

INTRODUCTION TO SNP GENOTYPING BY REAL-TIME PCR

OLIVIA MIHAELA POPA, MIHAI BOJINCĂ, VIOLETA BOJINCĂ,
CLAUDIA CIOFU, CONSTANTIN BĂRĂ, LUIS OVIDIU POPA

Abstract. In this work we present an introduction in the SNP genotyping technique through real-time PCR. SNP genotyping is largely used in medicine, but it is becoming increasingly important in various fields of today biology, as molecular ecology, evolution, biological conservation. The real-time PCR is a high throughput genotyping technique, and efficient protocols were recently developed, both from the technical and financial point of view.

Résumé. On présente une introduction dans la technique de genotypage des polymorphismes ponctuels (SNP) avec la technologie de PCR en temps réel. Cette dernière technologie est largement utilisée en médecine, mais il devient de plus en plus important dans les études d'écologie moléculaire, d'évolution et de conservation biologique. La technique Real-Time PCR permet une analyse rapide (en temps réel) et simultanée des polymorphismes SNP, tandis que les progrès enregistrés ces derniers temps permettent le développement de protocoles efficaces tant du point de vue technique que financier.

Key words: SNP, Real-Time PCR, Genotyping.

INTRODUCTION

The term genotyping refers to a variety of applications used to analyze differences in genomic DNA between individuals, applications associated with human, animals, plants, microbes or viral samples. There are a lot of techniques and platforms suitable for genotyping, but they are all based on the principles of a few basic technologies:

- PCR-based methods and primer extension technologies;
- Hybridization technologies including arrays;
- Fragment length polymorphism analysis;
- Sequencing.

No matter what method is used and for what type of organism, the challenges associated with genotyping applications are similar and include important factors such as sample quality and amount, sensitivity, reliability, time to result and overall cost.

A single nucleotide polymorphism (SNP) is a DNA polymorphisms at the level of a single nucleotide. This sort of DNA variation occurs with a general frequency of about 1 every 1000bp in the human genome (Sachidanandam et al., 2001). The SNPs are not evenly distributed across the human genome, being more frequent in non-coding regions than in coding regions (Li & Sadler, 1991). The SNPs in the non-coding regions can be used as molecular markers in evolutionary studies. The SNPs in the coding regions of the genome can affect gene functions, protein structure or expression and for these reasons they are used as markers in genetic disease studies (Kim & Mishra, 2007). Because of this use of SNPs as molecular markers efforts have been made to develop high-throughput genotyping technologies.

REAL-TIME PCR BASICS

In molecular biology, real-time polymerase chain reaction, also called quantitative real time polymerase chain reaction (Q-PCR/qPCR) or kinetic polymerase chain reaction is a technique of high sensitivity, enabling amplification and quantification of a specific nucleic acid sequence with detection of the PCR product in real time (Higuchi et al., 1992). The procedure follows the general principle of polymerase chain reaction; its key feature is that the amplified DNA is quantified as it accumulates in the reaction in *real time*, after each amplification cycle (Higuchi et al., 1993). Although real-time quantitative polymerase chain reaction is sometimes incorrectly abbreviated as RT-PCR, it should not be confused with reverse transcription polymerase chain reaction, also known as RT-PCR.

Real-time PCR is highly suited for a wide range of applications, such as gene expression analysis, SNP genotyping and allelic discrimination, determination of viral load, detection of genetically modified organisms (GMOs) (Logan et al., 2009). Two common methods of quantification are: the use of fluorescent dyes that intercalate with double-stranded DNA, and the use of modified DNA oligonucleotide probes that fluoresce when hybridized with a complementary DNA. When intercalating dyes are used to measure real-time PCR products, the major disadvantage is that they can detect accumulation of both specific and nonspecific PCR products. The same disadvantage occurs when using non-specific hybridization probes to detect the signal during the real-time PCR reaction.

*METHODS OF SIGNAL GENERATION WITH SEQUENCE-SPECIFIC OLIGONUCLEOTIDES**TaqMan probes*

TaqMan probes or Hydrolysis probes are oligonucleotides constructed with a fluorescent reporter dye (Tab. 1) attached at the 5' end of the probe, and a quencher at the 3' end. When the probe is intact, the quencher reduces the fluorescence emitted by the reporter dye. When the specific target sequence is present, the probe anneals between the primer sites and, during the extension phase of PCR, the probe is cleaved (hydrolyzed) by the 5' → 3' exonuclease activity of Taq DNA polymerase (Bustin, 2000) (Fig. 1).

Table 1

Dyes frequently used in real-time PCR with TaqMan probes.

Dye	Excitation (nm)	Emission (nm)
FAM™	494	518
TET™	521	538
VIC®	538	552
NED™	546	575
ROX™	587	607
JOE™	520	548
HEX	535	553

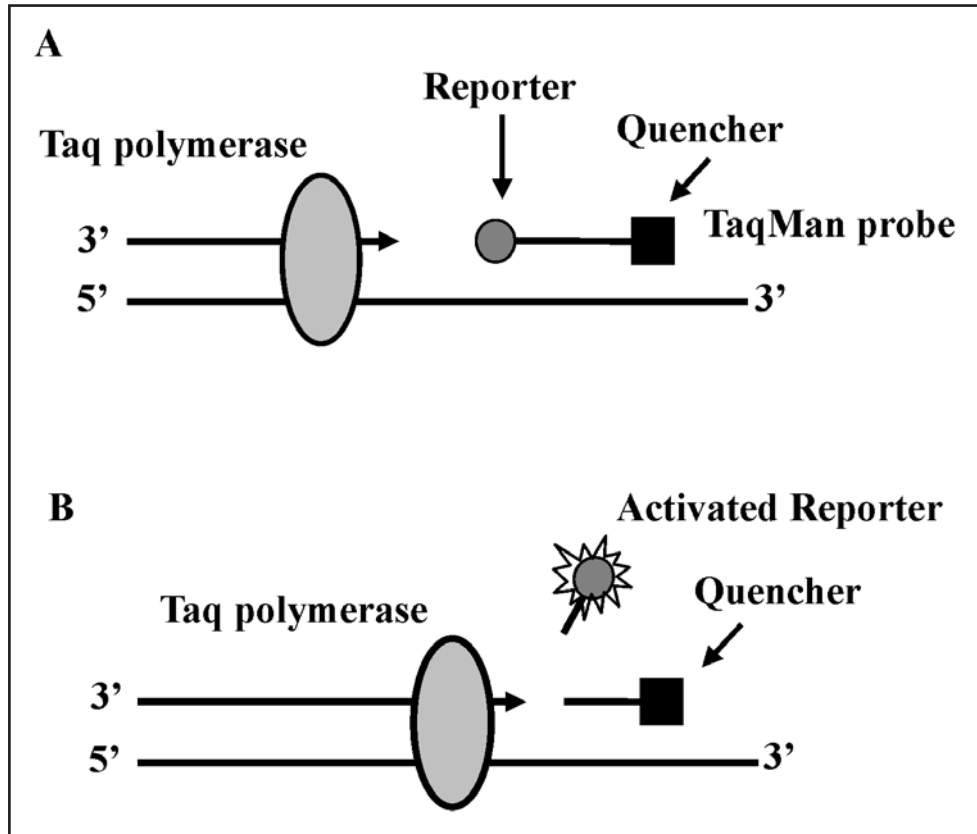


Fig. 1 - The principle of TaqMan assay. A. Before cleavage by Taq polymerase, the TaqMan probe is intact and the quencher prevents the fluorescent reporter from emitting a signal. B. After the TaqMan probe is cleaved, the reporter molecule emits the detectable fluorescent signal.

The cleavage of the probe has two consequences: first, it removes the probe from the target DNA strand in order that the primer extension can continue; second, it separates the reporter dye from the quencher, allowing the increase of the fluorescence signal which can be detected. This process is repeated each cycle, resulting in an increase in fluorescence intensity proportional to the amount of the PCR product.

In order to perform SNP genotyping, two specific probes labeled with different dyes are used, the first for the wild type allele and the second for the mutant allele. If the assay results in the generation of only the first fluorescent color, then the individual is homozygous wild type at that locus. If the assay results in the generation of only the second fluorescent color, then the individual is homozygous mutant. And finally, if both fluorescent colors are produced, then the individual is heterozygous.

At the end of the reaction, hydrolysis probes are digested. The quality of a hydrolysis probe is given by the hybridization efficiency, the quenching of the intact probe and the cleavage activity of Taq polymerase (Kutyavin et al., 2000).

Molecular beacon probes

Molecular beacons are single-stranded oligonucleotides that contain a fluorescent reporter (FAM, TAMRA, TET, ROX) and a quenching dye (typically DABCYL or BHQ) at either end, and which are designed to form a stem-and-loop structure. The loop is the probe sequence that can hybridize with the target DNA. The stem structure is formed by the annealing of the complementary arms that are located on either side of the probe according to Marras et al. (2002) and Drake & Tan (2004).

In solution, the close proximity of the quencher and the dye does not allow fluorescence (Fig. 2 A), but when the probe hybridizes with the target sequence, a more stable double-stranded structure is formed. The molecular beacon structure undergoes a conformational change that restores fluorescence (Fig. 2 B). Molecular beacons are added to the assay mixture before the PCR amplification, they remain intact during PCR and must rebind to target every cycle for fluorescence emission. This will correlate to the amount of PCR product available. It is possible to detect simultaneously different targets in the same reaction by labeling molecular beacons with different dyes (Marras et al., op. cit.; Dvorak, 2006).

Molecular beacons are very specific. They can discriminate target sequences that differ from one another by a single nucleotide substitution, because they can exist in two different physical states. This property makes molecular beacons ideal probes for SNP detection and other applications, by using two different molecular beacons, one complementary to one allele, and the other complementary to the second allele. The scoring of the allele follows the same principle as in the case of TaqMan probes.

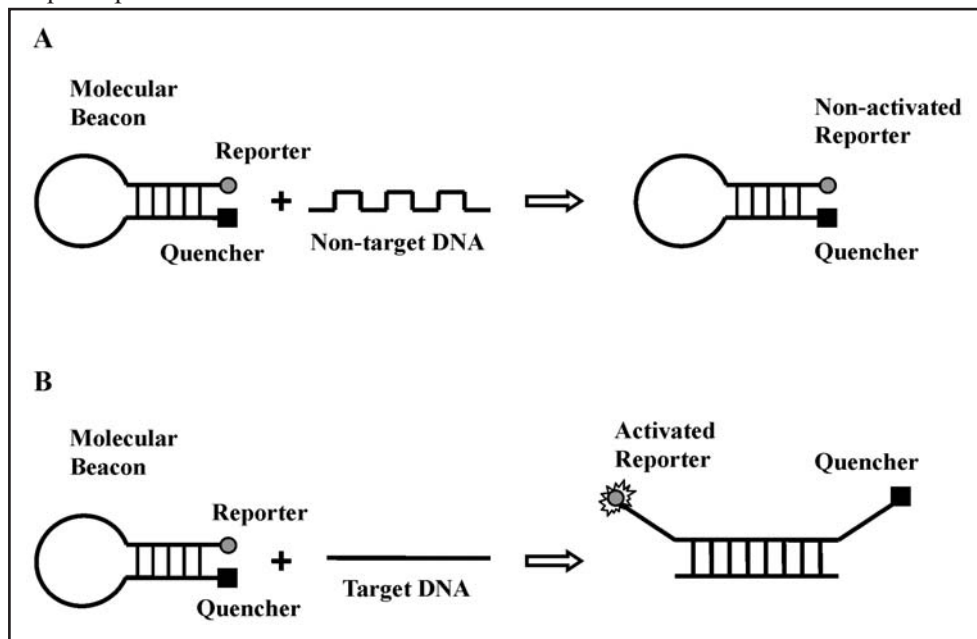


Fig. 2 - The molecular beacon probe principle. A. In the absence of target DNA, the probe retains his secondary structure and the reporter is inhibited by the quencher. B. In the presence of target DNA, the probe hybridizes to the target DNA and the reporter is activated, by no longer being in the proximity of the quencher.

Scorpion primers/probes

By using Scorpion primers/probes, sequence-specific priming and PCR product detection is obtained using a single oligonucleotide. The Scorpion primer is a specific probe sequence that is held in a hairpin loop configuration by complementary stem sequences at the 5' and 3' ends of the probe (Fig. 3 A). A fluorophore is attached to the 5'-end and is quenched by a moiety (normally methyl red) joined to the 3'-end of the loop. The hairpin loop is linked to the 5'-end of a primer via a PCR stopper (non-amplifiable monomer). After extension of the primer during PCR amplification, the specific probe sequence is able to bind to its complement within the same strand of DNA and, as a consequence, the hairpin structure is opened so that the fluorescence is no longer quenched and an increase in signal is observed (Fig. 3 B). The read-through that could lead to the detection of non-specific PCR products is prevented by the PCR stopper. Simultaneous detection of both normal and mutant alleles in a single reaction is possible by combining two Scorpions in a multiplex reaction (Thelwell et al., 2000).

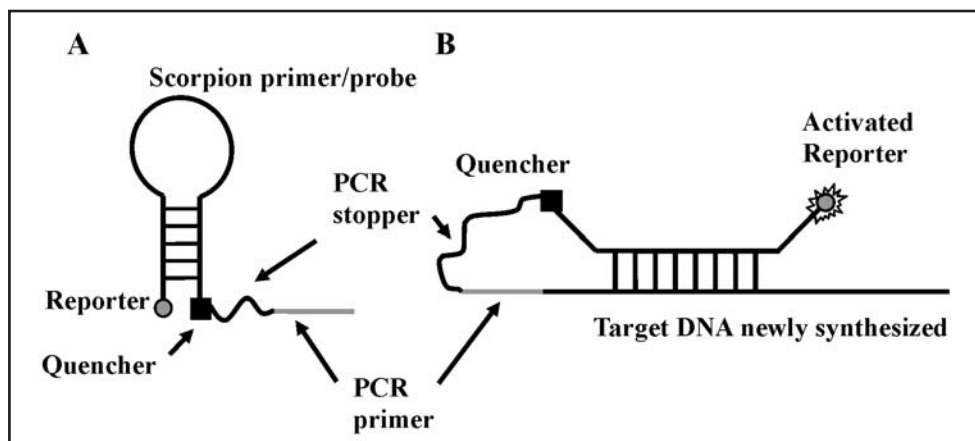


Fig. 3 – A, The scorpion primer/probe structure; B, The scorpion primer/probe attached to its target DNA sequence which was generated from the PCR reaction with the primer incorporated in the scorpion probe. The reporter becomes activated because it is no longer in the close proximity of the quencher.

SNP DETECTION ON APPLIED BIOSYSTEMS PRISM SEQUENCE DETECTION SYSTEM

Applied Biosystems Company offers the largest collection of ready-to-use human and mouse single nucleotide polymorphisms (SNP) assays available with TaqMan MGB probes. Applied Biosystems Company offers two types of TaqMan probes: TaqMan probes with TAMRA™ dye as quencher and FAM™, TET™ or VIC® as 5' label dye; TaqMan MGB (minor groove-binding) probes with non-fluorescent quencher (NFQ) and FAM™, TET™, NED™ or VIC® as 5' label dye, recommended when the probes exceed 30 nucleotides. The non-fluorescent quencher allows the instrument to measure the reporter dye contributions more precisely. The MGB probes have greater differences in melting temperature values between matched and mismatched probes, which allow more accurate allelic discrimination (Applied Biosystems 2006 b).

Human TaqMan® SNP Genotyping Assays collection consists of ~ 30,000 assays for SNPs in gene-coding regions, ~ 160,000 gene centric assays and also >3 million genome-wide assays that include known disease mutations and SNPs in protein domains associated with drug binding regions. Mouse TaqMan® Pre-designed SNP Genotyping Assays provide more than 10,000 SNP loci across 48 inbred mouse strains (Applied Biosystems, 2006 a). For any other organisms, SNP Genotyping Assays can be ordered on the base of the sequence surrounding the SNP of interest.

These products are pre-designed and optimized assays that used genomic DNA as template. The reaction needs only three components: genomic DNA template, SNP Genotyping Assay and PCR Master Mix (TaqMan® Universal PCR Master Mix) which have to be mixed according to the producer specifications. The standard protocol of PCR amplification is valid for each assay: 1 cycle for 10 min at 95°C to activate the AmpliTaq Gold enzyme and 40 cycles of 15 sec at 92°C, 1 min at 60°C (Applied Biosystems, 2006 b). After a pre-read procedure (which is necessary to eliminate the background of the reaction components) and the amplification step, the endpoint read allows obtaining the results (Fig. 4).

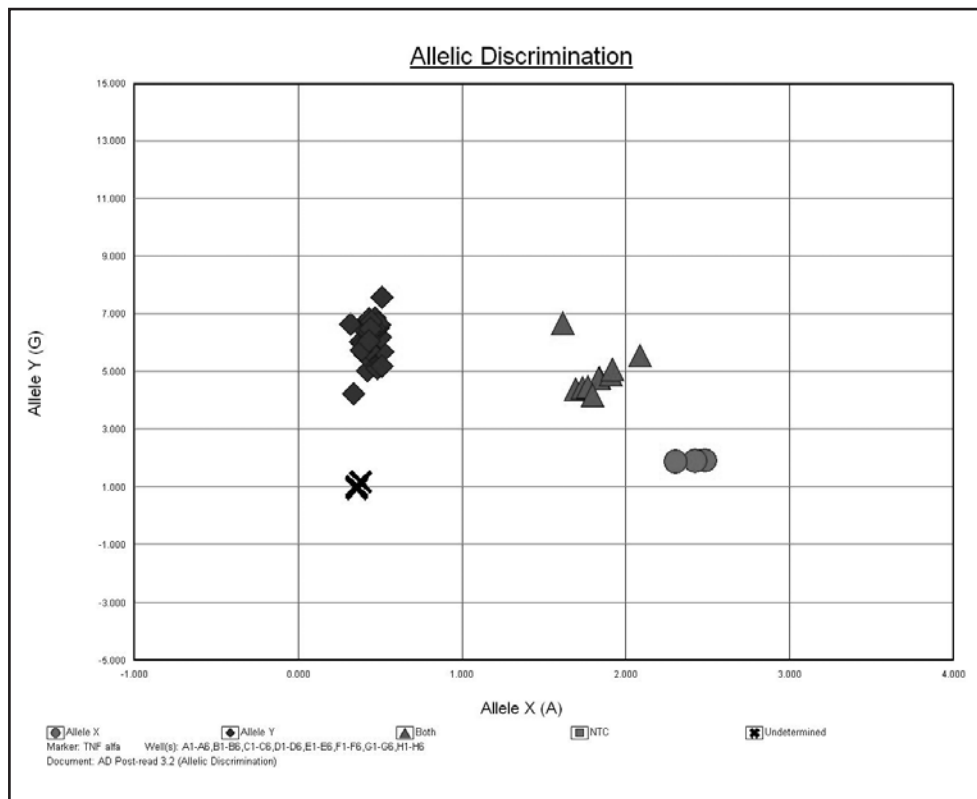


Fig. 4 - SNP Genotyping. Real-time PCR with genomic DNA template was carried out on the Applied Biosystems 7300 Real Time PCR System using TaqMan® SNP Genotyping Assay C_7514879_10 for human TNF α gene (tumor necrosis factor). The allelic discrimination plots clearly indicate the homozygotes for each allele (circles for Allele X or squares for Allele Y) and the heterozygotes (triangles for Both) (O. M. Popa, personal results, University of Medicine and Pharmacy „Carol Davila”, Bucharest, Romania).

ACKNOWLEDGEMENTS

This work was supported by the CNCSIS IDEI grant ID_695 no. 311/2007 allotted to Mihai Bojincă.

INTRODUCERE ÎN TEHNICA GENOTIPĂRII POLIMORFISMELOR SNP
PRIN TEHNICA REAL-TIME PCR

REZUMAT

Lucrarea prezintă o introducere în principiile și metodologia genotipării prin utilizarea tehnicii Real-Time PCR, cu accent pe folosirea platformei Applied Biosystems Sequence Detection System. Genotiparea polimorfismelor punctuale (SNP, Single Nucleotide Polymorphism) este larg folosită în medicină, dar devine din ce în ce mai importantă în studii de ecologie moleculară, evoluție, conservare biologică. Tehnica Real-Time PCR permite analiza rapidă (în timp real) și simultană a polimorfismelor SNP, iar progresele înregistrate în ultimul timp permit dezvoltarea de protocoale eficiente atât din punct de vedere tehnic, cât și financiar.

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Received: October 7, 2008
Accepted: October 21, 2008

Olivia Popa, Constantin Bără
University of Medicine and Pharmacy "Carol Davila" Bucharest,
Department of Immunology and Physiopathology,
Str. Dionisie Lupu no. 37, 020021, București, Romania
e-mail: oliviapopa@yahoo.com
caravi.bara@gmail.com

Mihai Bojincă, Violeta Bojincă, Claudia Ciofu
University of Medicine and Pharmacy "Carol Davila" Bucharest,
Department of Rheumatology and Internal Medicine,
Str. Dionisie Lupu no. 37, 020021, București, Romania
e-mail: vmbojinca@yahoo.com
claudia.ciofu@gmail.com

Luis Ovidiu Popa
National Museum of Natural History "Grigore Antipa",
Șos. Kiseleff no. 1, 011341 București 2, Romania
e-mail: popaluis@antipa.ro