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Restriction Fragment Length Polymorphism Analysis of PCR-Amplified Fragments (PCR-RFLP) and Gel Electrophoresis – Valuable Tool for Genotyping and Genetic Fingerprinting

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1. Introduction

PCR-restriction fragment length polymorphism (RFLP)-based analysis, also known as cleaved amplified polymorphic sequence (CAPS), is a popular technique for genetic analysis. It has been applied for the detection of intraspecies as well as interspecies variation. There exist several techniques that are related with PCR-RFLP and also involve gel electrophoresis including techniques for DNA fingerprinting and expression profiling. This chapter describes PCR-RFLP and related techniques.

1.1 Genetic variation

There are different types of genetic variations. The so-called small-scale genetic variation includes single nucleotide polymorphisms (SNPs), multi-nucleotide polymorphisms (MNPs) and microindels. MNPs are multiple, consecutive nucleotide variations of a single common length such as double nucleotide polymorphisms (DNPs) and triple nucleotide polymorphisms (TNPs) with two and three variable nucleotides, respectively (Figure 1). Microindels are deletions, duplications and combinations thereof involving the gain or loss of 1 to 50 nucleotides (Gonzalez et al., 2007).

The human genome contains more than 3 million SNPs located with an average distance of approximately 1000 bp (International HapMap Consortium 2005, Levy et al., 2007). The frequency of DNPs and TNPs, the most common forms of MNPs, amounts to ~1% of the total number of SNPs (Rosenfeld et al., 2010).

Most likely, the genome-wide occurrence of small insertions and deletions has been underestimated, probably reflecting an inaccuracy of the current sequencing techniques (Krawitz et al., 2010). One study identified 400,000 indels of 1-16 bp in length from a

personal genome (Bentley et al., 2008). Another study detected approximately 150,000 indels with a size of 1 – 3 bp (Wang et al., 2008).

ACTGCGTG**C**TGAGGTA
ACTGCGTG**A**TGAGGTA   *A SNP*

ACTGCGTG**CT**GAGGTA
ACTGCGTG**AG**GAGGTA   *A DNP*

ACTGCGT**GCT**GAGGTA
ACTGCGT**CAG**GAGGTA   *A TNP*

Fig. 1. *Small-scale genetic variation.* Small-scale genetic variation includes SNPs, DNPs and TNPs. Adapted with permission from Rosenfeld et al., (2010).

SNPs, microindels and other types of small-scale genetic variations are implicated in monogenic as well as complex diseases (Bessenyei et al., 2004; Ball et al., 2005). They are also involved in individual drug responses. For example, SNPs and microindels in genes encoding drug metabolising enzymes such as CYP2D6 may lead to the loss of enzyme activity and slow metabolism of a variety of important drugs (Ingelman-Sundberg & Sim, 2010; Eichelbaum et al., 2006). Consequently, large efforts have been invested to develop techniques for genotyping of small-scale variations, in particular SNPs, resulting in the emergence of an abundance of techniques for this purpose including PCR-RFLP.

2. PCR-restriction fragment length polymorphism (RFLP) analysis

2.1 Overview

PCR-restriction fragment length polymorphism (RFLP)-based analysis is a popular technique for genotyping. A search in PUBMED as of October 30, 2011, using the search term "RFLP and PCR" produced 15,725 hits. The technique exploits that SNPs, MNPs and microindels often are associated with the creation or abolishment of a restriction enzyme recognition site (Narayanan, 1991). The first step in a PCR-RFLP analysis is amplification of a fragment containing the variation. This is followed by treatment of the amplified fragment with an appropriate restriction enzyme. Since the presence or absence of the restriction enzyme recognition site results in the formation of restriction fragments of different sizes, allele identification can be done by electrophoretic resolvement of the fragments (Figure 2).

Important advantages of the PCR-RFLP technique include inexpensiveness and lack of requirement for advanced instruments. In addition, the design of PCR-RFLP analyses generally is easy and can be accomplished using public available programs (see below). Disadvantages include the requirement for specific endonucleases and difficulties in identifying the exact variation in the event that several SNPs affect the same restriction enzyme recognition site. Moreover, since PCR-RFLP consists of several steps including an electrophoretic separation step, it is relatively time-consuming. Finally, the technique is not

suitable for the simultaneous analysis of a large number of different SNPs due to the requirement for a specific primer pair and restriction enzyme for each SNP. This limits its usability for high throughput analysis. Important advantages and disadvantages of PCR-RFLP are listed in Table 1.
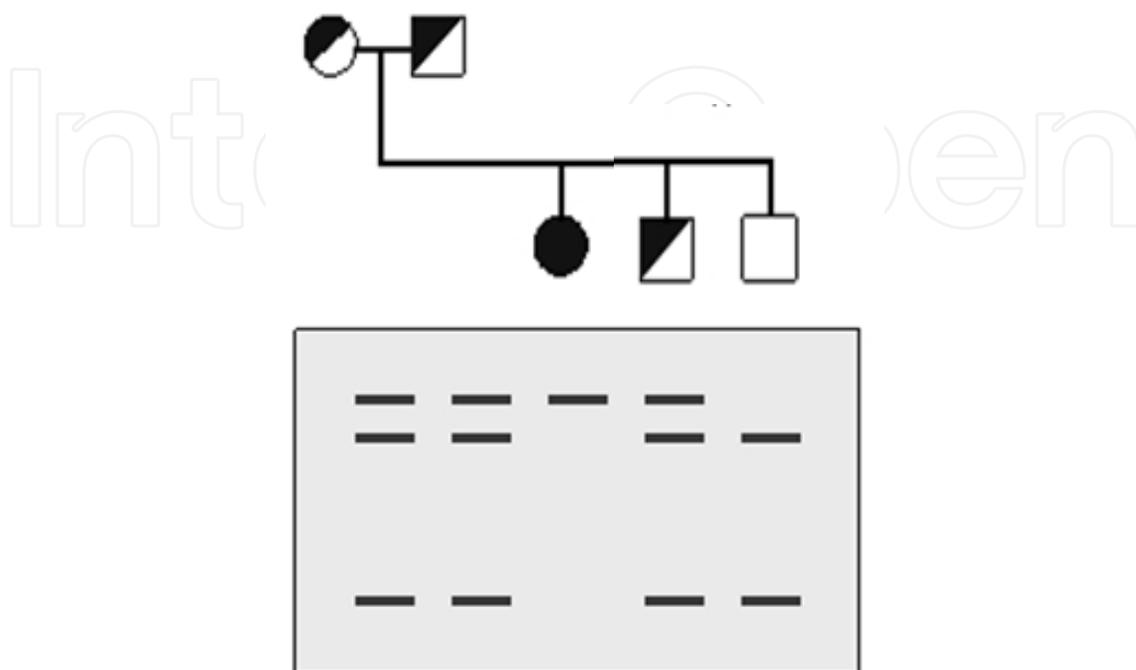

Fig. 2. *Genotyping a biallelic RFLP marker in a family.* One of the RFLP alleles lacks the restriction enzyme recognition site and manifests a single fragment (black in the family tree). The other allele harbours the restriction enzyme recognition site and displays two restriction fragments (white in the family tree). RFLP markers are inherited in a Mendelian fashion.

Advantages	Inexpensive Easy to design Applicable to analysis of single nucleotide polymorphisms as well as microindels No requirement for expensive instruments No requirement for extensive training of laboratory staff Miniaturisable
Disadvantages	Requires that a variation generates or abolishes a restriction enzyme recognition site Some restriction enzymes are expensive Exact genotyping cannot be achieved in the event that there is more than one nucleotide variation in a restriction enzyme recognition site Requires relatively large amounts of hand-on-time Long time from start to completion of the analysis Not suitable for high-throughput analysis

Table 1. Advantages and disadvantages of PCR-RFLP.

PCR-RFLP is an extremely valuable technique for genotyping of species-specific variations. It is the most commonly used reference standard for genotyping of Factor V Leiden and prothrombin G20210A (Emadi et al., 2009). It has also been used for a variety of other purposes including detection of the JK allele associated with a Kidd-null phenotype (Horn et al., 2011), and determination of apolipoprotein E (APOE) alleles (Jiang et al., 2011). A PCR-RFLP analysis of the gene encoding deoxycytidine kinase is shown (Figure 3).
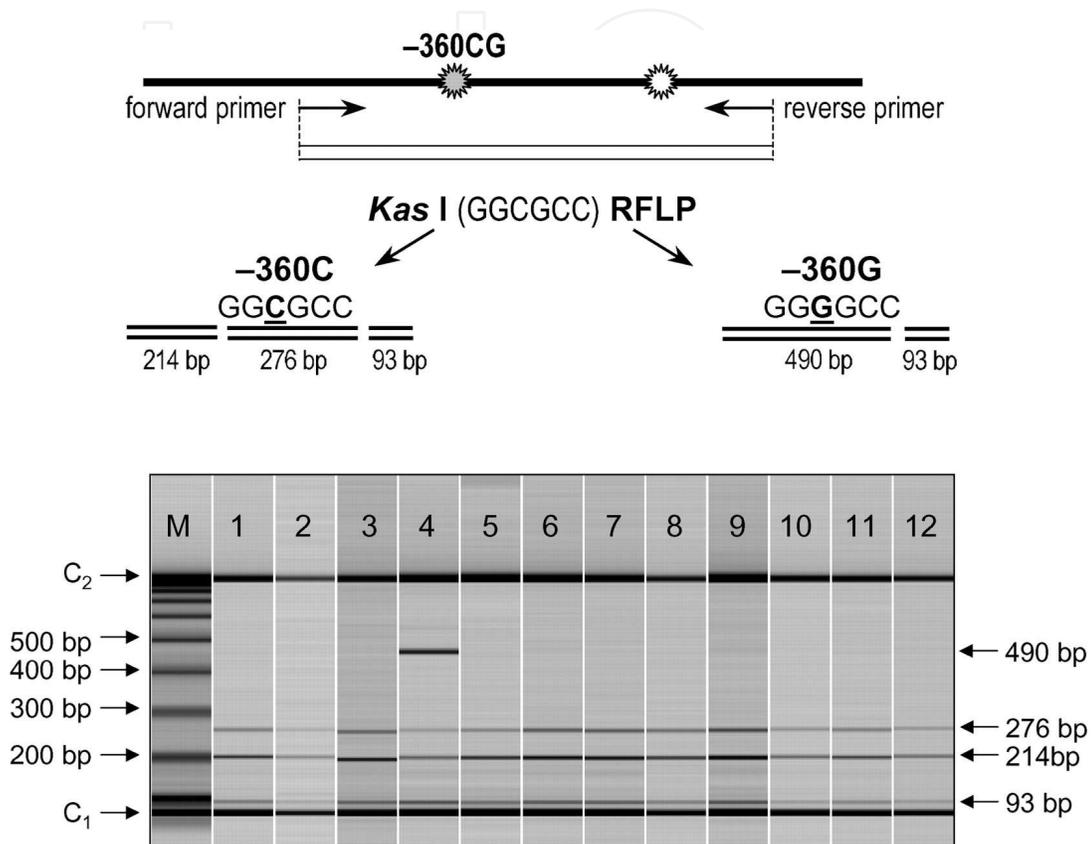

Fig. 3. *PCR-RFLP for genotyping -360C/G in the gene encoding deoxycytidine kinase.* In the upper part of the figure, the amplified fragment and the restriction fragments for the two alleles produced by treatment with *Kas*I are shown. Note that the amplified fragment contains a non-polymorphic *Kas*I recognition site. Fragment sizes are 214, 276 and 93 bp for the *Kas*I-positive allele and 490 and 93 bp for the other allele. In the lower part of the figure, the restriction fragments are shown in gel view format. There is one heterozygote (lane 4); all others are homozygotes. C1 and C2 are internal calibration markers. Adapted with permission from Szantai et al., (2006).

Besides being valuable for the determination of intraspecies variation, the PCR-RFLP technique is very popular for species identification and differentiation. Until recently, it was the preferred technique for identification and differentiation of mycobacterial species (Sankar et al., 2011). Moreover it has been used for differentiation of game bird species by amplification of a conserved region of the mitochodrial D-loop (Rojas et al., 2009). Using primers targeting a conserved region in the 12S rRNA gene, followed by restriction enzyme treatment and electrophoretic separation, closely related poultry species could be differentiated (Saini et al., 2007). A similar approach has been used to differentiate game animals such as roe deer, red deer and mouflon from domesticated ruminants (Fajardo et al.,

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2006; Fajardo et al., 2009). Use of a single restriction enzyme may be sufficient for species differentiation by PCR-RFLP (Fajardo et al., 2009). However, often the ability to differentiate between closely related species requires application of more than one restriction enzyme (Rojas et al., 2009).

PCR-RFLP consists of several separate steps including design of primers, identification of an appropriate restriction enzyme, amplification, restriction enzyme treatment of amplified products and electrophoresis to resolve the restriction fragments. Below a description of PCR-RFLP is provided. In this description attention will be focused upon the design of primers, identification of appropriate restriction enzymes and electrophoretic techniques.

2.2 Design of primers and identification RFLP enzymes

Previously, the design of primers and identification of a restriction enzyme allowing allele discrimination were carried out in two separate steps. That is, the primers were designed by one program such as Primer3 (Rozen & Skaletsky, 2000), followed by *in-silico* analysis of the segments defined by the designed primers to identify an appropriate restriction enzyme. Identification of a restriction enzyme allowing allele discrimination by *in-silico* analysis can be done using the program designated NEBcutter V2, which has an option for viewing fragments of an *in-silico* digest (Vincze et al., 2003).

There are now several programs for the design of PCR-RFLP in which the selection of primers and restriction enzymes has been integrated (Table 2). The design of PCR-RFLP using such programs may save significant amounts of time. Furthermore, these programs are very efficient in the identification of appropriate restriction enzymes and most of them permit the design primers for both natural PCR-RFLP as well as mismatch PCR-RFLP, also known as primer-introduced restriction analysis (PIRA) or forced PCR restriction fragment length polymorphism (F-PCR-RFLP).

Name	Functions	Reference and URL[1]
PIRA-PCR	Design of primers for mismatch PCR-RFLP	Ke et al., 2001 http://cedar.genetics.soton.ac.uk/public_html/primer2.html
SNPicker	Design of primers for natural and mismatch PCR-RFLP	Niu & Hu, 2004
SNP Cutter	Design of primers for natural and mismatch PCR-RFLP	Zhang et al., 2005 http://bioapp.psych.uic.edu/SNP_cutter.htm
SNP-RFLPing	Design of primers for natural and mismatch PCR-RFLP. Accepts bi-allelic, tri-allelic, and tetra-allelic SNPS, in addition to sequences with multiple SNPs.	Chang et al., 2010
Prim-SNPing	Design of primers for natural PCR-RFLP, mismatch PCR-RFLP, and other purposes including regular PCR	Chang et al., 2009

[1] The programs are freely available on the internet.

Table 2. Programs for design of PCR-RFLP.

Mismatch PCR-RFLP is based on the introduction of one or more mismatches in the 3' end of a primer to create an artificial enzyme recognition site. Programs for the design of this type of PCR-RFLP are valuable since not all small-scale variations are associated with the creation or abolishment of a restriction enzyme recognition site. One program "PIRA PCR designer" (Ke et al. 2001) has been specifically developed to select primers and restriction enzymes for design of mismatch PCR-RFLP.

Several of the programs for design of PCR-RFLP possess important additional functions. This includes Prim-SNPing, which has an in-built facility for improvement of PCR efficiency by evaluation of the potential for the formation of secondary structures of the primers such as hairpins (Chang et al., 2009). The program designated SNP-RFLPing 2 has other important options, e.g. on-line retrieval of SNPs from a variety of different species and acceptance of tri- and tetra-allelic SNPs and indels (Chang et al., 2010).

The design of specific primers can be difficult in the event that there are sequences closely related with the target in the material subjected to amplification. For example, the design of primers for amplification of a gene belonging to a multi-gene family can be challenging. Also the design of primers for amplification of genes with closely related pseudogenes may impose difficulties. Except for Prim-SNPing, which assesses PCR specificity by a BLAST search of the primer sequences, none of the commonly used programs for design of PCR-RFLP has an in-built option for test of primer specificity. Consequently, subsequent *in-silico* examination of the specificity of the primers may be required. This can be done the using the option in the UCSC browser for *in-silico* PCR (http://genome.ucsc.edu/), PrimerBlast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) or a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). In the event of mispriming due to pseudogenes or other sequences, the program called RExPrimer can be useful (Piriyapongsa et al., 2009). This program is specifically designed for amplification of challenging regions but is not capable of identifying RFLP enzymes. Guidelines for design of PCR-RFLP are presented (Table 3).

Advice	Comments
High specificity of amplification primers, absence of genetic variation in the regions targeted by the primers and high yield of amplified product	Specificity of amplification primers can be assessed by *in-silico* PCR or a BLAST search. The program Prim-SNPing has an in-built option for assessment of primer specificity and primer secondary structure formation
Presence of a non-polymorphic recognition site of the RFLP enzyme in the amplicon serving as an internal digestion control	If such non-polymorphic site is not present, design of mismatched amplification primers is a possibility

Table 3. Guidelines for the design of PCR-RFLP.

2.3 Digestion of amplicons

2.3.1 Internal digestion controls

The reliability of a PCR-RFLP analysis depends upon the complete cleavage of the fragments containing the recognition sequence of the restriction enzyme used. In order to

assess whether the digestion mixture has been prepared correctly and the reaction conditions are suitable, the use of an internal digestion control is recommended. In the event that the amplified fragment contains a non-polymorphic recognition site of the restriction enzyme used for the genotyping of an RFLP marker, this site may serve as an internal digestion control (Griffioen et al., 2005; Szantai et al., 2006). If such non-polymorphic site is not present in the amplified fragment, the possibility of artificially creating a recognition site for the used RFLP enzyme by the design of a mismatched primer should be considered. For example, this approach was used in a PCR-RFLP analysis for identification of K-*ras* gene mutations (Mora et al., 1998). Alternatively, the digestion reaction can be spiked with a DNA fragment that contains the relevant restriction enzyme recognition site and produces fragments of other sizes than those of the amplicon (Lima-Neto et al., 2009). Plasmides have been used as such internal digestion control and are suitable for this as they contain recognition sites for many of the commonly used restriction enzymes. For PCR-RFLP carried out with a fluorescent-labelled primer, a fragment labelled with another fluorescent dye and containing the relevant restriction enzyme recognition site, can be used as internal digestion control fragment (Nielsen et al., 2007).

2.3.2 Multiple SNPs in the amplified fragment

Occasionally, an amplified fragment contains several RFLP markers. In that event simultaneous digestion with more than one restriction enzyme in the same reaction mixture is possible (Szantai et al., 2006). Restriction enzymes with significant differences in temperature and buffer preference require digestion in separate reactions.

2.4 Electrophoretic separation and visualisation of fragments

After completion of digestion of amplicons with the selected restriction enzyme(s), the resultant fragments are resolved by electrophoresis. Frequently, this is done using slab gel electrophoresis with polyacrylamide or agarose as molecular sieving matrix. Recently, capillary electrophoresis and microchannel electrophoresis have become increasingly popular. They offer higher resolving power and throughput than conventional slab gels (Stellwagen et al., 2009; Sinville & Soper, 2007).

Visualisation of the restriction enzyme-treated amplicons can be done using fluorescent-labelled amplification primers. However, most frequently PCR-RFLP analyses are conducted with unlabelled primers. In that event, visualisation of restriction fragments is accomplished by complexation of DNA fragments with ethidium bromide or another fluorescent dye during the electrophoresis ("*in migratio*"). For PCR-RFLP analysis with covalently labelling of primers, the restriction-enzyme treated fragments are usually heated and analysed by denaturing electrophoresis in a single-stranded state to determine fragment sizes adopting the procedure for genotyping of microsatellites. The advantage of this is that the size determination primarily depends upon fragment lengths. In contrast, size determination of DNA fragments using electrophoresis under non-denaturing conditions may be influenced by the configuration of the DNA fragments.

PCR-RFLP analysis with covalently labelling of primers has been used for a variety of purposes. This includes genotyping of protein tyrosine phosphatase non-receptor type 22 using FAM as label (Nielsen et al., 2007).

2.4.1 Conventional slab gel electrophoresis

The electrophoretic analysis of PCR-RFLP fragments, is commonly accomplished using vertical or horizontal slab gels. Horizontal "submarine" gels are prepared from agarose. These gels are submerged in the electrophoresis buffer between the two electrodes of an electrophoresis chamber. Hence, buffer and gel form a bridge between the two electrodes allowing an electric current to pass. Agarose gels do not have the same resolving power as polyacrylamide gels. However, chemically modified agaroses have significant higher resolving power than standard agaroses approaching that of polyacrylamide gels (MacDonell et al., 1987; Highsmith, 2006). Precast agarose and polyacrylamide gels are commercially available.

Advantages of conventional slab gel electrophoresis include the lack of requirement for expensive equipment and low costs of reagents for preparation of the gels. Furthermore, slab gels are easy to prepare. A disadvantage of the conventional slab gel electrophoresis is that it is time-consuming. Moreover, conventional slab gels frequently suffer from lack of resolution. Reduction in the time for electrophoresis may increase the throughput of PCR-RFLP significantly. For this purpose the technique known as microplate array diagonal gel electrophoresis (MADGE) has been developed. MADGE is an electrophoresis system with a large number of wells placed askew in a separating matrix of polyacrylamide or agarose (Gaunt et al., 2003). The number of wells can be as large as 384 or 768 permitting rapid transfer of samples from microplates.

2.4.2 Ultrathin-layer slab gel electrophoresis

Ultrathin-layer gel electrophoresis refers to electrophoresis in gels with a thickness of 20-200 µm. Major advantages of ultrathin-layer electrophoresis gels over that of conventional slab gels include lower temperature gradient across the gel and more efficient heat dissipation resulting in a reduction in the band distortion (Stegemann et al., 1991). Automated electrophoretic analysis using an ultrathin-layer slab gel format has been applied for high-throughput genotyping of factor V Leiden mutation (Lengyel et al., 1999).

2.4.3 Capillary gel electrophoresis

Capillary gel electrophoresis is a miniaturised format of the conventional rod (tube) gel electrophoresis. The small inner diameter of the capillaries typically ranges from 2 to 100 µm (Ferrance & Landers, 2001). Such small diameter, permits efficient heat dissipation and application of high voltages (10 - 30 kV) leading to fast separation and high resolution of the analytes. Application of capillary gel electrophoresis in the analysis of DNA polymorphisms has been reviewed elsewhere (Mitchelson, 2003).

Basically, a system for capillary gel electrophoresis consists of two buffer reservoirs, a capillary filled with a separating matrix, a high-voltage power supply, a sample introduction device and an output device (Figure 4). Often capillary gel electrophoresis systems are also equipped with a device for on-line detection of the analytes during their migration in the capillary. Inclusion of several capillaries in the separation unit allows the analysis of multiple samples in parallel, thereby increasing the throughput. Some systems
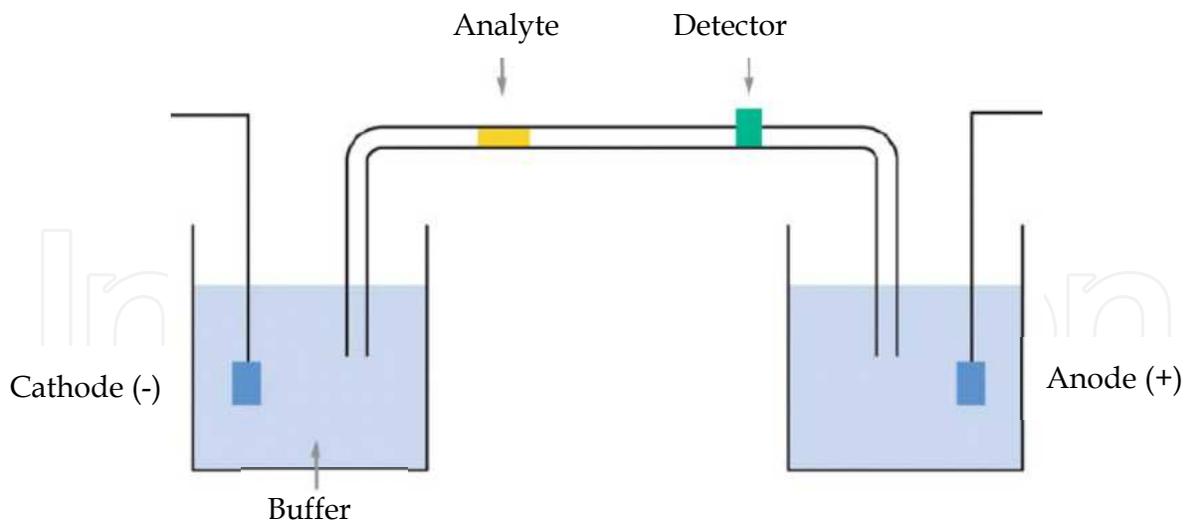
Fig. 4. *Schematic representation of the basic setup of a capillary electrophoresis system.* Note the on-line detection device. Adapted with permission from Ghosal (2006).
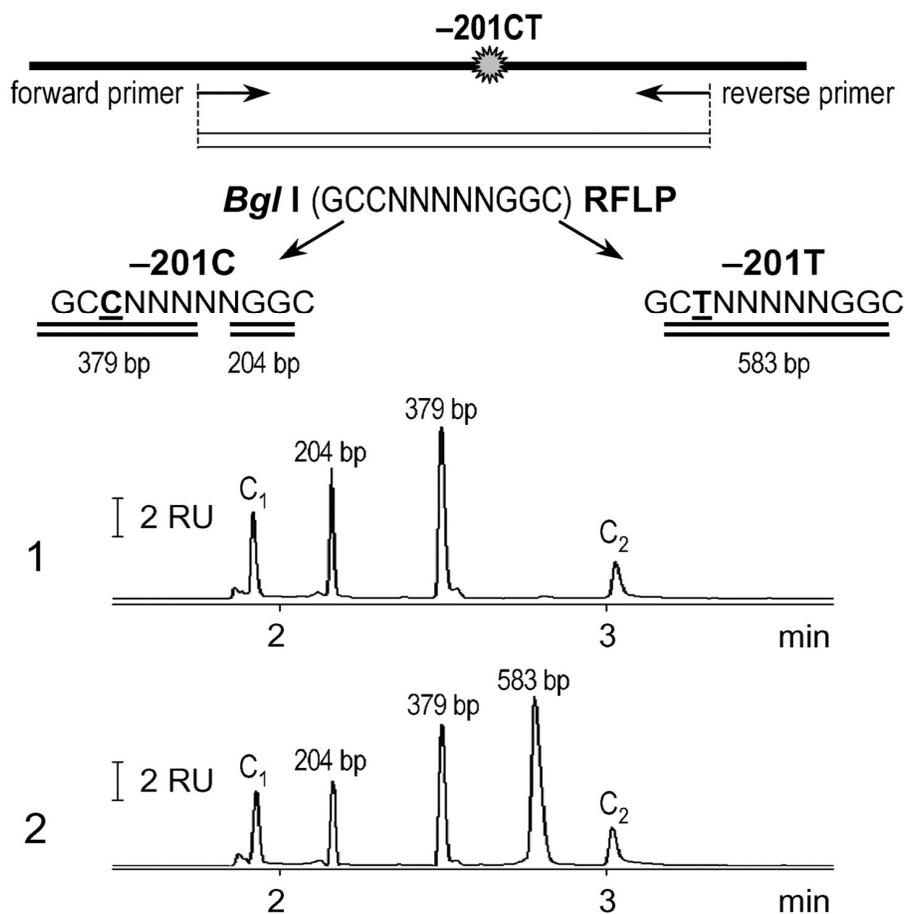

Fig. 5. *PCR-RFLP for genotyping -201C/T in the gene encoding deoxycytidine kinase.* In the upper part of the figure the amplified fragment and the polymorphic restriction site of *BglI* are shown. Below there is an electropherogram with a *BglI*-positive homozyote in panel 1 and a heterozygote in panel 2. C1 and C2 are internal calibration markers; RU denotes relative units of fluorescence. Adapted with permission from Szantai et al., (2006).

permit direct transfer of samples from 96- or 384-well plates into the electrophoretic separation channels. Multi-capillary electrophoresis has commonly been used for fragment separation in PCR-RFLP analysis. Results from a PCR-RFLP analysis using multi-capillary electrophoresis for genotyping of deoxycytidine kinase are shown (Figure 5).

Based upon determination of the peak area, capillary electrophoresis offers possibilities for quantification of analytes. For example, it has been used for analysis of restriction enzyme-treated amplified fragments to quantify quasi-species of mumps virus (Gulija et al., 2011). Consequently, capillary electrophoresis may be used to monitor the progression of a viral infection.

2.4.4 Microchip electrophoresis

Gel electrophoresis can be miniaturised and conducted in microchips. Electrophoresis in microchips and microfluidic systems provides significant lower separation time and higher throughput compared to other types of electrophoretic systems (Sinville & Soper, 2007). Another advantage of the microchip format is that electrophoresis can be integrated with the other steps of a PCR-RFLP procedure.

The so-called conventional microchips are made of glass, quartz or silicon. Microchips can also be produced using a variety of polymers (Sinville & Soper, 2007). Microchips are fabricated using techniques from the semiconductor and plastics industries (Fiorini & Chiu, 2005). There are several commercially available microfluidic systems for electrophoretic separation of DNA fragments. Some of these systems are compatible with 96- and 384-well plates.

The decrease in separation time provided by microfluidic devices can be substantial. For example, separation of nucleic acids and proteins can be completed in less than 120 sec using on-chip electrophoresis (Hawtin et al., 2005). Moreover, an electrophoresis system consisting of 110 μm wide and 630 μm deep microchannels with an effective separation distance of 3 cm was capable of resolving an amplicon digest, composed of fragments with sizes up to 184 bp, in 280 sec (Qin et al., 2005).

A large number of studies have reported application of microchip electrophoresis in PCR-RFLP. For example, this approach is useful in the quality control and regulatory screening in enforcement laboratories including screening to detect undeclared admixture of fish species in seafood products (Dooley et al., 2005). Lab-on-a-chip capillary electrophoresis of digested amplicons can also be applied for authentication of coffee beans (Spaniolas et al., 2006).

Using PCR-RFLP in combination with the commercially available Agilent 2100 microchip electrophoretic separation system, allele frequencies in DNA pools could be reliably estimated by measurement of fluorescence intensities of the enzymatically digested DNA fragments (Wang et al., 2009). Thus, PCR-RFLP can be used to achieve provisional information about the allele frequencies in different groups of individuals and to guide the design of large genetic association studies.

Recently, a high performance system for determination of ABO blood genotypes, combining restriction enzyme treatment and electrophoresis on a chip was reported (Akamine et al., 2009). This system provided good accuracy and high resolution for fragments within a size

range of 25 to 300 bp. Since digestion was accomplished in 20 mins and electrophoretic separation was done in 7 mins, the two steps together required less than ½ hour. Another study reported robotic spotting of PCR amplicons and restriction enzymes from 96-microwell plates onto the tabs of a membrane loader, where digestion took place, followed by the transfer of the digested products to a 96-lane microchip gel electrophoresis unit with a separation matrix containing ethidium bromide (Guttman et al., 2002). Using this analytical system the time required for the digestion and electrophoretic separation generally was below 20 minutes.

The integration of all processes of a PCR-RFLP analysis including amplification, restriction enzyme treatment and electrophoresis into a lab-on-a-chip may increase throughput and decrease the amount of hands-on time significantly. A portable device containing all these functions has been developed and applied for the genotyping of thiopurine S-methyltransferase to detect defective alleles associated with adverse reactions during treatment with thiopurine (Chowdhury et al., 2007). This device addresses the need for rapid, inexpensive and accurate pharmacogenetic testing of the single patient required for individualisation of the drug treatment.

3. Techniques related with PCR-RFLP

Several variants of PCR-RFLP have emerged. This includes a technique in which the electrophoretic separation has been replaced by a gel-free method in addition to techniques for DNA fingerprinting and species determination (Table 4).

Technique	Characteristics and usage	Reference
PCR combined with restriction fragment melting temperature (PCR-RFMT) analysis also known as melting curve analysis of SNPs (McSNP)	Genotyping of SNPs and indels exploiting differences in the heat dissociation profiles of restriction enzyme-treated fragments	Akey et al., 2001 Ye et al., 2002 Jahangir Tafrechi et al., 2007
Amplified fragment length polymorphism (AFLP)	Amplification of subsets of fragments of genomic DNA using a limited set of generic primers. Used for profiling of microbial communities	Vos et al., 1995 Meudt & Clarke, 2007
Terminal restriction fragment length polymorphism (T-RFLP)	Amplification using end-labelled primers followed by restriction enzyme treatment of amplified fragments and electrophoretic separation. Used for profiling of microbial communities and differentiation of animal species	Dunbar et al., 2001 Wang et al., 2010
Inverse PCR-based amplified restriction fragment length polymorphism (iFLP)	PCR-based identification of low-level somatic mutations associated with creation of a restriction enzyme recognition site	Liu et al., 2004

Table 4. Techniques related with PCR-RFLP.

3.1 Restriction fragment melting temperature analysis of amplified products (PCR-RFMT)

Restriction fragment melting temperature analysis of amplified products (PCR-RFMT), also known as melt curve analysis of SNPs (McSNP), is a gel-free and inexpensive approach for genotyping of SNPs and other types of small-scale genetic variation (Jahangir Tafrechi et al., 2007; Akey et al., 2001; Ye et al. 2002). It exploits differences in the dissociation temperature of the digested amplicons for fragment identification and genotyping. In melt curve analysis the restriction enzyme-treated fragments are gradually heated in the presence of SYBR Green I, a DNA fluorescent dye with specificity for doubled-stranded DNA and negligible binding to single-stranded DNA. When this dye is bound to double-stranded DNA, it emits fluorescence. As the temperature is increased, the two complementary strands in the double-stranded DNA fragments dissociate resulting in an abrupt loss in fluorescence. Since fragments of different size and nucleotide composition dissociate at different temperatures, melt curve profiling can be used for identification of fragments.

PCR-RFTM has been used to assess the load of the pathogenic A3243G mitochodrial mutation (Jahangir Tafrechi et al., 2007) and for genotyping of SNPs present in genomic DNA (Ye et al., 2002). Compared to PCR-RFLP with conventional slab gel electrophoresis, PCR-RFMT is less labour intensive. Moreover, the risk of contamination with PCR products in PCR-RFMT is low since the only post-PCR processing step in this procedure that requires opening of the reaction tubes, is addition of restriction enzyme to the amplified products. However, the ability of melt curve analysis to resolve fragments of nearly identical sizes is lower than that of a gel electrophoresis. This limits its usability.

3.2 Amplified fragment length polymorphism PCR (AFLP-PCR)

Amplified fragment length polymorphism PCR (AFLP, or AFLP-PCR) is a DNA fingerprinting technique based upon amplification of subsets of fragments of genomic DNA using a limited set of generic primers (Vos et al., 1995). The initial step in AFLP is digestion of genomic DNA. This is done using two restriction enzymes, a rare cutter and a frequent cutter, generating fragments with sticky ends. After ligation of adaptors to these ends, subsets of the digested fragments are selectively amplified. The amplification primers are complementary to the adaptors and the restriction site sequences but have an extension of one to four nucleotides in their 3′ ends. The selectivity in the amplification leading to a reduction in the complexity of the amplicons is conferred by these extensions and reflects that primer-template mismatches in the 3′ end of a primer prevent or significantly reduce elongation by commonly used DNA polymerases.

Previously, the amplification step in AFLP was done using radioactively labelled primers followed by slab gel electrophoresis of the amplified material. Now, fluorescence-labelled primers and capillary gel electrophoresis is preferred (Meudt & Clarke, 2007).

The complexity of the amplified fragments in AFLP can be controlled by the choice of restriction enzyme. Moreover, the number of different fragments can be restricted by changing the number of selective nucleotides in the 3′ end of the primers. Alternatively, variants of AFLP, which have been specifically designed to produce lower number of different amplicons, can be applied. This includes the variant termed three endonuclease-AFLP (TE-AFLP), which uses three restriction enzymes for digestion of genomic DNA

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rather than two (van der Wurff et al. 2000). Since only two adaptors are used in TE-AFLP, selective ligation is achieved resulting in reduction in the number of potentially amplifiable fragments. Incorporation of selective nucleotides in the 3' end of the amplification primers is not necessary with TE-AFLP. A more recent variant of AFLP, amplified ligation selected fragment length polymorphism (ALIS-FLP) for DNA fingerprinting of microbial organisms, distinguishes itself by using only one restriction enzyme, *Tsp*RI (Brillowska-Dabrowska et al., 2008). Moreover, ALIS-FLP does not require labelled primers and electrophoresis can be accomplished using conventional agarose gels.

AFLP has been used for a variety of purposes including genomic profiling of bacteria (Macdonald et al. 2011), phylogenetic analysis (Arrigo et al., 2011), quantitative trait locus mapping (Heidari et al., 2011), and population genetics (Chybicki et al., 2011). A major disadvantage of AFLP is that it is relatively laborious. Moreover, successful outcome of this technique is highly dependent upon complete digestion of the samples.

cDNA-AFLP is a variant of AFLP used to study gene expression and quantify differences in gene expression levels (Bachem et al., 1996). Applications of cDNA-AFLP include identification of genes associated with sexual maturation (Kang et al., 2011) and identification of genes differentially expressed as a result of the development of antifungal resistance in *Candida albicans* (Levterova et al., 2010). Recently, cDNA-AFLP was combined with mRNA differential display to produce an improved variant of this technique, robust ordered mRNA differential display (RoDD), for global gene expression profiling (Liu et al., 2011).

3.3 Terminal restriction fragment length polymorphism (T-RFLP)

Terminal restriction fragment length polymorphism (T-RFLP) is a molecular genetic profiling technique for assessment of species diversity in a sample (Liu et al. 1997). It uses a pair of amplification primers of which one or both are 5' end-labelled with a fluorescent dye. Broad specificity of the primer pair permits amplification of a representation of the different templates in a pool. The principle of this technique is similar to that of PCR-RFLP with fluorescent-labelling of primers, but extended to analysis of multiple species by the use of primers with a high degree of species cross-reactivity.

The terminal restriction fragments containing the fluorescent dye label are subjected to denaturing capillary gel electrophoresis. Since fragments amplified from different species typically differ in nucleotide sequence, they produce fragments of different sizes on digestion with a restriction enzyme. Often several restriction enzymes are used to increase the discriminative ability. The most frequent use of T-RFLP is for profiling of microbial communities. This includes study of the effect of dietary changes on the microbial diversity in the rumen of cattle (Lillis et al., 2011) and characterisation of the stomach microbiobial flora in gastric cancer (Dicksved et al., 2009). In addition T-RFLP has been used to profile the fungus flora of beetle galleries (Endoh et al., 2011) and for characterisation of nematode communities (Donn et al., 2008). Furthermore, it is a valuable tool for authentication of meat products (Wang et al., 2010).

Overall, T-RFLP is a relatively simple and inexpensive procedure for molecular genetic profiling of complex samples containing multiple different DNAs. Recently, a variant of T-RFLP using two-dimensional electrophoresis for separation of fragments emerged (Wang et al., 2011). This novel procedure provides higher resolution than conventional T-RFLP.

3.4 Inverse PCR-based amplified restriction fragment length polymorphism (iFLP)

Inverse PCR-based amplified restriction fragment length polymorphism (iFLP) has been designed to detect low-level somatic mutations in cancers (Liu et al., 2004; Wang et al., 2005). The technique combines PCR-RFLP with denaturing high performance liquid chromatography exploiting that a given segment of DNA contains a variety of sequence patterns that can be converted into a recognition site of a restriction enzyme by somatic mutation. For example, a DNA fragment of 500 bp without recognition sites for the four-base cutter *Taq*I contains 80-100 sites, which can potentially be converted into the recognition site of this enzyme by substitution, deletion or insertion of single nucleotides.

The first step in iFLP is digestion of genomic DNA with *Mse*I or a corresponding enzyme and circularisation of digested fragments by ligation. After digestion of the circularised fragments with another restriction enzyme such as *Taq*I, and ligation of adaptors to the fragments, they are amplified using primers targeting the adaptors. Subsequently, gene-specific primers targeting regions that normally do not harbour recognition sites for the second restriction enzyme are used for amplification of the library. Using high-precision liquid chromatography low frequency somatic mutations associated with genomic instability at the single nucleotide level can be discovered and their occurrence quantified by comparison with mutation-negative samples spiked with known amounts of mutated fragments.

Application of more than one restriction enzyme for digestion of the circularised fragments increases the possibility of detecting low-level somatic mutations due to substitutions, deletions and insertions of single nucleotides. By using methylation-sensitive restriction enzymes it is likely that iFLP can be modified to allow the discrimination of methylated from non-methylated DNA fragments.

iFLP has been used for identification of low-level mutation signatures in cancer cell lines and surgical samples of colon and breast cancers. The ability to detect somatic mutations in cancers is important as this may help individualise cancer therapies.

4. Concluding remarks

PCR-RFLP is an easy-to-design analysis. Hence PCR-RFLP analysis can be implemented in the laboratory within a short time. Furthermore, this technique is inexpensive without a requirement for advanced equipment. A disadvantage of the PCR-RFLP technique is that it requires relative large amounts of hands-on-time. Although this technique has been miniaturised and throughput increased by integration of the steps of amplification, restriction enzyme treatment and electrophoresis into a microchip, other genotyping procedures are more amenable for automation and high-throughput analysis. Overall, PCR-RFLP is best suited for investigation of low numbers of samples. Platforms in which several steps of PCR-RFLP have been integrated may be suited for the analysis of large number of samples for a single marker or a limited number of markers.

Variants of PCR-RFLP such as T-RFLP are useful for DNA profiling purposes, including DNA fingerprinting of microbial communities and authentication of foods. These variants are significantly less expensive than next-generation sequencing procedures and are able to provide results significantly faster than most of the currently used next-generation sequencing platforms.

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In conclusion, PCR-RFLP and variants thereof are valuable analytical procedures. In particular, the variants used for DNA profiling and DNA fingerprinting purposes may provide detailed information that cannot be obtained at similar costs using other techniques.

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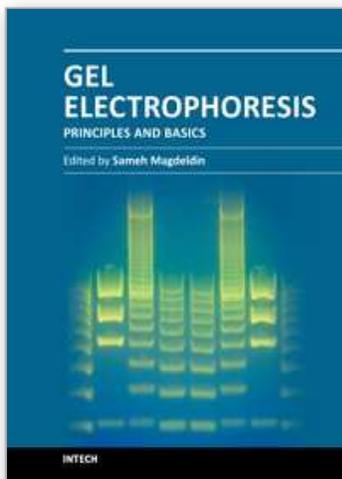
Gel Electrophoresis - Principles and Basics

Edited by Dr. Sameh Magdeldin

Most will agree that gel electrophoresis is one of the basic pillars of molecular biology. This coined terminology covers a myriad of gel-based separation approaches that rely mainly on fractionating biomolecules under electrophoretic current based mainly on the molecular weight. In this book, the authors try to present simplified fundamentals of gel-based separation together with exemplarily applications of this versatile technique. We try to keep the contents of the book crisp and comprehensive, and hope that it will receive overwhelming interest and deliver benefits and valuable information to the readers.

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