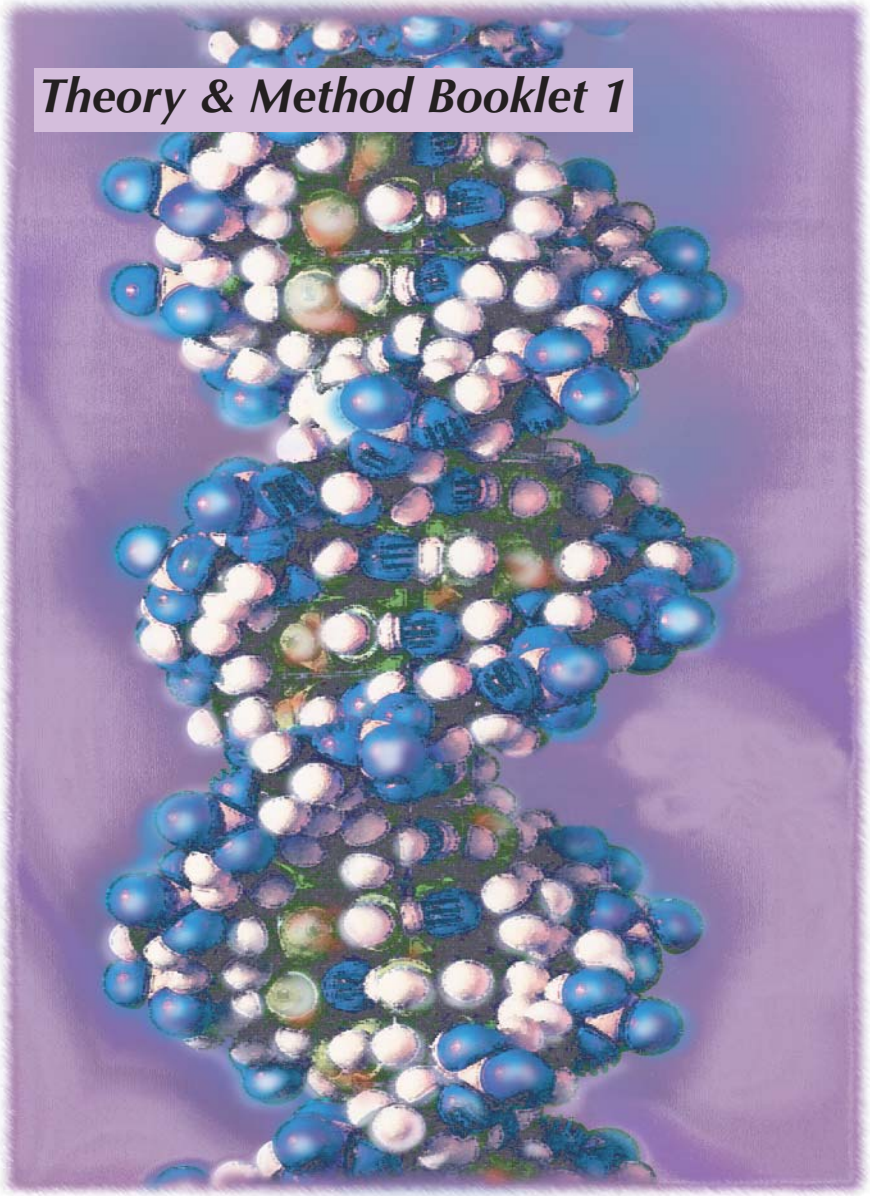
Theory & Method Booklet 1



Molecular Applications:
Oligonucleotides, PCR© & RT-PCR©

Vol.1

BIOSOURCE INTERNATIONAL

Molecular Applications:
Oligonucleotides, PCR© & RT-PCR©

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Introduction

In the last twenty years, the great strides made in molecular biology are directly attributable to the wide spread use of DNA sequencing and PCR sequence amplification. Key to the performance of these technologies is the synthetic DNA oligonucleotide. This is due to the specificity of the synthetic oligo that resides in its ability to base pair to a precise region of its target. This specificity enables the oligo to be used either as a primer for DNA synthesis extension or as a probe for detection or targeting of specific gene sequences. Table I is a list of general applications for synthetic oligonucleotides.

Table I

General Applications of Synthetic Oligonucleotides

Type of Oligo	Application
Primers	DNA Sequencing, PCR
Probes	Colony Hybridization, Diagnostic Hybridization
Gene Synthesis	Synthetic Genes
Therapeutic	Antisense
Physiochemical Studies	NMR, X-ray crystallography

While DNA oligo synthesis dates back more than forty years, it has only been the last twenty years that DNA oligos have become readily available to the research community. This is the result of the development of beta-cyanoethyl phosphoramidite chemistry (1), robust automated synthesis protocols and equipment, and availability of inexpensive reagents.

One of the earliest uses of DNA oligos was as hybridization probes. Oligos were labeled with radioactive ^{32}P and used to probe Southern blots or to screen recombinant libraries for specific genes. Recently, oligos have been used more often as primers for DNA sequencing and PCR amplification. While the use of isotopically labeled oligos as probes has diminished, fluorescent labeled oligos, such as Molecular Beacons (2) or TaqMan probes (3), have increased sharply in use as real-time PCR quantitation technologies and genetic detection in homogeneous solutions become more popular. Also the increased use of antisense oligos as therapeutic agents for the inhibition of either gene transcription or mRNA translation, has spurred the development of a new generation of oligo chemistries and modifications. BioSource International, Inc. continues to play an active role as a supplier of custom oligonucleotides and of research molecular kits and reagents for these new technologies.

BioSource Custom Oligonucleotide Service

BioSource Custom Oligonucleotide Service's main goal has been to provide the highest-quality, low-cost DNA primers to the molecular biology community. This commitment to quality and service has resulted in the constant in-house development of new or improved synthesis instrumentation, reagents, and synthesis and purification protocols. These new technologies provide not only standard oligonucleotides of the highest quality, but complex modified oligos of the most demanding purity. Among the services BioSource provides are oligos with unique reporter and linkage groups, enzyme conjugated oligos, and fluorescent labeled oligos used in energy transfer technologies such as Molecular Beacons and TAQMAN®.

The commitment to quality and service has also led to the development of the BioSource Custom Oligo Quality Assurance Program. This Quality Assurance Program begins with your product order and finishes with your product delivery. Central to this program is our integrated information management system that tracks your order from receipt, transfer to synthesizer, in-process monitoring, post synthesis testing, packaging, labeling and shipping. Our system insures that you get the right oligo, on time, every time.

Ordering: BioSource oligos may be ordered by fax, email or online at our WEB site. For 100% order accuracy, all fax orders (1-800-786-4362) are double confirmed using our computer voice replay system. Email and WEB orders (www.keydna.com) are transferred directly to our system without alteration or manipulation. All electronic orders are confirmed by email.

Synthesis: All reagents used are of the highest quality available. Phosphoramidites, CPG and activator are QC tested extensively before use. All our synthesizers and synthesis protocols have been modified and optimized to ensure coupling efficiencies in excess of 99%. Each day every instrument is monitored to safeguard that the highest coupling efficiencies are maintained.

In-Process Monitoring: All syntheses are monitored for coupling efficiencies using trityl analysis methods. Each wash step during the synthesis cycle is monitored for the complete removal of either trityl or oxidation products to ensure minimal failure sequences.

Post Synthesis Testing: Following trityl monitoring, representative oligos are selected and analyzed by either HPLC or Capillary Electrophoresis to ensure the quality of each machine's synthesis run. OD_{260} values are determined for all synthesized oligos before lyophilization to guarantee that synthesis yields are in-line with theoretical yields determined by trityl analysis.

Packaging, Labeling and Shipping: After post synthesis processing, computer aided workstations process the oligos through lyophilization, capping, labeling and shipping. Each step is tracked by our information management system. A Certificate of Analysis is generated for each oligo which provides molecular weight, extinction coefficient and melting temperatures based on exact sequence, primer name, sequence, and amount in OD_{260}, micrograms and picomoles.

Priority Overnight Delivery: Every order is shipped by Priority Overnight Federal Xpress to ensure rapid and dependable delivery to you. All EconoPURE oligos are shipped 24 hours after receipt of order.

Customer Service and Technical Support: BioSource's well-trained Customer Service and Technical Support staff will provide a prompt and knowledgeable response to all of your questions. Should any part of your order be unclear, our staff will contact you by phone or email to resolve the question. We will also make every effort to contact you should your order be delayed for any reason. BioSource Technical Support staff has twenty-five years combined experience in the synthesis and modification of oligonucleotides. Our staff is available to provide technical assistance in the selection of synthesis protocols, modifications and applications of your oligo.

Synthesis and Purification Services Offered: BioSource offers a wide range of scales of synthesis, modifications, and purifications. Standard phosphodiester and phosphorothioate syntheses are routinely done. Please inquire about RNA, 2-O-methyl RNA and methyl phosphonate syntheses. Custom synthesis modifications are not limited to a small menu selection, but rather to any reagent commercially available including biotin derivatives and fluorescent dyes. Oligo-enzyme conjugate syntheses are standard. For a comprehensive list of modification reagents commercially available, check the Glen Research web site (www.glenres.com). Keeping pace with the latest technologies, BioSource offers dual labeled fluorescent (FRET) probes including those used in the PE-ABI 7700 Detection System. Standard EconoPURE oligos are desalted and ready to use. For those occasions when greater purity is required, we offer a full range of options from reverse phase cartridge and HPLC to denaturing PAGE purification.

BioSource Oligo Guarantee: Our oligos are guaranteed to be the correct sequence as specified, and to meet our strict QC specifications. If your oligo fails to perform or you are not completely satisfied, we will replace the product free of charge or issue a credit without question.

Fax Orders:	800-786-4362
Email Orders:	dna@keydna.com
WEB Orders:	www.keydna.com

Chapter 1: Chemistry of Synthetic DNA Synthesis

The beta-cyanoethyl phosphoramidite method of DNA synthesis is the current standard in the field. This chemistry provides high coupling yields and the absence of side products that may adversely affect the biological activity of the product oligomer. In the basic synthetic cycle, a solid support with the initial 3′ nucleotide monomer, derivatized to small controlled-pore glass, is contained in a reaction column. Reagents and solvents are pumped through the column to effect the addition of successive protected nucleotide monomers (phosphoramidites). Each cycle is actually composed of several main steps including deblocking, activation, coupling, oxidation and capping. Intervening wash steps remove excess reactants and by-products of reaction. After chain elongation is complete, the oligo is cleaved from the support and fully deprotected.

Monomers: The phosphoramidite monomers are protected at the 5′ hydroxyl position with dimethoxytrityl (Trityl) protecting group. Different protecting groups are also attached to the exocyclic amines of the bases. In addition, the phosphorous atom is protected with beta-cyanoethyl and diisopropylamine groups.

Deblocking: In the first step of the synthesis cycle, the Trityl group of the support bound monomer is removed with a mild acid treatment. The resulting Trityl cation is orange in acid solution. This orange Trityl fraction can be collected and quantified colorimetrically to determine the stepwise coupling efficiencies.

Coupling: After deblocking of the 5′-hydroxyl group of the support-bound nucleotide, the next protected monomer is delivered to the reaction column along with Activator. Activator is a weak acid that protonates the amino group of the diisopropylamine protecting group of the in-coming protected monomer. As a result, the diisopropylamine moiety becomes a good leaving group. The coupling mechanism is a nucleophilic attack, by the free 5′hydroxyl group of the column attached monomer, on the phosphorous of the incoming activated monomer. The coupling efficiencies of this reaction are very high, thereby allowing synthesis of long oligos.

Oxidation: The most recently added monomer is now linked to the growing chain by a trivalent phosphite triester bond. This bond is not as stable as the pentavalent bond found in native DNA; therefore, an iodine solution is used to oxidize this bond to the pentavalent state.

Capping: As much as 1% of the free 5′-hydroxy groups may not undergo coupling. These unreacted chains (failure sequences) must be capped to prevent further elongation in the next cycles. This capping procedure minimizes the length of impurities and thereby greatly simplifies the purification process.

Cycling: Following the capping step, the cycle of reactions is repeated again, beginning with the deblocking step, until the elongation is complete.

Post Synthetic Processing: After the specified sequence has been assembled, the oligo must be removed (cleaved) from the support and fully deprotected prior to use. Typically, treatment with ammonium hydroxide at 55ºC for 16 hours is used to cleave the oligo from the support, to deprotect the phosphorous by beta-elimination of the cyanoethyl group, and to remove the acetyl capping groups and the base protecting groups. After cleavage/deprotection, the resulting crude mixture contains the product oligomer, the truncated failure sequences with free 5′-hydroxy ends, by-products of deprotection and silicates from hydrolysis of the glass support.

Purification of Crude Oligos

Purification of the desired oligonucleotide from the crude mixture involves separation of the oligo from the short failure sequences and the by-products of deprotection. Effective methods for purification include desalting, disposable reverse phase columns, high performance liquid chromatography using either reverse phase or anion-exchange column packing, and gel electrophoresis. Time constraints, sequence length, and the degree of purity desired will determine the best method for purification. The procedures used at BioSource are described in the following sections. Although they are general in nature, they should prove to be effective in almost all cases.

EconoPURE: Desalting

Desalting is often not necessary, but may be required for some applications. Desalting is primarily used to remove inhibitors that may interfere with DNA synthesis and sequencing reactions. The major culprit responsible for these inhibitions is the ammonium ion. Desalting of the mixture can be accomplished by ethanol precipitation, size-exclusion chromatography, or reverse-phase chromatography.

A general procedure for the ethanol precipitation of oligos is as follows.:

1. Dry the oligo in a microcentrifuge tube.
2. Resuspend oligo in 0.3 mL of 4 M LiCl.
3. Add 4 volumes of (1:1) ethanol: acetone.
4. Incubate 1 hour at -20ºC.
5. Collect precipitate by centrifugation, 10,000 x g for 5 minutes.
6. Carefully remove the supernatant without disturbing the pellet.
7. Add 1 mL of cold 70% ethanol to tube.
8. Centrifuge at 10,000 x g for 5 minutes.
9. Carefully remove the supernatant.
10. Dry pellet and resuspend in working buffer.

Size exclusion chromatography can be done using NAP 10 (part #17-0854-02) columns from Pharmacia Biotech. Likewise, Sep-Pak C_{18} columns (part #WAT 020805) for reverse phase desaltng may be obtained from Waters.

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CartridgePURE: Poly-Pak Columns

Purification with Poly-Pak Columns is a fast batch elution procedure that can be used to purify oligomers up to 40 nucleotides long. Between 10 and 50 OD_{260} units of crude material can be loaded on a single Poly-PAK column. The final product is of suitable purity for use as primers, probes, and similar applications.

The basis of separation is the relative hydrophobicity of the different species present in the crude mixture. The least hydrophobic molecules elute first. The more hydrophobic molecules are retained on the column longer. For this method, the oligo must be synthesized such that the 5′-Trityl group is left ON the oligomer. The Trityl group renders the full length product more hydrophobic. With the appropriate selection of wash buffer, the less hydrophobic failure sequences may be washed from the cartridge. The cartridge is then treated with mild acid to cleave the Trityl group from the attached full-length oligo. Likewise, appropriate buffer is selected to elute the newly deblocked full length oligo from the cartridge, leaving the highly hydrophobic cleaved Trityl group on the column.

HplcPURE: Reverse Phase and Anion-Exchange HPLC

HPLC methods require less than an hour to perform. HPLC provides a higher degree of purity than the Poly-Pak cartridge columns and is recommended for applications such as diagnostic probes and antisense probes.

Reverse phase HPLC methods can be used to purify oligomers up to 40 nucleotides in length. The reverse phase columns are available with capacities of up to 100 OD_{260} units of crude mixture. Reverse phase columns work on the same principle as the Oligo-Pak cartridge columns. Hence, the oligo should be synthesized with the 5′-Trityl group left ON the oligo. The desired product oligo, with the 5′-Trityl group present, elutes later than the failure sequences with free 5′-hydroxy groups. The collected fraction of Trityl containing oligo is treated with mild acid. The resulting mixture of full-length oligo and cleaved Trityl group is desalted to obtain the purified full length oligo.

Anion-exchange HPLC resins are limited to purification of sequences approximately 35 nucleotides in length. Due to this limit of resolution, these columns are sensitive to sample capacity. Only 20-40 OD_{260} units can be loaded onto the recommended anion-exchange column. Anion-exchange columns separate molecules on the basis of overall charge. With anion-exchange columns, the shorter failure sequences elute first. The full-length product oligo (with the greatest number of negatively charged phosphate groups) is retained longer. In order for anion-exchange columns to be effective, the crude mixture must be deblocked prior to the separation (i.e., the oligo must be synthesized such that the 5′-Trityl group is OFF). Usually anion-exchange is used to "polish" off oligos previously purified by Trityl-ON reverse phase methods.

PagePURE: Denatured Gel Electrophoresis

Denatured gel electrophoresis provides a higher degree of purity than HPLC and is recommended for applications such as site directed mutagenesis, construction of genes, etc. Electrophoresis can be used to purify oligos of any length but requires 6-10 hours to perform. Generally, up to 10-30 OD_{260} units of crude material can be loaded in a single lane of a preparative gel. The basis of separation is molecular size and charge. Using denaturing solvents to disrupt secondary structures of the crude oligo, the gel matrix then acts essentially as a sieve. The smaller molecules move more rapidly through the polymeric network of the gel. Larger molecules do not penetrate the gel network as easily and therefore migrate more slowly. Hence, the product molecule is the uppermost band in the gel. In order for gel electrophoresis to be effective, the crude mixture must be deblocked prior to running the gel. After the gel is run, the product is visualized by UV shadowing and excised. The band is cut from the gel, placed into a tube and crushed. Warm buffer is added to the crushed gel to elute the oligo. The extracted oligo is removed from the gel by centrifugation and concentrated.

For oligos longer than 50 bases, significant amounts of truncated failure sequences are present in the crude mixture. Even with coupling efficiencies greater than 99%, 30% or more of the crude oligo mixture may be failure sequences. For those applications where the presence of truncated sequences may hinder the experiment, oligos greater than 40 bases should be purified by PAGE. Reverse phase-based purification protocols are not reliable for the purification of oligos greater than 40 bases. See Table II for the theoretical yield of various length syntheses at different coupling efficiencies.

Table II

Theoretical Yield: Oligo Length versus Coupling Efficiencies

Coupling Efficiencies	Theoretical Yields: %			
Length	98.0%	98.5%	99.0%	99.3%
40	45.5	55.5	67.6	76.0
50	37.2	47.7	61.1	70.9
60	30.4	41.0	55.3	66.1
70	24.8	35.2	50.0	61.6
80	20.3	30.3	45.2	57.4
90	16.6	26.1	40.9	53.5
100	13.5	22.4	37.0	49.9
120	9.0	16.6	30.2	43.3
140	6.0	12.2	24.7	37.7
160	4.0	9.0	20.2	32.7

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Table III is a list of recommended BioSource purifications for various applications using oligonucleotides.

Table III

Recommended Scales of Synthesis and Purification

Application	Scale of Synthesis	Purification
For Non-modified Oligos		
DNA sequencing	0.2 µm	EconoPURE
PCR (general amplification)	0.2 µm	EconoPURE
PCR (diagnostic application)	0.2 µm	HplcPURE
Subcloning, site-directed mutagenesis or cDNA synthesis	0.2 µm	CartridgePURE, HplcPURE or PagePURE
Gene Construction: <80mer	0.2 µm	PagePURE
Gene Construction: >80mer	1.0 µm	PagePURE
Antisense	1.0 µm or more	HplcPURE
NMR & X-RAY crystallography	15 µm or more	HplcPURE
For Modified Oligos		
Modified bases and chemical linkers	0.2 µm	CartridgePURE
Reporter groups (biotin, DIG or fluorescent dyes)	0.2 µm	HplcPURE

Chemical Modifications of Oligos: Direct or Indirect Labeling

Oligos may be chemically modified to include reporter groups such as biotin or digoxigenin, fluorescent labels such fluorescein or rhodamine, and chemical linkers such as amines, thiols or phosphates. These modifications may be located at either the 5′ or 3′ end of the oligo or, when reagents are available, internally as adducts to the bases or stand alone moieties within the oligo chain. Modifications are not restricted to the bases, but may also include the phosphate backbone, as phosphothioates and methyl-phosphonates, and sugar moieties such as 2′-O-methyl modifications.

One of the major issues related to labeling of oligonucleotides is the choice of the tag, and more specifically, its availability under one or another chemical form that would make it sufficiently user-friendly for synthesis. Ideally, the chosen tag should be available as a fully protected monomer phosphoramidite. This would allow the direct synthesis of the modified oligo on the DNA synthesizer. Using this "direct labeling" method on DNA synthesis instrument will result in the direct and efficient incorporation of the label at the desired position in the oligonucleotide being synthesized. The advantage of this approach

is that the number of steps involved in obtaining the labeled oligo is significantly reduced when compared to the "indirect labeling" method. More importantly, direct labeling of modifications is much less expensive than the indirect procedure.

The indirect labeling method should be used when the chosen tag is not available as a modified phosphoramidite. This method, which should be considered as a "default" method, would still require that the tag be presented under a form compatible with easy coupling to the synthetic oligonucleotide. Typically, the tag would be added as an activated group (e.g., NHS-ester) to an oligonucleotide into which a primary amino group has been previously incorporated. The amine group could be at either the 5′ or 3′ end or as part of modified base such as amino dT. The indirect procedure would require the use of the same "labeling" method during the initial synthesis, for the purpose of incorporating the primary amino group, via a modified phosphoramidite. Some purification would be needed prior to setting up the actual coupling reaction with the tag.

There are advantages and disadvantages to both methods. The main advantage of the direct labeling approach is that fewer steps are involved in obtaining the labeled oligonucleotide, which has a positive impact on both the overall yield of the procedure, the amount of time and labor required, and cost. The major inconvenience is that this method implies the use of modified phosphoramidites, which are substantially more expensive and less stable than their standard, unmodified counterparts. This last statement applies to practically all modified phosphoramidites currently available commercially, regardless of the nature of the modifying group.

The indirect labeling method currently requires the incorporation of a primary amino group, most of the time at the 5' end of the oligonucleotide. This is typically achieved by adding a so-called amino-link or amino-modifier phosphoramidite as the last step in the synthesis, using the "labeling" method on the synthesizer. The major advantage of this approach is that, once purified and 5′-deblocked, this amino-oligonucleotide preparation can be further labeled according to the user's specific needs. In other words, a small aliquot of the preparation could be labeled with fluorescein, while another could be labeled with biotin, etc. Overall, although more cumbersome and involving more steps, the indirect labeling approach offers a higher level of flexibility with regard to labeling oligonucleotides without the reagent cost associated with the use of labeled phosphoramidites. However labor costs are higher than the direct method.

General Procedure for Indirect Labeling Amino-Modified Oligonucleotides

This general procedure can be used to conjugate amino-modified oligonucleotides with NHS active esters of compounds that are not suitable for use as cyanoethyl phosphoramidites, such as certain fluorescent dyes, digoxigenin, and cholesterol. At pH 9.0, conjugation occurs virtually exclusively at the amino group and not at all at the exocyclic amino groups of the bases. Since the amino-modified oligo must be free from ammonium ion, the amine oligo should be desalting by either ethanol precipitation or reverse phase cartridge purification and dried down.

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1. Dissolve the amino oligo from a 200 nmol scale synthesis of amino-modified oligonucleotide (i.e., approximately 0.1-0.2 µmoles of free primary amines) in 0.7 mL of sterile distilled water.
2. Add 0.1 mL of 10 X Buffer containing 1M $NaHCO_3/Na_2CO_3$, pH 9.
3. Freshly prepare a 10 mg/mL solution of the NHS active ester in DMF. Add 0.2 mL of the solution to the reaction mixture.
4. Allow the coupling mixture to incubate at least 2 hours. (Note: Overnight reactions may be more convenient.)
5. Desalt the reaction mixture on a Poly-Pak cartridge or a Sephadex G-25 or G-50 column to remove excess label. If necessary purify the product using reverse phase HPLC. Many times the added conjugated oligo is much more hydrophobic than the starting material, and as result will elute off later than the unreacted starting material.

Description of Modifications and Their Uses

Biotin Labeling

Description: Biotin is a non-aromatic heterocyclic compound. Many of the derivatives of biotin are available in which the biotin moiety is connected to some linker molecule that can be attached directly to an oligonucleotide.

Applications: The biotin moiety has a remarkable affinity towards streptavidin and related proteins. A biotin-labeled oligo can be used to link the oligo to streptavidin-protein conjugates, streptavidin affinity columns, or labeled streptavidin. For example, streptavidin-HRP conjugate (horseradish peroxidase) couples to a biotinylated oligo to form an HRP-oligo, useful in various chemiluminescent assays.

Fluorescent Labeling

Description: Fluorescein and various fluorescein analogs are the most frequently used fluorescent compounds to label oligonucleotides. Fluorescein is a multi-ring aromatic compound that is strongly fluorescent. The two fluorescent cyanine dyes, Cy3 and Cy5, are also available as phosphoramidites for machine labeling the 5′ ends.

Applications: Fluorescent dye-labeled oligos are used in automated DNA sequencing with fluorescent detection. A combination of four different dyes (ABI dyes) are used with the Applied Biosystems sequencers. Fluorescent-labeled oligos are also useful in PCR quantification, detection of amplified products in homogeneous assays, and as probes for *in situ* hybridization.

Amino Modifiers

Description: The amino modifier reagents are used to introduce a primary amino group into the oligo. A variety of different modifications are available and can be selected depending on individual design requirements.

Applications: A primary amino group is useful in a variety of coupling reactions that use NHS-esters to attach various labels to the oligo. NHS-esters are activated compounds that readily couple with primary amino groups.

Phosphate

Description: This is the inorganic phosphate group. It occurs automatically (as a phosphate diester) between the nucleosides in a standard oligo, but standard oligos contain 5'- and 3'-terminal hydroxyl groups. As an option, phosphate can be attached to the 5'- and/or 3'-hydroxyl groups of the oligo.

Applications: Oligonucleotides require a 5'-phosphate in order to be ligated by a ligase. This phosphate can be added enzymatically using polynucleotide kinase, or can be added chemically during oligonucleotide synthesis. 3' labeled phosphate oligos are also useful in preventing the hydrolysis of the oligo by exonucleases and can also be used to prevent primer elongation by DNA polymerases.

Deoxyuridine

Description: This is a deoxynucleoside with the base uracil.

Applications: The uracil base within a DNA sequence behaves much like a thymine base. However, the enzyme Uracil-N-glycosylase can specifically remove uracil to create baseless sites at the deoxyuridine positions. This property can be used to generate specific strand breaks in a DNA structure.

Halogenated Nucleosides

Description: Many of the standard deoxynucleosides are available as modifications with bromine, iodine, or fluorine atoms covalently attached to the bases.

Applications: Halogenated bases within an oligo can be activated by light to cross-link the oligo to the DNA, RNA, or protein to which it is associated. 5-Bromo-dU and 5-Iodo-dU are often used for this purpose. Halogenated nucleosides may also be useful in crystallographic studies of oligonucleotide structure.

Antisense Activities

Antisense research is of particular interest. Antisense oligonucleotides are designed to be complementary to critical regions on mRNA of a targeted gene. They act by binding to mRNA and blocking the translation of sequence information into protein synthesis. This is accomplished either directly through translation arrest, or indirectly through the activation of RNase H, an enzyme that degrades RNA in RNA/DNA duplexes, thereby reducing levels of the target RNA.

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Phosphorothioates (S-oligos)

Description: A phosphorothioate group is a modified internucleotide phosphate group with one of the nonbridging oxygen atoms replaced by a sulfur atom. In a phosphorothioated oligo (often called an "S-oligo") phosphorothioate groups replace some or all of the internucleotide phosphate groups.

Applications: The modified "backbone" of an S-oligo is resistant to the action of most exonucleases and endonucleases. Such S-oligos may be useful in some "antisense" applications because of their enhanced stability to nucleases.

The C-5 propyne modification has been shown to have higher affinities, higher specificity, and less toxicity than standard S-oligos. Antisense oligo phosphorothioates, in which all dC and dT residues have been replaced with pdC and pdU, have been shown to be effective inhibitors of gene expression as a result of enhanced binding to the target RNA sequence.

Chimeric Antisense Oligos™ which are DNA oligos containing phosphorothioates at specific positions for the purpose of blocking gene expression are available from Integrated DNA Technologies (IDT) under U.S. patent # 5,491,133. BioSource is not licensed to sell Chimeric Antisense Oligos™.

Alternatives to phosphothioate DNA and Chimeric Antisense Oligos™ are 2'-OMe RNA Analogs and Chimeric RNA analogs. These analogs and chimeras have been shown to have advantages over DNA for antisense studies. They have increased protection against nucleases and greater affinity for their complementary sequence than DNA. DNA\2'-OMe RNA chimeras are considered to be more stable than natural RNA and have a longer retention of RNase H^+ activity. BioSource provides full-length 2'-OMe RNA analogs and DNA\2'-OMe RNA chimeras synthesized at a 1 µM scale and PAGE purified. Yields are dependent on the length of the oligonucleotide. Oligo-2'-OMe-nucleotides are partially resistant to a variety of ribo- and deoxyribonucleases. As well as being stable to normal handling and nuclease resistant, oligo-2'-OMe-nucleotides form more stable hybrids with complementary RNA strands than equivalent DNA and RNA sequences.

Degeneracy

Description: Standard "wobbles" are equimolar mixtures of two or more different bases at a given position within the sequence.

Applications: Wobbles are often incorporated into oligonucleotide probes designed to hybridize to an unknown gene that encodes a known amino acid sequence. Oligos with wobbles are also useful in random mutagenesis and combinatorial chemistry. The following table lists the International Union of Biochemistry (IUPAC) codes for degenerate bases used for "wobbles".

Table IV

IUPAC Codes for Bases

Code	Bases Coded	Code	Bases Coded
R	A,C	D	G,A,T
Y	C,T	H	A,T,C
M	A,C	B	G,T,C
W	A,T	V	G,A,C
S	G,C	N	A,C,G,T
K	G,T		

Poisoned Oligos

Description: Instead of equal molar mixtures of different bases at a given position within the sequence, poisoned oligos contain unequal mixtures of different bases (i.e., 94% A, 2% each C, G and T).

Applications: Poisoned oligos also known as dosed oligos are used to introduce low frequency mutations into selected regions of known genes for mutagenesis studies.

Deoxyinosine

Description: This is a deoxynucleoside with the base hypoxanthine.

Applications: When used within a hybridization probe, deoxyinosine residues can form base-pairs with dA, dC, dG, or dT residues on the target strand. Deoxyinosine can be used in place of "wobbles" without increasing the degeneracy of the oligo. The use of deoxyinosine in applications in which this base must serve as part of a template strand (e.g., PCR primers) is discouraged, since DNA polymerases may fall off when encountering this residue in a template.

Oligo Enzyme Conjugates

Description: Enzymes such as Alkaline Phosphatase, Horseradish Peroxidase or Beta-Galactosidase are coupled to the oligo.

Applications: Colorimetric or chemiluminescent techniques can be used for visualization of hybridized conjugated oligos. Using chemiluminescence, sensitivity similar to ^{32}P have been obtained without the problems associated with radioactive labeling.

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Frequently Asked Questions (FAQ) and Frequently Encountered Problems (FEP) with Oligo Synthesis.

1. **What is scale of synthesis?**
   Scale of synthesis refers to the amount of starting CPG (controlled-pore glass) support-bound monomer used to initiate the DNA synthesis, not the amount of final material synthesized. As an example, a 20mer synthesized at a 200 nanomole scale of synthesis will produce approximately 80 nanomoles. The losses occur during synthesis, post-synthetic processing, transfer of material, and quality control.

2. **Do I need to have my oligo purified?**
   It depends on whether or not modifications are requested and what the applcation will be. Failure sequences may be generated both during the synthesis and post-synthesis processing. We recommend that all modifications be purified either by cartridge or HPLC. For recommended purity and scale (based upon application), please see Table III.

3. **How much do I get or what scale of synthesis should I order?**
   Refer to the Table V.

Table V

Scale of Synthesis, Purification and Yields

Scale of synthesis	Purification	Yield
50 nanomole	Desalt	100 µg: actual yield is sequence and length dependent
	Reverse phase cartridge	Average yield: 50-75 µg
	Reverse phase HPLC	Average yield: 50-75 µg
	Anion-exchange HPLC	Average yield: 50-75 µg
200 nanomole	Desalt	Average yield: 600 µg
	Reverse phase cartridge	Average yield: 300 µg
	Reverse phase HPLC	Average yield: 300 µg
	Anion-exchange HPLC	Average yield: 300 µg
	PAGE: less than 40mer	Average yield: 25-75 µg
	Greater than 40mer	Average yield: 10 µg
1 micromole	Desalt	Average yield: 2 mg
	Reverse phase cartridge	Average yield: 1 mg
	Reverse phase HPLC	Average yield: 1 mg
	Anion-exchange HPLC	Average yield: 1 mg

4. **What do I re-suspend my oligo in and what concentration should I make it?**
TSE (25 mM Tris, pH 8, 100 mM NaCl, 0.1 mM EDTA), PBS or any biological buffers are acceptable as diluents. The recommended diluent volume is 100 µL - 1 mL, the concentration depending on the application to be used and the yield of the resulting product. Standard concentration for PCR primers is 0.1 mM.

5. **How do I determine my concentration?**
Concentration is determined by measuring the OD_{260} of the diluted oligo. Prepare a dilution of the resuspended oligo and measure the OD_{260}. Determine the concentration as follows:

(µg or pmoles / OD_{260}) X dilution factor = final concentration / mL.

6. **How stable is my oligo once I have resuspended it?**
If sterile diluent is used to resuspend the oligo, it will be stable at 4°C for about a month. If stored frozen at -20°C or -70°C, it will remain stable for 2-3 months. Repeated freeze-thaw should be avoided, as it will denature the oligo. Avoid the use of distilled water, since solution pH may be as low as 4-5.

7. **Does my oligo have a phosphate on the 5' end?**
Unless requested, oligos are synthesized without either 3′ or 5′phosphate. The 5′ phosphate modification is available, normally as an additional charge.

8. **My annealed oligos will not ligate. What is the problem?**
Ligation reactions require a 5′ phosphate. If your oligos do not contain a 5′phosphate, ligation will not occur. The problem can be addressed without ordering an additional oligo pair: phosphorylate your oligos enzymatically with kinase before use in ligation reactions.

9. **How do you calculate the molecular weight of my oligo?**
The molecular weights for oligos are the sum of the component molecular weights of all bases, with mixed bases contributing proportionately. The component molecular weights of the bases vary as to their salt form. Many times desalted oligos are ammonium salts, while cartridge, HPLC and PAGE purified oligos are sodium salts. The molecular weights used in the calculations are listed in the following Table VI.

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Molecular Weight Calculations
Table VI

Molecular Weights of the Bases

	Sodium Salt		Ammonium Salt
	DNA	Thioate	DNA
W_A	313.21	329.27	330.24
W_C	289.18	305.25	306.24
W_G	329.21	345.27	346.24
W_T	304.19	320.26	321.23
W_{CORR}	61.96	61.96	96

Molecular Weight Calculation: $(P_A * W_A) + (P_C * W_C) + (P_G * W_G) + (P_T * W_T) + (P_{mod} * W_{mod}) - W_{CORR}$
Where P_A is the number of A's (adenine) and W_A is the component weight of A and P_{mod} is the number of modifications, and W_{mod} is the component weight of the added modification.

Please refer to Table VII for the molecular weights of common modifications.

Table VII

Molecular Weights of Common Modifications

Modification	Molecular Weight	Modification	Molecular Weight
5′-Biotin	405.45	3′-TAMARA	623.60
5′-(6 FAM)	537.46	3′-Dabcyl	498.49
5′-HEX	744.13	3′-Fluorescein-dT	815.71
5′-TET	675.24	3′-(6 FAM)	569.46
5′-Cy5	533.63	3′-Amino Modifier C3	153.07
5′-Cy3	507.59	3′-Amino Modifier C7	209.18
5′-Dabcyl	430.18	3′-Thiol Modifier C3	154.12

10. **What is coupling efficiency?**
Coupling efficiency is a measure of the DNA synthesizer's ability to couple each new monomer to the growing chain. If all the monomers coupled completely to the growing chain, the coupling efficiency would be 100%. If 1% of the growing monomer chain fails to react, then the coupling efficiency of that step is only 99%. The coupling efficiency for the complete synthesis of the oligo is usually determined from the yields of full-length sequence after the first and last cycle. Coupling efficiencies greater than 99.0% are essential for good oligo product with minimum purification.

11. How is the coupling efficiency determined?

Following the first coupling step, the amount of Trityl released during deblocking is directly proportional to the amount of full-length oligo made in the previous cycle. When the Trityl is cleaved during the deblocking step, the resulting Trityl cation is orange in color. The intensity of this color can be measured by UV spectrophotometry. By comparing the intensities of the Trityl produced after the first and last coupling, one can calculate the average successful base coupling per cycle and hence the coupling efficiencies.

12. I sequenced a clone I prepared with one of your 100 mer primers and the sequence for the primer region was different from the one I ordered. Why?

In general, the longer the oligo, the greater the probability of side reactions accumulating along with increased chances of incomplete deprotection. Base insertions are attributed to a small amount of detritylated amidite present during coupling, while deletions are probably due to failure sequences that are not capped and subsequently extended.

However, a better explanation for the observation of altered sequences is the incomplete deprotection of the oligo. With protecting groups still on a few positions when the annealed and ligated oligos were transformed into *E. coli*, the host mismatch repair system would try to resolve these bumps. Sometimes the repair results in the wrong base. The most likely culprit for incomplete deprotection is the isobutyryl protected dG's. These are the hardest deprotection groups to remove. We recommend that if you are using long oliogos (>80 mers) for gene construction, request that your oligos be deprotected a second time to remove residual protection groups.

13. Why are some modified oligos so expensive in relation to the cost of the modifying reagent?

The limited reagent stability (most <48 hours) and lower coupling efficiencies of the reagent require that excess modifying reagent be used to insure adequate quantities of full length product are made. As a result, higher cost is incurred in synthesis.

14. Why are the yields lower for modified bases?

Many of the modified amidites are unstable and do not couple as efficiently as the unmodified bases (even though longer coupling procedures may be used), thus, failure sequences are more abundant than in normal synthesis. Consequently, all modified oligos should be purified either by cartridge or HPLC to remove the more abundant failure sequences. Yields are reduced as a result of purification. The end product, although with a lower yield, is much more pure.

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15. Why isn't the yield for 1 µm scale synthesis five times greater than 0.2 µm scale synthesis?

For 0.2 µm scale, the monomer coupling is done at a 40- to 50-fold excess. To do so for larger scale syntheses (such as 1.0 µm scale) would be cost-prohibitive. Large-scale syntheses are done at 10-fold mole excess of amidites. However, to increase the yields for these larger scale syntheses, the coupling times are extended to increase coupling efficiencies.

16. What is the longest length an oligo can be synthesized?

The real answer lies in the limit of resolution of the purification method and the coupling efficiency of the DNA synthesizer. It is not unusual to synthesize oligos in excess of 150 bases and to obtain sufficient quantities by PAGE purification to do successful gene construction. It should be remembered that the longer the oligo, the greater the chance of accumulated sequence errors.

Chapter 2: Physical Chemical Properties of DNA Oligonucleotides

Oligo Quantitation and Extinction Coefficient Calculations

Oligonucleotides are quantitated using UV absorbance. The absorbance of the synthetic oligonucleotide is determined at 260 nm. This absorbance reading can then be converted into a concentration employing one of two commonly used methods.

Oligonucleotides are most often quantitated in " A_{260}" or absorbance units. One unit is the quantity of dissolved oligo that has an absorbance of 1.0 at 260 nm when measured in a 1 cm pathlength cuvette. A commonly used approximation of the concentration of an oligo in solution assumes that all oligomers have the same extinction coefficient. The concentration of an oligonucleotide can then be determined using the following conversion factor:

1 A_{260} unit of single-stranded DNA oligo = 33 µg/mL

Where one unit is that quantity of oligo which has an absorbance of 1.0 at 260 nm when dissolved in 1 mL of phosphate buffer at pH 7.0 and measured in a 1 cm path length cuvette.

A more accurate estimation of the concentration from the absorbance can be obtained using a "nearest neighbor" calculated extinction coefficient (4). The extinction coefficient for a single-stranded oligo of base sequence ApBpCpDp……KpL may be calculated according to the formula:

$$\Sigma_{ApBpCpDp\cdot\ KpL} = [2(\Sigma_{ApB} + \Sigma_{BpCp} + \Sigma_{CpD} + \Sigma_{KpL}) - (\Sigma_{pB} + \Sigma_{pCp} + \Sigma_{pD} + \Sigma_{pK})]$$

Where L is not a base like A, C, G, or T, but a math symbol for the last base.

The millimolar extinction coefficients for the deoxy mono- and dinucleotides are listed below in Table VIII.

Table VIII

Millimolar Extinction Coefficients at 260 nm, 25ºC and Neutral pH, per Average Residue (5)

Σ260		Σ260		Σ260	
dpdA	15.4	dApdA	13.7	dGpdA	12.6
dpdC	7.4	dApdC	10.6	dGpdC	8.8
dpdG	11.5	dApdG	12.5	dGpdG	10.8
dpdT	8.7	dApdT	11.4	dGpdT	10.0
		dCpdA	10.6	dTpdA	11.7
		dCpdC	7.3	dTpdC	8.1
		dCpdG	9.0	dTpdG	9.5
		dCpdT	7.6	dTpdT	8.4

An example of a calculation of the extinction coefficient for an oligo with the sequence 5′- AGA-CTC-GTC-CGG-GT-3′ is given in Table IX.

Table IX

Example of Extinction Coefficient Calculation

		Sequence:	A	G	A	C	T	C	G	T	C	C	G	G	G	T
dXpdX	dpdX															
12.5		AG	x	x												
12.6	11.5	GA		x	x											
10.6	15.4	AC			x	x										
7.6	7.4	CT				x	x									
8.1	8.7	TC					x	x								
9	7.4	CG						x	x							
10	11.5	GT							x	x						
8.1	8.7	TC								x	x					
7.3	7.4	CC									x	x				
9	7.4	CG										x	x			
10.8	11.5	GG											x	x		
10.8	11.5	GG												x	x	
10	11.5	GT													x	x
Sum	126.4	119.9														

Millimolar Extinction Coefficient = 2*(126.4) - (119.9) = 132.9/ mmol·cm

The "nearest neighbor" method for the calculation of extinction coefficients does not include any provisions for oligos with fluorescent dyes. If the oligo is labeled at either the 3′ or 5′ end, determine the extinction coefficient for the sequences without the terminal dyes. To this calculated value, add the millimolar extinction coefficient of the dye. Table X contains millimolar extinction coefficient for several commonly used fluorescent dyes.

Table X

Millimolar Extinction Coefficients for Commonly used Fluorescent Dyes

Residues	1/(mM-cm)
6′ FAM	20.96
TET	16.25
HEX	31.58
TAMRA	31.98

Thermal Melting Temperature

Binding between a specific nucleic acid sequence and the DNA oligo leading to a stable hybrid molecule is mediated by specific interactions between complementary purine and pyrimidine bases forming A:T and G:C base pairs. The specificity of the hybrid formation is influenced primarily by the geometry of the bases responsible for the hydrogen bonds between A and T (2 hydrogen bonds) and G and C (3 hydrogen bonds). G:C base pairs are more stable than A:T pairs since G:C pairs have one additional hydrogen bond. Therefore, G:C rich sequences are more stable than A:T rich sequences, and thus require more energy to disassociate the formed hybrid.

However, the stability of the hybrid is not only the result of the generation of a variable number of hydrogen bonds, but also of the effects of electrostatic as well as hydrophobic interactions. Electrostatic forces are due predominantly to the phosphate molecules of the nucleic acid backbone. The double stranded hybrid results in the placement of phosphate groups from each strand into close juxtaposition. As a result, the same ionic charged chains repel each other, and tend to destabilize the hybrid. Since salt cations mask the phosphate charges, increasing ionic strength stabilizes double-stranded sequences. Hydrophobic interactions between the staggered bases also contribute to hybrid stability. The Tm, or melting temperature, characterizes the stability of the DNA hybrid form between an oligonucleotide and its complementary strand.

The melting temperature or Tm is the temperature at which 50% of a given oligo in solution is hybridized to its complementary strand. The Tm is a critical factor for determining the optimal temperature to use an oligo as a primer in PCR applications (annealing temperature) and as probes for *in situ* hybridization, Southern, Northern or Dot blot analyses. There are three major parameters upon which Tm is dependent:

1. The sequence: G:C rich sequences have a higher melting temperature, since more hydrogen bonds are involved in hybrid formation.
2. The strand concentration: high oligo concentrations favor hybrid formation, which results in higher melting temperature.
3. The salt concentration: high ionic strength results in a higher Tm as cations stabilize the DNA duplex.

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Several methods have been used to determine Tm values of DNA oligos. Three methods will be discussed briefly.

Method 1 is the Wallace rule for short oligos: Tm = 2 x (A + T) + 4 x (G + C). This method is simple but is only accurate for oligos shorter than 18 bases (6).

Method 2 is based on the %GC content and length of the oligo.

Tm = 81.5 + 16.6 (log10 [Na+]) + 0.41 (%GC) – (625/N) where N is the length of the oligo (7).

It is important to note that this equation estimates the "reversible" Tm defined by hyperchromicity measurements in solution. This value should be used for determining PCR annealing temperatures. The "irreversible" Tm is more important for solid phase hybridization such as Southern, Northern and Dot blots, and is usually 7° to 10° higher. The equation for "irreversible" Tm is

Tm = 81.5 + 16.6 (log10 [Na+]) + 0.41 (%GC) – (500/N) (8)

Method 3 is a thermodynamic calculation based on entropy, enthalpy, free energy and temperature (9). This analysis involves determining the specific enthalpy and entropy contribution to the free energy of the duplex made by each "nearest neighbor" in the sequence. The nearest neighbor starts at the 5′ end and the enthalpy and entropy contributions are additive.

$\Delta H = \Sigma \Delta hi$ where Δhi is the individual nearest neighbor enthalpy contribution

$\Delta S = \Sigma \Delta si$ where Δsi is the individual nearest neighbor entropy contribution

$Tm = \Delta H/(\Delta S + R \cdot \ln(1/[primer])$

R is the molar gas constant (1.987 cal/[K°-mol])

Adjustments to the above formula are 3.4-kcal free energy change during the transition from single stranded to B-form DNA (10), salt concentration, and conversion factor for Kelvin to Centigrade.

$Tm = \{(\Delta H - 3.4 \text{ kcal/K°-mol})/(\Delta S + R \cdot \ln(1/[primer])\} + 16.6 * \log10([Na+]) - 273.2$

Tables XI and XII are the nearest neighbor contributions for enthalpy and entropy. Table XIII is an example of Nearest Neighbor calculation of Tm.

Table XI

ΔH for nearest neighbor calculation kcal/K°mol

Second base / First base	A	C	G	T
A	8.0	9.4	6.6	5.6
C	8.2	10.9	11.8	6.6
G	8.8	10.5	10.9	9.4
T	6.6	8.8	8.2	8.0

Table XII

ΔS for nearest neighbor calculation cal/mol

Second base / First base	A	C	G	T
A	21.9	25.5	16.4	15.2
C	21.0	28.4	29.0	16.4
G	23.5	26.4	28.4	25.5
T	18.4	23.5	21.0	21.9

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Table XIII

Example of Tm Calculation Using Nearest Neighbor Approach

Tm = [(delta H)-3.4]*1000/[(delta S) +1.98*ln(1/[primer])] +16.6*LOG[Na]-273.2

		Sequence:	**A**	**G**	**A**	**C**	**T**	**C**	**G**	**T**	**C**	**C**	**G**	**G**	**G**	**T**
ΔH	**ΔS**															
6.6	16.4	AG	x	x												
8.8	23.5	GA		x	x											
9.4	25.5	AC			x	x										
6.6	16.4	CT				x	x									
8.8	23.5	TC					x	x								
11.8	29.0	CG						x	x							
9.4	25.5	GT							x	x						
8.8	23.5	TC								x	x					
10.9	28.4	CC									x	x				
11.8	29.0	CG										x	x			
10.9	28.4	GG											x	x		
10.9	28.4	GG												x	x	
9.4	25.5	GT													x	x
SUM	124.1	323.0														

The Tm for 50 nM of an oligo with the sequence AGACTCGTCCGGGT in 50 mM NaCl is [(124.1-3.4)*1000]/[323+1.98 ln {1/(5x10^{-8})}]+16.6 log (5x10^{-2})-273.2 = 43.7°C.

While the Tm value provided by any of these methods does not take into account any nucleotide modification, like biotin, digoxigenin, fluorescent dyes or amine, the calculated values serves as a good estimate for use in determining starting annealing or hybridization temperatures.

Hybrid Formation

The formation of stable hybrids between oligonucleotides and their target nucleic acid is dependent upon several factors. These include base composition, salt concentrations and base mismatches - all parameters that influence Tm. Typically, oligonucleotides are hybridized under conditions that are 5-10ºC below Tm. While these stringent conditions reduce the number of mismatched hybrid formations, these conditions reduce the rate at which perfect hybrids form. Hybrids formed with short oligos under these conditions are somewhat unstable and are easy to unwind. As a result, the hybridization reaction can be considered reversible. Therefore, when high concentrations (0.1-1.0 pmole/mL) of oligo are used, the hybridization reaction should not exceed 3-4 hours.

The effects of base mismatches on hybrid stability are not easily quantitated since different types of mismatch (mispairing between single bases, loopouts on either strand, multiple mismatches closely or distantly spaced) have different effects on hybrid stability. For instance, a single mismatch in the center of a short oligo will prevent any possible hybridization under normal conditions, while a mismatch at either end of the same oligo will not have any detectable effect. An adjustment to the calculated Tm can be used to estimate the effect of base mismatching on the Tm. For each % of base mismatch, one degree can be subtracted from the calculated Tm.

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Chapter 3: Oligonucleotides as Probes

An oligonucleotide probe may be a single oligonucleotide of defined sequence or a degenerate probe or pool of oligonucleotides whose sequences are degenerate. A probe consisting of a single oligonucleotide of defined sequence usually targets a previously determined sequence. These probes normally match the target sequences perfectly and are of sufficient length to allow discrimination between target sequences and closely related sequences.

The length of the probe may vary between 18-40 nucleotides. Based on complexity considerations (11), oligos 14-15 bases in length are expected to be represented only once in mammalian genomes. However, since the distribution of nucleotides in the coding sequences is non-random, longer oligonucleotide probes normally are used to increase specificity of hybridization. A good start for the development of a hybridization probe is a 20mer with GC content between 40-60%. Probes designed in this manner will permit discrimination between target sequences and single base mismatches. As a general rule, the Tm for a mismatched hybrid is reduced 1-1.5ºC for every 1% of mismatch (12). For a 20mer, a single internal mismatch would reduce the Tm by 5-7.5ºC. This range is sufficient to be used to discriminate between perfectly matched and internally mismatched hybrids. With longer probes, mismatches would not lower the Tm as much, and as a result, they are likely to be stable under conventional conditions of hybridization.

For most purposes involving hybridization with membranes or solid phase, the hybridization temperature selected for oligo probes is usually 5-15ºC below Tm, unlike the standard 25ºC below Tm used for 200 base pair DNA fragment probes. However, the specificity of the hybrid formed is predominately influenced by the stringency of the final washing steps after hybridization. Stringency is enhanced during the final washing steps by increasing the temperature again to 5-15ºC below Tm and lowering the salt concentration from 5 X SSC (0.75 M Na^+) to 0.1 X SSC (0.015 M Na^+). Nonetheless, the optimal washing temperature has to be determined experimentally, and compromise has to be made with respect to the washing conditions in which the probe binds strongly to the target and weakly to closely related sequences.

A degenerate probe is a pool of oligoncleotides that, to a certain degree, represent all DNA sequences that can code for a given sequence of amino acids. The degeneracy of this pool is a consequence of the degeneracy of the genetic code itself. With 64 possible codons and only 20 amino acids, most amino acids are coded for by more than one codon. Therefore, for a given sequence of amino acids, several different oligonucleotides can represent that amino acid sequence. Degenerate probes to a given amino acid sequence may be generated by inserting "wobbles" in the probe DNA sequence where there is more than one possible codon for the same amino acid.

For a degenerate pool of probes it is difficult to estimate a consensus Tm. This is due to the variation of GC content of the individual oligo sequences. For hybrid formation, the hybridization temperature is frequently chosen to be 2º below the calculated Tm of the least G/C rich-member of the pool (13). The disadvantage to this method is that a large

number of false positives may be generated. These false positives are the result of mismatched hybrids of higher GC content being more stable than a perfectly matched hybrid formed by the correct sequence. However, the number of false positives may be reduced by the use of hybridization solutions containing quarternary alkylammonium salts such as tetramethylammonium chloride (TMAC). In this solvent, the Tm of a hybrid is independent of base composition and primarily dependent upon on its length (14). Therefore, mismatches may be avoided by selecting a temperature for hybridization appropriate for the lengths of the oligonucleotides in the pool. The recommended hybridization temperature for 17mers in 3M TMAC is 48-50ºC; for 19mers, it is 55-57ºC; and for 20mers, it is 58-66ºC. These higher temperatures also suppress nonspecific adsorption of the probe to solid supports. One note on the use of TMAC solvents, significant advantages of TMAC over sodium ions only occurs when the length of the oligonucleotide exceeds 16 bases.

The purpose of degenerate probes is not to target unambiguous sequences, but to detect a series of clones that can be tested further as "new" genes or gene families.

FRET Probes

More recently, fluorescent modified oligos are being used as probes in homogeneous solutions. Two such probes, FRET and Molecular Beacons, are being increasingly used in real time PCR quantitation and diagnostic molecular analysis.

The FRET oligos are constructed with a fluorescence reporter dye in proximity to a quenching molecule. While the probes are intact, the proximity of the quencher greatly reduces the fluorescence emitted by the reporter dye by Förster Resonance Energy Transfer (FRET) through space. Fluorescence of the reporter occurs only when quencher is released through some event, such as displacement or nuclease activity by Taq polymerase during PCR. The FRET probe technology has been used in conjunction with the PCR reaction to produce the 5′ fluorogenic nuclease assay or TaqMan® assay.

The development of instrumentation for detecting fluorescent signal during thermal cycling provided an ideal platform for real-time detection of PCR product using FRET probes. In the 5′ fluorogenic nuclease assay or TaqMan assay, the FRET probe is included in the PCR reaction. During the annealing phase of the PCR cycle, the FRET probe binds to a sequence between the PCR primers. With both dyes intact in the probe, the reporter dye emission is quenched. During each extension cycle, the Taq polymerase cleaves the reporter dye from the probe. Once separated from the quencher, the reporter dye emits its characteristic fluorescence.

BioSource offers complete FRET probe synthesis service. FRET probes from BioSource / Keystone are available with a choice of 5′ reporter fluorescent dyes: FAM, TET or HEX. The 3′ quencher dye is TAMRA. All probes are HPLC and PAGE purified. The PAGE purification step ensures that all unwanted synthesis failure sequences are removed. These failure sequences are one of the major contributors to high assay background; and their removal results in greater sequence-specific signal discrimination.

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Molecular Beacons

Molecular Beacons are hairpin shaped molecules with an internally quenched fluorescent dye whose fluorescence is restored when they bind to a target nucleic acid. They are designed in such a way that the loop portion of the molecule is a probe sequence complementary to a target nucleic acid molecule. The stem is formed by the annealing of complementary arm sequences on the ends of the probe sequence. A fluorescent dye is attached to the end of one arm and a quenching moiety is attached to the end of the other arm. The stem keeps these two groups in close proximity to each other, causing the fluorescence of the dye to be quenched by energy transfer. When the probe encounters a target molecule, it forms a hybrid that is longer and more stable than the stem. As a result of this new hybrid formation, the probe ends become separated from each other. As a result, the fluorophore and quencher are removed from each other and the reporter dye can now emit its characteristic fluorescence. Molecular Beacons can be synthesized with a broad range of different fluorophores. DABCYL, a non-fluorescent chromophore, serves as the universal quencher in Molecular Beacons.

Chapter 4: Oligonucleotides as Primers

The use of oligos as primers for PCR and DNA sequencing account for more than 70% of the use of oligonucleotides. All enzymatic-derived DNA synthesis requires a 3′ hydoxyl group from an exogenous source of either RNA or DNA. The oligo primer provides the necessary 3′ hydoxyl group. The specificity of the oligo primer to base-pair to a defined region of its target also insures that the enzymatic primer directed DNA synthesis is anchored precisely and that the synthesis product is fixed at its 5′end. Without this precise anchoring afforded by the oligo primer, such techniques as enzymatic DNA sequencing or PCR would not be possible.

General considerations for the selection of primers are: a) the oligo should be 18-24 bases in length; b) the Tm should be between 50-65ºC; c) the GC content should be 40-60%; d) primer should only bind to one site of the template; e) avoid sequences that would produce internal secondary structure; and f) the primer has a free 3′ hydroxyl.

PCR Primer Design

The primers for PCR determine the size of the product, the sequence location of the PCR product and the Tm of the amplified region. Well-designed primers avoid generation of background and non-specific products. We recommend that a computer-aided program be used during primer design in order to avoid certain fatal design flaws. Several commercial programs are available, as well as several internet WEB sites, whose function is to select PCR primer pairs that balance specificity of amplification and efficiency of amplification. Primer specificity is a measure of the frequency with which mispriming events occur, while primer efficiency is a measure of how close to theoretical optimum of twofold increase of product for each PCR cycle a primer can amplify a product.

PCR specificity is controlled by length and annealing temperature of PCR, with annealing temperature being dependent upon primer Tm. The following general primer pair design guidelines have proven to be successful:

- Primer pairs between 18-24 nucleotides in length tend to be target-specific.
- The primer pairs should be complementary to particular sites on the template DNA.
- Each primer should bind to only one of the two template strands.
- The optimum distance between primer annealing sites is generally 100-1000 base pairs.
- The GC content of the primers should be between 40-60%.
- Ideally, both primers should anneal at the same temperature. At minimum, the primers should have Tm′s within 5ºC of each other. The optimal annealing temperature is dependent upon the primer with the lowest melting temperature. As a rule, 20mer primer pairs with 50% GC content have Tm′s between 56-62ºC.
- Primer sequence should start and end with 1-2 purine bases.
- The placement of the 3′ end is critical for success. Perfect base pairing between the primer 3′ end and template is optimal. If a conserved amino acid sequence can be defined, use the first two bases of the codon as 3′ end.
- There should be minimal mismatch within the last 5-6 nucleotides at the 3′ end.
- Avoid three G or C nucleotides in a row near the 3′-end of the primer.
- Simple rule: choose regions that are deficient in a single nucleotide.

In general, addition of unrelated sequences at the 5′ end does not alter annealing of the sequence-specific portion of the primer. Many times these additions are recognition sequences for restriction enzymes. If restriction sites are to be added, include 2-3 non-specific extra bases 5′ to the recognition site to allow more efficient cutting by the enzyme. If a significant number of unrelated bases is added, 4-5 cycles of amplification at a lower annealing temperature, followed by cycles with optimal annealing temperature, may be used.

Keep the primers and product sequences within the coding region of the mRNA. Place primers on different exons so RNA-specific PCR is different in size from DNA contamination.

If no primer candidates survive the above criteria, relax the stringency of the selection requirements. The best test of a good primer is only in its use, and not all working primers can be accurately predicted by the above general rules.

Degenerate PCR primers

The use of degenerate PCR has proven to be a very powerful tool to find "new" genes or gene families. Most genes come in families that share structural similarities. By aligning the protein sequences from a number of related proteins, one can find shared conserved sequences which can be used as a starting point to make degenerate PCR primers. Several general rules for the construction of degenerate pools of primers from conserved amino acid motifs are:

1. Use codon bias of the appropriate species for translation.
2. Use two blocks of conserved amino acid for primer pairs.
3. Use 4-6 amino acid sequences as bases for degenerate primers. The length of the primers should be a minimum of 20 nucleotides.
4. Avoid degenerate bases at 3′ end, omit the last base of terminal codon unless the amino acid is met or trp.
5. Consider using deoxyinosine to reduce degeneracy.
6. Primers ending in T are efficiently extended even when mismatched with T, G or C. 3′ terminal mismatches A-G, G-A, C-C, and G-G reduce PCR yields 100 fold, with A-A mismatch reducing yield by 20 fold.

Automated DNA Sequencing Primer Design

When designing primers for automated sequencing, use the same general criteria used for designing PCR primers. While almost any sequence primer can be made to work, by following several general rules of primer design, your chance of generating high quality DNA sequence by using cycle sequencing protocols will be greatly enhanced.

The steps in primer design are as follows:

1. If designing a primer from a sequence chromatogram, pick an area of which you are 100% sure. Avoid designing primers using regions of poorer quality (i.e., areas beyond single peak resolution).
2. Choose a priming site that is greater than 50 bases away from the position where new sequence is needed. When targeting sequences at very specific regions, position the primer so the desired sequence falls about 80-150 nucleotides away from the primer.
3. Identify candidate primers that form stable base pairing (18-24 bases in length, 40-60% GC with Tms between 55-75ºC).
4. Discard candidate primers that demonstrate undesirable self-hybridization. Avoid primers that can form 4 or more consecutive bonds with itself, or 8 or more bonds total.
5. Verify the site-specificity of the primer. Avoid primers where alternative priming sites are present with more than 90% identity to the primary site or that match at more than seven consecutive bases at the 3'end.
6. Choose among the candidate primers that are more A-T rich at the 3'end. Thesetend to be slightly more specific in action.

Like the PCR primer design criteria, be aware that no set of guidelines will accurately predict the success of a primer. Some primers may fail for no apparent reason, and primers that appear to be poor candidates may work very well.

WEB sites for designing primers

PCR Primers

Oligos-U-Like Primer Design

http://www.path.cam.ac.uk/cgi-bin/primer3cgi

The Oligos-U-Like program allows the user to specify up to 56 different parameters. Fortunately, defaults are provided. Just enter the DNA sequence and a list of primer pairs and results including length, starting base number, Tm, %GC and PCR product size for each pair are given.

CODEHOP-PCR primer designed from protein multiple sequence alignments

http://www.blocks.fhcrc.org/codehop.html

The CODEHOP program designs PCR primers from protein multiple-sequence alignments. The program is intended for cases where the protein sequences are distant from each other and degenerate primers are needed. The multiple-sequence alignments should be of amino acid sequences of the proteins and be in the Blocks Database format. Proper alignments can be obtained by different methods. The results of the CODEHOP program are suggested degenerate sequences of DNA primers that you can use for PCR. You have to choose appropriate primer pairs, get them synthesized and perform the PCR.

BIOSOURCE

Web Primer

http://genome-www2.stanford.edu/cgi-bin/SGD/web-primer

PCR primers:
There are many factors that influence the success of a pair of primers. Some of the properties of primers which can affect the outcome of PCR include: the GC / AT ratio, length, melting temperature, and the extent of annealing between primers. The location of a primer also heavily influences its usefulness. All of these variables are able to be influenced by the user.

Sequencing Primers:
Sequencing primers are also highly customizable. Potential valid primers are evenly spaced along the DNA of interest starting at each 5' end. The user is allowed to specify the area of DNA to be sequenced, how many strands to order sequencing primers for, which strand to order primers for, the approximate distance between primers, the length and percent GC content of the primers, and the maximum self annealing of the primers.

Chapter 5: Basic PCR

The PCR process (15) is an *in vitro* method for enzymatically synthesizing and amplifying defined DNA sequences. A typical amplification reaction includes the target DNA, a thermostable DNA polymerase, two oligonucleotide primers, deoxynucleotide triphosphates (dNTPs), reaction buffer and magnesium. The components of the reaction are mixed and the reaction is placed into a thermal cycler. The thermal cycler takes the reaction through a series of different temperatures for varying amounts of time. This series of temperature and time adjustments is referred to as one cycle of amplification.

The initial step in a cycle denatures the target DNA by heating it to 95°C or higher for 15 seconds to 2 minutes. In the denaturation process, the two strands of DNA separate from each other and produce the necessary single-stranded DNA template for the thermostable polymerase.

In the next step of a cycle, the temperature is reduced to 40-60°C. At this temperature, the oligonucleotide primers anneal with the separated target DNA strands and serve as primers for DNA synthesis by a thermostable DNA polymerase. This step lasts approximately 30-60 seconds.

The synthesis of new DNA begins when the reaction temperature is raised to the optimum for the thermostable DNA polymerase. For most thermostable DNA polymerases this temperature is approximately 72°C. Extension of the primer by the thermostable polymerase lasts approximately 1-2 minutes. This step completes one cycle, and the next cycle begins with a return to 95°C for denaturation.

Since the primer extension product synthesized in a given cycle can serve as a template in the next cycle, the number of target DNA copies (amplicons) approximately doubles every cycle. Ten cycles can multiply the amplicon by a factor of about one thousand; 20 cycles, by a factor of more than a million in a matter of hours. After 20-40 cycles, the amplified nucleic acid may then be analyzed for size, quantity, sequence, etc., or used in further experimental procedures (e.g., cloning).

PCR Optimization

Although the PCR concept is simple, successful performance of a PCR reaction depends on a number of factors. This review presents a brief overview of some of these factors and strategies for optimizing them.

PCR has several characteristics that vary qualitatively: specificity, sensitivity, efficiency and fidelity (error rate). The design of the reaction determines which of these characteristics is most important. For instance, diagnostic testing requires sensitivity, specificity and reproducibility. For preparative PCR, used for synthesis of probes or template for sequencing, the efficiency of the reaction determines its overall success. By modifying the physical conditions and chemical components of the reaction, the PCR characteristics can be optimized. For instance, the elimination or reduction of nonspecific reactions can optimize both the sensitivity and specificity for diagnostic testing by removing competing side reactions.

BIOSOURCE

Primer Design

An essential prerequisite for successful PCR is the design of optimal primer pairs. See Chapter 4 (page 30) for a discussion of PCR primer design. In designing primers for PCR, the following rules have proved to be useful:

- Individual primers should be between 18-24 bases. Longer primers (30-35 bp) seem to work in similar cycling conditions compared with shorter primers.
- The two primers should have close Tms (within 5ºC). If Tm difference between the two primers is high, the lower Tm can be increased by increasing the length of that primer at the 3' end (this keeps the size of the amplified locus constant) or the 5' end.
- Maintain G:C content between 40-60%.
- Primer sequence should start and end with 1-2 purine bases.
- Each primer pair should be tested for primer-primer interactions due to 3′ end complementarity.
- Primer sequences should be checked against all DNA sequences entered in the databases using the BLAST (www.basic.nwu.edu/biotoools/oligocal.html) program. Also primer sequences with similarities with repetitive sequences or with other loci elsewhere in the genome should be avoided.
- Cycling conditions and buffer concentrations should be optimized for each primer pair, such that amplification of the desired locus is specific with no secondary products. If this is not possible, the sequences of the primers should be either elongated with 4-5 bases or changed.

The sequence of the primers can also include regions at the 5´-ends that can be useful for downstream applications. For example, restriction enzyme sites can be placed in the primer pair design if the desired PCR product is to be subsequently cloned.

Usually an equal molar concentration of both primers is required to avoid asymmetric reaction conditions. Regardless of primer choice, the final concentration of the primer in the reaction must be optimized. We recommend 1 µM final concentration (100 pmol/100 µL reaction) as a starting point for optimization. An excess of primers during PCR is required for optimum reproducibility. Normally this can be determined when residual, non-incorporated primers are visible on agarose gel analysis following PCR.

Magnesium Concentration

In the absence of adequate free magnesium ion (Mg^{++}), Taq DNA polymerase is inactive. Therefore, magnesium concentration is a crucial factor affecting the performance PCR. Many of the reaction components, including template DNA, chelating agents present in the sample (e.g., EDTA or citrate), dNTPs and proteins, bind magnesium and as a result can affect the amount of free magnesium present in the reaction. On the other hand,

excess free magnesium decreases enzyme fidelity and may increase the level of nonspecific amplification (16). Therefore, optimal $MgCl_2$ concentration for each set of reactions should be determined.

When optimizing Mg^{++} concentration, the $MgCl_2$ stock should be thoroughly thawed and vortexed. Since magnesium solutions form a concentration gradient when frozen, vortexing will insure a uniform solution. Also, it should be noted that there have been reports that pre-made reaction buffers containing 1.5 mM final concentration $MgCl_2$ have shown performance variability. This variability has been attributed to the precipitation of magnesium chloride as a result of multiple freeze/thaw cycles. Heating the stock buffer at 90°C for 10 minutes will restore the homogeneity of the solution (17).

Enzyme Choice

Several choices are available for selection of thermostable DNA polymerase. The choice of the correct enzyme(s) to use in the PCR reaction is determined by several factors. Taq DNA polymerase, the first thermostable enzyme used for PCR, possesses relatively high processivity and is the least expensive thermostable. However, Taq DNA polymerase generates single dA overhangs on the 3´-ends of the PCR product. Yet, these overhangs allow easy cloning into vectors that contain "T" overhangs complementary to those on the PCR product. When high fidelity is required, select an enzyme that has 3´→5´ exonuclease ("proofreading") activity. Enzymes with "proofreading" capabilities are recommended to ensure accurate amplification of the PCR product. These enzymes normally generate blunt-ended PCR products.

Table XIV

Comparison of Thermostable DNA Polymerases

	Taq	Tfl	Tth	Tli	Pfu	Pwo
5'-3' Exonuclease Activity	Yes	Yes	Yes	No	No	No
3'-5' Exonuclease Activity	No	No	No	Yes	Yes	Yes
Reverse Transcriptase Activity	Weak	Yes	Yes	No	n.a.	n.a.
Resulting DNA Ends	3′A	3′A	3′A	>95% Blunt	n.a.	Blunt

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Enzyme Concentration

The optimal enzyme concentration may vary between 1.0-5 units per reaction. It is especially important to titrate the Mg^{++} concentration and the amount of enzyme required per assay. It should be noted that Pwo polymerase has higher activity with $MgSO_4$ than with $MgCl_2$. Also, those enzymes with "proofreading" capabilities, in the absence of dNTPs, may degrade template and primer DNA. Therefore, a hot start technique should be used with these enzymes.

For most applications, enzyme excess does not significantly increase product yield. However, increased amounts of enzyme and excessively long extension times may increase the frequency of artifacts. These degradation artifacts are generated by the endogenous 5´-3´ exonuclease activity associated with Taq DNA polymerase, which results in smearing of the PCR products in agarose gels (18).

Often, when additional enzyme is added, the associated glycerol also added may have a detrimental effect on amplification. Pipetting errors are the most frequent cause of excessive enzyme levels. Accurate dispensing of sub-microliter volumes of enzyme solutions in 50% glycerol is nearly impossible. The use of reaction master mixes is strongly recommended. While the use of master mixes will increase the initial pipetting volume of reactants, it will reduce pipetting errors.

Template Considerations

Successful amplification of the region of interest is dependent upon the amount and quality of the template DNA. The template DNA should be free of potent inhibitors of DNA polymerases. Frequently inhibitors are carryover of reagents commonly used to purify nucleic acids. These include salts, guanidine, proteases, organic solvents and SDS. A final ethanol precipitation of the nucleic acid sample before use will eliminate most of these inhibitory agents.

The amount of template required for successful amplification is dependent upon the complexity of the DNA sample. The complexity is the sum of the nucleotide pairs that occur in single-copy sequences and those that occur in one copy of each repeated sequence. For instance, *E. coli* has a complexity of ~4.0×10^6, while mammals have a complexity of ~1.8×10^9. If possible, start with >10^4 copies of the target sequence to obtain a signal in 25-30 cycles. The final DNA concentration of the reaction should be no greater than 2 ng/µL. Table XV relates the number of unique molecules per microgram of various sources of DNA.

TABLE XV

Source	Molecules per microgram
1kb RNA	1.77×10^{12}
1kb dsDNA	9.12×10^{11}
pGEM® Vector DNA	0.85×10^{11}
Lambda DNA	1.90×10^{10}
E. coli genomic DNA	2.00×10^{8}
Human genomic DNA	3.04×10^{5}

Thermocyclers

A number of different types of thermocyclers and associated PCR reaction tubes are available. Since the same PCR program may work slightly different on different thermocyclers, PCR results on different machines using the same primer pair may vary. However, with proper cycling adjustments, similar results may be obtained on most thermocyclers. Many new PCR machines utilize thin-walled 0.2 mL PCR vials (and/or 96 well microtiter dishes). For these tubes, the differences in tube to tube results are minimal. Older machines, which use 0.5 or 1.5 mL tubes, often exhibit tube to tube variations that include reduction in product or no amplification. Slight differences in shape and wall thickness of these PCR tubes lead to imperfect contact between the tube and the metal block of the thermocycler. As a result of uneven heat transfer between the thermocycler and PCR tube, significant variations in PCR amplification occur.

First PCR program

The requirement of an optimal PCR reaction is amplification of a specific locus in relatively large amounts without any nonspecific by-products. Therefore, to ensure specificity, annealing must occur at a sufficiently high temperature to allow only the perfect DNA-DNA matches to take place. As a first step in the PCR cycle, the DNA template must be denatured. A denaturing time of 30-60 seconds at 94ºC is sufficient to achieve good PCR products. Too long a denaturing time will increase the time the Taq polymerase is subjected to high temperatures, and increases the percentage of polymerase molecules that lose their activity. A 2-5 minute initial denaturing step before the actual cycling starts may be used. This helps denature the target DNA better (especially the hard to denature templates). Some researchers omit this step, since they have found that this step does not change the outcome of the PCR reaction.

The subsequent PCR annealing and elongation steps are selected on the basis of the composition (GC content) of both the primers and PCR product and of the length of the expected PCR product. The annealing temperature can be chosen based on the melting temperature of the primers. Typically the annealing temperature chosen is 5ºC below the lower Tm of the primer pair. The optimal annealing temperature (T_a^{OPT}) also may be estimated using the following formula (19):

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$$T_a^{OPT} = 0.3 * T_m^{primer} + 0.7 * T_m^{product} - 14.9$$

in which T_m^{primer} is the calculated T_m of the less stable primer-template pair and $T_m^{product}$ is the T_m of the PCR product. The formula for $T_m^{product}$ is a modification of Baldino, et al.(7):

$$T_m^{product} = 81.5 + 0.41*(\%GC) + 16.6 \log[K] - 625/l$$

where l is the length of the PCR product in base pairs. [K] is the K^+ concentration of the PCR reaction (50 mM).

The annealing time of 30-60 seconds is sufficient for most primer pairs.

If reliable Tm values for the primers are not available, a general rule when using primer pairs 20 bp or more is to start with 54ºC as the initial annealing temperature. If nonspecific products result, increase the temperature. If the reaction is specific (only the expected product is synthesized), the temperature can be used as is. It is normal for the two primers to have Tms within 5ºC. Should the Tm difference be greater than 5ºC, the lower Tm can be increased by lengthening the primer at the 3' end or the 5' end.

In the majority of the cases, the expected amplified product is relatively small (from 0.1 to 1 kb). Since the extension activity of the Taq polymerase is about 2000 nucleotides/minute at optimal temperature (72-78ºC), the extension time can be calculated accordingly. An easy rule to use is to select an extension time (in minutes) equal to the number of kb of the product. Later the extension time may be reduced. Also, a final last extension time of 10-20 minutes may be used to finish the elongation of PCR products initiated during the last cycle. In general, 30 cycles should be sufficient for a usual PCR reaction. An increased number of cycles in most cases will not dramatically change the amount of product.

Table XVI

Designing First PCR program

Name	Temperature	Time
First denaturing	94ºC	Optional 2-5 minutes
Denaturing	94ºC	30-60 sec
Annealing	54ºC	30-60 sec
Extension	72ºC	30-90 sec
Last extension	72ºC	Optional 10-20 minutes

Unwanted side reactions, such as nonspecific amplification and primer-dimer formation, frequently occur in PCR. They usually begin at room temperature when all reaction components are mixed. These side reactions can be avoided by incorporating one of several "hot start" methods. In general, hot start techniques limit the availability of one necessary reaction component until a higher temperature (>60°C) is reached. This can be done manually by the addition of the critical component when the reaction mixture reaches the higher temperature. This method is labor intensive and can increase the chances of contamination. Other methods incorporate the critical substance in a wax bead, which melts at the higher temperature, releasing the missing component. Another technique uses an antibody to the polymerase that, at lower temperatures, binds the polymerase, preventing polymerization. At higher temperatures, the antibody binding is reversed, releasing a functional polymerase.

Nucleic Acid Cross-Contamination

It is important to minimize the potential for cross-contamination between samples and to prevent carryover of RNA and DNA from one experiment to the next. Workflow in the laboratory must proceed in an unidirectional manner. Each laboratory must establish their own dedicated areas for reagent preparation, specimen preparation, and amplification/detection. There should be no overlap of equipment or supplies between the reagent preparation, specimen preparation and amplification/detection areas. Each area should have dedicated equipment and disposable supplies. The equipment and supplies in each area should remain in the assigned area at all times. Use positive displacement pipettes or aerosol resistant tips to reduce cross-contamination during pipetting. Wear gloves and change them often. Use UNG (uracil n-glycosylase) or another sterilization technique to prevent DNA carryover to subsequent reactions.

Example PCR Protocol

Materials Required:

- Nuclease-Free Water
- 10X Reaction Buffer
  500 mM KCl; 100 mM Tris-HCl (pH 8.3); 15 mM $MgCl_2$ (the final concentrations of these ingredients in the PCR mix are: 50 mM KCl; 10 mM Tris-HCl; 1.5 mM $MgCl_2$). The buffer should be prepared in a larger volume (10-15 mL), aliquoted and stored at -20ºC. The buffer is good for years when stored at -20ºC.
- dNTP Mix: (1.25 mM of each dNTP)
- Upstream and Downstream Oligonucleotide Primer: (25 pmol/µL each)
- Taq DNA Polymerase
- Template: genomic DNA (20 ng/µL)
- Nuclease-Free Light Mineral Oil (Sigma); Do Not Autoclave

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Pipetting and DNA template:

- Prepare Master Mix by pipetting water first, followed by the other ingredients except the template.
- Keep the vials on ice while pipetting the ingredients of the reaction to minimize the chance of primer binding to the DNA template and to prevent the polymerase from working prior to the first denaturing step.
- Pipetting should be done under a laminar flow hood when plasmids are commonly used in the lab or at the bench when the template DNA is genomic DNA or when a larger amount of DNA is used.
- To be on the safe side, always use aerosol resistant tips for every PCR reaction. This is especially true when plasmids, phages or cosmids are used as templates in PCR. When using complex templates like genomic DNA such precaution may not be necessary.
- Care should be taken when pipetting small volumes (1-2 µL) of a complex DNA sample (like genomic DNA) when preparing working stocks since small variations in volume may have a significant impact on product yields.

Amplification:

1. Place 5 µL of genomic DNA template into PCR reaction tube.
2. The remaining reagents may be combined to form a Master Mix that may then be aliquoted into the reaction tubes. To make the Master Mix, multiply the number of reactions being done by the following:
   66.5 µL Water
   10 µL 10x Reaction Buffer
   16 µL dNTP Stock (1.25 mM each of dATP/dGTP/dCTP/dTTP)
   2 µL Primer Pair (25 pmol/µL)
   0.5 µL Taq Polymerase (5 units/µL)
3. When running many samples it is helpful to make a Master Mix that is slightly larger than required, (e.g., multiplying by 11 when only doing 10 reactions). This will ensure having enough reagent for each reaction you are running.
4. Aliquot 95 µL of PCR Master Mix into each tube containing the DNA template.
5. Gently tap the tubes to mix all the reagents. Briefly centrifuge tubes to deposit all of the liquid at the bottom of the reaction tube, place the PCR tubes into the PCR Thermal Cycler. If required, overlay the reaction with 1-2 drops (20-40 µL) of nuclease-free mineral oil to prevent condensation and evaporation.
6. Follow the manufacturer's instructions for programming. After an initial denaturing step at 94ºC for 2.5 minutes run the following temperature profile for 30 cycles:
   Denaturing step: 94ºC 1 minute
   Annealing step: 54ºC 1 minute
   Extension step: 72ºC 1 minute
7. For the final step incubate at 72ºC for 10 minutes followed by cooling to 4ºC. If the PCR products are not used immediately, freeze the samples. Completed PCR samples are stable for at least one week at 4ºC and at least one month at -20ºC.
8. Analyze 10-15 µL of each PCR reaction by agarose gel electrophoresis.

Detection of PCR Products:

Analyze 10-15 µL of each PCR product by 2% agarose gel electrophoresis.

1. A 2% agarose gel is prepared as follows (suitable for 7x10 cm gel):
   - 1 gram agarose
   - 5 mL 10x TBE (0.89 M TRIS base, 0.89 M boric acid, 0.02 M EDTA, pH 8.0)
   - H_2O to 50 mL

   Boil until all of the agarose is dissolved.
   Cool to approximately 50°C.
2. Add ethidium bromide so that the concentration will be 0.5 µg/mL (2.5 µL of a 10 mg/mL stock solution). (Caution: ethidium bromide is a mutagen and should be handled with care and disposed of properly.)
3. Pour gel into casting tray and allow the gel to harden at least 30 minutes.
4. Once hardened, remove the comb carefully.
5. Place gel in electrophoresis apparatus containing enough 1x TBE and 0.5 µg/mL ethidium bromide to cover the gel.
6. Mix 15 µL of PCR sample with 3 µL of 6x gel loading buffer (0.25% Bromophenol Blue, 30% glycerol).
7. Load the sample into the wells.
8. Run the samples at ~100 volts until the dye has migrated ~ 3/4 the length of the gel.
9. Examine the gel with UV transilluminator.
10. If desired, photograph the gel.
11. Store reaction products at -20°C until needed. The reaction products can be further purified using a number of procedures.

Note: To facilitate optimization, troubleshooting and validation of any PCR reaction, we strongly recommend including both positive and negative control reactions. If possible, start with $>10^4$ copies of the target sequence to obtain a signal in 25-30 cycles. Keep the final DNA concentration of the reaction at ≥2 ng/µL. Less than 10 copies of a target can be amplified, but more cycles may be required to detect a signal by gel electrophoresis. Additional cycles may increase nonspecific amplification, evidenced by smeared bands upon gel electrophoresis.

Human Cytokine Primers

Description	Part Number	Size
GM-CSF	GHC2011	2.5 nmols
TIMP-1	GHC1491	2.5 nmols
TIMP-2	GHC1141	2.5 nmols
Fas	GHS9501	2.5 nmols
Fas Ligand	GHU0011	2.5 nmols
IFN-β	GHC4041	2.5 nmols
IFN-γ	GHC4022	2.5 nmols
IL-1α	GHC0811	2.5 nmols
IL-1β	GHC0012	2.5 nmols
IL-2	GHC0021	2.5 nmols
IL-3	GHC0031	2.5 nmols
IL-4	GHC0041	2.5 nmols
IL-6	GHC0061	2.5 nmols
IL-9	GHC0091	2.5 nmols
IL-10	GHC0101	2.5 nmols
IL-11	GHC0111	2.5 nmols
IL-12p35	GHC0121	2.5 nmols
IL-12p40	GHC0122	2.5 nmols
IL-13	GHC0131	2.5 nmols
IL-15	GHC0151	2.5 nmols
IL-16	GHC0161	2.5 nmols
TNF-α	GHC3012	2.5 nmols
TGF-α	GHG0051	2.5 nmols
TGF-β	GHG0101	2.5 nmols
β-Actin	GHL1001	2.5 nmols
β-Actin cDNA template	GHL2001	100 ng

Human Chemokine Receptor Primers

Description	Part Number	Size
CCR3	GHR1511	2.5 nmols
CCR5	GHR1501	2.5 nmols
CXCR4	GHR1461	2.5 nmols

Human Chemokine Primers

Description	Part Number	Size
6CKINE	GHC1471	2.5 nmols
SDF	GHC1351	2.5 nmols
BCA	GHC1481	2.5 nmols
Eotaxin	GHC1431	2.5 nmols
IL-8	GHC0081	2.5 nmols
Lymphotactin	GHC1181	2.5 nmols
MCP-1	GHC1011	2.5 nmols
MCP-3	GHC1571	2.5 nmols
MDC	GHC1201	2.5 nmols
MIP-1α	GHC1021	2.5 nmols
MIP-1β	GHC1031	2.5 nmols
Neurotactin	GHC1171	2.5 nmols
RANTES	GHC1051	2.5 nmols

Human Primer Panels

Description	Part Number	Size
IL-8, MCP-1, RANTES	GHM1013	50 tests
IL-1β, IL-6, TNF-α	GHM0013	50 tests
IL-2, IL-10, IFN-γ	GHM0023	50 tests

Mouse Cytokine Primers

Description	Part Number	Size
IFN-γ	GMC4022	2.5 nmoles
IL-1α	GMC0912	2.5 nmoles
IL-1β	GMC0012	2.5 nmoles
IL-2	GMC0021	2.5 nmoles
IL-3	GMC0031	2.5 nmoles
IL-4	GMC0041	2.5 nmoles
IL-5	GMC0051	2.5 nmoles
IL-6	GMC0061	2.5 nmoles
IL-7	GMC0071	2.5 nmoles
IL-10	GMC0101	2.5 nmoles
IL-12 p35	GMC0121	2.5 nmoles
IL-12 p40	GMC0122	2.5 nmoles
TNF-α	GMC3011	2.5 nmoles
GM-CSF	GMC2011	2.5 nmoles
β-Actin	GML1001	2.5 nmoles
β-Actin cDNA template	GML2001	100 ng

Mouse Chemokine Primers

Description	Part Number	Size
MCP-1	GMC1011	2.5 nmoles
MIP-1α	GMC1021	2.5 nmoles
MIP-1β	GMC1031	2.5 nmoles
MIP-2	GMC1111	2.5 nmoles
RANTES	GMC1051	2.5 nmoles
BCA-1	GMC1481	2.5 nmoles

Rat Cytokine Primers

Description	Part Number	Size
IFN-γ	GRC4021	2.5 nmoles
IL-1β	GRC0011	2.5 nmoles
IL-2	GRC0021	2.5 nmoles
IL-4	GRC0041	2.5 nmoles
IL-6	GRC0061	2.5 nmoles
IL-10	GRC0101	2.5 nmoles
IL-12p35	GRC0121	2.5 nmoles
IL-12p40	GRC0122	2.5 nmoles
TNF-α	GRC3011	2.5 nmoles
TGF-β1	GRG0101	2.5 nmoles
β-Actin	GRL1001	2.5 nmoles
β-Actin cDNA template	GRL2001	100 ng

Rat Chemokine Primers

Description	Part Number	Size
CINC-1	GRC1121	2.5 nmoles
CINC-2	GRC1451	2.5 nmoles
CINC-3/MIP-2	GRC1111	2.5 nmoles
Eotaxin	GRC1431	2.5 nmoles
Gro	GRC1061	2.5 nmoles
Lymphotactin	GRC1181	2.5 nmoles
MCP-1	GRC1011	2.5 nmoles
MIP-1α	GRC1021	2.5 nmoles
MIP-1β	GRC1031	2.5 nmoles
Neurotactin	GRC1171	2.5 nmoles
PF-4	GRC1381	2.5 nmoles
RANTES	GRC1051	2.5 nmoles

Rat Primer Panels

Description	Part Number	Size
IL-4, IL-10, IL-12 p35	GRM0013	50 tests
CINC-1,CINC-3, MIP-1α	GRM1013	50 tests
MCP-1, MIP-1β, RANTES	GRM1023	50 tests
ICE, iNOS, TIMP-2	GRM5013	50 tests

Swine Cytokine Primers

Description	Part Number	Size
IFN-γ	GSC4021	2.5 nmoles
IL-1α	GSC0811	2.5 nmoles
IL-1β	GSC0011	2.5 nmoles
IL-2	GSC0021	2.5 nmoles
IL-4	GSC0041	2.5 nmoles
IL-6	GSC0061	2.5 nmoles
IL-8	GSC0081	2.5 nmoles
IL-10	GSC0101	2.5 nmoles
IL-15	GSC0151	2.5 nmoles
TGF-β	GSG0051	2.5 nmoles
TNF-α	GSC3011	2.5 nmoles

(These products are for research only. Not for use in diagnostic procedures.)

Chapter 6: Introduction to RT-PCR

Numerous techniques have been developed to measure gene expression in tissues and cells. Of these methods, RT-PCR is the most sensitive and versatile. The adaptation of PCR methodology to the investigation of RNA provides the researcher with a method featuring speed, efficiency, specificity and sensitivity. Since RNA can not serve as a template for PCR, reverse transcription is combined with PCR to make complementary DNA (cDNA) suitable for PCR. This combination of both technologies is referred to as RT-PCR. The technique can be used to determine the presence or absence of a transcript, to estimate expression levels and to clone cDNA products without the necessity of constructing and screening a cDNA library.

As originally described, RT-PCR employed avian myeloblastosis virus (AMV) or moloney murine leukemia virus (MMLV or MuLV) reverse transcriptases for first strand cDNA synthesis. Second strand cDNA synthesis and subsequent PCR amplification was performed with Taq DNA polymerase. Most RT-PCR protocols recommend a first strand cDNA synthesis reaction followed by inactivation of the reverse transcriptase and dilution of the first strand reaction mixture. This step was incorporated to eliminate the inhibitory effects of the reverse transcriptase upon Taq DNA polymerase. Second strand synthesis and PCR amplification required a separate reaction that involved the addition of the newly synthesized cDNA to a PCR reaction mix containing Taq DNA polymerase, buffer and primers.

In an effort to decrease both hands-on time and the likelihood of introducing contaminants into the reaction, several one-tube RT-PCR methods have been developed. The observation that the thermostable DNA polymerase *Thermus thermophilus* (Tth) DNA polymerase possesses reverse transcriptase activity in the presence of manganese led to the development of protocols for single-enzyme reverse transcription and PCR amplification (20). Finally, the discovery that bicine buffers containing manganese ions with Tth DNA polymerase supported both reverse transcription and subsequent DNA amplification facilitated the development of one-step, one- buffer RT-PCR method (21). One major drawback to the Tth polymerase/ bicine buffer system is the reduced fidelity of base incorporation. Consequently, the bicine buffer system is not recommended for experiments requiring high fidelity such as gene cloning and expression.

RT-PCR Optimization

A number of factors should be considered when selecting the optimal system for RT-PCR. Important considerations include the selection of the appropriate reverse transcriptase, optimal temperature for reverse transcription, choice of primers to initiate reverse transcription, and the level of sensitivity required.

Enzyme and Temperature

Three different enzymes with reverse transcriptase activity are available commercially. These are the viral reverse transcriptase from avian myoblastosis virus (AMV) and moloney murine leukemia virus (M-MuLV) and the heat stable DNA polymerase derived from *Thermus thermophilus* (Tth). All enzymes require different pH, salt concentrations, and incubation temperatures for optimal activity.

RNA transcripts exhibiting significant secondary structure must be denatured before efficient reverse transcription can occur. This step occurs before the addition of viral reverse transcriptase since it is inactivated at elevated temperatures. With the viral enzymes the first strand synthesis reactions must be performed at 37°-48°C. The maximum recommended reaction temperature for MuLV is 42°C. AMV RT, however, is active at 48°C. The thermostable DNA polymerase Tth, which exhibiting intrinsic reverse transcriptase activity, reaches optimal activity at much higher temperatures (60°-70°C). This is advantageous for eliminating RNA secondary structure during cDNA synthesis. However, recent experimental evidence demonstrates that the viral reverse transcriptases offer greater sensitivity than Tth DNA polymerase (22). As mentioned earlier, the Tth reverse transcriptase requirement for manganese also may result in reduced enzyme fidelity. Therefore, use of either MuLV or AMV reverse transcriptase is recommended when fidelity is a critical factor (e.g., when the amplified DNA is to be used for cloning).

Primer Design

There are three types of primers that may be used for reverse transcription:

1. Oligo(dT)$_{12\text{-}18}$ binds to the poly (A) tail at the 3′ end of mammalian RNA. This primer often produces full length cDNA products.
2. Random hexanucleotides bind to mRNA at any complementary site and will give partial length cDNAs. This primer may be better for overcoming difficulties caused by template secondary structure. Also, the random primers may transcribe more 5′ regions of the RNA.
3. Specific oligo primers can be used to selectively prime the RNA sequence of interest.

Selection of an appropriate primer for reverse transcription is dependent upon the size and secondary structure of the target mRNA.

A primer that anneals specifically to the 3´-end of the transcript (a sequence-specific primer or oligo(dT)) may be problematic with long mRNAs or molecules that have significant secondary structure. Under these conditions, the reverse transcriptase must extend the primer over long distances and/or through significant secondary structure. On the other hand, random hexamers prime reverse transcription at multiple points along the transcript. For this reason, they are useful for either long mRNAs, or transcripts with significant secondary structure. First strand cDNA synthesis with random hexamers should be conducted at room temperature due to the low Tm of the hexamers. At the reduced temperatures required for random primer-initiated first strand synthesis, thermostable enzymes exhibit minimal activity. Therefore the AMV and MuLV reverse transcriptases are recommended for use with random hexamers.

Whenever possible, use sequence specific primers that anneal only to defined sequences in particular RNAs, rather than to the entire RNA population in the sample (e.g., random hexamers or oligo(dT)). To differentiate between amplification of cDNA and amplification of contaminating genomic DNA, use primers that anneal to sequences in exons on opposite sides of an intron. An amplification product derived from genomic DNA will be larger than the product amplified from the target cDNA. This size difference not only makes it possible to differentiate the two products by gel electrophoresis, but it also favors the synthesis of the smaller cDNA-derived product (PCR favors the amplification of smaller fragments).

Regardless of primer choice, the final concentration of the primer in the reaction must be optimized.

Reaction Parameters

A denaturation step to reduce the effects of RNA secondary structure may be incorporated before cDNA synthesis by incubating a separate tube containing the primers and RNA template at 94°C for 2 minutes. (Do not incubate the AMV reverse transcriptase at 94°C as it will be inactivated). Alternatively, the primers and template may be heated to 65ºC for 10 minutes. The template/primer mixture can then be cooled to 42°C and added to the RT-PCR reaction mix for the standard AMV reverse transcription incubation at 42°C.

Efficient first-strand cDNA synthesis can be accomplished in a 20-60 minute incubation at 37-42°C using AMV reverse transcriptase. Perform the reverse transcription reaction at 42°C for 60 minutes as a starting point. The higher temperature will minimize the effects of RNA secondary structure and encourage full length first cDNA synthesis.

Following the reverse transcription incubation, a 2 minute incubation at 95°C is recommended to denature the RNA/cDNA hybrid, inactivate the AMV Reverse Transcriptase and dissociate the AMV from the cDNA. Most cDNA samples can be detected using 30-40 cycles of amplification. If the target cDNA is rare or if only a small amount of starting material is available, it may be necessary to increase the number of cycles to 45 or 50. Optimization of the parameters for each target RNA is highly recommended.

The following protocols for RNA purification and cDNA syntheses have been found to be fairly robust.

Extraction of RNA

The following protocol is taken from the Trizol™ LS Reagent. This procedure is based on the method developed by Chomczynski and Sacchi (23). Please follow the instructions that are provided with the RNA isolation reagent you choose to use.

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Reagents and Equipment Required but not Supplied:

Trizol™ LS Reagent, Gibco-BRL (or any other total RNA reagent)
Chloroform (without any additives, such as isoamyl alcohol)
Refrigerated microcentrifuge
RNase-free water: Prepare by adding diethylpyrocarbonate (DEPC) to a final concentration of 0.1%, shaking the solution vigorously until the DEPC is in solution and then autoclaving to inactivate the DEPC

Precautions for Preventing RNase Contamination:

In an effort to prevent accidental RNase contamination during the isolation procedure, the following guidelines should be observed:

Always wear disposable gloves
Use sterile, disposable plasticware
Use automatic pipettes that are reserved for RNA work and use cotton-plugged pipette tips
Non-disposable glassware should be baked at 150ºC for 4 hours to destroy RNases

Homogenization

Biological Fluids: The volume ratio of Trizol™ LS Reagent to sample should always be 3:1; for each 0.25 mL of sample add 0.75 mL of Trizol™ LS Reagent. Use at least 0.75 mL of Trizol™ LS Reagent per 5 to 10 x 10^6 cells. The cells are lysed by pipetting the suspension several times.

Tissues: Approximately 0.75 mL of Trizol™ LS Reagent should be used per 50 to 100 mg of tissue. The tissue sample should be homogenized in the presence of the reagent.

Cells Grown In Monolayer: Cells grown in monolayer are lysed directly in the culture dish by adding 0.3 to 0.4 mL of Trizol™ LS Reagent per 10 cm^2 of the culture dish. Pipette the suspension several times washing the bottom of the dish each time.

Cells Grown in Suspension: The cells should first be pelleted by centrifugation. Add 0.75 mL of Trizol™ LS Reagent per 5 to 10 x 10^6 cells and pipette several times to lyse the cells.

Phase Separation

Allow the samples to incubate at room temperature for 5 minutes.
Add 0.2 mL of chloroform per 0.75 mL of Trizol™ LS Reagent used. Cap the tubes tightly and vortex vigorously for one minute.
Incubate the samples at room temperature for 10 to 15 minutes.
Centrifuge the tubes at 12,000 x g for 15 minutes at 4ºC.
Transfer the upper aqueous layer to a new RNase free tube, being careful not to disturb the interphase layer.

RNA Precipitation

Add 0.5 mL of isopropyl alcohol to the samples for each 0.75 mL of Trizol™ LS Reagent used.
Incubate at room temperature for 10 minutes.
Centrifuge at 12,000 x g for 10 minutes at 4ºC.
Carefully aspirate the supernatant.
Wash the RNA pellet with 1 mL of 75% ethanol per 0.75 mL of Trizol™ LS Reagent used.
Centrifuge at 7500 x g for 10 minutes at 4ºC.
Carefully aspirate the supernatant.
Dry the pellets briefly by vacuum or air. Be careful not to overdry the pellet; this would result in difficulties in re-dissolving the RNA.
The yield and purity of the RNA can be determined by measuring the OD_{260} to OD_{280} ratio. RNA purified by this method should result in an $OD_{260/280}$ ratio of >1.7.
At this point the isolated RNA should be kept on ice when in use and quick frozen on dry ice and stored at -80ºC when not in use.

High-quality RNA will greatly increase the efficiency of RT-PCR. If at least 100 ng of purified RNA are available, its integrity can be checked on a gel by verifying the absence of 18S and 28S rRNA degradation. The 28S and 18S bands should be clearly visible; the larger 28S band should be about twice as intense as the 18S band after staining with ethidium bromide. The smear between these two bands is the mRNA. Smears and bands below the 18S band generally result from degradation of mRNA.

Production of cDNA

It is recommended that a reverse transcriptase kit, containing all the reagents necessary for the production of cDNA, be used for this section. Such a kit is available from InVitrogen called the cDNA Cycle™ Kit for RT-PCR. **Please follow the instructions that are provided with the reverse transcriptase kit you choose to use**.

Reagents and equipment required but not supplied:

Ice and ice bucket
Water baths at 42ºC, 65ºC, and 95ºC
Refrigerated microcentrifuge
Absolute ethanol at -20ºC
Dry ice

Reverse Transcription:

The isolation of mRNA is not necessary for the production of cDNA. Add approximately 5 to 10 µg of total RNA to a microcentrifuge tube.
Add 1 µL of oligo dT or specific primer (0.2 µg/µL) to the total RNA and bring the volume to a total of 11.5 µL.
Heat at 65ºC for 10 minutes to remove secondary structure.
Incubate the tube at room temperature for 2 minutes and then spin briefly to bring the solution to the bottom of the tube.

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Add 1 µL of RNase inhibitor.
Add 4 µL of 5x RT buffer.
Add 1 µL of 400 mM dNTP mix.
Add 1 µL of 80 mM sodium pyrophosphate.
Add 0.5 µL of AMV reverse transcriptase.
Mix gently and incubate at 42ºC for 1 hour.
Incubate at 95ºC for 2 minutes, spin briefly, then quickly place on ice.
The cDNA production is completed with a phenol extraction and ethanol precipitation.

a. Add 1 µL of 0.5 M EDTA, pH 8.0.
b. Add 20 µL of phenol/chloroform mix, vortex well.
c. Spin 5 to 10 minutes in a microcentrifuge.
d. Transfer the upper aqueous layer.
e. Add an equal volume of 4 M ammonium acetate.
f. Add 2 to 3 volumes of absolute ethanol at -20ºC.
g. Place on dry ice for at least 30 minutes.
h. Spin at least 15 minutes at top speed at 4ºC.
i. Carefully aspirate the supernatant, dry briefly.
j. Resuspend the pellet in 20 µL water.

Checking Integrity of RNA Extraction and cDNA Synthesis Processes

RNA extraction and reverse transcription often give variable yields. GAPDH mRNA is present in all cellular samples. Take an aliquot of the cDNA and perform PCR reaction using GAPDH, β-actin, or equivalent housekeeping marker, primers. The ability to amplify GAPDH in the cDNA demonstrates the integrity of the RNA extraction and reverse transcription processes.

Appendix

Troubleshooting Guide for PCR and RT-PCR

Symptom: Low yield or no amplification product (PCR or RT-PCR)

Possible Causes:	**Comments**:
Insufficient number of cycles	Return reactions to thermal cycler for 5 or more cycles.
Template degraded	Verify the integrity of the DNA by electrophoresis after incubation in the presence of Mg^{2+}.
Thermal cycler programmed incorrectly	Verify that times and temperatures are correct.
Temperature too low in some positions of thermal cycler	Perform a set of control reactions to determine if certain positions in the thermal cycler give low yields.
Inhibitor present	Reduce the volume of sample in the reaction. Ethanol precipitate to remove inhibitors.
Improper reaction conditions	Reduce the annealing temperature and/or allow longer extension times for longer amplicons.
Missing reaction component	Check the reaction components and repeat the reaction.
Poor primer design	Make sure primers are not self-complementary or complementary to each other. Try a longer primer.
Incorrect primer specificity	Verify that the primers are complementary to the appropriate strands.
Primer concentration too low	Verify primer concentration in the reaction. Increase primer concentration in the reaction.
Suboptimal reaction	Optimize Mg^{2+} concentration, annealing temperature and time. Always vortex the Mg^{2+}. Store solution prior to use. Verify that primers are present in equal concentration.
Nucleotides degraded	Keep nucleotides frozen in aliquots, thaw quickly and keep on ice once thawed. Avoid multiple freeze/thaw.
Target sequence not present in DNA	Redesign experiment or try other sources of target DNA.
AMV Reverse Transcriptase thermally inactivated	If an initial denaturation/annealing step is introduced into the cDNA protocol, be certain to add the enzyme mix containing AMV Reverse Transcriptase after the denaturation step and subsequent 48°C equilibrium.
Primer specificity	Verify that the "downstream" primer sequence is complementary to the downstream sequence of the RNA.
Primer annealing	If oligo(dT) was used as a "downstream" primer, verify that the annealing incubation was carried out at an appropriate temperature prior to reverse transcription.
RNA purification problem	Carryover of reagents (e.g., SDS, NaCl, heparin, guanidine thiocyanate) from some RNA purification methods can interfere with RT-PCR. Reduce the volume of target RNA, perform additional purification steps or change the purification method.

BIOSOURCE

Symptom: Amplification product has a higher than expected molecular weight (RT-PCR)

Possible Causes:	**Comments**:
Genomic DNA sequences related to the RNA template contaminate the RNA preparation	Treat the RNA sample with RNase-Free DNase.

Symptom: Multiple, nonspecific amplification products (PCR or RT-PCR)

Possible Causes:	**Comments**:
Suboptimal reaction conditions	Optimize $MgSO_4/MgCl_2$ concentration, annealing temperature, size extension time and cycle number to minimize non specific priming.
Poor primer design	Make sure primers are not self-complementary or complementary to each other, especially near the 3'-ends. Try a longer primer. Avoid using three consecutive G or C nucleotides at the 3'-end of a primer.
Primer concentration too high	Verify primer concentration in the reaction. Try a lower concentration in the reaction.
Contamination by another target RNA/DNA	Use positive displacement pipettes or aerosol-resistant tips to reduce cross-contamination during pipetting. Use a separate work area and pipettor for pre- and post-amplification. Wear gloves and change them often. Use UNG or another sterilization technique to prevent DNA carry-over to subsequent reactions.
Multiple target sequences exist in target	Design new primers with higher specificity to target DNA sequence.

Symptom: Low yield or no first strand product (RT-PCR)

Possible Causes:	**Comments**:
RNA degraded	Verify the integrity of the RNA by denaturing agarose gel electrophoresis. Ensure that reagents, tips and tubes are RNase-free. Isolate the RNA in the presence of a ribonuclease inhibitor.

BIOSOURCE

Suggested Vendors for Reagents

Thin wall PCR tubes 0.2 mL
- PGC Scientifics, catalog #502-079
- Perkin Elmer, catalog #N801-0537

Taq Polymerase with 10X buffer
- Perkin Elmer, catalog #N808-0160
- Fisher Scientific, catalog #FB-6000-60

Deoxynucleotides
- Pharmacia Biotech, catalog #27-2305-01

Agarose Gel Electrophoresis System
- VWR, catalog #IB1000
- BioRad, catalog #170-4466

Agarose
- FMC, catalog #50013

Ethidium Bromide
- Sigma, catalog #E1510
- BioRad, catalog#16-0433

cDNA Production
- Gibco-BRL, Superscript™, catalog #18089-011
- InVitrogen, cDNA Cycle Kit, catalog #L1310-01

RNA isolation-Trizol™ LS Reagent
- Gibco-BRL, catalog #10296-010

Mineral Oil, Nuclease-Free
- Sigma, catalog #M5904

References

1. Beaucage, S.L. and Iyer, P.R., (1992) Advances in the synthesis of oligonucleotides by the phosphoramidite approach. Tetrahedron 48:2223-2311.
2. Tyagi, S. and Kramer, F.R., (1996) Molecular beacons: probes that fluoresce upon hybridization. Nature Biotechnology 14:303-308.
3. Lee, L.G., Connell, C.R. and Bloch, W., (1993) Allelic discrimination by translation PCR with fluorogenic probes. Nucleic Acids Res. 21:3761-3766.
4. Cantor, C.R., Warshaw, M.M. and Shapiro, H., (1970) Oligonucleotide interactions: 3 circular dichromism studies of the conformation of deoxyligonucleotides. Biopolymers 9:1059-1077.
5. Data from Handbook of Biochemistry and Molecular Biology, Volume 1: Nucleic Acids, Fasman, G.D., editor, 3rd Edition CRC Press 1975, p 589.
6. Itakura, K., et al., (1984) Expression in *Escherichia coli* of a chemically synthesized gene for the hormone somatostatin. Science 198:1056-1063.
7. Baldino, F., Jr., Chesslet, M.F. and Lewis, M.E., (1989) High-resolution *in situ* hybridization histochemistry. Methods Enzymol. 168:761-777.
8. Kessler, C. in Non Isotopic Probe Techniques, ed. Larry Krika, Academic Press 1992, Chapter 2, p 29-92.
9. Breslauer, K.J., Frank, R., Blocker, H. and Marky, L.A., (1986) Predicting DNA duplex stability from the base sequence. Proc. Natl. Acad. Sci. USA 83:3746-3750.
10. Sugimoto, N., et al., (1996) Improved thermodynamic parameters and helix initiation factor to predict stability of DNA duplexes. Nucleic Acids Res. 24:4501-4505.
11. Laird, C.D., (1971) Chromatid structure: relationship between DNA content and nucleotide sequence diversity. Chromosoma 32:378-406.
12. Bonner, T.I., Brenner, D.J., Neufeld, B.R. and Britten, R.J., (1973) Reduction in the rate of DNA reassociation by sequence divergence. J. Mol. Biol. 81:123-135.
13. Suggs, S.V., et al., (1981) Use of synthetic oligodeoxyribonucleotides for the isolation of specific cloned DNA sequences. ICN-UCLA Symp. Mol. Cell Biol. 23:683.
14. Jacobs, K.A., et al., (1988) The thermal stability of oligonucleotide duplexes is sequence independent in tetraalkylammonium salt solutions: application to identifying recombinant DNA clones. Nucleic Acids Res. 16:4637-4650.
15. Mullis, K., et al., (1986), Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. Cold Spring Harb. Symp. Quant. Biol. 51(Part 1):263-273.
16. Eckert, K.A. and Kunkel, T.A., (1990) High fidelity DNA synthesis by the Thermus aquaticus DNA polymerase. Nucleic Acids Res. 18:3739-3744.
17. Hu, C.Y., Allen, M. and Gyllensten, U., (1992) Effect of freezing of the PCR buffer on the amplification specificity: allelic exclusion and preferential amplification of contaminating molecules. CR Methods Appl. 2:182-183.
18. Sardelli, A.D., (1993) Amplifications 9:1.
19. Rychlik, W., Spencer, W.J. and Rhoads, R.E., (1990) Optimization of the annealing temperature for DNA amplification *in vitro*. Nucleic Acids Res. 18:6409-6412.
20. Myers, T.W. and Gelfand, D.H., (1991) Reverse transcriptase and DNA amplification by Thermus thermophilus DNA polymerase. Biochem. 30:7661-7666.
21. Myers, T.W. and Sigua C.L., (1994) Amplifications 12:1.
22. Cusi, M.G., Valassina, M., and Valensin, P.E., (1994) Comparison of M-MLV reverse transcriptase and Tth polymerase activity in RT-PCR of samples with low virus burden. Biotechniques 17:1034-1036.
23. Chomczynski, P.,et al., (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156-159.