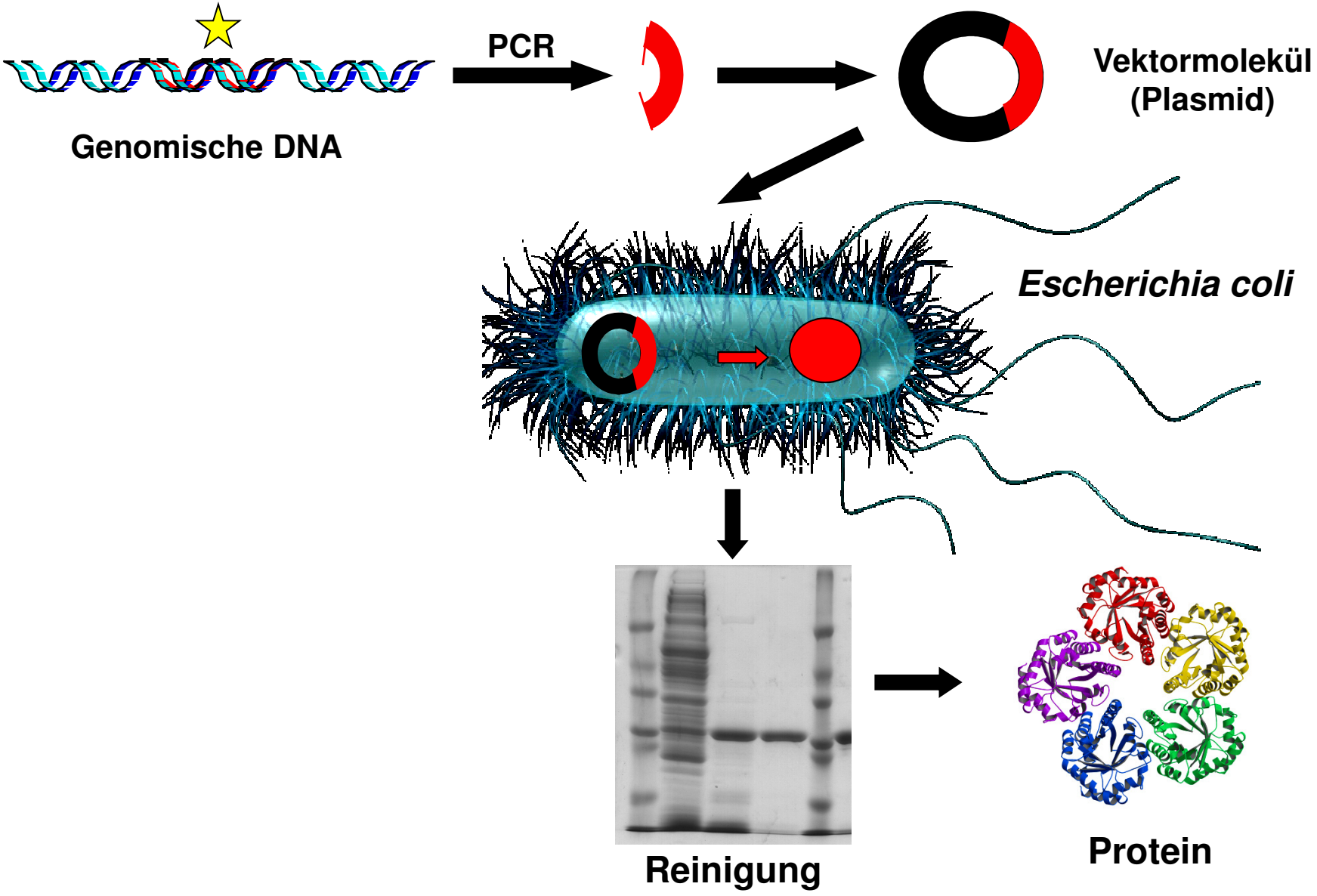
Praktikum Biochemie

„Biotechnologie“

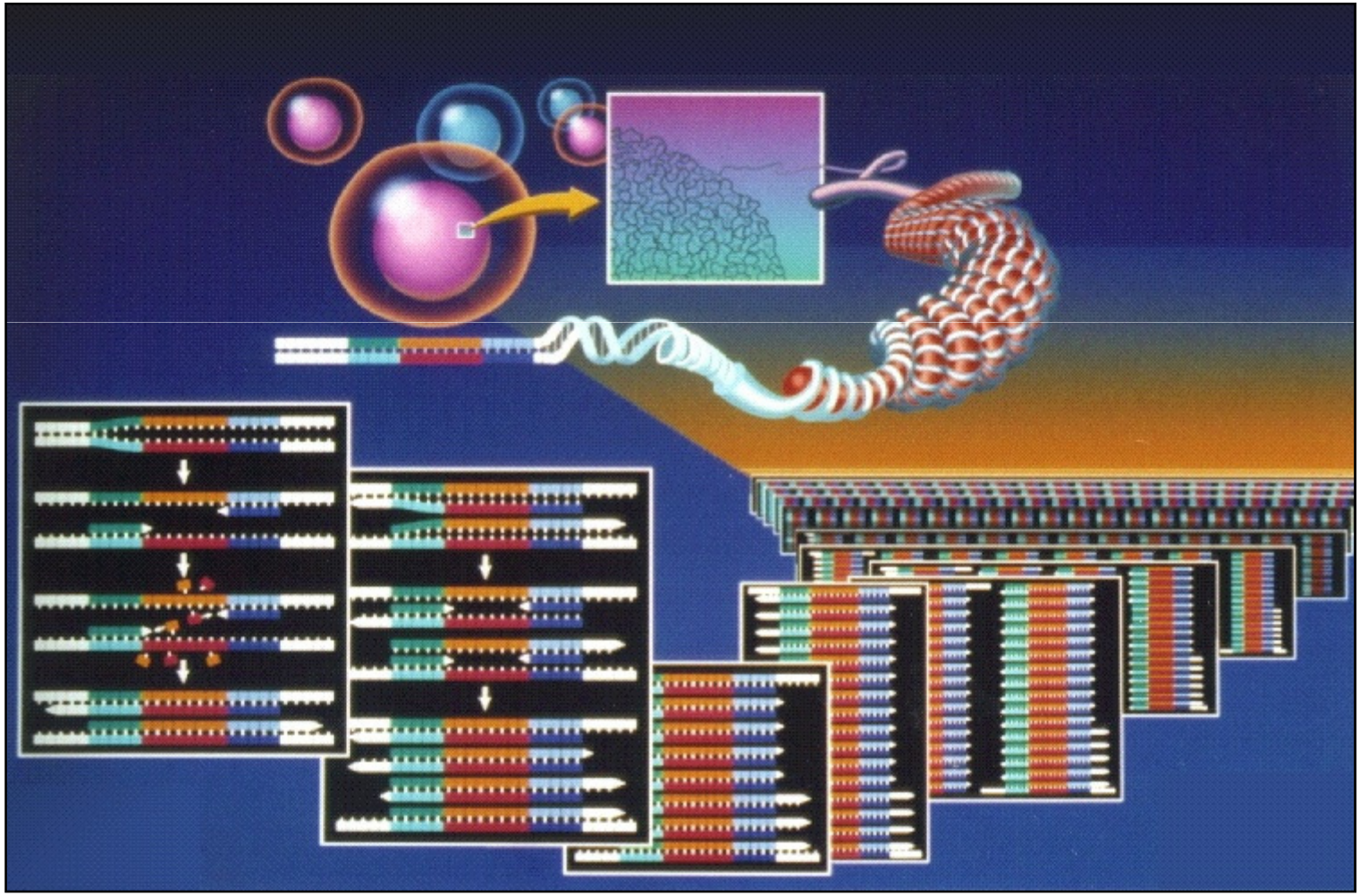
(Molekularbiologie & Biochemie)

Bettina Siebers

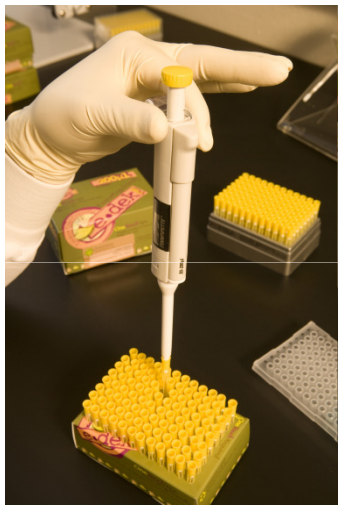
Protein Expression



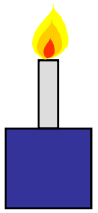
(1) Kolonie-PCR
Polymerase Chain Reaction



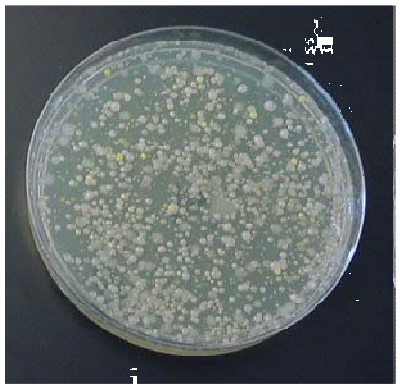
Picken der Klone

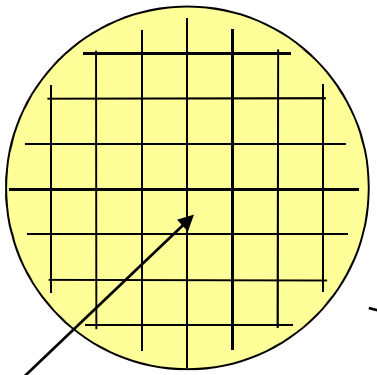


Eine Kolonie wird mit einer sterilen Spitze abgenommen und zunächst auf eine Agarplatte überführt.

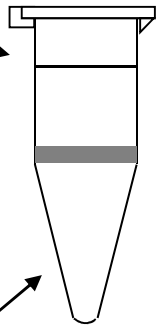


In der **Nähe des Bunsen-brennsers** arbeiten

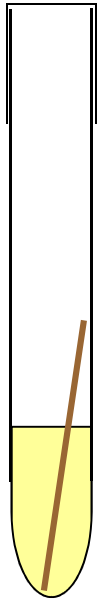




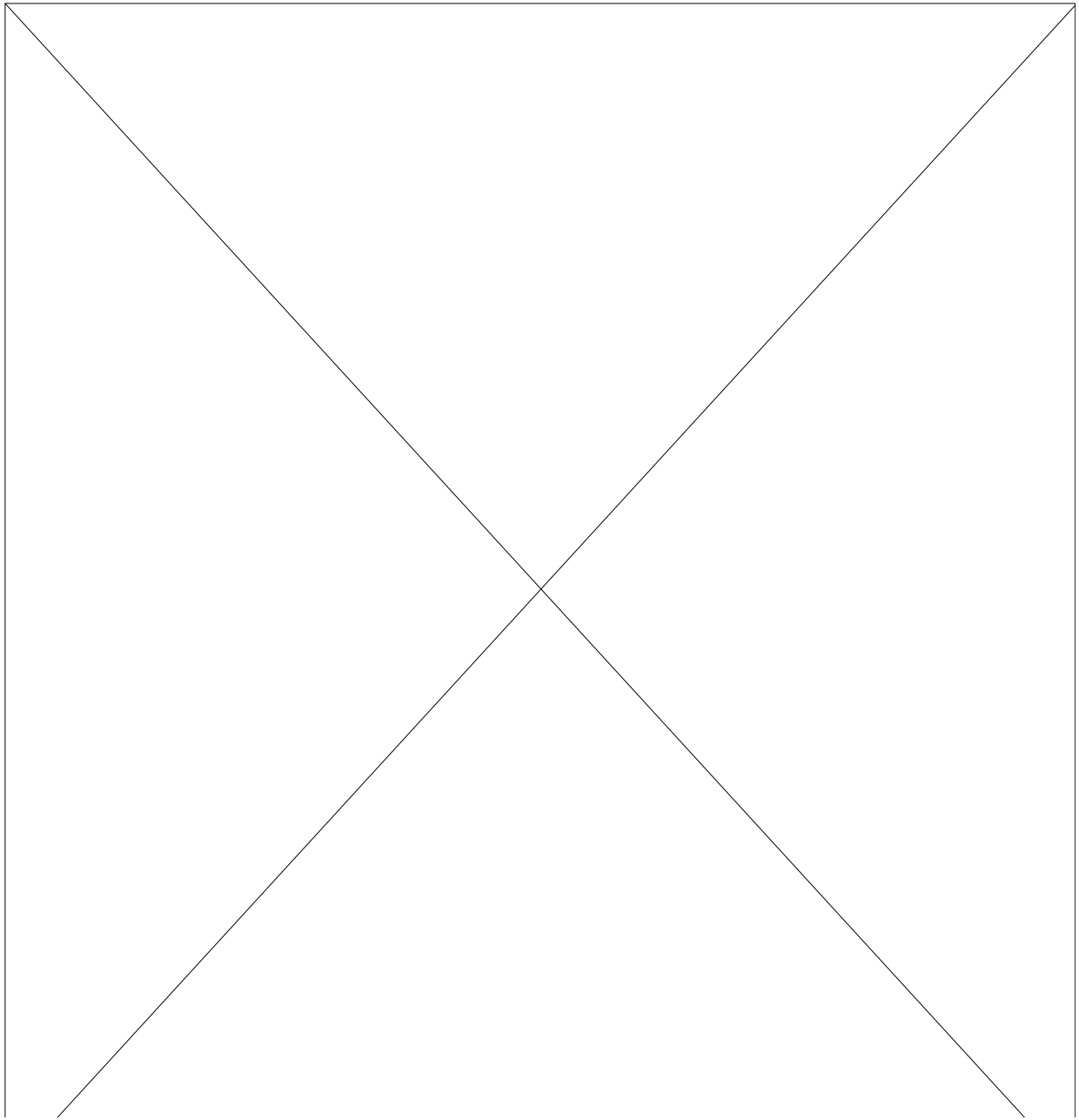
Denken sie an die korrekte **Beschriftung** von Platte, Tube und Reagenzglas!



Kolonie-PCR



Plasmid Präparation



(2) Agarose Gelelektrophorese

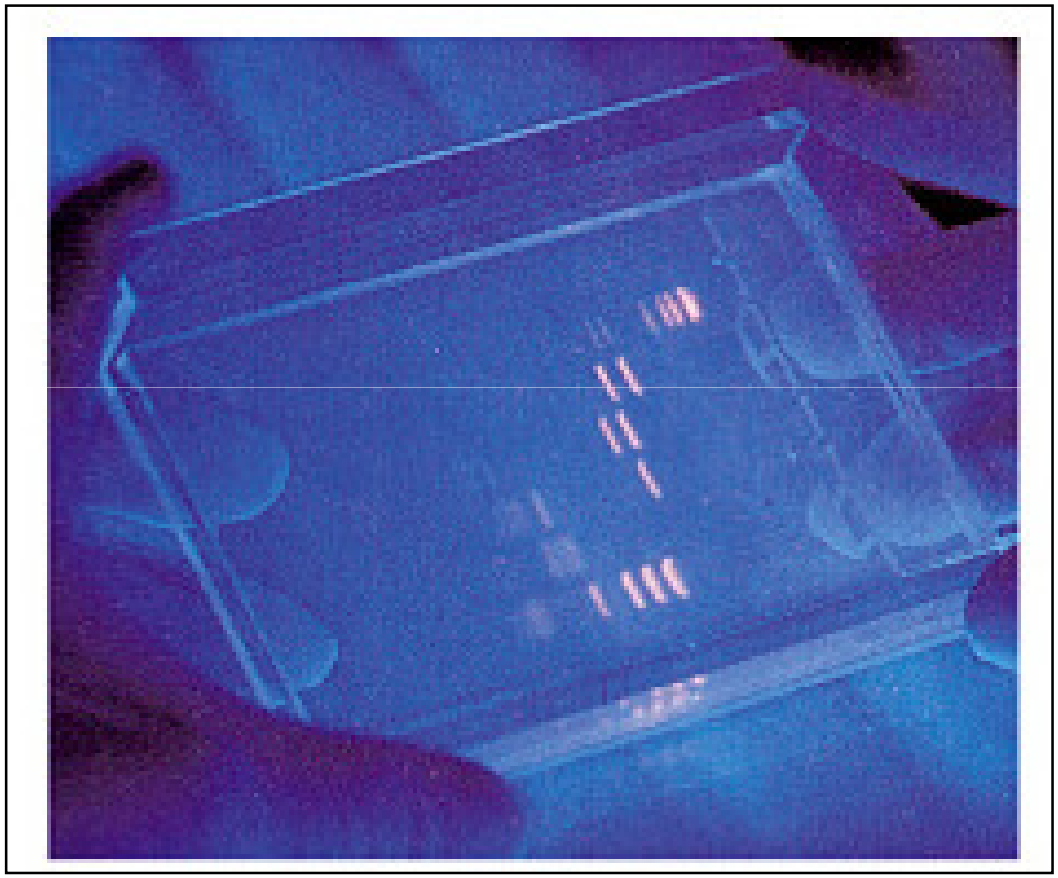


FIGURE 20.9c Biology 6/e

Marker = Größenstandard

- Die **Größe linearer DNA-Fragmente** im Agarosegel ist proportional zur Laufstrecke.
- Mit Hilfe bekannter **Längenstandards (Marker)** kann so eine genaue Größenbestimmung erfolgen.
- Als Größenmarker wird der Gene Ruler 1 kb DNA-Ladder der Firma Fermentas verwendet

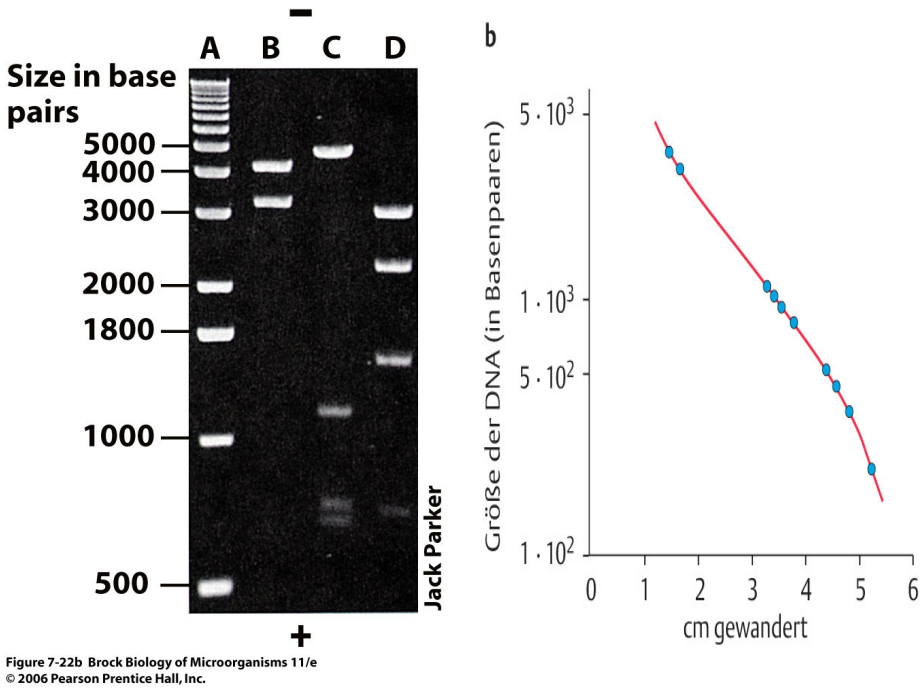
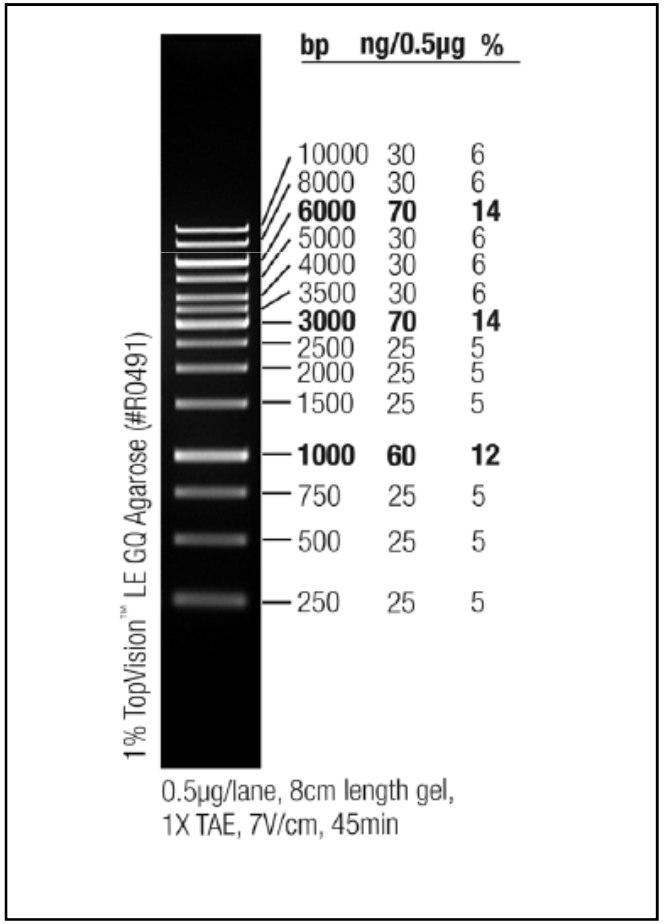
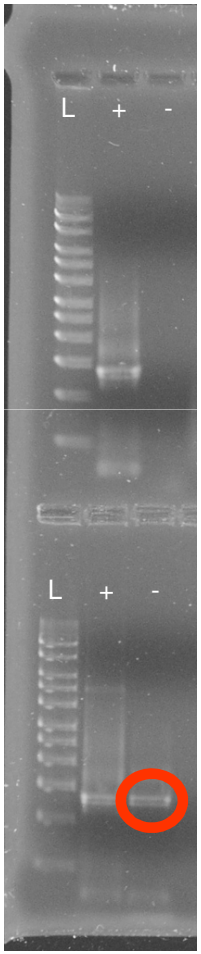


Figure 7-22b Brock Biology of Microorganisms 11/e
© 2006 Pearson Prentice Hall, Inc.

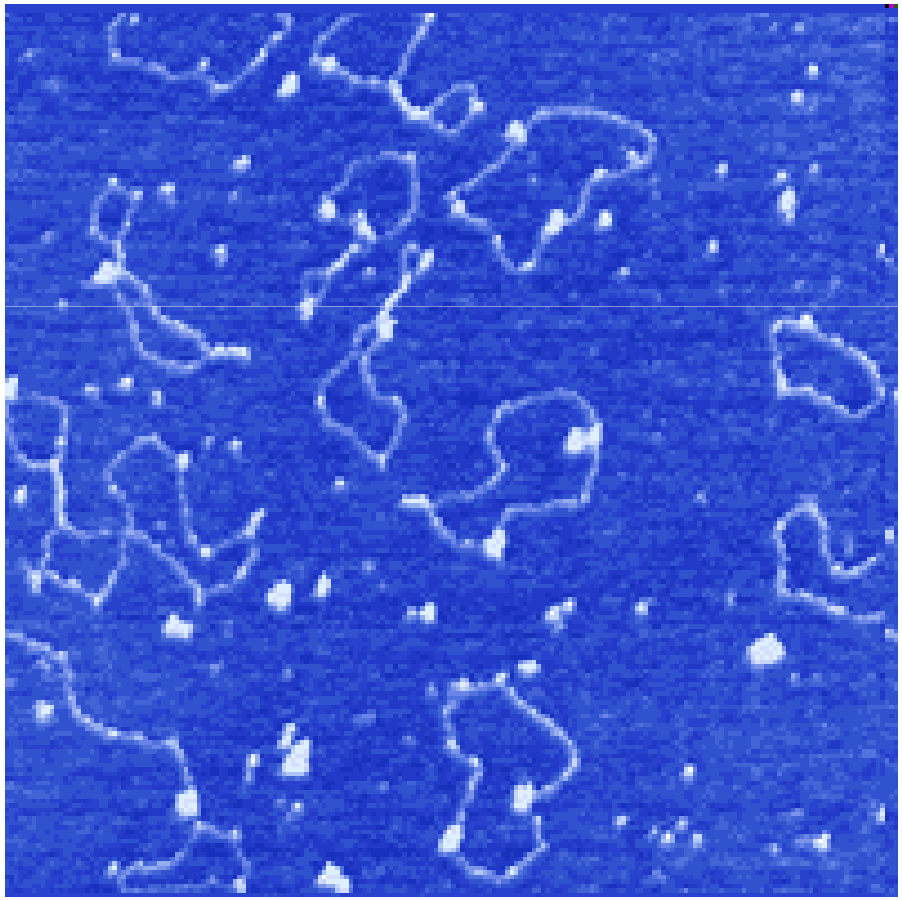


PCR



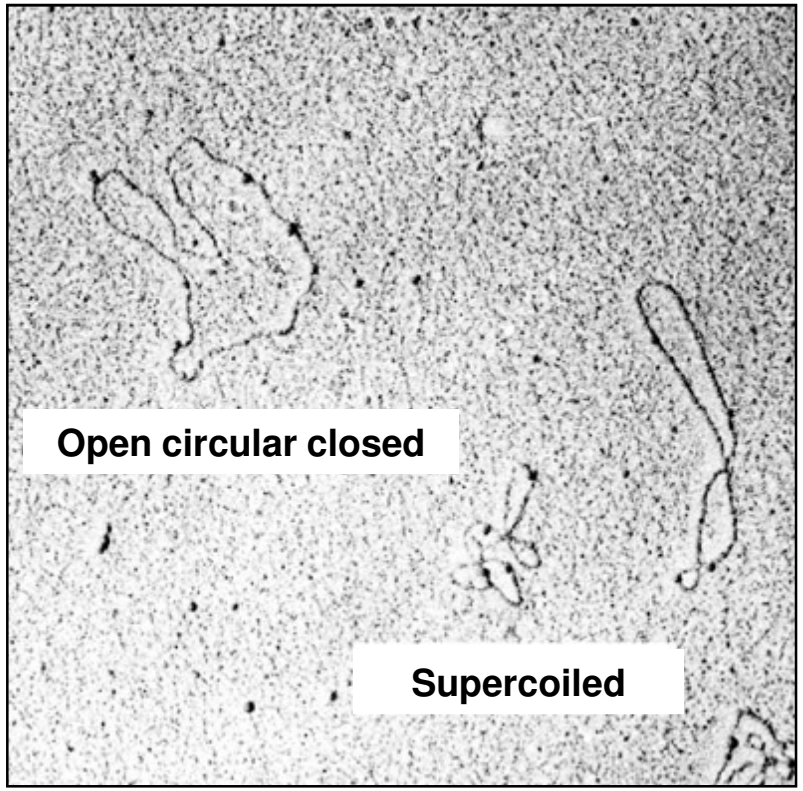
- Negativ Kontrolle mit allen Komponenten außer „template“ DNA.
- Bande in der negativ Kontrolle:
  - Ausversehen DNA in der negativ Kontrolle
  - Pipettierfehler beim Beladen des Gels
  - Kontamination einer der PCR Komponenten mit DNA

(1) Plasmidpräparation & Restriktion



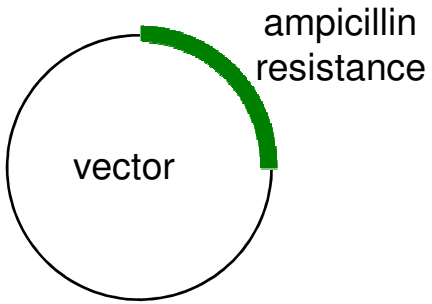
Vectors: Plasmids

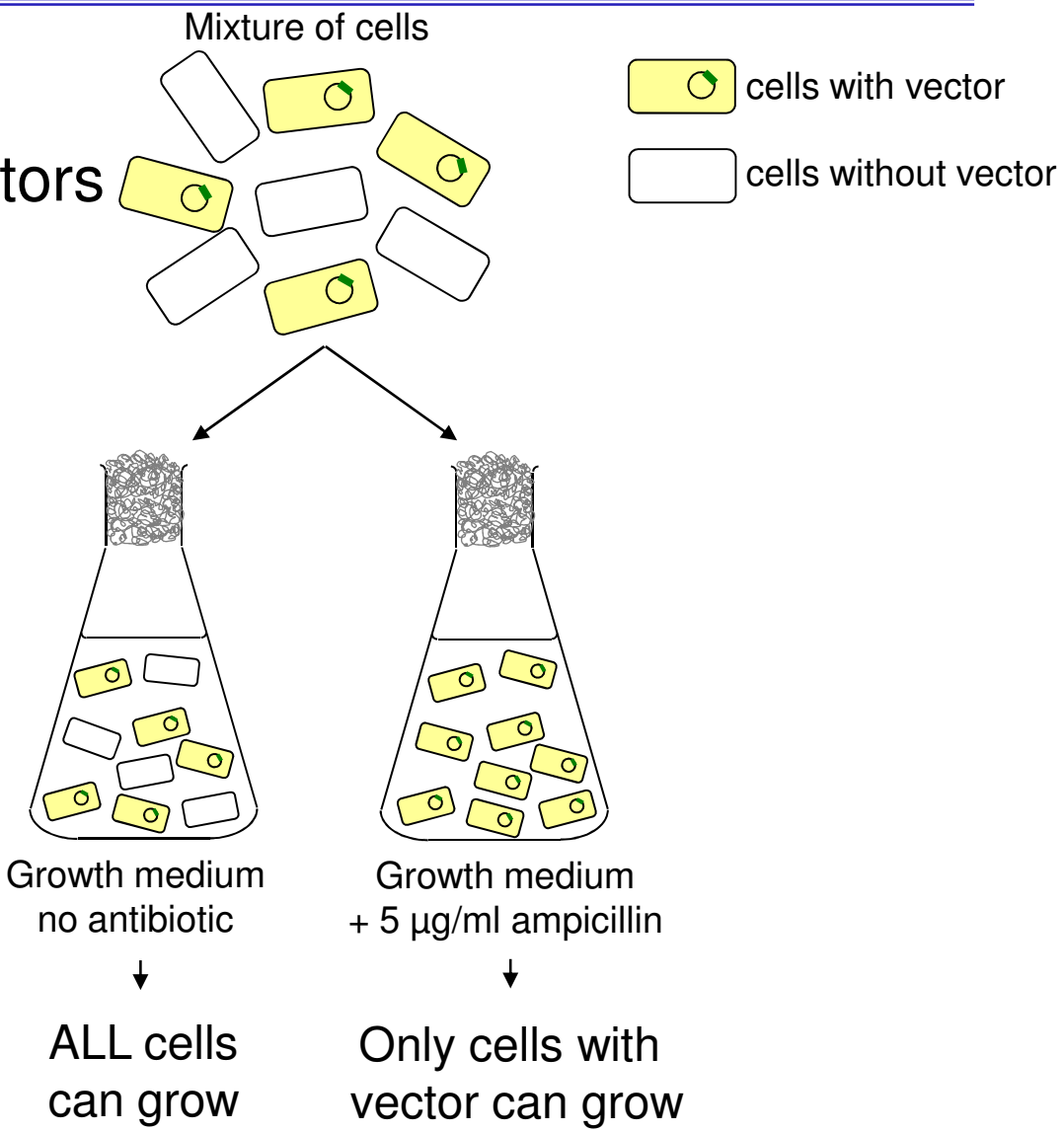
- Mostly circular double stranded DNA
- Variable in size
- Variable in copy number in the cell
- Can multiply in the cell independently (not always)
- For replication an origin (*ori*) of replication is required



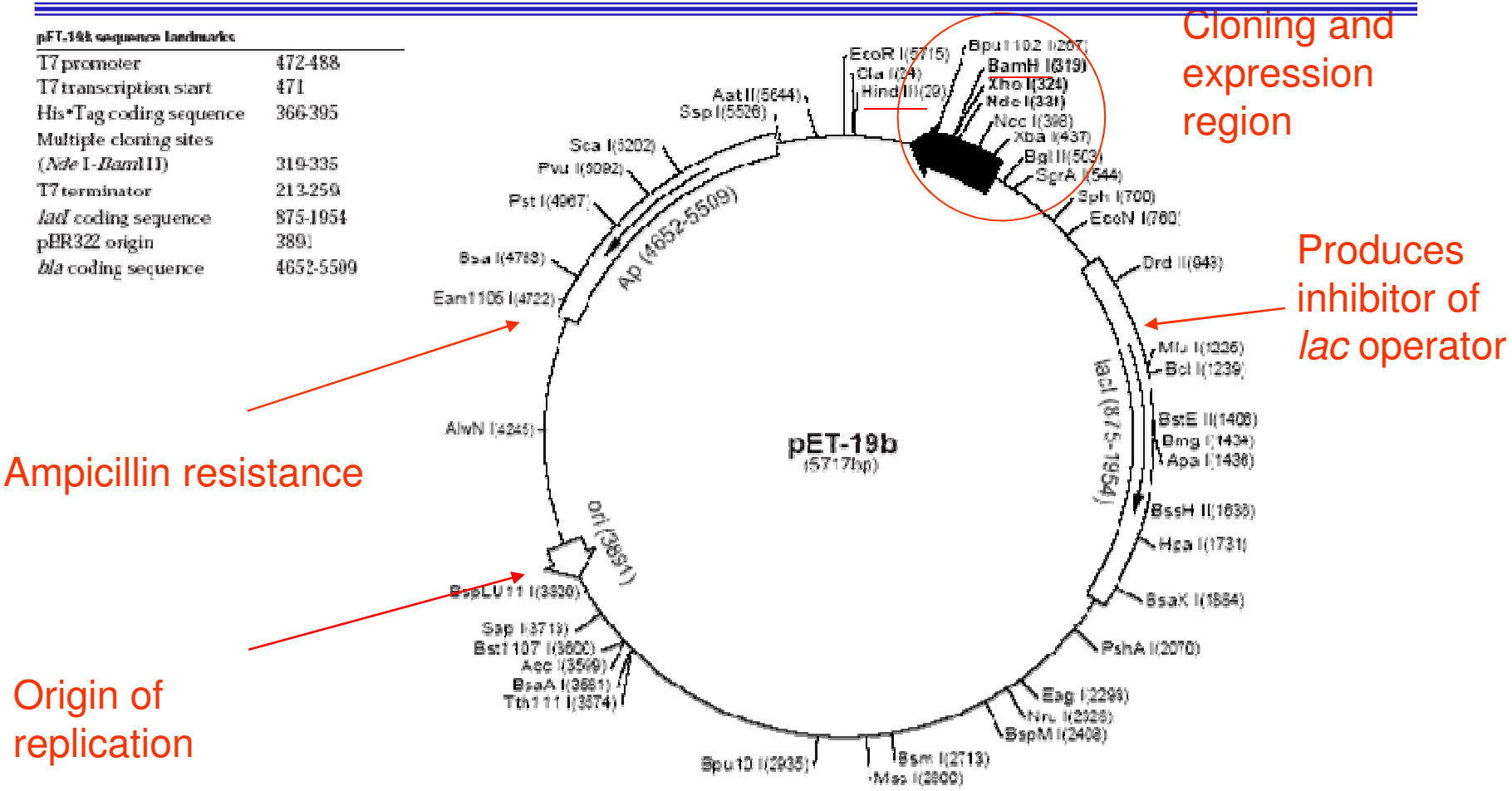
Vectors

- Genetic markers on vectors
  - Antibiotic resistance





Expressionsvektor: pET-System

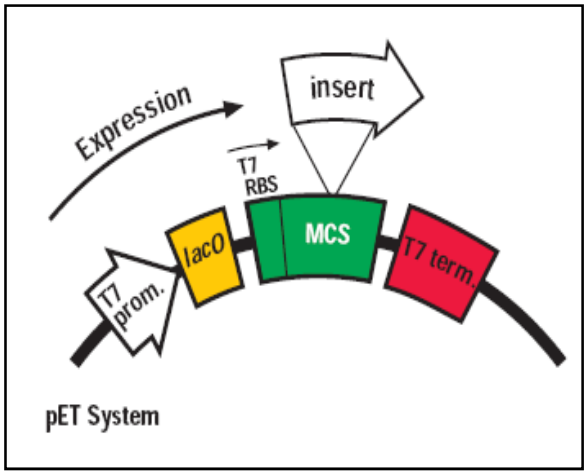


pET-19b sequence landmarks

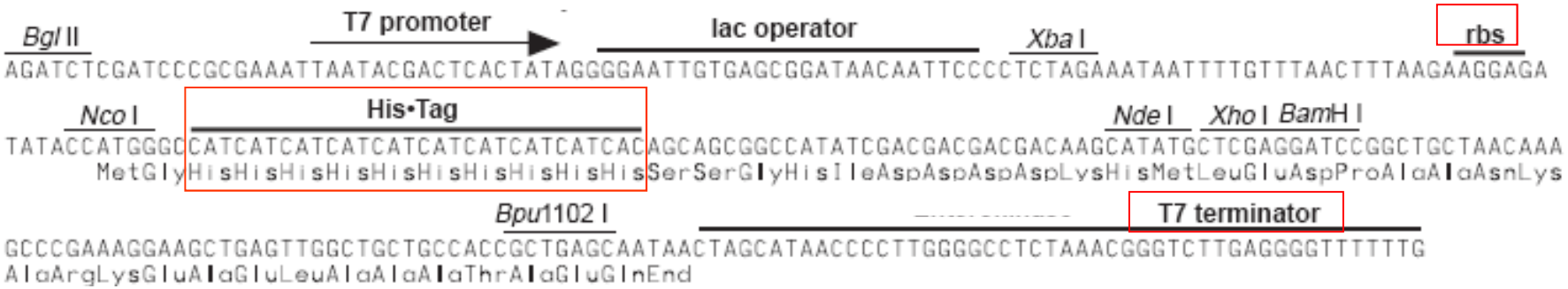
T7 promoter	472-488
T7 transcription start	471
His•Tag coding sequence	366-395
Multiple cloning sites (*Nde* I-*Bam*H I)	319-335
T7 terminator	213-259
lacI coding sequence	875-1954
pBR322 origin	3891
bla coding sequence	4652-5509

- Expression vector for production of proteins with an N-terminal His-tag

pET-19b Klonierungs & Expressions Region

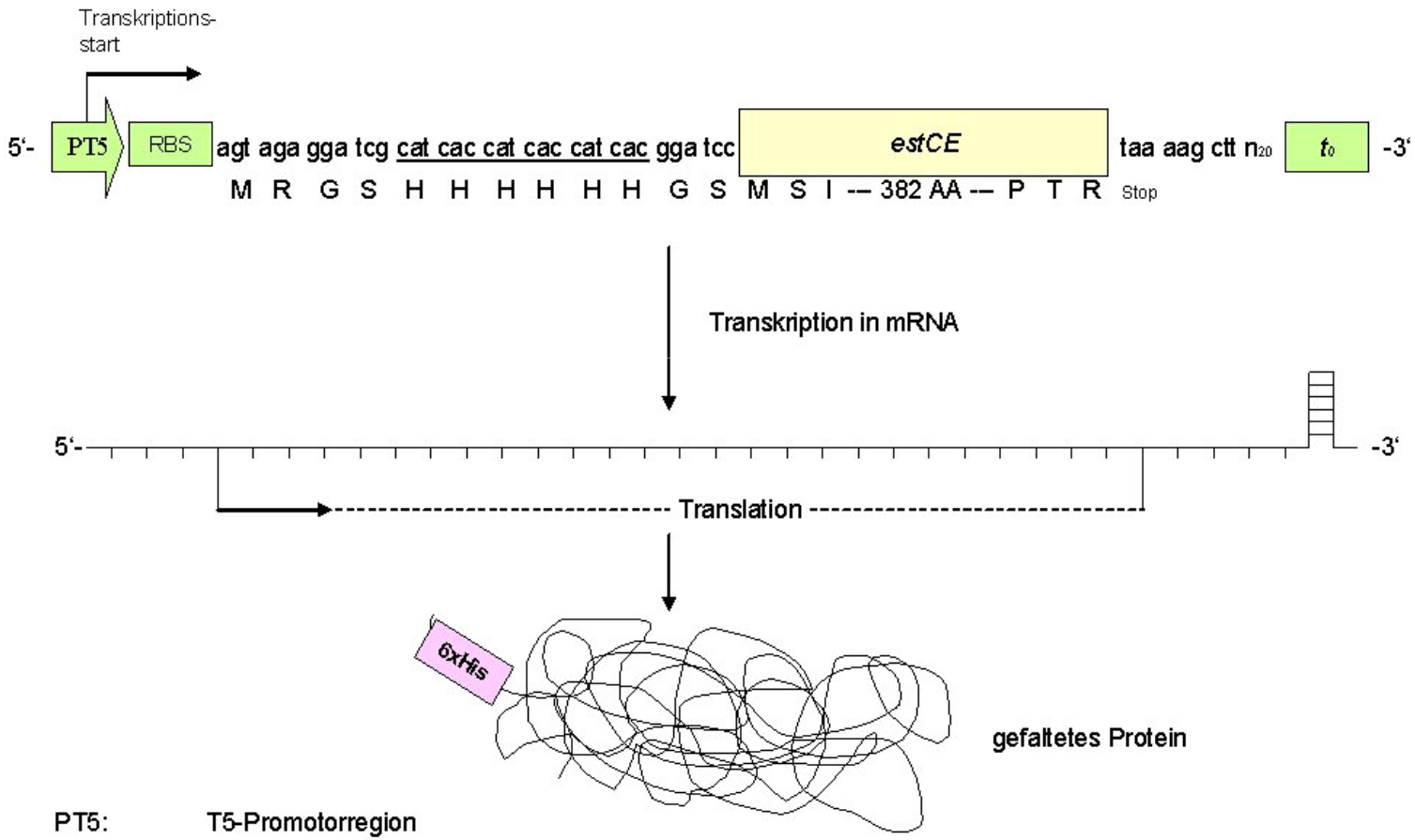


Where the inhibitor binds



- IPTG binds the inhibitor and prevents it from blocking the operator = gene is expressed.

N-terminaler His-Tag



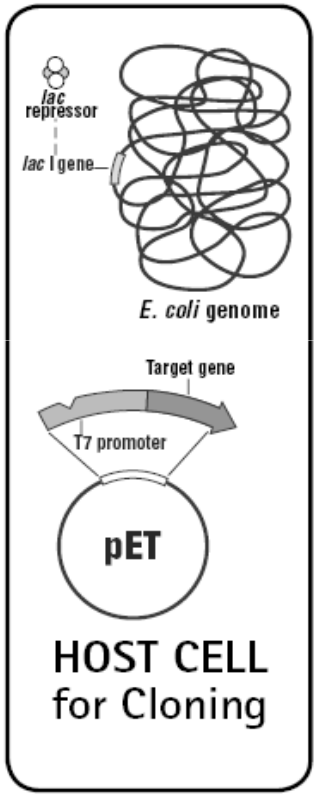
- The His-tag does not interfere with the activity of the protein.

Expression Vector

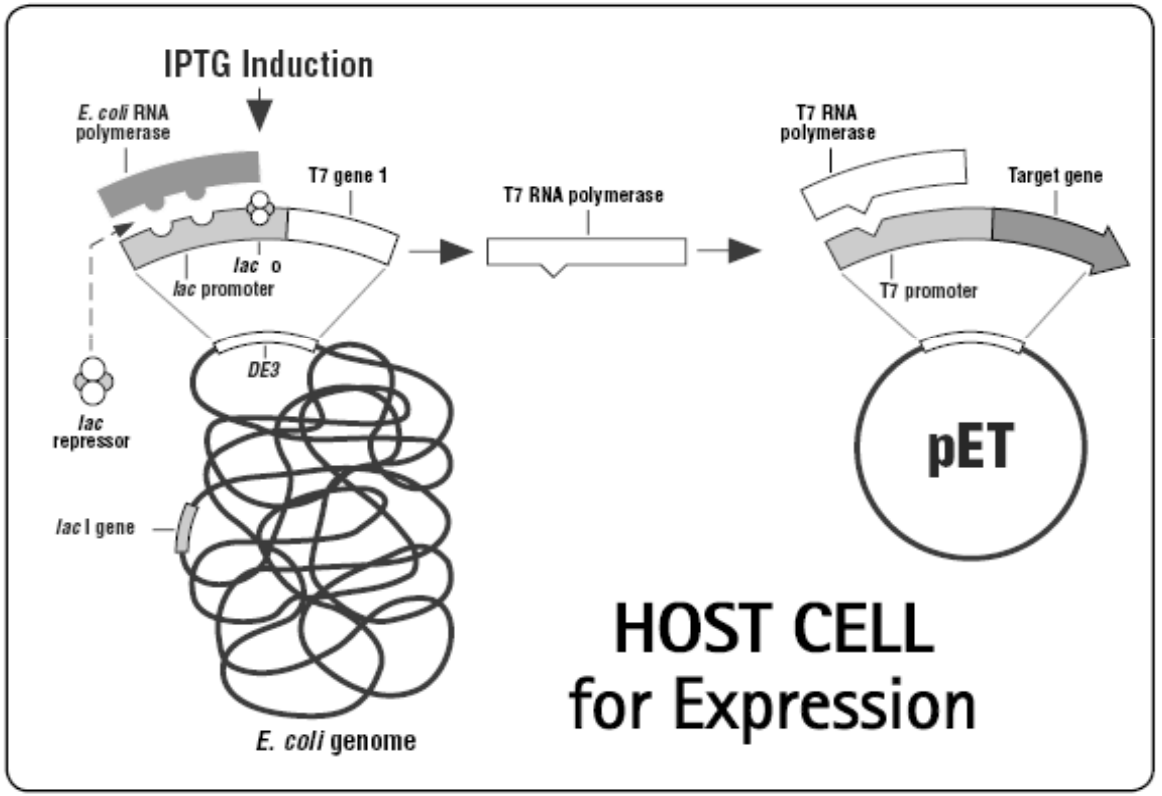
- Antibiotic resistance
- Origin of replication (ori)
- Multiple cloning site (MCP)
- Inducible Promoter (T7 Polymerase, lac operator, *lacI* gene (encodes lac repressor), Transcription)
- Ribosome binding site (rbs, Translation)
- Terminator
- Tag for purification (e.g. histidine tag)

pET Expressions System

Hosts for cloning



Hosts for expression



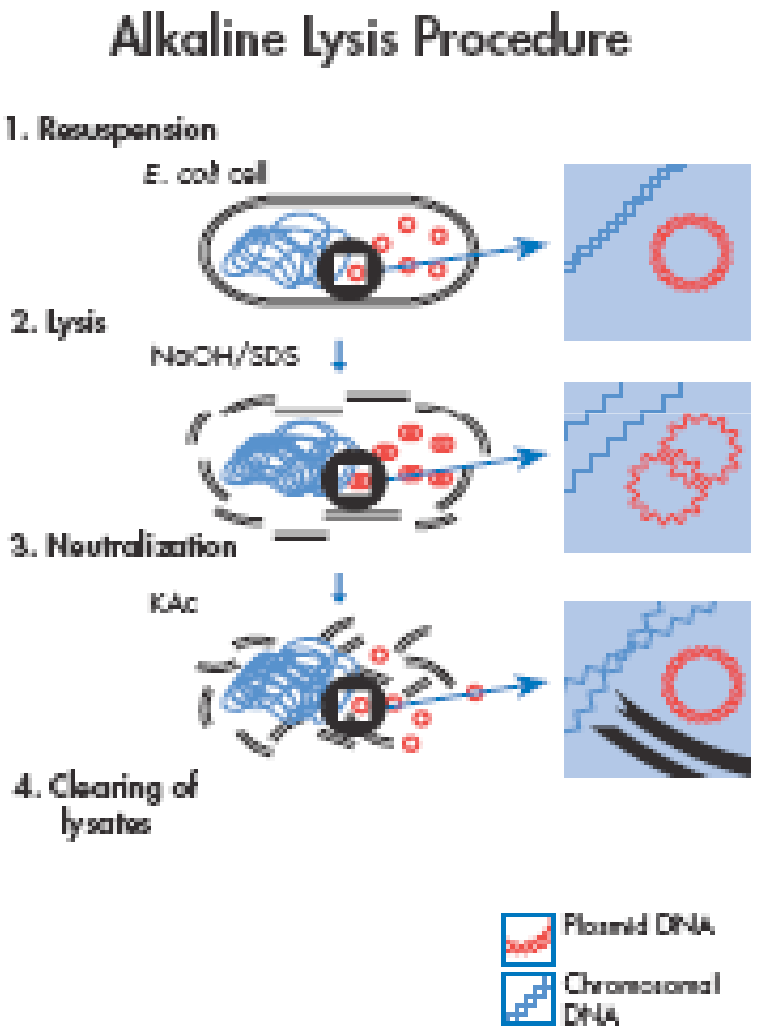
Novagen-pET System Manual-11th edition

"Spezielle Expressionsstämme mit chromosomaler **T7 RNA polymerase gene (λDE3)** (BL21 DE3)"

Plasmid isolation

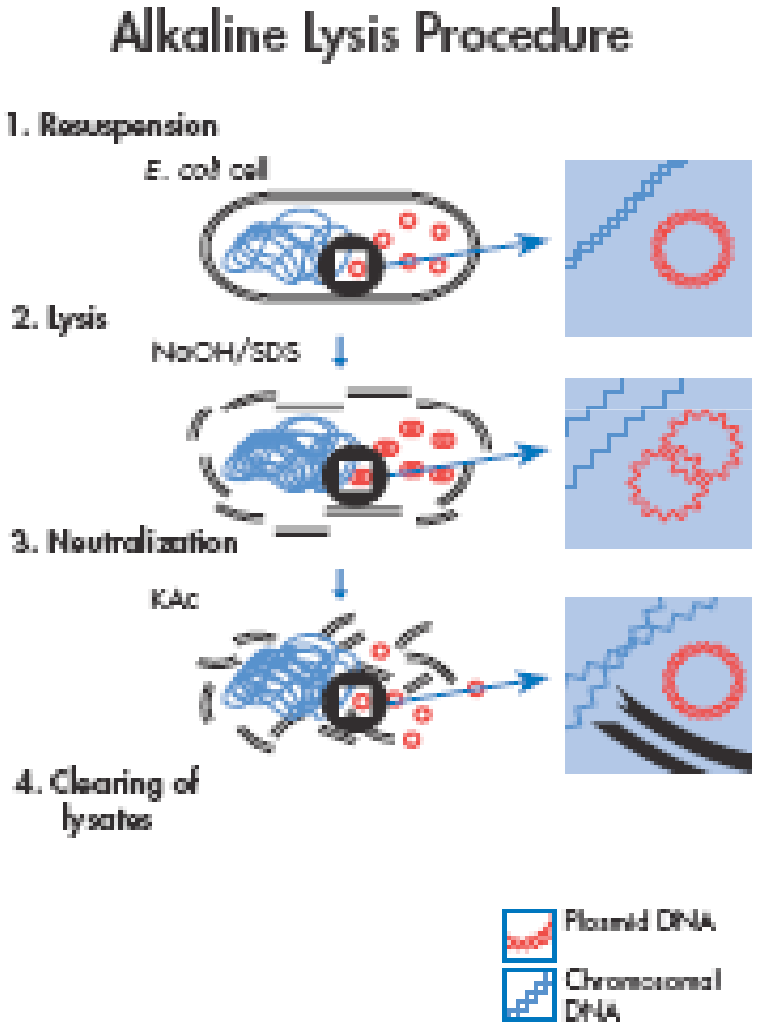
- The plasmid is within an *Escherichia coli* cell. As the *E. coli* culture grows, the quantity of the plasmid present increases.
- Plasmid is isolated using **alkaline lysis** method.
  - This method relies on the fact that plasmid DNA is closed and circular in structure.
  - Because of this it can be denatured under alkaline conditions, the same as chromosomal DNA.
  - The difference is that plasmid DNA can renature and stay soluble when the solution it is in is neutralised.

Plasmidpräparation



- 2 ml der Übernachtkultur in 2 ml Eppendorf Cup
- 2 min zentrifugieren (13.000 rpm, RT), Überstand verwerfen
- Resuspendieren des Zellpellets in 200 µl Puffer **P1** (TE Puffer + RNase A)
- Zugabe von 200 µl Puffer **P2** (NaOH, SDS) mischen; **Zell-Lyse**
- Zugabe von 200 µl Puffer **P3** (KAc^-) mischen; **Neutralisation**
- 5 min bei Raumtemperatur inkubieren
- 5 min bei 13000 rpm zentrifugieren
- Überführung des Überstandes in ein neues 1.5 ml Eppendorf Cup

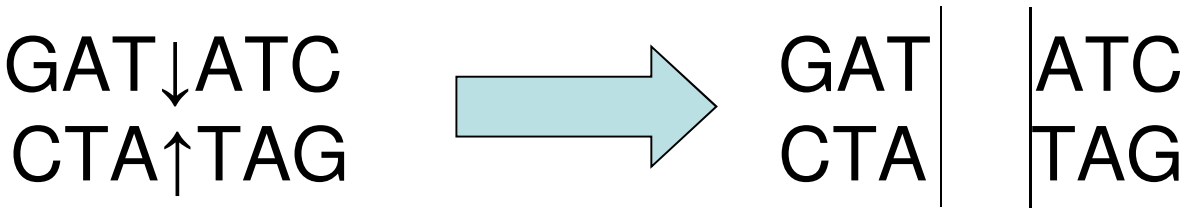
Purification of plasmid DNA



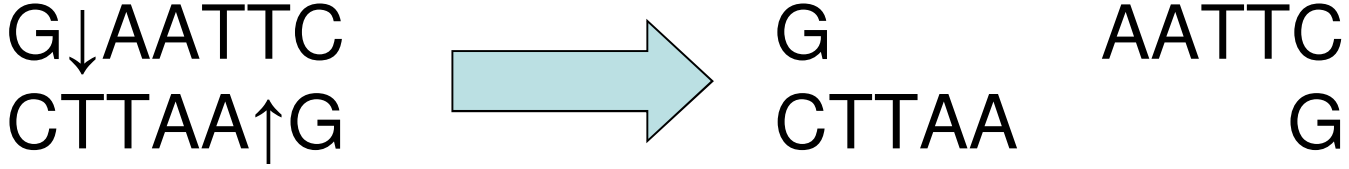
- Fällung der DNA, 1 ml Eis-kaltes Isopropanol (5 min auf Eis)
- 20 min bei 4 °C zentrifugieren.
- Isopropanol (Überstand) mit einer Pipette vorsichtig abnehmen
- 1 ml 70% Ethanol zugeben und für 10 min bei Raumtemperatur inkubieren
- 5 min zentrifugieren, Ethanol (Überstand) mit einer Pipette vollständig abnehmen
- Das Pellet für ca. 10 min bei 50 °C im Heizblock trocknen
- Lösen sie ihre DNA in 40 µl H_2O durch Inkubation für 10 min bei 50 °C

Plasmid restriction

- **Restriction enzymes** recognize specific short sequences in DNA and make breaks in the DNA
- ***Eco*RV** is a blunt cutter. This means that when the DNA is restricted (cut) with this enzyme the cut ends looks like this: **blunt = straight**

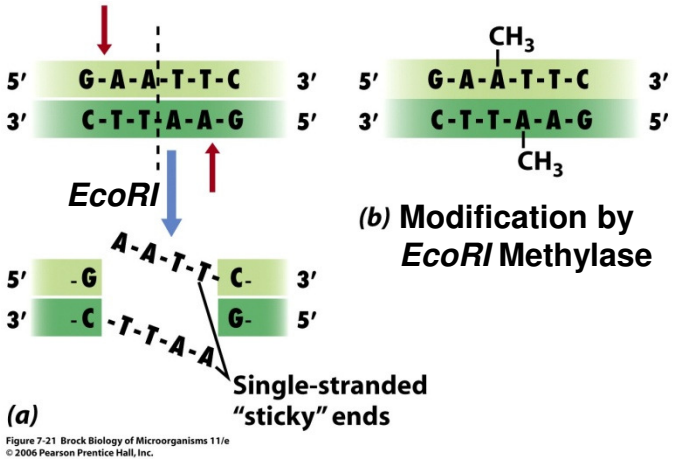


- An example of an enzyme which cuts and leaves **single-stranded, sticky ends** is *Eco*RI



Restriction enzymes

- Restriction endonucleases
- Wide-spread among prokaryotes
- Important tool in molecular biology
- Type II enzymes recognize sequences with two-fold symmetry „**Palindrome**“ (two strands have the same sequence if one is read from the left and the other from the right side



- Function:
  - Protection from invasion by foreign DNA
  - The cell protects ist own DNA from destruction by a modification system (typically methylation, e.g. *EcoRI* methylase)

Table 7.4 Recognition sequences of a few restriction endonucleases

Organism	Enzyme designation[a]	Recognition sequence[b]
Bacillus globigii	*Bgl*II	A↓GATCT
Bacillus subtilis	*Bsu*RI	GG↓CC
Brevibacterium albidum	*Bal*I	TGG↓CCA
Escherichia coli	*Eco*RI	G↓AATTC[c]
Haemophilus haemolyticus	*Hha*I	GCG↓C
Haemophilus influenzae	*Hind*II	GTPy↓PuAC
Haemophilus influenzae	*Hind*III	A↓AGCTT
Klebsiella pneumoniae	*Kpn*I	GGTAC↓C
Nocardia otitidiscaviarum	*Not*I	GC↓GGCCGC
Proteus vulgaris	*Pvu*I	CGAT↓CG
Serratia marcescens	*Sma*I	CCC↓GGG
Thermus aquaticus	*Taq*I	T↓CGA

[a] Nomenclature: The first letter of the three letter abbreviation of a restriction endonuclease designates the genus from which the enzyme originates, the second two letters, the species. The roman numeral designates the order of discovery of enzymes in that particular organism, and any additional letters are strain designations.

[b] Arrows indicate the sites of enzymatic attack. Asterisks indicate the site of methylation (modification). G, guanine; C, cytosine; A, adenine; T, thymine; Pu, any purine; Py, any pyrimidine. Only the 5′ → 3′ sequence is shown.

[c] See Figure 7.21*a*

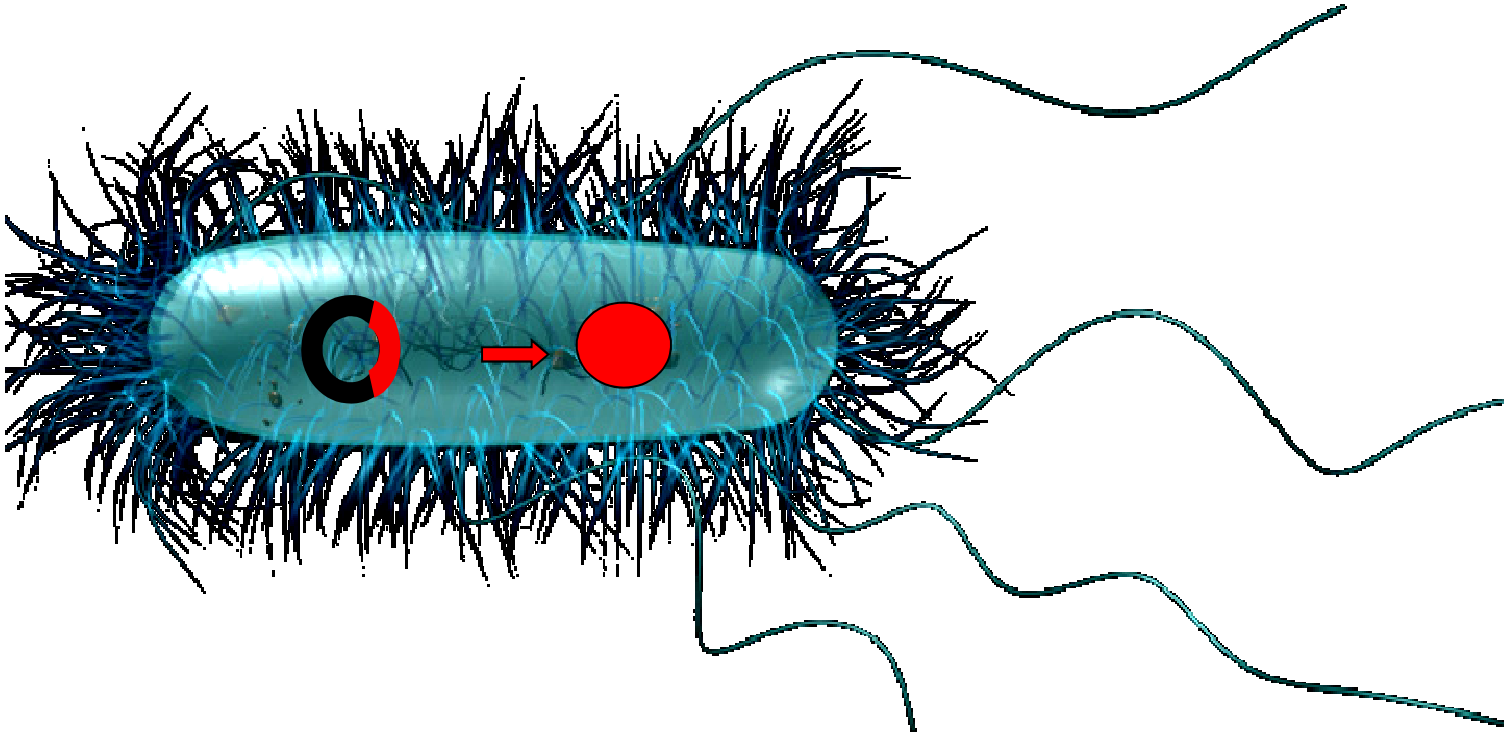
Restriktionsanalyse

- 17.5 µl DNA
- 2.0 µl Restriktions Puffer
- 0.5 µl *BamH*I Restriktions-Enzym

- Vorsichtig mischen und kurz anzentrifugieren
- Inkubation für 2h bei 37°C (ÜN)
- Abstoppen der Enzymreaktion durch 10-minütige Inkubation bei 70°C
- Die Proben können entweder direkt mittels **Agarose-Gelelektrophorese** analysiert werden oder aber bei 4°C/-20°C gelagert werden (Morgen)

(2) Rekombinante Expression

einer Esterase in *E. coli*



Heterologe Expression in *E. coli*

- Esterase Gen kloniert in den Expressionsvektor pET-19b in einem *E. coli* Expressionsstamm