Near East University
DESAM Research Institute

One Kit One Product

Workshop

Optimization/Analysis/Troubleshooting

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Experimental Design

PrimerF_H131 AATCCCAGAAATTCTCCCA

probe CACAAGCAAACCACAGTCACAGT

AATCCCAGAAATTCTCCCATTTGGATCCCACCTTCTCCATCCCACAAGCAAACCACAGTCACAGTGGTGA

TTAGGGTCTTTAAGAGGGTAAACCTAGGGTGGAAGAGGTAGGGTGTTCGTTTGGTGTCAGTGTCACCACT

TTACCACTGCACAGGAAACATAGGCTACACGCTGTTCTCATCCAAGCCTGTGACCATCACTGTCCAAGGT

AATGGTGACGTGTCCTTTGTATCCGATGTGCGACAAGAGTAGGTTCGGACACTGGTAGTGACAGGTTCCA

GTGACAGGTTCCA PrimerR_H131/R131

PrimerF_H131 AATCCCAGAAATTCTCCCG

probe CACAAGCAAACCACAGTCACAGT

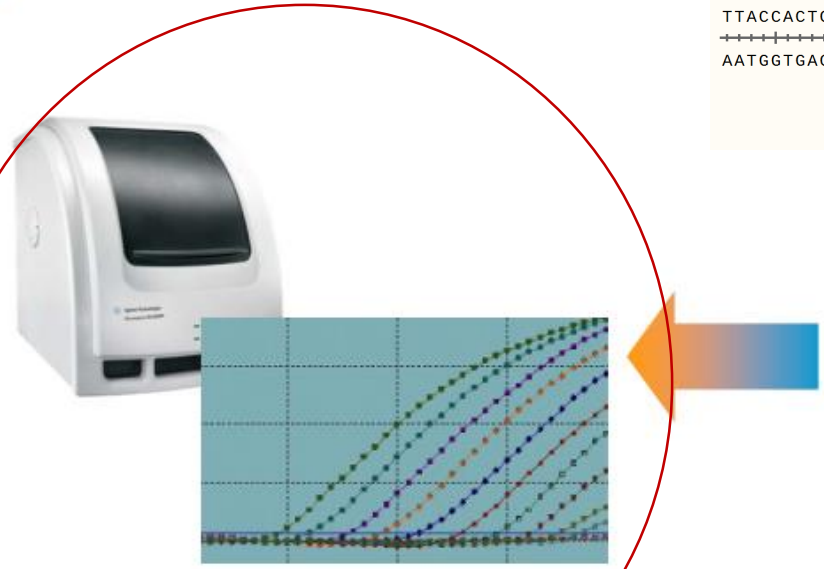
AATCCCAGAAATTCTCCCATTTGGATCCCACCTTCTCCATCCCACAAGCAAACCACAGTCACAGTGGTGA

TTAGGGTCTTTAAGAGGGTAAACCTAGGGTGGAAGAGGTAGGGTGTTCGTTTGGTGTCAGTGTCACCACT

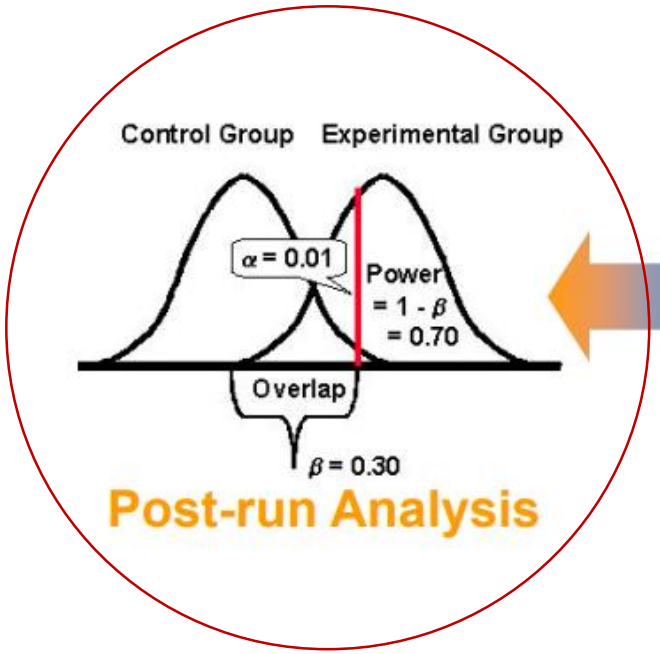
TTACCACTGCACAGGAAACATAGGCTACACGCTGTTCTCATCCAAGCCTGTGACCATCACTGTCCAAGGT

AATGGTGACGTGTCCTTTGTATCCGATGTGCGACAAGAGTAGGTTCGGACACTGGTAGTGACAGGTTCCA

GTGACAGGTTCCA PrimerR_H131/R131



Real time QPCR



Post-run Analysis

Optimization

- Optimizing your assay can help you to
- Increase specificity: Get rid of unspecific amplification eg. primer dimers
- Increase sensitivity: Get earlier Ct values, detect lower concentrations
- Increase reproducibility: Low replicate variability, high amplification efficiency

Optimization

- Assay optimization is essential and it is required to ensure that the assay is as sensitive as is required and that it is specific to the target of interest.
    - Pathogen detection or expression profiling of rare mRNAs require high sensitivity;
    - SNP detection requires high specificity
    - Viral quantification needs both high specificity and sensitivity.
- Assays requiring high specificity are particularly vulnerable when performed without optimization and adequate controls.
- Similarly, when multiple targets are to be detected simultaneously in multiplex reactions, assay conditions must be optimized to detect all targets equally.

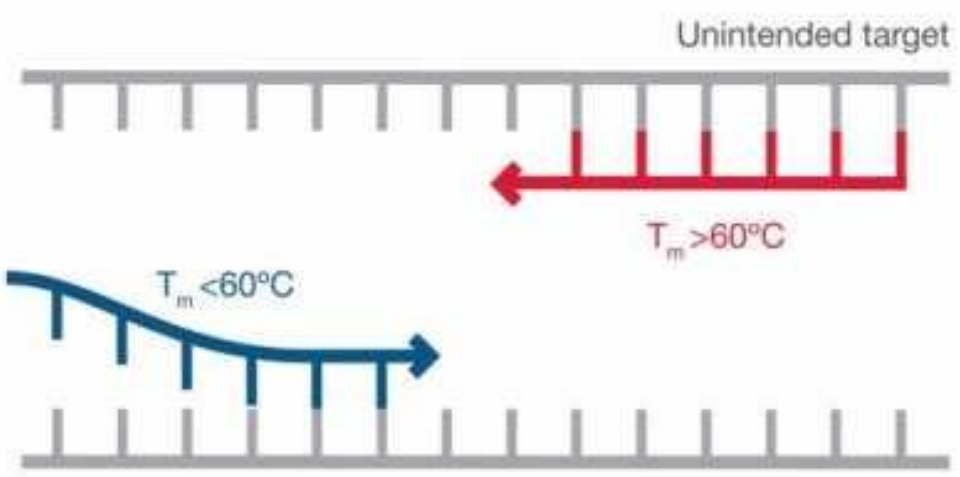
Optimization

- There are a number of factors that can be altered to obtain optimum assay performance and thereby lead to higher molecular sensitivity and specificity.
- Regardless of whether the target is DNA (qPCR) or RNA (RT-qPCR), the following preliminary steps use successful quantification:

  - Optimizing Primer Annealing Temperature
  - Optimizing Primer/Probe Concentrations
  - Optimizing Reaction Components

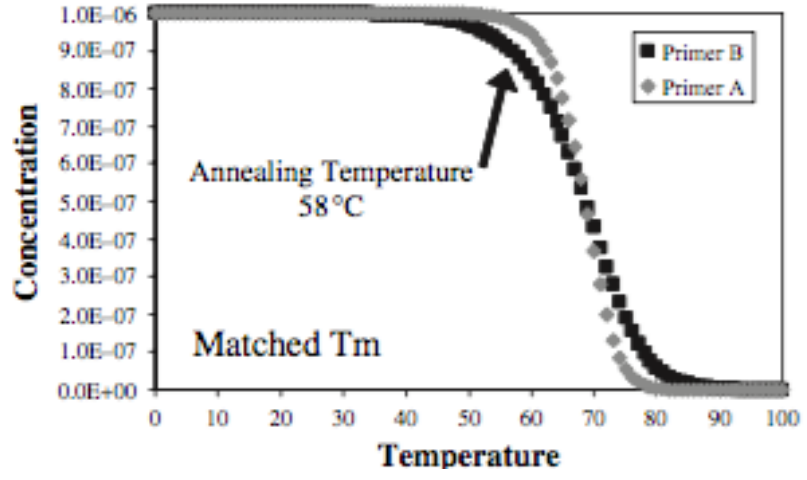
Optimizing Primer Annealing Temperature

- Primer annealing is a critical step in PCR.
- In this step, the primers bind to flanking sequences of the target DNA or cDNA for amplification.
- The annealing temperature of this step should be determined from the melting temperature (Tm) of the selected primers to help ensure specificity of primer binding and target amplification.



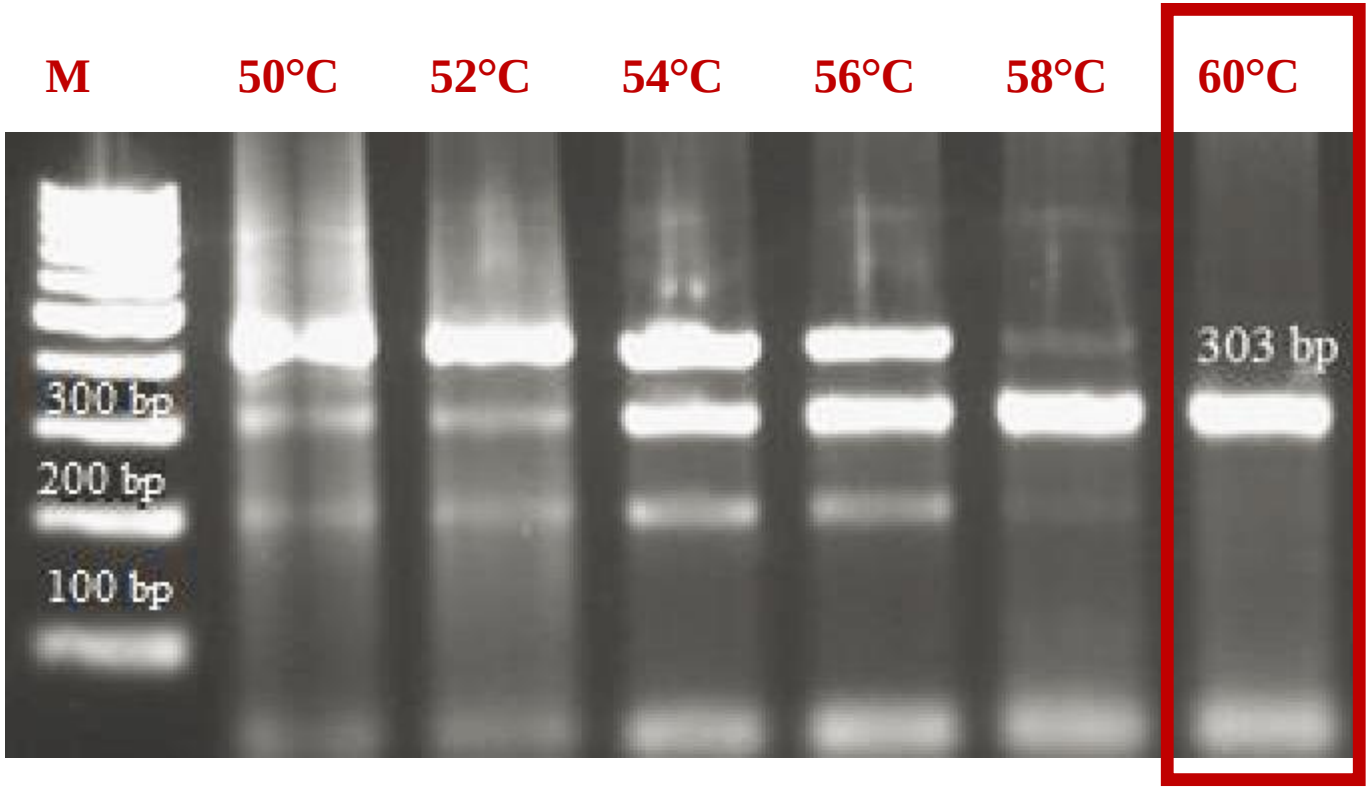
Optimizing Primer Annealing Temperature

- The recommended Tm of PCR primers is usually in the range of 55°C to 70°C and within 5°C of each other.
- In that case, the primer with the higher Tm could bind to unintended targets,
- While the primer with the lower Tm would have difficulty binding at an annealing temperature chosen for these primers
- Typical annealing temperatures are 5°C below the lowest primer's Tm and often fall in the range of 50-60°C



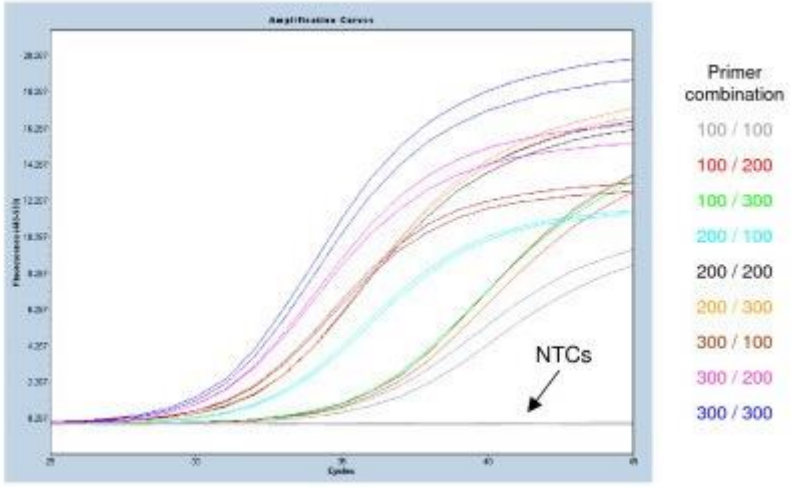
Optimizing Primer Annealing Temperature

- To identify the optimal annealing temperature for the primer pair can optimize by the use of gradient thermal cycler by increasing the temperature in 2-degree steps.



Optimizing Primer/Probe Concentrations

- Optimal concentration of primer pairs and probes is the lowest concentration that results in
  - The lowest Ct
  - Minimal variation between replicates
  - Adequate fluorescence

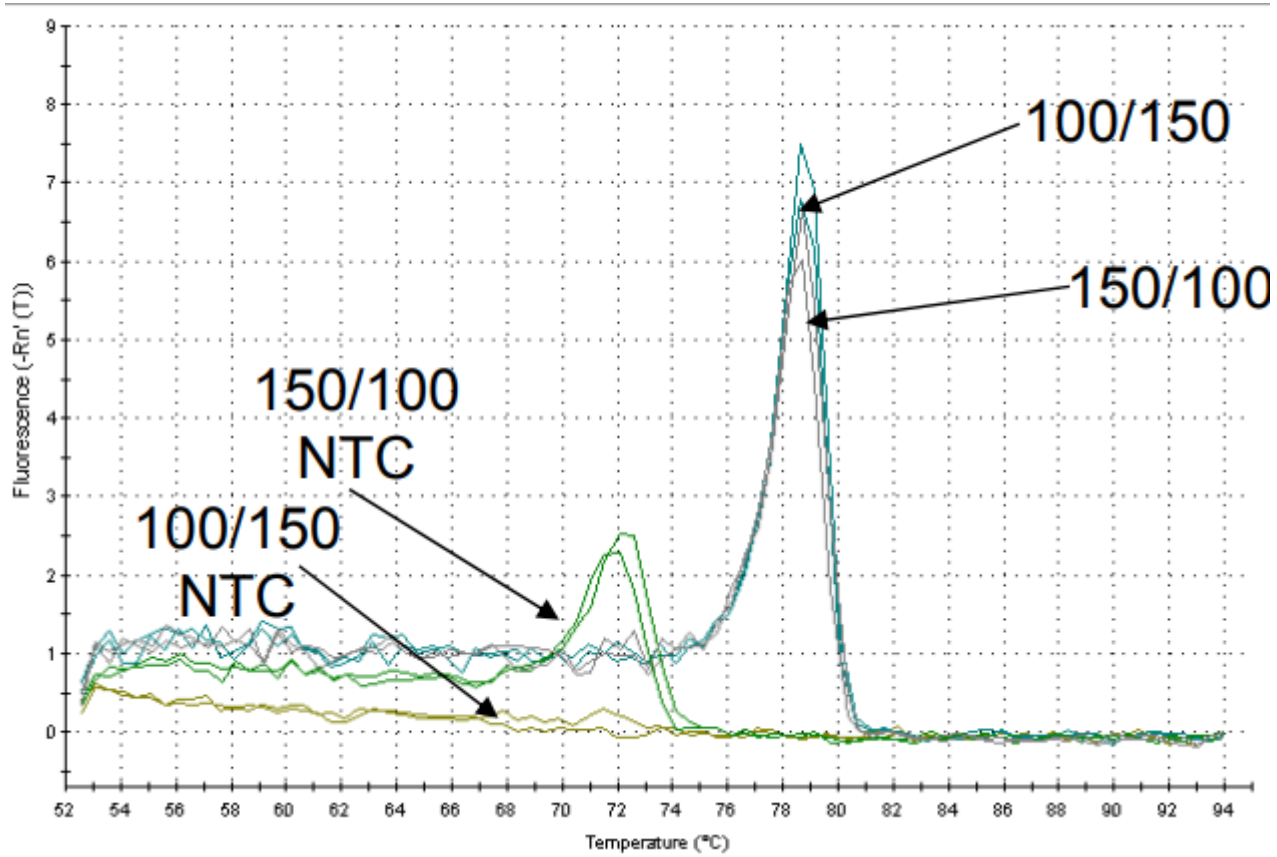


Optimizing Primer/Probe Concentrations

- Once primers and probes have been designed and obtained, it is necessary to optimize their concentration for each RT-PCR assay.
    - What concentration should primers and probe be for qPCR?
- A final concentration of 200 nM per primer and 250 nM for probe is effective for most reactions.
- Optimal results different concentrations of primers and probe must be try!!!
- Following amplification of the template DNA, amplification plots are compared.

Optimizing Primer/Probe Concentrations

- Aims:
- Low Ct values
- No unspecific amplification or primer dimers
- Low inter-replicate variability
- High efficiency of Amplification



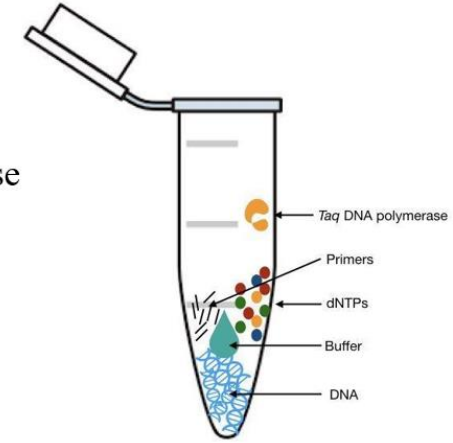
Optimizing Reaction Components

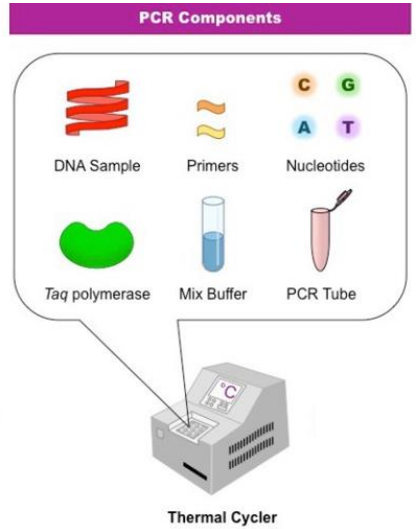
- Required for enzyme activation and amplification
- It stabilizes dsDNA and raises the Tm
- Mg^{2+} concentration controls the specificity of the reaction.
- Mg++ ions
  - Essential co-factor of DNA polymerase
  - Too little: Enzyme won't work.
  - Too much: DNA extra stable, non-specific priming, band smearing

Reagents for PCR

What we need in the laboratory:

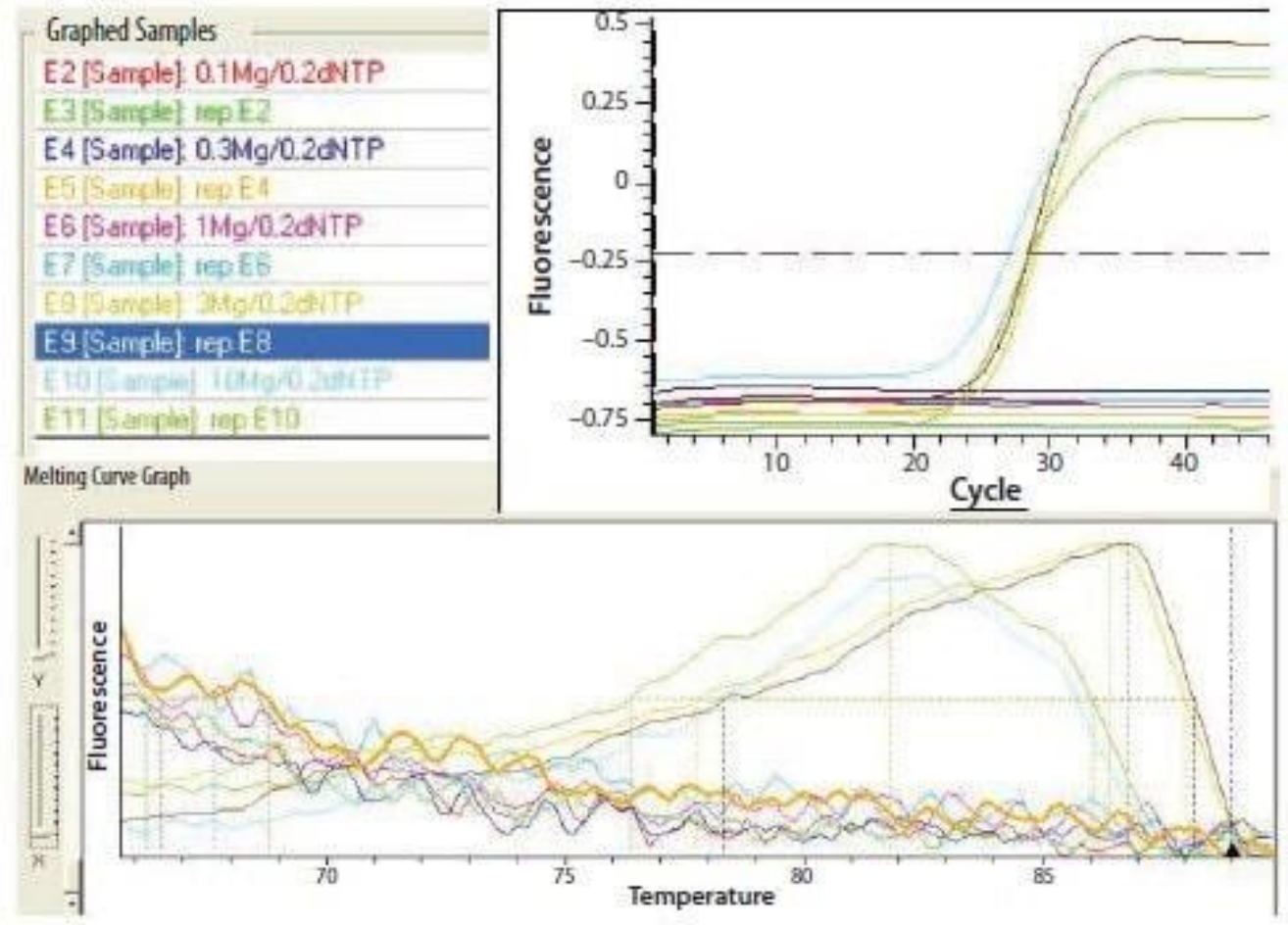
1. DNA template
2. Primers
3. DNA polymerase
4. Buffer
5. dNTPS (bases)
6. $MgCl_2$





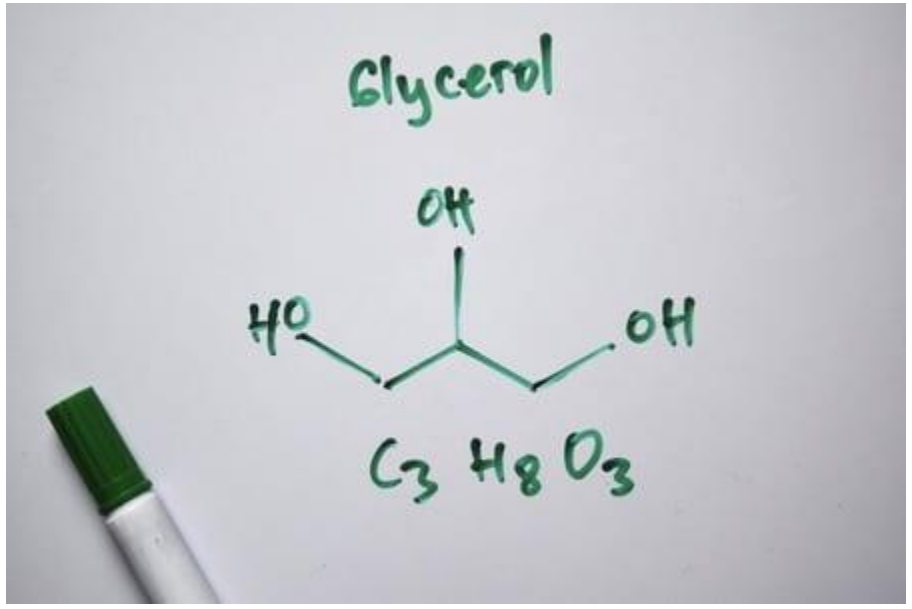
Optimizing Reaction Components

- In contrast to conventional PCR assays which use 1.5–2 mM standard $MgCl_2$ concentrations, qPCR assays require higher concentrations of around 3–5 mM to achieve efficient cleavage of the probe
- Optimization of $MgCl_2$ concentrations becomes more important when running multiplex reaction

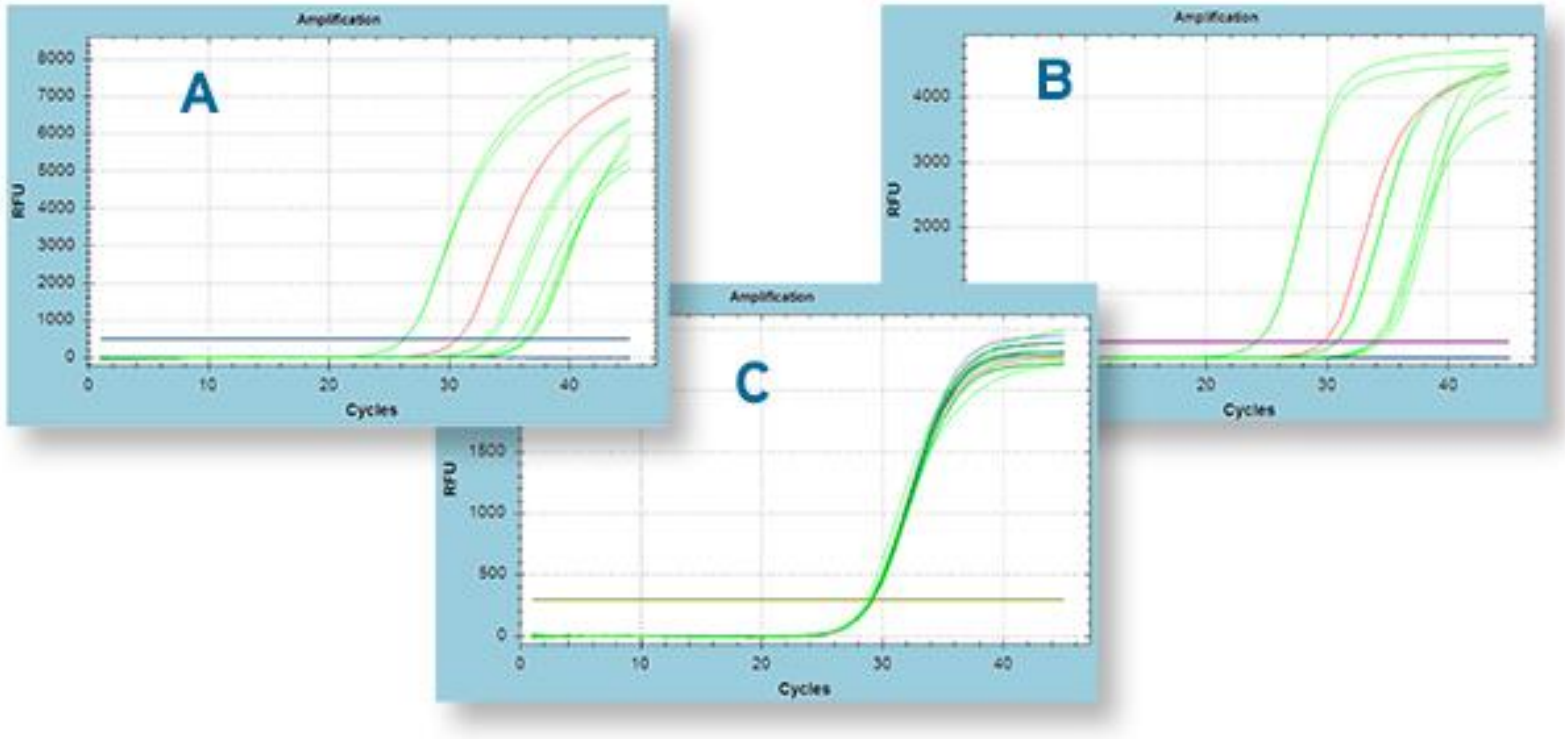


Optimizing Reaction Components

- Why glycerol is used in PCR?
- The addition of glycerol to PCR reaction enhance the specificity of the primer and reduce the formation of secondary structures caused by GC-rich regions.
- Glycerol can also lower the strand separation temperature of DNA, thus facilitating amplification.

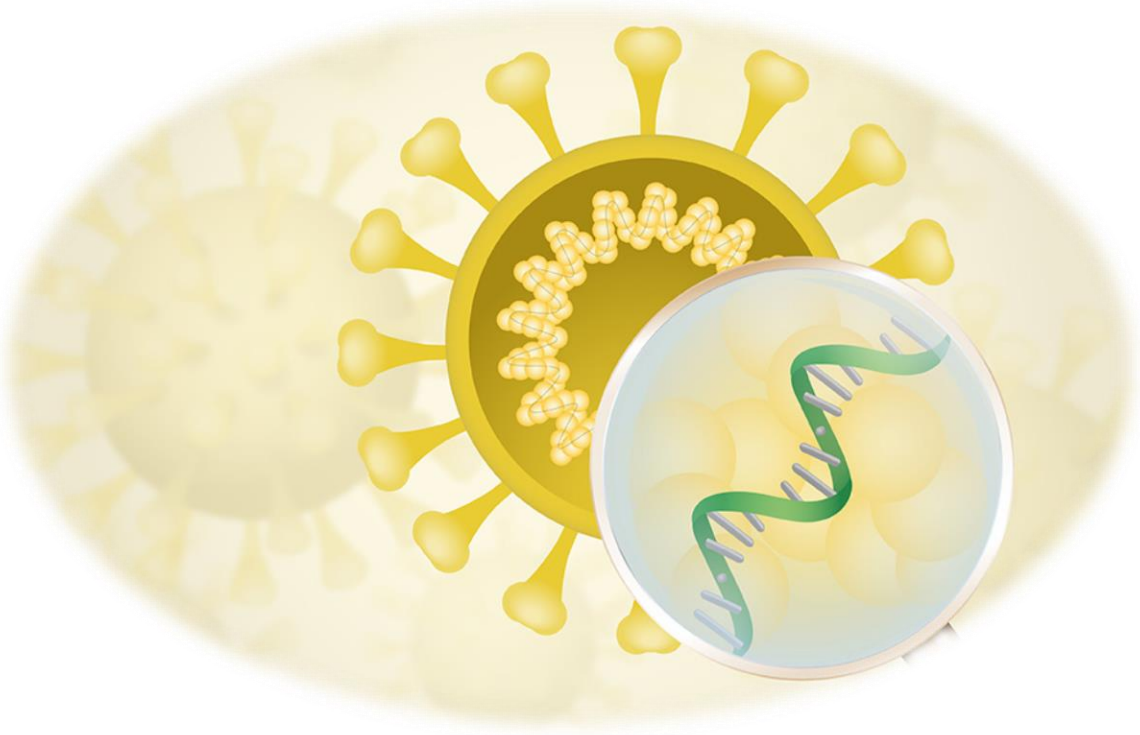


Analysis


Amplification
A
RFU
8000
7000
6000
5000
4000
3000
2000
1000
0
0
10
20
30
40
Cycles
Amplification
B
RFU
4000
3000
2000
20
30
40
Cycles
Amplification
C
RFU
1500
1000
500
0
0
10
20
30
40
Cycles

Analysis

- SARS-CoV-2 RT-PCR detection kit indicates the **presence** or **absence** of **SARS-CoV-2** viral genome and also can allow us to predict the viral load.



Analysis

	Hedef Organizma	Hedef Gen	Kanal
UniMM Mastermix	SARS-CoV-2	N1	FAM
	SARS-CoV-2	N2	FAM
	SARS-CoV-2	Orf1ab	FAM
	İnsan	Rnase-P	HEX

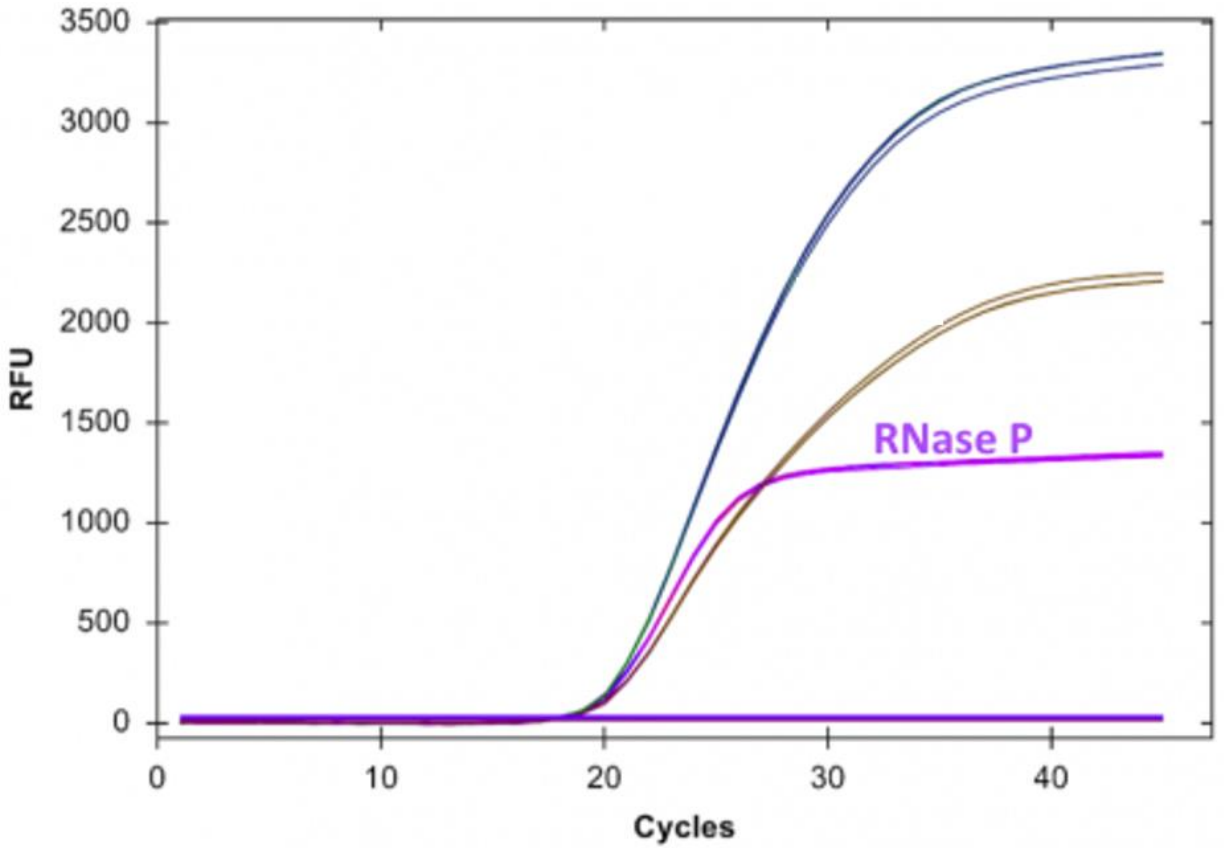
internal controls that check for successful nucleic acid extraction

Analysis

There is amplification for internal control

There is amplification for positive control

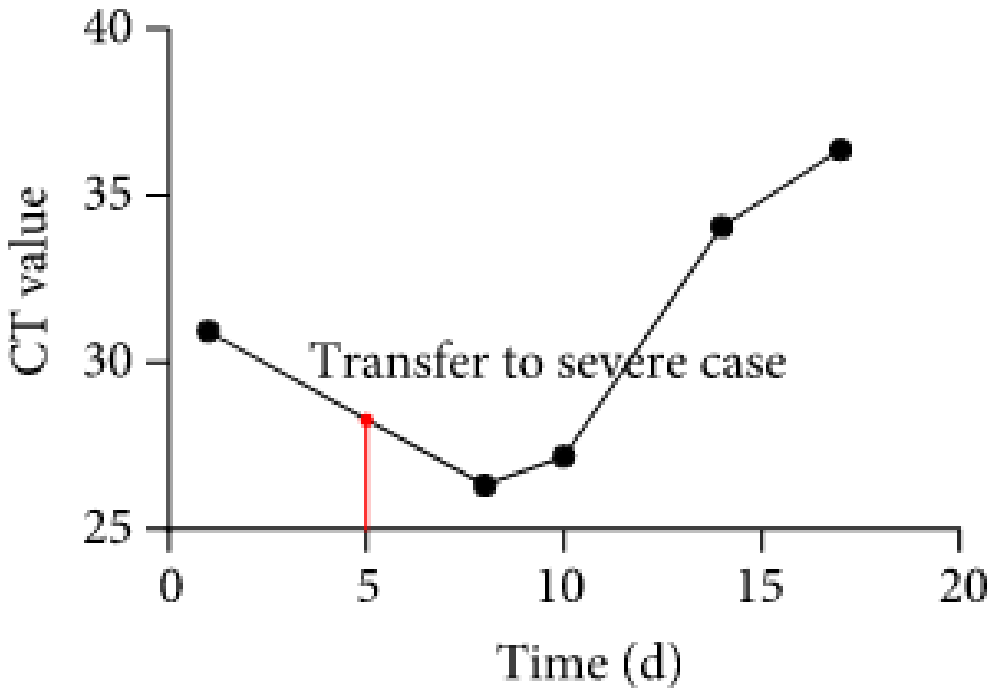
There is no amplification for negative control



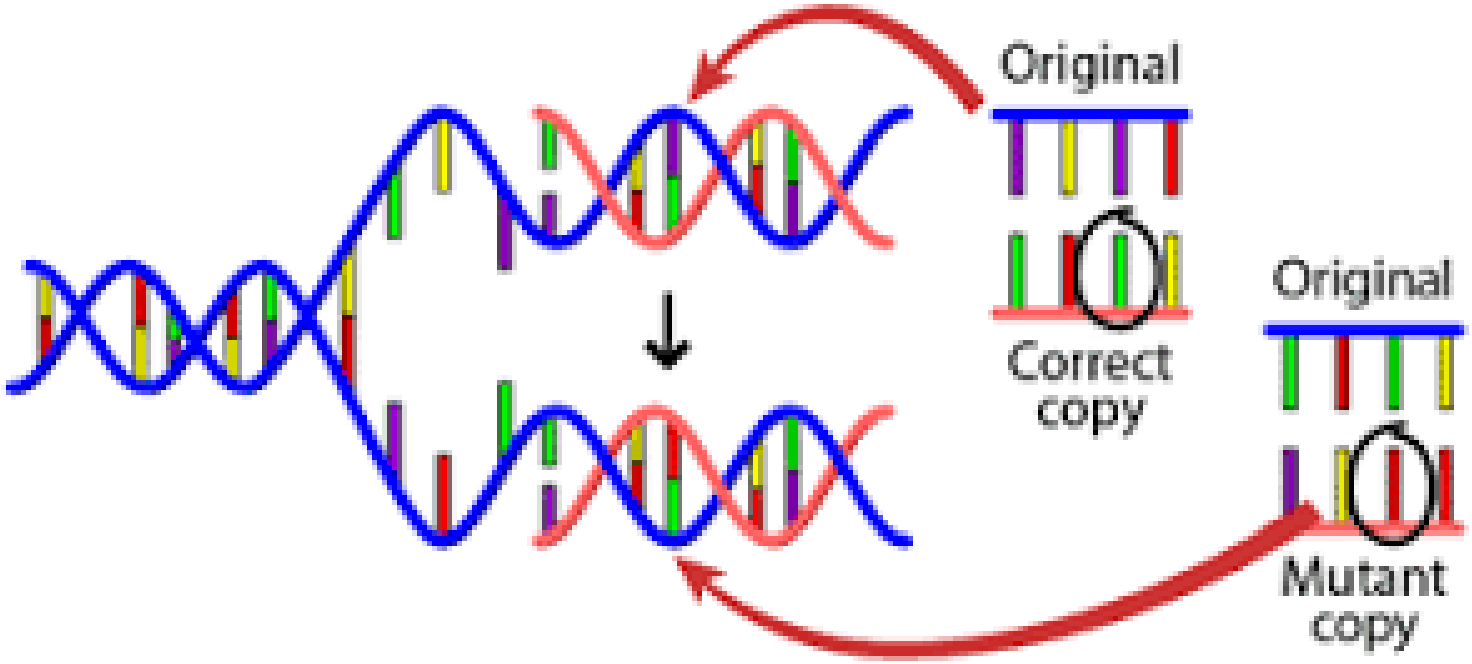
All samples which show amplification are infected with SARS-CoV-2

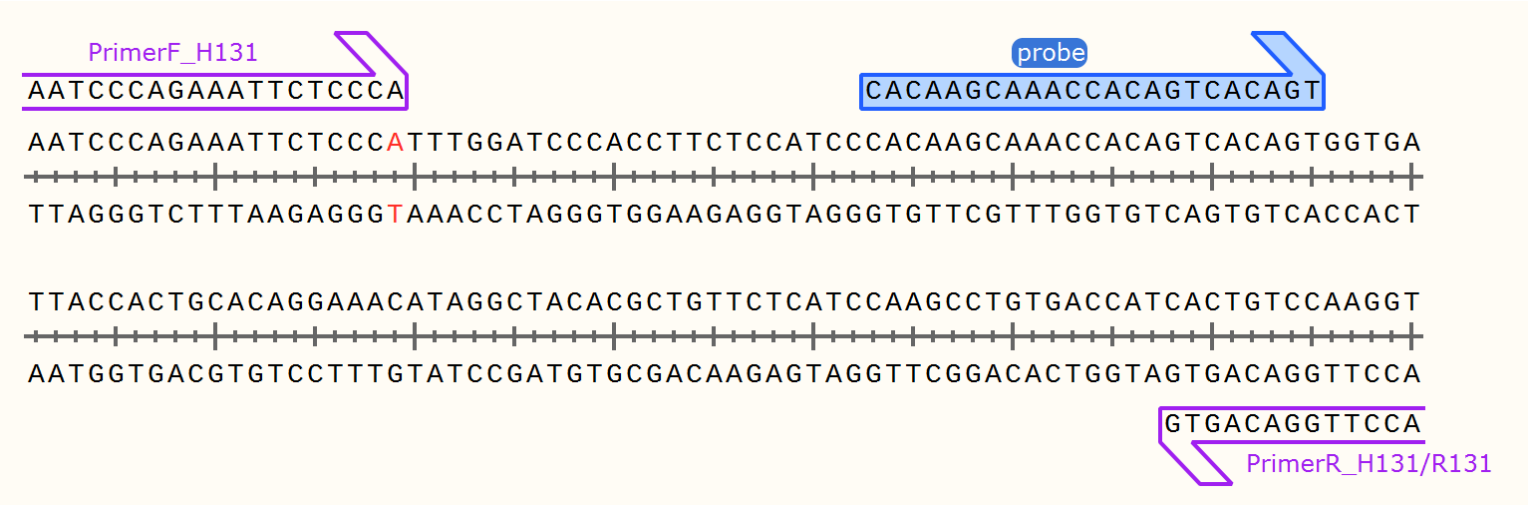
Analysis

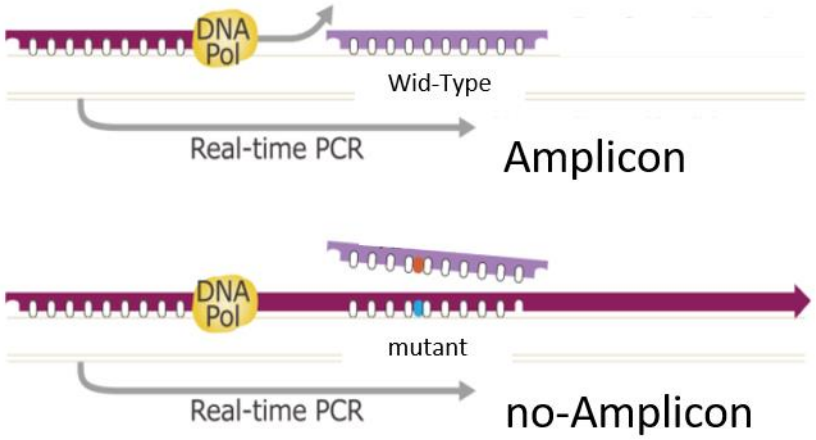
- The cycle threshold (Ct) is the number of replication cycles required to produce a fluorescent signal.
- viral RNA in the nasopharyngeal swab as measured by the Ct becomes detectable.
- The lower Ct values representing higher viral loads,
- while higher Ct values representing lower viral load.

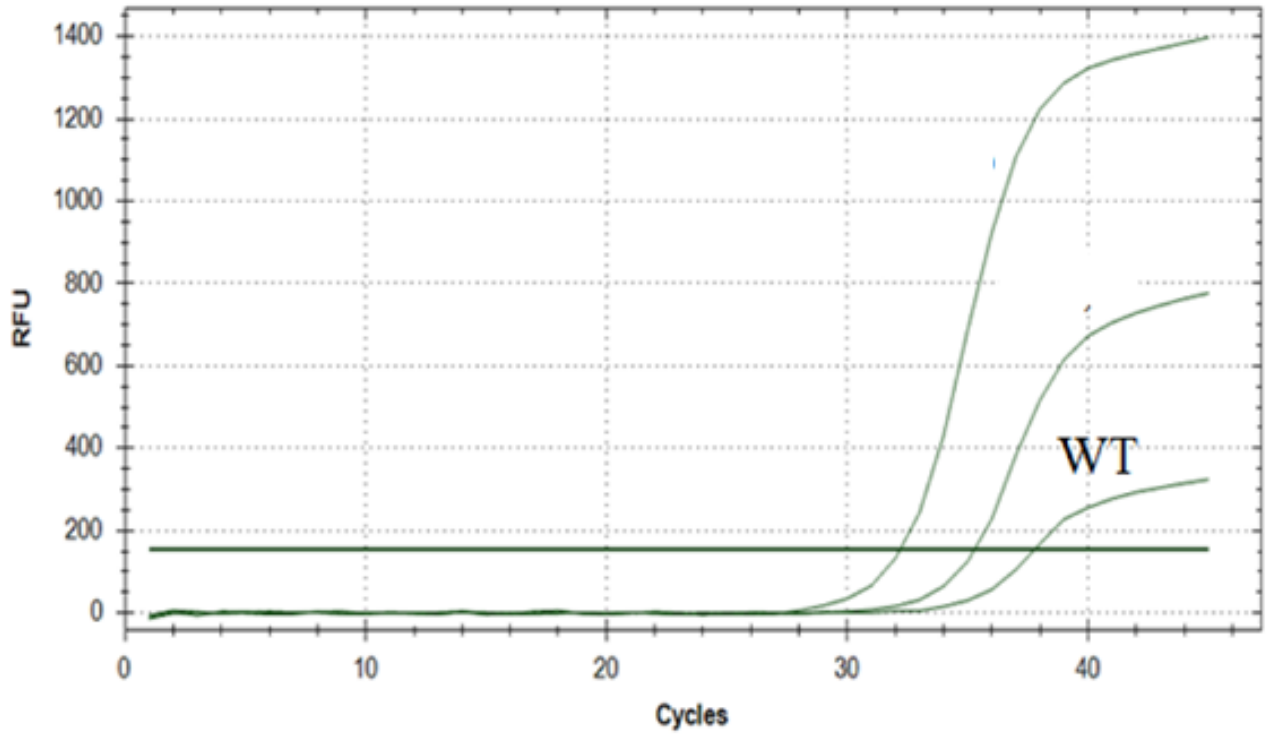


Known mutation detection and variant determination




PrimerF_H131
AATCCCAGAAATTCTCCCA
probe
CACAAGCAAACCACAGTCACAGT
AATCCCAGAAATTCTCCCATTTGGATCCCACCTTCTCCATCCCACAAGCAAACCACAGTCACAGTGGTGA
TTAGGGTCTTTAAGAGGGTAAACCTAGGGTGGAAGAGGTAGGGTGTTCGTTTGGTGTCAGTGTCACCACT
TTACCACTGCACAGGAAACATAGGCTACACGCTGTTCTCATCCAAGCCTGTGACCATCACTGTCCAAGGT
AATGGTGACGTGTCCTTTGTATCCGATGTGCGACAAGAGTAGGTTCGGACACTGGTAGTGACAGGTTCCA
GTGACAGGTTCCA
PrimerR_H131/R131


DNA Pol
Wid-Type
Real-time PCR
Amplicon
DNA Pol
mutant
Real-time PCR
no-Amplicon


RFU
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
Cycles
WT


PrimerF_H131
AATCCCAGAAATTCTCCCG
probe
CACAAGCAAACCACAGTCACAGT
AATCCCAGAAATTCTCCCATTTGGATCCCACCTTCTCCATCCCACAAGCAAACCACAGTCACAGTGGTGA
TTAGGGTCTTTAAGAGGGTAAACCTAGGGTGGAAGAGGTAGGGTGTTCGTTTGGTGTCAGTGTCACCACT
TTACCACTGCACAGGAAACATAGGCTACACGCTGTTCTCATCCAAGCCTGTGACCATCACTGTCCAAGGT
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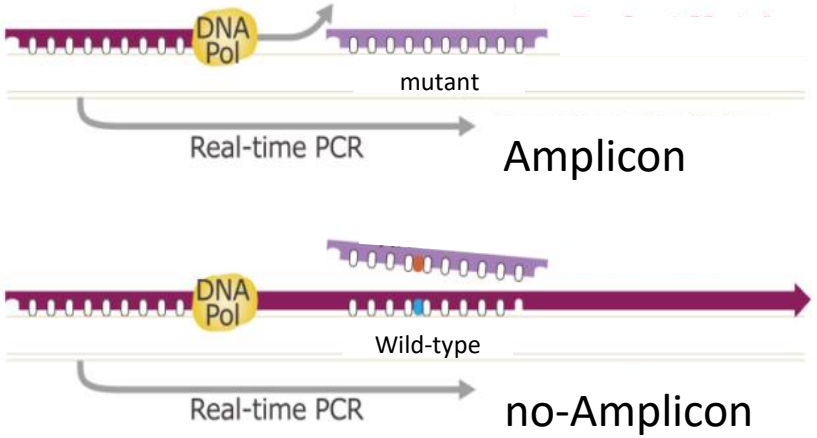
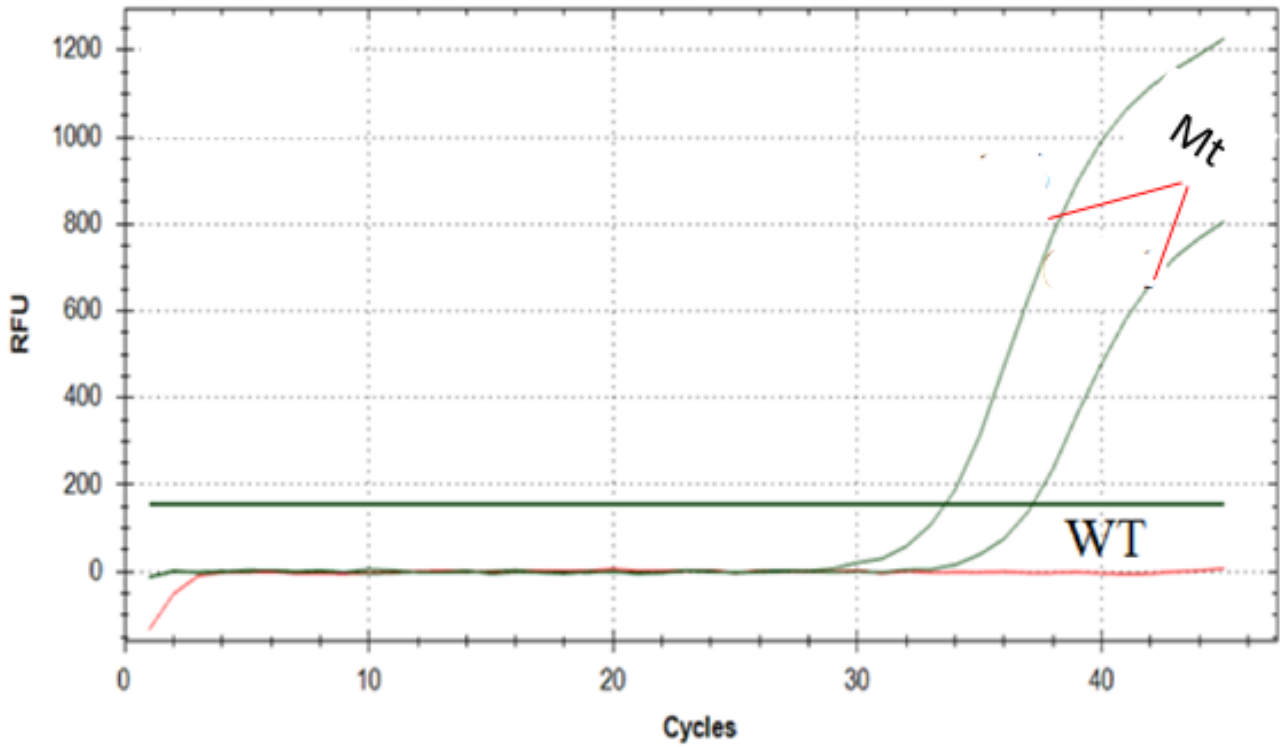
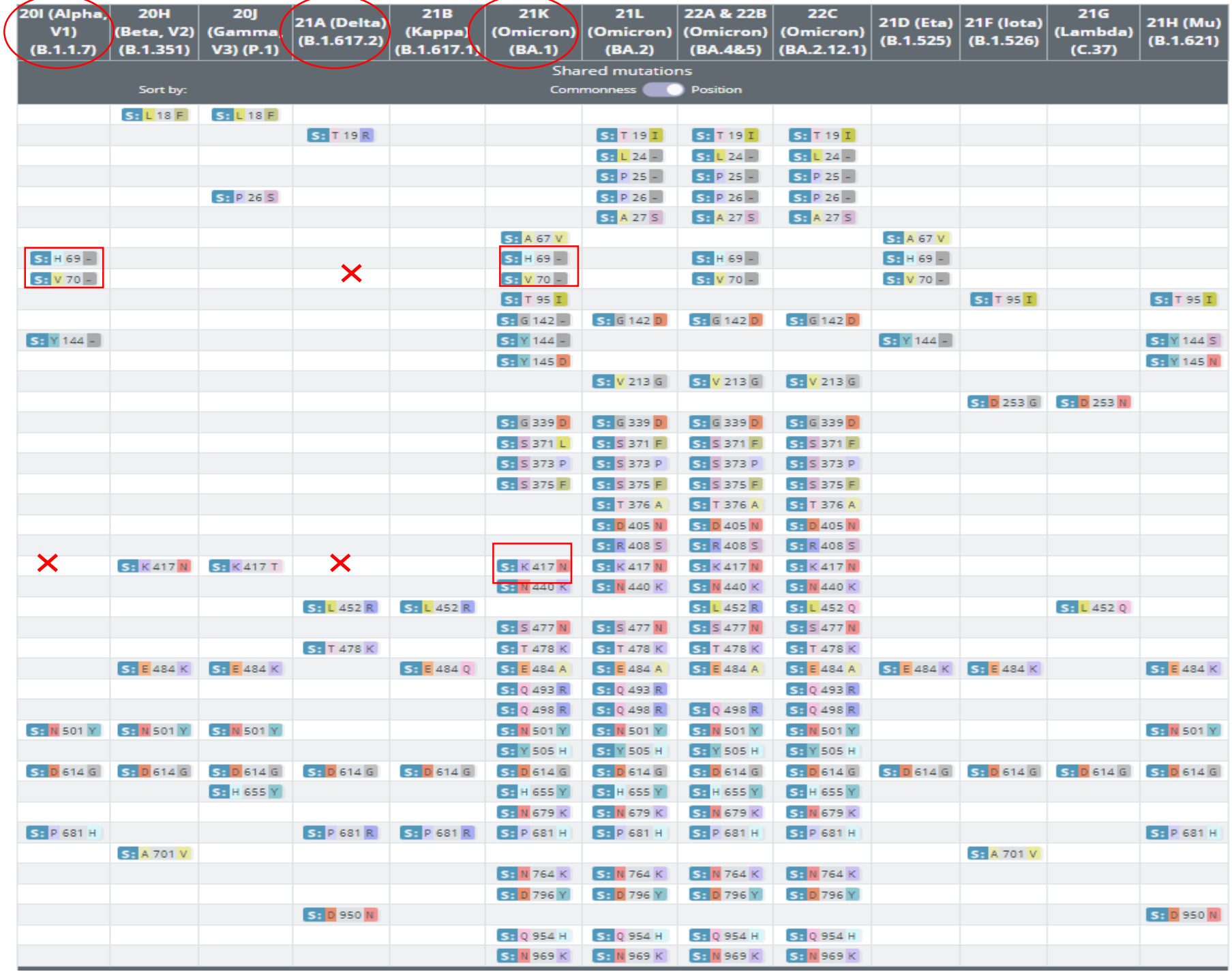
GTGACAGGTTCCA
PrimerR_H131/R131


DNA Pol
mutant
Real-time PCR
Amplicon
DNA Pol
Wild-type
Real-time PCR
no-Amplicon


RFU
1200
1000
800
600
400
200
0
0
10
20
30
40
Cycles
Mt
WT


20I (Alpha, V1) (B.1.1.7)
20H (Beta, V2) (B.1.351)
20J (Gamma, V3) (P.1)
21A (Delta) (B.1.617.2)
21B (Kappa) (B.1.617.1)
21K (Omicron) (BA.1)
21L (Omicron) (BA.2)
22A & 22B (Omicron) (BA.4&5)
22C (Omicron) (BA.2.12.1)
21D (Eta) (B.1.525)
21F (Iota) (B.1.526)
21G (Lambda) (C.37)
21H (Mu) (B.1.621)
Shared mutations
Sort by:
Commonness
Position

S gene mutations	Alpha (B.1.1.7)	Delta (B.1.617.2)	Omicron (B.1.1.529)
HV 69-70 deletion	+	×	+
K417N	×	×	+

Troubleshooting

Ensuring the Accuracy of testing Results?

internal control

positive control

negative control

Troubleshooting

- **There is no amplification for internal control**
- An additional potential source of false negatives could stem from insufficient sample collection or sample extraction.

Type of Control	Problem identified
Internal	Did the sample extraction work?

Troubleshooting

- **There is no amplification for positive control**
- A positive control is expected to have amplification of the assay specific SARS-CoV-2 target regions. The resulting signaling show that the reagents are working properly. If something was inhibiting the reaction, then the positive control would not be able to make amplicons. Likewise, if the reagents for the reaction were not made or mixed properly, the positive control would also not work as expected.

Type of Control	Problem identified
Postitive	Did the RT-PCR reaction work?

Troubleshooting

- **There is amplification for negative control**
- The negative control is expected to result in no amplification of the target regions. Due to the sensitivity of the primer/probe sets for RT-PCR, if amplicons were made and signal is shown for the SARS-CoV-2 target genes, then contamination of the PCR experiment with foreign DNA has occurred.

Type of Control	Problem identified
Negative	Is the run contaminated?