Rapid Detection and Characterization of Foodborne Pathogens by Molecular Techniques

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Dedication

This volume is dedicated to the memories of Max Levine, PhD, without whose early inspiration this volume would not have been possible, and Eugene Levine, MD, who departed this world too soon.

Contents

Preface

During the three decades following the development of the polymerase chain reaction (PCR), a significant array of associated techniques has been developed, along with the enormous expansion of Genbank. This has now made it possible to rapidly detect low numbers of all known pathogenic microorganisms in foods, water, and environmental samples without the traditional methods of cultivation and phenotypic characterization, which are labor intensive and require several days to complete. This volume has been written specifically with the intent that it be of significant utility to individuals in the field. Readers unfamiliar with the details of real-time PCR will find that Chapter 1 is comprehensive and presents the theoretical and operational aspects of this method in complete detail. Each of the following chapters deals with a different bacterial pathogen associated with foods.

The more recent development of intercollating dyes, such as ethidium bromide monoazide, has notably expanded the application of PCR to allow the selective amplification of DNA from only viable bacterial cells, which greatly enhances the public health utility of PCR in detecting only viable pathogens. The recent development of loop-mediated isothermal amplification of DNA has allowed the quantitative yield of amplicons to be increased by three orders of magnitude, has eliminated thermal cycling, and has reduced amplification time to 60 minutes.

The purpose of this volume is to serve as a comprehensive presentation of the literature and methodology pertaining to the use of these and other molecular techniques to detect and quantify foodborne pathogenic bacteria, and to characterize isolates of a given species below the species level. Each chapter presents a comprehensive list of DNA primers and probes with respect to conventional and real-time PCR for the convenience of the reader.

The Author

The author, Robert E. Levin, is professor of food microbiology in the Department of Food Science at the University of Massachusetts, Amherst. He obtained his BS degree in biology from Los Angeles State University, his MS degree in bacteriology from the University of Southern California, and his PhD in microbiology from the University of California, Davis. His early academic career involved the isolation and study of obligately psychrotrophic bacteriophages, characterization of seafood food spoilage enzymes of bacterial origin, and fermentative production of microbial gums. His present research program focuses on the conventional and real-time PCR quantification of viable bacterial pathogens in foods.

CHAPTER 1

Molecular Techniques for Detecting, Quantifying, and Subspecies Typing of Foodborne Pathogenic Bacteria

I. THE POLYMERASE CHAIN REACTION (PCR)

A. Introduction

PCR is one of the most powerful analytical techniques ever developed. It allows segments of minute amounts of double-stranded DNA to be amplified several millionfold in several hours. Its most notable application to foods is for the detection of low numbers of foodborne pathogenic and toxigenic bacteria in a wide variety of food products, in addition to confirming the identification of such organisms isolated from food.

B. Requirements for PCR

PCR uses repeated temperature cycling involving template denaturation, primer annealing, and the activity of DNA polymerase for extension of the annealed primers from the 3′-ends of both DNA strands (Figure 1.1). This results in exponential amplification of the specific target DNA sequence. The availability of the thermostabile *Taq* DNA polymerase, from the extreme thermophile *Thermus aquaticus,* greatly facilitates repeated thermal cycling at ~95°C for template denaturation without having to repeatedly add a less thermally stabile DNA polymerase after each cycle. The notably high optimum temperature for *Taq* polymerase activity (75–80°C) allows high extension temperatures (72–75°C), which, when coupled with a high annealing temperature (50–65°C) and denaturation at 95°C, increase specificity, yield, and sensitivity of the PCR reaction (Innis and Gelfand, 1990). PCR reactions are usually performed in 0.5-ml or 0.2-ml thin-walled polyethylene PCR tubes containing 50 µl total reaction volume. The availability of second-generation thermal cyclers with heated lids has eliminated the previous need for overlaying the reaction volumes with

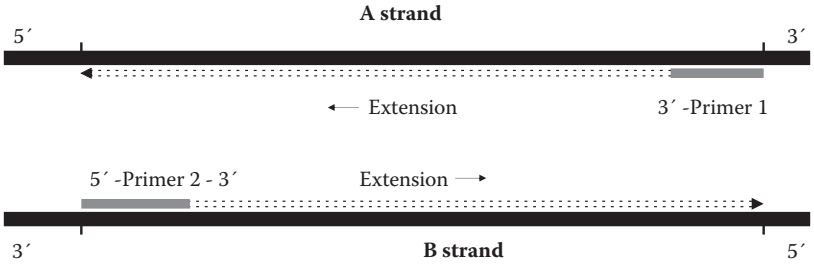

Figure 1.1 Amplification of a known target sequence with a set of two primers.

Table 1.1 Typical PCR Reaction Components

1. Template DNA	1–10 µl
2. Tris-HCl (pH 8.3 at 20°C)	20 mM
3. KCl	25 mM
4. Triton X-100	0.1%
5. dNTPs (dATP, dCTP, dGTP, dTTP)	50 mM each
6. *Taq* polymerase	1.0 unit
7. $MgCl_2$	1.5 mM
8. Primers	10 pmoles each

50 µl of mineral oil to prevent evaporation. The four deoxynucleotide triphosphates (Table 1.1) are presently available commercially premixed. Variables that require optimization include components 5–8 in Table 1.1. The concentration of $MgCl_2$ is particularly critical. Innis and Gelfand (1990) have discussed the optimization of PCRs in detail. Most thermal cycling of PCRs encompasses 35 cycles; rarely are more than 35 cycles of benefit.

A typical thermal cycling protocol is given in Table 1.2. After an initial denaturation step at 95°C, steps 2–4 are then sequentially performed for 35 cycles followed by a final extension (step 5) at 72°C to ensure that the final round of strand synthesis at high substrate concentration is completed. The sixth step involving reduction of the temperature to 4°C is used to terminate all reactions for convenient holding until agarose gel electrophoresis is performed. The time required to traverse from one temperature to another is referred to as the ramp time and usually contributes

Table 1.2 Typical PCR Thermal Cycling Protocol

Step	Process	Temperature	Time
1	Initial denaturation	94°C	3 min
2	Denaturation	94°C	1 min
3	Annealing	60°C	1 min
4	Extension	72°C	1 min
5	Final extension step	72°C	4–7 min
6	Terminate reactions and hold	4°C	—

significantly to the total thermal cycling time. Most 35-cycle amplification protocols for the PCR are completed within 3 to 4 hr. The total cycling time can often be significantly reduced to less than 90 min by lowering each holding time to 10–20 s.

C. Sample Preparation without Enrichment

A variety of components in foods and various tissues is capable of inhibiting the PCR. Sample preparation is therefore one of the more critical steps that can adversely influence the PCR. Meat and cheese products are particularly challenging. With ground beef, a 25-g sample is stomached with 225 ml of 0.01 M phosphate buffered saline (PBS, pH 6.0) in a Wirl-pak stomacher bag with a mesh insert and homogenized at normal speed for 60 s. A majority of the homogenate (~200 ml) is then centrifuged at low speed (160 g, or 1000 rpm) for 3 min at 4°C to pellet tissue debris. Most of the supernatant (~190 ml) is then centrifuged at high speed (16,000 g or 10,000 rpm) for 10 min at 4°C to pellet bacterial cells. The supernatant is discarded and the pellet is resuspended in a minimum volume of saline. Bacterial cells are then lysed and the DNA is purified.

D. Lysing of Cells and Isolation of Bacterial DNA

The author has found that most or all gram-negative bacterial cells are readily lysed using TZ lysing solution (Abolmaaty et al., 2000) at 2× concentration (5.0 mg/ml of sodium azide and 4.0% Triton X-100 in 0.2 M Tris-HCl buffer, pH 8.0. One ml of 2× TZ lysing solution is added to the resuspended pellet from above and heated at 100°C for 10 min to achieve cell lysis. The lysate is then centrifuged at 10,000 g for 10 min to remove cellular debris.

Gram-positive bacteria are notably more difficult to lyse than gram-negative organisms. The method of Wiedman, Barany, and Batt (1995) has been found to be effective for lysing *Listeria monocytogenes* and also *Staphylococcus aureu*s. Cells are suspended in 100 µl of PCR buffer containing 2 mg/ml of lysozyme followed by incubation at room temperature for 15 min. Proteinase K (1 µl, 20 mg/ml) is then added followed by incubation for 1 hr at 55°C. The sample is then boiled for 10 min to inactivate the proteinase K and to lyse the cells.

Bacterial DNA is then isolated from the above lysate with the "Wizard" DNA clean-up system (Promega Co., Madison, Wisconsin) by mixing 0.45 ml of the lysate with 1.0 ml of the DNA clean-up resin. The mixture is then passed through a "Wizard" microcolumn, washed with 2.0 ml of 80% isopropanol, and dried by centrifugation (2 min at 10,000 g). DNA is then eluted from the column with 0.45 µl of sterile deionized water dH_2O. The eluate is then mixed with 1 µl of pellet paint (Novogen Co., Madison, Wisconsin), which is a visible fluorescent-dye-labeled carrier to facilitate visualization of the ultimate DNA pellet, followed by the addition of 5 µl of 3 M sodium acetate and 102 µl of ice cold 100% ethanol. The preparation is then stored at 4°C for 30 min and then centrifuged at 14,000 g at 4°C for 10 min. The supernatant is removed, the pellet air dried and dissolved in 20 µl of sterile deionized water, and the entire preparation incorporated into the PCR reaction.

E. PCR for Identification of Pure Cultures

Confirmation of the identity of pure cultures using PCR is most readily achieved by picking up a small visible amount of cells from an agar culture on the end of a needle and suspending the cells in 20 µl of dH_2O. Gene releaser (20 µl, Bioventure, Inc., Murfreesboro, Tennessee) is then added and the heating protocol of the manufacturer is used to lyse the cells. Then 1.0 µl of the resulting cell lysate is added directly to the PCR reaction mixture.

F. Quantitative PCR

The author has found that the methodology described below is ideally suited for quantitative assessment of target DNA incorporating an internal standard with the use of a conventional thermal cycler.

The operational assumption is that PCR yields an exponential rate of increase of the initial number of target DNA molecules. This is described by the following equation:

$$P = T\,(2^n)$$

where P is the number of PCR product molecules formed, T is the number of input target sequences, and n is the number of amplification cycles. This exponential equation, however, does not apply to the entire amplification process. During the first few cycles, when the number of initial target molecules is very low, the rate of amplification can be anticipated to be low (Diaco, 1995). During the last few cycles, when the ratio of PCR product molecules to unreacted primer and *Taq* polymerase molecules has greatly increased, the overall amplification rate can be expected to decrease significantly from an exponential rate. This is illustrated in Figure 1.2. It is only along the linear portion of the plot that a reliable quantitative relationship between the number of input molecules and final accumulated products can be derived. A direct approach to determine when amplification enters the plateau phase is to increase the number of input target molecules and measure the number of product molecules after cycling (Figure 1.2). It is important with quantitative PCR assays that the initial number of target DNA molecules be low, because the linear range of amplification is represented by no more than about a two log increase in DNA. This will often result in a working range of input target colony-forming units (CFU) of about 1.5×10^2 to 1.5×10^4 (Guan and Levin, 2002a). If an enrichment step (usually 4.5–6.0 hr) is used, then an appropriate dilution of the resulting CFU should be used for the PCR to ensure that the number of input target molecules falls within the linear PCR amplification range. There are several advantages to the use of nonselective enrichment cultivation of food samples:

1. Only viable CFU are increased.
2. Detection of the initial viable CFU is lowered to 0.5 to 1.0 CFU/g of sample (Guan and Levin, 2002b).
3. Dilution of PCR inhibitors in the food sample. It is important to keep in mind that a 10% reduction in the efficiency of amplification will result in a reduction of PCR product accumulation by more than 95%.

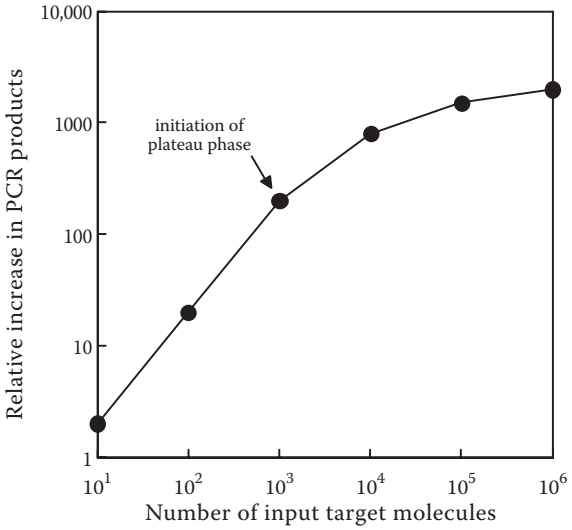

Figure 1.2 Dependence of PCR product accumulation on number of input targets from 35 amplification cycles.

4. The inclusion of an internal standard involving an identical set of primers and a similar target can be used to eliminate variations in amplification efficiency, allowing reliable PCR quantitation.

G. Use of an Internal Standard for Quantitative PCR

The application of an internal standard is based on the coamplification of the target sequence and an internal DNA standard, which is amplified with equal efficiency as the target sequence by using the same primer pair. The internal standard contains the same primer binding sites as the target, and the two DNAs compete for reaction reagents to produce PCR products of different sizes, which can be separated in an agarose gel. The log ratio of intensities of amplified target DNA to internal standard is determined by the following equation given by Zarchar, Thomas, and Goustin (1993):

$$-\text{Log}\,(N_{n1}/N_{n2}) = \log\,(N_{01}/N_{02}) + n\,\log\,(\text{eff}_1/\text{eff}_2)$$

where

N_{n1} = number of resulting target molecules after amplification.
N_{n2} = number of resulting internal standard molecules after amplification.
N_{01} = number of initial target molecules.
N_{02} = number of initial internal standard molecules.
n = a given cycle.
eff_1 = efficiency of amplification of target molecules.
eff_2 = efficiency of amplification of internal standard molecules.

If the efficiencies of amplification (eff_1/eff_2) are equal, the ratio of amplified products (N_{n1}/N_{n2}) is dependent on the log ratio of starting reactants (N_{01}/N_{02}). Even if the efficiencies of the two reactions are not equal, the values for N_{n1}/N_{n2} still hold assuming that eff_1/eff_2 is constant and amplification is in the exponential phase (Zarchar, Thomas, and Goustin, 1993). With this technique, varying amounts of target DNA are coamplified with a constant amount of internal standard. The resulting log ratio of intensities of PCR products is plotted against the log of target CFU for the construction of a standard curve that is used for determining the number of CFU per gram of food product.

H. Construction of an Internal Standard for Quantitative PCR

The ideal internal standard should consist of a nucleotide sequence derived from the target sequence and should utilize the same two primers and primer binding sites so that when the internal standard is added to the PCR mix, amplification occurs in a competitive mode. An internal DNA standard can be synthesized by PCR using a hybrid primer and the reverse primer (Rupf, Merte, and Eschrich, 1997). The hybrid primer contains two components: a 5′ portion consisting of the original forward primer, and a 3′ portion consisting of a small DNA fragment derived from the target sequence. A 20-nucleotide sequence about 200 to 300 nucleotides in from the 5′-terminal of the target sequence is identified and a hybrid primer is synthesized (Figure 1.3) for use in the construction of an internal DNA standard. Complete details are given by Guan and Levin (2002b).

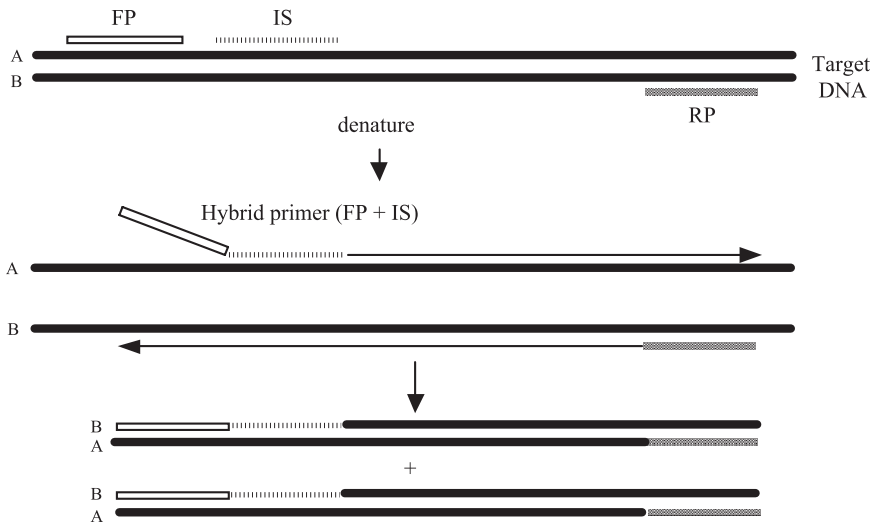

Figure 1.3 Construction of an internal DNA standard for quantitative PCR. FP, forward primer; RP, reverse primer; IS, internal sequence.

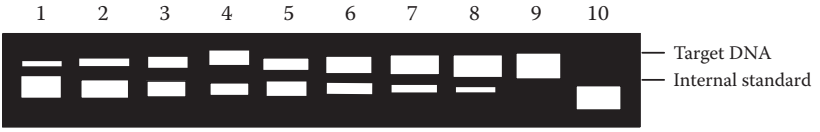

Figure 1.4 Idealized representation of competitive amplification of a constant amount of internal standard and varying amounts of target DNA. Lane 9, target DNA alone. Lane 10, internal standard alone.

I. Construction of a Calibration Curve for Quantitative PCR

Varying amounts of target DNA from a pure culture are coamplified with a constant amount of the internal standard (IS). The amplified co-PCR products are separated by electrophoresis in a 1.5% agarose gel (Figure 1.4). The gel images are then captured by a digital camera and analyzed with National Institutes of Health (NIH) Image 1.61 software. To correct differences in the intensity of fluorescence of ethidium bromide-stained PCR fragments of different sizes, the intensity of the internal standard is multiplied by the ratio of the number of nucleotides in the target sequence to that of the internal standard. The log of the ratio of fluorescence intensity of the amplified target sequence to that of the internal standard is then plotted against the log of CFU to establish a standard curve, using a constant amount of internal standard and varying the number of CFU (Figure 1.5). It is important to note that the calibration curve is operational over little more than a one log cycle of input target sequences (CFU).

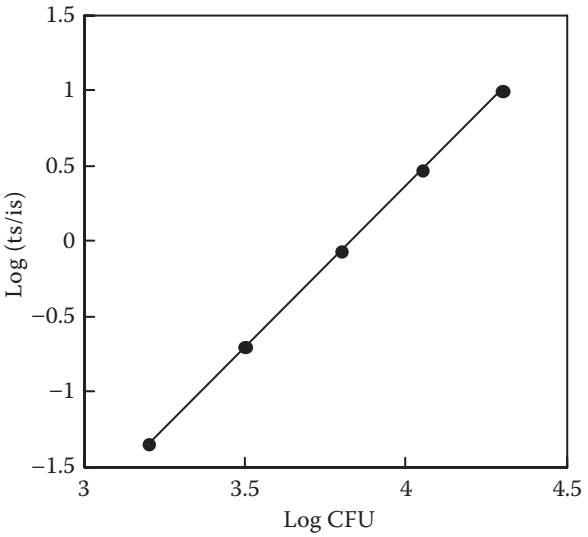

Figure 1.5 Standard curve for quantitative PCR.

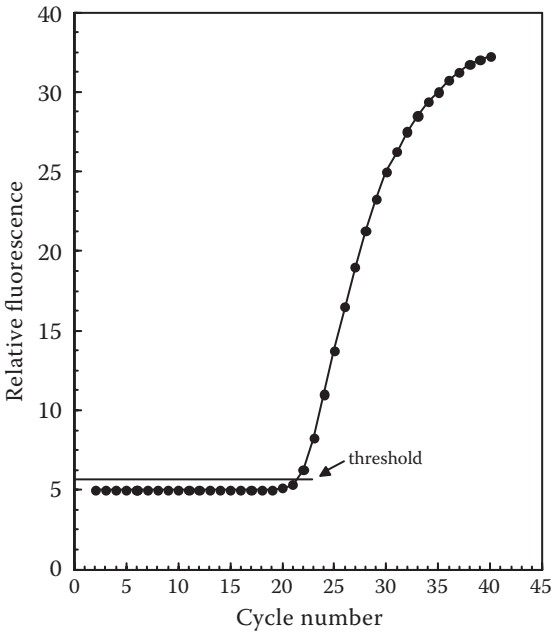

Figure 1.6 Real-time PCR screen display of DNA target amplification.

II. REAL-TIME PCR (RTI-PCR)

A. Introduction

Real-time PCR (Rti-PCR) refers to the detection of PCR-amplified target DNA (amplicons) usually after each PCR cycle. The signal is readily followed on a computer screen where each point is automatically plotted and the extent of amplification is followed as an ongoing continual direct graphical plot (Figure 1.6). Computer software handles all of the preprogrammed calculations and plotting of data. Table 1.2 lists the major Rti-PCR thermal cyclers presently available and the manufacturers. Table 1.3 lists a number of commercial sources of primers and fluorescent probes.

B. Advantages of Rti-PCR

Conventional thermocyclers often require 2 to 3 hr to complete 35–40 "thermal" cycles. Much of this time is consumed by the "ramp" time required to traverse from one temperature to another. Rti thermocyclers incorporating air heating and cooling, capillary sample systems, and thermoelectrically controlled blocks have greatly reduced ramp times. The use of shortened target DNA sequences (60–70 bp) in Rti-PCR results in more efficient amplification than standard PCR where amplicons are required to be at least 200 bp in length to allow detection by electrophoretic separation and also allows reduced extension times. Short PCR product yields are

Table 1.3 Real-Time PCR Instrumentation

Name of Instrument	Manufacturer	Heating/ Cooling	Cycling Time	Observation of Data	Number of Wells
IQ5	BioRad	Thermoelectric	~2 hr	Each PCR cycle	96
Smart Cycler	Cepheid	Ceramic heating plate	~40 min to 1 hr	Each PCR cycle	6–96
Gene Amp 5700 & Prism 7700	Applied Biosystems	Heating block	~2 hr	End-point	96–384
Rotor Gene	Corbett Research	Air	~50 min	Each PCR cycle	32
ICycler iQ	BioRad	Heating block	~2 hr	Each PCR cycle	60, 96, 384
MX 4000	Stratagene	Heating block	~90 min	Each PCR cycle	96
LightCycler	Roche Applied Biosystems	Air	~20 min to 1 hr	Each PCR cycle	32

significantly improved by lower denaturation temperatures (Yap and McGee, 1991) so that a denaturation temperature of 90°C instead of 95°C is preferred. This also reduces ramp time. In addition, conventional PCR requires visualization of amplified products after agarose gel electrophoresis, which usually involves an additional 30–60 min. Rti-PCR completely eliminates this step through the use of a fluorometer built into the Rti-PCR thermal cycler that measures the intensity of fluorescence after each amplification cycle, unless one wishes to confirm the identity of the products on the basis of molecular size. Agarose gel electrophoresis is now usually replaced with Rti-PCR systems by programmed generation of a thermal denaturation curve of the amplified product after the PCR that allows automatic calculation of the T_m value of the amplicon when SYBR Green is the fluorescent reporter molecule. This is most useful in confirming the identity of an amplicon.

The quantitative range with conventional PCR is no more than 1.5–2.0 log cycles, whereas with Rti-PCR an operational range of at least 5–6 log cycles is usually achieved. Conventional thermocyclers can presently be acquired for $2000 to $3000. In contrast, Rti-PCR systems presently range in price from $20,000 to $40,000. An inexpensive approach is the use of a fluorescence-activating microplate reader where final fluorescence is measured after amplification in a conventional thermal cycler. Rti-PCR can be performed with units furnishing 16 wells, with systems accommodating 96 or 384 well microplates, with chains of eight linked PCR tubes, or with individual PCR tubes. Because of the significantly increased cost of reagents, particularly the fluorescent probes and dyes compared to reagents used with conventional PCR, the reaction volume is usually reduced from 50–100 µl to 10–20 µl.

In addition to detection of amplicons, Rti-PCR units can quantitate amplified target DNA and differentiate alleles (determine point mutation or sequence variation). Allelic variation is assessed on the basis of T_m variations derived from analysis of melting curves of duplexes formed by fluorescent probes and amplicons. In addition,

two or more different PCR reactions (multiplex PCR) amplifying different target sequences can be followed and quantifed in the same PCR tube or well. Cockerill and Uhl (2002) have extensively discussed the advantages of Rti-PCR.

The GeneAmp 5700 and PRISM 7700 (Applied Biosystems) and the LightCycler system (Roche Applied Science; Table 1.3) can be obtained with coupled automated nucleic acid extraction instruments resulting in completely automated DNA extraction, amplification, and detection. Rti-PCR has great potential for the meat-processing industry where massive recalls have occurred during the past few years due to the presence of *Escherichia coli* O157:H7 and *L. monocytogenes* in various meat products. More recently, costly recalls in vegetables due to *E. coli* O157:H7 and outbreaks of salmonellosis from tomatoes have occurred The present state of Rti-PCR technology should allow processors to detect product contamination from the production line in essentially near real-time to reduce such massive recalls by allowing detection prior to shipment of the product.

C. Mechanisms of Rti-PCR

Rti-PCR depends on the emission of a an ultraviolet (UV)-induced fluorescent signal that is proportional to the quantity of DNA that has been synthesized. Several fluorescent systems have been developed for this purpose and are discussed below.

1. SYBR Green

The simplest, least expensive, and most direct fluorescent system for Rti-PCR involves the incorporation of the dye SYBR green whose fluorescence under UV greatly increases when bound to the minor groove of double-helical DNA (Figure 1.7).

SYBR green lacks the specificity of fluorescent DNA probes but has the advantage of allowing a DNA melting curve to be generated and software calculation of

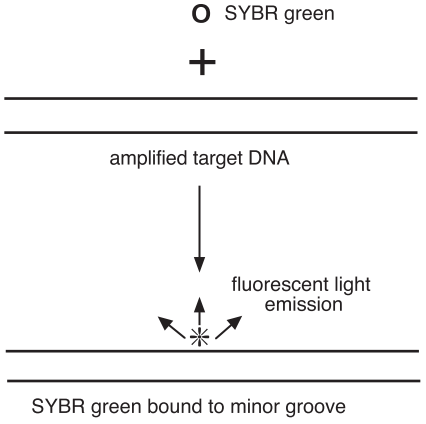

Figure 1.7 Mechanism of SYBR green fluorescence. SYBR green dye binds to the minor groove of the DNA double helix. The unbound dye emits little fluorescence, which is greatly enhanced when bound to DNA.

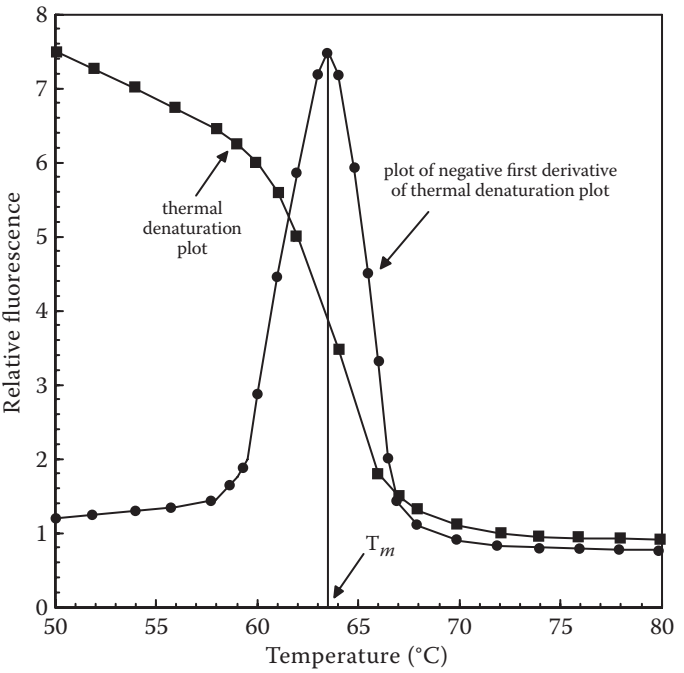

Figure 1.8 Thermal denaturation curve of double-stranded amplified target DNA with SYBR green I as reporter dye. As the double helix is denatured to the single-strand state, increasing amounts of SYBR green dissociate from the double-stranded DNA resulting in a linear decrease in fluorescence. Bell-shaped curve results from plotting the second derivative of the thermal denaturation plot with the apex coincident with the T_m value.

the T_m of the amplicon after the PCR (Figure 1.8). This allows identification of the amplified product and its differentiation from primer–dimers, which also result in a fluorescent signal with SYBR green but which usually have a lower T_m value. The fluorescent signal is measured immediately after the extension step of each cycle because thermal denaturation yielding single-stranded DNA eliminates fluorescence. A software plot of the negative first derivative of the thermal denaturation plot yields a bell-shaped symmetrical curve, the midpoint of which yields the T_m value for the amplified product (Figure 1.8). Interference of the amplicon's signal by the signal resulting from primer–dimer formation can be eliminated by raising the temperature to a critical point that is above the T_m of the primer–dimer formed (resulting in thermal denaturation of the primer–dimers) but below the T_m of the amplicons prior to measuring the intensity of fluorescence emission.

2. TaqMan™ Probes

The TaqMan probes are proprietary double-dye probes synthesized by Perkin Elmer Applied Biosystems. A variety of such double-dye probes is available from a

number of commercial sources (Table 1.4). The TaqMan system makes use of the 5′ 3′-exonuclease activity of *Taq* polymerase to produce a fluorescent signal. A custom-synthesized TaqMan probe is incorporated into the PCR containing a sequence of nucleotides homologous to a specific nucleotide sequence of one strand of the amplicon internal to both primers. The probe harbors a fluorophore (reporter dye) such as 6-carboxyfluorescein (FAM) as the reporter dye at the 5′ end and 6-carboxytetramethyl-rhodamine (TAMRA) as the quenching dye at the 3′ end (Figure 1.9), which are close enough to prevent emitted fluorescence of the reporter. A phosphate molecule is usually attached to the terminal 3′-thymine residue to prevent extension of the bound probe during amplification. The fluorescent emission spectrum of FAM is 500–650 nm. The fluorescent intensity of the quenching dye TAMRA changes very little over the course of PCR amplification. The intensity of TAMRA dye emission therefore serves as an internal standard with which to normalize the reporter (FAM) emission variation. Following each thermal denaturation step, the temperature is lowered to allow annealing of the probe to single-stranded amplicons. Increasing amounts of the single-stranded amplicons will bind increasing amounts of the probe. During primer extension, *Taq* polymerase cleaves the probe from the 5′ to the 3′ direction releasing the reporter dye, which then emits fluorescence as a result of its increased distance from the quencher. Fluorescence is then measured following each extension stage of every cycle.

Eclipse probes are proprietary double-dye probes available from Epoch Biosciences and Fluoresentric (Table 1.4). They differ from TaqMan probes in that the fluorophore is bound to the 3′ end and the quencher to the 5′ end. Prior to binding, the fluorophore is in close proximity to the quencher. The presence of the quencher at the 5′ position prevents digestion by *Taq* polymerase. Fluorescence is measured during the annealing step. Black hole quenchers are molecular species that exhibit no inherent fluorescence themselves that are used in conjunction with fluorogenic reporter dyes with dual-labeled probes. Primer–dimers are not detected by any of these dual-labeled probes, which constitutes an additional advantage in their use.

3. Fluorescent Resonance Energy Transfer (FRET)

FRET involves the incorporation of two different custom-synthesized oligonucleotide probes into the PCR. One probe (light donor) harbors a fluorescein label at its 3′ end and the other probe (light acceptor) is labeled with LightCycler Red 640 at its 5′ end (Figure 1.10). The sequences of the two probes are selected so that they can hybridize to the same strand of an amplicon in a head-to-tail orientation internal to both primers resulting in the two fluorescent dyes coming into close proximity to each other. Under UV, the fluorescein emits a green light, which then excites the Red 640 dye because of their close proximity, which in turn emits a red light proportional to the amount of amplicon present. The red emission is measured at 640 nm. This energy transfer is referred to as FRET and occurs when no more than one to five nucleotides separate the two dyes. Fluorescence is measured after each annealing step of every cycle. After annealing, the temperature is raised and the hybridization probes are displaced by the *Taq* polymerase. Primer–dimers are not detected.

Table 1.4 Commercial Sources of Real-Time PCR Primers and Fluorescent Probes

Source	Double Dye[a]	FRET Probes	Molecular Beacons	Other
BioNexus Inc./ABP www.bionexus.net	+	+	+	
Biosearch Technologies www.biosearchtech.com	+	+	+	Black hole quenchers
Proligo (formerly Genset) www.proligo.com	+	+	+	
Epoch Biosciences www.epochbio.com	–	–	–	Eclipse probes
Qiagen Operon www.operon.com	+	+	+	Black hole quenchers
Eurogentec SA www.eurogentec.com	+	+	+	Scorpions primers
Molecular Research Laboratories www.molecula.com	+	+	+	
Gene Link Inc. www.genelink.com	+	+	+	
Integrated DNA Technologies www.idtdna.com	+	+	+	
Midland Certified Reagent Co. www.mcrc.com	+	+	+	
Sigma-Genosys www.sigma-genosys.com	+	+	+	
TriLink BioTechnologies www.trilinkbiotech.com	+	+	+	
Applied Biosystems www.appliedbiosystems.com	+	+	+	
Invitrogen www.invitrogen.com	+	+	–	Lux primers
Fluoresentric www.fluoresentric.com	+	+	+	Eclipse primers
Roche Applied Science www.Roche-Applied-Science.com	+	+	+	
Molecular Probes www.probes.com	+	+	+	
Fluorosentric www.fluoresentric.com	+	+	+	Eclipse probes

[a] TaqMan™ probes are proprietary double-dye probes synthesized by Perkin Elmer Applied Biosystems.

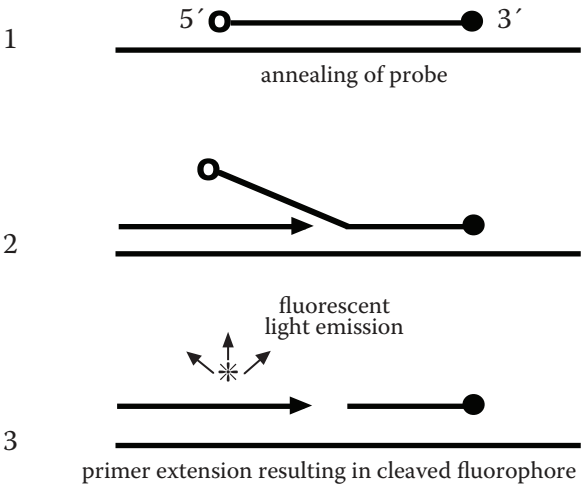

Figure 1.9 Mechanism of TaqMan fluorescence probes. The 5′ nuclease activity of *Taq* DNA polymerase is utilized to cleave a TaqMan probe during DNA amplification. (1) The TaqMan probe is annealed to the target DNA sequence. The probe contains a reporter dye (o) at the 5′ end of the probe and a quencher dye (●) at the 3′ end of the probe. (2) During PCR amplification, a complementary strand of DNA is synthesized and the 5′ exonuclease activity of *Taq* polymerase excises the reporter dye. (3) Fluorescence of the reporter dye (o) occurs when it is separated from the quencher dye and the intensity is measured.

An interesting variation of FRET involves the use of a single probe labeled at the 5′-end with a reporter dye LCRed64 or Cy5 and the addition of SYBR green I to the PCR mix (Loh and Yap, 2002). The labeled probe hybridizes to the homologous sequence of the amplified target strand and then SYBR green I binds to the resulting double-stranded DNA so as to excite the reporter dye. Primer–dimers are distinguished from the amplified target DNA on the basis of different T_m values obtained after the PCR.

4. Molecular Beacons™

Molecular Beacons™ is the proprietary trade name of custom-synthesized nucleotide probes available from Stratagene with GC-rich complementary terminal nucleotides that form a hairpin configuration with a hybrid stem (Figure 1.11).

A variety of such probes is available from a number of commercial sources (Table 1.4). The probes harbor a reporter dye at one end and a quencher dye at the other end. The reporter dye or fluorophore is close to the quencher dye and is nonfluorescent in the hairpin configuration. The energy taken up by the fluorophore is transferred to the quencher and released as heat rather than being emitted as light. When the probe hybridizes to its homologous nucleotide sequence of the amplicons, a rigid double helix is formed that separates the quencher from the reporter dye resulting in emission of UV-induced fluorescence. Fluorescence is then measured after the annealing step of each cycle.

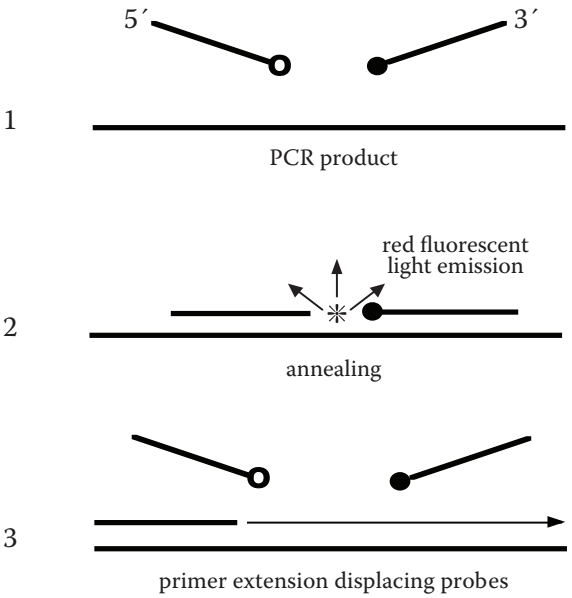

Figure 1.10 Mechanism of resonance fluorescence energy transfer (FRET). Two different oligonucleotide labeled probes are depicted. Probe 1 carries a fluorescein label at its 3′ end and probe 2 is labeled with Light Cycler Red 640 or another suitable fluorescent dye such as Cyanin 5 at its 5′ end (frame 1). The sequences of the two probes are chosen so that they can hybridize to the PCR product in a head-to-tail orientation, resulting in close proximity of the two fluorescent dyes (frame 2). The first dye (fluorescein) is excited by the cycler's light source and emits a green fluorescent light. Close proximity (within one to five nucleotides) of the two dyes results in the emitted green fluorescence exciting the Light Cycler Red 640 dye attached to the 5′ end of the second probe, which in turn emits a red fluorescent light that is measured. This energy transfer is known as FRET. Increasing amounts of DNA resulting from the PCR yields a proportional increase in red fluorescence. Because red fluorescence at 640 nm occurs only when both probes are hybridized, fluorescence is measured after annealing. During primer extension, the hybridized probes are displaced by the *Taq* polymerase and are then too distant from each other for FRET to occur (frame 3).

5. Unique Fluorogenic Primers

Nazarenko et al. (2002) reported on the development of unique fluorescent primers for use with multiplex quantitative Rti-PCR (QRti-PCR). Fluorogenic primers are labeled with a single fluorophore on a base close to the 3′ end with no quencher. A tail of 5–7 nucleotides is added to the 5′ end of the primer to form a blunt-end hairpin loop when the primer is not incorporated into a PCR product. This design provides a low initial fluorescence of the primers that increases up to eightfold upon formation of the PCR product. The hairpin oligonucleotide primers provide additional specificity to the PCR by preventing primer–dimer formation and mispriming. Invitrogen custom-synthesizes such single-fluorophore hairpin loop primers under the proprietary designation LUX™ primers. No probes are required.

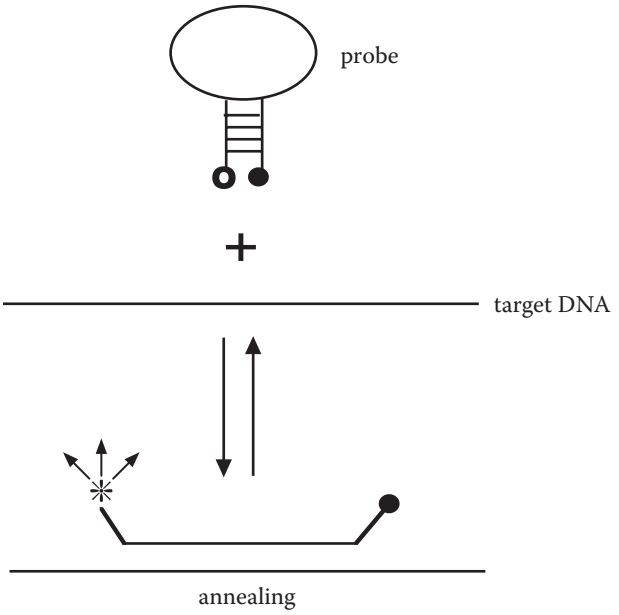

Figure 1.11 Mechanism of molecular beacon probes. The molecular beacon probe in its hairpin configuration is nonfluorescent because of the close proximity of the fluorescent dye such as fluorescein (6-FAM) (o) and quencher dye such as DABCYI (●) maintained by the hybrid stem. Hybridization of the probe sequence in the loop with its homologous target sequence results in physical separation of the two dyes allowing fluorescence to occur. Fluorescence is measured after thermal denaturation and annealing of the probe at a low temperature (~41°C), notably lower than the optimum temperature for primer annealing (~52°C).

Scorpions™ are PCR primers with a "stem-loop" tail containing a fluorophore and quencher (Figure 1.12). The "stem-loop" tail is separated from the PCR primer sequence by a "PCR blocker" that prevents the *Taq* polymerase from copying the stem-loop sequence. During PCR, Scorpion primers are extended to form PCR products. During the annealing phase, the probe sequence in the Scorpion's tail curls back to hybridize to the target sequence in the newly formed PCR product. Because the tail of the Scorpions and PCR product are now part of the same strand, the interaction is considered intramolecular and the incorporated fluorophore is at a distance from the quencher. The recommended length of the "loop" is 20 to 35 nucleotides.

D. Theory of Quantitative Real-Time PCR (QRti-PCR)

QRti-PCR requires the design and use of proper controls for quantitation of the initial target sequences. Heid et al. (1996) were the first to develop such QRti-PCR methodology and made use of the TaqMan reaction with FAM as the reporter dye and TAMRA as the quencher. The software calculates a value termed ΔRn or ΔRQ from the following: $\Delta Rn = (Rn^+) - (Rn^-)$, where Rn^+ = emission intensity of reporter/

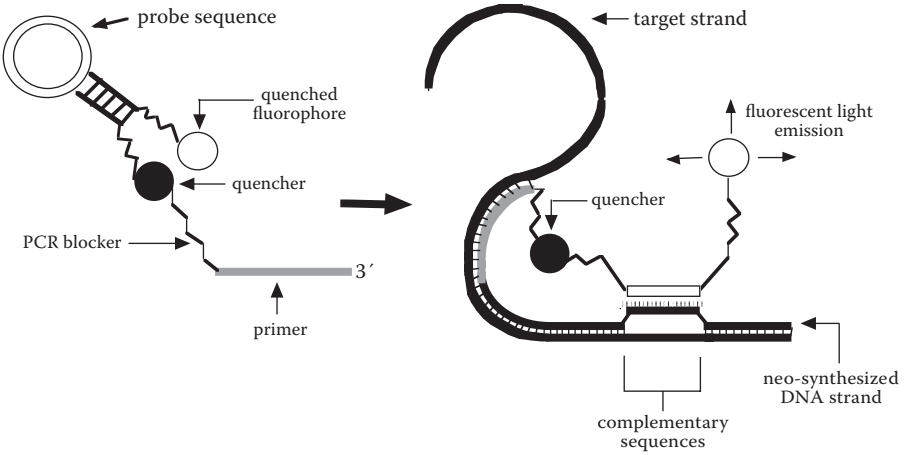

Figure 1.12 Mechanism of Scorpions™ labeled primers. During PCR, Scorpions primers are extended to form PCR products. During annealing the probe sequence in the Scorpion's tail curls back to hybridize to the target sequence in the newly formed PCR product so that the fluorophore (reporter dye) (o) is incorporated into the newly synthesized strand at a considerable distance from the quencher (●).

emission intensity of quencher at any given time in a reaction tube, and $Rn^- =$ emission intensity of reporter/emission intensity of quencher measured prior to PCR amplification in the same reaction tube. The ΔRn mean values are plotted on the *y*-axis, and the number of cycles is plotted on the *x*-axis. During the early cycles, the ΔRn remains at baseline.

When a sufficient amount of hybridization probe has been cleaved by the 5′-nuclease activity of *Taq* polymerase, the intensity of reporter fluorescence emission increases. A threshold level of emission above the baseline is selected, and the point at which the amplification plot crosses the threshold is defined as C_T (threshold cycle) and is reported as the number of cycles at which the log phase of product accumulation is initiated (Figure 1.6). The threshold is usually set at 10 times the standard deviation of the baseline. By setting up a series of wells containing a 4–5 log span of genomic DNA concentration (each concentration in triplicate wells) a series of amplification plots is generated by the software in real time (Figure 1.13). The amplification plots shift to the right as the quantity of input target DNA is reduced. Note that the flattened slopes and early plateaus do not influence the calculated C_T values. The C_T values decrease linearly with increasing target quantity. A plot of the resulting C_T values on the *y*-axis versus the log of the ng of input genomic DNA yields a straight line (Figure 1.14), which is then used as a standard curve for quantitation of samples with unknown levels of genomic DNA. This approach and the original nomenclature of Heid et al. (1996) have been universally adapted for quantitative PCR. However, this methodology does not address the issue that PCR inhibitors may be present in a DNA sample derived from a complex food product.

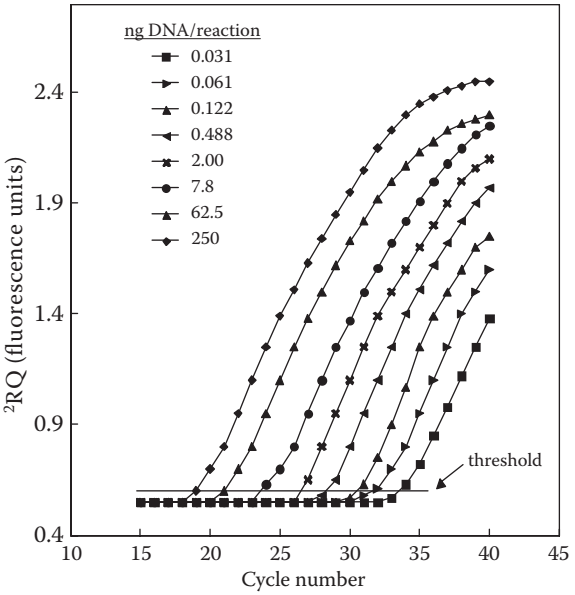

Figure 1.13 Appearance of real-time PCR screen display of thermal cycling plots of target DNA amplification. Decreasing the quantity of target DNA results in an increased number of cycles required to detect fluorescence above the threshold level (shift of the plot to the right).

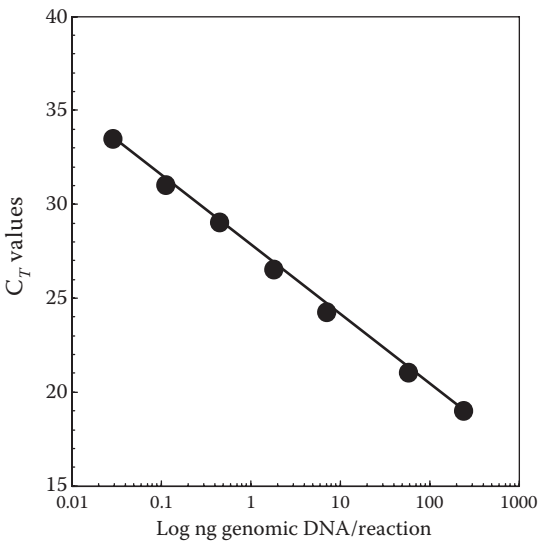

Figure 1.14 Standard curve for real-time PCR quantification of target DNA.

Detection of PCR inhibition and normalization of C_T values can be accomplished with the use of either of two methods: (1) splitting the sample into two parts, where one portion is subjected to QRti-PCR and the second portion is used to amplify an external control standard, or (2) quantitative comparative PCR using a normalization or "housekeeping" gene contained within the sample for QRti-PCR for correction of the observed C_T values. If equal amounts of nucleic acid are analyzed for each sample and if the amplification efficiency is identical for each sample, then the internal control (normalization gene or competitor) should give equal signals for all samples (Heid et al., 1996).

E. Problems and Limitations of QRti-PCR

Wilhelm, Hahn, and Pingoud (2000) found that with the LightCycler instrument unexpected variations in QRTi-PCR experiments occurred. This unit is a rapid thermal cycler that utilizes temperature-controlled airflow for heating and cooling samples. The PCRs were performed in glass capillaries placed along the perimeter of a circular carousel with 32 positions. The shape and amplitude of fluorescence curves as well as the calculated amounts of target DNA exhibited wide variations that were found to be dependent on the position of a particular capillary in the carousel. Interestingly, the presence of capillaries in the carousel was found to alter the temperature stability or airflow inside the carousel chamber. Revised software (version 5.32) ensuring the continued rotation of the sample carousel significantly reduced temperature variation in the carousel and eliminated the large variations in results.

The incorporation of an internal control in a multiplex format to detect inhibition of amplification is critical for the detection of false negative results. However, if QRti-PCR is to be successfully used for enumerating the initial number of gene targets, then a control is necessary to detect the extent to which positive amplification may be partially inhibited. Such a "quantitative" control can assume several formats. The simplest and most direct approach is to split the sample into two PCR tubes, where one tube is used to amplify the target sequence to be enumerated and the second tube is used to amplify a different control target sequence but with ideally identical primers (external control). Standard curves are prepared for both targets. The extent to which the external control deviates from its standard curve is then used to correct and normalize the C_T value for the target being quantified.

A second approach is to establish a multiplex PCR incorporating primers for the primary target sequence of interest and primers for a control sequence plus a known number of control targets to be simultaneously amplified. The use of TaqMan probes differing with respect to the fluorophores and their emission wavelengths will then allow the amplification of both target sequences to be followed. For example, if 100 control target sequences in a PCR that normally have a C_T of 30 cycles without inhibition yield a C_T of 35 cycles, then a corresponding correction factor can be applied to the C_T derived from the target being quantified. Alternatively, both TaqMan probes can be labeled identically and the multiplex PCR performed in two tubes: one tube containing the TaqMan probe for the primary target and the second tube containing the other TaqMan probe for the internal control sequence. Such multiplex reactions

with an internal control are predicated on an abundance of *Taq* polymerase and dNTP precursors such that they do not become limiting as a result of competitive PCR.

The use of universal primers for QRti-PCR of Eubacteria using the highly conserved 16S or 23S rRNA gene sequences is ideally suited for samples containing large numbers of bacteria (>10^4/g). The detection or quantitation of low numbers of bacteria with nonspecies- or nongenus-specific primers is fraught with problems arising from the presence of low numbers of contaminating organisms and DNA from PCR reagents. It is well documented that *Taq* DNA polymerase preparations frequently contain contaminating DNA as a result of incomplete purification during manufacture (Böttger, 1990; Rand and Houck, 1990).

Taq DNA polymerase is commonly produced as a recombinant protein in *E. coli* or is obtained as a native enzyme from *T. aquaticus*. Corless et al. (2000) examined this problem in detail with Rti-PCR using a set of primers targeting conserved flanking sequences of the eubacterial 16S rRNA gene and a TaqMan probe yielding an 87-bp amplicon from *E. coli*. The study included a comparison of four *Taq* DNA polymerases from three commercial sources with respect to their ability to produce amplicons in Rti-PCR assays without added target DNA (negative controls). All four *Taq* DNA polymerases yielded false Rti-PCR positive results. More than one amplicon was identified by DNA sequencing, reflecting contamination by DNA from more than one organism. The authors concluded that the problem is exacerbated by the highly sensitive nature of the Rti-PCR process and the fact that the 16S rRNA gene is present in multiple copies (up to seven) in the genomes of many bacteria. The study indicated that low-DNA LD Ampli*Taq* polymerase (Applied Biosystems) yielded the highest C_T values in Rti-PCR assays, among the four *Taq* enzymes examined, without added DNA, reflecting the lowest amount of contaminating bacterial DNA.

III. NESTED PCR

A. Introduction

The use of a pair of "nested primers" flanking a DNA sequence internal to the sequence encompassed by the external pair of primers allows a greater level of amplification than is normally achieved with a single set of primers. A target sequence is initially amplified by an external pair of primers for 20–40 cycles. A small aliquot of this reaction is then amplified a second time for 20–40 cycles using the internal or nested primer set (Figure 1.15). The inner pair of primers anneals to complementary sequences internal to the initially amplified product. This has been shown to result in greater amplification than reamplifying with the same initial pair of primers (Albert and Fenyo, 1990). The number of nucleotides flanked by the internally nested primers determines the final product size. The nucleotide sequence of the nested product of the second stage of amplification is always shorter than the primary PCR product.

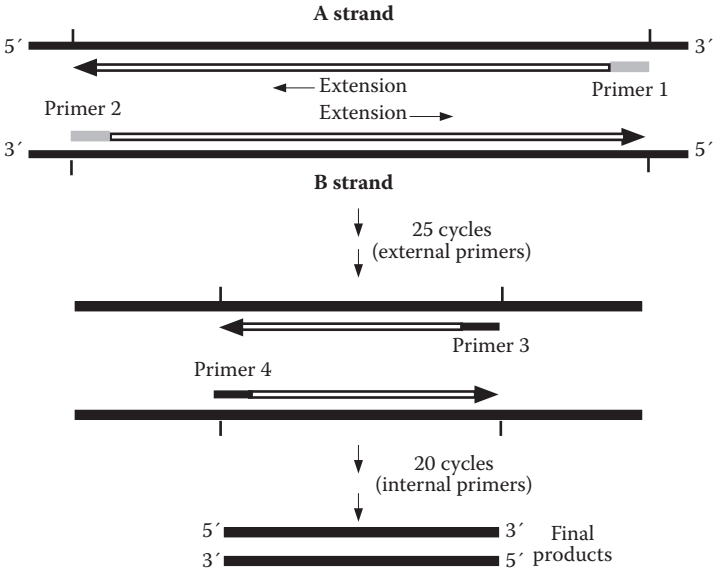

Figure 1.15 Two-tube nested PCR. Amplification with external primers for 25 cycles followed by amplification of an aliquot for 25 cycles using internal nested primers.

Nested PCR is of particular value with foods in the absence of enrichment cultivation where inhibitors present from the food may significantly reduce the efficiency of amplification. In addition, the nested primers serve as a control for the specificity of the amplified external sequence and can therefore improve both sensitivity and specificity of DNA amplification (Jackson, Hayden, and Quirke, 1991). A major advantage of nested PCR is that the second round of amplification can result in an increase in the sensitivity of detection of several orders of magnitude over that achieved with just primary amplification alone. An additional advantage is that of enhanced specificity in that it is unlikely that any nonspecific product of amplification in the primary PCR will contain sequences complementary to the primers of the secondary PCR.

Nested PCR, however, introduces an additional risk of amplification of contaminating DNA when used routinely. This problem can be circumvented by using a "one-tube nested PCR" involving the use of two primer pairs of different melting points (T_m values) in a single reaction mixture. Annealing of the nested primers during the first set of cycles is prevented by their lower T_m. The larger external fragment should ideally have a higher G+C content so that it will denature at a slightly higher temperature than the nested fragment. This difference in T_m values for the two amplified products has to be critically determined for both the larger and the nested fragment. A number of mathematical approaches have been developed for calculating the T_m based on either the G+C content of the fragments or slightly more accurate values based on nearest-neighbor thermodynamics (Breslauer et al., 1986; Freier et al., 1986; Rychlik, Spencer, and Rhoads, 1990). Other calculations of T_m

values for primers are based on formulas developed for nucleotides over 100 bp in length (McConaughy, Laird, and McCarthy, 1969). The equation of Suggs et al. (1981), $T_m = 2°C \times (A + T) + 4°C \times (G + C)$ is widely used for its convenience and approximate determination of T_m values of primers. In addition, the GCG software program "Prime" will readily determine T_m values for primers.

Nested PCR has been applied to the detection of a variety of pathogens in a number of foods (Ozbas et al., 2000; Lindquist, 1999; Waage et al., 1999; Gilgen et al., 1998; Kapperud et al., 1993). The use of immunomagnetic capture of target cells prior to nested PCR has been found to increase the sensitivity of detection of *E. coli* O157:H7 from ground beef without enrichment cultivation from 110 CFU/10 g to 24 CFU/10 g (Guan and Levin, 2002a) presumably due to magnetic separation of captured cells from PCR inhibitors.

IV. LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) ASSAY

A. Introduction

LAMP was first developed by Notomi et al. (2000) and utilizes a DNA polymerase isothermally at 60–65°C and a set of four primers that recognize a total of six distinct sequences on the target DNA. An inner primer containing sequences of the sense and anti-sense strands of the target DNA initiates LAMP. The resulting strand displacement DNA synthesis primed by an outer primer releases a single strand of DNA. This then serves as a template for DNA synthesis primed by the second inner and outer primers that hybridize to the other end of the target, which produces a stem-loop DNA structure. In subsequent LAMP cycling one inner primer hybridizes to the loop on the product and initiates displacement DNA synthesis, yielding the original stem-loop DNA and a new stem-loop DNA with a stem twice as long. The cycling reaction continues with an accumulation of 10^9 copies of target in less then 60 min. LAMP reactions usually result in about 10^3-fold or higher levels of amplification than conventional PCR. The final products are stem-loop DNAs.

With an additional one or two primers termed "loop" primers, the reaction can be accelerated. The method is capable of yielding an unusually large amount of DNA, more then 500 μg. At the completion of amplification a white precipitate forms that has been identified as magnesium pyrophosphate (Mori et al., 2001), which can be used to confirm amplification. The yield of synthesized DNA, can be quantified by measuring the intensity of fluorescence using ethidium bromide in agarose gels or in an Rti-PCR unit or alternatively by measuring turbidity. The level of turbidity has been found to correlate with the amount of synthesized DNA, which is related to the number of initial target sequences. The identity of the DNA polymerase was found to be a critical factor for efficient amplifcation. *Bst* polymerase (Takara) is preferred along with the presence of betaine or L-proline, which were found to stimulate the rate of amplification and to increase selectivity (Notomi et al., 2000). DNA amplification can be further greatly accelerated by the use of two additional primers termed loop primers resulting in detection of 600 templates in 13 min (Nagamine, Hase, and Notomi, 2002).

V. PULSED-FIELD GEL ELECTROPHORESIS (PFGE)

A. Introduction

PFGE is presently considered the method of choice and is frequently referred to as the "gold standard" for discriminating genetic differences and lineage among strains of the same bacterial species. The method is particularly useful in epidemiology and has also been applied to the detection of clonal strains of pathogens that have been found to persist in food manufacturing facilities (Johansson et al., 1999; Autio et al., 1999; Brett, Short, and McLauchlin, 1998).

B. Mechanism of PFGE

PFGE is based on the use of low-frequency restriction nucleases to generate a family of high molecular weight fragments derived from genomic DNA and their subsequent resolution based on size using changes in the direction of the electric field during agarose electrophoresis. Large fragments stretch out linearly in the direction of the electric field. When the direction of the field changes, the fragments undergo an initial relaxation in conformation and then form multiple undulations or kinks in the direction of the new field, followed by linearization (Gurrieri et al., 1990). At present the most widely adapted system is the contour-clamped homogeneous electric field (CHEF) apparatus, involving an hexagonal distribution of electrodes that undergo periodic alternate uniform electric fields with an angle of 120° (Figure 1.16). CHEF instruments are available from Bio-Rad, Pharmacia, and BRL. A longer pulse time increases the size of the fragment that can be separated but results in a decrease in resolution of fragments of similar size.

Progressive increases in pulse time (pulse time ramping) can significantly increase resolution. A ramp from 5 to 40 s will allow optimal separation of DNA fragments from 50 kb to 600 kb, and increasing the pulse time to 75 s will extend the separation

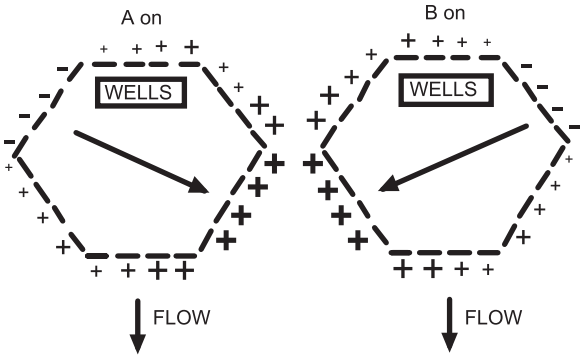

Figure 1.16 Voltage clamping by the CHEF-DR-II system. A: Relative electrode potentials when the +60 volt field vector is activated. B: Relative electrode potentials when the –60 volt field vector is activated.

to 1 Mb (Struelens, De Ryck, and Deplano, 2001). PFGE is usually performed at 12–15°C for enhanced band resolution and to prevent the development of temperature gradients in the gel. Agarose of high gel strength and low electro-endosmosis (EEO) is used. DNA fragments from 50 kb to 1 Mb are usually separated with 1.0% agarose. Reducing the agarose concentration will increase the pore size and allow separation of larger fragments. Higher concentrations of agarose (1.2 to 1.6%) reduce pore size and will increase resolution and sharpness of bands, but reduce separation of larger fragments and result in longer running times (Struelens, De Ryck, and Deplano, 2001). Birren et al. (1988) have reported on optimized conditions for PFGE and the effects of a variety of variables on the resolution of DNA fragments.

The restriction nucleases used with PFGE are selected on the basis of rarity of their recognition sequence in the target genome. Struelens, De Ryck, and Deplano (2001) and Tenover et al. (1995) have listed restriction nucleases useful for PFGE analysis of a number of bacterial genera and species.

The usual methodology for DNA extraction is unsuitable for PFGE because the large size of genomic bacterial DNA (~5×10^9 Da) results in rapid shearing during pipetting to ~1×10^7 Da. To prevent such rupture of large DNA molecules, intact cells are embedded in an equal volume of 2.0% nuclease-free, low-melting-temperature agarose plugs. The cells are then lysed in situ, immersing the plugs first in lysozyme and then proteinase K, or other suitably lytic enzymes for the specific organism involved. To save time, lysozyme can be added to the agarose–cell mixture before solidification. The agarose plugs are then placed in wells for PFGE. Uniform cell densities of $1–5 \times 10^9$ cells/ml are critical for valid comparison of resolved bands. Adjusting cell suspensions to a uniform A_{600} value is reliably effective and convenient, provided each culture is of the same age. To ensure adequate alignment and normalization of banding patterns and accurate fragment size estimates, appropriate DNA ladders should be included in at least every fifth lane (Struelens, De Ryck, and Deplano, 2001).

C. Interpretation of PFGE Banding Patterns

Distinctions in banding patterns are based on the size and shape of resolved bands. The problem of how minor differences in banding patterns should be interpreted has been dealt with in detail by Tenover et al. (1995) with the establishment of well-reasoned criteria based on the resolution of at least ten distinct fragments derived from each culture being compared. The utility of strain typing using PFGE is based on the assumption that isolates derived from the same reference strain are of recent lineage from the original strain. A limitation occasionally arises when unrelated isolates may have similar or indistinguishable genotypes, especially if there is limited genetic diversity within a species or subtype (Barrett et al., 1995). It should also be assumed that random genetic events, including endpoint mutations and deletions and insertions of nucleotides, will alter PFGE patterns of DNA from progeny derived from an original reference strain. An isolate is interpreted to be closely related to a reference strain if its PFGE pattern differs from the reference strain by

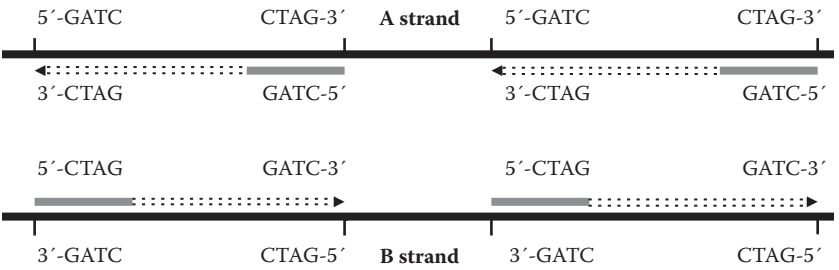

Figure 1.17 Random amplification of polymorphic sequences with a single random primer.

changes reflecting a single genetic event, that is, a point mutation or a frame shift mutation involving the insertion or deletion of one or more sequential nucleotides.

Such changes usually result in two to three band differences. For example, a single spontaneous mutation that creates a new genomic restriction site will split one restriction fragment into two smaller ones. The loss of the original large fragment plus the appearance of two new smaller fragments will result in a three-band difference between the reference strain and its direct progeny that has undergone such a single mutational event (Tenover et al., 1995). In addition, variations of two to three bands have been reported in strains repeatedly cultured or isolated multiple times from the same patient (Arbeit et al., 1993; Sader et al., 1993). Patterns that are distinctly different from an outbreak or reference strain by only two or three fragments should therefore be considered subtypes of the same clonal lineage. An isolate is considered to be possibly related to a reference strain if there are four to six band differences. An isolate should be considered unrelated to a reference strain if its PFGE pattern differs by seven or more bands reflecting three or more independent genetic events.

VI. RANDOM AMPLIFICATION OF POLYMORPHIC DNA (RAPD)

A. Introduction

The use of a single random primer with RAPD will yield significantly fewer DNA bands than PFGE with a single restriction nuclease and is therefore notably less discriminating then PFGE in terms of distinguishing various isolates of the same species. However, when the results derived from the use of three random primers independently are combined, the discriminatory power is usually equal to that of PFGE and is still less costly and time consuming.

RAPD, also referred to as arbitrarily primed PCR (AP-PCR) analysis, has been found to be a rapid and valuable technique for distinguishing different strains of the same species (Lawrence, Harvey, and Gilmour, 1993) with a high level of strain discrimination (Lawrence and Gilmour, 1995). It is a particularly useful technique for genetic typing of human pathogens from foods, processing plants, and food-borne outbreaks. In addition, it has been found to be a powerful method that can

advantageously replace other more cumbersome typing methods such as serotyping, ribotyping, multilocus enzyme electrophoresis, restriction enzyme analysis, and phage typing, and has been found to be very efficient in differentiating strains while still allowing the clear recognition of clusters (Boerlin et al., 1995). RAPD has been found capable of distinguishing strains of a given species with identical 16s rDNA sequences (Czajka et al., 1993). This high level of discrimination should allow RAPD to be used in establishing the persistence of a specific strain in foods and in processing plants and its distinction from transient strains of the same species.

B. Mechanism of RAPD

In conventional PCR, a known DNA sequence is amplified by using two primers, one that anneals to the 3′ end of the sequence on the A strand and is extended inward with *Taq* polymerase from the 3′ end of the primer. The second primer anneals to the 3′ end of the B strand and is also extended inward from the 3′ end (Figure 1.1). After the first cycle and denaturation, four target sequences are then available for duplication. The sequence is usually highly specific for the target gene and occurs only once (monomorphic) in the genomic DNA of the organism.

With RAPD a single random primer of about ten nucleotides is used with no known target sequence being required, and the first round of amplification results in single strands having palindromatic termini (Figure 1.17). The single randomly chosen primer targets specific but unknown sites in the genomic DNA, which are polymorphic (repeating) with respect to the terminal sequences to which the single primer anneals. During subsequent cycles a number of different target sequences are amplified, many of which will be of differing base pair length so as to generate a variety of DNA agarose bands resulting in a specific DNA banding pattern for each culture (Figure 1.18).

The ratio of primer to template in the RAPD reaction is critical. Template DNA concentration should be carefully titered against a standard concentration of primer so as to reveal the most reproducible amplified products (del Tufo and Tingey, 1994). A hazy smear obscuring the amplified bands on the agarose gel is usually caused by failure to saturate the DNA template with primer. This is easily corrected by adjusting the ratio of primer to template DNA (del Tufo and Tingey, 1995). Meuner and Grimont (1993) expressed concern about the reproducibility of RAPD profiles but concluded that reproducibility was excellent with standardized methodology. They found that reproducibility of banding patterns was dependent on the make of the thermal cycler, with variations in patterns occurring with less rigid temperature control. Reproducibility of RAPD with whole cells is critically dependent on all strains being in the same stage of growth (Boerlin et al., 1995). Mazurier and Wernars (1992) obtained reproducibility in RAPD profiling of strains of *L. monocytogenes* by using suspensions of washed whole cells grown overnight in broth that were adjusted to an absorbance at 600 nm of 1.5 yielding 7.5×10^6 CFU/ml 5 µl of the cell suspension and that were then incorporated directly into a 50-µl PCR reaction volume without a prior cell lysis step. The same cell density among isolates

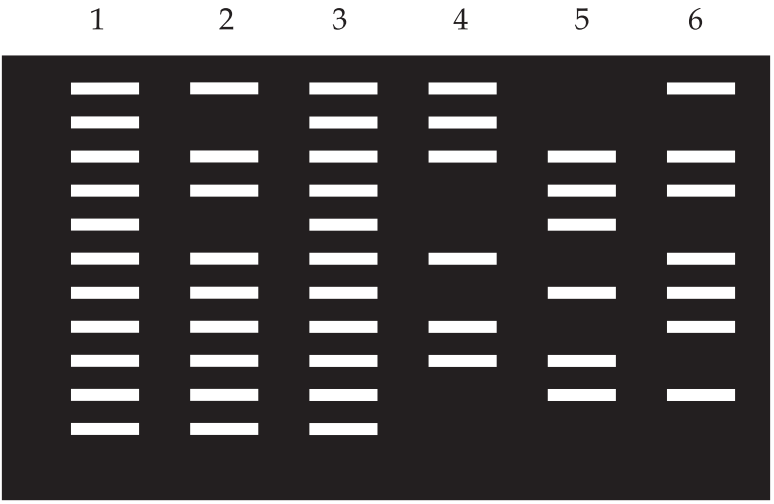

Figure 1.18 RAPD banding profiles of six bacterial isolates of the same species. Isolates corresponding to lanes 1 and 3 have identical banding patterns and are therefore considered of identical clonal origin. Isolates corresponding to lanes 2 through 6 are distinguishable from each of the other isolates.

being subjected to RAPD comparison is particularly important for reproducibility of faint bands.

VII. MULTILOCUS SEQUENCE TYPING (MLST)

A. Introduction

MLST makes use of the PCR to amplify sequences of several housekeeping genes (see Table 11.2 in Chapter 11). The resulting amplicons are then subjected to sequence analysis, and the sequences derived from the various isolates are compared with respect to each individual gene for differences.

VIII. RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) AND PCR-RFLP

A. Introduction

RFLP refers to the application of one or more restriction nucleases directly to the genomic DNA of a bacterium and the resulting products are separated electrophoretically as bands in an agarose gel. PCR-RFLP refers to the initial amplification of a specific gene sequence by PCR followed by restriction of the resulting amplicons with one or more restriction nucleases and separation of the products

electrophoretically in an agarose. With both techniques the numbers of bands from each isolate and their number of base pairs are used for distinguishing isolates.

IX. AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP)

A. Introduction

AFLP involves digestion of purified genomic DNA using a restriction nuclease such as *Hind*III, followed by the ligation of the resulting fragments to a double-stranded oligonucleotide adapter that is complementary to the base sequence of the restriction site using T4 DNA ligase. The adapters are designed such that the original restriction sites are not restored after ligation, thus preventing further restriction digestion. Because the adapters are not phosphorylated, adapter-to-adapter ligation is prevented. Selective amplification by PCR of sets of these fragments is achieved using primers corresponding to the contiguous base sequences in the adapter restriction site plus one or more nucleotides in the original target DNA. The resulting DNA fragments amplified by PCR are then resolved by gel electrophoresis.

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CHAPTER 2

Escherichia coli O157:H7

I. CHARACTERISTICS OF THE ORGANISM

Enterohemorrhagic *E. coli* (EHEC) of serotype O157:H7 causes hemorrhagic colitis, which is characterized by microangiopathic haemolytic anaemia, thrombocytopenia, and central nervous system symptoms (Karmali, 1989) that may develop into life-threatening renal failure involving hemolytic-uremic-syndrome (HUS), particularly with children. *E. coli* isolates are serologically distinguished on the basis of three major surface antigens: somatic (O), flagella (H), and capsule (K) antigen. Although more than 100 *E. coli* serotypes produce Shiga-like toxins (SLTs), O157:H7 is the predominant serotype implicated in foodborne diseases. *E. coli* O157:H7 strains are sorbitol negative, β-D-glucuronidase negative, exhibit poor or no growth at 44.5°C, possess an attaching and effacing gene (*eae*), possess a 66-MDa plasmid, and express an uncommon 5000 to 8000 mol. wt. outer membrane protein (OMP; Padhye and Doyle, 1991). The infectious dose is considered to be about 1 CFU/g of raw food (Centers for Disease Control and Prevention, 1995; Griffin et al., 1994; Zhao, Doyle, and Wang, 1994).

II. VIRULENCE FACTORS

A. Hemolysins

Two different plasmid-encoded hemolysins have been described for Shiga toxin-producing *E. coli* (STEC). Alpha hemolysin encoded by the *hlyA* gene is formed by porcine edema disease-causing STEC strains that produce Stx variant 2e (Imberechts, De Greve, and Lintermans, 1992) and by *E. coli* causing urinary tract infections and septicemia (Korhonen et al., 1985; Orskov and Orskov, 1985). The alpha hemolysin produces a broad zone of hemolysis on enterohemolysin agar (Beutin et al., 1989). The second hemolysin encoded by the *elyA* gene is secreted exclusively by human STEC strains and produces a narrow, turbid, hemolytic halo on enterohemolysin agar (Beutin et al., 1989) and is 62% to 64% homologous to the *hlyA* gene (Kuhnert

et al., 1997; Schmidt and Karch, 1996). The two genes *hlyA* and *elyA* harbored by STEC were found to be readily distinguished by PCR-restriction fragment length polymorphism (PCR-RFLP) analysis (Lehmacher et al., 1998). *Alu*I, *eco*RI, and *Mlu*I PCR-RFLP showed that all 93 human isolates of STEC examined harbored only the *elyA* gene and not the *hlyA* gene. Primers *hlyA*-start/*hlyA*-end homologous to both genes were used to amplify a 202-bp sequence of the *hlyA* gene and a 199-bp sequence of the *elyA* gene.

B. Intimin

Intimate adherence to intestinal cells is mediated by intimin, a 94-kda OMP encoded by the *eaeA* locus and results in effacement of underlying microvilli resulting in adhesion and effacement (AEA) lesions. The *eae* gene locus houses the *eaeA*, *eaeB*, and *sep* genes termed LEE for locus of enterocyte effacement and is absent in commensulate strains of *E. coli.*

C. Shiga-Like Toxins

Virulence of *E. coli* O157:H7 involves a number of factors including the production of one or more bacteriophage encoded SLTs, also called verotoxins (VTXs), that are lethal to Vero cells. VT1 and VT2 are synonymous with SLT-I and SLT-II and have been renamed Stx1 and Stx2. Stx1 from various strains are homogeneous whereas Stx2 exhibits heterogeneity among toxin-producing strains resulting in subgroups designated Stx2b, Stx2c, Stx2d, Stx2e, and Stx2f. Stxs consist of linked A and B subunits. The B subunits bind specifically to galactose moieties of globoglycolipids on the eukaryote cell surface. The A subunit is then cleaved from the B subunit, enters the cell, and proceeds to enzymatically rupture an N-glycoside bond of ribosomes so as to prevent the binding of tRNA resulting in the inhibition of protein synthesis.

D. Locus for Enterocyte Effacement

Among the various mechanisms involved in the virulence of *E. coli* O157:H7 is the ability to intimately adhere to enterocytes by an attaching and effacing mechanism. Attaching and effacing lesions are encoded on a pathogenicity island termed the locus for enterocyte effacement (LEE). LEE encodes a type III secretion system and *E. coli* secreted proteins that deliver effector molecules to the host cell and disrupt the host cytoskeleton (Donnenberg, Kaper, and Finlay, 1997; Elliot et al., 1998; Perna et al., 1998). LEE also carries the *eae* gene, which encodes an outer membrane protein (intimin) required for intimate attachment to epithelial cells (Yu and Kaper, 1992) and has been used as a convenient diagnostic marker for LEE-positive STEC strains (Gannon et al., 1993; Louie et al., 1994; Paton and Paton, 1998a). However, the presence of *eae* is not absolutely linked to human virulence in that HUS can be caused by LEE-negative strains (Paton and Paton, 1998b). SLT-II is associated with an increased risk of developing HUS (Boerlin et al., 1999; Kleanthous et al., 1990; Ostroff et al., 1989). Paton et al. (2001) described a gene designated *saa*, which is

carried on a large plasmid of certain LEE-negative but not LEE-positive strains. This gene encodes a novel outer membrane protein that appears to function as an autoagglutinating adhesin.

E. Extracellular Serine Protease

Brunder, Schmidt, and Karch (1997) identified and characterized a novel extracellular protease designated EspP encoded by the large ~90-kb plasmid that cleaves human coagulation factor V required for prothrombin and clot formation. A specific immune response against EspP was detected in sera from patients suffering from EHEC infections. The degradation of factor V is thought to contribute to the prolonged mucosal hemorrhage observed in patients with hemorrhagic colitis.

F. Additional Virulence Factors

All *E. coli* O157:H7 strains harbor a large plasmid of about 66 MDa. However, several other SLT-producing *E. coli* serotypes also harbor this plasmid (Fratamico et al., 1995). An enterohemorrhagic toxin is encoded by this plasmid. In addition, a 90-kb plasmid encodes potential virulence factors, such as an enterohaemolysin (Schmidt, Kernbach, and Karch, 1996), a catalase-peroxidase (Brunder, Schmidt, and Karch, 1996), a serine protease (Brunder, Schmidt, and Karch, 1997), and a type II secretion pathway system (Schmidt, Henkel, and Karch, 1997). PCR detection relying solely on sequences specific for these plasmids will not guarantee detection of EHEC O157:H7 strains.

III. PHENOTYPIC VARIATION OF *E. COLI* O157:H7

Phenotypic variation has been found to occur in strains of *E. coli* O157:H7. Feng (1995) reported the recovery of sorbitol-fermenting isolates of *E. coli* O157:H7 from sorbitol-containing foods. A case of blood diarrhea in 1995 was reported to yield a β-D-glucuronidase-producing strain of *E. coli* O157:H7 (Hays et al., 1995). Ware, Abbott, and Janda (2000) reported on the percentage of phenotypically aberrant strains of *E. coli* O157:H7 among a total of 657 isolates. Typical reactions observed were as follows: no acid from rhamnose (1.2%), no acid from sucrose (0.8%), no acid from lactose (0.8%), no acid from glucose (0.8%), positive hydrolysis of urea (0.5%), negative production of lysine decarboxylase (0.3%), negative production of ornithine decarboxylase (0.3%), acid from D-sorbitol (0.3%), and negative production of indole (0.3%). None of the strains analyzed possessed multiple aberrant properties, nor were aberrant phenotypes associated with outbreaks with the exception of six strains that failed to ferment L-rhamnos linked to a daycare center outbreak. In addition, 21 strains were found to be nonmotile after incubation at 35°C for 96 hr in motility deeps. Many nonmotile isolates required multiple passages (4–17) before the presence of H7 antigen could be detected. In some instances, the H7 gene could only be demonstrated using the PCR. The authors concluded that such aberrant

reactions could result in misidentification of O157:H7 isolates as some other species and discarded.

Hara-Kudo, Miyahara, and Kumagai (2000) found that strains of *E. coli* O157:H7 were culturable on agar media after being left in dH_2O at 18°C for 21 months. However, a number of cells had lost their O157 O antigenicity. The lost O157 O antigenicity was not recovered by growth in tryptic soy broth (TSB). Other phenotypic characteristics of O157:H7 strains were retained along with the SLT gene.

Wetzel and LeJeune (2007) reported on the isolation of a number of *E. coli* O157:H7 isolates that were PCR-negative for the presence of *slt-I* and various derivatives of *slt-II* but positive for the presence of the *eae* and *ehxA* genes. These isolates were experimentally lysogenized by Stx2-converting bacteriophage to a positive SLT-II state.

A. Conventional PCR

Genes used for PCR detection of *E. coli* O157:H7 and various phenotypic properties of isolates are presented in Table 2.1. Table 2.2 presents primer and probe sequences utilized with *E. coli* O157:H7 PCR assays.

Gannon et al. (1992) developed a PCR assay for detection of *slt-I* and *slt-II* genes in SLT-I- and SLT-II-producing strains of *E. coli* seeded into ground beef. When 25 g of ground beef were cultured in 225 ml of mTSB for 4 hr, 8 CFU/g were detected. With an enrichment time of 6 hr, 0.8 CFU/g was detected. DNA extraction and purification were essential to achieve these levels of detection.

Gilgen et al. (1998) developed a PCR assay for detection of verotoxin-producing *Escherichia coli* (VTEC) in ground beef. Ground beef (10 g) was stomached with 75 ml of homogenization buffer (10 mmolar Tris-HCl at pH 8.0, 150 mmolar NaCl, 2 mmolar ethylenediaminetetraacetic acid (EDTA), 0.1% sodium dodecyl sulfide (SDS), and 0.5 mg/ml pronase with A mesh insert. The bags were then incubated for 30 min at 37°C to release bacterial cells adsorbed to the meat surface. The sample (50 ml) was then centrifuged at 30,000 × g for 30 min. The pellet was then lysed in 2 ml of lysis buffer (10 mmolar Tris-HCl at pH 8.0, 50 mmolar KCl, 1.5 mmolar $MgCl_2$ containing 4 mg/ml of lysozyme, and 0.2 mg/ml proteinase K for 20 min at room temperature followed by 60 min at 60°C. The lysate (1 ml) was then centrifuged for 10 min at 14,500 × g and 450 µl used for nucleic acid isolation with the "Wizard" DNA resin. Ten µl of the 50-µl eluate were then incorporated into a 100-µl PCR reaction volume. Two nested sequential assays were used to detect the genes encoding VT1 and VT2 in various strains of *E. coli* regardless of serotype. The primers I-1/I-2 (Table 2.2) amplified a 614-bp sequence of the *vt1* gene. The internally nested primers I-3/I-4 (Table 2.2) amplified a 347-bp sequence of the *vt1* gene. The primers II-1/II-2 (Table 2.2) amplified a 779-bp sequence of the *vt2* gene. The internally nested primers II-3/II-4 (Table 2.2) amplified a 372-bp sequence of the *vt2* gene. The detection limit in ground beef was 110 CFU/10 g. The use of a 6 hr enrichment resulted in a detection limit of 1 CFU/10 g.

Karch and Meyer (1989) developed a single pair of primers MK1/MK2 (Table 2.2) capable of amplifying a 227-bp and a 224-bp sequence from *slt-I* and

Table 2.1 Genes Used for the PCR Identification of *E. coli* O157:H7

Gene	Description
eaeA	Chromosomal gene encoding intimin, which is involved in attaching and effacing lesions of enterocytes with LEE-positive strains
saa	Large plasmid-bearing gene that encodes an outer membrane protein that functions as an autoagglutinating adhesin in LEE-negative strains but not LEE-positive strains
stx (*slt*)	Encodes the Stx toxin
stx_1 (*slt-I)*	Encodes the Stx_1 toxin
stx_2 (*slt-II)*	Encodes the Stx_2 toxin
stx_{2b} (*slt-IIb)*	Encodes subtype of corresponding Stx_2 toxin
stx_{2c} (*slt-IIc)*	Encodes subtype of corresponding Stx_2 toxin
stx_{2d} (*slt-IId*)	Encodes subtype of corresponding Stx_2 toxin
stx_{2e} (*slt-IIe*)	Encodes subtype of corresponding Stx_2 toxin
stx_{2f} (*slt-IIf*)	Encodes subtype of corresponding Stx_2 toxin
$flic_{H7}$	Encodes the flagellin of H7 serotype
rfbE	Encodes an enzyme necessary for O-antigen synthesis
uidA	Encodes β-glucuronidase
Sil_{O157}	A small 2634 inserted locus (SIL) present in O157 serotypes that encodes an OMP designated IHP1
hly_{933}	Encodes the enterohemolysin of human O157:H7 strains
hly_{21}	Encodes the allelic enterohemolysin of bovine O157:H7 strains
ehxA	Encodes the enterohemolysin
$orfU_{leeO157}$	Encodes the origin of replication for the locus of enterocyte effacement with O157 strains
EspP	Encodes an extracellular serine protease produced by O157:H7 isolates that cleaves human coagulation factor V and thought to result in prolonged hemorrhagic colitis

slt-II, respectively. The primers amplified sequences from a conserved region of both *slt-I* and *slt-II* genes.

Pollard et al. (1990) developed primers for the detection of the *slt-I* and *slt-II* genes harbored by strains of *E. coli*. The primers VT1a/VT1b (Table 2.2) amplified a 130-bp sequence of the *slt-I* gene and primers VT2a/VT2b (Table 2.2) amplified a 346-bp sequence of the *slt-II* gene. Detection of these genes was restricted to strains of *E. coli* with the exception that *slt-I* was detected in five strains of *Shigella dysenteriae*.

Li and Drake (2001) developed a quantitative competitive PCR assay for detection and quantification of *E. coli* O157:H7 in skim milk. The advantage of a competitive PCR assay is that it automatically corrects for partial inhibition of the PCR by components from a food. The primers TXAF/TXAR (Table 2.2) amplified a 401-bp sequence of the *slt-II* gene. The primer TXAF1 in conjunction with TXAR amplified a competitive 275-bp sequence of the *slt-II* gene. Target cop numbers were obtained from the standard curve where the log of the ratio of fluorescence intensities of the competitive band and the target band were plotted against the log of the concentration of competitive molecules and the use of equivalent fluorescent

Table 2.2 PCR Primers and DNA Probes[a]

Primer or Probe	Sequence (5′ → 3′)[a]	Size of Sequence (bp)	Amplified Gene or DNA Target Sequence	Reference
MFS1F	ACG-ATG-TGG-TTT-ATT-CTG-GA	166	60-MDa plasmid	Fratamico et al. (1995)
MFS1R	CTT-CAC-GTC-ACC-ATA-CAT-AT			
MK1	TTT-ACG-ATA-GAC-TTC-TCG-AC	227 and 224	*slt-I* & *slt-II*	Fratamico et al. (1995) from Karch and Meyer (1989)
MK2	CAC-ATA-TAA-ATT-ATT-TCG-GTC			
AE19	CAG-GTC-GTC-GTG-TCT-GCT-AAA	1087	*eaeA*	Fratamico et al. (1995) from Gannon et al. (1993)
AE20	TCA-GCG-TGG-TTG-GAT-CAA-CCT			
LEE	CCA-TAA-TCA-TTT-TAT-TTA-GAG-GGA	633	upstream of *eaeA*	Meng et al. (1997)
LEE	GAG-AAA-TAA-ATT-ATA-TTA-ATA-GAT-CGG-A			
SLTI	TGT-AAC-TGG-AAA-GGT-GGA-GTA-TAC-A	210	*slt-I*	Meng et al. (1997)
SLTI	GCT-ATT-CTG-AGT-CAA-CGA-AAA-ATA-AC			
SLTII	GTT-TTT-CTT-CGG-TAT-CCT-ATT-CC	484	*slt-II*	Meng et al. (1997)
SLTII	GAT-GCA-TCT-CTG-GTC-ATT-GTA-TTA-C			
LP30	CAG-TTA-AAG-TGG-TGG-CGA-AGG	348	*slt-I*	Cebula, Payne, and Feng (1995)
LP31	CAC-CAG-ACA-ATG-TAA-CCG-CTG			
LP43	ATC-CTA-TTC-CCG-GGA-GTT-TAC-G	584	*slt-II*	Cebula, Payne, and Feng (1995)

LP44	GCG-TCA-TCG-TAT-ACA-CAG-GAG-C			
PT-2	GCG-AAA-ACT-GTG-GAA-TTG-GG	252	*uidA* allele	Cebula, Payne, and Feng (1995)
PT-3	TGA-TGC-TCC-ATA-ACT-TCC-TG			
O157BF	AAA-TAT-AAA-GGT-AAA-TAT-GTG-GGA-ACA-TTT-GG	149	*rfbe*	Fortin, Mulchandani, and Chen (2001)
O157Br	TGG-CCT-TTA-AAA-TGT-AAA-CAA-CGG-TCA-T			
O157rfbe	FAM-CGC-TAT-GGT-GAA-GGT-GGA-ATG-GTT-GTC-ACG-AAT-AGC-DABCYL			
TXAF	TTA-AAT-GGG-TAC-TGT-GCC-T	401	*slt-II*	Li and Drake (2001)
TXAR	CAG-AGT-GGT-ATA-ACT-GCT-GTC			
TXAF1	TTA-AAT-GGG-TAC-TGT-GCC-TTC-AGG-GGA-CCA-CAT-CGG-T	275	*slt-II*	Li and Drake (2001)
TXAR	CAG-AGT-GGT-ATA-ACT-GCT-GTC			
MK1	CAG-CTC-TTC-AGA-TAG-CAT-TT	227 and 224	*slt-I* and *slt-II*	Karch and Meyer (1989)
MK2	CAG-CTC-TTC-AGA-TAG-CAT-TT			
SZ-I	CCA-TAA-TCA-TTT-TAT-TTA-GAG-GGA	632	*eaeA*	Oberst et al. (1998)
SZ-II	GAG-AAA-TAA-ATT-ATA-TTA-ATA-GAT-CGG-A			
SZI-97	FAM-TTG-CTG-CAG-GAT-GGG-CAA-CTC-TTG-TAMRA			

Continued

Table 2.2 PCR Primers and DNA Probes[a] (*Continued*)

Primer or Probe	Sequence (5′ → 3′)[a]	Size of Sequence (bp)	Amplified Gene or DNA Target Sequence	Reference
Forward	TTA-AAT-GGG-TAC-TGT-GCC-T	401	*slt-II*	McKillip and Drake (2000)
Reverse	CAG-AGT-GGT-ATA-ACT-GCT-GTC			
Probe	FAM-GCG-AGT-TGA-CCA-TCT-TCG-TCC-TCG-C-DABCYL			
VT1-A	CGC-TGA-ATG-TCA-TTC-GCT-CTG-C	302	*slt-I*	García-Sánchez et al. (2007) from Blanco et al. (2003)
VT1-B	CGT-GGT-ATA-GCT-ACT-GTC-ACC			
VT2-A	CTT-CGG-TAT-CCT-ATT-CCC-GG	516	*slt-II*	García-Sánchez et al. (2007) from Blanco et al. (2003)
VT2-B	CTG-CTG-TGA-CAG-TGA-CAA-AAC-GC			
HlyA1	GGT-GCA-GCA-GAA-AAA-GTT-GTA	1551	*ehxA*	García-Sánchez et al. (2007) from Schmidt, Beutin, and Karch (1995)
HlyA4	TCT-CGC-CTG-ATA-GTG-TTT-GGT-A			
EAE-1	GAG-AAT-GAA-ATA-GAA-GTC-GT	775	*eaeA*	García-Sánchez et al. (2007) from Blanco et al. (2003)
EAE-2	GCG-GTA-TCT-TTC-GCG-TAA-TCG-CC			
EAE-19	CAG-GTC-GTC-GTG-TCT-GCT-AAA	1087	*eae_1*	García-Sánchez et al. (2007) from Desmarchelier et al. (1998)
EAE-20	TCA-GCG-TGG-TTG-GAT-CAA-CCT			
O157-AF	AAG-ATT-GCG-CTG-AAG-CCT-TTG	497	$rfbe_{O157}$	

O157-AR	CAT-TGG-CAT-CGT-GTG-GAC-AG			
H7-F H7-R	GCG-CTG-TCG-AGT-TCT-ATC-GAG-C CAA-CGG-TGA-CTT-TAT-CGC-CAT-TCC	625	*fliC*$_{h7}$	García-Sánchez et al. (2007) from Gannon et al. (1997)
H7-F H7-R	TAC-CAC-CAA-ATC-TAC-TGC-TG TAC-CAC-CTT-TAT-CAT-CCA-CA	560	*fliC*$_{h7}$	Nagano et al. (1998)
O157-F O157-R	AAC-GGT-TGC-TCT-TCA-TTT-AG GAG-ACC-ATC-CAA-TAA-GTG-TG	678	O157	Nagano et al. (1998)
O157 A-F O157 A-R	AAG-ATT-GCG-CTG-AAG-GCT-TTG CAT-TGG-CAT-CGT-GTG-GAC-AG	497	*rfbe*$_{O157}$	Fach et al. (2003) from Desmarchelier et al. (1998)
O157 P-F8 O157 P-R8	CGT-GAT-GAT-GTT-GAG-TTG AGA-TTG-GTT-GGC-ATT-ACT-G	420	*rfbe*$_{O157}$	Fach et al. (2003) from Maurer et al. (1999)
P1EH P2EH	AGG-CGA-CTG-AGG-TCA-CT ACG-CTG-CTC-ACT-AGA-TGT	476	*eaeA*$_{O157}$	Fach et al. (2003) from Louie et al. (1994)
FLIC H7-F FLIC H7-R	GCG-CTG-TCG-AGT-TCT-ATC-GAG-C CAA-CGG-TGA-CTT-TAT-CGC-CAT-TCC	625	*fliC*$_{H7}$	Fach et al. (2003) from Gannon et al. (1997)

Continued

Table 2.2 PCR Primers and DNA Probes[a] (*Continued*)

Primer or Probe	Sequence (5′ → 3′)[a]	Size of Sequence (bp)	Amplified Gene or DNA Target Sequence	Reference
Sz I	CCA-TAA-TCA-TTT-TAT-TTA-GAG-GGA	632	$Orfu_{LEEO157}$	Fach et al. (2003) from Meng et al. (1996)
SZII	GAG-AAA-TAA-ATT-ATA-TTA-ATA-GAT-CGG-A			
RJD3	TTA-AAA-CCG-GTG-ACG-TGA-TGA-TGG-TG	125	SIL_{O157}	Fach et al. (2003) from Perelle et al. (2002)
SG7	AGC-AAC-AGG-CGC-AGA-TCG-TAG-CCA-C	578	SIL_{O157}	Perelle et al. (2002)
SF6	CGC-AGA-AAT-ACC-GGC-TTT-AAG-TAC-C			
VS8	GGC-GGA-TTA-GAC-TTC-GGC-TA	120	*eaeA*	Kawasaki et al. (2005) from Shama, Dean-Nystrom, and Casey (1999)
VS9	CGT-TTT-GGC-ACT-ATT-TGC-CC			
SLTI-F3	GGC-ATT-AAT-ACT-GAA-TTG-TCA-TC	416	*slt-I*	Witham et al. (1996) from Gannon et al. (1992)
SLTI-R	CTG-AAT-CCC-CCT-CCA-TTA-TG			
SLT 102	FAM-CAG-AAT-GGC-ATC-QTGA-TGA-GTT-TCC-TAMRA			
VT1a	GAA-GAG-TCC-GTG-GGA-TTA-CG	130	*slt-I*	Pollard et al. (1990)
VT1b	AGC-GAT-GCA-GCT-ATT-AAT-AA			
VT2a	TTA-ACC-ACA-CCC-ACG-GCA-GT	346	*slt-II*	Pollard et al. (1990)
VT2b	GCT-CTG-GAT-GCA-TCT-CTG-GT			
RfbF	GTG-TCC-ATT-TAT-ACG-GAC-ATC-CAT-G	292	*rfb*	Zang and Meitzler (1999)
RfbR	CCT-ATA-ACG-TCA-TGC-CAA-TAT-TGC-C			