

Separation and Detection of Nucleic Acids

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Objectives

- Electrophoresis and its variants
 - Gel Electrophoresis
 - Restriction Fragment Length Polymorphism
 - Capillary Electrophoresis
- Real-Time PCR and its Variants
 - Quantitative and qualitative real-time PCR
 - Digital PCR
 - Melt Curve Analysis
- MALDI-TOF
 - Use in Clinical Microbiology
 - Use in Molecular Diagnostics

References and Additional Resources

- Buckingham, Lela. *Molecular Diagnostics: Fundamentals, Methods, and Clinical Applications*. 3rd ed., F.A. Davis, 2019.
 - Chapter 4: Resolution and Detection of Nucleic Acids
 - Chapter 5: Analysis and Characterization of Nucleic Acids and Proteins
 - Chapter 6: Nucleic Acid Amplification
 - Chapter 8: Gene Mutations

- Rifai, Nader, et al. *Principles and Applications of Molecular Diagnostics*. 3rd ed., F.A. Davis, 2019.
 - Chapter 4: Nucleic Acid Techniques

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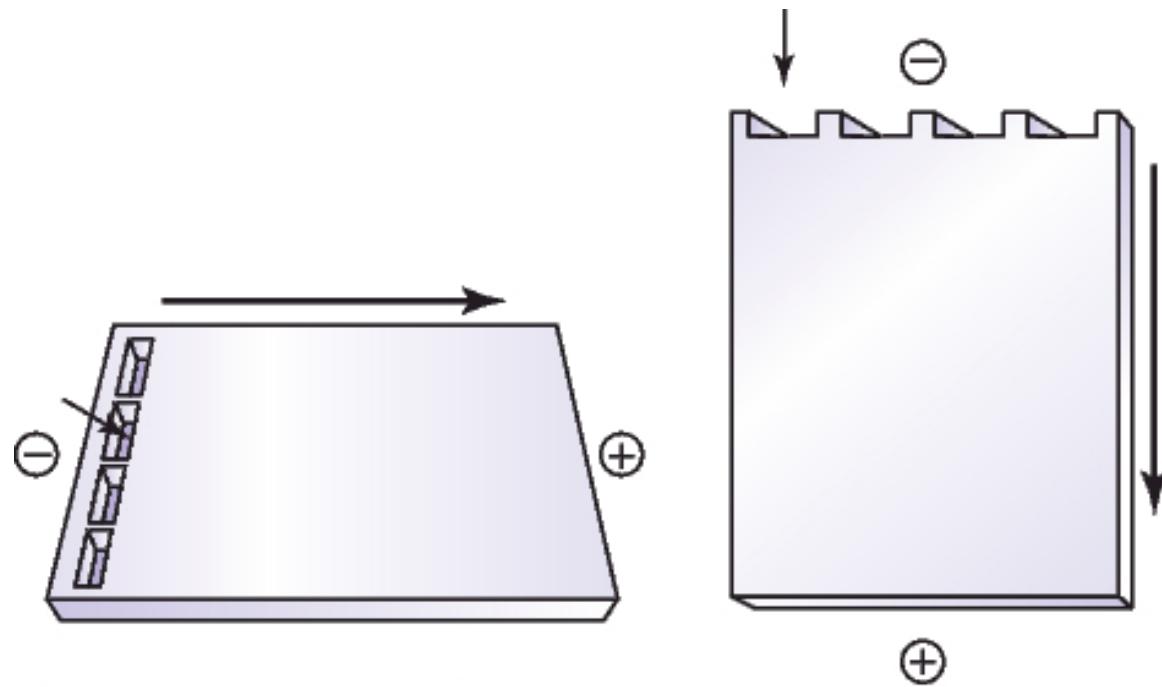
Electrophoresis and its Variants

- Gel Electrophoresis
- Restriction Fragment Length Polymorphism (RFLP)
- Capillary Electrophoresis
- Pulsed-Field Gel Electrophoresis

Electrophoresis utilizes an electric current to mobilize molecules through a matrix.

Phosphate group of the sugar-phosphate backbone gives nucleic acids a negative charge.

This means that when an electric current is applied, DNA and RNA will migrate **towards the positive pole** (anode) and **away from the negative pole** (cathode).



Mobility

Mobility is the ability of a molecule to migrate through the sieve-like matrix of an electrophoretic system.

The mobility of DNA and RNA molecules is primarily affected by three factors:

- **Molecular weight**
 - High-molecular weight molecules will travel slower than smaller molecules
 - dsDNA travels slower than ssDNA and ssRNA
- **Pore size**, or how much space is between the individual molecules of the matrix
 - The more densely-packed a matrix is, the harder it is for big molecules to migrate.
- **Charge-to-mass ratio**
 - In practice, charge-to-mass ratio remains constant across DNA/RNA of varying sizes

In general, DNA/RNA fragment mobility is inversely proportional to fragment size (bp).

- Small fragments migrate faster than large fragments.

Gel Systems

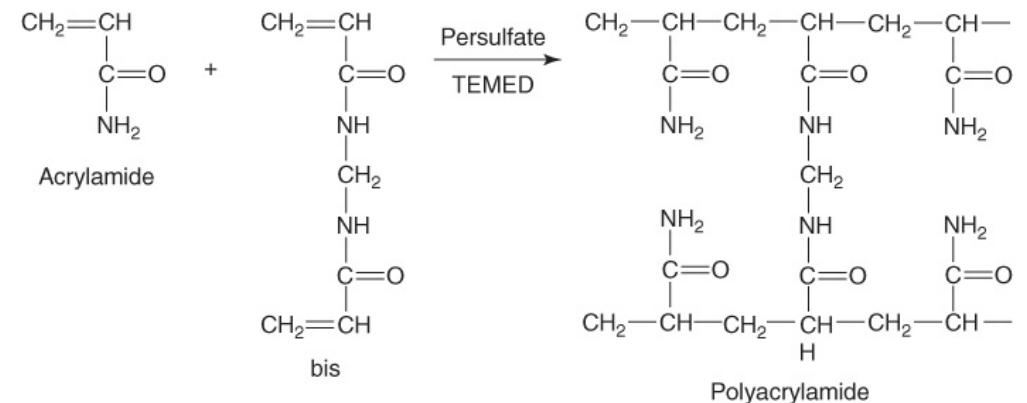
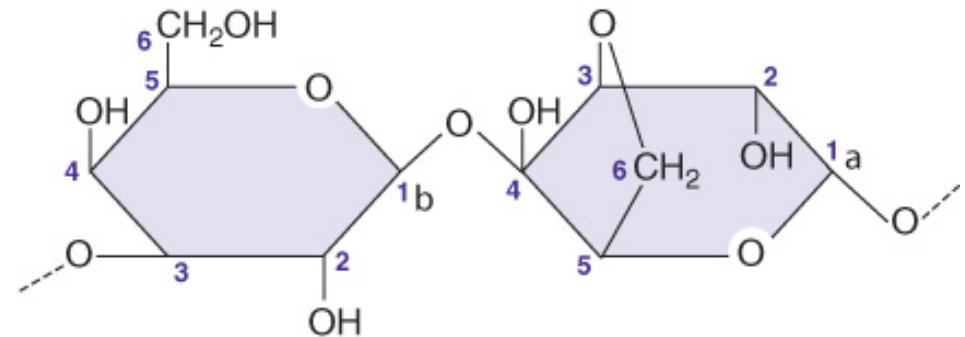
There are two primary gel systems used for electrophoresis.

Agarose gels are composed of a polysaccharide polymer extracted from seaweed.

- Non-toxic, cheap and easy to make
- Poorer banding resolution relative to PAGE.

Polyacrylamide gels (PAGE) are composed of a synthetic polymer of crosslinking acrylamide and bis-acrylamide molecules.

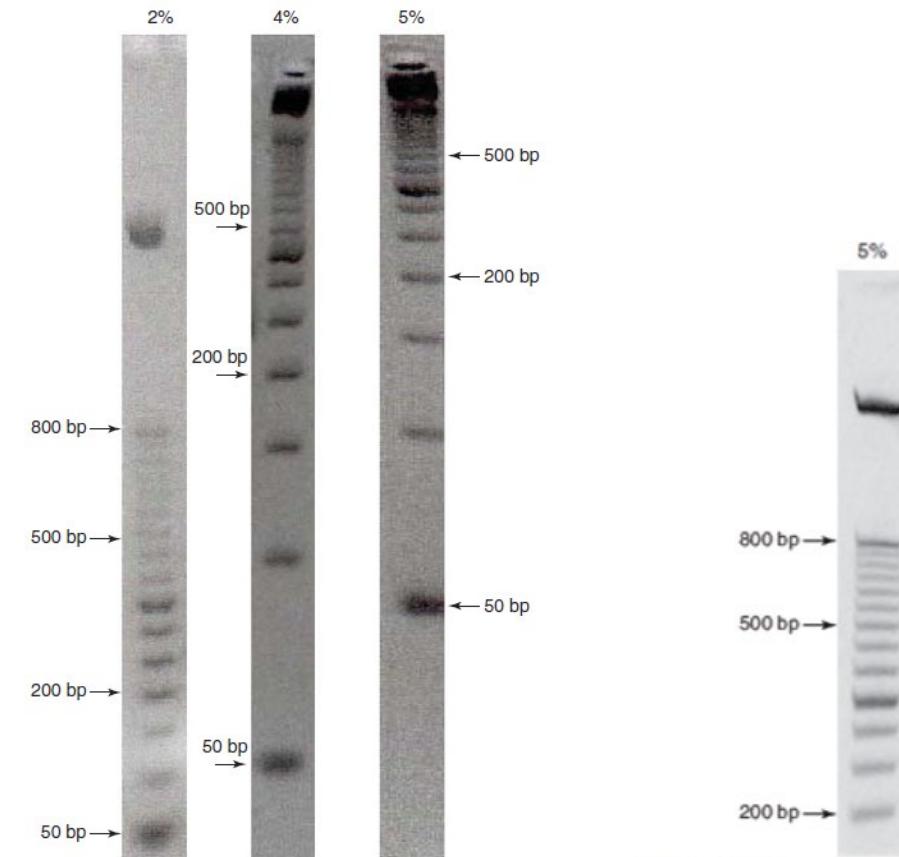
- More expensive, difficult to manufacture, and toxic.
- Offers higher and more consistent resolution than agarose, especially for smaller bp fragments.



Pore Size

For both gel types, increasing gel concentration will decrease pore size.

- Too low of a concentration and fragments will not separate during migration because pore size is too large.
- Too high of a concentration and DNA will not migrate at all because pore size is too small.



■ **Figure 5-3** Resolution of double-stranded DNA fragments on 2%, 4%, and 5% agarose.

■ **Figure 4-6** Resolution of double-stranded DNA fragments on a 5% polyacrylamide gel.

Gel Concentration

Choice of Agarose Concentration for DNA Gels

Agarose Concentration (%)	Separation Range (size in bp)
0.3	5000-60000
0.6	1000-2000
0.8	800-10000
1.0	400-8000
1.2	300-7000
1.5	200-4000
2.0	100-3000

Choice of Acrylamide Concentration for DNA Gels

Acrylamide Concentration(%)	Separation Range (size in bp)
3.5	100-1000
5.0	80-500
8.0	60-400
12.0	40-200
20.0	10-100

In general...

0.5-5% for agarose gels

3.5-20% for polyacrylamide gels

Buffer Systems

A **buffer** is a solution of a weak acid and its conjugate base, and serves several functions in gel electrophoresis:

- Maintains pH of the gel
- Protects nucleic acid molecules from damage
- Carries the electric current through buffer ions

Increasing buffer concentration increases conductivity and should be met with a proportional reduction in voltage.

Tris - and EDTA-based buffers are commonly used for nucleic acid electrophoresis.

- Tris borate EDTA (TBE)
- Tris phosphate EDTA (TPE)
- Tris acetate EDTA (TAE)

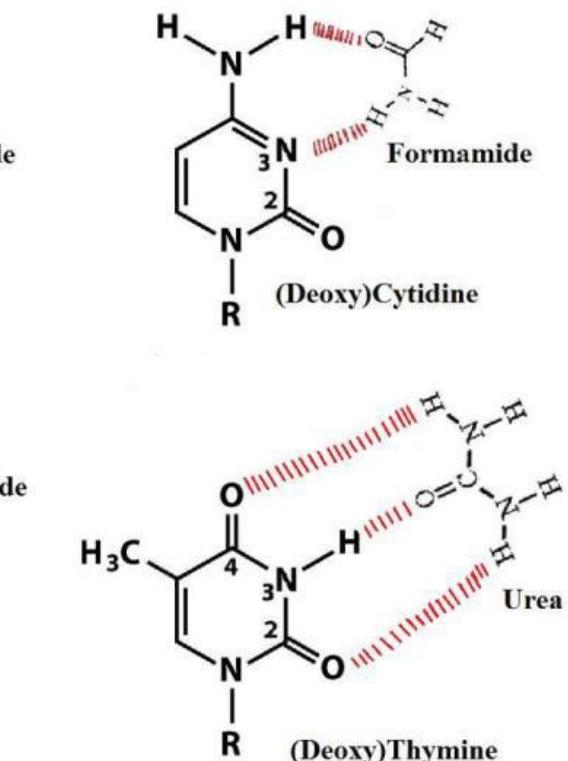
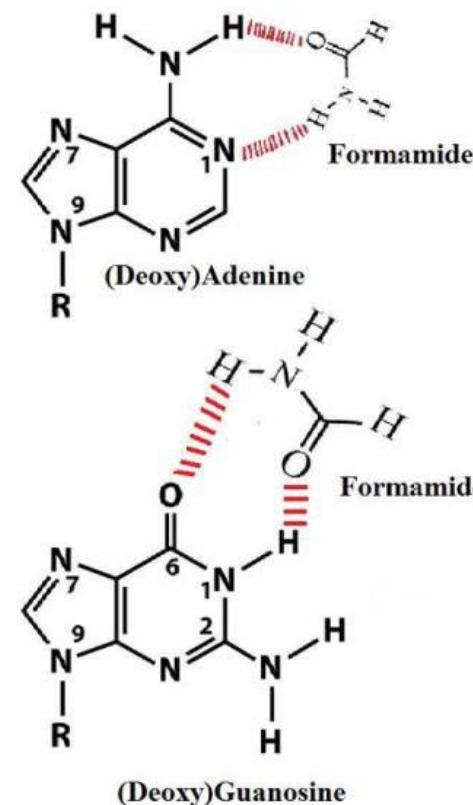
Denaturing Agents

As previously described, dsDNA vs. ssDNA and linear vs. non-linear ssRNA have different mobilities.

To adjust for this, PCR product is usually heat denatured prior to electrophoresis to ensure a linearized single-stranded structure.

Incorporation of denaturing agents helps maintain single-stranded structure by preventing complementary base pairing.

- DNA – **formamide** or urea
- RNA – methylmercuric hydroxide (MMH)



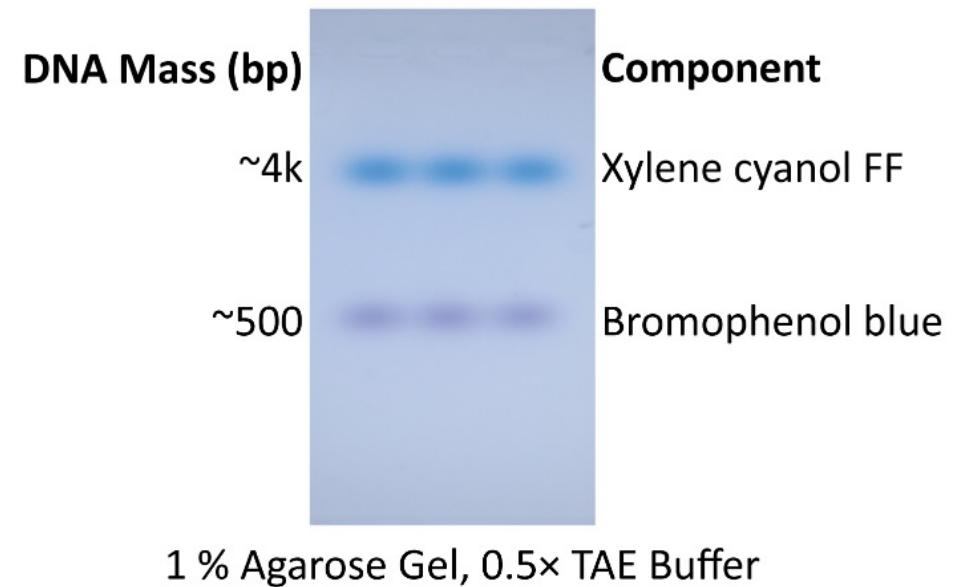
Tracking Dyes

Nucleic acids are colorless, making their migration difficult to monitor. Running a gel for too long can result in the loss of bands as they run off the bottom of the gel.

To prevent this, tracking dyes are loaded into sample wells as a visual way of monitoring overall migration.

- Bromophenol blue
- Xylene Cyanol

Tracking dyes ideally migrate ahead of smallest sample fragments so that electrophoresis can be stopped prior to band loss.

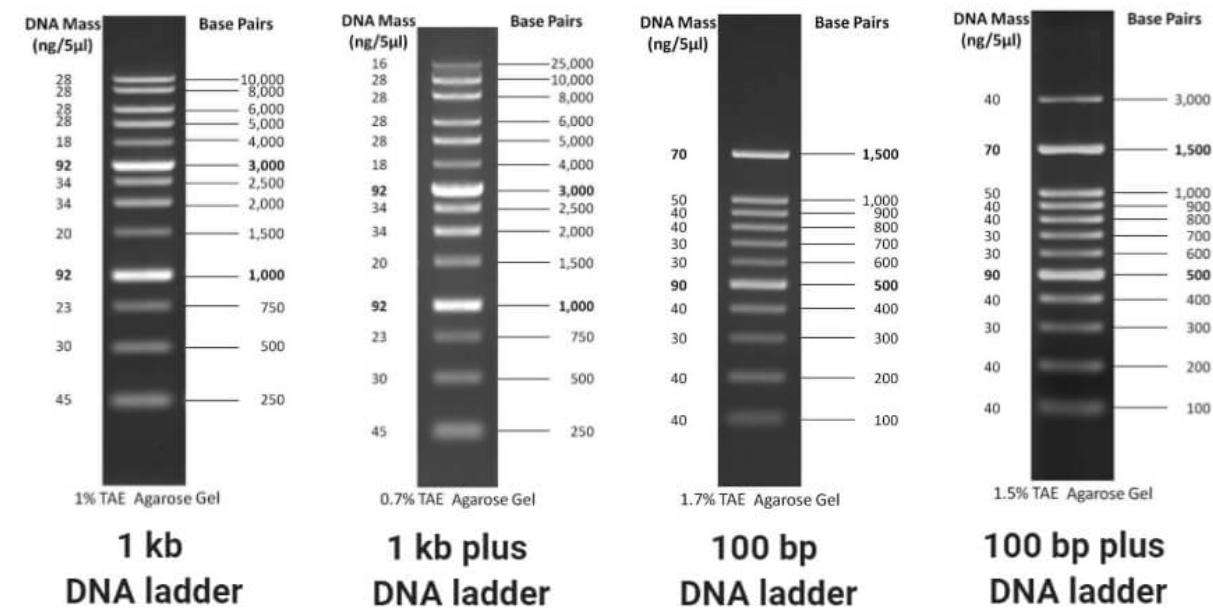


Sizing Ladder

Wells on the side(s) of the gel are designated for a **sizing ladder**.

The sizing ladder is a manufactured standard with a known number of fragments of known base pair size.

Size of patient sample fragments can be determined by comparison to the ladder.



Fluorescent Detection

Fluorescent dyes are commonly used to visualize nucleic acids as/after they migrate during electrophoresis.

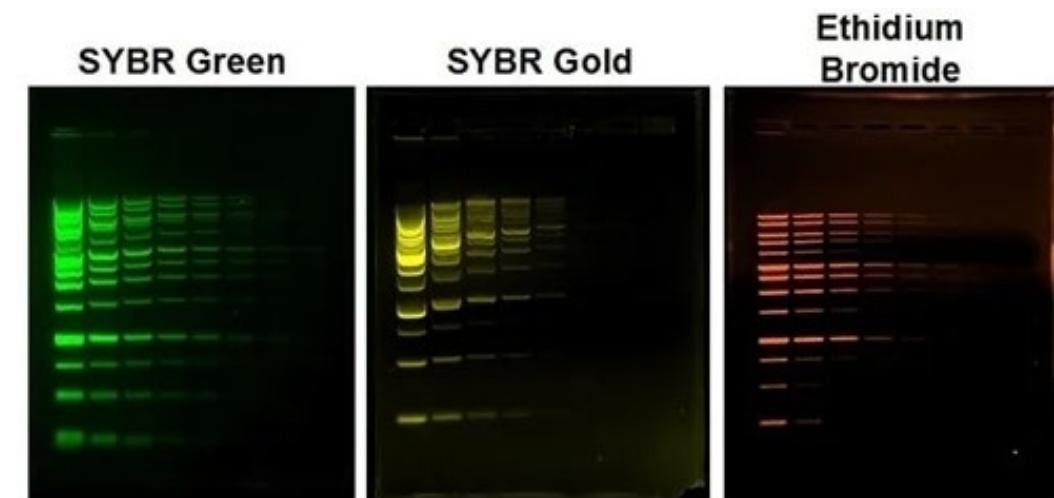
Ethidium bromide (**EtBr**) intercalates between successive nitrogenous bases .

- Intercalating nature makes it a potent carcinogen, mostly replaced by less hazardous fluorophores

SYBR dyes bind to the minor grooves of nucleic acid chains.

- More sensitive than EtBr and non-mutagenic

Dye	Method	Target	Spectrum (nm)
EtBR	intercalation	DNA/RNA	300 – 590
SYBR Green I	minor groove	dsDNA	300 – 520
SYBR Green II	minor groove	ssDNA/ssRNA	300 – 520
SYBR Gold	minor groove	DNA/RNA	300 – 537



Colorimetric Detection

Silver Stain is a highly sensitive, colorimetric method of visualizing protein and DNA.

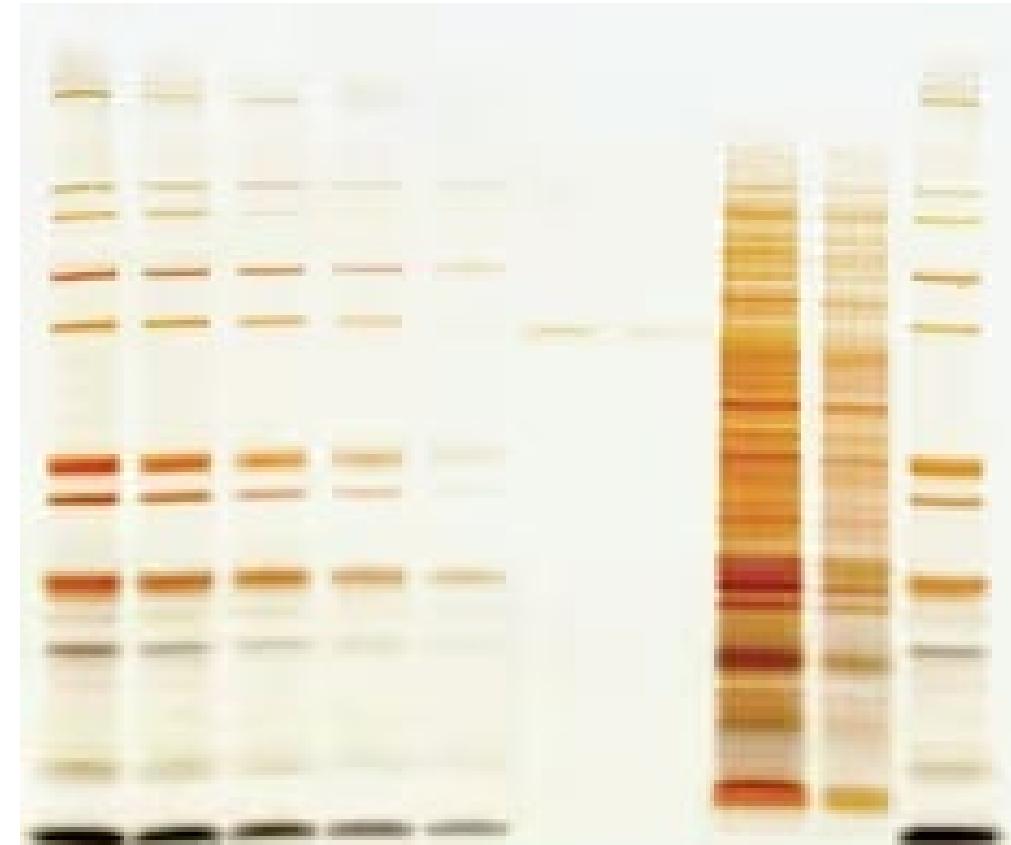
- Following migration, silver diamine or silver nitrate is applied to gel in a weakly acidic solution.
- Silver ions interact with protein/DNA target and form insoluble black precipitate.

Pros:

- More sensitive than fluorescent dyes
- Non-mutagenic
- No need for UV light to visualize

Cons

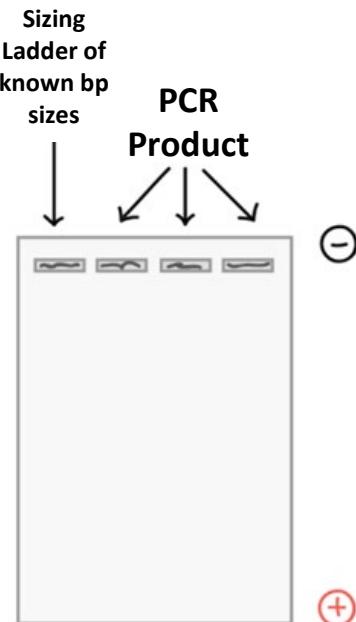
- More complicated staining process



Gel Electrophoresis – The Process

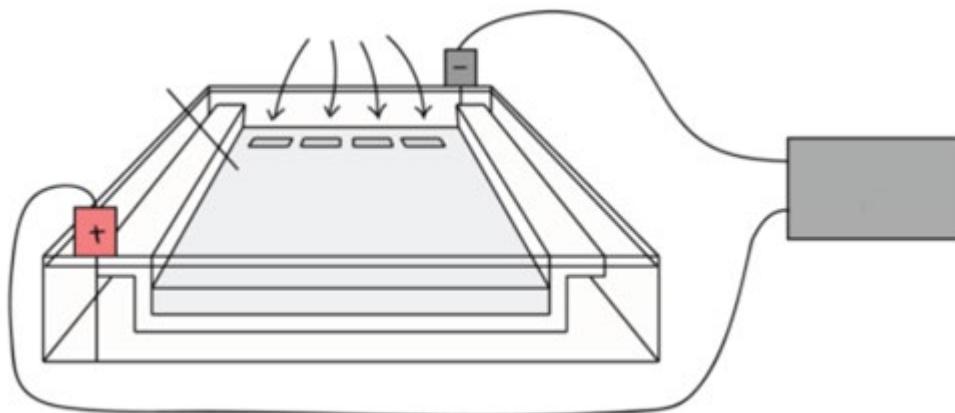
Step 1

Sizing ladder and samples are mixed with tracking dye and added to loading wells of the gel near the cathode.



Step 2

Power source is turned on, creating a current between the cathode and anode. Negatively-charged DNA travels toward the positively-charged pole (anode).



Step 3

Application of UV light allows for visualization of fluorescently stained nucleic acid bands. Comparison to the sizing ladder allows for visual determination of base pair length of each sample fragment.



Restriction Enzymes

Restriction enzymes are endonucleases that recognize specific base sequences and break/restrict the DNA polymer at the sugar-phosphate backbone.

REs are named after the bacteria from which they're derived:

- *EcoRI* = *Escherichia coli* RY13
- *SmaI* = *Serratia marcescens* Sb_a
- *PstI* = *Providencia stuartii*

The **recognition site**, or **binding site**, is a 4-8bp sequence that is recognized by the restriction enzyme. It's presence in a sequence is what allows a RE to bind and cut dsDNA.

- Many are **palindromic**, meaning the sequence is read the same 5'-3' on both strands.

The **cut site** is the location where the enzyme restricts the DNA. Depending on the specific RE, this can be within the recognition site or thousands of base pairs away.

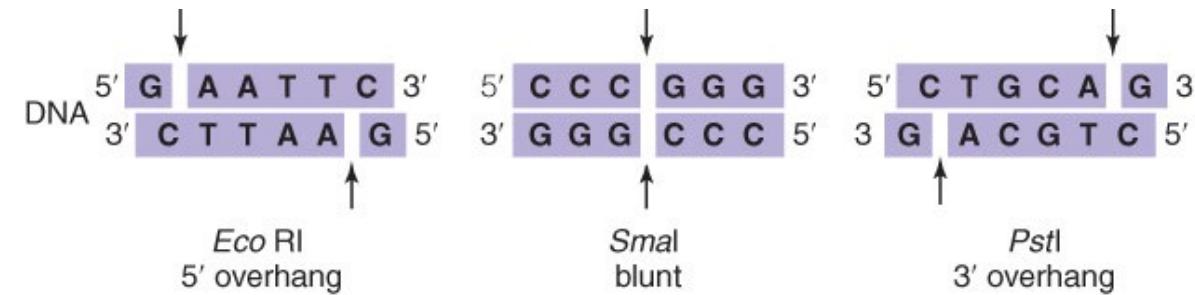
Restriction Enzymes

Cuts can be differentiated by whether the ends of DNA are left “sticky” or “blunt” after cutting.

- **Sticky ends** are made by unequal cuts that leave one DNA strand longer than the other, such that the longer strand has unpaired bases.
- **Blunt ends** are made by equal cuts that leave both strands the same length, such that no strand has unpaired bases after cutting.

Examples:

- *EcoRI* = produces “sticky” ends with a 5’ overhang
- *SmaI* = produces blunt ends
- *PstI* = produces “sticky” ends with a 3’ overhang



Restriction Enzymes

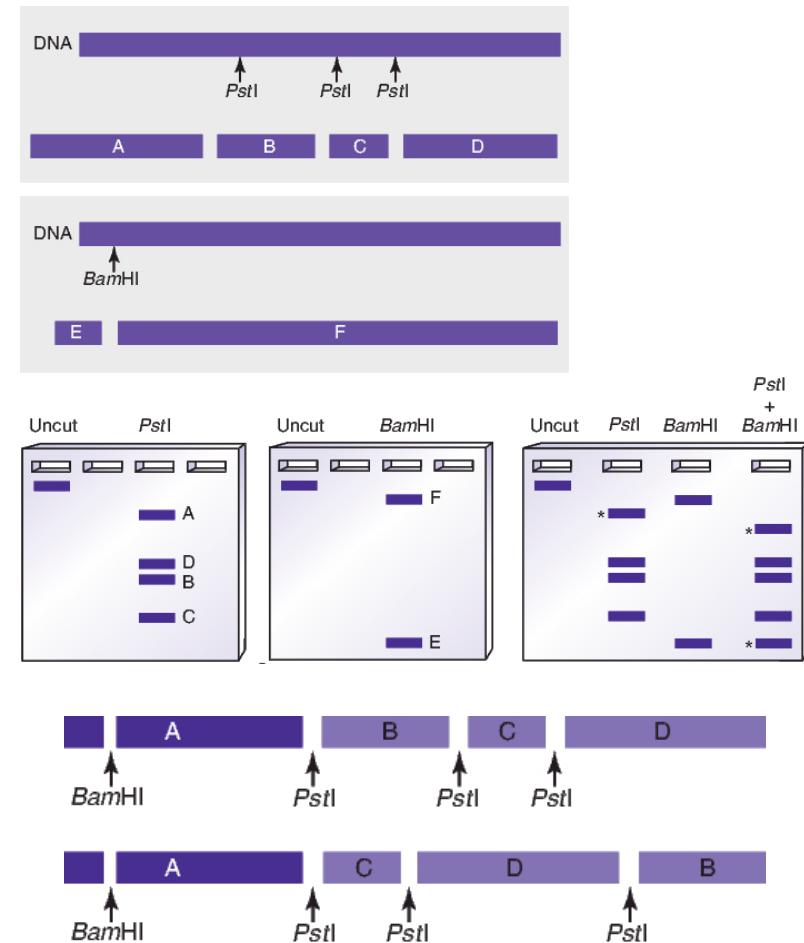
Type	Methylation Activity	Binding Site	Binding Site Example	Example	Cut Site
I	+	Asymmetrical	5'-A ^{Me} CNNNNNNNGT -3' 3'-T GNNNNNNCA ^{Me} -5'	EcoK	Remote (>1000bp) and variable distance from binding site
II	-	Palindromic	5'-CCCGGG-3' 3'-GGGCC-5'	SmaI	Within or close (1-20bp) to binding site
III	+	Asymmetrical	5'-CTGAT G-3' 3'-GACTA ^{Me} C-5'	PstI III	25-27bp 3' to binding
IV	+	Asymmetrical	5'-CTCAG-3' 3'-GAGTC-5'	BseM II	Asymmetrical, with one strand cut one distance from recognition site (i.e., 8bp) and the other strand cut another distance (i.e., 10bp)

RFLP and Restriction Mapping

Restriction enzyme digestion of DNA generates fragments of different lengths, depending on how many binding sites are present in sequence.

Sample differences in the number of RE binding sites, and thus the number and size of fragments produced by RE digestion, are called **restriction fragment length polymorphisms (RFLP)**.

Restriction mapping of RFLP patterns can be used for human identity testing and epidemiological testing of microorganisms.



PCR-RFLP

PCR-RFLP testing utilizes restriction enzymes to determine sequence polymorphisms among patient samples.

Particularly useful for targeted SNP analysis.

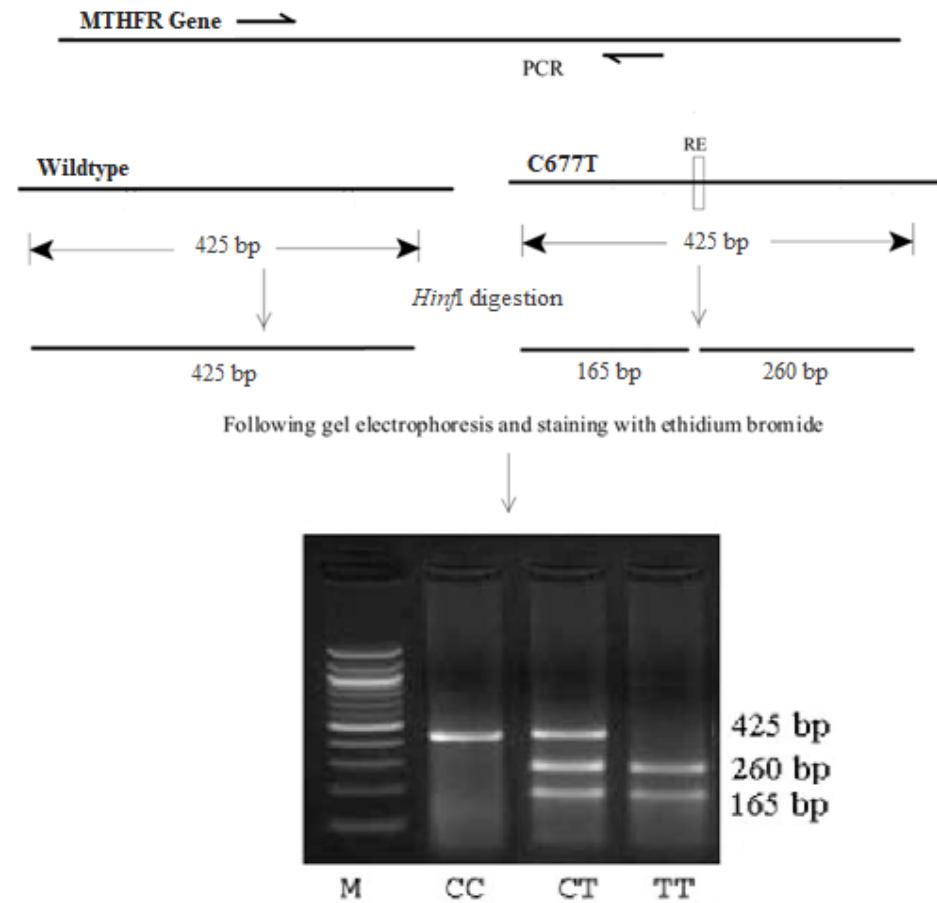
Process:

- Target region is amplified by primer-driven PCR.
- PCR product is then digested by restriction enzyme.
 - RE should have binding site at same locus as mutation
 - Mutation can add or remove binding site(s)
- Digested product is then resolved by electrophoresis.
- Genotype (WT, HET, HOM) can be interpreted from the number and size of fragments visualized.

PCR-RFLP: Two Scenarios

Scenario One: Mutation Adds Binding Site

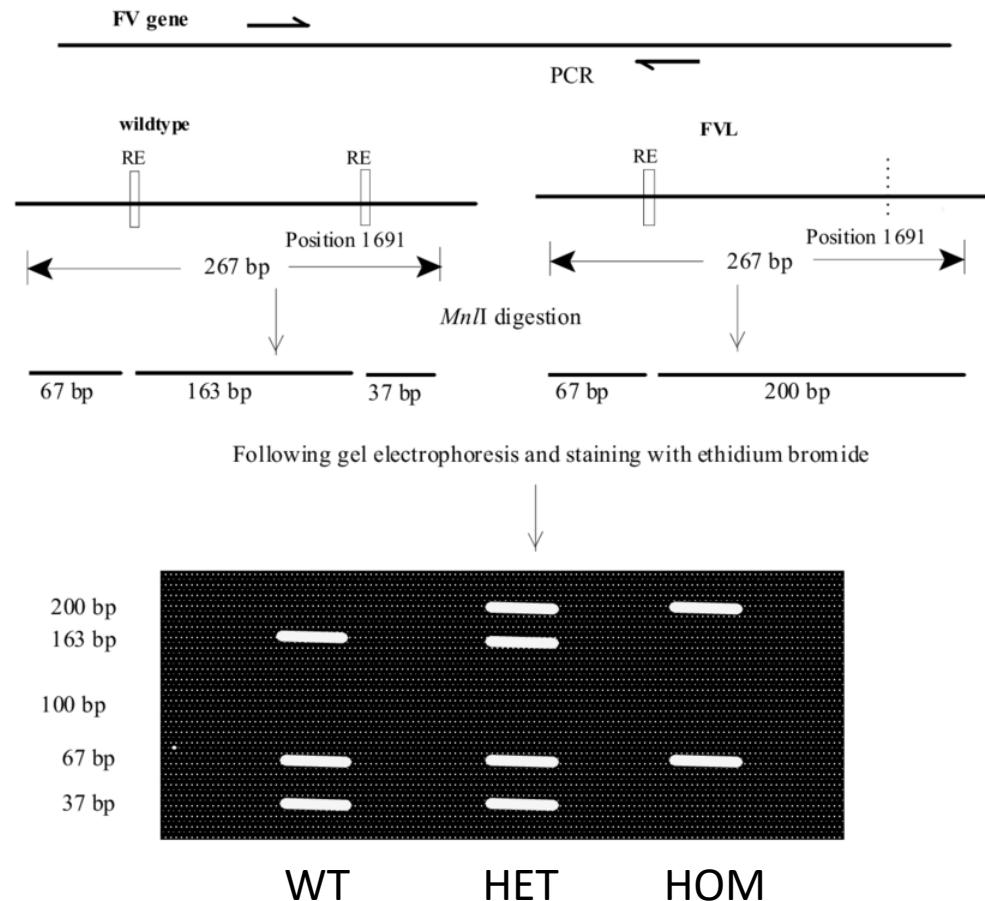
- Primer-driven amplification of a 425bp target sequence of the MTHFR gene normally contains no *HinfI* binding sites.
 - Wildtype patients: no cuts are made by *HinfI*, so only one band is generated (425bp)
- Point mutation (677C>T) in the MTHFR gene results in the addition of one *HinfI* binding site.
 - Homozygous patients: one cut on both mutant copies of the gene generates two bands (165bp and 260bp)
 - Heterozygous patients: one cut on mutant allele generates two bands (165bp and 260bp) while no cuts on wildtype allele generates one band (425bp) for a total of three visible bands (165bp, 260bp, 425bp)



PCR-RFLP: Two Scenarios

Scenario Two: Mutation Removes Binding Site

- Primer-driven amplification of a 267bp target sequence of the Factor V gene normally includes two binding sites for *MnII*.
 - Wildtype patients: two cuts on each copy of the gene generate three bands (67bp, 163bp, and 37bp)
- Point mutation (1691G>A) in the Factor V gene results in the removal of the second *MnII* binding site.
 - Homozygous patients: one cut on both mutant copies of the gene generates two bands (67bp and 200bp)
 - Heterozygous patients: one cut on mutant allele generates two bands (67bp and 200bp) while two cuts on wildtype allele generates three bands (67bp, 163bp, and 37bp) for a total of four visible bands (37bp, 67bp, 163bp, and 200bp)



Capillary Electrophoresis

In **capillary electrophoresis**, nucleic acids are separated within thin glass capillaries containing a fluid-polymer matrix.

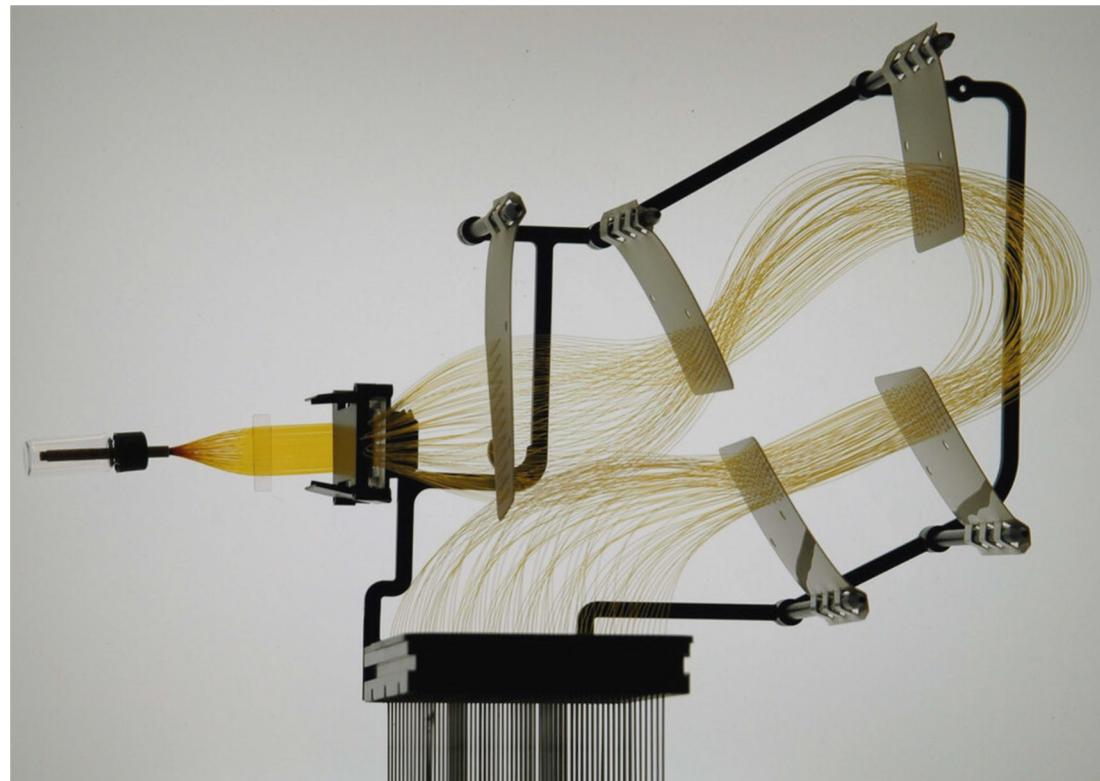
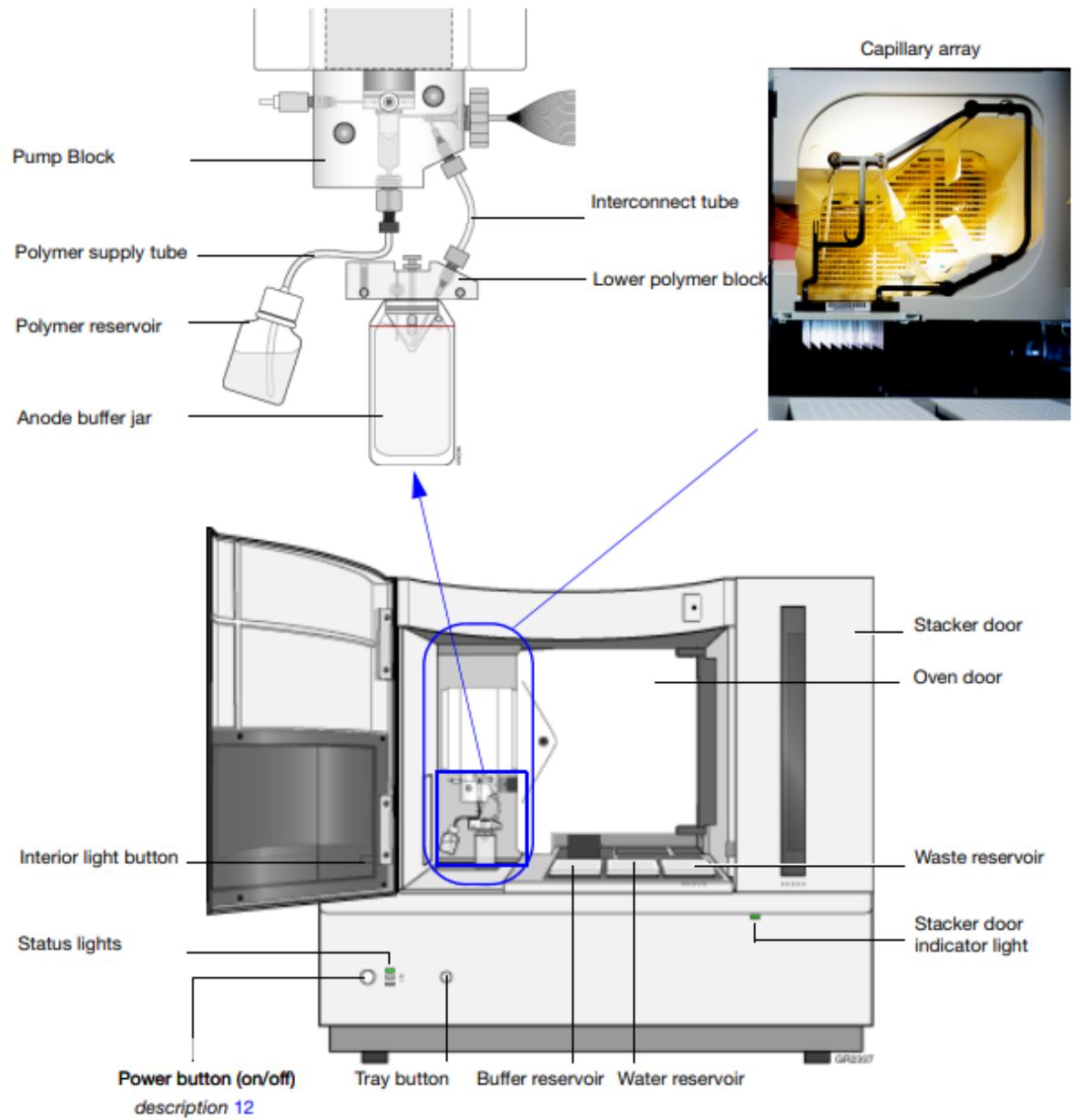
Similar principles to gel electrophoresis:

- Negatively charged DNA migrates toward the anode (positive pole).
- Larger fragments migrate slower, smaller fragments migrate faster.
- ssDNA travels faster than dsDNA, so denaturation (heat, formamide) is still necessary.



Applied Biosystems 3730 DNA Analyzer

Polymer Delivery Pump (PDP)



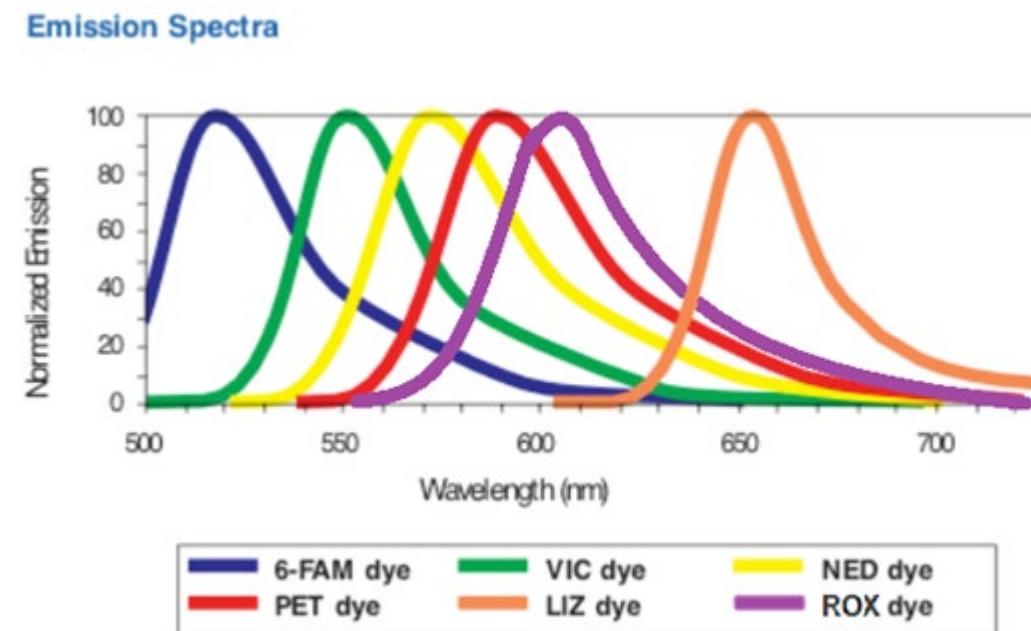
Capillary Electrophoresis – The Process

DNA is first amplified by PCR

- Fluorescently-labelled **dye primers** target sequences of interest.
- If target sequence is present, amplification will occur.
- Fluorescently tagged fragments will be detectable by CE instrument

Using multiple fluorophores allows for **multiplexing** of the reaction.

- Different primer sets, targeting different sequences, tagged with different fluorophores, detected at different wavelengths.
- Detects multiple targets in a single reaction well



Capillary Electrophoresis – The Process

CE plate is loaded with the following:

- Fluorescently-labelled PCR product
- Formamide, to maintain denaturation
- Sizing ladder, labelled with a unique non-interfering fluorescent dye

Plate is denatured under high temperature to prevent secondary structure of nucleic acids from forming.

Plate is then loaded onto the CE instrument



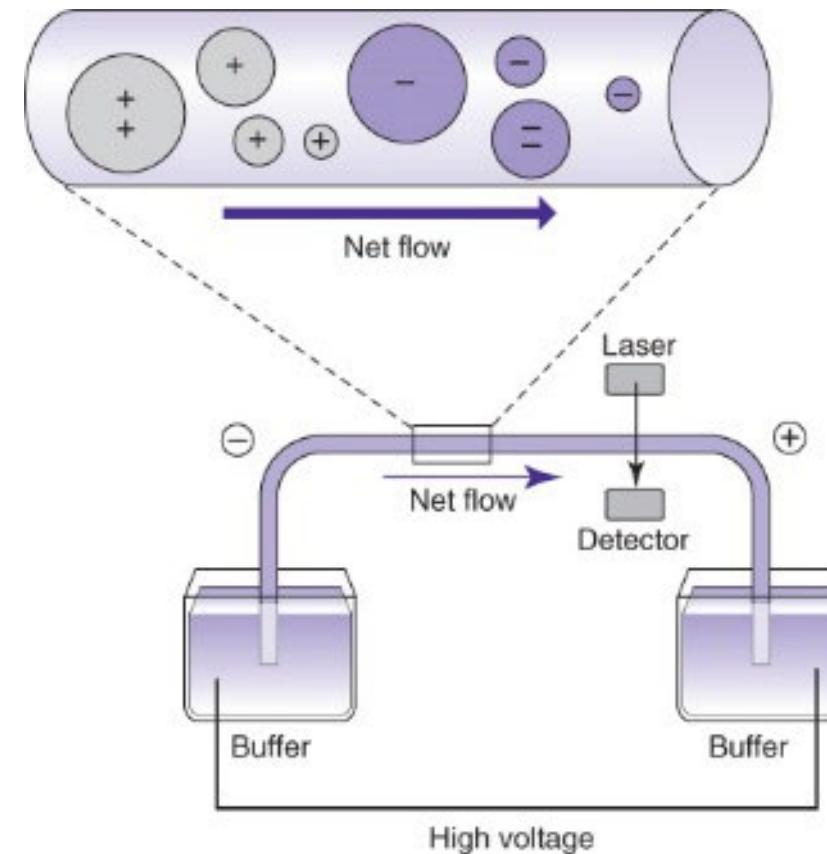
Capillary Electrophoresis – The Process

Samples (with ladder) are applied to the capillary by **electrokinetic injection**.

- Current is applied to platinum-tipped capillaries to create a transient positive charge that draws DNA out of solution and into the capillary.

Electro-osmotic flow within capillaries is then generated, causing DNA fragments to migrate from injection site toward the anode.

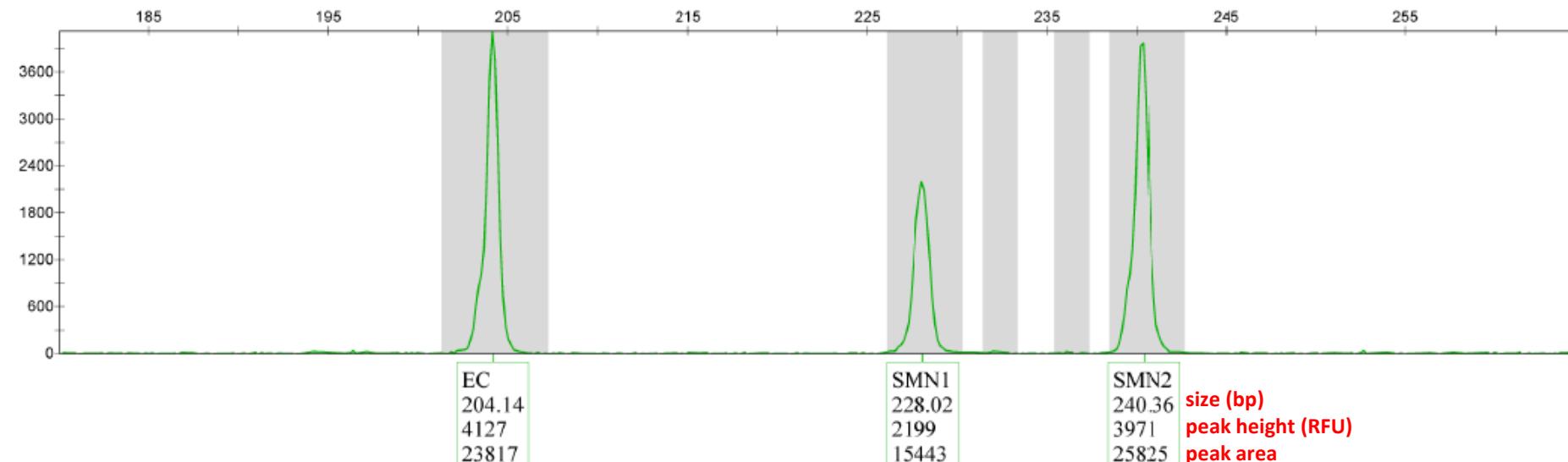
Near the end of the capillary, a detection unit reads fluorescently-tagged fragments as they pass through a laser and are excited.



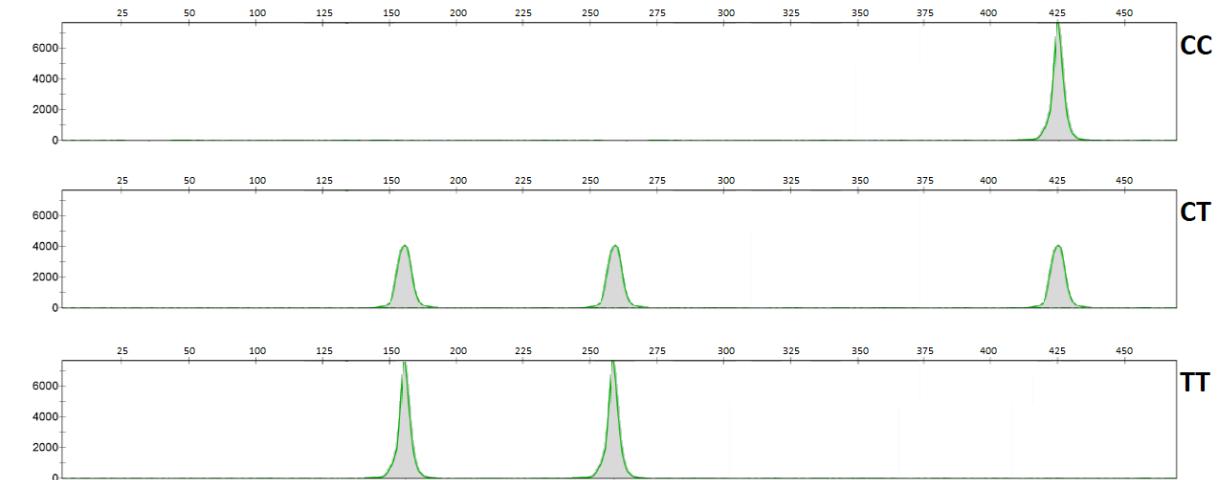
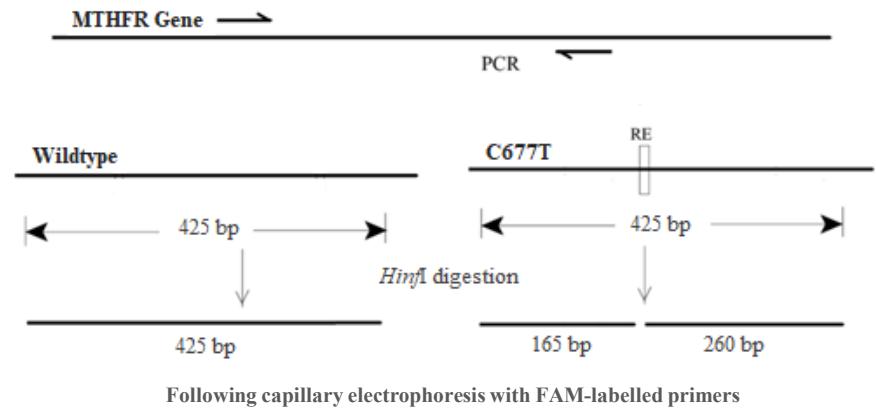
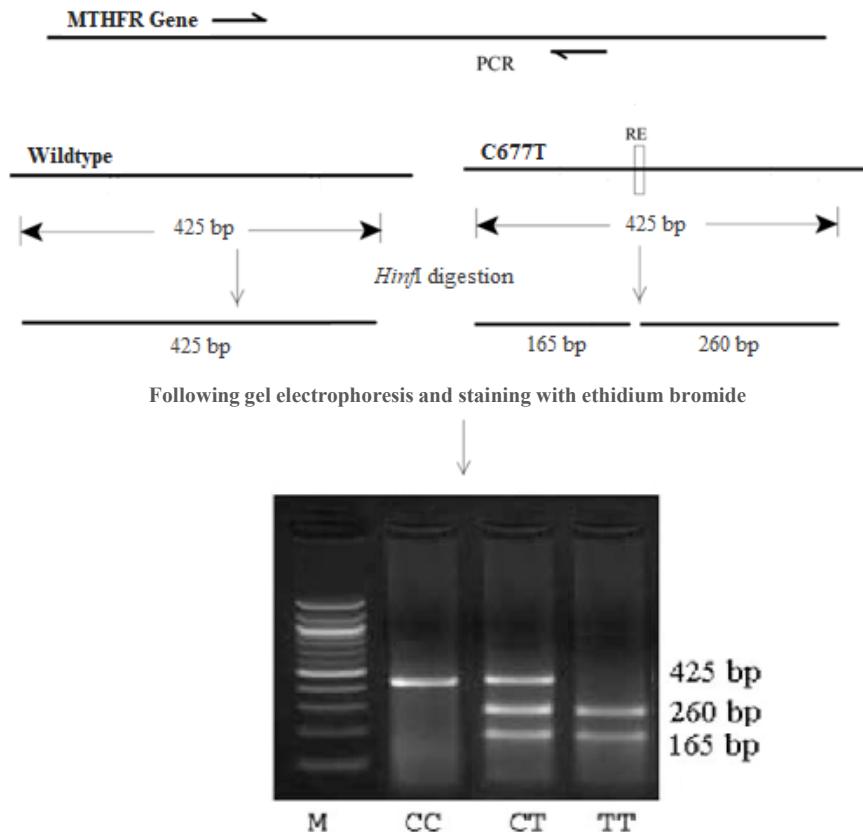
Capillary Electrophoresis – The Process

Analysis software generates an **electropherogram** of the CE run data.

- Fragments appear as peaks.
- Comparison of peak to size ladder of known base pair sizes allows software to auto-calculate base pair size of each fragment along the x-axis.
- Intensity of fluorescent signal for each fragment, measured in **relative fluorescent units (RFU)** along the y-axis, is demonstrated by the height of the peak and the area underneath it.



Comparing Capillary and Gel Reads



Fragment Analysis – FLT3-ITD

Let's use FLT3-ITD testing as a case study.

Why we test FLT3-ITD:

- FLT3 is a member of the class III receptor tyrosine kinase family and is primarily expressed on hematopoietic stem cells.
- Internal tandem duplication (ITD) of the FLT3 gene results in uncontrolled cell proliferation of leukemic cells.
- FLT3-ITD mutation is associated with poorer prognosis.

What we need to know to gauge prognosis:

- Is a FLT3-ITD mutation present?
- How large is the ITD fragment?
- What is the allelic ratio of WT to MUT?

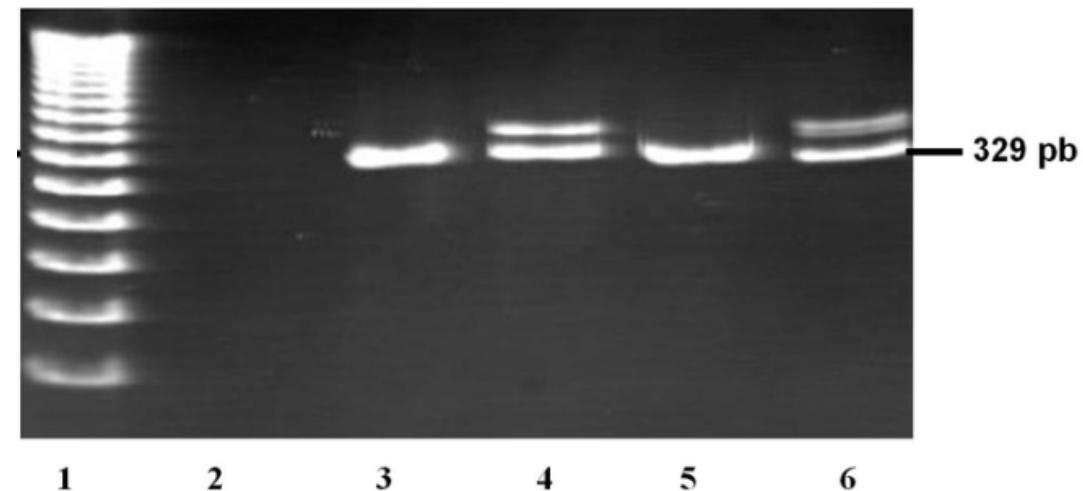
FLT3-ITD – Gel Electrophoresis

Primers are designed to flank the area of the FLT3 gene where ITD mutation occurs.

When no mutation is present, amplification should produce a single 329bp product.

Image shows results for five samples run on 12% polyacrylamide gel and stained with EtBr

- Lane 1 = sizing ladder
- Lane 2 = NTC, no amplification
- Lane 3 = WT control, no mutation detected
- Lane 4 = 50% POS control, mutation detected
- Lane 5 = Patient, no mutation detected
- Lane 6 = Patient, mutation detected



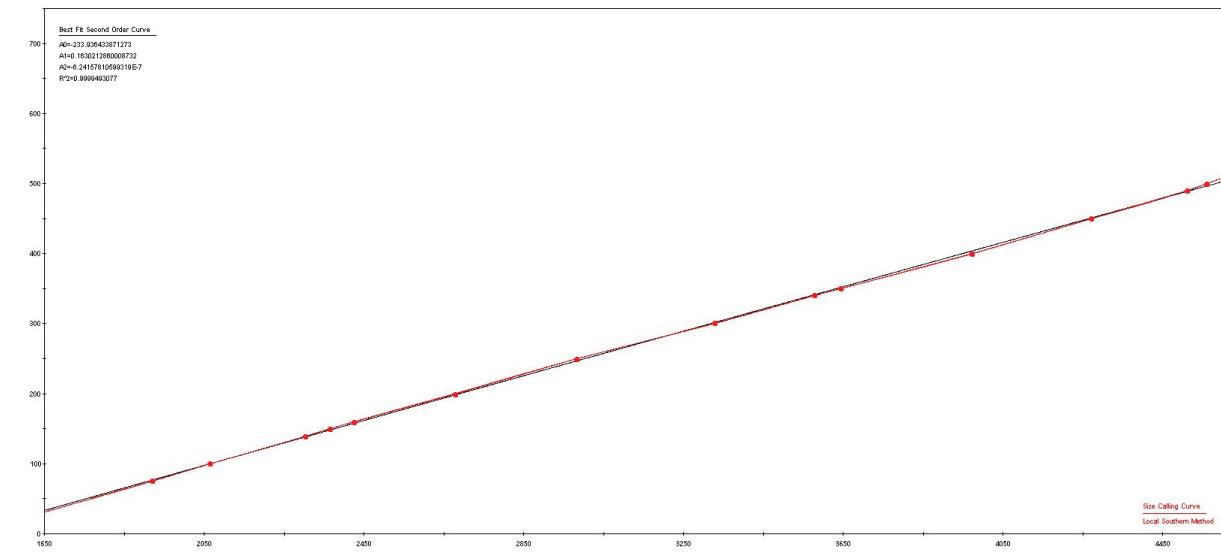
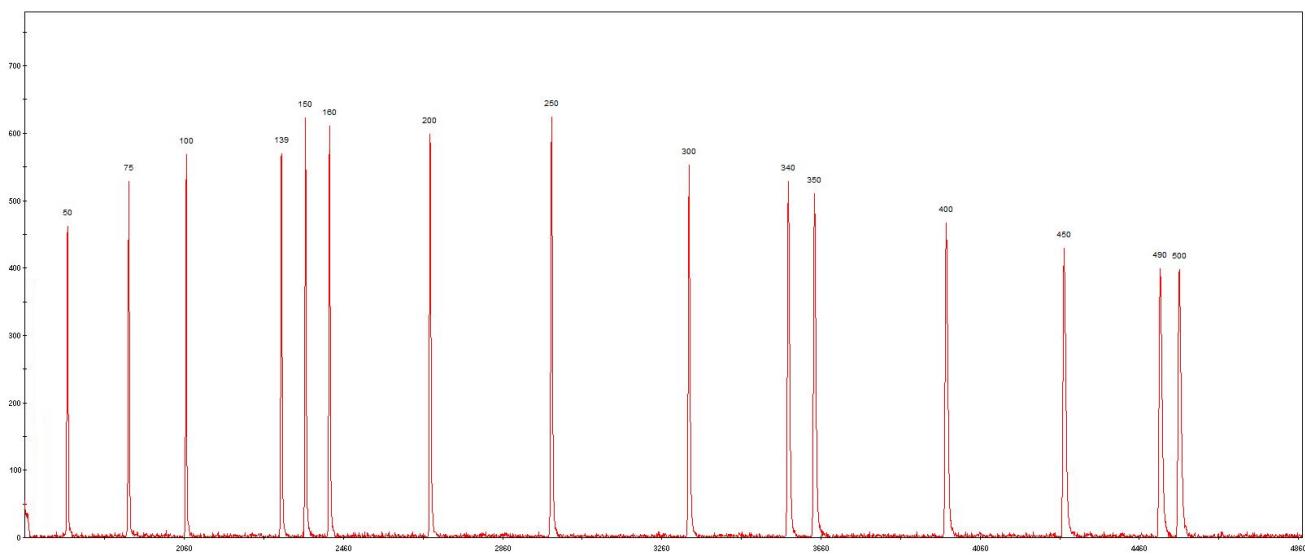
FLT3-ITD – Capillary Electrophoresis

Same primer set and target region, but now fluorescently labelled.

Prior to CE, each sample PCR product is combined with formamide and a ROX-labelled sizing ladder.

Detection unit identifies all 15 ROX-labelled fragments as they pass the laser and fluoresce at 604nm.

Base pair sizes of each fragment is known (50, 75, 100, 139, 150, 160, 200, 250, 300, 350, 400, 450, 490, and 500bp) allowing the instrument software to auto-generate a standard curve.



FLT3-ITD – Capillary Electrophoresis

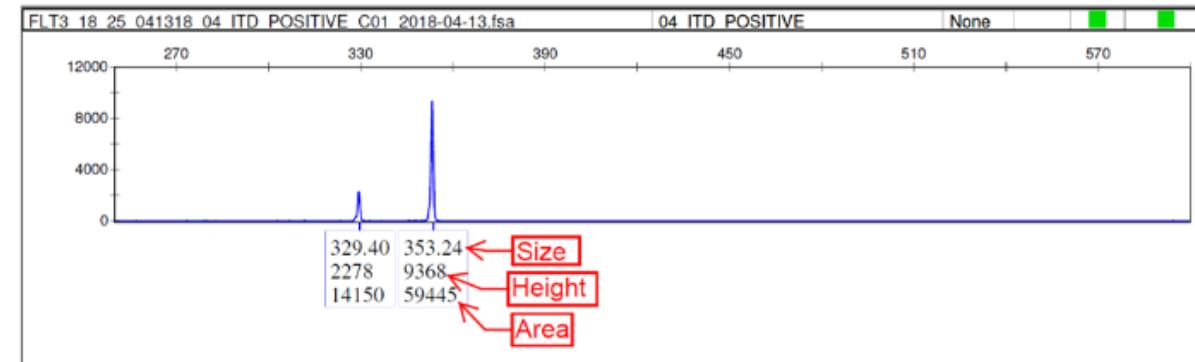
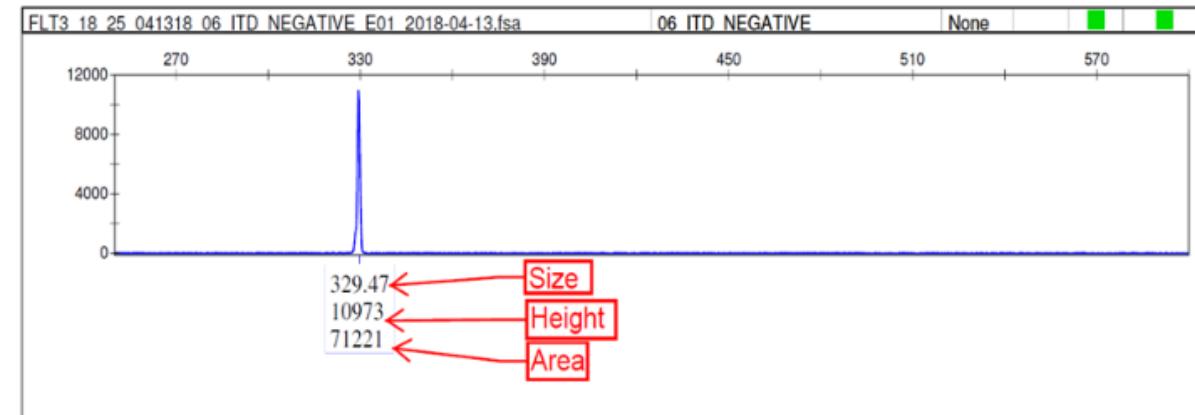
Amplicon is easily distinguished from the sizing ladder because FAM-labelled primer set is read at 517nm.

For each sample peak, three quantifiers that can be used to interpret the patient result:

- Fragment size (bp)
- Peak height (RFU)
- Peak area

All testing questions easily answered:

- Mutation present? (peaks larger than 329bp)
- ITD size? (MUT peak minus WT peak)
- Allelic ratio?(MUT peak area/WT peak area)



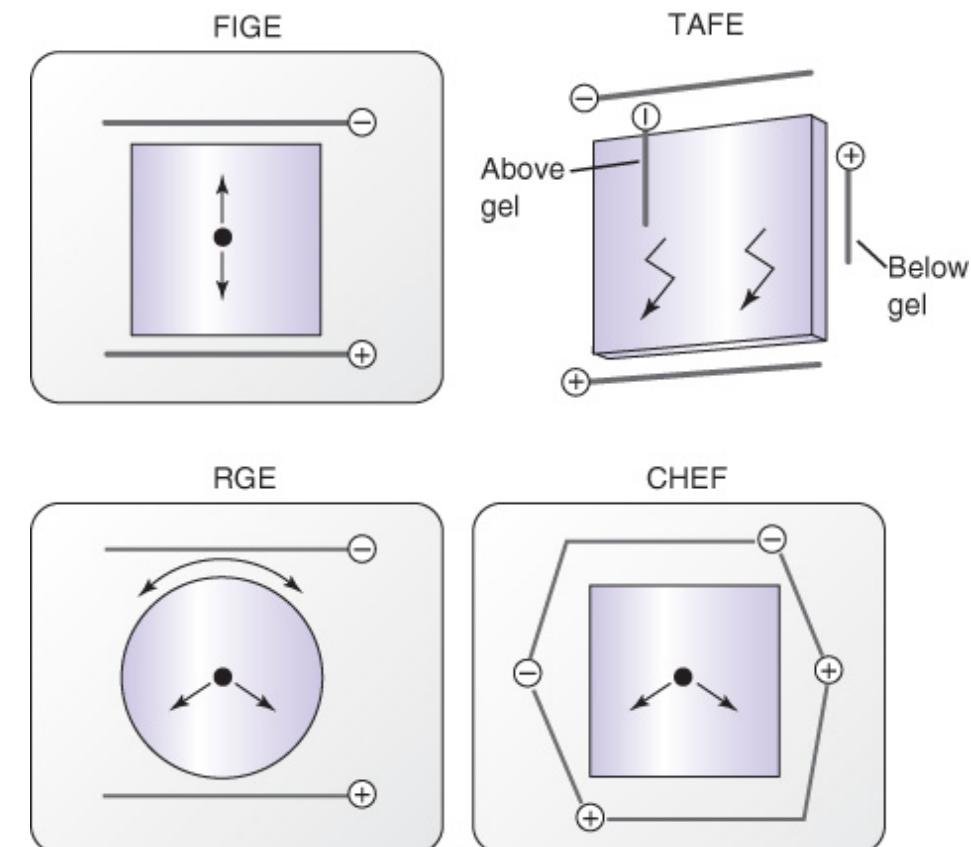
Pulsed-Field Gel Electrophoresis

Very large DNA molecules (>50,000bp) are better resolved by pulsed-field gel electrophoresis (**PFGE**) systems.

- Current is applied to gel in alternating dimensions to enhance migration of large DNA fragments.

Four kinds:

- Field-inversion gel electrophoresis (**FIGE**) - alternating positive and negative poles
- Transverse alternative field electrophoresis (**TAFE**) - transverse angle reorientation of poles on a vertical gel
- Rotating gel electrophoresis (**RGE**) - rotating gel with fixed poles
- Contour-clamped homogenous electric field (**CHEF**) - alternating polarity in an electrode array

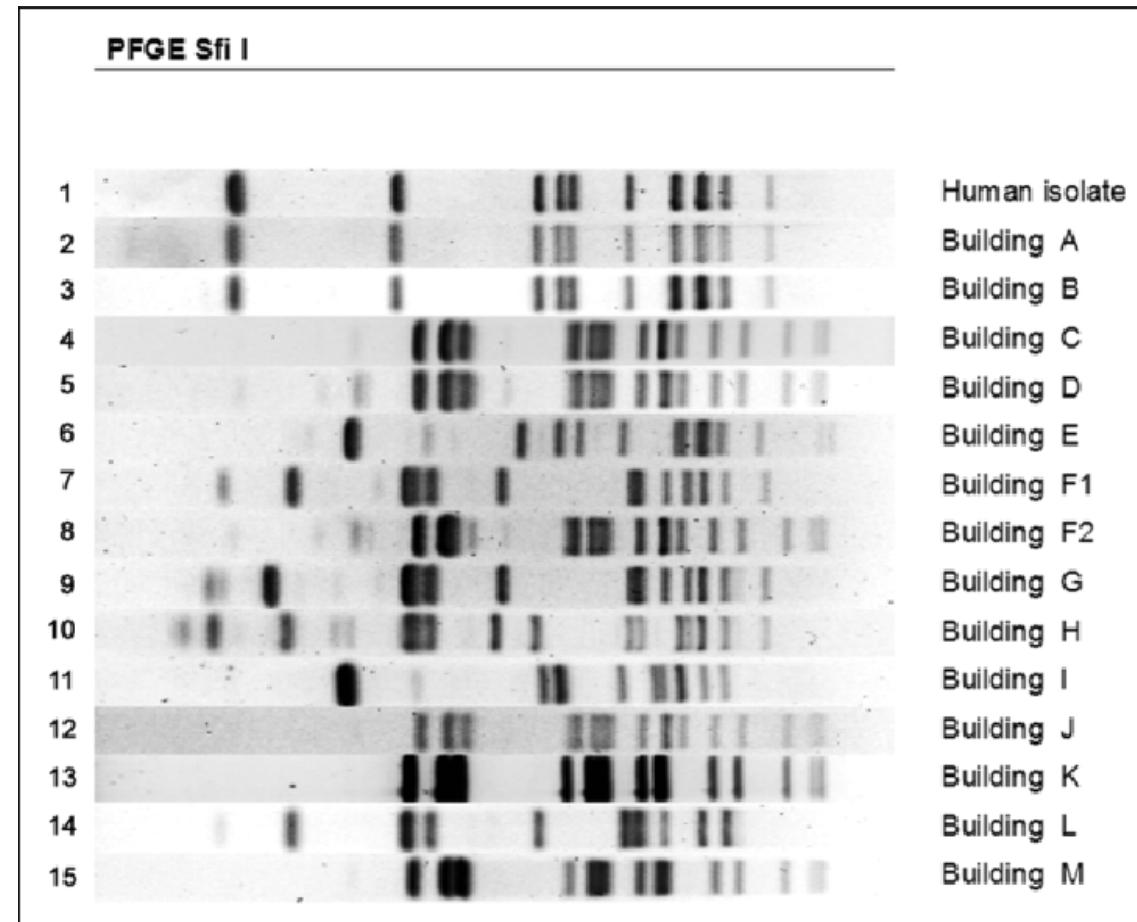


Pulsed-Field Gel Electrophoresis

PFGE is especially useful for epidemiological investigation of infectious pathogens.

Image: PFGE patterns for *Legionella pneumophila* isolates collected during a 2015 outbreak of Legionnaires disease.

- Extracted DNA was digested with *Sfi I*
- Human isolate lane = PFGE pattern representative of strain of *L. pneumophila* that infected 138 patients
- Remaining lanes are for *L. pneumophila* cultured from cooling towers on 16 buildings in the area
- Banding patterns indicate that Buildings A and B are likely the sources of the outbreak.



Real-Time PCR and its Variants

- qPCR
- Digital PCR
- Melt Curve Analysis

Recall the ingredients of a generic PCR amplification: polymerase, dNTPs, buffer, primers.

Real-Time PCR, or qPCR, adds one more component to the mastermix: **fluorescent probes.**

UV excitation of the fluorescent probe generates detectable fluorescence, but only if the fluorescent probe is able to hybridize to its target.

- Fluorescence = presence of target sequence
- No fluorescence = absence of target sequence

Fluorescence is detected by instrumentation, allowing techs to visualize DNA amplification in “real-time” as it occurs.

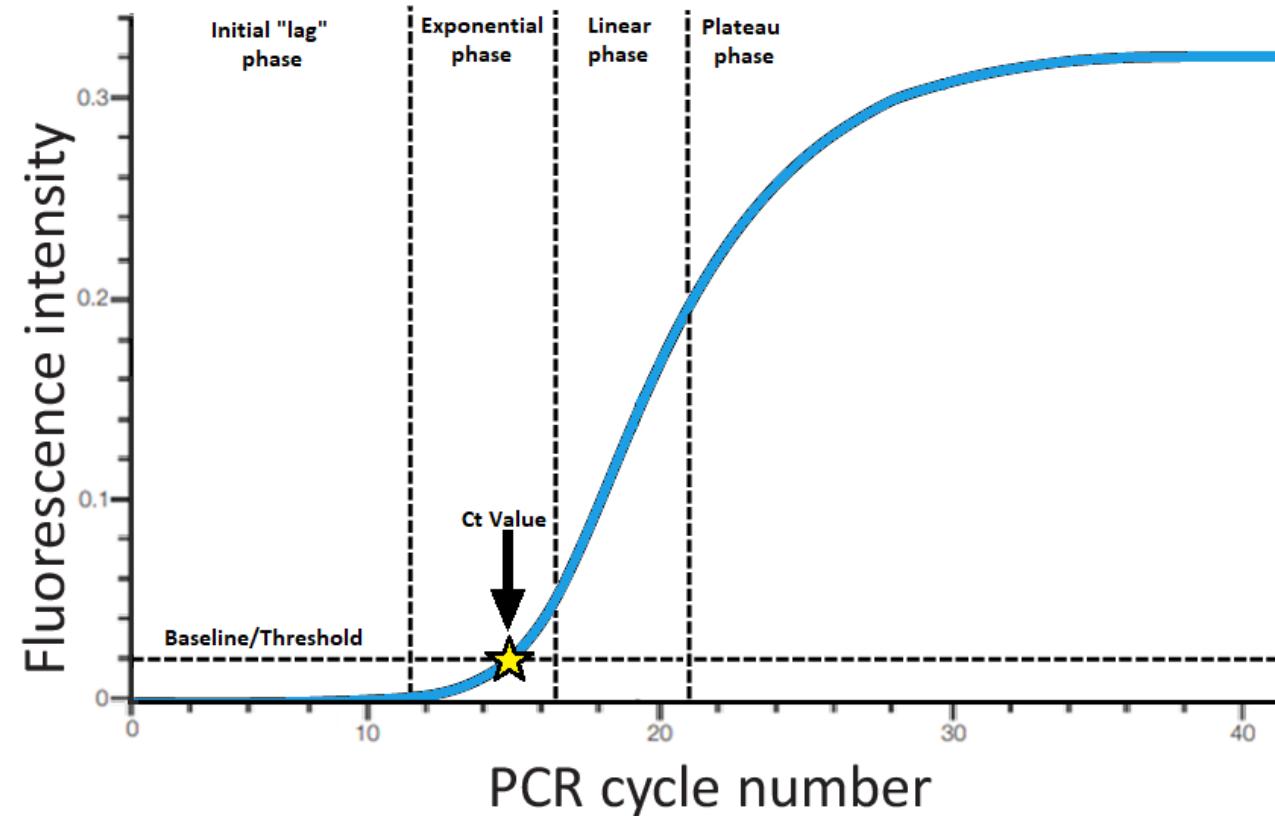
Real-Time PCR

Sample fluorescence (y-axis) is graphed against PCR cycle (x-axis).

If target sequences are replicated during PCR, more and more probe can bind and generate fluorescence.

Accumulation of fluorescence gives qPCR a characteristic **sigmoidal curve** with four phases:

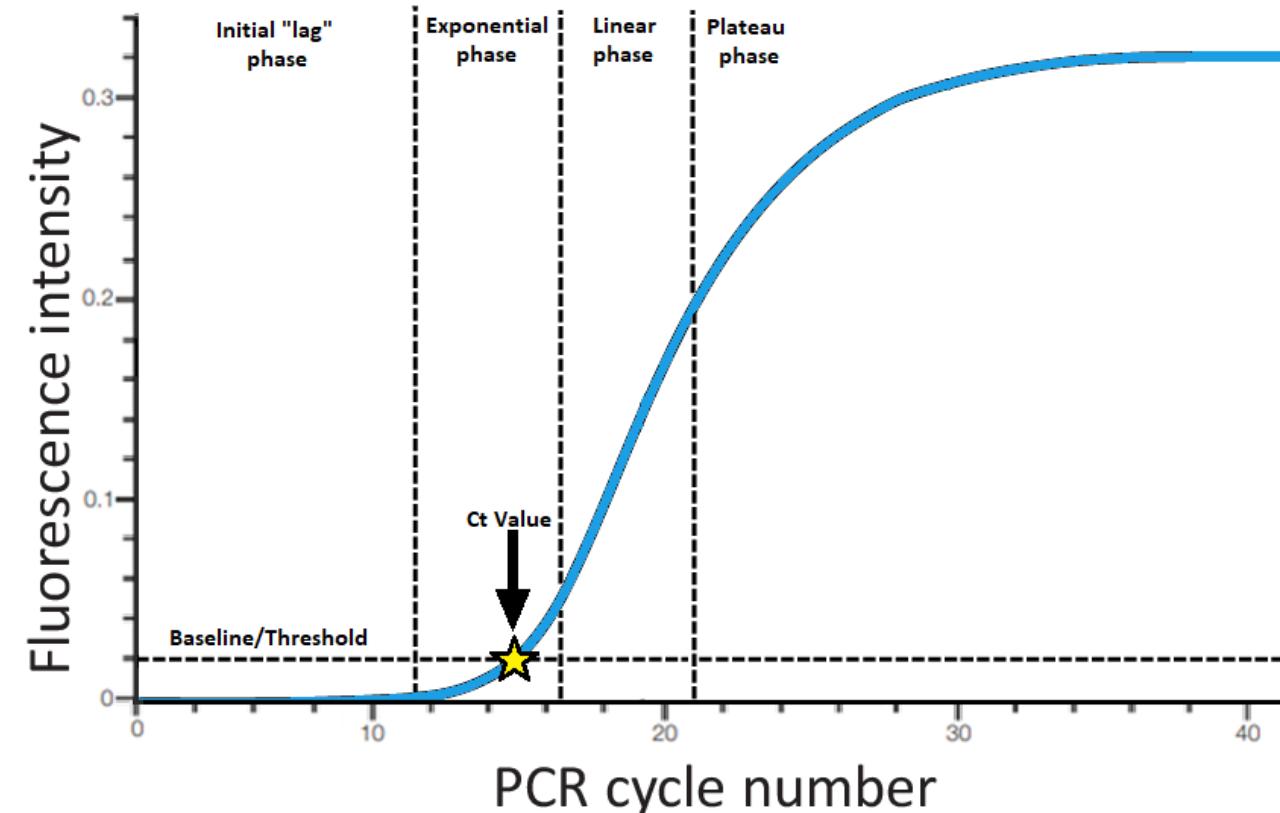
- Initial
- Exponential
- Linear
- Plateau



Real-Time PCR

Phases of qPCR:

- **Initial “lag” phase:** reaction has not yet produced enough amplicons to generate detectable fluorescence above the baseline
- **Exponential (log) phase:** maximally efficient logarithmic growth, with each cycle doubling the amount of amplicons
- **Linear phase:** less efficient linear growth, with each cycle producing the same amount of additional amplicons
- **Plateau phase:** some reaction component (dNTP, probe, primer) is depleting or depleted



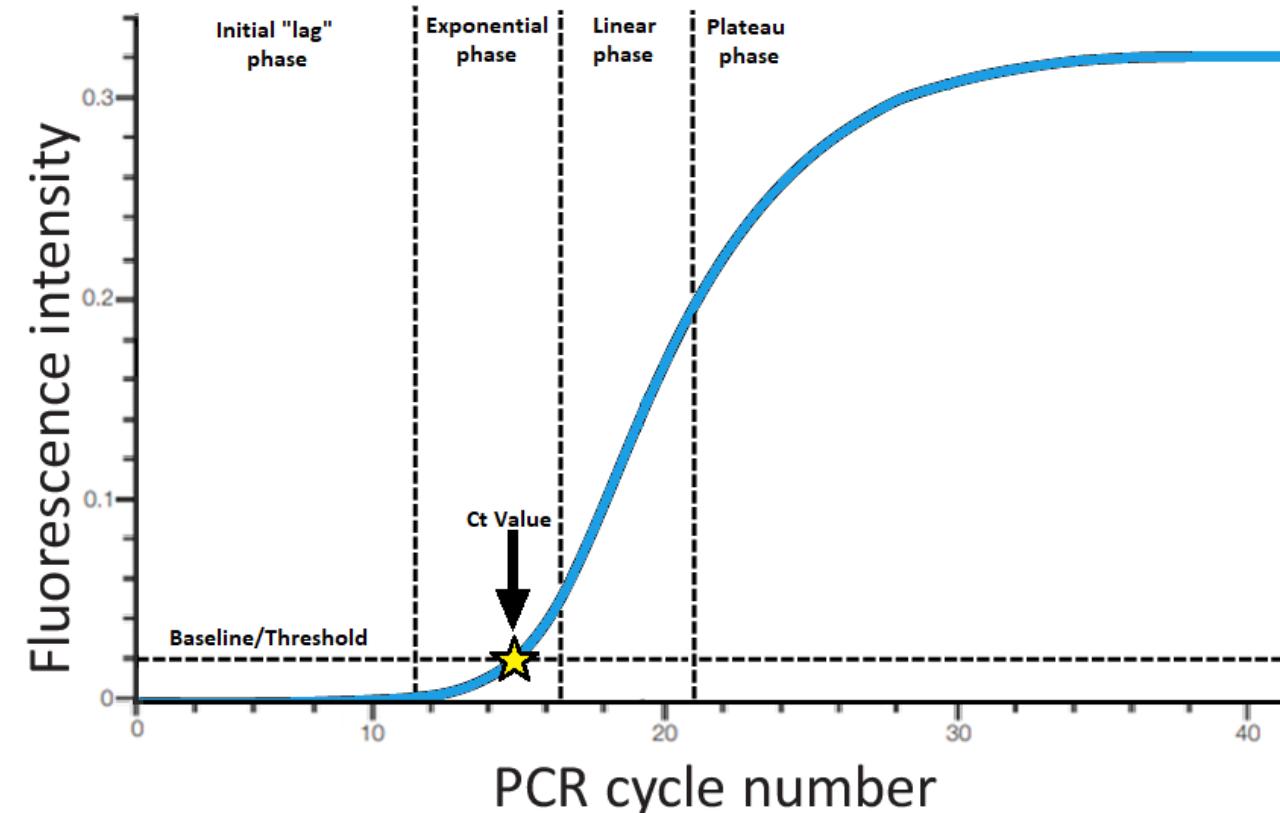
Real-Time PCR

The **threshold level**, set during validation, represents the normal baseline fluorescence of a test absent sample amplification.

Threshold cycle (C_T) is the cycle of PCR at which sample fluorescence exceeds the threshold level.

C_T is **inversely proportional** to the amount of target present in the sample prior to PCR

- High initial target = low C_T
- Low initial target = high C_T
- No initial target = no amplification, no C_T



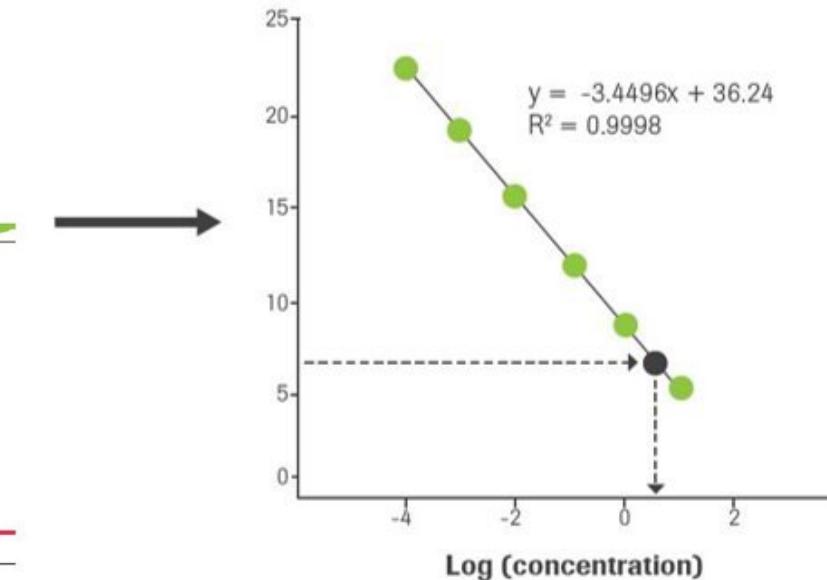
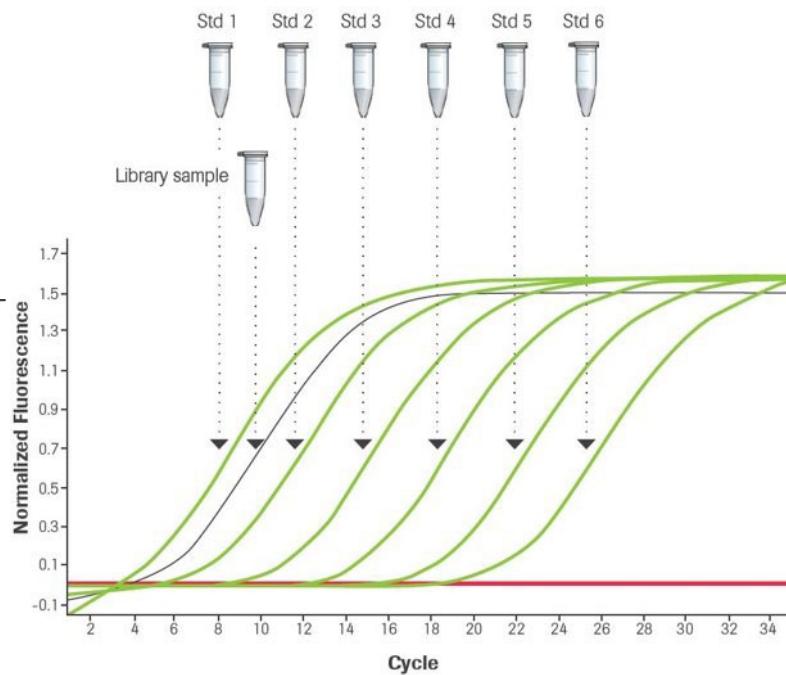
Real-Time PCR – Quantitative Application

Quantitative (Real-Time) PCR

- Standards of known concentrations are used to generate a standard curve.
- Linear regression equation can then be used to calculate initial concentration of target template from the measured C_T value of a patient sample.

Useful in establishing:

- Variant allele fraction (VAF) for mutation detection
- Viral load



Real-Time PCR – Quantitative Application

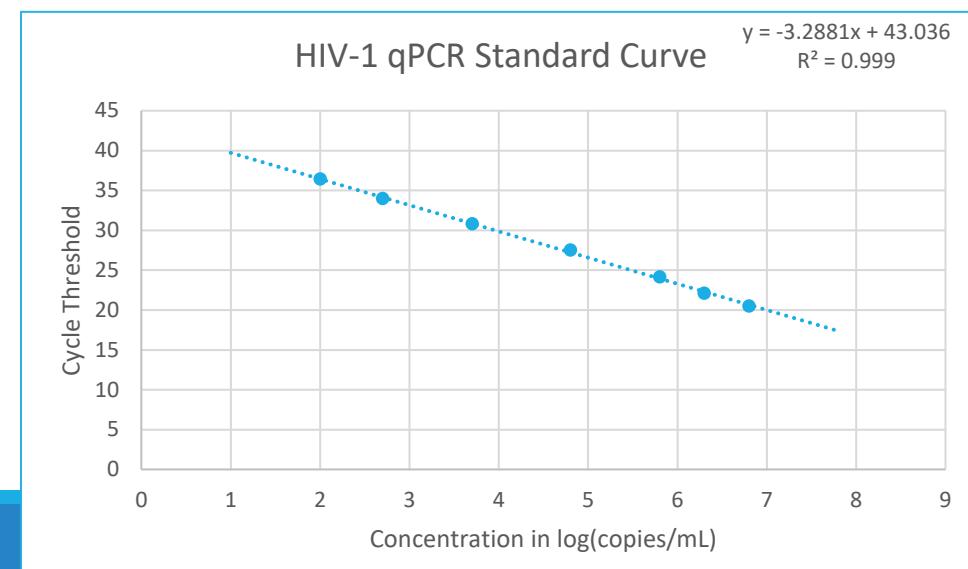
Let's use an HIV-1 qPCR assay as an example.

HIV-1 viral load is quantified through RT-qPCR amplification of the highly conserved *gag*-gene and LTR-region of the HIV-1 RNA genome.

To establish the standard curve, 7 commercially purchased standards covering the analytical measurement range with known viral loads (copies/mL) are tested.

Standard curve generated from results by plotting known concentration (x-axis) against measured cycle threshold (y-axis).

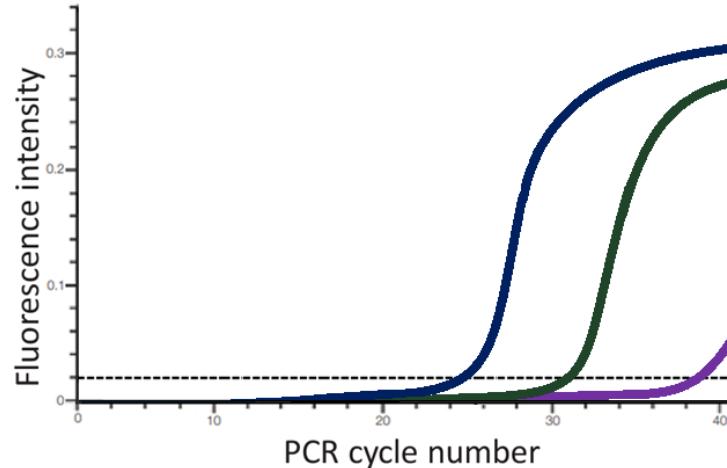
Standard	Concentration (copies/mL)	Concentration log(copies/mL)	Measured Ct
#1	100	2.0	36.44
#2	600	2.7	34.02
#3	6,000	3.7	30.82
#4	60,000	4.8	27.56
#5	600,000	5.8	24.17
#6	2,000,000	6.3	22.14
#7	6,000,000	6.8	20.53



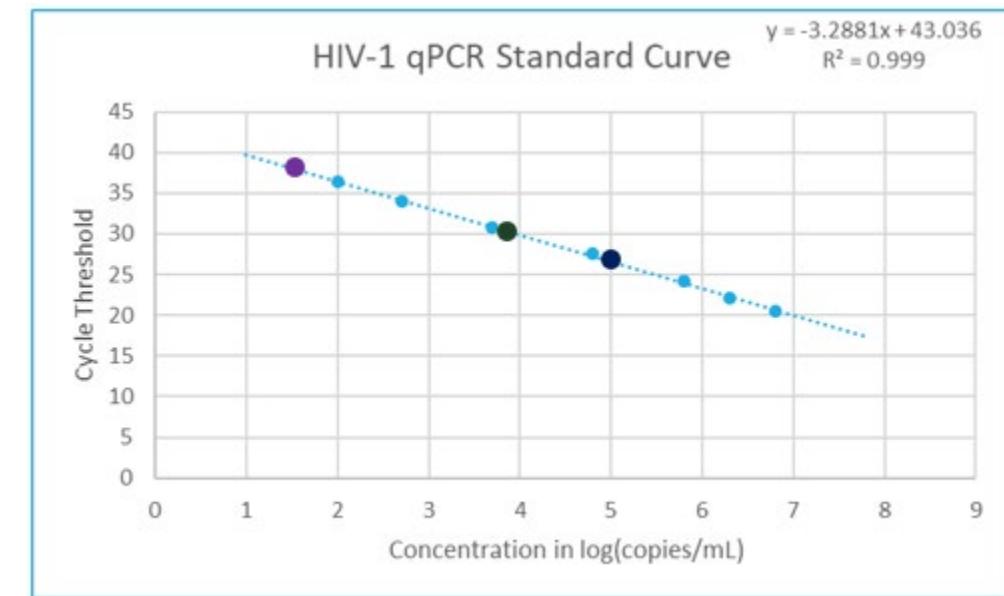
Real-Time PCR – Quantitative Application

Subsequent analysis of patient samples have their concentration calculated by plotting measured C_t along the standard curve:

- Sample 1 $C_t = 26.46$
- Sample 2 $C_t = 30.5$
- Sample 3 $C_t = 38.1$



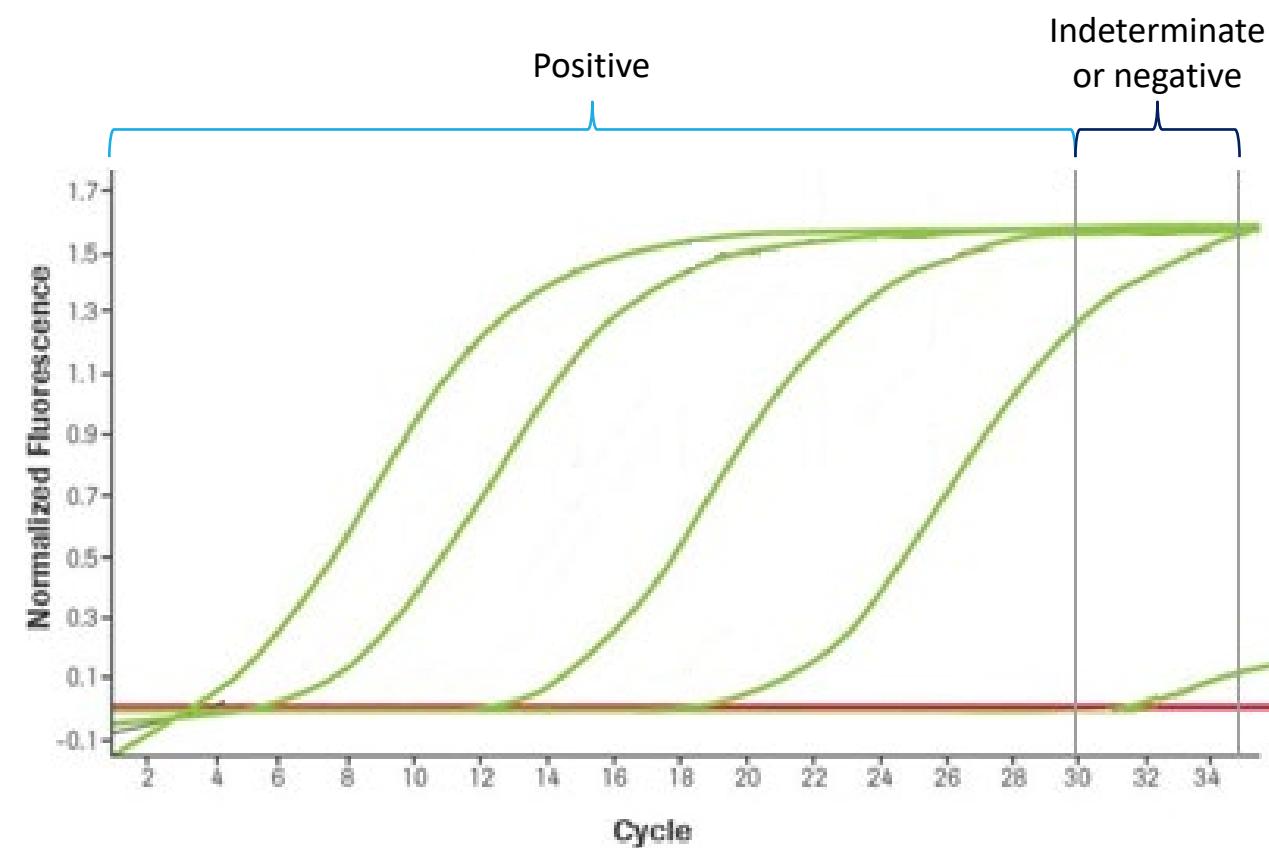
Patient	Measured Ct (copies/mL)	Concentration log(copies/mL)	Concentration (copies/mL)
#1	26.46	5.0	100,000
#2	30.5	3.8	6,309.57
#3	38.1	1.5	31.62



Real-Time PCR – Qualitative Application

qPCR can also be used to qualitatively detect the presence of target without explicitly quantifying the amount of target present in the original sample.

Ranges set during validation are used to interpret C_t values as positive, negative, or indeterminate.



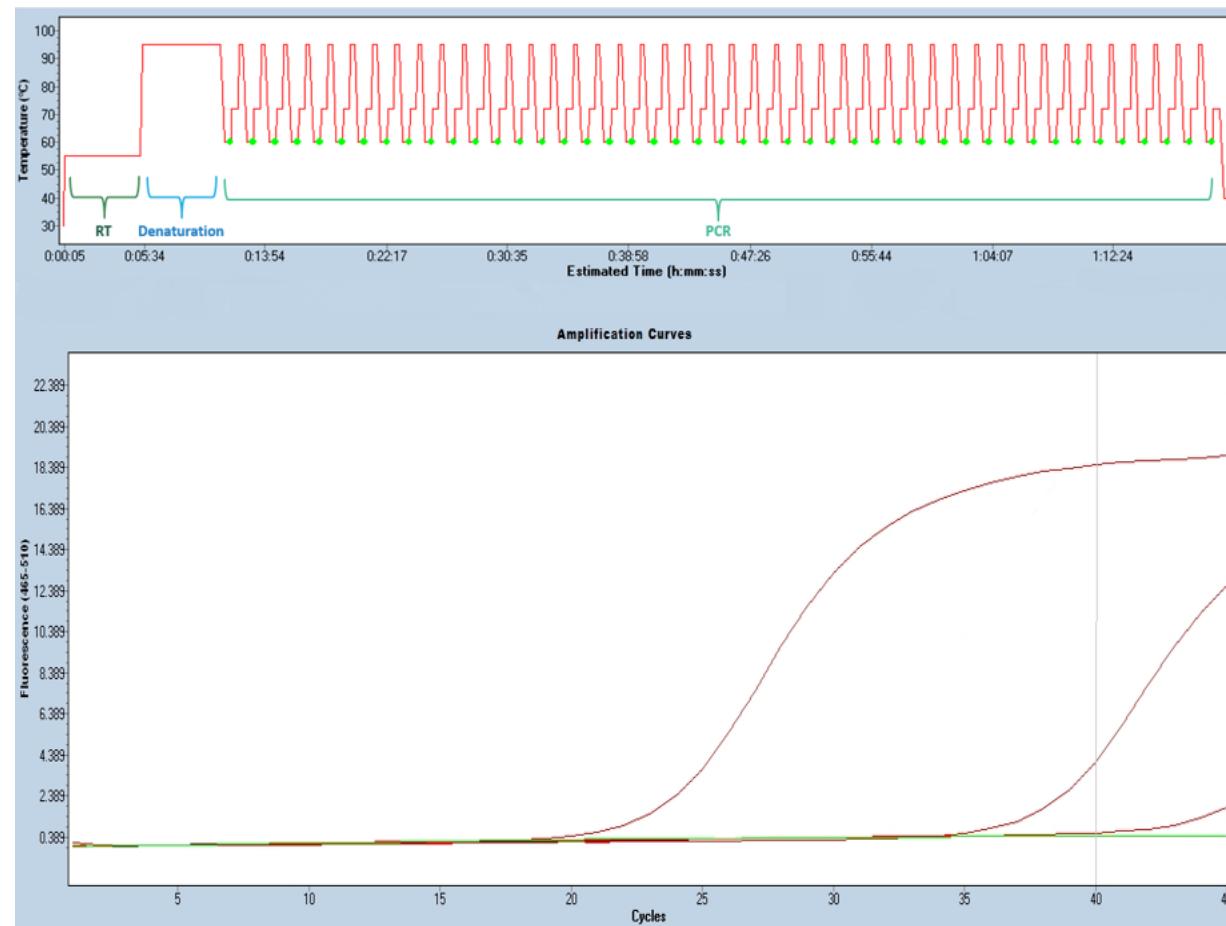
Real-Time PCR – Qualitative Application

Let's use an old SARS-CoV-2 assay as an example of qualitative detection.

SARS-CoV-2 is qualitatively detected through a 45-cycle amplification of the RdRP gene, with the following ranges initially used for interpretation:

- $C_T < 40$ = Positive
- $C_T \geq 40$ = Negative
- No C_T = Negative

Sample	C_T	Interp
#1	20	POS
#2	36	POS
#3	>40	NEG
#4	None	NEG



qPCR Probe Categories

Three major fluorescent probe categories:

Non-sequence-specific dyes = fluorescence is generated when dye binds nonspecifically to nucleic acids

- EtBr and SYBR green

Hydrolysis probes = fluorescence is generated at the extension step

- TaqMan probes

Hybridization probes = fluorescence is generated at the hybridization step

- Molecular beacons
- Scorpion probes
- Dual hybridization probes

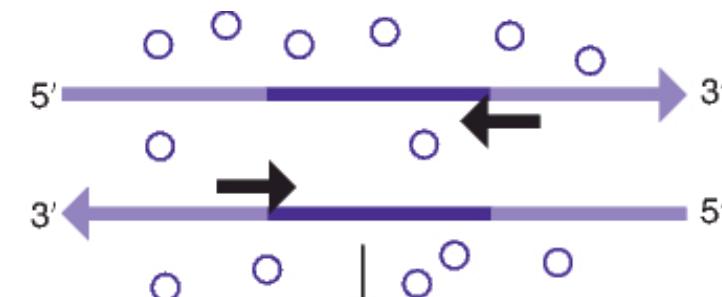
Non-sequence-specific Dyes

Fluorescent dyes that bind to dsDNA generally but don't target any specific sequence.

As nucleic acid amplifies during PCR, more target is available for binding and fluorescence increases.

Fluorescent dyes

- Ethidium Bromide (EtBr)
 - Intercalates between successive nucleotides in sequence
 - Mutagenic
- SYBR green
 - Binds to the minor groove of the double helix
 - Reduced toxicity compared with EtBr



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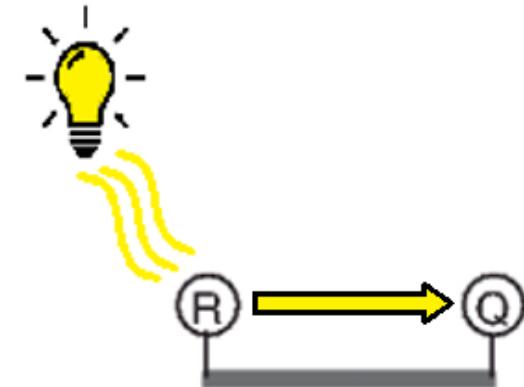
FRET

Fluorescence resonance energy transfer is the underlying principle of both hydrolysis and hybridization probes.

FRET generically refers to the transfer of energy between two fluorophores.

Two scenarios in real-time PCR probes:

- A quenching fluorophore transfers energy from a reporting fluorophore to itself, preventing detectable fluorescence from the reporting fluorophore.
 - TaqMan, Molecular Beacon, Scorpion
- A donor fluorophore transfers energy to a reporting fluorophore, generating detectable fluorescence from the reporting fluorophore.
 - Dual Hybridization



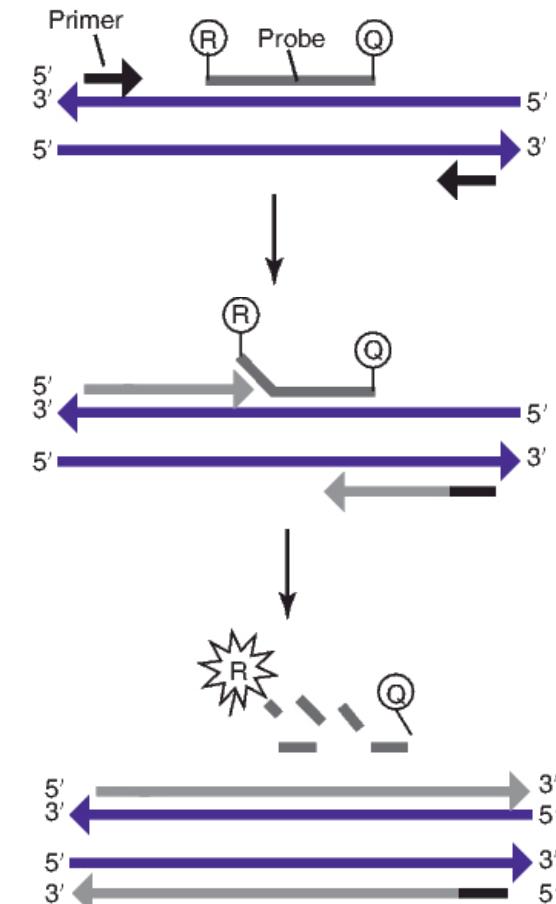
TaqMan Probes

Oligonucleotide probe has a 5' fluorescent **reporter** molecule, and 3' **quencher** molecule that negates the fluorescence of the reporter.

If target sequence is present, probe will hybridize between primer binding sites.

Polymerase will hydrolyze the probe molecule during extension step.

Reporter, now dissociated from the quencher, can freely emit detectable fluorescence.



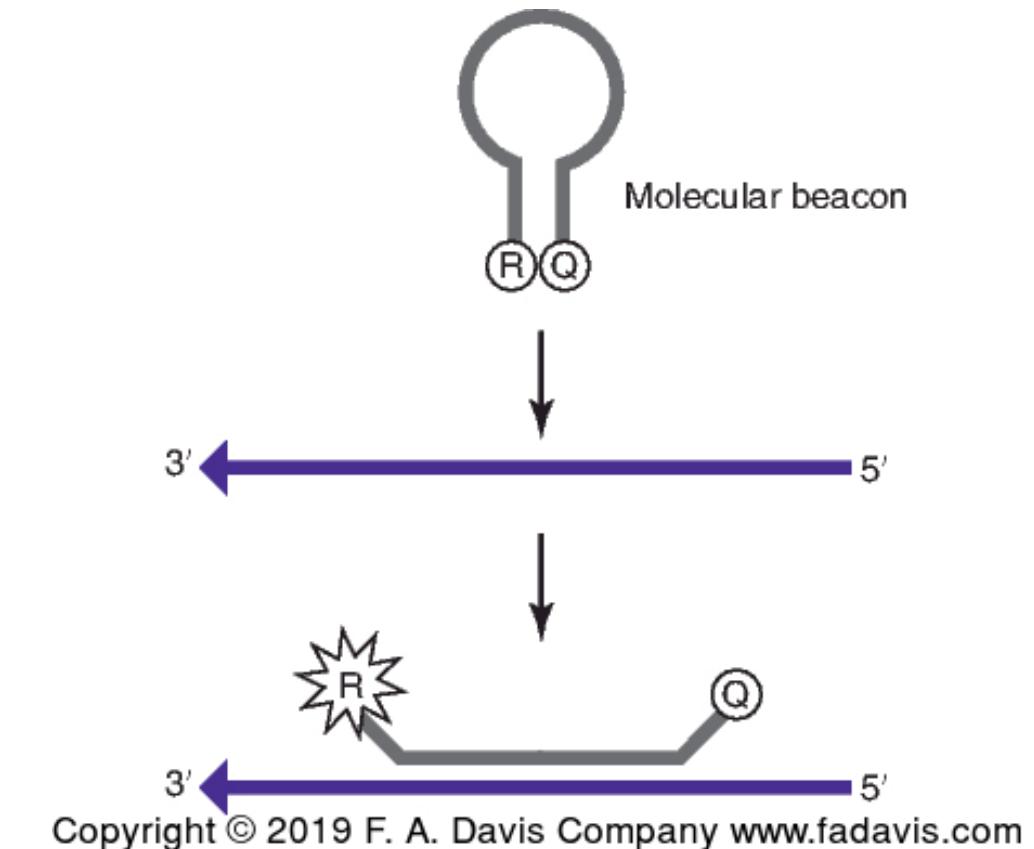
Molecular Beacons

When unbound, oligonucleotide probe forms a stem-and-loop/hairpin structure where 5' **reporter** fluorescence is negated by the 3' **quencher**.

Stem sequences on either end of the probe are complementary to one another.

Loop sequence is positioned between stem sequences and is complementary to target

If target is present, loop sequence will hybridize and force the stem sequences to denature. This dissociates the quencher from the reporter, generating detectable fluorescence



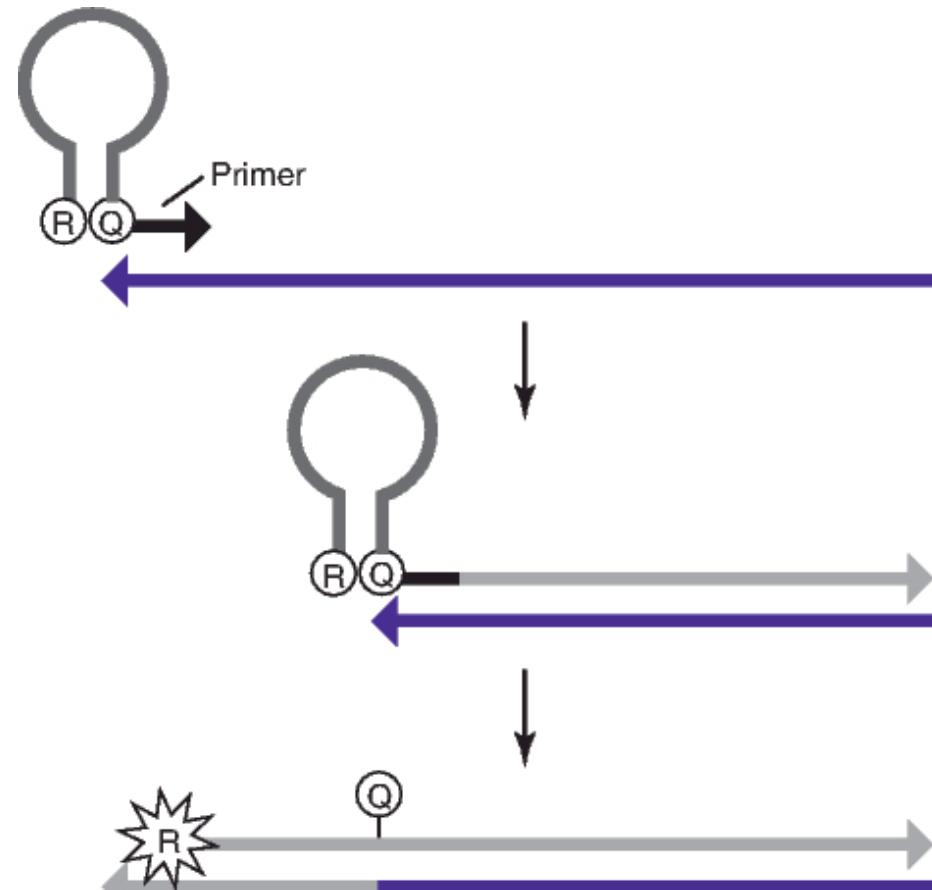
Scorpion Probes

A variation of the molecular beacon design that combines the oligonucleotide probe and PCR primer into one molecule.

Primer hybridizes and allows extension of a new DNA strand.

Subsequent denaturation step linearizes molecular beacon.

Linearized loop sequence is will hybridize intra-molecularly to the newly synthesized DNA strand, dissociating the quencher from the reporter and generating detectable fluorescence.



Dual Hybridization Probes

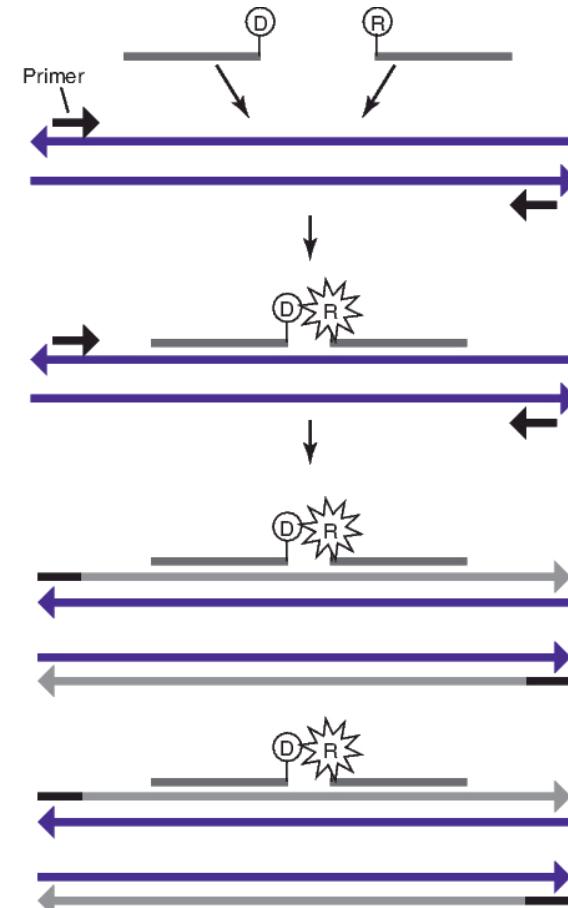
Dual hybridization probes, which used to be generically named *FRET probes*, involve two separate oligonucleotide probes with adjacent targets ~1 to 5 bases apart

- Probe one = has a **donor** fluorophore
- Probe two = has an **acceptor/reporter** fluorophore

Reporter is reliant on the donor to fluoresce.

- Donor emission = Reporter excitation

If targets are present, probes will hybridize next to one another. This allows donor to transfer energy to the acceptor and generate detectable fluorescence.



Multiplexed Real-Time PCR

Multiplexing of real-time PCR can be achieved by labelling probes targeting different sequences with different fluorophores.

CCF's Human Papillomavirus (HPV) Genotyping assay uses this method to detect and differentiate high risk HPV Types.

Internal control, Type 16, and Type 18 can all be uniquely differentiated.

Remaining Types measured in Channel 4 cannot be differentiated from each other.

Channel	Target(s)
Channel 1	β -Globin (IC)
Channel 2	Type 16
Channel 3	Type 18
Channel 4	Types 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68

qPCR Instruments Aplenty at CCF



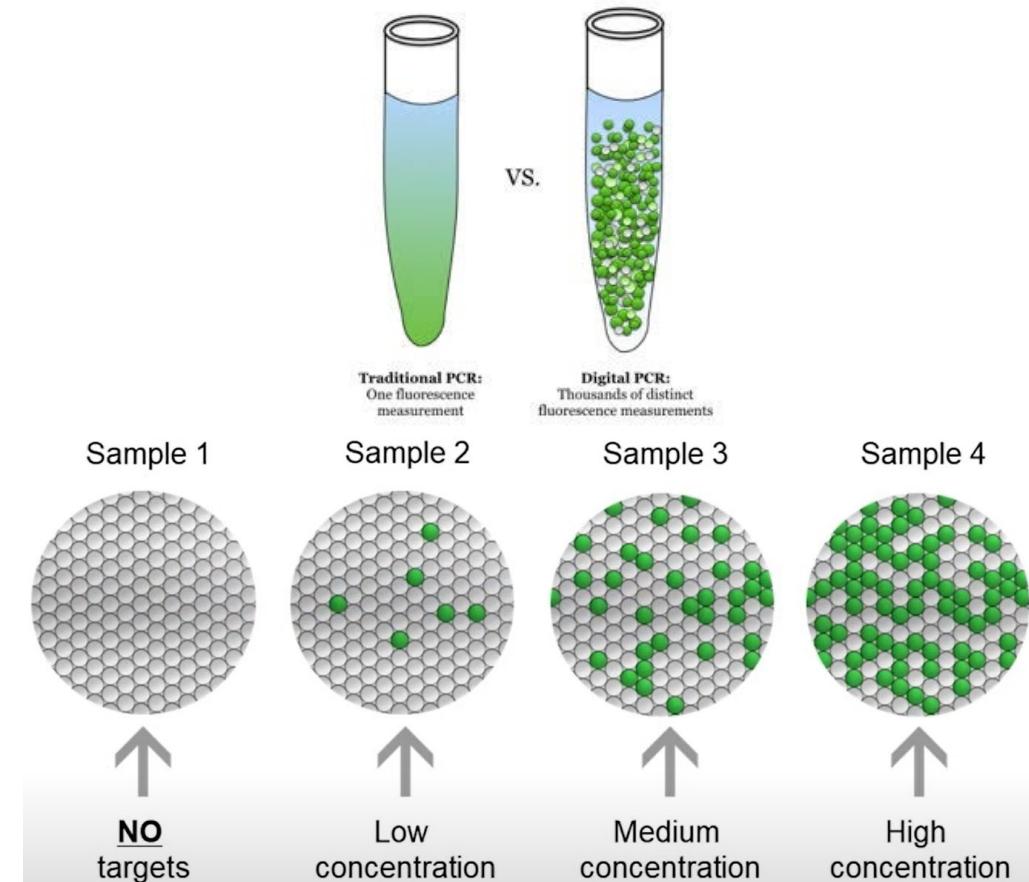
Digital PCR

Traditional qPCR provides only a single fluorescent measurement for the entire specimen, which is then used to extrapolate the amount of starting template.

Digital PCR, or dPCR, improves on this by partitioning individual copies of template DNA and discretely capturing every single amplification event.

By comparing the number of “digits” that fluoresce to those that don’t, dPCR can provide an absolute quantitative measurement of initial target without needing to generate a standard curve.

- Uses Poisson distribution calculation (don’t worry about it)



Droplet Digital PCR

Droplet Digital PCR (**ddPCR**) is one example of dPCR technology that is used by CCF to detect and quantify the JAK2 V617F mutation.

- A normal qPCR reaction is prepared using TaqMan probes.
- Using the AutoDG droplet generator instrument, mastermix is partitioned into 10,000-20,000 individual oil droplets for every reaction well of a 96-well PCR plate.
- Appears as a fine mist on the inside of the reaction well



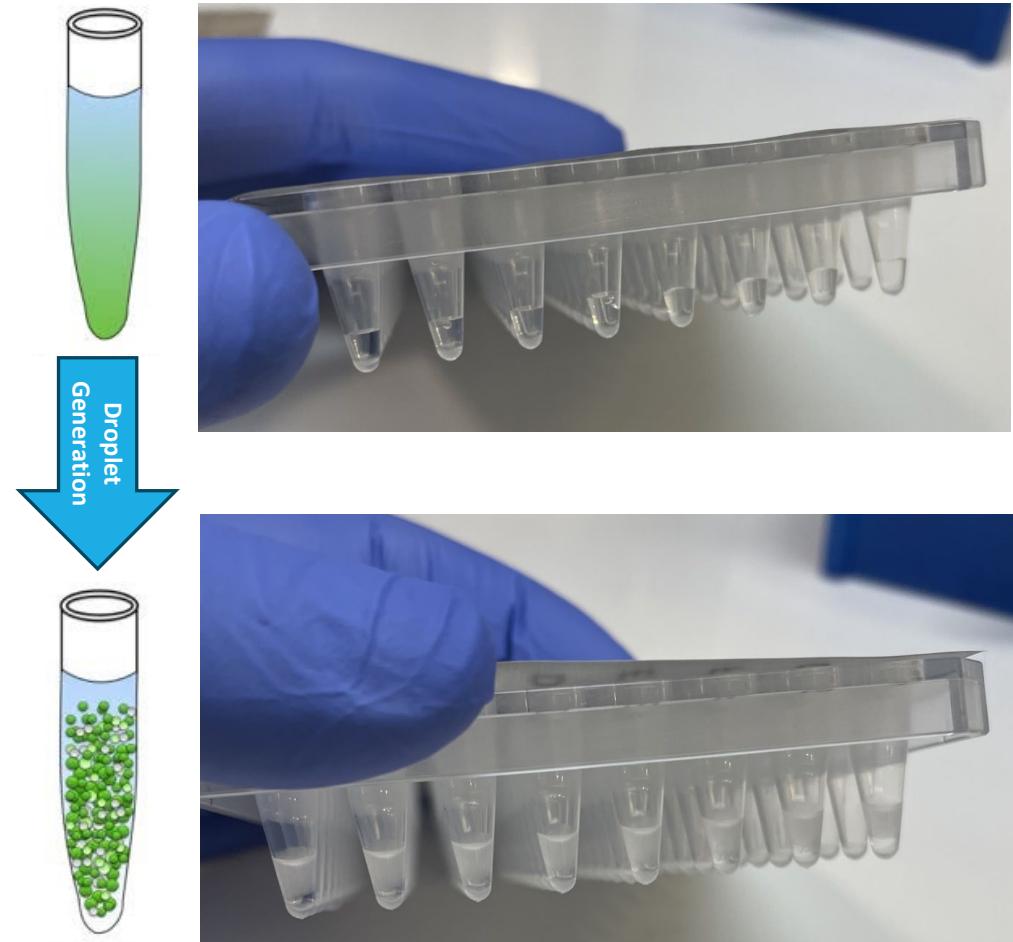
AutoDG
Droplet Generator

QX200
Droplet Reader

Droplet Digital PCR

The process:

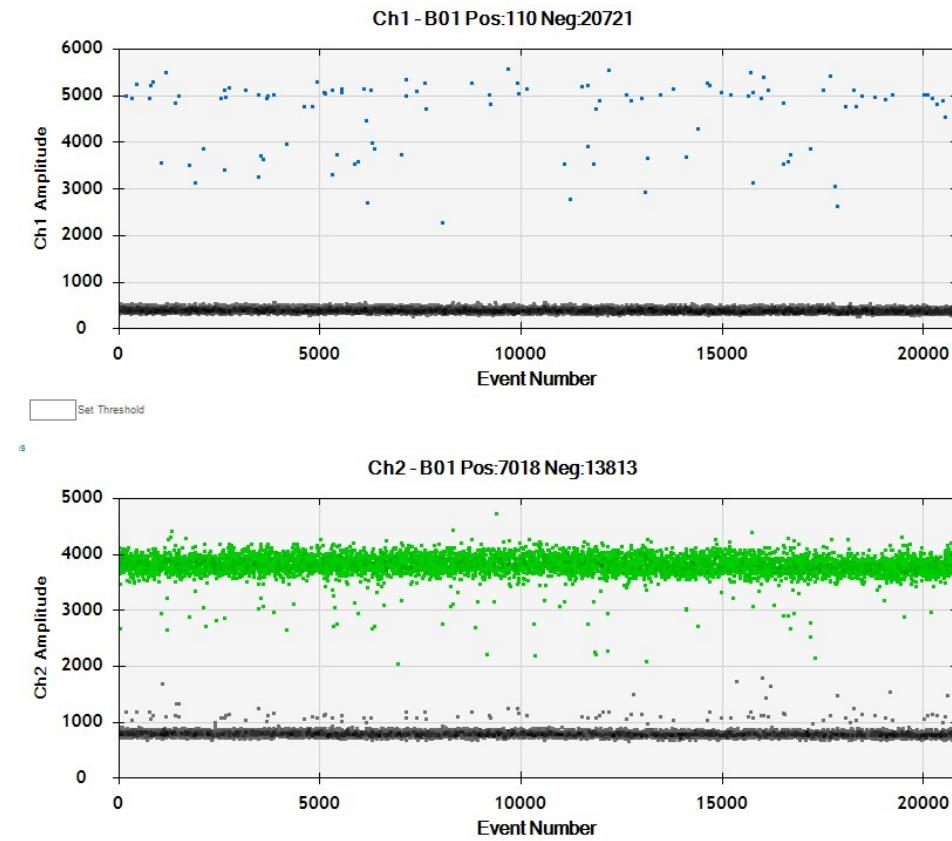
- A multiplexed qPCR reaction is prepared in a 96-well plate using TaqMan probes.
 - FAM Probe = targets WT sequence
 - HEX Probe = targets MUT sequence
- Using the AutoDG droplet generator instrument, mastermix is partitioned into 10,000-20,000 individual oil droplets for every reaction well of a 96-well plate.
 - Appears as a fine mist on the inside of the reaction well
- 96-well plate undergoes a 35 cycle PCR inside of a thermalcycler.
 - No fluorescent detection at this step.



Droplet Digital PCR

The process:

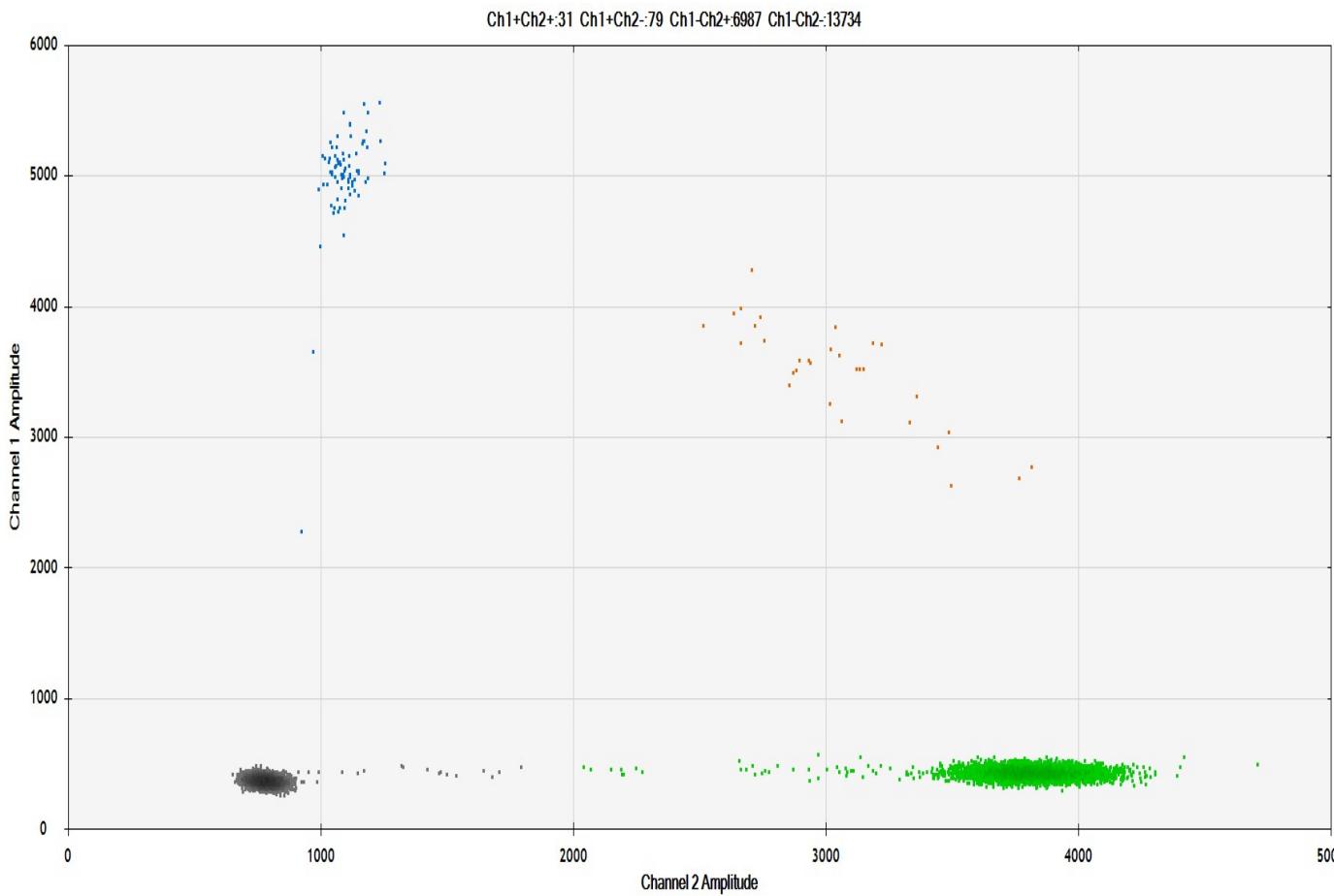
- Following PCR, plate is loaded on QX200 Droplet reader.
- One well at a time, Droplet Reader uses drive oil to push sample droplets through a thin capillary.
- Droplets pass by a detection unit which captures fluorescence at both wavelengths.



Droplet Digital PCR

The process:

- Final scatterplot result plots FAM fluorescence against HEX fluorescence.
- Creates four distinct quadrants:
 - Lower Left = unamplified droplets
 - Top Left = FAM fluorescence, MUT only
 - Bottom Right = HEX fluorescence, WT only
 - Top Right = FAM-HEX fluorescence, MUT and WT
- Graph to the right shows a positive JAK2 mutation detection result.
 - Total droplets = 20,831
 - MUT droplets = 110
 - WT droplets = 7066
 - Unamplified droplets = 13734
 - **Calculated VAF = 0.013%**

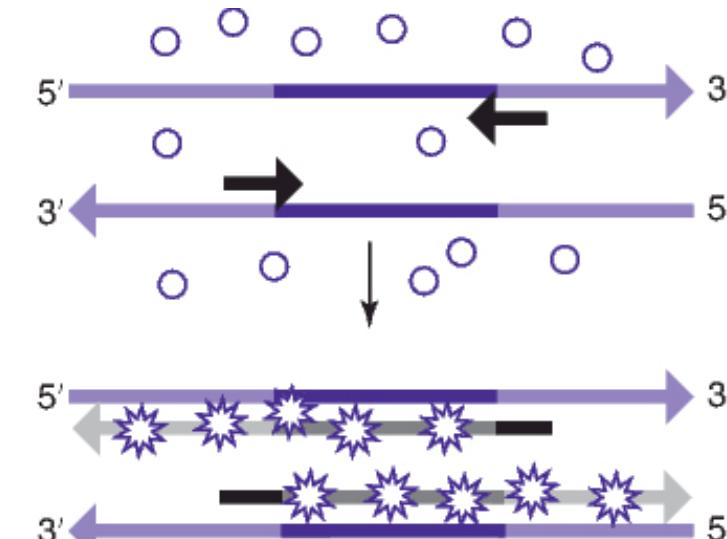


Melt-Curve Analysis (MCA)

Melt-curve analysis (MCA) is a post-amplification step of real-time PCR used to differentiate DNA duplexes based on their sequence-based denaturation temperatures.

Utilizes non-sequence-specific dyes to monitor dsDNA denaturation in real time.

- EtBR
- SYBR green
- LC green

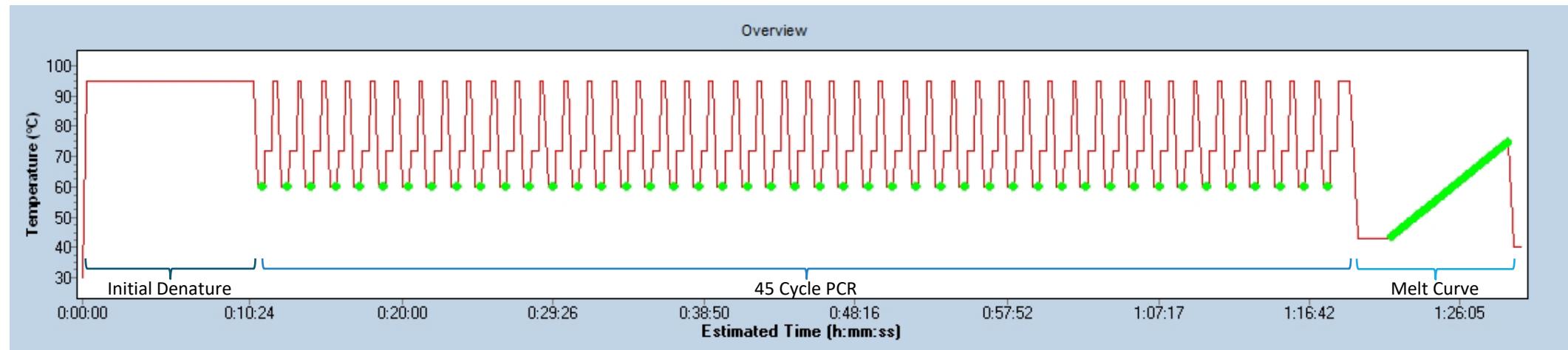


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Melt-Curve Analysis (MCA)

The process:

- Following normal PCR amplification, sample is dropped to a low temperature (50-60°C) where DNA anneals and is mostly double-stranded.
- Non-sequence-specific dyes will bind and fluoresce under UV excitation.
- Temperature is slowly raised, causing dsDNA to denature into single strands.
- Non-sequence-specific dyes are released as DNA denatures, causing fluorescence to decrease.

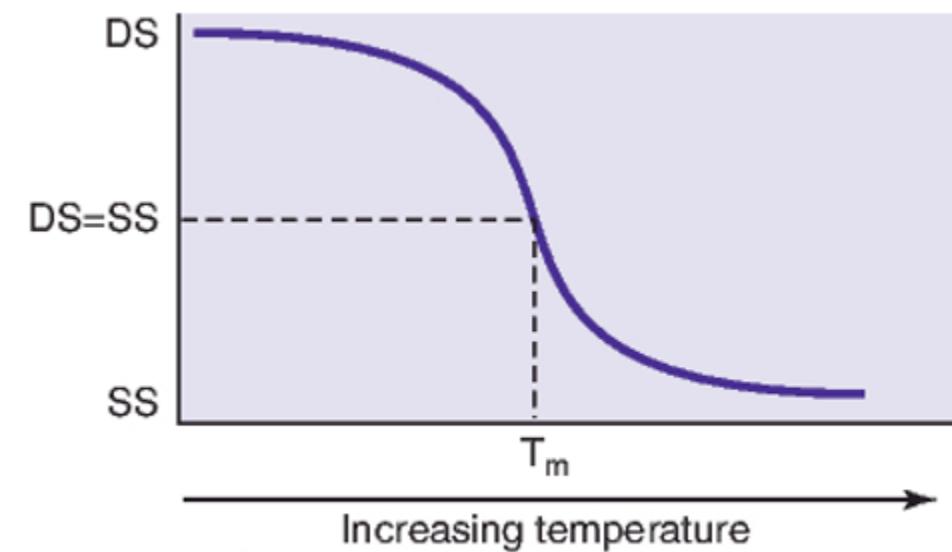


Melt-Curve Analysis (MCA)

Recall that **melting temperature (T_m)** is the point at which exactly half of a double-stranded sequence becomes single stranded.

- A way to express the amount of energy (heat) required to denature a probe from its target sequence.
- For short sequences (14-20bps), based almost entirely on sequence:

$$T_m = (4^\circ\text{C} \times \# \text{ of GC pairs}) + (2^\circ\text{C} \times \# \text{ of AT pairs})$$



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Melt-Curve Analysis (MCA)

Mismatches between the target sequence and the probe decreases the T_m .

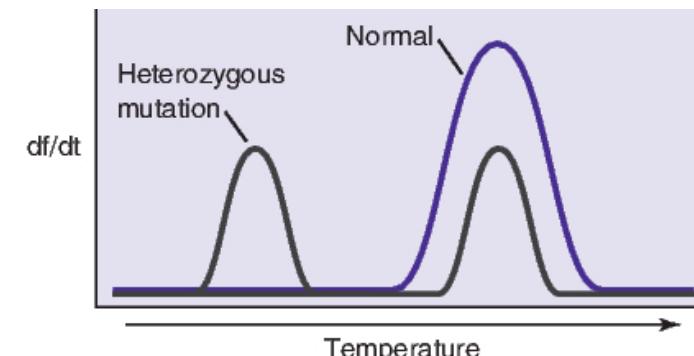
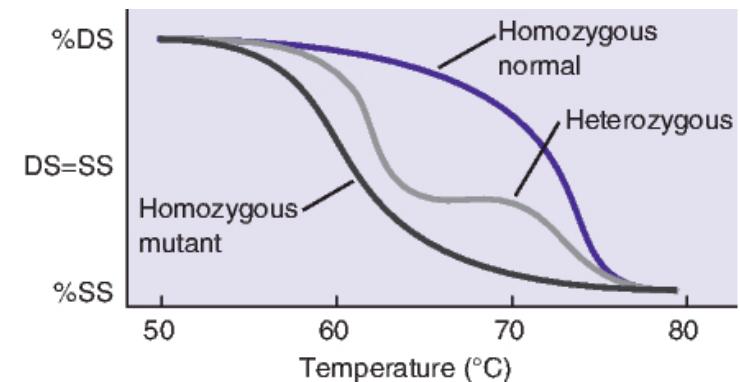
- Fewer bp matches = fewer hydrogen bonds = less energy required to denature

For every 1% difference in sequence, T_m decreases by $\sim 1.5^\circ\text{C}$.

For MCA, final results are graphed as change in fluorescence (df/dt) against temperature ($^\circ\text{C}$).

Produces easily interpretable peaks for SNPs

- Wildtype
- Heterozygous
- Homozygous



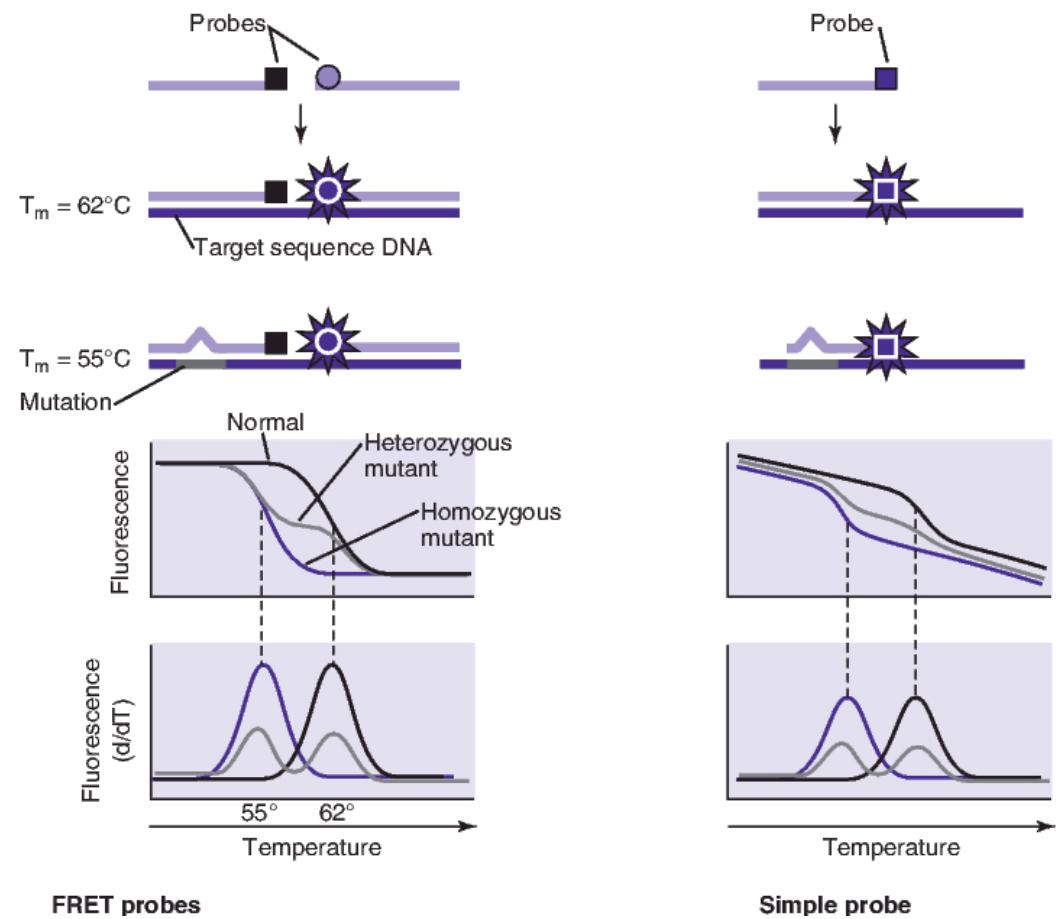
High Resolution-MCA (HR-MCA)

High-resolution melt-curve analysis (HR-MCA) further improves the specificity of the test system.

Probes used are fluorescently-labelled oligonucleotides that report only when bound to target sequence.

Commonly used probe designs:

- Simple probe format = single molecule probe
- FRET probe format = dual hybridization probes

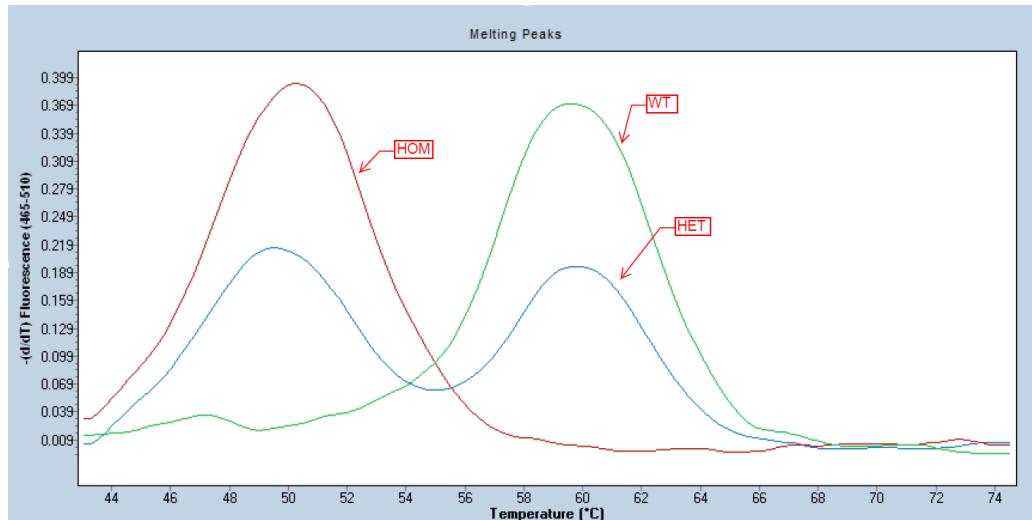


High Resolution-MCA (HR-MCA)

Factor II Gene, c. G20210A mutation

Probe targets the *wildtype* sequence

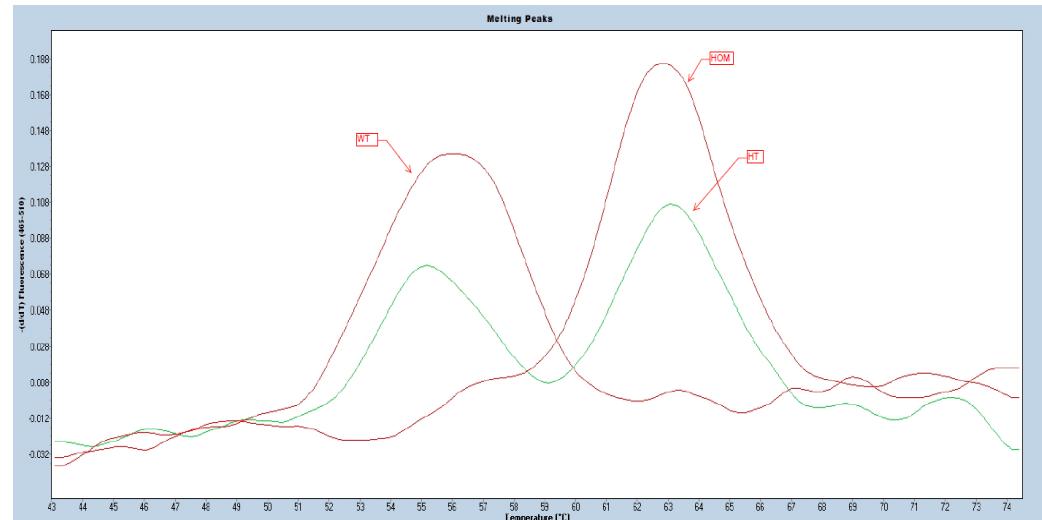
- Homozygous WT = one peak at higher temp
- Heterozygous MUT = two peaks
- Homozygous MUT = one peak at lower temp



HFE Gene, p. H63D mutation

Probe targets the *mutant* sequence

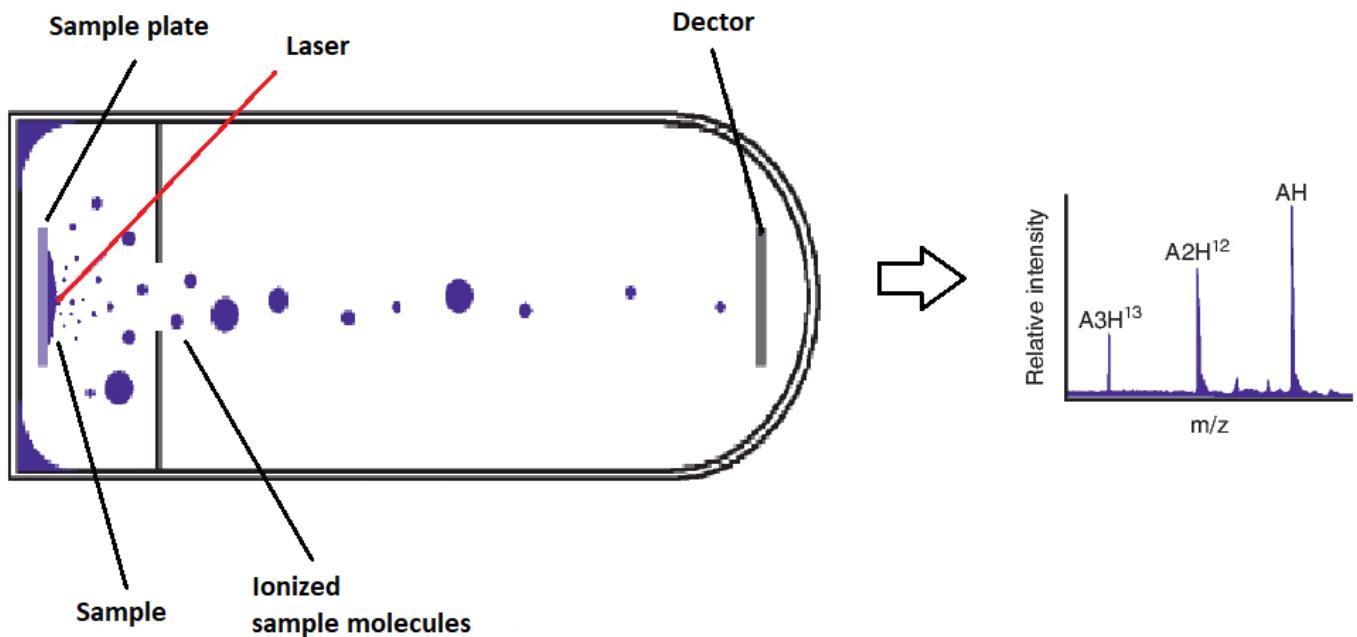
- Homozygous WT = one peak at lower temp
- Heterozygous MUT = two peaks
- Homozygous MUT = one peak at higher temp



MALDI-TOF

- Basics
- Application in Microbiology
- Application in Molecular Dx

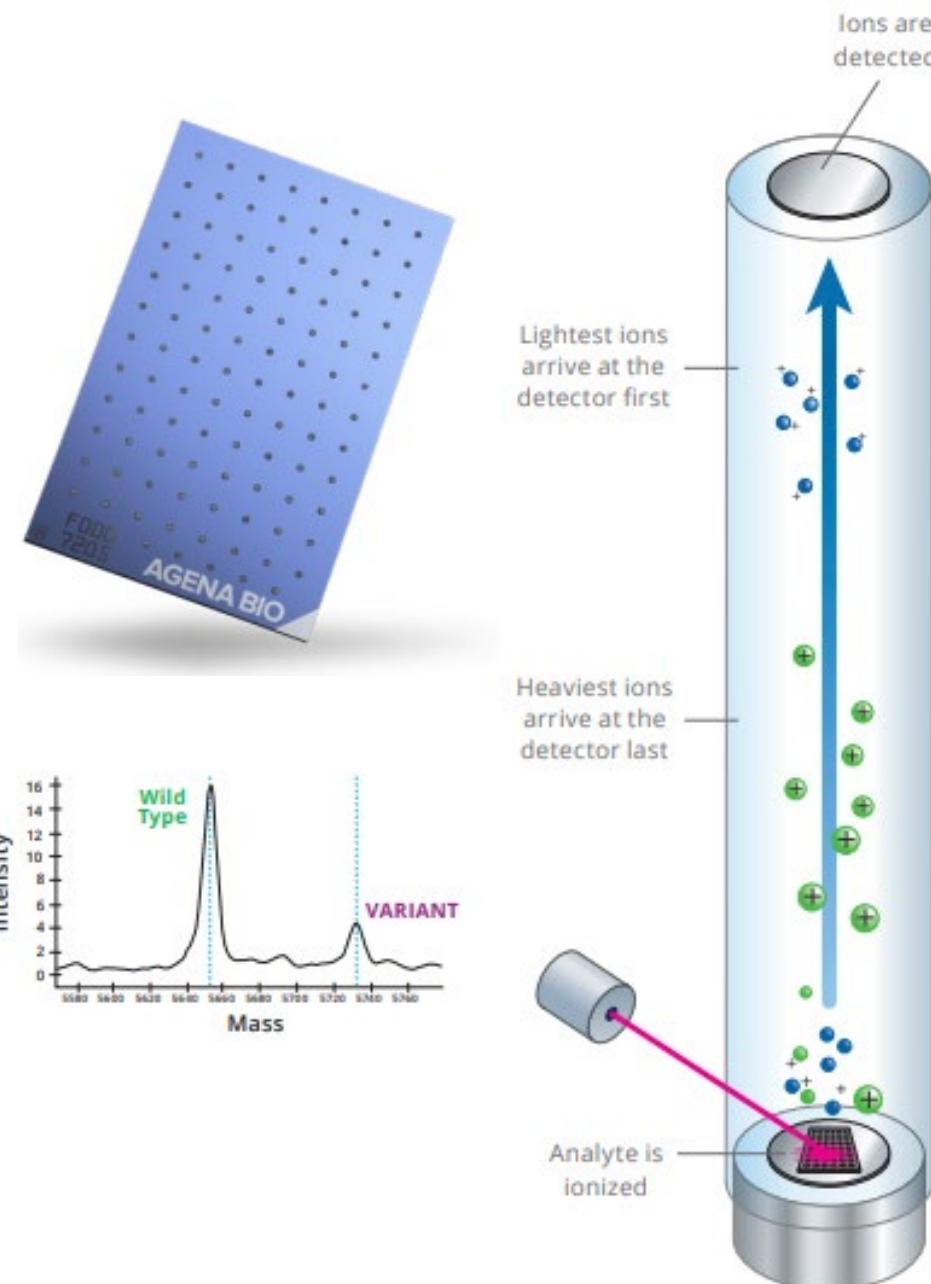
Matrix Assisted Laser Desorption Ionization – Time of Flight, or (MALDI-TOF), is a form of mass spectrometry that separates and detects sample particles by mass and charge.



MALDI-TOF

Basic principle:

- Sample analyte is loaded (adsorbed) onto an organic **matrix**
- A **laser** strikes the matrixed analyte, releasing a cloud of positively charged ions (**desorption**)
- Magnetic field drives the ions up a vacuum tube towards the detector. Ions separate by their m/z ratio as they travel
 - High-molecular-weight molecules travel slower than lighter molecules
- Detector measures each ion collision and graphs the ionic profile of the sample based on how long it takes each molecule to hit the detector, or “**time of flight**”
 - No need for fluorescent dyes to detect
- Graph is compared to a database of known profiles to identify genotype/microorganism



MALDI-TOF at CCF

Cleveland Clinic laboratories use MALDI-TOF in two distinct ways, depending on the department.

- Clinical Microbiology: pathogen identification using the Vitex MS MALDI-TOF instrument
- Molecular Diagnostics: variant detection using the Agena MassArray instrument



Agena MassArray



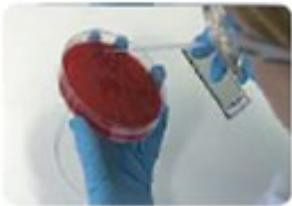
Vitek MS MALDI-TOF

MALDI-TOF - Micro

Clinical Microbiology uses MALDI-TOF to generate a spectral profile of an unknown organism.

A quick procedure, where an organism growth colony can be smeared directly onto the target slide and loaded for analysis.

Laser desorption produces a cloud of protein ions, which travel to the detector at different speeds based on their m/z ratio.



Pick Colony



Smear on the target slide



Add Matrix and dry



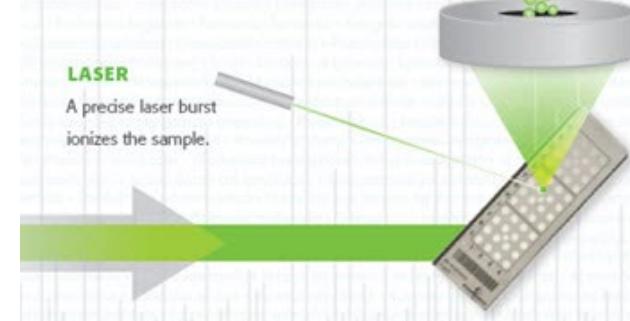
Load into the VITEK® MS

How It Works

MALDI-TOF has become state-of-the-art technology for identification of common bacteria and yeast in microbial applications because of its:

- rapid time to result (in minutes)
- capacity to work with small samples
- improved workflow
- specific, reliable and accurate results.

MALDI-TOF principles are simple, and the entire sample acquisition process typically takes less than a minute:



DETECTOR

Proteins are detected with a sensor to create a spectrum that represents the protein makeup of each sample.

SPECTRUM

A spectra from a particular sample is carefully digitized using bioMérieux's proprietary Advanced Spectra Classifier, which uses a Weighted Bin Matrix to identify sample spectra.

This approach is extremely robust, because no spectra comparisons are made. This process enables very rapid time-to-result and high accuracy.

TIME OF FLIGHT

After passing through the ring electrode, the proteins' Time of Flight is recorded using a formula:

$$\frac{\text{Mass}}{\text{Charge}} = \frac{2 \cdot (\text{elementary charge}) \cdot (\text{acceleration voltage})}{\text{path length}^2} = \text{time}^2$$

RING ELECTRODE

A "cloud" of proteins is released and accelerated by an electric charge.

SAMPLE

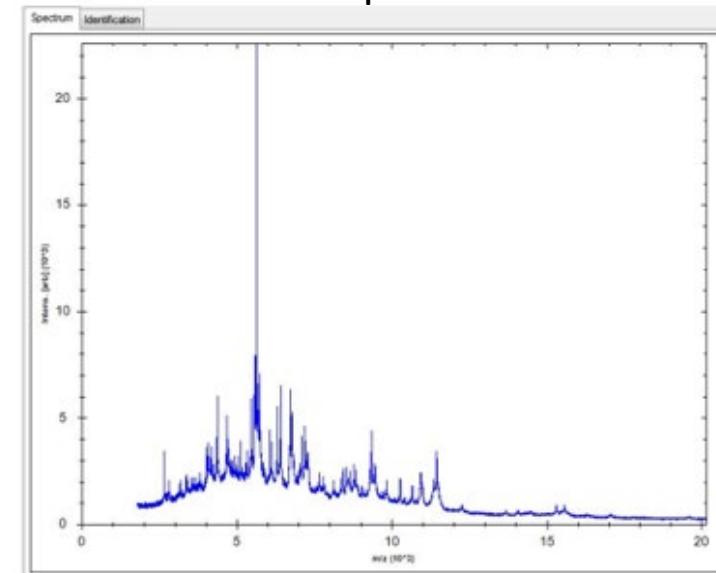
The target slide is prepared and introduced to a high-vacuum environment.

MALDI-TOF - Micro

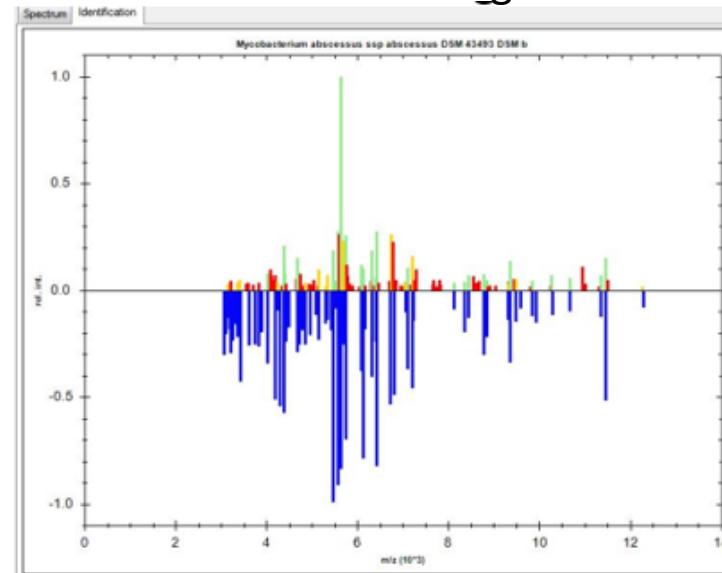
The raw spectrum produced is then compared to an electronic library of characteristic spectrums for organisms.

Spectrum peaks are matched up to reference spectrums by the analysis software, and a list of best matches is generated.

Raw Spectrum



Peak Matching



Best Matches in Database

Mo	Detected Species	Log(Score)
●	Mycobacterium abscessus ssp abscessus DSM 43493 DS...	2.180
●	Mycobacterium abscessus ssp abscessus BER2 DCSS b	2.160
●	Mycobacterium abscessus AFB034 MCW b	2.130
●	Mycobacterium abscessus ssp bolletii Meche 51906 DCSS b	2.080
●	Mycobacterium abscessus NIH S004	2.080
●	Mycobacterium abscessus RV423 C I 2011 MVD b	2.030
●	Mycobacterium abscessus NIH ATCC 19977T	1.990
●	Mycobacterium abscessus CCUG 55245 CCUG b	1.980
●	Mycobacterium abscessus ssp massiliense DSM 45103T D...	1.980
●	Mycobacterium abscessus NIH S005	1.960

MALDI-TOF – MDx

MDx uses MALDI-TOF as a way of identifying mutational variants in the CFTR gene that cause cystic fibrosis.

- A much lengthier procedure
- Used as a carrier screen for expecting parents

142 targeted variants are first amplified through a large multiplex PCR reaction.

- Each specimen is amplified across 8 reaction wells, with 10-20 primer sets/well

Cystic fibrosis (CFTR, RefSeq# NM_000492.3) Legacy names of 142 pathogenic variants: 1078delT, 1154insTC, 1213delT, 1248+1G>A, 1259insA, 1341+1G>A, 1461ins4, 1525-1G>A, 1548delG, 1677delTA, 1717-1G>A, 1717-8G>A, 1811+1.6kbA>G, 1812-1G>A, 1898+1G>A, 1898+3A>G, 1898+5G>A, 1898+5G>T, 2055del9>A, 2143delT, 2183AAtoG, 2184delA, 2184insA, 2307insA, 2347delG, 2585delT, 2622+1G>A, 2711delT, 2789+5G>A, 394delTT, 3007delG, 3120+1G>A, 3120G>A, 3121-1G>A, 3199del6, 3272-26A>G, 3659delC, 3791delC, 3849+10KbC>T, 3876delA, 3905inst, 405+1G>A, 406-1G>A, 4005+1G>A, 4016insT, 4209TGTT>AA, 4382delA, 457TAT>G, 574delA, 621+1G>T, 663delT, 711+1G>T, 711+3A>G, 711+5G>A, 712-1G>T, 852del22, 935delA, A455E, A559T, CFTRdelE2,3, CFTRdelE22,23, D110H, D1152H, deltaF508, deltaI507, E1104X, E585X, E60X, E822X, E831X, E92K, E92X, F508C, G1244E, G1349D, G178R, G330X, G542X, G551D, G85E, G970R, H199Y, I336K, K710X, L1065P, L1077P, L206W, L467P, L732X, L927P, M1101K, M1V, N1303K, P205S, P67L, Q1313X, Q220X, Q39X, Q493X, Q525X, Q552X, Q890X, Q98X, R1066C, R1066H, R1158X, R1162X, R117C, R117H, R334W, R347H, R347P, R352Q, R553X, R560K, R560T, R709X, R75X, R764X, R851X, S1196X, S1251N, S1255X, S341P, S466X, S489X, S492F, S549N, S549R-CGT, S549R-AGG, S945L, T338I, V520F, W1089X, W1204X, W1204X, W1282X, W401X, W846X, Y1092X, Y1092X, Y122X (conditionally reported variants: Poly T, I506V, I507V).

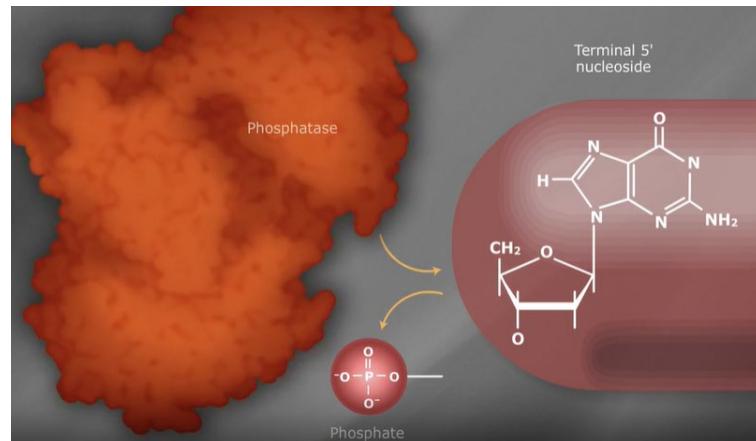
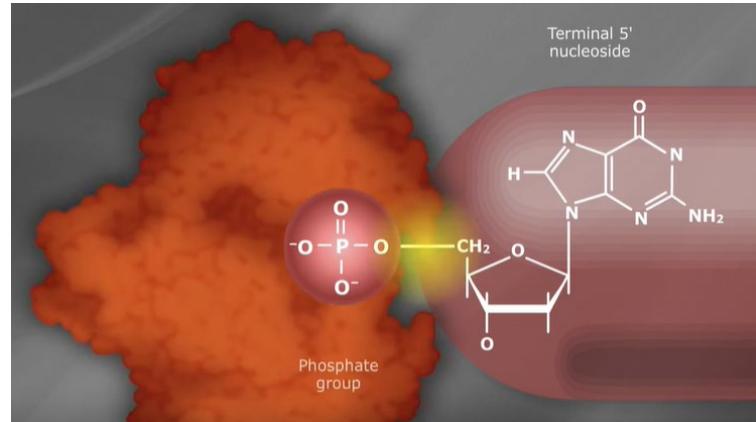
MALDI-TOF – MDx

Following initial amplification, PCR products undergo a shrimp alkaline phosphatase (SAP) digestion.

- SAP enzyme dephosphorylates unextended primers and unincorporated dNTPs.
- Necessary to remove prior to final thermocycling program: single base extension.



*Pandalus
borealis*

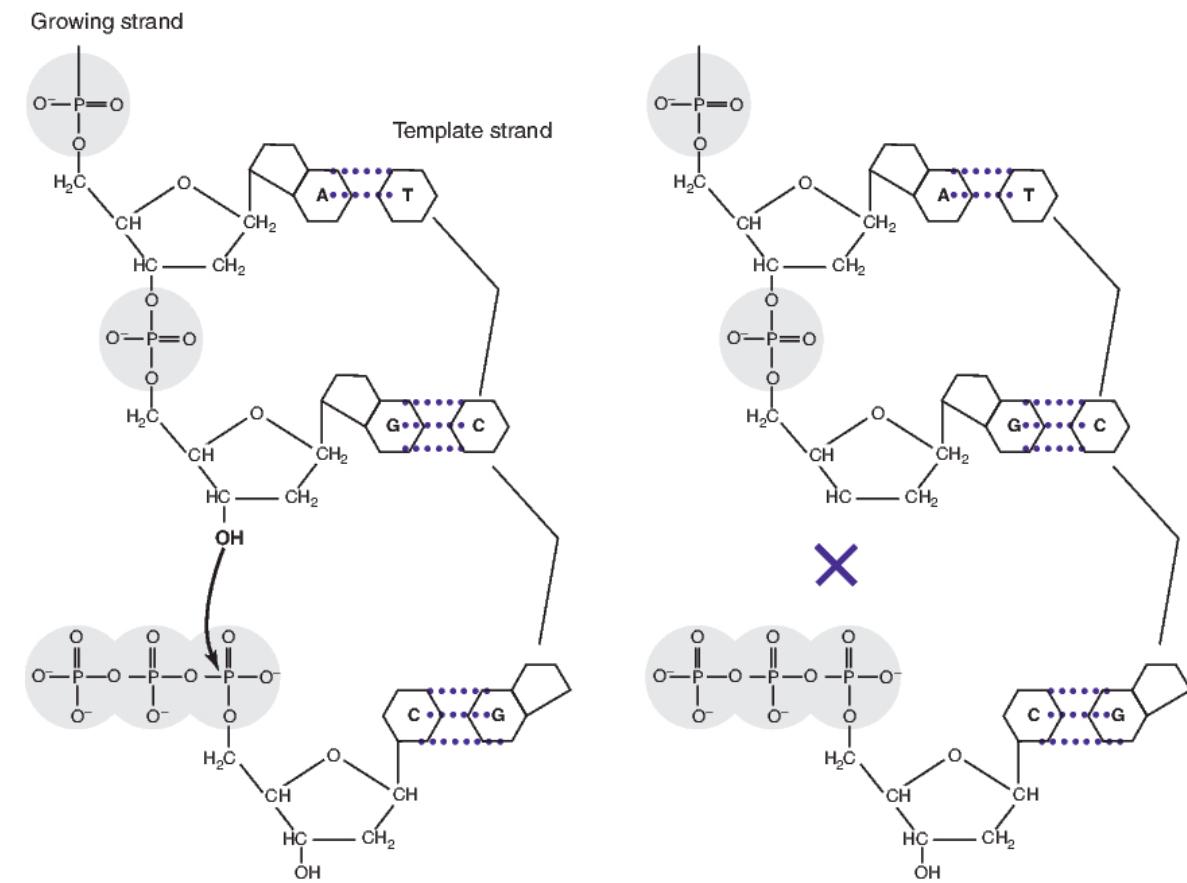
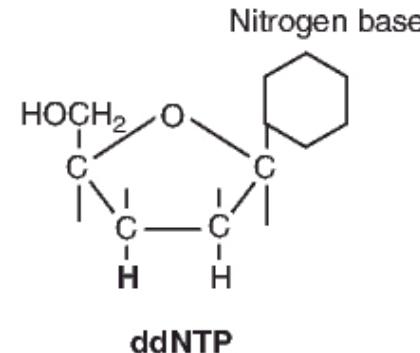
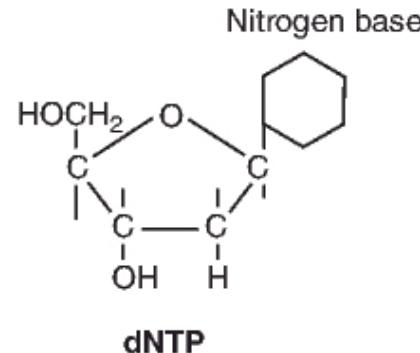


MALDI-TOF – MDx

Following SAP cleanup, samples then undergo a **single base extension** reaction.

Utilizes dideoxynucleotides (**ddNTPs**) instead of dNTPs to stop DNA synthesis after a single base addition.

- Lack the 3' hydroxyl group
- Prevents 5' phosphate group of incoming nucleotide from forming a phosphodiester bond

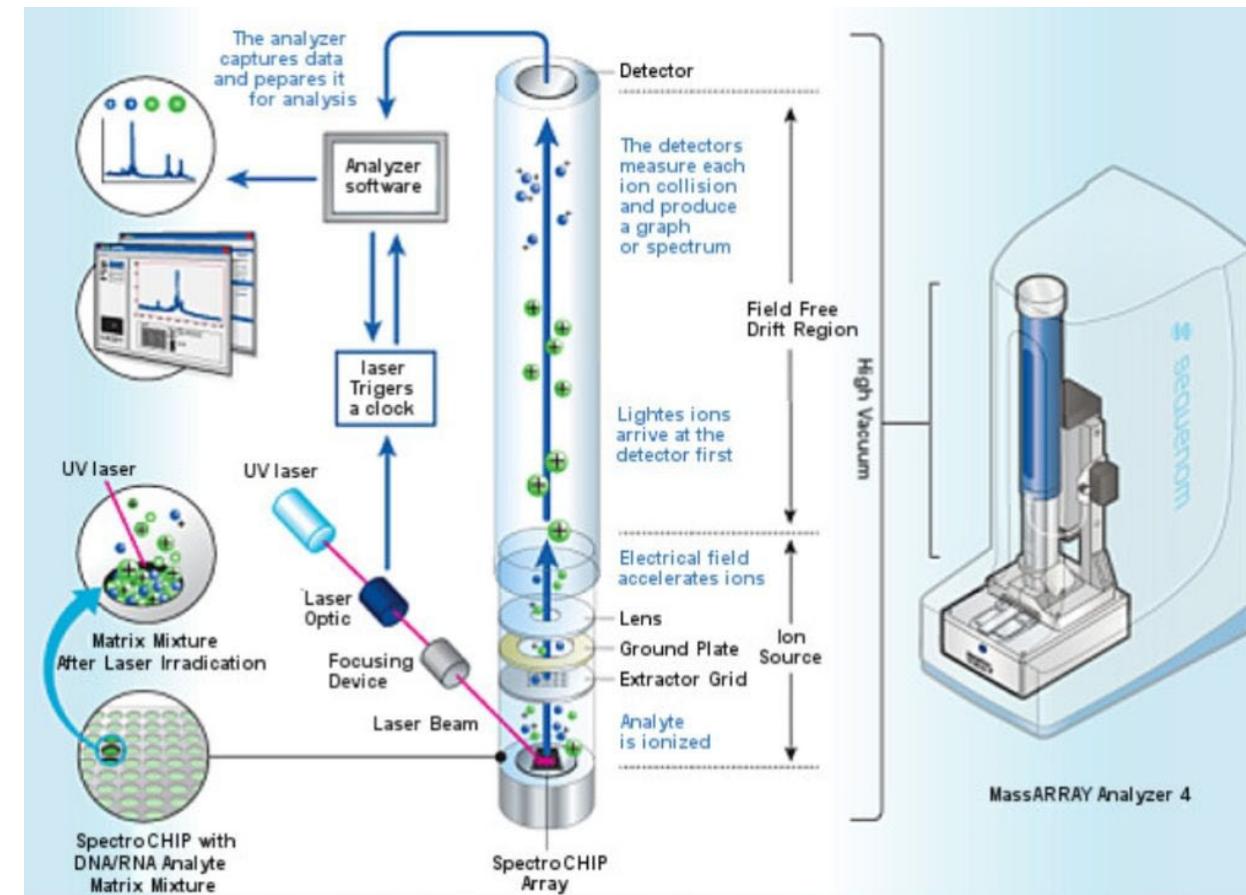


MALDI-TOF – MDx

Following single base extension, all samples are then applied to spectrochip matrices and placed in the MALDI-TOF instrument for analysis.

Once laser is fired, a timer begins. Every ion collision with the detector generates an electrical signal graphed on a spectrum.

Since nitrogenous bases are electrostatically neutral, TOF from laser-to-detector is proportional to the mass of the amplicon.



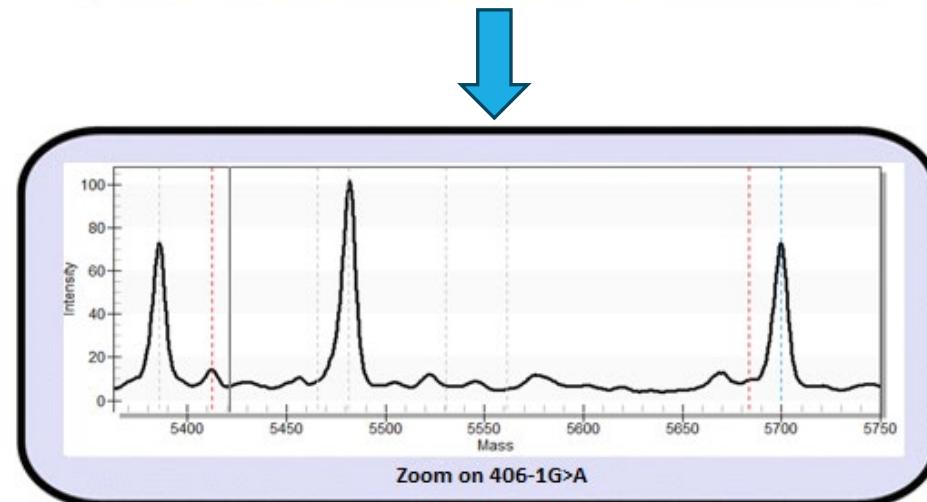
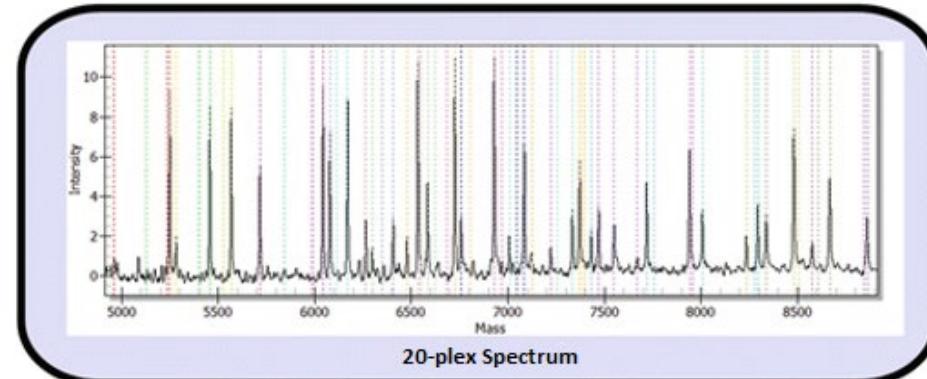
The MALDI-TOF Process in the MassARRAY Analyzer 4

MALDI-TOF – MDx

Spectrum generated from each “zap” of the laser will show every ion present in an individual reaction well.

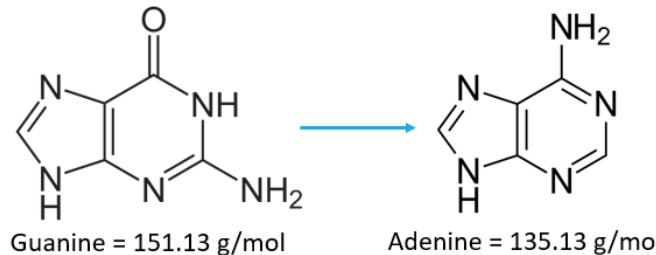
- Wildtype amplicons for all primer sets
- Mutant amplicons for all primer sets
- Unextended primers for all primer sets

Knowing the sequence (and therefore the mass) of the extension primer and the possible single base additions allows laboratorian to zoom in on amplicons from a specific primer set and interpret the genotype.



MALDI-TOF – MDx

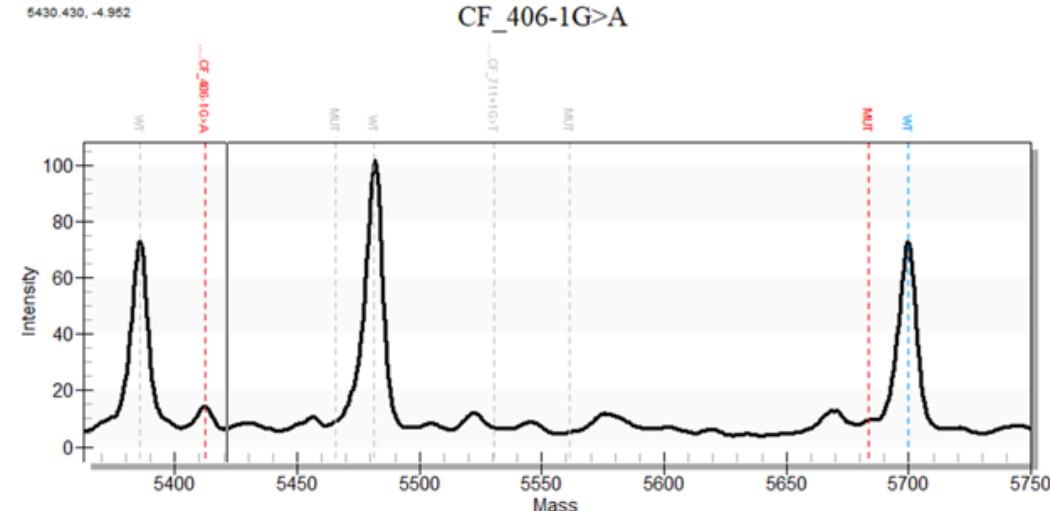
One variant, 406-1G>A, is a single base substitution of guanine with adenine



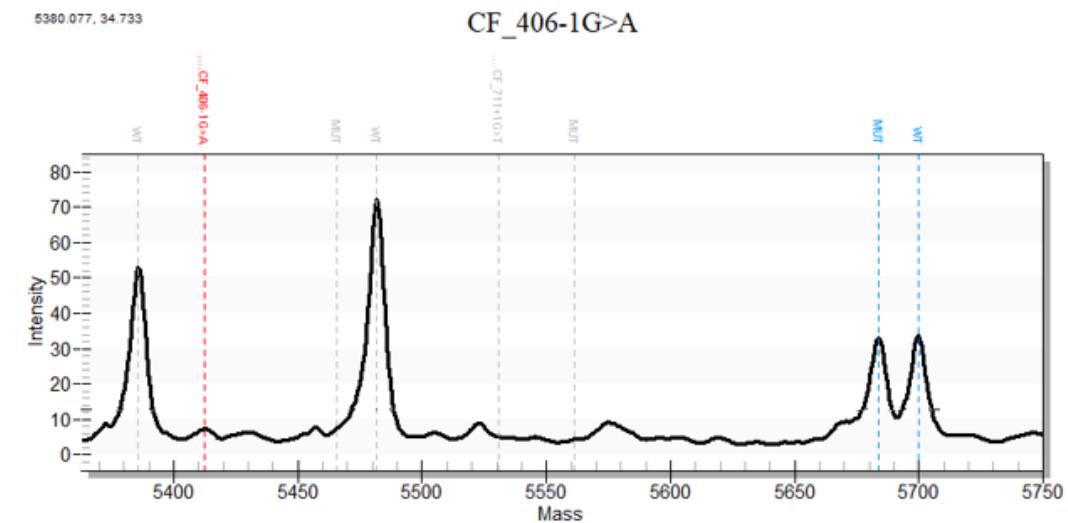
Extension primer is complementary to sequence just prior to the 406-1G>A SNP, so the single base addition of ddGTP or ddATP is easily resolved.

- Ext Primer = 5'-TGTTTCCCCTTTGTA-3'
- WT Sequence = 5'-TGTTTCCCCTTTGTG-3'
- MUT Sequence = 5'-TGTTTCCCCTTTGTA-3'

Variant is not detected (WT-WT)



Variant is detected (MUT-WT, aka HET)



Questions?

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This concludes the presentation.

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