Advanced PCR – Based Technology

i.e.: Nested PCR;
Immunocapture-PCR;
Reverse Transcriptase-PCR
Real time PCR (Taqman);
Low density PCR array;
Diagnostics tools

Conventional tools

- Biological Indexing
- ELISA

Molecular tools

- PCR
- Real time PCR (TaqMan)
- Low Density Array
PCR

**FIRST CYCLE**
- Double-stranded DNA
  - Heat to separate strands
  - Hybridization of primers

**STEP 1**
- DNA strands and add primer
- DNA oligonucleotide primers
- Region of double-stranded chromosomal DNA to be amplified

**STEP 2**
- DNA synthesis

**STEP 3**
- Separate DNA strands and anneal primer
- DNA synthesis

**SECOND CYCLE**
- Producing four double-stranded DNA molecules

**THIRD CYCLE**
- Producing eight double-stranded DNA molecules

The Polymerase Chain Reaction (PCR)

- **Denaturation**: 94°C for 30 sec
- **Annealing**: 55°C for 30 sec
- **Extension**: 72°C for 60 sec

35 cycles
The Polymerase Chain Reaction

- DNA amplification technique
- Can detect as few as 10 copies of target DNA in clinical samples (low copies available - unable to detect using hybridization)
- Principle: Amplification of a specific region of target DNA that lies between two regions of known sequence
- Primers - fragments of synthesized DNA made complementary to the ends of the target DNA
- Three steps of PCR
  - Heat denaturation
  - Annealing
  - Extension (using heat-resistant polymerase - ex. Taq or Pfu polymerase)
Thermo-cycler for PCR

An older model three-temperature

New model
Primer Selection and Design

- **Primer design** is critical for successful long PCR.
- **Length**: Primers are usually 21 to 34 bases in length and designed to have a GC content of 45-50%.
- **Melting temperatures (Tm)**:
  - In general, primers should have a Tm of at least 70 °C if a 68 °C annealing/extension step is used.
  - Optimally, the melting temperatures of the forward and reverse primers should be within 3 °C of each other and the Tm of the primers should be between 65 °C and 72 °C.
- Primers **should not** have any internal base-pairing sequences (i.e., potential hairpins) or any significant length of complementary regions between the two PCR primers.
- It may helpful to design primers with a final CC, GG, CG, or GC on the 3’ end of the primers in order to increase priming efficiency.
Primer Selection and Design

1. Database Searching for gene sequences
2. Design the primers taking into consideration the proper parameters
3. Apply the experiment
The Polymerase Chain Reaction (PCR)– Based Technology.

- Conventional PCR
- Nested PCR
- IC-PCR
- RT-PCR
  - Two Steps RT-PCR
  - One Step RT-PCR
- Multiplex PCR
- Quantitative PCR
- Real time PCR (TagMan)
- Low density PCR
Conventional PCR
Nested PCR

- Set of Primes are designed to anneal at the target nucleic acids in both directions.
- A second nested primer designed to anneal immediately adjacent to first primer or at sequences located further upstream of first one within the cDNA product.
- Other sequentially nested primers may be required depending on the efficiency and specificity of the primary PCR.
- In general, these primers should be highly specific for their target sequences, able to form stable duplexes with their target sequences, and free of
Nested PCR

One-Step RT-PCR

Nested PCR
IC-PCR

- **Trapping** the pathogen with specific antibodies.
  - Precoating with Protein A or G is usually done to increase the sensibility of the tube to the specific antibodies
  - MAbs are usually used
  - Washing with PBS + 0.05% Tween 20
- **Adding Sample** preparation and incubate for 1 h.
  - Washing with PBS + 0.05% Tween 20
- **Nucleic acid liberation** with 0.1% SDS and incubate at 50°C for 2 min with vortexing.
- **PCR**
- Gel Electrophoresis Analysis
RT-PCR

RT-PCR is used for the detection and amplification of RNA genomes.
- RT-PCR has been used in plant viruses using partially purified nucleic acids

RT-PCR technique consists of two steps:
- **Reverse transcription**: where RNA is used by reverse transcriptase (i.e. M-MLV) as template for cDNA production, in presence of specific primers complementary (anti-sense primer) to target sequence.
- **Extension**: that performed by employing thermo-resistant DNA polymerase, in presence of specific primers, identical (sense primer) or complementary (anti-sense primer) to the target sequences.

It is PCR.
RT-PCR

- It could be:
  - Two steps PCR
  - One step PCR

- In One Tube RT-PCR reaction mix is placed in the thermal cycler and initiate the following program:
  - 52 °C – 30 minutes (Reverse Transcription)
  - 94 °C – 30 “
  - varies – 45 “ - 35 cycles (PCR)
  - 72 °C – 60 “
  - 72 °C – 7 minutes
  - 4-16 °C – ∞
• One Tube RT-PCR mix
  • Sterile distilled water
  • PCR buffer
  • sucrose/cresol red buffer
  • dithiothreitol (DTT)
  • C-primer
  • V-primer
  • MgCl₂
  • RNasin (RNase inhibitor)
  • dNTP mix
  • Superscript II Rtase (200 U/ml)
  • AmpliTaq Taq Polymerase

• Reagent
Multiplex PCR

- Using more than set of primers for detection of several genes of the same or different target genome.
  - It consists of multiple **primer** sets within a single PCR mixture to produce **amplicons** of varying sizes that are specific to different DNA sequence
  - Multiplex PCR **Primer** Design must be highly specific
  - Multiplex **primers** for standard PCR assays can be for up to 30 sequences
Quantitative PCR
Quantitative PCR

- **Real-Time PCR** is designed to collect data as the reaction is proceeding.
  - It combines PCR with the power of fluorescent detection

- This reaction rely on the *fluorescence resonance energy transfer* (FRET) between either:
  - **Fluorogenic labels**: PCR can be quantified by using *fluorescent dye* (i.e. PicoGreen, SYBR-Green 1, ..etc) which fluorescence's specifically upon binding to dsDNA, and the intensity of that is measured by a fluorometer.
  - **Fluorophore & Quencher**: More sophisticated high-technology approaches of *Real-Time PCR/Taqman*, based on incorporation of fluorescent dyes into PCR mixes have been developed.
Positive Real-time PCR Results May Be Visualized By Two Means:

- **An amplification plot** represents the number of cycles on the X-axis, versus the amplification intensity of the fluorescence emitted by the reporter.
  - The fluorescence of the reporter molecule increases as products accumulate with each successive round of amplification.
  - The point or the cycle at which the accumulation of product results in a measurable change in the fluorescence of the reaction fluorescence rises appreciably above background has been called the *threshold cycle*.

- **The C$_T$ value**, which is the point at which the fluorescence passes from insignificant levels to clearly detectable levels, is called *threshold cycle* ($C_T$).
  - This value is used in the calculation of template quantity during quantitative real-time PCR.
Real-Time PCR Results Visualization

Real-time Positive results can be monitored through:

**Amplification Plot:**
- The generation of the reporter dye during amplification is proportional to the formation of PCR products.

**Threshold cycle (Cₜ):**
- The PCR cycle in which the gain in fluorescence generated by accumulation of amplicon exceeds 10 standard deviation of the mean baseline fluorescence.
- Cₜ is proportional to the amount of PCR product produced.
The different Real-time PCR instruments currently present in the market differ in:
- their probe chemistry,
- fluorescence detection,
- meaning what range of wavelength that they can detect,
- detection software
- representation of results and sample format
Real-Time PCR Instrumentation, Block Platforms

- ABI 7000
- Biorad’s iCycler
- MJ’s Opticon/2
- Stratagene’s MX3000P
- Stratagene’s MX4000
- ABI 7900HT
- MJ’s Opticon/2
iCycler iQ Real-Time PCR Detection System

- Laser coupled thermal cycler
- 5 unique excitation and emission filter pairs
- Intensifier to amplify light signal 96 well microplate
- Molecular Beacon Design software
Different Real-Time chemistry

- Hybridization Probes
  - *Taq*Man Probes
  - Molecular Beacons
  - Scorpions
**TaqMan Probes**

- The **TaqMan probe** is designed to anneal to a specific sequence of template between the forward and reverse primers.
  - The probe is labeled with a reporter **fluorophore** at the 5’ end and a **quencher** fluorophore at the 3’ end.
  - As the **Taq DNA polymerase** starts to copy DNA and reaches the annealed probe 5’
  - **Exonuclease activity** of the enzyme cleaves the probe and the reporter molecules start to **emit fluorescence** at a distinctive wavelength resulting in an increase in reporter fluorescence intensity which is recorded by the instrument.
Concept of TaqMan™ PCR
Conventional PCR Cycle

Cycling protocol:
- **Denaturation** (D): Single strand 94°C
- **Annealing** (A): Primers binding (55-75°C)
- **Elongation** (E): TaqDNA Polymerase active at 72°C

TaqMan® PCR Cycle

Cycling protocol:
- **Carry-over prevention** (U): 50°C for 2’
- **Activation of AmpliTaq Gold DNA polymerase** (G): 95°C for 10’

Cycling protocol:
- 40 cycles of each
  - **Annealing** (A): 60°C for 60 sec
  - **Denaturation** (D): 95°C for 15 sec
Conventional vs. TaqMan PCR

- For the conventional PCR protocol, the process can be summarized in three steps:
  - dsDNA denaturation at temperature >90°C
  - Primer annealing at 50-75°C
  - Strand extension at 72°C

- The TaqMan PCR protocol is similar, with the addition of:
  - a cycle which is 50°C for 2 min for carry over prevention,
  - then activation of Taq Gold DNA polymerase at 95°C for 10 min,
  - then denaturation 95°C for 15 sec
- There is no strand elongation in TaqMan PCR.
TaqMan probes

- Fluorogenic labeled probes
  - 5’ reporter fluorophore
  - 3’ quencher fluorophore
  - Fluorescence Resonance Energy Transfer (FRET)

Chemistry is based on the 5’-3’ exonuclease activity of Taq DNA polymerase
TaqMan PCR Chemistry

Reverse Transcription

Probe Annealing

Primer Annealing/Extension

Probe cleavage

Polymerization

Fluorescence absorbed
The TaqMan probe capitalizes on the 5' exonuclease activity of *Taq polymerase* to cleave a labeled hybridization probe during the extension phase of PCR.

In a fluorescent TaqMan assay, the probe is labeled at the 5' end with a fluorescent reporter molecule such as fluorescein and at the 3' end with another fluorescent molecule, which acts as a quencher for the reporter.
• When the 2 fluorophores are fixed at opposite ends of the 20–30 nt probe and the reporter fluorophore is excited by an outside light source, the normal fluorescence of the reporter is absorbed by the nearby quencher, and no reporter fluorescence is detected.

• When *Taq* polymerase encounters the bound probe during extension from one of the primers, it digests the probe, freeing the reporter from the quencher, and the reporter fluorescence can be detected and measured.
TaqMan Primer/Probe Design Efficiency

- Amplification of PCR products.
- Sequencing of PCR products.
- Multiple alignment of PCR products with Gen-Bank sequence.
- Design TaqMan Primers and Probe.
TAQMAN Primer/Probe Design Conditions

- Smaller sized PCR product.
- TaqMan probe longer than primers.
- GC content of probe 40-60%.
- No repeated sequence motif in probe.
- Absence of hybridization or overlap between probe and primers.
- Binding of TaqMan probe prior to the primers.
- Stability of probe at elevated temp. (Melting temp. of TaqMan probe is 100°C > than that of the primers).
- Distance proximity of fluorophores in the probe (20-30 nt).
3 generations of TaqMan probes:

- **Conventional TaqMan Probe**: 20 – 40 bp

- **Turbo TaqMan probes**: 15 – 20 bp
  - 5-propyne-2’deoxyurididine in place of thymidine

- **Minor Groove Binder**: MGB TaqMan probes: 12-14 bp
  - fluorescent dye labeling (R, Q)
  - High melting temperatures (65 - 70 ºC)
  - AT-rich sequences: probes > 40 nt
Quantitative PCR (Real-Time PCR)

- The *Taqman* assay
- Molecular Beacon
- Scorpion primers
- Low Density PCR Array
Molecular Beacons

- Molecular beacons are a novel non-radioactive method for detecting specific sequences of nucleic acids involves using "Molecular Beacon Probe".

- **Stem and loop shaped hybridization probes.**

- A fluorescent molecule and a quenching molecule are covalently attached to each end of the stem.

- Probes undergo a conformational change = increased fluorescence.
Molecular Beacons

- A typical molecular beacon probe is characterized, by the following:
  - 25 nt. long, the middle 15 nucleotides are complementary to target DNA and do not base pair with one another, and the five nt. at each end are complementary to each other and not to target DNA.
  - The fluorescent chromophore is attached to the 5’ end, and the non fluorescent chromophore is attached to the 3’ end.
  - Quencher will absorb the energy emitted by fluorophore before it fluoresces
  - At room temperature the conformation of the molecular beacon ensure that the Fluorophore and Quencher are close to each other that the fluorophore is quenched
  - In real-time PCR molecular beacons are attached with a primers by blocker
Molecular Beacon probes

1. Quenching of the fluorescence
2. Emission of the fluorescence
Scorpions

- Scorpions are bifunctional molecules
- PCR primer covalently linked to probe.
- A fluorophore that can interact with a quencher to reduce fluorescence.
• Scorpions are bi-functional molecules containing a PCR primer covalently linked to a probe.
• The molecules also contain a fluorophore that can interact with a quencher to reduce fluorescence.
• During amplification, Scorpion primer starts to extend on target sequence, the quencher dissociates.
• The probe sequence in the Scorpion tail curls back to hybridize to the target sequence in the PCR product.
• This hybridization event opens the hairpin loop so that fluorescence is not longer quenched and an
Scorpions are PCR primers with a "Stem-Loop" tail containing a fluorophore and a quencher.
The Stem-Loop tail is separated from the PCR primer sequence by a "PCR stopper", a chemical modification that prevents the PCR from copying the stem-loop sequence of the Scorpions primer.
During PCR, the Scorpions primers are extended to form PCR products.
As the tail of the scorpion and the PCR product are now part of the same strand of DNA, the interaction is intermolecular.
The target sequence is typically chosen to be within 3 bases of the 3'end of the Scorpion primer.
Low Density PCR Array

Micro fluidics Card: 384-well format

- Primers and TaqMan® probes are dried into 384-well based card.
- The reaction chambers allow PCR reaction volumes of 1 - 5 µl.
- The sample + PCR master mix manually pipetted into a loading chamber.
- The whole micro fluidic card is centrifuged in upright position.
- The sample/master mix travels along the microfluidic channels into reaction chambers.
- The card is then sealed, the loading ports cut away, storing at 4°C.
- **Real-time TaqMan® PCR analysis** in 7900 HT ABI platform.
High-throughput pathogen detection using the Low density PCR Arrays

Sample grinding

Automated Nucleic Acid Extraction

RT-reactions: Universal cDNA pool

Low Density Arrays

Raw data extraction

Transfer

ABI® ANAE 6700 Liquid handling system

Real-time TaqMan® PCR set-up for ABI PRISM platform

ABI PRISM 7900 HT SDS platform for real-time TaqMan® PCR
384-well cards designed for fluidic micro analyzing gene expression pattern in many samples across a defined set of genes target

The analysis work by 7900HT ABI Platform-
Device uses in Low Density PCR
7900HT Micro Fluidic Card Workflow

Step 1.
Load the Sample

Step 2.
Fill the Micro Fluidic Card Wells

Step 3.
Seal the Micro Fluidic Card

Step 4.
Prepare the Micro Fluidic Card for Loading into 7900HT System

Step 5.
Perform Real-time PCR

Step 6.
View and Analyze Data
Detection of Pathogens in Grapevines

detection of the human papillomavirus

Evaluation of suitable reference genes for normalization of real-time reverse transcription PCR analysis in colon cancer
RNA Extraction Methodology

- Two methods of RNA extraction:
  - Qiagen kit and fully automated ABI extraction method.
- **The Qiagen** relies on the binding of your target RNA to Silica membranes.
  - The disadvantage of this method is that the genomic DNA and the viral RNA compete for the same silica membrane and the DNA genome usually out-competes the viral RNA.
  - The extraction of the RNA is performed in an as closed system with the immediate conversion to cDNA.
- **The ABI fully automated workstation** allows for the total size separation of the desired RNA from the other host genomic DNA and cellular debris through propriety membranes.
Comparison of different RNA Methodology

ABI PRISM™ 6700 Nucleic Acid Automated Workstation

RNeasy Plant Mini Kit

1X ABI lysis buffer
2X ABI lysis buffer
Corbett lysis buffer

X-tractor Gene™
automated nucleic acid extraction