La infección natural mediante bacteriofagos (vectores) aumenta la eficiencia de la transformación e introducción del DNA exógeno

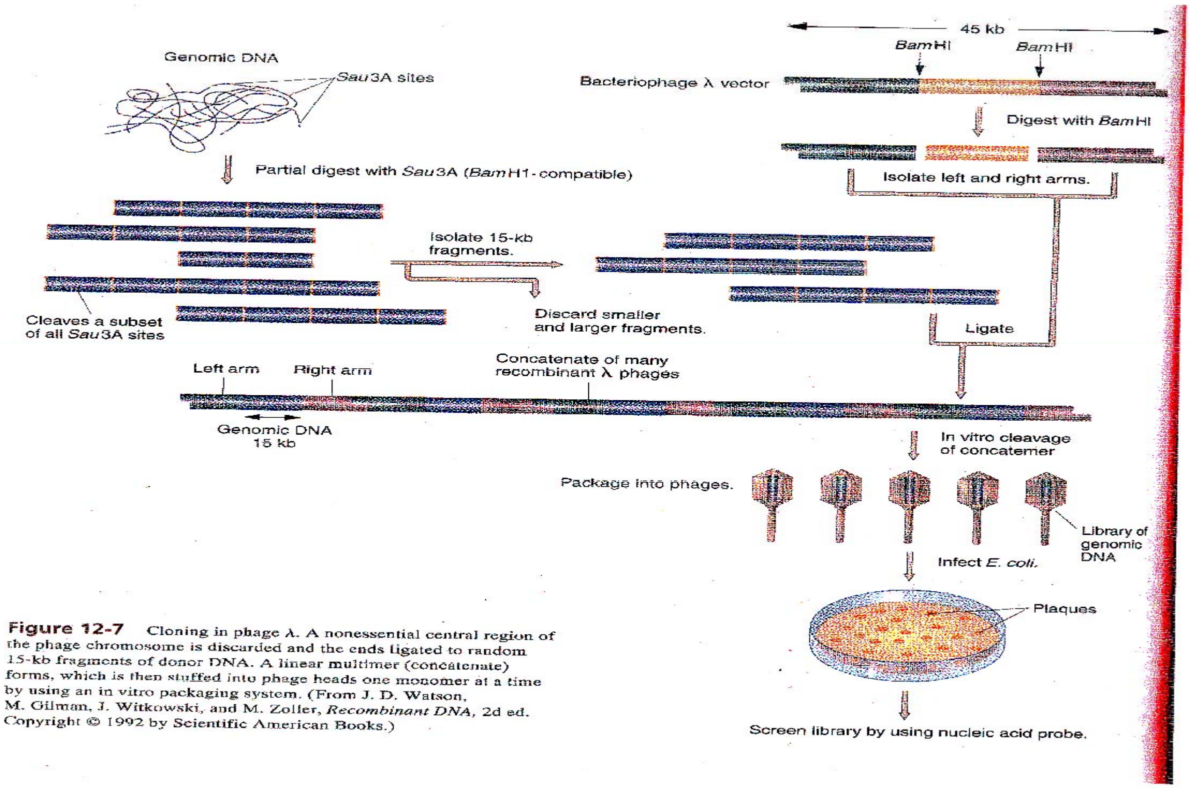
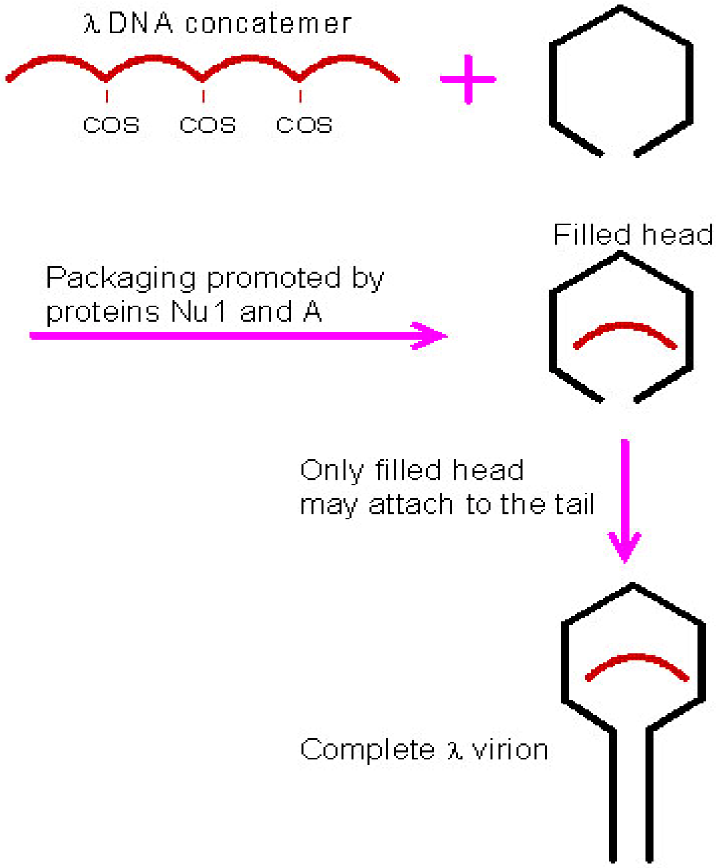
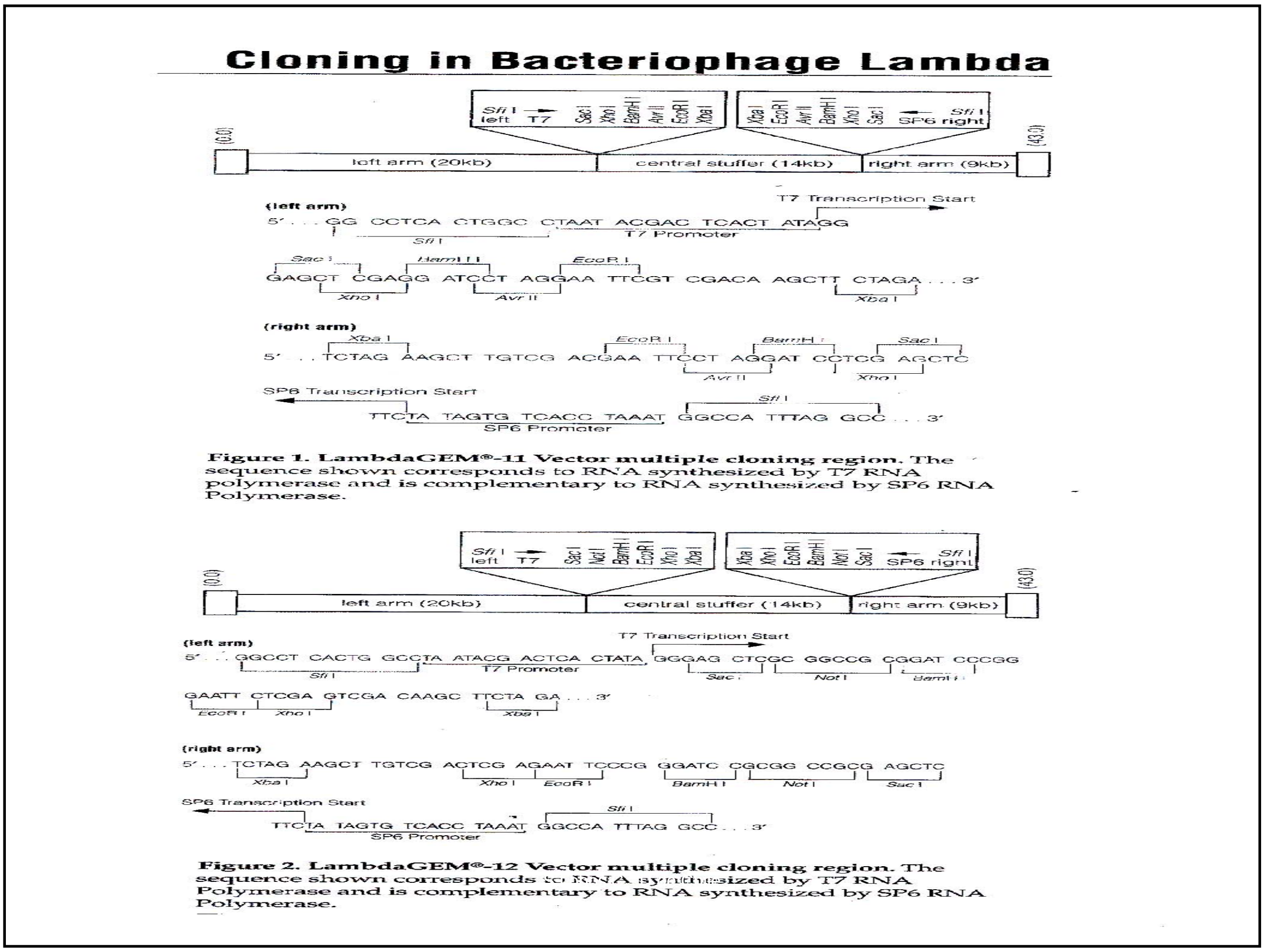

45 kb
Genomic DNA
*Sau*3A sites
*Bam*HI
*Bam*HI
Bacteriophage λ vector
Digest with *Bam*HI
Partial digest with *Sau*3A (*Bam*H1-compatible)
Isolate left and right arms.
Isolate 15-kb fragments.
Discard smaller and larger fragments.
Cleaves a subset of all *Sau*3A sites
Ligate
Left arm
Right arm
Concatenate of many recombinant λ phages
Genomic DNA 15 kb
In vitro cleavage of concatemer
Package into phages.
Library of genomic DNA
Infect *E. coli*.
Plaques
Screen library by using nucleic acid probe.

Figure 12-7 Cloning in phage λ. A nonessential central region of the phage chromosome is discarded and the ends ligated to random 15-kb fragments of donor DNA. A linear multimer (concatenate) forms, which is then stuffed into phage heads one monomer at a time by using an in vitro packaging system. (From J. D. Watson, M. Gilman, J. Witkowski, and M. Zoller, *Recombinant DNA*, 2d ed. Copyright © 1992 by Scientific American Books.)

Proteins Nu1 and A can recognize the COS site, directing the insertion of the λ DNA between them into an empty head. The filled head is then attached to the tail, forming a complete λ virion. The whole process normally takes place in the host cell. However, to prepare the λ virion carrying recombinant λ DNA, the following **in vitro assembly system** is commonly used.


λ DNA concatemer
COS
COS
COS
+
Filled head
Packaging promoted by proteins Nu1 and A
Only filled head may attach to the tail
Complete λ virion

The assembly process of the λ virion.



Cloning in Bacteriophage Lambda

Sfi I left
T7
Sac I
Xho I
BamH I
Avr II
EcoR I
Xba I
Xba I
EcoR I
Avr II
BamH I
Xho I
Sac I
Sfi I SP6 right
(0.0)
left arm (20kb)
central stuffer (14kb)
right arm (9kb)
(43.0)

(left arm)

T7 Transcription Start

5′ . . . GG CCTCA CTGGC CTAAT ACGAC TCACT ATAGG

Sfi I
T7 Promoter

GAGCT CGAGG ATCCT AGGAA TTCGT CGACA AGCTT CTAGA . . . 3′

Sac I
BamH I
EcoR I
Xho I
Avr II
Xba I

(right arm)

5′ . . . TCTAG AAGCT TGTCG ACGAA TTCCT AGGAT CCTCG AGCTC

Xba I
EcoR I
BamH I
Sac I
Avr II
Xho I

SP6 Transcription Start

TTCTA TAGTG TCACC TAAAT GGCCA TTTAG GCC . . . 3′

Sfi I
SP6 Promoter

Figure 1. LambdaGEM®-11 Vector multiple cloning region. The sequence shown corresponds to RNA synthesized by T7 RNA polymerase and is complementary to RNA synthesized by SP6 RNA Polymerase.

Sfi I left
T7
Sac I
Not I
BamH I
EcoR I
Xho I
Xba I
Xba I
Xho I
EcoR I
BamH I
Not I
Sac I
Sfi I SP6 right
(0.0)
left arm (20kb)
central stuffer (14kb)
right arm (9kb)
(43.0)

(left arm)

T7 Transcription Start

5′ . . . GGCCT CACTG GCCTA ATACG ACTCA CTATA GGGAG CTCGC GGCCG CGGAT CCCGG

Sfi I
T7 Promoter
Sac I
Not I
BamH I

GAATT CTCGA GTCGA CAAGC TTCTA GA . . . 3′

EcoR I
Xho I
Xba I

(right arm)

5′ . . . TCTAG AAGCT TGTCG ACTCG AGAAT TCCCG GGATC CGCGG CCGCG AGCTC

Xba I
Xho I
EcoR I
BamH I
Not I
Sac I

SP6 Transcription Start

TTCTA TAGTG TCACC TAAAT GGCCA TTTAG GCC . . . 3′

Sfi I
SP6 Promoter

Figure 2. LambdaGEM®-12 Vector multiple cloning region. The sequence shown corresponds to RNA synthesized by T7 RNA Polymerase and is complementary to RNA synthesized by SP6 RNA Polymerase.

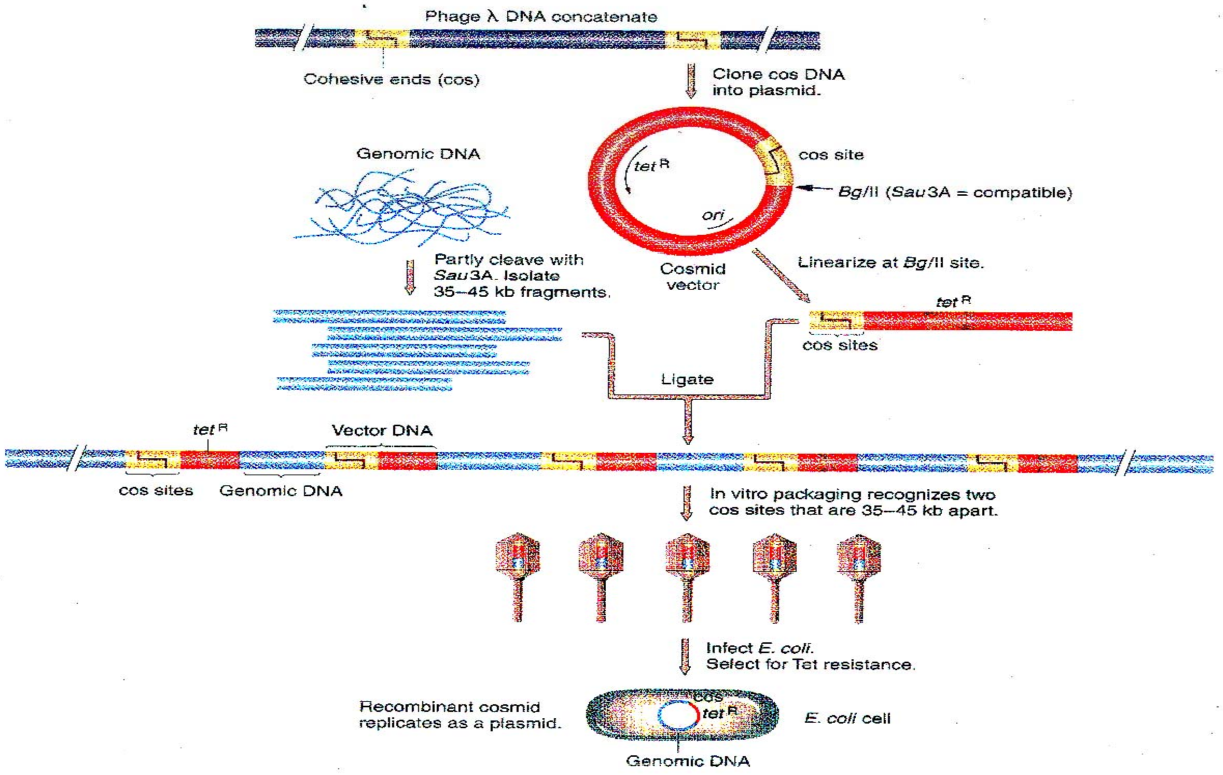
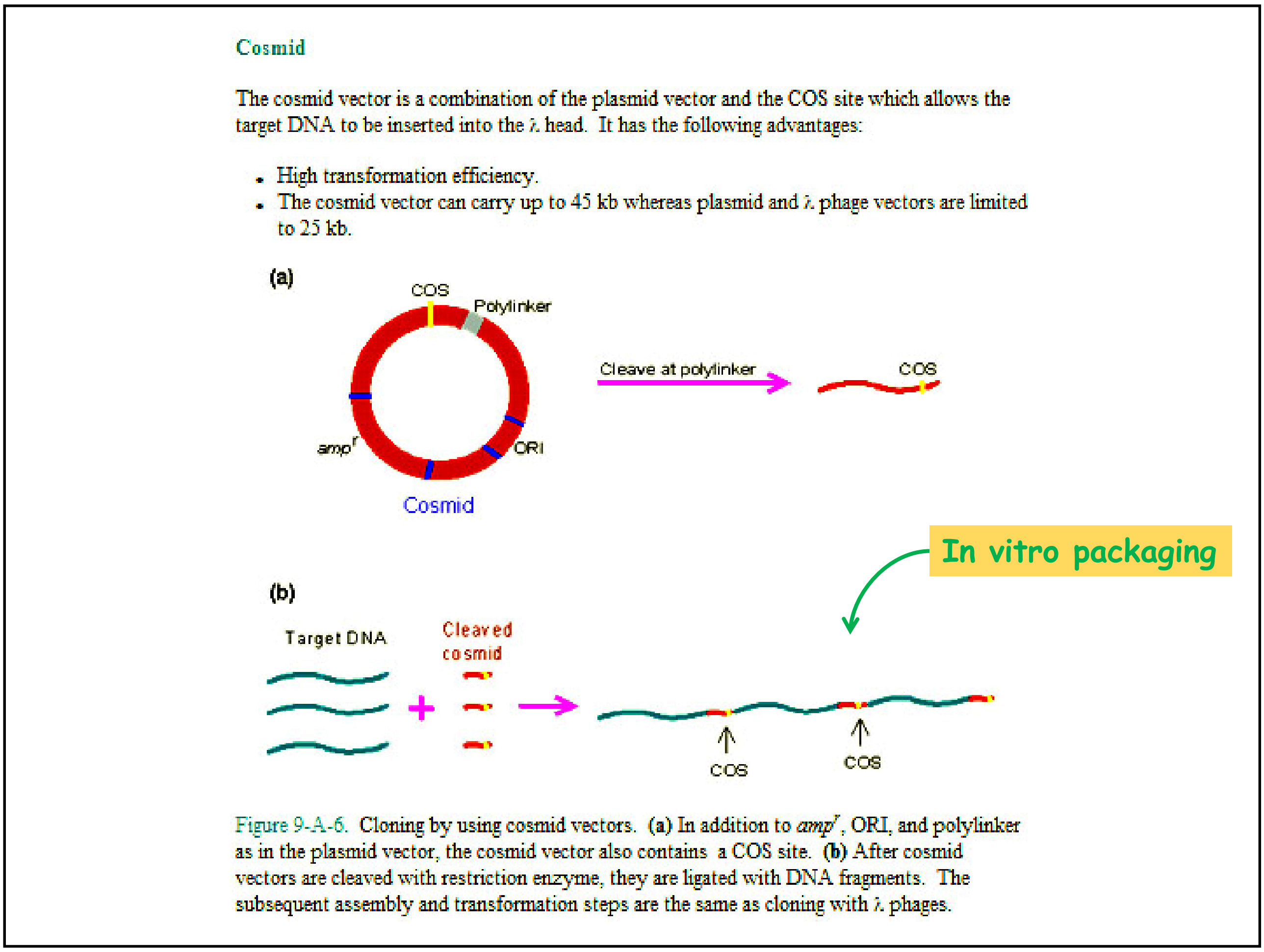

Phage λ DNA concatenate
Cohesive ends (cos)
Clone cos DNA into plasmid.
Genomic DNA
tet R
cos site
Bg/II (Sau3A = compatible)
ori
Partly cleave with Sau3A. Isolate 35–45 kb fragments.
Cosmid vector
Linearize at Bg/II site.
tet R
cos sites
Ligate
tet R
Vector DNA
cos sites
Genomic DNA
In vitro packaging recognizes two cos sites that are 35–45 kb apart.
Infect E. coli. Select for Tet resistance.
Recombinant cosmid replicates as a plasmid.
cos
tet R
E. coli cell
Genomic DNA

Figure 12-8 Cloning by cosmids. The cosmid is cut at a *BglII* site next to the cos site. Donor genomic DNA is cut by using *Sau*3A, which gives sticky ends compatible with *BglII*. A tandem array of donor and vector DNA results from mixing. Phage is packaged in vitro by cutting at the cos site. The cosmid with insert recircularizes after it is in the bacterial cell. (From J. D. Watson, M. Gilman, J. Witkowski, and M. Zoller, *Recombinant DNA*, 2d ed. Copyright © 1992 by Scientific American Books.)



Cosmid

The cosmid vector is a combination of the plasmid vector and the COS site which allows the target DNA to be inserted into the λ head. It has the following advantages:

- High transformation efficiency.
- The cosmid vector can carry up to 45 kb whereas plasmid and λ phage vectors are limited to 25 kb.

(a)
COS
Polylinker
amp^r
ORI
Cosmid
Cleave at polylinker
COS
(b)
Target DNA
Cleaved cosmid
+
COS
COS

In vitro packaging

Figure 9-A-6. Cloning by using cosmid vectors. **(a)** In addition to *amp*^{r}, ORI, and polylinker as in the plasmid vector, the cosmid vector also contains a COS site. **(b)** After cosmid vectors are cleaved with restriction enzyme, they are ligated with DNA fragments. The subsequent assembly and transformation steps are the same as cloning with λ phages.

Trabajando Con Gigantes:

The BAC Vector

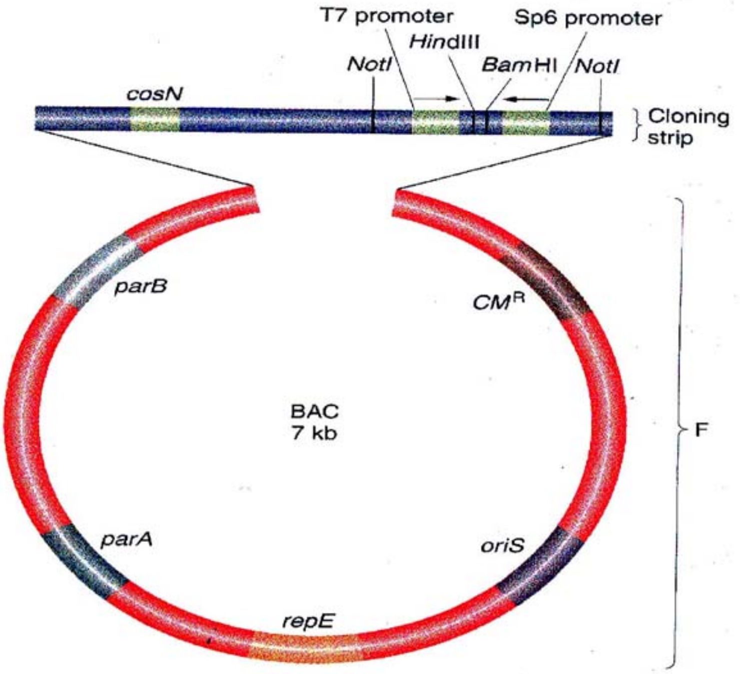
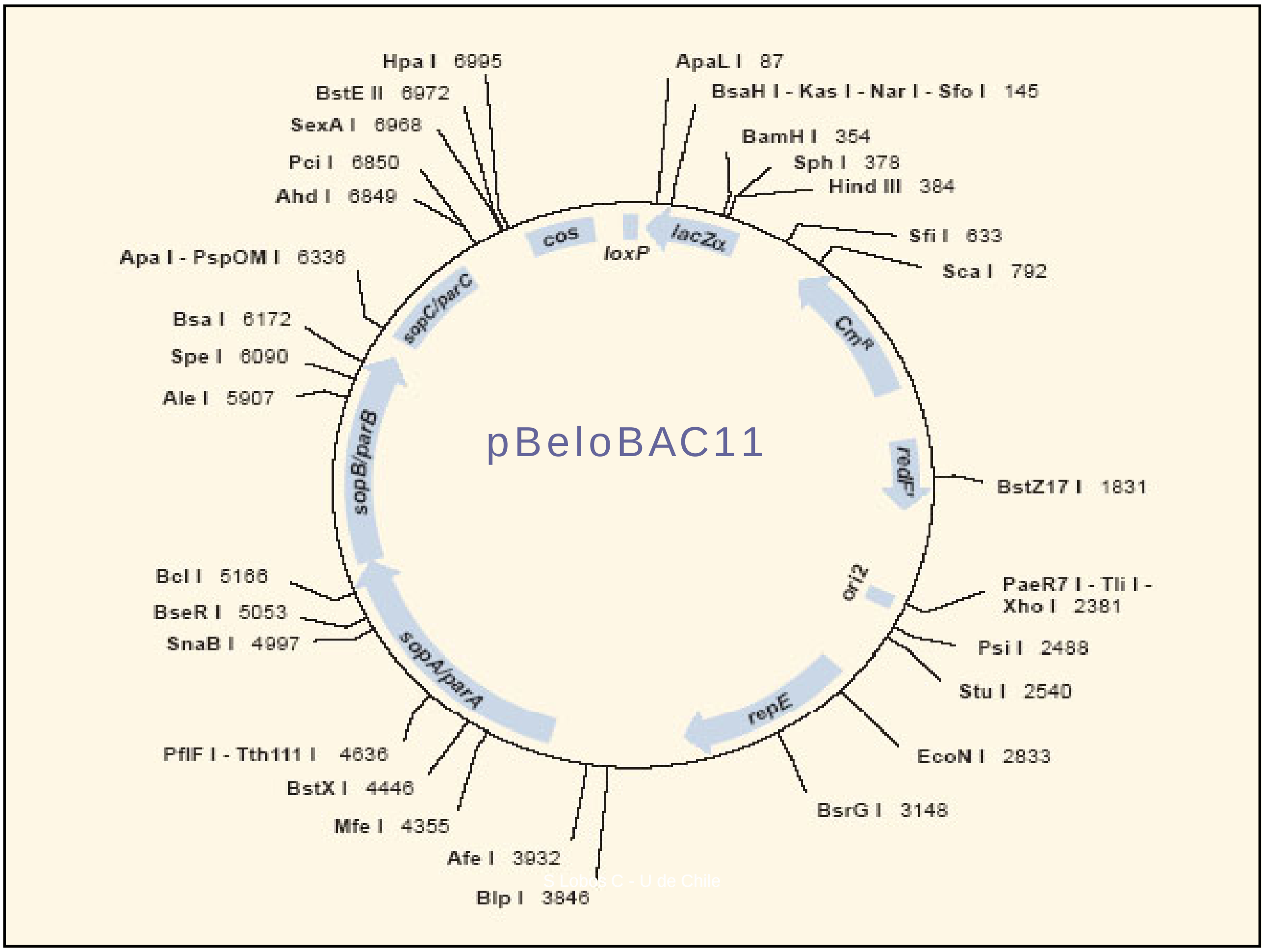
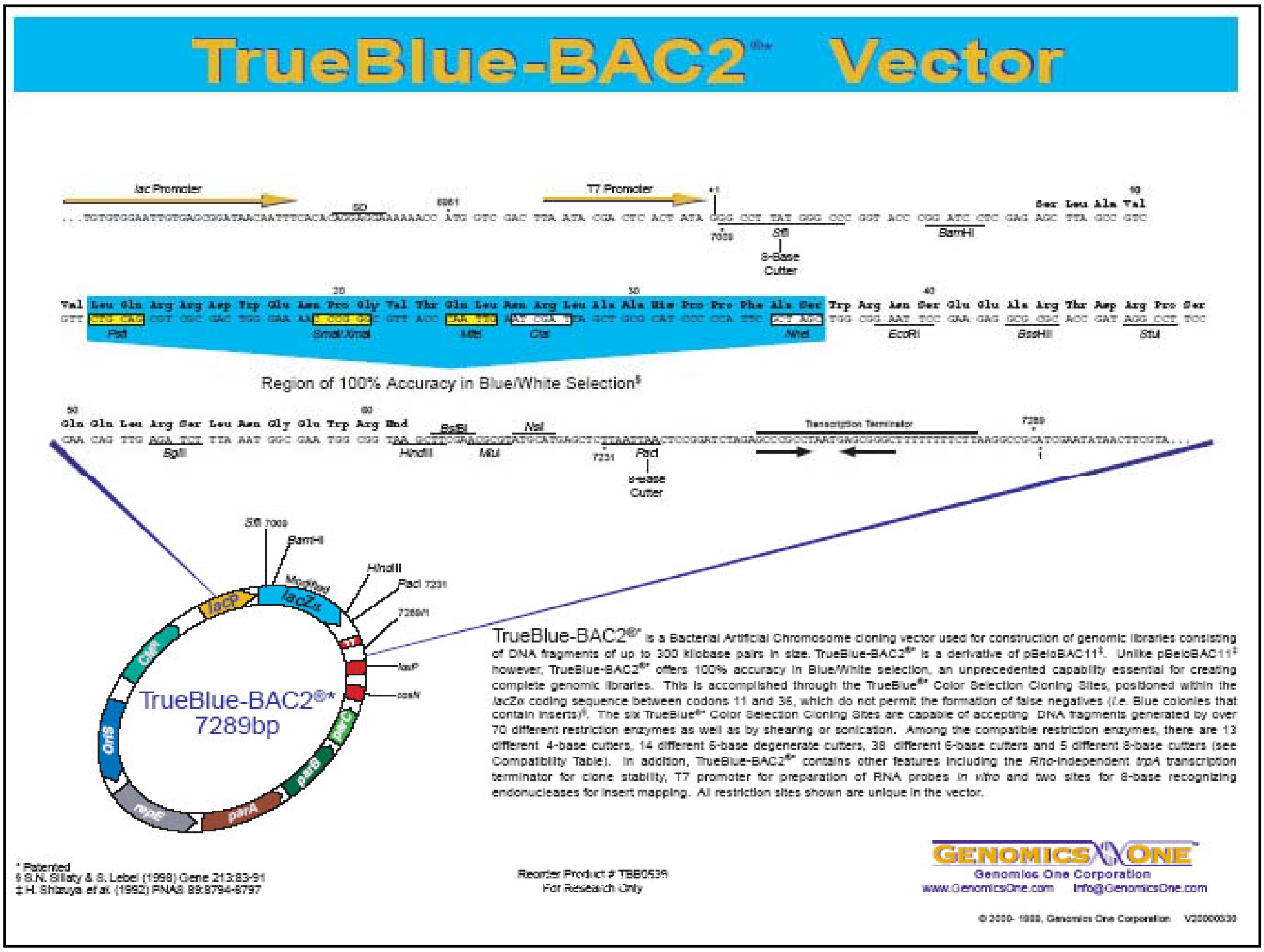

T7 promoter
Sp6 promoter
HindIII
NotI
BamHI
NotI
cosN
Cloning strip
parB
CMR
BAC
7 kb
F
parA
oriS
repE

Figure 14-12 Structure of a bacterial artificial chromosome (BAC), used for cloning large fragments of donor DNA. CM^R is a selectable marker for chloramphenicol resistance. *oriS, repE, parA,* and *parB* are F genes for replication and regulation of copy number. *cosN* is the *cos* site from λ phage. *Hin*dIII and *Bam*HI are cloning sites at which foreign DNA is inserted. The two promoters are for transcribing the inserted fragment. The *NotI* sites are used for cutting out the inserted fragment.


pBeloBAC11
Hpa I 6995
BstE II 6972
SexA I 6968
Pci I 6850
Ahd I 6849
Apa I - PspOM I 6336
Bsa I 6172
Spe I 6090
Ale I 5907
Bcl I 5166
BseR I 5053
SnaB I 4997
PflF I - Tth111 I 4636
BstX I 4446
Mfe I 4355
Afe I 3932
Blp I 3846
ApaL I 87
BsaH I - Kas I - Nar I - Sfo I 145
BamH I 354
Sph I 378
Hind III 384
Sfi I 633
Sca I 792
BstZ17 I 1831
PaeR7 I - Tli I - Xho I 2381
Psi I 2488
Stu I 2540
EcoN I 2833
BsrG I 3148
cos
loxP
lacZα
CmR
redF
ori2
repE
sopA/parA
sopB/parB
sopC/parC

S Lobos C - U de Chile



TrueBlue-BAC2®* Vector

lac Promoter
T7 Promoter
SD
6981
+1
10
Ser Leu Ala Val
...TGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAGGAAAAAACC ATG GTC GAC TTA ATA CGA CTC ACT ATA GGG CCT TAT GGG CCC GGT ACC CGG ATC CTC GAG AGC TTA GCC GTC
7009
SfiI
8-Base Cutter
BamHI
20
30
40
Val Leu Gln Arg Arg Asp Trp Glu Asn Pro Gly Val Thr Gln Leu Asn Arg Leu Ala Ala His Pro Pro Phe Ala Ser Trp Arg Asn Ser Glu Glu Ala Arg Thr Asp Arg Pro Ser
GTT CTG CAG CGT CGC GAC TGG GAA AAC CCG GGC GTT ACC CAA TTG AAT CGA TTA GCT GCG CAT CCC CCA TTC GCT AGC TGG CGG AAT TCC GAA GAG GCG CGC ACC GAT AGG CCT TCC
PstI
SmaI/XmaI
MfeI
ClaI
NheI
EcoRI
BssHII
StuI
Region of 100% Accuracy in Blue/White Selection§
50
60
Gln Gln Leu Arg Ser Leu Asn Gly Glu Trp Arg End
CAA CAG TTG AGA TCT TTA AAT GGC GAA TGG CGG TAA GCTTCGAACGCGTATGCATGAGCTCTTAATTAACTCGGGATCTAGAGCCCGCCTAATGAGCGGGCTTTTTTTTCTTAAGGCCGCATCGAATATAACTTCGTA...
BglII
HindIII
BstBI
MluI
NsiI
7231
PacI
8-Base Cutter
Transcription Terminator
7289
1
SfiI 7009
BamHI
Modified lacZα
HindIII
PacI 7231
7289/1
loxP
cosN
lacP
CmR
OriS
repE
parA
parB
parC
TrueBlue-BAC2®*
7289bp

TrueBlue-BAC2®* is a Bacterial Artificial Chromosome cloning vector used for construction of genomic libraries consisting of DNA fragments of up to 300 kilobase pairs in size. TrueBlue-BAC2®* is a derivative of pBeloBAC11‡. Unlike pBeloBAC11‡ however, TrueBlue-BAC2®* offers 100% accuracy in Blue/White selection, an unprecedented capability essential for creating complete genomic libraries. This is accomplished through the TrueBlue®* Color Selection Cloning Sites, positioned within the *lacZα* coding sequence between codons 11 and 36, which do not permit the formation of false negatives (*i.e.* Blue colonies that contain inserts)§. The six TrueBlue®* Color Selection Cloning Sites are capable of accepting DNA fragments generated by over 70 different restriction enzymes as well as by shearing or sonication. Among the compatible restriction enzymes, there are 13 different 4-base cutters, 14 different 6-base degenerate cutters, 38 different 6-base cutters and 5 different 8-base cutters (see Compatibility Table). In addition, TrueBlue-BAC2®* contains other features including the *Rho*-independent *trpA* transcription terminator for clone stability, T7 promoter for preparation of RNA probes *in vitro* and two sites for 8-base recognizing endonucleases for insert mapping. All restriction sites shown are unique in the vector.

* Patented
§ S.N. Slilaty & S. Lebel (1998) Gene 213:83-91
‡ H. Shizuya *et al.* (1992) PNAS 89:8794-8797

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SCREENING DE GENOTECAS
Búsqueda de un Gene específico.

- Sondas de DNA Genómico
- Sondas de cDNA
- Oligonucleótidos sintéticos
- Anticuerpos

Homólogas, heterólogas, degeneradas
marcadas con radiactividad o
quimioluminiscencia

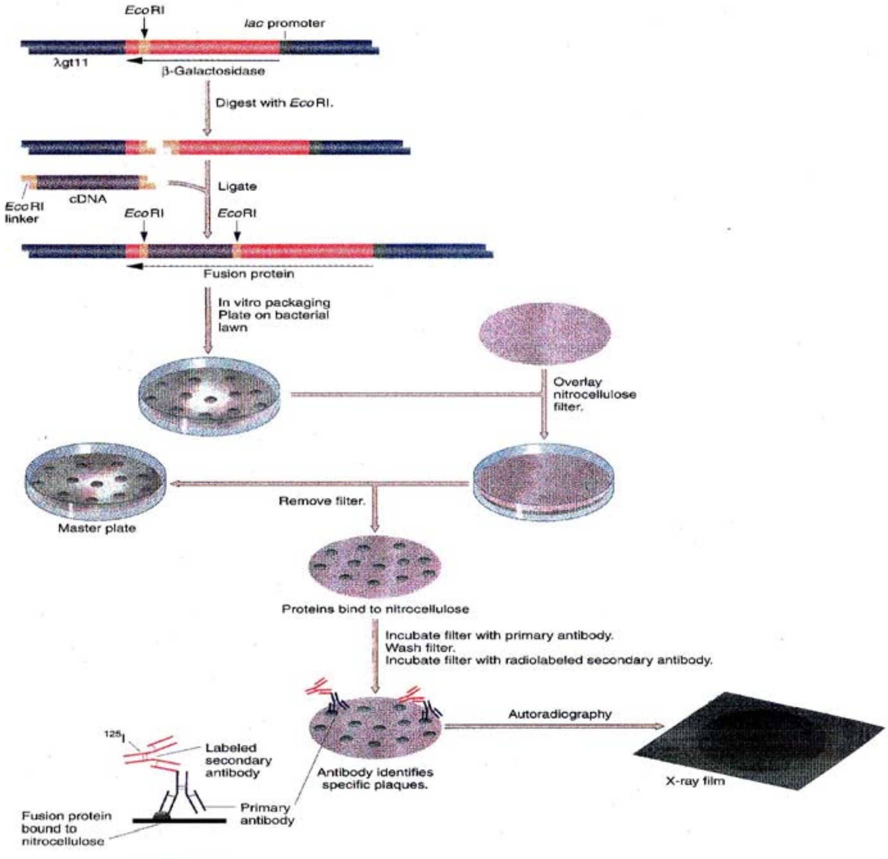

EcoRI
lac promoter
λgt11
β-Galactosidase
Digest with EcoRI.
Ligate
EcoRI linker
cDNA
EcoRI
EcoRI
Fusion protein
In vitro packaging
Plate on bacterial lawn
Overlay nitrocellulose filter.
Remove filter.
Master plate
Proteins bind to nitrocellulose
Incubate filter with primary antibody.
Wash filter.
Incubate filter with radiolabeled secondary antibody.
Autoradiography
125I
Labeled secondary antibody
Antibody identifies specific plaques.
X-ray film
Fusion protein bound to nitrocellulose
Primary antibody

Figure 12-12 Finding the clone of interest by using antibody. An expression library made with phage derivative λgt11 is screened with a protein-specific antibody. After the unbound antibodies have been washed off the filter, the bound antibodies are visualized through binding of a radioactive secondary antibody. (From J. D. Watson, M. Gilman, J. Witkowski, and M. Zoller, *Recombinant DNA*, 2d ed. Copyright © 1992 by Scientific American Books.)

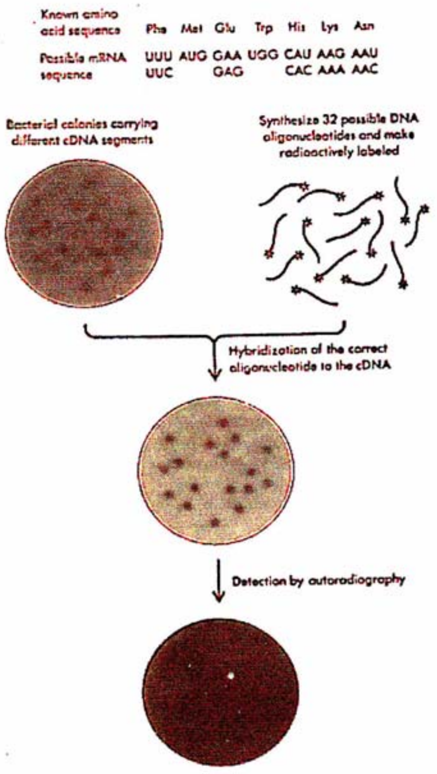

Known amino acid sequence
Phe Met Glu Trp His Lys Asn
Possible mRNA sequence
UUU AUG GAA UGG CAU AAG AAU
UUC GAG CAC AAA AAC
Bacterial colonies carrying different cDNA segments
Synthesize 32 possible DNA oligonucleotides and make radioactively labeled
Hybridization of the correct oligonucleotide to the cDNA
Detection by autoradiography

Figure 19-18, above
Cloning genes by use of a probe designed from a known amino acid quence. Given the amino acid sequence, it is possible to synthesize oligonucleotides for the possible mRNA sequences. The correct oligo otide will hybridize to the appropriate cDNA that we wish to identi is carried in the plasmid. Since the oligonucleotide probe is radioacti labelled, the hybrid DNA can be detected by autoradiography.

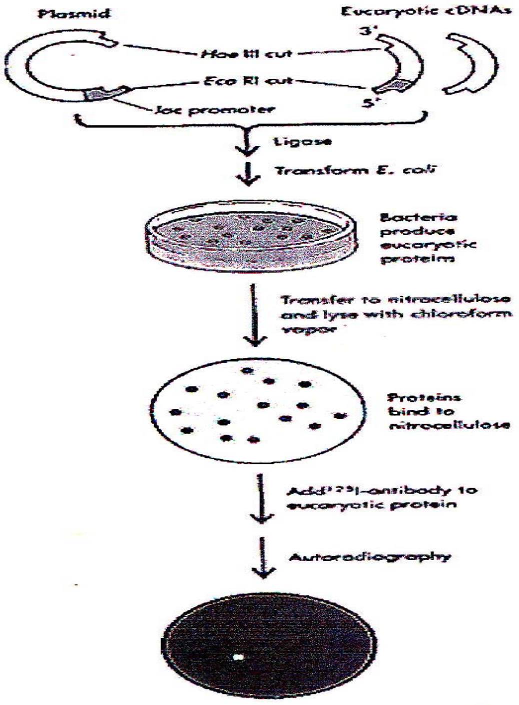

Plasmid
Eucaryotic cDNAs
3'
5'
Hae III cut
Eco RI cut
lac promoter
Ligase
Transform E. coli
Bacteria produce eucaryotic proteins
Transfer to nitrocellulose and lyse with chloroform vapor
Proteins bind to nitrocellulose
Add ^{125}I-antibody to eucaryotic protein
Autoradiography

Figure 19-20
Immunological screening of expression vectors to detect desired eucaryotic-bacterial hybrid proteins. Proteins produced by bacteria are transferred to nitrocellulose filters and treated with chloroform to make them permeable to antibodies. An antibody specific for the hybrid protein we want to isolate is radioactively labeled and added to the filter, specifically binding to the correct protein. The protein can then be detected by autoradiography.

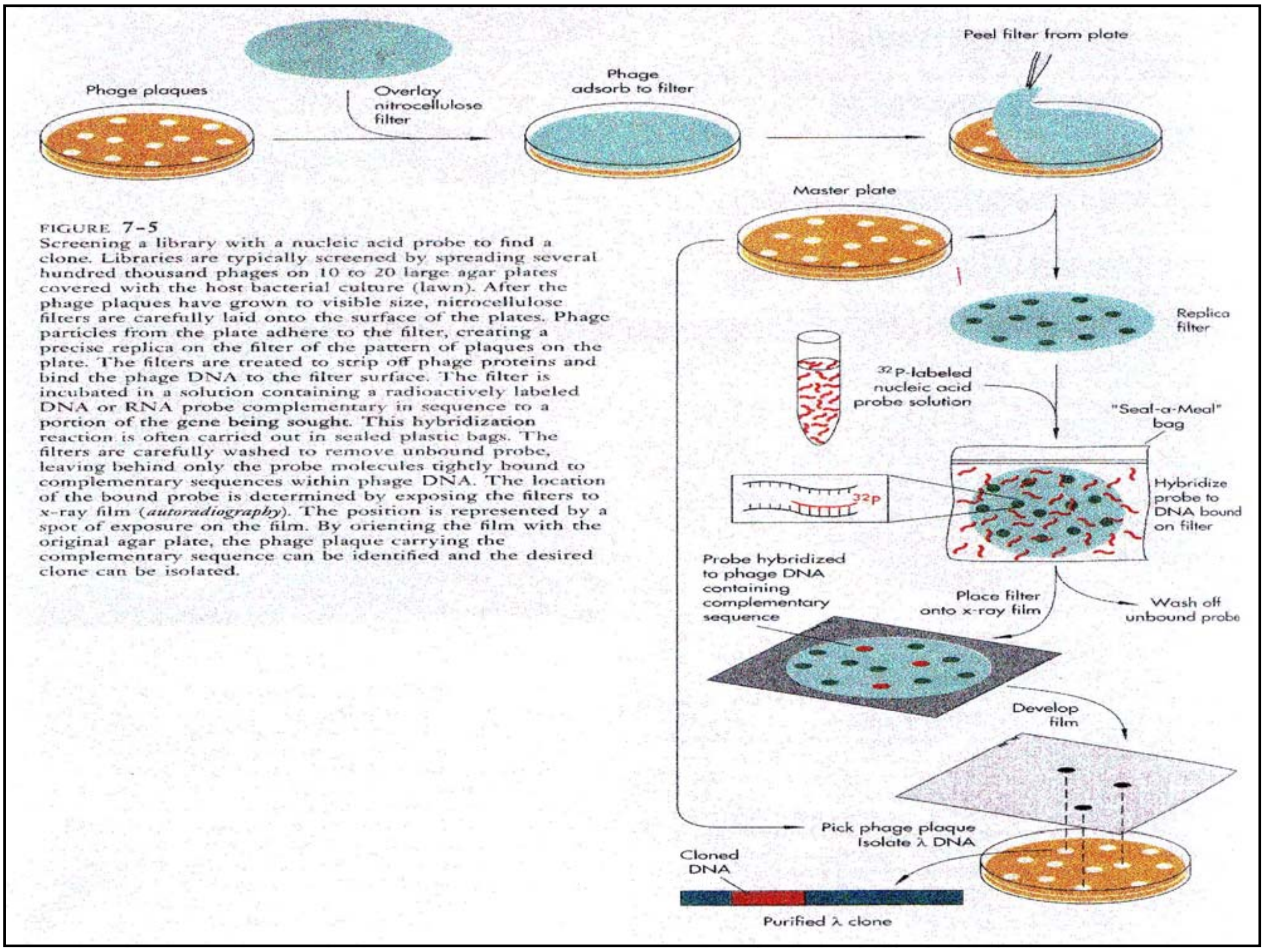
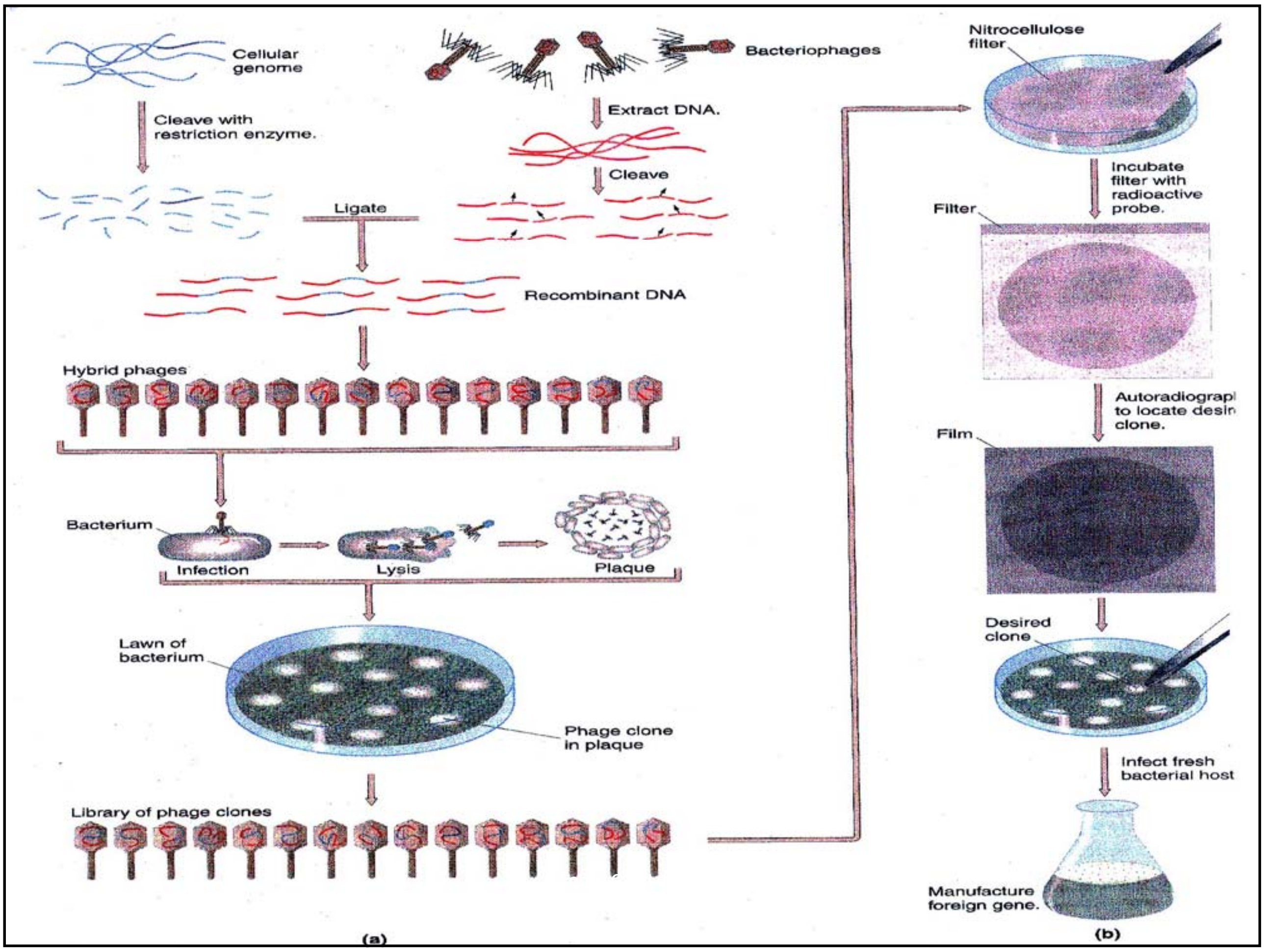

Peel filter from plate
Phage plaques
Overlay nitrocellulose filter
Phage adsorb to filter
Master plate
Replica filter
^{32}P-labeled nucleic acid probe solution
"Seal-a-Meal" bag
32P
Hybridize probe to DNA bound on filter
Probe hybridized to phage DNA containing complementary sequence
Place filter onto x-ray film
Wash off unbound probe
Develop film
Pick phage plaque Isolate λ DNA
Cloned DNA
Purified λ clone

FIGURE 7–5
Screening a library with a nucleic acid probe to find a clone. Libraries are typically screened by spreading several hundred thousand phages on 10 to 20 large agar plates covered with the host bacterial culture (lawn). After the phage plaques have grown to visible size, nitrocellulose filters are carefully laid onto the surface of the plates. Phage particles from the plate adhere to the filter, creating a precise replica on the filter of the pattern of plaques on the plate. The filters are treated to strip off phage proteins and bind the phage DNA to the filter surface. The filter is incubated in a solution containing a radioactively labeled DNA or RNA probe complementary in sequence to a portion of the gene being sought. This hybridization reaction is often carried out in sealed plastic bags. The filters are carefully washed to remove unbound probe, leaving behind only the probe molecules tightly bound to complementary sequences within phage DNA. The location of the bound probe is determined by exposing the filters to x-ray film (*autoradiography*). The position is represented by a spot of exposure on the film. By orienting the film with the original agar plate, the phage plaque carrying the complementary sequence can be identified and the desired clone can be isolated.


Cellular genome
Cleave with restriction enzyme.
Bacteriophages
Extract DNA.
Cleave
Ligate
Recombinant DNA
Hybrid phages
Bacterium
Infection
Lysis
Plaque
Lawn of bacterium
Phage clone in plaque
Library of phage clones
(a)
Nitrocellulose filter
Incubate filter with radioactive probe.
Filter
Autoradiograp
to locate desir
clone.
Film
Desired clone
Infect fresh bacterial host
Manufacture foreign gene.
(b)

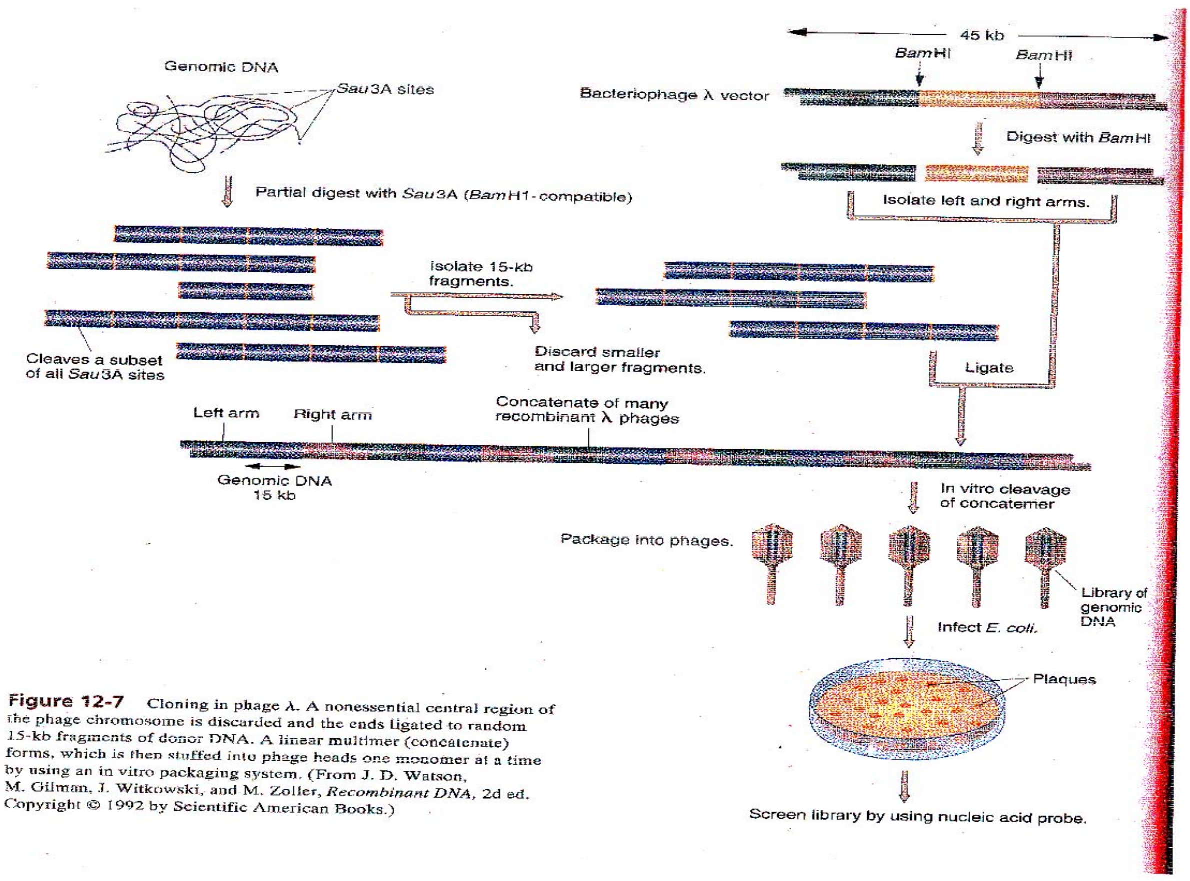
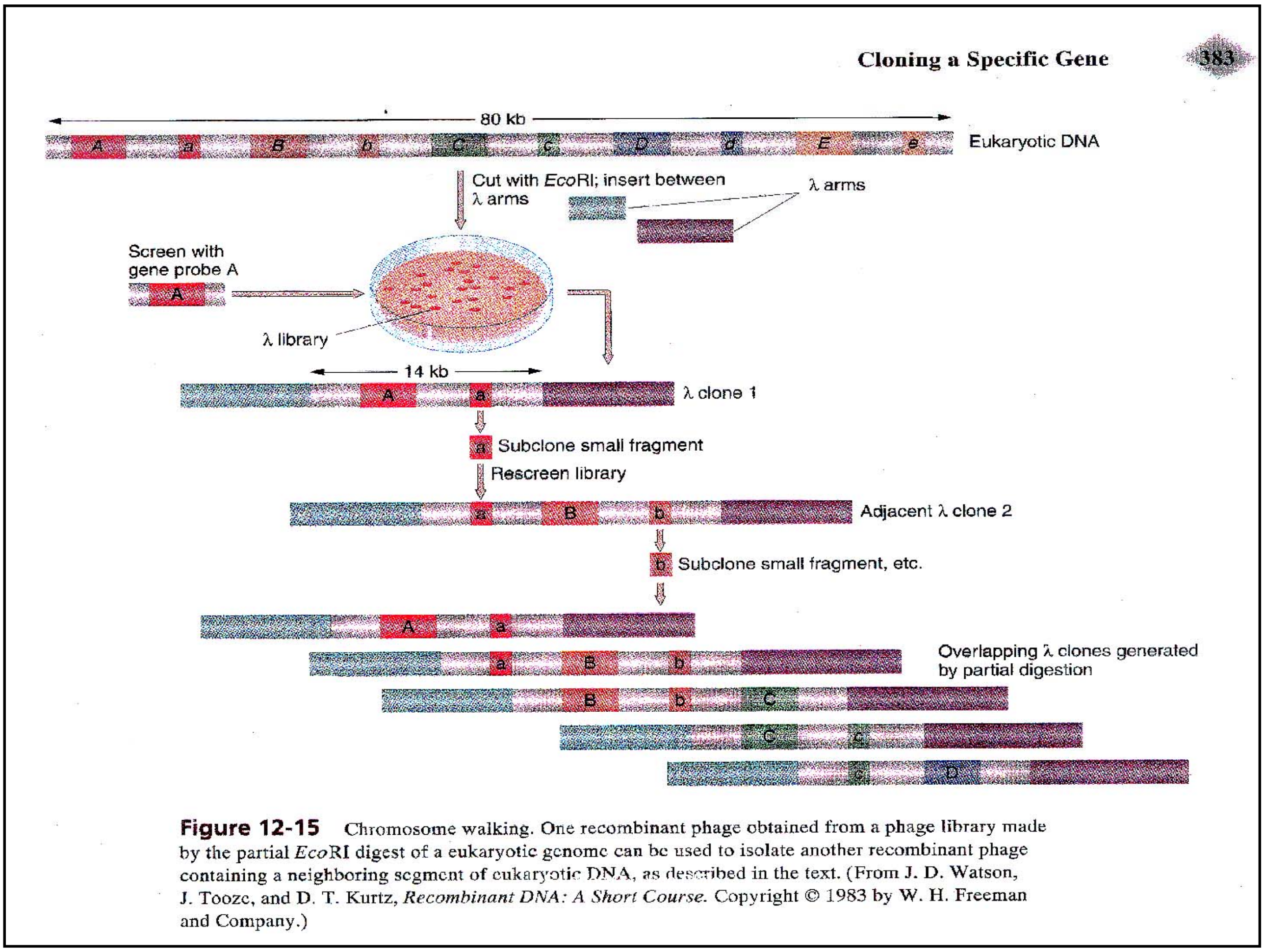

45 kb
BamHI
BamHI
Genomic DNA
Sau3A sites
Bacteriophage λ vector
Digest with BamHI
Partial digest with Sau3A (BamH1-compatible)
Isolate left and right arms.
Isolate 15-kb fragments.
Discard smaller and larger fragments.
Cleaves a subset of all Sau3A sites
Ligate
Left arm
Right arm
Concatenate of many recombinant λ phages
Genomic DNA 15 kb
In vitro cleavage of concatemer
Package into phages.
Library of genomic DNA
Infect E. coli.
Plaques
Screen library by using nucleic acid probe.

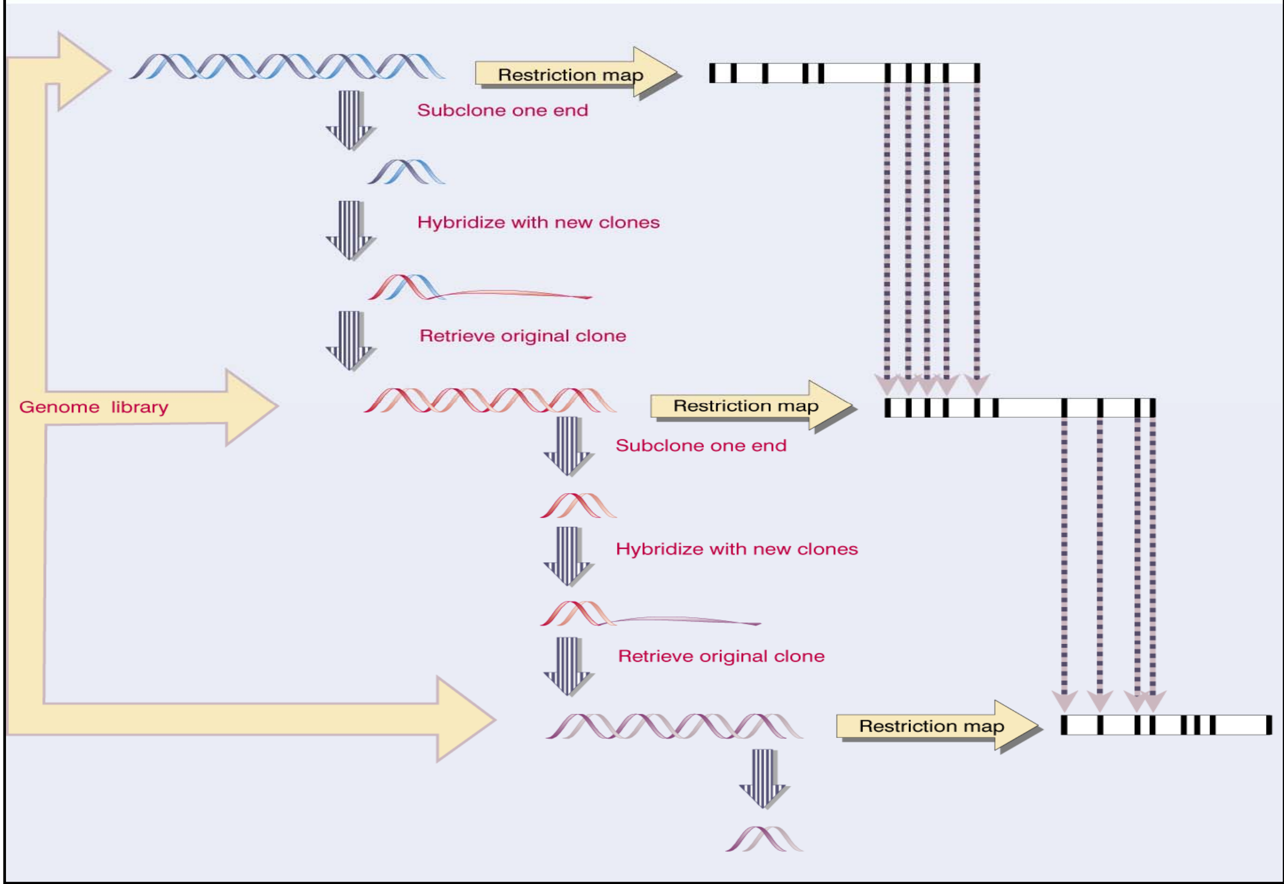
Figure 12-7 Cloning in phage λ. A nonessential central region of the phage chromosome is discarded and the ends ligated to random 15-kb fragments of donor DNA. A linear multimer (concatenate) forms, which is then stuffed into phage heads one monomer at a time by using an in vitro packaging system. (From J. D. Watson, M. Gilman, J. Witkowski, and M. Zoller, *Recombinant DNA*, 2d ed. Copyright © 1992 by Scientific American Books.)



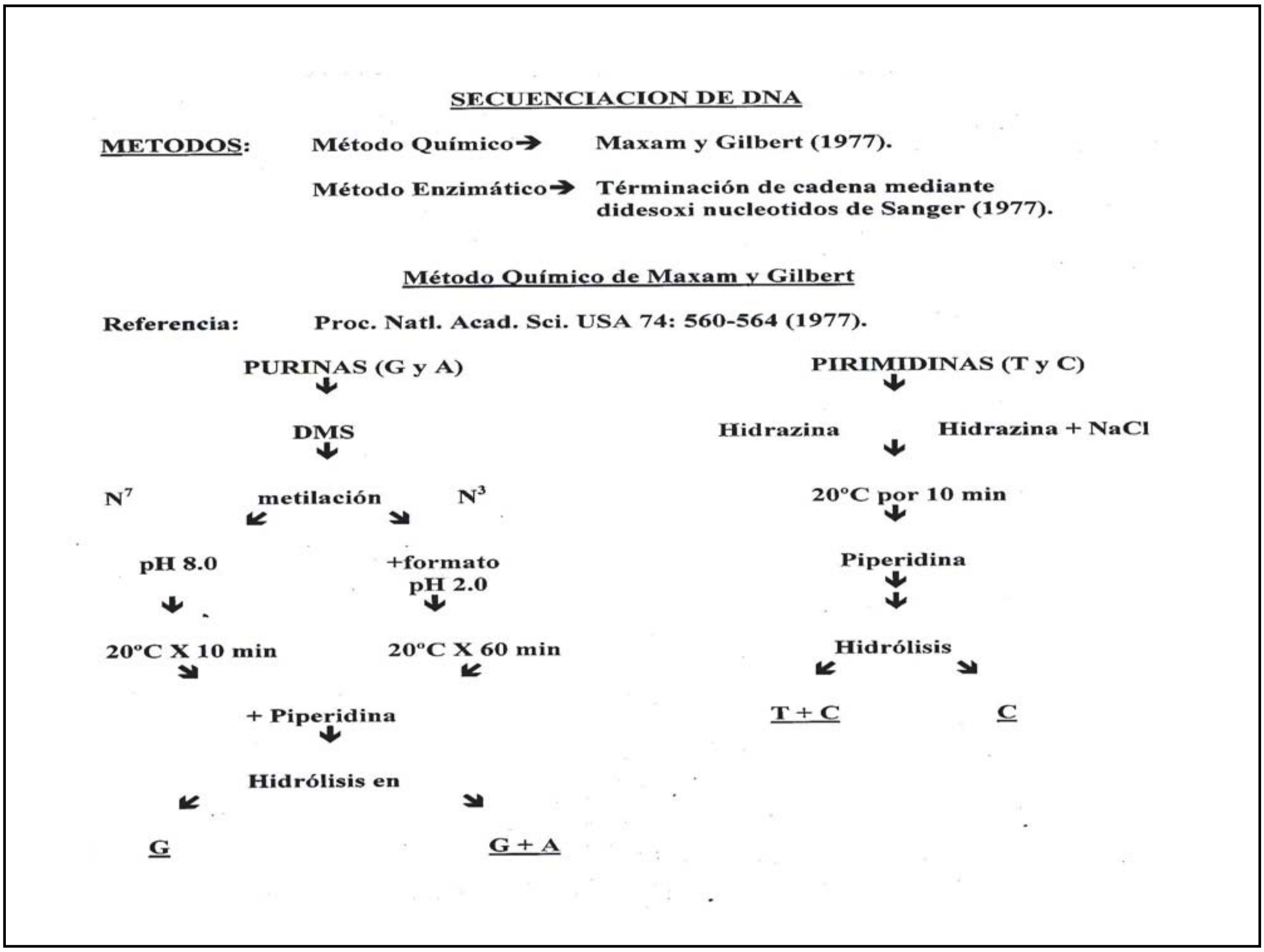
80 kb
A
a
B
b
C
c
D
d
E
e
Eukaryotic DNA
Cut with *Eco*RI; insert between λ arms
λ arms
Screen with gene probe A
A
λ library
14 kb
A
a
λ clone 1
a
Subclone small fragment
Rescreen library
a
B
b
Adjacent λ clone 2
b
Subclone small fragment, etc.
A
a
a
B
b
Overlapping λ clones generated by partial digestion
B
b
C
C
c
c
D

Figure 12-15 Chromosome walking. One recombinant phage obtained from a phage library made by the partial *Eco*RI digest of a eukaryotic genome can be used to isolate another recombinant phage containing a neighboring segment of eukaryotic DNA, as described in the text. (From J. D. Watson, J. Tooze, and D. T. Kurtz, *Recombinant DNA: A Short Course.* Copyright © 1983 by W. H. Freeman and Company.)

Figure 2.16 Chromosome walking is accomplished by successive hybridizations between overlapping genomic clones.


Restriction map
Subclone one end
Hybridize with new clones
Retrieve original clone
Genome library
Restriction map
Subclone one end
Hybridize with new clones
Retrieve original clone
Restriction map

SECUENCIACIÓN
Y
MUTAGÉNESIS DE DNA



SECUENCIACION DE DNA

METODOS: Método Químico→ Maxam y Gilbert (1977).

Método Enzimático→ Términación de cadena mediante didesoxi nucleotidos de Sanger (1977).

Método Químico de Maxam y Gilbert

Referencia: Proc. Natl. Acad. Sci. USA 74: 560-564 (1977).

PURINAS (G y A)

DMS

N^7 metilación N^3

pH 8.0

+formato
pH 2.0

20°C X 10 min

20°C X 60 min

+ Piperidina

Hidrólisis en

G

G + A

PIRIMIDINAS (T y C)

Hidrazina

Hidrazina + NaCl

20°C por 10 min

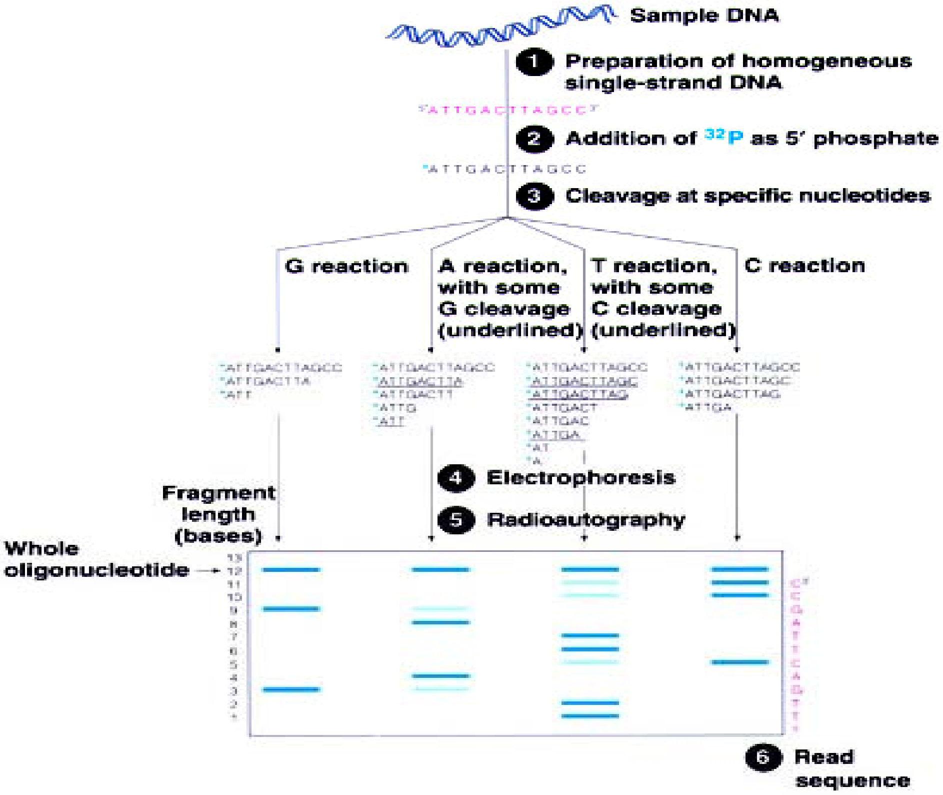
Piperidina

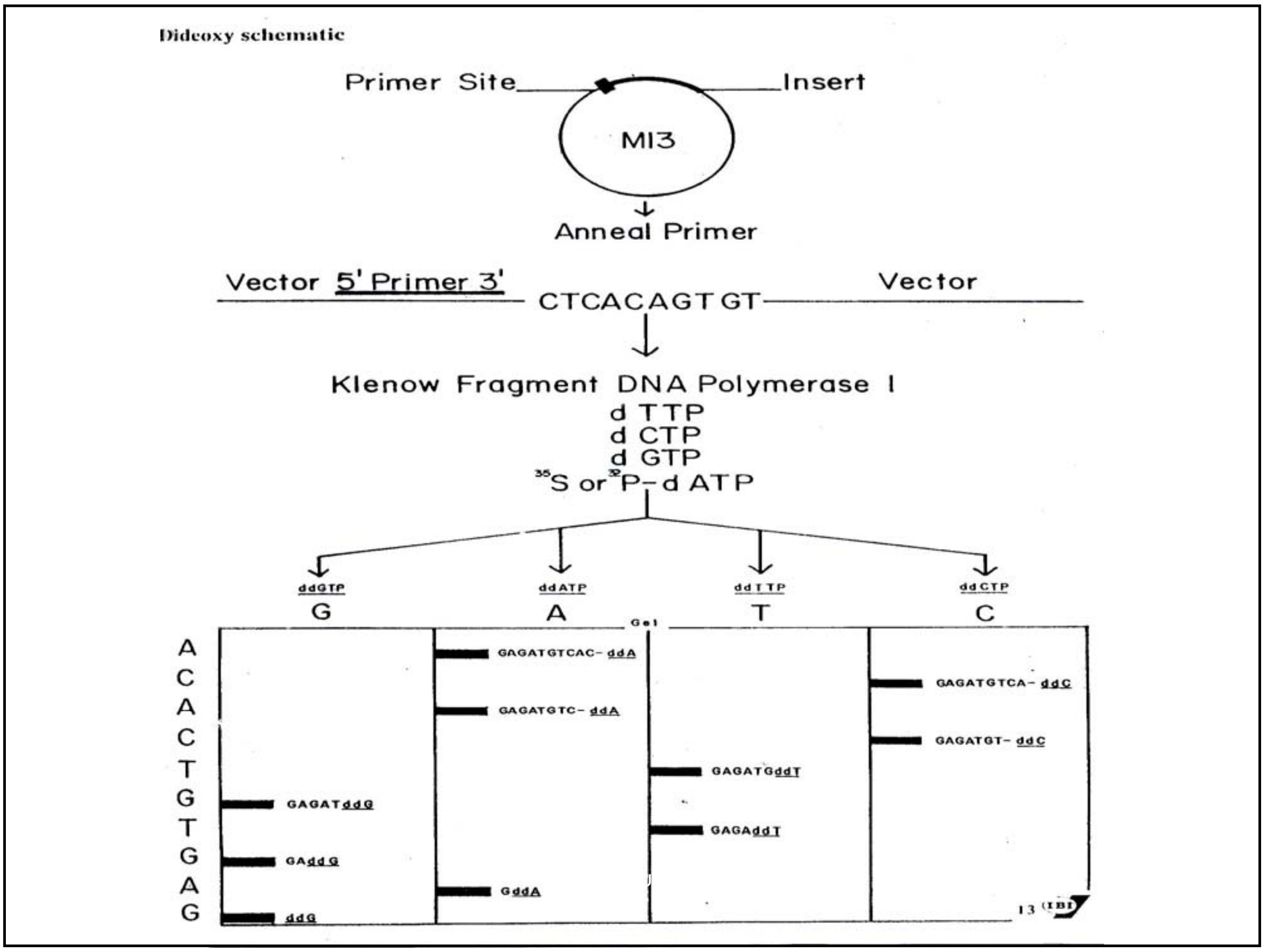
Hidrólisis

T + C

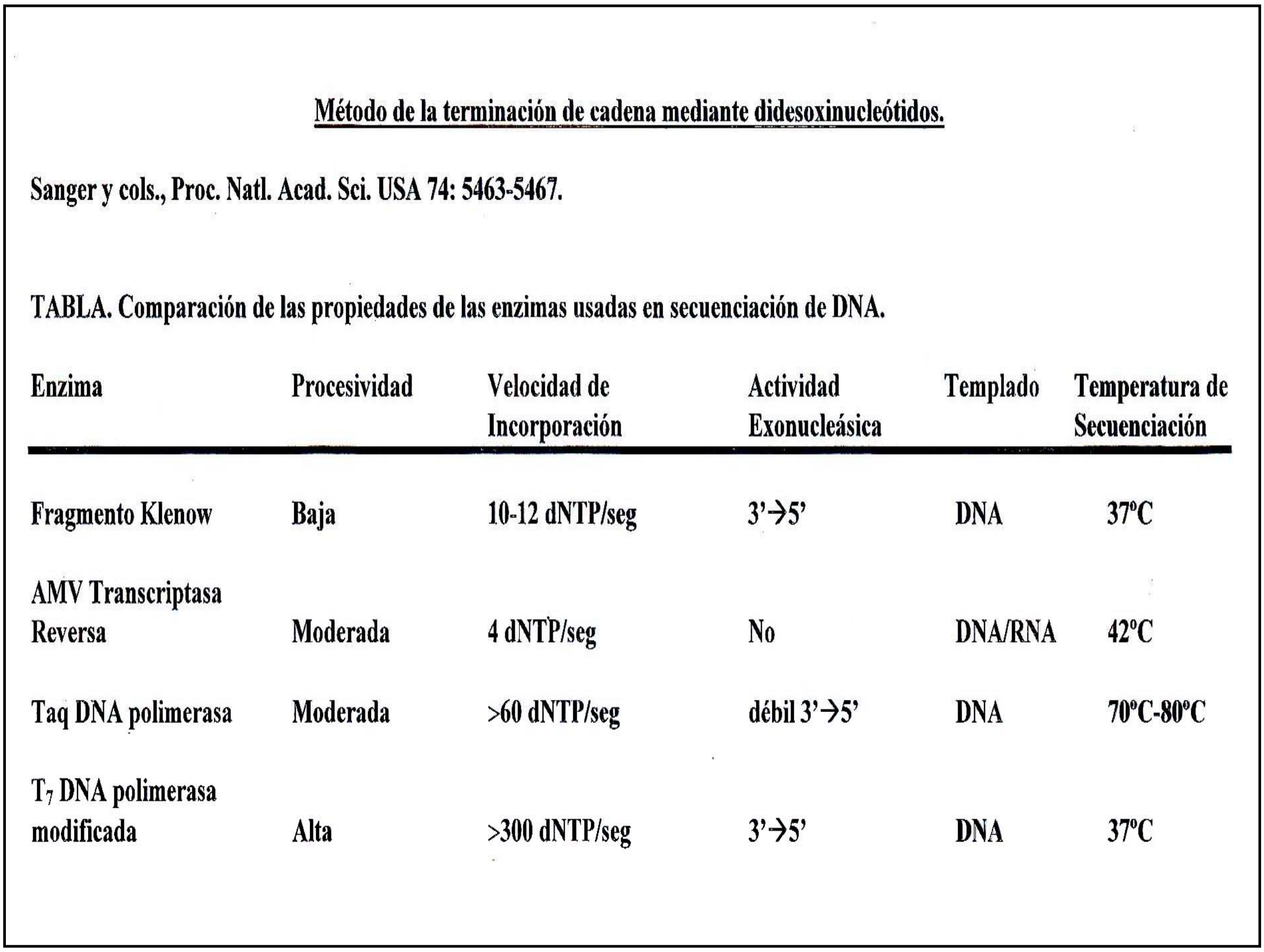
C

Figure 4A.4 Sequencing an oligonucleotide by the Maxam-Gilbert method


Sample DNA
1 Preparation of homogeneous single-strand DNA
5'ATTGACTTAGCC3'
2 Addition of 32P as 5′ phosphate
*ATTGACTTAGCC
3 Cleavage at specific nucleotides
G reaction
A reaction, with some G cleavage (underlined)
T reaction, with some C cleavage (underlined)
C reaction
*ATTGACTTAGCC
*ATTGACTTA
*ATT
*ATTGACTTAGCC
*ATTGACTTA
*ATTGACTT
*ATTG
*ATT
*ATTGACTTAGCC
*ATTGACTTAGC
*ATTGACTTAG
*ATTGACT
*ATTGAC
*ATTGA
*AT
*A
*ATTGACTTAGCC
*ATTGACTTAGC
*ATTGACTTAG
*ATTGA
4 Electrophoresis
5 Radioautography
Fragment length (bases)
Whole oligonucleotide
13
12
11
10
9
8
7
6
5
4
3
2
1
CCGATTCAGTTA
6 Read sequence


Dideoxy schematic

Primer Site
Insert
M13
Anneal Primer
Vector 5' Primer 3'
CTCACAGTGT
Vector
Klenow Fragment DNA Polymerase I
d TTP
d CTP
d GTP
35S or 32P-d ATP
ddGTP
G
ddATP
A
Gel
ddTTP
T
ddCTP
C
A
C
A
C
T
G
T
G
A
G
GAGATGTCAC- ddA
GAGATGTC- ddA
GddA
GAGATddG
GAddG
ddG
GAGATGddT
GAGAddT
GAGATGTCA- ddC
GAGATGT- ddC
13 (IBI)



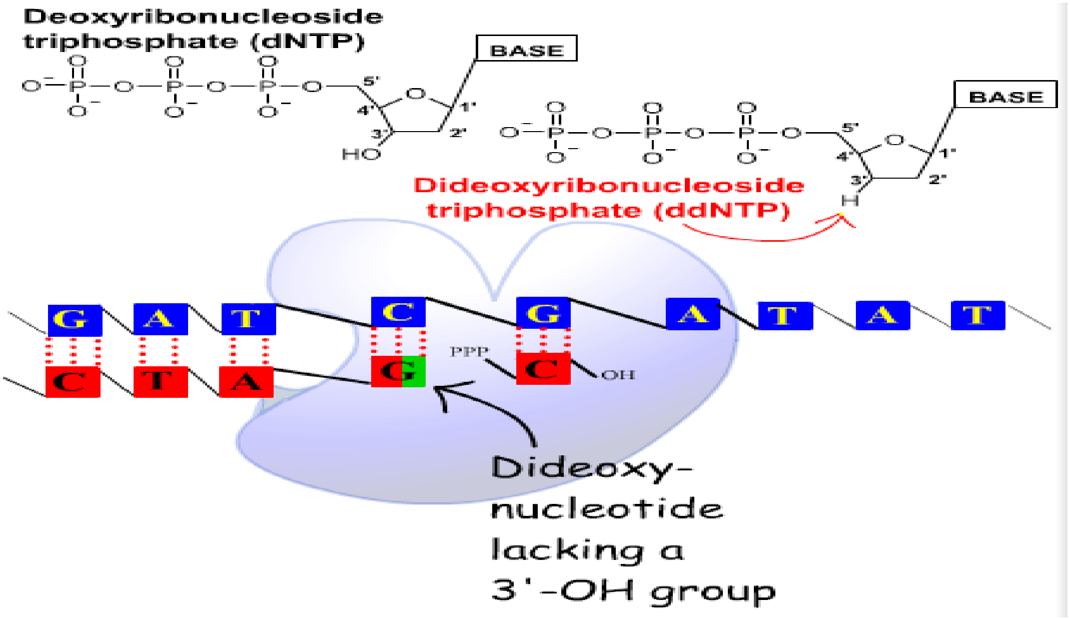
Método de la terminación de cadena mediante didesoxinucleótidos.

Sanger y cols., Proc. Natl. Acad. Sci. USA 74: 5463-5467.

TABLA. Comparación de las propiedades de las enzimas usadas en secuenciación de DNA.

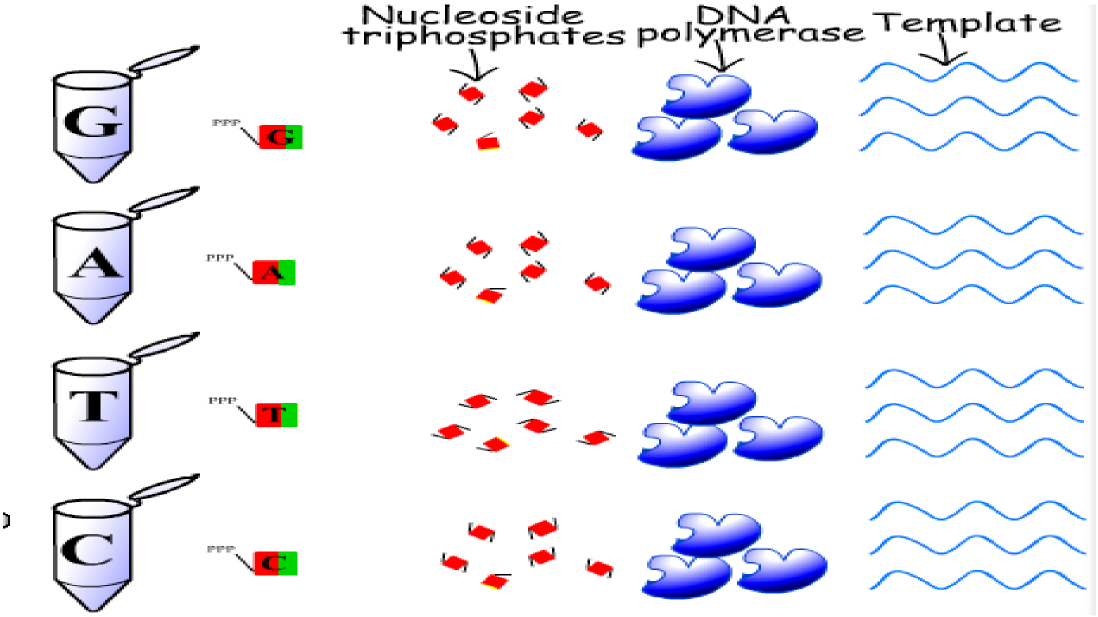
Enzima	Procesividad	Velocidad de Incorporación	Actividad Exonucleásica	Templado	Temperatura de Secuenciación
Fragmento Klenow	Baja	10-12 dNTP/seg	3'→5'	DNA	37ºC
AMV Transcriptasa Reversa	Moderada	4 dNTP/seg	No	DNA/RNA	42ºC
Taq DNA polimerasa	Moderada	>60 dNTP/seg	débil 3'→5'	DNA	70ºC-80ºC
T_7 DNA polimerasa modificada	Alta	>300 dNTP/seg	3'→5'	DNA	37ºC

Secuenciación de DNA


Deoxyribonucleoside triphosphate (dNTP)
BASE
5'
4'
3'
HO
1'
2'
BASE
Dideoxyribonucleoside triphosphate (ddNTP)
5'
4'
3'
H
1'
2'
G A T C G A T A T
C T A G C
PPP
OH
Dideoxy-
nucleotide
lacking a
3'-OH group

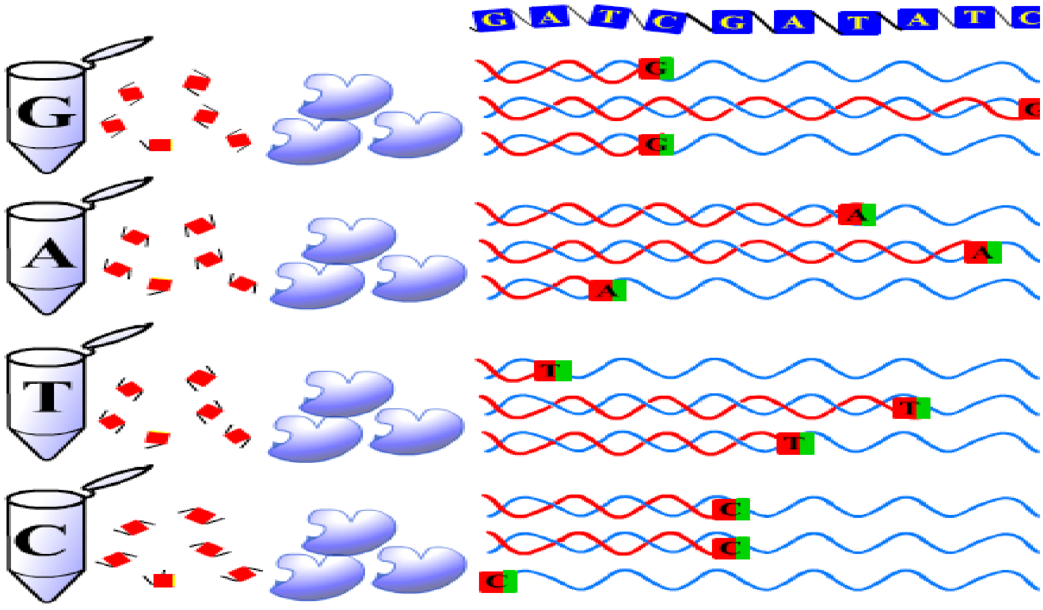
La adición de un dideoxinucleosido trifosfato, que no tiene el grupo hidroxilo 3', termina la síntesis de DNA

Secuenciación de DNA


Nucleoside triphosphates
DNA polymerase
Template
G
A
T
C
PPP
G
PPP
A
PPP
T
PPP
C

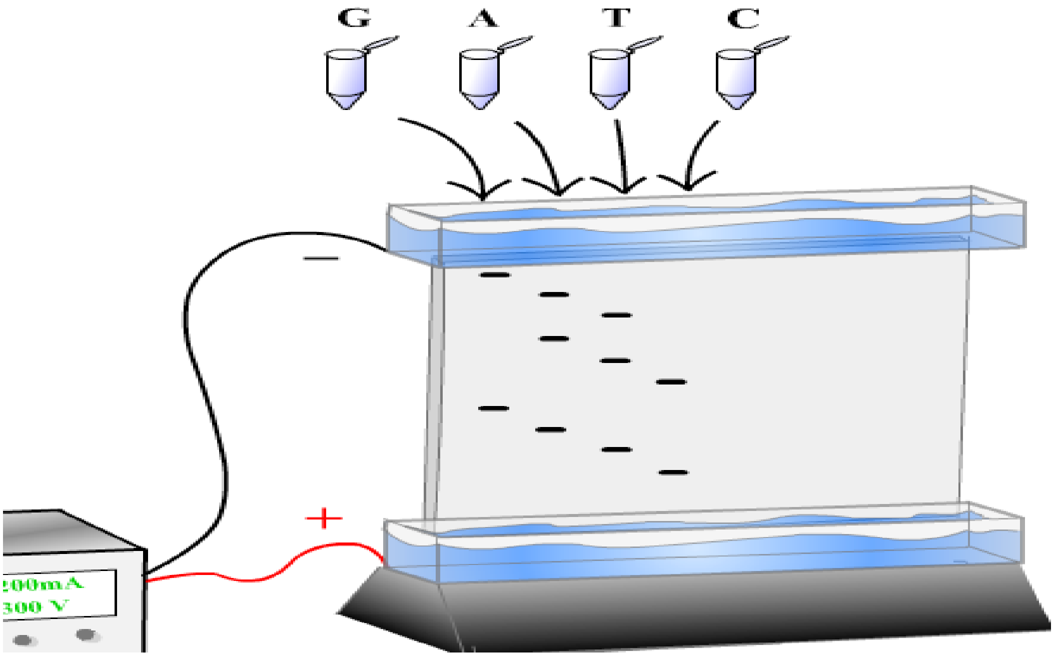
Se realizan 4 reacciones separadas - Cada una contiene DNA molde, polimerasa, los cuatro dNTPs, y uno de los cuatro dideoxi nucleotidos (ddNTP)

Secuenciación de DNA


G
A
T
C
G
A
T
C
G
A
T
A
T
C
G
G
C
G
A
A
A
T
T
T
C
C
C

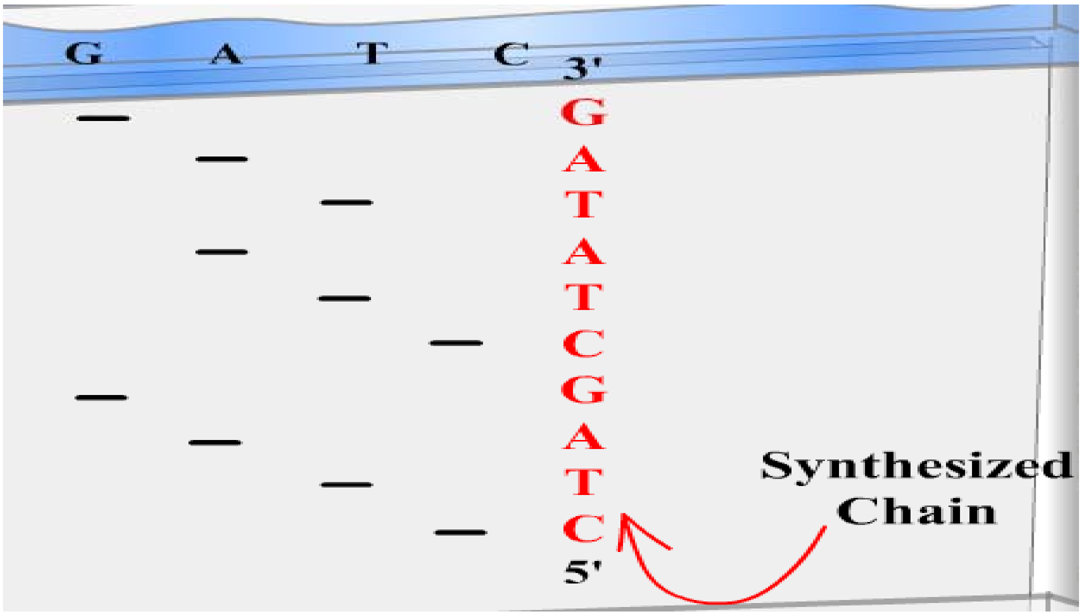
Ocasionalmente se incorpora un ddNTP y se detiene la extension. Cada reacción contendrá una colección de cadenas de DNA de todas las longitudes posibles que acaben en la base que lleva en ddNTP de esa reacción

Secuenciación de DNA


G
A
T
C
−
+
00mA
300 V

Las cadenas de DNA se separan mediante electroforesis según sus tamaños

Secuenciación de DNA



G
A
T
C
3'
G
A
T
A
T
C
G
A
T
C
5'
Synthesized Chain

La secuencia de DNA puede determinarse leyendo el gel para ver cual nucleótido termina la cadena en cada posición sucesiva

Método Sanger dideoxy

Requerimientos

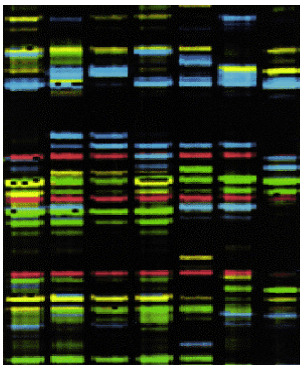
- DNA
  - calidad
  - cantidad
- Primer
- Enzima
  - Capacidad de incorporar nucleótidos modificados
  - Estabilidad térmica
  - procesividad
- Nucleótidos
  - marcaje
  - ratios
- Buffer

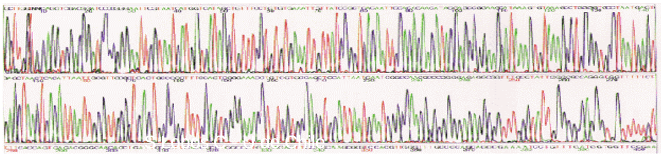
Cada ddNTP está marcado con un fluoroforo distinto

ddATP
ddCTP
ddGTP
ddTTP

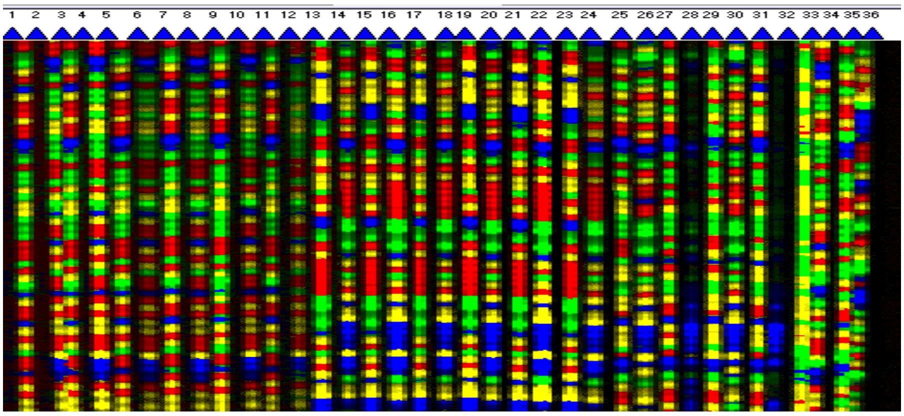
Analizadores automáticos de fragmentos de DNA (PE-ABD/Pharmacia)

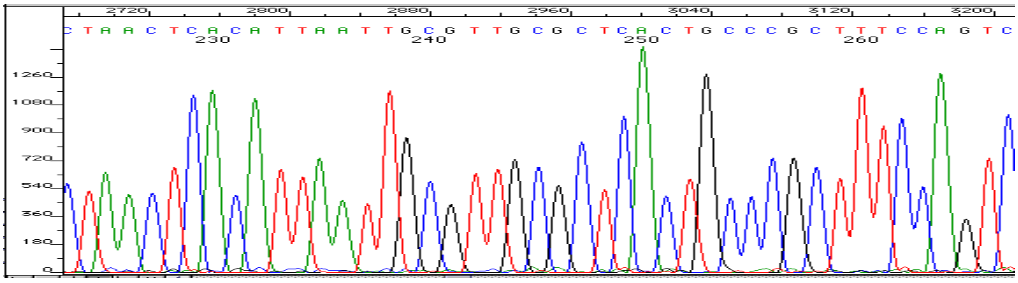
- Electroforesis gel/capilar
- Excitación por laser de fluorocromos
- Detección CCD cámara
- Colección automática de datos e identificación de alelos





Gel y cromatograma

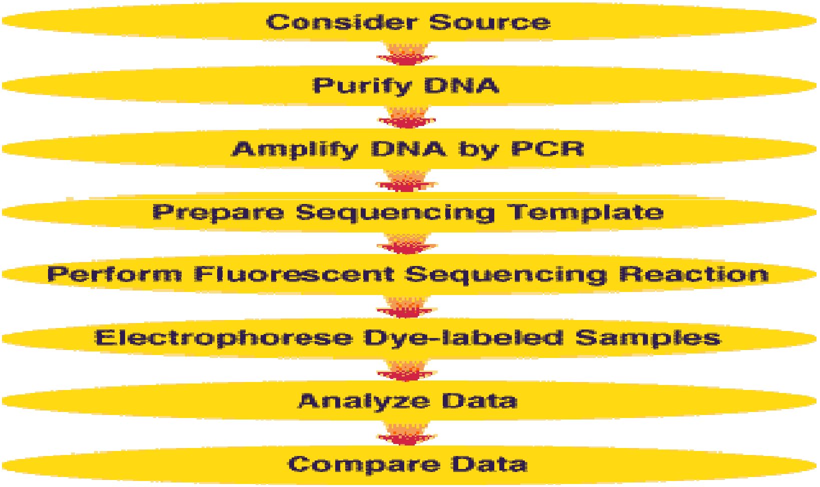

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36



Electroferograma

Secuenciación Automática de DNA

Is really not-so automated!



Consider Source

Purify DNA

Amplify DNA by PCR

Prepare Sequencing Template

Perform Fluorescent Sequencing Reaction

Electrophorese Dye-labeled Samples

Analyze Data

Compare Data

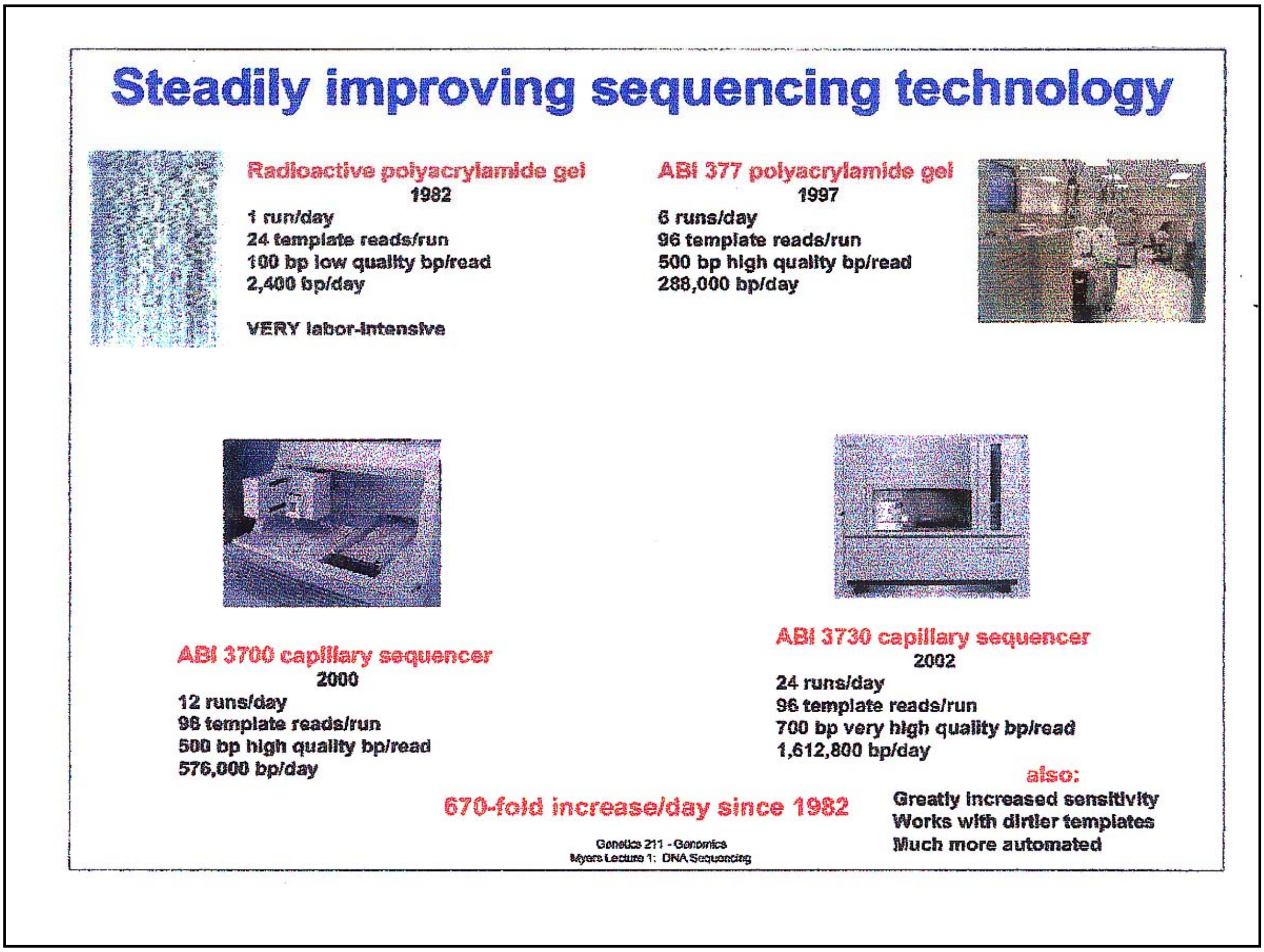
Nuevas tecnologías de secuenciación de DNA

- Mejora de la separación de los productos de reacción
  - Electroforesis capilar
    - Maquinas de 96 capilares (ABI 3700 y Molecular Dynamics MegaBace) disponibles
    - Más de 1000 columnas capilares por run con detección por microscopía confocal
- Mass-spectrometry
  - especialmente MALDI (matrix-assisted laser desorption /ionization)
- Single molecule DNA-sequencing
  - flow-cytometry

Nuevas tecnologías de secuenciación de DNA

- Mejora de reacciones y estrategias de secuenciación
  - Incremento de la procesividad del enzima
  - Mejora de fluorocromos

- **Secuenciación por hibridación a paneles de oligonucleótidos de secuencia conocida**



Steadily improving sequencing technology

Radioactive polyacrylamide gel
1982
1 run/day
24 template reads/run
100 bp low quality bp/read
2,400 bp/day

VERY labor-intensive

ABI 377 polyacrylamide gel
1997
6 runs/day
96 template reads/run
500 bp high quality bp/read
288,000 bp/day

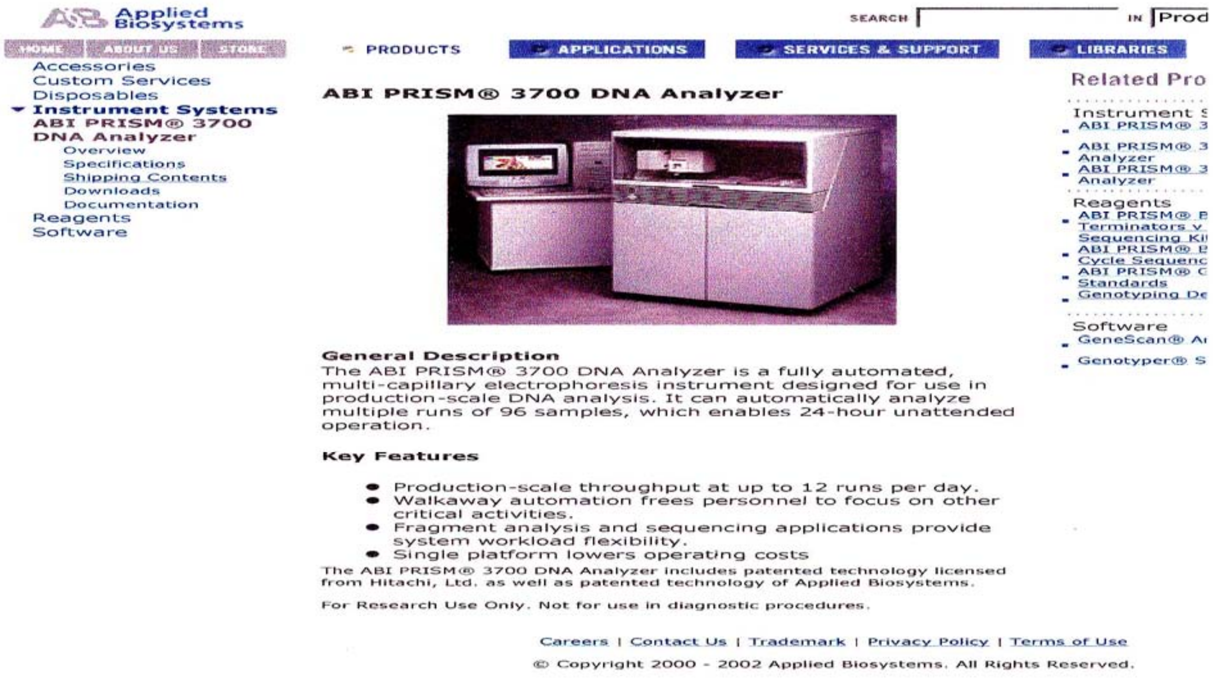
ABI 3700 capillary sequencer
2000
12 runs/day
96 template reads/run
500 bp high quality bp/read
576,000 bp/day

ABI 3730 capillary sequencer
2002
24 runs/day
96 template reads/run
700 bp very high quality bp/read
1,612,800 bp/day

670-fold increase/day since 1982

also:
Greatly increased sensitivity
Works with dirtier templates
Much more automated

Genetics 211 - Genomics
Myers Lecture 1: DNA Sequencing


Applied Biosystems
SEARCH
IN Prod
HOME
ABOUT US
STORE
PRODUCTS
APPLICATIONS
SERVICES & SUPPORT
LIBRARIES
Accessories
Custom Services
Disposables
Instrument Systems
ABI PRISM® 3700 DNA Analyzer
Overview
Specifications
Shipping Contents
Downloads
Documentation
Reagents
Software
Related Pro
Instrument S
ABI PRISM® 3
ABI PRISM® 3 Analyzer
ABI PRISM® 3 Analyzer
Reagents
ABI PRISM® B Terminators v Sequencing Ki
ABI PRISM® B Cycle Sequenc
ABI PRISM® G Standards
Genotyping De
Software
GeneScan® A
Genotyper® S
Careers | Contact Us | Trademark | Privacy Policy | Terms of Use

ABI PRISM® 3700 DNA Analyzer

General Description

The ABI PRISM® 3700 DNA Analyzer is a fully automated, multi-capillary electrophoresis instrument designed for use in production-scale DNA analysis. It can automatically analyze multiple runs of 96 samples, which enables 24-hour unattended operation.

Key Features

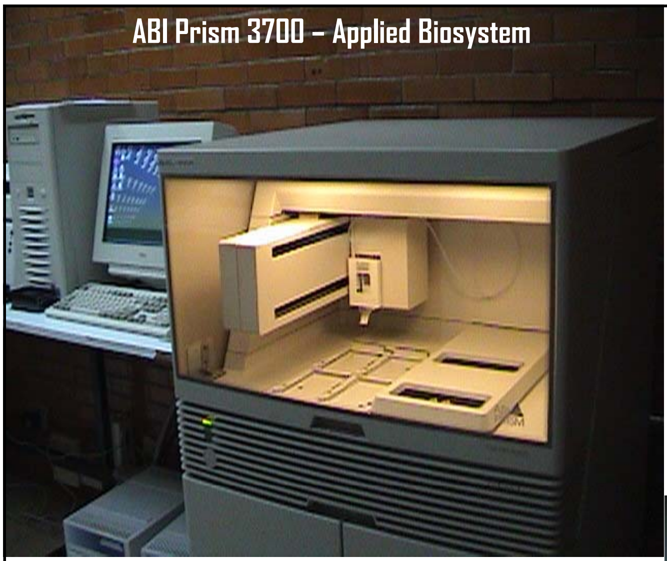
- Production-scale throughput at up to 12 runs per day.
- Walkaway automation frees personnel to focus on other critical activities.
- Fragment analysis and sequencing applications provide system workload flexibility.
- Single platform lowers operating costs

The ABI PRISM® 3700 DNA Analyzer includes patented technology licensed from Hitachi, Ltd. as well as patented technology of Applied Biosystems.

For Research Use Only. Not for use in diagnostic procedures.

© Copyright 2000 - 2002 Applied Biosystems. All Rights Reserved.

Automated DNA-sequencing instruments (DNA sequencers) can sequence up to 384 DNA samples in a single batch (run) in up to 24 runs a day



ABI Prism 3700 – Applied Biosystem

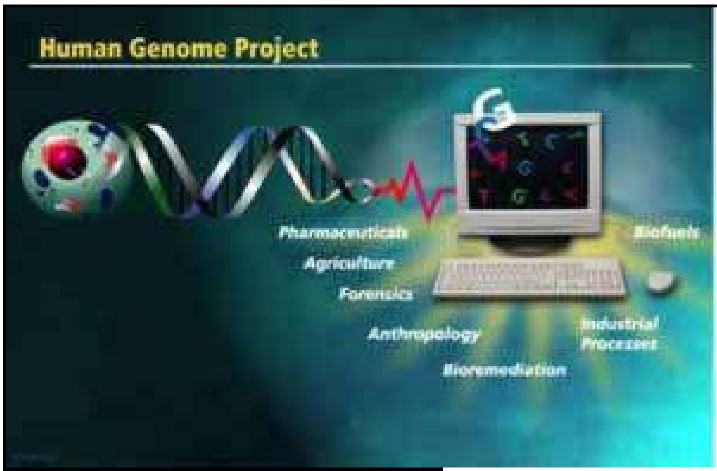
eppendorf
vapo.protect
eppendorf
Mastercycler
pro S

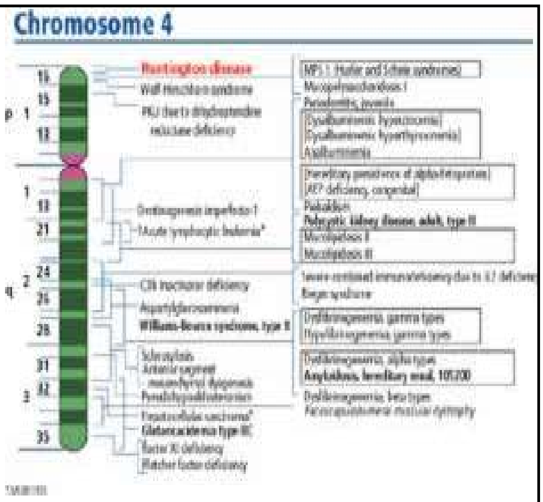
Thermo cycler Master cycler eppendorf

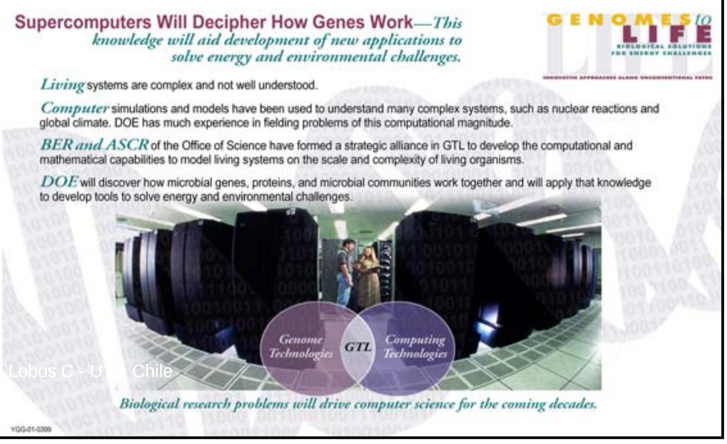
DNA Thermal Cycler Perkin Elmer

BECKMAN
Oligo 1000

Oligo 1000 - Beckman


Human Genome Project
Pharmaceuticals
Agriculture
Forensics
Anthropology
Bioremediation
Industrial Processes
Biofuels


Chromosome 4
p
1
16
15
13
q
1
13
21
2
24
26
28
3
31
32
35
Huntington disease
Wolf-Hirschhorn syndrome
PKU due to dihydropteridine reductase deficiency
Dentinogenesis imperfecta 1
Acute lymphocytic leukemia
C3b inactivator deficiency
Aspartylglucosaminuria
Williams-Beuren syndrome, type II
Sclerotylosis
Anterior segment mesenchymal dysgenesis
Pseudohypoaldosteronism
Hepatocellular carcinoma
Glutaricacidemia type IIC
Factor XI deficiency
Fletcher factor deficiency
MPS 1 Hurler and Scheie syndromes
Mucopolysaccharidosis I
Periodontitis, juvenile
Dysalbuminemic hyperzincemia
Dysalbuminemic hyperthyroxinemia
Analbuminemia
Hereditary persistence of alpha-fetoprotein
AFP deficiency, congenital
Piebaldism
Polycystic kidney disease, adult, type II
Mucolipidosis II
Mucolipidosis III
Severe combined immunodeficiency due to IL7 deficiency
Rieger syndrome
Dysfibrinogenemia, gamma types
Hypofibrinogenemia, gamma types
Dysfibrinogenemia, alpha types
Amyloidosis, hereditary renal, 105200
Dysfibrinogenemia, beta types
Facioscapulohumeral muscular dystrophy



Supercomputers Will Decipher How Genes Work—*This knowledge will aid development of new applications to solve energy and environmental challenges.*

Living systems are complex and not well understood.

Computer simulations and models have been used to understand many complex systems, such as nuclear reactions and global climate. DOE has much experience in fielding problems of this computational magnitude.

BER and ASCR of the Office of Science have formed a strategic alliance in GTL to develop the computational and mathematical capabilities to model living systems on the scale and complexity of living organisms.

DOE will discover how microbial genes, proteins, and microbial communities work together and will apply that knowledge to develop tools to solve energy and environmental challenges.

GENOMES to LIFE
BIOLOGICAL SOLUTIONS FOR ENERGY CHALLENGES
Genome Technologies
GTL
Computing Technologies

Biological research problems will drive computer science for the coming decades.

Lobos C - U Chile

Tipos de secuencias del DNA eucarionte

Secuencias de copia única
Encontradas una vez en el genoma

DNA medianamente repetitivo
Encontrado de 10 a 1000 veces en el genoma
Puede variar de 100-300 bp a 5000 bp y puede estar disperso en el genoma.

DNA altamente repetitivo
Secuencias más abundantes
Encontradas de 100,000 a 1 millón de veces en el genoma
Puede variar de unos pocos a varios cientos de bases en longitud. Tiende a ordenarse como repeticiones en tandem.

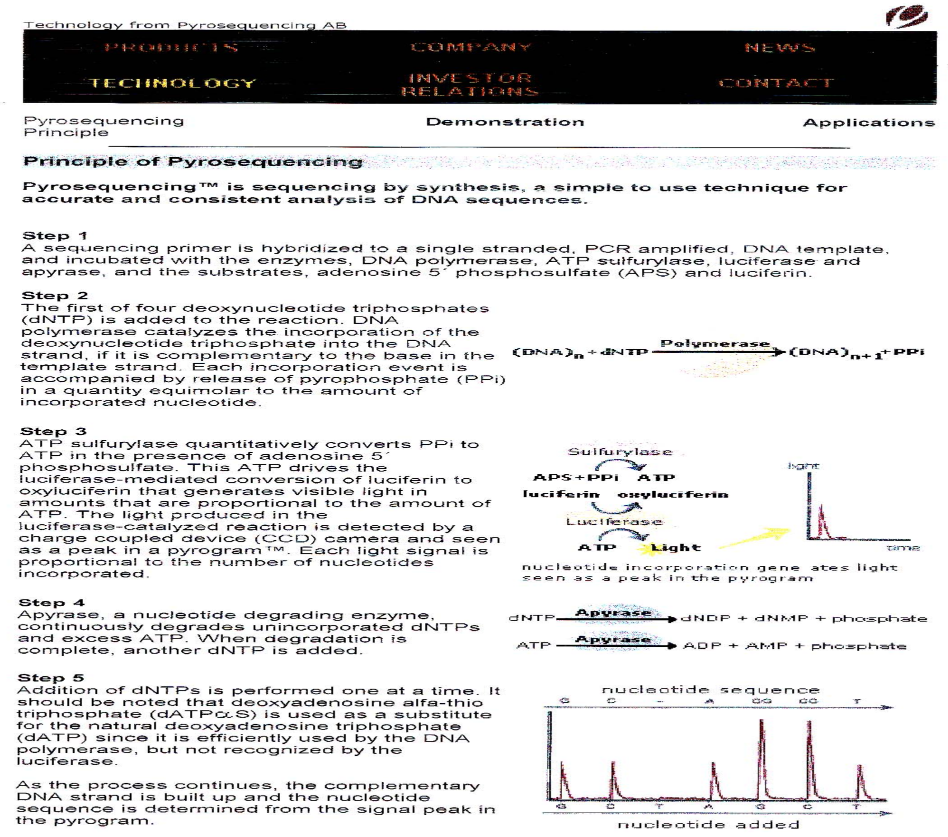
Tarea a desarrollar:

¿Cuántos picogramos de DNA contiene una célula bacteriana?

¿Cuántos picogramos de DNA contiene una célula humana?

¿Cuáles son sus longitudes respectivamente?

Pyrosequencing


Technology from Pyrosequencing AB
PRODUCTS
COMPANY
NEWS
TECHNOLOGY
INVESTOR RELATIONS
CONTACT
Pyrosequencing Principle
Demonstration
Applications

Principle of Pyrosequencing

Pyrosequencing™ is sequencing by synthesis, a simple to use technique for accurate and consistent analysis of DNA sequences.

Step 1
A sequencing primer is hybridized to a single stranded, PCR amplified, DNA template, and incubated with the enzymes, DNA polymerase, ATP sulfurylase, luciferase and apyrase, and the substrates, adenosine 5´ phosphosulfate (APS) and luciferin.

Step 2
The first of four deoxynucleotide triphosphates (dNTP) is added to the reaction. DNA polymerase catalyzes the incorporation of the deoxynucleotide triphosphate into the DNA strand, if it is complementary to the base in the template strand. Each incorporation event is accompanied by release of pyrophosphate (PPi) in a quantity equimolar to the amount of incorporated nucleotide.

$$(DNA)_n + dNTP \xrightarrow{\text{Polymerase}} (DNA)_{n+1} + PPi$$

Step 3
ATP sulfurylase quantitatively converts PPi to ATP in the presence of adenosine 5´ phosphosulfate. This ATP drives the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP. The light produced in the luciferase-catalyzed reaction is detected by a charge coupled device (CCD) camera and seen as a peak in a pyrogram™. Each light signal is proportional to the number of nucleotides incorporated.

Sulfurylase
APS+PPi
ATP
luciferin
oxyluciferin
Luciferase
ATP
Light
light
time

nucleotide incorporation gene ates light seen as a peak in the pyrogram

Step 4
Apyrase, a nucleotide degrading enzyme, continuously degrades unincorporated dNTPs and excess ATP. When degradation is complete, another dNTP is added.

$$dNTP \xrightarrow{\text{Apyrase}} dNDP + dNMP + \text{phosphate}$$

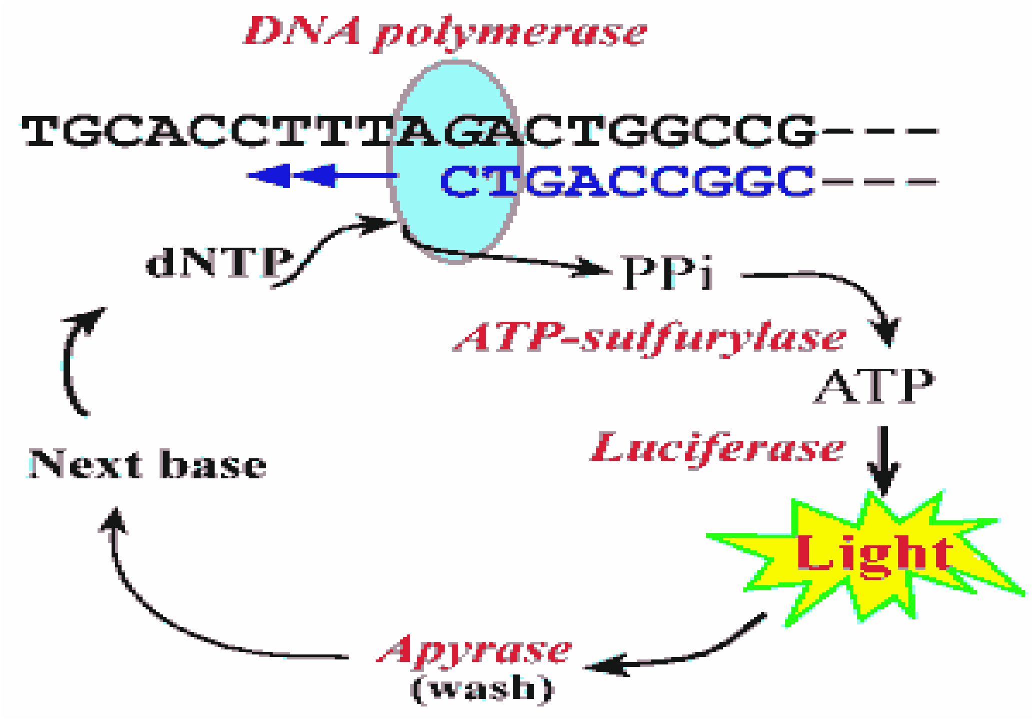
$$ATP \xrightarrow{\text{Apyrase}} ADP + AMP + \text{phosphate}$$

Step 5
Addition of dNTPs is performed one at a time. It should be noted that deoxyadenosine alfa-thio triphosphate (dATPαS) is used as a substitute for the natural deoxyadenosine triphosphate (dATP) since it is efficiently used by the DNA polymerase, but not recognized by the luciferase.

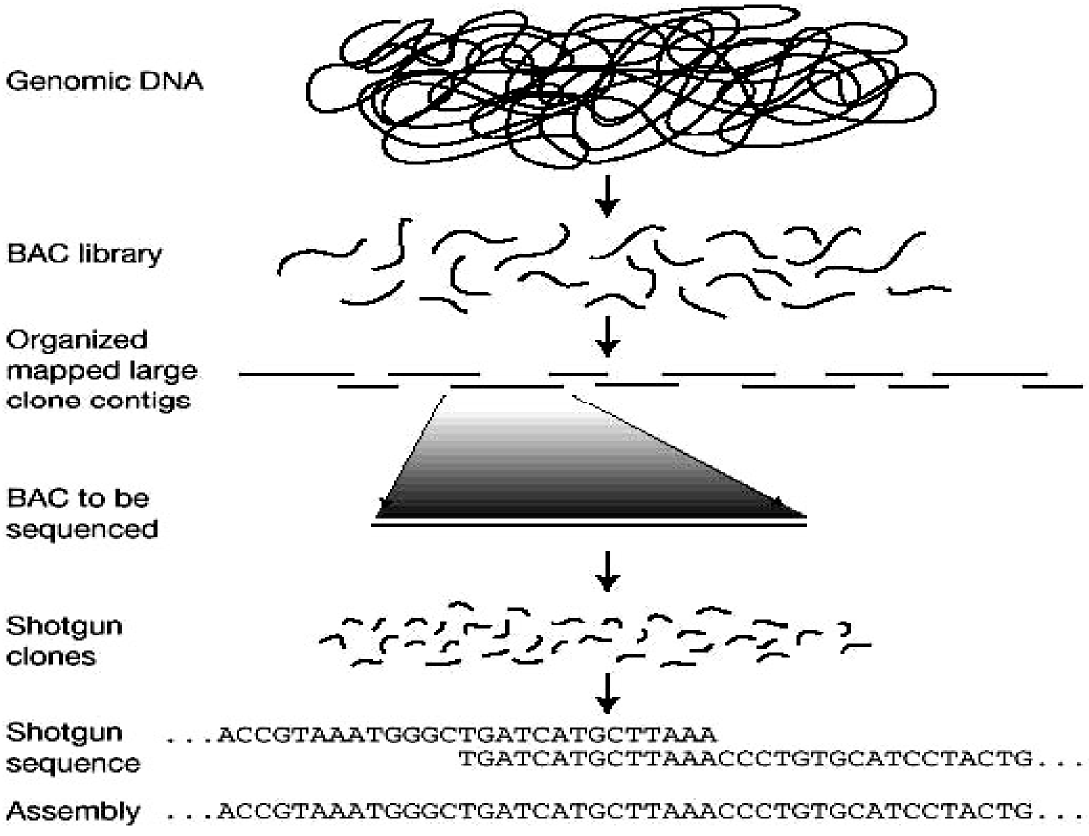
As the process continues, the complementary DNA strand is built up and the nucleotide sequence is determined from the signal peak in the pyrogram.

nucleotide sequence
G C – A GG CC T
G C T A G C T
nucleotide added

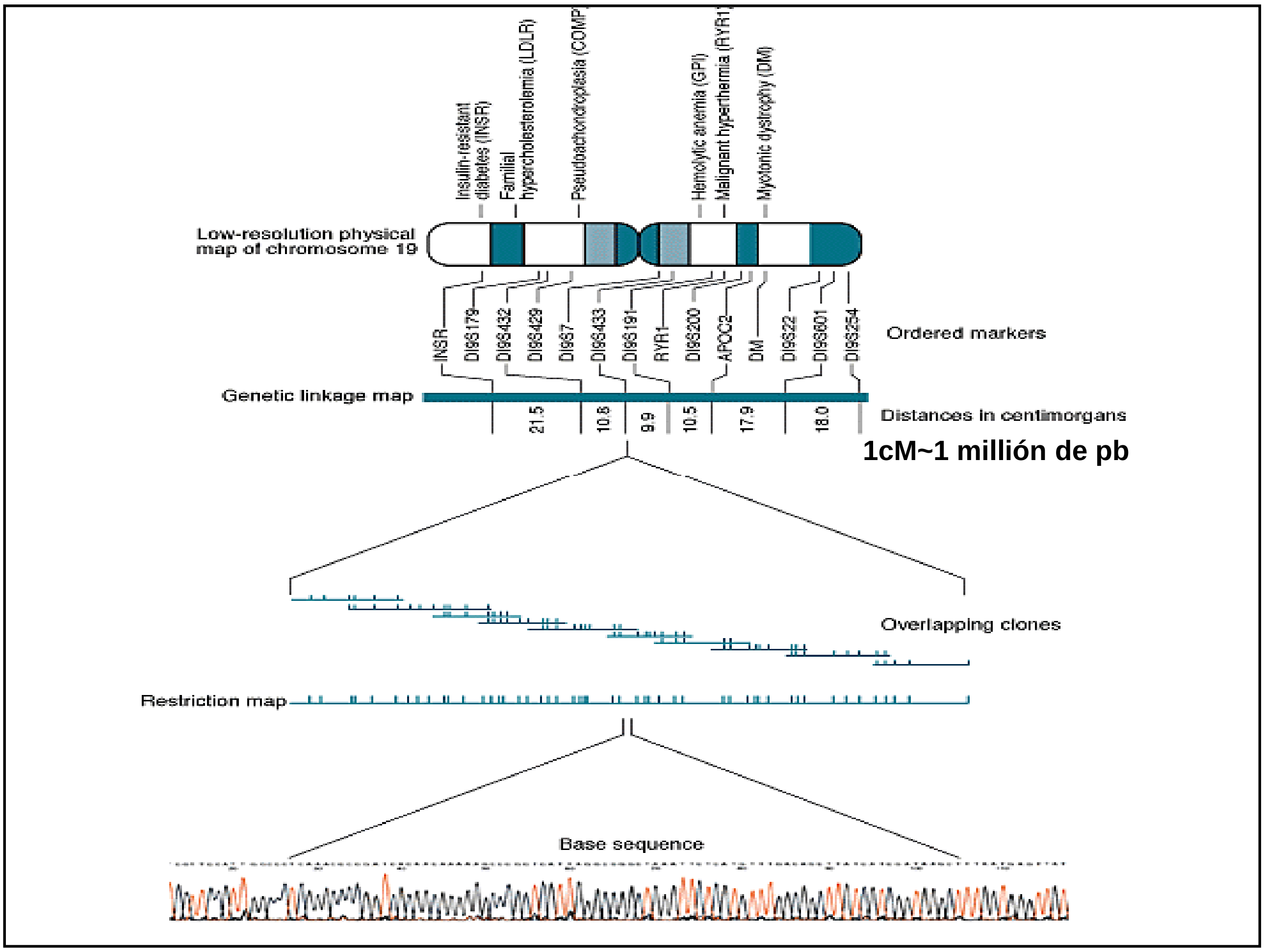
Pyrosequencing system


DNA polymerase
TGCACCTTTAGACTGGCCG---
CTGACCGGC---
dNTP
PPi
ATP-sulfurylase
ATP
Luciferase
Light
Apyrase
(wash)
Next base

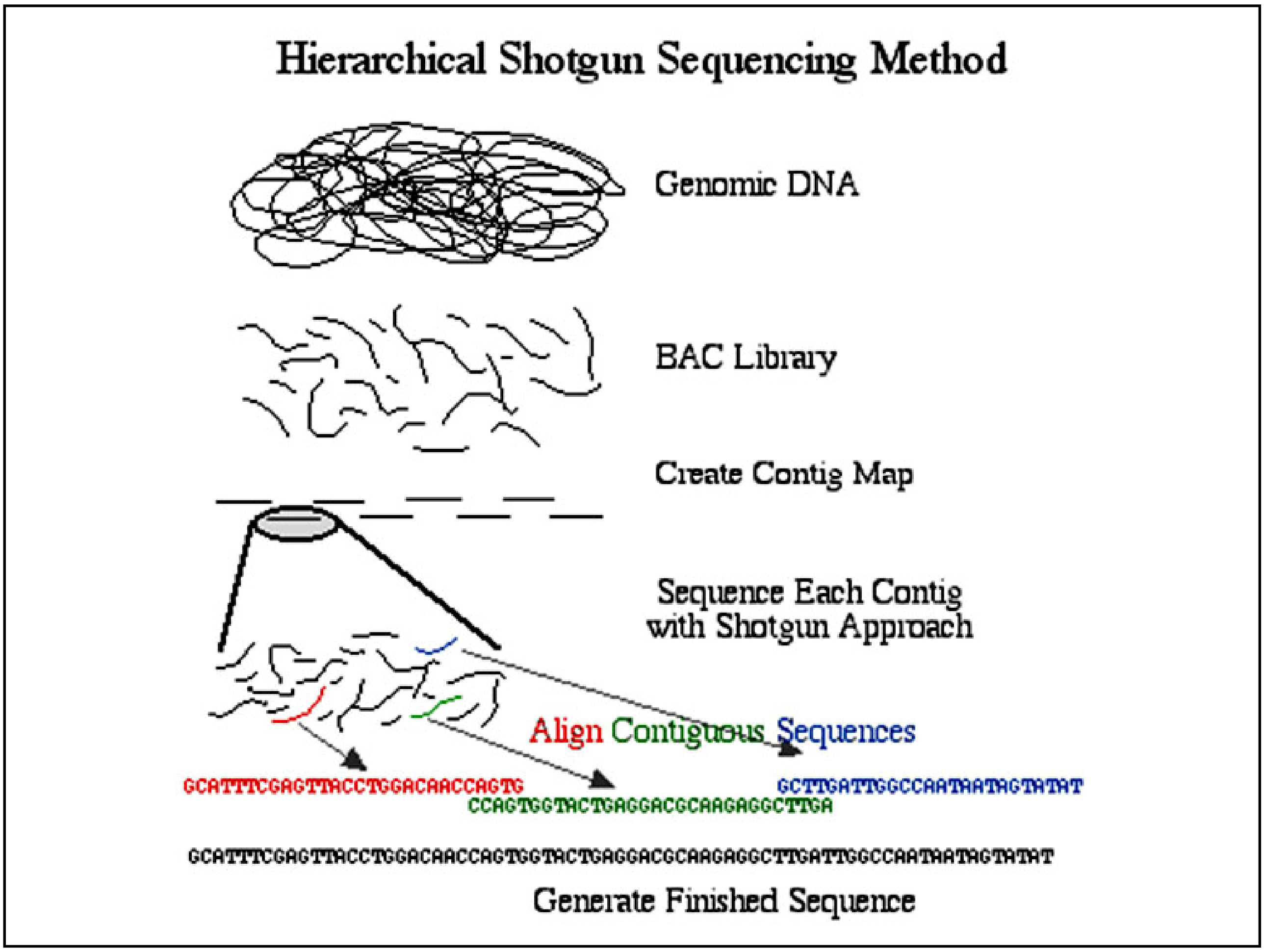
Hierarchical shotgun sequencing


Genomic DNA
BAC library
Organized mapped large clone contigs
BAC to be sequenced
Shotgun clones
Shotgun sequence
...ACCGTAAATGGGCTGATCATGCTTAAA
TGATCATGCTTAAACCCTGTGCATCCTACTG...
Assembly
...ACCGTAAATGGGCTGATCATGCTTAAACCCTGTGCATCCTACTG...

Nature Vol. 409 Feb. 2001. HGP


Insulin-resistant diabetes (INSR)
Familial hypercholesterolemia (LDLR)
Pseudoachondroplasia (COMP)
Hemolytic anemia (GPI)
Malignant hyperthermia (RYR1)
Myotonic dystrophy (DM)
Low-resolution physical map of chromosome 19
INSR
D19S179
D19S432
D19S429
D19S7
D19S433
D19S191
RYR1
D19S200
APOC2
DM
D19S22
D19S601
D19S254
Ordered markers
Genetic linkage map
21.5
10.8
9.9
10.5
17.9
18.0
Distances in centimorgans
Overlapping clones
Restriction map
Base sequence

1cM~1 millión de pb



Hierarchical Shotgun Sequencing Method

Genomic DNA

BAC Library

Create Contig Map

Sequence Each Contig with Shotgun Approach

Align Contiguous Sequences

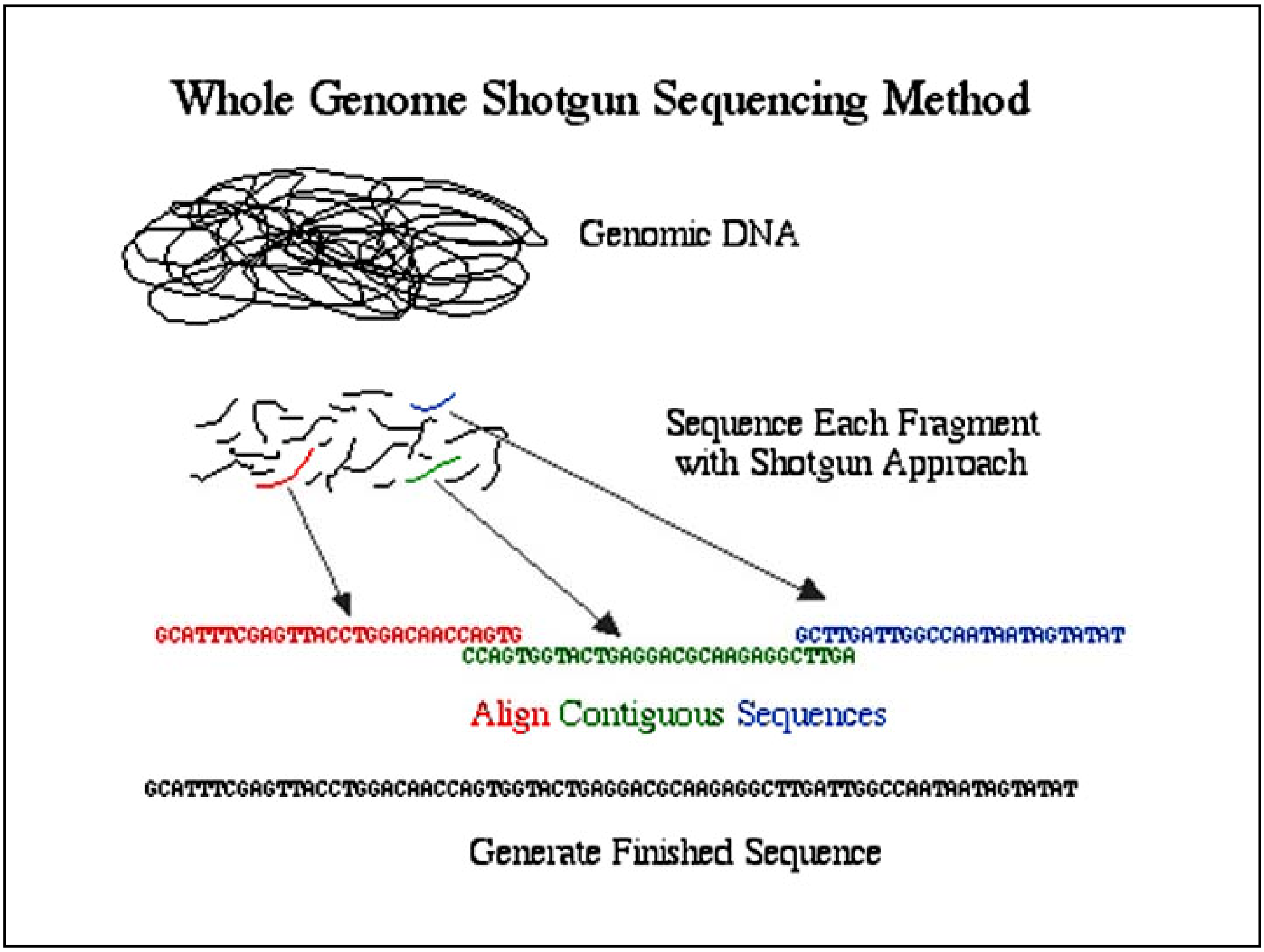
GCATTTCGAGTTACCTGGACAACCAGTG

CCAGTGGTACTGAGGACGCAAGAGGCTTGA

GCTTGATTGGCCAATAATAGTATAT

GCATTTCGAGTTACCTGGACAACCAGTGGTACTGAGGACGCAAGAGGCTTGATTGGCCAATAATAGTATAT

Generate Finished Sequence



Whole Genome Shotgun Sequencing Method

Genomic DNA

Sequence Each Fragment
with Shotgun Approach

GCATTTCGAGTTACCTGGACAACCAGTG

CCAGTGGTACTGAGGACGCAAGAGGCTTGA

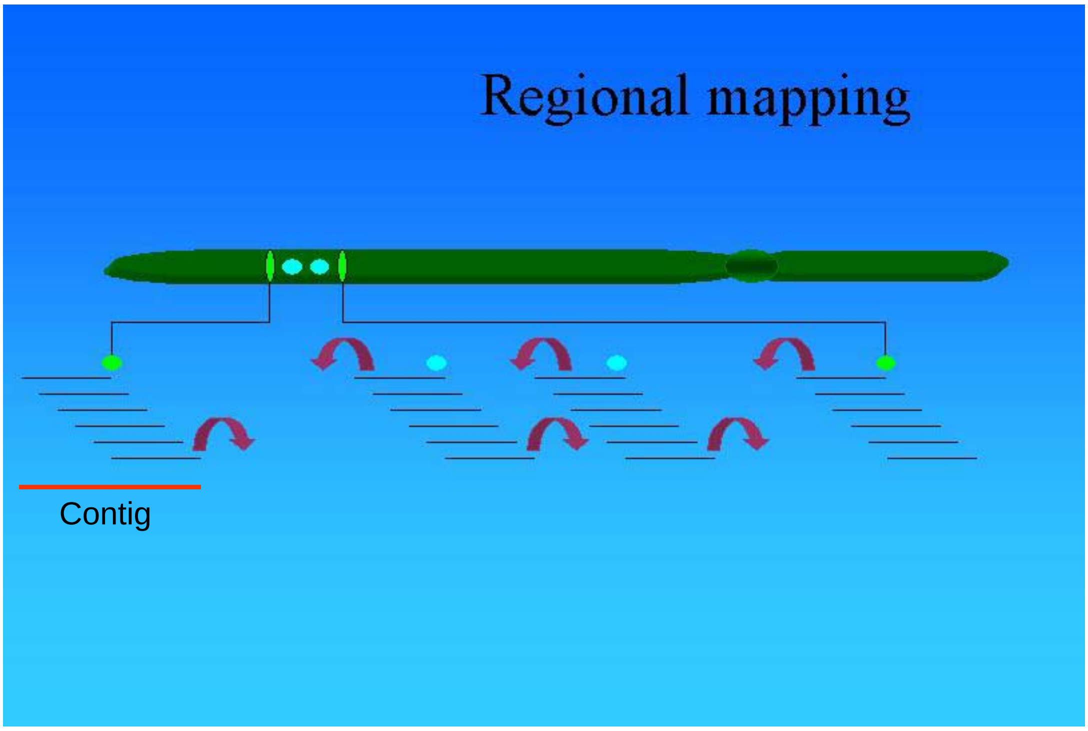
GCTTGATTGGCCAATAATAGTATAT

Align Contiguous Sequences

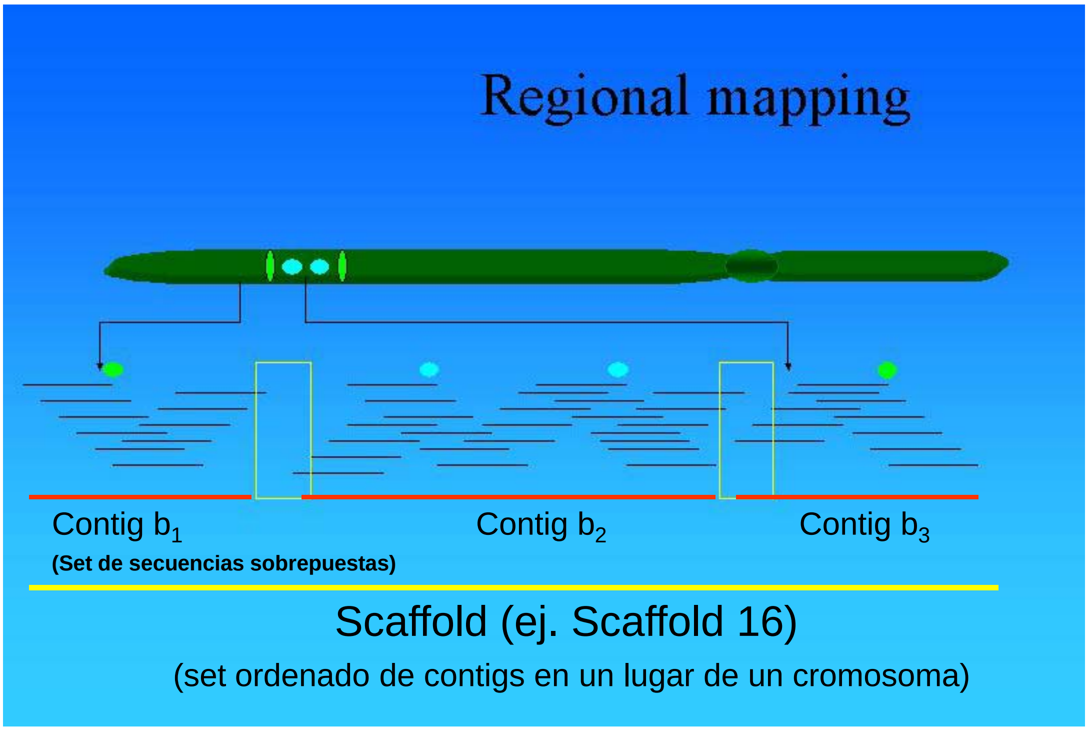
GCATTTCGAGTTACCTGGACAACCAGTGGTACTGAGGACGCAAGAGGCTTGATTGGCCAATAATAGTATAT

Generate Finished Sequence

Contig Scaffolding (ensamblaje)


Regional mapping
Contig

Uso de algoritmos computacionales para ensamblaje



Regional mapping

Contig b_1

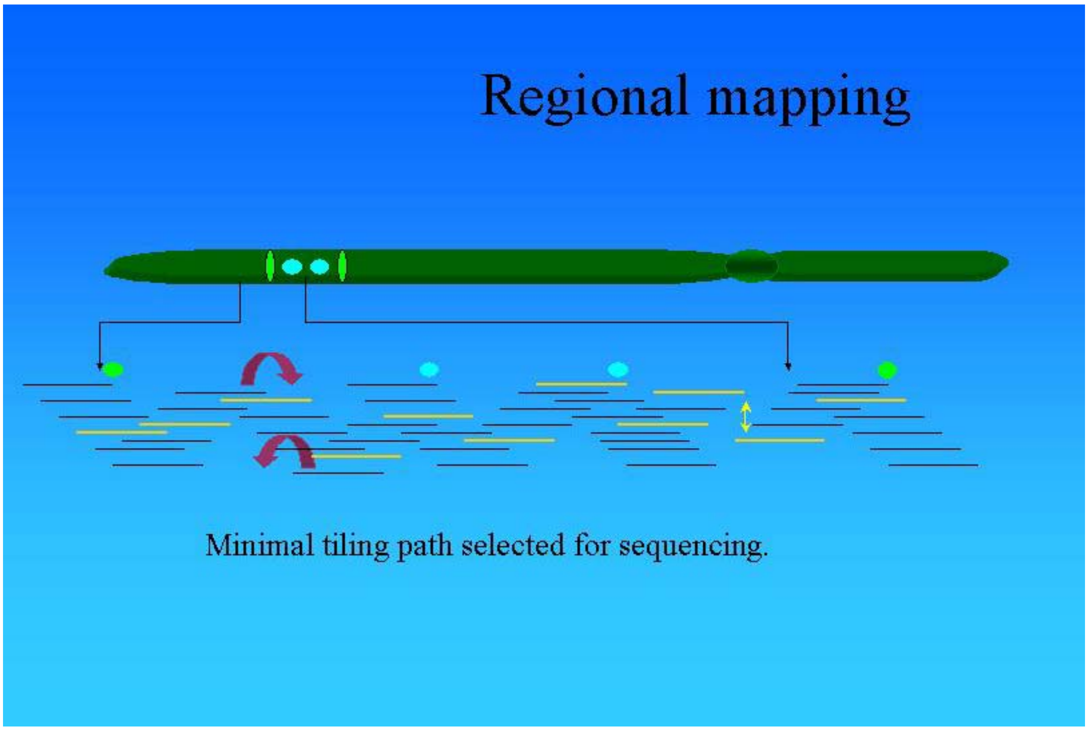
(Set de secuencias sobrepuestas)

Contig b_2

Contig b_3

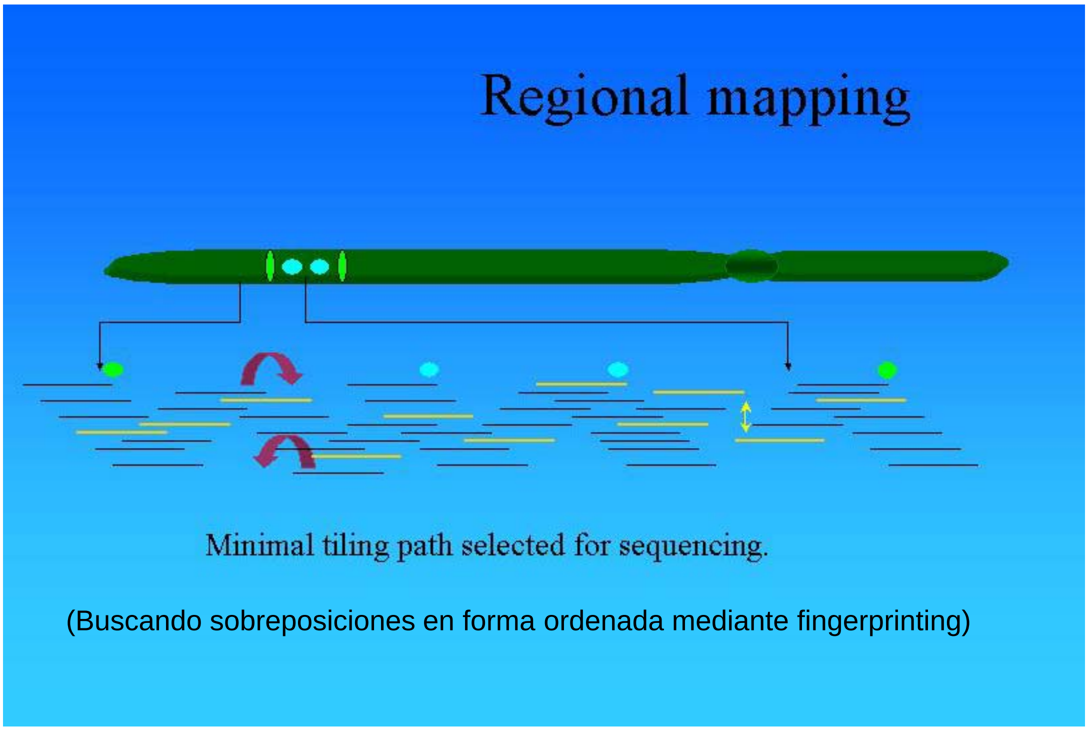
Scaffold (ej. Scaffold 16)

(set ordenado de contigs en un lugar de un cromosoma)



Regional mapping

Minimal tiling path selected for sequencing.



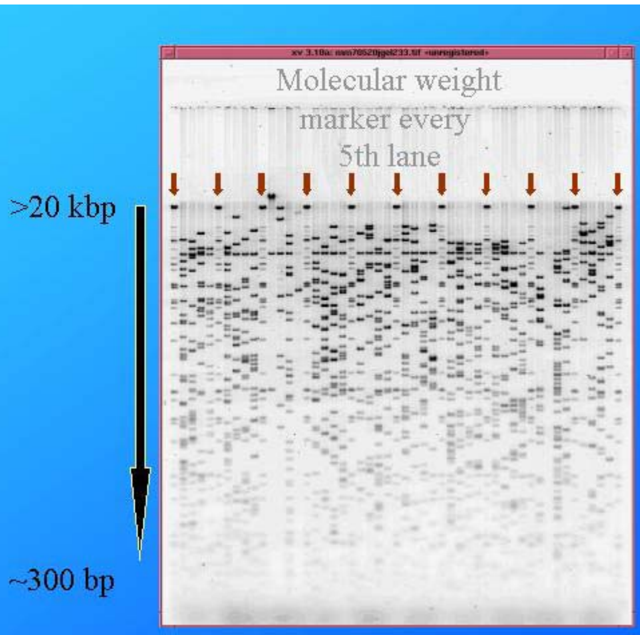
Regional mapping

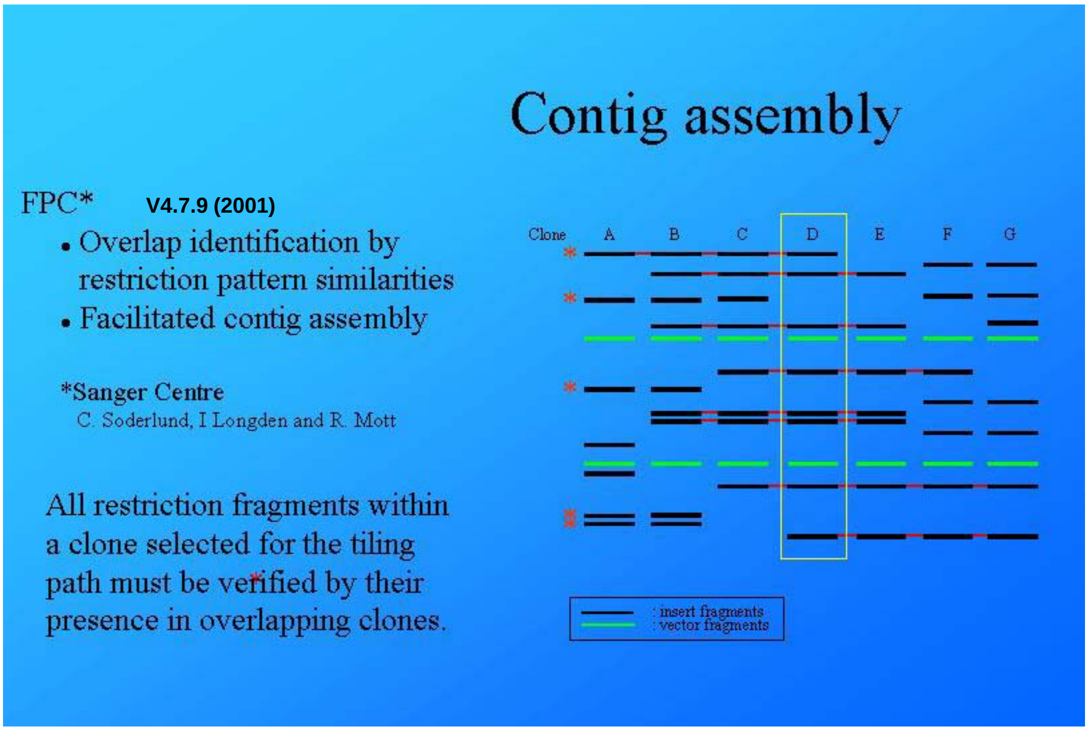
Minimal tiling path selected for sequencing.

(Buscando sobreposiciones en forma ordenada mediante fingerprinting)

Restriction fragment fingerprinting

- BAC clones are grown in 96-well format
- Hind III digest
- 1% agarose


Molecular weight marker every 5th lane
>20 kbp
~300 bp



Contig assembly

FPC* V4.7.9 (2001)

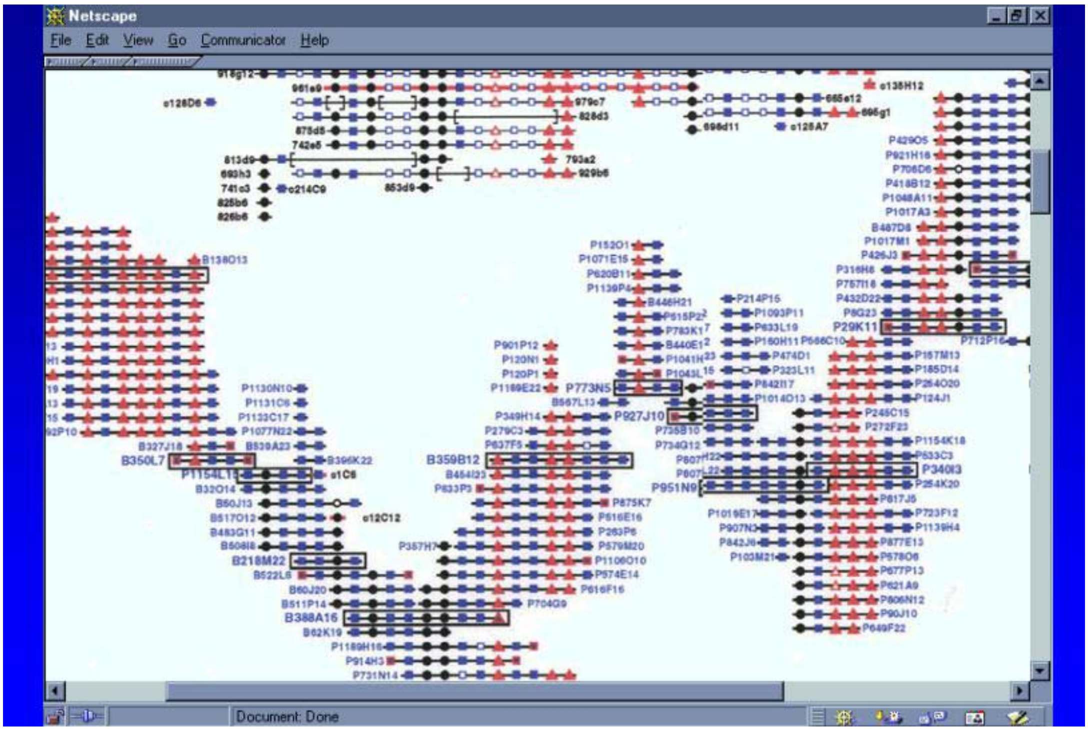
- Overlap identification by restriction pattern similarities
- Facilitated contig assembly

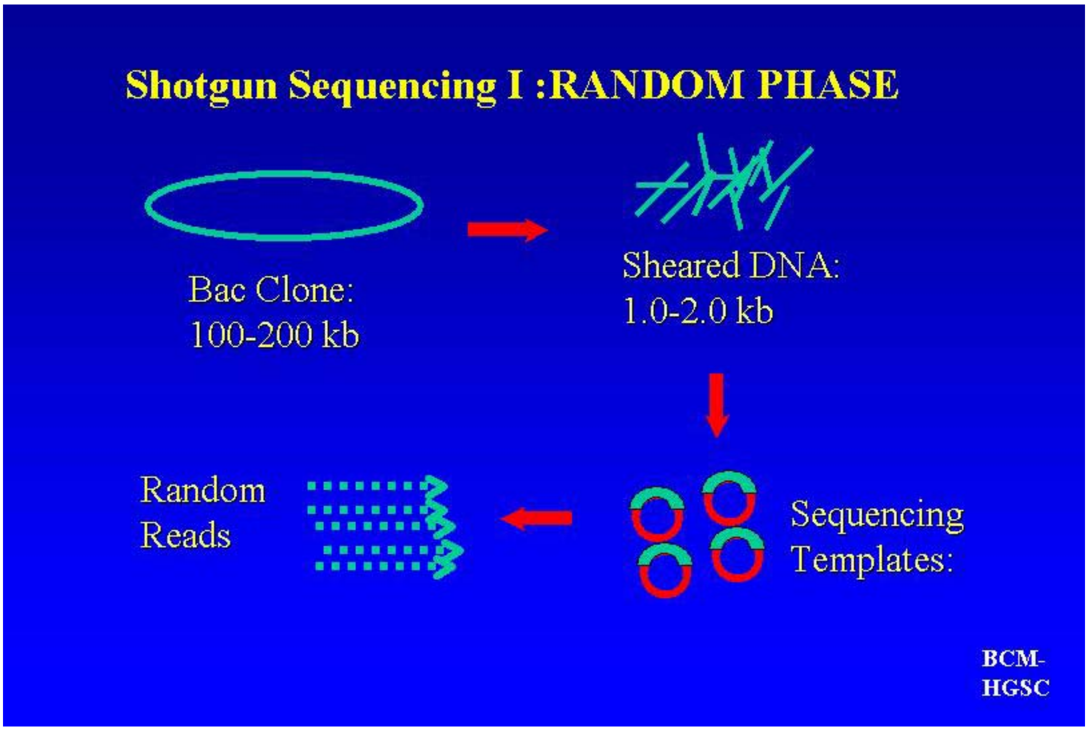
*Sanger Centre

C. Soderlund, I Longden and R. Mott

All restriction fragments within a clone selected for the tiling path must be verified by their presence in overlapping clones.

Clone
A
B
C
D
E
F
G
: insert fragments
: vector fragments


Netscape
File
Edit
View
Go
Communicator
Help
B350L7
B359B12
P773N5
P927J10
P951N9
P340I3
P29K11
B218M22
B388A16
Document: Done



Shotgun Sequencing I :RANDOM PHASE

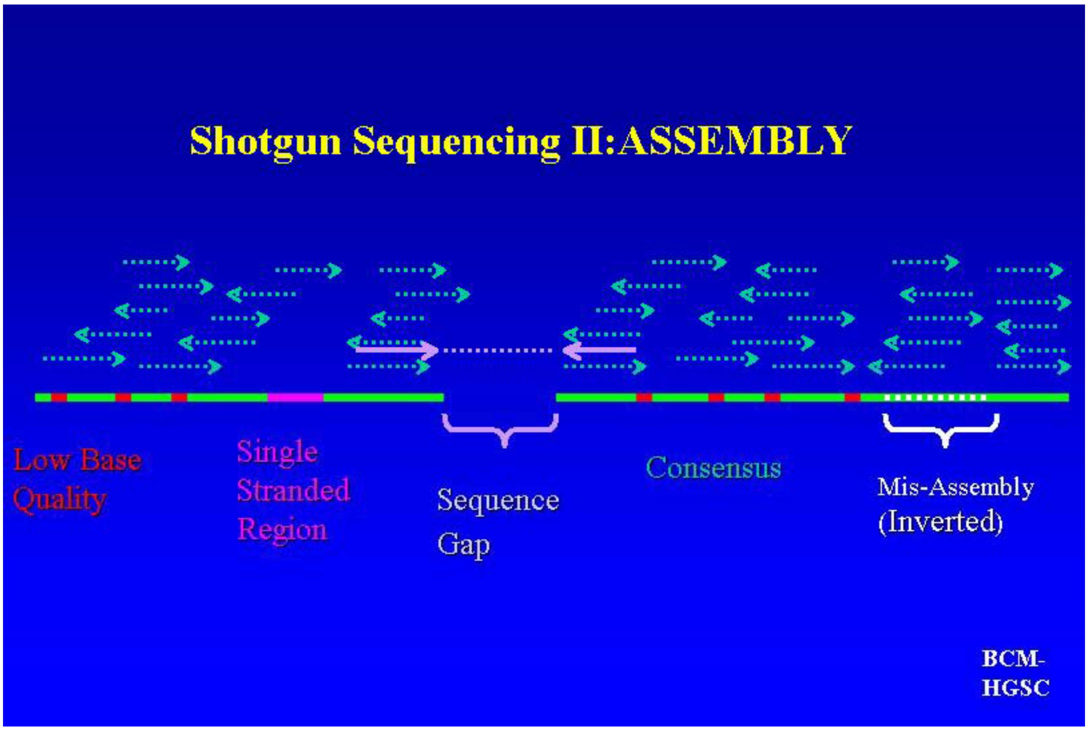
Bac Clone:
100-200 kb

Sheared DNA:
1.0-2.0 kb

Sequencing
Templates:

Random
Reads

BCM-
HGSC



Shotgun Sequencing II:ASSEMBLY

Low Base Quality

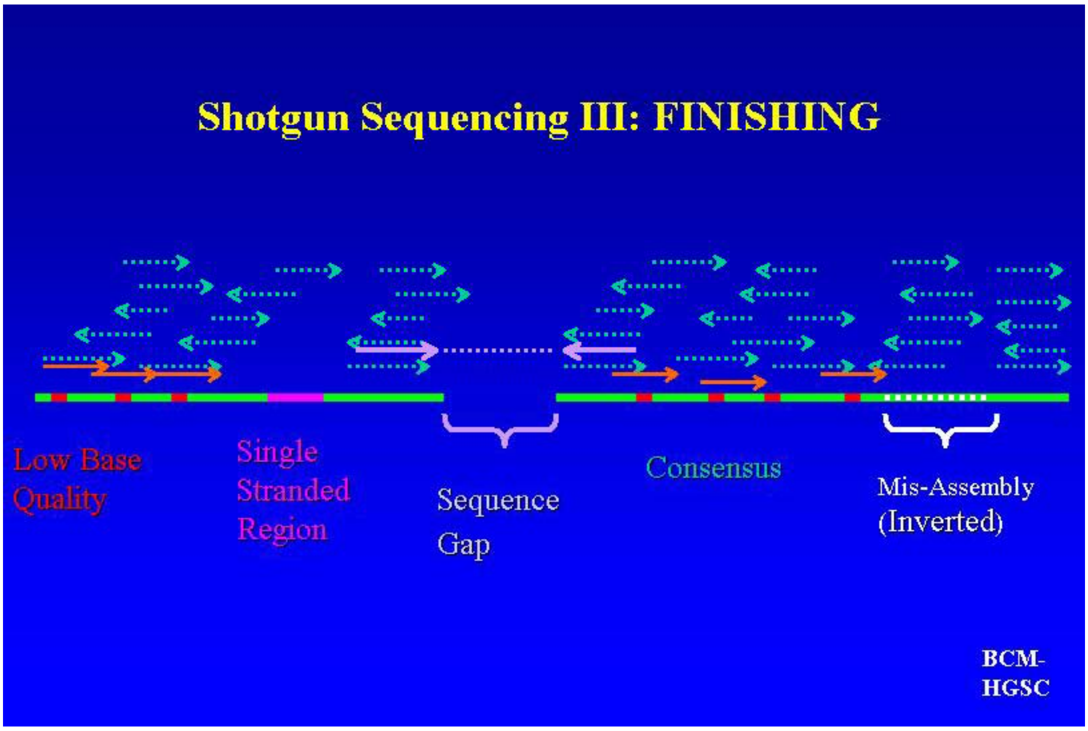
Single Stranded Region

Sequence Gap

Consensus

Mis-Assembly (Inverted)

BCM-HGSC



Shotgun Sequencing III: FINISHING

Low Base Quality

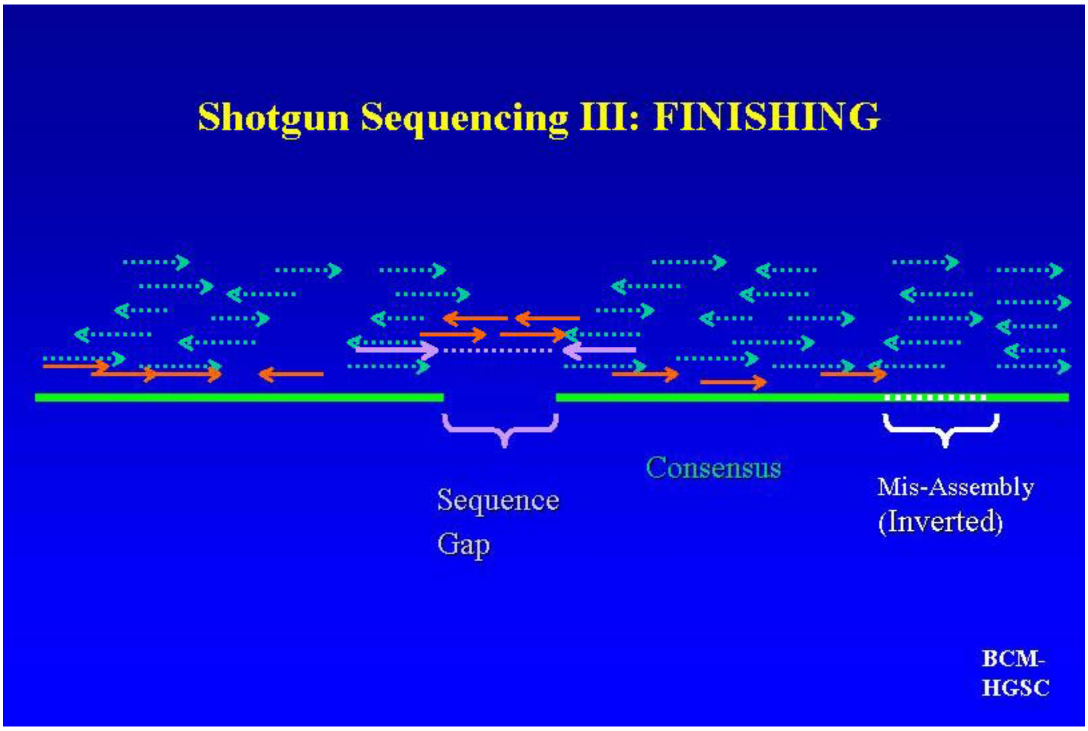
Single Stranded Region

Sequence Gap

Consensus

Mis-Assembly (Inverted)

BCM-HGSC



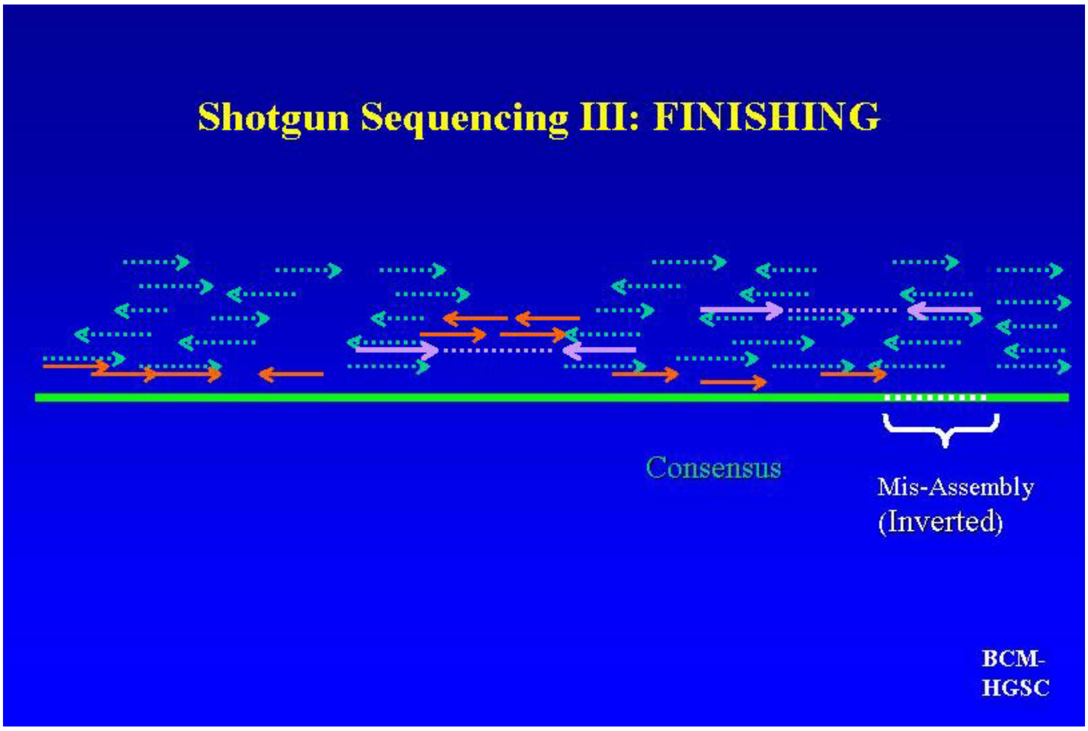
Shotgun Sequencing III: FINISHING

Sequence Gap

Consensus

Mis-Assembly (Inverted)

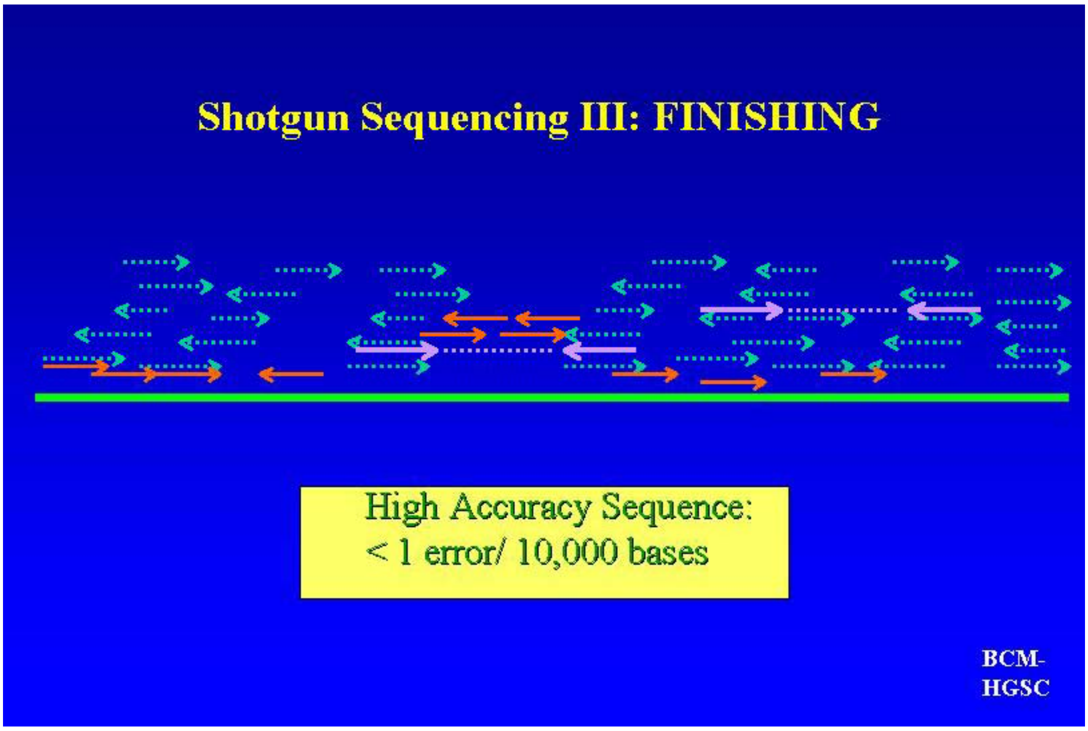
BCM-HGSC



Shotgun Sequencing III: FINISHING

Consensus
Mis-Assembly
(Inverted)

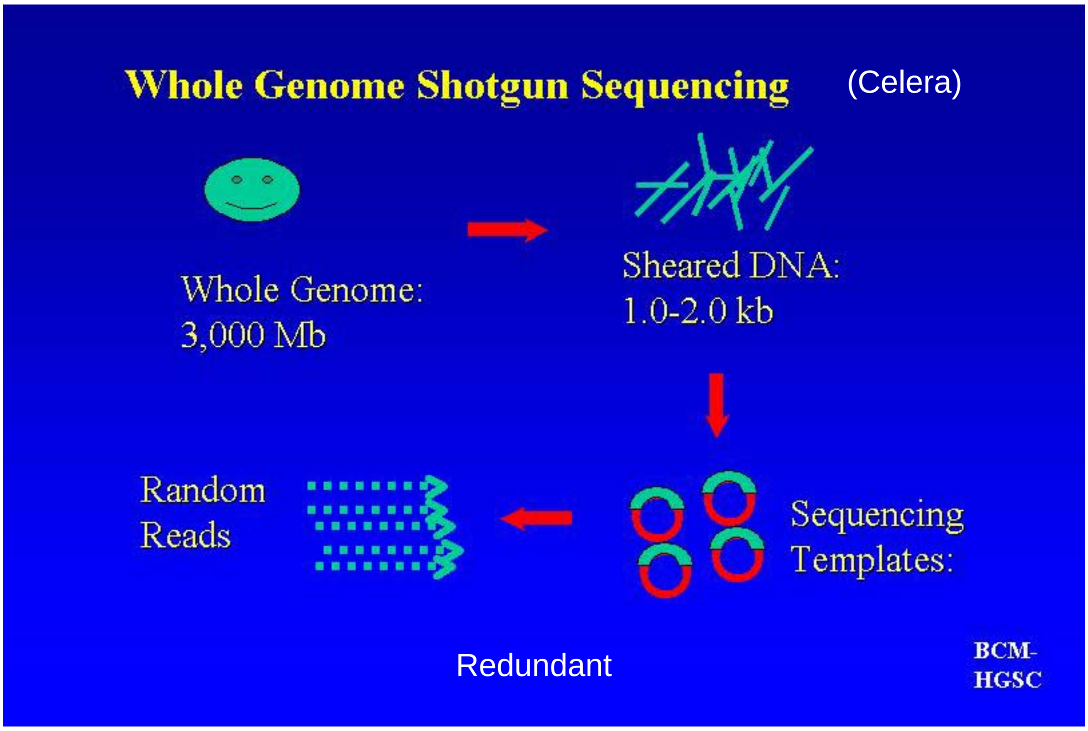
BCM-
HGSC



Shotgun Sequencing III: FINISHING

High Accuracy Sequence:
< 1 error/ 10,000 bases

BCM-
HGSC



Whole Genome Shotgun Sequencing (Celera)

Whole Genome:
3,000 Mb

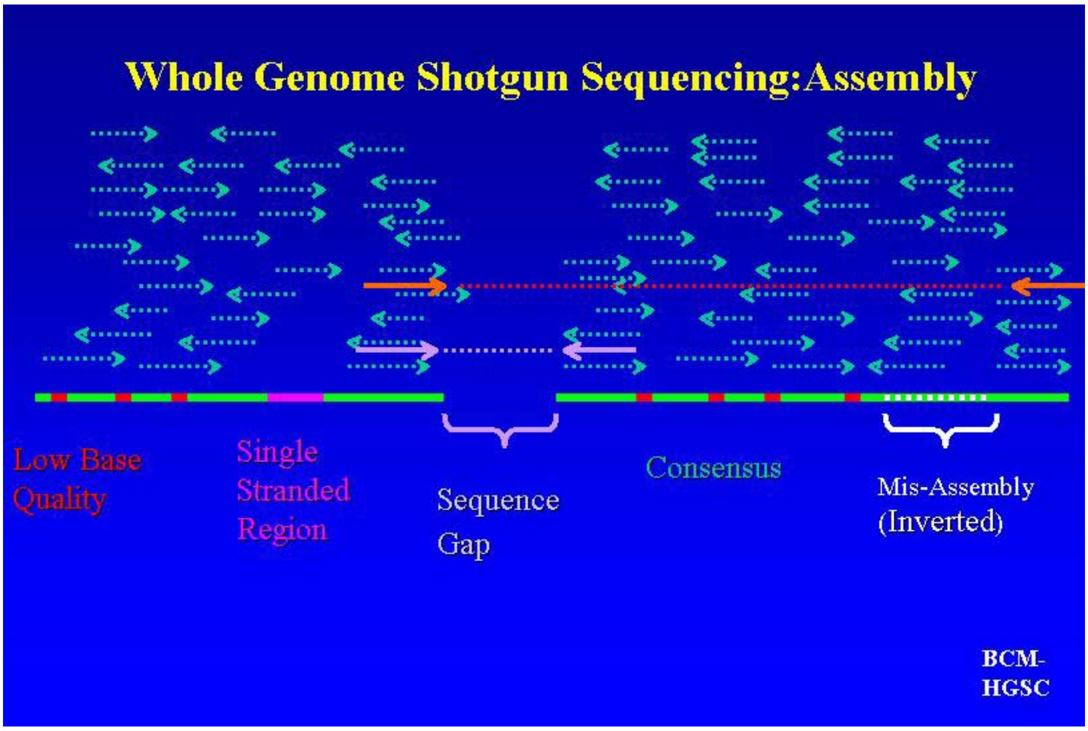
Sheared DNA:
1.0-2.0 kb

Sequencing
Templates:

Random
Reads

Redundant

BCM-
HGSC



Whole Genome Shotgun Sequencing:Assembly

Low Base Quality

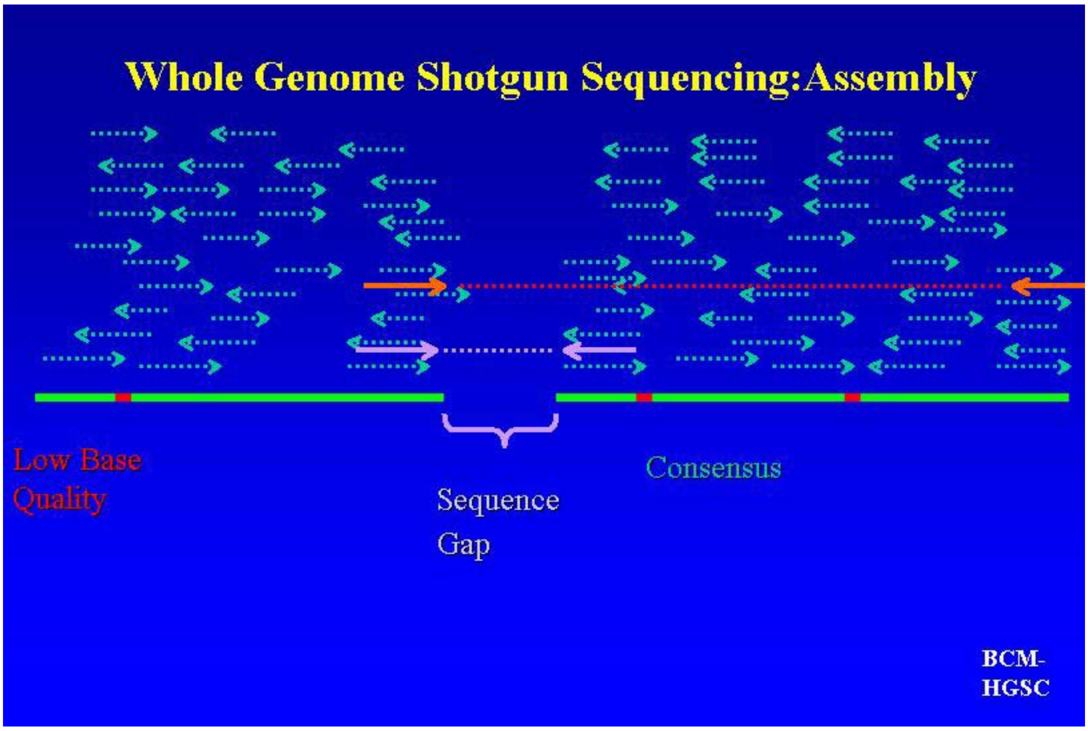
Single Stranded Region

Sequence Gap

Consensus

Mis-Assembly (Inverted)

BCM-HGSC



Whole Genome Shotgun Sequencing:Assembly

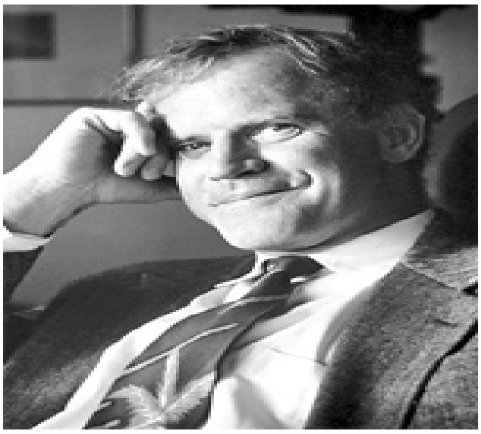
Low Base Quality

Sequence Gap

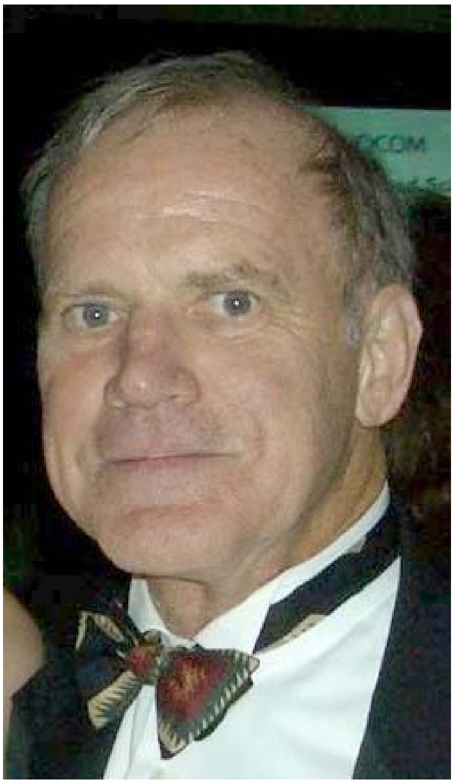
Consensus

BCM-HGSC

PCR
Polymerase Chain Reaction



Kary B. Mullis inventó el PCR en 1983.
Ganó el Premio Nobel de Química en 1993.



Kary Banks Mullis, Nobel Prize winning chemist, was born on December 28, 1944, in Lenoir, North Carolina.

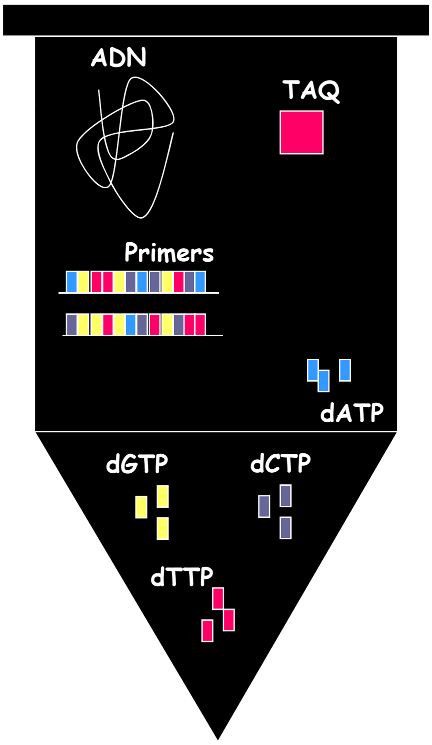
Nobel Prize Winner for Chemistry in 1993 and inventor of PCR, a method for detecting even the smallest amount of DNA in ancient materials. "Would I have invented PCR if I hadn't taken LSD? I seriously doubt it," he says. "I could sit on a DNA molecule and watch the polymers go by. I learnt that partly on psychedelic drugs."

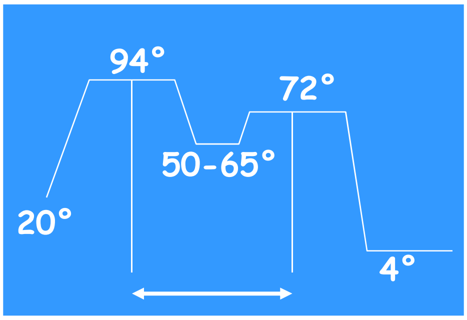
PCR

Amplificación IN VITRO de secuencias determinadas de ADN basada en el uso de:

- -Polimerasa
- -Partidores
- -dNTPs

PCR


ADN
TAQ
Primers
dATP
dGTP
dCTP
dTTP

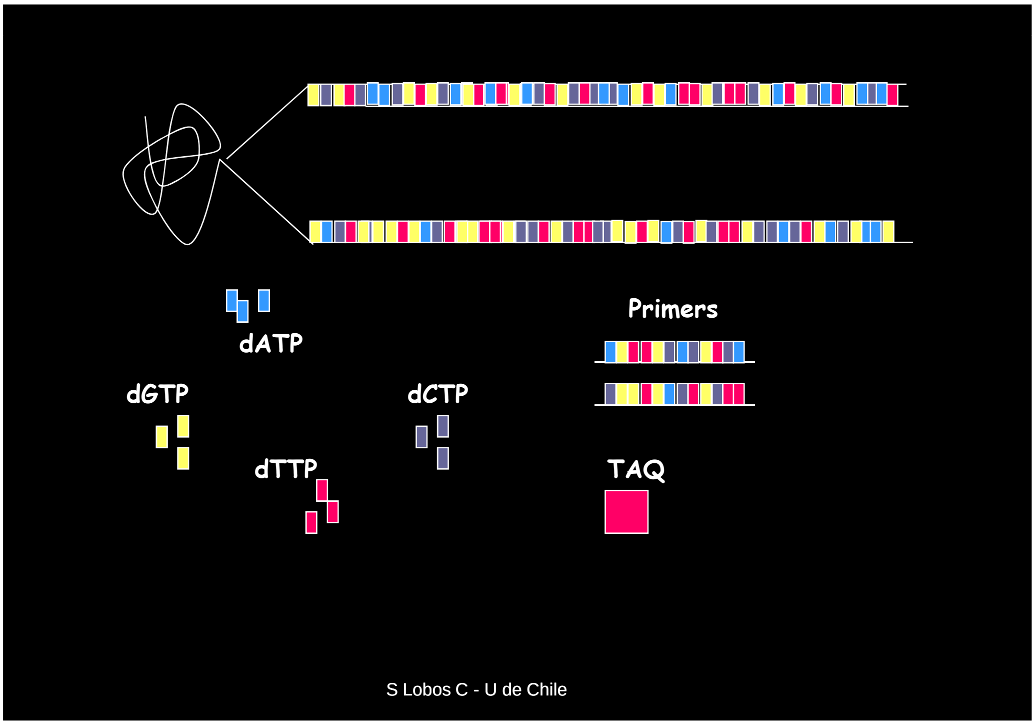

94°
72°
50-65°
20°
4°

30 ciclos

1ª FASE: Desnaturalización

94°C

CICLO 1

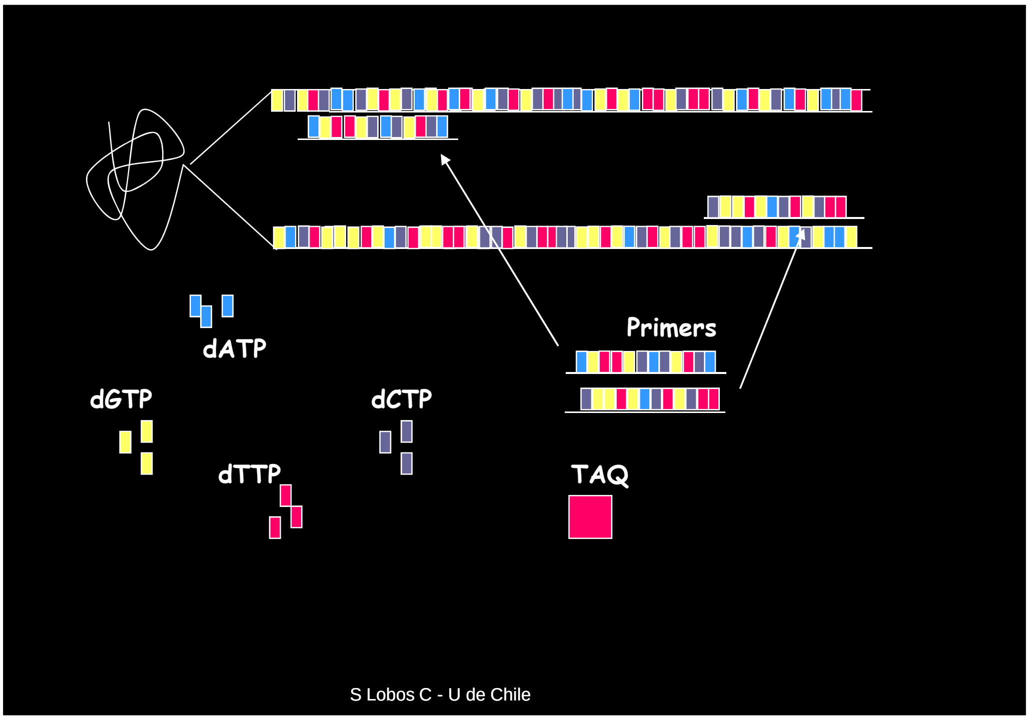

Primers
dATP
dGTP
dCTP
dTTP
TAQ

S Lobos C - U de Chile

2ª FASE: Apareamiento

50-65°C

CICLO 1

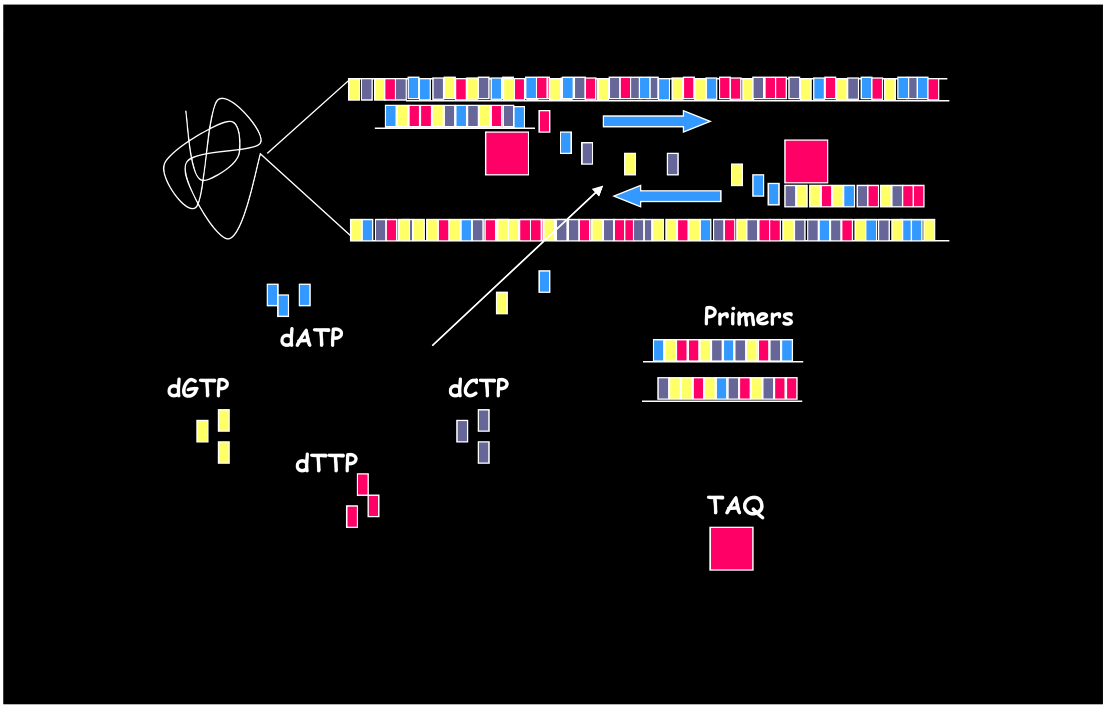

Primers
dATP
dGTP
dCTP
dTTP
TAQ

S Lobos C - U de Chile

3ª FASE: Extensión

72°C

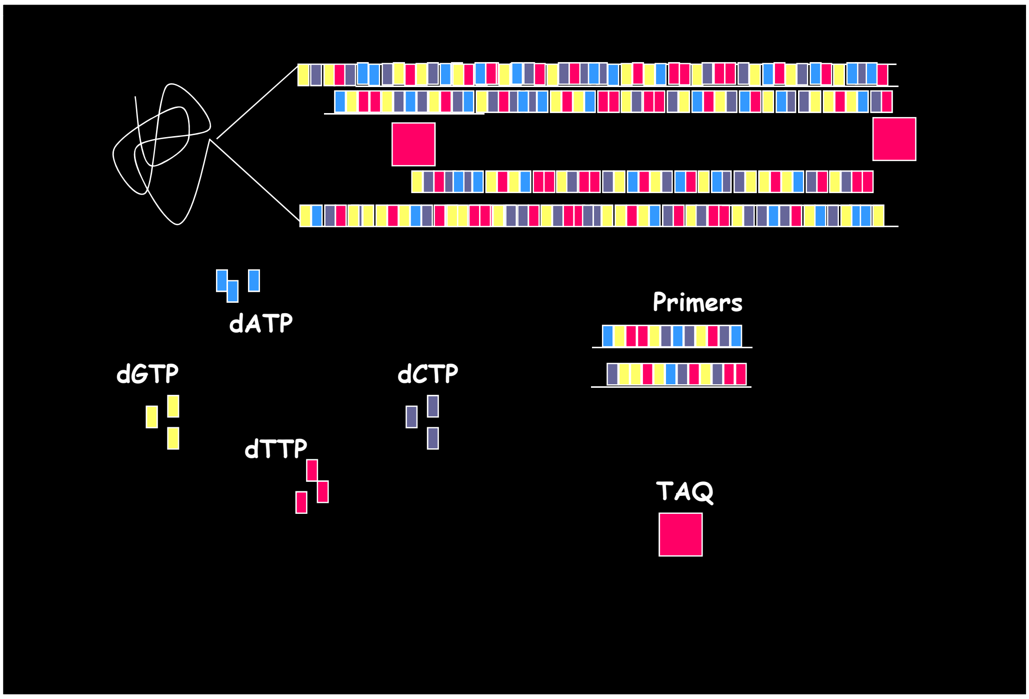
CICLO 1


dATP
dGTP
dCTP
dTTP
Primers
TAQ

3ª FASE: Extensión

72°C

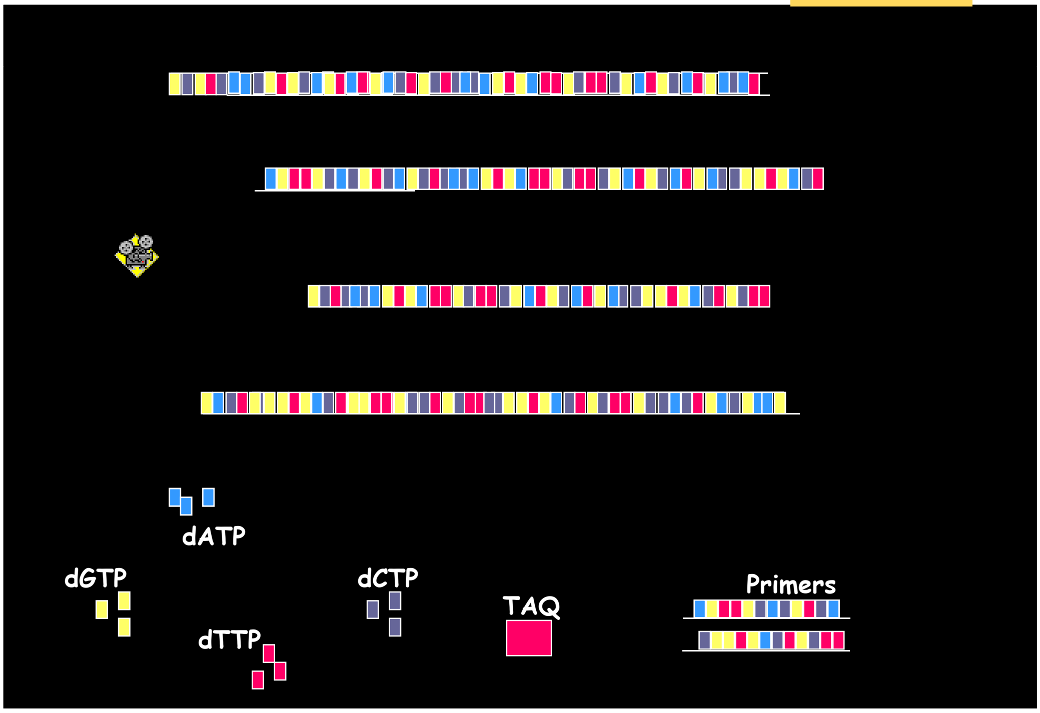
CICLO 1


dATP
dGTP
dCTP
dTTP
Primers
TAQ

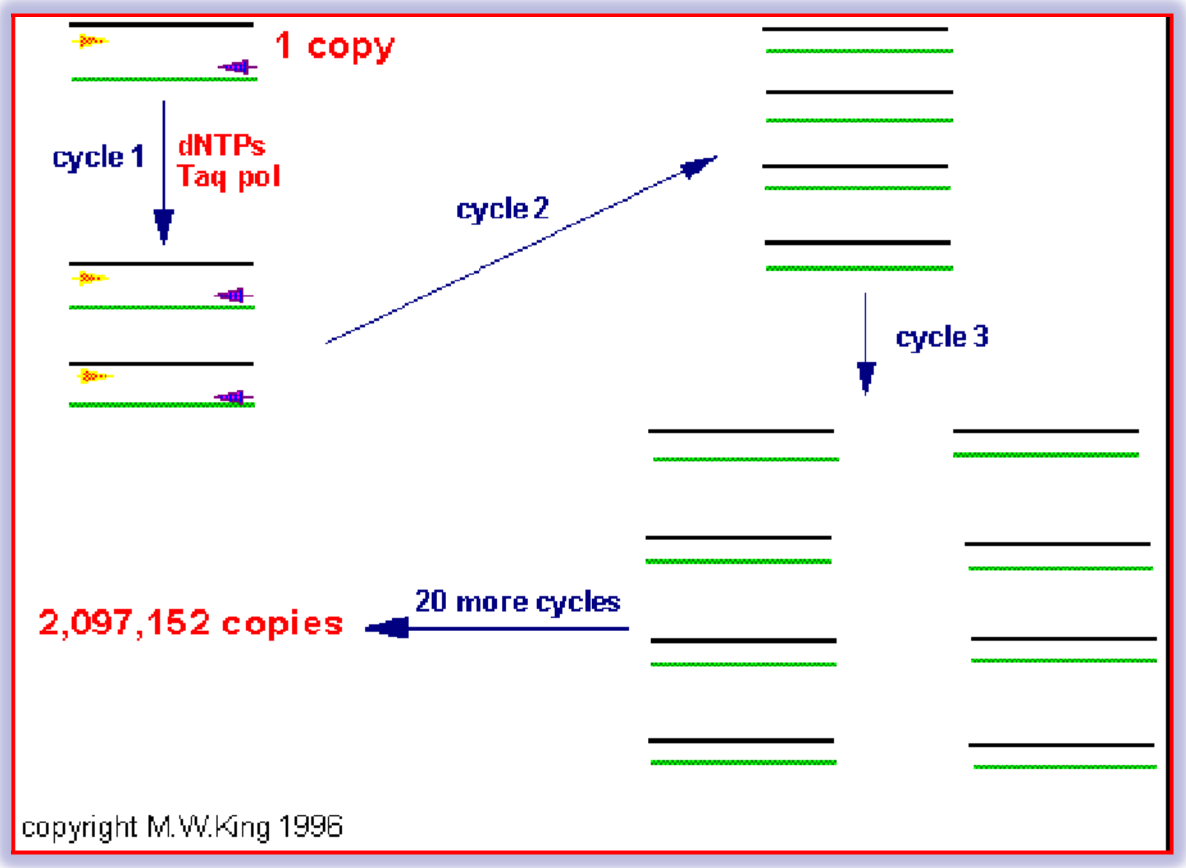
1ª FASE: Desnaturalización

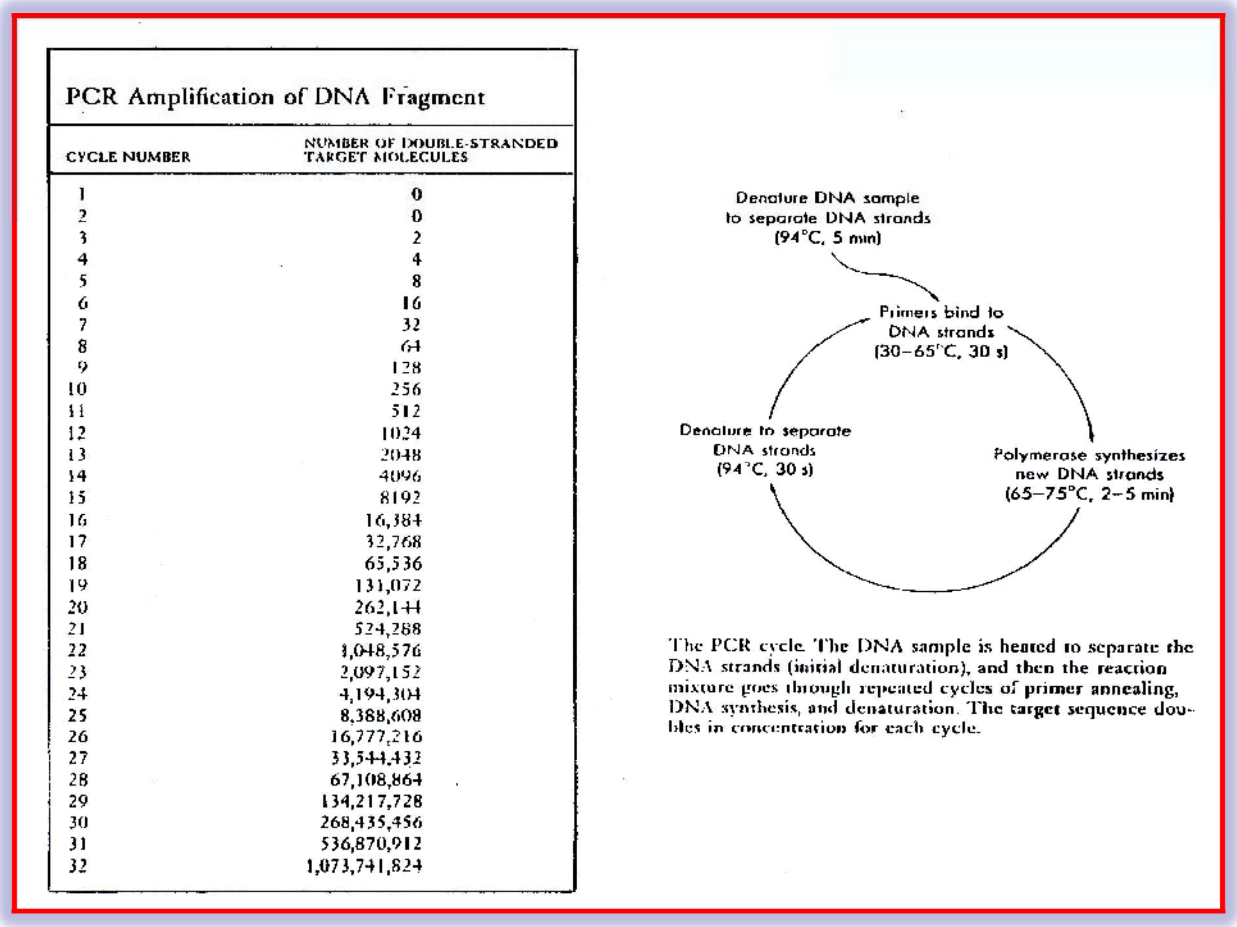
94°C

CICLO 2


dATP
dGTP
dCTP
TAQ
Primers
dTTP

PCR


1 copy
cycle 1
dNTPs
Taq pol
cycle 2
cycle 3
2,097,152 copies
20 more cycles
copyright M.W.King 1996



PCR Amplification of DNA Fragment

CYCLE NUMBER	NUMBER OF DOUBLE-STRANDED TARGET MOLECULES
1	0
2	0
3	2
4	4
5	8
6	16
7	32
8	64
9	128
10	256
11	512
12	1024
13	2048
14	4096
15	8192
16	16,384
17	32,768
18	65,536
19	131,072
20	262,144
21	524,288
22	1,048,576
23	2,097,152
24	4,194,304
25	8,388,608
26	16,777,216
27	33,544,432
28	67,108,864
29	134,217,728
30	268,435,456
31	536,870,912
32	1,073,741,824

Denature DNA sample to separate DNA strands (94°C, 5 min)
Primers bind to DNA strands (30–65°C, 30 s)
Polymerase synthesizes new DNA strands (65–75°C, 2–5 min)
Denature to separate DNA strands (94°C, 30 s)

The PCR cycle. The DNA sample is heated to separate the DNA strands (initial denaturation), and then the reaction mixture goes through repeated cycles of primer annealing, DNA synthesis, and denaturation. The target sequence doubles in concentration for each cycle.

PCR

Aplicaciones

- -Obtener copias de una secuencia determinada
- -Introducir modificaciones en la secuencia:
    - -Mutaciones
    - -Secuencias para enzimas de restricción
    - -Elementos reguladores

Ventajas

- -Cantidad mínima de DNA molde
- -No es necesario DNA de calidad

PCR

Inconvenientes

-Limitación de tamaño

-Necesidad de conocer secuencias flanqueantes

Problemas

-Problemas de contaminación

-Presencia de inhibidores

-Fallos por variación genética en sitios de apareamiento

PCR

Optimización

-Perfil de temperaturas

-Reactivos:

- DNA
- primers
- Buffer: pH, Cl_2Mg, K
- Inhibidores
- dNTPs
- Taq polimerasa

Montaje de Reacciones de PCR

Setting Up PCR Reactions

10X Buffer Taq DNA polymerase	10 µL
25mM $MgCl_2$	4 µL
10mM dNTPs mix	2 µL
Primer 1	? (10pmoles/µL)
Primer 2	? (10pmoles/µL)
DNA	ng a µg
H_2O_{dd} estéril	? llevar a Vt
Taq DNA polymerase	1-5 Unidades
Volumen Total	100 mL

Un oligonucleótido primer de **17 nucleótidos=17 mer**, equivale a una concentración de **5 ng/pmol**

Especificidad PCR

Factores que afectan la especificidad en el PCR:

1.- Primers: Mal diseño. Se debe considerar longitud, contenido G+C (no mayor a 50-60%), Tm>55ºC y posibilidad de formación de dímeros de primers.

2.- Temperatura de apareamiento de los primers: Tm Considerar Tm sobre 55ºC y similar para ambos primers.

3.- Denaturación incompleta: Falta de “Hot Start”

T_m = temperatura de fusión es la temperatura a la cual la ½ de las moléculas complementarias se encuentran unidas

T_m teórica = $4(G+C) + 2(A+T)$

a una concentración 1M NaCl

Siendo la **T_m (temperatura de desnaturación del dúplex**) un parámetro crítico para PCR, se debe ser más riguroso con el cálculo para este oligonucleótido. Por ejemplo, a una concentración determinada de Na^+ se debe aplicar la siguiente fórmula:

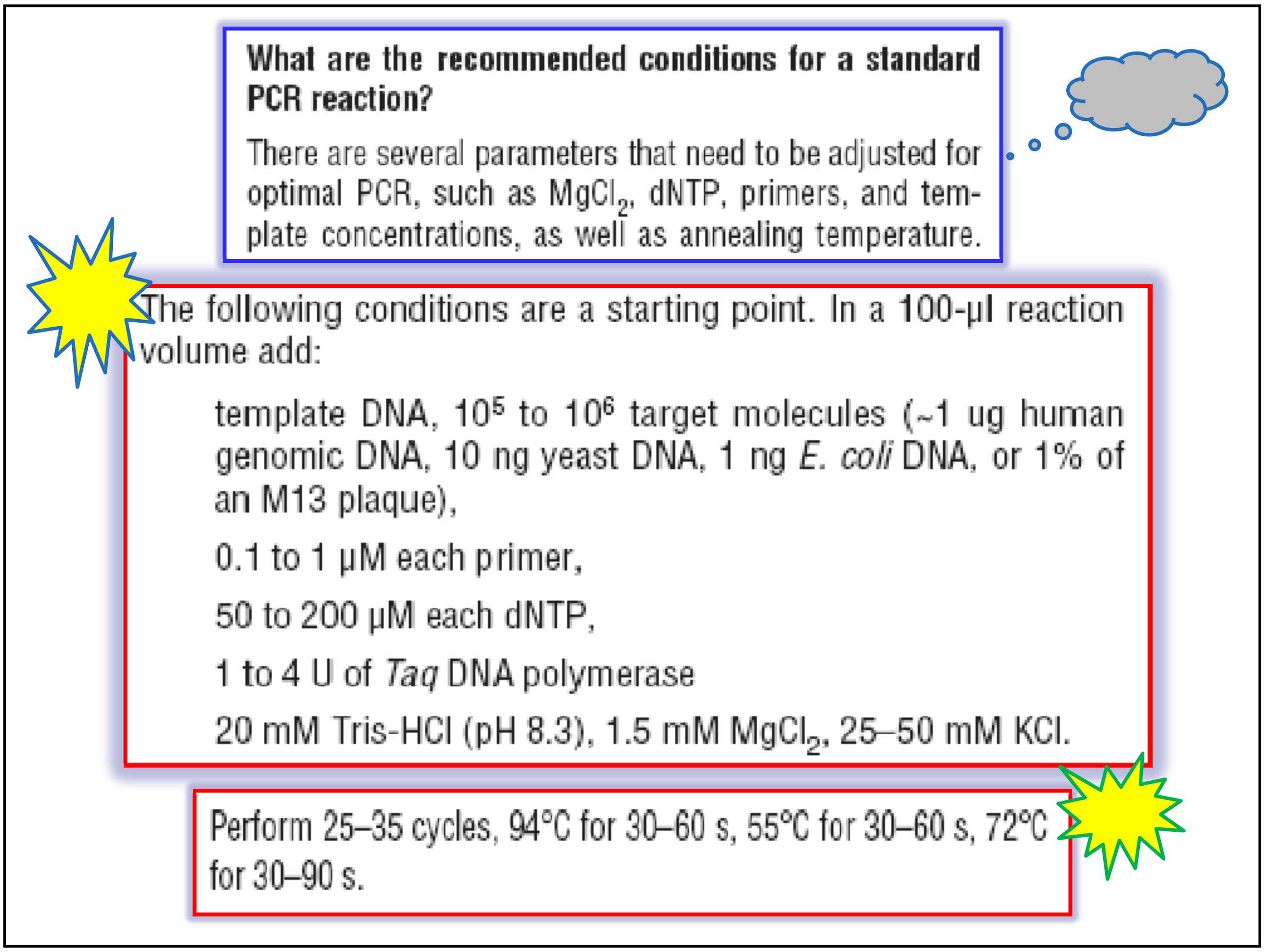
$$T_m=81.5+ [(16.6) (\log_{10}\{ [Na^{+\,]}/1.0+0.7[Na^{+\,]\}})] +[0.41(\%G+C)] -500 /D- P$$

Donde **D** es la longitud del dúplex y **P** es el porcentaje de apareamiento incorrecto. Para un buen resultado en la reacción de PCR se puede partir utilizando la T_m calculada menos 5ºC.

Controles de especificidad en el PCR

1.- DNA + Primer 1 (Fw) + Primer 2 (Rev) (+)
2.- DNA + Primer 1 (Fw) (-)
3.- DNA + Primer 2 (Rev) (-)
4.- DNA sin primers (-)
5.- Primer 1 + Primer 2 sin DNA (-)

Contaminación!! → PCR carry over!!



What are the recommended conditions for a standard PCR reaction?

There are several parameters that need to be adjusted for optimal PCR, such as $MgCl_2$, dNTP, primers, and template concentrations, as well as annealing temperature.

The following conditions are a starting point. In a 100-µl reaction volume add:

template DNA, 10^5 to 10^6 target molecules (~1 ug human genomic DNA, 10 ng yeast DNA, 1 ng *E. coli* DNA, or 1% of an M13 plaque),

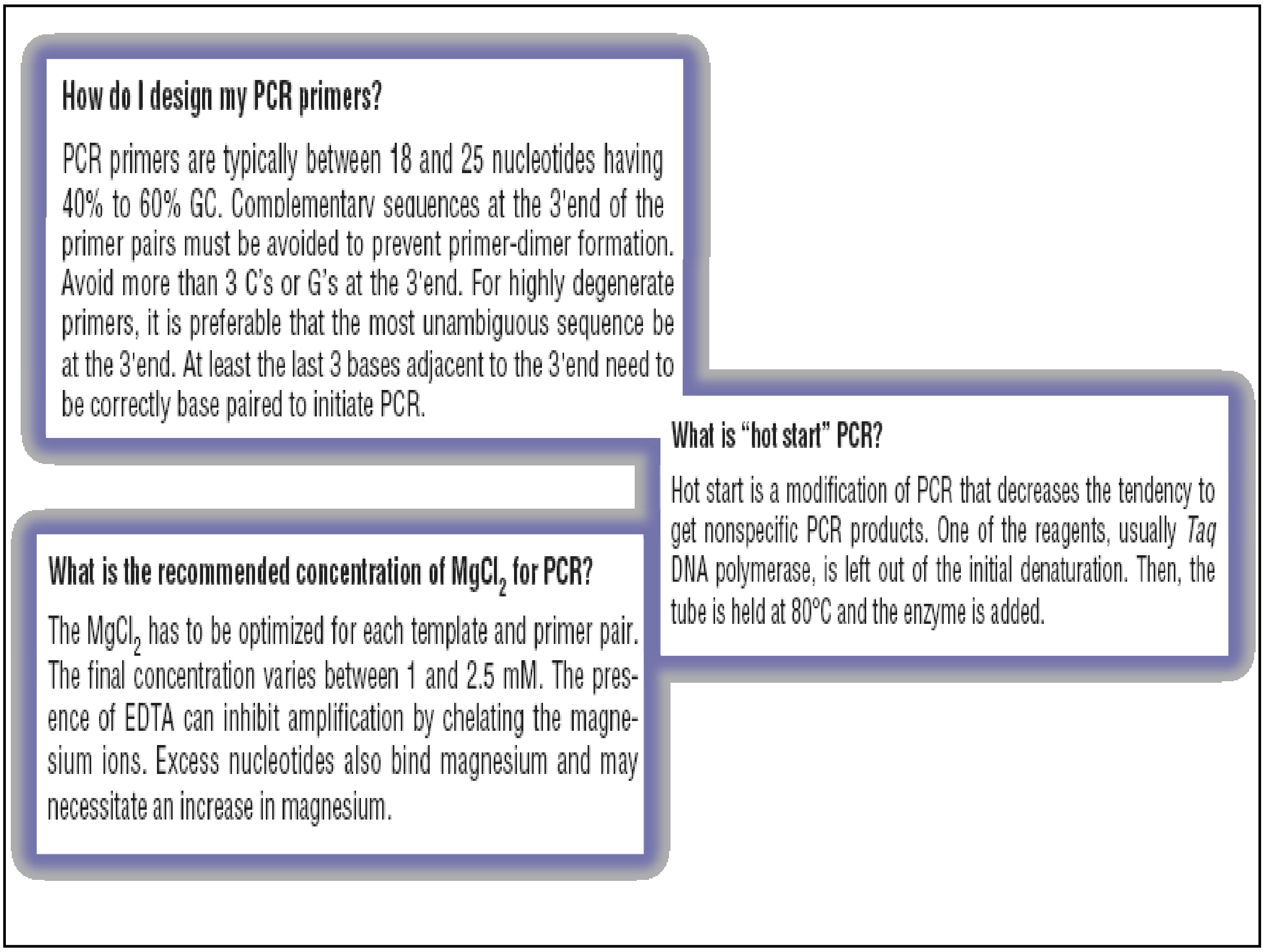
0.1 to 1 µM each primer,

50 to 200 µM each dNTP,

1 to 4 U of *Taq* DNA polymerase

20 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 25–50 mM KCl.

Perform 25–35 cycles, 94°C for 30–60 s, 55°C for 30–60 s, 72°C for 30–90 s.



How do I design my PCR primers?

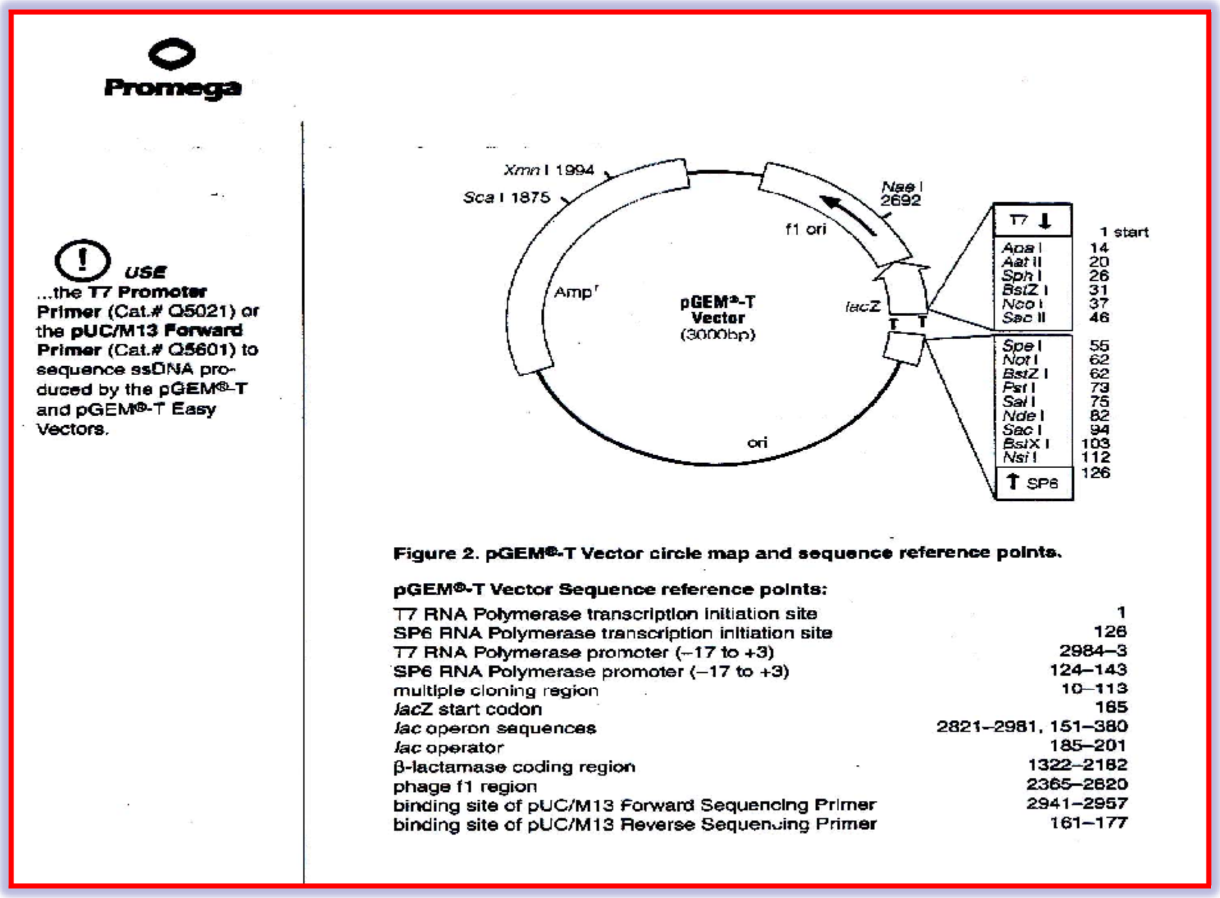
PCR primers are typically between 18 and 25 nucleotides having 40% to 60% GC. Complementary sequences at the 3'end of the primer pairs must be avoided to prevent primer-dimer formation. Avoid more than 3 C's or G's at the 3'end. For highly degenerate primers, it is preferable that the most unambiguous sequence be at the 3'end. At least the last 3 bases adjacent to the 3'end need to be correctly base paired to initiate PCR.

What is "hot start" PCR?

Hot start is a modification of PCR that decreases the tendency to get nonspecific PCR products. One of the reagents, usually *Taq* DNA polymerase, is left out of the initial denaturation. Then, the tube is held at 80°C and the enzyme is added.

What is the recommended concentration of $MgCl_2$ for PCR?

The $MgCl_2$ has to be optimized for each template and primer pair. The final concentration varies between 1 and 2.5 mM. The presence of EDTA can inhibit amplification by chelating the magnesium ions. Excess nucleotides also bind magnesium and may necessitate an increase in magnesium.


Promega
Xmn I 1994
Sca I 1875
Nae I
2692
f1 ori
Amp^r
pGEM®-T
Vector
(3000bp)
lacZ
T
T
ori
T7
1 start
Apa I 14
Aat II 20
Sph I 26
BstZ I 31
Nco I 37
Sac II 46
Spe I 55
Not I 62
BstZ I 62
Pst I 73
Sal I 75
Nde I 82
Sac I 94
BstX I 103
Nsi I 112
126
SP6

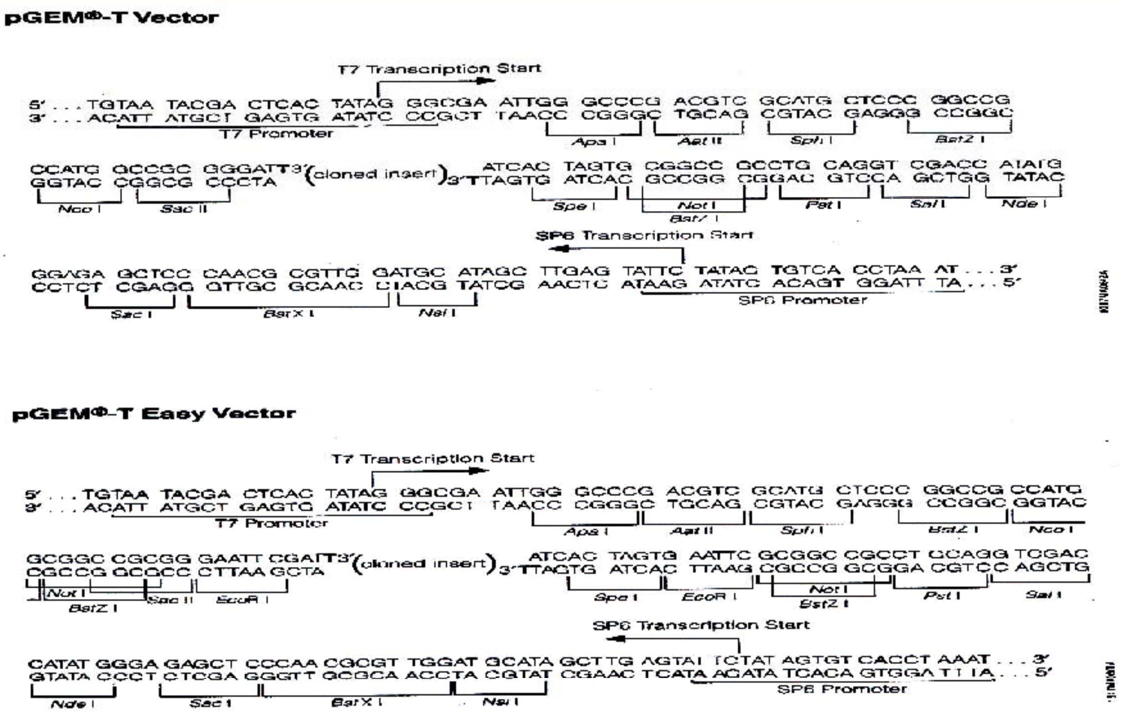
USE

...the **T7 Promoter Primer** (Cat.# Q5021) or the **pUC/M13 Forward Primer** (Cat.# Q5601) to sequence ssDNA produced by the pGEM®-T and pGEM®-T Easy Vectors.

Figure 2. pGEM®-T Vector circle map and sequence reference points.

pGEM®-T Vector Sequence reference points:

T7 RNA Polymerase transcription initiation site	1
SP6 RNA Polymerase transcription initiation site	126
T7 RNA Polymerase promoter (–17 to +3)	2984–3
SP6 RNA Polymerase promoter (–17 to +3)	124–143
multiple cloning region	10–113
lacZ start codon	165
lac operon sequences	2821–2981, 151–380
lac operator	185–201
β-lactamase coding region	1322–2182
phage f1 region	2365–2620
binding site of pUC/M13 Forward Sequencing Primer	2941–2957
binding site of pUC/M13 Reverse Sequencing Primer	161–177



pGEM®-T Vector

```
5' . . . TGTAA TACGA CTCAC TATAG GGCGA ATTGG GCCCG ACGTC GCATG CTCCC GGCCG
3' . . . ACATT ATGCT GAGTG ATATC CCGCT TAACC CGGGC TGCAG CGTAC GAGGG CCGGC

CCATG GCCGC GGGATT3'(cloned insert)   ATCAC TAGTG CGGCC GCCTG CAGGT CGACC ATATG
GGTAC CGGCG CCCTA                  3'TTAGTG ATCAC GCCGG CGGAC GTCCA GCTGG TATAC

GGAGA GCTCC CAACG CGTTG GATGC ATAGC TTGAG TATTC TATAG TGTCA CCTAA AT . . . 3'
CCTCT CGAGG GTTGC GCAAC CTACG TATCG AACTC ATAAG ATATC ACAGT GGATT TA . . . 5'
```

T7 Transcription Start
T7 Promoter
Apa I
Aat II
Sph I
BstZ I
Nco I
Sac II
Spe I
Not I
BstZ I
Pst I
Sal I
Nde I
SP6 Transcription Start
Sac I
BstX I
Nsi I
SP6 Promoter

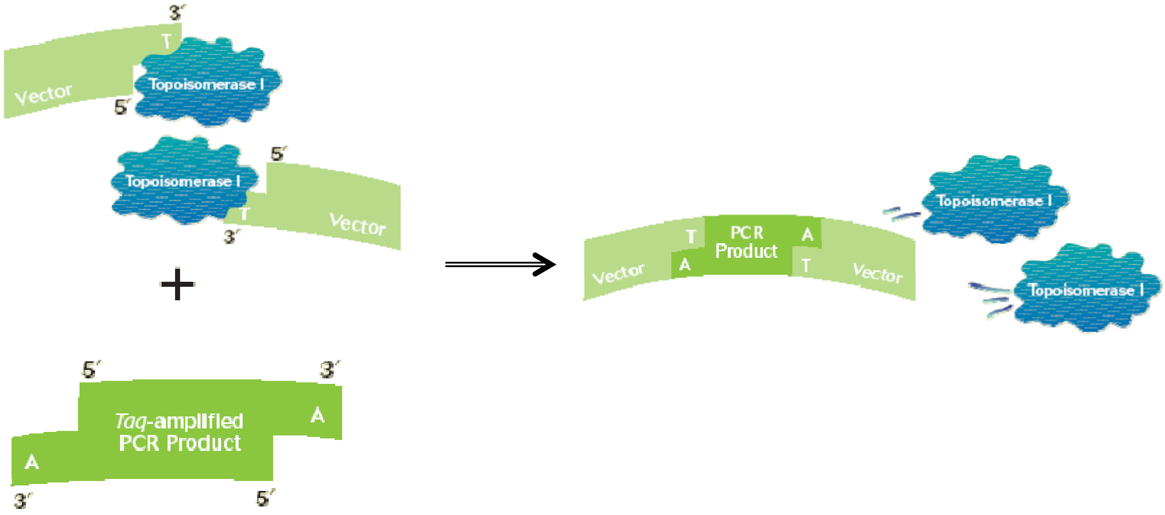
pGEM®-T Easy Vector

```
5' . . . TGTAA TACGA CTCAC TATAG GGCGA ATTGG GCCCG ACGTC GCATG CTCCC GGCCG CCATG
3' . . . ACATT ATGCT GAGTG ATATC CCGCT TAACC CGGGC TGCAG CGTAC GAGGG CCGGC GGTAC

GCGGC CGCGG GAATT CGATT3'(cloned insert)   ATCAC TAGTG AATTC GCGGC CGCCT GCAGG TCGAC
CGCCG GCGCC CTTAA GCTA                  3'TTAGTG ATCAC TTAAG CGCCG GCGGA CGTCC AGCTG

CATAT GGGA GAGCT CCCAA CGCGT TGGAT GCATA GCTTG AGTAT TCTAT AGTGT CACCT AAAT . . . 3'
GTATA CCCT CTCGA GGGTT GCGCA ACCTA CGTAT CGAAC TCATA AGATA TCACA GTGGA TTTA . . . 5'
```

T7 Transcription Start
T7 Promoter
Apa I
Aat II
Sph I
BstZ I
Nco I
Not I
BstZ I
Sac II
EcoR I
Spe I
EcoR I
Not I
BstZ I
Pst I
Sal I
SP6 Transcription Start
Nde I
Sac I
BstX I
Nsi I
SP6 Promoter

Figure 1. The promoter and multiple cloning sequence of the pGEM®-T and pGEM®-T Easy Vectors. The top strand of the sequence shown corresponds to the RNA synthesized by T7 RNA Polymerase. The bottom strand corresponds to the RNA synthesized by SP6 RNA Polymerase.

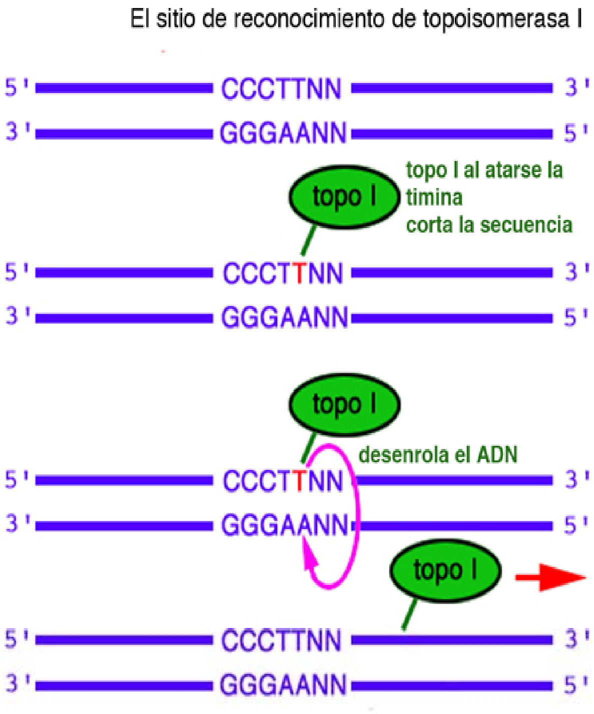
TOPO TA Cloning Kit
Dual Promoter (Invitrogen)


3′
T
Vector
Topoisomerase I
5′
Topoisomerase I
5′
T
3′
Vector
+
5′
3′
Taq-amplified
PCR Product
A
A
3′
5′
Vector
T
A
PCR
Product
A
T
Vector
Topoisomerase I
Topoisomerase I

Clonamiento de los productos PCR: topoisomerasa

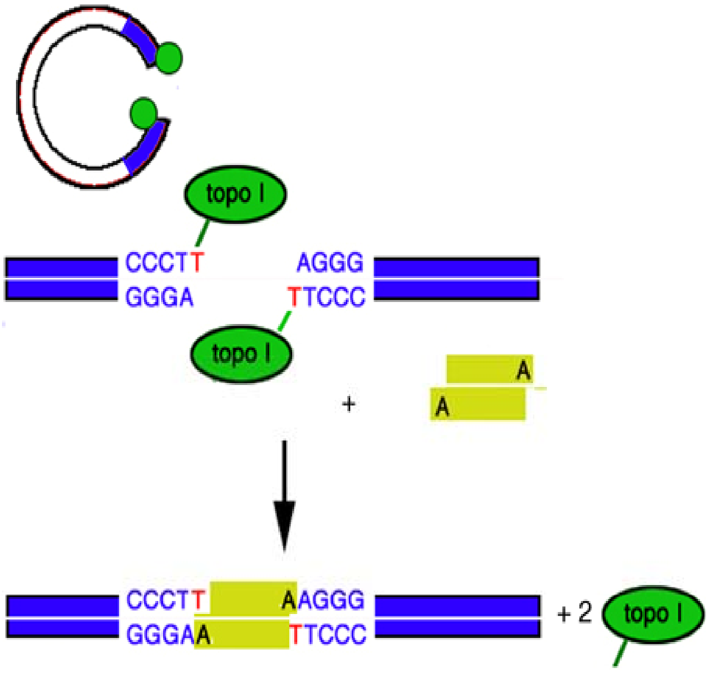
Para clonar los productos PCR, se puede utilizar la DNA topoisomerasa, una enzima que corta y enrolla-desenrolla DNA en el curso de la replicación. La topoisomerasa separa las hebras de DNA, permitiendo a la hebra separada desenrollarse alrededor del eje de la hebra no separada. La enzima liga entonces los extremos de la hebra separada y desprendida de DNA.

Su funcionamiento se parece a la vez a una enzima de restricción y una ligasa. La topoisomerasa I del virus *Vaccinia* reconoce la secuencia 5'-(C/T)CCTT-3' en una hebra doble de DNA, y se une de manera covalente a un 3'-fosfato de la timina situado al final de esta secuencia.

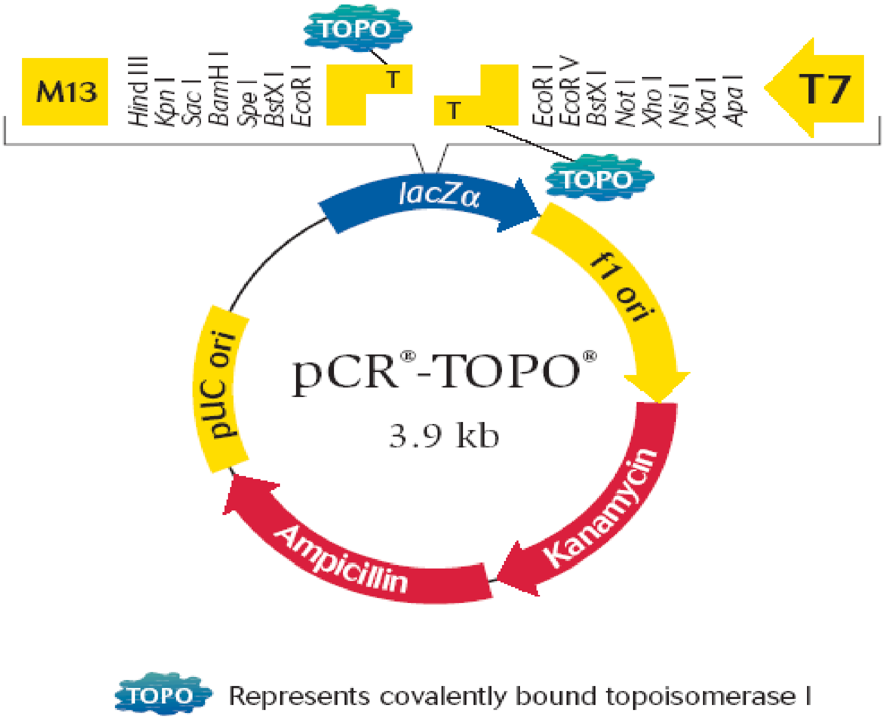

El sitio de reconocimiento de topoisomerasa I

5'
CCCTTNN
3'
3'
GGGAANN
5'
topo I
topo I al atarse la timina corta la secuencia
5'
CCCTTNN
3'
3'
GGGAANN
5'
topo I
desenrola el ADN
5'
CCCTTNN
3'
3'
GGGAANN
5'
topo I
5'
CCCTTNN
3'
3'
GGGAANN
5'

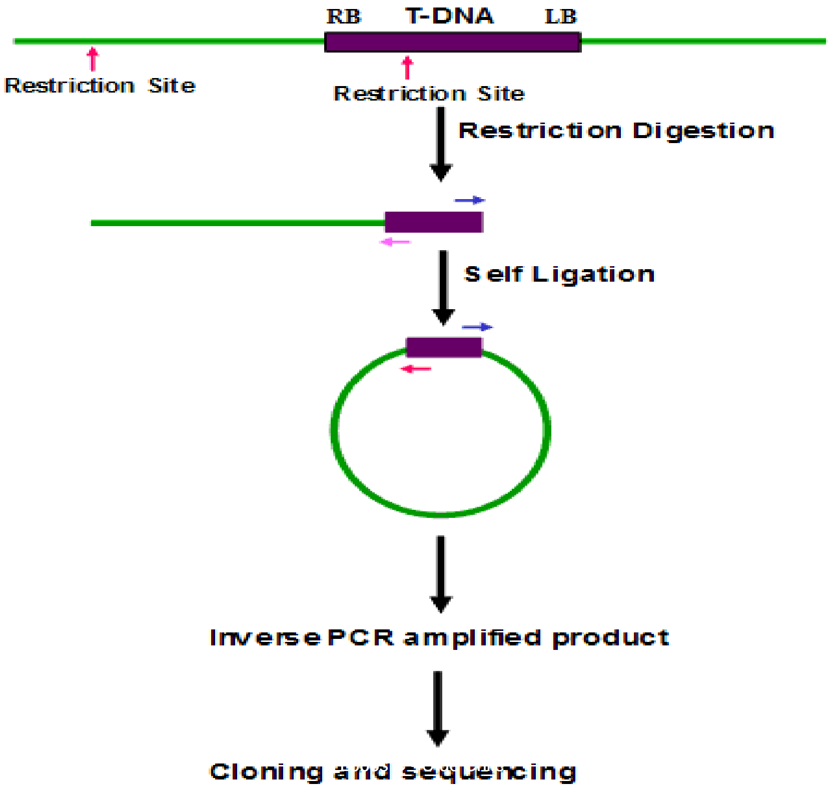
La secuencia de reconocimiento de la topoisomerasa se coloca en cada extremo de un plásmido linearizado. Cuando el vector es mezclado con la topoisomerasa, la enzima se une de manera covalente al extremo 3'-fosfato del extremo del vector. Esta acción protege al vector linearizado de su degradación por exonucleasas y evita su religado o recircularización. Se obtiene así un vector activado por la topoisomerasa. Los productos PCR que carecen de extremo 5'-fosfato sirven de sustrato de la enzima y son unidos al vector por la topoisomerasa en 5 minutos a la temperatura ambiente.


topo I
CCCTT
AGGG
GGGA
TTCCC
topo I
A
+
A
CCCTT
AAGGG
GGGAA
TTCCC
+ 2
topo I

pCR®2.1-TOPO®


TOPO
M13
Hind III
Kpn I
Sac I
BamH I
Spe I
BstX I
EcoR I
T
T
EcoR I
EcoR V
BstX I
Not I
Xho I
Nsi I
Xba I
Apa I
T7
TOPO
lacZα
f1 ori
Kanamycin
Ampicillin
pUC ori
pCR®-TOPO®
3.9 kb
TOPO
Represents covalently bound topoisomerase I

PCR Inverso



RB T-DNA LB

Restriction Site

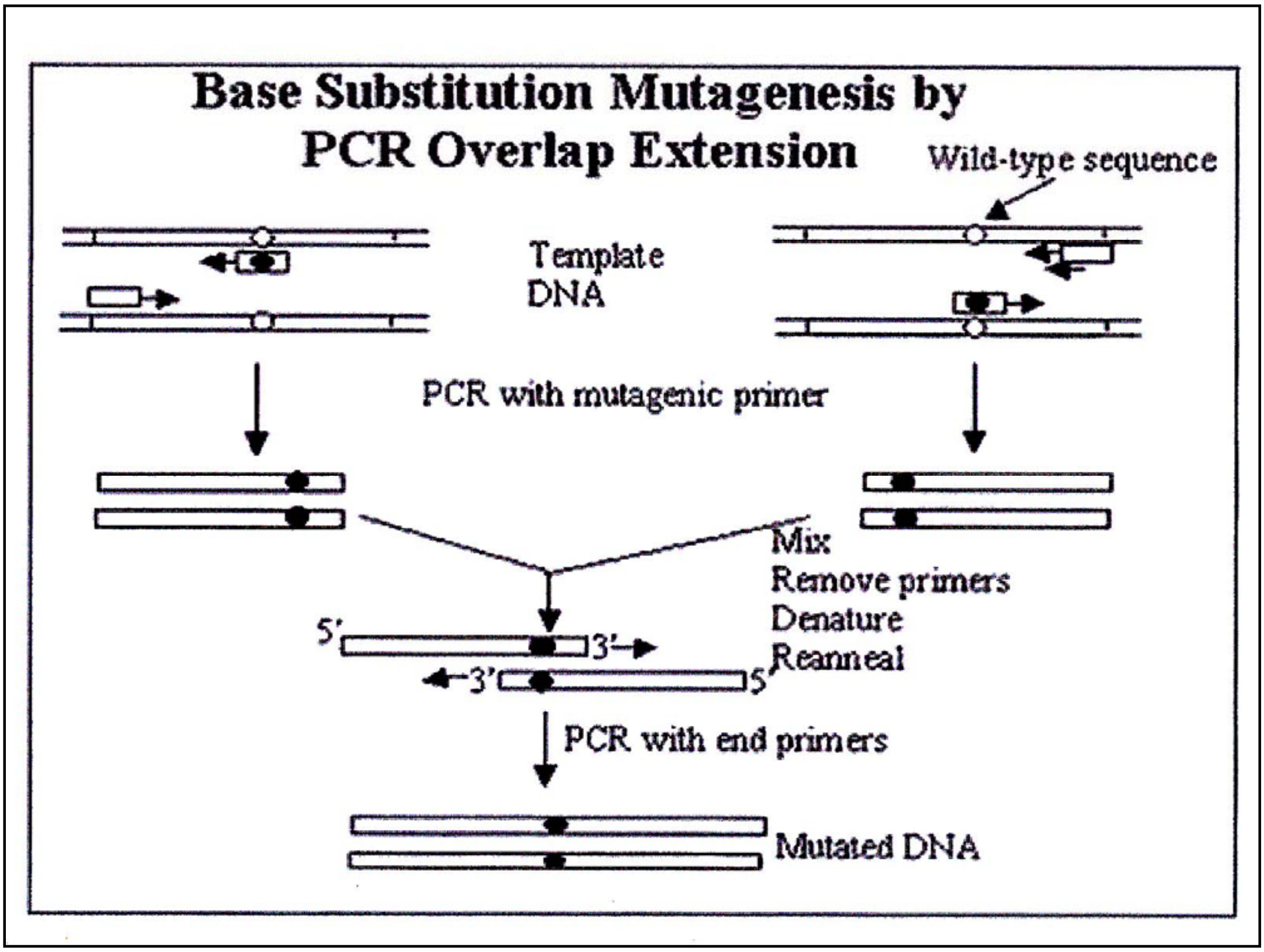
Restriction Site

Restriction Digestion

Self Ligation

Inverse PCR amplified product

Cloning and sequencing



Base Substitution Mutagenesis by PCR Overlap Extension

Wild-type sequence
Template DNA
PCR with mutagenic primer
Mix
Remove primers
Denature
Reanneal
5'
3'
3'
5'
PCR with end primers
Mutated DNA