Gene technologies I

EVROPSKÁ UNIE
Evropské strukturální a investiční fondy
Operační program Výzkum, vývoj a vzdělávání

MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY

Tools of gene technologies

1. Restriction enzymes
   Restriction enzymes (restriction endonucleases) are commonly used to digest DNA in specific sites for analytical as well as preparatory purposes.
2. Other enzyme tools
   Other enzymes in gene technologies include DNA ligase, reverse transcriptase and others.
3. Polymerase chain reaction
   Polymerase chain reaction (PCR) is one of most common DNA technology. It can be used to detect, isolate, quantify or label nucleic acids in research, diagnostics, forensics, biotechnologies and other fields.
4. Sequencing
   Determination of nucleic acid sequence underwent a huge progress since its introduction, especially in terms of throughput and price per base.
5. Microarrays
   Microarrays have been used to parallel measurement of concentration of specific nucleic acids, especially mRNA.
6. Gene cloning and recombinant protein expression
   Isolation of DNA (e.g. gene) from one organism and its insertion to another organism is nowadays routinely used in research, biotechnology and other areas, recombinant protein expression.
7. RNAi
   RNA interference can be used to specifically knock out a gene.
8. Gene editing
   CRISPR/Cas system can be used in gene editing to knock out or modify a gene.
9. Gene therapy
   Modification of human genome for disease treatment.

Tools of gene technologies

1. Restriction enzymes

Restriction enzymes (restriction endonucleases) are commonly used to digest DNA in specific sites for analytical as well as preparatory purposes.

Various bacteria have evolved mechanism to specifically cleave viral DNA. They specifically cleave DNA containing certain sequence. At the same time they mask such sequences in their own DNA by methylation, binding of proteins etc. Restriction endonucleases most commonly used in research are type II restriction endonucleases. They recognize a palindomatic sequence and they cleave both strands within this sequence. They are abbreviated by the name of organism, e.g. *Eco*RI is from *E. coli*, *Bam*HI from *Bacillus amyloliquefaciens* etc. They are often produced recombinantly (will be explained later). They are supplied as solutions with high concentration of glycerol, so they are liquid at –20 ºC and they can be repeatedly used without thawing and freezing.

Practically, they are used to verify a sequence. If we know the sequence of some fragment, but we are not sure, it is possible to predict size of fragments produced by cleavage by endonuclease and compare them with sizes determined by electrophoresis (usually agarose gel electrophoresis). Restriction enzymes can be used to specifically cleave DNA into fragments, which can be combined by DNA ligase or other enzymes into a longer constructs. This can be used in gene cloning.

*Eco*RI (from *E. coli*) and *Hind*3 (from *Haemophilus influenza*) are examples of enzymes producing sticky 5'-overhanging ends. *Sma*I (from *Serratia marcescans*) is an example of enzymes producing blunt ends. *Kpn*I (from *Klebsiella pneumoniae*) is an example of enzyme producing sticky 3'-overhanging ends. *Nde*I (from *Diplococcus pneumoniae*) cleaves only methylated DNA.

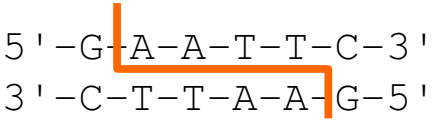
Tools of gene technologies

1. Restriction enzymes

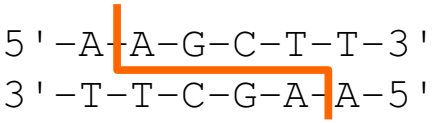
Restriction enzymes (restriction endonucleases) are commonly used to digest DNA in specific sites for analytical as well as preparatory purposes.

Examples:

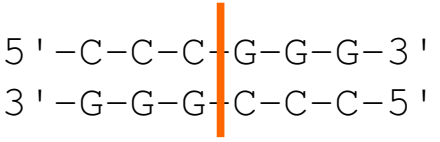
*Eco*RI (from *E. coli*)



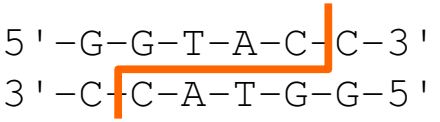
*Hind*3 (from *Haemophilus influenza*)



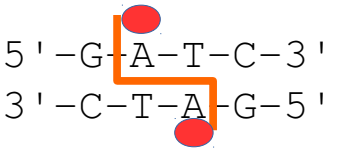
*Sma*I (from *Serratia marcescans*)

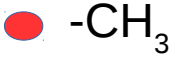


*Kpn*I (from *Klebsiella pneumoniae*)



*Nde*I (from *Diplococcus pneumoniae*)





Tools of gene technologies

2. Other enzyme tools

Other enzymes in gene technologies include DNA ligase, reverse transcriptase and others.

DNA ligase joints two fragments of DNA. Energy required for this is obtained by cleavage of ATP. Natural role of this enzyme is in DNA replication or repair. Typically T4-phage DNA ligase is used in laboratory practice. DNA ligase can easily join two fragments with complementary sticky ends, typically fragments produced by one restriction enzyme. It can also join two fragments with blunts ends, but this is more complicated and different protocol is needed.

Reverse transcriptase (RNA-directed DNA polymerase) is a viral enzyme produced by retroviruses. It can be used to rewrite RNA into a complementary DNA. It is usually applied in sequencing, cloning and quantification of mRNA, but also in other applications. For example, most amino acid sequences of protein were not determined by direct sequencing of proteins. Instead, mRNA was converted to a complementary DNA (cDNA) by reverse transcriptase, isolated and sequenced. The protein sequence was then obtained by simple translation. The cDNA can be inserted into a suitable vector for recombinant production of the corresponding protein.

Tools of gene technologies

3. Polymerase chain reaction

Polymerase chain reaction (PCR) is one of most common DNA technology. It can be used to detect, isolate, quantify or label nucleic acids in research, diagnostics, forensics, biotechnologies and other fields.

DNA polymerases are used by cell in DNA replication, repair and other processes. First DNA polymerase used to amplify DNA in vitro was Klenow fragment (DNA pol I from *E. coli* cleaved by protease to avoid 5'→3' exonuclease activity). This enzyme can synthesize the second strand to a single stranded DNA. Unfortunately it cannot survive denaturation of DNA by high temperature, because high temperature also denaturates the enzyme.

This problem was solved by introduction of thermostable enzymes from thermophilic microorganisms. PCR requires a DNA sample (template), DNA polymerase, magnesium cations, primers (short synthetic oligonucleotides defining the start and the end of the amplified DNA) and activated building blocks (dATP, dTTP, dGTP and dCTP). It is done in cycles (typically tens) comprising 1. denaturation of DNA by high temperature, 2. annealing of primers onto the template, 3. extension of primers by DNA polymerase. All these steps are done in programmable thermostats (thermocyclers). Amplified DNA is selected by design of primers. Various companies sell custom primers for quite low costs.

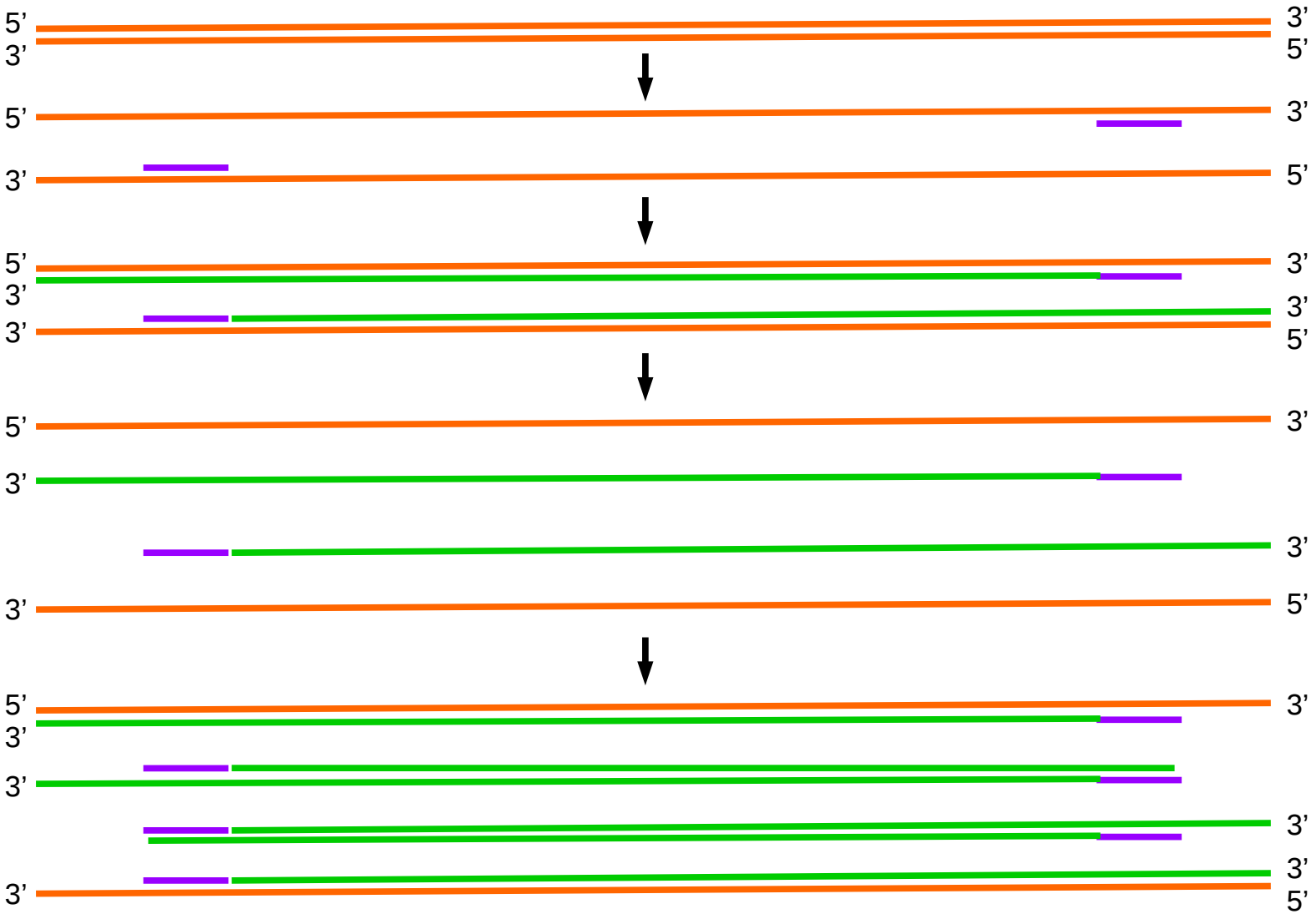
Very often DNA polymerases have not only 5'→3' polymerase but also the 3'→5' and 5'→3' exonuclease activity for "proofreading" and "nick translation".

PCR can be used to detect (e.g. in forensics or diagnostics), amplify (e.g. isolate gene from an organism), quantify, label, mutate or shuffle DNA.

Tools of gene technologies

3. Polymerase chain reaction

Polymerase chain reaction (PCR) is one of most common DNA technology. It can be used to detect, isolate, quantify or label nucleic acids in research, diagnostics, forensics, biotechnologies and other fields.



Tools of gene technologies

3. Polymerase chain reaction

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A quantitative variant of PCR has been developed. It uses different approaches to quantify the product. For example, the amount of the product can be monitored by a fluorescent dye that binds onto a double-stranded DNA and is fluorescent only when bound. The level of fluorescence indicates the concentration of the product and can be used to estimate the concentration of the template.

Alternatively, it is possible to exploit the 5'→3' exonuclease activity. Beside the two primers, another oligonucleotide complementary to the middle of the amplified fragment is added to the reaction mixture. This oligonucleotide is labeled by a fluorescent dye and a quencher of fluorescence. This oligonucleotide is not fluorescent because of proximity of both moieties. Cleavage by the exonuclease activity releases the fluorescent dye which becomes really fluorescent. This phenomenon is known as fluorescent resonance energy transfer (FRET).

Quantitative PCR is used to quantify certain biological material in another biological material (e.g. contamination and adulteration of one food by another food) or in diagnostics. It is also used together with reverse transcription to quantify RNA, mostly mRNA.

Tools of gene technologies

4. Sequencing

Determination of nucleic acid sequence underwent a huge progress since its introduction, especially in terms of throughput and price per base.

The first DNA sequencing was done by Sanger using DNA polymerase, radioactively labeled primers and dideoxynuclotide triphosphates (ddNTP). The reaction was carried out in parallel in four test tubes. Each contained a template, labeled primer, all four dNTP and one of ddNTP (ddATP, ddTTP, ddGTP and ddCTP). ddNTP cannot be extended and causes termination of DNA synthesis. Finally, each reaction is analysed by electrophoresis. Termination by specific nucleotides makes it possible to read the DNA sequence.

DNA sequencing was later improved by fluorescent labeling (replacement of each ddNTP by a color-coded terminating building block) and capillary electrophoresis. This technology was used to sequence human genome.

In last decade, new parallel techniques were introduced. These techniques provide high throughput by parallelization of sequencing reactions. They use reversible fluorescently labeled terminators (Illumina), detection of polymerase reaction using luciferase (454 system) of electrochemistry (Ion Torrent). DNA-polymerase-independent technologies include DNA-ligase-based sequencing (SOLiD) and nanopore-based sequencing (MinION). These modern technologies made it possible to sequence with much higher throughput and at much lower costs.

Tools of gene technologies

4. Sequencing

Determination of nucleic acid sequence underwent a huge progress since its introduction, especially in terms of throughput and price per base.

Template

```
5′–GCGCATGCGCCAATTATAGCTACGATCGATCGGCATCCGATCCGCT–3′
3′–CGCGTACGCGGTTAATATCGATGCTAGCTAGCCGTAGGCTAGGCGA–5′
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Primer

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 5′–CGCATG–3′
3′–CGCGTACGCGGTTAATATCGATGCTAGCTAGCCGTAGGCTAGGCGA–5′
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Products with ddCTP

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CGCATGC
CGCATGCGC
CGCATGCGCC
CGCATGCGCCAATTATAGC
CGCATGCGCCAATTATAGCTAC
CGCATGCGCCAATTATAGCTACGATC
CGCATGCGCCAATTATAGCTACGATCGATC
CGCATGCGCCAATTATAGCTACGATCGATCGGC
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CGCATGCGCCAATTATAGCTACGATCGATCGGCATCC
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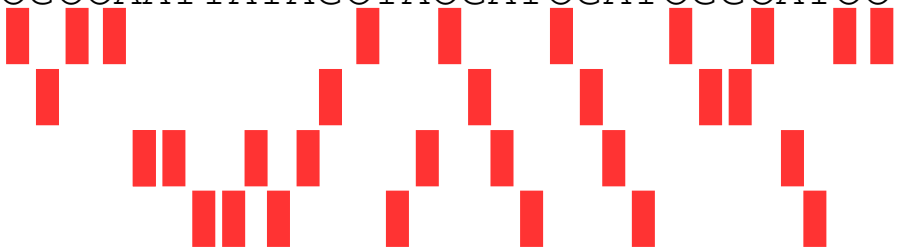


ddCTP
ddGTP
ddATP
ddTTP

Tools of gene technologies

5. Microarrays

Microarrays have been used to parallel measurement of concentration of specific nucleic acids, especially mRNA.

DNA microarrays (or DNA chips) have been used to determine concentration of various nucleic acids in parallel. A DNA microarray contains numerous spots. Each spot contains a different single stranded DNA with known sequence. The sample with labeled nucleic acids is loaded onto the microarray. DNA on the microarray hybridizes with the DNA in the sample. The chip is then scanned to measure intensity of label on each spot. This intensity is proportional to the concentration of the nucleic acid in the sample.

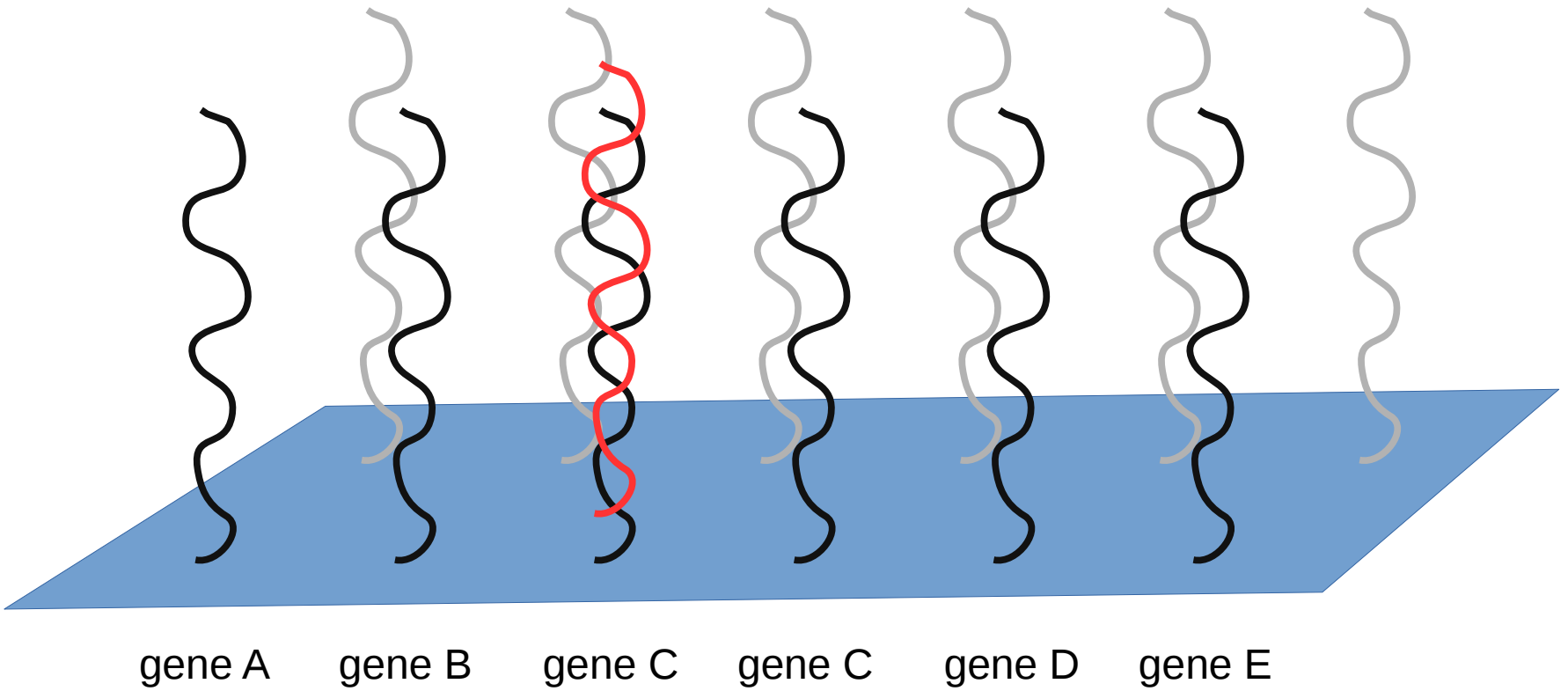
DNA microarrays can be produced by separate synthesis of DNA probes and their application on the microarray surface using micropipetting. Alternatively, DNA probes can be synthesized in situ by photochemically activated DNA synthesis.

DNA microarrays are mostly used to measure mRNA concentration (“gene expression”). mRNA is first converted into cDNA and labeled by reverse transcriptase and then hybridized onto a DNA microarray. Less frequently it can be used to measure DNA content (e.g. to study genome instability in cancer) or to identify DNA molecules recognized by proteins by their coprecipitation by antibodies.

Tools of gene technologies

5. Microarrays

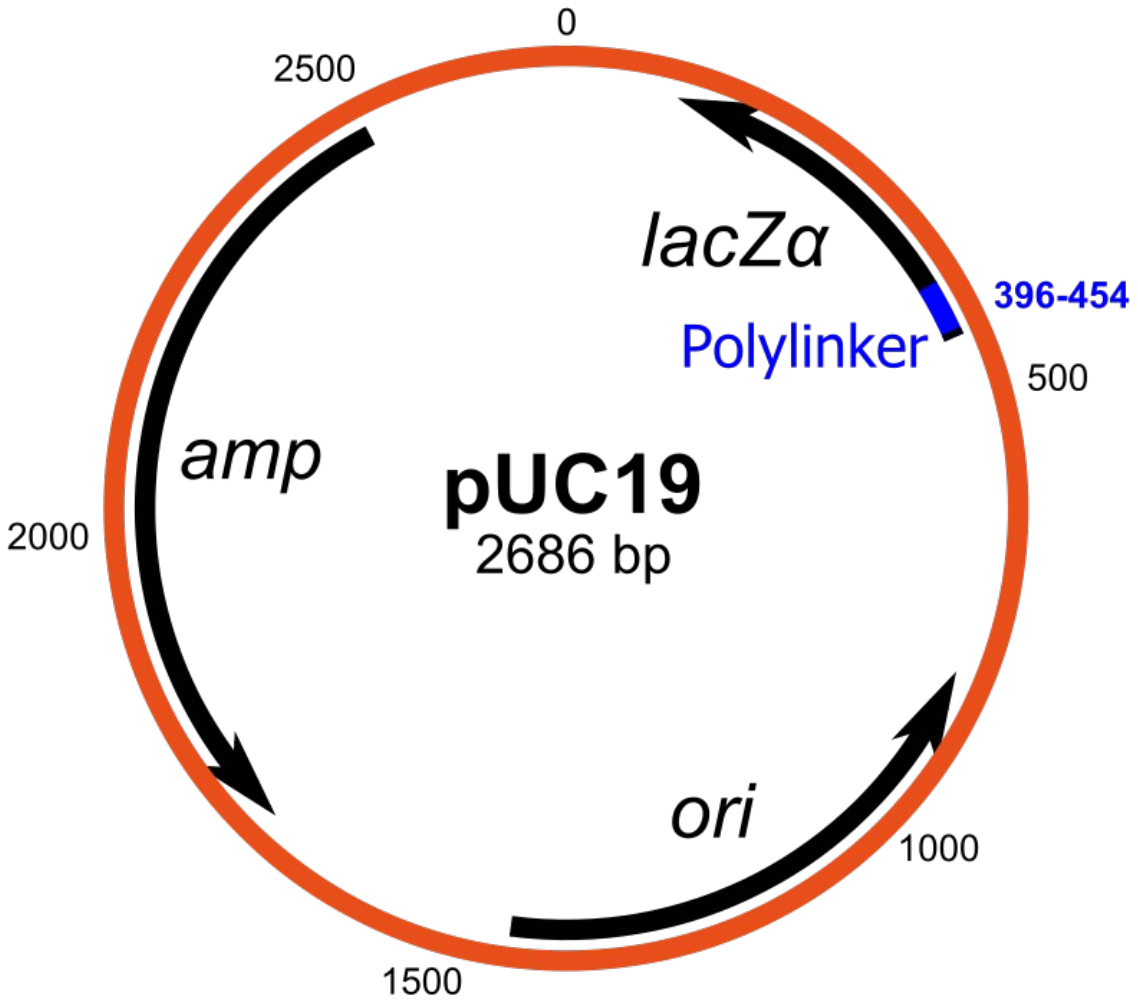
Microarrays have been used to parallel measurement of concentration of specific nucleic acids, especially mRNA.



Tools of gene technologies

6. Gene cloning

Isolation of DNA (e.g. gene) from one organism and its insertion to another organism is nowadays routinely used in research, biotechnology and other areas.

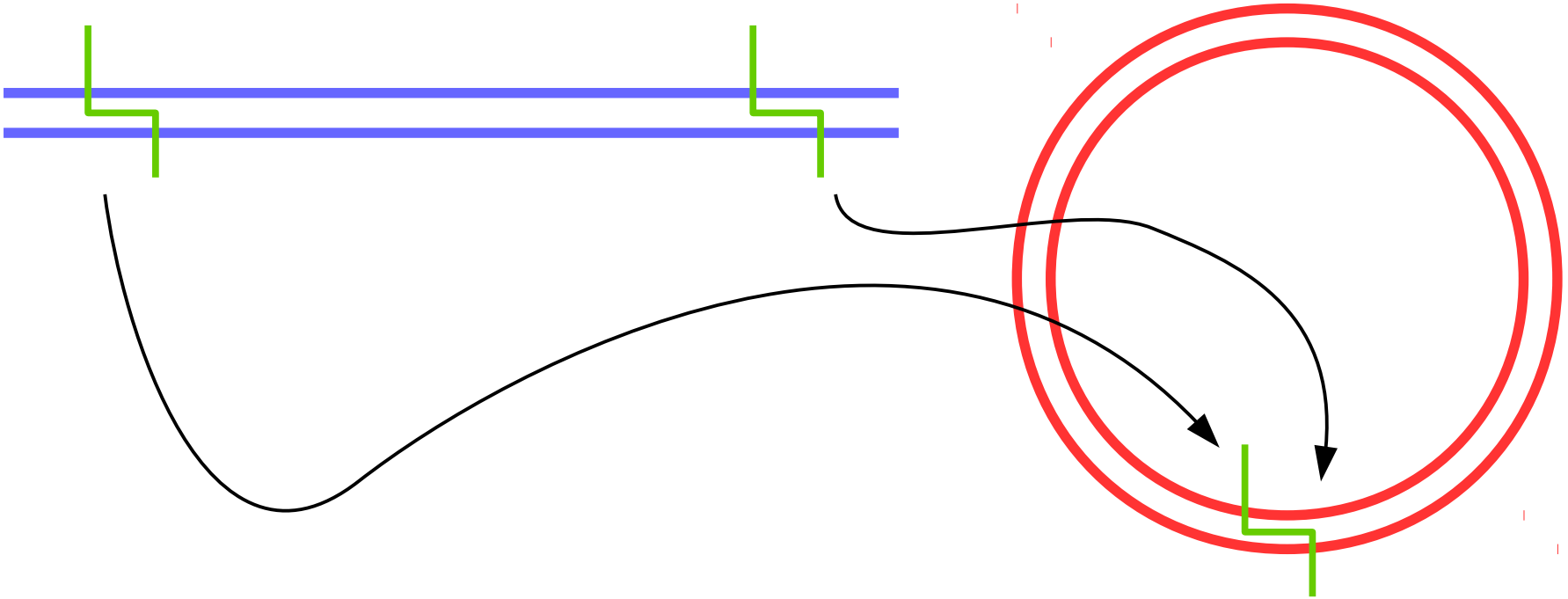


Tools of gene technologies

6. Gene cloning

Isolation of DNA (e.g. gene) from one organism and its insertion to another organism is nowadays routinely used in research, biotechnology and other areas.

DNA can be isolated directly from an organism (by use of proteases and RNases, detergents, heat, chromatography etc.) or by PCR. Next, it can be digested by a restriction endonuclease. Also a vector DNA can be digested by the same restriction endonuclease. These fragments have compatible sticky ends so they can be ligated by DNA ligase. Alternatives for a DNA ligase reaction exist. The resulting construct can be introduced into an organism.



Tools of gene technologies

6. Gene cloning and recombinant protein expression

Isolation of DNA (e.g. gene) from one organism and its insertion to another organism is nowadays routinely used in research, biotechnology and other areas, recombinant protein expression.

Gene cloning can be used to produce recombinant proteins. Isolation of a protein from a natural source is often very difficult or impossible. First, natural material is often difficult to obtain. Second, a cell contains hundreds or thousands of proteins. Complicated series of chromatographic and other separations are necessary. The result can be hampered by losses due to protein instability. As an alternative, the gene coding a protein can be inserted into a suitable vector and produced recombinantly. Usually a molecule of mRNA is converted to cDNA and then inserted to vector to avoid problems with splicing. Prokaryotic expression systems provide high yields, but they are not good for “spoiled” proteins (difficult to fold, with post-translational modifications etc.). As an alternative, it is possible to use yeast (*Pichia*), insect (*Spodoptera*) or mammalian (e.g. chinese hampster ovary – CHO) cells. Protein isolation can be simplified by adding a sequence tag at N- or C- terminus of the protein. Oligo-His, maltose-binding protein (MBP) or glutathion-S-transferase (GST) are most popular sequence tags. They can be isolated by affinity for nickel ions (oligo-His), amylose (MBP) or glutathion (GST). The tag can be cleaved.

Gene technologies are used to produced enzymes or antibodies used in biotechnology or as drugs (biotech drugs).

Tools of gene technologies

6. Gene cloning

Isolation of DNA (e.g. gene) from one organism and its insertion to another organism is nowadays routinely used in research, biotechnology and other areas.

Biotech drugs

Insulin – porcine (1923), chemically or enzymatically humanized (seventies) and recombinant (1982) hormone for diabetes treatment

Bevacizumab (Avastin) – recombinant humanized mouse monoclonal antibody against VEGF (vascular endothelial growth factor) for treatment of colon cancer (inhibits angiogenesis)

Infliximab (Remicade), Adalimumab (Humira) – humanized mouse and human, respectively, antibody against TNF-α (tumour necrosis factor) for treatment of rheumatoid arthritis and other autoimmune diseases

Etanarcept (Enbrel) – fusion protein of TNF-α receptor and Fc fragment of antibody

Epoetin α (Epogen, Procrit, Eprex, Espo) – recombinant human erythropoietin for treatment of anaemia

Tools of gene technologies

6. Gene cloning

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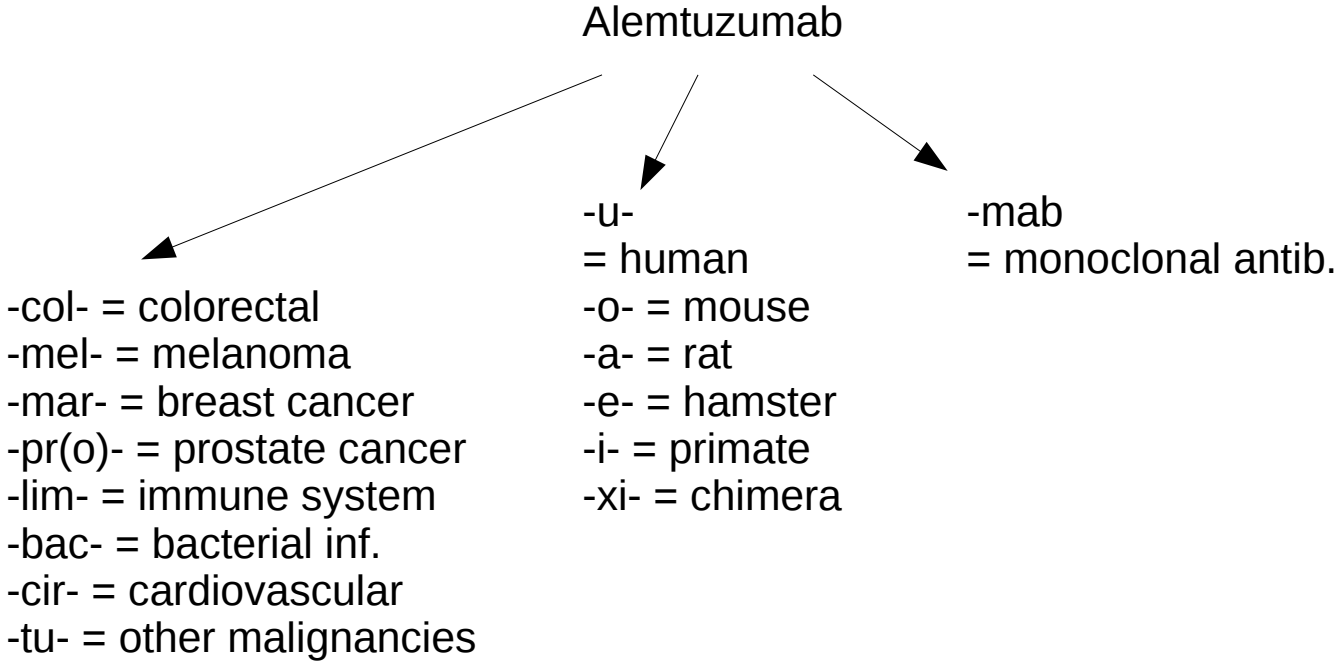
Recombinant antibodies are biotech drugs with high potential

Nomenclature:



Tools of gene technologies

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Isolation of DNA (e.g. gene) from one organism and its insertion to another organism is nowadays routinely used in research, biotechnology and other areas.

Other special applications of gene cloning:

Generation of fusion proteins – two or more proteins can be fused into a single polypeptide chain for research purposes

Fusing with green fluorescent protein (GFP) – example of fusion is fusion with GFP or other fluorescent proteins. GFP is naturally produced by a jellyfish. It can be used to label proteins and to visualize them or track their trafficking or degradation in the cell.

Yeast two-hybrid system – This is a special system to detect protein-protein interactions

Mutagenesis – mutation, random or specific, can be introduced into a gene in a suitable vector

Tools of gene technologies

7. RNAi

RNA interference can be used to specifically knock out a gene.

Neutralization of mRNA by a complementary DNA or RNA used to be a popular as a tool to reduce expression of genes. When doing similar experiments, Craig C. Mello and Andrew Fire found that it is not necessary the neutralizing strand, but it is a double-stranded RNA which causes reduction of gene expression by triggering its degradation. The double stranded RNA is recognized and cleaved by a RISC complex.

This phenomenon can be used to selectively and reversibly knock-down a gene of interest. This can be used to develop model organisms in order to test hypothesis on drug targets. For example, if researchers want to treat some disease by inhibition of an enzyme, they can use RNAi to mimic enzyme inhibition by knocking-down the gene coding the enzyme. It is been also being tested in clinical trials to treat viral diseases or cancer.

Tools of gene technologies

8. Gene editing

CRISPR/Cas9 system can be used in gene editing to knock out or modify a gene.

It was found that bacterial DNA contains repetitive sequences (Clustered Regularly Interspaced Short Palindromic Repeats – CRISPR). They are formed by repeating alternation of variable and conserved blocks. Later it was found that variable blocks are similar to DNA of viruses infecting bacteria. It was shown that this is a bacterial adaptive immune system! Bacteria infected by a virus can digest and incorporate a piece of its DNA into its genome into CRISPR. These fragments are inherited to the offspring of the bacteria. The CRISPR codes short RNA molecules (guide RNA – gRNA) containing a variable and constant part of each repeat. In case of viral infection this RNA can bind to a viral DNA and target it to Cas9 nuclease.

It can be used to specifically knock-out the gene by introducing the gRNA into the cell or into the genome. Several technologies have been developed to modify (introduce deletions, insertions or modifications) into the targeted gene.

This technology is currently used to modify DNA of various organisms or to correct DNA in therapy.

Tools of gene technologies

9. Gene therapy

Modification of human genome for disease treatment.

Gene therapy has been considered for a long time as a viable strategy to treat various diseases. It is possible to replace nonfunctional gene (due to its absence or mutation) by a functional one. It is also possible to introduce genes neutralizing unwanted genes. It is also possible to introduce a “suicide” gene into the diseased tissue.

Unfortunately the progress of gene therapy was very slow. This is due to fact that introduction of some gene into sensitive areas of genome can cause other complications, even a cancer. This was the case of the clinical trial for treatment of X-linked severe combined immunodeficiency in 2003. Several patients treated by this therapy developed T-cell leukemia. Since then, specificity of gene delivery was improved. Several gene therapies have been approved since 2016 (Strimvelis, Tisagenlecleucel, Yescarta, Luxturna).