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PCR-RFLP and Real-Time PCR Techniques in Molecular Cancer Investigations

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1. Introduction

1.1 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is a rapid scientific method for generating a 10^6 - 10^7 -fold increase in the number of copies of discrete DNA or RNA sequences (Boehm,1989; Imboden et al,1993). The use of PCR technology has greatly increased the ability to study on genetic material. PCR is a rapid and reliable molecular biology technique that allows quick replication of mainly DNA, the starting material can be a single molecule of rRNA or mRNA. It was developed by Kary Mullis in 1983, and he was awarded the Nobel Prize in 1993. PCR method is useful in situations of limited amount of DNA sample as in forensics, prenatal testing, because it amplifies a single or a few copies of DNA creating millions of copies of the region(1). The ability to quickly produce large quantities of genetic material has enabled significant scientific advances including DNA fingerprinting and sequencing of the human genome. As PCR technology allows taking specimen of genetic material even from just one cell, copy its genetic material several times, this facilitates genetic studies. Currently, besides research purposes, PCR technology is heavily used in diagnosis and patient management especially for viral diseases such as AIDS and hepatitis. Other than detection of infectious organisms, this technology is also useful for determination of genetic polymorphisms or mutations of individuals (Stahlberg,2011).

The method relies on thermal cycles of repeated heating and cooling of the reaction for DNA melting. Double stranded DNA can be disrupted by heat or high pH, giving rise to single stranded DNA. The single stranded DNA serves as a template for synthesis of a complementary strand by replicating enzymes, DNA polymerases. In order to imitate the accelerated form of DNA replication for a gene region, a special form of DNA polymerase is used. This DNA polymerase should be resistant to the thermal denaturation. Most of the PCR applications employ Taq polymerase, an enzyme isolated from the bacterium *Thermus aquaticus*, but there are some other heat-stable DNA polymerases used by the same purpose. Most polymerases require short regions of double stranded nucleic acid to initiate synthesis. For in vitro PCR reactions, this can be provided by synthetic oligonucleotides of about 21-25 bp that are complementary to the negative strand of main DNA molecule. Those

oligonucleotide sequences are known as 'primer' and chosen due to the DNA region that we want to amplify. In PCR, two synthetic primers that flank the region of interest are used; one primer is complementary to the negative strand of DNA and second primer to the positive strand. The primers must be oriented that DNA synthesis proceeds across the regions defined by the primers. By this way, only a single region of giant DNA molecule can be amplified. As only one amplification is not enough, PCR is a cyclic process to generate 10^6 - 10^7 -fold increase in a gene region; each PCR cycle contains three steps. Those thermal cycling steps are necessary separate two strands in the DNA double helix at a high temperature by a process called DNA melting. There are three main sequentially repeating steps of PCR:

- *Denaturation* of DNA duplex (94-98°C),
- *Annealing* of primers (37-60°C),
- *Extention* (elongation) of primers by polymerase reaction (~72°C)

In the *denaturation* step, the purpose is to separate strand to be ready for replication, denaturation temperature is higher than the other steps. In the *annealing* step, at a lower temperature, each strand is used as templates for DNA synthesis. The selectivity of PCR results from this step by the usage of primers complementary to the targeted DNA region under specific thermal cycling conditions. After this, there is *extention* step continuing by the heat-stable DNA polymerase to amplify the target DNA region (Boehm, 1989).

After 20 cycles of amplification, a million copies of DNA can be generated from a single copy. After several rounds of amplification (about 40 times), the PCR product is analysed on an agarose gel and is abundant enough to be detected with an ethidium bromide stain.

After this stage, to detect the changes on the DNA sequence, the classical PCR-RFLP method (the next heading) can be used. But also specific DNA sequences can be detected without opening the reaction tube (Higuchi, 1992). Recently, after first preliminary studies the technique developed to get both structural and quantitative informations about the amplified DNA region by real-time PCR devices using fluorescent dyes, as we will mention in following headings.

1.2 PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) method

In contemporary, there are several forms of PCR that are extensively modified to perform a wide array of genetic manipulations. PCR-RFLP (PCR-restriction fragment length polymorphism) is one of those that was preliminary to most of classified PCR methods. RFLP is a technique referring to a difference between restriction enzyme sites on DNA samples, broken into pieces (digested) by those restriction enzymes and the resulting fragments are separated according to their lengths by gel electrophoresis.

Restriction endonucleases are specific enzymes that can cleave specific nucleotide sequences; because of that property, it is possible for them to discriminate nucleotide changes in DNA. Sometimes they can effect the loci other than the target one, but the important part of the procedure is the possible polymorphism or mutation loci to be detected whether the cleavage site is intact or not. If there is a change in the cleavage site of restriction endonuclease, it will not cleave the site, or by addition of the mutation, there may occur a previously not existing cleavage site.

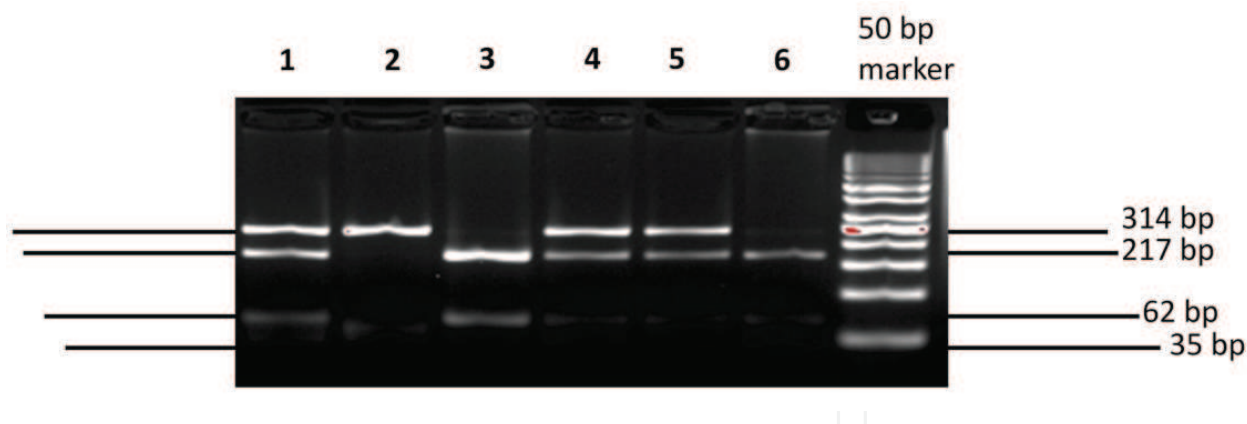


Fig. 1. An example of RFLP results from our laboratories. As shown in the figure, there is a 50 bp marker to compare with our own results and detect the basepair (bp) length. After treatment and incubation with specific restriction enzyme:

- The uncut homozygote cases (having the same alleles) were expected to be having only one 314 bp band (as in number 2).
- The cut homozygote cases (having the same alleles) were expected to be having three bands of 217, 62, 35 bp (as in number 3 and 6)
- The heterozygote cases (having two separate alleles) were expected to be having four bands of 252, 217, 62, 35 bp (as in number 1, 4 and 5)

1.3 Types of PCR

- **Conventional PCR:** This is the DNA-based PCR, primers target specific sequences on DNA and amplification follows the usual steps of denaturation, annealing and elongation.
- **Reverse transcription-PCR:** mRNA or rRNA can be the main material to be amplified. The first step is the enzymatic 'reverse transcriptase' reaction to transcribe RNA to cDNA. Subsequent steps are similar to conventional PCR steps (Tania et al, 2006).
- **Asymmetric PCR:** It can be used for generation of single strand for sequencing studies. This can be done by adjusting primer concentrations to favor one strand; by this way after first cycles, only the strand complementary to the first strand continues to be copied.
- **Nested PCR:** In this type of PCR, there are two stages of the procedure; in the first part, by using a set of primers, a fragment is amplified. After this, by using another primer set, a sub-region of the previously amplified region is re-amplified. Main aim is to increase sensitivity and specificity.
- **Real-time PCR.**

2. Real-time PCR

Real-time PCR (PCR with real time) is also known as kinetic PCR, QPCR, QRT-PCR. Automated thermal cycling devices have been improved by using Taq DNA polymerase which is thermostable and continued to be developed by fluorescence luminescence techniques (Higuchi et al, 1992; Logan et al, 2009.). Real-time PCR is easy to perform, providing reliable results with high accuracy as well as rapid quantification. Quantification of polymorphic DNA regions and genotyping single nucleotide polymorphisms are detected by using the real-time PCR reaction. For gene expression analyses, the mRNA

levels can be done quantitatively by reverse transcriptase-PCR (RT-PCR) reaction (Tanie Eet al, 2006). By this way, it is possible to monitor gene outputs numerically in many different fields, from the drug-resistant tumor cells to the chemotherapy scanning and also to the molecular determination of tumor stages. The use of gene expression analysis is getting increased in many notable fields of biological research. Gene profiling opens new possibilities to classify the disease into subtypes and guide a differentiated treatment.

This method has been preferred especially in the samples, the analysis of which cannot be possible, or in the samples, the cytogenetic analysis of which are turned out as auxiliary techniques to the molecular analysis. Therefore, it has become one of the indispensable methods. The introduction of real-time PCR technology has significantly improved and simplified the quantification of nucleic acids, and this technology has become a valuable tool for many scientist working in different disciplines. Especially in the field of molecular diagnosis, real-time PCR-based assays took their advantage (Pfaffl, 2004).

2.1 Real-time PCR protocols

Real-time PCR has been preferred as one of the favored methods in molecular studies and in routine analyses, since the process takes short time as 20-30 minutes, it provides fast heating and cooling cycles of 30-40 times, in addition to these, it benefits the control of PCR reaction on a computer monitor (Wittwer, 1997). High sensitivity of real-time PCR makes the technique applicable to very small samples, such as fine needle aspirates. Real-time PCR instruments can simultaneously amplify and detect, eliminating the need to open tubes containing PCR products and therefore reducing the risk of future contamination (Lyon, 2009). Additionally nested PCR and touchdown PCR can be performed using real-time PCR Machine. There are various real-time PCR machines that are used mostly in laboratory experiments:

- ABI Prism 7700
- LightCycler2/Lightcycler 480 Probes (Roche, Mannheim, Germany)
- i-cycler (BioRad)

2.2 Probing techniques

Today, fluorescence is exclusively used as the detection method in real-time PCR. The fluorescent reporters can be divided into two categories: nonspecific and sequence-specific labels (Wilhelm, 2003).

Nonspecific labels: These are DNA-binding dyes such as SYBR Green I (Wittwer et al, 1997; Zipper et al, 2004.) and BEBO (Bengtsson et al, 2003), which become strongly fluorescent when they are bound to double-stranded DNA. SYBR Green I binds all double-stranded DNA molecules regardless of their sequence. The Double-stranded DNA binding dye SYBR Green I is proven to be effective. Maximum excitation of SYBR Green I dye occurs at 497 nm. Maximal emission of DNA stained with SYBR Green I occurs at 521 nm. The specificity and sensitivity of SYBR Green I detection can be monitored by performing a melting curve analysis after using the amplification reaction with external standard.

Differentiation of single point mutant alleles from wild type allele is not possible with SYBR Green I but it is possible to detect small deletions/insertions (10 to 20 bp).

2.2.1 Hybridization probes (pair of sequence-specific, single-labeled probes)

Sequence-specific probes are based on oligonucleotides or their analogs that one or two fluorescent dyes are coupled.

There are some types of probes with two dyes (Holland et al 1991; Tyagi et al, 1996; Tyagi et al, 1998; Caplin et al 1999): a) hydrolysis probes (TaqMan® probes), b) molecular beacons, c) hybridization probes.

- a. **Hydrolysis probes:** This probe is a single oligonucleotide labeled with two different fluorophores. The fluorophore near the 3' end(acceptor) acts as a fluorescence emission "quencher" of the other one near the 5' end(donor) (Holland et al 1991). As soon as Taq DNA polymerase hydrolyzes the probe via its 5' exonuclease activity during a combined annealing/extension step, the 5' fluorophore (donor) is liberated. Therefore, its emission can no longer be suppressed by the quencher and can be measured in the fluorimeter. TaqMan real-time PCR is one of the two types of quantitative PCR methods, and uses a fluorogenic probe which is a single stranded oligonucleotide of 20-26 nucleotides and is designed to bind only the DNA sequence between two PCR primers. In this case, two primers with a preferred product size of 50-150 bp, a probe with a fluorescent reporter or fluorophore such as 6-carboxyfluorescein (FAM) and tetrachlorofluorescein (TET) and quencher such as tetramethylrhodamine (TAMRA) covalently attached to its 5' and 3' ends are required, respectively (Giller et al, 2011).
- b. **Hybridization probes:** In this case, there are two oligonucleotides that hybridize to adjacent internal sequences of the same amplicon (Witther et al, 2011). For instance, the 5' oligonucleotide (donor) has a fluorescence in label at its 3' end. The 3' oligonucleotide(acceptor) has either LightCycler-Red 640 or LightCycler-Red 705 at its 5' end. Only after hybridization to the template DNA, two probes come in close proximity, resulting in fluorescence resonance energy transfer (FRET) between the two fluorophores. During FRET, fluorescein, the donor fluorophore, is excited especially by the light source of the LightCycler Instrument, and part of the excitation energy is transferred to either LightCycler-Red 640 or LightCycler-Red 705, the acceptor fluorophores. Emitted fluorescence of these acceptor fluorophores are then measured by the LightCycler Instrument. Specific detections are performed with these probes. For example, the mutation detections are analysed via the external and internal standards .
- c. **Molecular Beacon Probes:** A molecular beacon is one oligonucleotide labeled with two different fluorophores, an acceptor and a donor. Due to the specific secondary structure formed by the oligonucleotide (beacon), acceptor (quencher) and donor dyes are in close proximity. A molecular beacon unfolds while binding to the growing PCR product, thereby separating the dyes and enhancing the fluorescence of the donor dye. Four different fluorophores can be designed to detect different point mutations simultaneously (Vincent et al, 2005).

2.3 Melting curve analysis

At the beginning of a melting curve analysis, the reaction temperature is low and the fluorescence signal is high. As the temperature steadily increases, the fluorescence will suddenly drop as the reaction reaches the melting point (T_m) of each DNA fragment. More specific analysis of PCR reactions can be performed with SYBR Green I because of its specific melting behaviour, identification/differentiation of multiple specific PCR products

(multiplex PCR) with SYBR Green I, genotyping and mutation analyses with hybridization probes. Melting Curve Analysis has many advantages (Wittwer et al, 2009). Just like gel

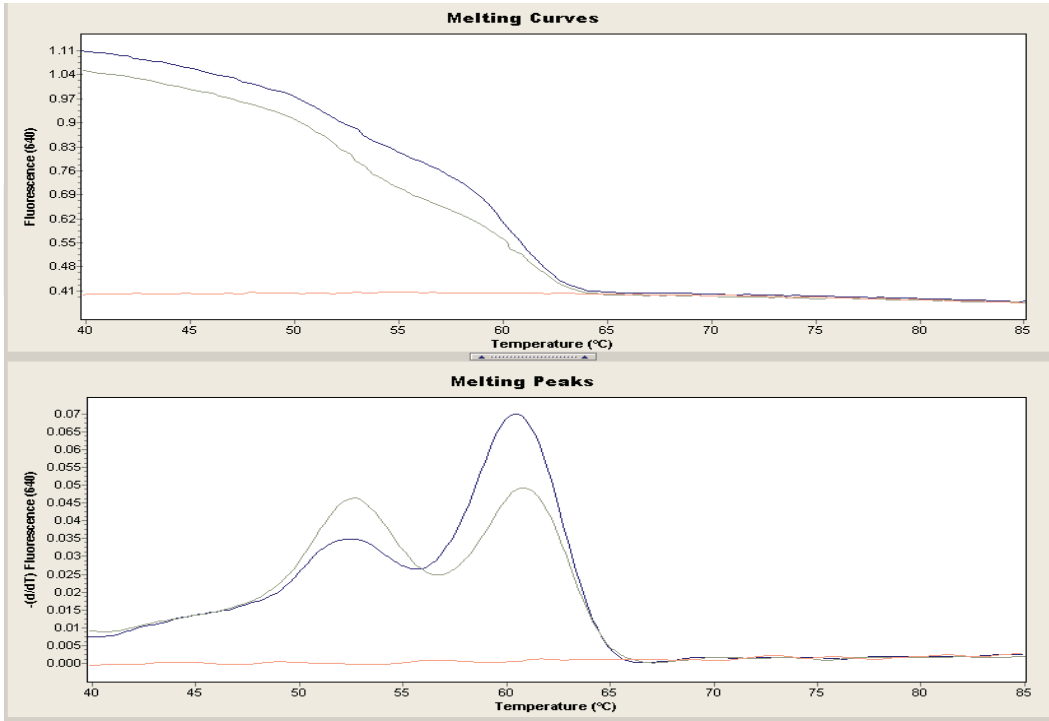


Fig. 2. Heterozygote result indicating two melting curves (53.0 °C and 62.0 °C)

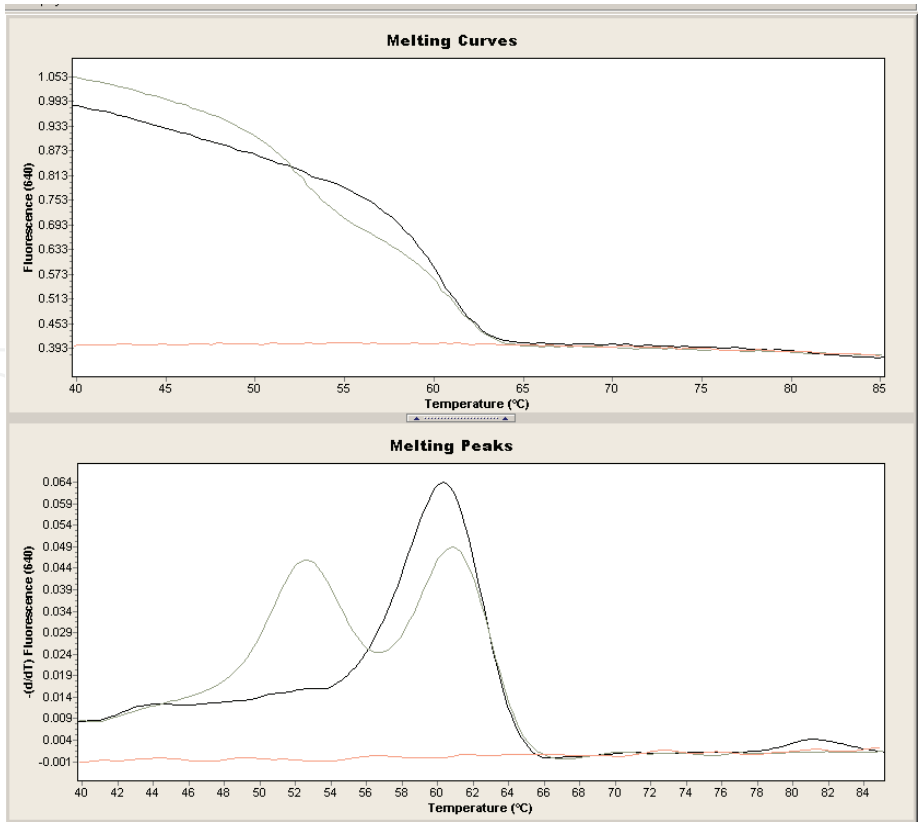


Fig. 3. Wild type result which indicate one melting curve (62.0 °C)

electrophoresis, melting point analysis permits clear identification of the amplicon, since each PCR product possesses a characteristic melting point. Moreover, nonspecific products (primer dimers) can also be identified by this method. If performed with hybridization probes, melting point analysis can also detect point mutations. For instance, the acquired Janus Kinase 2(JAK2) V617F point mutation can be found in more than 90% patient with polycythaemia, and in 50% of patients with other chronic myeloproliferative diseases. For instance in the figures 2-4, our own laboratory results are given. Myeloproliferative neoplasms JAK2V617F-mutation analysis results are shown as melting curve analyses. The genotype is identified by running a melting curve with specific melting points (T_m).

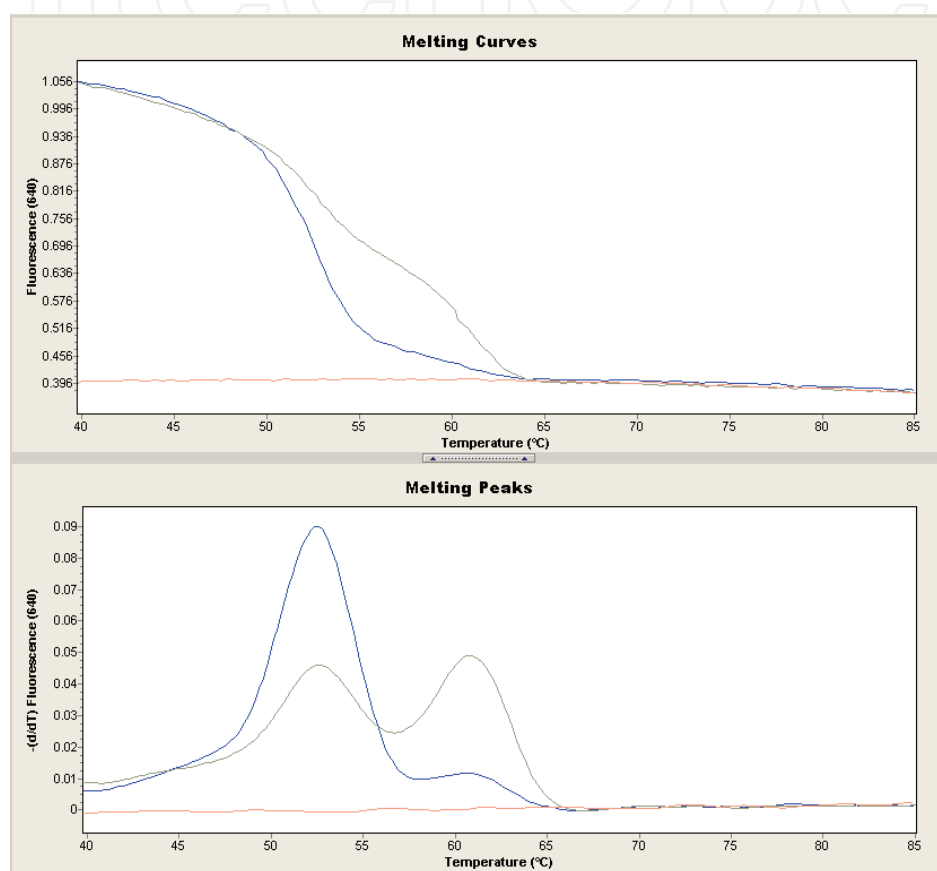


Fig. 4. Mutant result which indicate one melting curve (53.0 °C)

2.4 High-Resolution Melting Analysis (HRMA)

High resolution melting is a post-PCR-based method for detecting DNA sequence variation by measuring changes in the melting of a DNA duplex (Martin-Nunez et al, 2011). Melting analysis using new instruments have been designated for high-resolution melting curve analysis (HRM or HRMA) based on its ease of use, simplicity, flexibility, cost-effectivity, nondestructive nature, superb sensitivity, and specificity (Vossen et al, 2009). It enables researchers to rapidly detect and categorize genetic mutations and single nucleotide polymorphisms(SNPs), identify new genetic variants without sequencing (gene scanning) or determine the genetic variation in a population (e.g. viral diversity) prior to sequencing. SYBR®Green I is introduced into a sensitive conventional dye for PCR product melting analysis. High-resolution melting analysis have been used clinically to detect somatic

changes in select exons of oncogenes such as *EGFR*,⁵³ *KRAS*,⁵⁴ *PDGFRA*,⁵⁵ *KIT*,⁵⁶ *BRAF*,⁵⁷ and *TP53* (Bastien et al, 2008).

2.5 Gene expression analysis

Conventional microarrays have limitations in flexibility, speed, cost, and sensitivity. Gene expression analysis by microarray techniques and real-time PCR offers new possibilities to classify malignant tumors, such as lymphomas, into more distinct subtypes for diagnosis and treatment (Schmit et al, 2010; Bagg et al, 1999; Stahlberg et al, 2005.). The study of biological regulation usually involves gene expression assays and requires quantification of RNA frequently. In the past, conventional gel- or blot-based techniques were used for these assays. However, these techniques often have limitations in speed, sensitivity, dynamic range, and reproducibility required by current experimental systems. In contrast, real-time PCR methods, can easily meet these requirements. Reverse transcription PCR (RT-PCR) is a common and powerful tool for highly sensitive RNA expression profiling. Quantification by real-time PCR may be performed as either absolute measurements using an external standard, or as relative measurements, comparing the expression of a reporter gene with that of a presumed constantly expressed reference gene (Stahlberg et al, 2005).

A flow- chart, represents the steps of Real-time PCR and its applications, is given in Figure 5.

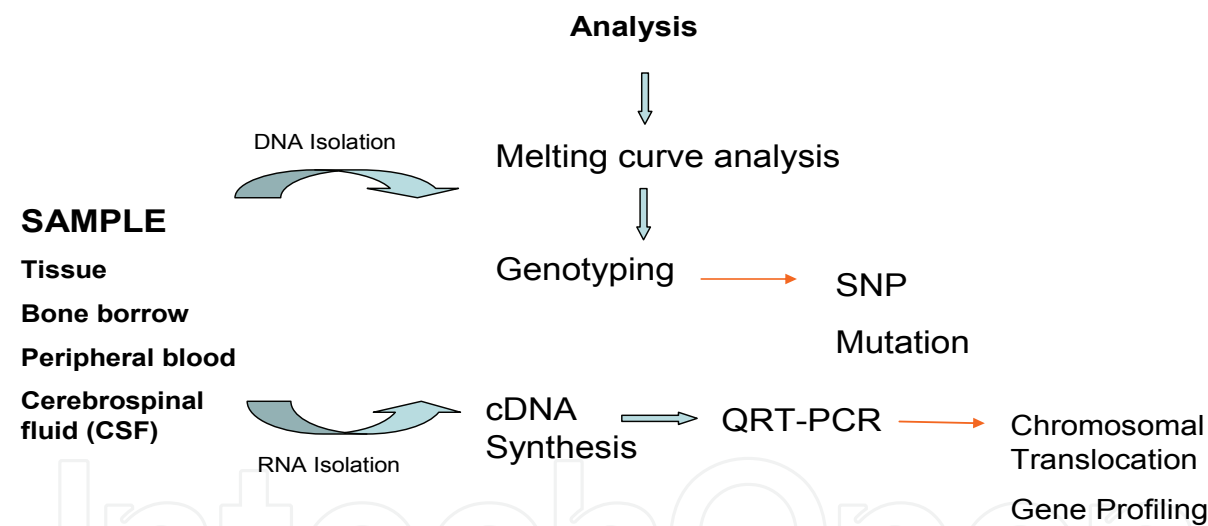


Fig. 5. Real-time PCR Flow-chart

2.6 Epigenetic studies with PCR

Epigenetic information is usually lost during the PCR because of the insensitivity of DNA polymerase, it cannot distinguish between methylated and unmethylated cytosines. After PCR, any methylated allele will be extremely diluted. Therefore, something must be done to preserve methylated form of DNA. Treatment with sodium bisulfite will deaminate cytosine to uracil, the rate of deamination of 5-methylcytosine to thymine is slower than the conversion of cytosine to uracil, thus it is assumed that the only cytosines remaining after sodium bisulfite treatment are derived from 5-methylcytosines. By this way, during subsequent PCR cycles, uracil residues are replicated as thymine residues, and 5-methylcytosine residues are replicated as cytosines. The efficiency of the method is about

99% in appropriate conditions, but this method needs intense attention while choosing primers and arranging study conditions (Gulley et al.).

3. PCR-based studies in cancer research

The advances in molecular techniques provide new molecular targets for diagnosis and therapy of cancer. These advances can provide both researchers and clinicians with precious information concerning the behavior of tumors. Therefore, these tumors can be detected at earlier stages when the tumor burden is smaller and be potentially more curable currently. After the human genome project has completed, the application of high-throughput technologies for polymorphism detection for explaining molecular mechanism for complex disease has created very important opportunities (Khouri, 1997).

Single nucleotide polymorphisms (SNPs) offers widespread use in gene mapping of genetic disorders, in the delineation of genetic influences in multifactorial diseases such as cancer, cardiovascular disease, in haplotype mapping, and as genetic markers to predict responses to drugs (Riddick et al, 2005). However, for example, there are some inconsistent results regarding the relationship between the presence of polymorphic forms of genes encoded detoxifying enzymes and chemotherapeutic response. It has been reported that the genetic polymorphism analysis in peripheral blood may not be enough representative for the status in tumour tissue. For instance, Uchida et al reported that individuals heterozygous for the 28-bp polymorphism in thymidylate synthase (TS) gene may have increased risk for cancer that are homozygous for this polymorphism due to loss of one allele during carcinogenesis (Uchida et al, 2004). They also showed that the response to 5-FU-based chemotherapy in these cases was comparable to cases where the individual was homozygous. Therefore, it may be excellent to determine the genotype of polymorphisms in tumour cells than in peripheral blood.

Some data obtained from combined genotype studies have demonstrated that these data may have significance for models of cancer prognosis or treatment. But, many researchers suggest that larger studies will be needed also to investigate the effect of specific treatment modalities in cancer. While investigating the post-initiation stages of cancer, four basic parts can be dedicated to gene polymorphisms affecting: (a) growth control of cell (cell proliferation, differentiation and death); (b) factors involved in tumour invasion and metastasis (immune and inflammatory responses, extracellular matrix remodelling, angiogenesis and cell adhesion); (c) effects of hormones and vitamins on growing tumours; (d) outcome of cancer therapy (cancer pharmacogenetics) (Loktionov, 2004). Quantitation of gene expression in tumor or host cells has another enormous importance for investigating the gene patterns responsible for cancer development, progression and response or resistance to therapy.

Analysis of transcriptional activity of tumor cells or detection of possible new tumor markers by polymerase chain reaction (PCR), reverse transcriptase PCR (RT-PCR) and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) techniques have the potential to change cancer diagnosis and treatment (Mocellin, 2003). New molecular techniques for diagnosis offers the promise of accurately matching patient with treatment. It has been shown that there is a resultant significant effect on improved disease outcome. Currently, the real-time reverse transcription polymerase chain reaction (qRT-PCR), has a

potential to become an important analytical technique for the mRNA detection in tissue biopsies or body fluids. qRT-PCR is especially promising in prognostic assays and monitoring response to treatment for cancer patients. It is known that histopathological staging in cancer defines patient prognosis. However, there are some limitations in the prognostic heterogeneity of patients within a given tumour stage. According to this view, not all patients with lymph node-negative are treated and not all patients with lymph node-positive tumours die from their cancer. So, more accurate staging protocols are needed for detection clinical tumour staging by using molecular techniques.

Gene expression analysis is one of the most important parameter that utilises the qRT-PCR assay's potential for generating quantitative data (Skrzypski, 2008; Schuster et al, 2004). It is reported that the detection of disseminated tumor cells in peripheral blood obtained from colorectal cancer patients by RT-PCR could be an effective method for identifying patients for adjuvant therapy. It is known that the mRNA for prostate specific antigen (PSA) is expressed only by prostatic cells. RT-PCR are suggested as a potentially more sensitive assay for the detection of cells expressing PSA mRNA in peripheral blood or in extraprostatic tissues. Some studies suggest that the molecular detection of circulating tumor cells (CTC) and micrometastases may help develop new prognostic markers in patients with solid tumors (Ghossein et al, 2000). It has been reported that prostatic tissue specific markers and melanoma related transcripts were detected by RT PCR in the peripheral blood, bone marrow and lymph nodes of patients with localized and advanced tumors. Currently, many reliable methods emerged with fast and efficient mechanisms for screening and monitoring large populations for genetically linked traits and for cancer-related genes discovery.

In addition to gene expression profiling, real-time PCR is also useful to detect chromosomal aberrations. Non-random chromosomal translocations are frequently associated with a variety of cancers, particularly hematologic malignancies and childhood sarcomas (Peter et al, 2006). For example t(15,17) translocation is found only in the leukemic cells. Only in patients with acute promyelocytic leukemia (APL) and the other forms of leukemia, t(1;19) translocation is found with B-cell precursor acute lymphoblastic leukemia (ALL). Quantitative analysis provide small number of remaining malignant cells (minimal residual disease, MRD) in patients to be revealed whose disease is in a clinical remission. Therefore, quantitative results are very important in terms of detection in malignancies and MRD. For example, *BCR-ABL* quantification monitors MRD and therapy of chronic myelogenous leukemia (Lyon et al, 2009). Using the real-time PCR Instrument as a closed tube, rapid amplification and real-time fluorescence detection system, for example quantitative measurement of the *BCR-ABL* expression level can be performed with a minimum risk of cross contamination. Relative expression levels of different samples may be calculated by standardizing the amount of *BCR-ABL* transcripts in a sample to the amount of an endogenous expressed housekeeping gene. The values for *BCR-ABL* and housekeeping gene for each sample are calculated by the real-time PCR software by the comparing the crossing points to the standard curve. A normalized target value (the ratio of *BCR-ABL*/housekeeping) is then derived by dividing the amount of *BCR-ABL* by the amount of housekeeping gene. The chromosomal aberration examples in various leukemia types can be detected by RNA quantification, shown in Table 1. On the other hand, melting analysis of the PCR product or the probe is used to confirm detection of the correct product.

ACUTE LYMPHOBLASTIC LEUKEMIA (ALL)			ACUTE NON-LYMPHOBLASTIC LEUKEMIA (ANLL)	
t(9;22)	BCR-ABL	Translocation	t(15;17)	PML-RAR α Translocation
t(1;19)	E2A-PRL	Translocation	t(8,21)	AML1-ETO Translocation
t(12;21)	TEL-AML1	Translocation	inv (16)	CBF β - MYH11 Inversion
t(4;11)	MLL-AF4	Translocation	CRONIC MYELOID LEUKEMIA (KML)	
Multidrug resistance 1 (MDR1)			t(9;22)	BCR-ABL Translocation

Table 1. The chromosomal aberrations that can be detected by RNA quantification.

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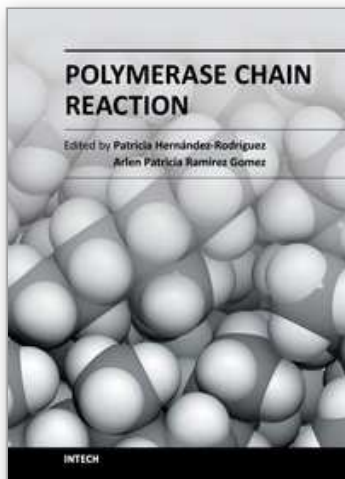
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