

## RT-PCR technique and its applications. State-of the-art\*

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### ABSTRACT

The reverse transcription polymerase chain reaction (RT-PCR) is one of the most sensitive methods for the detection and quantitation of mRNA. It is widely used for quantification of mRNA levels and is useful tool for basic research, agriculture, medicine and biotechnology. Introduction of Real-time technique significantly improves rapidity, sensitivity, specificity and reproducibility of RT-PCR method. Real-time PCR detects and quantifies nucleic acids even from live and dead pathogens or cells. This review discusses the background, advantages and limitations of conventional and Real-time RT-PCR methods for quantitation of gene expression and its application in basic, agricultural and biomedical research.

KEY WORDS: RT-PCR, Real-time PCR, gene expression, molecular diagnostics, mRNA

### INTRODUCTION

Many cellular processes concerning survival, growth and differentiation are reflected in altered patterns of gene expression and the ability to measure transcription levels of specific genes has always been central to any research into gene function. The emergence of molecular medicine and veterinary has resulted in the increased use of techniques able to quantitate levels of RNA. Such applications

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are wide-ranging and include procedures designed to quantify the regulation and expression of drug resistance markers in tumour cells (Ramachandran and Melnick, 1999), monitor responses to chemotherapy (Desjardin et al., 1999), provide a molecular assessment of tumour stage (Bustin and Dorudi, 1998), detect circulating tumour cells in cancer patients (Ghossein and Rosai, 1996), detect bacterial (Hill, 1996) and viral (Holodniy, 1994) pathogens. It is the most sensitive and the most flexible of the quantification methods (Wang and Brown, 1999) and can be used to characterize patterns of mRNA expression, to analyse RNA structure and clone full-length complementary DNA (cDNA). The focus on maximizing sensitivity has led to the development of semi-nested (Wasserman et al., 1999), nested (Israeli et al., 1994; Soeth et al., 1996), and even three-step nested (Funaki et al., 1998) RT-PCR techniques.

#### CHARACTERISTICS OF RT-PCR REACTION

RT-PCR analysis consists of four steps:

- isolation and purification of RNA
- cDNA synthesis based on RNA template
- amplification of a part of cDNA using PCR
- estimation of product quantity.

The scheme of analysis is presented on Figure 1. Isolation and purification of RNA are based on two methods. The first one is classical Chomczyński and Sacchi method (1987). RNA is deproteinized in mixture of guanidine thiocyanate and phenol. The second method is based on absorption of RNA on silica dioxide in solutions of wide range of ionic strength, washing to remove contaminants, and elution by solutions with low ionic strength and particular pH. This method allows purification of RNA from main inhibitors of enzymatic reaction, proteins and polysaccharides (Cheung et al., 1994; Rasool et al., 2002). Presently there are many modifications of these two methods. The comparing experiments had indicated that they assured similar quantity of RNA of comparable purity (Gaurino et al., 1997; Rasool et al., 2002). Till now many biochemical companies have designed kits for extraction either total DNA and mRNA from tissues and cells (Sigma Aldrich, Ambion, Quigen et al.). Moreover there are commercially available kits for RNA isolation directly from blood samples, for example RNAqueous-Blood Module (Ambion). The kit guaranteed yield of 200ng of total RNA from 200-300µl of blood.

The second step in RT-PCR analysis is the reverse transcription of the RNA template into cDNA by RNA-dependent DNA polymerase (reverse transcriptase, RT). RNA transcripts can exhibit significant secondary structure that affects the ability of the RNA-dependent reverse transcriptase to generate transcripts. There are com-

monly used reverse transcriptases of two origins: - a myeloblastosis virus reverse transcriptase (AMV-RT) and moloney murine leukaemia virus reverse transcriptase (MMLV-RT). AMV-RT is more robust than MMLV-RT (Brooks et al., 1995) and can help eliminate problems associated with RNA secondary structure. MMLV-RT have significantly less RNaseH activity than AMV-RT (Gerard et al., 1997) what allows synthesis of long amplicons and may be a better choice for amplification of full-length cDNA molecules. The RT step can be primed using specific primers, random hexamers or oligo(dT) primers. The use of specific primers decreases background priming, whereas the use of random and oligo(dT) primers maximizes the number of mRNA molecules that can be analysed from a small sample of RNA.

Obtained in the first step cDNA is used as a template for standard PCR reaction. Some experiments demands only detection of specific RNA molecule or statement of absence of that molecule. For this type of experiment a single tube ('one-enzyme/one-tube') system is useful, utilizing *Thermus thermophilus* (Tth) polymerase, a DNA polymerase with intrinsic RT, but no RNase H, activity. This assay uses bicine buffers containing  $Mn^{2+}$  ions that are compatible with both RT and subsequent PCR reaction (Chiocchia and Smith, 1997). There is also evidence that RT-PCR reactions using Tth polymerase may be more robust and resistant to inhibitors present in biological specimen (Poddar et al., 1998). The main drawbacks of this system are that sensitivity of Tth polymerase is two orders of magnitude lower than that of Taq polymerase (Grabko et al., 1996) so this system is not as sensitive as two-enzyme procedures, and presence of  $Mn^{2+}$  ions reduces the fidelity of nucleotide incorporation (Fromant et al., 1995). The aim of most experiments is quantitative measurement of target gene expression. Results of quantitative measurements can be presented as relation

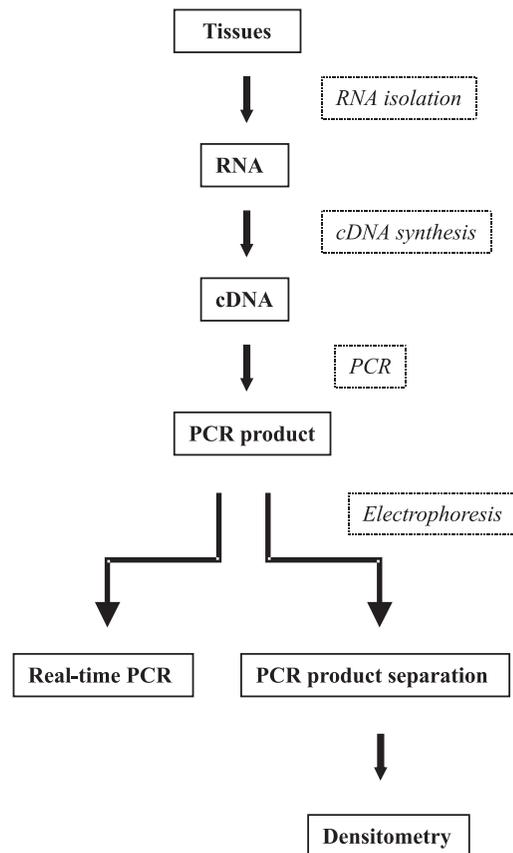


Figure 1. Standard RT-PCR reaction setup

of target gene to reference gene mRNA copy number or as absolute mRNA copy number. As references are chosen genes exhibiting constants expression level in the majority of tissues, for example: glyceraldehyde-3-phosphate dehydrogenase (GAPDH),  $\beta$ -actin, or 18S rRNA. One of examples of measurement of relative expression level of homeotic *Dlx5* gene in mice mammary gland is presented on Figure 2.

For measurement of absolute mRNA copy number is used competitive PCR, Real-time PCR and ELISA-PCR methods often called quantitative RT-PCR (QRT-PCR). Performing QRT-PCR analysis is necessary pay attention to contamination of RNA samples with DNA traces because synthesis of PCR products can begin not only on cDNA template but on genomic DNA as well. This problem can be solved for conventional or competitive, but not in real-time PCR by careful selection of primers. Selection of primers, localized in different exons of investigated genes, allows separation of PCR product synthesised on cDNA or genomic DNA template. Limitation of this approach is frequent appearance of retropseudogenes in genomic DNA. Recently, have been published poly(A) cDNA-specific RT-PCR technique (PACT RT-PCR) (Folz and Nepluev, 2000), and EXACT RT-PCR (Smith et al., 2000) promising design primers which can distinguish PCR product from cDNA and genomic DNA even for templates containing retropseudogenes.

Accurate measurement of mRNA copy number is possible during exponential stage of PCR reaction. This requires choosing of appropriate amount of cDNA and

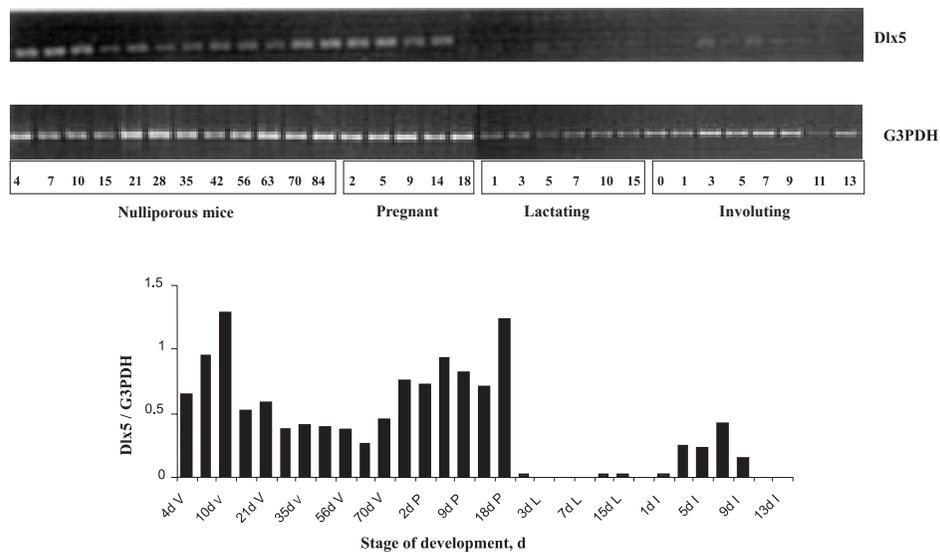


Figure 2. Measurement of relative expression of homeotic *Dlx5* gene in the mammary gland of mice

cycle number for PCR reaction. For quantifying mRNA by competitive PCR, internal standard RNA (competitor) is added in a defined quantity to the RNA sample prior to the RT reaction (Larrick, 1997). The resulting standard cDNA is coamplified with the same primers as the endogenous target sequence. To make a competitor PCR with a conventional downstream primer and a modified upstream primer is performed to give a sequence approximately 10% shorter than target sequence. The PCR product is isolated and cloned into a vector that contains strong DNA-dependent RNA-polymerase promoter sequence (T7, T3 or SP6). Appropriate RNA polymerase performs *in vitro* transcription of the cloned fragment. The internal standard RNA is treated with RNase-free DNase I to remove traces of plasmid DNA and finally measured by spectrophotometry. For the quantitation of the mRNA several RT reactions are prepared with the same amount of total RNA and different amounts of competitor. For the first measurements of a mRNA it is best to add competitor amounts which differ by factor 10 to determine the range in which the transcript amount is to be found. If that is known, factor 2-2.5 between the standard amounts gives more accurate results. This method allows measurement of small differences (as low as factor 2) in mRNA amount between RNA samples. The main advantage of the method is possibility of estimation of PCR products either during exponential reaction stage as well as during *plateau* stage what greatly broadens range of RNA quantities, which could be able to measure (Jungerman, 1997).

Considerable progress in QRT-PCR was achieved after introduction of Real-time PCR. The comparison of several QRT-PCR methods showed that, techniques based on gel electrophoresis and densitometry of PCR products had variability coefficient much greater (10-19%) than Real-time (even 2%) (Klein et al., 2001; Ylikoski et al., 2001). Real-time RT-PCR quantitates the initial amount of the template most specifically, sensitively and reproducibly, and actually is a preferable alternative to other forms of quantitative RT-PCR which detects the amount of final amplified product. Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle (i.e. in Real-time) as opposed to the endpoint detection by competitive PCR. Real-time PCR quantitation eliminates post-PCR processing of PCR products what helps to increase throughput, reduce the chances of carryover contamination and remove post-PCR processing as a potential source of error. Real-time PCR has also much wider dynamic range (up to  $10^7$ -fold comparing with  $10^3$ -fold of conventional RT-PCR) (Klein, 2002). This means that a wide range of ratios of target and normalizer can be assayed with equal sensitivity and specificity.

Three different technologies are commonly used for Real-time PCR detection. All of them 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 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. Non-sequence specific agents (SYBR-green I or ethidium bromide) intercalate in a minor groove of DNA. After intercalation fluorescence significantly increase and is proportional to amount of double-stranded DNA generated by PCR. Accurate quantitation can be obtained over a large range of initial template amounts if conditions are optimal. SYBR Green has been shown to be linear over a  $10^8$ -fold range of template concentrations, sensitive down to 10 copies (Bustin, 2000). This technology can be easily applied to already established PCR assays and does not need any additional fluorescence-labelled oligonucleotide. The major problem with SYBR Green-based detection is that non-specific amplifications cannot be distinguished from specific amplifications. Therefore, these assays require careful optimization of the PCR conditions and a clear differentiation between specific and non-specific PCR products using melting-curve analysis. The other two principles are based on the introduction of an additional fluorescence-labelled probe. The TaqMan probes (Heid et al., 1996) and molecular beacons (Tyagi and Kramer, 1996; Tan et al., 2000) use the fluorogenic 5' exonuclease activity of Taq polymerase to measure the amount of target sequences in cDNA samples. The TaqMan assay exploits the 3'-5' exonuclease activity of Taq polymerase to cleave a TaqMan probe during PCR. The TaqMan probe is a 20-30 bases long oligonucleotide that contains a reported fluorescent dye at the 5' and a quencher dye at the 3' end (because the 3' end is blocked, it cannot act as a primer). When irradiated, the excited fluorescent dye transfers energy to the nearby quenching dye molecule rather than fluorescing (this is called FRET, Förster or fluorescence resonance energy transfer). TaqMan probes are designed to anneal to an internal region of a PCR product. When the polymerase replicates a template on which a TaqMan probe is bound, its 5' exonuclease activity cleaves the probe. This ends the activity of quencher (no FRET) and the reporter dye starts to emit fluorescence, which increases in each cycle proportional to the rate of probe cleavage. Molecular beacons also contain fluorescent (FAM, TAMRA, TET, ROX) and quenching dyes (typically DABCYL) at either end but they are designed to adopt a hairpin structure while free in solution to bring the fluorescent dye and the quencher in close proximity for FRET to occur. It is designed to have two arms with complementary sequences that form a very stable hybrid or stem. The close proximity of the reporter and the quencher in this hairpin configuration suppresses reporter fluorescence. When the beacon hybridizes to the target during the annealing step, the reporter dye is separated from the quencher and the reporter fluoresces (FRET does not occur). Molecular beacons remain intact during PCR and must rebind to target every cycle for fluorescence emission. This will correlate to the amount of PCR product available (Knemeyer et al., 2000; Liu et al., 2002). Both TaqMan probes and molecular beacons allow detection of multiple DNA species (multiplexing) by use of different reporter dyes on different

probes/beacons. By multiplexing, both the target and endogenous control can be amplified in single tube.

Accumulation of PCR products is detected by monitoring the increase in fluorescence of the reporter dye. TaqMan assay uses universal thermal cycling parameters and PCR reaction conditions. Because the cleavage occurs only if the probe hybridizes to the target, the fluorescence detected originates from specific amplification. The process of hybridization and cleavage does not interfere with the exponential accumulation of the product. The slope of the log-linear phase is a reflection of the amplification efficiency. For the slope to be an indicator of real amplification (rather than signal drift), there has to be an inflection point. This is the point on the growth curve when the log-linear phase begins. It also represents the greatest rate of change along the growth curve. The important parameter for quantitation of PCR product is the threshold cycle ( $C_T$ ). The threshold cycle value is the cycle when the system begins to detect the increase in the signal associated with an exponential growth of PCR product during the log-linear phase. The higher the initial amount of cDNA, the sooner accumulated product is detected in the PCR process, and the lower the  $C_T$  value. Correct choice of threshold, which determine the  $C_T$  value, is one of the most important elements in Real-time PCR. It should be placed above any baseline activity and within the exponential increase phase. Software allows determination of the cycle threshold by a mathematical analysis of the growth curve.

#### APPLICATIONS OF RT-PCR IN MOLECULAR DIAGNOSTIC

Clinical microbiology, virology and food microbiology are important fields for Real-time PCR applications. Hundreds of assays have been published for the detection and quantification of various infectious agents (Jordan, 2000). However, critical and strict evaluation of newly developed assays is a prerequisite to obtain reliable data. Real-time PCR assays are less time- and labour-intensive (Hein et al., 2001). It detects and quantifies nucleic acids from live and dead pathogens, whereas classical microbiological assays count only live pathogens. Most infectious agents are characterized by a high mutation rate, which can influence the viral load estimation dramatically (Fend et al., 2000). This problem can be overcome by using highly conserved regions for assay development. 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 the advantage for pathogen quantification as up to four different fluorescent dyes can be detected and quantified simultaneously (Ohyama et al., 2000). 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 (Fend et al., 2000; Klein et al., 2002). Nevertheless, duplex or multiplex assays are complex and 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 (Fend et al., 2000; Ohyama et al., 2000; Klein et al., 2002). Internal standardization in multiplex approaches is also an important aspect in applications requiring quality assurance (Baugh et al., 2001).

#### *Applications in virology*

RNA is a genetic material of great number of viruses, which are etiological factors of human, animals and plants infectious diseases. Introduction of RT-PCR technique allowed achieving considerable progress in examination of diseases caused by RNA viruses, for example HIV, hepatitis virus, enteroviruses, rabies virus. The replication of nucleic acids is one of the first steps in virus development, thus RT-PCR method allows detecting of infection even before organism have begun to produce specific antibodies, thus before seroconversion. The measurement of virus RNA quantity allows the estimation of viremia level and the monitoring of antiviral therapy efficiency. In diagnostics of virus diseases RT-PCR was applied in three main cases:

- detection of invasion with short period between contact and appearance of disease symptoms
- lack of serological tests
- epidemiological investigation.

RT-PCR is often used for detection of enteroviruses caused diseases. The clinical picture does not allow differentiate diseases caused by enteroviruses and another kind of virus infection. Till RT-PCR has been introduced diagnosis of enteroviruses infection was based on isolation of virus from faeces or cerebrospinal fluid or on analysis of antibody titre in serum. Enteroviruses isolation is time-consuming process persisting at least 4 - 8 days (Chonmaitree, 1982). Serological diagnostics was found to be difficult because of great antigenic variation of enteroviruses, appearance of heterotypical immunological response and lack of mutual antigenic determinant – laboratory confirmation of infection takes at least two weeks. These methods are appropriated in epidemiological investigations but are not satisfactory for diagnosis. RT-PCR technique help solve many of these problems. High homology of RNA sequences on non-translating 5'-end of genome (5'-UTR) makes possible to design primers for detection of majority if not all of enteroviruses. Moreover it is

possible to design primers for detection of limited amounts of serotype (group- or strain specific primers). RT-PCR has been found even more sensitive in detection of enteroviruses than cell cultures (Rotbart, 1990; Sawyer, 1994). Short time of analysis is the main advantage of RT-PCR method. It was convinced that time from sample preparing till evaluation of reaction result takes about 5 h, at sensitivity 94.7%, specificity 94.7% and repeatability 99% (Rotbart, 1997). Additional advantage of RT-PCR is small volume of serum or cerebrospinal fluid is also essential for analysis - about 50  $\mu$ l (Kammerer, 1994). Introduction of heat labile form of the enzyme uracil DNA glycosylase (UNG) for amplicon contamination control and an RT-PCR enzyme mixture, enabling a one tube/one step amplification (Taggart et al., 2002) further increased capacities of RT-PCR method. Read et al. (1997) has analysed usefulness of RT-PCR for diagnostic of aseptic neuroinfection. Authors indicated high repeatability of the test and its exceptional usefulness in diagnostic of infection caused by coxackieviruses type A; 1 and 6, which does not proliferate in cell lines - it is impossible to use classical diagnostic method. RT-PCR is also used in diagnostics and monitoring of hepatitis virus type C and AIDS. Genetic material of hepatitis virus type C (HVC) is a single stranded RNA molecule 9500 base pair long. The main screening test for HCV laboratory diagnostic is ELISA assay, which detect HVC antigen. Although even use of III generation of ELISA test time between HVC infection and symptoms appearance or seroconversion takes 3 to 6 weeks (Decker and Troonen, 1997). During this period HVC could be detected only by RT-PCR. It is estimated that RT-PCR is able to detect as low as 10 molecules of HCV-RNA, even after one week post-infection. HCV-RNA detection in practise is used for screening of patients anti-HCV negative patients from high risk groups, detection of infection in patient whit normal aminotransferase activity, qualification of patients for anti-virus treatment and control of treatment efficiency. Recently, Roche Diagnostic Inc., designed COBAS AmpliScreen HCV and AMPLICOR HVC RT-PCR kits for diagnostic of HCV as well as kits for AIDS diagnostic, which were in 2001 year allowed by FDA for clinical applications on USA market. In year 2002 the permit was extended on COBAS TaqMan Analyser.

#### *Applications in oncology*

Major challenges of clinical oncology for assay techniques are sensitivity and 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 nucleic acid during sample preparation and per PCR reaction is the limiting step. One of example of successful application of RT-PCR in clinical oncology is monitoring of residual disease after leukaemia treatment (Kantarjian et al., 2003; Pine et al., 2003). The end of remission period is marked by appearance of malignant cells in blood. Appearance of malignant cells during remission period gave name of residual

disease. The detection and quantification of chromosomal translocations by Real-time PCR has been applied to monitor minimal residual disease (Pongers-Willems et al., 1998) or to show graft-versus lymphoma effects (Mandigers et al., 1998). More than 90% patients with chronic myeloid leukaemia (CML), 20% of adult and 5% of children with acute lymphoblastic leukaemia (ALL) has Philadelphia chromosome (Ph). This chromosome appears as result of reciprocal translocation of chromosome 22 in breakpoint cluster region (BCR) and chromosome 9 on the 5'-end of ABL gene. Joint product of fused BCR and ABL is transcribed, and transcription product is alternatively spliced. Alternative splicing of primary transcript generates three types of mRNA with following exon sequence: - e11-e12-e13(b2)-a2-a3-a4 (b2a2 product), e11-e12-e13(b2)-e14(b3)-a2-a3-a4 (b3a3 product), and e1-a2-a3-a4 (e1a2 product) (Jungerman, 1997). Scheme of alternative splicing of BCR-ABL primary transcript is presented at Figure 3. The majority of CML patients indicate presence of b2a2 or b3a2 splicing products and about 70% of patients with ALL-Ph(+) phenotype indicates presence of e1a2a. This is the excellent marker because jointed BCR-ABL product is present only in lymphocytes of patients suffering leukaemia. Expression of ABL gene proceeds in all leukocytes what makes possible to use its mRNA as an internal standard. Final result of RT-PCR analysis is presented as percentage BCR-ABL quantity in comparison to ABL. Value below 0.02% indicate that patient is still in remission, 0.02-0.5% indicate end of remission period and return of disease (Cross, 1993; Lion, 1993).

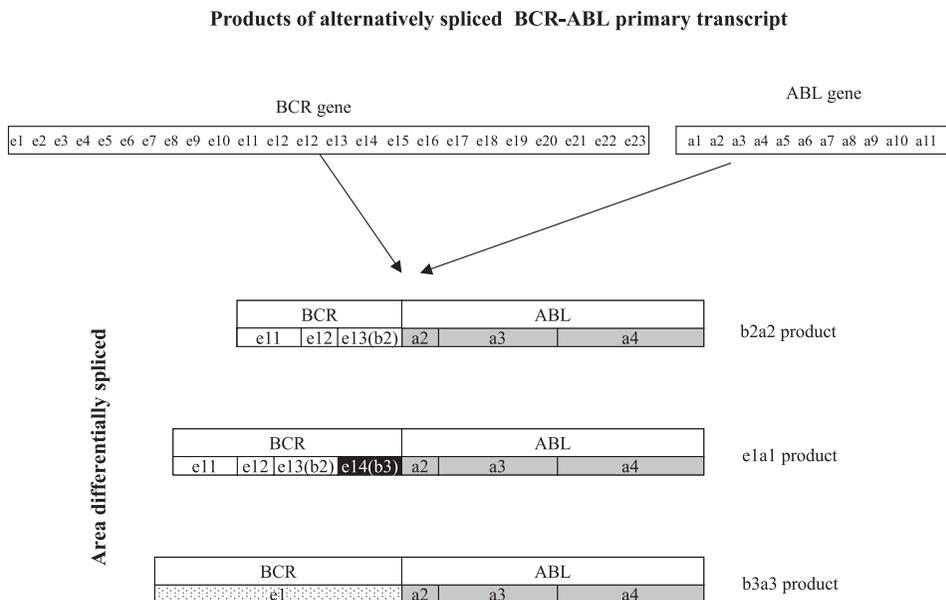


Figure 3. Alternative splicing of primary BCR-ABL transcript

Recently, new markers have been found that can detect different kinds of tumours, among them kallikreins are one of the most promising. There is strong but circumstantial evidence linking kallikreins and cancer. Prostate-specific antigen (PSA; hK3) and, more recently, human glandular kallikrein (hK2) are widely used tumour markers for prostate cancer. Three other kallikreins, hK6, hK10, and hK11, are emerging new serum biomarkers for ovarian and prostate cancer diagnosis and prognosis. Several other kallikreins are differentially expressed at both the mRNA and protein levels in various endocrine-related malignancies, and they have prognostic value (Diamandis et al., 2002). Tyrosinase can be a marker of melanoma. Tyrosinase mRNA in blood was detected in 76% of IIB and 66% stage III patients. The presence of tyrosinase mRNA in blood was correlated with a shorter survival ( $P: 0.03$ ) and in multivariate analysis was an independent prognostic factor for relapse (Gogas et al., 2002).

Real-time RT-PCR significantly simplifies and accelerates the process of mRNA quantification. The use of appropriate standard curves make possible easily calculates absolute copy numbers of mRNA. Moreover, Real-time RT-PCR is readily standardized making the comparison of results from different laboratories, templates from different samples and clinical samples from different patients easier and more reliable. Therefore, Real-time RT-PCR must be the method of choice for any experiments requiring sensitive, specific and reproducible quantification of mRNA

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## STRESZCZENIE

### Metody RT-PCR oraz możliwości ich zastosowania

Reakcja PCR połączona z odwrotną transkrypcją (RT-PCR ang. *reverse transcription polymerase chain reaction*) jest jedną z najbardziej czułych metod wykrywania obecności i pomiaru ilości mRNA. Metody RT-PCR są szeroko stosowane do pomiaru ilości mRNA w badaniach podstawowych, biotechnologii, medycynie oraz w rolnictwie. Zastosowanie techniki pomiaru ilości produktu PCR w czasie rzeczywistym (ang. *Real Time PCR*) znacznie zwiększyło szybkość, czułość, specyficzność oraz powtarzalność RT-PCR. Tematem pracy jest omówienie podstaw metody RT-PCR wykonywanej techniką tradycyjną oraz techniką Real Time PCR, ich zalet i ograniczeń oraz ich zastosowania do pomiaru ekspresji genów w badaniach podstawowych, rolniczych i biomedycznych.