Quantification of Gene expression

Dr. Sudipta Chakraborty
Assistant Professor
PG Department of Microbiology
Bidhannagar College

Table 9.1 Summary of some commonly used tools for analyzing gene expression.

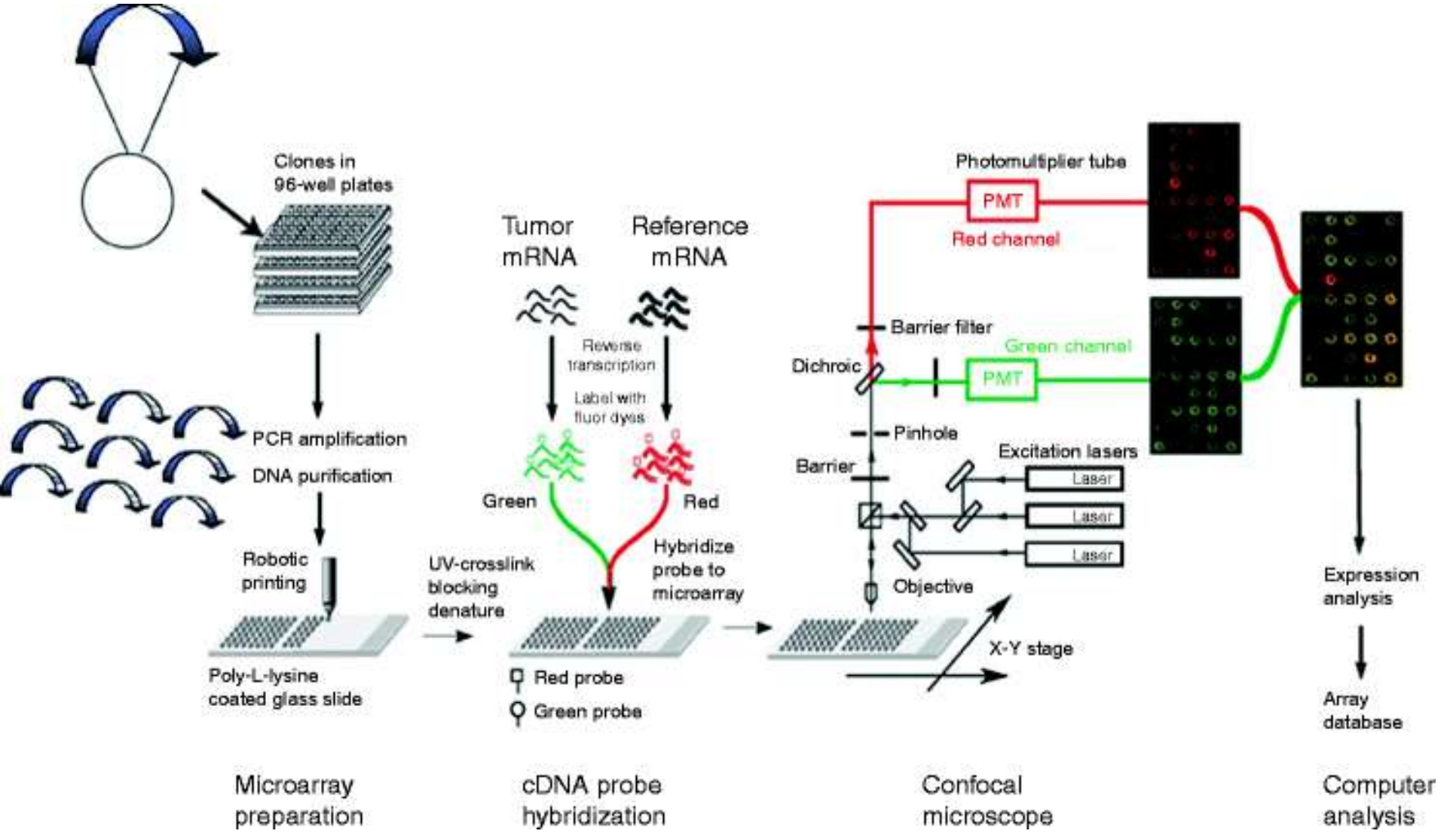
Level	Methods
Inhibition	*In vitro* mutagenesis Antisense oligonucleotides Expression of antisense RNA RNA interference (RNAi)
Transcription	Northern blot *In situ* hybridization RNase protection assay (RPA) Reverse transcriptase–PCR (RT-PCR)
Translation	Reporter gene enzyme activity Western blot *In situ* analysis Enzyme-linked immunosorbent assay (ELISA)
DNA–protein interactions	Electrophoretic mobility shift assay (EMSA) DNase I footprinting Chromatin immunoprecipitation (ChIP) assay
Protein–protein interactions	Pull-down assays Yeast two-hybrid assay Coimmunoprecipitation assay Fluorescence resonance energy transfer (FRET)
Protein structure	X-ray crystallography Nuclear magnetic resonance (NMR) spectroscopy Cryoelectron microscopy Atomic force microscopy (AFM)

Table 9.2 Various cell transfection methods.

Method	Comments
Chemical transfection	Many methods, e.g. calcium phosphate or DEAE-dextran transfection of animal cells. DNA is internalized by endocytosis
Lipofection	DNA complexed with cationic liposomes and taken up by endocytosis. Highly efficient method for transfecting animal cells, yeast spheroplasts, and plant protoplasts*
Electroporation	Naked DNA taken into cells through transient pores created by brief pulses of high voltage. Very efficient method for the transfection of yeast, plant, and animal cells
Direct injection	Labor-intensive but 100% efficient. Routinely applied to animal oocytes, eggs, and zygotes (see Fig. 15.2)
Microballistics (biolistics)	The use of microprojectiles, tungsten, or gold particles coated with DNA, which are fired into cells at high velocity using a gene gun. Gives efficient transfection of plant cells without removing cells walls. Can also be used to transfect whole plant and animal tissues (see Section 15.6 and Table 17.4)

* A spheroplast is a yeast cell from which the cell wall has been removed. A protoplast is a plant cell with the cell wall removed.

DNA Microarray



12.5
DNA Microarrays

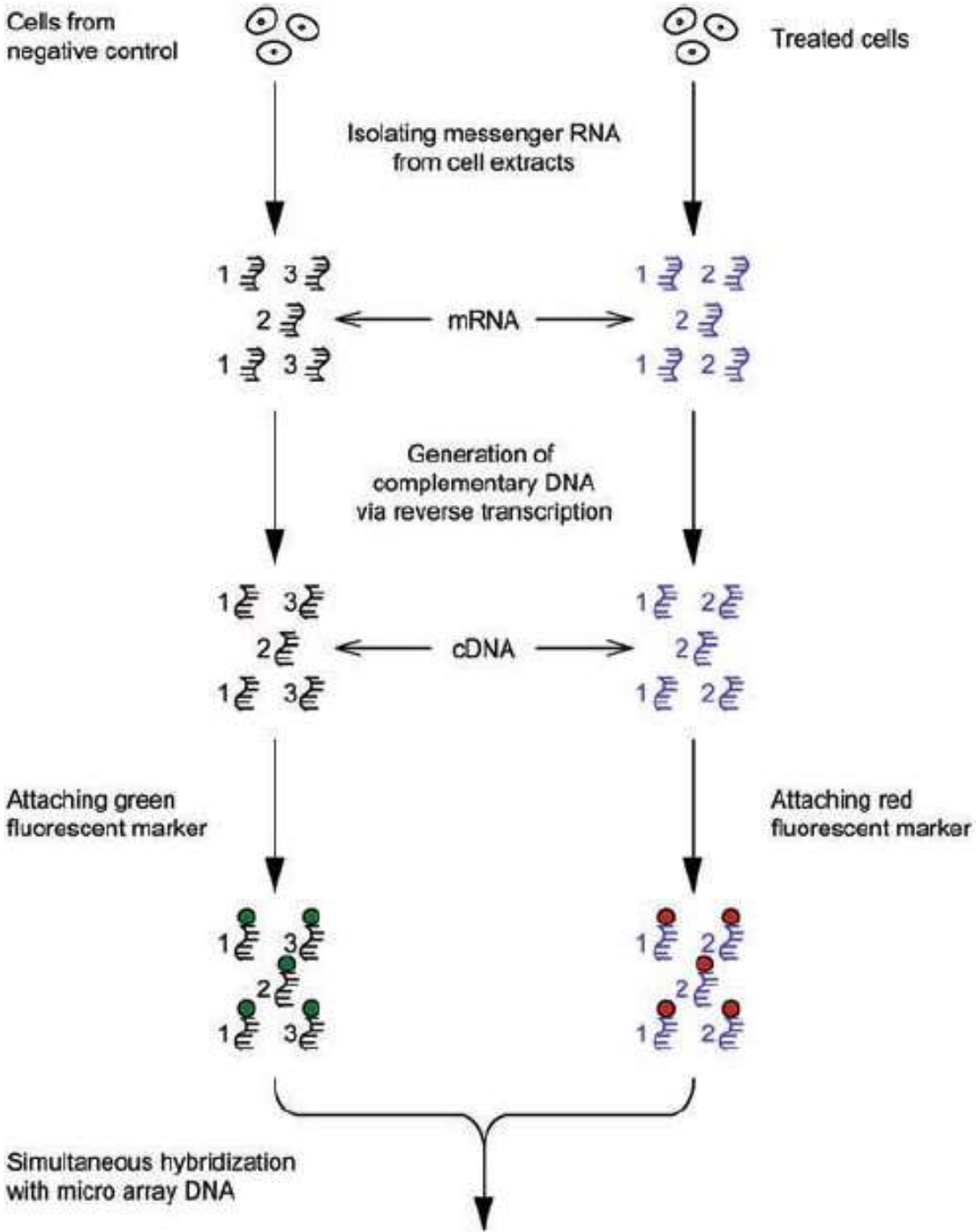
Even though cells carry a large set of DNA sequences encoding proteins (exons) not all proteins are generated in a cell simultaneously and to the same extent. Up- and downregulation of this gene expression is one of the most important mechanisms governing specifically the functioning of a cell. Often, it is important to know how distinct compounds influence the regulation of gene expression. This helps, for example, in understanding how a certain biological regulator or a potential drug candidate might affect the cell metabolism. But how can the gene expression for a large number of proteins be monitored simultaneously in a reasonable time? For such and similar problems DNA microarrays represent a very useful tool.

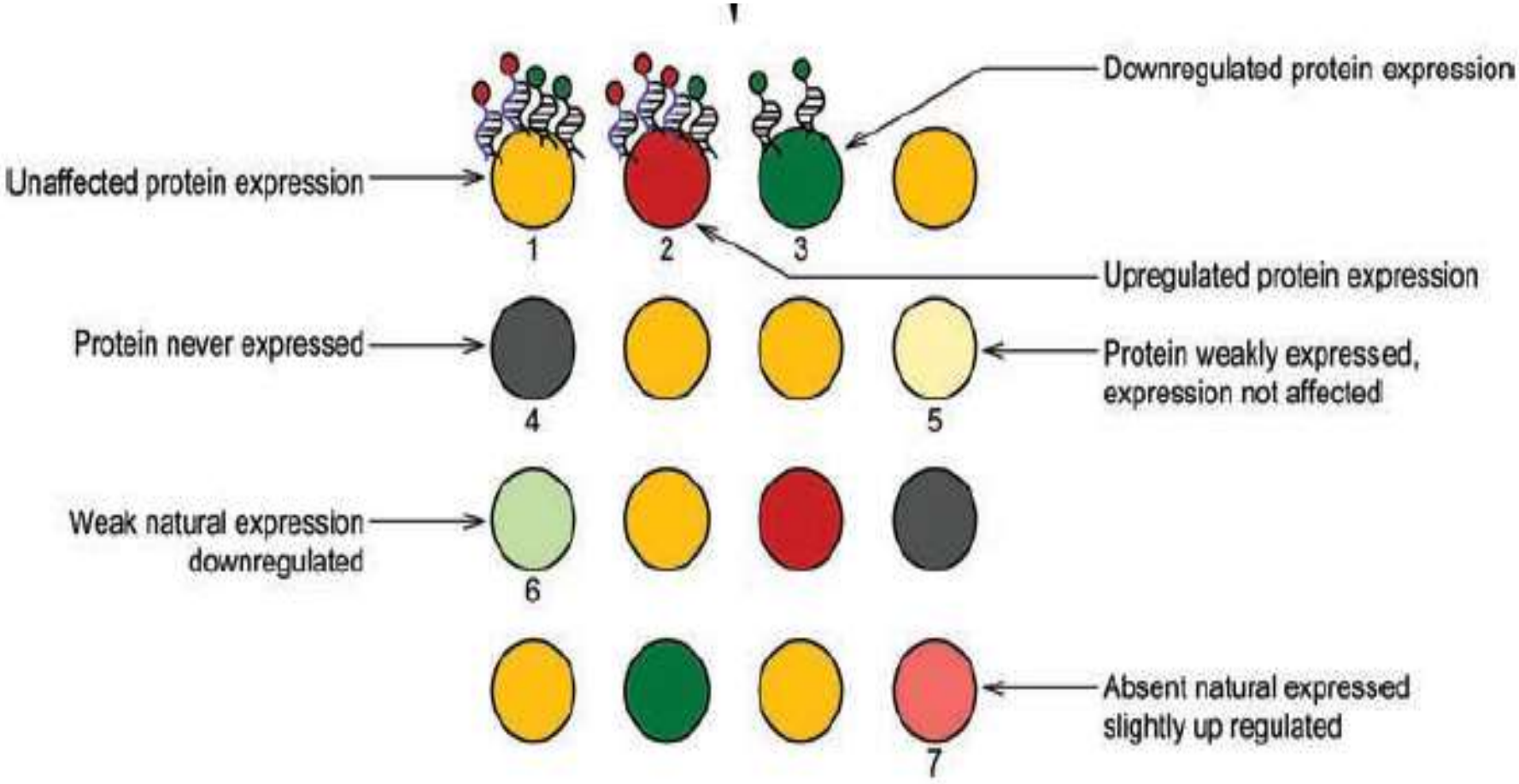
Figure 12.14 shows the principle of monitoring effects on the gene expression of cells using DNA microarrays. A DNA microarray is a small substrate having a regular pattern of spots that carry immobilized sequences of DNA. The DNA sequences at the different spots correspond to the sequence of different messenger RNA (mRNA) encoding important cell proteins. To monitor the regulation of gene expression by certain compounds, usually a procedure similar to the one described here is applied. First, a cell population is divided into two parts. One part is treated with the compound or any procedure for which the influence on the gene expression shall be found. The other part is not treated. After a certain incubation time the mRNA of both cell lines is isolated from their cell extracts. The amount of DNA transcribed

into mRNA is a very important parameter in regulation of gene expression. The more mRNA that is transcribed the more of the corresponding protein will be generated by translation in the cells ribosomes. In the case shown in Figure 12.14, for example, the treated cells contained three times more mRNA 2 but no mRNA 3. The treatment has upregulated the gene expression of mRNA 2 and completely downregulated mRNA 3. Only the gene expression of mRNA 1 is unaffected. In a next step, complementary DNA (cDNA) is generated from the isolated mRNA by the enzyme reverse transcriptase. One reason amongst others for this intermediate step is that DNA is far more stable than RNA. RNA is very quickly degraded by enzymes that are quite abundant in natural environments. Then, the cDNA of both cell-line parts is labelled with different fluorescence dyes (green and red in Figure 12.14). It is important that both cell populations are treated equally in order to properly compare them later on. In the next step, the DNA microarray is now exposed to both labelled cDNA preparations simultaneously. Finally, the fluorescence from each spot on the DNA microarray is imaged in one shot. From the fluorescence emission of individual spots it is immediately possible to determine the gene expression in the treated and untreated cells as well as the effect of treatment on the gene expression. The gene expression of mRNA 1, for example, was unaffected by the treatment. As a result, the corresponding spot on the DNA microarray carries about equal amounts of red and green fluorescence dye (spot 1 in the lower part of Figure 12.14). The additive mixture of these colours appears as a yellow spot. A yellow fluorescence spot indicates no effect on the gene expression of this particular gene.

In contrast, gene 2 (mRNA 2) was upregulated by a factor of three in comparison to the untreated cells. As a consequence, the corresponding spot on the DNA microarray shows an almost exclusively red fluorescence (spot 2 in Figure 12.14). A red fluorescence spot indicates upregulated genes. Finally, gene 3 was completely downregulated. Consequently, only green fluorescence can be detected at the corresponding spot (spot 3 in Figure 12.14). The detection using DNA microarrays provides further important information about the gene expression. The absence of any fluorescence on spot 4 in Figure 12.14 indicates, for example, that the protein corresponding to this gene is apparently never produced by the investigated cell line. The weaker yellow fluorescence at spot 5 indicates weak gene expression that is apparently unaffected by the treatment. The weak green fluorescence at spot 6 indicates a weak gene expression in the untreated cells that is downregulated by the treatment. Finally, the weak red fluorescence at spot 7 might indicate a gene expression that is absent in the untreated cells but is slightly upregulated by the treatment. Of course, it is also possible to measure the entire fluorescence spectrum of the spots to quantify the relative amounts of red and green fluorescence more precisely.

Often, the large amount of information deduced from such an analysis is compared with databases that contain typical regulation patterns. This helps to identify the functioning of certain genes, drug candidates or the toxicity of certain compounds. Also, very valuable information on the metabolic pathways in cells and the reasons for certain diseases can be identified using this information.


Cells from negative control
Treated cells
Isolating messenger RNA from cell extracts
mRNA
Generation of complementary DNA via reverse transcription
cDNA
Attaching green fluorescent marker
Attaching red fluorescent marker
Simultaneous hybridization with micro array DNA


Downregulated protein expression
Unaffected protein expression
1
2
3
Upregulated protein expression
Protein never expressed
Protein weakly expressed,
expression not affected
4
5
Weak natural expression
downregulated
6
Absent natural expressed
slightly up regulated
7

Nuclear Run-on transcription

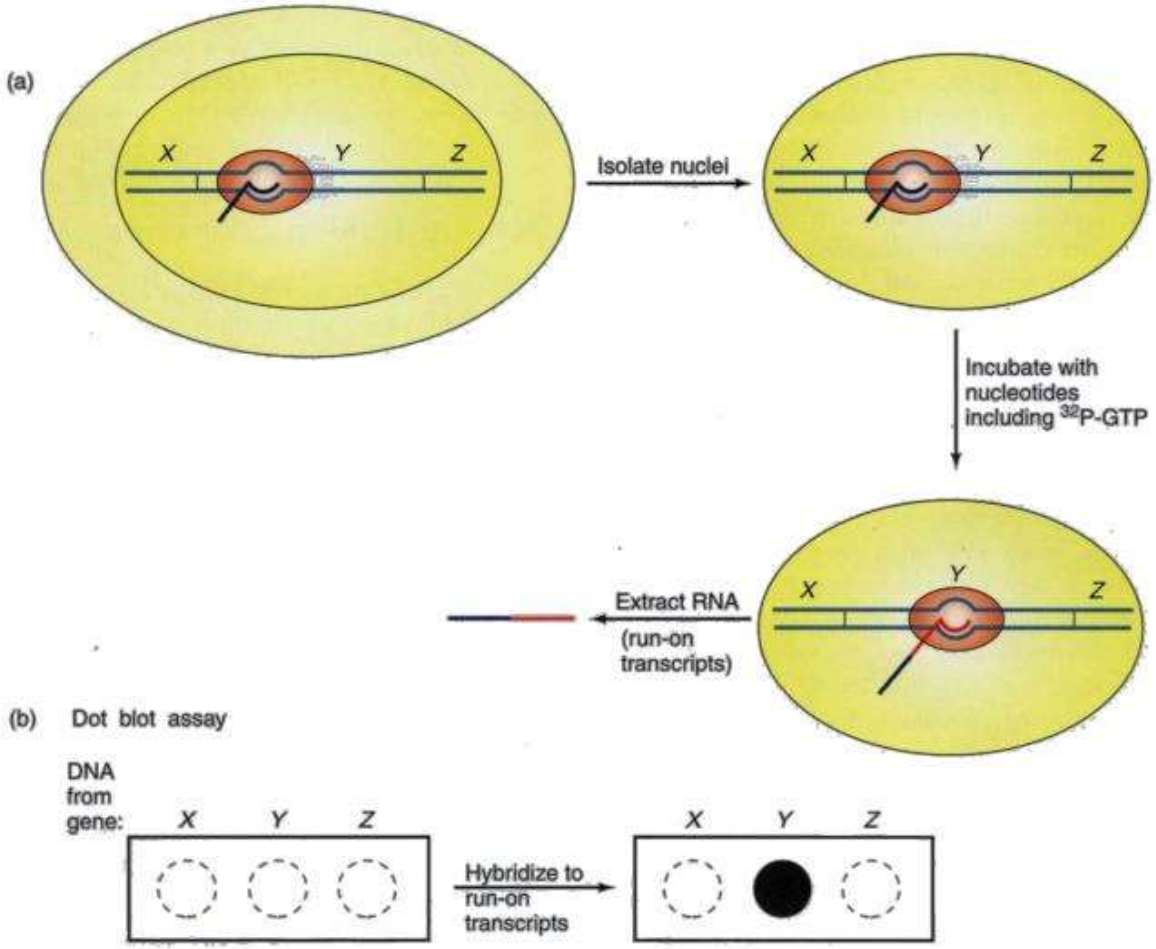


Figure 5.28 Nuclear run-on transcription. (a) The run-on reaction. We start with cells that are in the process of transcribing the *Y* gene, but not the *X* or *Z* genes. The RNA polymerase (orange) is making a transcript (blue) of the *Y* gene. We isolate nuclei from these cells and incubate them with nucleotides so transcription can continue (run-on). We also include a labeled nucleotide in the run-on reaction so the transcripts will become labeled (red). Finally, we extract the labeled run-on transcripts. (b) Dot blot assay. We spot single-stranded DNA from genes *X*, *Y*, and *Z* on nitrocellulose, or another suitable medium, and hybridize the blot to the labeled run-on transcripts. Since gene *Y* was transcribed in the run-on reaction, it will be labeled, and the gene *Y* spot will become labeled. On the other hand, since genes *X* and *Z* were not active, no labeled *X* and *Z* transcripts were made, so the *X* and *Z* spots remain unlabeled.

Real-Time qRT-PCR

(Real-Time Quantitative Reverse Transcription PCR) is a major development of PCR technology that enables reliable detection and measurement of products generated during each cycle of PCR process. This technique became possible after introduction of an oligonucleotide probe which was designed to hybridize within the target sequence. Cleavage of the probe during PCR because of the 5' nuclease activity of Taq polymerase can be used to detect amplification of the target-specific product.

Techniques to monitor degradation of the probe

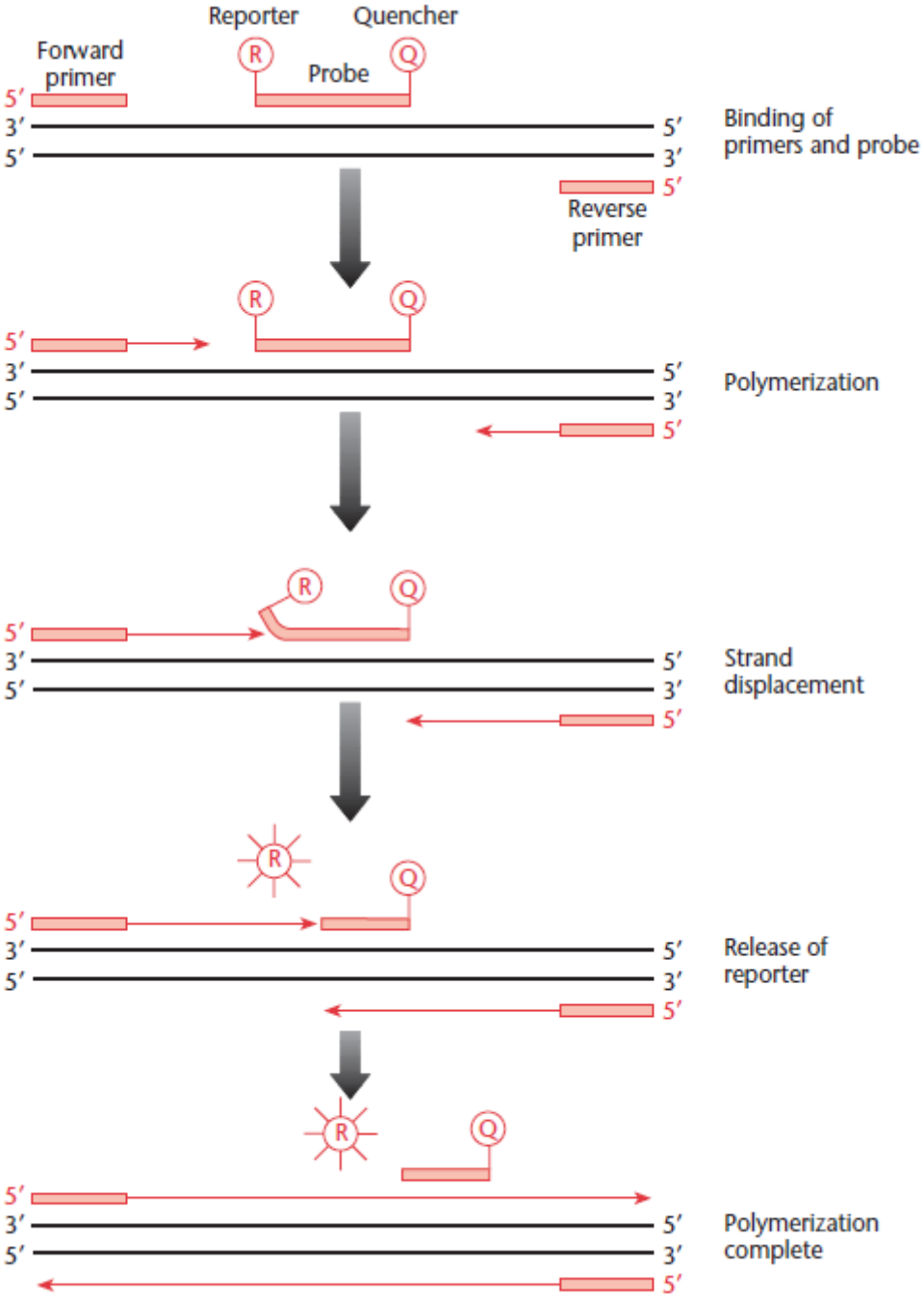
Intercalation of double-stranded DNA-binding dyes

^{32}P probe labeling

Labeling of the probe with fluorescent dyes

TaqMan

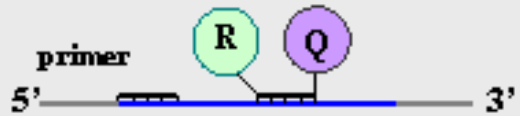
assay (named after*Taq*DNA polymerase) was one of the earliest methods introduced for real time PCR reaction monitoring and has been widely adopted for both the quantification of mRNAs and for detecting variation. The method exploits the 5' endonuclease activity of *Taq*DNA polymerase to cleave an oligonucleotide probe during PCR, thereby generating a detectable signal. The probes are fluorescently labeled at their 5' end and are non-extendable at their 3' end by chemical modification. Specificity is conferred at three levels: via two PCR primers and the probe.Applied Biosystemsprobes also include a minor groove binder for added specificity.


Reporter
Quencher
Forward primer
R
Q
Probe
5′
3′
5′
5′
3′
5′
Reverse primer
Binding of primers and probe
Polymerization
Strand displacement
Release of reporter
Polymerization complete

TaqMan® Applied Biosystems

Annealing

R is reporter fluorophore, which emits at a wavelength absorbed by the quencher fluorophore (**Q**).

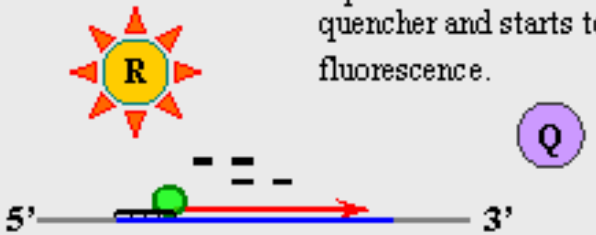


Probe displacement

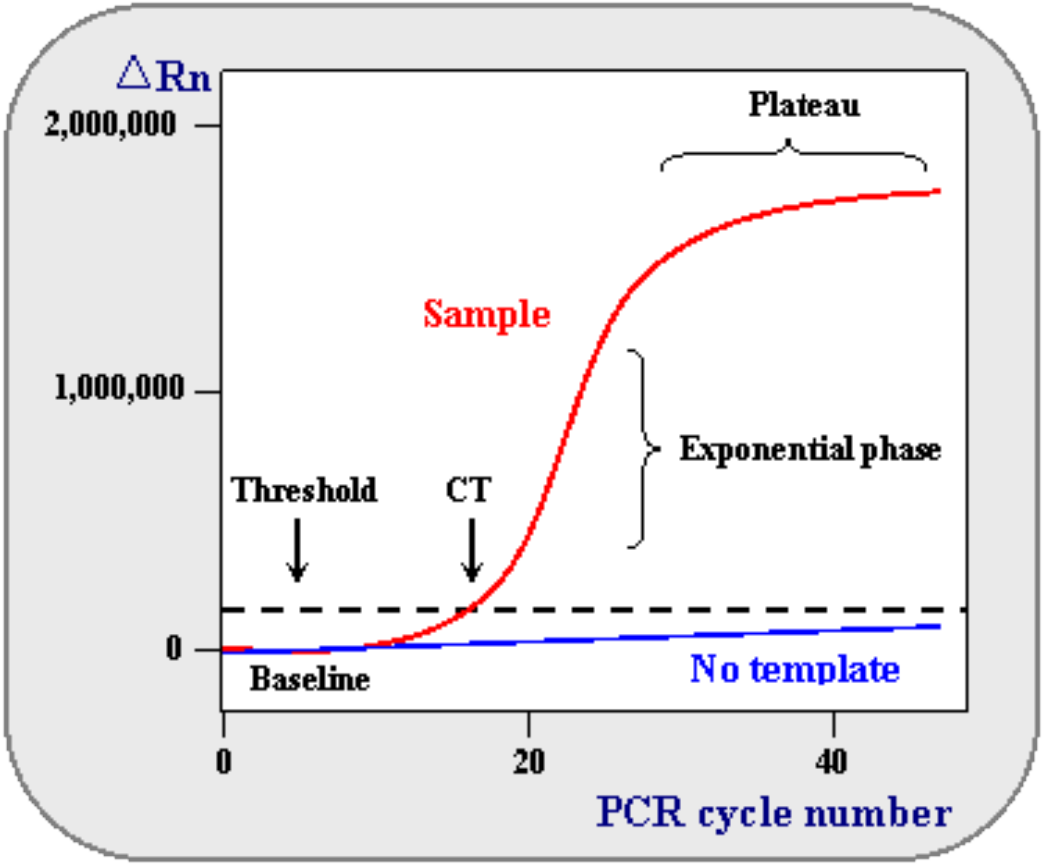
DNA polymerase starts extending primers moving toward the probe.

Probe cleavage

The probe is degraded. The reporter is released from the quencher and starts to emit fluorescence.



Model of real time quantitative PCR plot



Nomenclature used in RT-qRT-PCR

Baseline

is defined as PCR cycles in which a reporter fluorescent signal is accumulating but is beneath the limits of detection of the instrument.

ΔRn

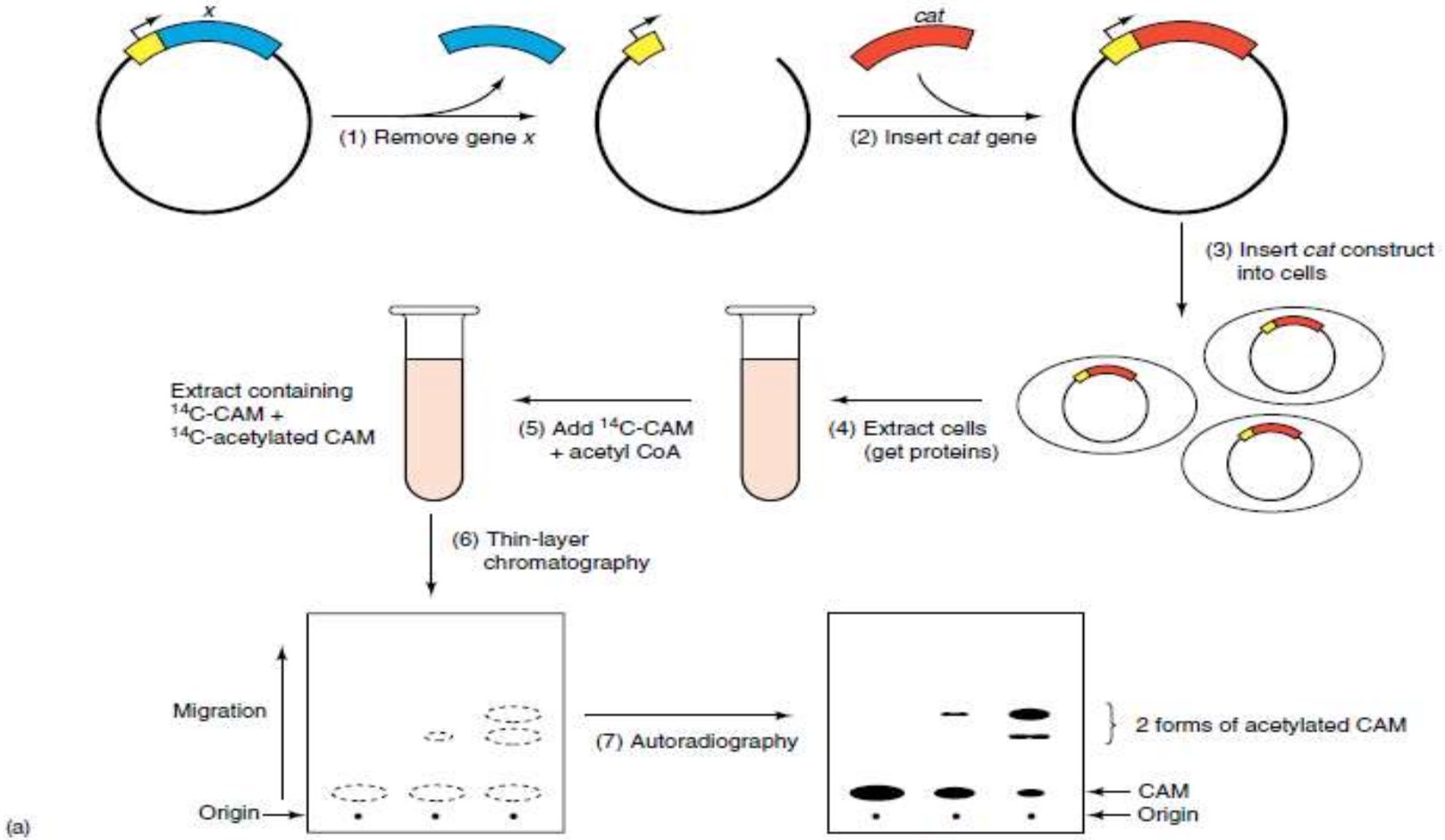
is an increment of fluorescent signal at each time point. The ΔRn values are plotted versus the cycle number.

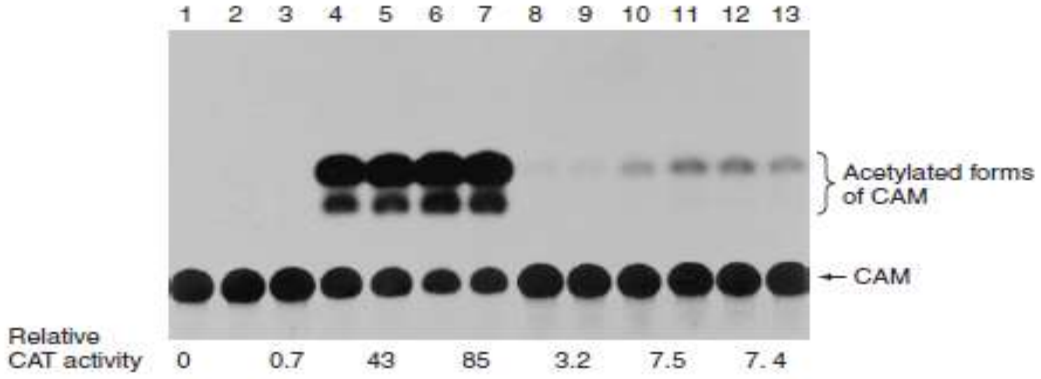
Threshold

is an arbitrary level of fluorescence chosen on the basis of the baseline variability. A signal that is detected above the threshold is considered a real signal that can be used to define the threshold cycle (Ct) for a sample. Threshold can be adjusted for each experiment so that it is in the region of exponential amplification across all plots.

Ct

is defined as the fractional PCR cycle number at which the reporter fluorescence is greater than the threshold. The Ct is a basic principle of real time PCR and is an essential component in producing accurate and reproducible data.


x
(1) Remove gene x
cat
(2) Insert cat gene
(3) Insert cat construct into cells
(4) Extract cells (get proteins)
(5) Add ^{14}C-CAM + acetyl CoA
Extract containing ^{14}C-CAM + ^{14}C-acetylated CAM
(6) Thin-layer chromatography
Migration
Origin
(7) Autoradiography
2 forms of acetylated CAM
CAM
Origin
(a)


1 2 3 4 5 6 7 8 9 10 11 12 13
Acetylated forms of CAM
CAM
Relative CAT activity
0 0.7 43 85 3.2 7.5 7.4
(b)

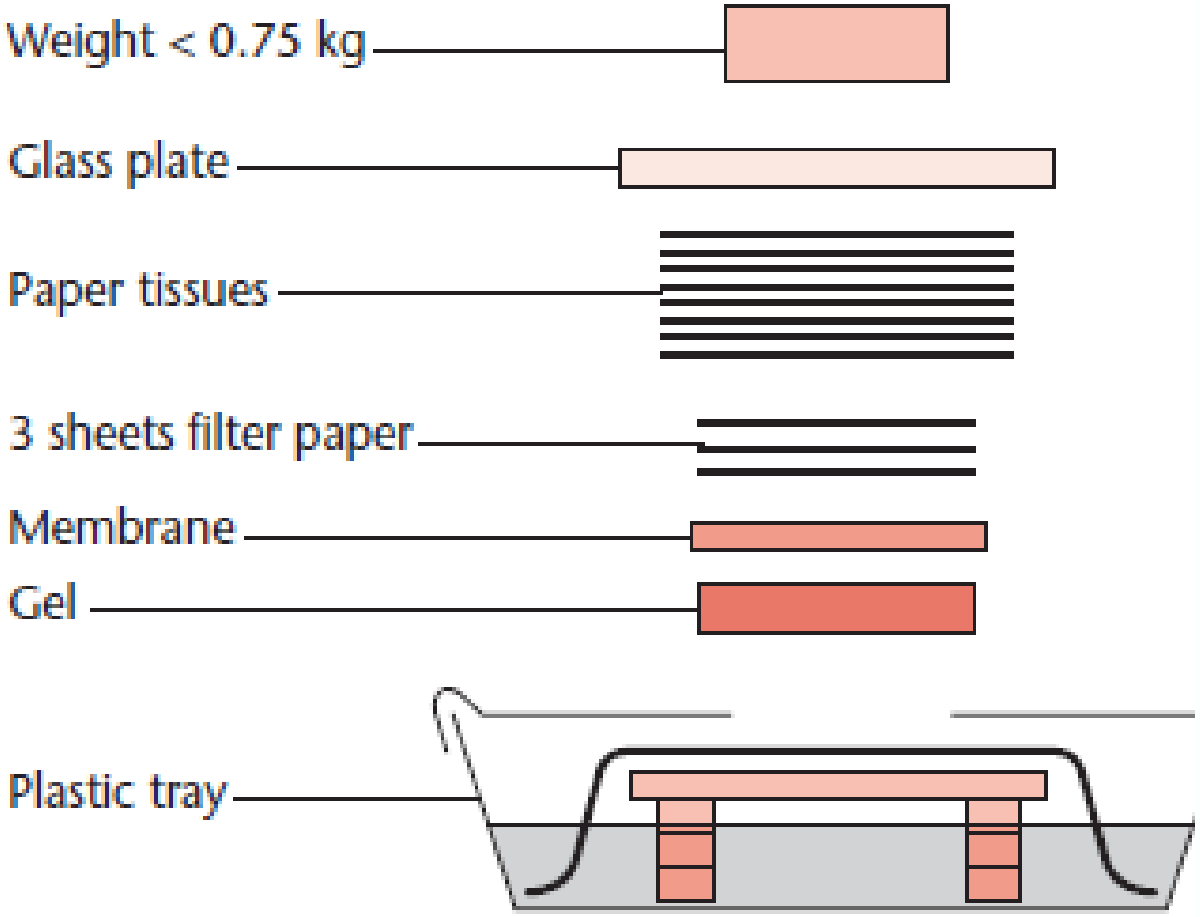
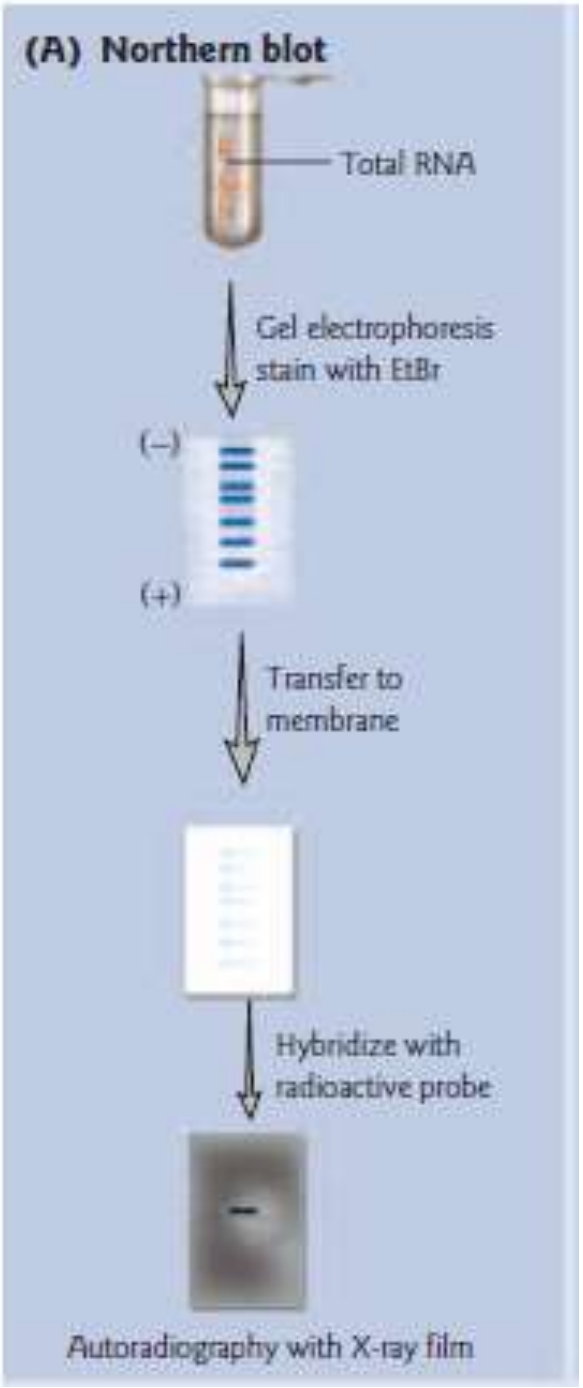


Fig. 2.5 A typical capillary blotting apparatus.



Rate enhancers	Dextran sulphate and other polymers act as volume excluders to increase both the rate and the extent of hybridization
Detergents and blocking agents	Dried milk, heparin and detergents such as sodium dodecyl sulphate (SDS) have been used to depress non-specific binding of the probe to the membrane. Denhardt's solution (Denhardt 1966) uses Ficoll, polyvinylpyrrolidone and bovine serum albumin
Denaturants	Urea or formamide can be used to depress the melting temperature of the hybrid so that reduced temperatures of hybridization can be used
Heterologous DNA	This can reduce non-specific binding of probes to non-homologous DNA on the blot

T_m is the temperature at which the probe and target are 50% dissociated. For probes longer than 100 base pairs:

$$T_m = 81.5°C + 16.6 \log M + 0.41\ (\%\ G + C)$$

where M = ionic strength of buffer in moles/litre. With long probes, the hybridization is usually carried out at $T_m - 25°C$. When the probe is used to detect partially matched sequences, the hybridization temperature is reduced by 1°C for every 1% sequence divergence between probe and target.

$$T_m = 4 \times (\text{number of GC base pairs}) + 2 \times (\text{number of AT base pairs})$$

- probe length – the longer the oligonucleotide, the less chance there is of it binding to sequences other than the desired target sequence under conditions of high stringency;
- oligonucleotide composition – the GC content will influence the stability of the resultant hybrid and hence the determination of the appropriate stringency washing conditions. Also the presence of any non-complementary bases will have an effect on the hybridization conditions.

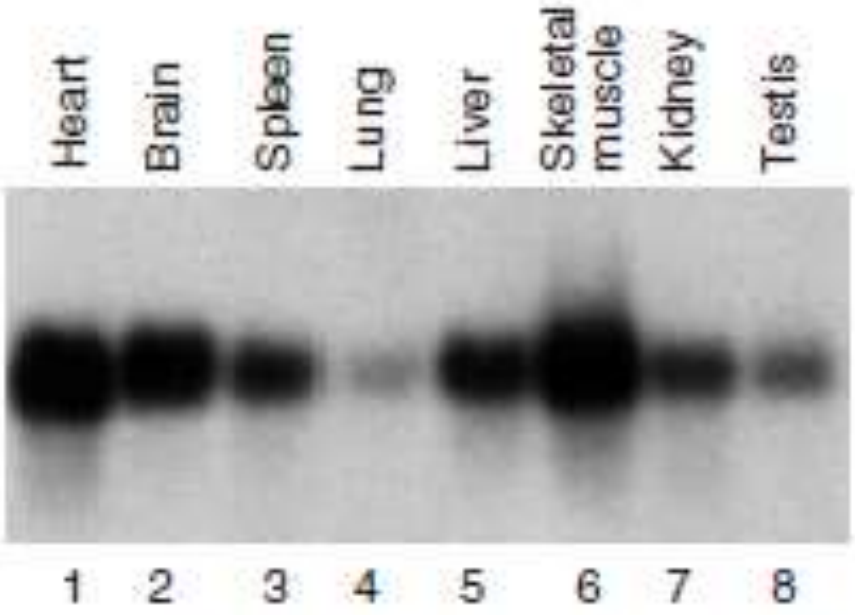
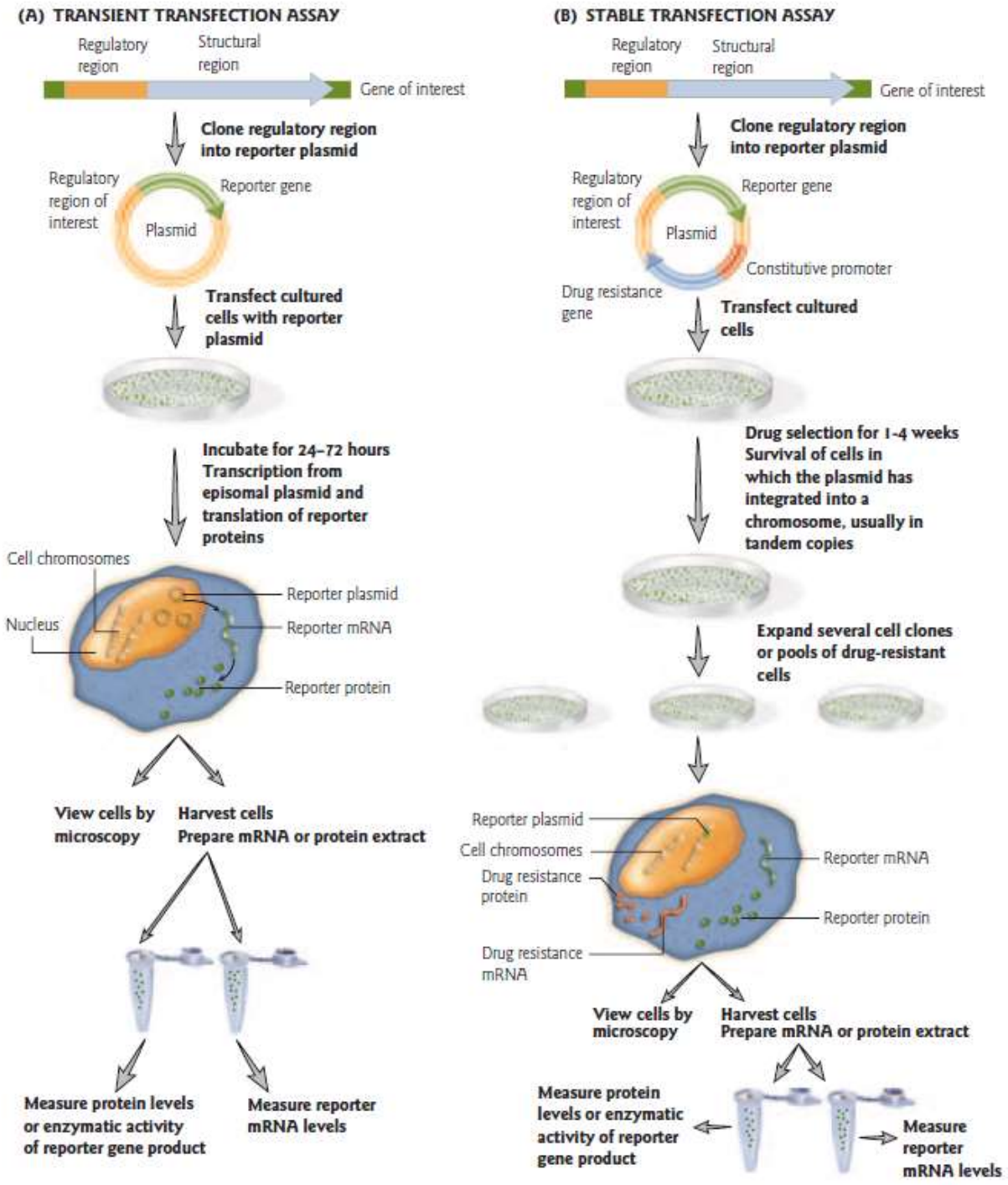
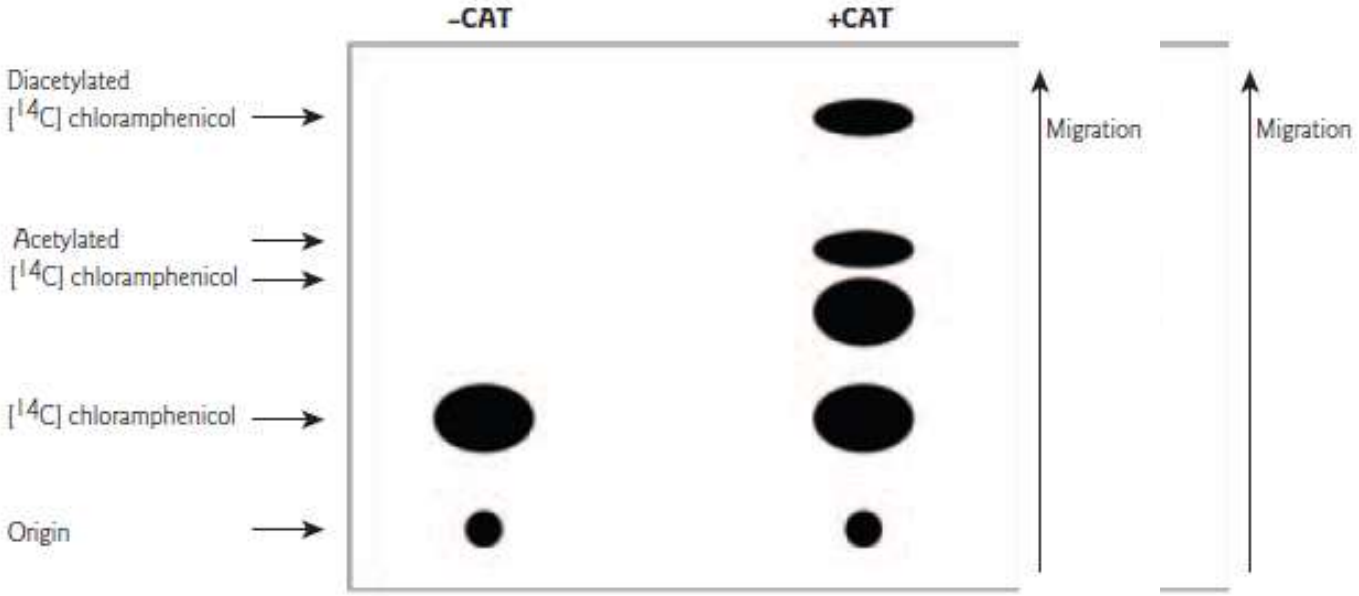


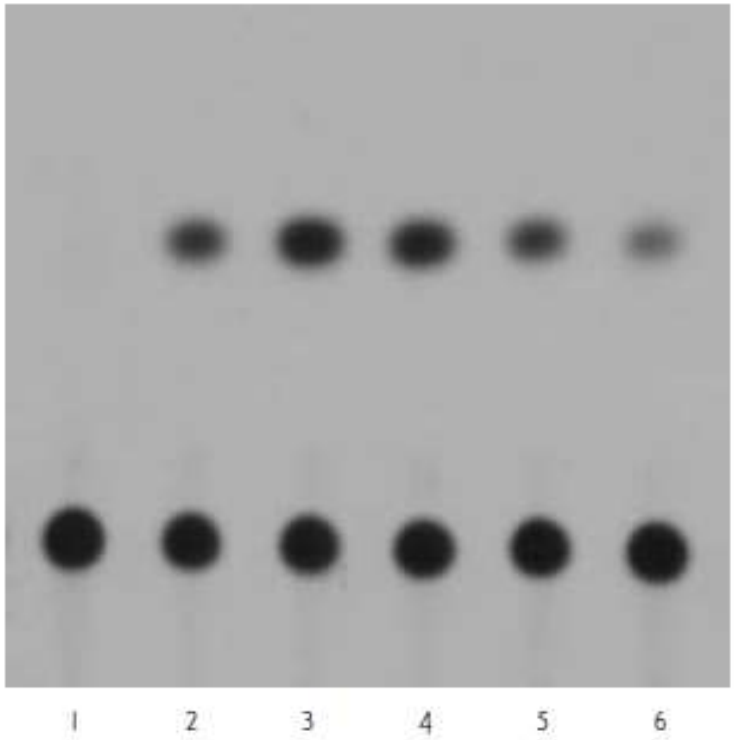
Figure 5.16 A Northern blot. Poly(A)$^+$ RNA was isolated from the rat tissues indicated at the top, then equal amounts of RNA from each tissue were electrophoresed and Northern blotted. The RNAs on the blot were hybridized to a labeled probe for the rat glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene, and the blot was then exposed to x-ray film. The bands represent the G3PDH mRNA, and their intensities are indicative of the amounts of this mRNA in each tissue. (*Source:* Courtesy Clontech.)



Transient and stable transfection assays

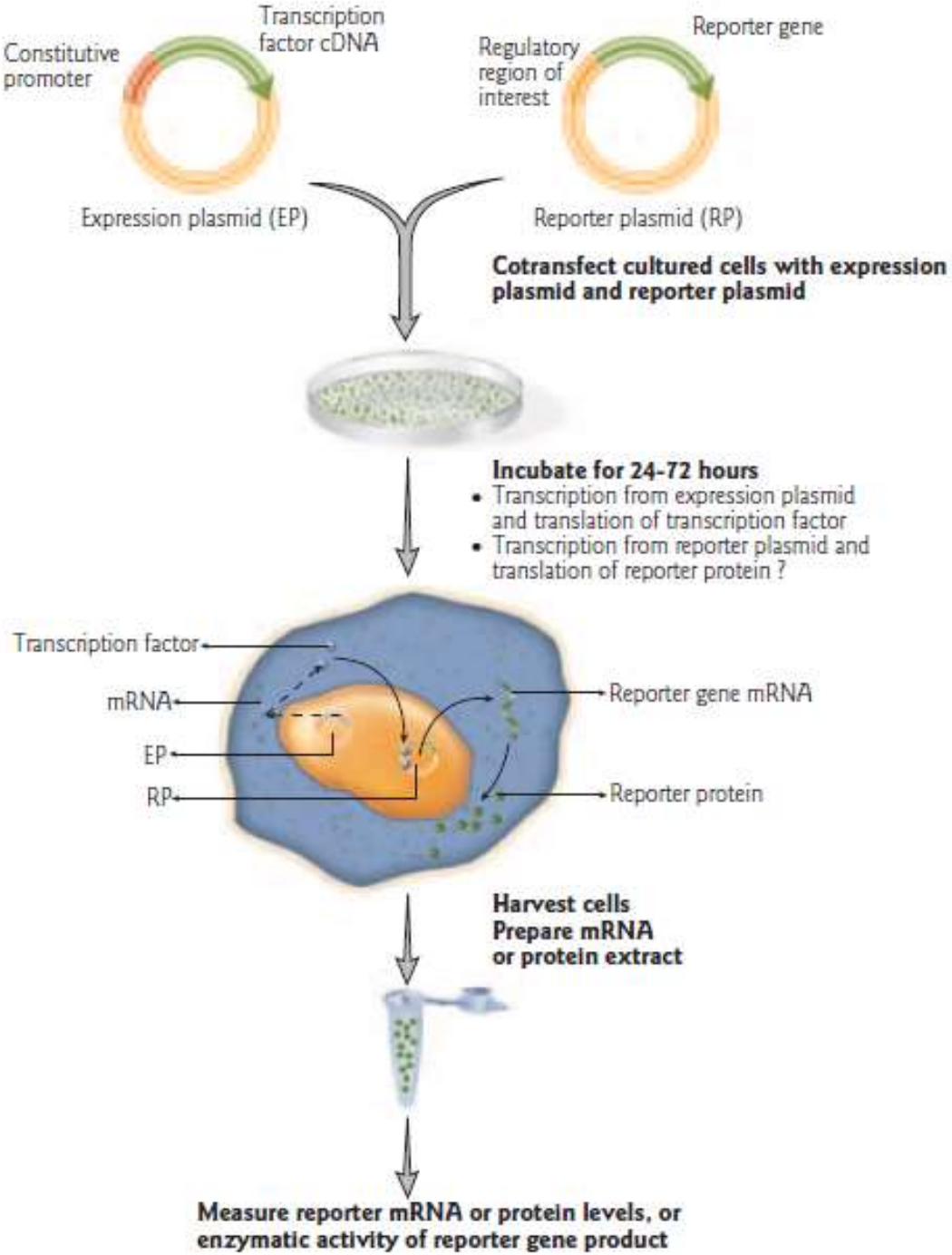


CAT reporter gene assay



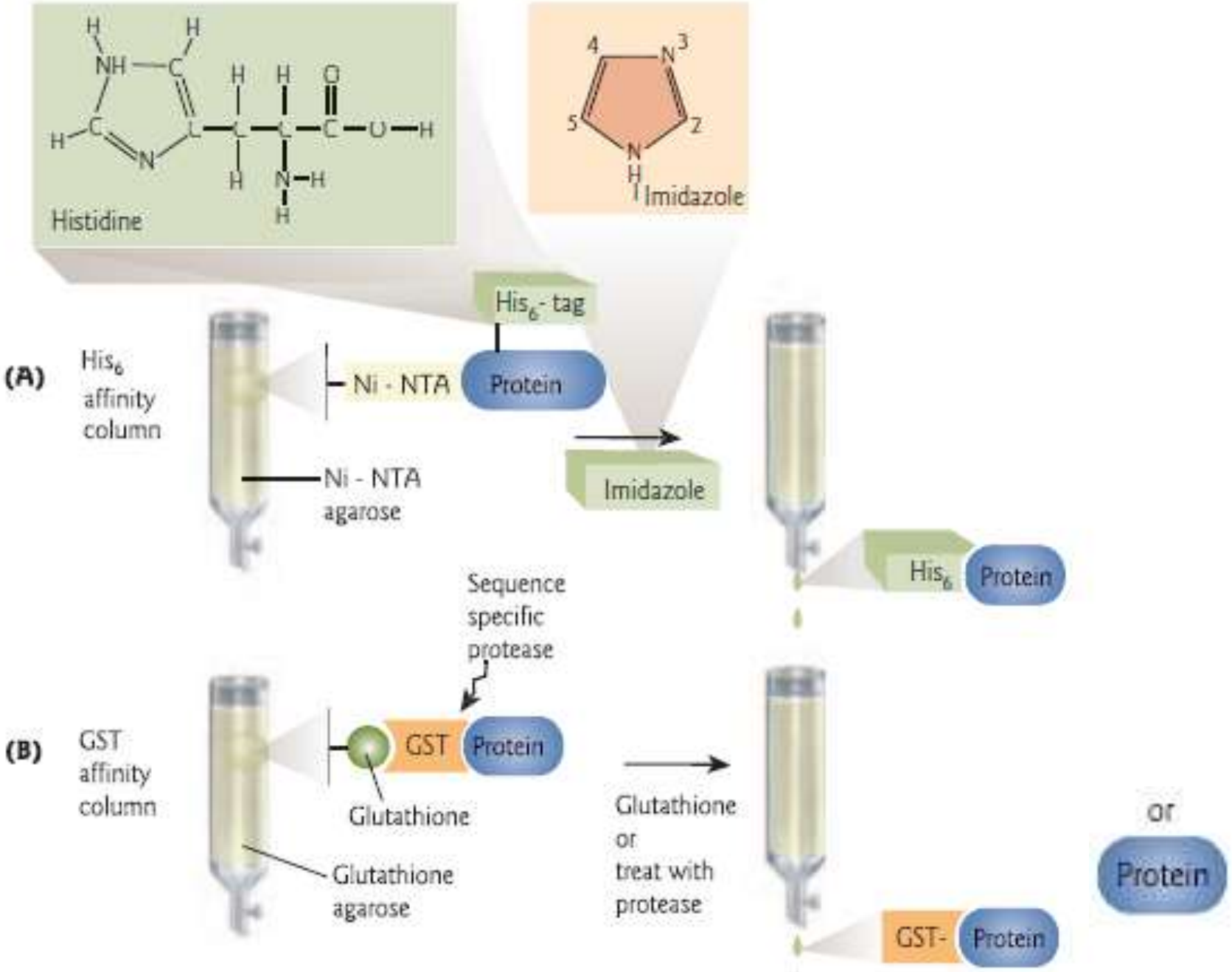
Commonly used reporter genes

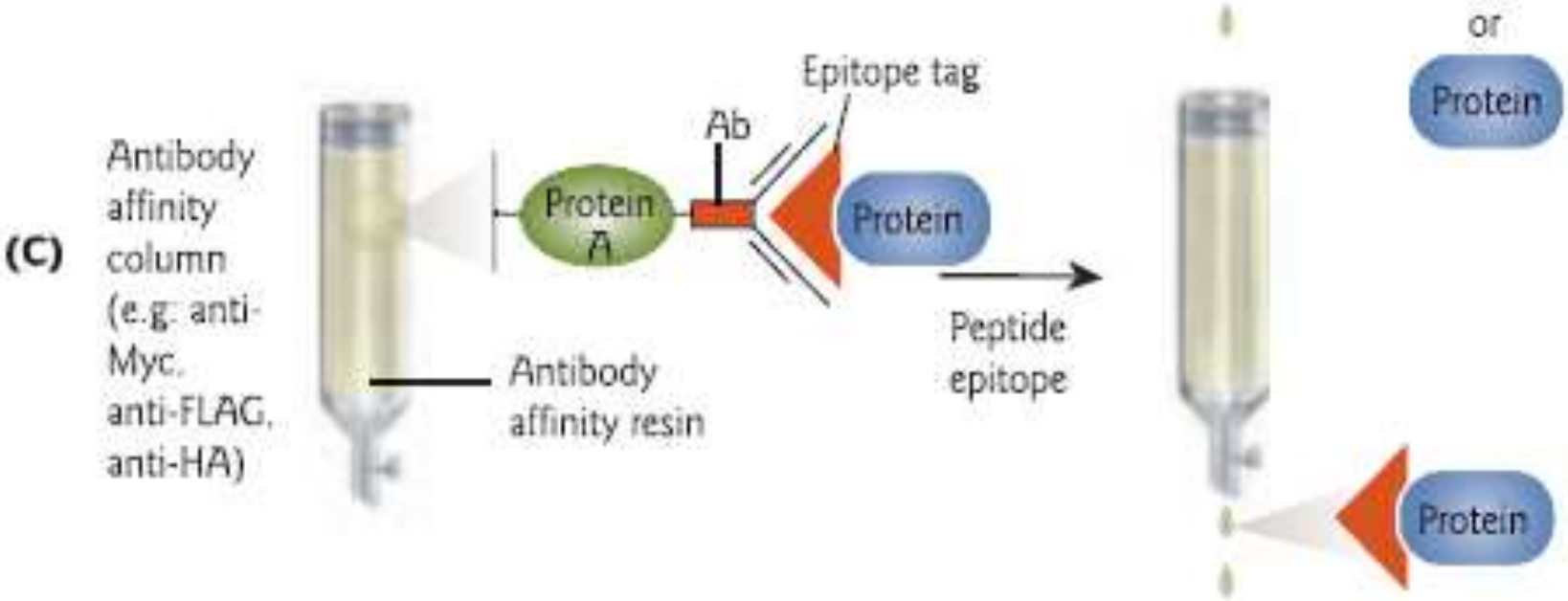
Lacz
Gus
gfp
cat
luc



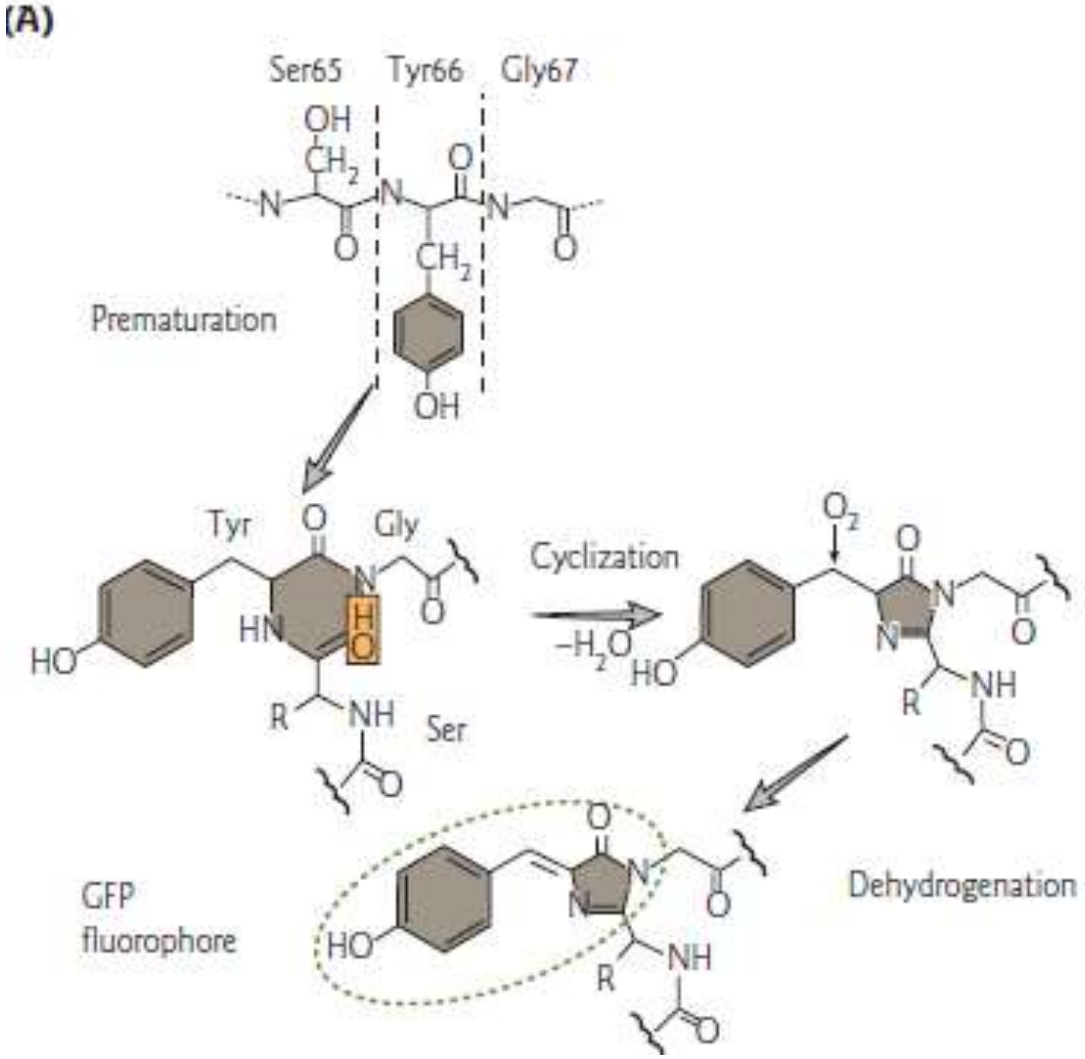
Cotransfection assay: Analysis Of gene regulation

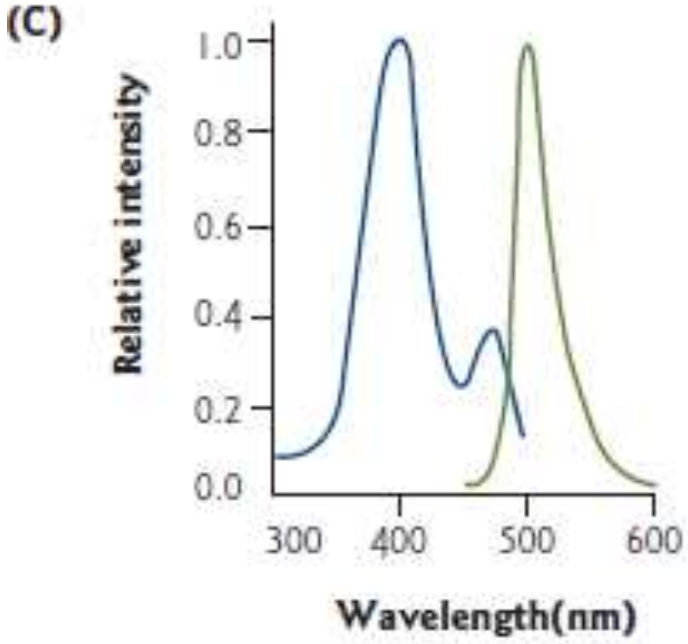
Commonly used purification and detection tags




(C)
Antibody affinity column (e.g. anti-Myc, anti-FLAG, anti-HA)
Protein A
Ab
Epitope tag
Protein
Antibody affinity resin
Peptide epitope
or
Protein
Protein

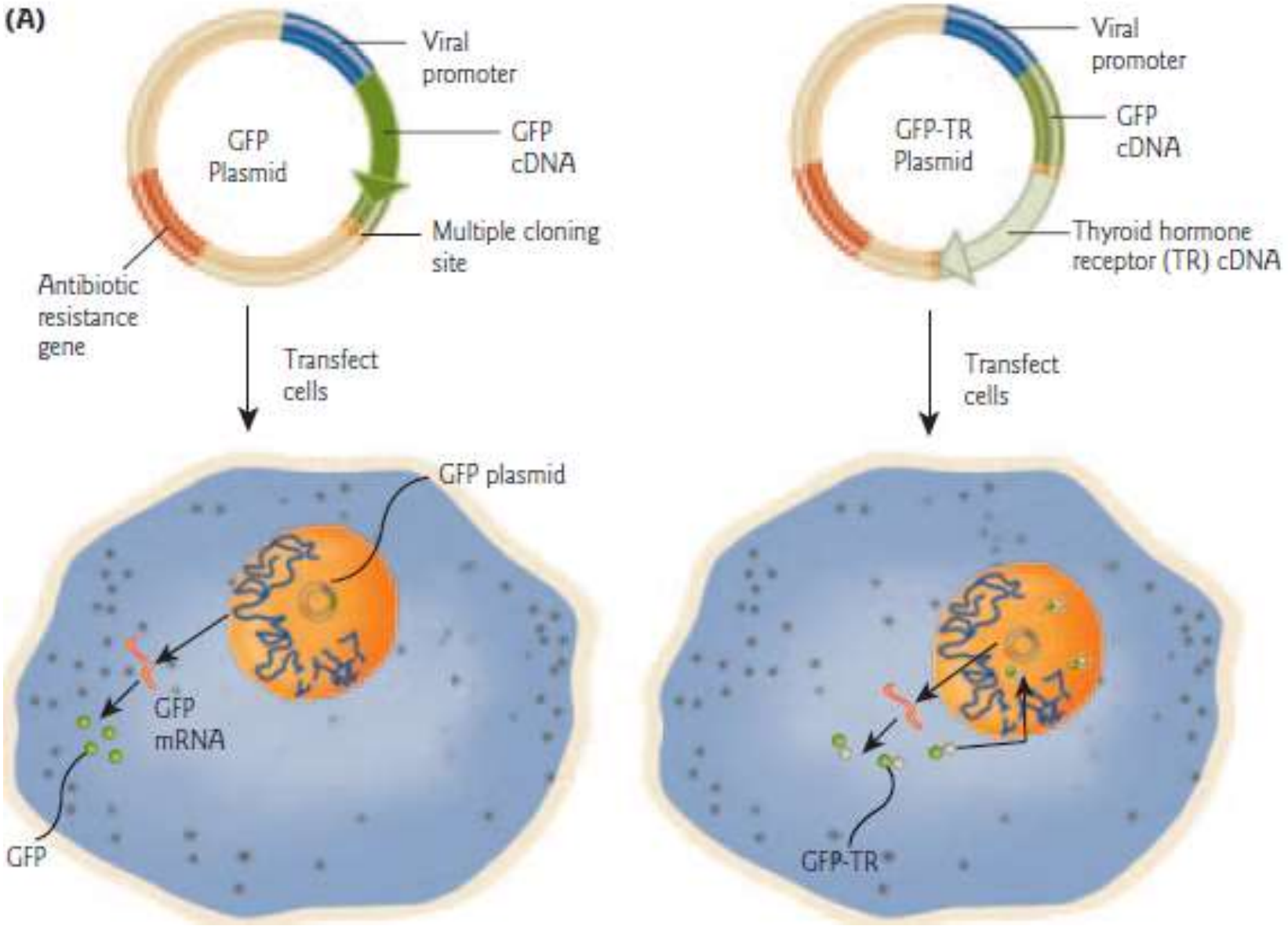
Properties of green fluorescent protein (GFP)



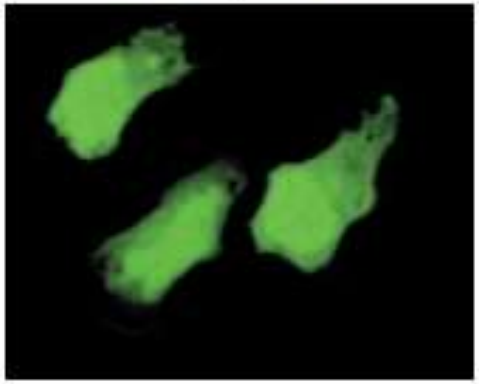


GFP-----CFP
RFP orDsRED
Discosoma striata

Use of Green fluorescent protein (GFP) fusion proteins



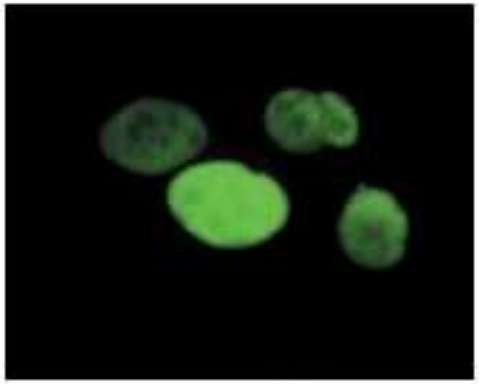
View by fluorescence microscopy



GFP

DAPI

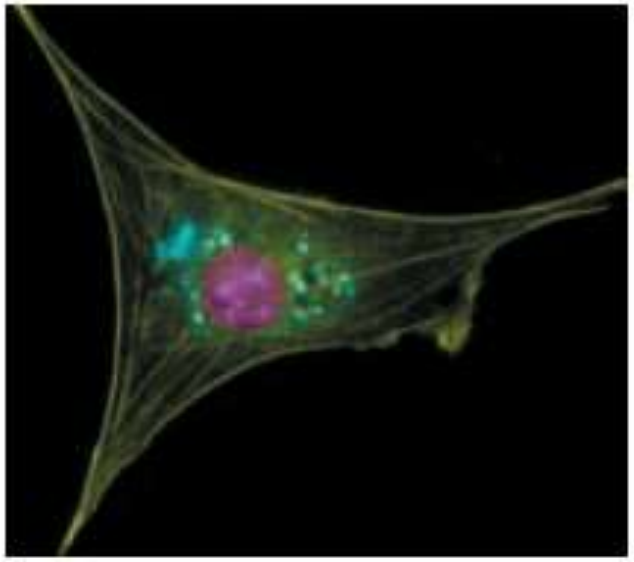
View by fluorescence microscopy

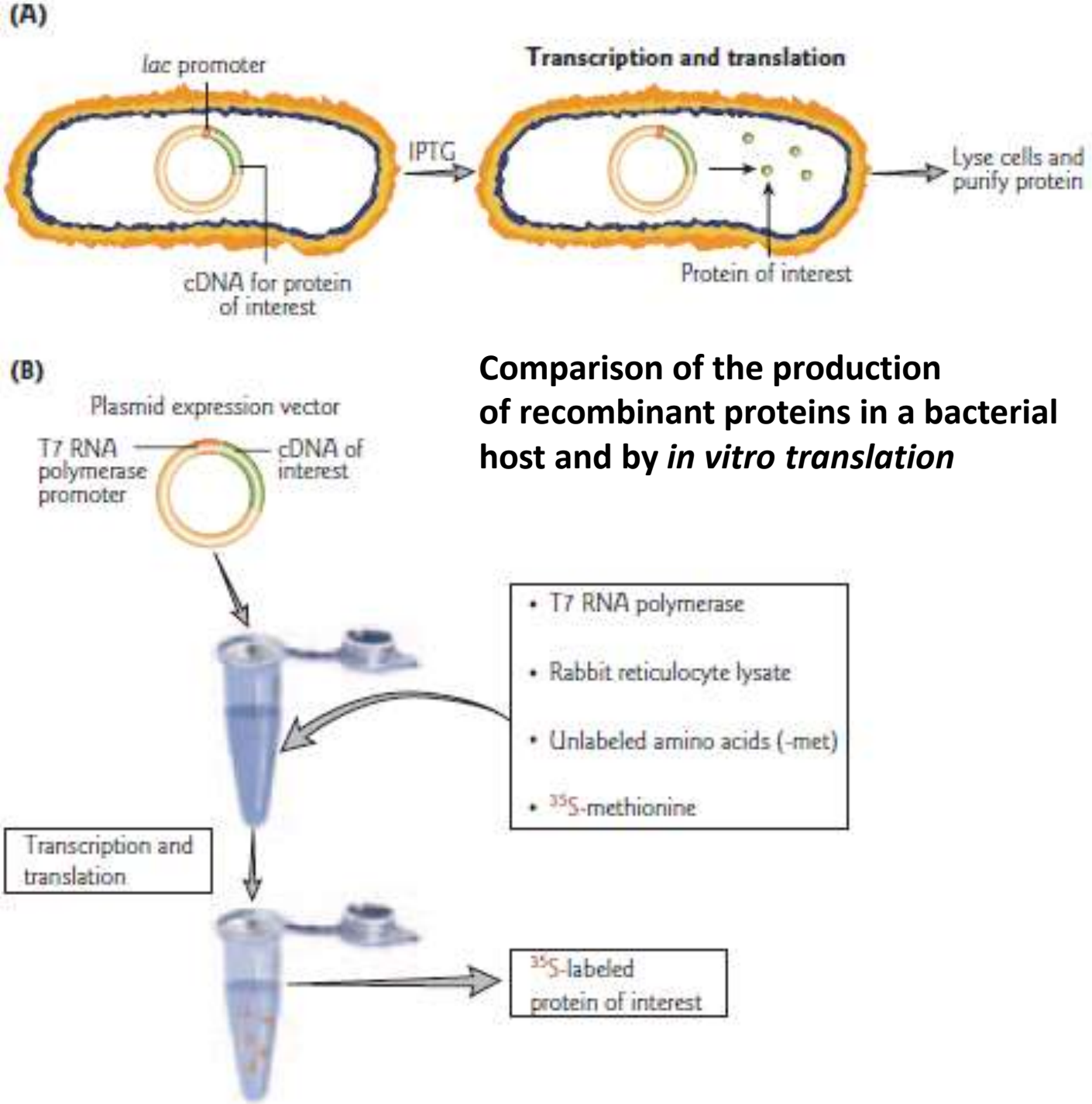


GFP-TR

DAPI

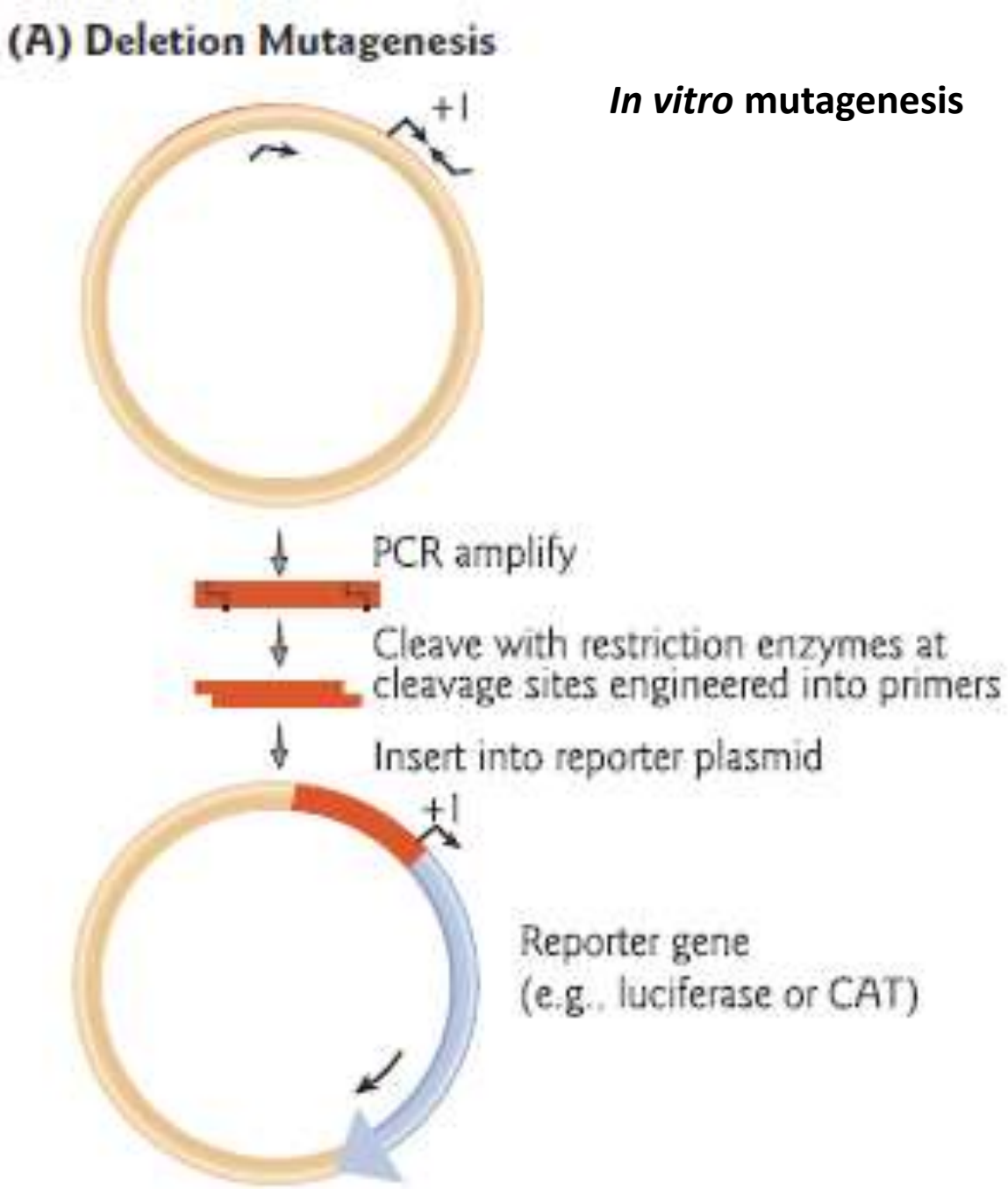
(B)



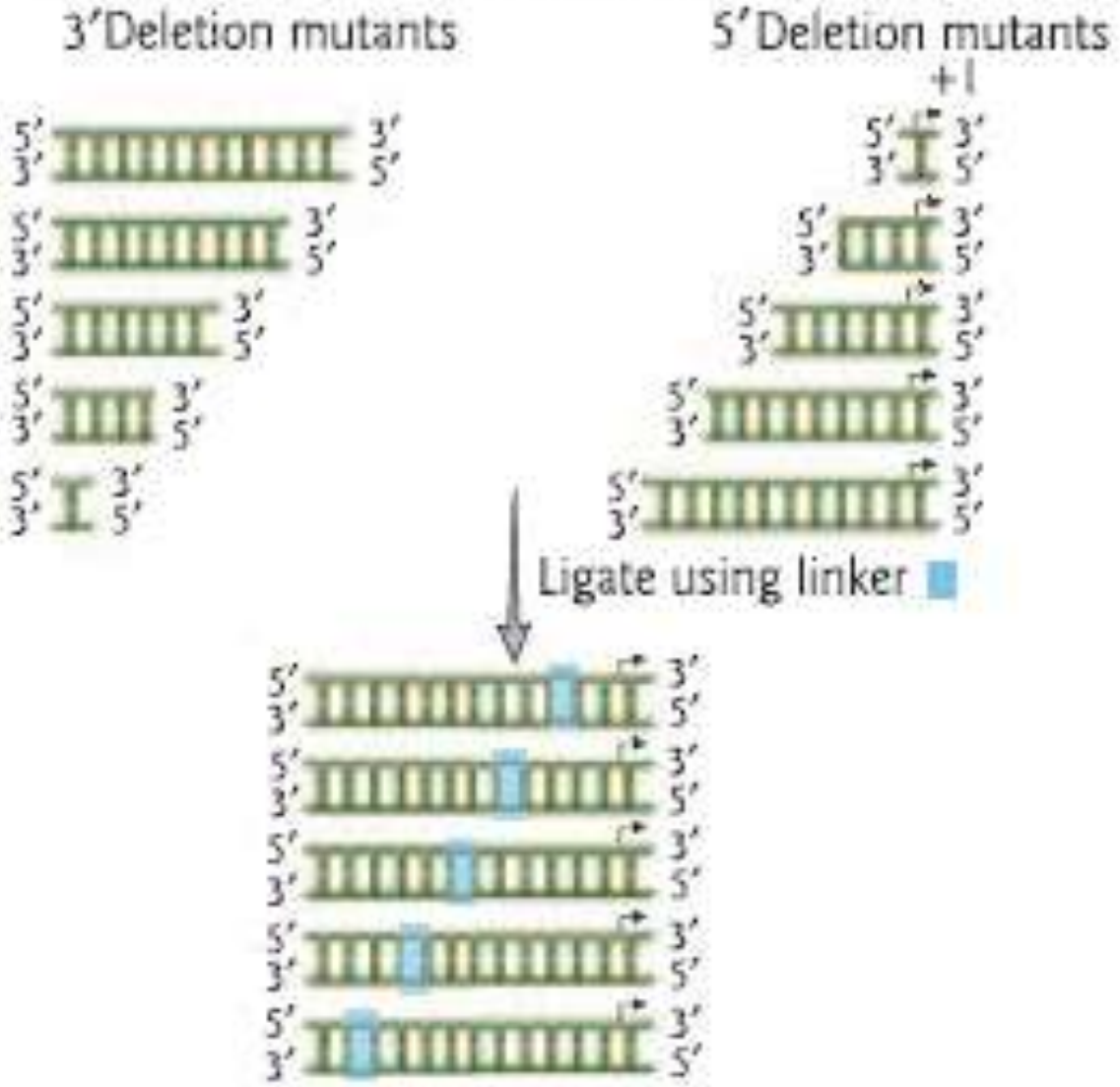


Comparison of the production of recombinant proteins in a bacterial host and by *in vitro translation*

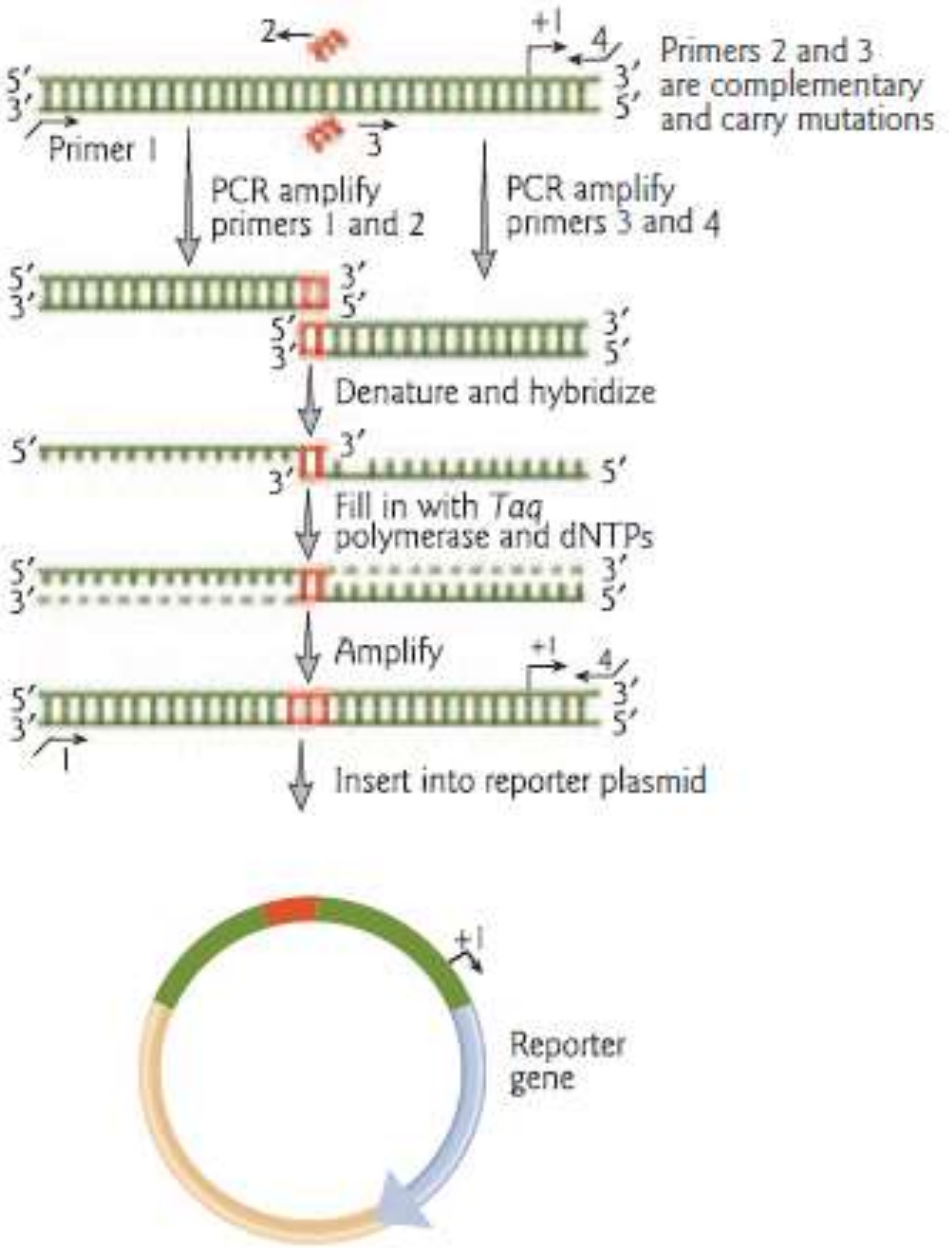
***In vitro* mutagenesis**



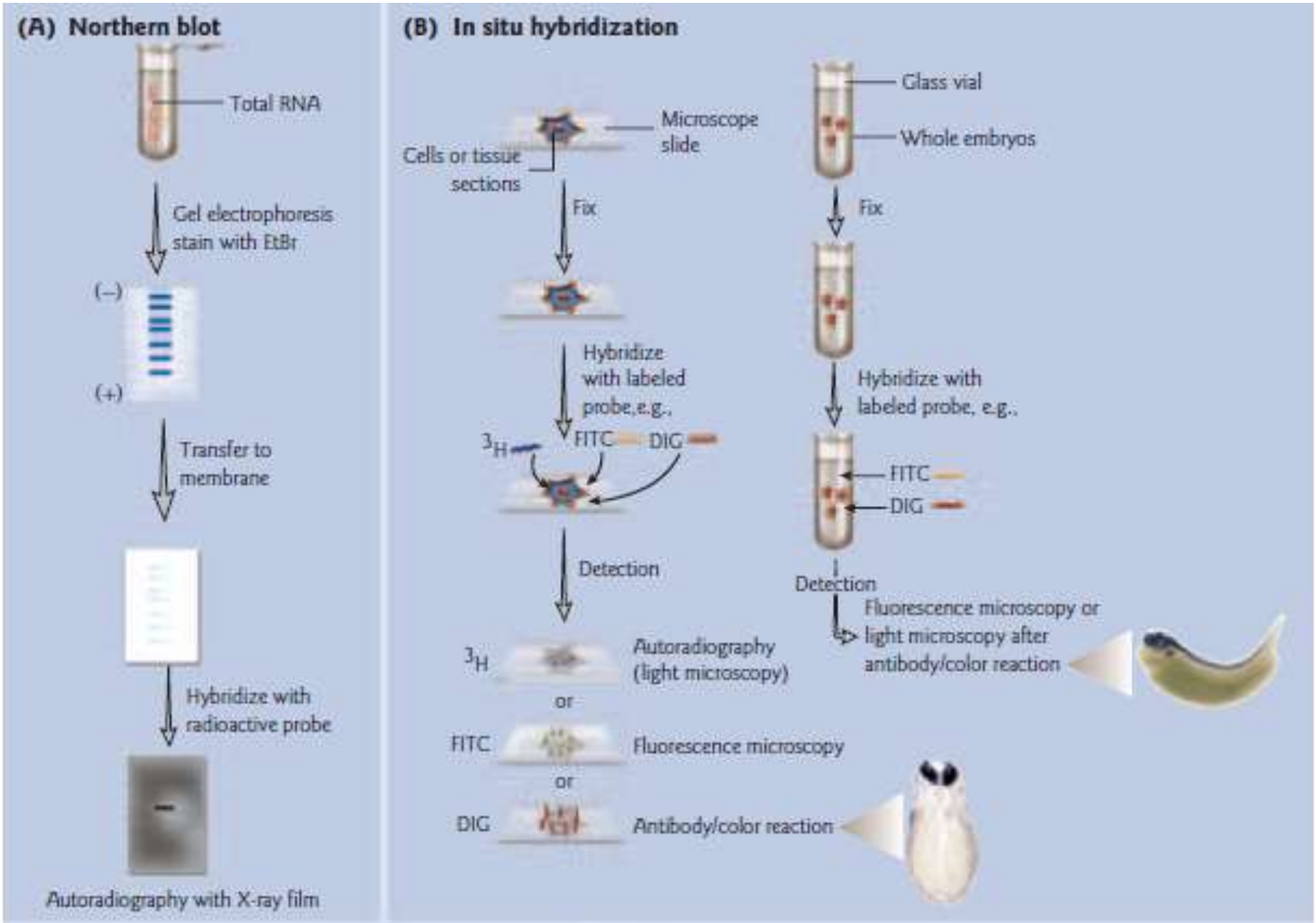
(B) Linker scanning Mutagenesis

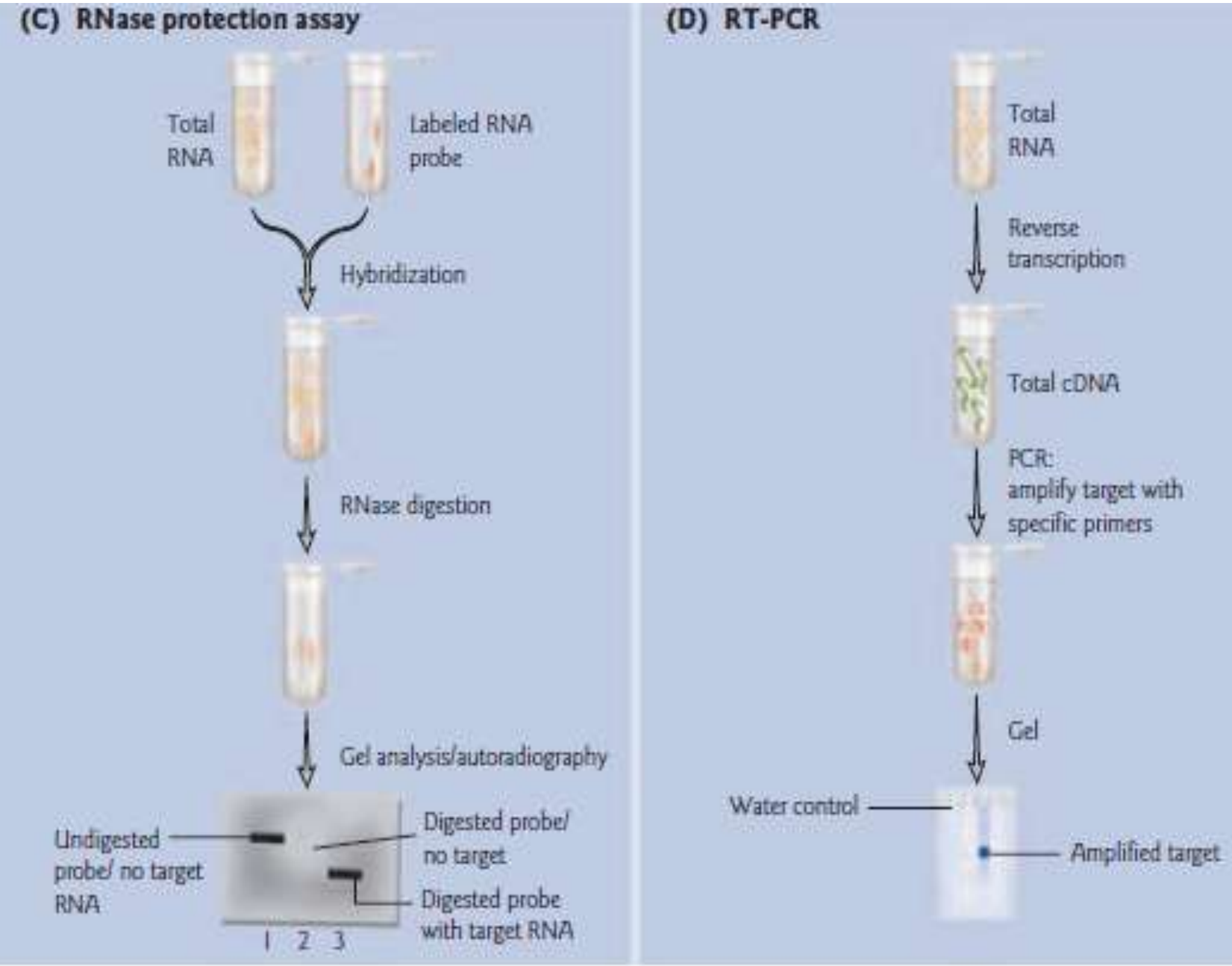


(C) Site-Directed Mutagenesis by PCR

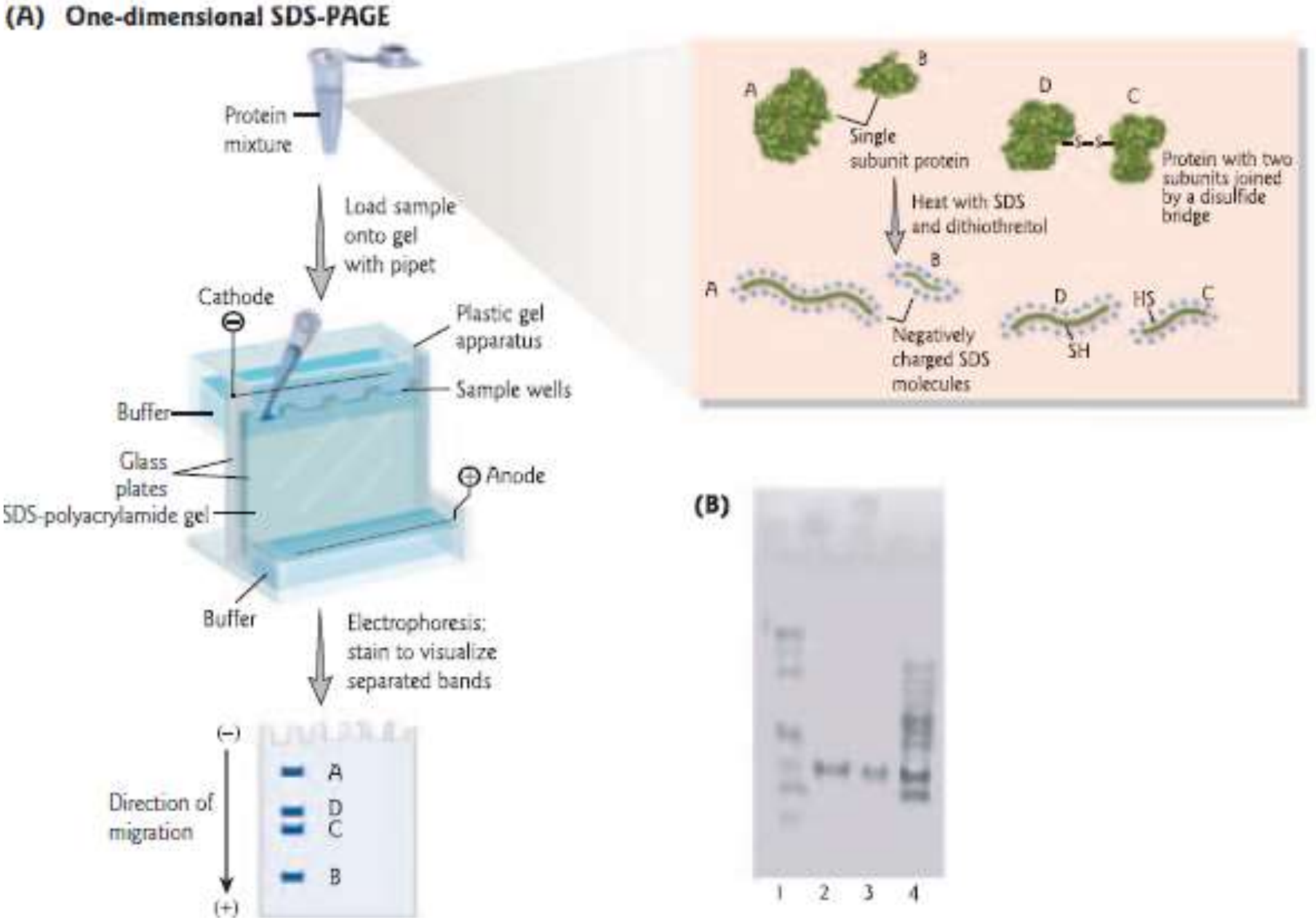


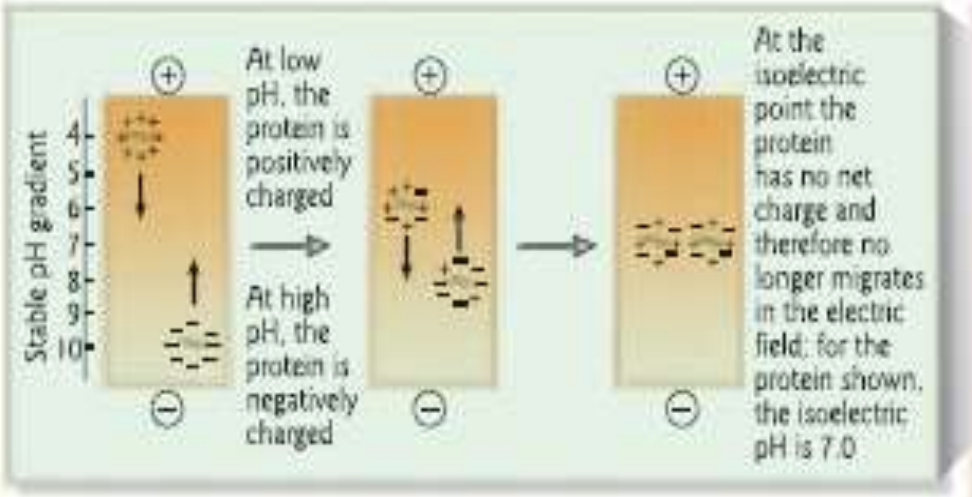
Analysis at the level of gene transcription: RNA expression and localization



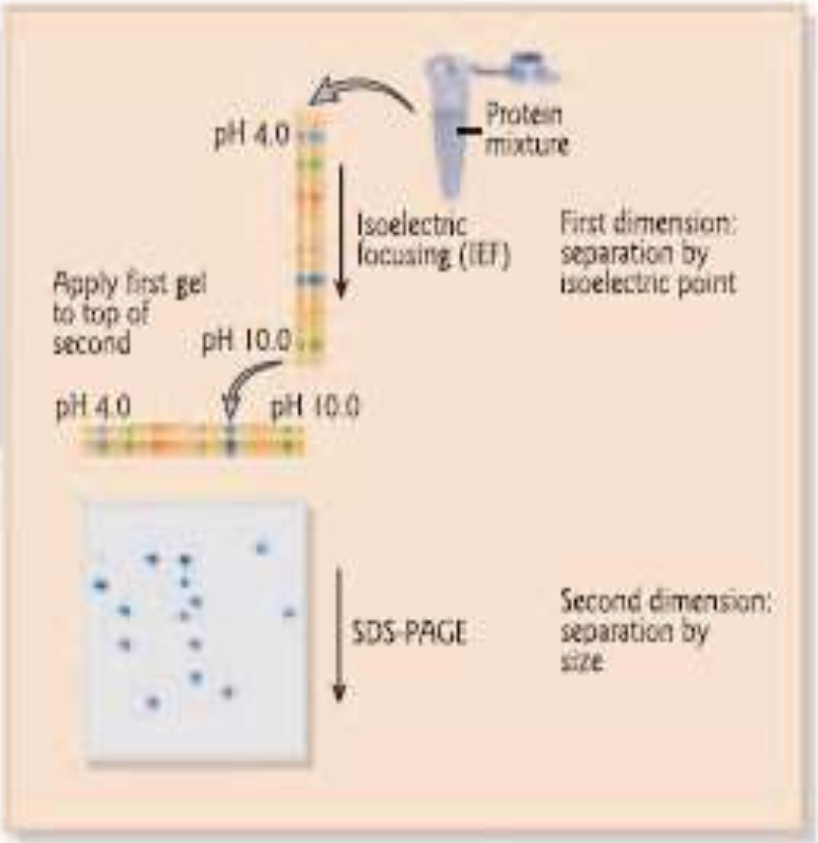

(C) RNase protection assay
Total RNA
Labeled RNA probe
Hybridization
RNase digestion
Gel analysis/autoradiography
Undigested probe/ no target RNA
Digested probe/ no target
Digested probe with target RNA
1 2 3
(D) RT-PCR
Total RNA
Reverse transcription
Total cDNA
PCR: amplify target with specific primers
Gel
Water control
Amplified target

Analysis at the level of translation: protein expression and localization

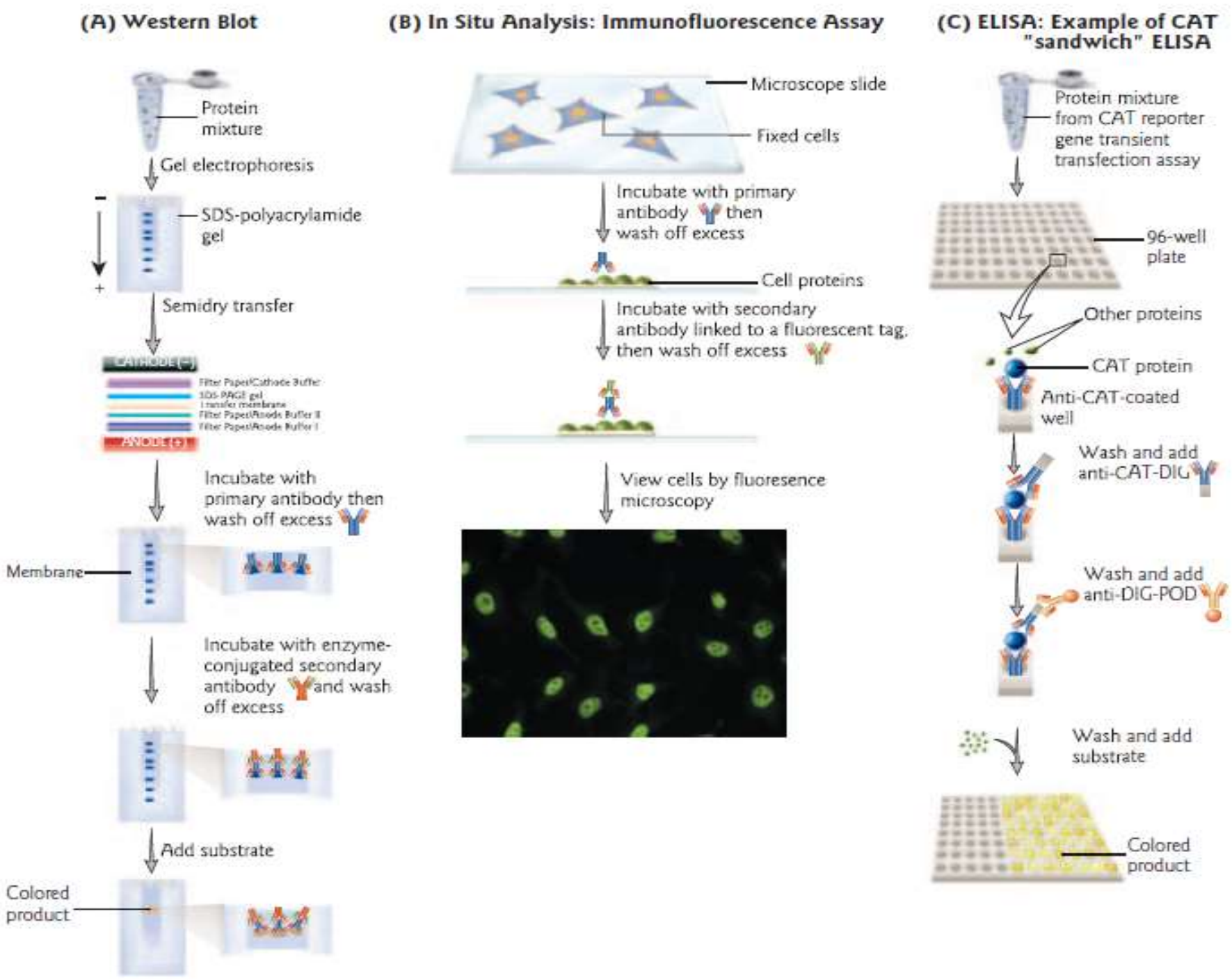




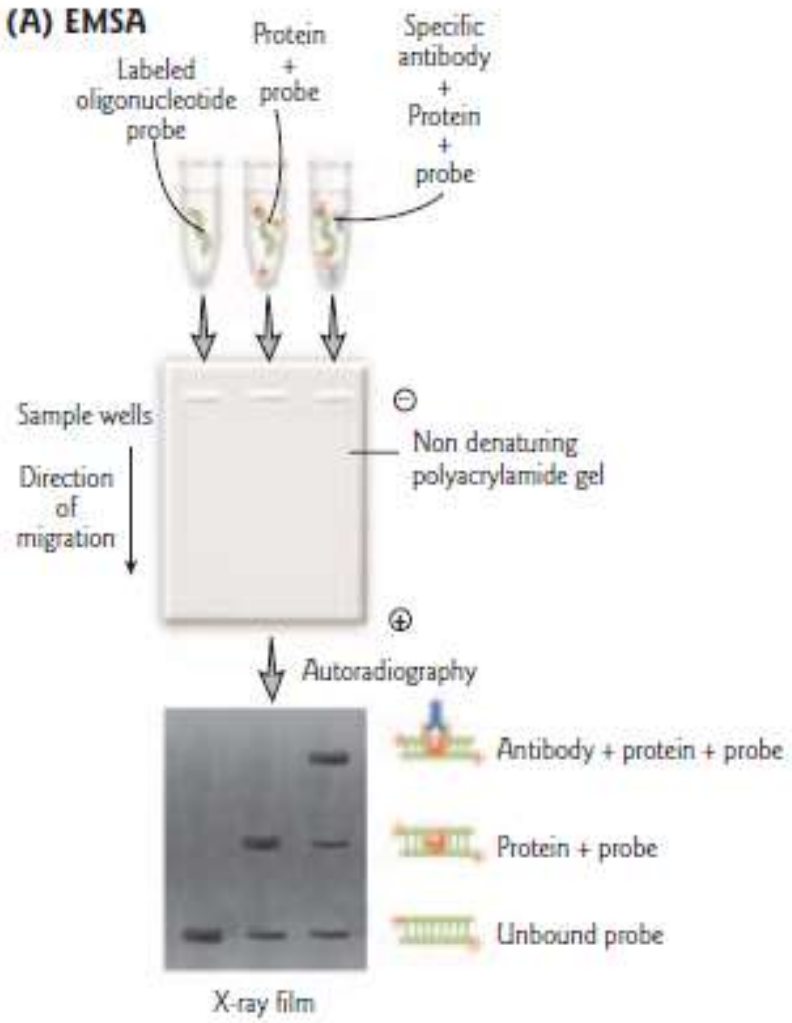
(C) **Two-dimensional PAGE**

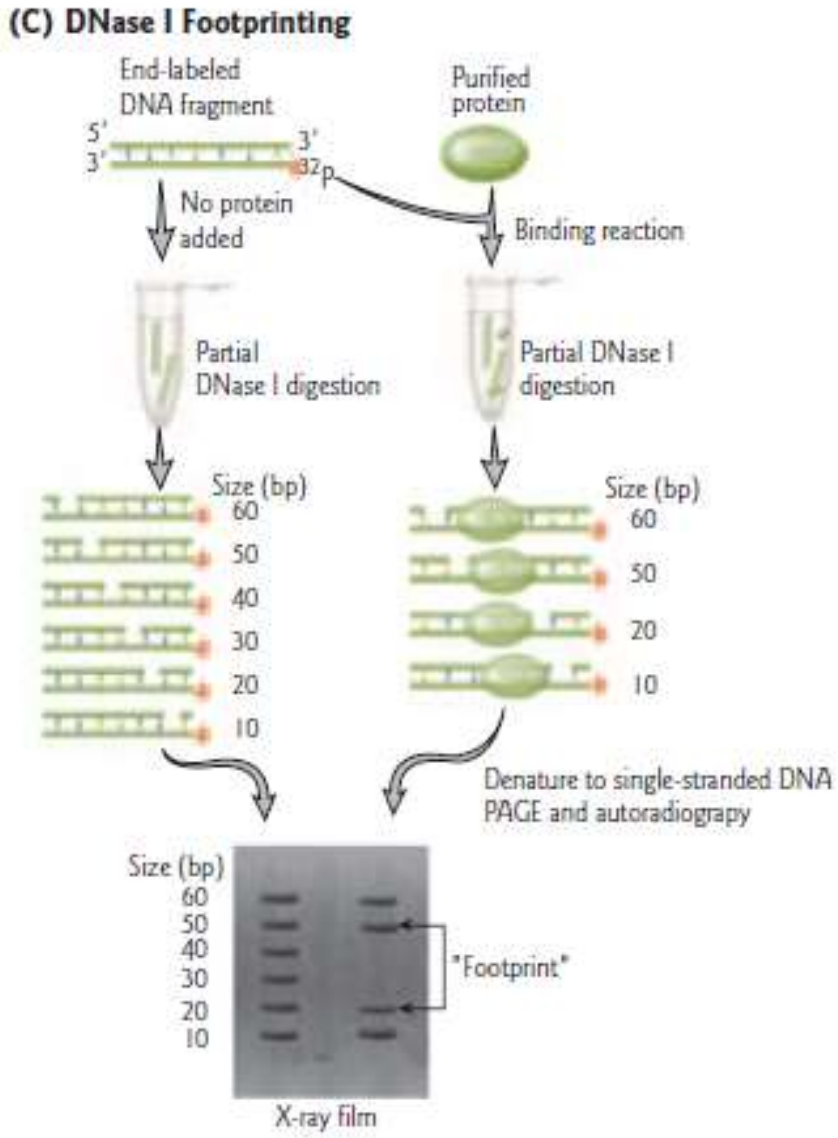


(D)

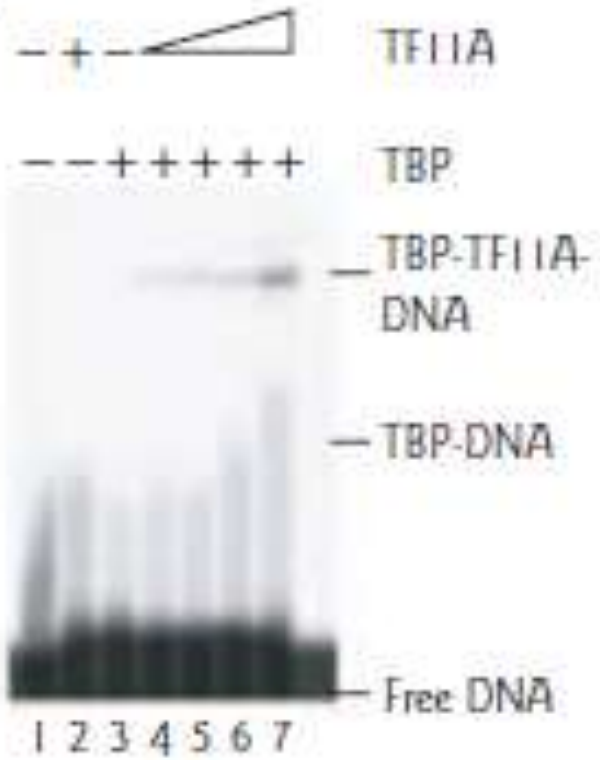

(A) Western Blot
Protein mixture
Gel electrophoresis
SDS-polyacrylamide gel
Semidry transfer
Incubate with primary antibody then wash off excess
Membrane
Incubate with enzyme-conjugated secondary antibody and wash off excess
Add substrate
Colored product
(B) In Situ Analysis: Immunofluorescence Assay
Microscope slide
Fixed cells
Incubate with primary antibody then wash off excess
Cell proteins
Incubate with secondary antibody linked to a fluorescent tag, then wash off excess
View cells by fluoresence microscopy
(C) ELISA: Example of CAT "sandwich" ELISA
Protein mixture from CAT reporter gene transient transfection assay
96-well plate
Other proteins
CAT protein
Anti-CAT-coated well
Wash and add anti-CAT-DIG
Wash and add anti-DIG-POD
Wash and add substrate
Colored product

Analysis of DNA–protein interactions

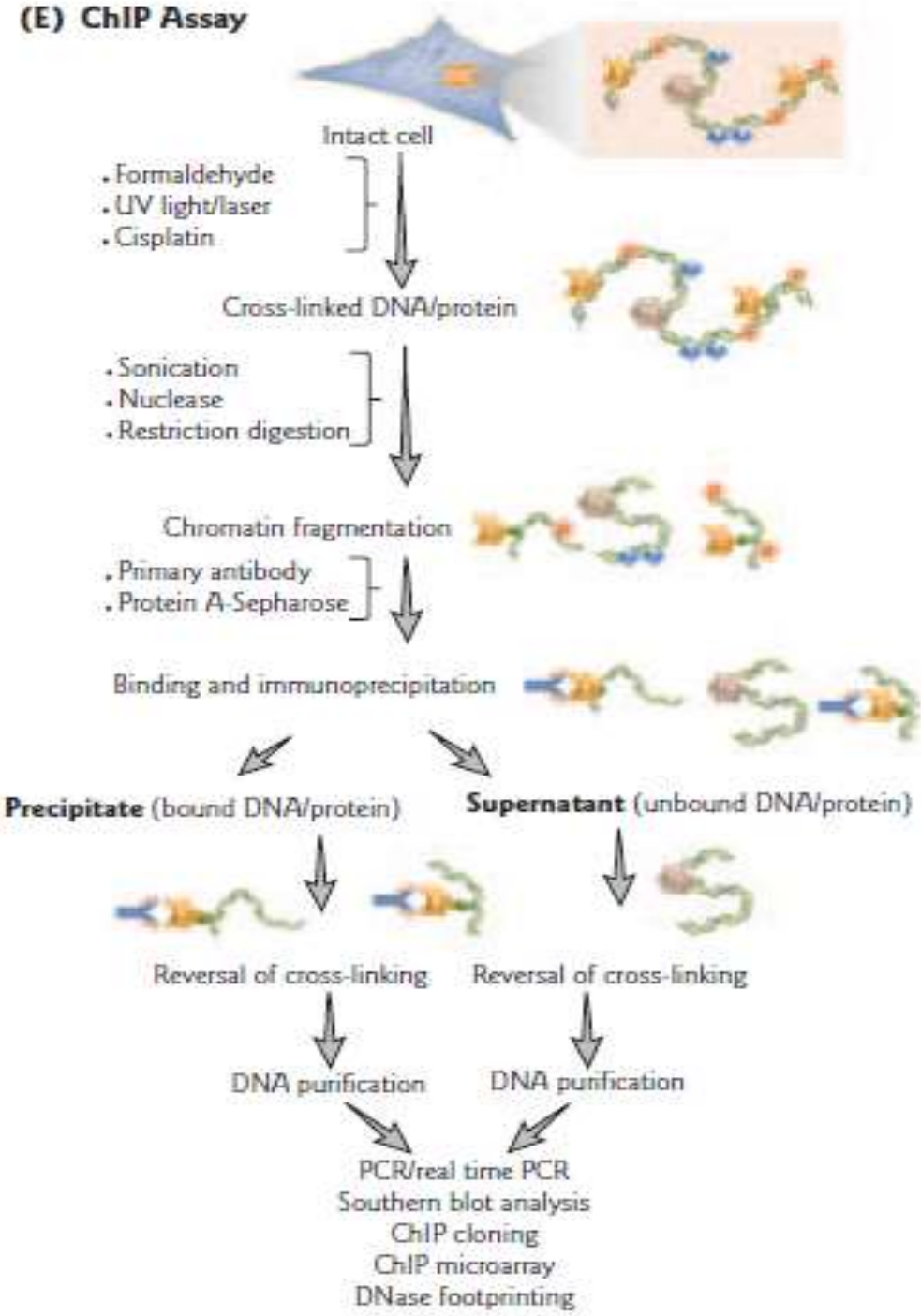




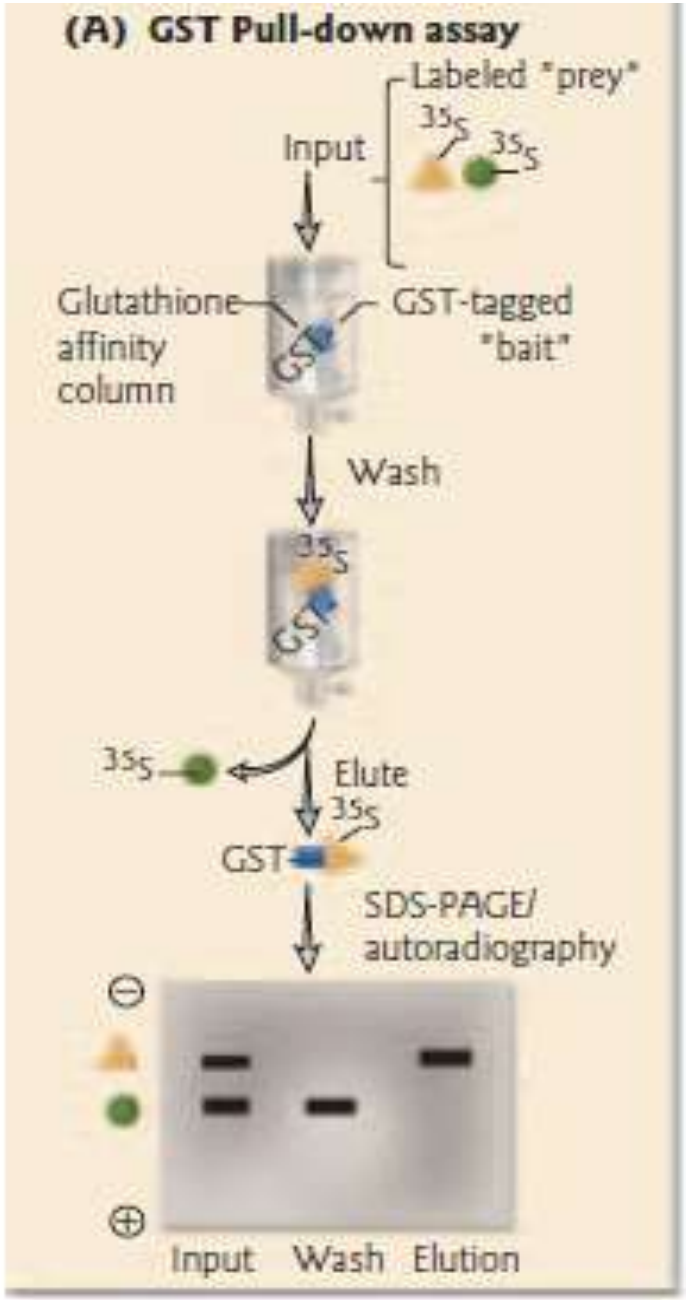
(B)

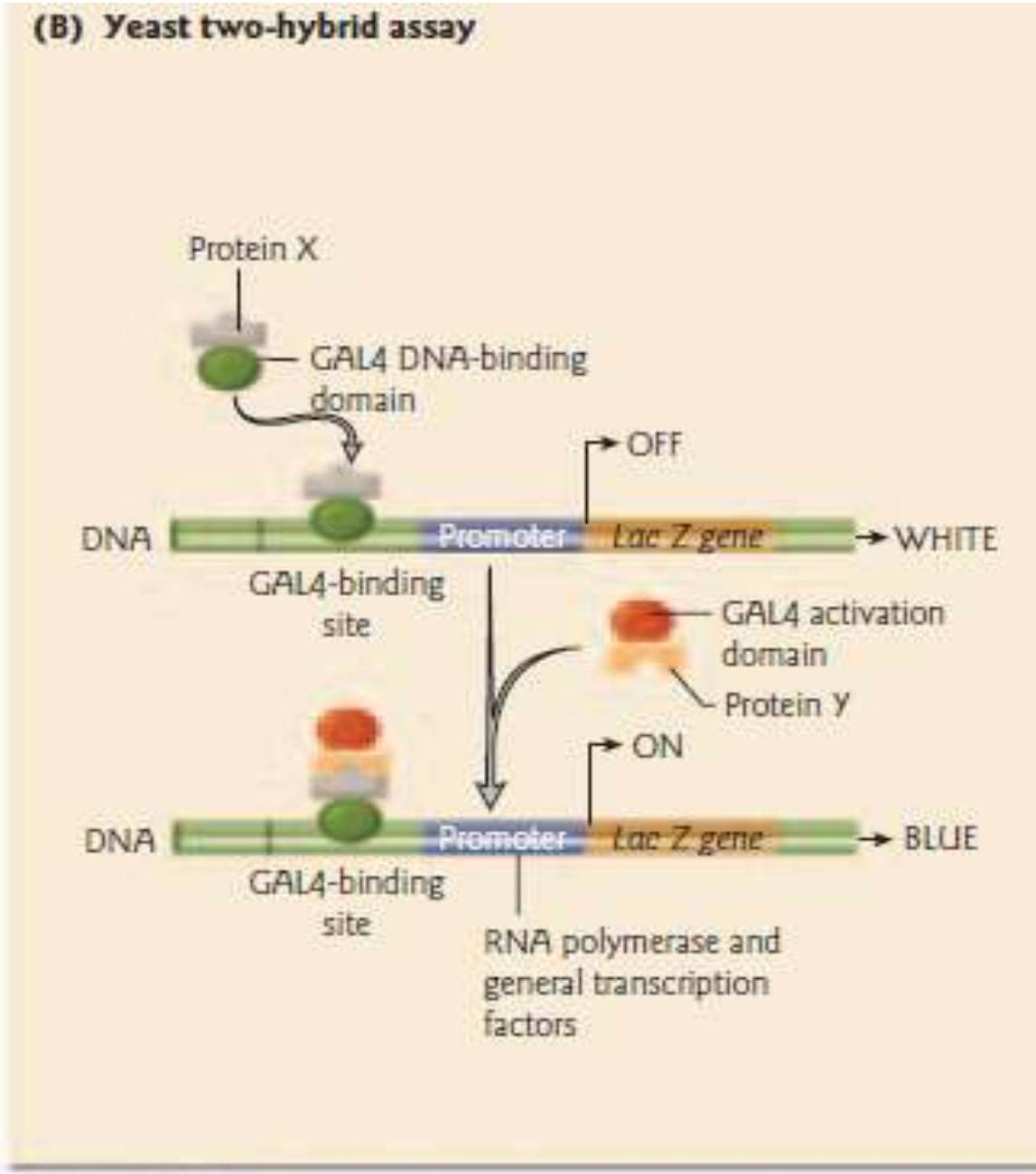


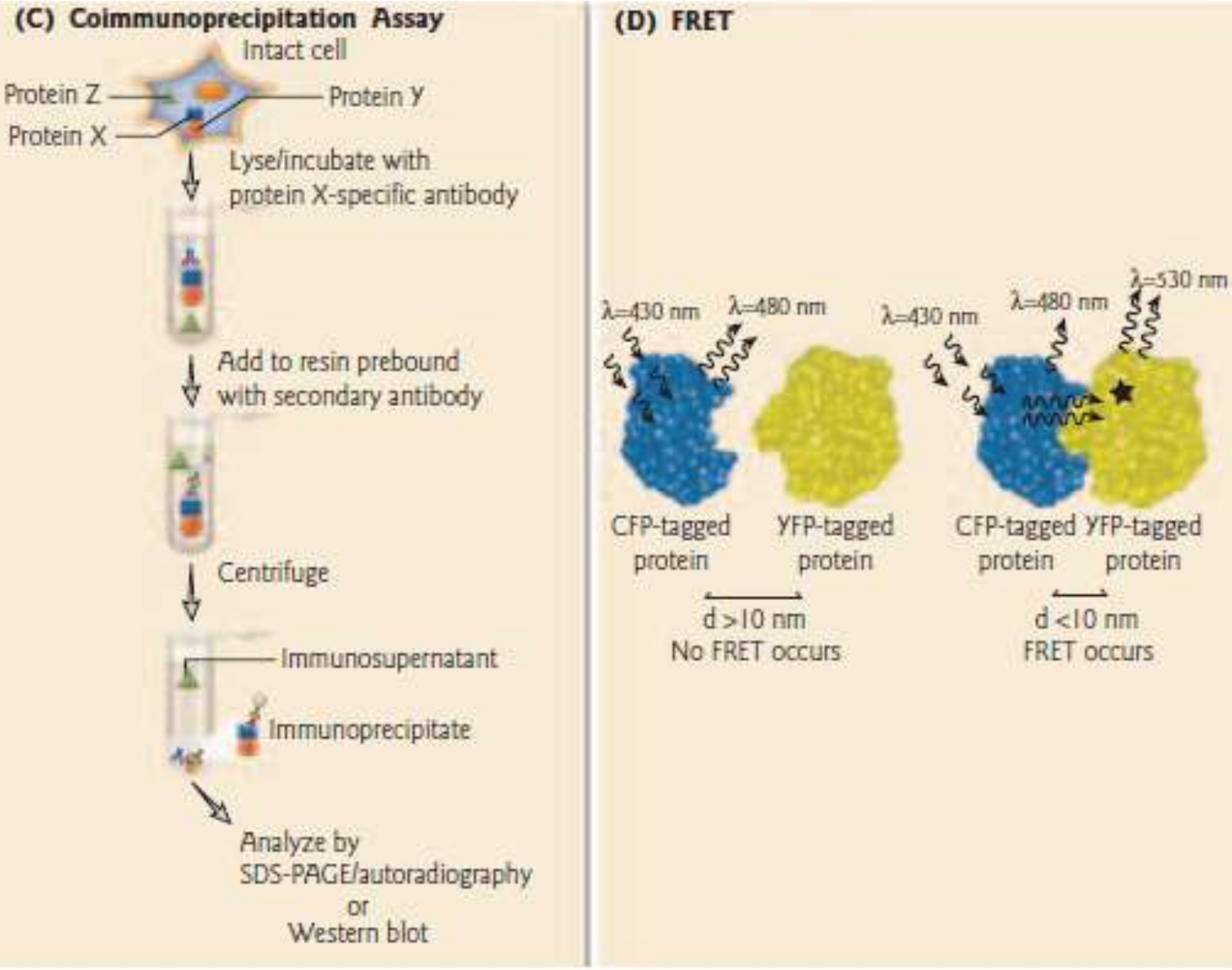
(D)


(E) ChIP Assay
Intact cell
• Formaldehyde
• UV light/laser
• Cisplatin
Cross-linked DNA/protein
• Sonication
• Nuclease
• Restriction digestion
Chromatin fragmentation
• Primary antibody
• Protein A-Sepharose
Binding and immunoprecipitation
Precipitate (bound DNA/protein)
Supernatant (unbound DNA/protein)
Reversal of cross-linking
Reversal of cross-linking
DNA purification
DNA purification
PCR/real time PCR
Southern blot analysis
ChIP cloning
ChIP microarray
DNase footprinting

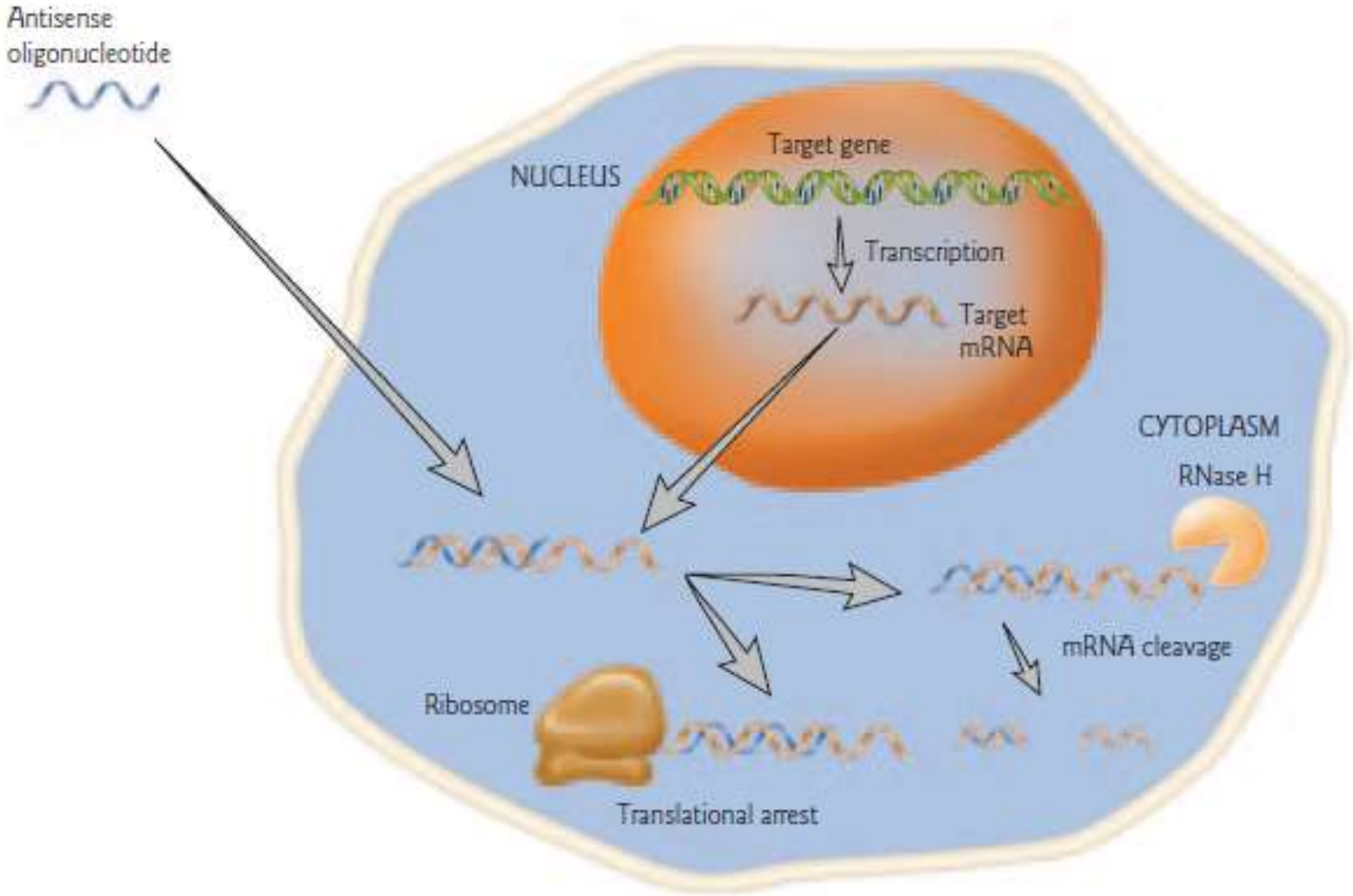
Analysis of protein–protein interactions






(C) Coimmunoprecipitation Assay
Intact cell
Protein Z
Protein Y
Protein X
Lyse/incubate with protein X-specific antibody
Add to resin prebound with secondary antibody
Centrifuge
Immunosupernatant
Immunoprecipitate
Analyze by SDS-PAGE/autoradiography or Western blot
(D) FRET
λ=430 nm
λ=480 nm
λ=530 nm
CFP-tagged protein
YFP-tagged protein
d >10 nm
No FRET occurs
d <10 nm
FRET occurs

Antisense technology



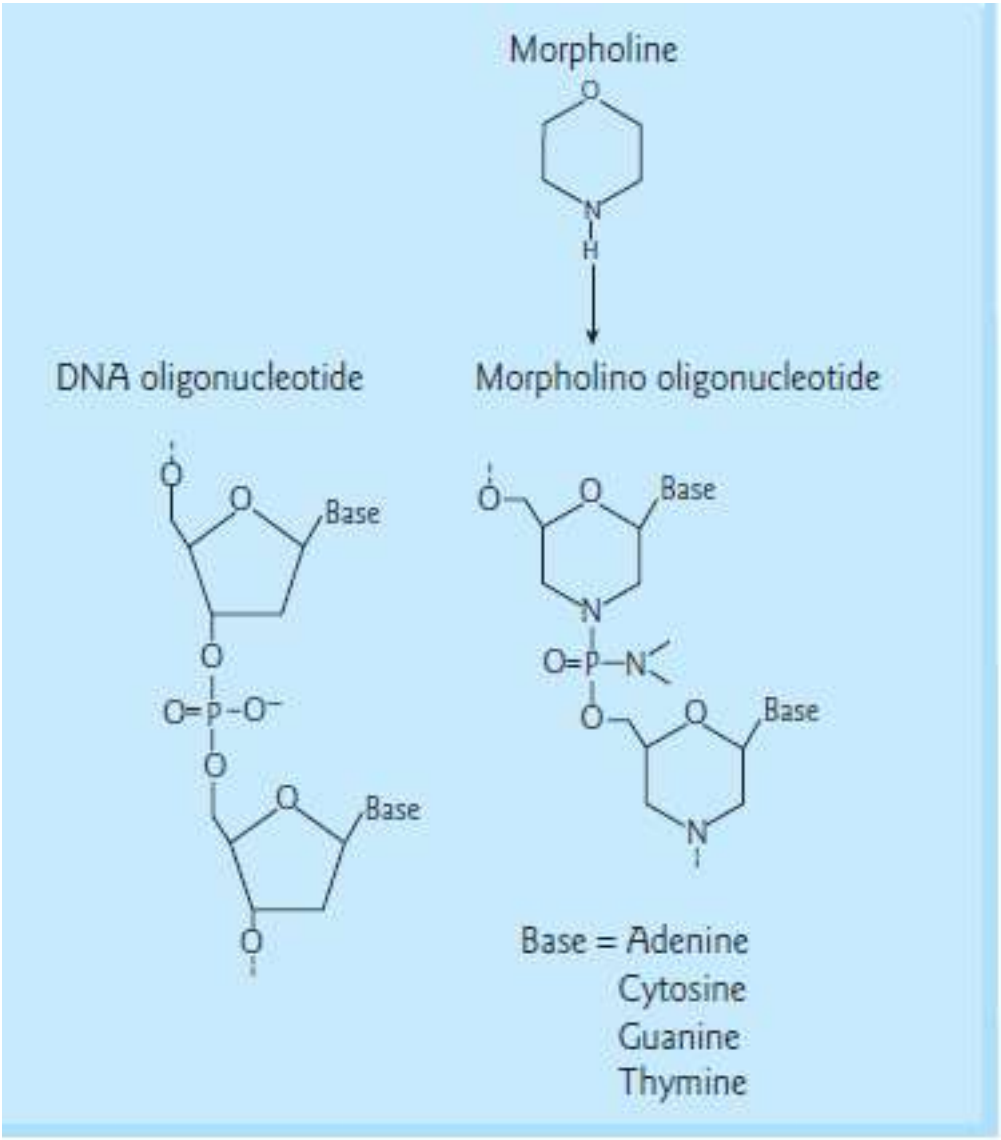
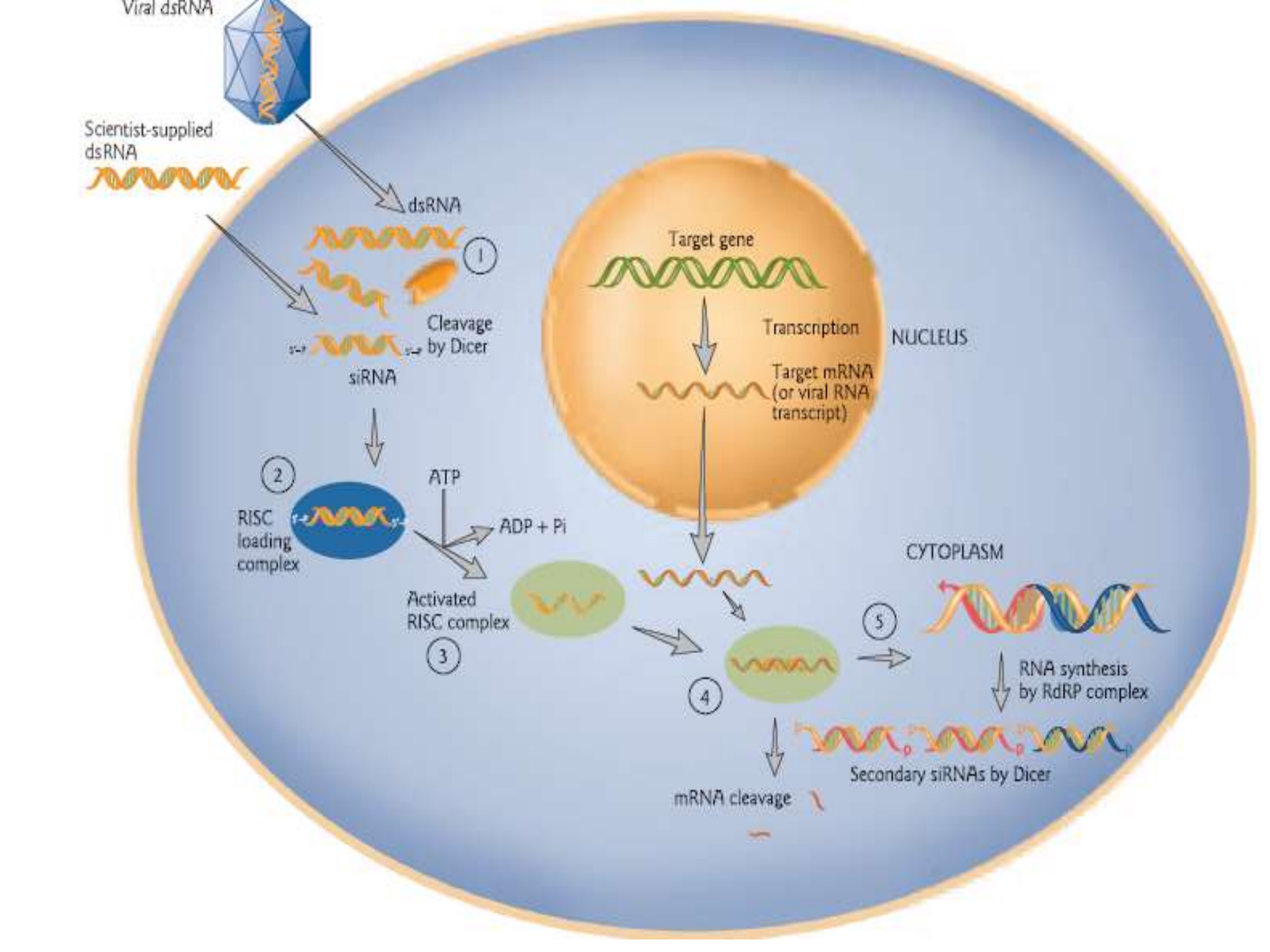


Figure 9.11 Structures of DNA and morpholino oligonucleotides.
Morpholino oligonucleotides are assembled from four different morpholine subunits, each of which contains one of the four bases (adenine, cytosine, guanine, and thymine) linked to a six-membered morpholine ring. Typically, 18–25 subunits are joined in a specific order by nonionic phosphorodiamidate intersubunit bonds to give a morpholino oligonucleotide, but they can be any length. The structure is compared with a DNA oligonucleotide in which the nucleotide subunits are joined by phosphodiester bonds.


Viral dsRNA
Scientist-supplied dsRNA
dsRNA
1
Cleavage by Dicer
siRNA
Target gene
Transcription
NUCLEUS
Target mRNA (or viral RNA transcript)
2
RISC loading complex
ATP
ADP + Pi
Activated RISC complex
3
CYTOPLASM
5
RNA synthesis by RdRP complex
4
Secondary siRNAs by Dicer
mRNA cleavage

Mechanism of RNA interference

The diagram depicts the RNAi pathway triggered by the introduction into cells of either viral doublestranded RNA (dsRNA) or scientist-supplied dsRNA.

(1) The ribonuclease Dicer processes long dsRNA into double-stranded small interfering RNAs (siRNAs), with two-nucleotide 3′ overhangs.

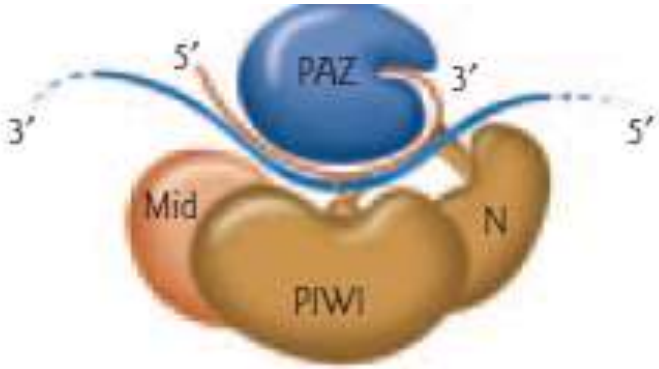
(2) The siRNAs trigger the formation of an RNA-induced silencing complex (RISC).

(3) The ATP-dependent unwinding of the siRNA duplex by helicase activity in the RISC loading complex (blue) leads to activated RISC (green).

(4) The single-stranded siRNA is used as a guide for target RNA (viral RNA or cellular mRNA) recognition. The complex targets RNAs of complementary sequence for cleavage by "Slicer" activity at the sitewhere the antisense siRNA strand is bound.

(5) In worms, flies, plants, and fungi, RNA-directed RNA polymerase (RdRP) uses the siRNA antisense strands as primers and targets RNA as a template to make new dsRNA. Dicer can then process the dsRNA to make more siRNA. This starts a new round of priming and siRNA amplification, and mRNA or viral RNA cleavage

siRNA-guided mRNA cleavage by Argonaute



siRNA (blue)
and mRNA (orange)