Army Of Identical Scientists Demands Legislative Support For Cloning

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DNA CLONING: amplification of unique DNA molecules

- *In vitro*-PCR
- *In vivo*-in different host cells
POLYMERASE CHAIN REACTION (PCR)
PCR

- THE POLYMERASE CHAIN REACTION (PCR) PROVIDES AN EXTREMELY SENSITIVE MEANS OF AMPLIFYING RELATIVELY LARGE QUANTITIES OF DNA

- FIRST DESCRIBED IN 1985, NOBEL PRIZE FOR KARY MULLIS IN 1993
PCR

- The length of the amplified DNA sequence can be as long as 40 KB.

- Only approx. 20 bp sequence from both ends of the DNA to be amplified is needed to be known.

- Since the human genome and many other genomes are fully sequenced, it is easy to amplify any wanted pieces of DNA.
The technique was made possible by the discovery of *Taq* polymerase, the DNA polymerase that is used by the bacterium *Thermus aquaticus* that was discovered in hot springs.
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BEFORE AND AFTER
THE DISCOVERY OF Taq POLYMERASE
COMPOSITION OF A PCR REACTION

- DEOXIRIBOSE NUCLEOTIDE TRIPHOSPHATES (dNTP-s), the building blocks for the new DNA
- TEMPLATE DNA, the DNA sequence that you want to amplify
- PRIMERS, single-stranded DNAs between 20 and 50 nucleotides long (oligonucleotides) that are complementary to a short region on either side of the template DNA
- HEAT STABLE DNA POLYMERASE that catalyzes the synthesis of new DNA
- BUFFER, WATER
PCR

There are three major steps in a PCR, which are repeated for 20 to 40 cycles.

This is done in an automated thermo cycler, which can heat and cool the reaction tubes in a very short time.
THE STEPS OF A PCR CYCLE

➢ **DENATURATION** at around 94 °C:
During the denaturation, the double strand melts open to single stranded DNA, all enzymatic reactions stop (for example the extension from a previous cycle).

➢ **ANNEALING** at around 54 °C:
Hydrogen bonds are constantly formed and broken between the single stranded primer and the single stranded template. If the primers exactly fit the template, the hydrogen bonds are so strong that the primer stays attached.

➢ **EXTENSION** at around 72 °C:
The bases (complementary to the template) are coupled to the primer on the 3' side (the polymerase adds dNTP's from 5' to 3', reading the template from 3' to 5' side, bases are added complementary to the template)
THE STEPS OF A PCR CYCLE

PCR: Polymerase Chain Reaction

30 - 40 cycles of 3 steps:

Step 1: denaturation
1 minute 94 °C

Step 2: annealing
45 seconds 54 °C
forward and reverse primers !!!

Step 3: extension
2 minutes 72 °C
only dNTP's

(Andy Vierstraete 1999)
PCR: Denaturation 94°C
EXPONENTIAL INCREASE OF THE NUMBER OF COPIES DURING PCR

Exponential amplification

\[ 2^1 = 2 \text{ copies} \]
\[ 2^2 = 4 \text{ copies} \]
\[ 2^3 = 8 \text{ copies} \]
\[ 2^4 = 16 \text{ copies} \]

\[ 2^{35} = 34 \text{ billion copies} \]
EXPONENTIAL INCREASE OF THE NUMBER OF COPIES DURING PCR

- EVERY CYCLE RESULTS IN A DOUBLING OF THE NUMBER OF STRANDS DNA PRESENT

- AFTER THE FIRST FEW CYCLES, MOST OF THE PRODUCT DNA STRANDS MADE ARE THE SAME LENGTH AS THE DISTANCE BETWEEN THE PRIMERS

- THE RESULT IS A DRAMATIC AMPLIFICATION OF THE DNA THAT EXISTS BETWEEN THE PRIMERS. THE AMOUNT OF AMPLIFICATION IS $2^N$; $N$ REPRESENTS THE NUMBER OF CYCLES THAT ARE PERFORMED.
OVERVIEW OF THE FIRST THREE CYCLES

Region to be amplified

5' 3' 3' 5' Target DNA

Primer 1
Add excess primers 1 and 2, dNTPs, and Taq polymerase
Heat to 95° to melt strands
Cool to 60° to anneal primers

Primer 2

Primers extended by Taq polymerase at 72°

Heat to 95° to melt strands
Cool to 60° to anneal primers

Cycle 1

Cycle 2

Cycle 3

Primers extended by Taq polymerase at 72°

Heat to 95° to melt strands
Cool to 60° to anneal primers
VERIFICATION OF PCR PRODUCT

Verification of PCR product on agarose or separeide gel

- Ladder markers: 383, 929, 1058, 1857
- PCR fragments: 1, 2, 3, 4, 5
PCR AND CONTAMINATION

The most important consideration in PCR is contamination.

Even the smallest contamination with DNA could affect amplification.

For example, if a technician in a crime lab set up a test reaction (with blood from the crime scene) after setting up a positive control reaction (with blood from the suspect) cross contamination between the samples could result in an erroneous incrimination, even if the technician changed pipette tips between samples. A few blood cells could volitilize in the pipette, stick to the plastic of the pipette, and then get ejected into the test sample.

Modern labs take account of this fact and devote tremendous effort to avoiding cross-contamination.
APPLIEDS OF PCR

DIAGNOSIS OF INFECTIONS

➢ design primers which amplify the pathogens sequences, but no human sequence

➢ perform PCR with sample from patient used as template (presumably containing the pathogen’s DNA)

➢ gel electrophoresis of the PCR reaction product

➢ if the expected size DNA is amplified, the infection is verified.

Diagnosis can be done in a FEW HOURS, before PCR it might have taken days/weeks
DIAGNOSIS OF BACTERIAL INFECTIONS

Appropriate primer pairs are commercially available for many pathogenic bacteria

- Neisseria gonorrhea
- Chlamydia trachomatis
- Mycobacterium tuberculosis (TBC)
- Treponema pallidum (Syphilis)
- Mycobacterium leprae (leprosy lepra)
DIAGNOSIS OF VIRAL INFECTIONS

- HIV
- HPV
- CMV
- EBV
- herpes viruses
- rota virus
- Calici virus
APPLICATIONS OF PCR

DIAGNOSIS OF GENETICALLY INHERITED DISEASES

Mutations in any gene can be detected if the gene is PCR amplified, then sequenced.

- CYSTIC FIBROSIS-CFTR (cystic fibrosis transmembrane conductance regulator)
- BREAST CANCER-BRCA
- RETIONOBLASTOMA-RB
- P53
DNA template for PCR can be obtained from the developing embryo by:

- Preimplantation Genetic Diagnosis
- Chorionic Villus Sampling
- Amniocentesis
PRAENATAL DIAGNOSIS OF GENETICALLY INHERITED DISEASES

IN VITRO FERTILIZATION, coupled with PREIMPLANTATION GENETIC DIAGNOSIS, can eliminate the risk that adults who are carriers of genetic diseases will produce affected children. It is possible to take a cell from a blastocyst at the 4- or 8-cell stage without damaging its developmental potential. The sampled cell can be subjected to molecular analysis to determine whether it carries the harmful gene.
CHORIONIC VILLUS SAMPLING

Information about genetic defects can be obtained from chorionic tissues. The fetus and placenta are imaged by a sonogram to guide a catheter, which samples a chorionic villus.
APPLICATIONS OF PCR

PRAENATAL DIAGNOSIS OF GENETICALLY INHERITED DISEASES

AMNIOCENTESIS: Cells slough off of the developing human embryo and float in the amniotic fluid that bathes it. A small sample of the amniotic fluid may be sampled with a needle as the first step of a process called. Cells from the fluid can be cultured and used for biochemical and genetic analyses that can reveal the sex of the fetus, as well as genetic diseases.
APPLICATIONS OF PCR

FORENSIC MEDICINE
APPLICATIONS OF PCR

FORENSIC MEDICINE

➢ ANY PERSON CAN BE IDENTIFIED BY THE PCR BASED VNTR TECHNIQUE.

➢ FOR TEMPLATE, A DROP OF BLOOD, SEMEN, A PIECE OF HAIR IS SUFFICIENT.
STR/ SHORT TANDEM REPEATS

- 2-7 base is repeated 7-40 times.

- The number of repeats might be different:

  VNTR (variable number of tandem repeat)

- 10 STR gives a person specific pattern.
<table>
<thead>
<tr>
<th>Locus</th>
<th>Repeat</th>
<th># Alleles</th>
<th>Population Match Probabilities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Caucasian American</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.12</td>
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<td></td>
<td></td>
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<td>0.195</td>
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<td></td>
<td>0.039</td>
</tr>
<tr>
<td>Product</td>
<td></td>
<td></td>
<td>$1.738 \times 10^{-3}$</td>
</tr>
<tr>
<td>One in</td>
<td></td>
<td></td>
<td>$5.753 \times 10^{14}$</td>
</tr>
</tbody>
</table>

One set of VNTR locus are inherited from the mother and one set from the father. The genes are amplified using PCR, and then run through electrophoresis. The position of the two bands on the electrophoresis gel depends on the exact number of repeats at the locus.
Three VNTR loci from suspects, along with the DNA from the scene of the crime are run through PCR amplification, and then through electrophoresis. This gives six bands, which can have common bands for some individuals, but the overall pattern is distinctive for each person.
DNA fragments from different sources can be joined together
DNA fragments from different sources can be joined together.
RECOMBINANT DNA TECHNOLOGY

1. Bacterium
   - Plasmid isolated
   - DNA isolated
   - Gene of interest
   - Gene inserted into plasmid

2. Cell containing gene of interest
   - DNA isolated
   - Gene of interest

3. Recombinant DNA (plasmid)
   - Plasmid put into bacterial cell

4. Recombinant bacterium
   - Cell multiplies with gene of interest

5. Copies of gene
   - Gene for pest resistance inserted into plants
   - Protein used to make snow form at higher temperature
   - Gene used to alter bacteria for cleaning up toxic waste
   - Protein dissolves blood clots in heart attack therapy
<table>
<thead>
<tr>
<th>Product category</th>
<th>Examples/uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anticoagulants</td>
<td>Tissue plasminogen activator (TPA) activates plasmin, an enzyme involved in dissolving clots; effective in treating heart attack victims.</td>
</tr>
<tr>
<td>Blood factors</td>
<td>Factor VIII promotes clotting and is deficient in hemophiliacs; use of factor VIII produced by recombinant DNA technology eliminates infection risks associated with blood transfusions.</td>
</tr>
<tr>
<td>Colony stimulating factors</td>
<td>Immune system growth factors that stimulate leukocyte production; used to treat immune deficiencies and to fight infections.</td>
</tr>
<tr>
<td>Erythropoietin</td>
<td>Stimulates erythrocyte production; used to treat anemia in patients with kidney disease.</td>
</tr>
<tr>
<td>Growth factors</td>
<td>Stimulate differentiation and growth of various cell types; used to promote wound healing.</td>
</tr>
<tr>
<td>Human growth hormone</td>
<td>Used to treat dwarfism.</td>
</tr>
<tr>
<td>Human insulin</td>
<td>Used to treat diabetes.</td>
</tr>
<tr>
<td>Interferons</td>
<td>Interfere with viral reproduction; used to treat some cancers.</td>
</tr>
<tr>
<td>Interleukins</td>
<td>Activate and stimulate different classes of leukocytes; possible uses in treating wounds, HIV infection, cancer, and immune deficiencies.</td>
</tr>
<tr>
<td>Monoclonal antibodies</td>
<td>Extraordinary binding specificity is used in: diagnostic tests; targeted transport (of drugs, toxins, or radioactive compounds to tumors as a cancer therapy); many other applications.</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>Prevents tissue damage from reactive oxygen species when tissues briefly deprived of $O_2$ during surgery suddenly have blood flow restored.</td>
</tr>
<tr>
<td>Vaccines</td>
<td>Proteins derived from viral coats are as effective in “priming” an immune system as the killed virus more traditionally used for vaccines, but are safer; first developed was the vaccine for hepatitis B.</td>
</tr>
</tbody>
</table>
THE RESTRICTION ENDONUCLEASES

ANTI PHAGE DEFENSE
THE RESTRICTION-MODIFICATION SYSTEM

RESTRICTION ENDONUCLEASE + MODIFICATION METHYLASE
THE RESTRICTION ENDONUCLEASES

A. Enzymes and reactions

Recognition site

modification methylase (M.EcoRI)

Restriction enzyme (R.EcoRI)

Double-strand break

B. Attack on unmethylated incoming DNA

Bacteriophage

Bacterial cell

Bacterial chromosome

Restriction modification gene complex

RECOGNITION/RESTRICTION SITE:
4-8 bp palindrome sequence
THE RESTRICTION ENDONUCLEASES

restriction endonuclease-DNA interaction at non-specific site

restriction endonuclease-DNA interaction at specific recognition site
EcoRI cuts the double stranded DNA at its recognition sequence.
THE RESTRICTION ENDONUCLEASES

- **Blunt ends**
  - AluL: 5' ...AGCT... 3'
    - 3' ...TCGA... 5'
  - HaeIII: 5' ...GGCC... 3'
    - 3' ...CCGG... 5'
  - BamHI: 5' ...GGATCC... 3'
    - 3' ...CCCTAGG... 5'
  - HindIII: 5' ...AAGCTT... 3'
    - 3' ...TTAGAA... 5'
  - EcoRI: 5' ...GAAATTC... 3'
    - 3' ...CTTAA... 5'

- **Sticky ends**
separation of DNA molecules according to their molecular weight
DNA CLONING

For cloning you need:

- the purified fragment (with ends produced by a known restriction enzyme)
- the vector DNA opened (linearized) by the same restriction enzyme
- DNA ligase enzyme
- Host cell

amplification of unique DNA molecules
DNA CLONING

Restriction Digestion

Ligation

Transformation

Recombinant Plasmid Isolation
DNA CLONING

Cloning vectors  expression vectors
DNA CLONING - LIGATION
DNA CLONING

Selection of the recombinant vector containing cells:

- **Plasmids with two antibiotic resistance genes**

- **Plasmids with LacZ**
DNA CLONING

Plasmids with LacZ gene of interest

R1

lacZ digestion

ligation

recombinant plasmid

transformation

spread on petri dish
with antibiotic 1 and X-gal

Isolate white colony

Grow ON

purify plasmid

white colony: no X-gal breakdown, recombinant plasmid

Plasmids with LacZ
RECOMBINANT PROTEIN PRODUCTION

(a)  

Recombinant proteins can be produced in bacteria in huge quantities. The protein encoding gene is cloned into a plasmid containing an inducible bacterial promoter.

(b)  

β-galactosidase

Desired proteins
The recombinant proteins are isolated by various methods (AFFINITY CHROMATOGRAPHY).
Many eukaryotic protein can not be produced in bacteria because of the posttranslational modifications (which are essential for the protein’s function) happening only in eukaryotes. In these cases eukaryotic expression systems are used.
RECOMBINATIONAL CLONING: CLONING WITHOUT RESTRICTION ENZYMES
RECOMBINATIONAL CLONING: CLONING WITHOUT RESTRICTION ENZYMES
RECOMBINATIONAL CLONING: CLONING WITHOUT RESTRICTION ENZYMES

1. Entry Clone (Km\(^R\))
   - Gene
   - L1
   - L2

2. Destination Vector (Ap\(^R\))
   - ccaB
   - R1
   - R2

3. LR CLONASE™
   - Ap\(^R\)

4. Expression Clone
   - Gene
   - B1
   - B2

5. By-Product
   - ccaB
   - P1
   - P2

6. Transform E. coli and select Ap\(^R\) transformants

7. Combining L x R gives B and P

8. >90-99% correct clones