When quantifying a double-stranded DNA sample (150 base pairs) in the lab using a spectrophotometer, the undiluted DNA gives an A260 of 0.100.

a)What is the concentration of your DNA sample, assuming that 1 A260 = 50 micrograms/milliliter DNA

b) If you have 85 microliters of this DNA sample left, how many nanograms of the DNA do you have ?

Amplification of DNA *in vitro*

I. Components of the PCR reaction
II. Applied PCR/amplification techniques
    a) Reverse transcription PCR (for RNA measurements)
    b) Quantitative real-time PCR
    c) PCR of long DNA fragments
    d) Detection of an RNA virus: SARS nCov-2
        a) PCR
        b) LAMP
    e) Whole genome amplification (WGA)

Guide to readings: PCR

1) "**Discovering Life in Yellowstone…** Where Nobody Thought It Could Exist": the story of the discovery of *Thermus aquaticus* by Brock and Freeze

2)15 MC4 PCR:

a) Introduction to PCR, DNA pols, primer design (p. 455-69)
b) Basic PCR protocol, and troubleshooting (p. 470-76)
c) PCR topics: Hot Start, Touchdown, Taq (p. 477-83, 533-6)
d) PCR primer design w/ Primer3Plus (p. 564-70)

3) ***16 MC4 quantitative PCR***: Theory and practice

4) ***Whole genome amplification*** (2003). Protocols for indiscriminate amplification of DNA

5) ***Guide to Primer3***. A short guide for the primer design program " Primer3" (complementary to section D above)

6) ***EUA-CDC-Panel-IFU***. Applied PCR: the CDC protocol for its kit that detects SARS nCoV 2 RNA

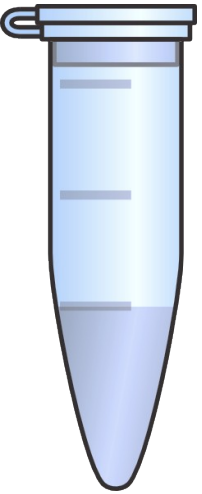
The need for specific DNA segments

- DNA for cloning and sequencing, or for *in vitro* studies
- Confirm the identity of engineered DNA constructs
- Monitor gene expression
- Detect a genetic disorder
- Detect a microbe
- Identify an individual
- Etc.

Multiple round DNA synthesis by thermal cycling -- PCR

- Polymerase Chain Reaction -- first described in 1971 by Kleppe and Khorana
- Re-description and first successful use in 1985 (Mullis)
- Massive amplification of specific sequences that have defined endpoints
- Fast, powerful, adaptable, and simple
- Many many applications

PCR: What you need in the tube



1. **Template DNA** that contains the " target sequence"

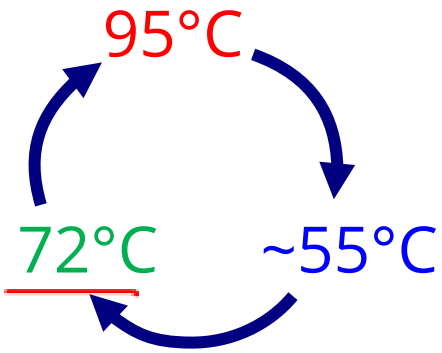
2. **Primers**: short oligonucleotides that define the ends of the target sequence

3. Thermostable **DNA polymerase**

4. Buffer, dNTPs

5. Temperature cycles (provided by a thermal cycler)

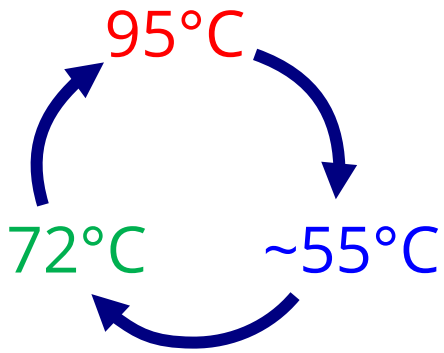
PCR temperature cycling



Denaturation:

- denature template strands (95°C for 2-5 minutes)
- can also add your DNA polymerase at this temp. for a "hot start", which prevents false priming in the initial round of DNA replication

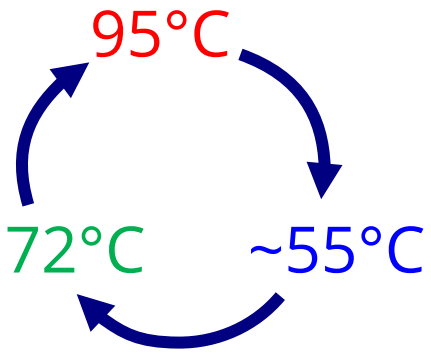
PCR temperature cycling



Annealing:

- The primers base pair (anneal, hybridize) to the template DNA
- The default T is around 55°C
- This temperature variable is the most critical one for getting a successful PCR reaction – the best variable to start with when trying to optimize a PCR reaction for a specific set of primers
- Annealing temperatures can go as low as 40-45°C, but non-specific annealing can be a problem

A typical PCR program (part II):



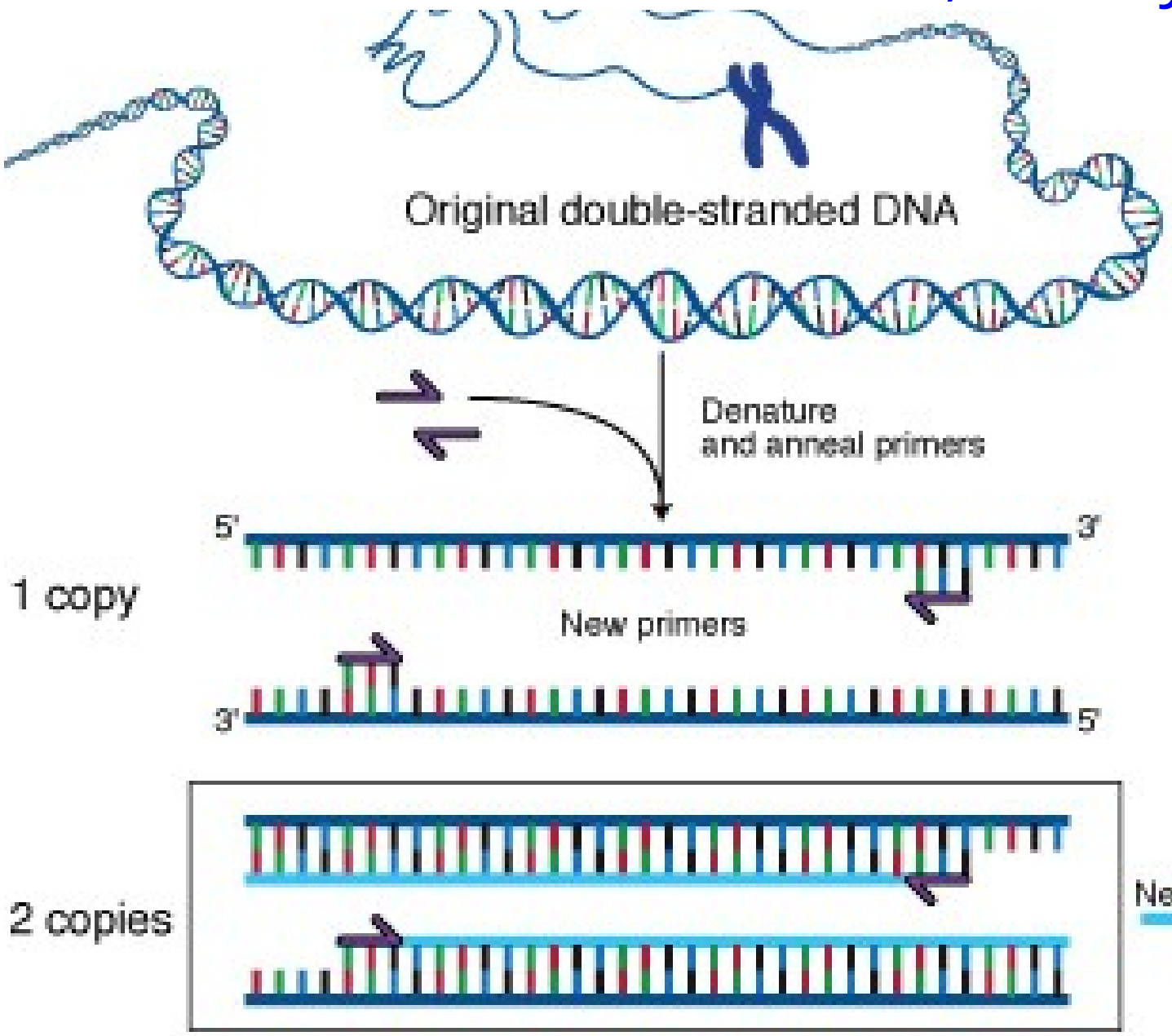
Extension:

- DNA polymerase extends the primer, making a copy of the DNA template
- Generally 72°C, allows enzymatic activity of many thermostable DNA polymerases

Number of cycles:

- Each cycle repeats the temperature series: 94→~55→72
- 20 to 30 cycles is typical

How it works: PCR reaction, first cycle



(Note: template DNA can also be single-stranded)

94°C

↓

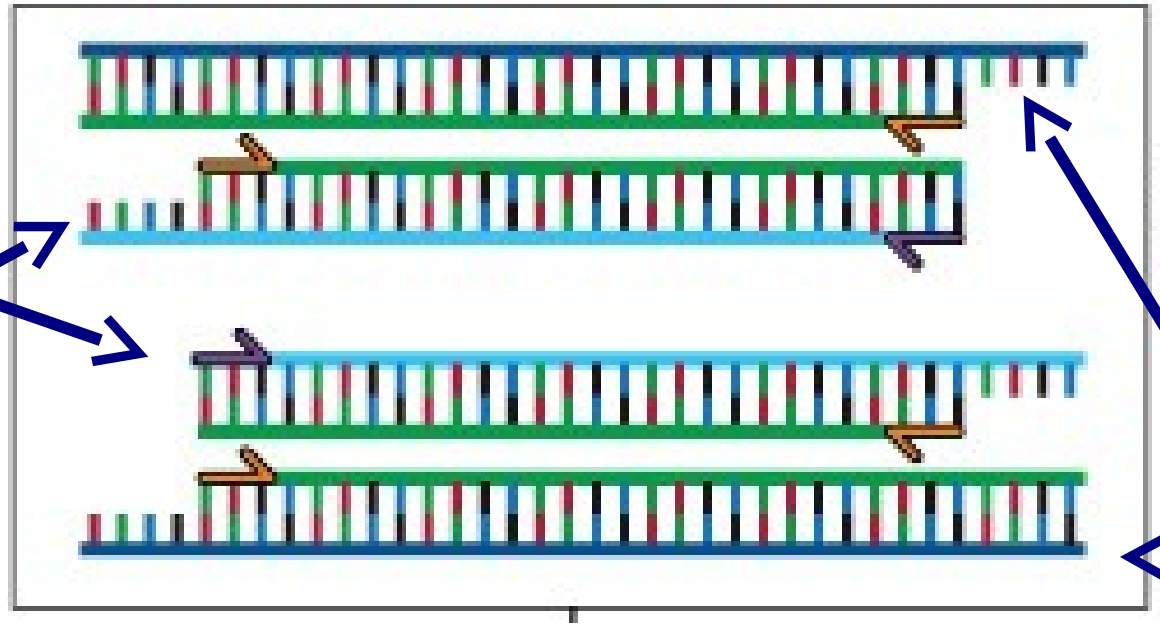
~55°C

↓

72°C

http://www.genome.gov/Glossary/index.cfm?id=159

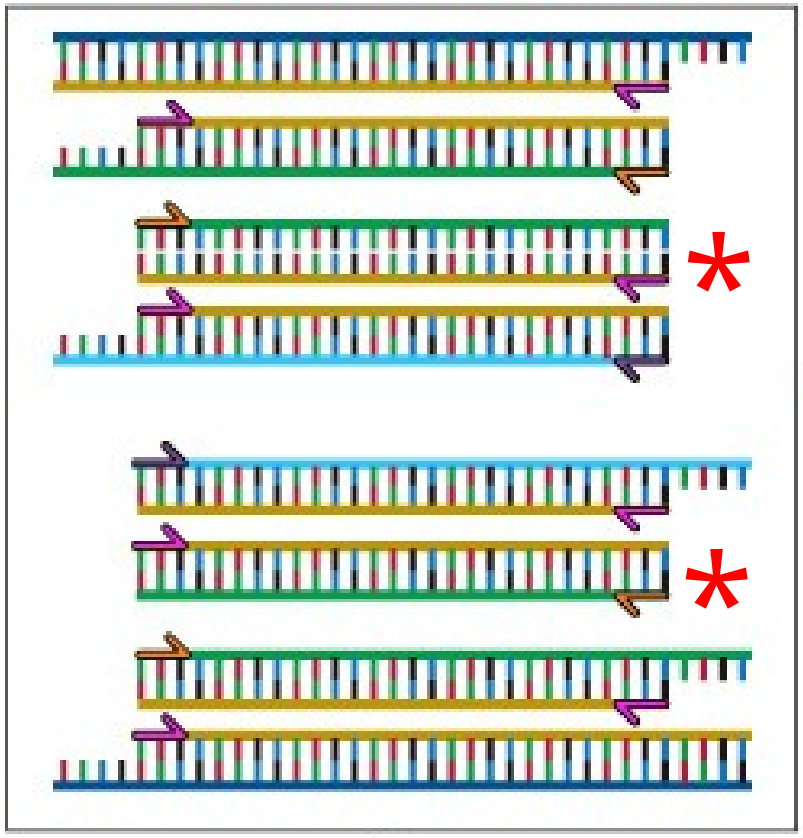
PCR reaction, second cycle



Template is product from first cycle

like first cycle

R reaction, third cycle: the first finished products appe



* These products serve as templates for future rounds: copies of copies

Cycle number	Number of double-stranded target molecules
1	0
2	0
3	2
4	4
5	8
29	134,217,728
30	268,435,456

PCR animation:
https://dnalc.cshl.edu/resources/animations/pcr.html

How to choose primers for PCR?

- Should be ~18-25 nucleotides (can be longer)
- Calculated melting temperature (T_m) should be nearly identical for both primers
- If possible, avoid inverted repeat sequences and self-complementary sequences in the primers
- If possible: avoid complementarity between primers (‘ primer dimers’)
- Use software to help, e.g. “ Primer3Plus” : you can choose “ detection” or “ cloning” to define the type of primers you want

http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/

Design a pair of primers (10 nucleotides each) that will amplify DNA from this template.

Label the 5′ and 3′ end of each primer

```
5′gtgttgtatt tgtctgaaga gtatccacct 3′
3′cacaacataa acagacttct cataggtgga 5′
```

The primers for PCR are often modified

1) Tags
   - Biotin
   - Fluorescent tag
   - See for example: IDT.com

2) Extra non-complementary sequences may be added to the primer (at the 5’ end only!)
   - For cloning purposes
   - For mutagenesis
   - To give a specific sequence identifier tag

Thermostable DNA polymerases from thermophile microbes

- Bacterial
  - Taq, from *Thermus aquaticus* (discovered by Dr. Tom Brock, see "Discovering Life in Yellowstone….")
  - High efficiency, but low fidelity
  - Excellent for routine reactions and small PCR products
- Archaeal
  - Pfu, from *Pyrococcus furiosus*
  - Lower efficiency, but high fidelity'
  - also good for routine reactions and best for cloning
    - 3' →5' exonuclease activity provides very high fidelity
    - Very stable to heat

Thermal cycling

I. Standard heat block

•“ ramp” times 5-10 seconds to change temperature, 30 cycle PCR lasts 2-3 hours.

• Advantage: easily automated, heat blocks can PCR up to 384 samples at a time

• Disadvantage: relatively slow (1-3 hours)

II. Capillary tubes with heat source

• heated and cooled by blasts of air

• 30 cycle-PCR done in < 30 minutes, but it’s limited to only a few samples at a time

III. Continuous flow

• channels force liquid through temperature gradients, *very* fast (seconds to minutes)

Super-fast chip-based PCR

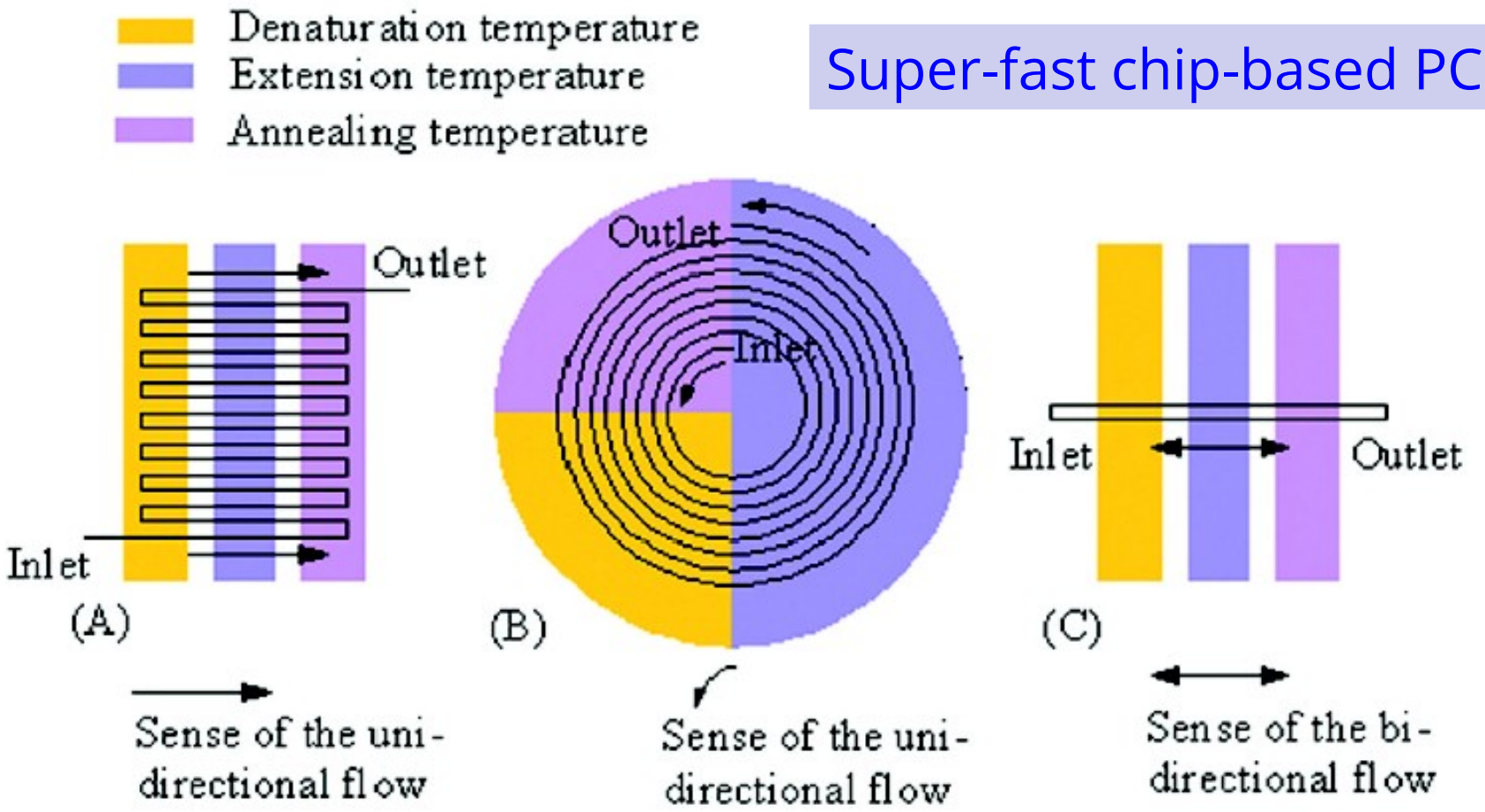


Figure 2. Continuous-flow PCR. (**A**) The serpentine channel continuous-flow PCR. (**B**) The spiral channel-based continuous-flow PCR. The sample is introduced at the inlet and pumped unidirectionally towards the outlet. (**C**) The straight channel oscillatory-flow PCR. The sample is introduced in the inlet and pumped back and forth in a straight channel. Temperature zones are provided by three heaters.

Zhang & Xing (2007) *Nucl. Ac. Res.* **35**, p 4223

Hot Start of PCR reactions

- non-specific priming occurs at low temperature (room temp.) -- the non-specific priming could give artifactual amplification as temperature rises in the PCR tube

- Withhold some component of the reaction until the denaturing temperature is reached (94°C)

  - Wait until 94°C to add enzyme
    or
  - Enzyme bound to an inactivating enzyme antibody that releases at high temperature
    or
  - Wax beads containing Mg++ that can only be released at high temp

Touchdown PCR: improve target specificity

Allows you to selectively amplify only the best sequences (with the least mismatches) while minimizing non-specific PCR products

- Start with 2 cycles at an annealing temperature about 5-10°C higher than the calculated primer melting temperatures.
- Progressively reduce the annealing temperature by 1°C at 1 or 2 cycle intervals
- Final cycles of PCR done at annealing temp 2-5°C lower than calculated annealing temp

Useful if your primers are not 100% complementary to your template DNA (e.g. degenerate oligos), or when there are

Difficult PCR? Be sure to include controls

	Primers	**Bystander DNA**	**template DNA**	**Known target DNA**	Expected result
Your template	+	-	+	-	?
Positive controls					
1	+	-	-	+	Band
2	+	+	-	+	Band
Negative controls					
3	+	-	-	-	No
4	+	+	-	-	No

Deviation from expected results gives ideas for protocol changes

Template DNA: The DNA being tested

Known target DNA: known to contain primer recognition sequences

Bystander DNA: not recognized by

Trouble-shooting a failed PCR

-- No PCR product
-- Very little product
-- Wrong-sized DNA bands on gel
-- Etc.

Remedies:

- Temperature or solution conditions may need changing
- Template DNA may have contaminants – repurify DNA
- Primers may need to be re-designed

(see 15 MC4 PCR, p. 473-6)

II. Specific applications for DNA amplification

A. Reverse transcription PCR (for RNA measurements)

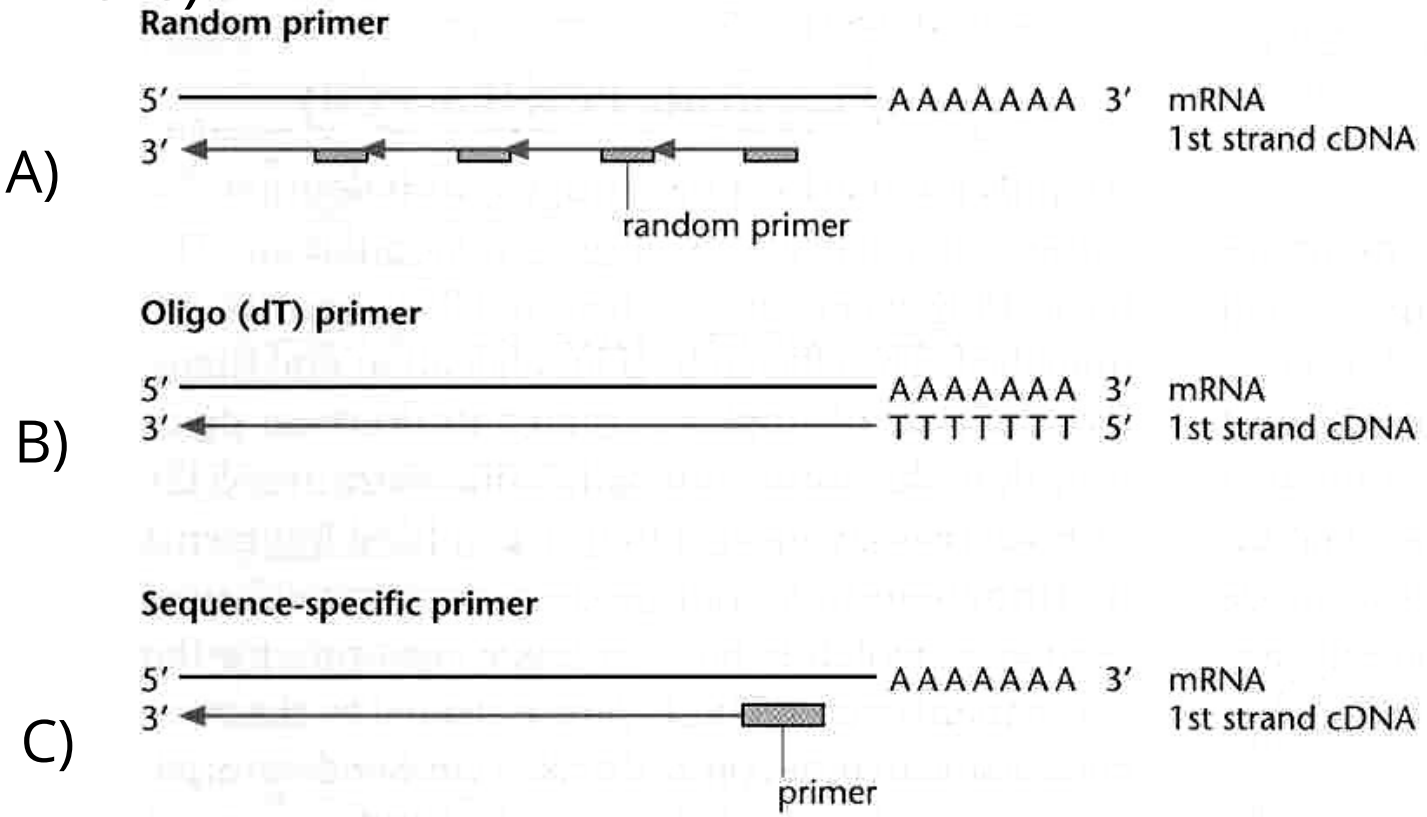
B. Quantitative (real-time) PCR

C. PCR of long DNA fragments

D. Whole genome amplification

Detection of RNA (gene expression, or RNA virus):
reverse transcription followed by PCR

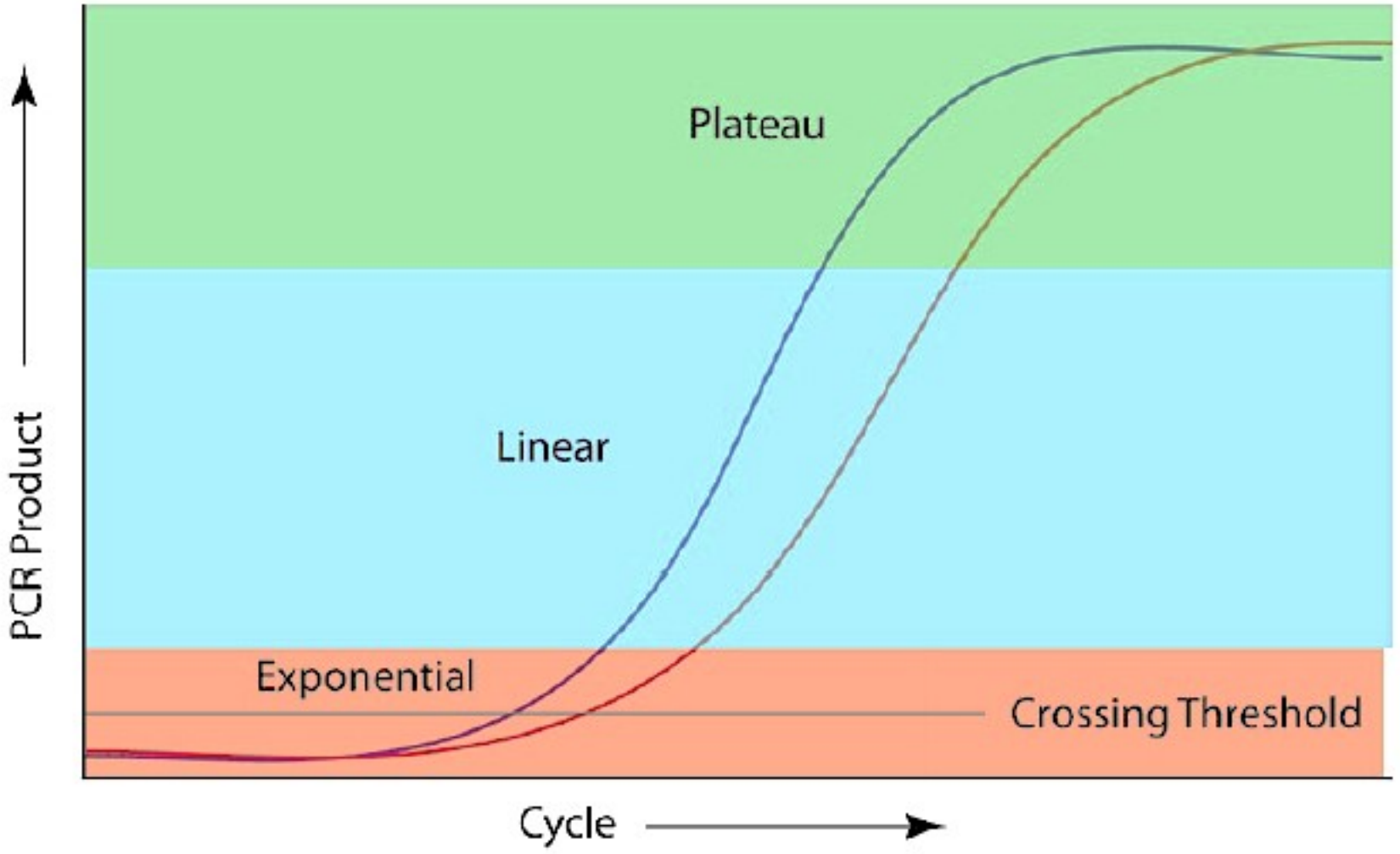
Step 1: make cDNA with reverse transcriptase (three ways shown here)



Step 2: normal PCR (from cDNA) using gene-specific primers

NOT QUANTITATIVE (end point DNA level doesn't report RNA levels)

PCR reaction progress as temperature cycling progresses

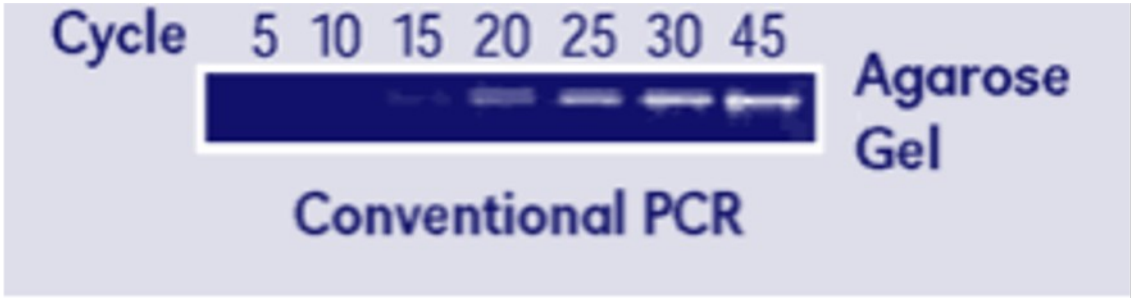


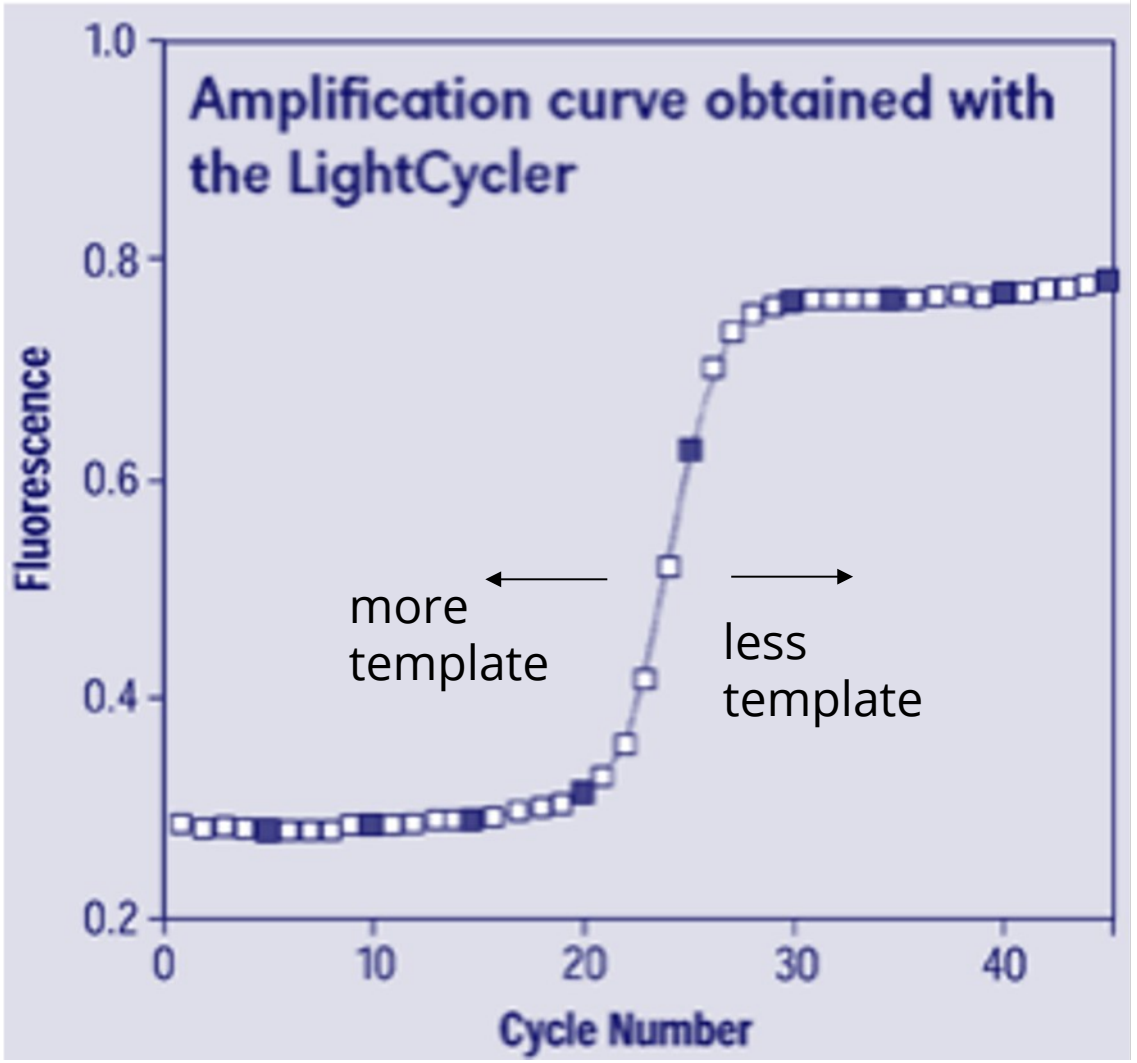
Each line represents a different template RNA/cDNA
Which one is present in the highest quantity?

Quantitative Real Time (QRT) PCR

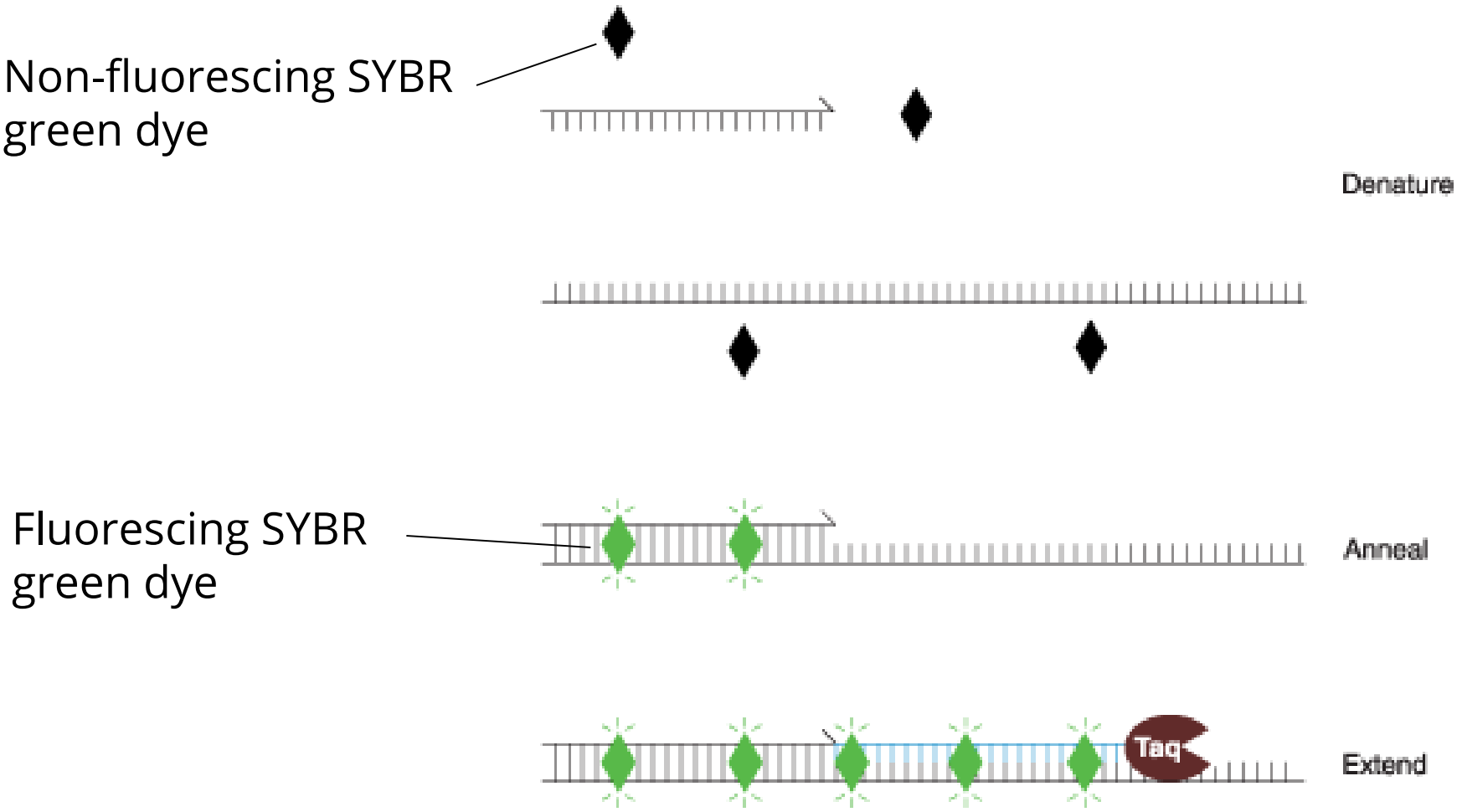
Fluorescence measurements are done simultaneously with PCR temperature cycles

Instantaneous measurement of product levels
Position of the center of the curve changes depending on the amount of template RNA/cDNA. *Variations of over 5 or 6 orders of magnitude can be*





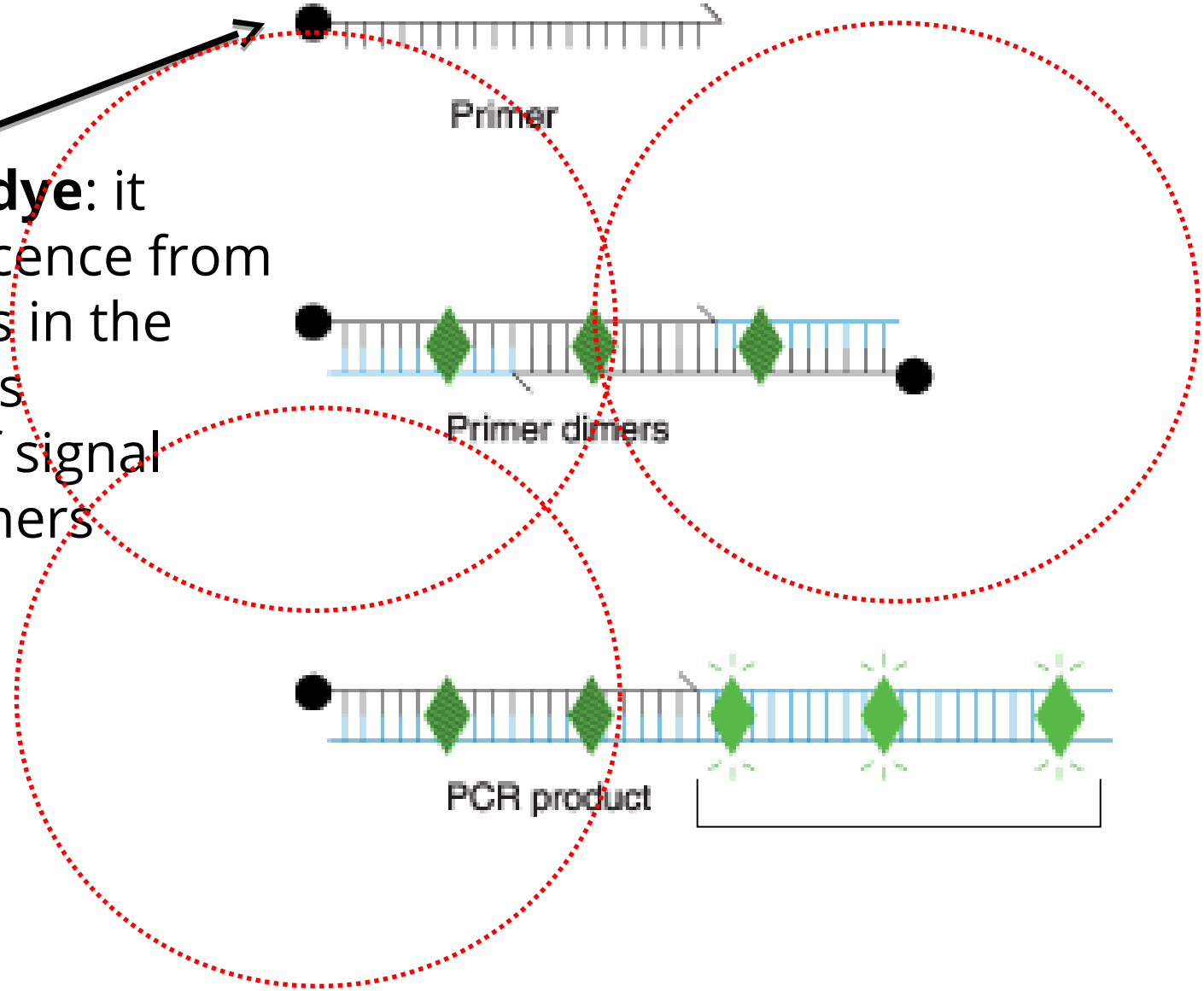
Dye-binding: more DNA, more fluorescence



Low cost, but detects all DNA, including artifacts

Primer dimers can give false signal with dye binding

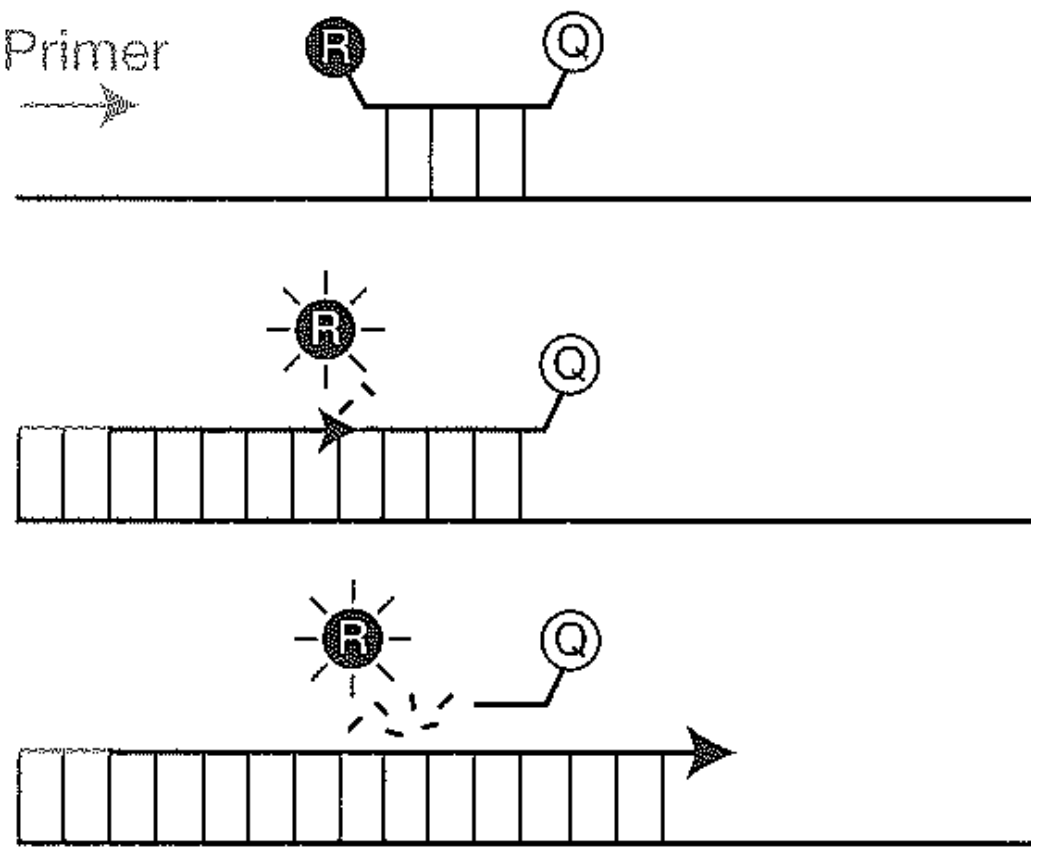
QSY quencher dye: it absorbs fluorescence from SYBR green dyes in the vicinity--prevents accumulation of signal from primer dimers



This can be avoided by short-range ‘quenching’ of fluorescence

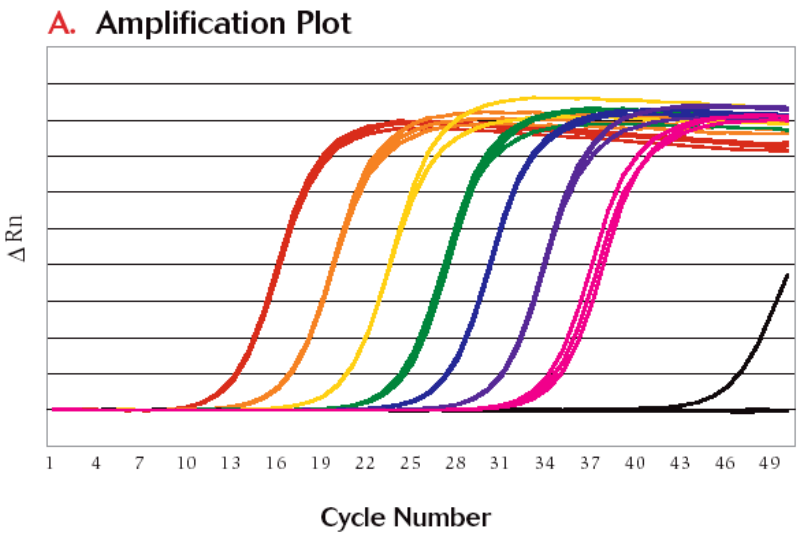
Fluorescent probes: removal of 'quencher' based on product accumulation

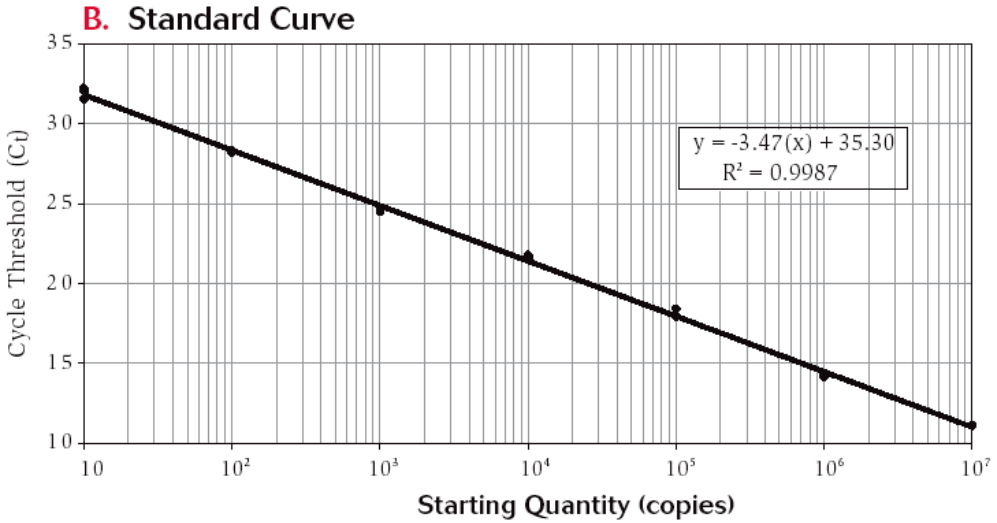
The more target DNA there is, the more probe anneals, the more it is cleaved (by Taq polymerase 5'→3' exonuclease activity), the more fluorescence is produced



Each experimental target needs its own probe

ontrol amplifications allow precise quantitation:





Real-time quantitative PCR of 10-fold serial dilutions (10^7 to 10 copies) of pCR2.1 plasmid were performed using primers specific to the Kanamycin resistance gene (200 nM each) with Platinum® SYBR® Green qPCR SuperMix-UDG and ROX Reference Dye. Reactions were incubated for 2 min. at 50°C, then 2 min. at 95°C, followed by 50 cycles of 95°C for 15 sec.; 60°C, 30 sec. using the ABI PRISM® 7700.

Standard curve: based on the cycle at which “ threshold” (of detection) is reached for a specific number of DNA molecules

(From the Invitrogen website)

Recommendations for the CDC regarding detection of SARS n-CoV 2 infection: https://www.cdc.gov/coronavirus/2019-ncov/hcp/testing-overview.html

- Many commercial tests available
- CDC offers its own test: https://www.cdc.gov/coronavirus/2019-ncov/lab/virus-requests.html
- RT-PCR based

Detection of 2019-nCov in patient samples (CDC protocol)

- Collect sample, extract RNA (Trizol reagent treatment)

- Add primers for
  - (step 1) Reverse transcriptase, making DNA copy of the RNA genome
  - (step 2) PCR amplification of nucleocapsid gene using primers specific to 2019-nCoV version of the gene
  - (step 3) Detection of the amplified DNA using a "TaqMan" probe approach (fluorophore revealed when probe binds target and gets degraded)

  - https://www.biosearchtech.com/support/videos/real-time-pcr-probe-animation-video

2019-Novel Coronavirus (2019-nCoV) Real-time rRT-PCR Panel Primers and Probes				
Name	**Description**	**Oligonucleotide Sequence (5'>3')**	**Label[1]**	**Working Conc.**
2019-nCoV_N1-F	2019-nCoV_N1 Forward Primer	5'-GAC CCC AAA ATC AGC GAA AT-3'	None	20 µM
2019-nCoV_N1-R	2019-nCoV_N1 Reverse Primer	5'-TCT GGT TAC TGC CAG TTG AAT CTG-3'	None	20 µM
2019-nCoV_N1-P	2019-nCoV_N1 Probe	5'-FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1-3'	FAM, BHQ-1	5 µM

Primers and probe for nucleocapsid protein gene

Control primers and probe for human RNAse P gene

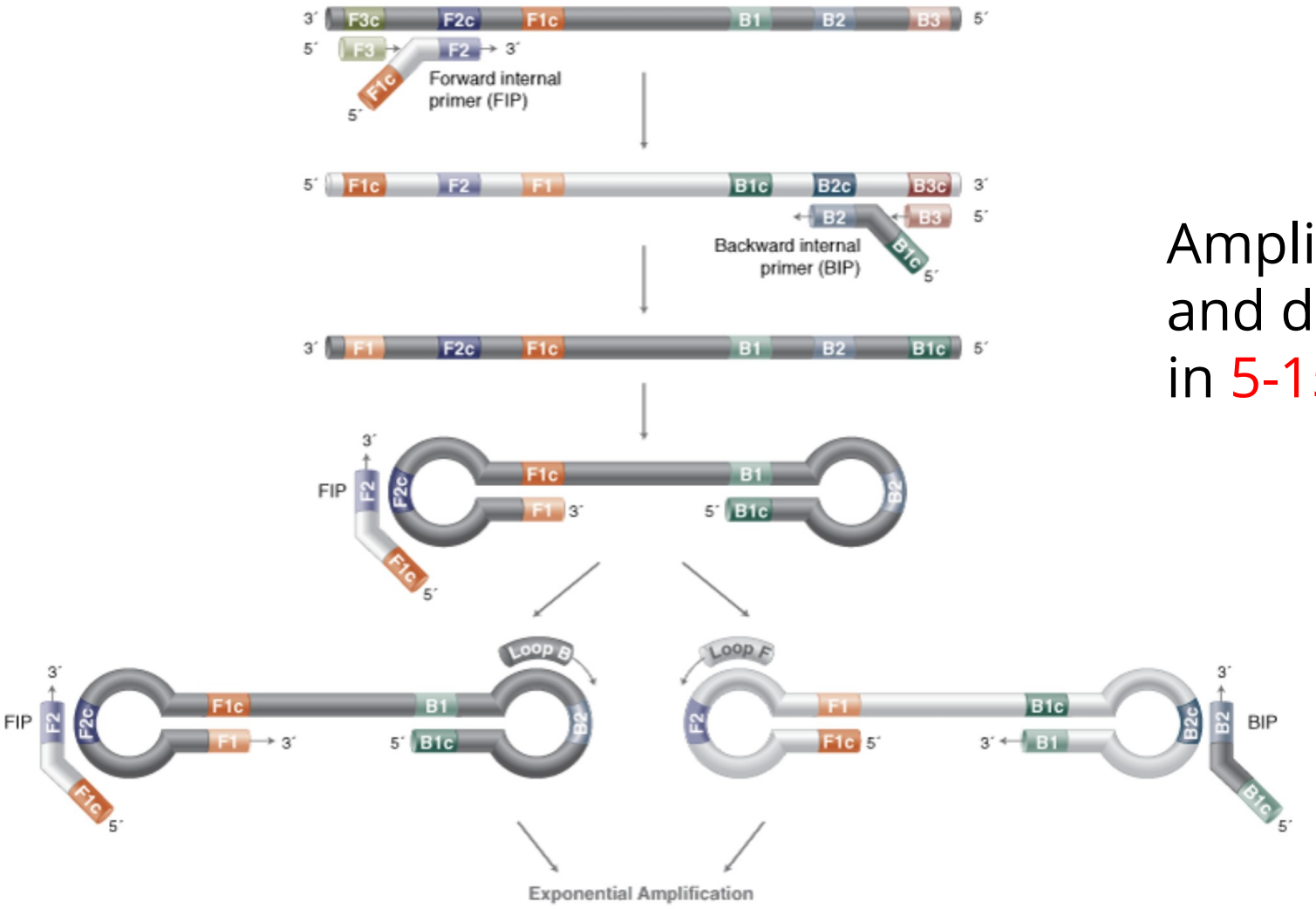
RP-F	RNAse P Forward Primer	5'-AGA TTT GGA CCT GCG AGC G-3'	None	20 µM
RP-R	RNAse P Reverse Primer	5'-GAG CGG CTG TCT CCA CAA GT-3'	None	20 µM
RP-P	RNAse P Probe	5'-FAM – TTC TGA CCT GAA GGC TCT GCG CG – BHQ-1-3'	FAM, BHQ-1	5 µM

[1]TaqMan® probes are labeled at the 5'-end with the reporter molecule 6-carboxyfluorescein (FAM) and with the quencher, Black Hole Quencher 1 (BHQ-1) (Biosearch Technologies, Inc., Novato, CA) at the 3'-end.

Note: Oligonucleotide sequences are subject to future changes as the 2019-Novel Coronavirus evolves.

Other tests: Isothermal amplification allowing rapid amplification of target DNA

LAMP: loop mediated isothermal amplification



Amplification and detection in 5-15 min

https://www.youtube.com/watch?v=L5zi2P4lggw

PCR of long sequences (>2 kb)

Long DNAs can be challenging to amplify

- Discontinuity (breakage) within the target DNA sequence reduces number of ‘good’ templates
- DNA polymerase is not 100% processive (it falls off or degrades before finishing)
- Misincorporation by error prone DNA polymerases causes mutations in the product: longer sequence = more mutations

PCR of long sequences (>2 kb)

Some changes to protocol to assist in long PCR

- Make sure DNA is exceedingly clean & prepared without breakage
- Use DNA polymerase “ cocktail” : Taq for high activity, and Pfu for proofreading activity (it can correct Taq’ s mistakes)
- Increase time of extension reaction (5-20 minutes, compared to the standard 1 minute for short PCRs). The rule of thumb is 1 minute per kilobase of DNA amplified
- Use DNA polymerases engineered to be more processive

Whole genome amplification: multiple displacement amplification (MDA)

<u>How it works:</u>

Strand-displacement amplification used by rolling-circle replication systems.

- Phi29 DNA polymerase (very low error rate)
- *Bacillus stearothermophilus* DNA polymerase (Bst) is also sometimes used (higher error rate)
- Primers: random hexamer (6 nucleotide)
- Incubation temperature: 30°C. No cycling.

Applications: forensics, *in utero* disease diagnosis, microbial diversity surveys, single cell genome

Whole genome amplification : multiple displacement amplification (MDA)

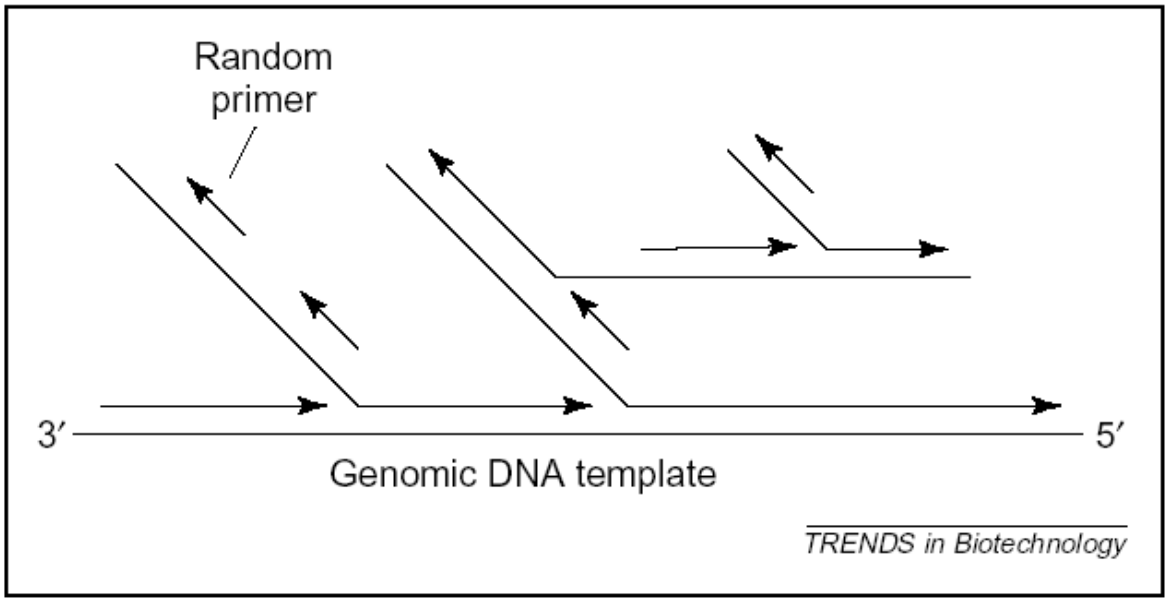


Figure 1. Multiple displacement amplification reaction. DNA synthesis is primed by random hexamers. Exponential amplification occurs by a 'hyperbranching' mechanism. Unlike PCR, which requires thermal cycling to repeatedly melt template and anneal primers, the ϕ29 DNA polymerase acts at 30°C to concurrently extend primers as it displaces downstream DNA products.

20-30 micrograms human DNA can be recovered from 1-10 copies of the human genome

Products arise from a random sampling of the available template – this is the least biased method for amplification of

In vitro amplification of DNA

I. Components of the PCR reaction

II. A few advanced applications of PCR

a) Reverse transcription PCR (for RNA measurements)

b) Quantitative real-time PCR

c) PCR of long DNA fragments

d) Whole genome amplification (WGA)

Design two PCR primers that will amplify the entire portion of this DNA fragment. Indicate the position of these primers relative to the DNA, the sequence of the primers, and their 5’ and 3’ ends.

5’GTGAATAAGCAAAAGGTTTGTCCTGCTTGTGAATCTGCGGAACTTATTTATGATCCA
GAGAGGGGGGAAATAGTCTGTGCCAAGTGCGGTTATGTAATAGAAGAGAACATAATTGA
TATGGGTCCTGAGTGGCGTGCTTTTGATGCTTCTCAAAGGGAACGCAGGTCTAGAACTG
GTGCACCAGAAAGTATTCTTCTTCATGACAAGGGGCTTTCAACTGAAATTGGAATTGAC
AGATCGCTTTCCGGATTAATGAGAGAGAAGATGTACCGTTTGAGGAAGTGGCAGTCCAG
ATTAAGAGTTAGTGATGCAGCAGAGAGGAACCTAGCTTTTGCCCTAAGTGAGTTGGATA
GAATTACTGCTCAGTTAAAACTTCCAAGACATGTAGAGGAAGAAGCTGCAAGGCTGTAC
AGAGAGGCAGTGAGAAAGGGACTTATTAGAGGTAGATCTATTGAGAGCGTTATGGCGGC
ATGTGTTTACGCTGCTTGTAGGTTATTAAAAGTTCCCAGGACTCTGGATGAGATTGCTG
ATATTGCTAGAGTTGATAAAAAGGAAATTGGAAGAAGTTACAGATTCATTGCGAGAAAT
CTCAATTTAACTCCCAAAAAACTATTTGTCAAGCCAACTGATTATGTAAATAAATTTGC
GGATGAGCTCGGATTAAGTGAAAAAGTTAGGAGAAGAGCTATTGAAATTCTTGATGAGG
CTTATAAAAGGGGGTTAACTAGTGGTAAGAGTCCAGCTGGTTTAGTAGCAGCAGCCCTA
TACATAGCTTCTTTATTGGAGGGAGAGAAGAGAACACAAAGAGAAGTTGCCGAAGTTGC
TAGAGTAACTGAAGTGACTGTGAGAAATAGATACAAGGAGCTCGTAGAGAAGTTGAAGA
TTAAAGTTCCTATAGCATGA 3’

Add extra sequences, containing restriction sites, to primers to help make them easier to clone. Indicate where those sequences should go.

One of the primers:

5′ 3′

You attempt the PCR, but you run the gel and you see no DNA products. Suggest two things that may have gone wrong.