SCST Genetics Super Workshop

Analysis of PCR

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What type of Analysis do I need?

Analysis vs. PCR Technology

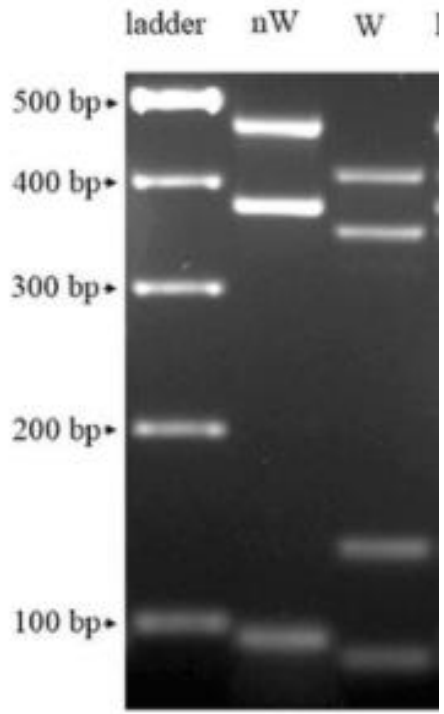
Analysis vs. PCR Technology

- Gel Electrophoresis
- Fluorescent Probes
  - Quantitative (Real-time)
  - End Point

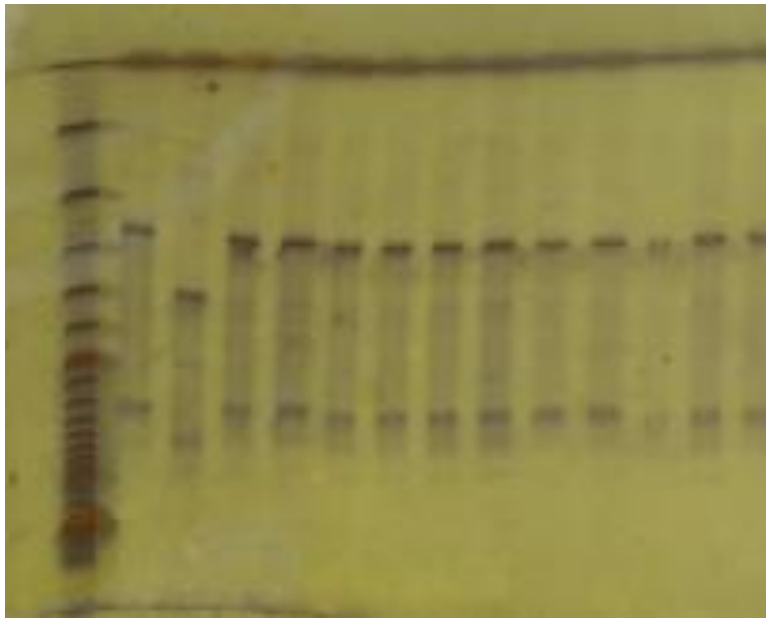
Electrophoresis

// PCR amplification products are physically removed from reaction plate/tube and ran on agarose or polyacrylamide gels and then stained

// This allows for a visual representation of the PCR product by size in comparison to control bands or “ladder”



https://www.researchgate.net/figure/Agarose-gel-electrophoresis-AGE-image-of-the-PCR-products-a-with-the-labelled-marker_fig1_344193749

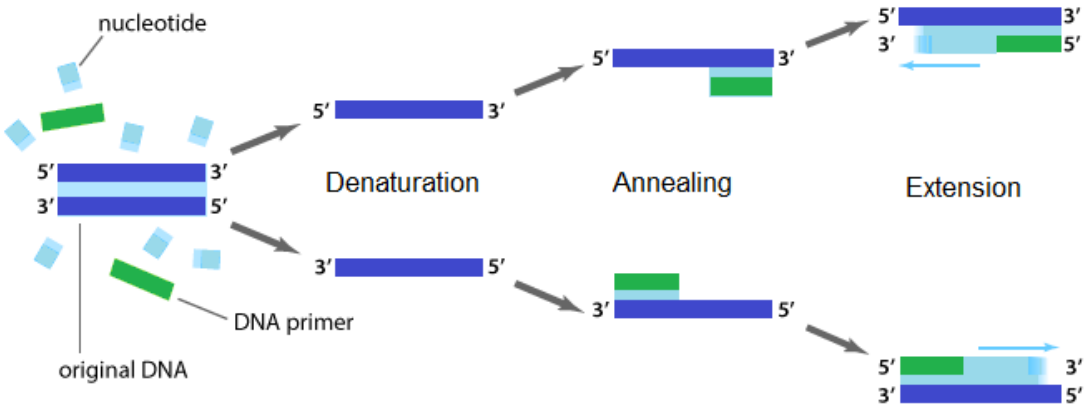


PCR Amplification

// If you can't visualize your PCR amplicons on a gel, how else might you visualize the success/failure of your reaction?

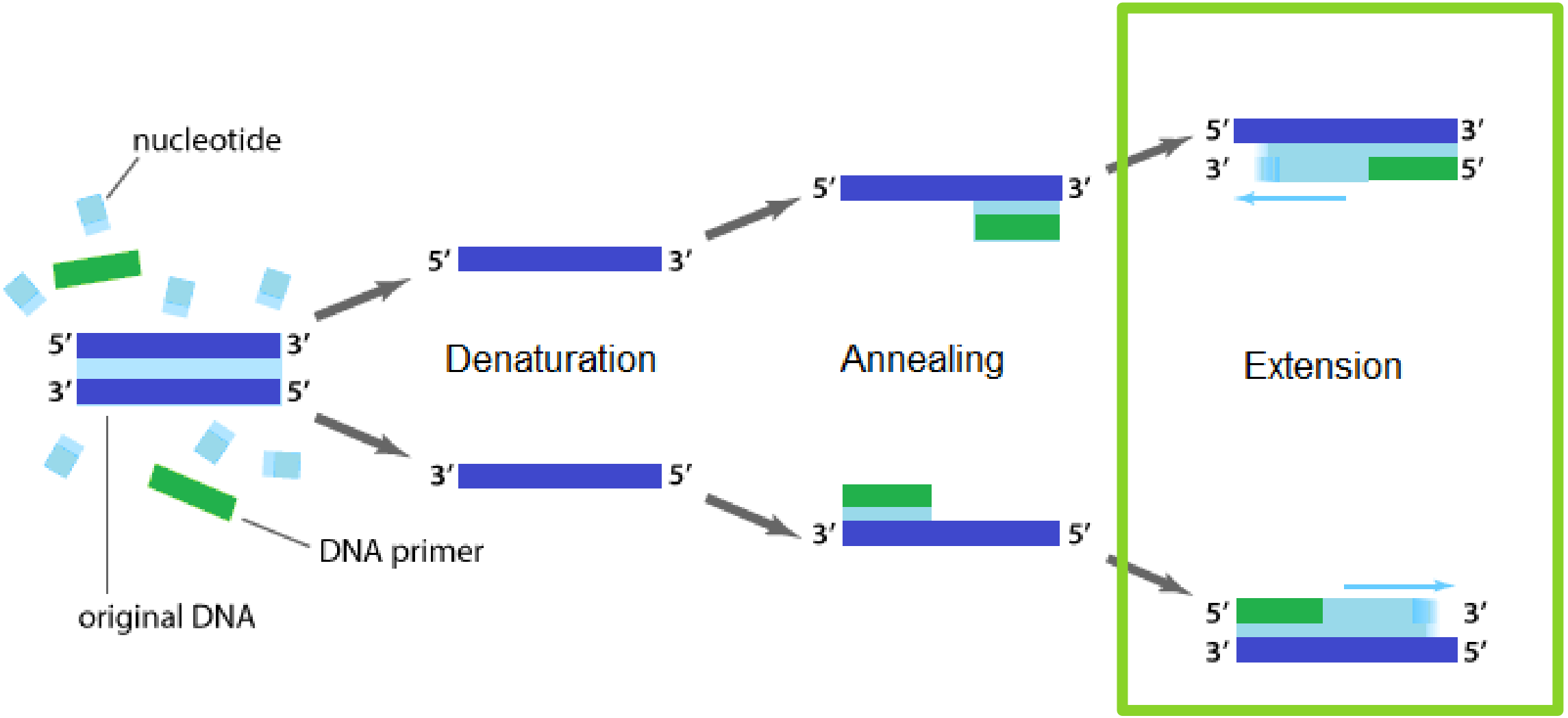
// A common solution is to include a dye/probe in your reaction.

  // These elements can produce a fluorescent signal upon successful creation of new (and hopefully intended) double-stranded DNA



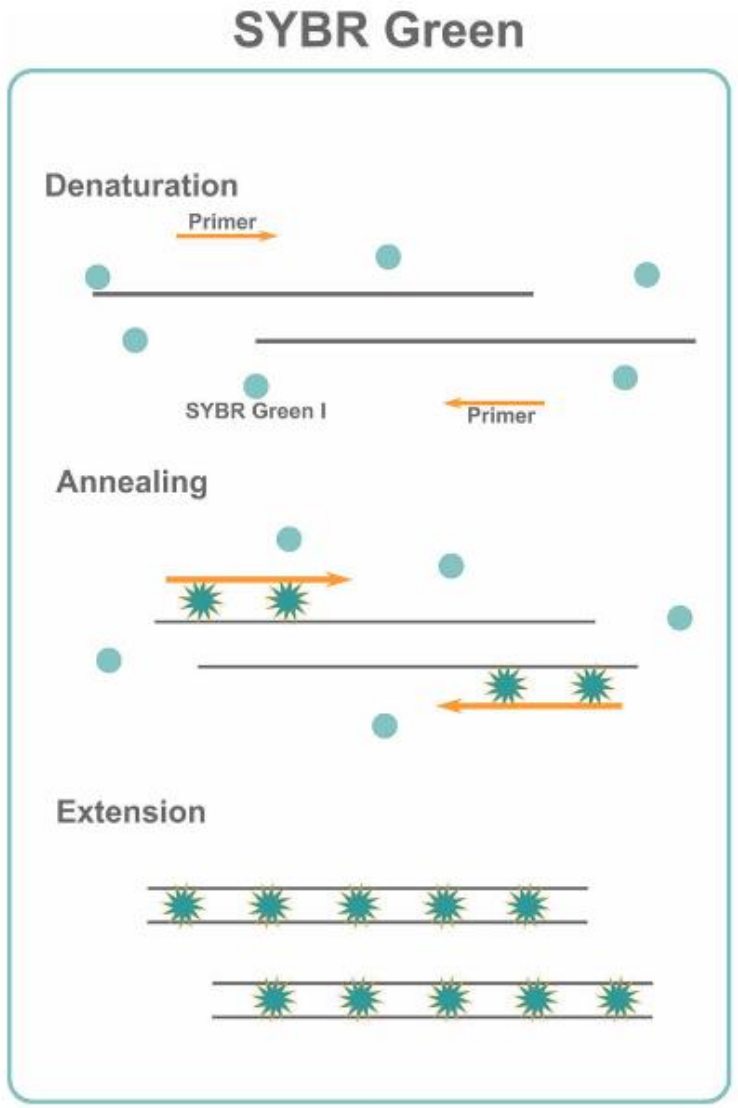
https://www.xxpresspcr.com

PCR Amplification



Probes and Dyes

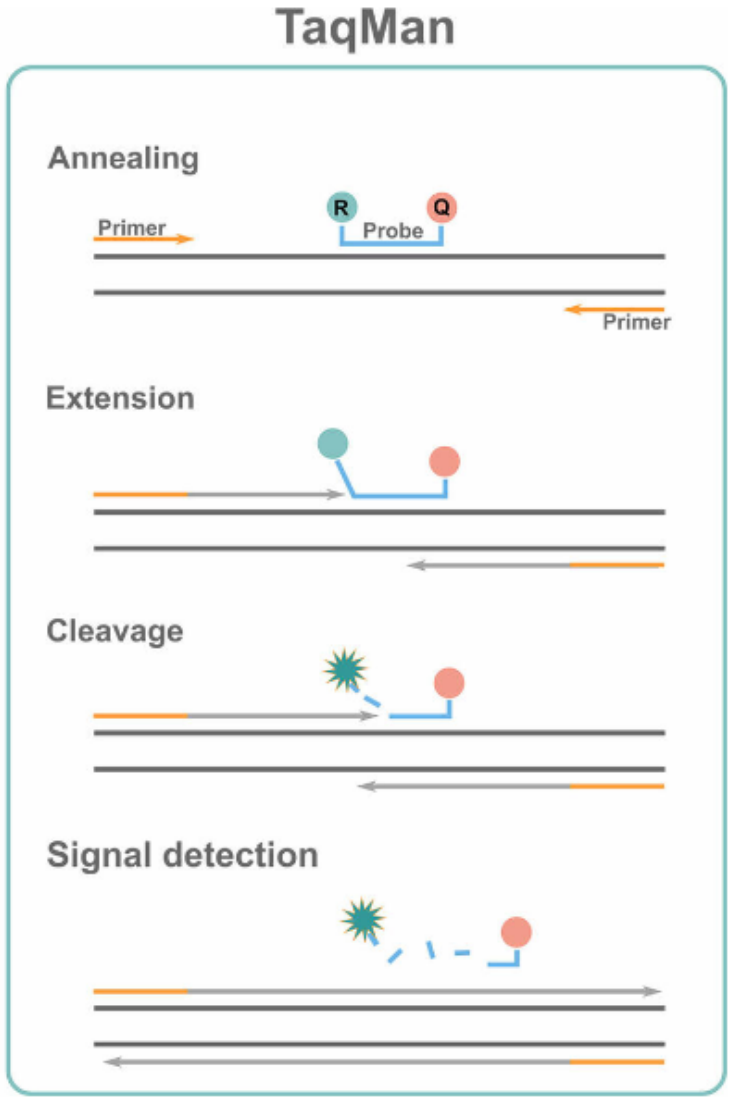
- It is possible to include things in your reaction that can be digitally detected to determine the success/failure of your PCR reaction
- One such choice is an intercalating dye such as SYBR® Green
  - This binds with double-stranded DNA
  - As your reaction progresses, more and more DNA is copied and synthesized, resulting in more dye being incorporated and visible to a fluorescent dye reader



https://www.integra-biosciences.com/france/en/blog/article/how-does-qpcr-work-sybrr-green-vs-taqmanr

Probes and Dyes (cont.)

- Another choice is via hydrolysis probes
  - One popular technology is called TaqMan
  - These probes are short oligonucleotides with a dye and quencher attached
    - FAM, VIC, HEX, TED, NED
  - During PCR, the Taq polymerase's 5' exonuclease activity results in the probe being "dissolved" and releasing the dye from the quencher into the free solution
  - As your reaction progresses, more and more probe is consumed and results in more and more dye being visible to a fluorescent reader



https://www.integra-biosciences.com/france/en/blog/article/how-does-qpcr-work-sybrr-green-vs-taqmanr

What will my results look like?

Analysis/Scoring Tools

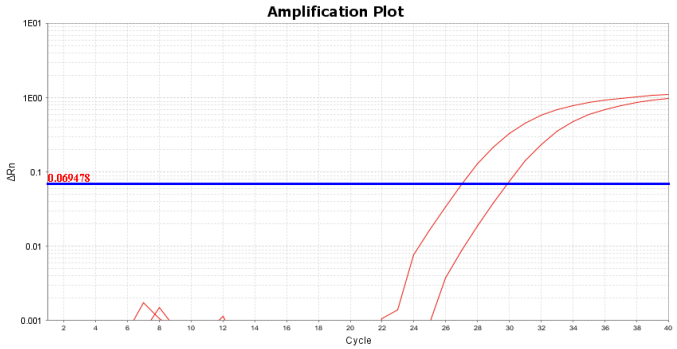
Scoring/Analysis Tools

- qPCR – Threshold curves
  - Quantitative
  - Very informative as a research or diagnostic tool

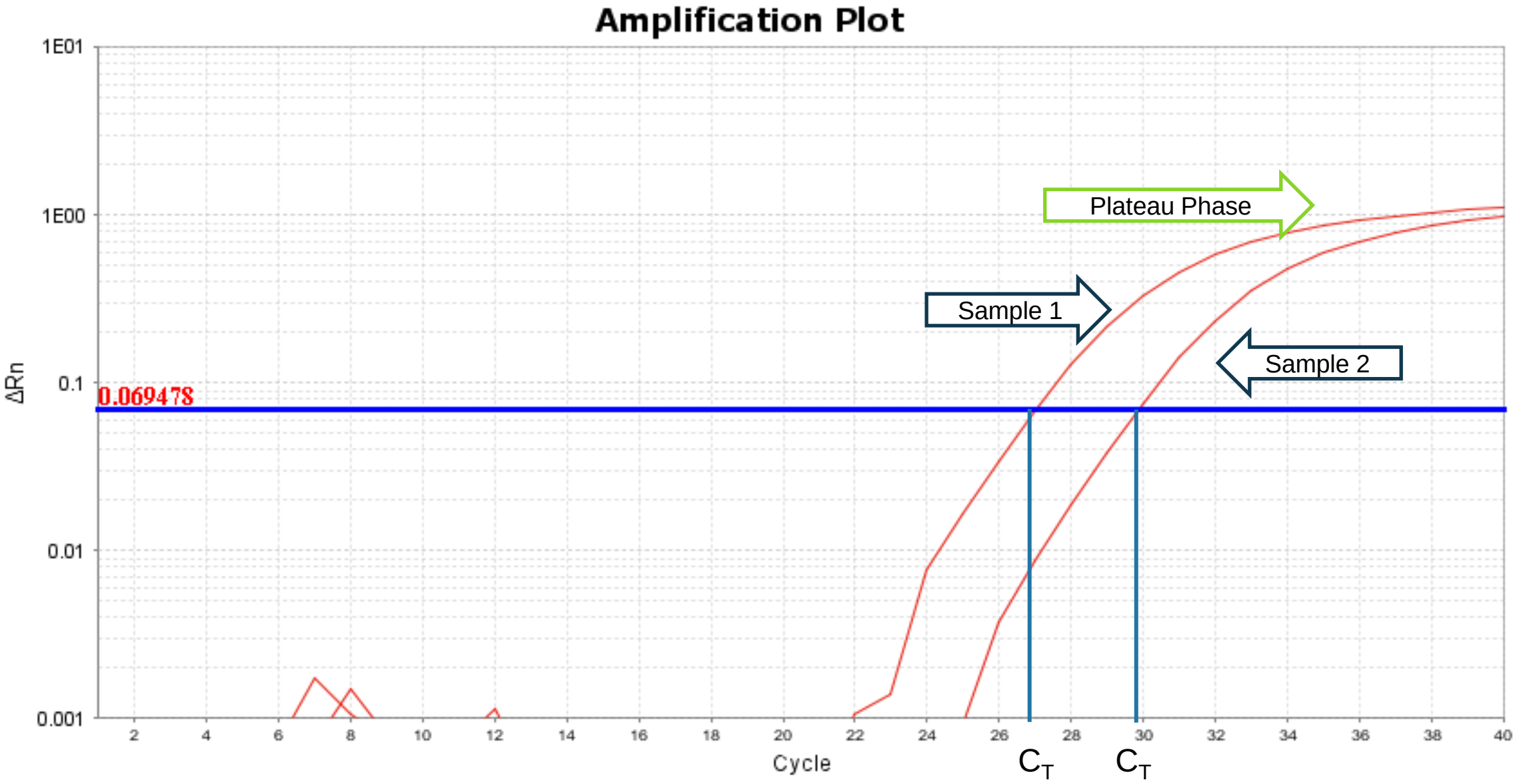
- End-point - Scatter Plot Scorers
  - Qualitative
  - Useful in comparing amplicons vs. specific control samples

qPCR data output

- qPCR output example to the right
  - For every well/reaction you get an amplification cycle followed by a fluorescent read
  - Each probe/dye of interest is read following each cycle
- These values are plotted on a curve (seen below)
  - These curves show the result of amplification after each cycle and the success and amount of DNA in your reaction can be determined.



Well	Cycle	Target	Rn
1	1	VIC	0.701914
1	1	FAM	0.931821
1	2	VIC	0.704537
1	2	FAM	0.937491
1	3	VIC	0.705486
1	3	FAM	0.91906
1	4	VIC	0.705754
1	4	FAM	0.926271
1	5	VIC	0.70383
1	5	FAM	0.964664
1	6	VIC	0.70722
1	6	FAM	0.957732
1	7	VIC	0.719632
1	7	FAM	0.954835
1	8	VIC	0.723635
1	8	FAM	0.959973
1	9	VIC	0.722601
1	9	FAM	0.955685
1	10	VIC	0.725126
1	10	FAM	0.955781
1	11	VIC	0.725414
1	11	FAM	0.964972
1	12	VIC	0.727352
1	12	FAM	0.974747
1	13	VIC	0.728112
1	13	FAM	0.977479
1	14	VIC	0.726119
1	14	FAM	0.98459
1	15	VIC	0.726743
1	15	FAM	0.99083
1	16	VIC	0.732761
1	16	FAM	0.996642
1	17	VIC	0.733663
1	17	FAM	1.001856
1	18	VIC	0.731023
1	18	FAM	0.990654
1	19	VIC	0.73487
1	19	FAM	0.972437
1	20	VIC	0.73713
1	20	FAM	0.971426
1	21	VIC	0.740647
1	21	FAM	1.00215
1	22	VIC	0.744839
1	22	FAM	1.017772
1	23	VIC	0.743357
1	23	FAM	1.018334
1	24	VIC	0.745773
1	24	FAM	1.027405
1	25	VIC	0.748063
1	25	FAM	1.051445
1	26	VIC	0.743999
1	26	FAM	1.040454
1	27	VIC	0.741811
1	27	FAM	1.032999
1	28	VIC	0.739786
1	28	FAM	1.027812
1	29	VIC	0.743339
1	29	FAM	1.031157
1	30	VIC	0.744617
1	30	FAM	1.054483
1	31	VIC	0.749272
1	31	FAM	1.050969
1	32	VIC	0.751105
1	32	FAM	1.021994
1	33	VIC	0.750543
1	33	FAM	1.017399
1	34	VIC	0.747948
1	34	FAM	1.038553
1	35	VIC	0.74543
1	35	FAM	1.054824
1	36	VIC	0.747478
1	36	FAM	1.060549
1	37	VIC	0.756229
1	37	FAM	1.058401
1	38	VIC	0.755765
1	38	FAM	1.060083
1	39	VIC	0.746331
1	39	FAM	1.065241
1	40	VIC	0.742072
1	40	FAM	1.068946

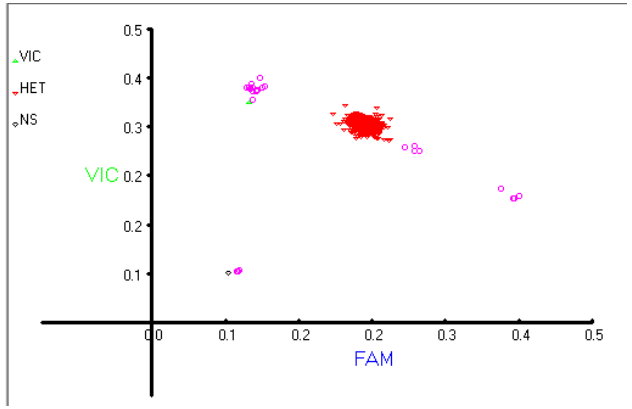

Amplification Plot
1E01
1E00
0.1
0.01
0.001
ΔRn
0.069478
Plateau Phase
Sample 1
Sample 2
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
Cycle
C_T
C_T

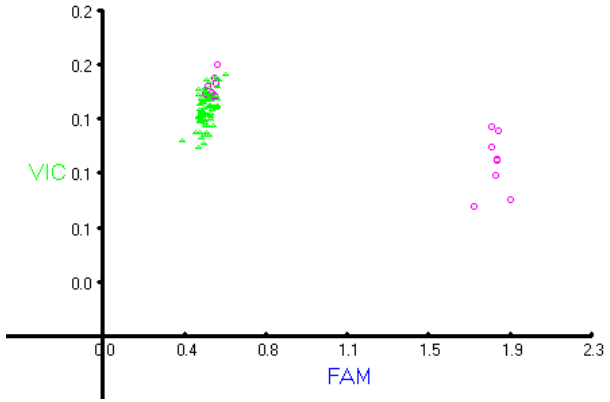
Endpoint data output

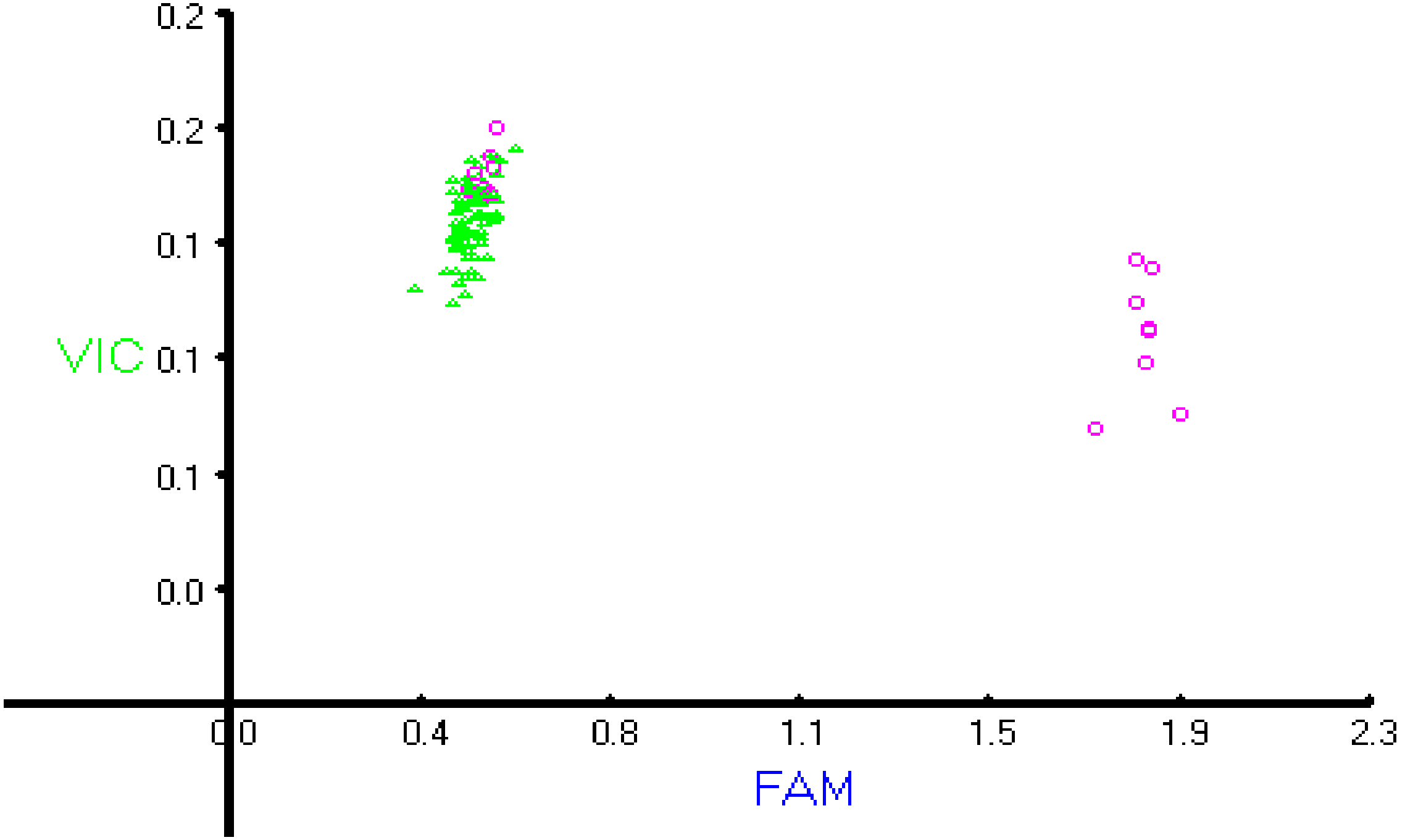
- Endpoint Fluorescent read to the right
  - For every well/reaction you get a single read after ALL amplification cycles have been completed
  - Each probe/dye gets a single read after a full amplification run

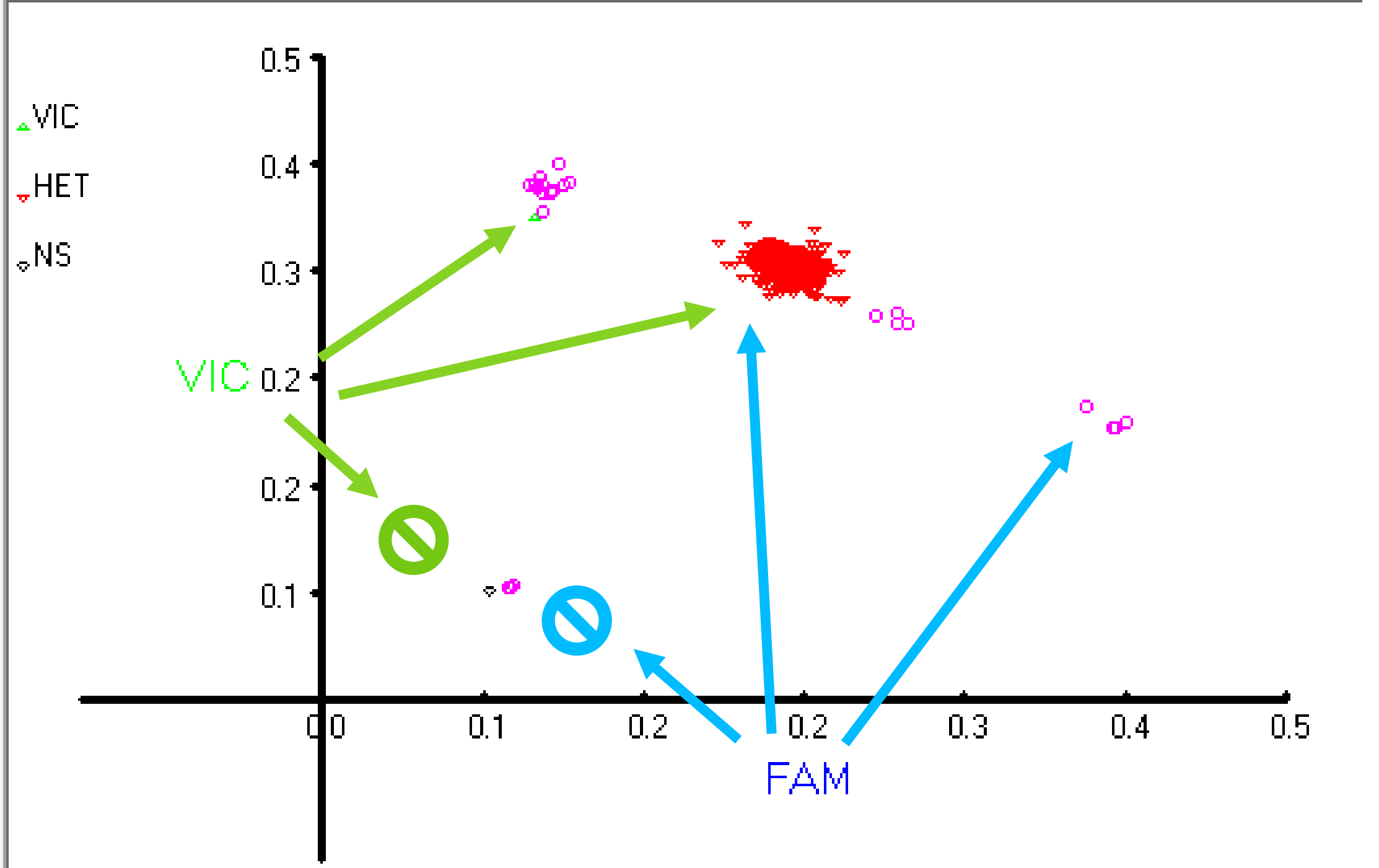
Well	FAM	VIC	ROX
A1	2812	1719	19036

- These values are placed on a scatter plot with your probes/dyes on each axis
  - These values indicate the amount of probe signal produced from the amplification and can be used to determine the presence of your target amplicon






0.2
0.2
0.1
VIC
0.1
0.1
0.0
0.0
0.4
0.8
1.1
1.5
1.9
2.3
FAM


VIC
HET
NS
0.5
0.4
0.3
0.2
0.1
VIC
0.0
0.1
0.2
0.2
0.3
0.4
0.5
FAM

What does my result mean?

Actual Data Analysis

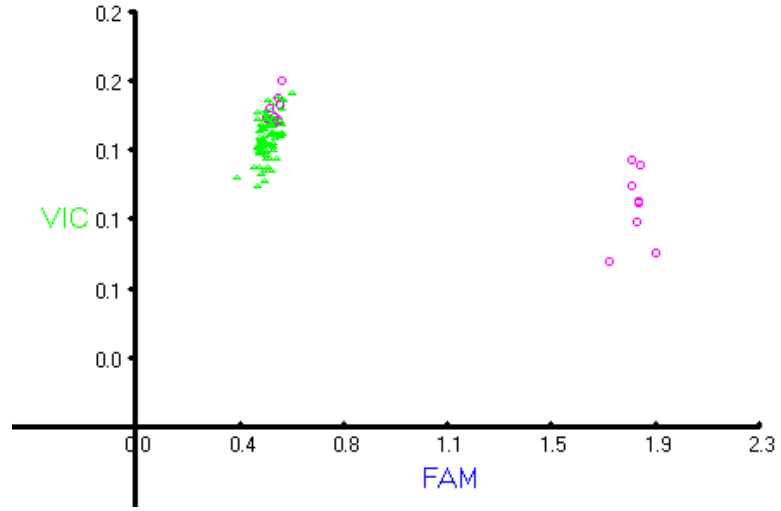
Actual Data Analysis

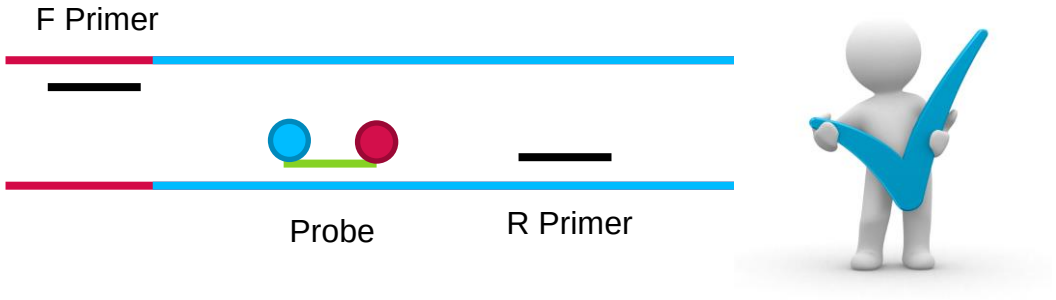
- What does my result mean?
  - AP/Trait
  - SNP

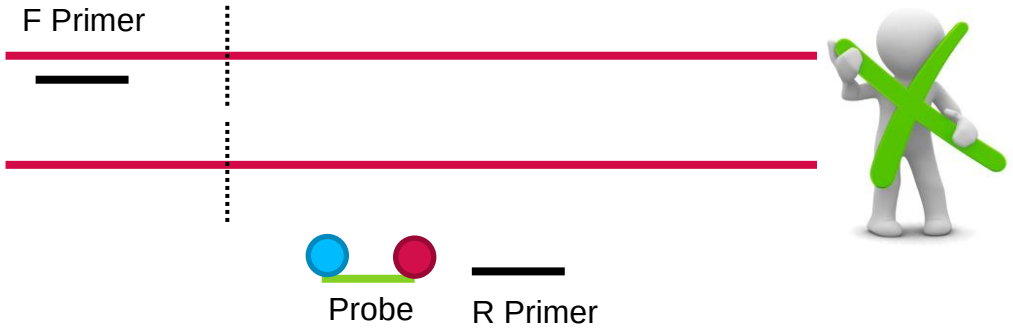
AP/Trait

Adventitious Presence/Trait

- These assay designs are commonly looking for the presence of an inserted transgene or native section of DNA within a specific genomic location
- Even just a single probe assay can help you determine the presence/absence of your target region
- On your plot, a positive detection of your target would be shown by an increased signal on your probe's axis.
  - Conversely, a negative detection would result in a lack of signal from your probe.
- NOTE! In this assay design, the difference between negative trait presence and a reaction failure look identical.

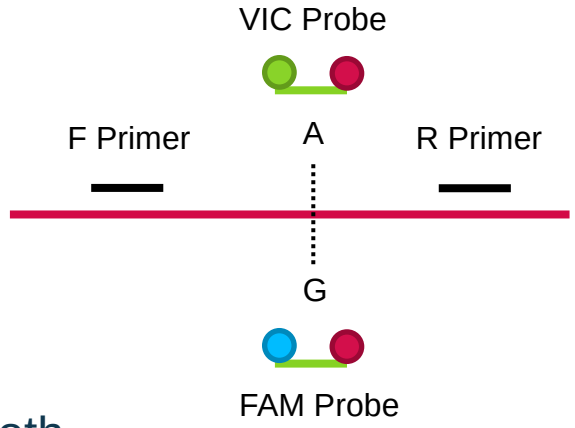




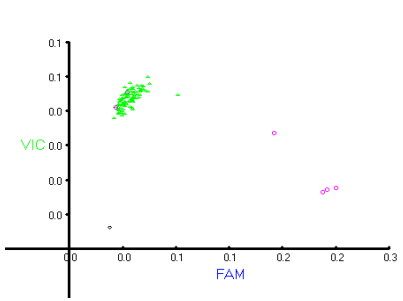


SNP

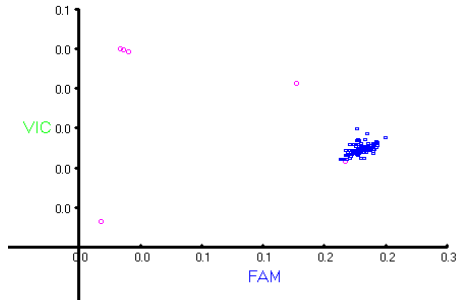
Single Nucleotide Polymorphism



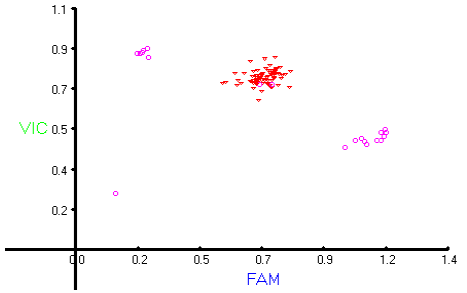
- These assays are designed for a specific region of DNA that flanks a SNP
- Since there are 2 copies of DNA present, you may get signal from one or both probes which would indicate which (or both) alleles are present
- Since each copy of DNA is inherited from a single parent, these results can be used to determine parental lineage of your sample
  - Provided you know the allele present in both parental samples



VIC
ACCG**C**CCGA
ACCG**C**CCGA



FAM
ACCG**T**CCGA
ACCG**T**CCGA



HETEROZYGOUS
ACCG**C**CCGA
ACCG**T**CCGA

SNP (cont.)

Marker	1	2	3	4	5	6	7	8	9	10
Parent 1	T:T	A:A	G:G	T:T	T:T	G:G	T:T	A:A	A:A	T:T
Parent 2	T:T	A:A	G:G	C:C	A:A	G:G	T:T	A:A	A:A	T:T
Unknown 1	T:T	A:A	G:G	T:C	T:A	G:G	T:T	A:A	A:A	T:T
Unknown 2	C:C	A:A	G:G	T:T	T:T	G:G	T:T	C:C	A:A	T:T

Able to determine the nature of your unknown sample:

- Female and Male Self
- Hybrid
- Out-cross (Pollenation from an undesired source)
- Error (A genotype that CAN'T be explained by outcrossing)