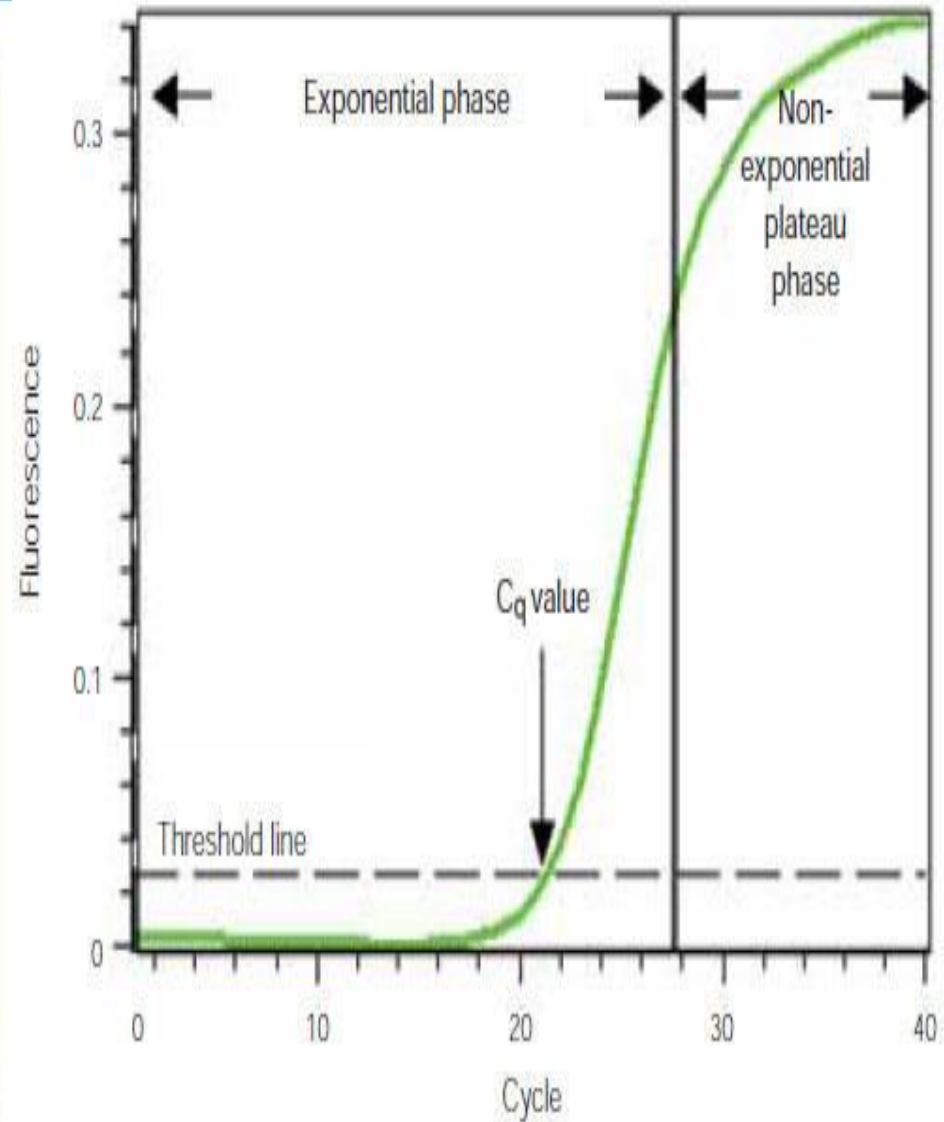
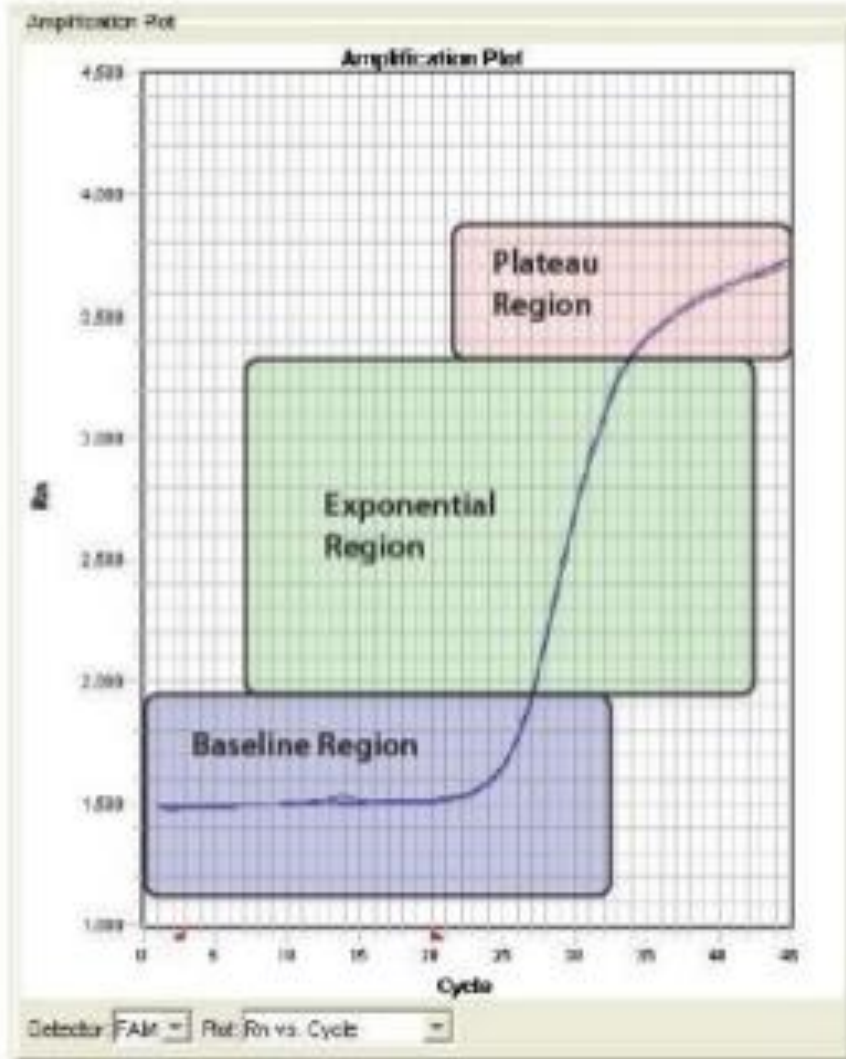


# Troubleshooting of Real Time PCR

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**Faculty of Vet. Medicine – Assiut University**

# How Does Real-Time PCR Work?



# Reaction efficacy

- Efficiency reflects whether DNA doubled every cycle.
- It takes 3.32 cycles for DNA to be amplified 10 fold.

CYCLE NUMBER	AMOUNT OF DNA
0	1
1	2
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1,024
11	2,048
12	4,096
13	8,192
14	16,384
15	32,768
16	65,536
17	131,072
18	262,144
19	524,288
20	1,048,576
21	2,097,152
22	4,194,304
23	8,388,608
24	16,777,216
25	33,554,432
26	67,108,864
27	134,217,728
28	268,435,456
29	536,870,912
30	1,073,741,824
31	1,400,000,000
32	1,500,000,000
33	1,550,000,000
34	1,580,000,000

# Troubleshooting

**It is always best to  
follow the  
recommendations of  
the manufacturer.**



**Troubleshooting in the real-time PCR reaction seems to be absent when, assuming proper assay design was taken into consideration.**

**Common real-time PCR difficulties can be grouped into four main areas:**

- **Formation of primer-dimers**
- **Storing primers and probes**
- **Real-time PCR inhibition and poor reaction efficiency**
- **Software analysis settings**

# Formation of primer-dimers

- **Primer-dimers form when partial sequence homology exists between the members of the primer pair.**
- **If the primers anneal to each other during the PCR reaction, the *Taq* DNA polymerase may be able to extend them.**
- **Depending on its length, it is also possible for a primer to fold upon itself and therefore set up a competitive environment with the template.**

# Problems caused by primer-dimers

The effect that primer-dimers mainly affected by the type of RT-PCR.

## Fluorogenic probe-based reactions:

Not greatly affected by primer-dimers → 

Because a probe annealing and being cleaved in a primer-dimer region is an extremely rare event.

# Problems caused by primer-dimers

Reactions that rely on double-stranded DNA-binding

dyes:- It is a serious problem→



Because the dye would bind to them nonspecifically and therefore contribute to fluorescence signal being monitored during the reaction.

This will shift the  $C_t$  and give false results.

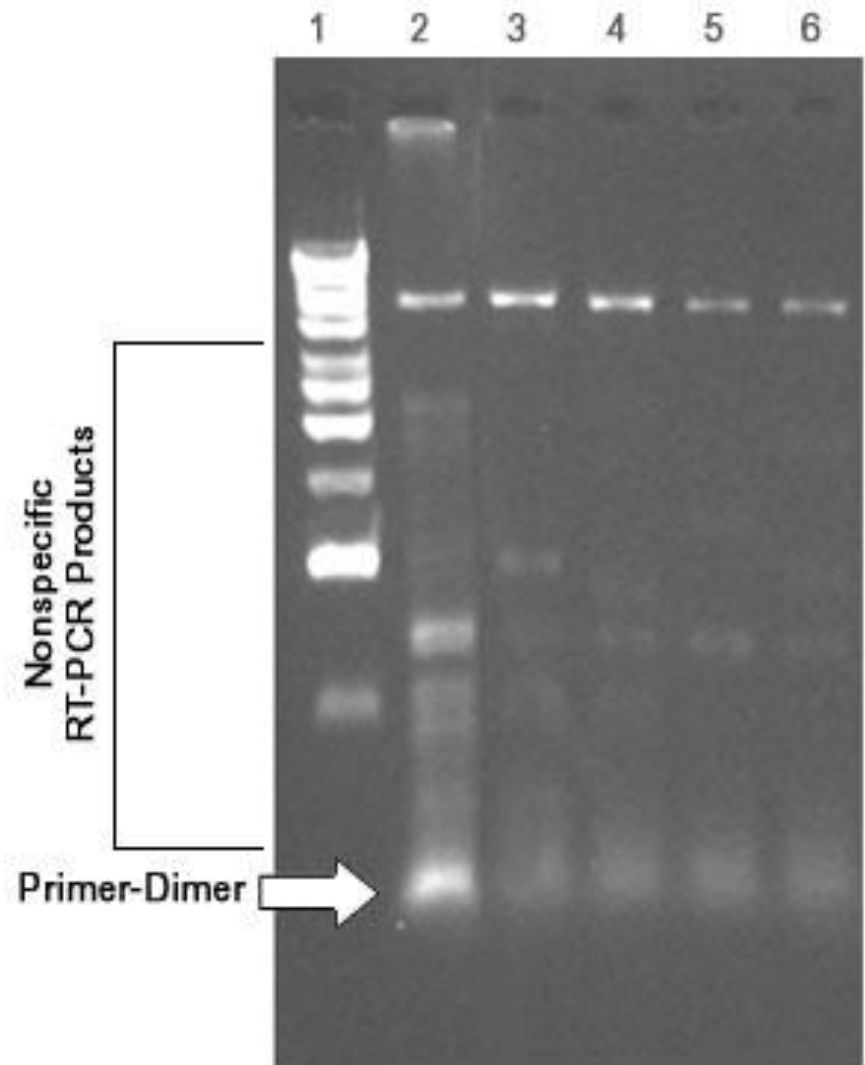
It is best to take simple precautions during primer design to avoid dimerization in the first place.



# Determining if primer-dimers are present

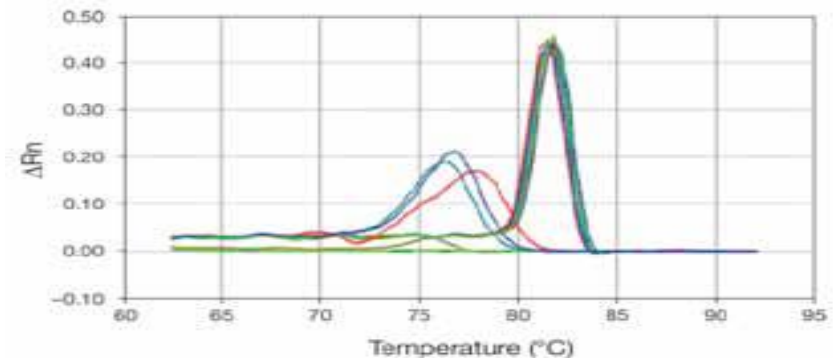
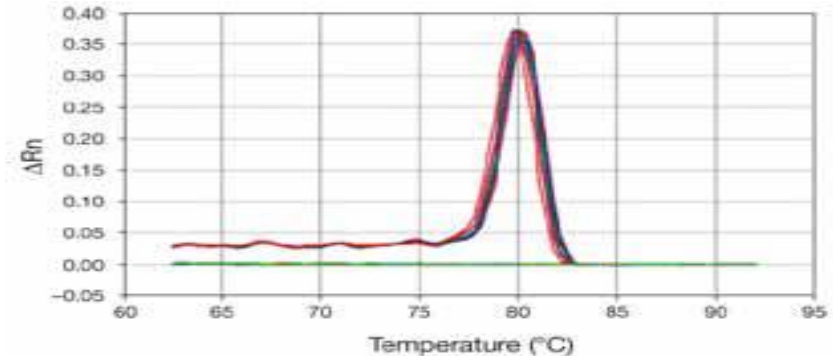
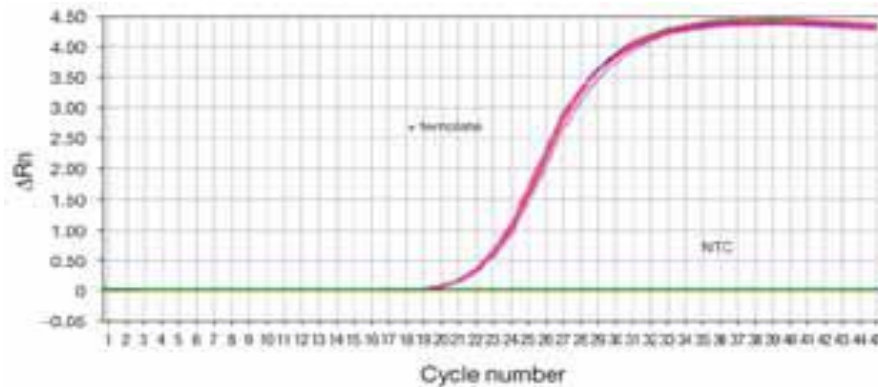
Gel electrophoresis is a great way to visualize primer dimers.

primer-dimers appear as diffuse bands near the bottom of the gel, usually below 100 bp.



# Determining if primer-dimers are present

Primer-dimers manifest themselves as lower fluorescence, broader “waves”.



# Reducing or removing primer-dimers

- 1. The first is optimization of the thermo cycling conditions, which mainly involves raising the annealing temperature.**
- 2. Primer concentration can always be lowered. In most cases, a final concentration of 200 nM per primer is ideal, but this can be reduced to 60 nM if necessary.**

# Reducing or removing primer-dimers

- 3. Magnesium is usually best at a concentration of about 3 mM, primer-dimers are favored at concentrations above this.**
- 4. If primers were not evaluated for their propensity toward dimerization, evaluate them and as usual, hot-start DNA polymerases and reaction setup on ice are also preferable.**

Keep in mind that dimers may be more of a concern in one-step qRT-PCR reactions due to the lower temperature of the RT reaction in the presence of the primer pair.



# Storing primers and probes

**Primer and probe storage can have a major effect on the success and consistency of a real-time PCR assay.**

**The main factors that affect primer and probe stability are:**

- 1. The storage temperature.**
- 2. The length of storage time.**
- 3. Prolonged exposure to light.**
- 4. The concentration of the stored primer or probe.**
- 5. The composition of the storage solution.**

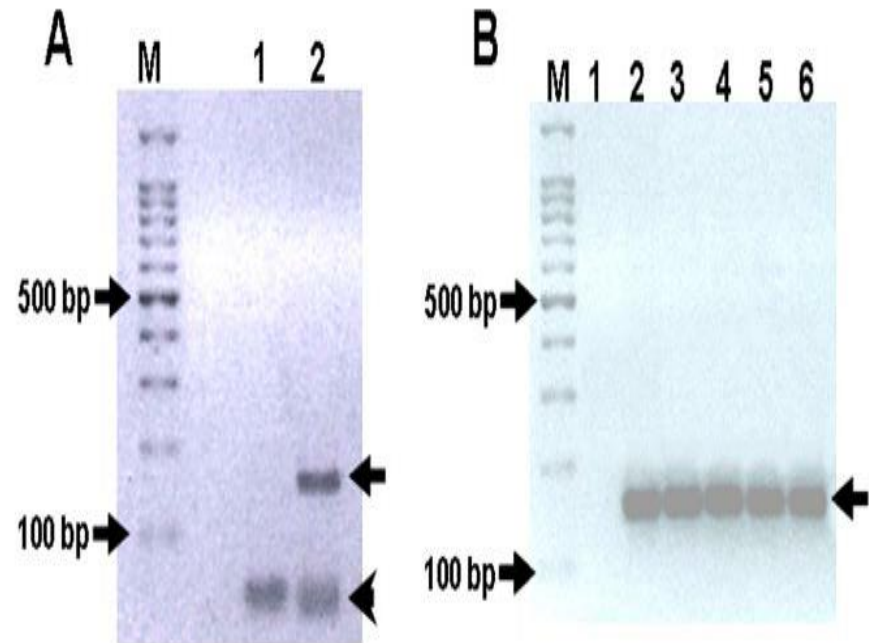
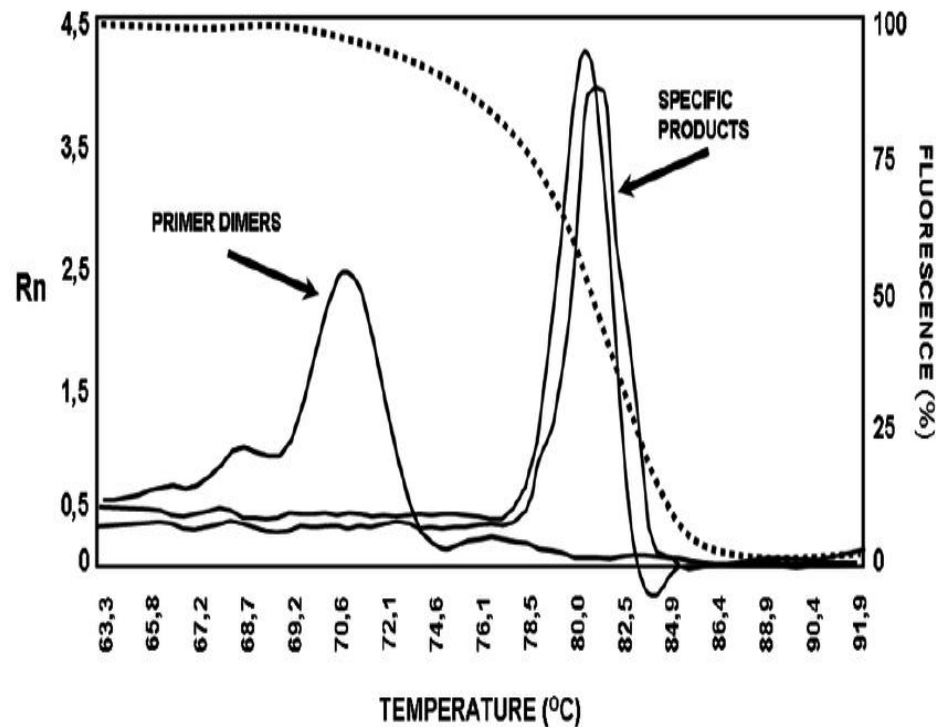
# **Problems caused by poor storage of primers and probes**

**Improper storage of primers and probes can cause them to degrade and lose specificity, which in turn affects the reaction efficiency.**

**In assays that rely on fluorescently labeled primers and probes, degraded probe releases free dye, which increases background and decreases the signal-to-noise ratio.**

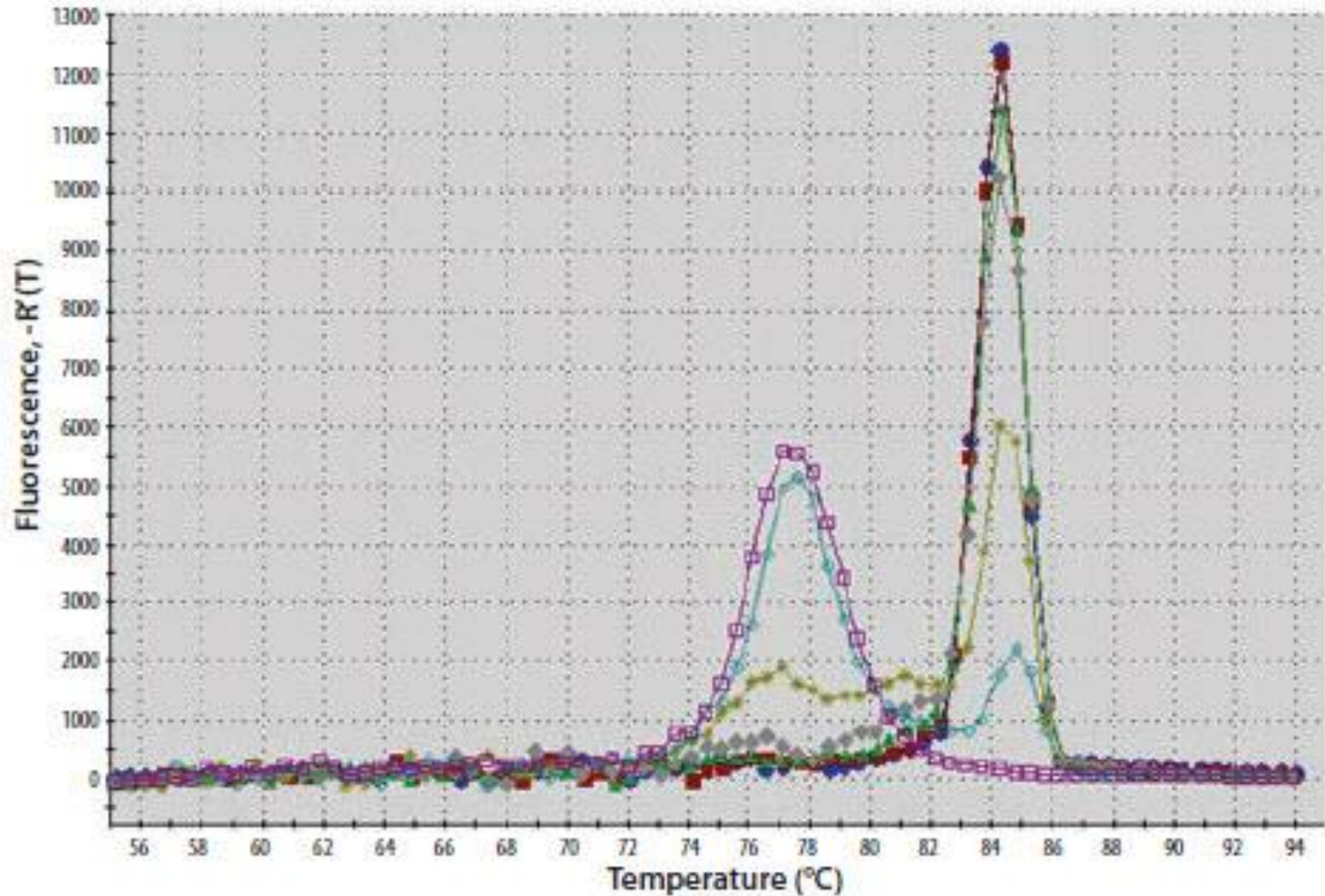
# Specificity and Melting Curve:


- Pure, homogenous PCR products produce a single, sharply defined melting curve with a narrow peak.
- Primer dimers melt at relatively low temperatures and have broader peak





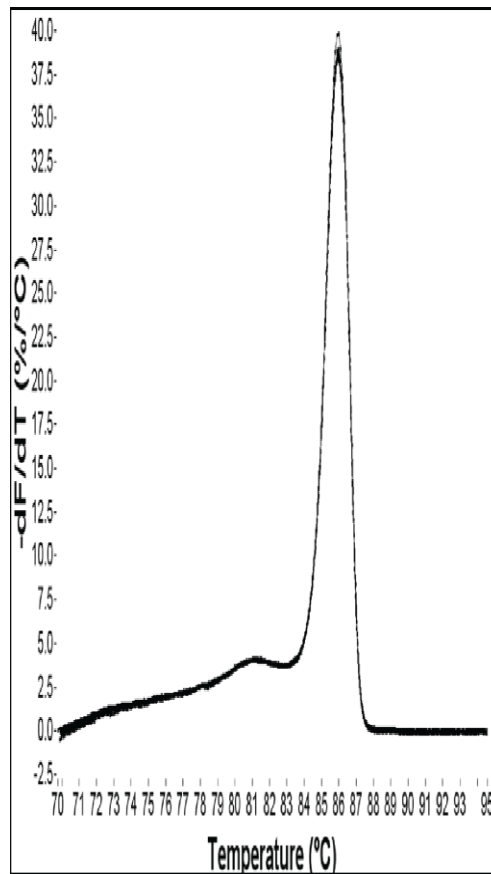
# Melting curve with primer dimer



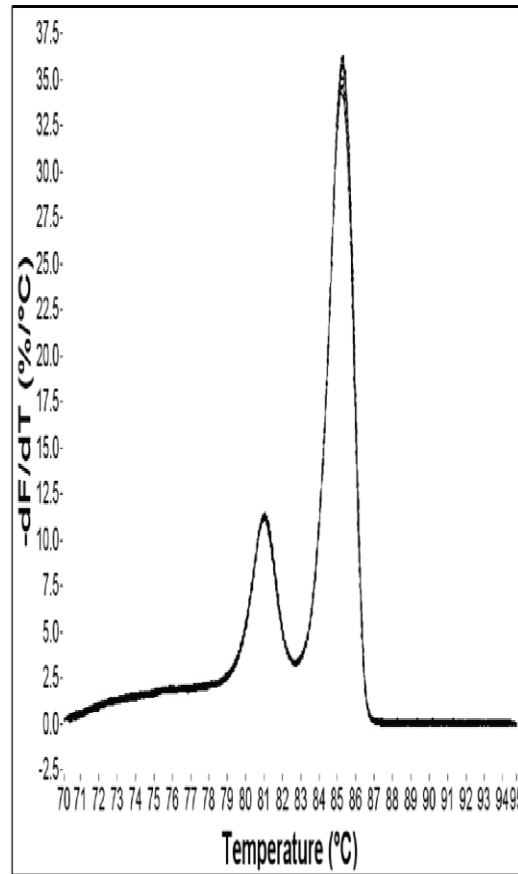


**Let's  
Take a  
Quiz**

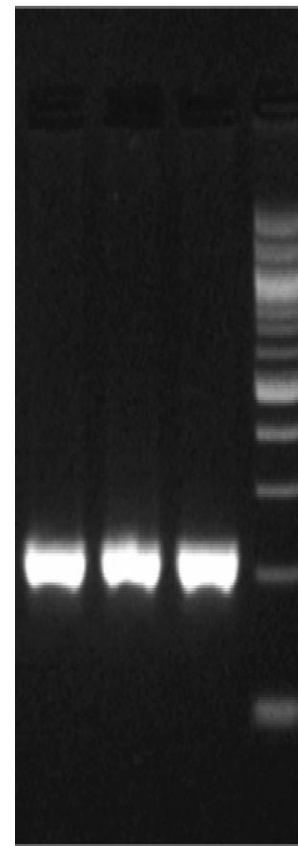
**As the dsDNA starts to melt, regions of the amplicon that are more stable (e.g., G/C rich) do not melt immediately. These stable regions maintain their dsDNA configuration until the temperature is sufficiently high to cause it to melt.**



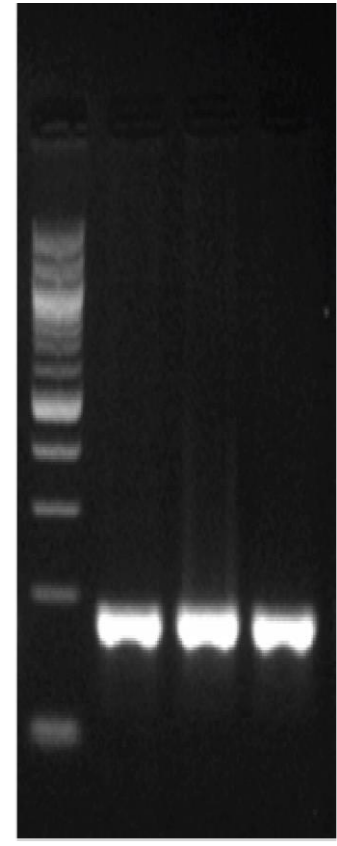
A. CFTR Exon 17b



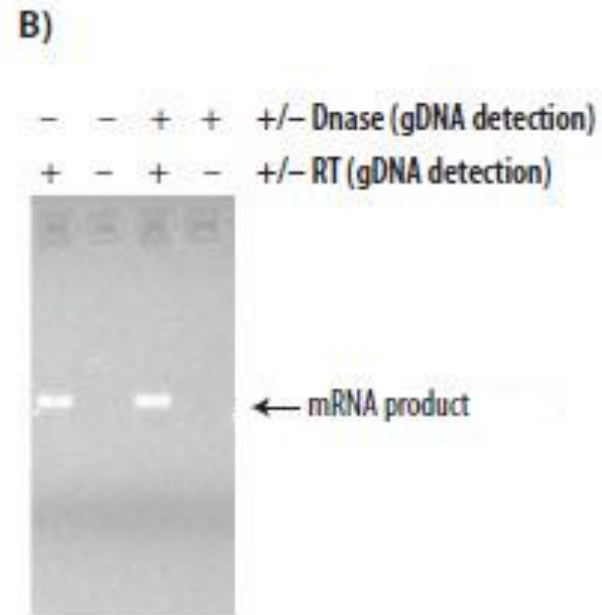
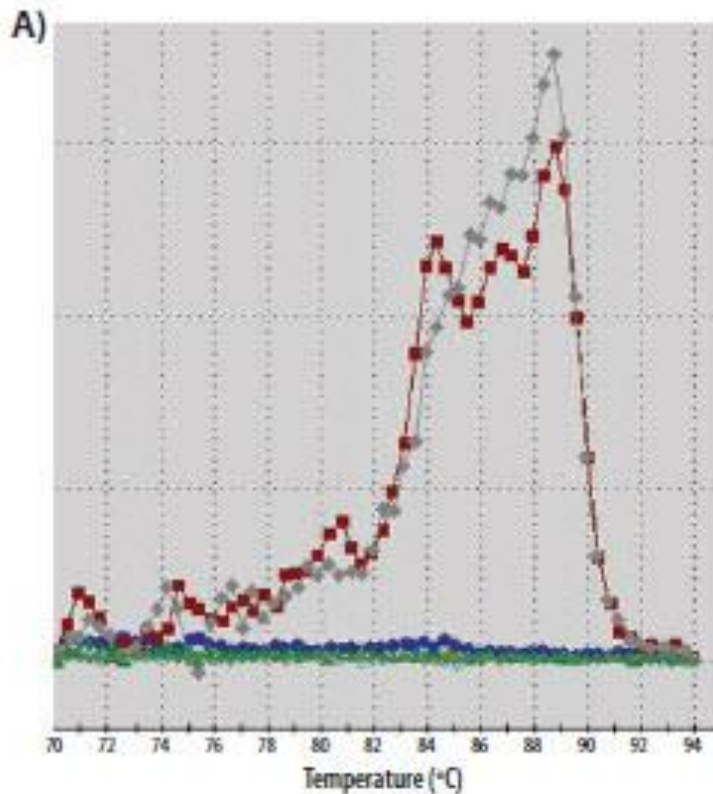
B. CFTR Exon 7



A. CFTR Exon 17b



B. CFTR Exon 7.



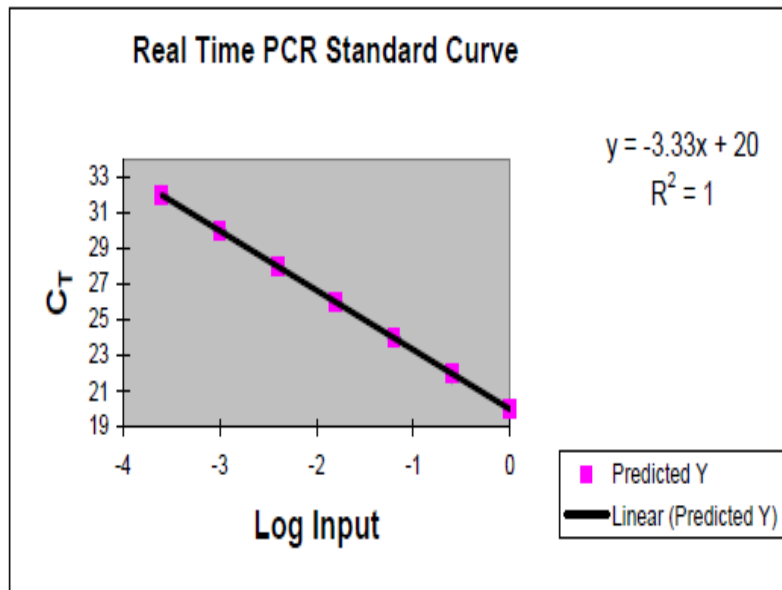
A) melt profile and B) agarose gel analysis of a SYBR green I reaction. The melt profile suggests products of varying  $T_m$ , the gel image indicates that a single amplicon is present. This is indicative of an amplicon sequence that contains GC rich regions.

# **Abnormal Standard curve**

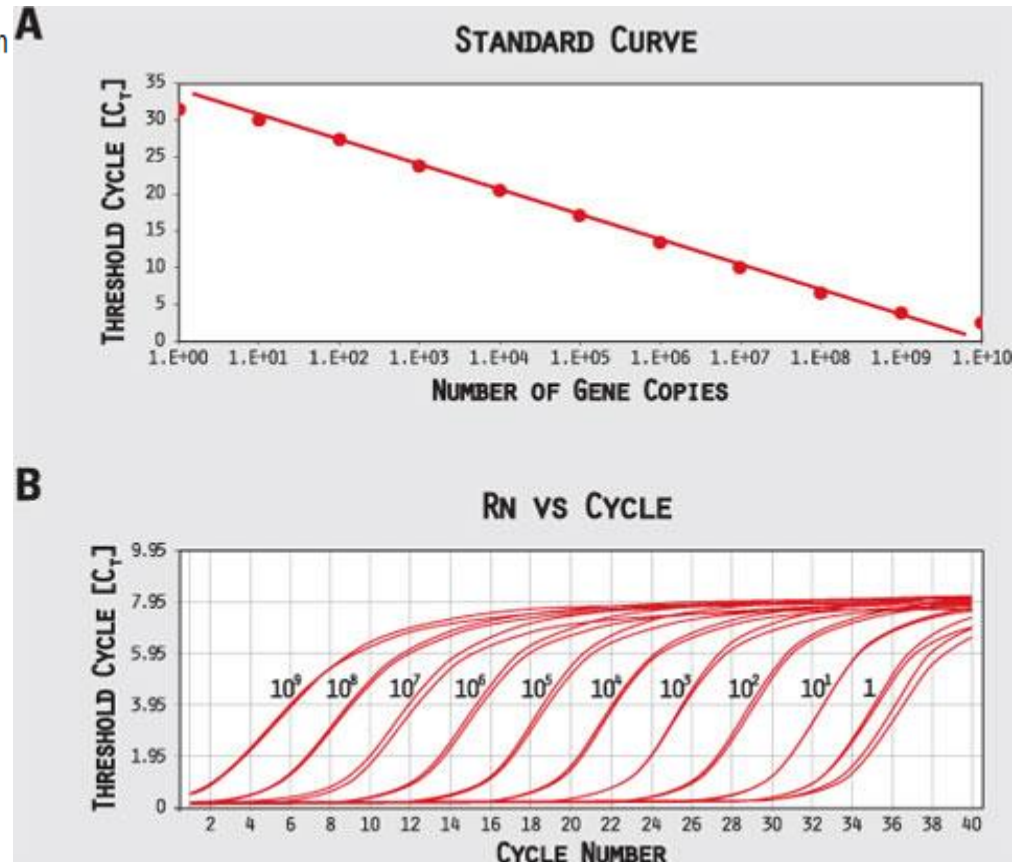


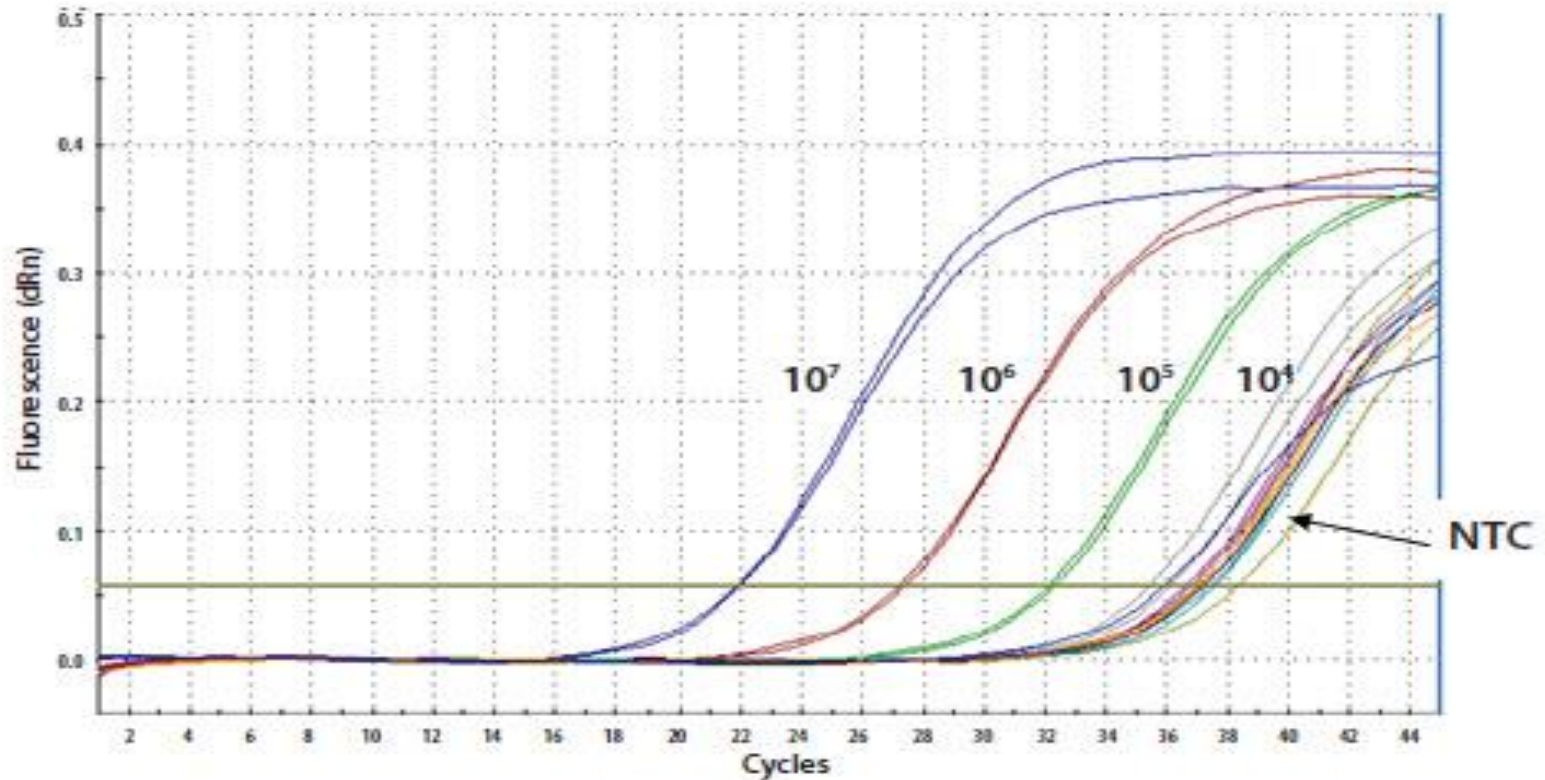
# Ideal standard curve

**Note:** A 100% efficient reaction will yield a 10-fold increase in PCR amplicon during the exponential phase of amplification ( $\log_2 10 = 3.3219$ ).

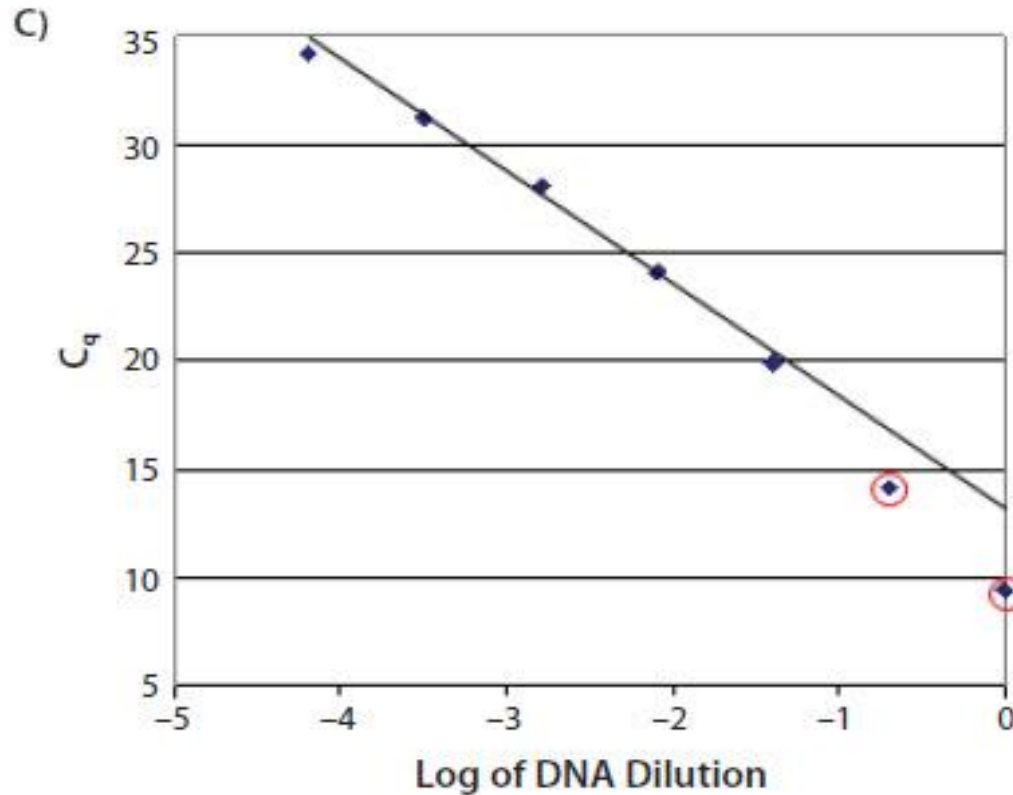


**Figure 1:** Real-Time PCR Standard Curve representing 100% PCR Efficiency





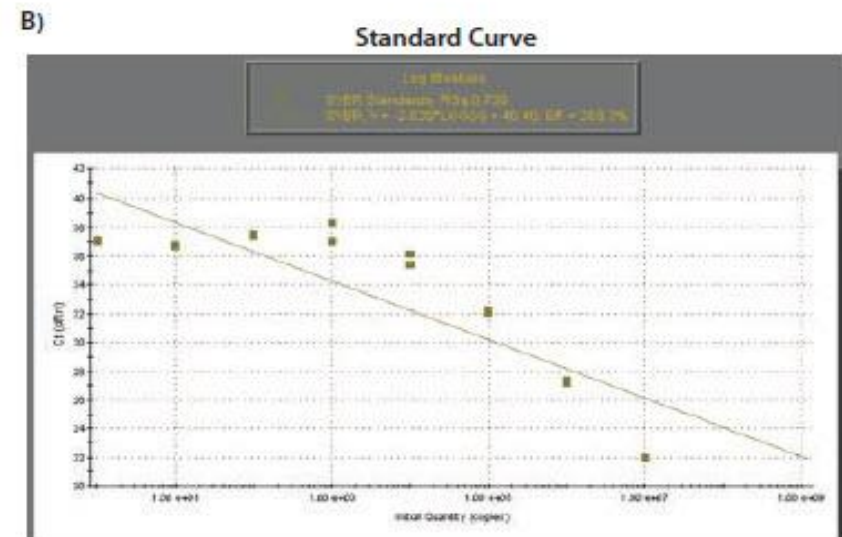
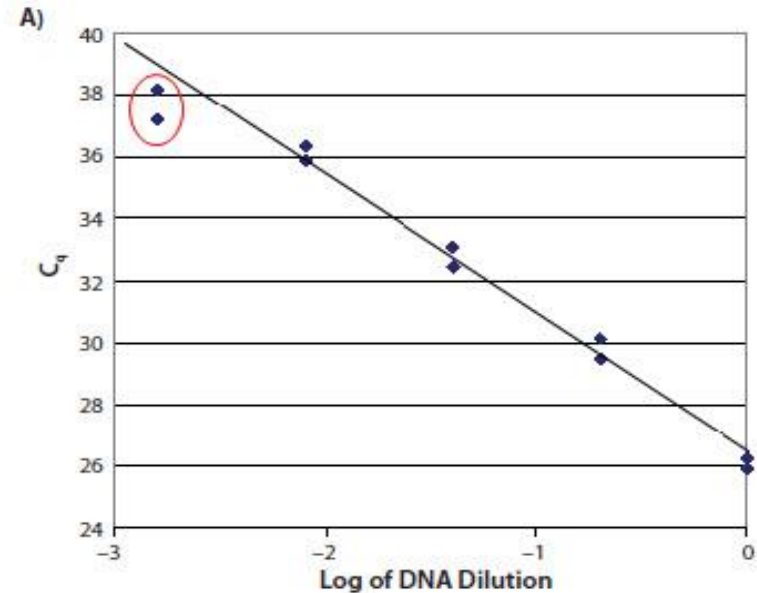
The data shows a positive signal in the no template control (NTC) indicating contamination or primer dimer formation.

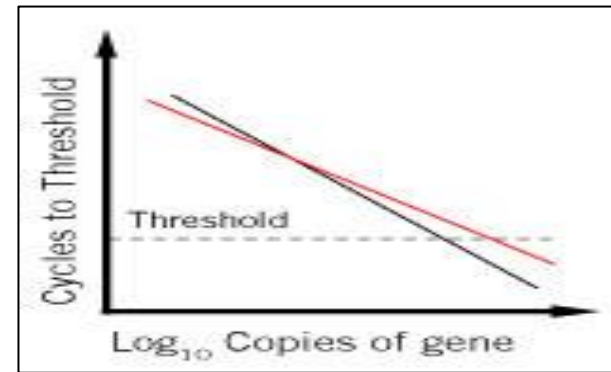
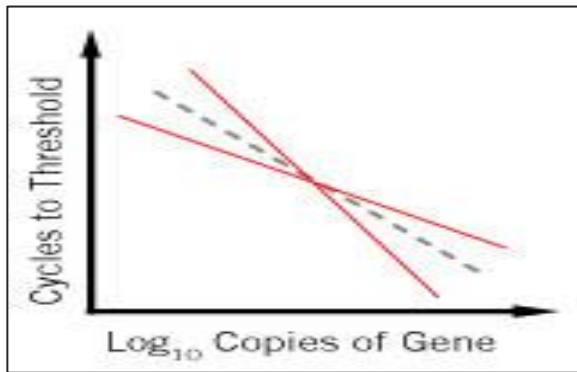


The samples with high concentrations of template do not lie on the standard curve. This is typical of reactions that are inhibited by template concentration.



The data points relating to the lower concentrations of target do not lie on the standard curve.





**Observation:** Slope of standard curve is more or less than  $-3.34$ .

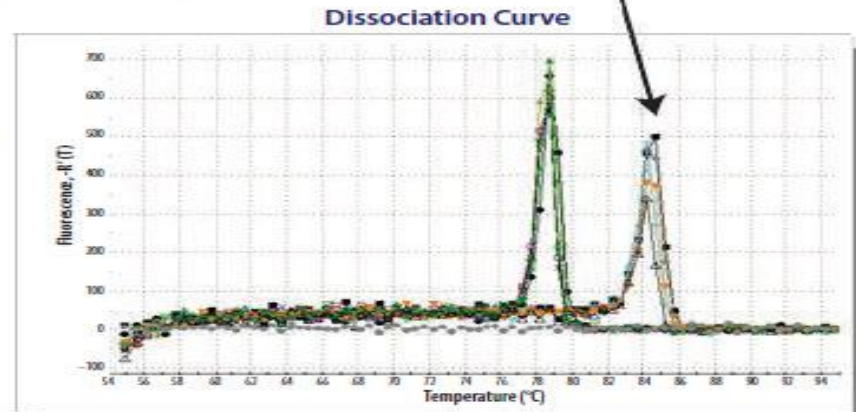
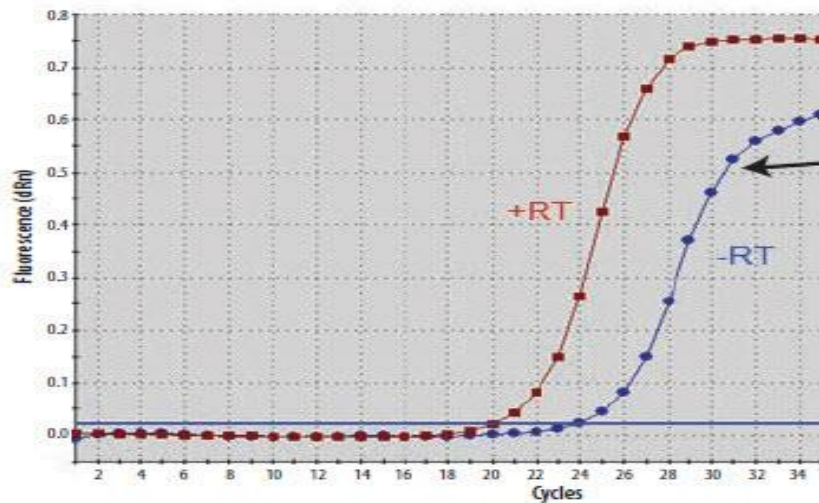
**Potential Causes:**

1. Inaccurate dilutions.
2. Efficiency of amplification is below 88% in one or both samples; differences in efficiency are  $> 5\%$ ;
3. RNA degradation

**Corrective Steps:**

1. Recalculate the standard concentration.
2. Make new stock solutions of the control standards.
3. Eliminate extreme concentrations.
4. Re-design primers for one or both genes.
5. Repeat experiment with fresh reagents and sample.

# **Amplification Curve**

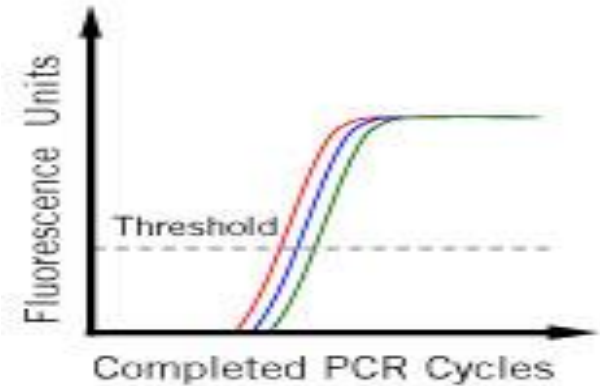
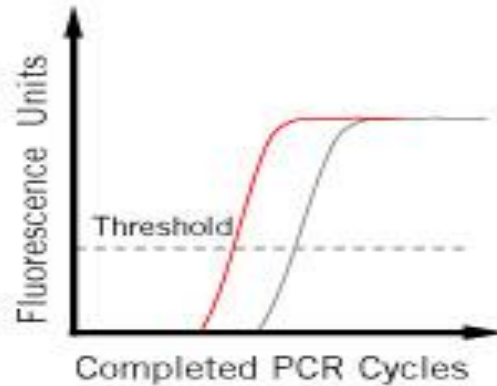
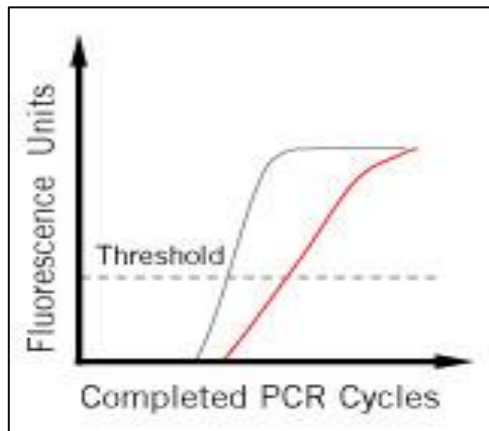


## Observation:

Exponential amplification in the no template control (NTC).

**Potential Causes:** Contamination of the reagent manufacture or possibly Laboratory.

**Corrective Steps:** Clear work area with 10% Bleach and nuclease-free water; order new reagent stocks; relocate reaction set-up to a clean lab

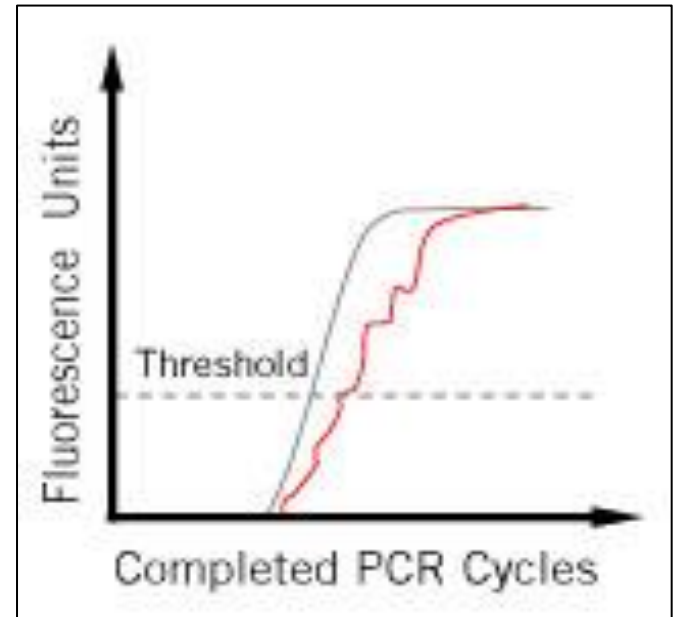


**Observation:** Technical replicates are not overlapping and have a difference in  $C_T$  values,  $C_T$  values occur later or earlier.

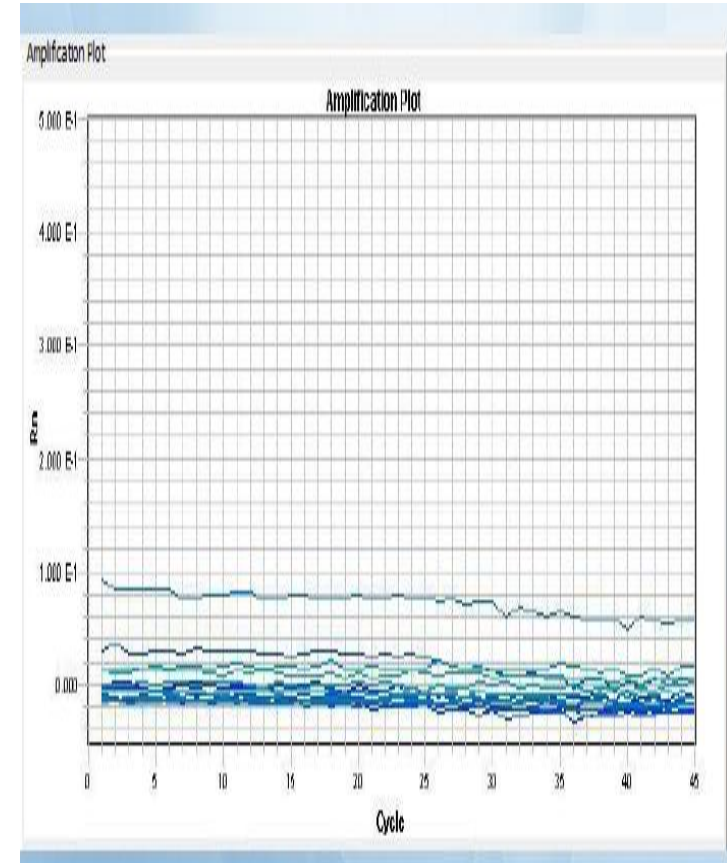
**Potential Causes:** Pipetting error; insufficient mixing of solutions.

**Corrective Steps:** Calibrate pipettes; use positive-displacement pipettes and filtered tips; mix all solutions thoroughly during preparation and during use; hold pipette vertically when aspirating solutions.

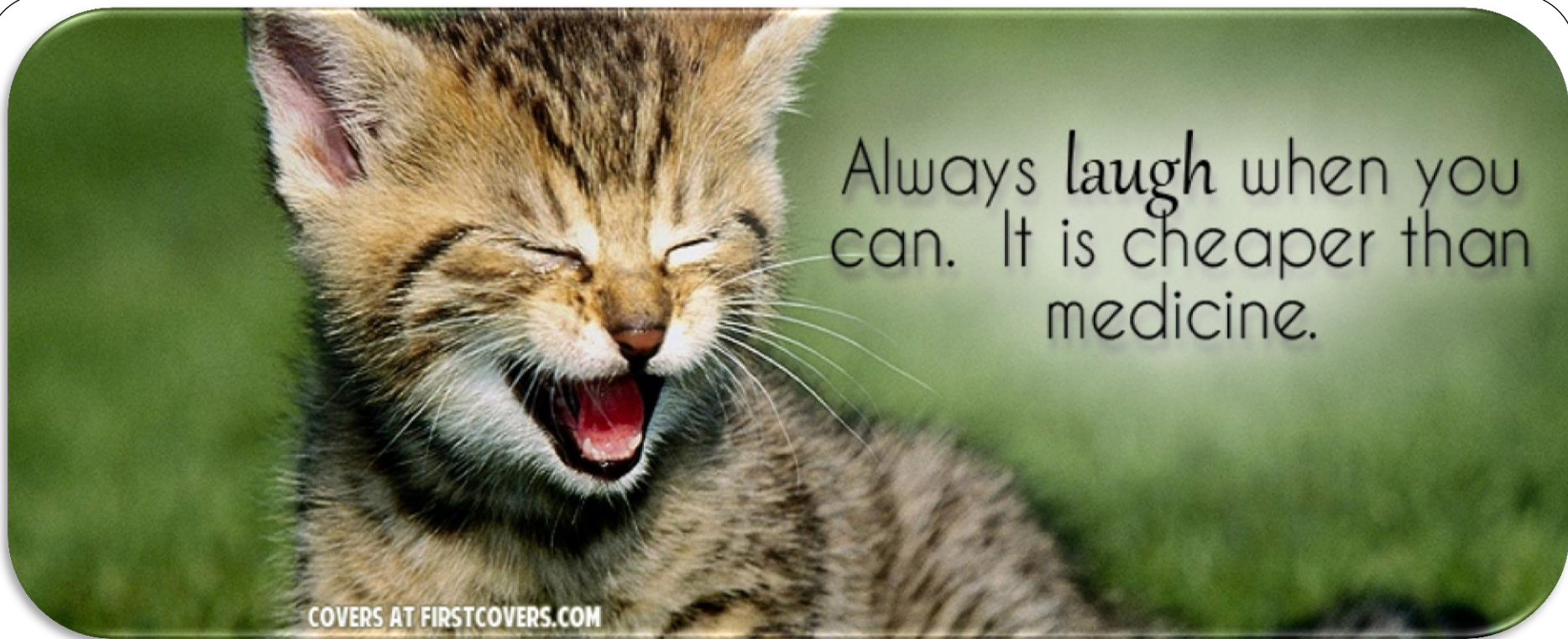
- **Observation:** Jagged signal throughout amplification plot
- **Potential Causes:** Mechanical error; buffer-nucleotide instability; poor amplification or weak probe signal.
- **Corrective Steps:** Contact equipment technician; warm master mix to room temperature and mix thoroughly before use; allow primers and probes to equilibrate for several minutes at room temperature before use; mix primer/probe/master solution thoroughly during reaction set up; redesign the probe and primer sequences.



- **Observation:** No data in selected wells
- **Potential Causes:** Wells not selected for analysis; wrong dye selection for analysis; failed first strand synthesis; no expression of target transcript
- **Corrective Steps:** Check settings for data collection and for data viewing; repeat experiment with new reagents; test assay performance against carefully quantified controls







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*Thanks a lot*

with my Best Regards and My Best wishes

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