**Molecular Diagnosis of**

**Microorganisms-**practical part

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* **Extraction of DNA**
* **genomic DNA**
* **plasmid DNA**
* **Polymerase Chain Reaction PCR**
* **Extraction of RNA**
* **Conversion of RNA to cDNA**
* **Real time-PCR**

**DNA Studies**

**Purification of total (genomic) DNA**

Genomic DNA can be extracted from the cell pellets of normal/cancer cell lines or tissues using DNeasy Blood & Tissue Kit (e.g. Qiagen) as per the manufacturer’s recommendations.

**Note**/ DNA can be extracted from different microorganisms, and manual techniques can be followed.

**Purification of DNA from living cells**

The procedure for total DNA preparation from a culture of bacterial cells can be divided into four stages:

1. A culture of bacteria is grown and then harvested.
2. The cells are broken open to release their contents.
3. This cell extract is treated to remove all components except the DNA.
4. The resulting DNA solution is concentrated.

**Preparation of a cell extract**

Techniques for breaking open bacterial cells can be divided into physical methods, in which the cells are disrupted by mechanical forces, and chemical methods, where cell lysis is brought about by exposure to chemical agents that affect the integrity of the cell barriers. Chemical methods are most commonly used with bacterial cells when the object is DNA preparation.

Chemical lysis generally involves one agent attacking the cell wall and another disrupting the cell membrane. The chemicals that are used depend on the species of bacterium involved, but with *E. coli* and related organisms, weakening of the cell wall is usually brought about by lysozyme, ethylene diamine tetra acetate (EDTA), or a combination of both. Lysozyme is an enzyme that is present in egg white and in secretions such as tears and saliva, and which digest the polymeric compounds that give the cell wall its rigidity. EDTA removes magnesium ions that are essential for preserving the overall structure of the cell envelope, and also inhibits cellular enzymes that could degrade DNA. Under some conditions, weakening the cell wall with lysozyme or EDTA is sufficient to cause bacterial cells to burst, but usually a detergent such as sodium dodecyl sulphate (SDS) is also added. Detergents aid the process of lysis by removing lipid molecules and thereby cause disruption of the cell membrane.

The final step in the preparation of a cell extract is the removal of insoluble cell debris. Components such as partially digested cell wall fractions can be pelleted by centrifugation, leaving the cell extracts as clear supernatant.

**Purification of DNA from a cell extract**

In addition to DNA, a bacterial cell extract contains significant quantities of protein and RNA. A variety of methods can be used to purify the DNA from this mixture. One approach is to treat the mixture with reagents, which degrade the contaminants, leaving a pure solution of DNA. Other methods use chromatography or some other fractionation process to separate the mixture into its various components, so the DNA is removed from the proteins and RNA in the extract.

**Removing contaminants by organic extraction and enzyme digestion**

The standard way to deproteinize a cell extract is to add phenol or a 1:1 mixture of phenol and chloroform. These organic solvents precipitate proteins but leave the nucleic acids (DNA and RNA) in aqueous solution. The result is that if the cell extract is mixed gently with the solvent, and the layers are then separated by centrifugation, precipitated protein molecules are left as a white coagulated mass at the interface between the aqueous and organic layers. The aqueous solution of nucleic acids can then be removed using a pipette.

With some cell extracts the protein content is so great that a single phenol extraction is not sufficient to completely purify the nucleic acids. This problem could be solved by carrying out several phenol extractions one after the other, but this is undesirable as each mixing and centrifugation step results in a certain amount of breakage of the DNA molecules. The answer is to treat the cell extract with a protease such as pronase or proteinase K before phenol extraction. These enzymes break polypeptides down into smaller unites, which are more easily removed using phenol.

Some RNA molecules, especially messenger RNA (mRNA), are removed by phenol treatment, but most remain with the DNA in the aqueous layer. The only effective way to remove the RNA is with the enzyme ribonuclease, which rapidly degrades these molecules into ribonucleotide subunits.

Other methods of purification include:

* **Using ion-exchange chromatography to purify DNA from a cell extract**
* **Using silica to purify DNA from a cell extract**

**Preparation of plasmid DNA**

The purification of plasmids from a culture of bacteria involves the same general strategy as the preparation of total cell DNA. A culture of cells, containing plasmids, is grown in liquid medium, harvested, and a cell extract prepared; the protein and RNA are then removed and the DNA probably concentrated by ethanol precipitation. However, there is an important distinction between plasmid purification and the preparation of total cell DNA. In a plasmid preparation it is always necessary to separate the plasmid DNA from the large amount of bacterial chromosomal DNA that is also present in the cells.

Separating the two types of DNA can be very difficult, but is nonetheless essential if the plasmids are to be used as cloning vectors. The presence of the smaller amount of contaminating bacterial DNA in a gene cloning experiment can easily lead to undesirable results. Fortunately, several methods are available for removal of bacterial DNA during plasmid purification, and the use of these- either individually or in combination- can result in the isolation of very pure plasmid DNA.

The methods are based on the several physical differences between plasmid DNA and bacterial DNA, the most obvious of which is size. The largest plasmids are only 8% of the size of the *E. coli* chromosome, and most are much smaller than this. Techniques that can separate small DNA molecules from large one should therefore effectively purify plasmid DNA.

In addition to size, plasmids and bacterial DNA differ in conformation. When applied to a polymer such as DNA, the term conformation refers to the overall spatial configuration of the molecule, with the two simplest conformations being linear and circular. Plasmids and the bacterial chromosome are circular, but during preparation of the cell extract the chromosome is always broken to produce linear fragments. A method for separating circular from linear molecules would therefore result in pure plasmids.

**Plasmid amplification**

The aim of plasmid amplification is to increase the copy number of a plasmid. Some multi-copy plasmids (those with copy numbers of 20 or more) have the useful property of being able to replicate in the absence of protein synthesis. This contrasts with the main bacterial chromosome, which cannot replicate under these conditions. This property can be utilized during the growth of a bacterial culture for plasmid DNA purification. After a satisfactory cell density has been reached, an inhibitor of protein synthesis (e.g., chloramphenicol) is added and the culture incubated for a further 12 h. During this time the plasmid molecules continue to replicate, even though chromosome replication and cell division are blocked. The result is that plasmid copy numbers of several thousand may be attained. Amplification is therefore a very efficient way of increasing the yield of multicopy plasmids.

**Preparation of bacteriophage DNA**

The key difference between phage DNA purification and the preparation of either total cell DNA or plasmid DNA is that, for phage, the starting material is not normally cell extract. This is because bacteriophage particles can be obtained in large numbers from the extracellular medium of an infected bacterial culture. When such a culture is centrifuged the bacteria are pelleted, leaving the phage particles in suspension. The phage particles are then collected from the suspension and their DNA extracted by a single deproteinization step to remove the phage capsid.

This overall process is more straightforward than the procedure used to prepare total cell or plasmid DNA. Nevertheless, successful purification of significant quantities of phage DNA is subject to several pitfalls. The main difficulty, especially with λ lambda phage, is growing an infected culture in such a way that the extracellular phage titre (the number of phage particles per ml of culture) is sufficiently high. In practical terms, the maximum titre that can reasonably be expected for λ is 1010 ml -1, yet 1010 λ particles will yield only 500 ng of DNA. Large culture volumes, in the range of 500-1000 ml, are therefore needed if substantial quantities of λ DNA are to be obtained.

**Calculating nucleic acid concentration**

Both RNA and DNA concentrations are determined using a NanoDrop® Spectrophotometer.

**Primers**

Primers or oligonucleotides are used for amplification of cDNA or fragments of gDNA sequences applying PCR, RT-qPCR etc. Primers can be designed using NCBI software or manually as below.

**Design primers manually**

Each one of a pair of PCR primers needs to be about 18-30 nt long and to have similar G+C content so that they anneal to their complementary sequences at similar temperatures. For short oligonucleotides (<25 nt), the annealing temperature (in °C) can be calculated using the formula:

Tm= 2(A+T) + 4(G+C)

Where Tm is the melting temperature and the annealing temperature is approximately 3-5°C lower.

The primers are designed to anneal on opposite strands of the target sequence so that they will be extended towards each other by addition of nucleotides to their 3’-ends. Short target sequences amplify more easily, so often this distance is less than 500 bp, but, with optimization, PCR can amplify fragments over 10kb in length. If the DNA sequence being amplified is known, then primer design is relatively easy. The region to be amplified should be inspected for two suitable sequences of about 20 nt with a similar G+C content, either side of the region to be amplified (e.g. the site of mutation in certain cancers). If the PCR product is to be cloned, it is sensible to include the sequence of unique restriction enzyme sites within the 5’-ends of the primers.

If the DNA sequence of the target is not known, for instance, when trying to clone a cDNA for a protein for which there is only some limited amino acid sequence available, then primer design is more difficult.

**Melting Temperature (Tm):** The melting temperature is the temperature at which an oligonucleotide duplex is 50% single-stranded and 50% double-stranded.

During the annealing step of PCR, primers and probes hybridize to targets, forming short duplexes. The stability of these duplexes is described by the melting temperature. Melting temperature depends on oligonucleotide sequence, oligonucleotide concentration, and cations found in the buffer, specifically salt concentrations (e.g. Na+ and Mg2+).

An annealing temperature of 60˚C can be used during PCR. The optimal melting temperature of the primers is between 60 and 64˚C, with an ideal temperature of 62˚C, which is based on the average conditions and factors associated with the PCR. The melting temperature of the two primers should not differ by more than 4˚C in order for both primers to bind simultaneously and efficiently amplify the product.

**GC content:** ensure that the primers are specific to the target and that they do not contain regions of four or more consecutive Gs. The GC content should be within the range of 35-65%, with an ideal content of 50%, which allows complexity. Avoid sequences that may create secondary structures, self-dimers, and heterodimers.

**General Design Considerations**

* It is important to understand your gene of interest, including knowledge of the transcript variants and their exon organization.
* Use databases such as Ensembl or GenBank to identify exon junctions, splice variants, and locations of single-nucleotide polymorphisms (SNPs).
* For genes that have multiple transcript variants, align related transcripts to understand exon overlap using program such as NCBI online tools (Gene Viewer).
* For transcript-specific designs, target primers and probes within exons unique to the transcripts of interest, and BLAST primer and probe sequences to ensure they do not occur in related transcripts or cross-react with other genes within the species.
* For splice-common designs, target primers and probes within exons found across all transcript variants.
* Avoid SNPs: A single mismatch between primer and target, due to a SNP, can significantly decrease the melting temperature of the hybrid (by up to 10 ˚C), affecting the efficiency of the PCR, and ultimately the interpretation of experimental results.
* Ensure that both the primers and the probe are specific to the target and not complementary to other sequences. Use BLAST to analyze the sequences to ensure their specificity. BLAST is a Basic Local Alignment Search Tool provided by NCBI that finds regions of local similarity between sequences.
* For two-step RT-qPCR protocols, the input amount of cDNA used for qPCR can be regulated to increase the amount of target available for detection. This is useful when working with low abundance targets when sample is not limited.
* When sample is limited, pre-amplification of RNA or first strand cDNA prior to qPCR, can increase the amount of detectable target for low abundance transcripts from minute amounts of sample. This extra step is incorporated when working with clinical samples or single cell analysis, fine needle biopsies…..
* **Primers**

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**Length:** use primer lengths of 18-30 bases with a balance between the melting temperature, purity, specificity, and secondary structure…

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* **Probes**

**Tm:** The melting temperature of the probe should be 6-8°C higher than the primers and should fall within the range of 66-70°C for a standard two-step protocol. If the melting temperature is too low, the probe will not bind to the target.

**Length:** The length of a single-quenched probe should be 20-30 bases to achieve an ideal Tm without increasing the distance between the dye and quencher such that the quencher will no longer absorb the fluorescence of the dye.

**GC content:** The GC content should be within the range of 35-65% and avoid G at the 5’ end to prevent quenching of the 5’ fluorophore. As with the primers, avoid sequences that may create dimers or secondary structures.

**Location:** Ideally, the probe should be in close proximity to the forward or reverse primer, but not overlap. Probes can be designed to bind to either strand of the target.

**Amplicons**

Design amplicons of 70-150 bp, which will allow the primers and probe to compete for hybridization and provide a sequence that is long enough for all components to bind. This length is most easily amplified using standard cycling conditions. Longer amplicons of up to 500 bases can be generated, but cycling conditions will need to be altered.

**Examples of PCR primers used for sequencing**

All oligonucleotides used to sequence the coding region of *E2F6* and *STAG3* promoter region were purchased from Eurofins, Germany. For *E2F6* sequencing, many different primers and some are unique for the transcript variant a (isoform 1) and others unique for variant b (isoform 2) were designed and listed below.

|  |  |
| --- | --- |
| Primer | Sequence |
| STAG3 Fwd  | CACCGATTCACCCCTAGATGTGT |
| STAG3 Rev  | TCCTCTCACACCTTCCCCAGAG |
| E2F6-b Fwd | ATGGAAGATGCTTTGGATGAG |
| E2F6 c,d,e Fwd | ATGGATCTTGTCAGATCTGCTCCC |
| E2F6 f 6F3 Fwd | ATGGAAGATGCTTTGGATGAG |
| 6R-common Rev | TCAGTTGCTTACTTCAAGCA |
| E2F6 Rem For | ATGAGTCAGCAGCGGCCGGCGA |
| E2F6 Rem Rev | GGTGATGTCATACACTCTCCGC |

**PCR**

PCR is used to amplify DNA samples in normal/cancer cell lines or tissues and also to incubate the reverse transcription reaction according to the instructions of the used kit. PCR is performed using Master cycler® or Theromcycler. The PCR reaction mixture used for DNA amplification is composed of mili Q water or ddH2O, 5× Hi-Fi reaction buffer, 10 mM dNTP mix, 10 µM of each forward and reverse primers, DNA template as required and velocity DNA polymerase enzyme. Standard cycling conditions are as below. The amplified PCR products are confirmed using agarose gel electrophoresis.

**PCR reaction**

|  |  |
| --- | --- |
| ddH2O | Up to 50µl |
| 5× Hi-Fi Reaction Buffer | 10 µl |
| 10 mM dNTP mix | 2 µl |
| Primers 10 µM each | 1 µl each |
| cDNA or DNA template | As required |
| Velocity DNA polymerase enzyme | 1 µl |

**PCR cycling conditions**

|  |  |  |  |
| --- | --- | --- | --- |
| **Step** | **Temperature** | **Time** | **Repeat** |
| Initial denaturation | 98ºC | 5 min | 1 |
| Denaturation | 95ºC | 30 s | 30-40 |
| Annealing | 50-68ºC\*  | 30 s |
| Extension | 72ºC | 30 sec- 4 min\*\* |
| Final extension | 72ºC | 4-10 min | 1 |
| Hold | 4ºC | forever |  |

\* depends on the primer annealing temperature, \*\* depends on the length of the desired amplicon.

For incubation of the reverse transcription reaction, the PCR cycling conditions are 37ºC for 60 min, and then stop the reaction by heating to 95ºC for 5 min and hold at 4ºC (following recommendations of High Capacity RNA-to-cDNA Kit), or use temperatures of: 25ºC for 10 min followed by 37ºC for 120 min and 5 min at 85ºC and hold at 4ºC according to the instructions of High Capacity cDNA Reverse Transcription Kits.

**Agarose gel electrophoresis**

PCR products, amplified DNA molecules, are separated and analysed by using agarose gel electrophoresis.1× TAE buffer (prepared as below) is used to dissolve 1% (w/v) agarose, which is heated using a microwave. The melted agarose gel is poured into its cast with putting a suitable comb to make holes where the samples loaded. The comb is then removed from the solidified gel, which in turn is immersed in the electrophoresis tank filled of 1× TAE. Before loading the DNA samples onto the gel, they are mixed with 6× DNA loading dye and diluted with ddH2O to a total volume of 12 µl. Finally, a suitable amount of Ethidium Bromide (Bio-Rad) is added to the TAE buffer, and the electrophoresis of samples is run at 85 V in TAE buffer for 1-2 h to separate DNA bands. PCR products are visualized by putting the gel on a UV transilluminator. Gel images are taken using a suitable equipment if available (the Kodak Electrophoresis Documentation and Analysis System 120 and Kodak 1D image analysis software). Sizes and concentrations of DNA are compared with 1 kb DNA Ladder (e.g. Biolab), which is loaded onto the gel.

**50× TAE buffer**

1 L 50× TAE buffer is made by dissolving 242 g Tris-base, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA pH 8.0. The ingredients are dissolved in 500 ml ddH2O on magnetic stirrer. The pH is amended by HCl to 7.6-7.8, and the volume completed to 1 L using ddH2O. The solution is stored at room temperature.

**PCR optimization**

PCR reactions are not usually 100% efficient, even when using cloned DNA and primers of defined sequence. Usually the reaction conditions must be varied to improve the efficiency. This is very important when trying to amplify a particular target from a population of other sequences, for example one gene from genomic DNA, or one cDNA from either a cDNA library or the products of a first strand cDNA synthesis reaction. This latter method of reverse transcribing mRNA and then PCR amplifying the first strand cDNA is called reverse transcriptase (RT)-PCR. If the reaction is not optimal, PCR often generates a smear of products on a gel rather than a defined band.

The usual parameters to vary include the annealing temperature and the Mg2+ concentration. Too low an annealing temperature favors mispairing. The optimal Mg2+ concentration varies with each new sequence, but is usually between 1 and 4 mM. The specificity of the reaction can be improved by carrying out nested PCR, where, in a second round of PCR, a new set of primers are used that anneal within the fragment amplified by the first pair, giving a shorter PCR product. If on the first round of PCR some nonspecific products have been produced, giving a smear or a number of bands, using nested PCR should ensure that only the desired product is amplified from this mixture as it should be the only sequence present containing both sets of primer-binding sites.

**PCR variations**

If multiple pairs of primers are added, PCR can be used to amplify more than one DNA fragment in the same reaction and these fragments can easily be distinguished on gels if they are of different lengths. This use of multiple sets of primers is called **multiplex PCR** and is often used as a quick test to detect the presence of microorganisms that may be contaminating food or water, or be infecting tissue. Modifications to the basic PCR make it possible to amplify (and hence clone) sequences that are upstream or downstream of the region amplified by the basic primer pair. For example, if genomic DNA is first digested by a restriction enzyme and then circularized by ligation, a pair of back-to-back primers can be used to amplify round the circle from the region of known sequence to obtain the 5’- and 3’-flanking regions up to the joined restriction sites. This is known as **inverse PCR**. When a fragment of cDNA has been produced by RT-PCR it is possible to amplify the 5’-flanking sequence by first using terminal transferase to add a tail, e.g. oligo (dC), to the first strand cDNA. This allows a gene specific primer to be combined with oligo (dG) primer to amplify the 5’-region. This technique is called rapid amplification of cDNA ends (**RACE**). 3’-RACE to amplify the 3’-flanking sequence of eukaryotic mRNAs uses a gene specific primer and an oligo (dT) primer, which will anneal to the poly (A) tail at the 3’-end of the mRNA. RNA can be used to make labeled probes to screen libraries or carry out blotting experiments, by adding radioactive or modified nucleotides in the later stages of the PCR reaction, or labeling the PCR product. PCR can be used to introduce specific mutations into a given DNA fragment.

**Multiplex qPCR**

In multiplex PCR, multiple targets are amplified in a single reaction tube. Each target is amplified by a different set of primers and a uniquely labeled probe that will distinguish each PCR amplicon. Multiplexing provides some advantages over single-reaction PCR, including requiring a lower amount of starting material, increased throughput, reduce reagent costs and less sample handling. However, the experimental design for multiplexing is more complicated because the amplification of each target can affect others in the same reaction. Therefore, careful consideration of design and optimization of the reactions is critical. In order to incorporate all necessary parameters, it is recommended to use a design tool for primers and probes.

1- Ensure that the primers and probe sets are not complementary to each other. Use BLAST to analyze the sequences and ensure they are non-complementary.

2- each target must be identified by a separate reporter dye. Select dyes with little or no overlap in their emission spectra. Some instruments are compatible with only certain dyes. As a general rule, it is a good idea to choose FAM for any low copy transcripts because of its strong signal. Lower signal fluorophores can then be used for the more abundant transcripts.

3- It is important to optimize the individual reactions and ensure that each has an efficiency >90%.

4- Validate the multiplex reactions by running a combined reaction alongside an individual reaction to ensure similar performance. Compare the standard curves and verify that the Cq/Ct values are similar at both the high and low ends. A good multiplex should have similar curves and similar limits of detection.

5- Optimize the multiplex reactions. Limit the primers for targets expressed at a high level to a 1:1 primer-to-probe ratio. Increase the primer-to-probe ratio for targets expressed at lower levels. Increasing the amount of enzyme and dNTPs added to the reaction may be essential. It is recommended to double the amount of these reagents.

**Multiplex qPCR**

In multiplex PCR, multiple targets are amplified in a single reaction tube. Each target is amplified by a different set of primers and a uniquely-labeled probe that will distinguish each PCR amplicon.

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**Sequencing reaction**

For example, cDNAs or gDNA from normal/cancer cell lines or tissues with many different primers are used to amplify the required coding regions or genomic DNA promoter region for instance ~1 kb from the transcription start site. Sequencing is performed by sequencing service or specific sequencing company. DNA templates to be sequenced and their forward and reverse primers are prepared as required by the facility. Sequences obtained have to be treated manually to confirm the sequence, aligned by LALIGN Server software or using 6-Frame Translation**.** Concerning the sequenced coding region, the nucleic acid sequence is translated to its corresponding protein sequence using Blast Basic Local Alignment Search Tool**.**

**RNA Studies**

**Comparison between methods used for RNA studies**

Quantitative real-time polymerase chain reaction (RT-qPCR) has become the most precise and accurate method for analyzing gene expression. Prior to qPCR, many methods were used for determining gene expression levels including: northern blotting, RNase protection assays, or traditional endpoint reverse transcription (RT) PCR. Endpoint RT-PCR was an improvement over the older methods because of the ease of its use and the much smaller amounts of RNA needed for the reaction. However, with this method, expression levels can only be observed by performing agarose gel electrophoresis on a sample of the product at the end of the entire reaction. While traditional RT-PCR can be useful for determining the presence or absence of a particular gene product, qPCR has the advantage of measuring the starting copy number and detecting small differences in expression levels between samples. qPCR allows investigators to observe PCR product accumulating over the entire amplification curve and eliminates the need to run a gel, which reduces the duration of the process and the chance of contamination. Amplification and quantification occur simultaneously.

A typical qPCR amplification plot has baseline, exponential, linear, and plateau phases. Amplification reaches a plateau as the reaction components are exhausted and PCR products self-anneal and thus prevent further amplification.

In endpoint PCR, amplification can only be viewed at the end of the reaction, and only the final plateau is observed. Any differences in initial abundance are obscured. In contrast, qPCR quantifies the PCR products while the amplification is in progress. Fluorescent reagents allow amplification to be measured while the reaction is occurring. This allows analysis of the entire amplification curve rather than only the end point.

**Intercalating Dyes**

Intercalating dyes are nonsequence-specific fluorescent dyes that exhibit a large increase in fluorescence emission when they intercalate into double-stranded DNA. Examples include SYBR Green I, Cyto, EvaGreen and LC dyes. During PCR, the primers amplify the target sequence and multiple molecules of the dye are inserted between bases of the double-stranded product, causing fluorescence.

* Intercalating fluorescent dyes are not specific to a particular sequence; thus they are both inexpensive and versatile.
* As they can bind to any double-stranded sequence, they will also bind to primer-dimer artifacts or incorrect amplification products. Therefore, when using intercalating dyes, it is essential to analyze the melting curve of the amplicon to ensure that the primers are amplifying a single product, observed as a single melting curve peak.
* These types of dyes cannot be used for multiplex analyses as the different PCR products would be indistinguishable.
* Because multiple dye molecules intercalate into a double-stranded product, the intensity of the fluorescent signal is dependent on the mass of the amplified product. E.g. assume you amplify long and short product of the same efficiency, a longer product will generate more signal than a shorter product. In contrast, probes are specific to a particular sequence and will emit the same amount of energy from a single fluorophore irrespective of the amplified product length.
* The choice of fluorescent dye will depend on the instrument used and the compatibility of the dye with the instrument.

**qPCR Workflow**

The typical qPCR experiment involves the following steps:

* Sample collection.
* RNA isolation and quality control.
* Reverse transcription.
* Real-time PCR.
* Assay validation and data analysis.

Collect sample isolate RNA reverse transcription reaction qPCR analyze qPCR.

**RNA Extraction and Quality control**

The first step in running qPCR assay is to collect the sample and isolate total RNA. The method of RNA isolation will depend on the sample type and experimental conditions. Following isolation, both the quantity and quality of the RNA should be assessed. It is important that the sample remain free of RNases and DNases.

To analyse gene expression at the mRNA level and also to sequence the coding region of any gene, after obtaining the cell pellets (when working on cell lines) as above, RNA is extracted using RNeasy® Mini Kit (e.g. Qiagen) or mammalian total RNA miniprep kit (Sigma-Aldrich). Manufacturer’s instructions are applied to obtain total RNA whose concentration is measured as described below.

1. **Isolate or extract RNA**

To analyse gene expression at the mRNA level and also to sequence the coding region of any gene, after obtaining the cell pellets, RNA can be extracted using RNeasy® Mini Kit (e.g. Qiagen) or mammalian total RNA miniprep kit (e.g. Sigma-Aldrich). Manufacturer’s instructions are applied to obtain total RNA whose concentration is measured as described above.

Otherwise, RNA can be isolated using organic extraction methods (TRIzol® reagent), QIAzol® reagent or a variety of solid phase RNA isolation kits that are available commercially from companies including Qiagen, Life Technologies…

The best method will depend on your sample type and the amount of RNA available for harvesting. For instance, small RNAs and miRNAs can only be efficiently isolated using organic extraction methods, while solid phase kits are suitable for high-throughput processing.

It is essential that the RNA be extracted from all samples using the same method, and that the resulting RNA be of high quality. Variations in either sampling or isolation methods can result in unwanted differences between samples. Surfaces and supplies should be free of RNases. Some samples may require DNase treatment to avoid contamination with genomic DNA. This step may not be necessary if the assay is designed to span exon junctions.

RNA is converted immediately to cDNA, or kept at -20˚C for short-term storage for a few months. For longer term storage, freeze and store the RNA at -80˚C, or precipitate the RNA and store it in ethanol at -20˚C.

1. **Quantity**

RNA can be quantified by several methods, including UV spectrophotometry, microfluidic analysis (capillary gel electrophoresis), or by the use of fluorescently-labeled RNA binding dyes. Absorbance measurements at 260 nm on standard spectrophotometers can be used for quantification when RNA is abundant, while the NanoDrop and DropSense instruments are useful for measuring limited quantities of sample.

1. **Check Quality**

It is important that the samples have similar quantities and quality of RNA. The quality of the RNA can affect the results of the experiment; poor RNA quality can compromise the entire experiment and result in wasted time and money. Moreover, differences in quality between two samples can result in misinterpretation of gene expression differences.

RNA quality can be assessed most accurately by calculating the integrity of the RNA. High-quality eukaryotic RNA have both 18S and 28S rRNA peaks, with the 28S region in greater abundance, and a low amount of 5S RNA (note/the 28S rRNA species is more susceptible to degradation). The RNA integrity value is determined from the shape of the resulting electropherogram curve and is based on several characteristics. The ideal integrity value will depend on the RNA source, as some tissues will provide higher quality RNA. For more information, see the reference below:

Fleige S and Pfaffl MW. (2006) RNA integrity and the effect on the real-time qRT-PCR performance. Mol Aspects Med, 27 (2-3): 126-139.

The ratio of 260/280 nm absorbance readings can give an indication of RNA quality. However, other contaminants may influence this ratio, making it less accurate than analysis of the RNA integrity value. A ratio of 1.8 indicates the RNA is of good quality. Lower ratios could be due to organic compound contamination. Turbidity or low pH can also lead to calculation errors. Alternative methods for determining quality include gel electrophoresis or a reference gene/target gene 3:5 integrity assay.

1. **Avoid RNases, DNases**

RNases and DNases are nucleases that can quickly degrade samples and oligonucleotide primers and probes. They are ubiquitous and can be difficult to eliminate. Therefore, it is very important to take precautions to ensure that samples are protected from degradation by these nucleases. RNase inhibitors can be added to block the action of some ribonucleases, and DNases can be inactivated by heat treatment.

**Guidelines for Maintaining a Contamination-Free Workplace**

Many guidelines are available.

1. **Sample**

For accurate comparison in the qPCR step, equal amounts of starting RNA should be used from each sample in the RT reaction. Large variations in the amount of RNA between RT reactions can cause fluctuations in RT efficiency. Poor RT may lead to loss of signal or failure to detect transcripts with low levels of expression.

1. **Primers choice**

The type of primers used will depend on the experimental goal. Both random primers and oligo (dT) will produce random cDNA, while gene-specific primers produce cDNA for a specific target.

* **Random hexamer and nonamer primers** bind to RNA at different complementary sites and lead to short, partial-length cDNA. These primers can be used when the template has extensive secondary structure. Random primers will produce the greatest yield, but the majority of the cDNA will be copies of ribosomal RNA, unless it is depleted prior to RT-PCR. The main advantage to using random primers is the preservation of the transcriptome so that any remaining cDNA can be used in other qPCR assays. The disadvantage is that low abundance messages may be under-represented due to consumption of reagents during cDNA synthesis of the more prevalent RNAs. Random hexamers produce a greater amount of cDNA, while random nonamers produce longer products.
* **Gene-specific oligonucleotide primers,** selectively bind the mRNA of interest, yield the least complex cDNA mixture and avoid reagent depletion. The main disadvantage to their use is that the cDNA produced cannot be used for assaying other genes.
* **Oligo(dT) primers** ensure that mRNA containing poly (A) tails are reverse transcribed. These primers are more commonly used when trying to limit the amount of ribosomal RNA being copied, or when the qPCR assays are designed to target the 3’ end of the RNA. If the mRNA is long, the 5’ end of the message may be under-represented.
1. **Replicates and Controls**

It is very important that all samples are treated the same including the input amount of RNA, the priming strategy, the enzyme type, the volume of the reaction, the temperature used, and the reaction time.

**Replicates:** for each experimental and control sample to be compared, it is recommended that at least three biological replicates are used. The number of technical replicates performed depend on the steps taken to minimize errors due to poor pipetting or uncalibrated equipment, and on the precision required.

**No RT Control:** for every reverse transcription control, it is important to incorporate a “no RT control” to identify erroneous signal due to genomic DNA contamination. This negative control contains all of the components of the other reactions except the reverse transcriptase is missing.

1. **cDNA Storage**

Aliquot the cDNA samples and store the first strand cDNA at -20˚C.

1. **One-Step versus Two-Step RT-qPCR**

One-step qPCR may be a good option if you plan to use the cDNA for only a limited number of assays. In contrast, two-step qPCR is recommended if you are interested in making a large amount of cDNA to use for multiple assays.

One-step qPCR can be less sensitive than two-step and prevent you from using various amounts of input cDNA.

For more information, see the article:

*Starting with RNA-One-Step or Two-Step RT-qPCR,* in the IDT DECODED 1.3, October 2011 newsletter at www.idtdna.com.

**RT-qPCR primers**

Primers are used in RT-qPCR to detect the expression of target genes in normal and cancer cell lines. Either the forward or reverse primer of each gene is placed at the junction between two exons to avoid contamination with amplified genomic DNA. The length of amplicons is about 100 nucleotides. All the oligonucleotides supplied are dried, and suitable amounts of milliQ water (ddH2O) to make a stock concentration of 100 µM are added as stated by the manufacturer. Except reference primers particularly 18S and Actin, which are used in 5 µM concentration, all the other oligonucleotides are then diluted to 10 µM concentration using ddH2O prior to use and stored at -20ºC. Here are **examples for some primers**:

|  |  |
| --- | --- |
| Primer | Sequence |
| FLI1#1 Fwd. | GAATTCTGGCCTCAACAAAAG |
| FLI1#1 Rev | CCCAGGATCTGATACGGATCT |
| FLI1#2 Fwd | ATCCAGCTGTGGCAATTCCT |
| FLI1#2 Rev | CATCGGGGTCCGTCATTTTG |
| 18S Fwd | AGAAACGGCTACCACATCCA |
| 18S Rev | CACCAGACTTGCCCTCCA |
| Actin Fwd | CAGCCATGTACGTTGCTATCCAGG |
| Actin Rev | AGGTCCAGACGCAGGATGGCATG |
| HSPCB Fwd | TCTGGGTATCGGAAAGCAAGCC |
| HSPCB Rev | GTGCACTTCCTCAGGCATCTTG |

Note/ Primer and probe design are crucial to the success of the experiment.

**Reverse Transcription**

Transcription is the synthesis of RNA from a DNA template; reverse transcription (RT) is the synthesis of DNA from an RNA template. DNA synthesized from RNA is often referred to as first-strand cDNA. The conversion of RNA to cDNA is necessary because PCR uses DNA-dependent polymerases. The exact reaction conditions depend on the particular kit or protocol used, but all contain the same basic components: the RNA to be converted, dNTPs to provide the nucleotides for cDNA synthesis, primers, buffer, DTT to stabilize the enzymes, RNase inhibitor to prevent RNA degradation, and a reverse transcriptase enzyme.

Following extraction, RNA is converted to cDNA using a High Capacity RNA-to-cDNA kit (AB Applied Biosystems) or High Capacity cDNA Reverse Transcription Kit (AB Applied Biosystems). The recommendations of the manufacturer are followed. The cDNA is kept at -20˚C. The cDNA is used for RT-qPCR to study gene expression, to sequence the coding region of target genes, and also can be used for other purposes*.*

**Quantitative PCR (RT-qPCR)**

**RT-qPCR** can determine the amount (number of molecules) of DNA in a test sample. One of the best methods of quantitative PCR involves adding known amounts of a similar DNA fragment, such as one containing a short deletion, to the test sample before amplification. The ratio of the two products produced depends on the amount of the deleted fragment added and allows the quantity of the target molecule in the test sample to be calculated. In asymmetric PCR only one strand is amplified (in a linear fashion) and when applied to DNA sequencing it is known as cycle sequencing. PCR can also be used to increase the sensitivity of DNA fingerprinting.

In **real time PCR** the thermal cycler can determine the amount of product that has been made as the reaction proceeds, for instance by detecting the increase in dye binding by the synthesized DNA, using a fluorometer. The advantages of real time PCR, apart from immediate information on the progress of the reaction, include high sensitivity, ability to cover a large range of starting sample concentrations, easy compensation for different efficiencies of sample amplification and the ease of processing many samples, since these do not necessarily need to be analyzed at the end by, for example, gel analysis. Unfortunately, the equipment is rather costly.

RT-qPCR is applied to study the expression of target genes, the reference genes along with the genes used as negative controls. Each reaction consists of 20 µl of the following: 10 µl of 2× SensiMix containing a mixture of: buffer, dNTP, HiRox, SYBR Green and modified Taq polymerase called “Hot start”. Additionally, the reaction includes: 3 µl of ddH2O sterile water, 2 µl of 10× primers with 5 µl cDNA as a template. Reactions are carried out in quadrate, triplicate or in duplicate technical repeats.

Analysis of melt curve is applied to check for presence of primer dimers as well as amplification of a single product. The cycling conditions are 95°C for 10 minutes for Taq polymerase activation followed by 40 cycles of denaturing at 95°C for 15 s, annealing at 58°C for 15 s and finally 30 s at 72°C for extension. PCR product quantity gained is proportional to the fluorescence signal. Using the software, CT (the threshold) value is determined for each cDNA sample in every reaction.

In order to assess whether the two amplicons of the target and reference genes have roughly the same amplification efficiency, 10-fold serial dilutions of 1/10, 1/100, 1/1000, 1/10,000 and 1/100,000 are prepared from cDNA samples. These cDNA serial dilutions were amplified by gene-specific primers using RT-qPCR and the Δ CT values are calculated for the dilutions. The amplification efficiency is determined using the formula E= 10 (-1/slope) (Pfaffl, 2001).

Relative gene-expression value is calculated according to (Livak and Schmittgen, 2001) using the formula: Δ CT = CT target gene- CT endogenous reference gene, allowing for the comparison of samples independently of the amount of total cDNA input. If the average amplification efficiency is 1.9, so that 1.9 –ΔΔCT parameter is applied to compare the expression level among genes in the same cell line or relative to the expression of same gene in a normal tissue or cell line. This denotes the expression fold of one gene with regard to a reference gene.

Concerning gene depletion in RNAi experiments, the remaining mRNA of the silenced gene is measured using the method described by Haimes and Kelley in Thermo Fisher Scientific (2010).