

2nd Course on Principles of Molecular Microbiological Diagnostics, ESCMID Postgraduate Education Course 

### Quantification

Jim Huggett

Science  
for a safer world



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### Quantification



In mathematics and empirical science, **quantification** (or quantitation) is the act of counting and measuring that maps human sense observations and experiences into members of some set of numbers. **Quantification** in this sense is fundamental to the scientific method.

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### Metrology



- **Metrology is the science of measurement**
  - **Scientific or fundamental metrology**

The establishment of quantity systems, unit systems, units of measurement, the development of new measurement methods, realisation of measurement standards and the traceability from these standards to society.
  - **Applied, technical or industrial metrology**

The application of measurement science to manufacturing, clinical and other applications and their use in society, ensuring instrument suitability, their calibration and quality control.
  - **Legal metrology**

Underpins results for statutory measurement to support the needs for (e.g.) protection of health, public safety, the environment, enabling taxation, protection of consumers and fair trade.

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**Measurement information needed** 

- Identity
  - Organism X is present
- Identity and Quantity
  - Organism X is present at abundance Y
- Identity, Quantity and Confidence (Uncertainty)
  - Organism X is present at abundance Y measured with confidence Z



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**When may quantification be necessary** 

- Diagnosis
- Stratification
- Prognostic monitoring

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**Quantitative methods used in Molecular Microbiological Diagnostics** 

- Nucleic acid amplification tests (NAAT)
  - qPCR
  - Quantitative isothermal amplification methods
- Sequencing
  - Next Generation(s) Sequencing
- Others
  - Arrays
  - Branched DNA

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**Relative vs absolute quantification**



- Relative
  - qPCR, NGS, Arrays, Fluorescence
- Absolute
  - CFU counting, Absorbance, digital PCR
- Convert 'relative' to 'absolute' using calibrators
- Calibrators represent a simple idea that is complicated to implement

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**Achilles heel of quantification (measurement)**



**Standardisation**

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**Standardisation**



- Enables
  - Comparison of measurement
  - Robust findings
  - Better conclusions
- Standardisation is essential to measurement
- Standardisation is often taken for granted

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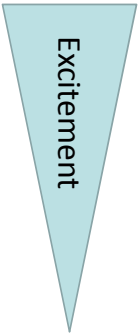
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**Molecular diagnosis research** 

- Developing novel methods
- Point of care
- Improve current approaches
- Challenges of standardisation




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**What unit?** 

- Colony (Plaque) Forming Units
- Genomes/Genome equivalents
- International Units (IU)
- Instrument specific units (e.g. Cq, or Ct, for qPCR)

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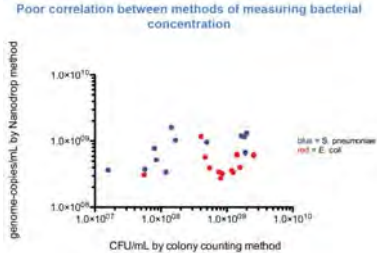
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**What if different units disagree?** 

Poor correlation between methods of measuring bacterial concentration



Spearman's  $\rho = 0.20$  (-0.44, 0.70)

Legend: blue = *S. pneumoniae*, red = *E. coli*

Source: *Making standards for quantitative real-time pneumococcal PCR* (Scott C. Moye et al., 2011)

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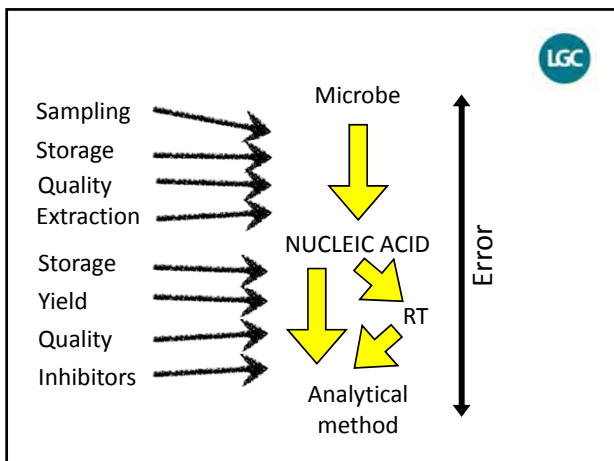
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What is the solution

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Journal of Clinical Virology 41 (2008) 307–311

Contents lists available at ScienceDirect

Journal of Clinical Virology

Journal homepage: www.elsevier.com/locate/jcv

Development of working reference materials for clinical virology

**Table 1**  
Summary of candidate run controls undergoing phase II evaluation

Run control	Strain	No. of responders/participants	$\Delta C_q$	2SD <sup>a</sup>	Overall $C_q$ value range <sup>b</sup>
Influenza A (H1N1)	A/Chonburi/1/2003 (H1N1)	11/13 (85)	$\Delta C_q=15$ ( $3.2 \times 10^4$ -fold)		25.30–40.66
Influenza A (H3N2)	A/Wyoming/2/2003 (H3N2)	11/13 (85)	$\Delta C_q=13$ ( $0.8 \times 10^4$ -fold)		24.30–37.88
Influenza A*	Rijnsburg/10/2003	12/13 (92)	$\Delta C_q=14$ ( $1.6 \times 10^4$ -fold)		22.46–38.75
Norovirus	Genogroup II	15/17 (88)	$\Delta C_q=21$ ( $2.1 \times 10^5$ -fold)		23.00–44.03
HSV-1	17	14/23 (61)	$\Delta C_q=21$ ( $2.1 \times 10^5$ -fold)		18.73–39.23
HSV-2	HCS2	14/23 (61)	$\Delta C_q=21$ ( $2.1 \times 10^5$ -fold)		20.83–39.00
HCMV	AD169	15/23 (65)	$\Delta C_q=19$ ( $5.2 \times 10^4$ -fold)		24.87–37.40
EBV	Risk	10/18 (56)	$\Delta C_q=13$ ( $0.8 \times 10^4$ -fold)		17.26–36.56
			$\Delta C_q=19$ ( $5.2 \times 10^5$ -fold)		

<sup>a</sup> More than one dataset was returned by some laboratories.  
<sup>b</sup> Excluding results that were negative or outside the cut-off for the assay.  
<sup>c</sup> Excluding results for one laboratory performing nested real-time PCR.

Overall in the performance of clinical target viruses. For the influenza and norovirus candidate run controls a number of assays failed to detect these targets in one or more runs, although in some cases this was reported to be due to inhibition of the PCR. For all run controls, a high level of both inter- and intra-assay performance variation was observed. The highest

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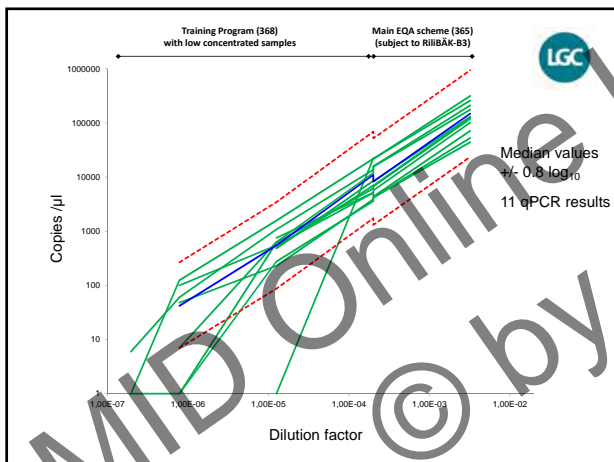
**Virus Genome Detection of CMV**  
Main-EQA Scheme (365) and Training Program (368) – September 2014

Main EQA scheme (365)		
Sample No.	Sample source	Dilution Dilution factor: 4 x Y
365069*	plasma pool of healthy blood donors spiked with a lysate of CMV infected cells (isolate of a patient)	1 : 313*
365070 = 365072	negative plasma pool of healthy blood donors	----
365071* <sup>5</sup>	plasma pool of healthy blood donors spiked with a lysate of CMV infected cells (isolate of a patient)	1 : 5 000* <sup>5</sup>
365072 = 365070	negative plasma pool of healthy blood donors	----


  

Training Program (368)		
Sample No.	Sample source	Dilution Dilution factor: 4 x Y
368005* <sup>5</sup>		1 : 5 000* <sup>5</sup>
368006*	plasma pool of healthy blood donors spiked with a lysate of CMV infected cells (isolate of a patient)	1 : 5 120 000*
368007*		1 : 1 280 000*
368008*		1 : 80 000*



What about bacteria?



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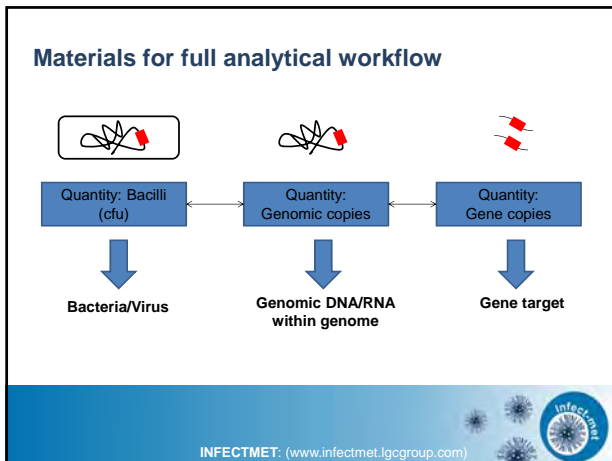
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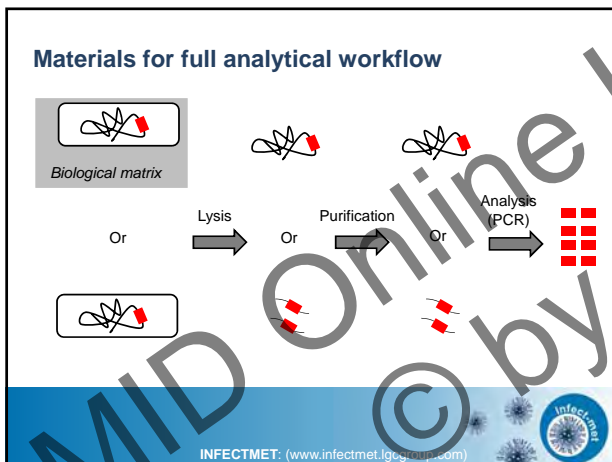
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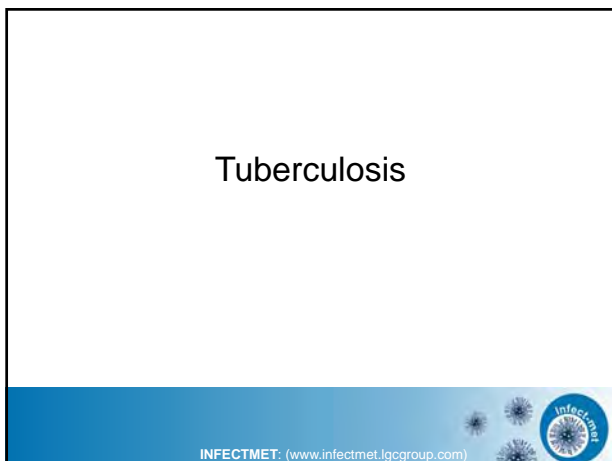
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The International Journal of Biochemistry & Cell Biology  
 Volume 35, Issue 10, October 2003, Pages 1407-1412

Medicine in focus  
**Tuberculosis: amplification-based clinical diagnostic techniques**

Jim F. Huggett<sup>a,\*</sup>, Timothy D. McHugh<sup>b,1</sup>, Alimuddin Zumla<sup>a,2</sup>

<sup>a</sup> Centre for Infectious Diseases, Royal Free and University College Medical School, University College London, Windeyer Building, 46 Cleveland Street, London W1T 4JF, UK  
<sup>b</sup> Department of Medical Microbiology, Royal Free and University College Medical School, University College London, Royal Free Campus, London NW3 2PF, UK

Available online 8 April 2003  
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Xpert RIF/MTB



Automated Real-time Nucleic Acid Amplification Technology for Rapid and Simultaneous Detection of Tuberculosis and Rifampicin Resistance Xpert MTB/RIF System

Cepheid

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analytical chemistry


Highly Reproducible Absolute Quantification of *Mycobacterium tuberculosis* Complex by Digital PCR

Alison S. Devonshire,<sup>a</sup> Isabella Honeyborne,<sup>a</sup> Alice Gutteridge,<sup>b,1</sup> Alexandra S. Whale,<sup>a</sup> Gavin Nixon,<sup>a</sup> Philip Wilson,<sup>a</sup> Gervyn Jones,<sup>a</sup> Timothy D. McHugh,<sup>a</sup> Carole A. Foy,<sup>a</sup> and Jim F. Huggett<sup>a,2,3</sup>

<sup>a</sup> Molecular and Cell Biology Team, LGC, Teddington, Middlesex TW11 0LX, United Kingdom  
<sup>b</sup> Centre for Clinical Microbiology, Department of Infection, Royal Free Campus, University College London, London NW3 2PF, United Kingdom  
<sup>3</sup> Statistics Team, LGC, Teddington, Middlesex TW11 0LX, United Kingdom

Supporting Information

ABSTRACT: Digital PCR (dPCR) offers absolute quantification through the limiting dilution of template nucleic acid molecules and has the potential to offer high reproducibility. However, the robustness of dPCR has yet to be evaluated using complex genomes to compare different dPCR methods and platforms. We used DNA templates from the pathogen *Mycobacterium tuberculosis* to evaluate the impact of template type, master mixes, primer pairs and extraction methods on dPCR performance. Performance was compared between the chip (BioMark) and droplet (Quanta) formats. In the absence of any external calibrators, dPCR measurements were generally consistent within ~2-fold between different master mixes and primers. Template DNA integrity could influence dPCR performance; high molecular



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### What is Digital PCR?

- Limiting dilution
  - Some reaction contain 0 templates
- PCR performed as normal using standard real-time PCR chemistry
- Absolute quantification
  - +ve or -ve reactions
  - Poisson statistics to account for multiple targets per partition (> 1)

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### Digital PCR

“The ability to measure extremely low concentrations of specific DNA sequences, independent of a standard curve, with high precision, in a complex background, is unique to dPCR”

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### M. bovis BCG in synthetic sputum

CFU data:  
Mean: 1.09E+07 ± 1.52E+06 (14%)

300 units prepared in synthetic sputum.

Molecular homogeneity & Short term stability complete, Long term stability ongoing

Journal of Medical Microbiology (2015), 54, 507–515. DOI: 10.1099/jmm.0.05991-0

Microcolony formation: a novel biofilm model of *Pseudomonas aeruginosa* for the cystic fibrosis lung

Dinesh D. Sasmal,<sup>1,2</sup> Heinrich Linsdorf,<sup>1</sup> Joseph S. Lam<sup>3</sup> and Ute Rostling<sup>1</sup>

<sup>1</sup>Microbiology and Tumor Biology Center (MTC), Karolinska Institutet, 141 77 Stockholm, Sweden  
<sup>2</sup>Department of Cell Biology and Immunology<sup>3</sup> and Department of Microbiology<sup>4</sup>, Gesellschaft für Strahlen- und Umweltforschung, 80334 München, Germany  
<sup>5</sup>Department of Microbiology, University of Guelph, Canada N1G 2W1

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**AMPLIRUN<sup>®</sup> TOTAL MTB CONTROL  
(SPUTUM)**  
For research use only

**MBTC013:** Inactivated *Mycobacterium tuberculosis* (MTB) cells formulated to mimic human sputum specimen and intended to validate and control sample processing, analysis and detection in nucleic acid assays using the product as an external run control.

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JCM  
Journal of Clinical Microbiology

Direct Comparison of Xpert MTB/RIF Assay with Liquid and Solid  
*Mycobacterium tuberculosis* Complex for Presumptive Tuberculosis and Rifampin Resistance

**A Multisite Assessment of the Quantitative Capabilities of the Xpert MTB/RIF Assay**

Robert Blakemore<sup>1</sup>, Pamela Nabeta<sup>10</sup>, Amy L. Davidow<sup>2</sup>, Viral Vadwai<sup>3</sup>, Rahn Tahiri<sup>4</sup>, Vanisha Munsamy<sup>2</sup>, Mark Nicol<sup>5</sup>, Martin Jones<sup>6</sup>, David H. Persing<sup>9</sup>, Doris Hillermann<sup>7</sup>, Sabine Ruesch-Geddes<sup>1</sup>, Felicity Leisegang<sup>8</sup>, Carlos Zamudio<sup>9</sup>, Camilla Rodriguez<sup>1</sup>, Catharina C. Boehme<sup>10</sup>, Mark D. Perkins<sup>10</sup>, and David Alland<sup>1</sup>


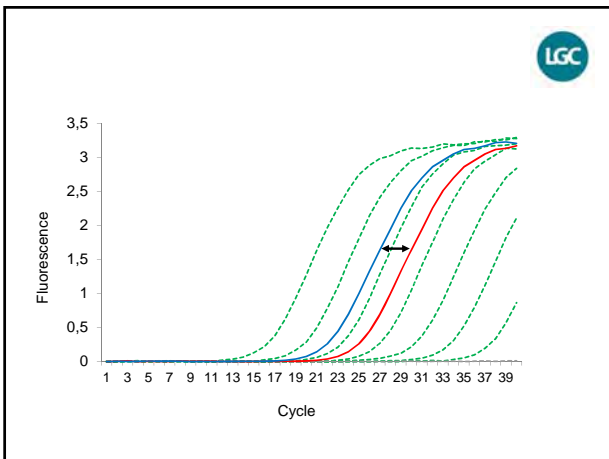
**Conclusions:** Xpert MTB/RIF quantitation offers a new, standardized approach to measuring bacterial burden in the sputum of patients with tuberculosis.

<sup>1</sup>Du; <sup>2</sup>Prc; <sup>3</sup>Frc; <sup>4</sup>Lal; <sup>5</sup>Me; <sup>6</sup>Sox

Azerbaijan; <sup>7</sup>Cepheid, Sunnyvale, California; and <sup>10</sup>Foundation for Innovative New Diagnostics, Geneva, Switzerland

**Abstract**  
Tuberculosis remains a leading cause of death globally, with approximately 1.5 million deaths annually. The Xpert MTB/RIF assay has shown a decrease in bacterial burden from mean baseline log CFU<sub>u</sub> = 5.00 (TP = 100.0) to day 14 (log CFU<sub>u</sub> = 0.26 ± 1.24, P = 0.001), day 28 (log CFU<sub>u</sub> = -0.55 ± 0.60, P = 0.0006), TTP = 18.0 (F = 80.000), and F = 4.37, P = 0.0020. The best discriminations between groups of effects was found with TTP on day 28 (F = 80.000, P < 0.0001), and F = 11.560, P < 0.0001, following log CFU<sub>u</sub> at day 28 (F = 48.000, P = 0.0001) and F = 7.257, P < 0.0001. F<sub>2</sub> was also significantly discriminative (F = 1.995, P = 0.001), and log CFU<sub>u</sub> at day 14 (F = 1.995, P = 0.001). Culture-based methods were superior to Xpert for the quantification of early antitubercular treatment effects in sputum.

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### Interlaboratory comparison

- Materials sent to eight clinical laboratories
  - Three perform qPCR
  - Six perform Xpert MTB/RIF

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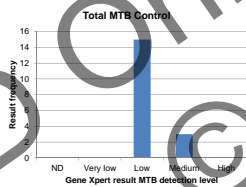
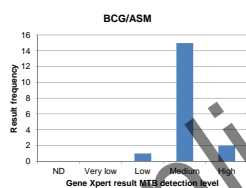
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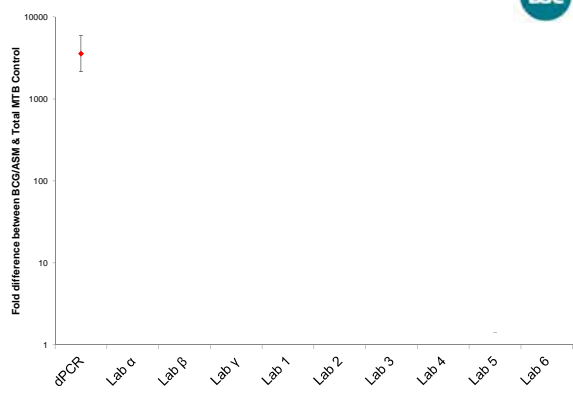
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Measured fold difference between materials




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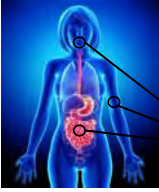
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### Metagenomics for microbial analysis



**The microbiome as a human organ**



- Oral ( $10^{10}$ ) bacteria
- Skin ( $10^{12}$ ) bacteria
- Intestine ( $10^{14}$ ) bacteria

Microbiomes play a crucial role in health  
 Implicated in many diseases  
 Microbiome analysis will define:  
 How they affect health and disease  
 Role in Diagnostics/Prognostic monitoring  
 Therapeutic manipulation

Human microbiome: 8,000,000+ genes  
 Human genome: 23,000 genes  
 >360 times

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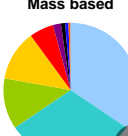
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### Method comparison




**Mass based**



**Method A**



**Method B**



- *Neisseria meningitidis*
- *Streptococcus pneumoniae*
- *Staphylococcus aureus*
- *Klebsiella pneumoniae*
- *Streptococcus pyogenes*
- *Streptococcus agalactiae*
- *Enterococcus faecalis*
- *Escherichia coli*
- *Pseudomonas aeruginosa*
- *Acinetobacter baumannii*

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### Accuracy?

*Int. J. Mol. Sci.* 2014, 15, 21476-21491; doi:10.3390/ijms151121476

OPEN ACCESS  
 International Journal of  
**Molecular Sciences**  
 ISSN 1422-0067  
 www.mdpi.com/journal/ijms

Article

#### Assessing the Accuracy of Quantitative Molecular Microbial Profiling

Dunise M. O'Sullivan <sup>1,\*</sup>, Thomas Laver <sup>2,†</sup>, Sachithan Temisk <sup>1,2</sup>, Nicholas Redshaw <sup>1</sup>, Kathryn A. Harris <sup>3</sup>, Carole A. Foy <sup>1</sup>, David J. Studholme <sup>2</sup> and Jim F. Hoggart <sup>1</sup>

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<sup>2</sup> Biosciences, University of Exeter, Geoffrey Pope Building, Stocker Road, Exeter EX4 4QD, UK; E-Mails: tw207@exeter.ac.uk (T.L.), d.j.studholme@exeter.ac.uk (D.J.S.)  
<sup>3</sup> Department of Microbiology, Virology and Infection Control, Great Ormond Street Hospital for Children NHS Trust, Great Ormond Street, London WC1N 3JH, UK; E-Mail: Kathryn.harris@gosh.nhs.uk

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**Summary. Quantification**

- Quantitative considerations influence all measurement.
- To develop useful quantitative molecular methods we need to understand technical sources of bias and variance.
- This will improve technical accuracy and allow us to determine what is biologically possible.
- For pre clinical methods to be successfully translated, we must consider technical reproducibility and mechanisms for standardisation.

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**Acknowledgements**

- LGC**
  - Alison Devonshire
  - Simon Cowen
  - Denise O'Sullivan
  - Alexandra Whale
  - Alice Gutteridge
  - Gerwyn Jones
  - Carole Foy
  - Helen Parkes
- University College London**
  - Tim Mchugh, Isobella Honeybourne
  - Jeremy Garson & Kathryn Harris
- Charite**
  - Heinz Zeichhardt, Hans-Peter Grunert
- Vircell**
  - Pablo Mendoza
- Bio-Rad**
  - Svilen Tzonev/Vresh Patel/DBC




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**Acknowledgements**

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- Great Ormond Street Hospital
  - NUI Galway
  - Forschungszentrum Borstel
  - KCMC/KCRI
  - Lancet Laboratories
  - San Raffaele Scientific Institute
  - TASK Applied Science
  - University College London



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**EMPIR**  **EURAMET**  
The EMPIR initiative is co-funded by the European Union's Horizon 2020 research and innovation programme and the EMPIR Participating States

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### Biomolecular Detection and Quantification

Focus on **qualitative and quantitative molecular methodologies** to all areas of clinical and life sciences.

To provide a forum for discussion and recommendation of guidelines designed to improve the accuracy of **molecular measurement**

To publish **molecular biology** based studies that adhere to best practice guidelines, both current and future.

Editor-in-Chief: Prof Stephen Bustin  
jim.huggett@lgcgroup.com



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