Genomics: more than genes

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Abstract. The fundamentals of Real-time Polymerase Chain Reaction, Automated capillary electrophoresis –Sanger sequencing and Fragment analysis– and "Next-generation" sequencing are reviewed. An overview of applications is presented using our own examples carried out in our facility.

1. Introduction

Genomics is the study of the nucleotide sequences – genes, regulatory sequences and non-coding regions – and their functions. The analysis of the pattern of expression and the regulation of the gene expression are also aims of this field. DNA sequencing was the origin and nowadays is the basis of genomics. Sanger sequencing technology has had an impressive influence in biology, and a revolution is occurring with the "Next-generation" Sequencing (NGS). However, other high-impact technologies in biology are closely related to Genomics as Real-time Polymerase Chain Reaction (Real-time PCR) (see Fig. 1). The three technologies presented in this chapter: Real-Time PCR, Automated Capillary Electrophoresis and NGS share some applications such as genotyping, species determination, and others. The best approach for a project is determined by many factors such as the number of samples, prior knowledge of the region or species, complexity of the samples and so forth. It should be highlighted that applications from these technologies reach other research areas since they provide useful genetic tools.

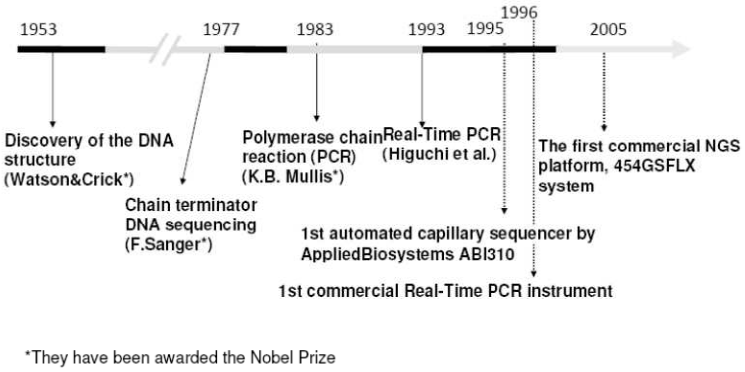

Figure 1: Chronology of technological advances in Genomics.

2. Real-Time PCR (qPCR)

2.1. Instruments of qPCR

The Genomic unit of the CCiTUB has five instruments of Real-Time PCR (qPCR), two SDS7700, two 7900HT and one LightCycler 480 II.

The LightCycler® 480 Real-Time PCR System (see Fig. 2) has an interchangeable 96 well and 384 well plate. The ABI PRISM 7700 has a unique sample block of 96 wells and the 7900HT instrument has an interchangeable 96 well, 384 well and TLDAs. The LightCycler® 480 and 7900HT, can analyze up to 384 samples simultaneously in 2 hours.

Figure 2: LigthCycler 480 II, Sequence Detector System 7900HT. ABI PRISM 7700

2.2. Methodology of qPCR

PCR was developed in 1983 by Kary Mullis[1]. It is a technique capable of generating large amounts of fragments of DNA from single or a few copies of this DNA. PCR, in brief, involves two oligonucleotide primers complementary to the ends of the sequence to be amplified and a heat-stable, Taq polymerase. The PCR consists in three repeated cycles: heat denaturation of DNA, annealing of primers to their complementary sequences of DNA template and a final step of

elongation of annealed primers with enzyme Taq Polymerase. The result is an exponential accumulation of the specific target of DNA, aproximately 2^n, where "n" is the number of cycles of amplification performed.

The quantitation and detection of this target of DNA is performed at endpoint analysis after the reaction has finished with an agarose gel electrophoresis. But this does not allow reliable quantification of the initial sample, probably because at the end point all samples have reached the same amount (plateau phase).

2.2.1. Real Time-PCR (qPCR)

This problem was solved in 1992 when Higuchi et al [2] described the Real-Time PCR (qPCR) with ethidium bromide. This system was an adapted thermal cycler used to irradiate the samples with ultraviolet light, and the detection of the fluorescence was performed with a computer-controlled cooled CCD camera. Amplification produces increasing amounts double-stranded DNA (dsDNA), which binds ethidium bromide, resulting in an increase of fluorescence detected by a CCD camera.

At present, we use other fluorescent molecules such as, TaqMan Probes, SyBr Green, LNA (Locked nucleic Acid), Molecular beacon Probes, Scorpions Primers, QuantiProbes, etc... Taqman Probes and SyBr green are the most widely used.

TaqMan® probe (see Fig. 3) is an oligonucleotide that is complementary to one of the strands of the amplicon and has a fluorescent reporter at the 5' end (FAM, VIC or NED..) and a quencher (TAMRA or Black Hole) at the 3' end. When Taq polymerase extends from the primer, it displaces the 5' end of the probe and it is degraded by 5'-3' exonuclease activitity of Taq polymerase. The reporter is separated from the quencher, and generates a fluorescent signal that increases with each cycle and is proportional to the amount of amplified product.

SyBr Green (see Fig. 4) is a dsDNA intercalating dye, that fluoresces once bound nonspecifically to the dsDNA. The fluorescence of SYBR Green I increases up to 1,000-fold when it binds dsDNA and this is proportional to the amount of dsDNA present.

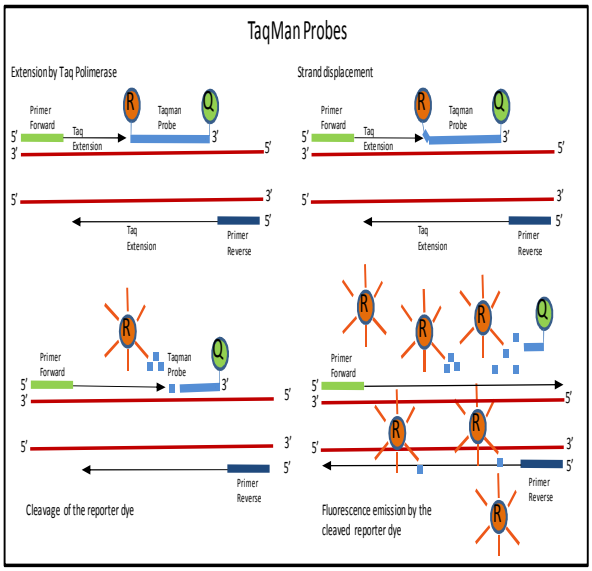

Figure 3: Taqman Probe Workflow

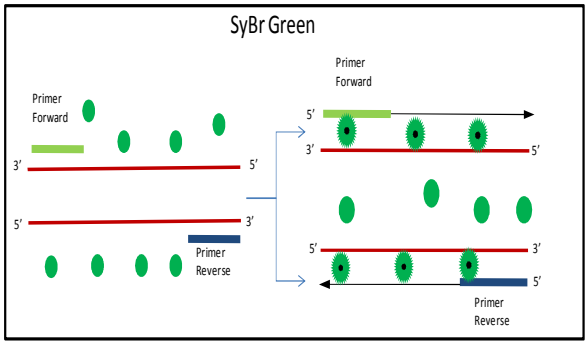

Figure 4: SyBr green workflow

The qPCR process can be divided in two phases; an exponential and a plateau phase (see Fig 5). At the beginning of PCR the fluorescence remains at background levels, there is amplification of DNA but the level of fluorescence is not detectable (cycles 1-15). In the exponential phase, there is enough amplified product to yield a detectable fluorescent signal. Finally, the PCR reaches the plateau phase where no fluorescence increases due to exhaustion of any reagents.

The threshold is the level of signal that reflects a statistically significant increase of fluorescence over the calculated baseline signal. The threshold cycle (Ct in Applied or Cp in Roche) is the intersection between an amplification curve and a threshold in the exponential phase. It is a relative measure of the concentration of target in the PCR reaction. The Ct value is inversely related to the amount of starting template. Therefore, small amounts of template have a high Ct and high amounts of template have a low Ct.

The most common method for relative quantitation is the Livak method [3] or $2^{-\Delta\Delta Ct}$ method, where:

$$\Delta C_{t(test)} = Ct(target) - Ct(endogenous) \qquad \Delta C_{t(control)} = Ct(target) - Ct(endogenous) \qquad \Delta\Delta C_t = \Delta C_{t(test)} - \Delta C_{t(control)}$$

This method relies on two assumptions. The first is that the reaction occurs with 100% efficiency (E = 10 [–1/slope]) and the second assumption is that there is an endogenous gene (or genes) that is expressed at a constant level between the samples.

When the efficiency is not 100% or there are differences between the genes, we use the Pfaffl method [4]:

$$Ratio = \frac{(E_{target\ gene})^{\Delta C_t \text{ target gene } (C_t \text{ Control- } C_t \text{ test})}}{(E_{endogenous\ gene})^{\Delta C_t \text{ endogenous gene}(C_t \text{ Control- } C_t \text{ test})}}$$

where E_{target} and $E_{endogenous}$ are the amplification efficiency of target gene and endogenous gene respectively. The result is the ratio of the target gene in the test sample to the calibrator sample, normalized to the expression of the reference gene.

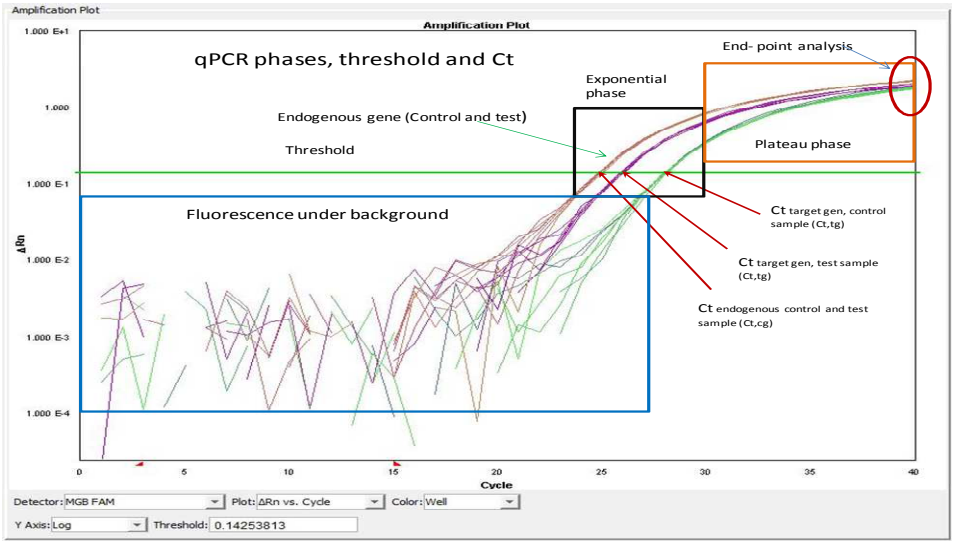

Figure 5: qPCR phases and concepts threshold and Ct

2.3. Applications of qPCR

qPCR systems can support different application or assay types. These assays can be divided into two categories (quantitative real-time PCR assays and endpoint assays) based on the time point during the assay at which data are being collected. Real time classical applications are absolute quantification or relative quantification.

2.3.1. Absolute Quantification

- *Microorganism quantification.* qPCR is considered as a method of choice for the detection and quantification of microorganisms. One of its major advantages is that is faster than conventional culture-based methods. It is also highly sensitive, specific and enables simultaneous detection of different microorganisms. For example, accurate quantification

is of prime importance for most food microbiology applications [5] or in correlating viral copy number with disease state.

- *Detection of genetically-modified organisms (GMO).* Several techniques have been developed for detection and quantification of GMO but qPCR is by far the most popular approach [6].

2.3.2. Relative Quantification

- *Gene expression.* In relative quantitation, changes in gene expression in a given sample relative to another reference sample (such as an untreated control sample) are analyzed. Because of the qPCR sensitivity and broad dynamic range, qPCR is frequently used to validate the results of microarray and next-generation sequencing-based gene expression profiling experiments [7].
- *MicroRNAs (miRNAs)* are a class of naturally-occurring noncoding RNAs that play a role in gene regulation. qPCR and microarray hybridization approaches as well as ultra high throughput sequencing of miRNAs (small RNA-seq) are popular and widely used profiling methods [8].
- *Copy-Number Variations (CNV).* Copy-number changes are known to be involved in numerous human genetic disorders. In this context, qPCR-based copy number screening may serve as the method of choice for targeted screening of the relevant disease genes and their surrounding regulatory landscapes [9].
- *Single cells.* Interest in single cell molecular analysis has risen dramatically over the last couple of years, chiefly because single cell molecular analysis is the only way to research genetic heterogeneity, i.e., differences in copy number or gene expression levels between individual cells, or genetically analyze very rare cells such as circulating tumor or fetal cells. Multiplex single-cell qPCR can be used to examine the expression of multiple genes within individual cells [10].

2.3.3. End-point detection

- *Allelic discrimination*: is a multiplexed end-point assay that detects variants of a single nucleic acid sequence. A common approach is to use hydrolysis (TaqMan) probes. The presence of two primer/probe pairs in each reaction allows genotyping of the two possible variants at the single-nucleic polymorphism (SNP) site in a target template sequence [11,12]. One fluorescent dye detector is a perfect match to the wild type (allele 1) and the other fluorescent dye detector is a perfect match to the mutation (allele 2).
- *High Resolution Melting (HRM)* analysis is a new, post-PCR analysis method used for identifying genetic variation in nucleic acid sequences (mutations, methylations, SNPs) in PCR amplicons. Simple and fast, HRM characterizes nucleic acid samples based on their disassociation (melting) behavior [13].

2.4. Examples of applications

2.4.1. Gene expression: Analysis of ubiquitin C-terminal hydrolase-1 expression levels in dementia with Lewy bodies

In the reported example [14], Real-Time PCR has been applied to test UCHL-1 gene expression in post-mortem frontal cortex of PD and DLB cases, compared with agematched controls. Parkinson disease and dementia with Lewy bodies are characterized by the accumulation of abnormal a-synuclein and ubiquitin in protein aggregates conforming Lewy bodies and Lewy neurites. Ubiquitin C-terminal hydrolase-1 (UCHL-1) disassembles polyubiquitin chains to increase the availability of free monomeric ubiquitin to the ubiquitin proteasome system (UPS) thus favoring protein degradation. TaqMan PCR assays demonstrated down-regulation of UCHL-1 mRNA in the cerebral cortex in DLB (either in pure forms, not associated with Alzheimer disease: AD, and in common forms, with accompanying AD changes), but not in PD, when compared with age-matched controls (Fig. 6).

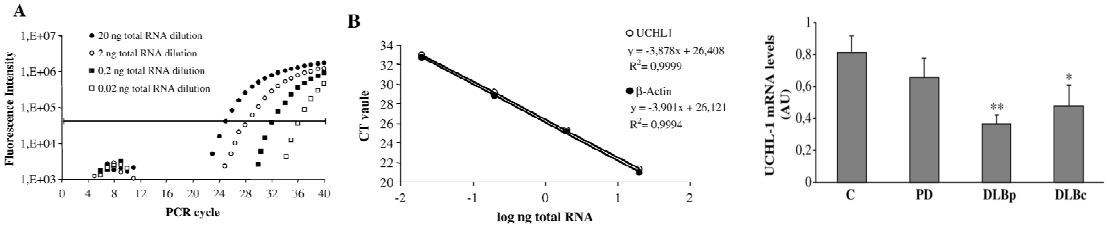

Figure 6: (A) Amplification plot of UCHL-1 using serial dilutions of control human brain RNA.The horizontal line represents the threshold line adjusted manually. (B) Representative standard curves for h-actin and UCHL-1 constructed from several concentrations of control human brain RNA. Ct values (y axis) vs. log of several RNA concentrations of control samples (x axis) show a reverse linear correlation. (C) Relative UCHL-1 gene expression in the frontal cortex of controls (C, n = 6), Parkinson's disease (PD, n = 6), Dementia with Lewy bodies, pure form (DLBp, n = 7) and common form (DLBc, n = 6). (C) UCHL-1 mRNA levels (mean T SEM) normalized with h-actin. *P < 0.05 and **P < 0.01 compared to control samples (ANOVA with post hoc LSD test).

2.4.1. Copy Number Variation and Allelic Discrimination: Lack of association between lipoprotein ((a) genetic markers and coronary heart disease in HIV-1-infected patients

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General population studies have shown associations between Copy Number Variation (CNV) of the LPA Kringle-IV type-2 (KIV-2) coding region, associated SNP and the CHD. The aim of the study was to confirm these associations in our HIV-1 cohort.

72 HIV patients were included. Genomic DNA was isolated from blood frozen at -20ºC. A multiplex qPCR was carried out using TaqMan technology for *LPA* KIV-2 and single-copy reference gene *RNaseP* in order to perform absolute quantifications. CopyCaller software (Applied Biosystems) was used for relative quantifications (See Fig 7). Allelic Discrimination was performed using TaqMan SNP Genotyping Assay (See Fig. 8). Fisher's exact test was used for comparisons.

No statistically significant differences were found between cases and controls in terms of CNV (p=0.66) neither in the SNP genotyping (p=0.58).

The clinical utility of these biomarkers to predict CHD in HIV population remains unclear.

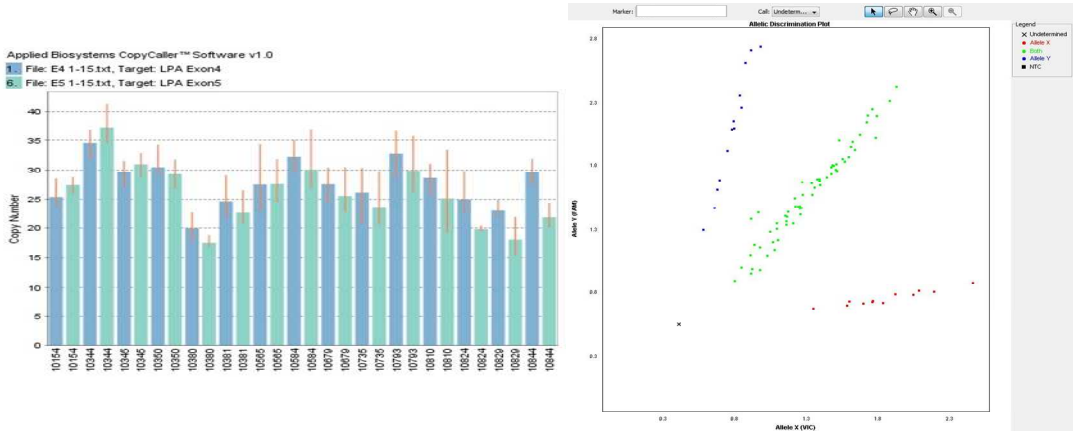

Figure 7: Copy Caller Software (Applied Biosystems) **Figure 8**: Plot of Allelic Discrimination

2.4.3. Quantitative detection of Lactarius deliciosus extraradical soil mycelium by real-time PCR and its application in the study of fungal persistence and interspecific competition

Real-Time PCR has been applied to quantify extraradical soil mycelium of edible ectomycorrhizal fungi [15,16,17,18]. Taqman probes and specific primers have been designed for the amplification of the ITSrDNA region of *Lactarius deliciosus*, *Boletus edulis* and *Tuber melanosporum*. The quantification of fungal mycelium allowed for undertaking studies on fungal persistence, seasonal

abundance of extraradical mycelium and interspecific competition. Basic protocols involved genomic DNA extraction to perform real-time PCR analysis. DNA extractions from soil mixed with known amounts of mycelium of the target fungus were used as standards. Significant correlations between mycorrhizas and ectomycorrhizal fungal mycelium were found whereas no relationship was observed between soil mycelium and fruit body production. Factors as soil type, soil depth and season influenced the amount of fungal mycelium recovered from soil.

Quantitative PCR is a powerful technique for extraradical mycelium quantification in studies aimed at evaluating the persistence of a target fungi in field plantations established with inoculated plants. Also, the positive relationship found between the vegetative phases of the symbiosis (mycorrhizas and extraradical mycelium) allowed to establish a non-destructive method for controlling fungal persistence in the field.

2.4.4. Identification of Variable Number Tandem Repeats (VNTRs) by High Resolution Melting (HRM)

The aim of this study is describe a screening method for the detection of both heterozygous and homozygous VNTR.

All samples were extracted with the same method from buccal swaps and were diluted to the same DNA concentrations. We test the optimum $MgCl_2$ with a dilution series, and the optimum is the lowest concentration resulting in high yield of target PCR products and no unspecific products. The HRM protocol was performed and monitored in a Light Cycler 480 II.

We show that it is possible to use HRM to distinguish heterozygous and homozygous VNTRs (See Fig. 9).

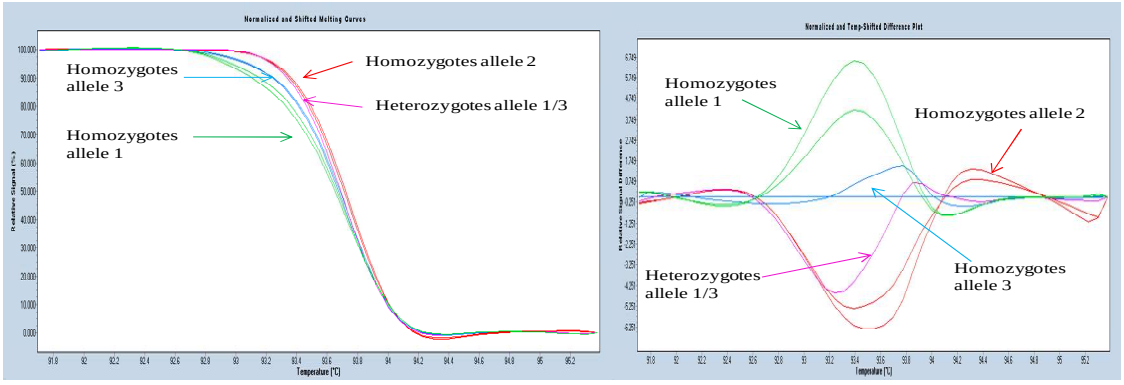

Figure 9: Plots of Normalized and shifted melting curve, and Normalized and Temp-Shifted Difference plot

3. Automated capillary electrophoresis (ACE)

Capillary electrophoresis and automated detection of fluorescently tagged DNA fragments (ACE) is the common instrumentation for DNA sequencing by Sanger chain terminator dideoxy method and Fragment analysis. Electrophoresis is a largely used technique in molecular biology. The application of an electric field produces the movement of the negatively charged DNA molecules through a polymer from the cathode to the anode and they are separated according to size. The smaller fragments run faster than the larger fragments. The DNA fragments have been previously labeled with fluorescent dyes so they are detected by the excitation of a laser beam at the end of the capillary (see Fig. 10). The Genomics Unit has two sequencers, a 48-capillary 3730 DNA Analyzer and a 96-capillary 3730*xl* DNA Analyzer (Fig. 11). The 3730*xl* can analyze up to 96 samples simultaneously in 2 hours (1152 samples/day, 980 Kbases with high quality 850 QV20 bases).

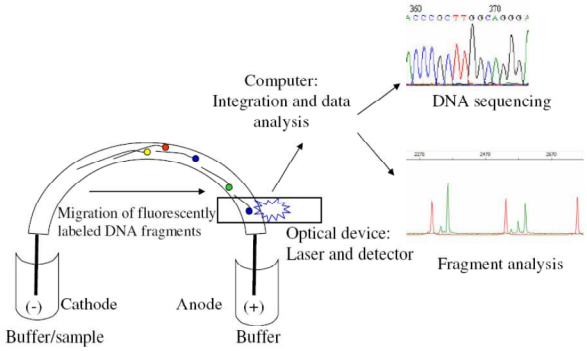

Figure 10: Scheme of the ACE and detection in automatic sequencers

Figure 11: 3730 and 3730*xl* DNA Analyzers at the CCiTUB.

3.1. Methodology and applications

3.1.1. Sanger dideoxy sequencing method

This method, also called the chain terminator method [19], is based on the synthesis of DNA in vitro with an optimized mix of deoxynucleotides (dNTPs) and modified nucleotides, dideoxynucleotides (ddNTPs). The incorporation of ddNTPs prevents the formation of the phosphodiester bridge with the previous dNTP so the DNA synthesis is stopped. Since there are billions of copies of the DNA template, the sequencing reaction results in a collection of DNA molecules of different length that differ in one nucleotide in size and terminate with a ddNTP. The electrophoresis separates the DNA molecules by size. DNA template can be PCR fragments, cDNA, gDNA or clone constructs.

The original method has been improved by the use of a thermally-stable DNA polymerase (PCR-like), the labelling with distinct fluorescent dyes and the ACE (see Fig. 12).

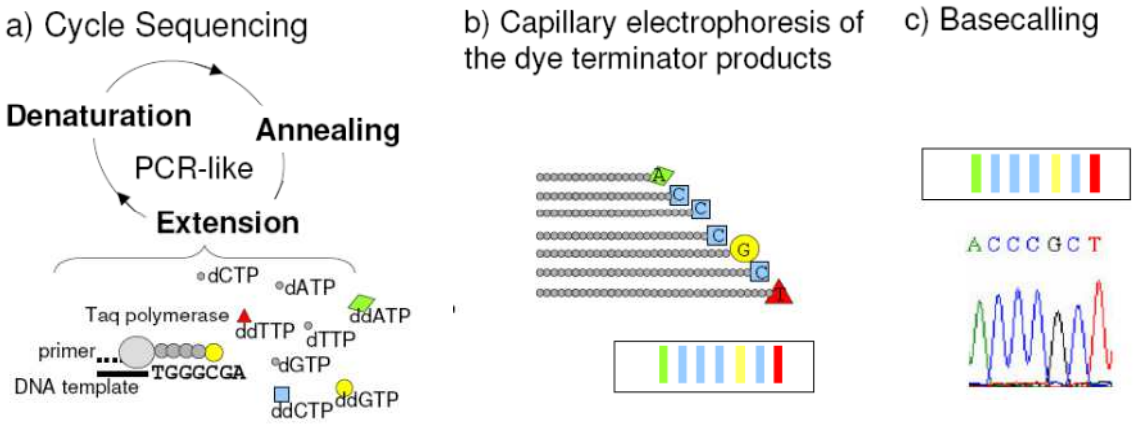

Figure 12: Schematic diagram of dye terminator cycle sequencing

3.1.2. Fragment analysis

Fragment analysis estimates the size for DNA fragments amplified by PCR with fluorescently labeled primers. The sample is pooled with a size standard of DNA fragments with known lengths, electrophoresed and detected together (see Fig.13).

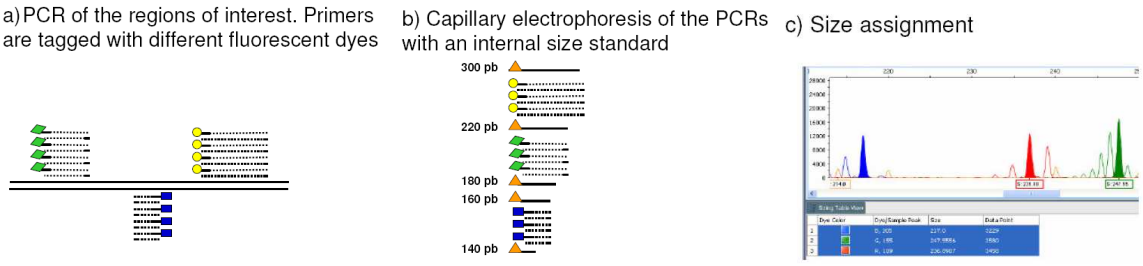

Figure 13: Schematic diagram of Fragment analysis

The Fragment analysis results are band patterns. The analysis of these profiles and their changes are used in a variety of applications as genotyping of individuals or strains, identification of species, linkage mapping studies, disease association studies, paternity testing and others (see Table 1).

Table 1: Main applications of ACE

	DNA sequencing	Fragment Analysis
	De novo sequencing of genomes Checking of clone constructs	
Genotyping and genetic variation	SNPs*, indels** and other structural variants	Microsatellites Analysis, Fragments Length Polimorphisms+
Gene Expression Analysis	mRNA sequencing: active genes, alternative splicing, ESTs§... Epigenetics :Methylation analysis	
Strains and Species identification	Resequencing	Microsatellites Analysis Fragments Length Polimorphisms
Linkage mapping and association studies	Resequencing	Microsatellites Analysis Fragments Length Polimorphisms

*SNPs (Single Nucleotides Polymorphisms) ** Indels (insertions-deletions), +Fragments Length Polimorphisms: AFLPs (Amplified fragment length polymorphisms), RFLPS (Restriction fragment length polymorphisms), MLPAs (Multiplex ligation-dependent probe amplifications), ESTs§ (Expression Sequences Tag).

3.2. Examples of applications

3.2.1. Nucleotide variation at the "Insuline-like" receptor (InR) gene in Drosophila

Population genetics studies the level and patterns of nucleotide variability. The major evolutionary processes are natural selection, genetic drift, mutation and migration. Most of the empirical data in this field comes from DNA sequencing. The *InR* gene is the first component of the highly conserved insulin-signaling pathway. It is known the influence of this pathway in processes as intermediary metabolism, reproduction, aging and growth. The variation of *Inr* might affect many phenotypic traits. The level and pattern of variation for the *InR* region has been analysed in *D. melanogaster*. PCR fragments of ~8-kb long (or alternatively two overlapping PCR fragments of ~5-kb long) that encompassed the *InR* gene were cycling sequenced and analysed by ACE [20] in eight lines of *D. melanogaster* and one line of *D. simulans* (see Fig. 14)

3.2.1. DNase I footprinting assay by ACE (DFACE)

This is an unusual application of ACE that combines Analysis of fragments and DNA Sequencing. DNase I Footprinting assay is used to study DNA protein interactions and identify the specific binding DNA sequence of the proteins. This technique is performed for analysis of the transcription factors in studies of regulation of gene expression. Briefly, a labelled probe of DNA (about 100-600 bp) that contains the region of interest is incubated with an extract of protein under test and followed by controlled digestion with DNase I. The enzyme only cuts the regions free of bounded protein. The digestion product is electrophoresed. The comparison of the DNase I digestion patterns with the protein extract, in the absence of protein and with a sequencing region of the DNA probe allows the identification of protected regions (seen as gaps in the footprint) due the interaction of the protein with the DNA.

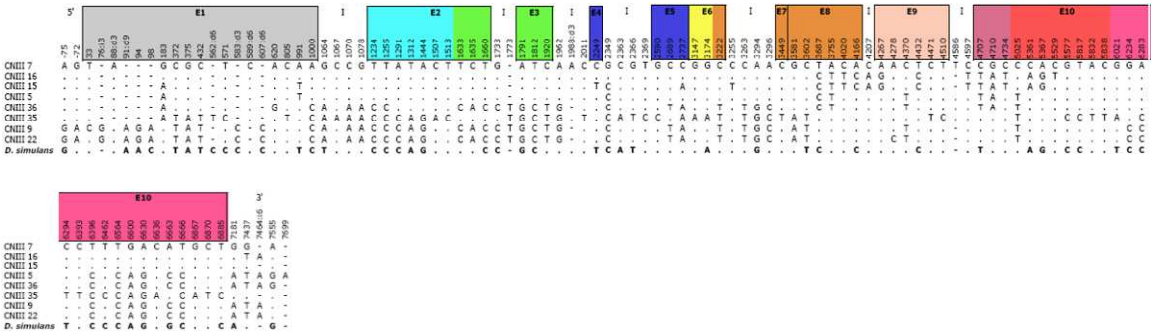

Figure 14: Nucleotide polymorphisms at the *InR* gene in .*D. melanogaster* and *D. simulans* Numbers at the top correspond to the position from the *InR* translation start codon. Dots indicate nucleotide variants identical to the first sequence. Dashes indicate gaps. For length polymorphisms, the position indicates the first affected site. d, deletion; i, insertion. E, exon; I, intron. Exons are denoted by numbers. Figure modified from [20].

In DFACE the classical radioactive label of the DNA probe and polyacrylamide gel electrophoresis is replaced by a fluorescent dye and ACE [21]. The analysis of the fragment pattern is performed with the Genemapper software. Figure 15 shows an example of validation of a DNAse I footprint assay of the transcription factor *Adf-1* of the *Adh* (Alcohol dehydrogenase) gene promoter in Drosophilidae [22].

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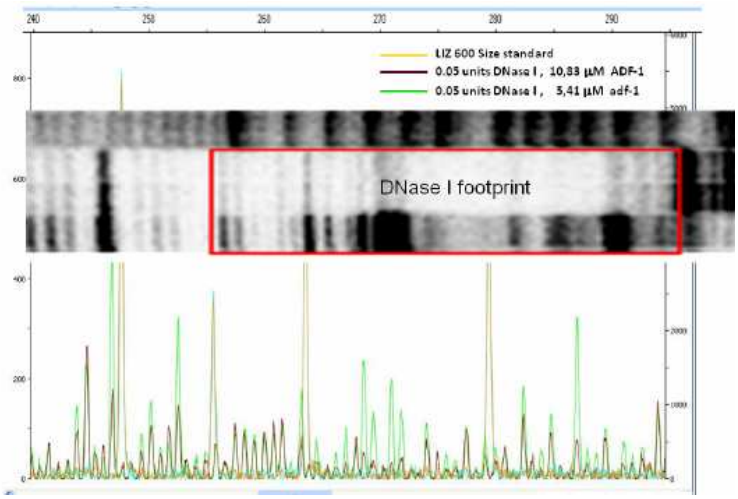

Figure 15: Comparison of DNase I footprinting of the transcription factor *Adf-1* of the *Adh* (Alcohol Dehydrogenase) gene promoter in Drosophilidae using a radioactive labeled probe and a polyacrylamide gel with the DNase I footprint obtained with the same FAM labeled probe and ACE in the 3730 DNA Analyzer. Figure is courtesy of E. Juan.

3.2.3. Microsatellites analysis in biodiversity and conservation studies

The use of molecular genetic techniques is common in other different fields of genomics and biomedicine. Fragment analysis is a useful tool for ecological studies. In particular microsatellites analysis are widely used in biodiversity and conservation studies. A microsatellite or short tandem repeat (STR) is a sequence of 2-7bp repeated tandemly distributed widely throughout the eukaryotic genomes. The number of repeats is highly variable.

The Genomics Unit has participated in the performance of a paternity determination of 12 microsatellites markers for 200 individuals, adults and chicks, from different locations of two subspecies of a game bird (see Fig. 16). Conclusions about mating choice, behavior, hybridization between wild and domesticated subspecies will arise from the post-analysis of these results (unpublished data). This kind of information is useful to game bird management.

4. Next-generation Sequencing

Sanger sequencing method has been preeminent in the last 30 years until the sudden appearance of the Next-generation sequencing (NGS). NGS, also called Massive Parallel Sequencing (MPS), are a group of diverse sequencing technologies that share two important features: no bacterial cloning in the preparation of the DNA template and a huge throughput of sequence data at a significantly decreased cost.

4.1. Instrumentation and chemistry

The Genomics Unit is equipped with the platform 454 Genome Sequencer FLX (Roche) (see Fig. 17). This platform is based in the miniaturization of pyrosequencing reactions [23] that occur simultaneously. One run produces 400Mb or more than 1 million high-quality reads of 400 bp of length. Each of the reads obtained comes from one molecule of DNA.

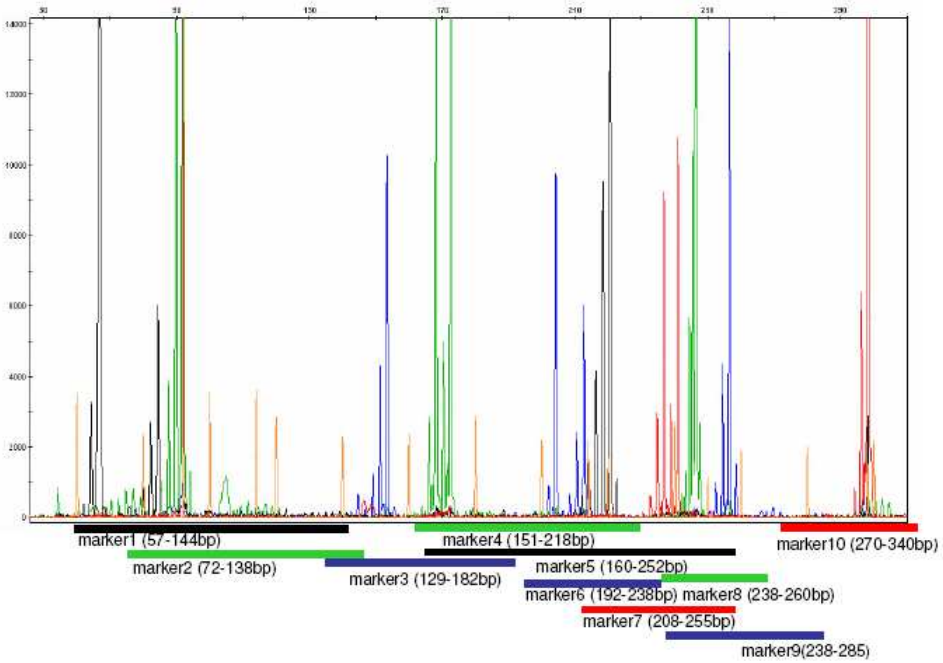

Figure 16. Plot of a multiplex PCR of 10 microsatellites markers of an individual. The colored bars below the peaks correspond to the size range of the microsatellites. Some ranges overlap but markers are distinguished by their fluorescent label.

Figure 17: 454 GS FLX (Roche)

The template preparation consists in the generation of a library of fragments with adaptors. Then they are bound to beads (one DNA molecule per bead) and clonally amplified by PCR into droplets of an oil-aqueous emulsion (emPCR). The adaptors contain the sequences needed to link to beads, to purify and enrich the amplified beads and to sequence them. After the emPCR, beads

carrying amplified DNA are purified and loaded into a special microwell plate (PTP, PicoTiterPlate), one bead per well. There 1 million of pyrosequencing reactions take place in parallel. The emissions of light from each well are recorded by a CCD camera, the image is processed and converted in flowgrams (see Fig. 18).

Bionformatics tools are needed in order to manage and analyse the overwhelming amount of obtained data.

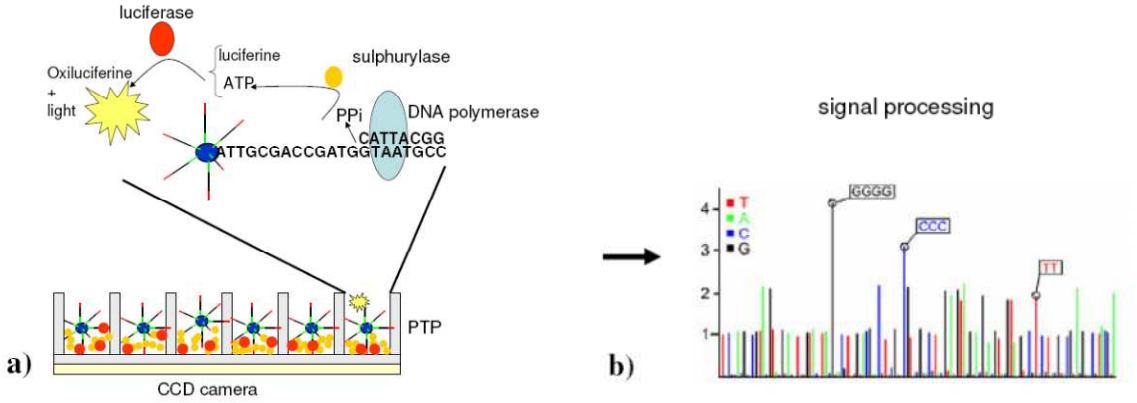

Figure 18: **a)** Schematics of the Pyrosequencing reaction. Cyclic flows of nucleotides are performed in a determined order. If the nucleotide flowed into the well is complementary to the synthesized DNA strand it is incorporated and a pyrophosphate (PPi) is released. An enzymatic cascade is coupled and generates a final emission of light whose intensity is directly proportional to the number of nucleotides incorporated in a single flow. Each bead contains millions of copies of a single fragment. **b)** The light signals are recorded and processed into flowgrams.

4.2. Applications

NGS technology has a broad range of applications in diverse fields of biology but the own special characteristics in read length, throughput and read accuracy of diverse platforms make some of them more suitable than others for particular applications. The greatest asset of 454 GS FLX is its long read length, about 450 bp up to 1000 bp with the latest improvements, so *de novo* genome and transcriptome sequencing are its main applications (see Table 2).

Table 2: Main applications of 454 GS FLX

De novo whole or partial genome
Resequencing
Ancient DNA and Paleobiology
Metagenomics: species identification and gene discovery
Transcriptome
Methylation/Epigenetics

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