Model systems (molecules to mammals)

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Investigative Medicine

Purpose

- Some idea of scientific method
- Make reading papers easier
- Allow critical assessment of work

Techniques covered

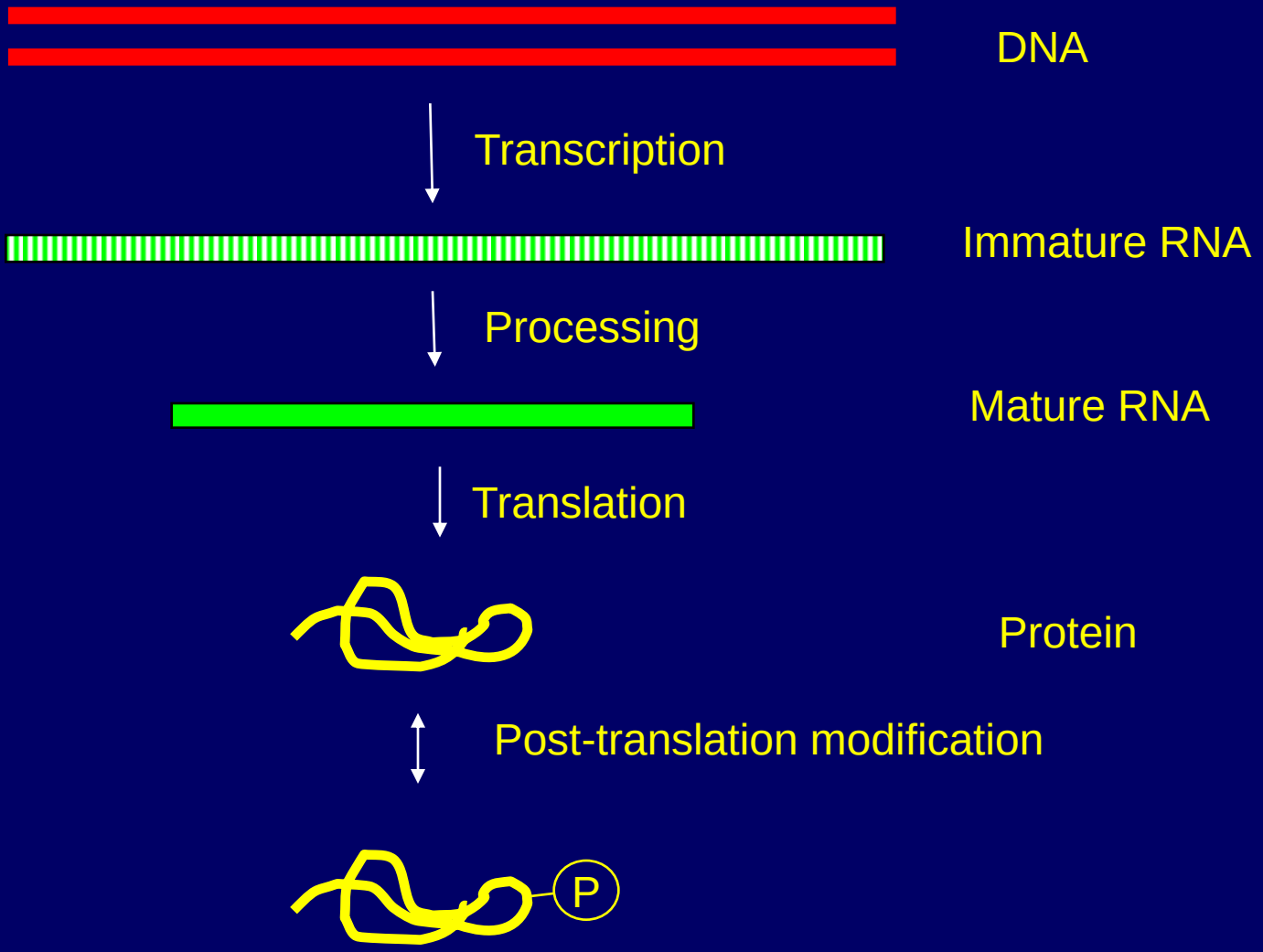
- Quantification
  - Northern blot RNA
  - Real time PCR RNA
  - Western blot PROTEIN
- Localisation
  - *in situ* hybridisation RNA
  - immunocytochemistry PROTEIN

Quantification

Quantification

- Give a snap-shot of what is happening at specific time.
- Can investigate the effect of changes on system
  - Give stimulus quantify effect.
  - E.g. pharmacological, physiological.
- Understand how Genes are controlled.
- Gives idea of function.

What to Quantify



RNA quantification

Principles detection nucleic acids

- DNA- double stranded.
- RNA single stranded, but still forms Watson-Crick base pairs
- If you know the sequence (>20bp) can make a specific probe or primers.

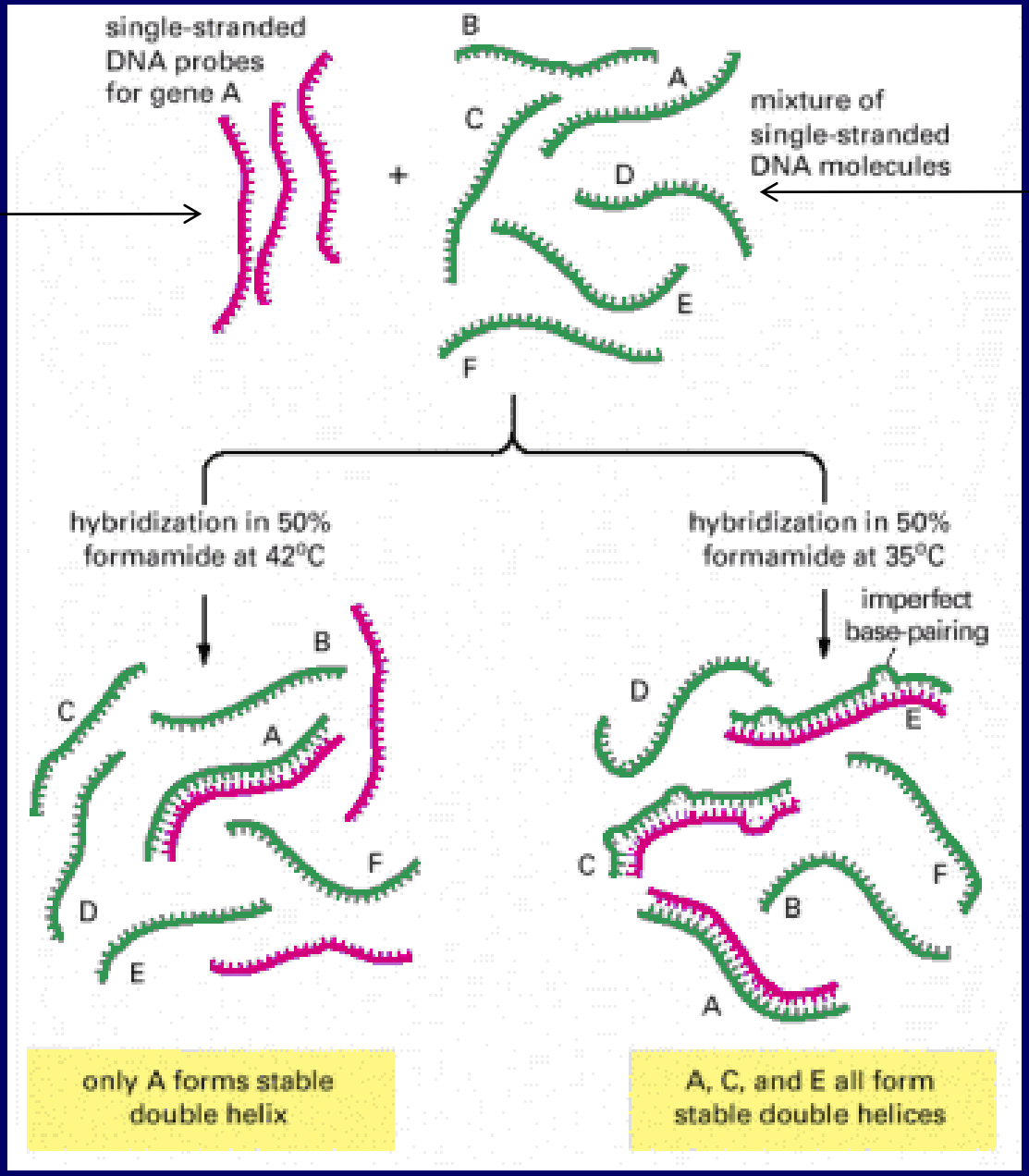
Principles detection nucleic acids

- DNA- double stranded
- RNA single stranded, but still forms Watson-Crick base pairs
- If you know the sequence (>20bp) can make a specific probe.

Principles detection nucleic acids (2)

- Base pairing can be disrupted by heat
- RNA and DNA can be synthesised in vitro incorporating a label (radioactive etc)
- manipulating temperature, chemicals and salt concentration changes specificity of binding two strands (hybridisation)

Labelled DNA or RNA probe



Mixed mRNA from tissue extract

RNA Quantification

Northern blot

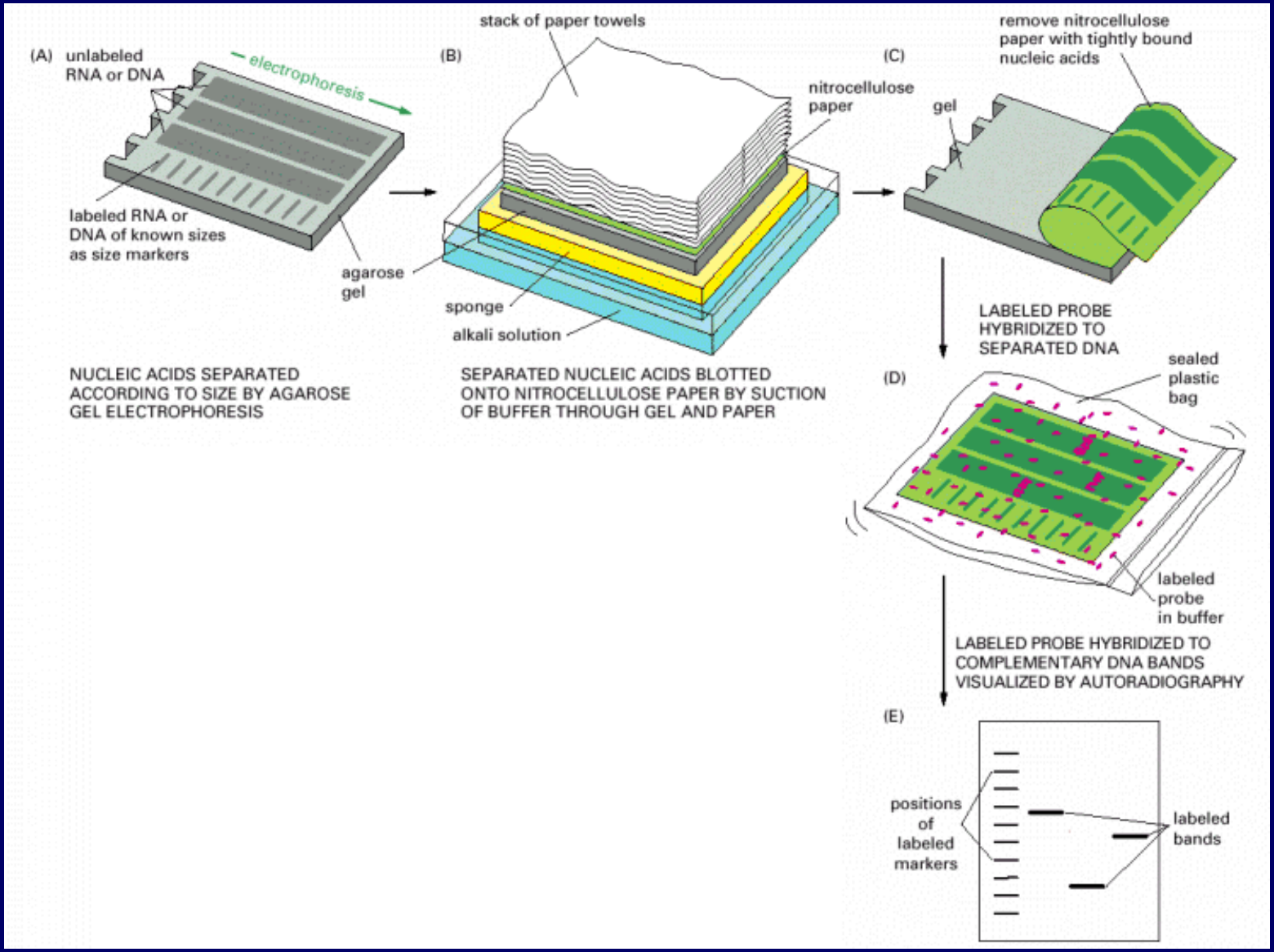
Quantitative PCR

Northern blot

Northern blot

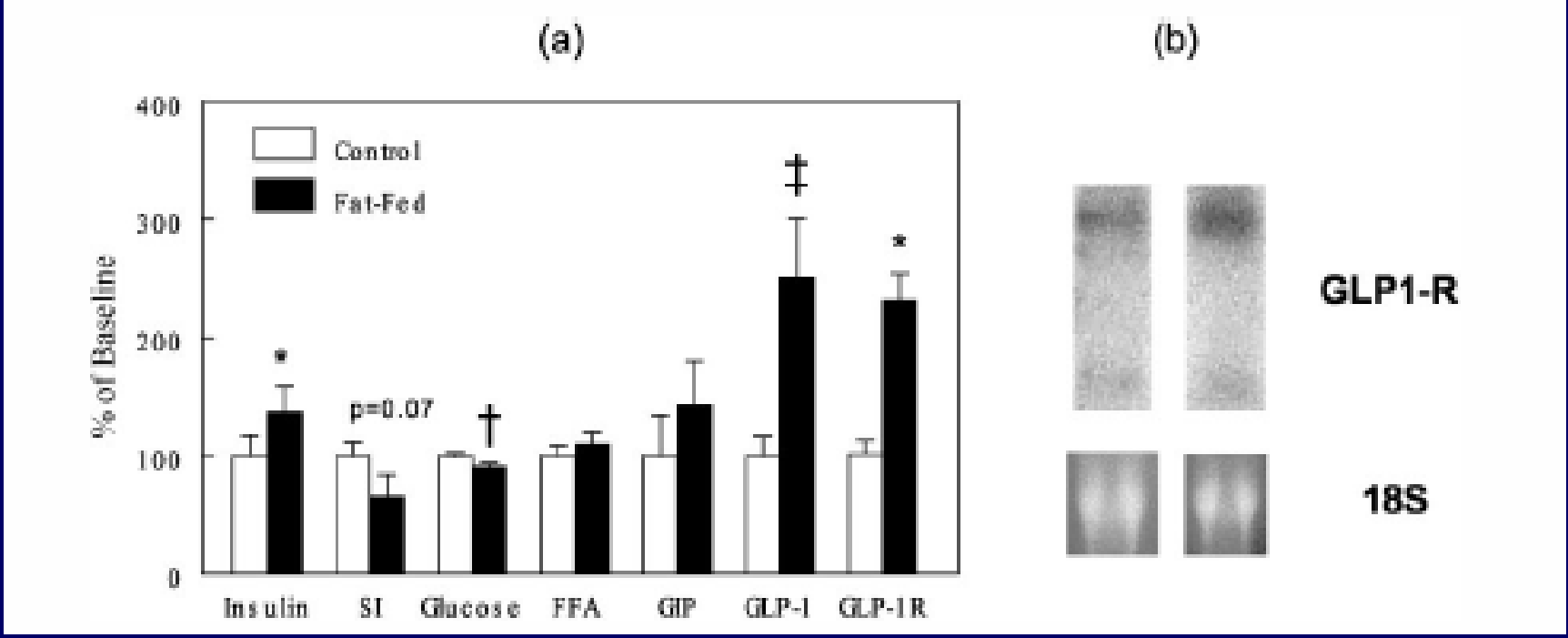
- RNA extracted from target tissue
- RNA run on agarose gel
- Transferred to nylon membrane (capillary action)
- Covalently cross linked
- Non specific binding blocked
- Presence of specific RNA species detected by specific probe
- Data normalised (18s, oligo dT, β-actin)

Northern blot

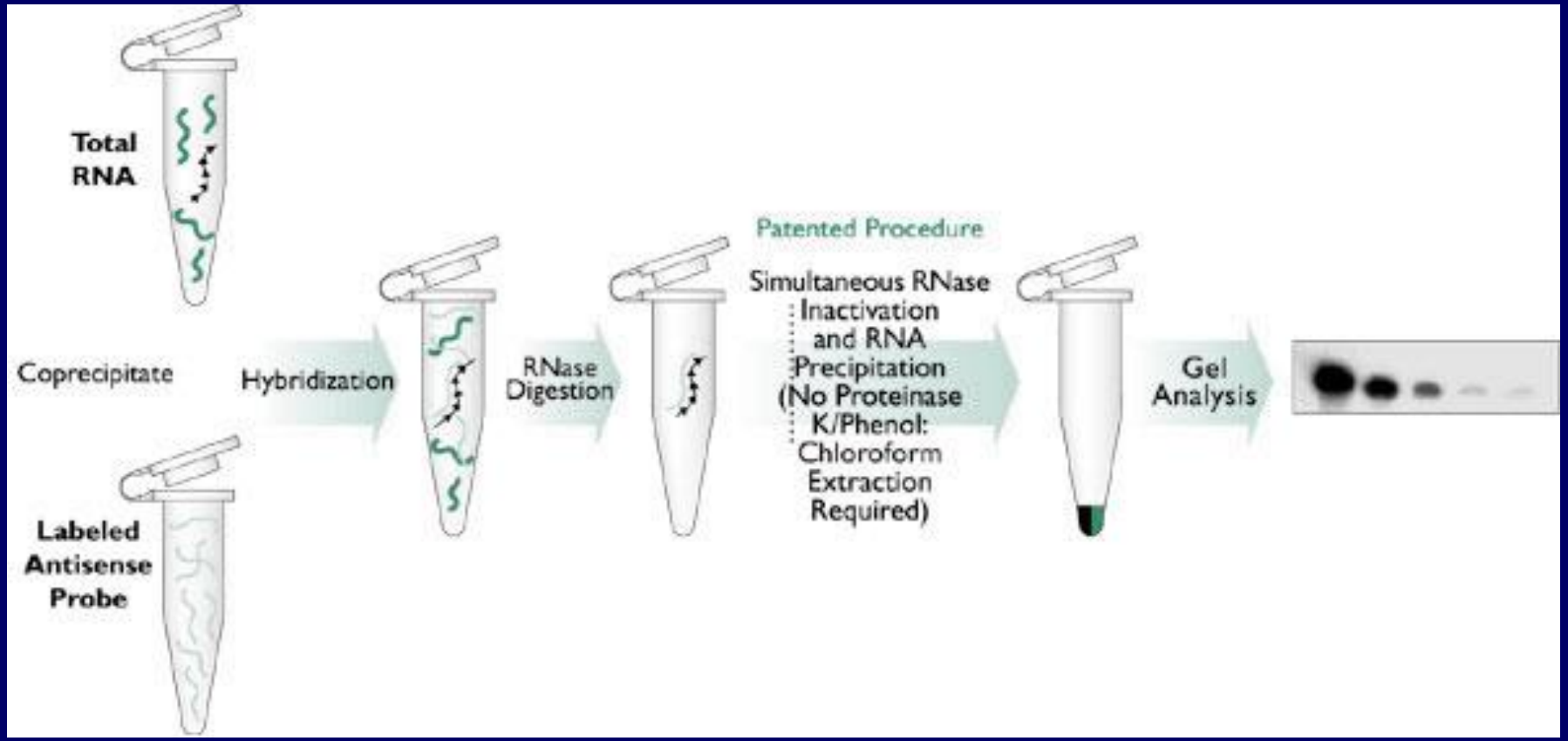


Northern blot

GLP-1 expression is increased when mice fed high fat diet.



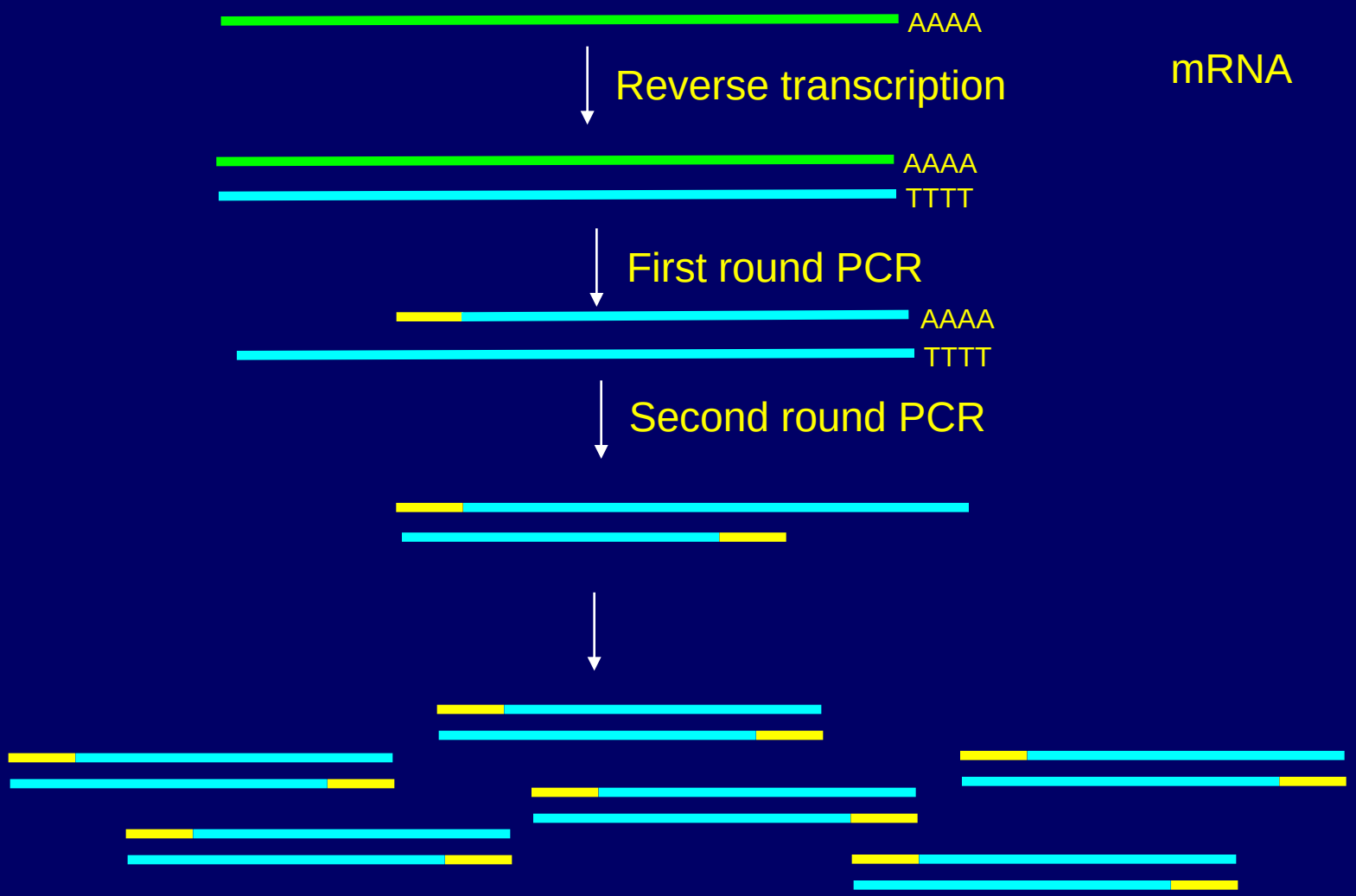
Rnase protection assay



Characteristics

- Robust
- Same filter can be used several times
- Cheap
- Simple
- Low sensitivity
  - Large amount of sample
- Time consuming
- Not amenable to large numbers of samples

Reverse transcriptase - PCR

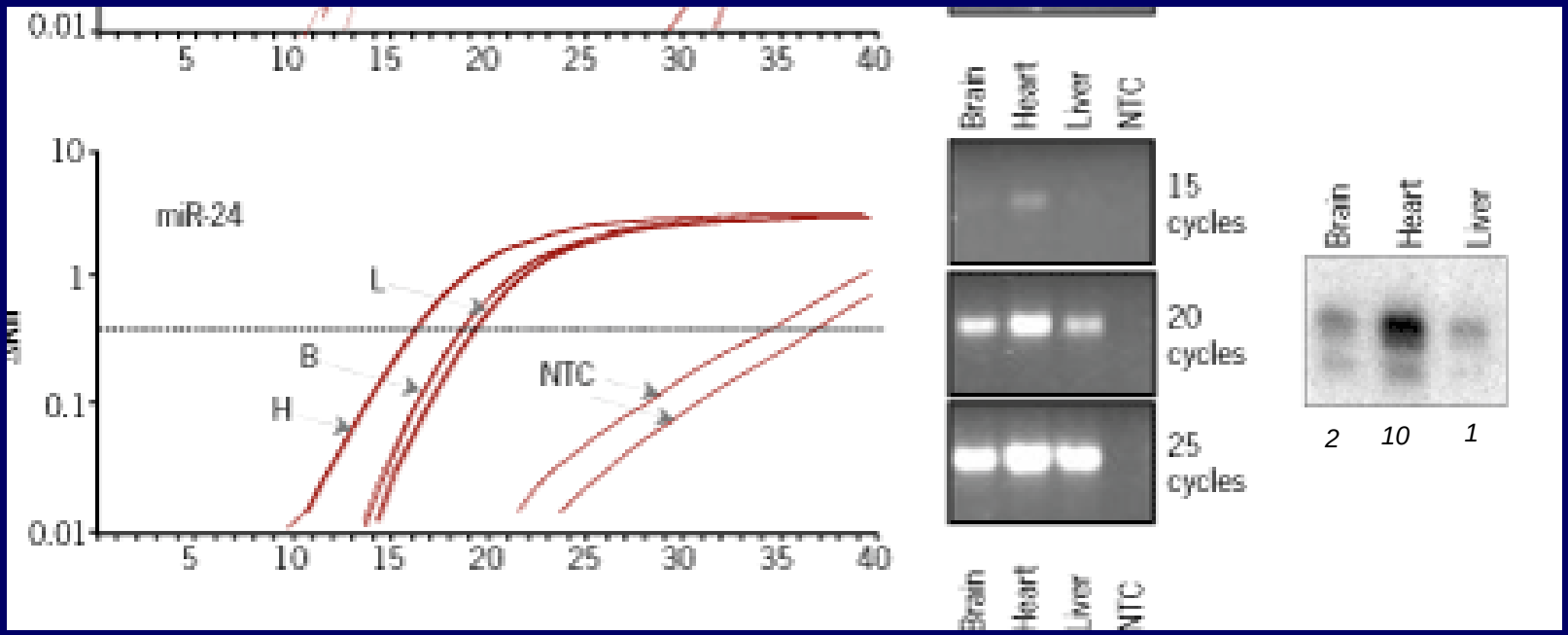


http://highered.mcgraw-hill.com/sites/0072437316/student_view0/chapter16/animations.html#

PCR

- Rapidly confirm presence of specific message
- Not quantitative
- Attempts to make quantitative
  - Real time PCR

PCR is not Quantitative



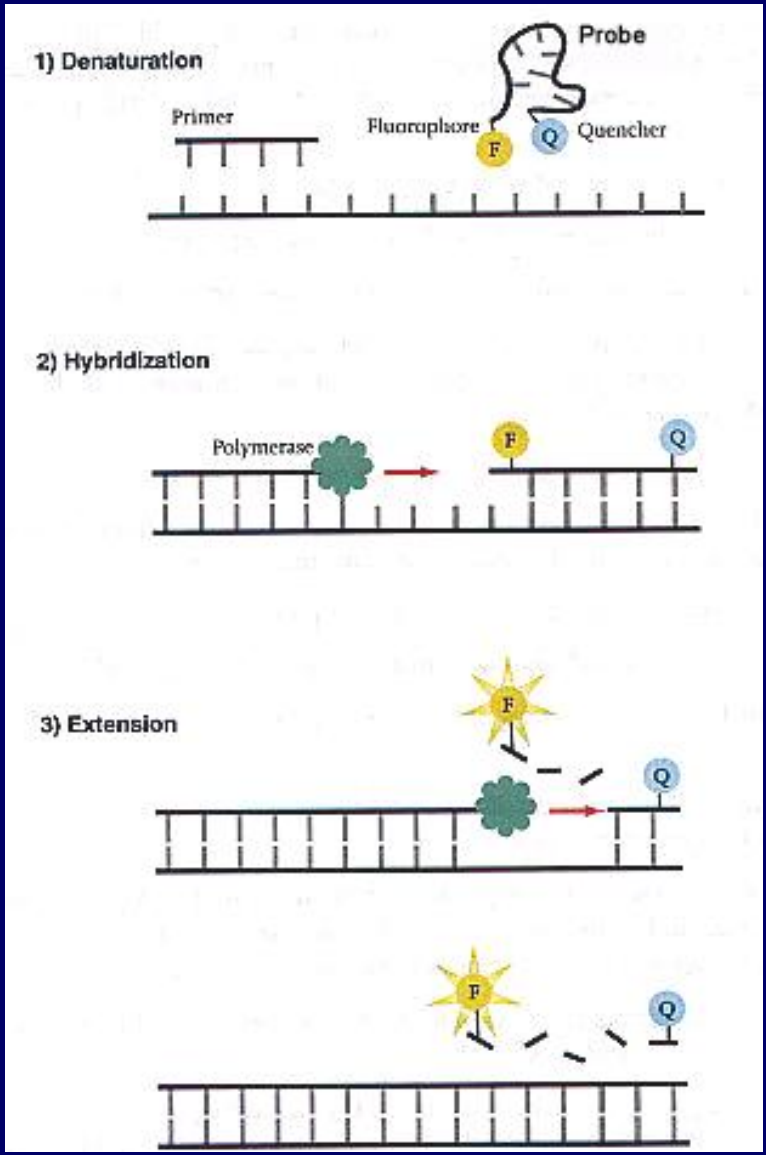
Real time PCR

- PCR in the presence of a fluorescent reporter
- Need to include control throughout the process

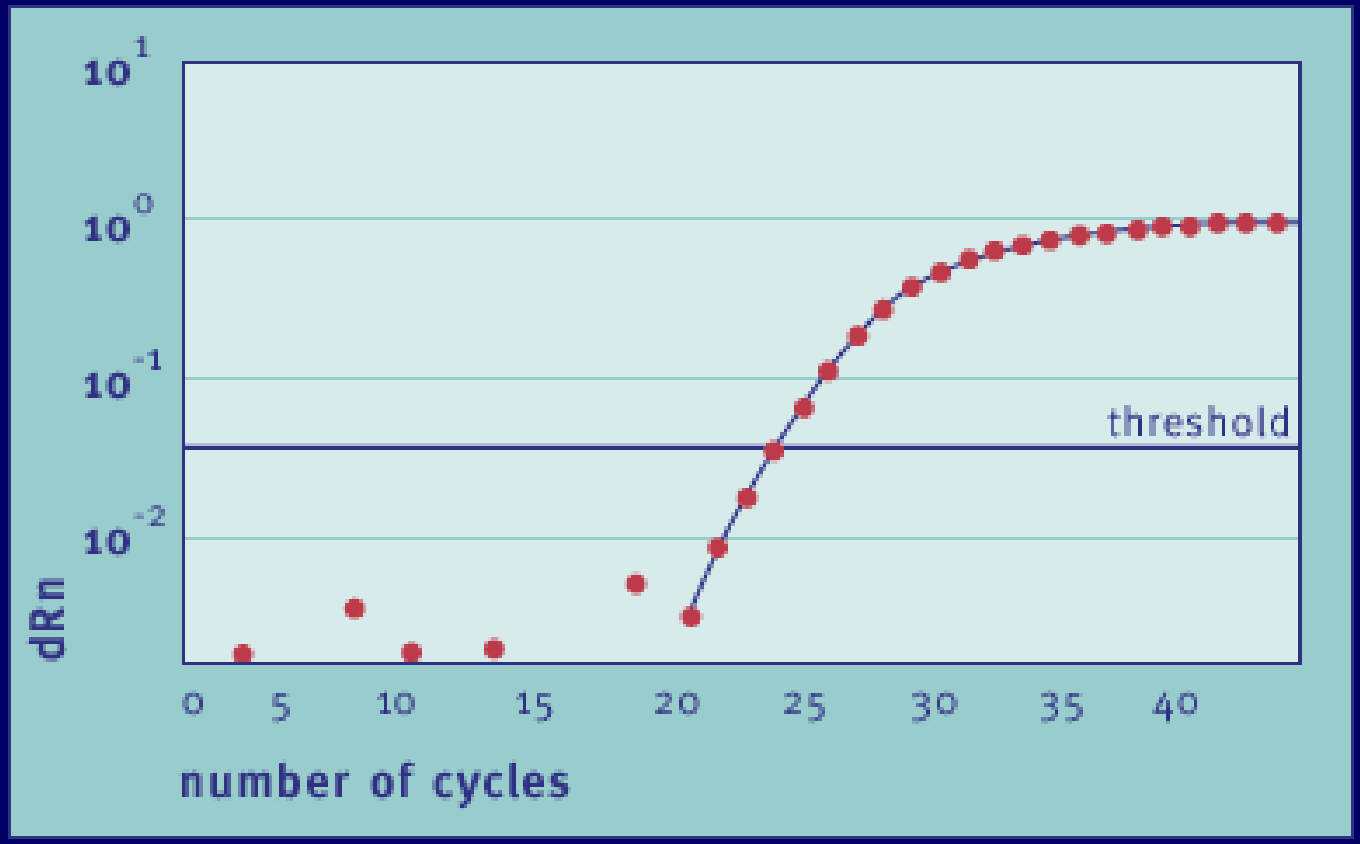
Taq1.mp4

http://www.lifetechnologies.com/featured-solutions/pcr/real-time-pcr-animation.html

Real time PCR (Taqman)



TaqMan Results

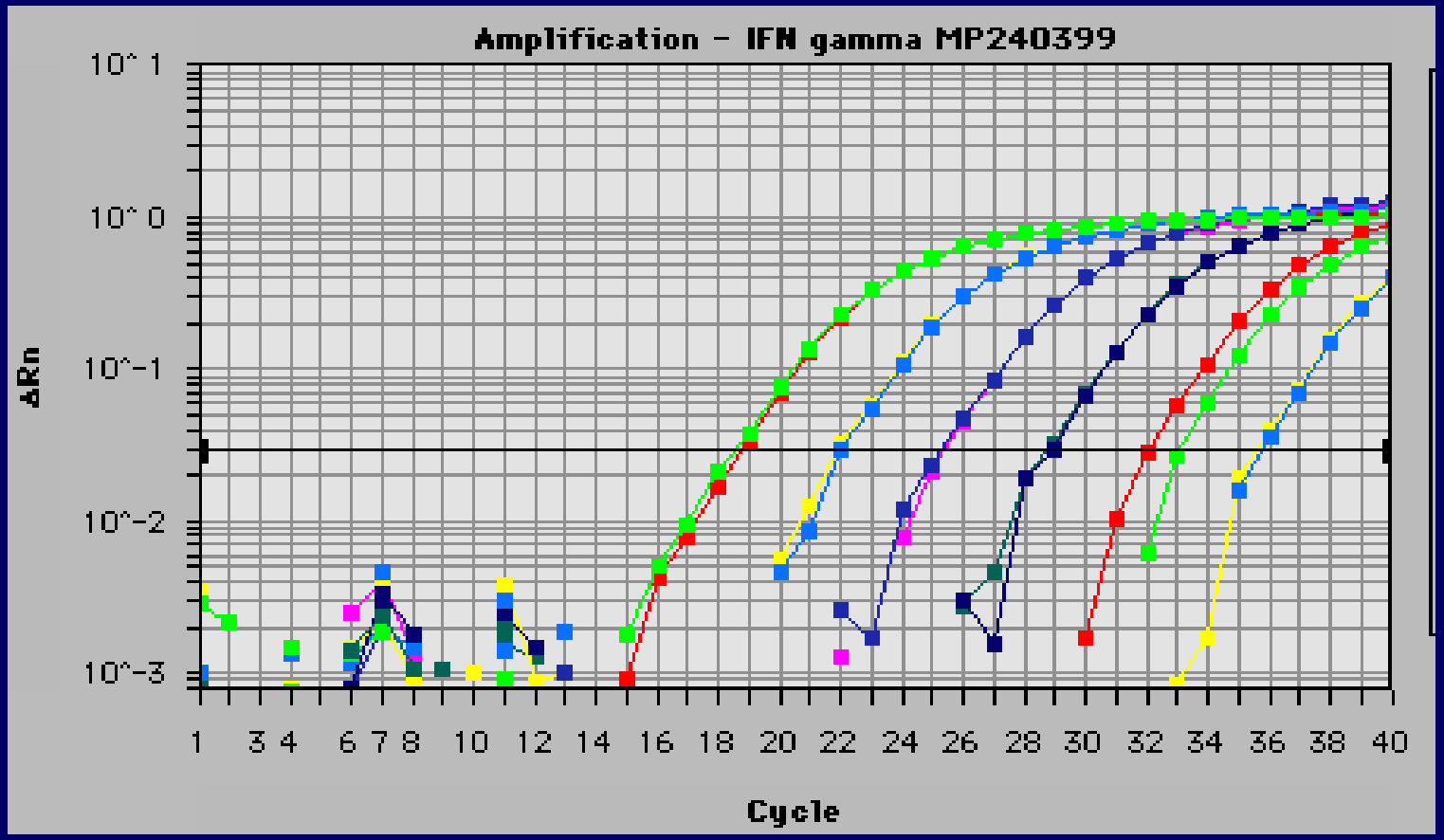


Normal PCR measured here

Real time PCR measured here

Curve shifts to the right as the amount of RNA increases

TaqMan Results - reproducible



Characteristics

- Very sensitive
- Rapid (once established)
- Easily scalable
- Expensive
- Tricky
- Needs extensive optimisation
- ? Robustness
- Destructive of sample

Comparison

- Sensitivity- qPCR>>northern
- Cost - qPCR>northern
- Difficulty - qPCR>northern
- Speed - qPCR>>northern
- Robustness northern>PCR

Protein quantification

Protein quantification

- RIA
- Elisa
- Western

Western Blotting

Western Blot

- Measure protein production
- Measure phosphorylation status

Principles of protein detection

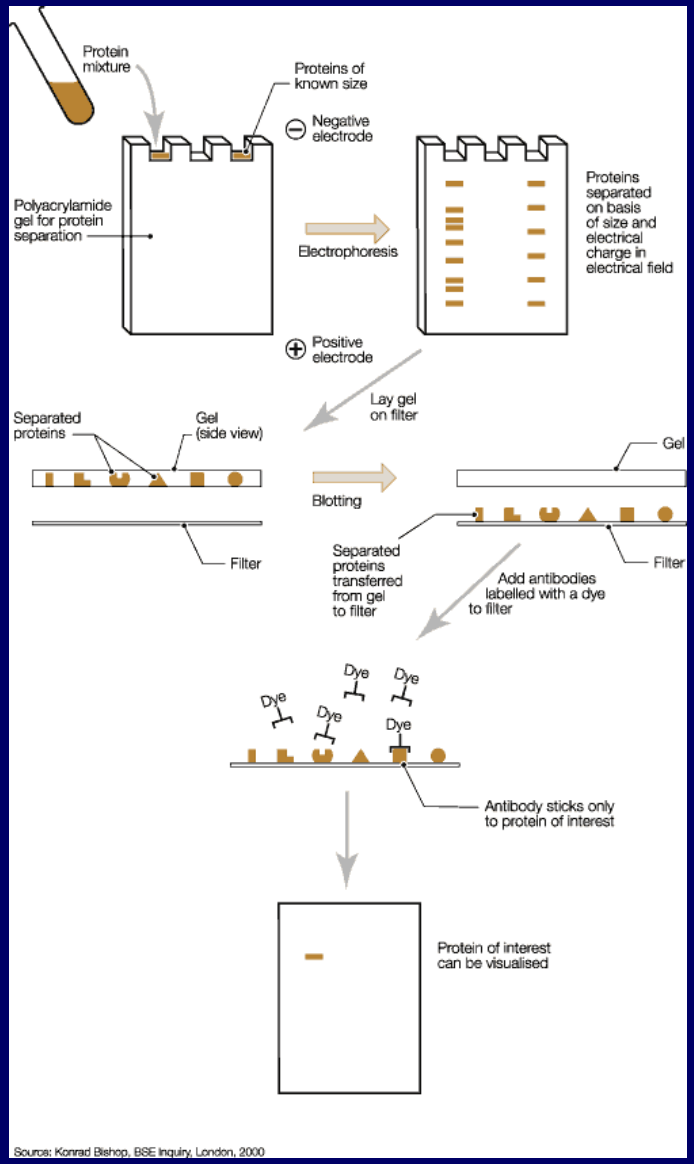
- Uses antibodies
  - Monoclonal (hybridoma (b-cell/ myoloma fused) cell line single antibody single epitope)
  - Polyclonal (in host eg sheep, rabbit, goat multiple antibodies, multiple epitopes)
- Specificity and sensitivity very much antibody dependent

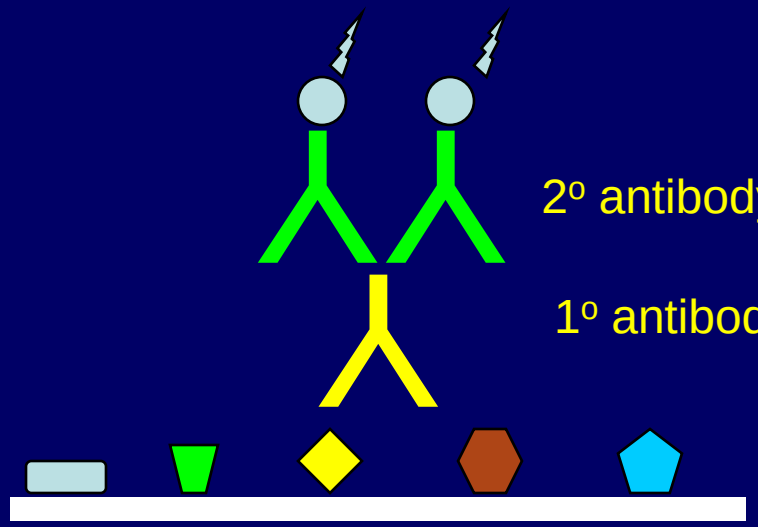
Basic principles

- Treat cells/animals (eg insulin treated mice) appropriate controls
- Protein extracted from tissue of interest cell line
- Proteins separated by polyacrylamide gel electrophoresis
- Electro-transferred to membrane
- Detected using specific antibodies
- Signal- Chemiluminescence, radioactive

immunoblotting.mov

Basic principles

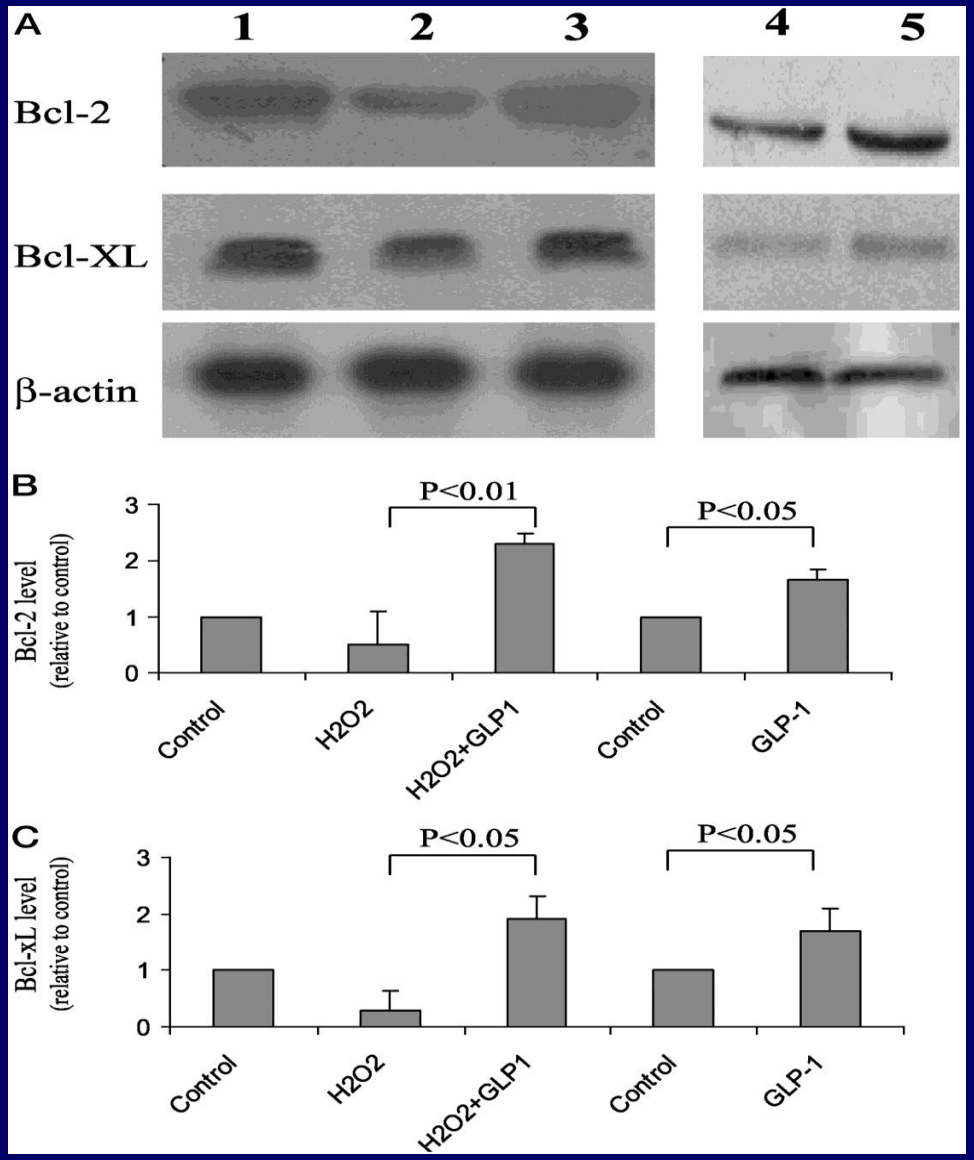




Protein measurement

- Measure changes in production

GLP-1 increases the expression of the antiapoptotic proteins Bcl-2 and Bcl-xL.



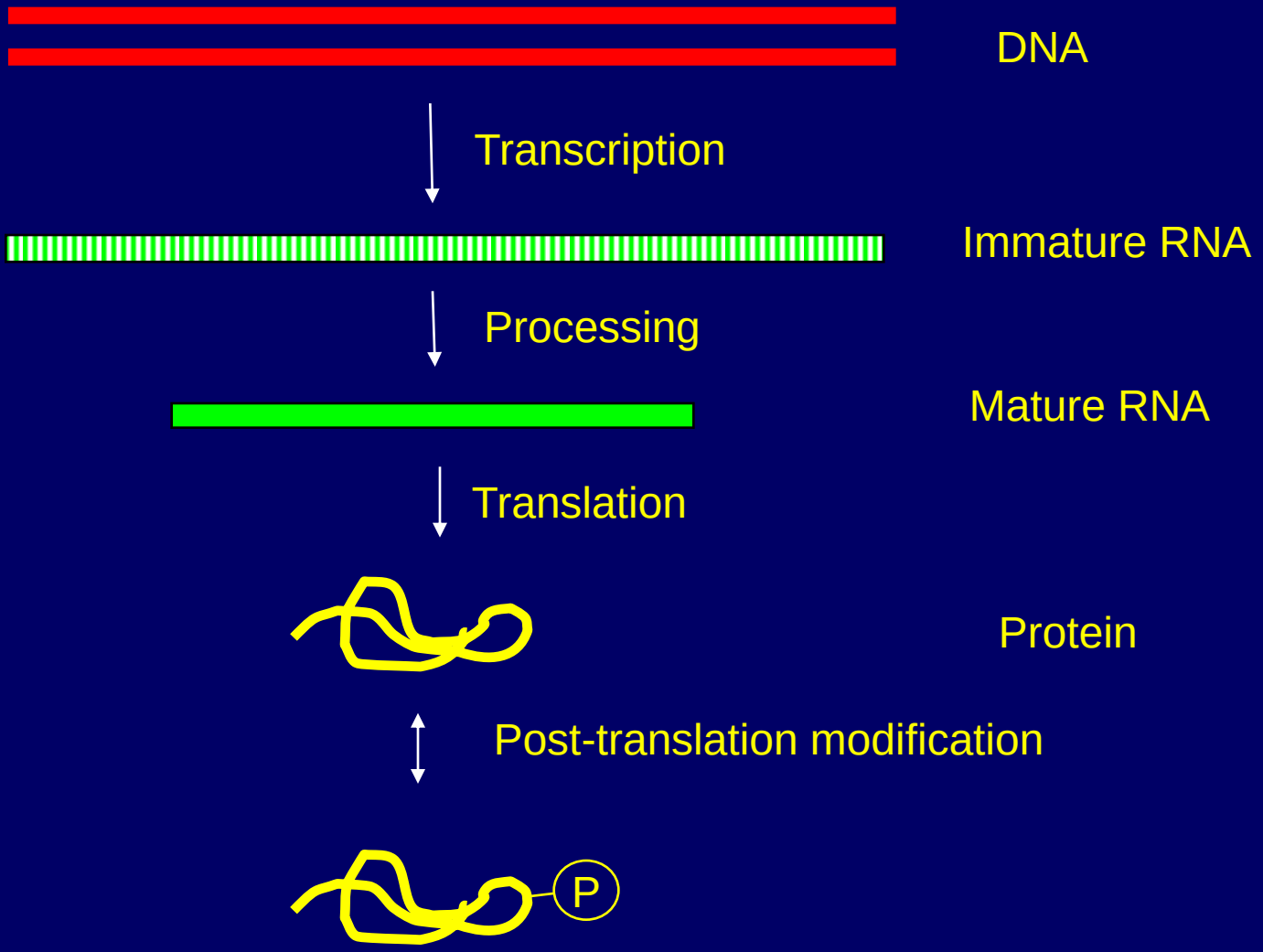
Hui H et al. Endocrinology 2003;144:1444-1455

Endocrinology

Characteristics

- Very sensitive
- Robust
- Fairly straight forward (protein specific)
- Very much antibody dependent
- Can be expensive

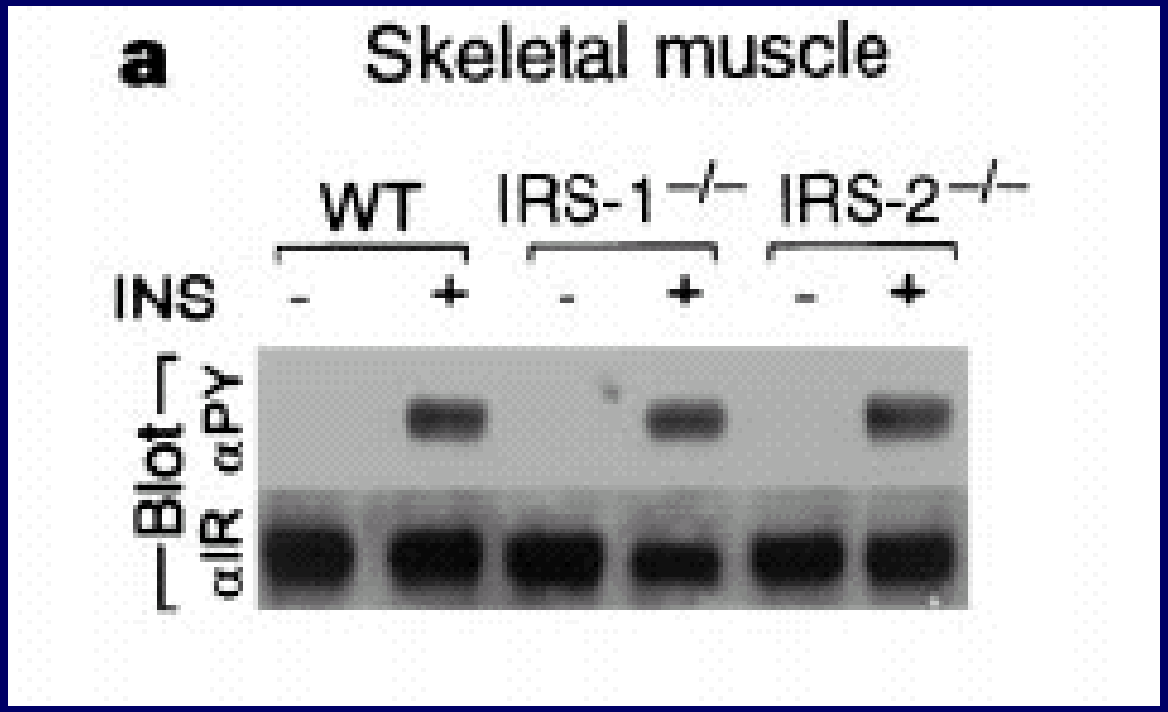
Quantification



Measurement of phosphorylation status

- Phosphospecific antibody
- Can be protein specific (insulin receptor)
- Can be non-specific (p-Tyr)
- Main use examining intracellular signalling

Measurement of phosphorylation status



Phosphorylation of the insulin receptor following insulin treatment

Characteristics

- Very sensitive
- Robust
- Very much antibody dependent
- Multiple phosphorylation sites

Limitations of all

- Give snapshot
- Changes in RNA not necessarily linked to protein
- Changes in protein not necessarily linked to changes in activity/action

Why measure RNA

- Simpler (easily detected)
- Cheaper
- For stored proteins (eg hormones) may be more meaningful than protein
- Often changes in RNA levels reflect changes in protein

Why measure protein

- Generally its what we are interested in
- Sometimes changes in protein not matched by changes in message
- Phosphorylation status is necessary for intracellular signalling (kinase mediated)

Generally

- Measure both
  - Much more information
  - Can give dynamic information

Localisation

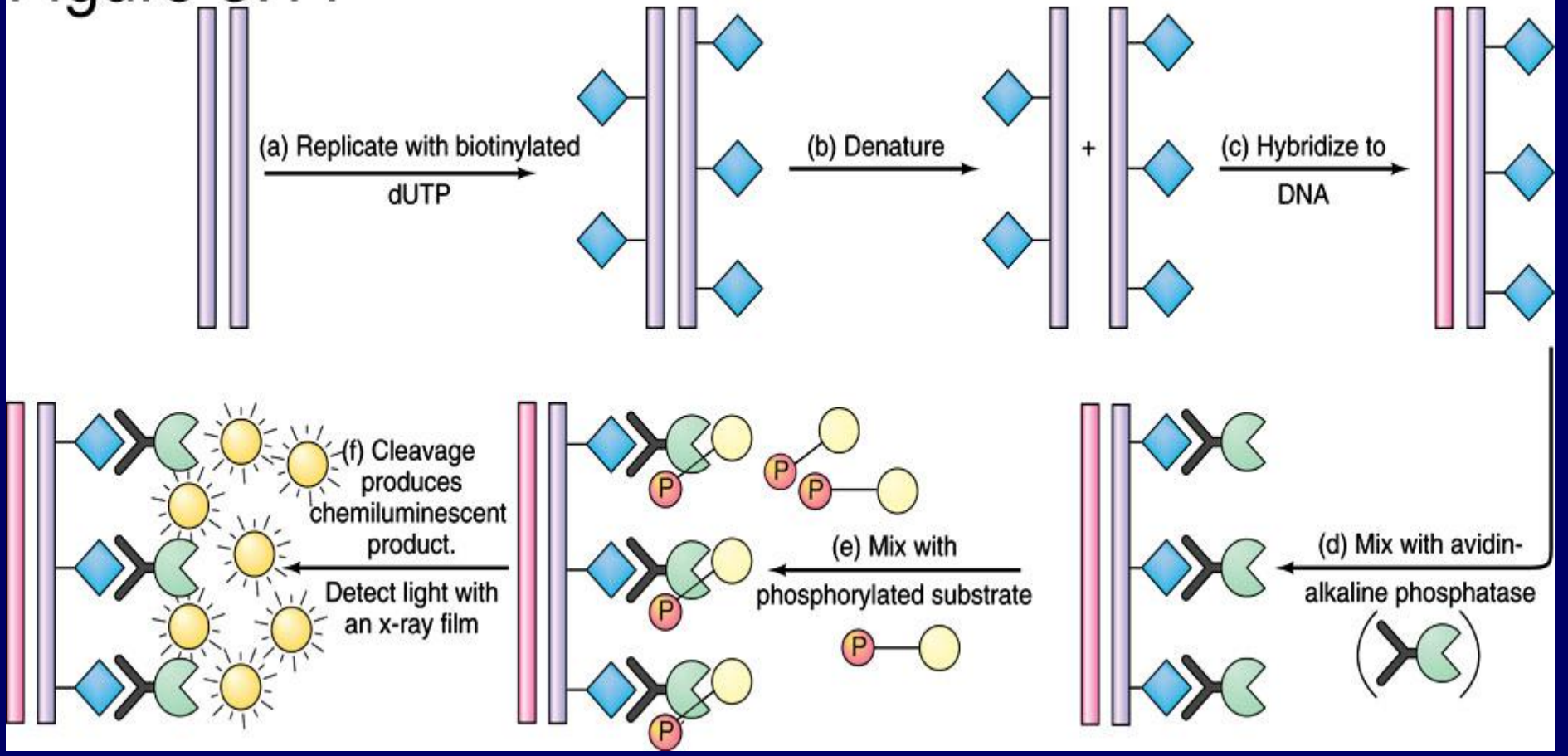
Localisation

- RNA
  - *in situ* hybridisation
- Protein
  - immunocytochemistry (ICC)
  - Immunohistochemistry (IHC)

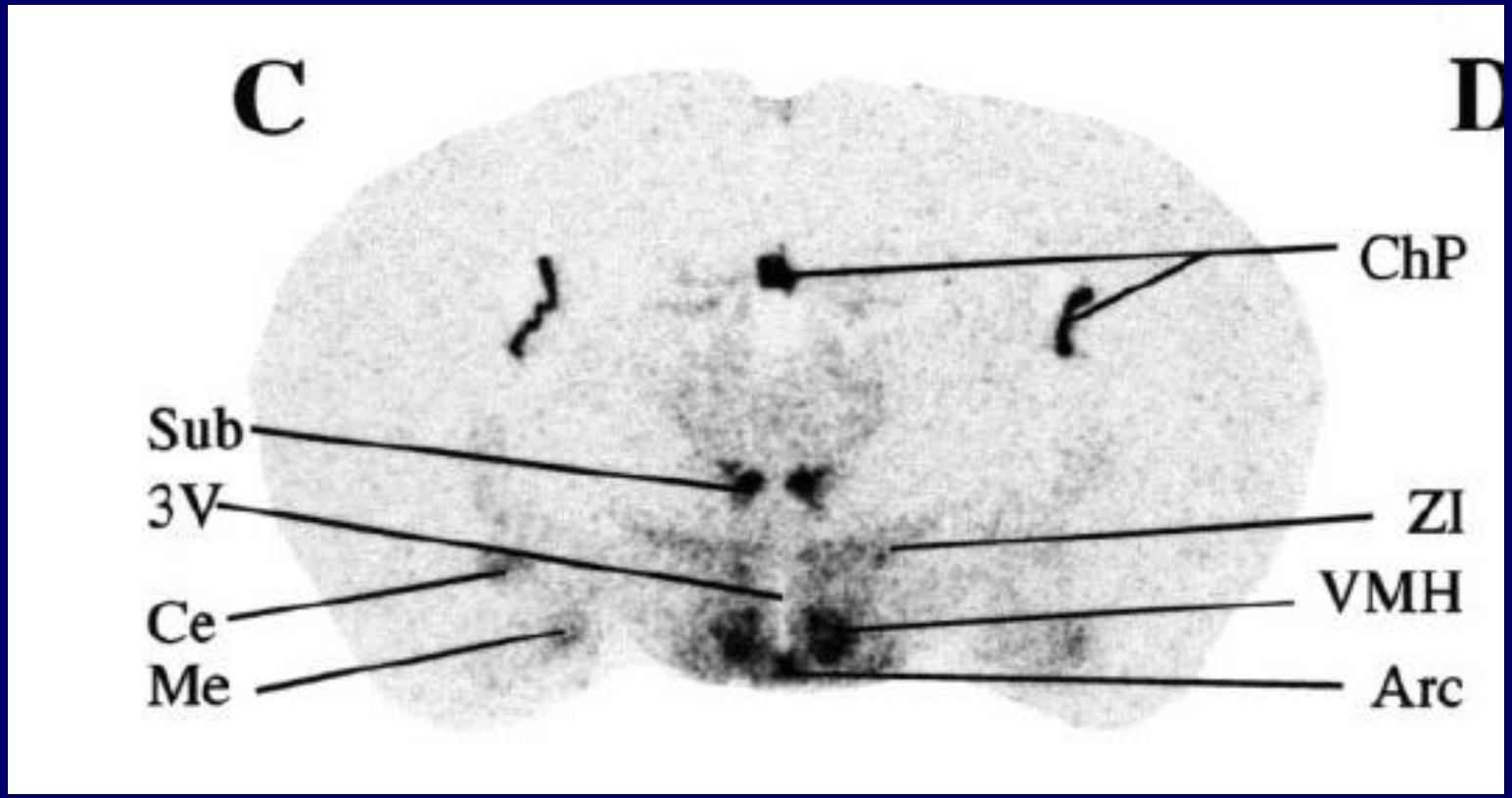
In situ hybridisation

- Tissue collected and frozen -150^{o}C
- Thin sections cut (12µm)
- Tissue lightly fixed
- Complementary labelled DNA or RNA probe made.
- Non specific binding blocked
- Probe hybridised to tissue section
- Non specific bound probe removed by washing

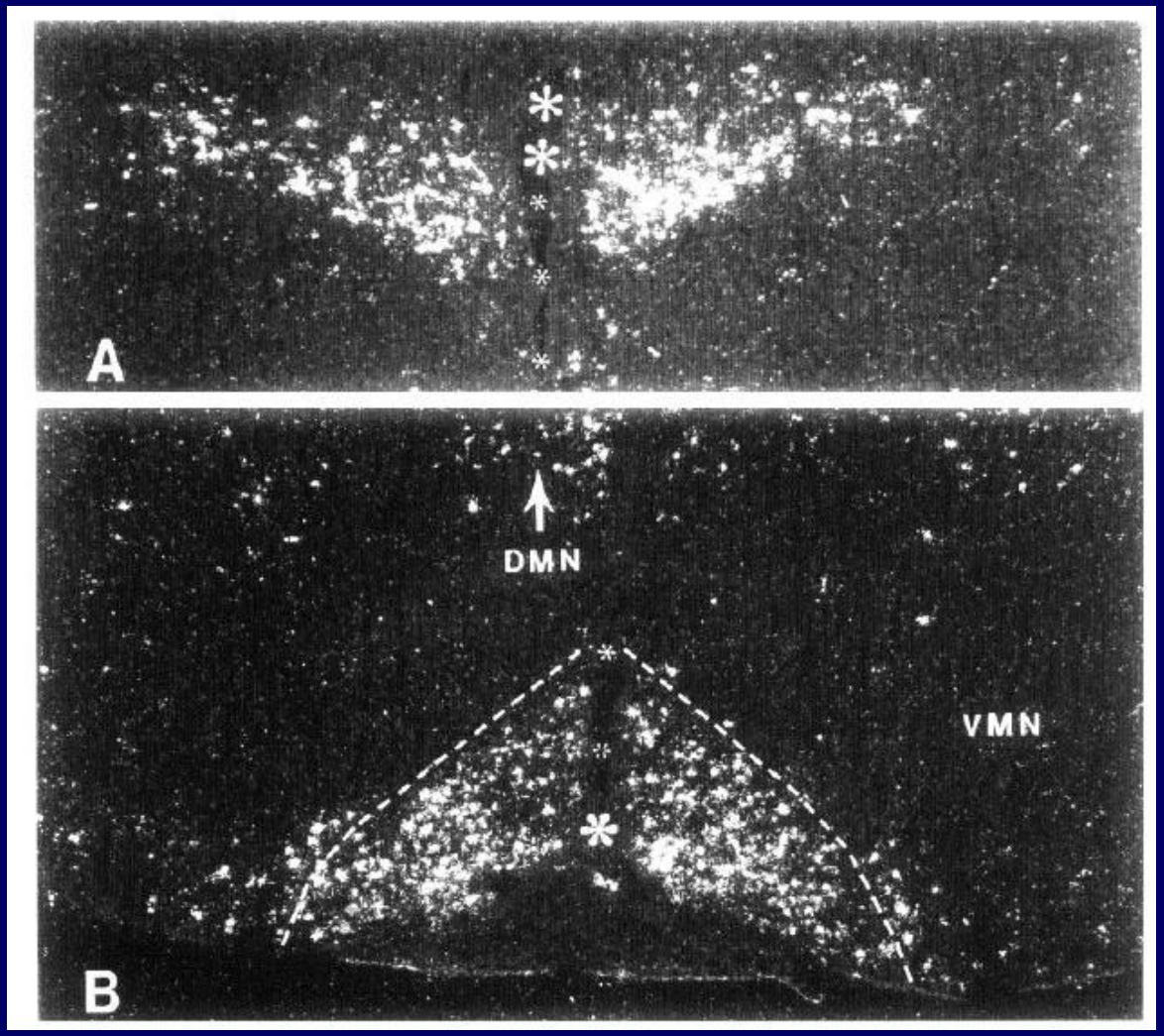
Figure 5.11



in situ hybridisation

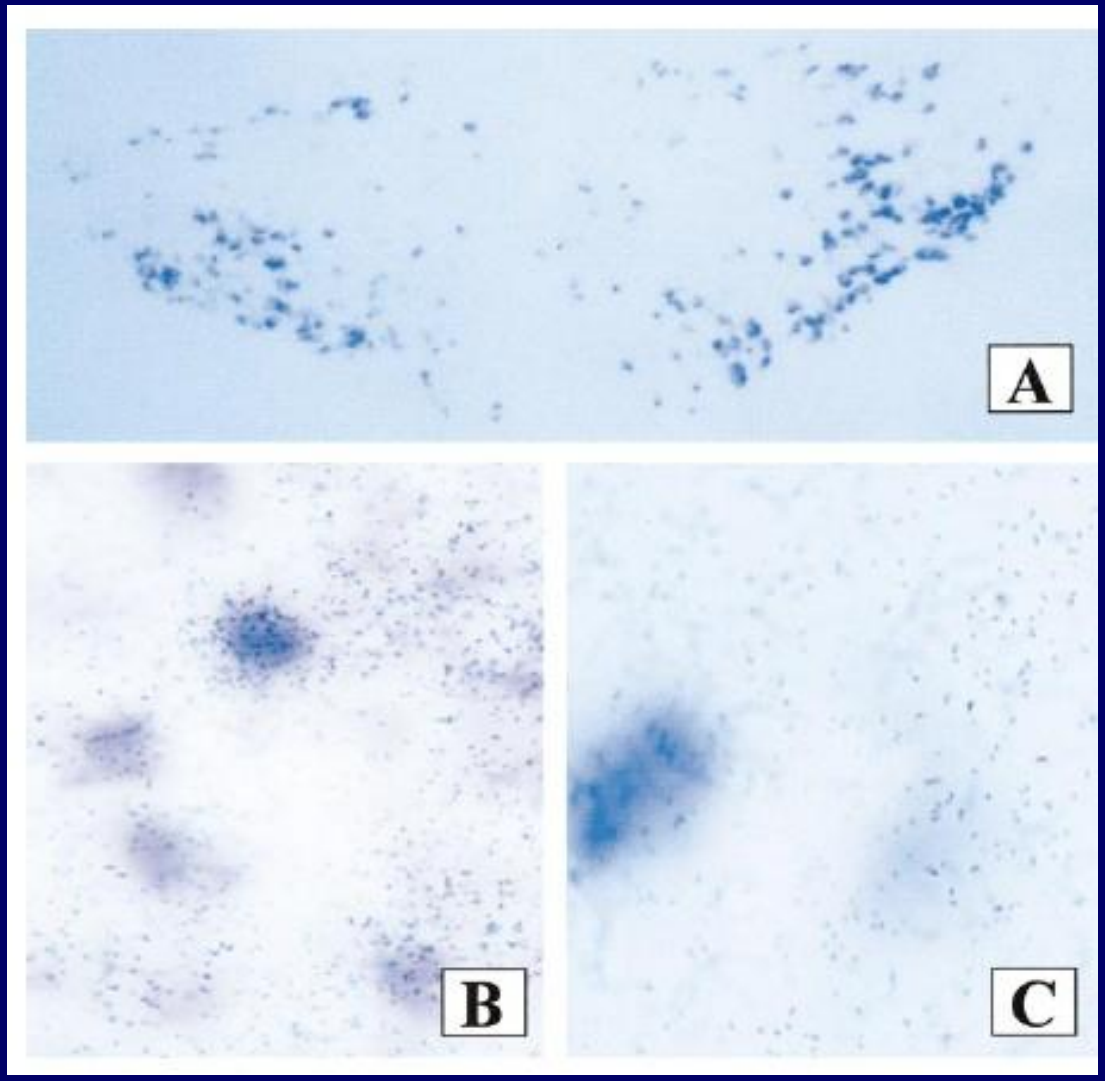


in situ hybridisation



GLP-1 receptor in hypothalamus

Double labelled in situ hybridisation



GLP-1 receptor in PVN co-localised with AVP.

Characteristics

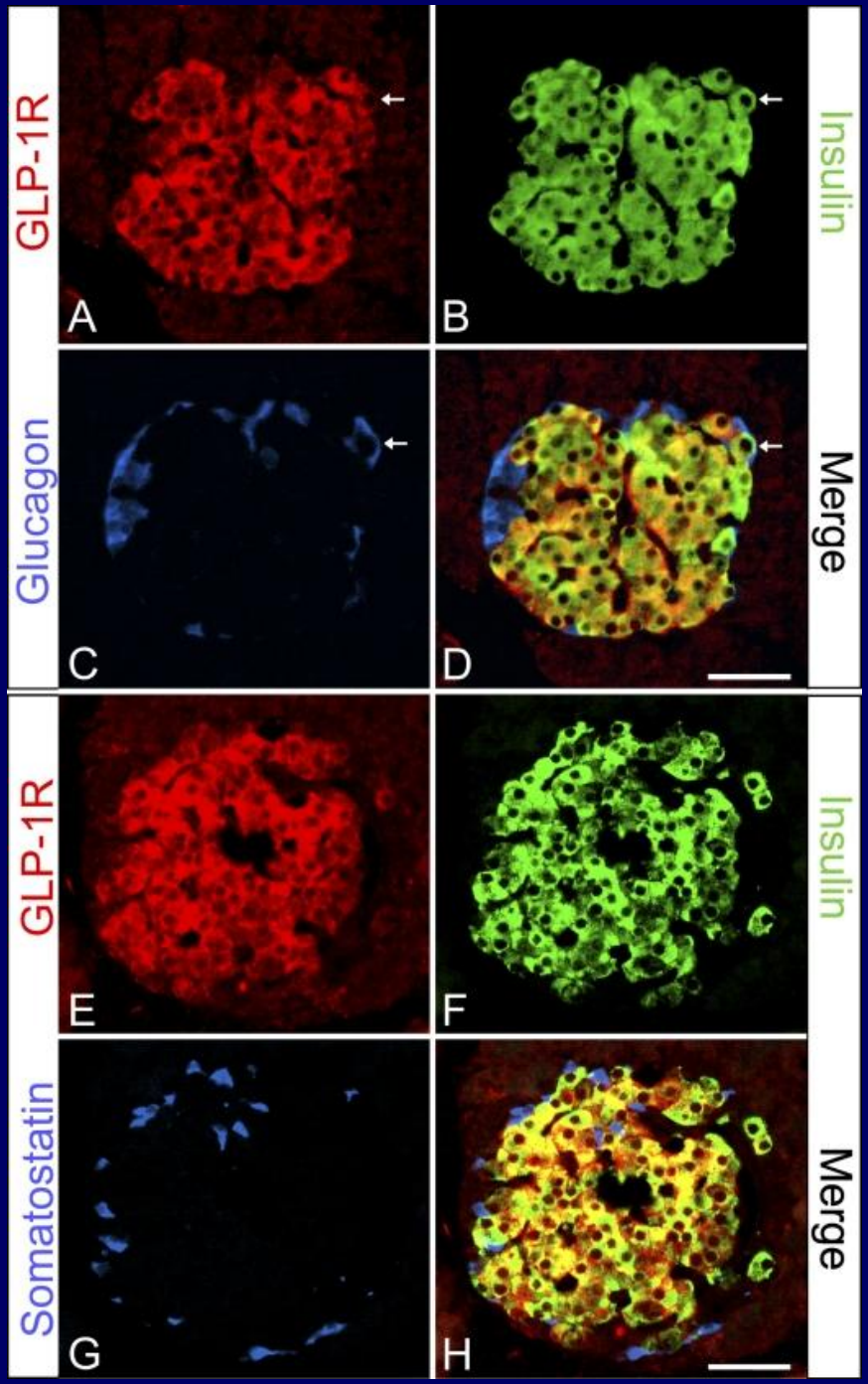
- Very good localisation of RNA
- Can be semi-quantitative
- RNA is localised in cytoplasm
- Can be used for co-localisation studies
- Sensitive
- Robust
- Time consuming (use small n numbers)

IHC

- Very flexible technique
  - Localisation of protein
  - Co-localisation
  - Cell activation
  - Cell characterisation
  - Diagnosis

IHC

- Tissue fixed
  - embedded in paraffin wax very thin sections >5um mounted on glass slides
  - frozen thick sections 50um
- Block non specific binding
- Incubated with appropriate antibody
- Sections extensively washed
- Detection system applied, enzyme fluorescence)



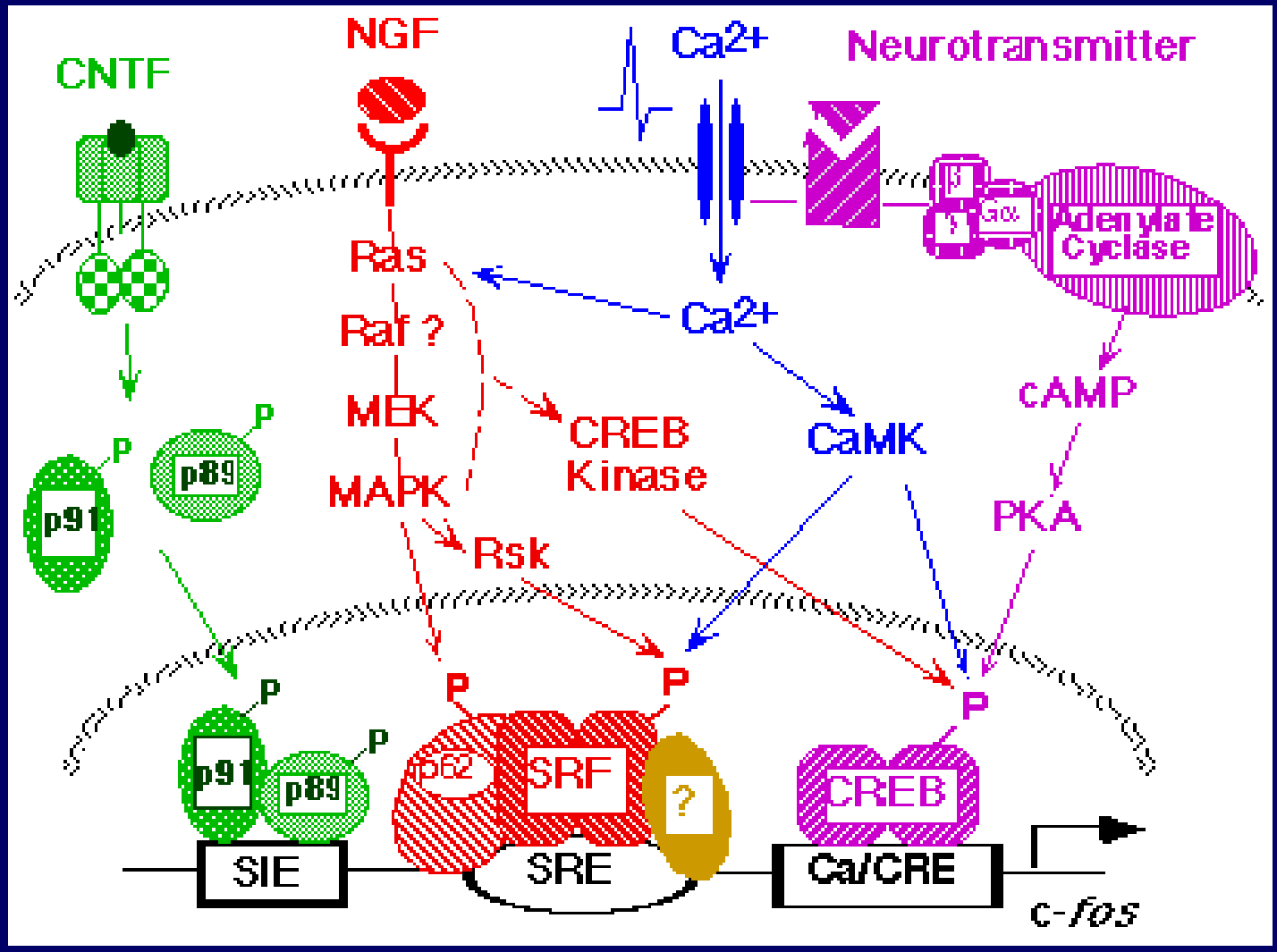
IHC (co-localisation)

Characteristics

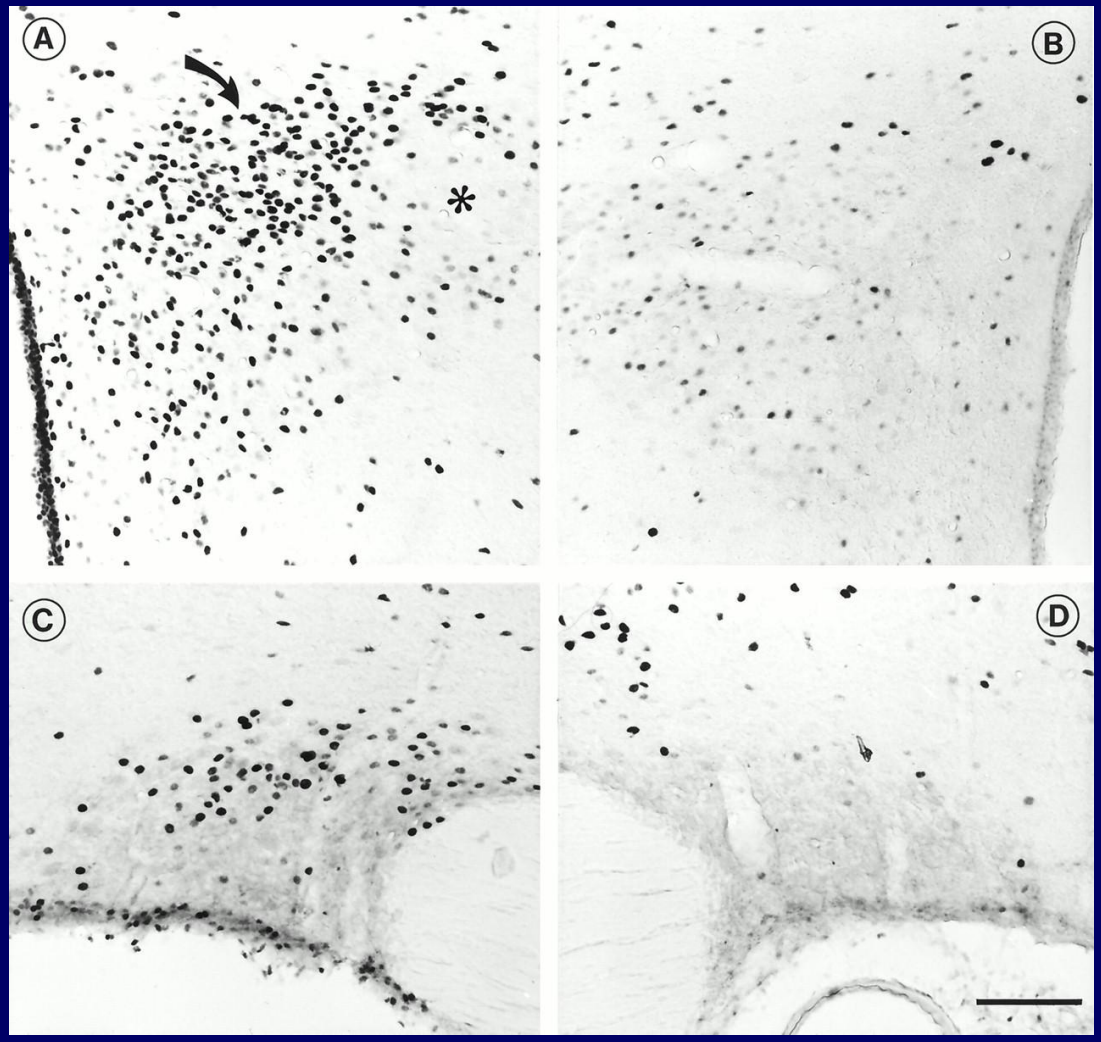
- Very antibody dependent
- Time consuming
- Proteins expressed in same cell can be spatially very distant (neurones)
- Can get non-specific staining

c-fos IHC

- c-fos immediate early gene
- Often expressed by activated neurones
- Can be used to identify neurones activated in response to stimulus

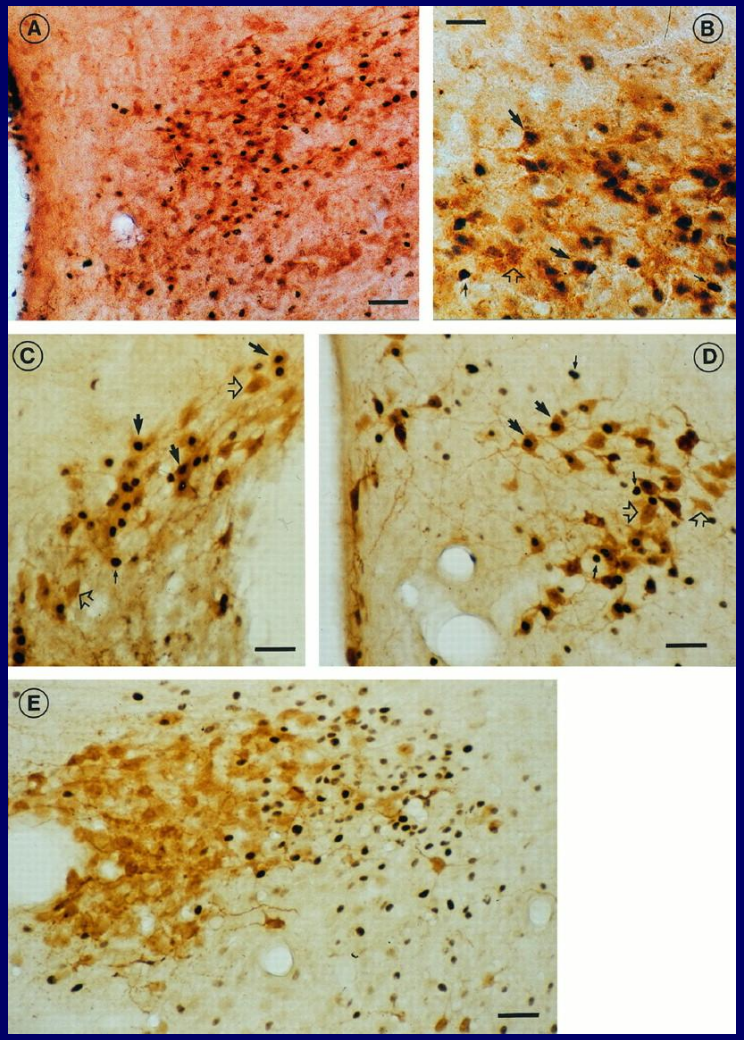

NGF
CNTF
Ca2+
Neurotransmitter
Ras
Raf ?
MEK
MAPK
Rsk
Ca2+
CREB
Kinase
CaMK
cAMP
PKA
Adenylate
Cyclase
Gα
P
p91
p89
p62
SRF
?
SIE
SRE
CREB
Ca/CRE
c-fos

Photomicrographs demonstrating c-fos-immunoreactive nuclei in the hypothalamic paraventricular (A and B) and supraoptic nuclei (C and D) 90 min after icv injection of GLP-1 alone (A and C) or GLP-1 preceded by a dose of exendin-(9–39) given 10 min earlier (...



Larsen P J et al. Endocrinology 1997;138:4445-4455

GLP-1 induced c-fos expression in neurochemically identified neurons in the PVN (A, B, D, and E) and SON (C), as shown in photomicrographs of sections double labeled for c-fos and CRH (A and B), for c-fos and OT (C and D), or for c-fos and AVP (E).



Endocrinology

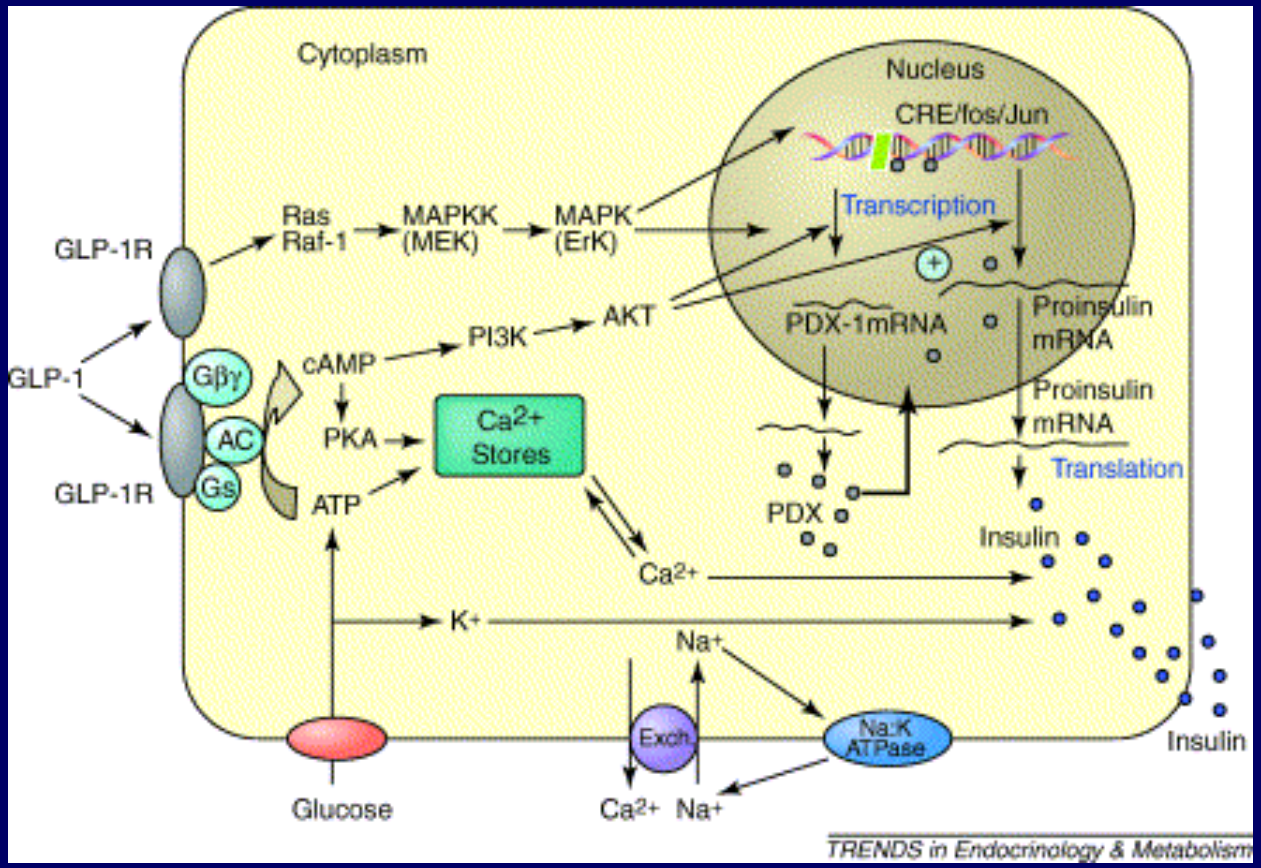
Larsen P J et al. Endocrinology 1997;138:4445-4455

Characteristics

- Time consuming
- Only see where you look
- Can be non specific
- Not all activated neurones express c-fos
- Inactivated neurones often do not express c-fos

Relevance

GLP-1 function in β-cell



GLP-1 analogues (exanatide, byetta) fastest growing treatment for type 2 diabetes