Tumor cells were identified by immunostaining for the epithelial cell adhesion molecule and isolated using micromanipulation. An unbiased global amplification was performed, and labeled PCR products were used to hybridize a commercially available DNA hybridization array. Interestingly, EMPIRIN, a stimulator of matrix metalloproteinase expression, was identified in 60% of single metastatic cells by gene expression and 80% by the presence of protein. Eventually, profiles of molecules like these could lead to better detection and monitoring of tumour spread.

Despite technical advances in single-cell gene expression analysis, future applications will have to address important experimental pitfalls. Better methods are needed to identify cells relevant to disease and preclude mRNA contamination from neighbouring cells. Sample handling must be optimized to avoid mRNA degradation and reduce genomic DNA contamination. Bias in the relative amplification of low-, medium- and highly-expressed transcripts needs to be better understood. Improved methods of gene-expression analysis with archival pathological specimens must be developed [24,25]. Finally, a more rapid means of linking disease-related transcriptional activity to cellular dysfunction must be developed.

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References

1 Ormerod, M. (2000) *Flow Cytometry: A Practical Approach*, Oxford Univerisity Press, Oxford, UK
2 Busche, S. *et al.* (2000) Expression of angiotensin AT(1) and AT(2) receptors in adult rat cardiomyocytes after myocardial infarction. A single-cell reverse transcriptase-polymerase chain reaction study. *Am. J. Pathol.* 157, 605–611
3 Clement-Sengewald, A. *et al.* (2000) Laser microdissection as a new approach to prefertilization genetic diagnosis. *Pathobiology* 68, 232–236
4 Schutze, K. and Lahr, G. (1998) Identification of expressed genes by laser-mediated manipulation of single cells. *Nat. Biotechnol.* 16, 737–742
5 Simone, N.L. *et al.* (1998) Laser-capture microdissection: opening the microscopic frontier to molecular analysis. *Trends Genet.* 14, 272–276
6 Emmert-Buck, M.R. *et al.* (1996) Laser capture microdissection. *Science* 274, 998–1001
7 Fend, F. *et al.* (2000) Laser capture microdissection: methodical aspects and applications with emphasis on immuno-laser capture microdissection. *Pathobiology* 68, 209–214
8 Rappolee, D.A. *et al.* (1989) Novel method for studying mRNA phenotypes in single or small numbers of cells. *J. Cell. Biochem.* 39, 1–11
9 Brady, G. *et al.* (1990) Representative *in vitro* cDNA amplification from individual hemopoietic cells and colonies. *Methods Cell Biol.* 2, 17–25
10 Dixon, A.K. *et al.* (1998) Expression profiling of single cells using 3 prime end amplification (TPEA) PCR. *Nucleic Acids Res.* 26, 4426–4431
11 Freeman, T.C. *et al.* (1999) Analysis of gene expression in single cells. *Curr. Opin. Biotechnol.* 10, 579–582
12 Eberwine, J. (1996) Amplification of mRNA populations using aRNA generated from immobilized oligo(dT)-T7 primed cDNA. *Biotechniques* 20, 584–591
13 Luo, L. *et al.* (1999) Gene expression profiles of laser-captured adjacent neuronal subtypes. *Nat. Med.* 5, 117–122
14 Ohyama, H. *et al.* (2000) Laser capture microdissection-generated target sample for high-density oligonucleotide array hybridization. *Biotechniques* 29, 530–536
15 Baugh, L.R. *et al.* (2001) Quantitative analysis of mRNA amplification by *in vitro* transcription. *Nucleic Acids Res.* 29, E29
16 Klein, C.A. *et al.* (2002) Combined transcriptome and genome analysis of single micrometastatic cells. *Nat. Biotechnol.* 20, 387–392
17 Al-Taher, A. *et al.* (2000) Global cDNA amplification combined with real-time RT-PCR: accurate quantification of multiple human potassium channel genes at the single cell level. *Yeast* 17, 201–210
18 Thakker-Varia, S. *et al.* (2001) Rab3A is required for brain-derived neurotrophic factor-induced synaptic plasticity: transcriptional analysis at the population and single-cell levels. *J. Neurosci.* 21, 6782–6790
19 Datson, N.A. *et al.* (1999) MicroSAGE: a modified procedure for serial analysis of gene expression in limited amounts of tissue. *Nucleic Acids Res.* 27, 1300–1307
20 Velculescu, V.E. *et al.* (2000) Analysing uncharted transcriptomes with SAGE. *Trends Genet.* 16, 423–425
21 Neilson, L. *et al.* (2000) Molecular phenotype of the human oocyte by PCR-SAGE. *Genomics* 63, 13–26
22 O'Dell, D.M. *et al.* (1999) Single-cell molecular biology: implications for the diagnosis and management of neurological disease. *Arch Neurol.* 56, 1453-1456.
23 O'Dell, D.M. *et al.* (1998) Amplification of mRNAs from single, fixed TUNEL-positive cells. *Biotechniques* 25, 566–570
24 Specht, K. *et al.* (2001) Quantitative gene expression analysis in microdissected archival formalin-fixed and paraffin-embedded tumor tissue. *Am. J. Pathol.* 158, 419–429
25 Gillespie, J.W. *et al.* (2002) Evaluation of non-formalin tissue fixation for molecular profiling studies. *Am. J. Pathol.* 160, 449–457

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Quantification using real-time PCR technology: applications and limitations

Dieter Klein

The introduction of real-time PCR technology has significantly improved and simplified the quantification of nucleic acids, and this technology has become an invaluable tool for many scientists working in different disciplines. Especially in the field of molecular diagnostics, real-time PCR-based assays have gained favour in the recent past. However, the wide use of real-time PCR methods has also highlighted some of the critical points and limitations of these assays. These aspects must be considered to increase the reliability of the obtained data.

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Four different principles are commonly used for real-time PCR detection. All four technologies are based on the measurement of fluorescence during the PCR. The amount of emitted fluorescence is proportional to the amount of PCR product and enables the monitoring of the PCR reaction. The resulting PCR curve is used

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to define the exponential phase of the reaction, which is a prerequisite for accurate calculation of the initial copy number at the beginning of the reaction.

The simplest and cheapest principle is based on intercalation of double-stranded DNA-binding dyes. This technology can be easily applied to already established PCR assays and does not need any additional fluorescence-labelled oligonucleotide. However, specific and nonspecific PCR products are both detected. Therefore, these assays require careful optimization of the PCR conditions and a clear differentiation between specific and nonspecific PCR products using melting-curve analysis.

The other three principles are based on the introduction of an additional fluorescence-labelled oligonucleotide. Sufficient amounts of fluorescence are only released either after cleavage of the probe (hydrolysis probes [1,2]) or during hybridization of one (molecular beacon [3]) or two (hybridization probes [4]) oligonucleotides to the amplicon. Introduction of the additional probe increases the specificity of the quantified PCR product and allows the development of multiplex reactions.

Beside these four main principles other technologies have been described. Scorpion primers contain an attached fluorescence-labelled tail that hybridizes to an amplified target [5]. This self-probing amplicon provides substantial benefits for rapid assays with short equilibration times.

Advantages of real-time PCR assays

Real-time PCR assays are characterized by a wide dynamic range of quantification of 7–8 logarithmic decades, a high technical sensitivity (< 5 copies) and a high precision (< 2% standard deviation) [6,7]. Another advantage of this method is that no post-PCR steps are required, thus avoiding the possibility of cross-contamination due to PCR products. This advantage is of special interest for diagnostic applications. Together with lower turn-around times and decreased costs it has revolutionized the field of molecular diagnostics [8]. New systems for field use, which can detect microorganisms in less than 10 minutes, have been developed [9]. Taken together, these advantages have enabled the shift of molecular diagnostics toward a high-throughput and automated technology (see Box 1).

Applications in molecular diagnostics

Clinical microbiology and food microbiology are important fields for real-time PCR applications (see Box 2). Hundreds of assays have been published for the detection and quantification of various infectious agents [8]. However, critical and strict evaluation of newly developed assays is a prerequisite to obtain reliable data. Comparison with other classical microbiological methods should be performed during the evaluation process. Real-time PCR assays have similar sensitivities as classical methods, but are less time- and labour-intensive [10]. However, they detect and quantify nucleic acids from live and dead pathogens, whereas classical microbiological assays count only live pathogens. Future analysis will tell which parameter correlates better with the relevant factor (e.g. disease induction, virulence of pathogen).

In clinical oncology the detection and quantification of chromosomal translocations by real-time PCR has been applied to monitor minimal residual disease [11] or to show graft-versus-lymphoma effects [12]. Predictive genetic testing and the identification of relevant single nucleotide polymorphisms (SNPs) is a second important area [5,13]. The possibility of using high-throughput real-time PCR assays together with the sequencing of the human and numerous other genomes provides an immense source for the development of new assays. Currently, major biotech companies are working on projects in which allele-specific assays are automatically developed for all SNPs identified during sequencing programmes. These assays are likely to become an important area of molecular diagnostics in the future.

A third major area for applications of real-time reverse transcriptase (RT)-PCR assays is the quantification of gene expression [6]. However, these applications harbour some drawbacks in diagnostic approaches, which will be discussed below.

Clinical microbiology

Most infectious agents are characterized by a high mutation rate, which can influence the viral load estimation dramatically [7]. This problem can be overcome by using highly conserved regions for assay development. However, for some pathogens (e.g. emerging pathogens, highly variable pathogens) this is not possible, and careful comparative genome investigations have to be performed before a reliable assay can be developed. On the other hand, sequence variations provide the basis for the development of subtype-specific assays. In combination with a multiplex approach this an elegant tool to monitor different strains within a patient or population.

Molecular beacons have also been used for pathogen quantification [14]. They have the advantage that a non-fluorescent quencher is used and up to four different fluorescent dyes can be detected and quantified simultaneously. However, molecular beacons are highly sensitive to mismatches in the binding region and should be used in cases where highly conserved regions are known.

Many applications necessitate the quantification of more than one nucleic acid. The simultaneous quantification of different targets decreases both the cost and turn-around time of the assay and, if the assay is carefully optimized, increases the reliability of the obtained data. The multiplex approach provides a benefit, in that pipetting errors are minimized and both nucleic acids are amplified simultaneously under the same conditions. In comparison with monoplex approaches, the duplex assay results in higher precision and accuracy of the obtained data [7,16]. Nevertheless, duplex or multiplex assays are complex and

Box 1. Advantages and limitations of real-time PCR

Advantages	Limitations
Wide dynamic range of quantification (7–8 log decades)	PCR product increases exponentially
High technical sensitivity (< 5 copies)	Variation increases with cycle number
High precision (< 2% CV of C_T values)	Increased variation after transformation to linear values
No post-PCR steps	Overlap of emission spectra[a]
Minimized risk of cross contamination	Maximal four simultaneous reactions[a]
High throughput	Increased risk of false negative results[b]
Multiplex approach possible	

[a]It is expected that new technology will improve this.
[b]In particular for pathogen detection (e.g. new emerging pathogens, highly variable pathogens).

Box 2. Applications of real-time PCR in molecular diagnostics[a]

Clinical microbiology
Viral load (e.g. HIV, HCV, HBV)
Bacterial load (e.g. EHEC, *Salmonella*, *Mycobacterium*)
Fungal load (e.g. *Candida*, *Cryptococcus*, *Aspergillus*)

Food microbiology
Bacterial load (e.g. *Listeria*, *Salmonella*, *Campylobacter*)

Veterinary microbiology
Viral load (e.g. FIV, CSFV, FCV)
Zoonotic agents

Clinical oncology
Minimal residual disease
Chromosomal translocations
Single nucleotide polymorphisms (SNPs)

Gene therapy
Gene transfer estimation
Biodistribution of vectors

Gene expression
Cytokines, receptors, etc.

[a]Note that this list is not exhaustive.

require a high degree of optimization work. Primer matrix assays for all primer pairs are necessary to increase the sensitivity of the assay and identify the lowest primer concentration that has no effect during the exponential phase of the reaction [7,14,16]. Internal standardization in multiplex approaches is also an important aspect in applications requiring quality assurance [15]. Taken together, optimized multiplex approaches increase the reliability of the data and provide a useful tool to screen several targets simultaneously.

Clinical oncology

In contrast to pathogen detection, sequence variations in primer and probe binding regions are of much less importance in assays for quantification of mammalian chromosomal genes. The two major challenges here are the sensitivity and the level of discrimination of the assay. The technical sensitivity of an optimized real-time PCR assay itself is high (< 5 copies per reaction), but the maximal input of DNA during sample preparation and per PCR reaction is the limiting step. This limitation must be considered in all applications, where sensitivity is of the utmost interest [e.g. monitoring of minimal residual disease [11] or transmission of endogenous pathogens during xenotransplantation [17]). In all applications that rely on the detection or quantification of SNPs, the level of discrimination between target and non-target allele is an important aspect to define the sensitivity of the assay. The discrimination level can be increased using a modified real-time PCR system. In this system the primer, instead of the probe, is placed at the polymorphic nucleotide. The introduction of an additional mutation increases the discrimination level and has been successfully used to detect mitochondrial heteroplasmy in cloned animals for the first time [18]. However, this system is mainly used for monoplex approaches. In cases where more than one allele has to be quantified, molecular beacons [19] or scorpion primers [5] provide an interesting alternative. However, the level of discrimination depends on the mismatch and has to be tested experimentally for every SNP assay.

Gene expression

Real-time RT-PCR assays have been widely used to estimate the expression level of genes of interest [6]. The reproducibility of an optimized real-time RT-PCR assay itself is nearly as good as in real-time PCR assays [7]. However, the critical issues defining the reliability of the obtained data are the choice of the housekeeping gene and sample preparation. An ideal housekeeping gene has always the same level of expression. However, not all proposed housekeeping genes fulfil this prerequisite. Therefore, the constant expression of the housekeeping gene chosen for the experimental set-up must be tested and demonstrated before any reliable data can be obtained. The influence of sample preparation (e.g. losses during storage or RNA extraction) can be monitored by using RNA to spike the samples. Companies have started to develop collection tubes, which already contain spiking RNA for this purpose. However, the stability of the spiked nucleic acid before sampling and the comparative degradation of spiked RNA and RNA in the sample are currently the critical problems.

For some applications (e.g. the respective influences of various factors on gene expression levels) a relative quantification is sufficient. In these cases, the laborious development of accurate RNA standards can be avoided by using a comparative quantification method ($\Delta\Delta C_T$-method). The method is based on the fact that the difference in threshold cycles (ΔC_T) between the gene of interest and the housekeeping gene is proportional to the relative expression level of the gene of interest. To ensure the accuracy of the obtained data, the reaction efficiencies of the two assays either should be similar or a correction factor must be introduced into the calculation.

In other applications, relative quantification is not sufficient and absolute numbers are required. Here, it should be kept in mind that the obtained 'absolute numbers' are always calculated relative to the standard (RNA, cDNA, plasmid DNA, genomic DNA) and largely depend on the accuracy of the used standard. Accurate estimation therefore necessitates that the same reaction conditions are used for a standard and for the sample. Duplex or multiplex approaches with internal standardization [15] provide a solution for this problem. Finally, the standardization of several other factors (e.g. common standard, same reagents, instrumentation and data analysis) must be performed before 'absolute numbers' obtained by different laboratories can be compared. This is one of the major tasks that needs careful and critical discussion to ensure the reliability of the data independent of the location (i.e. the lab).

Perspectives

Multiplex real-time PCR approaches have several advantages, but are limited by the availability of fluorescent dye combinations and the capacity of simultaneous PCR reactions. In some cases, it will be interesting to monitor more than four or five different targets simultaneously. DNA chip technology is currently used for these purposes. However, current microarray technologies necessitate a high amount of starting material and display only a limited dynamic range for quantification. Therefore, a synergistic combination of both technologies, in which the screening of the involved genes is performed by microarrays and the precise quantification and high-throughput screening is done by real-time PCR, would be currently the method of choice.

Concluding remarks

The introduction of real-time PCR technology into the field of molecular diagnostics has simplified the

quantification of nucleic acids. Enormous amounts of data can be generated within a short time. However, a certain amount of the time saved has to be spent on critically checking the data. Although most real-time PCR assays in themselves are characterized by high precision and reproducibility, the accuracy of the obtained data is largely depended on several other factors such as sample preparation, quality of the standard and choice of housekeeping gene. Therefore, the accuracy of the obtained data has to be checked during the establishment of the assay by comparison with other established assays. This is the major task for molecular diagnostics to guarantee reliable data in the future.

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References

1 Heid, C.A. *et al.* (1996) Real time quantitative PCR. *Genome Res.* 6, 986–994
2 Gibson, U.E.M. *et al.* (1996) A novel method for real-time quantitative RT-PCR. *Genome Res.* 6, 995–1001
3 Tyagi, S. and Kramer, F.R. (1996) Molecular beacons — probes that fluoresce upon hybridization. *Nat. Biotechnol.* 14, 303–308
4 Wittwer, C.T. *et al.* (1997) Continous fluorescence monitoring of rapid cycle DNA amplification. *Biotechniques* 22, 130–138
5 Whitcombe, D. *et al.* (1999) Detection of PCR products using self-probing amplicons and fluorescence. *Nat. Biotechnol.* 17, 804–807
6 Bustin, S.A. (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J. Mol. Endocrinol.* 25, 169–193
7 Klein, D. *et al.* (2001) Influence of preassay and sequence variations on viral load determination by a multiplex real-time reverse transcriptase-polymerase chain reaction for feline immunodeficiency virus. *J. Acquir. Immune Defic. Syndr.* 26, 8–20
8 Jordan, J.J. (2000) Real-time detection of PCR products and microbiology. *New Technologies for Life Sciences: A Trends Guide*, 61-66
9 Belgrader, P. *et al.* (1999) PCR detection of bacteria in seven minutes. *Science* 284, 449–450
10 Hein, I. *et al.* (2001) Detection and quantification of the *iap* gene of *Listeria monocytogenes* and *Listeria innocua* by a new real-time quantitative PCR assay. *Res. Microbiol.* 152, 37–46
11 Pongers-Willemse, M.J. *et al.* (1998) Real-time quantitative PCR for the detection of minimal residual disease in acute lymphoblastic leukemia using junctional region specific Taqman probes. *Leukemia* 12, 2006–2014
12 Mandigers, C.M. *et al.* (1998) Graft-versus-lymphoma effect of donor leucocyte infusion shown by real-time quantitative PCR analysis of t(14;18). *Lancet* 352, 1522–1523
13 Ibrahim, M.S. *et al.* (1997) The potential of 5′nuclease PCR for detecting a single-base polymorhism in Orthopxvirus. *Mol. Cell. Probes* 11, 143–147
14 Vet, J.A. *et al.* (1999) Multiplex detection of four pathogenic retroviruses using molecular beacons. *Proc. Natl. Acad. Sci. U. S. A.* 96, 6394–6399
15 Gruber, F. *et al.* (2001) Quantitation of viral DNA by real-time PCR applying duplex amplification, internal standardization, and two color fluorescence detection. *Appl. Environ. Microbiol.* 67, 2837–2839
16 Klein, D. *et al.* (2000) Accurate estimation of transduction efficiency necessitates a multiplex real-time PCR. *Gene Ther.* 7, 458–463
17 Paradis, K. *et al.* (1999) Search for cross-species transmission of procine endogenous retrovirus in patients treated with living pig tissue. *Science* 285, 1236–1241
18 Steinborn, R. *et al.* (2000) Mitochondrial DNA heteroplasmy in cloned cattle produced by fetal and adult cell cloning. *Nat. Genet.* 25, 255–257
19 Tyagi, S. *et al.* (1998) Multicolor beacons for allele discrimination. *Nat. Biotechnol.* 16, 49–53

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