PCR (polymerase chain reaction) is a technique for amplification of DNA in vitro using thermostable DNA polymerases. It involves repetitive cycling of three steps: denaturation, annealing and extension. Denaturation occurs at ~95 °C for 30 seconds to 1 min, causing ds-stranded DNA to be separated into ss-DNA. At annealing step, primers form base-pairing with the specific sites within the ss-DNA because of base complementarities. Following the attachment of the primers to the DNA, heat stable DNA polymerase catalyzes the incorporation of deoxyribonucleotides into DNA.

- 29 μl sterile, distilled H₂O
- 5 μl 10 × PCR buffer without Mg (final concentration 1 ×)
- 5 μl 25-mM MgSO₄ (final concentration 2.5 mM)
- 5 μl 2.5-mM dNTP mix (final concentration 250 μM)
- 1 μl 5-μM Forward oligonucleotide primer (final concentration 0.1 μM)
- 1 μl 5-μM Reverse oligonucleotide primer (final concentration 0.1 μM)
- 3 μl 10-μg ml⁻¹ human genomic DNA (final concentration 0.6 ng μl⁻¹)
- 1 μl 1-u μl⁻¹ Pfu DNA polymerase (final concentration 0.02 u μl⁻¹).
Synthesis of oligos

The Phosphoramidite Method

1. The dimethoxytrityl (DMTr) protecting group at the 5' end of the growing oligonucleotide chain (which is anchored via a linking group at its 3' end to a solid support, S) is removed by treatment with acid.

2. The newly liberated 5' end of the oligonucleotide is coupled to the 3'-phosphoramidite derivative of the next deoxynucleoside to be added to the chain. The coupling agent in this reaction is tetrazole.

3. Any unreacted 5' end (the coupling reaction has a yield of over 99%) is capped by acetylation so as to block its extension in subsequent coupling reactions. This prevents the extension of erroneous oligonucleotides.

4. The phosphite triester group resulting from the coupling step is oxidized to the phosphotriester, thereby yielding a chain that has been lengthened by one nucleotide.

FIGURE 28-62. The reaction cycle in the phosphite-triester method of oligonucleotide synthesis. Here B1, B2, and B3 represent protected bases, and S represents an inert solid phase support such as controlled-pore glass.
Design PCR Primers

1. Design specific primers:
   PCNA gene of the dinoflagellate *Pyrocystis lunula*
   
   **Forward or sense primer (24 nts):** 5'-ATGGCACTCGAGGCCCATCTCCAGCAGCGGCTCTCTGAAGAAAGTGGTGATGCATGCCATCAAGGACCTGTGCA
   3'-AGAGAGCGAGCACATGGAGATCC-3'
   
   **Reverse or antisense primer (22 nts):** 5'-TCACTCGTCGATCTTGGGAGCC-3'
   
   1. Primers are usually 17-30 nucleotides long.
   2. The 3' region of the primer should have the perfect match with the target sequence.
   3. The melting point temperature can be predicted based on the following equation:
   
   \[
   T_m = 64.9 \, ^\circ C + 41 \, ^\circ C \times \frac{(\#GC-16.4)}{n}
   \]
   
   Where \( n \) is the number of bases in the primer.
   
   4. Sequences with biased nucleotide composition should be avoided. For example, the sequence
   
   \[GGGGGCTTTGGGCCCGATGCCCCCGCTCC\]
   
   contains long stretches of Gs and Cs, thus lowering complexity of the primer.
   
   5. Primer dimers may form between two copies of a single oligonucleotide or two different oligos. Dimers are detrimental to PCR.

   **5'-AGAGAGCGAGCACATGGGAGATCC-3'**
   
<table>
<thead>
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</table>
   
   **5'-GTCTGCTCGTACCTTATCCTCG-3'**
Design PCR Primers

2. Design degenerate primers:
Degenerate primers are a mixture of primers, each of which contains alternative nucleotides at a given position. These are usually derived from amino acid sequences.

Degenerate primers for the peptide sequence, CKGFDYG, can be
5′-TGYA ARGGNT TYGAYTAYGG-3′.

Likewise, 5′-GCNGAYTCCATYTCCCARAA-3′ are degenerate primers for the peptide sequence, FWEMESG.

IUPAC symbols

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Equivalent Nucleotides</th>
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<tbody>
<tr>
<td>B</td>
<td>C+G+T</td>
</tr>
<tr>
<td>D</td>
<td>A+G+T</td>
</tr>
<tr>
<td>H</td>
<td>A+C+T</td>
</tr>
<tr>
<td>K</td>
<td>T+G</td>
</tr>
<tr>
<td>M</td>
<td>A+C</td>
</tr>
<tr>
<td>N</td>
<td>A+C+G+T</td>
</tr>
<tr>
<td>R</td>
<td>A+G</td>
</tr>
<tr>
<td>S</td>
<td>G+C</td>
</tr>
<tr>
<td>W</td>
<td>A+T</td>
</tr>
<tr>
<td>V</td>
<td>A+C+G</td>
</tr>
<tr>
<td>X</td>
<td>a minor base (specified elsewhere)</td>
</tr>
<tr>
<td>Y</td>
<td>C+T</td>
</tr>
</tbody>
</table>

PCR with degenerate primers are usually run at relatively low annealing temperatures (40-50 °C).

Inosine nucleotide can substitute any of other four nucleotides because it can pairs with any of them by hydrogen bonds.
In each PCR cycle, the numbers of DNA molecules would be doubled if the reaction proceeds ideally. The process can be described by the following equation: \( A = A_02^n \). For 35 cycles of PCR amplification of 1 kb DNA from 50 ng human genome (\(3 \times 10^9\) bps), what is the expected yield?

**MW of human genome**

\[ = 3 \times 10^9 \times 660 \]

\[ = 1.98 \times 10^{12} \text{ daltons} \]

The moles of DNA in 50 ng of DNA

\[ = \frac{50 \times 10^{-9}}{1.98 \times 10^{12}} \]

\[ = 2.5 \times 10^{-20} \]

After 35 cycles, the number of moles would be: 

\[ 2.5 \times 10^{-20} \times 2^{35} = 8.6 \times 10^{-10} \text{ moles} \]

One mole of 1 kb ds-DNA has a mass of 

\[ (1000 \times 660) = 6.6 \times 10^5 \text{ grams} = 6.6 \times 10^{11} \mu g \]

So, the yield = 

\[ 8.6 \times 10^{-10} \times 6.6 \times 10^{11} = 567.6 \mu g = 0.567 \text{ mg} \]

The number is much higher than the actual yield.

So, the actual values of cycling per cycle are less than 2, ranging 1.4-1.6. These values are determined by efficiency, thermostable enzymes, and concentration of nucleotides.

From working with DNA by Dr. Metzenberg
Thermostable DNA Polymerases

The first thermostable DNA polymerase, called Taq DNA polymerase, was purified from the hot springs bacterium *Thermus aquaticus*.

<table>
<thead>
<tr>
<th>Polymerase</th>
<th>3’-&gt;5’Exonuclease</th>
<th>Source and Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taq</td>
<td>No</td>
<td>Thermus aquaticus. Half-life at 95°C is 1.6 hours.</td>
</tr>
<tr>
<td>Pfu</td>
<td>Yes</td>
<td>Pyrococcus furiosus. Appears to have the lowest error rate of known thermophilic DNA polymerases.</td>
</tr>
<tr>
<td>Vent</td>
<td>Yes</td>
<td>Thermococcus litoralis; also known as Tli polymerase. Half-life at 95°C is approximately 7 hours.</td>
</tr>
</tbody>
</table>

PCR

Lecture 19: 9
12/11/2006

PCR fragments produced by Taq has a A-overhang while Pfu and Vent products are blunt-ended.

Extension time for Taq is 1 min for 1 kb; for Pfu, it is 2 min.

Long PCR Enzyme Mix is a unique blend of Fermentas Taq DNA Polymerase and a thermostable high fidelity DNA polymerase with proofreading activity. The two enzymes synergistically generate long PCR products with greater yield and fidelity than Taq DNA Polymerase alone. The fidelity of PCR with this enzyme mix is three-times higher than with Taq DNA Polymerase. The ratio of enzymes in the Long PCR Enzyme Mix is optimized for generation of very long amplicons: up to 47 kb with viral DNA and up to 21 kb with genomic DNA templates.
Optimization of PCR reactions

Mg2+ concentrations: MgCl2 (Taq) and MgSO4 (Pfu), from 1-6 mM have been used. 1.5 mM is a default concentration.

- dNTP concentrations: 50-250 µM. Km for Taq is ~13 µM; Vent: 50 µM, and Pfu and Kod: ?.
- Usually 200 µM is used.
- Make sure that four nucleotides have balanced concentrations.

Primer concentrations: specific primers are used at 200 nM. Higher concentrations are needed for degenerate primers (up to 1-5 µM). Thermostable polymerases that have 3'-5' exnuclease activity require higher concentration of nucleotides.

Templates: remove polysaccharides and phenols and proteins by ethanol precipitation or protease treatment.

Additives: Hotstart PCR with anti-Taq;
- DMSO (1-5%), formamide (1-5%), and glycerol (5-10%), can decrease the Tm of GC-rich DNA;
- BSA and Triton X-100 sometimes are found to be helpful;
- betaine (N,N,N-trimethylglycine) (0.2-2M) is also an useful agent for PCR
- >50mM TMAC (tetramethylammonium chloride), TEAC (tetraethylammonium chloride), and TMANO (trimethlamine N-oxide) can also be used.
- BSA (up to 0.8 µg/µl) can also improve efficiency of PCR reaction.

Manipulation of the cycling parameters:
- Annealing temperatures: 35-75 oC
- Step-down PCR: start at high annealing temperature, then decrease annealing temperature in steps to reduce non-specific PCR product. Can also be used to determine DNA sequence of known protein sequence.
Various PCR methods

Nested PCR - use to synthesize more reliable product - PCR using a outer set of primers and the product of this PCR is used for further PCR reaction using an inner set of primers.

Inverse PCR - for amplification of regions flanking a known sequence. DNA is digested, the desired fragment is circularise by ligation, then PCR using primer complementary to the known sequence extending outwards.

AP-PCR (arbitrary primed)/RAPD (random amplified polymorphic DNA) - methods for creating genomic fingerprints from species with little-known target sequences by amplifying using arbitrary oligonucleotides. It is normally done at low and then high stringency to determine the relatedness of species or for analysis of Restriction Fragment Length Polymorphisms (RFLP).

RT-PCR (reverse transcriptase) - using RNA-directed DNA polymerase to synthesize cDNAs which is then used for PCR and is extremely sensitive for detecting the expression of a specific sequence in a tissue or cells. It may also be use to quantify mRNA transcripts. See also Quantitative RT-PCR, Competitive Quantitative RT-PCR, RT in situ PCR, Nested RT-PCR.

RACE (rapid amplification of cDNA ends) - used where information about DNA/protein sequence is limited. Amplify 3' or 5' ends of cDNAs generating fragments of cDNA with only one specific primer each (+ one adaptor primer). Overlapping RACE products can then be combined to produce full cDNA. See also Gibco manual.

DD-PCR (differential display) - used to identify differentially expressed genes in different tissues. First step involves RT-PCR, then amplification using short, intentionally nonspecific primers. Get series of band in a high-resolution gel and compare to that from other tissues, any bands unique to single samples are considered to be differentially expressed.

Multiplex-PCR - 2 or more unique targets of DNA sequences in the same specimen are amplified simultaneously. One can be use as control to verify the integrity of PCR. Can be used for mutational analysis and identification of pathogens.
5' RACE

Figure 1. Overview of the 5' RACE Procedure.

Anneal first strand primer, GSP1, to mRNA

Copy mRNA into cDNA with SuperScrip™II RT

Degrade RNA with RNase Mix

Purify cDNA with GlassMAX Spin Cartridge

Tail purified cDNA with dCTP and TdT

PCR amplify dG-tailed cDNA using the Abridged Anchor Primer and nested GSP2

Reamplify primary PCR product using AUAP, or UAP, and nested GSP
3' RACE

Figure 1. Summary of the 3' RACE System procedure.
Modified RACEs

The GeneRacer™ method is described below. This technique is based on RNA ligase-mediated (RLM-RACE) and oligo-capping rapid amplification of cDNA ends (RACE) methods, and results in the selective ligation of an RNA oligonucleotide to the 5’ ends of decapped mRNA using T4 RNA ligase (Maruyama and Sugano, 1994; Schaefer, 1995; Volloch et al., 1994).

**Note:** If you are only interested in the 3’ ends of mRNA, skip Steps 1–3 and proceed directly to Step 4, reverse transcription.

1. Treat total RNA or mRNA with calf intestinal phosphatase (CIP) to remove the 5’ phosphates. This eliminates truncated mRNA and non-mRNA from subsequent ligation with the GeneRacer™ RNA Oligo. **Note:** CIP has no effect on full-length, capped mRNA.

   ![Diagram of mRNA structures with CIP treatment](image)

2. Treat dephosphorylated RNA with tobacco acid pyrophosphatase (TAP) to remove the 5’ cap structure from intact, full-length mRNA. This treatment leaves a 5’ phosphate required for ligation to the GeneRacer™ RNA Oligo.

   ![Diagram of mRNA structures with TAP treatment](image)
3. Ligate the GeneRacer™ RNA Oligo to the 5' end of the mRNA using T4 RNA ligase. The GeneRacer™ RNA Oligo will provide a known priming site for GeneRacer™ PCR primers after the mRNA is transcribed into cDNA.

4. Reverse-transcribe the ligated mRNA using Cloned AMV RT or SuperScript™ II RT and the GeneRacer™ Oligo dT Primer to create RACE-ready first-strand cDNA with known priming sites at the 5' and 3' ends. (If you are only interested in the 5' ends, you can reverse-transcribe using random primers or a gene-specific primer. If you are only interested in the 3' ends, reverse-transcribe the original, unligated mRNA or total RNA using the GeneRacer™ Oligo dT Primer.)

5. To obtain 5' ends, amplify the first-strand cDNA using a reverse gene-specific primer (Reverse GSP) and the GeneRacer™ 5' Primer (homologous to the GeneRacer™ RNA Oligo). Only mRNA that has the GeneRacer™ RNA Oligo ligated to the 5' end AND is completely reverse-transcribed will be amplified using PCR. If needed, perform additional PCR with nested primers.

6. To obtain 3' ends, amplify the first-strand cDNA using a forward gene-specific primer (Forward GSP) and the GeneRacer™ 3' Primer (homologous to the GeneRacer™ Oligo dT Primer). Only mRNA that has a polyA tail and is reverse-transcribed will be amplified using PCR. If needed, perform additional PCR with nested primers.
DNA fragments can be used as primers
Creation of a chimera gene
Real time PCR
Real time or quantitative PCR (qPCR) is a method that allows continual monitoring of product accumulation during the polymerase chain reaction.

1. SYBR Green qPCR:
Ds-stranded DNA binds to the fluorescent dye, STBR green. The fluorescence yield is proportional to the amount of ds-stranded DNA in the PCR reactions.

2. qPCR with Molecular beacons:
Molecular beacons are oligonucleotides with two functional groups (a fluorescent dye and a quencher) attached at each end. The dye emits fluorescence whose emission spectra overlaps with absorption of the quencher. If the two groups are positioned closely enough and in the right angles, energy can be transferred directly from one molecule to another, a phenomenon known as fluorescence resonance energy transfer (FRET). So, FRET causes the fluorescence emitted by the former to be diminished (quenched) by the absorption of the latter.

The molecular beacon is so designed that it has regions (stems) that can be self-annealed to form a short hairpin structure to bring the two groups at 5’- and 3’-ends of the oligonucleotide together. The close positioning of the two groups suppress the fluorescence. The sequence in the loop region can hybridize with the complementary sequence in the DNA to be detected, thereby separating the fluorescent dye from its quencher and increasing the fluorescent emission. The increase in fluorescence is a measure of the amount of PCR products.
3. qPCR using TaqMan method:
TaqMan method is also based on the concept of FRET. In this method, an oligonucleotide that pairs with the internal region of the DNA to be amplified carries the fluorescent dye at one end and the quencher at the other. The close proximity of the two groups reduces the fluorescence. During PCR amplification, the oligonucleotide with the two functional groups is annealed to the template and blocks DNA extension catalyzed by Taq DNA polymerase. The 5' to 3' exonuclease activity degrades the oligonucleotide, dissociating the dye and quencher and resulting in an increase in fluorescence. The more the PCR products, the higher the fluorescence. The method is diagrammed in the figure below. F: fluorescent group, Q: Quencher group.
Microarray (gene chip) is a glass on which hundreds or thousands or a complete set of genes of an organism are deposited as tiny spots of less than 10 μM in diameter. The array can then be probed by fluorescently labeled cDNAs derived from mRNAs of two samples. Since the dyes for labeling the two samples, Cy3 for one and Cy5 for another, have different fluorescence wavelengths (colors), the ratio of emission yields from two dyes can be quantified for each spot (so each gene), which reflects the relative abundance of the gene products in two samples. The microarray technology allows expression of many genes are compared simultaneously.
Phage display

Phage display is a molecular technique by which foreign proteins are expressed at the surface of phage particles. Such phages thereby become vehicles for expression that not only carry within them the nucleotide sequence encoding expressed proteins, but also have the capacity to replicate. Using phage display vast numbers of variant nucleotide sequences may be converted into populations of variant peptides and proteins which may be screened for desired properties.

Many types of phage have been used as vehicles for phage display including F1 filamentous phage, Lambda and T7. Each of these has advantages and disadvantages with respect to each particular application. The Ff1 phage family (M13 and its close relatives f1d and f1) are excellent cloning vehicles because their size is not constrained by the DNA contained within them. The insertion of foreign sequences within their genome is accommodated simply by the assembly of longer phage particles. On the other hand, the non-lytic propagation mechanism of F1 phage requires that the all the components of the phage coat be exported through the bacterial inner membrane prior to the assembly of the mature phage particle. As a consequence, only proteins that are capable of withstanding this export may be displayed. This limitation may be avoided by using the lytic phage Lambda and T7, in which capsid assembly occurs entirely in the cytoplasm prior to cell lysis. Furthermore, recent studies have shown that unlike T7, Lambda phage can tolerate the display of relative large proteins at high density.
A DNase footprinting assay is a technique from molecular biology that detects DNA-protein interaction using the fact that a protein bound to DNA will often protect that DNA from enzymatic cleavage. The method uses an enzyme, deoxyribonuclease (DNase, for short) to cut the radioactively end-labelled DNA, followed by gel electrophoresis to detect the resulting cleavage pattern.