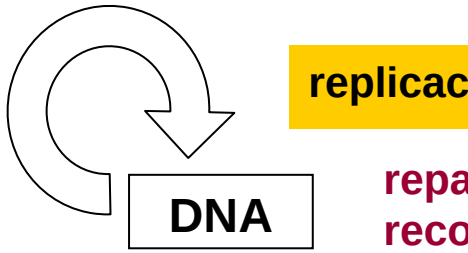
"Dogma central de la biología molecular"



DNA

replicación de DNA

reparación
recombinación

virus a DNA

transcripción

transcripción inversa

retrovirus y retroelementos

procesamiento de RNA

splicing, edición, modificación de nucleótidos y de extremos 5´y 3´

RNA

transcripción y replicación de RNA

virus a RNA

traducción

proteína

plegamiento (*folding*), procesamiento, direccionamiento (*targeting*)

Expresión de la información genética en eucariotas

transcripción

- similitudes y diferencias con procariotas
- metodologías de estudio

Víctor Romanowski, 2012

¿Qué se transcribe?

Los genes son segmentos del genoma que se transcriben y sus secuencias regulatorias

Productos de transcripción = "transcriptos"

RNAs codificantes para proteínas (son traducidos) = mRNAs

Hay 35 000 genes codificantes para proteínas en un genoma de mamífero típico, incluyendo el genoma humano.
Este número es mucho menor que lo que se pensaba (> 100 000)

RNAs no codificantes = ncRNAs

rRNA: RNAs ribosomales: hay 150-200 copias de los 28S - 5.8S - 18S + 200-300 copias del 5S .

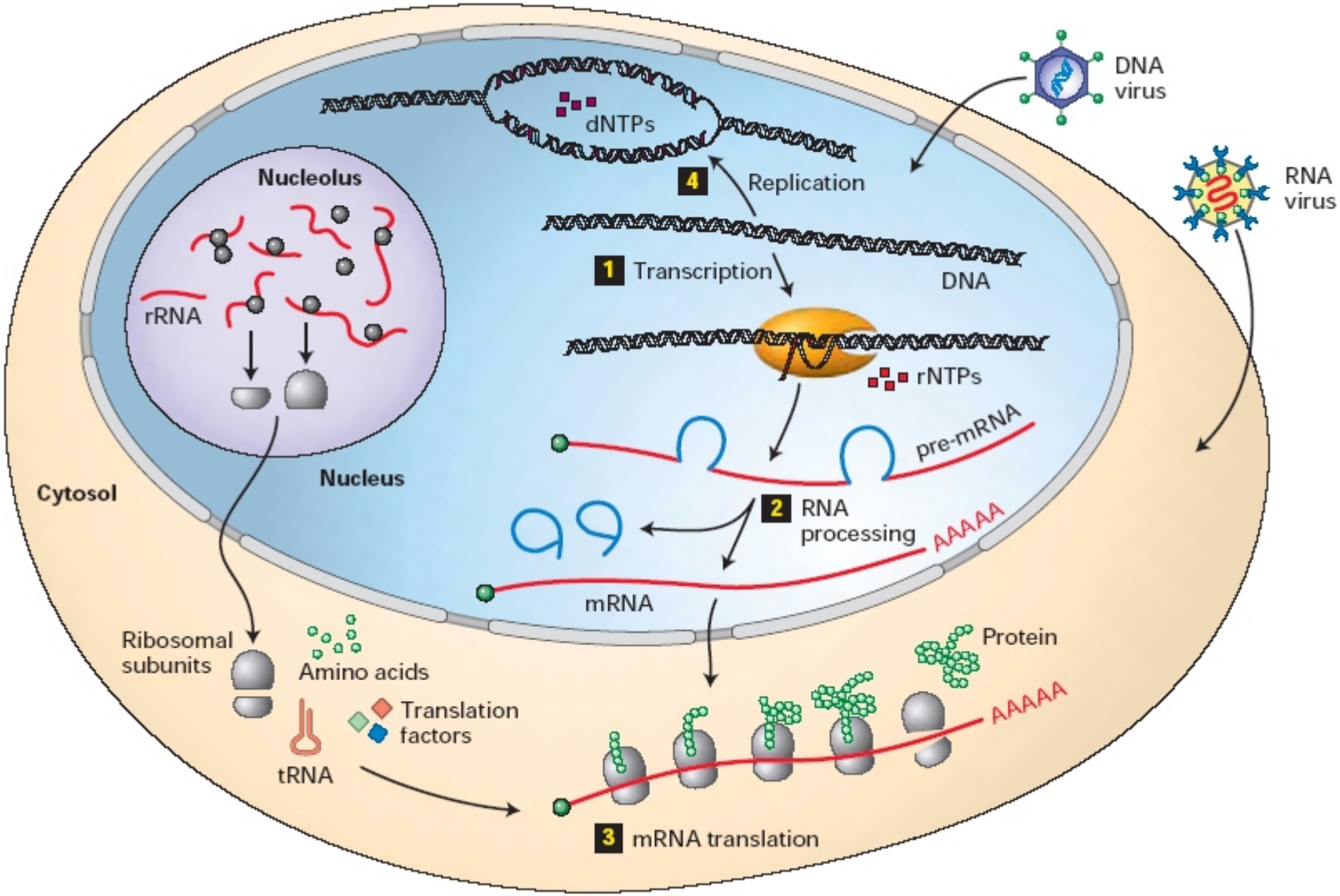
tRNA: RNAs de transferencia: >> 500 genes en el genoma humano.

snoRNA: small nucleolar RNAs; la mayor parte de los snoRNAs participan en modificación de rRNA

snRNAs: small nuclear RNAs: constituyen los RNA que forman el **spliceosoma**

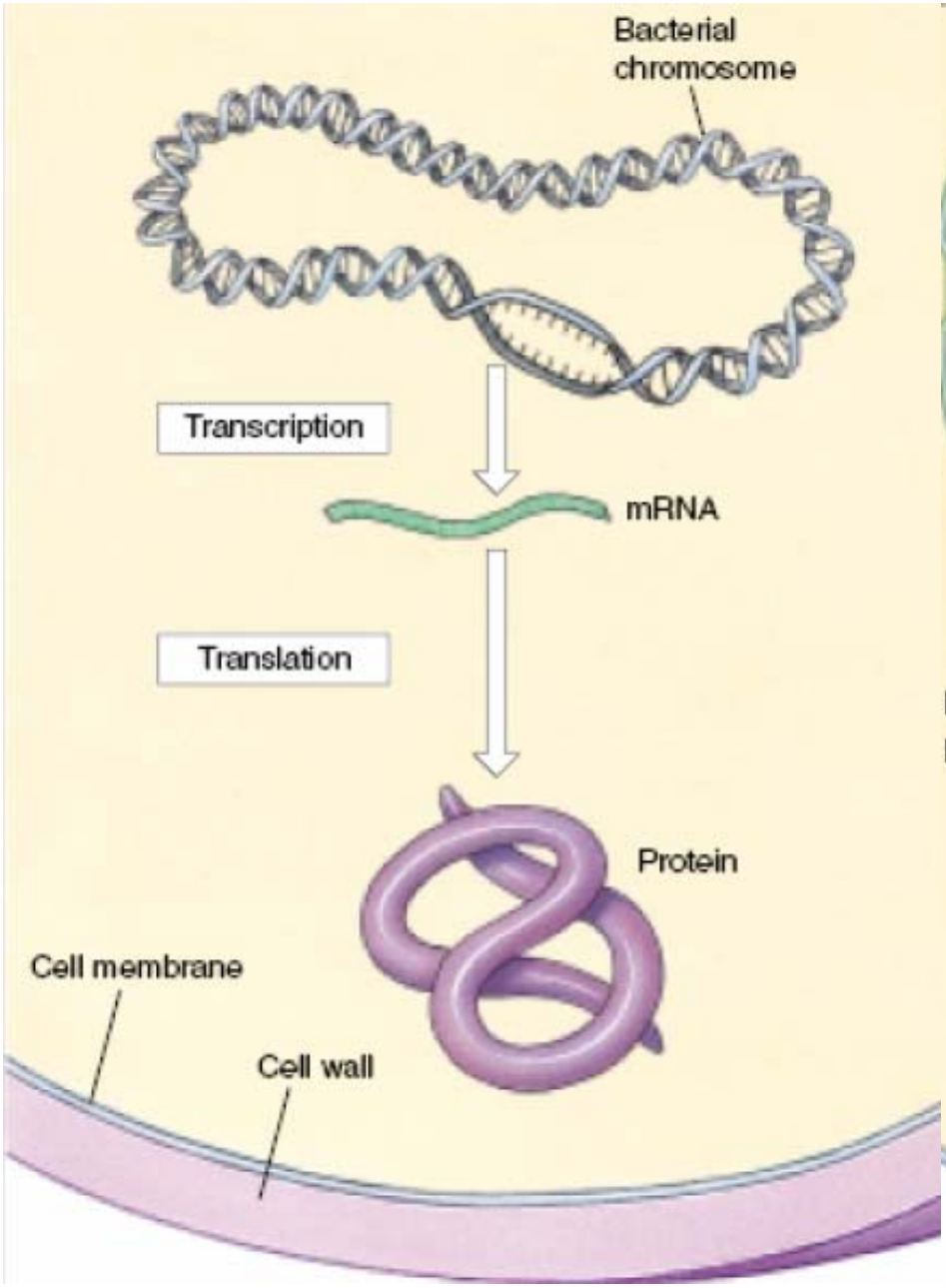
Otros: incluyen microRNAs (**miRNAs**), pequeños de interferencia (**siRNAs**), RNA citoplasmáticos pequeños (**scRNA**), telomerasa, ribonucleasa P, SRP, etc.

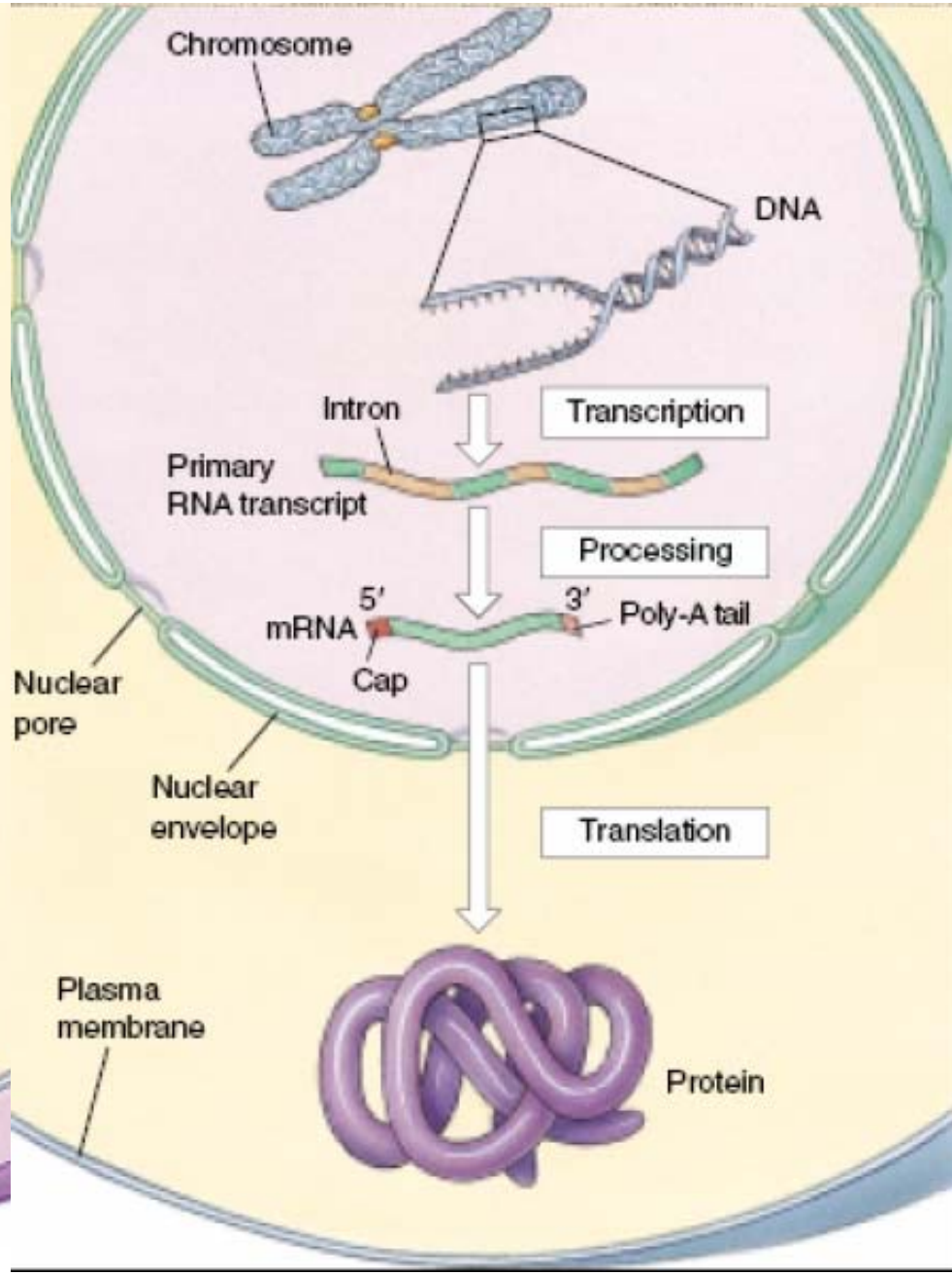
Célula eucariota



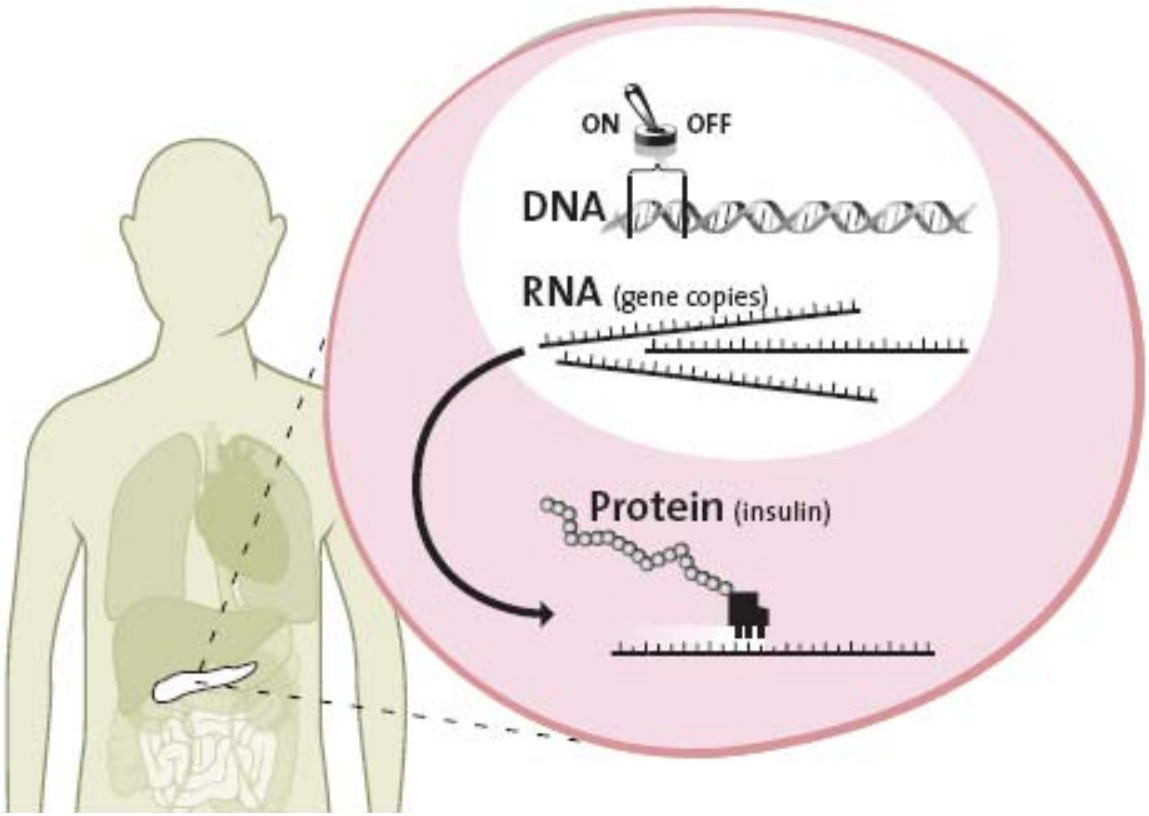
Procariotas

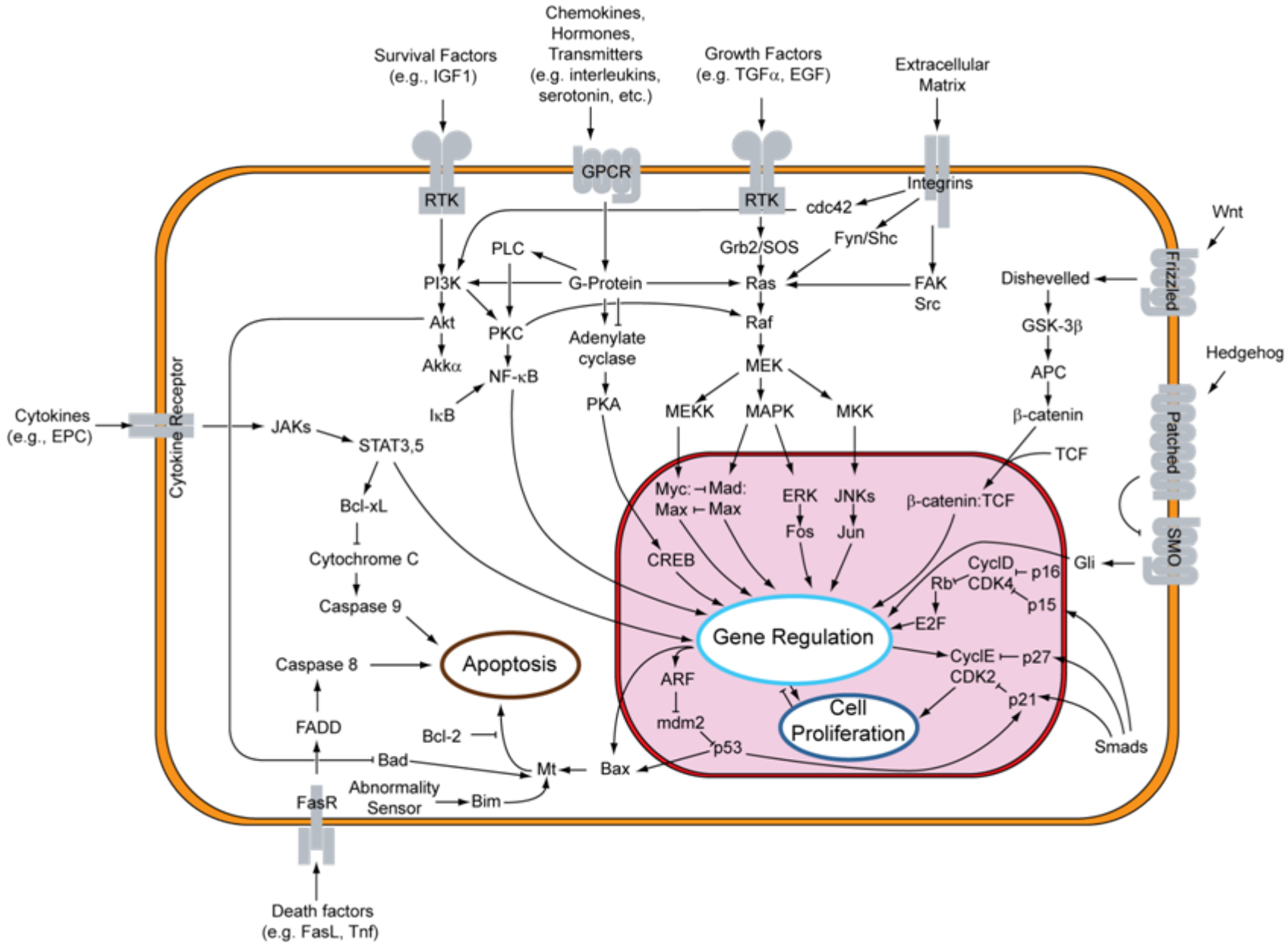
Eucariotas





Diferentes células expresan conjuntos distintos de productos génicos en diferentes situaciones





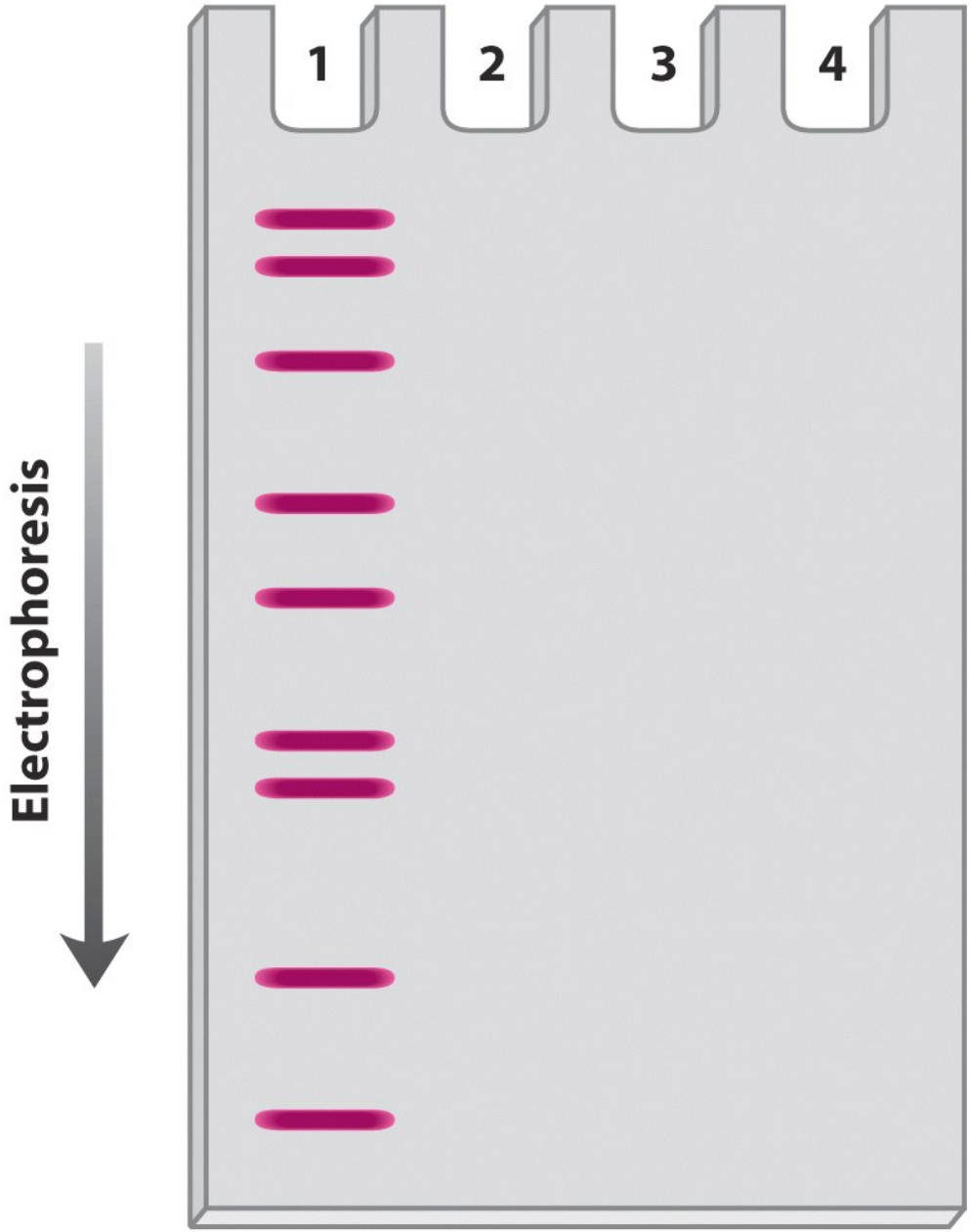
Señal ►►►transcripción ►expresión regulada ► respuesta… fenotipo…

Técnicas relacionadas con la transcripción

- 1- Niveles estacionarios de RNA
- 2- Síntesis de RNA
- 3- Interacciones DNA-proteína
- 4- Actividad de promotores
- 5- Inicio de la transcripción

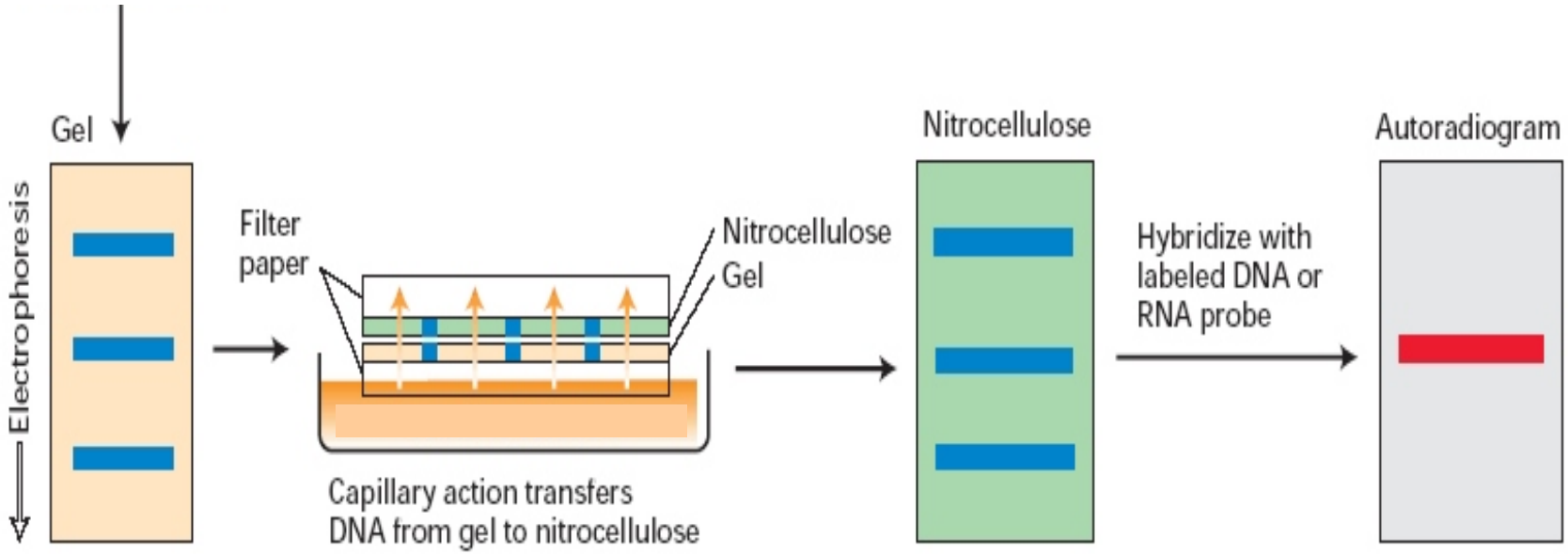
Técnicas para medir niveles estacionarios de RNA

- Northern blot
- Dot blot/Macroarray (macro-arreglo)
- Microarray (micro-arreglo)
- PCR en tiempo real (real time PCR)

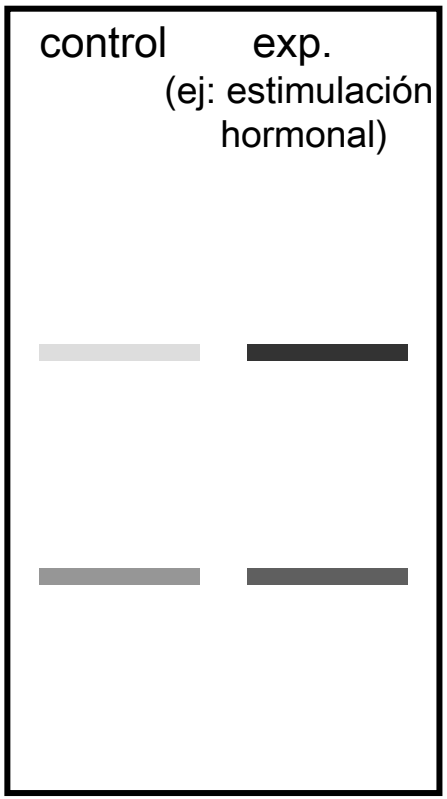

1
2
3
4
Electrophoresis

Northern blot

RNA total



NORTHERN blot



Gen de interés *(target gene)* **10X**

Control interno:
gen que se transcribe
sin modificaciones de nivel
en diferentes condiciones
y tipos celulares
Ej.: actina, GAPDH, RPLP0,
housekeeping genes, etc

2X

Corrected fold increase = 10/2 = 5

$$\text{Ratio target gene in experimental/control} = \frac{\text{fold change in target gene}}{\text{fold change in reference gene}}$$

Standards

- same copy number in all cells
- expressed in all cells
- medium copy number advantageous
  - correction more accurate

Standards

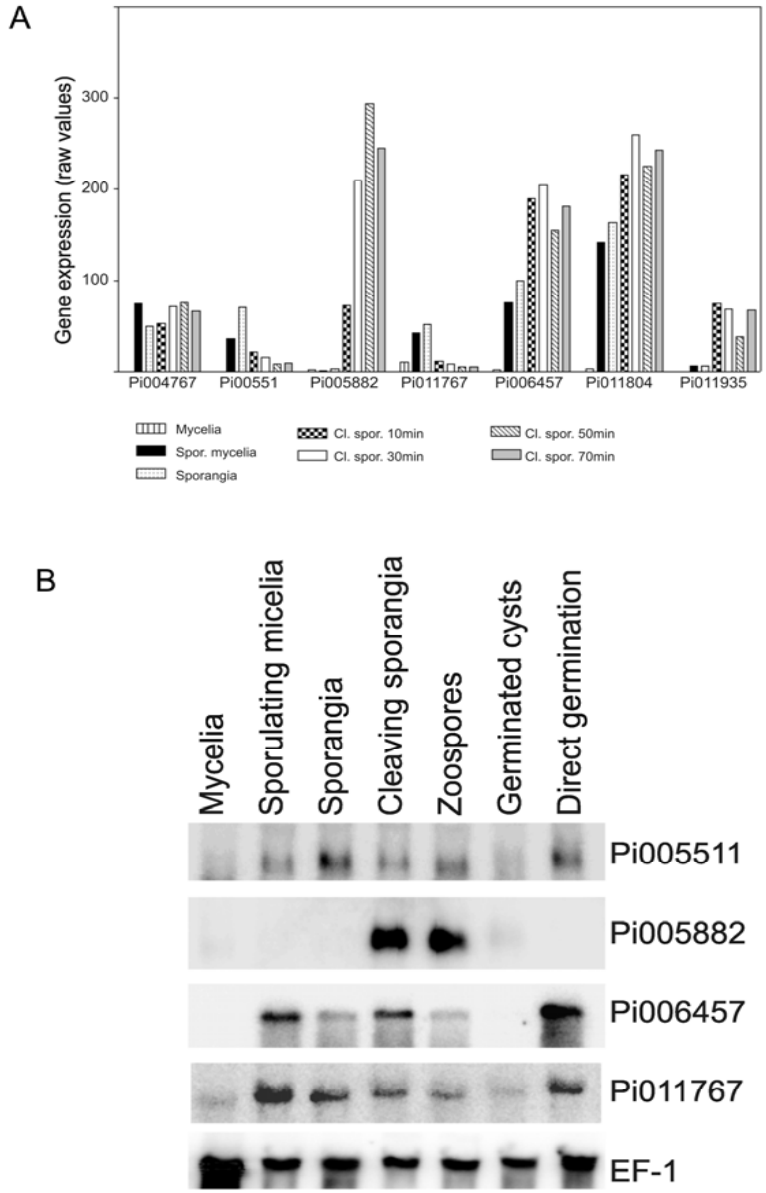
- **The perfect standard does not exist**

- Commonly used standards
  - Glyceraldehyde-3-phosphate dehydrogenase mRNA
  - Beta-actin mRNA
  - MHC I (major histocompatability complex I) mRNA
  - Cyclophilin mRNA
  - mRNAs for certain ribosomal proteins
    - *e.g.* RPLP0 (ribosomal protein, large, P0; also known as 36B4, P0, L10E, RPPO, PRLP0, 60S acidic ribosomal protein P0, ribosomal protein L10, Arbp or acidic ribosomal phosphoprotein P0)
  - 28S or 18S rRNA

Northern blot

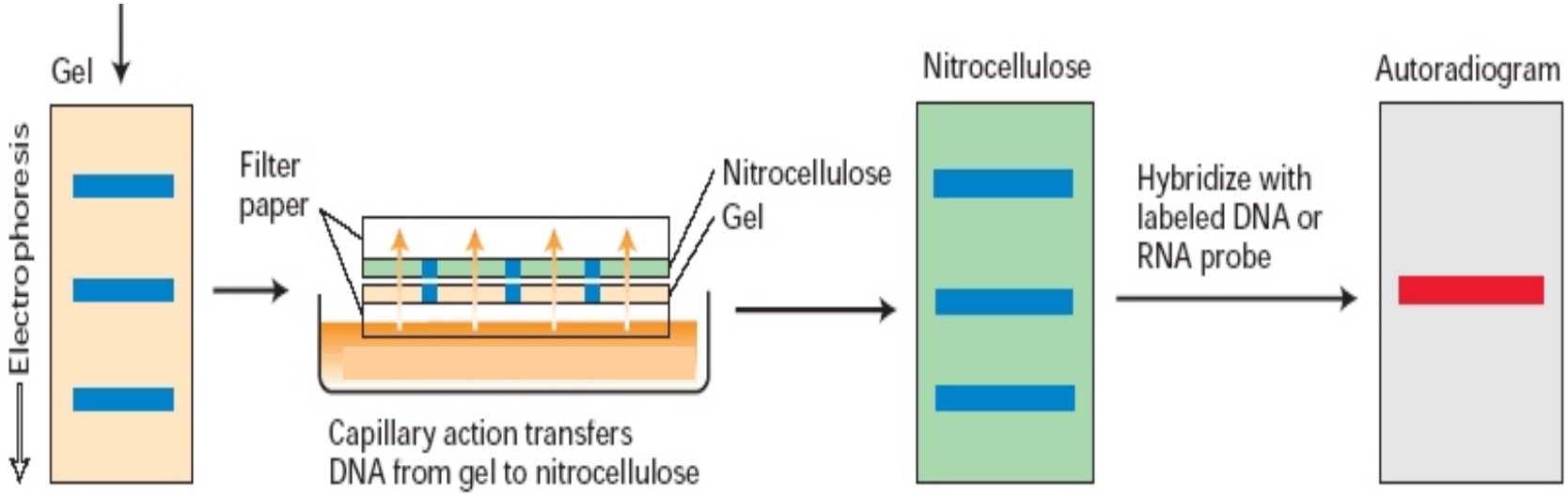
Ejemplo: expresión
de varios genes
en distintos estadíos
del desarrollo

Las mismas muestras
de RNA total hibridadas
con las sondas de los
diferentes genes de interés



Northern blot

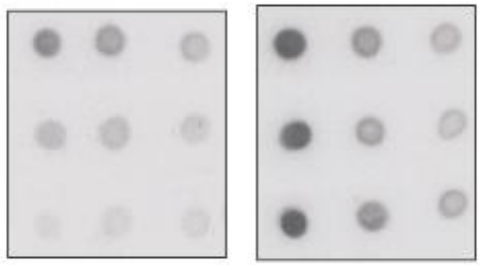
RNA total



Dot blot-Macroarray (expresión de varios genes)

RNA → cDNA (sonda)
^{32}P dNTPs

DNA spots



Dot blot

Alternativas:

1. Depositar en cada posición una muestra de RNA de células sometidas a diferentes estímulos e hibridar con una única sonda de DNA correspondiente al gen de interés (análisis de múltiples situaciones para un único gen).
2. Depositar en cada posición un exceso de DNA clonado con la secuencia de los genes de interés (uno diferente en cada posición) e hibridar con una sonda (mezcla de sondas) extraída de células incubadas en presencia de precursores radiactivos, o de cDNA (RNA extraído de células y usado para generar una sonda por transcripción reversa en presencia de precursores radiactivos)
3. otras

Specific RNAs can be quantitated and mapped on DNA by nuclease protection

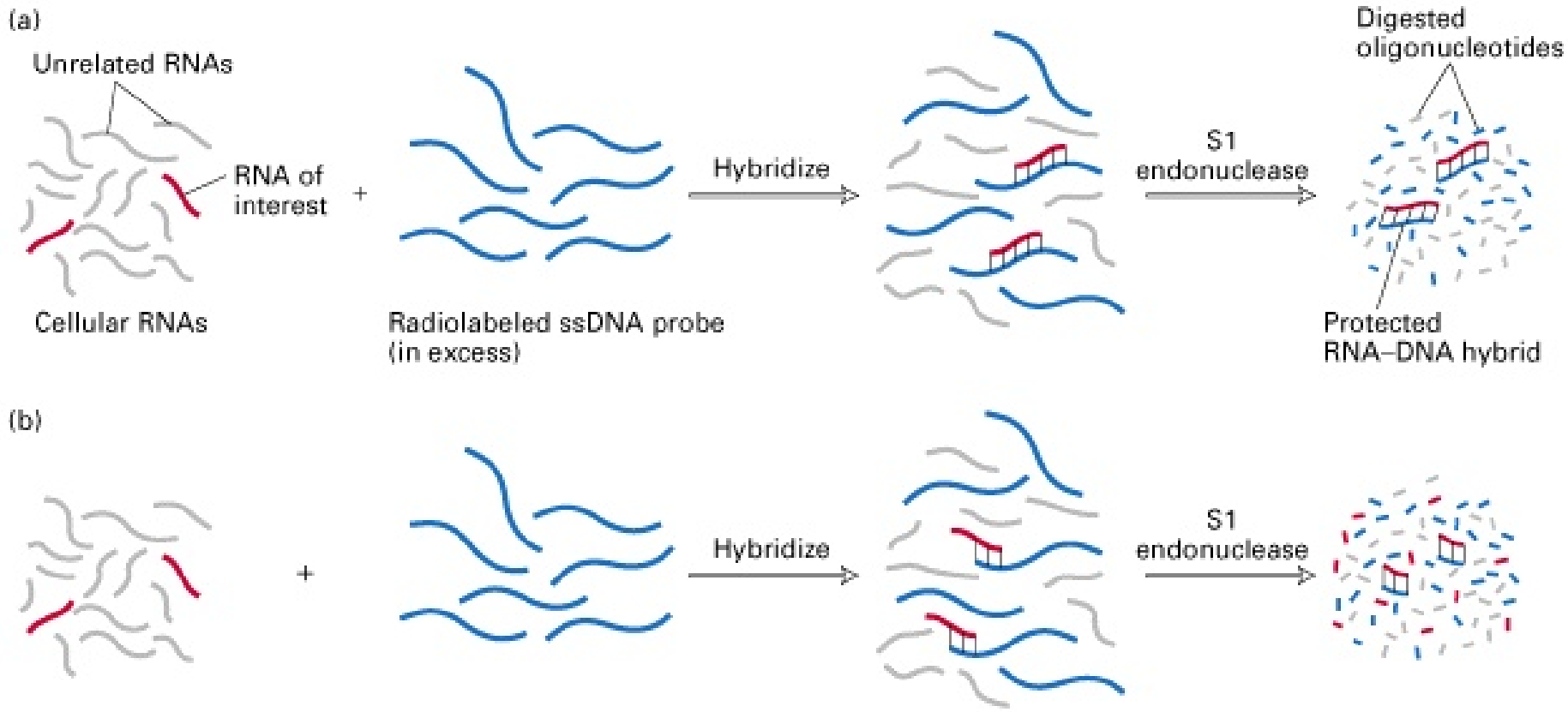
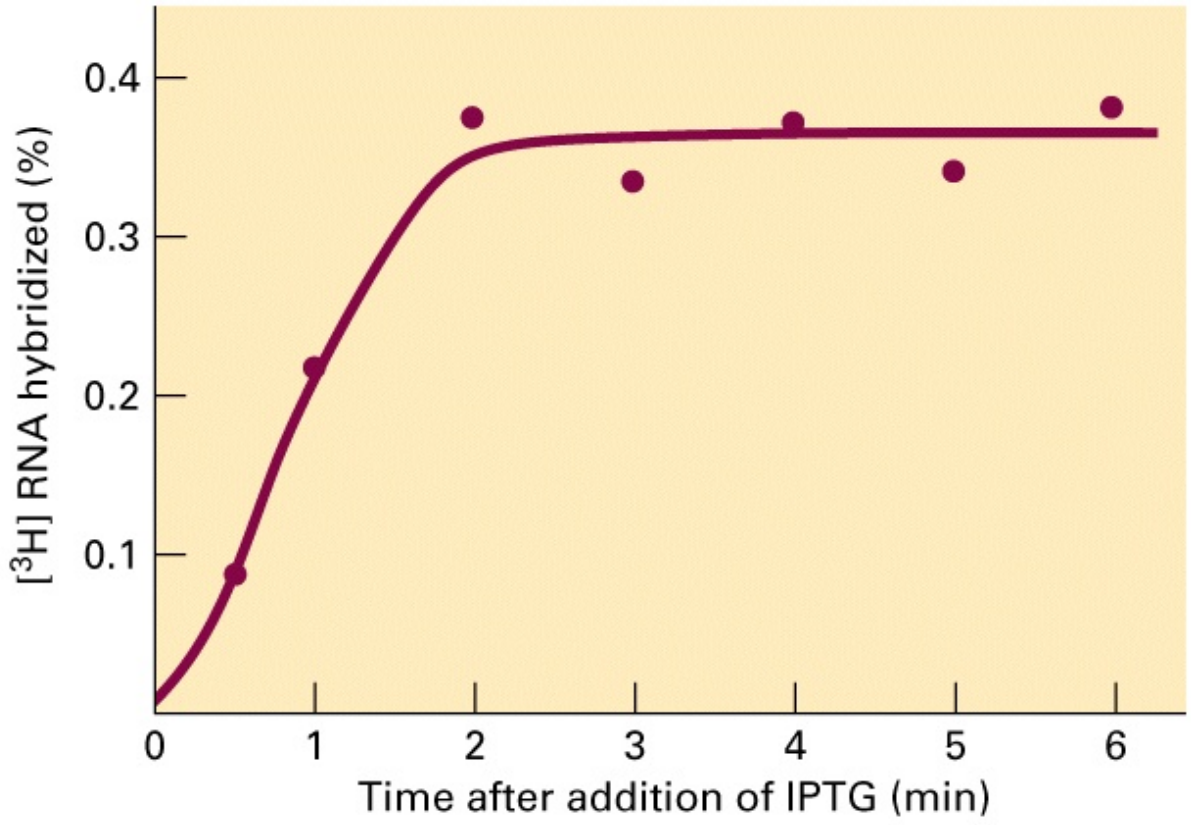


Figure 7-34a,b

Biochemical experiments confirm that induction of the *lac* operon leads to an increased synthesis of *lac* mRNA



Ver alternativas: sonda, *target* de hibridación (atrapa sonda).
Situaciones en las que puede incubarse el sistema con precursores radiactivos y otras en que esto no es posible

DNA microarrays: analyzing genome-wide expression

- DNA microarrays consist of thousands of individual gene sequences bound to closely spaced regions on the surface of a glass microscope slide
- DNA microarrays allow the simultaneous analysis of the expression of thousands of genes
- The combination of DNA microarray technology with genome sequencing projects enables scientists to analyze the complete transcriptional program of an organism during specific physiological response or developmental processes

Transcriptoma

conjunto de RNAs transcriptos en un tipo de célula

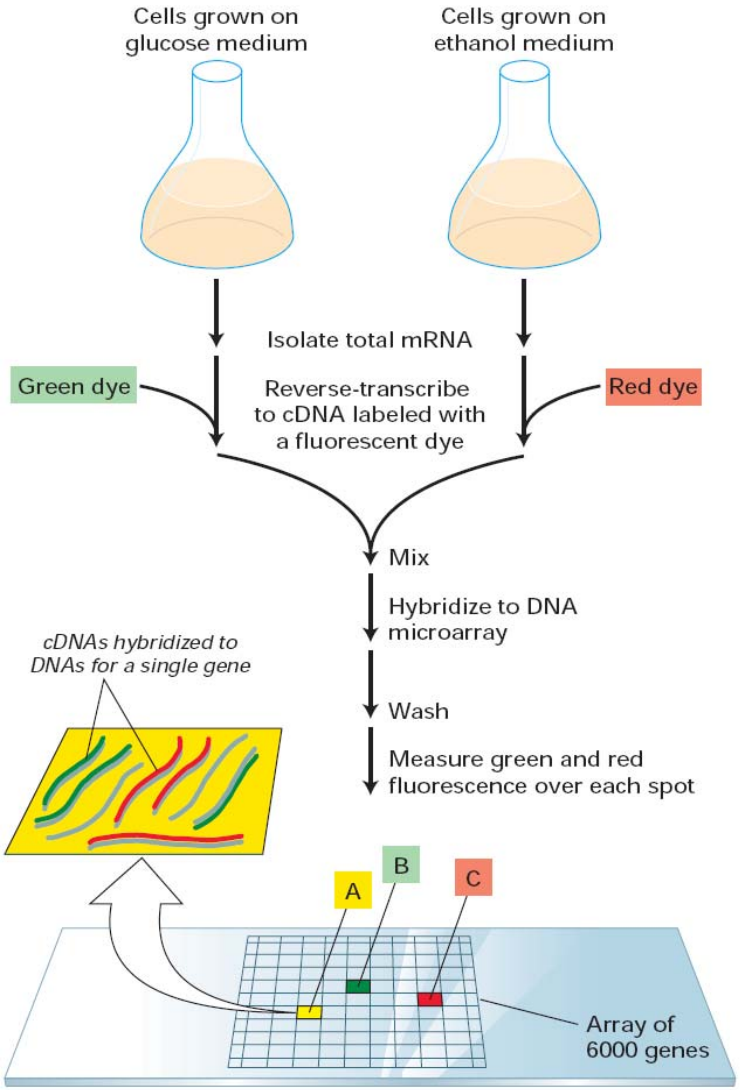
Las diferentes células de un organismo tienen el mismo genoma (DNA)

Las células son diferentes porque un conjunto distinto de genes se expresa en cada tipo celular

¿Qué genes se expresan en un tejido y cuáles en otro?

¿Qué genes se activan por estímulos hormonales?

¿Qué diferencias se observan a nivel de la expresión de genes en determinados estados patológicos?

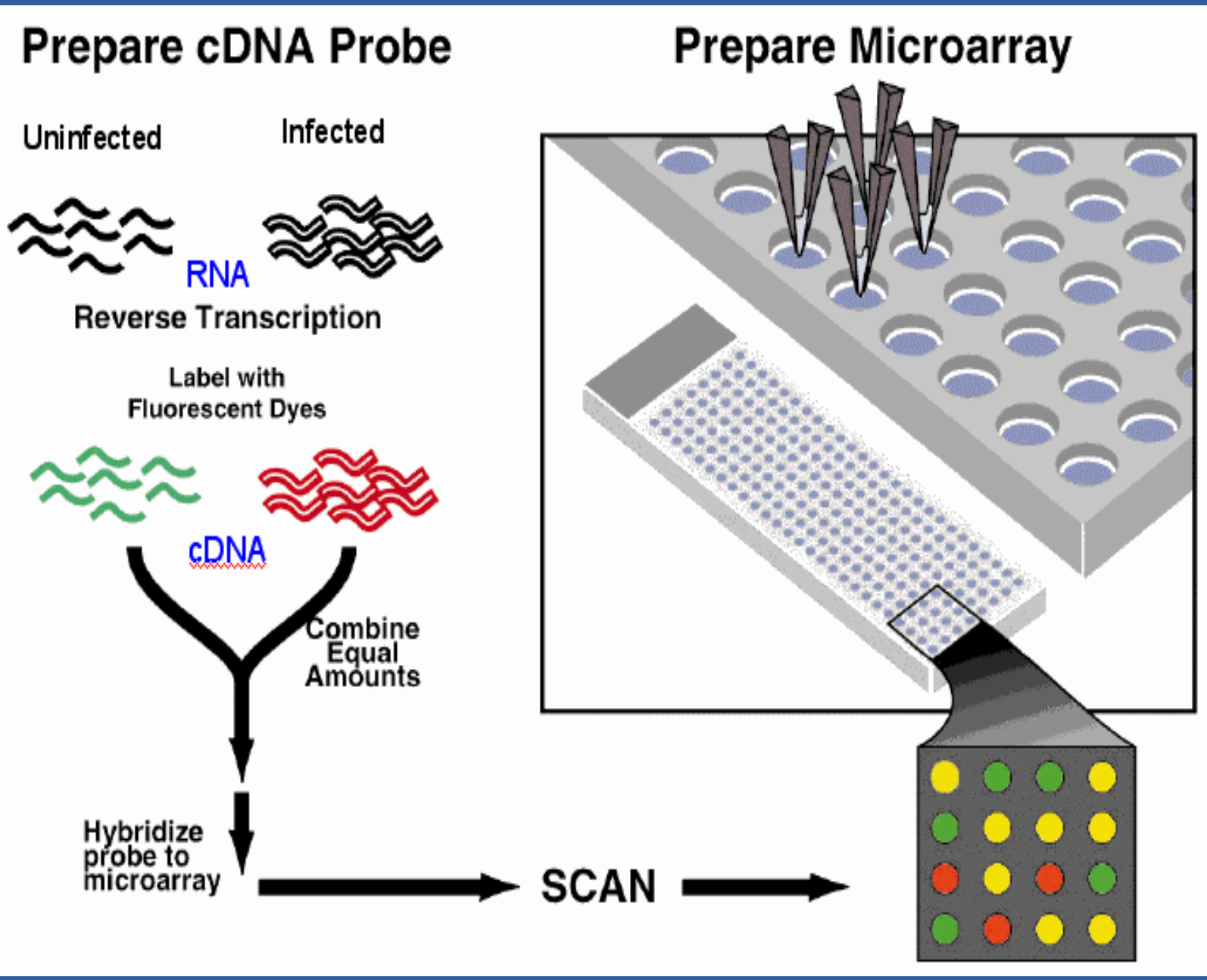


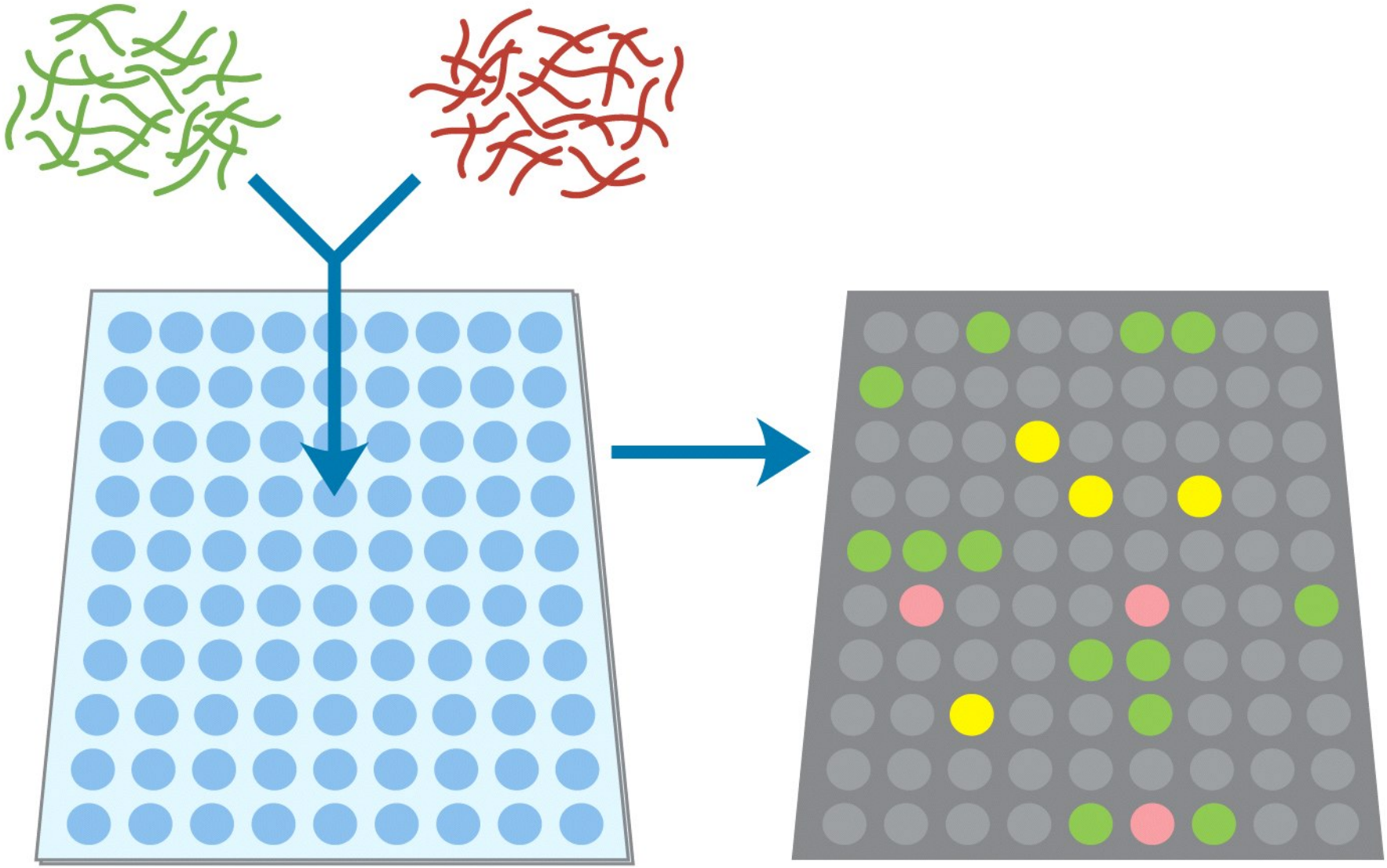
A If a spot is yellow, expression of that gene is the same in cells grown either on glucose or ethanol

B If a spot is green, expression of that gene is greater in cells grown in glucose

C If a spot is red, expression of that gene is greater in cells grown in ethanol

DNA microarrays can be used to evaluate the expression of many genes at one time

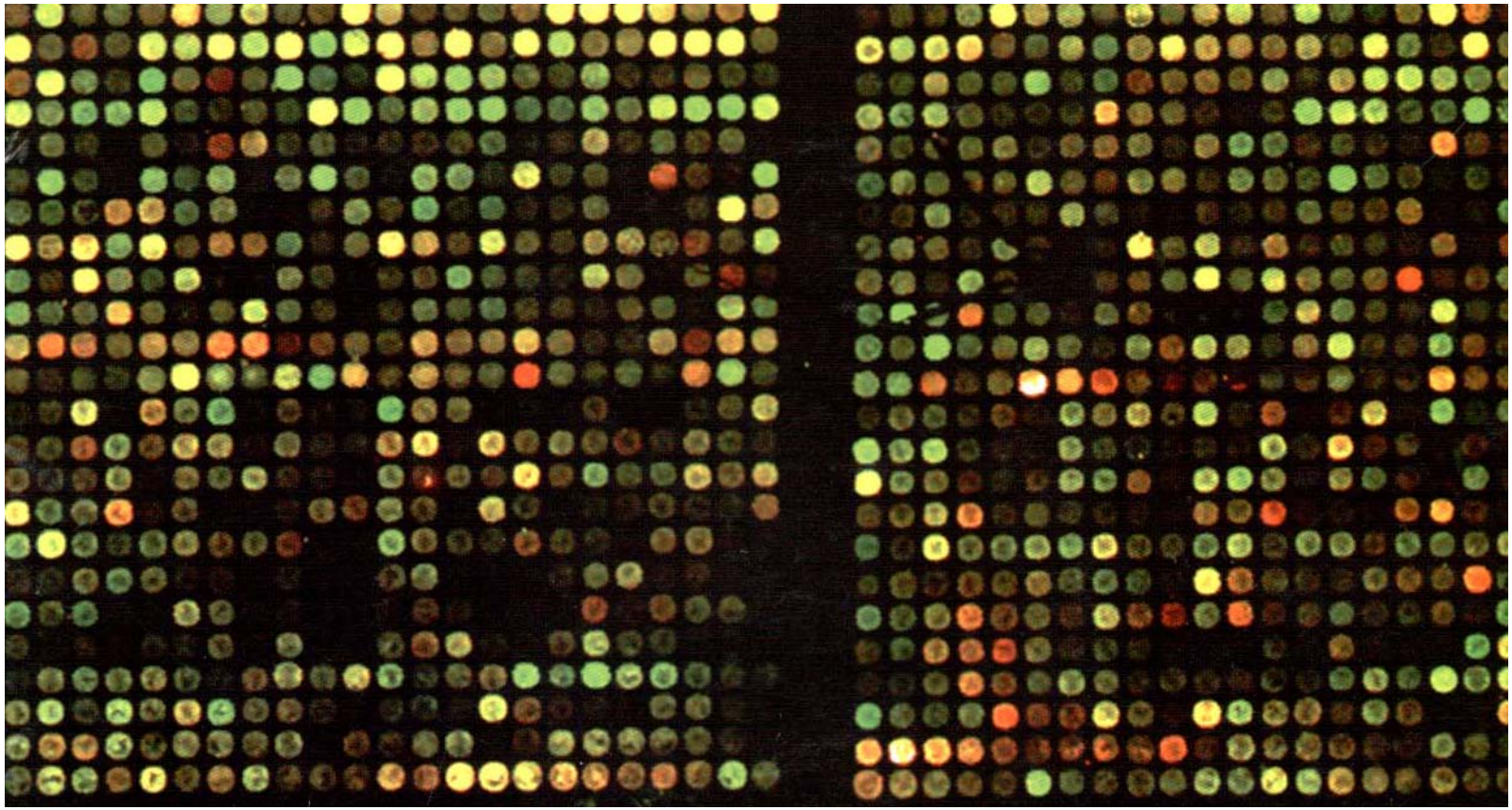

Prepare cDNA Probe
Prepare Microarray
Uninfected
Infected
RNA
Reverse Transcription
Label with
Fluorescent Dyes
cDNA
Combine
Equal
Amounts
Hybridize
probe to
microarray
SCAN



TRANSCRIPTOMA

la expresión miles de genes a nivel de la transcripción de puede estudiarse mediante

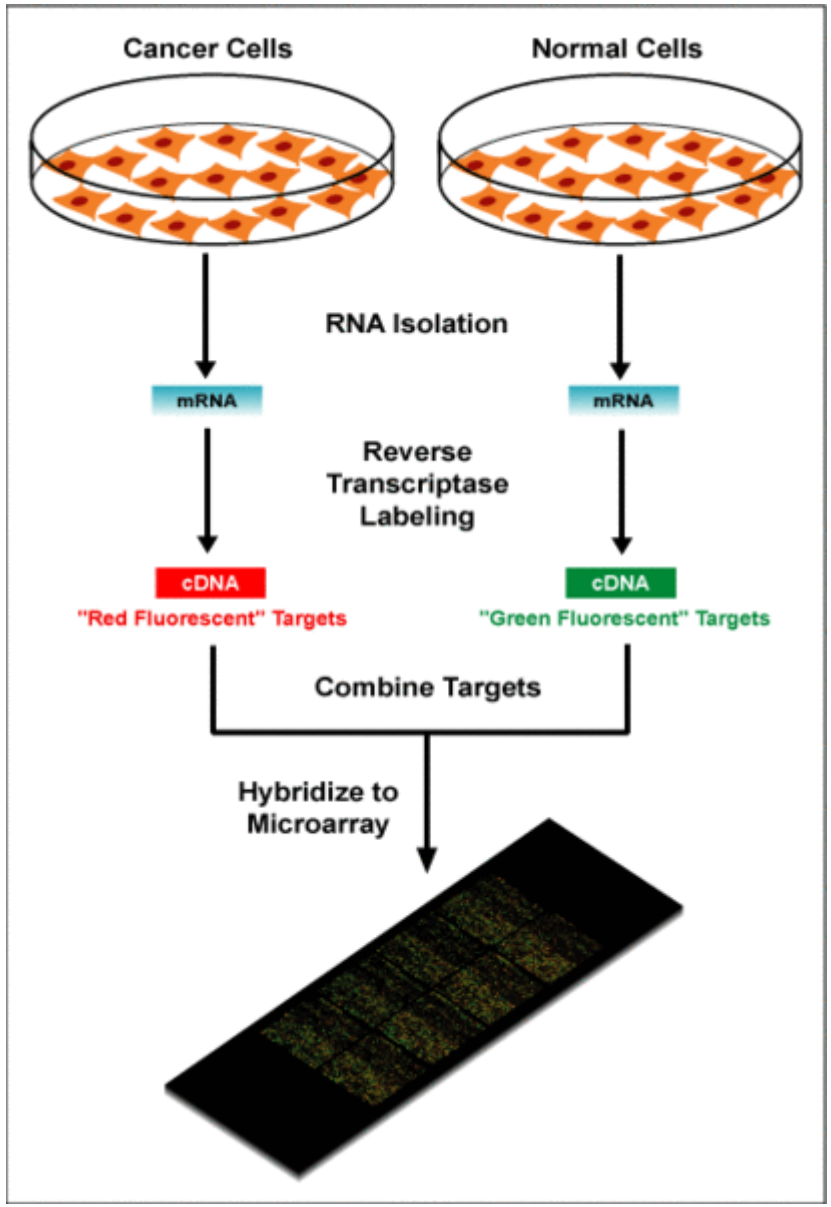
***microarrays* o *chips* de DNA**

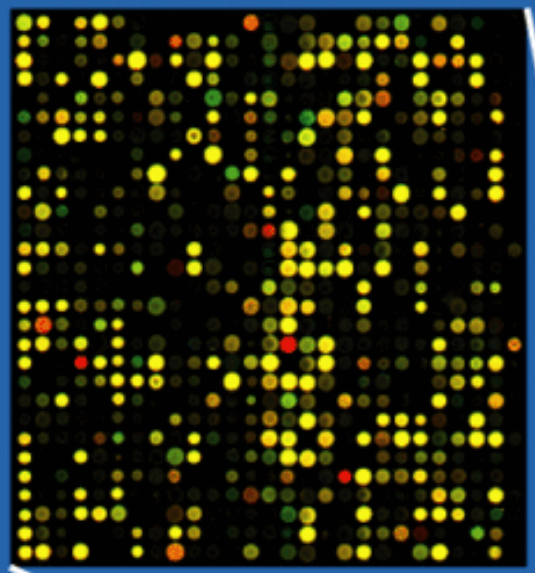


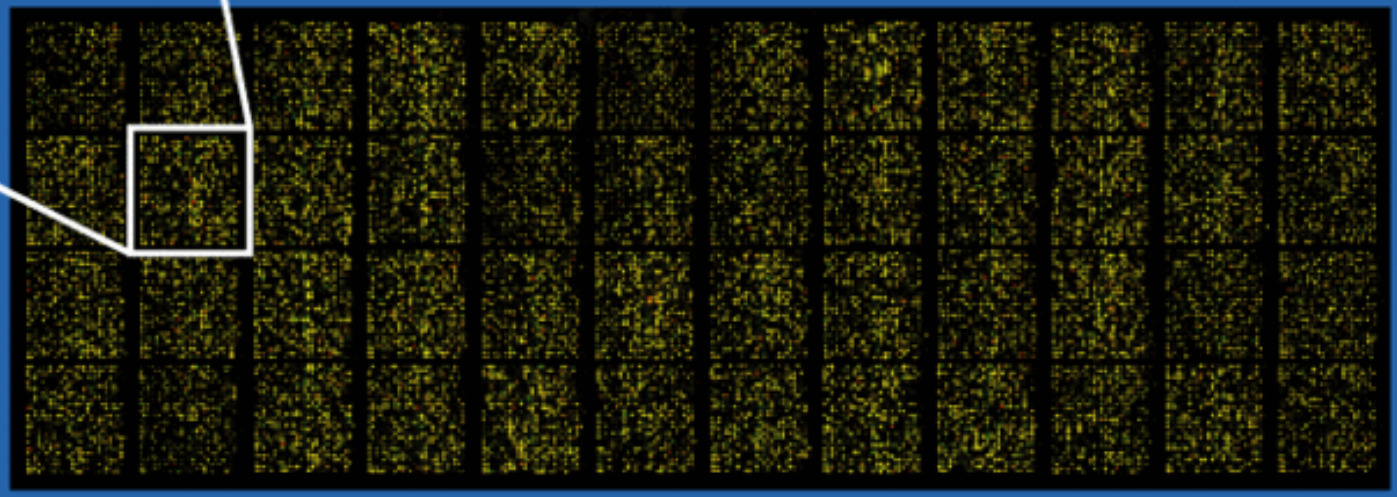
sonda roja: cDNA de hígado

sonda verde: cDNA de cerebro

Microarray (microarreglo)





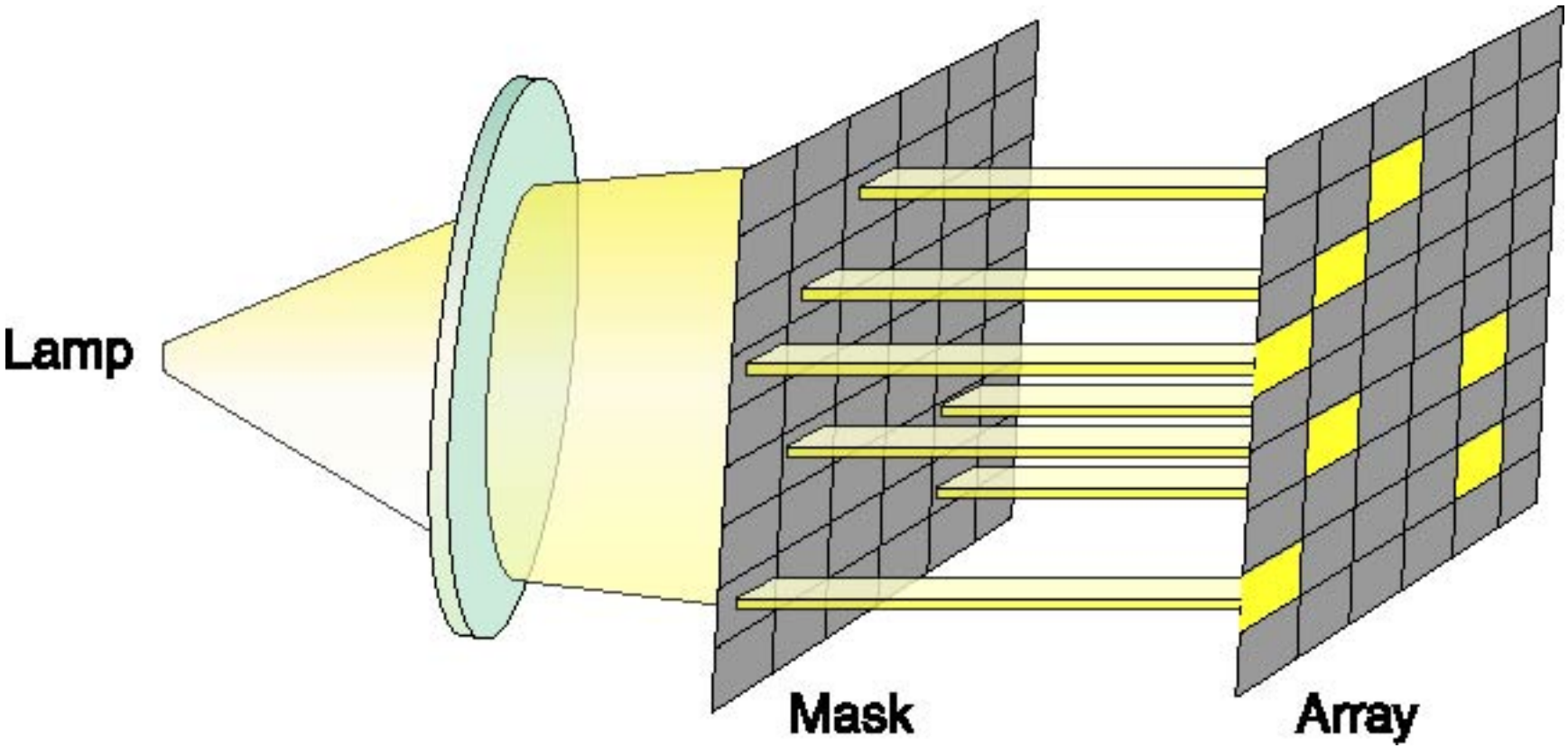


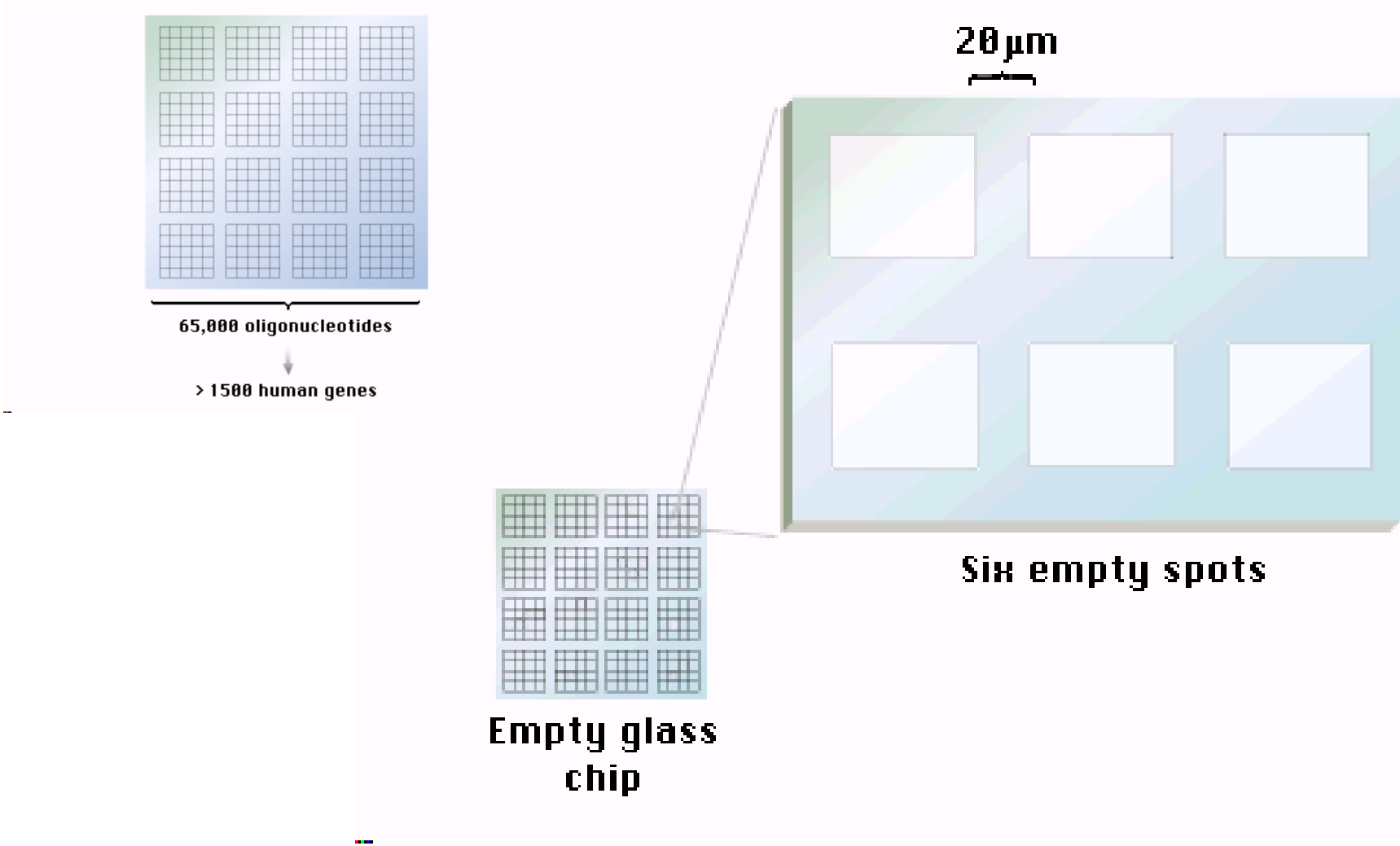
DNA chips


AFFYMETRIX
GeneChip®
Human Genome
U133 Plus 2.0
P/N: 520019
Lot #: 4010008
Exp. Date: 05/07/06
For Research Use Only
AFFYMETRIX
GeneChip®
Mouse Genome
430 2.0 Array
P/N: 520029
Lot #: 4015112
Exp. Date: 11/24/06
For Research Use Only


AFFYMETRIX

Síntesis de oligonucleótidos anclados al vidrio (Affimetrix)




65,000 oligonucleotides
> 1500 human genes
20µm
Six empty spots
Empty glass chip

DNA chips

microarrays

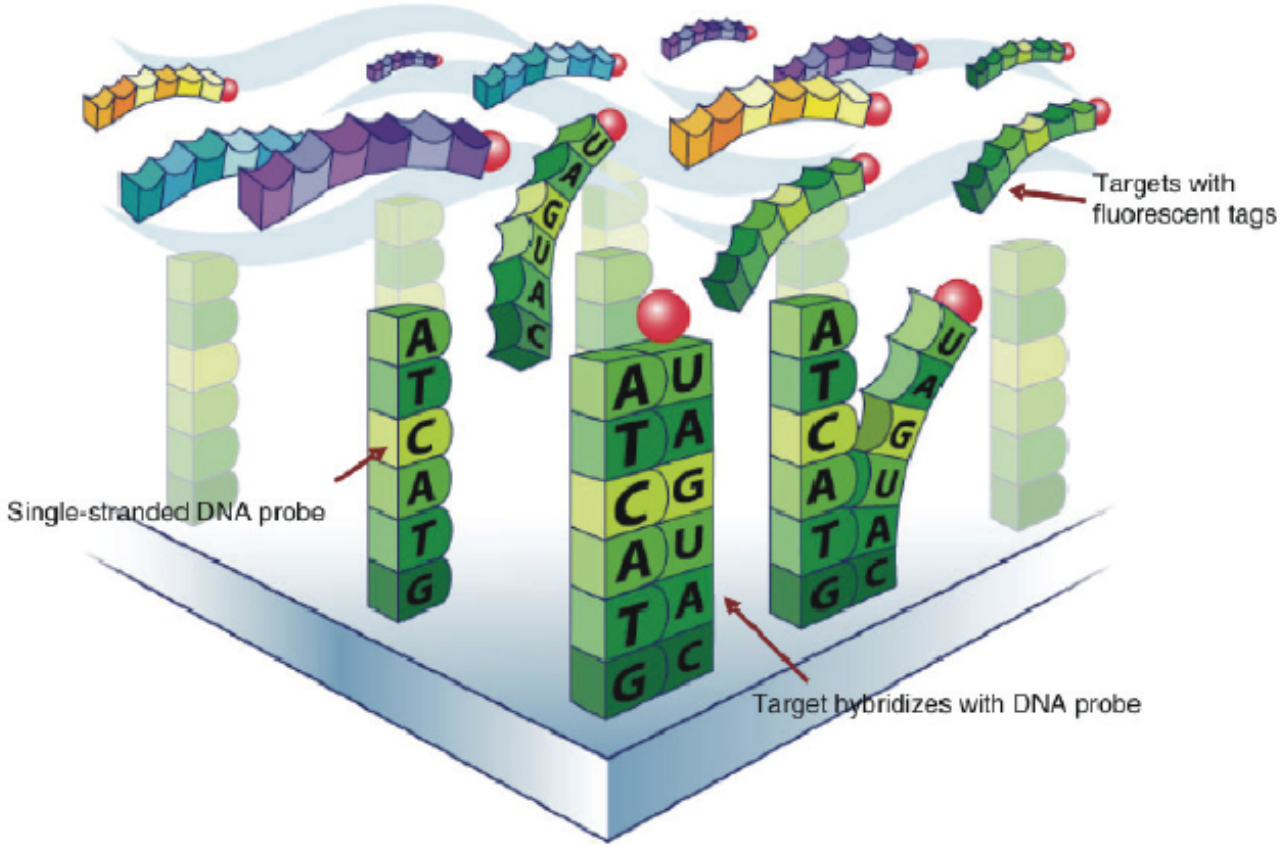
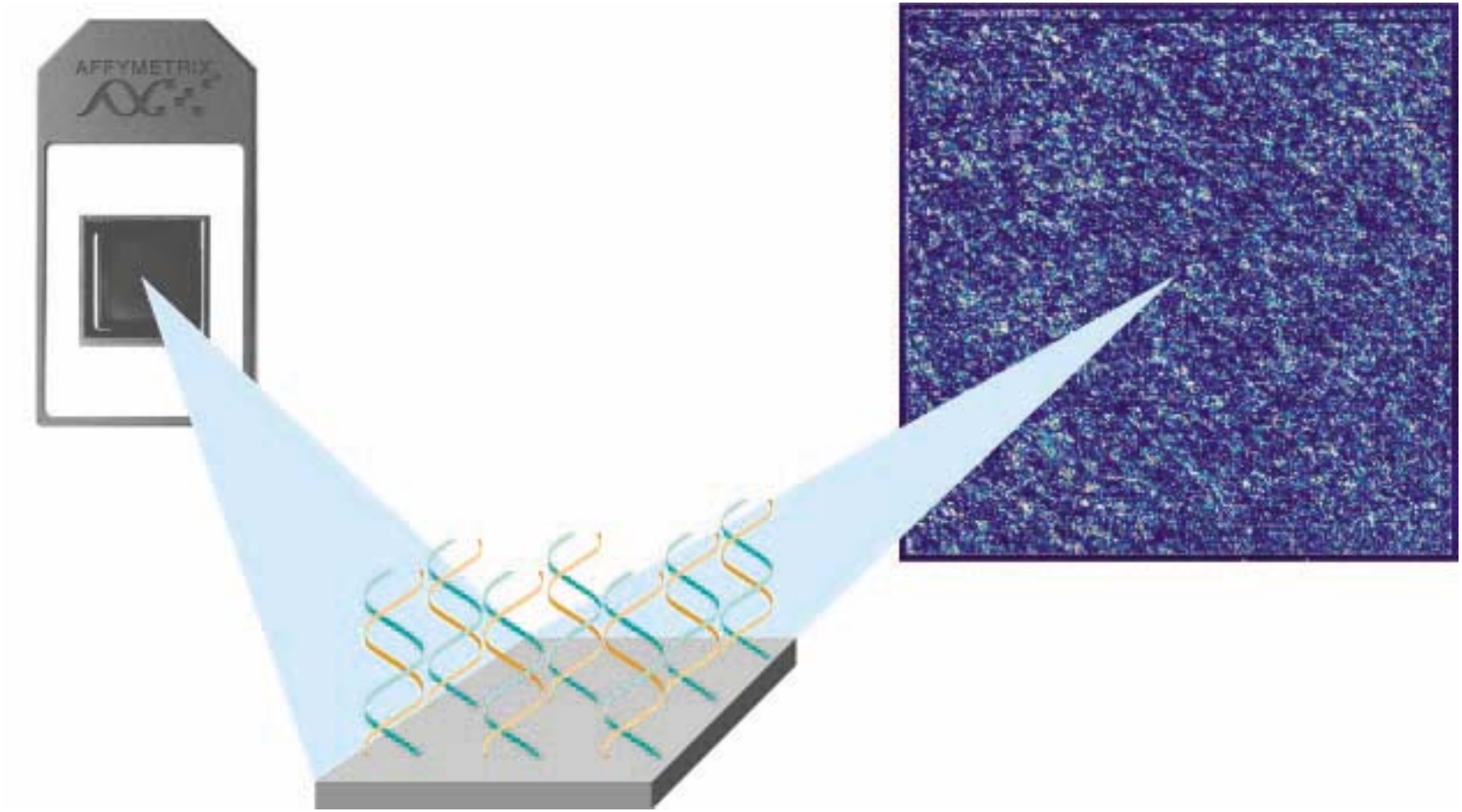
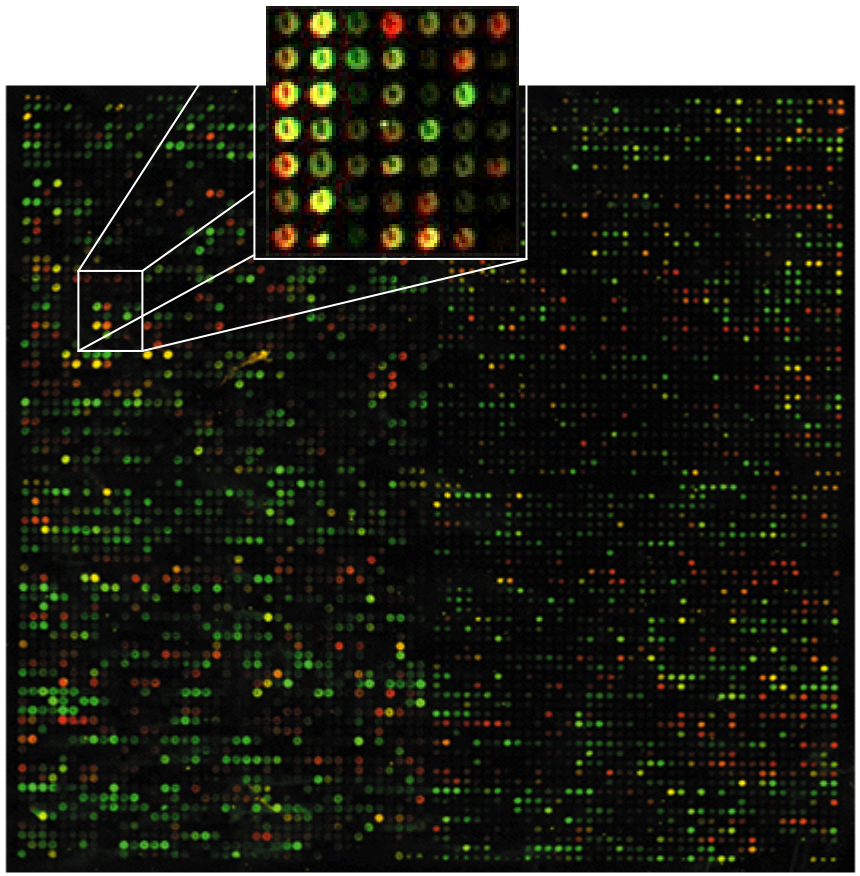


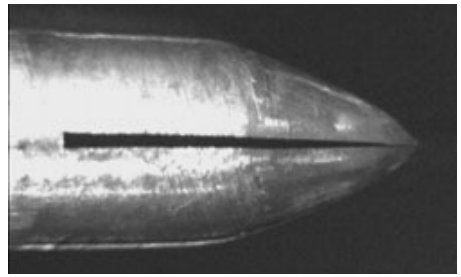
Figure 1-1. Cartoon illustration of DNA microarray probes and target hybridization (source: Wellcome Trust Centre for Human Genetics, http://www.well.ox.ac.uk/).

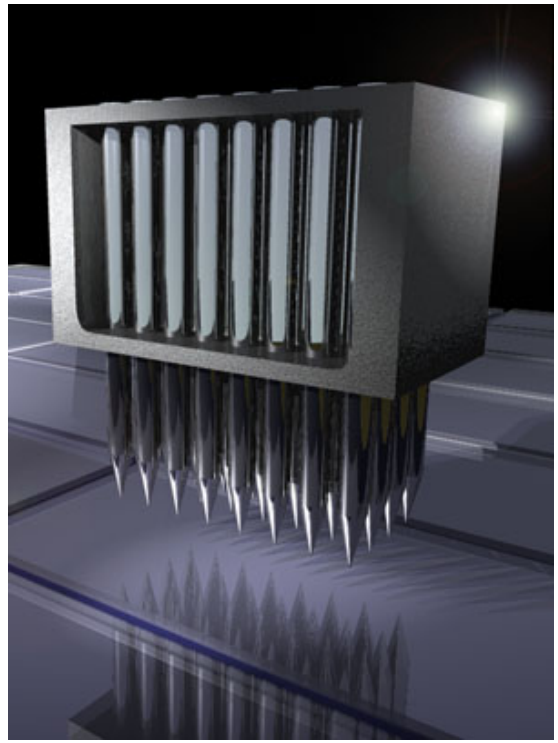

AFFYMETRIX

microarrays

(*spotter ~ ink jet printer*)







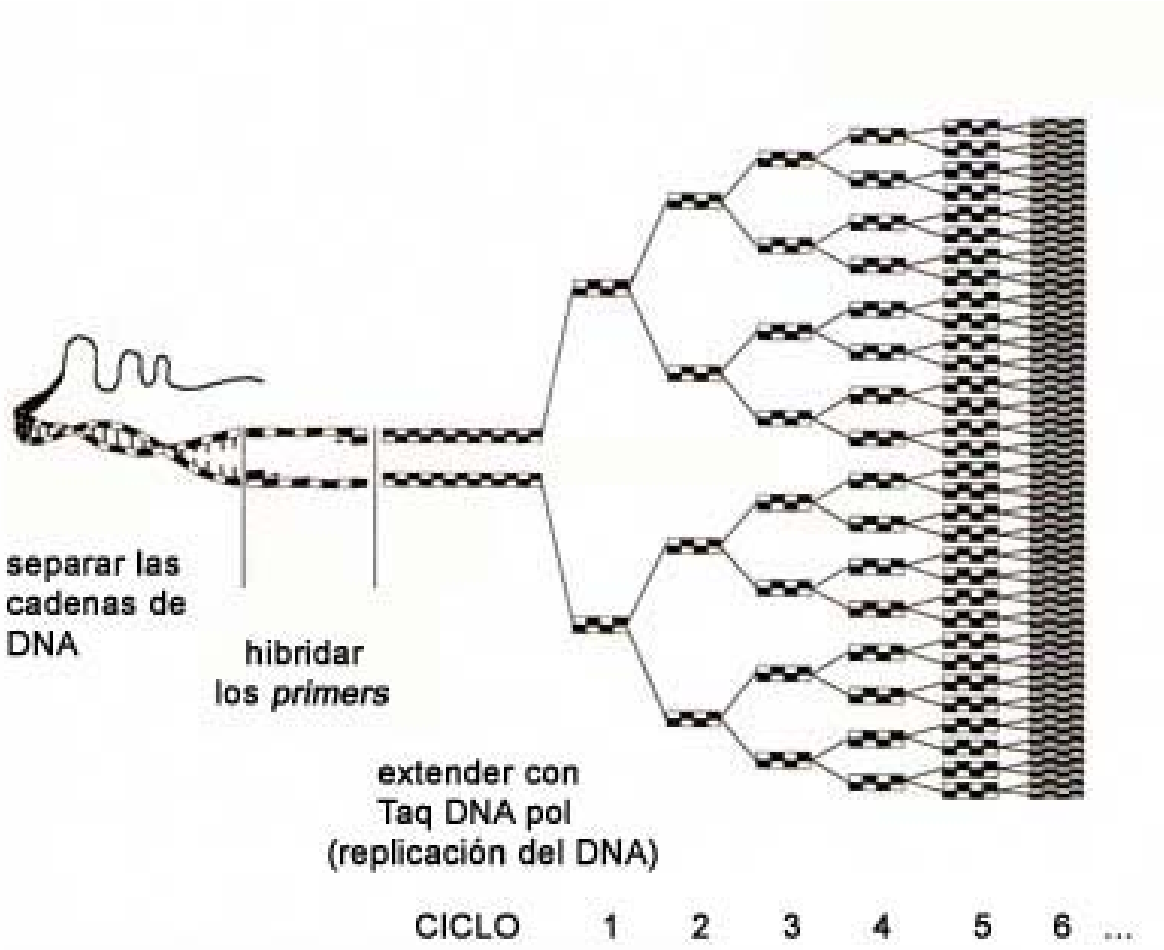
Polymerase chain reaction (PCR)

- The polymerase chain reaction (PCR) can be used to amplify rare specific DNA sequences from a complex mixture when the ends of the sequence are known

- PCR amplification of mutant alleles allows detection of human genetic diseases

- DNA sequences can be amplified by PCR for use in cloning, as probes, and in forensics

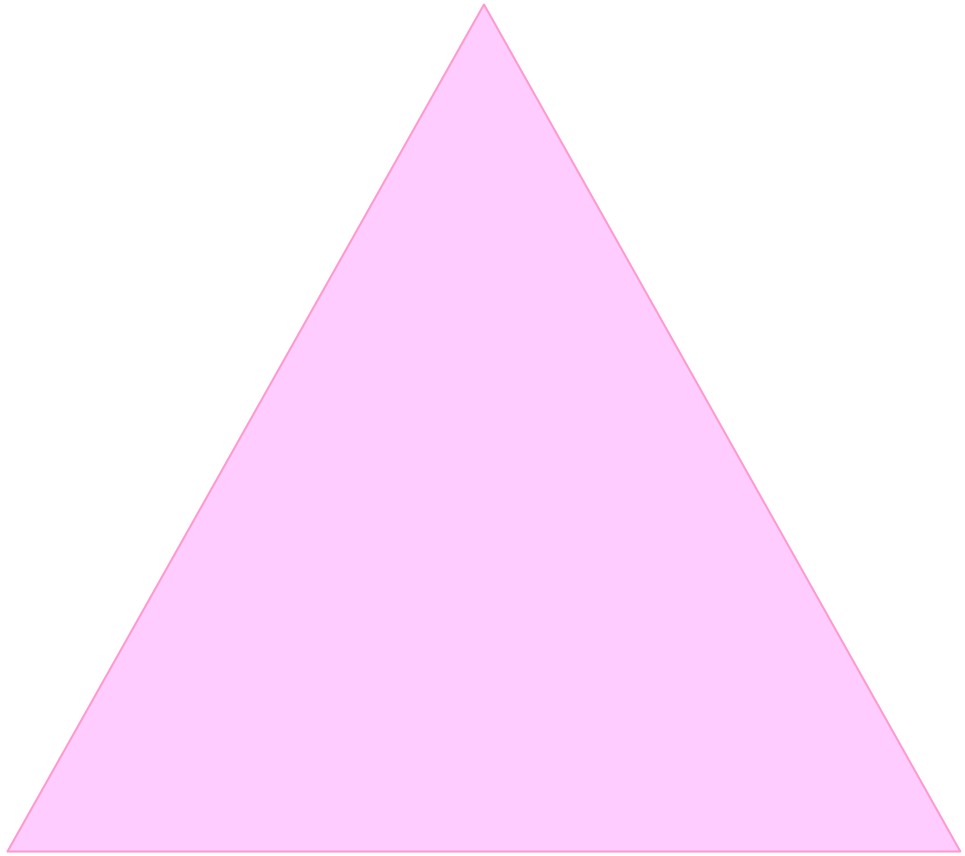
PCR: *Polymerase Chain Reaction*



Ciclos	Copias
1	2
2	4
4	16
10	1.024
15	32.768
20	1.048.576
25	33.554.432
30	1.073.741.824

CYCLE NUMBER	AMOUNT OF DNA
0	1
1	2
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1,024
11	2,048
12	4,096
13	8,192
14	16,384
15	32,768
16	65,536
17	131,072
18	262,144
19	524,288
20	1,048,576
21	2,097,152
22	4,194,304
23	8,388,608
24	16,777,216
25	33,554,432
26	67,108,864
27	134,217,728
28	268,435,456
29	536,870,912
30	1,073,741,824

Número de copias de la secuencia blanco (dsDNA)



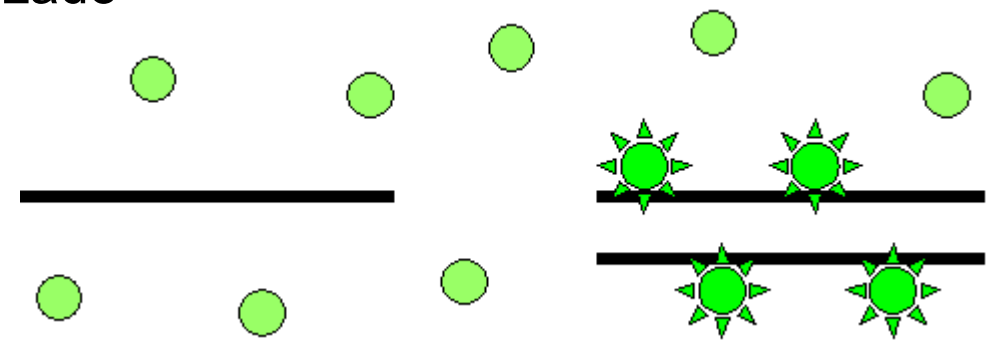
Real time RT-PCR
qRT-PCR

q = quantitative

Cuantificación directa y contínua **durante** la amplificación

Uso de fluoróforos utilizados como agentes intercalantes, acoplados a sondas o *primers*

Existe una proporcionalidad directa entre la cantidad moléculas de fluoróforo que emiten luz y la cantidad de moléculas de DNA sintetizado



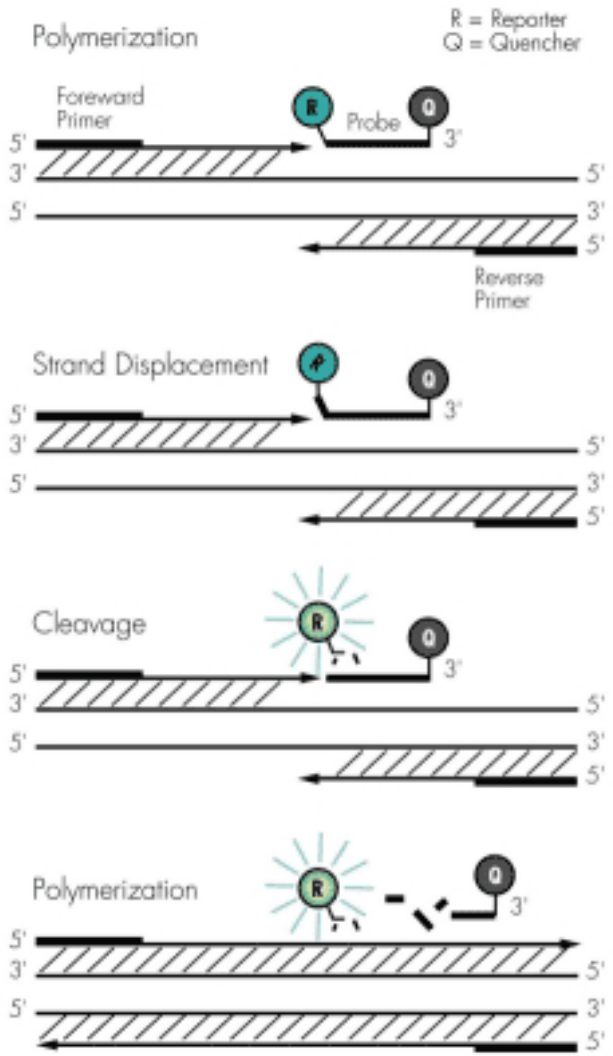
SYBR green

Real time RT-PCR

Cuantificación directa y contínua **durante** la amplificación

Uso de fluoróforos utilizados como agentes inte
sondas o *primers*

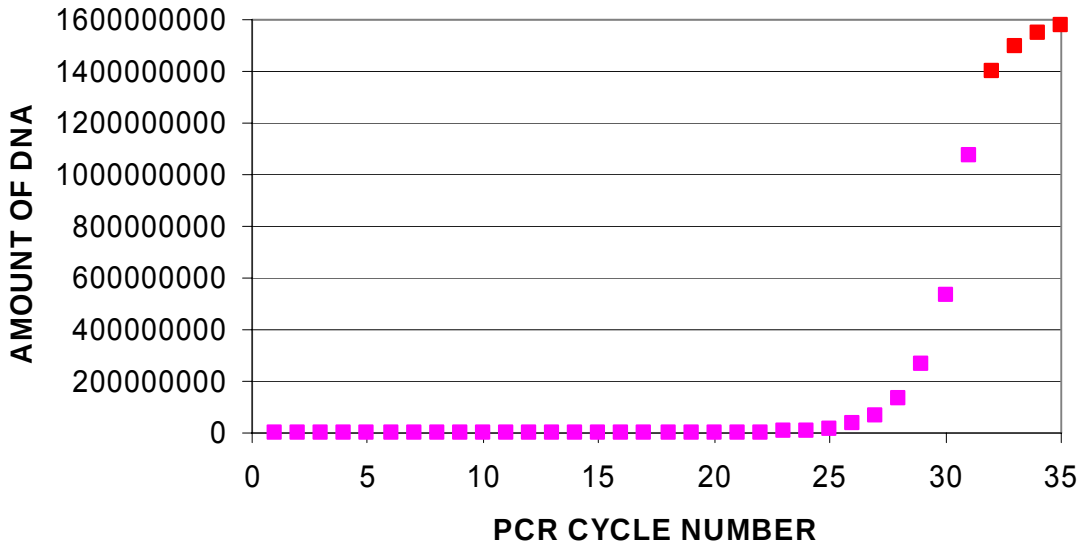
Existe una proporcionalidad directa entre la can
moléculas de fluoróforo que emiten luz y la can
de DNA sintetizado

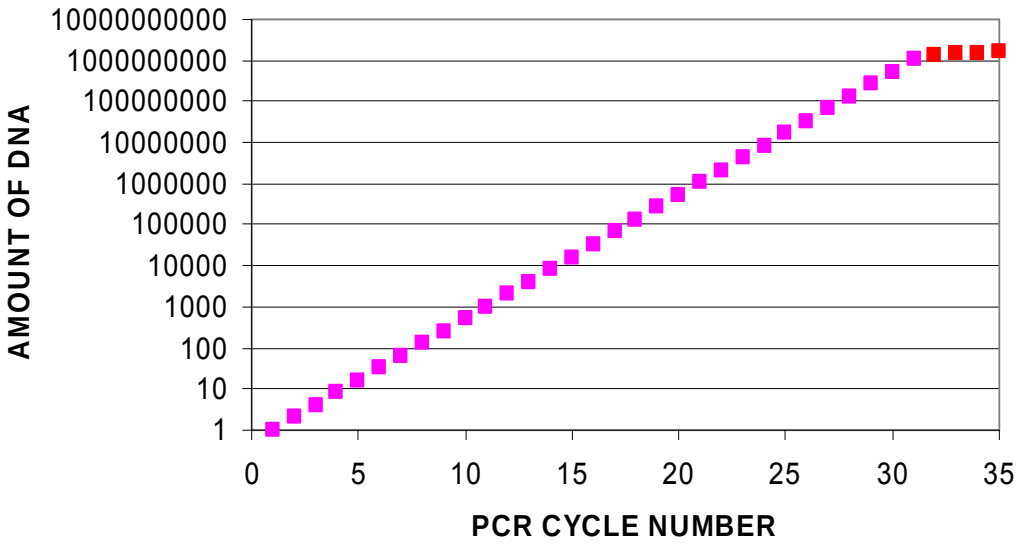


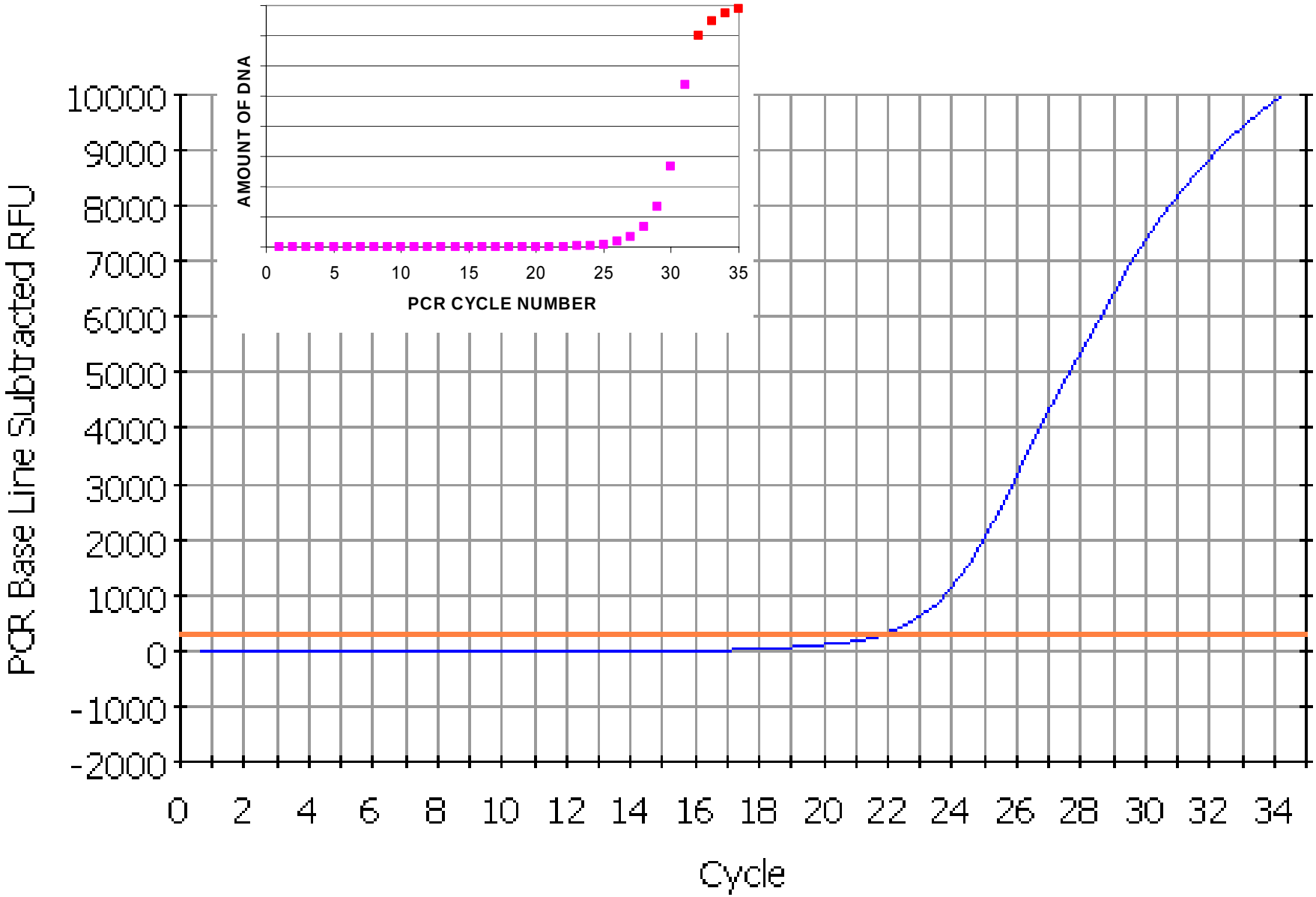
Reporter-quencher

Liberación por exonucleasa 5’

CYCLE NUMBER	AMOUNT OF DNA
0	1
1	2
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1,024
11	2,048
12	4,096
13	8,192
14	16,384
15	32,768
16	65,536
17	131,072
18	262,144
19	524,288
20	1,048,576
21	2,097,152
22	4,194,304
23	8,388,608
24	16,777,216
25	33,554,432
26	67,108,864
27	134,217,728
28	268,435,456
29	536,870,912
30	1,073,741,824
31	1,400,000,000
32	1,500,000,000
33	1,550,000,000
34	1,580,000,000





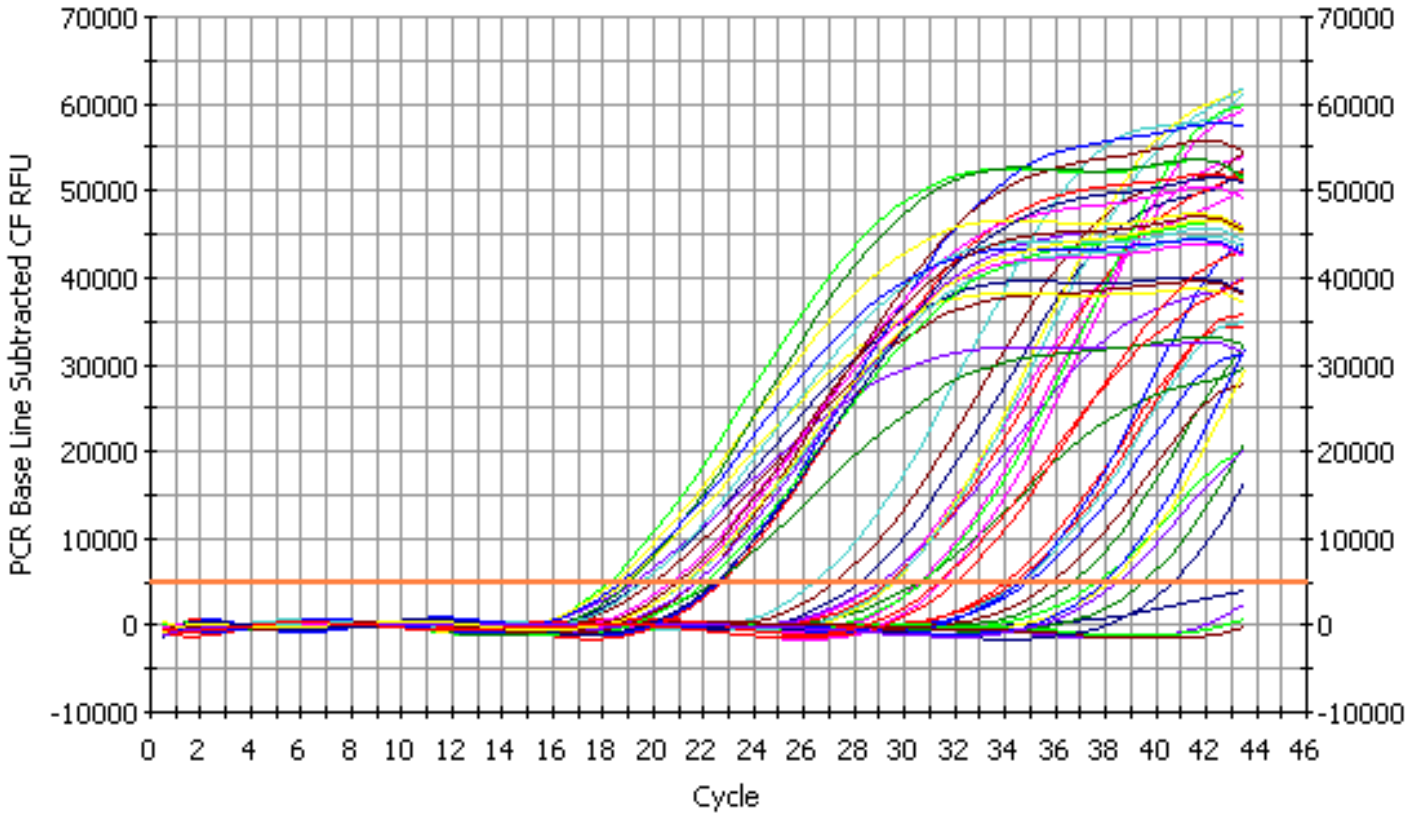


real-time PCR trace for a single well on a 96-well plate, cycles are shown along the X-axis, and arbitrary fluorescence units (actually these are fold increase over background fluorescence) are shown on the Y-axis

PCR en tiempo real (Real time PCR o qPCR)

RNA → cDNA → PCR con cebadores específicos en presencia de un compuesto fluorescente

iCycler



BIO-RAD

2- Síntesis de RNA

Run-on transcription assay (núcleos aislados)

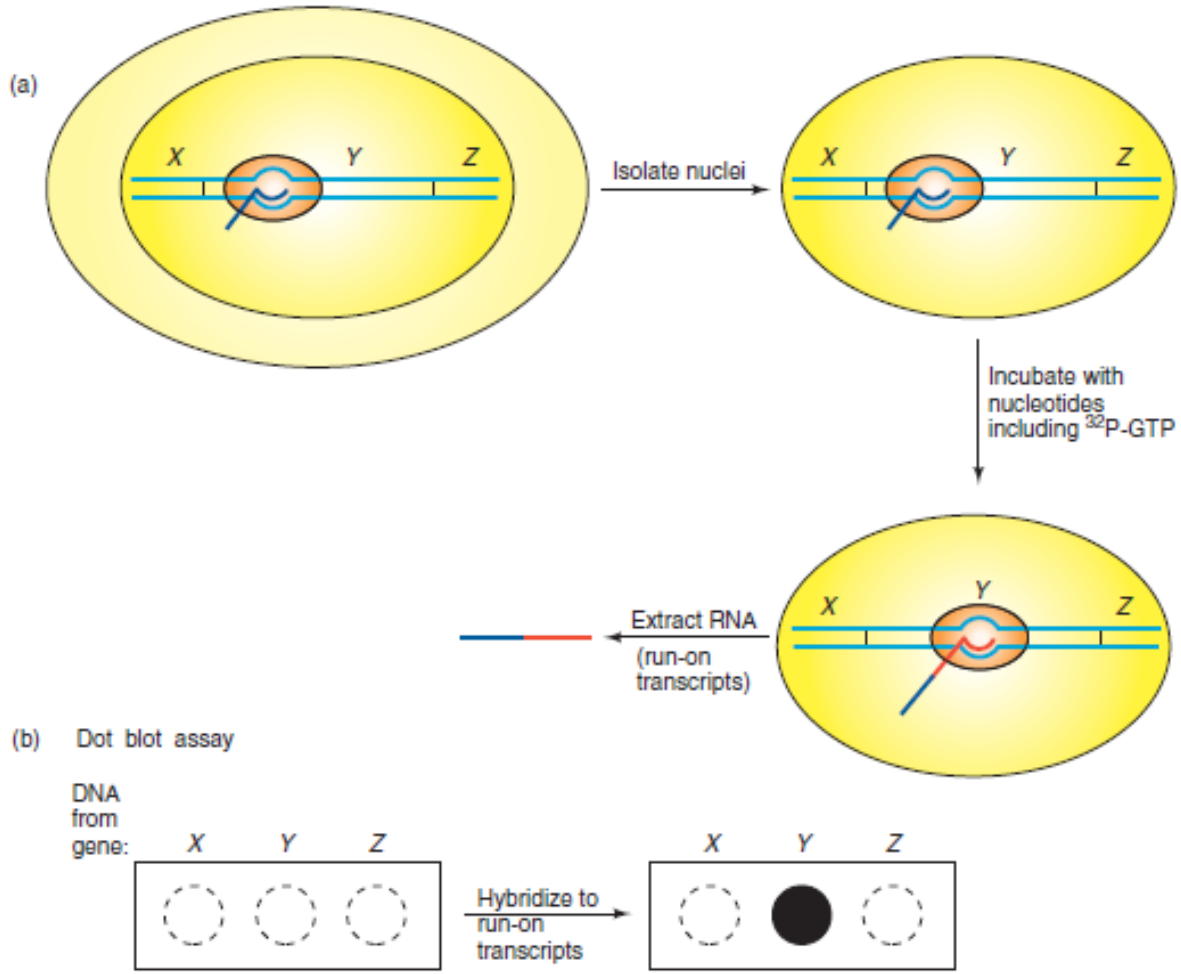


Figure 5.33 Nuclear run-on transcription. (a) The run-on reaction. Start with cells that are in the process of transcribing the *Y* gene, but not the *X* or *Z* genes. The RNA polymerase (orange) is making a transcript (blue) of the *Y* gene. Isolate nuclei from these cells and incubate them with nucleotides so transcription can continue (run-on). Also include a labeled nucleotide in the run-on reaction so the transcripts become labeled (red). Finally, extract the labeled run-on transcripts. **(b)** Dot blot assay. 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. Because gene *Y* was transcribed in the run-on reaction, its transcript will be labeled, and the gene *Y* spot becomes labeled. The more active the transcription of gene *Y*, the more intense the labeling will be. On the other hand, because genes *X* and *Z* were not active, no labeled *X* and *Z* transcripts were made, so the *X* and *Z* spots remain unlabeled.

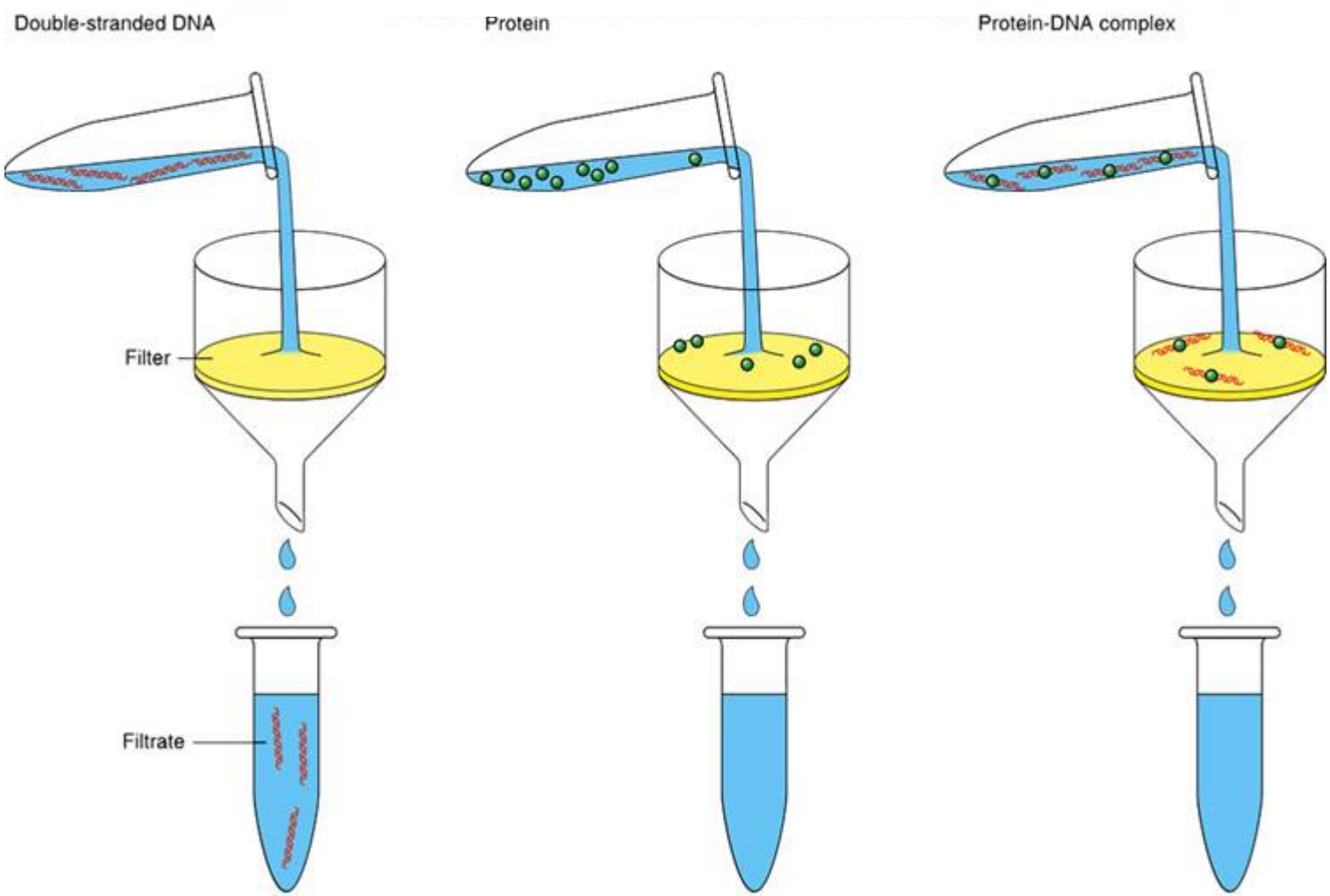
Técnicas para identificar interacciones DNA-proteína

Unión a filtros de nitrocelulosa (*filter binding*)

EMSA: *electrophoretic mobility shift assay* (*gel shift*, *band shift*, retardo en gel,etc.)

Footprinting

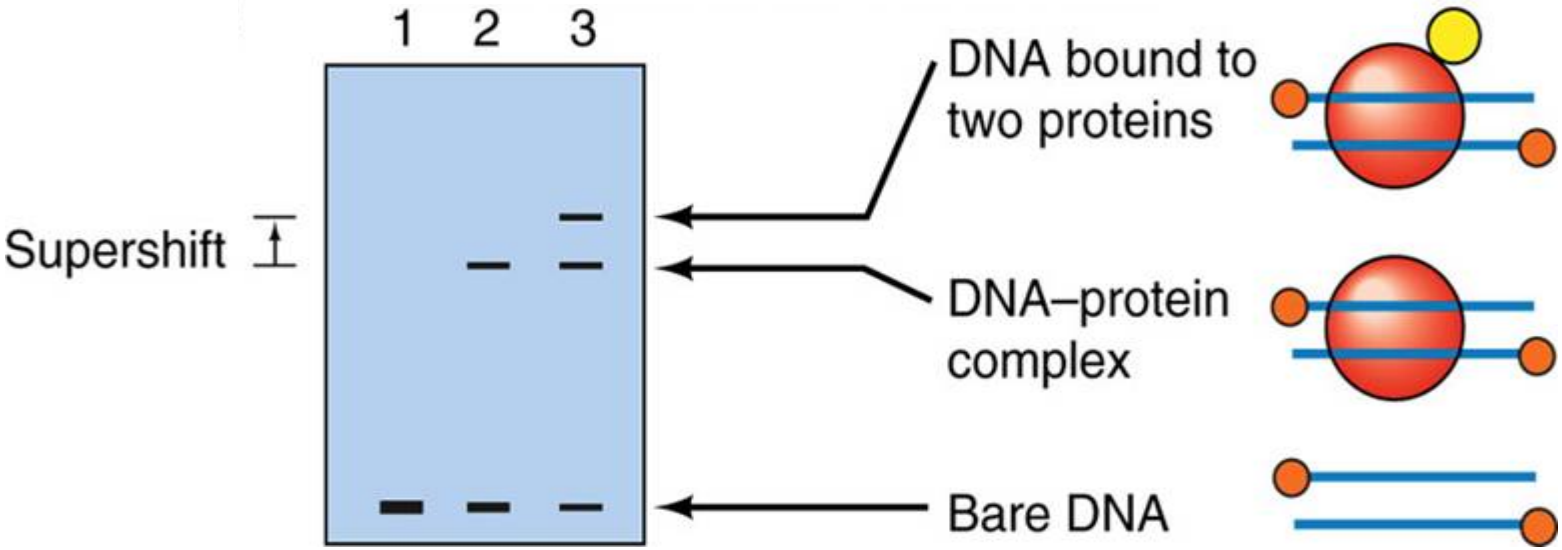
NITROCELLULOSE FILTER BINDING ASSAY



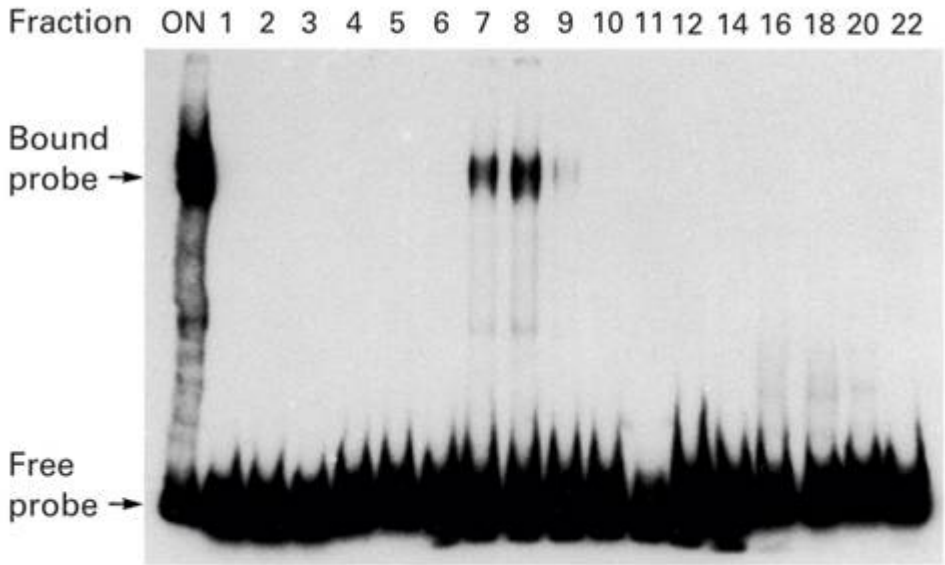
DNA alone passes through the filter while protein is retained; because of this, retained DNA must be bound to protein

WEAVER: FIG. 5.34

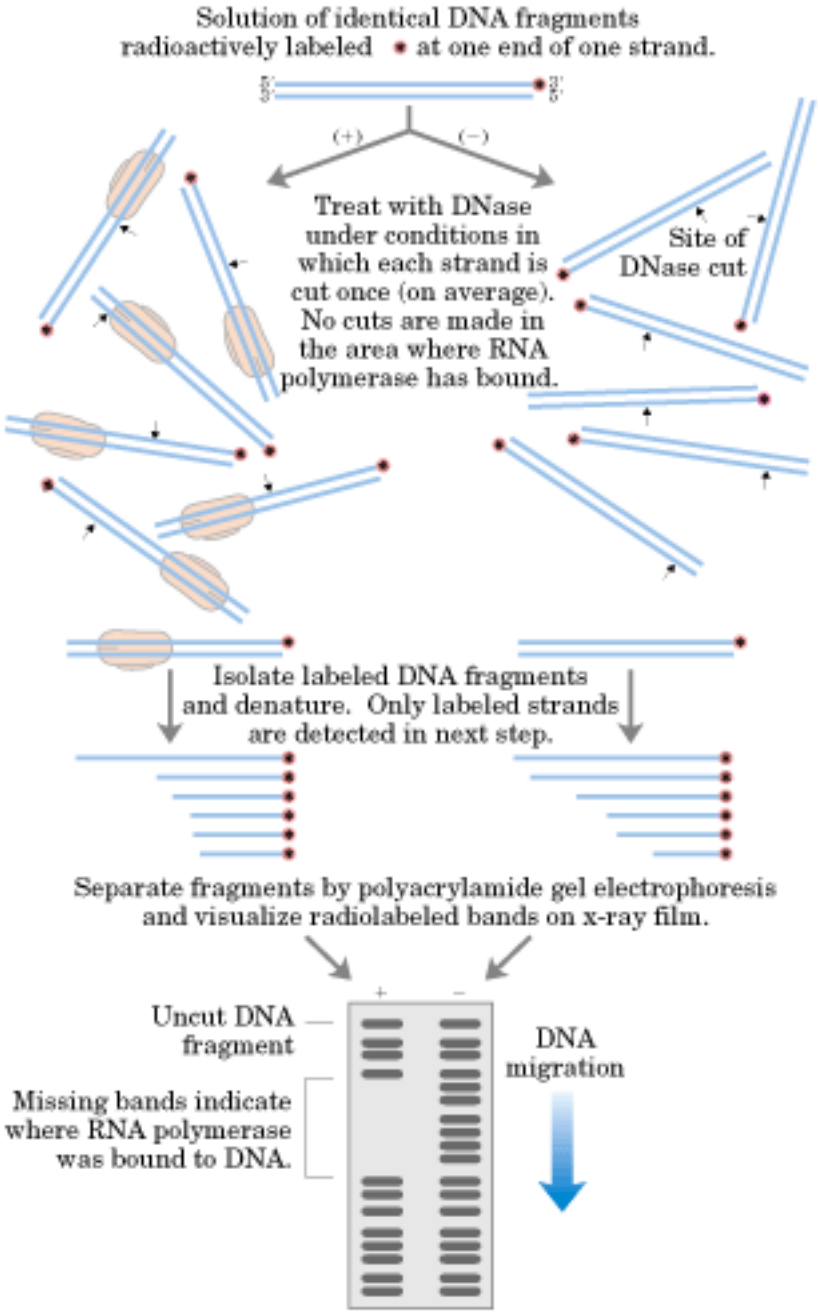
ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

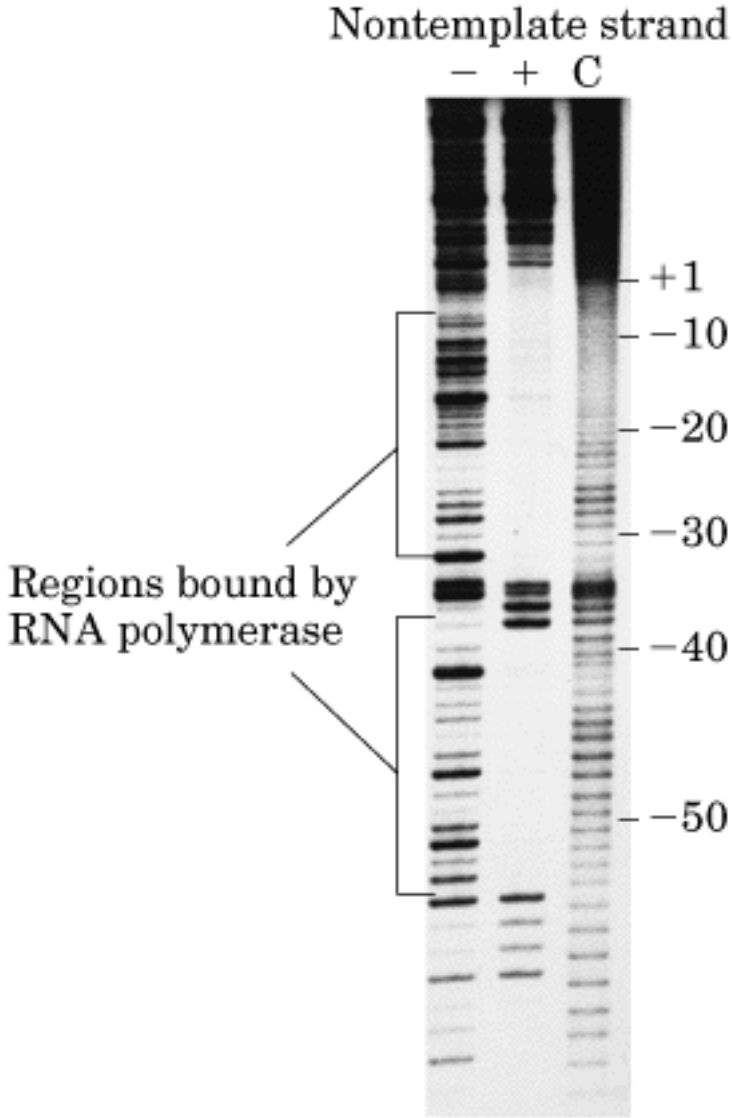


La movilidad electroforética del DNA es retrasada por la unión de la(s) proteína(s)

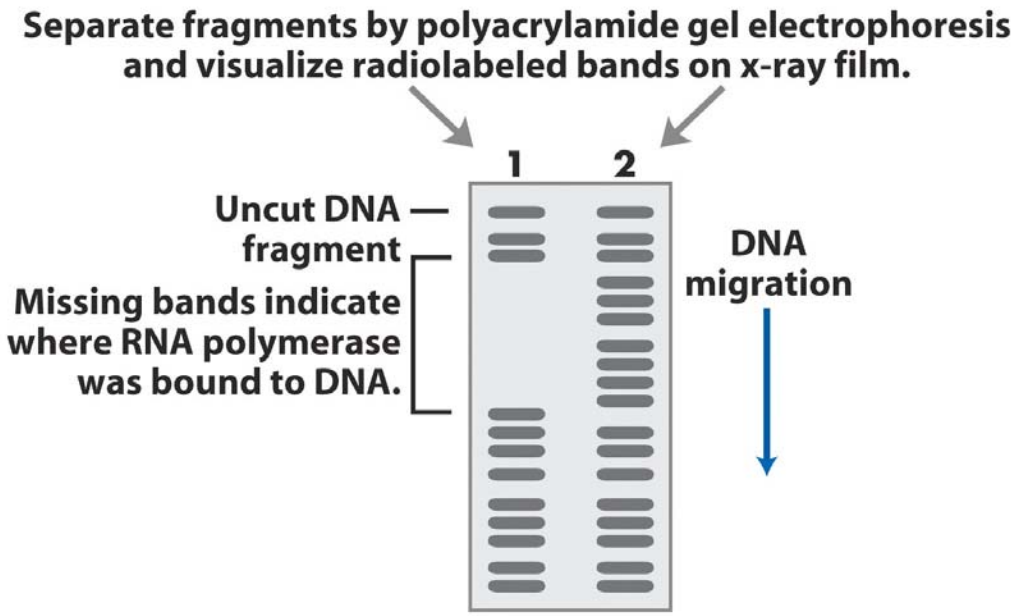


Ensayos de protección (*footprinting)*






Isolate labeled DNA fragments
and denature. Only labeled strands
are detected in next step.


Separate fragments by polyacrylamide gel electrophoresis
and visualize radiolabeled bands on x-ray film.
1
2
Uncut DNA fragment
Missing bands indicate where RNA polymerase was bound to DNA.
DNA migration

Técnicas para identificar interacciones DNA-proteína

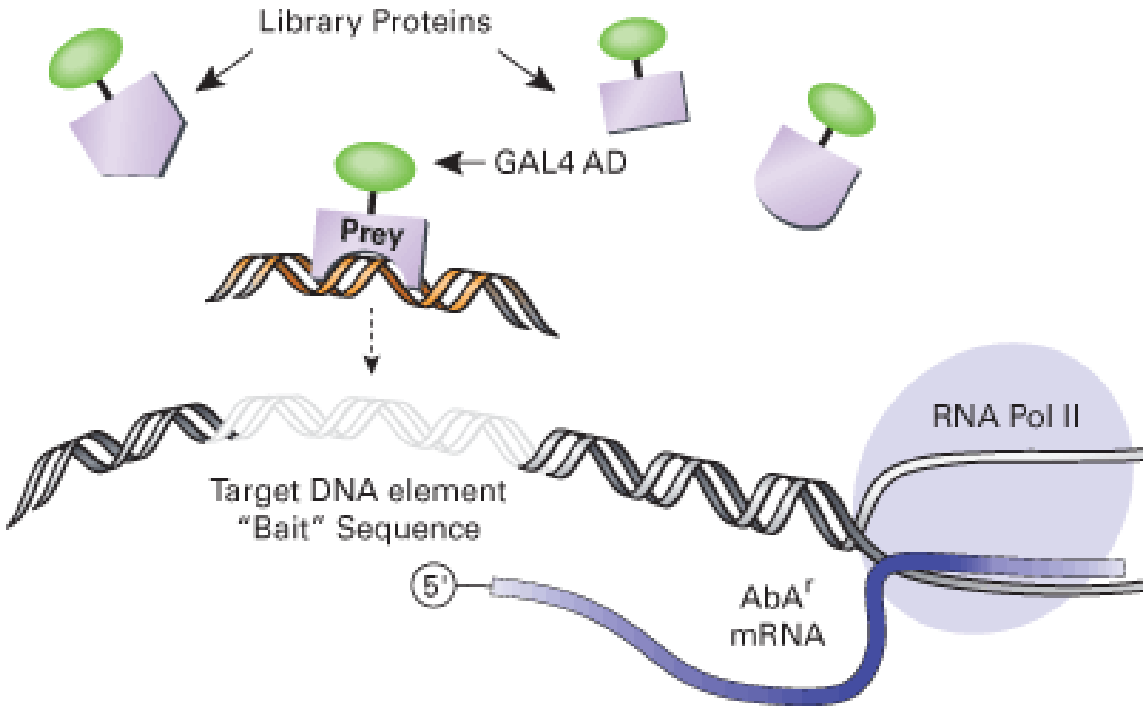
y otras interacciones entre macromoléculas

- **Yeast-one-hybrid system**
- Yeast-two-hybrid system
- Yeast-three-hybrid system

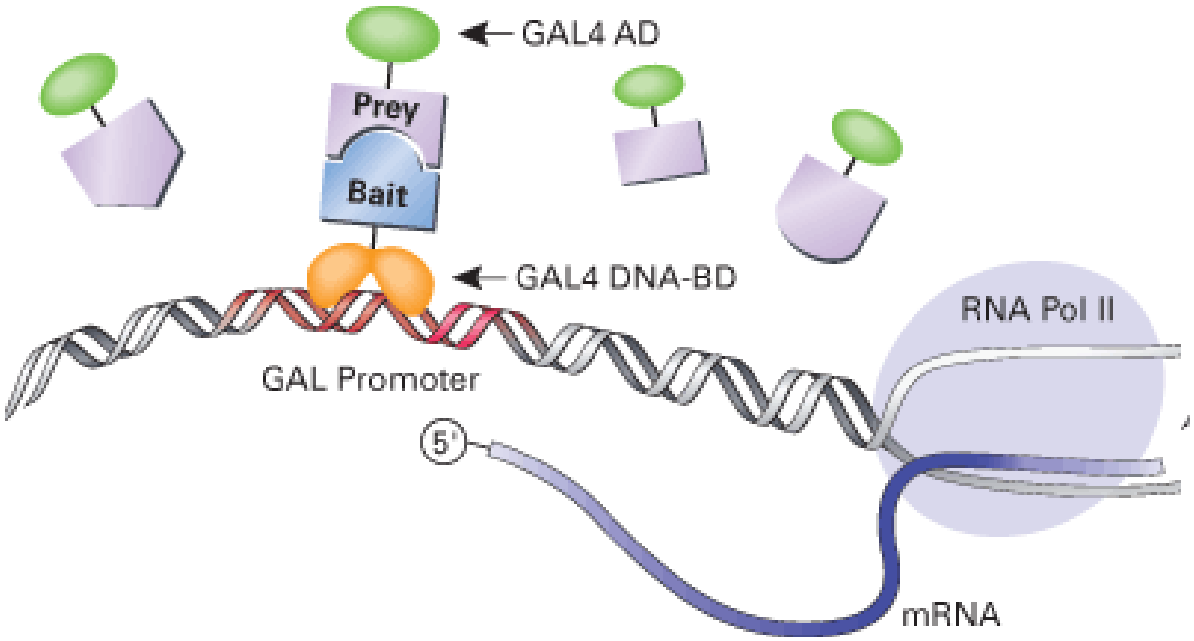
Factores de transcripción (dominios)

***Reporter genes* = genes indicadores**

Yeast-one-hybrid



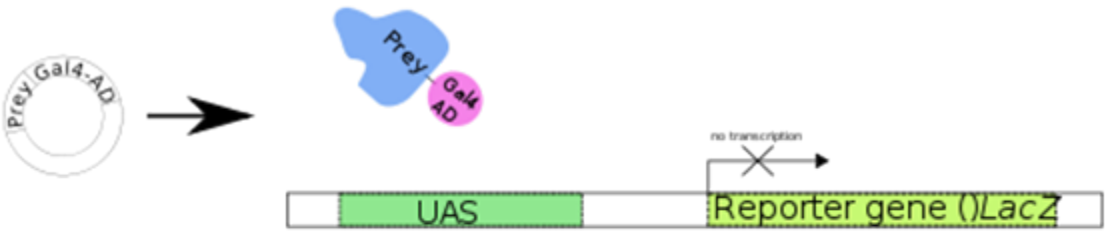
Yeast-two-hybrid



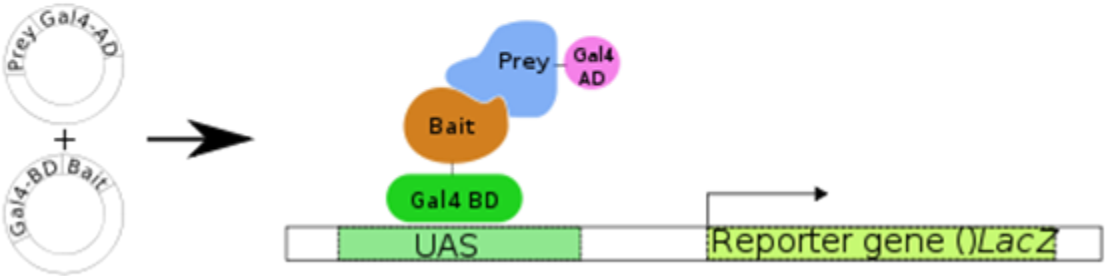
Yeast-two-hybrid

A. Regular transcription of the reporter gene

B. One fusion protein only (Gal4-BD + Bait) - no transcription

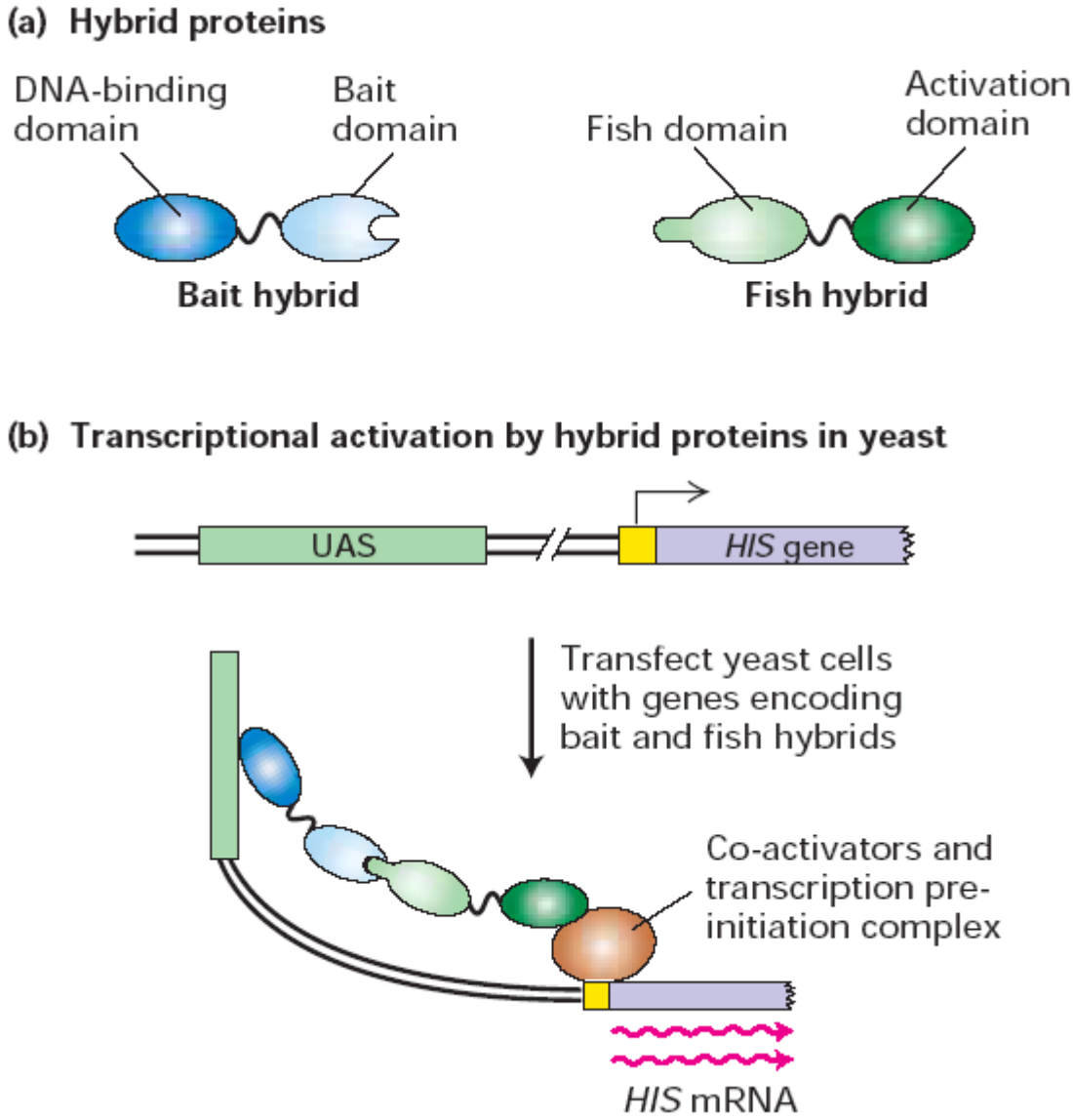


C. One fusion protein only (Gal4-AD + Prey) - no transcription



D. Two fusion proteins with interacting Bait and Prey

Yeast two hybrid system



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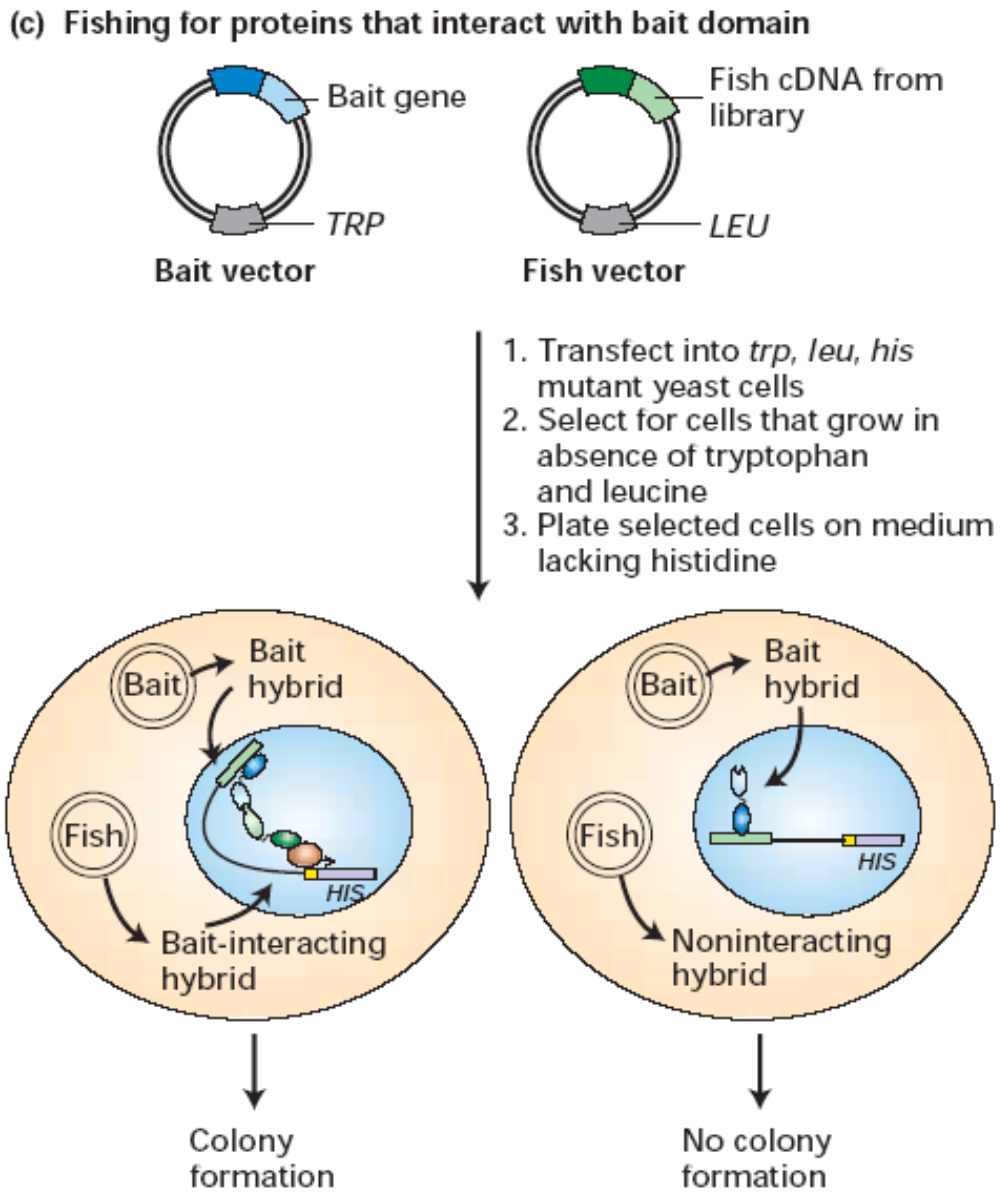
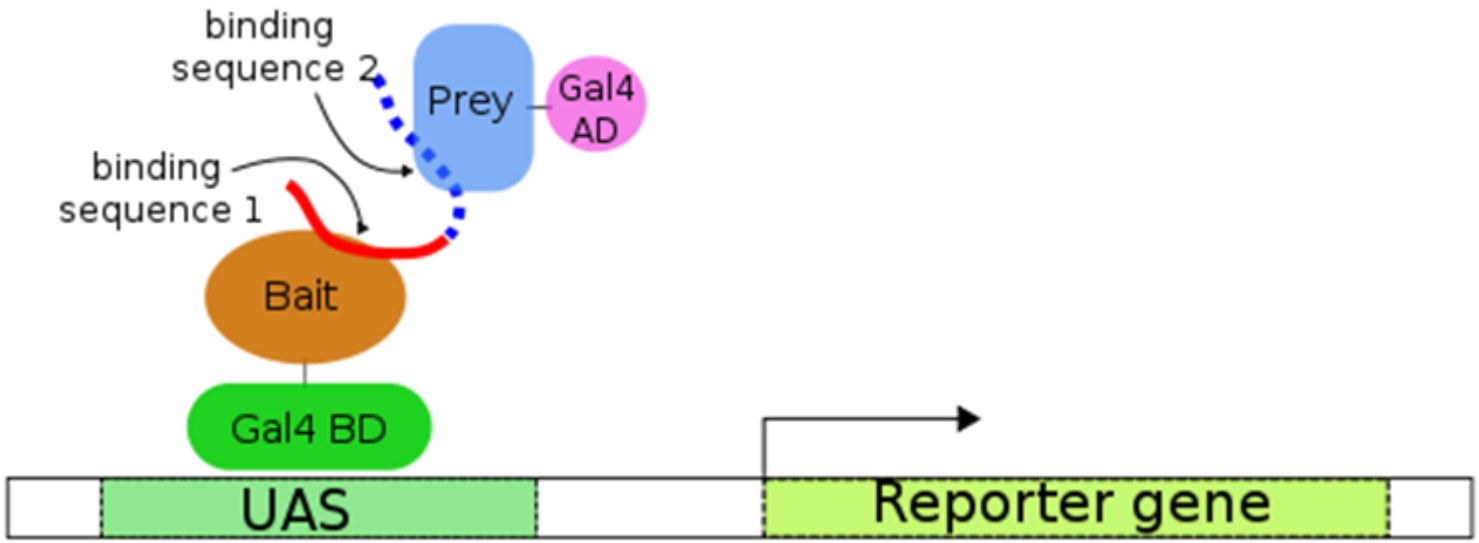
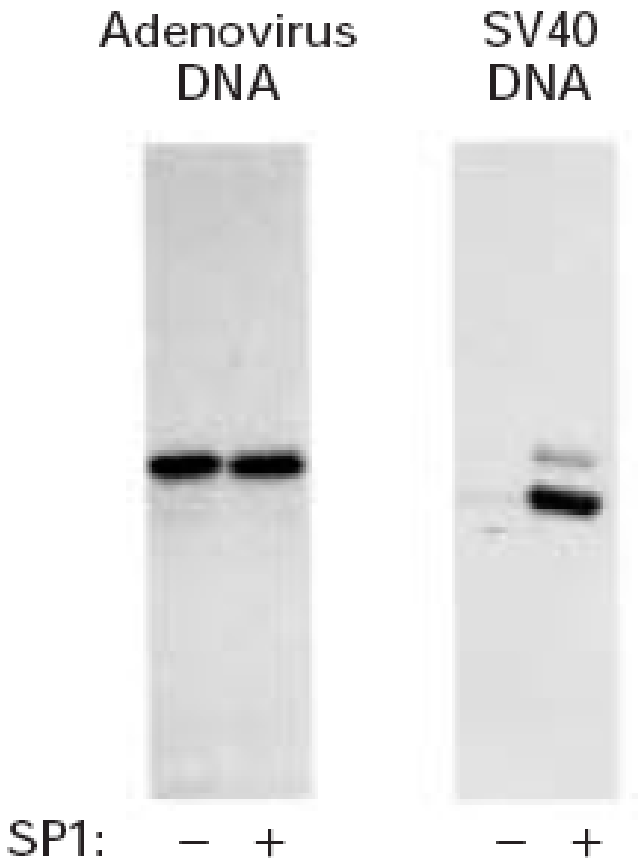
Yeast two hybrid system



Yeast-three-hybrid





EXPERIMENTAL FIGURE 11-15

Transcription factors can be identified by *in vitro* assay for transcription activity.

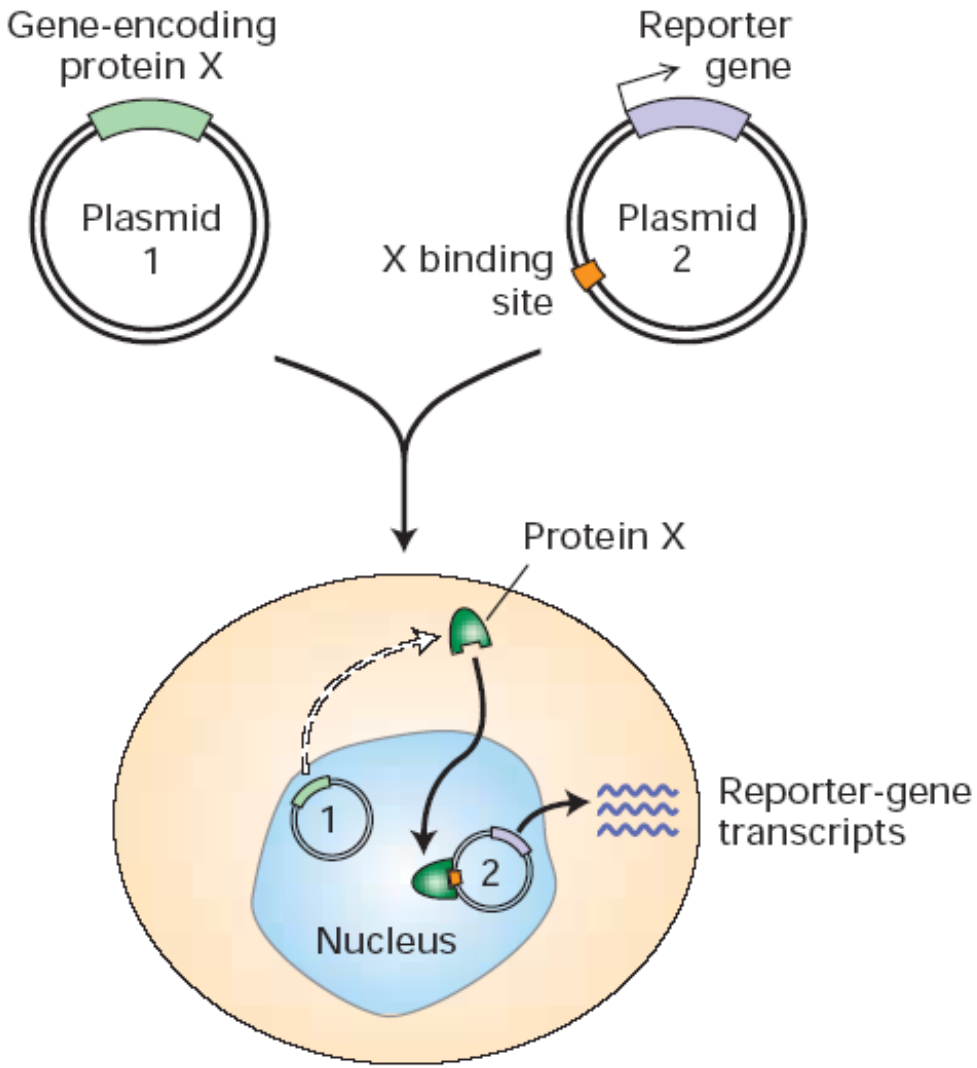
SP1 was identified based on its ability to bind to a region of the SV40 genome that contains six copies of a GC-rich promoter-proximal element and was purified by column chromatography. To test the transcription-activating ability of purified SP1, it was incubated in vitro with template DNA, a protein fraction containing RNA polymerase II and associated general transcription factors, and labeled ribonucleoside triphosphates. The labeled RNA products were subjected to electrophoresis and autoradiography. Shown here are autoradiograms from assays with adenovirus and SV40 DNA in the absence (-) and presence (+) of SP1. SP1 had no significant effect on transcription from the adenovirus promoter, which contains no SP1-binding sites. In contrast, SP1 stimulated transcription from the SV40 promoter about tenfold. [Adapted from M. R. Briggs et al., 1986, *Science* **234**:47.]

Lodish 2003, p.455

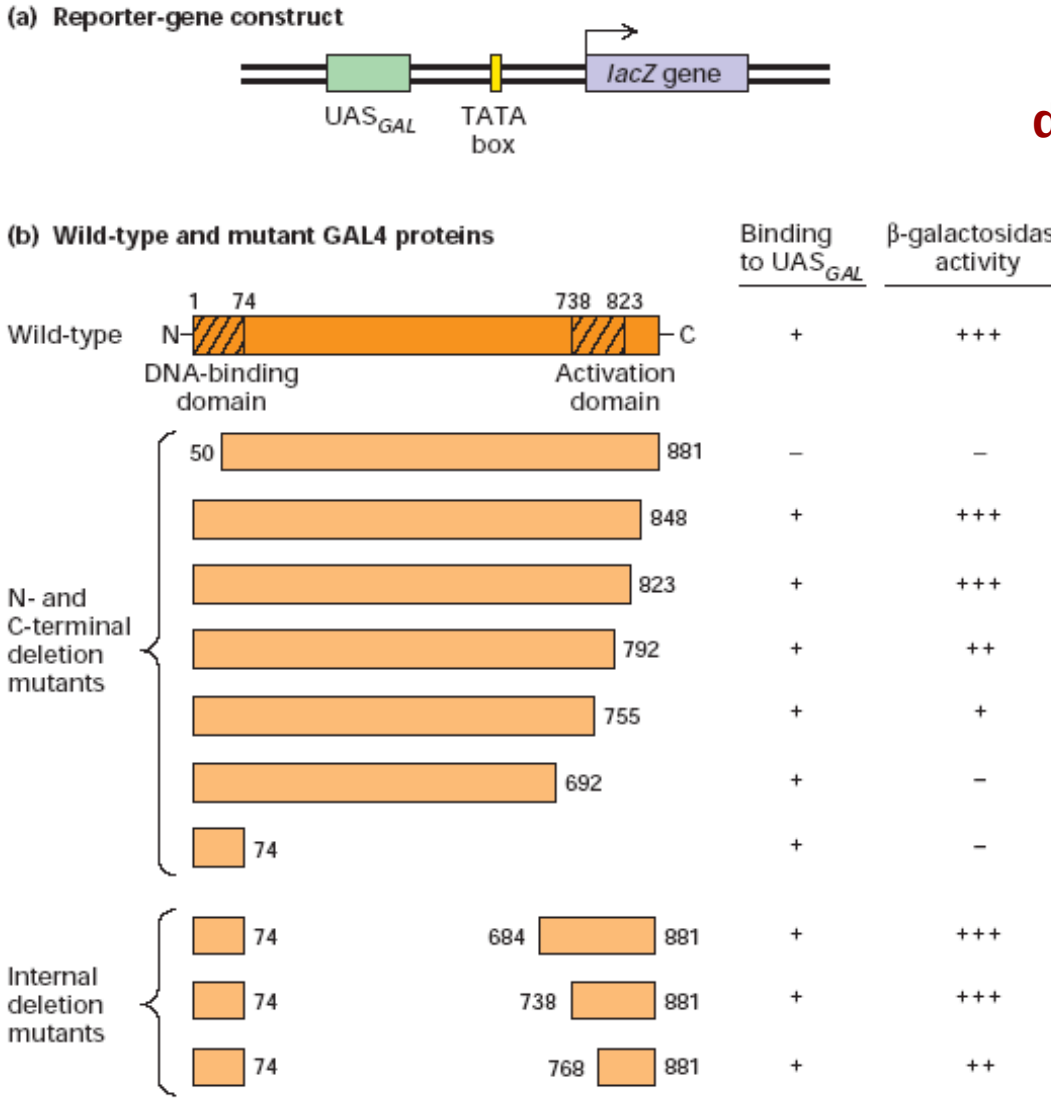
▲ **EXPERIMENTAL FIGURE 11-16**

In vivo transfection assay measures transcription activity to evaluate proteins believed to be transcription factors.

The assay system requires two plasmids. One plasmid contains the gene encoding the putative transcription factor (protein X). The second plasmid contains a reporter gene (e.g., *lacZ*) and one or more binding sites for protein X. Both plasmids are simultaneously introduced into cells that lack the gene encoding protein X. The production of reporter-gene RNA transcripts is measured; alternatively, the activity of the encoded protein can be assayed. If reporter-gene transcription is greater in the presence of the X-encoding plasmid, then the protein is an activator; if transcription is less, then it is a repressor. By use of plasmids encoding a mutated or rearranged transcription factor, important domains of the protein can be identified.

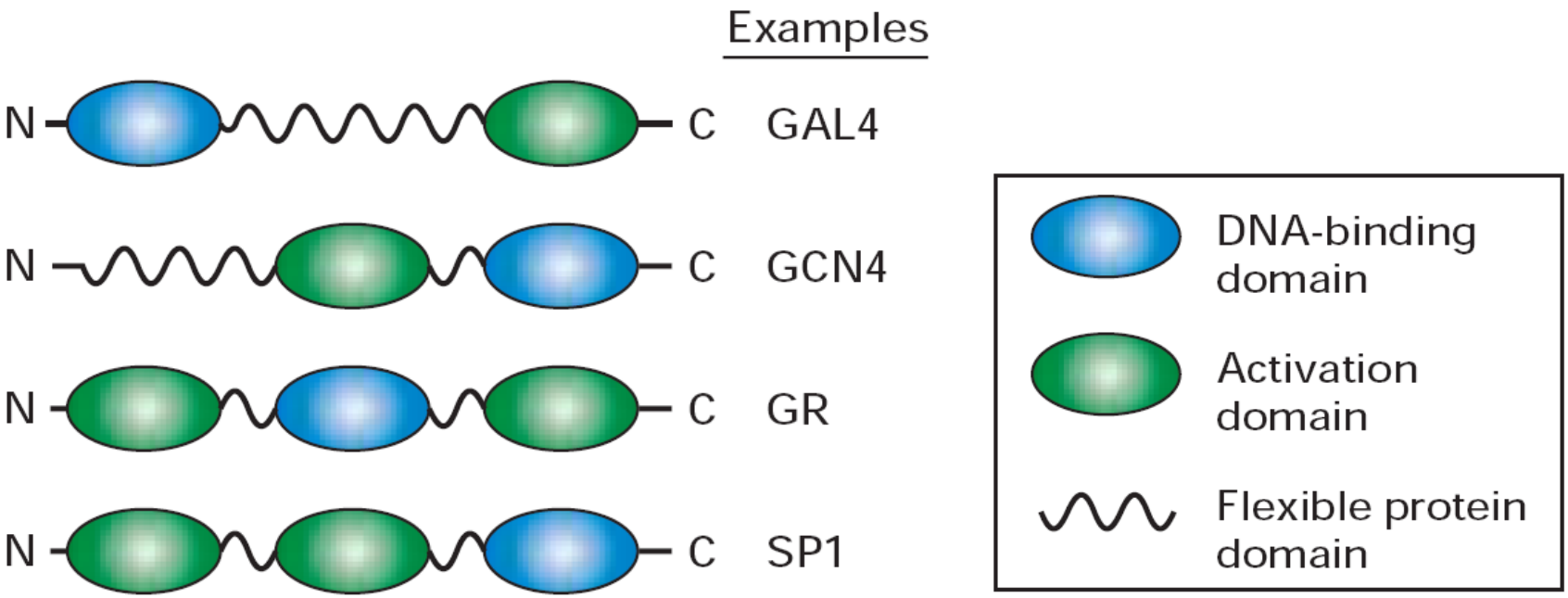


Deletion mutants of the GAL4 gene in yeast with a UAS*GAL* reporter-gene construct demonstrate the separate functional domains in an activator.



▲ EXPERIMENTAL FIGURE 11-17 Deletion mutants of the GAL4 gene in yeast with a UAS*GAL* reporter-gene construct demonstrate the separate functional domains in an activator. (a) Diagram of DNA construct containing a *lacZ* reporter gene and TATA box ligated to UAS*GAL*, a regulatory element that contains several GAL4-binding sites. The reporter-gene construct and DNA encoding wild-type or mutant (deleted) GAL4 were simultaneously introduced into mutant (*gal4*) yeast cells, and the activity of β-galactosidase expressed from *lacZ* was assayed. Activity will be high if the introduced *GAL4* DNA encodes a functional protein. (b) Schematic diagrams of wild-type GAL4 and various mutant forms. Small numbers refer to positions in the wild-type sequence. Deletion of 50 amino acids from the N-terminal end destroyed the ability of GAL4 to bind to UAS*GAL* and to stimulate expression of β-galactosidase from the reporter gene. Proteins with extensive deletions from the C-terminal end still bound to UAS*GAL*. These results localize the DNA-binding domain to the N-terminal end of GAL4. The ability to activate β-galactosidase expression was not entirely eliminated unless somewhere between 126–189 or more amino acids were deleted from the C-terminal end. Thus the activation domain lies in the C-terminal region of GAL4. Proteins with internal deletions (*bottom*) also were able to stimulate expression of -galactosidase, indicating that the central region of GAL4 is not crucial for its function in this assay. [See J. Ma and M. Ptashne, 1987, *Cell* **48**:847; I. A. Hope and K. Struhl, 1986, *Cell* **46**:885; and R. Brent and M. Ptashne, 1985, *Cell* **43**:729.]

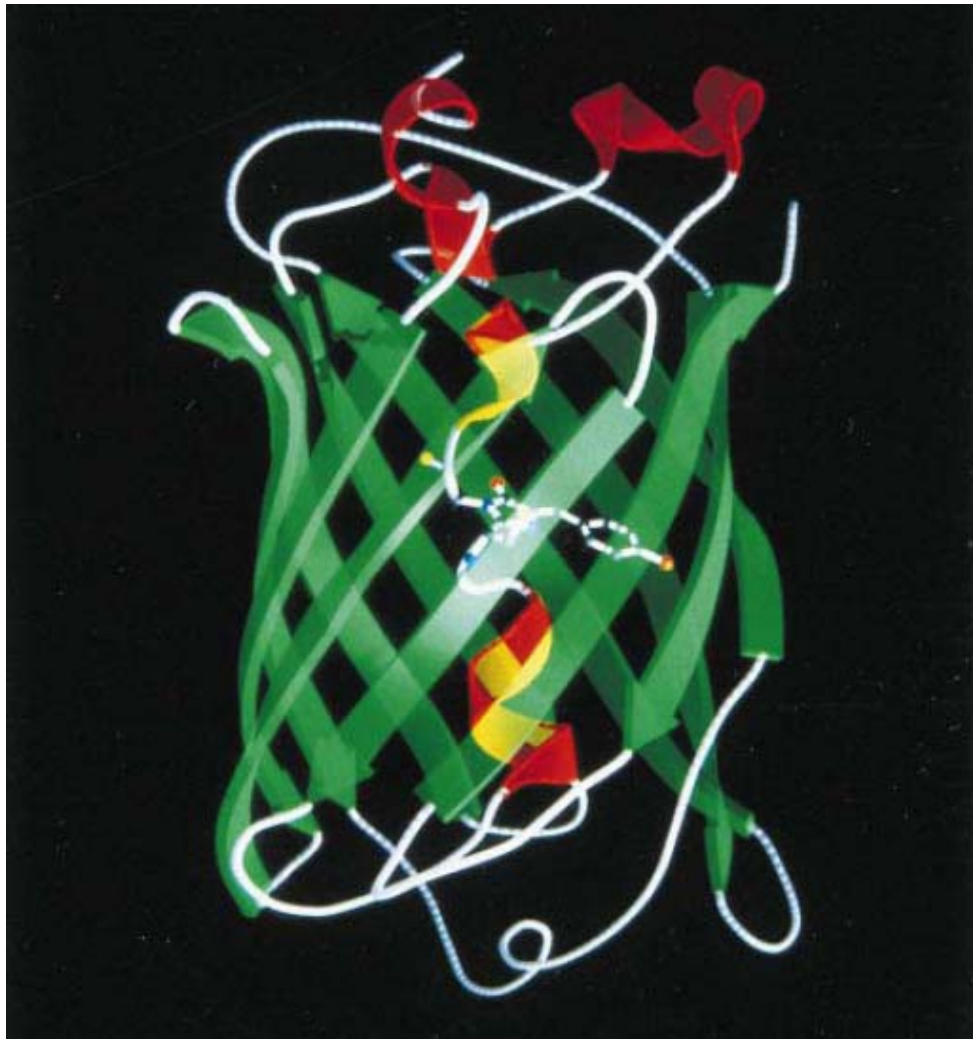
modular structure of eukaryotic transcription activators



Schematic diagrams illustrating the modular structure of eukaryotic transcription activators.
These transcription factors may contain more than one **activation domain** (**AD**) but rarely contain more than one **DNA-binding domain** (**DBD**).
GAL4 and GCN4 are yeast transcription activators. The glucocorticoid receptor (GR) promotes transcription of target genes when certain hormones are bound to the C-terminal activation domain. SP1 binds to GC-rich promoter elements in a large number of mammalian genes.

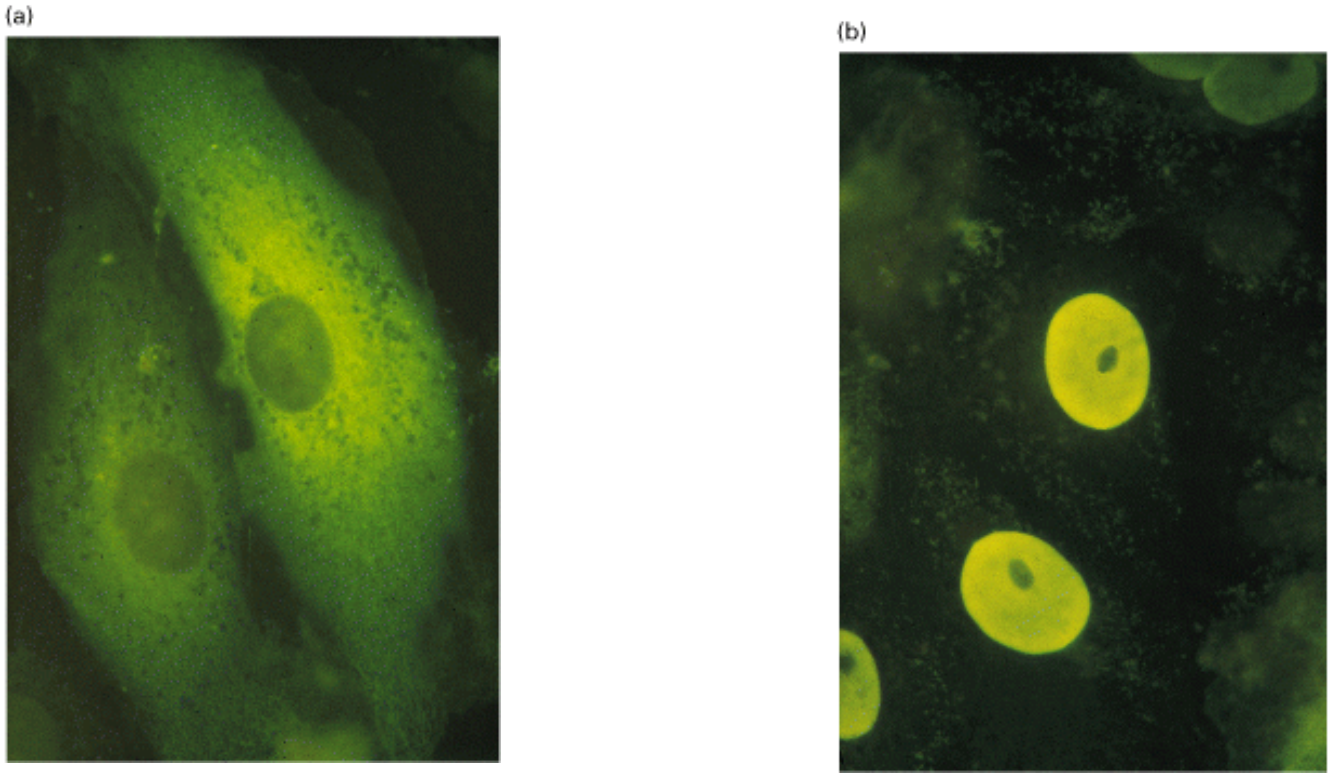
Uso de “reporter genes” o genes indicadores

GFP



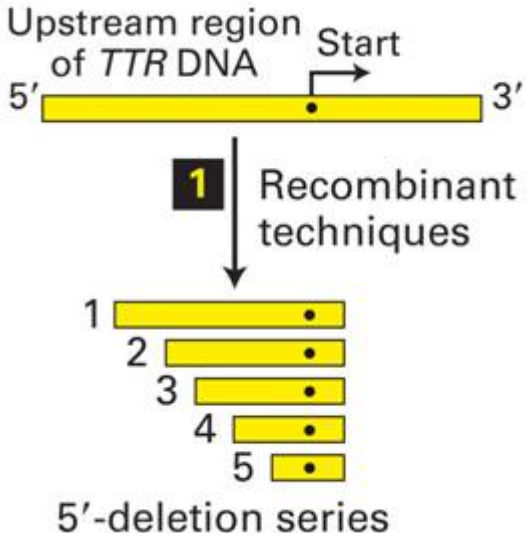
Localización de proteínas

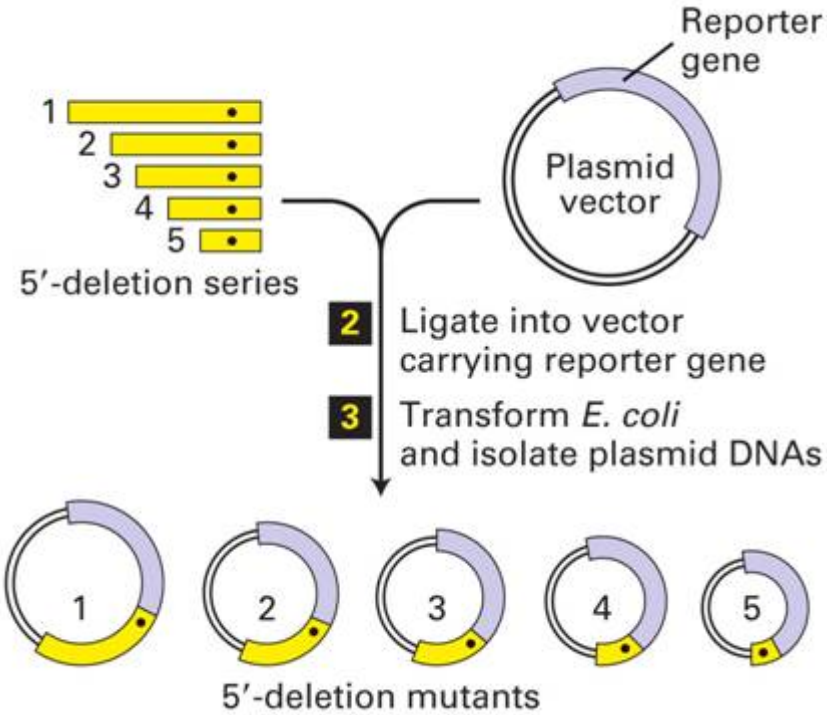
(señal de localización nuclear NLS)

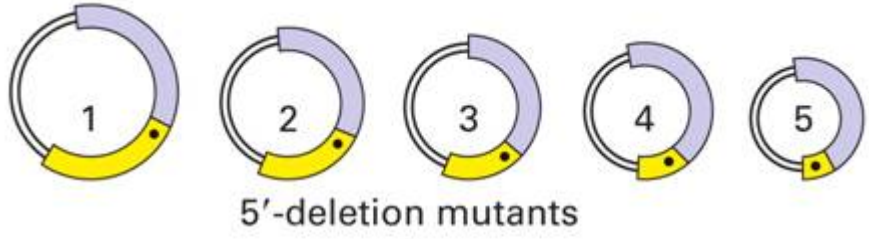


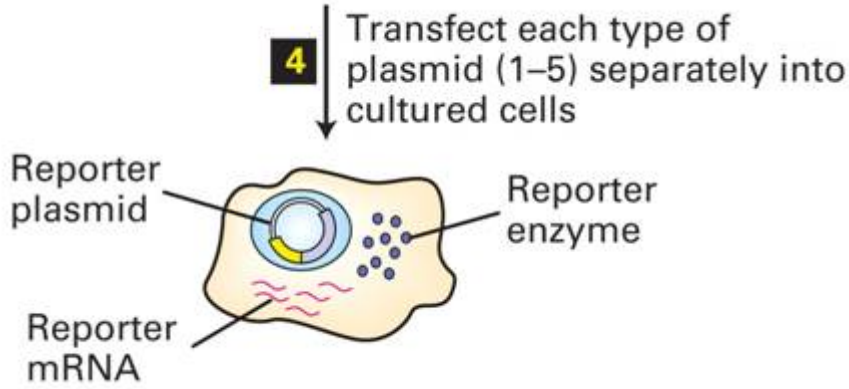
4- Estudio de actividad de promotores

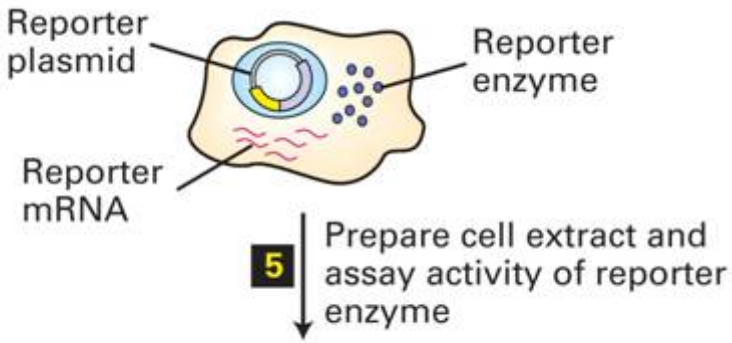
Estudio de promotores









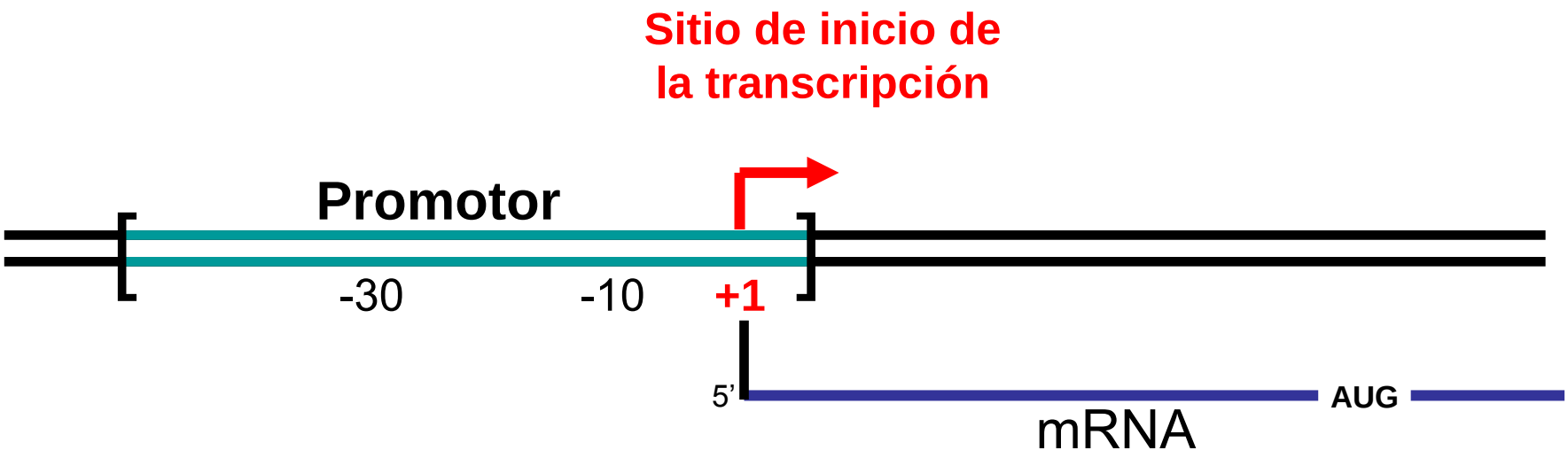


Plasmid no.	Reporter-gene expression
1	+++
2	+++
3	+
4	+
5	–

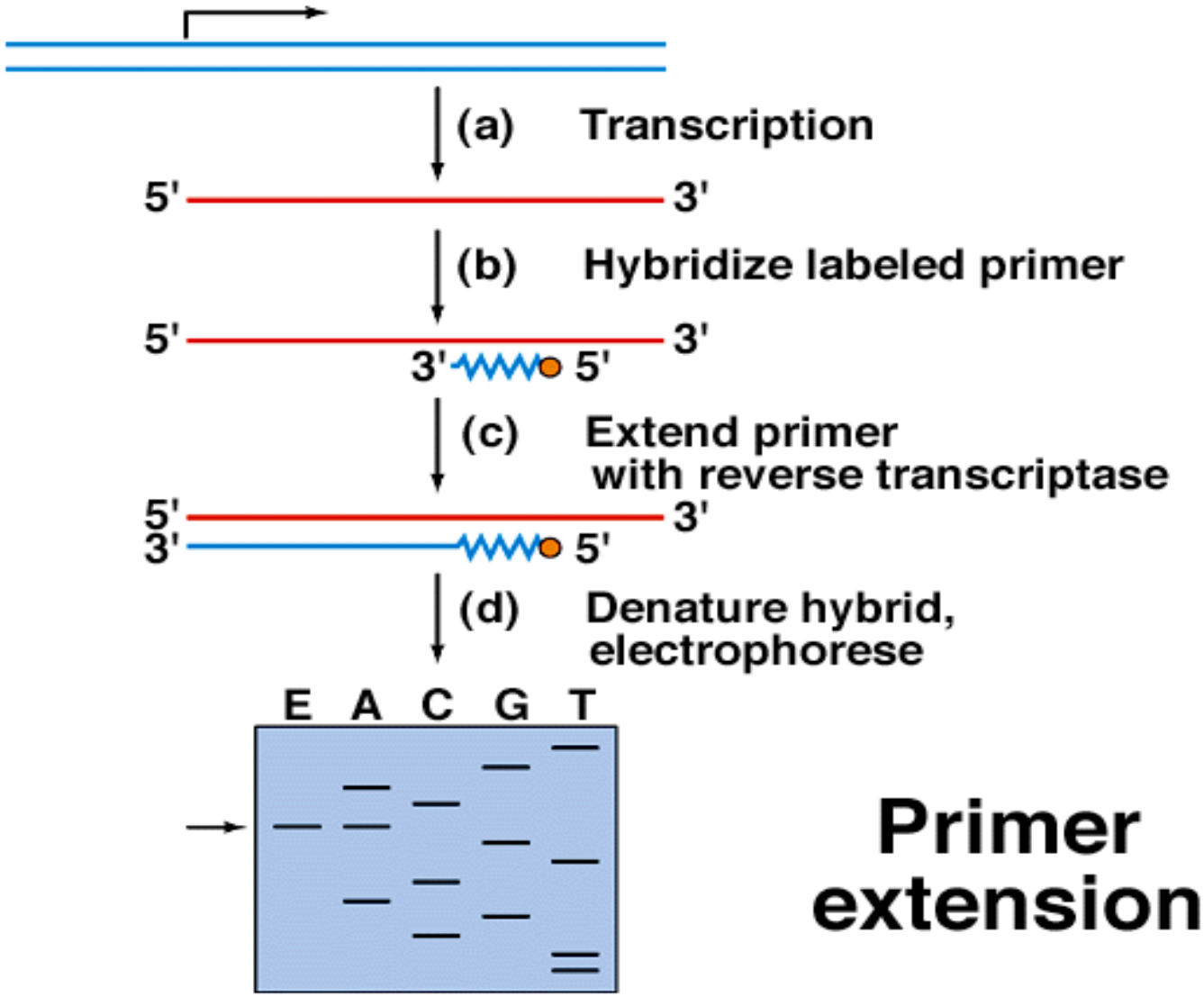
5- Identificación del inicio de la transcripción

Primer extension
Race: *rapid amplification of cDNA ends*
Mapeo con S1
Run off

¿Cómo se determina el sitio de inicio de la transcripción?



Determinación del sitio de inicio de la transcripción



WEAVER: FIG. 5.29

Mapeo de extremo 5´ mediante extensión de cebador (*primer extension*)