Techniques used in the diagnosis of viruses and phytoplasmas

ELISA tests, DNA isolation, PCR and its variants, RFLP analysis, agarose gel electrophoresis,

DNA sequencing

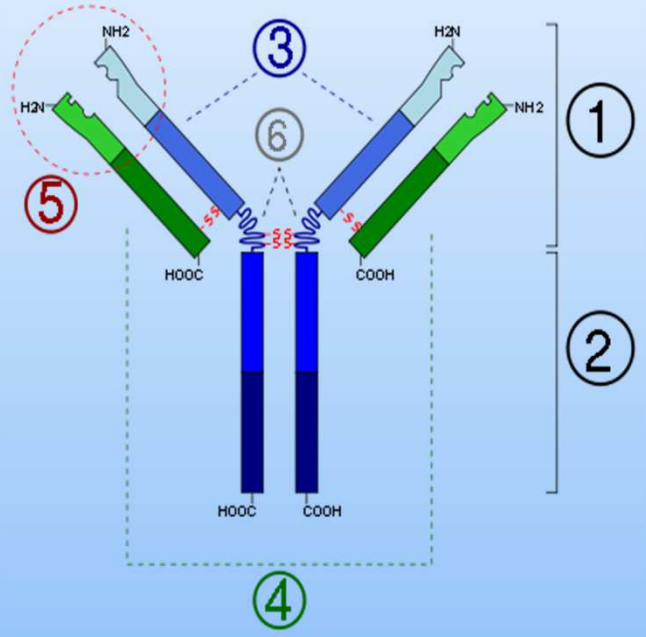
Sarajevo 7-11 th September 2015 r.

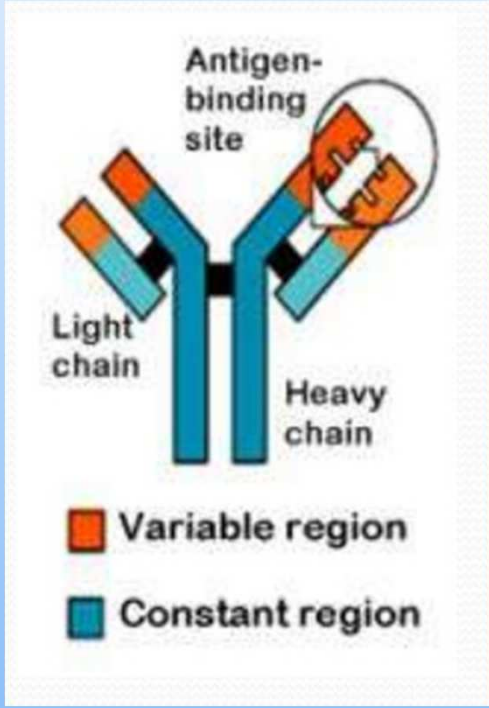
ELISA tests

ELISA – serological basics

- ELISA (Enzyme-Linked ImmunoSorbent Assay) or enzyme immunoassay (EIA)- serological method used to detect molecules like peptides, proteins, immunoglobins or hormons.
- highly specyfic interaction **antigen-antibody**
- Used in routin virological tests

Antygen - a scheme of structure

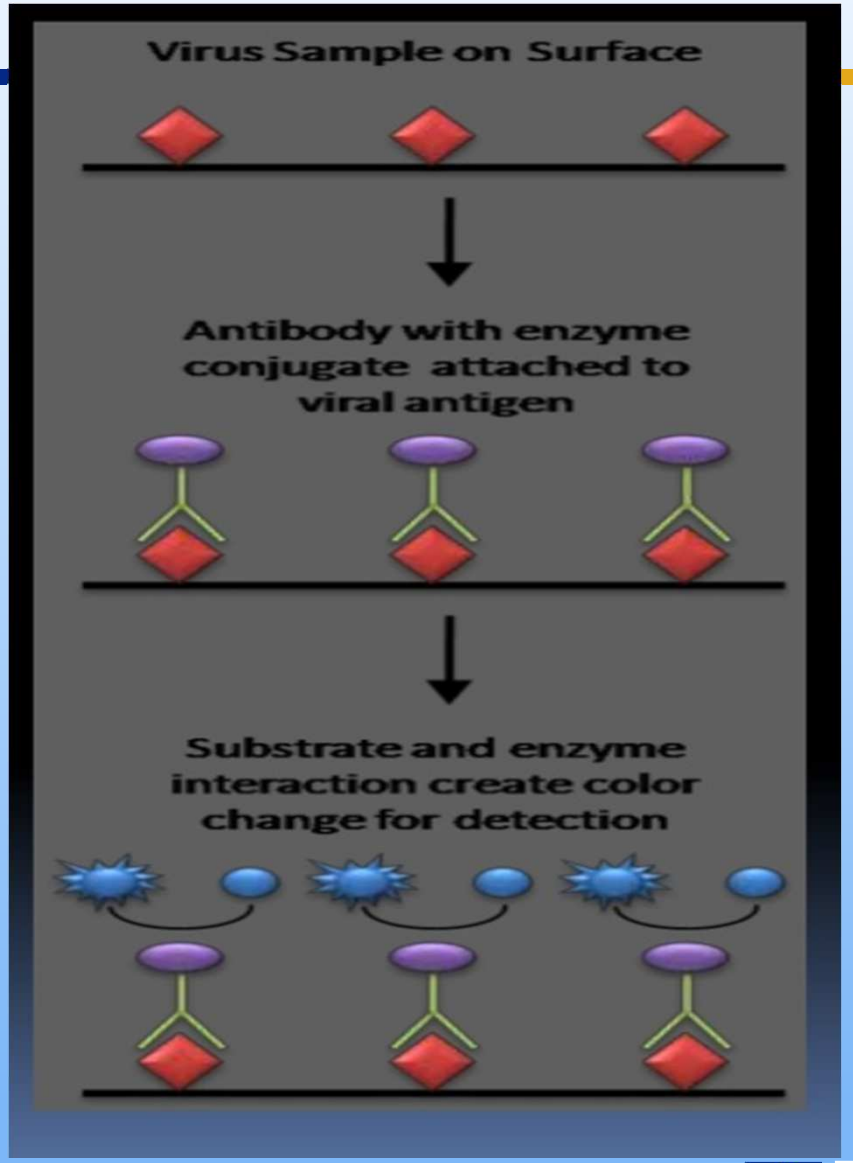




A scheme of structure of immunoglobulins:
1. Fab fragment
2. Fc fragment
3. Heavy chain (contains VH, CH1, hinge, CH2 and CH3 regions: starting from N-end)
4. Light chain (contains VL and CL regions: starting from N-end)
5. Antigen binding site
6. Hinge regions
(*) -S-S- disulfide bridges

Selected variants of ELISA assay

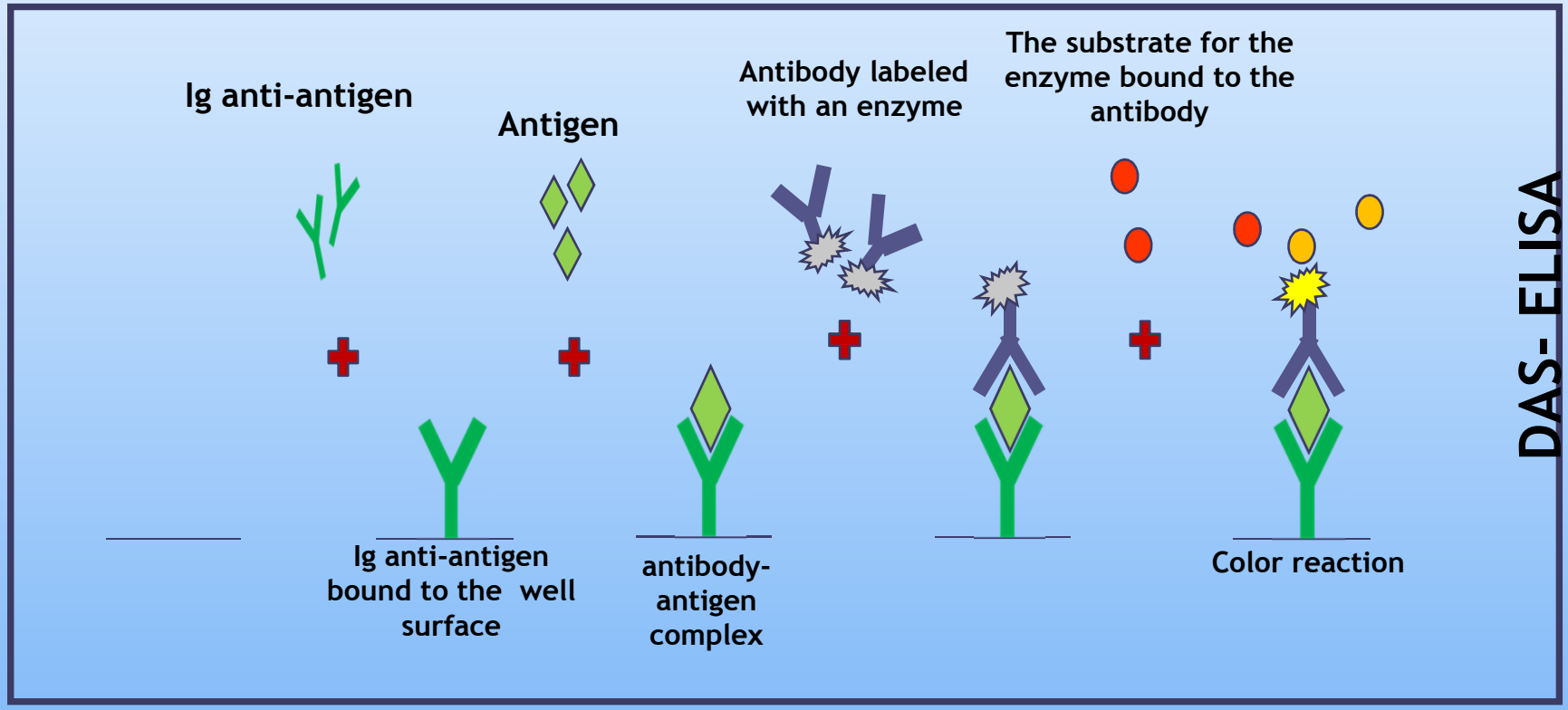
Direct ELISA



http://www.slideshare.net

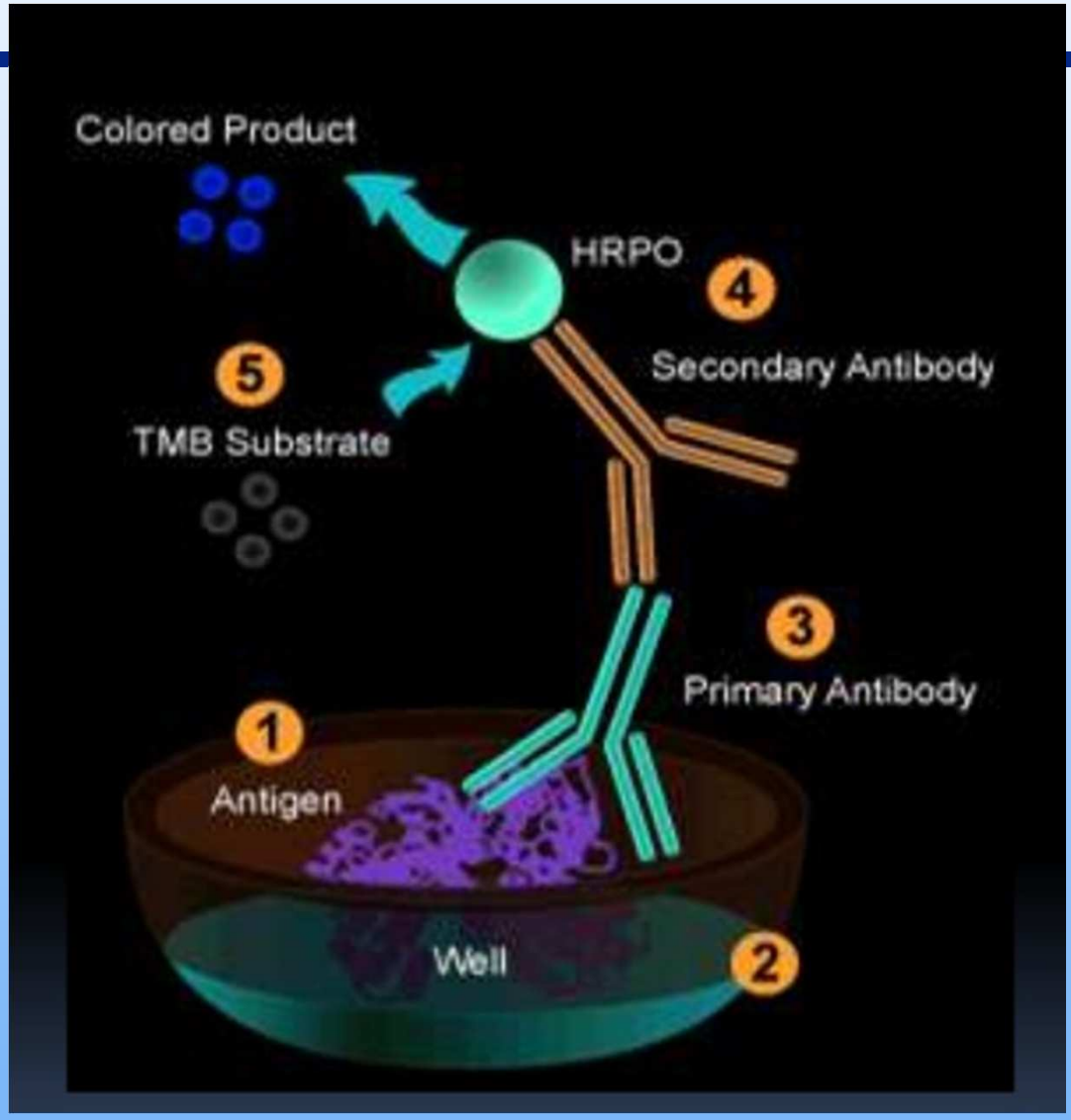
Selected variants of ELISA assay

DAS-ELISA (direct Double Antibody Sandwich ELISA)



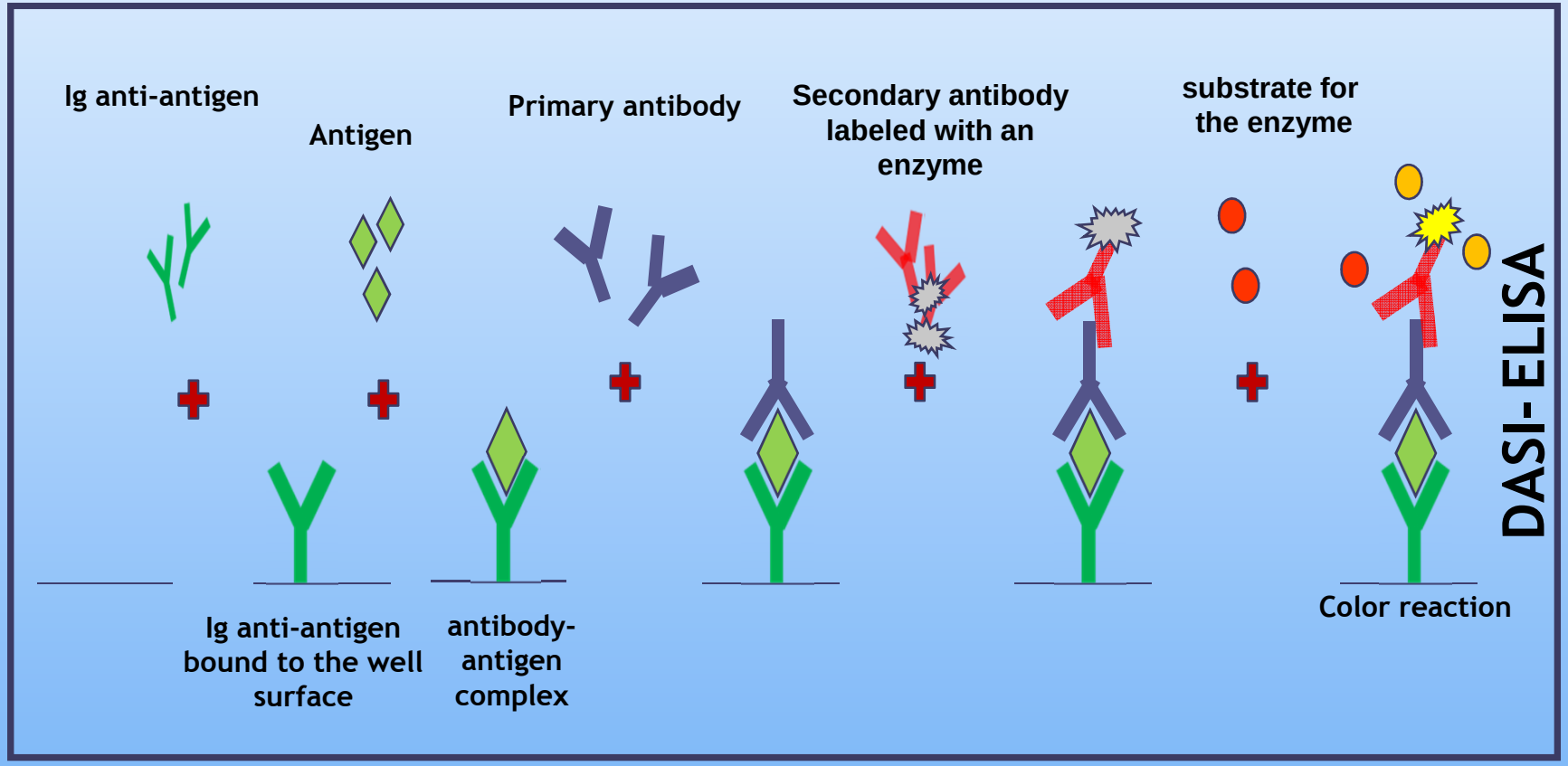
Selected variants of ELISA assay

Indirect ELISA

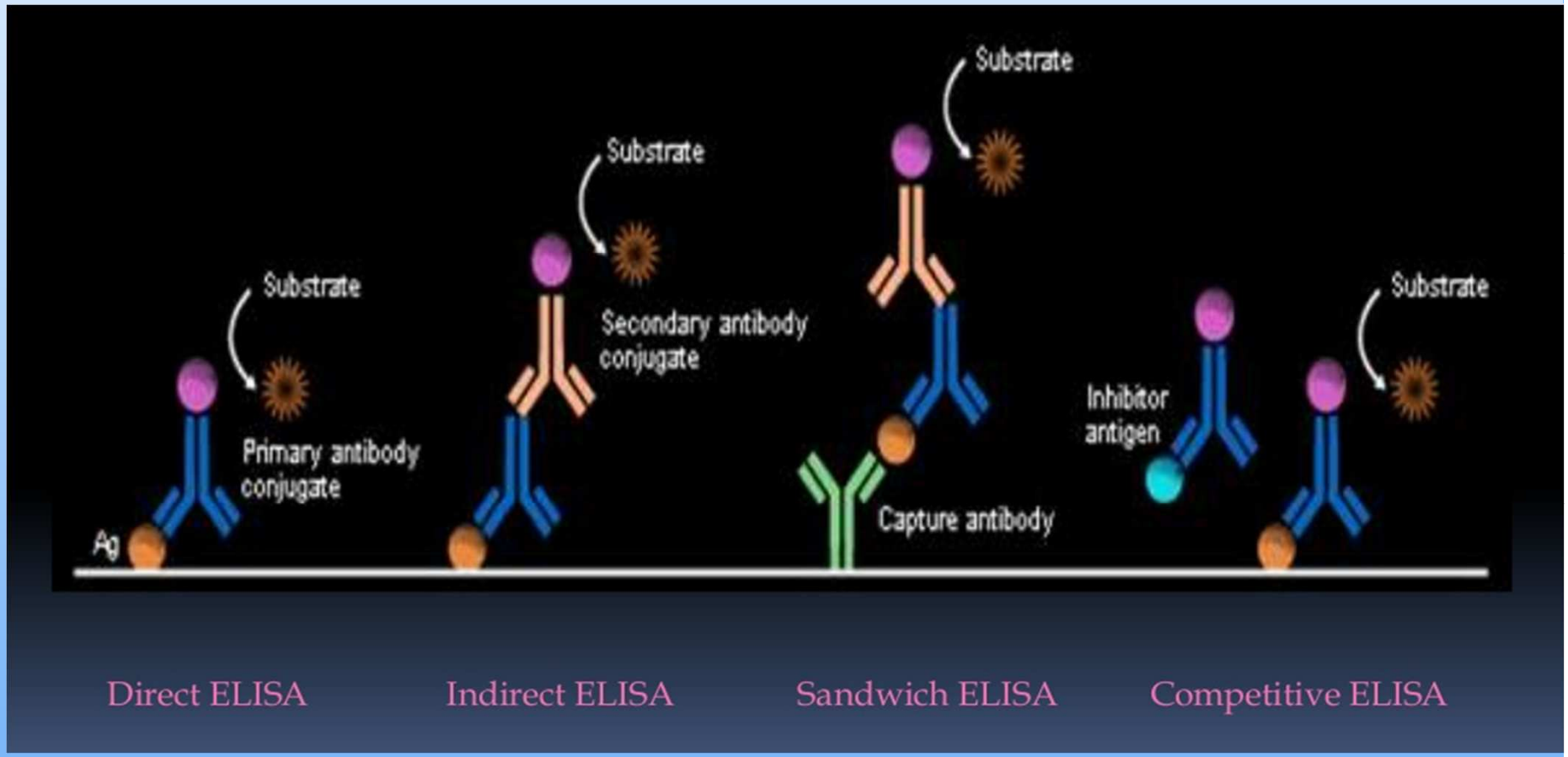


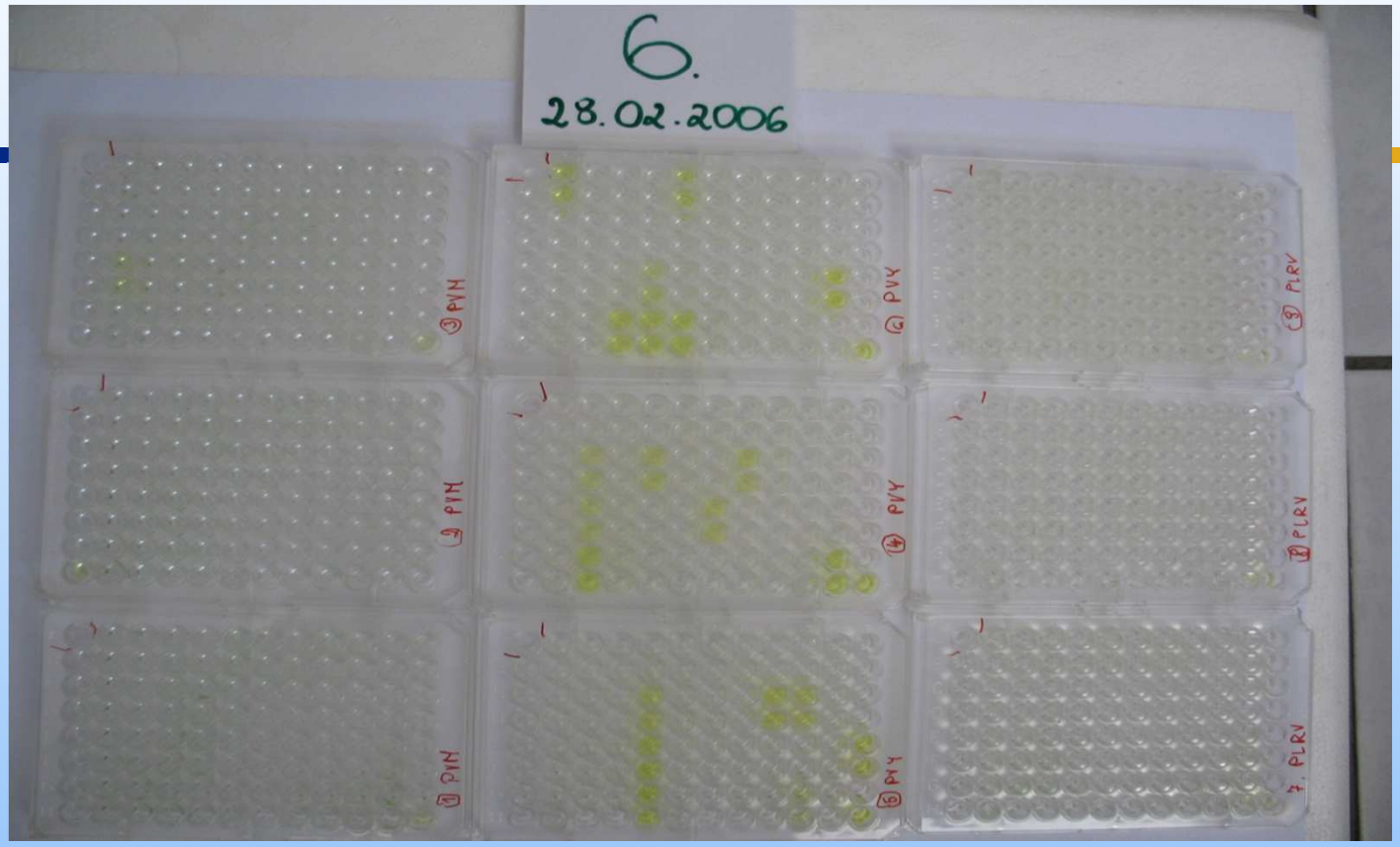
Selected variants of ELISA assay

- DASI-ELISA (Indirect Double Antibody Sandwich ELISA)



Different types of ELISA assays- comparison





Diagnostic protocol EPPO PM7/XXX(1)

ELISA tests for viruses

Troubleshootings in ELISA

Dirty plate

No signal/low OD value

- Improperly performed procedure (omitting the stage, improper incubation times)
- incorrect buffer (pH, contamination)
- calculation error or improper intake of liquids
- storage and quality of the reagents
- no infected samples
- drying out wells between stages
- quality of a plate, filter-its condition, the quality of the substrate.
- Too long storage of already coated plates.
- Improper wavelength, ambient temperature too low, reagents and plates
- old reagents, mixed batches
- he excessive dilution of the positive control, target present below the LOD

Irregular coloration or nonspecyfic reactions

- Incorrect washing
- wrong concentration of components
- mistake in ELISA steps
- edge effect – wrong temperature conditions
- weels dried out between steps
- a quality of chemicals and accesories/plate

Troubleshootings in ELISA

High background

- Wells are insufficiently washed
- Contaminated wash buffer
- Too much detection reagent
- Blocking buffer ineffective (e.g. detection reagent binds blocker; wells not completely blocked)
- Salt concentration of incubation/wash buffers
- Waiting too long to read plate after adding stop solution
- Non-specific binding of antibody
- High antibody concentration
- Substrate incubation carried out in light
- Precipitate formed in wells upon substrate addition
- Dirty plate

Edge effect

- Uneven temperature of working surface
- uneven lighting between the outer and the central part of the plate
- setting plates in piles (one on the other)
- use of buffers directly from a refrigerator (temperature) and immediate incubation at an increased temperature

Interpretation of ELISA Results

Simple data analysis

Technical Information

Simple ELISA Data Analysis

BIOREBA

Suggestion for programming the cut-off in the microtiter plate reader

For each ELISA reagent in our catalogue, lyophilized positive and negative controls are available that are primarily intended to verify the assay performance. In most cases, OD readings are obtained that correspond to values which can be expected typically also for natural samples. However, pathogen concentration varies considerably and depends on a multitude of factors such as plant species and variety, season, physiological age, kind of tissue, storage, and extraction procedure. Therefore, we strongly recommend for any calculation of the cut-off to use your own positive and negative controls employing the same kind of tissue as you are analyzing.

Furthermore, depending on reagent, purity of chemicals, type of microtiter plate, handling (especially washing), and incubation conditions, background values may vary not only from reagent to reagent but also from plate to plate even within a series of plates testing the same pathogen. Determination of the cut-off should therefore be made individually for each plate.

There are many methods of calculating or setting the cut-off. In a previous information sheet «Technical Information - ELISA Data Analysis» we suggested to sort all data obtained from each plate individually in ascending order. In the resulting histogram, negative or background values could then easily be discriminated from potential positive values which were characterized by a sudden increase of the OD value. The values of the lower side of this «step» were then taken for the calculation of the cut-off using the formula «**mean value + 3 x standard deviation + 10%**». This method has the advantage that it discriminates potential positive samples, which have relative low OD values from evenly distributed negative or background values. The drawback is that this method is quite laborious to perform, relatively complicated and not applicable for programmable microtiter plate readers.

Alternatively, we can suggest another formula, which gives in most cases satisfactory results as well and is suited to be programmed in microtiter plate readers:

Add to wells A1 to D1 extracts of different healthy (negative) samples. The formula for the cut-off value can now be programmed as three times the mean value of these four wells:

Cut-off = mean value (of A1-D1) x 3

Interpretation of results: all values above this cut-off can be regarded as potential positive.

Restriction: The negative samples in wells A1-D1 should produce typically OD values in the range of 0.080 - 0.150 (after 60 min substrate incubation and blanking against air). If these values are > 0.150, we recommend to perform the data analysis using the more precise calculation «mean value+ 3 x standard deviation + 10%» described in «Technical Information - ELISA Data Analysis».

Caution: This «3 x mean value method» is less stringent than the more sophisticated method «mean value + 3 x standard deviation + 10%». In some cases, some samples with low OD values due to low pathogen concentration will be interpreted as negative with the «3 x mean value method» while being distinguished as low positive with the statistically more sophisticated method «mean value + 3 x standard deviation + 10%».

In any case and regardless which method you are ever choosing, we strongly suggest to repeat samples that are close to the cut-off.

ELISA data analysis

Technical Information

ELISA Data Analysis

One of the major difficulties in ELISA tests can be the determination of the cut-off or threshold value, which discriminates positive results from background readings. The background or the reaction of healthy samples in ELISA-tests depends on a variety of different factors such as reagents, chemicals (purity of pNPP!), type of microtiter plate, incubation conditions, kind of plant tissue, but also on handling (especially washing!). Even if all these parameters are kept as constant as possible, it may happen that there are differences from plate to plate in the same test series. Therefore, it is not advisable to work with a fix OD value as cut-off, but to calculate it for each microtiter plate.

Any method of setting the cut-off of OD values is arbitrary. Here, we describe a statistical analysis that has proven its usefulness in our laboratory and can easily be calculated for each microtiter plate. We recommend to use a spreadsheet program such as MS-Excel allowing the programming of macros for the presented formulas and the sorting of data. For an automated processing of data, we use a fix design for the distribution of test samples in a 96-well microtiter plate. Below, an example is given for distributing 40 samples in duplicate wells, together with a positive and a negative control and a buffer blank (Fig. 1).

Fig. 1. Distribution of 40 duplicate samples in a 96-well ELISA plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Extraction buffer	sample	sample	sample	sample	sample	sample	sample	sample	sample	sample	positive
B		1	5	9	13	17	21	25	29	33	37	control
C		sample	sample	sample	sample	sample	sample	sample	sample	sample	sample	negative
D		2	6	10	14	18	22	26	30	34	38	control
E		sample	sample	sample	sample	sample	sample	sample	sample	sample	sample	
F		3	7	11	15	19	23	27	31	35	39	
G		sample	sample	sample	sample	sample	sample	sample	sample	sample	sample	
H		4	8	12	16	20	24	28	32	36	40	

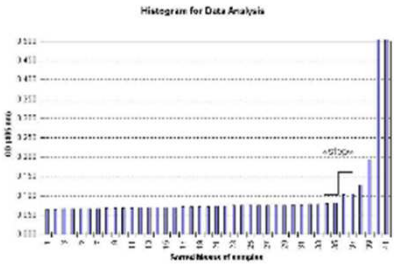
Tab. 1. These raw data are a typical example of our test service for a nursery where we have tested pelargonium plants for the presence of the pathogen *Xanthomonas campestris* pv *pelargonii* (Xcp).

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.069	0.064	0.067	0.068	0.068	0.102	0.071	0.072	0.068	0.065	0.063	2.098
B	0.070	0.064	0.067	0.066	0.068	0.102	0.066	0.068	0.065	0.070	0.067	2.026
C	0.073	0.069	0.073	0.068	0.077	0.070	0.068	0.071	0.072	0.557	0.068	0.069
D	0.075	0.074	0.077	0.071	0.082	0.072	0.070	0.074	0.076	0.592	0.071	0.074
E	0.078	0.200	0.082	0.074	0.076	0.072	0.134	0.075	0.075	0.076	0.089	0.071
F	0.075	0.188	0.075	0.071	0.075	0.071	0.120	0.073	0.073	0.078	0.074	0.073
G	0.073	0.065	0.070	0.071	0.073	0.073	0.104	0.074	0.076	0.067	0.067	0.069
H	0.073	0.069	0.070	0.071	0.075	0.077	0.106	0.076	0.077	0.069	0.064	0.071

By eye, a quite even distribution of butter and sample values of approx. 0.070 OD can be observed. Some of the values, however, are slightly increased, e.g. 6A/6B with OD values of 0.102. The following procedure allows to decide if this sample (sample 17) is positive i.e. can be distinguished from the background of «healthy» samples.

First, calculate the means of the two readings of each sample and sort these means in ascending order. By making a histogram, data belonging to the background can be recognized as a slight linear increase of OD values. A 'step' distinguishes potential positive samples (at least the positive control) from the preceding background values. In Fig. 2, an example is given with the data of Tab.1.

Fig. 2. Histogram of sorted mean values.



In this histogram, means 1 - 35 show a linear increase of OD values. The 36th value exhibits a clear increment compared to the foregoing values. This «step» has to be determined for each processed microtiter plate. The calculation of the cut-off then indicates if the next values on the right side of the step (indicated with an arrow) can be considered as positive or not; in other words, if these values are significantly different from the preceding «background». In this example, the calculation of the cut-off has been done with mean values 1-35 using the following formula (Tab. 2):

Tab. 2. Formula for calculation of the cut-off value

cut-off = (mean + 3s) x 1.1
mean: mean of the mean values up to the step (mean values 1-35) = 0.072 **s**: standard deviation of first 35 mean values = 0.004 **cut-off** = (0.072 + (3 x 0.004)) x 1.1 = 0.092

Tab. 3. Calculation of means and comparison with cut-off value (values in mOD)

	1	2	3	4	5	6	7	8	9	10	11	12
mean	73	64	67	67	68	102	69	70	67	68	66	2062
A		64	67	68	68	102	71	72	68	65	63	2098
B		64	67	66	68	102	66	68	65	70	67	2026
result		-	-	-	-	positive	-	-	-	-	-	positive
mean		72	76	70	80	71	69	73	74	676	70	72
C		69	73	68	77	70	68	71	72	557	68	69
D		74	77	71	82	72	70	74	76	592	71	74
result		-	-	-	-	-	-	-	-	positive	-	-
mean		194	79	73	76	72	127	74	74	77	82	72
E		200	82	74	76	72	134	75	75	76	89	71
F		188	75	71	75	71	120	73	73	78	74	73
result		positive	-	-	-	-	positive	-	-	-	-	-
mean		67	70	71	74	76	106	76	77	68	66	70
G		65	70	71	73	73	104	74	78	67	67	69
H		69	70	71	75	77	108	76	77	69	64	71
result		-	-	-	-	-	positive	-	-	-	-	-

In Tab. 3, all means were compared with the cut-off value of 92 mOD. The means of the samples 3 (194 mOD), 17 (102 mOD), 23 (127 mOD), 24 (105 mOD), and 34 (575 mOD) were interpreted as positive. To avoid false positives, check the single values as well. In some cases, one value can be relatively high (e.g. caused by insufficient washing) and therefore giving a mean that corresponds not to the reality. Therefore, as a rule, check if both values contributing to the calculation of the mean are above the cut-off.

Recommendation: The level of background can be different for different plant tissues (e.g. leaves, sprouts, and tubers) or for different species/varieties of plants. In order to obtain an even distribution of the unspecific reactions contributing to the background, use as homogenous samples as possible in one microtiter plate.

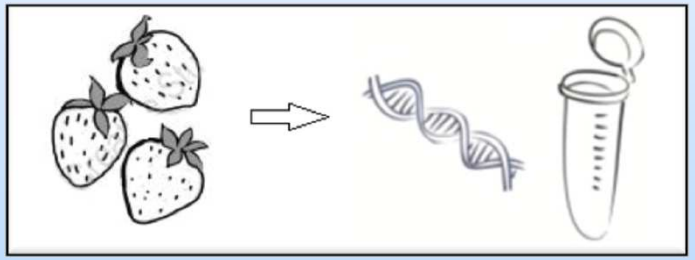
Conclusion: This data interpretation helps to avoid false negatives in case of weak positive samples with an antigen concentration near the detection limit. It allows therefore taking full advantage of the sensitivity of ELISA tests.

Molecular techniques

PCR & RT-PCR

DNA Isolation

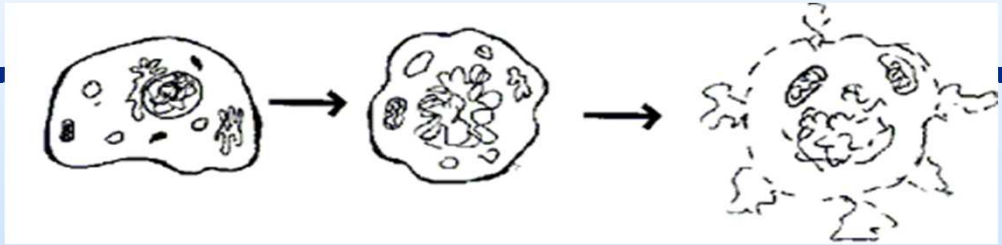
- compromise between using methods enough invasive to disintegrate analyzed material and isolate the DNA/RNA and at the same time delicate to avoid destruction of the isolated nucleic acid



- DNA isolation consists of three main steps: cell lysis, nucleic acid purification and main isolation/precipitation of nucleic acid

- there are a lot of protocols available for DNA isolation (by means of SDS and CTAB) but ready-to-use kits are becoming more popular (e.g. Roche, Qiagen, Promega)

DNA Isolation

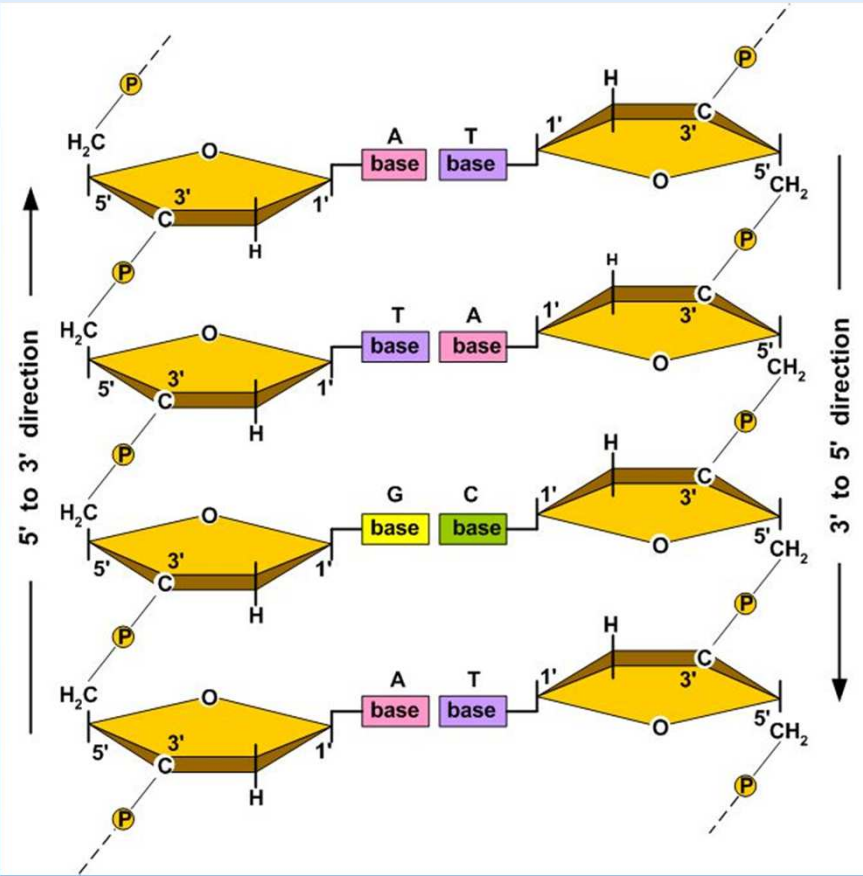


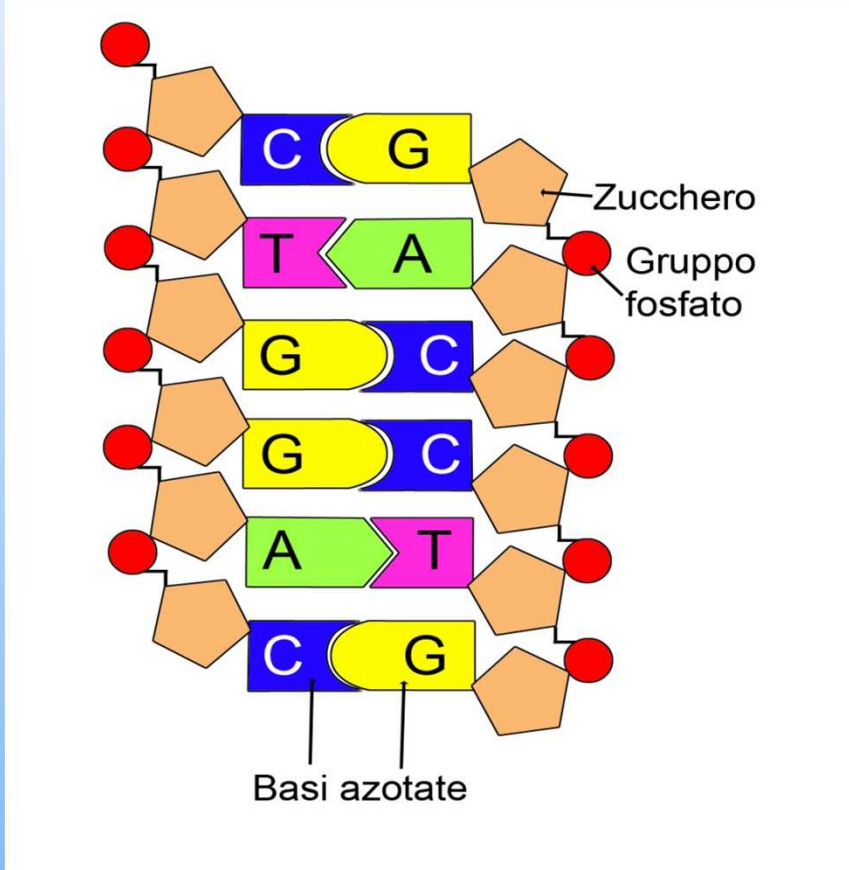
- **cell lysis**
- mechanical – grinding
- chemical – ionic detergents (SDS, CTAB)
  non-ionic (Triton X-100)
- enzymatic – proteinase K

- **purification of the DNA in a solution**
- chloroform-isoamyl extraction (protein impurities removal)
- chromatographic techniques (selective binding of the DNA)

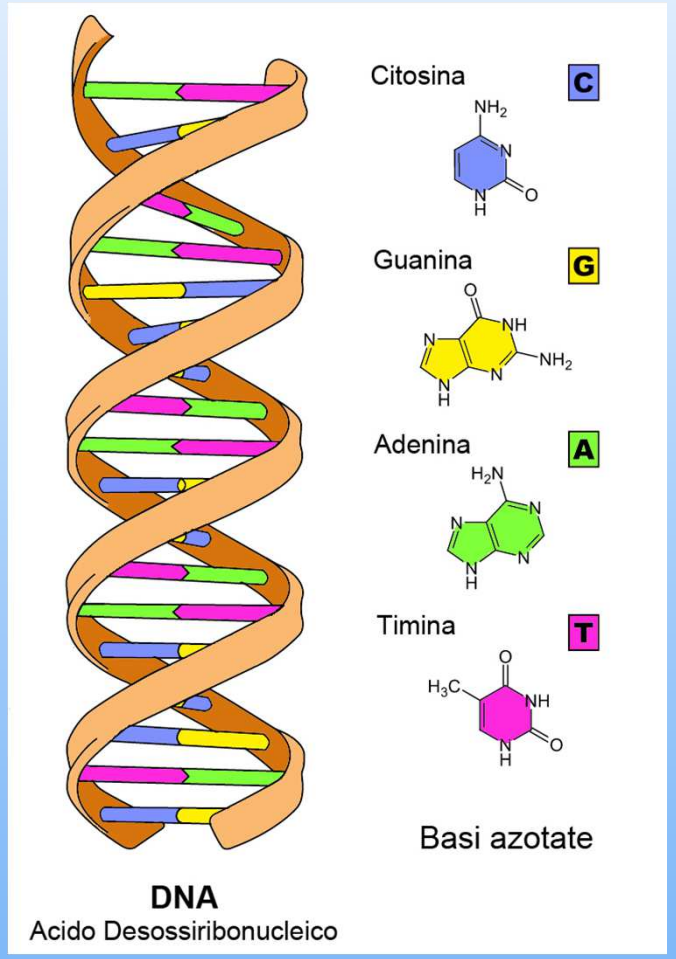
- **DNA precipitation**
- isopropanol or ethanol
- salting-out (e.g. higher concentrations of sodium acetate)

DNA

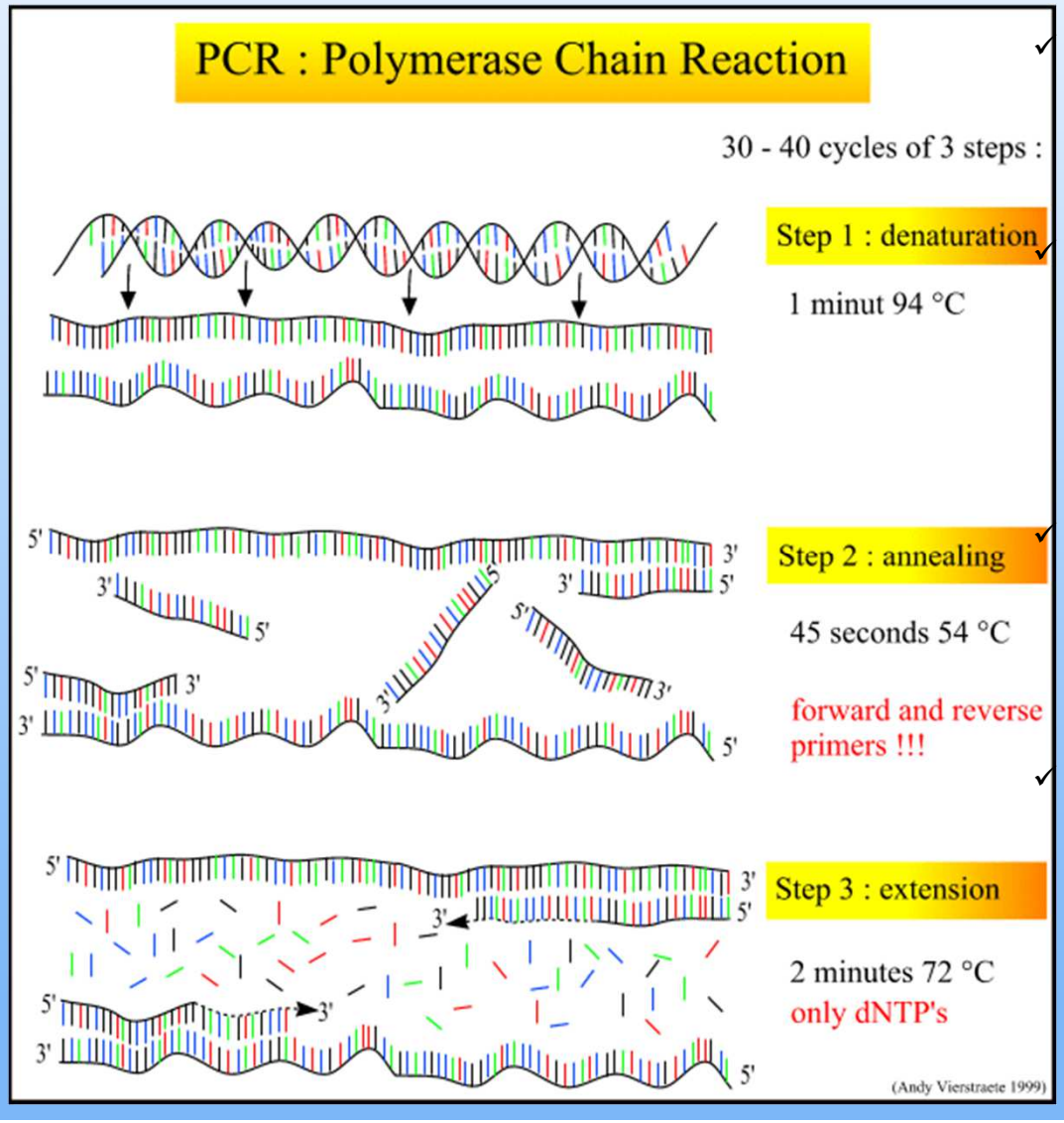

5' to 3' direction
3' to 5' direction
A
T
G
C
base
P
O
H
C
1'
3'
5'
H_2C
CH_2


C
G
T
A
G
C
G
C
A
T
C
G
Zucchero
Gruppo fosfato
Basi azotate

DNA


Citosina
C
Guanina
G
Adenina
A
Timina
T
Basi azotate
DNA
Acido Desossiribonucleico

PCR reaction



denaturation step: melting of the DNA double helix at high temperature about 95°C – the hydrogen bonds break and the helix separates into two single-stranded DNA molecules

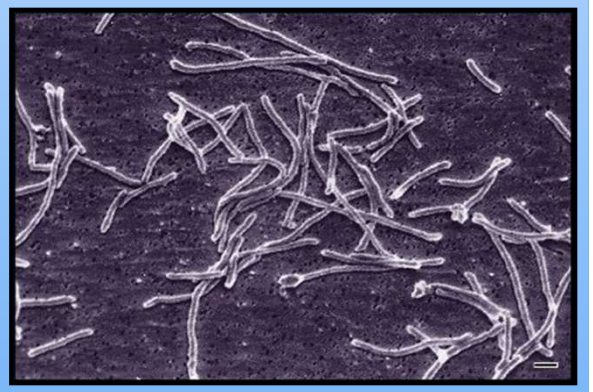
annealing step: annealing of the primers to the single-stranded DNA. This step occurs at a temperature specified for each pair of primers (usually 45 – 60°C). Excess of primers in relation to the matrix prevents the formation of hybrid DNA – DNA

elongation step: synthesis and amplification of the desired DNA sequence.
At a temperature of about 70 – 80°C polymerase complex binds to the primer-template hybrid and begins DNA formation

cycles repeating: the matrix are all previously synthesized DNA molecules – the reaction proceeds more rapidly, causing the increase of copies of DNA

PCR – important informations

- method relies on cycles of **repeated heating and cooling** of the DNA (Nobel prize in1993r.)
- used enzyme: a **thermostable** (up to 97,5°C!) **polymerase** from ***Thermus aquaticus*** bacteria living in the hot waters of Yellowstone Park
- there is no need to know the whole sequence of the tested gene – knowing the sequence of the **flanking sequences** is **sufficient**
- very s**pecific** – with a suitable choice of primer sequences only one desired DNA fragment is amplified
- very **sensitive** – amplification of even one single molecule of DNA!
- possible **uses of PCR**: clinical diagnostics, research on the genome and cloning of genes, identification of missing persons, forensics, paleontology and many others



PCR

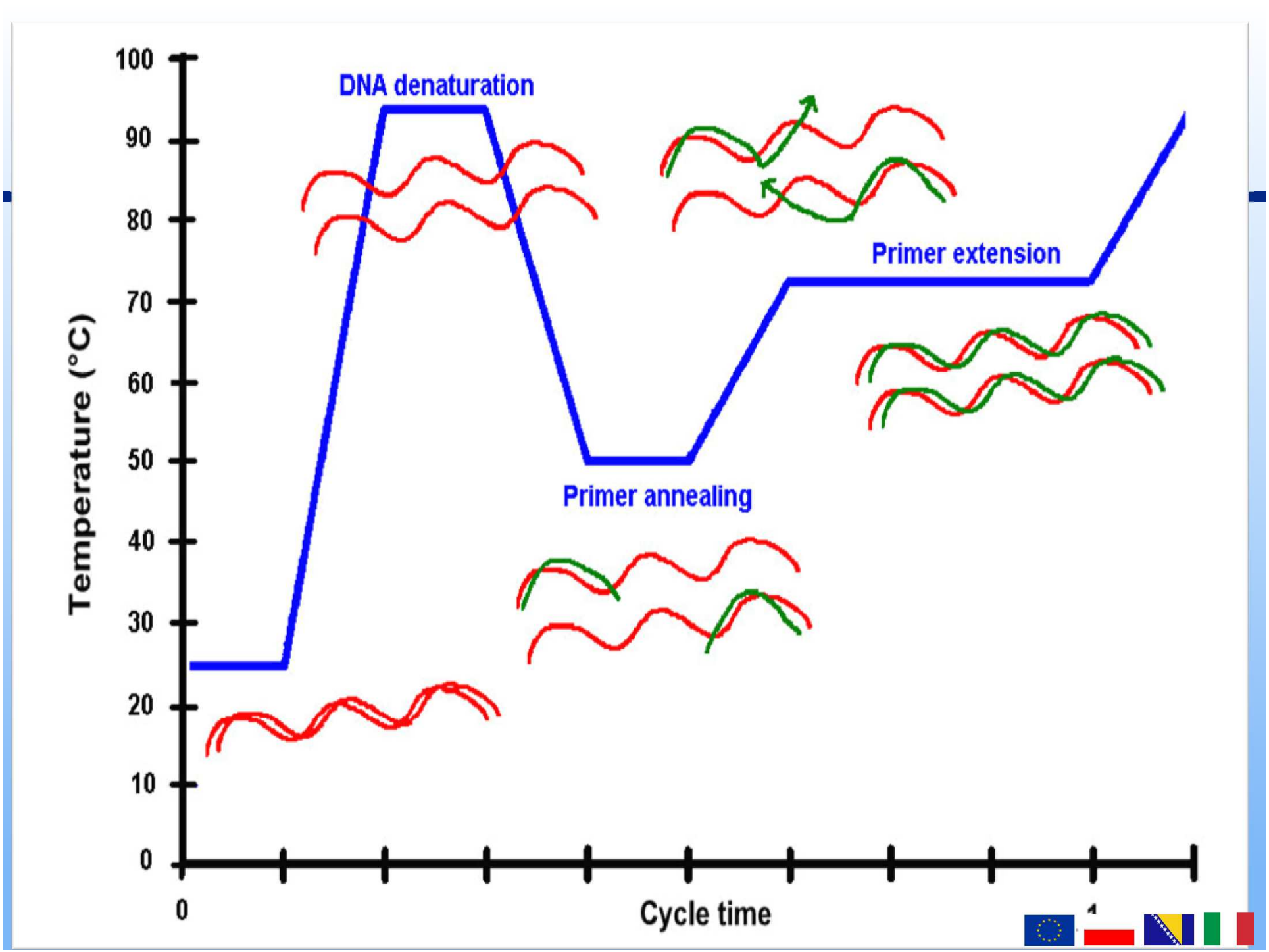
Reaction components:

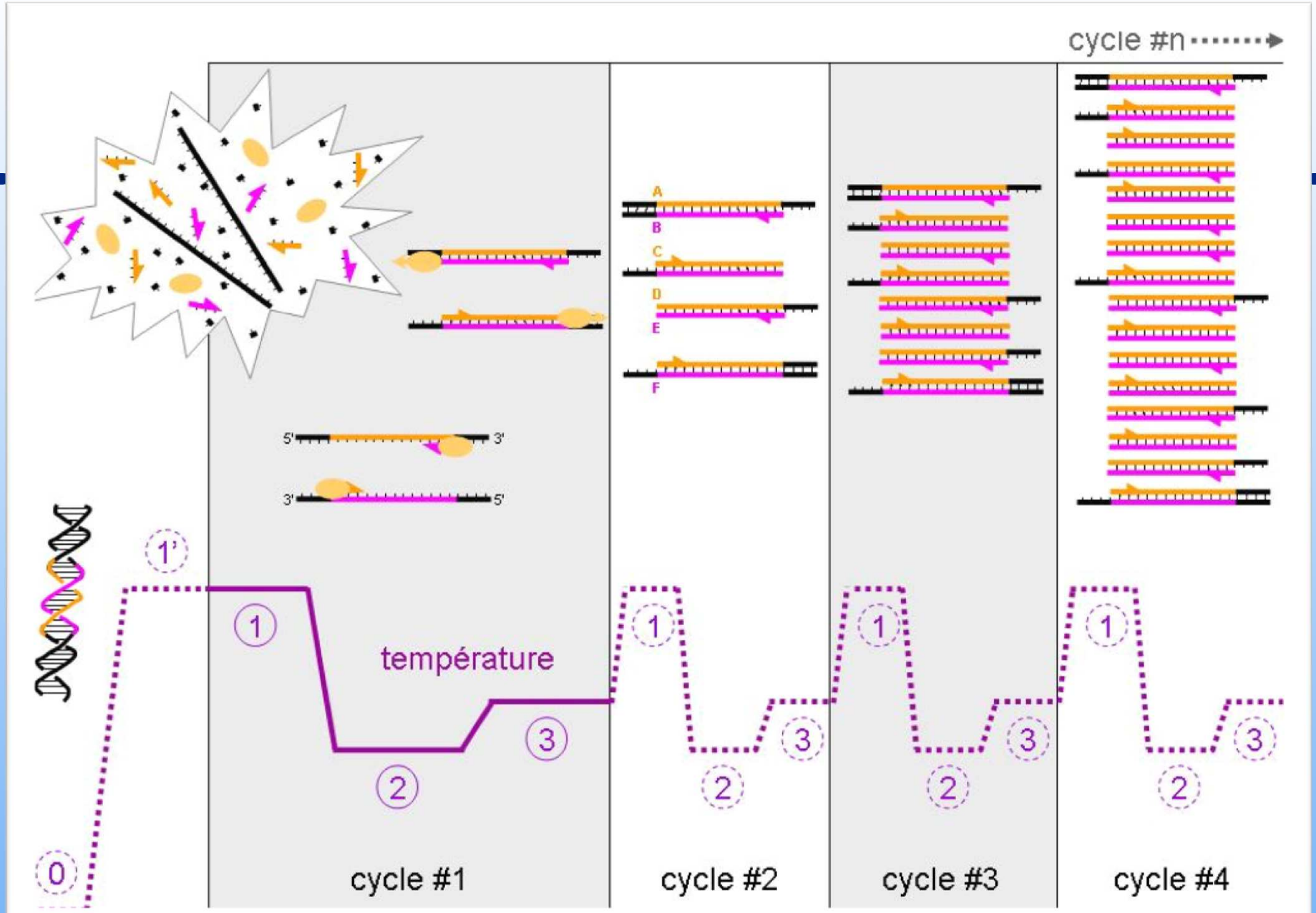
- DNA template free nucleotides
- Primers
- *Taq* polymerase
- polymerase buffer
- Mg^{2+}
- water for molecular biology

Factors affecting PCR reaction

primers	▫ length and sequence: 15 – 25 nt and no CG blocks, too short primers will not be specific, too long will be difficult to attach to the DNA strand; no secondary structures and concatemers, no complementary to each other (no hairpin structures)
$MgCl^{2+}$, dNTP concentration	▫ little concentration results in low reaction yield, too big results in the formation of non-specific products and polymerase mistakes in nucleotide substitution
***Taq* polymerase concentration and type**	▫ sensitivity and stability – standard *Taq* polymerase or recombinant ''hot start'' type; in some cases increase of concentration may improve the reaction efficiency but usually it significantly increases the formation of non-specific products
reaction temperature profile	▫ properly chosen annealing temperature can be calculated using software such as OLIGO (high temperature is high specificity primer-template, but low efficiency; lower temperature is lower specificityn but higher efficiency)
inhibitors	▫ compounds used for the DNA isolation – proteinase K, salts, ionic detergents, phenol, etc. – it is necessary to clean the mixture

PCR Amplification


DNA denaturation
Primer extension
Primer annealing
Temperature (°C)
100
90
80
70
60
50
40
30
20
10
0
0
Cycle time


cycle #n
A
B
C
D
E
F
5'
3'
3'
5'
1'
1
2
3
0
température
1
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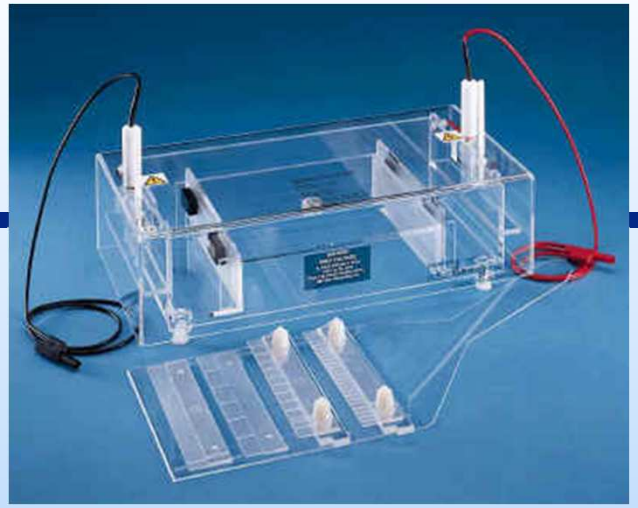
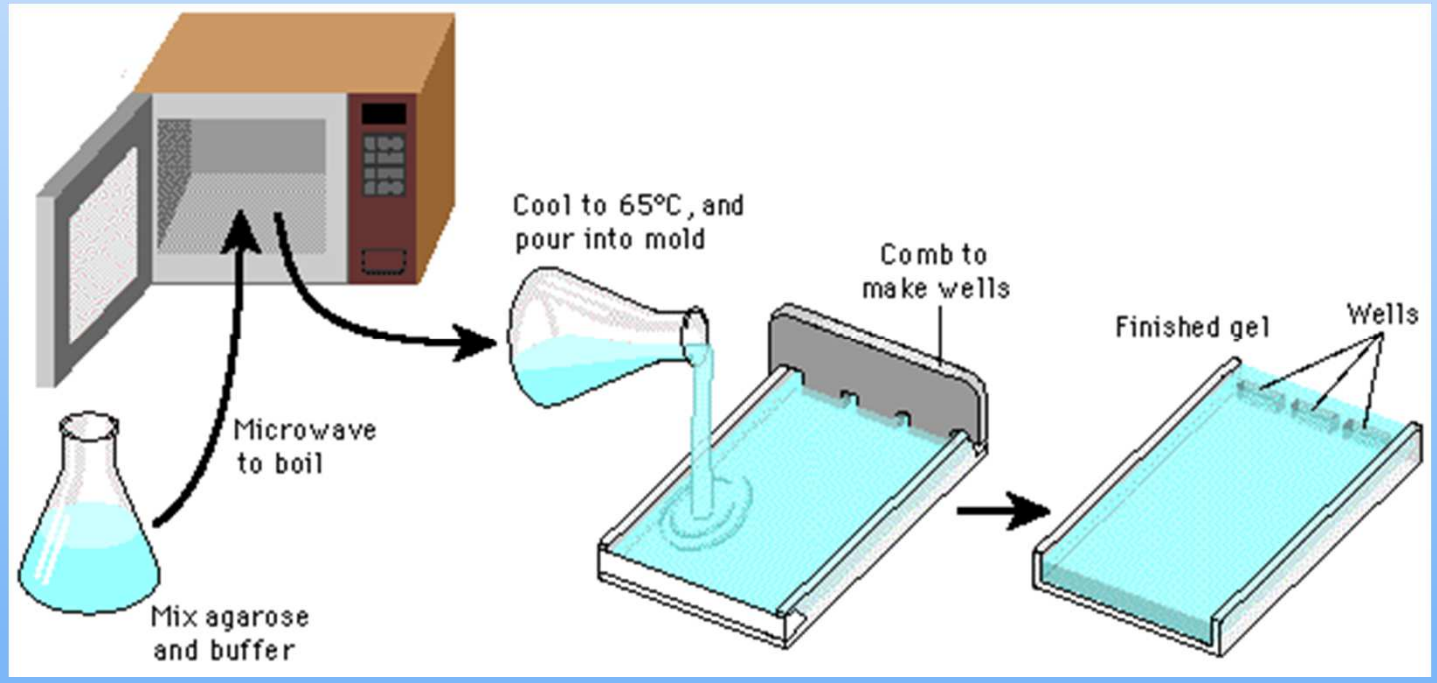
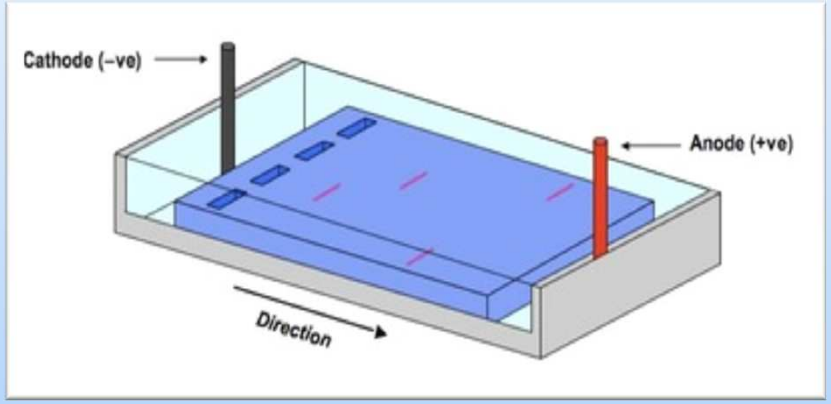
cycle #1
cycle #2
cycle #3
cycle #4

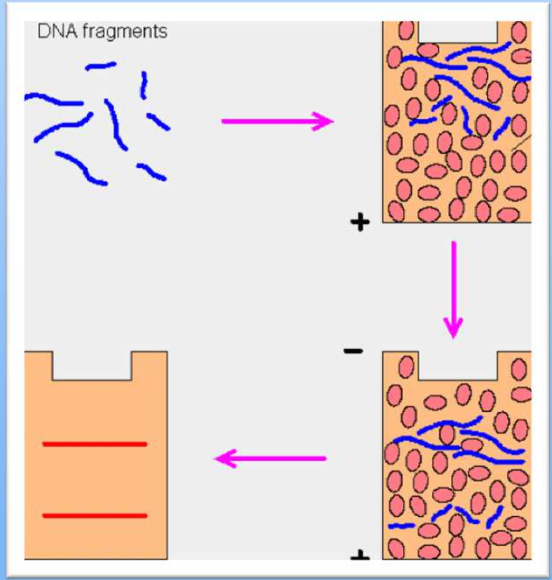


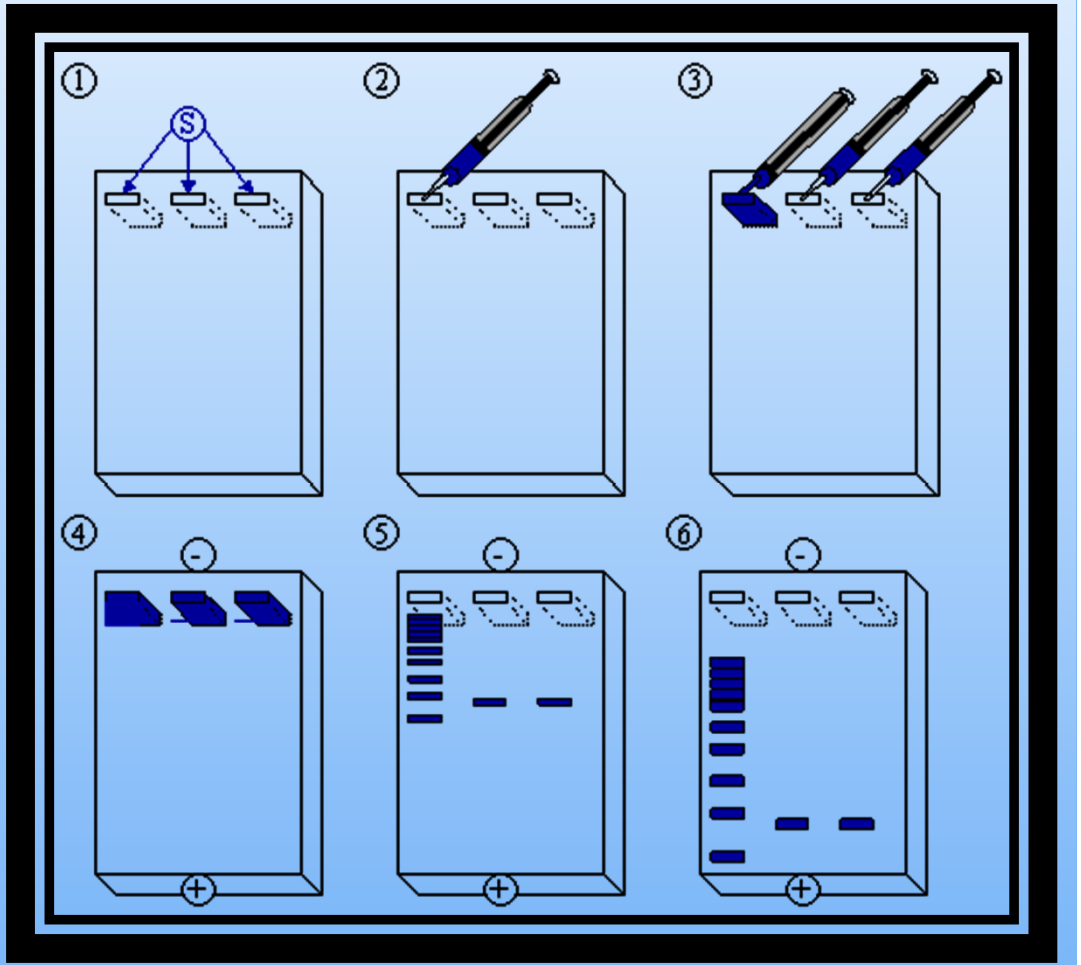
Agarose gel electrophoresis



Agarose gel electrophoresis

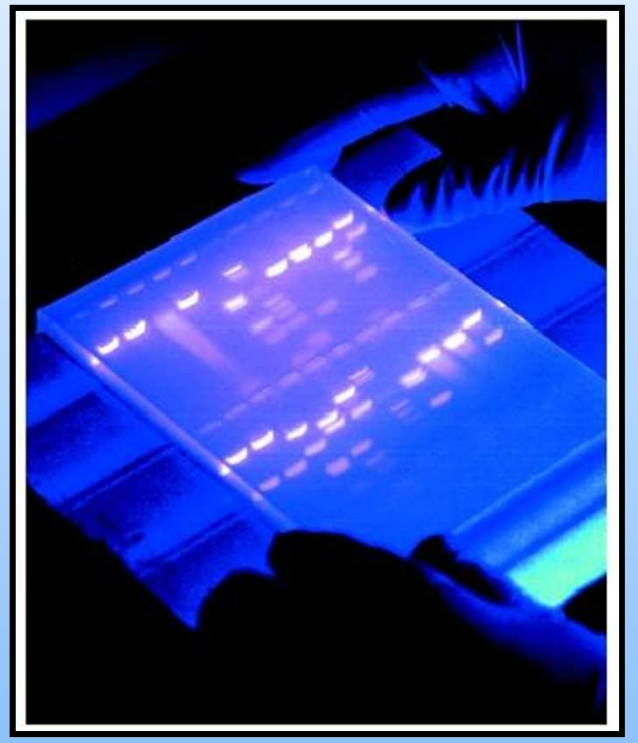


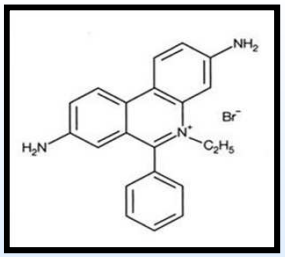




Agarose gel electrophoresis

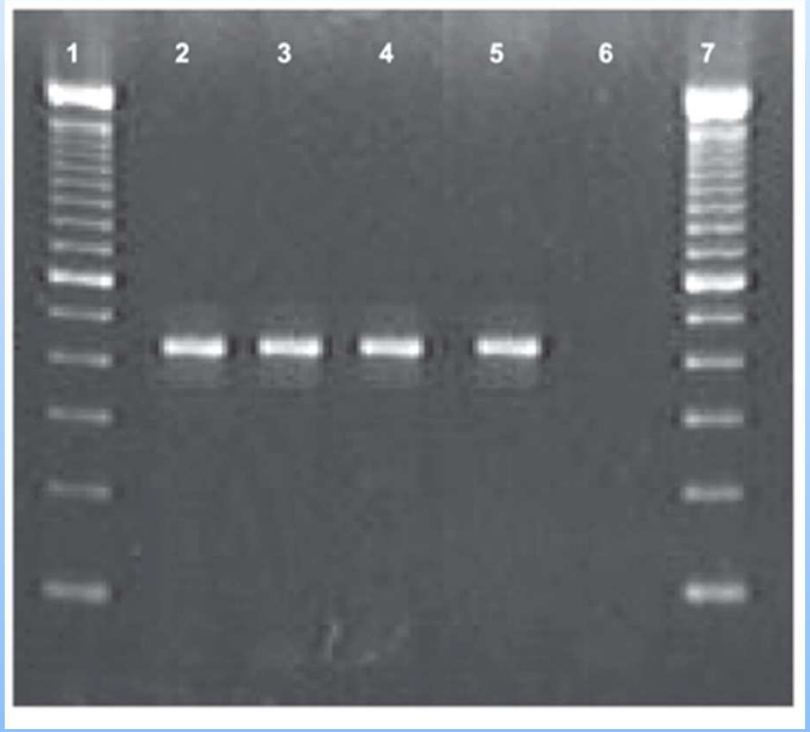
- **agarose** – polysaccharide, when dissolved in water/buffer and cooled to room temperature polymerizes to a gel
- **electrophoresis** – separation of the mixture of components in agarose gel, under the electrical voltage
- because of the presence of the phosphate residues, each **DNA** molecule has a **negative charge** and migrates towards the positive electrode in the electric field
- **mobility** of the DNA molecules depend on the **size** – the smaller molecule, the easier it goes through the gel, and migrates to a further distance





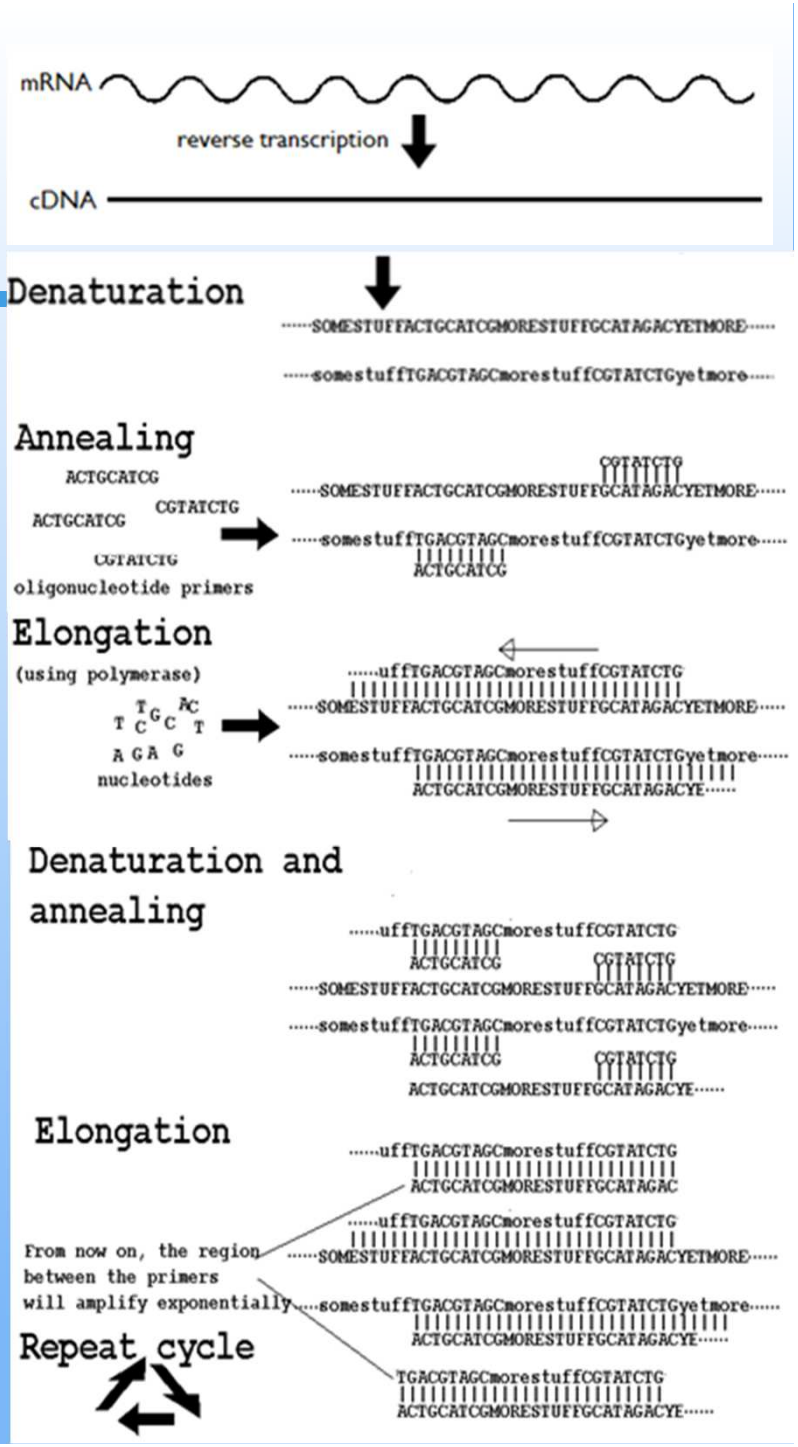
- all work with ethidium bromide: in a designated area and with **nitryle gloves** on!
- ethidium bromide is a strong **mutagen** and **carcinogen**
- for **1,5% agarose gel**: add agarose to 1x TAE buffer (e.g. 1,5 g of agarose to 100 ml of 1xTAE buffer), bring to boil in the microwave, mix thoroughly and cool to approximately 65°C
- add **EtBr** (final concentration10 mg/ml) and mix
- pour the gel to a **casting tray**, remove any air bubbles (if present) and put a comb
- allow to **polymerize** for at least 30 minutes

Agarose gel electrophoresis



RT PCR (reverse trascription PCR)

- Working with RNA is quite difficult, hence the idea to "prescribe" RNA into more stable DNA and use it as a template in a standard PCR reaction
- transcribed mRNA is called. cDNA (Eng. Coding DNA) by the enzyme reverse transcriptase and the resulting cDNA serves as a template in a conventional PCR reaction
- both steps may occur one after the other in the same reaction tube (ie. one-step RT-PCR) - in a PCRmixture outside the buffer, $MgCl_2$, dNTPs, primers, template, and polymerase there is also enzyme reverse transcriptase
- reverse transcription and PCR can also be performed in separate tubes (ie. two-step RT-PCR), which will increase the sensitivity of the method, but at the same time increases the risk of sample contamination
- Example of use RT PCR: diagnostics of Potato spindle tuber viroid (PSTVd)

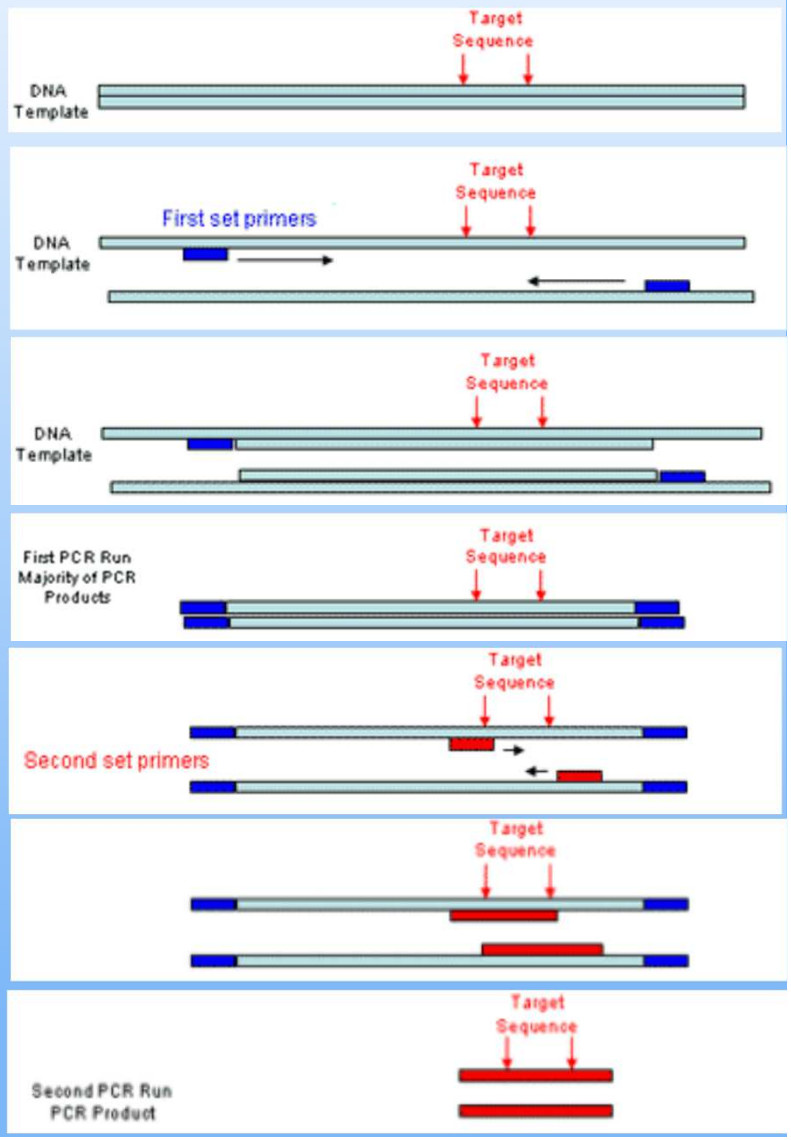


RNA isolation

- RNA is more difficult for the isolation than DNA: highly reactive and unstable, sensitive to nucleases and pollution, quickly degrades
- it is necessary to discipline the whole process of work - possibly the most sterile conditions, RNase-free reagents and gloves
- the first step: the introduction of tissue into a liquid phase in TRIzol (a mixture of phenol, isoamyl and guanidine isothiocyanate, the mystery of the manufacturer) - chaotropic salts denature protein (ubiquitousRNases)
- second step: extraction with chloroform - separating the aqueous phase with RNA from an interphase with DNA and from organic phase containing proteins and other cellular debristrzeci
- the step of precipitating RNA with isopropanol and suspended in autoclaved and DEPC-treated water
- You can also isolate RNA using comercially available kits

Nested PCR

- the method consists of carrying out two PCR reactions: in the first reaction except of the desired DNA fragment there are present also flanking sequences so that produced product is unspecific
- Second PCR is based on primers located within the first stage amplified DNA fragment and multiplies an interesting targeted sequence
- two reactions allow a greater number of cycles, and that results in obtaining more of the desired product (after the first PCR should be supplemented reagents include polymerase
- double reaction increases the sensitivity and efficiency of amplification
- Example of use nested PCR: diagnostics of phytoplasmas (universal primers P1/P7, followed by R16F2n/R16R2)



RFLP Analysis (restriction fragment leng polymorphism)

- analysis of the digest patterns of DNA using restriction enzymes
- DNA is digested only in specific for particular restriction enzymes cleavage sites
- DNA of organisms differs, therefore, for each species, there are other cleavage sites and thus different „pattern" of restriction digestion (the entire genome or its fragment)
- Usually targeted DNA is amplified in PCR reaction, product is digested with use of restriction enzymes, and the digested DNA is separated on agarose gel
- based on the pattern of bands after electrophoresis it is defined a membership species of the test organism
- the method is sometimes ambiguous - mismatched digestion time, the possibility of appearance of non-specific products of digestion, intraspecific variability

Example of use RFLP: diagnostics of *Candidatus Phytoplasma mali*

- First stage: PCR amplification of targeted sequence using primers r01 / f01
- restriction digestion of the resulting PCR product (~ 1050-1070 bp): using two enzymes SspI and BfmI in temp37°C for 2 h.
- electrophoresis on a 2% agarose gel
- Result

Molecular techniques

Real-time PCR

&

real-time RT-PCR

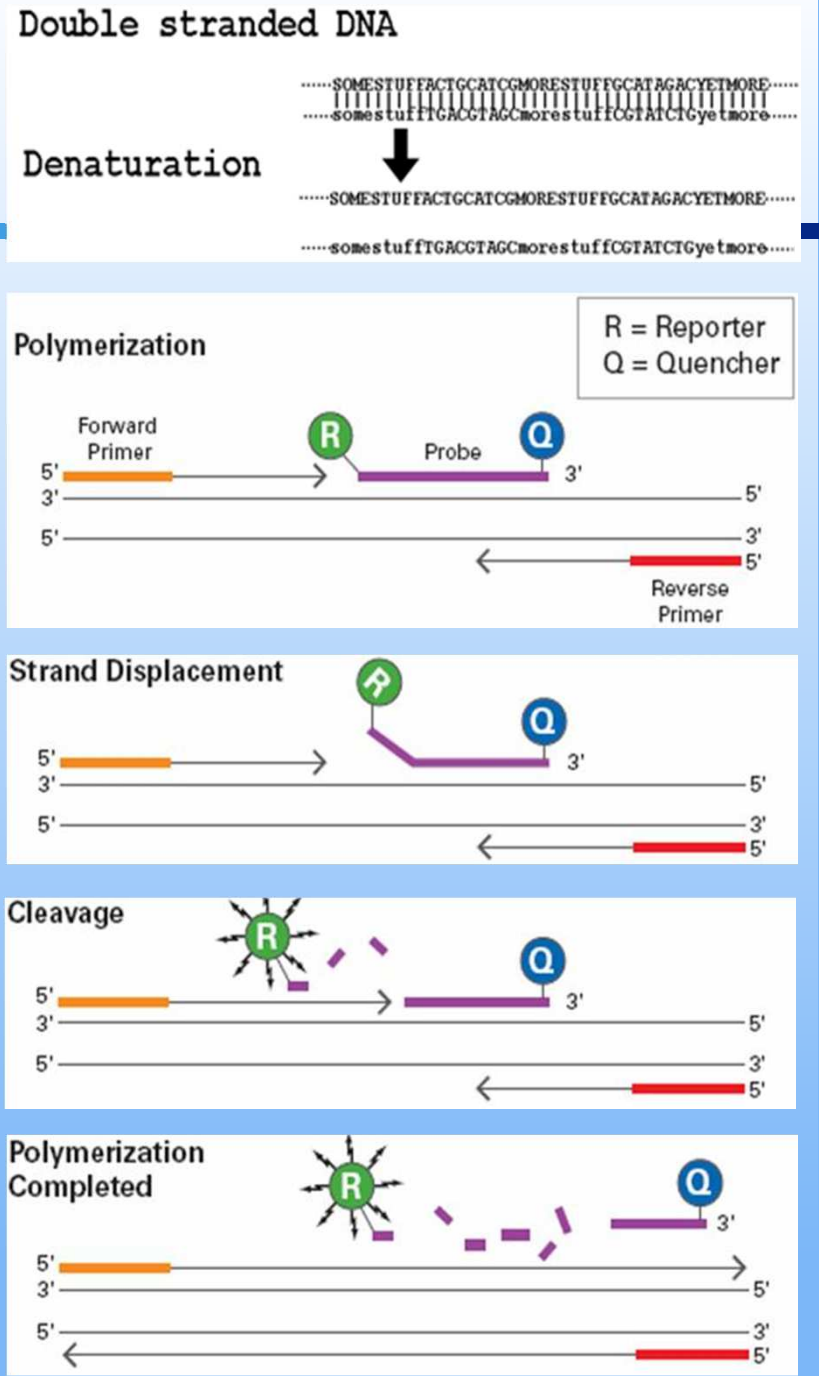
Real-Time PCR

- DNA amplification with addition of the fluorescent dye allows detection of PCR product in real time – a fluorescence of each sample is proportional to the amount of the synthesized product (the stronger fluorescence, the more copies of the tested DNA sequence)
- the most widely used markers in Real-Time PCR are a TaqMan probes - DNA fragments length 20 - 40 nucleotides with the reporter (emitter) and fluorescence quencher
- Real-Time PCR allows fast and accurate analysis of the tested sequence without work and time-consuming electrophoresis
- A modification is the RT Real-Time PCR (additional step of reverse transcription allows the detection of RNA viruses)
- multiplex Real-Time PCR - probes with different fluorophores for the detection of amplified DNA using different primers

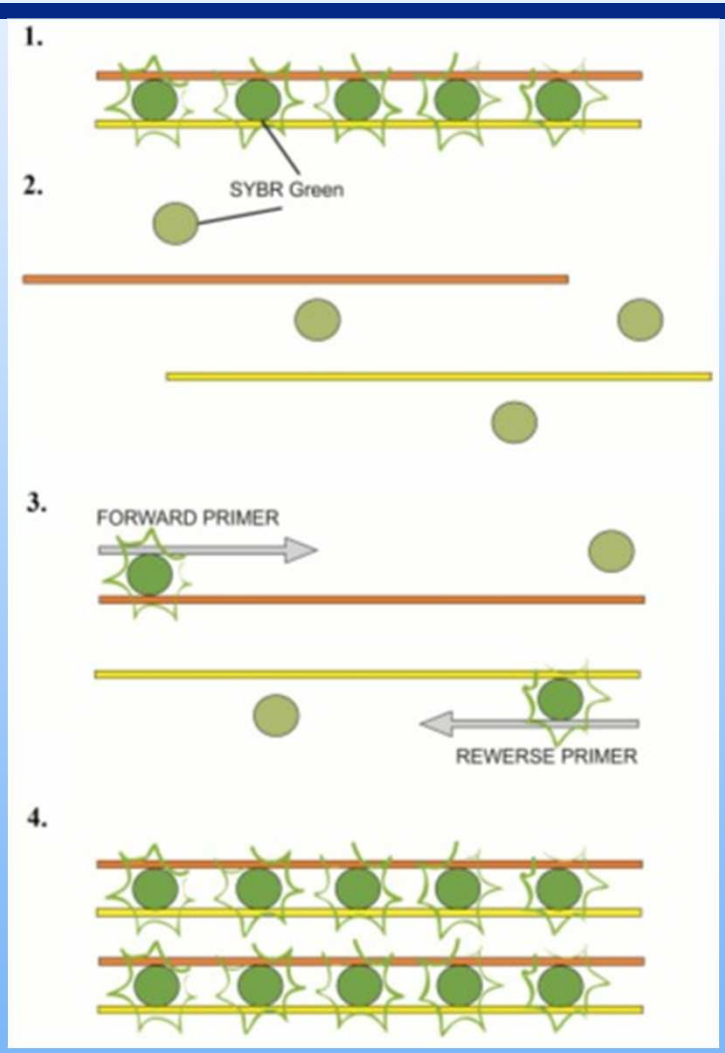
Real-Time PCR

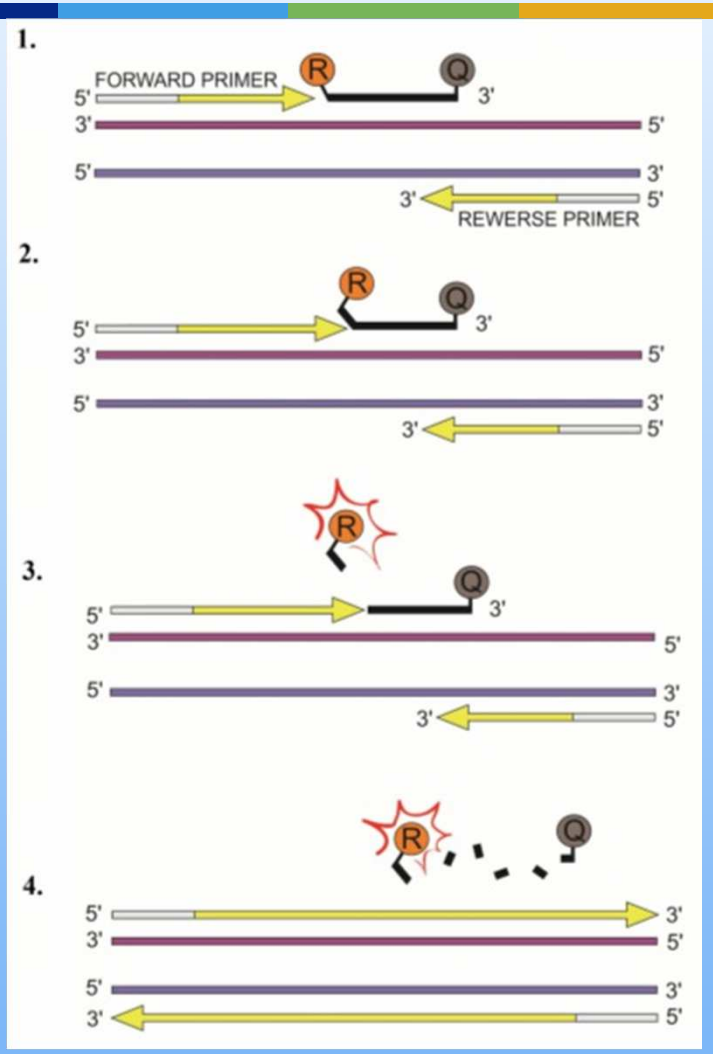
- Reaction components: templated DNA, free nucleotides, primers, polymerase buffer, Mg^{2+} ions, *Taq* polymerase, molecular grade water, labeled probe (e.g. TaqMan)
- denaturation
- annealing of primers and probes fluorophore is connected with a quencher - no lightening
- Extension of a strand DNA synthesis and amplification of targeted gene. Cutting off the fluorophore from the quencher by Taq polymerase results in emission of light
- Cycles repeating**:** a probe is attached to each single stranded copy of a DNA, so in each cycle the probe is attached to the DNA strand, and then degraded by *Taq* polymerase activity what release fluorophore from a close proximity of a quencher.

  Effect – increasing of fluorescence

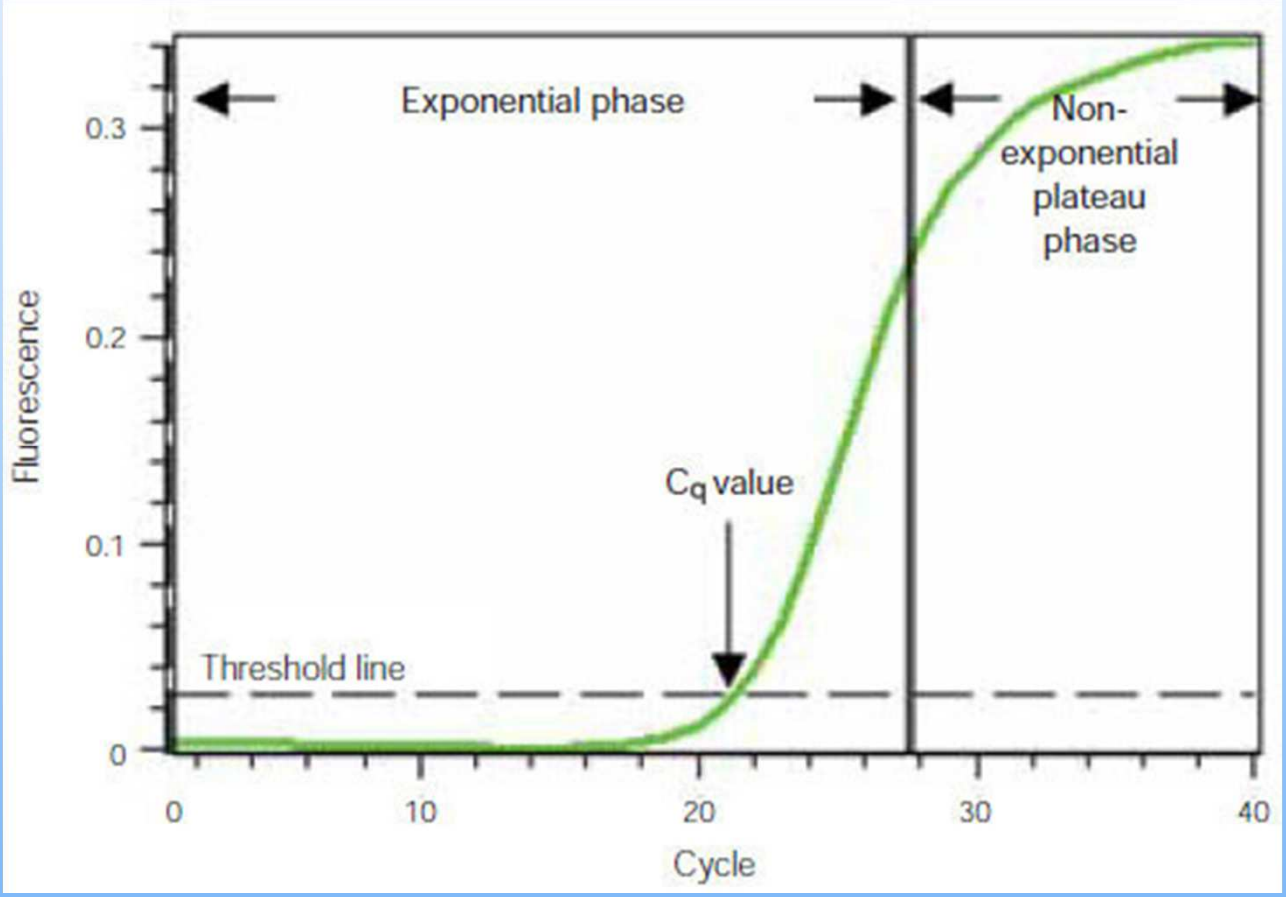


real-time PCR





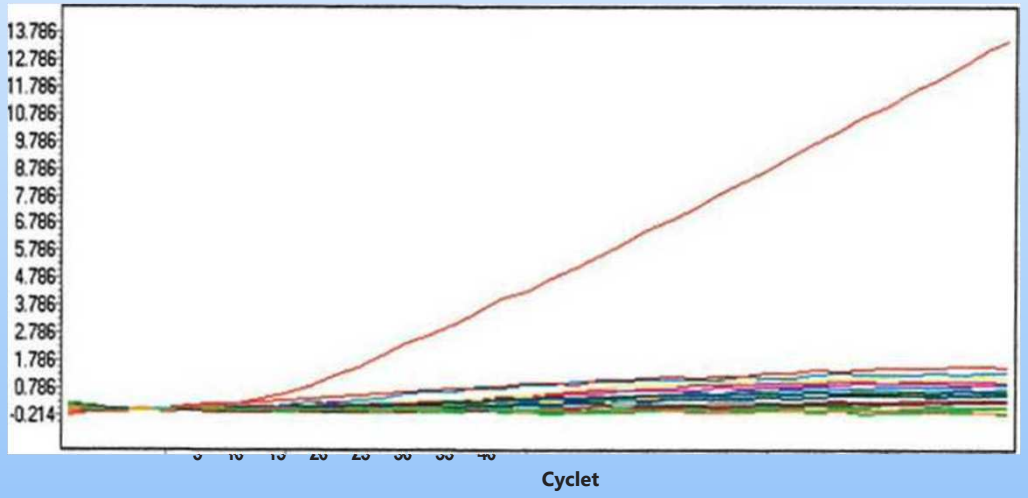
Real-time PCR result



Example of use ***Real-Time*** RT PCR: diagnostics of *Pepino Mosaic Virus* (PepMV)

Amplfc*tcfi Curv«

— A1: FF/3190/12	— A2: FF/3190/12	-A3: FF/3190/12	— A4. FF/3190/12
-A5: FF/3190/12	A6: FF/3190/12	-A7 FF/3190/12	-A8: FF/3190/12
-A9: FF/3190/12	A10: FF/3190/12	-Ali: KN	-A12: KP FW/W/KP-PCd?
-B1 FF/3190/12	-82- FF/3190/12	-B3. FF/3190/12	-84: FF/3190/12
-85: FF/3190/12	86: FF/3190/12	-87: FF/3190/12	-B8'FF/3190/12
-09 FF/3190/12	B10: FF/3190/12	-811: KN	812: H20



- Is amplification has taken place at all?

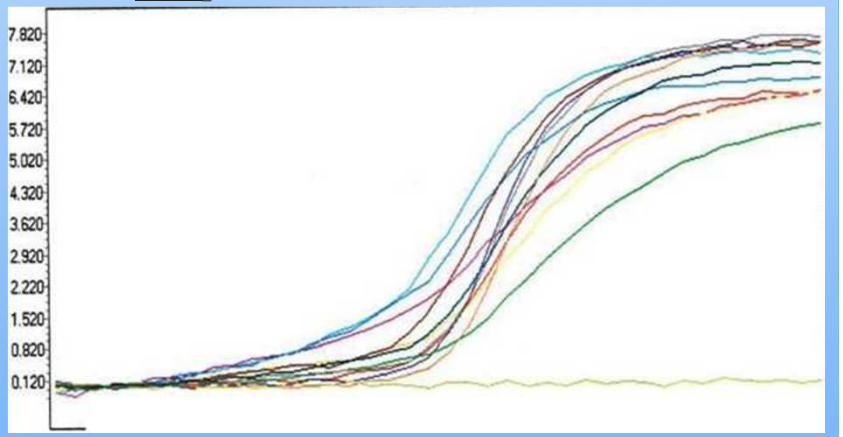
  The internal control for each sample

- lack of reaction product for no template control

- amplification curves - lack of reaction product for tested samples
- the reaction product is visible only for the positive control

Amplfication Curves

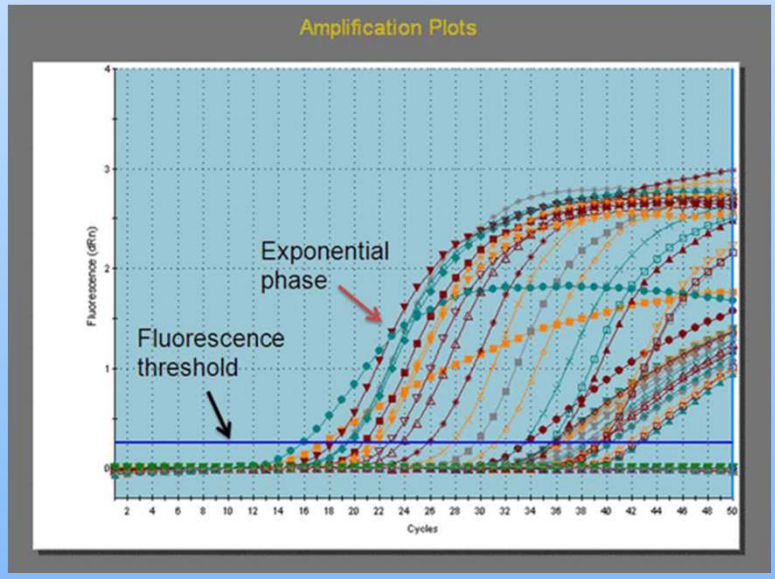
— C1: FF/3190/12 cox —C2: FF/3130/12 cox —C3: FF/3190/12 cox —C4: FF/3190/12 cox —C5: FF/3190/12 cox CS: FF/3190/12 ccx -C7: FF/3190/12 cox —C8: FF/3190/12 cox —C9: FF/3190/12 cox —C10: FF/3190/12 cox
-C11:KPcox C12: H20



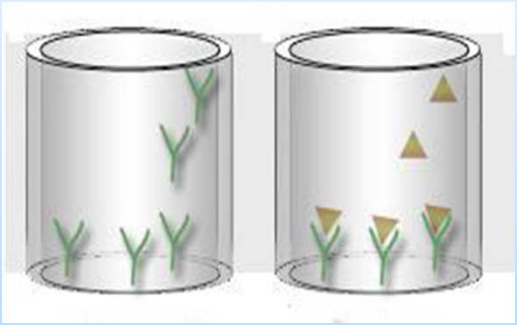
PCR vs real-time PCR

PCR

Real-time PCR



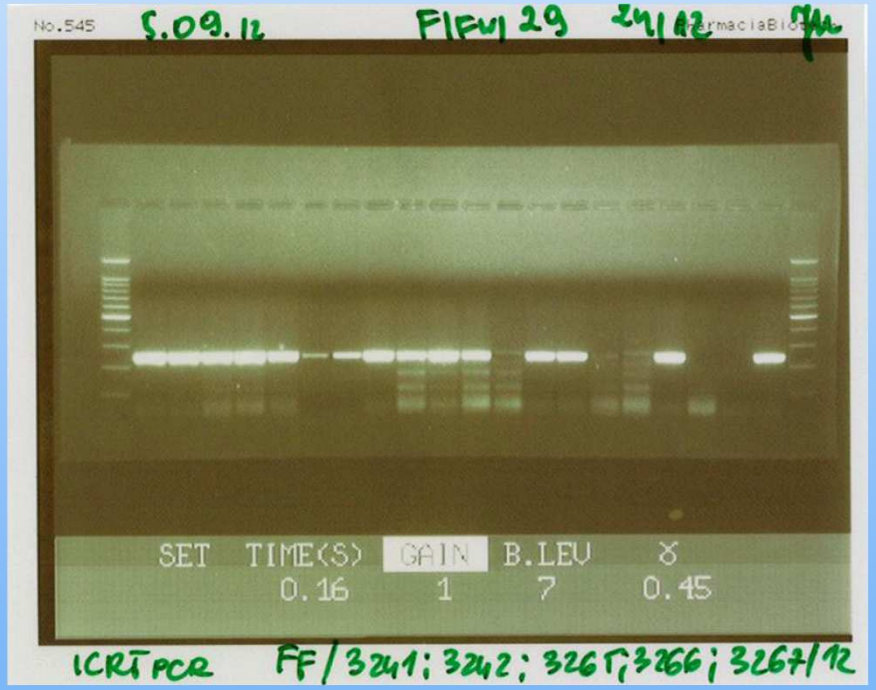
IC/RT-PCR (immunocapture RT-PCR)



- a methode consists of trapping of viral particles on antiserum-coated wall of a tube followed by washing to remove inhibitors of plant origin
- this allows the detection of pathogens in the material at a very low concentration (antibodies are highly specific for the virus searched)
- big advantage of this method is no need to isolate RNA from the capsid of the virus to be able to start RT-PCR
- after catching the viral particles reverse transcription of RNA into cDNA is carried out, followed by standard PCR

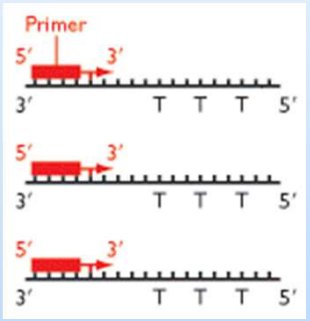
Example of use IC/RT-PCR: diagnostics of *Plum Pox Virus* (*PPV*)

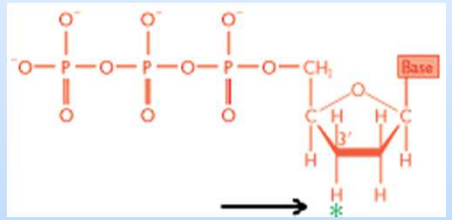
- tubes/reaction plates are coated with antibodies IgG specific for Plum Pox Virus
- Plant sap prepared in appropriate way is placed in coated tubes/plates and incubated 3h in 37°C
- After incubation a plant extract is removed and tubes/plates are washed RT-PCR MIX is prepares
- included in the mix appropriate detergents degrade capsid „exposing" viral RNA and reverse transcriptase rewrites RNA to DNA
- The last stage is PCR reaction and gel electrophoresis

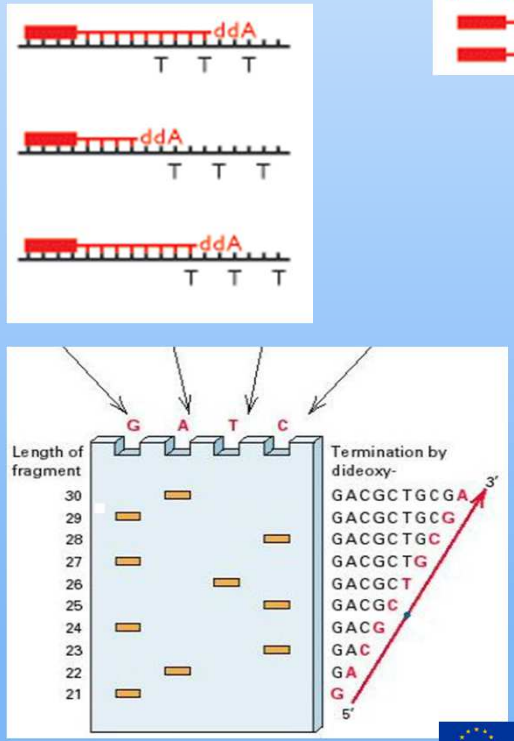


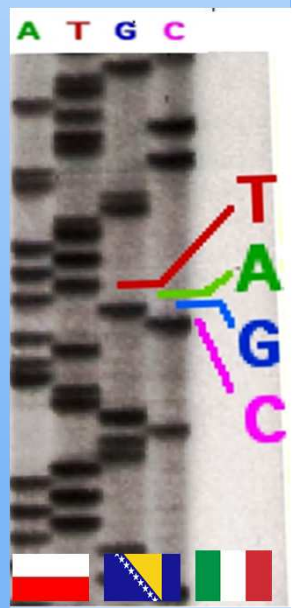
DNA sequencing (met. Sangera)

- the first step is to obtain high quality DNA template - treatment eg. using comercially available kits, then denaturation of a strand
- then synthesis of a new strand by polymerase, primer, dNTP and one ddNTP (each reaction in a separate tube for ddATP etc.).
- ddNTP causes the end of the synthesis, which is why for example in a test tube with ddATP each fragment will start at starter and ended with adenosine (fragments of different lengths)
- Likewise with other ddNTP
- the mixture of fragments is applied to the polyacrylamide gel and separated under denaturing conditions (urea + temp. 55 - 70°C)
- To read a results primers or ddNTP are labeled e.g. Isotopes, and then applied to X-ray film
- now: fluorescence →automation



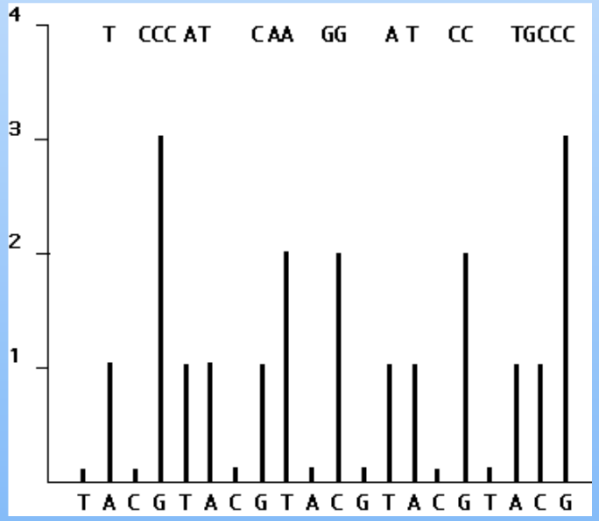






DNA pyrosequencing

- each dNTP is added singly with nucleotidase which degrades unused nucleotide
- turned nucleotide accompanied by a flash of chemiluminescence caused by the release of pyrophosphate

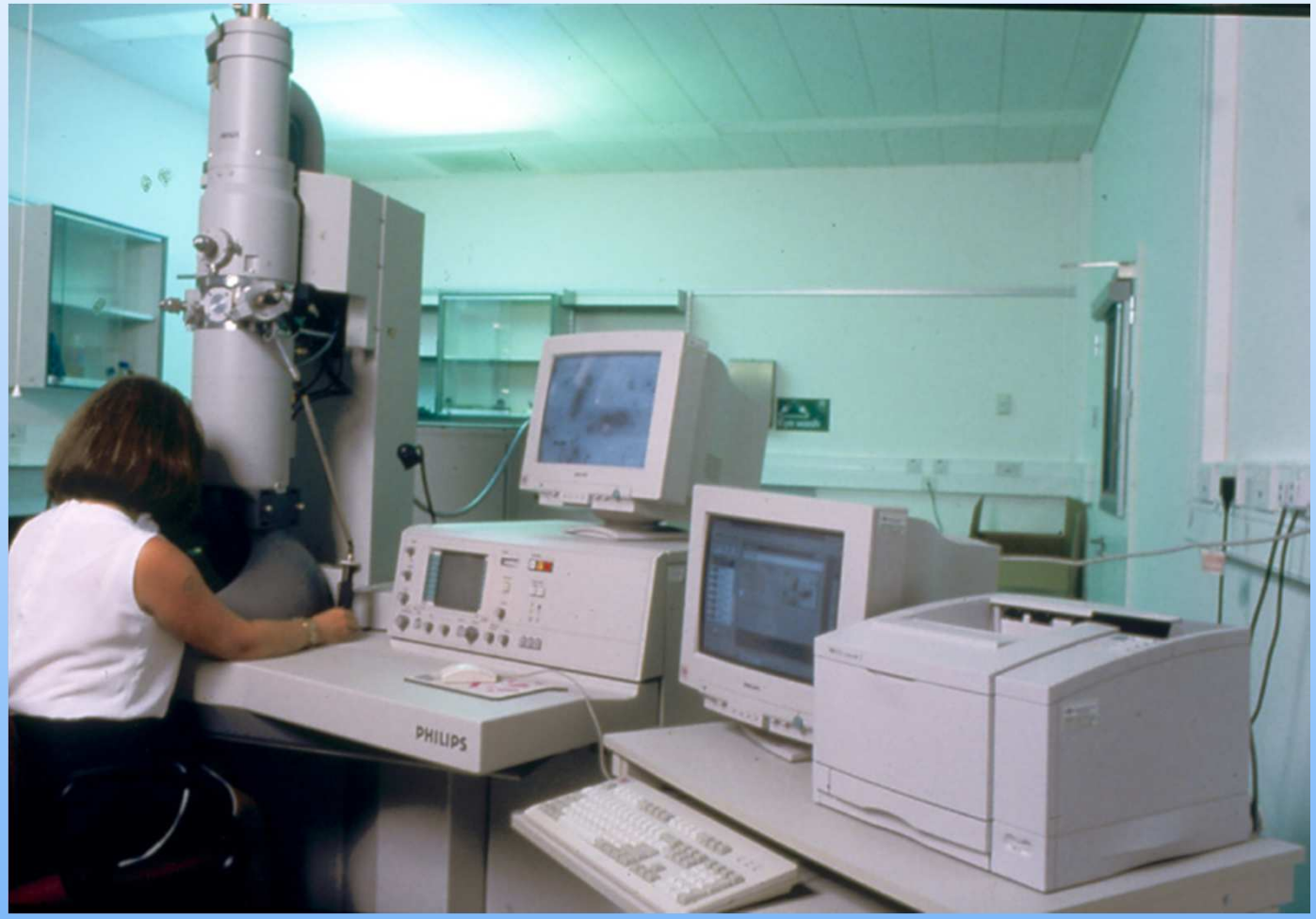


- method used for sequencing on a large scale

Mechanical inoculation-indicator plants

Local damages on indicator plant

Electron microscopy



Thank you