european pharmaceutical review

PCR

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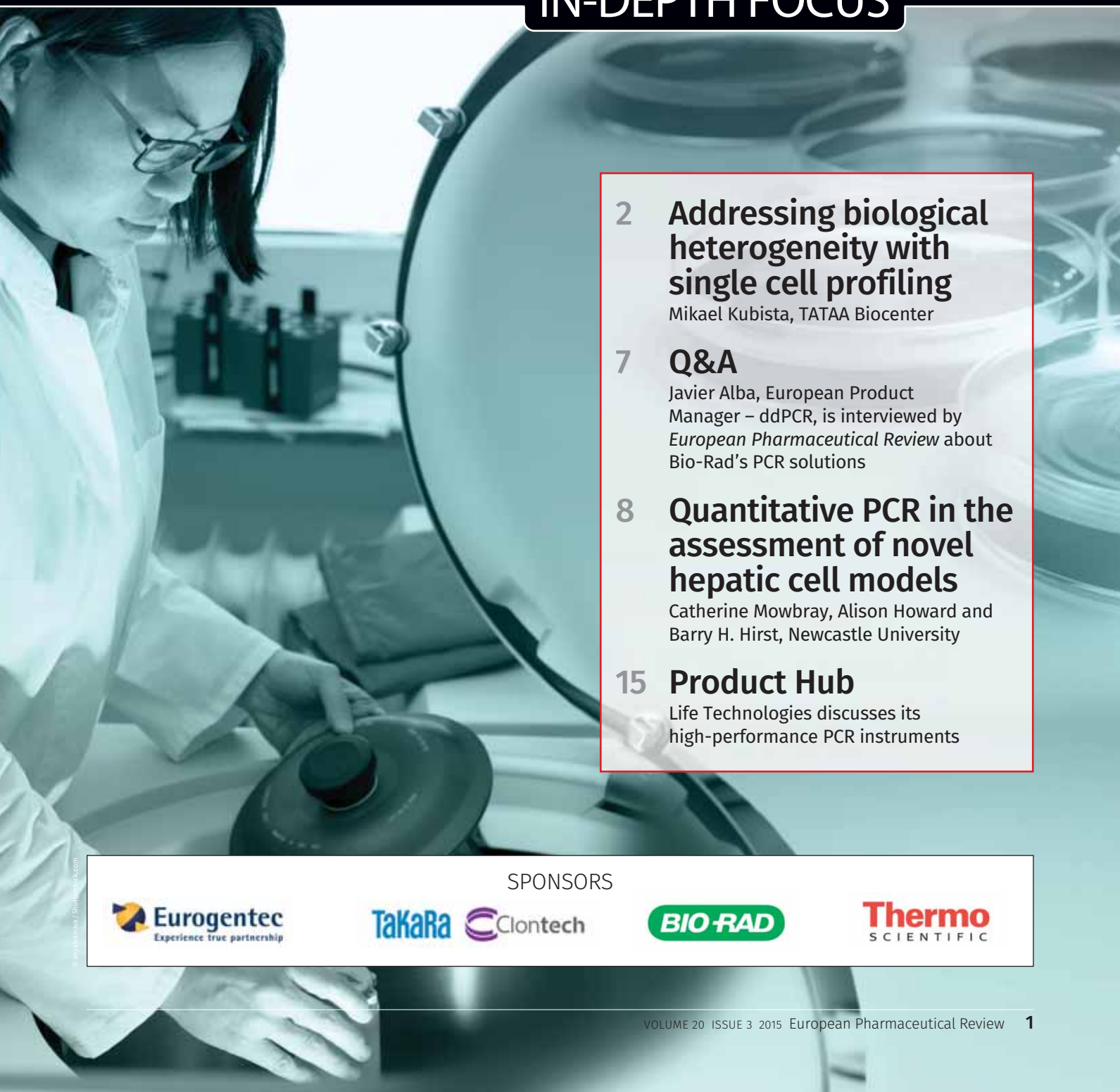

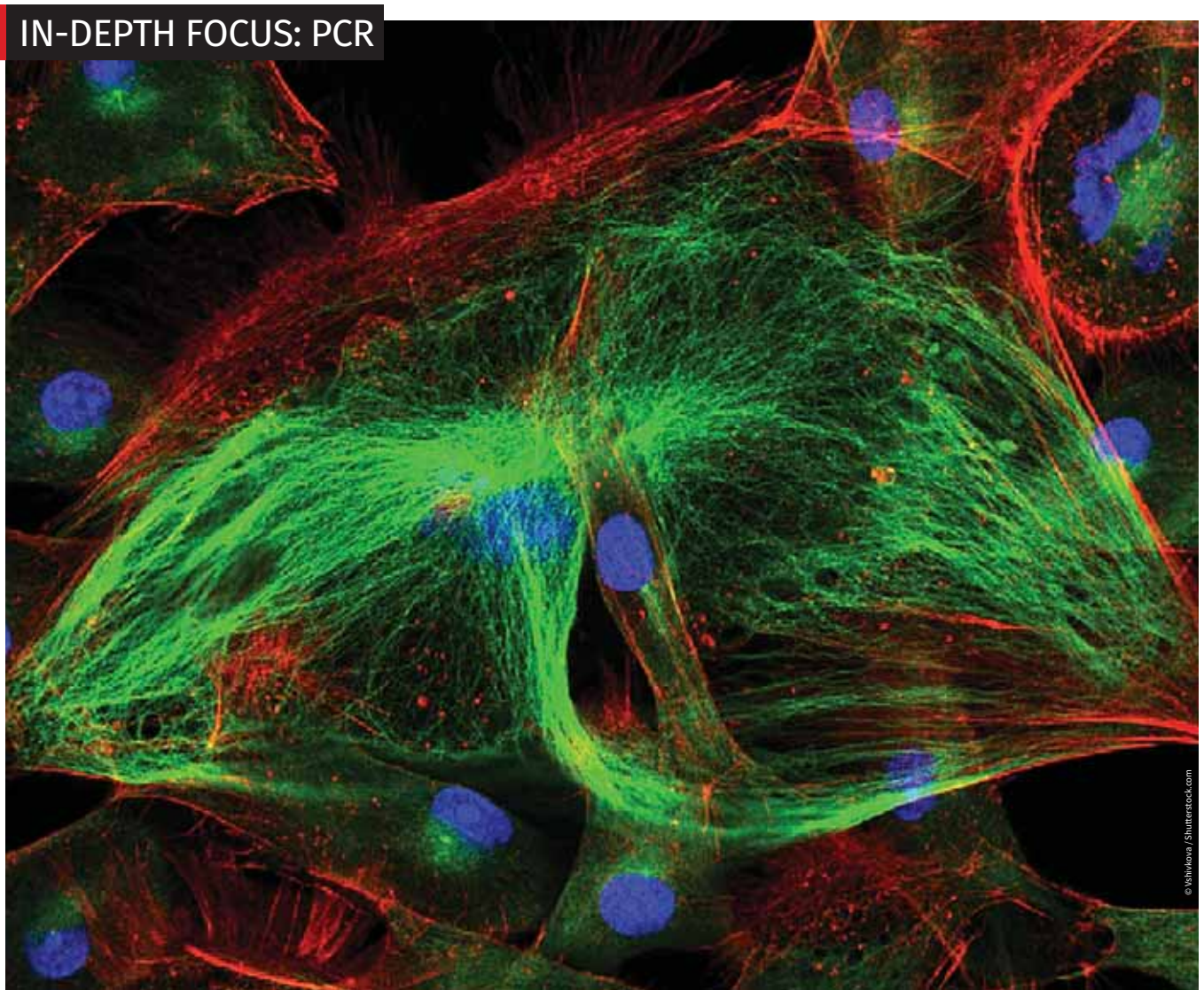

Addressing biological heterogeneity with single cell profiling

Mikael Kubista
TATAA Biocenter

The more we study biology, the more complexity we discover. Organs, for example, are composed of many different cell types and several tumour types are thought to be polyclonal. It is the individual cells that make up tissues and organs that we need to study in order to better understand biological responses and communications, and during the past few years we have seen important developments in techniques for single cell profiling and in methods to analyse such data.

Kary Mullis, a scientist at the Cetus Corporation, conceived in 1983 the polymerase chain reaction (PCR) as a method to copy DNA and produce large amounts of any selected sequence. A team of Cetus scientists were successful in implementing those ideas into practice, studying the ß-globin sequence variation in sickle cell anaemia and publishing the first PCR paper in 1985.[1] Just three years later, the first publication of PCR of single human cells came about.[2] This was an analysis of DNA in somatic and sperm cells and demonstrated the

sensitivity of PCR, however, it did not reflect any variable features of the cells. The first gene expression profiling study of single cells came in 2005.[3] It was performed on beta cells and revealed unexpectedly large heterogeneity in the amount of transcript across the cells, with the majority of cells containing just few transcripts, while some cells had very large levels (**Figure 1**). The spread could be modelled with a normal distribution in logarithmic scale (**Inset, Figure 1**). Since this first observation, lognormal distribution of transcripts across like-cells has been observed for all kinds of transcripts in almost all tissues; currently the only exception is *Xenopus laevis* oocytes, which lack mRNA metabolism. Hence, in a classical sample made up of many cells, most transcripts come from just a handful of cells.

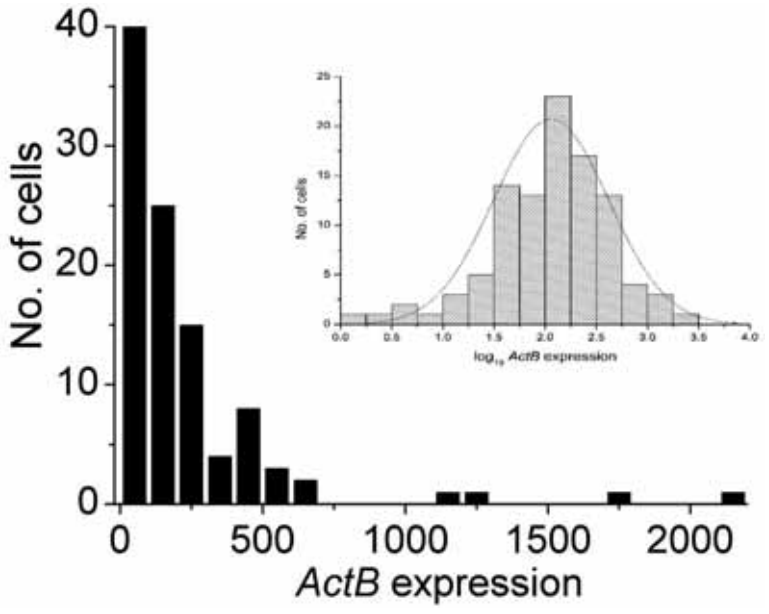

Figure 1: Frequency diagram showing the distribution of beta actin transcripts among individual beta cells from a MIN6 cell line. Inset shows the same data presented with logarithmic scale on the x-axis.

The mechanism behind this was revealed by a series of excellent fluorescence *in situ* hybridisation studies of life cells which revealed that expression on a cell level is stochastic.[4] Expression bursts are seen, leading to very large variation in transcript levels over time in each cell. Taking a snapshot of many individual cells, the burst kinetic leads to a distribution of transcripts across cells that is consistent with the observed lognormal distribution. At each time point different cells will be bursting but, over time, the integrated expression in each cell should be the same. The dynamic behaviour of single cell transcription has the consequence that a cell type cannot be identified by the amount of a particular transcript it contains at any given time, since even the most expressed gene does not give rise to transcripts in all cells every time.

> "A series of excellent fluorescence *in situ* hybridisation studies of life cells revealed that expression on a cell level is stochastic"

So the question is: how can we tell different cell types apart? It seems that while transcriptional bursts for most genes are independent, some genes show highly correlated bursts. Hence, by measuring levels of many transcripts in each cell, different types of cells can be distinguished by their expression profiles, which reflect groups of active genes. This requires the ability to measure the expression of several genes per cell. Even in the first single cell profiling study of the β cells, expression of five genes per cell was measured. Indeed, three genes showed no correlation of transcript amounts on a single cell level with any of the other genes, demonstrating that their bursts are completely independent, while the two insulin genes showed almost perfect correlation, showing that they are expressed at the same time in the same cell (**Figure 2**).

	ActB	*Ins1*	*Ins2*	*Abcc8*	*Kcnj11*
ActB	1				
Ins1	0.15	1			
Ins2	0.12	0.90	1		
Abcc8	−0.02	−0.01	0.06	1	
Kcnj11	0.11	−0.02	0.24	−0.15	1

Figure 2: Correlation between genes' expressions in individual beta cells expressed as Pearson correlation coefficients.

Real-time PCR (qPCR) has very limited multiplex capability. With standard approaches a maximum of four-to-five targets can be simultaneously measured and then usually with compromised sensitivity and dynamic range. Analysis of larger numbers of transcripts requires the sample to be split into aliquots with each then being analysed for a single transcript. This causes a problem when analysing single cells. Each cell contains only very few transcripts of each type and if the content were to be divided into, say, 100 aliquots (which is pretty close to the 96 markers commonly analysed in most workflows), it may not be possible to obtain a transcript in each aliquot and one may fail detecting it. In fact, some 300 transcripts are needed to get at least one in 100 aliquots with 95% probability (equivalent to a confidence level of 95%). Apart for the most extreme expressors, no gene will have 300 transcripts in the majority of cells at any given time. Still, even if we had a total of 300 transcripts for a gene in a cell and, hence, could detect its expression with 95% confidence, we would not be able to quantify its expression level with any precision. Precise quantification requires there are some 35 copies per aliquot.[5] Hence, in analysing 100 genes, we would need each of them to have 3,500 transcripts in the cell for accurate quantification. Normal cells do not have this many high expressors. Clearly, a different approach is needed.

Preamplification method

During the past couple of years a workflow for single cell expression profiling based on preamplification has been developed (**Figure 3**, page 4). Individual cells are harvested by some means. Most popular are fluorescent activated cell sorting (FACS), aspiration, which can

be automated using, for example, the CellCelector[6], and laser capture. The cells are deposited into a direct lysis reagent, such as the Cellulyser[7], which opens the cell and stabilises the RNA. The direct lysis reagent is compatible with downstream analyses, which eliminates the need for washing and minimises losses. After reverse transcription, the cDNA is preamplified by highly multiplexed PCR using limiting concentrations of primers for a limited number of cycles (typically 12-20, depending on reaction volumes used in the downstream singleplex PCR), such that the reactions do not compete for reagents.

Highly optimised PCR assays should be used to suppress the formation of aberrant products, commonly referred to as primer-dimers. The preamplified DNA is then aliquoted for singleplex PCRs, which are typically run on one of the high-throughput instruments such as the BioMark (Fluidigm), OpenArray (Life Technologies) or LC1536 (Roche). The workflow typically includes ValidPrime to correct for genomic DNA (gDNA) background.[7,8] One might think this should not be needed, considering a single cell usually only contains two copies of each chromosome and most assays are designed spanning introns to avoid amplifying the genomic gene sequence.

> “While transcriptional bursts for most genes are independent, some genes show highly correlated bursts”

In practice, signal from gDNA may be substantial for some assays. This is because of the presence of pseudogenes. About 15% of the human genes have pseudogenes that may be present in up to several hundred copies, and about half of the pseudogenes have been formed via retrotransposons and lack introns. These are readily amplified by intron-spanning primers giving rise to substantial background signal. The MIQE guidelines explain that one should test for gDNA background and recommends running RT- controls.[9] This is, however, impractical in the single cell workflow. Instead the ValidPrime assay is included throughout (i.e., also in pre-amplification). It is designed to specifically amplify a non-transcribed sequence present in exactly one copy per haploid genome. Hence, the ValidPrime does not amplify cDNA and measures only the amount of residual preamplified gDNA.

Knowing the gDNA background is not sufficient for correction. In addition to including the ValidPrime as assay, a gDNA standard is included as sample. The gDNA standard is analysed via preamplification with all the PCR assays, which reflects their sensitivity for gDNA and depends on the presence of pseudogenes. The combined

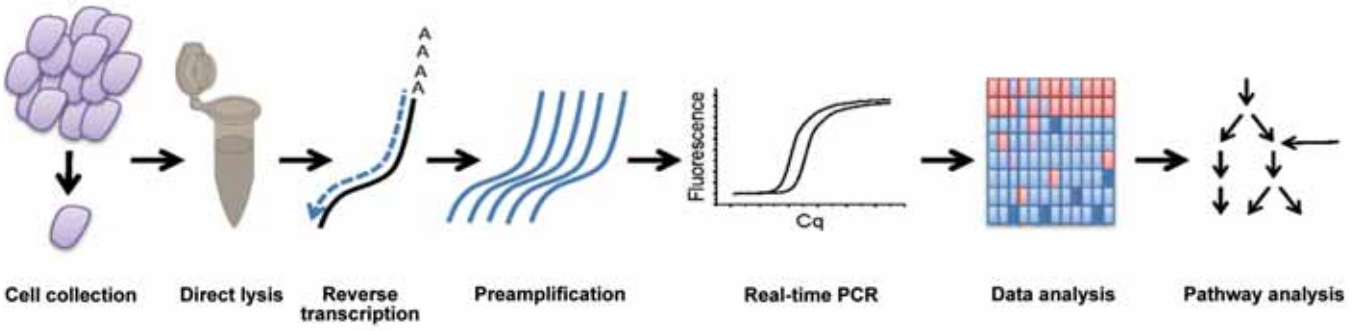

Figure 3: Single cell workflow based on preamplification

information from the measurements with the ValidPrime assay and on the gDNA standard allows correcting for the gDNA background in all of the samples for all assays. Correction is reliable for up to 50% gDNA background. Measured data from all major instruments are conveniently corrected and analysed with the GenEx software.[10]

Several exciting studies using single cell expression profiling have appeared during the last few years, including early embryonic development in animal models, human sperm and oocytes, stem cells, blood cells, tumour cells etc. Such studies often reveal previously unnoticed heterogeneity, clearer responses to environmental changes and to drugs, and more distinct interactions between gene networks. One example is a study of the activation of astrocytes in response to brain trauma in the form of focal cerebral ischaemia using a mouse model.[11] In this model astrocytes express green fluorescent protein under the control of the astrocyte marker glial fibrillary acidic protein (GFAP). Mice were sacrificed at zero, three, seven and 14 days after trauma, and astrocytes were prepared and analysed for genes' expression using the single cell workflow. The cells were then classified based on their global expression patterns using dynamic principal component analysis (PCA), which is a multivariate tool to classify the cells based on the combined expression of the genes, while filtering them to select the most informative.[10]

Astrocytes collected before trauma, and at three, seven, and 14 days after trauma separate in the PCA, reflecting gene expression changes taking place as the astrocytes are reactivated in response to the trauma. Notably, the astrocytes collected at 14 days after trauma form two distinct clusters revealing a heterogeneous population is created (**Figure 4**). Genes that are important for the activation could be identified from the PCA, as well as genes differentiating between the two subpopulations of the reactive astrocytes. Finally, correlation between the genes' expressions on the single cell level defined networks that produce transcripts at the same time in the same reactive astrocyte.

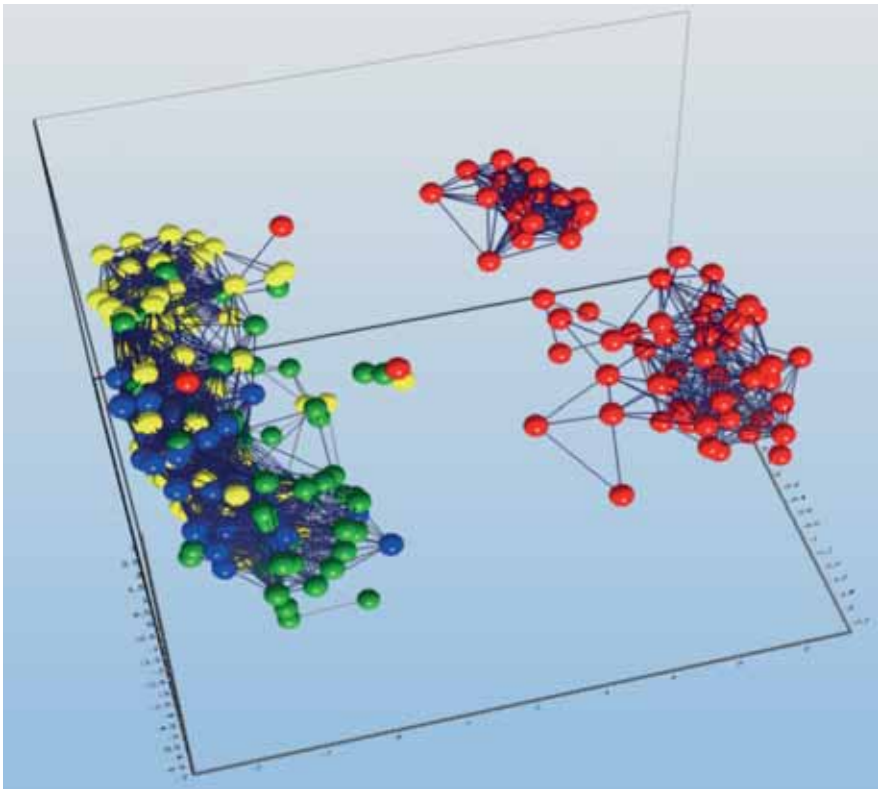

Figure 4: Multivariate classification of single astrocytes collected from mouse brains before trauma (blue), and at three (yellow), seven (green), and 14 days (red) after trauma based on expression profiles. Close lying cells are connected for visualization of the clusters.

Mikael Kubista had an interest in life sciences his entire life. He obtained a BSc in chemistry at the University of Göteborg, Sweden, in 1984, before working at Astra Hässle (today part of AstraZeneca), studying the K+/H+-ATPase inhibitor omeprazole. He returned to academia joining Chalmers University of Technology in Göteborg and received in 1986 a Technology Licentiate in Chemistry and in 1988 a PhD in physical chemistry on studies of nucleic acid interactions with polarised light spectroscopy. He did a postdoc at La Trobe University, Australia, on transcriptional foot-printing, and then at Yale University, USA, studying chromatin and epigenetic modulation of nucleosomes. Returning to Gothenburg in 1991, Kubista started his own research group studying DNA-ligand interactions. In 2001 he set up the TATAA Biocenter as center of excellence in qPCR and gene expression analysis with locations in Gothenburg, Sweden, Prague, Czech Republic, and Saarbrücken, Germany. In 2014 Kubista introduced non-invasive prenatal testing in Sweden founding the company Life Genomics AB. He also co-authored the MIQE guidelines for RT-qPCR analysis, which receives an average of 25 citations per week, and became member of the CEN/ISO working group that developed the guidelines for the pre-analytical process in molecular diagnostics that came in force during 2015.

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Joshua Fenrich, Product Manager – ddPCR, talks to *European Pharmaceutical Review* about Bio-Rad's PCR solutions.

How have RT-qPCR products evolved to support the pharmaceutical industry?

Improvements have been made over the years that enable real-time PCR technology to be used in high-throughput environments where there is a need to screen hundreds to thousands of samples.

Bio-Rad's CFX Automation System II is an example of this advancement for qPCR automation. This instrument allows for continuous unattended processing of qPCR plates using one or two CFX Real-Time PCR Detection Systems.

The reagents that support the use of qPCR automation have evolved as well. Sample preparation has been streamlined by using products like Bio-Rad's SingleShot™ Cell Lysis Kits that eliminate the need for RNA purification when analysing expression in tissue culture. The supermixes used for qPCR also provide features that improve throughput, from one-step RT-qPCR supermixes like Bio-Rad's iTaq™ Universal Probes One-Step Kit that bypass the need for a discrete reverse transcription step to room temperature-stable qPCR supermixes like Bio-Rad's SsoAdvanced™ Universal SYBR® Green Supermix that facilitates queuing plates on a qPCR automation system.

What are the benefits of using predesigned, wet-lab validated real-time PCR gene expression assays?

By using assays like Bio-Rad's PrimePCR™ gene expression assays, scientists can rapidly and confidently initiate projects to analyse recently identified gene targets without wasting time and money on multiple rounds of assay design and optimisation. Assays developed using the stringent criteria that were applied to PrimePCR provide scientists with assurance that assays have been properly designed and validated.

Designing an assay for real-time PCR can be a tedious task to perform properly. The multiple criteria that need to be considered while designing primers can easily be omitted when using an online design tool such as Primer-BLAST. Assays should be designed to target all relevant transcript variants while avoiding the detection of closely-related genes or genomic DNA. In addition, features that may compromise performance, such as SNPs under priming sites, interactions within and between primers, and strong secondary structure throughout the sequence must also be avoided.

Once designed, an assay must also be validated in the laboratory to determine whether it amplifies efficiently, has an acceptable dynamic range, detects genomic DNA, or produces non-specific amplification or primer-dimers. For PrimePCR gene expression assays, this validation was taken a step further to include the use of next-generation sequencing to confirm specificity.

Given all of these requirements for developing new gene expression assays, many scientists find it beneficial to select commercially available assays – knowing these assays will be both reliable and reliably available.

Which quantitative PCR technology is best for my project: real-time PCR or Droplet Digital™ PCR (ddPCR™)?

This depends on your particular application and the specific questions you are trying to answer.

For absolute quantification of nucleic acids (e.g., mutation detection, copy number determination, viral load determination, etc.), digital PCR is preferred because of its superior precision, reproducibility and discrimination over real-time PCR. In many cases, digital PCR can also be more sensitive than real-time PCR, particularly when working with compromised samples or in the presence of PCR inhibitors.

However, real-time PCR is more cost effective than ddPCR when the sensitivity and precision of ddPCR is not needed for gene expression analysis. Also, real-time PCR automation and support for 384-well plates allows this technology to achieve higher throughput than ddPCR. So if you do not need extreme sensitivity or precision and your project involves thousands of samples, real-time PCR may be a better choice for you.

Why is droplet digital PCR the right validation tool for genome editing projects?

Successful genome editing relies on the balance of efficiency and specificity. Droplet digital PCR enables the sensitive detection and quantification of low-level (typically <1%), but highly specific genome editing events. Other commonly-used methods including real-time PCR and NGS are unable to reliably detect such editing events.

What are the key advantages of droplet digital PCR over other technologies?

Droplet digital PCR is more precise and reproducible (intra-run and inter-run) as compared with other nucleic acid quantification technologies. ddPCR provides a direct and absolute measurement without the need for a calibration/standard curve to determine target nucleic acid (DNA or RNA) concentrations. It delivers between 10- and 100-fold increased sensitivity for applications where low frequency targets (mutations, foreign sequences, etc.) are present in an excess (1000-fold or higher) of background nucleic acids.

Quantitative PCR in the assessment of novel hepatic cell models

Catherine Mowbray, Alison Howard and Barry H. Hirst
Newcastle University

The process of amplifying DNA by exploiting the polymerase chain reaction (PCR) was first reported in the 1980s by Mullis and colleagues[1]. 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 Cq, Ct or Cp). 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 ΔΔCt to determine relative changes in expression of a gene (see [2] for explanation).

Although this is essentially straightforward, it is in fact often over-simplified 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[3], 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 challenge or treatment in a variety of cell lines, or in the preliminary assessment of a model.

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

The search for appropriate cell lines

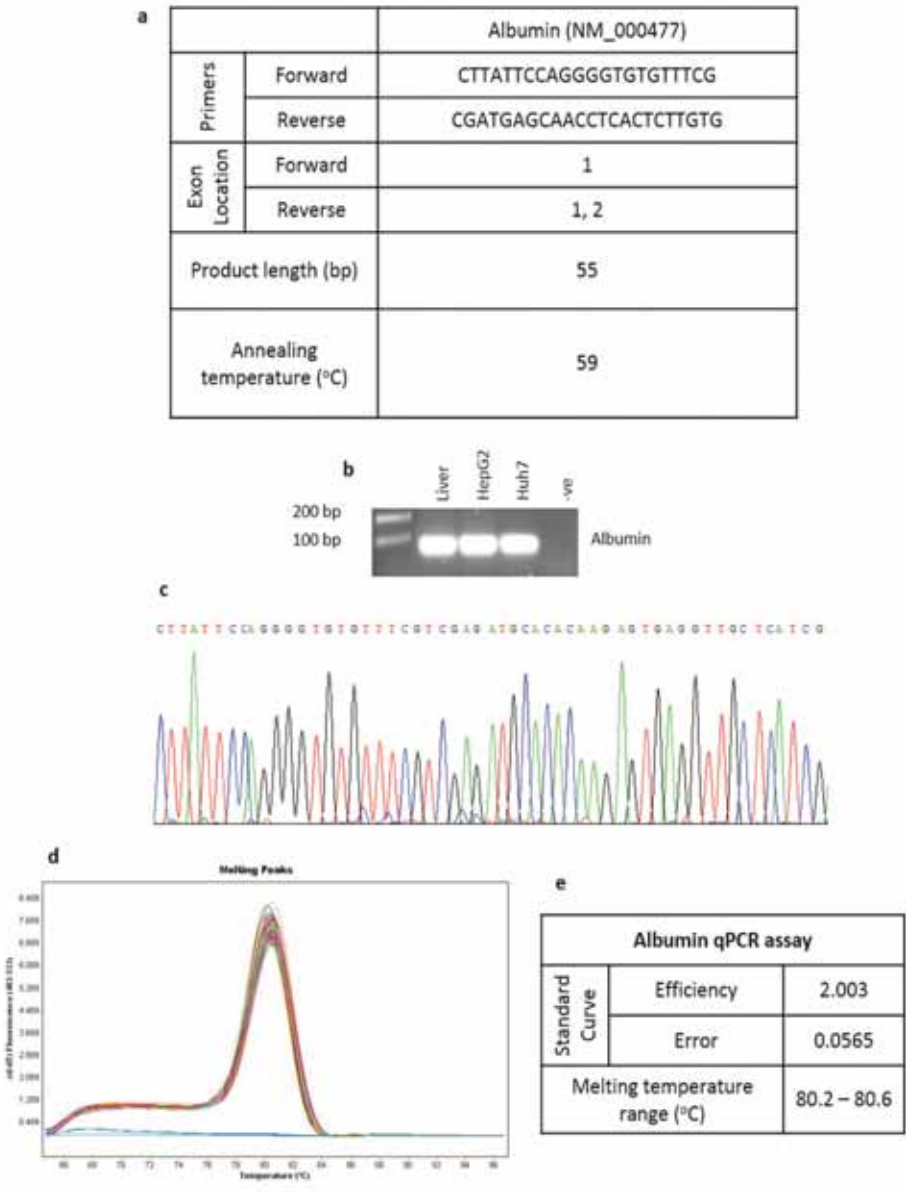
Drug development is an expensive process, especially considering the fact that a compound can pass through initial tests unscathed but then produce toxic effects in later studies or even in patients. The cost of bringing a drug to market has been estimated to be $800 million to $2 billion[4,5] so any 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 cryopreserved 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[6-11]. A fairly recently established cell line, HepaRG, is potentially phenotypically much closer to the *in vivo* hepatocyte[12-16]. 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[17].

a

		Albumin (NM_000477)
Primers	Forward	CTTATTCCAGGGGTGTGTTTCG
	Reverse	CGATGAGCAACCTCACTCTTGTG
Exon Location	Forward	1
	Reverse	1, 2
Product length (bp)		55
Annealing temperature (°C)		59



e

Albumin qPCR assay		
Standard Curve	Efficiency	2.003
	Error	0.0565
Melting temperature range (°C)		80.2 – 80.6

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

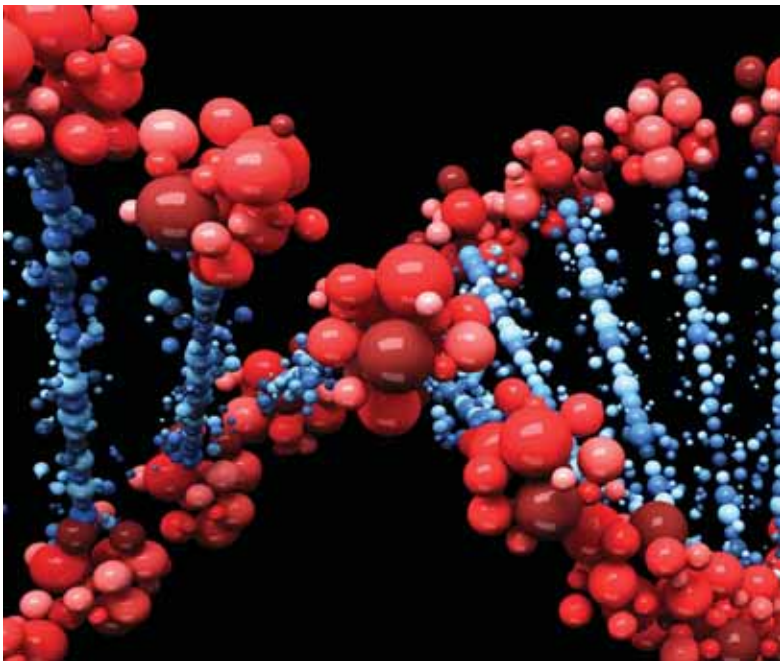
Hepatic cell line experiment

Here, we investigated DMSO treatment of

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?

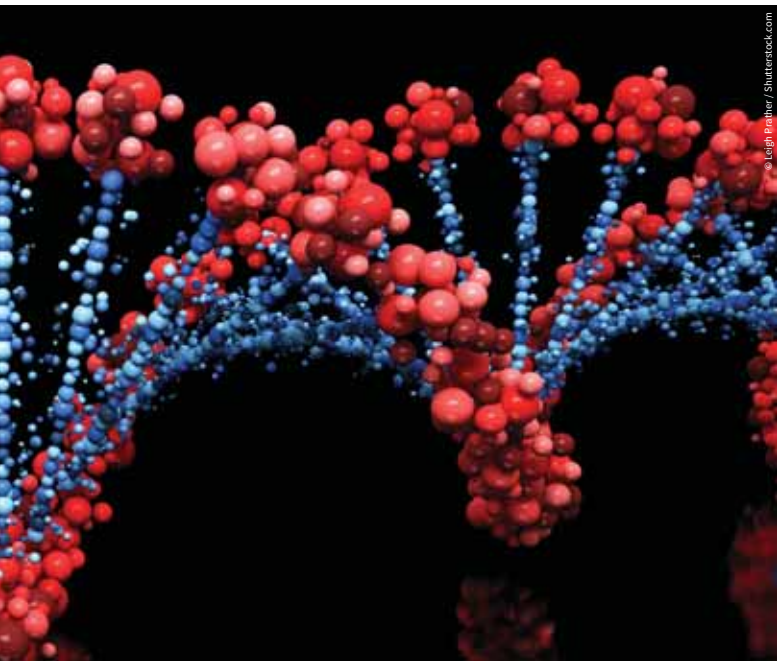
The industry requires rapid, sensitive and accurate detection of any RNA. Takara Clontech offers a unique, complete product line for one- and two-step RT-qPCR based on best-in-class enzymes. PrimeScript RTase was developed to express high strand-displacement activity, efficient cDNA synthesis up to 13kb 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_ms 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[18], 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[19-22] 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

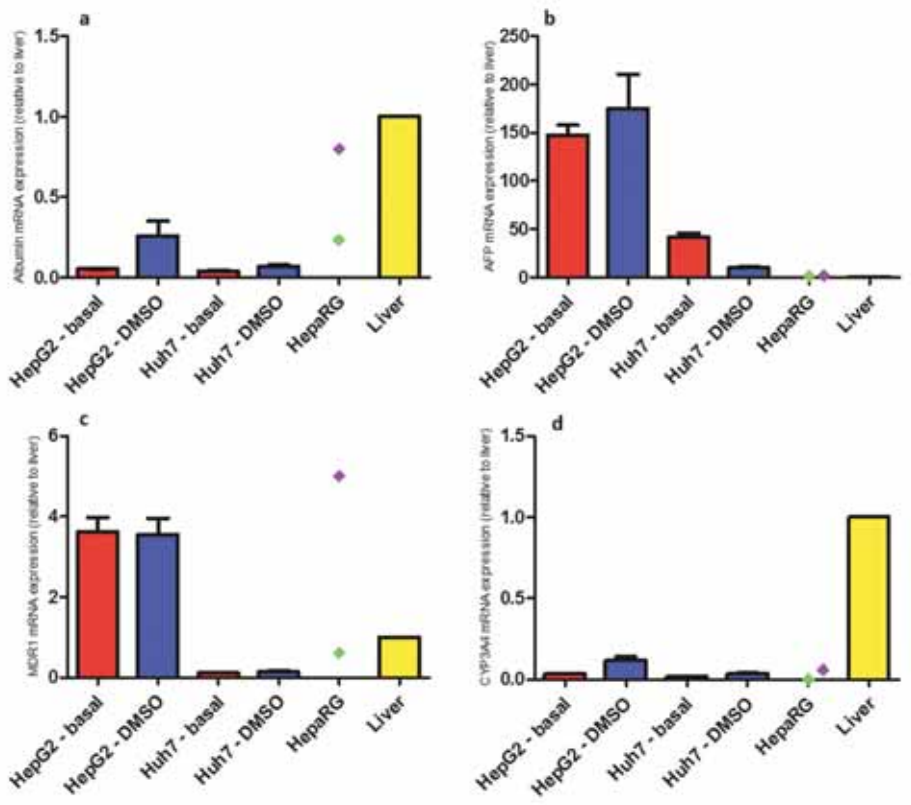


Figure 2: mRNA expression, shown relative to that in the liver, of basal and DMSO-related HepG2 and Huh7 cells assessed by qPCR for albumin (a), AFP (b), MDR1 (c) and CYP3A4 (d). A minimum of N=3 experiments were performed for HepG2 and Huh7 results. A literature search was performed to obtain data for 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[23,24]. 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[12,14,25] (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*[12,14,25].

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

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[26]. 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 cells varied between levels similar to those in HepG2 and comparable to liver[14,27-29]. 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)[12,14,25,28,29].

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[30] 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.*[31] 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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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 secetory efflux systems contribute to limitation of drug absorption in the gut and contribute to drug secretion.

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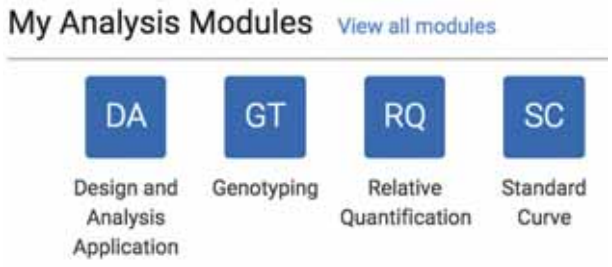

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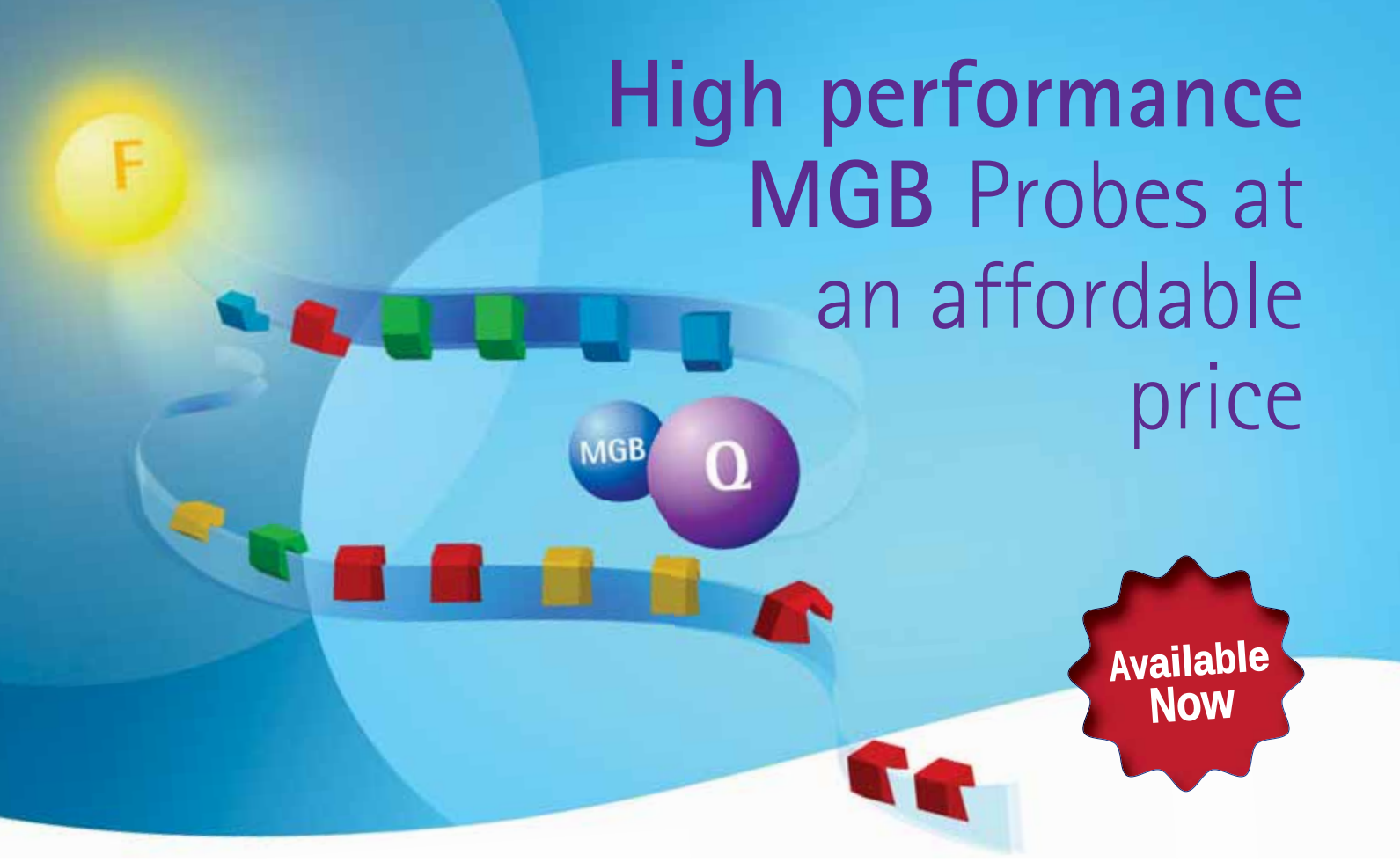

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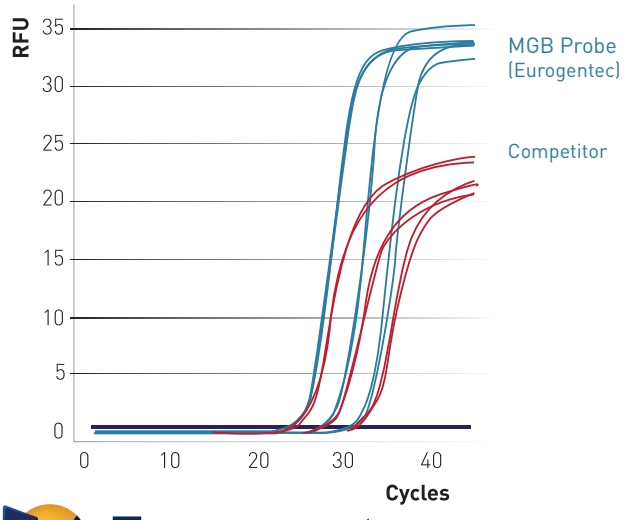

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