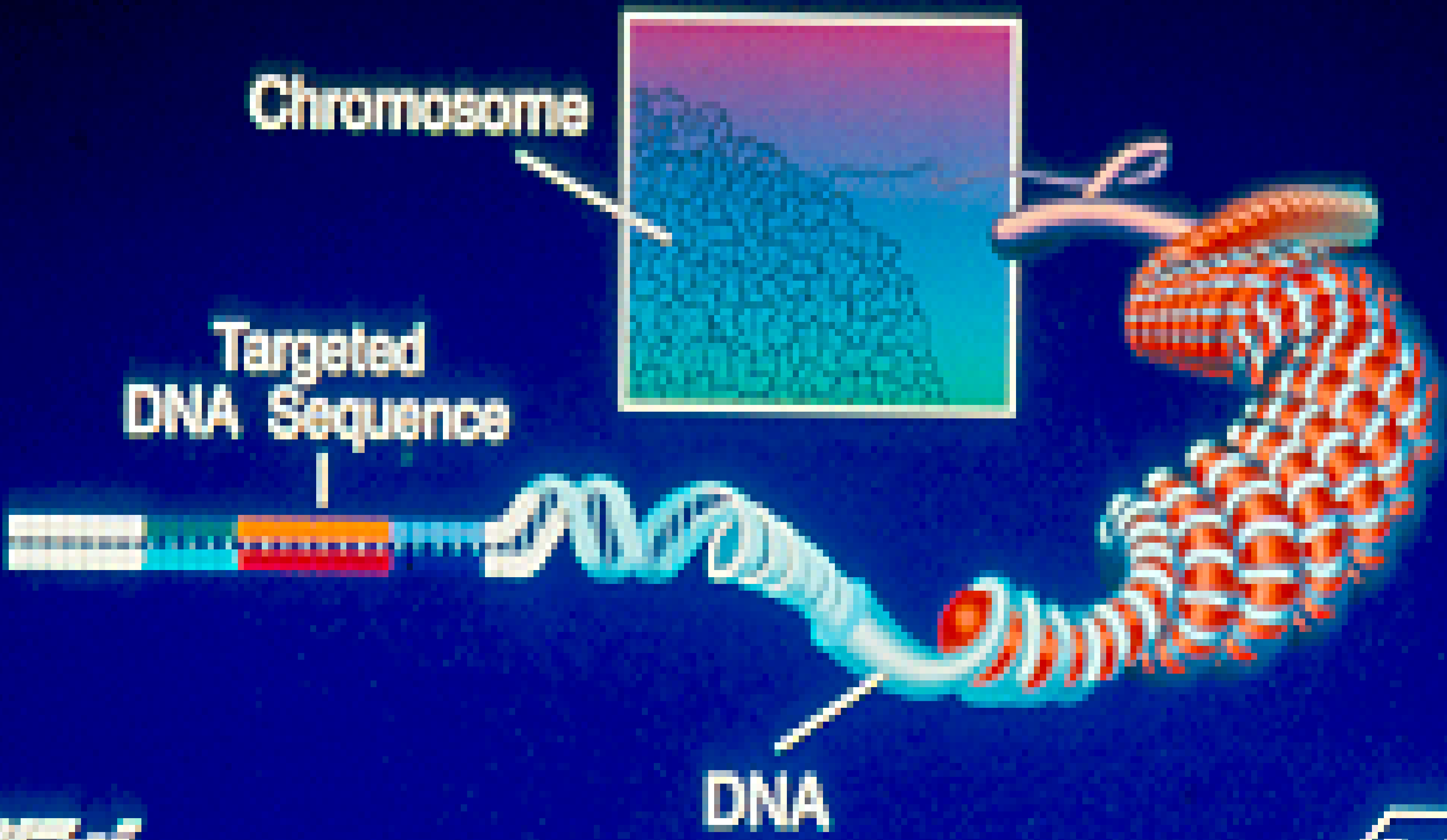
PCR, RT-PCR ,NESTED-PCR, MULTIPLEX PCR

QUANTITATIVE PCR

PCR AMPLIFIES A TARGETED SEQUENCE



Chromosome

Targeted DNA Sequence

DNA

PCR PRIMERS

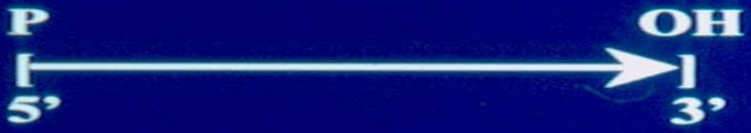
PRIMER SELECTION

- Empirical (visual)
- Computer software assisted

PRIMER PRODUCTION

PRIMER PARAMETERS

- Primer Length
- Base Constitution
- Concentration



ANNEAL CONDITIONS

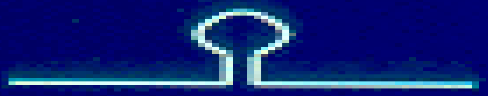
PRIMER PARAMETERS

Primer Length

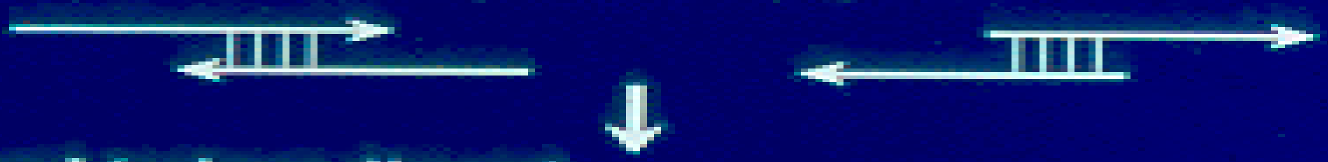
- 15-30 Nucleotides (20-25)

Base Constitution

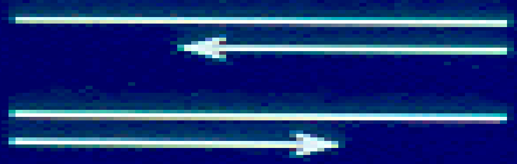
- Specific for a single specific sequence within the target
- 50% G/C nucleotides
- No secondary structure



- No complementarity within the primer



○ 3'-end (primer dimers)



ANNEALING CONDITIONS

Annealing depends on:

- Temperature and Time
- Primer Concentration
- Target Concentration
- Primer Length
- Base Constitution
- Tm

Tm (18-28 nucleotides)

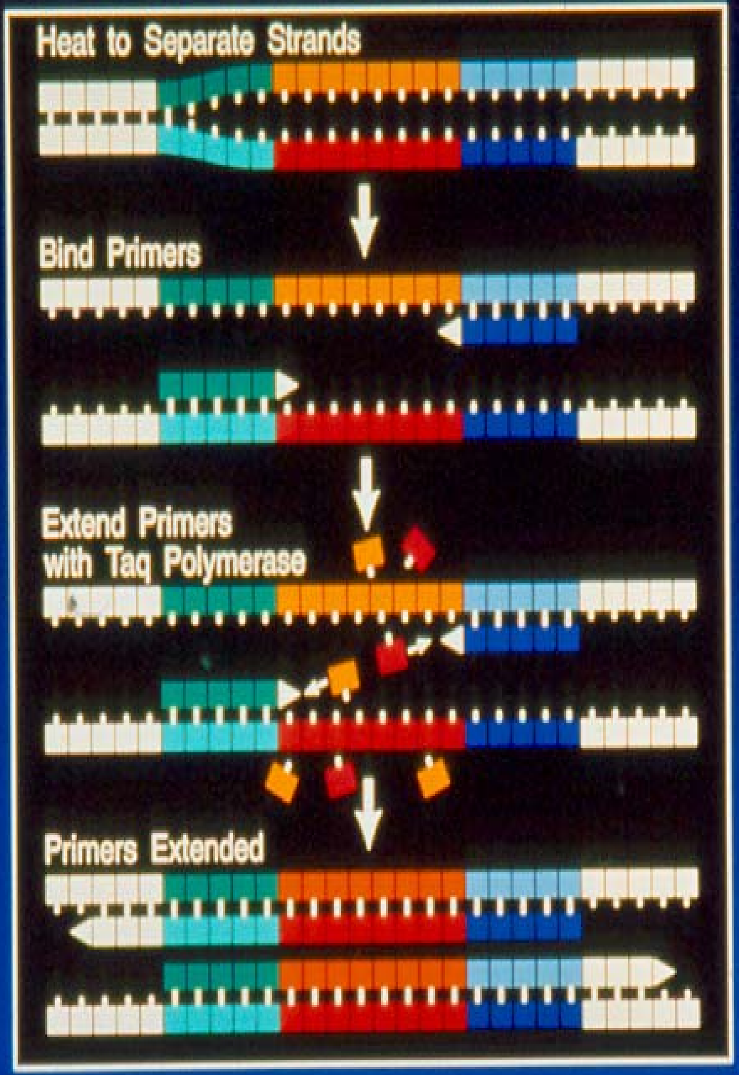
- Thein & Wallace, 1986 rule of thumb 2°C A/T, 4°C G/C
- Often lower than calculated under standard conditions [50mM NaCl)
- Tm 55-80°C is desired
- Should be equal for both primers, else lowest Tm
- Optimal annealing is empirical
- Ta = Tm minus 5-10°C

A SPECIFIC AMPLIFICATION

Conditions

- T ↓
- Salt ↑
- [Primer] ↑
- [Target DNA] ↑

FIRST PCR CYCLE



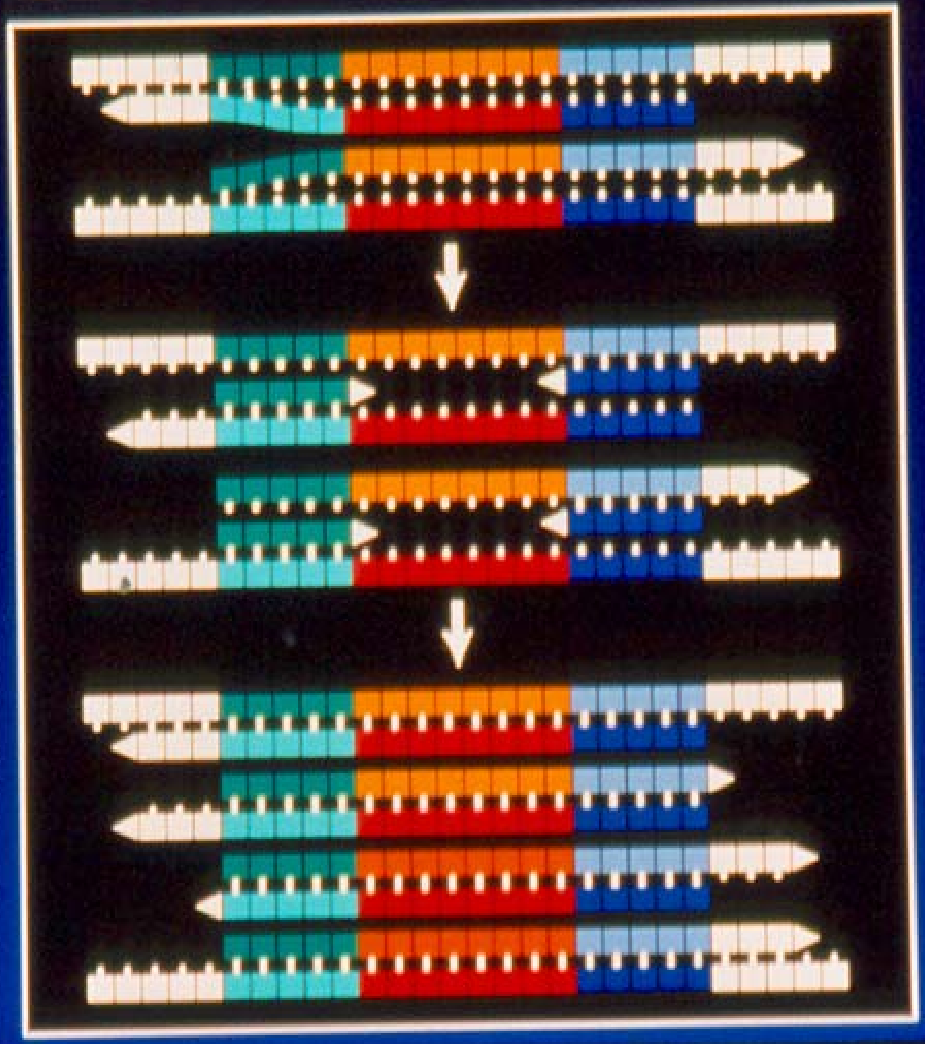
Heat to Separate Strands

Bind Primers

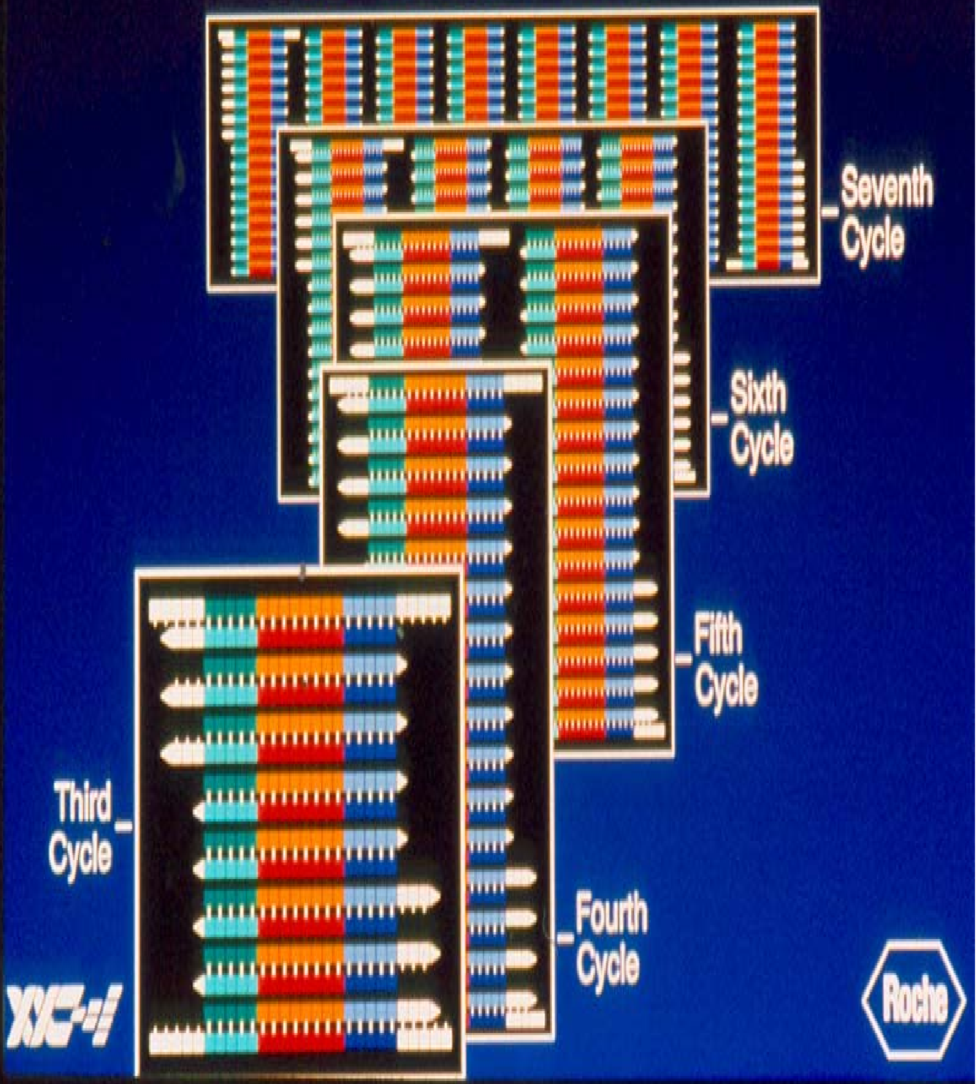
Extend Primers
with Taq Polymerase

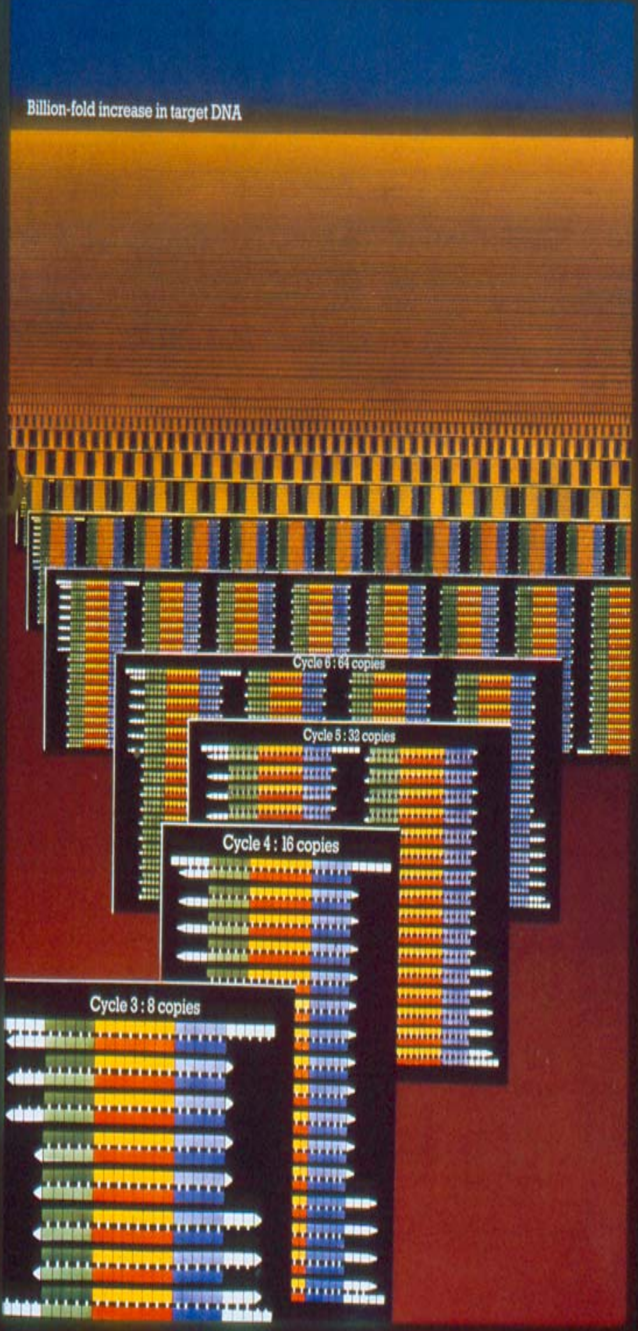
Primers Extended

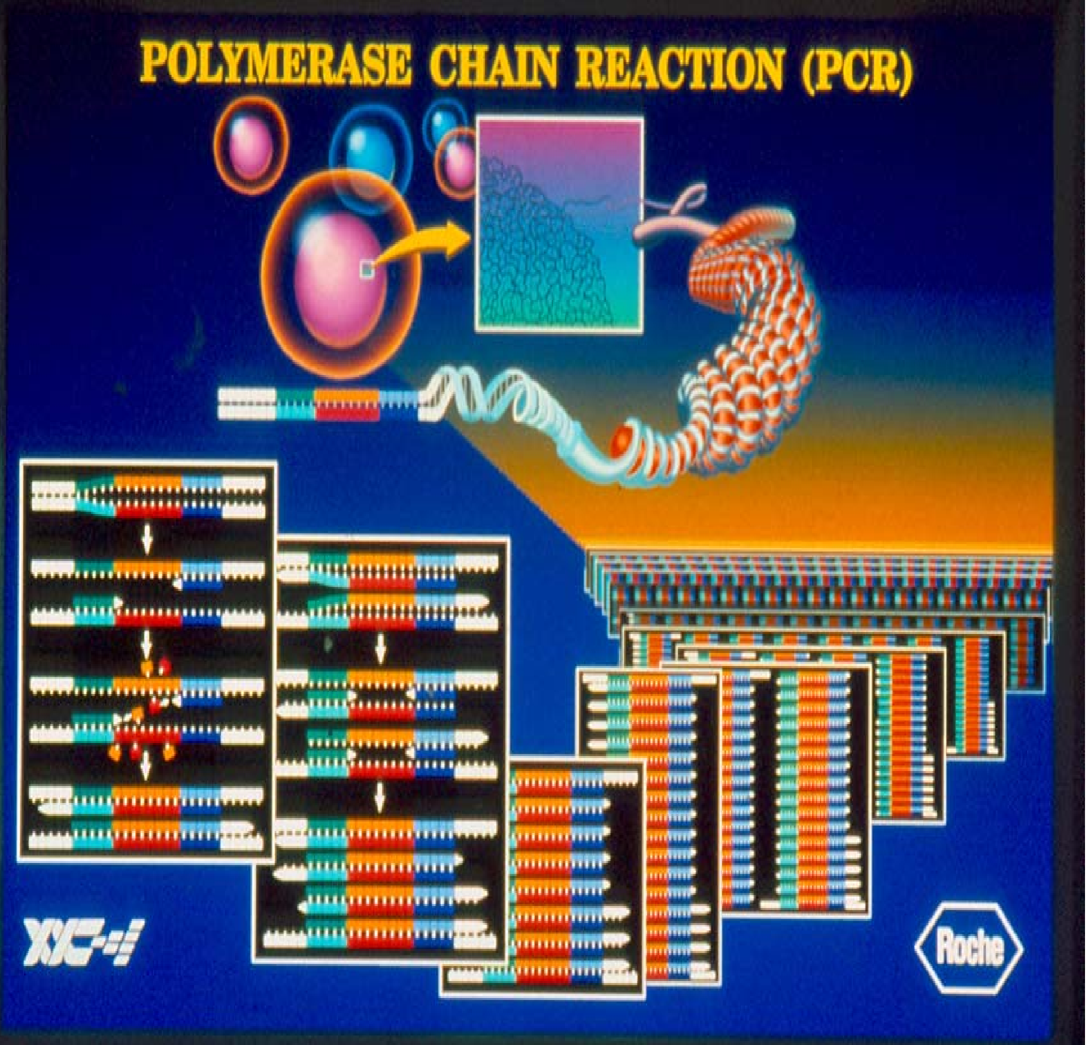
SECOND PCR CYCLE



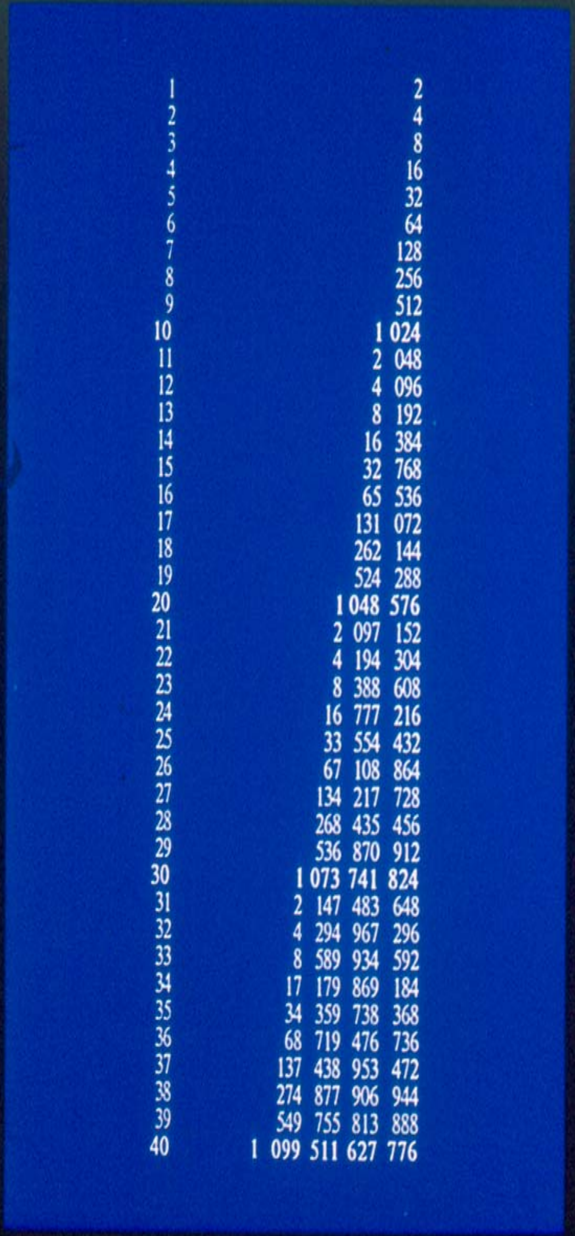
EXPONENTIAL AMPLIFICATION OF TARGET DNA


Seventh Cycle
Sixth Cycle
Fifth Cycle
Third Cycle
Fourth Cycle
Roche

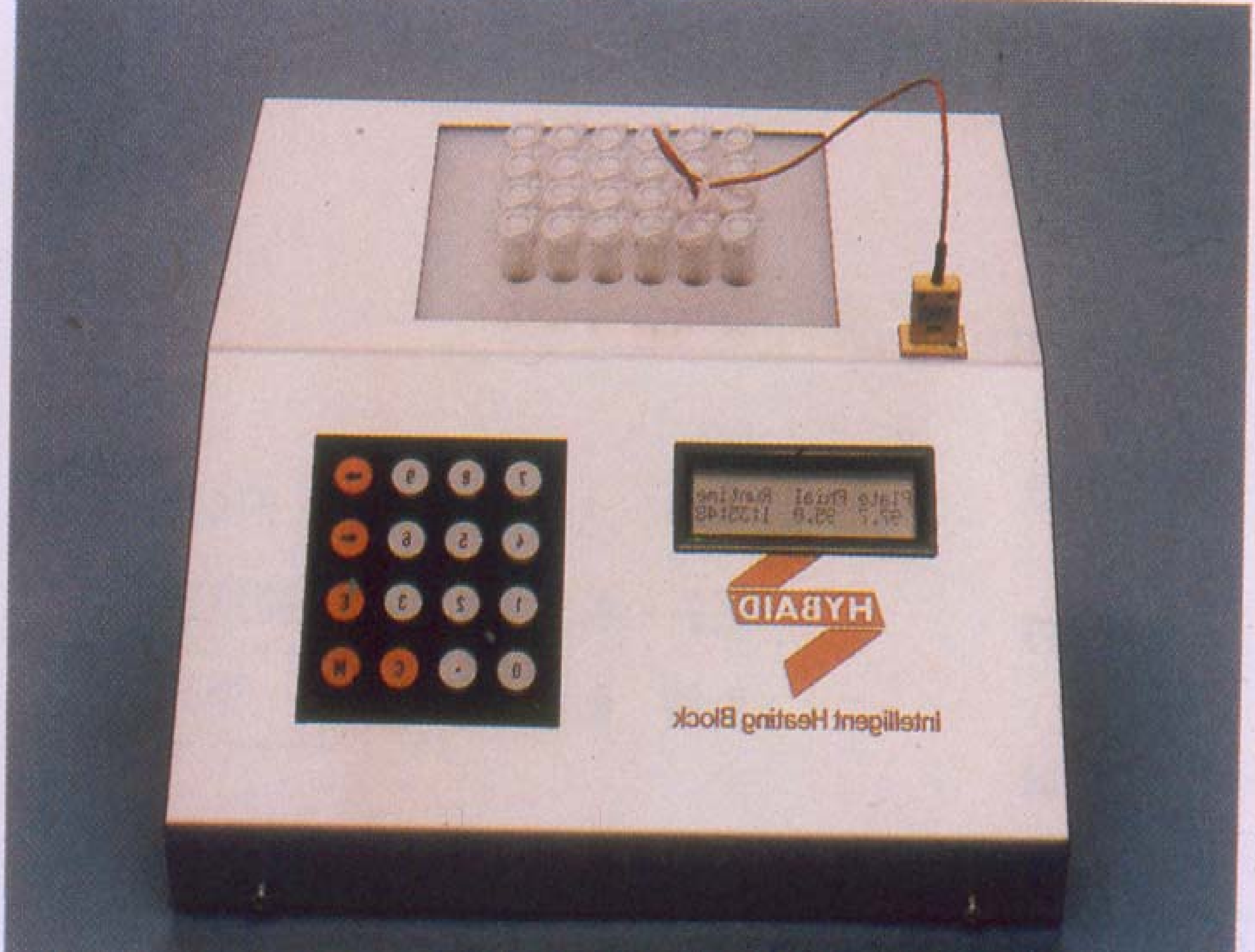

Billion-fold increase in target DNA
Cycle 6 : 64 copies
Cycle 5 : 32 copies
Cycle 4 : 16 copies
Cycle 3 : 8 copies

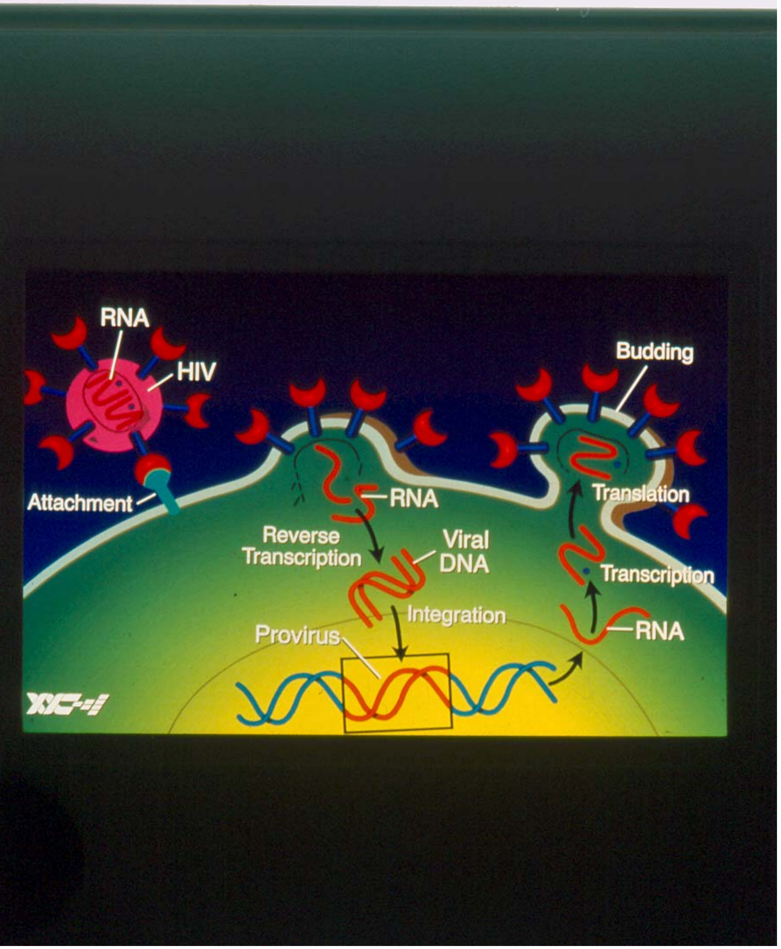


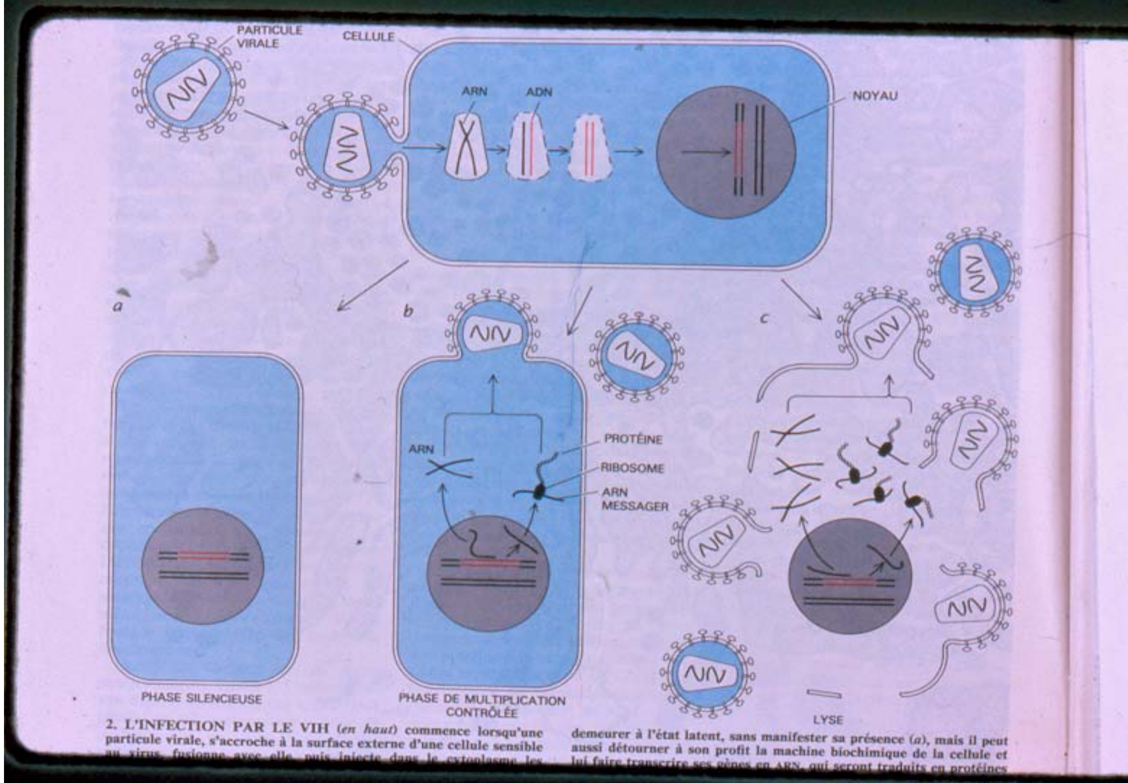
POLYMERASE CHAIN REACTION (PCR)



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	2 147 483 648
32	4 294 967 296
33	8 589 934 592
34	17 179 869 184
35	34 359 738 368
36	68 719 476 736
37	137 438 953 472
38	274 877 906 944
39	549 755 813 888
40	1 099 511 627 776

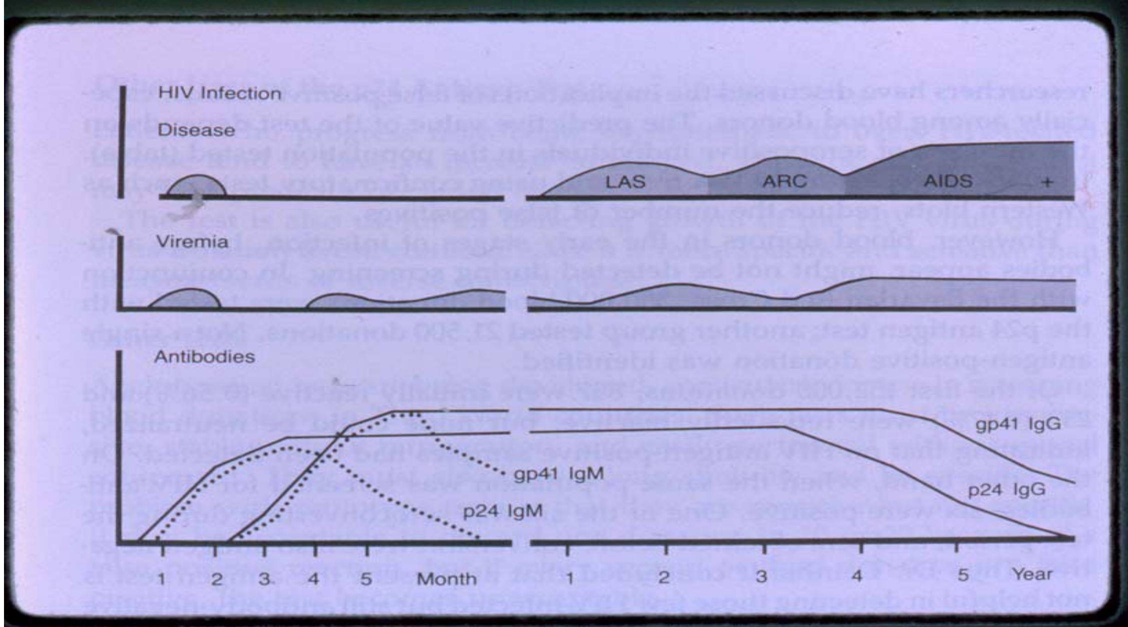

HYBAID
Intelligent Heating Block


RNA
HIV
Attachment
Budding
RNA
Reverse Transcription
Viral DNA
Integration
Provirus
Translation
Transcription
RNA


PARTICULE VIRALE
CELLULE
ARN
ADN
NOYAU
a
b
c
ARN
PROTÉINE
RIBOSOME
ARN MESSAGER
PHASE SILENCIEUSE
PHASE DE MULTIPLICATION CONTRÔLÉE
LYSE

2. L'INFECTION PAR LE VIH (*en haut*) commence lorsqu'une particule virale, s'accroche à la surface externe d'une cellule sensible au virus, fusionne avec elle, puis injecte dans le cytoplasme les

demeurer à l'état latent, sans manifester sa présence (*a*), mais il peut aussi détourner à son profit la machine biochimique de la cellule et lui faire transcrire ses gènes en ARN, qui seront traduits en protéines



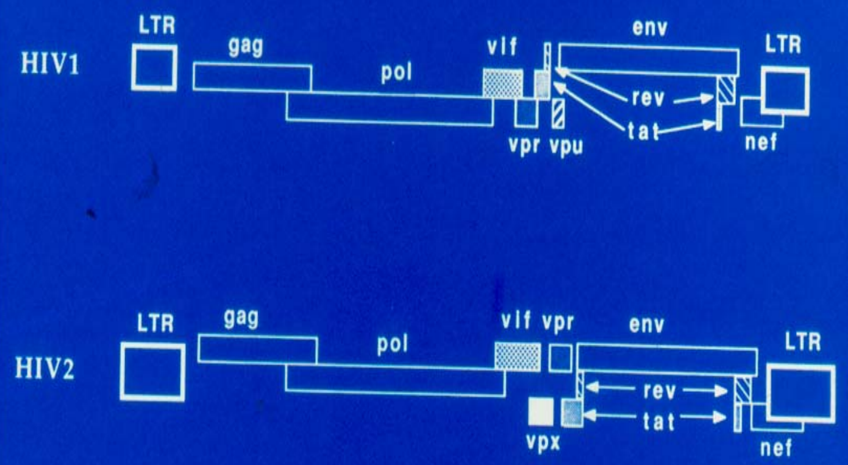
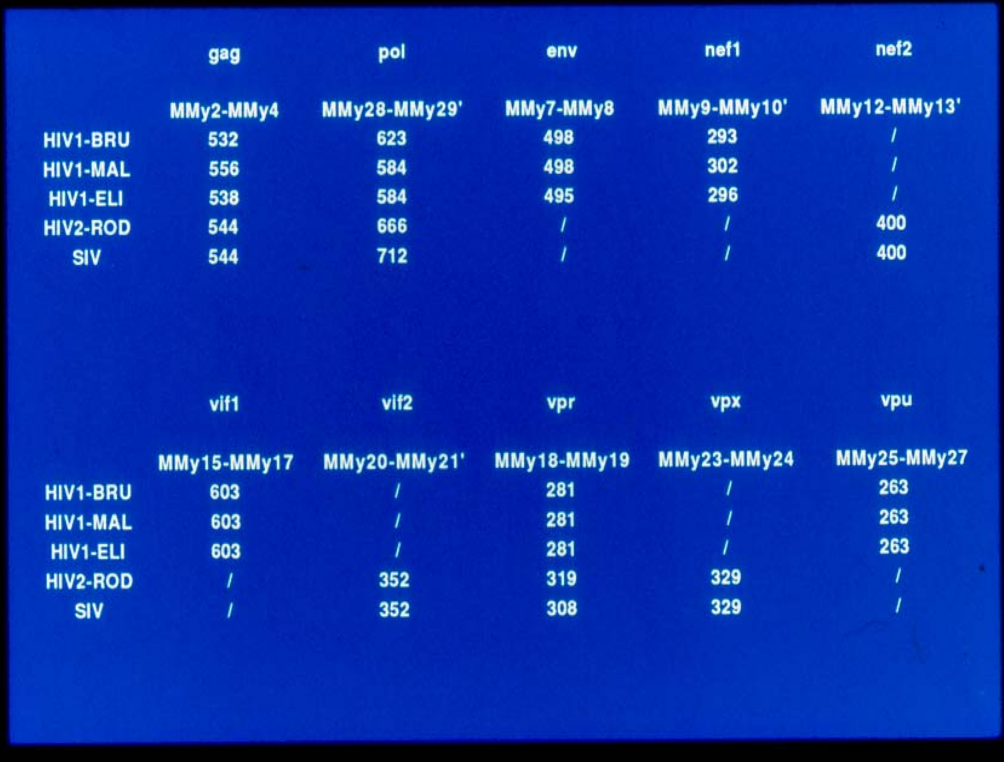
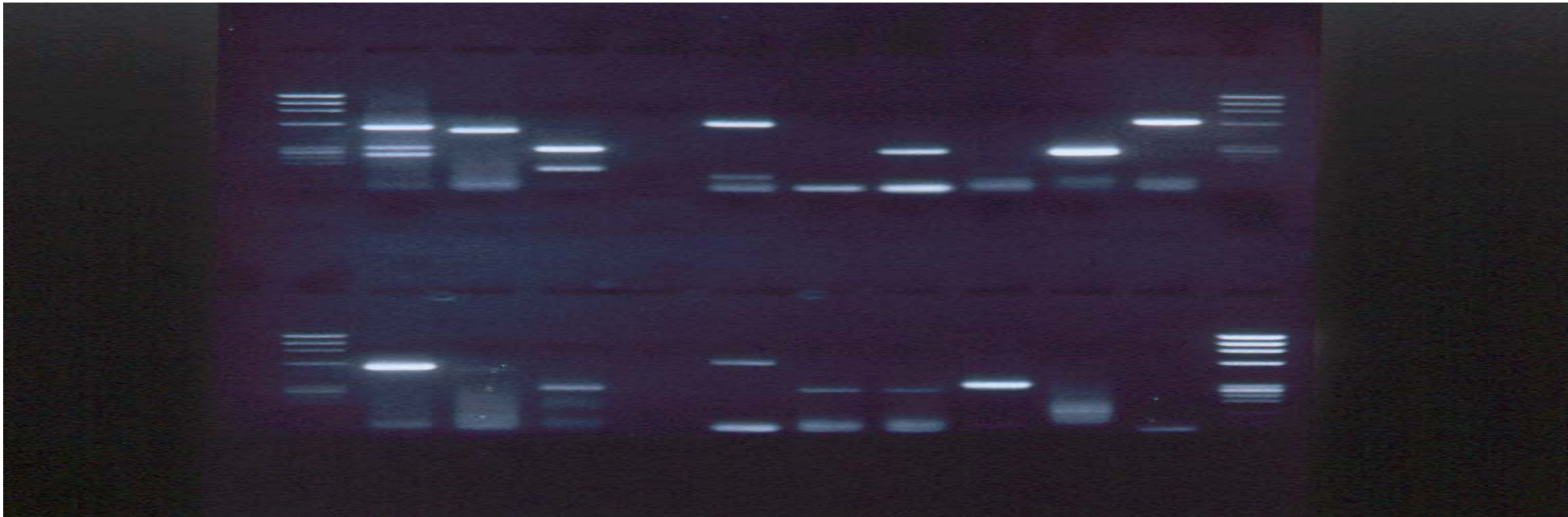

HIV1
LTR
gag
pol
vif
env
LTR
rev
tat
vpr
vpu
nef
HIV2
LTR
gag
pol
vif
vpr
env
LTR
rev
tat
vpx
nef

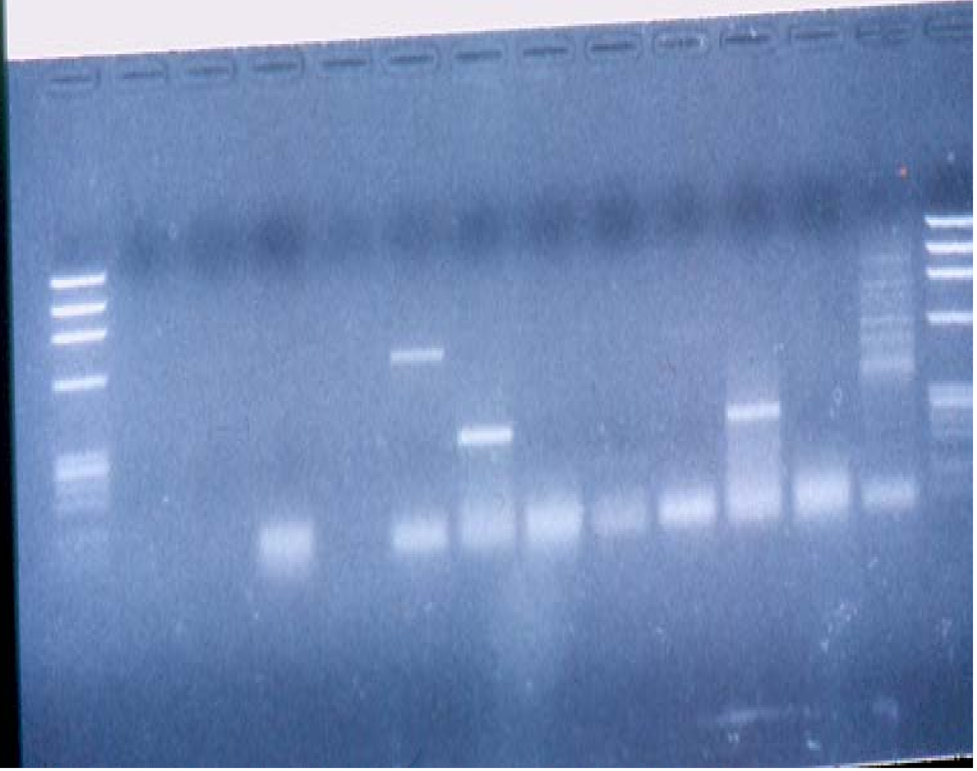
Figure 12: Organisation génomique des virus HIV1 et HIV2

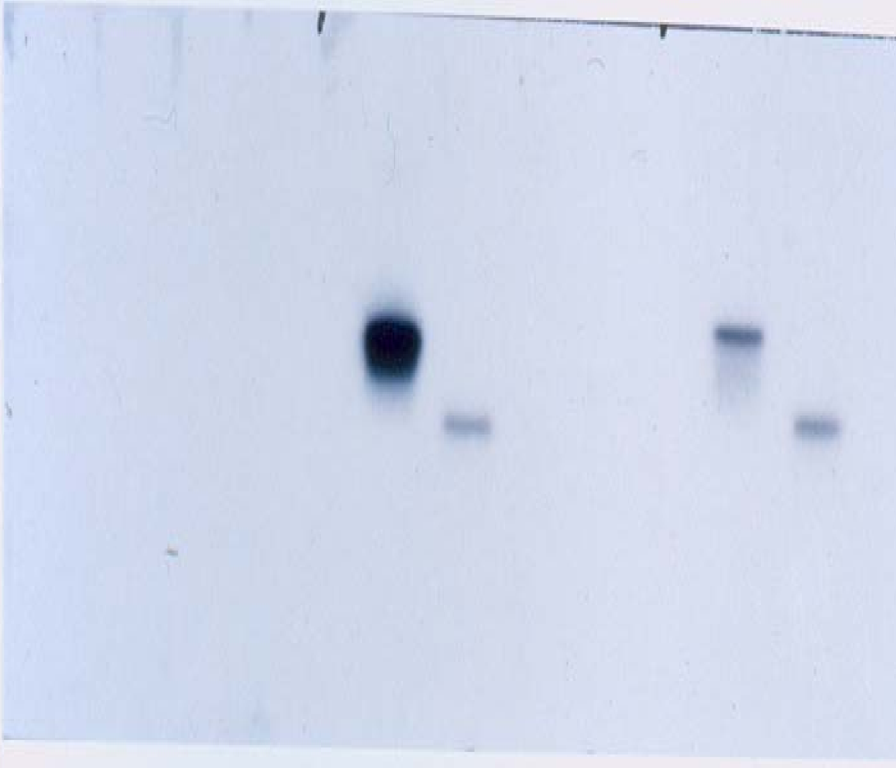


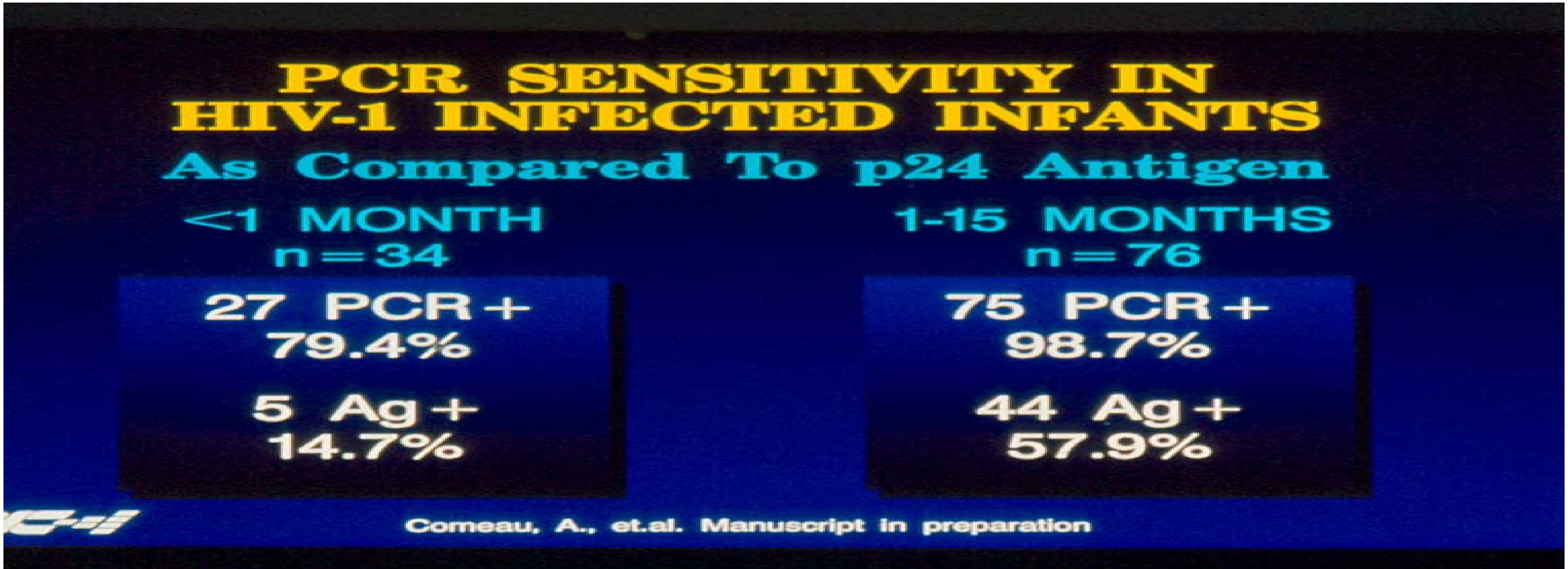
	gag	pol	env	nef1	nef2
	MMy2-MMy4	MMy28-MMy29'	MMy7-MMy8	MMy9-MMy10'	MMy12-MMy13'
HIV1-BRU	532	623	498	293	/
HIV1-MAL	556	584	498	302	/
HIV1-ELI	538	584	495	296	/
HIV2-ROD	544	666	/	/	400
SIV	544	712	/	/	400

	vif1	vif2	vpr	vpx	vpu
	MMy15-MMy17	MMy20-MMy21'	MMy18-MMy19	MMy23-MMy24	MMy25-MMy27
HIV1-BRU	603	/	281	/	263
HIV1-MAL	603	/	281	/	263
HIV1-ELI	603	/	281	/	263
HIV2-ROD	/	352	319	329	/
SIV	/	352	308	329	/







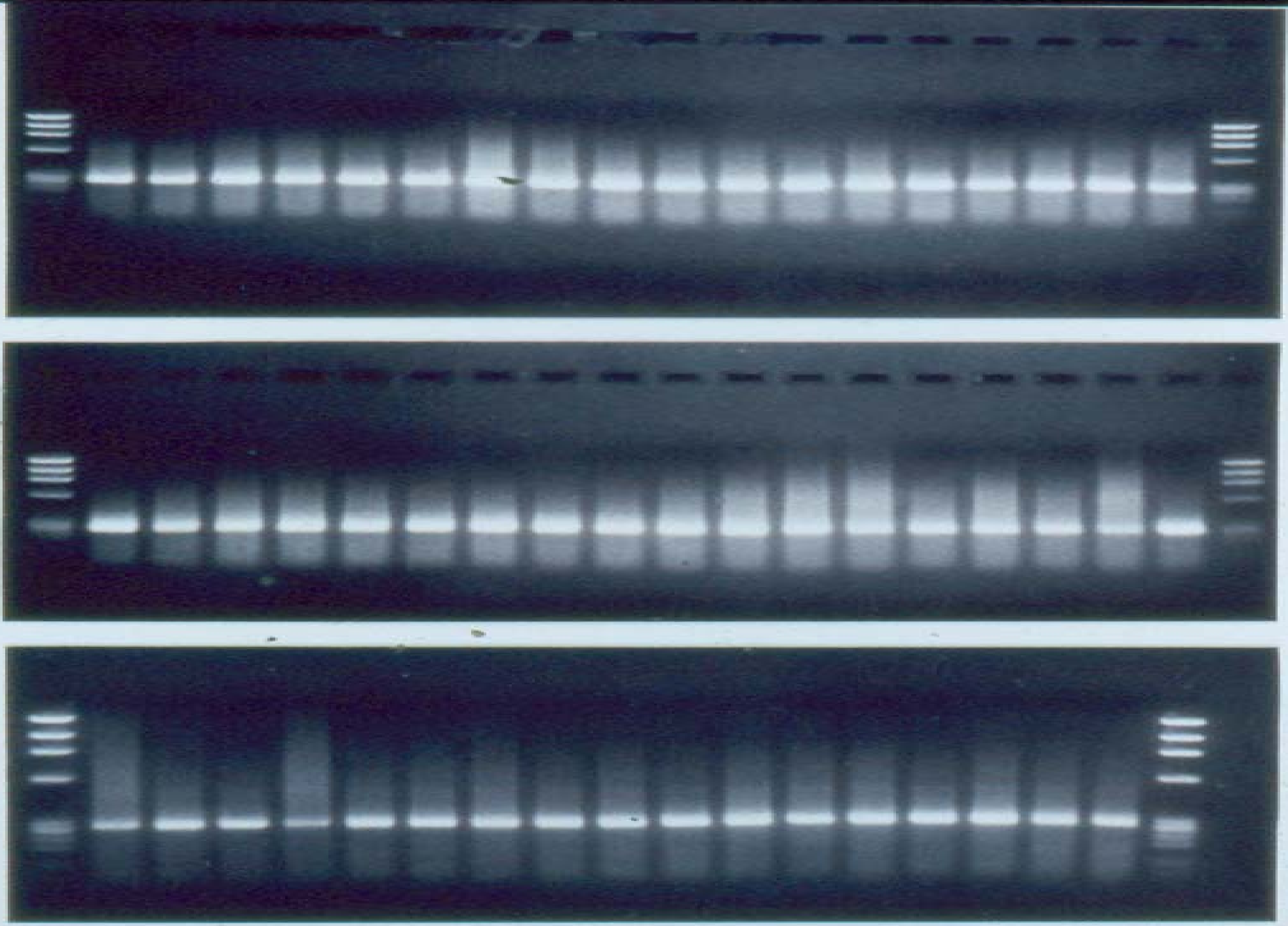


PCR SENSITIVITY IN HIV-1 INFECTED INFANTS

As Compared To p24 Antigen

<1 MONTH n=34	1-15 MONTHS n=76
27 PCR+ 79.4%	75 PCR+ 98.7%
5 Ag+ 14.7%	44 Ag+ 57.9%

Comeau, A., et.al. Manuscript in preparation



CALIBRATION OF THE PHC-2 THERMO-CYCLER.

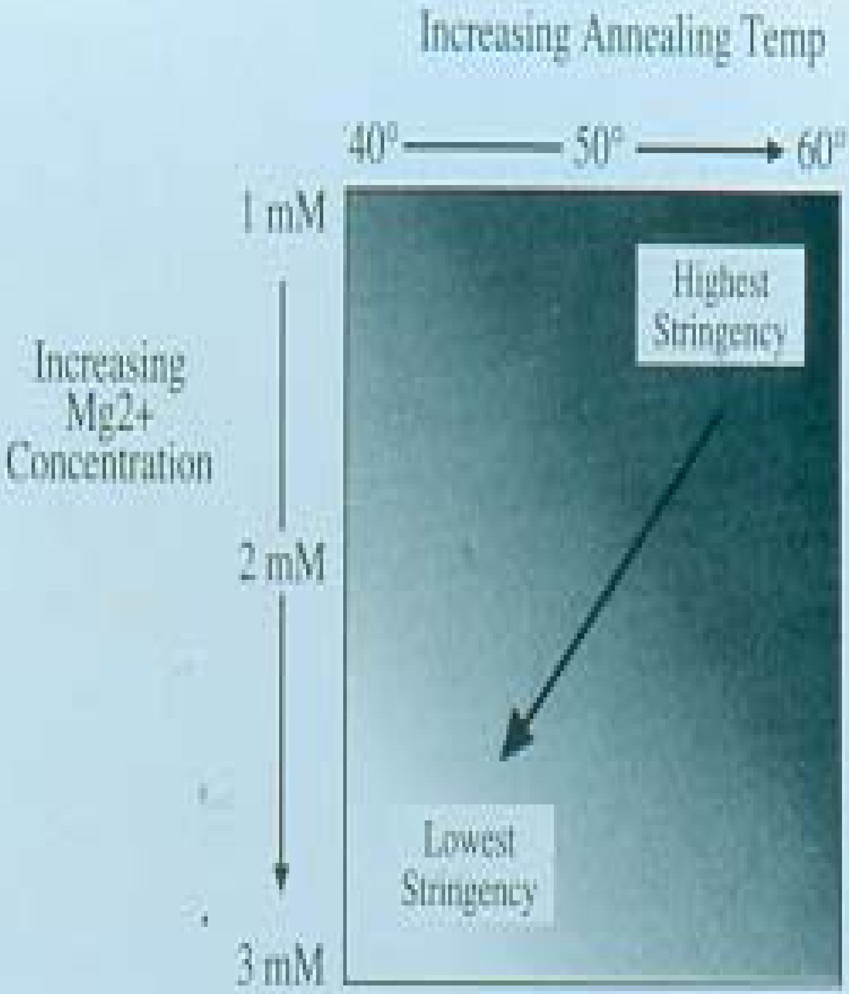
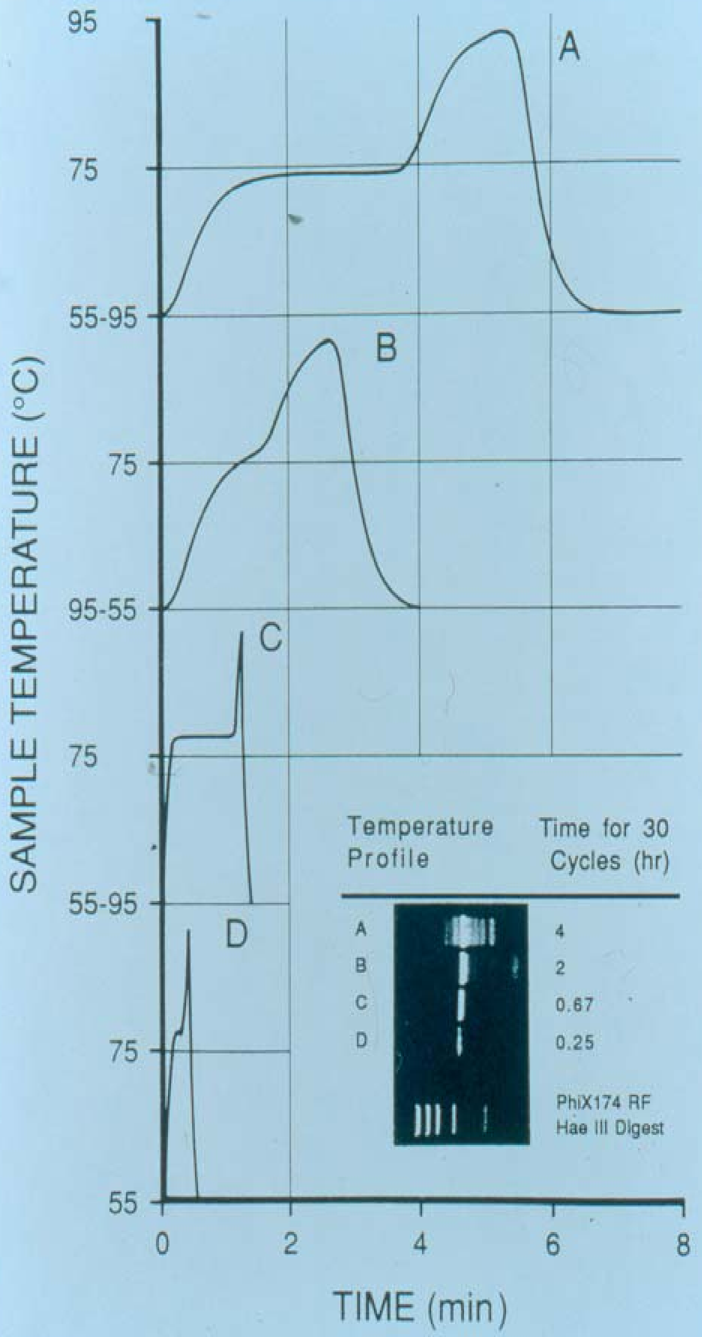

Increasing Annealing Temp
40° 50° 60°
1 mM
2 mM
3 mM
Increasing Mg2+ Concentration
Highest Stringency
Lowest Stringency

Figure 1. Effect of Mg^{2+} concentration and annealing temperature on stringency.



Temperature Profile	Time for 30 Cycles (hr)
A	4
B	2
C	0.67
D	0.25

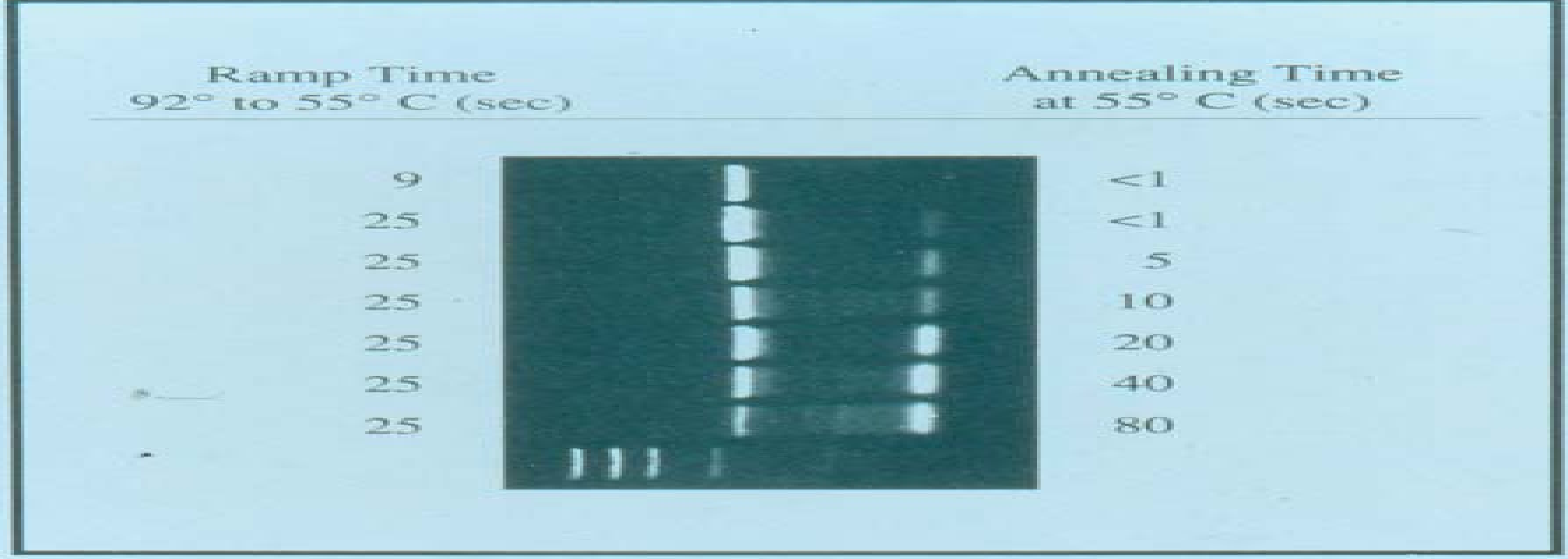

Ramp Time
92° to 55° C (sec)
Annealing Time
at 55° C (sec)
9
25
25
25
25
25
25
<1
<1
5
10
20
40
80

Figure 5. Effect of annealing time on DNA amplification reaction's specificity and yield.

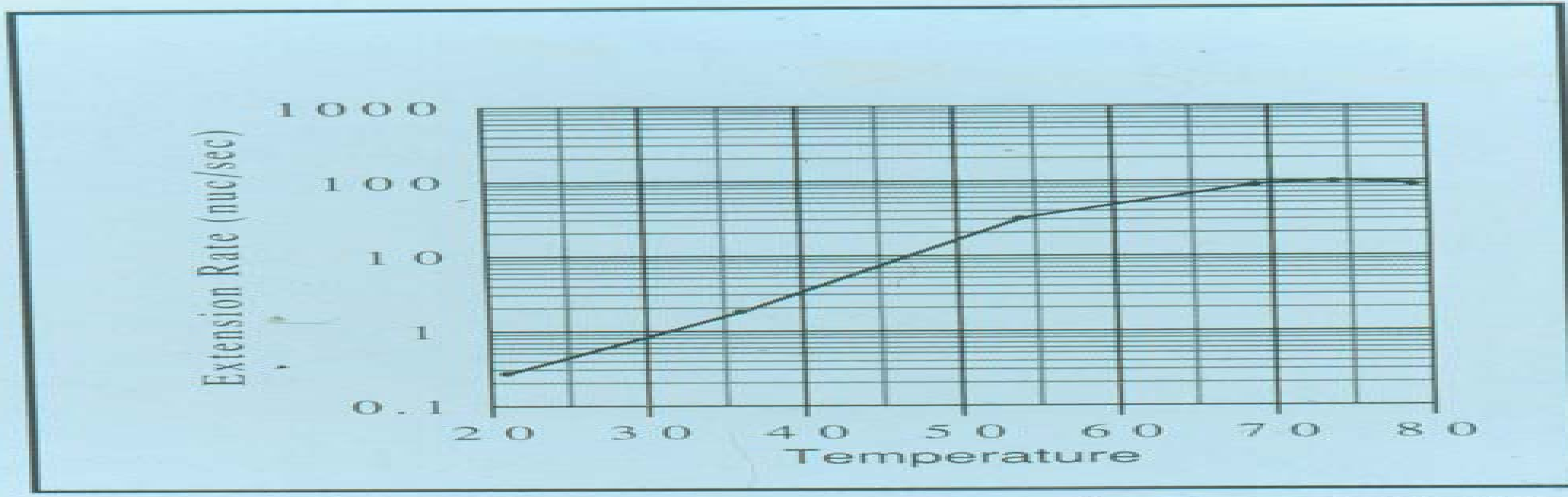


Figure 2. Extention rate vs temperature for Taq polymerase.

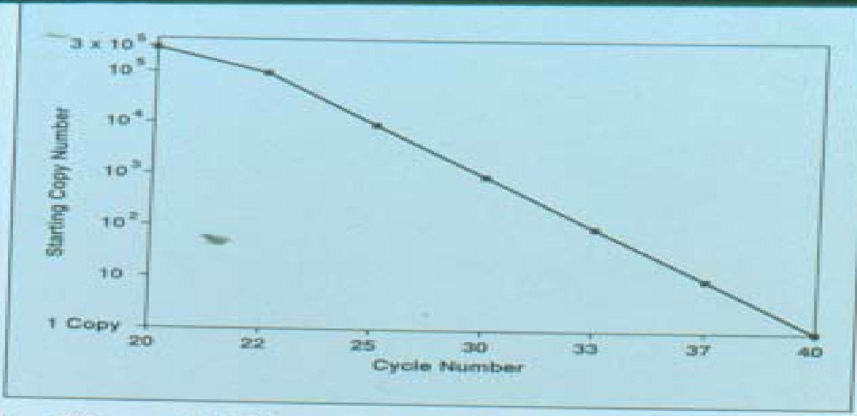


Figure 5. *When does the PCR product concentration reach 10^{-9} M? Graph shows when PCR product concentration reaches enzyme concentration. Two units of AmpliTaq DNA Polymerase is equivalent to 10^{-9} M. Assuming 85% efficiency in PCR, one copy, for example, will reach 10^{-9} M after approximately 40 cycles.*

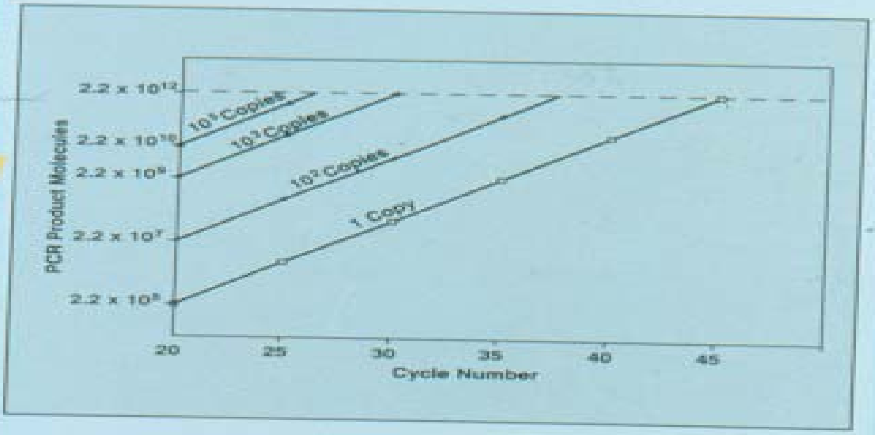


Figure 6. *PCR product copy number as a function of starting copy number of target. Saturation of the PCR amplification process occurs at 10^{12} copies of target. This graph shows the cycle at which saturation will occur with various starting target copy numbers.*

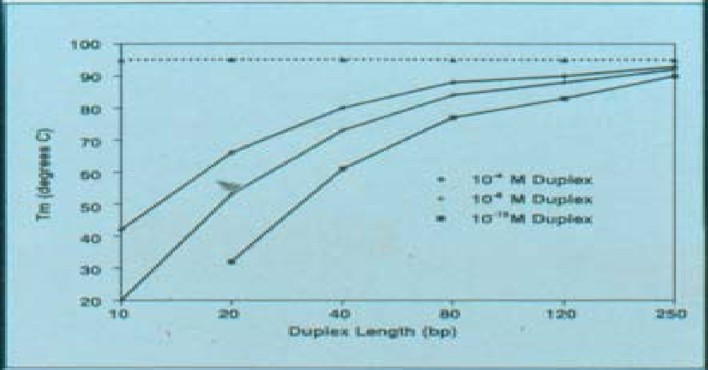


Figure 3. *Variation in duplex T_m as a function of length at various concentrations. Graph of the variation in duplex T_m as a function of length at various concentrations. At 10^{-15} M, the T_m of a 120-bp fragment is about 81°C. The T_m of that same 120-bp duplex when it reaches 10^{-4}M is about 90°C.*

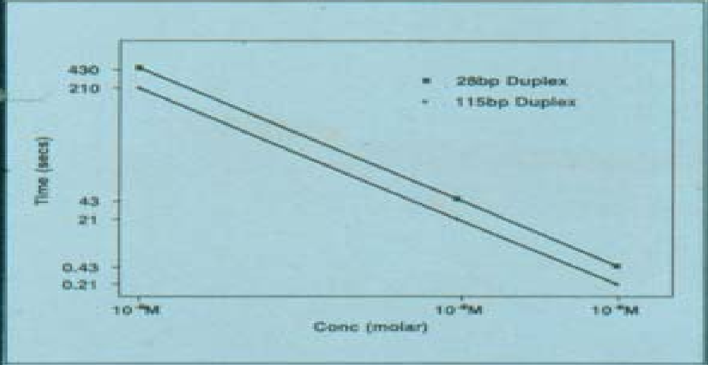
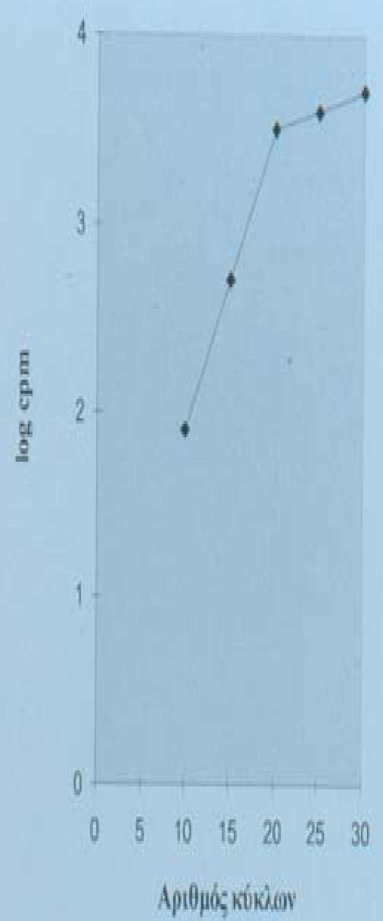
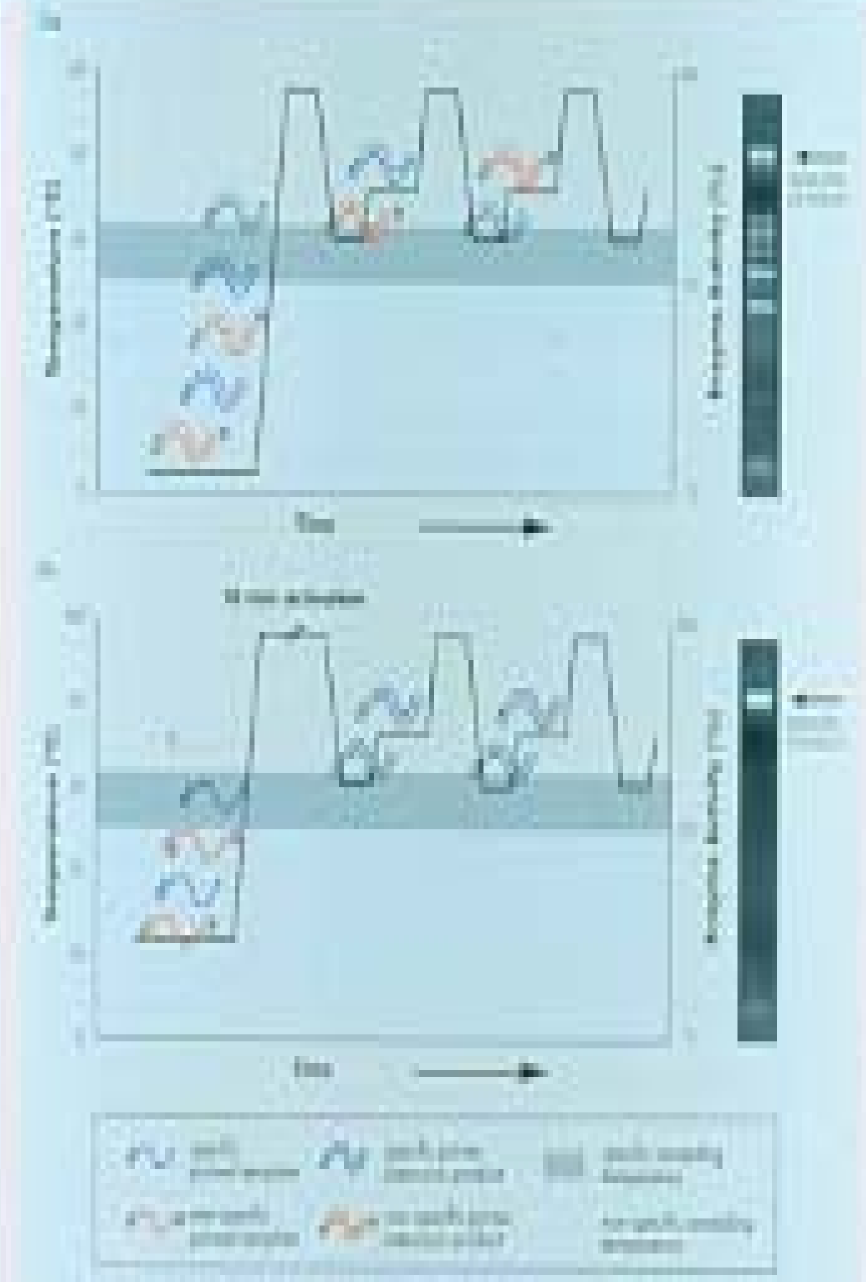
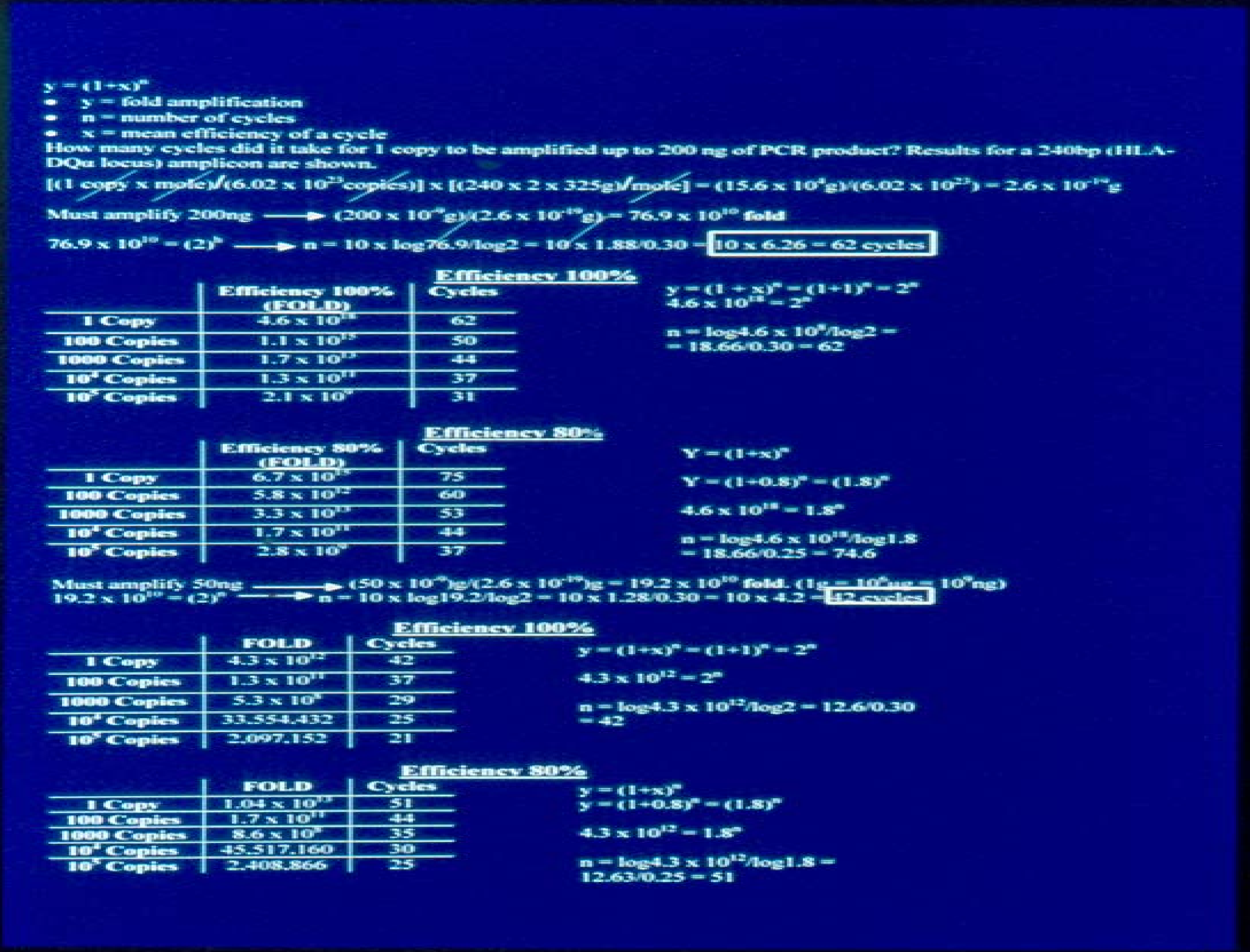


Figure 4. *Renaturation times for duplexes as a function of concentration. Graph showing the effect of DNA concentration on renaturation times for duplexes of various lengths. The assumption is that primer-template annealing can be related to the formation of a 28-bp duplex. The kinetics of reannealing is determined by the molecule in the greatest concentration. As the reaction proceeds, the primer concentration decreases, and it takes a longer time for the*



Εικόνα 6. Η γραφική παράσταση απεικονίζει τον log των cpm σε σχέση με τον αριθμό των κύκλων (από το 10ο έως τον 30ο). Η εκθετική φάση αύξησης του προϊόντος της γονιδιακής επέκτασης (♦) με μια σταθερή απόδοση λαμβάνει χώρα μόνο για ένα περιορισμένο αριθμό κύκλων μετά τον οποίο (στο συγκεκριμένο παράδειγμα μετά τον 20ο κύκλο) παύει να είναι εκθετική και φθάνει σε ένα plateau





$y = (1+x)^n$

- y = fold amplification
- n = number of cycles
- x = mean efficiency of a cycle

How many cycles did it take for 1 copy to be amplified up to 200 ng of PCR product? Results for a 240bp (HLA-DQα locus) amplicon are shown.

$[(1\ \cancel{\text{copy}} \times \cancel{\text{mole}})/(6.02 \times 10^{23}\ \cancel{\text{copies}})] \times [(240 \times 2 \times 325\text{g})/\cancel{\text{mole}}] = (15.6 \times 10^4\text{g})/(6.02 \times 10^{23}) = 2.6 \times 10^{-19}\text{g}$

Must amplify 200ng $\longrightarrow (200 \times 10^{-9}\cancel{\text{g}})/(2.6 \times 10^{-19}\cancel{\text{g}}) = 76.9 \times 10^{10}$ **fold**

$76.9 \times 10^{10} = (2)^n \longrightarrow n = 10 \times \log 76.9/\log 2 = 10 \times 1.88/0.30 = \boxed{10 \times 6.26 = 62 \text{ cycles}}$

Efficiency 100%

	Efficiency 100% (FOLD)	Cycles
1 Copy	4.6×10^{18}	62
100 Copies	1.1×10^{15}	50
1000 Copies	1.7×10^{13}	44
10^4 Copies	1.3×10^{11}	37
10^5 Copies	2.1×10^{9}	31

$y = (1 + x)^n = (1+1)^n = 2^n$
$4.6 \times 10^{18} = 2^n$

$n = \log 4.6 \times 10^{8}/\log 2 =$
$= 18.66/0.30 = 62$

Efficiency 80%

	Efficiency 80% (FOLD)	Cycles
1 Copy	6.7×10^{15}	75
100 Copies	5.8×10^{12}	60
1000 Copies	3.3×10^{13}	53
10^4 Copies	1.7×10^{11}	44
10^5 Copies	2.8×10^{9}	37

$Y = (1+x)^n$

$Y = (1+0.8)^n = (1.8)^n$

$4.6 \times 10^{18} = 1.8^n$

$n = \log 4.6 \times 10^{18}/\log 1.8$
$= 18.66/0.25 = 74.6$

Must amplify 50ng $\longrightarrow (50 \times 10^{-9})\text{g}/(2.6 \times 10^{-19})\text{g} = 19.2 \times 10^{10}$ **fold.** $(1\text{g} = 10^6\mu\text{g} = 10^9\text{ng})$
$19.2 \times 10^{10} = (2)^n \longrightarrow n = 10 \times \log 19.2/\log 2 = 10 \times 1.28/0.30 = 10 \times 4.2 = \boxed{42 \text{ cycles}}$

Efficiency 100%

	FOLD	Cycles
1 Copy	4.3×10^{12}	42
100 Copies	1.3×10^{11}	37
1000 Copies	5.3×10^{8}	29
10^4 Copies	33.554.432	25
10^5 Copies	2.097.152	21

$y = (1+x)^n = (1+1)^n = 2^n$

$4.3 \times 10^{12} = 2^n$

$n = \log 4.3 \times 10^{12}/\log 2 = 12.6/0.30$
$= 42$

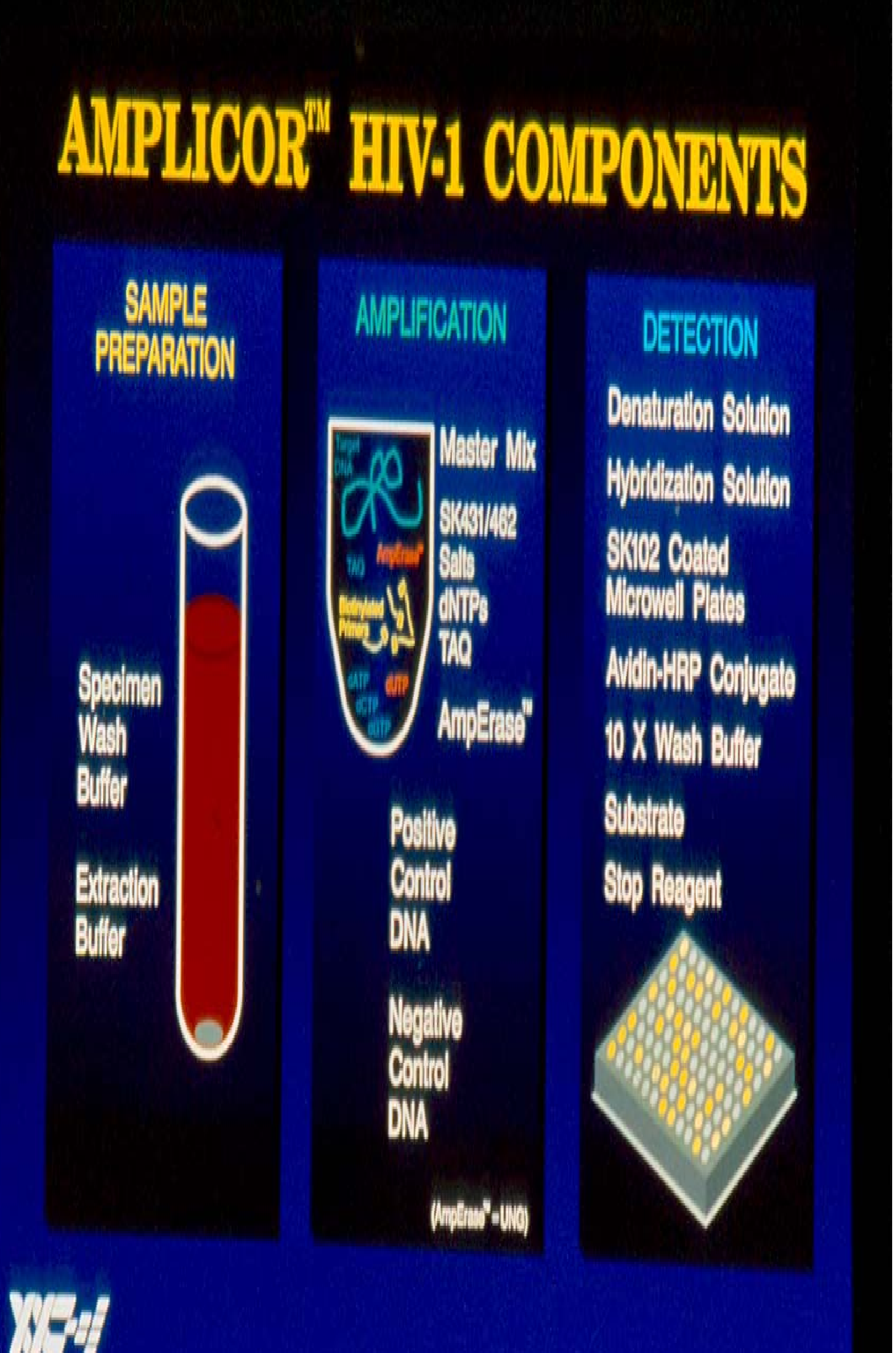
Efficiency 80%

	FOLD	Cycles
1 Copy	1.04×10^{13}	51
100 Copies	1.7×10^{11}	44
1000 Copies	8.6×10^{8}	35
10^4 Copies	45.517.160	30
10^5 Copies	2.408.866	25

$y = (1+x)^n$
$y = (1+0.8)^n = (1.8)^n$

$4.3 \times 10^{12} = 1.8^n$

$n = \log 4.3 \times 10^{12}/\log 1.8 =$
$12.63/0.25 = 51$



AMPLICOR™ HIV-1 COMPONENTS

SAMPLE PREPARATION

Specimen Wash Buffer

Extraction Buffer

AMPLIFICATION

Target DNA
TAQ
AmpErase
Biotinylated Primers
dATP
dCTP
dGTP
dUTP

Master Mix

SK431/462
Salts
dNTPs
TAQ

AmpErase™

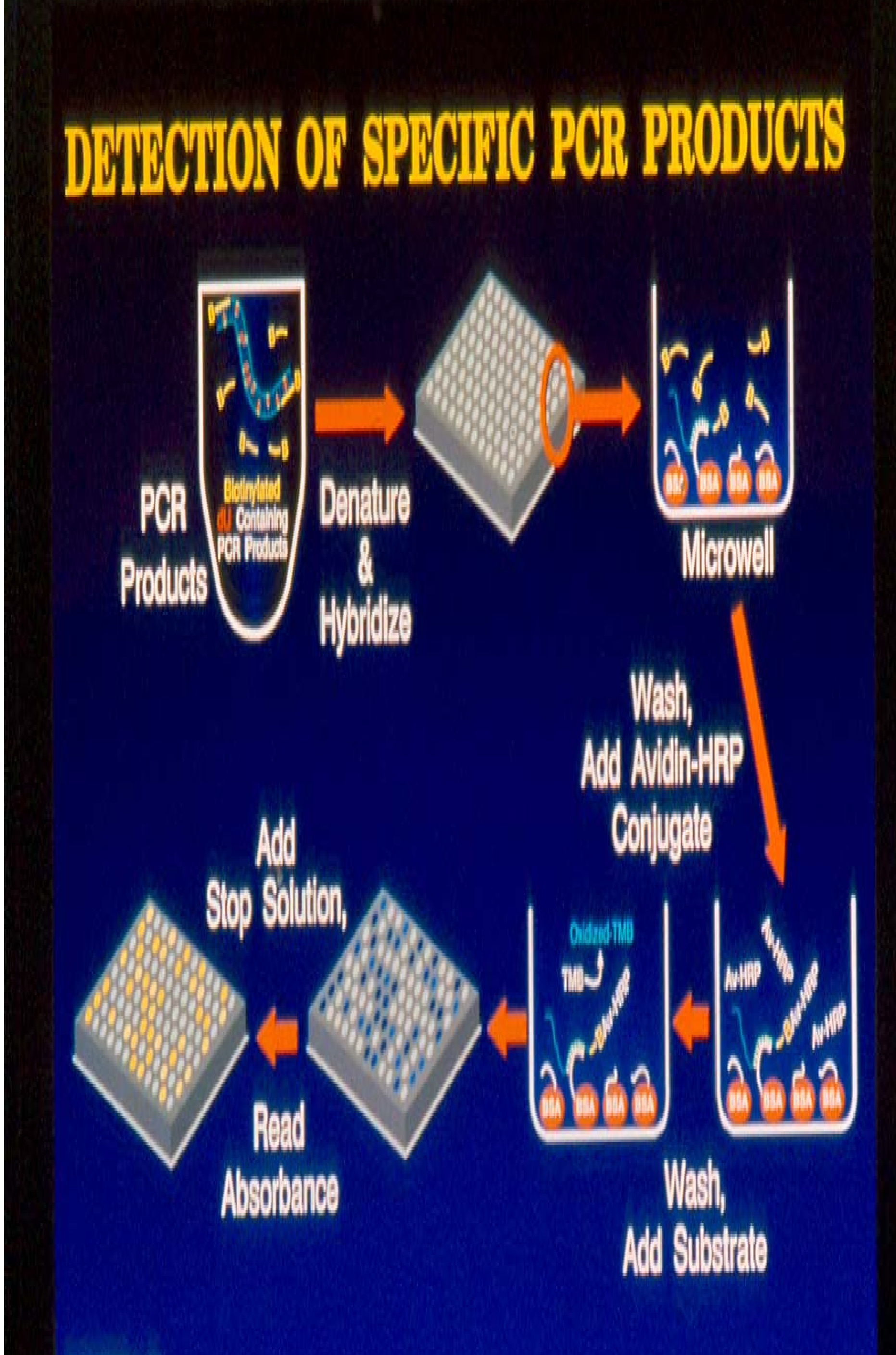
Positive Control DNA

Negative Control DNA

(AmpErase™ = UNG)

DETECTION

- Denaturation Solution
- Hybridization Solution
- SK102 Coated Microwell Plates
- Avidin-HRP Conjugate
- 10 X Wash Buffer
- Substrate
- Stop Reagent



DETECTION OF SPECIFIC PCR PRODUCTS

Biotinylated dU Containing PCR Products
BSA
Oxidized-TMB
TMB
Av-HRP

PCR Products

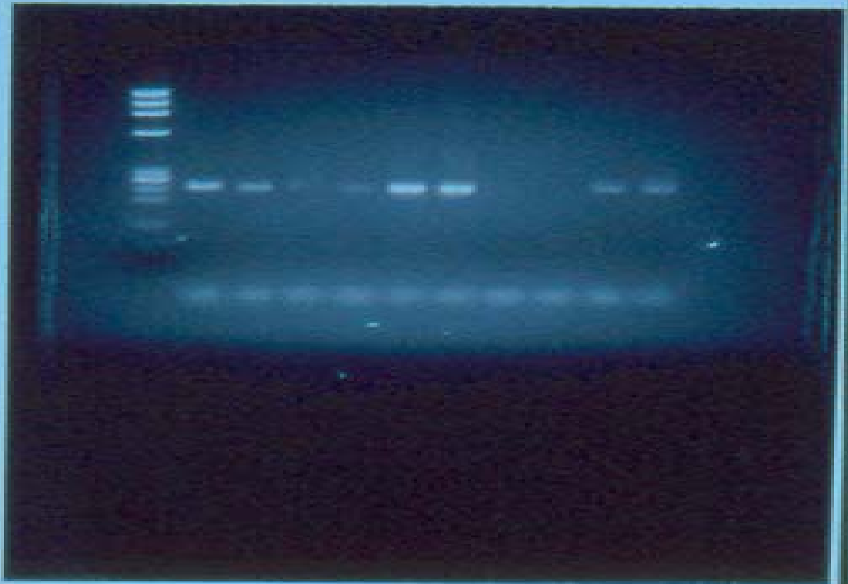
Denature & Hybridize

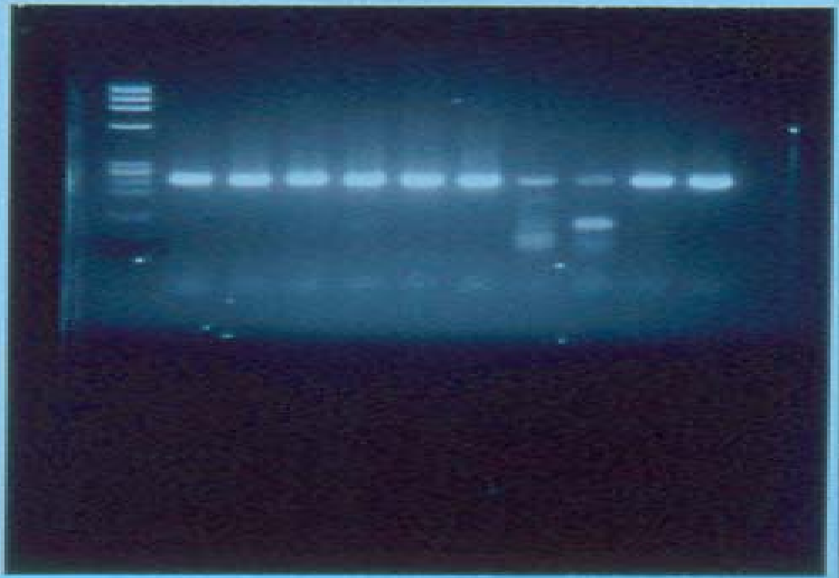
Microwell

Wash, Add Avidin-HRP Conjugate

Wash, Add Substrate

Add Stop Solution, Read Absorbance





Nested Primers for PCR

PCR is a powerful method to amplify specific sequences of DNA from a large complex mixture of DNA. For example, you can design PCR primers to amplify a single locus from an entire genome. From a single template molecule, you can produce over 1 billion copies of the PCR product very quickly. However, the capacity to amplify over one billion fold also increases the possibility of amplifying the wrong DNA sequence over one billion times.

The specificity of PCR is determined by the specificity of the PCR primers. For example, if your primers bind to more than one locus , then more than one segment of DNA will be amplified. To control for these possibilities, investigators often employ nested primers to ensure specificity.

Nested PCR means that two pairs of PCR primers were used for a single locus (figure 1). The first pair amplified the locus as seen in any PCR experiment. The second pair of primers (nested primers) bind within the first PCR product (figure 4) and produce a second PCR product that will be shorter than the first one (figure 5).

The logic behind this strategy is that if the wrong locus were amplified by mistake, the probability is very low that it would also be amplified a second time by a second pair of primers.

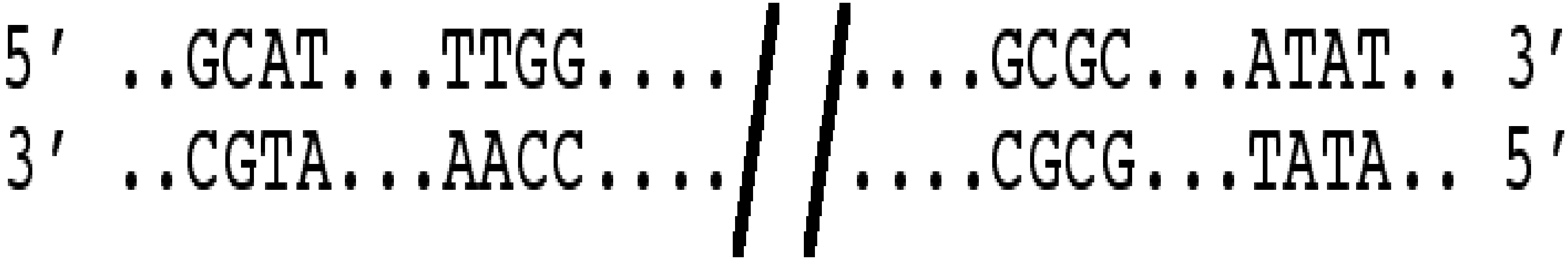

5′ ..GCAT...TTGG....
....GCGC...ATAT.. 3′
3′ ..CGTA...AACC....
....CGCG...TATA.. 5′

Figure 1. Nested PCR strategy. Segment of DNA with dots representing nondiscript DNA sequence of unspecified length. The double lines represent a large distance between the portion of DNA illustrated in this figure. The portions of DNA shown with four bases in a row represent PCR primer binding sites, though real primers would be longer.

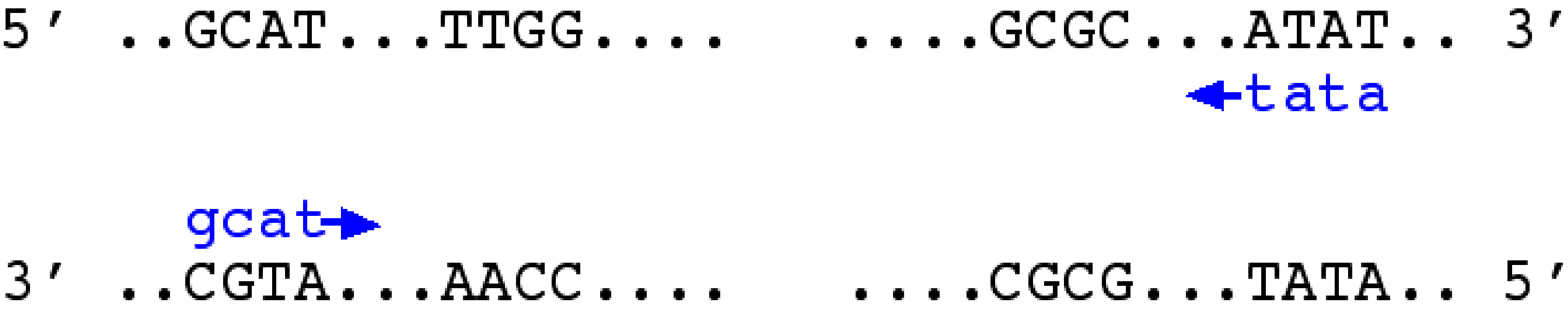
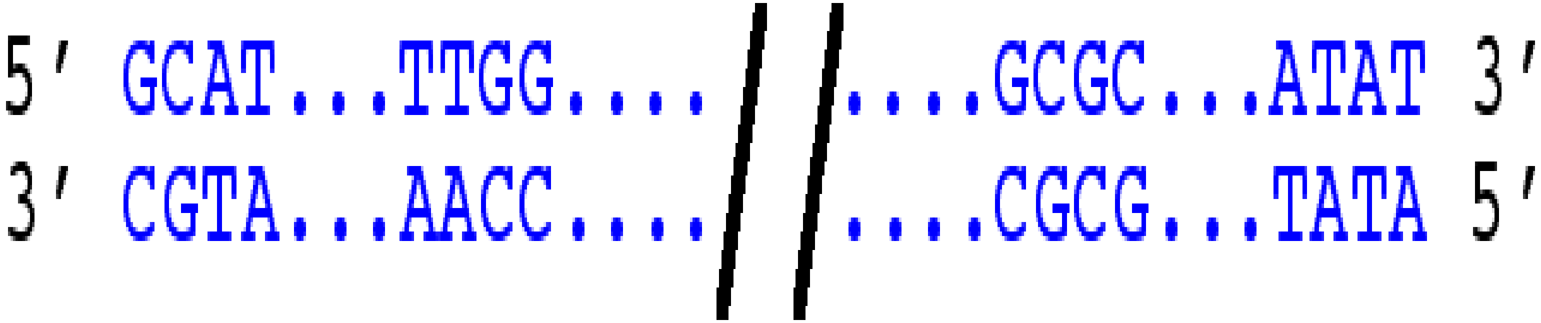

5′ ..GCAT...TTGG....
....GCGC...ATAT.. 3′
tata
gcat
3′ ..CGTA...AACC....
....CGCG...TATA.. 5′

Figure 2. The first pair of PCR primers (blue with arrows) bind to the outer pair of primer binding sites and amplify all the DNA in between these two sites.


5′ GCAT...TTGG.... ....GCGC...ATAT 3′
3′ CGTA...AACC.... ....CGCG...TATA 5′

.

Figure 3. PCR product after the first round of amplificaiton. Notice that the bases outside the PCR primer pair are not present in the product.

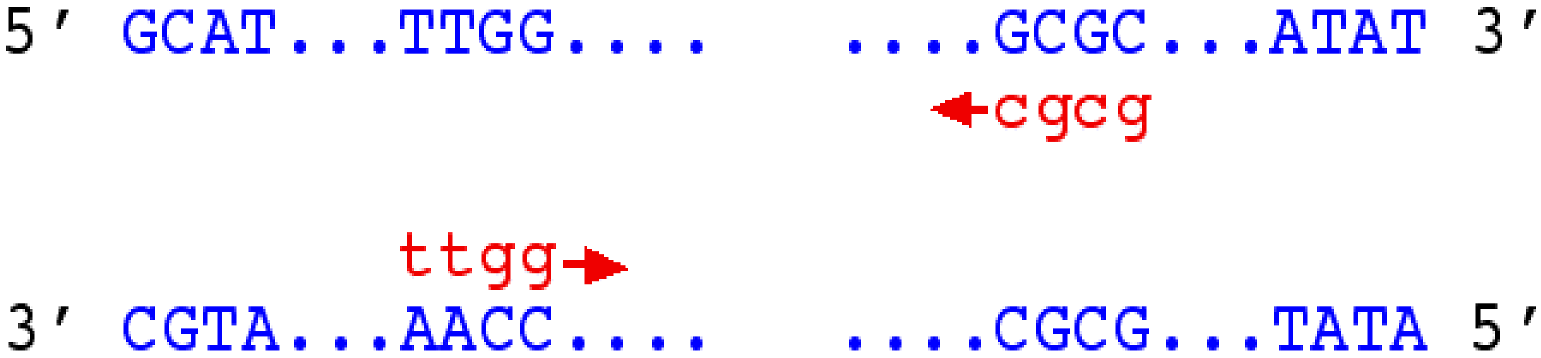

5′ GCAT...TTGG.... ....GCGC...ATAT 3′
cgcg
ttgg
3′ CGTA...AACC.... ....CGCG...TATA 5′

Figure 4. Second pair of **nested primers** (red with arrows) bind to the first PCR product. The binding sites for the second pair of primers are a few bases "internal" to the first primer binding sites

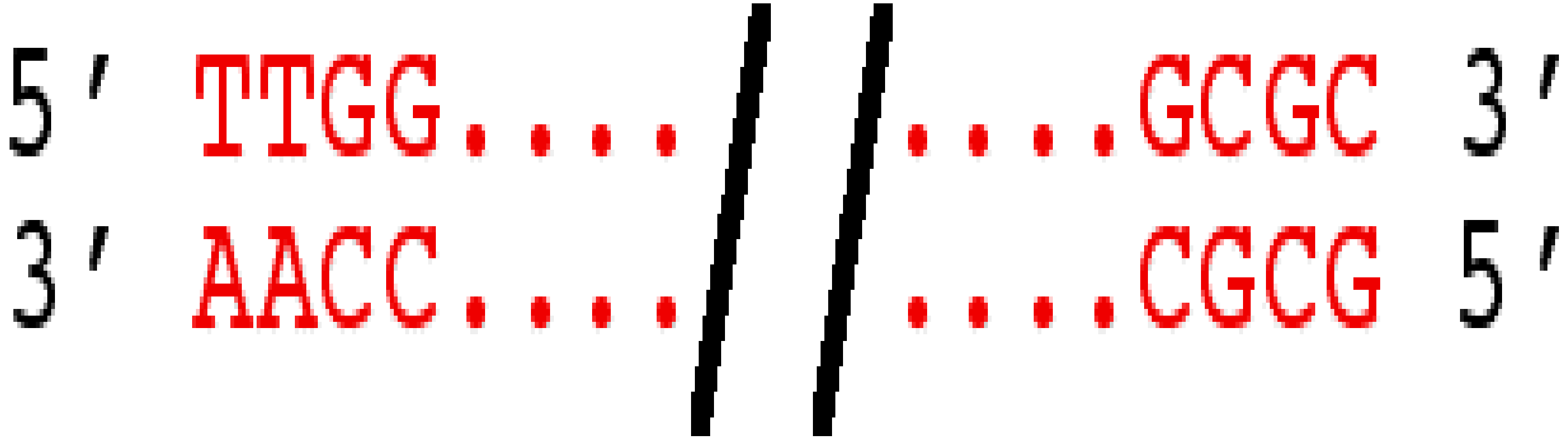
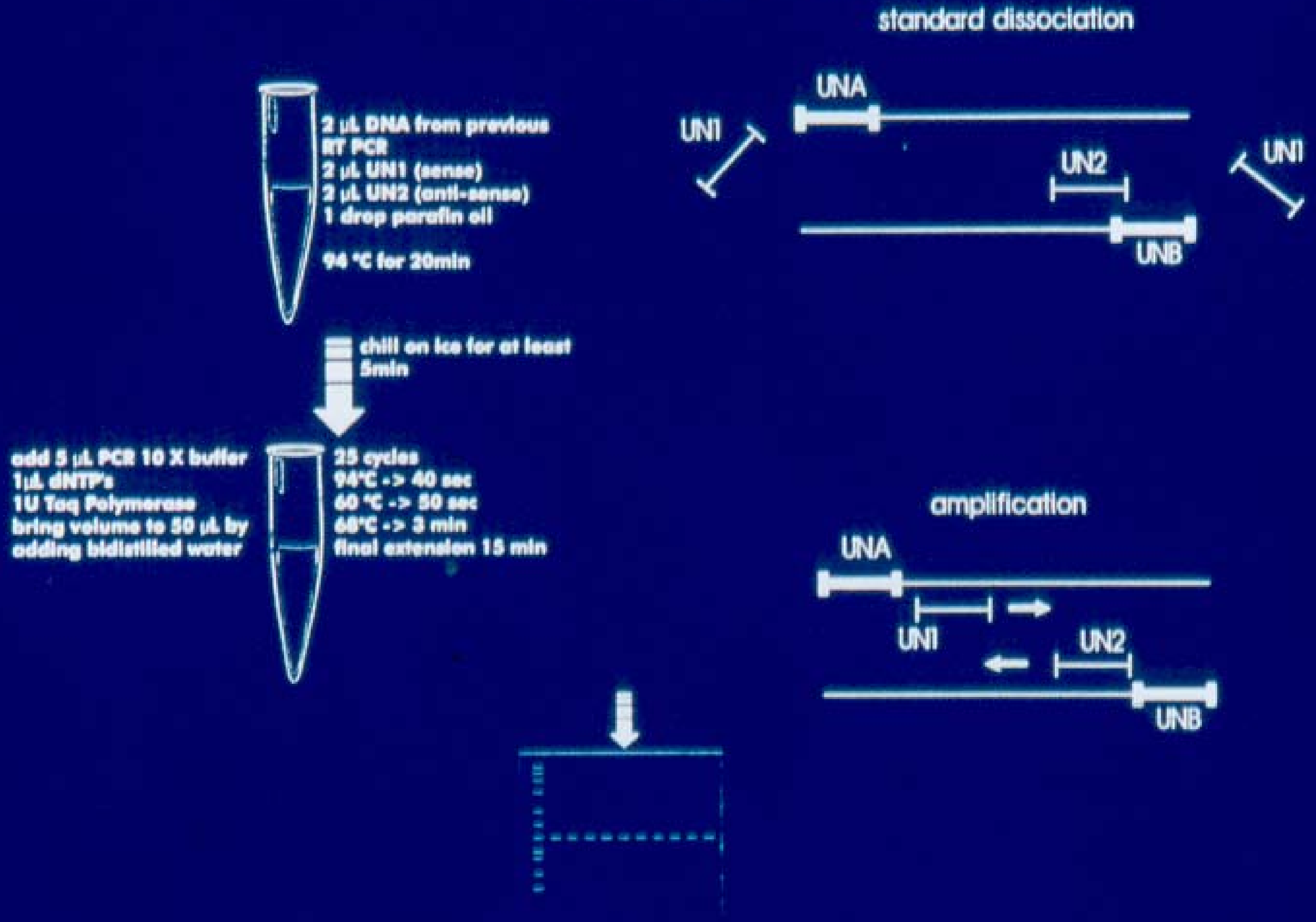


Figure 5. Final PCR product after second round of PCR. The length of the product is defined by the location of the internal primer binding sites.

When a complete genome sequence is known, it is easier to be sure you will not amplify the wrong locus but since very few of the world's genomes have been sequenced completely, nested primers will continue to be an important control for many experiments.

Nested PCR



2 µL DNA from previous RT PCR
2 µL UN1 (sense)
2 µL UN2 (anti-sense)
1 drop parafin oil

94 °C for 20min

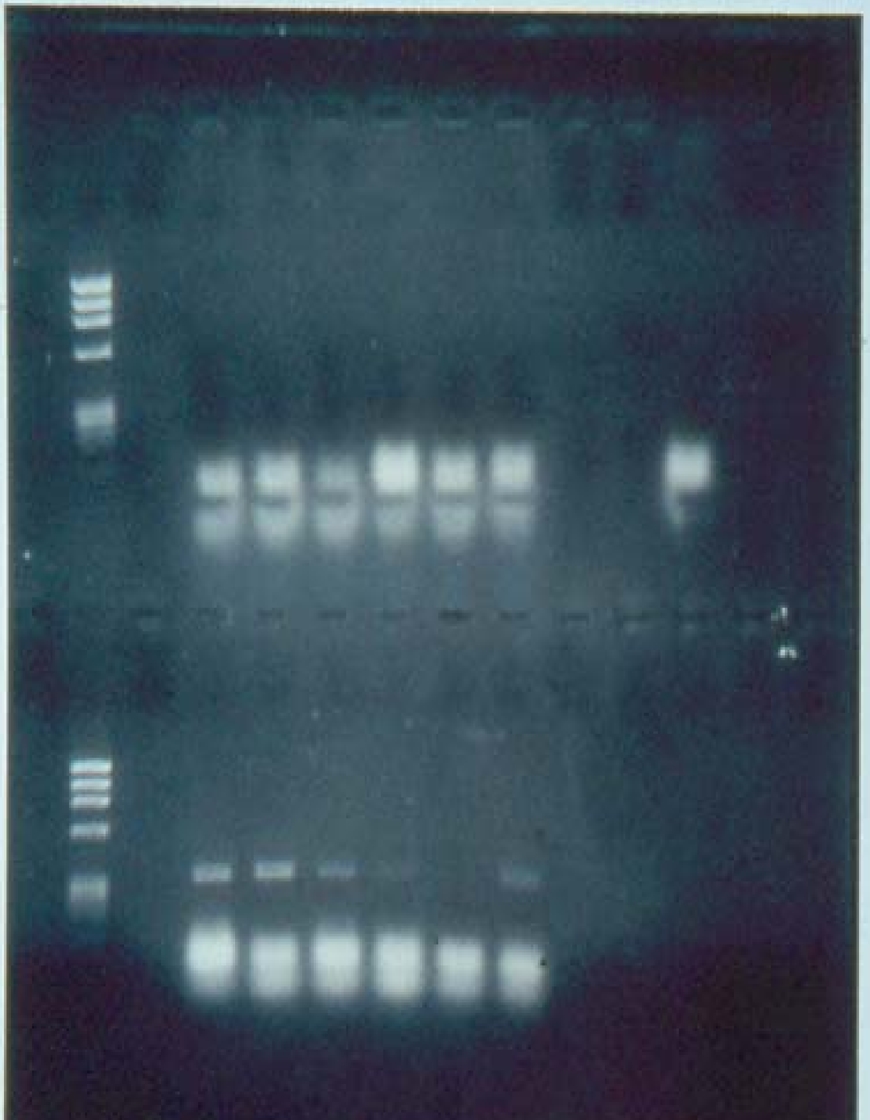
chill on ice for at least 5min

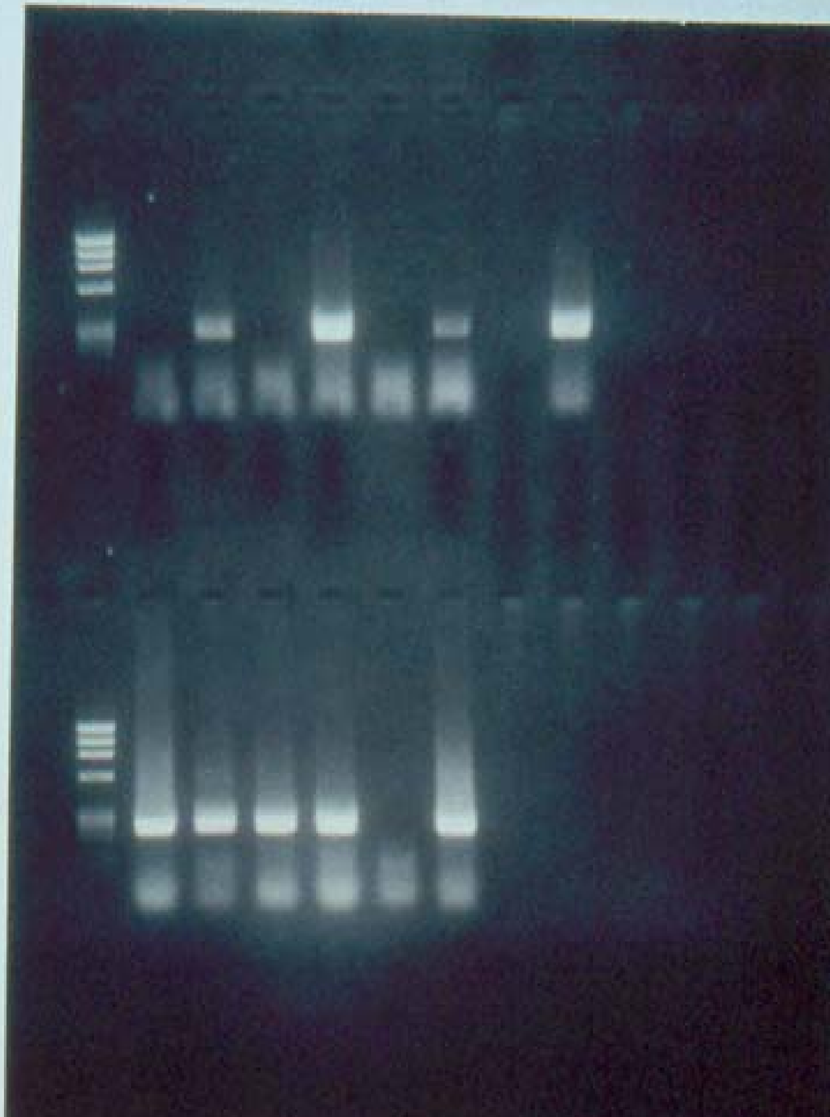
add 5 µL PCR 10 X buffer
1µL dNTP's
1U Taq Polymerase
bring volume to 50 µL by adding bidistilled water

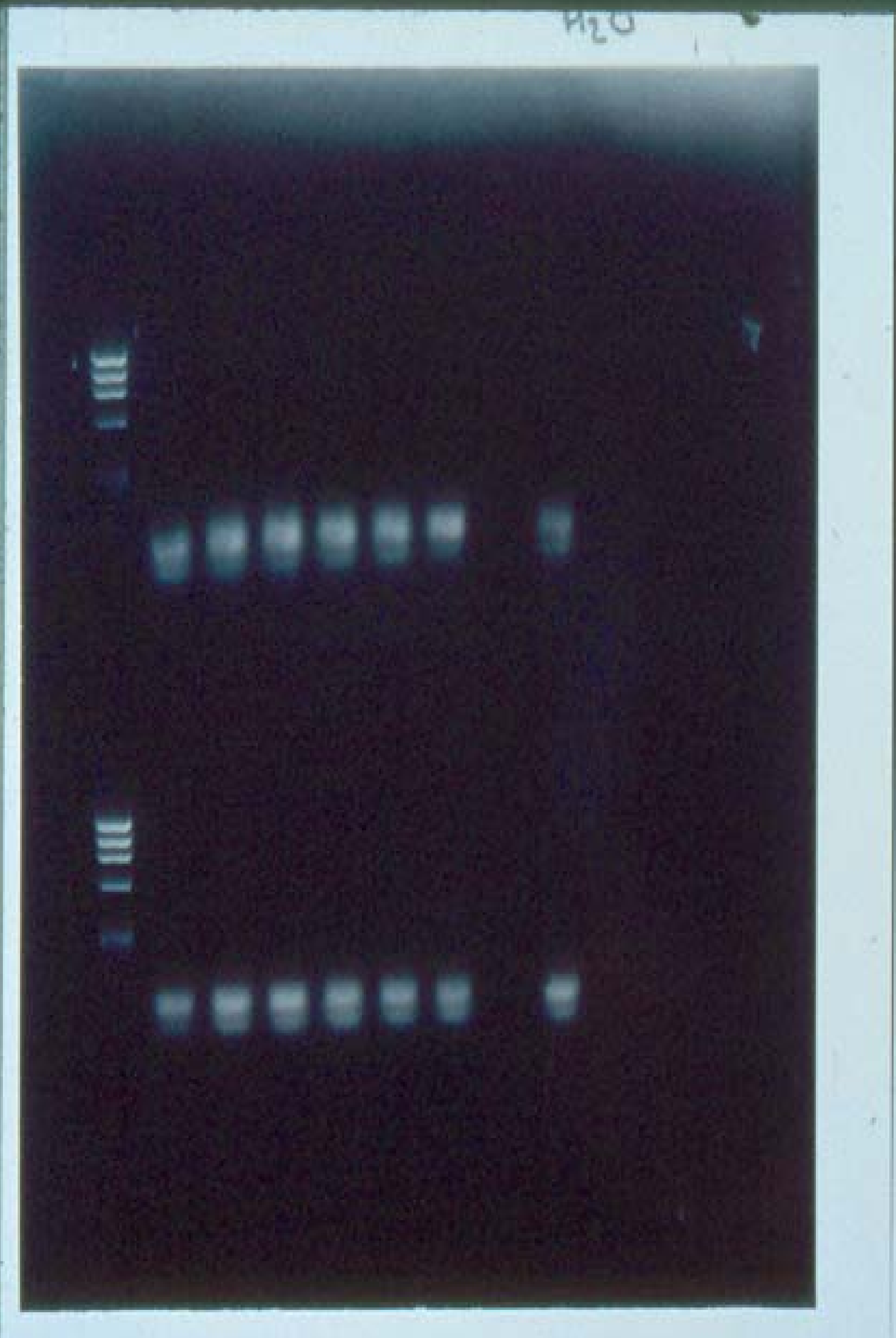
25 cycles
94°C -> 40 sec
60 °C -> 50 sec
68°C -> 3 min
final extension 15 min

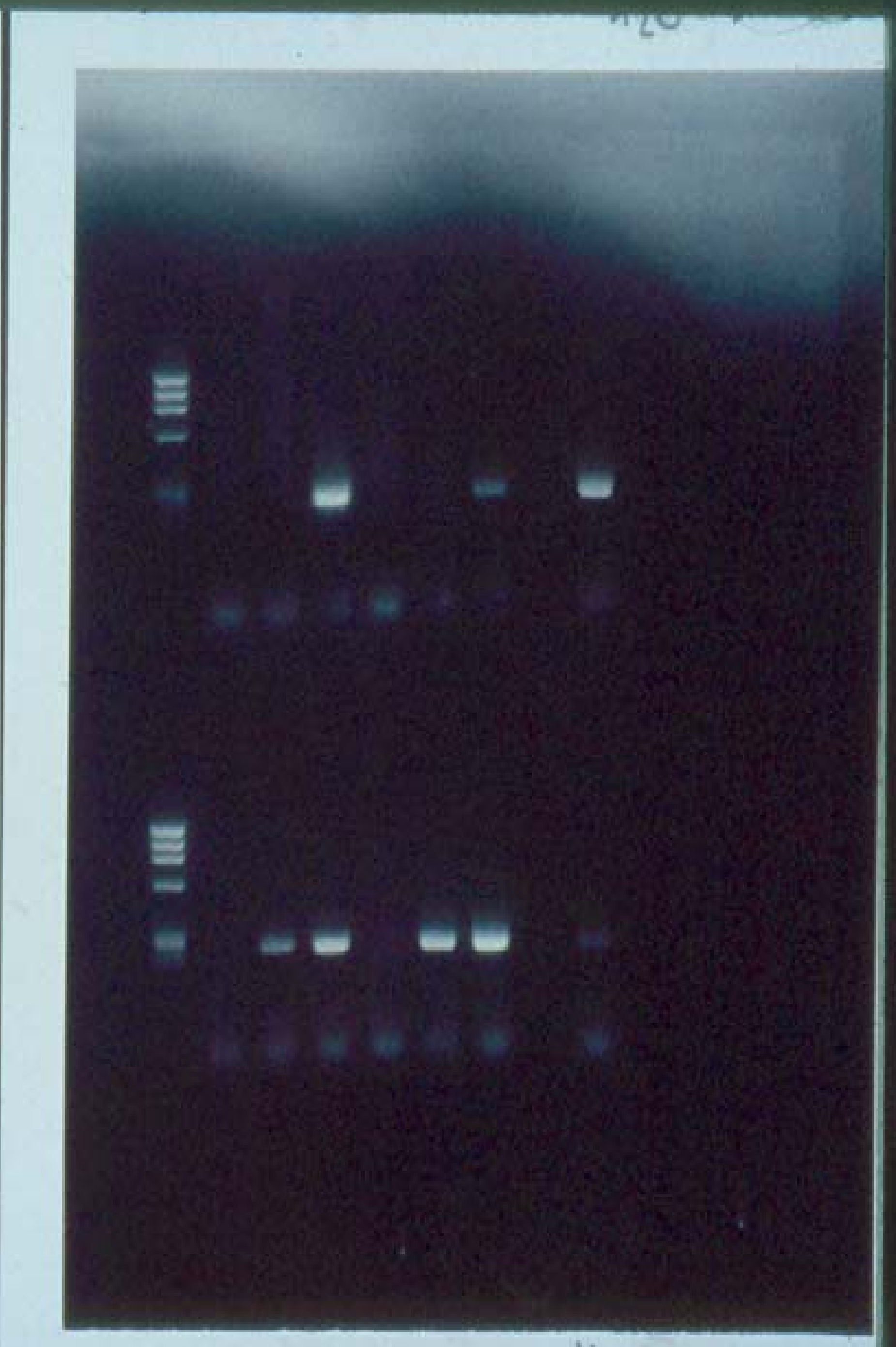
standard dissociation

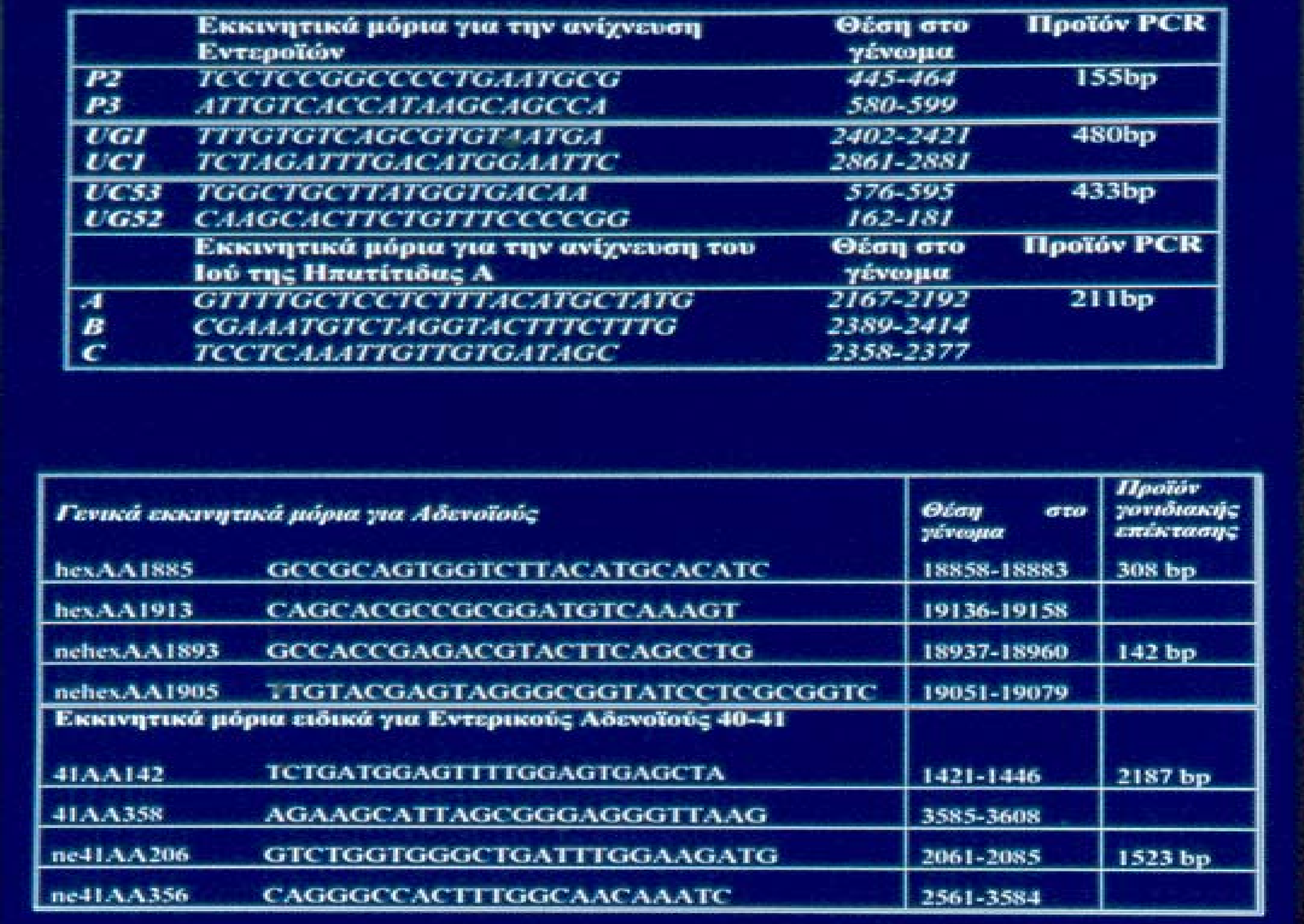
amplification





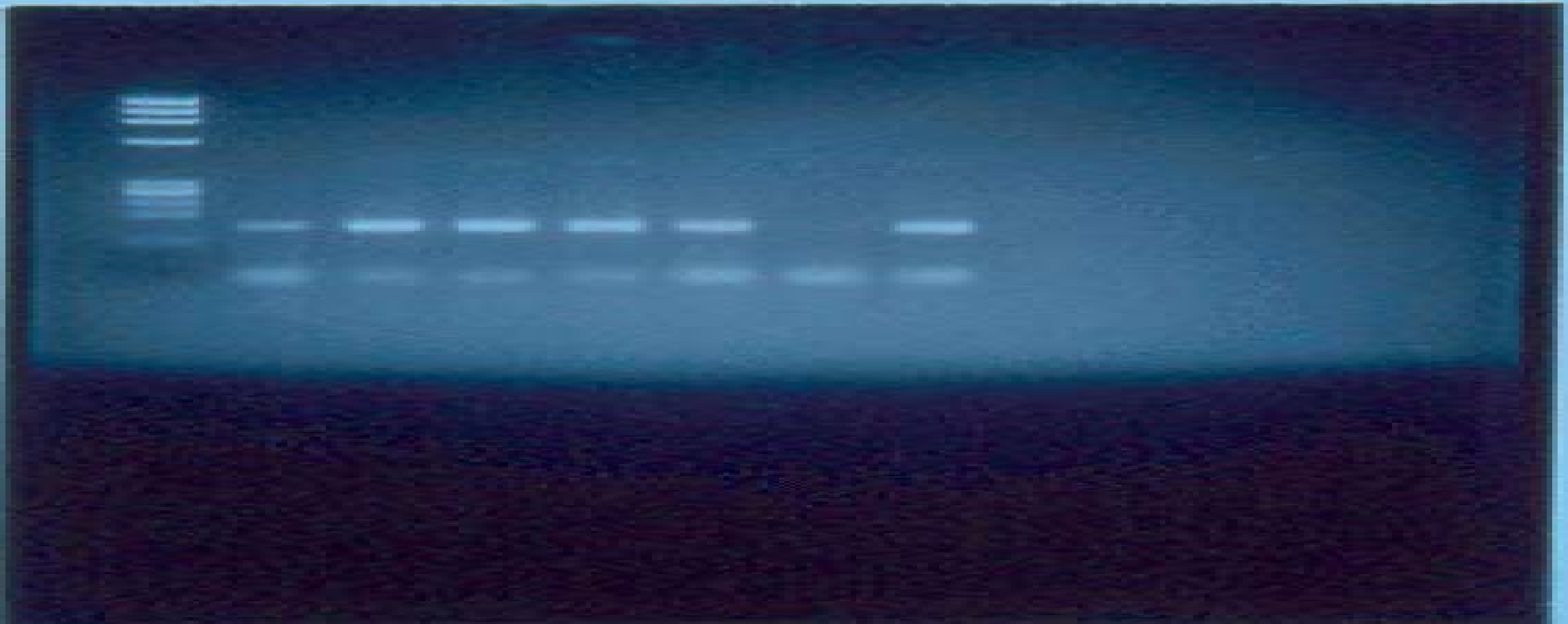

H₂O

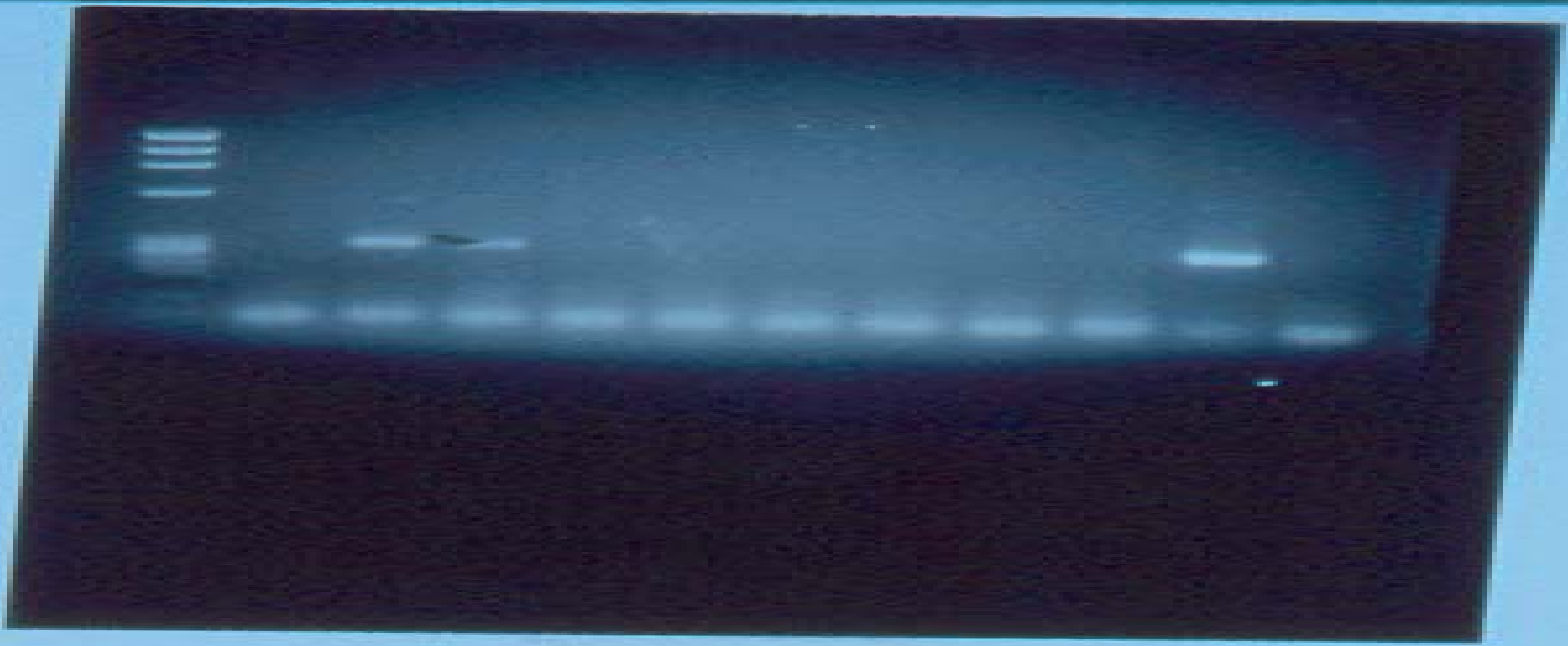

H₂O

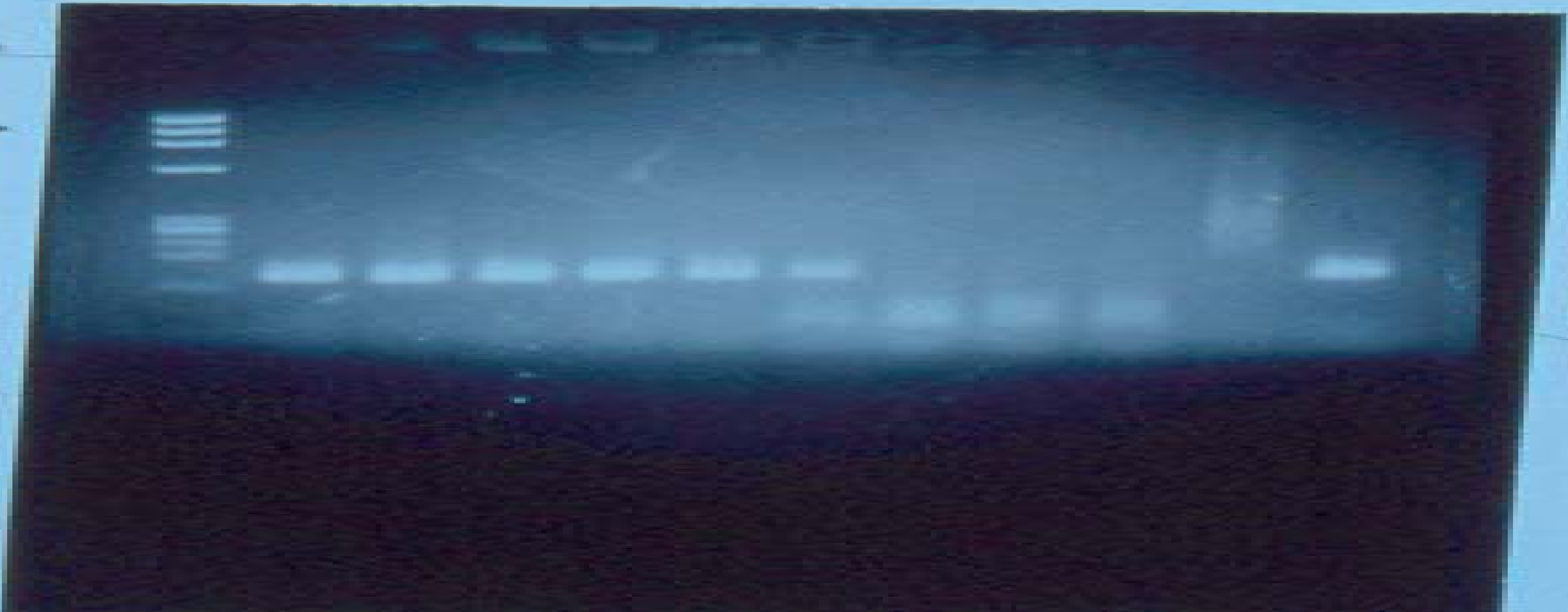


	Εκκινητικά μόρια για την ανίχνευση Εντεροϊών	**Θέση στο γένωμα**	**Προϊόν PCR**
P2	*TCCTCCGGCCCCTGAATGCG*	*445-464*	**155bp**
P3	*ATTGTCACCATAAGCAGCCA*	*580-599*	
UG1	*TTTGTGTCAGCGTGTAATGA*	*2402-2421*	**480bp**
UC1	*TCTAGATTTGACATGGAATTC*	*2861-2881*	
UC53	*TGGCTGCTTATGGTGACAA*	*576-595*	**433bp**
UG52	*CAAGCACTTCTGTTTCCCCGG*	*162-181*	
	Εκκινητικά μόρια για την ανίχνευση του Ιού της Ηπατίτιδας Α	**Θέση στο γένωμα**	**Προϊόν PCR**
A	*GTTTTGCTCCTCTTTACATGCTATG*	*2167-2192*	**211bp**
B	*CGAAATGTCTAGGTACTTTCTTTG*	*2389-2414*	
C	*TCCTCAAATTGTTGTGATAGC*	*2358-2377*	

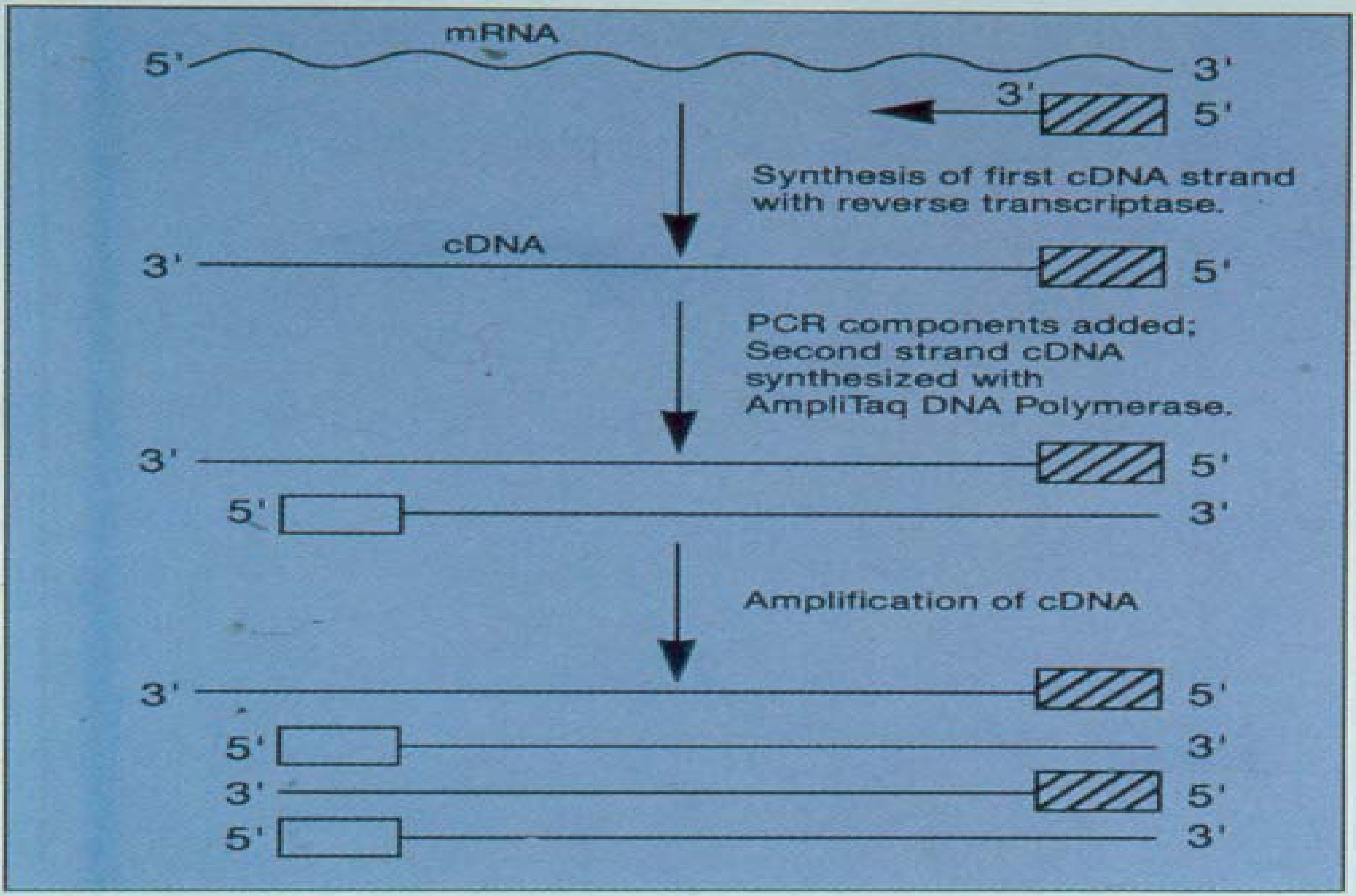
Γενικά εκκινητικά μόρια για Αδενοϊούς		***Θέση στο γένωμα***	***Προϊόν γονιδιακής επέκτασης***
hexAA1885	GCCGCAGTGGTCTTACATGCACATC	18858-18883	308 bp
hexAA1913	CAGCACGCCGCGGATGTCAAAGT	19136-19158	
nehexAA1893	GCCACCGAGACGTACTTCAGCCTG	18937-18960	142 bp
nehexAA1905	TTGTACGAGTAGGGCGGTATCCTCGCGGTC	19051-19079	
Εκκινητικά μόρια ειδικά για Εντερικούς Αδενοϊούς 40-41			
41AA142	TCTGATGGAGTTTTGGAGTGAGCTA	1421-1446	2187 bp
41AA358	AGAAGCATTAGCGGGAGGGTTAAG	3585-3608	
ne41AA206	GTCTGGTGGGCTGATTTGGAAGATG	2061-2085	1523 bp
ne41AA356	CAGGGCCACTTTGGCAACAAATC	2561-3584	







RNA PCR Process



Synthesis of first cDNA strand with reverse transcriptase.

PCR components added; Second strand cDNA synthesized with AmpliTaq DNA Polymerase.

Amplification of cDNA

Figure 1: *Reverse transcription of RNA to cDNA and subsequent amplification of the synthesized cDNA, using the GeneAmp PCR process.*

cDNA Production

Central dogma states that biological information goes from DNA to RNA to protein (figure 1). However, there are times when information goes from RNA to DNA. Viruses such as HIV have RNA genomes that can be converted into DNA by an enzyme called reverse transcriptase. Molecular biologists realized that they could use reverse transcriptase to convert mRNA into complementary DNA and thus was born the term cDNA

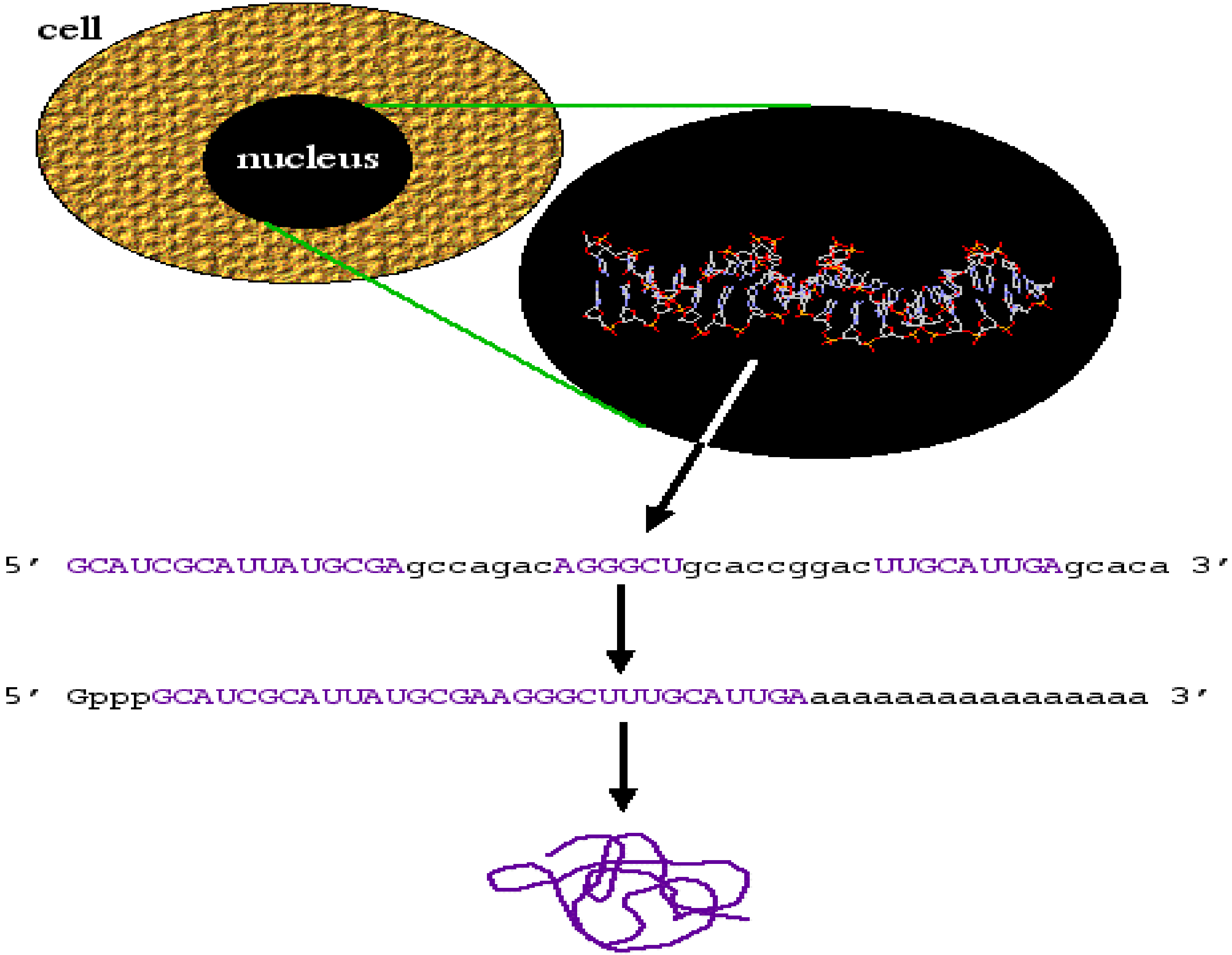

cell
nucleus
5′ GCAUCGCAUUAUGCGAgccagacAGGGCUgcaccggacUUGCAUUGAgcaca 3′
5′ GpppGCAUCGCAUUAUGCGAAGGGCUUUGCAUUGAaaaaaaaaaaaaaaaa 3′

Figure 1. Central dogma: DNA to RNA to mRNA to protein. Coding sequence (purple) exons are spliced together and the 5' cap and 3' polyA tail is added to produce a mature mRNA molecule from the primary transcript. The mRNA is translated into protein.

cDNA is a more convenient way to work with the coding sequence than mRNA because RNA is very easily degraded by omnipresent RNases. This the main reason cDNA is sequenced rather than mRNA. Likewise, investigators conducting DNA microarrays often convert the mRNA into cDNA in order to produce their probes. Let's see what is required to produce cDNA.

By definition, cDNA is double-stranded DNA that was derived from mRNA which can be obtained from prokaryotes or eukaryotes. Once the mRNA is isolated, you need a few more reagents: dNTPs (dGTP, dCTP, dATP and dTTP), primers, and reverse transcriptase which is a DNA polymerase (figure 2). Mix the mRNA with the other reagents and allow the polymerase to make a complementary strand of DNA (first strand synthesis). Next, the mRNA must be removed and the second strand of DNA synthesized. There are many technical details in these steps, but we do not need to focus on them at this time.

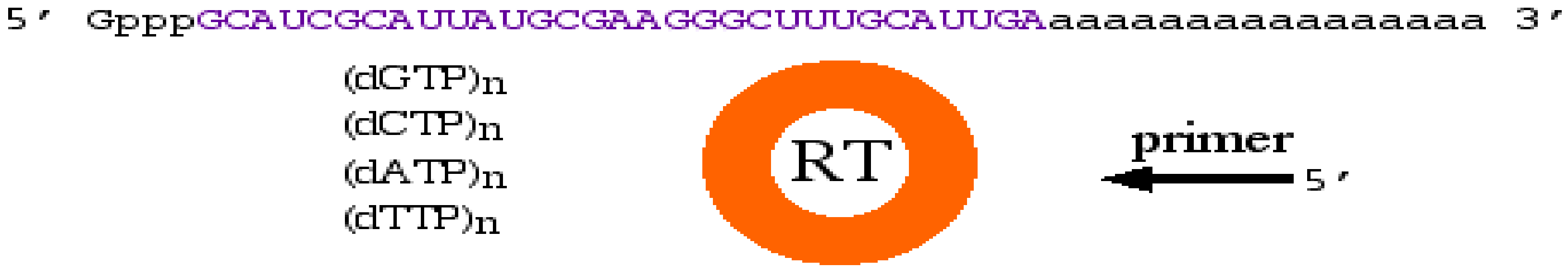


Figure 2. Four basic reagents needed to produce cDNA: mRNA as template, dNTPs, reverse transcriptase and primers.

The only issue worth mentioning now is that three different types of primers can be used (figure 3). 1) If the mRNA has a poly-A 3' tail, then an oligo-dT primer can be used to prime all mRNAs simultaneously. 2) If you only wanted to produce cDNA from a subset of all mRNA, then a sequence-specific primer could be used that will only bind to one mRNA sequence. 3) If you wanted to produce pieces of cDNA that were scattered all over the mRNA, then you could use a random primer cocktail that would produce cDNA from all mRNAs but the cDNAs would not be full length. The major benefits to random priming are the production of shorter cDNA fragments and increasing the probability that 5' ends of the mRNA would be converted to cDNA. Because reverse transcriptase does not usually reach the 5' end of long mRNAs, random primers can be beneficial

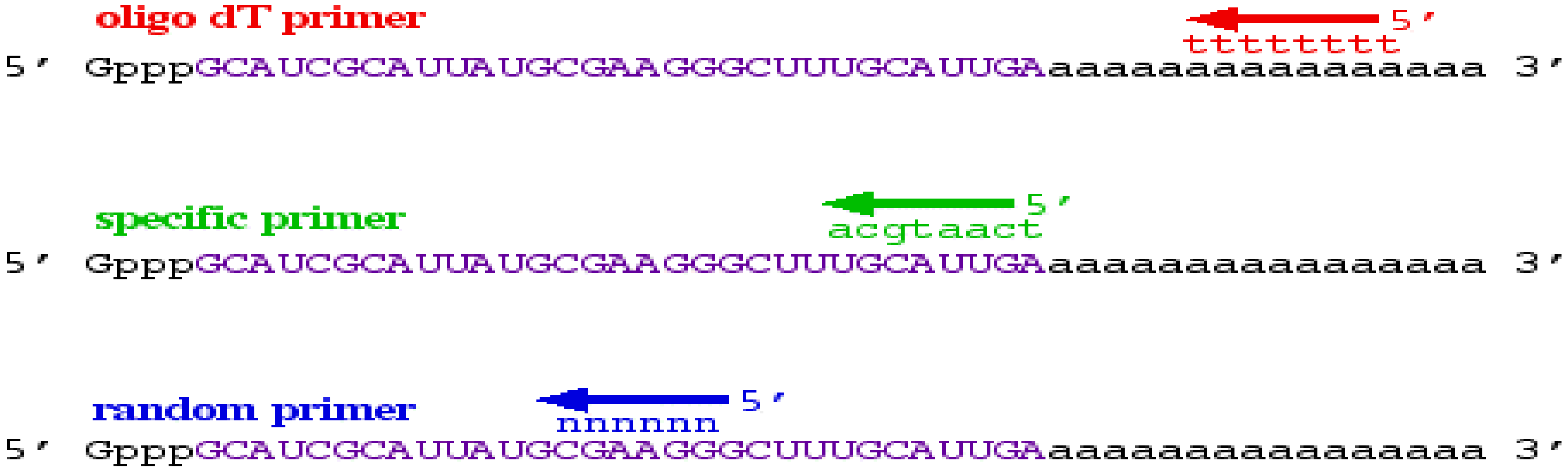

oligo dT primer
5'
tttttttttt
5' GpppGCAUCGCAUUAUGCGAAGGGCUUUGCAUUGAaaaaaaaaaaaaaaaa 3'
specific primer
5'
acgtaact
5' GpppGCAUCGCAUUAUGCGAAGGGCUUUGCAUUGAaaaaaaaaaaaaaaaa 3'
random primer
5'
nnnnnnn
5' GpppGCAUCGCAUUAUGCGAAGGGCUUUGCAUUGAaaaaaaaaaaaaaaaa 3'

Figure 3. Three ways to prime the production of cDNA: oligo-dT primer (red), sequence-specific primer (green), random primer (blue).

Random Priming Technique

How can you produce a complementary strand of DNA when you don't know the sequence or you want to produce many short DNA copies of every section of DNA in a complex mixture?
The solution is the random primer which is so simple . Random primers are short segments of single-stranded DNA (ssDNA) called oligonucleotides, or oligos for short. These oligos are only 6, 8, 9 nucleotides long and they consist of every possible combination of bases which means there must be for a octamer 4^8 = 65,536 different combinations in the mixture. Because every possible -amer is present, these primers can bind to any section of DNA.

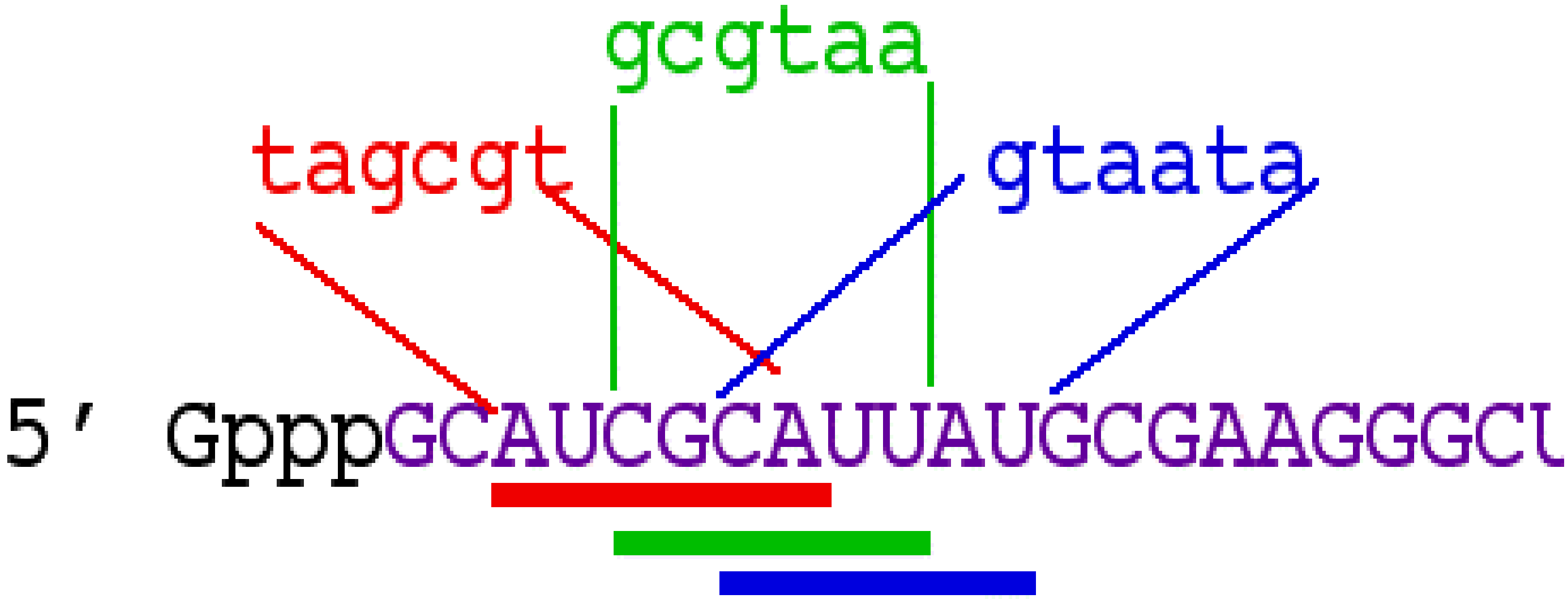
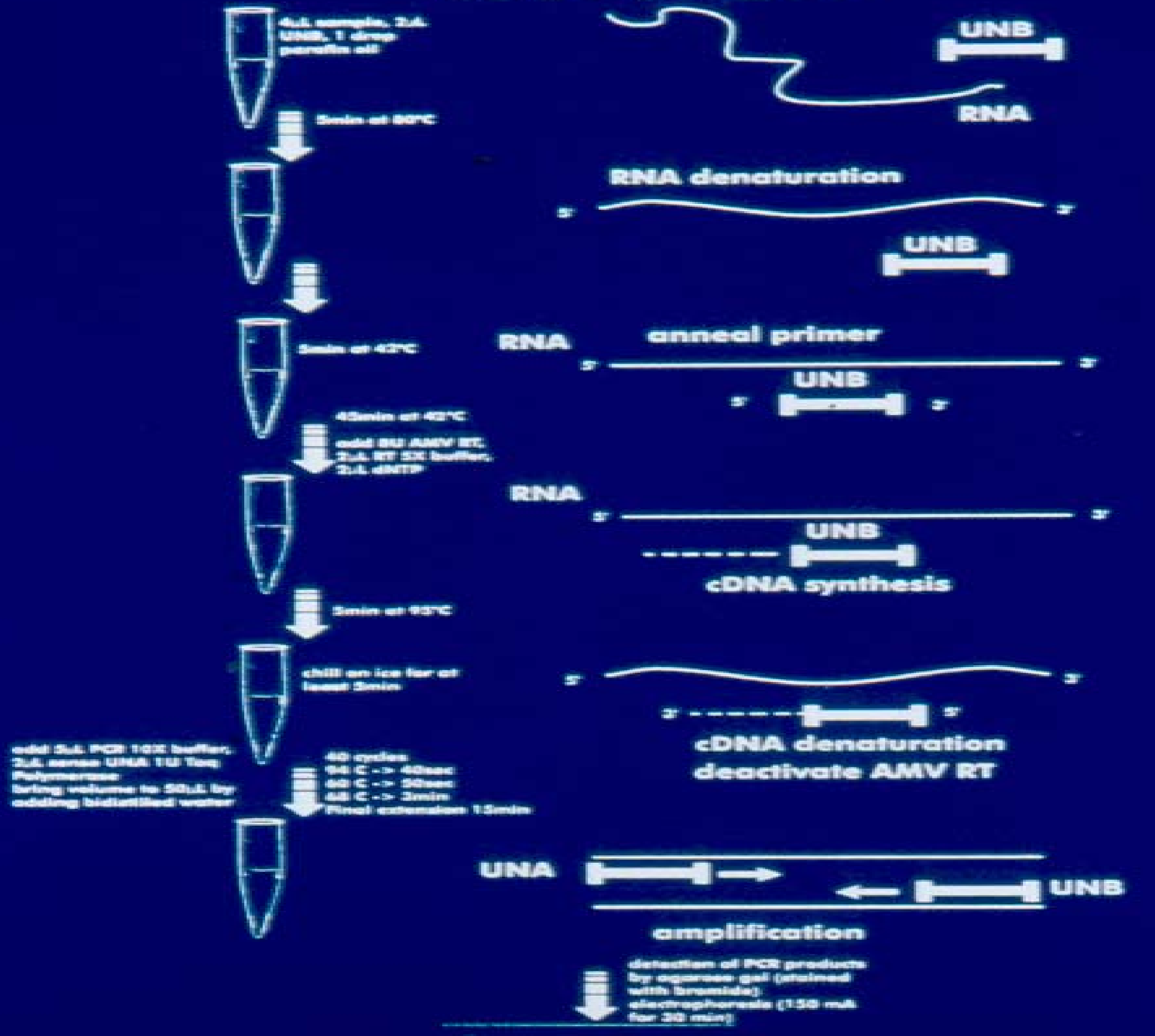

gcgtaa
tagcgt
gtaata
5′ GpppGCAUCGCAUUAUGCGAAGGCU

Figure 1. Three examples of hexamers from the mixture of all possible hexamers in random primers. These three particular primers could bind to three overlapping portions of this mRNA to prime the production of cDNA. The primer that arrives first will bind and the other two will have to find another segment of DNA (either another copy of the same mRNA or from a different locus) to bind.

The only other point to consider is that their short length means that they do no bind to a segment of ssDNA with much force since there are very few hydrogen bonds holding the two strands together (template and oligo). Nevertheless, the method works amazingly well and is still in use to produce random pieces of DNA for probe production. These probes can be used on blots or DNA microarrays.

RT-PCR



4µL sample, 2µL UNB, 1 drop paraffin oil

UNB

RNA

5min at 80°C

RNA denaturation

5' 3'

UNB

5min at 42°C

RNA anneal primer

5' 3'

UNB

5' 3'

45min at 42°C

add 8U AMV RT, 2µL RT 5X buffer, 2µL dNTP

RNA

5' 3'

UNB

cDNA synthesis

5min at 95°C

chill on ice for at least 2min

5' 3'

3' 5'

cDNA denaturation

deactivate AMV RT

add 5µL PCR 10X buffer, 2µL sense UNA 1U Taq Polymerase
bring volume to 50µL by adding bidistilled water

40 cycles
94 C -> 40sec
60 C -> 50sec
68 C -> 2min
Final extension 15min

UNA

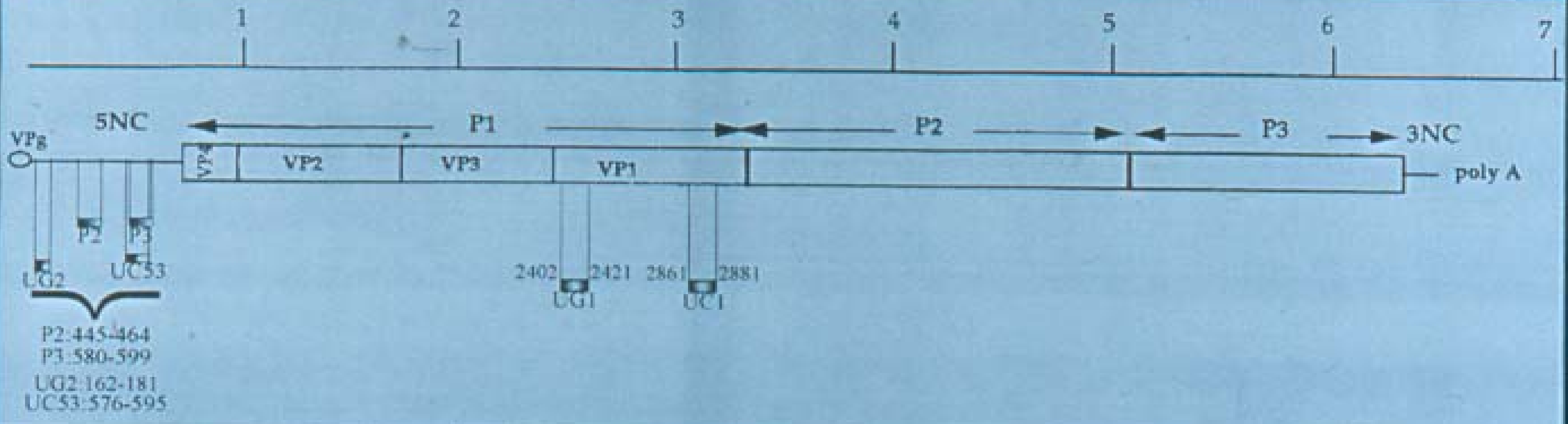
UNB

amplification

detection of PCR products by agarose gel (stained with bromide) electrophoresis (150 mA for 30 min)

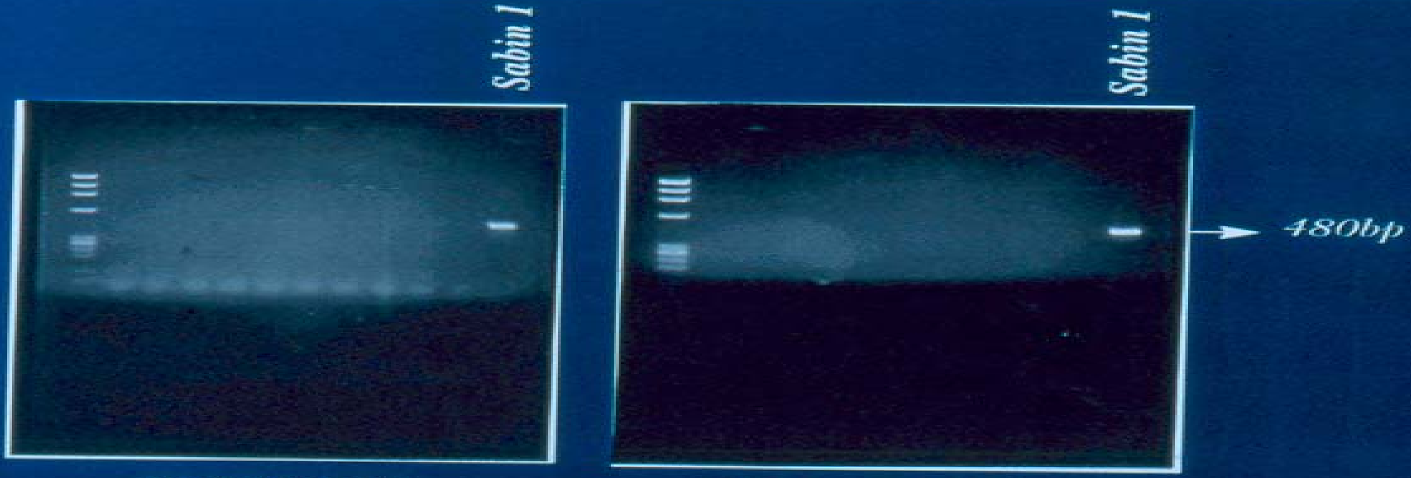
PRIMERS POSITION ON POLIOVIRUSES GENOME

P2	TCCTCCGGCCCCTGAATGCG	445-464	155bp
P3	ATTGTCACCATAAGCAGCCA	580-599	
UG1	TTTGTGTCAGCGTGTAATGA	2402-2421	480bp
UC1	TCTAGATTTGACATGGAATTC	2861-2881	
UC53	TGGCTGCTTATGGTGACAA	576-595	434bp
UG52	CAAGCACTTCTGTTTCCCCGG	162-181	


1
2
3
4
5
6
7
VPg
5NC
P1
P2
P3
3NC
VP4
VP2
VP3
VP1
poly A
P2
P3
UG2
UC53
2402
2421
2861
2881
UG1
UC1

P2:445-464
P3:580-599
UG2:162-181
UC53:576-595

RT-PCR USING PRIMERS UC1/UG1


Sabin 1
Sabin 1
480bp

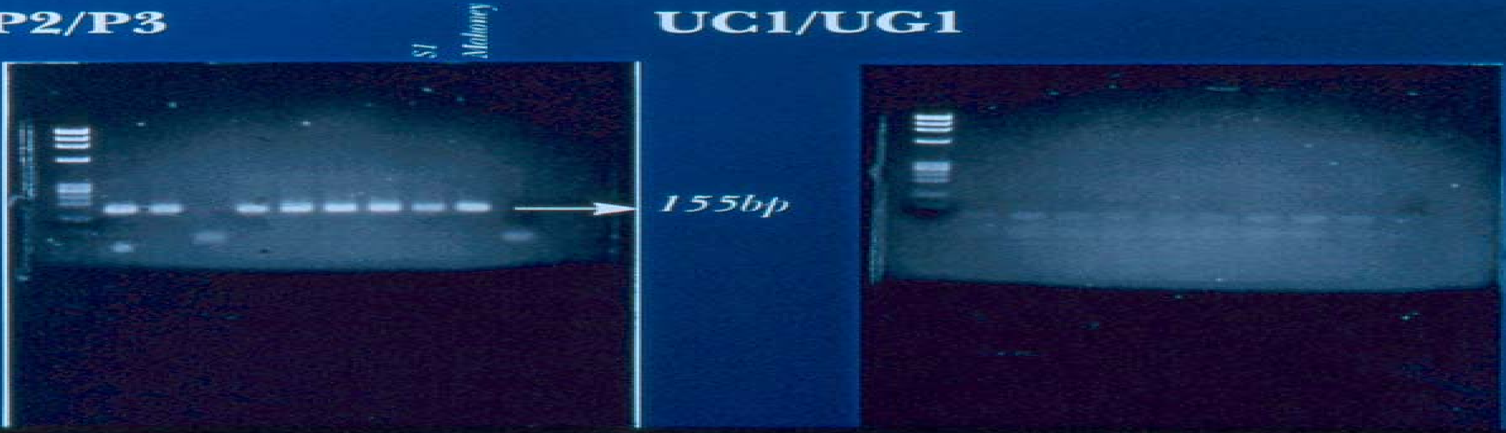
30 CYCLES

50 CYCLES

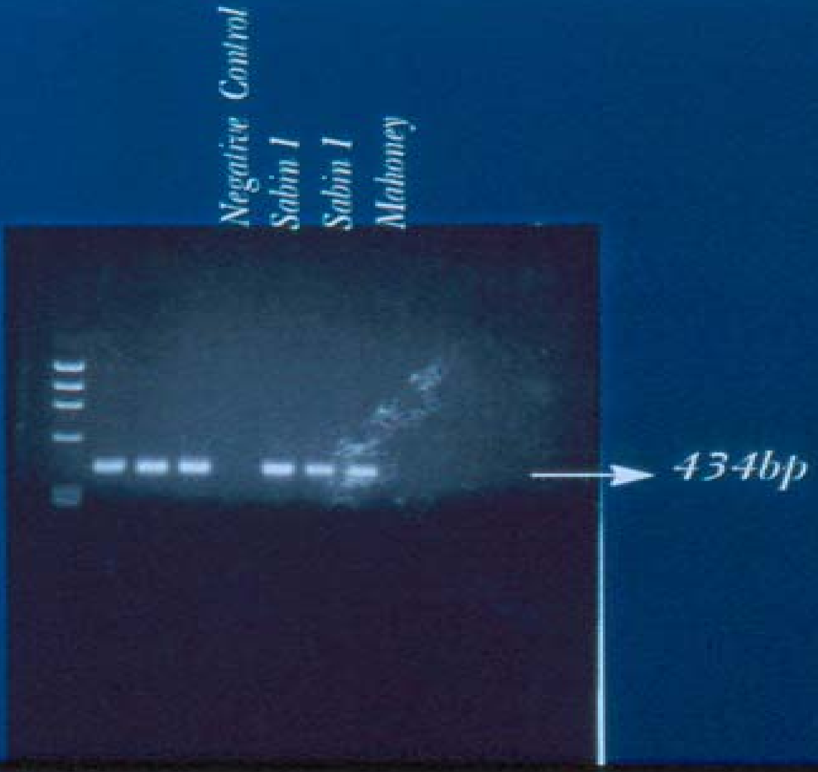
RT-PCR USING PAIRS OF PRIMERS P2/P3 & UC1/UG1

P2/P3

UC1/UG1


S1
Mahoney
155bp

RT-PCR USING PRIMERS UC53/UG52


Negative Control
Sabin 1
Sabin 1
Mahoney
434bp

Multiplexing primer pairs

First step in designing a multiplex PCR is choosing the primer pairs which can be combined. One important requirement is to find a PCR program allowing optimal amplification of all loci when taken individually . This is achieved by adjusting the annealing and extension time and temperature.

Multiplexing equimolar primer mixtures. The next step is combining the desired primer pairs in multiplex mixture(s), using equimolar amounts of each primer. PCR amplification of the multiplex mixtures can be performed, first using exactly the same PCR program as with individual primer pairs. Very often, this will results in preferential amplification of some loci. Such a situation will require further adjustment in cycling conditions and primer concentration.

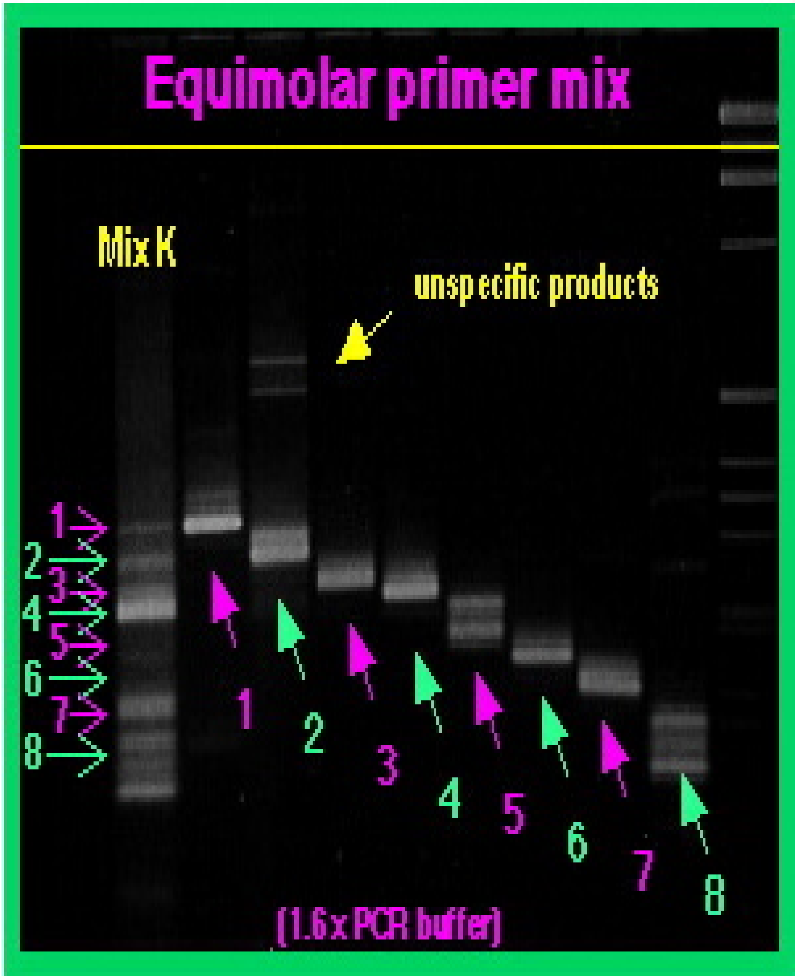

Equimolar primer mix
Mix K
unspecific products
1
2
3
4
5
6
7
8
1
2
3
4
5
6
7
8
(1.6x PCR buffer)

Fig. 7 (duplicate). Single locus PCR and multiplex PCR with equimolar amounts of primers from mixture K, performed in the same cycling conditions. In Some products of mixture K become weak or invisible, requiring further adjustment of primer amount(s) and of cycling conditions. Primers used in mixture K amplify polymorphic loci, explaining the appearence of multiple bands on a nondenaturing agarose gel.

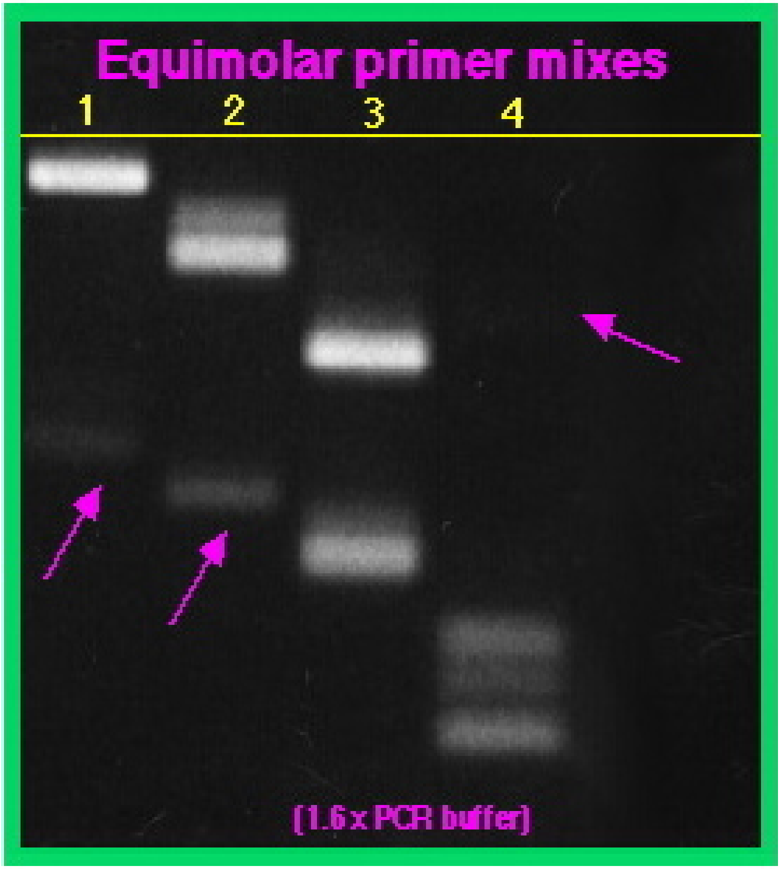

Equimolar primer mixes
1
2
3
4
(1.6 x PCR buffer)

Fig. 10. Equimolar amounts of the same primers used for mixture K (see also Fig. 7 above), where amplified in pairs. In lanes 1, 2 and 4, one locus was amplified less efficiently than the other one (arrows). As mentioned before, amplification of the "weaker" loci can be improved increasing the amount of primers or adjusting the reaction conditions

Adjustment of cycling conditions

- annealing time and temperature
- extension time and temperature

For example, figure 11 illustrates the influence of the extension temperature. Equimolar primer mixtures A-D were amplified using two different PCR programs, one at 65º C (yellow lanes) and the other at 72º C (green lanes) extension temperature. In general, there is a higher yield of PCR products for A, B and D when program A was used. This shows that the 72º C extension temperature, negatively influenced amplification of some loci (pink arrows),while also making some unspecific products visible (yellow arrows).

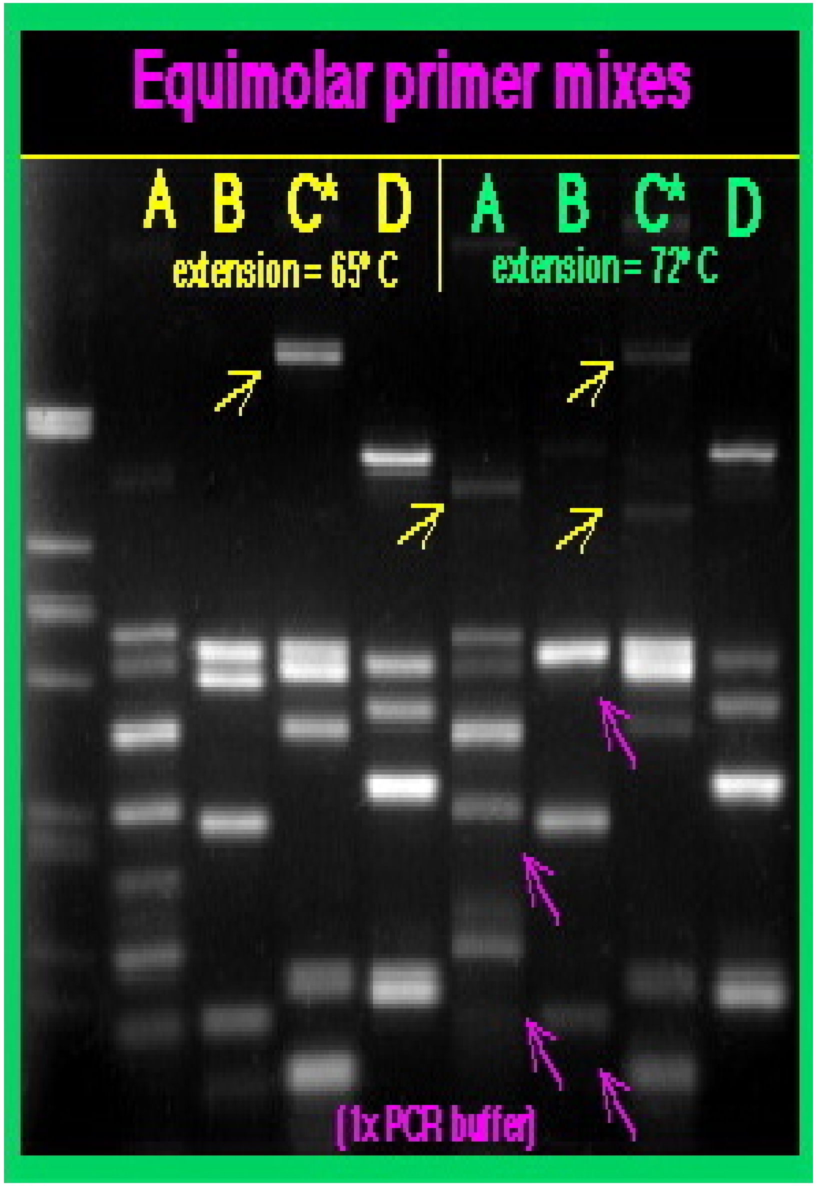

Equimolar primer mixes
A B C* D
extension= 65° C
A B C* D
extension= 72° C
(1x PCR buffer)

Fig. 11. Example of the influence of extension temperature. Multiplex PCR with mixtrues A-B using two different PCR programs. Reactions on the right side (green) were performed in identical cycling conditions with Fig. 9, whereas reactions on the left side (yellow) were performed using cycling conditions in which extension temperature was dropped from 72 º C to 65 º C. Reaction worked more efficiently with the lower extension temperature (pink arrow show missing products, yellow arrows show unspecific products).

Primer amount and buffer concentration. To improve the amplification of some of the DNA products from Fig. 11 above, the amount of primers was increased 2-5x for those loci. At the same time, the PCR buffer concentration was increased to 2x. These modifications allowed a much more efficient and reproducible amplification, with no unspecific products.

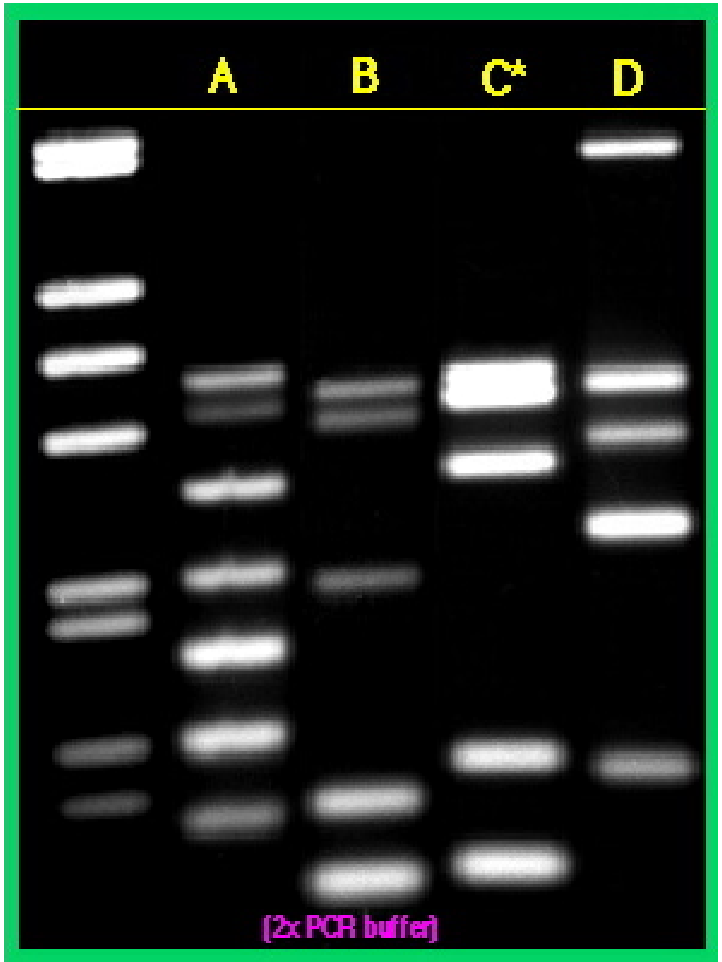
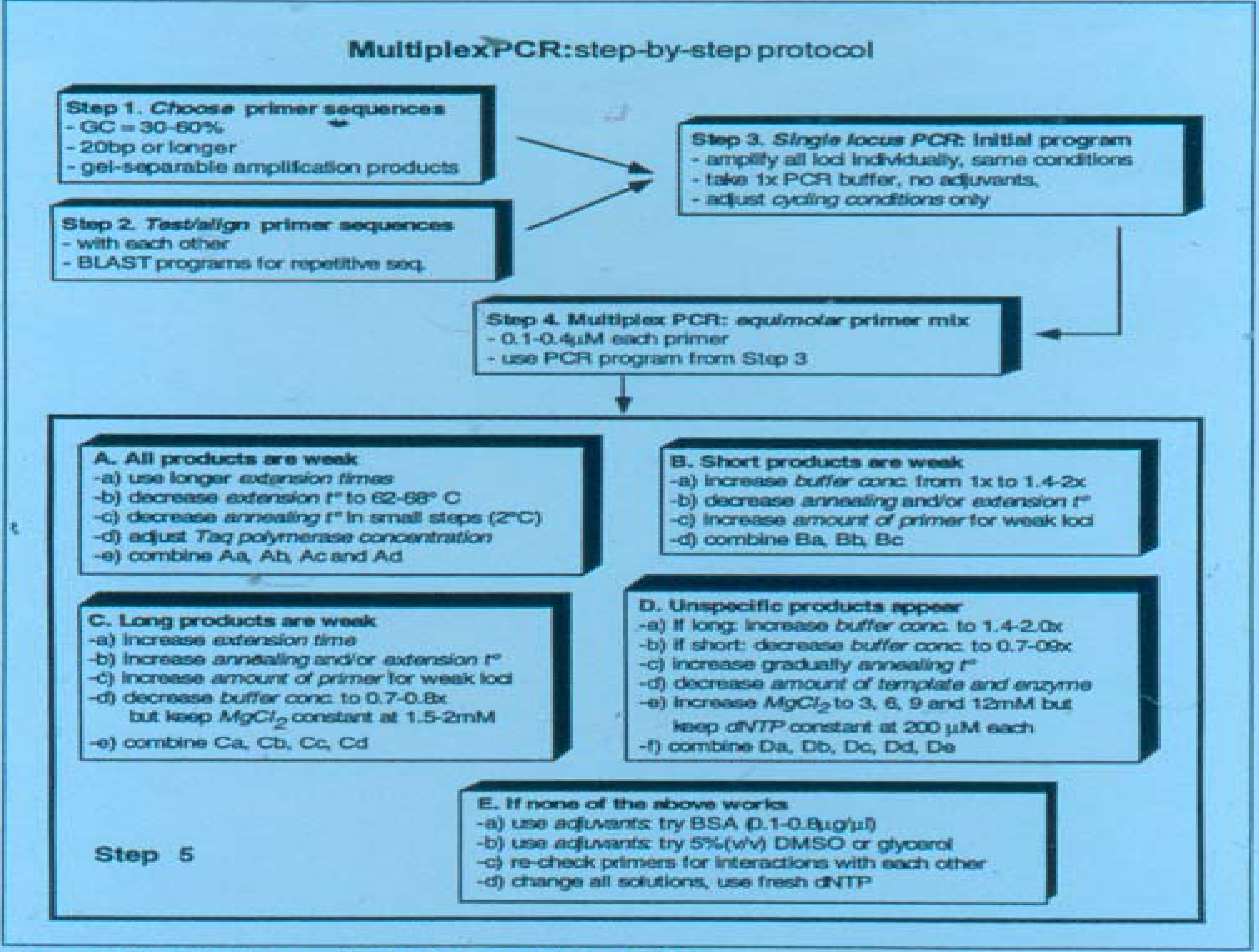


Fig. 12. Multiplex PCR with mixtures A-D, in cycling conditions similar to the ones on the left side of Fig. 11 above (annealing at 65 º C), but using 2x PCR buffer. The amount of primer pairs was increased for some of the weak products from Fig. 11. Cleaner and more efficient amplifications were obtained.



MultiplexPCR: step-by-step protocol

Step 1. ***Choose* primer sequences**
- GC = 30-60%
- 20bp or longer
- gel-separable amplification products

Step 2. ***Test/align* primer sequences**
- with each other
- BLAST programs for repetitive seq.

Step 3. ***Single locus PCR:* initial program**
- amplify all loci individually, same conditions
- take 1x PCR buffer, no adjuvants,
- adjust *cycling conditions* only

Step 4. Multiplex PCR: ***equimolar* primer mix**
- 0.1-0.4μM each primer
- use PCR program from Step 3

A. All products are weak
- a) use longer *extension times*
- b) decrease *extension t°* to 62-68° C
- c) decrease *annealing t°* in small steps (2°C)
- d) adjust *Taq polymerase concentration*
- e) combine Aa, Ab, Ac and Ad

B. Short products are weak
- a) increase *buffer conc.* from 1x to 1.4-2x
- b) decrease *annealing* and/or *extension t°*
- c) increase *amount of primer* for weak loci
- d) combine Ba, Bb, Bc

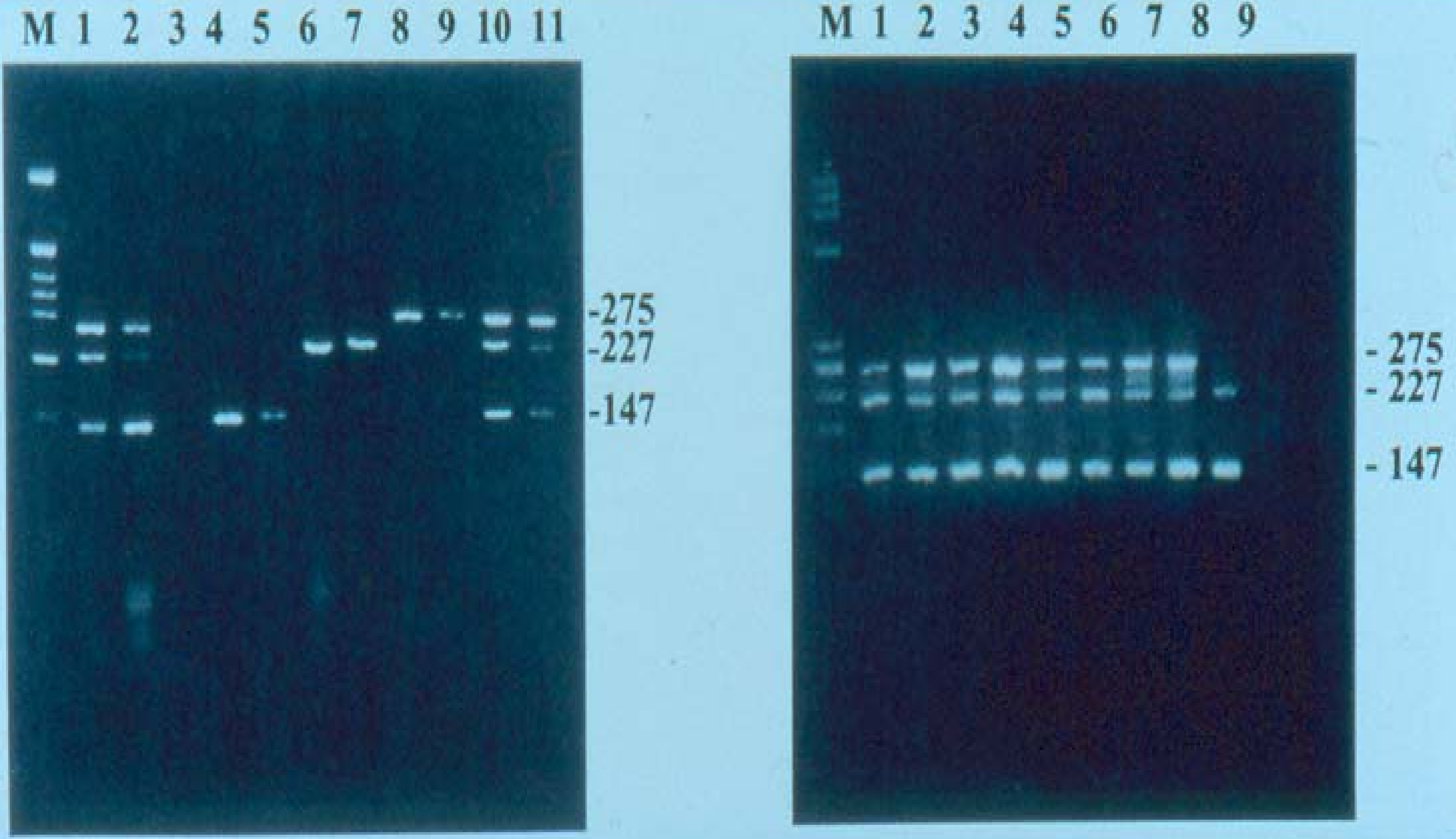
C. Long products are weak
- a) increase *extension time*
- b) increase *annealing and/or extension t°*
- c) increase *amount of primer* for weak loci
- d) decrease *buffer conc.* to 0.7-0.8x but keep *$MgCl_2$* constant at 1.5-2mM
- e) combine Ca, Cb, Cc, Cd

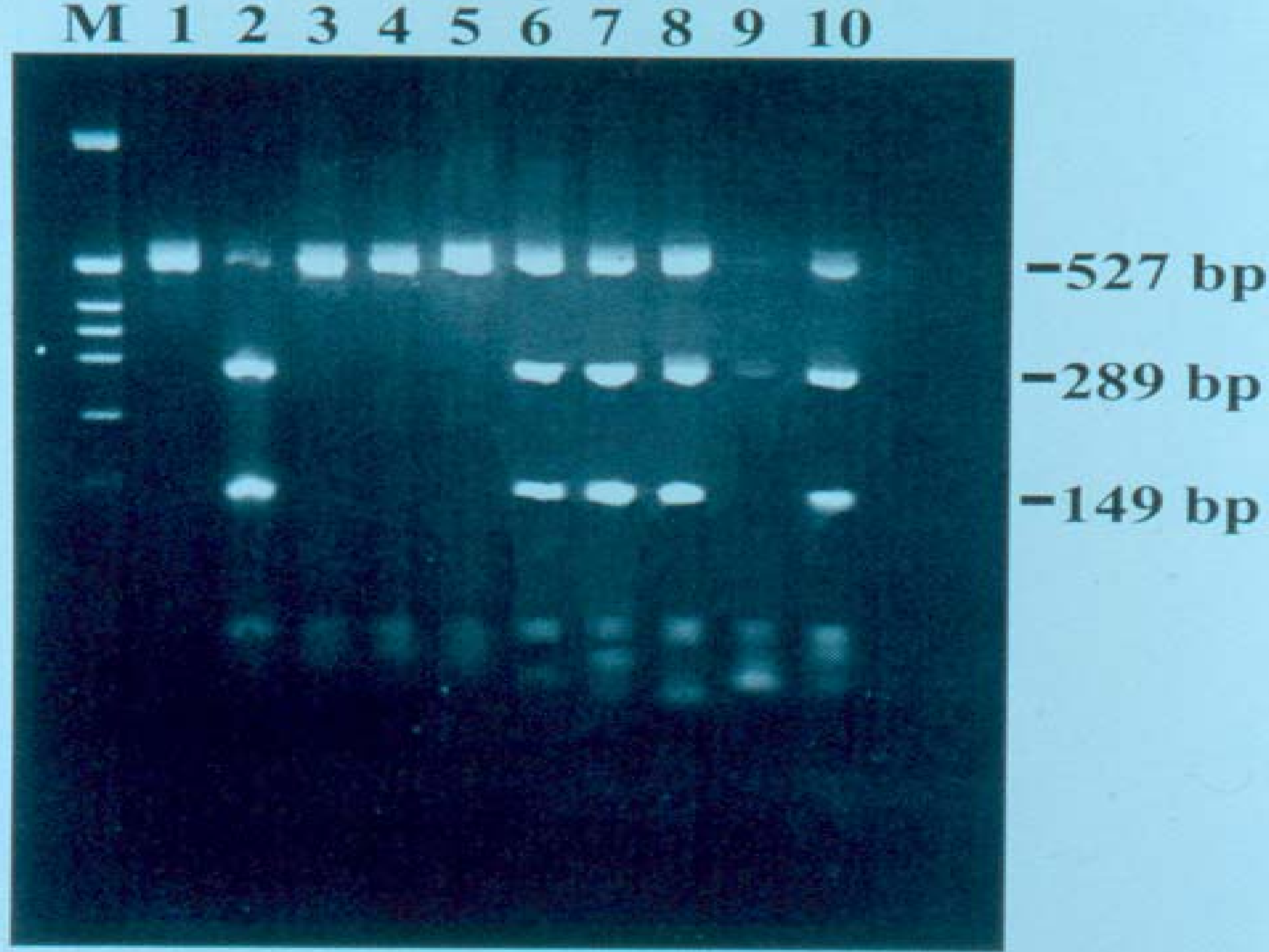
D. Unspecific products appear
- a) if long: increase *buffer conc.* to 1.4-2.0x
- b) if short: decrease *buffer conc.* to 0.7-09x
- c) increase gradually *annealing t°*
- d) decrease *amount of template and enzyme*
- e) increase *$MgCl_2$* to 3, 6, 9 and 12mM but keep *dNTP* constant at 200 μM each
- f) combine Da, Db, Dc, Dd, De

E. If none of the above works
- a) use *adjuvants:* try BSA (0.1-0.8μg/μl)
- b) use *adjuvants:* try 5%(v/v) DMSO or glycerol
- c) re-check primers for interactions with each other
- d) change all solutions, use fresh dNTP

Step 5

Figure 1. Step-by-step protocol for the multiplex PCR.


M 1 2 3 4 5 6 7 8 9 10 11
-275
-227
-147
M 1 2 3 4 5 6 7 8 9
- 275
- 227
- 147



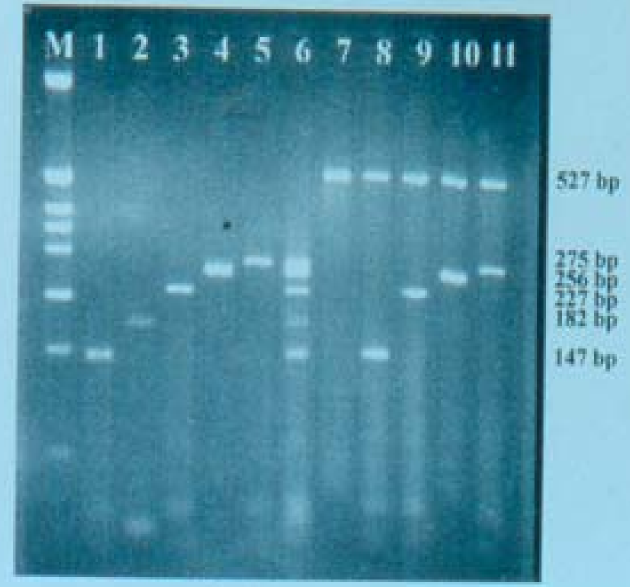
M
1
2
3
4
5
6
7
8
9
10
527 bp
289 bp
149 bp

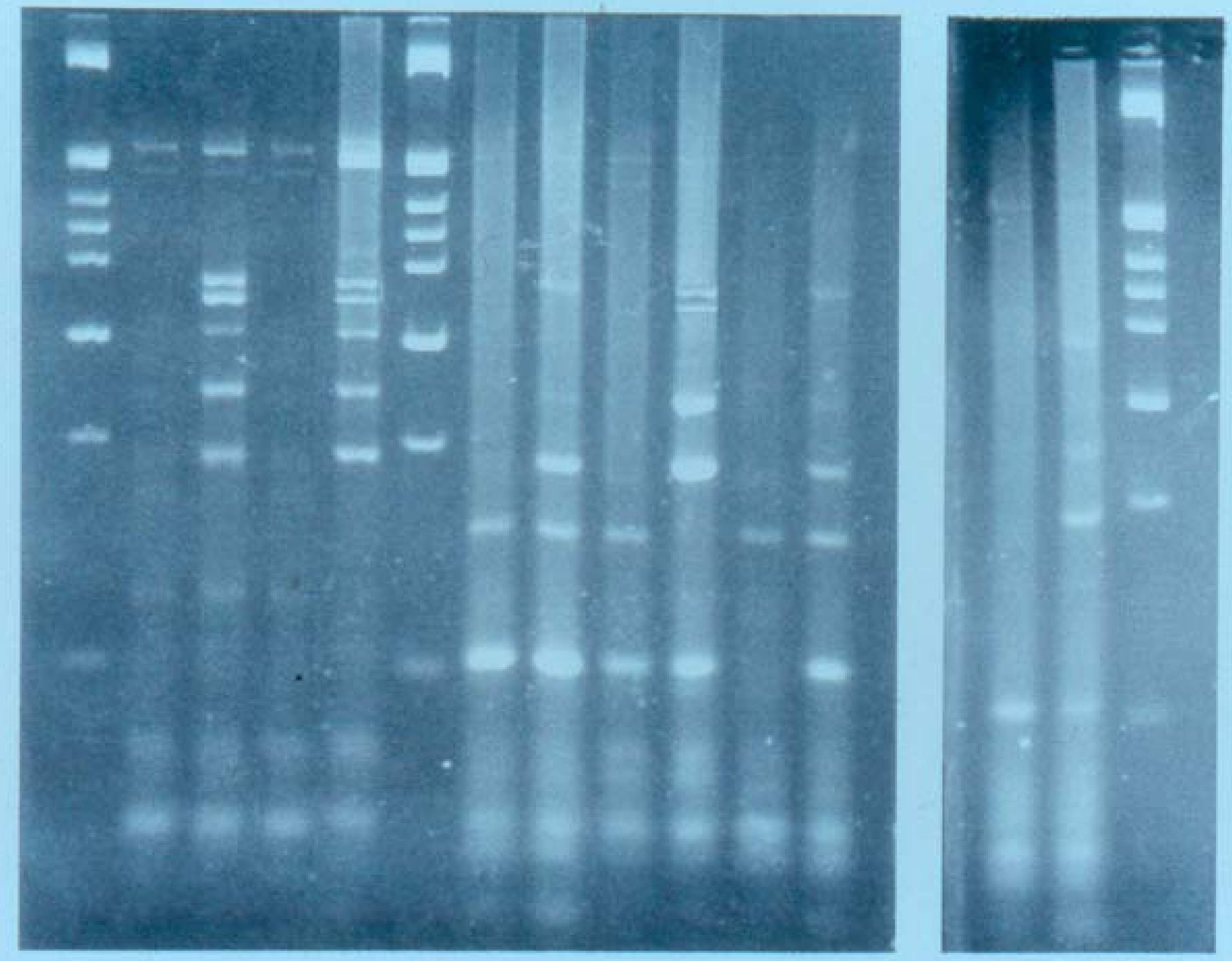
TABLE II. Sensitivity of the Multiplex PCR for the detection of HSV-1, HSV-2, VZV, CMV and EBV.

Virus	Log infectivity ($TCID_{50}$/ml)[a]	Log $PCRD_{50}$/ml[b]	$TCID_{50}$/ $PCRD_{50}$	Serial dilutions of quantitated PCR products
HSV-1	8	10	0,01	—
HSV-2	5,5	7	0,3	—
VZV	—	—	—	14 attogram
CMV	—	—	—	18 attogram
EBV	—	—	—	160 attogram

[a]: $TCID_{50}$, TCID at which 50% of inoculated monolayers become infected.

[b]: The reciprocal of the highest dilution positive by PCR adjusted to concentration per milliliter after DNA extraction.


M 1 2 3 4 5 6 7 8 9 10 11
527 bp
275 bp
256 bp
227 bp
182 bp
147 bp



PCR PRIMER DESIGN AND REACTION OPTIMISATION

Factors Affecting the PCR:

Denaturing Temperature and time

The specific complementary association due to hydrogen bonding of single-stranded nucleic acids is referred to as "annealing": two complementary sequences will form hydrogen bonds between their complementary bases (G to C, and A to T or U) and form a stable double-stranded, anti-parallel "hybrid" molecule. One may make nucleic acid (NA) single-stranded for the purpose of annealing by heating it to a point above the "melting temperature" of the double- or partially-double-stranded form, and then flash-cooling it: this ensures the "denatured" or separated strands do not re-anneal.

Additionally, if the NA is heated in buffers of ionic strength lower than 150mM NaCl, the melting temperature is generally less than 100oC - which is why PCR works with denaturing temperatures of 94-96 oC.

Taq polymerase is given as having a **half-life of 30 min at 95oC**, which is partly why one should not do more than about **30 amplification cycles**: however, it is possible to **reduce the denaturation temperature** which means one may do as many as **40 cycles** without much decrease in enzyme efficiency.

"**Time at temperature**" is the main reason for denaturation / loss of activity of Taq: thus, if one reduces this, one will **increase the number of cycles that are possible**, whether the temperature is reduced or not. Normally the denaturation time is **1 min at 94oC**: it is possible, for short template sequences, to **reduce this to 30 sec or less**. Increase in denaturation temperature and decrease in time may also work: **96oC for 15 sec**.

Annealing Temperature and Primer Design

Primer length and sequence are of critical importance in designing the parameters of a successful amplification: the melting temperature of a NA duplex increases both with its length, and with increasing (G+C) content: a simple formula for calculation of the Tm is

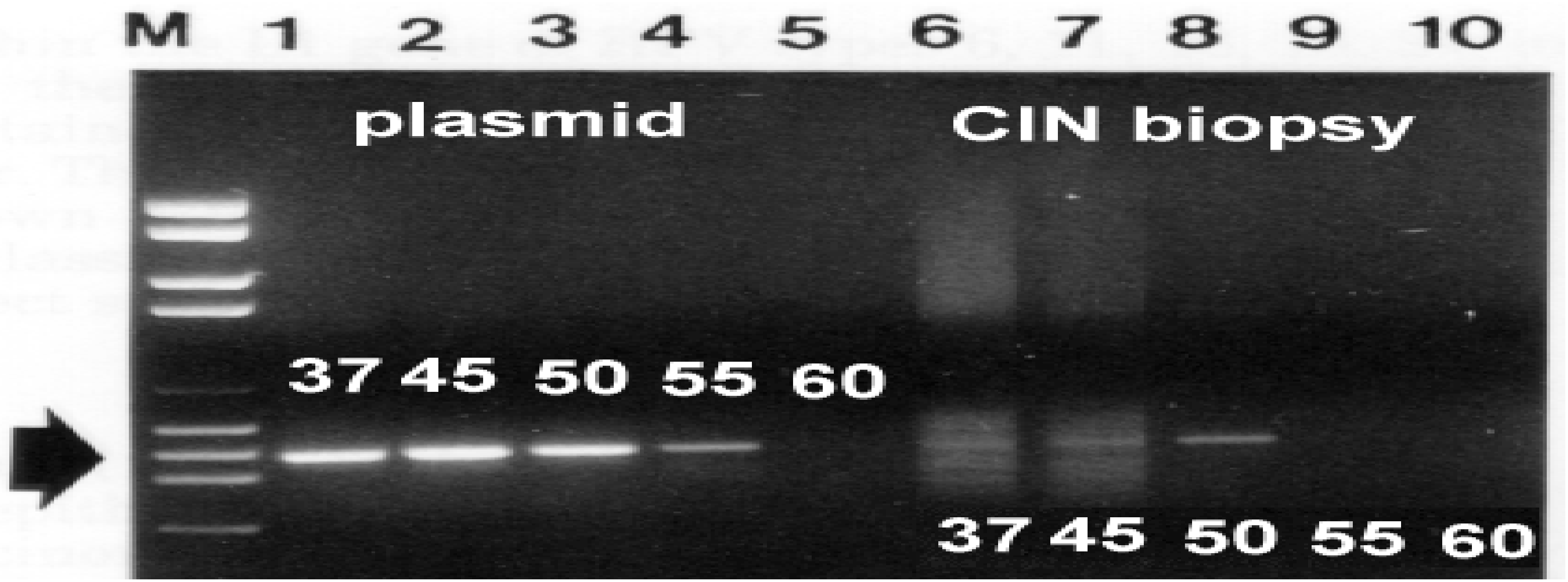
Tm = 4(G + C) + 2(A + T)oC.

Thus, the annealing temperature chosen for a PCR depends **directly on length and composition** of the primer(s). One should aim at using an annealing temperature (Ta) about **5oC below the lowest Tm of ther pair of primers to be used**; however, it can lead to **"non-specific" amplification**

and consequent reduction in yield of the desired product.

Annealing does not take long: most primers will **anneal efficiently in 30 sec or less**,

An illustration of the effect of annealing temperature on the specificity and on the yield of amplification of *Human papillomavirus type 16* (HPV-16) is given below


M 1 2 3 4 5 6 7 8 9 10
plasmid
CIN biopsy
37 45 50 55 60
37 45 50 55 60

Plasmid and biopsy sample DNA templates were amplified at different annealing temperatures as shown: note that while plasmid is amplified from 37 to 55ºC, HPV DNA is only specifically amplified at 50ºC.

Primer Length

The optimum length of a primer depends upon its (A+T) and (G+C) content, **and so** the Tm .A **prime consideration is that the primers should be complex enough so that the likelihood of annealing to sequences other than the chosen target is very low.**

For example, there is a ¼ chance of finding an A, G, C or T in any given DNA sequence; there is a 1/16 chance of finding any dinucleotide sequence (eg. AG); a 1/256 chance of finding a given 4-base sequence. Thus, a **sixteen base sequence will statistically be present only once in every 4 294 967 296, or 4 billion bases)**: this is about the size of the human or maize genome, and 1000x greater than the genome size of *E. coli*. Thus, the association of a greater-than-17-base oligonucleotide with its target sequence is an extremely sequence-specific process.Consequently, **17-mer or longer primers are routinely used for amplification from genomic DNA of animals and plants.**

Degenerate Primers

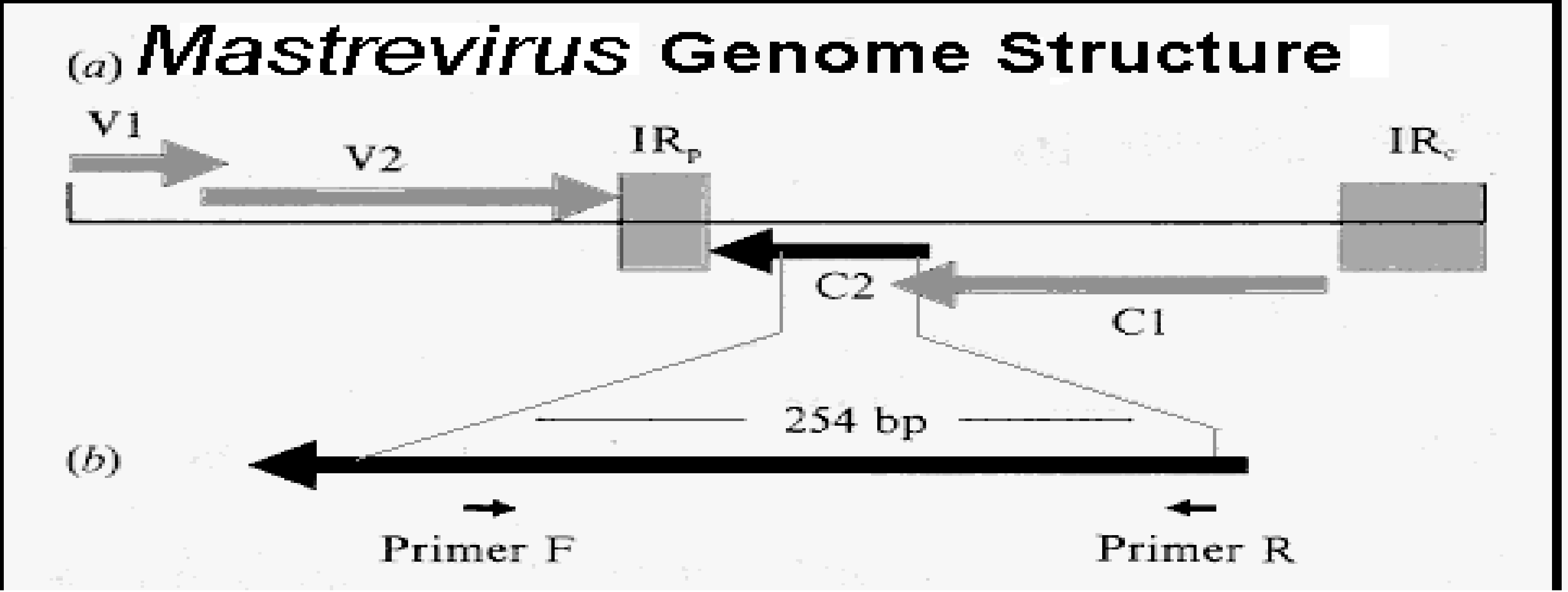
For amplification of cognate sequences from different organisms, or for "evolutionary PCR", one may increase the chances of getting product by **designing "degenerate" primers:** these would in fact be **a set of primers which have a number of options at several positions in the sequence so as to allow annealing to and amplification of a variety of related sequences**.

For example, Compton (1990) describes using 14-mer primer sets with 4 and 5 degeneracies as forward and reverse primers, respectively, for the amplification of glycoprotein B (gB) from related herpesviruses. The reverse primer sequence was as follows:

TCGAATTCNCCYAAYTGNCCNT

where Y = T + C, and N = A + G + C + T, and the 8-base 5'-terminal extension comprises a ***Eco*RI site** (underlined) and flanking spacer to ensure the **restriction enzyme can cut the product . Degeneracies obviously reduce the specificity of the primer(s),** meaning mismatch opportunities are greater, and background noise increases; also, **increased degeneracy means concentration of the individual primers decreases;**

(a) *Mastrevirus* Genome Structure



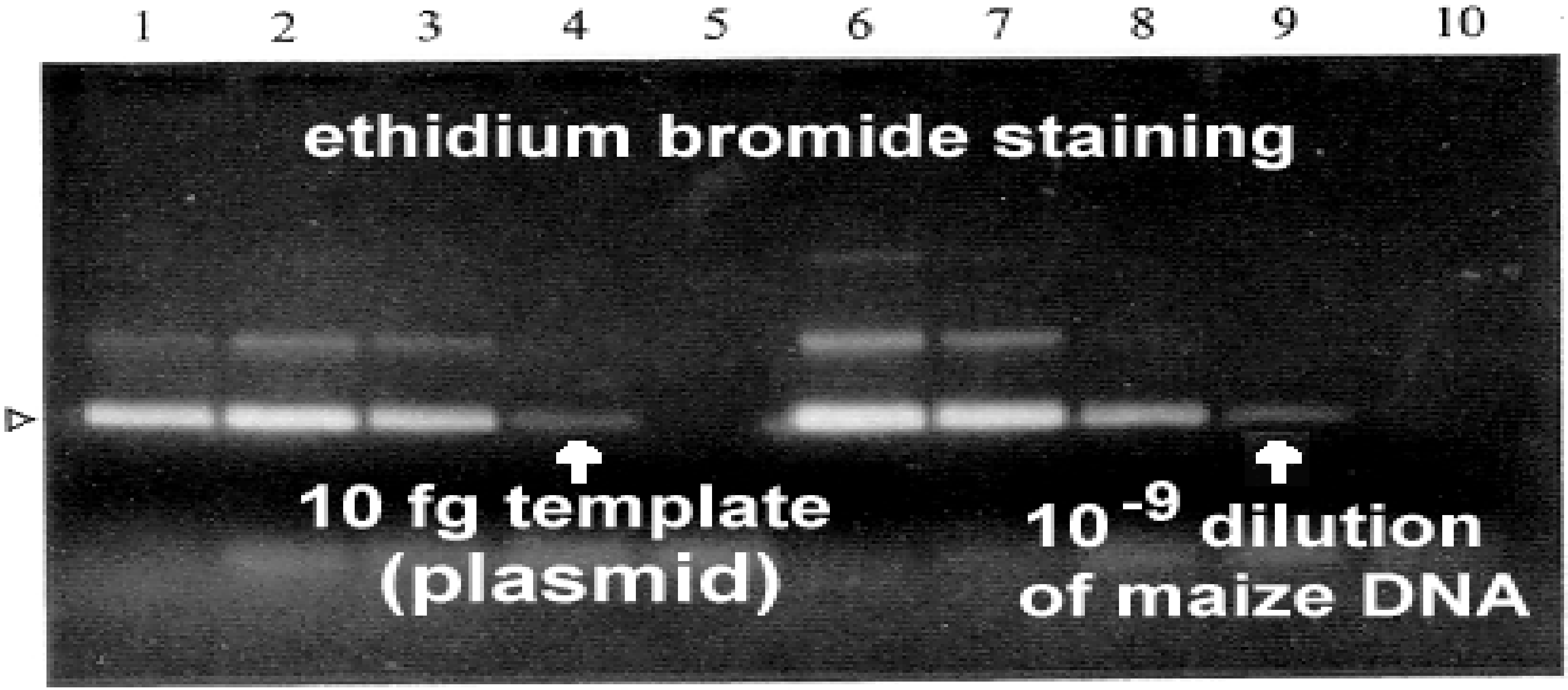
```
MSV   TTCATCCAATCTTCATC.....220 b....GCCCAAGTAGATTTTCC
WDV   TTGAGCCAATCTTCGTC.....217 b....GCCCAGGAAGTCTTTCC
CSM   TGCAGCCAGTCTTCATC.....220 b....GCCCAAGAAGTCTTTCC
DSV   TTCATCCAATCTTCATC.....220 b....GCCCAAGTAGACTTTCC
       ** *   *     *                     * *  **
```

Primer sequences

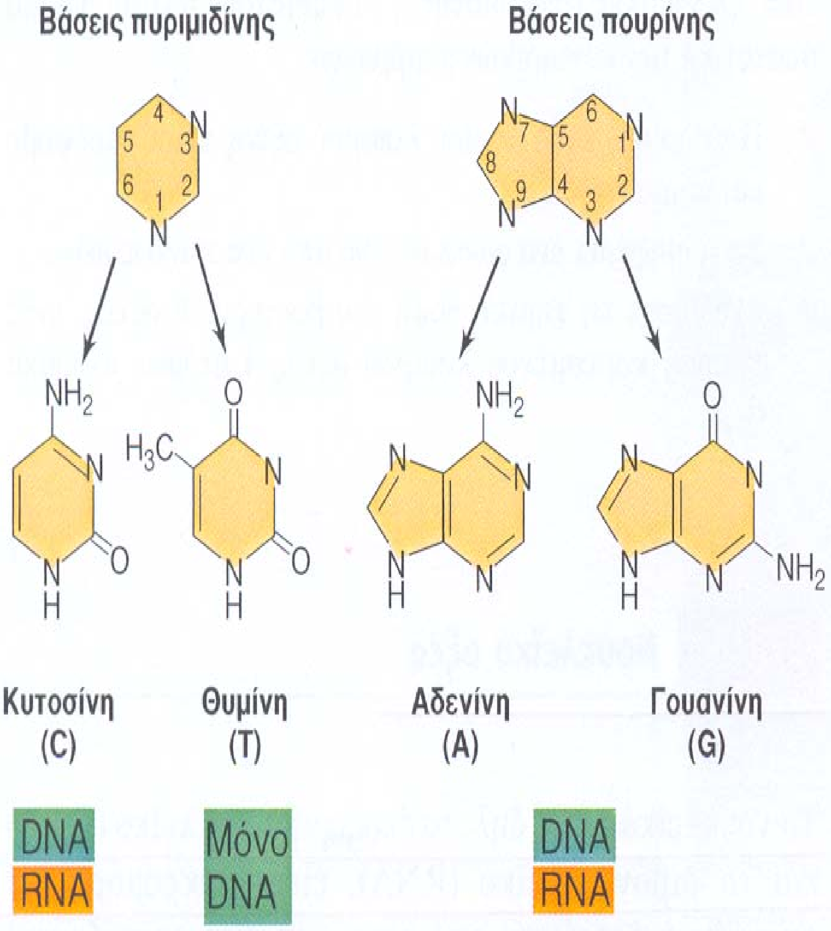
```
F:     5'-T**A*CCA*TCTTC*TC-3'
R:     5'-GGAAA**CT*C*TGGGC-3'
```

Primer sequences were derived from multiple sequence alignments; the mismatch positions were used as 4-base degeneracies for the primers (shown as stars; 5 in F and 4 in R), as shown above. Despite their degeneracy, the primers could be used to amplify a 250 bp sequence from viruses differing in sequence by as much as 50% over the target sequence, and 60% overall.

They could also be used to very sensitively detect the presence of *Maize streak virus* DNA against a background of maize genomic DNA, at dilutions **as low as $1/10^9$ infected sap / healthy sap** (see below).


1
2
3
4
5
6
7
8
9
10
ethidium bromide staining
10 fg template (plasmid)
10^{-9} dilution of maize DNA

Some groups use deoxyinosine (dI) at degenerate positions rather than use mixed oligos: this base-pairs with any other base, effectively giving a four-fold degeneracy at any postion in the oligo where it is present. This lessens problems to do with depletion of specific single oligos in a highly degenerate mixture, but may result in too high a degeneracy where there are 4 or more dIs in an oligo.


Βάσεις πυριμιδίνης
Βάσεις πουρίνης
NH2
O
H3C
N
H
NH2
Κυτοσίνη
(C)
Θυμίνη
(T)
Αδενίνη
(A)
Γουανίνη
(G)
DNA
RNA
Μόνο
DNA
DNA
RNA

Εικόνα 3.9 Δομές των βάσεων του DNA και του RNA. Δεν παρατίθεται στην εικόνα η ουρακίλη (U), της οποίας η δομή είναι όμοια με της θυμίνης με τη μόνη διαφορά ότι στερείται της μεθυλομάδας στον C-5. Παρατηρήστε το σύστημα αρίθμησης των δακτυλίων. Κατά την ένωση τους με τον άνθρακα 1′ του φωσφοσακχάρου για τον σχηματισμό νουκλεοτιδίου (Εικόνα 3.8), οι βάσεις πυριμιδίνης συνδέονται μέσω του N-1 του δακτυλίου και οι βάσεις πουρίνης μέσω του N-9.

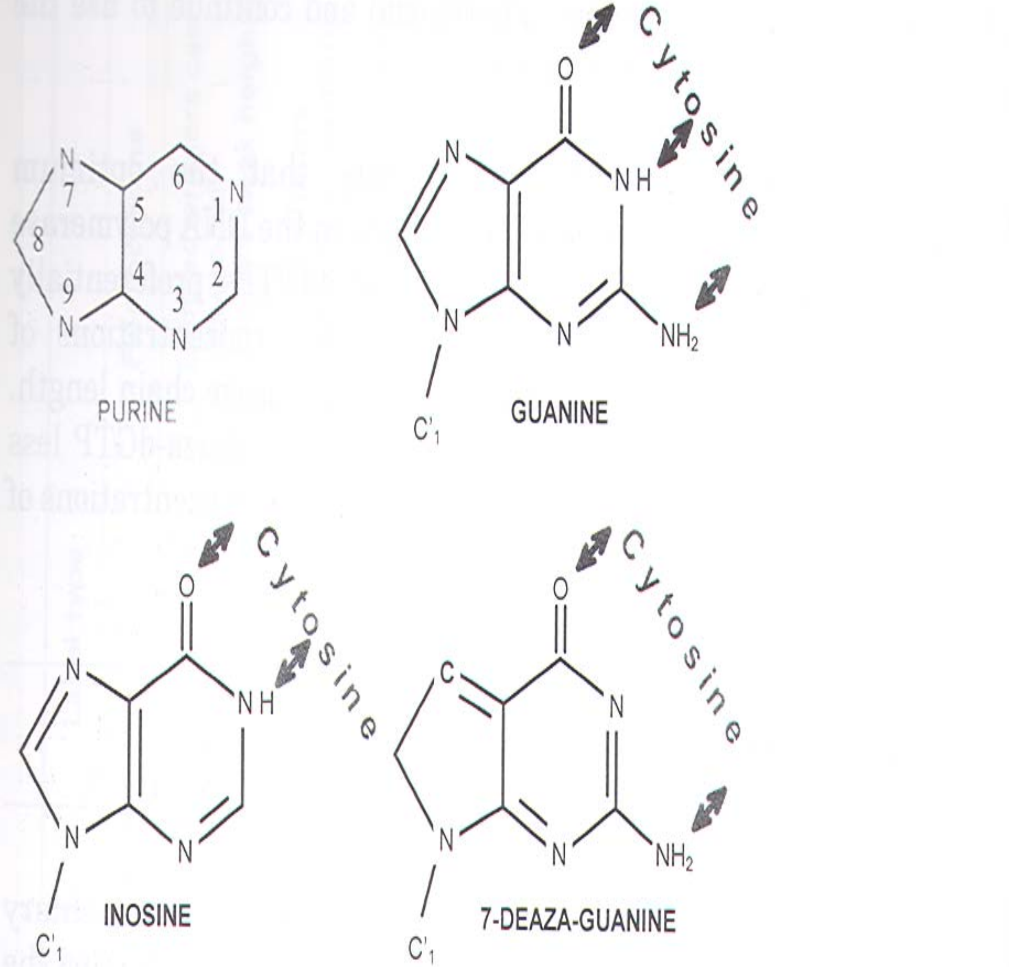

PURINE
GUANINE
INOSINE
7-DEAZA-GUANINE
Cytosine
Cytosine
Cytosine
NH
NH2
C'1

FIGURE 4.5: *The structure of base analogs used to resolve compressions. Inosine and 7-deaza-guanine form only two hydrogen bonds when base pairing with cytosine. These base pairs are therefore less stable than G–C base pairs, which form three hydrogen bonds. Secondary structure is therefore less stable when dITP or 7-deaza-dGTP is used in place of dGTP in the sequencing reactions. This minimizes compressions.*

Degenerate Primers - What are they?

Primers which have a number of options at several positions in the sequence so as to allow annealing to and amplification of a variety of related sequences.

eg:

5'-TCG AAT TCI CCY AAY TGR CCN T-3'

Y = pYrimidines = C / T (degeneracy = 2X)

R = puRines = A/ G (degeneracy = 2X)

I = Inosine = C / G / A / T

N = Nucleotide = C / G / A / T (degeneracy = 4X)

Why... use degenerate primers?

to amplify (fish out) conserved sequences of a gene or genes from the genome of an organism.

to get the nucleotide sequence after having sequenced some amino acids from a protein of interest

When...to use degenerate primers?

there is evidence of highly conserved regions or motifs of amino acids that can be designed into degenerate primers; these regions may be conserved interspecies. Degenerate primers can then be used to fish out these sequences. Sequences amplified this way can then be sequenced to confirm that the sequence is correct. They can then be used as probes to fish out the gene of interest from a genomic library (prokaryotic) or a cDNA library (eukaryotic).

Degenerate Primer Design

1. **Sequence alignment**
   The aa sequences of similar or homologous proteins can be retrieved from a database such as GenBank . These sequences are then aligned. At least 2 blocks of conserved amino acids should be present to enable the design of PCR primers. A further alignment can be done at nucleotide level if desired. Should a base be conserved throughout the alignment then it can be 'guessed' that this particular base will also be the same in the case of interest. The primers should be 20-30 mer in length (min 20 mer). The sequence alignment will also give the expected size of the PCR product.
2. **Terminal aa sequence information**
   Should this data be available then it can also be used as a starting point for primer design other than using the alignmnet method above.
3. **Primer degeneracy**
   The degeneracy can be lowered with the use of inosines for substituting 4 base instead of using all 4 base substiutions. Another factor that must also be taken into consideration is that as the degeneracy of the primers increase, the concentration of a specific primer will decrease.

The PCR Reaction

1. Template consideration

   It is recommended that a cDNA template be used for eukaryotes.

2. The PCR cocktail

   A concentration increase may be necessary to compensate for the degeneracy. If the primers used are quite degenerate, 50 pmoles could be used as a starting point and optimised from there.

3. The PCR cycles (thermocycling)

   A lot of experimentation and optimisation is required here. As always, start with the standard conditions, then proceed by optimising the primer annealing temperature. Start with about 35 cycles and increase to 40 if necessary. Remember to consider *Taq* viability for 40 cycles!

Common Problems

Competitive inhibition due to high primer degeneracy .Primers anneal to the correct template but are not extended by the polymerase due to unstable 3' ends resulting in the first few PCR cycles being highly inefficient - can be overcome by increasing PCR cycles or running a standard 25-30 cycles followed by another reaction of 30-35 cycles using the product of the first as the new template.

Low specific primer concentration due to high degeneracy - can easily be corrected by increasing primer concentration

DNA polymerase with 3'--->5' exonuclease activity should not be used as they degrade the primers (*Taq* polymerase will work fine)

False priming / unspecific smearing due to highly degenerate primers or your template containing many annealing sites - try annealing temperature optimization or redesign primers

Sequencing

After the band of the expected size have been excised from the agarose gel, the product can be directly sequenced or cloned first prior to sequencing. Even though degenerate primers can be used directly for sequencing, it may result in unspecific priming for sequencing depending on the template. Cloning of the products first enable the use of the primer sites usually situated on the flanks of the vector MCS.

Controls

If you're fishing for your gene using primers designed from an alignment - it would be wise to do a simple dot blot consisting of a positive control, your template DNA, a few related gDNA and a distant gDNA to check for possible contamination. This should be done after sequencing and having ensured that you have the sequence you want by doing a database searxh (eg. BLASTX). Check that the BLASTX results are in frame to the aa seq of your degenerate primers.

Elongation Temperature and Time

This is normally 70 - 72oC, for 0.5 - 3 min. Taq actually has a specific activity at 37oC At around 70oC the activity is optimal, and **primer extension occurs at up to 100 bases/sec.** About **1 min is sufficient for reliable amplification of 2kb sequences** (Innis and Gelfand, 1990). Longer products require longer times: **3 min is a good bet for 3kb and longer products**. Longer times may also be helpful in later cycles when product concentration exceeds enzyme concentration (>1nM), and when dNTP and / or primer depletion may become limiting.

Reaction Buffer

Recommended buffers generally contain :
10-50mM Tris-HCl pH 8.3,
up to 50mM KCl, 1.5mM or higher MgCl2,
primers 0.2 – 1µM each primer,
50 – 200µM each dNTP,
gelatin or BSA to 100µg/ml,
and/or non-ionic detergents such as **Tween-20 or Nonidet P-40 or Triton X-100** (0.05 - 0.10% v/v)
Higher than 50mM KCl or NaCl inhibits Taq.

[Mg2+] affects primer annealing; Tm of template, product and primer-template associations; product specificity; enzyme activity and fidelity. Taq requires *free* Mg2+, so allowances should be made for dNTPs, primers and template, all of which chelate and sequester the cation; of these, dNTPs are the most concentrated, so [Mg2+] should be 0.5 - 2.5mM *greater* than [dNTP]. **A titration should be performed with varying [Mg2+] with all new template-primer combinations**, as these can differ markedly in their requirements, even under the same conditions of concentrations and cycling times/temperatures.

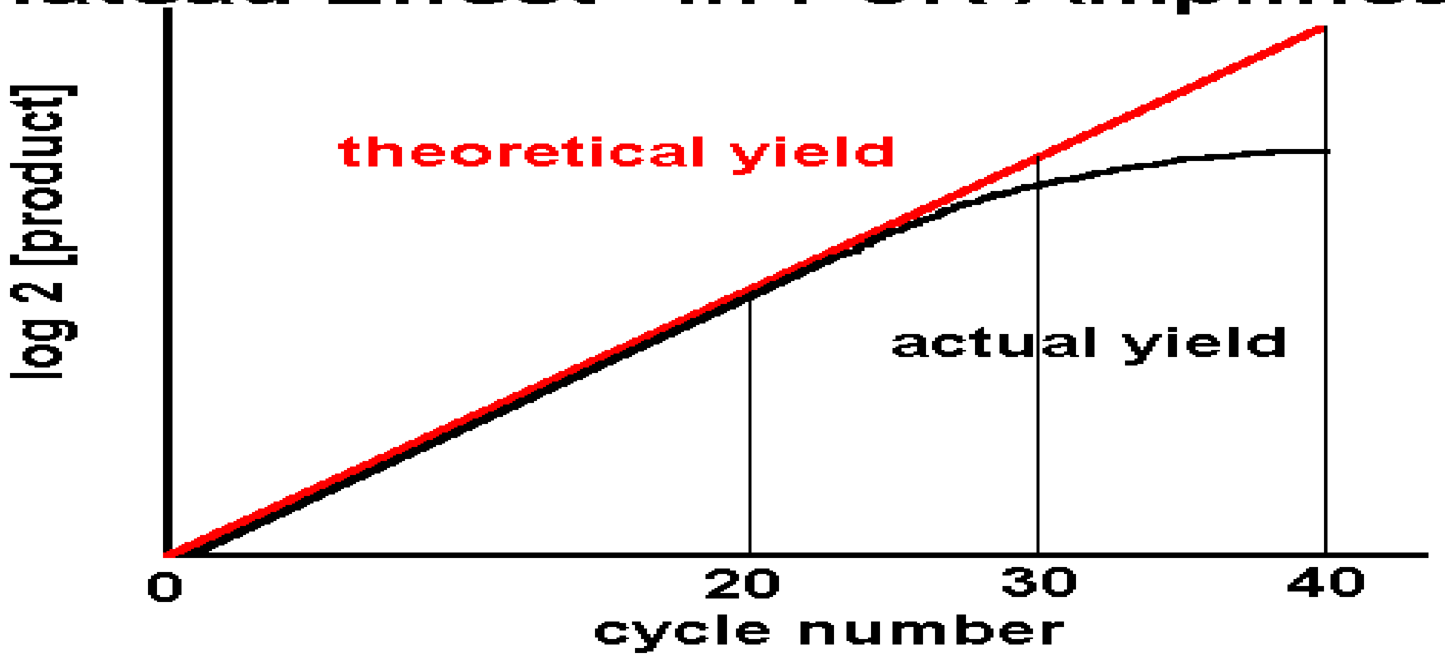
Some enzymes do not need added protein, others are dependent on it. Some enzymes work markedly better in the presence of detergent, probably because it prevents the natural tendency of the enzyme to aggregate.

Primer concentrations should not go above 1µM unless there is a high degree of degeneracy; 0.2 µM is sufficient for homologous primers.

Cycle Number

The number of amplification cycles necessary to produce a band visible on a gel depends largely on the starting concentration of the target DNA: 40 - 45 cycles to amplify 50 target molecules, and **25 – 30cycles to amplify 300.000 molecules** to the same concentration. This non-proportionality is due to a so-called ***plateau effect***, which is the attenuation in the exponential rate of product accumulation in late stages of a PCR, when product reaches 0.3 - 1.0 nM. This may be caused by degradation of reactants (dNTPs, enzyme); reactant depletion (primers, dNTPs); end-product inhibition ; competition for reactants by non-specific products; competition for primer binding by re-annealing of concentrated (10nM) product .

"Plateau Effect" in PCR Amplification

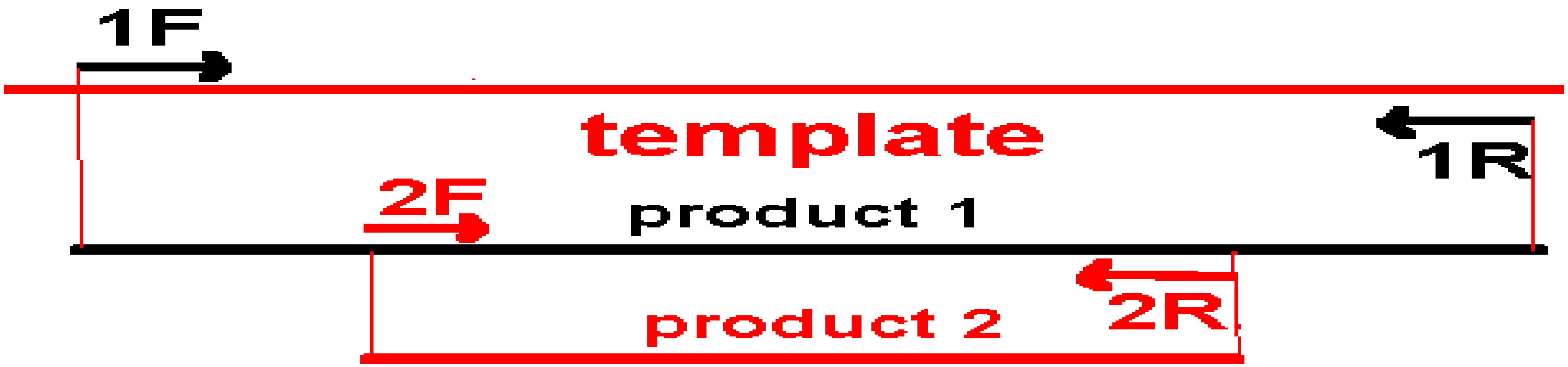


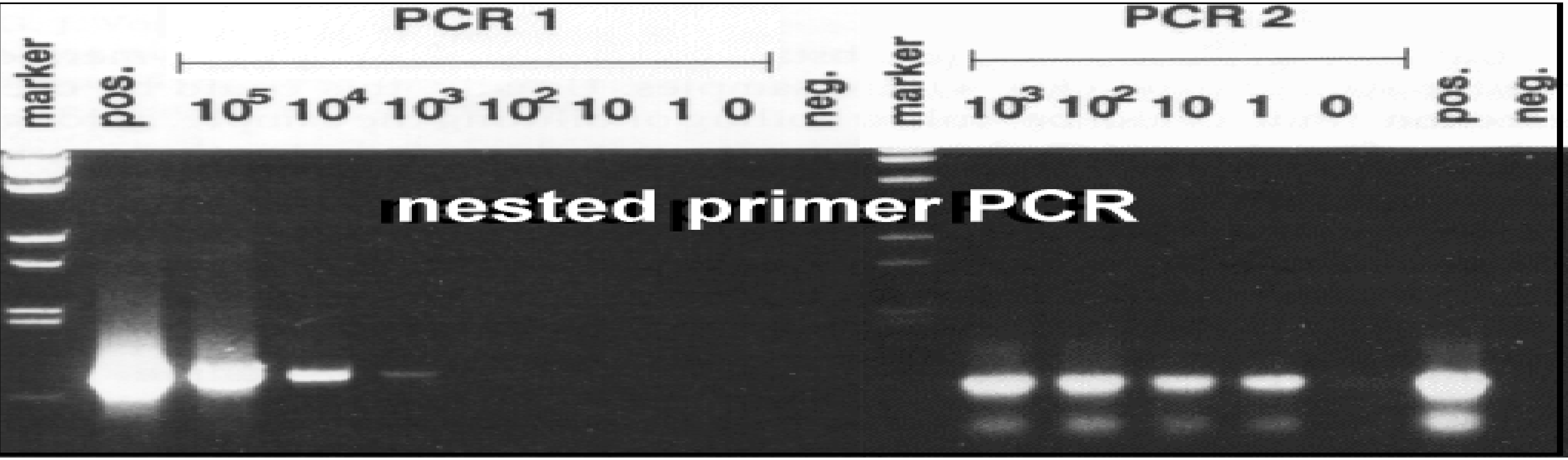
If desired product is not made in 30 cycles, take a small sample (1ul) of the amplified mix **and re-amplify 20-30x** in a new reaction mix rather than extending the run to more cycles: in some cases where template concentration is limiting, this can give good product where extension of cycling to 40x or more does not.

A variant of this is **nested primer PCR**: PCR amplification is performed with one set of primers, then some product is taken - with or without removal of reagents - for re-amplification with an internally-situated, "nested" set of primers. This process adds another level of specificity, meaning that all products non-specifically amplified in the first round will not be amplified in the second.

This is illustrated below:

NESTED PRIMER PCR:


1F
template
1R
2F
product 1
2R
product 2


PCR 1
PCR 2
marker
pos.
10^5
10^4
10^3
10^2
10
1
0
neg.
marker
10^3
10^2
10
1
0
pos.
neg.
nested primer PCR

This gel photo shows the effect of nested PCR amplification on the detectability of *Chicken anaemia virus* (CAV) DNA in a dilution series: the PCR1 just detects 1000 template molecules; PCR2 amplifies 1 template molecule

Helix Destabilisers / Additives

With NAs of high (G+C) content, it may be necessary to use “denaturation” conditions. For example, one may incorporate up to **10% (w or v/v) :**
dimethyl sulphoxide (DMSO),
dimethyl formamide (DMF),
urea
or formamide
in the reaction mix: **these additives are presumed to lower the Tm of the target NA**, although DMSO at 10% and higher is known to **decrease the activity of Taq by up to 50%**

Additives may also be necessary in the amplification of long target sequences: DMSO often helps in amplifying products of >1kb. **Formamide** can apparently dramatically improve the specificity of PCR (Sarkar *et al*., 1990), while **glycerol** improves the amplification of high (G+C) templates (Smith *et al*., 1990).

Polyethylene glycol (PEG) may be a useful additive when DNA template concentration is very low: it promotes macromolecular association by solvent exclusion, meaning the pol can find the DNA.

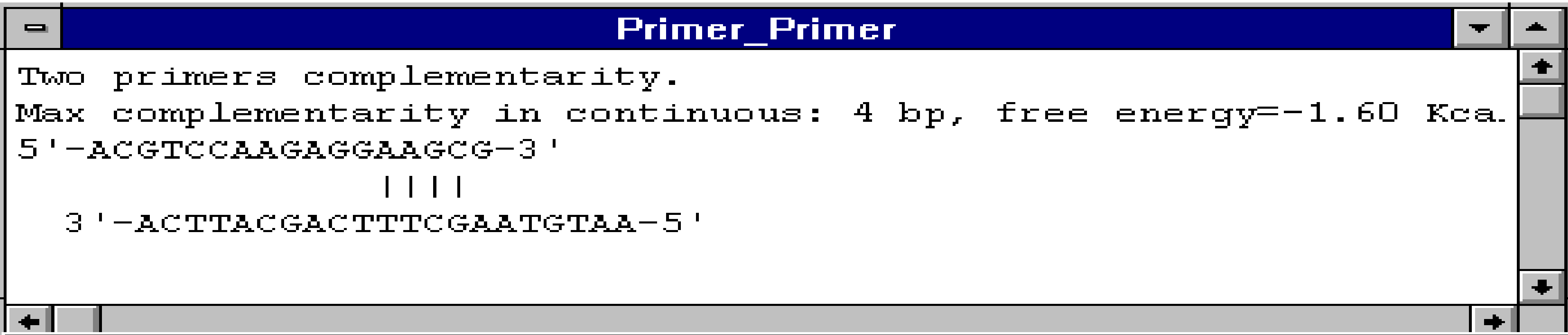
A simple set of rules for primer sequence design is as follows :

primers should be 19-25 bases in length;
base composition should be 50-60% (G+C);
primers should end (3') in a G or C, or CG or GC: this prevents "breathing" of ends and increases efficiency of priming;
Tms between 55-60oC are preferred;
runs of three or more Cs or Gs at the 3'-ends of primers may promote mispriming at G or C-rich sequences (because of stability of annealing), and should be avoided;

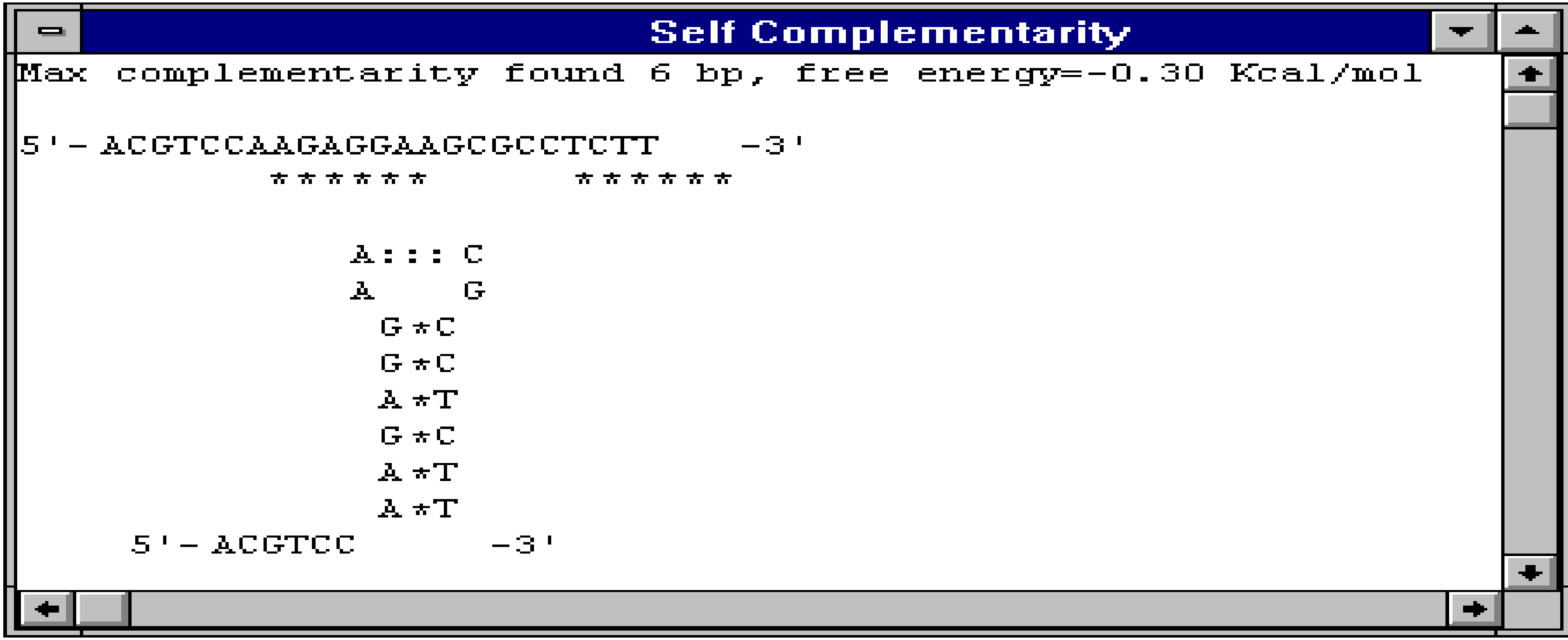
3'-ends of primers should not be complementary (ie. base pair), as otherwise primer dimers will be synthesised preferentially to any other product;

primer self-complementarity (ability to form secondary structures such as hairpins) should be avoided.

Examples of inter- and intra-primer complementarity which would result in problems:



```
Two primers complementarity.
Max complementarity in continuous: 4 bp, free energy=-1.60 Kca
5'-ACGTCCAAGAGGAAGCG-3'
               ||||
  3'-ACTTACGACTTTCGAATGTAA-5'
```



```
Max complementarity found 6 bp, free energy=-0.30 Kcal/mol

5'- ACGTCCAAGAGGAAGCGCCTCTT    -3'
          ******      ******


              A::: C
              A    G
               G*C
               G*C
               A*T
               G*C
               A*T
               A*T
     5'- ACGTCC     -3'
```

Recommended Reagent Concentrations:

Primers: 0.2 - 1.0 μM
Nucleotides: 50 - 200 μM EACH dNTP
Dimethyl sulphoxide (DMSO): 0 - 10% (v/v)
Taq polymerase: 0.5 - 2.0 Units/50ul rxn
Target DNA: 1 ng - 1 μg (NB: higher concentration for total genomic DNA; lower for plasmid / purified DNA / virus DNA target)
Buffer: use proprietary or home-made 10x rxn mix. This should contain: minimum of 1.5mM Mg2+, usually some detergent, perhaps some gelatin or BSA.
25mM MgCl2, may be used to allow user-specified [Mg2+] for reaction optimisation with different combinations of primers and targets.
MAKE POOLED MASTER MIX OF REAGENTS IN ABSENCE OF DNA using DNA-free pipette, then dispense to individual tubes (using DNA-free pipette), and add DNA to individual reactions USING PLUGGED TIPS.

OVERLAY REACTIONS WITH 50UL OF HIGH-QUALITY LIQUID PARAFFIN OR MINERAL OIL to ensure no evaporation occurs:

USE PLUGGED PIPETTE TIPS: prevents aerosol contamination of pipettes.
Use of detergents is recommended only for some Taq Polymerases (up to 0.1% v/v, Triton X-100 or Tween-20).
DMSO apparently allows better denaturation of longer target sequences (>1kb) and more product.
DO NOT USE SAME PIPETTE FOR DISPENSING NUCLEIC ACIDS AS YOU USE FOR DISPENSING REAGENTS

Remember **sample volume should not exceed 1/10th reaction volume**, and sample DNA/NTP/primer concentrations should not be too high as otherwise all available Mg2+ is chelated out of solution and enzyme reactivity is adversely affected. **Any increase in dNTPs over 200μM means [Mg2+] should be re-optimised.**
AVOID USING EDTA-CONTAINING BUFFERS AS EDTA CHELATES Mg2+

Recommended Reaction Conditions:
Initial Conditions:
Initial denaturation at start: 94 - 96oC for 3 - 5 min. If you denature at 96oC, denature sample only; add rest of mix after reaction cools to annealing temperature (prevents premature denaturation of enzyme).
Initial annealing temperature: as high as feasible for 1 min (eg: 50 - 65oC). Stringent initial conditions mean less non-specific product, especially when amplifying from eukaryotic genomic DNA.
Initial elongation temperature: 72oC for 1-2 min. This allows complete elongation of product on rare templates.

Temperature Cycling:

94 - 95oC for 30 - 60 sec (denature)
37 - 65oC for 30 - 60 sec (anneal)
72oC for 30 - 60 sec (elongate) (60 sec per kb target sequence length)
30 - 40 cycles only (otherwise enzyme decay causes artifacts)
72oC for 5-15 min at end to allow complete elongation of all product DNA

NOTE:

"Quickie" PCR is quite feasible: eg, [94oC 10 sec / 45oC 10 sec / 72oC 10 sec] x 30, for short products (200 - 300 bp).

DON'T RUN TOO MANY CYCLES: if you don't see a band with 30 cycles you probably won't after 40; rather take an aliquot from the reaction mix and re-PCR with fresh reagents.

"Hot Start" PCR:

In certain circumstances one wishes to **avoid mixing primers and target DNA at low temperatures in the presence of Taq** polymerase: **Taq pol is almost as efficient as Klenow pol at 37oC**; consequently, if primers mis-anneal at low temperature prior to initial template denaturation, **"non-specific" amplification may occur**. This may be avoided by only adding enzyme after the initial denaturation, before the reaction cools to the chosen annealing temperature.

This is most conveniently done by putting wax "gems"TM into the reaction tube after addition of everything except enzyme, then putting enzyme on top of the gem: the wax melts when the temperature reaches +/-80oC, and the enzyme mixes with the rest of the reaction mix while the molten wax floats on top and seals the mix, taking the place of mineral oil.

Asymmetric PCR for ssDNA Production:

Simply use a 100:1 molar ratio of the two primers (eg: primer 1 at 0.5uM, primer 2 at 0.005uM). This allows production of mainly ssDNA of the sense of the more abundant primer, which is useful for sequencing purposes or making ssDNA probes.

Detecting Products:

Take 1/10th - 1/3rd of the reaction mix **CAREFULLY** from under the oil or from under the Vaseline or solidified wax, using a micropipette with plugged tip,

IN AN AREA AWAY FROM YOUR PCR PREPARATION AREA!

Mix this with some gel loading buffer(1:1 - 1:5 mix:loading buffer): this is TBE containing 10 - 20% glycerol or sucrose and a dash of bromophenol blue (BPB) tracking dye.

Load 5 - 30ul of sample into wells of 0.8 - 3.0% submarine agarose gel made up in TBE, **preferably containing 50ng/ml ethidium bromide**.

Run at 80 -120 volts (not too slow or small products diffuse; not too fast or

bands smear) until BPB reaches end of gel
Use DNA markers going from 2kb down to 100 bp or less

View on UV light box at 254 - 300 nm, photo 1 - 5 sec.

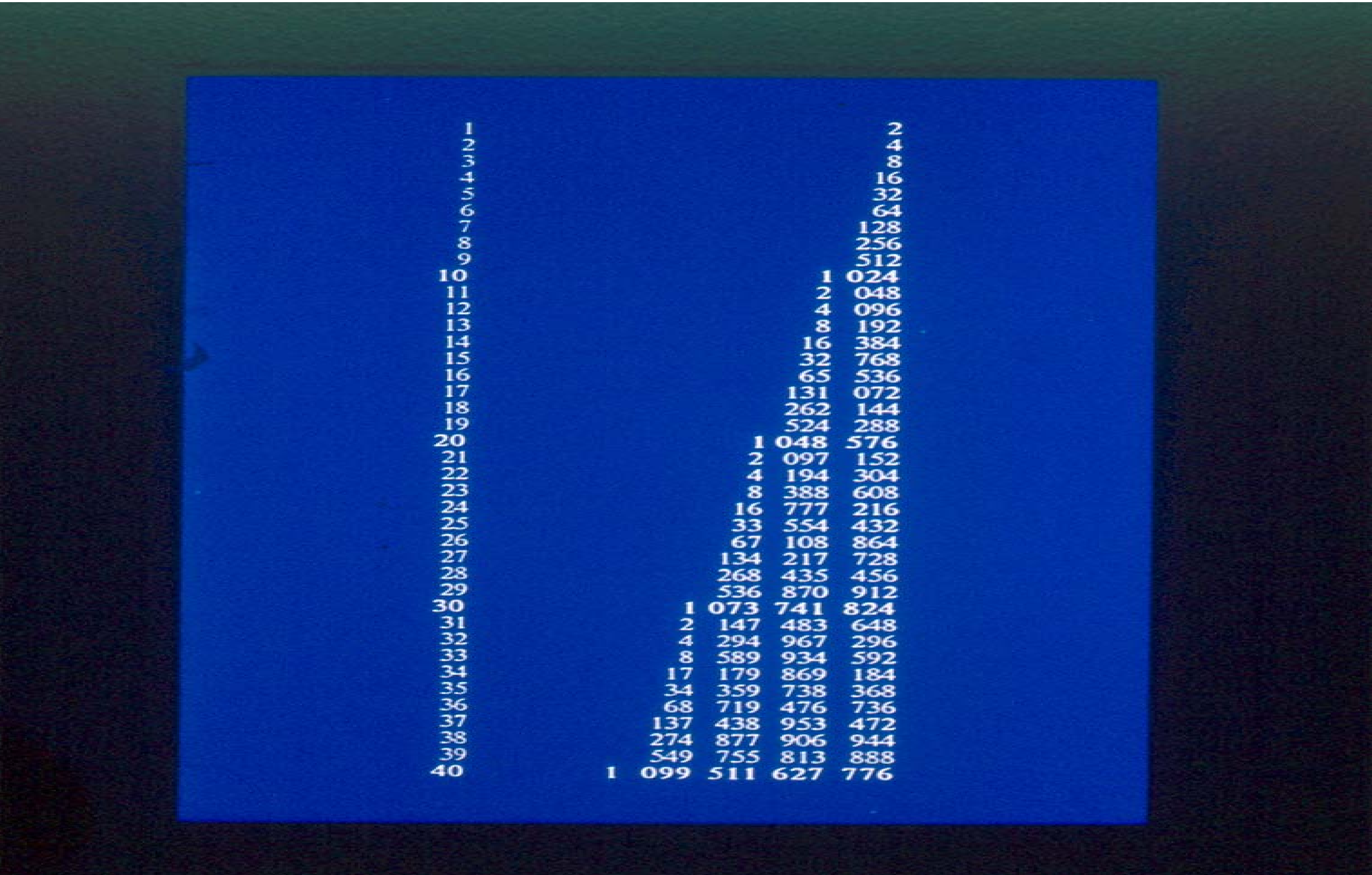
Small products are best seen on 3% agarose gels that have been **run fast** (eg: 100 volts), with BPB run to ½ - 2/3 down the gel. **It is best to include EthBr in the gel AND in the gel buffer**, as post-electrophoresis staining can result in band smearing due to diffusion, and if there is no EthBr in the buffer the dye runs backwards out of the gel, and smaller bands are stripped of dye and are not visible.

NUSIEVE TM gel (FMC Corp) can also be used for small products - better resolution than agarose.

Polyacrylamide gels can be silver stained by simple protocols for extreme sensitivity of detection.

Gels can be blotted directly after soaking in 0.5M NaOH / 1.5M NaCl for 10-20 min: "dry blotting" works well (eg: gel is over- and under-layered with paper towel stacks and pressed; bands transfer up and down), as does classic "Southern" blotting. Bands blotted in this way are already covalently fixed onto nylon membranes, and simply need a rinse in 5xSSPE before prehybridisation.

Στο τέλος κάθε κύκλου ο στόχος πολλαπλασιάζεται 2n όπου n ο αριθμός των κύκλων .Π.χ. στο τέλος του 3ου κύκλου ο στόχος έχει πολλαπλασιασθεί 8 φορές , στο τέλος του 4ου 16 κ.ο.κ.



```
 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        2 147 483 648
32        4 294 967 296
33        8 589 934 592
34       17 179 869 184
35       34 359 738 368
36       68 719 476 736
37      137 438 953 472
38      274 877 906 944
39      549 755 813 888
40    1 099 511 627 776
```

MOLECULAR WEIGHT CONVERSIONS FOR NUCLEIC ACIDS

- MW of a double-stranded DNA molecule = number of base pairs x **649** daltons/base pair
- MW of a single-stranded DNA molecule = number of bases x **325** daltons/base
- MW of a single-stranded RNA molecule = number of bases x **341** daltons/base
- MW of a DNA oligonucleotide:
  $MW = (N_A \times 312.2) + (N_C \times 288.2) + (N_G \times 328.2) + (N_T \times 303.2)$, where $N_{Adenine}$, $N_{Cytosine}$, $N_{Guanine}$, $N_{Thymidine}$ is the number of the respective nucleotide within the oligonucleotide.

SPECTROPHOTOMETRIC CONVERSIONS

1 A_{260} Unit	**Concentration (µg/ml)**
Double-stranded DNA	50
Single-stranded DNA	33
Single-stranded RNA	40
Oligonucleotide	20-30 Depends on the number of bases

Example: MW of an oligonucleotide of 20 bases

$MW = 325 \times 20 = 6500g$

$1M = 6500 \times 10^6 \mu g$

$x; \quad 1\mu g$

$x = 1/(6.5 \times 10^9) = 0.153 \times 10^{-9}M = 153 \times 10^{-12}M$

1µg of 20 bases Oligo = 153pmol

$1\mu g \longrightarrow 0.153nmol$

$x; \quad 5nmol$

$x = 5/0.153 = 32.7\mu g$

1 mole = 6,02X 1023 μόρια ή αντίγραφα πολλαπλασιασθέντων DNA αλληλουχιών .

Η απόδοση του συστήματος δίνεται από τον τύπο:

Y= (1+X)n

Y=φορές πολλαπλασιασμού του στόχου

n = αριθμός των κύκλων PCR

X = μέση απόδοση για τον κάθε κύκλο

ΠΑΡΑΔΕΙΓΜΑ ΥΠΟΛΟΓΙΣΜΟΥ

Πόσοι κύκλοι απαιτούνται ώστε ένα αντίγραφο του στόχου να πολλαπλασιασθεί και να δώσει ένα τελικό προϊόν PCR 200ng; Τα αποτελέσματα για ένα προϊόν PCR 240 ζεύγη βάσεων (bp) παρουσιάζεται κατωτέρω όταν η απόδοση του συστήματος είναι:

100% (X=1) και

80% (X=0,8).

$[(1\ \cancel{\text{copy}} \times \cancel{\text{mole}})/(6.02 \times 10^{23}\cancel{\text{copies}})] \times [(240 \times 2 \times 325\text{g})/\cancel{\text{mole}}] = (15.6 \times 10^{4}\text{g})/(6.02 \times 10^{23}) = 2.6 \times 10^{-19}\text{g}$

Must amplify 200ng $\longrightarrow (200 \times 10^{-9}\cancel{\text{g}})/(2.6 \times 10^{-19}\cancel{\text{g}}) = 76.9 \times 10^{10}$ **fold**

$76.9 \times 10^{10} = (2)^{h} \longrightarrow n = 10 \times \log 76.9/\log 2 = 10 \times 1.88/0.30 = \boxed{10 \times 6.26 = 62 \text{ cycles}}$

Efficiency 100%

	Efficiency 100% (FOLD)	Cycles
1 Copy	4.6×10^{18}	62
100 Copies	1.1×10^{15}	50
1000 Copies	1.7×10^{13}	44
10^4 Copies	1.3×10^{11}	37
10^5 Copies	2.1×10^{9}	31

$y = (1 + x)^{n} = (1+1)^{n} = 2^{n}$
$4.6 \times 10^{18} = 2^{n}$

$n = \log 4.6 \times 10^{8}/\log 2 =$
$= 18.66/0.30 = 62$

Efficiency 80%

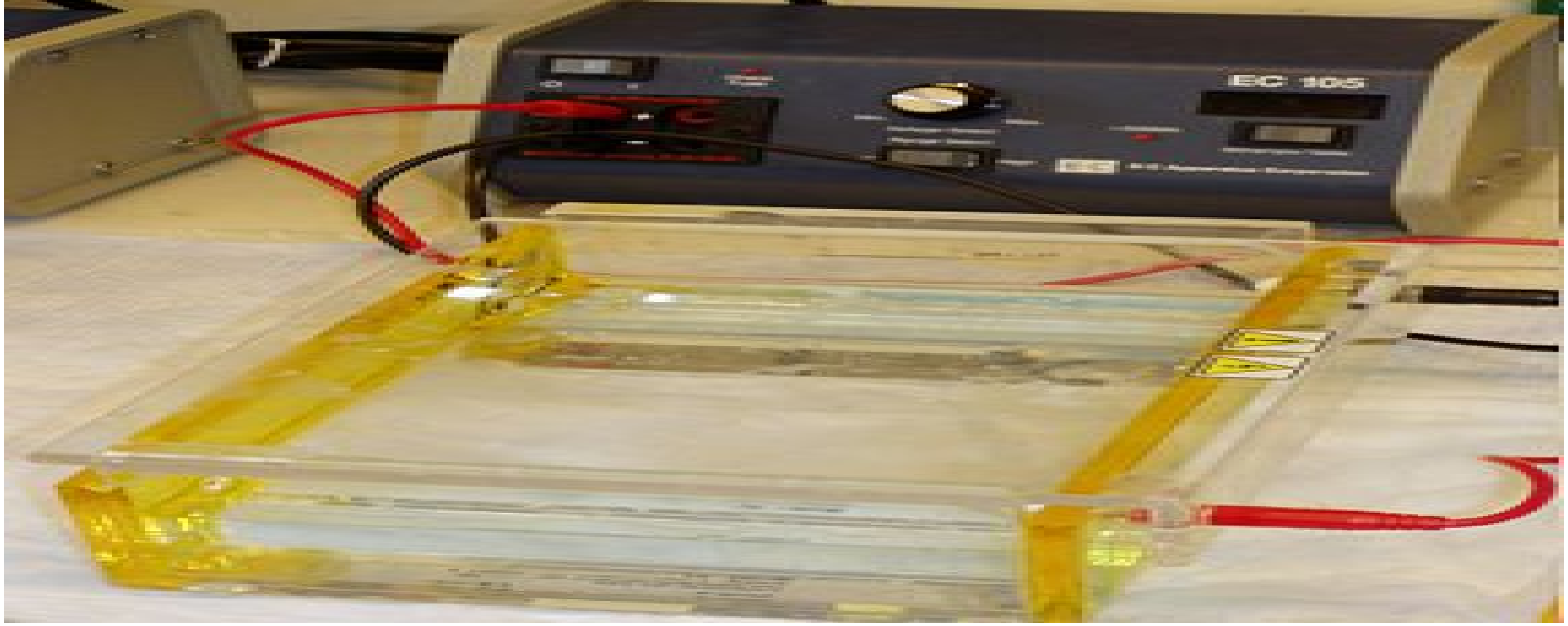
	Efficiency 80% (FOLD)	Cycles
1 Copy	6.7×10^{15}	75
100 Copies	5.8×10^{12}	60
1000 Copies	3.3×10^{13}	53
10^4 Copies	1.7×10^{11}	44
10^5 Copies	2.8×10^{9}	37

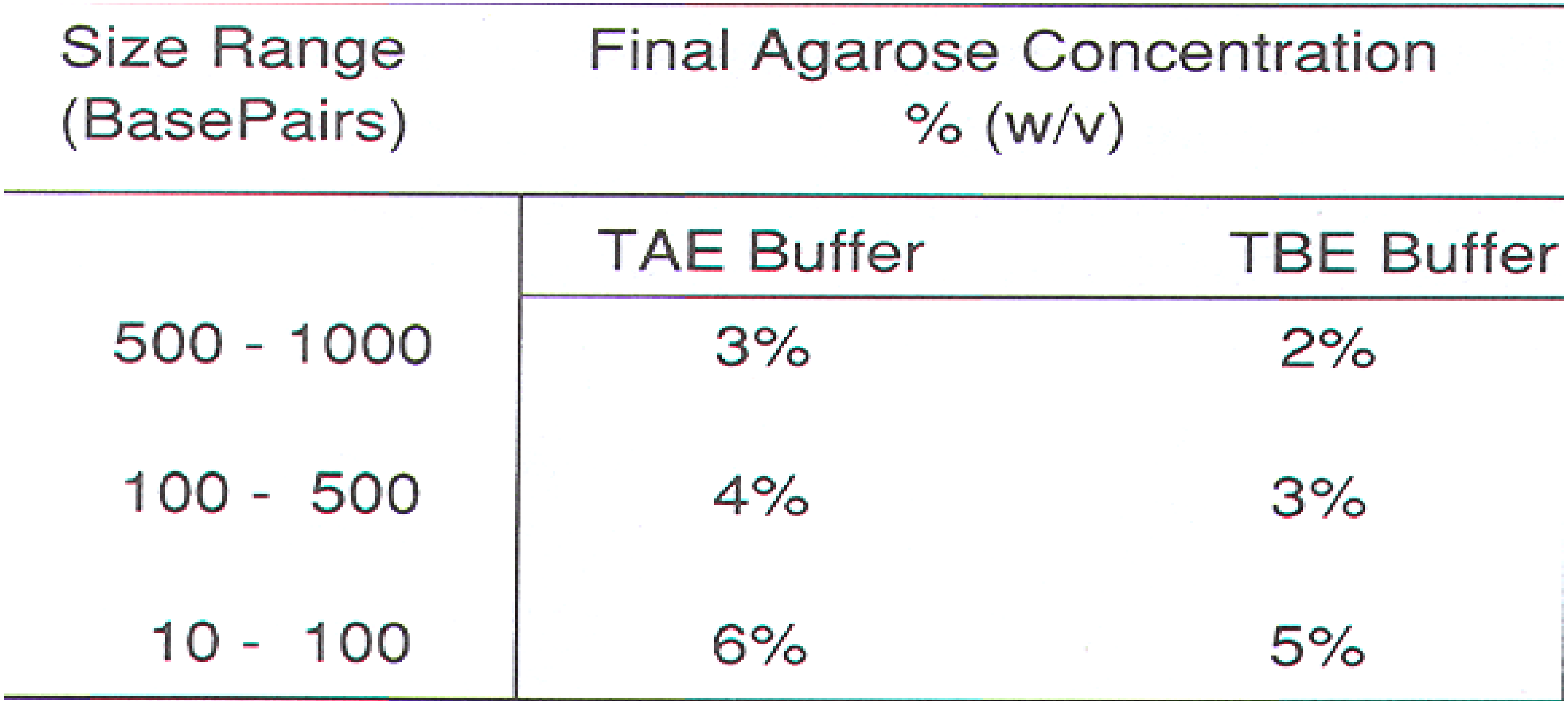
$Y = (1+x)^{n}$

$Y = (1+0.8)^{n} = (1.8)^{n}$

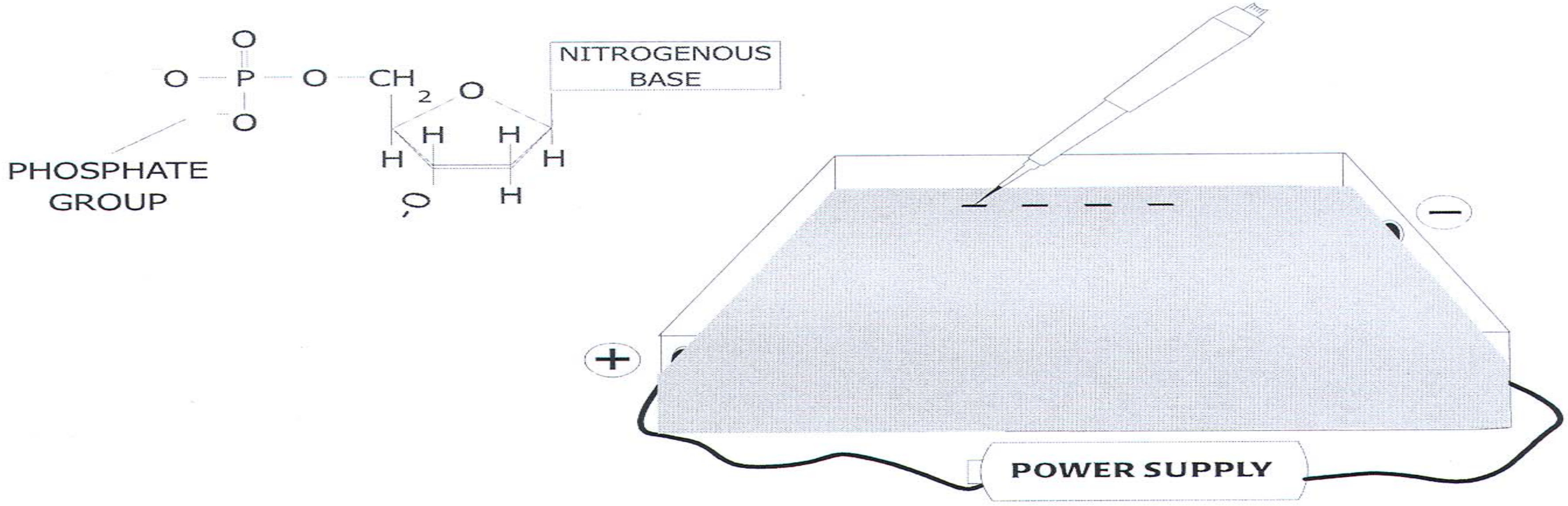
$4.6 \times 10^{18} = 1.8^{n}$

$n = \log 4.6 \times 10^{18}/\log 1.8$
$= 18.66/0.25 = 74.6$





Size Range (BasePairs)	Final Agarose Concentration % (w/v)	
	TAE Buffer	TBE Buffer
500 - 1000	3%	2%
100 - 500	4%	3%
10 - 100	6%	5%



Τα δείγματα PCR τα οποία πρόκειται να υποβληθούν σε ηλεκτροφόρηση αναμειγνύονται πριν την ηλεκτροφόρηση με 2-5 μl “Gel loading buffer” το οποίο περιέχει :

0,25% bromophenol blue

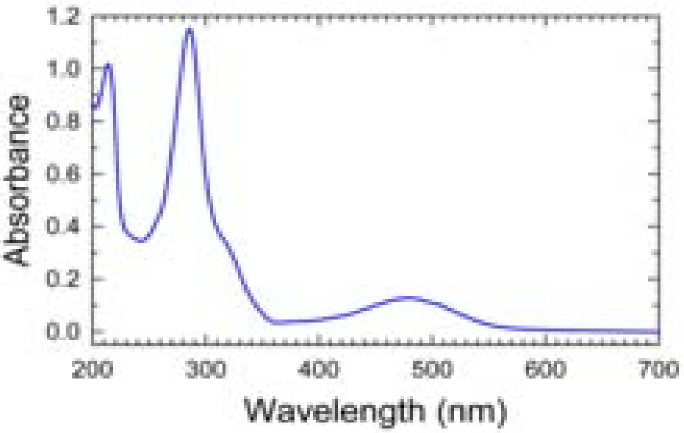
0,25% xylene cyanol

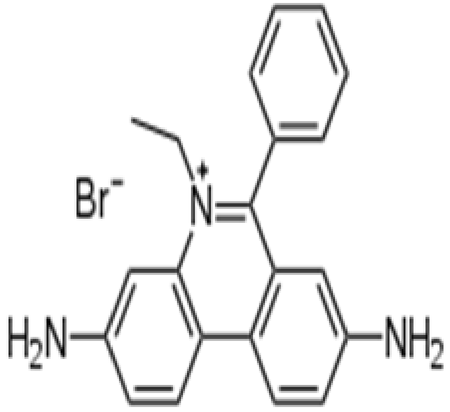
και οι δυο αυτές χρωστικές μας βοηθούν να παρακολουθήσουμε την εξέλιξη της ηλεκτροφόρησης μια που για μια δεδομένη συγκέντρωση αγαρόζης «ταξιδεύουν» μαζί με τον αντίστοιχο αριθμό ζευγών βάσεων του προιόντος PCR , όπως παρουσιάζεται στον κατωτέρω πίνακα.

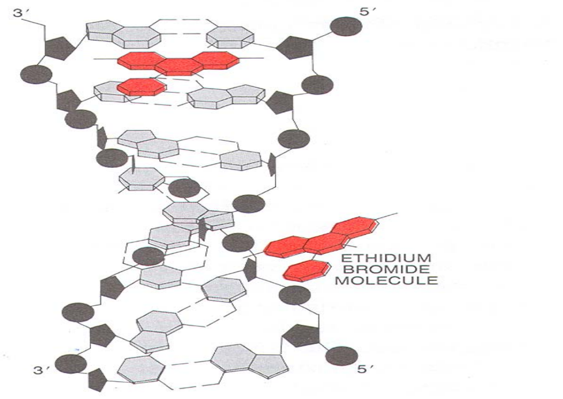
25% Ficoll 400 ή 50% σουκρόζη .Οι ουσίες αυτές βοηθούν ώστε το DNA να «κάθεται» στις οπές οι οποίες δημιουργούνται μετά την αφαίρεση της «κτένας» από το πήκτωμα αγαρόζης.

Agarose Conc.	1X TAE Buffer		1X TBE Buffer	
	Bromophenol Blue	Xylene Cyanol	Bromophenol Blue	Xylene Cyanol
2.5%	130 bp	950 bp	70 bp	700 bp
3.0%	80 bp	650 bp	40 bp	500 bp
4.0%	40 bp	350 bp	20 bp	250 bp
5.0%	30 bp	200 bp	8 bp	140 bp
6.0%	20 bp	120 bp	4 bp	90 bp

Στο πήκτωμα αγαρόζης ενσωματώνουμε βρωμιούχο αιθίδιο (φθορίζουσα χρωστική) σε συγκέντρωση 1μg/ml το οποίο ενσωματώνεται στο δίκλωνο μόριο του προιόντος της PCR και τοποθετώντας το πήκτωμα αγαρόζης σε συσκευή UV (με μέγιστο απορρόφησης μεταξύ 270-320 nm) εντοπίζουμε τα προιόντα PCR με την βοήθεια 1-2 μαρτύρων μοριακού βάρους (γραμμές 1 & 2 στην κατωτέρω φωτογραφία) και τέλος φωτογραφίζουμε το πήκτωμα ώστε να υπάρχει στο αρχείο μας.

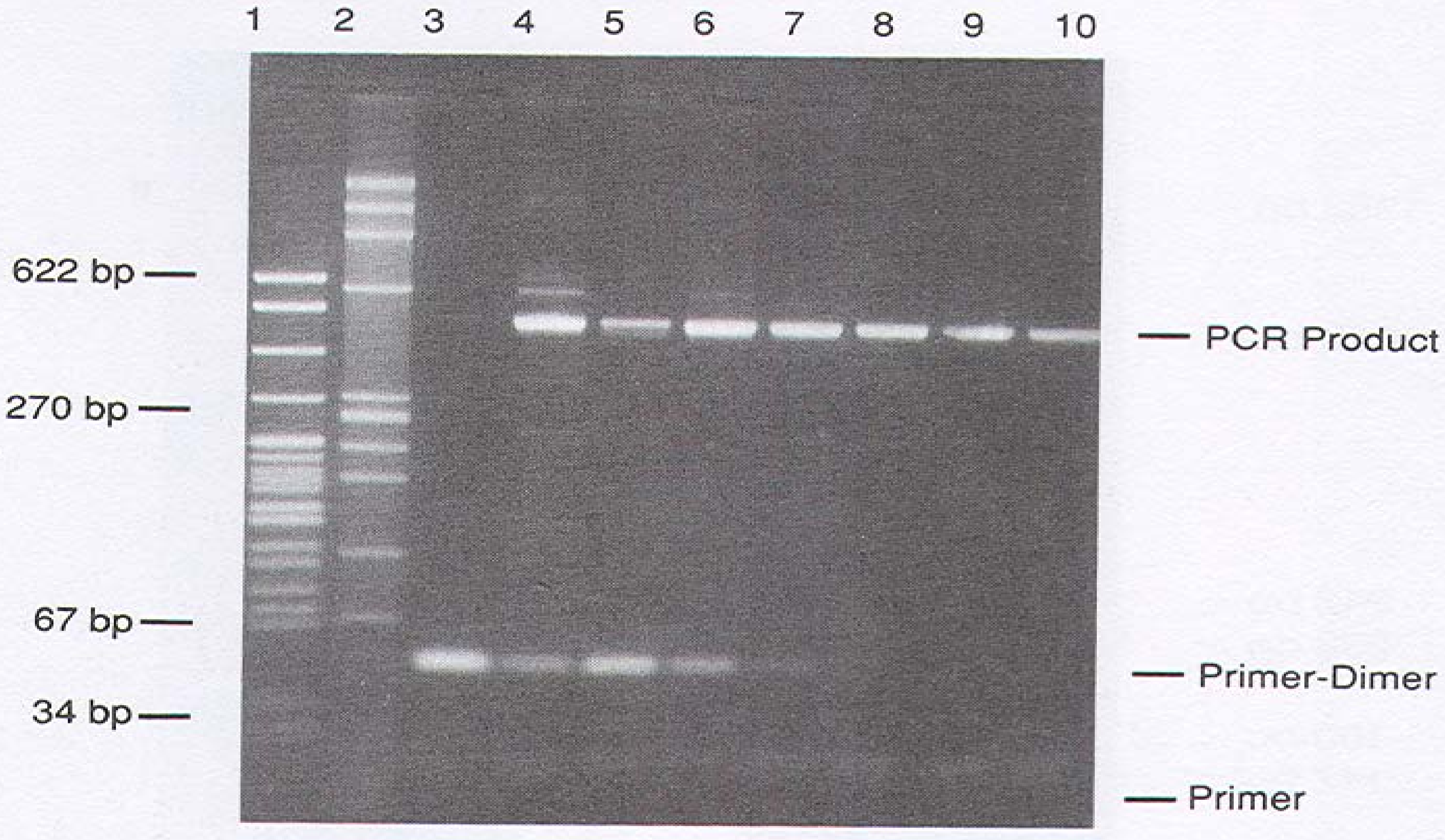


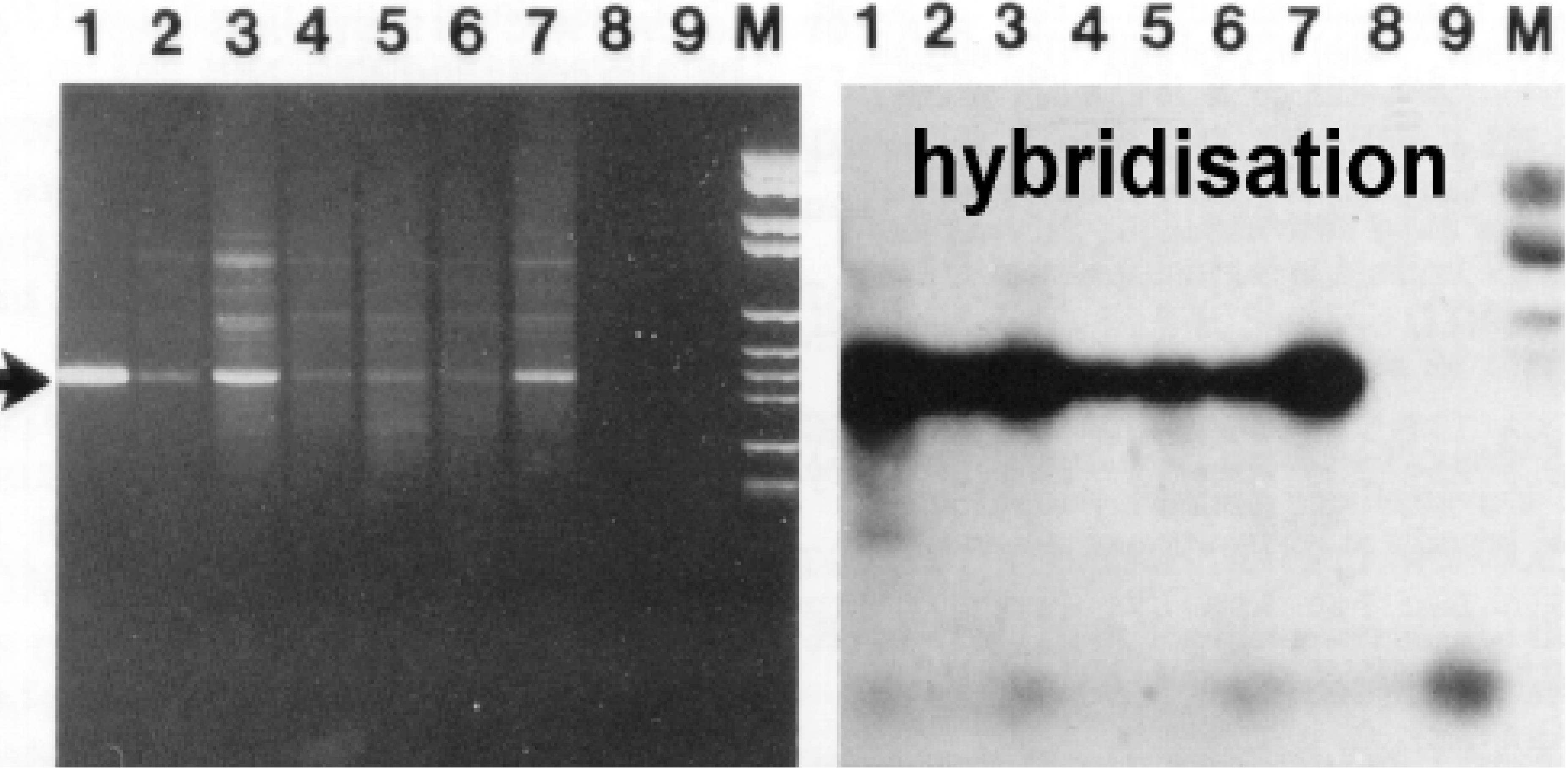




Intercalation of Ethidium Bromide into DNA Helix

ΘΕΣΕΙΣ 1&2 ΜΑΡΤΥΡΑΣ ΜΟΡΙΑΚΟΥ ΒΑΡΟΥΣ
ΘΕΣΗ 3 ΑΡΝΗΤΙΚΟ ΔΕΙΓΜΑ
ΘΕΣΕΙΣ 4-10 ΘΕΤΙΚΑ ΔΕΙΓΜΑΤΑ
ΓΥΡΩ ΣΤΙΣ 40 bp ΕΝΤΟΠΙΖΟΝΤΑΙ ΤΑ ΔΙΜΕΡΗ ΤΩΝ ΕΚΚΙΝΗΤΩΝ
Η ΗΛΕΚΤΡΟΦΟΡΗΣΗ ΕΓΙΝΕ ΣΕ ΑΓΑΡΟΖΗ NuSieve 3:1 ΚΑΙ ΣΥΓΚΕΝΤΡΩΣΗΣ 4%


1
2
3
4
5
6
7
8
9
10
622 bp
270 bp
67 bp
34 bp
PCR Product
Primer-Dimer
Primer



The example shown is of detection of *Human papillomavirus* type 16 (HPV-16) DNA amplified from cervical biopsy samples Detection of genital human papillomaviruses by polymerase chain reaction amplification with degenerate nested primers. The left panel is a photo of an EthBR-stained 2% agarose gel; the right is an autoradiograph of a Southern blot probed with ^{32}P-labelled HPV-16 DNA. Note how much more sensitive blotting is, and how much more specific the detection is.

Labelling PCR Products with Digoxigenin

PCR products may be very conveniently labelled with digoxigenin-11-dUTP (Boehringer-Mannheim) by incorporating the reagent to 10-35% final effective dTTP concentration in a nucleotide mix of final concentration 50-100µM dNTPs . This allows substitution to a known extent of probes of exactly defined length, which in turn allows exactly defined bybridisation conditions. It is also the most effective means of labelling PCR products, as it is potentially unsafe and VERY expensive to attempt to do similarly with 32P-dNTPs, and nick-translation or random primed label incorporation are unsuitable because the templates are often too small for efficient labelling.

Make a DIG-dNTP mix for PCR as follows:

DIG NUCLEOTIDE MIX CONCENTRATIONS

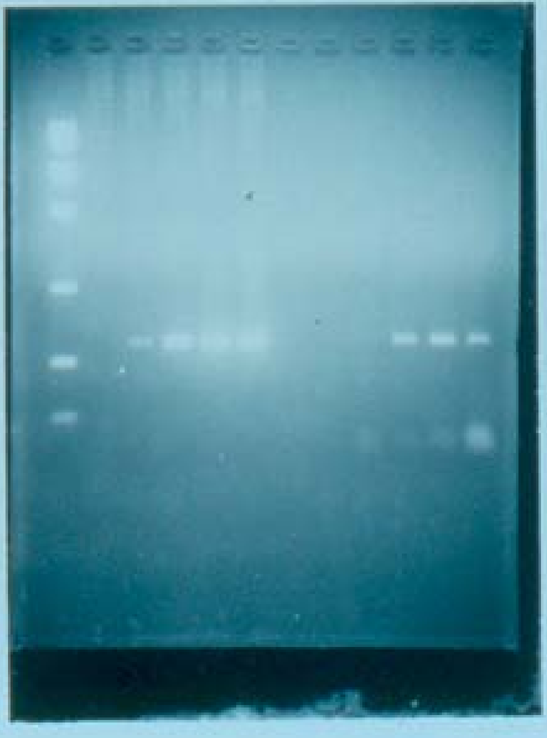
Dig-11-dUTP 350 µM
dTTP 650 µM
dATP 1 mM
dCTP 1 mM
dGTP 1 mM

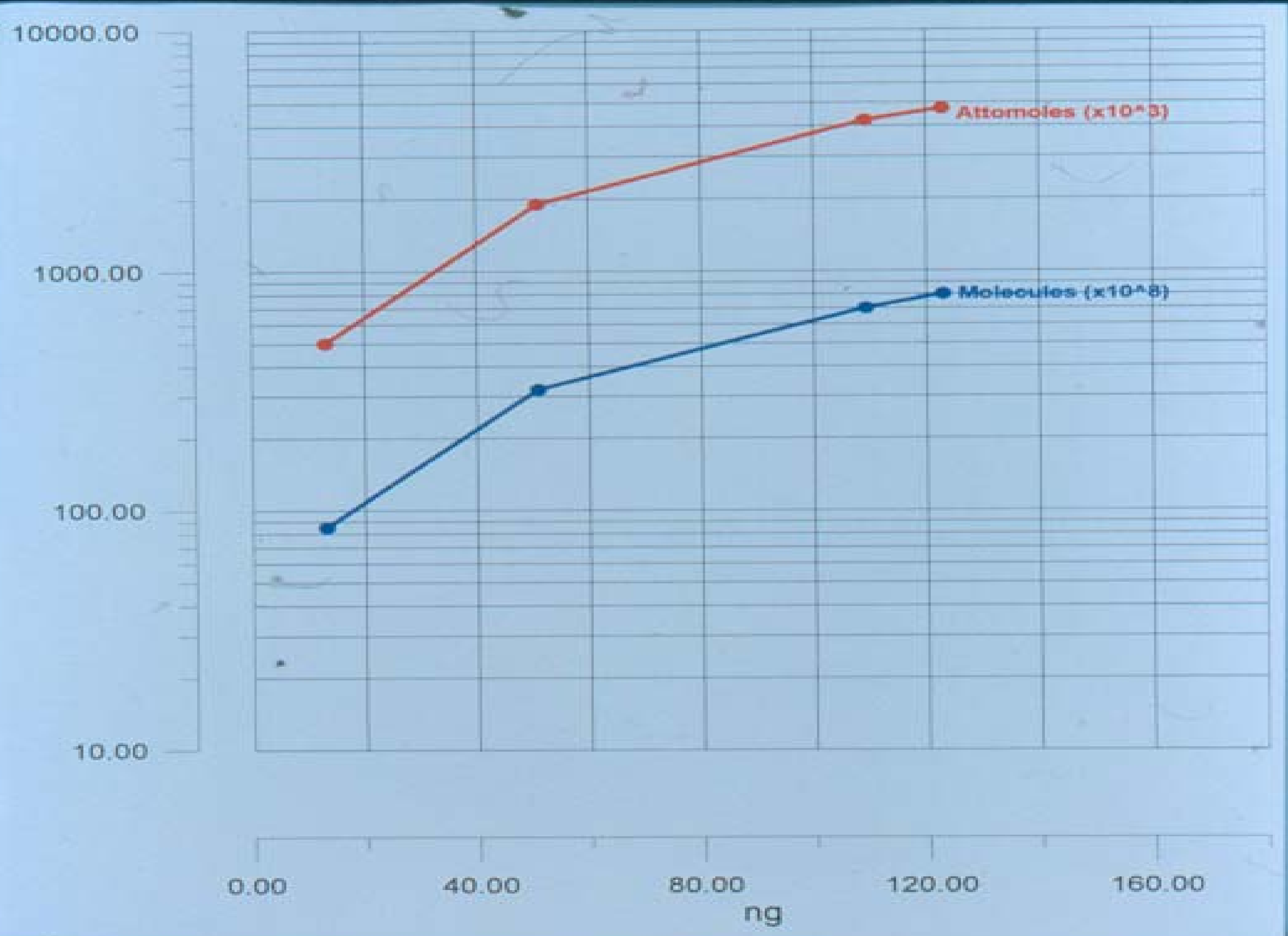
For each 50 ul of probe synthesized, a 1/10 dilution is made of the DIG-nucleotide mix when added to the other reagents as described above. The products may be analyzed by agarose gel electrophoresis - **NOTE: PRODUCTS ARE LARGER THAN NON-SUBSTITUTED PRODUCT** - and detected directly on blots immunologically. Probes can be used as 5-10 ul aliquots directly from PCR product mixes, mixed with hybridisation mix and denatured. Probes can be re-used up to 10 times, stored frozen in between experiments and boiled to denature.

AND ALWAYS REMEMBER:

- WORK CLEAN
- TITRATE MAGNESIUM
- DON'T USE TOO MUCH TEMPLATE DNA
- DON'T USE PCR PRODUCTS IN PCR PREPARATION AREAS
- *ALWAYS* INCLUDE MANY NEGATIVE CONTROLS AND VERY DILUTE POSITIVE CONTROLS IN EVERY EXPERIMENT
- WEAR GLOVES
- USE PLUGGED TIPS

Lanes: Bands	**Lane 1 (amount)**	**Lane 2 (amount)**	**Lane 3 (amount)**	**Lane 4 (amount)**	**Lane 5 (amount)**	**Lane 6 (amount)**	**Lane 7 (amount)**	**Lane 8 (amount)**
1	200	13.123	50.961	109.32	123.31	15.928	25.237	14.341
2	120.05							
3	80.064							
4	40.010							
5	20.013							
6	10							
Sum (ng)	470.13	13.123	50.961	109.32	123.31	15.928	25.237	14.341
In Lane: Attomoles		83.8×10^3	325.7×10^3	699.5×10^3	789.1×10^3			
Molecules		503×10^8	1954.5×10^8	4197×10^8	4734.7×10^8			




10000.00
1000.00
100.00
10.00
Attomoles (x10^3)
Molecules (x10^8)
0.00
40.00
80.00
120.00
160.00
ng

STANDARDIZATION OF HLA/HIV COAMPLIFICATION

- Compare the relative sensitivity of amplification versus coamplification
- Compare the results of coamplification for HIV-1 and HLA-DQ-α sequences in the presence of a concstant quantity of DNA derived from 10^6 uninfected PBMC
- Determine the range (normally 2-3 log) suitable for quantitation

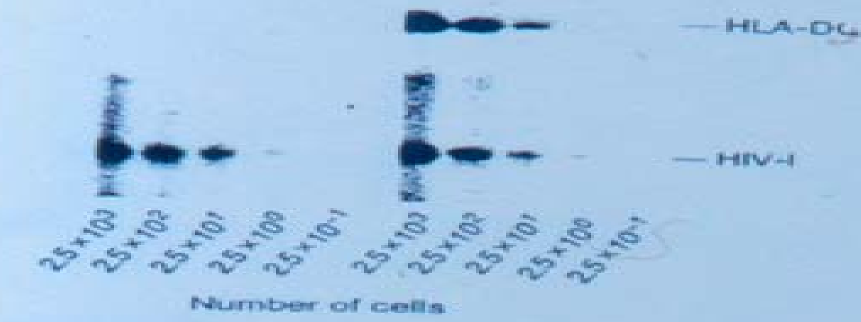
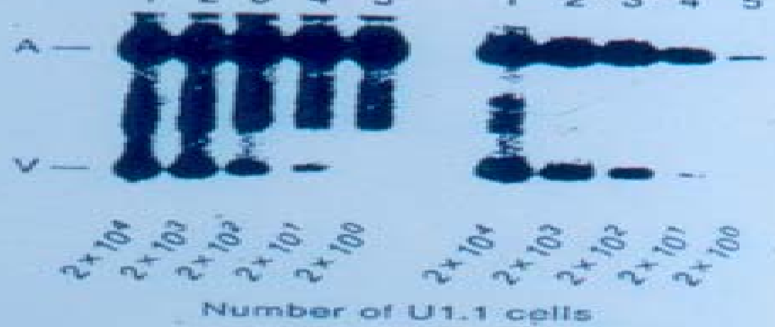
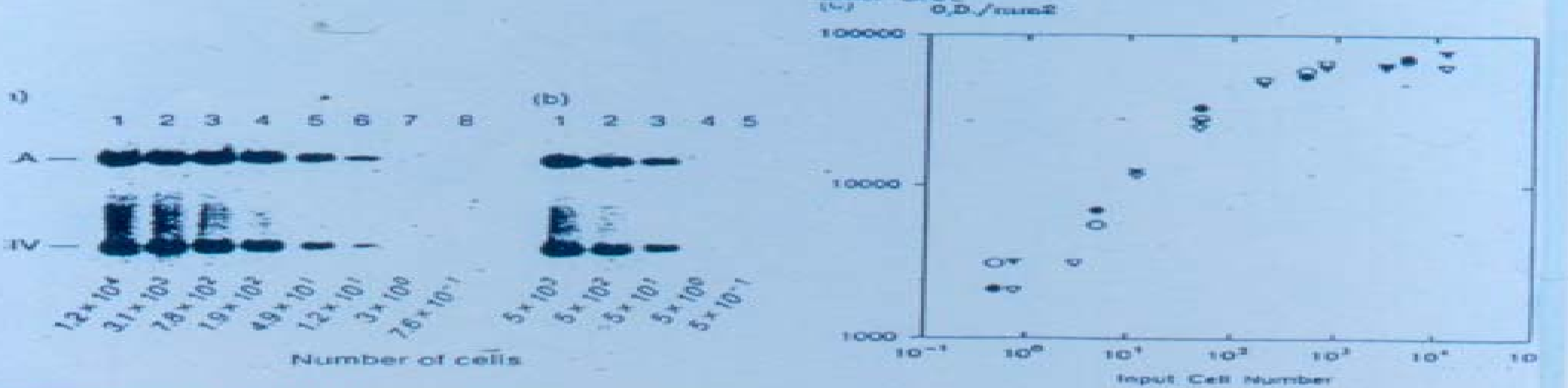


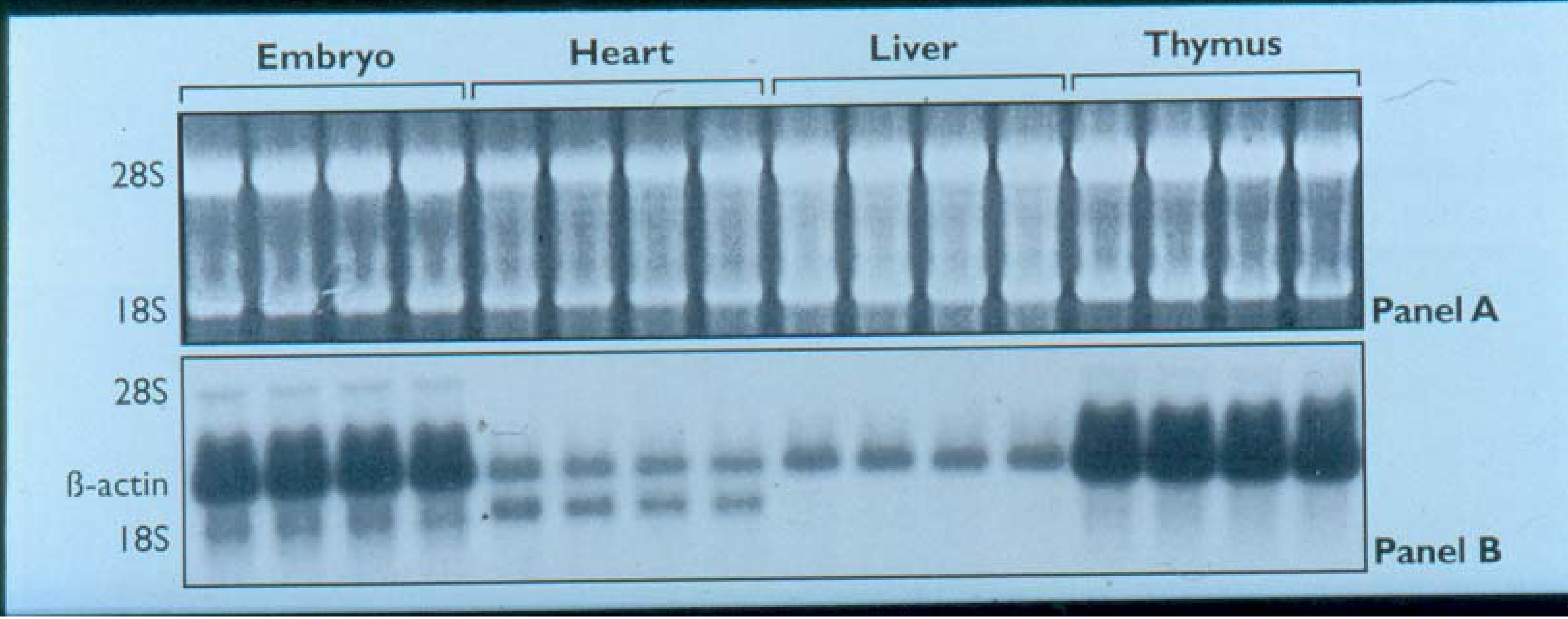
Fig. 2. Comparison of discrete amplification of HIV-1 sequence with coamplification for histocompatibility (HLA)-DQ-α and HIV 1 genes. A U1.1 cell lysate was serially diluted 10-fold into buffe (solution A and solution B) and subjected to both discrete ampli fication for HIV-1 gag genes only (lanes 1–5) and coamplificatior for HLA-DQ-α and HIV-1 gag genes (lanes 6–10).

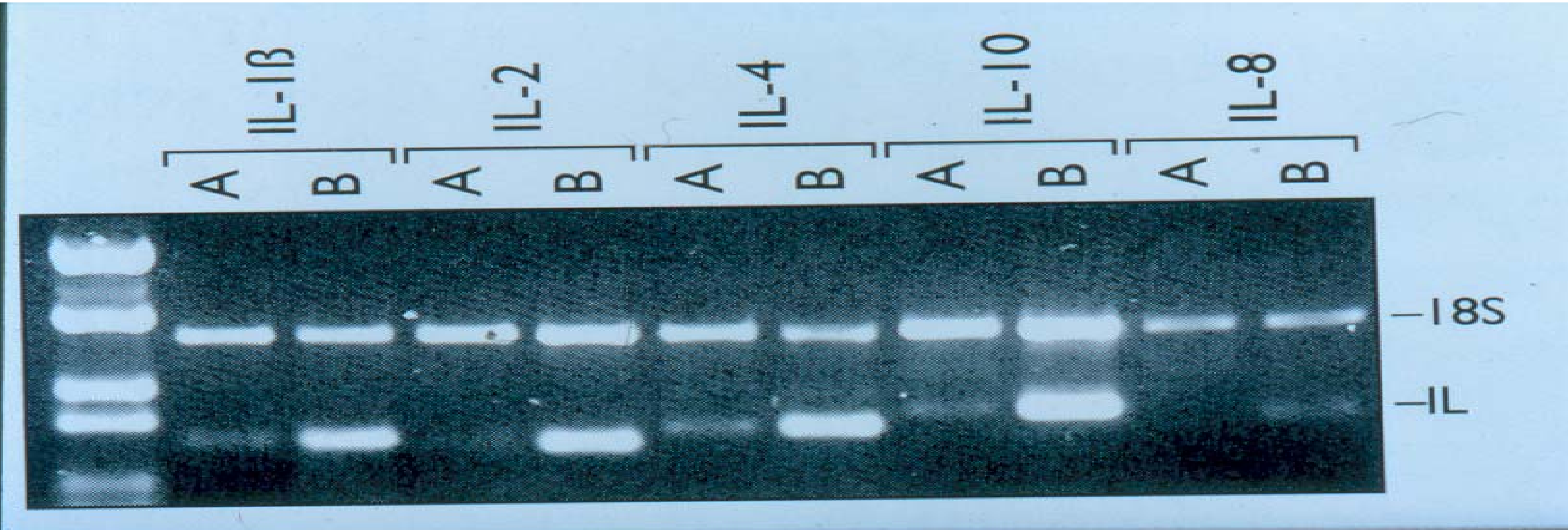


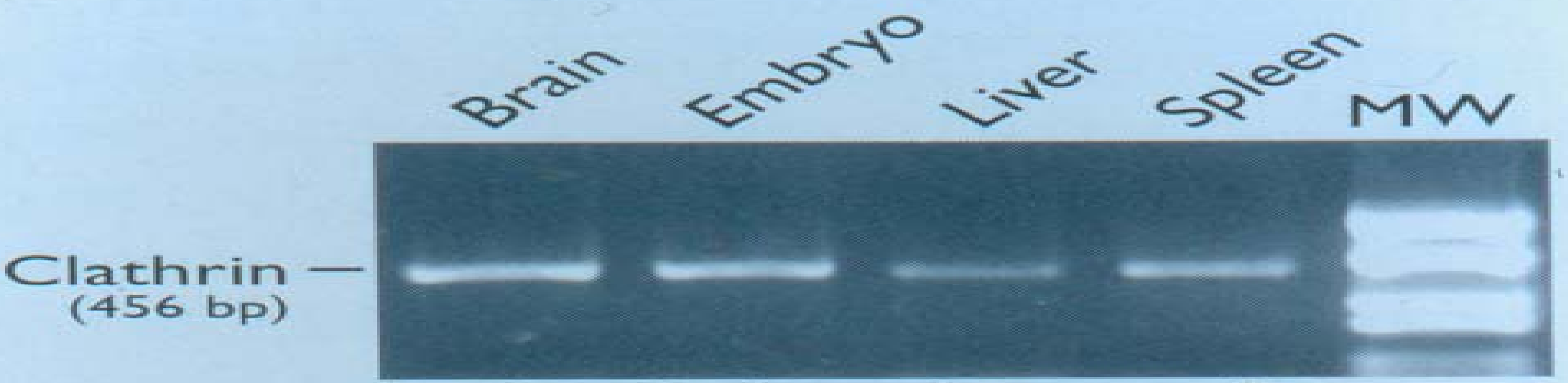
3. Comparison of results obtained following coamplifica- of histocompatibility (HLA)-DQ-α and HIV-1 gag genes us- 2 × 10⁴ U1.1 cells diluted into a lysate containing 1.25 × 10⁶ fected peripheral blood mononuclear cells (a), versus those ined following dilution into buffer (b). Note that the intensity e HIV-1 bands at each dilution are similar, and that the end- t sensitivity is two cells irrespective of presence or absence arrier DNA.



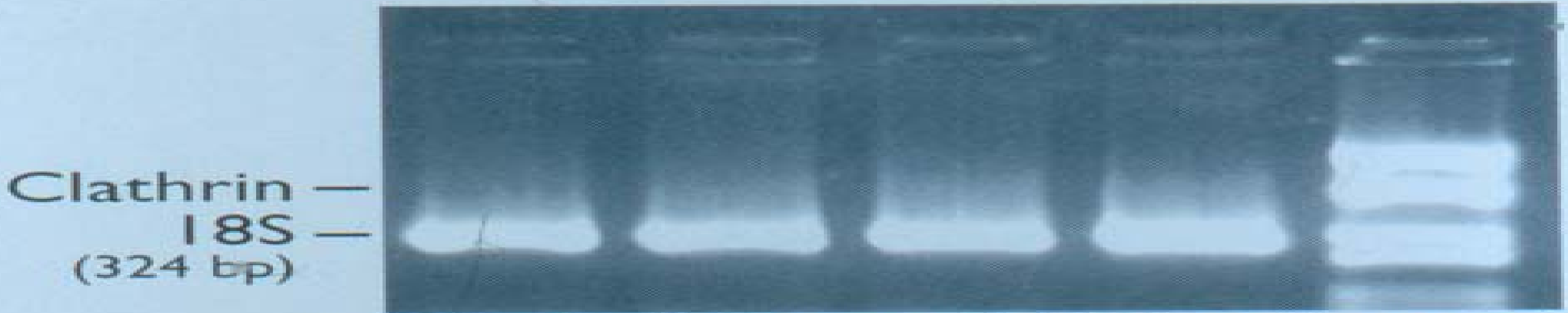
4. Reproducibility of HIV histocompatibility (HLA) coamplification. The figure shows polymerase chain reaction (PCR) results of rep ntative fourfold (a) and 10-fold (b) dilutions of a lysate of U1.1 cells into buffer. Each dilution was coamplified for HIV-1 gag an -DQ-α genes. Autoradiographs from coamplification of four sets of serial dilutions of this control lysate were analyzed using th ogical Vision 4000 Image Analysis System. The results of HIV band intensity versus input cell number are plotted in (c). Note tha the amplification conditions, primer concentrations and detection system used here, the assay is approximately linear from 10⁰ t


Embryo
Heart
Liver
Thymus
28S
18S
Panel A
28S
ß-actin
18S
Panel B

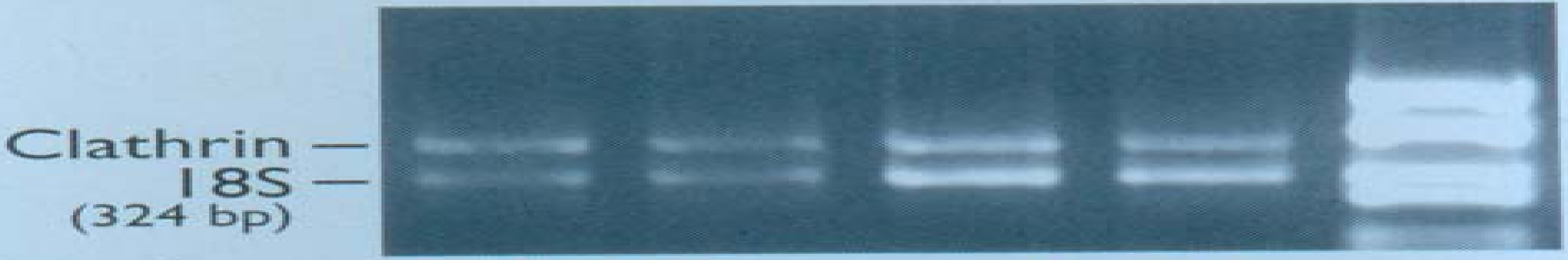

IL-1ß
IL-2
IL-4
IL-10
IL-8
A
B
A
B
A
B
A
B
A
B
–18S
–IL


Brain
Embryo
Liver
Spleen
MW
Clathrin
(456 bp)

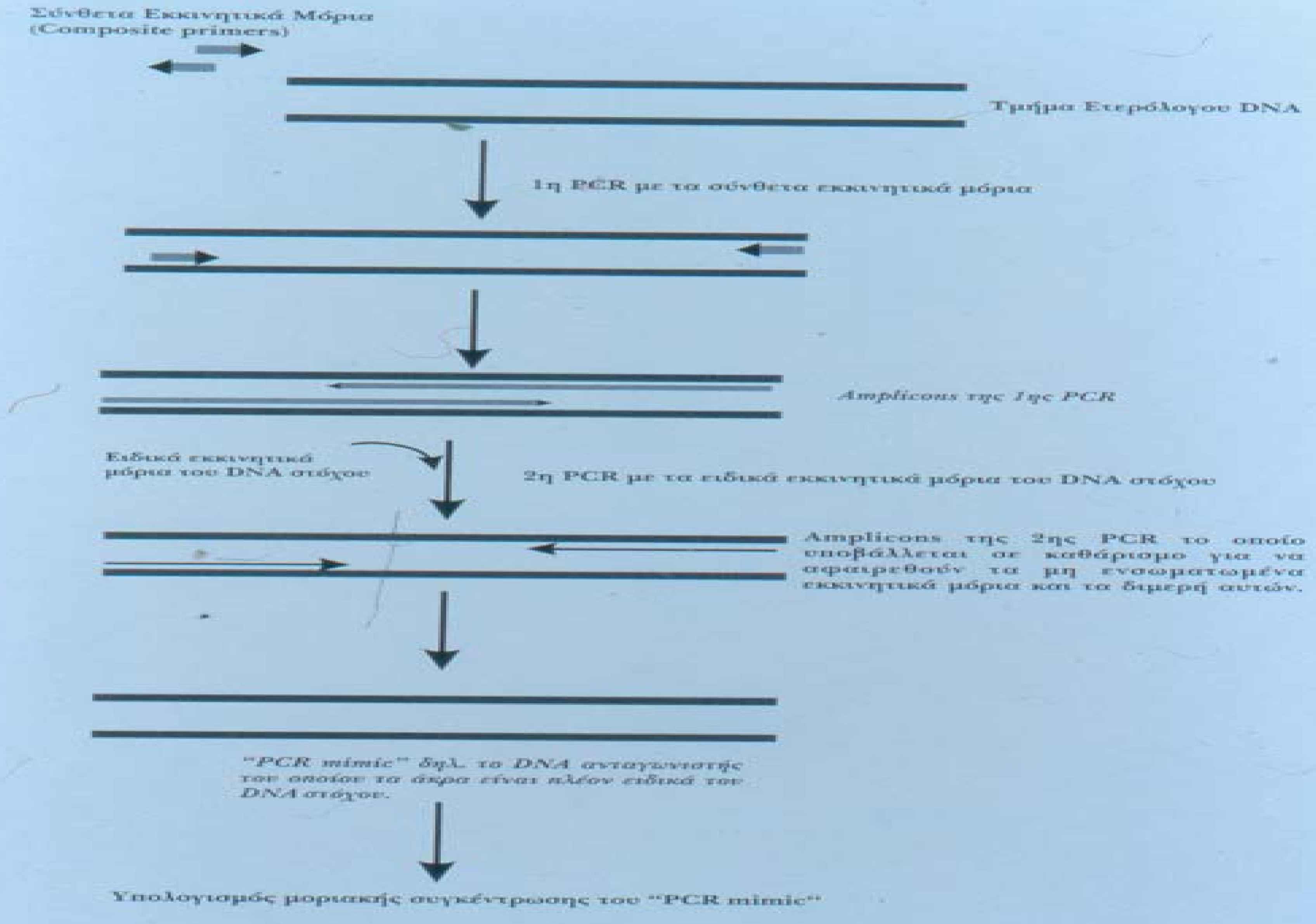
A. Clathrin primers alone


Clathrin
18S
(324 bp)

B. Clathrin and 18S primers; no competimers


Clathrin
18S
(324 bp)

C. Clathrin and 18S 3:7 competimers:primers



Σύνθετα Εκκινητικά Μόρια
(Composite primers)

Τμήμα Ετερόλογου DNA

1η PCR με τα σύνθετα εκκινητικά μόρια

Amplicons της 1ης PCR

Ειδικά εκκινητικά μόρια του DNA στόχου

2η PCR με τα ειδικά εκκινητικά μόρια του DNA στόχου

Amplicons της 2ης PCR το οποίο υποβάλλεται σε καθάρισμο για να αφαιρεθούν τα μη ενσωματωμένα εκκινητικά μόρια και τα διμερή αυτών.

"PCR mimic" δηλ. το DNA ανταγωνιστής του οποίου τα άκρα είναι πλέον ειδικά του DNA στόχου.

Υπολογισμός μοριακής συγκέντρωσης του "PCR mimic"

Εικόνα 2. Σχηματική παρουσίαση της κατασκευής ενός "PCR mimic".

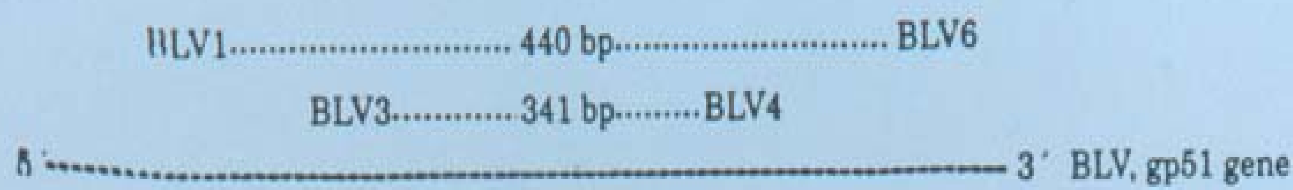

BLV1 .... 440 bp BLV6
BLV3 .... 341 bp BLV4
5′
3′ BLV, gp51 gene

Fig. 1. Schematic arrangement of the primers in the BLV genome. External BLV primers are BLV1 and BLV6, while internal primers are BLV3 and BLV5.

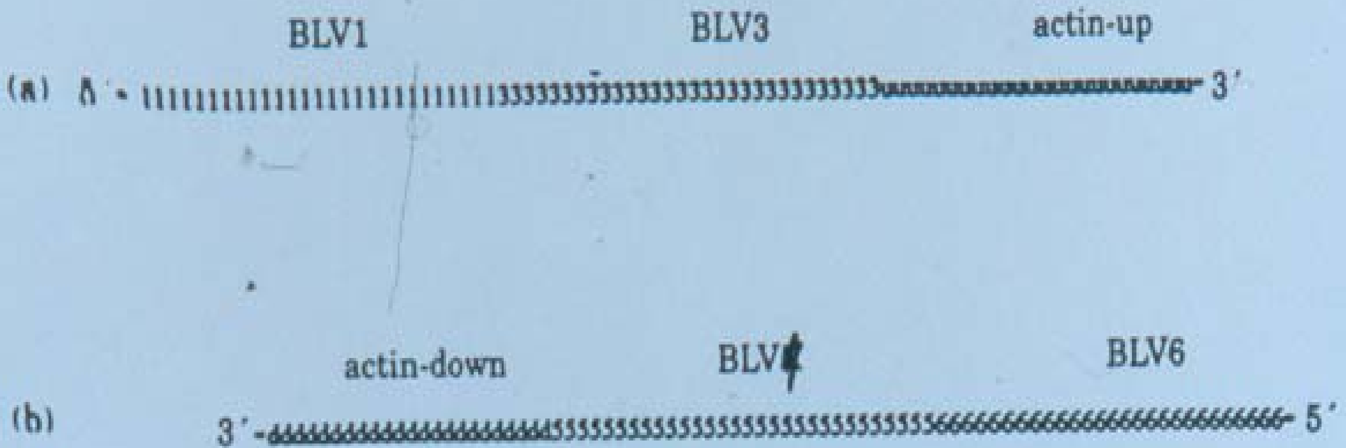

BLV1
BLV3
actin-up
(a) 5′
3′
actin-down
BLV4
BLV6
(b) 3′
5′

Fig. 2. Composition of the upstream (a) and downstream (b) primers used in MP-PCR to produce BLV-mimic.

Table 1. Sequences of p53-, mdm2- and GAPDH-primers for construction of deletion mutants (T7-P1-P3/oligo(dT)-P5) and for competitive PCR (P1/P5)

5'-Primer	T7	P1	P3
p53	5'-GGATCCTAATACGACTCACTATAGGGAGG	CATCTACAAGCAGTCACAGC	GTACCACCATCCACTACAAC-3'
mdm2	5'-GGATCCTAATACGACTCACTATAGGGAGG	CCTACTGATGGTGCTGTAAC	GATTTGTTTGGCGTGCCAAG-3'
GAPDH	5'-GGATCCTAATACGACTCACTATAGGGAGG	CACCCATGGCAAATTCCATG	GCCAAAAGGGTCATCATCTC-3'

3'-Primer	Oligo(dT)	P5
p53	5'-TTTTTTTTTTTTTTT	GCCCTTCTGTCTTGAACATG-3'
mdm2	5'-TTTTTTTTTTTTTTT	CCCTTATTACACACAGAGCC-3'
GAPDH	5'-TTTTTTTTTTTTTTT	GTAGAGGCAGGGATGATGTTC-3'

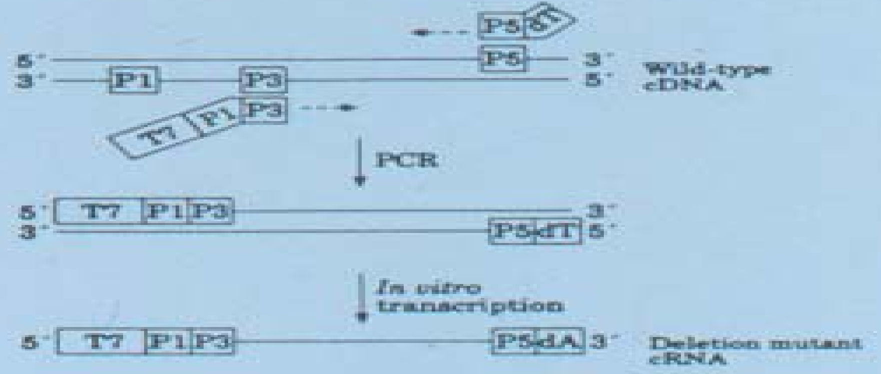
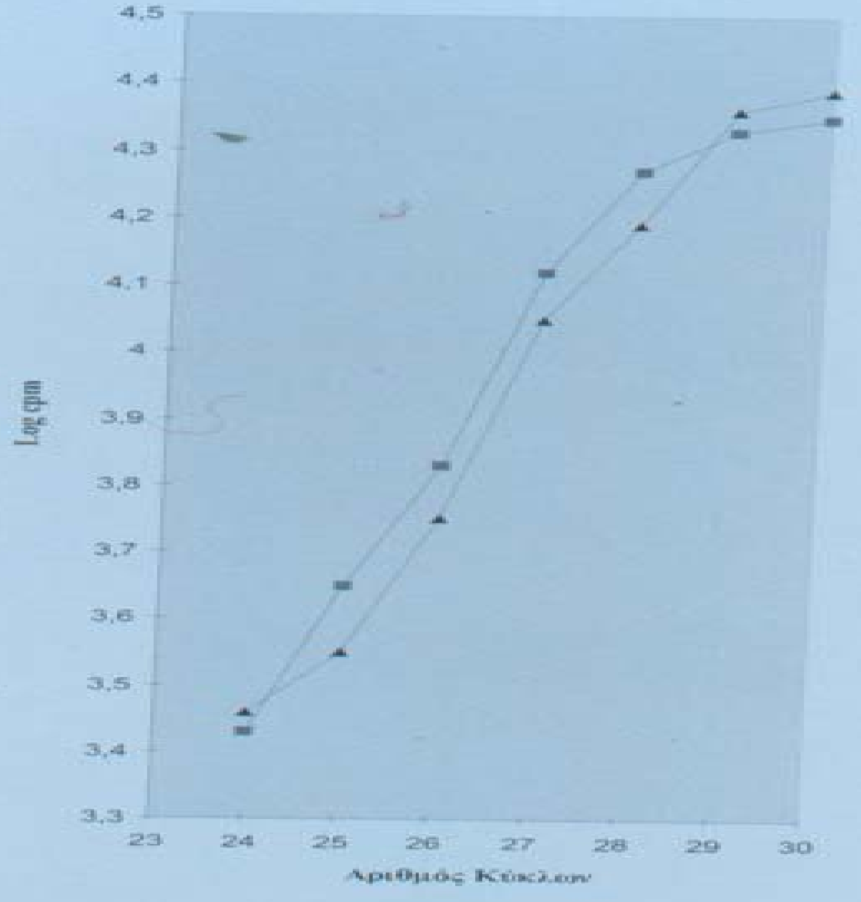


Fig. 1. Synthesis of the internal standards. Wild-type cDNA is amplified with the 5'-primer T7-P1-P3 and the 3'-primer oligo(dT)-P5 (see Table 1). By this procedure the nucleotides between P1 and P3 are deleted. T7 indicates a modified T7 promoter sequence that allows *in vitro* transcription with T7 RNA polymerase. Oligo(dT) in front of the 3'-primer generates a cRNA with a poly(dA) tail at the 3'-end.

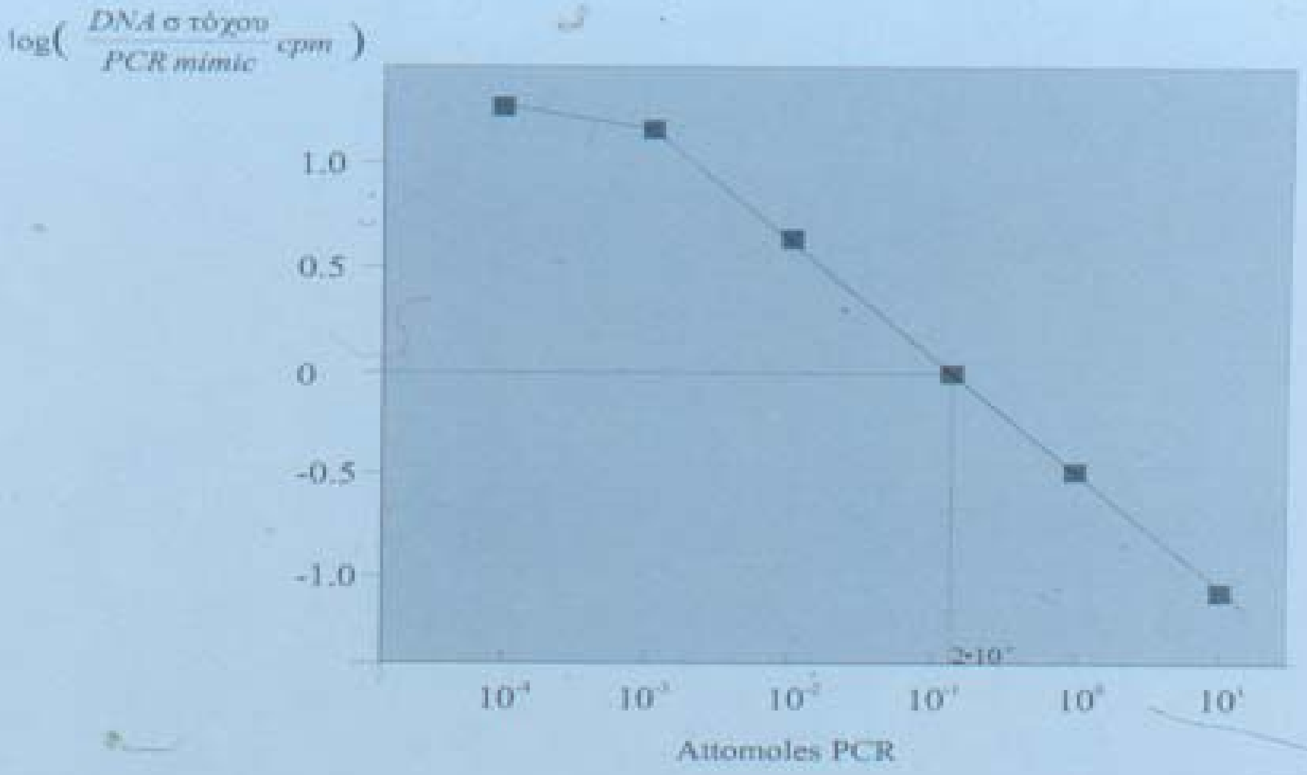
Table 2. Size of target and competitor cDNA for p53, mdm2 and GAPDH

Gene	Target	Competitor
p53	686 bp	503 bp
mdm2	586 bp	381 bp
GAPDH	480 bp	307 bp

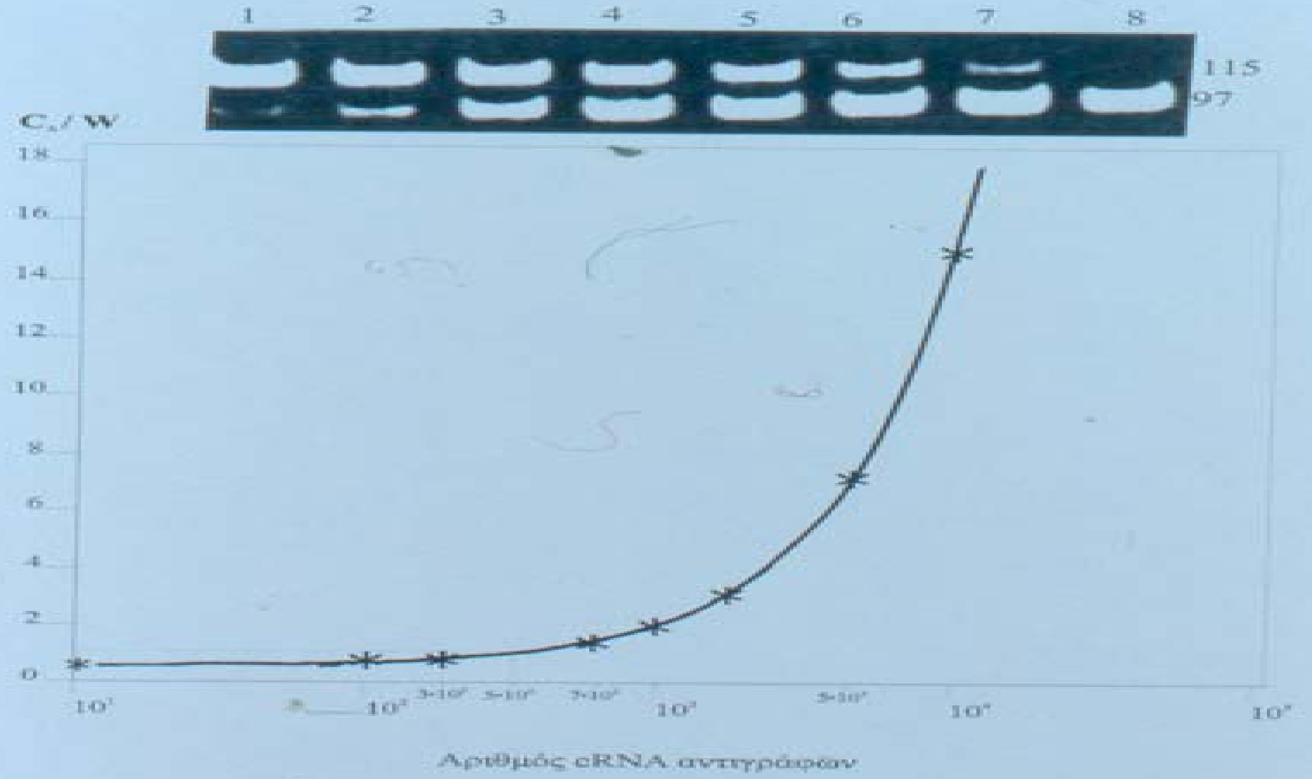
The use of T7-P1-P3 generated a cDNA differing from the wild-type cDNA by having a deletion.The T7 promoter sequence incorporated into the 5’-end of the 5’-primers was necessary for the in vitro transcription with T7 RNA polymerase.Oligo (dT)-P5 leads to a poly (dA) tail at the 3’-end.The mimic-PCR product will be purified from agarose gel and will be used for in vitro transcription , to produce the mimic RNA.



Εικόνα 3: Κινητική ανάλυση του DNA στόχου (■) και του "PCR mimic"(▲). Η γραφική παράσταση απεικονίζει τον log των cpm σε σχέση με τον αριθμό των κύκλων (από 24ο έως και τον 30ο). Τα ευθύγραμμα τμήματα και των δύο καμπυλών έχουν παρόμοια κλίση υποδηλώνοντας ότι το DNA στόχος και το "PCR mimic" έχουν όμοια απόδοση γονιδιακής επέκτασης.



Εικόνα 4. Διαδοχικές υποδεκαπλάσιες αραιώσεις του "PCR mimic" υποβάλλονται σε σύγχρονη γονιδιακή επέκταση σε μία σταθερη συγκέντρωση του DNA στόχου. Η γραφική παράσταση: $\log\left(\frac{\text{DNA στόχου}}{\text{PCR mimic}}\,cpm\right)$ έναντι γνωστών συγκεντρώσεων του PCR mimic επιτρέπει επακριβώς τον προσδιορισμό της συγκέντρωσης του DNA στόχου, διότι όταν ο λογαριθμικός λόγος είναι ίσος με μηδέν τότε η γνωστή συγκέντρωση του PCR mimic είναι ίση με την άγνωστη συγκέντρωση του DNA στόχου.



Εικόνα 7. Μια σταθερή συγκέντρωση γενωμικού HIV-1 RNA υποβάλλεται σε αντίστροφη μεταγραφή και εν συνεχεία σε σύγχρονη γονιδιακή επέκταση με έναν αυξανόμενο αριθμό cRNA αντιγράφων (*). Το πήκτωμα αγαρόζης μετά από χρώση με βρωμιούχο αιθίδιο υποβάλλεται σε σάρωση, C το εμβαδόν του cRNA και W το εμβαδόν του γενωμικού HIV-1 RNA. Η τιμή του C διορθώνεται λόγω του μικρότερου αριθμού bp του cRNA ως εξής: $C_c=C\ (115/97)=C\ 1,1855$. Η διόρθωση αυτή είναι απαραίτητη ώστε να αντισταθμιστεί η λιγότερη ενσωμάτωση του βρωμιούχου αιθιδίου στο cRNA. Ο λόγος C_c/W υπολογίζεται για τις γραμμές 1-8 του πηκτώματος, και για κάθε γραμμή αναφέρεται στον άξονα των Y σε σχέση με τον αντίστοιχο αριθμό των cRNA αντιγράφων στον άξονα των X. Όταν ο λόγος $C_c/W=1$, τότε το ποσό του RNA στόχου είναι ίσο με το ποσό του cRNA ανταγωνιστή. Από την καμπύλη αυτή υπολογίζεται κατά συνέπεια και ο αριθμός αντιγράφων του αγνώστου δείγματος. Στην συγκεκριμένη περίπτωση ο αριθμός HIV-1 αντιγράφων είναι ίσος με 500.

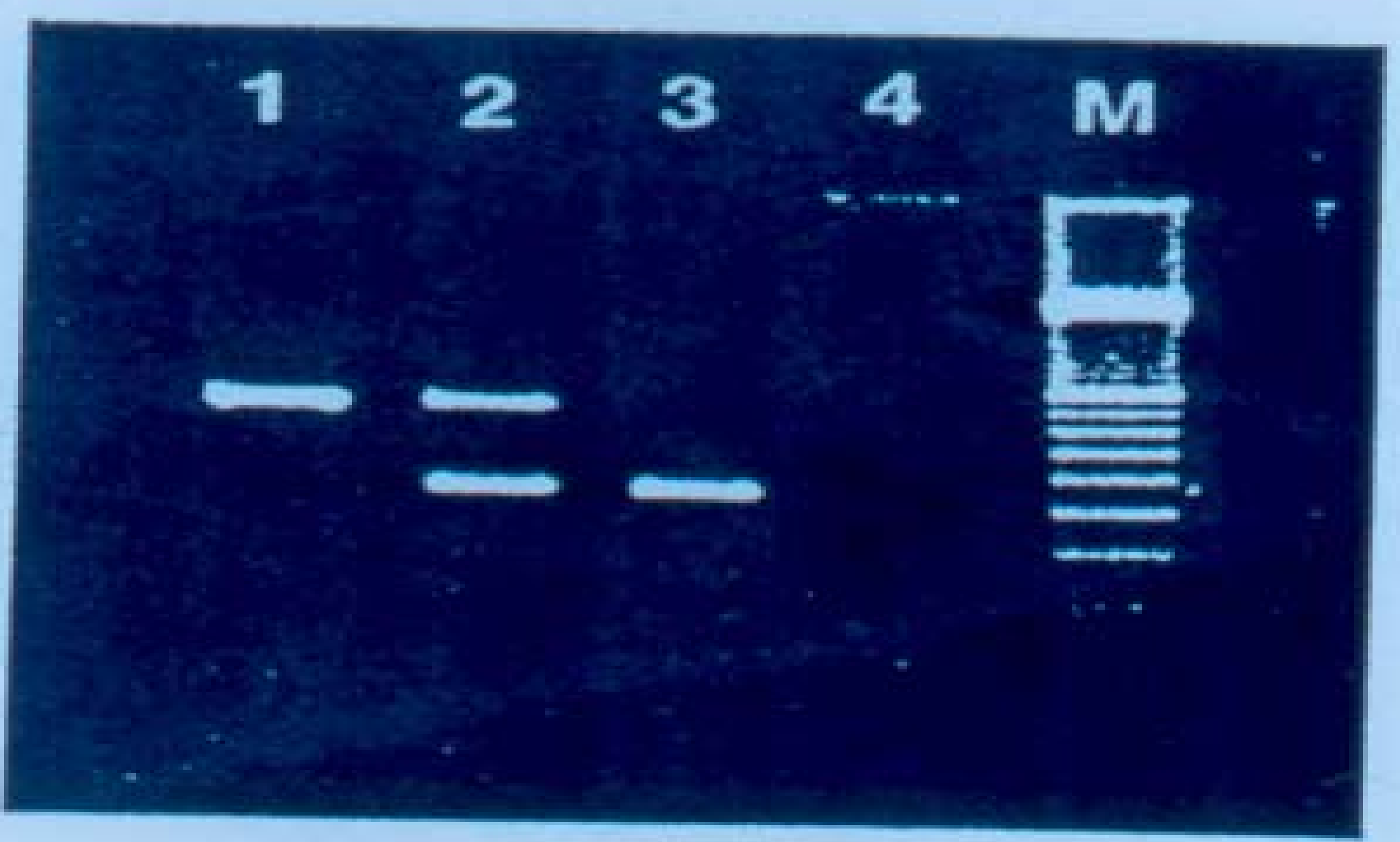
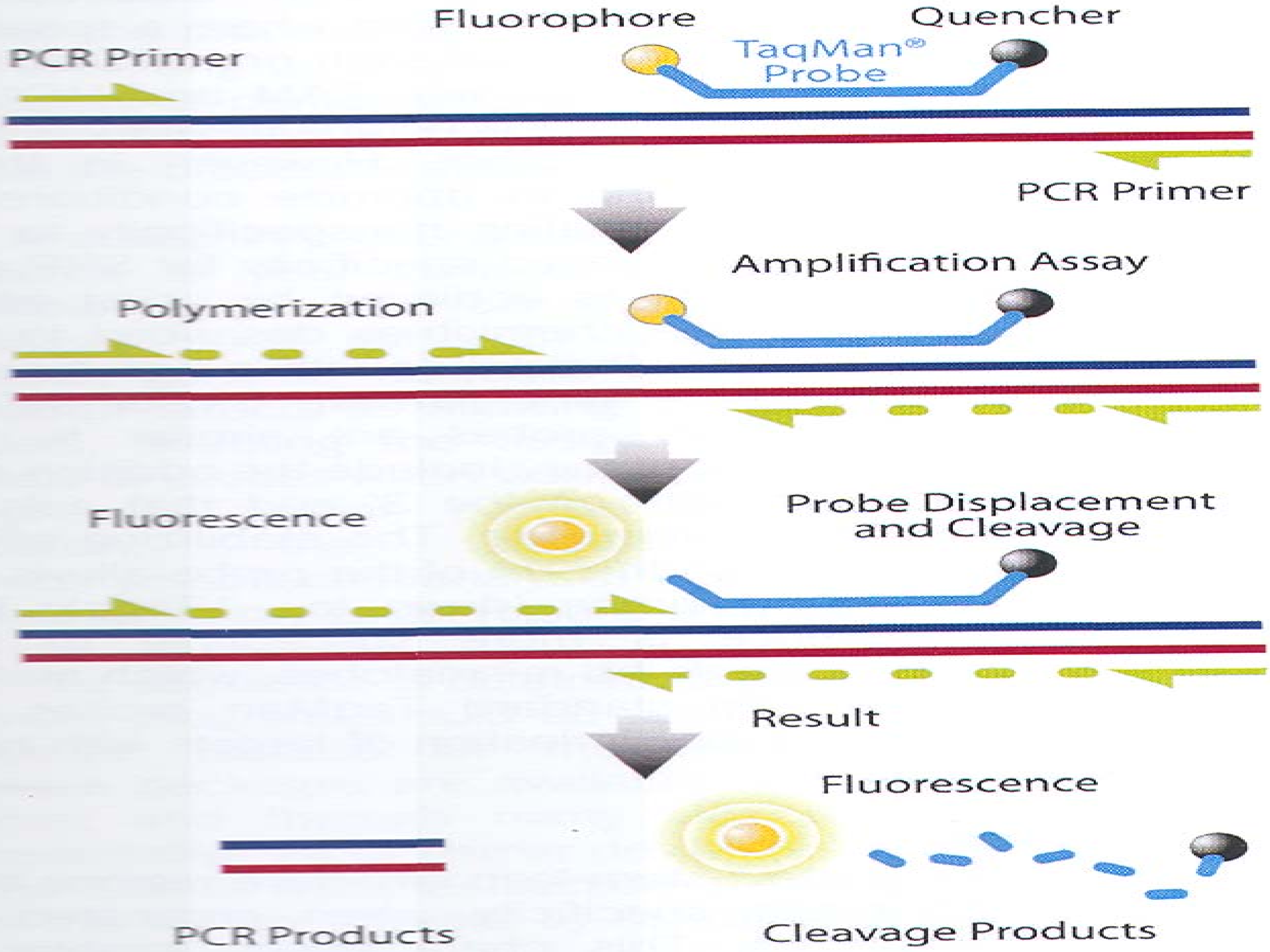
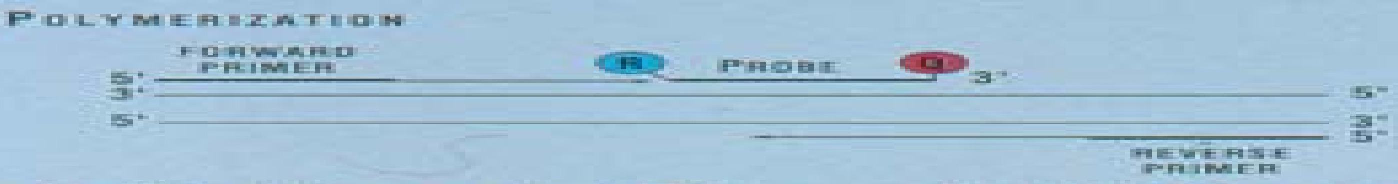


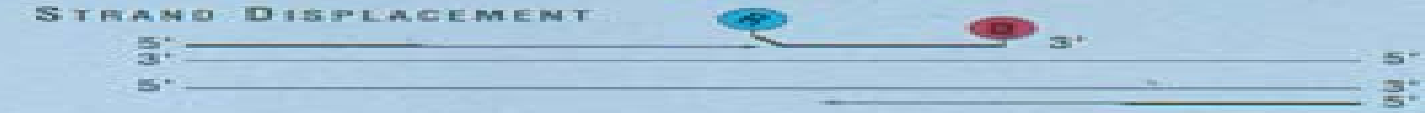
Fig. 5. Co-amplification of the mimic with BLV provirus in nested PCR. Lane 1: Approx. 10 mimic molecules without BLV infected lymphocytes; lane 2: Approx. 10 mimic molecules with approx. 10 BLV infected lymphocytes; lane 3: Approx. 10 mimic molecules with approx. 1000 BLV infected lymphocytes; lane 4: Approx. 10 mimic molecules with an extremely high amount of BLV infected lymphocytes (approx. 5×10^6 lymphocytes); lane M: 100 bp ladder as DNA size control (Pharmacia, Uppsala, Sweden).


Fluorophore
Quencher
PCR Primer
TaqMan®
Probe
PCR Primer
Amplification Assay
Polymerization
Fluorescence
Probe Displacement
and Cleavage
Result
Fluorescence
PCR Products
Cleavage Products

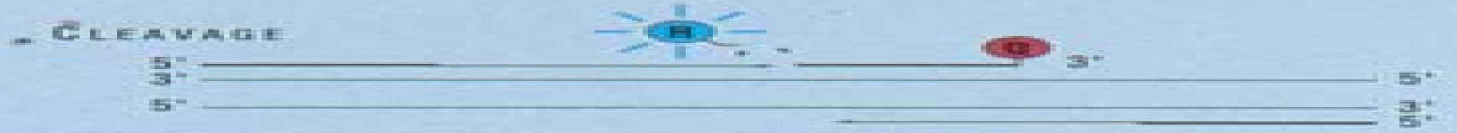
The fluorogenic 5' nuclease assay is a convenient, self-contained process. The assay uses a fluorogenic probe consisting of an oligonucleotide to which a reporter dye and a quencher dye are attached. During PCR, the probe anneals to the target of interest between the forward and reverse primer sites. During extension, the probe is cleaved by the 5' nuclease activity of the DNA polymerase. This separates the reporter dye from the quencher dye, generating an increase in the reporter dye's fluorescence intensity. (See diagram.)


POLYMERIZATION
FORWARD PRIMER
R
PROBE
Q
3'
5'
3'
5'
5'
3'
5'
REVERSE PRIMER

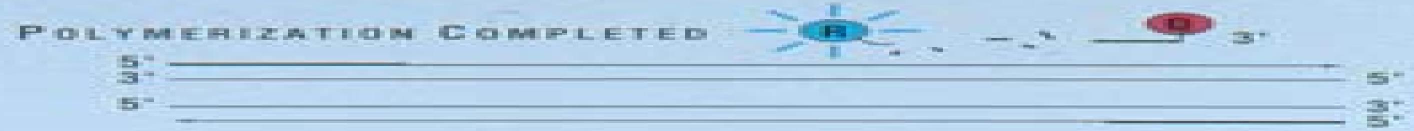
The reporter (R) and the quencher (Q) dyes are attached to the probe.


STRAND DISPLACEMENT
R
Q
3'
5'
3'
5'
5'
3'
5'

When both dyes are attached to the probe, reporter dye emission is quenched.

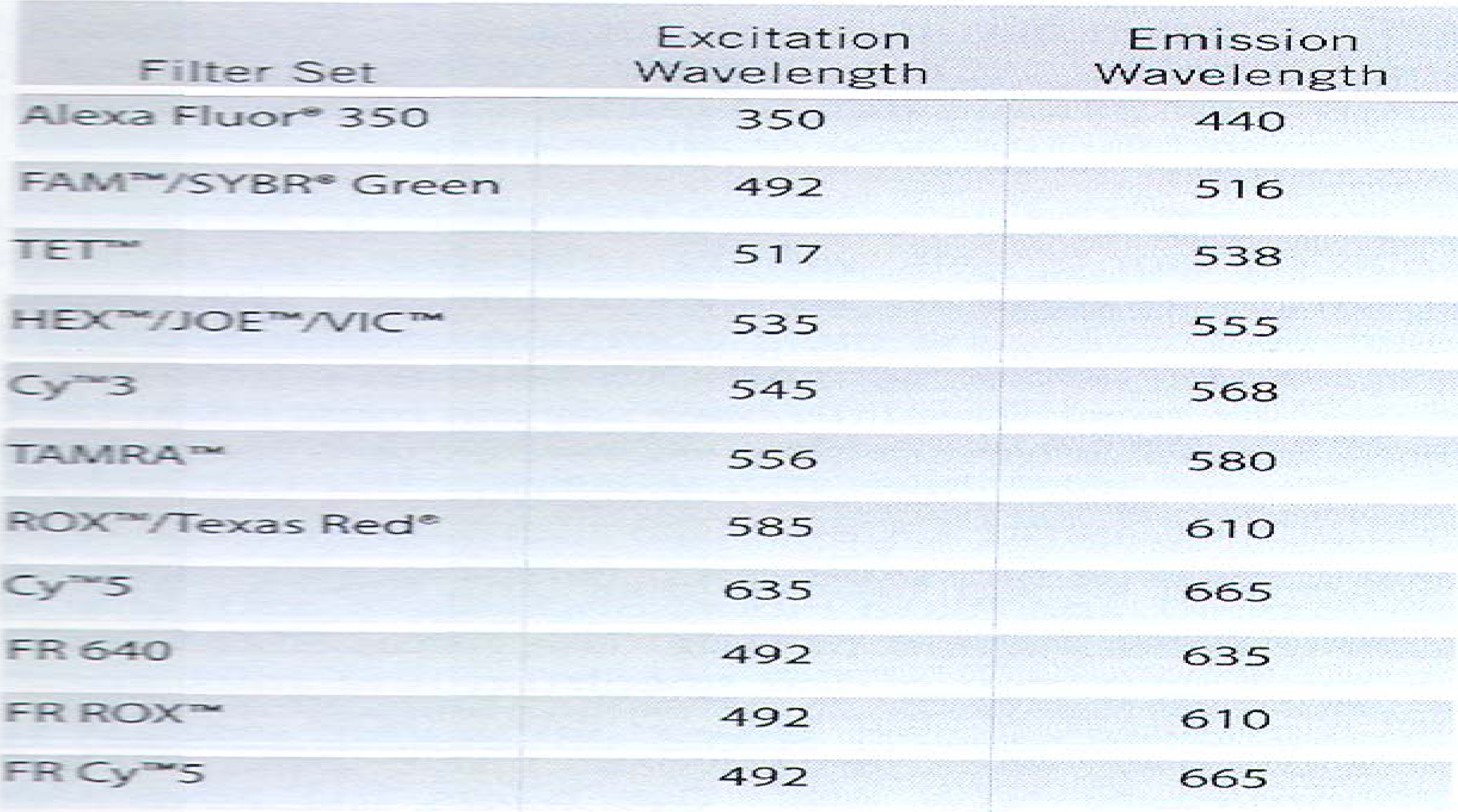

CLEAVAGE
R
Q
3'
5'
3'
5'
5'
3'
5'

During extension, DNA polymerase cleaves the reporter dye from the probe.


POLYMERIZATION COMPLETED
R
Q
3'
5'
3'
5'
5'
3'
5'

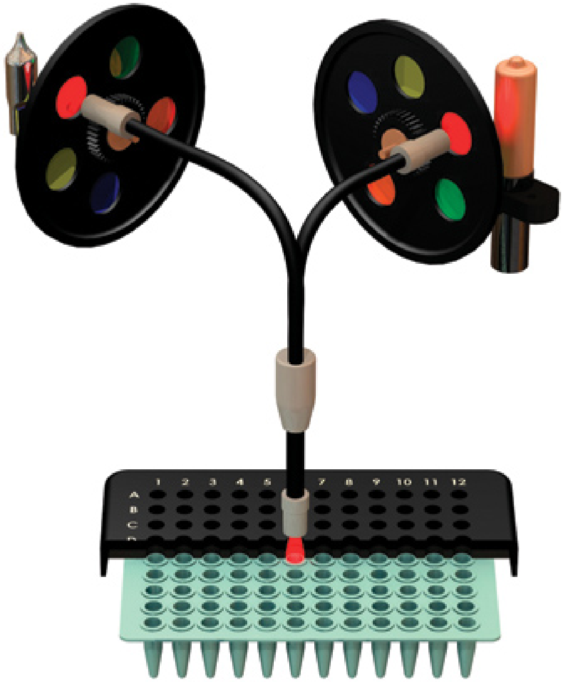
Once separated from the quencher, the reporter dye emits its characteristic fluorescence. The ABI Prism™ 7700 and GeneAmp™ 5700 Sequence Detection Systems measure the increase in the reporter dye's fluorescence during the thermal cycling of the PCR. This data is then used by the sequence detection software to generate quantitative results.

Ως μόρια ανταποκριτές μπορούν να χρησιμοποιηθούν οι 4,7,2΄,4΄,5΄,7΄-εξαχλωρο-6-καρβοξυφλουορεσκεΐνη (HEX), οι 4,7,2’,7΄-τετραχλωρο-6-καρβοξυφλουορεσκεΐνη (TET) ή 6-καρβοξυφλουορεσκεΐνη (FAM), ενώ ως μόριο αποσβέστης μπορεί να χρησιμοποιηθεί η ροδαμίνη ανάλογα με τα φίλτρα διέγερσης και εκπομπής που διαθέτει η συσκευή ποσοτικής PCR.



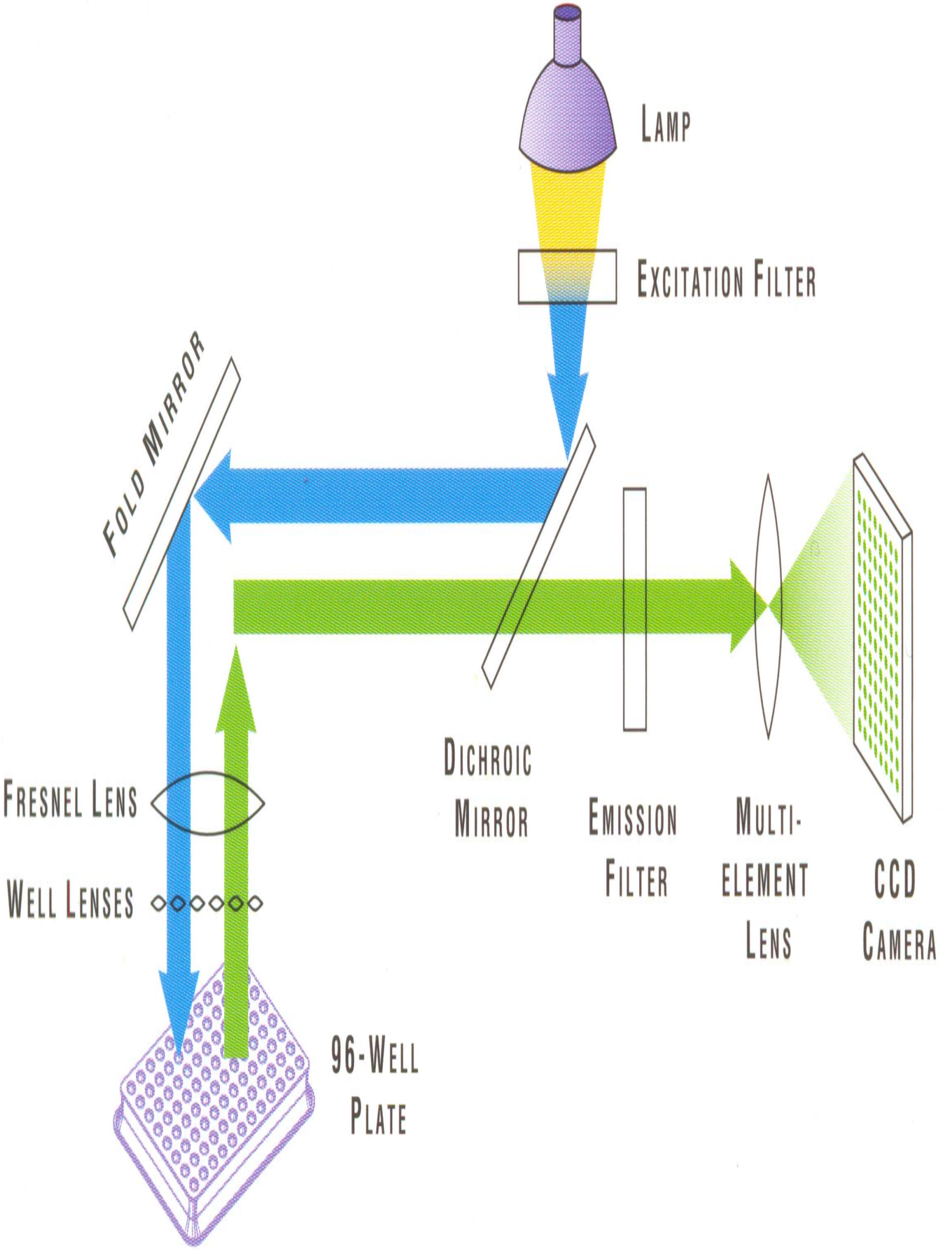
Filter Set	Excitation Wavelength	Emission Wavelength
Alexa Fluor® 350	350	440
FAM™/SYBR® Green	492	516
TET™	517	538
HEX™/JOE™/VIC™	535	555
Cy™3	545	568
TAMRA™	556	580
ROX™/Texas Red®	585	610
Cy™5	635	665
FR 640	492	635
FR ROX™	492	610
FR Cy™5	492	665

STRATAGENE
Mx3005P


1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D

ΣΥΣΚΕΥΗ ΠΟΣΟΤΙΚΗΣ PCR 96 ΘΕΣΕΩΝ Η ΟΠΟΙΑ ΔΙΑΘΕΤΕΙ ΦΩΤΟΠΟΛΛΑΠΛΑΣΙΑΣΤΗ ΚΑΙ ΚΑΤΑΛΛΗΛΟ ΛΟΓΙΣΜΙΚΟ ΕΧΟΝΤΑΣ ΤΗΝ ΔΥΝΑΤΟΤΗΤΑ ΝΑ ΠΑΡΑΚΟΛΟΥΘΗΣΕΙ ΤΗΝ ΑΝΤΙΔΡΑΣΗ ΣΕ ΚΑΘΕ ΦΡΕΑΤΙΟ/ΑΝΑ ΚΥΚΛΟ , ΝΑ ΤΗΝ ΚΑΤΑΓΡΑΨΕΙ ΚΑΙ ΜΑΛΙΣΤΑ ΔΥΝΑΤΑΙ ΝΑ ΧΡΗΣΙΜΟΠΟΙΗΣΕΙ ΜΕΧΡΙ ΚΑΙ 5 ΔΙΑΦΟΡΕΤΙΚΕΣ ΧΡΩΣΤΙΚΕΣ ΔΗΛ. ΝΑ ΠΡΑΓΜΑΤΟΠΟΙΕΙ ΑΝΑ ΦΡΕΑΤΙΟ ΜΕΧΡΙ ΚΑΙ 5 ΔΙΑΦΟΡΕΤΙΚΕΣ ΑΝΤΙΔΡΑΣΕΙΣ PCR

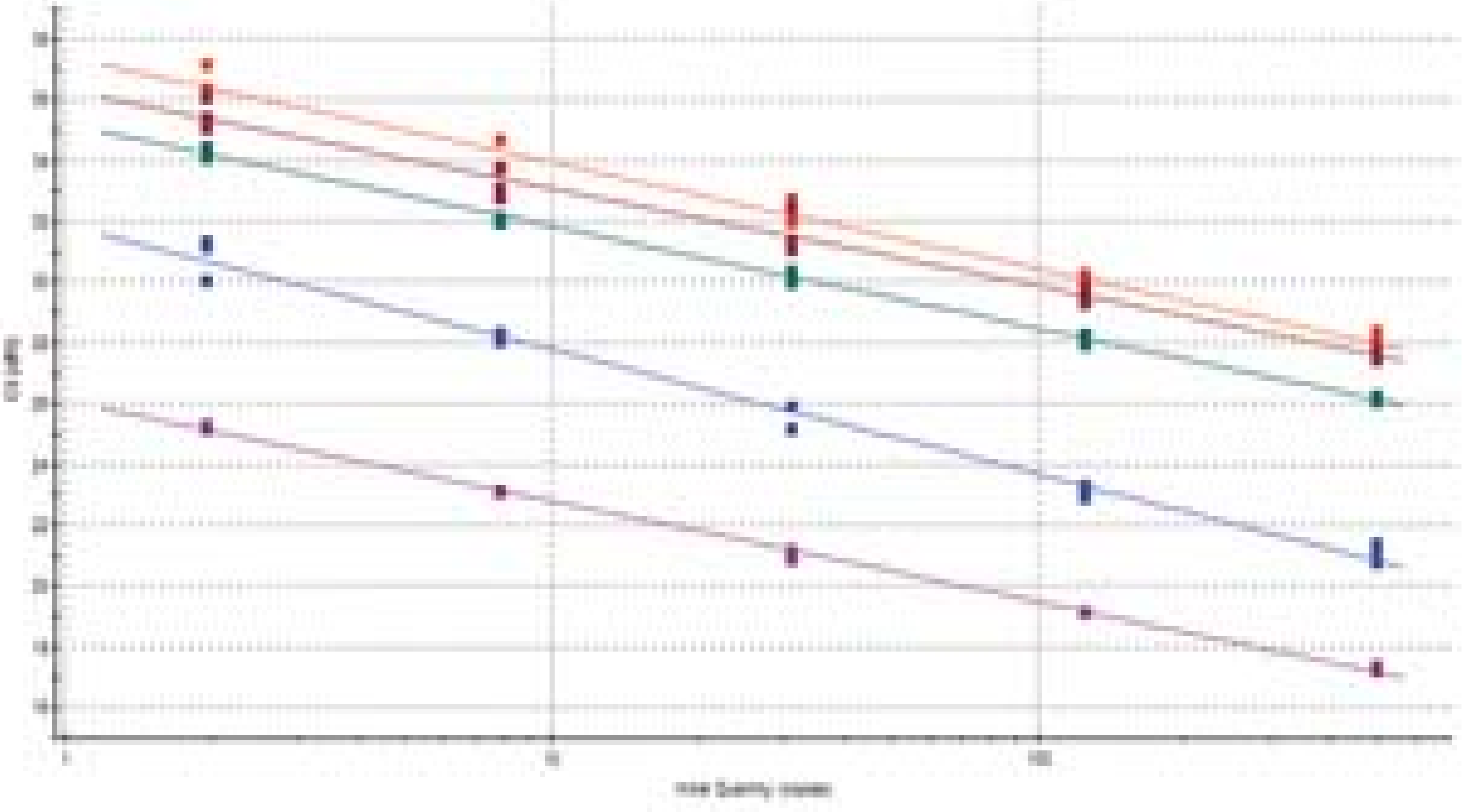
(MULTIPLEX QUANTITATIVE PCR)

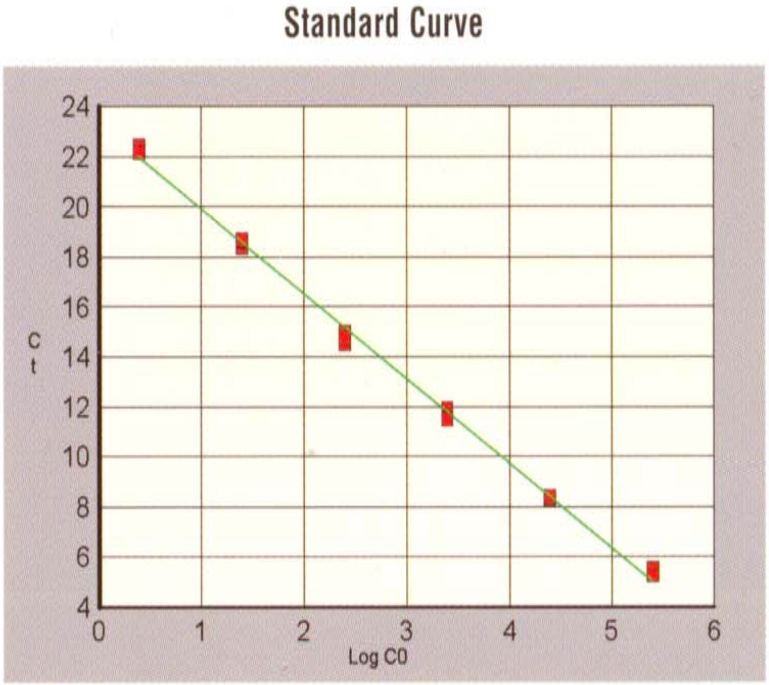

Lamp
Excitation Filter
Fold Mirror
Dichroic Mirror
Emission Filter
Multi-Element Lens
CCD Camera
Fresnel Lens
Well Lenses
96-Well Plate

The GeneAmp® 5700 system accurately monitors the fluorescence emission (530-590 nm) from all 96 sample wells after each cycle using precision optics and a CCD camera. The CCD camera collects the fluorescence intensity from all 96 wells in parallel and transfers the information to a computer for analysis. The software monitors the data throughout the PCR at every cycle and generates an amplification plot for each reaction.

ΧΡΗΣΙΜΟΠΟΙΩΝΤΑΣ STANDARDS ΓΝΩΣΤΩΝ ΑΝΤΙΓΡΑΦΩΝ ΤΟΥ DNA ΣΤΟΧΟΥ ΚΑΤΑΣΚΕΥΑΖΕΤΑΙ Η ΠΡΟΤΥΠΗ ΚΑΜΠΥΛΗ ΣΤΗΝ ΟΠΟΙΑ ΑΝΑΦΕΡΟΜΑΣΤΕ ΓΙΑ ΤΟΝ ΠΡΟΣΔΙΟΡΙΣΜΟ ΤΩΝ ΑΝΤΙΓΡΑΦΩΝ ΤΟΥ ΠΡΟΣ ΕΞΕΤΑΣΗ ΔΕΙΓΜΑΤΟΣ

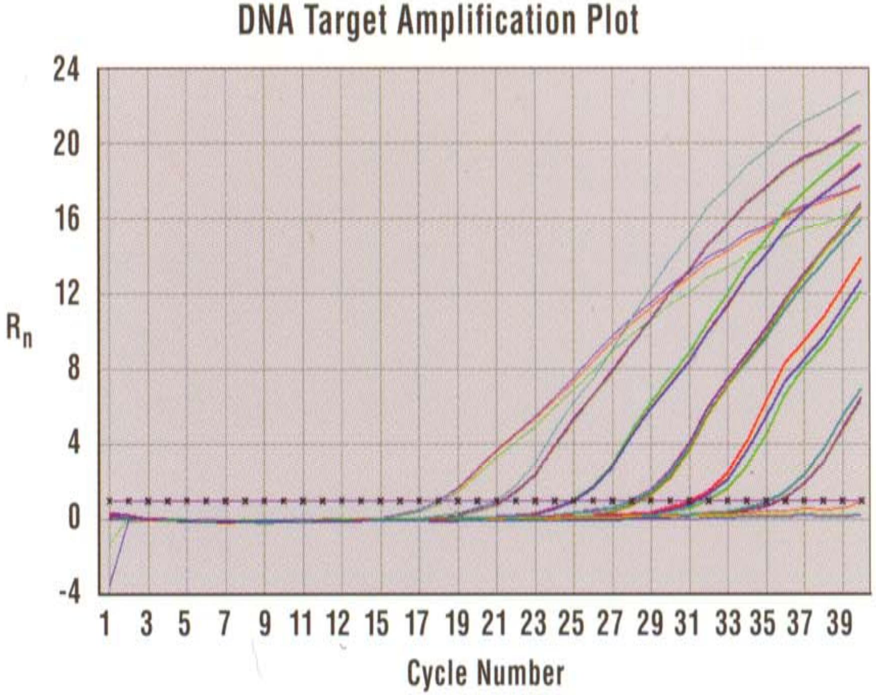
ΠΡΟΤΥΠΕΣ ΚΑΜΠΥΛΕΣ ΧΡΗΣΙΜΟΠΟΙΩΝΤΑΣ 5 ΔΙΑΦΟΡΕΤΙΚΕΣ ΧΡΩΣΤΙΚΕΣ ΣΕ ΚΑΘΕ ΦΡΕΑΤΙΟ (MULTIPLEX QUANTITATIVE PCR)





Room to grow: Amplification plots (right) from PCRs of several serial dilutions of samples with known starting copy number are used to generate a standard curve (above). This provides at least five orders of linear dynamic range for both DNA and RNA samples.

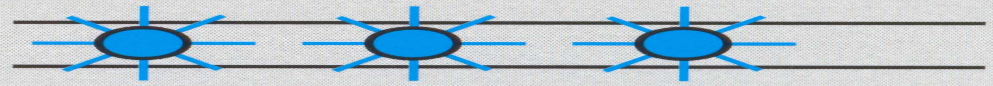
Multiple chemistries offer versatile assay development



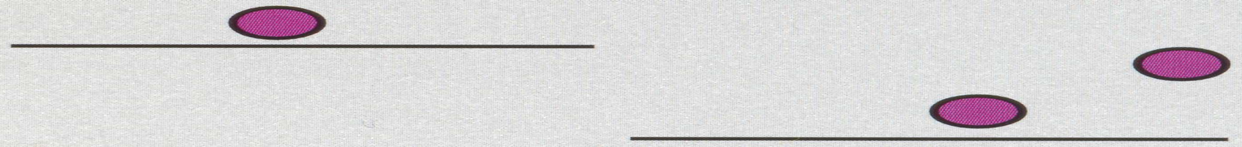
These amplification plots show real-time, cycle-to-cycle increases in signal from three replicates of ß-actin at different starting copy numbers: 1,000; 2,000; 5,000; 10,000; and 20,000. The software calculates the threshold cycle (C_t) where the amplification plot crosses a defined fluorescence threshold. The overlapping plots at the threshold demonstrate the precision of the 5700 system.

The incorporation of SYBR Green 1 dye into a real-time PCR reaction allows the detection of any double-stranded DNA generated during PCR. This provides great flexibility because no target specific probes are required, however both specific and non-specific products will generate signal. The use of the hot-start enzyme AmpliTaq Gold DNA Polymerase in all SYBR Green 1 reagent kits allows the highest performance available by minimizing non-specific product formation. SYBR Green 1 assays are ideal for use in target identification (screening assays), or when only a small number of reactions are required for a given assay.

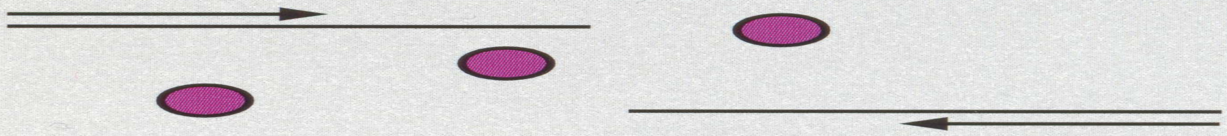
SYBR Green 1 assay



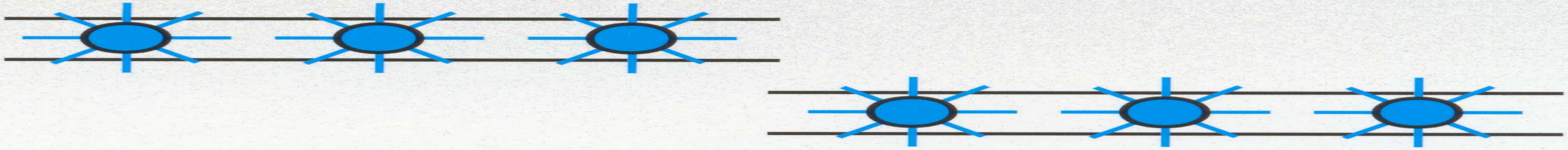
SYBR Green 1 dye fluoresces when bound to double-stranded DNA.



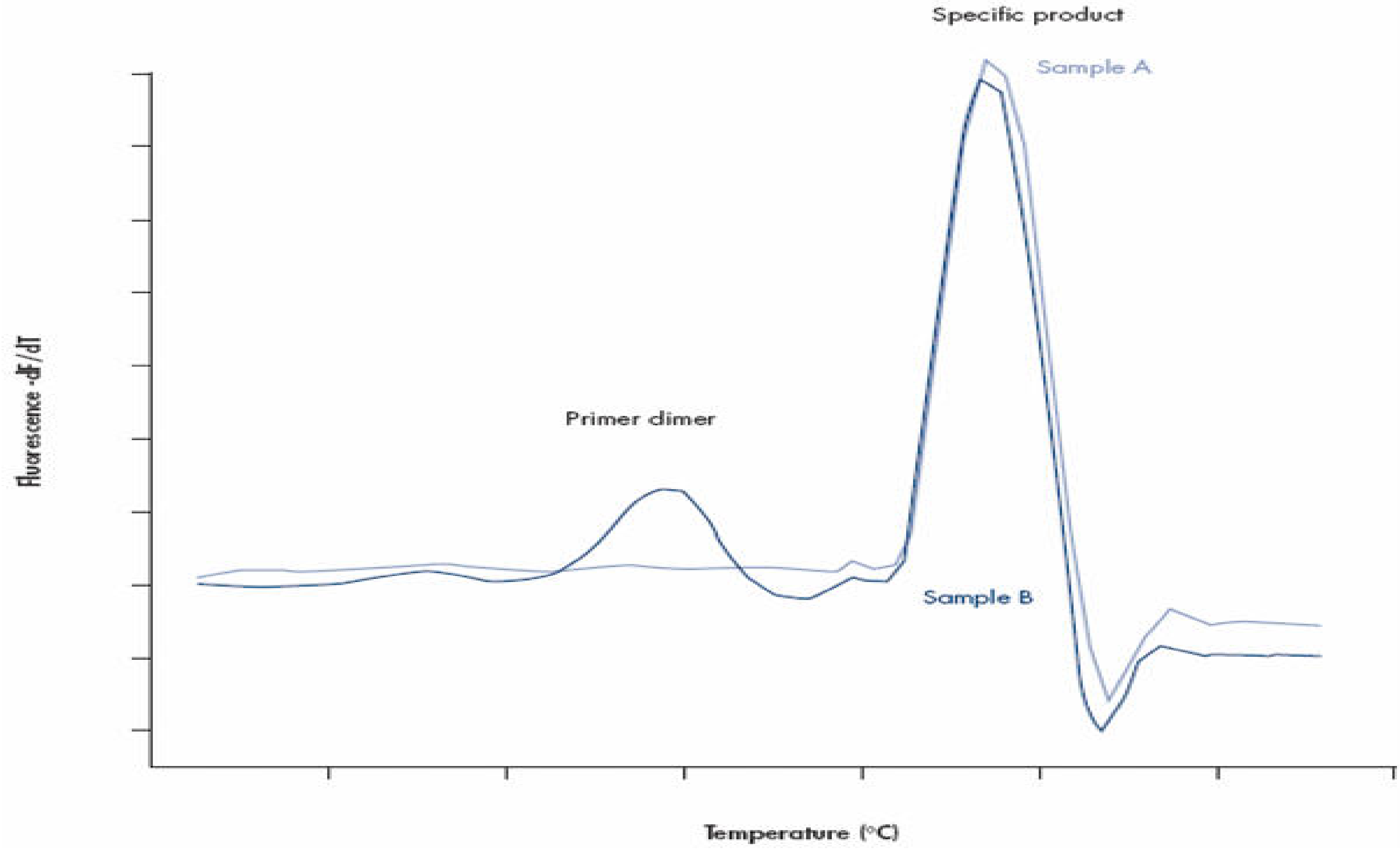
When DNA is denatured, the SYBR Green 1 dye is released and fluorescence is drastically reduced.



During extension phase, primers anneal and PCR product is generated.



Polymerization is complete and SYBR Green 1 dye binds, resulting in a net increase in fluorescence.



Sample A yields only 1 peak resulting from the specific amplification product (primer-dimers not coamplified). Sample B shows a peak from the specific product and a peak at a lower temperature from amplification of primer-dimers.

Real-Time PCR Method

Real-time PCR is able to detect sequence-specific PCR products as they accumulate in "real-time" during the PCR amplification process. As the PCR product of interest is produced, real-time PCR can detect their accumulation and quantify the number of substrates present in the initial PCR mixture before amplification began.

There are a few different variations of the procedure, but the one illustrated here is called molecular beacon <www.molecular-beacons.org/>. Molecular beacons are short segments of single-stranded DNA (Figure 1).

The sequence of each molecular beacon must be customized to detect the PCR product of interest. In figure one, you can see there are nine bases on one end of the molecular beacon that can base pair with nine bases on the other end of the beacon. This complementation permits the molecular beacon to form a hairpin structure. The loop portion of the molecular beacon is composed of bases (shown as pink lines) that are complementary to one strand of the PCR product the investigator wants to detect and quantify.

Attached to opposite ends of the beacon are a fluorescent reporter dye and a quencher dye. When the molecular beacon is in the hairpin conformation, any fluorescence emitted by the reporter is absorbed by the quencher dye and no fluorescence is detected.

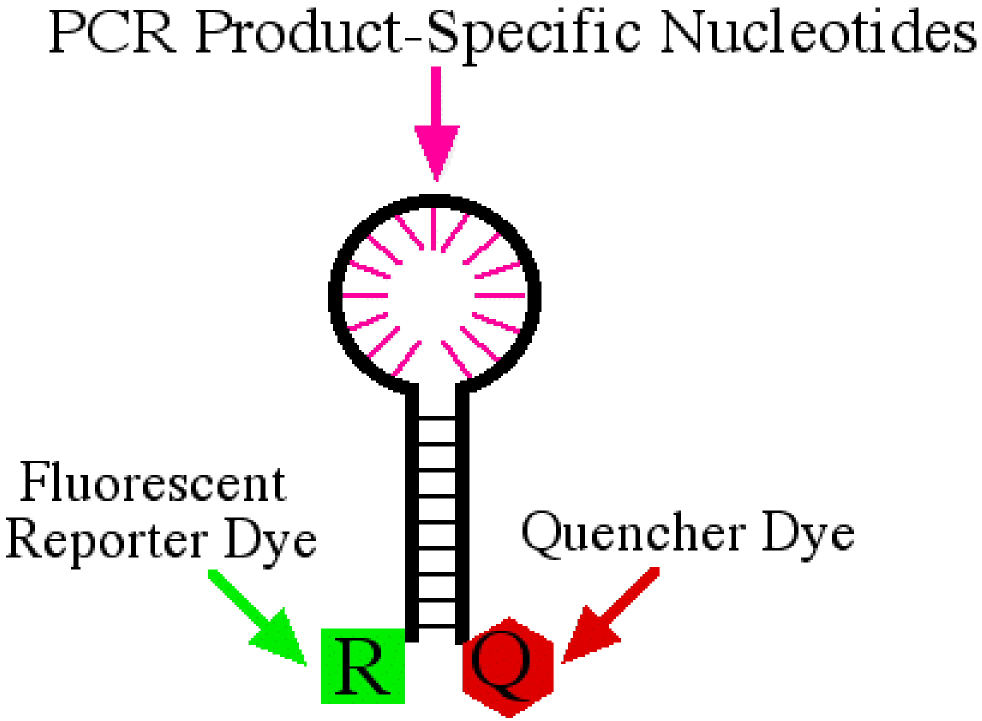


Figure 1. Diagram of molecular beacon. This beacon is 33 nucleotides long with a reporter dye attached to the 5' end and a quencher attached to the 3' end. The nine 5' bases are able to form base pairs with the nine 3' bases which brings the reporter and quencher in very close proximity. Therefore, when the reporter is excited by the appropriate light, its emission is absorbed by the quencher and no fluorescence is detected. The pink lines represent nucleotides that can form base pairs with the PCR product under investigation.

The PCR portion of real-time PCR is standard. Two PCR primers are used to amplify a segment of DNA (Figure 2).

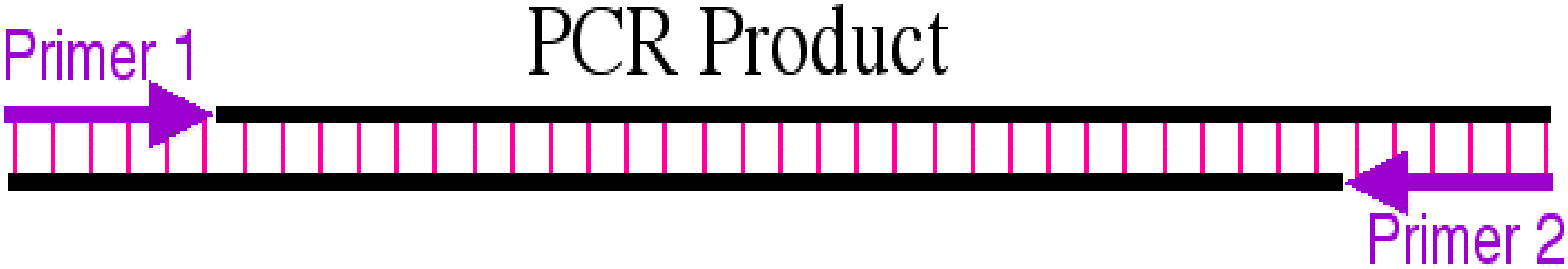


Figure 2. PCR product of interest. The two primers are show as purple arrows and the base pairing between the two strands are shown in pink.

As the PCR continues, the newly synthesized PCR products are denatured by high temperatures. As each strand of the product are separated, the molecular beacon also is denatured so the hairpin structure is disrupted. As the temperatures cool for the next round of primer annealing, the molecular beacon is capable of forming base pairs with the appropriate strand of the PCR product (Figure 3). Any molecular beacons that do not bind to PCR product reform the hairpin structures and thus are unable to fluoresce. However, molecular beacons that bind to PCR product remove the ability for the quencher to block fluorescence from the reporter dye. Therefore, as PCR product accumulates, there is a linear increase in fluorescence.

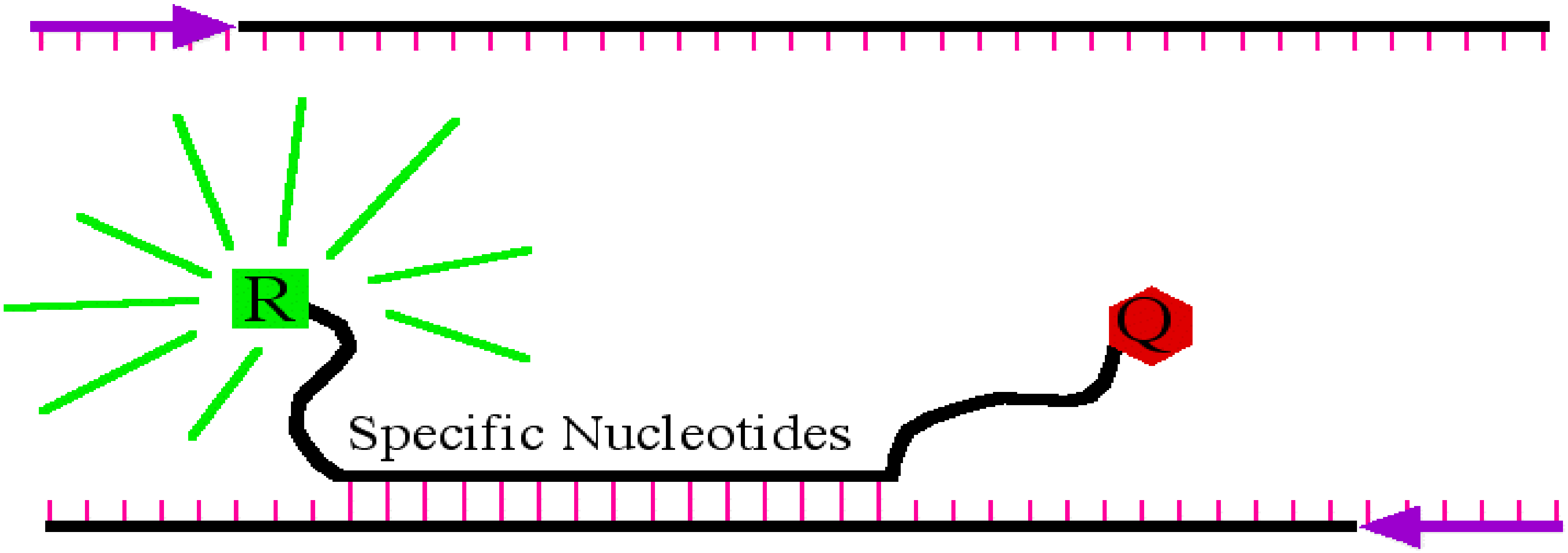


Figure 3. Detection of PCR product by molecular beacon. When the beacon binds to the PCR product, it is able to fluoresce when excited by the appropriate wavelength of light. The amount of fluorescence is directly proportional to the amount of PCR product amplified.

Real-time PCR can be performed in a "multiplex" format which means that more than one PCR product can be detected in a single reaction tube. For each sequence, there is a unique color of fluorescent dye and therefore, each PCR product is associated with its own color which is detected by the real-time PCR machine.