Analisi dell’espressione e localizzazione dei trascritti e dei loro prodotti

- Expression
  - Transcripts (Northern blot, Reverse transcription-Real Time PCR)
  - Proteins (Western blot)

- Localisation
  - Transcript (promoter::reporter fusions, *in situ* hybridization)
  - Proteins (protein-reporter fusions, immunolocalisation)

- Expression
  - Transcripts (Northern blot, Reverse transcription-Real Time PCR)
  - Proteins (Western blot)

- Localisation
  - Transcript (promoter::reporter fusions, *in situ* hybridization)
  - Proteins (protein-reporter fusions, immunolocalisation)

Northern blot

This is a technique used to study gene expression.

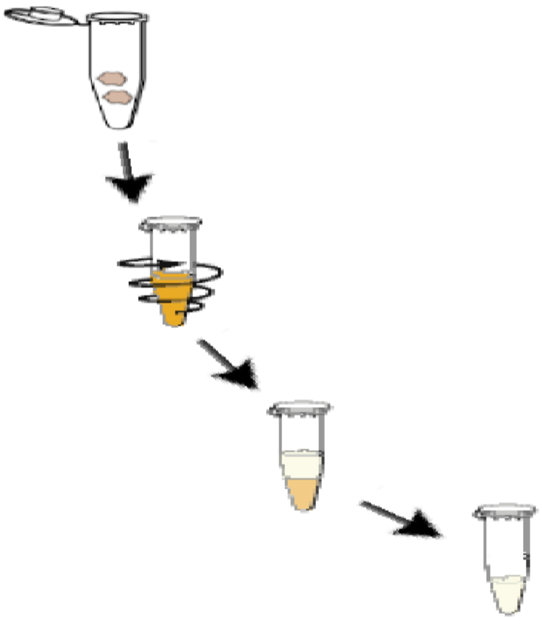
It takes its name from its similarity to the Southern blot technique, named for biologist Edwin Southern.

This technique uses electrophoresis and detection with a hybridization probe.

The gels may be run on either agarose or denaturing polyacrylamide, Formaldehyde is added to the gel and acts as a denaturant to agarose. For polyacrylamide, urea is the denaturant.

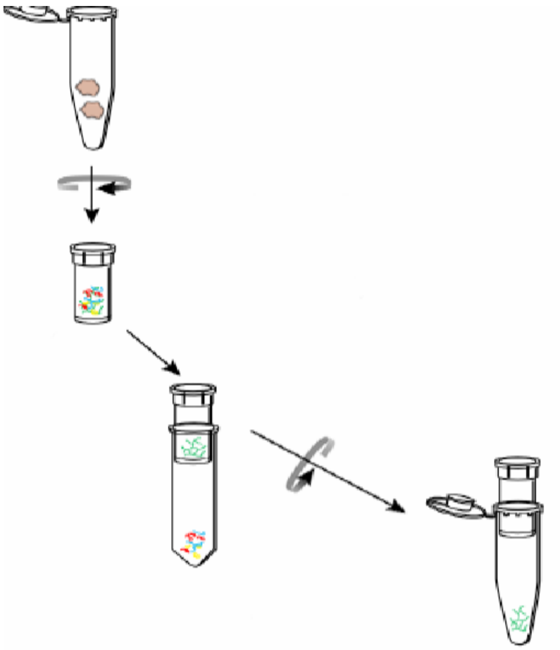
The hybridization probe may be made from DNA or RNA.

Organic RNA Extraction



1. Lyse/homogenize cells
2. Add phenol:chloroform:isoamyl alcohol to lysed sample, and centrifuge
3. Organic phase separates from aqueous phase
   - Organic solvents on bottom
   - Aqueous phase on top (contains total RNA)
   - Cellular debris and genomic DNA appears as a "film" of debris at the interface of the two solutions
4. Remove RNA solution to a clean tube; precipitate RNA and wash with ethanol, then resuspend RNA in water

Affinity Purification of RNA



1. Lyse cells, and spin to remove large particulates/cell debris
2. Apply lysate (containing nucleic acids and cellular contaminants) to column with glass membrane
3. Wash with alcohol to remove contaminants; nucleic acids stick to glass membrane while contaminants wash through. Treat with DNase enzyme to remove contaminating DNA.
4. Apply water to the column; purified RNA washes off the glass and is collected

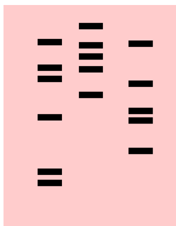
Isolation of PolyA (messenger) RNA

- Only 1-5%
- mRNA molecules have a tail of A's at the 3' end (polyA tail)
- Oligo(dT) probes can be used to purify mRNA from other RNAs
- mRNA can be eluted from oligo(dT) matrix using water or low-salt buffer

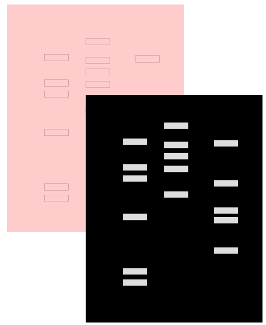
Spectrophotometry

- Sample absorbances are determined on the spectrophotometer at 260nm and 280nm
  - nucleic acid absorb light at 260nm
  - protein absorbs light at 280 nm and 230nm
- A260/A280 ratio (ideally 2) is a measure of RNA purity
- The absorbance at 260 nm is directly proportional to the concentration of RNA.

Schematic of the blotting process



Prepare gel, load RNA and run to separate fragments.



Transfer Nucleic acid tomembrane support and fix.

Label probe and hybridise to nucleic acid on the membrane. Wash to remove non-specific sequences

Expose film to label hybridised to the filter. Develop to reveal specific signal

Reverse transcription-Quantitative Polymerase Chain Reaction

RT-qPCR

Benefits of qPCR

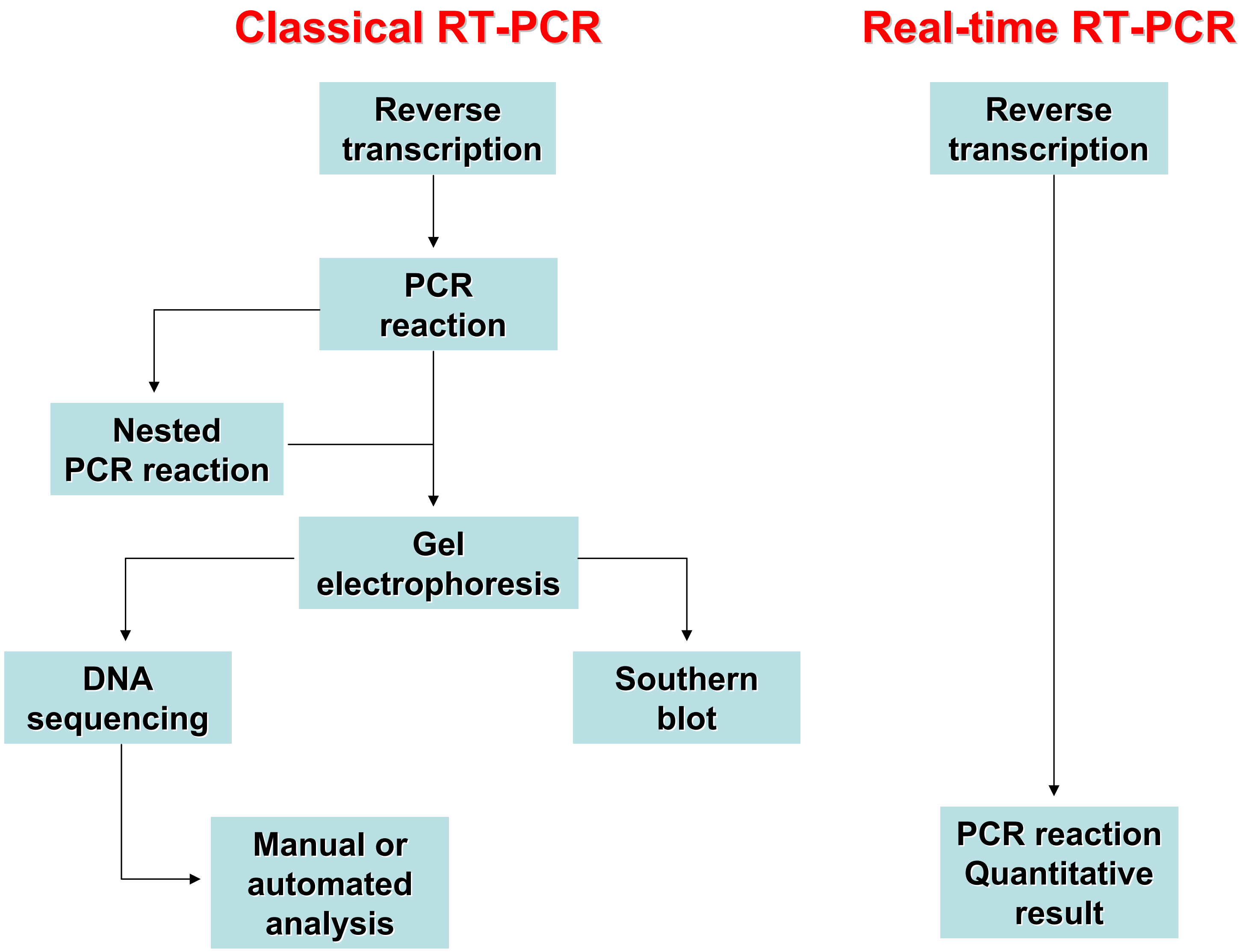
Accurate and reproducible quantification of nucleic acids

Large dynamic range of detection

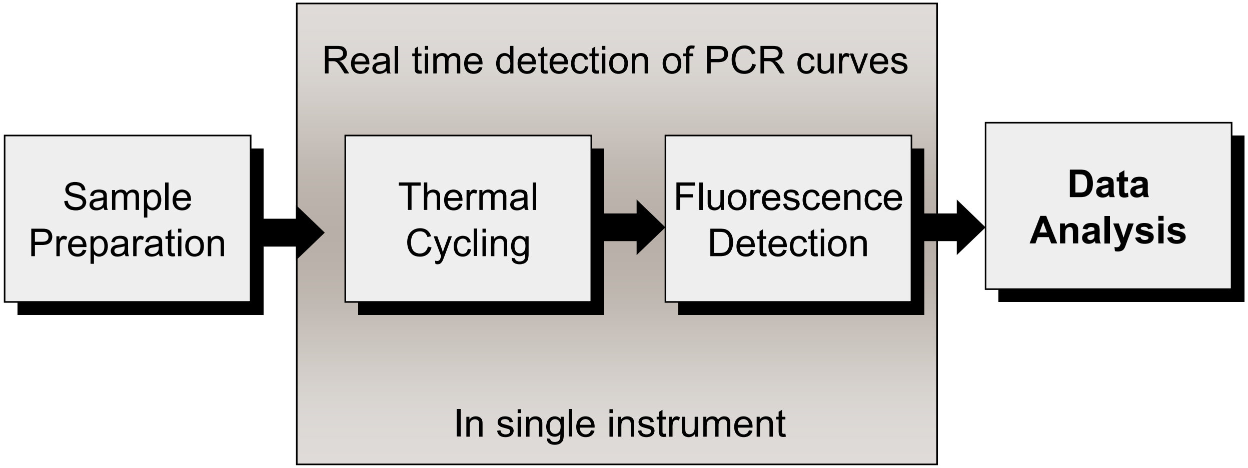
Closed tube chemistry

- No electrophoresis
- No post PCR processing

High throughput sample processing


Classical RT-PCR
Real-time RT-PCR
Reverse transcription
PCR reaction
Nested PCR reaction
Gel electrophoresis
DNA sequencing
Southern blot
Manual or automated analysis
Reverse transcription
PCR reaction Quantitative result

How does RT-qPCR work ?

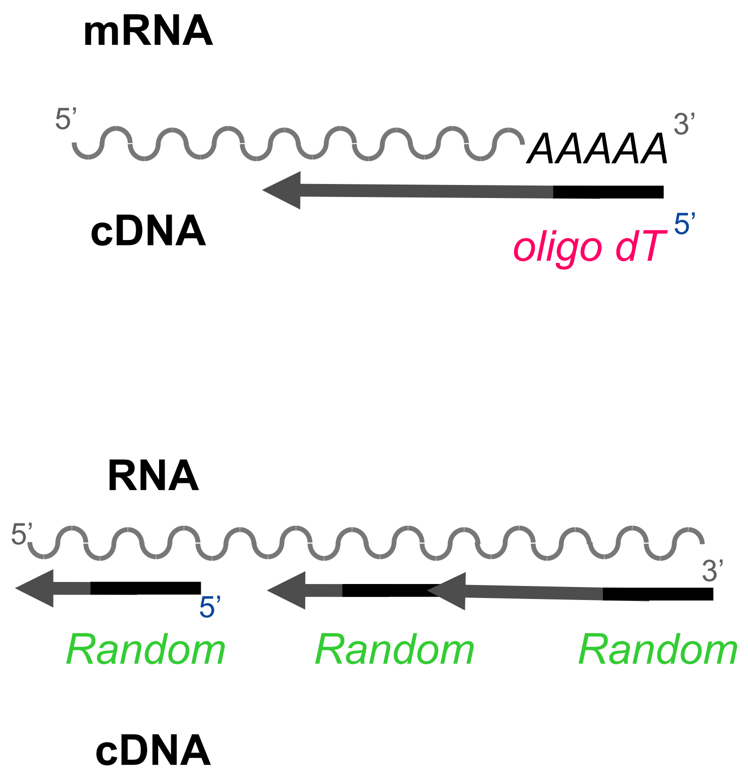


RT

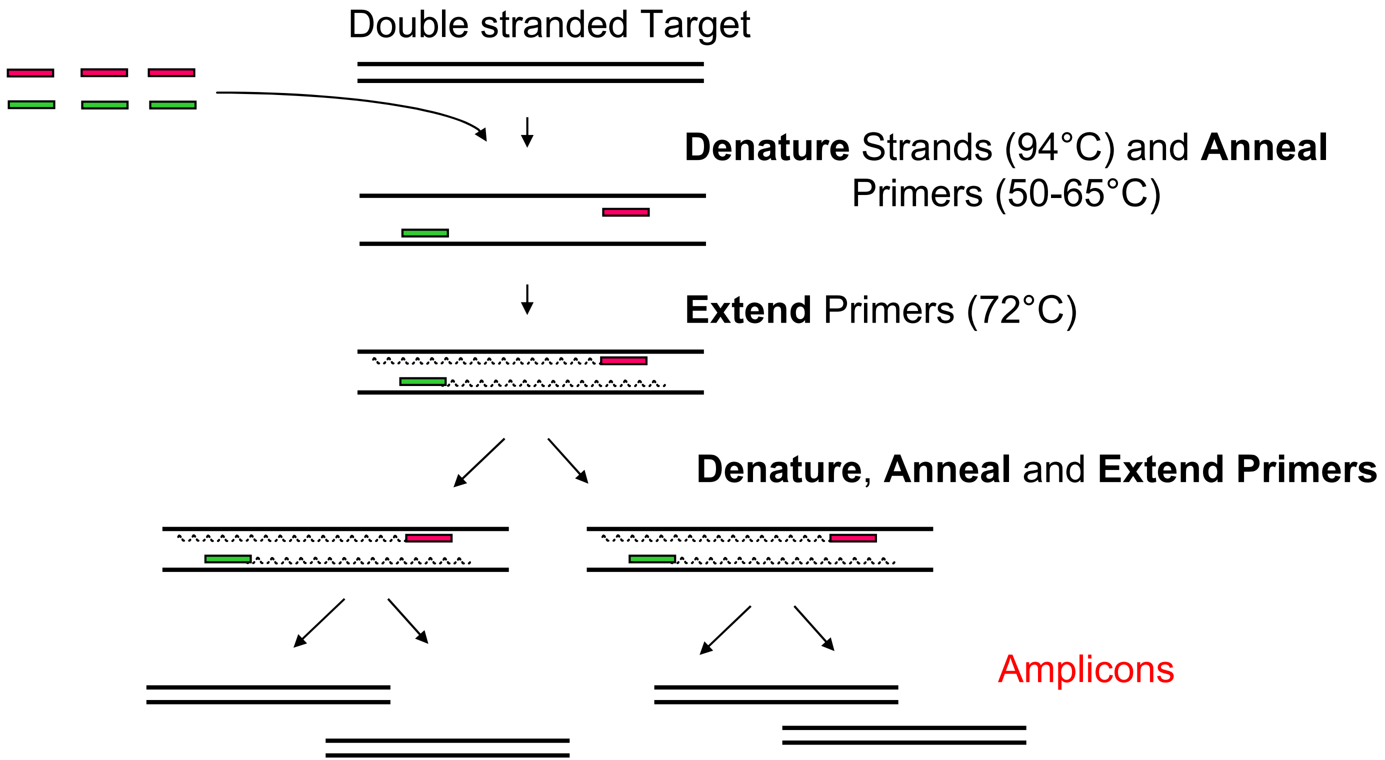
qPCR

Sample preparation

- ✓ Isolate pure RNA (total or polyA$^+$)
- ✓ Treat RNA with DNAse
- ✓ Make cDNA using RNA as template, oligo dT or random hexamers as primers and dNTPs
- ✓ Reverse transcribe



Thermal Cycling: Principle

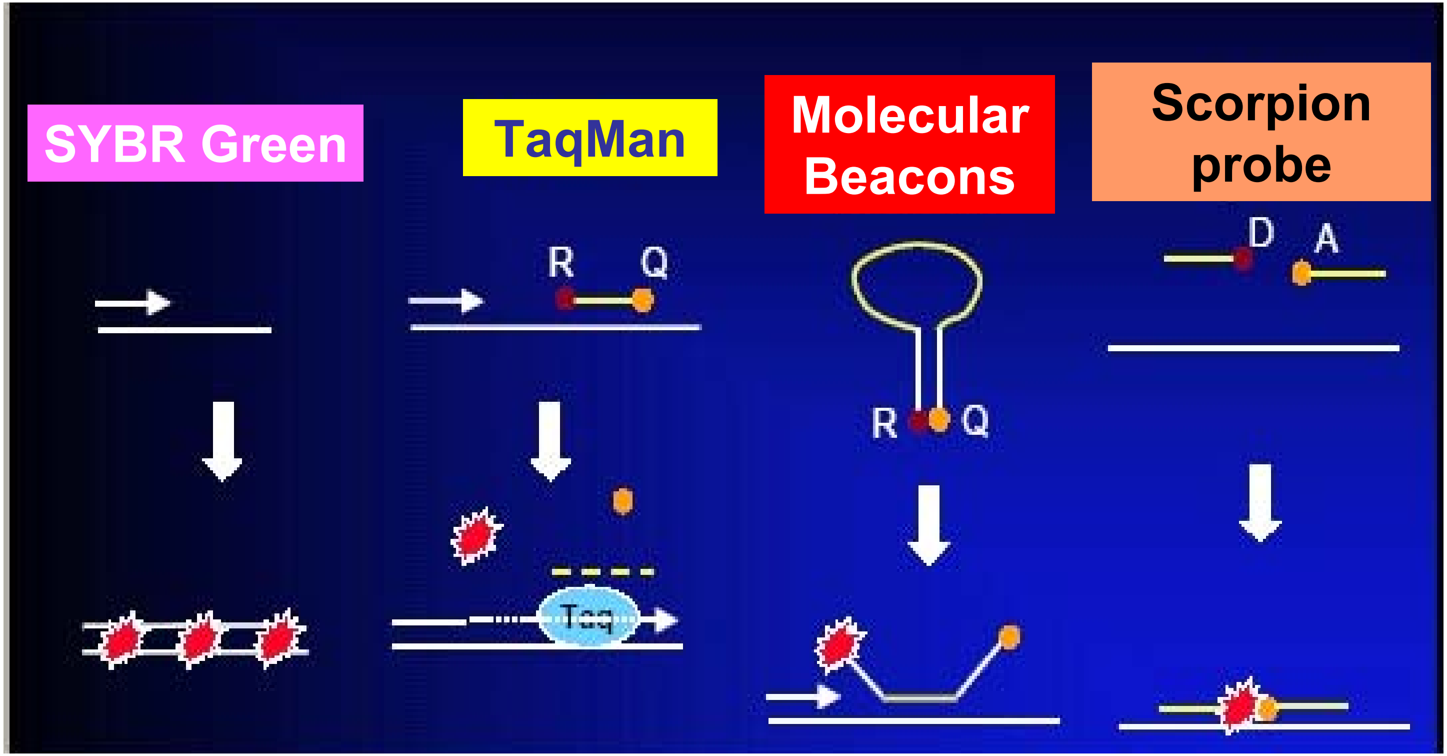


Theorical yield: **2^n**

$P=(2)^n T$: Product (P) increases exponentially every PCR cycle (n)

The PCR product depends on T, which is the starting copy number of template DNA

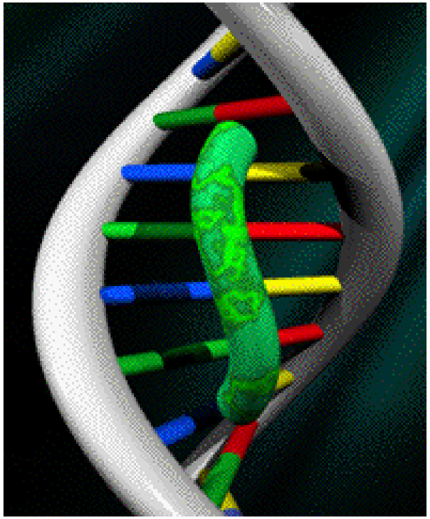
Detection methods



SYBR green Dye

SYBR® green DYE

- SYBR dye fluoresces upon binding to double stranded DNA
- Any double stranded DNA → NON-specific
- Used for target identification (screening) assays
- Low cost
- Drawback: Primer dimers or a-specific amplicons also fluoresce (see further)

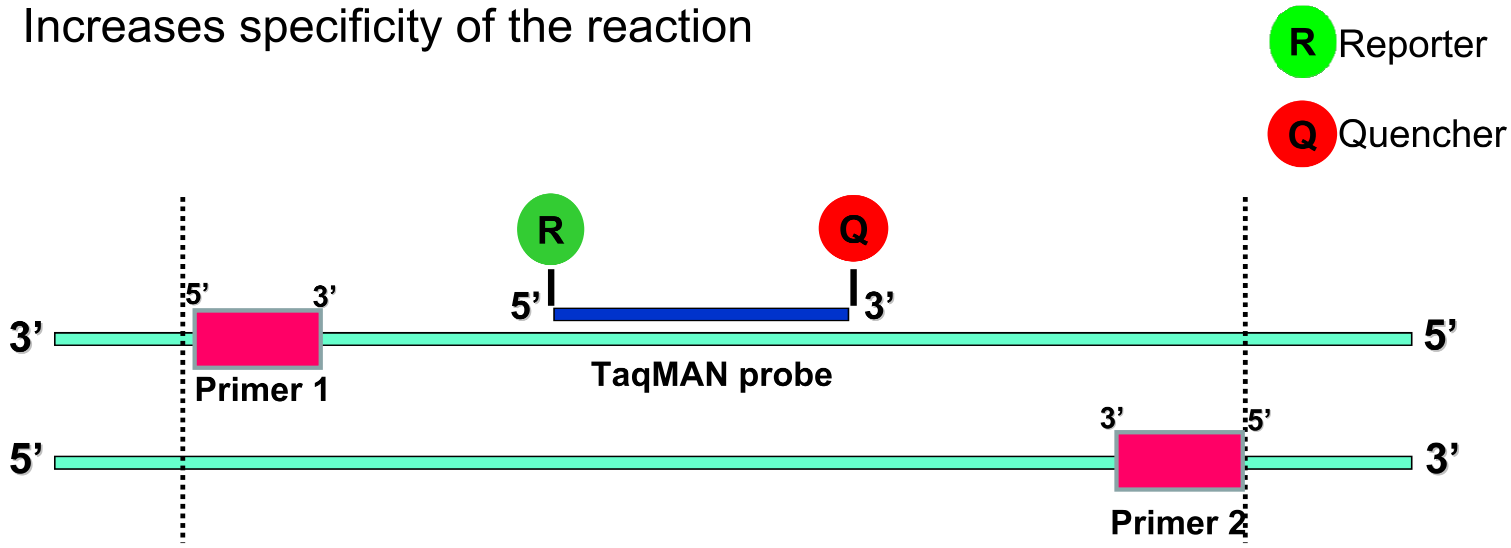


TaqMan probes

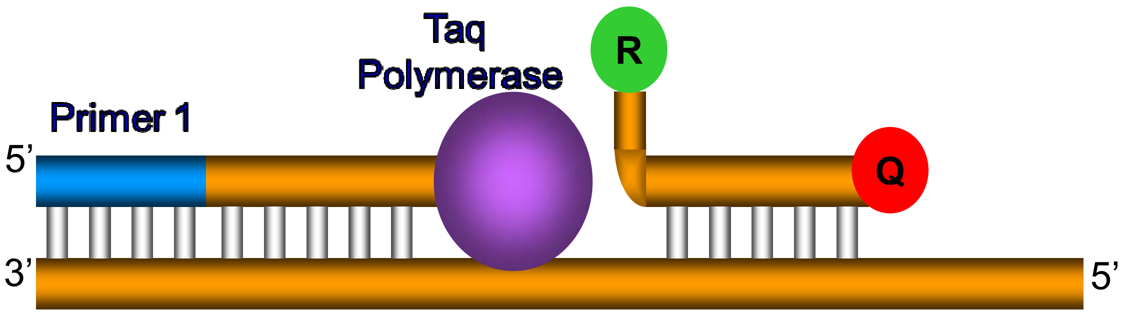
Fluorescent Taqman Probe

- Unique for each gene target
- Anneals between 2 primers at 10°C higher than primers

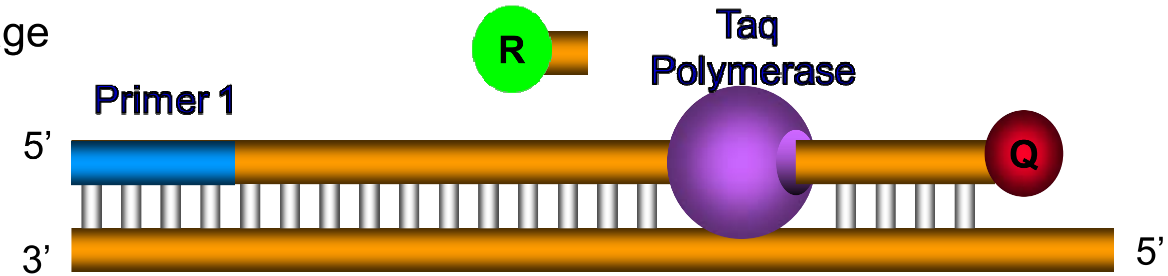
Increases specificity of the reaction



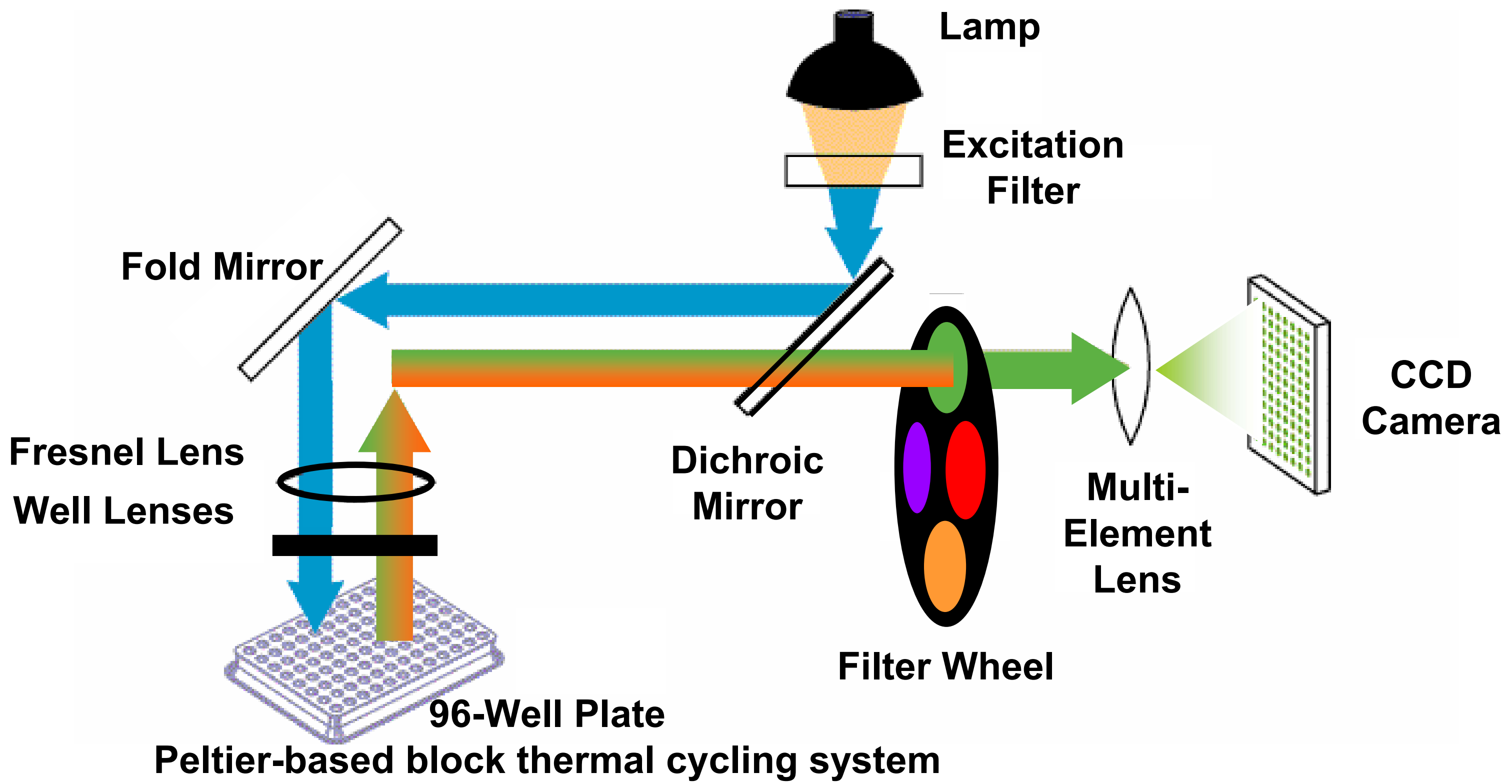
Polymerization and Displacement



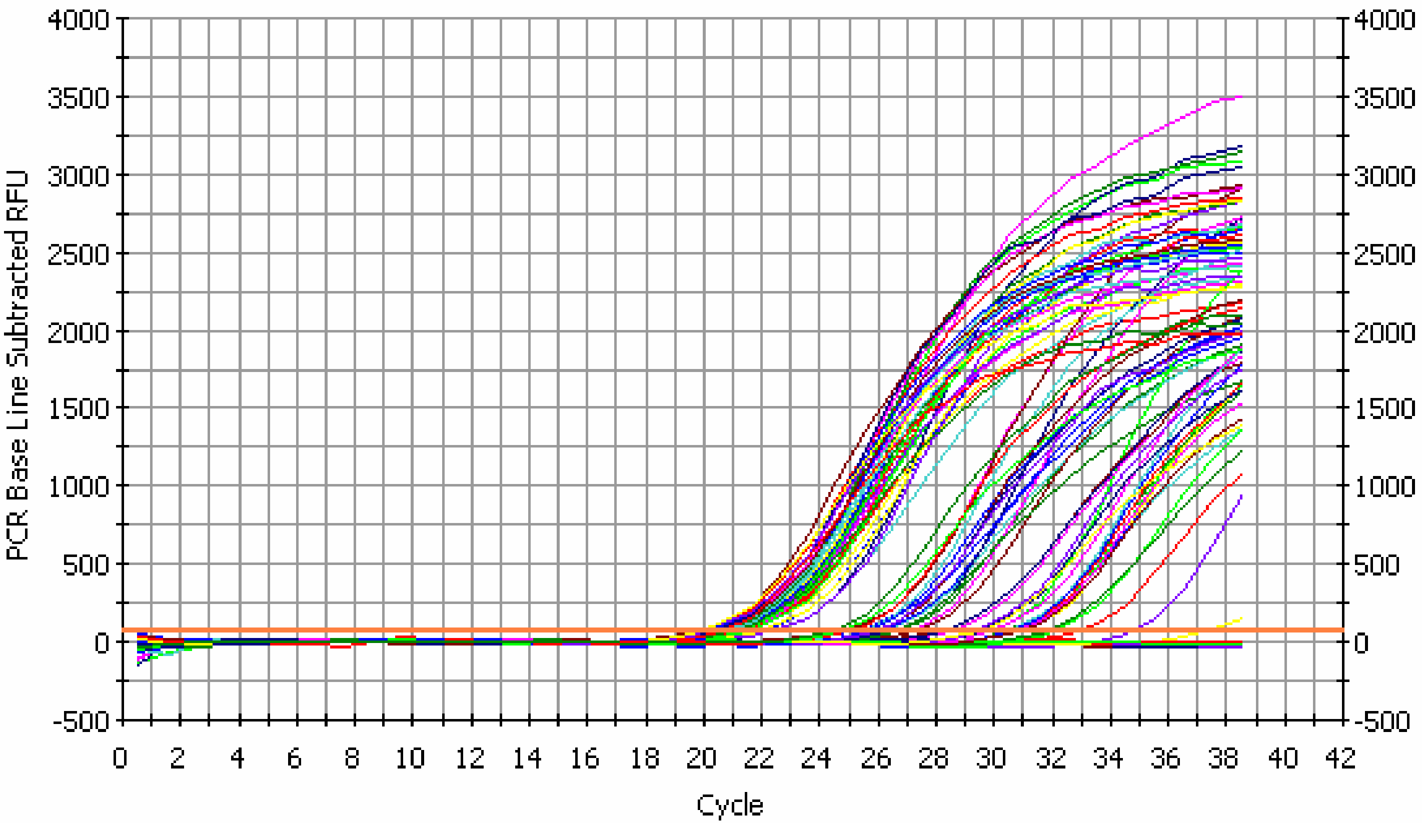
Cleavage



Fluorescence detection



Data analysis



- Expression
  - Transcripts (Northern blot, Reverse transcription-Real Time PCR)
  - Proteins (Western blot)

- Localisation
  - Transcript (promoter::reporter fusions, *in situ* hybridization)
  - Proteins (protein-reporter fusions, immunolocalisation)

Western blot

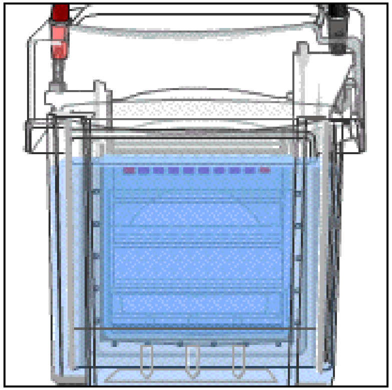
The **WB** (or **immunoblot**) is an analytical technique used to detect specific proteins in a given sample of tissue homogenate or extract.

It uses gel electrophoresis to separate by the length of the polypeptide (denaturing conditions, SDS) or by the 3-D structure of the protein (native/ non-denaturing conditions).

The proteins are then transferred to a membrane (typically nitrocellulose or PVDF), where they are probed (detected) using antibodies (Abs) specific to the target protein.

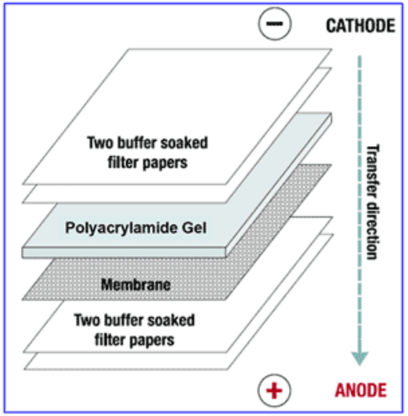
Procedures

- Extract proteins and quantify
- Prepare polyacrylamide gels
- Load samples
- Run the gel
- Stain the gel can either with Coomassie Brilliant Blue dye or with Silver Nitrate or use it for a Western Blot



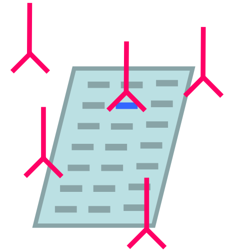
Western Blot

- The gel is placed into a sandwich clamp used for western blotting:
  - Sponge
  - Filter paper
  - Gel
  - Nitrocellulose Membrane
  - Filter paper
  - Sponge

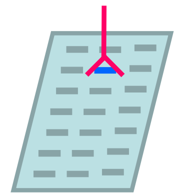


- Sandwich clamp is then placed into the transfer apparatus
- Proteins are transferred to membrane

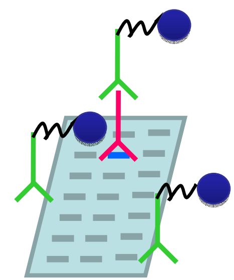
- Blocking of nonspecific binding sites,



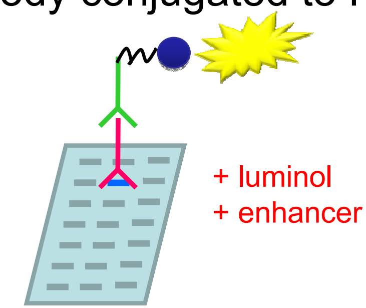
- Incubation with primary antibody,



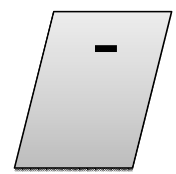
- Wash to get rid of unbound primary antibody,



- Incubation with secondary antibody conjugated to HRP,



- Wash and react with substrate

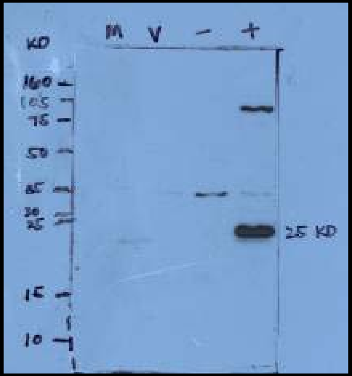


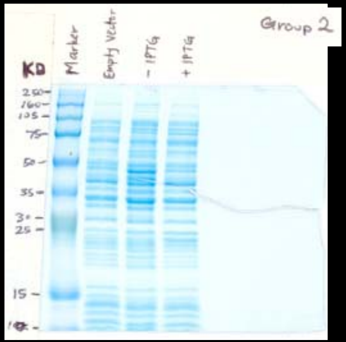
- Light emitted from luminol is detected with X-ray film.

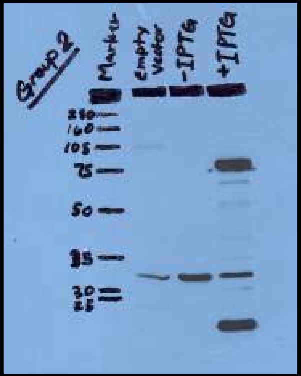
Results

Coomassie

WB







- Expression
  - Transcripts (Northern blot, Reverse transcription-Real Time PCR)
  - Proteins (Western blot)

- Localisation
  - Transcript (promoter::reporter fusions, *in situ* hybridization)
  - Proteins (protein-reporter fusions, immunolocalisation)

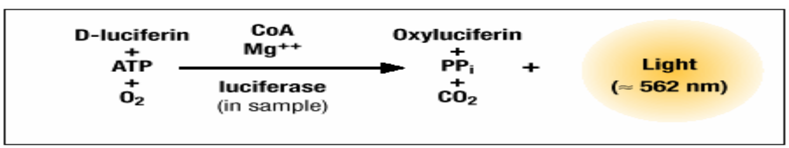
Reporter genes and assays

- Detection of physical presence and/or localisation of a gene
- The typical strategy is to engineer a DNA construct that reports the activity of a promoter
- common reporter genes utilized - typically these are enzymes.
  - chloramphenicol acetyl transferase (CAT)
  - luciferase (luc)
  - β-galactosidase (β-gal)
  - β-glucuronidase (β-gus)
  - β-lactamase
  - growth hormone (GH)
  - green fluorescent protein (GFP)

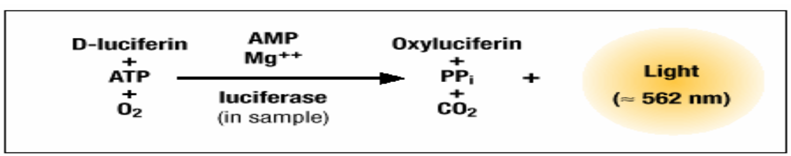
Reporter genes 1 - luciferase

- Luciferase (luc) gene encodes an enzyme that is responsible for bioluminescence in the firefly. This is one of the few examples of a bioluminescent reaction that only requires enzyme, substrate and ATP.

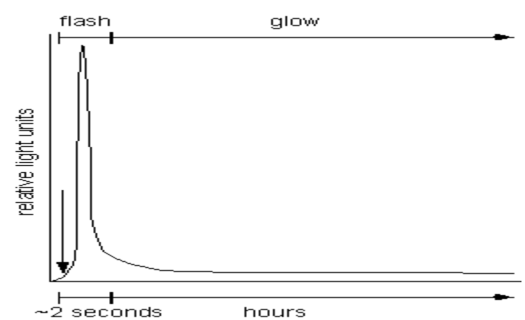
- Two phases to the reaction, flash and glow. These can be used to design different types of assays.

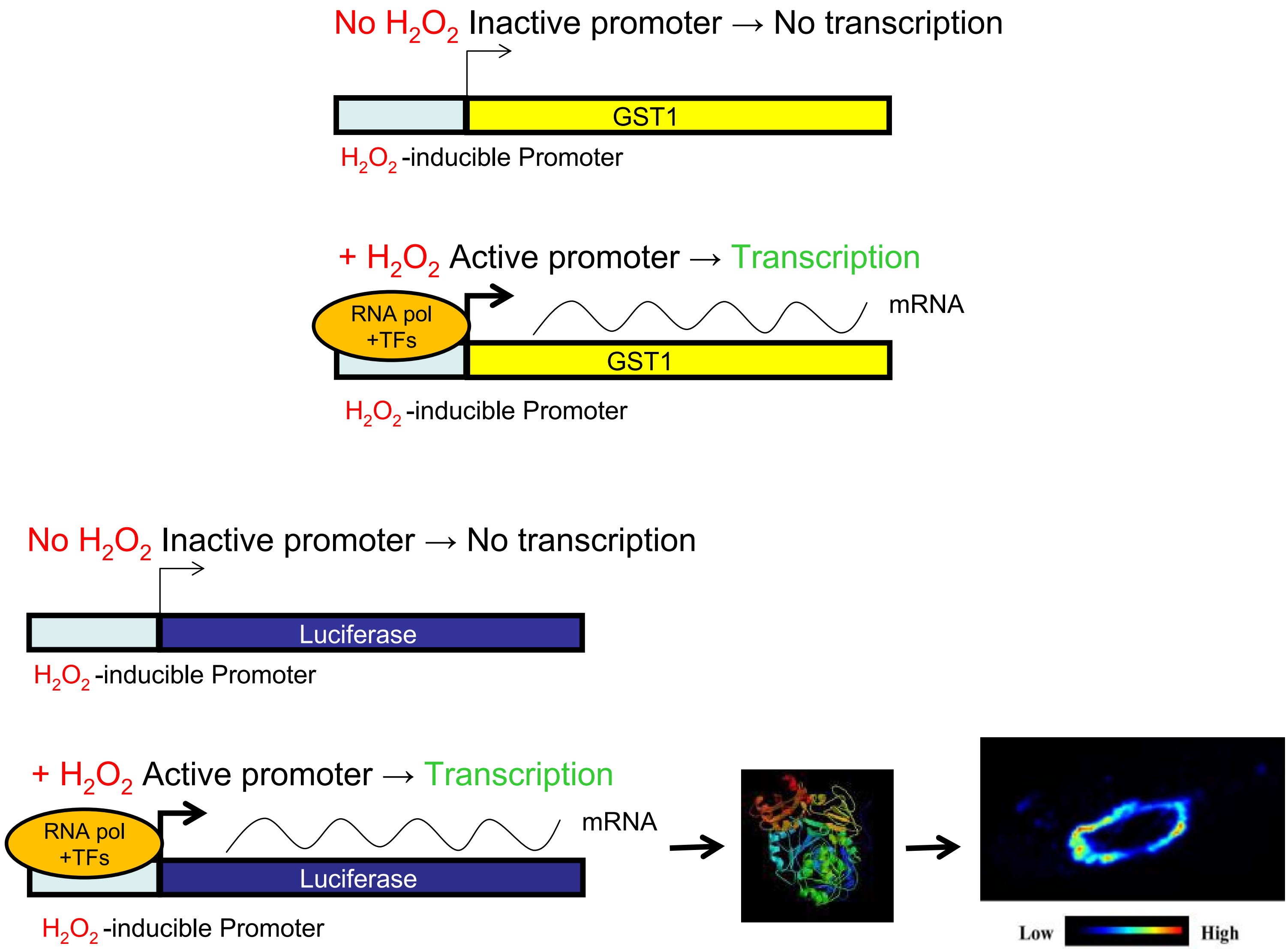


Flash reaction



Glow reaction



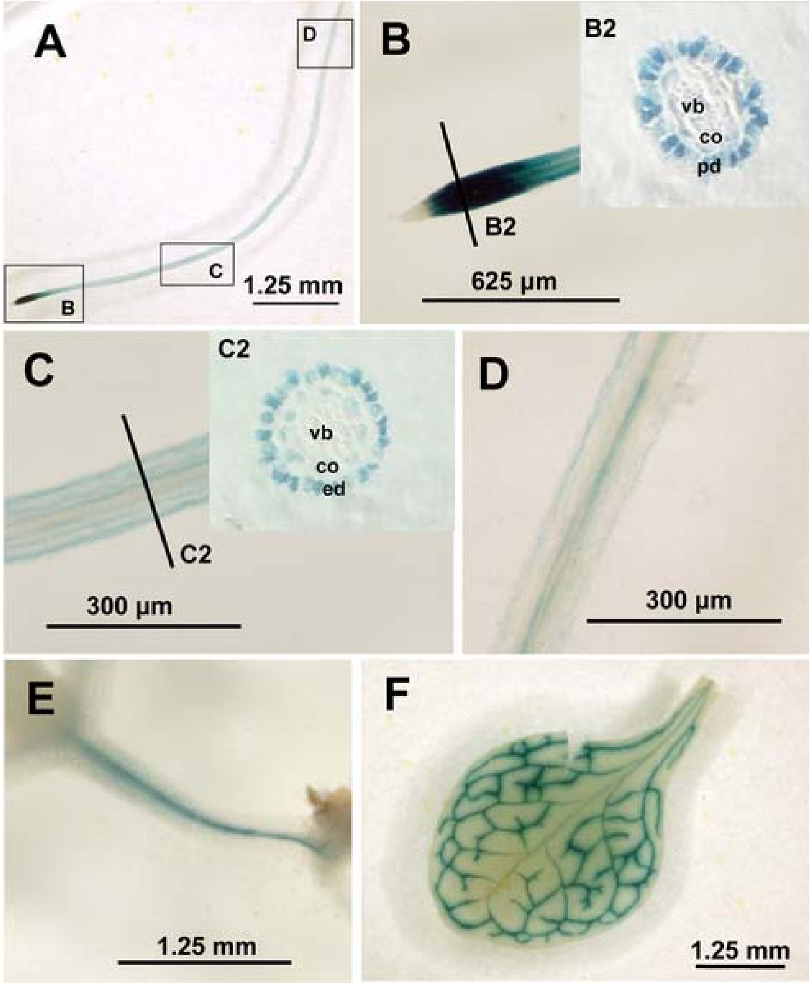

No H_2O_2 Inactive promoter → No transcription
GST1
H_2O_2 -inducible Promoter
+ H_2O_2 Active promoter → Transcription
RNA pol +TFs
mRNA
GST1
H_2O_2 -inducible Promoter
No H_2O_2 Inactive promoter → No transcription
Luciferase
H_2O_2 -inducible Promoter
+ H_2O_2 Active promoter → Transcription
RNA pol +TFs
mRNA
Luciferase
H_2O_2 -inducible Promoter
Low
High

Reporter genes 2 - β-glucuronidase

- Very stable enzyme tetramer similar to β-galactosidase
- cleaves β-D glucuronide linkage
- simple biochemical reaction

- advantages
  - low background
  - can require little equipment (spectrophotometer)
  - stable enzyme at 37ºC

- disadvantages
  - sensitive assays required
  - stability of the enzyme makes it a poor choice for reporter in transient transfections (high background = low dynamic range)



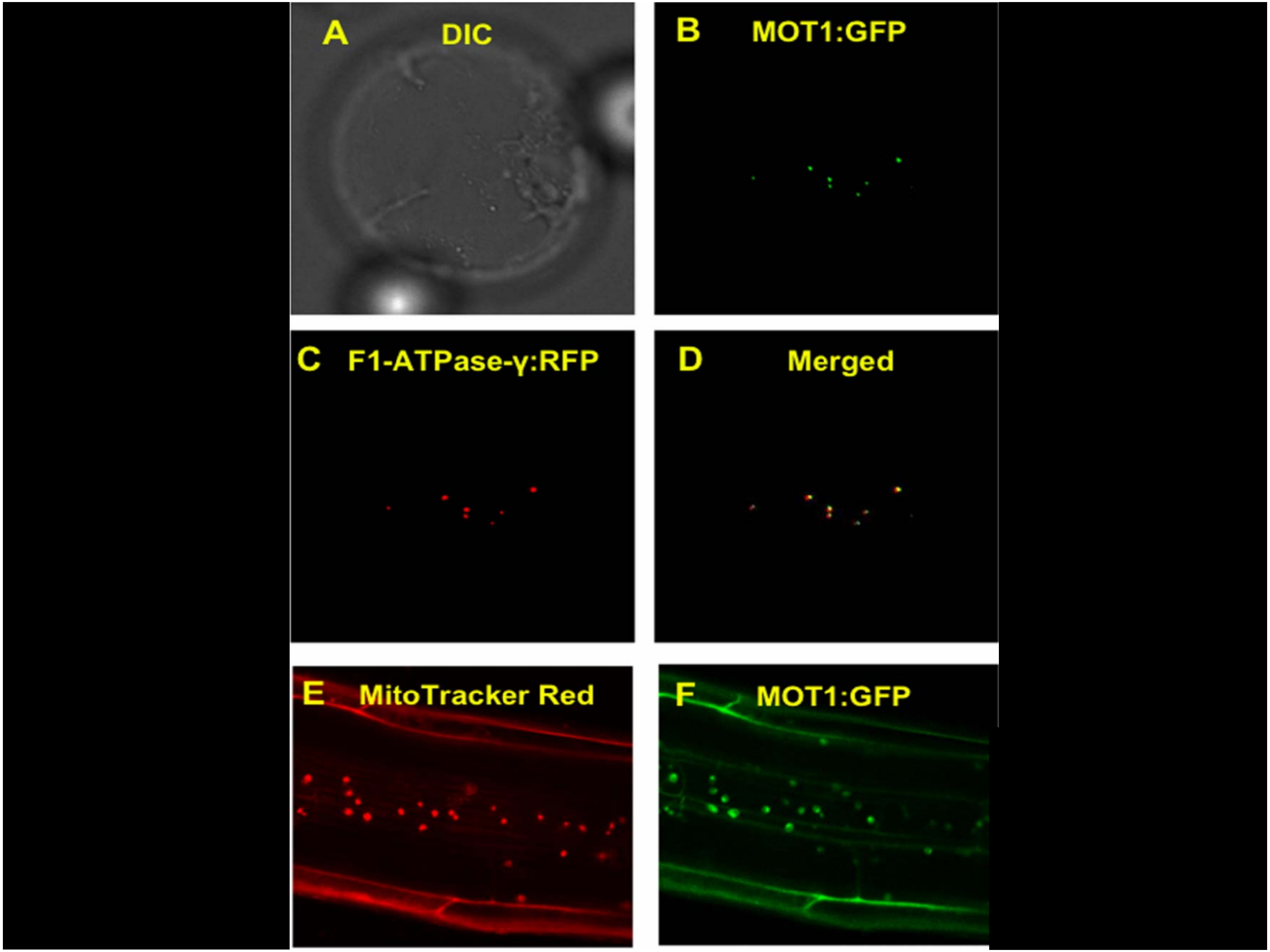
A
D
C
B
1.25 mm
B
B2
vb
co
pd
B2
625 µm
C
C2
vb
co
ed
C2
300 µm
D
300 µm
E
1.25 mm
F
1.25 mm

Reporter genes 3 - green fluorescent protein (GFP)

- Source is bioluminescent jellyfish *Aequora victoria*
- absorbs UV (~360 nm) and emits visible light.
  - has been engineered to produce many different colors (green, blue, yellow, red)

- advantages
  - can detect in living cells
  - inexpensive (no substrate)

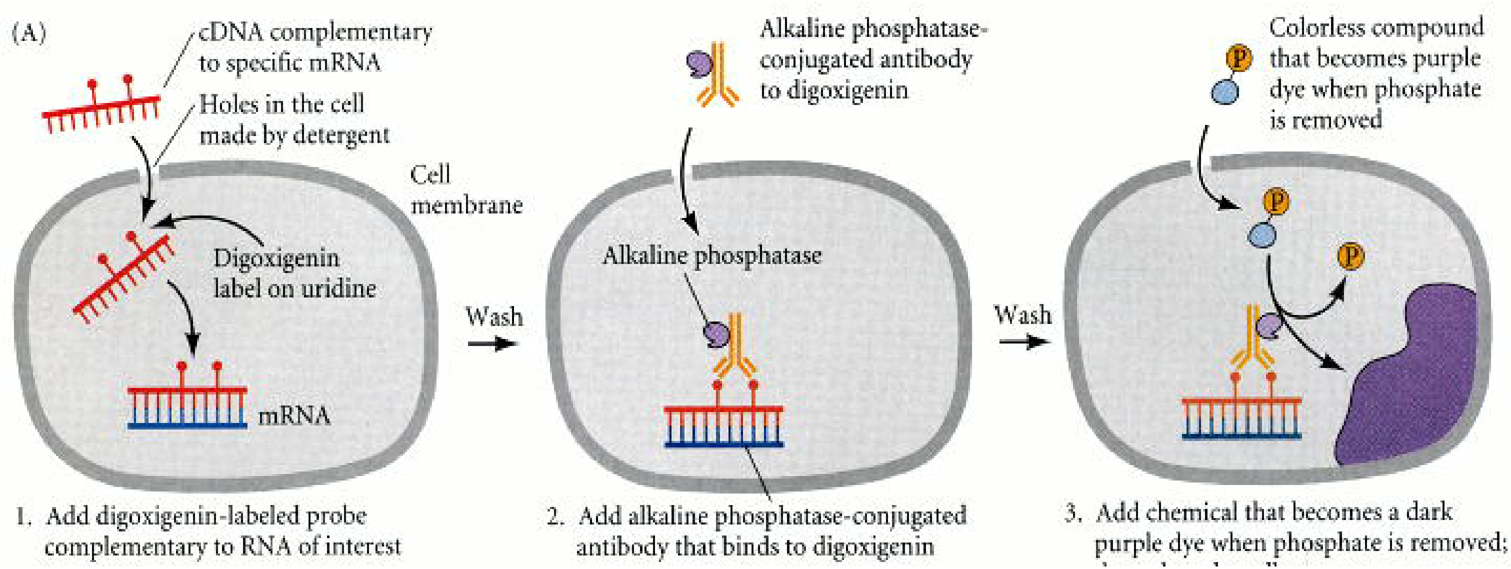
- disadvantages
  - low sensitivity and dynamic range
  - equipment requirements

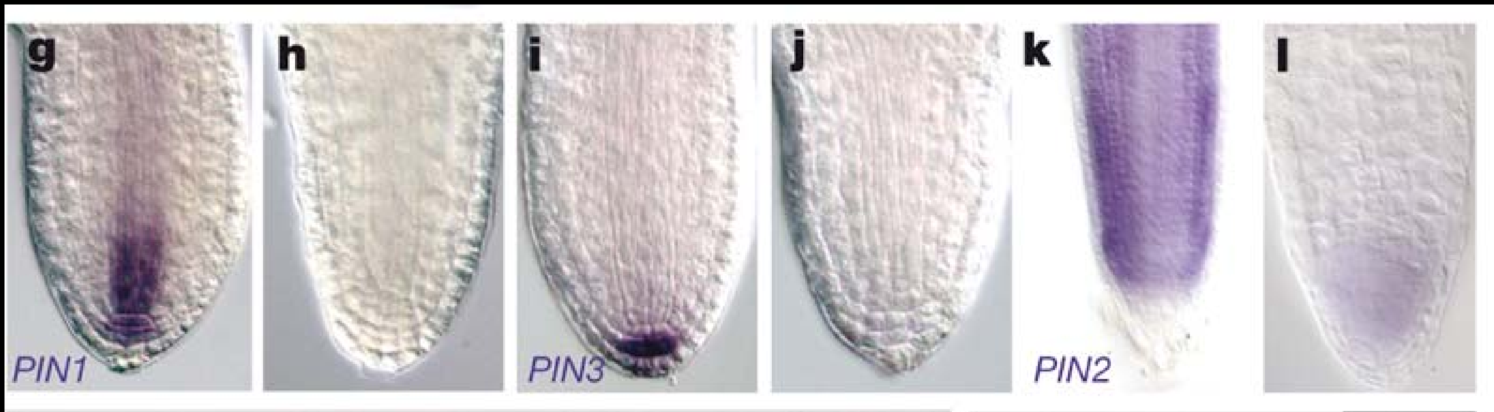

A
DIC
B
MOT1:GFP
C
F1-ATPase-γ:RFP
D
Merged
E
MitoTracker Red
F
MOT1:GFP

In situ hybridization

It is a type of hybridization that uses a labeled complementary DNA or RNA probe to localize a specific RNA sequence in a portion or section of tissue (*in situ*), or, if the tissue is small enough (e.g. plant seeds, Drosophila embryos), in the entire tissue (*whole mount*).

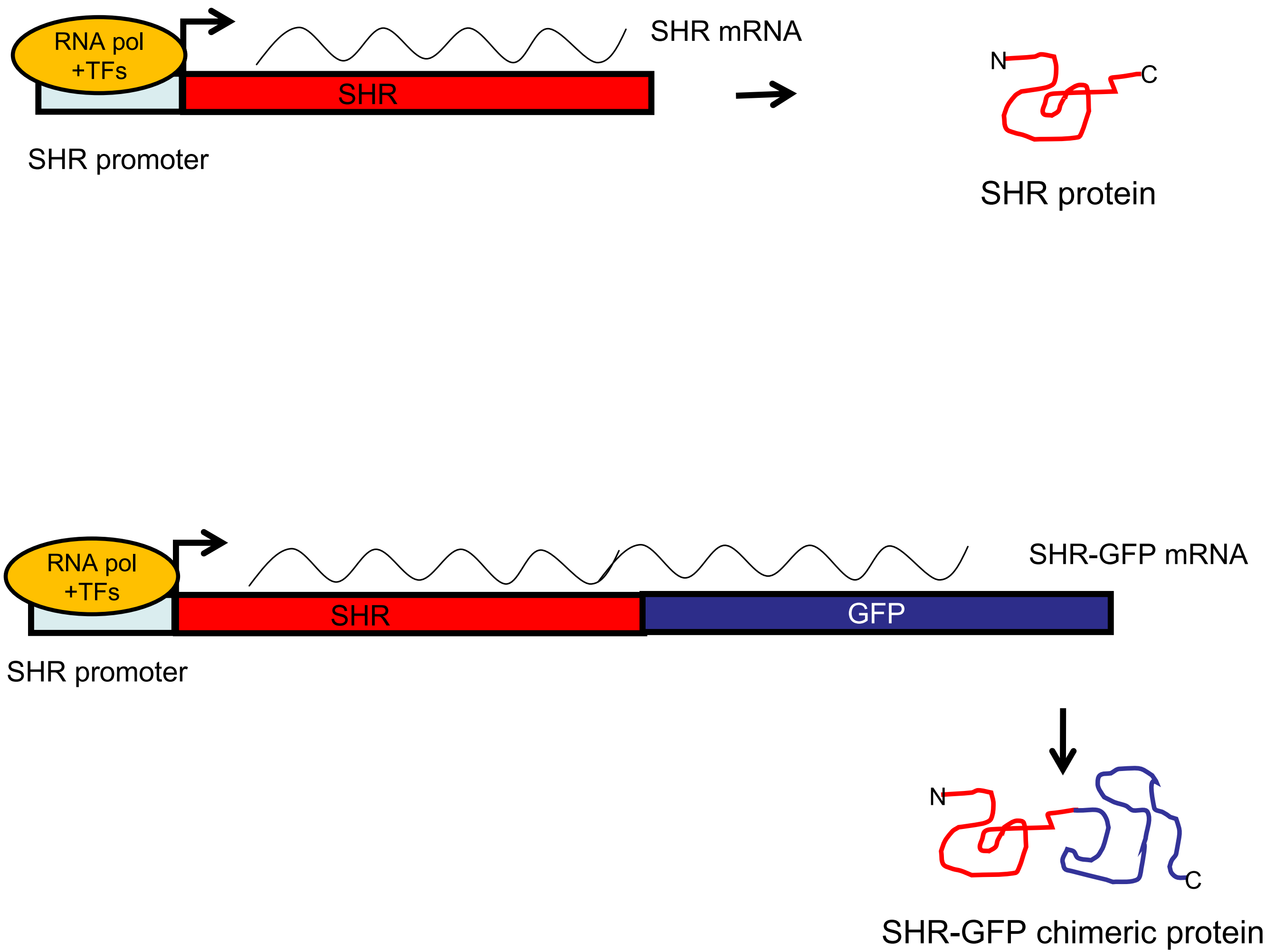
Whole mount in situ hybridization



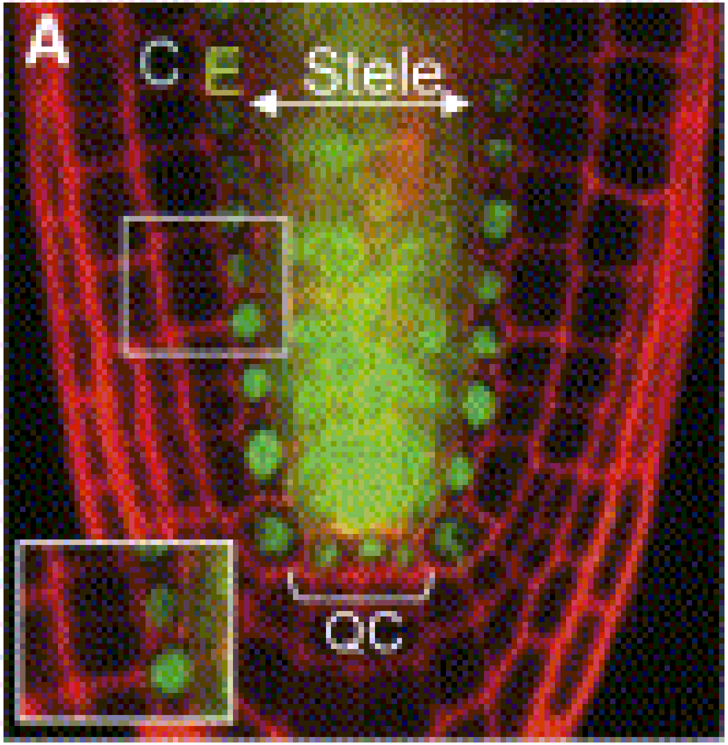

g
h
i
j
k
l
PIN1
PIN3
PIN2

- Expression
  - Transcripts (Northern blot, Reverse transcription-Real Time PCR)
  - Proteins (Western blot)

- Localisation
  - Transcript (promoter::reporter fusions, *in situ* hybridization)
  - Proteins (protein-reporter fusions, immunolocalisation)


RNA pol
+TFs
SHR
SHR mRNA
SHR promoter
N
C
SHR protein
RNA pol
+TFs
SHR
GFP
SHR-GFP mRNA
SHR promoter
N
C
SHR-GFP chimeric protein

Protein-reporter fusions

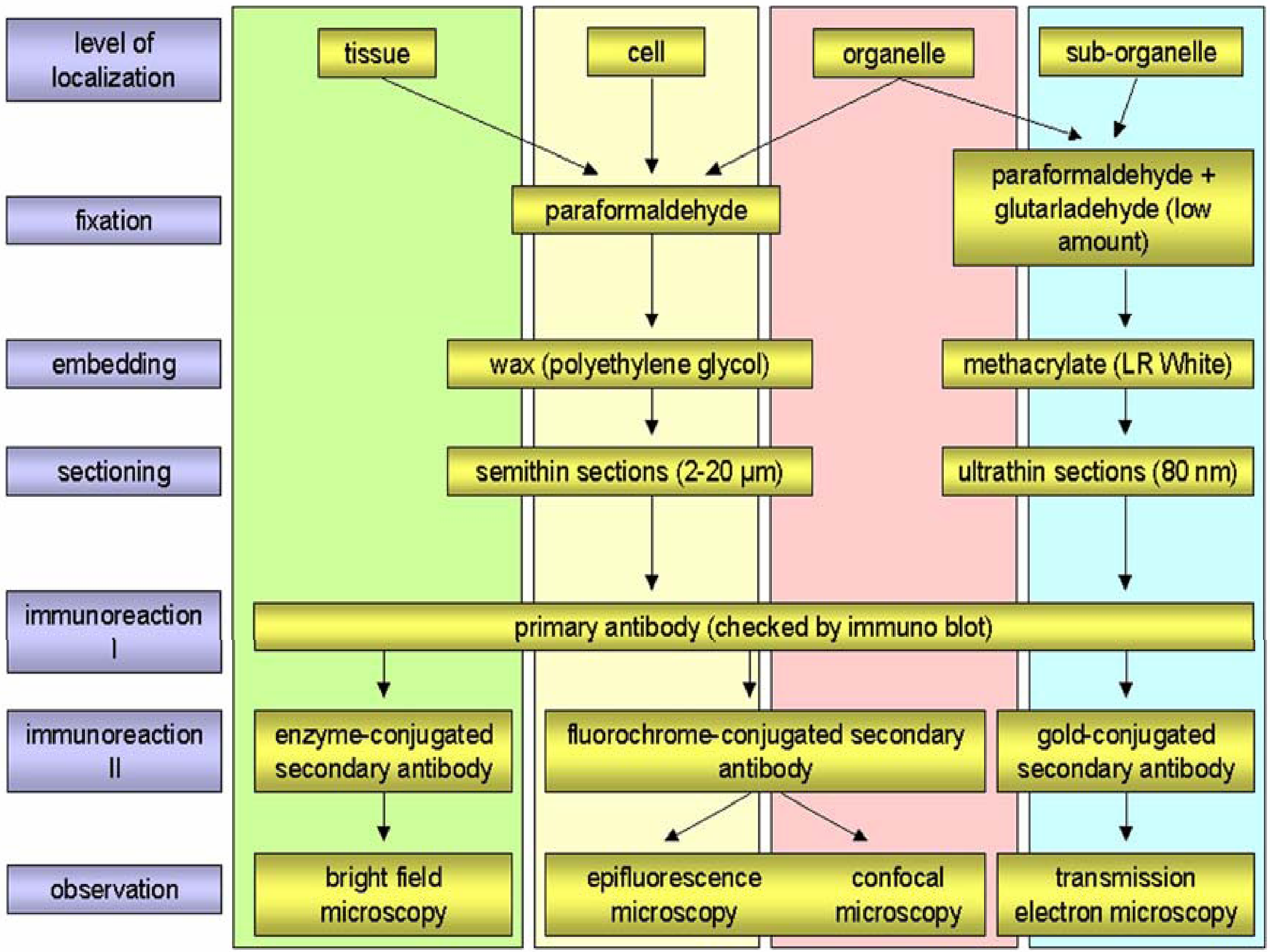


SHR::SHR-GFP

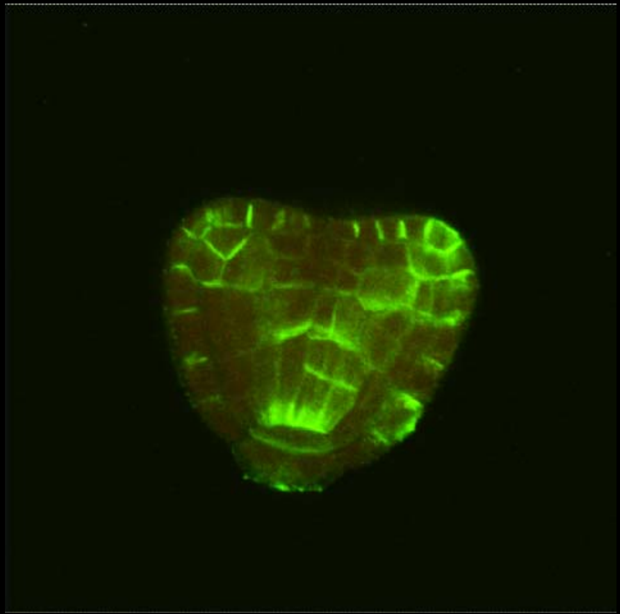
Immunolocalisation

The goal of immunocytochemistry is to define the cellular location of biochemically defined antigens.

Immunocytochemical techniques can be applied to all types of cells. Can be applied to a section of a tissue or, if the tissue is small enough (e.g. plant seeds, Drosophila embryos), in the entire tissue (*whole mount*).

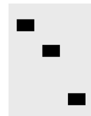


level of localization
tissue
cell
organelle
sub-organelle
fixation
paraformaldehyde
paraformaldehyde + glutarladehyde (low amount)
embedding
wax (polyethylene glycol)
methacrylate (LR White)
sectioning
semithin sections (2-20 µm)
ultrathin sections (80 nm)
immunoreaction I
primary antibody (checked by immuno blot)
immunoreaction II
enzyme-conjugated secondary antibody
fluorochrome-conjugated secondary antibody
gold-conjugated secondary antibody
observation
bright field microscopy
epifluorescence microscopy
confocal microscopy
transmission electron microscopy

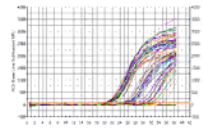


Expression

Transcripts

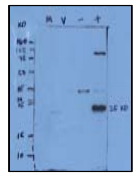


Northen blot



RT-qPCR

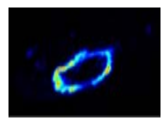
Proteins



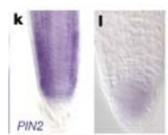
Western blot

Localisation

Transcripts

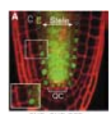


Promoter::reporter fusions

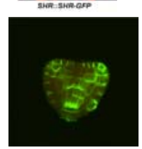


In situ hybridization

Proteins



Protein-reporter fusions



Immunolocalisation