Techniques in Molecular Biology

Course: Molecular Biology (02022312)

Instructor: Dr. M A Srour

Textbook:
Watson J, Baker TA, Bell SP, Gann A, Levine M, Losick R (2008). Molecular Biology of the Gene, 6th ed. Chap 21/ pp739-82

Gene expression

Why would we want to express a gene?

To study gene structure & expression

To make a large quantity of the gene's product, either for investigative purposes or for profit

If the goal is to use bacteria to produce an eukaryotic gene> use of cDNA works better

If an eukaryotic cell is used > cDNA usually is preferred

Applications of gene expression: recombinant protein production (Biotechnology)

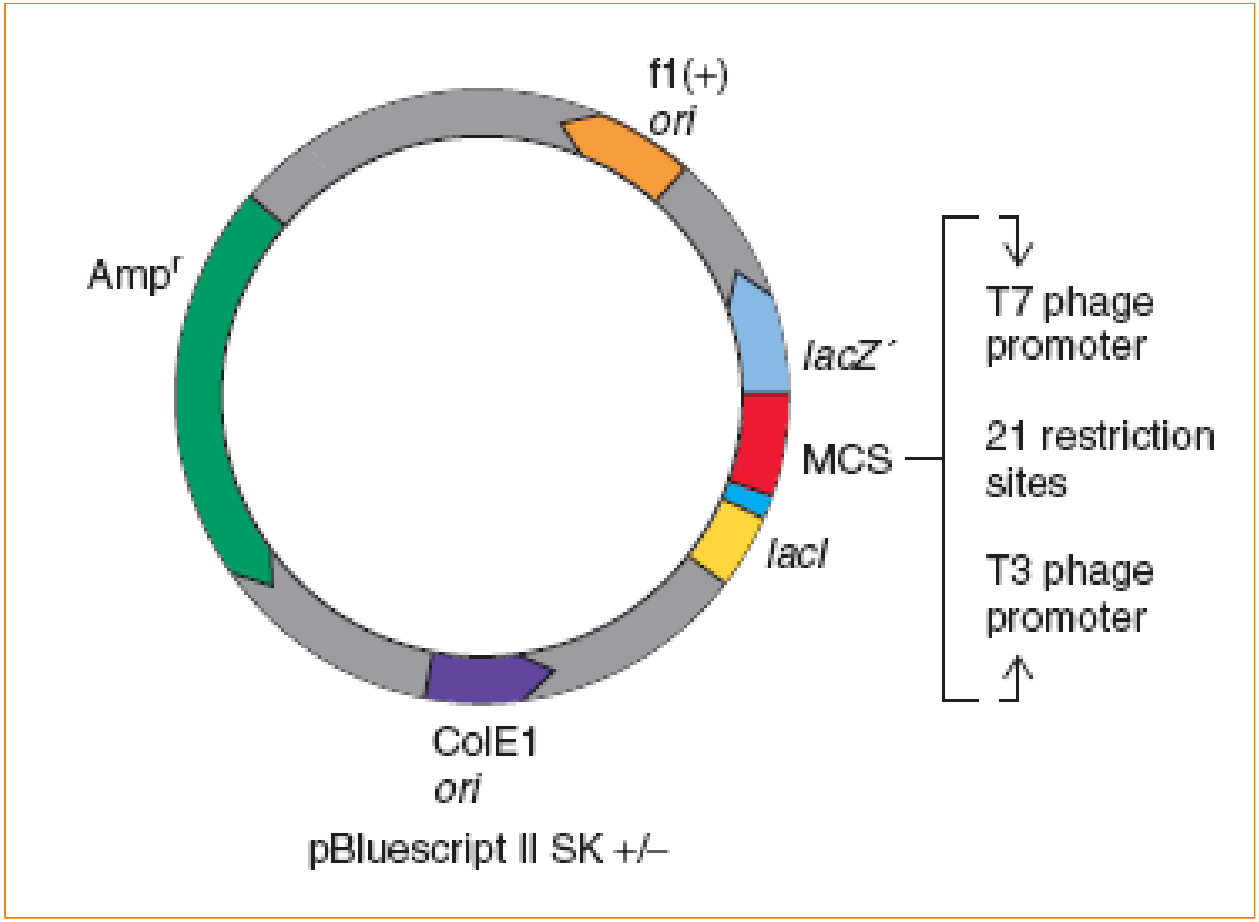
Prokaryotic Expression vectors

Ampicillin resistance gene

Origin of replication (ori)

Phage f1 origin of replication

MCS contains 21 unique restriction sites



The pBluescript vector

MCS is situated between two phage RNA polymerase promoters (T7 and T3)

MCS is embedded in an *E. coli lacZ′ gene (blue), so the* uncut plasmid will produce the β-galactosidase N-terminal fragment when an inducer such as isopropylthiogalactoside (IPTG) is added to counteract the repressor made by the *lacI gene (yellow). Thus, clones* bearing the uncut vector will turn blue when the indicator X-gal is added.

By contrast, clones bearing recombinant plasmids with inserts in the MCS will have an interrupted *lacZ′ gene, so no functional* β-galactosidase is made. Thus, these clones remain white.

Expression systems

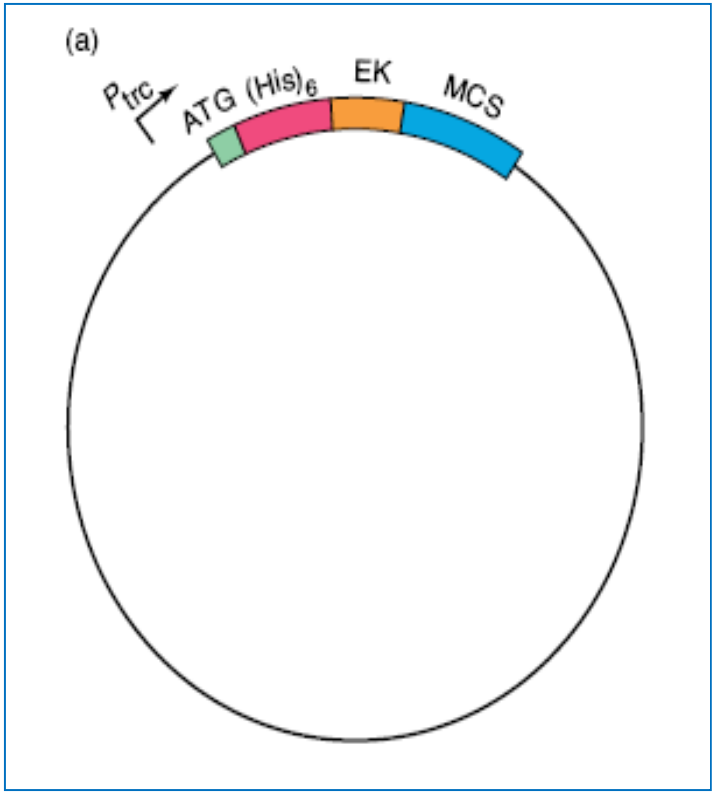
Produce recombinant proteins, e.g, therapeutic proteins like GH, G-CSF, insulin

Eukaryotes vs. prokaryotes

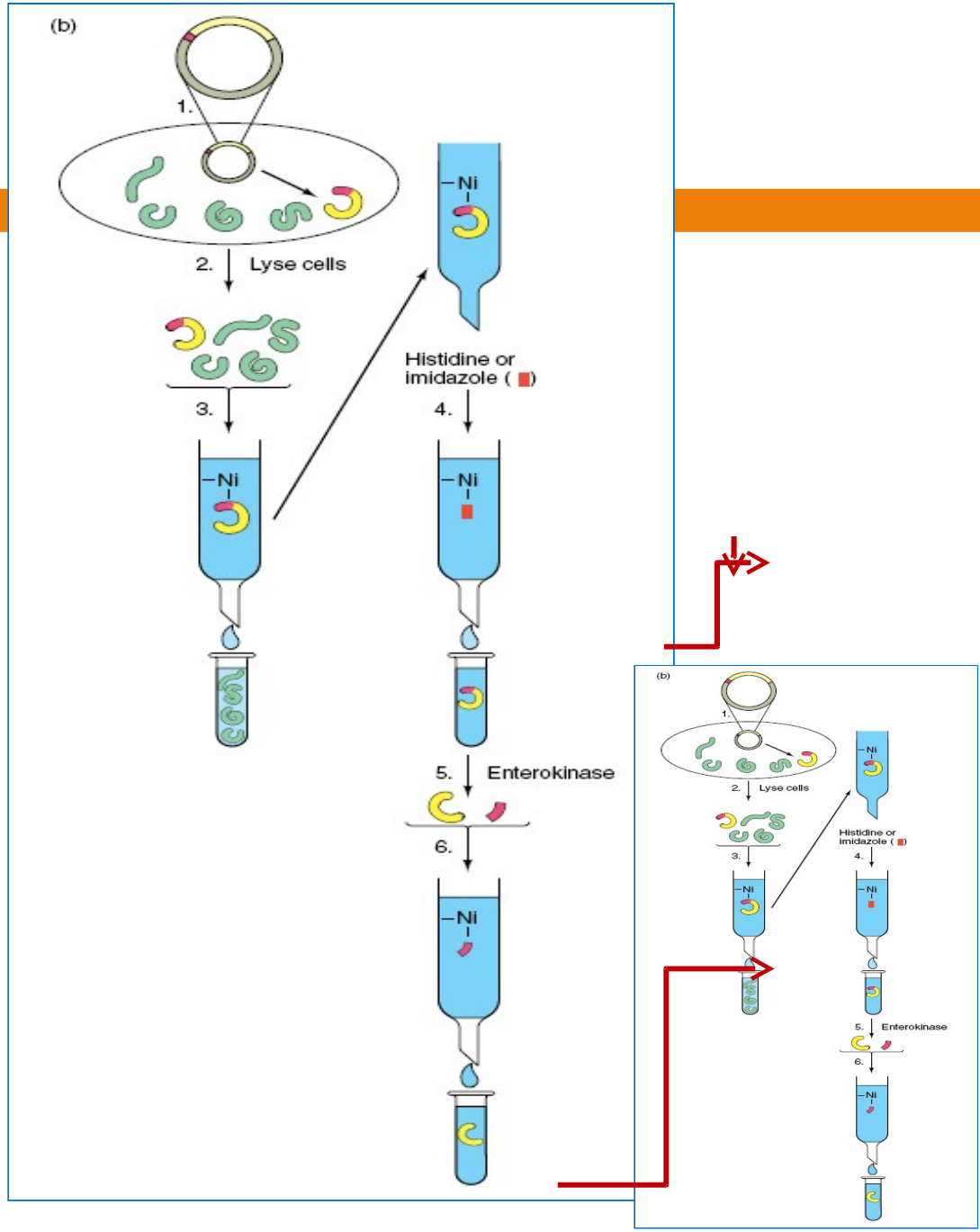
Prokaryotes: lac promoter- induce with IPTG (lactose analog) > inducible promoter

6-His tag (epitope Tag) at C- or N-terminus for purification: bind to matrix with chelated nickel and release with low pH

Prokaryotic expression vectors that produce fusion proteins



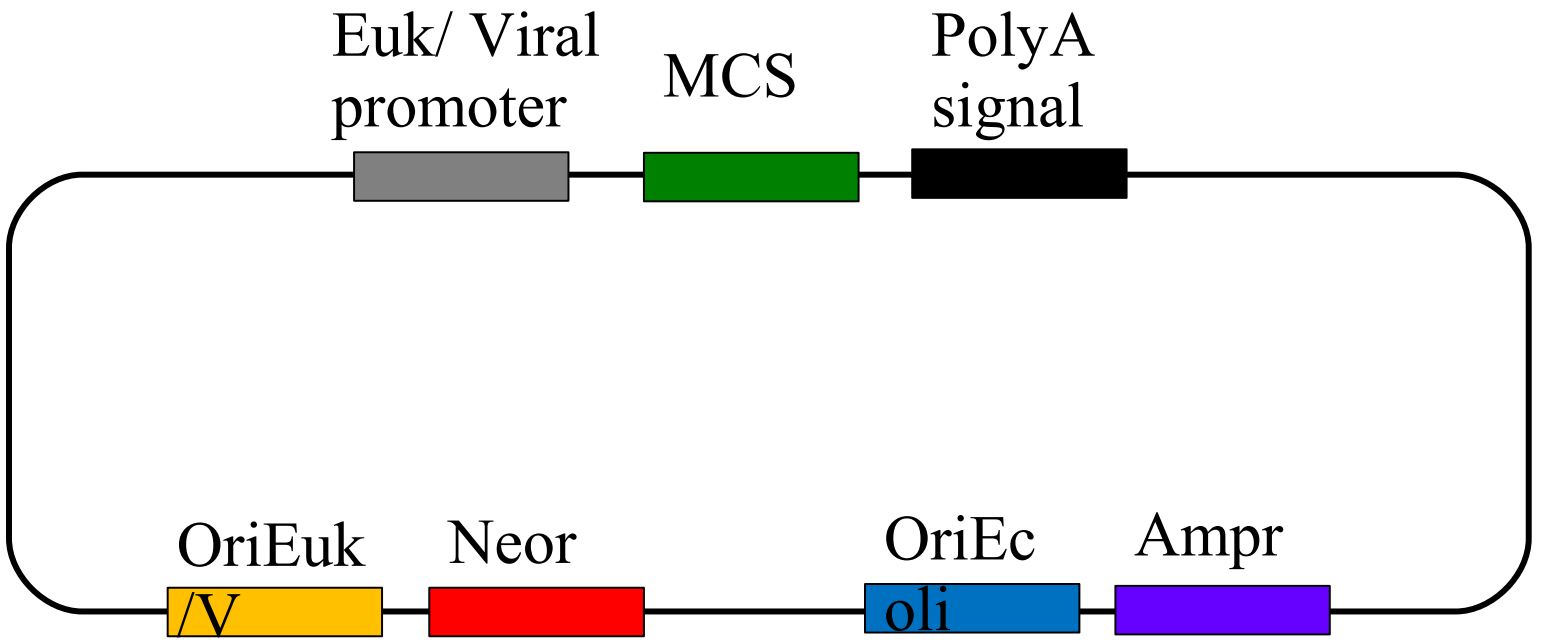
EK: a recognition site for the proteolytic



Using an oligohistidine (6xHis) expression vector

1. The gene of interest (yellow) is inserted into the vector in frame with the 6xHis (red), > Transform host cells > Cells produce the fusion protein (red and yellow), plus other proteins (green).
2. Lyse the cells, releasing the mixture of proteins.
3. Pour the cell lysate through a nickel affinity chromatography column, which binds the fusion protein but not the other proteins.
4. Release the fusion protein from the column with histidine or with imidazole, a histidine analogue, which competes with 6xHis for binding to the nickel
5. Cleave the fusion protein with Enterokinase (EK)
6. Pass the cleaved protein through the nickel column once more to separate 6xHis from the desired protein

Eukaryotic Expression vectors



Basic elements of an Eukaryotic Expression vector

Euk/Viral promoter: eukaryotic or viral promoter, inserted upstream of MCS, e.g HCMV promoter

MCS: multiple cloning site, used to insert the gene or cDNA of interest

PolyA signal: termination signal for transcription

Ori euk/Viral: eukaryotic or viral ori, allows the plasmid to replicate inside eukaryotic cells ((Optional))

NeoR: neomycine resistance gene, allows selection of plasmid inside eukaryotic cells

Ori E. coli & AmpR: allows replication of plasmid inside E coli cells and selection, respectively

Production of eukaryotic proteins

Advantages of eukaryotic systems over prokaryotic systems:

Eukaryotic proteins made in eukaryotic cells tend to be properly folded & not aggregated into insoluble inclusion bodies

Eukaryotic proteins made in eukaryotic cells are modified in a eukaryotic manner

Production of recombinant proteins

Obtain cDNA

Insert cDNA into plasmid/ expression vector

Transfect/transform plasmid into appropriate host cells

Downstream processing

Production of Eukaryotic proteins in ***E. coli*** from plasmid vectors containing **lac promoter**.

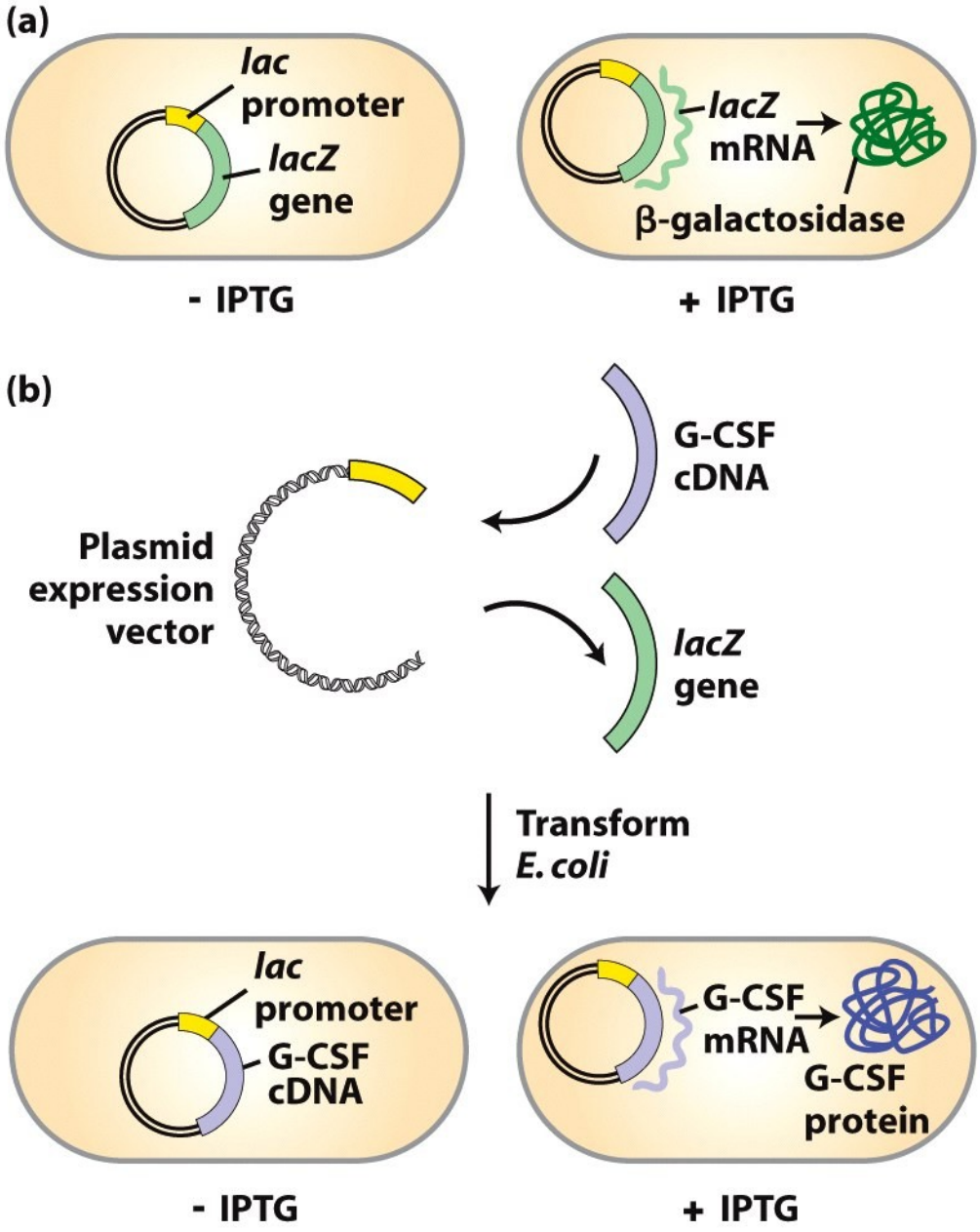


Figure 5-31
Molecular Cell Biology, Sixth Edition

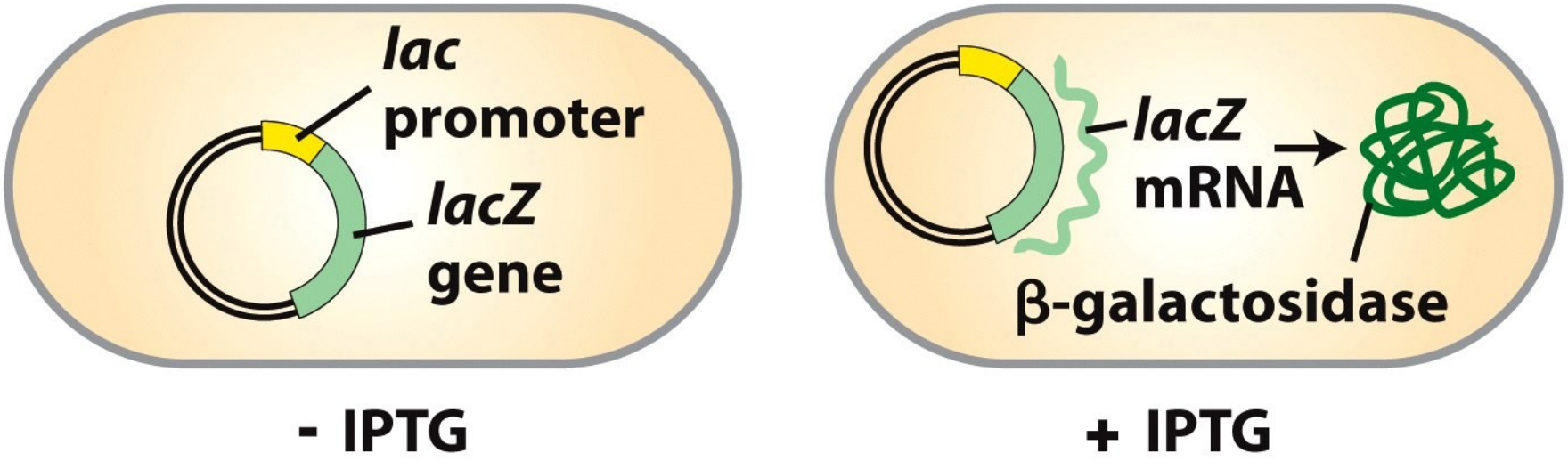


Figure 5-31a
Molecular Cell Biology, Sixth Edition

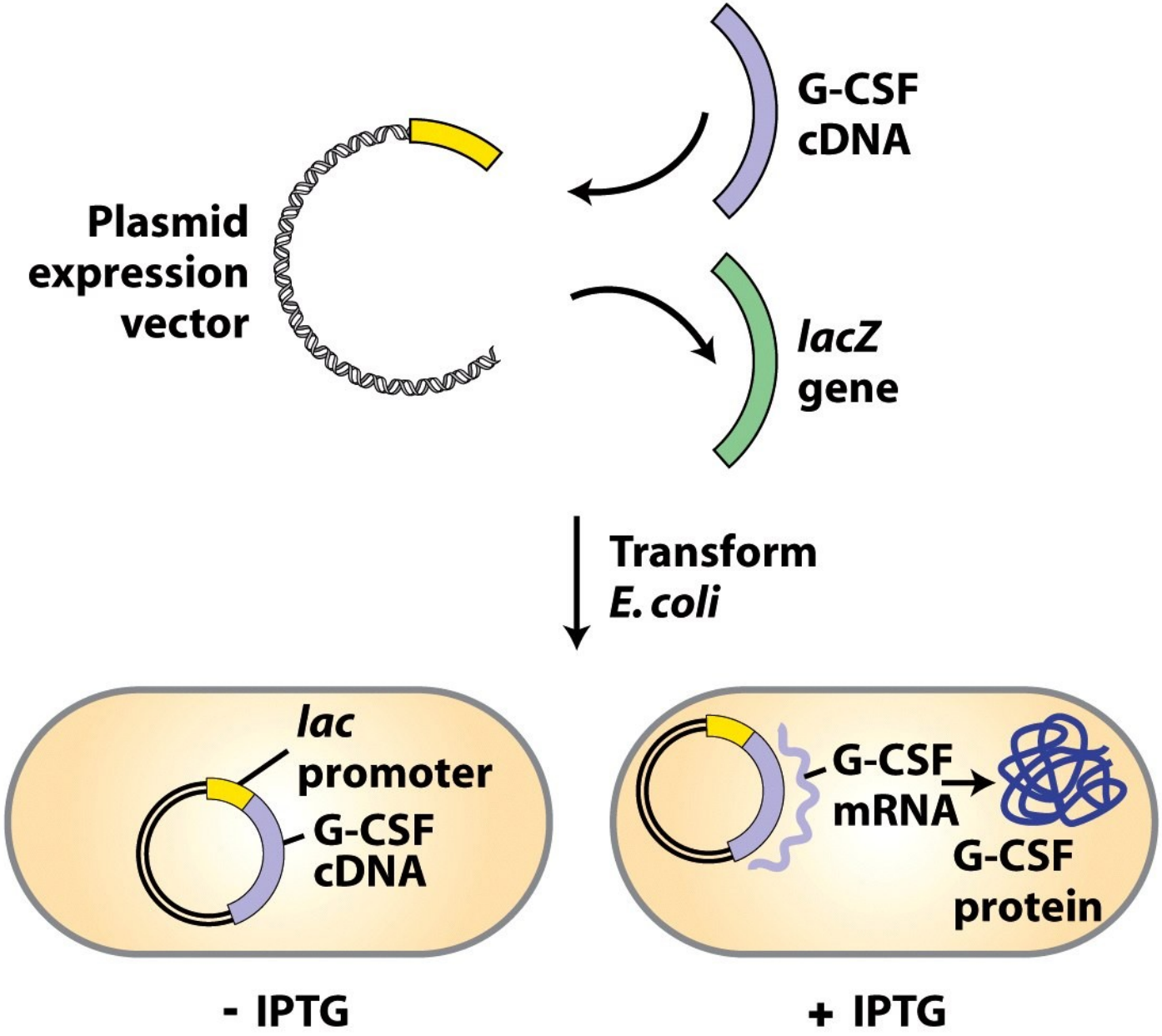


Figure 5-31b
Molecular Cell Biology, Sixth Edition

Transfection of animal cells

Method: Calcium phosphate; liposomes; electroporation; or by direct physical methods like microinjection using fine glass pippettes or firing metallic microparticles coated with DNA using “gene gun”.

Transient transfection: short-term expression from strong promoter, plasmid lost during cell division

Stable transfection: vector integrates into host chromosome; cells selected using selectable markers like neor (neomycin phosphotransferase; select with Geneticin or G-418)

Transient transfection

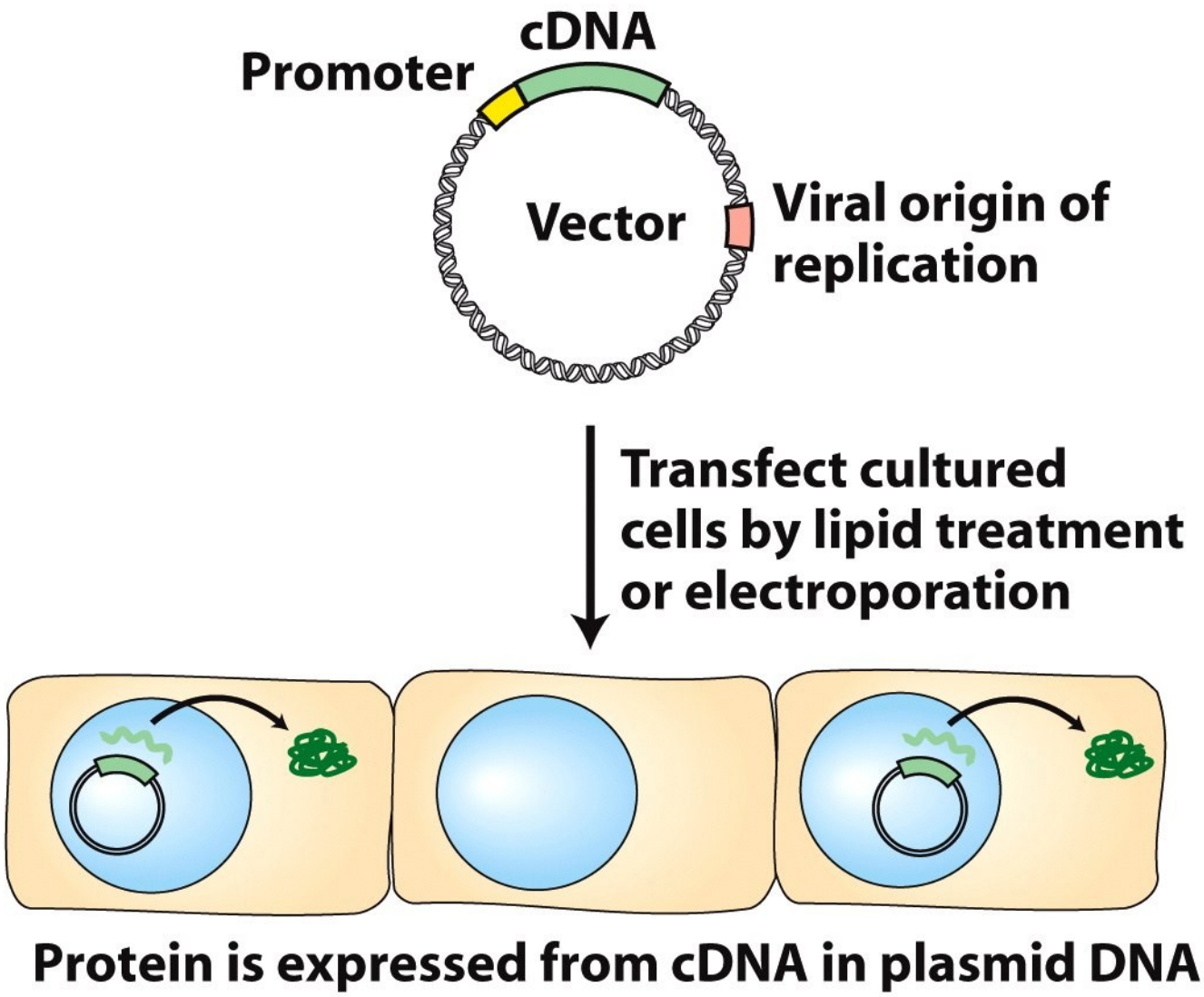


Figure 5-32a
Molecular Cell Biology, Sixth Edition

Stable transfection (transformation)

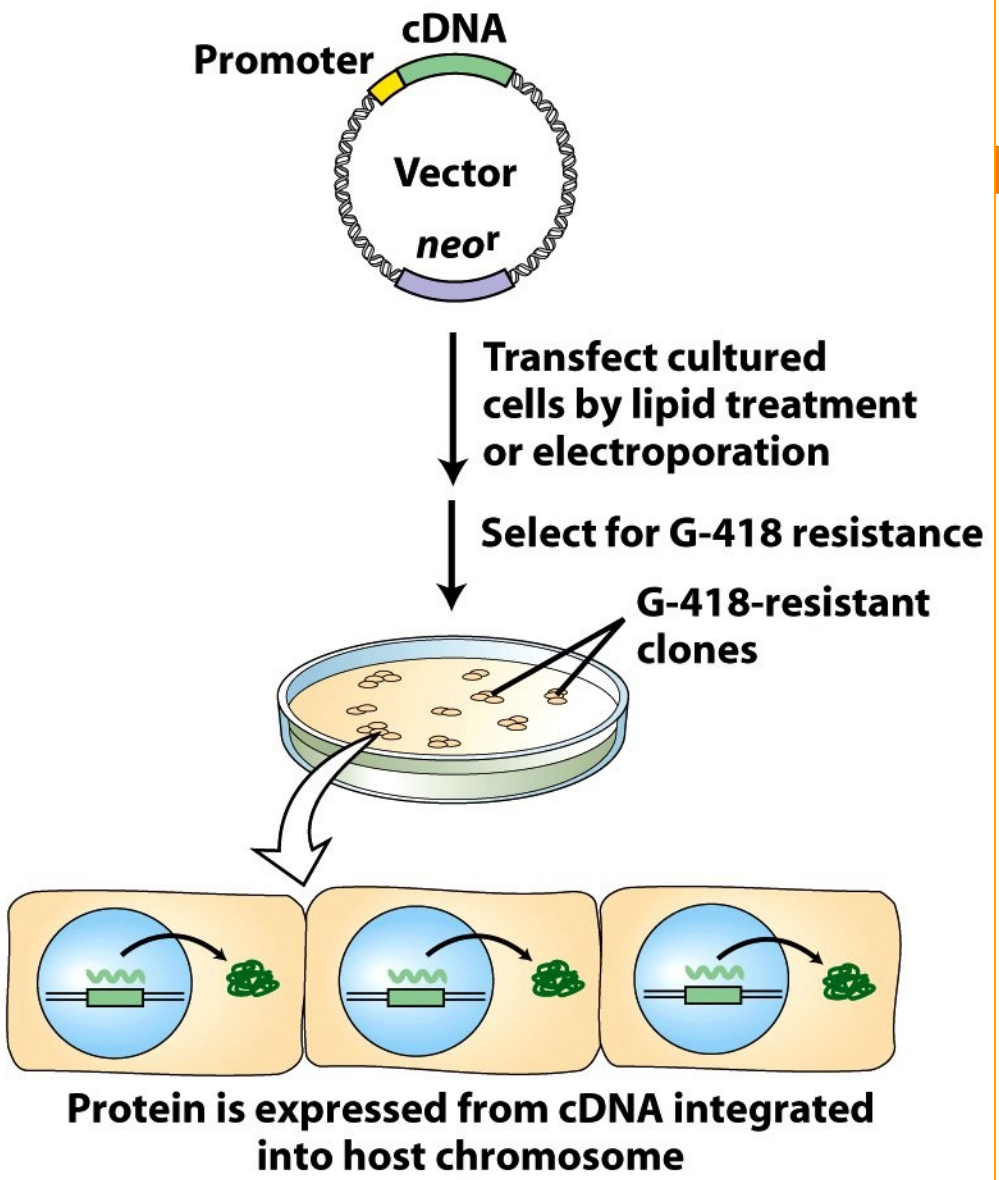


Figure 5-32b
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Genomic & cDNA libraries

Genomic library: a collection of DNA molecules each cloned into a vector molecule (e.g, λ-clones) representing all DNA sequences in the genome of an organism

cDAN library: a collection of cDNA molecules each cloned into a vector molecule (e.g, λ-cDNA clones) representing all the mRNAs expressed in a cell type (tissue-type specific)

cDNA: DNA molecule copied from an mRNA by reverse transcription

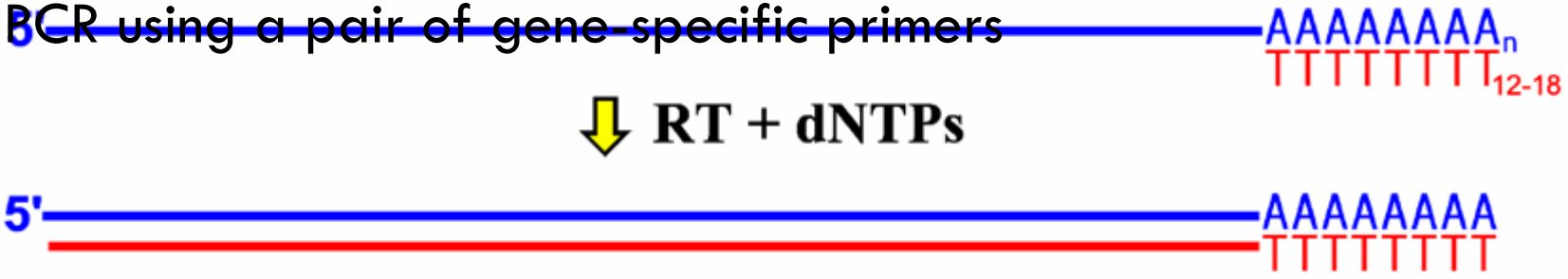
Preparation of cDNA

Preparation of mRNA (or total RNA) from target tissue

Synthesis of DNA-RNA hybrid (first strand) using

- Reverse transcriptase (RNA dependent DNA Polymerase)
- Oligo-dT primer or random priming (hexamers) or gene-specific primers
- RNAse inhibitor

Synthesis of second DNA strand & amplification by conventional PCR using a pair of gene-specific primers



Synthesis of cDNA

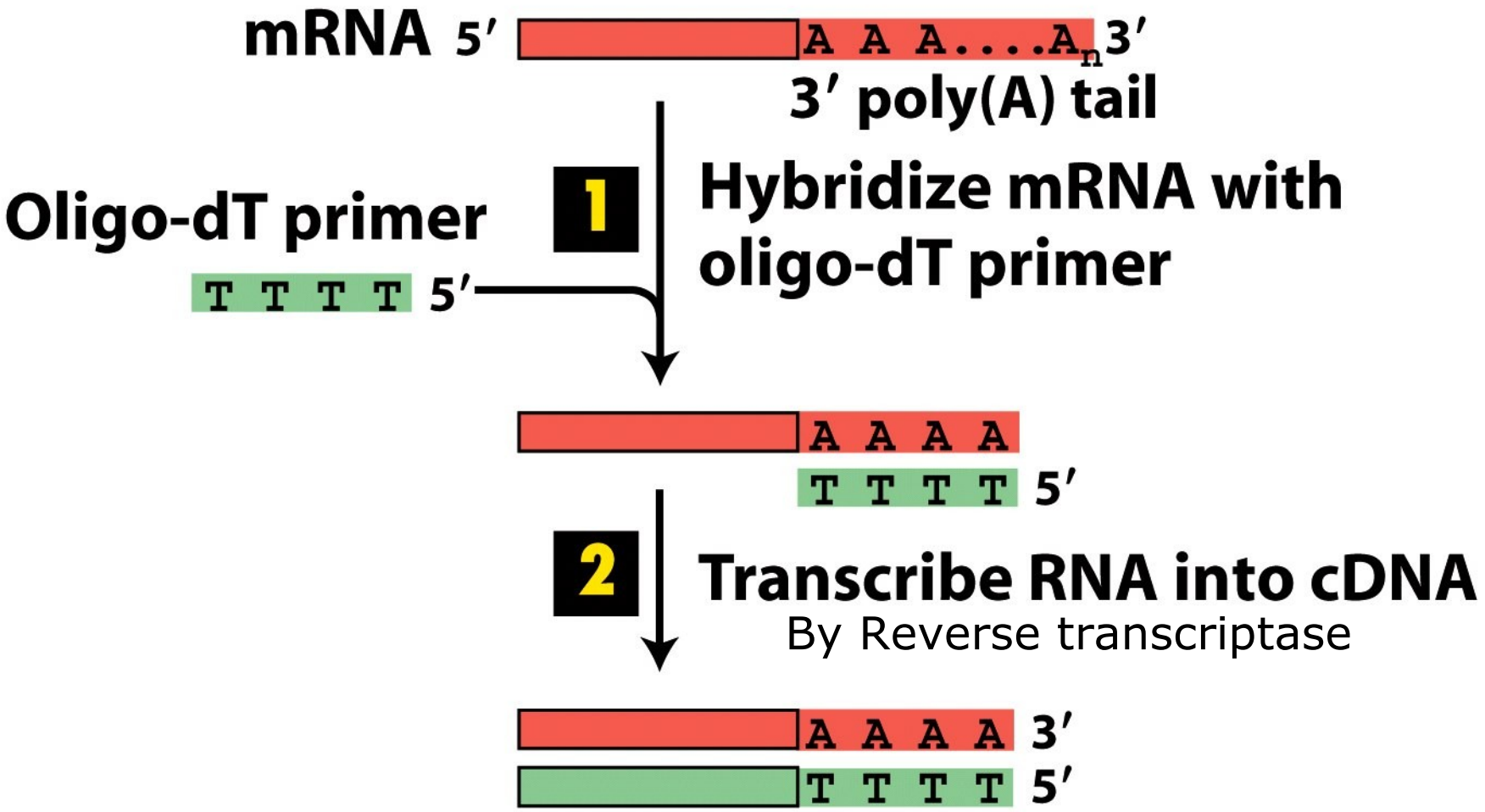


Figure 5-15 part 1
Molecular Cell Biology, Sixth Edition

Applications of cDNA

cDNA can be used for:

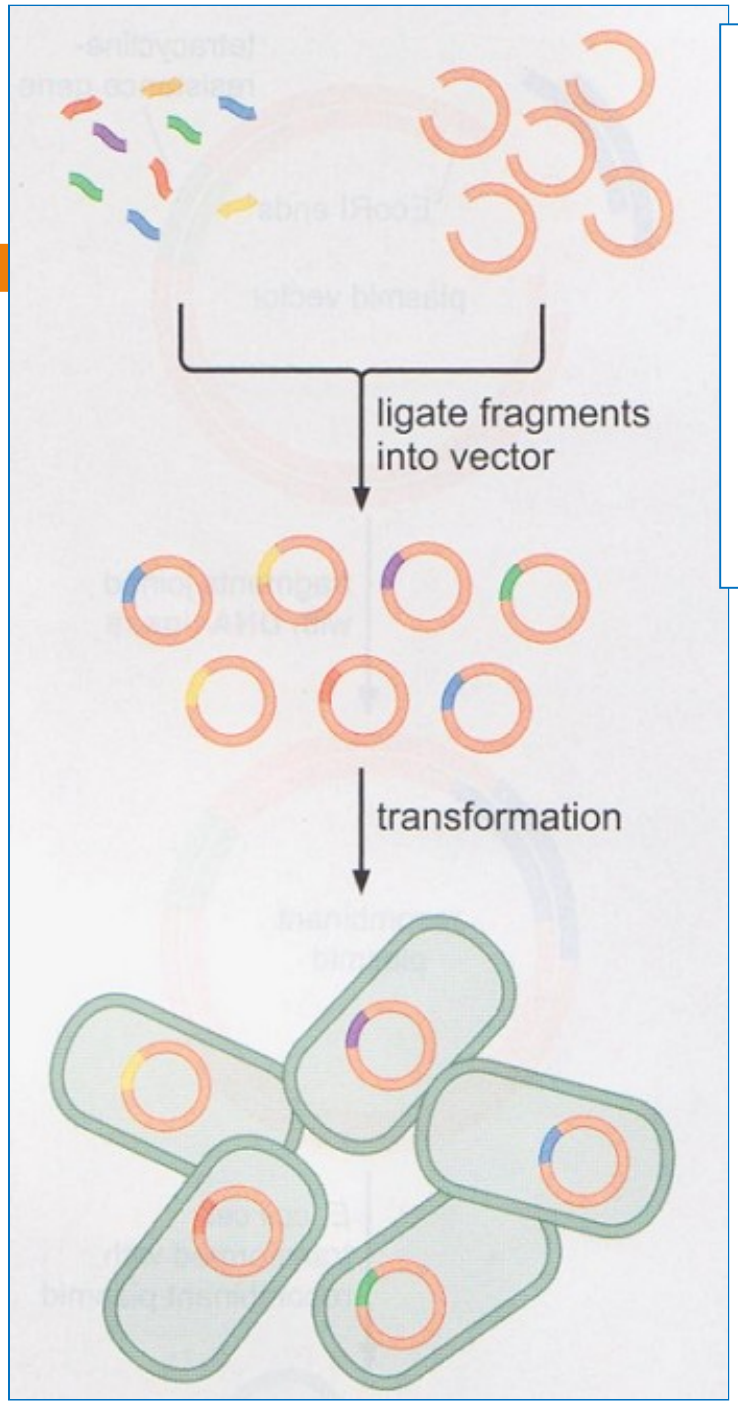
Cloning

DNA sequencing

Generation of cDNA library

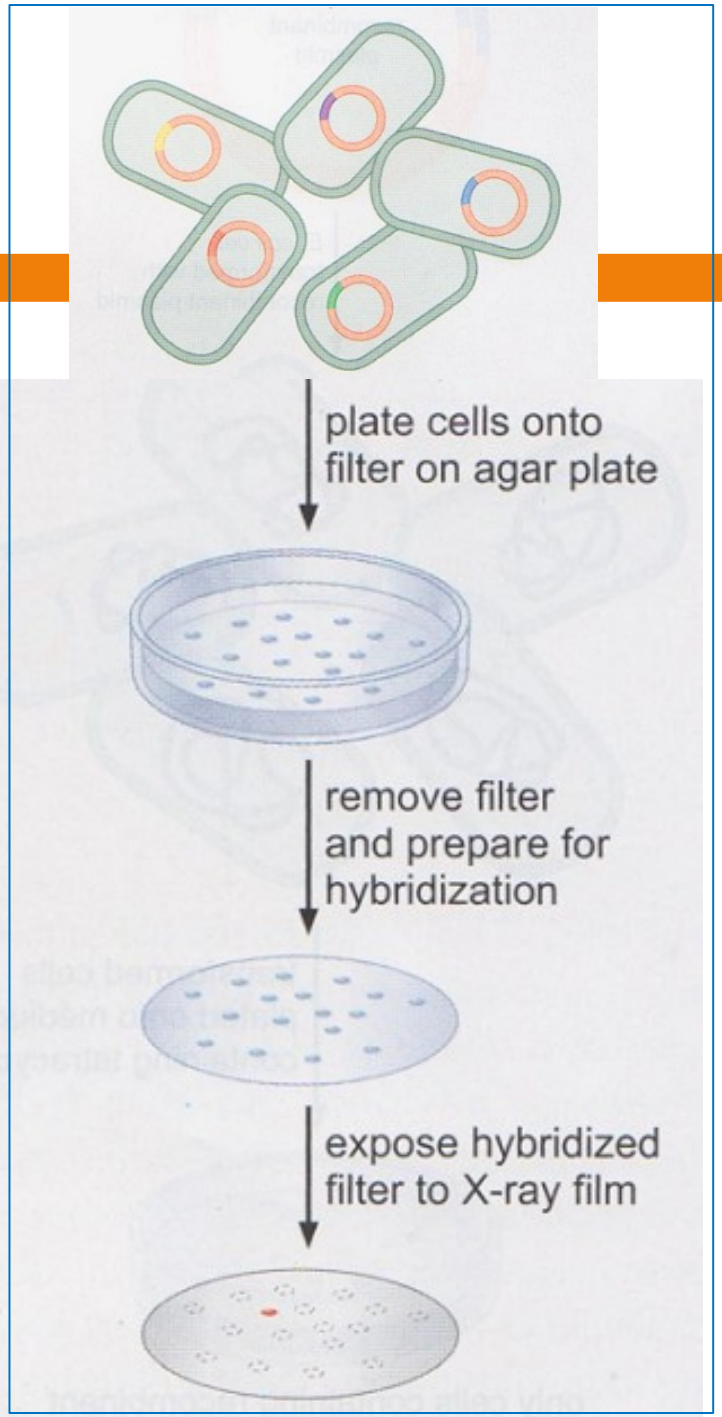
Analysis of gene expression

Construction of a Genomic DNA library

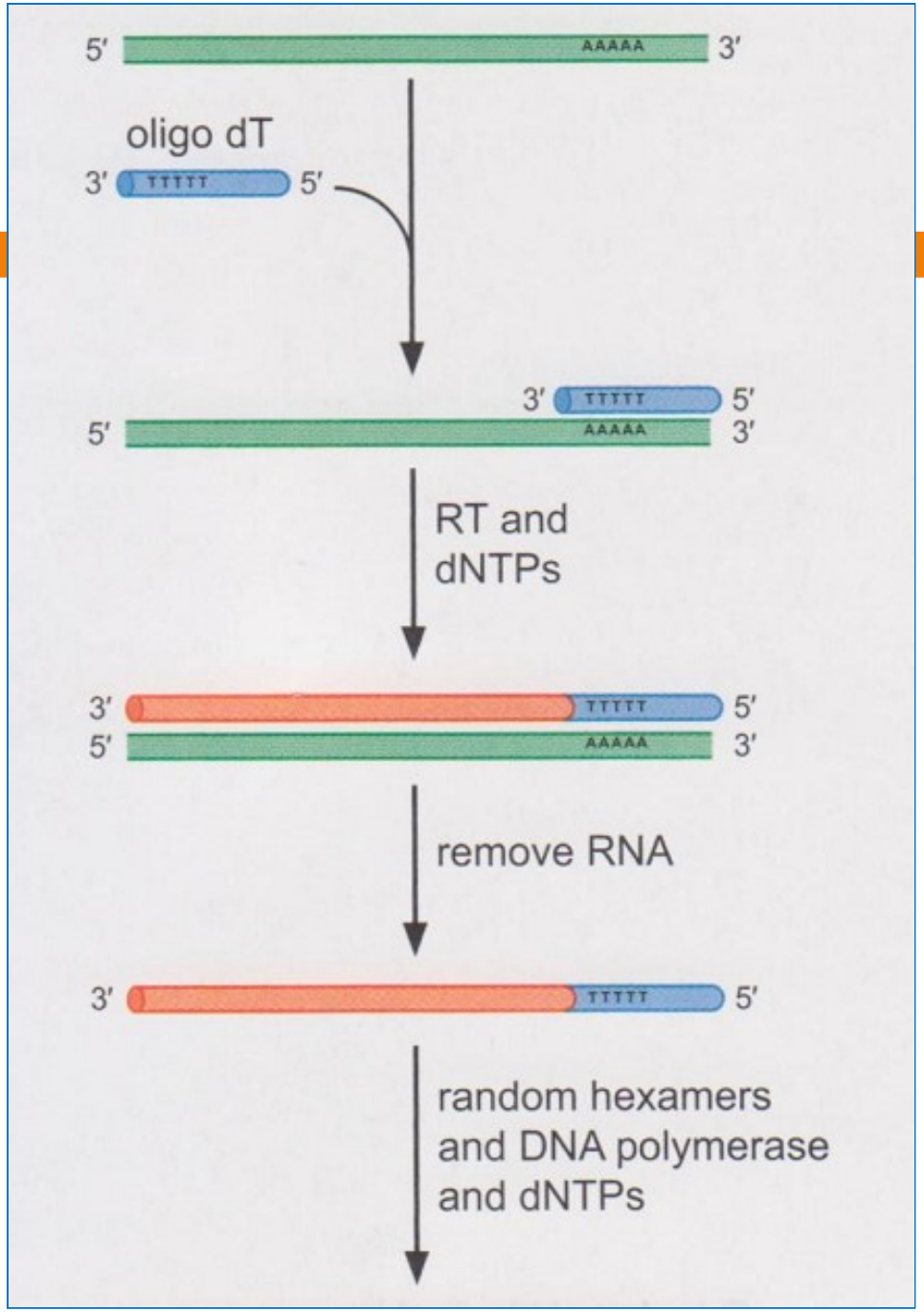


Partial digestion of genomic DNA, & digestion of vector by same RE

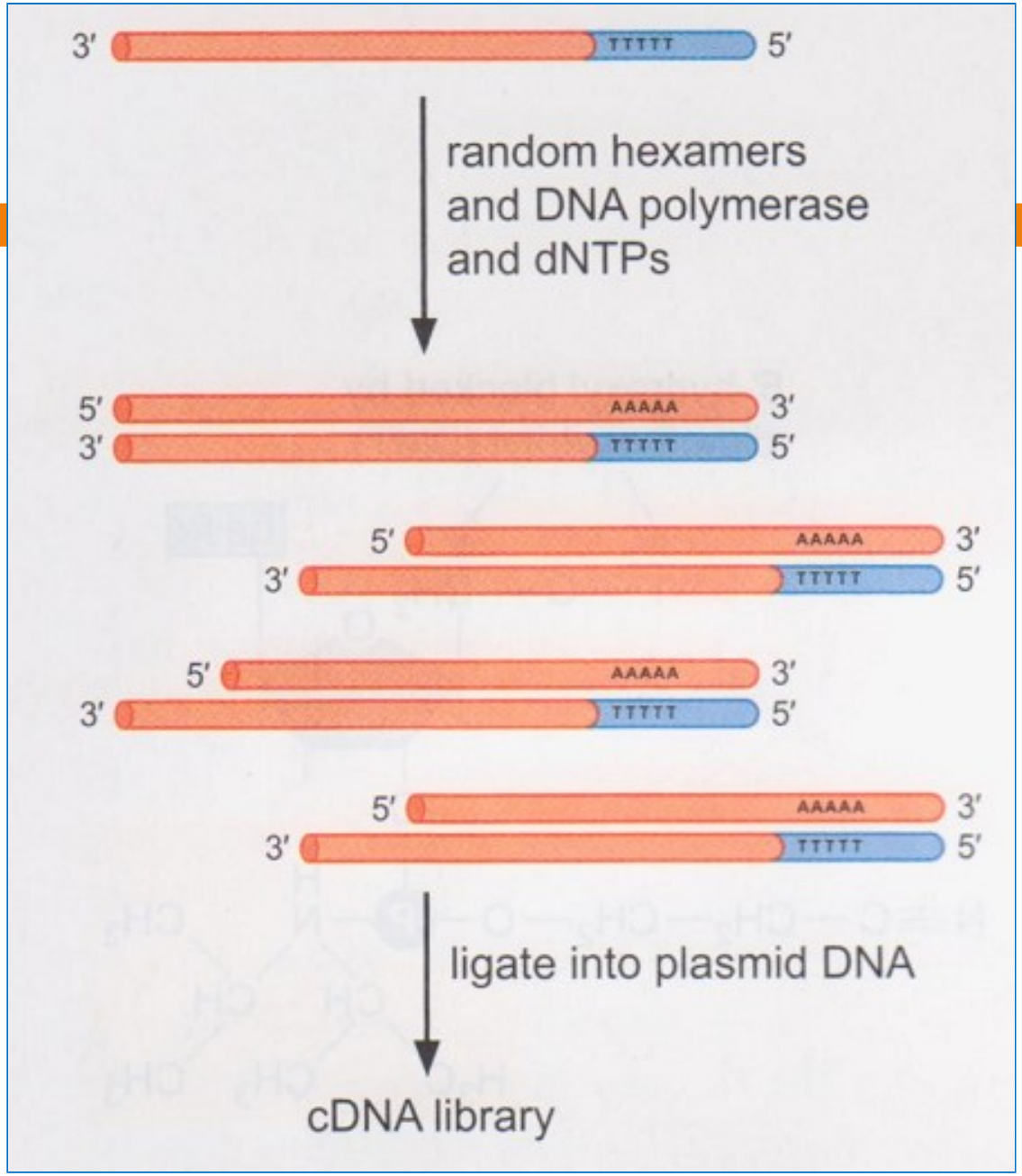
Construction of a Genomic DNA library



Construction of a cDNA library



Construction of a cDNA library



Screening of a cDNA library by ***Hybridization*** to a labeled oligonucleotide p

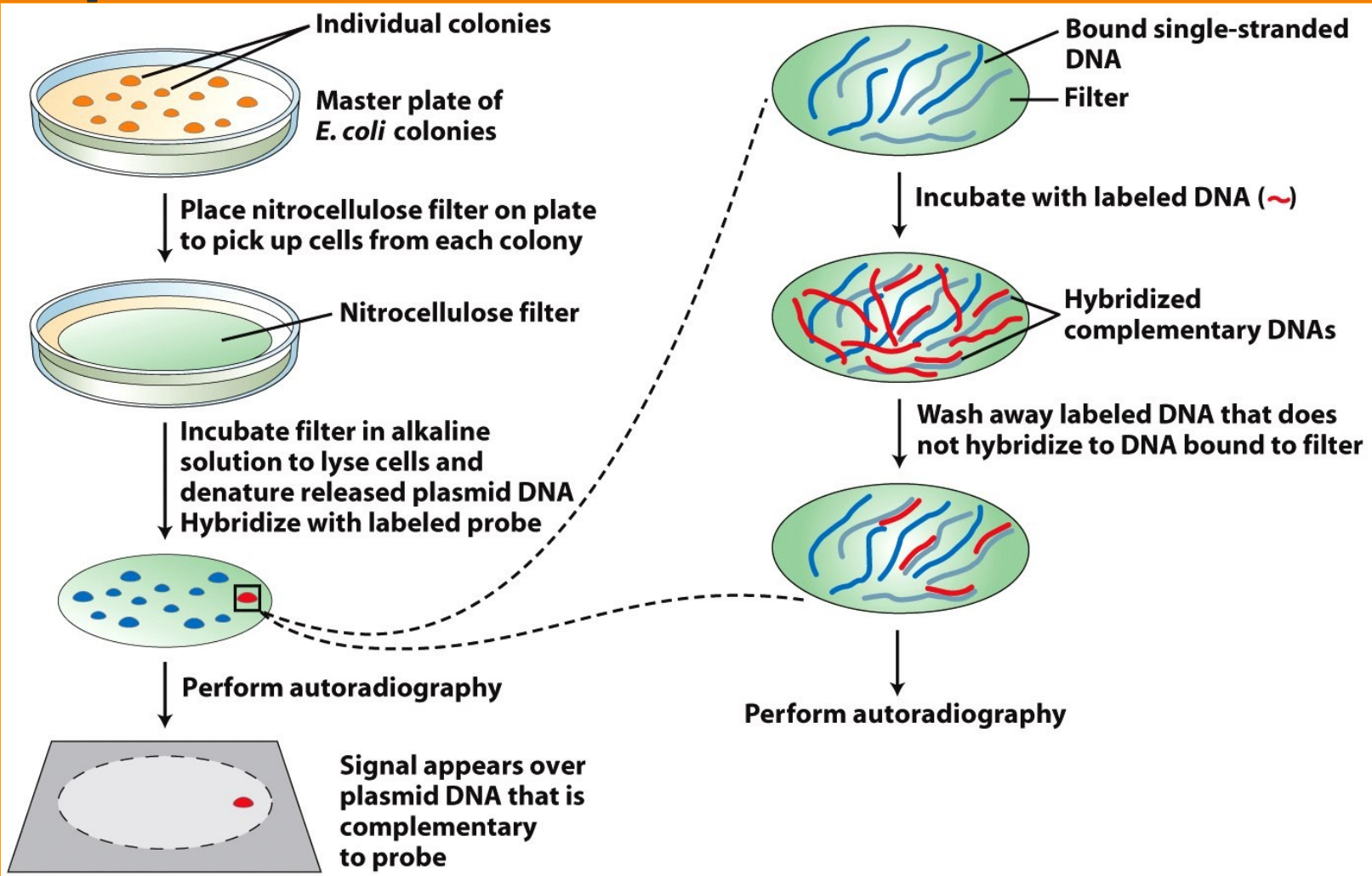


Figure 5-16
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From protein to DNA; or from DNA to protein?

Synthesis of a probe based on aa sequence

6	6	1	2	1	3	2	4	1	6
Arg-	Leu-	Met-	Glu-	Trp-	Ile-	Cys-	Pro-	Met-	Leu

How many different 17-mers are needed?

5aa+first 2 bases of 6th aa,

Met-Glu-Trp-Ile-Cys (15 nts)> 1X2X1X3X2=12 probes;

Plus first 2 bases of Pro (CC; CCU/A/C/G)