrylamide polymerization, but copper metal and other metals initiate polymerization. Ions can cause ionic interactions between the macromolecules in your sample, perhaps causing aggregation of certain proteins, with band smearing the result. The organic contaminants can also cause loss of resolution. The effects on staining the samples in the gel are also significant, as impurities in the water can bind the stain, causing bad background. A detailed discussions about preventing background in a stained gel is provided below. The principle here is that impurities in the water cause problems, and the purest water available should be used for electrophoresis to help prevent these problems.

Bacteria in your water purifier can also cause artifacts, such as vertical pinpoint streaks in your gel or on blots stained for total protein. Bacteria migrating up the hose from the sink to the filter cartridges is a common cause of contamination. Note that bacteria can grow in dishwater left to sit in the sink, so be careful where you place the end of the hose that carries water from the water purifier.

Another possible source of contamination in your water is the maintenance department in your institution, especially if your water purifier lacks a charcoal filter for removing organic contaminants. The maintenance department may add organic amine compounds to the distilled water system at your institution to keep scale off the walls of the pipes providing distilled water to your lab. This is commonly done every six months or so. Such contaminants will cause background problems in your stained gels, among other artifacts. The water used to prepare solutions for electrophoresis and staining procedures should be charcoal column-purified and deionized.

Reagents

Impure reagents—from gel components to buffer salts, stains, and dyes—can create problems similar to impure water. Gels will not be reproducible, resolution may be poor, and background staining may be substantial. For reproducible results and good resolution, always use the purest components available, electrophoresis grade.

WHICH GEL SHOULD YOU USE? SDS-PAGE, NATIVE PAGE OR ISOELECTRIC FOCUSING?

The strategy you choose depends on your goal, of course. If you want to determine the molecular weight of your protein, use SDS-PAGE. If you want to measure the isoelectric point of your protein, choose isoelectric focusing (IEF). For proteomics work, use 2-D electrophoresis (IEF followed by SDS-PAGE). Native PAGE is used to assay enzyme activity, or other biological activity, for example, during a purification procedure. Each kind of protein PAGE has issues to consider, and these issues are addressed in the next section. Improving gel resolution is addressed in a separate section below.

Will Your SDS Gel Accurately Indicate the Molecular Weight of Your Proteins?

Estimation of the molecular weight of the protein of interest, accurate to within 2000 to 5000 daltons, requires the protein band(s) to run within the middle two-thirds of the gel. This is illustrated in the graph of the log of the molecular weight of a set of standard proteins vs. the relative mobility of each one (Figure 12.4). Note that the proteins with a relative mobility below 0.3 or above 0.7 fall off the linear portion of the curve. Thus the most accurate molecular weight values are obtained when the relative mobility of the protein of interest is between 0.3 and 0.7. This means that if your protein doesn't enter the gel very well, you must change the gel %*T* before you can get a good molecular weight value. The sample may require a different (better) solubilization procedure also. (See comments on sample preparation, below.)

Should You Use a Straight % Gel or a Gradient Gel?

If you want to resolve proteins that are within a few thousand daltons of each other in molecular weight, then use a straight percent gel (the same concentration of acrylamide throughout the gel). To get baseline resolution for such proteins, that is, to get clear, unstained space between bands, you may need to use a

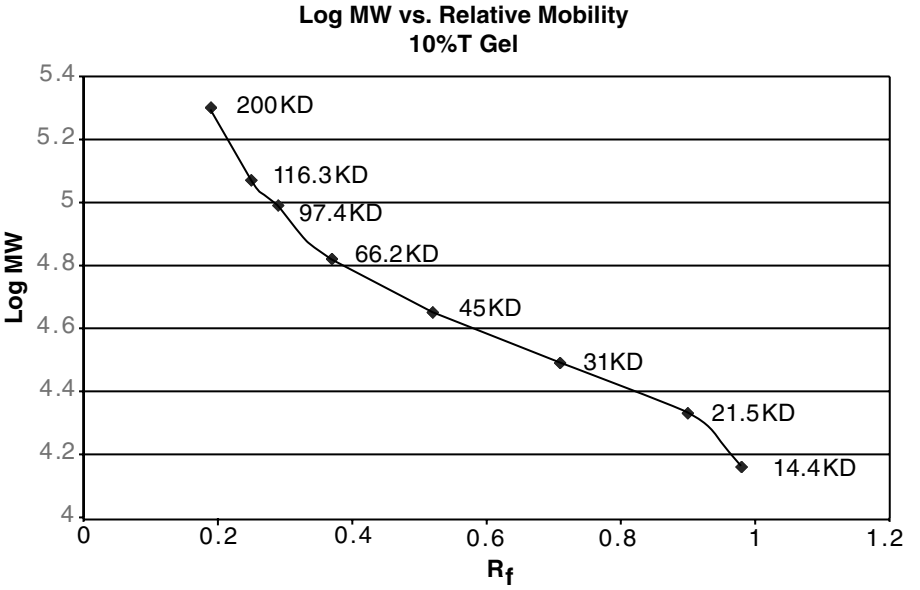

Figure 12.4 Log of the molecular weight (in daltons) of a protein versus the relative mobility. Reproduced with permission from Bio-Rad Laboratories.

longer gel. Mini gels have 6 to 8 cm resolution space. Large gels have 12 to 20 cm space. The closer the bands are in molecular weight, the longer the gel must be.

A gradient gel is used to resolve a larger molecular weight range than a straight percent gel. A 10% gel resolves proteins from 15 to 100 kDa, while a 4% to 20% gradient gel resolves proteins from 6 to 300 kDa, although the restriction about good molecular weight determination discussed above still holds. Accurate molecular weights can be determined with gradient gels (Podulso and Rodbard, 1980).

What Issues Are Relevant for Isoelectric Focusing?

Isoelectric focusing (IEF) measures the isoelectric point, or pI, of a protein. The main problem for IEF is sample solubility, seen as streaking or in-lane background on the stained IEF gel, or horizontal streaking on a 2-D gel. Sample solubilization should be optimized for each new sample; searching the scientific literature to identify protocols used for similar samples is a good starting point. Information on sample preparation is included below in the discussion about improving resolution.

At present there are two kinds of IEF gels in use: gels formed with carrier ampholytes, and gels formed with acrylamido buffers, known as IPG gels (immobilized pH gradient gels).

The two kinds of gels suffer from problems specific to each kind of gel. For gels formed with carrier ampholytes, the main problem is cathodic drift, the movement of the pH gradient off the basic part of the IEF gel with time. With cathodic drift, the pH gradient gradually drifts off the basic side of the gel, forming a plateau in the center of the pH gradient. Cathodic drift occurs after long focusing times. The drift is controlled by determining the optimum time of focusing in volt-hours, and then always, reproducibly, focusing your gels for the determined number of volt-hours. The optimum time of focusing is determined by performing a time course, setting up identical gels, and then taking them down one by one as time passes, and determining from the results when the proteins have reached the optimum resolution. Gels formed with carrier ampholytes are also limited in the amount of protein that can be focused, since with an overloaded gel, the gradient will deform before all the protein has moved to its pI.

Cathodic drift is completely avoided by the use of IPG gels for isoelectric focusing. The pH gradient is cast into the polyacrylamide gel, which is supported by a plastic backing. There is no cathodic drift because the pH gradient is fixed during the gel-casting step, rather than formed during the first part of the electrophoresis, as with carrier ampholyte gels.

There are major additional advantages to IPG gels: they are much more reproducible than carrier ampholyte gels, and they can focus much more protein than carrier ampholyte gels, up to 5 mg or more, because the fixed pH gradient cannot be overbuffered as above, and because electrophoresis can be carried out at much higher voltage potentials (up to 10,000 volts) and for much longer volt-hours (up to 100,000 volt-hours for 17–18 cm IPG gels). Proteins isolated using 2-D electrophoresis can be sequenced or analyzed by mass sprectrometry, and thus identified. The problems with IPG strips are still being identified. One problem for 2-D electrophoresis seems to be the loss of some hydrophobic (membrane) proteins during transfer of the proteins from the IPG strip to the SDS-PAGE gel (Adessi et al., 1997; Molloy, 2000). Very low and very high molecular weight proteins may also be problematic, as well as basic proteins. Procedures to avoid these problems must be worked out for each sample.

How Can You Resolve Proteins between Approximately 300 and 1000 kDa?

We suggest you use a composite gel for very large proteins. Composite gels are made of 1% acrylamide and 1% low melt agarose. The agarose makes the acrylamide strong enough to

handle, and the acrylamide makes the pores in the agarose gel small enough to resolve proteins above about 300 kDa. Composite gels are tricky to pour, as the gel cassette must be warmed to about 40°C, and the gel mixture must be cooled to just above the agarose gelling point before pouring. The mixture must be introduced into the gel casette within a few seconds of adding the catalysts, as acrylamide polymerization takes place within one or two minutes at elevated temperatures. Andrews (1986) has a general procedure for composite gels.

Another option for very large proteins is the use of PAGE with some additive that may enlarge the pore size and thus permit the separation of very large proteins. We have not tested this option, and thus have no recommendations, but Righetti et al. (1992) have used PEG with a standard 5%*T* gel to form much larger pores than normal.

WHAT ISSUES ARE CRITICAL FOR SUCCESSFUL NATIVE PAGE?

Sample Solubility

Native PAGE is performed under conditions that don't denature proteins or reduce their sulfhydryl groups. Solubilizing samples for native PAGE is especially challenging because most nondenaturing detergents do not solubilize complex samples well, and the unsolubilized proteins stick on the gel origin and bleed in, causing in-lane background.

Location of Band of Interest

Sample proteins move in a native gel as a function of their charge as well as their mass and conformation, and because of this, the location of the protein band of interest may be difficult to determine. For instance, in some buffer systems, BSA, at 64 kDa, will move in front of soybean trypsin inhibitor, at 17 kDa (Garfin, 2000). The easiest way to detect the protein of interest is to determine its location by Western blotting. Alternatively, the protein's location can be monitored by enzyme activity or bioassay, which usually requires elution from the gel. Elution is discussed below.

How Can You Be Sure That Your Proteins Have Sufficient Negative Charge to Migrate Well into a Native PAGE Gel?

To determine this, it is useful to have some idea of the pI of the protein of interest. The pH of the buffer should be at least 2 pH units more basic than the pI of the protein of interest. An alter-

native is to use an acidic buffer system, and reverse the polarity of the electrodes. This works well for very basic proteins.

Buffer Systems for Native PAGE

Buffer systems for native PAGE are either continuous or discontinuous. Discontinuous buffer systems focus the protein bands into thin fine lines in the stacking gel, and these systems are preferred because they provide superior resolution and sample volumes can be larger and more dilute. In a discontinuous buffer system, the buffers in the separating gel and stacking gel, and the upper and lower tank buffers, may all be different in concentration, molecular species, and pH. The reader should initially try the standard Laemmli SDS-PAGE buffer system without the SDS and reducing agent. That buffer system is relatively basic, so most proteins will be negatively charged and run toward the anode. If this is not successful for your protein, consult Chrambach and Jovin (1983), who have published a set of discontinuous buffer systems covering the whole range of pH, for additional discontinuous buffer systems.

Continuous buffer systems have the same buffer throughout the gel, sample and running buffer. Continuous buffer systems can be found in McLellan (1982). Continuous buffer systems are easier to use. For protein gels, the choice between continuous and discontinuous buffer systems is usually made on the basis of what works, and the pI of the protein(s) of interest.

Nucleic acid gels, both PAGE and agarose gels, use the same buffer in all parts of the system: in the gel, in the sample and in the running buffer (urea, which is uncharged, may be omitted from the running buffer). The pH, type of buffer, and buffer concentration are the same throughout the system in most methods of nucleic acid electrophoresis. This makes the gels easy to pour and to run.

The disadvantage of a continuous buffer system is that the samples must be low volume, because the bands in such a system will be as tall or thick as the height of the sample in the well, in a vertical and horizontal slab gel. This is true of both protein or nucleic acid samples.

WHAT CAN GO WRONG WITH THE PERFORMANCE OF A DISCONTINUOUS BUFFER SYSTEM?

In protein electrophoresis, the Laemmli buffer system used for SDS-PAGE has four different buffers, all different in pH, compo-

sition, and concentration. Of course, the main voltage potential across the whole gel drives the proteins into and through the gel. However, the differences in buffer pH and concentration set up small voltage potentials within the cell voltage potential. These small voltage potentials form across areas in a lane where the number of ions is lower than elsewhere in the lane, causing the mobility of the macromolecules to increase or decrease, depending on the voltage potential in that specific location in the lane. This is the basis of the "stacking condition" (Hames and Rickwood, 1981).

If the discontinuous buffer protocol is not carried out properly, the small voltage potentials can occur in the wrong places, causing the protein bands to spread out sideways into the next lane, or causing the lane to narrow into a vertical streak of unresolved protein. Thus it is important to make up the buffers for a discontinuous buffer system properly. For instance, in the Laemmli buffer system, the resolving gel buffer is TRIS, pH 8.8 (some authors use pH 8.9). TRIS base is dissolved, and pH'd to the correct value with 6N HCl. If the pH is made too low, and base is added to correct the error, then the total ionic strength of the separating gel buffer will be too high, and the lanes in the gel will narrow. Or, if the pH is too high (not enough HCl), the bands will broaden and smear. (A TRIS-based separating gel buffer takes about 30 minutes to pH correctly. It is best to proceed slowly so that the buffer is made correctly.)

WHAT BUFFER SYSTEM SHOULD YOU USE FOR PEPTIDE ELECTROPHORESIS?

The most favored buffer system currently is that described by Schägger and von Jagow (1987). This discontinuous buffer system uses much higher concentrations of buffer salts, but the ratios of the salts are balanced. So the movement of the small proteins (peptides) is slowed, and they are separated behind the dye front. The results with this buffer system are excellent, and it has been widely used for several years for peptides and proteins up to 100 kDa.

POWER ISSUES

Macromolecules move through a polyacrylamide or agarose gel because they carry a charge at the pH of the buffer used in the

system, and the voltage potential put across the cell by the power supply drives them through the gel. This is the effect of the main voltage potential, set by the power supply.

Constant Current or Constant Voltage—When and Why?

The choice of constant current or constant voltage depends on the buffer system, and especially on the size of the gel. Historically constant voltage was used because constant current power supplies were not available. However, currently available programmable power supplies, with constant voltage, constant current, or constant power options, permit any power protocol to be used as needed.

Generally speaking, constant current provides better resolution because the heat in the cell can be controlled more precisely (The higher the current, the higher the heat, and the poorer is the resolution, due to diffusion of the bands.) However, constant current runs will take longer than constant voltage runs (Table 12.2).

Table 12.2 Use of Power Supply Parameters

Procedure	Size of cell or inter–electrode distance	Buffer System	Power Parameter
SDS-PAGE	Mini cell: gel 6–8 cm long	Discontinuous	Constant voltage used routinely; better resolution with constant current
SDS-PAGE	Large cell: gel 16–20 cm long	Discontinuous	Constant current required; use of constant voltage degrades resolution significantly in the bottom $\frac{1}{3}$ of the gel
Native PAGE	Large or mini cell	Discontinuous	Constant current required; use of constant voltage degrades resolution significantly in the bottom $\frac{1}{3}$ of the gel
Native PAGE	Large or mini cell	Continuous	Constant voltage (no advantage to constant current; cooling recommended for good resolution)

Note: Recommended power conditions can vary among manufacturers.

Why Are Nucleic Acids Almost Always Separated via Constant Voltage?

Nucleic acids are usually separated with a continuous buffer system (the same buffer everywhere). Under these conditions, the runs take the same time with constant voltage as with any other parameter held constant, and the resolution is not improved using another parameter as constant. This is usually true for both agarose and polyacrylamide gel electrophoresis.

The use of continuous buffers in nucleic acid electrophoresis makes the gels easy to pour and to run. As with protein separation, small sample sizes must be utilized within continuous buffer systems, particularly when using vertical systems, to prevent bands from overlapping.

Why Are Sequencing Gels Electrophoresed under Constant Power?

Sequencing gels are run under constant voltage or constant power, at a temperature between 50 and 55°C. If constant voltage is used, then the voltage must be changed during the run, after the desired temperature is reached. If constant power is used, the power can be set, and the voltage and current will adjust as the run proceeds, maintaining the elevated temperature required for good band resolution. Elevated temperature and the urea in the sequencing gel maintain the DNA in a denatured condition.

Should You Run Two Sequencing Cells off the Same Power Supply under Constant Power?

If the power supply can draw enough current (power) to accommodate two sequencing cells, one might conclude that two sequencing gels could be run off the same power supply. *Don't do this!* If something happened to one cell, for instance, if the buffer level fell below the level of the gel so that the circuit in that cell was interrupted, then the other cell would carry the power needed for two. The buffer in the second cell would boil away, and the cell would likely catch fire. In practice, it is very difficult to get each cell to carry exactly the same current load through the entire run. When the current loads differ, a vicious cycle/runaway condition can arise, where one cell requires more current to maintain the voltage, causing the power supply to increase its output, but the second cell, because of its lower resistance, receives the additional power. It just isn't safe to run two sequencing cells on one power supply under constant power.

It is acceptable to run two sequencing cells under constant voltage from the same power supply, as long as the power supply

can provide the needed current. It is urgently recommended that you remain in the room while the run is proceeding, in case a problem occurs.

IMPROVING RESOLUTION AND CLARITY OF PROTEIN GELS

How Can You Generate Reproducible Gels with Perfect Bands Every Time?

High-quality, reproducible results are generated by using pure, electrophoresis grade chemicals and electrophoresis grade water, by preparing solutions the same way every time and with exact measurement of volumes, by correctly polymerizing your gels the same way every time as discussed above, and by preparing the samples so that they enter the gel completely, without contaminating components that can degrade the resolution. The most important factors for good band resolution and clarity are correct sample preparation and the amount of protein loaded onto the gel, and they are discussed in greater detail below. Finally, the detection procedure must be followed carefully, with attention to detail and elapsed time.

SAMPLE PREPARATION—PROBLEMS WITH PROTEIN SAMPLES

Some samples require exceptional patience and work to determine an optimal preparation protocol. Beyond what follows, a literature search for procedures that worked for proteins similar to yours is recommended.

What Procedures and Strategies Should Be Used to Optimize Protein Sample Preparation?

Consider the cellular location of your protein of interest, and attempt to eliminate contaminating materials at the earliest stages of the purification. If it is a nuclear binding protein, first isolate the nuclei from your sample, usually with differential centrifugation, and then isolate the proteins from the nuclei. If it is a mitochondrial protein, use differential centrifugation to isolate mitochondria (spin the cell lysate at $3000 \times$ g to remove nuclei, then at $10{,}000 \times g$ to bring down mitochondria). If the protein is membrane bound, use a step gradient of sucrose or other centrifugation medium to isolate the specific membrane of interest. For soluble proteins, spin the cell lysate at $100{,}000 \times$ g to remove all cellular membranes and

use the supernatant. Note that nucleic acids are very sticky; they can cause proteins to aggregate together with a loss of electrophoretic resolution. If you have smearing in your sample, add 1 μg/ml of DNase and RNase to remove the nucleic acids.

Is the Problem Caused by Sample Preparation or by the Electrophoresis?

If a nonprestained standard runs well in a gel, producing sharply defined, well-shaped bands, then any problems in the sample lanes lie in sample preparation or in the sample buffer. For this reason we urge you to run a standard on every gel.

Is the Problem Caused by the Sample or the Sample Buffer?

For lyophilized standards, make fresh standard buffer. Sometimes it is difficult to determine whether the problem is in the sample or the sample buffer. Run the standard both with and without the sample buffer to determine this. It is best to prepare the sample buffer without reducing agent—dithiothreitol (DTT), beta-mercaptoethanol (BME), or dithioerythritol (DTE)—freeze it into aliquots, and add the reducing agent to the aliquot before use. All these reducing agents evaporate readily from aqueous solution. Adding the reducing agent fresh for each use means the reducing agent will always be fresh and in full strength.

Buffer components may separate out during freezing, especially urea, glycerol, and detergents. Aliquots of sample buffer must be mixed thoroughly after thawing, to make sure the buffer is a homogeneous solution.

How Do You Choose a Detergent for IEF or Native PAGE?

Triton X-100 is often used to keep proteins soluble during IEF or native PAGE, but it may solubilize only 70% of the protein in a cell (Ames and Nikaido, 1976). SDS is the best solubilizer, but it cannot be used for IEF because it imparts a negative charge to the proteins. During the IEF, it is stripped off the proteins by the voltage potential, and the formerly soluble proteins precipitate in the IEF gel, resulting in a broad smear. Of course, SDS cannot be used in native PAGE because it denatures proteins very effectively. Some authors state that SDS may be used in combination with other detergents at 0.1% or less. It may help solubilize some proteins when used this way (Molloy, 2000). However, this is not recommended, as the protein loads must remain low, and other problems may result (Molloy, 2000).

Many non-ionic or zwitterionic detergents can be used for IEF or native PAGE to keep proteins soluble. CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) is most often used, as it is a very good solubilizer, and is nondenaturing. It should be used from 0.1% up to 4.0%. Another very effective solubilizer is SB 3-10 (decyldimethylammoniopropanesulfonate), but it is denaturing (Rabilloud et al., 1997). Other detergents, designed especially for IEF on IPG gels, have recently been designed and used successfully (Chevallet et al., 1998; Molloy, 2000). The minimum detergent concentration for effective solubilization must be determined for each sample (Rabilloud et al., 1999). Again, to learn what detergent might be effective for your sample, we suggest a literature search.

What Other Additives Can Be Used to Enhance Protein Solubility?

Some proteins are very difficult to solublize for electrophoresis. Urea can be used, from 2 to 8 M or 9.5 M. Thiourea can be used at up to 2 M; it greatly enhances solubility but cannot be used at higher concentration. This is because above 2 M, the urea, thiourea, or detergent may precipitate out (Molloy, 2000). The total urea concentration (urea + thiourea) cannot be above approximately 7.0 M if thiourea is used with a bis gel due to these solubility constraints.

AGAROSE ELECTROPHORESIS

What Is Agarose?

Agarose, an extract of seaweed, is a polymer of galactose. The polymer is 1,3-linked (beta)-D-galactopyranose and 1,4-linked 3,6-anhydro-(alpha)-L-galactopyranose. The primary applications are electrophoresis of nucleic acids, electrophoresis of very large proteins, and immunoelectrophoresis.

What Is Electroendosmosis ($-M_r$ or EEO)?

$-M_r$ is a measure of the amount of electroendosmosis that occurs during electrophoresis with a particular grade of agarose. Electroendoosmosis is the mass movement of water toward the cathode, against the movement of the macromolecules, which is usually toward the anode. High $-M_r$ means high electroendosmosis. The mass flow of water toward the cathode is caused by fixed negative charges in the agarose gel (sulfate and carboxyl groups on the agarose). Depending on the application, electroendosmo-

Table 12.3 Agarose Preparations of Different $-M_r$ Values

Application	Kind of Agarose
Chromosome separation	Pulsed field grade or chromosomal grade; each kind of agarose—molecular biology grade, pulsed field grade, or chromosomal grade—will result in different run times in a pulsed field run, depending on the size of the chromosomes.
Size separation and recovery of DNA or RNA	Low-melt agarose melts at 65°C, and nucleic acids can be recovered with a syringe filter above gelling temperature (35°C).
Isoelectric focusing of proteins	Zero $-M_r$ agarose
Immunoelectrophoresis of proteins (for a review of the many kinds of immunoelectrophoresis, see Axelsen et al., 1973)	Standard low $-M_r$ agarose

sis causes loss of resolution, or it can enable certain kinds of separations to occur, for instance, during counterimmunoelectrophoresis. Applications for agarose preparations of different $-M_r$ values are shown in Table 12.3.

Are Double-Stranded Markers Appropriate for Sizing Large Single-Stranded (Not Oligonucleotide) DNA?

A full discussion is given below under "Standardizing Your Gels."

What Causes Nucleic Acids to Migrate at Unexpected Migration Rates?

Supercoiled DNA is so twisted about itself that it has a smaller Stoke's radius (hydrated radius), and moves faster than some smaller DNA fragments. If supercoiled DNA is nicked, it will unwind or start to unwind during the electrophoresis, and become entangled in the agarose. As this occurs, the DNA slows down its migration, and produces unpredictable migration rates.

What Causes Commercial Preparations of Nucleic Acid Markers to Smear?

There are several reasons why nucleic acid markers smear:

1. Too much marker was added to the lane.
2. The markers were electrophoresed too fast (too hot).
3. The markers were contaminated with DNase.
4. The higher molecular weight markers were sheared by rough pipeting.

What Causes Fuzzy Bands?

The sample might have been degraded by endogenous DNase or that present in the enzymes or reagents used in sample preparation. You may see, "beards" or tails on the bands. For pulsed field samples (in agarose blocks), wash the gel blocks longer and at higher temperatures.

The gel may be running too hot, or the buffer may have been used up, causing high currents that overheat the gel. Turn the voltage down, and remake your buffers, paying careful attention to the dilution and mixing of the stock solution.

Samples loaded too high in the well (overloading) can also produce fuzzy results. DNA near the surface of the gel will run faster than the DNA remaining in solution within the well. The bands will run as inclined planes (\) rather than vertically (|). If the bands are viewed or imaged from directly above they will appear fuzzy. When viewed from a slight angle, the bands will appear normal. The sample should not fill the entire well. Rather, it should occupy half or less of the well. Also the samples should be level and parallel to the surface of the gel in the wells.

Poor-quality agarose can also contribute to a fuzzy appearance. Molecular biology grade or good-quality agarose will prevent this.

Bio-Rad technical support has had a report of a contamination in the user's water that was breaking down the DNA. When the water used for the preparation of the gel and buffers was autoclaved, the problem was eliminated.

ELUTION OF NUCLEIC ACIDS AND PROTEINS FROM GELS

Table 12.4 summarizes the features, benefits and limitations of different elution strategies. DNA purification and elution is also discussed in Chapter 7.

DETECTION

What Should You Consider before Selecting a Stain?

There are several factors to consider before selecting a stain, primary among them the sensitivity needed. Tables 12.5 and 12.6

Table 12.4 Comparison of Elution Strategies

Medium or Macromolecule	Feature	Benefit	Limitation
Agarose			
Nucleic acids	Freeze and Squeeze—Cut out the band of interest from the gel, put it in an Eppendorf microtube, and freeze it. This destroys the structure of the agarose gel. Then cut off the bottom of the Eppendorf tube, put the microtube into a slightly larger tube, and spin it down. The liquid containing the band of interest will be in the larger tube, and the agarose will remain in the smaller tube.	Easy and fast	Such kits don't work with oligos or very large nucleic acids
	Electroelution	None—not recommended	Recoveries low; nucleic acids bind to dialysis membrane
Oligonucleotides	Freeze and squeeze kits	Easy and fast	Not good below 30 bp, which don't electrophorese in an agarose gel
Proteins	Freeze and squeeze kits	Easy and fast	Buffer systems not worked out for very large proteins
Polyacrylamide			
Nucleic Acids	BAC crosslinkers	Excellent recoveries	Require subsequent column to separate nucleic acids from decrosslinked polyacrylamide
Oligonucleotides	Crush gels in an equal volume of elution buffer; let sit overnight	Easy to do, requires no equipment	Best reovery no more than 50%
Proteins	Electroelution	Excellent recoveries	Some proteins bind to dialysis membrane
	Crush gel piece in an equal volume of elution buffer, let sit overnight	Relatively easy to do, requires no equipment	Best recovery no more than 50%

(continued)

Table 12.4 (*Continued*)

Medium or Macromolecule	Feature	Benefit	Limitation
	BAC, DADT, DHEBA crosslinkers (significant amounts of acrylamide remain in polymerized gels with DADT and DHEBA being a safety issue). Not recommended.	Good recoveries possible with certain proteins, depending on subsequent application	Require subsequent column to separate protein from decrosslinked polyacrylamide; periodate oxidizes sulfhydryl containing amino acid sidechains; polypeptides with sulfhydryl groups bind to BAC-crosslinked matrix
	Preparative Electrophoresis	Excellent recoveries	May require fraction collector, peristaltic pumps, chillers, other accessories
Peptides	Electroelution	Excellent recoveries possible, depending on nature and size of peptide.	Time and power conditions must be optimized for especially small peptides to prevent their being driven into the dialysis membrane

provide a general guide to stain sensitivity, and mention other considerations.

Will the Choice of Stain Affect a Downstream Application?

This is an important question. Colloidal Coomassie and Sypro® Ruby can be used on 2-D gels when mass spectrometry (mass spec) is the detection procedure. Certain silver stains can also be used to stain samples for mass spec analysis because of improvements in the sensitivity of mass spectrometers. Sypro Red covers three orders of magnitude, Coomassie covers two, and silver stains provide coverage over one magnitude. Not all silver stains give good mass spectrometry results and those which are used are not as good as Coomassie or Sypro Ruby (Bio-Rad Laboratories, R&D).

For amino acid sequencing, the gel is usually blotted to PVDF, stained for the protein of interest, and then sequenced. Immunodetection or other more sensitive methods can be used, but usually the sequencing requires at least 1 μg of protein. For

Table 12.5 Common Protein Stains

Stain	Application	Sensitivity	Benefits/Limitations
Coomassie brilliant blue R-250 (with MeOH/HOAc)	SDS-PAGE	1 μg protein per band	Easy, traditional stain; low sensitivity, high disposal costs
Coomassie brilliant blue G-250 (colloidal, low or no MeOH)	SDS-PAGE, 2-D, native PAGE, IEF	100 ng per band	Much better sensitivity, easy disposal; long staining times for best results
Silver stain	SDS-PAGE, 2-D, native PAGE, IEF	10 ng per band	Excellent sensitivity, tricky to perform, requires excellent quality water
Copper stain (requires SDS to work)	SDS-PAGE only	10–100 ng per band	Fast and easy, good before blotting
Zinc stain (requires SDS to work)	SDS-PAGE only	10–100 ng per band	Fast and easy, good before blotting
Sypro Orange (requires SDS to work)	SDS-PAGE 2-D	10 ng per band	Published sensitivities may be difficult to attain; SDS concentration critical
Sypro Ruby	SDS-PAGE 2-D	10 ng per band	Easy to use, expensive, stain of choice for 2-D and subsequent mass spectrometry and quantitative analysis

Table 12.6 Common Nucleic Acid Stains

Stain	Application	Sensitivity
Ethidium bromide	Sub-cell gels. Note that this stain is carcinogenic and is viewed only on a UV light box. Good safety practices are mandatory with this stain. Disposal is also an issue.	1–10 ng
Silver stain	PAGE gels, agarose gels with certain silver stains. Disposal is an issue.	1–10 ng
Stains all	Stains various cell components with different colors.	100 ng–1 μg

this reason we suggest that you stain your blot with Coomassie. This does not interfere with sequencing. Note that if you want to blot your gel after staining, only reversible stains such as copper stain and zinc stain can be used with good success. If you stain your gel with Coomassie or silver, the proteins are fixed in the gel and are very difficult to transfer to a membrane. Only copper or zinc stains are recommended before blotting a gel for immune detection.

Is Special Equipment Needed to View the Stain?

A light box is helpful for viewing the colored stains—Coomassie, silver, copper, and zinc—on gels. Digitizing the stained image from the gel is the best way to store the data for silver-stained gels, as they darken when dried. Fluorescent stains require at least a UV light box, and may require a fluorescent imager or other specialized scanner, depending on the excitation and emission wavelengths of the chosen stain.

How Much Time Is Required for the Various Stains?

The speed of staining is quite variable depending on the quality of water, the temperature, and how closely the staining steps are timed. Gels stained with Coomassie can be left in stain from 30 minutes to overnight, but longer staining times will require much longer destaining times, and more changes of destain solution. Colloidal Coomassie may require several days in the stain for optimum sensitivity and uniformity of staining. Silver stain must be timed carefully for best results. There are many silver staining protocols; most can be completed in 1.5 to 4 hours. Both copper and zinc staining require only 5 to 10 minutes. The fluorescent stains have various time requirements, usually from a few minutes to an hour at most. It is recommended that the protocols for fluorescent staining be followed carefully for best results.

What If You Need to Quantify Your Stained Protein?

The amino acid composition of the protein of interest will affect stain performance. No general rules are available, but some proteins stain better with Coomassie, for instance, and others stain better with silver. Both of these stains are adequate for relative quantitation of your protein (i.e., "The treated band is 2× denser than the untreated sample."). It is useful to consult the literature for information on the staining characteristics of your protein of interest.

If you must obtain the absolute amount of your protein, the best standard to use is the protein of interest itself. If the protein of interest is not available in purified form to run in a separate lane in a known amount, then bovine gamma globulin gives a better standard curve than bovine serum albumin with Coomassie brilliant blue R-250 or G-250. BSA is stained much more densely with Coomassie than other proteins at the same concentration, restricting its use as a standard. We do not recommend any silver stain for quantitation, unless you are sure your protein of interest responds the same way to silver as the protein chosen as the standard.

Note also that most silver stains provide only one absorbance unit of linearity, whereas Coomassie will provide 2 to 2.5 absorbance units of linearity. Sypro Ruby is linear over 3 absorbance units. These generalizations may or may not apply to your protein of interest; the amount of linearity of a stain on a particular protein must be assessed anew for each protein.

What Causes High Background Staining?

Impure Reagents and Contaminants from Earlier Procedures

The effect of chemical impurities was discussed above. If the SDS within the PAGE gel is contaminated with C10, C14, or C16 forms of the detergent, Coomassie brilliant blue and silver may stain the background of the gel. These and other detergents, urea, carrier ampholytes, and other gel components may also be stained. They should be removed by fixation before the stain is applied.

Certain buffer and gel components can also contribute to background staining, which can be prevented if a gel is fixed before staining. Which fixative to use depends on the gel type and the stain. When using Coomassie (or colloidal Coomassie), SDS-PAGE gels should be fixed in the same solution used to prepare the stain. The several osmotic potentials that exist between the fixing solution and the buffers within the gel cause the TRIS, glycine, and SDS to leave the gel, making for a much cleaner background.

IEF gels should be fixed in 10% trichloroacetic acid, 40% MeOH, and if possible, 2.5% sulfosalicylic acid, since the latter helps remove carrier ampholytes. Immobilized pH gradient gels, IPG gels, are not usually stained with silver, but they can be stained with colloidal Coomassie. It is sometimes useful to stain the IPG strips as an aid in diagnosis of problems with the 2-D slab gels.

Will the Presence of Stain on Western-Blotted Proteins Interfere with Subsequent Hybridization or Antibody Detection Reactions?

Proteins can be detected on a blot after staining the blot with a general protein stain such as Coomassie or colloidal gold, but the interference with subsequent immunodetection will be high (Frank Witzman, 1999). The interference can be 50% or more, but this may not matter if the protein of interest is in high abundance.

Proteins which have been stained in the gel will not transfer out of the gel properly, and it is unlikely that an immuno detection procedure will be successful. It is usual to run duplicate gels or run duplicate lanes on the same gel and cut the gel in half, if you want to both stain and blot the protein of interest.

Does Ethidium Bromide Interfere with the Common Enzymatic Manipulation of Nucleic Acids?

Ethidium bromide does not usually interfere with the activities of most common DNA modifying enzymes. However, ethidium bromide has been shown to interfere with restriction endonucleases (Soslau and Pirollo, 1983; Parker et al., 1977).

STANDARDIZING YOUR GELS

What Factors Should Be Considered before Selecting a Molecular Weight Marker?

Ask yourself whether you need exact or approximate molecular weight values. If you need exact values, you must use a standard that will form thin tight bands at the same location from batch to batch. Most pre-stained standards do not form such thin, tight bands, and are good for only "ball park" molecular weight values and assessing transfer efficiencies.

You might also ask whether you will run native or denatured gels. Denatured gels, usually SDS-PAGE gels, provide exact molecular weights because of the elimination of the charge on the protein as a factor in the electrophoresis. (Negatively charged SDS coats the proteins, hiding the native charge on the proteins, and providing a constant charge to mass ratio.)

Native gels provide results which reflect the charge, size and shape of the proteins. It is not acceptable to measure molecular weight by native electrophoresis, because more than one parameter is measured during this technique. Some companies sell "molecular weight standards" for native gels, but these standards have

no scientific validity. Molecular weights can be determined for native gels by means of a Fergusson plot (Andrews, 1986). Proteins can be used to measure whether the electrophoresis is reproducible, and can provide information on the relative separation of various bands from each other. However, because more than one parameter influences the movement of the proteins in the gel, they cannot be used to measure molecular weight.

Another factor that affects the migration rate in any kind of gel is the protein's amount and type of posttranslational modification. Proteins with significant glycosylation will run more slowly than their total molecular weight might suggest (Podulso, 1981). It is also possible to use gradient gels for molecular weight determination (Lambin and Fine, 1979; Podulso and Rodbard, 1980).

Are Double-Stranded Markers Appropriate for Sizing Large (Not Oligonucleotide) Single-Stranded DNA? If Not, Which Markers Are Recommended?

Double-stranded DNA size markers are not appropriate for sizing large single-stranded DNAs. Most labs with need of such markers obtain single-stranded DNA (usually phage DNA), calibrate it for size by sequencing it, and use that as a single-stranded DNA marker. Since the mobility of many single-stranded nucleic acids is variable, it is recommended to cross-calibrate with a second single-stranded source (e.g., a different phage).

Can a Pre-stained Standard Be Applied to Determine the Molecular Weight of an Unknown Protein?

Pre-stained protein standards usually run as broad, fuzzy bands, making them useful for approximate, but not exact, molecular weight determinations. Thus they can be used to report only approximate molecular weights (within 10,000 daltons of the molecular weight as determined by an unstained standard). The molecular weight values of most pre-stained standards vary from batch to batch because the conjugation reaction between marker protein and dye marker is not perfectly reproducible.

Some vendors now offer pre-stained recombinant proteins of known, reproducible molecular weights. The bands in these protein standards form thin, tight bands, and they can be used for accurate molecular weight determination.

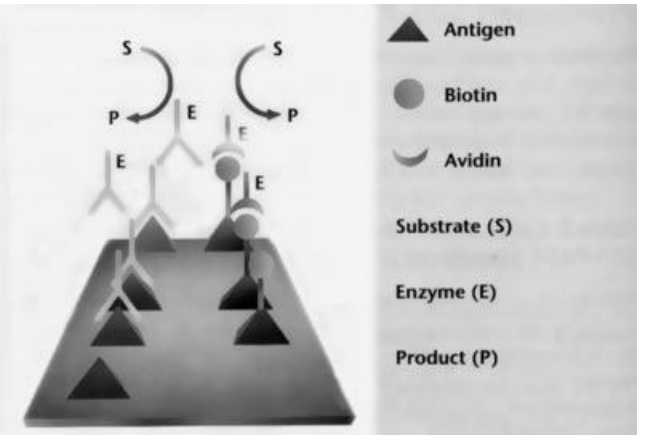

Figure 12.5 Use of biotinylated protein standards to calculate molecular weight on Western blots. Permission to use this Figure has been granted by Bio-Rad Laboratories, Inc.

How Do You Determine Molecular Weight on a Western Blot?

Use biotin-labeled molecular weight markers, and detect them with streptavidin-conjugated horseradish peroxidase or alkaline phosphatase. The streptavidin conjugate that will detect the markers is added to the solution containing the labeled secondary antibody (e.g., horseradish peroxidase or alkaline phosphatase) that will subsequently react with the sample proteins (Figure 12.5). These markers will provide precise molecular weight values.

The pre-stained recombinant proteins of known, reproducible molecular weights discussed above can also determine the molecular weights of proteins on a blot.

Some researchers will cut off the molecular weight standard lane from the blot and stain it with Coomassie or Amido Black, and then realign the stained standards with the rest of the blot once it has been processed. The problem with this approach is that the nitrocellulose can slightly shrink or swell, causing the bands to misalign. Other researchers simply feel uncomfortable about the prospect of perfectly aligning the segments after cutting, so this is not recommended.

What Are the Options for Determining pI and Molecular Weight on a 2-D Gel?

There are several ways to do this:

1. Add proteins of known (denatured) pI and MW to your sample and electrophorese the standards within the same gel. The added proteins are often difficult to detect within the

2-D spot pattern, which usually makes this method unsatisfactory. It may be appropriate for 2-D of *in vitro* translation products.

2. Use a 2-D standard comprised of proteins of known pI and MW, and run it on a separate gel, with the assumption that the gels will run identically. This is also problematic, since it is difficult to get the gels to run identically. The use of IPG strips and pre-cast slab gels helps, but drying artifacts may cause unacceptable variation between gels.

3. Measure the pH gradient of the IEF gel with a pH electrode (see below and Chapter 4, "How to Properly Use and Maintain Laboratory Equipment,") and use a MW standard in the second dimension to determine MW.

4. Carbamylate a protein of known (denatured) pI, and add it to the sample (Tollaksen, 1981). A protein with a MW not seen in the sample should be used. The carbamylated protein will run as a series of spots starting with the spot of known pI. Each spot to the acidic side will be 0.1 pH unit more acidic than the one to the basic side. Carbamylated proteins are also commercially available.

5. If you are electrophoresing a well-characterized sample, such as *E. coli* or mouse liver, compare your pI and MW data to online databases such as those available at *http://www.expasy.ch/*. This is the preferred option if your sample is present in such a database. If such a database is not available for your sample, you should use 2 of the above methods.

How Do You Measure the pH Gradient of a Tube IEF Gel or an IPG Gel?

Several methods are presented here. None are very satisfactory, as there are problems with them all.

To document the pH gradient, measure the migration distance for several proteins of known pI, and create a standard curve by plotting the pI value of your marker against the R_f value. You will need to normalize your standard proteins so that you can compare gels.

Several commercial products, comprised of colored proteins of known pI, are available for native IEF. However, these standards cannot be used for 2-D gels, since native pI values differ from the pI value of the same protein under denaturing conditions. The native pI value is based on the surface charge and conformational effects of the protein. In 2-D gels all amino acid side chains are

exposed and affect the migration of the protein in denaturing conditions, thus altering the pI.

A second approach is to directly measure the pH throughout the length of the gel (this works only with carrier ampholyte tube gels). Slice the gel into 1, 5, or 10 mm sections, and put the pieces into numbered tubes. Next, add 1.0 ml of 50 mM KCl to each tube, place them inside a vacuum dessicator without dessicant, and draw a vacuum on the tubes. Incubate overnight at room temperature, and measure the pH of the ampholyte solution, starting from the acidic end, after 24 hours. Incubation for 24 hours is recommended to ensure that equilibrium of the ampholyte concentration in the gel piece and the liquid has occurred. The potassium chloride and vacuum are required to prevent atmospheric CO_2 from affecting the pH of the solutions. The potassium chloride also helps the pH electrode work more easily in solutions with low concentrations of ampholytes. The problem with this procedure is that it is difficult to cut the gel into exact, reproducibly sized sections.

As decribed in Chapter 4, "How to Properly Use and Maintain Laboratory Equipment," electrodes are available that can directly measure the pH of a gel. There are two kinds: flat-bottomed electrodes, suitable for a flat strip gel, and microelectrodes, which must be inserted into the (tube) gel. Flat-bottomed electrodes usually have the reference electrode to the side, as a little piece of glass sticking out. The reference electrode must be parallel with the main electrode, at the same pH in use. The microelectrode has the reference electrode in a circular shape around the main electrode. Both types require some getting used to, but provide good results when used carefully and in a reproducible manner.

Veteran proteomics researchers identify proteins in their samples by comparison of their spot patterns to those in Web-based 2-D databases, and choose known proteins to sequence and measure by mass spectrometry. Once those proteins have been compared and identified for sure, they can be used as internal pI and MW standards. Usually constituitive proteins that do not vary in concentration are used. (Wilkins et al., 1997) Most 2-D data analysis software packages can establish a pH gradient once spots of known pI are specified.

Some groups report the use of pH paper to get a very rough idea of the pH gradient (personal communication from Bio-Rad customers), but this is not recommended because it lacks precision.

In the case of IPG strips, you may assume that if you have a pH 3 to 10 gel, that you can measure the length of the gel from end to end, and divide it up into pH units. This is valid only for a rough

idea of the pI of a protein of interest. Manufacturers' specifications for the length of the gels ranges from ±5 to ±2 mm, and the pH gradient on the gel may also vary enough to change the location of a pH on the gel.

TROUBLESHOOTING

What Is This Band Going All the Way across a Silver-Stained Gel, between Approximately 55 and 65 kDa?

The band most likely contains skin keratin, originating from fingers, flakes of skin, or hair dander (dandruff) within the gel solutions or running buffer. This band, which may be quite broad, is usually detected only with more sensitive staining methods, such as silver. There is usually only one band and the molecular weight varies depending on the type of skin keratin. Ochs (1983) demonstrates conclusively that this band is due to skin keratin contamination.

How Can You Stop the Buffer Leaking from the Upper Chamber of a Vertical Slab Cell?

The upper chamber should be set up on a dry paper towel before the run with the upper buffer in it, and let stand for up to 10 minutes to determine if there are any leaks from the upper chamber. In some cells the leaks can be stopped by filling up the lower chamber to the same height as the liquid in the upper chamber. This eliminates the hydrostatic head causing the leak, and the run can proceed successfully. Otherwise, make sure the cell is assembled correctly, and if the problem persists, contact the cell's manufacturer.

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APPENDIX A

PROCEDURE FOR DEGASSING ACRYLAMIDE GEL SOLUTIONS

Degas your acrylamide solution in a side-arm vacuum flask with a cork that is wider han the flask opening for 15 minutes with gentle stirring (Figure 12.6). Use at least a bench vacuum to degas (20–23 inches of mercury in most buildings); a water aspirator on the sink is not strong enough (at most 12–16 inches of mercury). A vacuum pump (>25 inches of mercury) is best. When the solution bubbles up and threatens to overflow into the side arm, release the vacuum by quickly removing the cork from the top of the flask. Then replace the cork, swirl the solution, and continue the procedure. The solution will bubble up four or five times, and then most of the air will be removed. Continue degassing for 15 minutes total. The degassing is a convenient time to weigh out 0.1 g of APS in a small weigh-boat and to test its potency as described in the text.

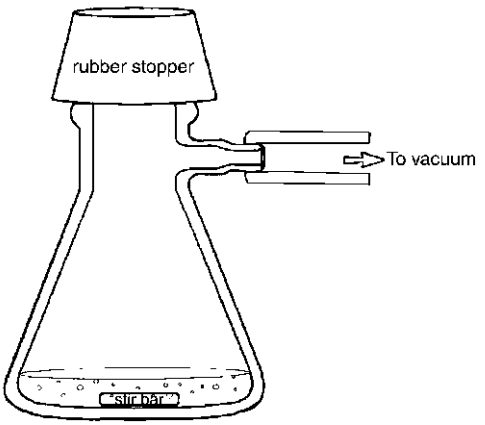

Figure 12.6 Vacuum flask strategy to eliminate dissolved oxygen from acrylamide solutions. Reproduced with permission from Bio-Rad Laboratories.