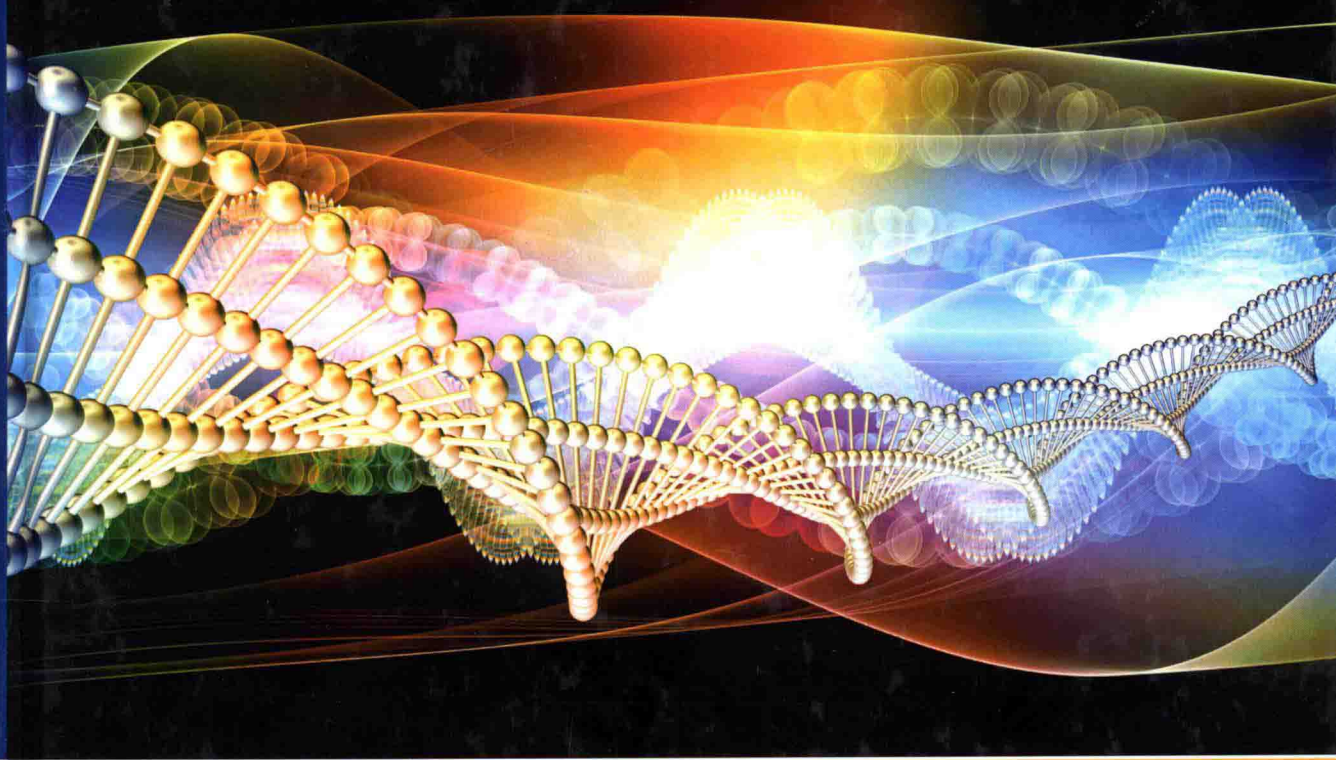
Third Edition

PCR Technology

Current Innovations



Edited by
Tania Nolan
Stephen A. Bustin

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Preface

It is astonishing to consider how much innovation surrounds the PCR, a technique that is, superficially, as simple as you can get. But the concepts discussed in *PCR Technology: Current Innovations, Third Edition* demonstrate numerous innovative tweaks, adaptations, formulations, concepts, and applications. Indeed, it is its very simplicity that allows PCR to be continuously expanded and reinvigorated, placing this technology firmly at the top of molecular techniques and, one could argue, making it the most important scientific tool ever invented.

It is curious, then, that the origin of the PCR is somewhat controversial and that the first public report of its use (1985) is predated by a long way by its theoretical description (1971). Kleppe and Gobind Khorana,[1] the Nobel laureate, published a description of a technique that represented the basic principles of a method for nucleic acid replication: "The principles for extensive synthesis of the duplexed tRNA genes which emerge from the present work are the following:

1. The DNA duplex would be denatured to form single strands. This denaturation step would be carried out in the presence of a sufficiently large excess of the two appropriate primers.
2. Upon cooling, one would hope to obtain two structures, each containing the full length of the template strand appropriately complexed with the primer.
3. DNA polymerase will be added to complete the process of repair replication. Two molecules of the original duplex should result.
4. The whole cycle could be repeated, there being added every time a fresh dose of the enzyme."

The authors go on to posit that "it is, however, possible that upon cooling after denaturation of the DNA duplex, renaturation to form the original duplex would predominate over the template–primer complex formation. If this tendency could not be circumvented by adjusting the concentrations of the primers, clearly one would have to resort to the separation of the strands and then carry out repair replication. After every cycle of repair replication, the process of strand separation would have to be repeated." This passage has been quoted many times and extensively scrutinized by patent lawyers, since it appears to be a clear description of the forerunner to the process now recognized as PCR.

Of course, we now know that the vast excess of primers favors the template–primer complex formation and drives the PCR. We could speculate that had the authors carried out an empirical assessment of their idea, the PCR would have been invented 14 years earlier. However, the concept proposed by Kleppe and coworkers was simply way ahead of the available technology in 1971. First, oligonucleotide synthesis was an expensive process performed only by organic chemists, of whom Khorana's group were among the few. Synthetic oligonucleotides were not as readily available as in 1985 and have become a low-cost commodity item we do not even think about any longer. Second, the description preceded the invention of DNA sequencing by either Gilbert and Maxam[2] or Sanger[3,4] and so the target sequence required for primer design was not readily available. This is of course a crucial issue and sometimes forgotten by those who criticize Kary Mullis.

As a consequence, he was the first person to actually amplify DNA and so was awarded the Nobel Prize for the invention of the technique and acknowledged this pioneering work of Kleppe and Khorana: "He [Kleppe] almost had it. He saw the problems but didn't realise how fast things happen [in the PCR]."[5]

In his Nobel Prize acceptance speech, Mullis described the journey of discovery and development of PCR, summarizing: "With two oligonucleotides, DNA polymerase and the four nucleoside

triphosphates I could make as much of a DNA sequence as I wanted and I could make it on a fragment of a specific size that I could distinguish easily."[6]

Originally, Mullis had assumed that when primers were added to denatured DNA, they would be extended, the extension products would then become unwound from their templates, be primed again, and the process of extension repeated. Unfortunately, these events do not simply occur by diffusion. The DNA must be heated to almost boiling after each round of synthesis, in order to denature the newly formed, double-stranded DNA. This inactivated the Klenow fragment of DNA polymerase I enzyme that was being used for synthesis and so more enzyme was required at the start of each cycle (as predicted by Kleppe, 1971). Arguably, the critical development leading to the universal adoption of the PCR technique was the concept of using the thermal stable DNA polymerase that could tolerate the high temperature of the repeated denaturation steps. The switch was made to the now commonly used *Taq* DNA polymerase that is extracted from the bacterium *Thermus aquaticus*,[7] which lives in thermal hot springs, and is resistant to permanent inactivation by exposure to high temperature.[8,9] This means that several rounds of amplification can be carried out in a closed reaction tube using the same batch of enzymes. In addition, using a thermally stable enzyme allowed for amplification of larger fragments and for the reaction to be performed at a higher temperature. The adoption of higher temperature reactions was sufficient to increase replication fidelity, reduce nonspecific product formation, and allow the products to be detected directly on ethidium bromide-stained, agarose gels.[10–12] An interesting aside is that the authors initially surmised that this enzyme might be useful as a reverse transcriptase, as it would be able to read through RNA secondary structure at elevated temperatures. They were not far wrong, since a related enzyme, *Tth* polymerase from *Thermus thermophiles*, does exactly that.

By 1989, the PCR technique was being used in all areas of modern biological sciences research, including clinical and diagnostic studies of detection of HIV in AIDS patients. The adoption of the PCR technique has been perhaps the major force driving the revolution in life sciences and associated fields we have all been privileged to witness.

The standard PCR is a deceptively simple process: low concentrations of template DNA, from a theoretical single copy to approximately 10^{11} copies, are combined with oligonucleotide primers in a reaction buffer containing variations on a basic composition consisting of ammonium sulfate, Tris, EDTA, BSA, β-mercaptoethanol, dNTPs, $MgCl_2$, KCl, NaCl, and DNA polymerase. Application of heat and a differential temperature profile, repeated 30–40 times, results in the amplification of the original target, which can then be visualized, purified, and used for a wide range of downstream manipulations.

In reality, there are numerous considerations for each of these components that transform the simple PCR concept into a series of steps that need to be carefully considered, optimized, and validated. The purification and handling of the template, prior to inclusion in the reaction, can have a profound effect on results. This is particularly important when a quantitative assessment is required. These factors are discussed in detail in Chapters 1 and 2. While the oligonucleotides may be considered a standard component of the reaction, these must be designed with care and the reaction conditions optimized to ensure that the results represent the true underlying biology, as described in Part IV. In addition, the manufacture and purification of the oligonucleotide can contribute to PCR behavior (Chapter 3) and so the appropriate synthesis and purification should be selected.

The absolute, optimum buffer composition is dependent upon the DNA polymerase used; different enzymes can affect PCR efficiency and therefore product yield.[13] In Chapter 4, Ernie Mueller presents a detailed discussion of the potential components for reaction buffers and in Chapter 8, new alternatives to the standard dNTPs are described.

As PCR reaches later cycles, it enters the plateau phase. This deviation from exponential amplification makes quantification inaccurate when based on estimates from end point product yield. Quantification using PCR requires that a modification to the basic PCR technique is made. This can involve digital PCR approaches or taking measurements earlier in the process, prior to the plateau phase. Higuchi et al.[14] recognized that the process of PCR could be tracked by including a

fluorescent label into the reaction that could bind to the accumulating PCR product. As the PCR product increases, the fluorescent emission and therefore intensity of the signal also increase. In current real-time quantitative PCR (qPCR) technology, these signals are generated by inclusion of either fluorescent DNA-binding dyes or additional oligonucleotide probes. Fluorescent DNA-binding dyes, such as SYBR Green I, and related derivatives such as BEBO or BOXTO,[15,16] are included in the PCR buffer along with the DNA primers. As the target is amplified, the dye binds to the DNA product and adopts an alternative conformation. This conformational change results in an increase in fluorescent emission. Alternatively, a labeled primer can be included in the reaction, as described in Chapter 5. Additional specificity is achieved by including an additional oligo probe situated between the two primers. This oligo probe is labeled (Chapter 5) and, in most cases, also quenched. Various probe options are available but the most popular are the linear hydrolysis probe (also referred to as TaqMan probes),[17] Molecular Beacons,[18] Scorpions[19] (Chapter 6), and LightCycler® probes.[20] Linear hydrolysis probes are oligos with a fluorescent label on the 5′ end and a quencher molecule on the 3′ end. These are designed to have an annealing temperature above that of the primers and therefore, as the reaction is cooled from the melting temperature to the annealing temperature of the primers, the probe hybridizes to the target sequence. On further cooling, the primers hybridize and the new strand is elongated until the DNA polymerase reaches the 5′ of the probe. The probe is then cleaved by 5′–3′ exonuclease activity of the enzyme, releasing the fluorescent label. In this way, a fluorescent label should be released with each amplicon synthesized. In reality, between 4% and 47% of amplicons are detected using the linear probe system. Alternative probe systems may be used to increase detection sensitivity or specificity. Different probe methods provide different sensitivities of detection due to greater efficiency in separating the fluorescent label from the quencher.[21] For example, we have observed that incorporation of the locked nucleic acid (LNA™)-modified nucleotides (Chapter 7) residues into a linear hydrolysis probe can increase detection sensitivity by up to 10-fold.

Scorpion probes are a structured detection system. These combine the forward primer and detection probe into a single molecule, also holding the fluorophore and quencher in close proximity with a stem structure. Initially the primer region hybridizes and elongates from the single-stranded target. After melting away the template, the Scorpion opens and the probe region hybridizes to the target region, separating the label from the quencher. In Chapter 6, David Whitcombe provides a detailed description of how to design these molecules and also how to apply them to genotyping experiments.

The sensitivity of any assay is, in part, determined by the assay design. Much has been written about the design and optimization of qPCR assays.[22] In Chapters 12 through 14 guidelines are detailed that would enable any bioscientist to design and optimize their own PCR or qPCR assay regardless of the application. Chapter 15 contains a description of a case study that illustrated the dangers of ignoring best practice and working with poorly defined assays.

The replicative power of PCR is phenomenal. In theory, a single-template molecule should be replicated during each PCR cycle. Assuming absolute, perfect replication at each cycle, this would lead to 2^{40} or 1^{12} amplicon molecules after 40 rounds of amplification. By now, there could be something approaching a similar number of applications for the technique and a description of just a few of these is included in Part VI of this book. The sensitivity achieved using PCR allows infectious diseases such as HIV, TB, and malaria to be detected with tremendous sensitivity and treatment efficacy monitored; however, there is a requirement for this to be performed within the highly restrictive environment of the developing world. In Chapter 25, Clare Watt and Jim Huggett share their experience of setting up diagnostic services in more challenging situations.

In addition to genomic sequence analysis, it has long been believed that investigating specific mRNA sequences can be informative about the biology of the cell. Investigating gene quantity changes, for example, between normal tissues and diseased cells or looking for changes in gene expression in response to drug treatments is being used to understand how regulation of gene expression is a part of the complex system of control of cellular processes. Measuring mRNA requires an additional step in order to convert RNA to a DNA template that is suitable for PCR amplification.

This is carried out using a reverse transcriptase enzyme and extension from one or more oligo primers. Priming of the reverse transcription may be from a sequence-specific primer, from a series of random primers that hybridize along the length of the mRNA, or from a primer directed toward a tract of adenosines that is added to the 3′ end of most messenger RNA sequences, referred to as the poly A tail. After elongation from the primer, a double-stranded hybrid of RNA and DNA, called single-strand cDNA, is produced. This cDNA is then a suitable template for PCR and relative quantities of specific RNA templates are determined carefully in a process of semi-qPCR or qPCR (as described above).

The availability of vast amounts of sequence data from the various genome and transcriptome sequencing projects has also enabled the identification of small RNA species (ncRNA), including miRNA genetic markers that are characteristic for specific diseases. Using PCR to amplify these targets is particularly challenging because they are so small and do not have natural poly A tails. In Chapter 22, Castoldi et al. present a novel technique to quantify all RNA species in a sample, including miRNA and precursor molecules, thus enabling the components in a miRNA pathway to be quantified. These miRNA genetic markers can be tracked and used to monitor patient relapse, along with effectiveness of drug and transplant treatments (Chapter 18).

When launching into a project requiring PCR, it is important to ensure that instrumentation is appropriate and functioning correctly. PCR instruments have become notorious due to variability between instruments and within a single block. It is not unheard of to have a reaction that functions only in the central wells and not around the edges, due to lack of thermal uniformity (described in detail in Chapter 9). There is now a huge effort to develop and refine instrumentation. Drives to miniaturize (Chapter 11) and also to increase the uniformity and rate of reactions (Chapter 10) are described which give a wonderful insight into the world of engineering that addresses the biological need to push PCR further. Similarly, the fundamentals of the PCR technique are being adapted to quantify proteins using proximity ligation assay (Chapter 27) or to identify small sequence changes such as single-nucleotide polymorphisms (SNPs) using differences in the melting behavior of the PCR amplicon, referred to as high-resolution melting analysis (Chapter 28).

In order to protect against uncertainties due to reaction variability, it is important to include a series of controls alongside all samples for any experiment.

The choice of controls and whether to include them should always depend only upon the nature of the study. Factors such as the need to complete a paper/report/thesis/presentation, the cost of the experiment or the date of the next grant body's review meeting may be important to scheduling but they should not influence the experimental design. An inexpensive experimental design, run very quickly but without controls to verify that the findings are genuine, is not just completely worthless, but if published enters the peer-reviewed literature and becomes virtually impossible to erase. It amounts to a false economy of all resources to run inadequate experiments that could even result in retractions. Some controls must be obligatory and honest, original data should be presented for inspection, especially when the results of the study or analysis lead to life and death decisions. It is worth recognizing that the simple disregard for the data from controls in a study claiming a link between autism and the MMR (measles, mumps, rubella) vaccination lead to the distress of parents of children affected by the disorder and even to loss of life as a result of parents refusing to have their children vaccinated. The sobering story is told by Tania Nolan and Stephen Bustin in Chapter 15 as they outline the journey and justification for the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines.[23] It is easy for scientists who are engrossed in a research project and chained to a laboratory bench for 16 h/day to lose sight of their responsibility to the scientific community. While there is a constant and substantial pressure to publish, this should never be to the detriment of the best-quality experiments possible or without absolute consideration for the complete truth.

In conclusion, the work presented in this third edition of *PCR Technology: Current Innovations* demonstrates that there are still numerous new developments affecting the "simple" PCR and we would hazard a guess that when the fourth and fifth editions are published, the same will be true. We

hope that readers of this book draw inspiration from the details presented by the numerous authors working in a wide range of fields and at the same time remember that while innovation is good, we must never lose sight of basic concepts of good science that can get overlooked in the rush to hop on the next bandwagon.

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Editors

Tania Nolan, BSc (Hons) PhD, earned a first class honors degree and was awarded the Excellence in Research Prize from undergraduate studies at Salford University and then a PhD in genetics from Manchester University, UK. She took an AstraZeneca Fellowship to study genetic regulation in breast cancer before moving to industry as an application scientist supporting the launch of a qPCR program. Dr. Nolan is an internationally recognized molecular biologist with a reputation for expertise in the field of mRNA quantification using RT-qPCR. She was a significant coauthor of the specialist textbook *The A–Z of Quantitative PCR* (ed. S.A. Bustin, IUL Press) along with numerous other book chapters and peer-reviewed papers. Among the publications are those describing the "SPUD" and "3′/5′mRNA integrity" assays. Dr. Nolan is an enthusiastic teacher and regularly organizes qPCR workshops. She has presented several plenary lectures and chaired sessions at major international qPCR meetings. Currently, she is a global manager, Technical and Application Support Team, Sigma Custom Products.

Stephen A. Bustin, BA(Mod) PhD FSB, obtained his PhD from Trinity College Dublin and has been working in the field of PCR (polymerase chain reaction) since 1987. He was professor of molecular science at Queen Mary University of London until 2012 and is currently professor of allied health and medicine at Anglia Ruskin University as well as visiting professor of molecular biology at the University of Middlesex. He acquired his first qPCR instrument in 1997 and has published numerous peer-reviewed papers that describe and use this technology. He wrote and edited the *A–Z of Quantitative PCR* (2004), universally acknowledged as the "qPCR Bible," edited *The PCR Revolution* (2011), and has written a series of *Definitive qPCR ebooks* (www.qPCRexpert.com). He led the international consortium that drew up the MIQE guidelines (2009) and is in constant demand as a speaker and teacher at international qPCR meetings and workshops. At the 2007 Autism trial at the Office of Special Masters of the U.S. Court of Federal Claims, he was an expert witness for the Department of Justice. Professor Bustin has extensive editorial involvements as editor-in-chief, *Gene Expression, International Journal of Molecular Sciences*, section editor, *Gene Expression, BMC Molecular Biology*, member of the editorial board of *Gene Regulation and Systems Biology* (Libertas Academica) and member of the editorial board of *Biomarkers in Medicine* (Future Science Group).

Contributors

Olga Adelfinskaya
TriLink Biotechnologies, Inc.
San Diego, California

Oto Akanji
Institute of Bioengineering
Queen Mary University of London
London, United Kingdom

Maria Galli de Amorim
Laboratory of Medical Genomics
AC Camargo Cancer Centre
São Paulo, Brazil

Ditte Andreasen
Exiqon A/S
Vedbaek, Denmark

Elena Hidalgo Ashrafi
TriLink Biotechnologies, Inc.
San Diego, California

Tzachi Bar
Labonnet
Ramat-Hasharon, Israel

Christiane Becker
Institute of Animal Nutrition
Liesel-Beckmann-Strasse
München, Germany

Vladimir Benes
European Molecular Biology Laboratory
Heidelberg, Germany

Einar S. Berg
Division for Infectious Disease Control
National Institute of Public Health
Oslo, Norway

Anders Bergkvist
Sigma-Aldrich Sweden AB
Stockholm, Sweden

Nick Burroughs
BJS Biotechnologies Ltd.
Perivale, Greenford
London, United Kingdom

Claire A. Bushell
LGC
Teddington, United Kingdom

Stephen A. Bustin
Postgraduate Medical Institute
Anglia Ruskin University
Chelmsford, United Kingdom

Mirco Castoldi
Clinic of Gastroenterology, Hepatology and Infectiology
Heinrich-Heine-University
Dusseldorf, Germany

Shiaw-Min Chen
Life Technologies, Inc.
Foster City, California

Tina Chowdhury
Institute of Bioengineering
Queen Mary University of London
London, United Kingdom

Paul Collier
European Molecular Biology Laboratory
Heidelberg, Germany

Theresa Creasey
Sigma-Aldrich
The Woodlands, Texas

Peter Damaschke
Department of Computer Science and Engineering
Chalmers University
Göteborg, Sweden

Philip Day
The Manchester Institute of Biotechnology
University of Manchester
Manchester, United Kingdom

Norha Deluge
Sigma-Aldrich
The Woodlands, Texas

Emmanuel Dias-Neto
Laboratory of Medical Genomics
AC Camargo Cancer Centre
and
Department and Institute of Psychiatry
Universidade de São Paulo
São Paulo, Brazil

Søren M. Echwald
Exiqon A/S
Vedbaek, Denmark

Malin Edling
NeuroSearch Sweden AB
Göteborg, Sweden

Giordana Feriotto
Department of Morphology
Ferrara University
Ferrara, Italy

Yannick Fillon
Cubist Pharmaceuticals
Lexington, Massachusetts

Simon Fredriksson
Olink Bioscience AB
Uppsala, Sweden

Roberto Gambari
Department of Life Sciences and Biotechnology
Ferrara University
Ferrara, Italy

Jorge A. Garcés
Genmark Dx
San Diego, California

Ray H. Gavin
Department of Biology
Brooklyn College–City University of New York
Brooklyn, New York

Michael Gotesman
Fish Medicine and Livestock Management
University of Veterinary Medicine
Vienna, Austria

Ashley R. Heath
Sigma-Aldrich
The Woodlands, Texas

Johannes Hedman
Department of Applied Microbiology
Lund University
Lund, Sweden

and

Department of Biology
Swedish National Laboratory of Forensic Science (SKL)
Linköping, Sweden

Tom Hendrikx
CYCLERtest BV
Landgraaf, the Netherlands

Jim Huggett
Nucleic Acid Metrology, Molecular and Cell Biology
LGC
Teddington, United Kingdom

Ehsan Karimiani
The Manchester Institute of Biotechnology
University of Manchester
Manchester, United Kingdom

Emmanouil Karteris
Department of Biosciences
Brunel University
Uxbridge, United Kingdom

Rickard Knutsson
Department of Bacteriology
National Veterinary Institute (SVA)
Uppsala, Sweden

Vlasta Korenkova
Institute of Biotechnology AS CR
Prague, Czech Republic

Kaarel Krjutškov
Estonian Genome Center
University of Tartu
Tartu, Estonia

Mikael Kubista
Institute of Biotechnology AS CR
Prague, Czech Republic

and

TATAA Biocenter
Göteborg, Sweden

Liu Xiao Kun
Temasek Life Sciences Laboratory
National University of Singapore
Singapore

Ulf Landegren
Department of Immunology, Genetics and Pathology/Molecular Medicine
Uppsala University
Uppsala, Sweden

Tony Le
TriLink Biotechnologies, Inc.
San Diego, California

Charlotta Löfström
National Food Institute
Technical University of Denmark
Søborg, Denmark

Maria Lövenklev
Department of Microbiology and Process Hygiene
Swedish Institute for Food and Biotechnology (SIK)
Göteborg, Sweden

Amelia Markey
School of Chemical Engineering and Analytical Science
Manchester Institute of Biotechnology
University of Manchester
Manchester, United Kingdom

Carlos Martinez
Technology Development and Engineering
Sigma Life Science–Custom Products
Sigma-Aldrich
The Woodlands, Texas

Andres Metspalu
Estonian Genome Center and Department of Biotechnology
Institute of Cell and Molecular biology
University of Tartu
Tartu, Estonia

Peter Mouritzen
Exiqon A/S
Vedbaek, Denmark

Ernest J. Mueller
Sigma-Aldrich Center for Biotechnology
St. Louis, Missouri

Azam Sheikh Muhammad
Department of Computer Science and Engineering
Chalmers University
Göteborg, Sweden

Gavin J. Nixon
LGC
Teddington, United Kingdom

Tania Nolan
Sigma-Aldrich Custom Products
Haverhill, United Kingdom

Xinghua Pan
Department of Genetics
Yale University School of Medicine
New Haven, Connecticut

Natasha Paul
TriLink Biotechnologies, Inc.
San Diego, California

Michael W. Pfaffl
Institute of Physiology
Center of Life and Food Sciences Weihenstephan
Technical University of Munich
Freising, Germany

Peter Rådström
Department of Applied Microbiology
Lund University
Lund, Sweden

Maido Remm
Department of Bioinformatics
University of Tartu
Tartu, Estonia

Irmgard Riedmaier
Institute of Physiology
Center of Life and Food Sciences Weihenstephan
Technical University of Munich
Freising, Germany

David W. Ruff
Fluidigm Corporation
South San Francisco, California

Vendula Rusnakova
Institute of Biotechnology AS CR
Prague, Czech Republic

Donald Salter
Institute of Genetics and Molecular Medicine
University of Edinburgh
Edinburgh, United Kingdom

Mark E. Shannon
Genomic Assays R&D
Life Technologies, Inc.
Foster City, California

Gregory L. Shipley
Shipley Consulting LLC
Missouri City, Texas

Sabrina Shore
TriLink Biotechnologies, Inc.
San Diego, California

Mary Span
CYCLERtest BV
Landgraaf, the Netherlands

Boel Svanberg
NeuroSearch Sweden AB
Göteborg, Sweden

David Svec
Institute of Biotechnology AS CR
Prague, Czech Republic

and

TATAA Biocenter
Göteborg, Sweden

Ales Tichopad
Charles University
Pilsen, Czech Republic

and

Biotechnology Institute
Academy of Science of the Czech Republic
Prague, Czech Republic

Alexander E. Urban
Department of Psychiatry and Behavioral Sciences
and
Center for Genomics and Personalized Medicine
Stanford University School of Medicine
Stanford, California

Marc Verblakt
CelsiusLabs
Landgraaf, the Netherlands

Clare Watt

Sherman M. Weissman
Department of Genetics
Yale University School of Medicine
New Haven, Connecticut

David Whitcombe
Clarity Now Limited
Northwich, United Kingdom

Selwyn A. Williams
Department of Biology
New York City College of Technology
Brooklyn, New York

Petra Wolffs
Department of Medical Microbiology
Maastricht University Medical Center
Maastricht, the Netherlands

Qin Xiang
Sigma-Aldrich
The Woodlands, Texas

Hong Yan
Temasek Life Sciences Laboratory
National University of Singapore
Singapore

Contents

PART III Instruments

PART IV Design, Optimization, QC, and Standardization

PART V Data Analysis