Session 3

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Outline

General instrument layout

Fluorescent dyes

AB Real-Time instruments

Various other Real-Time instruments

Real-Time PCR Instrumentation

Basics

Light hits the tube/vessel containing the PCR (once per cycle)

Fluorescent dye(s) emit light corresponding to their spectral characteristics

The emitted light is focused onto a detector

The computer-software interface interprets the detector signal

Real-Time PCR Instrumentation

Excitation light source range
- Visible range 330 – 1100 nm (bulb)
- Laser 488 nm (Argon ion)
- Light Emitting Diodes (specific wavelength)

Emission (fluorescence) range
- Common fluorescent dyes
- 500 – 700 nm
- Filters allow light of a specific wavelength onto detector

Real-Time PCR Instrumentation

Source: laser, LED, tungsten-halogen lamp
- Excite the fluorescent dye

Detector: CCD (charge coupled device), PMT (photomultiplier tube)
- Detect the light emitted from the excited dye

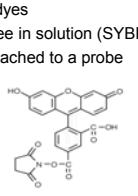
Heating/Cycling
- Traditional heat block (plate)
- Convection (fan oven) (capillaries, single tubes)

How many dyes can be detected?
- Determines the level of multiplexing

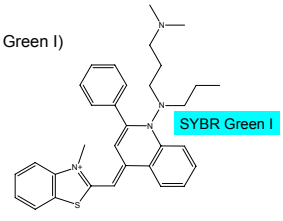
Fluorescence Detection

As the amount of amplified DNA in the PCR increases there is a change in the amount of fluorescence

Organic dyes
- Free in solution (SYBR Green I)
- Attached to a probe



FAM

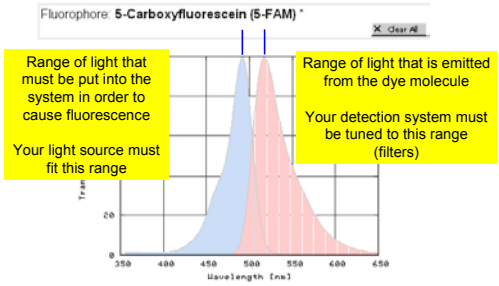


SYBR Green I

Zipper et al., Nucleic Acids Research 2004 32: e103

Dye Characteristics

Absorption and Emission Spectra for 5-FAM



https://www.omegafilters.com/front/curvomatic/spectra.php

Dye Characteristics

Fluorescent dyes commonly used in qPCR

Dye	Excitation (nm)	Emission (nm)
SYBR	497	520
FAM	495	520
TET	521	536
JOE	520	548
VIC		~555
HEX	535	556
R6G	524	557
Cy3	550	570
TAMRA	555	576
NED		~576
Cy3.5	581	596
ROX	575	602
Texas Red	583	603
Cy5	649	670
Cy5.5	675	694

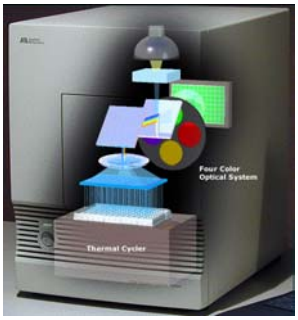
Detecting Multiple Dyes

Multiplexing from an instrument perspective

Dye	Excitation (nm)	Emission (nm)	
SYBR	497	520	
FAM	495	520	1
TET	521	536	2
JOE	520	548	
VIC		~555	
HEX	535	556	
R6G	524	557	
Cy3	550	570	3
TAMRA	555	576	
NED		~576	
Cy3.5	581	596	4
ROX	575	602	5
Texas Red	583	603	
Cy5	649	670	6
Cy5.5	675	694	7

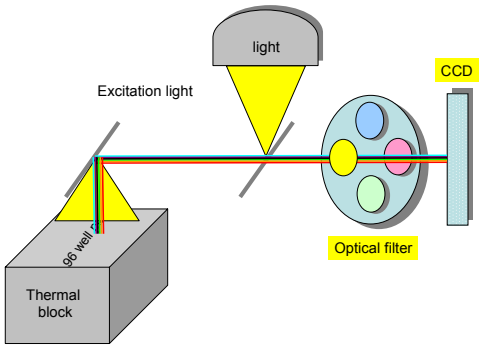
Singleplex - FAM
Duplex - FAM,VIC
Triplex - FAM,VIC,NED

General Schematic of Instrumentation



AB 7000

General Schematic of Instrumentation



AB 7500

AB 7500 is the successor to the 7000
The AB 7300 is similar
7500 can be fitted for 'high speed thermal cycling'
96 well format
5 color detection
Peltier heating block

- Quantifiler
- Quantifiler Y

Picture courtesy of Michelle Shepherd at AB

AB 7500

Tungsten-halogen lamp

Fluorescence emitted from dyes is focused onto a CCD (charge-coupled device)

- Range 500 - 660 nm
- Cycler similar to an AB 9700

Detecting Multiple Dyes

Ability to detect different emission wavelengths

AB 7300	AB 7500
FAM/SYBRI	FAM/SYBRI
VIC/JOE	VIC/JOE
TAMRA	NED/TAMRA/Cy3
ROX	ROX/Texas Red
	Cy5

ROX is typically used as passive reference on AB instruments to correct for variance between wells

AB 7900

High throughput instrument

96 and 384 well format

488 nm argon-ion laser excitation source

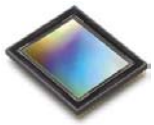
Interfaced with a robotics system

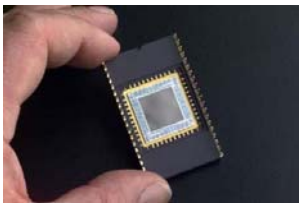
Fluorescence emitted from dyes is focused onto a CCD (charge-coupled device)

- Range 500 - 660 nm

CCD Charge-Coupled Device

A charge-coupled device (CCD) is a light-sensitive integrated circuit that stores and displays the data for an image in such a way that each pixel (picture element) in the image is converted into an electrical charge





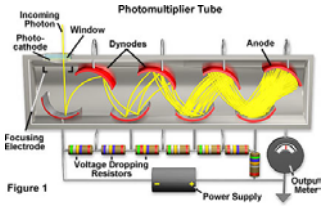
http://mcdonaldobservatory.org/research/instruments/instrument.php?i_id=3

Photomultiplier Tube (PMT)

A photomultiplier tube, useful for light detection of very weak signals

The absorption of a photon results in the emission of an electron

These detectors work by amplifying the electrons



http://micro.magnet.fsu.edu/primer/digitalimaging/concepts/photomultipliers.html

PMT vs CCD

CCD converts light into a digital signal

A photomultiplier tube is typically more sensitive

Choice will depend on the cost and intended use of the instrument

Calibration (AB 7500)

Region of Interest Calibration - ROI plate
- Map fluorescence to specific wells
- Perform for each filter

Background Calibration plate
- Measure ambient fluorescence inherent in the system

Optical Calibration Plate
- On 7500 only
- To compensate for the extra filter

Calibration (AB 7500)

Pure Dye Calibration
- Plates for various dyes (FAM, JOE, NED, ROX, SYBR, TAMRA, VIC, CY-3, CY-5, and Texas Red)
- ~8 min per plate
- Review spectral data

RNase P gene Plate
- Run to complete installation
- Assay is already set up on plate
- Results evaluated to confirm that the instrument in running properly

Other Instrumentation

Other instrumentation exists!
- Different methods of sample heating
- Flexibility (heating – dye detection)
- Portability
- Speed of thermal cycling
- Different light sources
- Cost (initial and consumables)
- Different calibration/maintenance requirements

iCycler - BioRad

Excitation range	475–495 nm
Light source	Tungsten-halogen lamp
Optical detection	CCD camera
Detection range	515–700 nm

http://www.bio-rad.com/iCycler/

iCycler - BioRad

MyIQ: detection of FAM/SYBR Green I

IQ5: multiplexing of up to 5 fluorescent dyes

Peltier heat block

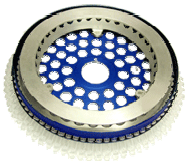
Robust, reliable thermal cycling performance and real-time PCR thermal **gradient** for rapid assay optimization

http://www.bio-rad.com/iCycler/

Rotor-Gene - Corbett

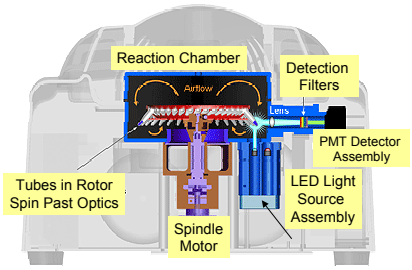
Rotor-Gene 3000 (4 channel)
Rotor-Gene 6000 (6 channel)

Uses a rotor platform to achieve temperature uniformity between wells
Shorter hold times due to rapid temperature equilibration
36 & 72 well rotors
No reference dye (ROX) is needed



http://www.corbettlifescience.com/

Rotor-Gene - Corbett



http://www.corbettlifescience.com/

Rotor-Gene - Corbett

Excitation Source: 470nm, 530nm, 585nm, 625nm LED high power diodes

Not the traditional 96 well format (a Corbett robot can be used to set up the rotor?)

Tube sizes 0.1 and 0.2 mL (10/25 μL min volume)

Emission detection ~500-670 nm range

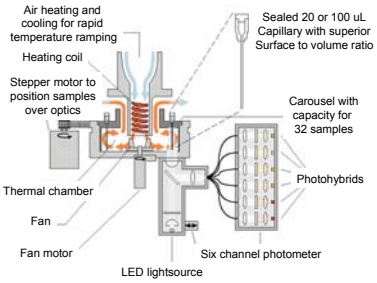
http://www.corbettlifescience.com/

LightCycler - Roche

Uses glass capillaries instead of tubes/plates
Ver 1.5 singleplex and duplex
Ver 2.0 multiplexing (6 colors)
Fast - 40 cycles in 45 min
20 or 100 uL capillaries
32 cap in a single run
LED light source
– 470 nm

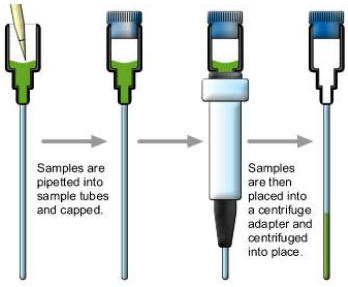
https://www.roche-applied-science.com/sis/rtpcr/lightcycler/index.jsp

LightCycler - Roche



https://www.roche-applied-science.com/sis/rtpcr/lightcycler/index.jsp

LightCycler - Roche



Glass capillaries have the potential to break

https://www.roche-applied-science.com/sis/rtpcr/lightcycler/index.jsp

SmartCycler - Cepheid

- Rapid cycling
- Somewhat portable – field work
- Flexible

http://www.cepheid.com/

SmartCycler - Cepheid

Solid state heater and forced-air cooling at each site

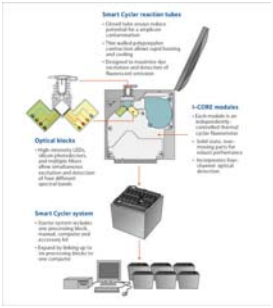
Up to 96 different cycling protocols can be performed simultaneously in one system

Multiple experimental runs can be started at different times, allowing several operators to use the system concurrently

Ver 2.0 multiplexing
25 or 100 uL volumes
16 samples per block – 1-6 blocks per set up
LED light source – 4 channel detection

http://www.cepheid.com/

SmartCycler - Cepheid



Modular approach

A single tube fits into a I-CORE module

16 I-CORE modules can be placed into a single processing block

Each I-CORE module can be independently controlled

Up to 6 processing blocks can be controlled by the software

http://www.cepheid.com/

SmartCycler - Cepheid

Useful for running different protocols simultaneously

Flexibility to start runs independently

R.A.P.I.D. – Idaho Technologies

RAPID Ruggedized Advanced Pathogen Identification Device

http://www.idahotech.com/rapid/index.html

R.A.P.I.D. – Idaho Technologies

A specialty instrument for military field hospitals, first responders and other rough environments

Integrates Idaho Technology's LightCycler® Instrument technology into a portable, impact resistant package

Distinctive software allows simple "push button" use of the R.A.P.I.D. system by field personnel with minimal training

Allows for field identification of possibly dangerous pathogens quickly, safely, and accurately

http://www.idahotech.com/rapid/index.html

R.A.P.I.D. – Idaho Technologies

Cycling protocols are developed and can be selected from a simple menu

http://www.idahotech.com/rapid/index.html

R.A.P.I.D. – Idaho Technologies

50 lbs in a backpack

Can be dropped from 1 meter
- 15 cm while running

Build to withstand: environmental conditions including smoke, dust, rain, salt spray, high humidity, temperature extremes, reduced atmospheric pressure, and sand.

http://www.idahotech.com/rapid/index.html

Summary

Process
- Excitation Light (sources)
- Thermal cycling (platforms)
- Fluorescence (filter, ranges)
- Detection (CCD or PMT)

The number of dyes detected = potential multiplexing of assays

There are many different Real Time Instruments with unique characteristics

All instruments come with a software package for data analysis