Performing Real-Time PCR

K. J. Edwards

Abstract

Optimisation of the reagents used to perform PCR is critical for reliable and reproducible results. As with any PCR initial time spent on optimisation of a real-time assay will be beneficial in the long run. Specificity, sensitivity, efficiency and reproducibility are the important criteria to consider when optimising an assay and these can be altered by changes in the primer concentration, probe concentration, cycling conditions and buffer composition. An optimised real-time PCR assay will display no test-to-test variation in the crossing threshold or crossing point and only minimal variation in the amount of fluorescence. The analysis of the real-time PCR results is also an important consideration and this differs from the analysis of conventional block-based thermal cycling. Real-time PCR provides information on the cycle at which amplification occurs and on some platforms the melting temperature of the amplicon or probe can be determined.

Optimisation of Real-Time PCR Assays

Real-time PCR assays require optimisation in order that robust assays are developed which are not affected by normal variations in the target

DNA, primer or probe compositions. A robust assay is defined as an assay in which these ‘normal’ variations cause no effect on the crossing threshold (CT) also known as crossing point (CP) and have only a minimal effect on the observed amount of fluorescence. The important criteria for optimisation are specificity, sensitivity, efficiency and reproducibility. It is important to decide before commencing optimisation which type of assay is required for a particular application. For example, there is little point in developing a quantitative assay if a simple qualitative assay will be just as informative. Melting curve analysis may also be required for product differentiation and in this case should be considered at the planning stage. If the real-time assay is based on conversion of an existing block-based assay it is important to note that the cycling conditions used for conventional block-based thermal cycling may not always translate easily to a real-time format and so it is important to consider re-optimisation of the assay (Teo *et al*., 2001).

The same principles of optimisation apply to assays run on all real-time platforms. The following criteria should be optimised: buffer composition, cycle conditions, magnesium chloride ($MgCl_2$) concentration, primer concentration, probe concentration and template concentration. Commercial master mixes, which are widely available, simplify the optimisation and are convenient. In this chapter all aspects of optimisation and analysis of real-time PCR results will be discussed.

PCR Master Mix

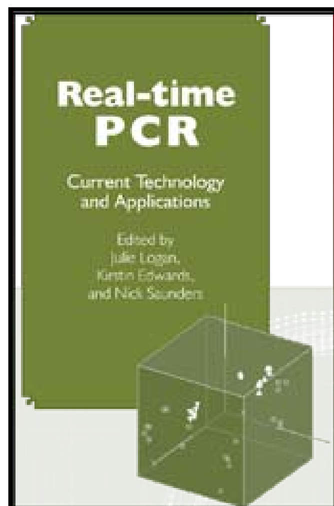
Commercial master mixes are available for most of the real-time platforms and although some are marketed for specific instruments and probe formats, they often work equally well on other instruments. Mixes are provided in easy-to-use formats and often contain additional features such as use of dUTP allowing the enzyme uracil-DNA glycosylase to be used to prevent cross-over contamination. Some commercial master mixes contain the uracil-DNA glycosylase as well as the dUTP and carry-over contamination never needs to be considered. However, it is important to note that the use of dUTP

©Horizon Scientific Press

REAL-TIME PCR
Current Technology and Applications

Edited by: **Julie Logan, Kirstin Edwards and Nick Saunders**

c. 262 pp., January 2009
ISBN 978-1-904455-39-4 $310/£150
Published by: Caister Academic Press www.caister.com



Real-time PCR (RT-PCR) technology is highly flexible and many alternative instruments and fluorescent probe systems have been developed recently. The decreased hands-on time, increased reliability and improved quantitative accuracy of RT PCR methods have contributed to the adoption of RT PCR for a wide range of new applications.

This essential manual presents a comprehensive guide to the most up-to-date technologies and applications as well as providing an overview of the theory of this increasingly important technique. Renowned experts in the field describe and discuss the latest PCR platforms, fluorescent chemistries, validation software, data analysis, and internal and external controls. This timely and authoritative volume also discusses a wide range of RT-PCR applications including: clinical diagnostics, biodefense, RNA expression studies, validation of array data, mutation detection, food authenticity and legislation, NASBA, molecular halotyping, and much more.

An essential book for all laboratories using PCR.

www.caister.com

Table of Contents

- An Introduction to Real-Time PCR *N. A. Saunders*
- An Overview of PCR Platforms *J. M. J. Logan and K. J. Edwards*
- Homogeneous Fluorescent Chemistries for Real-Time PCR *M. A. Lee, D. J. Squirrell, D. L. Leslie and T. Brown*
- Reference Gene Validation Software for Improved Normalization *J. Vandesompele, M. Kubista and M. W. Pfaffl*
- Data Analysis Software *M. W. Pfaffl, J. Vandesompele, M. Kubista*
- Performing Real-time PCR *K.J. Edwards and J.M.J. Logan*
- Internal and External Controls for Reagent Validation *M. A. Lee, D. L. Leslie and D. J. Squirrell*
- Introduction to the Applications of Real-Time PCR *N.A. Saunders*
- Analysis of mRNA Expression by Real-Time PCR *Stephen A. Bustin and Tania Nolan*
- Validation of Array Data *Elisa Wurmbach*
- Mutation Detection by Real-Time PCR *Elaine Lyon, Rong Mao and Jeffrey Swensen*
- Real-Time NASBA *Julie D. Fox, Catherine Moore and Diana Westmoreland*
- Applications in Clinical Microbiology *Andrew David Sails*
- Diagnosis of Invasive Fungal Infections *D.S. Perlin*
- Biodefense *Christina Egan, Nick M. Cirino and Kimberlee A. Musser*
- Real-Time PCR: Application to Food Authenticity and Legislation *Gordon Wiseman*
- Molecular Haplotyping by Real-time PCR *Genevieve Pont-Kingdon, Alison Millson and Elaine Lyon*

Order from:
- ISBS, Inc., 920 NE 58th Avenue, Suite 300, Portland, OR 97213-3786, **USA** Tel: 503 287-3093; Fax: 503 280-8832 http://usa.caister.com
- Book Systems Plus, BSP Hse, Station Road, Linton, Cambs, CB1 6NW, **UK** Tel: 01223 894870; Fax: 01223 894871 http://uk.caister.com

Quantity	Title	ISBN	Cost

Add carriage per copy:
UK £5; Europe £8; USA $5.50; Rest of World please call

Total

Name

Address

E-mail

Tel. Fax.

☐Visa ☐Mastercard ☐Bill me

Exp. date Security number

Cardholder

Signature Date