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Abbreviations

ACR	Acute cellular rejection
ALL	Acute lymphoblastic leukemia
ARMS	Amplification refractory mutation system
ARP	Acidic ribosomal phosphoprotein
ASO	Allele-specific oligonucleotide
ATP	Adenosine triphosphate
β2m	β2-microglobulin (B2M)
BC	Background control (in immuno-PCR)
BHQ	Black hole quencher
BKV	BK virus
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
CCD	Charge-coupled device
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
CML	Chronic myeloid leukemia
CMV	Cytomegalovirus
COX	Cytochrome c oxidase
C_p	Crossing point (LightCycler® terminology)
CRS	Cambridge Reference Sequence
C_t	Threshold cycle (ABI terminology)
CTAB	Cetyltrimethylammonium Bromide
CV	Coefficient of variation (standard deviation divided by the mean)
CVS	Chorionic villi sampling
CYC	cyclophilin
DABCYL	4-(dimethylamino)azobenzene-4′-carboxylic acid
DABSYL	4-(dimethylamino)azobenzene-4′-sulfonyl chloride
DART	Data analysis for real-time PCR
DFA	Direct fluorescence assay
DGGE	Denaturing gradient gel electrophoresis
ds	Double-stranded
dTTP	Deoxythymidine triphosphate
dUTP	2′ deoxyurindine 5′ triphosphate
EAC	Europe against cancer
EBV	Epstein–Barr virus
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescent-assisted cell sorting
FAM	6-carboxy fluorescein
FDM	First derivative maximum
FISH	Fluorescence in-situ hybridization
FITC	Fluorescein
FRET	Fluorescence resonance energy transfer
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
gDNA	Genomic DNA
GOI	Gene of interest
GUS	β-glucuronidase
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HEP	Human tpigenome Project
HEX	Hexachloro-carbonyl-fluorescein
HKG	House keeping gene
HPLC	High-performance liquid chromatography
HPRT	Hypoxanthine phosphoribosyl-transferase 1
HSV	Herpes simplex virus
GOI	Gene of interest
IC	Internal control (normalizer, reference)
IFN	Interferon
IL	Interleukin
IPC	Internal positive control
IVF	In-vitro fertilization
JOE	2, 7-dimethoxy-4, 5-dichloro-6-carboxy-fluoroscein
KOD	Kinetic outlier detection

LATE PCR	Linear after the exponential PCR
LCM	Laser capture microdissection
LED	Light emitting diode
LiCl	Lithium Chloride
LNA	Locked nucleic acid
LUX™	Light upon eXtension
^{m}C	Methylated cytosine
MDR1	Multidrug resistance protein 1
MGB	Minor groove binders
MHC	Major histocompatibility complex
MMF	Mycophenolate mofetil
MRD	Minimal residual disease
mRNA	Messenger RNA
MSE	Mean squared error (nothing to do with SEM)
MSP	Methylation-specific PCR
mtDNA	Mitochondrial DNA (see also nDNA)
NAC	No amplification control
NASBA	Nucleic acid sequence based amplification
nDNA	Nuclear DNA (as opposed to mtDNA)
NF	Normalization factor
NTC	No template control (no target control)
OD	Optical density
OD_{260}	Optical density at 260 nm
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PCV	Packed cell volume
PGD	Preimplantation genetic diagnosis
PGK	Phosphoglycerokinase
PMT	Photomultiplier tube
PND	Prenatal diagnosis
PTLD	Post-transplant lymphoproliferative disorders
qPCR	Quantitative PCR
r	(Pearson's) coefficient of correlation (varies between –1 and +1)
r^2	R-squared or coefficient of determination (varies between 0 and +1)
rCRS	Revised Cambridge Reference Sequence
REST	Relative expression software tool
REST-MCS	REST – multiple condition solver
REST-RG	REST Rotor-Gene
RFLP	Restriction fragment length polymorphism
ΔRn	Normalized reporter signal minus background fluorescence
–RTC	Minus reverse transcriptase control
Rn	Normalized raw fluorescence
ROS	Reactive oxygen species
ROX	6-carboxy-X-rhodamine
rRNA	Ribosomal RNA
RSV	Respiratory syncytial virus
RT	Reverse transcription
SD	Standard deviation
SDM	Second derivative maximum
SDS	Sequence Detection Software
SEM	Standard error of the mean
SMCC	Succinimidyl-4-(N-maleimidomethyl) cyclohexane-l-carboxylate
SNP	Single nucleotide polymorphism
STR	Short tandem repeat
TAMRA	6-carboxy-tetra-methyl-rhodamine
TBP	TATA-binding protein
TCA	Trichoroacetic acid
TCR	T-cell receptor
TE buffer	Tris-EDTA buffer
TfR	Transferrin receptor
Tm	Melting temperature
TREC	T-cell receptor excision circles
tRNA	Transfer RNA
UNG	Uracil-N-glycosylase
y-int	y-intercept

Preface

Polymerase chain reaction (PCR) has secured its place in biomedical history as a revolutionary method. Many techniques that have derived from PCR have come and gone. Real-time PCR is based on the conventional principles of PCR and since its beginnings about a decade ago its popularity has kept growing. With the simple shift of emphasis from the end-product to the whole course of the PCR process, real-time PCR has established itself as the most sensitive and specific quantitative PCR method. The real-time PCR concept has also contributed to the development of high-throughput allelic discrimination assays. With a variety of detection chemistries, an increasing number of platforms, multiple choices for analytical methods and the jargon emerging along with these developments, real-time PCR is facing the risk of becoming an intimidating method, especially for beginners. This book aims to provide the basics, explain how they are exploited to run a real-time PCR assay, how the assays are run and where these assays are informative in real life. The book does not intend to cover every aspect of real-time PCR in an encyclopedic fashion but instead to address the most practical aspects of the techniques with the emphasis on 'how to do it in the laboratory'. Keeping with the spirit of the Advanced Methods Series, most chapters provide an experimental protocol as an example of a specific assay. It is left with the reader to adapt the presented protocol to their individual needs.

The book is organized in two parts. The first part begins with a general introduction which is followed by chapters on the basics of data analysis, quantification, normalization and principles of primer and probe design, all contributed by leaders in the field. This part is concluded by chapters on more applied aspects of real-time PCR including an overview of high-resolution melting analysis by the most experienced users of this method. The second part of the book covers specific applications including the less recognized uses of real-time PCR: methylation detection, mitochondrial DNA analysis and immuno-PCR. The following chapters summarize many uses of real-time PCR in the clinic with examples. Clinical microbiology and virology, solid organ and bone marrow transplantation, and prenatal genetic diagnosis are among the topics covered. These chapters have been written with the laboratory and practical uses in mind and the contributors share their most valuable experiences. A comprehensive glossary and index supplement the 17 chapters and aim to make the book more accessible.

Real-time PCR is, like any other modern method in molecular genetics, expanding, with potential applications even in proteomics. This book is expected to provide the basic principles of applied real-time PCR and provide a firm grounding for those who wish to develop further applications. The selection of the chapters reflects the acknowledgment of inevitable future developments. We look forward to covering those in future editions of the book. Real-time PCR is surely here to stay and hopefully this book will help current users of this technique and also help develop further applications for it.

M. Tevfik Dorak

Glossary

This glossary has been adapted from the Editor's Real-Time PCR webpage (http://www.dorak.info/genetics/realtime.html). The online version includes references and hyperlinks.

Absolute quantification: The absolute quantitation assay is used to quantitate unknown samples by interpolating their quantity from a standard curve (as in determination of viral copy number).

Allelic discrimination assay: Assays designed to type for gene variants. Either differentially labeled probes (one for each variant) or a single probe and melting curve analysis can be used for this purpose. Alternatively, dsDNA-binding dyes can be used in combination with melting curve analysis.

Amplicon: The amplified sequence of DNA in the PCR process.

Amplification plot: The plot of cycle number versus fluorescence signal which correlates with the initial amount of target nucleic acid during the exponential phase of PCR.

Anchor & reporter probes: Two partnering LightCycler® probes that hybridize on the target sequence in close proximity. The anchor probe (donor) emits the fluorescein to excite the reporter probe (acceptor) to initiate fluorescence resonance energy (FRET). In allelic discrimination assays, it is important that the reporter probe spans the mutation and has a lower Tm than the anchor probe.

Baseline: The initial cycles of PCR during which there is little change in fluorescence signal (usually cycles 3 to 15).

Baseline value: During PCR, changing reaction conditions and environment can influence fluorescence. In general, the level of fluorescence in any one well corresponds to the amount of target present. Fluorescence levels may fluctuate due to changes in the reaction medium creating a background signal. The background signal is most evident during the initial cycles of PCR prior to significant accumulation of the target amplicon. During these early PCR cycles, the background signal in all wells is used to determine the 'baseline fluorescence' across the entire reaction plate. The goal of data analysis is to determine when target amplification is sufficiently above the background signal, facilitating more accurate measurement of fluorescence.

Calibrator: A single reference sample used as the basis for relative-fold increase in expression studies (assuming constant reaction efficiency).

Coefficient of variance (CV): Used as a measure of experimental variation. It is important that a linear value (e.g., copy numbers) is used to calculate the CV (but not C_t values which are logarithmic). Intra-assay CV quantifies the amount of error seen within the same assay (in duplicates) and inter-assay CV quantifies the error between separate assays.

C_t (threshold cycle): Threshold cycle reflects the cycle number at which the fluorescence generated within a reaction crosses the threshold. It is inversely correlated to the logarithm of the initial copy number. The C_t value assigned to a particular well thus reflects the point during the reaction at which a sufficient number of amplicons have accumulated. Also called crossing point (C_p) in LightCycler® terminology.

Derivative curve: This curve is used in Tm analysis. It has the temperature in the x axis and the negative derivative of fluorescence (F) with respect to temperature (T), shown as dF/dT, on the y axis. The reproducibility of a derivative melting curve is high with a standard deviation of only 0.1°C between runs.

dsDNA-binding agent: A molecule that emits fluorescence when bound to dsDNA. The prototype is SYBR® Green I. In real-time PCR, the fluorescence intensity increases proportionally to dsDNA (amplicon) concentration. The problem with DNA-binding agents is that they bind to all dsDNA products: specific amplicon or non-specific products (misprimed targets and primer-dimers included). For this reason, analysis using DNA-binding agents is usually coupled with melting analysis.

Dynamic range: The range of initial template concentrations over which accurate C_t values are obtained. If endogenous control is used for $\Delta\Delta C_t$ quantitation method, dynamic ranges of target and control should be comparable. In absolute quantitation, interpolation within this range is accurate but extrapolation beyond the dynamic range should be avoided. The larger the dynamic range, the greater the ability to detect samples with high and low copy number in the same run.

Efficiency of the reaction: The efficiency of the reaction can be calculated by the following equation: $E = 10^{(-1/\text{slope})} - 1$. The efficiency of the PCR should be 90–100% meaning doubling of the amplicon at each cycle. This corresponds to a slope of 3.1 to 3.6 in the C_t vs log-template amount standard curve. In order to obtain accurate and reproducible results, reactions should have efficiency as close to 100% as possible (e.g., two-fold increase of amplicon at each cycle) and, in any case, efficiency should be similar for both target and reference (normalizer, calibrator, endogenous control, internal control). A number of variables can affect the efficiency of the PCR. These factors can include length of the amplicon, presence of inhibitors, secondary structure and primer design. Although valid data can be obtained that fall outside of the efficiency range, if it is <0.90, the quantitative real-time PCR should be further optimized or alternative amplicons designed.

Endogenous control: This is an RNA or DNA that is naturally present in each experimental sample. By using an invariant endogenous control as an active 'reference', quantitation of a messenger RNA (mRNA) target can be normalized for differences in the amount of total RNA added to each reaction and correct for sample-to-sample variations in reverse transcriptase PCR efficiency.

Exogenous control: This is a characterized RNA or DNA spiked into each sample at a known concentration. An exogenous active reference is usually an in vitro construct that can be used as an internal positive control (IPC) to distinguish true target negatives from PCR inhibition. An exogenous reference can also be used to normalize for differences in efficiency of sample extraction or complementary DNA (cDNA) synthesis by reverse transcriptase. Whether or not an active reference is used, it is important to use a passive reference dye (usually ROX–6-carboxy-X-rhodamine) in order to normalize for non-PCR-related fluctuations in fluorescence signal.

FAM: 6-carboxy fluorescein. Most commonly used reporter dye at the 5' end of a TaqMan® probe.

Fluorescence resonance energy transfer (FRET): The interaction between the electronic excited states of two dye molecules. The excitation is transferred from one (the donor) dye molecule to the other (the acceptor) dye molecule. FRET is distance dependent and occurs when the donor and the acceptor dye are in close proximity.

Housekeeping gene: Genes that are widely expressed in abundance and are usually used as reference genes for normalization in real-time PCR with the assumption of 'constant expression'. The current trend is first to check which housekeeping genes are suitable for the target cell or tissue and then to use more than one of them in normalization.

Hybridization probe: One of the main fluorescence-monitoring systems for DNA amplification. LightCycler® probes are hybridization probes and are not hydrolyzed by Taq Polymerase. For this reason, melting curve analysis is possible with hybridization probes.

Hydrolysis probe: One of the main fluorescence-monitoring systems for DNA amplification. TaqMan® probes are an example. These kinds of probes are hydrolyzed by the 5' endonuclease activity of Taq Polymerase during PCR.

Internal positive control (IPC): An exogenous IPC can be added to a multiplex assay or run on its own to monitor the presence of inhibitors in the template. Most commonly the IPC is added to the PCR master mix to determine whether inhibitory substances are present in the mix. Alternatively, it can be added at the point of specimen collection or prior to nucleic acid extraction to monitor sample stability and extraction efficiency, respectively.

Log-dilution: Serial dilutions in powers of 10 (10, 100, 1000 etc).

LUX™ (Light upon eXtension) primers: Created by Invitrogen, LUX™ primer sets include a self-quenched fluorogenic primer and a corresponding unlabeled primer. The labeled primer has a short sequence tail of 46 nucleotides on the 5' end that is complementary to the 3' end of the primer. The resulting hairpin secondary structure provides optimal quenching of the fluorophore. When the primer is incorporated into double-stranded DNA during PCR, the fluorophore is dequenched and the signal increases by up to ten-fold. By eliminating the need for a quencher dye, the LUX™ primers reduce the cost.

Melting curve (dissociation) analysis: Every piece of dsDNA has a melting point (Tm) at which temperature 50% of the DNA is single stranded. The temperature depends on the length of the DNA, sequence order, G:C content and Watson–Crick pairing. When DNA-binding dyes are used, as the fragment is heated, a sudden decrease in fluorescence is detected when Tm is reached (due to dissociation of DNA strands and release of the dye). This point is determined from the inflection point of the melting curve or the melting peak of the derivative plot (what is meant by derivative plot is the negative first-derivative of the melting curve). The same analysis can be performed when hybridization probes are used as they are still intact after PCR. As hydrolysis probes (e.g., TaqMan®) are cleaved during the PCR reaction, no melting curve analysis possible if they are used. Mismatch between a hybridization probe and the target results in a lower Tm. Melting curve analysis can be used in known and unknown (new) mutation analysis as a new mutation will create an additional peak or change the peak area.

Minor groove binders (MGBs): These dsDNA-binding agents are attached to the 3' end of TaqMan® probes to increase the Tm value (by stabilization of hybridization) and to design shorter probes. Longer probes reduce design flexibility and are less sensitive to mismatch discrimination. MGBs also reduce background fluorescence and increase dynamic range due to increased efficiency of reporter quenching. By allowing the use of shorter probes with higher Tm values, MGBs enhances mismatch discrimination in genotyping assays.

Minus reverse transcriptase control (–RTC): A quantitative real-time PCR control sample that contains the starting RNA and all other components for one-step reaction but no reverse transcriptase. Any amplification suggests genomic DNA contamination.

Molecular beacons: These hairpin probes consist of a sequence-specific loop region flanked by two inverted repeats. Reporter and quencher dyes are attached to each end of the molecule and remain in close contact unless sequence-specific binding occurs and reporter emission (FRET) occurs.

Multiplexing: Simultaneous analysis of more than one target. Specific quantification of multiple targets that are amplified within a reaction can be performed using a differentially labeled primer or probes. Amplicon or probe melting curve analysis allows multiplexing in allelic discrimination if a dsDNA-binding dye is used as the detection chemistry.

Normalization: A control gene that is expressed at a constant level is used to normalize the gene expression results for variable template amount or template quality. If RNA quantitation can be done accurately, normalization might be done using total RNA amount used in the reaction. The use of multiple housekeeping genes that are most appropriate for the target cell or tissue is the most optimal means for normalization.

Nucleic acid sequence based amplification (NASBA): NASBA is an isothermal nucleic acid amplification procedure based on target-specific primers and probes, and the co-ordinated activity of THREE enzymes: AMV reverse transcriptase, RNase H and T7 RNA polymerase. NASBA allows direct detection of viral RNA by nucleic acid amplification.

No amplification control (NAC, a minus enzyme control): In mRNA analysis, NAC is a mock reverse transcription containing all the RT-PCR reagents, except the reverse transcriptase. If cDNA or genomic DNA is used as a template, a reaction mixture lacking Taq polymerase can be included in the assay as NAC. No product should be synthesized in the NTC or NAC. If the absolute fluorescence of the NAC is greater than that of the NTC after PCR, fluorescent contaminants may be present in the sample or in the heating block of the thermal cycler.

No template control (NTC, a minus sample control): NTC includes all of the RT-PCR reagents except the RNA template. No product should be synthesized in the NTC or NAC; if a product is amplified, this indicates contamination (fluorescent or PCR products) or presence of genomic DNA in the RNA sample.

Normalized amount of target: A unitless number that can be used to compare the relative amount of target in different samples.

Nucleic acid target (also called 'target template'): DNA or RNA sequence that is going to be amplified.

Passive reference: A dye that provides an internal reference to which the reporter dye signal can be normalized during data analysis. Normalization is necessary to correct for fluctuations from well to well caused by changes in concentration or volume. ROX is the most commonly used passive reference dye.

Quencher: The molecule that absorbs the emission of fluorescent reporter when in close vicinity. Most commonly used quenchers include 6-carboxy-tetra-methyl-rhodamine (TAMRA), (DABCYL) and black hole quencher (BHQ).

R: In illustrations of real-time PCR principles, R represents fluorescent **r**eporter (fluorochrome).

r coefficient: Correlation coefficient, which is used to analyze a standard curve (ten-fold dilutions plotted against C_t values) obtained by linear regression analysis. It should be ≥0.99 for gene quantitation analysis. It takes values between zero and –1 for negative correlation and between zero and +1 for positive correlations.

R^2 coefficient: Frequently mixed up with 'r' but this is R-squared (also called coefficient of determination). This coefficient only takes values between zero and +1. R^2 is used to assess the fit of the standard curve to the data points plotted. The closer the value to 1, the better the fit.

Rapid-cycle PCR: A powerful technique for nucleic acid amplification and analysis that is completed in less than half an hour. Samples amplified by rapid-cycle PCR are immediately analyzed by melting curve analysis in the same instrument. In the presence of fluorescent hybridization probes, melting curves provide 'dynamic dot blots' for fine sequence analysis, including single nucleotide polymorphism (SNPs). Leading instruments that perform rapid-cycle PCR are RapidCycler®2 (Idaho Technology) and LightCycler® (Roche).

Real-time PCR: The continuous collection of fluorescent signal from polymerase chain reaction throughout cycles.

Reference: A passive or active signal used to normalize experimental results. Endogenous and exogenous controls are examples of active references. Active reference means the signal is generated as the result of PCR amplification.

Reference dye: Used in all reactions to obtain normalized reporter signal (Rn) adjusted for well-to-well variations by the analysis software. The most common reference dye is ROX and is usually included in the master mix.

Reporter dye (fluorophore): The fluorescent dye used to monitor amplicon accumulation. This can be attached to a specific probe or can be a dsDNA binding agent.

Relative quantification: A relative quantification assay is used to analyze changes in gene expression in a given sample relative to another reference sample (such as relative increase or decrease, compared to the baseline level, in gene expression in response to a treatment or in time, etc). Includes comparative C_t ($\Delta\Delta C_t$) and relative-fold methods.

Ribosomal RNA (rRNA): Commonly used as a normalizer in quantitative real-time RNA. It is not considered ideal due to its expression levels, transcription by a different RNA polymerase and possible imbalances in relative rRNA-to-mRNA content in different cell types.

Rn (normalized reporter signal): The fluorescence emission intensity of the reporter dye divided by the fluorescence emission intensity of the passive reference dye. Rn+ is the Rn value of a reaction containing all components, including the template and Rn is the Rn value of an unreacted sample. The Rn value can be obtained from the early cycles of a real-time PCR run (those cycles prior to a significant increase in fluorescence), or a reaction that does not contain any template.

ΔRn (delta Rn, dRn): The magnitude of the signal generated during the PCR at each time point. The ΔRn value is determined by the following formula: (Rn+) – (Rn).

ROX (6-carboxy-X-rhodamine): Most commonly used passive reference dye for normalization of reporter signal.

Scorpion: Another fluorescence detection system consists of a detection probe with the upstream primer with a fluorophore at the 5' end, followed by a complementary stem-loop structure also containing the specific probe sequence, quencher dye and a PCR primer on the 3' end. This structure makes the sequence-specific priming and probing a unimolecular event which creates enough specificity for allelic discrimination assays.

Slope: Mathematically calculated slope of standard curve, e.g., the plot of C_t values against logarithm of ten-fold dilutions of target nucleic acid. This slope is used for efficiency calculation. Ideally, the slope should be 3.3 (3.1 to 3.6), which corresponds to 100% efficiency

(precisely 1.0092) or two-fold (precisely, 2.0092) amplification at each cycle. Also called gradient.

Standard: A sample of known concentration used to construct a standard curve. By running standards of varying concentrations, a standard curve is created from which the quantity of an unknown sample can be calculated.

Standard curve: Obtained by plotting C_t values against log-transformed concentrations of serial ten-fold dilutions of the target nucleic acid. Standard curve is obtained for quantitative PCR and the range of concentrations included should cover the expected unknown concentrations range.

Sunrise™ primers: Created by Oncor, Sunrise™ primers are similar to molecular beacons. They are self-complementary primers which dissociate through the synthesis of the complementary strand and produce fluorescence signals.

TAMRA (6-carboxy-tetra-methyl-rhodamine): Most commonly used quencher at the 3' end of a TaqMan® probe.

TaqMan® probe: A dual-labeled specific hydrolysis probe designed to bind to a target sequence with a fluorescent reporter dye at one end and a quencher at the other.

Threshold: Usually 10X the standard deviation of Rn for the early PCR cycles (baseline). The threshold should be set in the region associated with an exponential growth of PCR product. It is the numerical value assigned for each run to calculate the C_t value for each amplification.

Unknown: A sample containing an unknown quantity of template. This is the sample of interest whose quantity is being determined.

Links to software and web resources cited

geNORM (download)
http://medgen.ugent.be/~jvdesomp/genorm

qBASE (download)
http://medgen.ugent.be/qbase

DART (download)
http://nar.oxfordjournals.org/cgi/content/full/31/14/e73/DC1

REST (download)
http://www.gene-quantification.de/rest.html

Best Keeper (download)
http://www.gene-quantification.de/bestkeeper.html

Mfold (nucleic acid folding and hybridization prediction)
http://www.bioinfo.rpi.edu/applications/mfold

Exiqon OligoDesign (LNA Primers)
http://lnatools.com

D-LUX Designer
http://orf.invitrogen.com/lux

PrimerBank
http://pga.mgh.harvard.edu/primerbank

RTPprimerDB
http://medgen.ugent.be/rtprimerdb

Quantitative PCR Primer Database (QPPD)
http://web.ncifcrf.gov/rtp/GEL/primerdb

Human Genome Project Database
http://genome.ucsc.edu/cgi-bin/hgGateway?org=human

NCBI Entrez GENE (Replaced Locus Link)
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene

NCBI Blast
http://www.ncbi.nlm.nih.gov/blast

MITOMAP (Human Mitochondrial Genome Database)
http://www.mitomap.org

TATAA Biocenter
www.tataa.com

Gene Quantification Web Resources
http://www.gene-quantification.info

An introduction to real-time PCR

1

Gregory L. Shipley

1.1 Introduction

There are several ways to interrogate a cell for changes induced by artificial or natural agents during a biological process. One way is to look for changes in cellular transcript levels that may indicate changes in the corresponding proteins. In another instance, the focus may be on the presence or absence of a viral or bacterial pathogen. In this case, detecting not only the presence but also the level of the pathogen provides valuable information in devising a treatment regimen. Alternatively, looking for an increase in the level of expression from a transgene or the inhibition of expression of an endogenous gene by an siRNA may be the question of interest. In all cases, quantitative real-time PCR technology can be utilized to provide the required information. Successful implementation of the technology requires users to have a basic background in the theoretical principles of real-time PCR as well as their practical application to the project at hand. The goal of this introductory chapter is to provide a basic foundation in the use of real-time PCR. Some of the following chapters will expand on topics presented here and add necessary new information required for a full understanding of this powerful technique.

1.2 A brief history of nucleic acid detection and quantification

Initially, nucleic acid quantification meant the addition of radiolabeled UTP or deoxythymidine triphosphate (dTTP) to cell cultures or one of many possible *in vitro* experimental preparations and measuring their incorporation into nucleic acids by TCA (trichoroacetic acid) precipitation. Although radioactive incorporation is a quantitative technique and gave the investigator an idea of the global changes in the nucleic acid population of their experimental system, it was not satisfactory for identifying or quantifying specific genes or transcripts. The first breakthrough in the identification of specific genes came with the development of the Southern transfer method (Southern, 1975). This was followed by the Northern blot for RNA (Alwine *et al.*, 1977). In both cases, it was now possible to specifically identify a particular gene or transcript by hybridization of a radioactively labeled probe to a membrane bearing DNA restriction fragments or RNA. Neither of these methods were quantitative techniques despite the best efforts to extract quantitative information from their use.

The next improvement in RNA transcript quantification came with the RNase-protection experiment. In this method, a short (<500 bases) highly radioactive anti-sense RNA was made from a plasmid construct utilizing T7 RNA polymerase. *In vitro* transcribed RNA was synthesized for the transcript(s) of interest along with a probe used for loading normalization. The radioactive anti-sense probes were then combined with each total RNA sample and hybridized to completion in liquid. The resulting double-stranded RNA product(s) were protected from the subsequent addition of a cocktail of single strand-specific nucleases. The protected products were then separated on a gel via denaturing polyacrylamide gel electrophoresis (PAGE) and exposed to film. This method had the advantage of hybridization in liquid rather than a solid surface and did not require the transfer of the RNAs to a membrane. However, the inherent short dynamic range when using film was the same. Again, the phosphoimager improved greatly on quantification for these experiments due to the expanded dynamic range and improved software for analysis. The down side to RNase protection experiments was the probes had to have high radioactive specific activity and required great care for safety reasons.

The procedure for performing the polymerase chain reaction (PCR) was first introduced by Kerry Mullis in 1983 (Mullis, 1990) for which he won the Nobel Prize in 1993. It is hard to think of another laboratory technique that has had a greater impact on so many different facets of biological research than PCR. In combining the reverse transcriptase (RT) reaction with the PCR, identification of a specific RNA transcript was now possible from very low copy numbers of starting material. Quantification of transcripts from sample unknowns became possible with the advent of competitive RT-PCR (Vu *et al*., 2000). In this method, a truncated version of the target region of interest lies between the same primer-binding sites as the target transcript sequence within a plasmid clone. The easiest method for making a smaller competitive target was to digest the cloned region between the primer-binding sites with a restriction enzyme and then ligate the resulting sticky ends, dropping out a short section of sequence. The requirements for the quantification construct were that it be a similar, but different, size than the target PCR product and quantified. The plasmid contains a T7, SP6 or T3 RNA promoter sequence up stream of the cloned target sequence. Utilizing the RNA promoter, truncated *in vitro* transcribed RNA could be made and quantified. Known amounts of the RNA product were spiked into the RT reaction and converted into cDNA along with the target sequence within the unknown sample. Subsequently, both the truncated standard and unknown target sequences were amplified using the PCR. The amplified DNAs were separated using denaturing polyacrylamide gel electrophoresis. In some methods a radioactive deoxynucleotide base was added for labeling the amplified DNA and quantified using either film or a phosphoimager. In other methods, the products on the gel were imaged following staining with ethidium bromide or SYBR® Green I. Quantification of the unknown target band was determined by comparison to signal from the spiked and quantified DNA standard. Although this method was the most accurate to date it still suffered from the detection problems mentioned earlier. However, most of the criticism centered around the spiked DNA standard. The concern was that the DNA standard was competing for reagent

resources and primers with the unknown target during the PCR and might, therefore, alter the final result.

In 1996, Applied Biosystems (ABI) made real-time PCR commercially available (Heid *et al.*, 1996) with the introduction of the 7700 instrument. Real-time quantitative PCR has become the most accurate and sensitive method for the detection and quantification of nucleic acids yet devised. This method has overcome most of the major shortcomings of the preceding ones. Using the specificity and sensitivity of PCR combined with direct detection of the target of choice utilizing fluorescently labeled primers, probes or dyes, the inherent problems of gels, transfer to a membrane, radioactive probe hybridization and the limitations of film as a detector have been eliminated. There are two problems for real-time PCR that still linger. They are 1) methods of quantification, i.e. kind of standard, assay quality and calculation methodology used and 2) how to properly normalize different samples to correct for differences in nucleic acid input from sample to sample. Both of these areas are the topic of much study and will be discussed in more detail in following chapters.

1.3 Real-time quantitative PCR – a definition

What exactly is real-time quantitative PCR? Some believe you have to be able to watch the growth of the amplification curves during the PCR on a computer monitor in order to be truly 'real-time'. This of course is not the case. The ABI 7700 SDS software, the original real-time program, does not allow the visualization of the amplification curves as they progress throughout the run. This was primarily due to the way the SDS software performs data analysis utilizing the final data set from the whole plate rather than analyzing each reaction individually. Thus for some instruments, the final data have to be present for data analysis to proceed. For other instruments, it does not. The latter case allows the software to follow the progress of the amplification curve in each well in real time, which can be visualized on the computer monitor. This ability does not make these instruments exclusively 'real-time instruments'.

Thus, real-time PCR is the continuous collection of fluorescent signal from one or more polymerase chain reactions over a range of cycles. Quantitative real-time PCR is the conversion of the fluorescent signals from each reaction into a numerical value for each sample.

1.4 Practical and theoretical principles underlying real-time PCR

RNA quantification begins with the making of cDNA (complementary DNA) by reverse transcriptase. There are two kinds of RT enzymes readily available on the market, AMV (Peters *et al.*, 2004) and MMLV (Gerard *et al.*, 1997). AMV is a dimeric protein from the avian myeloblastosis virus. MMLV is derived from the Moloney murine leukemia virus and is a monomeric protein. Both enzymes have RNase H activity, which is the ability to degrade RNA in an RNA–DNA hybrid. However, AMVs have higher RNase H activity compared to MMLV enzymes. In both enzymes, the RNase H activity can be separated from the RNA-dependent DNA polymerase activity