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Professor Mikael Kubista was among the pioneers in qPCR. Starting in 1991 his laboratory developed dyes and probes and founded LightUp Technologies (www.lightup.se) as one of the first companies in the world focusing on qPCR based diagnostics. His team then developed experimental approaches for accurate measurements of expression levels by real-time PCR, and they pioneered the fields of single cell expression profiling and multidimensional expression profiling by qPCR. Kubista also developed methods and approaches to analyse gene expression data and founded the company MultiD Analyses (www.multid.se) that develops the popular software GenEx for qPCR data analysis. Working as an advisor for Unesco he introduced qPCR in Africa and in the Middle East.

In 2001 Kubista founded the TATAA Biocenters (www.tataa.com), as the world leading service providers and organisers of hands-on training in qPCR. Regular training courses are held all over Europe, US, Africa and Asia. The TATAA courses world-wide are supported by leading instrument manufacturers and reagents suppliers in the qPCR field. The TATAA Biocenters organise the leading QPCR symposia in Europe (www.qpcr2009.net) as well as in USA (www.qpcrsymposium.com). Most recently Kubista set up the first high throughput qPCR expression profiling centre in Europe at the new institute of Biotechnology in Prague (www.ibt.cas.cz).

What the future holds for real-time PCR

TATAA Biocenters, located in Gothenburg, Sweden, Prague, Czech Republic, Freising outside Münich in Germany, and Sunnyvale, California[1], work with leading instrument manufacturers and reagents companies in the quantitative real-time PCR (qPCR) field on new applications, making the know-how available through hands-on courses worldwide. Every year new courses are launched based on the most recent developments in the field. The year 2008 has been very active in the qPCR area, with several important advancements that provide solid ground for future development of new research and diagnostic tools.

Most important has been the development of high throughput qPCR platforms, today available from Fluidigm, Biotrove and Roche. In 2008 Biotrove signed licensing and collaboration agreements with Applied Biosystems to develop a high throughput genotyping platform based on their OpenArray that runs 3072 parallel reactions. Fluidigm launched the 96.96 Dynamic Array, which is a microfluidic platform that combines 96 primer pairs with 96 samples to 9216 parallel reactions, and Roche presented the hitherto most powerful microtiter plate based qPCR instrument for 1536 reactions. These platforms open for exciting expression profiling studies, including most powerful multiway profiling[2], that have not been feasible on conventional 96-well plate instruments. In multiway, profiling samples are characterised as functions of multiple factors, such as time, genetic makeup, drug load, etc., by their expression of reporter genes. The experiment generates a cube or even hypercube of data from which biological information can be extracted using multivariate analysis tools available, for example, from MultiD Analyses. Among the most exciting applications is qPCR tomography. A solid sample, which can dissect tissue, an organ, whole embryo or even a single egg cell, is sliced in a cryostat and optionally further cut into smaller pieces with laser microdissection. The pieces are analysed by qPCR expression profiling producing a spatially resolved expression map, which reflects any sample heterogeneity. The technique was recently used to demonstrate intracellular mRNA gradients in the *Xenopus laevis* oocyte[3]. In the future we expect qPCR tomographic studies to be performed also as function of time generating spatiotemporal expression maps of, for example, a developing embryo.

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In diagnostics we have high expectations qPCR based multimarker tests may be the tool needed to approach complex diseases. Particularly promising is multimarker therapy monitoring, prognostics and early detection of relapse. A qPCR test based on 21 markers (15 disease markers and six reference genes) is available for breast cancer from Genomic Health. The test is offered to women with node-negative, estrogen-receptor positive tumours, and is based on tissue biopsies. From the measured profile a score is calculated indicating the likelihood of cancer recurrence. Similar tests are being developed for other indications, and for other diseases. Most interesting are tests applicable on body fluids such as blood/plasma/serum, urine, saliva, scrapes, faeces, sweat and tears, which reduces sampling complexity. In Europe framework VI project SmartHEALTH aims to develop cancer monitoring diagnostic that eventually will be useable in a localised and more available setting than hospitals[4].

In 2005 we pioneered eukaryotic single cell expression[5]. Using state of the art qPCR technology of those days we measured transcript levels of five genes per cell. This was sufficient to reveal high variability in genes' expression among cells in a

seemingly homogeneous population of pancreatic beta cells that could be modeled with a log normal distribution. We also showed that correlation between genes' expressions on single cell level (Single Cell Expression Correlation, SCEC) indicates common regulatory mechanism and, hence, related functions. Today efficient and robust pre-amplification strategies are available to multiply the targeted transcripts in the individual cells prior to quantification by qPCR, which allows for the analysis of hundreds of genes per cell in high throughput format[6]. At the 2nd qPCR symposium in California (www.qpcrsymposium.com) it was mentioned some laboratories are pre-amplifying up to 1600 genes, which is approaching the size of smaller genomes. Other very important developments boosting this area are new reagents for 1-step extraction, RT and qPCR of single cells, eliminating losses at washing steps in conventional protocols[7]. Single cells can be harvested using a cell picker, using fluorescent assisted cell sorting (FACS) or by laser capture directly into the novel lysis reagent, reverse transcribed, optionally pre-amplified, and then analysed for multiple genes in the new qPCR platforms. The steps are amendable for automation and soon we expect results from high throughput SCEC exploring expression pathways, elucidating mechanisms of action of drugs and other stimulatory compounds, modes of cellular differentiation and many other aspects of the dynamic cell. Particularly exciting will be approaches related to circulating tumor cells (CTC's), which usage was ranked no. 1 among medical innovation for 2009 by the Cleveland Clinic[8]. Not all CTC's are capable of forming metastasis, rather rare circulating tumor stem cells are thought to be the bad guys. SCEC is promising for the identification of tumor stem cells and may also guide the selection of therapy to obliterate them.

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The high throughput platforms also enhance the performance of digital PCR originally developed in 1999[9]. By automatically distributing an already rather diluted sample into a large number of chambers (760 in the BIOMARK digital array), such that each chamber contains an average of about 0.5 copies, the total number of target molecules in the sample can be determined with an accuracy that far exceeds conventional qPCR. The approach is most powerful for studies of copy number variations[10], including prognostic markers such as HER2 amplification in cancer[11]. Another application is to reduce background by the distribution to find rare mutations[12], or identify rare cells, such as fetal cells in maternal blood[13]. Future applications will include counting of different cell types in various body fluids and tissues, and identify genetic variants among bacteria and viruses.

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High resolution melting (HRM), which was first described already in 2003[14], had also its breakthrough in 2008, mainly because more instruments and dyes became available. In HRM minor differences in DNA duplex stability due to small sequence variations are exploited. The targeted DNA is amplified by (optionally conventional) PCR in the presence of a suitable dye. The sample is then heated to strand separate the amplicons, and rapidly cooled to renature them under relaxed stringency. If the sample is heterogeneous, because the original template showed some sequence variation, the renaturation will lead to some heteroduplex formation that has lower thermal stability than the fully complementary homoduplexes formed by the PCR. The sample is now heat denatured again, this time in small temperature increments, and the fluorescence is monitored. As the duplexes melt the fluorescence drops. From the melting temperature it is possible to identify most single nucleotide polymorphism (SNP), and homo- and heterozygotic samples are readily distinguished from the shape of the melting curve. HRM can also be combined with bisulfite treatment for methylation analysis. With the new high throughput platforms HRM offers lowest cost per reaction for analysis of sequence variations and we expect the technique to rapidly grow in popularity.

Although novel and promising techniques for expression analysis are being developed, many with advantages to qPCR for certain applications, the overall sensitivity, accuracy, wide dynamic range and ease of use of qPCR, now combined also with high-throughput performance and multiplex capability through pre-amplification, which boosts its cost performance, will continue to make qPCR the platform of choice for more and more research and diagnostic laboratories. qPCR is here to stay. In fact, we are still far from exploring its full capacity.

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