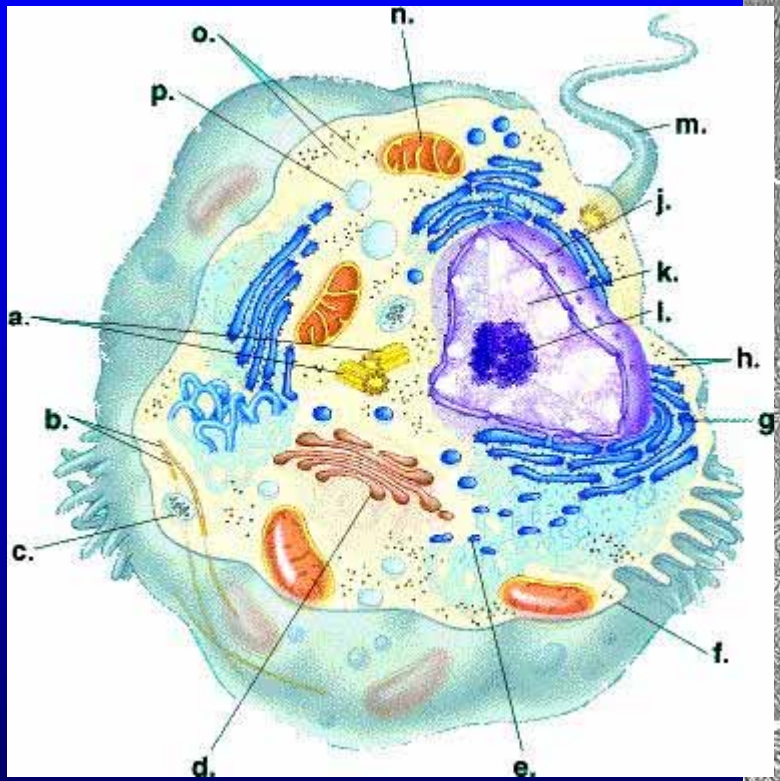
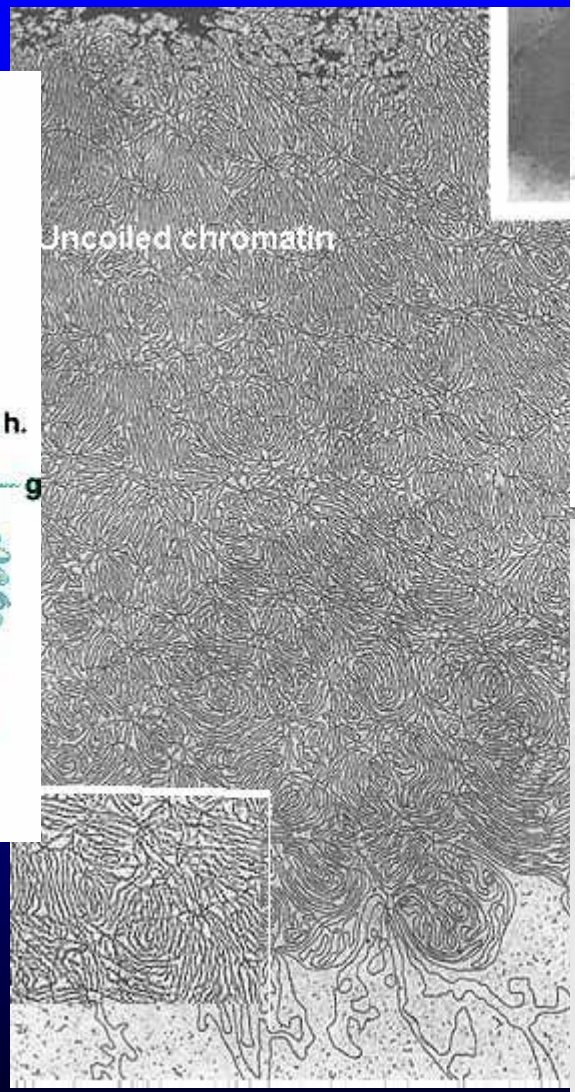
Methods of Biomaterials Testing
Lesson 3-5

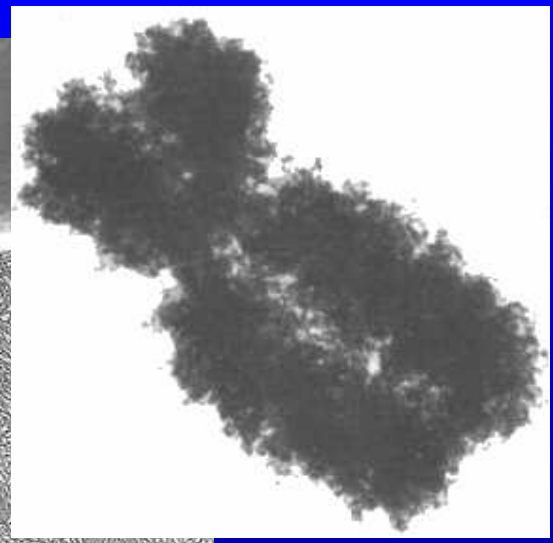
Biochemical Methods

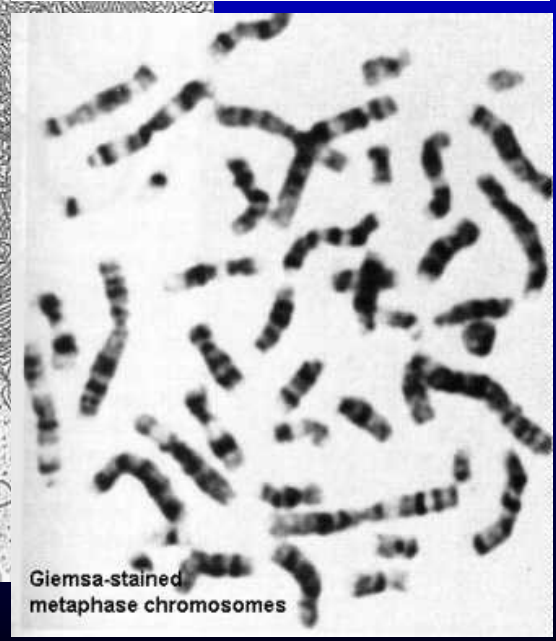
- Molecular Biology -

Chromosomes in the Cell Nucleus

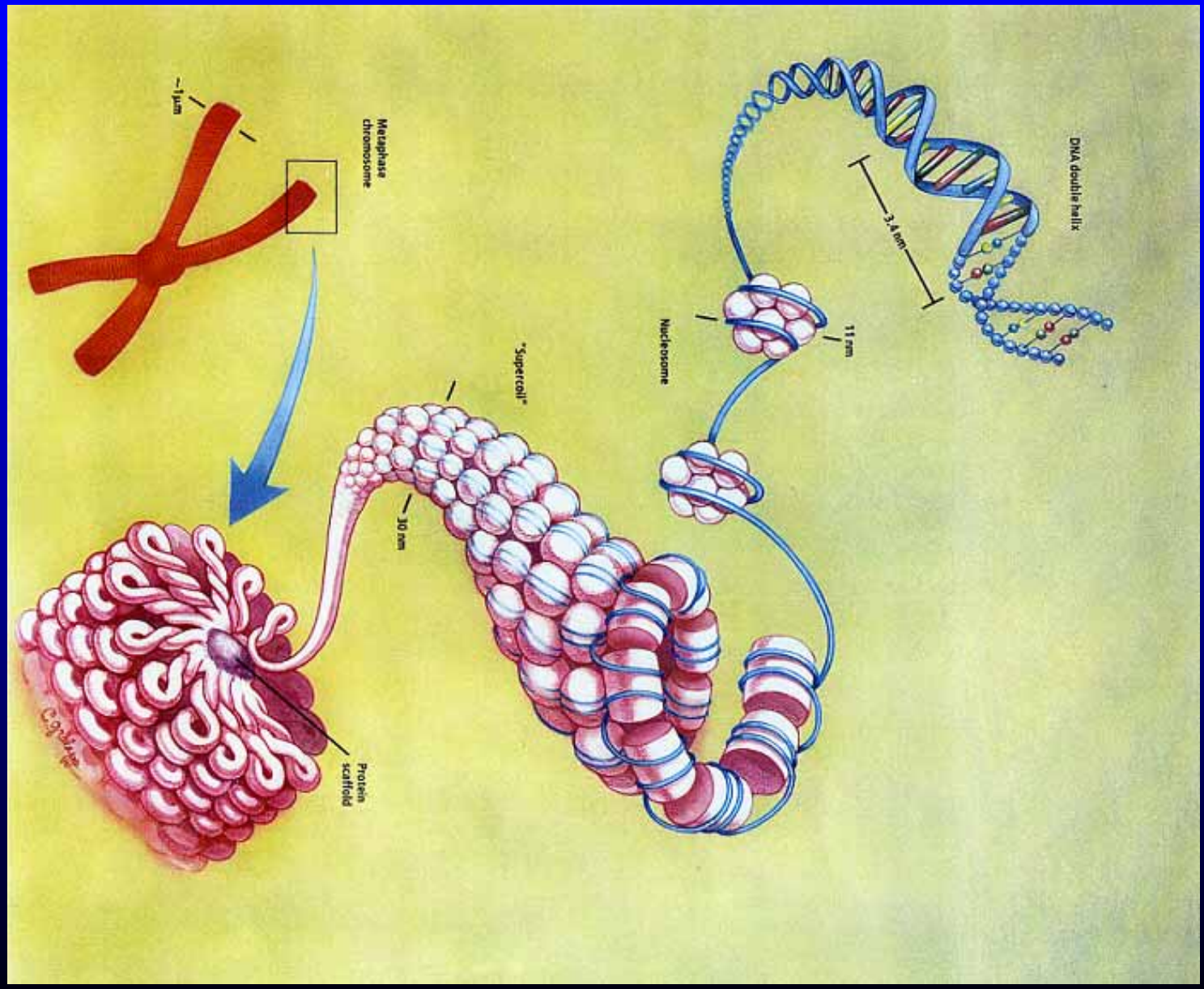




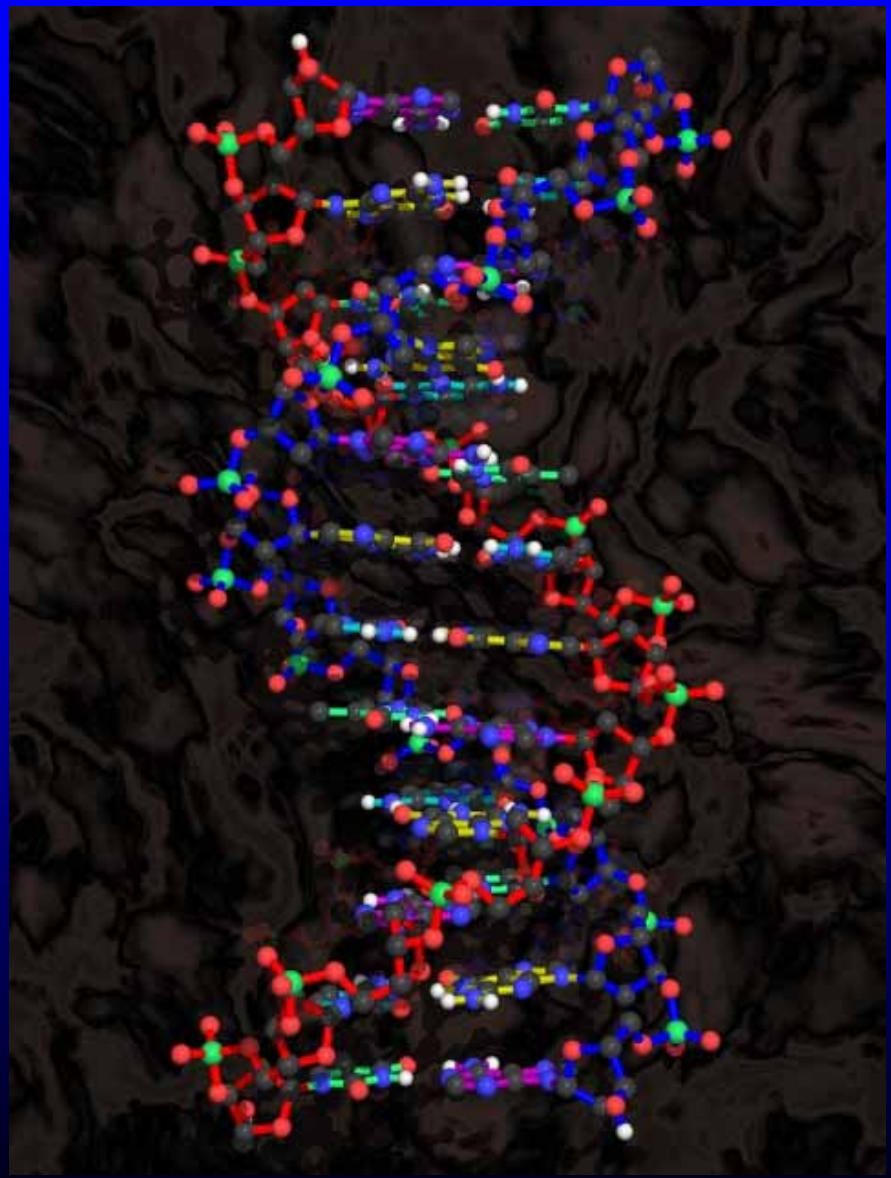


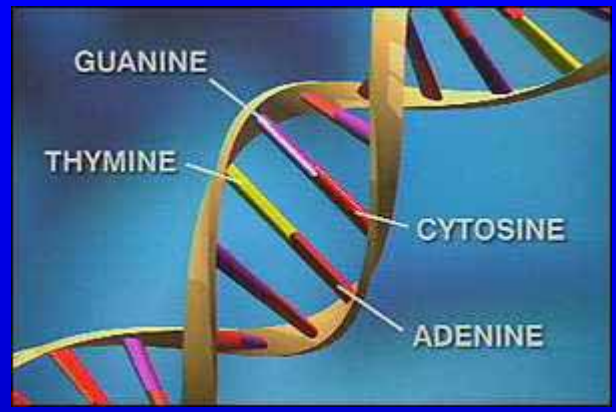


DNA in the Chromosome



Deoxyribonucleic Acid (DNA)





- DNA has double-helix structure
- The genetic information is stored in the sequence of the base-pairs
  - Adenine – Thymine
  - Guanine – Cytosine
  - Thymine – Adenine
  - Cytosine – Guanine
- Other pairings are not possible
- Pair formation by (weak) H bondings
- Each of the two columns carries the complete information, either in real or complementary

DNA Melting/ Denaturation

ATGGCTGAATCCGGTACCCTTTGAAG
TACCGACTTAGGCCATGGGAAACTTC **native**

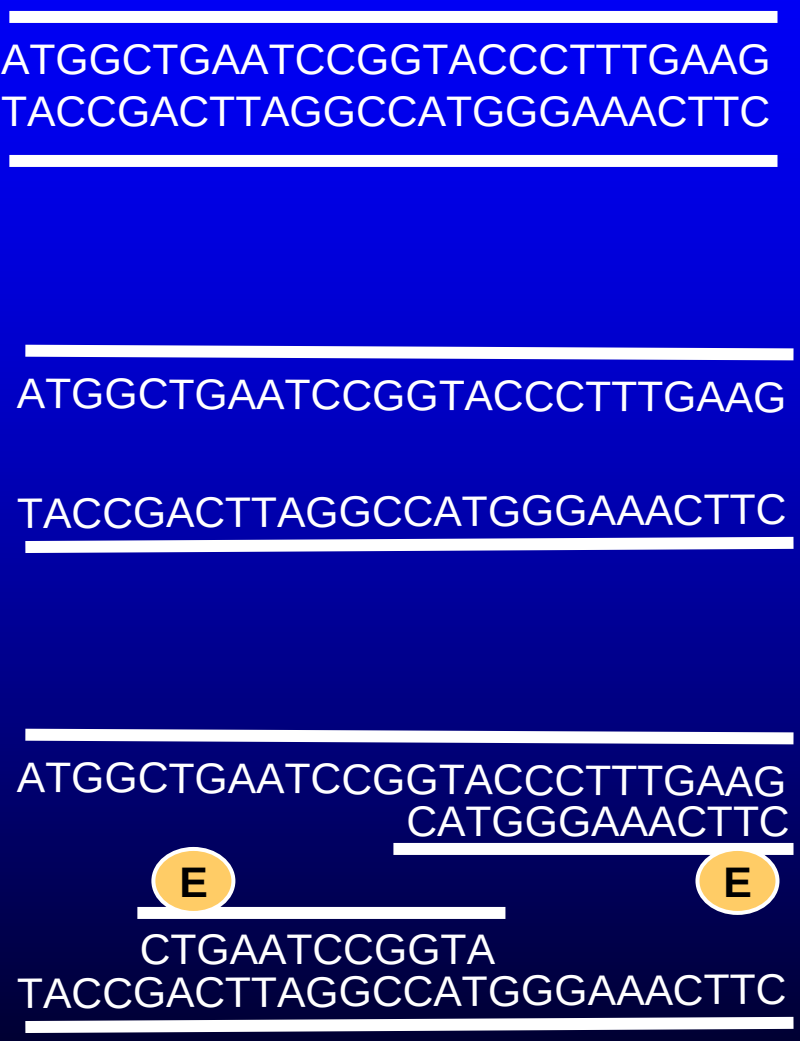
ATGGCTGAATCCGGTACCCTTTGAAG
TACCGACTTAGGCCATGGGAAACTTC **> 90°C**

ATGGCTGAATCCGGTACCCTTTGAAG **> 90°C**

TACCGACTTAGGCCATGGGAAACTTC **Single Strands**

- At high temperature (~90 - 100°C, dependent of sequence and length) the H bondings in the double helix break up and the strands separate
- The melting process is reversible at low temperature

In Situ Hybridization

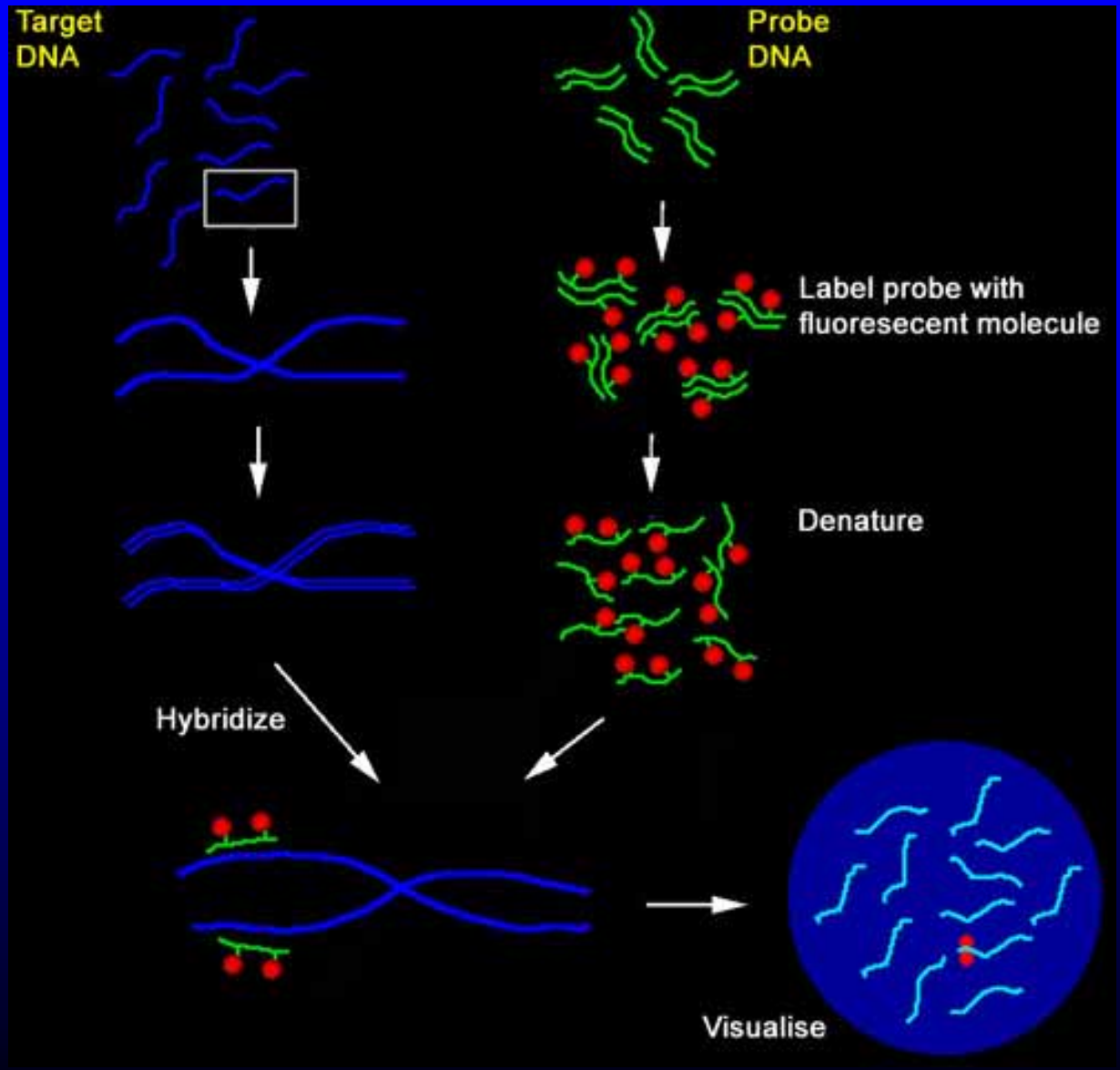


Purpose: Localization of certain, defined DNA sequences in tissue/ histological section (mainly for tumor histology, identification/ counting of chromosomes)

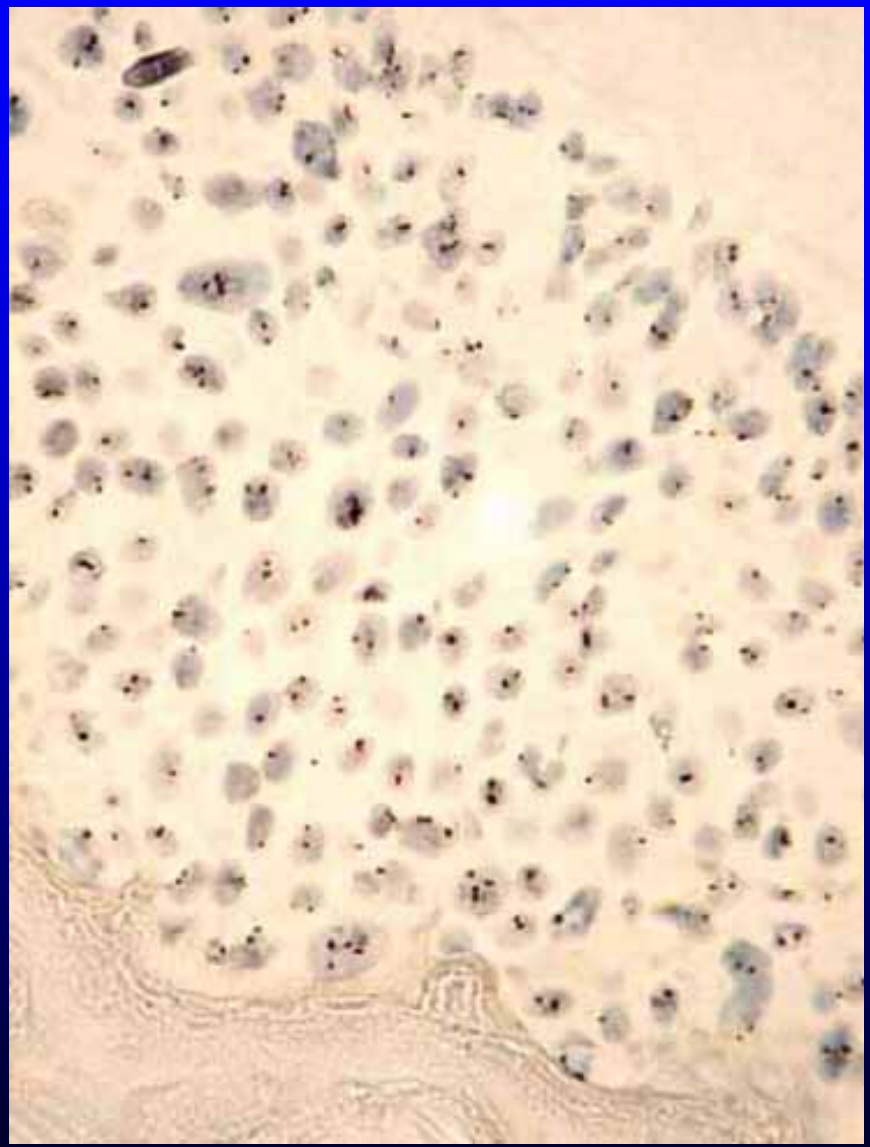
Principle:

- Melt (denature) the DNA (high temperature)
- Add labeled, specific single-strand DNA sequences
- Anneal at lower temperature (~54°C)
- Color reaction or fluorescent detection (= FISH)

In Situ Hybridization



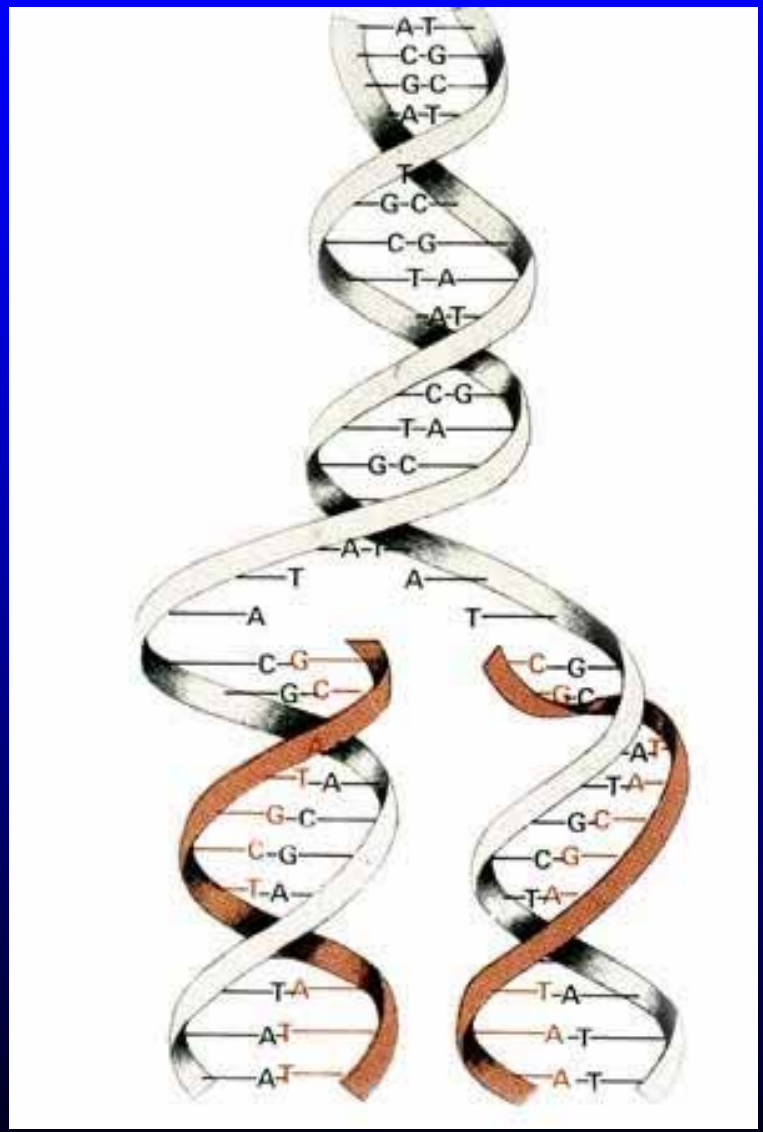
In Situ Hybridization - Example



"Alpha satellite sequences, whilst highly repetitive, are specific to each individual chromosome. These sequences flank the centromeres and can present a target measured in megabases. In this protocol a biotin or digoxigenin labelled DNA probe is detected using HRP-conjugated antibodies. The signal is visualised with diaminobenzidine (DAB). Normal, healthy nuclei show two spots. Aneusomic nuclei show 1, 3, 4 or more spots."

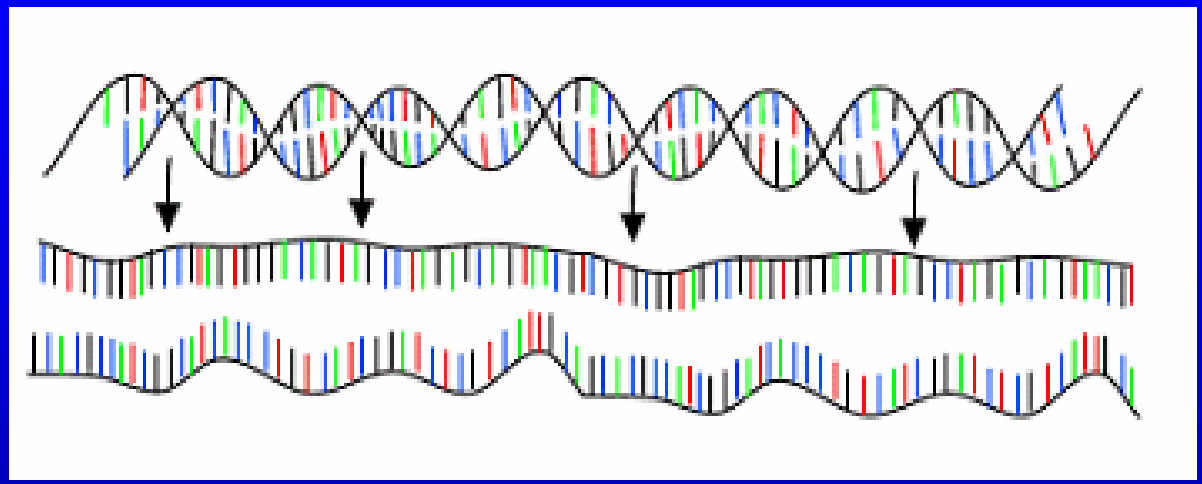
http://www.methodbook.net/probes/insitu.html

DNA Amplification for Cell Division



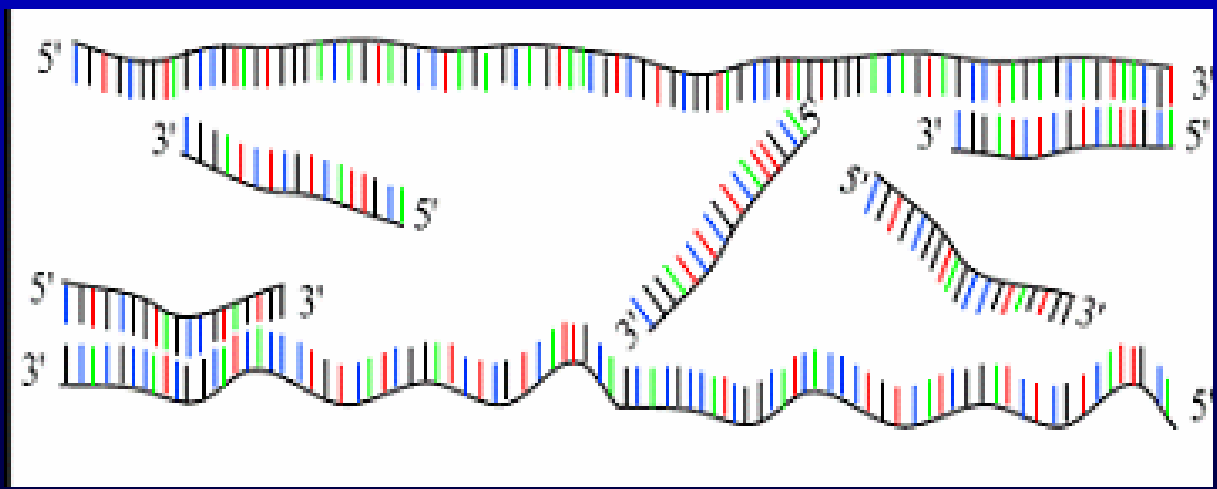
- The base pairings of the DNA split up
- The second, parallel column is formed by an enzyme (DNA polymerase) according to the defined pairings.
- Mistake in the graphic: The DNA polymerase works only in one direction, other strand/ direction is done step-wise
- DNA polymerase needs a short starting string with double-strand DNA (primer)

Polymerase Chain Reaction (I)



Step 1: Denaturation.
1min, ~94°C

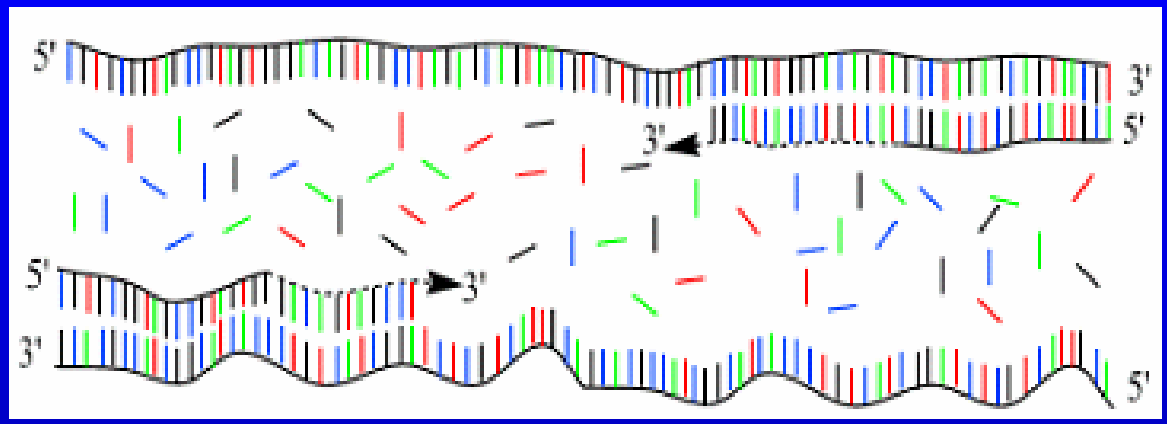
Double strand DNA splits up to two single strands



Step 2: Annealing.
1min, ~54°C

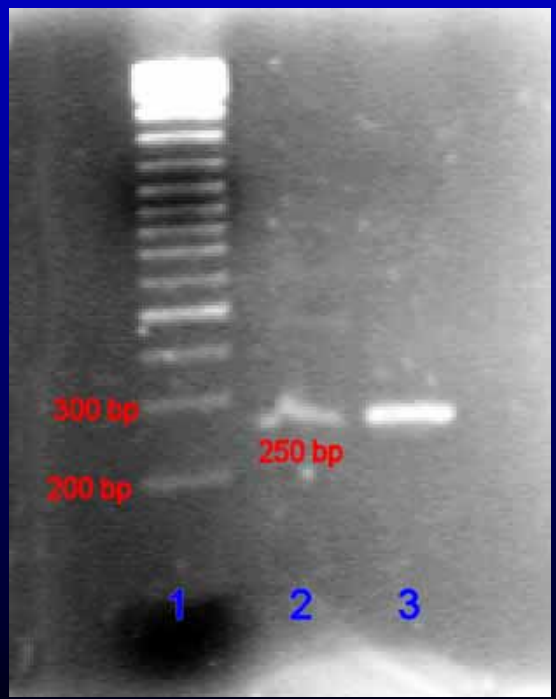
Forward and reverse primers bind to the DNA

Polymerase Chain Reaction (II)



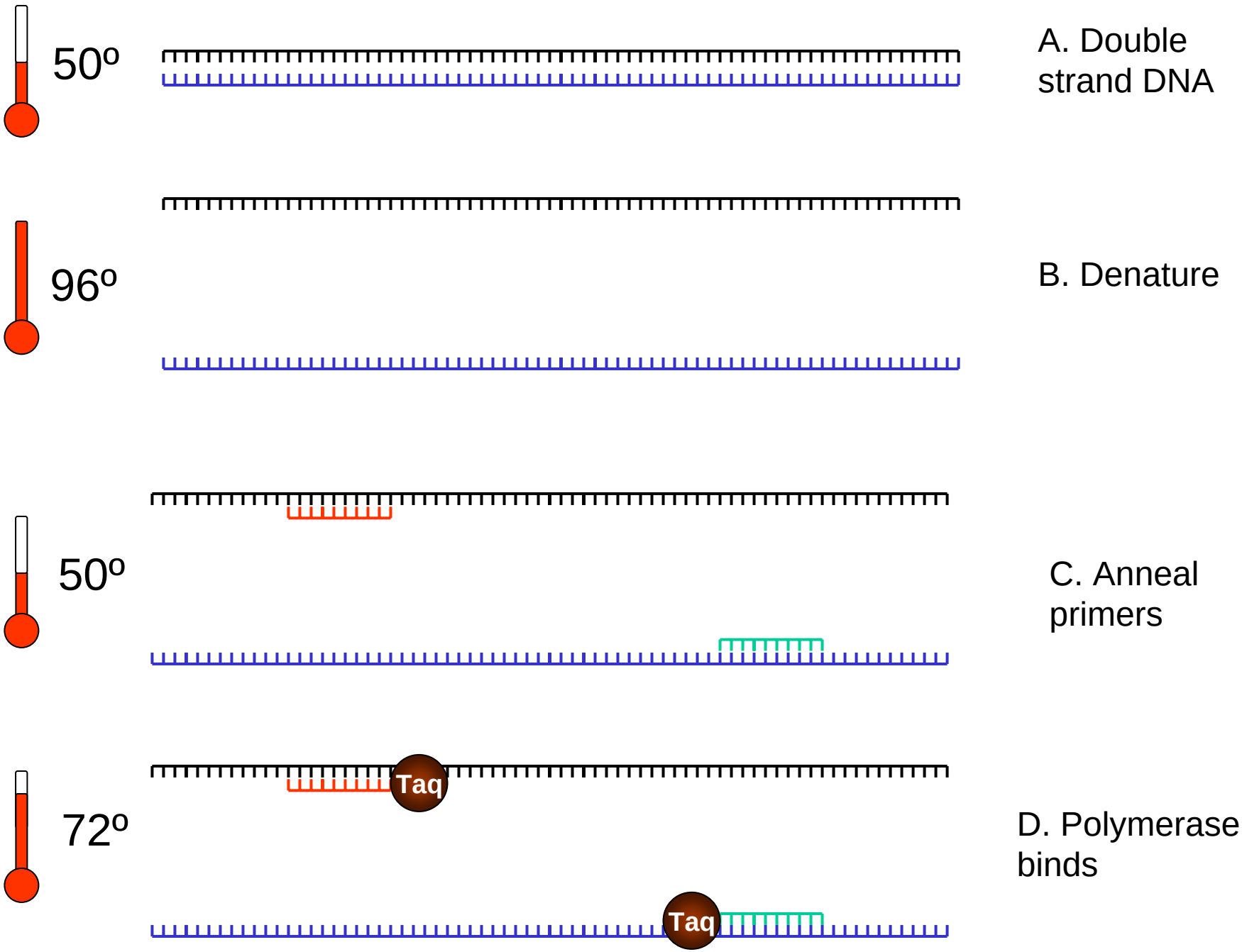
Step 3: Extension.
2min, ~72°C

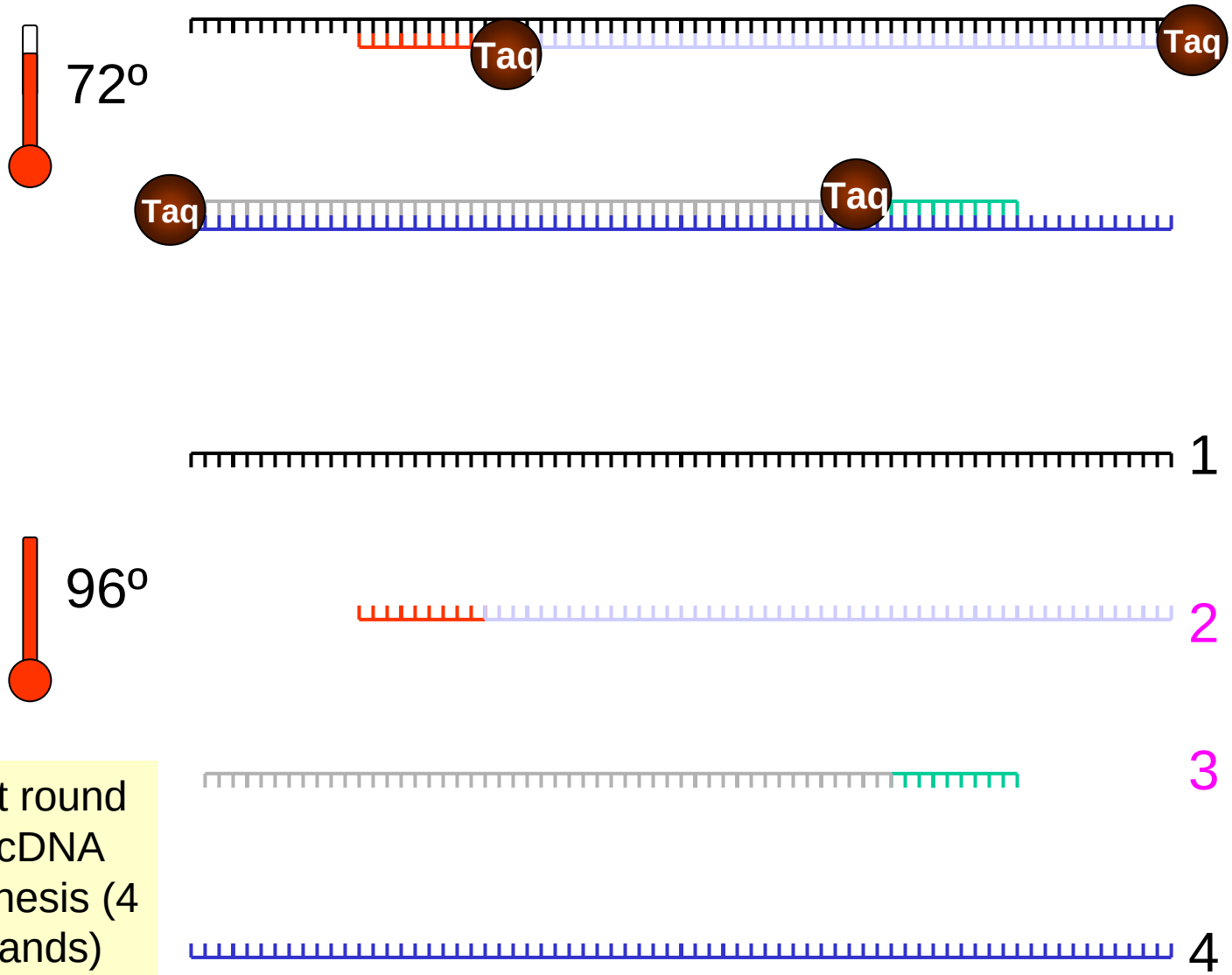
DNA Polymerase expands the primes using the dNTP's



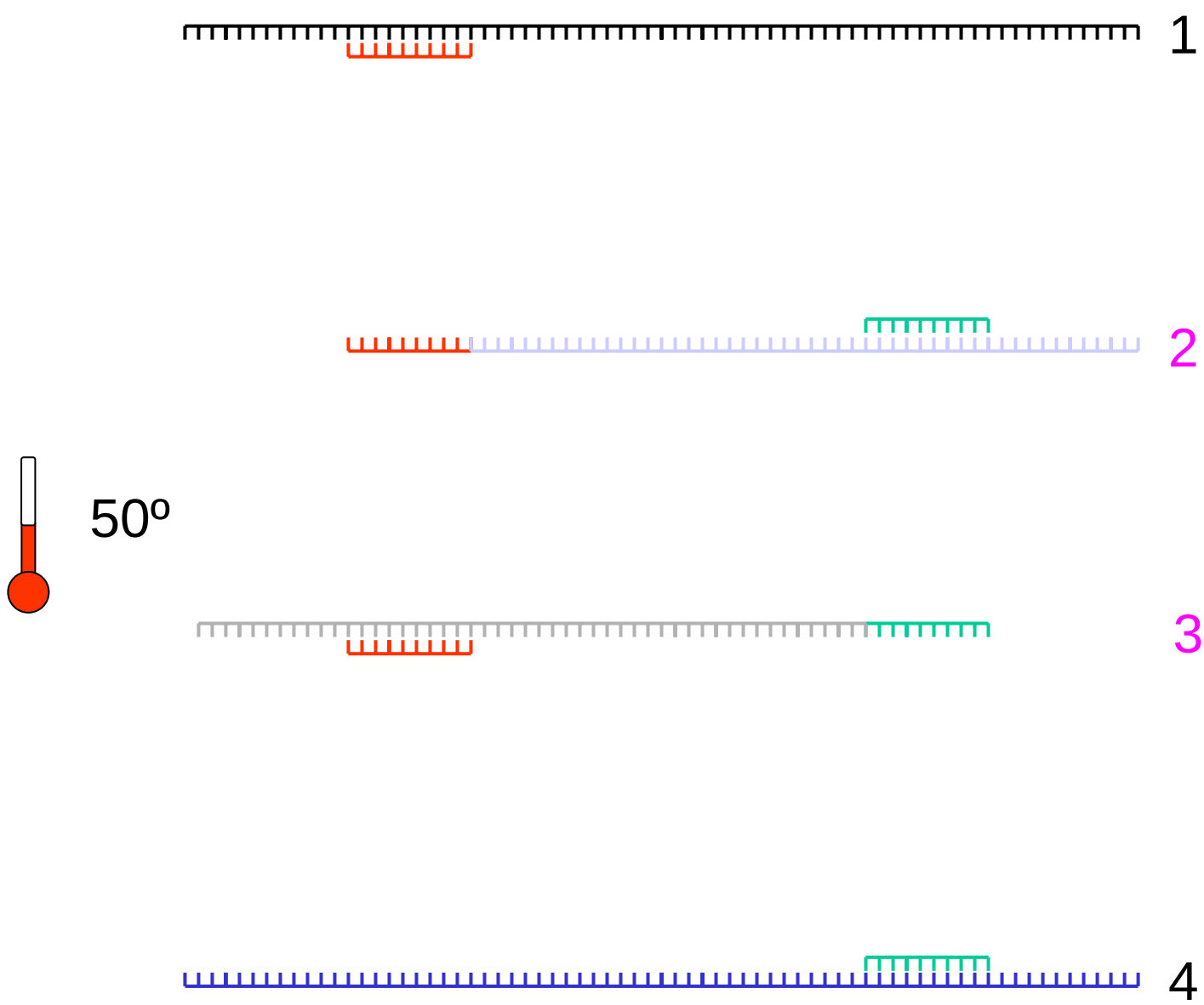
Step 4: return to step 1.
Typically 20-30 repeats

Only the sequence between the primers will amplify exponentially

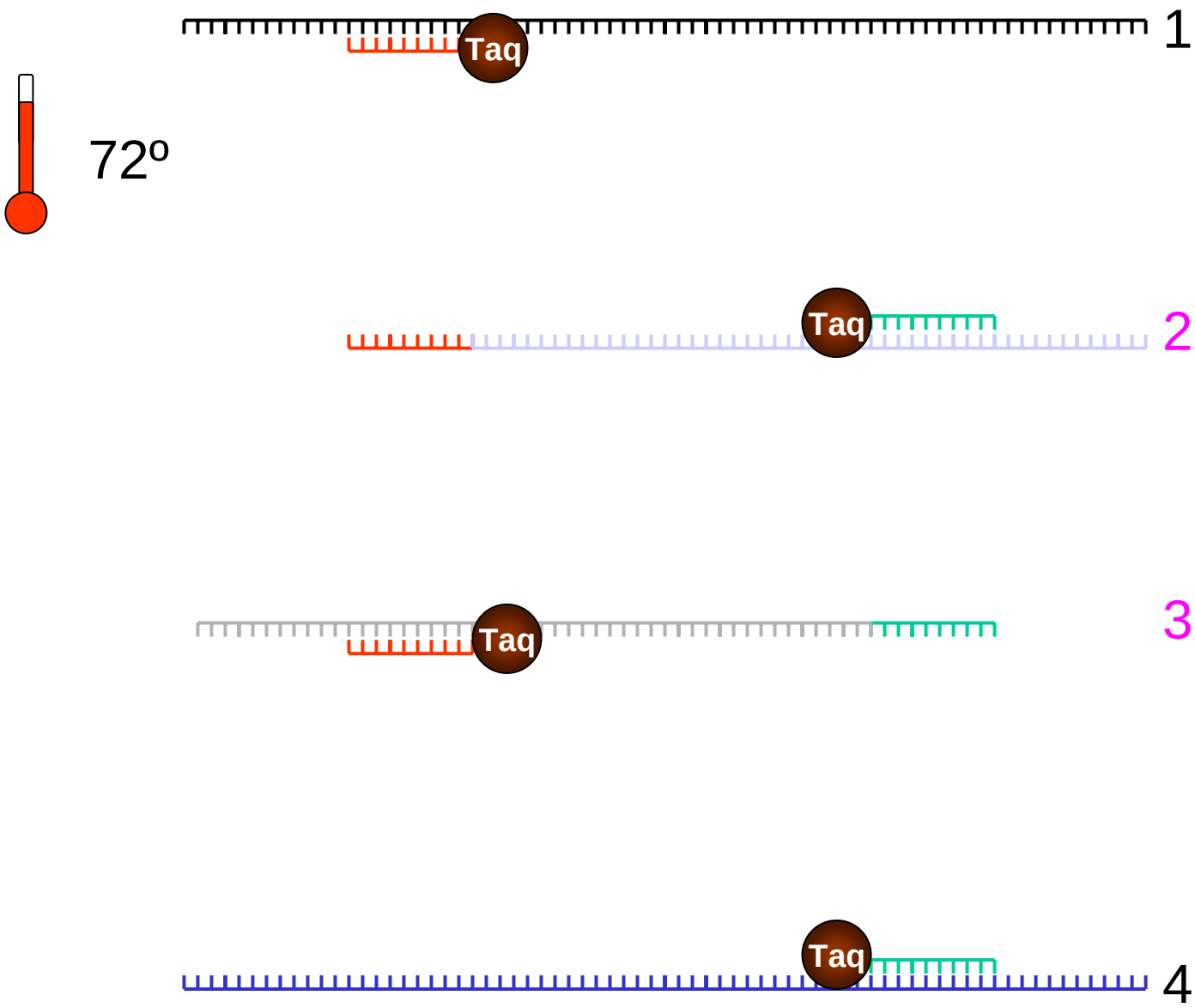

50º
A. Double strand DNA
96º
B. Denature
50º
C. Anneal primers
Taq
72º
D. Polymerase binds
Taq

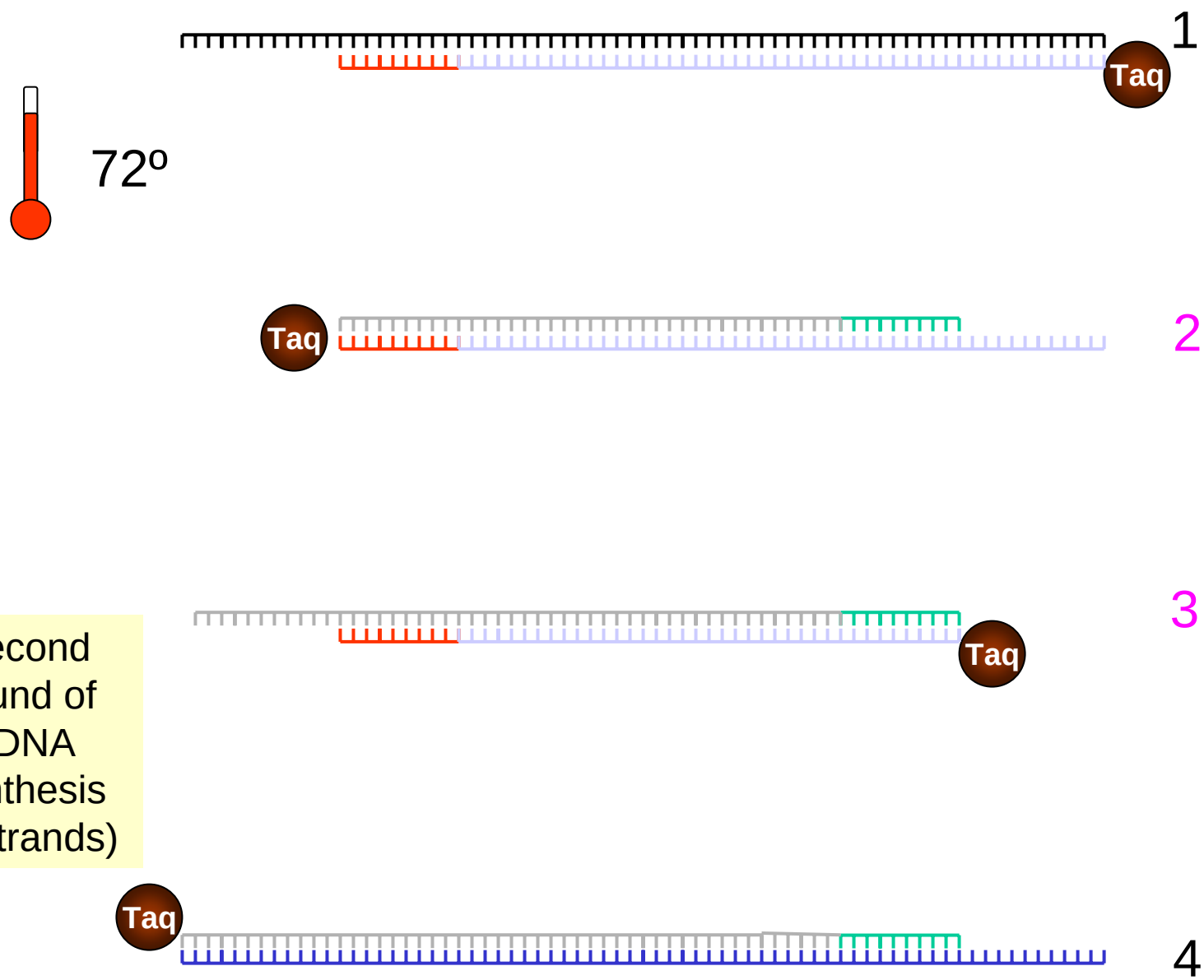


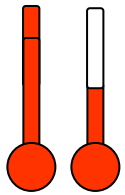
First round of cDNA synthesis (4 strands)



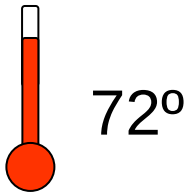
G. Anneal primers


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


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




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



72º

K. Bind polymerase (not shown) and copy strands

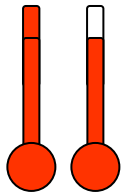
1

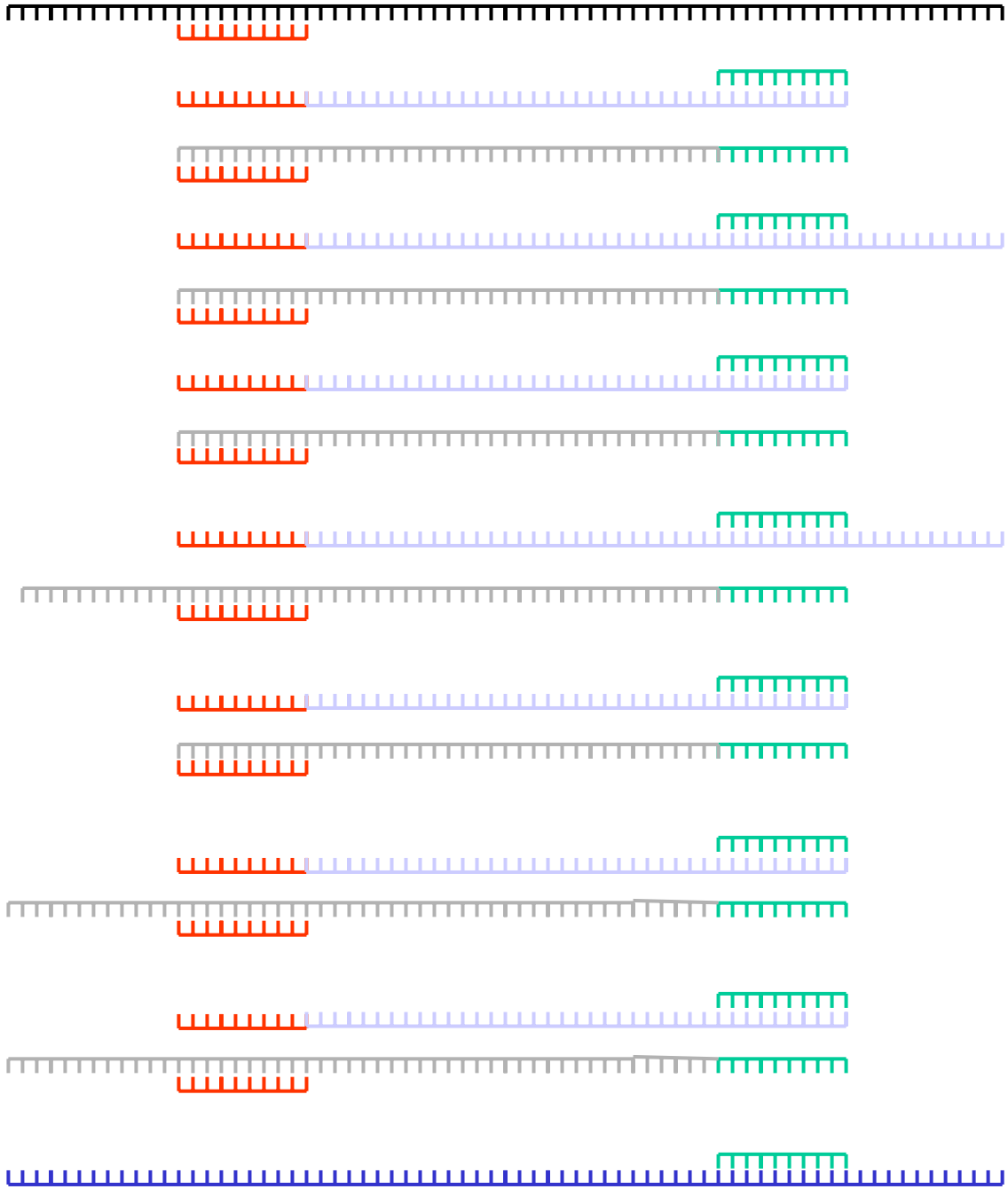
2

3

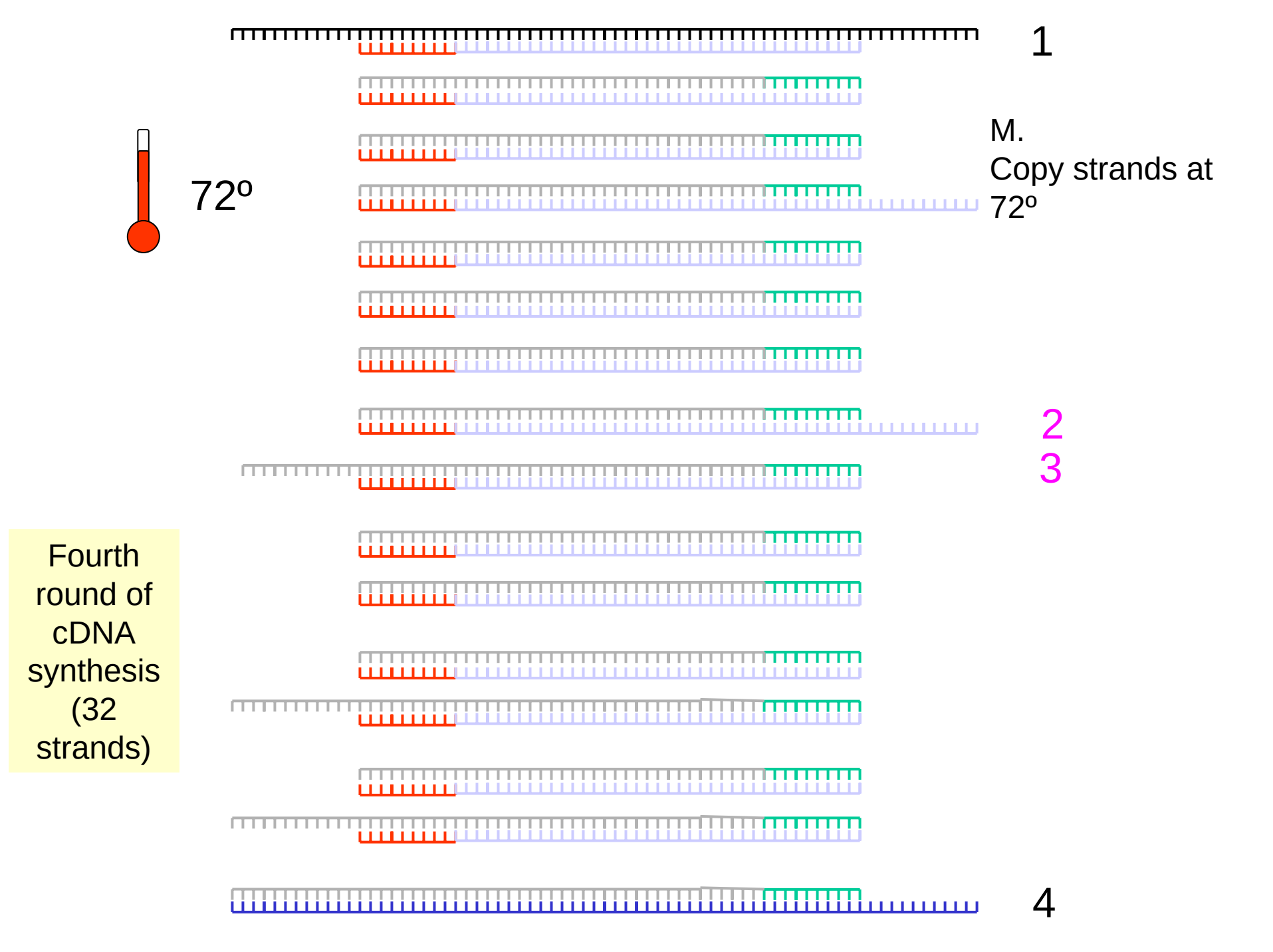
4

Third round of cDNA synthesis (16 strands)

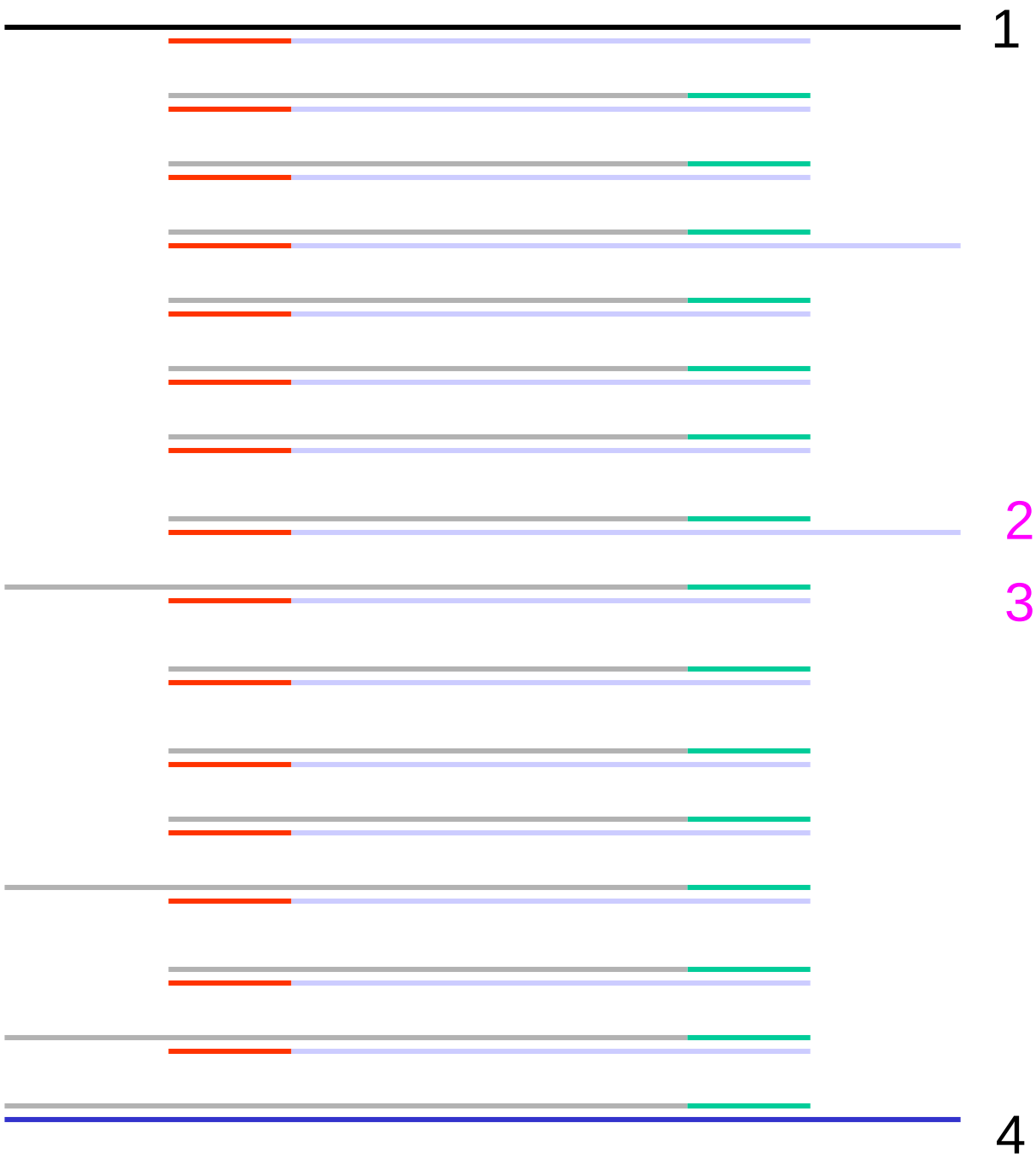




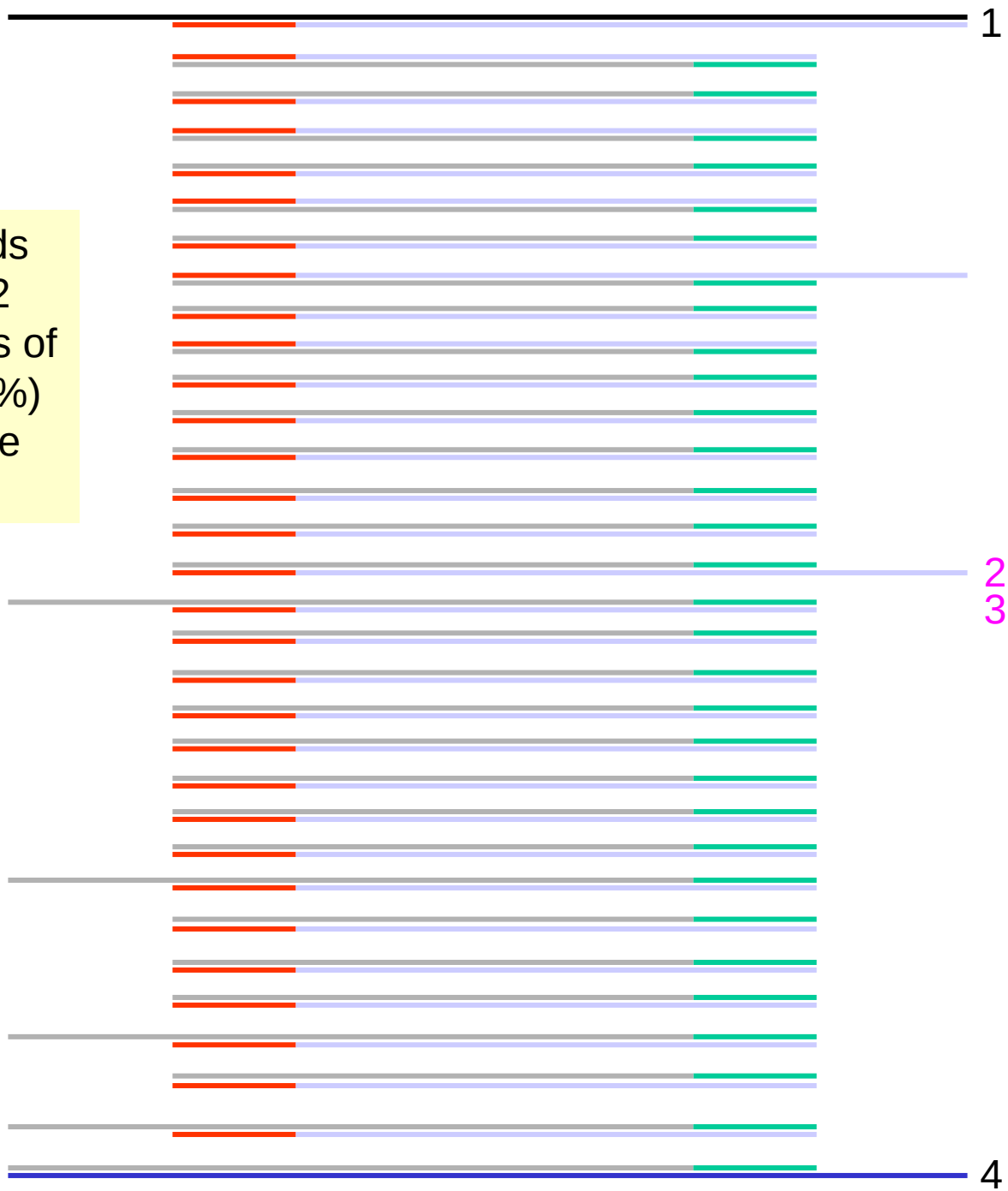
L.
Denature at 96º
Anneal primers
at 50º


1
72º
M.
Copy strands at 72º
2
3
Fourth round of cDNA synthesis (32 strands)
4

cDNA strands (32) are now shown as lines



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



Polymerase Chain Reaction (III)

- Result of PCR: Huge (theoretically 10^9 times for 30 cycles) amplification of a specific DNA sequence in the sample material
- Purpose
  - Detection: presence of the sequence (yes/no)
  - Quantification (at lower amplification)

As the human genome is completely analyzed and biomaterials do not change the DNA genome ("library"), the use of ordinary PCR in biomaterials research is very limited.

PCR Requirements

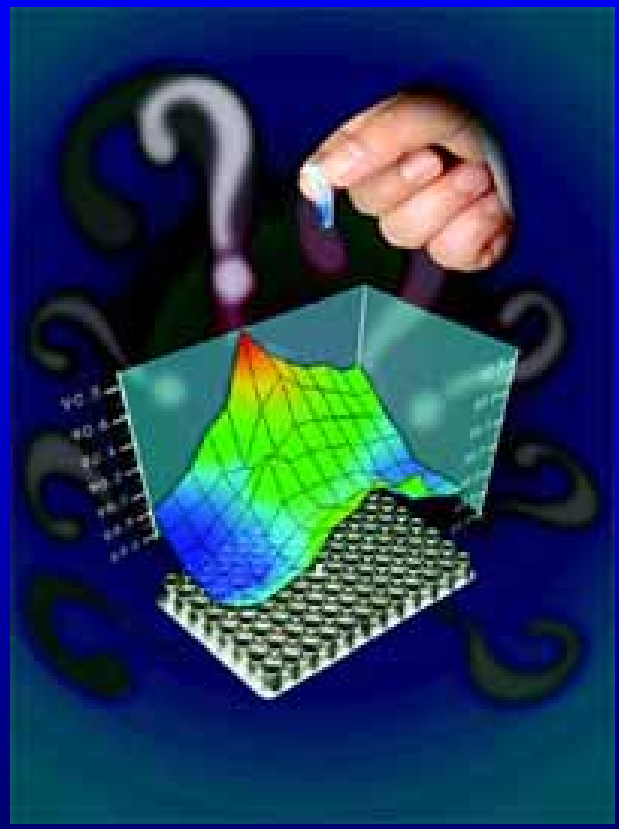
- **DNA** Sample
- DNA Polymerase (usually a heat resistant polymerase from the thermophil bcterium *thermus aquaticus*: Taq-polymerase)
- Primer-pair (sense and anti-sense). Length ~20 bases each; sequence of interest in between (typically 200-1000 basepairs)
- Single bases ("DNA-monomers"): dNTP's: dATP, dTTP, dGTP, dCTP

Total reagent mix: typically 10-50µl

- Required equipment: Thermocycler ("PCR-machine") for fast and controlled changes of the temperature

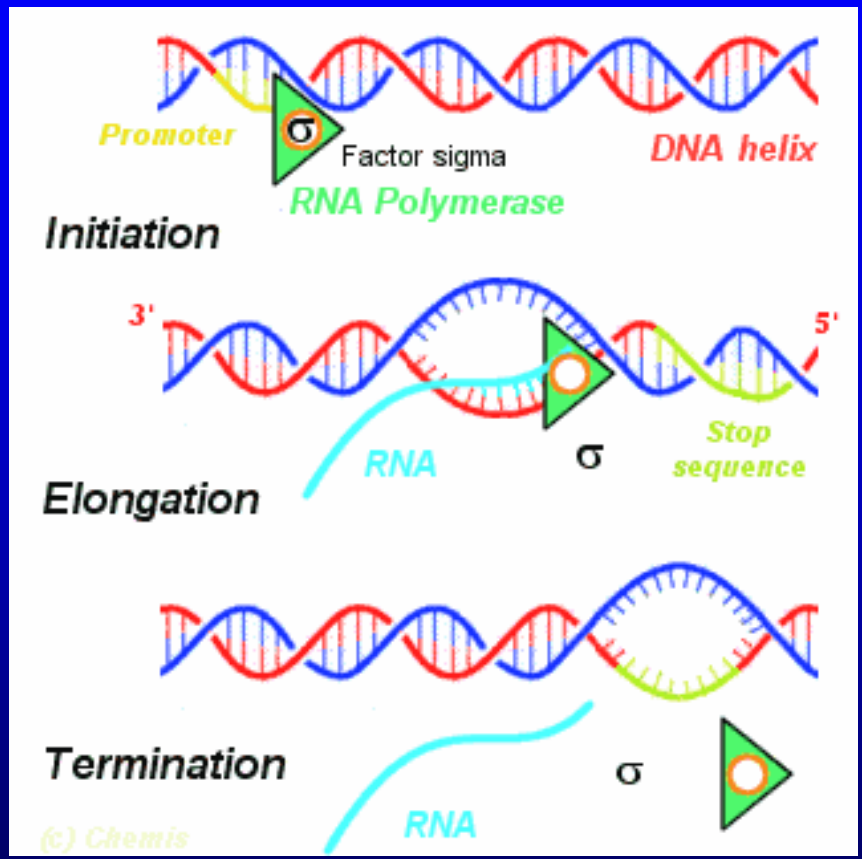
Thermocycler

…what it looks like



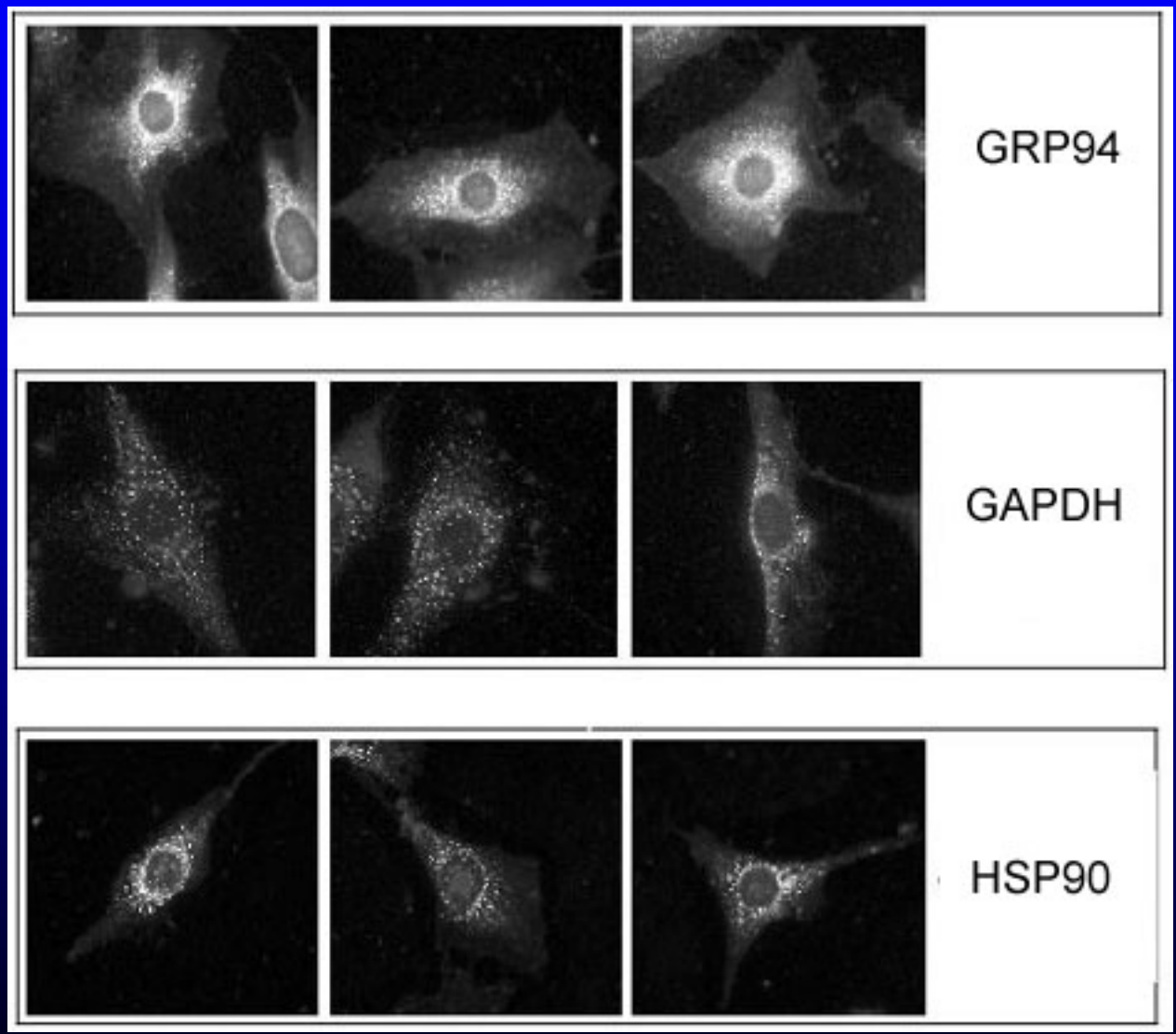
Temperature constance over the whole plate is important

RNA Transcription



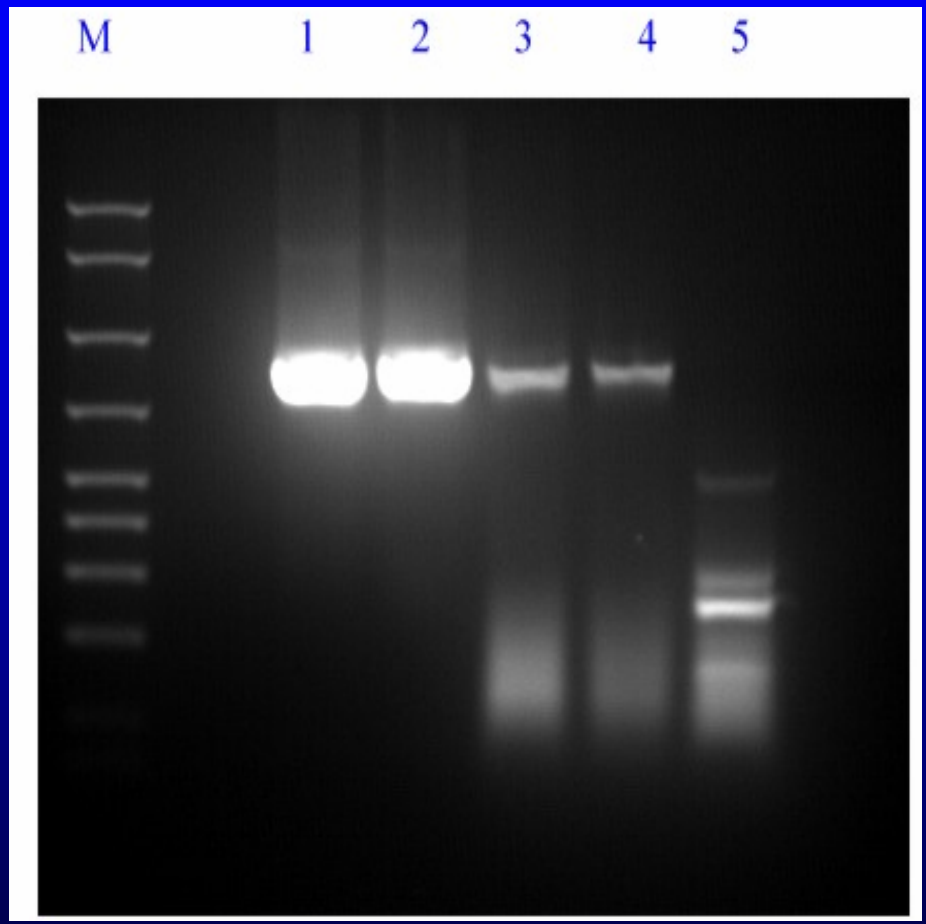
- Transcription is the way of making “working copies” of the genes coded in the DNA
- The RNA (“working copy”) can leave the cell nucleus and go freely into the cell (messenger RNA, mRNA)
- RNA is single stranded and has very similar chemistry as DNA
- RNA is degraded very quickly by RNase

RNA *in situ* Hybridization



- Also the RNA associates with complementary strands
- This can be used for *in situ* hybridization
- As mRNA is the "working copy" for making proteins, this gives information, where a specific protein is formed.
- Requirements at the samples material (degradation of the RNA) are very high

Reverse Transcription PCR (rt-PCR)



- Step 0: Extract the RNA
- Step 1: Degrade residual DNA
- Step 2: Use RNA is transcribed to DNA by the enzyme “reverse transcriptase”
- Step 3… PCR as usual

Reverse Transcription PCR (rt-PCR) (II)

Purpose

- Amplification and **quantification** of mRNA (semi-quantitatively)
- Cells need the RNA to produce proteins. The amount of protein produced is roughly proportional to the amount of mRNA
- It is cheaper and simpler to produce DNA-Primers than producing antibody (pairs) for ELISA quantification of the protein
- The sequence of all (common) genes in human and (common) animals is published and available for free at: http://www.ncbi.nlm.nih.gov

Pitfalls with rt-PCR

Improper Performance of the Method

- Fast degradation of the RNA
- Improper DNA degradation → Amplification of genomic DNA
- Wrong primer selection (Introns in the Gene)

Principle Problem

- Between the detection limit and saturation effect of PCR are only few (~5) cycles → problems with quantification

Improper interpretation of the results

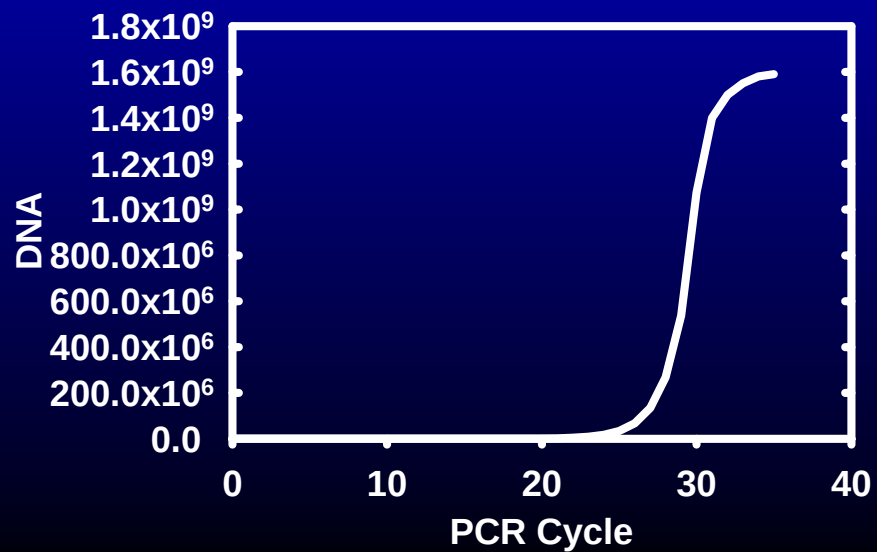
- mRNA degradation is not constant
- Degradation of the product protein is not constant → Conclusion from mRNA to protein concentration is critical (especially comparison between different types of proteins or one protein in different cell types)
- Protein may be produced in an inactive form or or only for storage → amount of mRNA says nothing about the protein activity

Cycle	DNA
0	1
1	2
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1024
11	2048
12	4096
13	8192
14	16384
15	32768
16	65536
17	131072
18	262144
19	524288
20	1048576
21	2097152
22	4194304
23	8388608
24	16777216
25	33554432
26	67108864
27	134217728
28	268435456
29	536870912
30	1073741824
31	1400000000
32	1500000000
33	1550000000
34	1580000000
35	1590000000

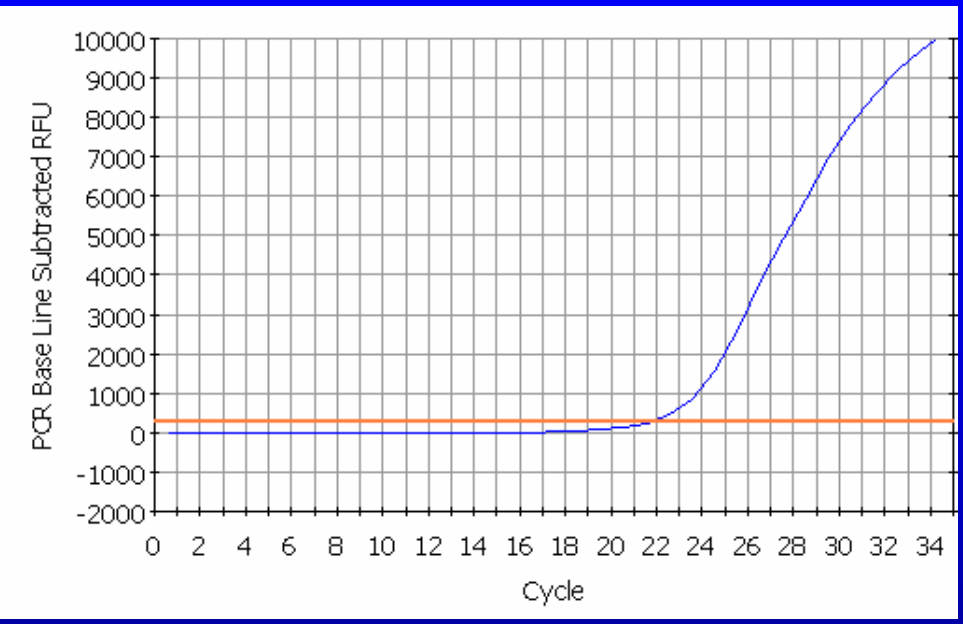
Real Time PCR

Normal (rt-)PCR is not very quantitative

- Low distance between detection limit and saturation effect
- Non-linearity of the detection method

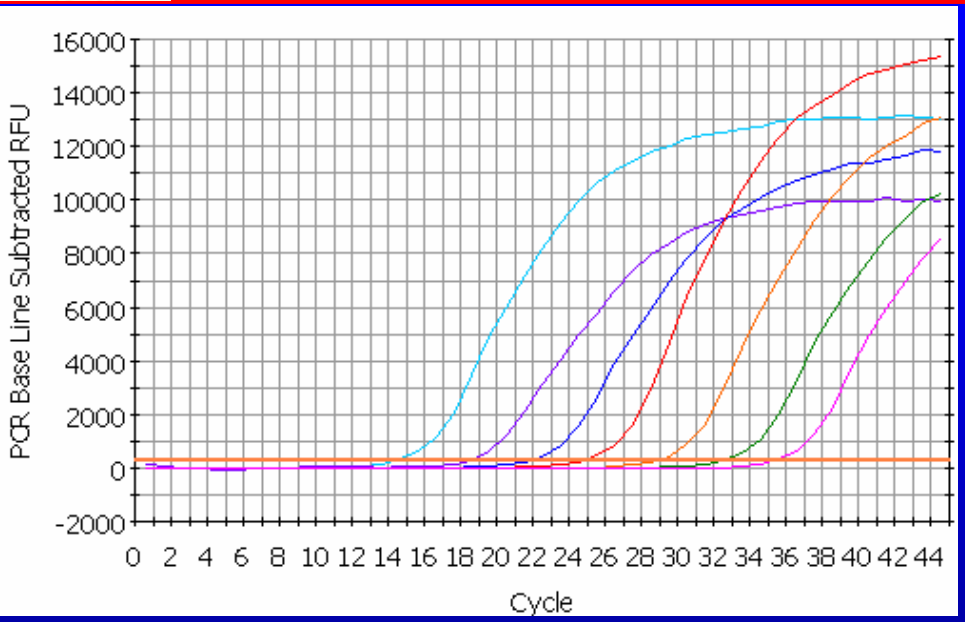


Real Time PCR



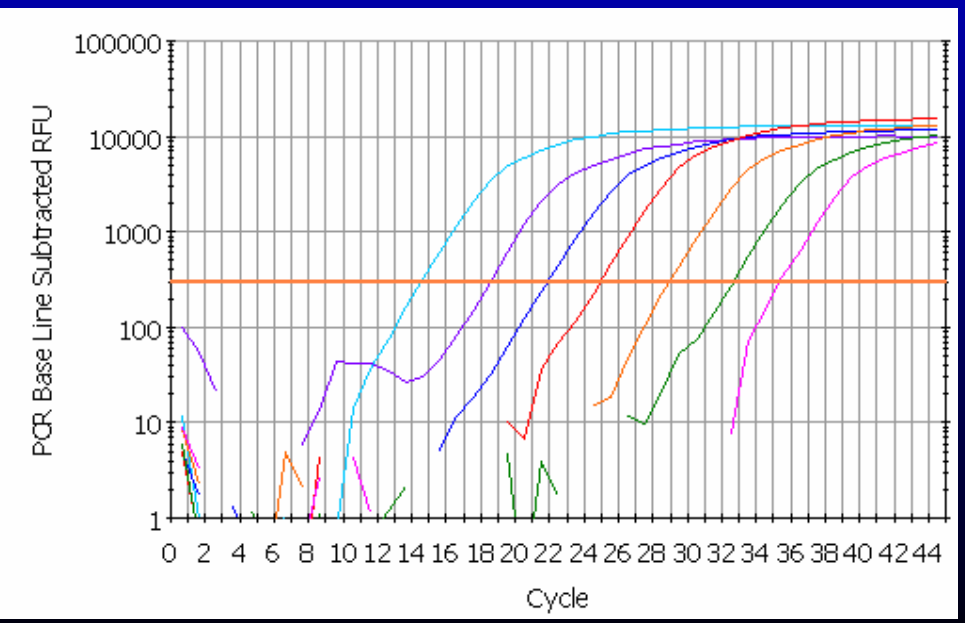
- Sybr Green (and some other dyes) show no/ very low fluorescence as free dye, but very high fluorescence when bound to double strand DNA
- Detection of the fluorescence in real time during the PCR

Real Time PCR



Real time PCR of a series of 10-times dilutions of a DNA sample

Linear plot of the fluorescence



Log. plot of the fluorescence

Microarray

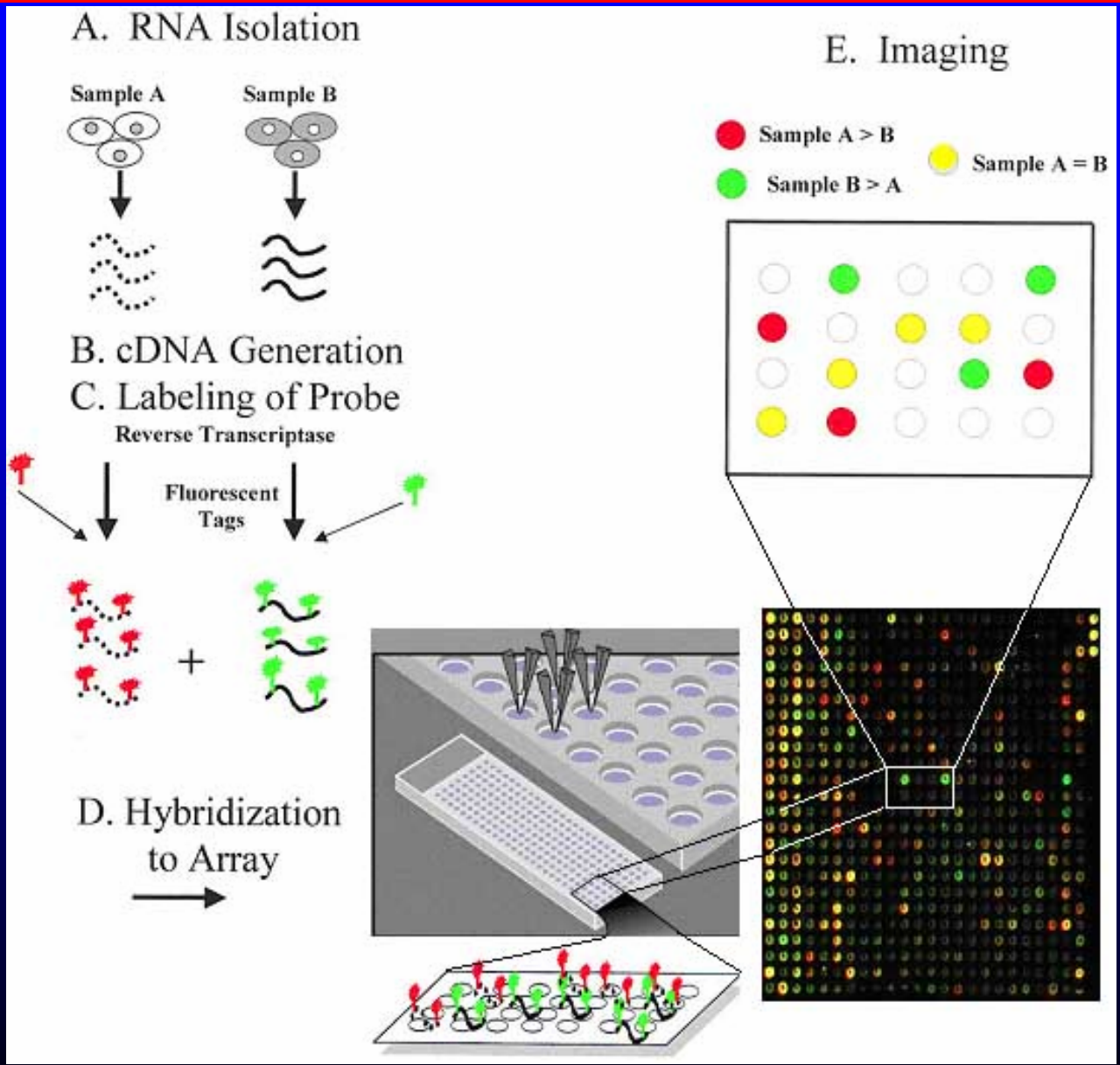
Purpose

- Detection and (semiquantitative) quantification of very many genes (whole genome) on RNA level

Principle

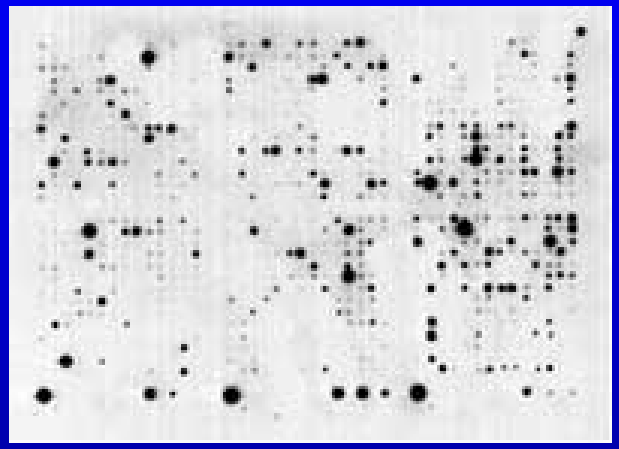
- Characteristic DNA sequences (single strand) of the genes of interest are put in small spots on a microscope slide (commercially provided)
- RNA is extracted from the sample(s)
- Reverse transcription to cDNA
- cDNA is labeled with a a fluorescent dye
- Hybridisation with the DNA on the slide
- Optical reading of the fluorescence

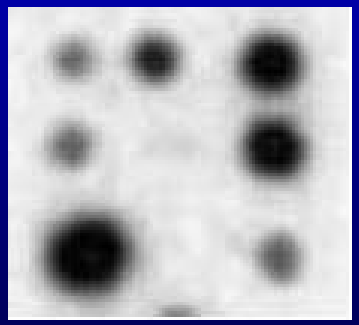
Microarray


A. RNA Isolation
Sample A
Sample B
B. cDNA Generation
C. Labeling of Probe
Reverse Transcriptase
Fluorescent Tags
+
D. Hybridization to Array
E. Imaging
Sample A > B
Sample B > A
Sample A = B

Microarray – What it looks like

Filter (Macro-Array)

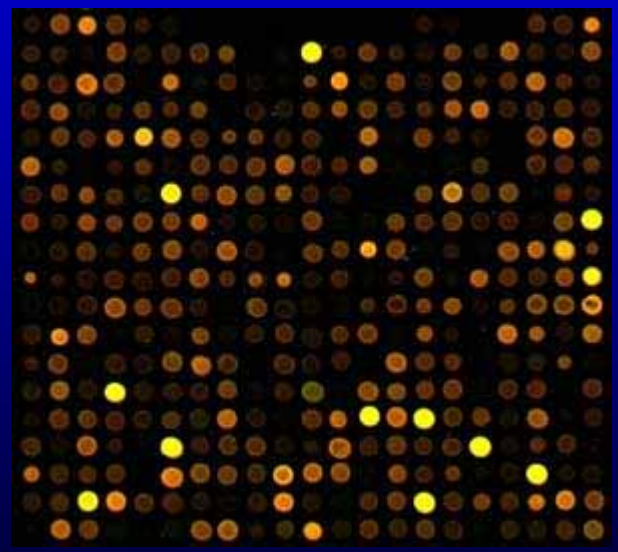




2.400 Sequences per array

Radioactive labelling

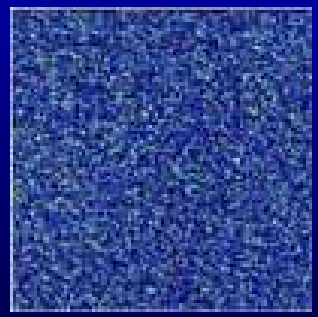
Glas Slide (Micro Array)



10.000 Sequences per array

Fluorescent labelling

Oligonucleotide Chip



30.000 Sequences per chip

Fluorescent labelling