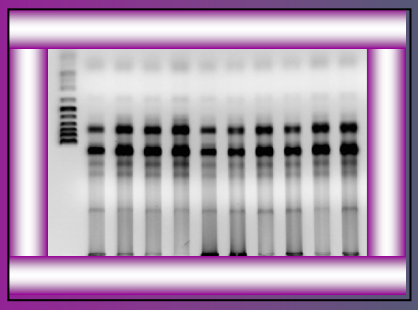
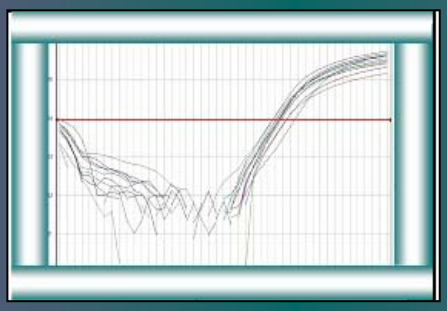
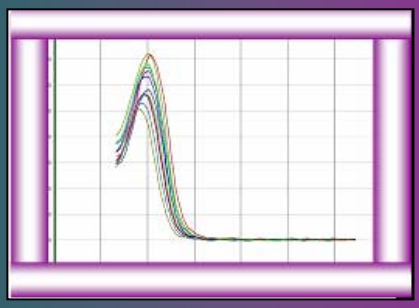
Faculty of Agriculture, University of Belgrade







Laboratory manual
Application of molecular methods in microbiology, biochemistry and plant phisiology

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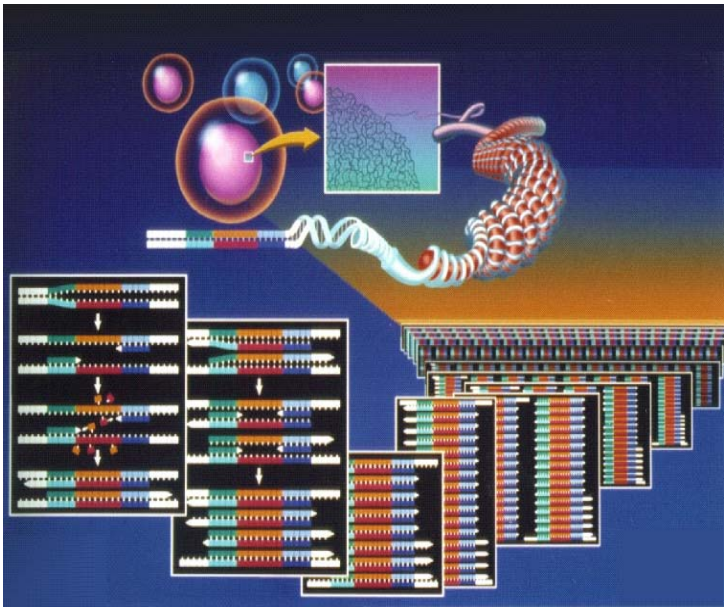
Content:

1. Polymerase chain reaction (PCR)

1.1. Principle of the method

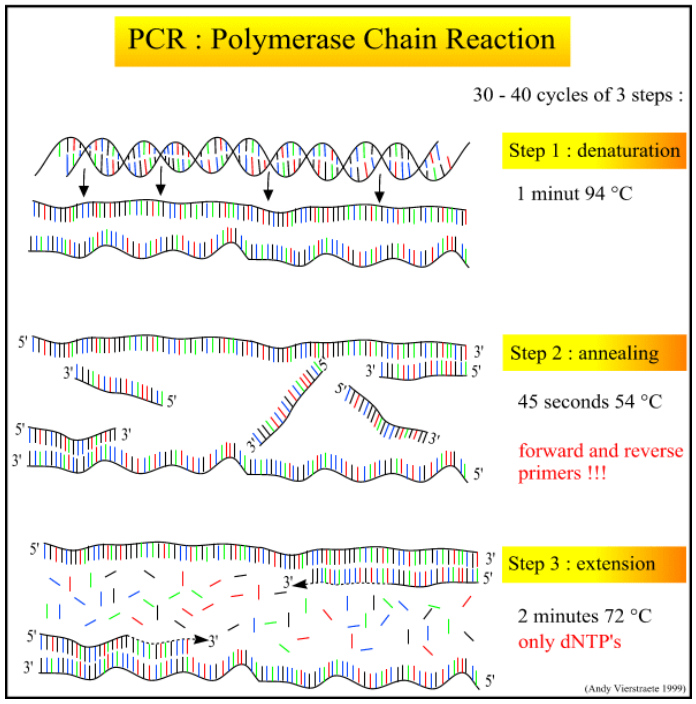
PCR (polymerase chain reaction) is a technique which affords multiplying specific DNA sequence (isolated from bacterial, animal or plant cells). The method is based on the complementary recognition between short segment of a nucleotide chain (called primer) and a fragment in the sample DNA. Transcription of the template part for which primer is specific follows. Template is DNA chain in which recognition between primer and target sequence took place. Sequence of a primer used in the PCR reaction can already be known if a target gene has previously been examined (data on such genes can be found in scientific journals) or if there is a unknown "new" gene, primer can be synthesized on the basis of its sequence (following the rule A –T, C-G) using one of computer programs designed for such a purpose.



Picture 1. Shematic drawing of PCR reaction

Recognition between primer and target gene is a process which involves formation of hydrogen bonds. In base pairs one purine base is always bonded to one pirimidine base. Adenine (A) is bonded to thymine (T) by two hydrogen bonds and cytosine (C) to guanine (G) by three

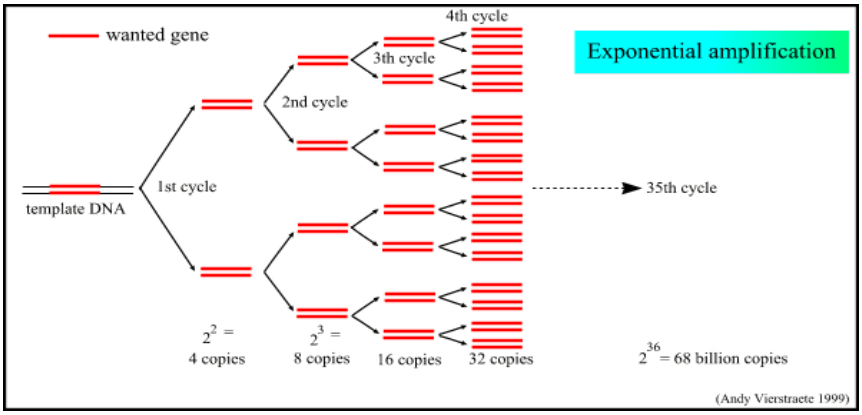
hydrogen bonds. If we divide overall process on phases the first step would be denaturation of double helix at high temperature. Denaturation happens because at higher temperatures atoms move faster (so-called Braunian movement) and hydrogen bonds between base pairs are broken.
To prevent inactivation of an enzyme on high temperatures it is necessary to use thermo stabile DNA polymerase such as Taq polymerase (isolated from bacteria *Thermophilus aquatics*). The next step is annealing process, in which recognition between primers and targeted gene in both DNA chains occurs. Each DNA chain is used as template. This step is needed because DNA polymerase only uses double helix as a substrate. It can ´t perform *de novo* synthesis. Since there are forward and reverse primers, each one complementary to the sequence of one end of the targeted gene, transcription of the DNA molecule between two primers occurs. DNA polymerase only moves on DNA in 3'-5' direction.



Picture 2. Steps in PCR reaction

Denaturation of DNA hybrids composed of one newly synthesized chain and a template chain follows. Then there are multiple cycles of denaturation and renaturation (usually between 25 and 45). Final products of PCR reaction are multiple copies of the targeted gene. This number can

be calculated using the formula: 2^n where n is the number of cycles and number 2 originates from the fact that there are two template chains in each step).



Picture 3. Exponention amplification of copy number

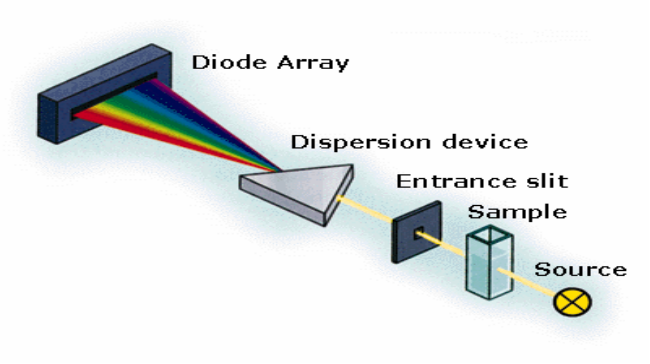
1.2. Parameters that are monitored through the isolation of DNA

Specificity*:** the effect of other components in the mixture on validity of the method; specific method is defined as such method in which other components in the mixture have no influence. ***Limit of detection: lowest concentration of analyte that can be determent (although not precisely). ***Repeatability:*** closeness of the results from two measurements of the same sample taken under the same conditions. ***Reproducibility*:** closeness of the results from two measurements of the same sample but under different conditions. ***Matrix effect*:** any increase in the detection limit or practical limit of quantitative determination of the measured component as a result of the presence of other components in the mixture. ***Positive deviation***: when alternative method gives positive results and reference method negative ones. ***Negative deviation*:** when alternative method gives negative results and reference method positive ones. **"*Relative proofness*":** closeness between results of alternative and reference method expressed as percentage (reference method is determent by National organization for standards, ISO or IDF).

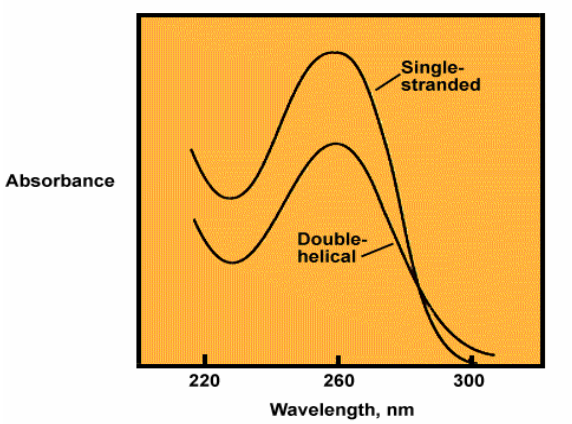
1.3. Size and purity of the isolated DNA

Different methods for isolation of DNA will give fragments of different size. Usual size of fragments is between 20-25 kb and they are completely denaturatued in PCR reaction. Purity of the isolated DNA is determined as the difference of absorbance at 280 nm (wavelength at which amino

acids from proteins absorb) and at 260 nm (absorption maximum of bases from DNA). Pure DNA is characterized by A_{260}/A_{280} ratio between 1.7 and 1.9. In order to obtain correct absorbance readings the value of the absorbance must be between 0.1 and 1.0 because this is a region of Lambert – Beer´s law linearity). Pick symmetry at 260 nm is the proof of isolated DNA purity.



Picture 4. Principles of spectrophotometric determination



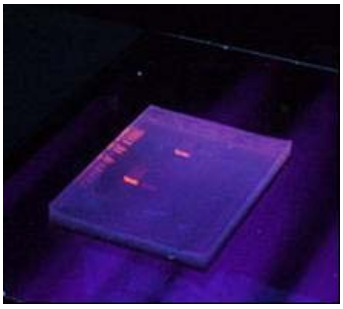
Picture 5. Differences in spectrums of ss and ds DNA

1.4. Visualization of products of PCR reaction

The purity of isolated fragments can be determent by agarose gel electrophoresis (agarose gels are used, because the size of pores is similar to fragment´s size).

Separation of fragments is based on their size differences. In an electrical field shorter fragments migrate faster through the gel. It is possible to determine molecular mass of a fragment by comparing its migration with the position of a fragment of known molecular mass. Molecular mass markers are commercially available.

Visualization is done by incorporating intercalating dyes into fragments. Most usually used dye for agarose gel electrophoresis is ethidium- bromide. Under the UV light, fragments are seen as fluorescent sites on dark background.



Picture 6. Agarose gel with products of PCR reaction

1.5. PCR Protocol for Identification of bacterial strains

♦Put 1 µl of DNA or 1 small colony of bacteria in a sterile 200 µl ependorf test tube

♦Add 12.5 µl Go Taq Green Master Mix (Promega)

♦Add 1 µl primer F

♦ Add 1 µl primer R

♦Add 9.5 µl sterile dH_2O

♦Centrifuge the sample

♦PCR Program: 1 cycle (94 ºC – 1 min and 45 sec.); 30 cycles (94 ºC – 15 sec.); 66 ºC – sec.; 72 ºC – 1 min.; 1 cycle (94 ºC – 15 sec.); 66 ºC – 15 sec. ; 72 ºC – 10 min.

♦Prepare 1% agarose gel (50 ml), add 5 µl gel-red, mix it, pour the gel into the tank and wait for it to solidify

♦Put 5 µl sample in the small well or 10 µl in the big well, add markers in the well and run gel 30 min at 120 V, 400 mA.

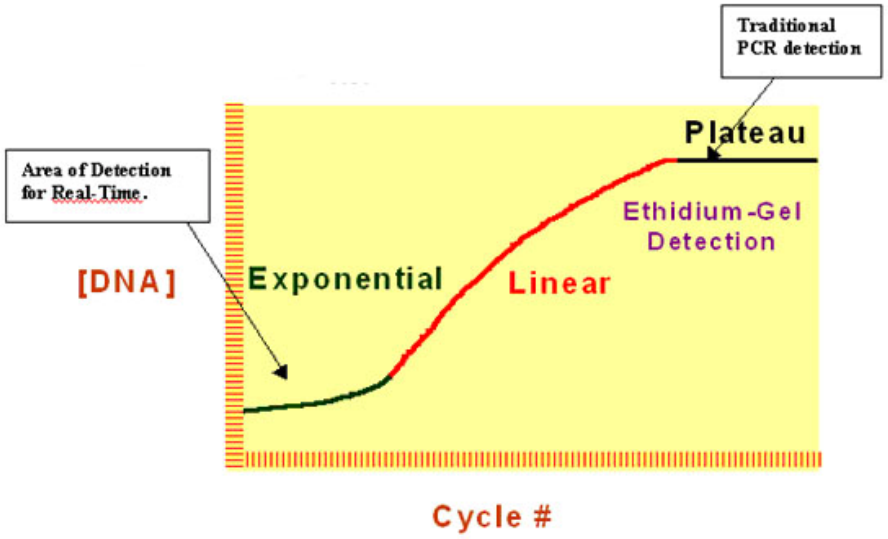
♦ Look at the gel under UV light and observe the results.

2. Real time PCR

2.1. Introduction

Cells in all organisms regulate gene expression and turnover of gene transcripts. The number of gene transcripts in a cell or tissue is determined by the rate of its expression and degradation.

PCR is a technique for amplifying DNA. Real-Time PCR (also called *quantitative real time polymerase chain reaction* (Q-PCR/qPCR) or *kinetic polymerase chain reaction)* is identical to a simple PCR except that the progress of the reaction is monitored by a camera or detector in "real-time". Its key feature is that the amplified DNA is quantified as it accumulates in the reaction in *real time* after each amplification cycle.



Picture 7. Differences in detection of PCR and Real time PCR method

Theoretically, there is a quantitative relationship between amount of starting target sample and amount of PCR product at any given cycle number. Real-Time PCR detects the accumulation of amplicon during the reaction. The data is then measured at the exponential phase of the PCR reaction. In the exponential phase, exact doubling of product is accumulating at every cycle (assuming 100% reaction efficiency). The reaction is very specific and precise. So the exponential phase is the optimal point for analyzing data.

Real-Time chemistry provides fast, precise and accurate results. Real-Time PCR is designed to collect data as the reaction is proceeding which is more accurate for DNA and RNA quantitation and does not require laborious post PCR methods. Traditional PCR

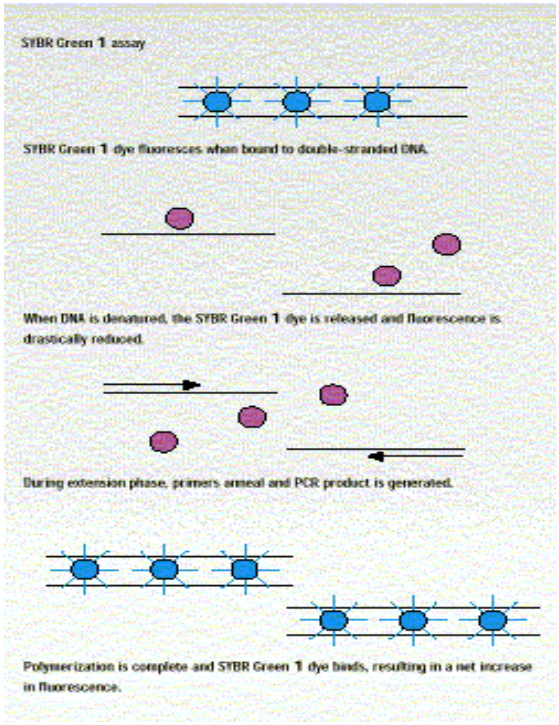
methods for quantization use agarose gels or other post PCR detection methods which are not as precise.

Real-time polymerase chain reaction is often combined with reverse transcription to quantify messenger RNA (mRNA) in cells or tissues.

2.2. Principles of quantization

There is a number of techniques that are used to allow the progress of a PCR to be monitored. Each technique uses some kind of fluorescent marker which binds to the DNA. Hence as the number of gene copies increases during the reaction so the fluorescence increases. This is advantageous because the efficiency and the rate of the reaction can be seen.

Methods of monitoring DNA amplification in "real-time" – There are two principle ways of monitoring DNA amplification: by using fluorescent dyes or fluorescent probes. Although many types of fluorescent probes are available the one most frequently used is Taqman-type probe.

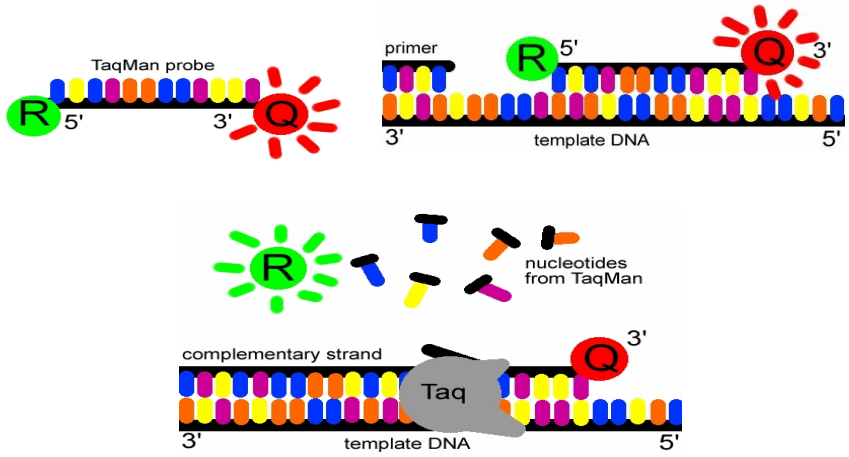


Picture 8. Assay with SYBR Green probe

Fluorescent dyes – The use of intercalating fluorescent dyes (e.g. SYBR green) is the simplest and cheapest way to monitor a PCR in real-time. These dyes fluoresce only when bound to double-stranded DNA. As the number of copies of DNA increases during the reaction so the fluorescence increases. The major disadvantage of using such a dye is the lack of specificity. This dye will report the amplification of any DNA not just the gene of interest.

Types of fluorescent probes

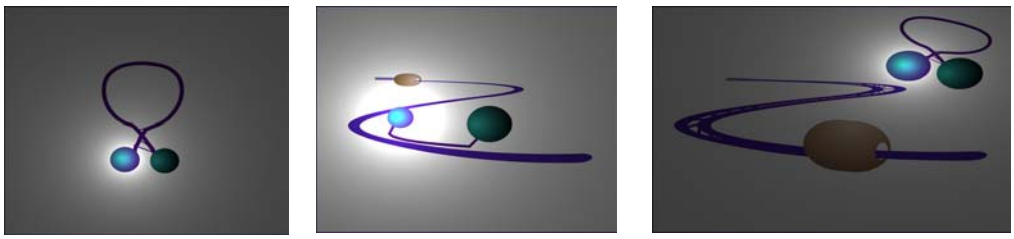
Fluorescent probes - Fluorescent probes are pieces of DNA complementary to targeted gene of interest that are labeled with a fluorescent dye.



Picture 9. Assay with Taqman probe

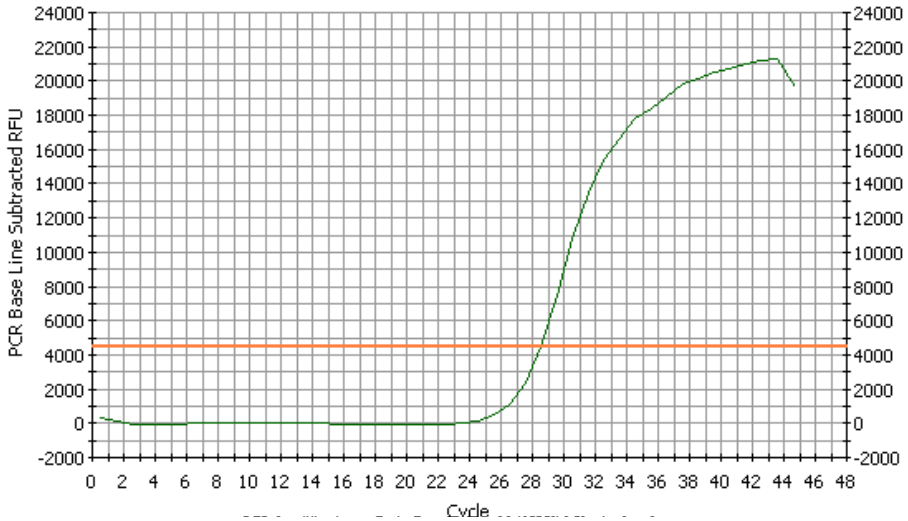
Taqman-type probe - The simplest and most commonly used type of probe is the Taqman-type probe. These probes are labeled with a fluorescent reporter molecule at one end and a quencher molecule (capable of quenching the fluorescence of the reporter) at the other. FRET or Florescent Resonance Energy Transfer technology is the basic principle utilized in this assay. The principle is that when a high-energy dye (reporter) is in close proximity to a low-energy dye (quencher) a transfer of energy from high to low happens. Under the normal circumstances the fluorescent emission from the probe is low while during the PCR reaction the probe binds to the gene of interest and DNA polymerase cleaves the reporter dye from the probe. Once separated from the quencher, the reporter dye emits its characteristic fluorescence and the overall fluorescence increases.

"Molecular beacon" - Another commonly used type of probe is the "molecular beacon". Again, these are small pieces of DNA complimentary to the gene of interest labeled with a fluorescent reporter and a quencher molecule on opposite ends. These probes are designed to fold on to themselves to bring the reporter and quencher to closer proximity and minimize fluorescent emission. However, when the probe binds to the gene of interest the probe takes up a linear confirmation and the reporter and the quencher are separated. This results in the desired increase in fluorescence. Molecular beacon probes are not cleaved by the polymerase but are simply "knocked off" from the probe.



Picture 10. Assay with "molecular beacon" probes

Scorpions - With Scorpion probes, sequence-specific priming and PCR product detection is achieved using a single oligonucleotide. The Scorpion probe maintains a stem-loop configuration in the unhybridized state. The fluorophore is attached to the 5' end and is quenched by a moiety coupled to the 3' end. The 3' portion of the stem also



Picture 11. Amplification plot

contains sequence that is complementary to the extension product of the primer. This sequence is linked to the 5' end of a specific primer via a non-amplifiable monomer. After the extension of the Scorpion primer, the specific probe sequence is able to bind to its complement within the extended amplicon thus opening up the hairpin loop. This prevents the fluorescence from being quenched and a signal is observed.

2.3. Presentation of the results

The output from a real-time PCR reaction is in the form of a graph showing the number of PCR cycles (1 cycle = 90°C, 50°C, 72°C) against the increasing fluorescence. This is known as an amplification plot (Picture 11).

The horizontal line on the graph represents a "threshold" set by the user. The point at which the amplification plot crosses this threshold is known as the Ct (cross threshold) value. Logic dictates that the lower the Ct value for a sample the greater the starting amount of DNA is present in the sample.

2. 4. Methods of quantitation

Two strategies are commonly employed to quantify the results obtained by real-time RT-PCR; the standard curve method and the comparative threshold method. These are briefly discussed below.

Standard Curve Method

In this method, a standard curve is first constructed from the RNA of known concentration. This curve is then used as a reference standard for extrapolating quantitative information for mRNA targets of unknown concentrations. Though RNA standards can be used, their stability can be a source of variability in the final analyses. In addition, using RNA standards would involve the construction of cDNA plasmids that have to be in vitro transcribed into the RNA standards and accurately measured which is a time-consuming process. However, the use of absolutely quantitated RNA standards will help generate the absolute copy number data.

In addition to RNA, other nucleic acid samples can be used to construct the standard curve including purified plasmid dsDNA, in vitro generated ssDNA or any

cDNA sample expressing the target gene. Spectrophotometric measurements at 260 nm can be used to assess the concentration of these DNAs, which can then be converted to a copy number value based on the molecular weight of the sample used. cDNA plasmids are the preferred standards for standard curve construction. However, since cDNA plasmids are not controlled for variations in the efficiency of the reverse transcription step, this method will only yield the information on relative changes in mRNA expression. This, and variations introduced due to variable RNA inputs, can be corrected by normalization to a housekeeping genes.

Comparative C_t Method

Another quantization approach is termed the comparative C_t method. This involves comparing the C_t values of the sample of interest with a control or calibrator such as a non-treated sample or RNA from normal tissue. The C_t values of both the calibrator and the samples of interest are normalized to an appropriate endogenous housekeeping gene. The comparative C_t method is also known as the $2^{-[\Delta][\Delta]Ct}$ method, where

$$[\Delta][\Delta]C_t = [\Delta]C_{t,\ sample} - [\Delta]C_{t\ ,reference}$$

Here, $[\Delta]C_{T,\ sample}$ is the C_t value for any sample normalized to the endogenous housekeeping gene and $[\Delta]C_{t,\ reference}$ is the C_t value for the calibrator also normalized to the endogenous housekeeping gene.

For the $[\Delta][\Delta]C_t$ calculation to be valid, the amplification efficiencies of the target and the endogenous reference must be approximately equal. This can be established by looking at how $[\Delta]C_t$ varies with template dilution. If the plot of cDNA dilution versus delta C_t is close to zero, it implies that the efficiencies of the target and housekeeping genes are very similar. If a housekeeping gene whose amplification efficiency is similar to the target cannot be found then the standard curve method is preferred.

2.5. Isolation of RNA from plant tissue

♦ Cutting and homogenization of plant material in liquid nitrogen

♦ Measure 50-100mg of homogenized plant material in an ependorf tube (the amount of measured material can vary depending from the kind of plant and concentration of *RNA* that can be isolated)

♦ Kits are used for isolation of *RNA* (e.g. *RNA-ase plant mini kit-a*, *Quiagen, Germany*). Isolation is done by the protocol given by the kit manufacturer. During planning of the experiment and before ordering kits one must check, on the basis of experience of other laboratories and using literature data, which kit will be the most useful for work with a particular kind of plant).

♦ Spectrophotometric determination of concentration and quality of isolated *RNA* is done with sample that is diluted 100x in *RNAse*-free water. (e.g. 10µl of sample in 490µl *RNAse-free* H_20). The reading of the absorbance is done at 260nm on a spectrophotometer. After reading the absorbance, calculations are done to determine the concentration of *RNA* in each sample by formula: spectrophotometric conversion x absorption at 260nm x factor of dilation- $RNA_{conc.}= 40 \times A_{260} \times 50$. Total *RNA* yield can also be calculated by multiplying concentration of *RNA with* total sample volume.

Synthesis of cDNA

♦Kits are used for synthesis of *cDNA* (e.g. *First strand cDNA synthesis kit , Fermentas-Lithuania)*. Also, type of kit used depends from the kind of plant and type of tissue. Synthesis is done according to the procedure given by the kit manufacturer.

Design of primers

Before starting an experiment it is necessary to design and analyze primers for targeted genes. This step should be done in one of the programs available from the Internet (e.g. *Primer3* with blast of *National Center for Biotechnology Information:* http://www.ncbi.nlm.nih.gov), or in program that can be given with Real Time RT-PCR apparatus.

Coating the plate

♦A plate has 96 wells for application. It should be properly orientated and it should contain at least two standards (for example place blanc standards, which contain all that is

in sample, except *cDNA*). After that, applay on the plate: 25µl TagMan universal PCR master mix (2x) *or SYBER Green* + 5µl forward primers + 5µl reverse primers + 5µl TagMan probe + 5µl cDNA sample + 5µl RNAse-free H_2O.

Chart 1. *Terminal conditions for two- steps RT PCR :*

1. RT-step	10min./25°C	120min./37 °C	5sec/85 °C	
2. PCR-step	**activ. UNG**	**activ. DNA pol.**	**Each of 40 PCR cycles**	
	2min/50 °C	10min/95 °C	15sec/95 °C (mel.)	1min/60°C (ann.)

Chart 2. *Terminal conditions for one- step RT PCR:*

RT transc.	**activ. DNA pol.**	**Each of 40 PCR cycles**	
30min/48 °C	10min/95 °C	15sec/95 °C (mel.)	1min/60°C (ann.)

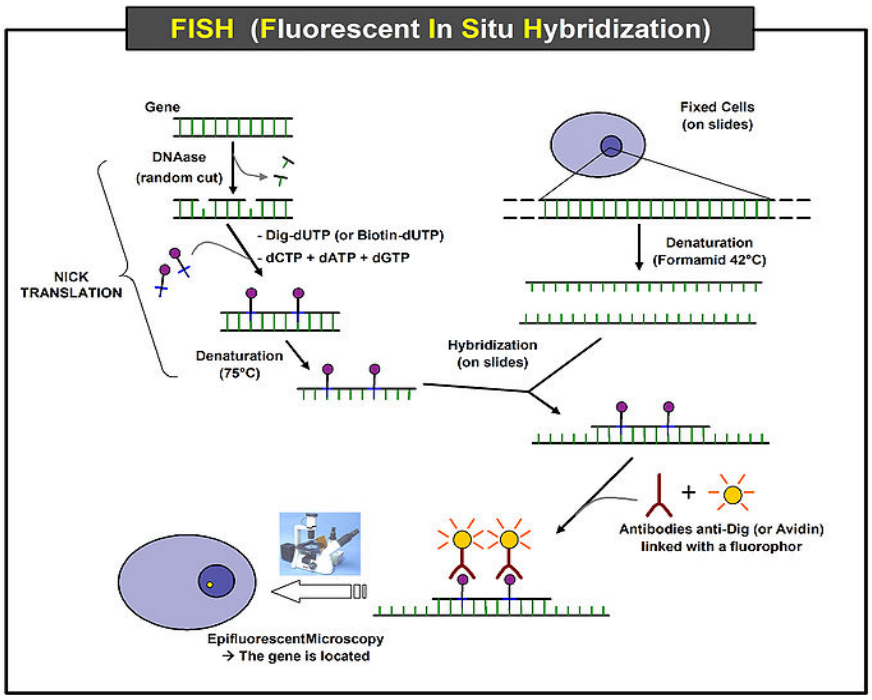
♦Cover the plate with light transparent foil and centrifuge for few seconds (it is important to avoid air bubbles). After centrifugation, place the plate in apparatus close it, choose the working program and mark spots on plate in it. Then determine specific temperature conditions, the number of cycles and start the program. Thermal cycles of RT *PCR* can be done by one-step or two-step method. (Charts 1 and 2). Two-step method enables first completition and then amplification of *cDNA* while in one-step method these two processes are done simultaneously.

♦ After the amplification, save the data and do the analysis of the results.

3. Fluorescence *In Situ* Hybridization (FISH)

3.1. Principles of Fluorescence *In Situ* Hybridization (FISH)

Fluorescence *in situ* hybridization (FISH) is a method used for detection and localization of certain DNA sequences on chromosomes. It is based on sequence similarities between the fluorescent probe and the part of targeted DNA molecule.



Picture 12. Principle of FISH method

Probes are fragments of DNA that were isolated, purified, amplified and labeled with fluorophore, with sites for interaction with antibody or avidin. So constructed florescent probe forms hybrids with sequences in the single-stranded molecule of DNA (mRNA can also be used) with which they show high degree of similarity. Probes can vary in length from a few base pairs for synthetic oligonucleotides to larger than one Mbp. Probe size is important because longer probes hybridize more specifically than shorter ones. There are 3 main types of probes for FISH: locus specific probes**,** centrometric repeat probes and whole chromosome probes. Probes of different types can be used to detect distinct DNA types. Locus specific probes bind to the specific locus in the chromosome. Centrometric repeat probes are usually PCR-amplified repeated DNA sequences, oligonucleotides specific for repeat elements, or cloned repeat elements that are used to detect clusters of repetitive DNA in heterochromatin blocks or centromeric regions of individual chromosomes. Whole chromosome probes are actually collections of smaller probes each of them specifically binding to a different sequence along the

length of a given chromosome. These probes mixtures are often used to detect deletion mutations or translocations in chromosomes. When combined with a specific color, a locus-specific probe mixture is used to detect very specific translocations. If each probe in a mixture is labeled with different fluorescent dye every chromosome can be seen in its own unique color. The resulting full-color map of the chromosomes is known as a spectral karyotype. Fluorescent dyes that are usually used for fluorescence *in situ* hybridization are: Cy 5 (color of fluorescence – far red), Cy 3 (orange), FLUOS (green).

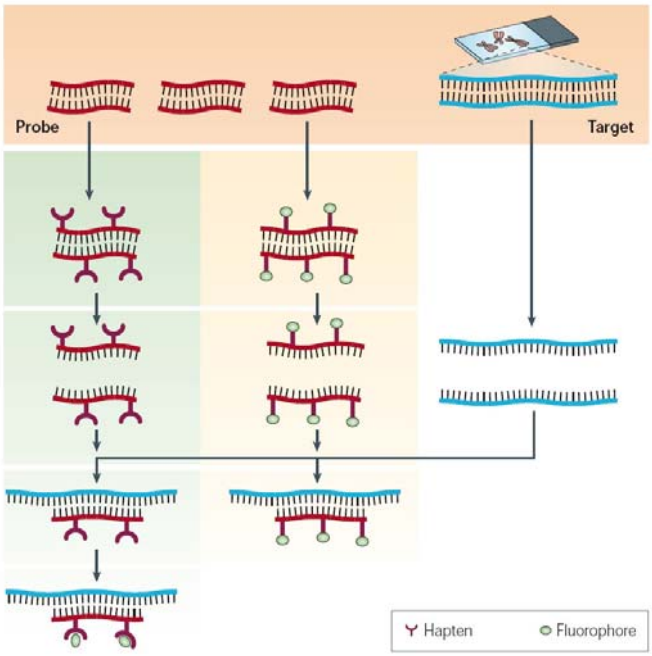
3.2. Procedure

The first step in FISH protocol is construction of a fluorescent probe. The probe is prepared by culturing bacteria or yeasts that contain desired cloned sequence in the selective media. Then cells are harvested lysed and DNA clone is purified and amplified. There are two basic methods for probe labeling: direct and indirect. In direct method fluorophores are associated by chemical conjugation to the probes while in indirect method nucleic acids are chemically conjugated with nonfluorescent molecule that after hybridization binds fluorescent material. In indirect labeling molecule that is directly attached to nucleic acid is usually biotin or hapten (e.g. dinitrophenol or dioxigenin). Tagging of probe can be done in various ways such as nick translation, random priming or PCR using tagged nucleotides.

Because of the existence of BAC (bacterial artificial chromosome) library, sequence of a probe which is specific for particular region of DNA is already known.

The second step is preparation of chromosome mixture. Either intherphase or metaphase chromosome preparation can be used but for interphase chromosome preparation denaturation is needed (at 72 ° C). Molecules of RNA can also be used for the reaction (e.g. for monitoring gene expression in cells or tissue). The mixture of chromosomes is then firmly attached to a substrate, usually glass slide. Pre-treatment of slide is optional and is necessary if the slide is dirty or not dry enough.

To minimize non-specific binding of probe(s), repetitive DNA sequences must be blocked by adding short fragments of DNA to the sample.



Picture 13. Steps in FISH method

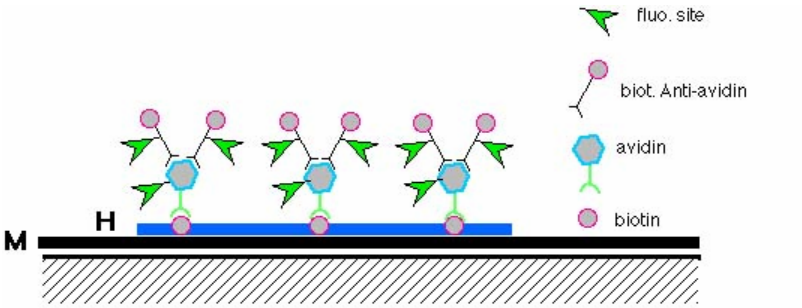
In the third step, the probe or the probe mixture is applied to a mixture of nucleic acids on a slide. This is a hybridization step and it takes about 12 hours to finish. Several wash steps are needed to remove all unhybridized or partially-hybridized probes.

The final step of fluorescent *in situ* hybridization is detection of the probe(s). Probes are recognized by fluorescent antibodies corresponding to the antigens incorporated in the probes. In the biotin-avidin system, the probe has modified nucleotidic sites, which possess a biotin molecule, to which fluorescent streptavidin molecules spontaneously bind. These molecules are then recognized by anti-avidin antibodies with a fluorescent site and a biotin arm. The same construction can be used several times (so-called sandwich of detection system). For large target sequences, nucleic acid probes labeled directly with fluorochromes are used. This direct method is not so time consuming as indirect, but signal of lower intensity is generated.

Visualization and quantization of the results is done by using an epifluorescent microscope that is capable of exciting the dye and recording images.

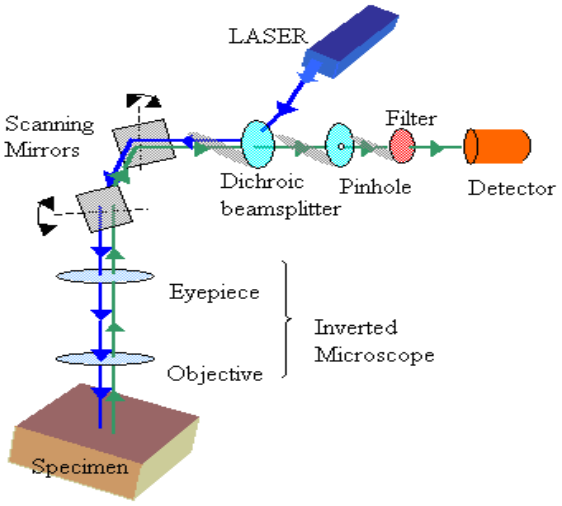
Lamp is a source of the white light. The white light from the source is filtered so that only the wavelengths which can cause excitation of the fluorescent molecules arrive

to the sample. Because the emitted light is of lower energy and longer wavelength a filter between the objective and the detector separates emitted light from the much brighter excitation light. Hybridization sites are seen as fluorescing areas against dark background. High-speed color film or CCD (charge coupled device) cameras are used for collection of data. Quantitation of the hybridization signals is performed using an automated image analyzer.



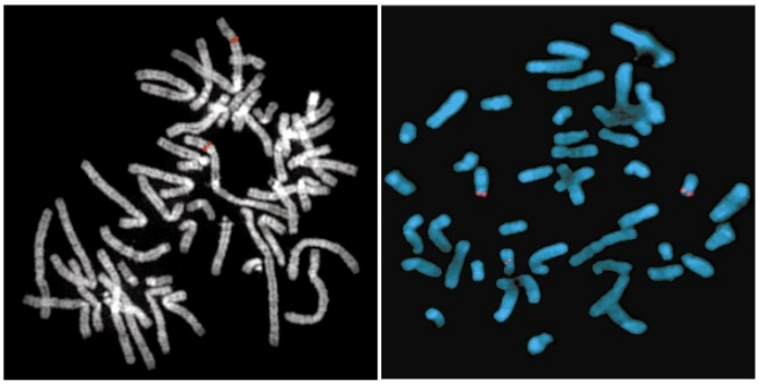
Picture 14. Detection of FISH probe

The strength of fluorescent signal depends on many factors such as type of the probe and type of the dye, probe labeling efficiency, methods used for labeling, number and type of secondary components (antibody or streptavidin).



Picture 15. Principle of analysis by confocal microscope

The main reason for low sensitivity is the fact that the efficiency of hybridization site detection decreases with decreasing probe size. Higher sensitivity can be obtained by building layers of detection reagents, resulting in amplification of the signal.

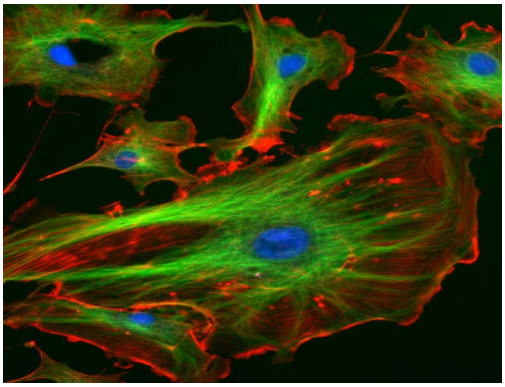


Picture 16. Visualization of results

3.3. Modification of the original technique

There are many variation from original method, such as fiber FISH, multicolor FISH, chromosome orientation fluorescence *in situ* hybridization, centromere -specific FISH. In fiber FISH, interphase or metaphase chromosome preparations, rather than being tightly coiled, as in conventional FISH, are attached to a slide in such a way that they are stretched out in a straight line. This is done by applying mechanical force along the length of the slide. The major improvement of this method is that extended conformation of the chromosomes allows dramatically higher resolution (to few kilobases). Multicolor FISH allows simultaneous detection of labeled sequences in individual cell(s). In this method, each probe is labeled with different color and each simultaneously hybridizes with its complementary sequence. Difference between standard FISH and chromosome orientation FISH is ability of CO-FISH to make hybridization strand-specific. This is accomplished by culturing cells for a single round of replication in the presence of the thymidine analog 5-bromo-2'-deoxyuridine (BrdU). BrdU is incorporated only into the newly synthesized daughter strands. Thus, CO-FISH allows determination of the relative orientation of two or more DNA sequences along a chromosome. Centromere-specific FISH is used for detection and identification of meiotic chromosome abnormalities. This

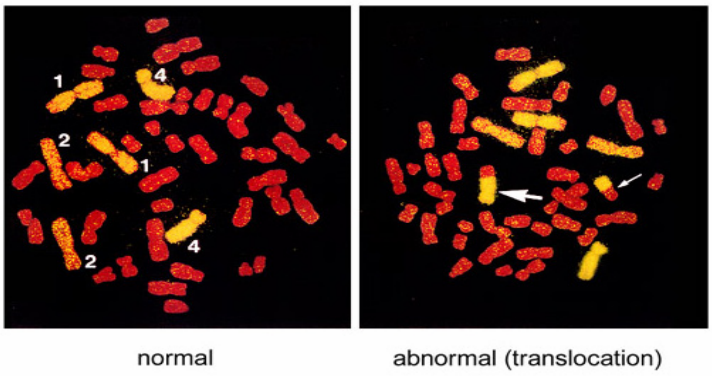
technique employs combination of centromeric satellite DNA probes labeled with five different fluorochromes to allow simultaneous identification of all human centromeres.



Picture 17. Application of multifore FISH method on endothelial cells

3.4. Application of the FISH method

In molecular biology, FISH is used for gene mapping, diagnosis of chromosomal abnormalities, studies of cellular structure and function. Chromosome painting has a major significance for detecting chromosome aberrations, which can cause diseases like Cri-du-chat. Because translocated chromosomes appear perfectly normal, both in shape and length traditional methods of chromosome analysis can not detect these changes.



Picture 18. Detection of chromosomal aberration by FISH method

FISH can be used in medicine to form a diagnosis, to make prognosis, or to evaluate remission of a disease such as cancer, for prenatal diagnosis of inherited chromosomal aberrations, postnatal diagnosis of genetic diseases carriers, diagnosis of

infectious diseases, viral and bacterial diseases, tumor cytogenetic diagnosis, and detection of aberrant gene expression, monitoring of therapy effects, detection of minimal residual disease, origin of cells after bone marrow transplantation, chromosomes abnormalities in non-dividing or terminally differentiated cells, determination of lineage involvement of clonal cell. In laboratory research, FISH is used for analyzing nuclear organization, study the evolution of genomes, analyzing nuclear organization… FISH is widely used in the field of microbial ecology to identify microorganisms. It can also be used in developmental biology to study the temporal expression of genes during differentiation and development.

3.5. Application of FISH method in microbiology for detection and identification of bacteria strains

When the sample is fixed, it can be used in FISH method. The procedure is as following:

Applay the sample on appropriate microscope plate with wells (coated or uncoated – depending of characteristics of cell in question) and let it to dry ;

♦Dehydratate the sample on microscope plate with wells with series of EtOH solutions of different concentrations

♦Place the solution for hybridization with nucleic acid probes on the sample

♦Perform hybridization step at appropriate temperature (ussualy at 46°C) for about 90 min.)

♦Wash the sample to remove unbond nucleic acid probes (at 46°C, for 10-20 min.)

♦Look at the sample under the microscope or store it in refrigerator until microcopy.

Procedure

♦Prepare the sample on microscopic plate with wells (if the sample is pure culure of bacteria) coated with teflone: place a few drops of fixated bacterial cells (2-15µl or more, depending from concentration of cells in the sample or if necessary, calculate the number of cells in the sample) and let it to dry over night or at 46°C for 20min. In the meantime, prepare buffer for hybridization and let it equilibrate at a room temperature.

♦Dehydrate the sample on microscopic plate by washing it with rising concentrations of ethanol solutions (50%, 80% and 100% solution of ethanol for 3 min.).

♦In the meantime melt oligonucleotide probes and desolve them in appropriate random solution (about 30 ng/µl for Cy3 and Cy5 liabeled probes or 50 ng/µl for FLUOS liabeled probes). Keep the probes in dark. It is not necessary to keep probes on the cold if they are not at a room temperature for more than 1h.

♦Place 8-10 µl hybridization buffer in well on microscopic plate but be careful not to scratch teflon surface

♦Add 1µl of each probe again avoiding scratching of teflon surface

♦Prepare hybridization tubes (50 ml falcon tube) – place a part of tissue in a tube and poar the rest of hybridization buffer over it.

♦Put microscopic plate in hybridization tube and place the tube in stirophore carrier, then put carrier in incubator (oven).

♦Incubate hibridization chamber with the sample in hybridization oven at 46°C for 90 min.

♦In the meantime, prepare wasing buffer and warm it at 48°C on wather bath.

♦Wash hybridization buffer from microscopic plate with washing buffer then incubate microscopic plate in washing buffer for 10-20 min at 48°C on wather bath. If quantitation of cells is to follow then the washing procedure should be the same for all the cells.

Rinse washing buffer with cold destilated wather (be careful not to wash the cells) and quickly dry the plate (under compresed air).

Preparation of buffers

Buffer for in situ hibridizion on 46º C: place in ependorfe tube (1ml): 5 M NaCl 360 µl + 1 M Tris/HCl pH 8,0 40µl

% Formamide (v/v)	Formamide (µl)	MQ H_2O (µL)
0	0	1600
5	100	1500
10	200	1400
15	300	1300
20	400	1200
25	500	1100
30	600	1000
35	700	900
40	800	800
45	900	700
50	1000	600
60	1100	500
65	1200	400
70	1300	300

Wasing buffer for in situ hibridization on 46°C: **(washing step – 20min on 48°C)**

mix in 50 ml Falcon tube: 1 ml 1 M Tris/HCl pH 8,0

% Formamide in buffer for hibridization	(NaCl) in mol/l	NaCl (µl) (from 20% formamide to higher concentration, by adding 500 µl 0,5 M EDTA)
0	0,900	9,000
5	0,636	6,300
10	0,450	4,500
15	0,318	3,180
20	0,225	2,150
25	0,159	1,490
30	0,112	1,020
35	0,080	700
40	0,056	460
45	0,040	300
50	0,028	180
55	0,020	100
60	0,008	40
70	0,000	do not add NaCl but only 350 µl EDTA

4. Literature

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