Techniques and strategies in molecular medicine

RNA detection and quantitation

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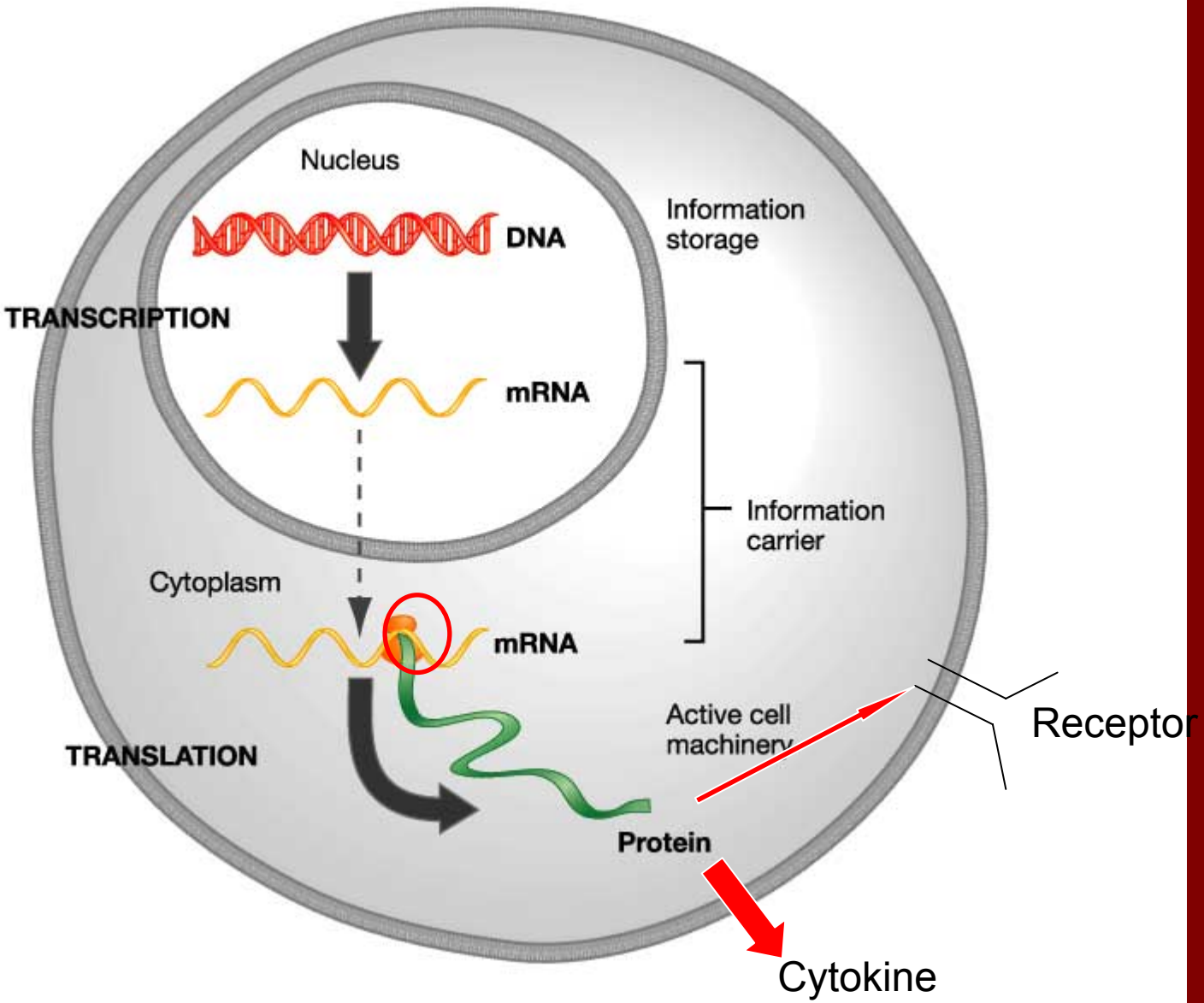
TCD

Central Dogma



Why and how RNA

- mRNA or transcript levels are immensely informative of the state of a cell.
- In general increases in the levels of a particular protein is reflected by increases in the corresponding mRNA transcript.
- Changes in gene expression is important in the cellular response to external stimuli and to basic cellular function.
- Completion of human genome sequencing projects has displayed detailed regions of hypothetical genes, EST and the location of many known genes.
- Far more proteins than genes, splice variation.
- Antibody production is laborious.
- RNA based molecular techniques can exploit data from the genome projects successfully.

Introduction to RNA

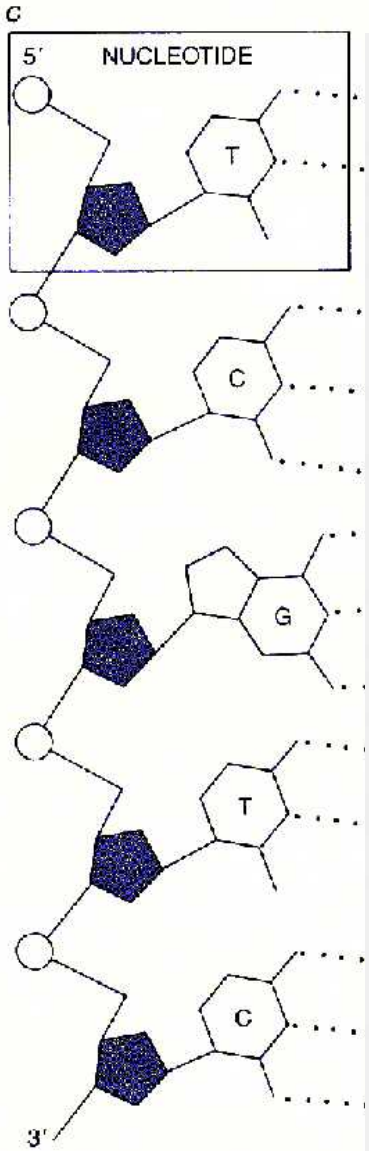
- Ribonucleic acid (RNA), a nucleic acid polymer consisting of many nucleotides
- Each nucleotide is made up of nitrogenous base, a ribose sugar and a phosphate.
- DNA deoxyribonucleic acid, ribose lacks one oxygen atom by comparison to RNA.
- RNA is usually single stranded as opposed to DNA double stranded.
- RNA utilises the nucleotide Uracil in place of thymine found in DCA
- 3 main types:-
  - Messenger RNA (mRNA)
  - Ribosomal RNA (rRNA)
  - Transfer RNA (tRNA)

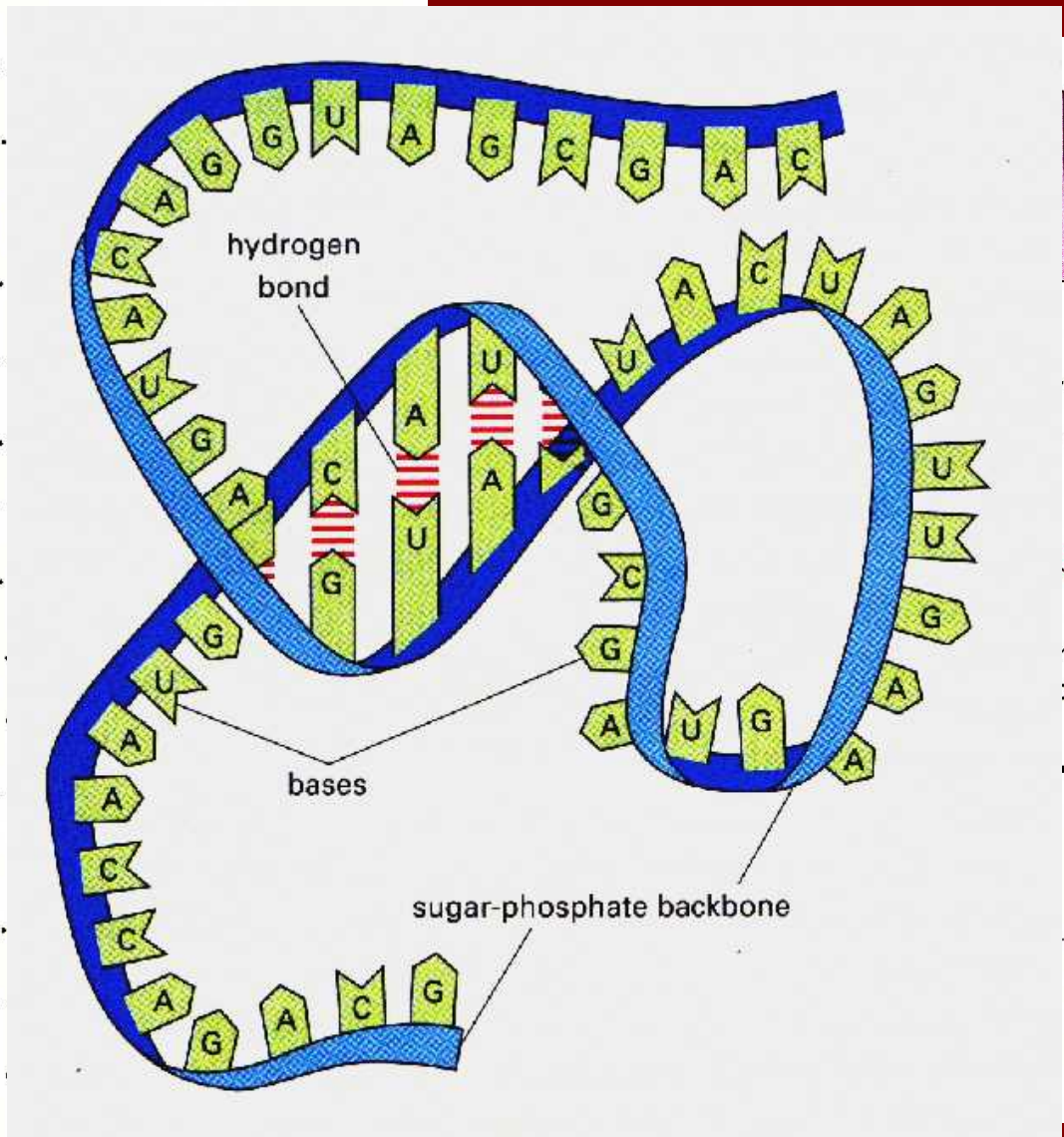
DNA Adenine-Thymine

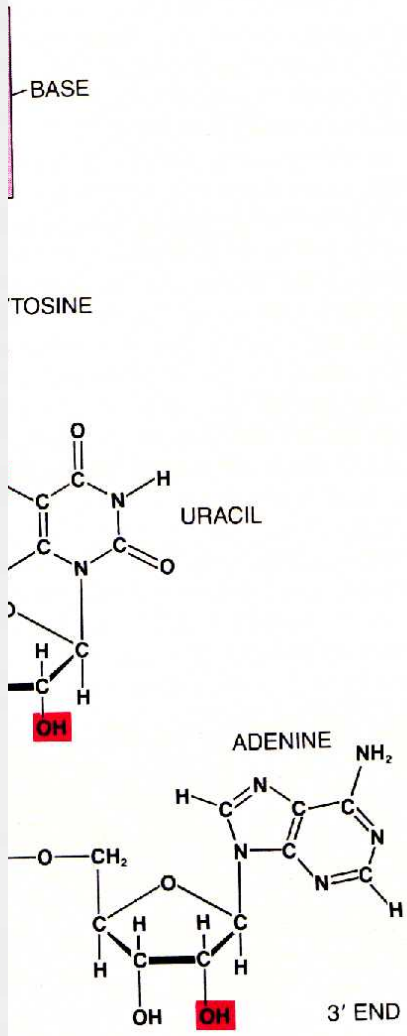
A—T

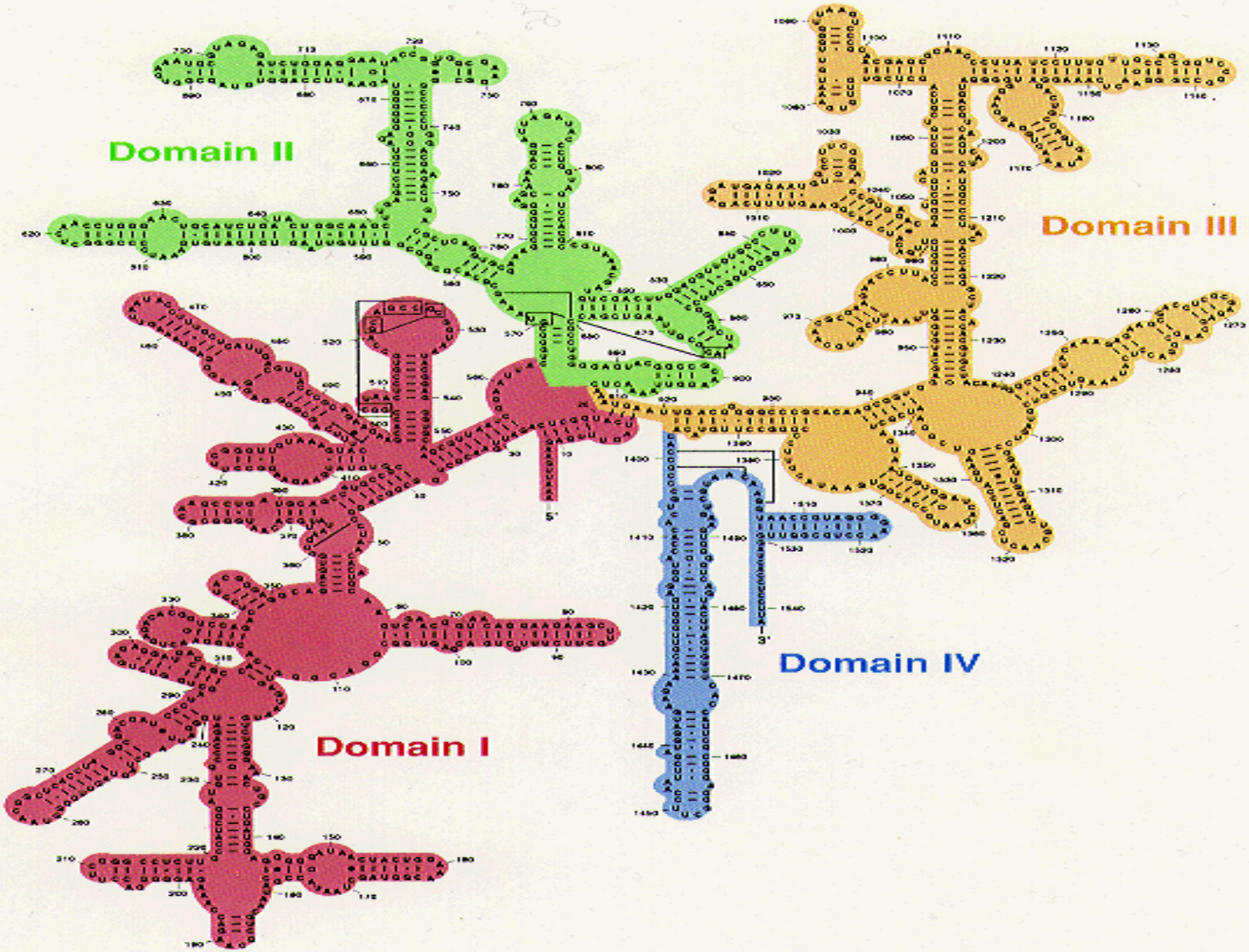
RNA Adenine-Uracil

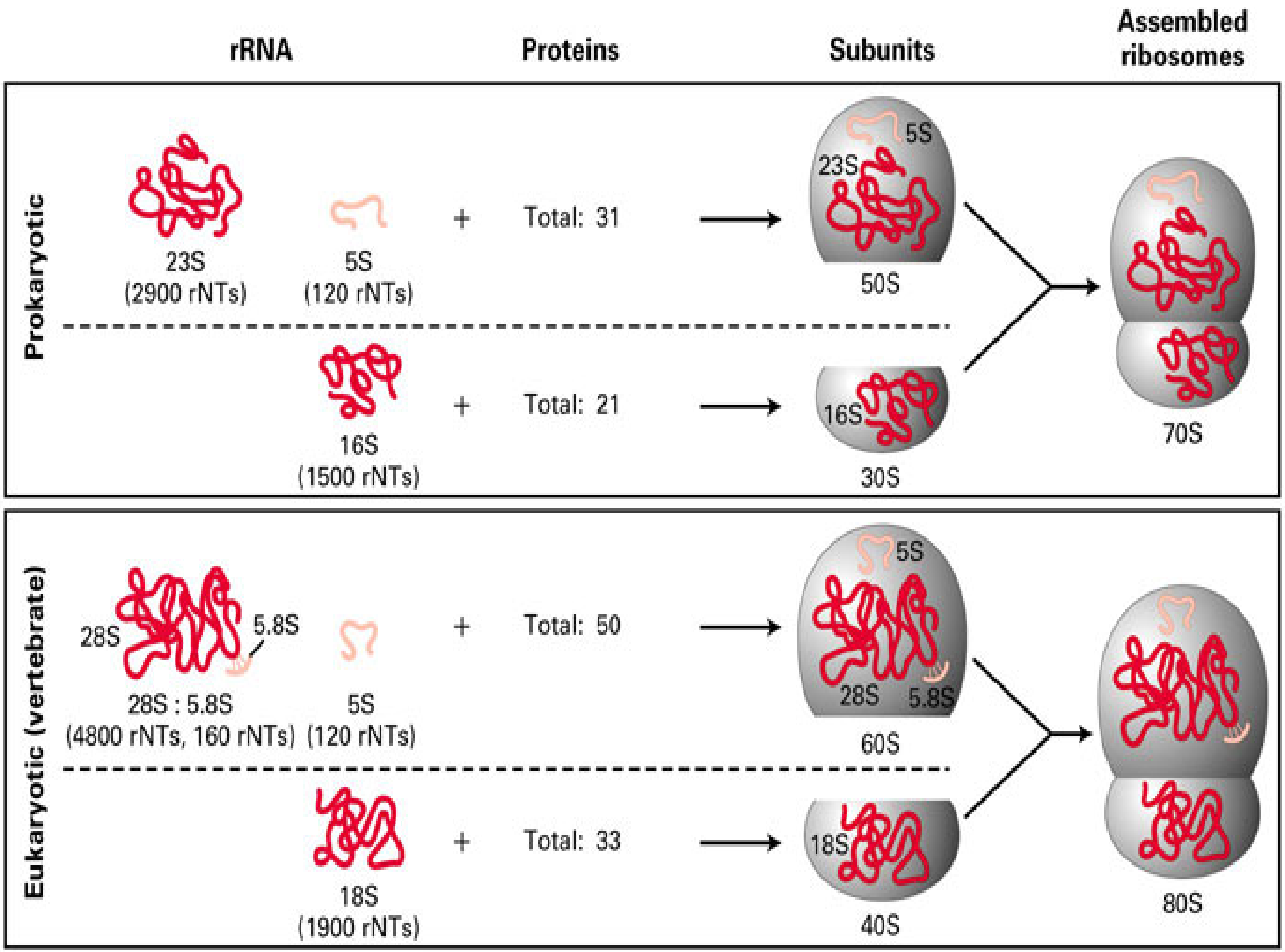
A—U


c
5′
NUCLEOTIDE
T
C
G
T
C
3′


hydrogen bond
bases
sugar-phosphate backbone


BASE
TOSINE
URACIL
ADENINE
3′ END




rRNA
Proteins
Subunits
Assembled ribosomes
Prokaryotic
23S
(2900 rNTs)
5S
(120 rNTs)
+
Total: 31
5S
23S
50S
16S
(1500 rNTs)
+
Total: 21
16S
30S
70S
Eukaryotic (vertebrate)
28S
5.8S
28S : 5.8S
(4800 rNTs, 160 rNTs)
5S
(120 rNTs)
+
Total: 50
5S
28S
5.8S
60S
18S
(1900 rNTs)
+
Total: 33
18S
40S
80S

RNA extraction and purification strategies

- Tissue storage
- Tissue disruption
- RNA storage

RNA extraction and purification strategies

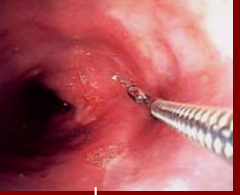
- Firstly DNA and RNA must be extracted from the cell or tissue of interest by:-
  - » Cell breakage (Lysis)
  - » Removal of protein or DNA or RNA
  - » Concentration of either DNA or RNA
  - » Determination of purity and quantity
- In modern labs nucleic acids may be purified using silica membranes and spin column centrifugation (High salt).
- Contaminating DNA and RNA can be removed by treating the membranes with appropriate DNAses or RNAses.
- Nucleic acid quantitation may be determined by UV spectrophotometry with absorption at 260 nm.
- Contaminating protein- 260:280 ratio.

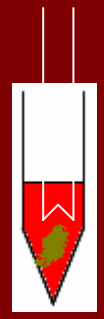
RNA isolation

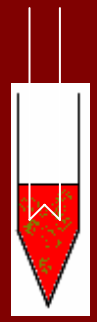
- Determinants of Cell lysis approach:-
  - Tissue type, Bone, connective, fatty tissues, Blood, urine, plant, Bacterial.
  - Viral, cellular RNA
  - Storage in RNAlater/ICE preservative
  - Storage in liquid nitrogen
  - Quantity and quality of RNA required for downstream applications
  - Cell lines generally not a problem
- Optimisation study definitely required.

Tissue disruption

TissueRuptor and disposable probes



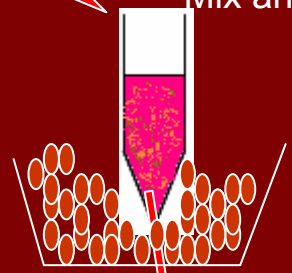




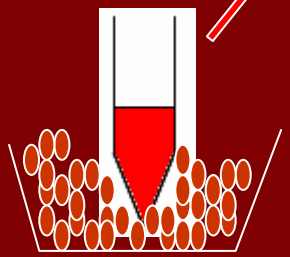
Add Choloform

Homogenisation

Mix and rest 5 mins

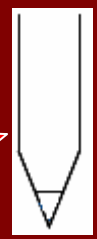


Centrifuge 15 mins



Pre-chilled
Tri-reagent

Spin
Pellet
RNA



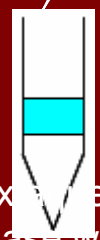
Re-suspend
In TE or H20

Aqueous phase
-RNA

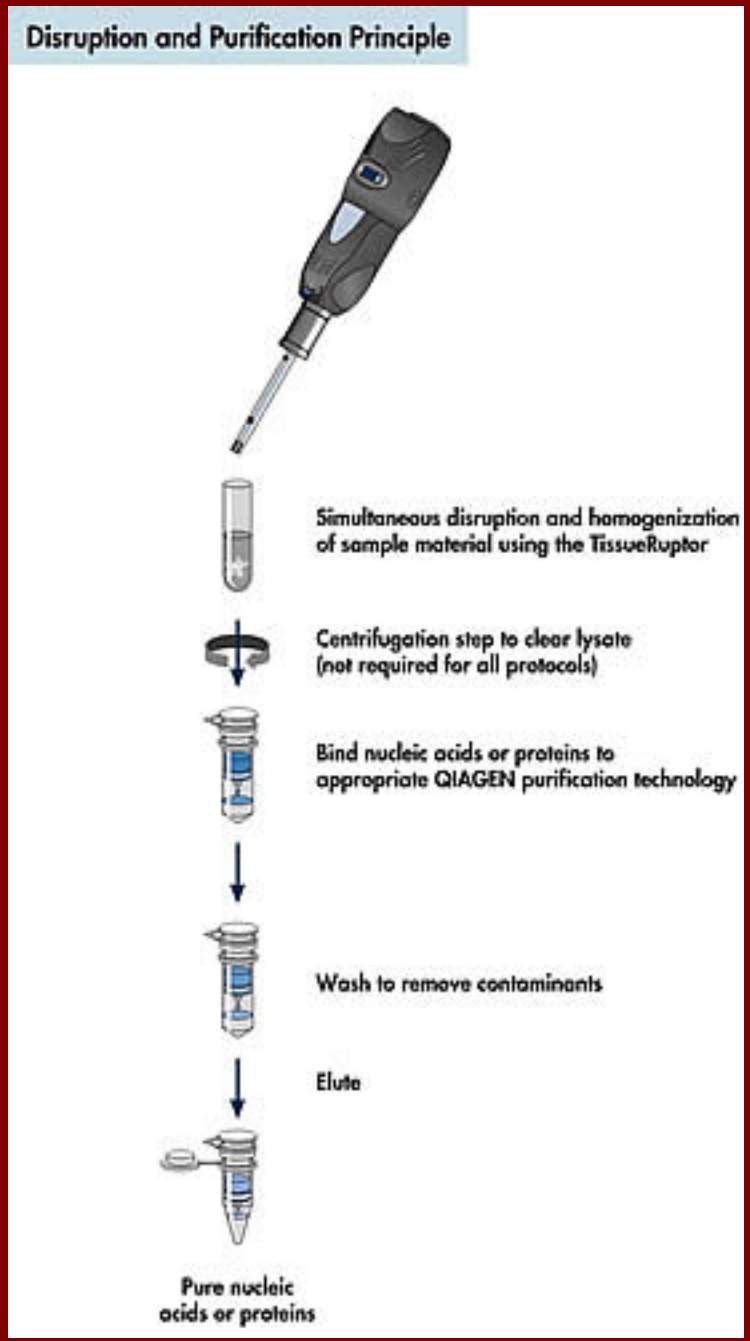
DNA precipitate

Organic phase
-Protein

Carefully remove
Aqueous phase



Mix [illegible]eous
Phase with
isopropanol

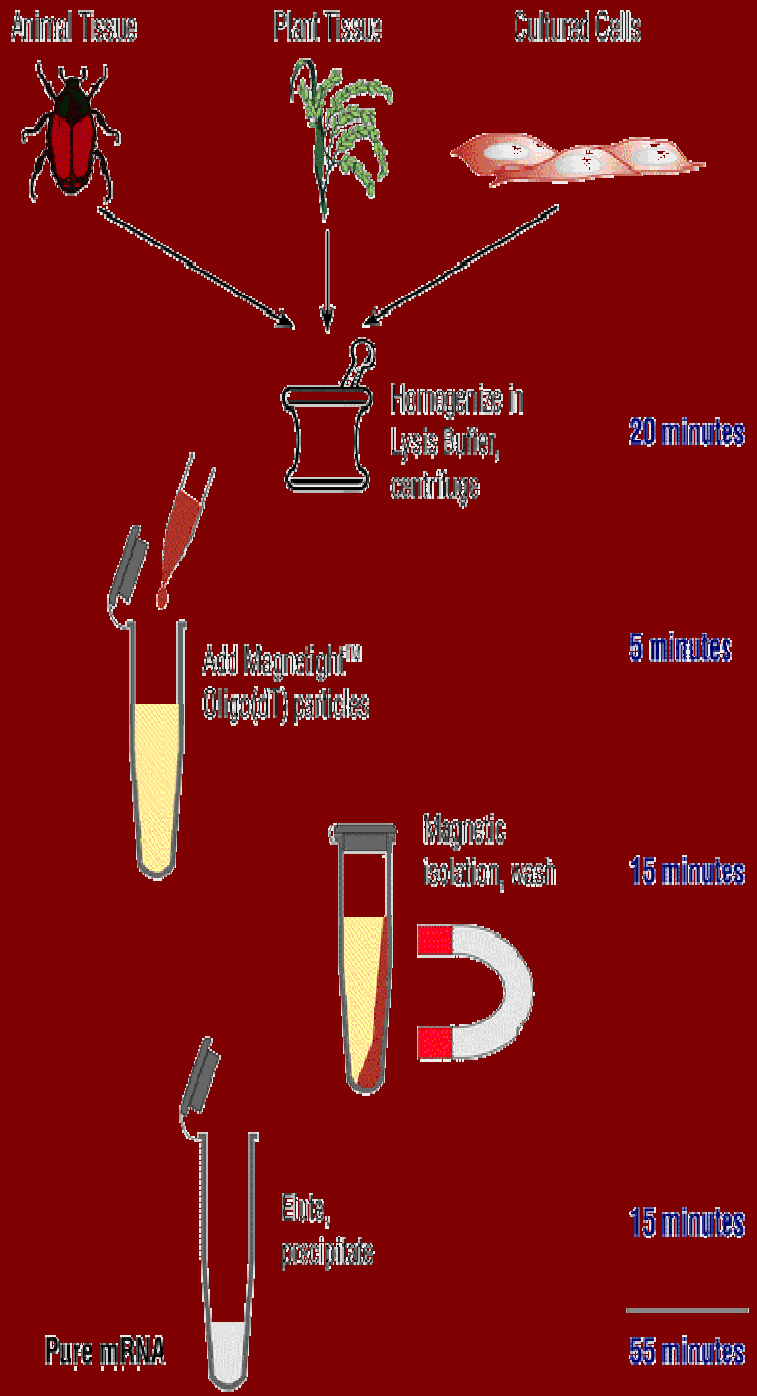


Spin column purification

- Great for cell lines
- Cleaning up after Tri-reagent
- Silica gel binding of RNA
- Ethanol salt washes
- Elute high quality RNa in H20
- General Nucliec acid
- Specific small RNA binding
- On column DNA digest

Rneasy MinElute Cleanup

- Ideal following Tri-reagent
- Concentrates RNA
- Fast, High-throughput

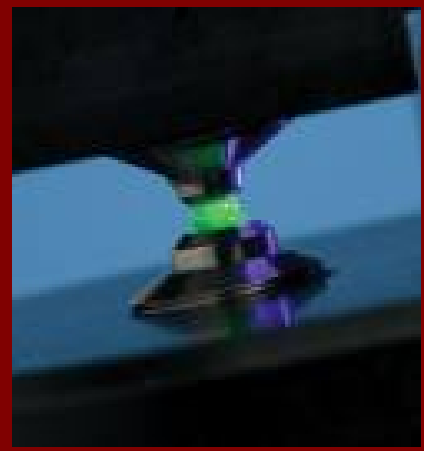


Poly(A)+ RNA isolation

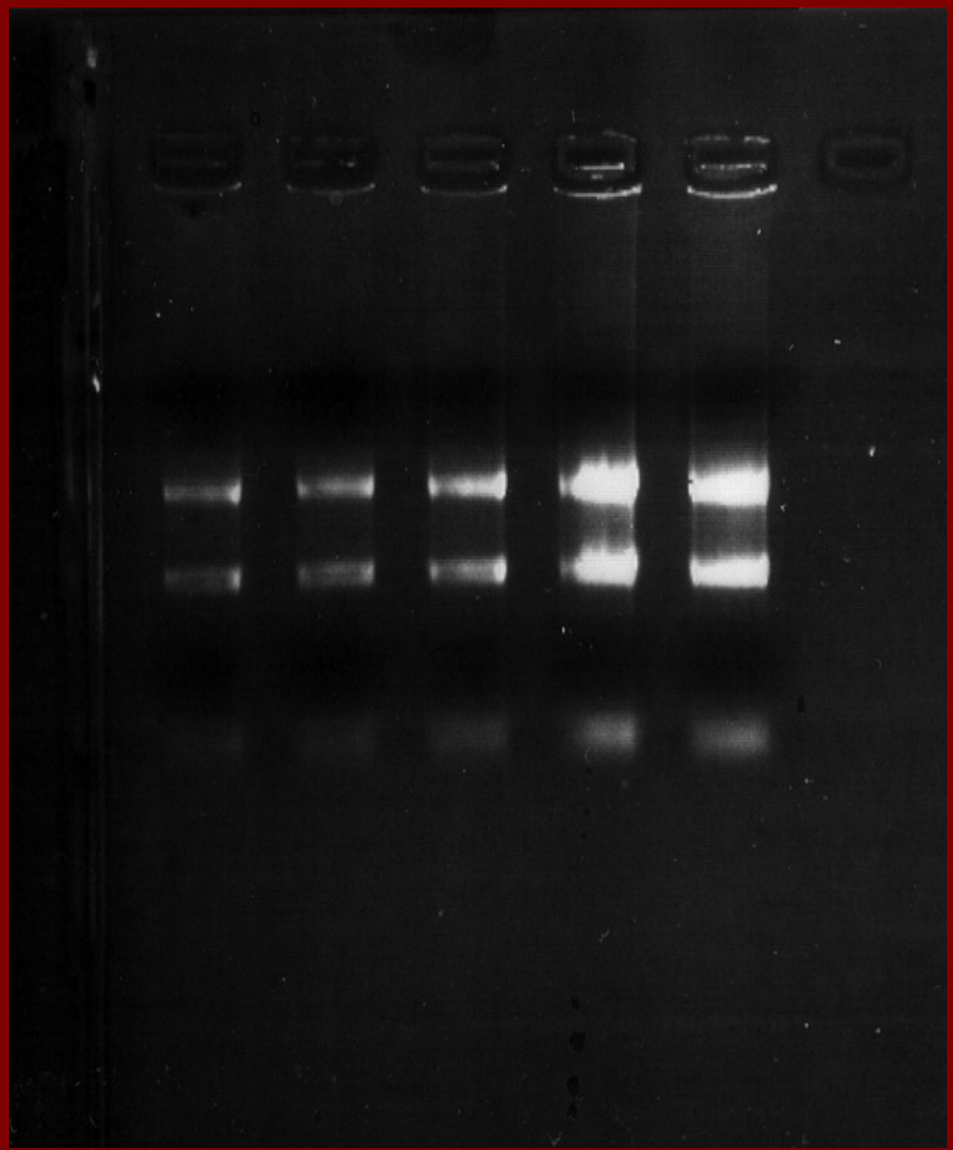
- Utilizes polydT coated magnetic beads
- Binds to poly A tail of mRNA
- Avoids DNA, protein, rRNA and tRNA
- Enriches transcripts by 30 fold
- Less prone to DNA contamination

RNA Quantitation

- UV spectroscopy
  - Consistant
  - Reliable
  - Easy
  - 260/280 ratio
  - 260/230 ratio
- Ribo green assay
  - RNA Standard curve
  - Accurate
- NanoDrop Flourospectrometer

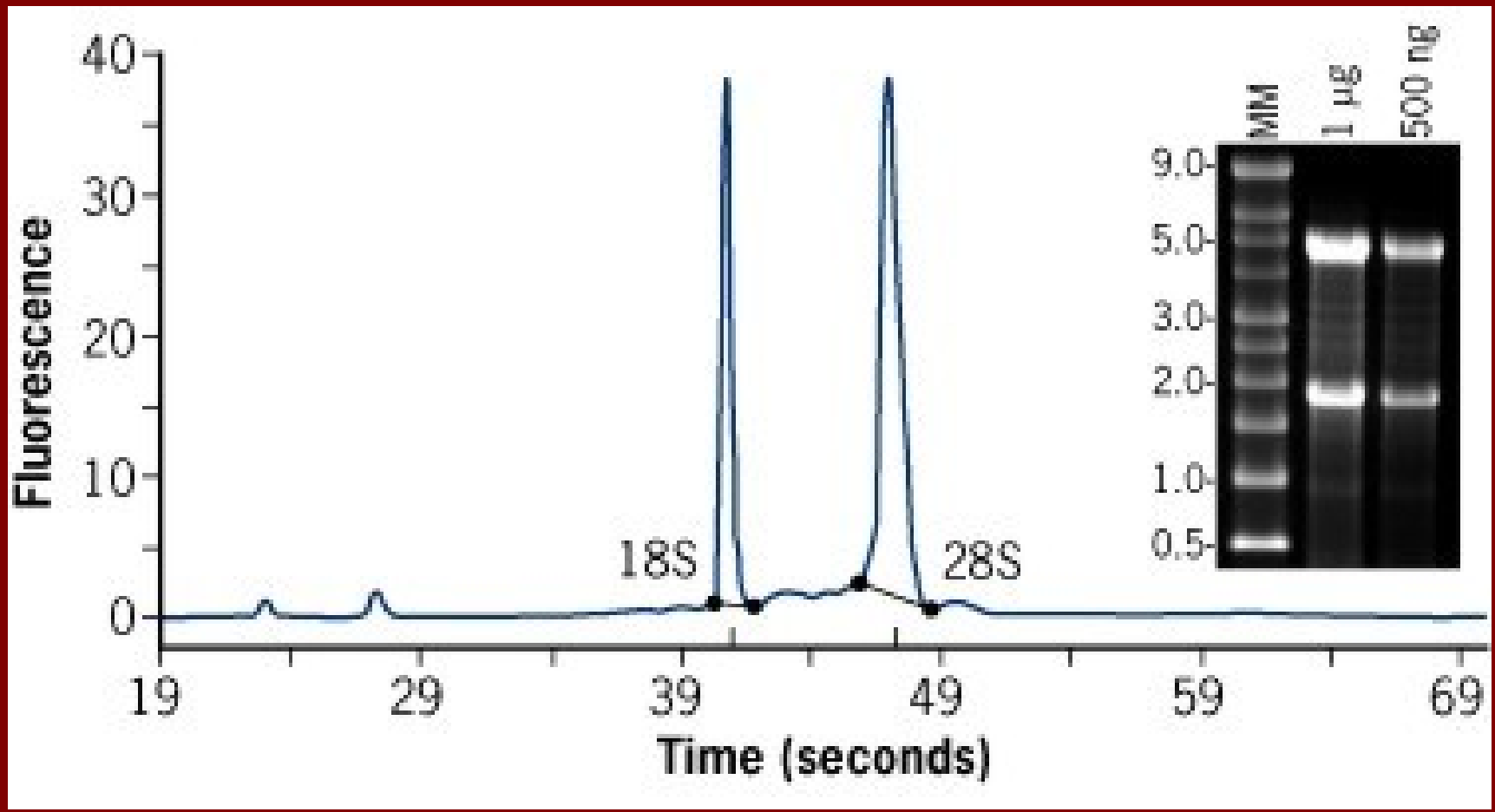


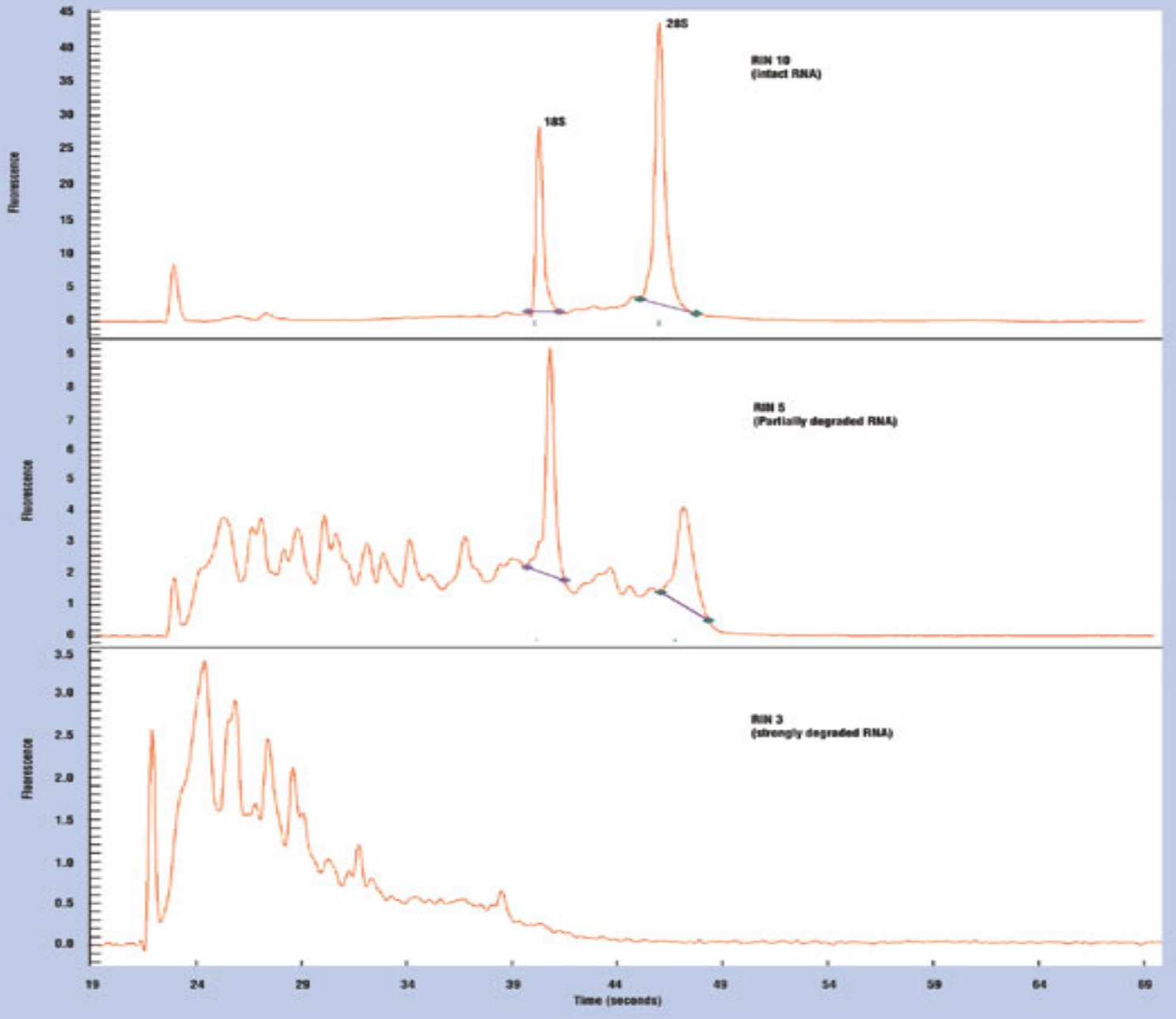
RNA qualitation



- MOPS gel eletrophoesis
  - Stain with ethdium bromide
  - Displaying ribosomal RNA units.
  - mRNA too dilute to see, but usually a smear when selected
  - rRNA ration good indicator of a healthy sample

RNA Qualitation

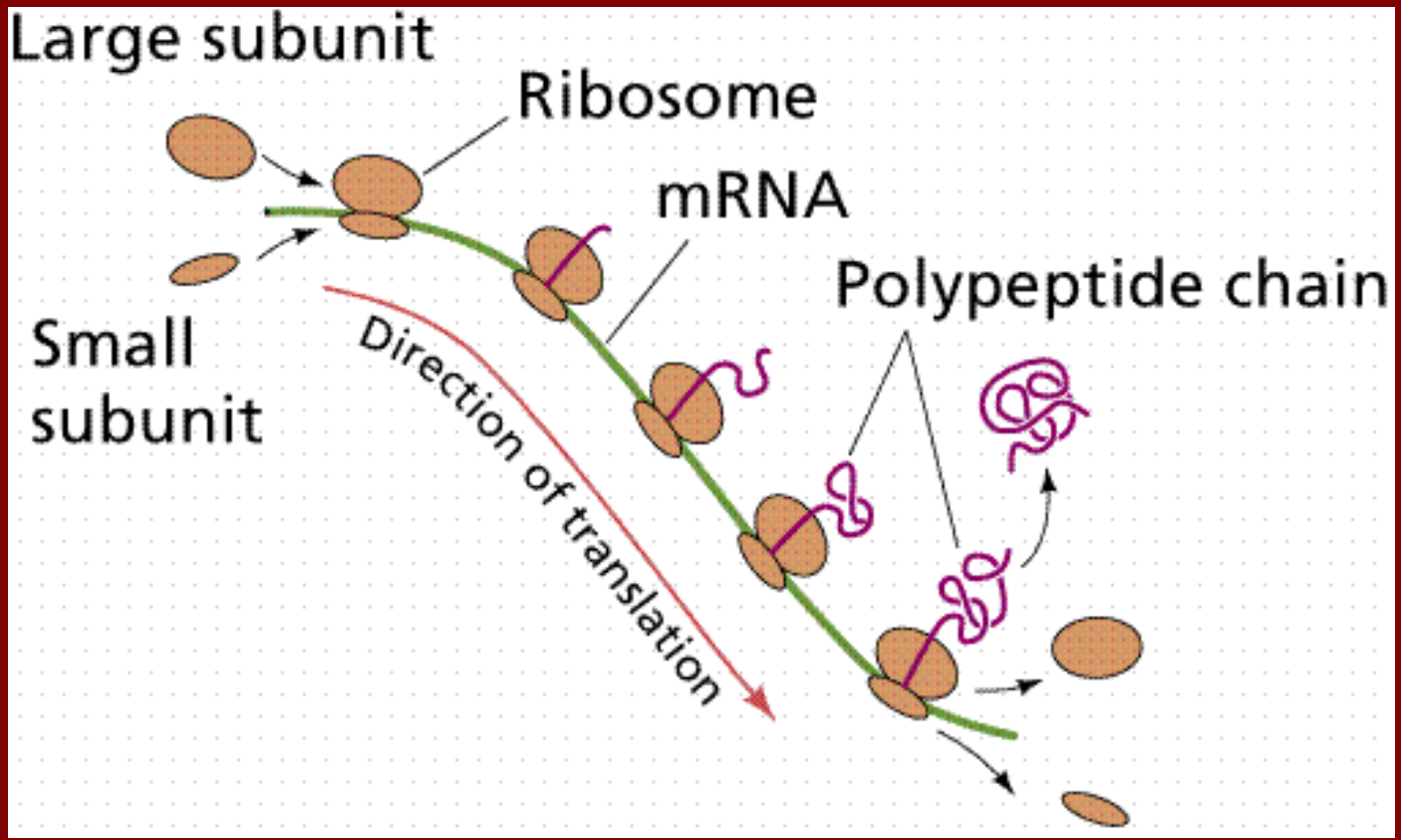



28S
18S
RIN 10
(intact RNA)
RIN 5
(Partially degraded RNA)
RIN 3
(strongly degraded RNA)
Fluorescence
Time (seconds)

Optimise RNA extraction protocol!

- Tissue or cell line
- Cell sorted (FACS, macs beads etc)
- Bio-bank or not
- Preservative (RNAlater) or liquid nitrogen
- Quantity and quality of RNA required for downstream application
- DNAse treatment required or correct primer design.

Polysomal RNA extraction!



Northern/Southern blotting

- Southern blotting is the name given to a technique, originally described by Southern et al, for the transfer of DNA from a gel to a filter.
- Blotting usually refers to the transfer of any molecules from an electrophoresis gel to a solid membrane support.
- DNA or RNA that has been separated and held in this manner can by probed by hybridisation of complementary sequences.
- This complementary sequences are usually radio-labelled by in-vitro transcription of radio-labelled nucleotides (dNTP) usually dCTP.

Northern blotting steps

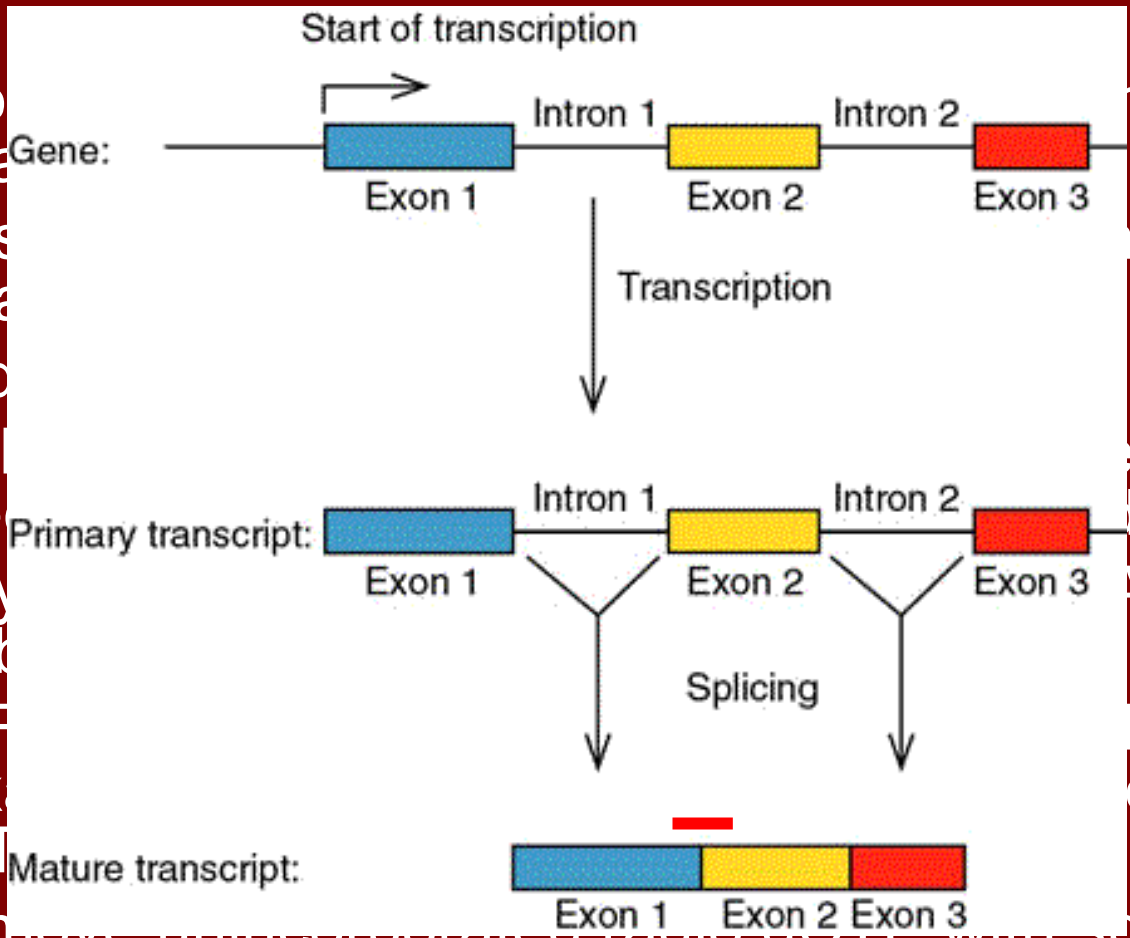
- RNA isolation
- Probe generation
- Denaturing agarose gel electrophoresis
- Transfer to solid support and immobilization
- Pre-hybridisation and hybridisation with probe
- Washing
- Detection
- Stripping and reprobing

Synthesis of radio-labelled probe

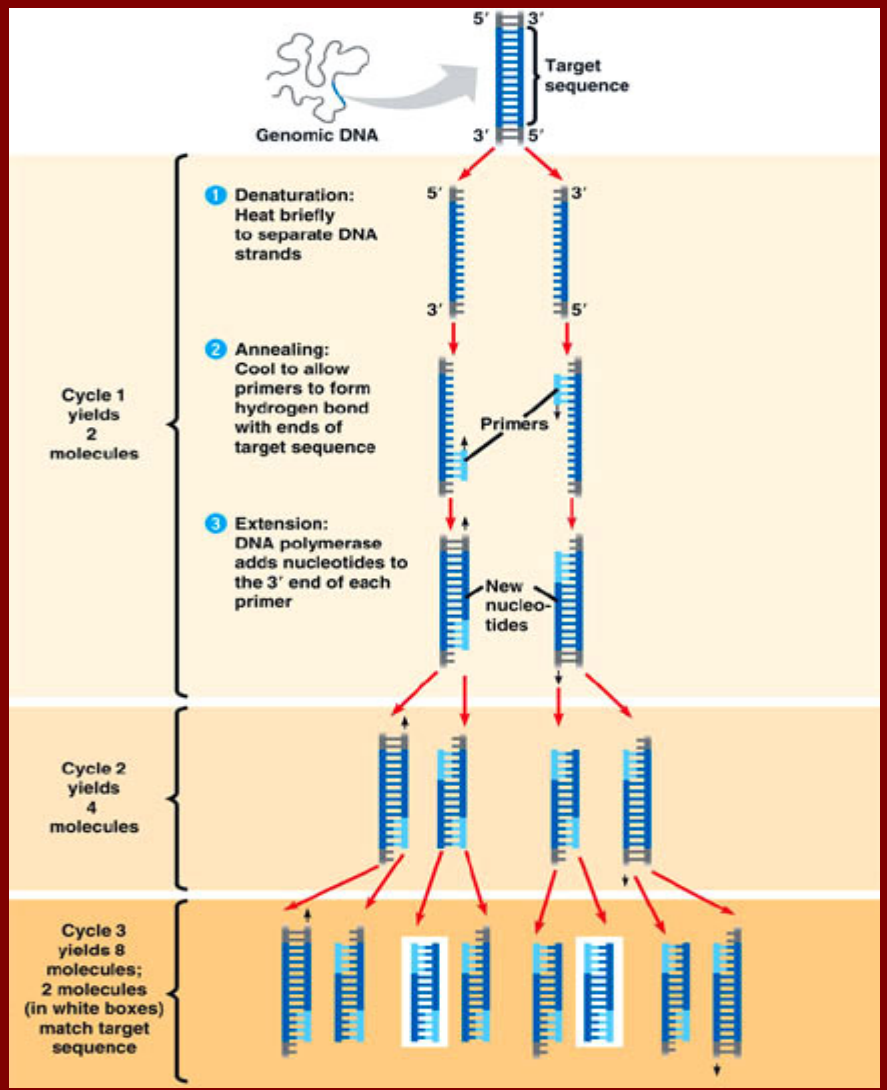
- Radioactively labelled
- Non-isotopically labelled (Biotin)
- 3 main methodologies
  - Random-priming of DNA probe
  - Asymmetric PCR probe generation
  - In vitro transcription of RNA
- Random priming is most traditional technique, best for radioactive probes.

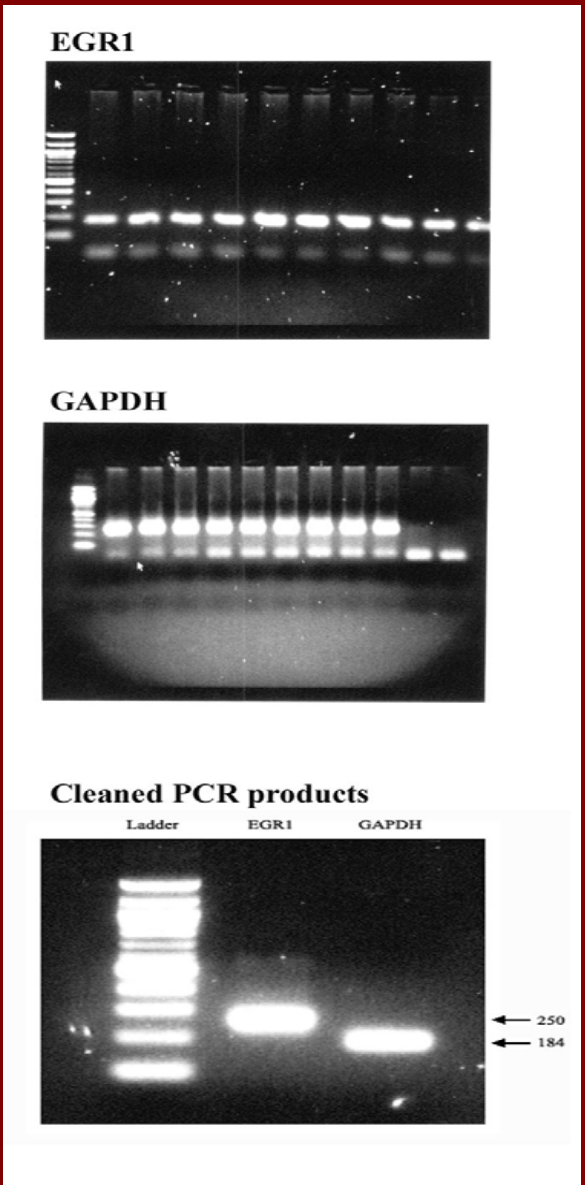
Gene sequence

- In o…mation must be a…
- Blas…e with grea…
- Coo…
- Prob…enough to be spe…lisation.
- Poly…A to generate prob…
- PCR…h random hex…o-labelled dCT…
- Remove unincorporated radio dCTP with spin columns

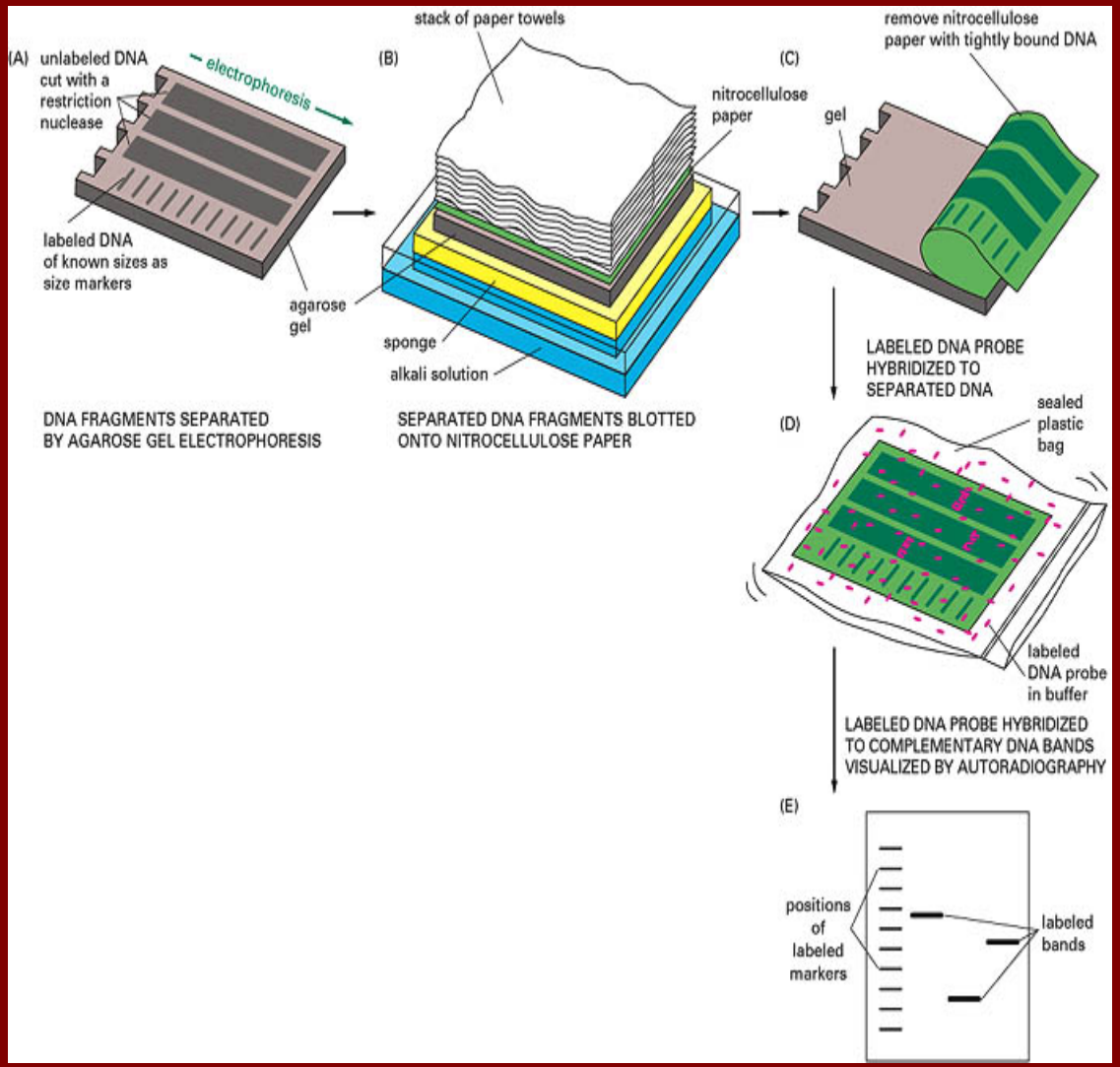


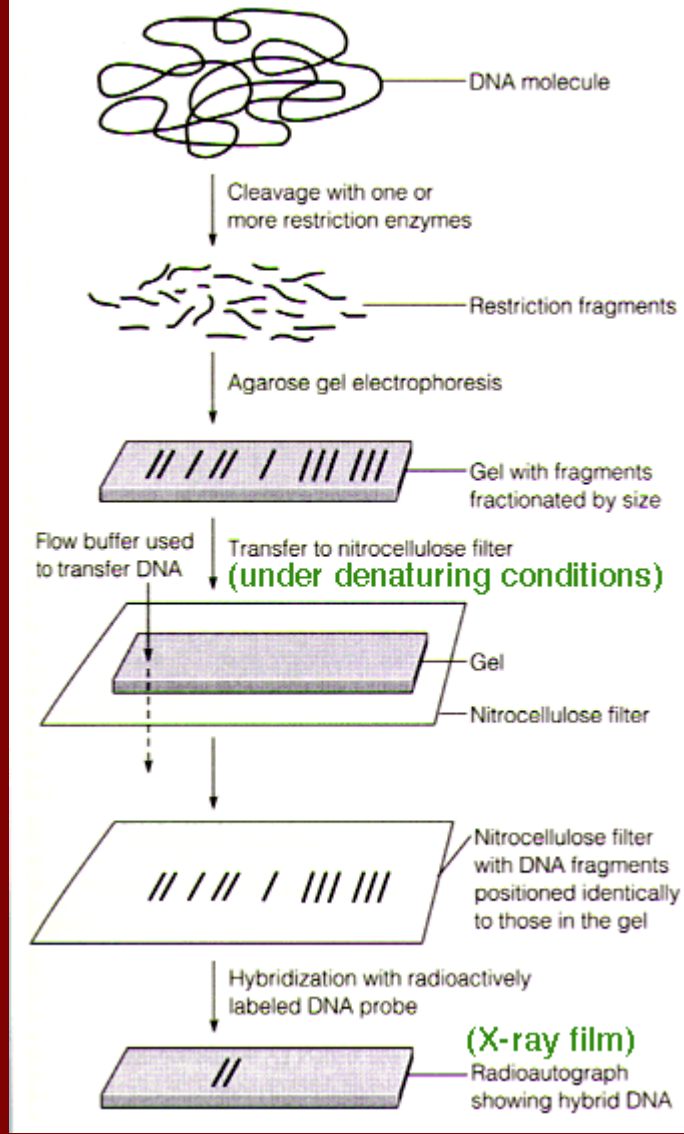
Synthesis of radio-labelled probe

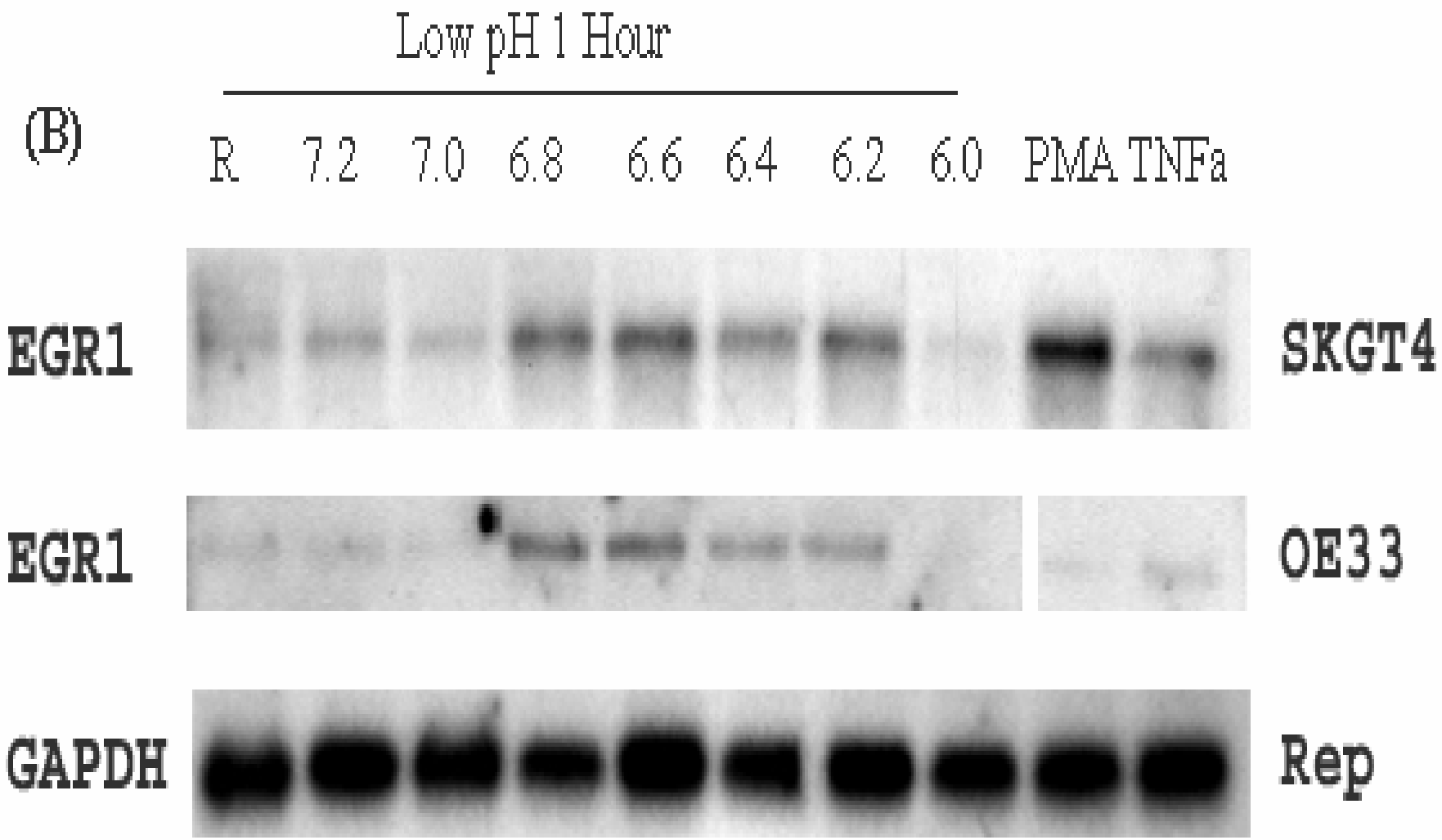


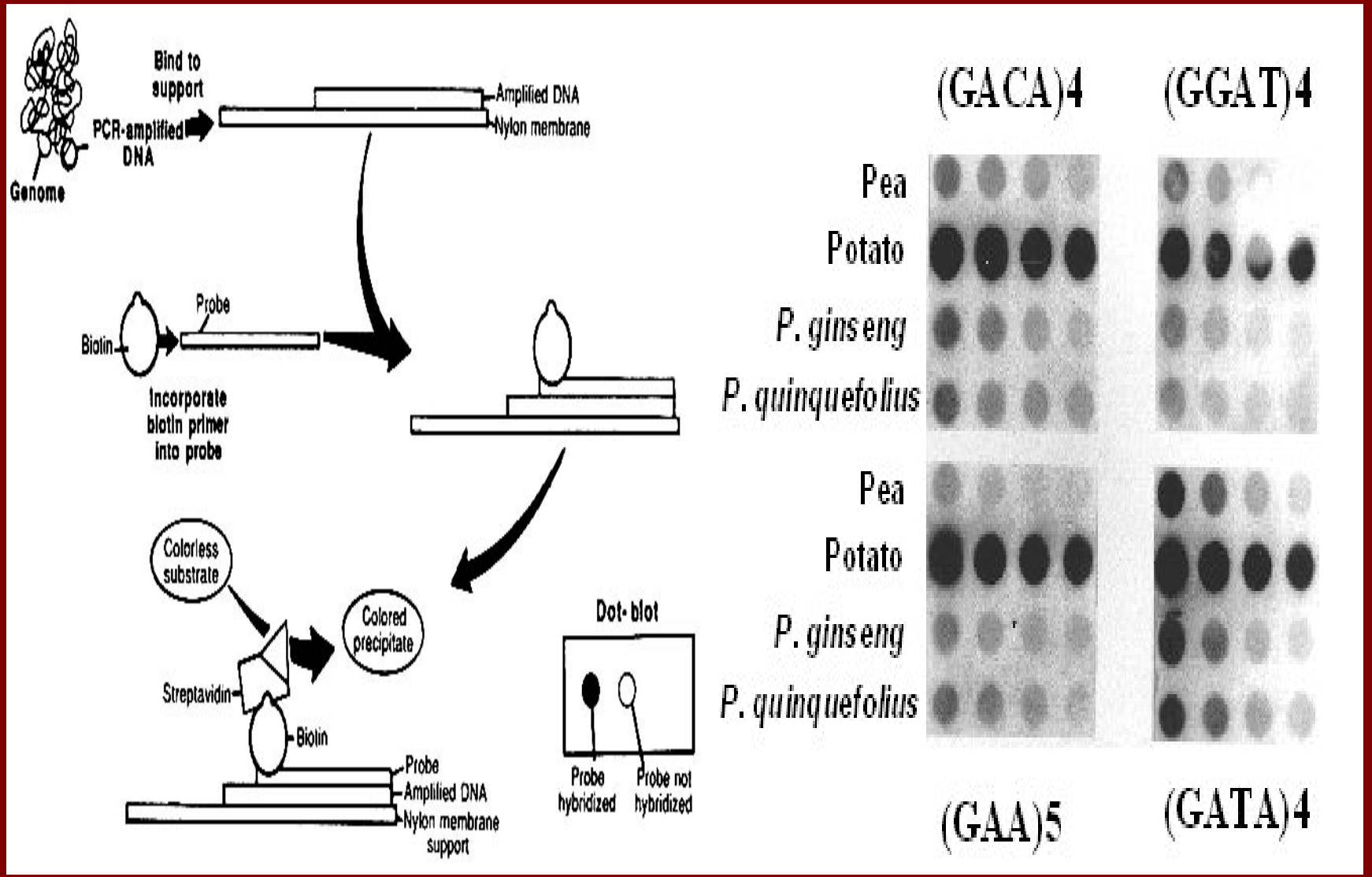


Blotting / Hybridisation





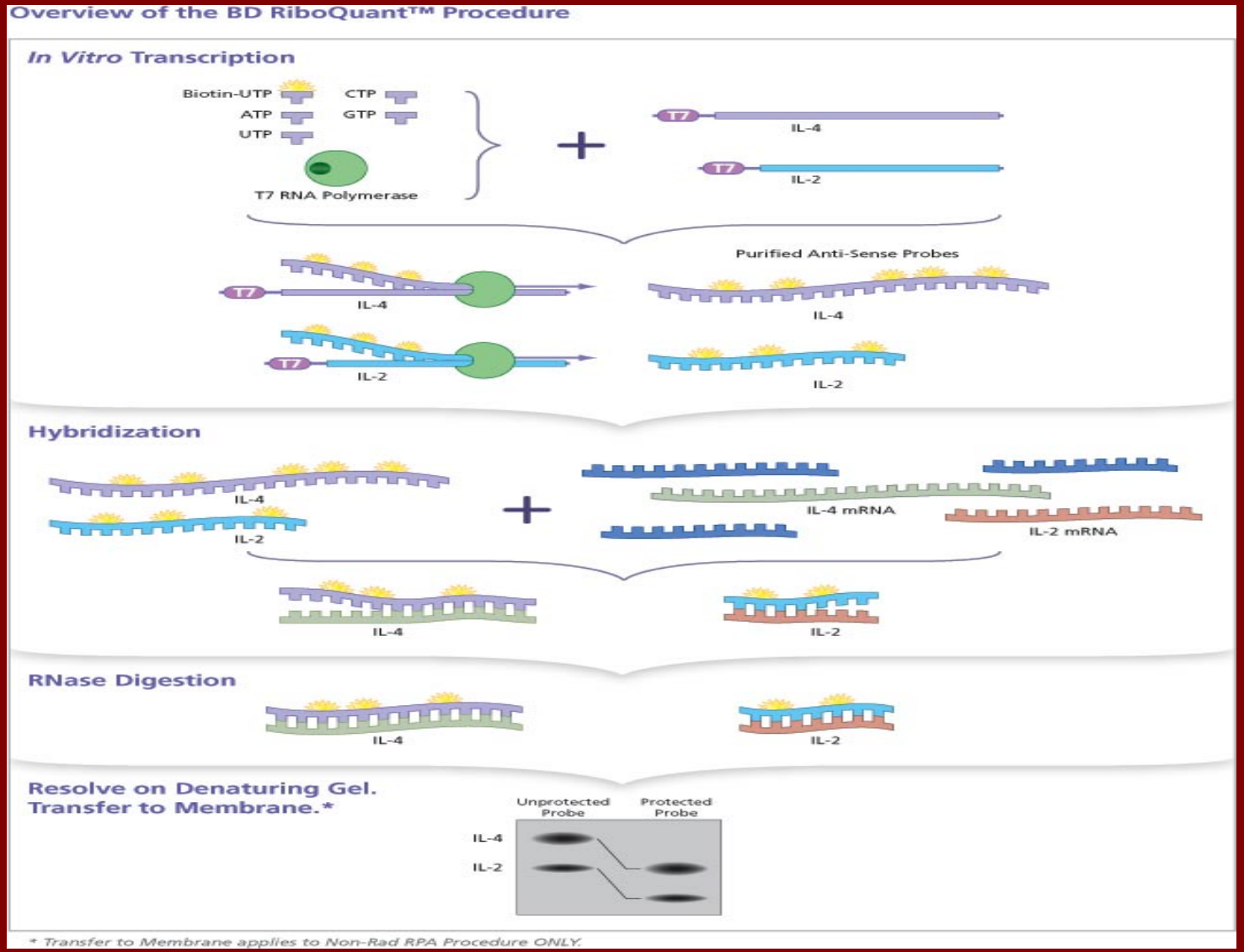

Low pH 1 Hour
(B)
R
7.2
7.0
6.8
6.6
6.4
6.2
6.0
PMA
TNFa
EGR1
SKGT4
EGR1
OE33
GAPDH
Rep

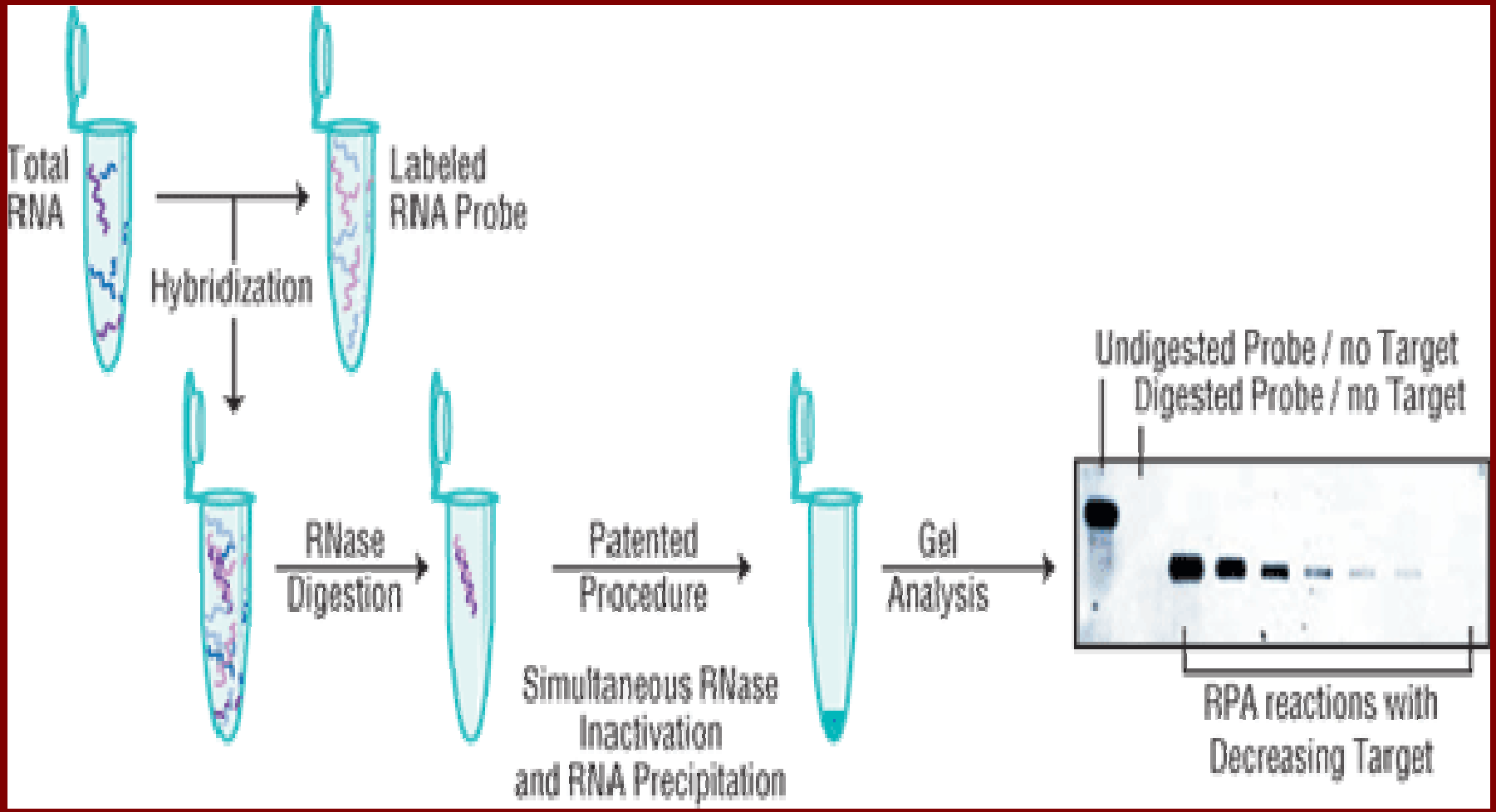

Genome
Bind to support
PCR-amplified DNA
Amplified DNA
Nylon membrane
Probe
Biotin
Incorporate biotin primer into probe
Colorless substrate
Colored precipitate
Streptavidin
Biotin
Probe
Amplified DNA
Nylon membrane support
Dot-blot
Probe hybridized
Probe not hybridized
(GACA)4
(GGAT)4
Pea
Potato
P. ginseng
P. quinquefolius
Pea
Potato
P. ginseng
P. quinquefolius
(GAA)5
(GATA)4

RNAse protection assay

- Northern in a tube
- Probes generated as before
- Increased sensitivity
- Hybridisation occurs in solution where target availability is maximal
- Multiple target/probe acqusition/utilisation is possible, determined by probe length.

RNAse protection assay




Total RNA
Labeled RNA Probe
Hybridization
RNase Digestion
Patented Procedure
Simultaneous RNase Inactivation and RNA Precipitation
Gel Analysis
Undigested Probe / no Target
Digested Probe / no Target
RPA reactions with Decreasing Target

RT-PCR

- Reverse transcription polymerase chain reaction (RT-PCR)
- A specific sequence is repeatedly amplified to generate enough copies of the transcript to be visualised by ethidium bromide staining.
- RNA samples are transcribed into cDNA by a reverse transcriptase and then amplified by DNA polymerase as in PCR for northern probe generation.
- Powerful for rare transcripts and more sensitive than northern blotting and RPA analysis.
- Can be both relative quantification and absolute quantification (competitive RT-PCR)

RT-PCR

- Reverse transcription is a process utilised by RNA viruses in the process of replication.
- Reterotransposons
- Reverse transcription is performed at 42ºC using a reverse transcriptase such as M-MLV (Moloney murine leukemia virus)
- An oligo dT primer is utilised to prime the reaction
- It can be performed with minute quantities of RNA.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

LON V. KENDALL, DVM AND LELA K. RILEY, PHD

Purpose: Reverse transcriptase polymerase chain reaction (RT-PCR) is similar to the PCR except its allows amplification of small amounts of ribonucleic acid (RNA). RT-PCR is used to detect viruses with an RNA genome and to detect RNA transcripts.

Method:

FIG. 1. RNA template. Prior to initiating reverse transcription the template RNA must be isolated from the sample to be tested. This figure shows a polyadenylated mRNA.

FIG. 2. Priming for reverse transcription. To generate cDNA using the enzyme reverse transcriptase (RT), a primer is annealed to the template RNA. The primer can be gene specific primers, random primers or oligo-dT primers for mRNA. In this example, oligo-dT primers are used to initiate cDNA synthesis from mRNA.

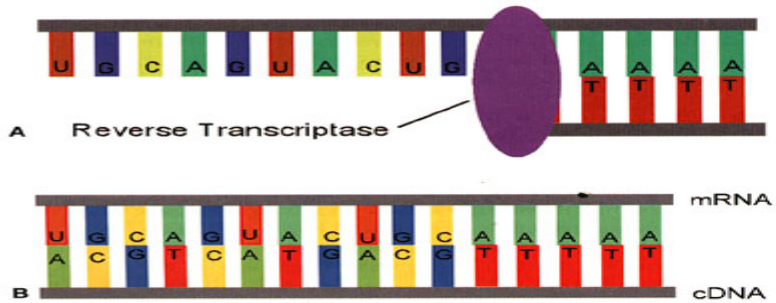


FIG. 3. First strand synthesis. The first strand of cDNA is synthesized using RT. Beginning at the primer annealing site (A), RT adds complementary nucleotide bases to the mRNA strand creating a strand of cDNA (B).

Research Animal Diagnostic and Investigative Laboratory, Department of Veterinary Pathobiology, University of Missouri, Columbia

FIG. 4. Removal of RNA. The template strand of RNA is removed by treatment with RNAse H. The cDNA can now be used for amplification by PCR.

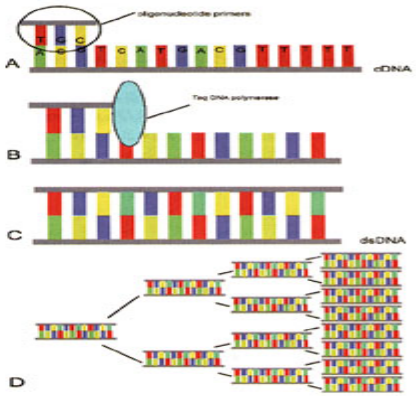


FIG. 5. The PCR reaction. The oligonucleotide primer is allowed to anneal to the template cDNA (A). *Taq* polymerase adds complementary nucleotides beginning at the primer annealing site (B). The resultant product is a double stranded cDNA (C). The three step process of denaturation, primer annealing and extension are repeated to yield a detectable PCR product (D). The product can be visualized on an ethidium bromide stained agarose gel following electrophoresis.

Alternative Techniques: Northern blot analysis, RNAse Protection Assay

Advantages: 1) RT-PCR has high sensitivity due to exponential amplification of the template RNA. 2) RT-PCR is very specific when using gene specific primers in the synthesis of cDNA. 3) The RT-PCR technique can be completed in one to two working days providing rapid results.

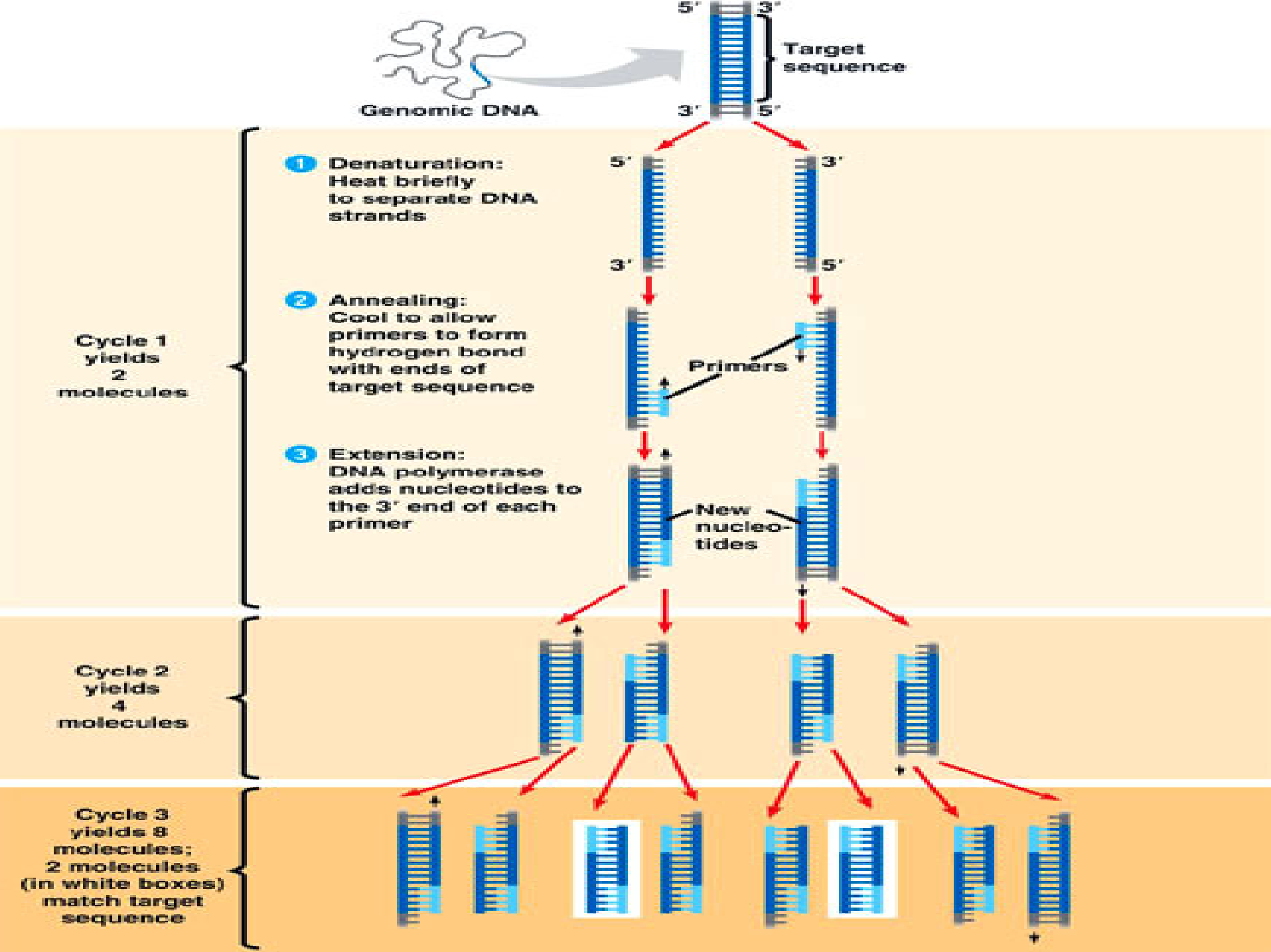
Disadvantages: 1) Similar to those seen with PCR. 2) RT-PCR detects transcripts, not functional protein.

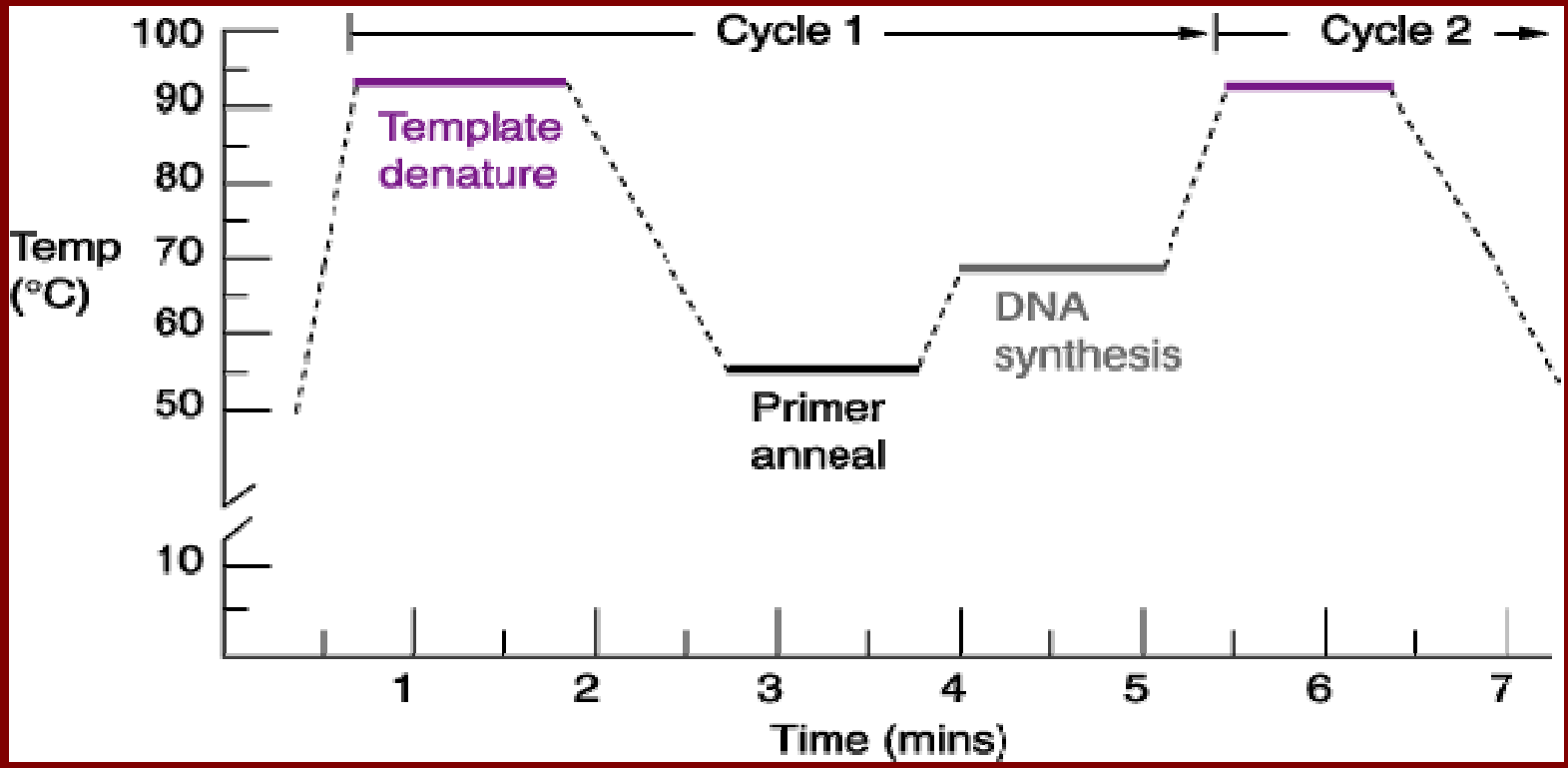
References

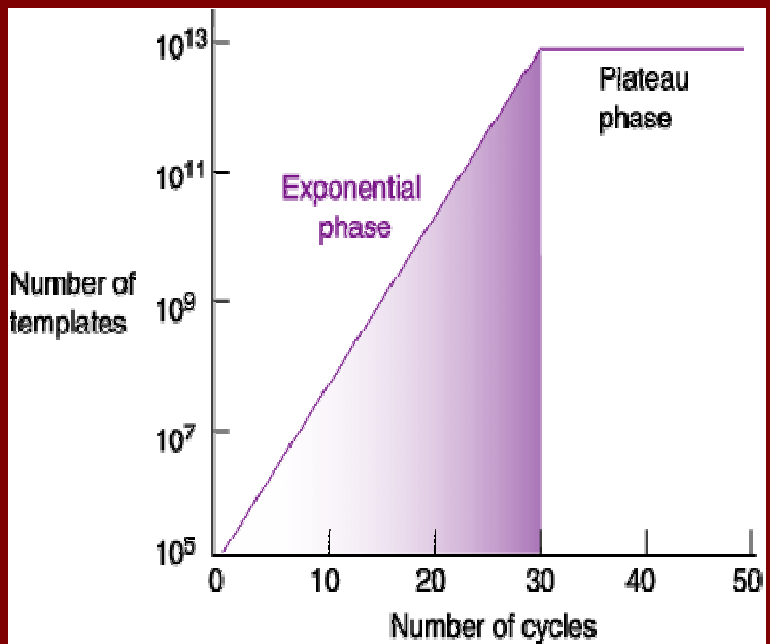
1. **Sellner, L. N. and G. R. Turbett.** 1998. Comparison of three RT-PCR methods. Biotechniques. **25(2):**230–234.
2. RNA-Dependent DNA polymerase. Contributed by Stanley Tabor. *In* Current Protocols in Molecular Biology. Ed. Ausubel F.M., Brent R., Kingston R.E. et al. Volume 1. Unit 3.7. John Wiley and Sons, Inc. 1997.

RT-PCR

- Following the generation of a cDNA template the gene of interest is amplified in a cyclical manner by DNA polymerase:-
  - Denaturation (94ºC) Double stranded DNA is made single stranded.
  - Annealing (Determined by primers 54ºC) Primers bind to complementary DNA sequences
  - Extension (72ºC) DNA pol. can now transcribe new complementary stretches of DNA starting at the site of primer annealing, incorporates nucleotides in a 5'to3' direction reading the sequence in a 3'to5' manner.


Genomic DNA
5′
3′
Target sequence
3′
5′
Cycle 1 yields 2 molecules
1 Denaturation: Heat briefly to separate DNA strands
2 Annealing: Cool to allow primers to form hydrogen bond with ends of target sequence
Primers
3 Extension: DNA polymerase adds nucleotides to the 3′ end of each primer
New nucleotides
Cycle 2 yields 4 molecules
Cycle 3 yields 8 molecules; 2 molecules (in white boxes) match target sequence

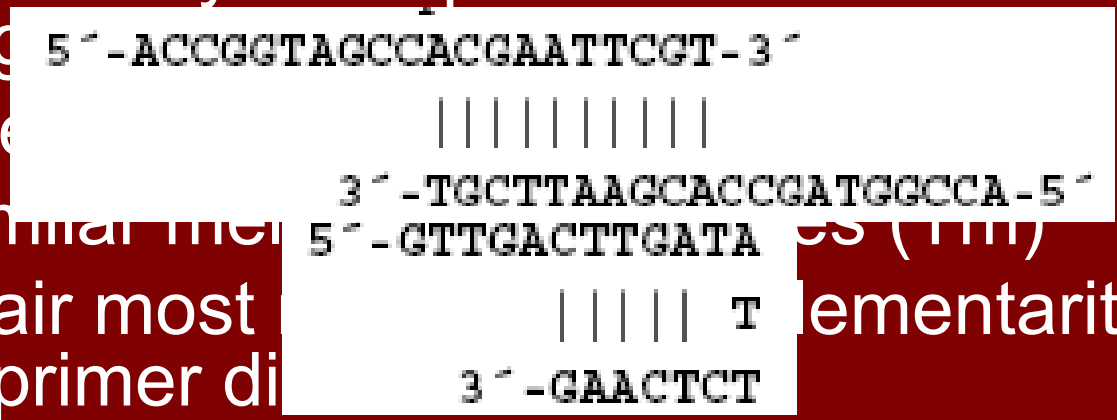




Cycle	Copies
1	2
2	4
4	16
10	1,024
15	32,768
20	1,048,576
25	33,554,432
30	1,073,741,824

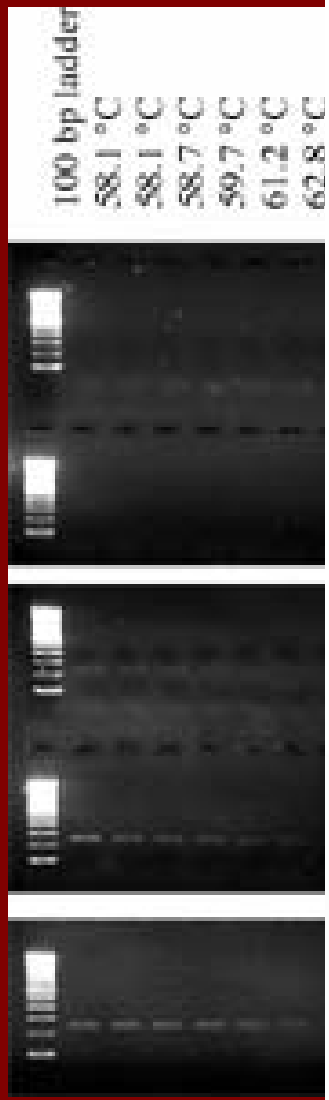
Primer design

- Complementary to sequence of interest! Blast searching
- Length be
- Share sim
- Primer pair most
- No palindromic sequences, hairpin loops.
- G/C content 45-50%
- Distance between primers, variable (150-500)
- Intron-exon boundaries!!!

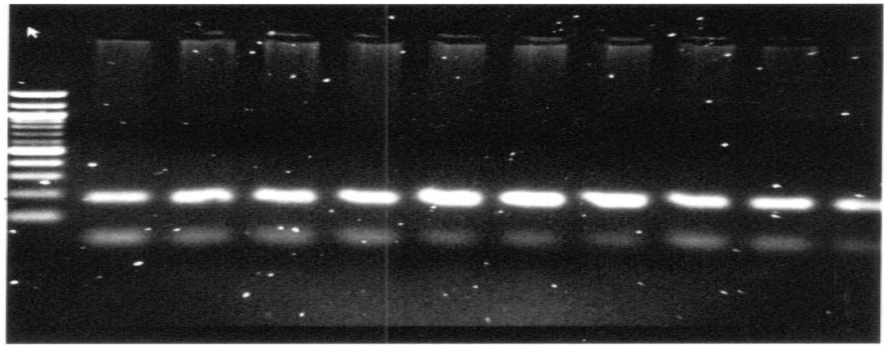


RT-PCR optimisation

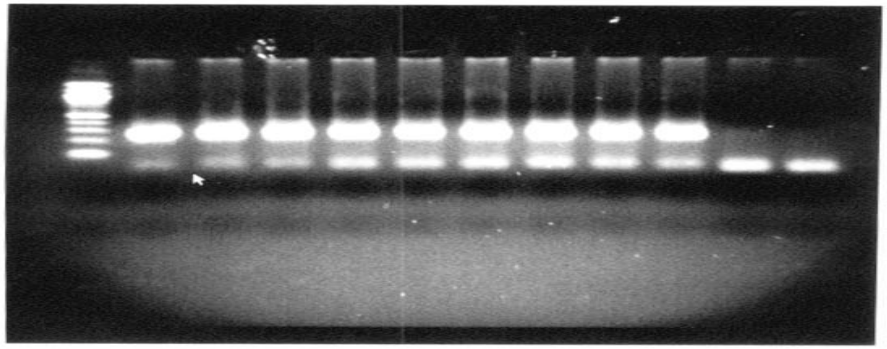
- Most of the component of a PCR reaction may be optimised to obtain maximum specificity and sensitivity:
  - Mg^{++} concentration
  - Template DNA concentration
  - *Taq* DNA polymerase used (proof reading exonuclease)
  - dNTPs concentration (high may inhibit)
  - Primer to template ratio
  - Thermal cycling (1000bp/min extension, increase denaturation if high GC content in primer)
- Controls:- no template, no primer.

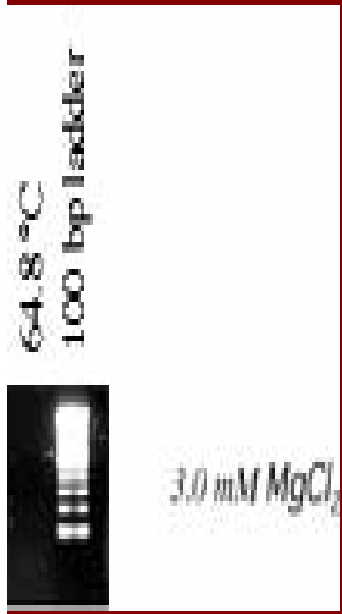


EGR1

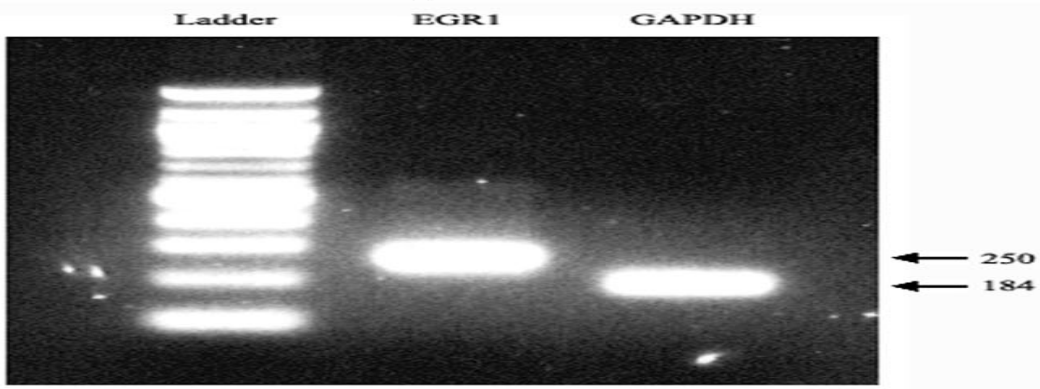


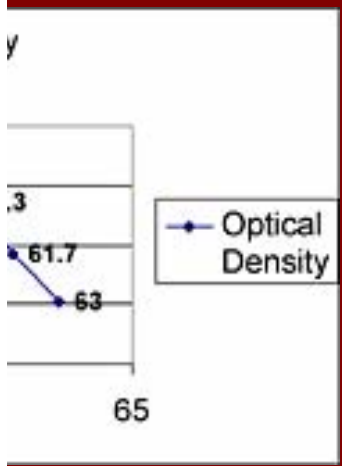
GAPDH



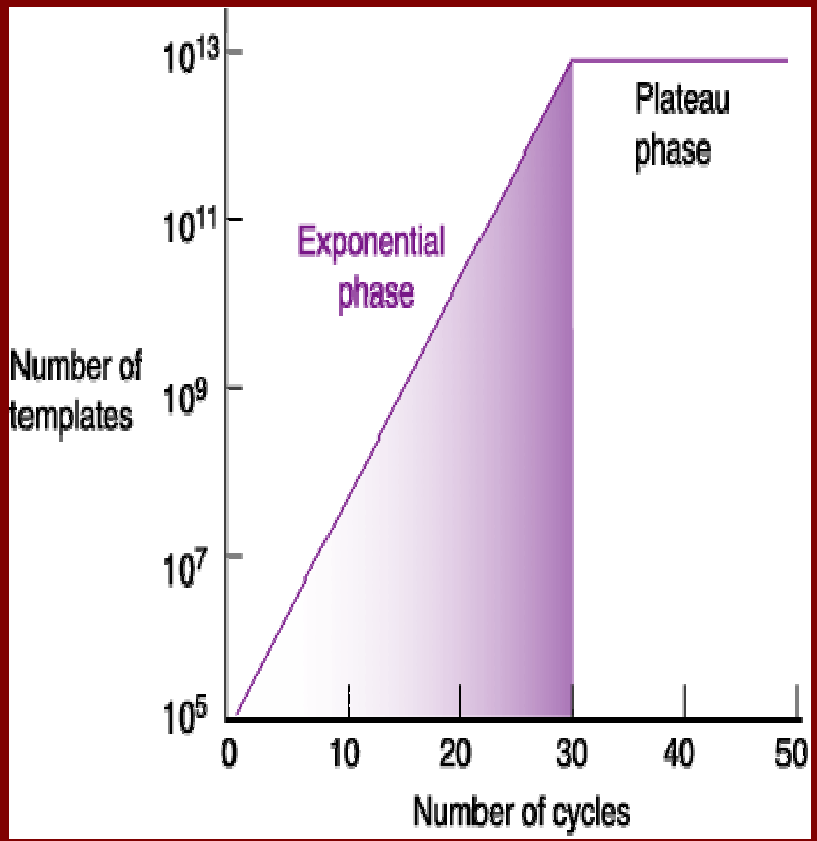


Cleaned PCR products

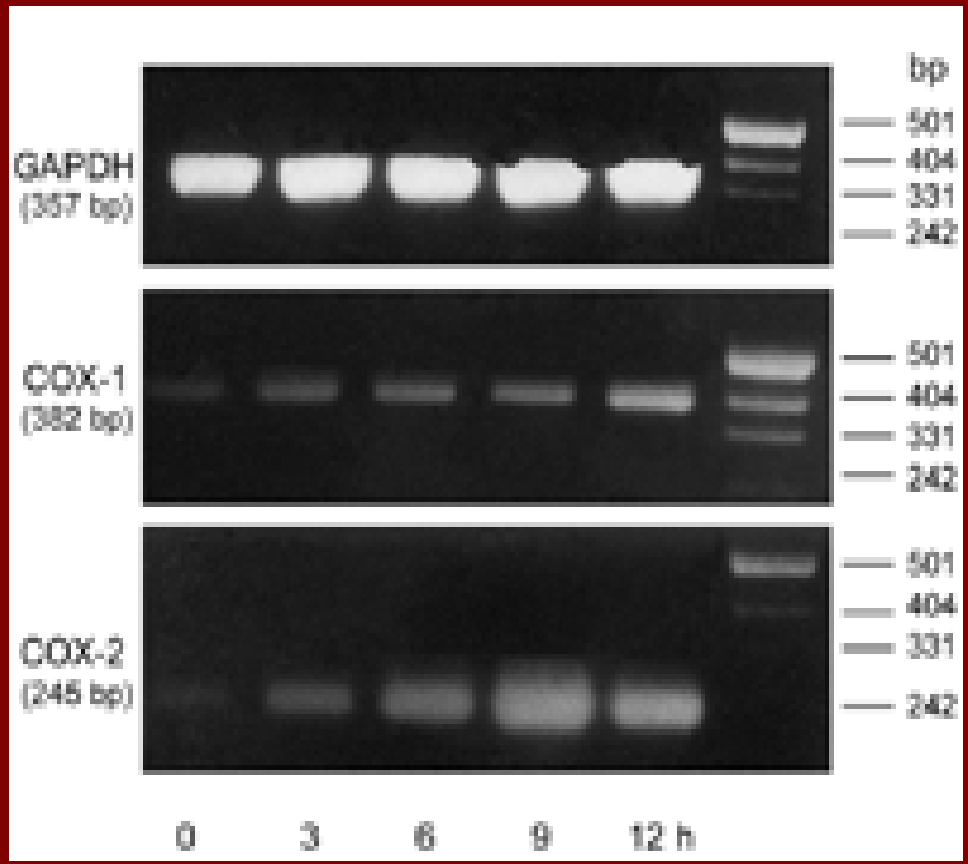




Cycle limitation



The Result

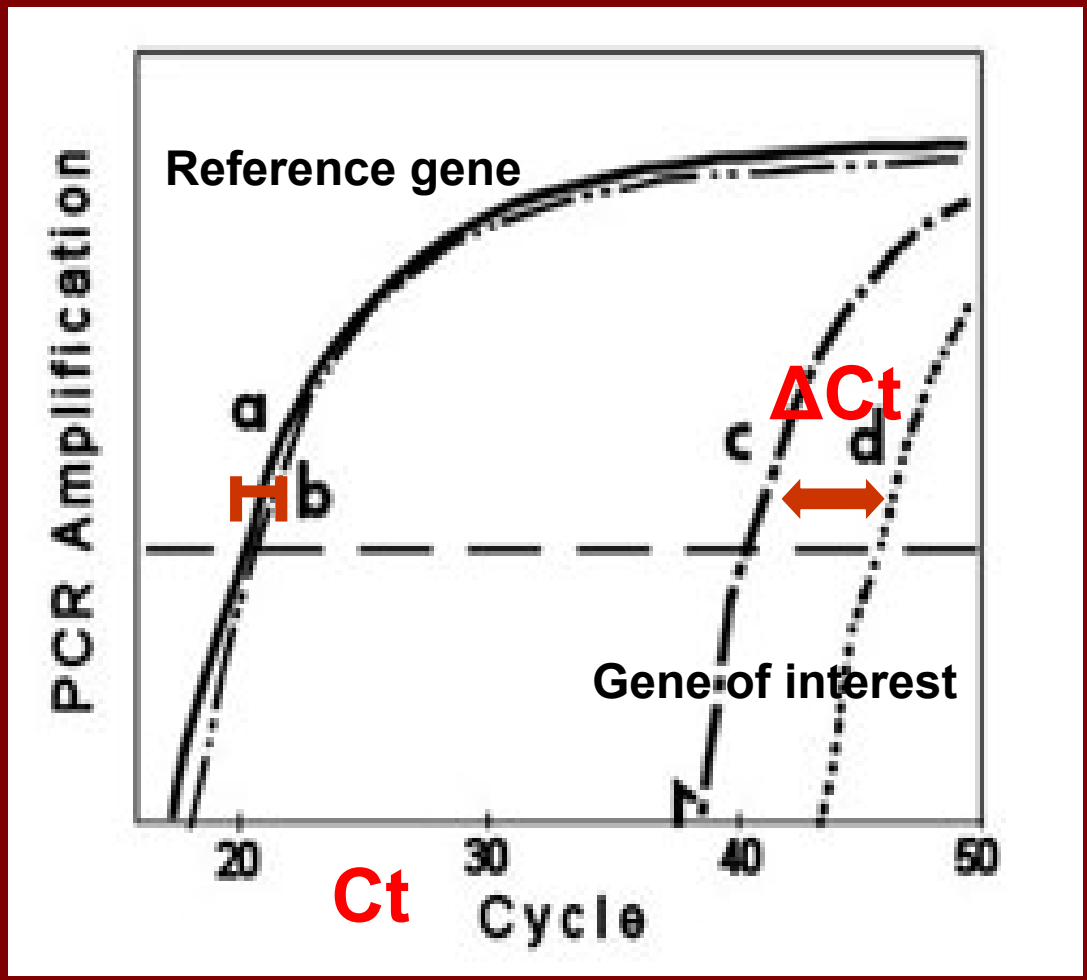


Competitive RT-PCR?

Real-time RT-PCR

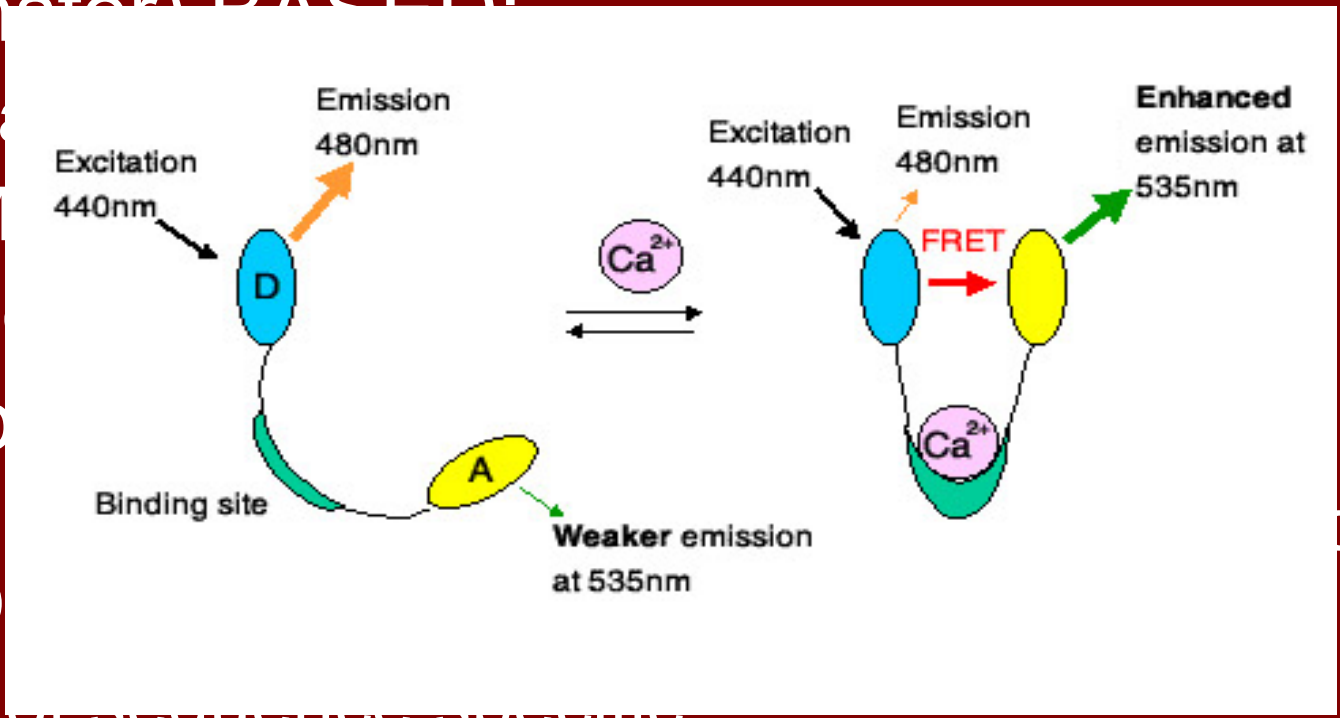
- Dramatic increase in sensitivity achieved!
- Fluorescent based version of RT-PCR
- Increases in PCR product is directly proportional to a fluorescent reporter.
- This reporter may be viewed in a live mode thus giving rise to real-time.
- A profile of the PCR is then generated charting the progress through the exponential phase to the plateau phase.
- A point in the linear phase may be chosen to examine gene expression differences between samples.
- The earlier this line is reached the more copies of that particular transcript was present.
- These movements are normalised by reference to a control house keeping gene if one is available.

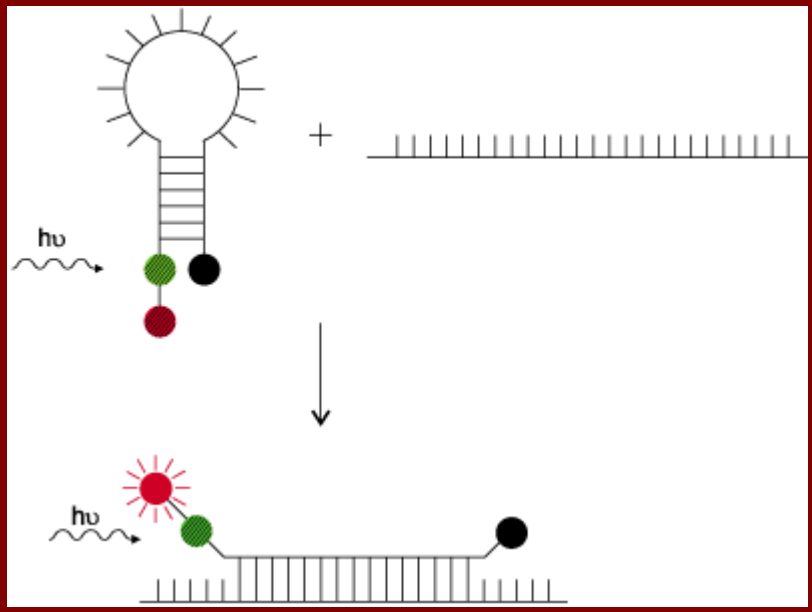
The concept

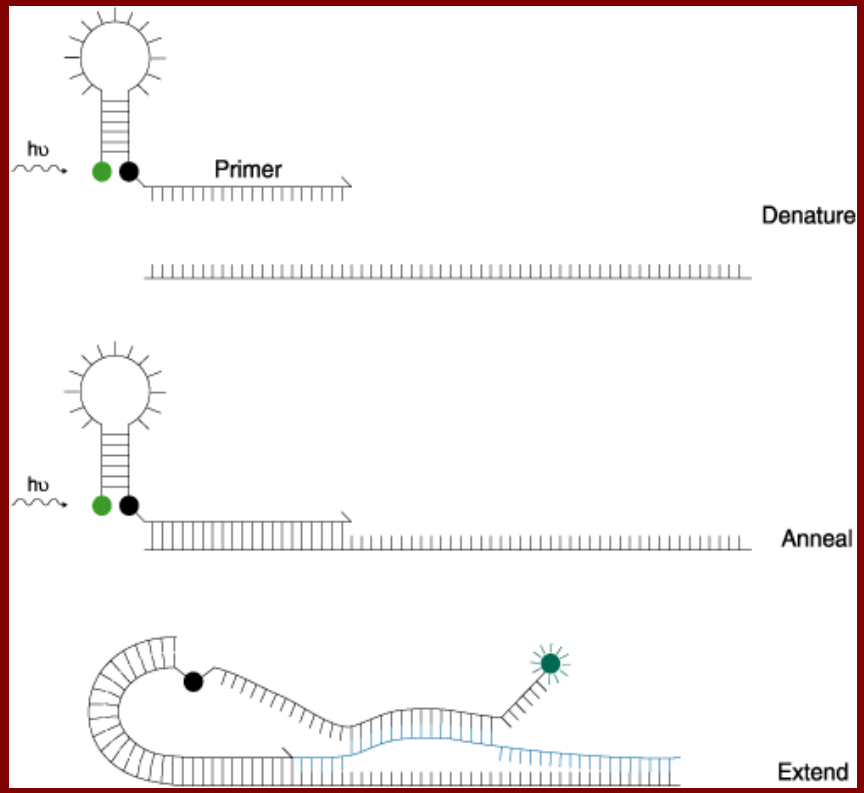

PCR Amplification
Reference gene
a
b
c
d
ΔCt
Gene of interest
20
30
40
50
Ct
Cycle

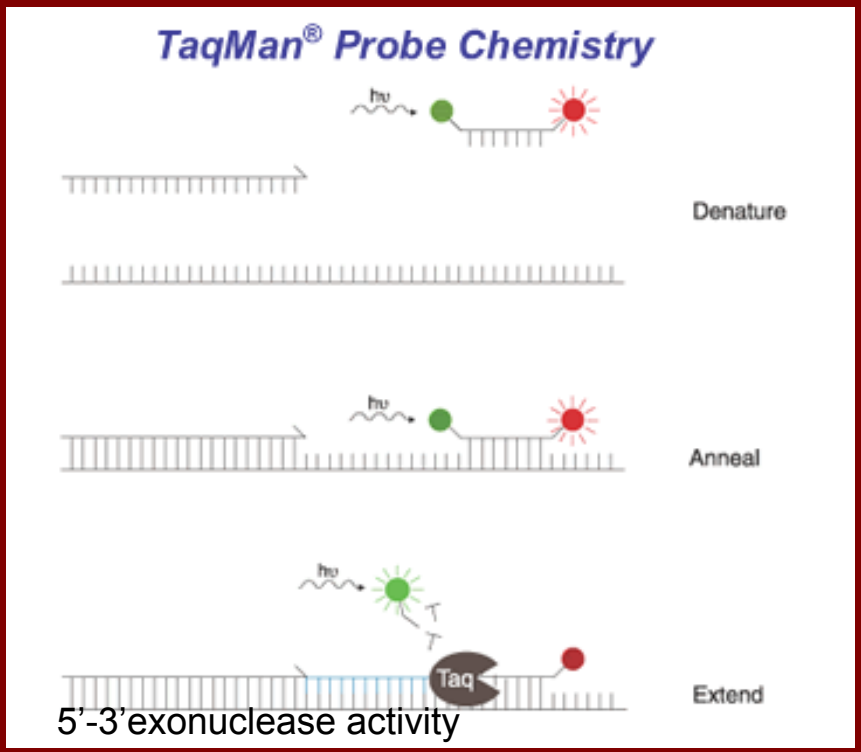
Examples

- FRET (Fluorescence resonance energy transfer) BASED:
  - Ta
  - M
  - S
- Syb
  - E
    D
  - Not sequence specific





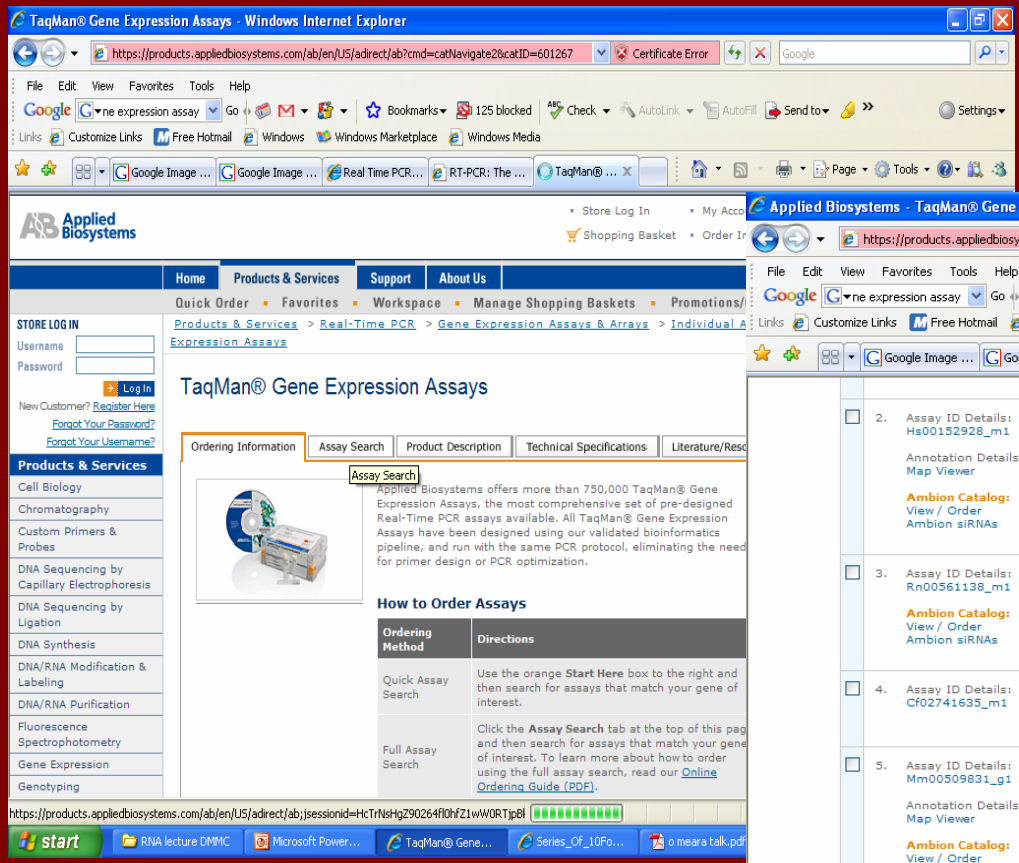


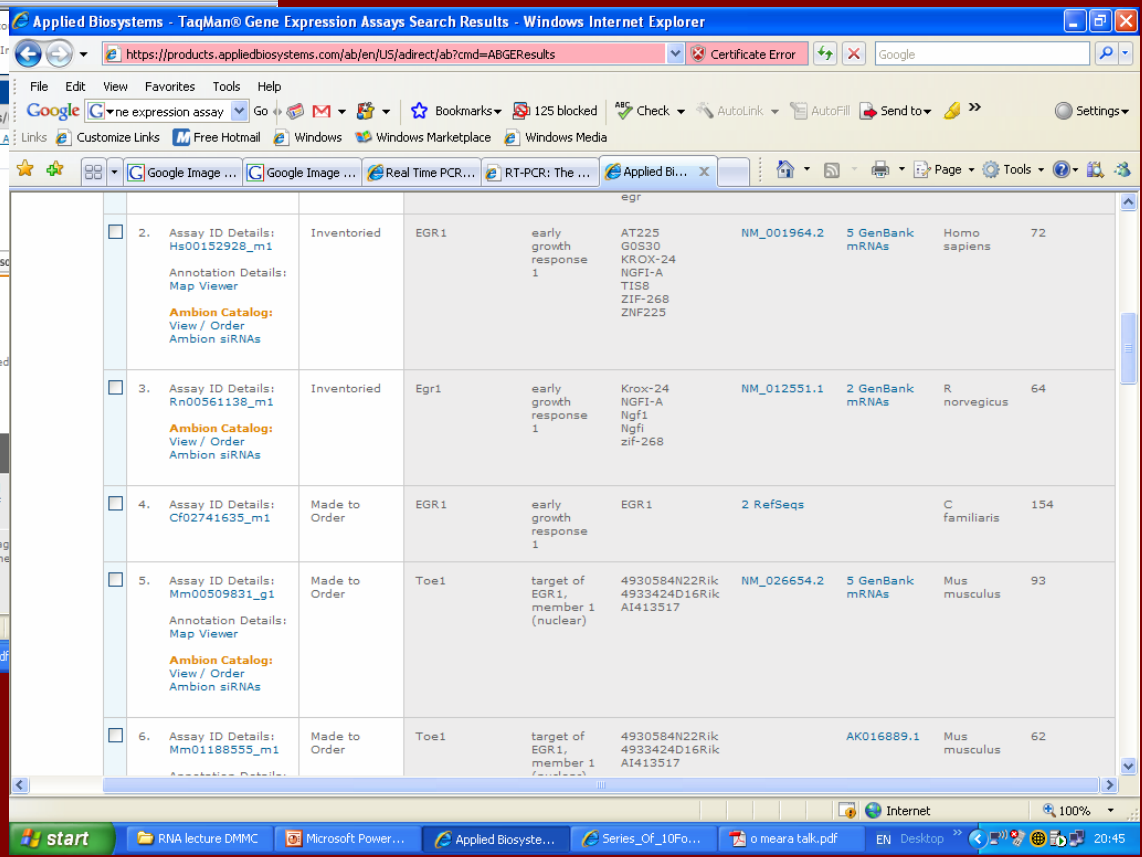


5’-3’exonuclease activity

Numerous dyes available
Reporter
•FAM, VIC, TET, JOE, HEX
Quencher
•TAMRA
Reference dye
•ROX, internal signal standard

Genes of interest





Efficiencies 100%

All function with identical PCR cycling conditions

Edit View Tools Instrument Analysis Window Help

Untitled 1 - Absolute Quantification

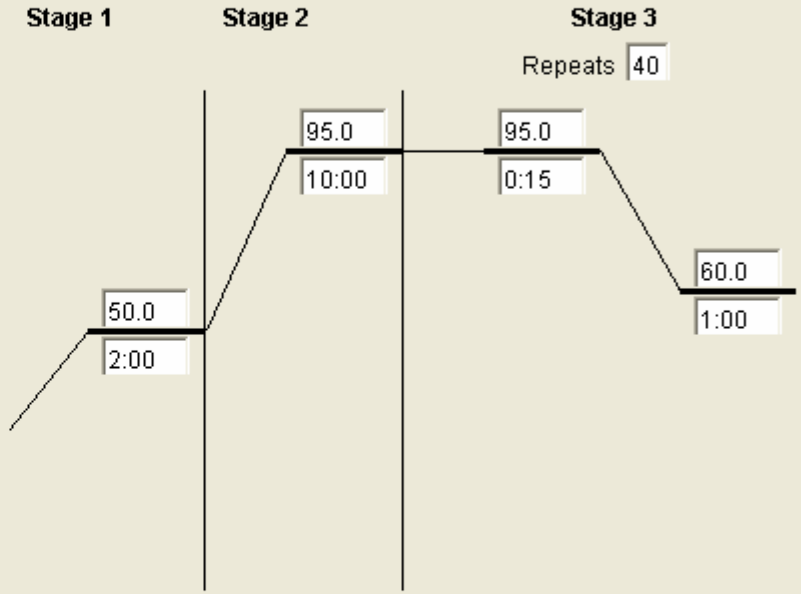
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20

tion	Sample	Detector	Task	Ct	Quantity
	A1				
	A2				
	A3				
	A4				
	A5				
	A6				
	A7				
	A8				
	A9				
	A10				
	A11				
	A12				
	A13				
	A14				
	A15				
	A16				

Setup | Instrument

Thermal Cycler Protocol

Thermal Profile | Auto Increment | Ramp Rate | Data Collection



Add Cycle | Add Hold | Add Step | Delete Step

Add Dissociation Stage

Sample Volume (µl

20

9600 Emulation

Real-Time | Queue

Start | Stop | Connect | Open/Close

Status: Disconnected

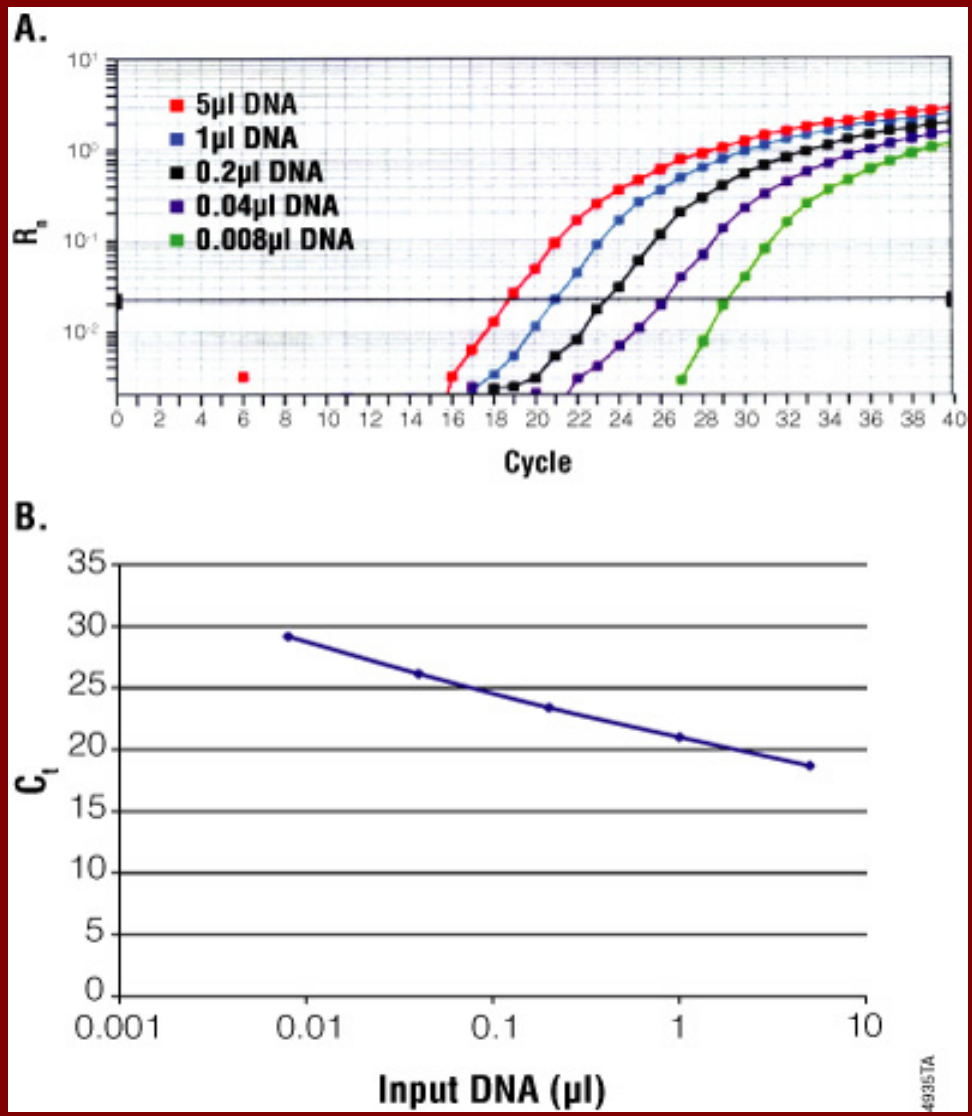
Time Remaining:

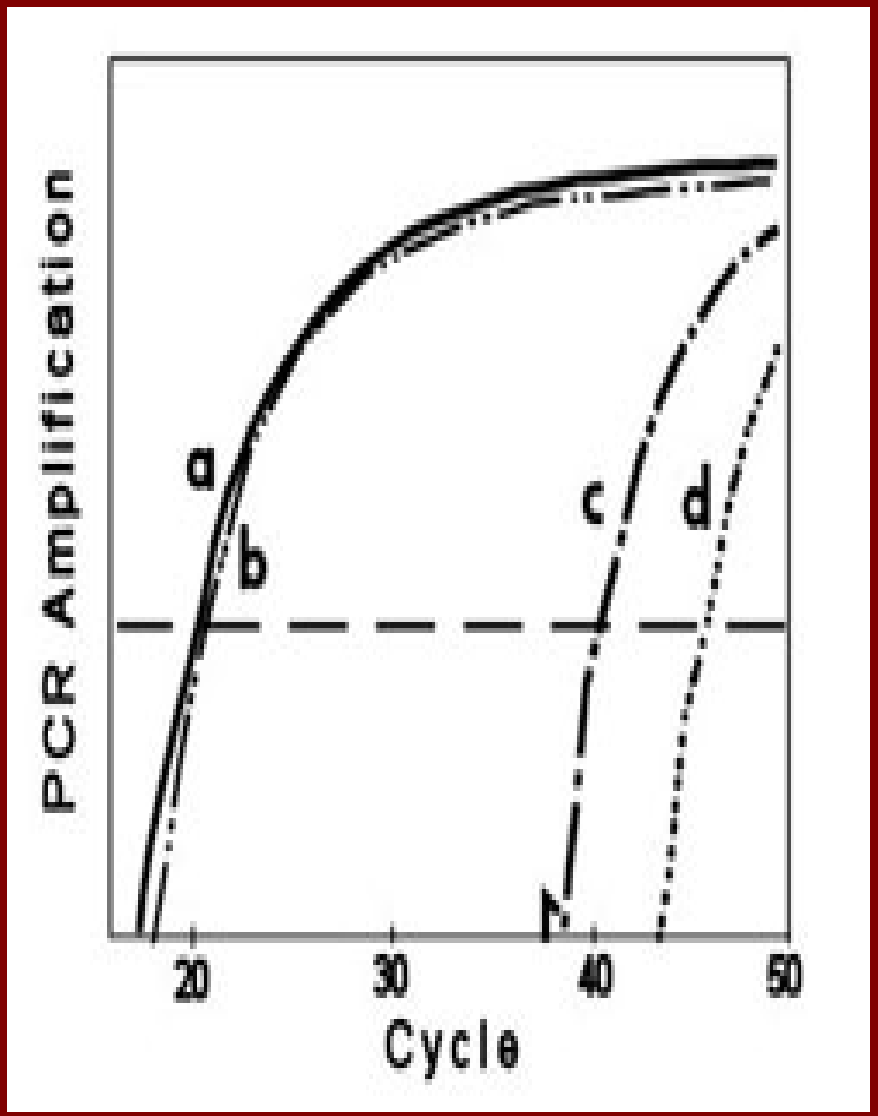
Disconnect

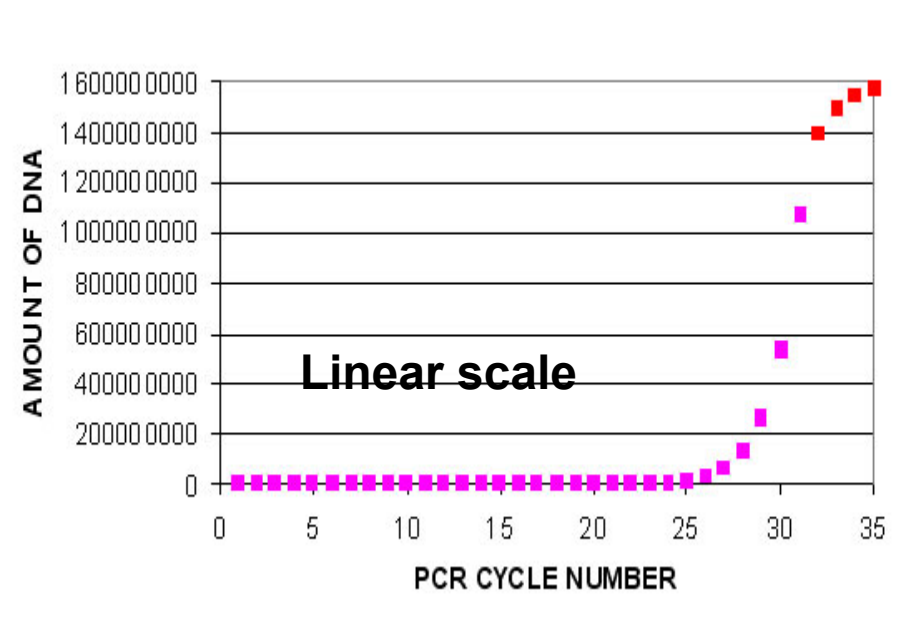
VIP

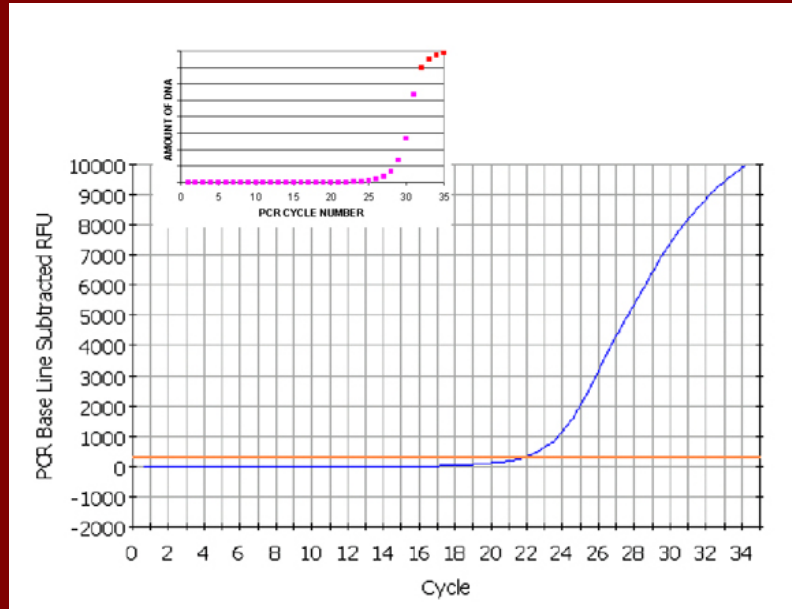
- Prior to performing real time RT-PCR expts it is important to determine an appropriate control housekeeping gene.
- Hypothesis, no such thing as a housekeeping gene????!
- Test a panel of non-changing genes GAPDH, Actin. 18srRNA etc
- Chose the gene with minimal variation across your samples when equal amounts of starting material has been used.

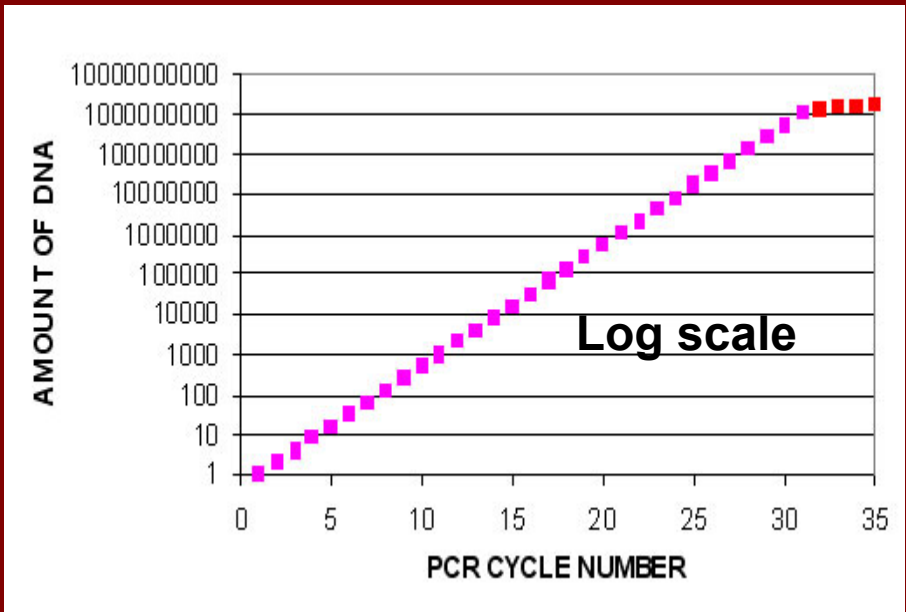
PCR profile

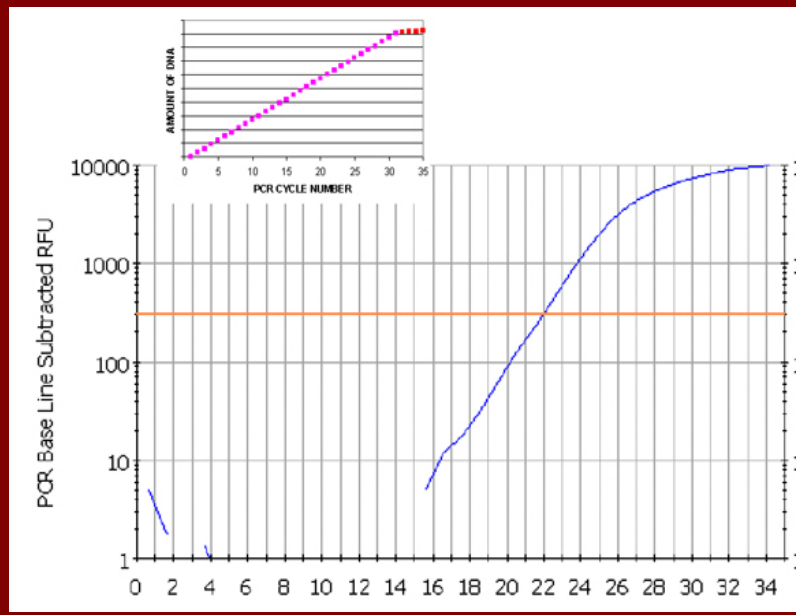

A.
5µl DNA
1µl DNA
0.2µl DNA
0.04µl DNA
0.008µl DNA
R_n
Cycle
B.
C_t
Input DNA (µl)
0.001
0.01
0.1
1
10

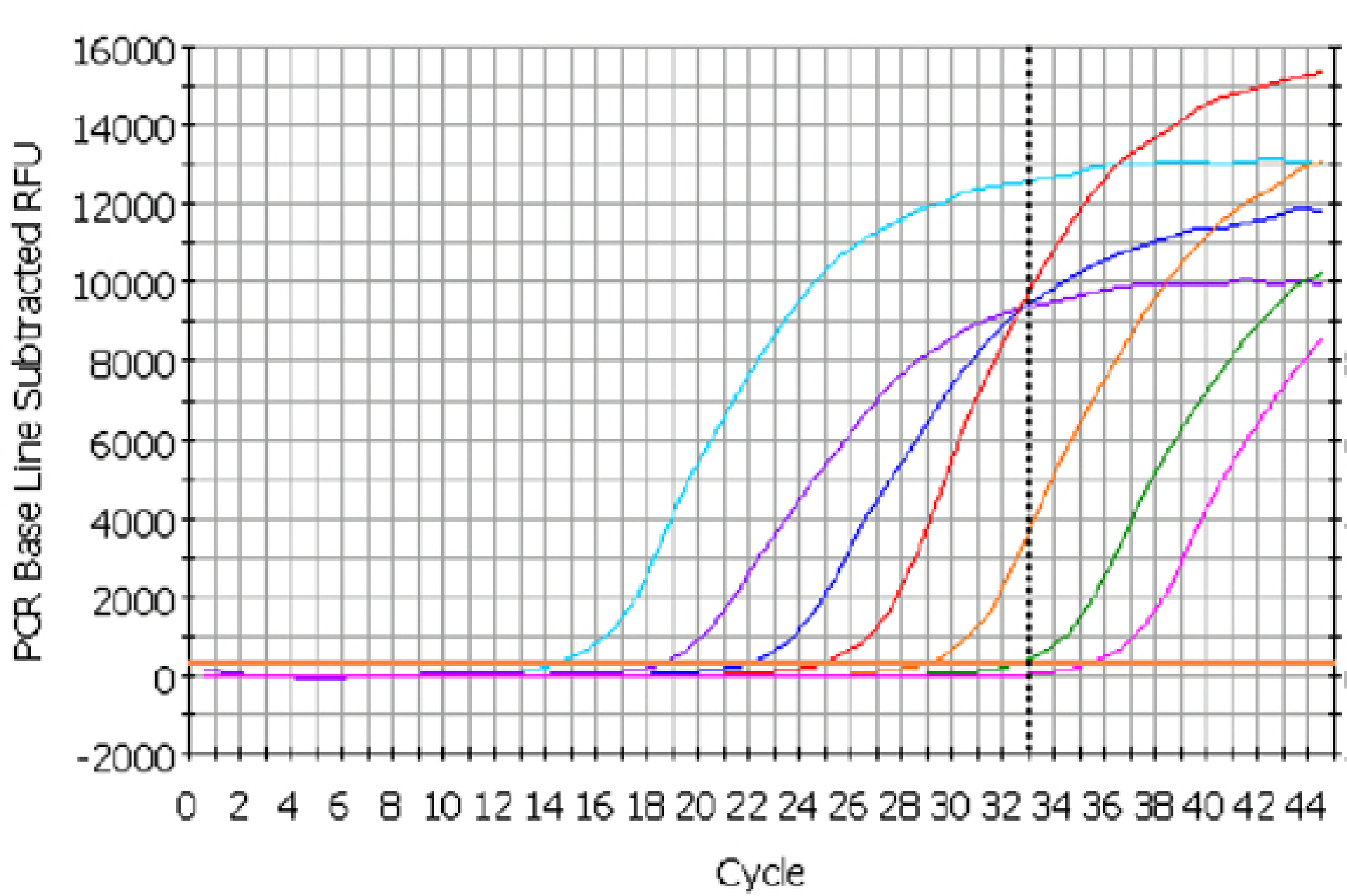

PCR Amplification
a
b
c
d
Cycle
20
30
40
50


AMOUNT OF DNA
160000 0000
140000 0000
120000 0000
100000 0000
80000 0000
60000 0000
40000 0000
20000 0000
0
Linear scale
0 5 10 15 20 25 30 35
PCR CYCLE NUMBER

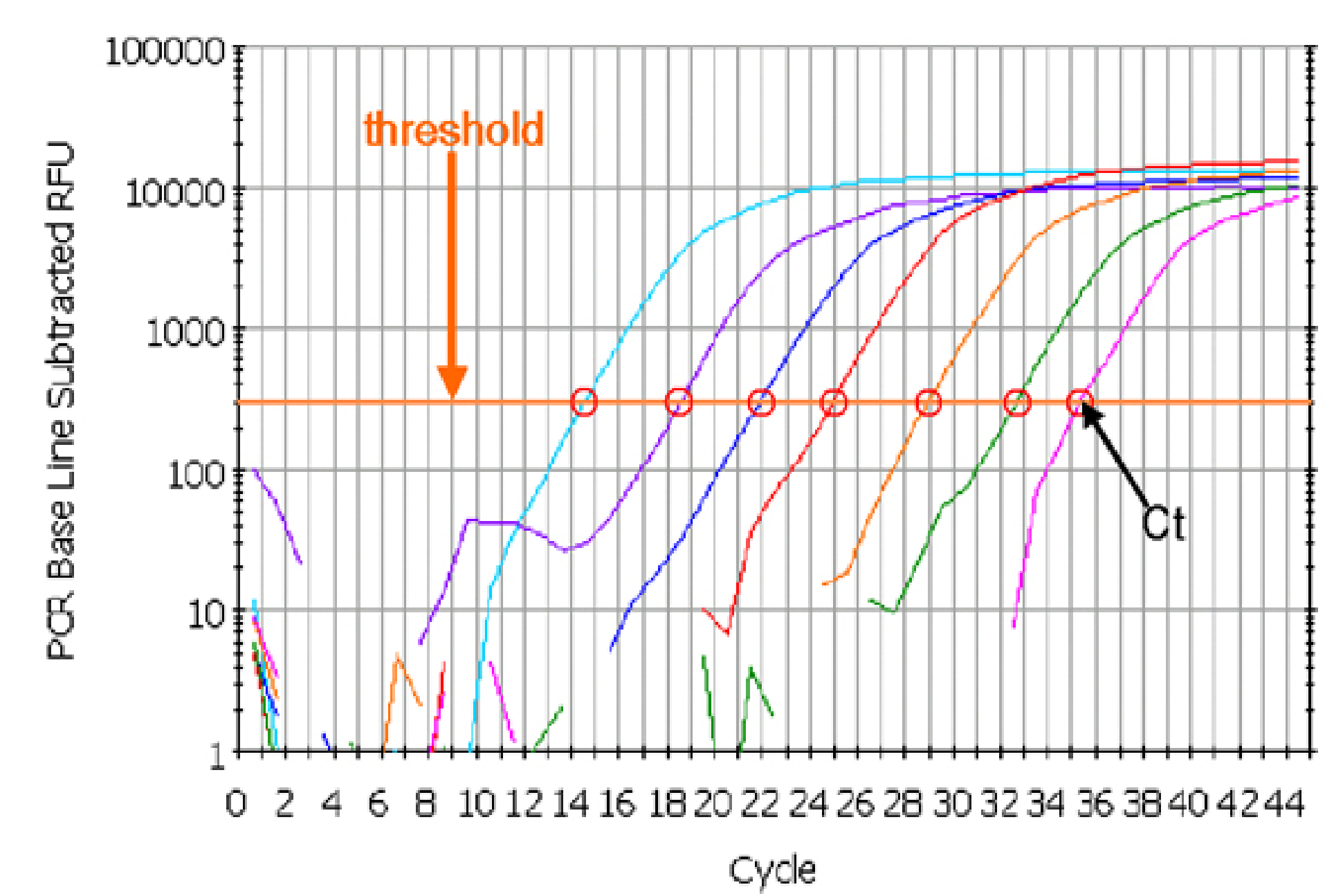

AMOUNT OF DNA
PCR CYCLE NUMBER
PCR Base Line Subtracted RFU
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
-1000
-2000
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34
Cycle


AMOUNT OF DNA
100000 00000
10000 00000
1000 00000
100 00000
10000000
1000000
100000
10000
1000
100
10
1
Log scale
0 5 10 15 20 25 30 35
PCR CYCLE NUMBER


AMOUNT OF DNA
PCR CYCLE NUMBER
PCR Base Line Subtracted RFU
10000
1000
100
10
1
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34



SERIES OF 10-FOLD DILUTIONS



SERIES OF 10-FOLD DILUTIONS

Primer optimisation Std/Dil curve

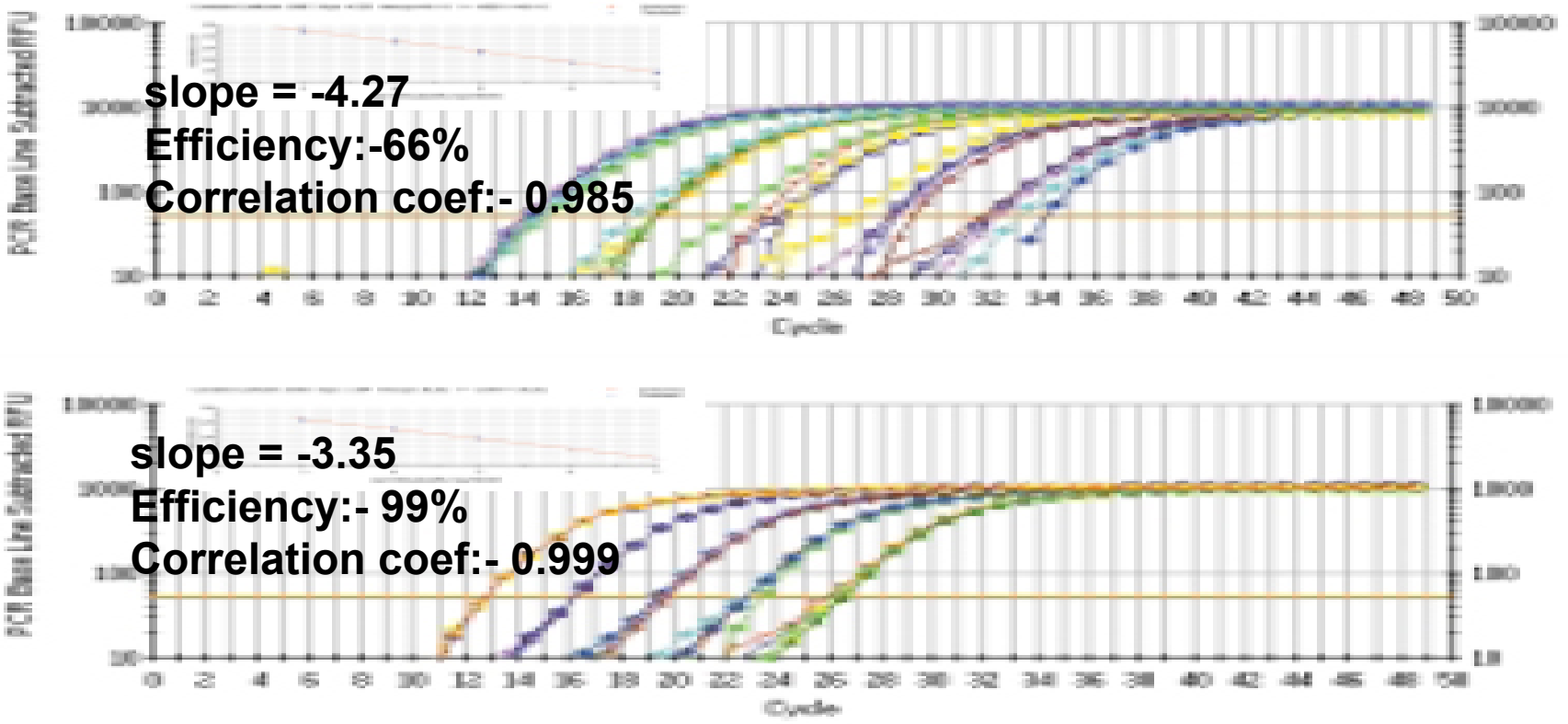
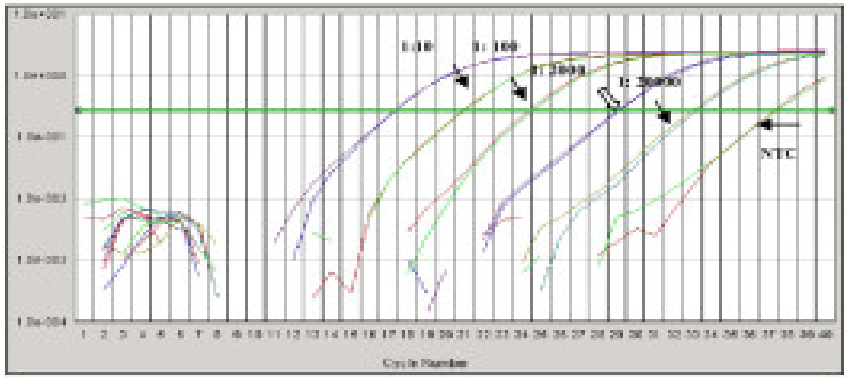
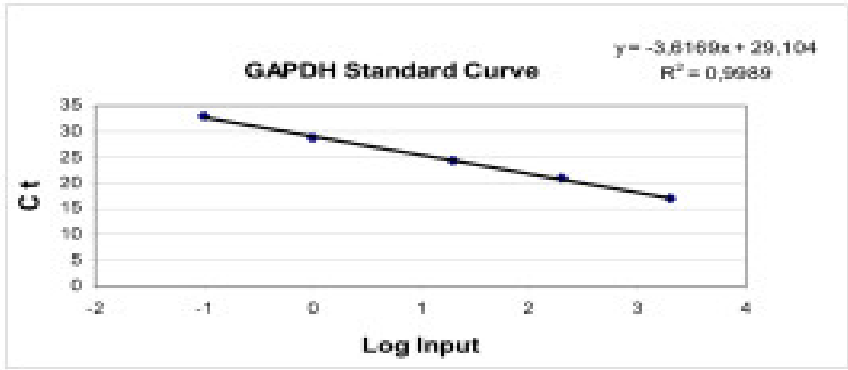


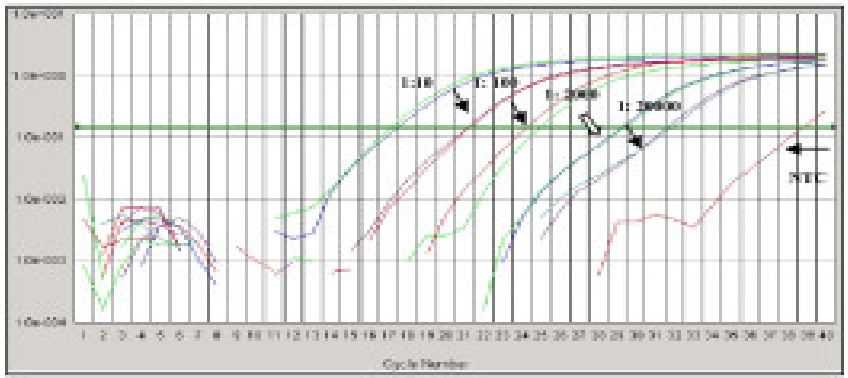
Fig. 2. Amplification plots of the 10x dilution series of the cyclophilin target with primer set A (upper panel) and primer set B (lower panel). The inset depicts the standard curve for each primer set. Data were analyzed with 95% of the data from the end of the cycle and the threshold was set at 55 RFU for both plots. Both experiments were performed on the same plate using the same plasmid dilution series.

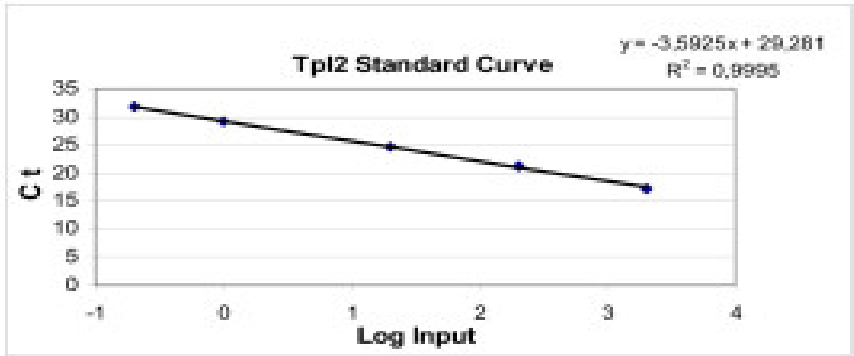
E = (10-1/slope)-1 to calculate efficiency (E),

A.

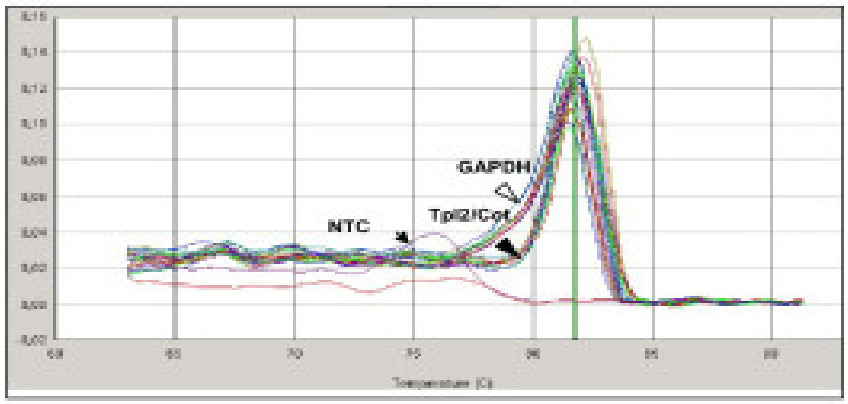

1:10
1: 100
1: 2000
1: 20000
NTC
Cycle Number


GAPDH Standard Curve
y = -3,8169x + 29,104
R² = 0,9989
Ct
Log Input


1:10
1: 100
1: 2000
1: 20000
NTC
Cycle Number


Tpl2 Standard Curve
y = -3,5925x + 29,281
R² = 0,9995
Ct
Log Input

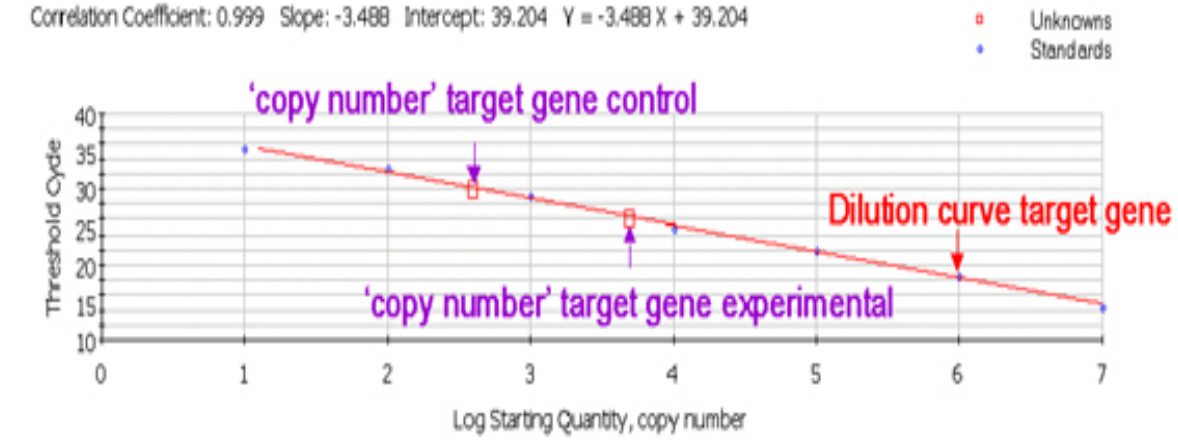
B.

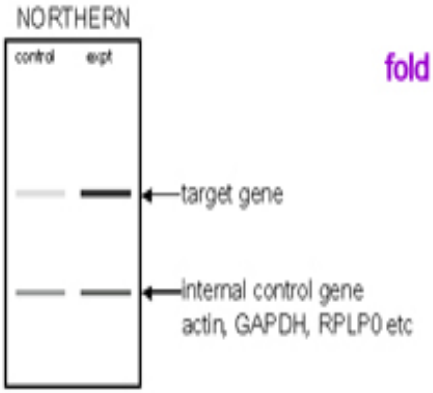

GAPDH
Tpl2/Cot
NTC
Temperature (C)

Interpretation of results

- Standard curve method (Similar to primer optimisation)
- PFAFFL method (incorporates efficiencies)
- ΔΔCt method
- Determine the amount of accuracy/power required to answer biological question.

Standard Curve





fold change in target gene= copy number experimental / copy number control

Ratio experimental/control = fold change in target gene / fold change in reference gene

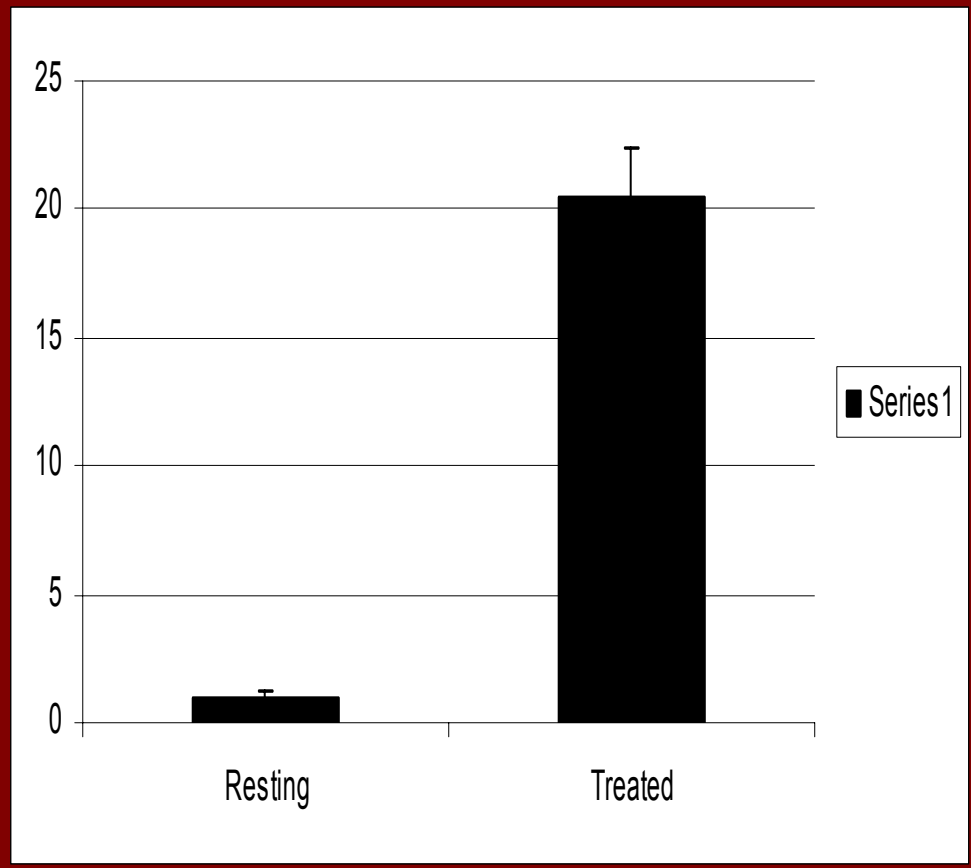
Std Curve
- Relative
  - Plasmid standard
  - cDNA dilution curve
- Quantitative
  - RNA standard
  - Copy number possible

Comparative Ct of ΔΔCt method

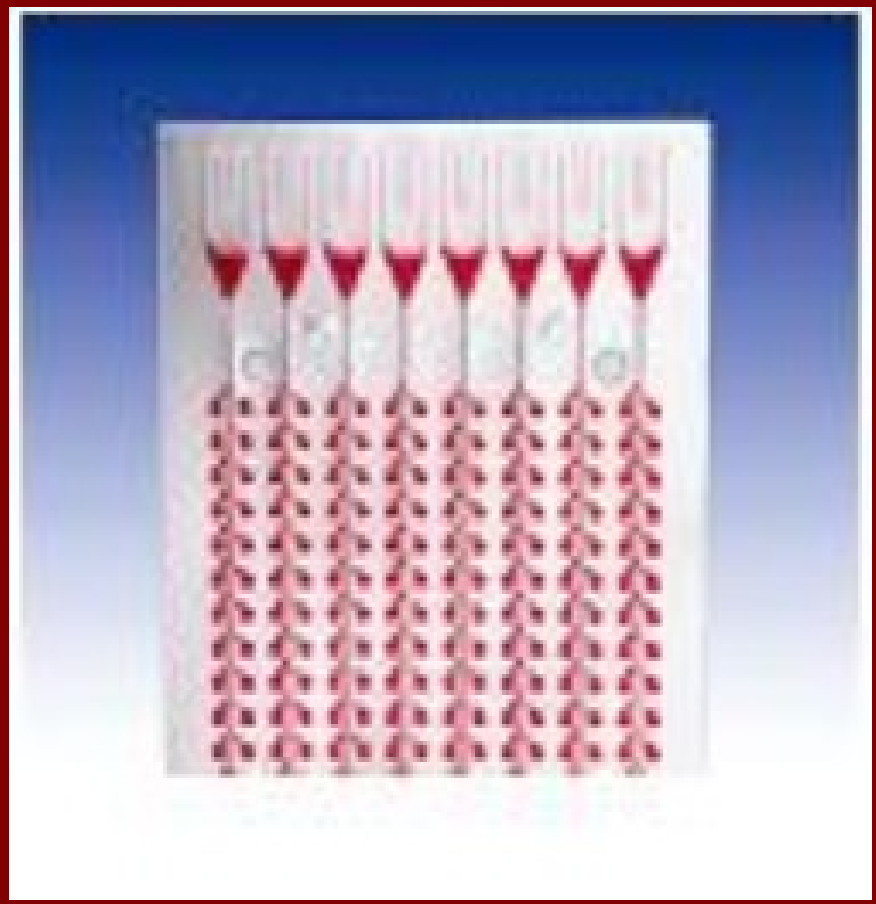
- For the ΔΔCt calculation to be valid, the amplification efficiencies of the target and the endogenous reference must be approximately equal.
- $\Delta Ct = \Delta Ct_{\text{target gene}} - \Delta Ct_{\text{control gene}}$ is determined for each sample
- Average ΔCt determined for calibrator (untreated sample)
- $\Delta\Delta Ct = \Delta Ct_{\text{sample}} - \Delta Ct_{\text{calibrator}}$ is change in Ct
- Relative fold change= $2^{(-\Delta\Delta Ct)}$

Example ΔΔCt method

	Resting	Treated
	17.67503	17.875984
GAPDH	17.79494	17.866985
	17.90083	17.892178
AVG	**17.79027**	**17.87838233**
	22.6176	17.93654
EGR1	22.29687	18.21179
	22.06322	18.041883
	-4.827333	-0.058157667
	-4.506605	-0.333407667
	-4.272958	-0.163500667
CAL Avg	**-4.535632**	
	0.291701	-4.477474
	-0.029027	-4.202224
	-0.262674	-4.372131
	1.224083	22.27686017
	0.980081	18.40752812
	0.833542	20.70821074
Rel FC	**1.012568**	**20.46419967**
StDEV	0.197287	1.94617283
	pvalue	**6.66796E-05**



Assay miniaturisation and automation



Thanks for your attention.