Lecture 18

PCR Technology

Growing PCR Industry

Basic PCR, Cloning of PCR product, RT-PCR, RACE, Quantitative PCR, Multiplex PCR, Hot start PCR, Touchdown PCR,PCR sequencing, Real-time RT-PCR (RT^2-PCR)…..

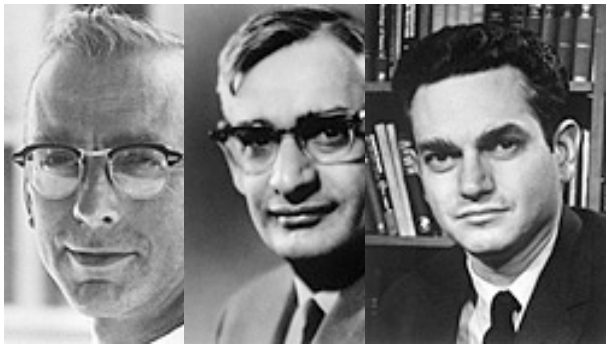
How PCR started

- The DNA duplex would be denatured to form single stands. This denaturation step would be carried out in the presence of a sufficiently large excess of the two appropriate primers. … DNA polymerase will be added to complete the process of repair …The whole cycle could be repeated.(H.G. Khorana, 1971, JMB 56, 341)

- I stopped the car again and started drawing lines of DNA molecules hybridizing and extending, the product of one cycle becoming the templates for the next in a chain reaction…(K.B. Mullis, 1990, Sci Am 262, 56

The Nobel Prize in Physiology or Medicine 1968

"for their interpretation of the genetic code and its function in protein synthesis"



Robert W. Holley	**Har Gobind Khorana**	**Marshall W. Nirenberg**
1/3 of the prize	1/3 of the prize	1/3 of the prize
USA	USA	USA
Cornell University Ithaca, NY, USA	University of Wisconsin Madison, WI, USA	National Institutes of Health Bethesda, MD, USA
b. 1922 d. 1993	b. 1922 (in Raipur, India)	b. 1927

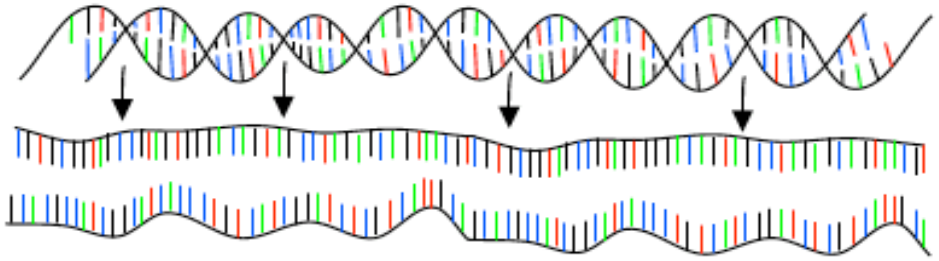
The Nobel Prize in Physiology or Medicine 1975

"for their discoveries concerning the interaction between tumour viruses and the genetic material of the cell"

David Baltimore	**Renato Dulbecco**	**Howard Martin Temin**
1/3 of the prize	1/3 of the prize	1/3 of the prize
USA	USA	USA
Massachusetts Institute of Technology (MIT) Cambridge, MA, USA	Imperial Cancer Research Fund Laboratory London, United Kingdom	University of Wisconsin Madison, WI, USA
b. 1938	b. 1914 (in Catanzaro, Italy)	b. 1934 d. 1994

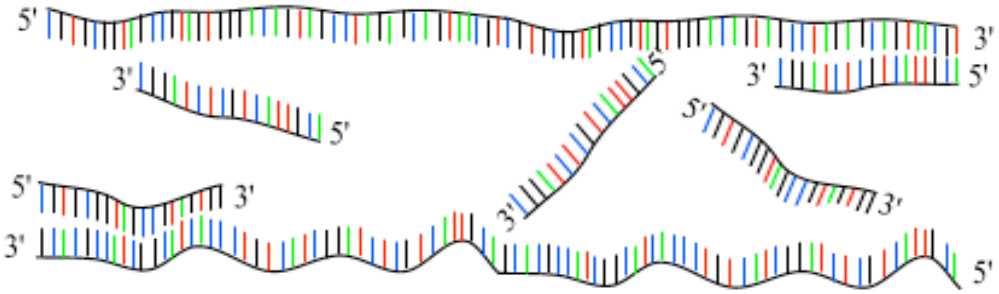
PCR : Polymerase Chain Reaction

30 - 40 cycles of 3 steps :



Step 1 : denaturation

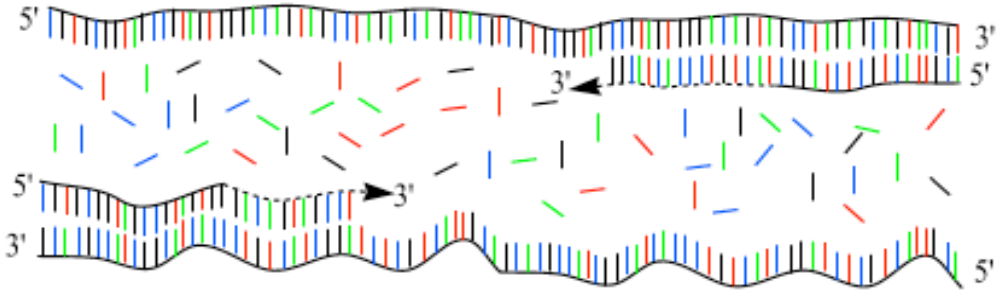
1 minut 94 °C



Step 2 : annealing

45 seconds 54 °C

forward and reverse primers !!!



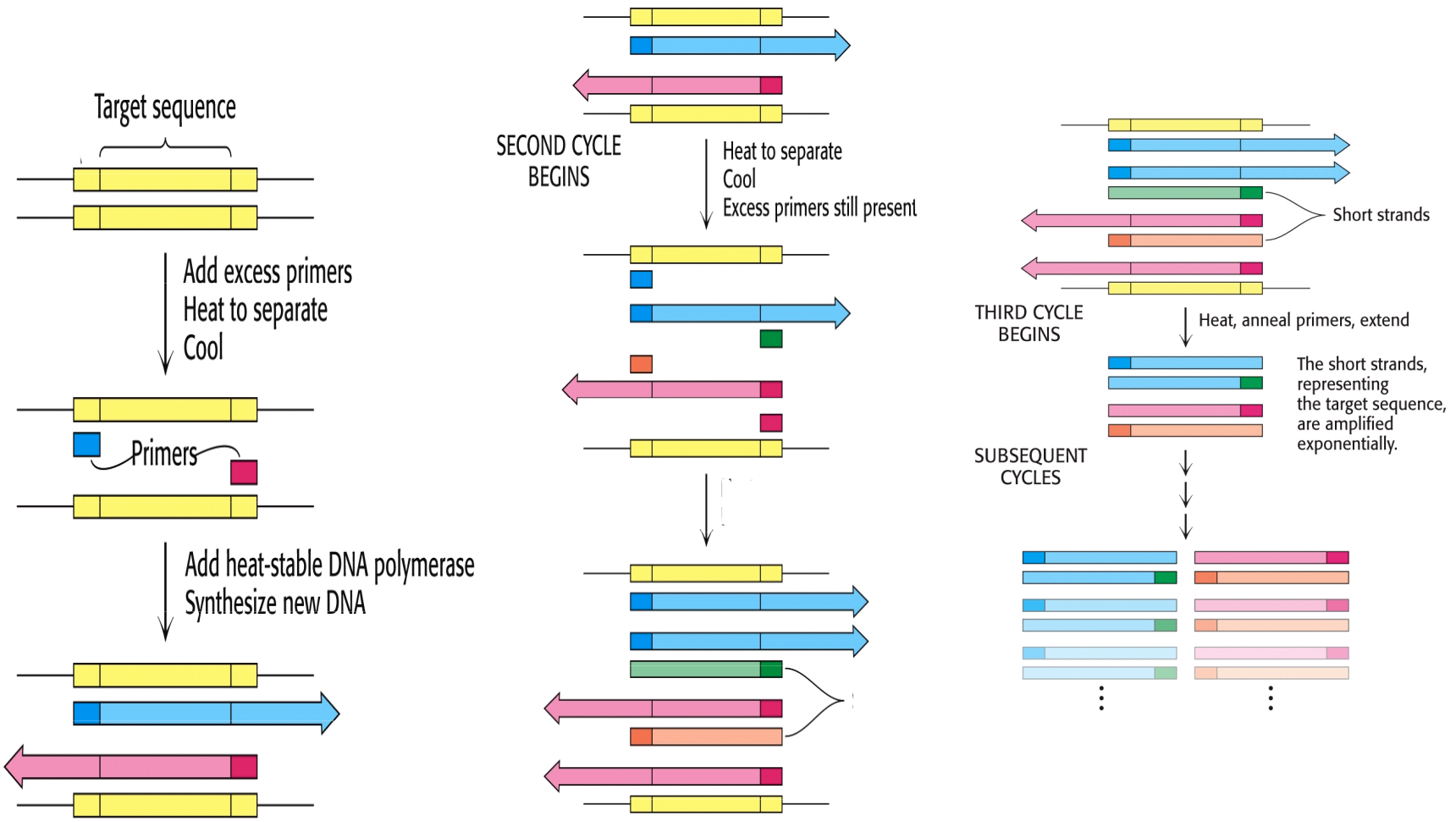
Step 3 : extension

2 minutes 72 °C

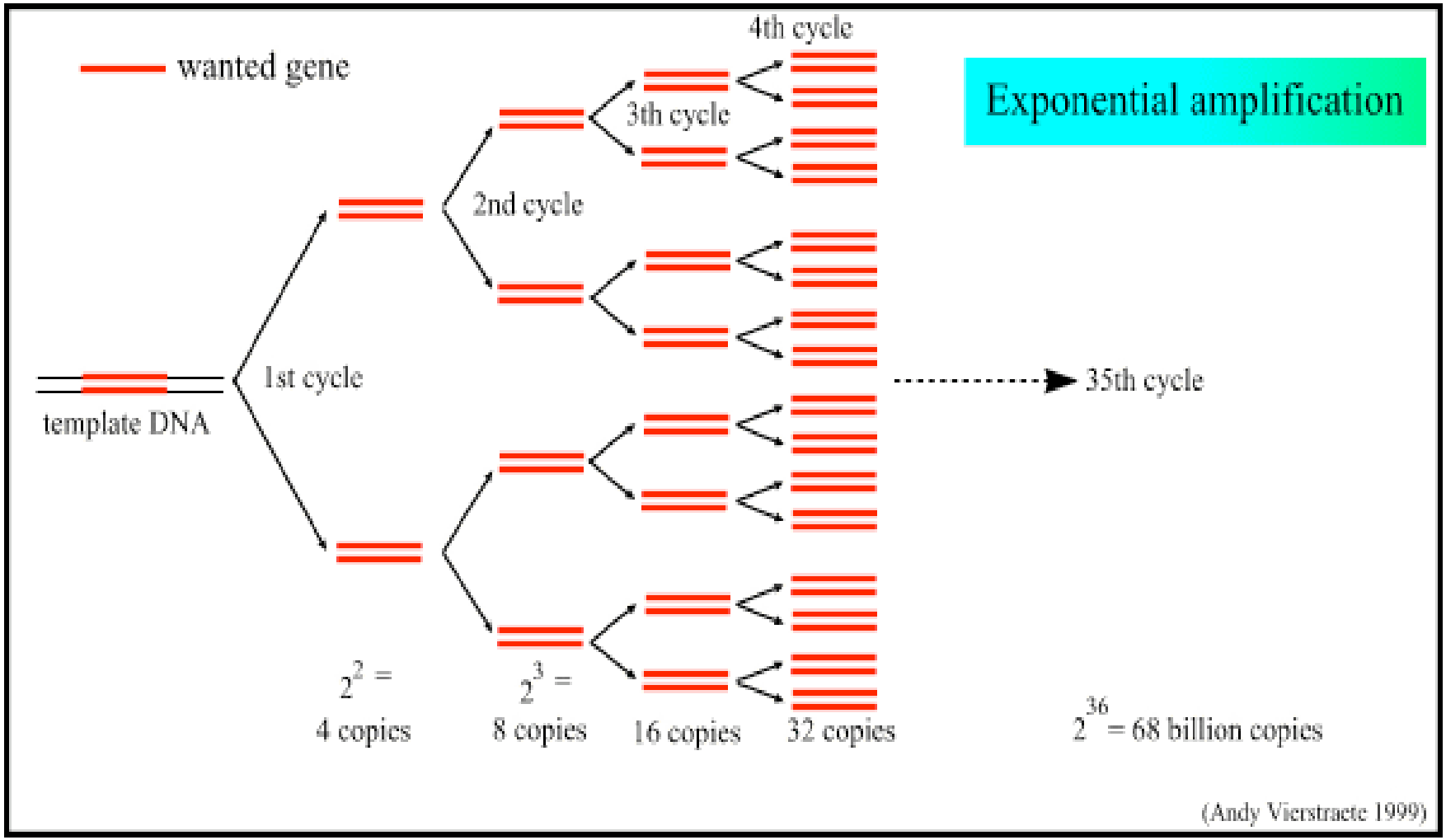
only dNTP's

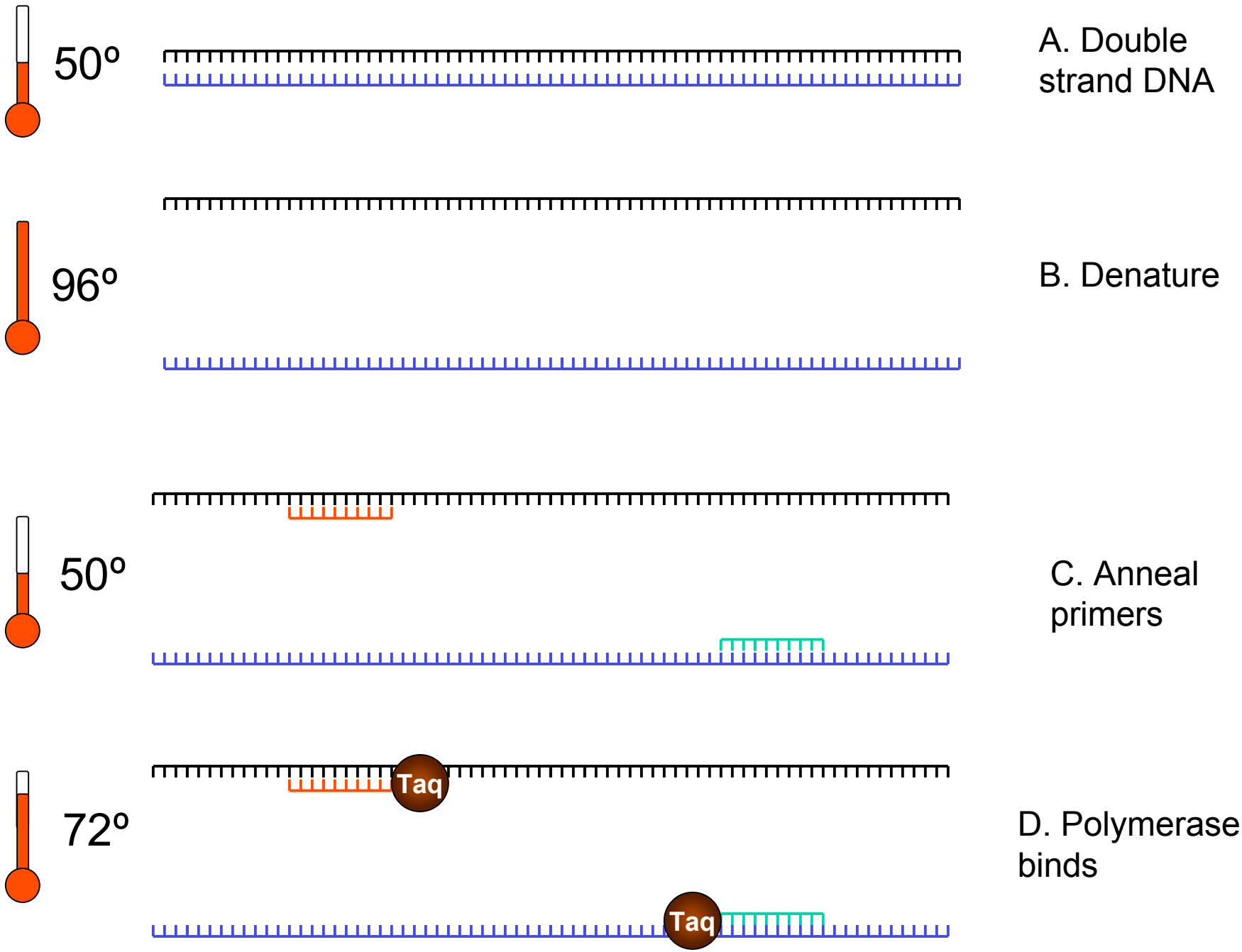
(Andy Vierstraete 1999)

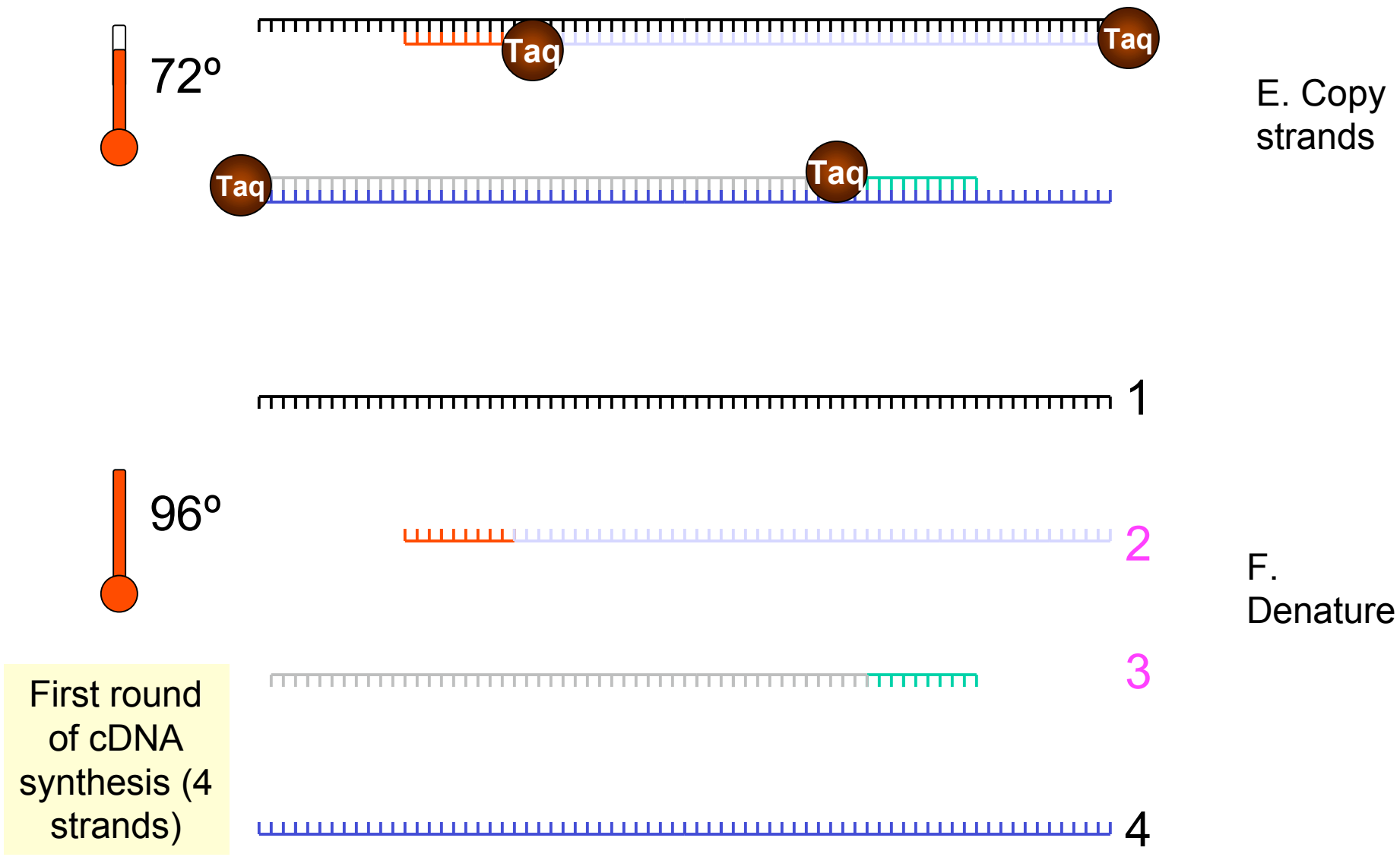
First three PCR cycles

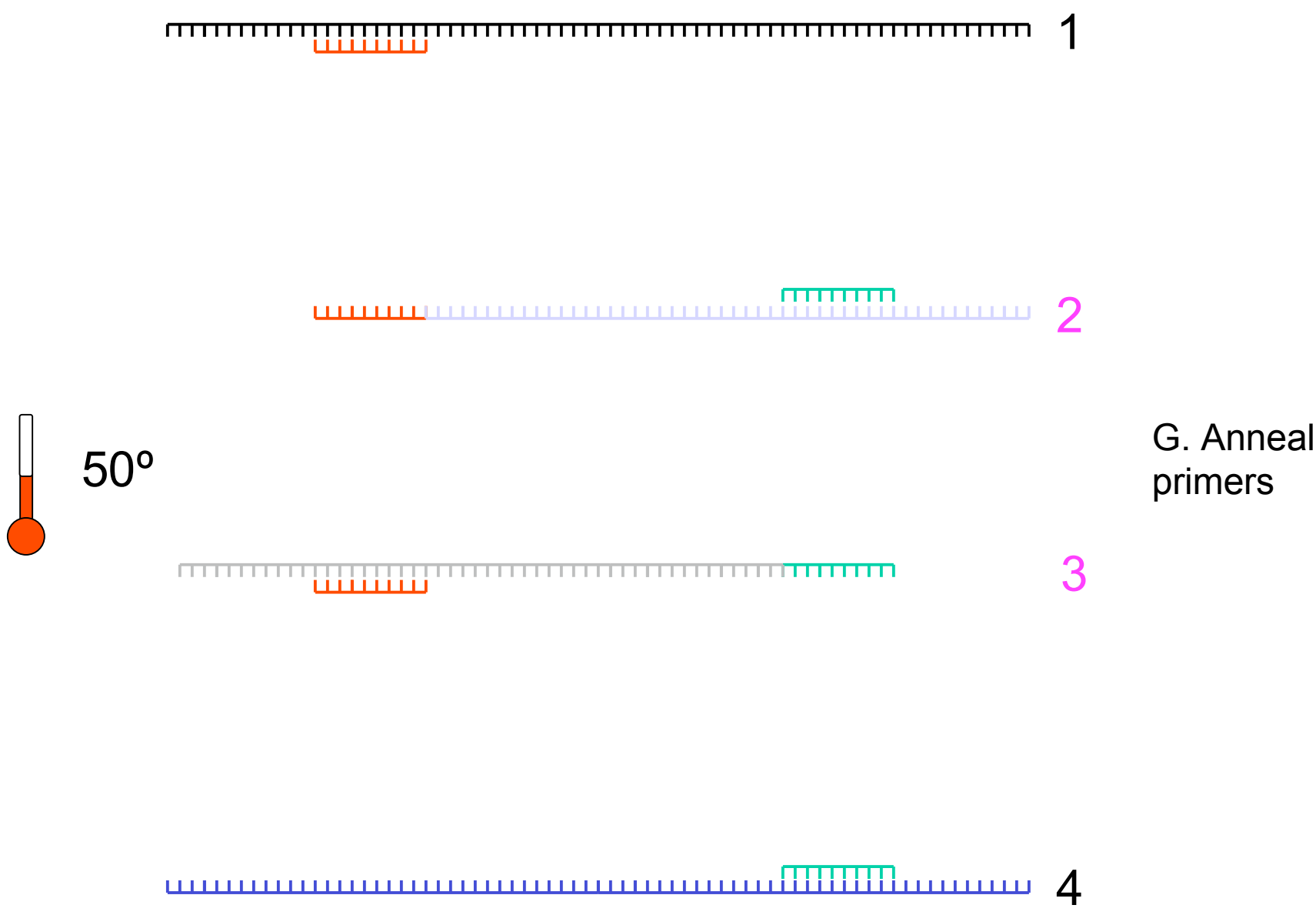


Exponential amplification

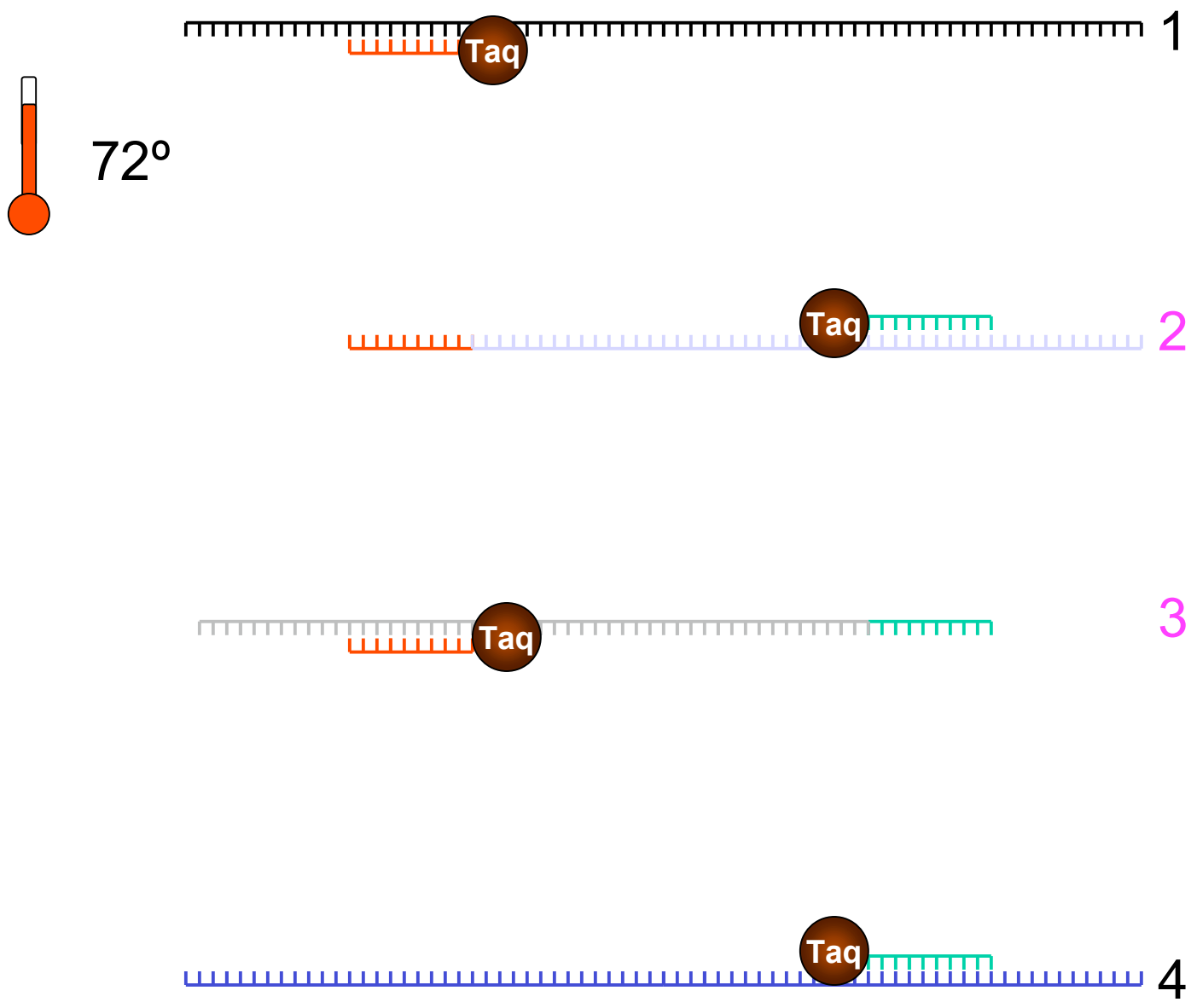


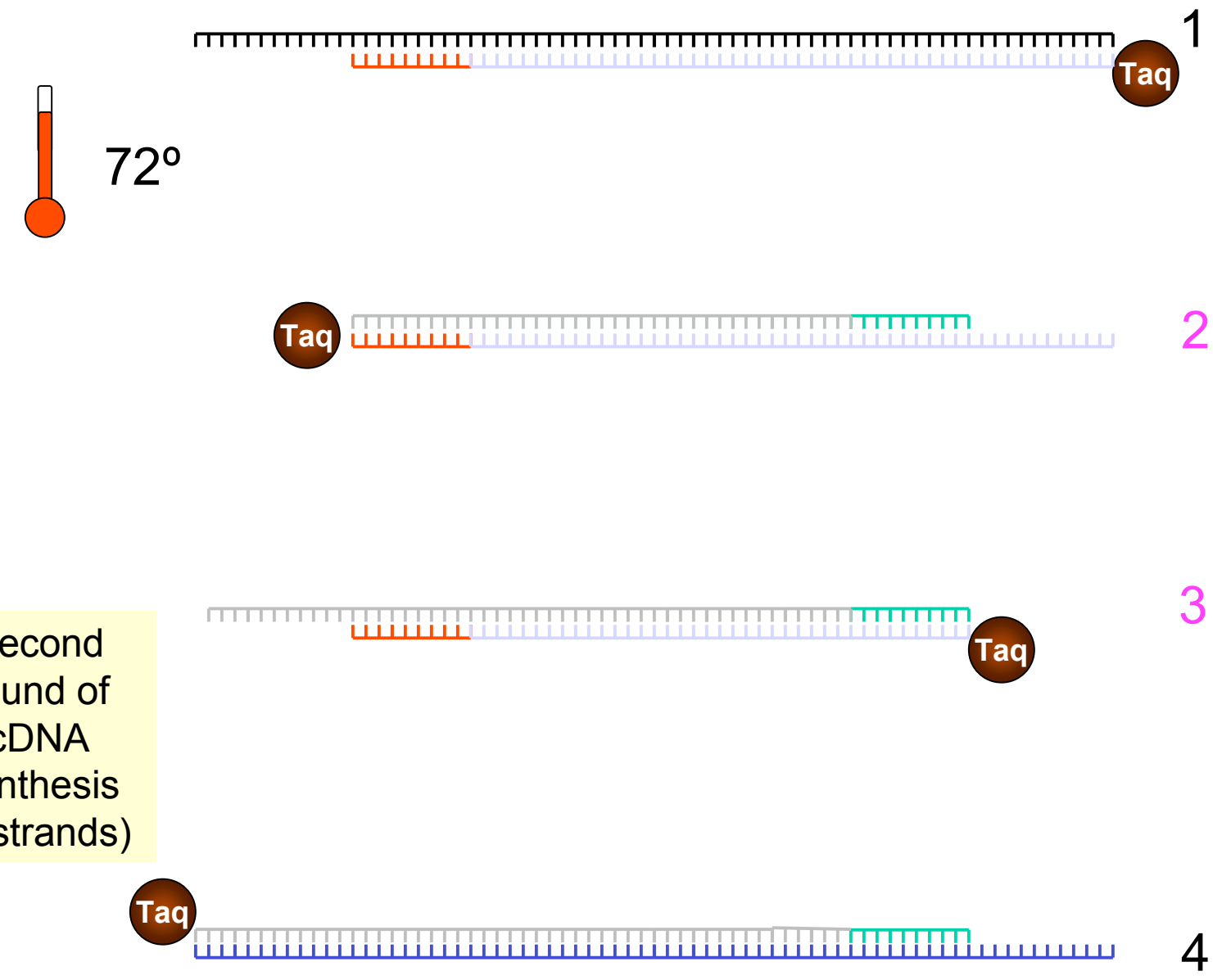

50°
A. Double strand DNA
96°
B. Denature
50°
C. Anneal primers
Taq
72°
D. Polymerase binds
Taq

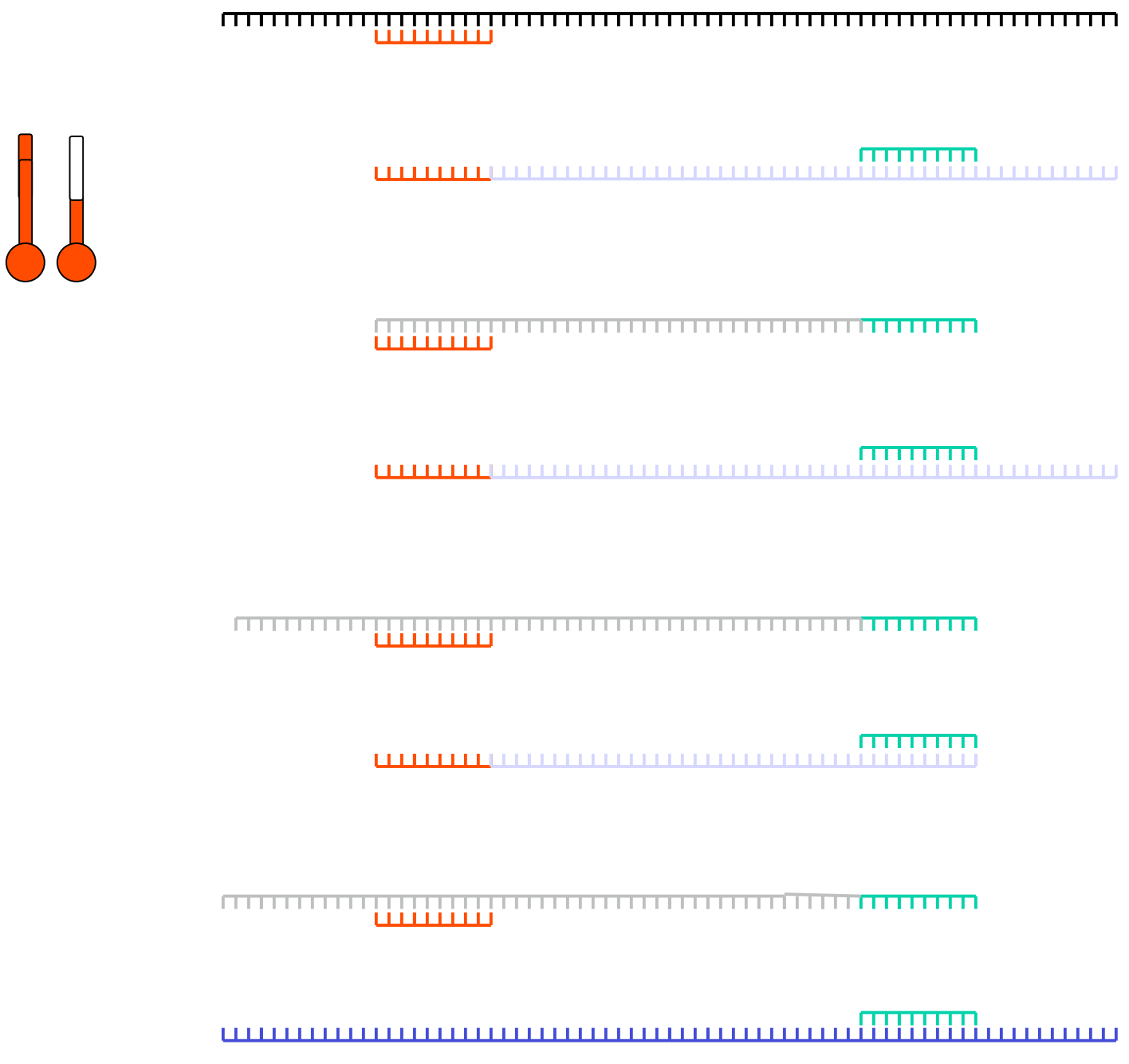

72º
Taq
Taq
Taq
E. Copy strands
1
96º
2
F. Denature
3
First round of cDNA synthesis (4 strands)
4

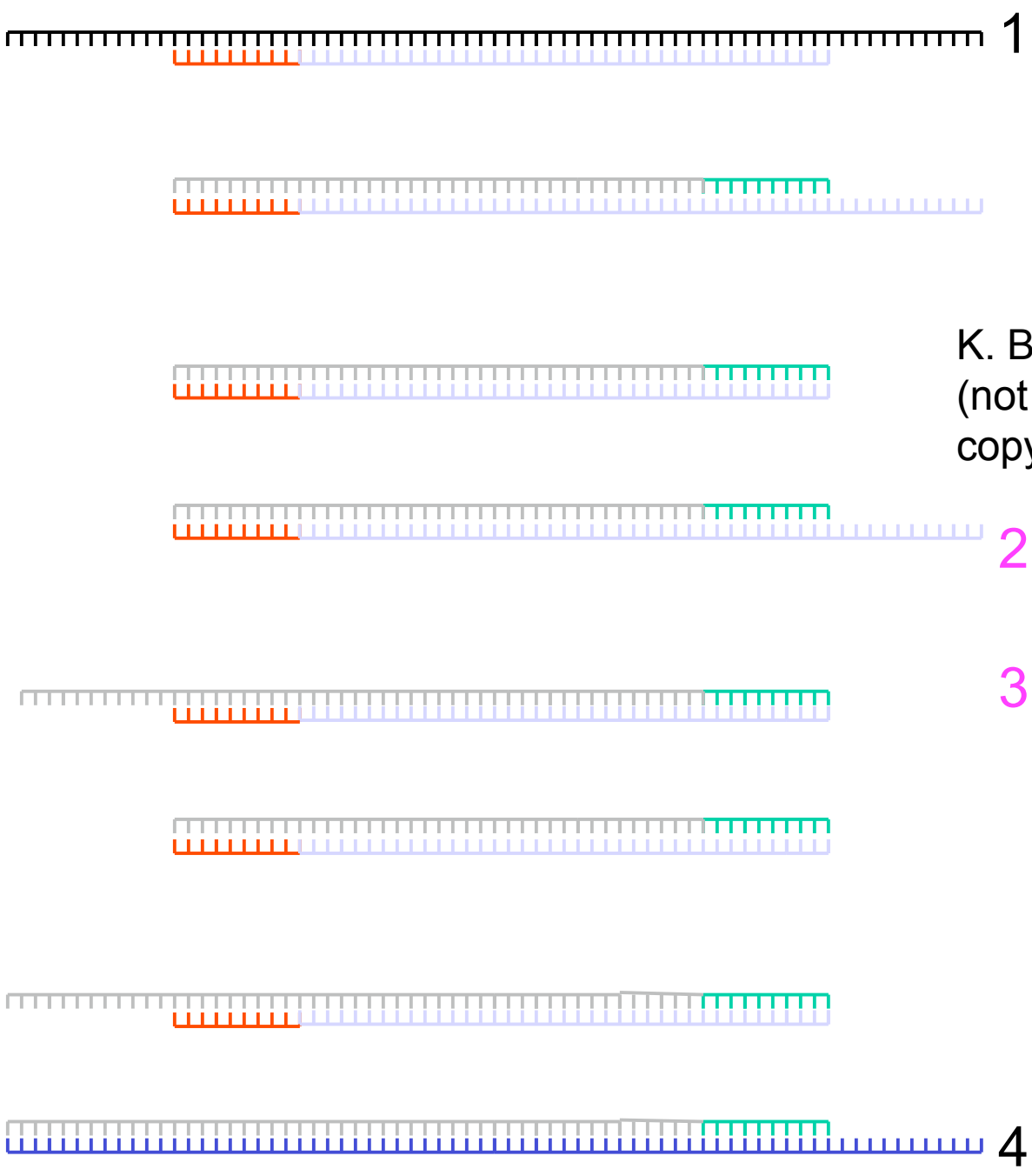


G. Anneal primers

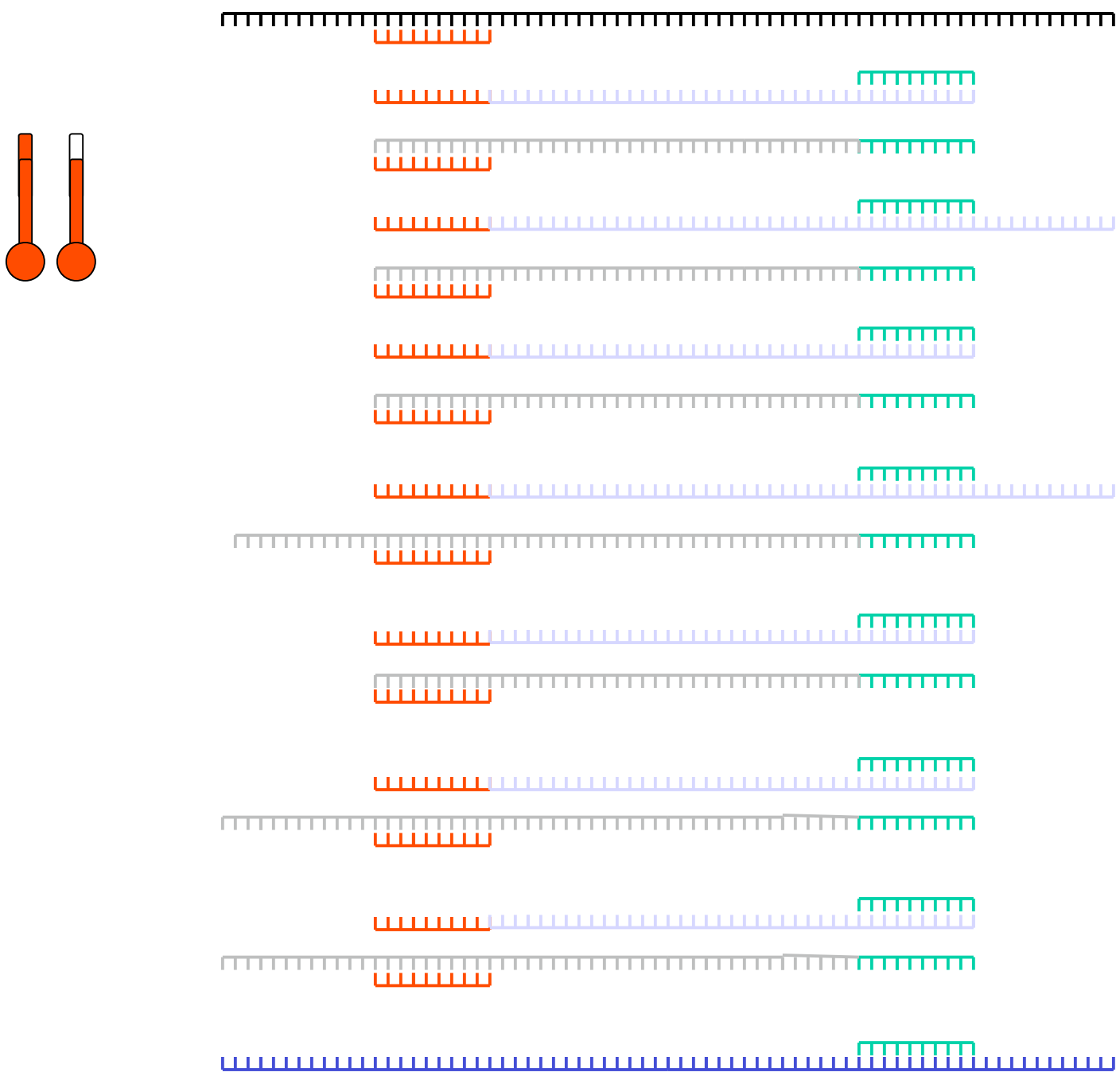

Taq
1
72º
Taq
2
H.
Polymerase
binds
Taq
3
Taq
4

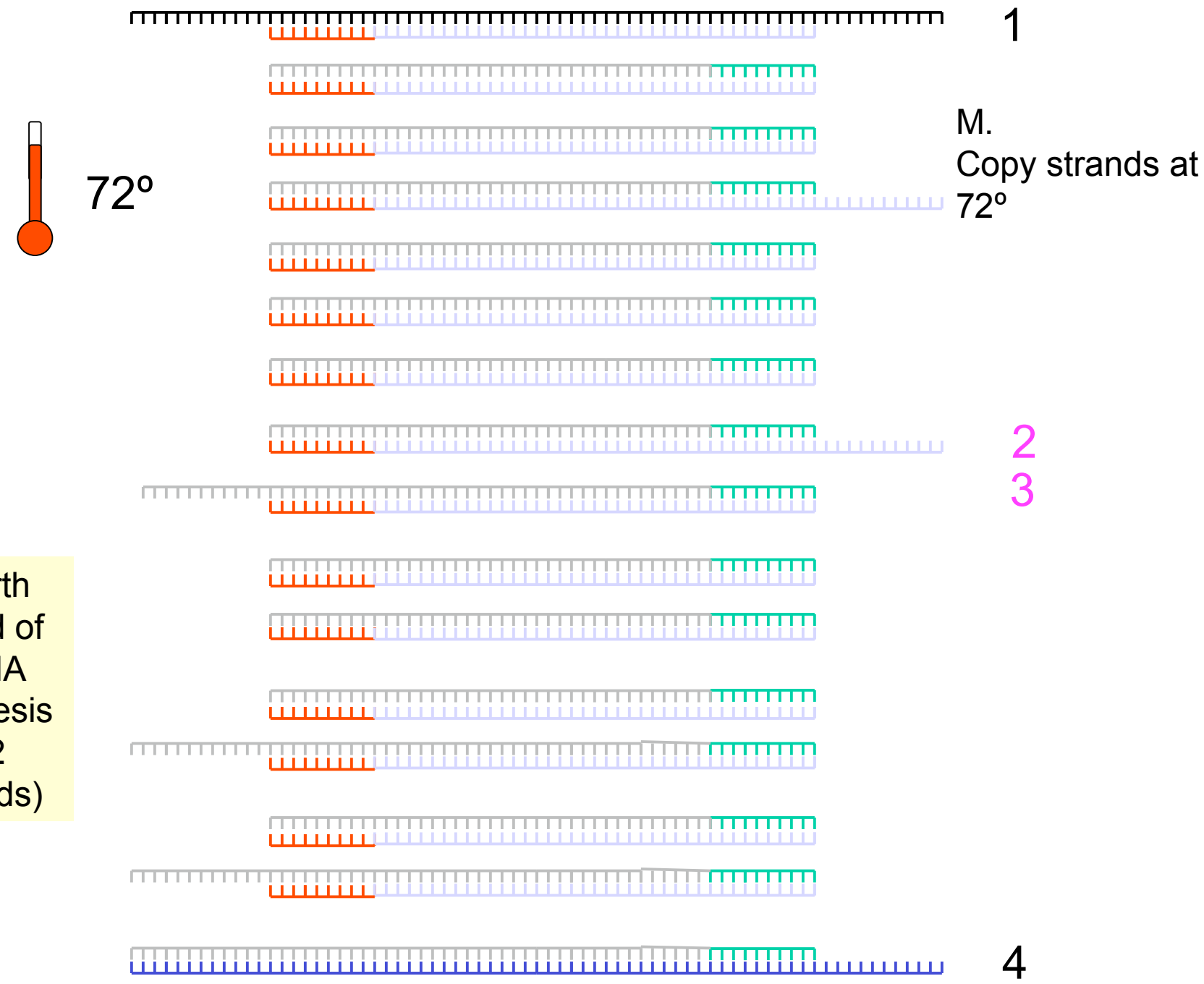

1
Taq
72º
Taq
2
I. Copy strands
3
Taq
Second round of cDNA synthesis (8 strands)
Taq
4


1
J.
Denature at 96º
Anneal primers
at 50º
2
3
4



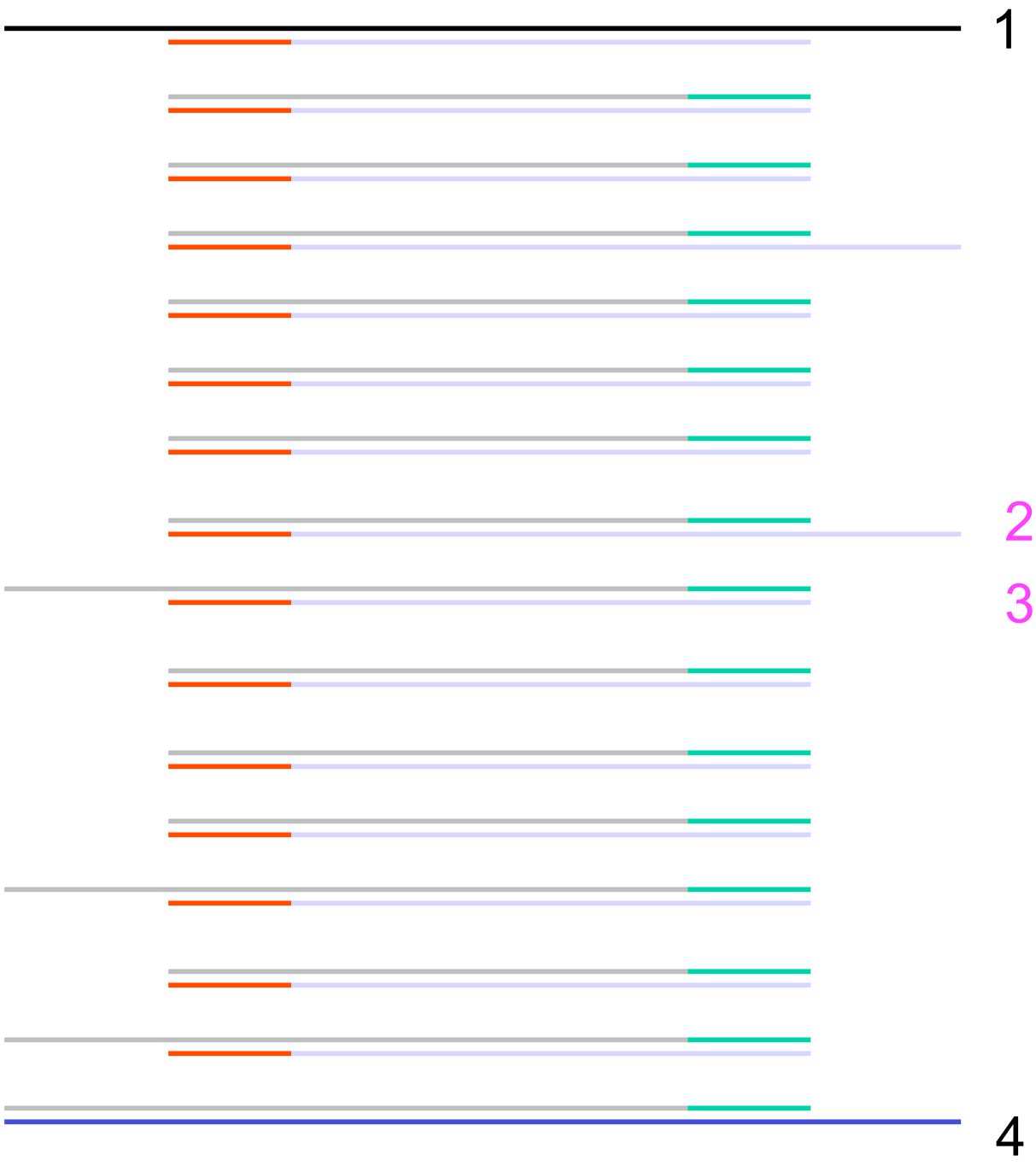
Third round of cDNA synthesis (16 strands)


1
L.
Denature at 96º
Anneal primers
at 50º
2
3
4

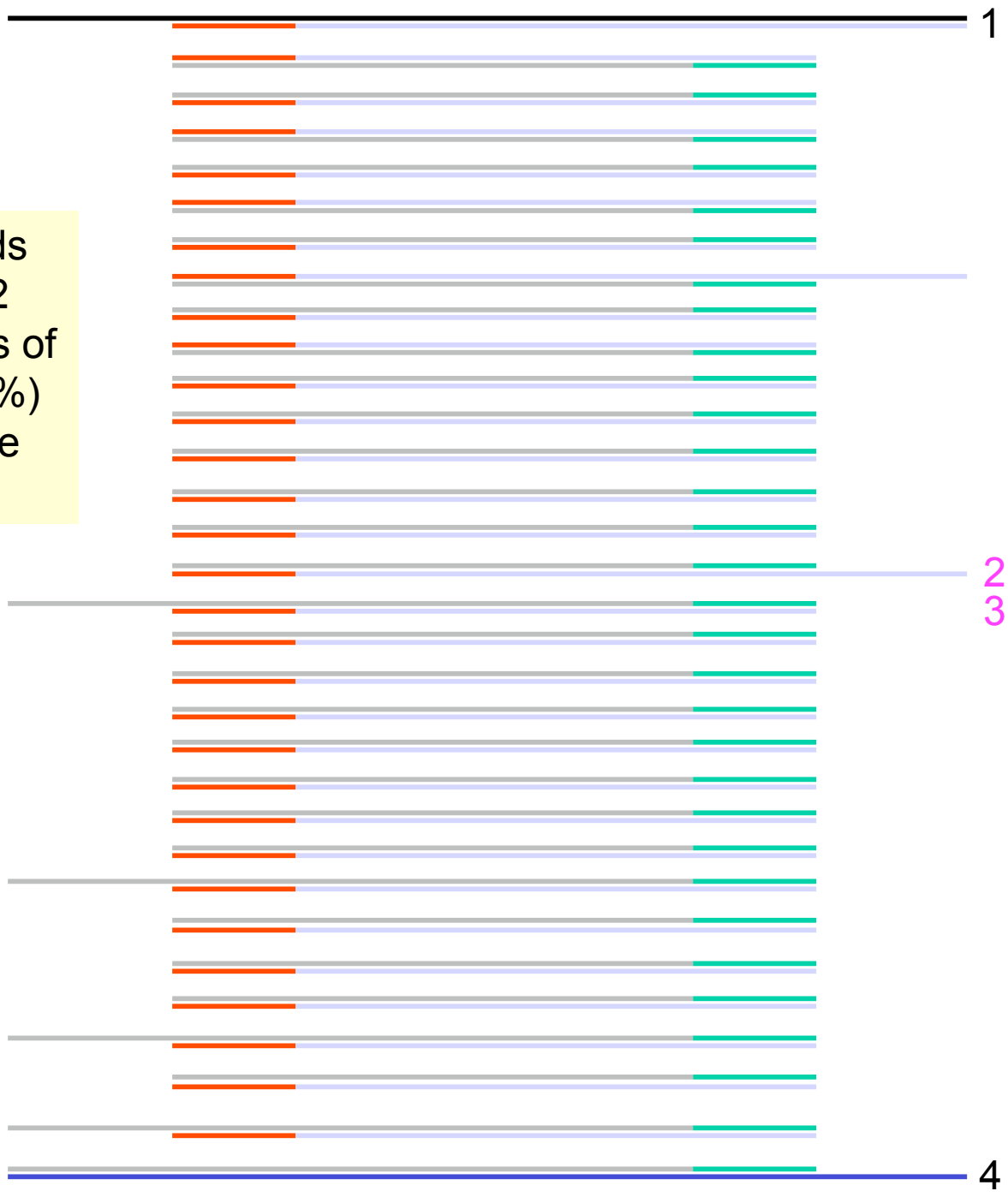


Fourth round of cDNA synthesis (32 strands)

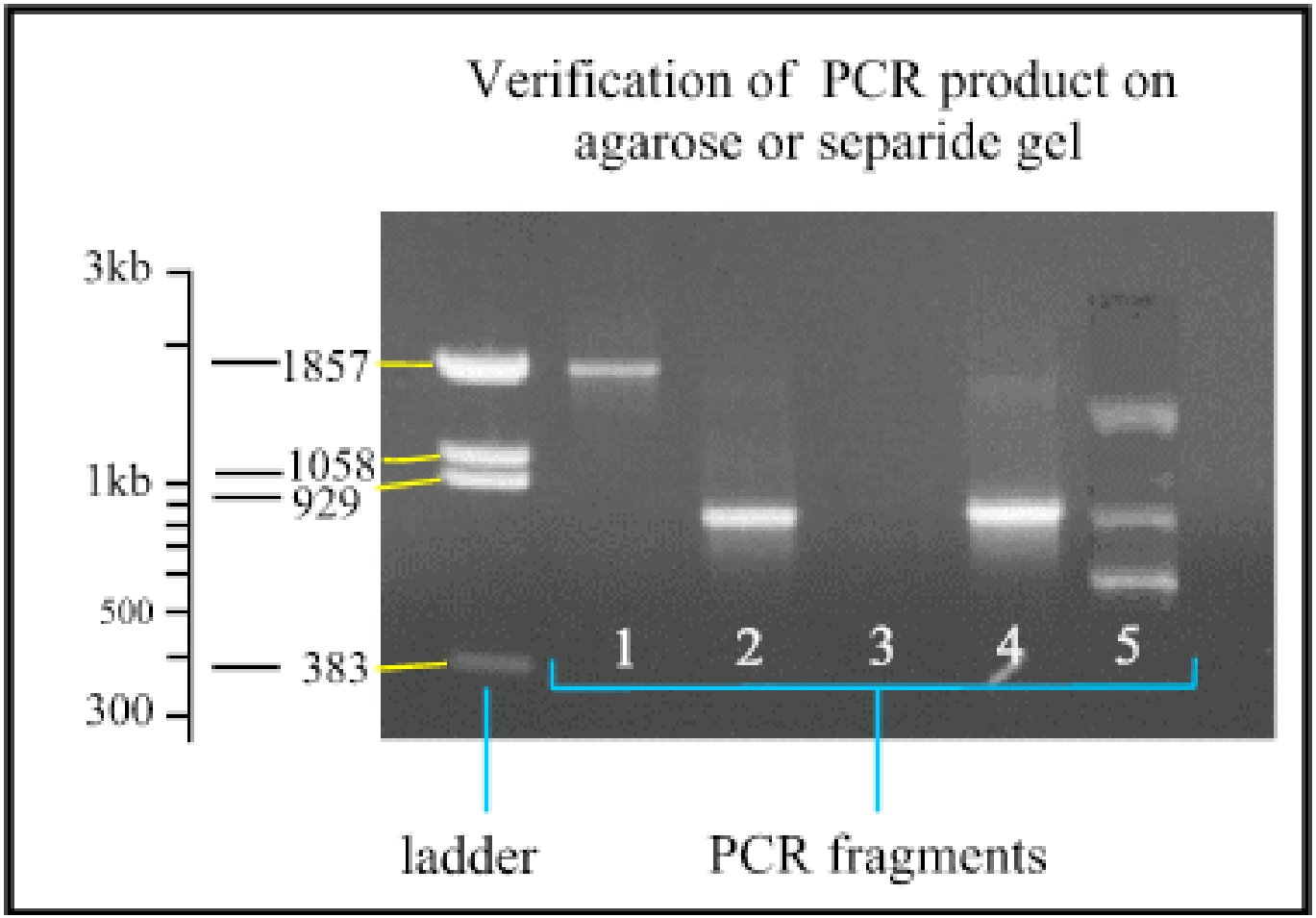
cDNA strands (32) are now shown as lines



After 5 rounds there are 32 double strands of which 24 (75%) are are same size



To see PCR Product



Basic Polymerase Chain Reaction (I)

- A thermostable DNA polymerase to catalyze template-dependent synthesis
- A pair of synthetic oligonucleotides to prime DNA synthesis
- Deoxynucleoside triphosphates (dNTPs)
- Divalent cations
- Buffer to maintain pH
- Monovalent cations
- Template DNA

- Template DNA (10^5 to 10^6 molecules)
- 20 pmol of each primer
- 20 mM Tris-HCl (pH 8.3 at 20°C)
- 1.5 mM MgCl2
- 25 mM KCl
- 0.05% Tween 20
- 100 μg/ml autoclaved gelatin or nuclease free BSA
- 50 μM dNTP each
- 2 units of Taq DNA polymerase
- Total volume 100 μl

Basic Polymerase Chain Reaction (II)

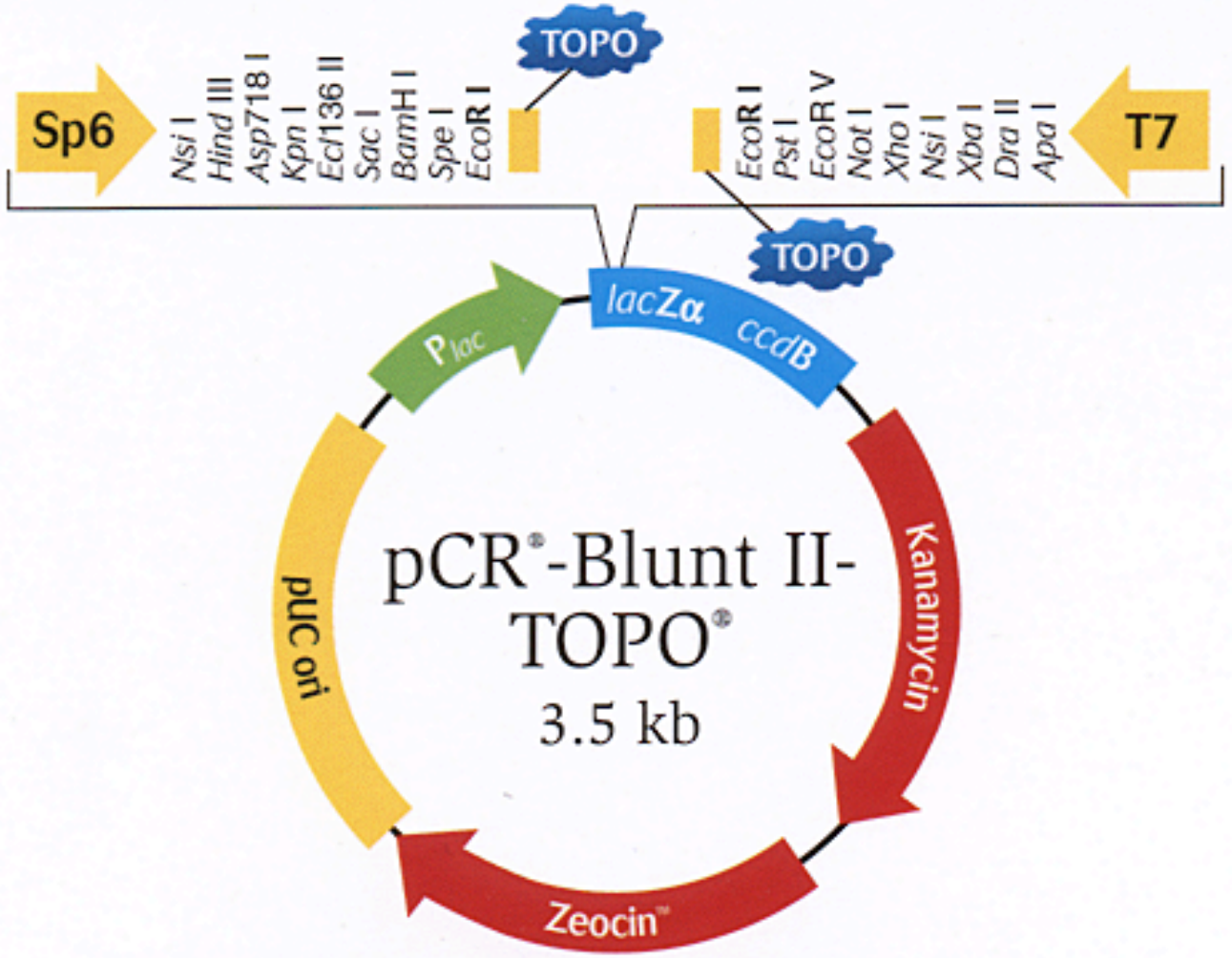
- (92-98 ˚C) Denaturation 96 ˚C for 15 sec
- (37-70 ˚C) Primer annealing 55 ˚C for 30 sec
- (70-74 ˚C) Primer extension 72 ˚C for 90 sec
- Repeat for 20 to 30 cycles
- (70-74 ˚C) Final extension 72 ˚C for 5 min
- Stop the reaction: Chill at 4 ˚C or adding EDTA to 10 mM
- PCR products gel purified for further work

Primer Design

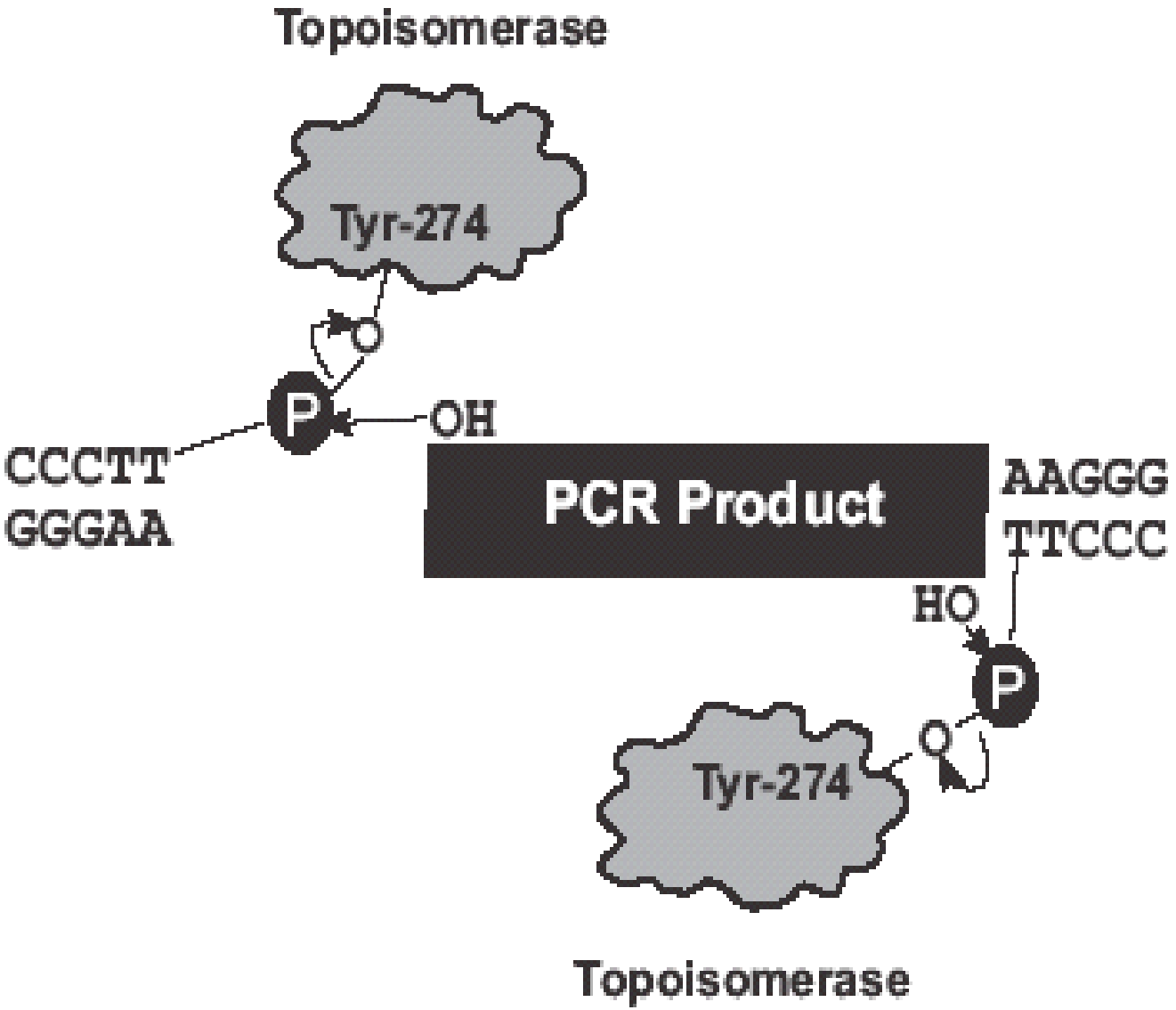
- Length: 18 to 28 nt
- GC content: 50 to 60%
- Tm: 55 to 80 ˚C
- Others: avoid 3'-end complementarity

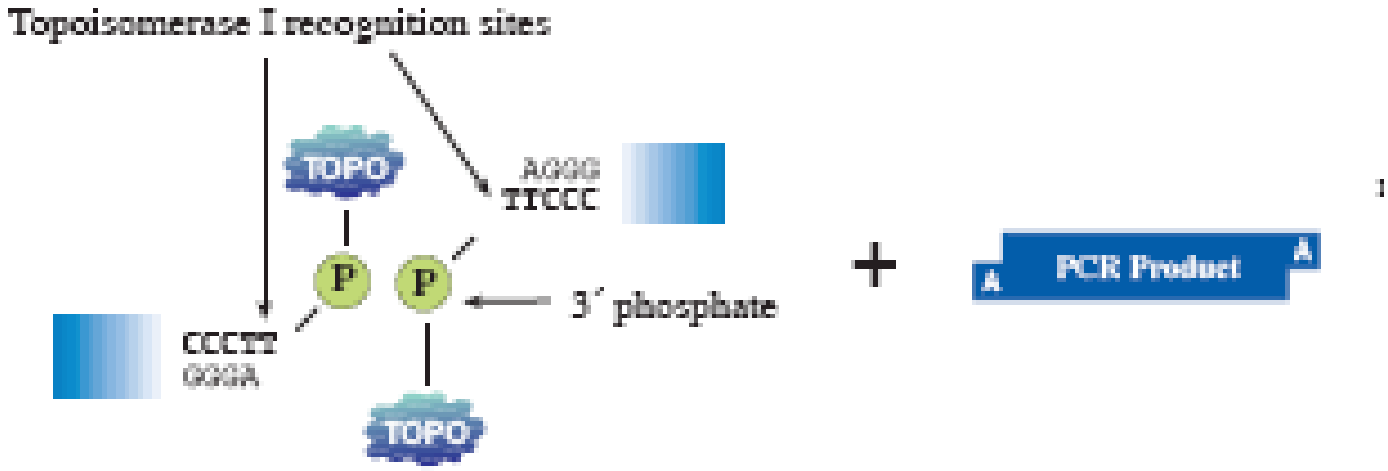
$$\text{Anneal Temperature} = 2 \times (A + T) + 4 \times (G + C)$$

Zero Blunt™ TOPO® Vector Map



TOPO cloning of PCR product



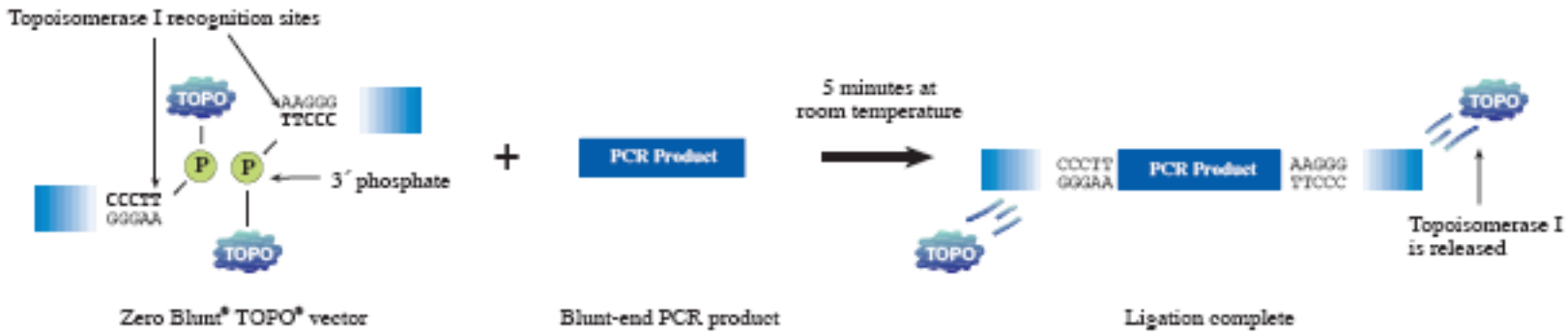


TOPO TA Cloning® vector

Taq-amplified PCR product

Ligation complete

Figure 2 - Zero Blunt® TOPO® Cloning of blunt-end DNA



DNA polymerase fidelity measured with the rpsL assay

Enzyme	Mutation Frequency (%)	Template Doubling (for 25 cycles)	Error Rate (× 10^{-6})	Relative Fidelity
Taq DNA Polymerase	4.00 ± 0.09	11	42 ± 19	1
PLATINUM *Taq* DNA Polymerase	3.4 ± 0.5	10	38 ± 5	1.1
Pfu DNA Polymerase	0.3 ± 0.1	10	3 ± 1	14
PfuTurbo DNA Polymerase	0.27 ± 0.07	11	2.4 ± 0.4	17
PLATINUM Pfx DNA Polymerase	0.14 ± 0.04	12	1.6 ± 0.5	26

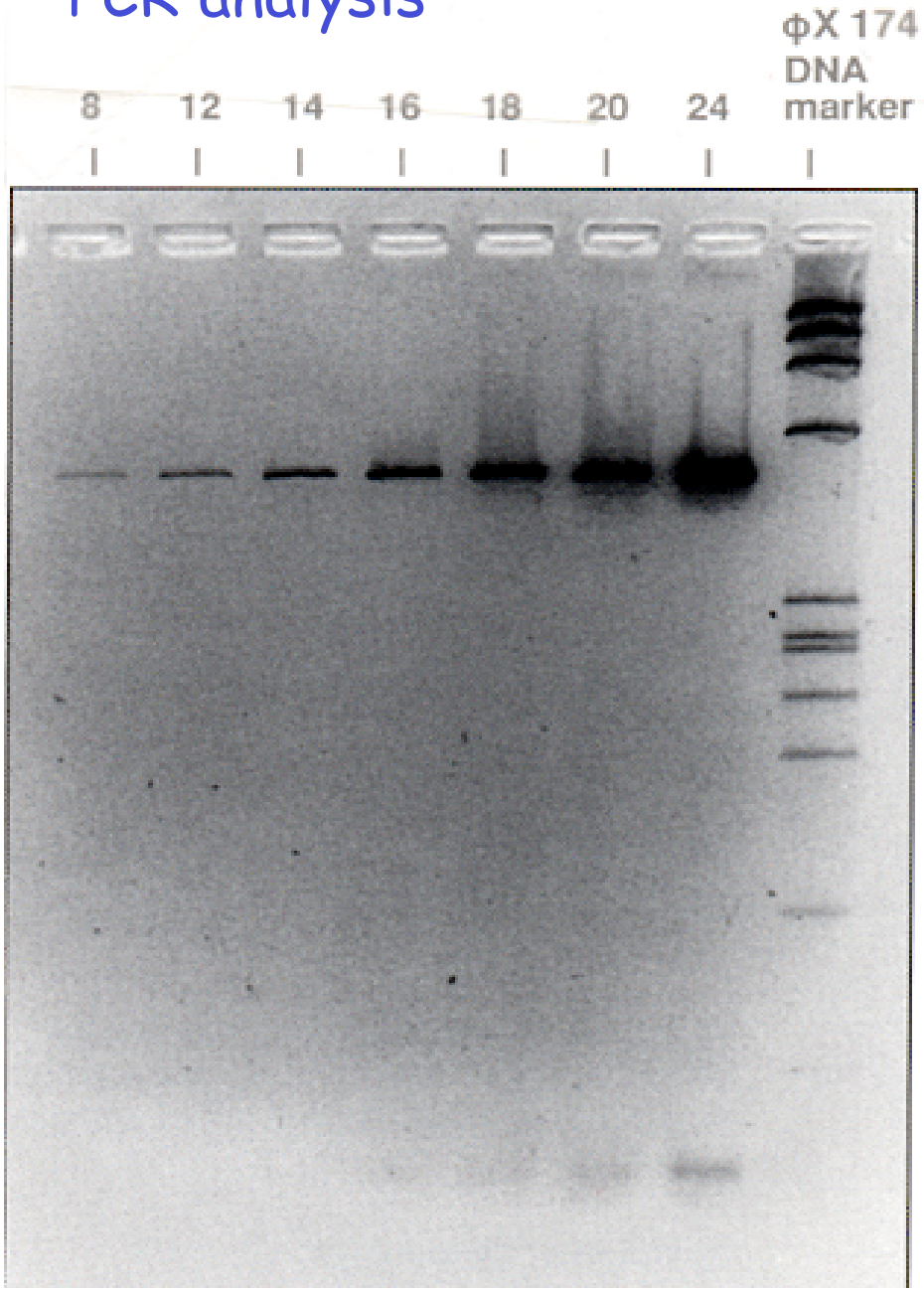
Results are the mean ± SD for 5 independent rpsL assays (2–25 cycles and 3–15 cycles). The number of mutant colonies and total colonies was Taq DNA polymerase (1,892/56,455), PLATINUM® Taq DNA Polymerase (2,043/57,695), Pfu DNA polymerase (1,033/395,469), PfuTurbo® DNA polymerase (1,068/395,469), and PLATINUM Pfx DNA Polymerase (960/584,230).

DNA polymerase fidelity measured with the *lac*Z assay

Enzyme	Mutation Frequency (%)	Template Doubling (for 25 cycles)	Error Rate (× 10^{-6})	Relative Fidelity
Taq DNA Polymerase	3.1 ± 0.3	14	19 ± 3	1
Pfu DNA Polymerase	0.18 ± 0.03	11	1.1 ± 0.4	15
PLATINUM Pfx DNA Polymerase	0.09 ± 0.01	11	0.67 ± 0.06	29

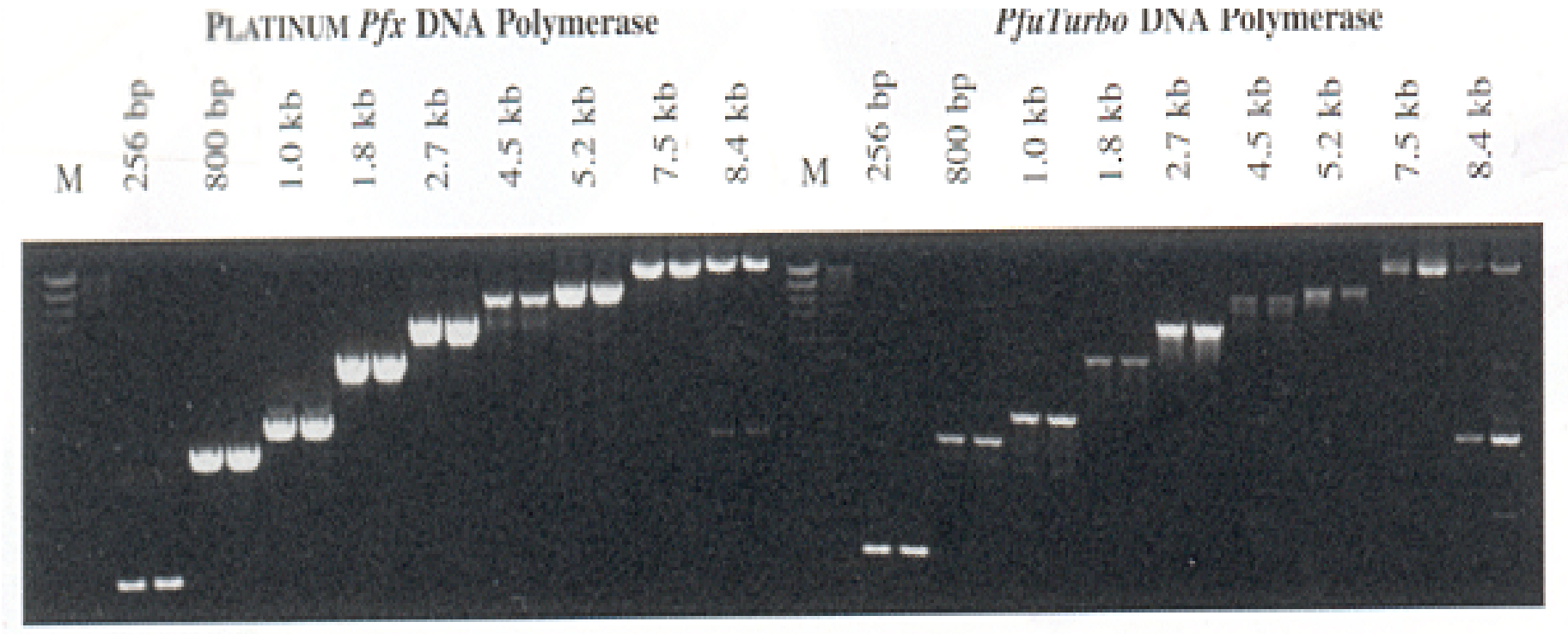
Results are the mean ± SD for 2 independent lacZ assays (1–25 cycles and 1–15 cycles). The number of mutant colonies and total colonies was Taq DNA polymerase (2,975/93,668), Pfu DNA polymerase (185/109,289), and PLATINUM® Pfx DNA Polymerase (96/107,665). All mutant colonies were repatched and grown overnight at 37°C for secondary true mutant confirmation.

PCR analysis



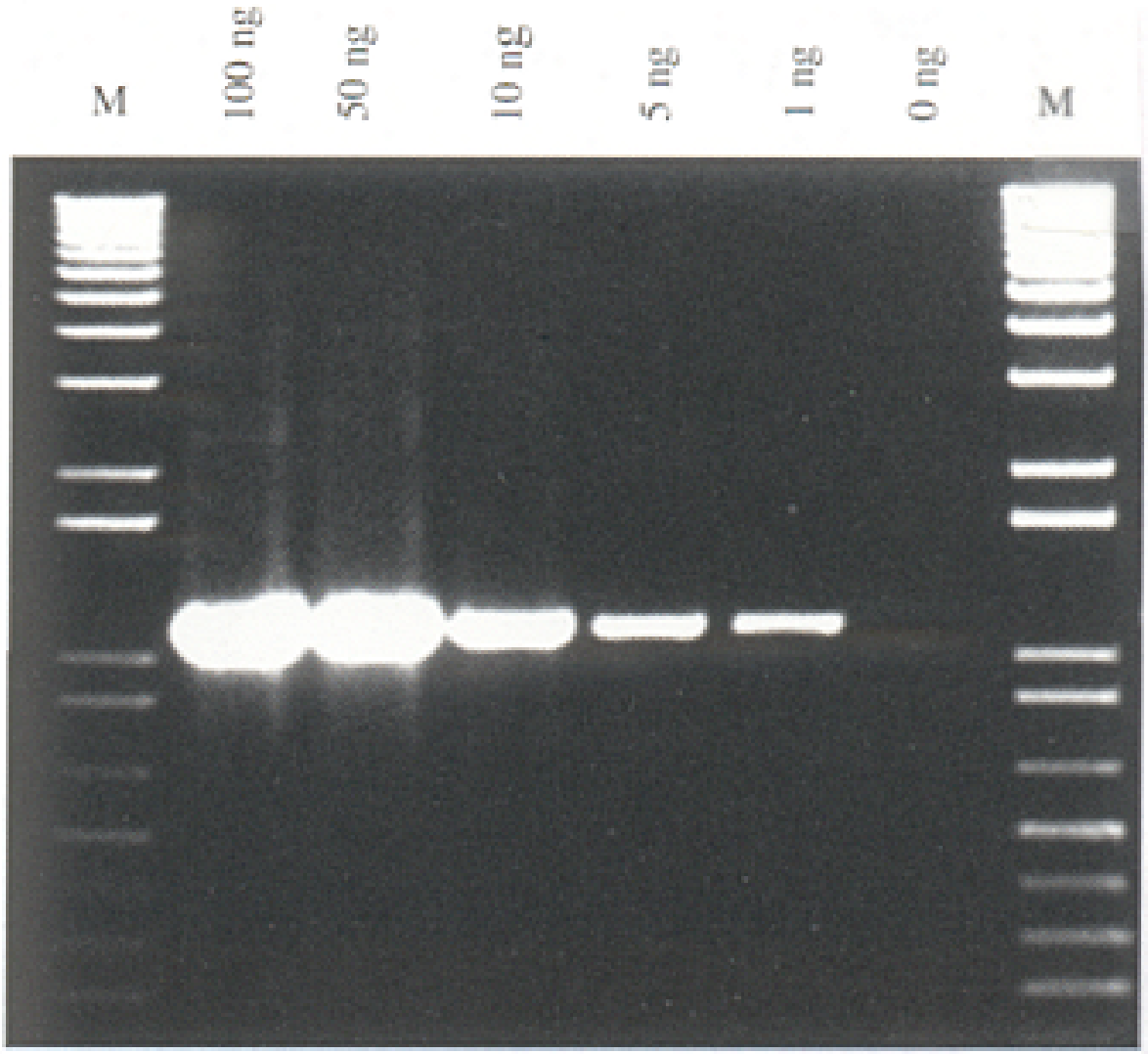
Aliquots of of PCR sample (10 μl) at indicated cycle numbers were withdrawn and run on 3% agarose gel in the presence of ethidium bromide

Large range PCR



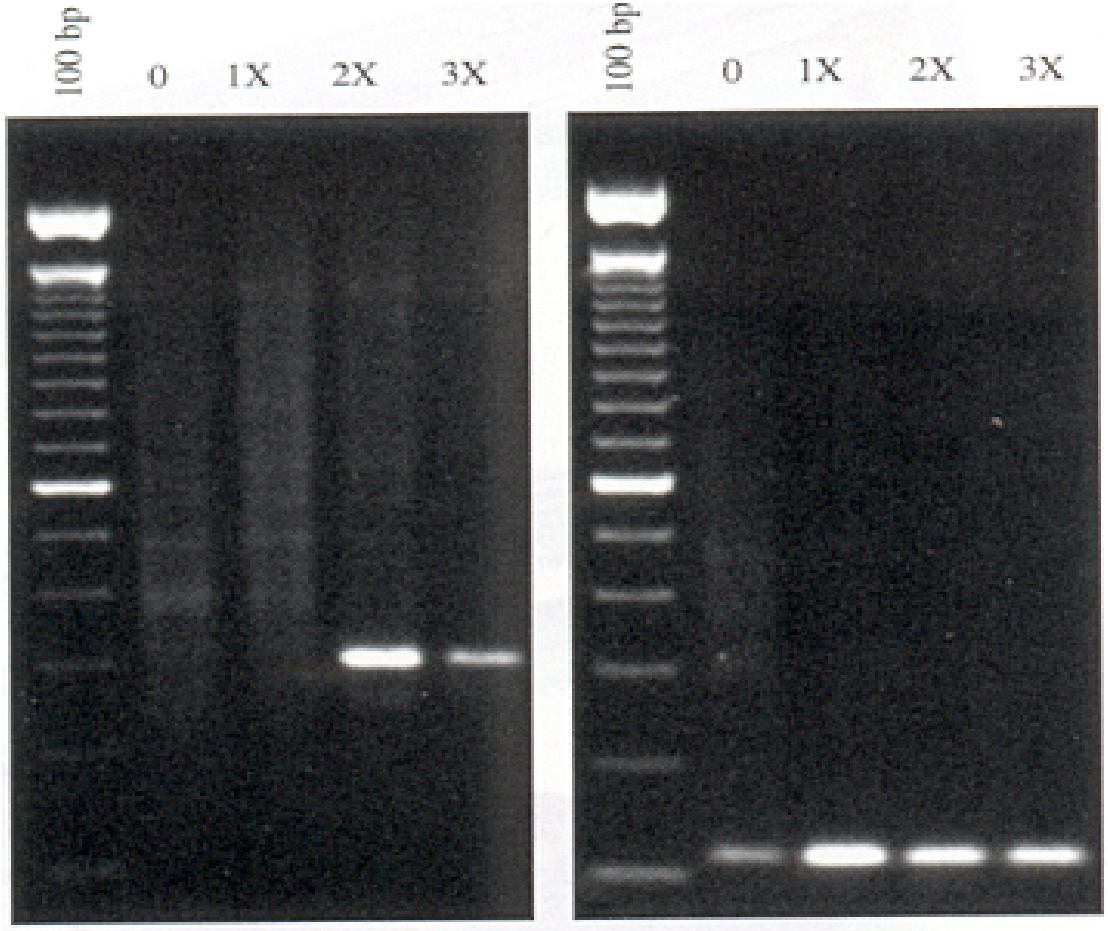
200 ng K562 genomic DNA amplified with 1.25 u PLATINUM Pfx DNA pol (assembled at rt) or 2.5 u PfuTurbo DNA pol (assembled on ice). Targets are beta-globin (256-bp), myosin heavy chain (1, 1.8, 5.2, 8.4 kb), myelin oligodendrocyte glycoprotein (2.7, 4.5, 7.5-kb), and thrombospondin fragments (802-bp).

High sensitivity



Genomic DNA, 0-100 ng, with 0.3 μM each primer (thrombospondin), 0.3 mM dNTPs, 1X Pfx buffer, 1 mM MgSO4, 1.25 u Pfx, 35 cycles

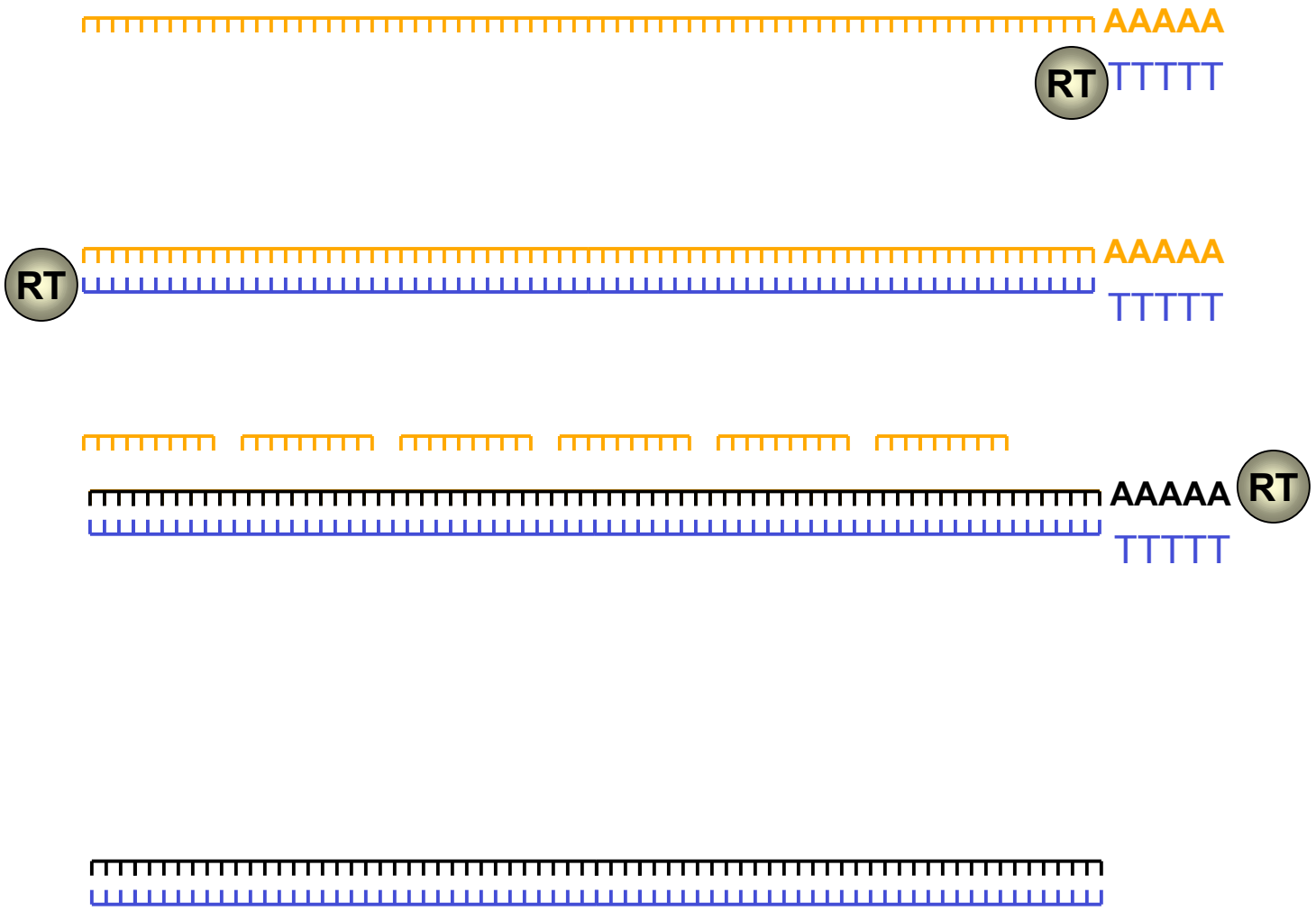
PCRx Enhancer Solution



Left: 316-bp, 77% GC-rich Fragile X chromosome, many tri-nt repeats. Right: 116-bp, 65% GC-rich AF064848 locus with GCC expansion region.
5 μl rx with 100 ng K562 DNA, 1 u Pfx and 0-3X PCRx Enhancer solution, 100-bp ladder

RT-PCR

- Reverse transcriptase-PCR is to amplify cDNA copies of RNA
- It is used to retrieve and clone the 5' and 3' termini of mRNA
- It is used to generate large cDNA libraries from very small amounts of mRNA
- It can be adapted to identify mutations and polymorphisms
- It can be used to measure the strength of gene expression



Conversion of mRNA to cDNA by Reverse Transcription

RT-PCR

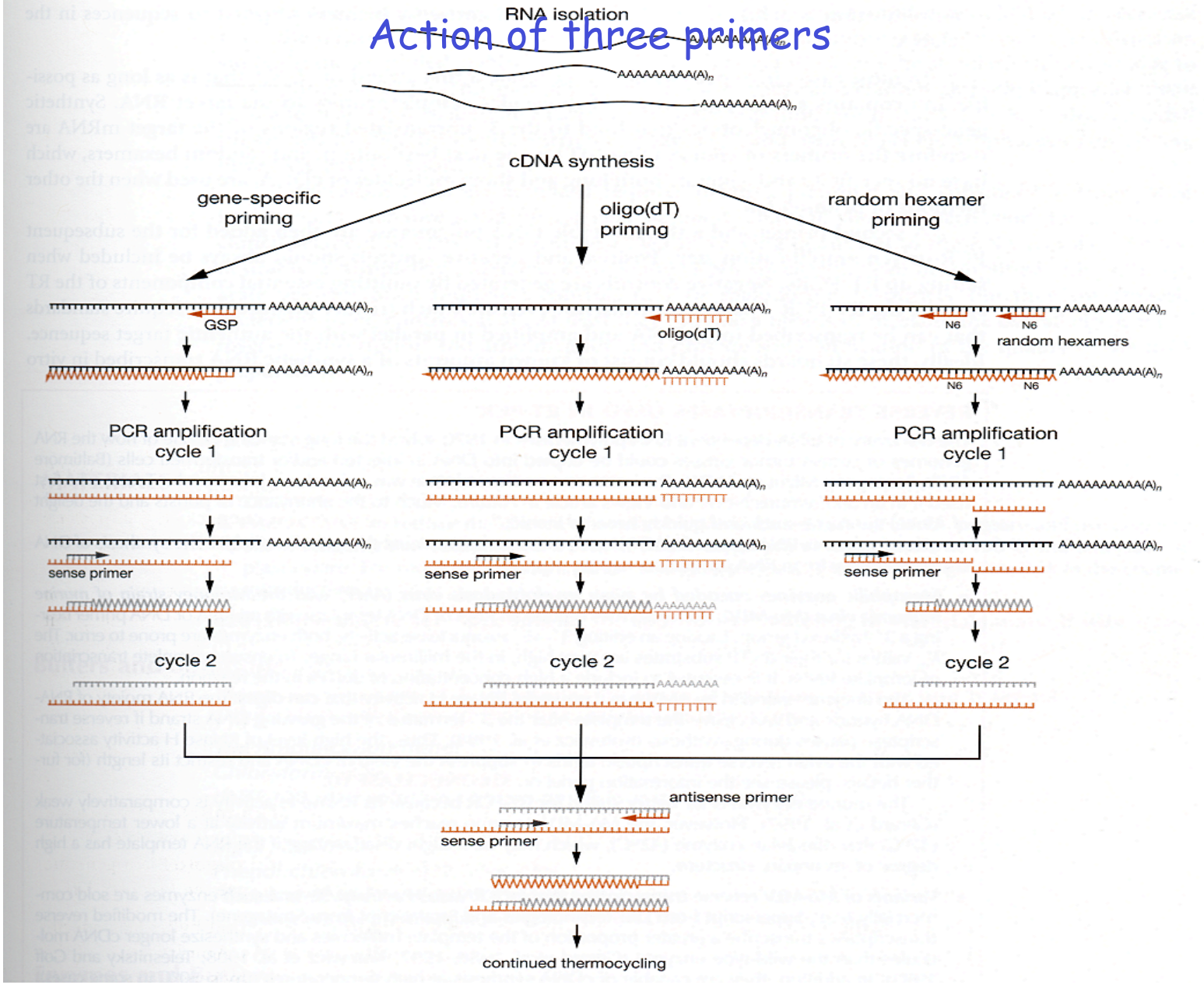
Reverse Transcriptase

- 1. Mesophilic enzymes encoded by avian myeloblastosis virus (AMV) or Moloney strain of murine leukemia virus (Mo-MLV).
- 2. Variants of Mo-MLV reverse transcriptase that lacks RNase H activity.
- 3, Thermostable Tth DNA polymerase (exhibit reverse transcriptase in the presence of Mn2+)

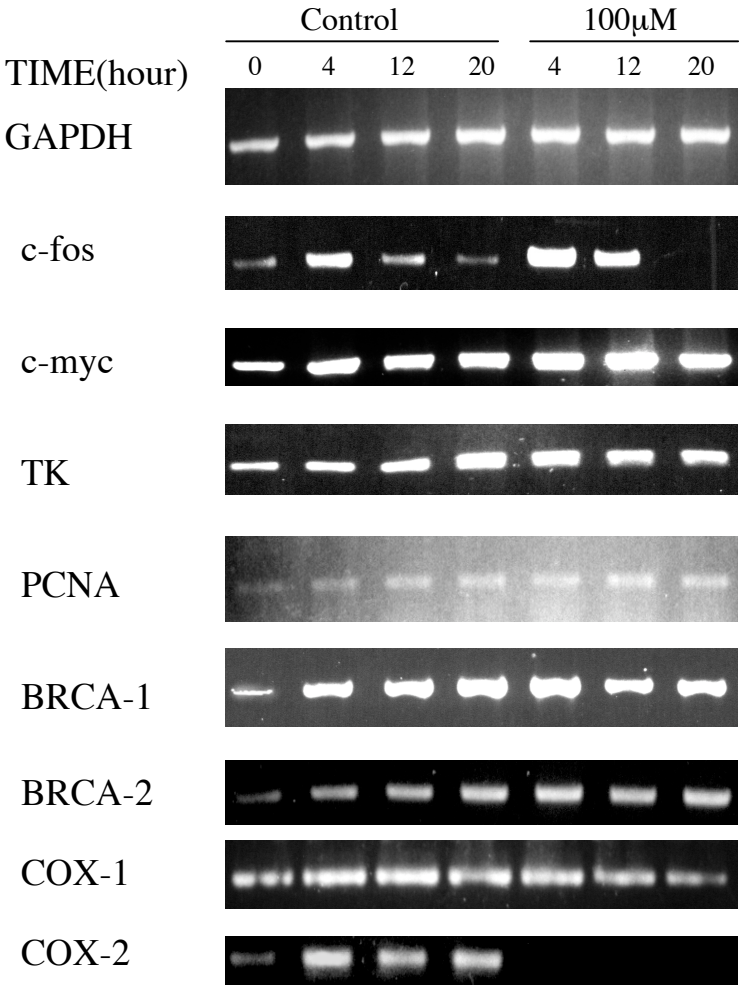
Three ways to amplify cDNA

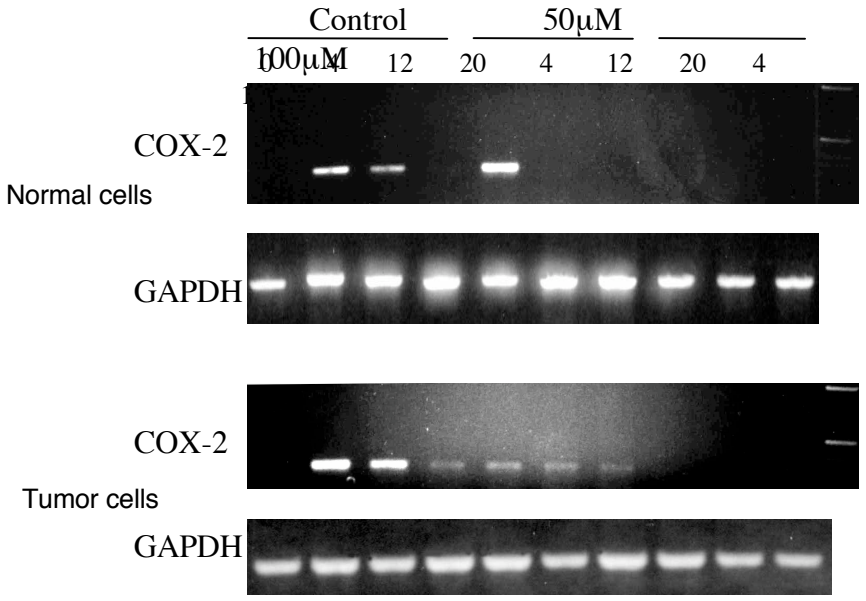
- Gene specific priming
- Oligo(dT) priming
- Random hexamer priming

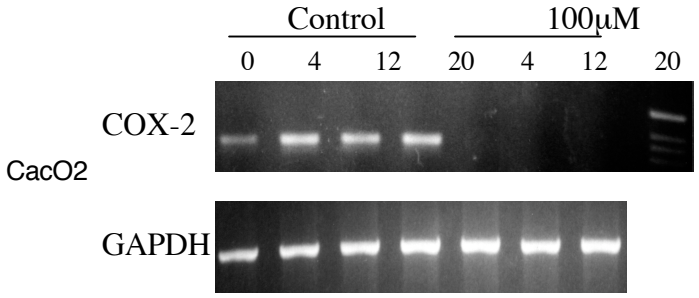
Action of three primers



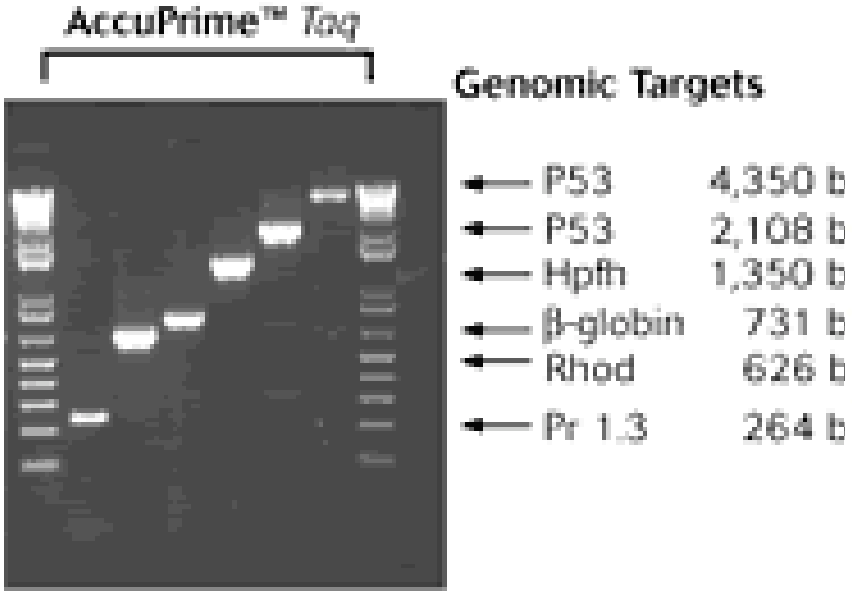
RT-PCR for gene expression

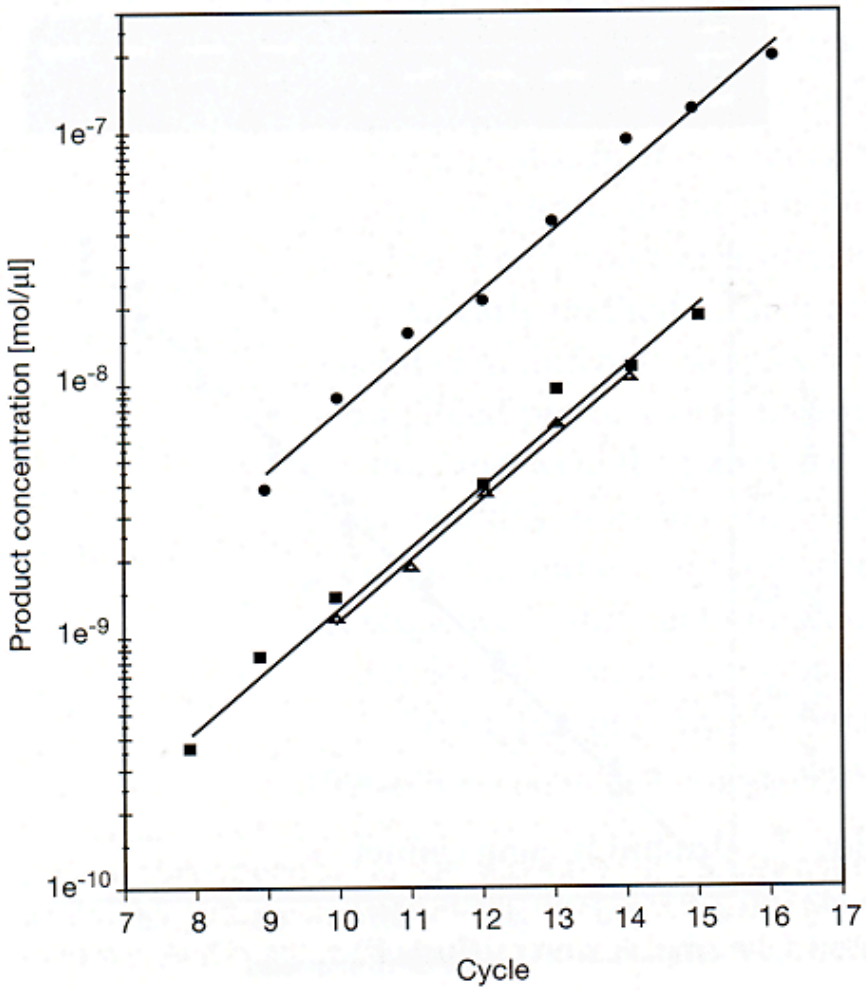






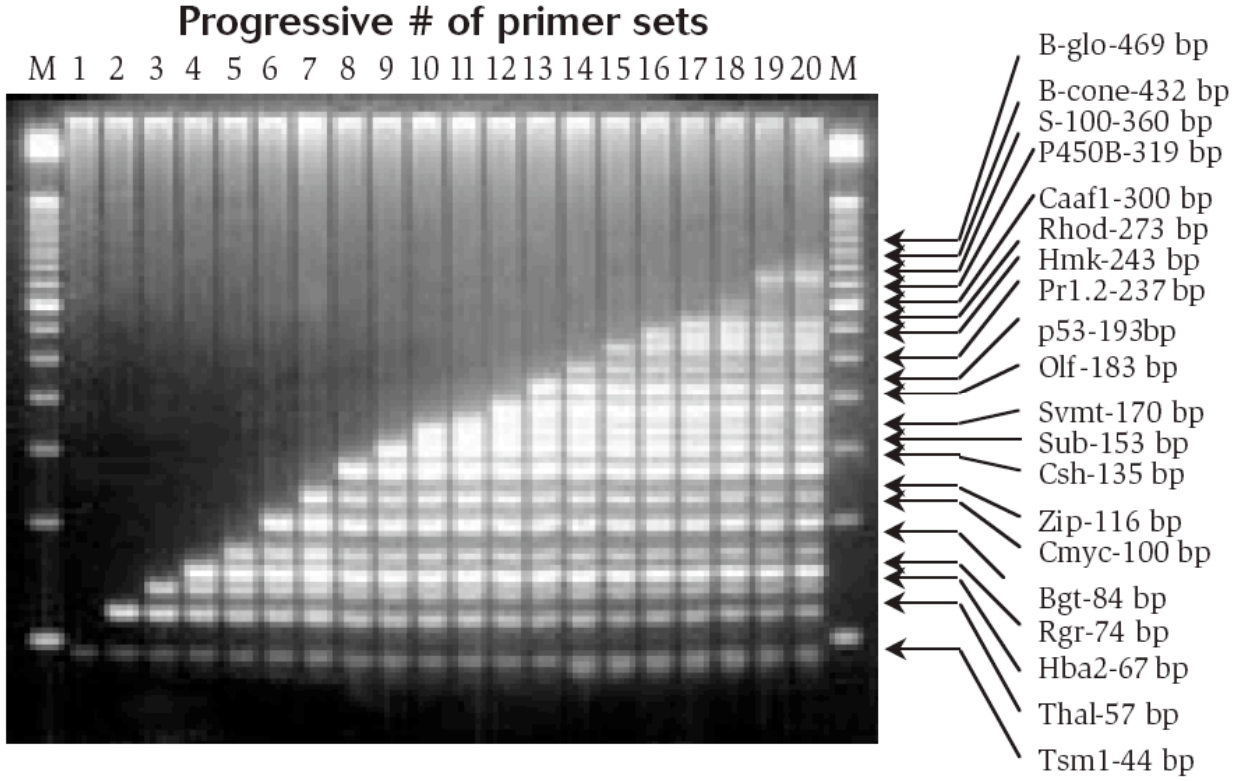
Multiplex PCR





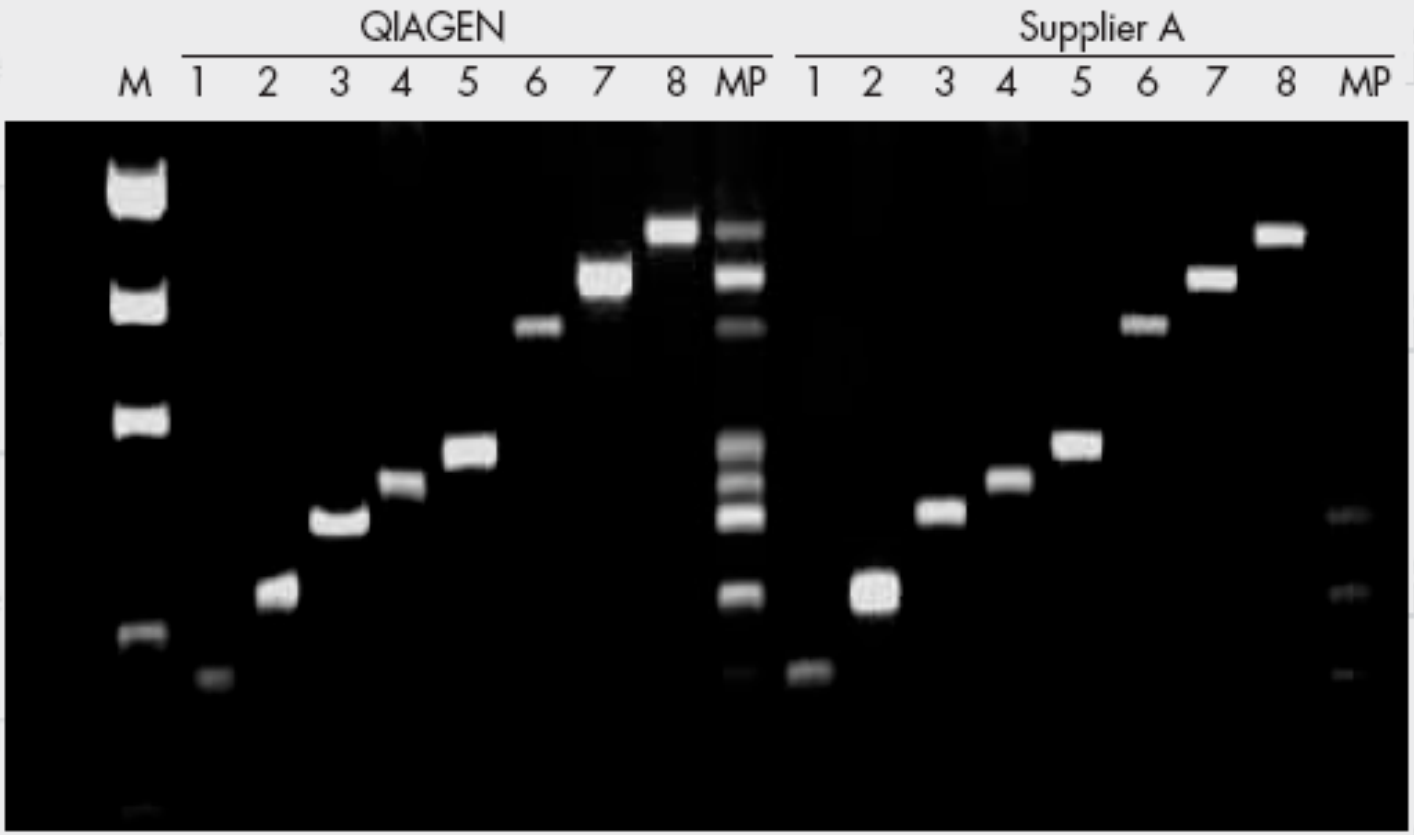
Multiplex PCR

Resolution of up to 20 specific PCR products oducts from a single-tube multiplex reaction



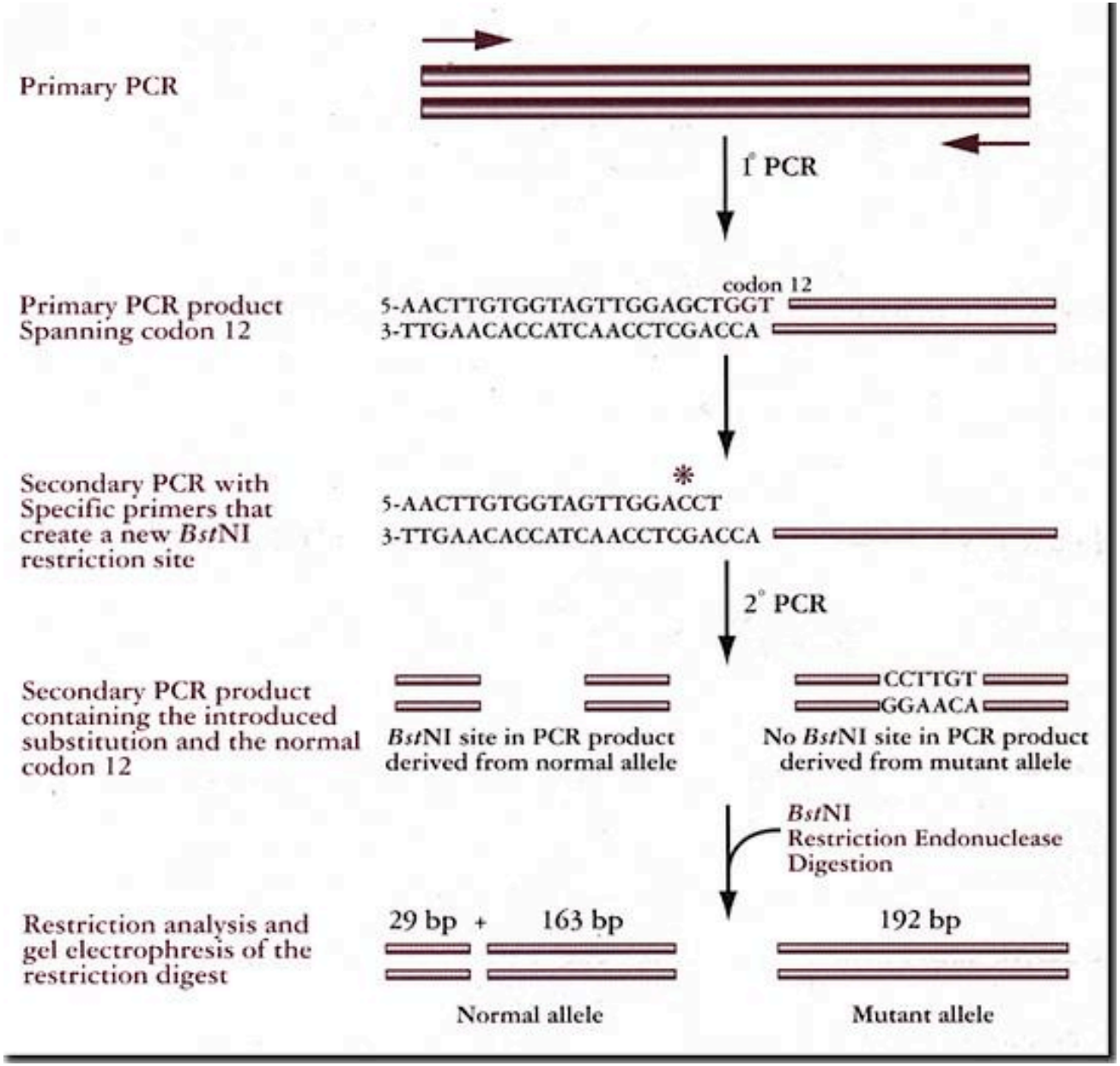
Each lane from left to right represents the progressive number of primer sets (1-20) included in a single-tube, 50 µl PCR reaction. PCR reactions were assembled on ice, using 200 ng K562 human genomic DNA, and five units AccuPrime™ *Taq* DNA Polymerase, and amplified for 35 cycles (94°C for 15 seconds, 60°C for 30 seconds, 68°C for one minute).

Multiplex PCR



*Comparison of exon-specific amplification reactions and multiplex PCR of the human HPRT gene using QIAGEN Taq DNA Polymerase and PCR Buffer (**QIAGEN**) or Taq DNA polymerase and PCR buffer from another supplier (**Supplier A**). **M**: markers; **1–8**: PCR with individual primer pairs; **MP**: multiplex PCR with all eight primer pairs. Individual PCR products correspond to **1**: exon 4 (334 bp); **2**: exon 6 (441 bp); **3**: exon 2 (572 bp); **4**: exon 1 (627 bp); **5**: exon 5 (708 bp); **6**: exon 3 (1059 bp); **7**: exon 9 (1278 bp); and **8**: a region spanning exons 7 and 8 (1533 bp).*

PCR-based restriction fragment length polymorphism (PCR-RFLP)



PCR-RFLP

PCR has greatly improved the sensitivity of detecting mutations in genomic DNA.

The general scheme of PCR-based restriction fragment length polymorphism (PCR-RFLP) includes amplification of DNA containing the mutated sequence using flanking primers, followed by enzyme restriction of the PCR product.

k-*ras* codon 12 mutations have been detected in gastric carcinomas by simple gel electrophoresis of the PCR product cleaved with the restriction endonuclease *Hpa* II.

Because any substitution of the first two nucleotides of codon 12 abolishes the restriction site for this enzyme, undigested product revealed by simple gel electrophoresis indicates a mutation.

As few as 10 copies of mutated k-*ras* gene in 10^9 wild-type sequences have been detected using such mutant-enhancement PCR-RFLP technology.

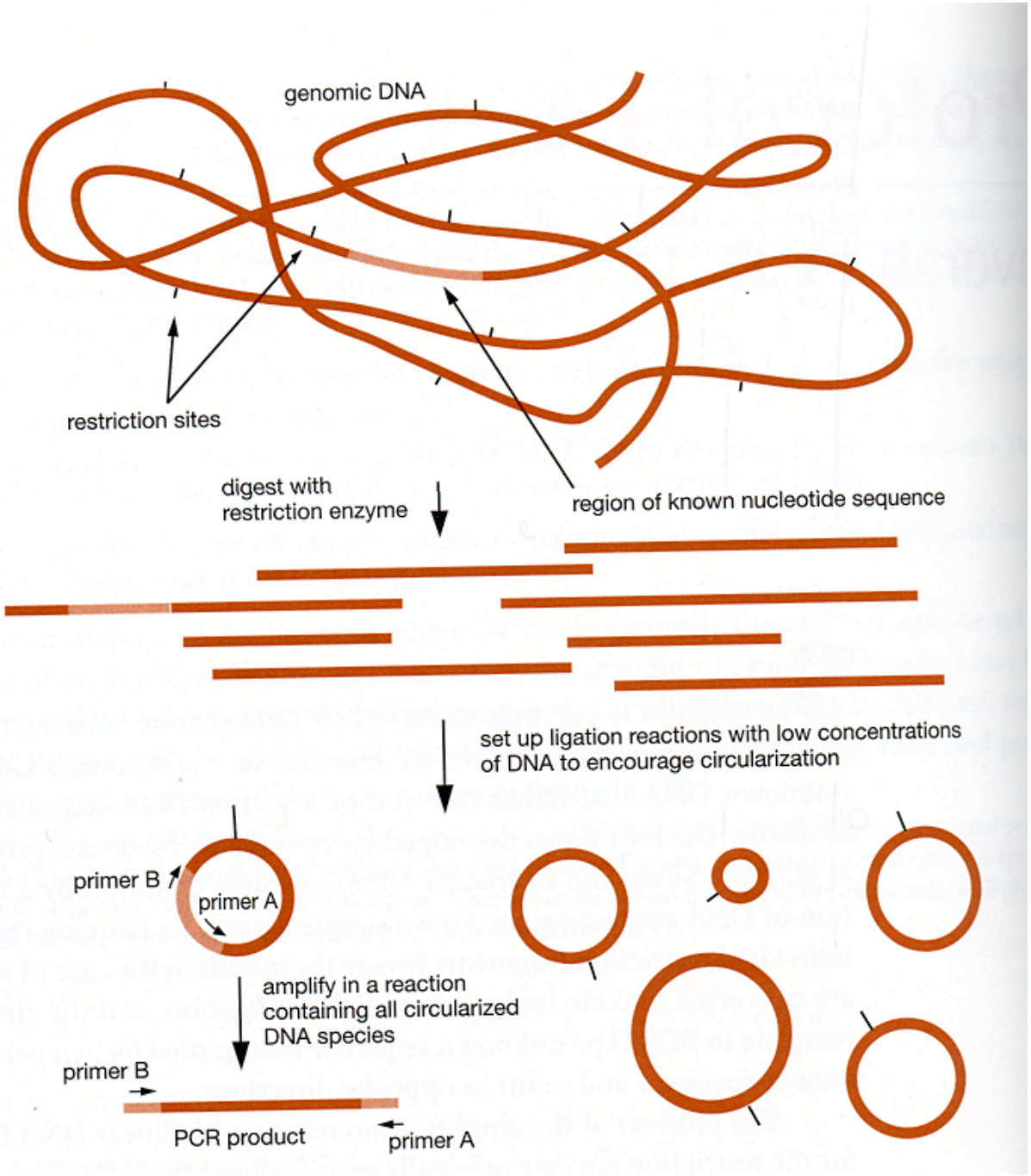
This way of transforming any alternation of DNA sequence into an allele-specific enzyme recognition site obviates the use of radioisotopic hybridization, and has been used successfully to detect multiple mutations in the cystic fibrosis gene as well as *ras* oncogenes in gastrointestinal cancers.

In addition, primer-mediated restriction polymorphism has enhanced by 20% the sensitivity of detection of codon 12 k-*ras* mutations in colorectal cancers, as compared to allele-specific oligonucleotide hybridization technique.

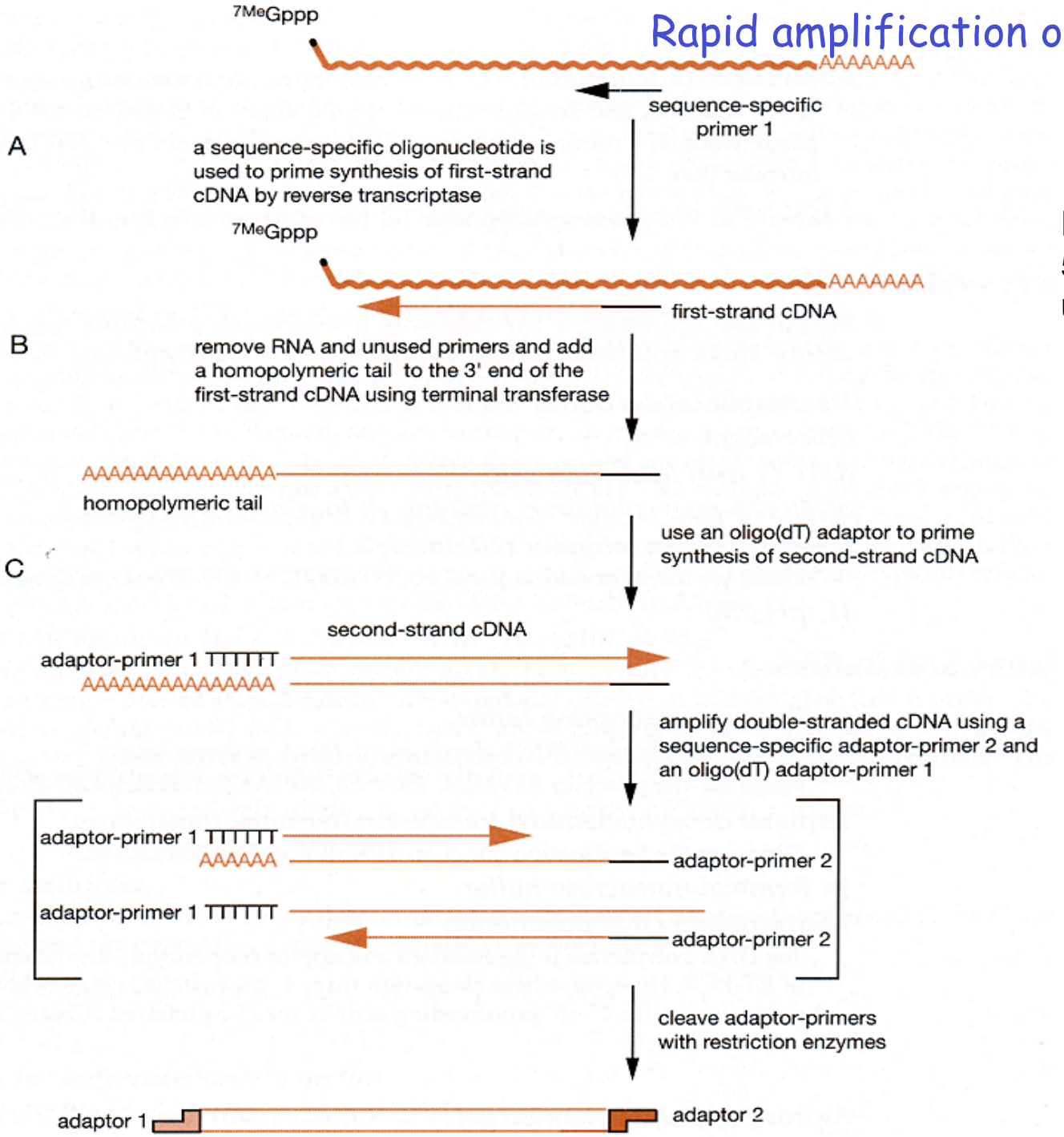
Inverse PCR

- Inverse PCR is used to amplify and clone unknown DNA that flanks one end of a known DNA sequence and for which no primers are available
- It is frequently used in chromosome walking

Inverse PCR is used to clone sequences flanking a known sequence. Flanking sequences are digested and ligated to make a circular DNA. PCR primers pointing away from the known sequences are used to amplify the flanking sequences. For Inverse PCR primer design, please visit Primo Inverse.

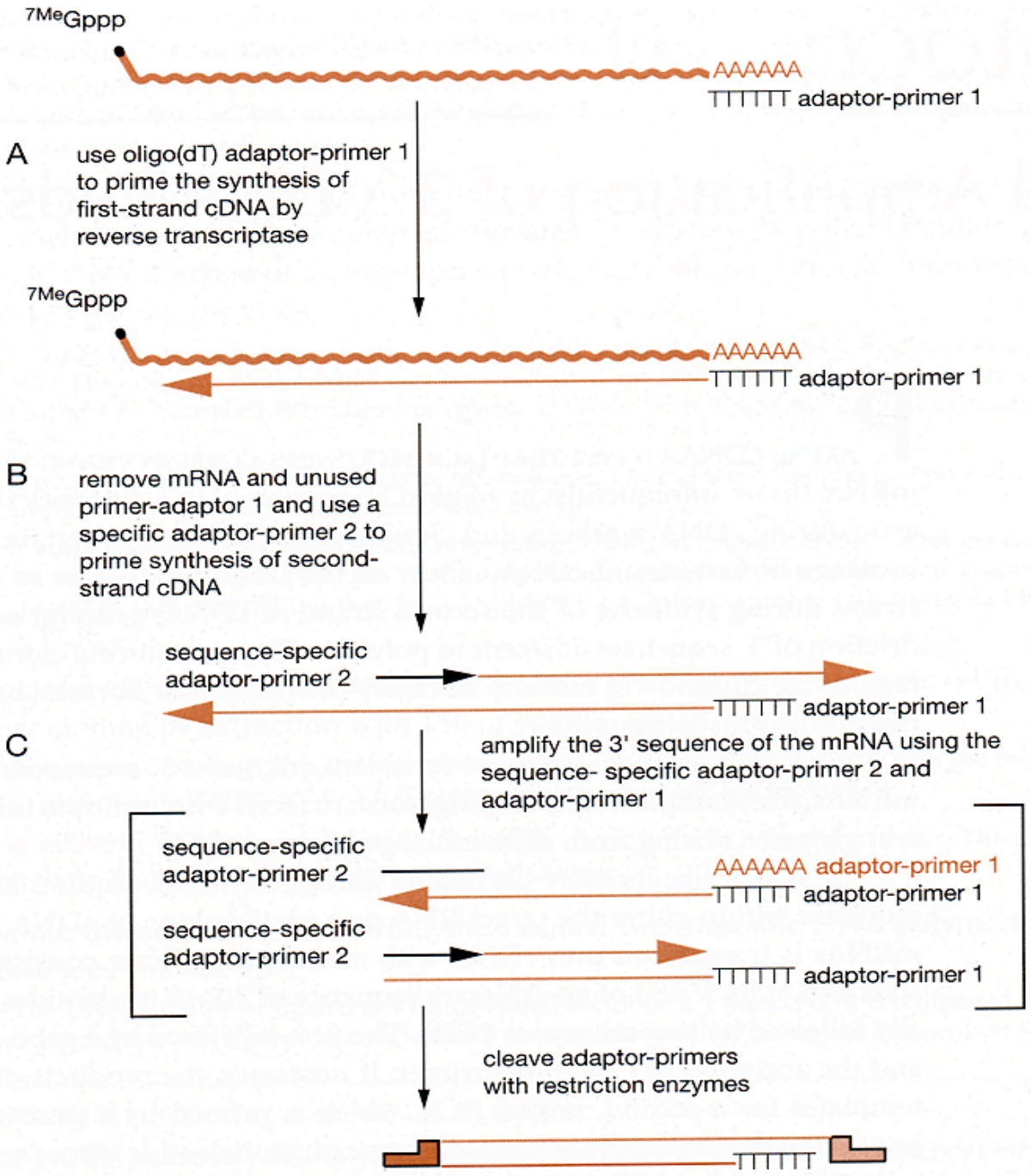


Rapid amplification of cDNA ends



Determination of the 5'-end of mRNA using 5'-RACE

3'-RACE



3'-RACE: The technique is used to isolate unknown 3' sequences and to map the 3' termini of mRNAs

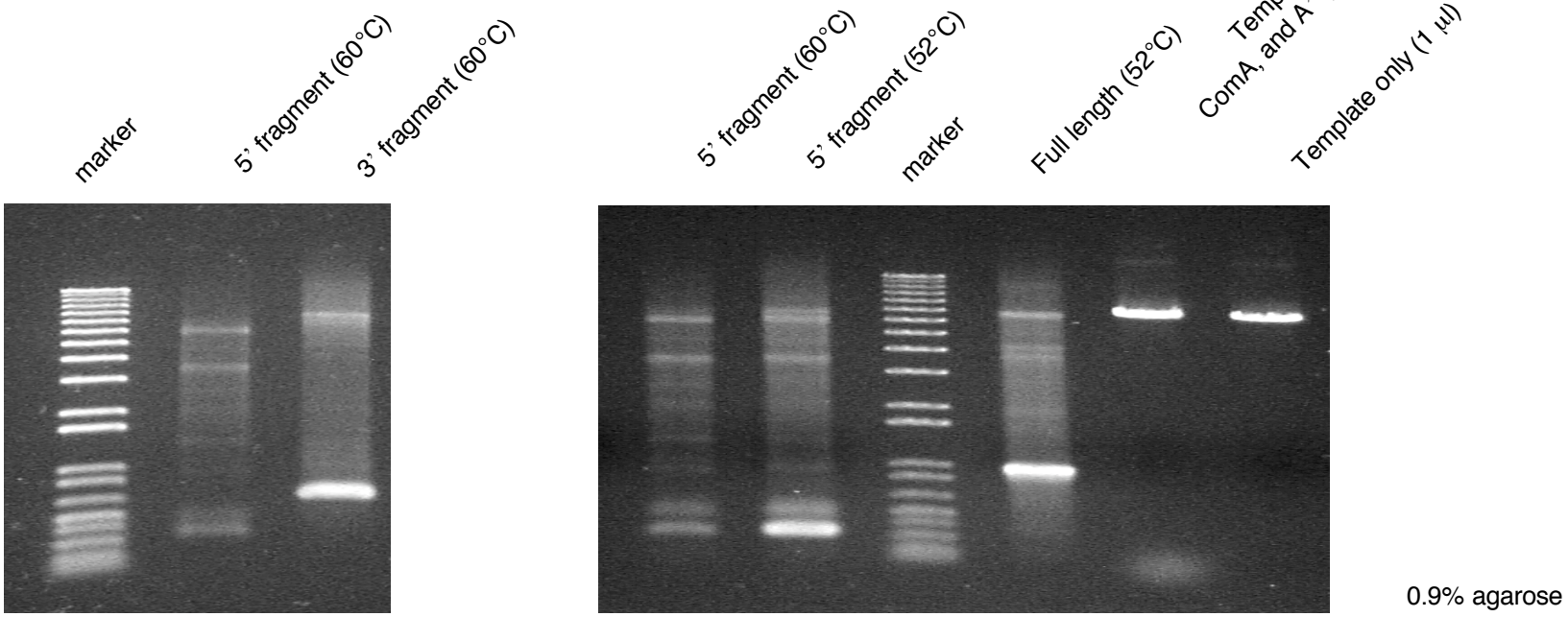
The technique requires knowledge of only a small region of sequence within the target mRNA or a partial clone of cDNA

A homopolymeric tail is added to the 3' termini of cDNAs

Two adaptor-primers are used to amplify the double-stranded cDNA

Synthesis of 5’ and 3’ fragments

PCR cycles
94°C, 4 min
94°C, 30 sec
60 or 52°C, 1 min
72°C, 1 min
72°C, 10 min 30 cycles
* Annealing temperature: 52°C for 5’ fragment; 60°C for 3’ fragment.
* Primer concentration is 0.1 μM in all PCR.

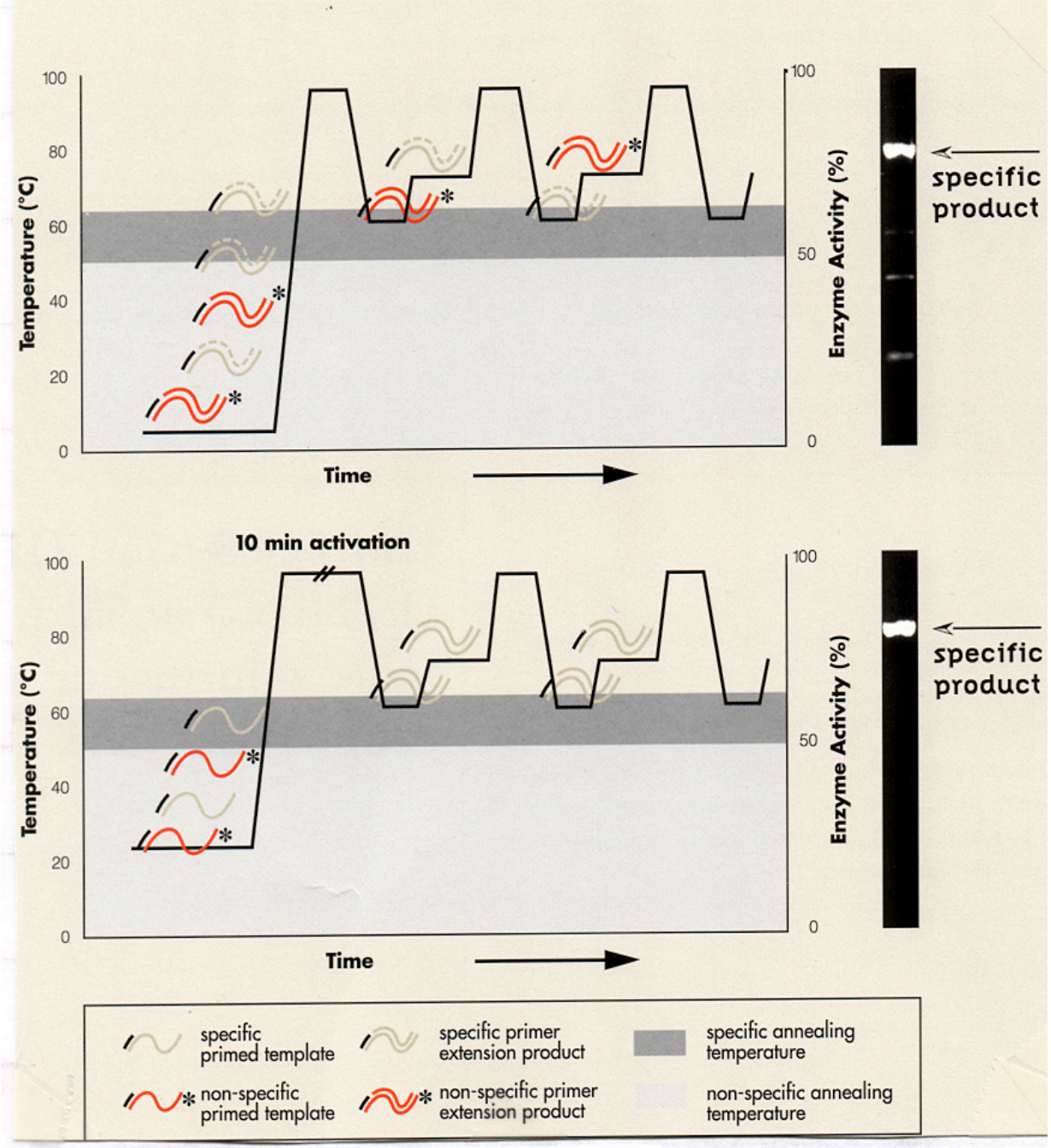


0.9% agarose

Purification of 5’ and 3’ fragments

The gel slices were dissolved in the Binding buffer of High Pure PCR Product Purification Kit (Roche) by incubated at 55°C for 10 min. The rest procedure was according to the company’s instruction. The purified fragments were dissolved in 100 μl TE, from which 5 μl was used in the following PCR to synthesize the full-length, mutagenized HSF1.

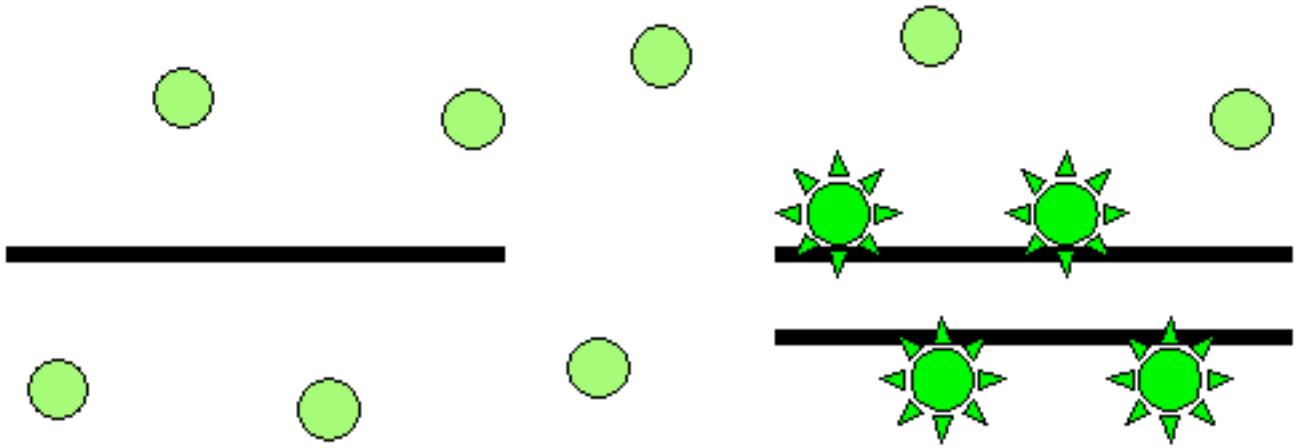
Hot start PCR



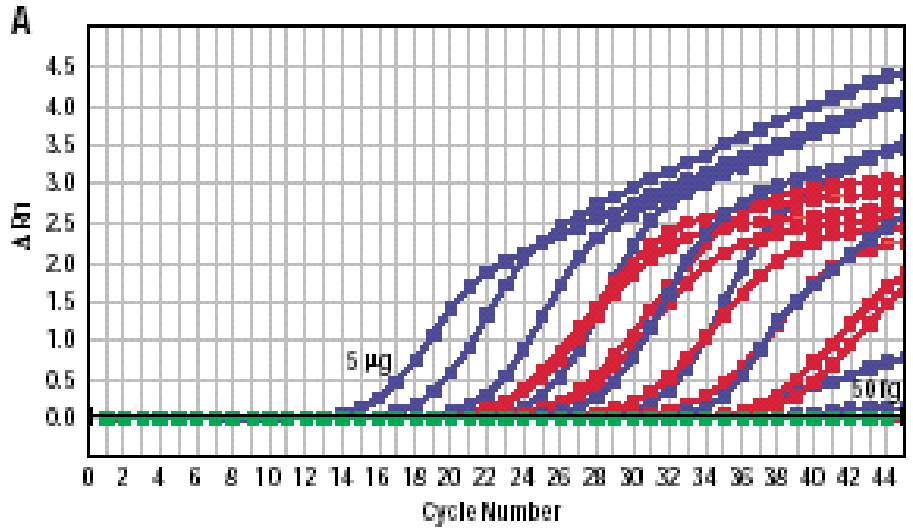
Top: in conventional PCR, mispriming events that occur on the initial up ramp can lead to amplification of non-specific products.

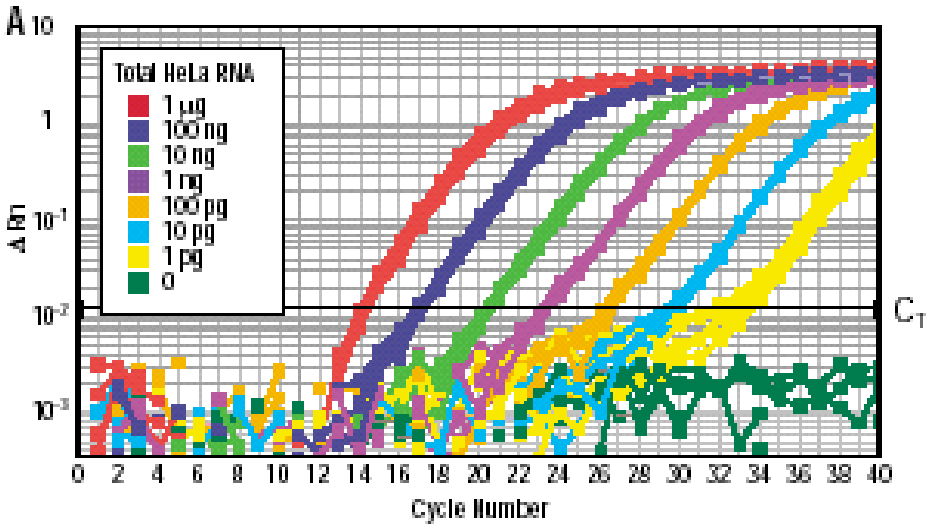
Bottom: in hot start PCR, active polymerase is present only at temperatures above the specific annealing temperature. The 10-min preincubation serves to fully denature any mispriming and to initiate activation of enzyme (AmpliTaq Gold enzyme from PE Applied Biosystem

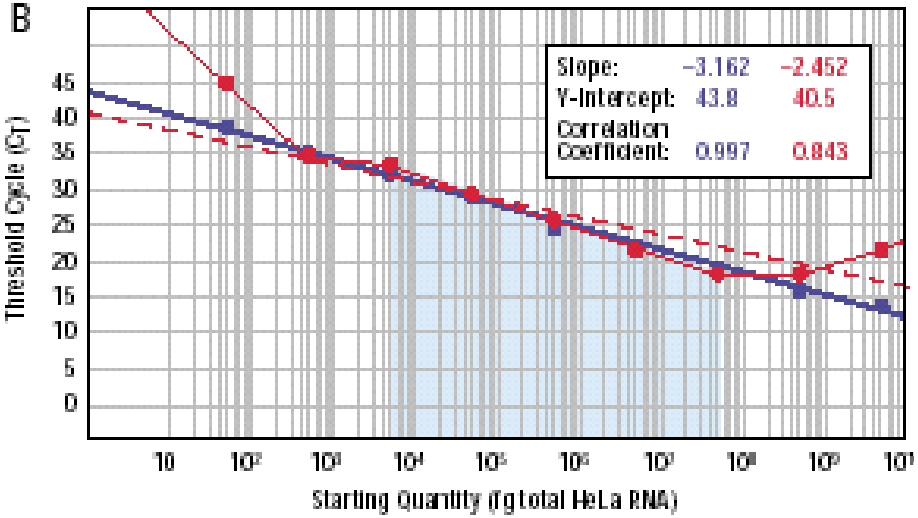
SYBR Green for RT^2-PCR

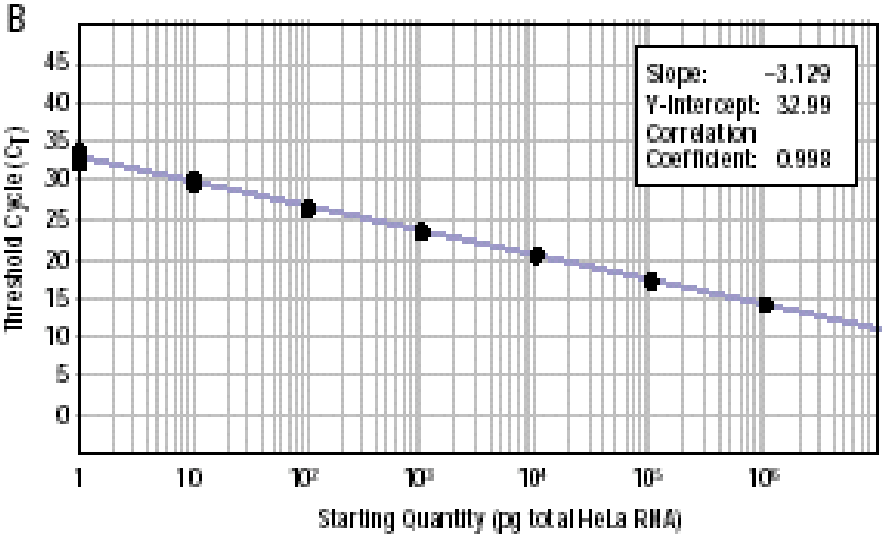


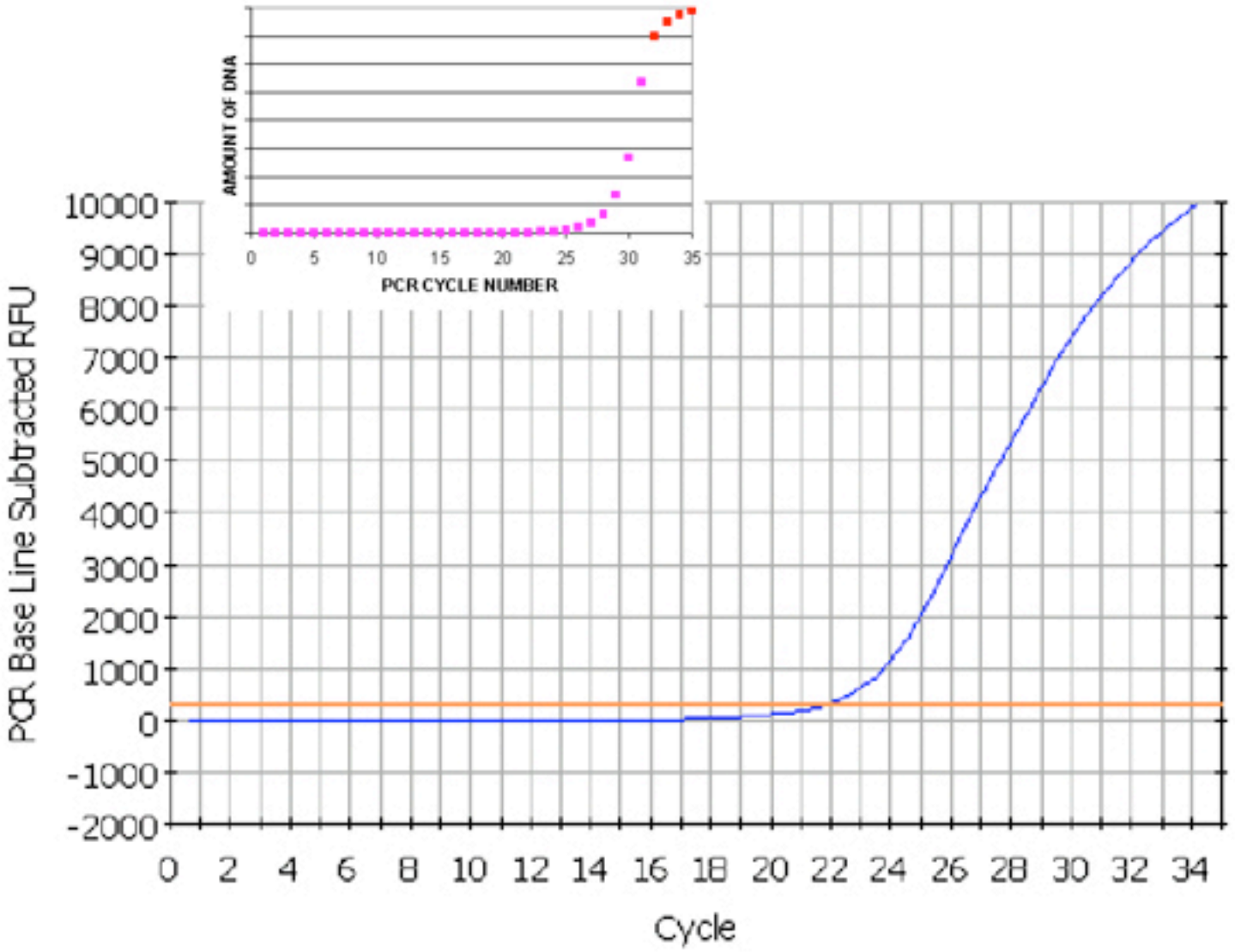
Real Time PCR : Ability to measure the concentrations of nucleic acids over a vast dynamic range; its high sensitivity; its capacity to process many samples simultaneously.

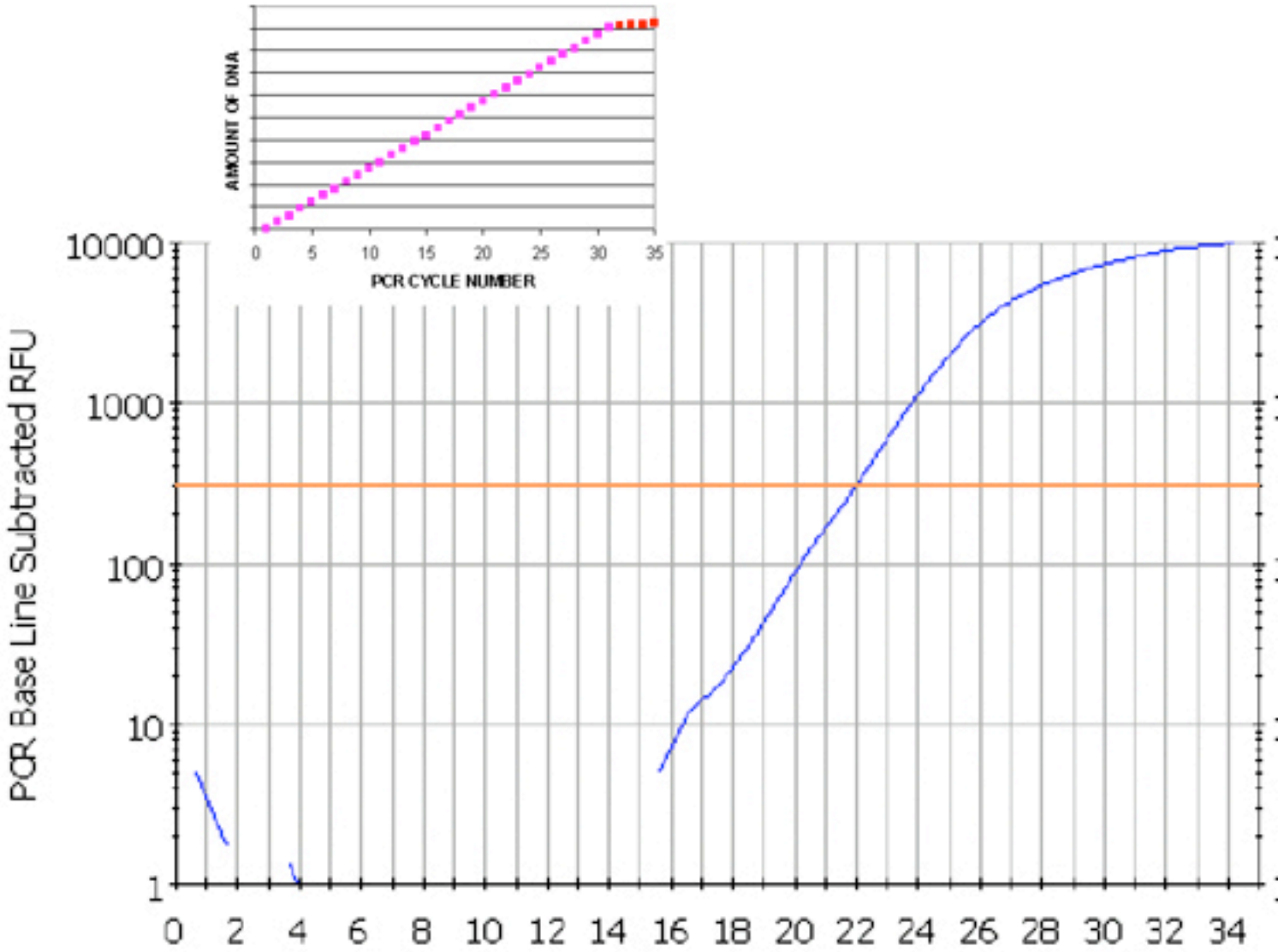


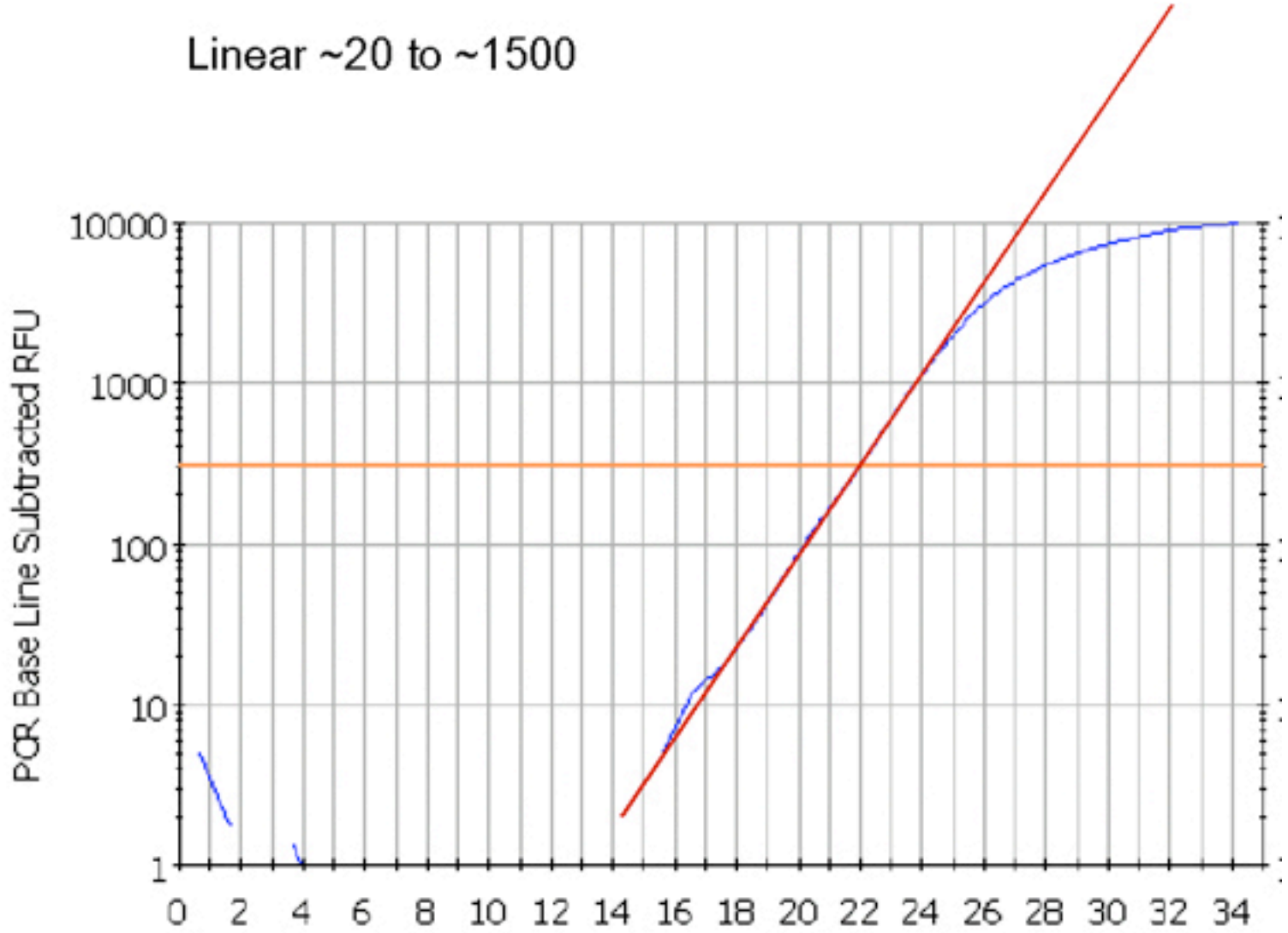




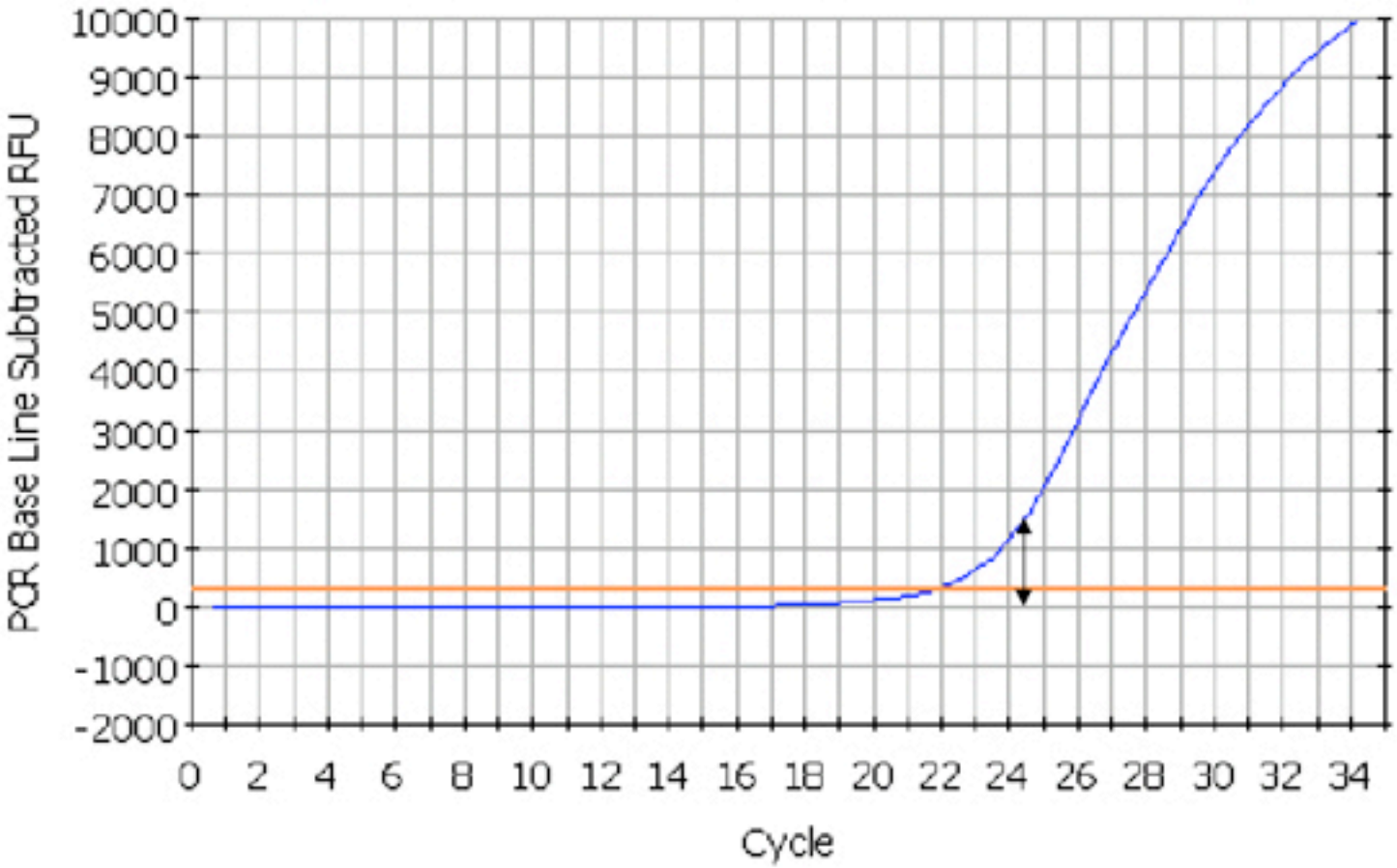



AMOUNT OF DNA
0
5
10
15
20
25
30
35
PCR CYCLE NUMBER
PCR Base Line Subtracted RFU
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
-1000
-2000
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
Cycle


AMOUNT OF DNA
0
5
10
15
20
25
30
35
PCR CYCLE NUMBER
PCR Base Line Subtracted RFU
10000
1000
100
10
1
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34


Linear ~20 to ~1500
PCR Base Line Subtracted RFU
10000
1000
100
10
1
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34

Linear ~20 to ~1500



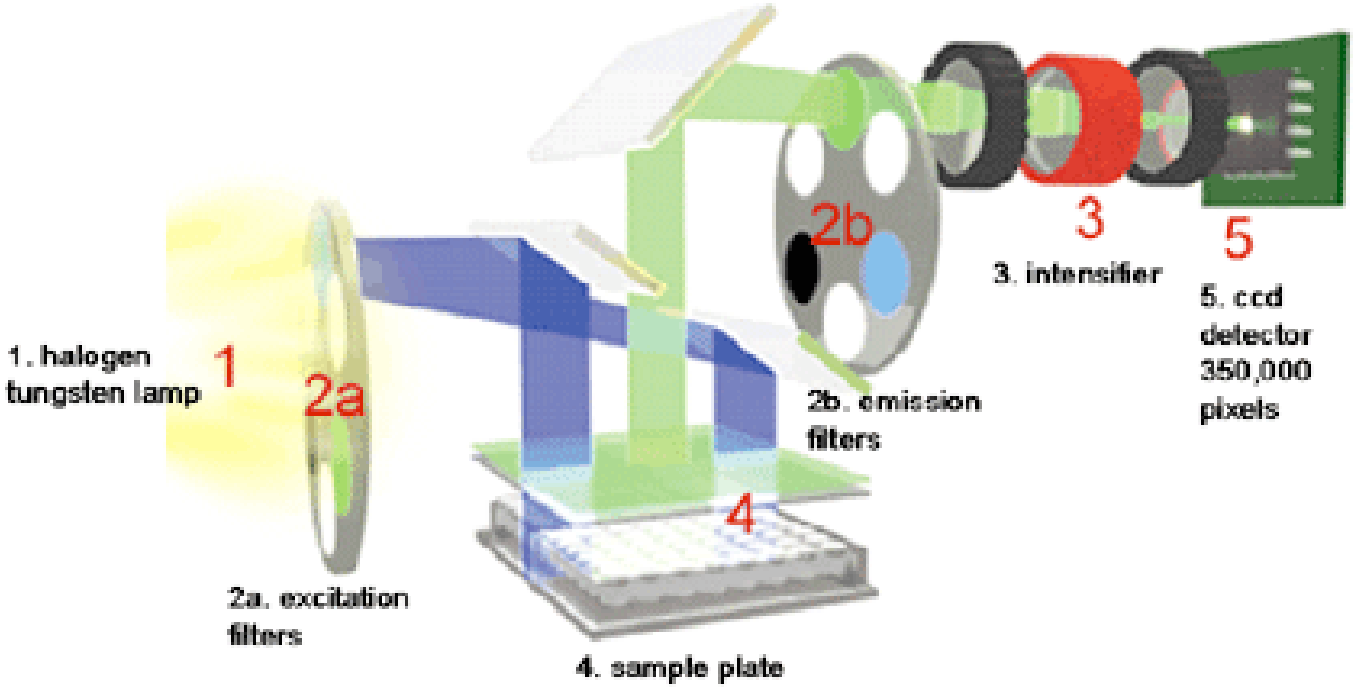
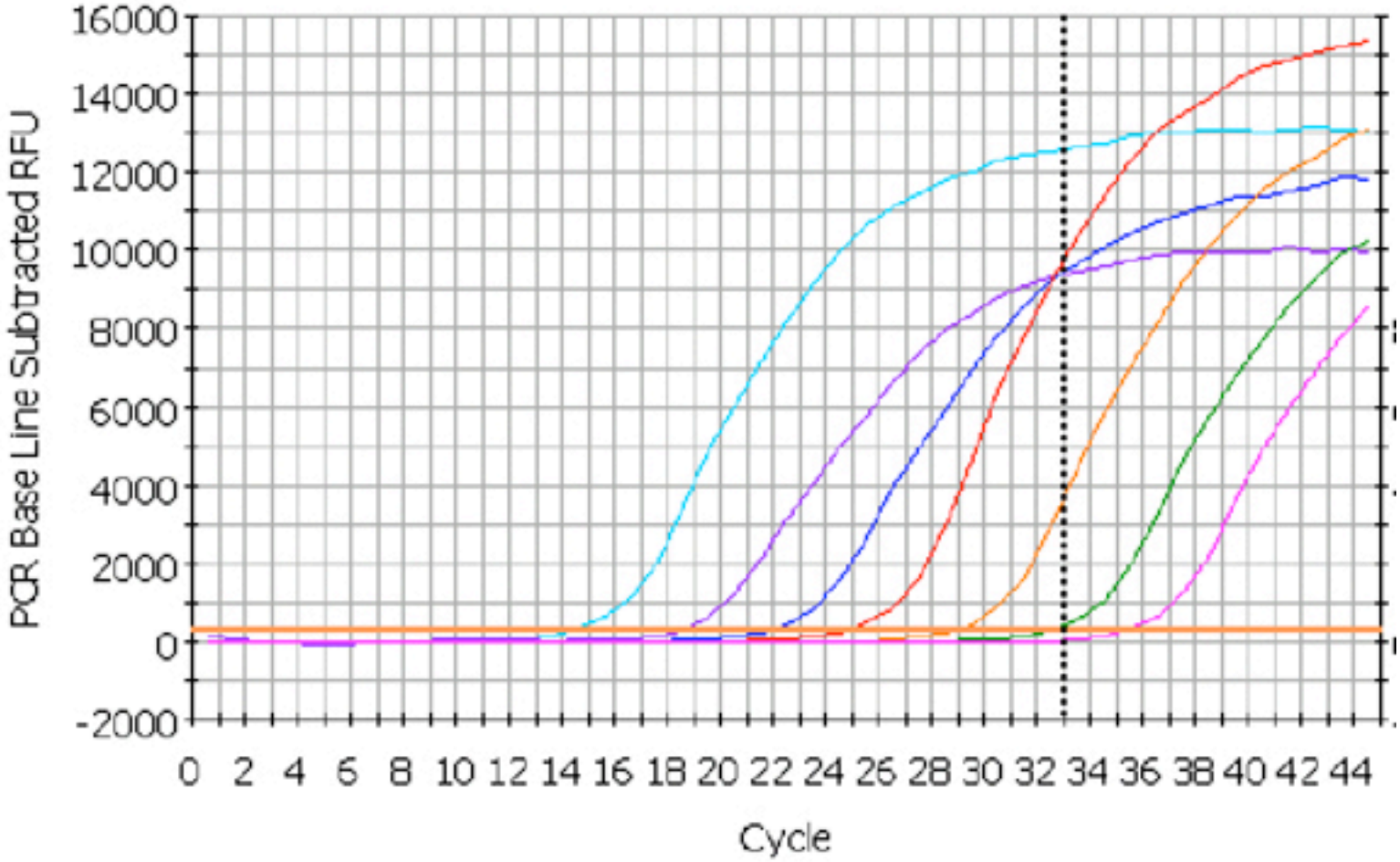
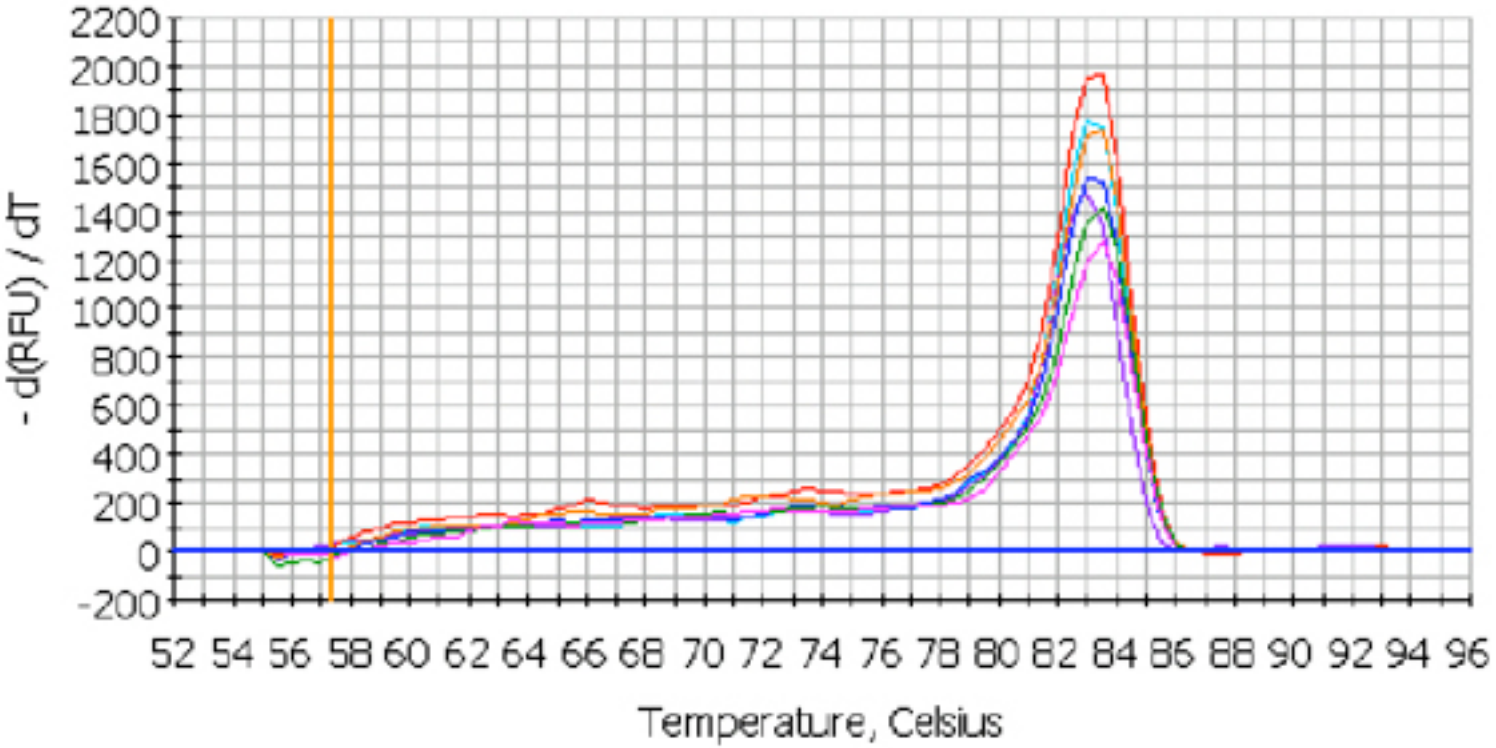


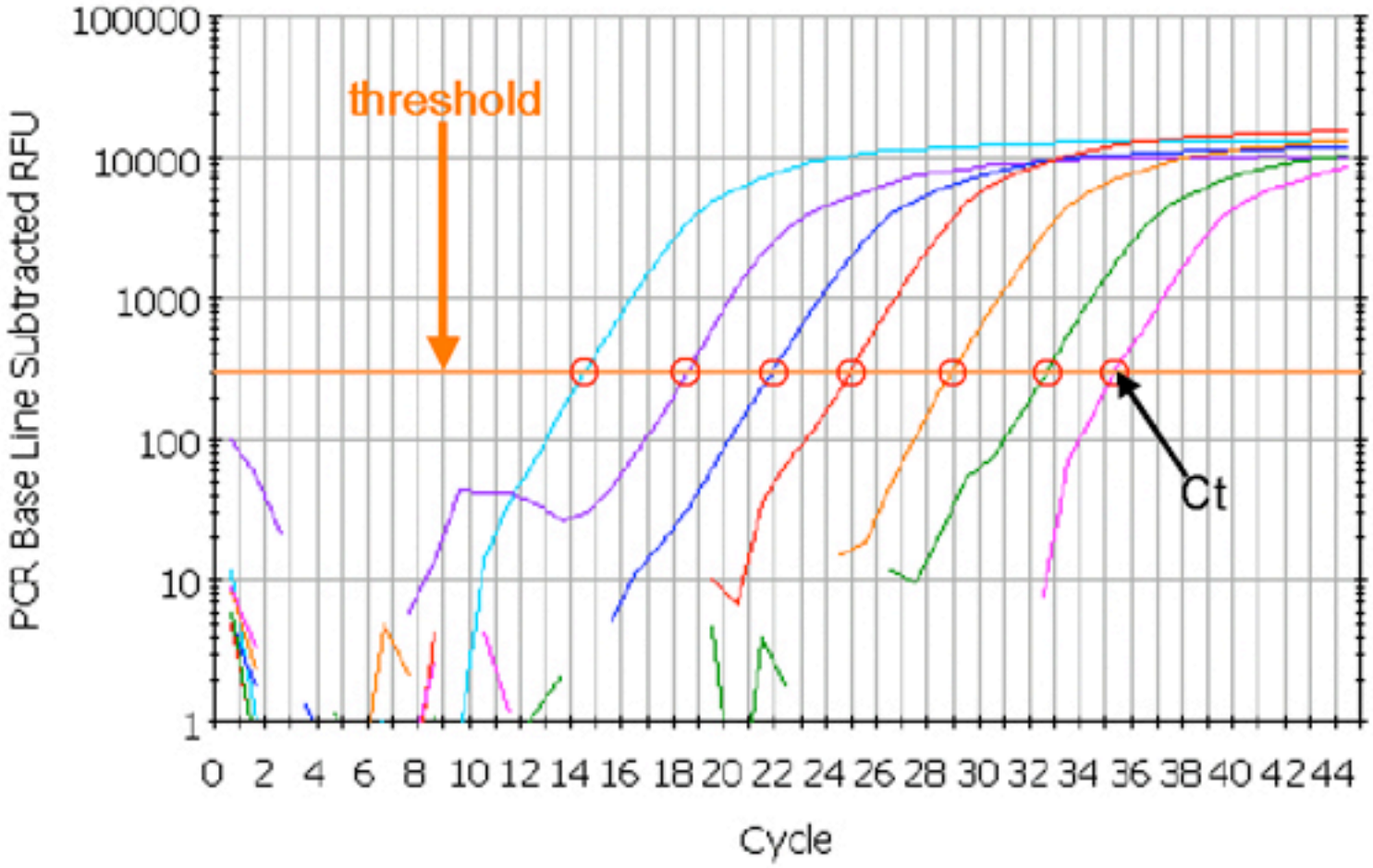
Fig. 1.2. Representation of Optical Detection System layout.

www.biorad.com

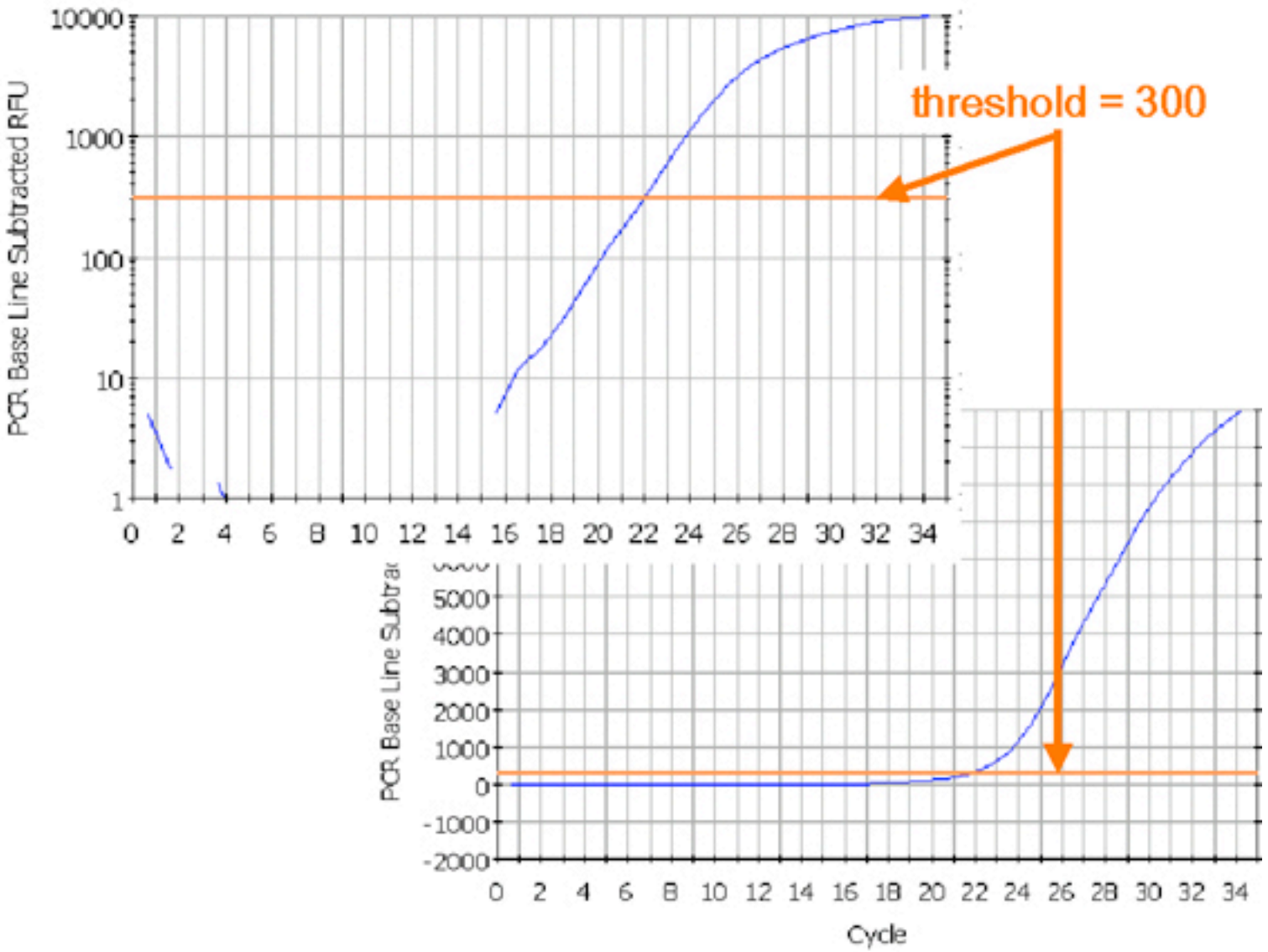


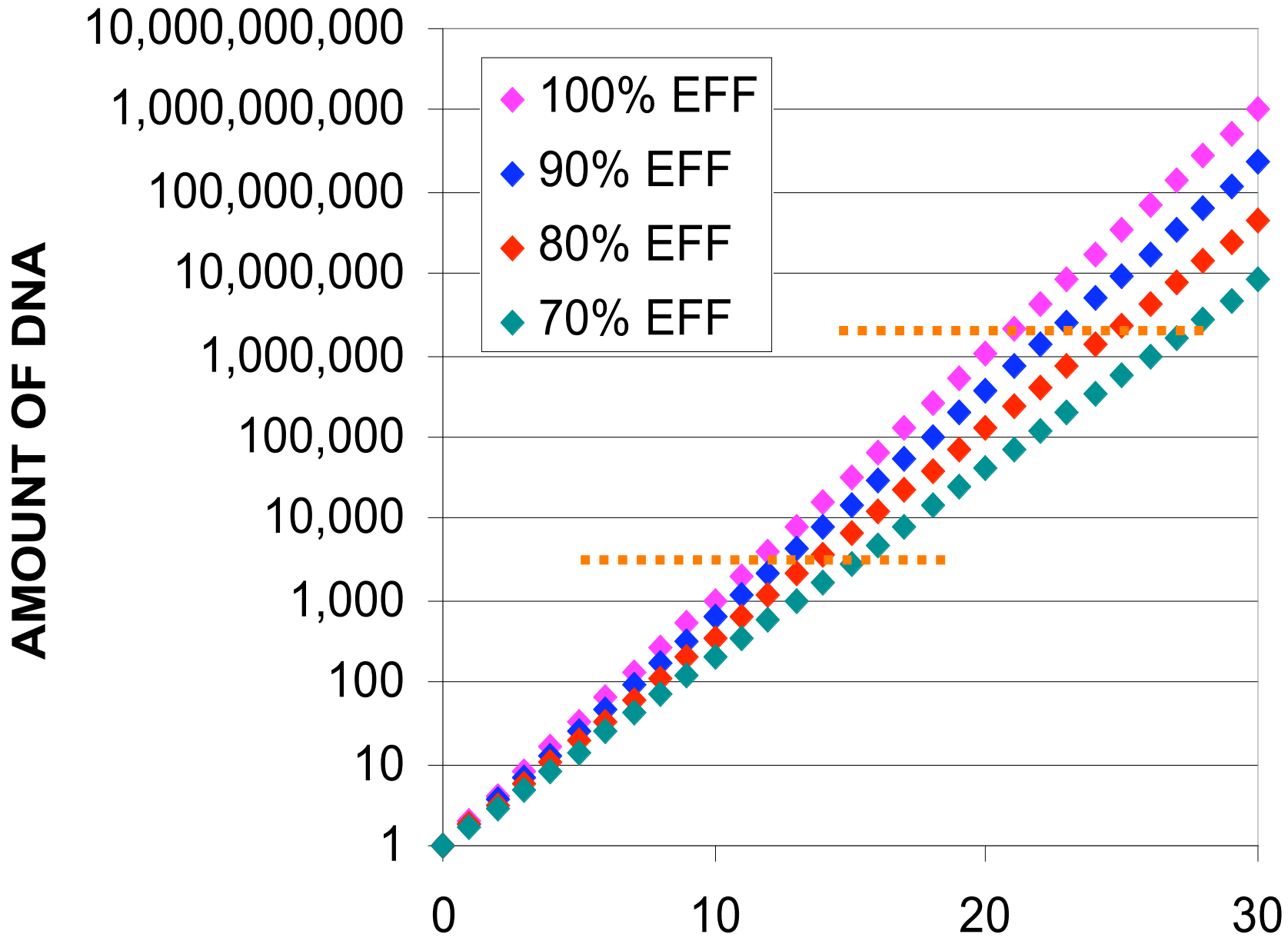
SERIES OF 10-FOLD DILUTIONS

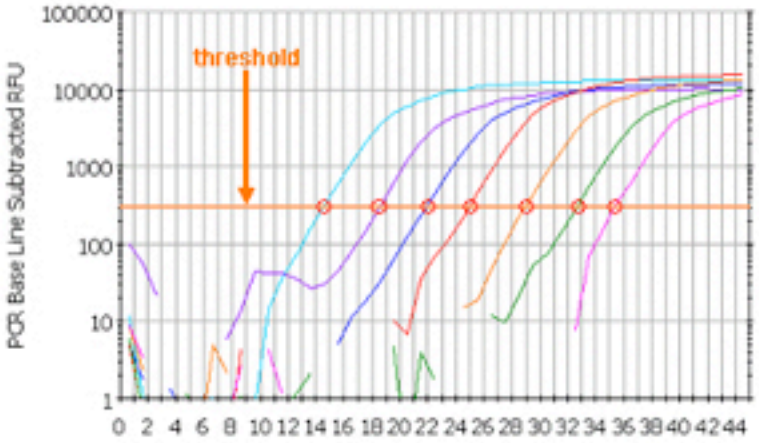

2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
-200
- d(RFU) / dT
52 54 56 58 60 62 64 66 68 70 72 74 76 78 80 82 84 86 88 90 92 94 96
Temperature, Celsius



SERIES OF 10-FOLD DILUTIONS

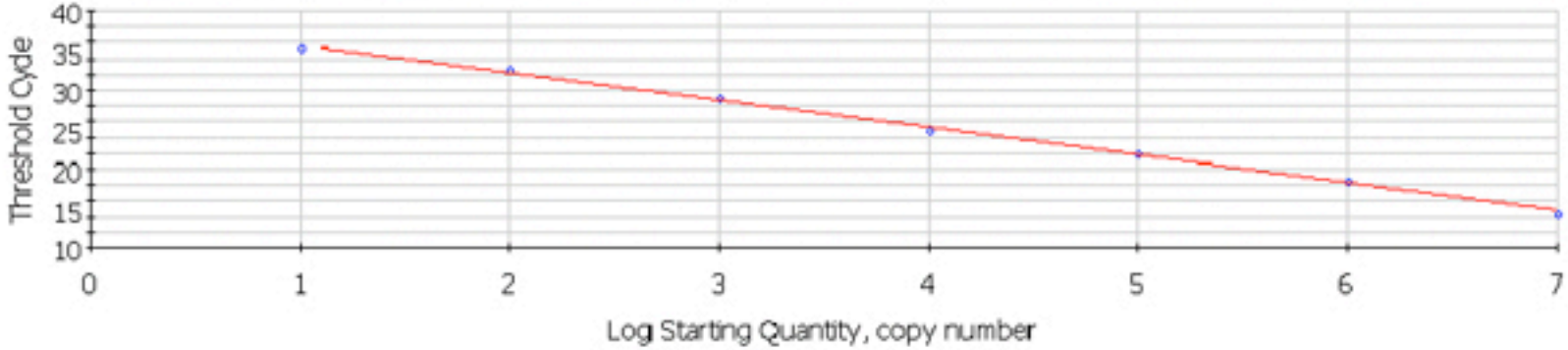

threshold = 300
PCR Base Line Subtracted RFU
10000
1000
100
10
1
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34
5000
4000
3000
2000
1000
0
-1000
-2000
Cycle


AMOUNT OF DNA
10,000,000,000
1,000,000,000
100,000,000
10,000,000
1,000,000
100,000
10,000
1,000
100
10
1
0
10
20
30
100% EFF
90% EFF
80% EFF
70% EFF

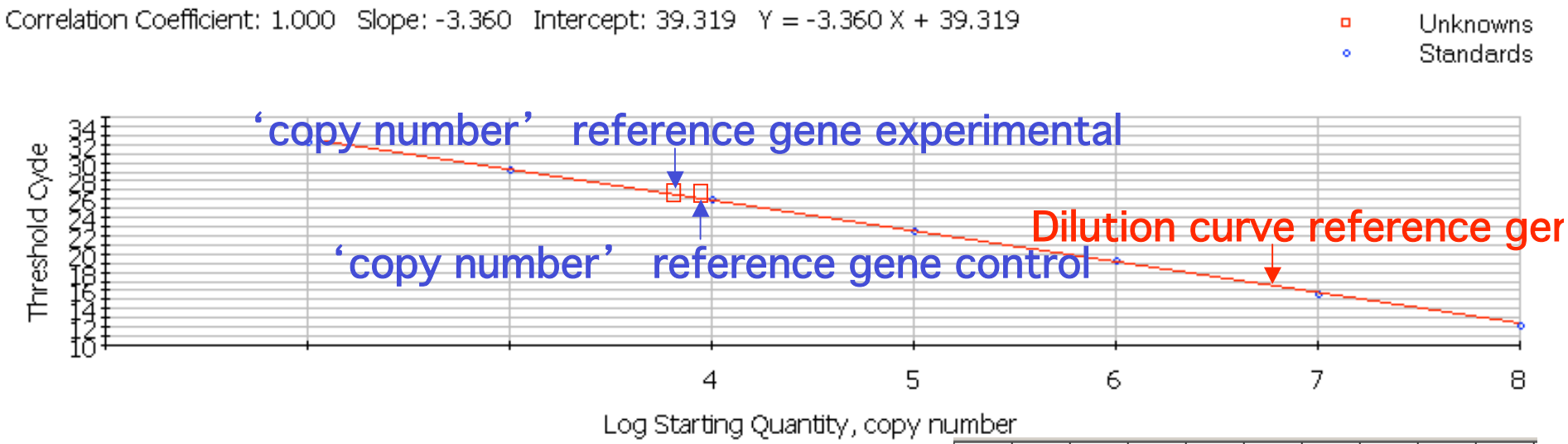


Correlation Coefficient: 0.999 Slope: -3.488 Intercept: 39.204 Y = -3.488 X + 39.204

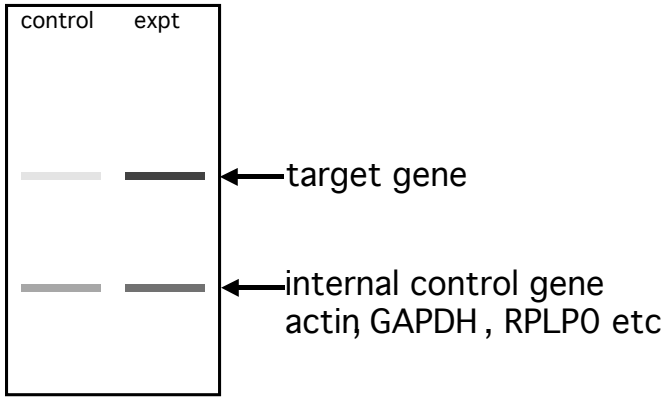
Unknowns
Standards



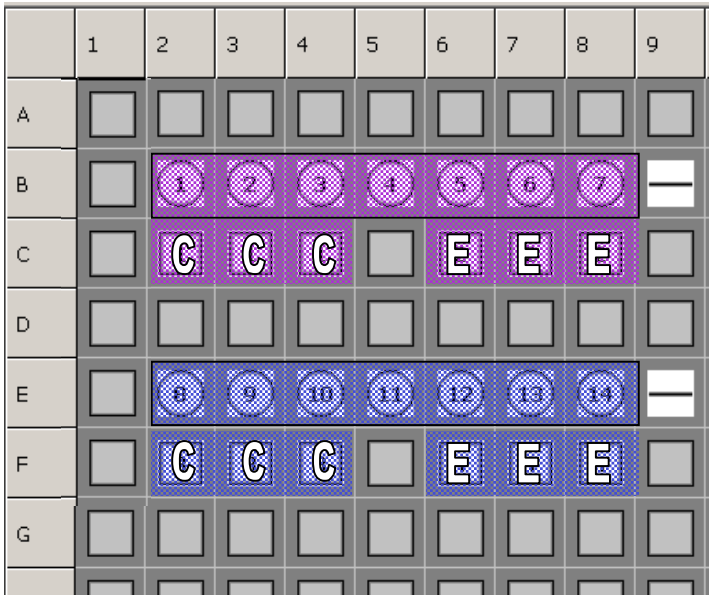
PCR Standard Curve: Data 27-Jan-03 1233ileff.opd

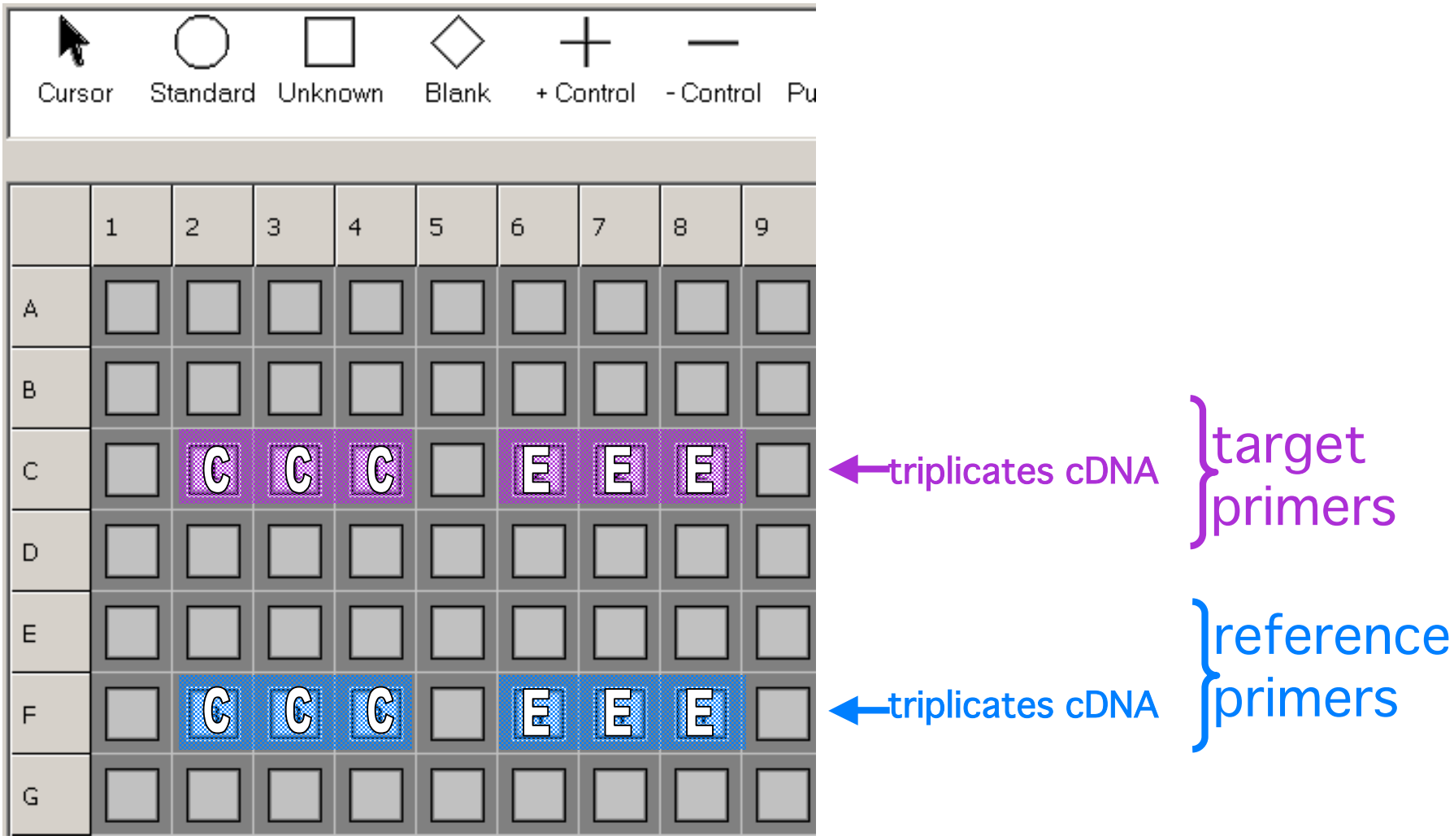


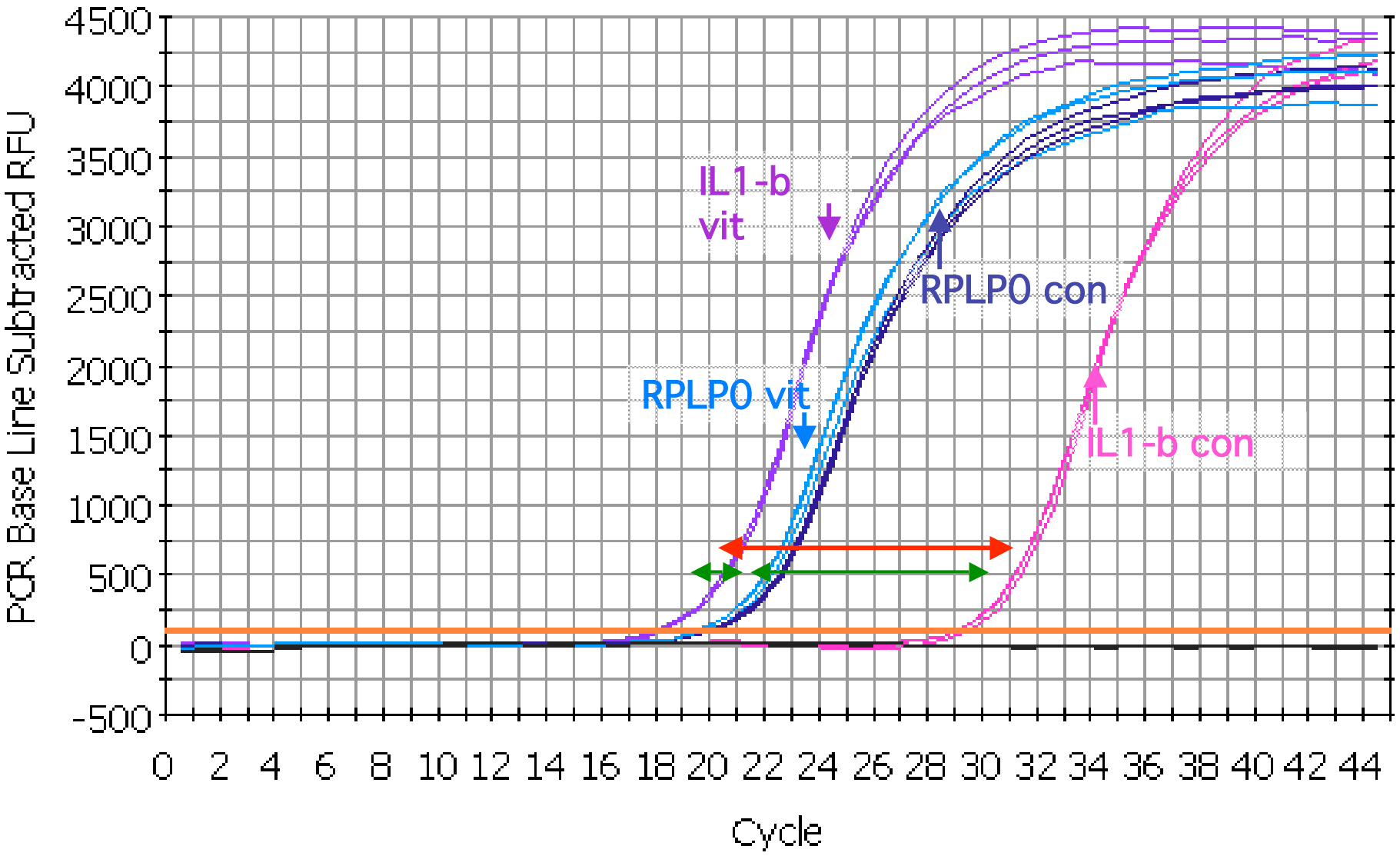
NORTHERN



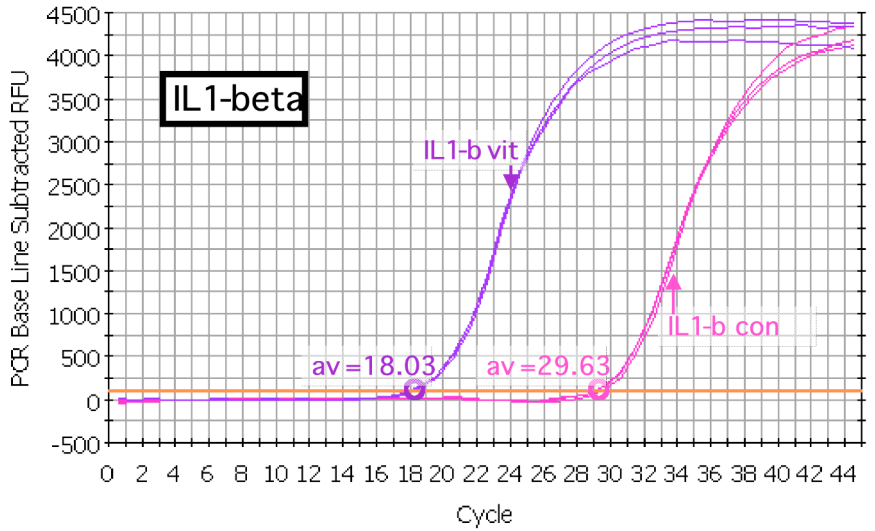
Ratio experimental/control = fold change in target gene / fold change in reference ge




Cursor
Standard
Unknown
Blank
+ Control
- Control
Pu
1
2
3
4
5
6
7
8
9
A
B
C
D
E
F
G
C C C
E E E
C C C
E E E
triplicates cDNA
target primers
triplicates cDNA
reference primers

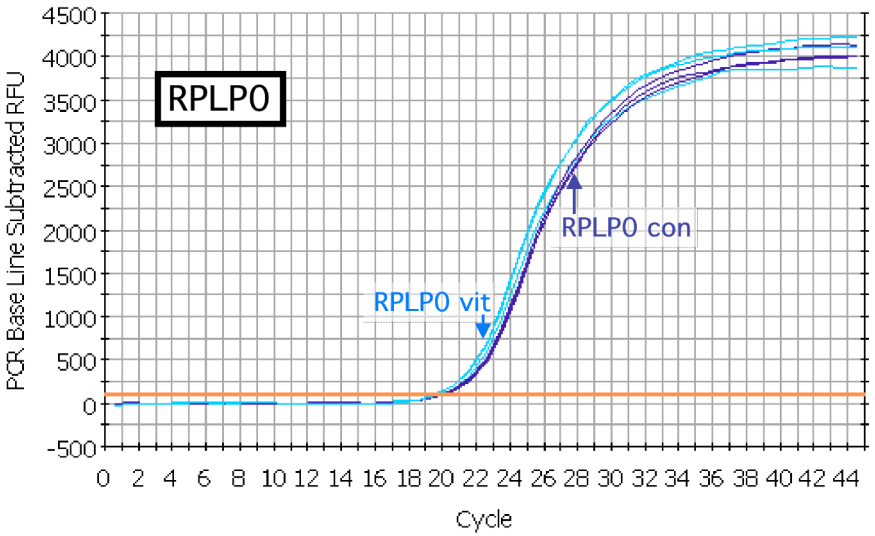


PCR Amplification vs Cycle: Data 26-Feb-03 1147.opd



AFTER N CYCLES: increase = (efficiency)^{N}

Ratio vit/con = $(1.93)^{29.63-18.03} = 1.93^{11.60} = 2053$



AFTER N CYCLES: increase = (efficiency)^{N}

Ratio vit/con = $(1.87)^{19.93-19.80} = 1.87^{7.13} = 108$

$$\text{ratio} = \frac{\text{change in IL1-B}}{\text{change in RPLP0}} = 2053/1.08 = 1901$$

$$\text{ratio} = \frac{(E_{target})^{\Delta Ct\ target\ (control-treated)}}{(E_{ref})^{\Delta Ct\ ref\ (control-treated)}}$$