Searching for microbes
Part XI.
Detection of nucleic acid

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To practical of VLLM0421c

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ICQ 242-234-100

Content of this slideshow

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Tale

- Whed Lipold, son of a peasant from Grillowitz, suburbs of Brno, died in 1184, his death was connected with suspicions. Was it really tuberculosis, what caused his death? People said, that his brother Diepold was jealous, because Lipold‘s part of family field was better and more sunny.
- More than eight centuries later, the truth will be found: Lipold‘s death was caused not by Diepold, but by *Mycobacterium tuberculosis*

What to learn from the tale

- Nucleic acid detection is a very sensitive and precise method – sometimes so sensitive, that its sensitivity has to be restricted artificially to avoid detection of accidentally found components
- An interesting fact: microbial DNA may be found in bones centuries old
- These methods are used mostly in situations, where other direct methods are complicated and poorly accessible

Methods of nucleic acid detection

Nucleic acid detection

- Methods without amplification (genetic probes). They are less sensitive, sometimes it is an advantage
- Polymerase chain reaction (PCR) is a very sensitive method, one molecule of DNA is remaining. It is possible to decrease the sensitivity artificially. *PCR was developped by Dr. K. Mullis, who got Nobel prize for PCR in 1993.*
- Ligase chain reaction (LCR) is very simillar (but implemented by another company)
- Detection of viral RNA is possible using adapted PCR

More classification of PCR with regard with various target molecules of amplification

- **specific PCR** (specific gene for an enzyme, pathogenicity factor etc.)
- **„multiplex PCR"** (several specific target sites in one reaction)
- **universal** (target site = a gene possessed by all bacteria – e. g. 16S rRNA)

Some more possibilities

Real-time PCR

- often used in practice
- product is fluorescence labelled (more way besides gel/ethidiumbromide and ELISA)

DNA mikroarray ("microchip")

- DNA chip (known also as gene chip, or biochip) is a set of microscopical dots of DNA placed on a solid surface (e. g. glass, plastic or quartz), used for express profiling, i. e. measuring of level of expression of thousend of genes in the same time.

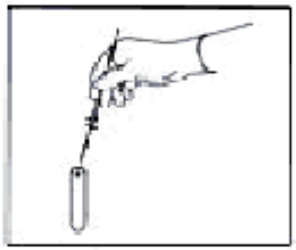
A genetic probe

www.pemed.com

Use of a genetic probe

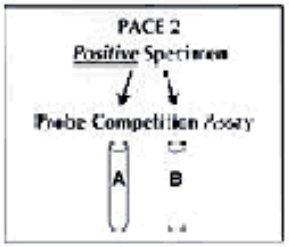
www.chlamydiae.com

Probe Competition Assay for *Chlamydia trachomatis* and *Neisseria gonorrhoeae*



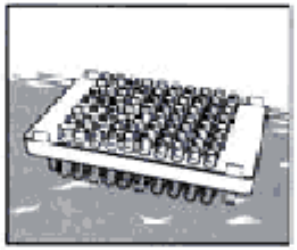
1. Sample Preparation

Vortex each specimen, express and discard swab.



2. Probe Competition Assay (PCA)

Run controls and specimens in duplicate using GEN-PROBE and PCA reaction tubes (A and B).

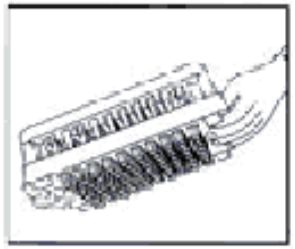


3. Hybridization

Pipette probe reagent into all tubes and incubate at 60°C for one hour.

4. Separation

Incubate with separation suspension in a 60°C for 10 minutes. Separate magnetic particles and decant.



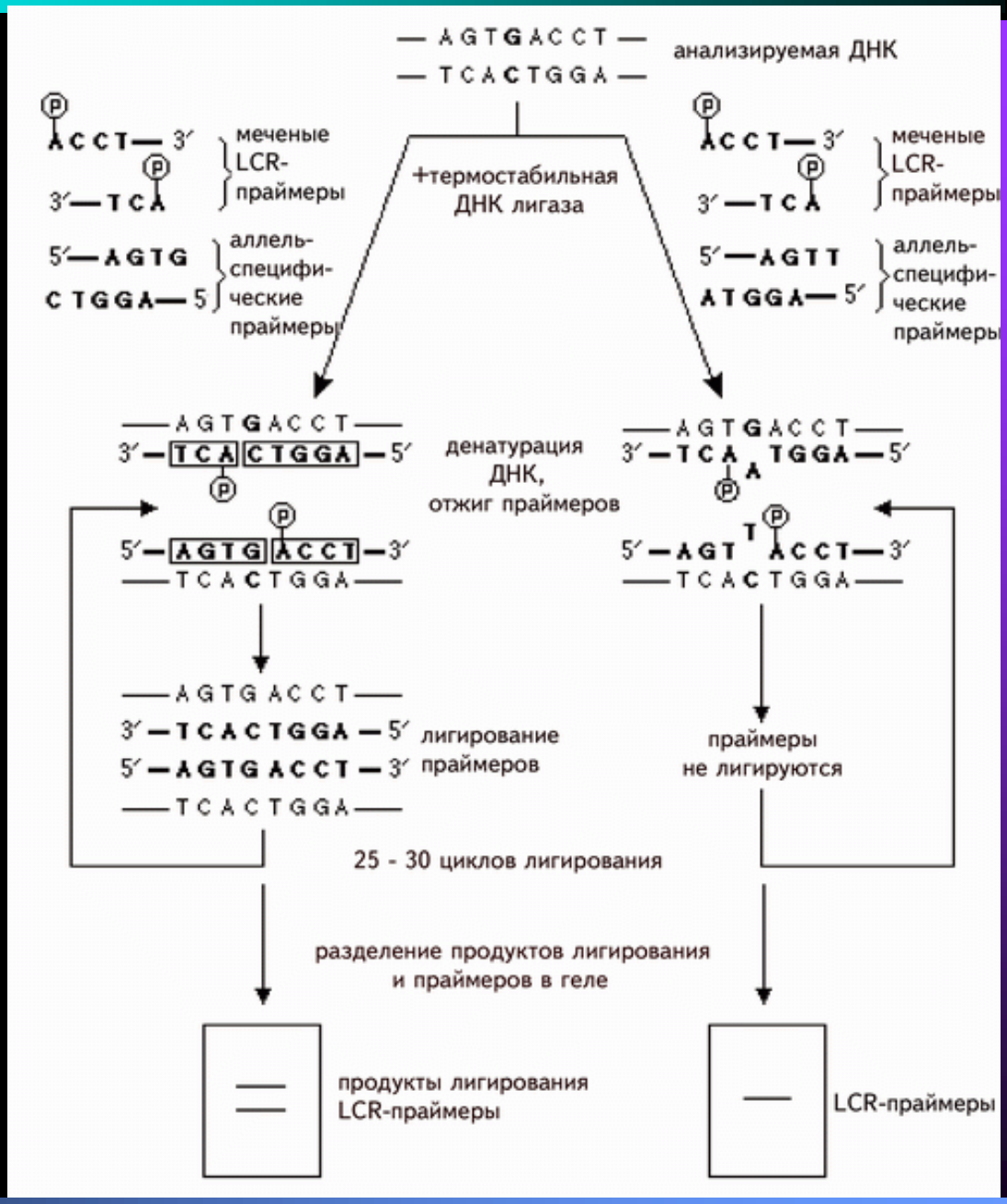
5. Wash

Fill tubes with wash solution; incubate at room temperature for 20 minutes.

6. Detection

Using appropriate protocol, read the chemiluminescent response with your GEN-PROBE luminometer.

LCR in Russian ☺

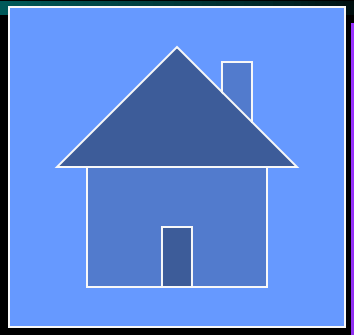


molbiol.edu.ru/review

Important note

- We are not going to teach you principles of molecular methods. You have it in other subjects.
- We are only going to show you how to use that methods in medical microbiology.
- *Individuals with more profound interest fot this problematic can register dr. Filip Růžička's subject VSMB081 subject in 3rd – 5th year (but until now in Czech only)*

Use of DNA (RNA) detection in medical microbiology



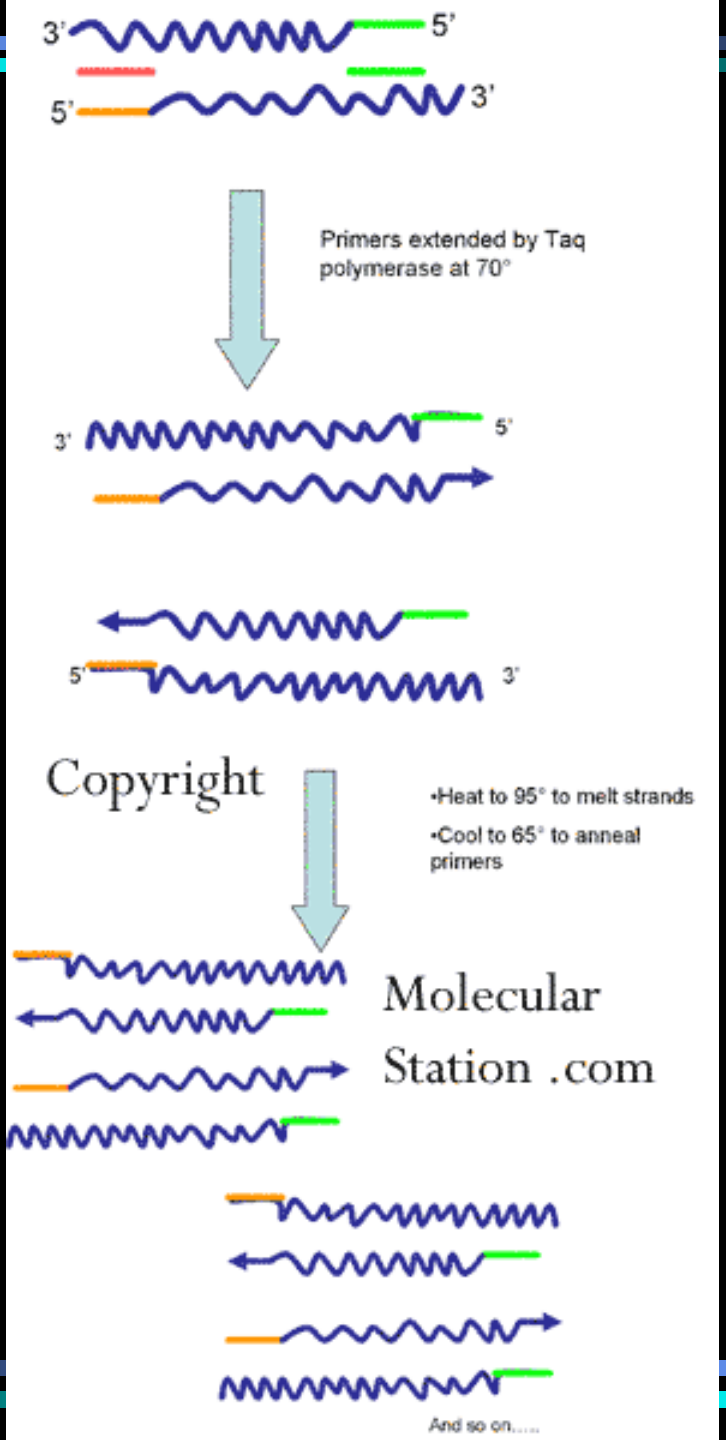
- The methods are used mostly in situations, where microscopic and culture diagnostic is difficult or impossible
- *A typical example is M. tuberculosis*

- It is not very useful for common, ubiquitous pathogens. Because of its sensitivity they would detect accidental molecules comming from environment
- The methods are neither useless, as some people think, nor all-problems-solving, as some other people suppose.

PCR principle

PCR process

toxics.usgs.gov



www.pcrstation.com/images

Basic scheme of PCR reaction

- In first phase we have to get isolated DNA. It is a complex process
- In second phase proper amplification runs (only if the specimen contains a part of DNA corresponding to a primer)
- In third phase amplification product should be detected by
  - gel electroforesis of by
  - ELISA method **(≠ serologic ELISA!!!)**

DNA isolation

- DNA isolation is the process that should precede the proper PCR. The cells should be lysed and their various components removed.

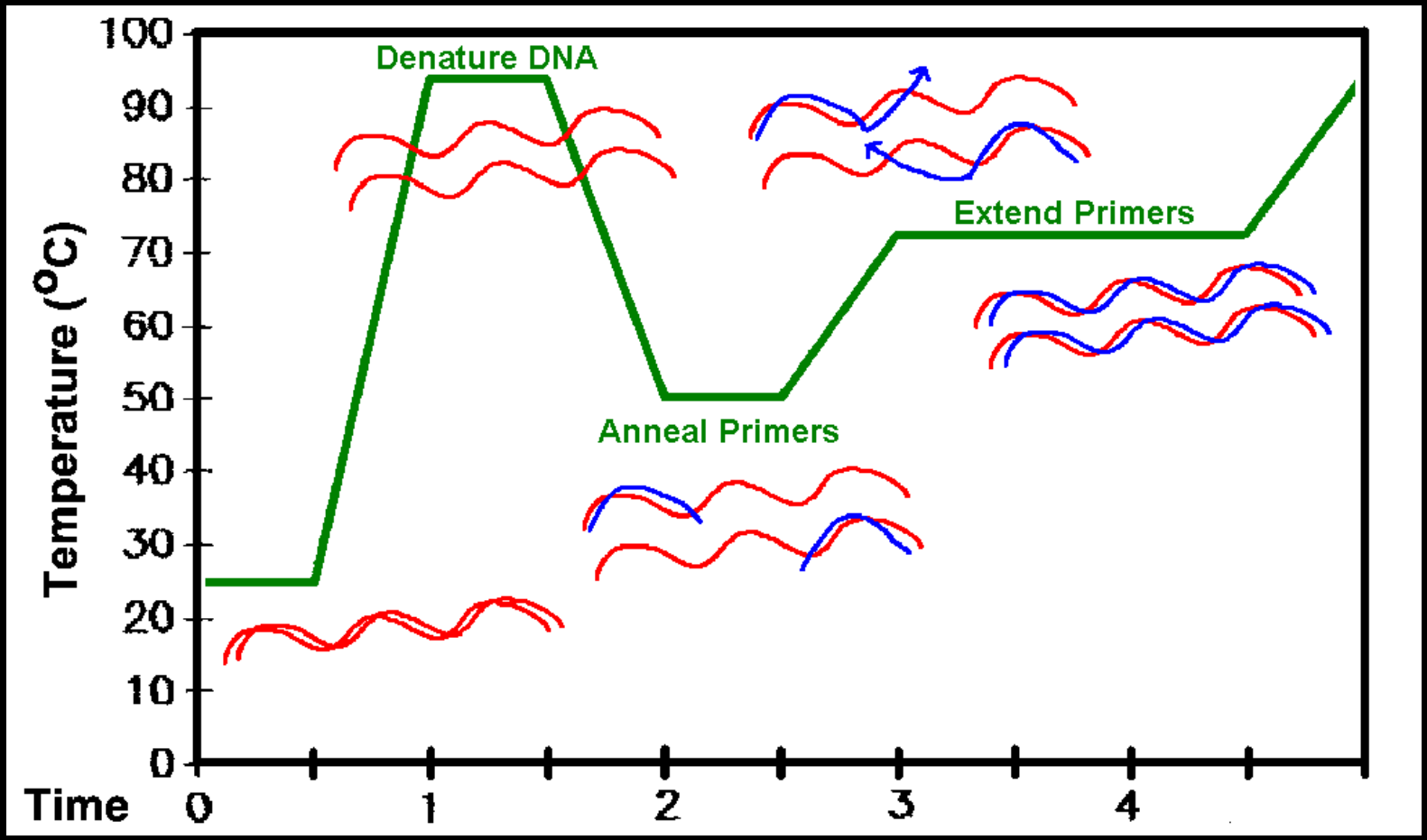
Amplification of specific sections of DNA using PCR method

- Amplification is the clue part of the PCR reaction. It uses Taq polymerase.

DNA isolation course

- Addition of Sarcosyl, vortexing
- Addition of Silica globules, centrifugation, supernatant →
- Addition of G2 solution, centrifugation, supernatant →
- Addition of 80% ethanol, centrifugation, supernatant →
- Addition of aceton, centrifugation, supernatant →
- Drying
- Addition of TE sollution, vortexing, centrifugation
- Removal of the pure DNA-solution to a micro-test tube

PCR and temperature



PCR development was enabled by research leading to finding Taq polymerase from a thermophilic bacterium *Thermus aquaticus*, being able to survive high temperatures.

Thermocyclers

kinich.cifn.unam.mx

http:///images/110.jpg

PCR practically

Why internal control is so important

- Very common situation is so named inhibition of reaction. This inhibition is caused by inferferring agents (talc from gloves)
- Therefore, the mixture for detection should contain not only the specimen and corresponding primers, but also control DNA + primers. Positivity of IC → no inhibition of reaction
- In highly positive specimens, IC might be negative (losing in nucleotide competition). It does not matter – inhibition of reaction is excluded by its positivity

Possible PCR results

The following is valid, not regarding the way of detection (gel electroforesis of ELISA)

- Positive result of a specimen is interpreted as positivity. IC result is usually positive, too, but in highly positive cases sometimes not.
- Negative result of a specimen at positive IC result = negative reaction result
- Both specimen and IC negative = inhibition of reaction

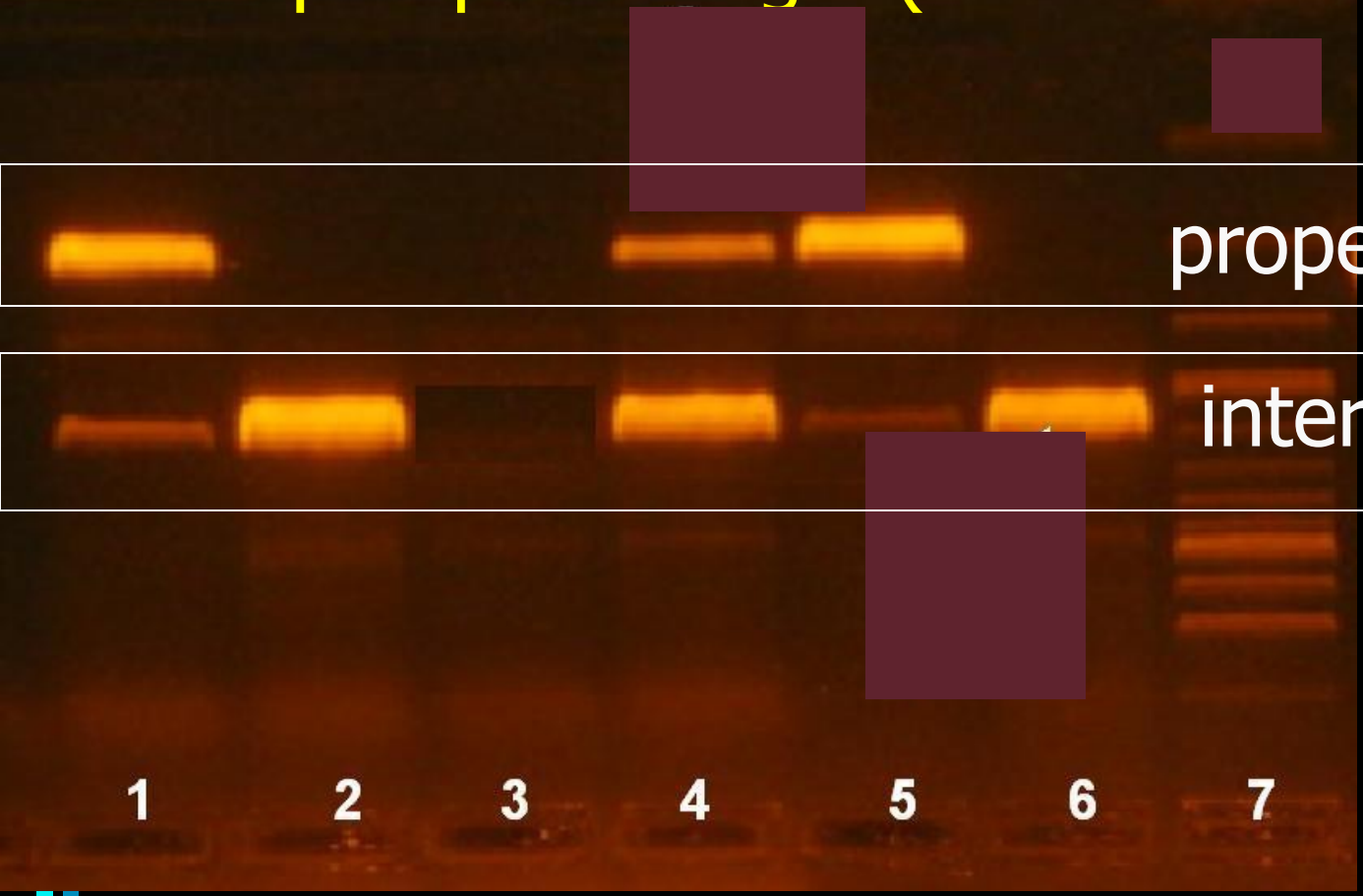
Survey of interpretation

Proper reaction	Internal control	Interpretation
negative	positive	negative
negative	negative	inhibition of reaction
positive	positive	positive
positive	negative	(highly) positive

Detection of PCR results using gel electroforesis

- Gel electroforesis is one of methods of PCR product detection
- The products difund through the gel from a cathode to an anode and they are visualised using a UV-transluminator
- Each specimen encomprises also an internal control (IC)
- Besides the specimens, we have also a ladder for measurement of molecular weight of a reaction product

An expample of a gel (www.medmicro.info)



Patients 1 and 4 – positive, patient 2 – negative, patient 3 – inhibition of reaction. 5 – positive control, 6 – negative control, 7 – ladder

Search for PCR product using ELISA method

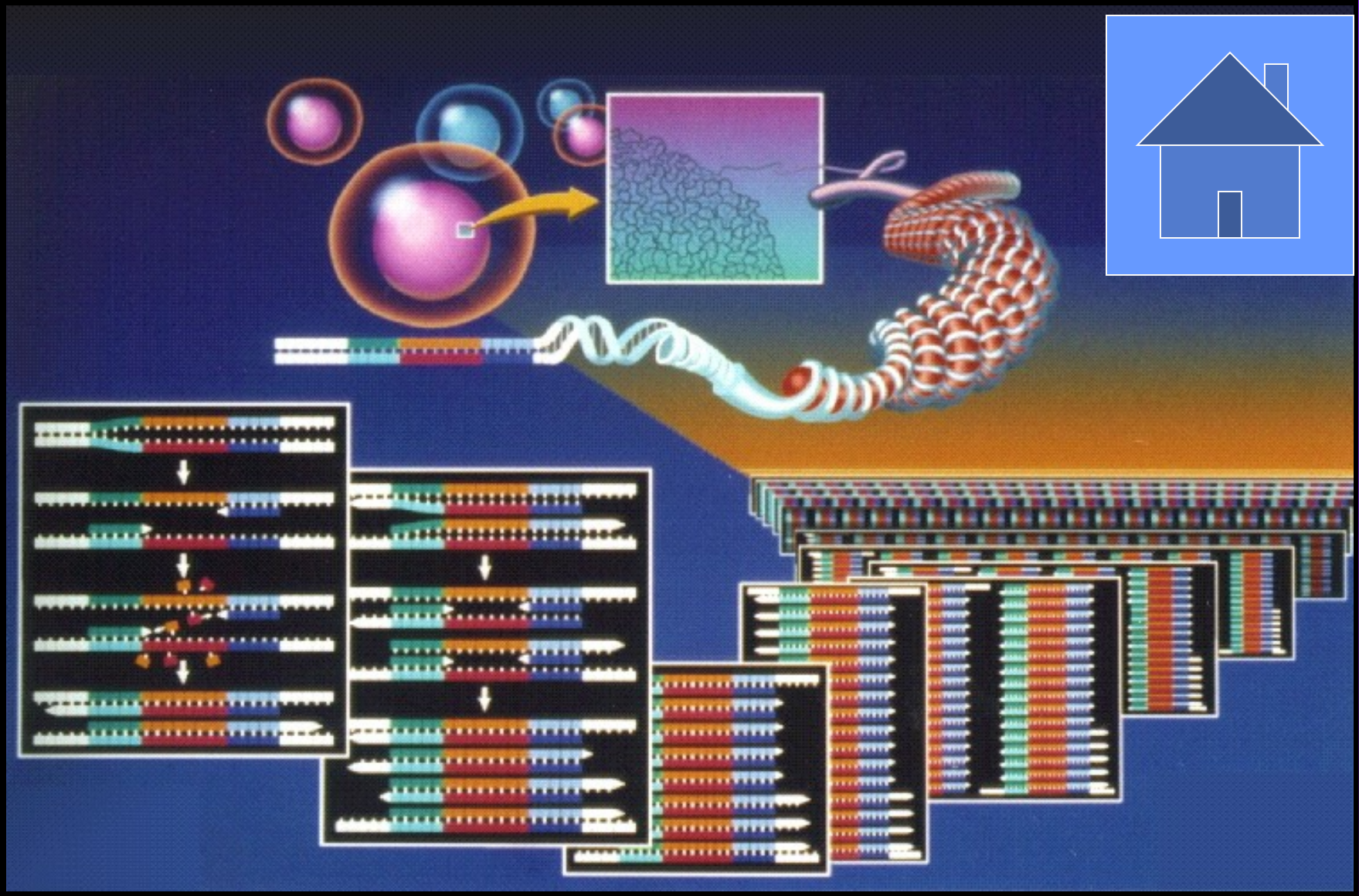
- In J10 practical we have learned, how to use ELISA reaction to antigen/antibody detection
- The approach, using ELISA reaction for PCR product detection, is different: it is not a „serological ELISA", but an ELISA-detection of PCR product. That is why besides the specimen, we have an internal control well, too, in the plate

Comparison of two reactions: antibody detection and PCR

- Result of one reaction is often not certain enough. Therefore, a combination of various reaction results is often used for interpretation.
- In our case, we have a PCR result and ELISA reaction result.
- We have to understand, that PCR is a direct diagnostic method, while ELISA for antibody detection is an indirect diagnostics, with all risks connected with this fact

The End

www.roche.com/pages/facets/1/chlamyde.htm.



Mycobacterium tuberculosis

- It is causative agent of all forms of tuberculosis
- Interesting for this microbe: it lives inside cells. One of consequences of this fact is that the immunity is mostly cellular and antibody response is weak and irregular. So indirect diagnostics is not used in practice.
- Microscopy is possible, but it requires special staining method including heating (Ziehl-Neelsen, see P08 practical session)
- Culture is also possible, but it requires special media and it durates several weeks
- Therefore, PCR is a good solution for diagnostics.