

How to
BECOME A
MOLECULAR
BIOLOGIST
in 4 days

DNA
& **RNA** **101**

Complete Reference Guide
from **Principles & Data Analysis**
to **Protocols & Troubleshooting**

- ✓ How to optimize & analyze PCR
- ✓ Protocols for DNA & RNA extraction
- ✓ Techniques for molecular cloning, library construction, and more!
- ✓ Tips for PCR and qRT-PCR primer design
- ✓ Complete with troubleshooting guide & FAQs section

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Introduction

Molecular biology has long been the basis for the understanding of each individual step in the biology central dogma: DNA replication, DNA transcription into RNA, and RNA translation into proteins. These molecules are responsible for giving information to cells of each organism on how to survive and reproduce according to the environmental conditions at each exact moment. All this information is stored in the genetic material of cells and transferred to progeny as well.

Manipulation and investigation of this genetic material is done through various techniques including traditional molecular cloning (library construction), Polymerase Chain Reaction (PCR), qRT-PCR, and much more. These molecular biology techniques have various broad and useful applications in our scientific community including:

- In molecular medicine for disease diagnosis, medical therapies and gene therapy
- Generation of new protein products and drug therapies
- Manipulation of organisms for desired phenotypic traits
- Understanding the actions and physiology of the cell

This comprehensive manual will guide you through the basic principles and techniques relevant to current molecular biology research, including protocols and troubleshooting solutions. If you would like a refresher course on the fundamental principles of molecular biology including DNA molecular structure, replication and transcription process mechanics, and more, please refer to the appendix at the end of this handbook.

We at Boster Biological Technology hope that this Molecular Biology ebook will be a useful reference to you at the lab bench. If you ever encounter questions that this guide does not answer, feel free to contact the Boster Support Team by email at support@bosterbio.com. Get better results with Boster!

Key Principles of Molecular Biology Techniques

A. Molecular Cloning Basics (Library Construction)

Molecular cloning is one of the most fundamental techniques of molecular biology used to study protein function and structure. Generally, in this technique, DNA coding for a protein of interest is cloned using PCR and/or restriction enzymes into a plasmid. All engineered plasmids or expression vectors, have 3 main distinctive features:

1. an origin of replication
2. a multiple cloning site (MCS) or multicloning site
3. a selectable marker (usually antibiotic resistance)

In the wild, a certain plasmid can be introduced into prokaryotic cells by transformation via uptake of naked DNA, by conjugation via cell-cell contact or by transduction via viral vector. On the other hand, the plasmid can also be inserted artificially into prokaryotic and eukaryotic cells, such as fungi and plant cells or complex animal cells. Introducing DNA into cells can be achieved by physical or chemical means, and is called transformation. Some available transformation techniques include electroporation, microinjection, calcium phosphate transfection, and liposome transfection. A stable transformation may result, wherein the plasmid is integrated into the genome. Or if the plasmid remains independent of the genome, we have what is called a transient transfection. For transient transfections, since the DNA introduced is usually not integrated into the nuclear genome, the foreign DNA will be diluted through mitosis or degraded over time.

In a more stable transformation, the transfected gene can be inserted in the genome of the cell, which guarantees its replication in the cell's progeny. Usually, a marker gene is co-transformed with the gene of interest, which gives the transformed cell some selectable advantage, such as antibiotic resistance. Some of the transformed cells will (by random chance) have integrated the foreign genetic material. When antibiotic is added to the cell culture, only those few cells with the marker gene integrated will be able to survive and proliferate, while other cells die. This is the process of screening. After applying this selective stress, over time, only the cells with a stable transfection survive and can be selected. Some examples of common agents for selecting stable transfection include: Ampicillin, Kanamycin, Zeocin, Puromycin, Blastidicin S, Hygromycin B, and G418, which is neutralized by the product of the neomycin resistance gene.

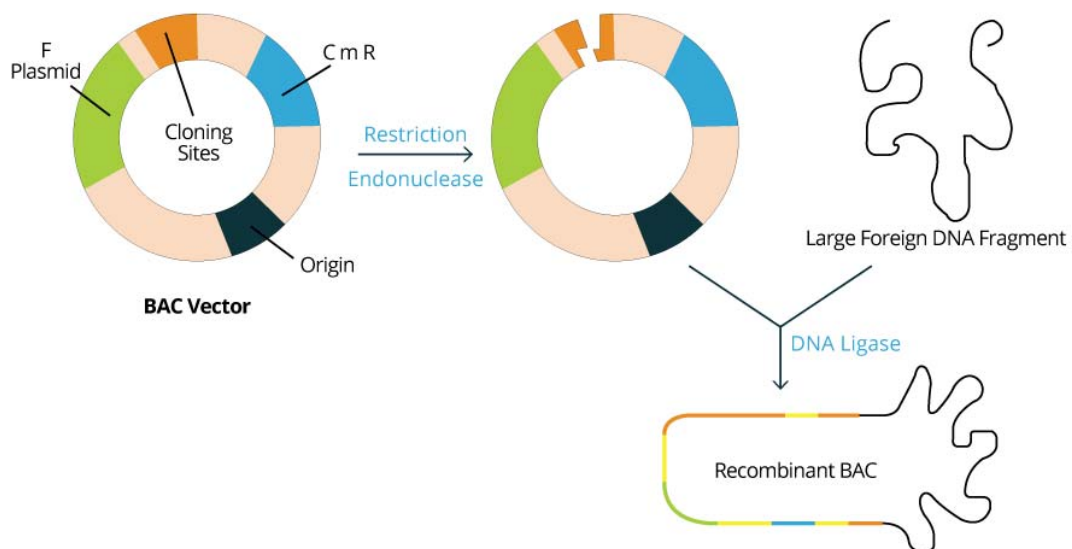
The protein of interest can now be expressed by the cell using the genetic information encoded in the inserted DNA. A variety of systems are readily available to help express this protein at high levels. This protein can then be tested for enzymatic activity under different situations, studied in the pharmaceutical industry, and/or the protein may be crystallized to study its tertiary structure. The possibilities are endless with molecular biology!

DNA library technology is a fundamental technique of current molecular biology, and the range of applications of these libraries vary depending on the source of the original DNA fragments. Molecular cloning techniques allow scientists to create and store a group of DNA fragments from different sources in a suitable microorganism

and take advantage of the cell machinery to protect and replicate these exogenous DNA fragments. In molecular biology, this procedure is known as DNA library construction, and we can store different kinds of genetic material, including: cDNA (formed from reverse-transcribed RNA), genomic libraries formed from genomic DNA, allele mutants, mitochondrial DNA, randomized mutant libraries, and more...

Usually the DNA fragments intended to be stored are inserted into cloning vectors or plasmids, and the type of vector to be used will depend on the host microorganism that is used as the biological library. For example, if the microorganism selected is a bacteria like *E. coli*, it is expected for one to use a BAC vector (Bacterial Artificial Chromosome vector). Whereas if a yeast such as *S. cerevisiae* is selected to store DNA fragments, then a YAC vector (Yeast Artificial vector) is used. The host cell machinery will be responsible for maintaining and replicating the exogenous DNA fragments according to the information contained in the vectors used. The population of bacteria or yeast will be constructed such that each organism contains on average one construct (vector + insert). As the population grows in culture, the DNA molecules contained within them are "cloned" (copied and propagated).

Here we have an example of a BAC vector which is commonly used:



List of vectors available for DNA libraries:

Vector	Properties	Size (kb)
BAC (Bacterial Artificial Chromosome)	Bacteria F factor and origin of replication	75 - 300
YAC (Yeast Artificial Chromosome)	Yeast centromere, and telomere	100 - 1000
MAC (Mammalian Artificial)	Mammalian centromere and telomere and origin of replication	100 - >1000

Chromosome)		
Plasmid	Multicopy plasmids	<10
Phage	Bacteriophage λ	5-10
Cosmid	Bacteriophage λ <i>cos site</i>	35-35

cDNA Libraries

This section goes over the typical experimental setup for cDNA library construction and basic principles. The cDNA library uses mRNA as the source of information, so it only includes the organism's expressed genes from a particular source. This mRNA can be extracted from the organism's cells, a specific tissue, or even an entire organism. mRNA needs to be converted into cDNA by reverse transcriptase, in order to allow the host organism to perform the correct replication and transcription processes for mRNA. This means that it represents the genes that were being actively transcribed in that particular source under the exact conditions (physiological, environmental and developmental) that existed at the time the mRNA was extracted and purified. cDNA libraries are useful in reverse genetics, although they only represent less than 1% of the overall genome in a given organism.

The main advantage behind the choice of mRNA as the source for the DNA library preparation is to avoid junk DNA from genomic DNA and introns from eukaryotic genes. This ensures that only expressed genes from a specific cell or tissue are being stored. Although bacterial cells do not process introns, the use of mature mRNA (which is already spliced) allows the successful expression of the cDNA genes in bacterial host cells for further physiological tests.

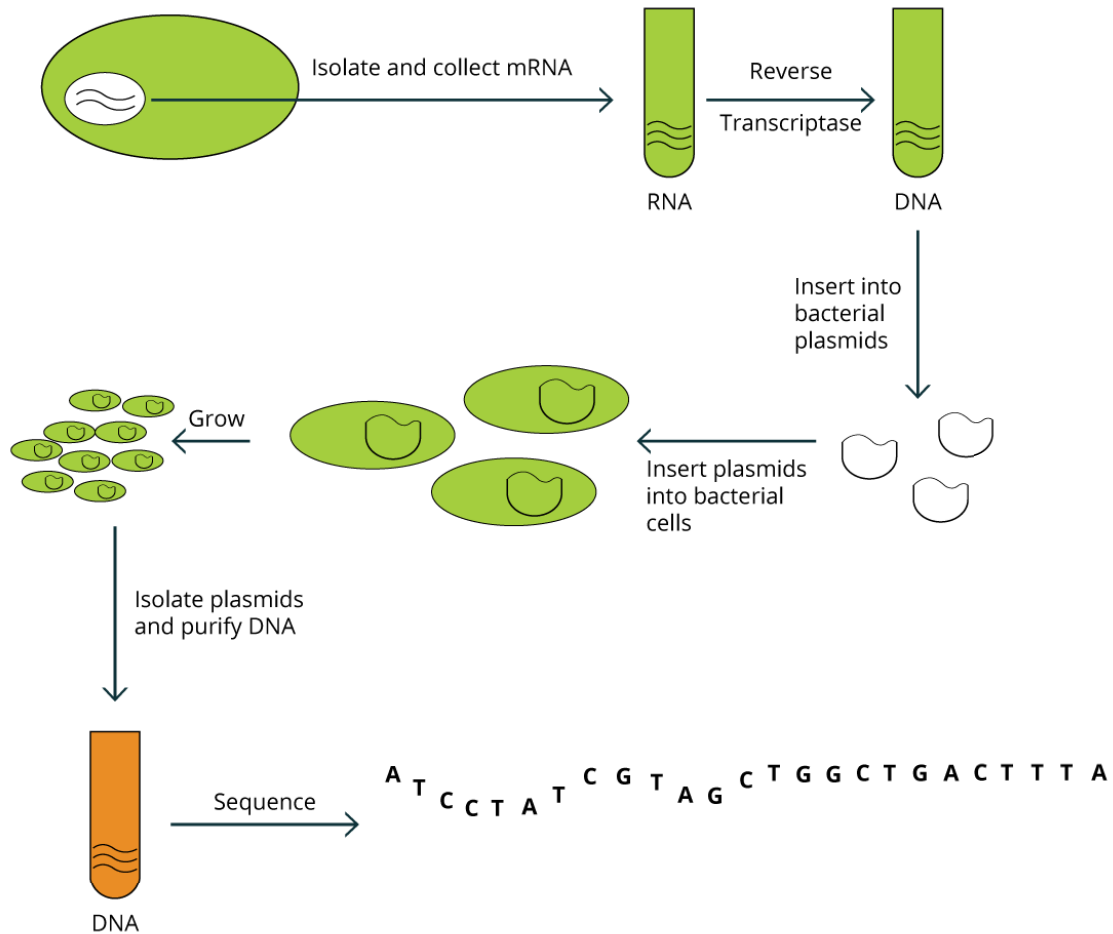
Common applications for DNA libraries are:

- Identification of new genes
- In vitro functional characterization of genes
- Transcriptomic analysis
- Identification of gene versions depending on alternative splicing

cDNA libraries are used to express eukaryotic genes in prokaryotes since it does not include introns, and therefore, can be expressed in prokaryotic cells. cDNA libraries remove the large numbers of non-coding regions from the library, and it is also useful for subsequently isolating the gene that codes for that mRNA.

The diagram below illustrates the general procedure when preparing a cDNA library.

Formation of a cDNA Library



Genomic Libraries

A genomic library is a collection of clones that together represent the total genomic DNA from an organism of interest. The DNA is stored in a population of identical vectors, each containing a different insert of DNA. The number of clones that make up a genomic library depends on:

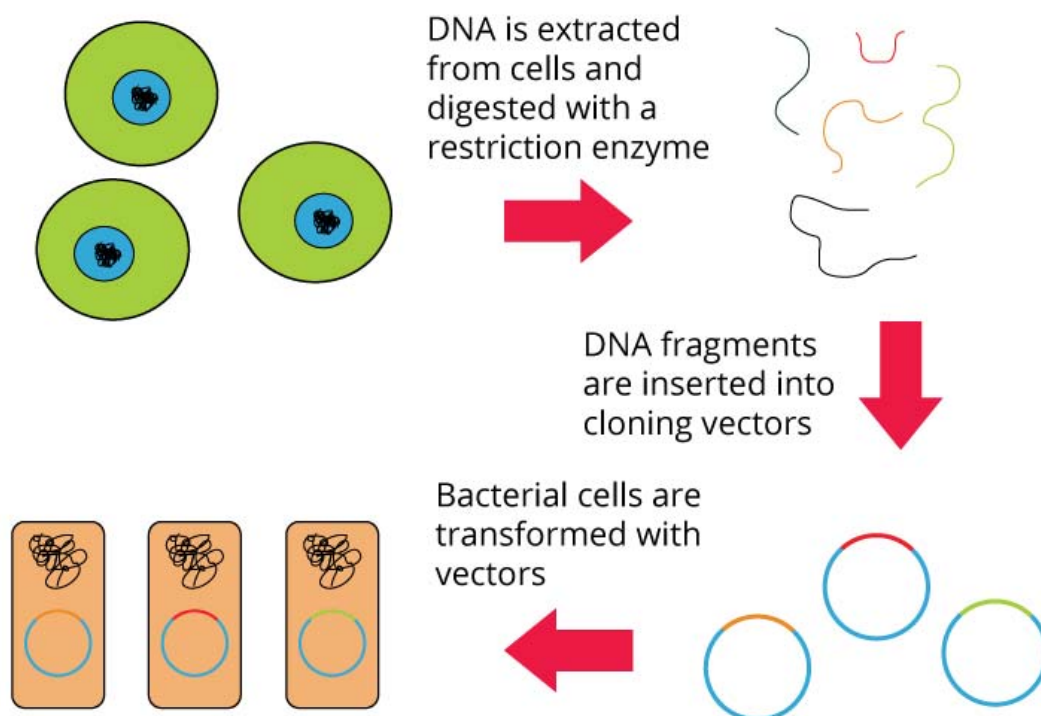
1. The size of the organism's genome
2. The insert size tolerated by the specific cloning vector system

The tissue source of the genomic DNA is usually not important because each cell contains the same DNA (with few exceptions). To construct a genomic library of a specific organism, several variables should be taken into account, such as: host organism, type of vector, cell transcription and translation machinery, etc. However, the most fundamental criteria is to use a host prokaryotic organism if the genome to be collected is from a prokaryotic organism, and the same principle if the genome is

extracted from a eukaryotic organism (which should be stored in eukaryotic host). This selection criteria will be critical to increase the chances of successful heterologous protein expression and allow further physiological tests. This procedure allows us to store the entire genome of a specific organism by inserting it in a specific vector with different fragment sizes depending on the restriction enzymes used. The vectors carrying small pieces of the genome are then transformed in host organism cells for further laboratory analysis.

Shown below are the steps for creating a genomic library from a large genome:

1. Extract and purify DNA
2. Digest the DNA with a restriction enzyme to cut the DNA into fragments of a specific size, each containing one or more genes
3. Using DNA ligase, insert the fragments of DNA into vectors that were cut with the same restriction enzyme. The enzyme DNA ligase anneals or seals the DNA fragments into the vector. This creates a large pool of recombinant molecules which are taken up by a host bacterium by transformation, creating a DNA library.



As an example, prokaryotic organisms from soil are usually targeted for genomic library procedures, largely because they are known for their ability to be resistant to several antibiotics. In vitro culture of these organisms is rather difficult, which impairs physiological tests to identify genes involved in antibiotic resistance.

The typical flowchart protocol for DNA library construction (described in previous image) should be followed. First extract genome from soil organisms, digest the genome, insert into an appropriate vector, and transform in a prokaryotic organism (e.g. *E. coli*). Then, *E. coli* transformants should be plated in LB culture medium containing a specific antibiotic, like kanamycin. Only the *E. coli* transformants that carry genes from soil organisms along with antibiotic resistance marker will be able to grow. Positive colonies should be selected to sequence the vector and identify genes that play a role in antibiotic resistance.

B. Polymerase Chain Reaction (PCR)

PCR Fundamental Principles

Molecular biologists realized that mimicking the cell mechanisms of DNA replication, they would be able to replicate DNA sequences of interest. In 1983, Kary Mullis developed the revolutionary *in vitro* procedure to reach large concentration of specific DNA fragments, which is called Polymerase Chain Reaction or PCR. The PCR procedure allows scientists to copy and amplify specific regions of a DNA molecule (like genes) exponentially.

In brief, PCR allows a specifically targeted DNA sequence to be copied and/or modified in predetermined ways. This reaction has the potential to amplify one DNA molecule to become over 1 billion molecules in less than 2 hours. This powerful and versatile technique can be used to introduce restriction enzyme sites to ends of DNA molecules, or to mutate particular bases of DNA (a technique called site-directed mutagenesis). PCR is also useful for determining whether a particular DNA fragment is found in a cDNA library. Today, PCR has been further developed to include many variations, like reverse transcription PCR (RT-PCR) for amplification of RNA, and quantitative PCR which allows for quantitative measurement of DNA or RNA molecules.

For different molecular procedures, several copies of a specific DNA sequence are required. Quantitative PCR methods allow scientists to estimate the amount of a given sequence present in a sample. This technique is often applied to quantitatively determine levels of gene expression, and is an established tool that measures the accumulation of DNA product after each round of PCR amplification.

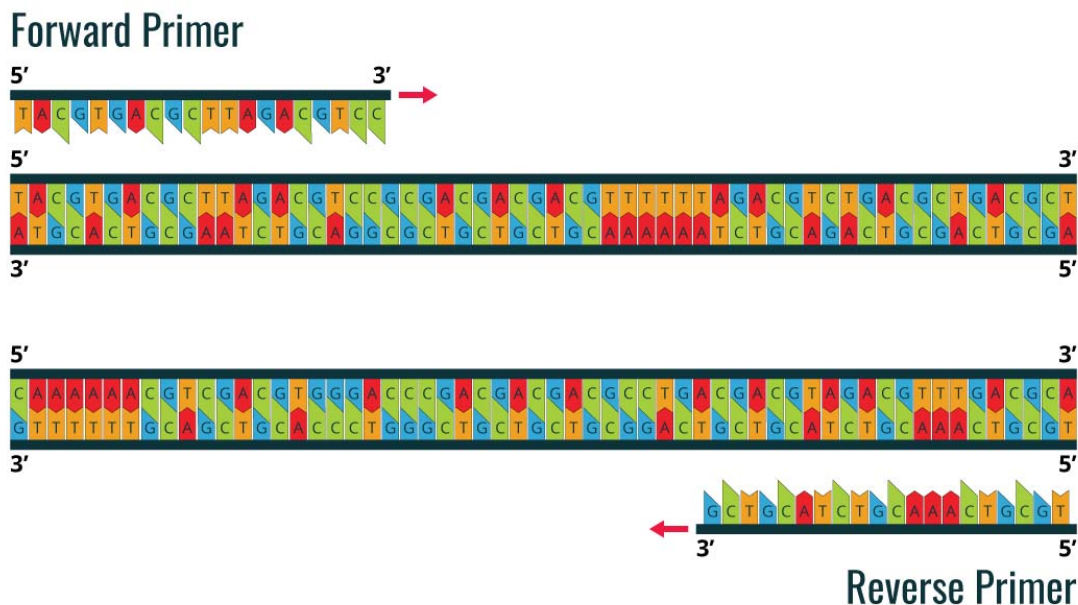
The first application of PCR was for analyzing the presence of genetic diseases mutations (genetic testing). A common application of PCR is the study of patterns of gene expression. Tissues and individual cells can be analyzed at different stages to see which genes are activated or inactivated, and quantitative PCR can be utilized to quantitate the actual levels of expression.

Because PCR amplifies the regions of DNA that it targets, it can be used to analyze extremely small amounts of sample available. This is important for many different applications. For example, PCR may be used in phylogenetic analysis of ancient DNA such as that found in bones of human ancestors or frozen mammoth tissues. In forensic analysis, often there is only a trace amount of DNA available as evidence and PCR amplification solves this problem. Viral DNA can also be detected by PCR, but the primers used must be specific to the targeted sequences in the DNA of the

virus. Furthermore, the high sensitivity of PCR permits virus detection soon after infection and sometimes even before disease onset. Such early detection may give physicians a significant lead time in treatment. PCR is also a critical tool to diagnose genetically rare diseases, such as Huntington disease.

PCR utilizes the DNA polymerase enzyme, which naturally catalyzes the synthesis of DNA sequences. All DNA polymerases synthesize DNA in the 5' to 3' direction. In order to start the synthesis process, it is necessary to have a pair of chemically synthesized oligonucleotide primers made of DNA (as the primers are synthesized by cells). These two primers are designed to flank the DNA fragment, which will be amplified.

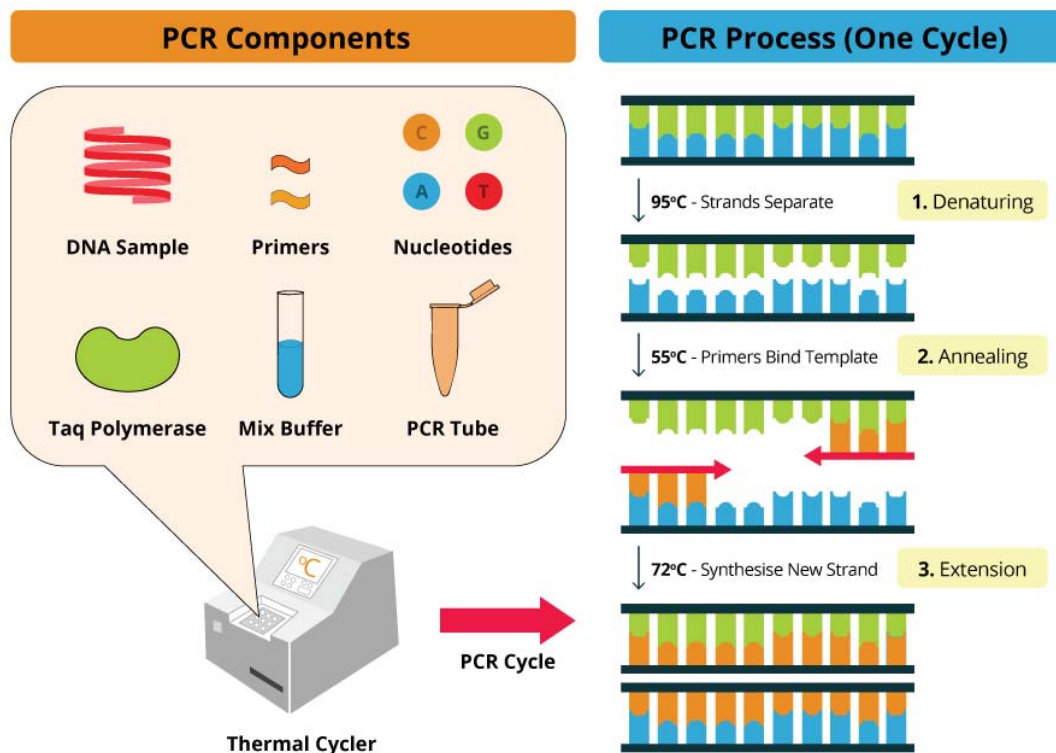
The image below is an example of a forward primer and reverse primer, which are designed before PCR amplification is performed for the double stranded DNA sample template shown in the middle.



A standard PCR requires four components:

- The DNA template molecule, from which the fragment will be amplified
- Two synthetic primers, usually denominated forward and reverse primers, which will flank the DNA region to be amplified (see image above)
- Synthetic deoxynucleotides (dNTPs)
- DNA polymerase enzyme

The following diagram illustrates the reagents needed for a standard PCR experiment, and the general steps of a PCR Cycle.

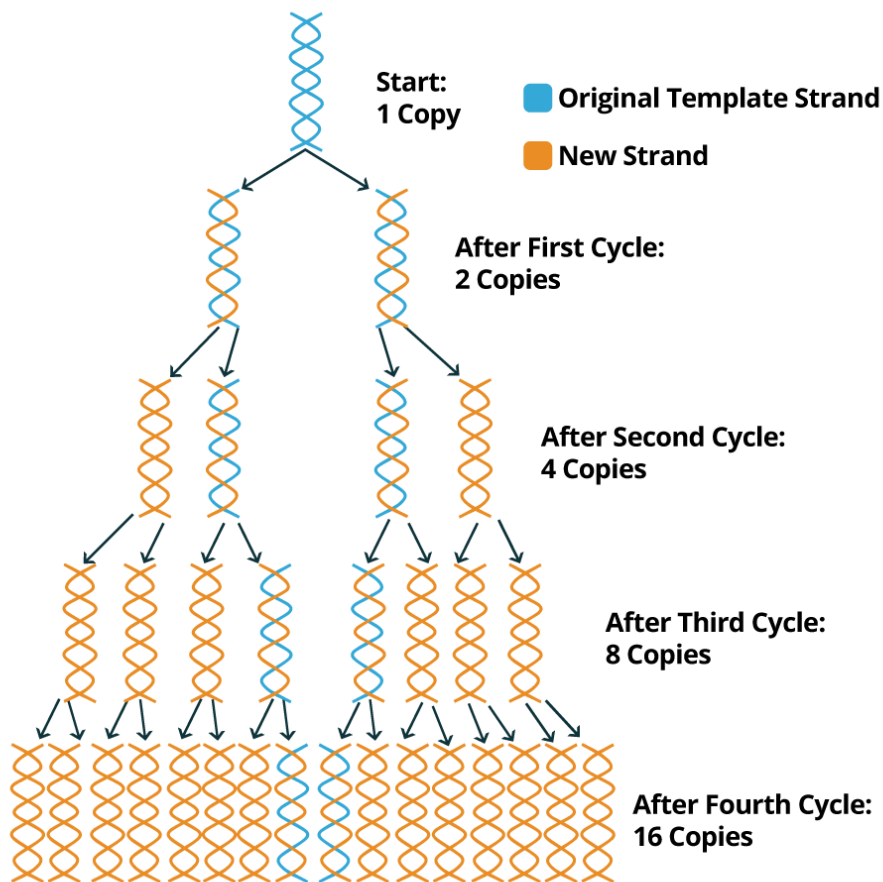


Upon high temperatures (around 95°C) the DNA template molecule will denature and the two strands will move apart, breaking the hydrogen bonds that link the double helix DNA strands. Then, using lower temperatures, also called annealing temperatures (55°C - 65°C) will trigger primer annealing with the DNA template at the complementary sequences. At optimal temperature, extension occurs and DNA polymerase will then synthesize DNA, starting in the primer annealing region. After a certain period of time, dependent on DNA polymerase synthesis rate, the PCR solution is again heated at denaturing temperatures to repeat the previous cycle for several times.

Since, high temperatures are required to denature the dsDNA molecule; the DNA polymerase in solution has to be able to maintain its catalytic activity after such extreme temperatures. Thus, most of the DNA polymerases used in PCR protocols are extracted and purified from extremophilic microorganisms accustomed to living in high temperature environments. As examples, there are the Taq-polymerase from *Thermophilus aquaticus* or the Pfu-polymerase which is from *Pyrococcus furiosus*. These can handle temperatures of 95°C and 100°C, respectively. There are dozens of DNA polymerase used in PCR protocols, each one with its own specificity. Some of them have proofreading activity, others exonuclease activity, and more...

At the end of each PCR cycle, the PCR product or amplicon will increase exponentially because the newly synthesized DNA sequences can be used as templates (in addition to the original DNA template). Usually, 20 to 30 standard PCR cycles are enough to promote an increment of 10^6 to 10^9 of the DNA fragments

flanked by the two primers. The following image depicts how the PCR amplification process works to exponentially create copies of the DNA sample template:



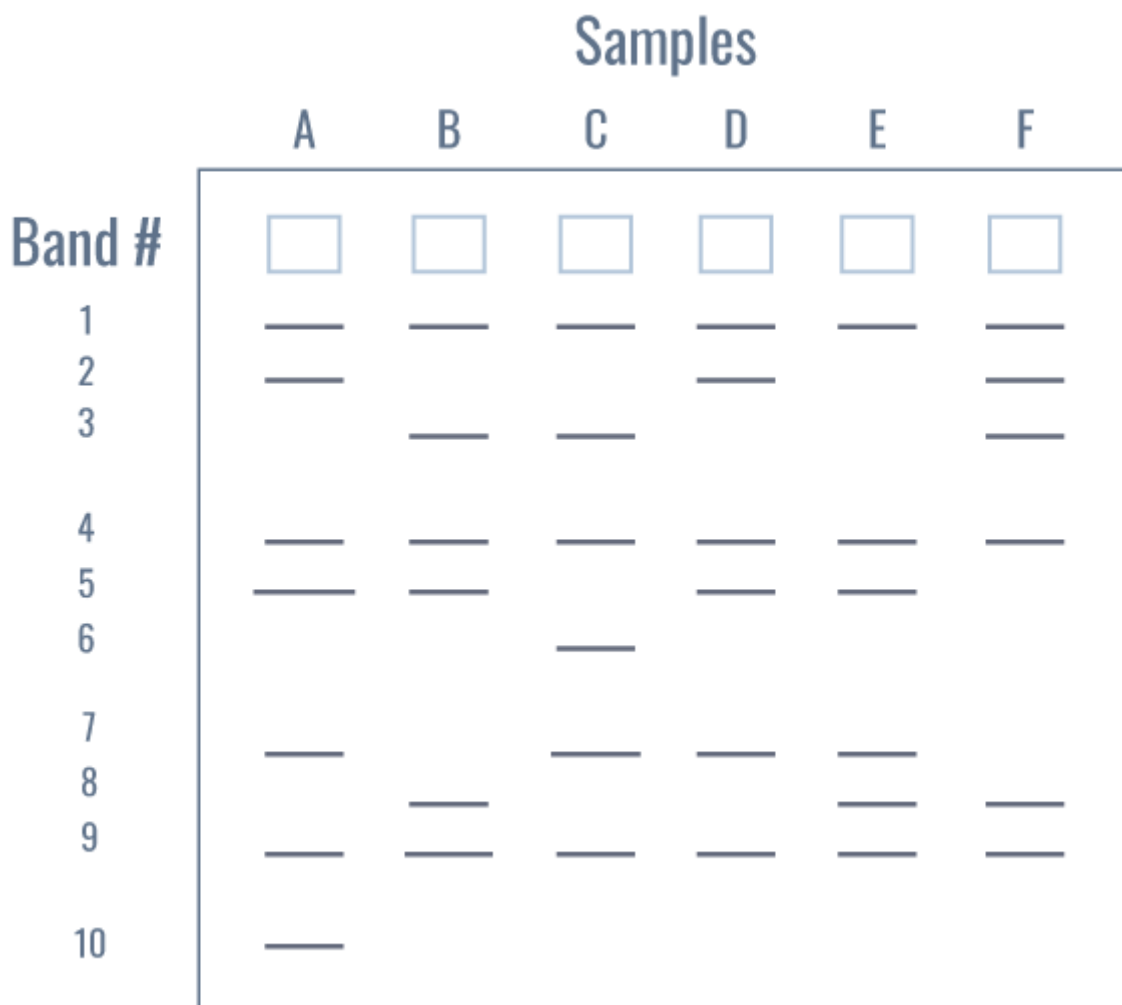
At the end of the PCR, the PCR product should be loaded into an agarose gel for electrophoresis. This will allow one to confirm the correct size of the amplicon, and if required, to purify it for further molecular experiments such as cloning. The PCR procedure can be useful for DNA cloning, DNA sequencing, phylogenetic studies, forensic analysis, and more!

Data Acquisition and Analysis: Gel Electrophoresis

Depending on the information needed, there are many ways to analyze the products of a PCR reaction. A common and rapid way of analyzing PCR products is by standard agarose gel electrophoresis. Gel electrophoresis is one of the principal tools of molecular biology. The basic principle is that DNA, RNA, and proteins can all be separated by utilizing an electric field and their size. In gel electrophoresis, DNA and RNA can be separated on the basis of size, by running the genetic material through an electrically charged agarose gel. Larger sized molecules will travel through this gel at a different rate than smaller molecules and thus become physically and visually separated. Proteins can also be separated on the basis of size by using an SDS-PAGE gel, or on the basis of size and their electric charge by using 2D gel electrophoresis.

Depending on the expected size of the amplified fragment, a fraction of your PCR reaction should be loaded onto a 0.8–3% agarose gel containing 1 µg ml⁻¹ ethidium bromide. Typically, one tenth or one-fifth of the reaction volume is loaded and the remainder is stored at 4°C or –20°C for future use. An aliquot of loading dye containing glycerol and a marker such as bromophenol blue should be added to the sample to assist both loading on the agarose gel and visualization of the sample migration through the gel. After gel electrophoresis is complete, the gel is visualized using UV light and special equipment. Non-toxic dyes such as SYBR® green can also be used to visualize the results. The intensity of the band can be used to estimate the amount of product of a particular molecular weight (relative to a molecular ladder). Gel electrophoresis also shows the specificity of the reaction, because the presence of multiple bands indicates secondary amplification products. A photo can be taken for this gel data, and then the results may be explained according to this data. See the Sample Preparation & Protocols section (**PCR Product Analysis: Gel Electrophoresis**) for more protocol details.

The diagram below represents an example result of gel electrophoresis.



Each sample of A, B, C, D, E and F was loaded into a separate lane of the agarose gel. After electrophoresis, each sample will be separated into a number of bands with each band representing a fragment of DNA. The larger sized fragments stay close to

the top, while the smaller sized fragments travel to the bottom. The similarity and difference between sample or genotypes depends on the presence or absence of bands.

qRT-PCR Experimental Basics

Advances in molecular biology allow us to diversify the applications for PCR procedure and the methods which molecular biologists study the genetic components of a cell. The reverse transcriptase enzyme is an enzyme that has a catalytic activity similar to DNA polymerase, but instead of DNA, it uses mRNA as the template to generate a DNA strand. This approach allows us to identify only genes that are being expressed in a specific cell at a specific time and environmental condition. After synthesis of complementary DNA or cDNA strand from the mRNA template by reverse transcriptase and using standard PCR protocols, it is possible to perform a qRT-PCR (quantitative real time PCR).

The qRT-PCR is based on the principle that higher or lower initial amounts of a specific DNA sequence will lead to higher or lower concentrations of amplicons respectively. Thus the PCR protocol will use a fluorescence dye to tag the newly synthesized sequences. Unlike a standard PCR, the qRT-PCR does not require purification and agarose gel analysis to analyze the amplicons. Since the amplicons are dyed, quantification of fluorescence will allow us to infer the amplicons concentrations. As an example, the SYBR green dye binds to dsDNA, but does not bind to single stranded DNA and RNA. When SYBR green dye is added to the PCR solution, it will radiate fluorescence only when it is bound to DNA, which uncovers the amplification of the DNA fragment flanked by the primers.

Data Acquisition and Analysis: Basic Calculations

The quantitative analysis of qRT-PCR products is achieved through analysis of quantification of cycle values (C_q)

A set of procedures and data normalization is required before doing the quantification analysis:

- **Baseline Correction**

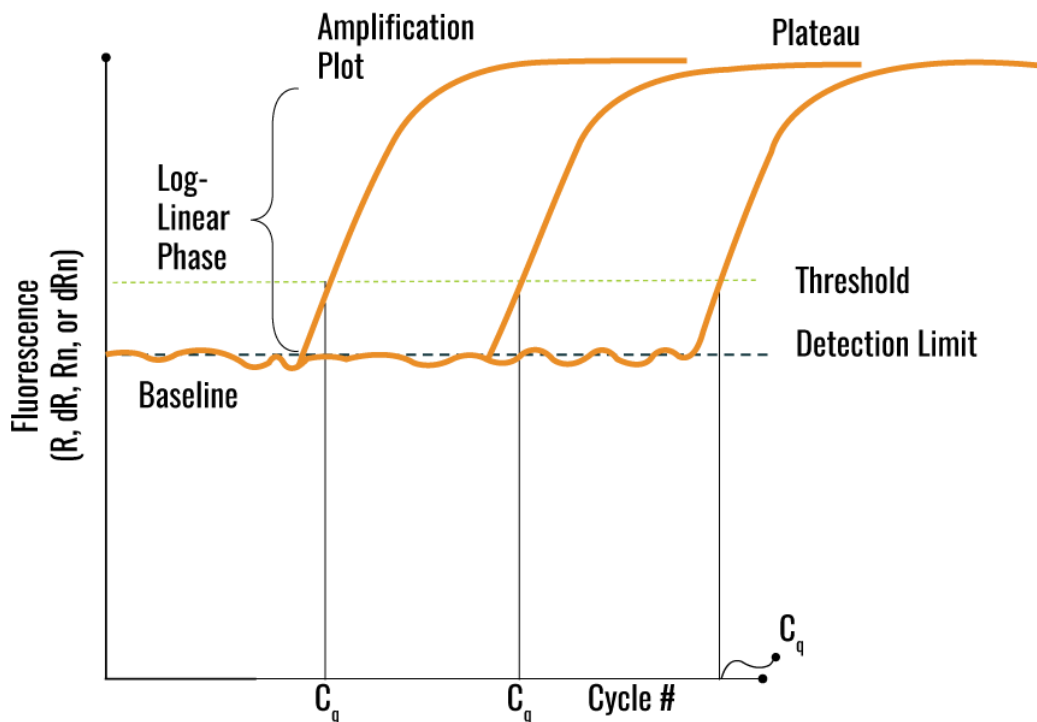
In order to avoid variation in background signal caused by external factors not related to the samples (*e.g.* plasticware, light leaking, fluorescence probe not quenched, etc...), it is recommended to select the fluorescence intensity from the first cycles, usually cycles 5-15 and uncover a constant and linear component of background fluorescence. This information will then be used to define the baseline for analysis.

- **Threshold setting**

It is also important to select a quantification zone that is over the detection limit of the instrument used. Usually the number of cycles needed is directly proportional to the sample copy number. Instead of using the intensity of minimum fluorescence detected by the instrument, a quantification zone with higher fluorescence intensity is selected and a threshold defined for this area. Here are some guidelines to define a correct threshold:

- Lies in the amplification log phase, and avoids the plateau phase
- Lies significantly over the background baseline
- Lies within a log phase range where sample amplification plots are parallel

Shown below is a reference diagram for threshold setting.



- **Standard Curve**

The standard curve calibration is obtained based on the known concentration of the standards used. As recommended during qRT-PCR experiments, serial dilutions of a standard should be prepared. Then the C_q is determined for each dilution of the standard and plotted against the concentration. As a result, a standard curve will be displayed and used to infer about the concentration of the samples. The threshold should be the same for standard samples as well as for samples tested.

- **Relative/Comparative Quantification**

Usually relative quantification is used to make the data interpretation more intuitive and clear. Instead of plotting the C_q values for each sample, also denominated as absolute quantification, the C_q differences between samples are plotted, which allows us to access the expression fold changes for a specific targeted gene.

Sample Preparation & Protocols

A. Extraction & Purification Protocols

The genomic DNA (gDNA) and total RNA are frequently the genetic elements targeted for molecular biology experiments, since these are the main sources of genetic information in an organism.

Genomic DNA and total RNA extraction have a straightforward methodology, requiring only cell lysis to accomplish the task. However, it is recommended to consider the type of organisms you are using in your lab research. Some organism's cells have a cell wall structure, which confers extra protection to cells. This is the case for most yeast, plants and bacteria species. For these organisms, it is recommended to include an extra step for cell lysis before disrupting the plasma membrane. This step is usually an enzymatic or mechanical process to disrupt the cell wall. The purification of DNA and RNA molecules is required to avoid contaminations with other intracellular components, such as proteins and metabolites. Some commonly used methods for DNA and RNA purification are precipitation with phenol-chloroform or isopropanol, or by spin columns with silica membrane.

However, if your target DNA is a plasmid, you should adapt your procedure to make sure the plasmid DNA (pDNA) is separated from gDNA. Alkaline lysis is the most current protocol used in the labs. The lysis buffer has in its composition sodium hydroxide and SDS, which will be responsible for denaturation of gDNA and pDNA. Subsequently, a neutralization buffer is added to renature the pDNA but not the gDNA. Because of the size of gDNA, it is much more difficult to renature again, unlike the pDNA. Then the same procedure of gDNA purification can be used for pDNA purification.

If your research is focused on gene expression analysis, then you should target total RNA. The same procedure used to extract and purify gDNA can be used for total RNA purification, although some additional steps are required, as you will see in the following RNA extraction and purification protocol. Boster recommends the following protocols for sample preparation depending on your initial sample source.

1. DNA Extraction and Purification

Reagents:

- lysis buffer: 1% SDS, 0.5 M NaCl
- isopropanol
- 70% (v/v) ethanol

1. Collect the cells for DNA extraction into 2 ml tubes.

- Note: If you are using cell suspensions you can move directly to the lysis procedure. If you are using samples, like cell tissues that are difficult to homogenize, you can use liquid nitrogen to create a homogeneous cell suspension. At the time you have a cell suspension, you should be careful about the membrane structure of your sample. Usually, for cells with cell wall, it is recommended a pre-lysis treatment to degrade this barrier. You can use an enzymatic method or a mechanical method.

2. Add 1ml of cell suspension into 2 ml tubes

3. Add 100 mg of 0.5 mm glass beads and vortex during 15 min.

- Note: Alternatively, you can use 10U of lyticase (for fungi samples) or 10U of lysozyme (for gram-negative bacteria).
4. Spin the cell suspension at top speed for 1 min using a centrifuge,
 5. Discard the supernatant and resuspend in 1000 μ l of the lysis buffer.
 6. Spin at top speed for 1 min and
 7. Transfer the supernatant into a new 2ml tube.
 8. Add 500 μ l of isopropanol and mix gently.
 9. Place the mixture on ice for 5 min.
 10. Spin at top speed 1min,
 11. Discard the supernatant and wash the DNA pellet with 500 μ l 70% (v/v) ethanol.
 12. Spin again at top speed during 1 min.
 13. Discard the supernatant.
 14. Let the pellet air-dry, placing the 2 ml tubes upside down in a paper-towel.
 15. Dissolve the DNA in 50 μ l ultrapure DEPC H₂O.
 16. Use the Nanodrop equipment to access DNA concentration and quality.
- Note: Nucleic acids absorb UV light at 260 nm, whereas proteins and phenolic compounds absorb at 280 nm. Many organic compounds as well as phenol, TRIzol, and chaotropic salts, have strong absorbances at around 230 nm. The A₂₆₀/A₂₈₀ ratio is used to identify protein contamination. For RNA and DNA, A₂₆₀/A₂₈₀ ratios should be around 2.1 and 1.8, respectively. The A₂₆₀/A₂₃₀ ratio indicates the presence of organic contaminants, such as phenol, TRIzol, chaotropic salts and other aromatic compounds. Samples with A₂₆₀/A₂₃₀ ratios below 1.8 have a significant amount of these contaminants.
17. Store the DNA sample at 4 °C for immediate use or -20 °C for long-term storage.

2. RNA Extraction and Purification

*Note: During this protocol always use new gloves and RNase free material.

1. Collect the cells into 2 ml tubes.
- Note: In the case you are using cell suspensions you can move directly to the centrifuge step before addition of lysis buffer. Although if you are using samples, like cell tissues that are difficult to homogenize, you can use liquid nitrogen to create a homogeneous cell suspension. For samples, in which cells have cell wall, add 1 ml of cell suspension into 2 ml tubes and add 100 mg of 0.5 mm glass beads and vortex for 15 min.
2. Spin the cell suspension at top speed for 1 min using a centrifuge,
 3. Discard the supernatant and resuspend in 1 ml of lysis buffer prewarmed at 65 °C.
 4. Add 900 μ l of acid phenol: chloroform and vortex for 10 s.
 5. Let the 2 ml tube in the bench for 10 min at room temperature.
 6. Spin at top speed for 10 min at 4 °C
 7. Transfer the supernatant to new 2 ml tube

8. Add 0.3 volume of 5 M sodium acetate and 0.7 volume of acid phenol:chloroform.
 9. Mix gently the tube and incubate on ice for 10 min.
 10. Top spin for 10 min at 4 °C
 11. Transfer the supernatant to fresh 2 ml tubes.
 12. Add 0.1 volume of 3 M sodium acetate and the same volume of isopropanol.
 13. Incubate the tubes at -20 °C for 1 h.
 14. Spin at top speed for 10 min at 4 °C and discard the supernatant.
 15. Wash the pellet with 500 µl ethanol (70 %)
 16. Centrifuge at 7,500 g for 5 min at 4 °C.10.
 17. Air dry the pellet for 5–10 min
 18. Dissolve in 25 µl RNase free water.
- Note: For further use of the RNA for expression analysis, it is highly recommended to treat the RNA sample with DNase, an enzyme that digests DNA.
19. Add into a 1ml tube 10ug of RNA,
 20. Add 5 µl of DNase buffer,
 21. Add 1 µl of DNase and complete the volume up to 50 µl with RNase free water.
 22. Incubate at 37 °C for 30 min.
 23. Add .500 µl ethanol (70 %)
 24. Centrifuge at 7,500 g for 5 min at 4 °C.10.
 25. Air dry the pellet for 5–10 min
 26. Use the nanodrop equipment to access RNA concentration and quality
 27. Store the RNA sample in 25 µl RNase free water at 4 °C for immediate use or -20 °C for long-term storage.
- Note: In order to remove DNase, that can destroy cDNA molecules in further qRT-PCR experiments, add 1ul of DNase inhibitor.

B. Molecular Cloning: DNA Library Preparation

If the goal is to create a genomic DNA library, this first step is to extract genomic DNA (please see the protocol for DNA extraction). Whereas the first step to generate a cDNA library relies on mRNA extraction (please see the protocol for RNA extraction). Afterward, the mRNA is converted to cDNA through the catalytic activity of reverse transcriptase enzyme (please see the protocol for conversion of mRNA into cDNA).

Once the cDNA is obtained, the use of restriction enzymes is required to create complementary ends in the vector and in the DNA fragments.

DNA digestion:

- In 200 µl tube add 2 µl of cDNA or genomic DNA
- Add 15 µl of DEPC-treated water
- Add 2 µl of restriction enzyme buffer (10x)

- Add 1 µl of restriction enzyme
- Incubate for 2h at proper temperature accordingly the restriction enzyme selected
- Inactivate the restriction enzyme at high temperature (usually 20 min at 65 °C)

Vector digestion:

- In 200 µl tube add 2 µl of vector (100 ng/µl)
- Add 15 µl of DEPC-treated water
- Add 2 µl of restriction enzyme buffer (10x)
- Add 1 µl of restriction enzyme
- Incubate for 2h at proper temperature accordingly the restriction enzyme selected
- Inactivate the restriction enzyme at high temperature (usually 20 min at 65 °C)

Cloning:

- In 200µl tube add 5 µl of digested cDNA
- Add 3 µl of digested vector
- Add 1 µl of ligase buffer (10x)
- Add 1 µl of ligase enzyme
- Incubate at 4°C overnight
- Use 5µl of the ligation solution to transform host competent cells
- Plate the cells in media with a selection marker, e.g. ampicillin, kanamycin, depending on the selection marker of the vector used.

DNA library screening

To functionally characterize and identify new genes, physiological tests can be carried out to detect phenotypic differences between the host organism and the host organism carrying DNA fragments of the DNA library. Usually growth tests are the most common approach to achieve this task, either by cultivating cells in different media formulations and/or different temperatures.

One real-life example is the use of renewable carbon sources aiming to produce biofuels as a recent initiative to find alternatives to fossil fuels. Most of the organisms used in this bioprocess, like *E. coli*, are not able to grow in these substrates, namely xylan. But the degradation product of xylan, which is xylose, can be assimilated and metabolized by industrial microbes (and used to produce biofuels). Thus, samples of microbes growing in substrates rich in xylan, were used to extract the RNA and prepare cDNA libraries as described previously, and to be transformed in *E. coli* competent cells. The transformed cells are subsequently plated in minimal media with xylan as sole carbon sources. This procedure will allow positive selection of transformants. Only transformants that carry genes in the vector that code for enzymes able to degrade xylan into xylose will grow in the plate. The vector of the transformants is then extracted and sequenced. This will allow us to identify which genes are responsible to allow growth in xylan media.

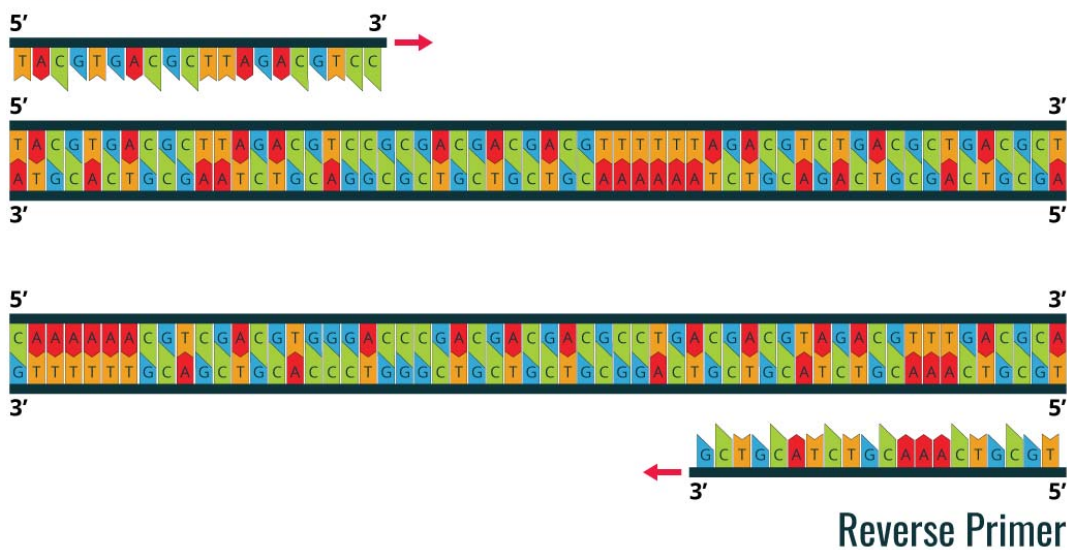
C. PCR Protocols

Once you have your genetic samples, one of the first steps of PCR is to design the primers required to perform the PCR reaction.

Primer Design

The first step to proceed with a standard PCR is the primer design. You should determine which fragment of the DNA template you are looking to amplify. Thus you need to know the DNA sequence of the template. The NCBI database is a web server with all DNA sequences known; you can look for your sequence there. After that, you should design two primers, the forward and the reverse primer. Primer design is a critical step in a PCR protocol. The set of primers should flank the fragment you intend to amplify from the DNA template. The forward primer will anneal with 3'-5' DNA strand and the reverse primer will anneal with the 5'-3' DNA strand. Refer to the diagram below.

Forward Primer



Many of the problems with PCR protocol are associated with wrong primer design. The most common mistakes in design include:

- Primer dimers
- Hairpin loop structures by primer self-annealing
- Non-specific annealing

The primer length should be around 18-30 bp, the GC content close to 50% and the melting temperature (T_m) between 55°C and 65°C. In order to calculate melting temperature, you can use the following equation $T_m = 2^\circ\text{C}(A+T) + 4^\circ\text{C}(G+C)$. Although most commercial DNA polymerases provide their own calculator and specific instructions for different conditions, several web platforms for assisting with primer design are available, such as <http://www.ncbi.nlm.nih.gov/tools/primer-blast/>.

Once you have successfully acquired your DNA samples and designed your forward and reverse primers, the PCR experiment can proceed.

General PCR protocol

Reagents –

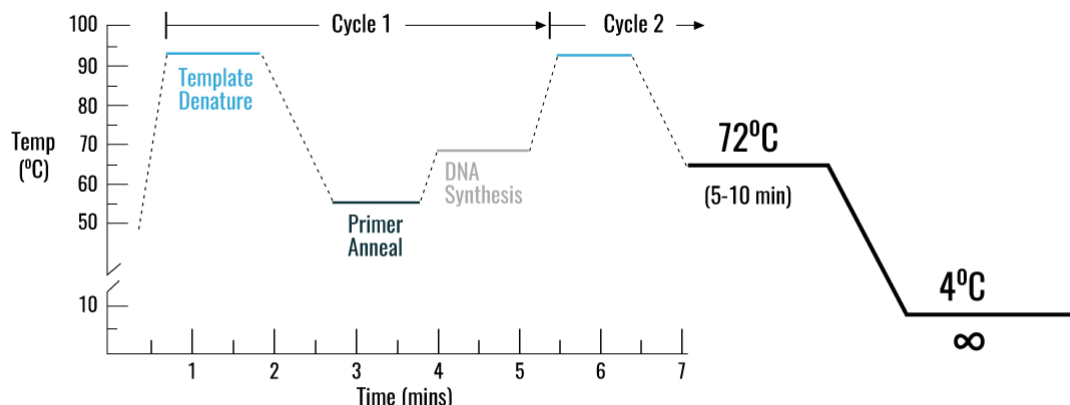
- Forward and reverse primers
- dNTPs
- DNA template
- DNA polymerase

Note: From the beginning of your PCR experiment until the end, you should always wear gloves in order to avoid DNA contamination. All the reagents, primers and enzymes should be kept in ice. Make sure that primers, DNA template, buffer are completely unfrozen before starting to prepare the PCR solution. It is important to create an experimental design in accordance with scientific guidelines, including positive control and a negative control. As the negative control, you can prepare a PCR deprived of DNA template. For the positive control you should use a set of primers and DNA template shown to work properly in previous experiments.

Depending on the DNA polymerase you use, final concentration of each reagent will vary. These are the standard volumes and concentrations taking into account a 50 μ l reaction:

1. In PCR tubes of 200 μ l:
 - a. Add 38 μ l sterile water
 - b. Add 2 μ l of forward primer (10 μ M)
 - c. Add 2 μ l of reverse primer (10 μ M)
 - d. Add 1 μ l of dNTPs (50 μ M)
 - e. Add 5 μ l of reaction buffer containing MgCl₂ (10X)
 - f. Add 1 μ l of DNA template (100 ng/ μ l)
 - g. Add 1 μ l of DNA polymerase (0.5 U/ μ l)
 - Note: If you are willing to do a significant number of PCR reactions it is recommended to prepare a reaction mix, excluding the reagents that will be different from experiment to experiment (usually the DNA template or the set of primers).
2. Pipette gently the reaction mixture to allow good homogenization.
 3. Short spin in a centrifuge is recommended.
 4. Make sure the caps of PCR tubes are closed.
 5. Insert the tube in the thermocycler.

Tip: Set up the PCR settings, such as temperature, time and cycles before your PCR reaction is ready. See the diagram below for reference of typical PCR reaction settings.



Note: Always pay attention to the guidelines of the DNA polymerase, since denaturation, annealing and extension temperature and time highly depend on it. In the thermocycler you should set up these steps subsequently (refer to diagram above):

1. An initial step of DNA denaturation at 94°C to 98°C for 3 to 5 min depending on the optimal temperature of the DNA polymerase
2. Denaturation temperature (the same used in the step before) for 30 seconds
3. Annealing temperature taking into account the T_m of the primers for 30 seconds
4. Extension temperature at 72°C and taking into account the size of the fragment to be amplified

These steps should be repeated for 25 to 35 rounds (cycles). A final step of extension is required to allow all the PCR products to be correctly synthesized, usually at 72°C for 10 min. Finally, the temperature should be reduced to 4°C to store the PCR product.

PCR Product Analysis: Gel Electrophoresis

Refer to Key Principles Section (Data Acquisition and Analysis: Gel Electrophoresis) for detailed explanation of how to analyze PCR results

Reagents:

- Add 5 μ l of the PCR product in a new PCR tube
- Add 1 μ l of DNA loading buffer

Common DNA loading buffer (6X) recipe:

- 30% (v/v) glycerol
- 0.25% (w/v) bromophenol blue
- 0.25% (w/v) xylene cyanol FF

1. Load the 6 μ l mixture in an agarose gel 1%.
2. Load 5 μ l of DNA marker in the same gel.
3. Use an UV transilluminator to visualize the PCR product in the agarose gel.

Note: In order to avoid staining with ethidium bromide, you can use Midori or Red Safe pre stained gels, which are less toxic compounds. Use a DNA marker to compare the correct size of the PCR product.

Reverse transcription protocol

Reverse transcription is the process by which RNA is transcribed into DNA, which will allow us to perform experiments such as qRT-PCR under the catalytic activity of a specific DNA polymerase that is only able to amplify fragments from DNA molecules.

Note: From the beginning of your PCR experiment until the end, you should always wear gloves in order to avoid DNA contamination. All the reagents, primers and enzymes should be kept in ice.

1. In a 200 μ l PCR tube:
 - Add 2 μ g of total RNA
 - Add 1 μ l of oligo dt (50 μ M)
 - Add 1 μ l of dNTP (10mM)
 - Add DEPC-treated water up to 10 μ l
2. Incubate at 65 °C for 5 min and then keep the tubes on ice for 1 min
3. Add 2 μ l of RT buffer (10X)
4. Add 4 μ l of MgCl₂
5. Add 2 μ l of DTT (0.1 M)
6. Add 1 μ l RNase inhibitor (40 U/ μ l)
7. Add 1 μ l of reverse transcriptase (200 U/ μ l)
8. Incubate at 50°C for 60 min, followed by 85°C for 5min
9. Afterward, keep the tube on ice
10. Add 1 μ l RNase H
11. Incubate for 30 min at 37°C
12. Store the PCR tube at -20°C
13. For RNA quantification and quality use a NanoDrop equipment to measure the RNA (260 nm), protein and salt concentrations

qRT-PCR

Primer design:

Similarly to standard PCR, a set of primers should be designed as described before in order to amplify each cDNA fragment of interest, usually corresponding to a specific gene. Moreover, it is also recommended to design primers specific for other cDNA genes for data normalization procedures, these are also known as “housekeeping” genes (e.g. *18S*, *GAPDH*, *ACTB*).

Note: Set up the PCR settings, such as temperature, time and cycles before your PCR reaction is ready.

For each cDNA gene, prepare the following reaction mix in 200 µl PCR tubes for a final volume of 50 µl:

- Add 25 µl of SYBR Green Mix (2x)
- Add 1 µl cDNA from the previous cDNA sample
- Add 1 µl Forward Primer (10 mM)
- Add 1 µl Reverse Primer (10 mM)
- Add 22.5 µl DEPC Water
- Insert the tubes in the thermocycler

When the PCR is finished, perform quantification analysis by comparative Ct method. The Ct method compares the Ct values of the samples with standards samples. The Ct values of the controls and the samples are normalized using the Ct values obtained for housekeeping genes. To validate the Ct calculation, it is required that the amplification efficiencies of the target should be close to endogenous. This can be accessed through the utilization of different template dilutions.

Troubleshooting Guide

The following guide serves as a checklist for the possible causes and solutions with respect to some of the most commonly encountered problems from molecular biology experimental techniques. We at Boster Bio are committed to helping our customers get better results. While the troubleshooting guide below covers a multitude of problems encountered while performing in the lab, we do not expect it to be the exclusive solution to any problems during your specific experiment. We hope that you will find the information beneficial to you and useful as a reference guide in troubleshooting any problems you may encounter. If you ever need more assistance with your experiments, please contact the Boster Support Team by email at support@bosterbio.com

DNA and RNA extraction

Problem	Possible Solutions
Low yields	Increase sample volume
	Increase lysis time or add enzymatic lysis step
	Increase lysis time in 10 min
	Make sure that vortex and resuspension steps allow a good homogenization
	Suspend DNA or RNA in less volume
Salt contamination	Repeat extraction protocol from precipitation process
Protein contamination	Increase lysis time or add enzymatic lysis step

PCR and qRT-PCR

Problem	Possible Solutions
No amplification	Perform a temperature gradient PCR
	Make new primer work solution
	Increase template concentration
	Decrease T _m temperature
	Increase cDNA concentration

	Check DNA template quality in Nanodrop
	Verify time and temperature settings
	Use new template
Non-specific amplification	Increase T_m temperature
	Avoid self-complementary sequences within primers
	Avoid stretches of 4 or more of the same nucleotide or dinucleotide repeats
	Lower primer concentration
	Follow general rules of primer design
	Decrease the number of cycles
Amplification in negative control	Use new reagents, namely buffer and polymerase
	"Homemade" polymerases usually contain genetic contaminants. Try a commercial polymerase instead.
	Make sure to use sterile tips
Low yields of PCR product	Increase number of cycles by 10
High quantification in standards	Use new diluted standards
	Check pipettes calibration
High variability in replicates	Verify pipettes calibration
	Use fresh diluted standards

Erratic curves	Calibrate optics of the system
	Repeat with rox-normalisation dye
Bands and smear are very intense	Reduce the number of cycles
Incorrect product size	Look for additional primer complementary sequences in the template
	Increase T _m temperature
	Make new primer work solutions

FAQs

1. How long does it take to complete the DNA/RNA extraction protocol?

It will depend on the number of samples you intend to use. Usually for 5-10 samples, 60 min is enough to perform the entire protocol. Before starting, be sure that all reagents and solutions are prepared.

2. What lysis method should I use to obtain DNA/RNA from my sample?

The lysis method that should be used will depend on the membrane and cell wall properties of the cells. If the cell does not have cell wall, you can use the lysis buffer only. Although if the cells have cell wall, we strongly recommend the use of enzymatic or mechanical processes to disrupt the membrane and cell wall, such as lyticase or beads, respectively.

3. What is the range of concentration expected in a DNA/RNA extraction protocol?

The DNA concentration will depend on the amount of DNA/RNA contained in cell sample. Usually 1-5 μ g is obtained from a typical DNA/RNA extraction protocol.

4. Is it possible to extract DNA/RNA from a small volume of cell sample?

Yes, it is. However, you should expect DNA concentrations to be less than 1 μ g of DNA.

5. Can I extract DNA/RNA from solid samples?

When you are using biological samples to perform DNA/RNA extraction you should add a previous step in the extraction protocol. Usually, it is recommended to use liquid nitrogen to break the solid sample into small volumes in order to obtain a cell suspension after resuspension buffer is added.

6. What are the quality standards for DNA/RNA?

When you check the DNA/RNA purity in Nanodrop you should expect to obtain A260/280 ratios around 2.1 and 1.8, for RNA and DNA respectively. The A260/230 ratio should be close to 2 and never below 1.8.

7. How long can I store the DNA/RNA?

Depending on your storage conditions, you can store at 4°C for immediate use or -20°C for 1-2 months.

8. Is it possible to perform a PCR reaction for different genes with primers that have different T_m?

It is possible. You just have to use the temperature gradient setting of the thermocycler and place the PCR tubes in the correct temperature row or column, depending on the thermocycler features.

9. Should I use the same polymerase for any PCR?

A huge number of commercial polymerases are available, and each one has different properties and applications. You should take into account what will be the main goal of your PCR reaction.

10. Can I use PCR products for molecular cloning?

One of the applications of PCR is to generate significant amounts of a specific DNA fragment to perform molecular cloning. However, it is recommended to perform a PCR cleanup before using the PCR product. PCR reagents can inhibit some enzymes used in molecular cloning.

11. Is it required to always perform DNA extraction before PCR?

In some situations, you do not need to perform DNA extraction, which is the case of colony PCR. Usually, colony PCR is used to check if a specific fragment is present in a sample without DNA extraction being done, typically after molecular cloning. This approach uses intact cells previously exposed to microwaves (which makes the cell membrane more permeable), and then the PCR reagents are added.

12. Can I use PCR to generate fragments flanked by restriction sites?

If you will use the PCR product for molecular cloning, you can choose which restriction enzymes you want to use and then add to the forward and reverse primer the sequence of each restriction site.

13. How can PCR be used in site directed mutagenesis?

The site directed mutagenesis method follows all the principles of a standard PCR, and the differences are: the set of primers should contain the nucleotide mutation to be generated; the polymerase should be a high-fidelity polymerase in order to avoid additional mutations.

14. Which “housekeeping genes” should be selected?

It is crucial to choose housekeeping genes that maintain the same expression levels in all the conditions tested. It is recommended to choose more than one housekeeping gene and make a preliminary test to verify that the expression levels are the same under the experimental conditions tested. Usually, these are the most commonly used housekeeping genes: *18S*, *GAPDH*, *ACTB*. However, it has been shown that in some samples and conditions even these genes have different expression patterns.

15. Which is the right number of replicates to be used in qRT-PCR?

The experimental system chosen will define the number of replicates to be used. At minimum, it is always recommended to perform 3 replicates for each sample.

16. What is the sensitivity range of qRT-PCR?

The sensitivity of qRT-PCR is highly dependent on the thermocycler used and experimental conditions. Usually it is possible to detect a minimal quantity of 10-20 copies of template.

17. What are relative and absolute quantification?

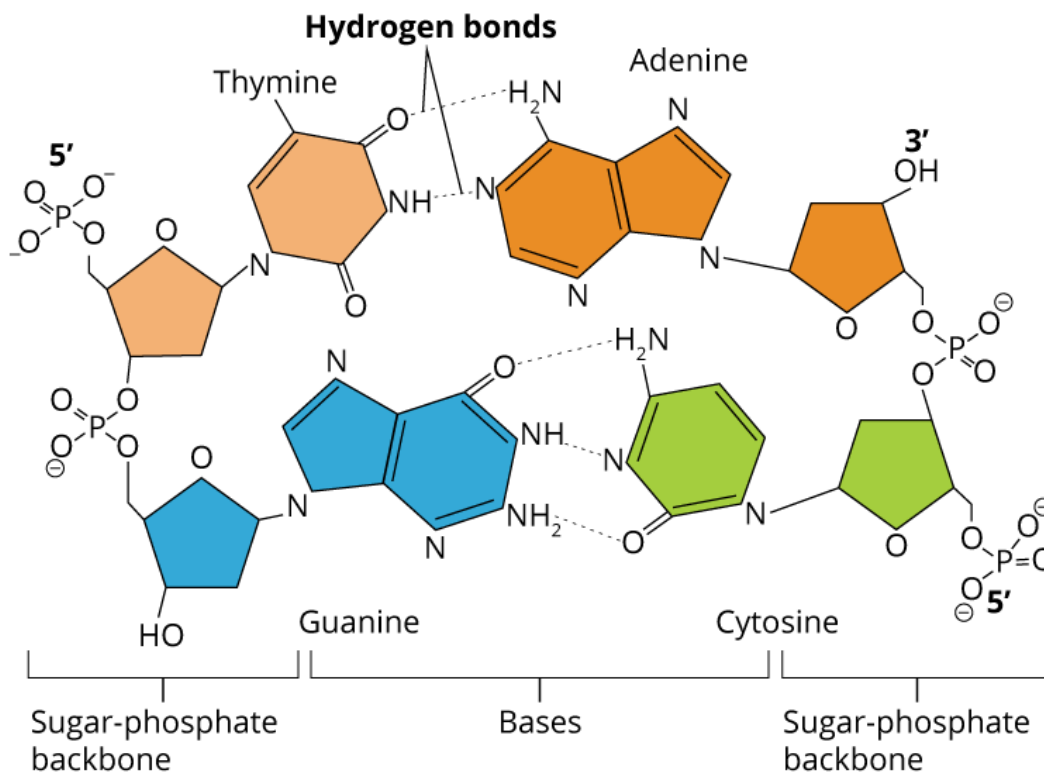
Absolute quantification calculates the total number of a specific target fragment when compared with a standard sample with known number of copies. The relative quantification calculates expression differences between samples; usually the sample expression profile is compared with the housekeeping gene expression levels.

Appendix: AN INTRODUCTION TO MOLECULAR BIOLOGY

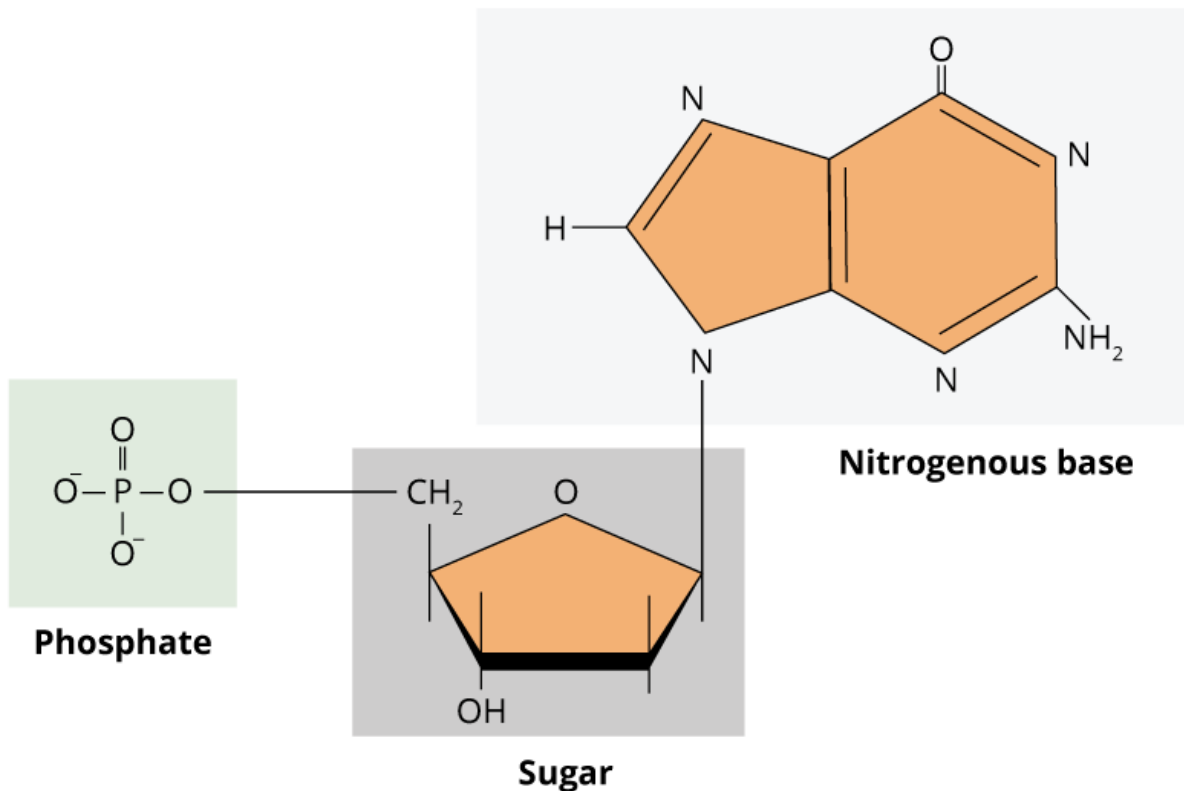
Molecular biology has been the basis for the understanding of each individual step in the biology central dogma: DNA replication, DNA transcription into RNA, and RNA translation into proteins. These molecules are responsible for giving information to cells of each organism on how to survive and reproduce according to the environmental conditions at each exact moment. All this information is stored in the genetic material of cells and transferred to progeny.

DNA and RNA – The Raw Materials of a Molecular Biologist

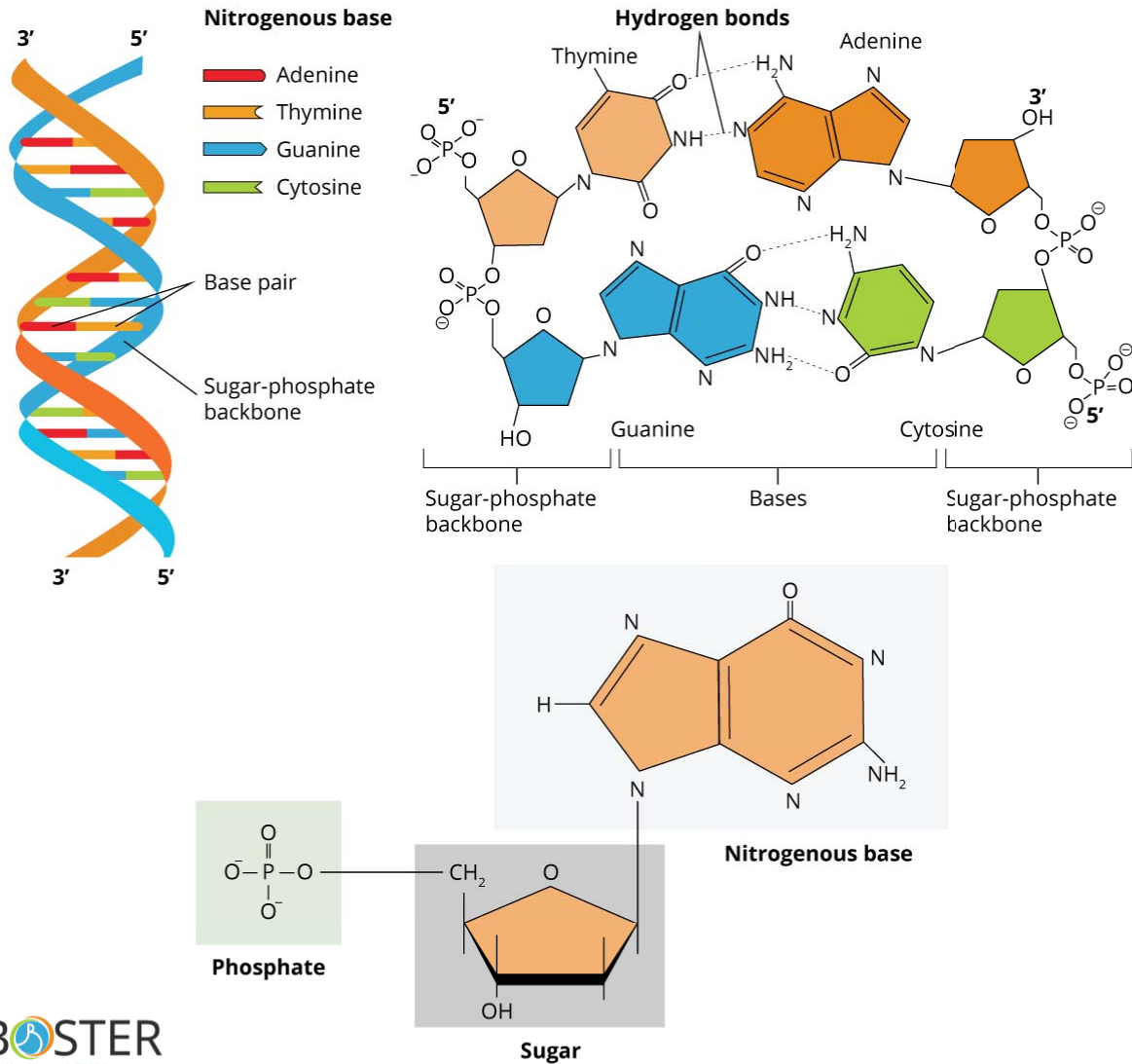
The genetic information in cells is present in nucleic acids, such as DNA and RNA. The deoxyribonucleic acid (DNA) carries the genetic code of a single cell, whereas the ribonucleic acid (RNA) is a molecule that converts this information into amino acid sequences of proteins. Genetic information relies on the sequence of monomers of nucleic acids. Thus, unlike polysaccharides and lipids that are normally formed by long repeated unities, nucleic acids are informational macromolecules. These monomers are known as nucleotides, and consequently, DNA and RNA are polynucleotides. A nucleotide is composed of three components: one pentose (ribose for RNA and deoxyribose for DNA), one nitrogenated base and one phosphate group. The structure of DNA and RNA nucleotides are very similar. The nitrogenated bases are purines (adenine and guanine, which contain two heterocyclic rings) or pyrimidines (thymine, cytosine and uracil, which contain one single heterocyclic ring).



Guanine, adenine, and cytosine are in the composition of DNA and RNA. Excluding few exceptions, thymine is present only in DNA and uracil is present only in RNA. The nitrogenated base is linked to sugar pentose by a glycosidic bond between the carbon atom of the sugar and the nitrogen atom from the base. When nitrogenated base is linked to a sugar, it is denominated nucleoside. For that reason, nucleotides, are nucleosides with an addition of one or more phosphate groups.



In 1953, based on results from x-ray diffraction studies done by Williams and Franklin, the scientists Watson and Crick proposed a structural model for DNA. This model embraces both chemical and biological DNA properties, namely the replication capacity of this molecule. According to this model, the DNA molecule is formed by two helicoidal strands linked by hydrogen bonds between the nitrogenated bases of each strand. When the nitrogenated bases face each other, hydrogen bonds are formed. These hydrogen bonds are more stable between adenine and thymine, and guanine and cytosine. Thus, the adenine of one strand pairs with a thymine of the other strand and the same for guanine and cytosine. The resultant double helix strands are antiparallel, which means that the inter bonds 3'-5' phosphodiester have opposite directions. Each round is composed by 10.5 pairs of nucleotides and measures 3.4 mm.



The size of a DNA molecule is defined as the number of nucleotide base pairs. Thus, a DNA molecule with 1000 nucleotides contains 1 kilobase (Kb). If the DNA molecule is in double helix structure, we use the base pairs nucleotide nomenclature. For instance, the bacteria *Escherichia coli* has around 4640 Kbp of DNA in its chromosome. Each base pair has 0.34 nanometers of length along the double helix, and each round is about 10 bp, which means that 1 Kbp of DNA represents 100 rounds, measuring 0.34 μm of length. The *E. coli* genome has 4640 bp and is 1.58 mm in total length. Since *E. coli* cells are only approximately 2 μm , the chromosome is much larger than its own cell size. For that reason, DNA needs to be compressed and packed to fit inside the cell.

The main genetic elements in cells are the chromosomes, but there are also other genetic elements such as viral genomes, plasmids, organelle genomes, and transposable elements. In prokaryotes, usually there is a single circular chromosome, while in the eukaryotes, genomes are organized in several chromosomes. Plasmids are genetic elements which replicate independently from the cell chromosomes. Usually plasmids are composed of double helix DNA molecules (circular or linear).

Transposable elements or jumping genes are DNA segments that are able to move from one site of a DNA molecule to another site of the same molecule or another distinct DNA molecule. Transposons are not found as individual DNA molecules, instead these elements are found inserted in other DNA molecules, such as chromosomes, plasmids, and viral genomes. Transposable elements are present in both eukaryotes and prokaryotes, and play an important role in genetic variation.

From Genes to Proteins

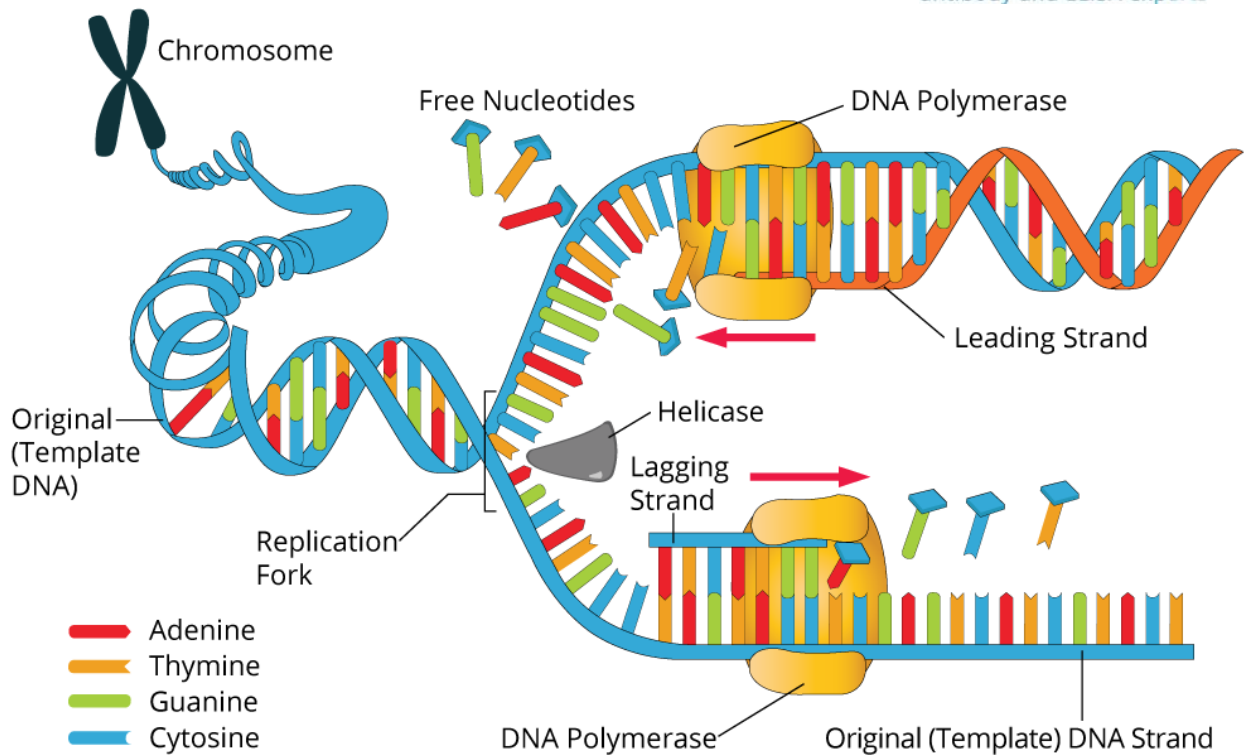
The gene is the basic and functional unit of genetic information. Genes are present in chromosomes or other big molecules, also referred to as genetic elements. In modern biology, the classification of organisms is made according to their genetic material composition and variability.

When genes are expressed, the genetic information stored in DNA is transferred to RNA. There are different RNA types, but only three cooperate for protein synthesis. The messenger RNA (mRNA) is a single stranded molecule that carries genetic information from the DNA to the ribosome, which is responsible for protein synthesis. The transfer RNA (tRNA) convert the genetic information of RNA nucleotides into amino acid sequences of proteins. The ribosomal RNA (rRNA) is an important catalytic and structural component of ribosomes.

The molecular processes of genetic information can be divided into three stages:

1. Replication: During replication, the DNA double helix is duplicated, producing two copies. Replication occurs through the action of the polymerase enzyme.
2. Transcription: The transference of genetic information from DNA to RNA is called transcription. Transcription occurs by the catalytic action of RNA polymerase.
3. Translation: Protein synthesis using genetic information contained in mRNA is denominated translation.

As shown in the image below, during replication, the DNA double helix is duplicated through the action of the DNA polymerase enzyme.

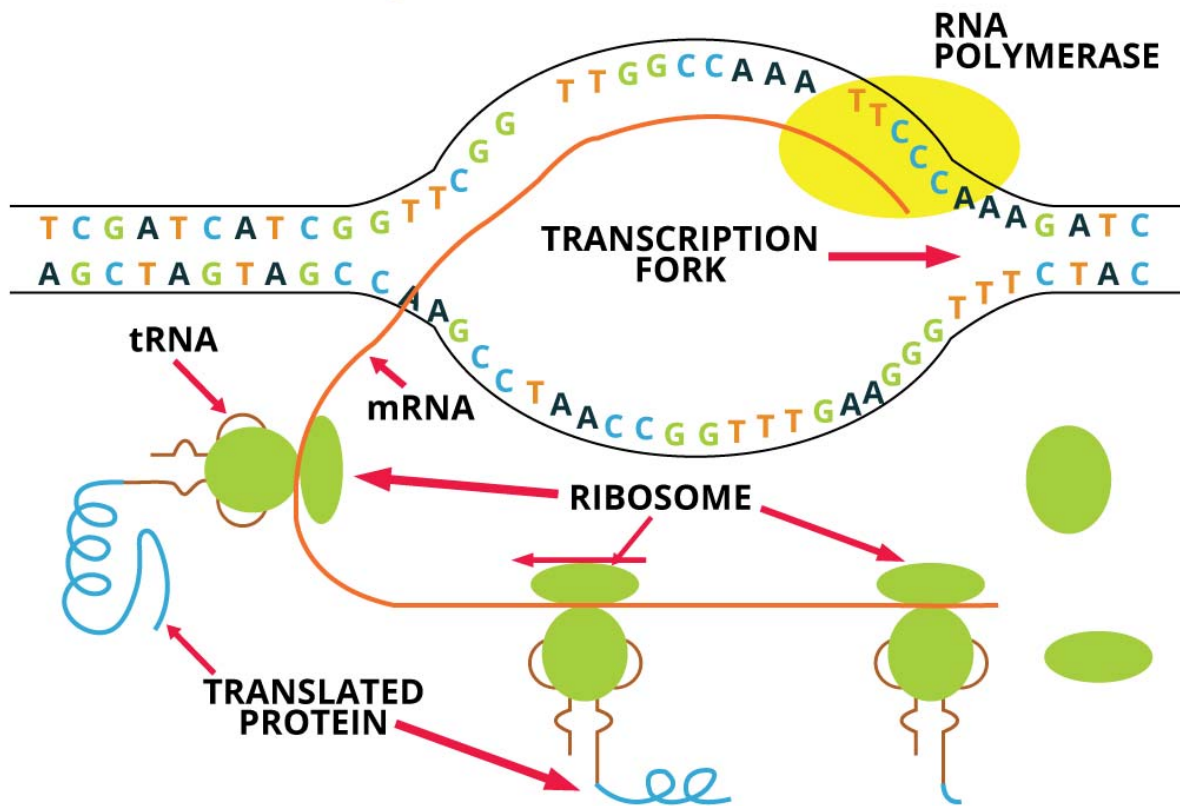


Replication

Many molecules different from RNA are transcribed from relatively short regions of DNA molecule. In eukaryotes, each gene is transcribed, generating mRNA, whereas in prokaryotes one single mRNA molecule can carry genetic information from several genes. There is a linear correlation between the nucleotide sequence of one gene and the amino acid sequence of a polypeptide. Each group of three nucleotides present in mRNA codes for one single amino acid, and each nucleotide triplet is referred to as a codon. Codons are translated into amino acid sequences by ribosomes, tRNA, auxiliary proteins, and translation factors.

Here is a simple graphic depiction of the transcription and translation processes:

Transcription and Translation

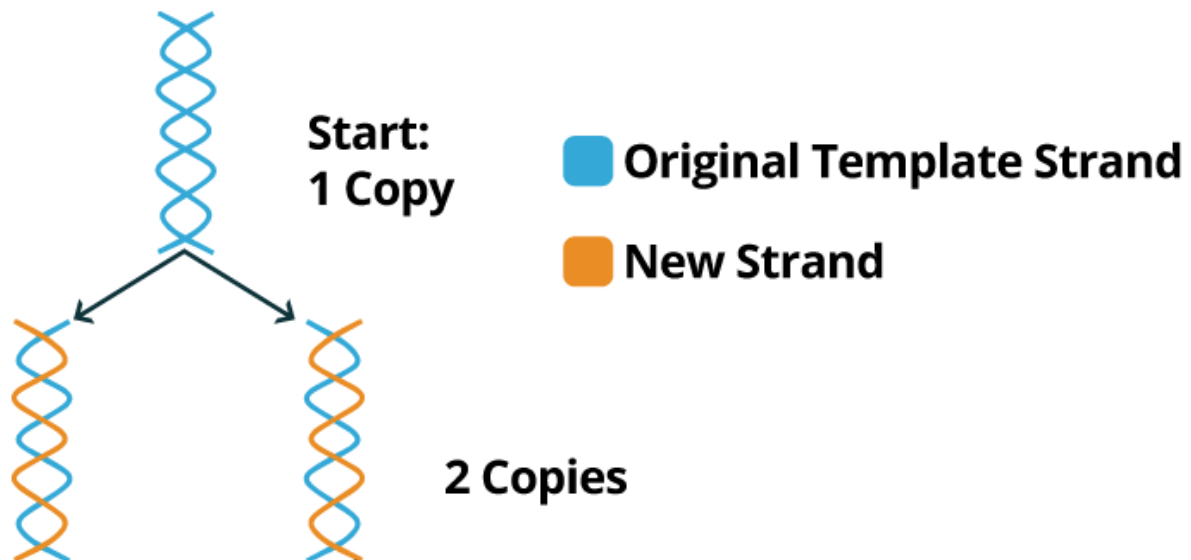


Now we will go into more detail about these processes, and briefly mention their differences.

Replication

DNA replication is used by cells to allow cell division, either in reproduction or in the duplication of new cells in multicellular or unicellular organisms. The complexity of DNA replication process requires the involvement of a great number of specific enzymes. DNA is present in cells as a double helix molecule and when this helix is unwinding, a newly synthesized strand emerges along with a parental strand. The DNA strand used to produce the complementary strand is referred to as the template strand, which is used to synthesize complementary strands of each parental strand. Thus, replication is a semi-conservative process.

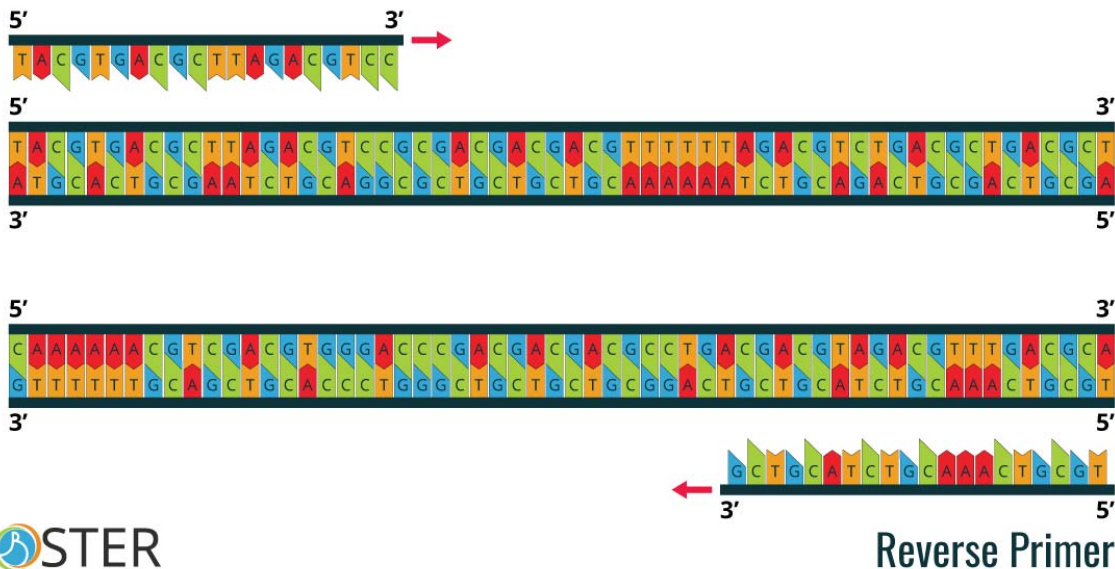
The following diagram shows a simple color coded depiction of this semi-conservative process.



The precursor of each new nucleotide in the DNA strand corresponds to a deoxynucleoside 5'-triphosphate. During replication, two terminal phosphates are removed and the internal phosphate is covalently bonded to the deoxyribose of the raising DNA strand. The nucleotide addition requires the presence of a free hydroxyl group, which is available only at the correct end, so the addition of the nucleotide phosphate group bonds with the 3'-hydroxyl(OH) of the previous nucleotide. The enzymes that catalyze the addition of deoxy ribonucleotides are denominated DNA polymerases. There are several types of these enzymes, each one with a specific role. All known DNA polymerases work in 5' to 3' direction, but none of them are able to start DNA synthesis alone. Since DNA polymerase can only add nucleotides to the 3'-OH, in order to start a new strand, it requires a primer.

The primer is a nucleic acid molecule in which the DNA polymerase can add a nucleotide to. Often, the primer is a small RNA fragment instead of DNA. When the double helix is unwinding at the beginning of replication, an enzyme of RNA polymerization (primase) synthesizes the RNA primer with 11-12 nucleotides, which is complementary to the DNA template strand. At the end of the RNA primer, there is a 3'-OH group in which the DNA polymerase adds the first deoxy ribonucleotide. Later the RNA primer will be removed and replaced by DNA.

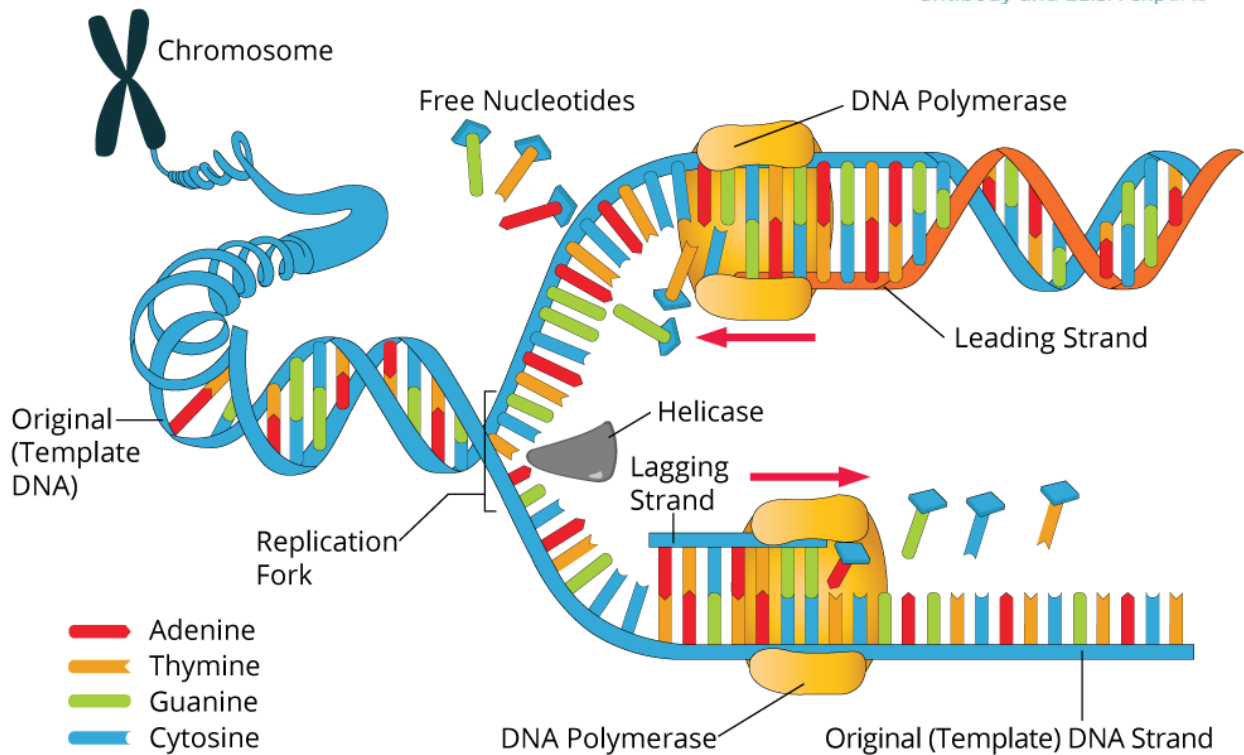
Forward Primer



Replication Fork

Before DNA polymerase synthesizes a new DNA strand, the existent DNA double helix needs to undergo an unwinding process to expose the template strand. The unpackaged region is where replication will start, and it is designated the replication fork. The DNA helicase enzyme is responsible unwinding and separating the DNA double helix strands in an ATP-dependent process, exposing a small region of a single strand. Helicase can move along the double helix structure right at the front of the replication fork. There are specific regions for replication to get started, also known as replication origins.

The replication process always happens in 5' to 3' direction, which means that a new nucleotide is added to the 3'-OH group of the raising DNA strand. For this reason, the strand being synthesized uses the 3'-5' strand as a template, and we call it the leading strand (continuous strand). DNA synthesis occurs continuously since there is always a free 3'-OH group. On the other hand, in the newly synthesized strand using as template the 5'-3' DNA strand, the DNA synthesis occurs in a discontinuous process. It does not have available a free 3'-OH for a nucleotide to be added. Thus, in the lagging strand, it is necessary for primase to synthesize the primer repeatedly to make available a free 3'-OH group. In the continuous strand only one primer is required at the beginning of DNA synthesis, but the lagging strand is synthesized in short fragments, also known as Okazaki fragments. These fragments are posteriorly fused, generating a continuous strand.



Replication

A complex of proteins including DNA polymerase attaches to the DNA strand at the replication fork, and slides along the DNA template strand. Two DNA polymerases and protein complexes are required for DNA replication (one for each strand) at the replication fork. After the synthesis of the leading strand and lagging strand, a DNA polymerase with exonuclease activity is necessary to remove the RNA primer and add complementary DNA nucleotides. The last phosphodiester ligation is done by a DNA ligase enzyme. Ligase enzyme joins the DNA cuts that contain a 5'-PO₄ and an adjacent 3'-OH group.

Transcription

Transcription is the synthesis of ribonucleic acid RNA using DNA as template. There are three significant chemical differences between RNA and DNA:

1. The RNA contains the ribose sugar instead of deoxyribose
2. RNA has uracil replacing the thymine from DNA
3. And excluding some viruses, RNA is not found as a double strand

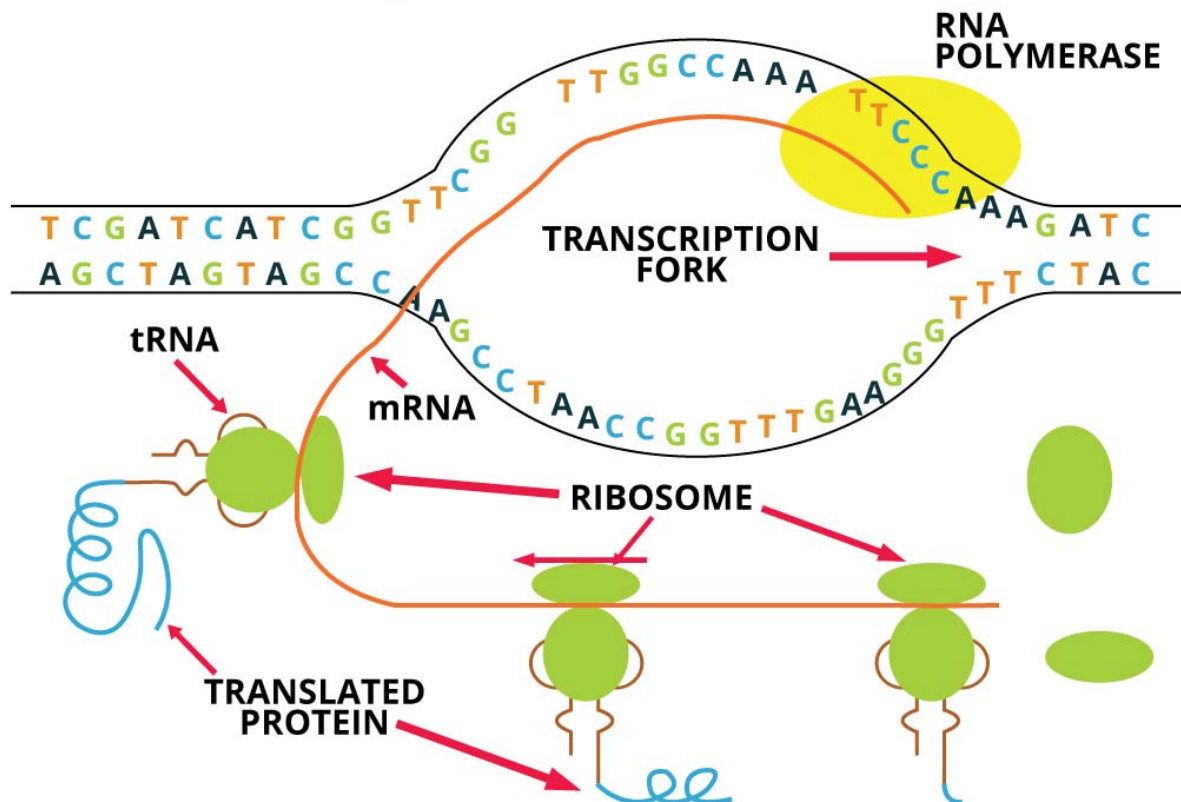
The replacement of deoxyribose by ribose affects the chemical properties of the nucleic acid and generally enzymes that catalyze reactions in DNA do not have any action in RNA (and vice-versa). Meanwhile, the substitution of thymine by uracil does not affect the base pairing, since both thymine and uracil pair with adenine with the same efficiency.

All RNA molecules are the product of DNA transcription. They play a role at two different levels, genetic and functional. At the genetic level, the mRNA carries the

genetic information from the genome to the ribosome. On the other hand, the rRNA play a functional and structural role in the ribosomes and the tRNA are responsible for transporting the amino acids for protein synthesis. Some RNA molecules, including rRNA, can have enzymatic activity.

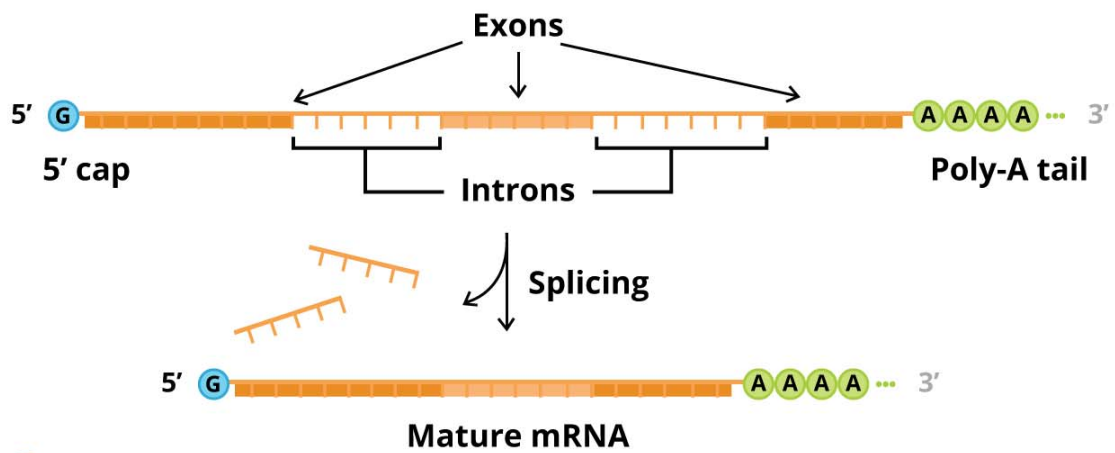
The transcription of genetic information is done by an RNA-polymerase enzyme in a similar fashion as DNA-polymerase does in DNA replication. RNA polymerase catalyzes the formation of phosphodiester between ribonucleotides. This is driven at the expense of energy released by hydrolysis of two phosphate bonds from ribonucleotides. Similarly to DNA synthesis, RNA synthesis is performed in the 5'-3' direction, with ribonucleotides being added to a free 3'-OH group from a previous ribonucleotide.

Transcription and Translation



Unlike DNA polymerase, RNA polymerase is able to start new strands independently. Consequently, there is no need for a primer. In order to start RNA synthesis, it is necessary that RNA polymerase recognizes the DNA initiation sequences, also referred to as promoters. After RNA polymerase binds the promoter, transcription is allowed to start. In this process, the DNA double helix at the promoter region is

residues, in a poly-A tail. This tail gives stability to the mRNA and its degradation will be required to allow RNA degradation.



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