Real Time PCR

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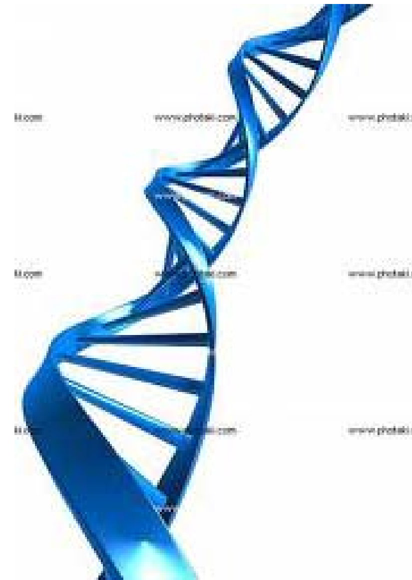
Goal

The aim is to construct a **Low-cost real time PCR machine for detection of diseases.** We will aim for the miniaturization of a working set-up for use in clinical laboratories in developing countries.

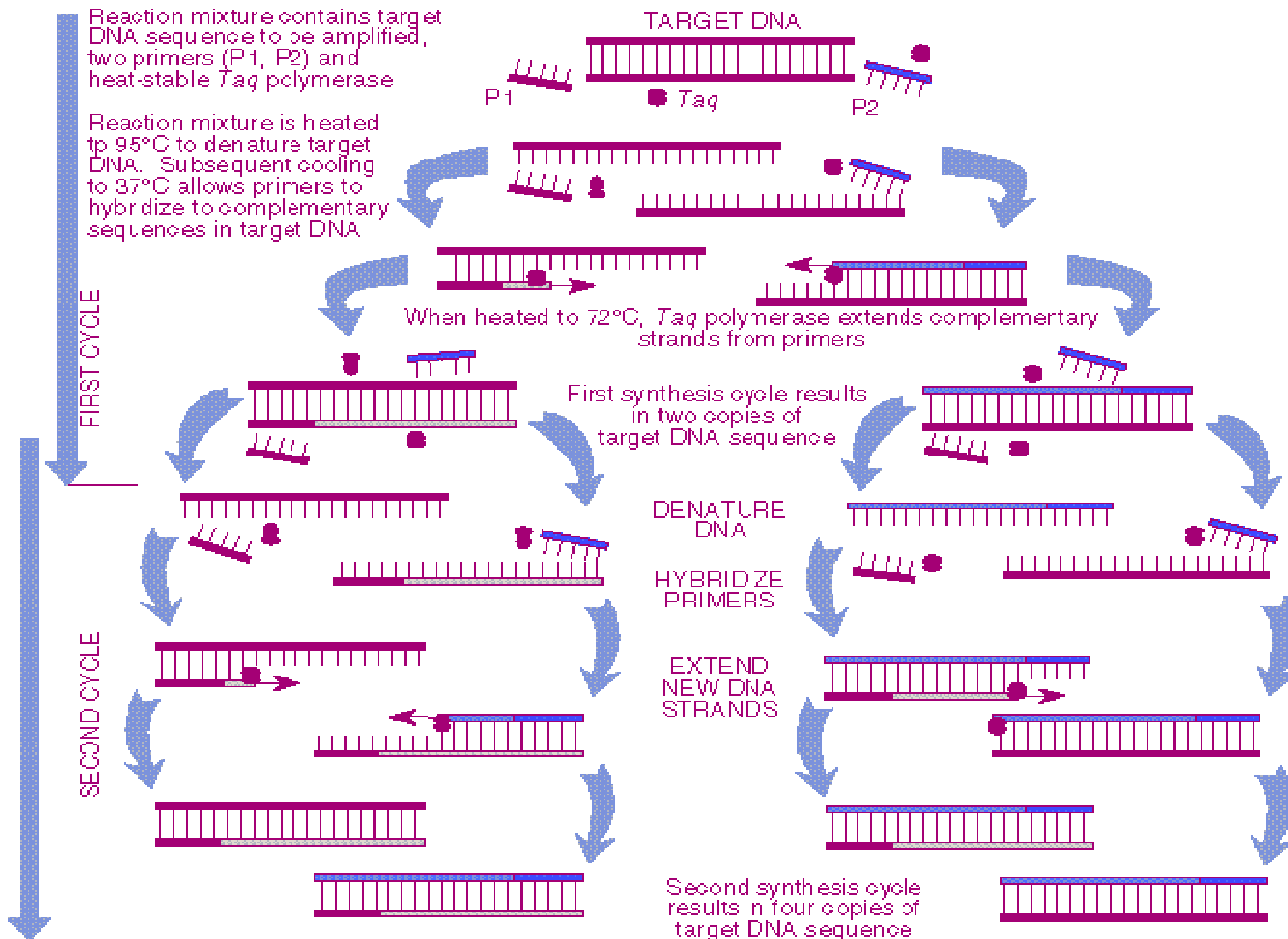
Overview

Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplified DNA production at each PCR cycle (in real time) as opposed to the endpoint detection

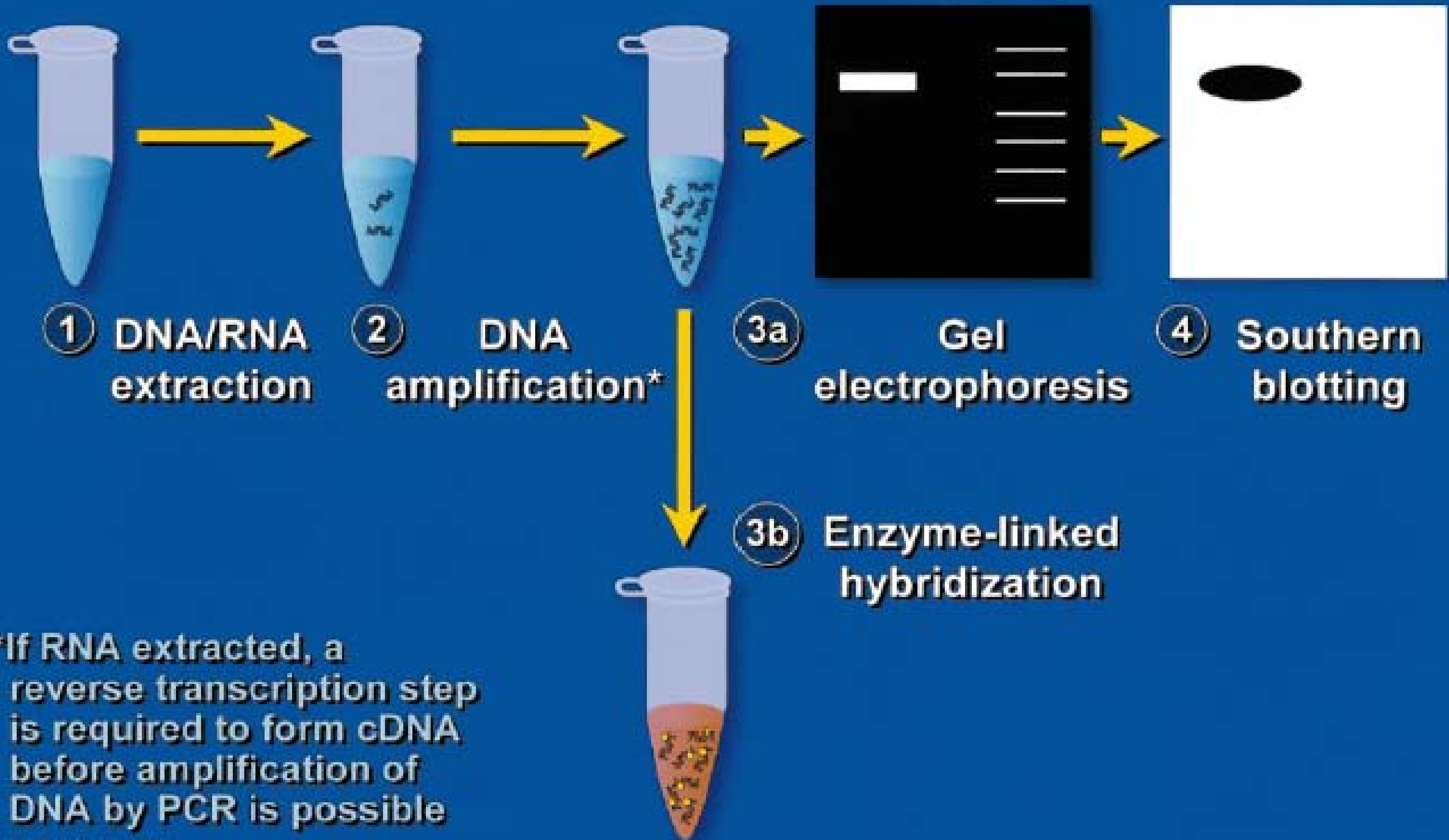


Normal PCR

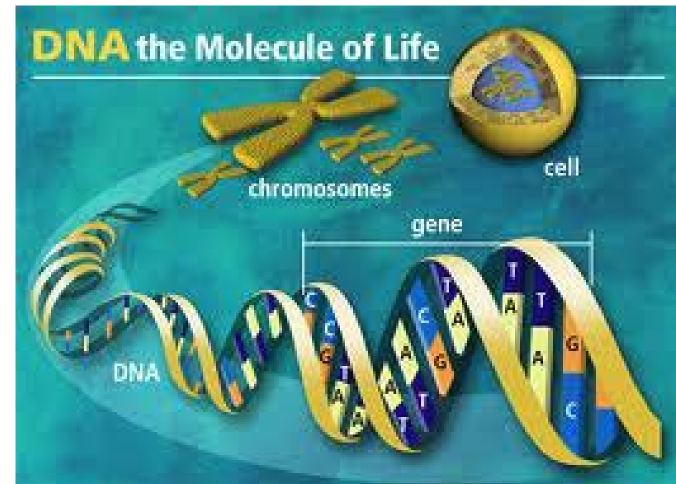
PCR (Polymerase Chain Reaction)



Conventional PCR-Based Testing Formats

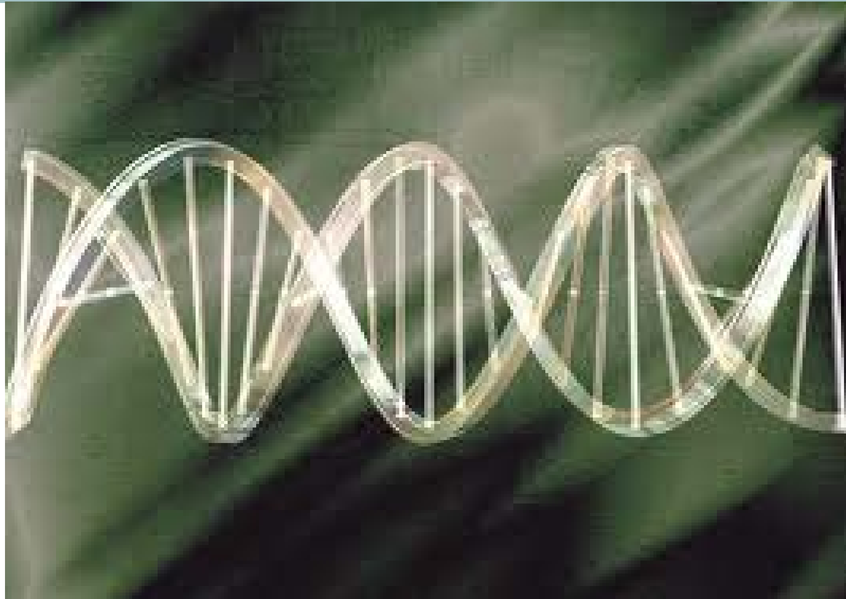


But it has issues…



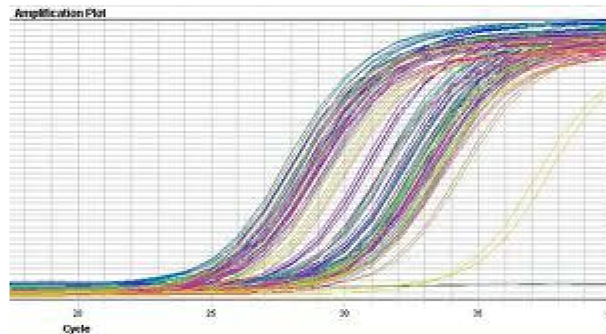
- Poor precision
- Low sensitivity
- Low resolution
- Non-automated
- Size-based discrimination only
- Ethidium bromide staining is not very quantitative

Going to Real Time PCR



Real Time PCR

- based on the detection and quantitation of a fluorescent reporter
- the first significant increase in the amount of PCR product (CT - threshold cycle) correlates to the initial amount of target template

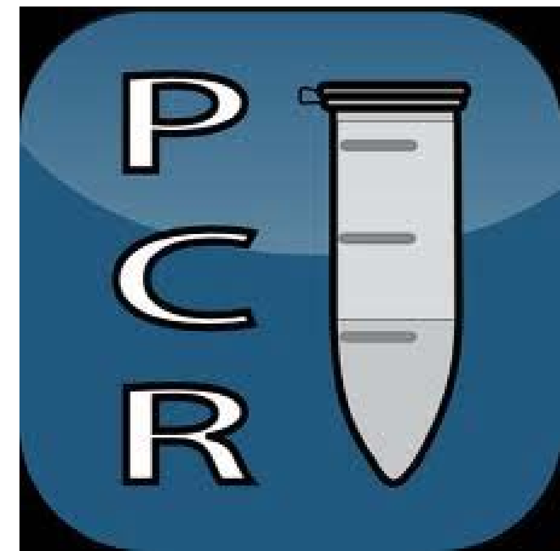


Real Time PCR

- amplification can be monitored real-time
- no post-PCR processing of products (high throughput, low contamination risk)
- detection is capable down to a two-fold change
- confirmation of specific amplification by melting curve analysis
- most specific, sensitive and reproducible
- not much more expensive than conventional PCR (except equipment cost)

Components

1. Thermal Cycler
2. Optical Arrangement (Flourimeter)
3. Data Acquisition
4. Photlithography (Time Permitting)



Basic Set-up

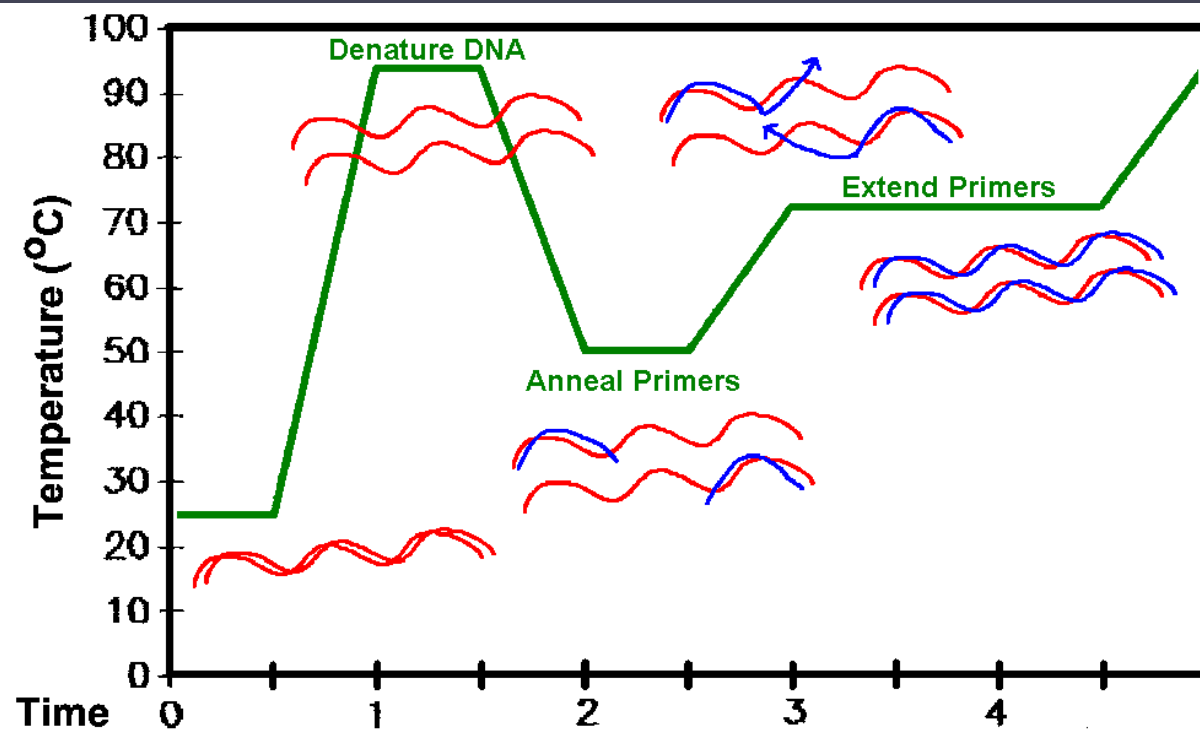
- The thermal cycler is used for the amplification of DNA.
- An excitation source (e.g. laser diode, led) to excite the fluorescent probe in the which has bound to double stranded DNA. And a receiver (camera, photo detector connected to optical filters) to capture the fluorescence emitted of the probe

Basic Set-up (continued)

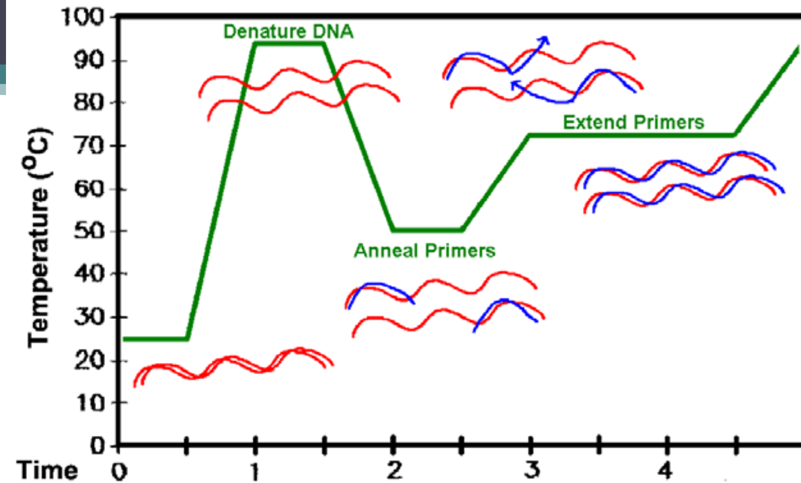
- A computer to regulate the heating cycle and record the data from the receiver

(this may be achieved through appropriate use of micro controller or FPGA)

Thermal Cycler



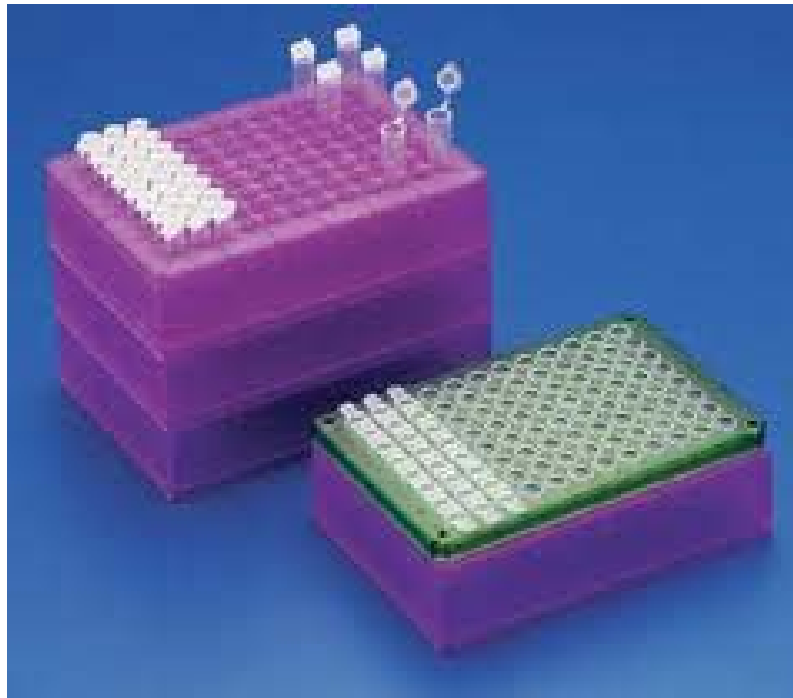
1. Thermal Cycler

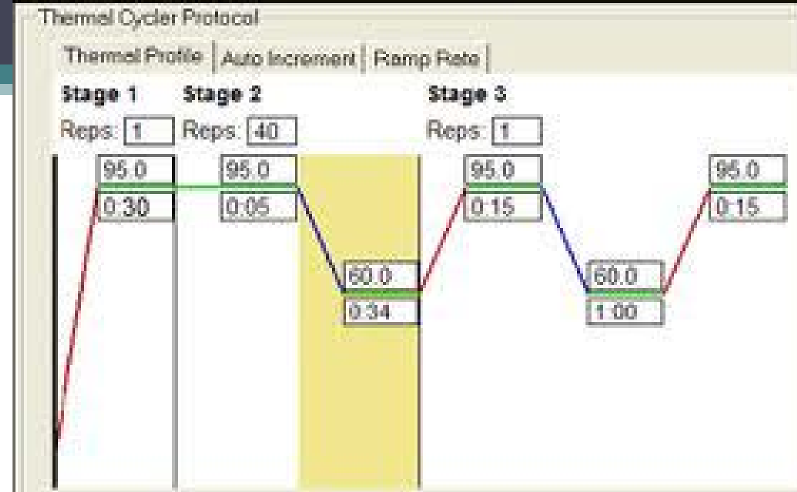


- The thermal cycler is one of the most important component in an PCR reaction. Our design of the thermal cycler hopes to achieve the following:

1. Speed : A complete PCR reaction requires 30-40 iterations of the cycle. rapid, precise cooling and heating will reduce the time needed to completely analyse a sample.
2. Precision Important to provide ideal temperatures to the molecules (primers, polymerase). So we will use feedback control , employing PID controllers.

3. Compatibility with Fluorimeter : it is necessary to find a way to be able to heat the sample in the tubes uniformly (from all sides), and at the same time be able to do imaging after each iteration.





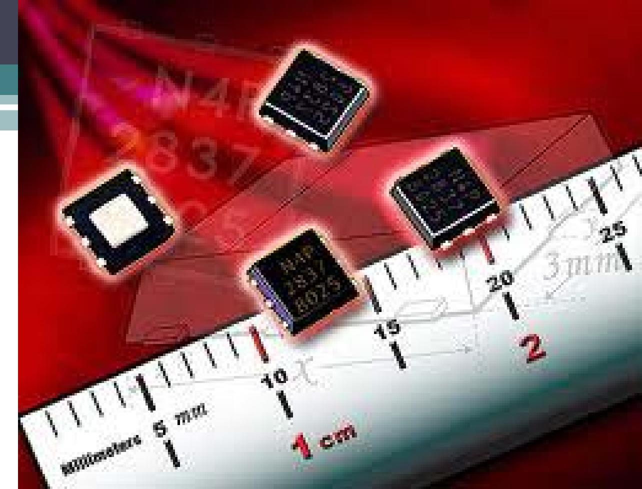
Desired temperature States:

- 1. **Denaturation Step : The reaction mixture needs to be heated to 94-98 °C, for 20–30 seconds.**
- 2. ***Annealing Step*: The reaction temperature is lowered to 50–65 °C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template.**
- 3 . **Extension step : Need to maintain at a temperature of about 72 °C, best suited for the polymerase being used.**

Temperature Sensor :

- For temperature control, we need a sensor in negative feedback. Once again, in choosing a sensor, our aim is to maximize sensitivity, accuracy, dynamic range, and speed of response. Potential sensors are :

1. Thermistors : resistance based sensors.
2. C-MOS based temperature sensors.
3. Thermocouple.



Control

- Control : we will implement our controller in a microcontroller, or an FPGA; depending on the need. We will implement a digital PID controller for optimal control.
- One potential design complication could be in the case we have multiple heaters and sensors , e.g. if we decide to place multiple samples on a large plate, then we will have to take into account the spatial locality of a particular sensor or actuator, in order to maintain a uniform temperature throughout the pate.

Heating Element

1. Thermoelectric Heaters (Peltier heater / cooler): are used in many commercial thermal cyclers. We can use them with heatsinks mounted on one side, to achieve rapid cooling when needed. They are also very effective heaters, providing a cheap solution.
2. Heated air (resistive wire heaters)

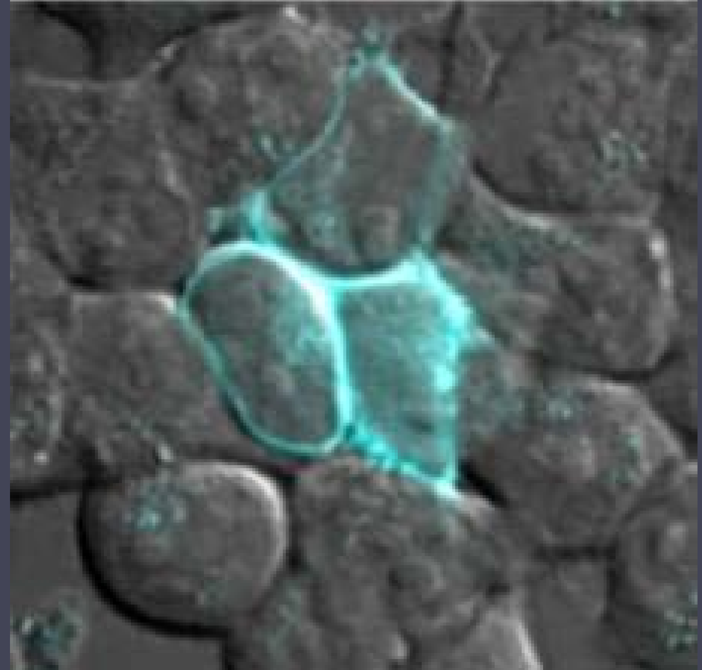
- Metal Casing: (to hold eppendorf tubes)
- We want to achieve rapid, uniform temperature control, so we need materials with high thermal conductivity:

1. Silver casing : 429 [W/(m·K)] , very good thermal conductor, can be used to hold the tubes. It is expensive, an inner coating may have to suffice.

2. Copper casing : 401 [W/(m·K)]
   good thermal conductor.

3. Aluminum casing : 237 [W/(m·K)]
   a cheap alternative.

Fluorescent Probes



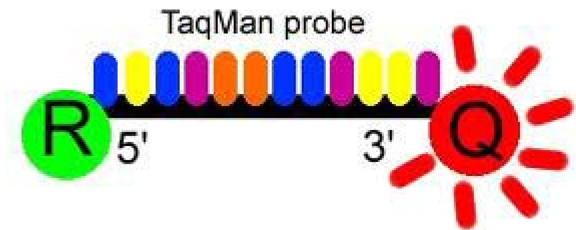
Generally there are two types of probes used to detect PCR products using real-time PCR instruments.

TaqMan® chemistry (also known as “fluorogenic 5' nuclease chemistry”)

SYBR® Green I dye chemistry

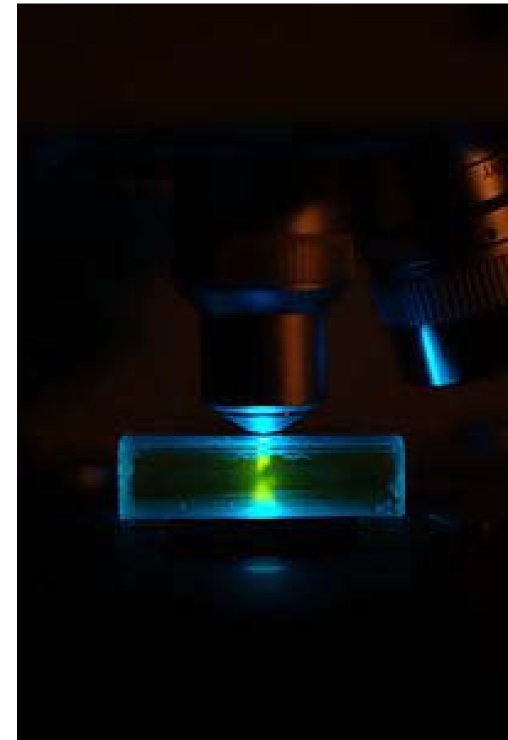
TaqMan-Based Detection

- Detects specific amplification products only.
- Specific hybridization between probe and target is required to generate fluorescent signal, significantly reducing background and false positives.
- BUT a different probe has to be synthesized for each unique target sequence.



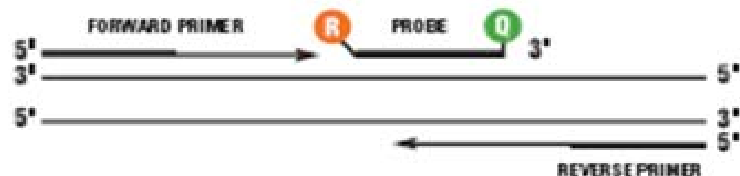
SYBR-Green Based Detection

- Enables you to monitor the amplification of any double-stranded DNA sequence.
- Multiple dyes can bind to a single amplified molecule, increasing sensitivity for detecting amplification products.

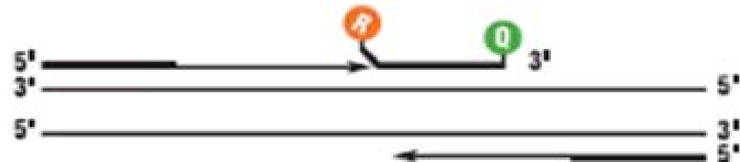


TAQMAN® PROBE-BASED ASSAY CHEMISTRY

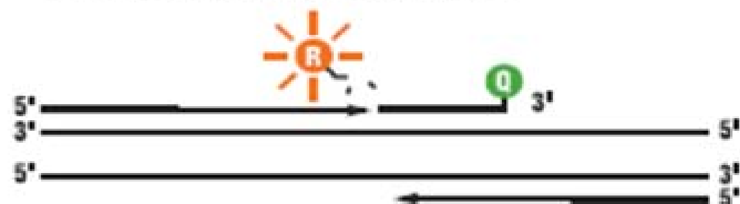
1. **Polymerization:** A fluorescent reporter (R) dye and a quencher (Q) are attached to the 5' and 3' ends of a TaqMan® probe, respectively.



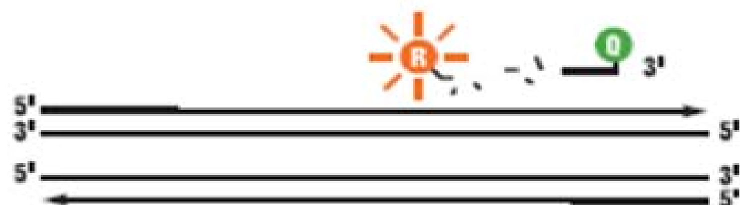
2. **Strand displacement:** When the probe is intact, the reporter dye emission is quenched.



3. **Cleavage:** During each extension cycle, the DNA polymerase cleaves the reporter dye from the probe.

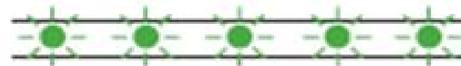


4. **Polymerization completed:** Once separated from the quencher, the reporter dye emits its characteristic fluorescence.



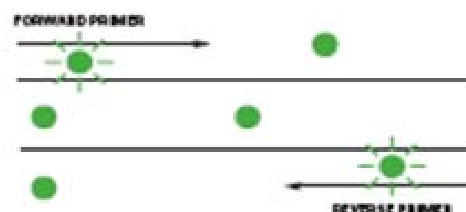
SYBR® GREEN I DYE ASSAY CHEMISTRY

1. **Reaction setup:** The SYBR® Green I Dye fluoresces when bound to double-stranded DNA.

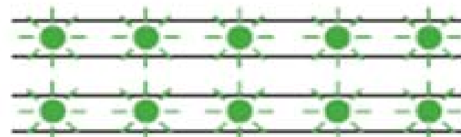


2. **Denaturation:** When the DNA is denatured, the SYBR® Green I Dye is released and the fluorescence is drastically reduced.

3. **Polymerization:** During extension, primers anneal and PCR product is generated.

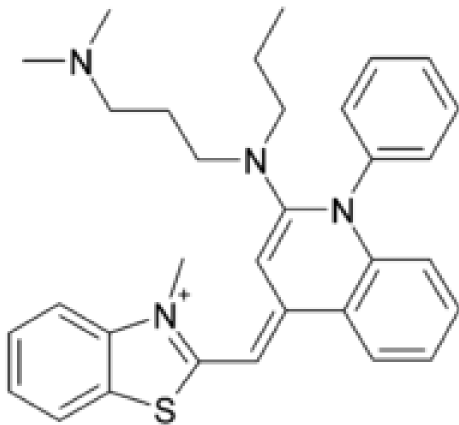


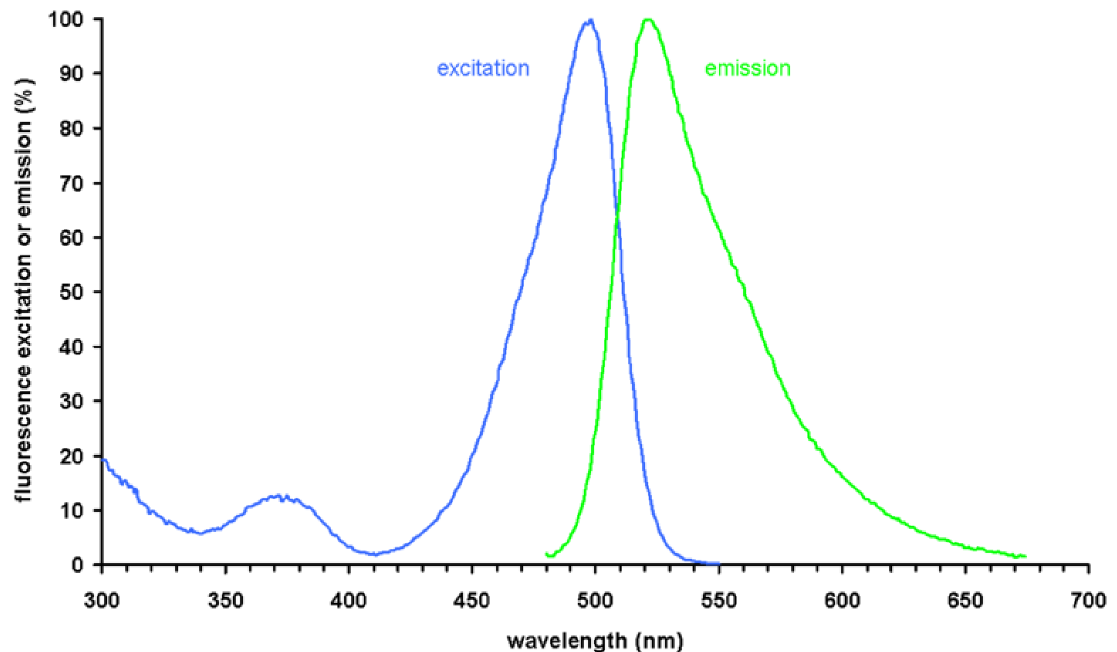
4. **Polymerization completed:** When polymerization is complete, SYBR® Green I Dye binds to the double-stranded product, resulting in a net increase in fluorescence detected by the 7900HT system.



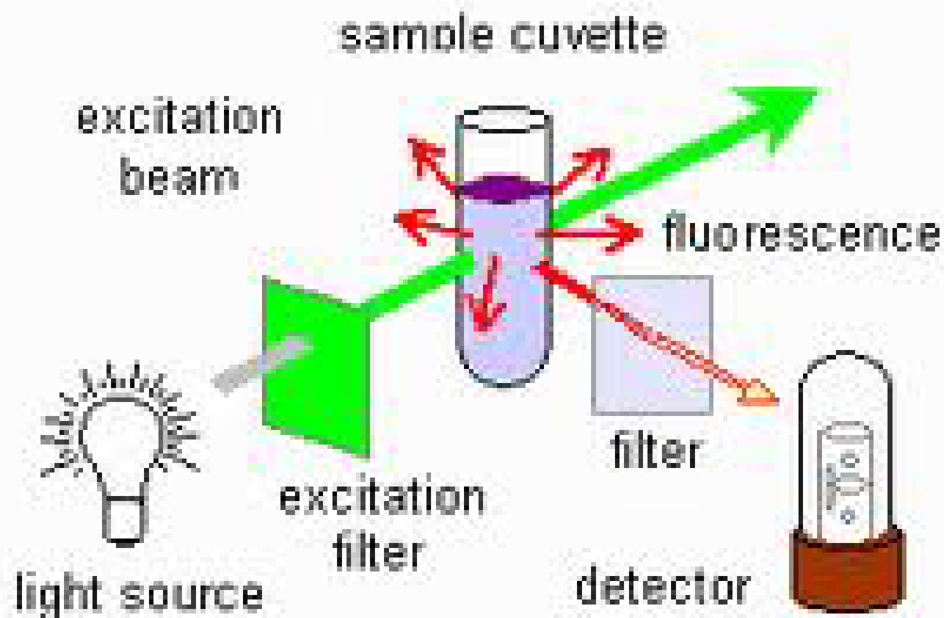
SYBR Green

- Detects all amplified double-stranded DNA, including non-specific reaction products and available at cheaper price





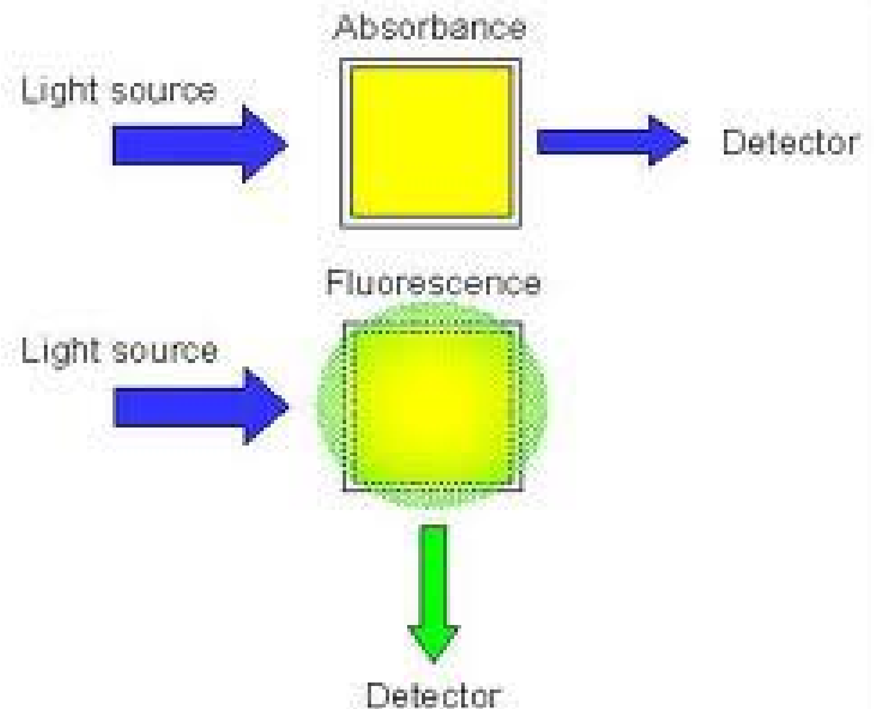
Fluorimeter



Components

Three are three main components of the fluorimeter:

i. Excitation light source.
ii. Optical Filter.
iii. Photo- detector



Excitation source

- We have three options for light sources:

1. High power, wide spectrum LED’s , or a narrowband LED, depending on the florescence agent.
2. Laser Diode : pros: coherent, precise wavelengths ; cons : expensive, very high powers can cause Photo-bleaching.
3. Quartz Tungsten halogen Lamp : very broad spectrum , will give flexibility in terms of probes/fluorescent dyes that can be used in the pcr reaction.

Optical Filter

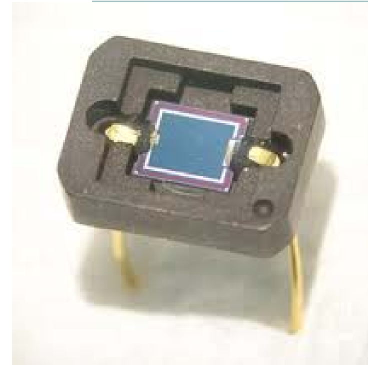
- The optical filter that we will use depends upon the wavelength of the fluorescent light, it will be used to block light of the excitation source, so that only relevant information goes into the photo detector.
- For a multiplex machine (one which uses different probes to detect different viruses at the same time), we can have an assembly of multiple filters, mounted on a rotatable assembly.

Photodetector

In choosing a photodetector, the parameters that need to be taken care of are:

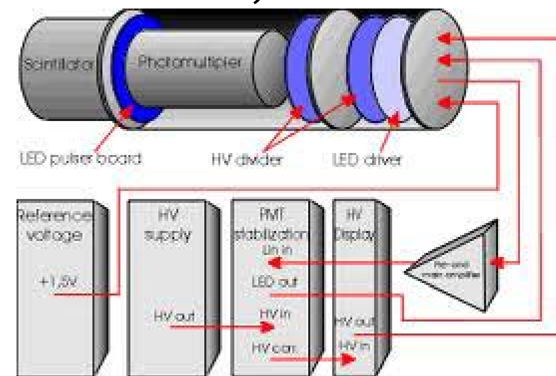
a) Linearity of output current with respect to intensity.
b) Sensitivity of the detector, in Ampere/Watt.
c) Noise levels, un-mounted laser diodes are cheap, but unless we take significant steps to shield them ourselves, they will give significant noise.

Photodiodes :



- these are to be placed after the optical filters. We could have them to be reverse biased, this increases sensitivity

PMT (Photo Multiplier Tubes):



- These are very sensitive, have a very fast response, and are used to detect very low levels of light. They are used in many commercial realtime PCR machines, they are very expensive, and we hope we will not need to use them.

CCD camera/ array

we can use sensors that are used in webcams, these should be a cheap option, they are quite sensitive. CCD cameras are used in many commercial machines. They typically require a PC for interfacing, so if we wish to incorporate this into an embedded system, using FPGA (field programmable gate arrays, or microcontrollers, then some one will need to design the interface, both software and hardware.

Data Analysis

DATA PROCESSING

- Initial goal is to detect fluorescence from a single sample, and ensure working of PCR process by measuring and displaying fluorescence intensity as a function of the number of thermal cycles
- The means of interfacing the detector with the computer will be decided once the detector is finalised. Possible interfaces include DAQ cards, USB ports, serial or parallel ports
- Next stage would be to determine initial concentration of DNA/mRNA in a given sample

Quantifying initial DNA/mRNA concentrations

- Multiple methods can be used to determine initial concentration in test sample
- Most methods involve measurement of fluorescence for standard samples, having known initial DNA/mRNA concentrations, and target samples
- Multiple standard samples with different dilutions are used in these methods
- Some of the methods have been discussed here. There are more which need to be investigated in more detail

Method1: Beer Lambart Law

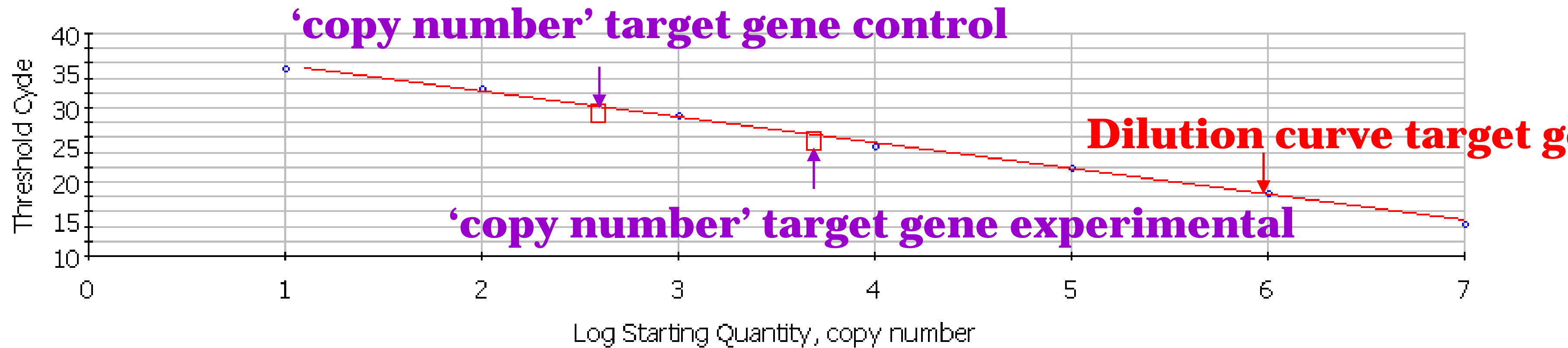
- Does not require a comparison with a standard
- Complex equation which needs to be modified according to our particular mechanical arrangement
- Advantage: Simpler mechanical assembly
- Disadvantage: Equation's parameters change with the reagent used; complication in developing a flexible product capable of detecting different viruses(different probes/dyes may be required for different viruses)

Method2: Standard Curve

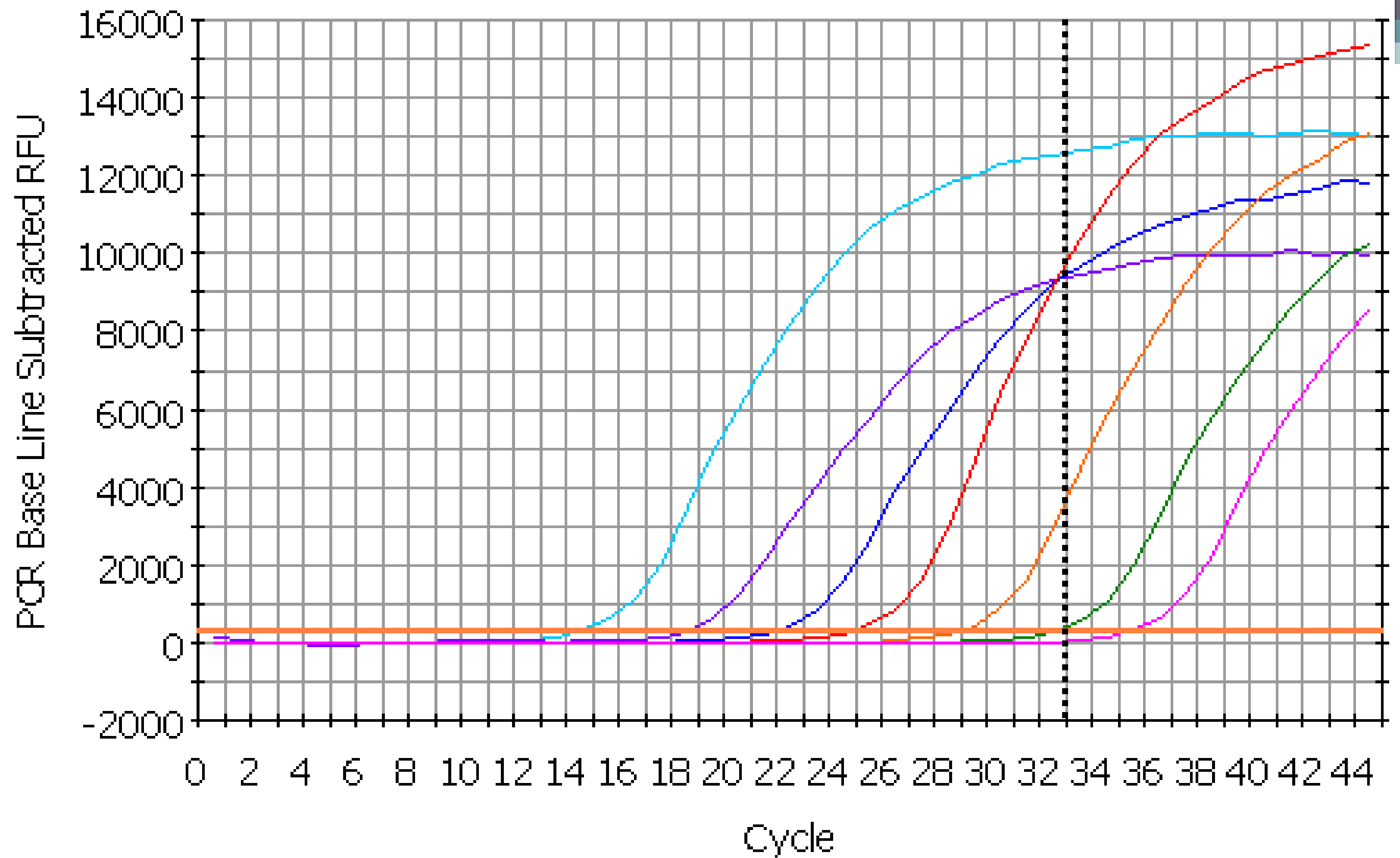
- Set a threshold fluorescence. Number of thermal cycles corresponding to threshold(Ct value) determined for standards with different dilutions differing by orders of ten, as well as for test sample
- Logarithmic scale plot of Ct values against concentration of standard samples
- Use this 'standard curve' to determine concentration of test sample
- Advantages:

  Any systematic errors would be catered for as the same error would occur in the standard curves with which the comparison is made

  Less data to handle. Only number of thermal cycles at the threshold fluorescence needs to be stored
- Threshold should be kept low; at higher thresholds, curves for different initial concentrations can intersect



Standard Curve Method
Above figure taken from a source which explains using RT-PCR to measure changes in mRNA expression in cells on exposure to certain experimental conditions; hence the 'control' and 'experimental' data



Slide taken from secondary source

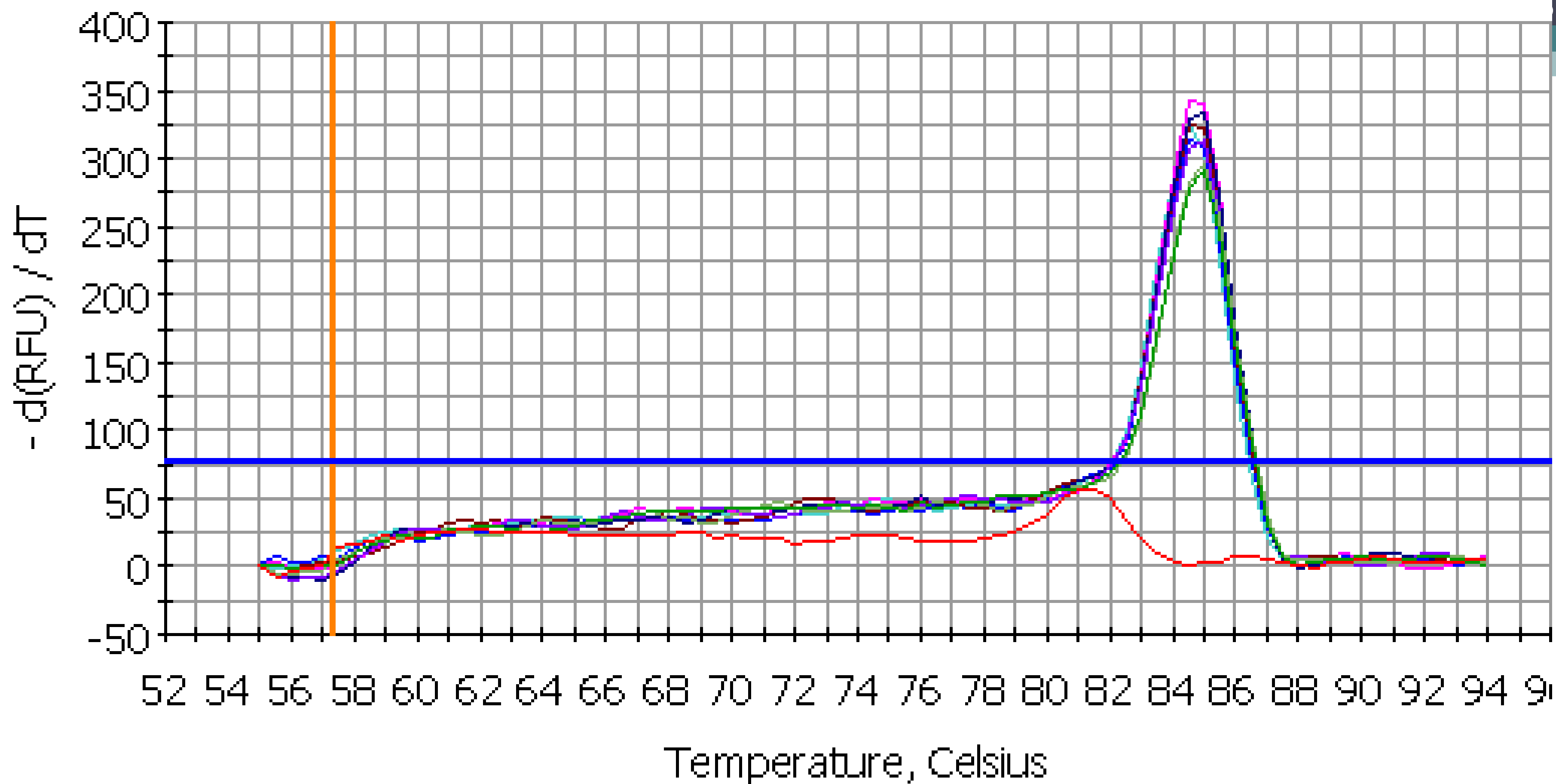
SERIES OF 10-FOLD DILUTIONS

Method3: Curve Fitting

- Similar to Standard Curve method, but here we have no threshold. Instead, we record several data points for fluorescence against thermal cycles to obtain complete curves for each standard sample, as well as for test sample
- Curve fitting techniques to match curve for test sample with those for standard samples. The standard curve which fits best is used to determine the concentration
- Advantage:Error mimimised as process dependent on several readings rather than just one reading at a threshold
- Disadvantage: Computationally taxing

A Few Essentials

- Data Acquisition from a negative control sample(containing no DNA) to ensure the PCR mix and/or dye is not contaminated
- The above precaution would also help determine whether primer-dimer artefacts form
- PCR products for the same primer pair should have the same melting point-a melting point analysis can ensure no contamination/imperfections have occurred in the process
- PCR efficiency(increase in DNA quantity per thermal cycle) has an impact on Ct value and slope of curve in logarithmic scale. By comparing slopes of standard curves amongst themselves and with the sample curve, it can be ensured that no error due to variation in PCR efficiency occurs
- Standard curves reproducible if PCR mix containing stabilizers for SYBR green used



Taken from secondary source

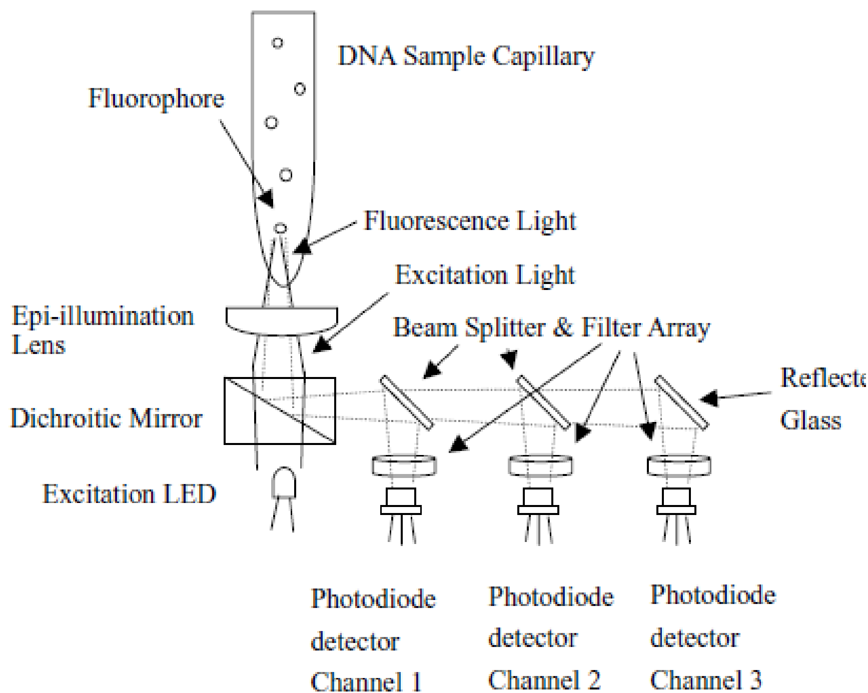
Dealing with Optical Noise

- Use filters
- Selecting probes that absorb and emit at longer wavelengths(>500 nm). Background noise is usually outside this range. Further study required about this

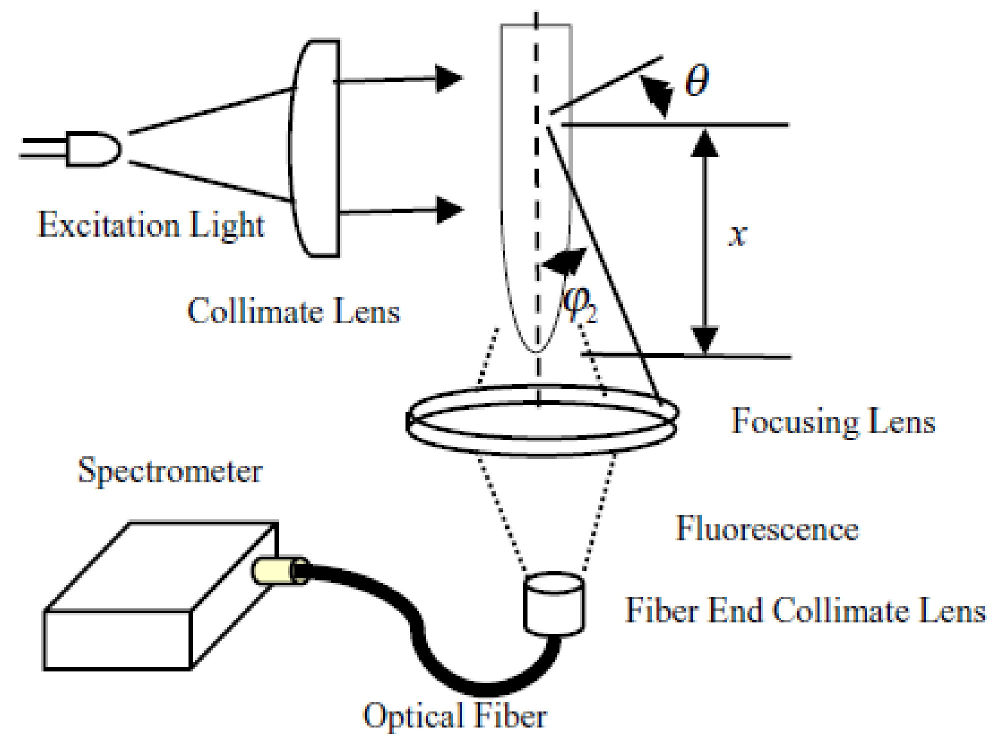
Arrangements

Realtime Detection Systems

a)



b)



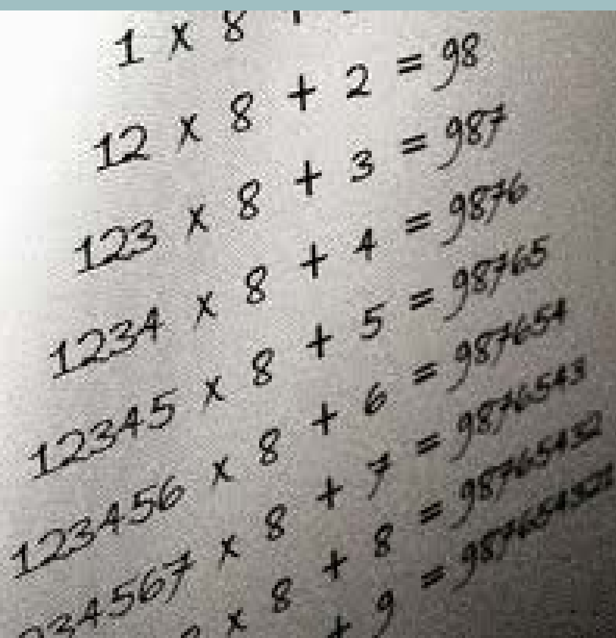
Issues

- No transparency from the sides
- Excitation from the top, and collection from the top of the eppendorf tube.

What do we do?

- We put the excitation source and the florescent collection setup together.
- For multiple eppendorf tubes, we use a moving assembly to scan over all the tubes during measurement.

Our Approach



Simulations

- Whichever arrangement we choose, first we will simulate its mathematical model using the beer0-lambert law.
- We'll use Matlab for this purpose. The relationship is

$$\Delta F = I_\lambda(0) \int_0^L \int_0^{2\pi} \int_{\varphi_1(x,\theta,\varphi)}^{\varphi_2(x,\theta,\varphi)} \sin\varphi \sum_{i=1}^{M} \phi_i a_{i,\lambda} c_i \times \left(\frac{I_\lambda(x)}{I_\lambda(0)}\right)\left(\frac{I_{\lambda'}(L)}{I_{\lambda'}(0)}\right) \mathrm{d}\varphi\, \mathrm{d}\theta\, \mathrm{d}x.$$

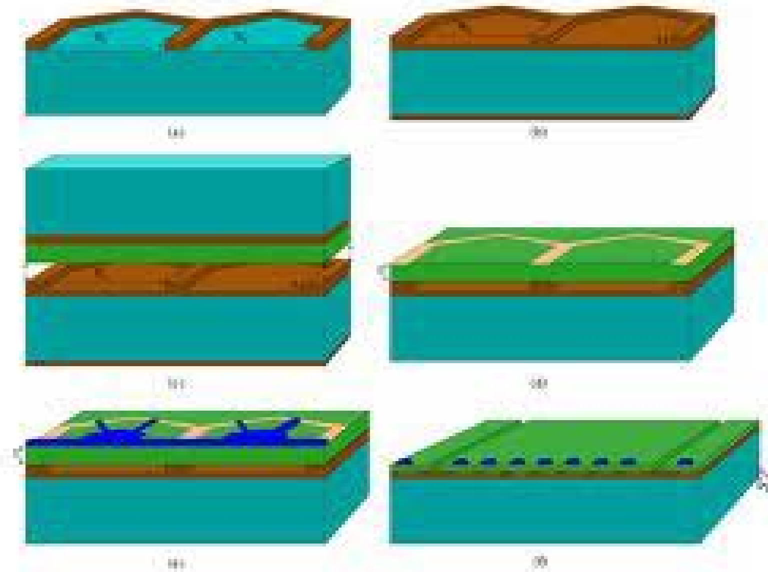
Hardware

- Then we will look to implement a simple arrangement involving one eppendorf tube, and a heater and the Flourimeter connected to a computer.
- The Flourimeter is right above the top of the tube, with the tube being heated from the sides.

Continuation

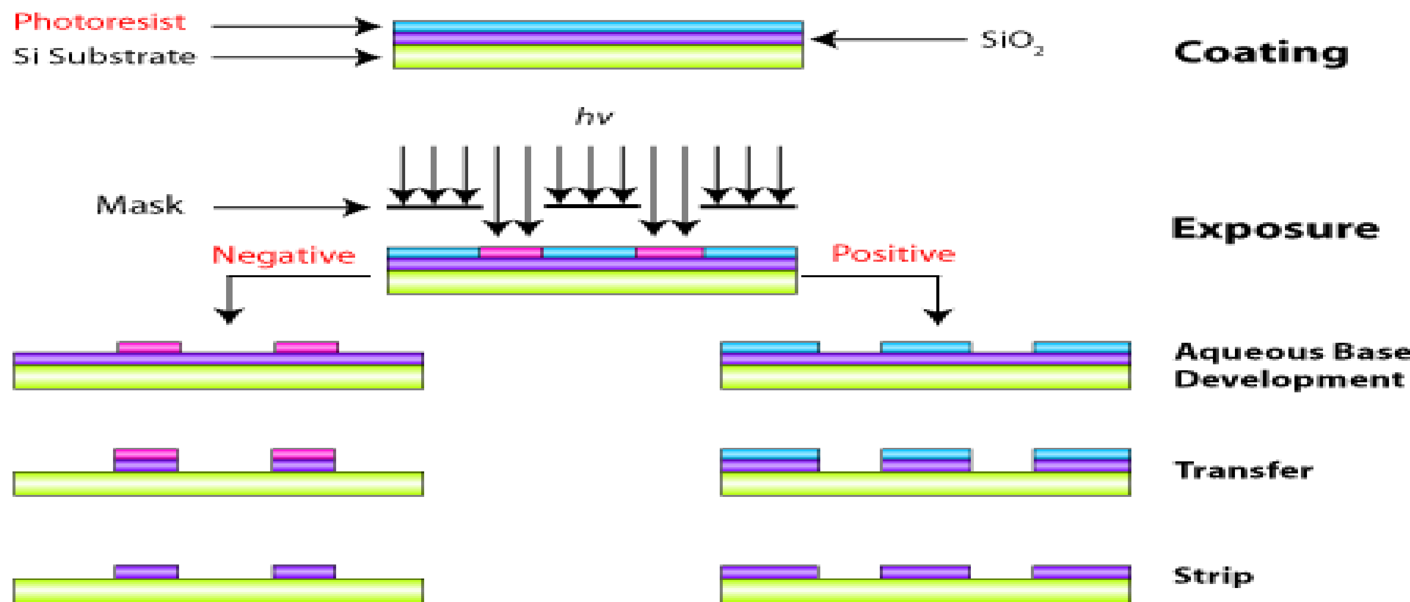
- Once we are successful in obtaining some positive results, we will then look to have multiple samples (multiple tubes) at the same time at different dilutions for more accurate values as to the initial concentration of the sample.
- We will then have an automated mechanical assembly including moving Flourimeter over the multiple tubes using stepper motors, one the samples have been put in place.

Photolithography



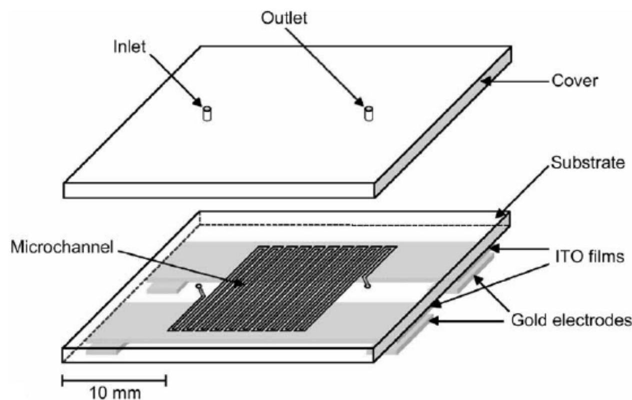
Photolithography

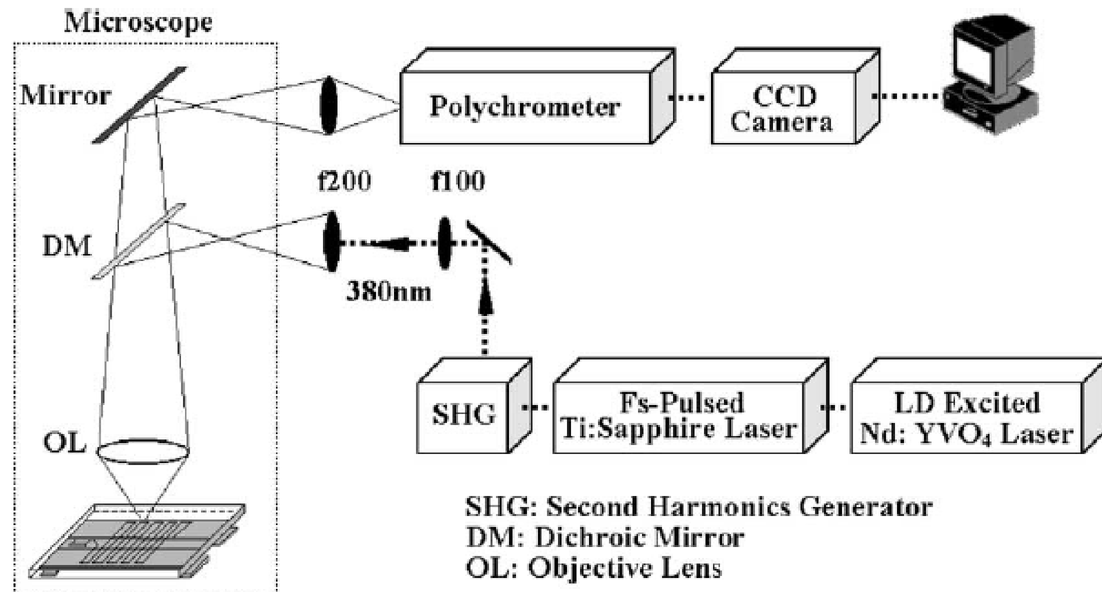
Photolithography is a process used in microfabrication to selectively remove parts of a thin film or the bulk of a substrate. It uses light to transfer a geometric pattern from a photo mask to a light-sensitive chemical on the substrate



Time Permitting…

- **Time permitting, once we have a working set-up in bulk, we will move onto the Photolithography part. Here are some examples below,**





Impact

Need

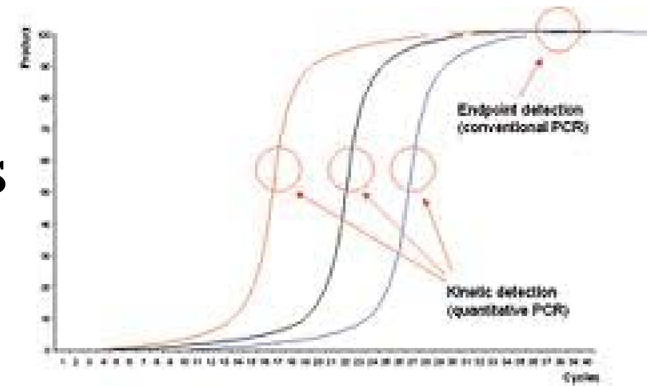
- A low cost set-up is vital in most developing countries, where treatment using such machines can have very high cost machinery.
- Being highlighted a few times in the news, rt-pcr machines would be a great asset especially to the rural areas of Pakistan.

Real-Time PCR Applications

- The introduction of real-time PCR assays to the clinical microbiology laboratory has significantly improved the diagnosis of infectious diseases, and is deployed as a tool to detect newly emerging diseases, such as new strains of flu, in diagnostic tests.
- Viral quantification
- Pathogen detection
- DNA damage (microsatellite instability) measurement

Other Applications

- Radiation exposure assessment
- In vivo imaging of cellular processes
- Mitochondrial DNA studies
- Methylation detection
- Detection of inactivation at X-chromosome
- Linear-after-the-exponential (LATE)-PCR: a new method for real-time quantitative analysis of target numbers in small samples, which is adaptable to high throughput applications in clinical diagnostics, biodefense, forensics, and DNA sequencing



The End

Questions?