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# Quantitative PCR in the assessment of novel hepatic cell models

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The process of amplifying DNA by exploiting the polymerase chain reaction (PCR) was first reported in the 1980s by Mullis and colleagues<sup>1</sup>. This formed the basis for the next step of PCR technology: using dyes to quantify the amount of DNA being produced after each cycle of the reaction. This process became known as quantitative real-time PCR (qPCR) and has been extensively used to assess the expression of genes from a huge variety of species, becoming a fundamental tool in investigative studies within molecular biology. Here, we discuss the use of qPCR to assess the differentiation of hepatic cell models at an early stage to inform model selection for drug development uses.

## Background

qPCR functions by generating a fluorescent reading with every cycle, which increases exponentially in line with the quantity of DNA present in each well. Generally, qPCR instruments report back the cycle number at which fluorescence first becomes significantly different from the background (variously referred to as C<sub>q</sub>, C<sub>t</sub> or C<sub>p</sub>). This is then used to calculate the concentration of the starting mRNA in the sample

by comparison to a standard curve or in calculation of the value of  $\Delta\Delta C_t$  to determine relative changes in expression of a gene (see <sup>2</sup> for explanation).

Although this is essentially straightforward, it is in fact often oversimplified and care is required in all aspects of sample preparation, storage of both samples and reagents, both design and verification of PCR assays and appropriate assessment of normalisation procedures to

ensure high quality data are produced. Without this verification, the end results can be at best disappointing and at worst entirely misleading. This is an area that needs recognition and significant improvements if qPCR studies are to have relevance in scientific reporting. The publication of inaccurate data due to elementary mistakes can and possibly has damaged the reputation of this technique for producing reliable and repeatable data. This was critically recognised by Steve Bustin and colleagues<sup>3</sup>, who published the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines in an attempt to improve qPCR standards. This provides a basic set of requirements for producing a reliable and repeatable qPCR assay to improve the quality of data reported. This is particularly important when qPCR data are being collated from the literature and used in comparisons, such as in the assessment of tissue responses to

“Currently, the gold standard model for *in vitro* testing of novel compounds is the primary hepatocyte”

challenge or treatment in a variety of cell lines, or in the preliminary assessment of a model.

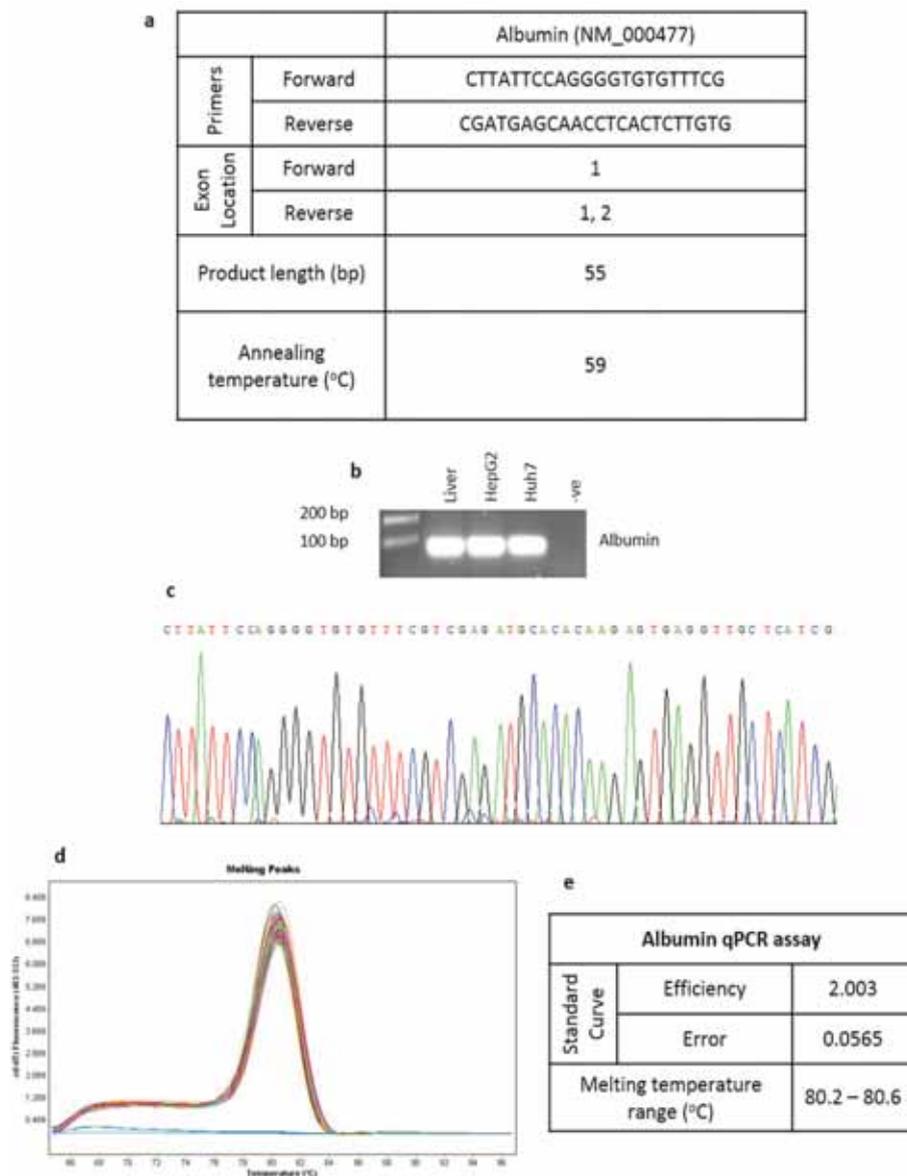
**The search for appropriate cell lines**

Drug development is an expensive process, especially considering the fact that a compound can pass through initial tests unscathed but then produce toxic effects in later studies or even in patients. The cost of bringing a drug to market has been estimated to be \$800 million to \$2 billion<sup>45</sup> so any improvement in the process of testing novel compounds to yield more reliable, relevant data in terms of predicting effects in patients would be of enormous benefit both to patients and in reducing costs overall.

The liver plays a critical role in drug metabolism and excretion in the body and so, currently, the gold standard model for *in vitro* testing of novel compounds is the primary hepatocyte. These are isolated directly

from human liver and used within a short time frame to provide the most relevant data currently possible from a cell model. However, together with the moral and ethical dilemmas of using such tissue, consistent and reliable supply can be an issue since the cells must be used within a short time frame to minimise de-differentiation away from the true *in vivo* hepatic phenotype. Primary hepatocytes are therefore a costly model to use and not ideally suited to initial high-throughput drug discovery phases. Although there are other systems such as cryo-preserved hepatocytes available, there continues to be a search for cell lines which could reliably predict toxicity and drug-drug interactions, with the potential to cut the costs of drug development dramatically.

Cell lines are relatively cheap and easy to maintain but do have their drawbacks. Well established hepatocyte-like or hepatocyte-derived cell lines such as HepG2 have been shown to differ markedly from the *in vivo* hepatocyte. This is especially evident in expression of metabolising enzymes like the cytochrome P450 (CYP) family, some of which are particularly important in drug metabolism<sup>46-48</sup>. A fairly recently established cell line, HepaRG, is potentially phenotypically much closer to the *in vivo* hepatocyte<sup>12-16</sup>. Further hepatocyte-like differentiation may be achieved by treatment with dimethyl sulphoxide (DMSO), which is also used in maintaining the differentiated state of isolated primary hepatocytes<sup>17</sup>.



**Figure 1:** Development of a qPCR assay for albumin. Part (a) shows primer and exon locations, melting temperature and product length, with endpoint PCR results shown in part (b). Part (c) shows sequencing results for the product. Data concerning the qPCR assay are shown in parts (d) and (e), where melt peaks, the corresponding melting temperatures and standard curve information are displayed

**Hepatic cell line experiment**

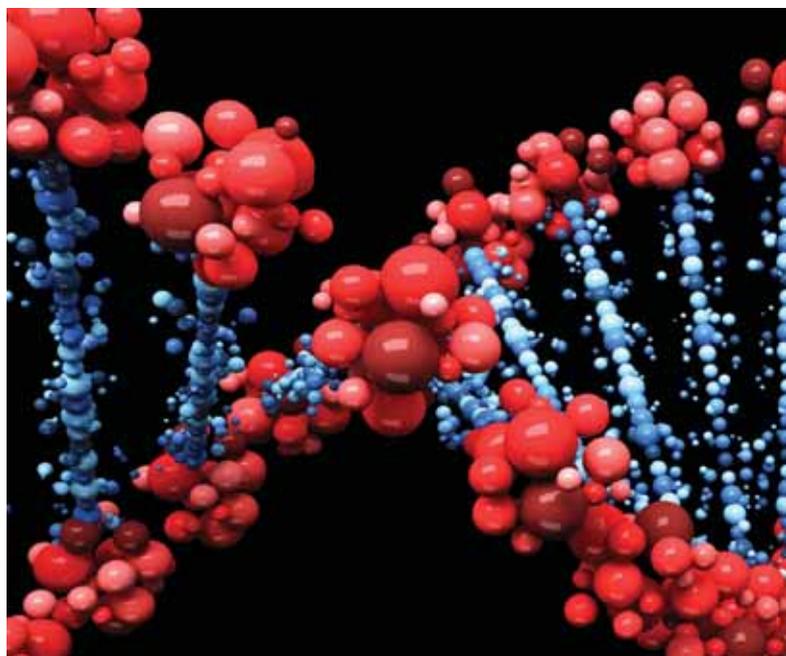
Here, we investigated DMSO treatment of

## IN-DEPTH FOCUS: PCR

two other established hepatocyte-derived human cell lines, HepG2 and Huh7. qPCR was used to assess expression of a variety of genes in relation to *in vivo* levels, which was then compared to available data from the literature for HepaRG cells.

HepG2 and Huh7 cells were grown on six-well plastic plates in normal growth media until roughly 90% confluent and subsequently treated with 1% DMSO (v/v) for up to 30 days. Initial expression data for markers of differentiation indicated an optimal exposure period of 15 days, which was used for all subsequent experiments. RNA was then extracted (SV Total RNA Isolation System, Promega), the quality assessed (2100 Bioanalyser, Agilent, California, USA), reverse transcribed (MMLV Reverse Transcriptase and RNasin, Promega), and qPCR performed for a range of genes (LightCycler 480, Roche). Sample quality is a key factor in the success of qPCR and in all experiments only RNA with a RIN of greater than eight was used.

Assay design and validation, as noted earlier, is vital in obtaining accurate and reliable qPCR data. **Figure 1** (page 9) illustrates the process of validation carried out for an albumin qPCR assay using Sybr green as the DNA marker; the process is essentially similar for each qPCR assay. Albumin is illustrated as a recognised marker of adult human hepatocyte differentiation. Primers were designed so that one of the pair crosses an exon-exon boundary, to remove the possibility of amplifying any contaminating genomic DNA, and total product length was under 150 base pairs where possible to ensure maximum PCR efficiency (**Figure 1a**, page 9). HPLC purification of primers can increase the accuracy of an assay where standard desalting purification results in an assay containing multiple products. Initially, the primers were used to conduct an end point PCR, analysed by agarose gel



electrophoresis (**Figure 1b**, page 29), to ensure that only one product was generated and to identify the presence or not of primer dimers. The products were extracted from the agarose gel and cloned using the pGEM T-easy vector system (Promega). This allowed both sequencing of the product to ensure the correct sequence was being amplified (**Figure 1c**, page 9) and generation of positive controls for use in the qPCR.

During all qPCR programs a melt curve was included as a final stage after amplification; this involves measuring fluorescence continuously

## Q&A

### **European Pharmaceutical Review interviews François-Xavier Sicot, PhD, Senior Product Manager at Takara Bio Europe, about his views on Reverse Transcription qPCR (RT-qPCR).**

#### **Why is it so important that the pharma industry and diagnostic companies adopt RT-qPCR?**

RT-qPCR has become the gold standard for RNA quantification. Following the whole transcriptome RNA-seq approach, RT-qPCR enables precise validation of differential gene expression information on selected genes. It is widely used by pharma to discover and monitor biomarkers in disease. In addition, RNA-virus diagnosis by RT-qPCR offers an accurate way of detecting pathogens and quantifying the viral load. Used as a companion diagnostic test, this technique can monitor the efficacy of therapeutic treatments against a targeted virus, cancer or chronic disease.

#### **How do your products compare to others on the market?**

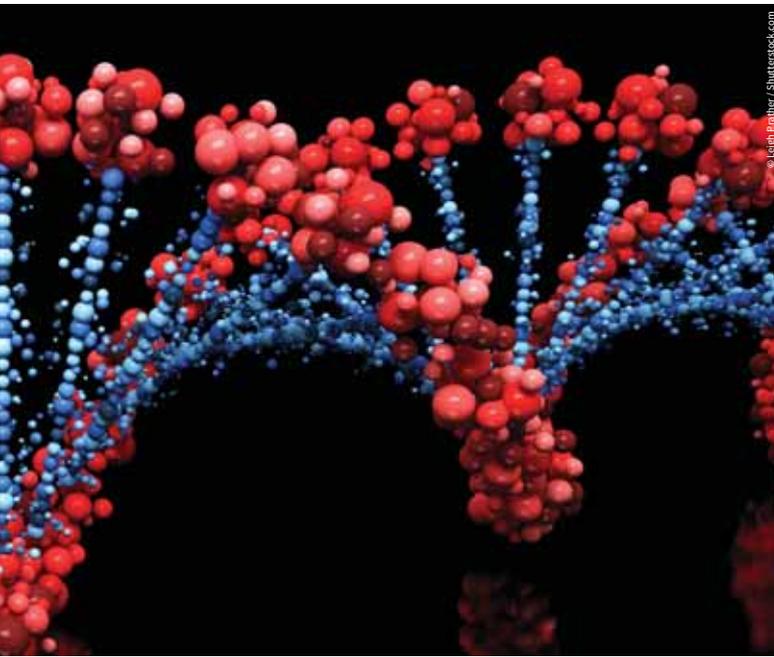
The industry requires rapid, sensitive and accurate detection of any RNA. Takara Clontech offers a unique, complete product line for one- and two-step RT-qPCR based on best-in-class enzymes. PrimeScript RTase was developed to express high strand-displacement activity, efficient cDNA synthesis up to 13kb and low error rates. Combining such a unique enzyme with the powerful ExTaq HS polymerase and a thermostable RNase H ensures accurate RNA quantification by removing any inhibition of PCR. This ultimately results in increased sensitivity, easy setup and less time expended, compared with competitors' solutions.

#### **There is currently a lack of consensus in the industry on how best to perform and interpret qPCR experiments. What are you doing to tackle this issue?**

Obtaining data from RT-qPCR is easy; interpreting them requires taking a step back from the experiment and identifying the bottlenecks. Our customers can rely on a team of experts in the setup and troubleshooting of RT-qPCR experiments. Takara Clontech produces consistent reagents knowing the requirements of the technology, e.g., by providing a RT reagent with a fast and efficient gDNA removal step, which avoids the quantification of contaminant genomic DNA; by including a special solution for accurate dilution of cDNA; and by addressing the specificity problems of SYBR detection with different reagents versions. As such we support researchers to help them produce reliable RNA analysis tools for diagnostic, prognostic and therapeutic validation of candidates.

#### **RT-qPCR technology is a field that is constantly evolving – can you keep up?**

Our motto is: That's GOOD Science!™. Our enzymologists are striving to constantly improve enzyme-buffer performance and kit formulation for gene expression analysis, anticipating commonly encountered issues and changing requirements of the technology. Production is performed under strict QC within our ISO certified facilities. Our products are already included in many CE-IVD or FDA-approved kits. 🧪



as the temperature is increased slowly from 65 to 95°C. SYBR green dye is initially bound by the double stranded DNA products which are the result of amplification; with the slow increase in temperature a point is reached at which the secondary structure denatures and the DNA strands separate, resulting in a sharp decrease in fluorescence as the SYBR green dissociates from the DNA. The temperature at which this occurs is specific to each product, allowing determination of whether the sample products are identical to that of positive or negative

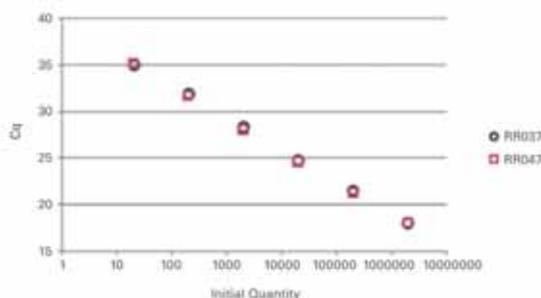
controls. **Figure 1d** (page 9) provides an example of the melting temperature (designated  $T_m$ ) graphic from the albumin qPCR assay, indicating that all products (including positive control) have the same  $T_m$ . Negative controls (illustrated as the flat blue lines along the bottom of the trace) indicate that any small amount of product present differs from that seen in the samples and positive controls. The range of  $T_m$ s observed in this assay is small, with good efficiency and small errors for the standard curve (a one in 10 serial dilution of the cloned PCR product over five orders of magnitude) (**Figure 1e**, page 9).

The efficiency, calculated from the slope of the standard curve, indicates that the amount of product detected in this assay increases by 2.003 with every cycle. Since an efficiency of exactly two, the theoretical value for qPCR, would indicate an exact doubling of product with each cycle, this assay is working well. For all assays used in this analysis of cell lines, efficiency values were as close as possible to two. If initial attempts showed efficiency significantly below this, optimisation of primer and template concentrations and annealing temperature were performed. Suitable reference genes for normalisation of the data were determined using GeNorm software<sup>18</sup>, and primer sets purchased from PrimerDesign (Southampton, UK) which were TOP1 and GAPDH for Huh7 cells, and hATP5b and GAPDH for HepG2 cells. Numerous studies<sup>19-22</sup> have indicated that many commonly used reference genes vary between tissue and sample types; therefore the MIQE guidelines suggest that better quality data are obtained using more than one reference gene.

Cell lines were compared before and following DMSO treatment as a proposed tool to enhance hepatocyte differentiation and compared to expression in a sample of whole liver cDNA comprised of a pool of three

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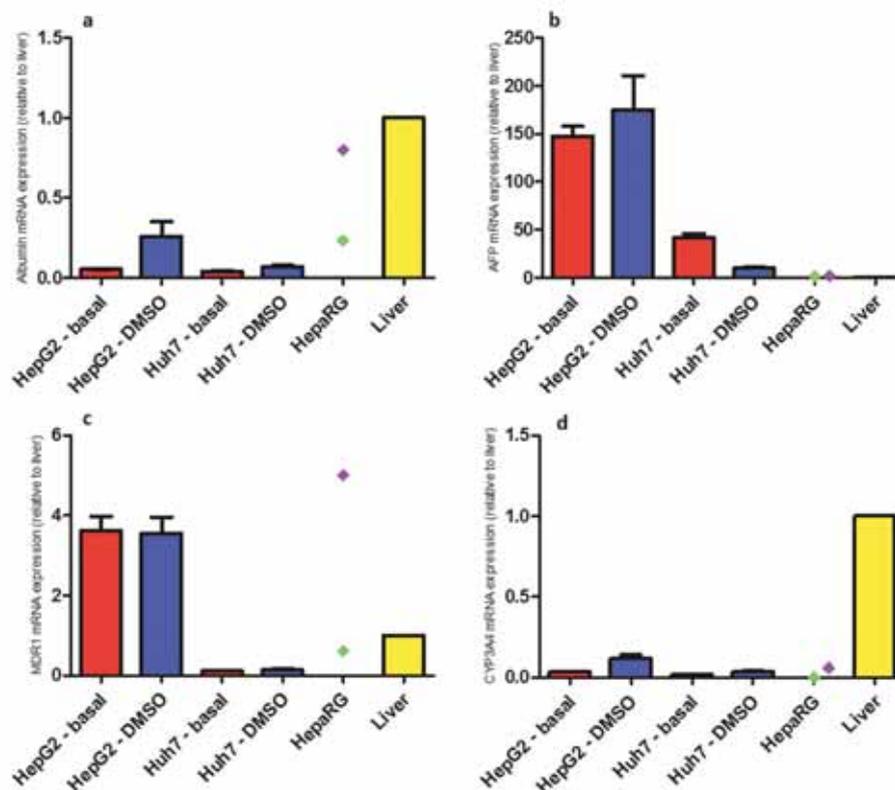
**2 step RT-qPCR on mouse Rsp18 gene.** 2pg-2µg total RNA was RTed with PrimeScript™ RT reagent (cat. no. RR037) or PrimeScript RT reagent with gDNA eraser (cat. no. RR047) for 15 minutes. With RR047, the RNA was spiked with 200 ng mouse gDNA and incubated for 2 minutes with gDNA eraser prior to the RT step. No significant change in detection is observed after gDNA eraser treatment compared with the RT alone.

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**Figure 2:** mRNA expression, shown relative to that in the liver, of basal and DMSO-related HepG2 and Huh7 cells assessed by qPCR for albumin (a), AFP (b), MDR1 (c) and CYP3A4 (d). A minimum of N=3 experiments were performed for HepG2 and Huh7 results. A literature search was performed to obtain data for levels of mRNA expression in the HepaRG cell line, with the highest (purple) and lowest (green) values shown

human liver samples (37 year old Caucasian male, 64 year old Caucasian male, 70 year old Caucasian female; purchased from PrimerDesign, UK). Albumin and alpha-feto protein (AFP) mRNA expression were determined to inform the relative hepatocyte-like differentiation of cells. Albumin expression is indicative of an adult-like differentiation comparable to hepatocytes *in vivo*, while AFP expression is indicative of de-differentiated foetal-like hepatocytes<sup>23,24</sup>. A slight increase in albumin mRNA expression following DMSO treatment was observed for both HepG2 and Huh7 cells, but this was much lower than the levels in liver (Figure 2a). Reported albumin mRNA expression in HepaRG cells ranged from close to liver to similar to that in HepG2 and Huh7 cells<sup>12,14,25</sup> (Figure 2a). AFP mRNA should decrease with differentiation; this was observed following DMSO treatment of Huh7 cells but not within HepG2 cells (Figure 2b). HepaRG cells are reported to express AFP at very low levels, similar to those in the liver *in vivo*<sup>12,14,25</sup>.

Results for two other genes of interest are illustrated in Figure 2; the multidrug resistance protein 1 (MDR1 or P-gp) transporter and the key drug-metabolising enzyme CYP3A4<sup>26</sup>. MDR1 is expressed extensively in the liver and increased expression can be associated with drug resistance in tumours; a high level of expression may lead to erroneous results in drug testing with cell models. HepG2 cells illustrated increased levels of MDR1 expression compared with liver under basal conditions, which was not reduced by DMSO treatment. MDR1 expression in Huh7 was lower than that found in liver and again was not altered by DMSO treatment. Reported expression of MDR1 in HepaRG

“ A qPCR assay would provide a relatively inexpensive, high-throughput means of determining the differentiation state of cells ”

cells varied between levels similar to those in HepG2 and comparable to liver<sup>14,27-29</sup>. Critically, there was a high level of expression of CYP3A4 in liver, but this was not replicated in any of the cell lines, with or without treatment with DMSO (Figure 2d)<sup>12,14,25,28,29</sup>.

At face value, while the cell lines under consideration here do not provide adequate models for differentiated adult hepatocytes, the data suggest that HepaRG may be the closest approximation. However, the results from these qPCR comparisons require some more detailed examination. Ceelen and colleagues<sup>30</sup> discussed HepaRG qPCR data and its compliance with the MIQE guidelines. They noted that many of the previous publications analysing mRNA levels in HepaRG cells utilised 18S rRNA as a reference gene. Upon conducting analysis of the suitability of reference genes in HepaRG cells with three different algorithms, including the GeNorm software, 18S rRNA was ranked among the least suitable by all three algorithms. Ceelen *et al.*<sup>31</sup> further analysed more than 20 previously published studies of qPCR in HepaRG cells, revealing that MIQE recommendations concerning appropriate housekeeping gene selection had not been taken into consideration. Thus, forming definitive conclusions on the superiority of HepaRG cells based on the evidence of such poorly controlled qPCR data alone is perhaps questionable.

### Conclusion

Quantitative PCR analysis of cell models is a powerful tool, if applied robustly. It has identified that neither HepG2 nor Huh7 cells are ideal



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## IN-DEPTH FOCUS: PCR

models to represent differentiated hepatocytes, with or without treatment with DMSO. HepaRG cells appear to provide a more suitable level of hepatocyte differentiation. However, in addition to ensuring appropriate application of qPCR standards, for any cell model to be validated fully also requires subsequent protein and functional characteristics to be determined. Once this has been achieved, and co-ordination between changes at the mRNA and protein/functional levels established, a qPCR assay would provide a relatively inexpensive, high-throughput means of determining the differentiation state of cells and thus their suitability as a model in drug discovery applications. 🦋



**Catherine Mowbray** is a Research Associate at Newcastle University. A very enthusiastic biology teacher and a subsequent degree in Human Genetics combined to spark an interest in human biology, which eventually led to a PhD investigating hepatic cell models. Since graduating from her PhD, Catherine has worked in several labs and acquired a large array of molecular biology techniques. She is currently investigating the role of anti-microbial peptides in innate immunity of both the gut and urinary tract, employing her expertise in quantitative PCR and ELISA development among other techniques to assess regulation and expression of these peptides.

**Alison Howard** is a research support specialist at Newcastle University. Following a PhD at London University investigating intestinal calcium absorption she spent many years researching transport processes in the intestine and has extensive experience in molecular biology and quantitative PCR. Her current research interests include regulatory pathways controlling expression of transporters involved in intestinal stress responses and she provides molecular biology expertise to many teams at Newcastle University's Institute for Cell and Molecular Bioscience.



**Barry Hirst** is Professor of Cellular Physiology at Newcastle University. Following BSc and PhD studies at Newcastle University, he was a NIH Fogarty Fellow at the University of California, Berkeley, before returning to a Faculty position at Newcastle. He has had a life-time interest in the molecular and cellular properties of epithelia, with particular reference to the gut. His studies on the basic physiology have translated in to applications in relation to drug absorption and secretion. A particular focus has been identifying how drug secretory efflux systems contribute to limitation of drug absorption in the gut and contribute to drug secretion.

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