

PCR Perfection: Insider tricks

The BioPark Hertfordshire, Welwyn Garden City, AL7 3AX: 3rd October 2008

- 10:00 – 10:30 **Registration**
- 10:30 – 10:45 **Introduction by the Chair:** Dr. David Whitehouse, UK
- 10:45 – 11:15 **Improving standards in real time PCR**
Dr Rob Powell, Southampton University, UK
It is easy to generate real time PCR data plots. However the correct interpretation depends on the fundamental characteristics of the assay that produced the data. Priming specificity and efficiency are critical components. The fundamental criteria for good primer and experimental design will be presented and illustrated with data from Southampton university and from the literature
- 11:15 – 11:45 **The PCR in medical bacteriology diagnostics and research**
Dr Craig Winstanley, University of Liverpool, UK
The talk will include an overview of the application of PCR-based techniques of relevance in medical microbiology. In addition, there will be a focus on the application of PCR-based technologies to the development and use of diagnostic PCR tests for specific cystic fibrosis pathogens.
- 11:45 – 11:50 **Speakers photo**
- 11:50 - 12:00 **Introduction to the Biopark**
- 12:00 – 13:00 **Lunch and Poster Viewing**
- 13:00 – 13:30 **qPCR – the SYBR Green solution**
Dr David Sugden, Kings College London
As a physiologist interested in making use of qPCR to measure expression of many different genes in a variety of different projects, I want assays that are easy to set-up, simple to use and reliable. This talk will describe our experience using the SYBR Green detection strategy for qPCR and will highlight the way we've approached assay design and set-up, preparation of standards and quantitative analysis of gene expression. The specificity, sensitivity, reliability of SYBR Green assays will be illustrated.
- 13:30 – 14:00 **RT-qPCR Assay Validation, Primer Selection, Reverse Transcription and mRNA Integrity**
Professor Stephen A. Bustin, Institute of Cell and Molecular Science, Queen Mary's School of Medicine and Dentistry, University of London
Preassay validation is a critical section of any experiment designed to quantitate cellular mRNA. Choice and localisation of RT primer, optimisation of PCR primers, choice of RT and assessment of mRNA quality are all essential if mRNA quantification is to have any real relevance. This presentation will provide a detailed analysis of the priming and reverse transcription steps and demonstrate the difficulties associated with measuring of RNA integrity.
- 14:00 – 14:30 **Afternoon Tea/Coffee and Last Poster Viewing**
- 14:30 – 15:00 **Detection of foot-and-mouth disease virus by RT-PCR: insider tricks**
Scott Reid, Institute of Animal Health, UK
Rapid and accurate diagnosis is essential for effective control of foot-and-mouth disease (FMD). I will describe the practical steps taken to use real-time RT-PCR (rRT-PCR) as a front-line laboratory diagnostic tool during the 2007 outbreaks of FMD in the United Kingdom. The practical considerations adopted for the processing of the samples, the deployment of rRT-PCR during the outbreaks and the advantages of the assay for laboratory diagnosis of FMD to support control decisions during the outbreaks will be discussed.
- 15:00 – 15:30 **Building quality control in a qPCR multi-user research facility**
Dr Frederique Ponchel, Senior Academic Research Fellow, Leeds University
This talk will describe the issues in running a multi-user facility in a research institute with multiple type of real time PCR applications and how to implement certain rules for assay validation, quality control and protection.
- 15:30 – 16:00 **Chairman's summing up**
- 16:30 **Soiree at *The Best Western Homestead Court Hotel for all the participants**

About the Chair:

Dr David Whitehouse is an experienced academic and commercial scientist. He has more than 20 years research experience in the university sector, mostly with the MRC Human Biochemical Genetics Unit in UCL focusing on protein detection, human molecular genetics and genomics and the development of rapid diagnostic tests using monoclonal antibodies. In 2000 he transferred to the commercial sector where he specialized in the development of optical and electrophoretic devices for microbial detection and new approaches to DNA based diagnostics. He is an experienced freelance manager of intellectual property including patent applications in the biotechnological and neurosciences fields. He writes, lectures, and devises presentations and learning modules in biotechnology and healthcare for the commercial and higher education sectors.

About the Speakers

Professor Stephen Bustin obtained his PhD from Trinity College, University of Dublin in molecular genetics in 1983. He carried out post-doctoral research at the Animal Virus Research Institute in Pirbright before his interest in biotechnology led him to join Corporate Research, Amersham International, as a senior research scientist, eventually becoming research manager, gene expression. His main achievements were producing a synthetic HRP gene, generating humanised antibodies, cloning a HIV-1 strain and inventing a lyophilized reagent system for PCR. He joined the London Hospital Medical College as a senior research fellow, aiming to apply his research in a more direct, practical setting. Following promotion to Senior Lecturer (1995) and Reader in Molecular Medicine (2002) he was awarded a personal chair by the University of London in 2004

Dr Scott Reid Obtained a BSc. Biochemistry from Edinburgh University followed by an MSc in Forensic Science from the University of Strathclyde. Worked for seven years as a research scientist in industry before taking up a lectureship in microbiology at the College of Health Sciences, Abha, Saudi Arabia. From there, joined the World Reference Laboratory for foot-and-mouth disease (FMD) at the Institute for Animal Health (IAH), Pirbright to develop improved RT-PCR procedures for rapid and sensitive detection of FMD virus and other vesicular disease-causing viruses. Recently completed a PhD thesis on the basis of published work carried out at the IAH.

Dr Craig Winstanley, has a BSc in Biochemistry 1983 (UMIST), PhD in Bacterial Genetics 1987 (Wales). He was a lecturer at Coventry University and the University of Bradford prior to becoming a Lecturer / Senior Lecturer in the School of Infection & Host Defence, University of Liverpool (since 1999). His major research interests include the study of genetic variation and the identification of virulence genes in Gram-negative bacterial pathogens and the development of diagnostic tests for bacterial pathogens in cystic fibrosis. He has published widely on various molecular aspects of bacterial pathogens.

Dr Rob Powell holds a PhD molecular virology and had an academic career working on Chronic fatigue syndrome and as a Wellcome trust fellow within Southampton University School of Medicine. PrimerDesign was established on his 10 years of research experience, including human virus diseases and asthma genetics. Dr Powell has been a consultant to the life science industry on matters relating to gene quantitation for the past three years.

Dr David Sugden obtained a BSc in Pharmacology from Leeds University then worked in the pharmaceutical industry for 6 years as a research scientist. He completed a PhD while working in industry then did a post-doc at Imperial College before taking a position as visiting associate at the National Institutes of Health, Bethesda, USA. He was tempted back to the UK to take up a Royal Society University Research Fellowship at King's College London, and was appointed to a Lectureship at King's in the Physiology Department in 1994, and promoted to Reader in 2003.

Dr Frederique Ponchel, Senior Academic Research Fellow, Leeds University. 2005/10 RCUK Senior Academic Research Fellow – Senior Lecturer, Leeds Institute of Molecular Medicine, The University of Leeds, Leeds, England. 2000/5 ARC Career Development Research Fellow Molecular Medicine Unit, The University of Leeds, Leeds, England. 1998/99, SENIOR Postdoctoral Fellow Molecular Medicine Unit, The University of Leeds, Leeds, England. 1995/98, EC Postdoctoral Position, YCRC p53 group, The University of York, York, England. 1994/95, Postdoctoral Position Laboratory of Molecular Oncology, INSERM 453, Lyon, France, 1994 Ph.D. – IFSBM : Doctoral Thesis in Fundamental Oncology Obtained with the highest distinction and special congratulations of the jury. Special program similar to an EMBL-Ph.D. University : PARIS XI Le Kremlin Bicetre.

*This meeting was **organised by Euroscicon** (www.euroscicon.com), a team of dedicated professionals working for the continuous improvement of technical knowledge transfer to all scientists. Euroscicon believe that they can make a positive difference to the quality of science by providing cutting edge information on new technological advancements to the scientific community. This is provided via our exceptional services to individual scientists, research institutions and industry. The event was hosted by **'BioPark'** (www.biopark.co.uk), a research and development centre in Welwyn Garden City providing specialist facilities and support for bioscience and health technology businesses to grow, and to develop new products and technologies*

Results from the 2007 proficiency testing scheme for RT-PCR methods used to detect foot-and-mouth-disease virus



Katja Ebert¹, Scott M. Reid¹, Yanmin Li¹, Geoff H. Hutchings¹, David J. Paton¹, Nigel P. Ferris¹, Donald P. King¹



¹Division of Epidemiology, Institute for Animal Health, Pirbright Laboratory, Ash Road, Pirbright, Woking, Surrey, UK. GU24 0NF

Introduction

The ability to recognise new outbreaks of exotic viral diseases is one of the most critical purposes of lab testing. Monitoring the equivalence of assay performance is therefore important to satisfy QA programmes and accreditation.

This poster presents the results of an inter-laboratory proficiency testing scheme (PTS), which was conducted during 2007 to evaluate RT-PCR assays used to detect foot-and-mouth-disease virus (FMDV). Of the 30 countries agreeing to participate in this study, 25 were from EU member countries and 5 from Non-EU countries. The study was organised by the Community Reference Laboratory (CRL) to assist European Union (EU) and other National FMD Laboratories in deploying accurate and reproducible tests for FMDV.

Materials and Methods

Two panels of coded samples were prepared and sent to participating laboratories (Figure 1) for testing by RT-PCR assays. The first ('live') panel was sent to 16 laboratories and consisted of 4 FMD viruses of serotype O (2x), A and Asia 1. In addition to RT-PCR, this panel was also used to assess the performance of virus isolation methods employed by the different laboratories. The second panel sent to 28 laboratories comprised 10 non-infectious FMDV samples prepared by binary ethylenimine inactivation and included FMD viruses of serotypes O, A, Asia 1, SAT 1 and SAT 2.

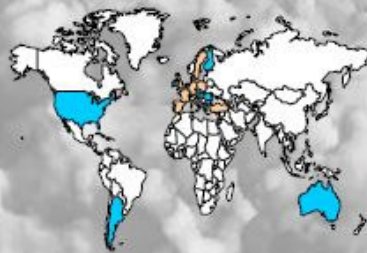


Figure 1: Participating laboratories
 EPIZONE partners
 other participating labs including additional EU Reference labs

Results

In total, 30 laboratories agreed to receive at least one of the panels for evaluation of their RT-PCR methods. Twenty-six were located in Europe, 11 of which were also partners in the EPIZONE project. Samples in Panel 1 were detected by all laboratories that reported results for RT-PCR; however dilution studies indicated that these assays differed in analytical sensitivity (Figure 2). Real-time RT-PCR methods are being increasingly adopted for routine use: in this study, 16 laboratories reported results for Panel 2 using this format (Table 1). Results for Panel 2 showed that 18/24 of the laboratories successfully detected FMDV in all the positive samples and correctly discriminated the 5 negative samples in the panel. Two laboratories failed to detect SAT viruses, possibly due to nucleotide mismatches with primers and/or probes, while 5 laboratories scored a false positive result for at least one of the negative samples. (Table 1) The majority of labs used conserved regions of the 5' UTR and/or 3D as the diagnostic target.

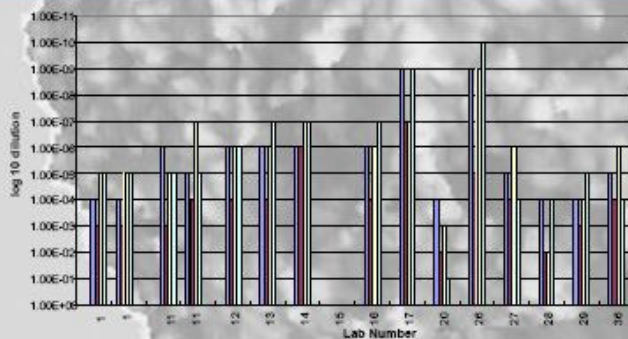


Figure 2: RT-PCR results of Panel 1
 HKN5/2005 IRN31/2005 IRN51/2005 IRN48/2005

Lab	O	A	SAT 1	SAT 2	Asia 1	SVD	Uninfected Cell Separation				Target
1	20.99	9.09	18.22	20.86	20.81	Reg	Reg	Reg	Reg	Reg	SUTR
2	21.19	11.29	19.29	19.29	20.93	Reg	Reg	Reg	Reg	Reg	3D
3	21.19	20.4	20.5	24.3	24.6	Reg	Reg	Reg	Reg	Reg	SUTR
4	22	20	20	20	20	Reg	Reg	Reg	Reg	Reg	SUTR
5	Reg	Reg	Reg	Reg	Reg	Reg	Reg	Reg	Reg	Reg	3D
7	20.26	17.1	24.8	27.3	21.4	Reg	Reg	Reg	Reg	Reg	SUTR
8	16	13	17	19	18	Reg	Reg	Reg	Reg	Reg	SUTR
9	20.26	18.9	25.2	26.9	27.7	Reg	Reg	Reg	Reg	Reg	3D
11	21.19	18.9	19.29	20.93	21.7	Reg	Reg	Reg	Reg	Reg	SUTR
12	21	17	19	20	21	Reg	Reg	Reg	Reg	Reg	3D
13	24	19	19	20	20	Reg	Reg	Reg	Reg	Reg	SUTR
14	19	19	Reg	Reg	Reg	Reg	Reg	Reg	Reg	Reg	3D
15	Reg	Reg	Reg	Reg	Reg	Reg	Reg	Reg	Reg	Reg	to confirm
16	Reg	Reg	Reg	Reg	Reg	Reg	Reg	Reg	Reg	Reg	3D
17	20.86	14.59	20.15	20.89	24.24	Reg	Reg	Reg	24.3	25.45	SUTR
18	Reg	Reg	border	border	Reg	Reg	Reg	Reg	Reg	Reg	3D
20	20.99	19.29	20.29	24.29	24.29	Reg	Reg	Reg	Reg	Reg	3D
21	22.18	22.28	22.28	24.28	24.45	Reg	Reg	Reg	Reg	Reg	3D
22	20.99	19.29	20.29	24.29	24.29	Reg	Reg	Reg	Reg	Reg	3D
23	19.82	15.4	16.32	18.73	23.28	Reg	Reg	Reg	Reg	Reg	to confirm
27	Reg	Reg	Reg	Reg	Reg	Reg	Reg	Reg	Reg	Reg	to confirm
27	17.25	17.25	28.22	22.56	27.48	Reg	Reg	Reg	Reg	Reg	3D
29	Reg (2)	Reg (2)	Reg (2)	Reg (2)	Reg (2)	Reg	Reg	Reg (2)	Reg (2)	Reg (2)	SUTR
31	Reg	Reg	Reg	Reg	Reg	Reg	Reg	Reg	Reg	Reg	3D
33	Reg	Reg (2)	Reg	Reg	Reg	Reg	Reg	Reg (2)	Reg (2)	Reg (2)	to confirm
35	25.89	18.24	19.89	23.21	26.89	Reg	Reg	Reg	21.23	Reg	3D
36	25.89	18.24	19.89	23.21	26.89	Reg	Reg	Reg	Reg	Reg	3D
37	22.18	20.15	19.89	19.22	25.89	Reg	Reg	Reg	Reg	Reg	3D
38	22.18	20.15	19.89	19.22	25.89	Reg	Reg	Reg	Reg	Reg	3D

Table 1: RT-PCR results of Panel 2
 CT-values highlighted in red were produced by EPIZONE partners

Discussion

The study was successful in that most of the participating laboratories correctly identified the positive and negative-FMDV samples. More significantly, these data will contribute to QA programmes and accreditation schemes in place in these laboratories. Future PTS will address important diagnostic activities of National Reference Laboratories and will be designed according to the guidelines set out in the proposed ISO 17043/43 standard. These guidelines will outline the PTS to the Community Reference Lab (CRL) and participating laboratories for FMDV and other exotic viral diseases. Prior to the start of each PT criteria for lab conformity must be set in order to identify poor-performing labs as stated in the EU policy to ensure the performance of important diagnostic activities of National Reference Laboratories. The primary goal of PT schemes is to evaluate lab (individual test and/or test system) performance against assigned values (qualitative or quantitative). The scope of the PT, therefore, needs to be fit for purpose and must be clearly defined and communicated to the participating labs.

Acknowledgements

This project was funded by EU CRL, FAO/EUFMD, EPIZONE (WP4.1) and LAB-ON-Site.

Comparison of Immunofluorescence (IF) and real-time PCR for the diagnosis of viral respiratory infections at Southampton General Hospital

Emma Andrews, Senior Biomedical Scientist (Virology), Molecular Diagnostics, HPA Southampton

A national strategy initiated by the HPA Molecular Diagnostics Forum (MDF) was to update respiratory diagnostics in the Regional Microbiology Network (RMN) by replacing current IF techniques with molecular methods. The MDF provided a pool of methods from which we chose suitable molecular assays that were compatible with existing local platforms and resources.

All samples received for IF between 1st January 2007 and 1st May 2007 were also tested by real time PCR. There were 200 samples tested in total. Assays deployed included influenza A and B, RSV, adenovirus, and parainfluenza 1, 2 and 3.

5 influenza A, 10 RSV, 11 adenovirus and 12 parainfluenza positives were detected by PCR in comparison to 2 influenza A, 5 RSV, 0 adenovirus and 2 parainfluenza positives detected by IF.

In summary, it was found that only 22.5% of the samples that were positive by PCR were also positive by IF. The majority of infections were found in the paediatric wards with a few cases in the oncology wards.

Changing to molecular methods for diagnosing respiratory viruses at Southampton General Hospital has greatly improved detection rates. Earlier detection by PCR will have the potential to contribute to reduced mortality and morbidity and preventing the spread of outbreaks at the hospital.

The development and clinical validation of a real-time duplex taqman assay for the simultaneous detection and quantification of CMV and EBV

Rivenberg WEN, Speirs A, Marsh P
HPA South East, Southampton Laboratory

The detection of both CMV and EBV DNA in immunocompromised (IC) patients by use of PCR has been the norm for many years. This is because IC patients lack the ability to synthesise the antibody used for diagnosis by serological techniques. Further, the serological evidence of both these virus' can be deceiving, hence the value of direct viral DNA detection.

Previously at Southampton General Hospital (SGH), leukaemic patients were monitored between once and twice per week depending on their post transplant status for CMV DNA using a quantitative monoplex real-time assay. With the highlighted importance of raised EBV DNA levels there is a need to monitor this routinely alongside CMV.

Primary infection or reactivation of either of these can have severe medical implications ranging from infectious mononucleosis-like illnesses to pneumonia and retinitis from CMV and Post Transplant Lymphoproliferation Disorders caused by EBV.

We therefore developed a multiplex real-time Taqman assay capable of specific CMV & EBV detection and discrimination. Furthermore, we have also trialled the assay as a multiplex quantitative test using a standard containing both CMV and EBV DNA allowing simultaneous viral load measurements on the same sample. This has produced very encouraging results when compared to a gold standard. This assay has improved sample-to-results turnaround time in simultaneous diagnosis of EBV/CMV status and viral load measurements in IC patients at SGH.