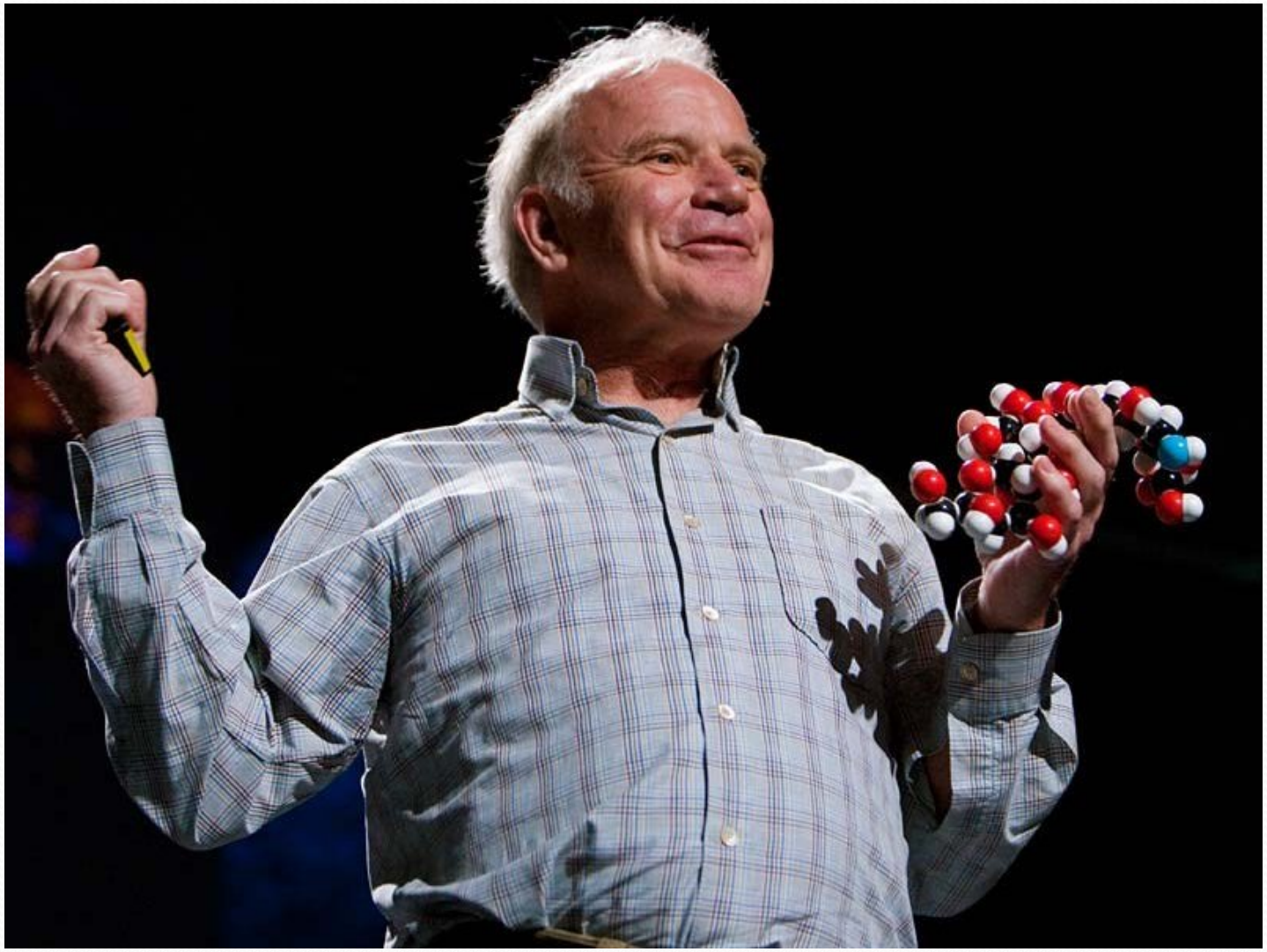
PCR (Theory and practice)

By

Mehdi Imani

بعد از اتمام این کارگاه شرکت کنندگان بایستی قادر به:

Kary Mullis



کاربرد و اهمیت

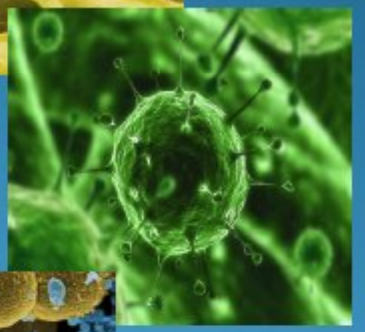
????

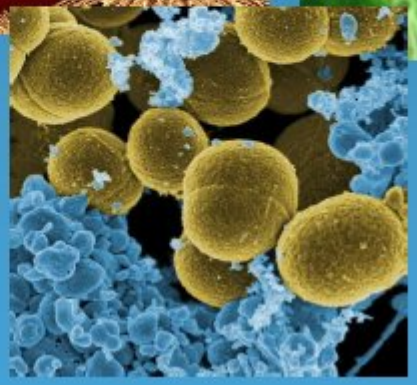
در سرم طیور برای تشخیص؟

Forensic

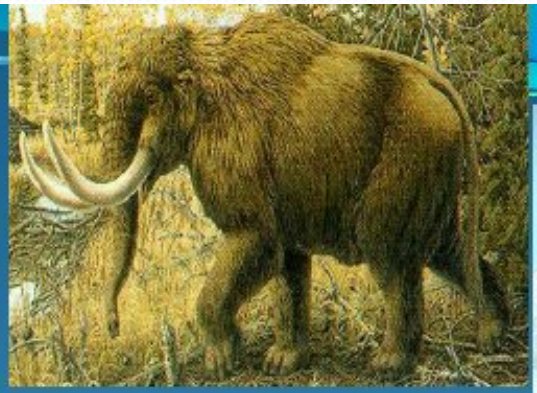
FORENSIC SCIENCE

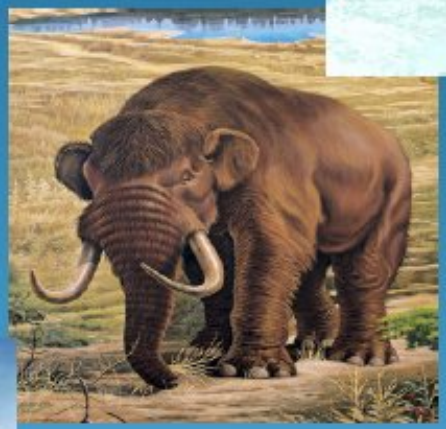
Diagnosis

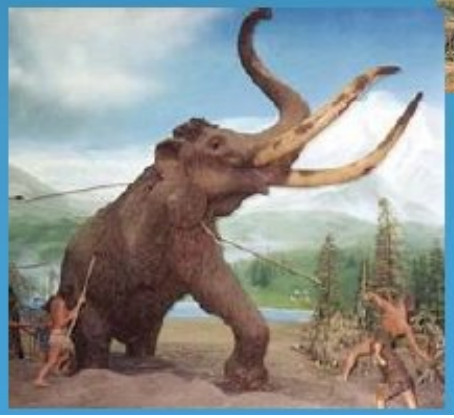




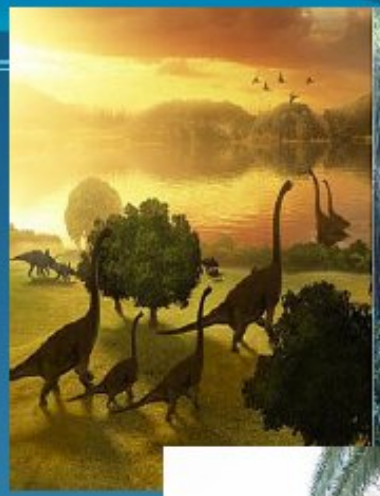
Studying evolution

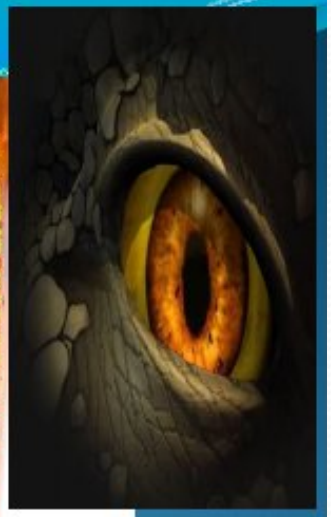


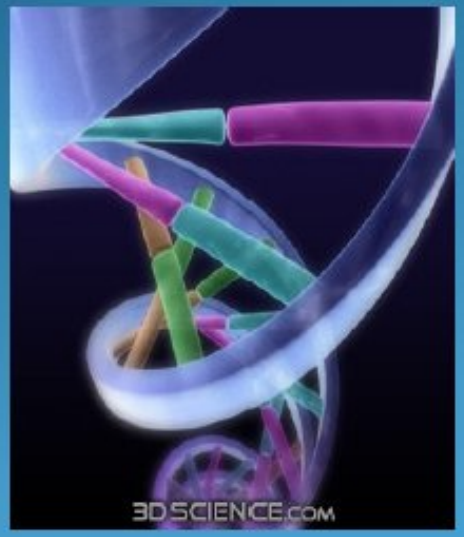




Fossils

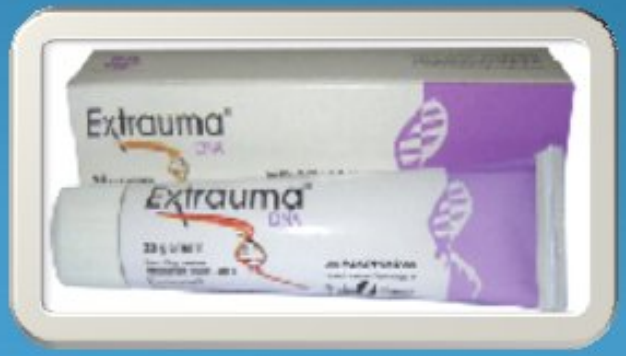




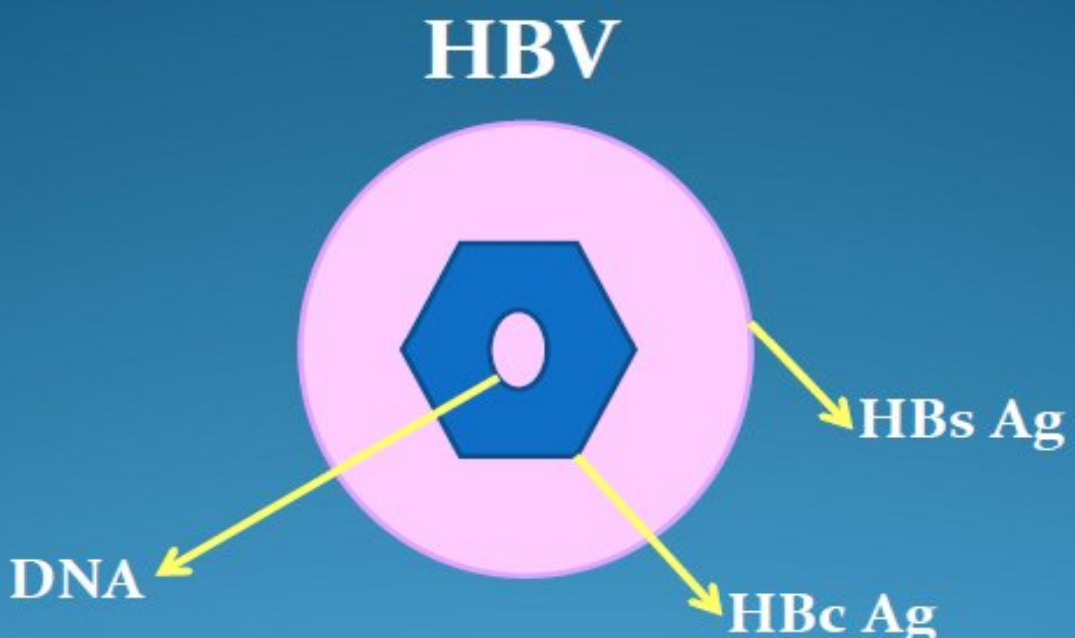


Drug

Thrombin inhibitor effectively treats hematoma and inflammation (oedema, redness, hotness).



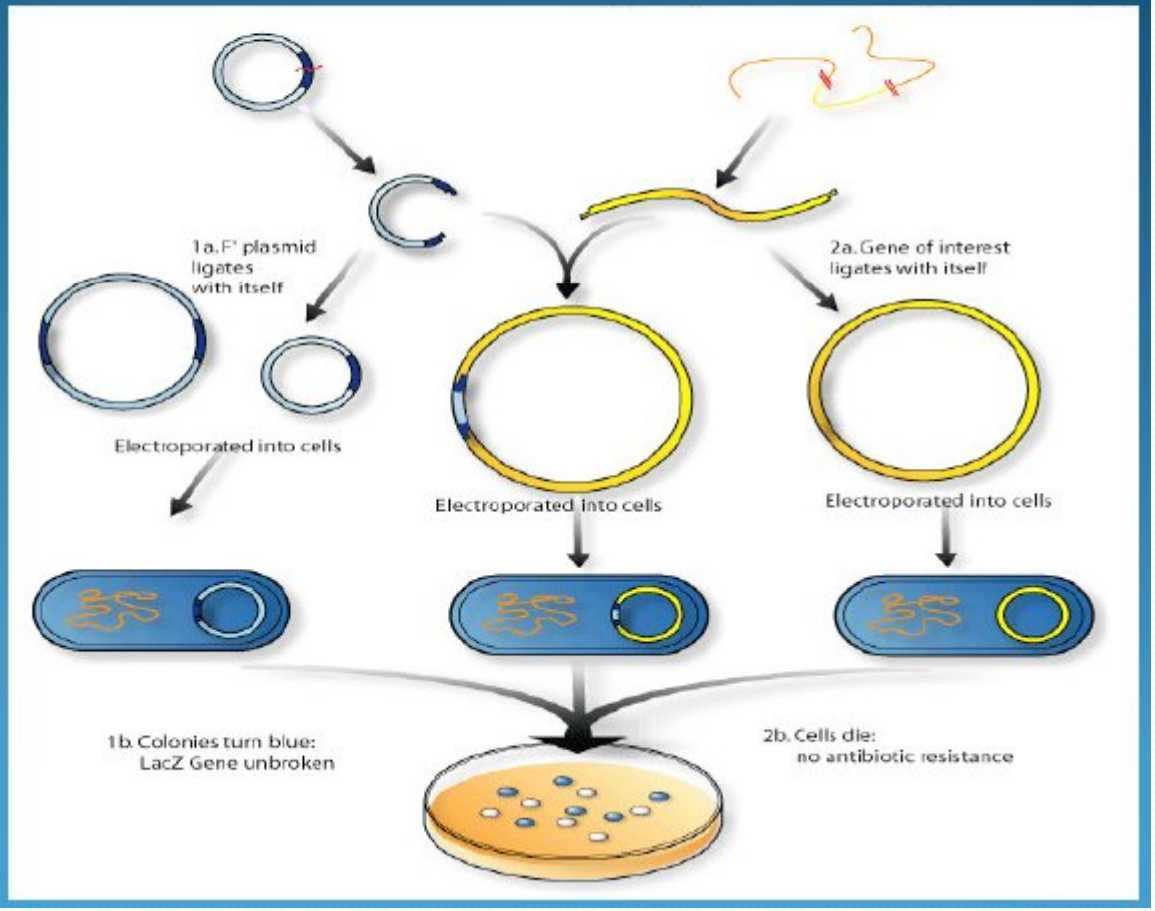
Vaccine



HBs Ag : surface antigen

HBc Ag : core antigen

Gene cloning & expression

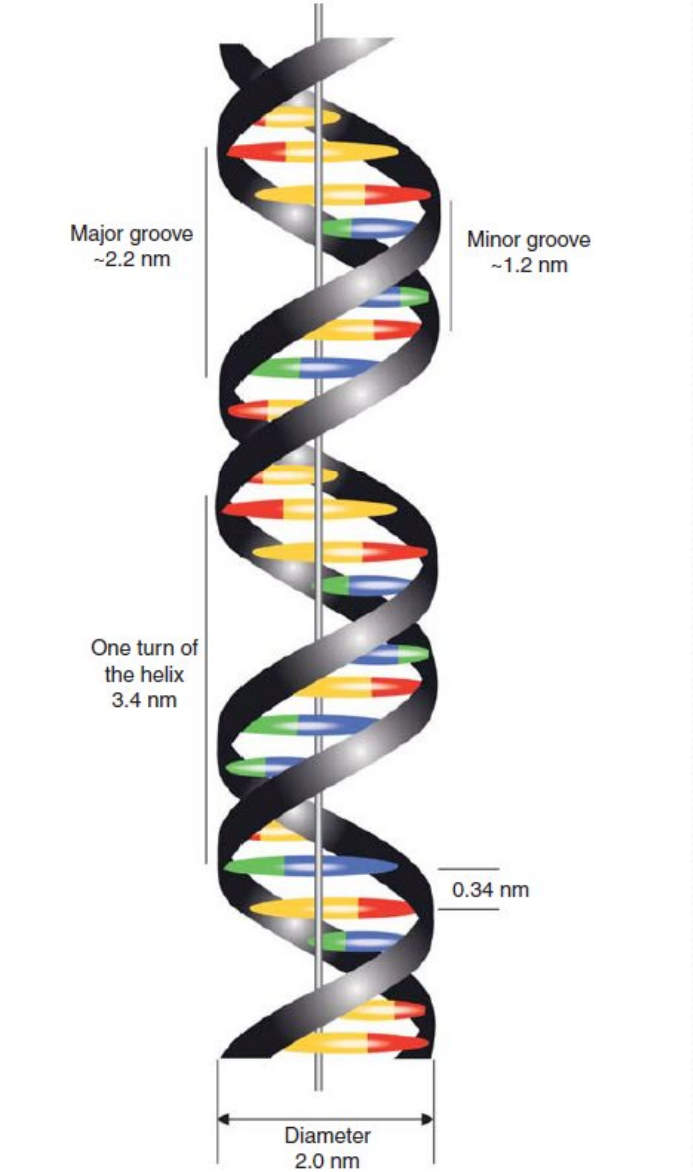


Creating mutation

Random or specific

ACCATCGGCCTGCATCA
TGGTAGCCTGACGTAGTCATGGTAGCCTGACT

DNA


Major groove
~2.2 nm
Minor groove
~1.2 nm
One turn of
the helix
3.4 nm
0.34 nm
Diameter
2.0 nm

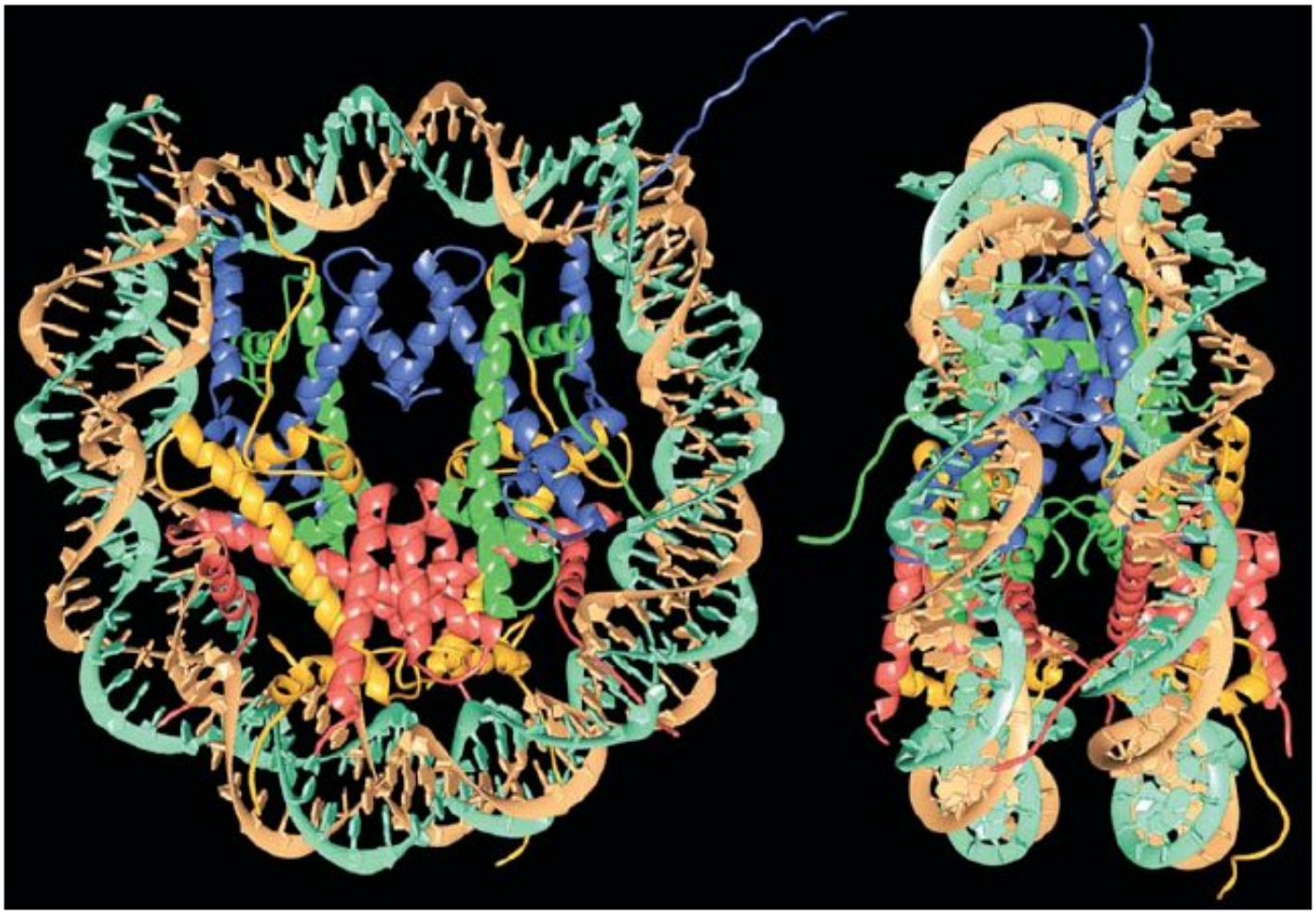
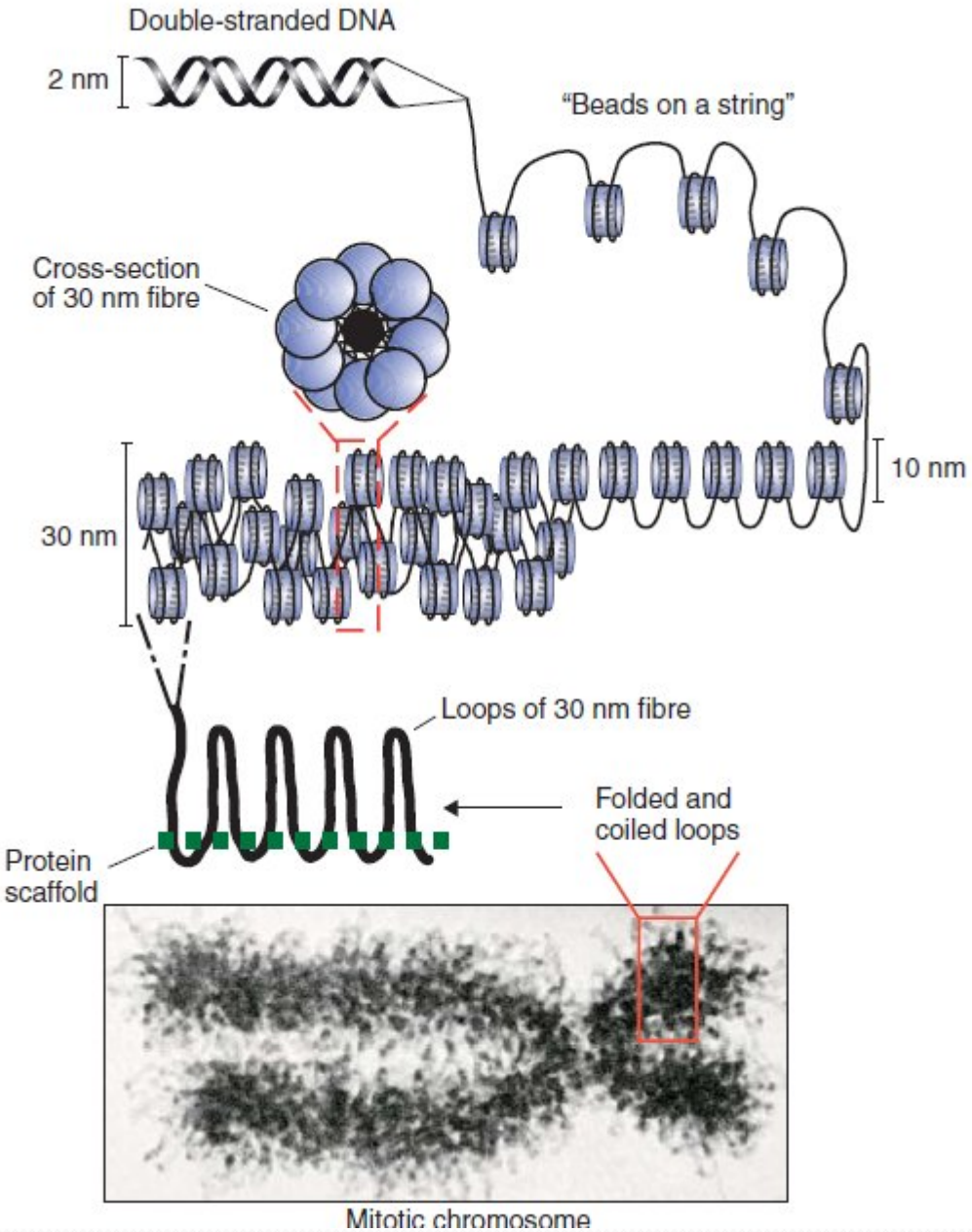


Figure 1.15. The structure of the nucleosome–DNA complex as solved by X-ray crystallography at high resolution. In both the top and side views of the complex, the strands of the DNA (shown in green and orange) wrap around the histone octamer. The protein core is almost exclusively internal with the exception of the amino-terminal histone tails that extend out from the complex. This figure was kindly provided by Tim Richmond (ETH Zurich) and is reprinted by permission from *Nature* (Luger *et al.*, 1997) copyright (1997) Macmillan Publishers Ltd


Double-stranded DNA
2 nm
"Beads on a string"
Cross-section
of 30 nm fibre
10 nm
30 nm
Loops of 30 nm fibre
Folded and
coiled loops
Protein
scaffold
Mitotic chromosome

همانندسازی DNA در باکتری

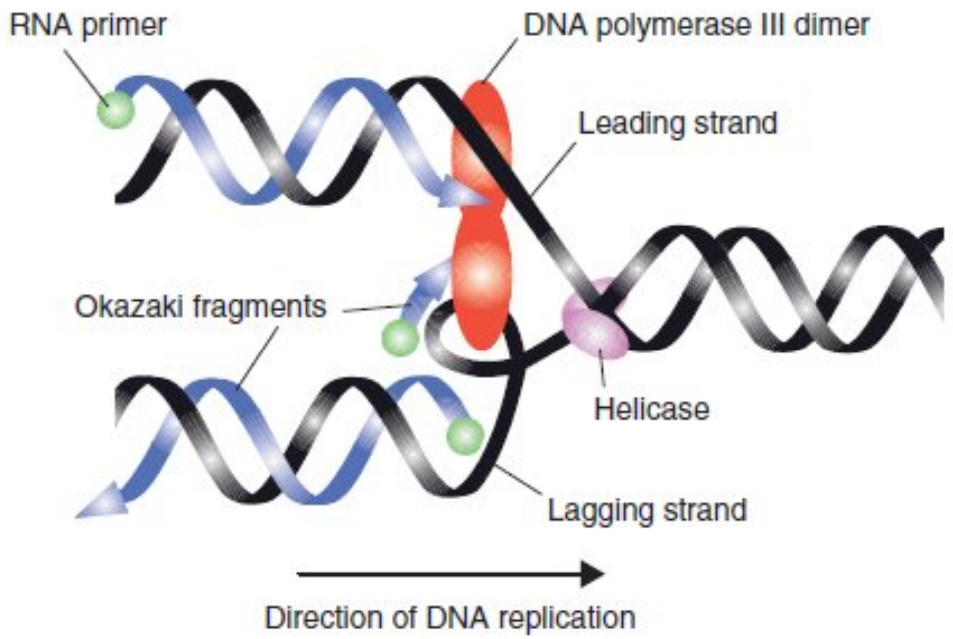


Figure 1.19. DNA replication by DNA polymerase III. The strands of the parent DNA molecule (black) are unwound by a helicase at the replication fork. The leading strand and the lagging strand are then fed into a dimer of DNA polymerase III. DNA replication on the leading strand occurs in a 5′ to 3′ direction by extending a single RNA primer. Many primers are required to produce the lagging strand in short sections (Okazaki fragments), which are later joined using DNA ligase

مواد مورد نیاز PCR

Component	25 µl reaction	50 µl reaction	Final Concentration
10X Standard *Taq* Reaction Buffer	2.5 µl	5 µl	1X
10 mM dNTPs	0.5 µl	1 µl	200 µM
10 µM Forward Primer	0.5 µl	1 µl	0.2 µM (0.05–1 µM)
10 µM Reverse Primer	0.5 µl	1 µl	0.2 µM (0.05–1 µM)
Template DNA	variable	variable	<1,000 ng
Taq DNA Polymerase	0.125 µl	0.25 µl	1.25 units/50 µl PCR
Nuclease-free water	to 25 µl	to 50 µl	

Master mix?

PCR requirements

- 10 fg–10 µg of chemically extracted DNA or reverse transcribed RNA in 1–10 µl of rehydration buffer or water
- 0.1–1.0 µM each of oligonucleotide primers, one complementary to the positive (sense) strand and the other complementary to the downstream (antisense) strand
- Approximately 200 µM of each of the four deoxyribonucleotide triphosphate DNA building blocks, i.e. adenosine-, cytosine-, guanosine- and thymidine triphosphate
- 0.2–2.0 units of Taq polymerase (other commercially available thermostable DNA polymerases may also be used but bear in mind differences in proof-reading ability and processivity)
- Tris-buffer (pH 8.3), 50 mM KCl (specific buffers are usually supplied free by the manufacturer of the thermostable enzyme used)
- 500–1,000 µg gelatin or Bovine Serum Albumin (BSA) per ml (optional).
- 0.5–5.0 mM of magnesium chloride salt solution ($MgCl_2$ is a DNA polymerase cofactor)

All prepared in a total typical PCR reaction volume of 50 µl (5–100 µl = the usual range).

Table 7.1 Fidelity and error rates for various thermostable DNA dependent DNA polymerases

Enzyme	Error rate ($\times 10^{6}$)	Accuracy ($\times 10^{-5}$)	3′-5′ Exonuclease activity
Klenow fragment	80	–	Yes
Pfu	1.3	7.7	Yes
Platinum *Pfx*	1.6	–	Yes
Pfu Turbo	2.4	–	–
DeepVent	2.7	3.6	Yes
Proofstart	3.6	–	Yes
Tli(Vent)	2.8	3.6	Yes
Tfu	90	–	Yes
Taq	180	1.2	No
UL*Tma*	55	0.2	Yes
Thermal Ace	60	–	Yes
FailSafe	8	–	Yes
MasterAmp	32	–	Yes

Some of the enzymes have been tested in the presence of stabilizing compounds. The error rate may deviate by approximately ±20%. The error rate is defined as the mutation frequency per basepair synthesized, whereas the accuracy describes the number of adequate incorporations before a misincorporation event occurs. Note that these rates are extremely sensitive to variation in the experimental conditions. (– = value not determined)

Different Hot-Start Methods

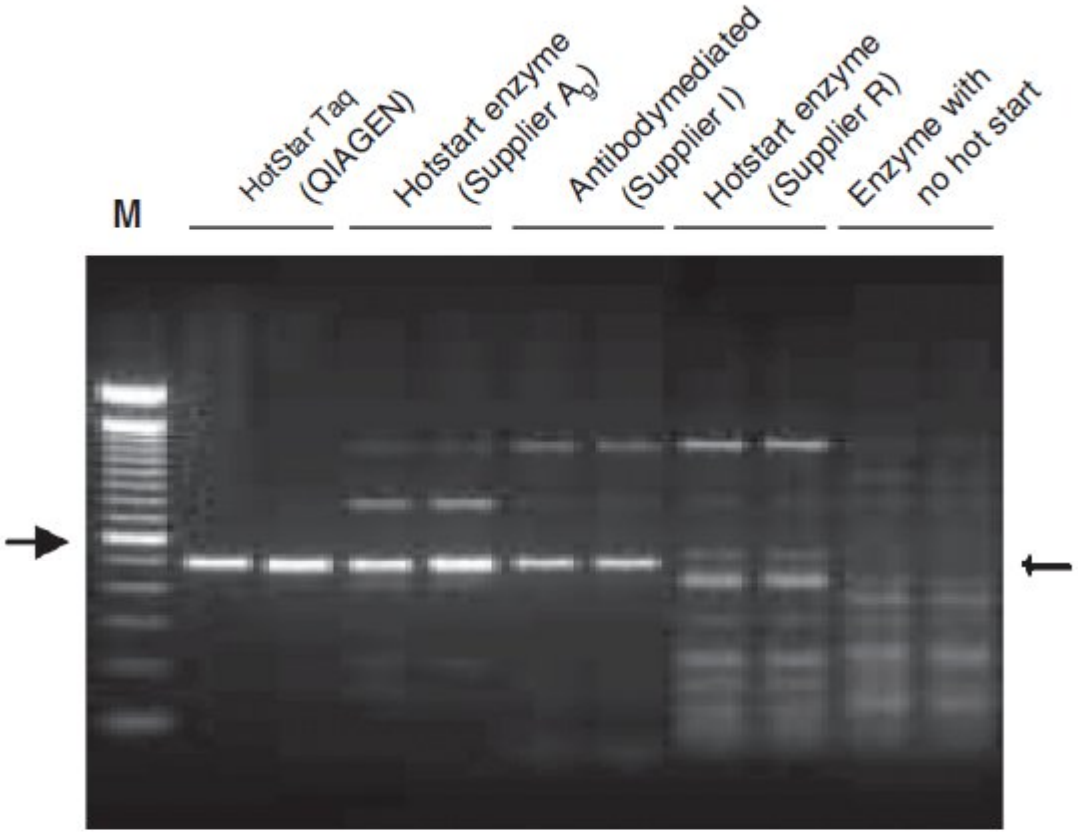


Fig. 7.4 Hot-start (time release) PCR and its effect on PCR quality. Lane 1 = molecular size marker; lanes 2 and 3 = the result of a time-release PCR of the HIV polymerase gene performed in duplicate; lane 4 = the same PCR without the time-release Taq formulation. Fifty copies of template were used in the presence of 1 µg of human DNA and the amplimers were analysed on a 2% agarose gel. The correct PCR product is 497 bp in length. The arrow on the left identifies a 600 basepair long marker in the lane marked M (From Critical Factors for Successful Real-Time PCR, www.qiagen.com)

Table 7.2 General characteristics of commercially available thermostable DNA dependent DNA polymerases

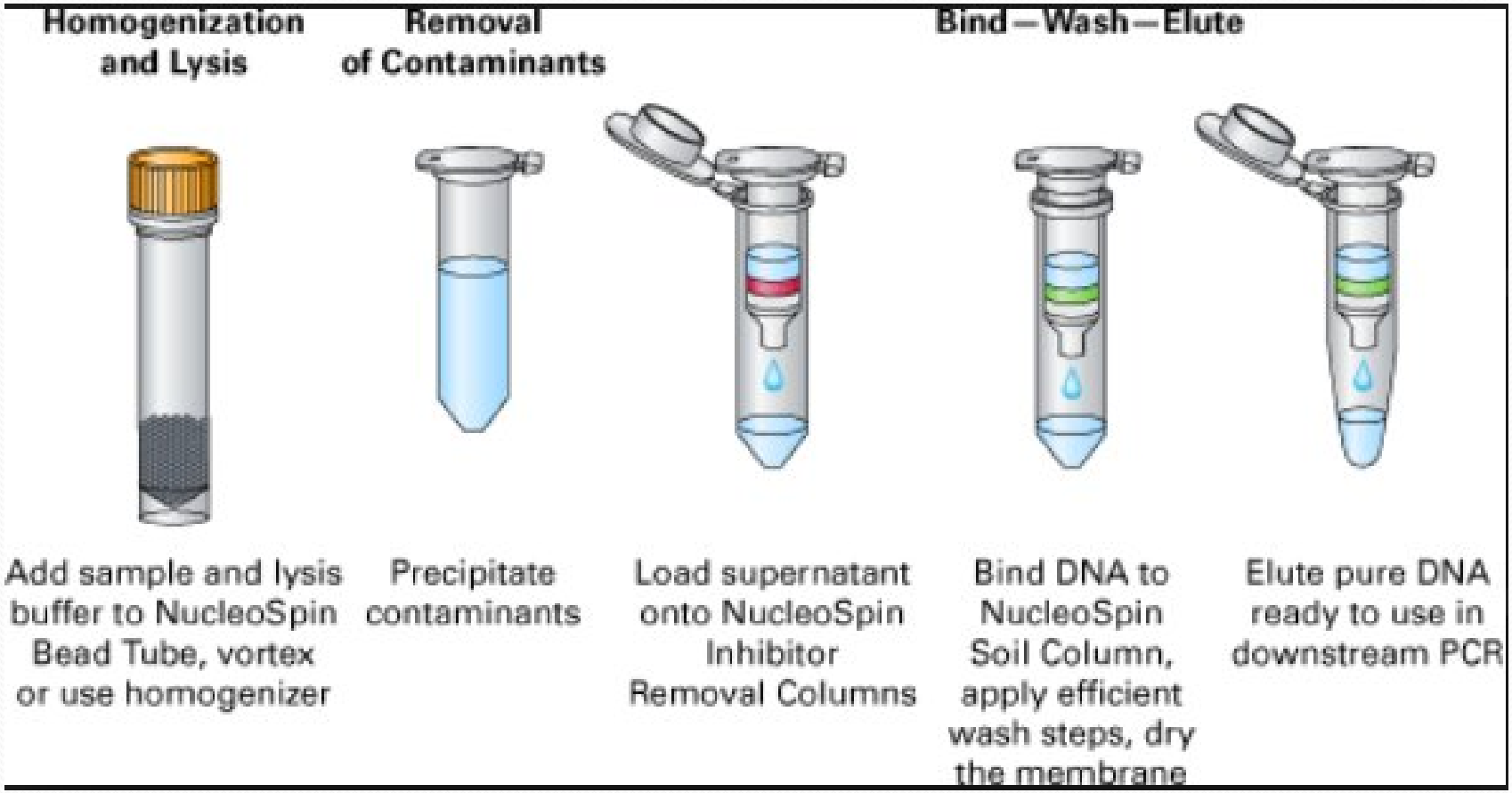
Enzyme	Thermostability (min at 95°C)	5′-3′ Proofreading	3′-5′ Proofreading	Extension speed (nucleotides/second)	pH optimum	Mg^{++} optimum ($\times 10^{-3}$)	Mol weight
AmpliTaq	40	Yes	No	75	7–7.5	1–4	94
Stoffel	80	No	No	>50	–	–	61
Ampliterm	–	No	No	–	–	1.5	–
Pyra	>300	No	No	–	7–7.5	3	90
TTH 94	20	Yes	No	>33	7–7.5	1.5–2.5	
Tfl	40	Yes	No	16	7–7.5	1–4	94
Tfu	300	No	Yes	64	–	–	–
DeepVent	400	No	Yes	>80	–	–	–
Vent	1380	No	Yes	–	–	–	–
Tli	400	No	Yes	33	7–7.5	2–6	90
Proofstart	>240	No	Yes	–	–	Broad	–
PFU 92	>120	No	Yes	60	8–9	2–6	
Pfx	–	–	Yes	–	–	–	–
Pwo	–	No	Yes	–	–	–	–
UL*Tma*	>50	No	–	–	–	–	70
ThermalAce	>4	–	Yes	–	–	–	–

–: not known; opt: optimum

الگو برای PCR

1. Genomic DNA
2. Plasmid
3. cDNA

جداسازی DNA


Homogenization and Lysis
Removal of Contaminants
Bind—Wash—Elute
Add sample and lysis buffer to NucleoSpin Bead Tube, vortex or use homogenizer
Precipitate contaminants
Load supernatant onto NucleoSpin Inhibitor Removal Columns
Bind DNA to NucleoSpin Soil Column, apply efficient wash steps, dry the membrane
Elute pure DNA ready to use in downstream PCR

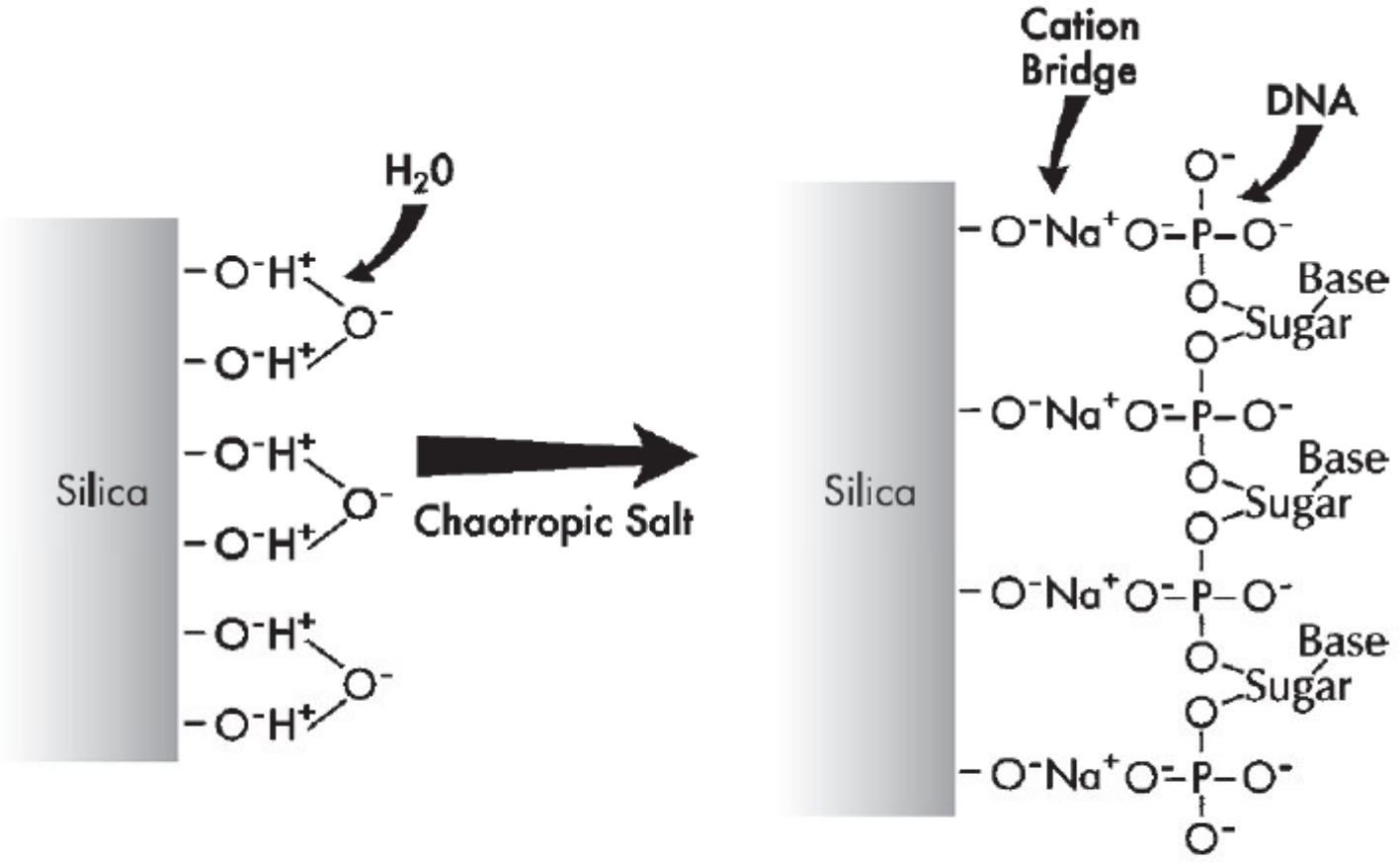


Fig. 4.5 Silica provides a universal medium for the immobilisation of nucleic acid molecules. In the presence of water and chaotropic salts the molecules interact ionically through sodium ions and the nucleic acid phosphate groups. After binding, contaminants may be removed by washing and centrifugation (From the Geneclean Guide to Protocols and Procedures, Bio101. Courtesy of MP Biomedicals LLC)

پرایمر یا الیگو

A primer is a short synthetic oligonucleotide which is used in many molecular techniques from PCR to DNA sequencing. These primers are designed to have a sequence which is the reverse complement of a region of template or target DNA to which we wish the primer to anneal.

طول پرایمر

- ***Proper length of primer***

Usually: 18 - 22 bases

Too short ⟶ Less specific

Too long ⟶ Wasting money

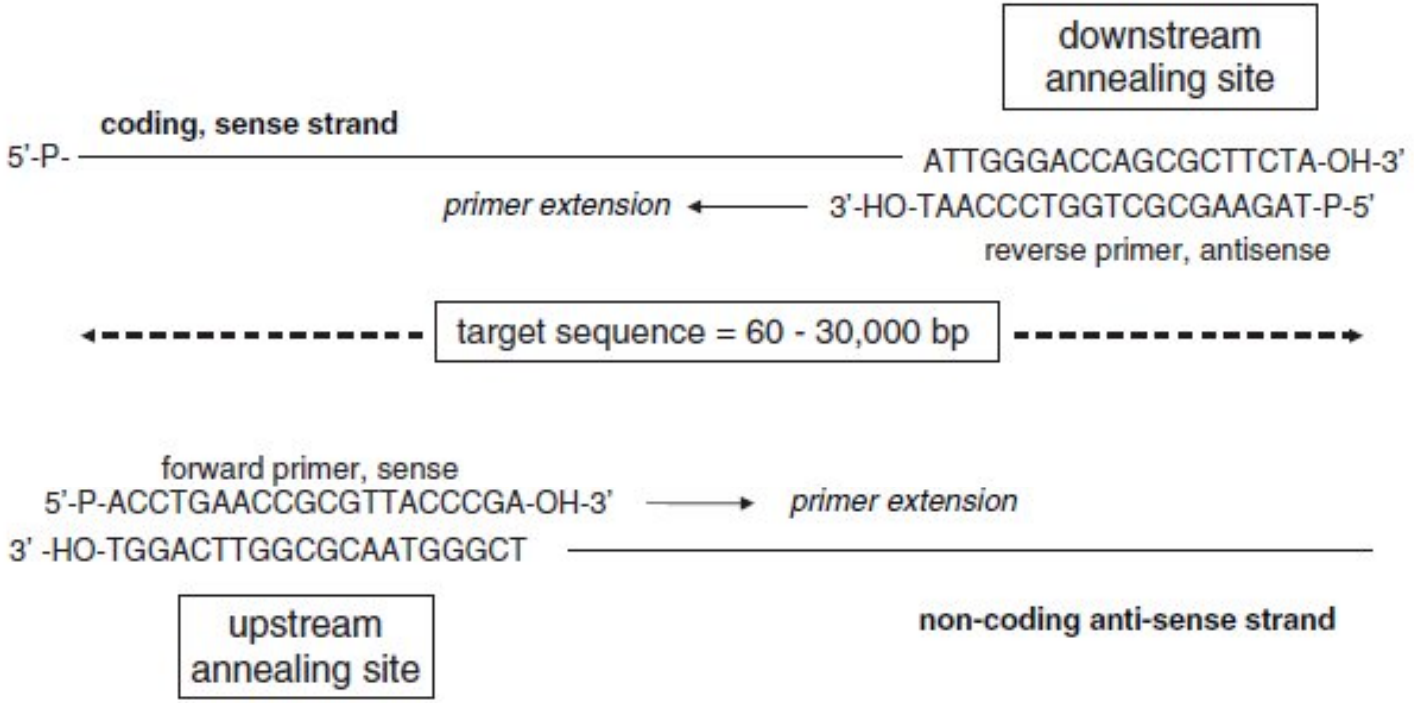
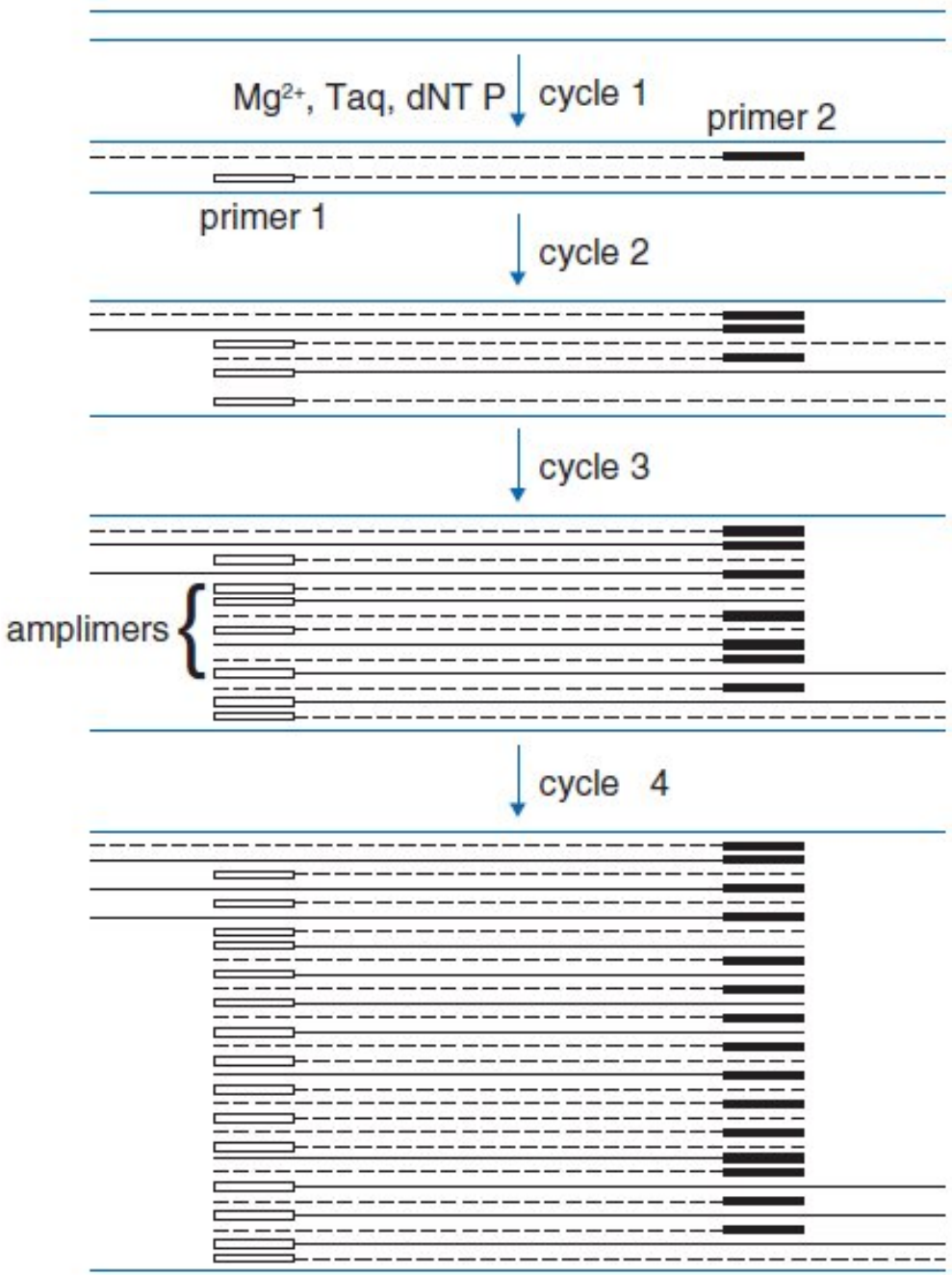
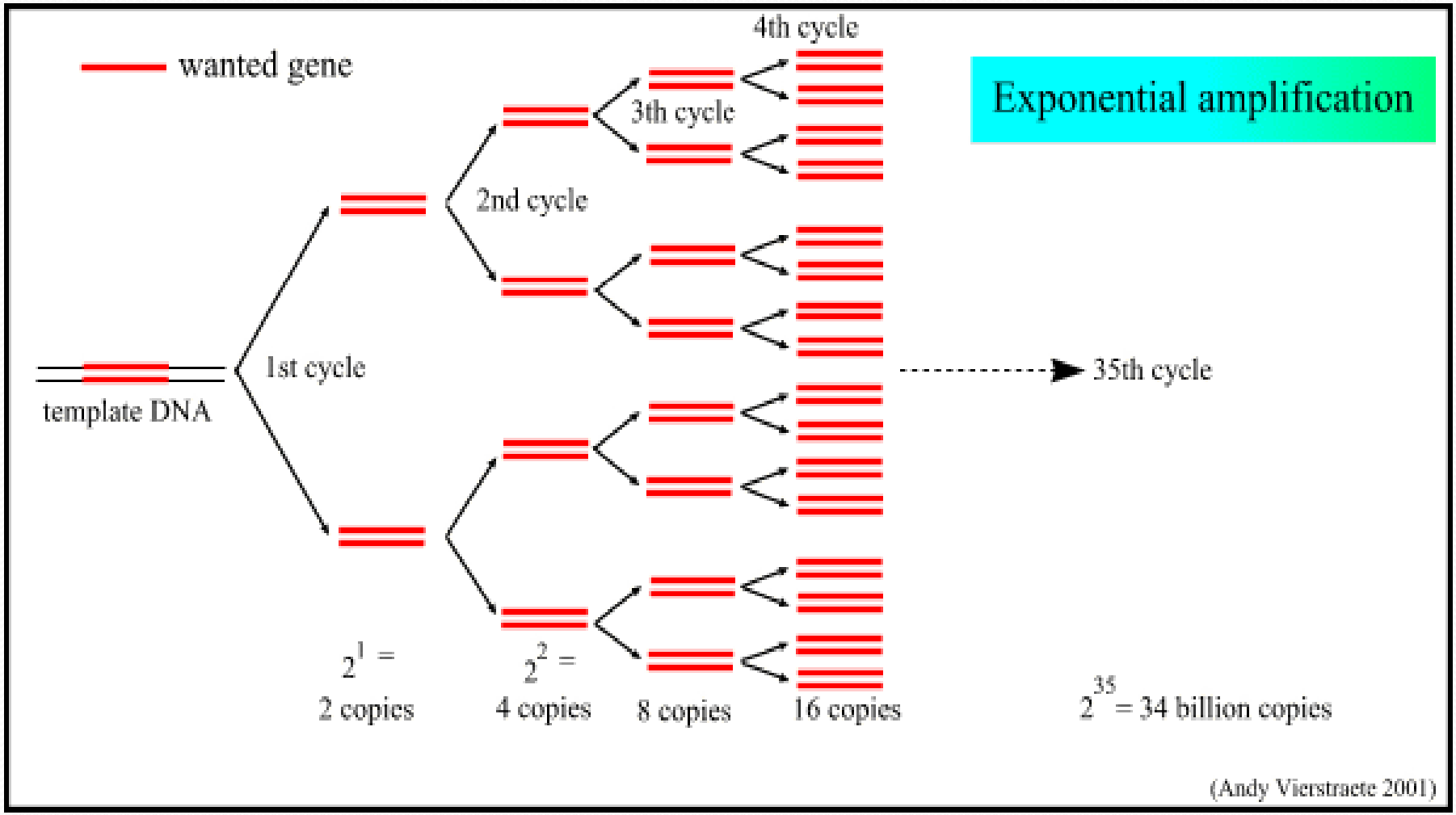


Fig. 1.1 The key components of PCR are the primers and the thermostable DNA polymerase. The forward and reverse primers determine the PCR specificity and the length of the amplification products. Primers need to be precisely complementary to their respective target sequences. Both the sense and anti-sense strands of the DNA double helix are copied and therefore amplified during PCR thermocycling. After target DNA disassociation, the primers are allowed to anneal to the target DNA so that strand amplification via a thermostable DNA polymerase can occur. The primer that hybridises nearest the "ATG" start codon of the gene (fragment) to be amplified is called the upstream or forward primer, whilst the primer which hybridises closest to the "stop" codon is referred to as the downstream or reverse primer


Mg2+, Taq, dNT P
cycle 1
primer 2
primer 1
cycle 2
cycle 3
amplimers
cycle 4

تکثیر

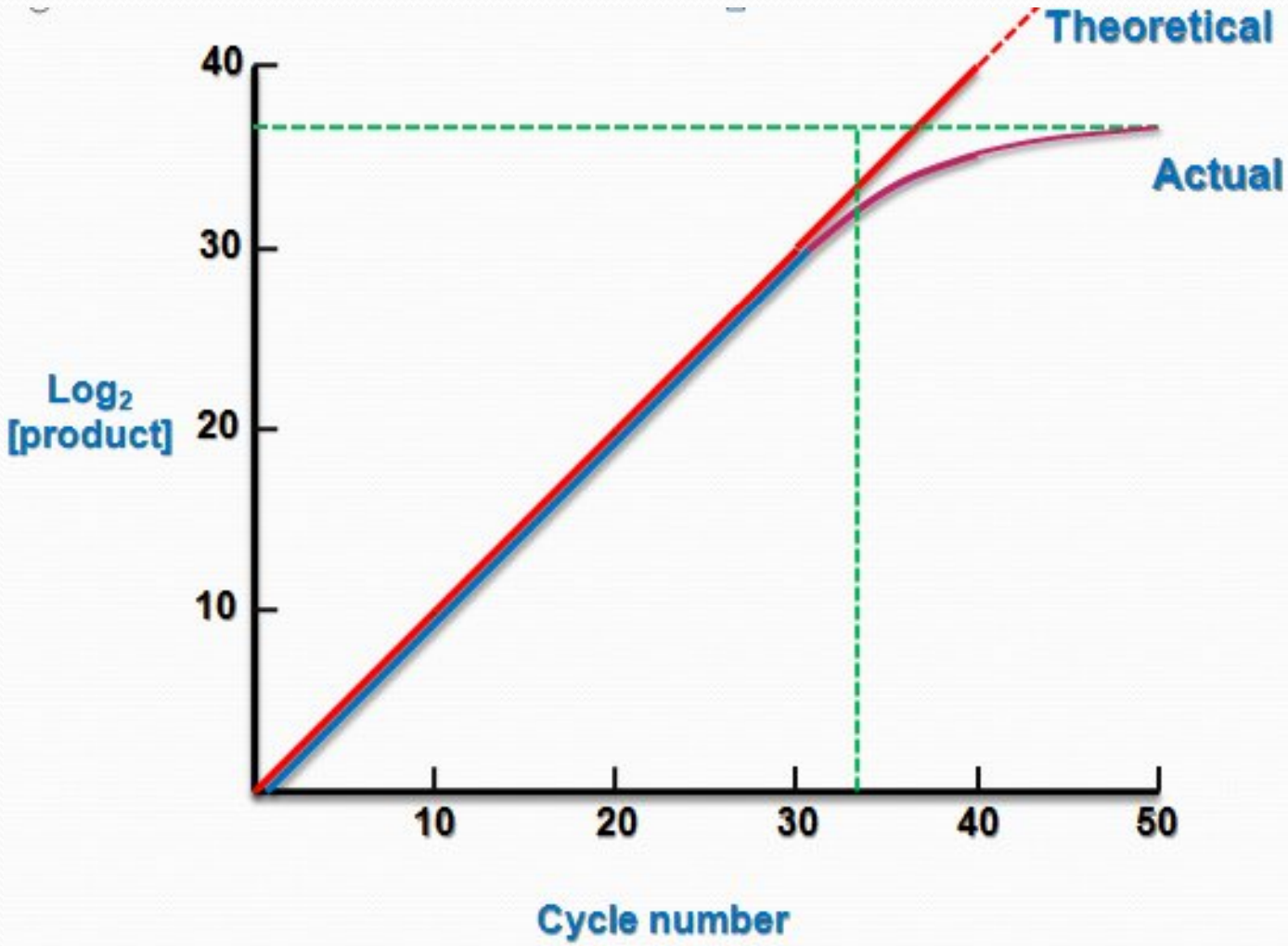

wanted gene
4th cycle
Exponential amplification
3th cycle
2nd cycle
1st cycle
template DNA
35th cycle
2^1 =
2 copies
2^2 =
4 copies
8 copies
16 copies
2^{35} = 34 billion copies
(Andy Vierstraete 2001)

How Many Cycles is Enough?

I. 30 cycles = 10^9-fold amplification
II. 40 cycles = 10^{12}-fold amplification
III. 50 cycles – 10^{15}-fold amplification….??

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

Plateau Effect in DNA Amplification



مراحل PCR

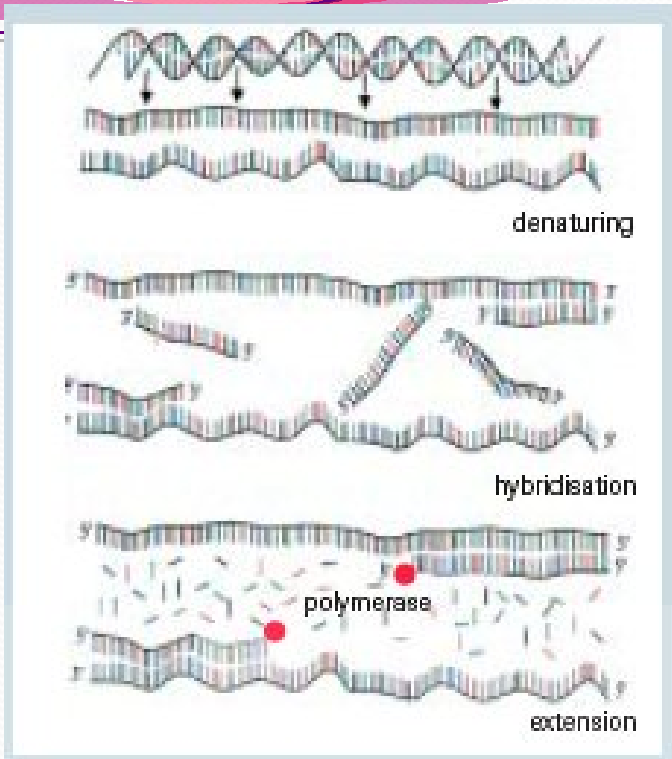
3 steps in a PCR repeated for 30-40 cycles.

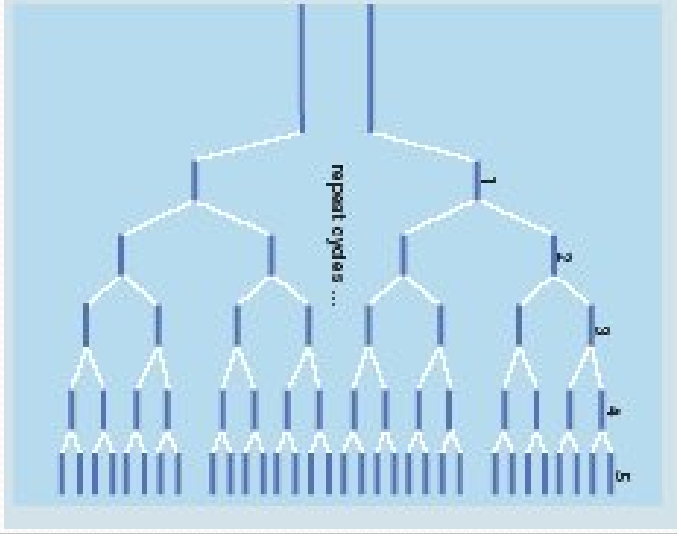
- **Denaturation** at 95˚C: the double stranded DNA melts open to single stranded DNA.

- **Annealing/hybridisation**: The primers anneal to the DNA.

- **Extension** 68˚C or 72˚C: The polymerase copies the template adding the dNTPs from 5’ to 3’.

Times of each step?????





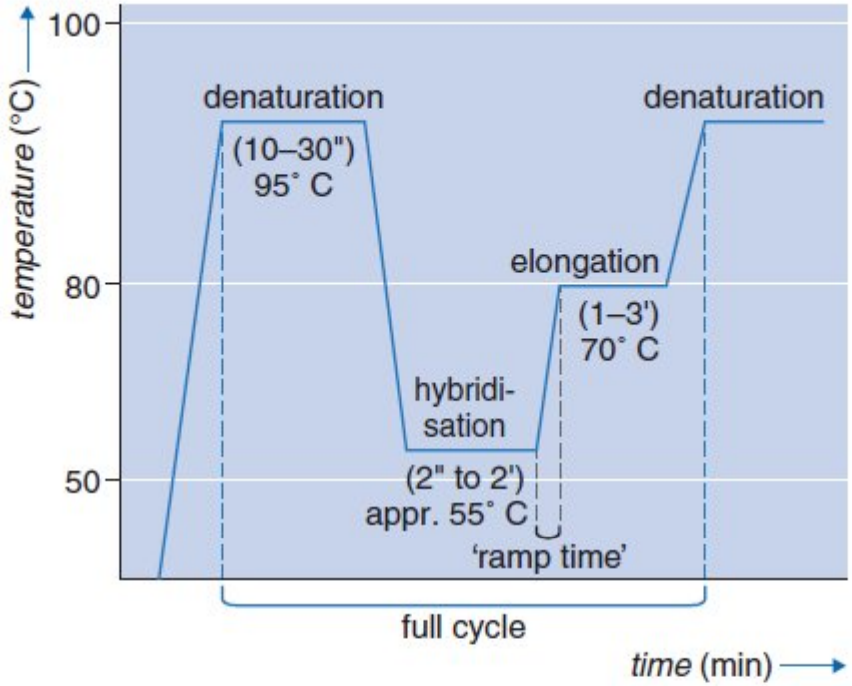


Fig. 3.1 Temperature profile of a typical three step PCR cycle. Initially, DNA is melted by an incubation step at 95–98°C (denaturation). Primers are then allowed to bind to the target DNA by lowering the temperature to the calculated annealing temperature of the primer pair used (usually between 45–65°C) (annealing). Subsequently, DNA extension takes place by increasing the temperature to the optimal temperature of the thermostable DNA dependent DNA polymerase, (usually around 70°C) (extension or replication). The following cycle of the PCR is then initiated by returning to the melting temperature of 95–98°C. Incubation times for each of these temperature steps may vary between 30 seconds and 2 minutes in an average PCR protocol

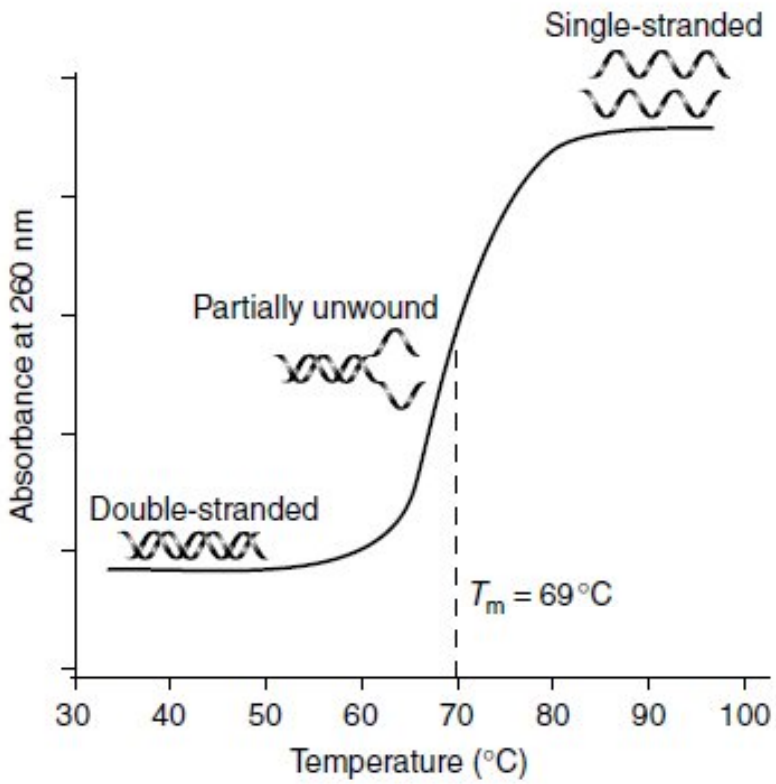


Figure 1.11. The effect of heating double-stranded DNA. As a double-stranded DNA molecule is heated above the ambient temperature, the non-covalent hydrogen bonds that hold the two strands together begin to break. This is accompanied by an increase in absorbance at 260 nm. At higher temperatures – above 90 °C – the two DNA strands will completely separate from each other. This process is reversible. If the temperature is reduced relatively slowly, the two complementary strands of DNA will reform to produce a correctly paired double-stranded DNA molecule. The melting temperature (T_m) is the temperature at which half the strands are separated

انواع پرایمر

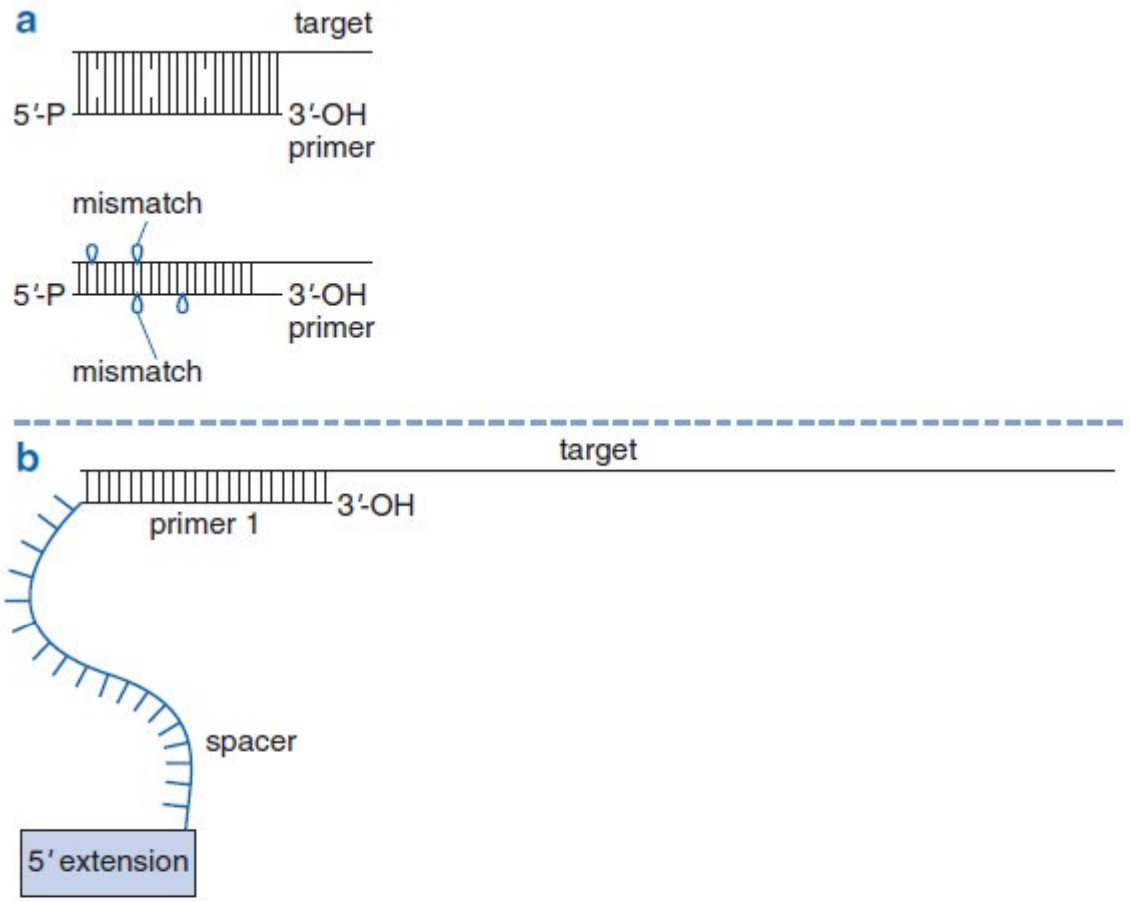


Fig. 5.2 A variety of modified PCR primers and their applications have been described. Exa

PCR machine

Primitive PCR machine

Stratagene RoboCycler 40 PCR Machine

Gradient PCR

Various Types of PCR

1. **Conventional PCR** – the basic PCR process, which produces up to a billion copies of a DNA or RNA strand; the results are only seen at the end of the process

2. **Long range PCR** – uses a mixture of polymerases to amplify longer stretches of DNA

3. **Multiplex PCR** – uses a number of pairs of primers to allow analysis of a number of fragments in a single sample
4. **Nested PCR** – after an initial 30-35 cycles of PCR, an additional round of PCR uses new primers 'nested' within the original primers, making the process more sensitive because it reduces the risk of unwanted products from primers binding to incorrect regions
5. **qPCR** – in quantitative PCR (also known as real time PCR, RT PCR or qRT-PCR), the DNA or RNA molecules are tagged using fluorescent probes, so that the concentration of amplified products can be monitored and quantified in real-time by tracking the level of fluorescence
6. **Hot start PCR** – inactivates the Taq polymerase until the reaction starts, using antibodies that are denatured by heat.
7. And so on.

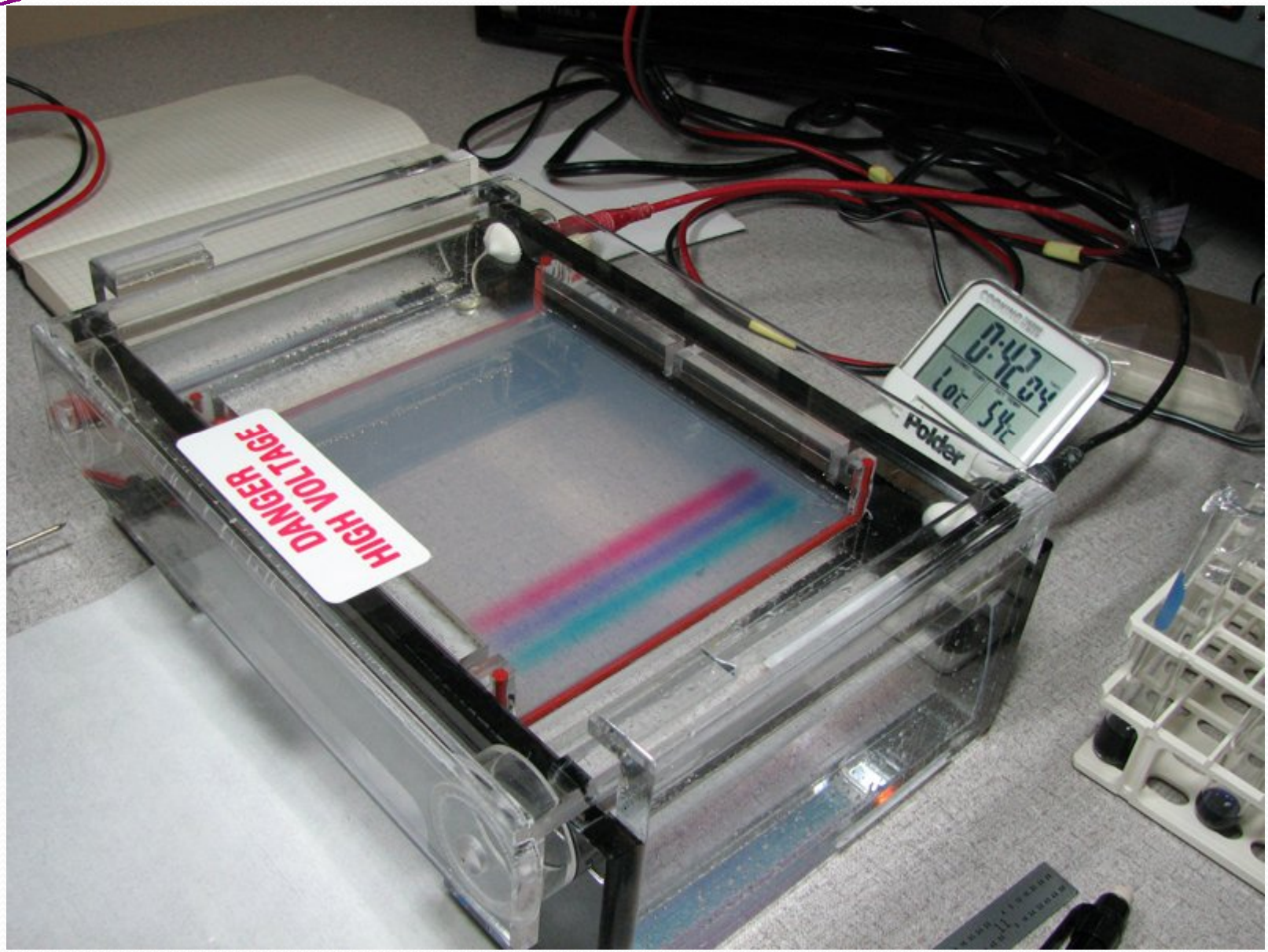
Visualization of PCR product

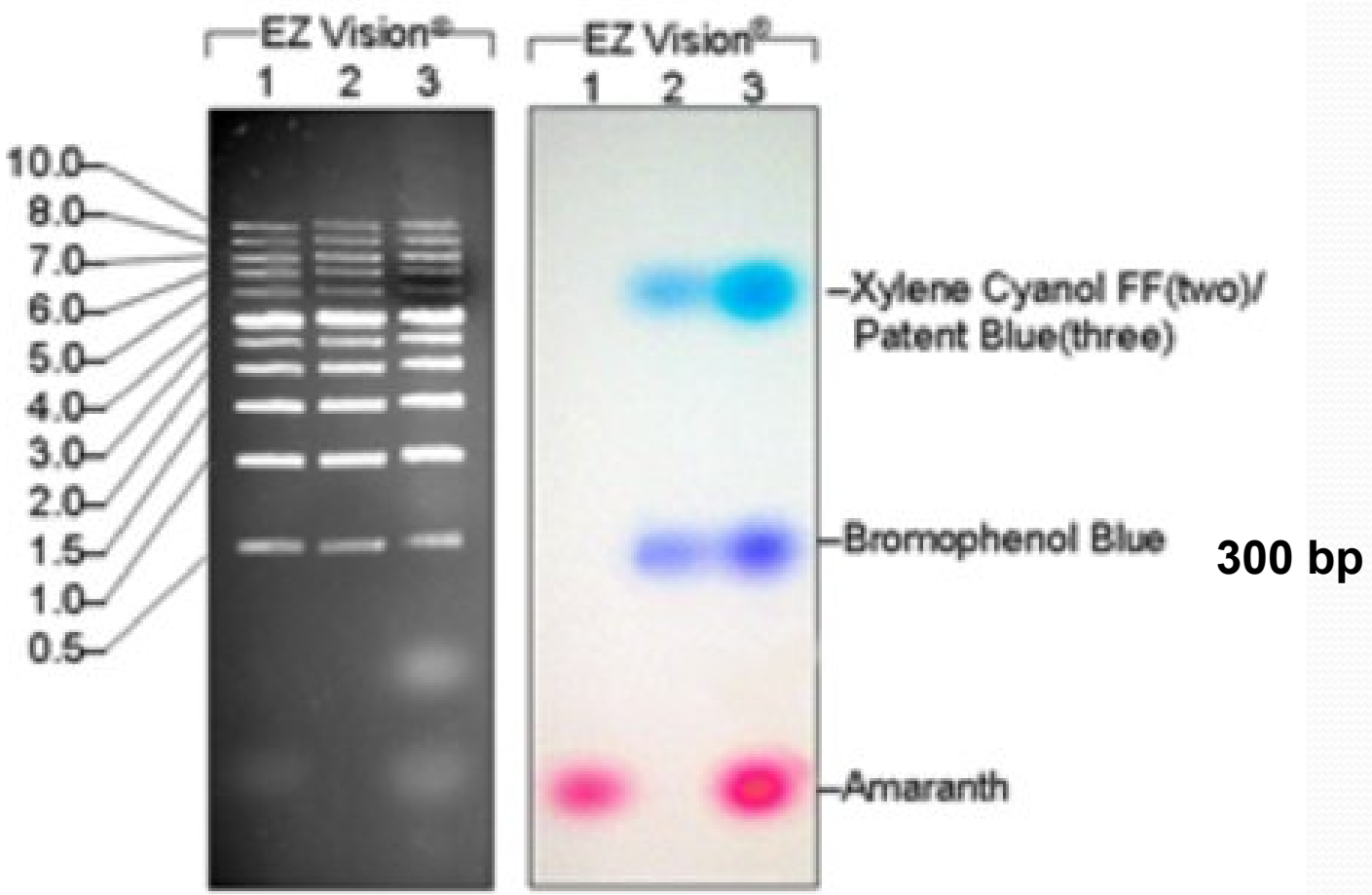
Detection of pcr product

Detection	Visualization
Agarose gel and/or polyacrylamide gel electrophoresis	-EtBr staining (UV transilluminator, image analyzer) -Southern blotting (hybridization with labeled probe) -Silver staining
Restriction endonuclease digestion	-Agarose or polyacrylamide gel -HPLC
Dot blots	Hybridization with labeled probe
High-pressure liquid chromatography	UV detection
Electrochemiluminescence	Voltage-initiated chemical reaction/photon detection
Direct sequencing	Radioactive or fluoescent-based DNA sequencing

انواع الکتروفورزِDNA

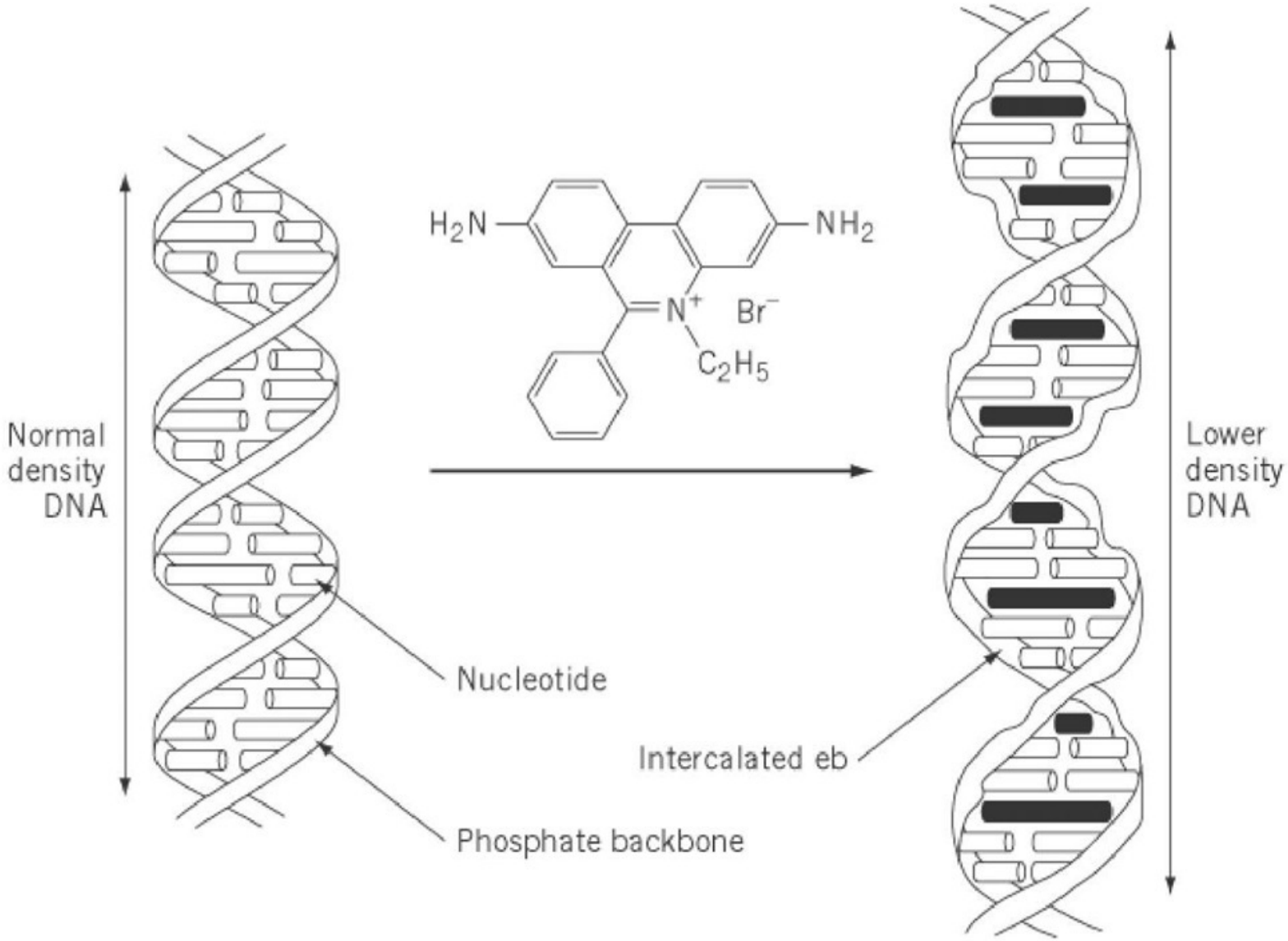
PAGE
Agarose


DANGER
HIGH VOLTAGE
Polder


EZ Vision®
1 2 3
EZ Vision®
1 2 3
10.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.5
1.0
0.5
Xylene Cyanol FF(two)/
Patent Blue(three)
Bromophenol Blue
Amaranth
300 bp

Dyes for DNA in agarose gel electrophoresis

- ethidium bromide - traditional DNA stain, quantum yield 0.2; 5g >98% pure **€73** 7870.2, **4x cheaper** than SYBR Gold, more stable than SYBR Green & Gold
- SYBR Gold - DNA & RNA, **10x more sensitive** than EtBr, quantum yield 0.6, 500µl 10'000x **€165** S-11494
- SYBR Green I - better for DNA, **20x more sensitive** than EtBr (60 pg per band with 300 nm transillumination), quantum yield 0.8; l000µl **690€** S-7585
- SYBR Green II - better for RNA, detection limit is 500 pg of RNA per band in non-denaturing gels with 300 nm transillumination, quantum yield 0.5
- SYBR Safe - **sensitivity similar** to EtBr; 400µl **77€** S33102
- GelRed, GelGreen producer website, GelRed is chemically equivalent to 2 molecules of EtBr joined by a linker, 500µl 10'000x **$105** 41003


Normal density DNA
H_2N
NH_2
N^+
Br^-
C_2H_5
Nucleotide
Phosphate backbone
Intercalated eb
Lower density DNA

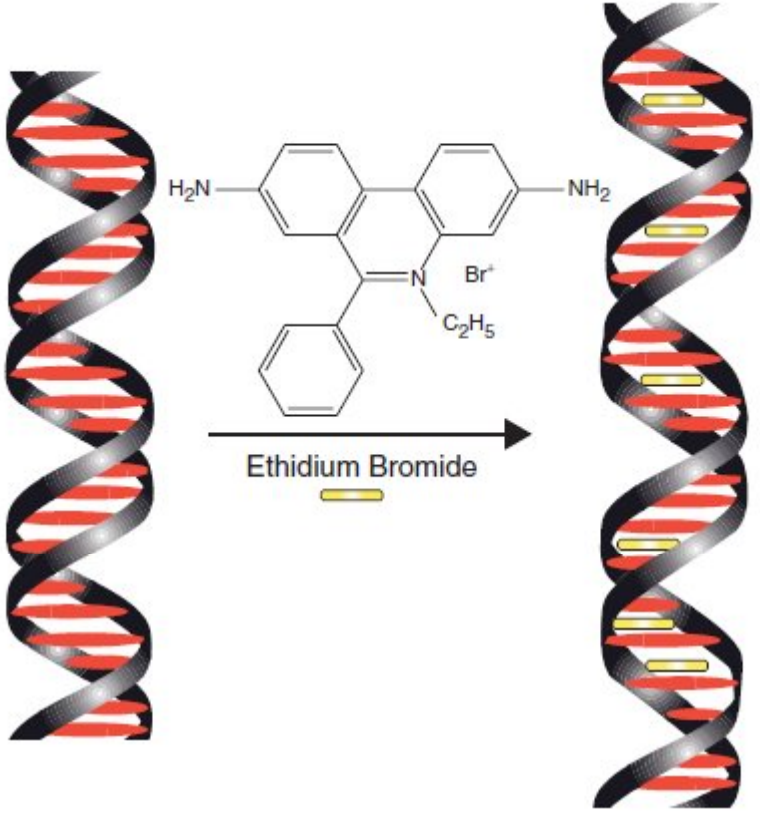
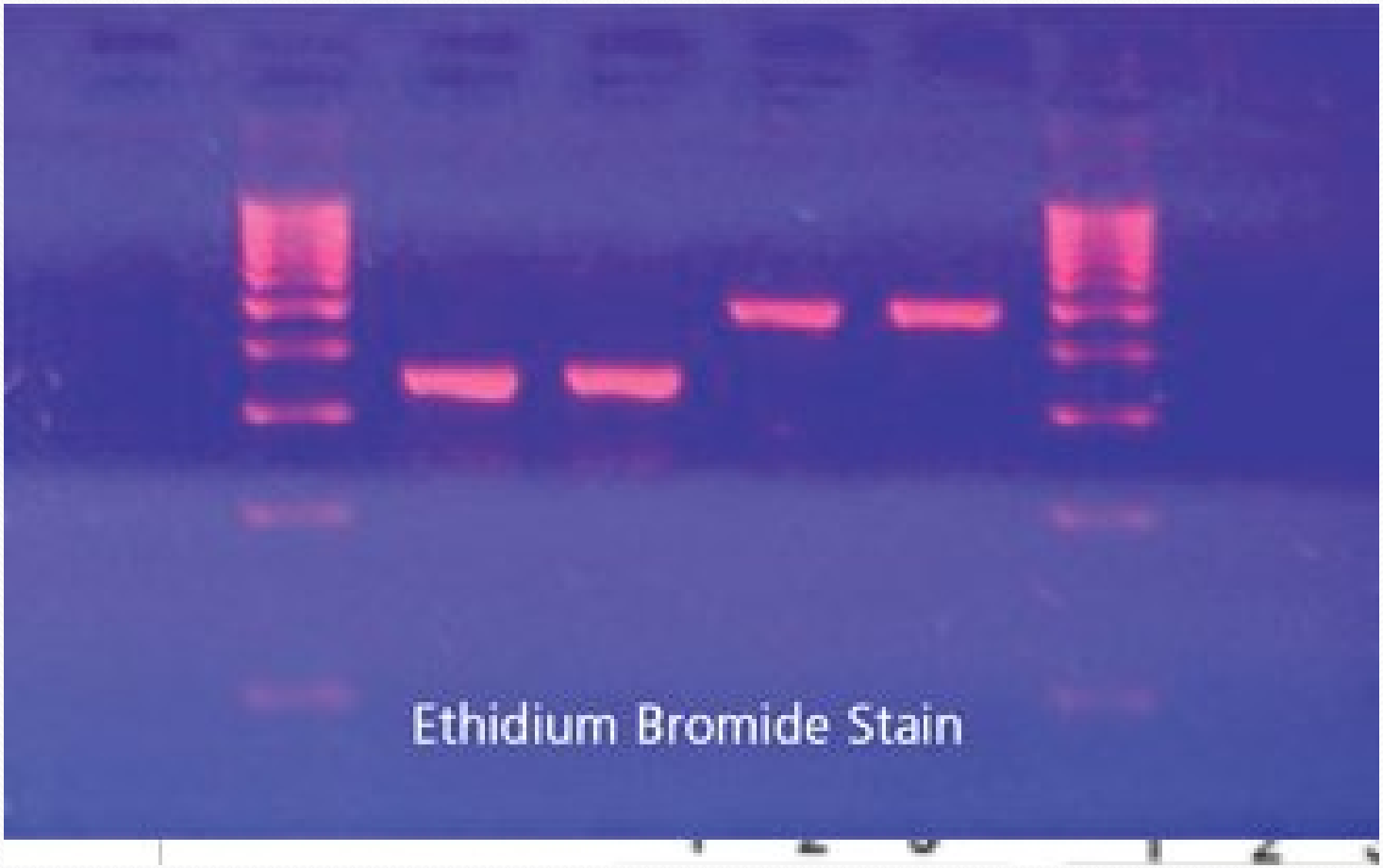
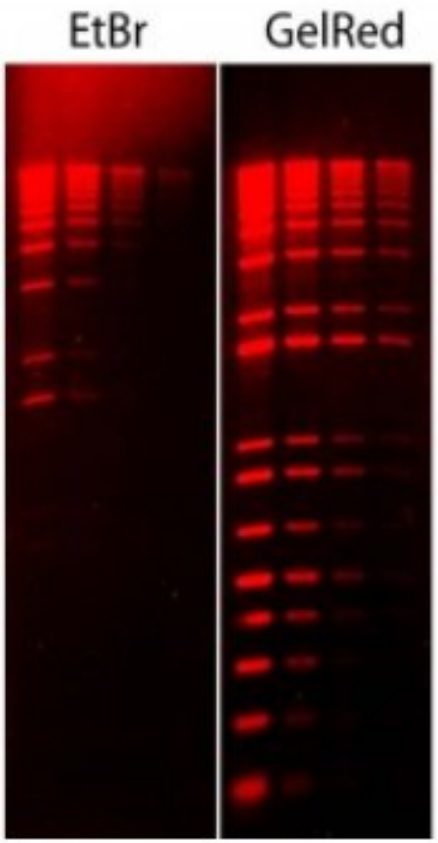


Figure 2.12. The binding of ethidium bromide to DNA. Ethidium bromide is a flat, planar molecule that is able to intercalate in between the stacked bases of double-stranded DNA. The binding of ethidium bromide distorts the double helix and increases its overall length. DNA to which ethidium bromide is bound fluoresces when viewed under ultraviolet light

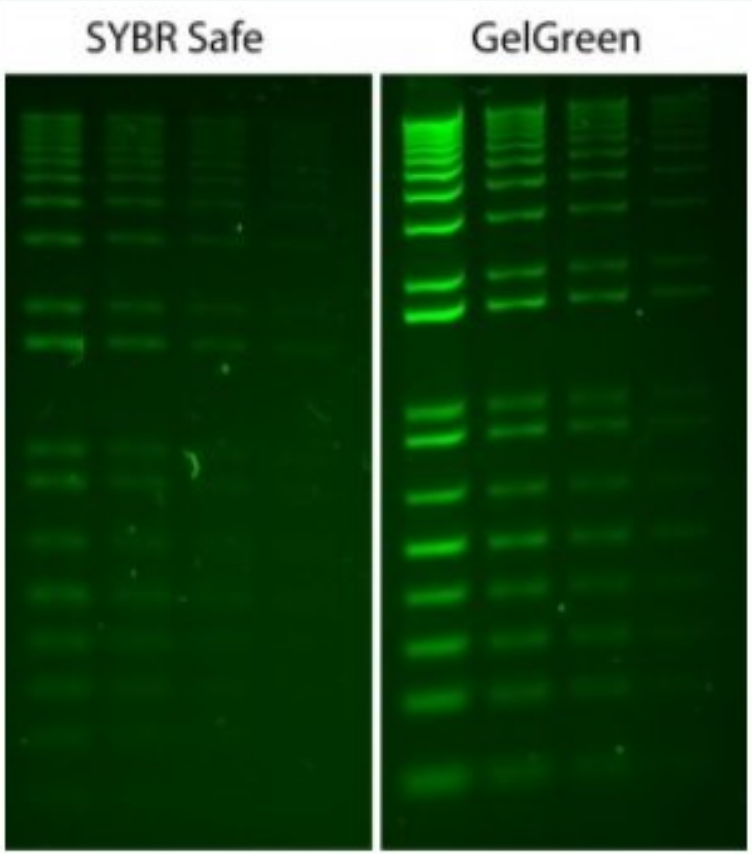

Ethidium Bromide Stain

GelRed™ Is Superior to EtBr

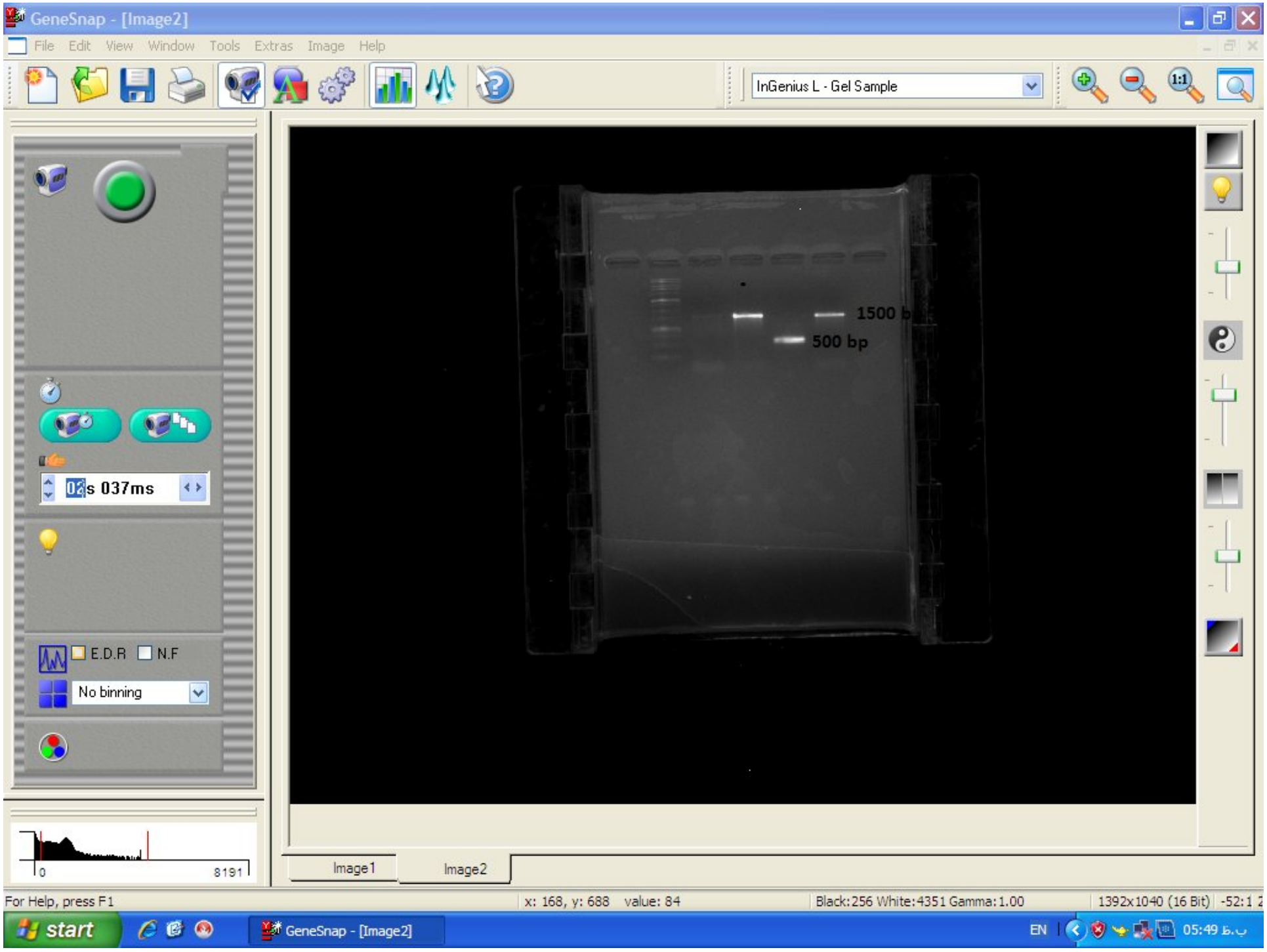


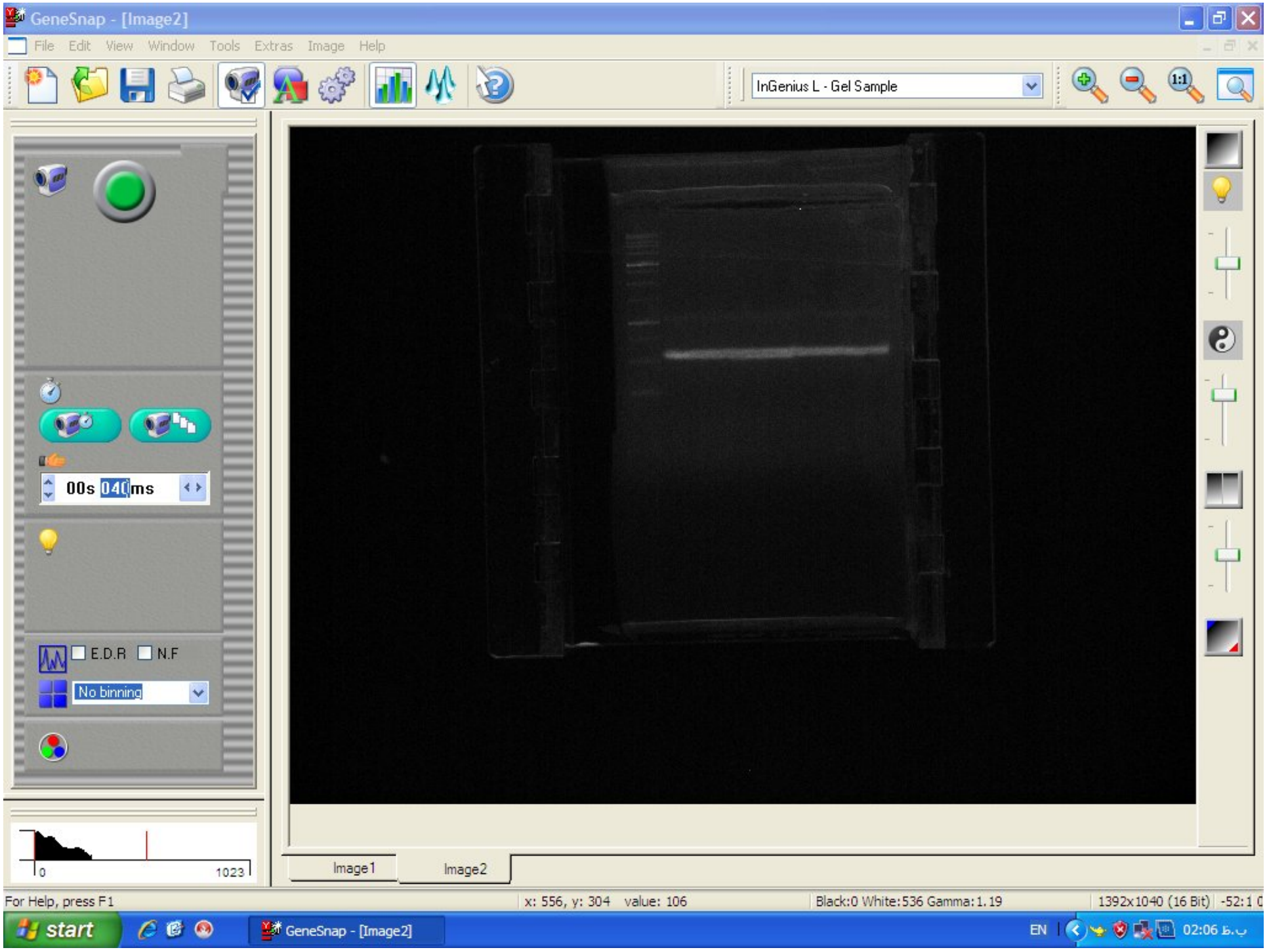
Comparison of ethidium bromide (EtBr) and GelRed in precast gel staining in 1% agarose/TBE gel. Two-fold serial dilutions of 1 kb Plus DNA Ladder (Invitrogen) were loaded in the amounts of 200 ng, 100 ng, 50 ng and 25 ng from left to right. Gels were imaged using 300 nm transilluminator and photographed with an EtBr filter.

GelGreen™ Is Unmatched by SYBR® Safe



Comparison of GelGreen and SYBR Safe in post-electrophoresis staining of 1% agarose/TBE gels. Two-fold serial dilutions of 1 kb Plus DNA Ladder (Invitrogen) were loaded in the amounts of 200 ng, 100 ng, 50 ng and 25 ng from left to right. Gels were imaged using 254-nm transilluminator and photographed with a SYBR filter.


GeneSnap - [Image2]
File Edit View Window Tools Extras Image Help
InGenius L - Gel Sample
1:1
1500 b
500 bp
02s 037ms
E.D.R
N.F
No binning
0
8191
Image1
Image2
For Help, press F1
x: 168, y: 688 value: 84
Black:256 White:4351 Gamma:1.00
1392x1040 (16 Bit)
start
GeneSnap - [Image2]
EN
05:49


GeneSnap - [Image2]
File Edit View Window Tools Extras Image Help
InGenius L - Gel Sample
00s 040ms
E.D.R
N.F
No binning
0
1023
Image1
Image2
For Help, press F1
x: 556, y: 304 value: 106
Black:0 White:536 Gamma:1.19
1392x1040 (16 Bit)
start
GeneSnap - [Image2]
EN
02:06

Gel percentage

Percent Agarose Gel (w/v)	DNA Size Resolution(kb = 1000)
0.5%	1 kb to 30 kb
0.7%	800 bp to 12 kb
1.0%	500 bp to 10 kb
1.2%	400 bp to 7 kb
1.5%	200 bp to 3 kb
2.0%	50 bp to 2 kb

Table 1: Correct Agarose Gel Concentration for Resolving DNA Fragments

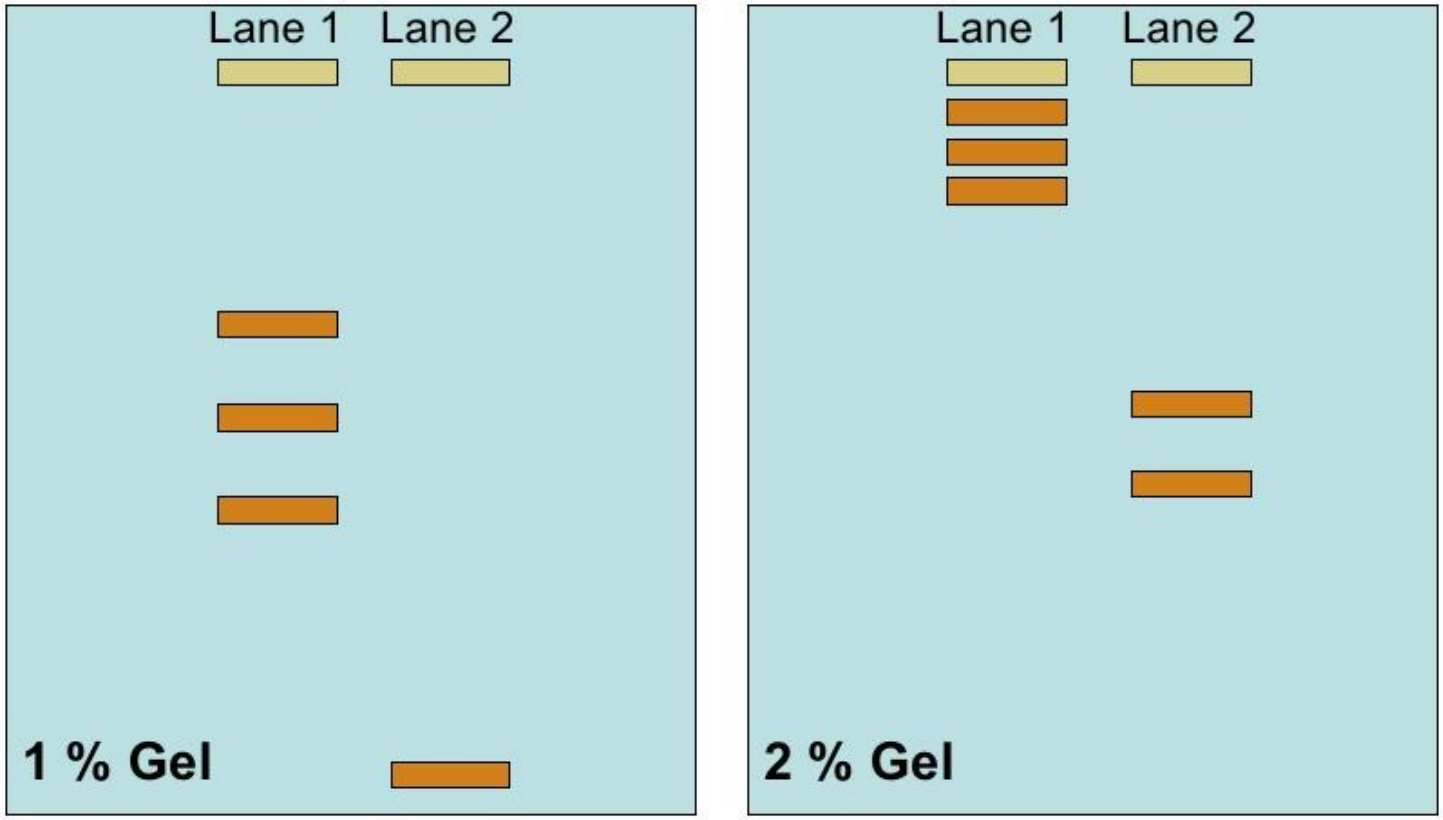
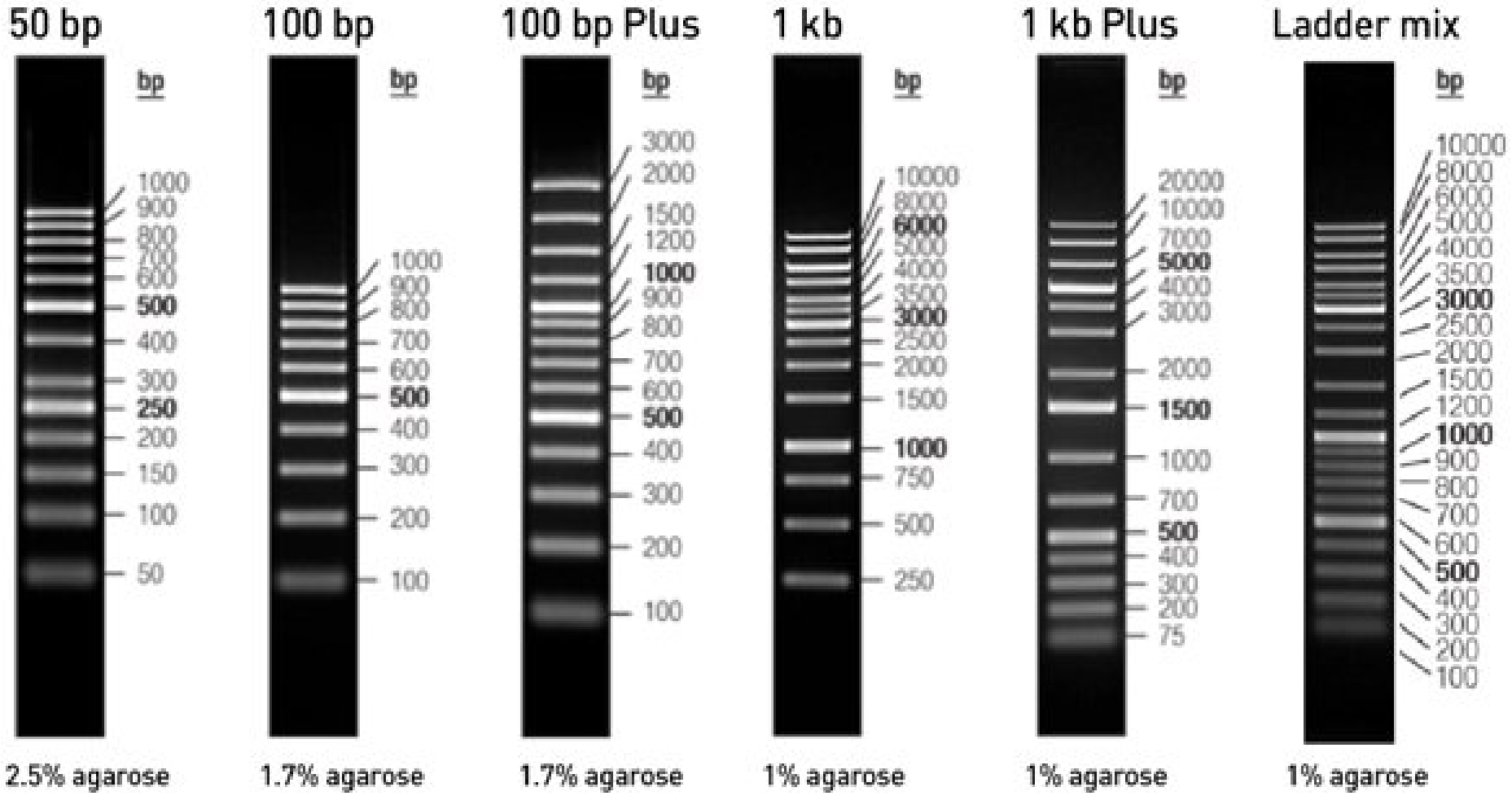
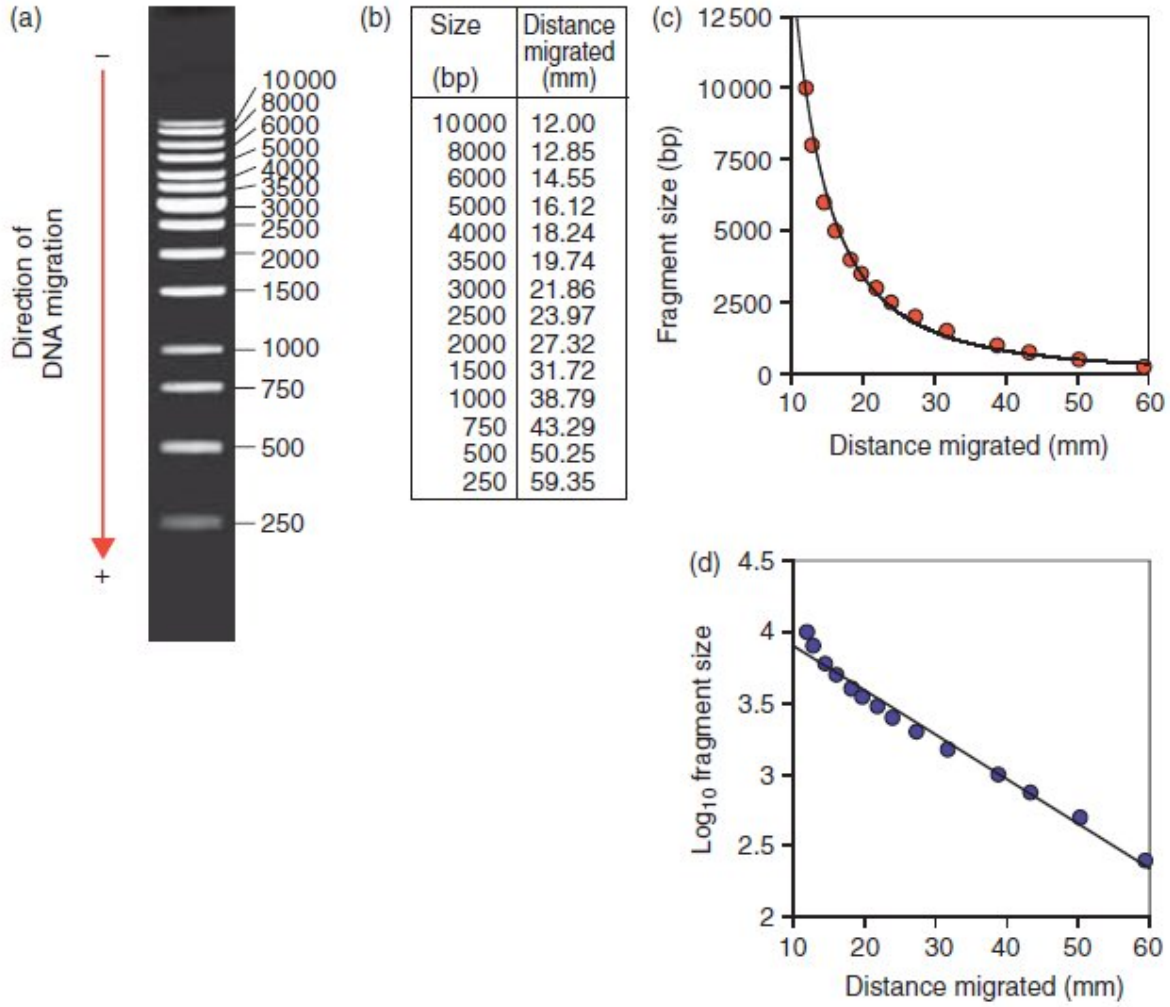


Figure 1: Comparing Resolution of 1 % Gel and 2 % Agarose Gel.

Lane 1 shows the separation of nicked, supercoiled and linear DNA of a 6 kb fragment. Lane 2 shows separation of 425 bp and 450 bp PCR fragments. The 1 % gel could not resolve the two PCR fragments. They appear as a single band. Gel purification would not eliminate either band. The PCR fragment would a produce a mixed sequencing result.

DNA ladder


50 bp
bp
1000
900
800
700
600
500
400
300
250
200
150
100
50
2.5% agarose
100 bp
bp
1000
900
800
700
600
500
400
300
200
100
1.7% agarose
100 bp Plus
bp
3000
2000
1500
1200
1000
900
800
700
600
500
400
300
200
100
1.7% agarose
1 kb
bp
10000
8000
6000
5000
4000
3500
3000
2500
2000
1500
1000
750
500
250
1% agarose
1 kb Plus
bp
20000
10000
7000
5000
4000
3000
2000
1500
1000
700
500
400
300
200
75
1% agarose
Ladder mix
bp
10000
8000
6000
5000
4000
3500
3000
2500
2000
1500
1200
1000
900
800
700
600
500
400
300
200
100
1% agarose



Size (bp)	Distance migrated (mm)
10 000	12.00
8000	12.85
6000	14.55
5000	16.12
4000	18.24
3500	19.74
3000	21.86
2500	23.97
2000	27.32
1500	31.72
1000	38.79
750	43.29
500	50.25
250	59.35

Figure 2.13. The migration of DNA fragments through an agarose gel. (a) An agarose gel showing the separation of DNA fragments of known size. (b) The size of a DNA fragment and the distance migrated through the gel. (c) A plot of fragment size against distance migrated taken from the data shown in (b). This indicates that the relationship between fragment size and distance migrated is not linear. (d) A plot of the log of fragment size against distance migrated. This indicates that there is a direct, inverse, relationship between distance migrated and the log of the size of a DNA fragment

50 X TAE buffer

Tris-base: 242 g

Acetate (100% acetic acid): 57.1 ml

EDTA: 100 ml 0.5M sodium EDTA

Add dH2O up to one litre.

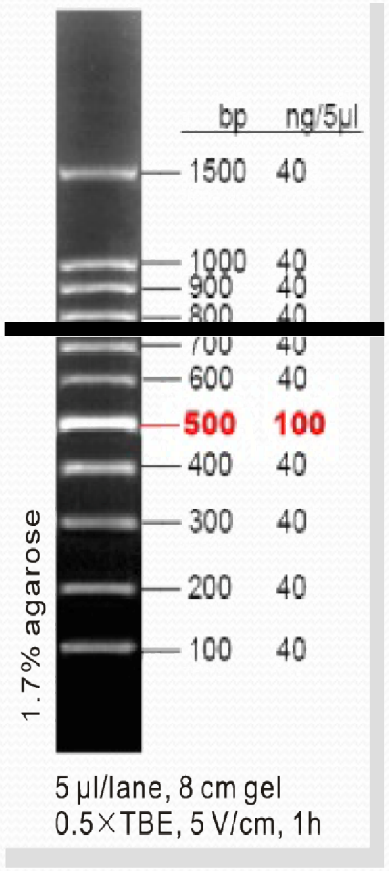
تخمین غلظت اسیدهای نوکلئیک

Table 5.4: Accepted Spectrophotometric Nucleic Acid Conversion Values	
1.0 A260 Unit of double-stranded DNA	50 µg/mL
1.0 A260 Unit of single-stranded DNA	37 µg/mL
1.0 A260 Unit of RNA	40 µg/mL

- **dsDNA concentration = 50 µg/mL × OD_{260} × dilution factor**
- **dsDNA concentration = 50 µg/mL × 0.65 × 50**
- **dsDNA concentration = 1.63 mg/mL**

100bp ladder

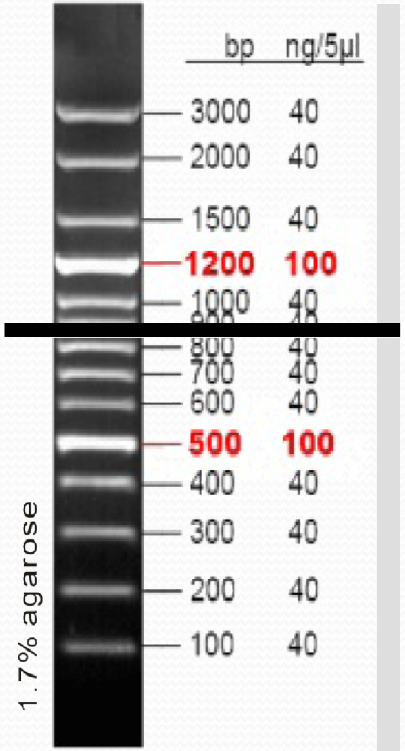
M1061/M1062



5 μl/lane, 8 cm gel
0.5×TBE, 5 V/cm, 1h

100bp ladder plus

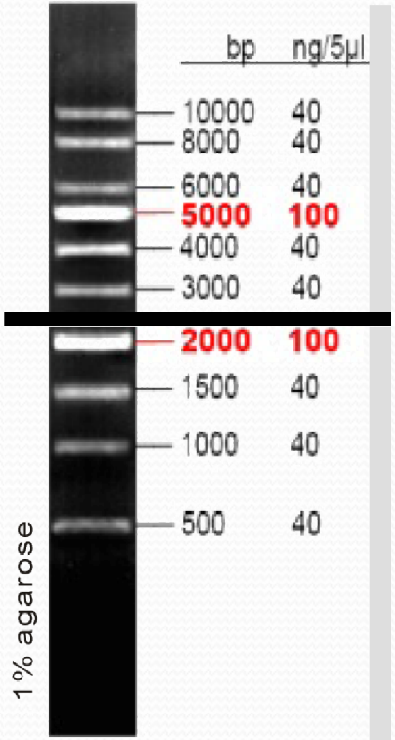
M1071/M1072



5 μl/lane ,8 cm gel
0.5×TBE, 5 V/cm, 1h

1kb ladder

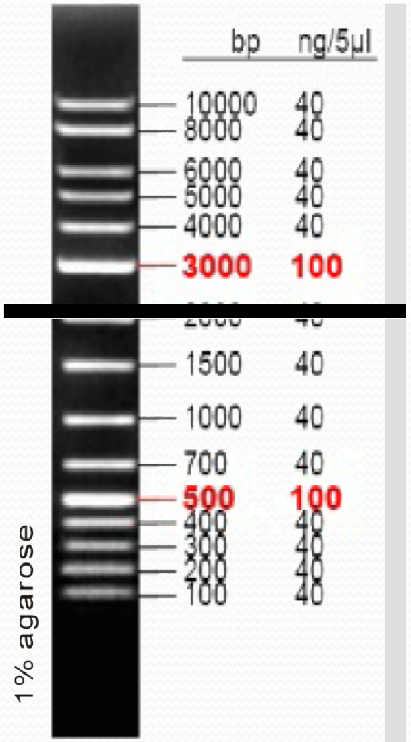
M1181/M1182



5 μl/lane, 8 cm gel
1×TAE, 7 V/cm, 45min

1kb ladder plus

M1191/M1192



5 μl/lane, 8 cm gel
1×TAE, 7 V/cm, 45min

ممنون از توجه شما