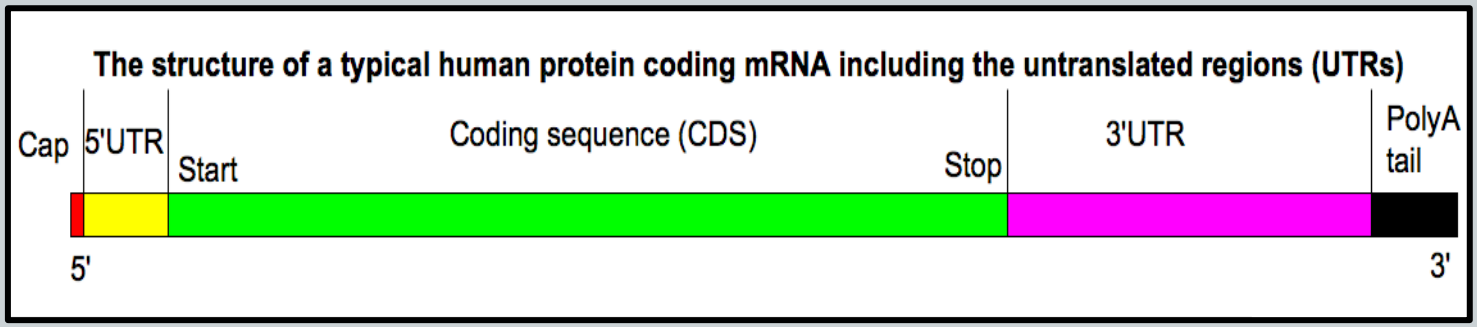


RNA Isolation

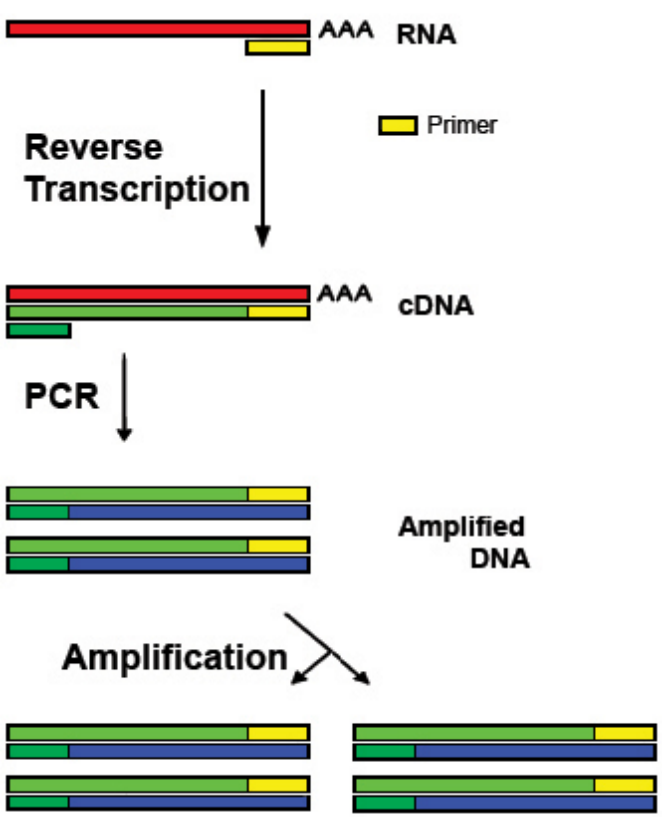
Step #1 of qRT-PCR

qRT-PCR Overview

Step 1: Isolate mRNA from cells (control and experimental)

Step 2: Convert mRNA into cDNA

Step 3: Amplify cDNA and quantify (relative) amount of starting mRNA in sample using qPCR (quantitiative/real-time polymerase chain reaction)



Several Types of RNA

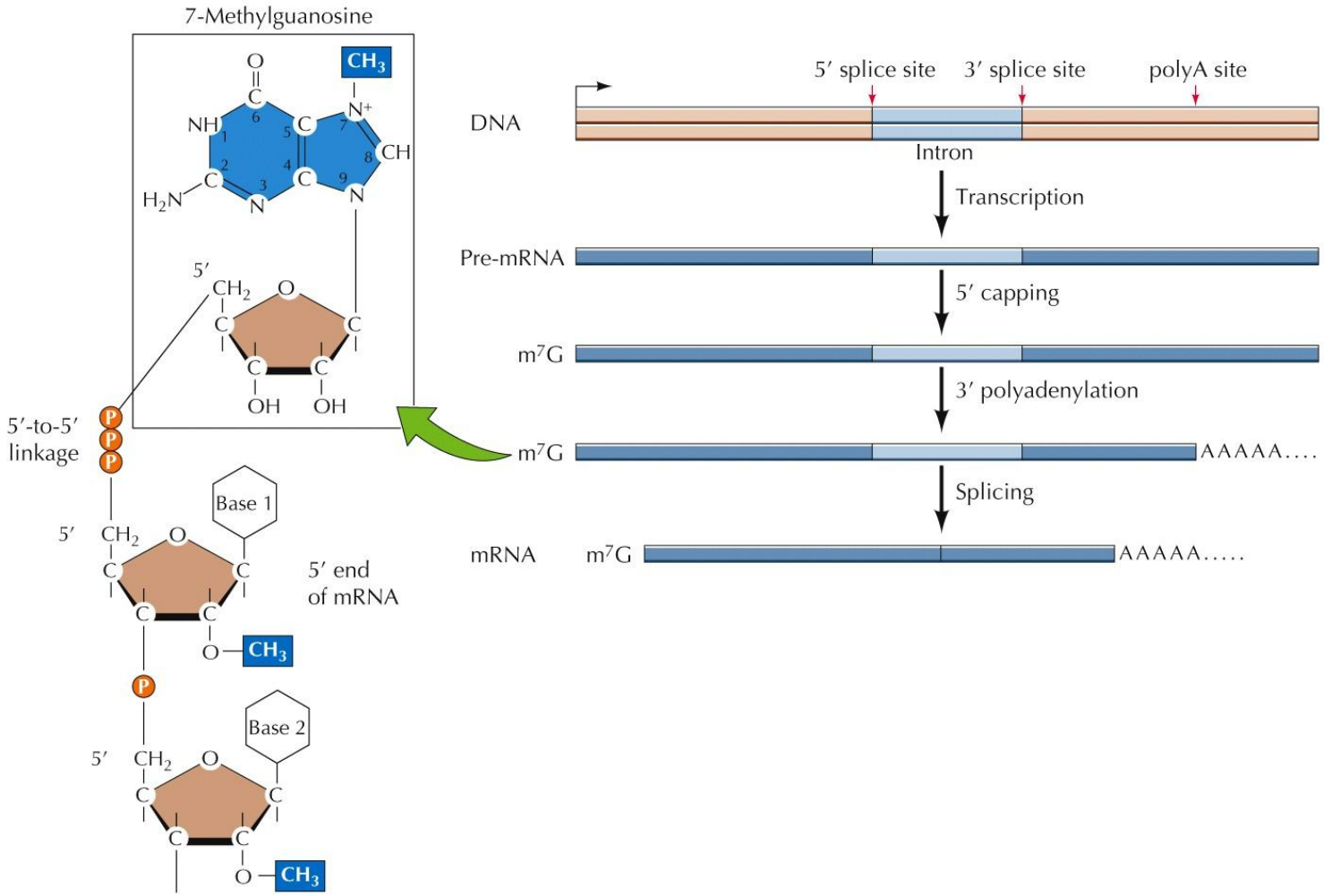
RNAs involved in protein synthesis:

mRNA → codes for protein (Average length of 2.2kb)
tRNA → translation
rRNA → translation

RNAs involved in post-transcriptional modifications and regulation:

snRNA → splicing
miRNA → gene regulation
siRNA → gene regulation

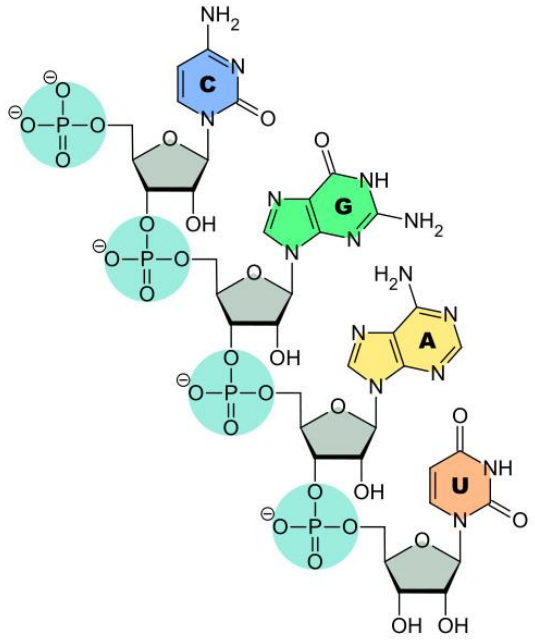
Pre-mRNA is modified prior to export from the nucleus



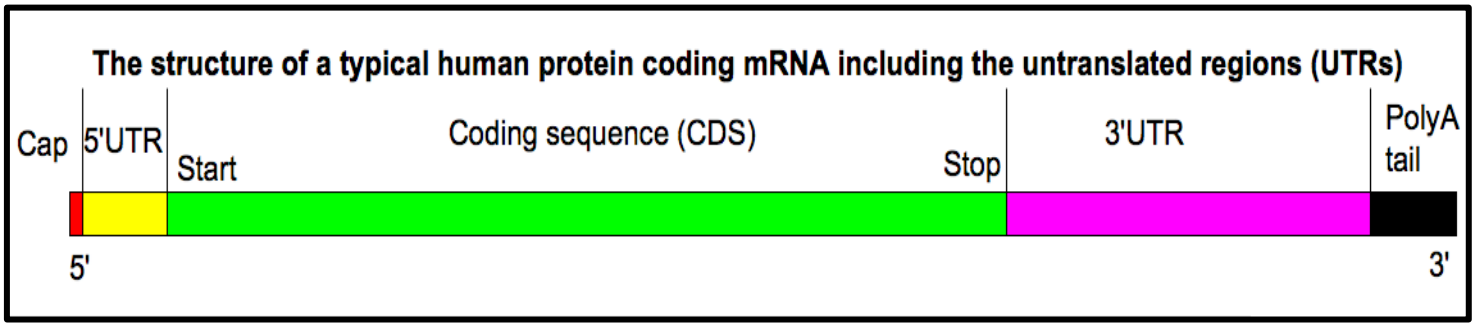
***THE CELL: A MOLECULAR APPROACH 6e*, Figure 7.44**

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Main Features of mRNA

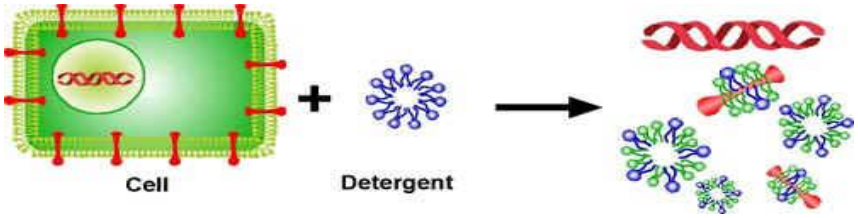


- Larger than other RNA types
- Smaller than genomic DNA
- More acidic than DNA
- Less stable than DNA

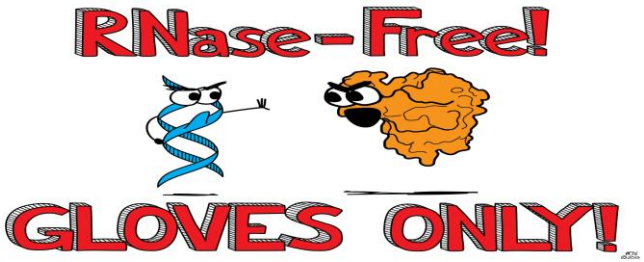


Overview of RNA Isolation

1. Cellular lysis and homogenization



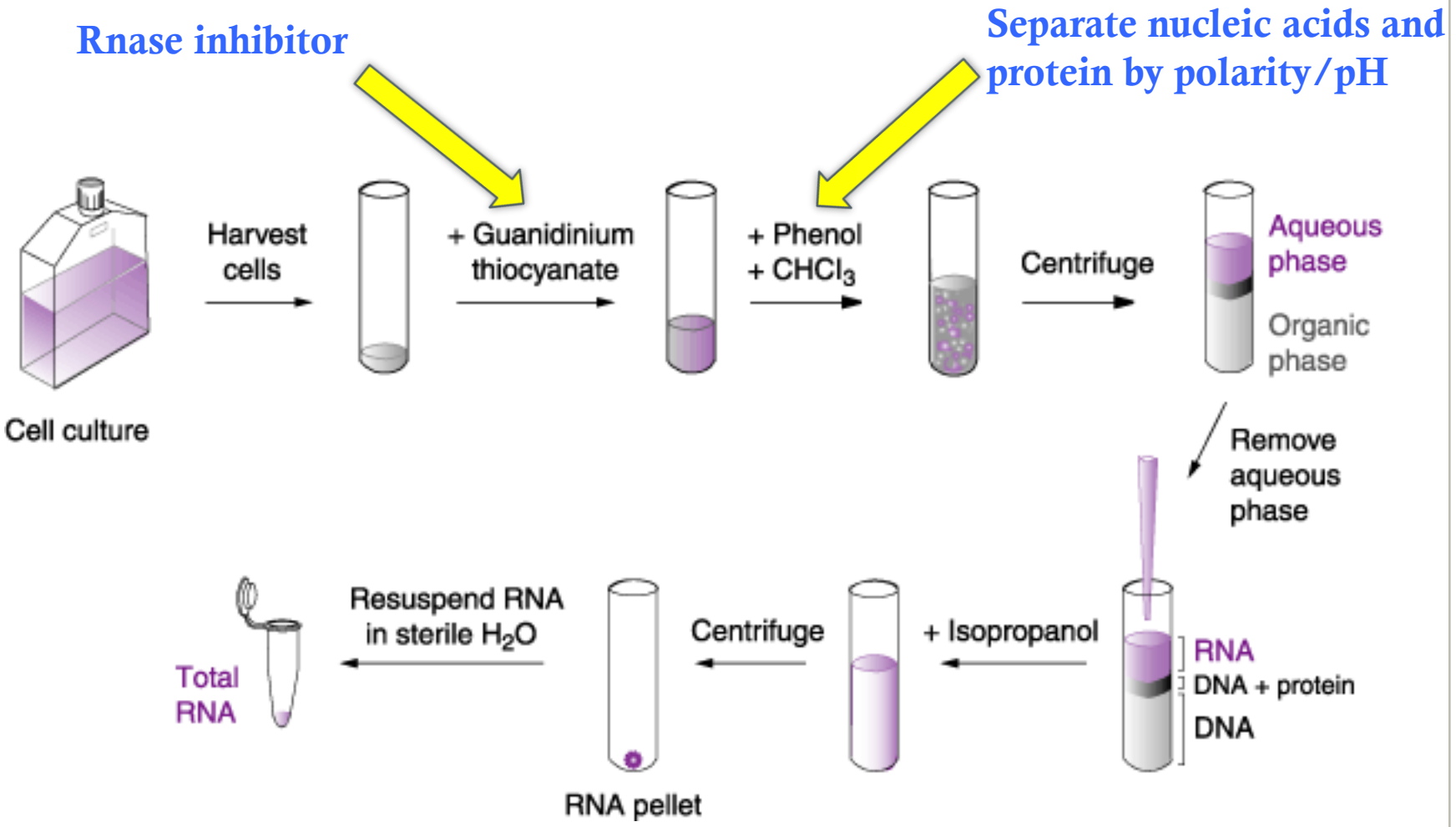
2. Protect from cellular nucleases (Rnase)



3. Separation of RNA (Organic or Filter)
   a) Removal of protein
   b) Removal of DNA
   c) Removal of other contaminants (sugars, salts, etc.)

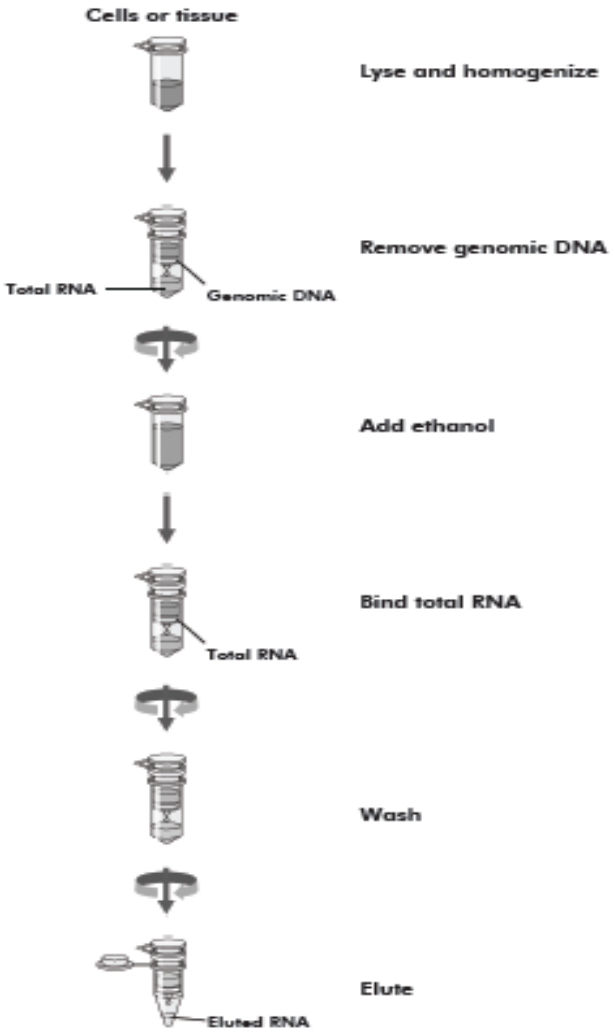
Overview of RNA Isolation

Organic Extraction Methods



Overview of RNA Isolation

Filter-based, Spin Basket Formats



1. Lyse cells and nucleus while inactivating cellular Rnase.
2. Remove genomic DNA with filter.
3. Add ethanol to bind RNA.
4. Run through membrane to trap and isolate larger RNA molecules (longer than 200bp, excludes most rRNA and tRNA)
5. Wash contaminants from the RNA.
6. Elute RNA with sterile water.

Lab: RNA Isolation

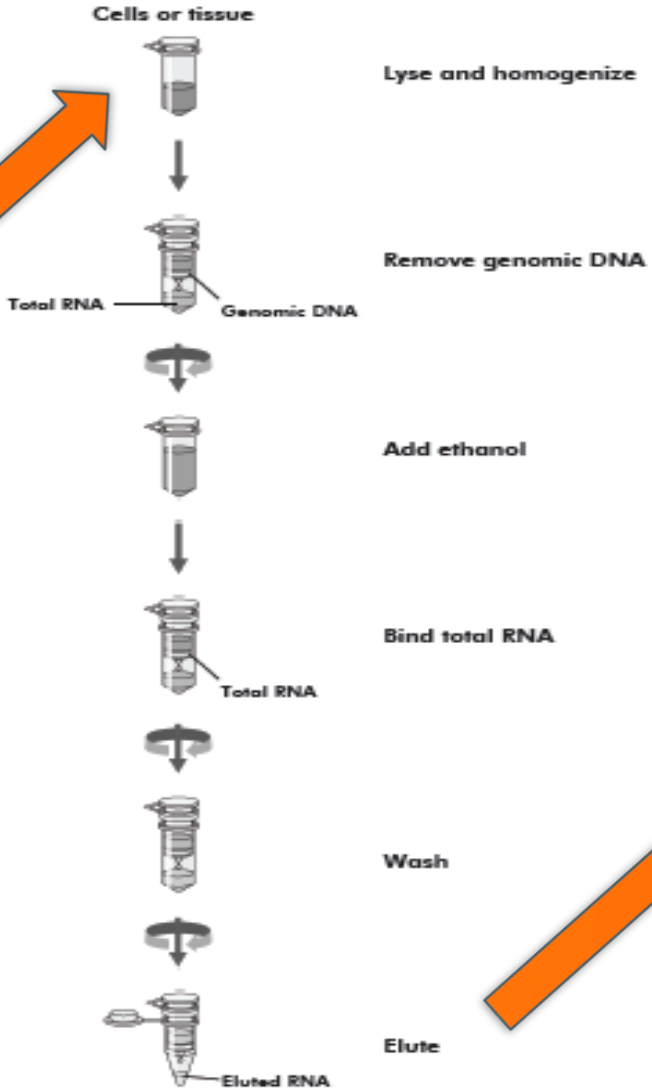
Goal: To isolate all the mRNA from a sample of control cells (untreated)

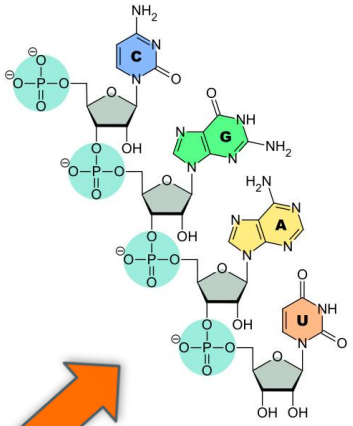
Technique: RNeasy Plus Mini Kit

End Product: Collection tube containing total cellular mRNA content with all other cellular components removed (including DNA).

mRNA Isolation







RNeasy Kit

Determining the amount of starting material

It is essential to use the correct amount of starting material in order to obtain optimal RNA yield and purity. The maximum amount that can be used is limited by:

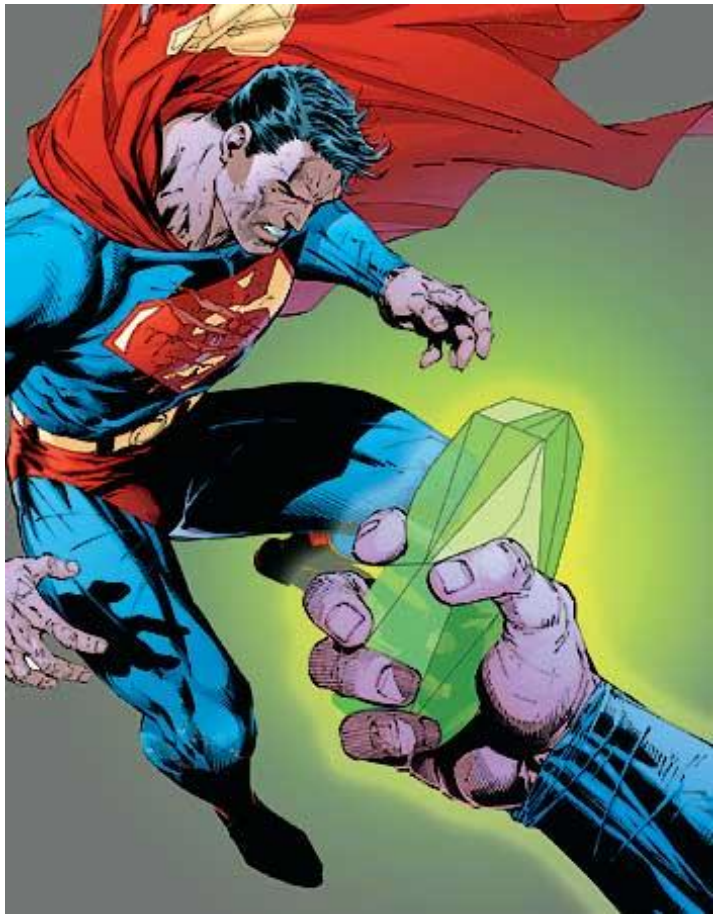
- The type of sample and its DNA and RNA content
- The volume of Buffer RLT Plus required for efficient lysis and the maximum loading volume of the RNeasy spin column
- The DNA removal capacity of the gDNA Eliminator spin column
- The RNA binding capacity of the RNeasy spin column

Harvesting our Cells

- In a T25, we expect ~$3x10^6$ cells.
- The Max Starting Material is $1x10^7$ cells.
- We will Trypsinize our T25 flasks and use all of the cells.

Trypsinize your flask at your benches. You will collect the resulting 3ml of media in a 15ml falcon and pellet the cells using the centrifuge.

Lab: RNA Isolation
handling



Rnase – Don't let it destroy your experiment!

- It's everywhere
- It's incredibly fast acting
- It's very hard to get rid of/inactivate

So how do we defeat it?

- Sterile equipment
- RNase Away
- Maintain sterility, change gloves frequently
- Quick, focused work
- Once RNA is converted to cDNA, you're safe!

**O.D. = optical density

Lab: RNA Isolation
analyzing

RNA content and purity is measured using a UV-vis spectrophotometer (we will be using the Nanodrop).

• Measures the amount of UV light absorbed by DNA or RNA at λ= 260nm. Cannot distinguish between DNA and RNA.

[RNA] = (OD_{260}) x (dilution factor) x (44ug/ml RNA)/(1 OD_{260} unit)

• Determines the purity of the sample by measuring UV absorption at 260nm (DNA/RNA) and at 280nm (protein)

-An A_{260}/A_{280} ratio less than 1.7 suggests significant protein contamination

Goal: ***RNA content of at least 100ng/ul and O.D. ratio of 1.7 or greater***

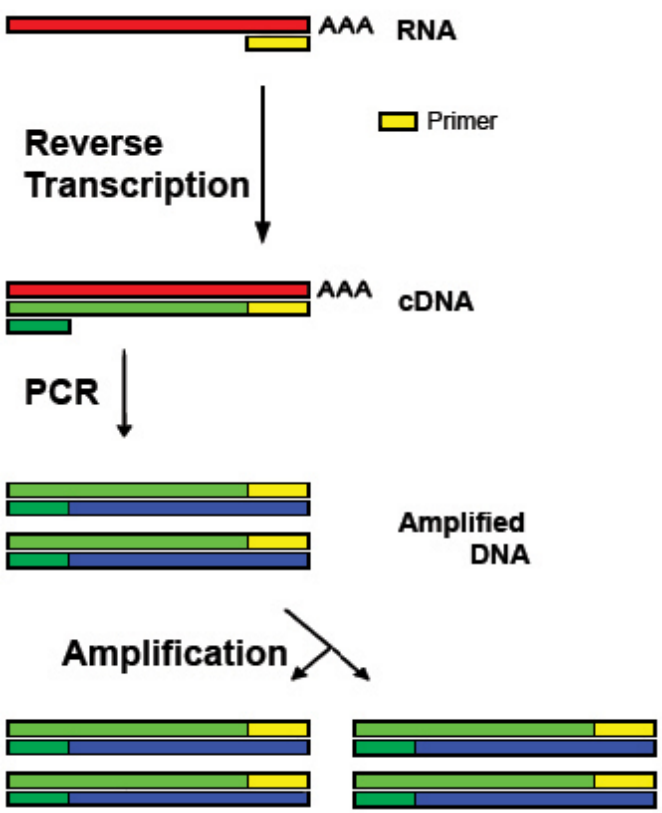


qRT-PCR Overview

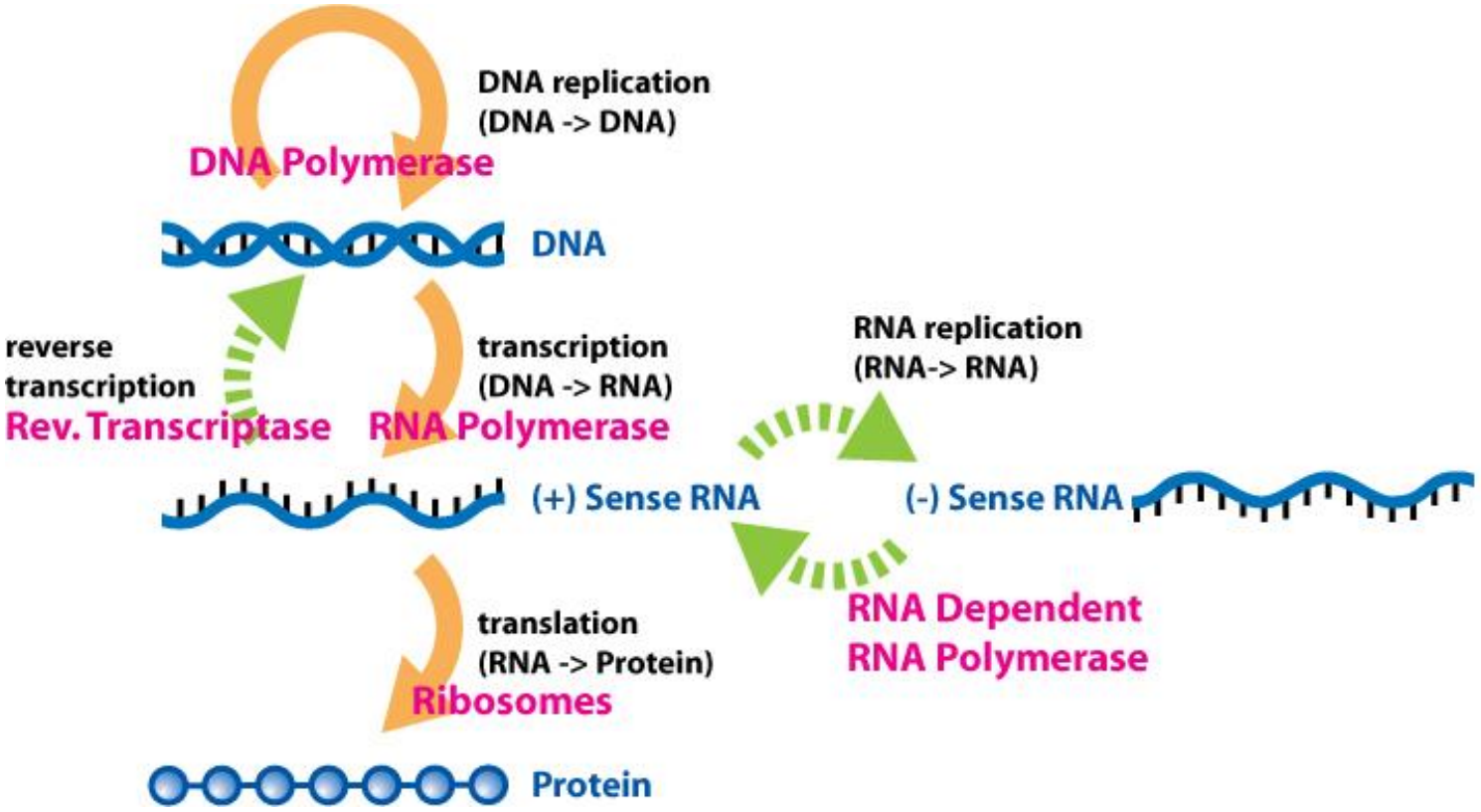
Step 1: Isolate mRNA from cells (control and experimental)

Step 2: Convert mRNA into cDNA

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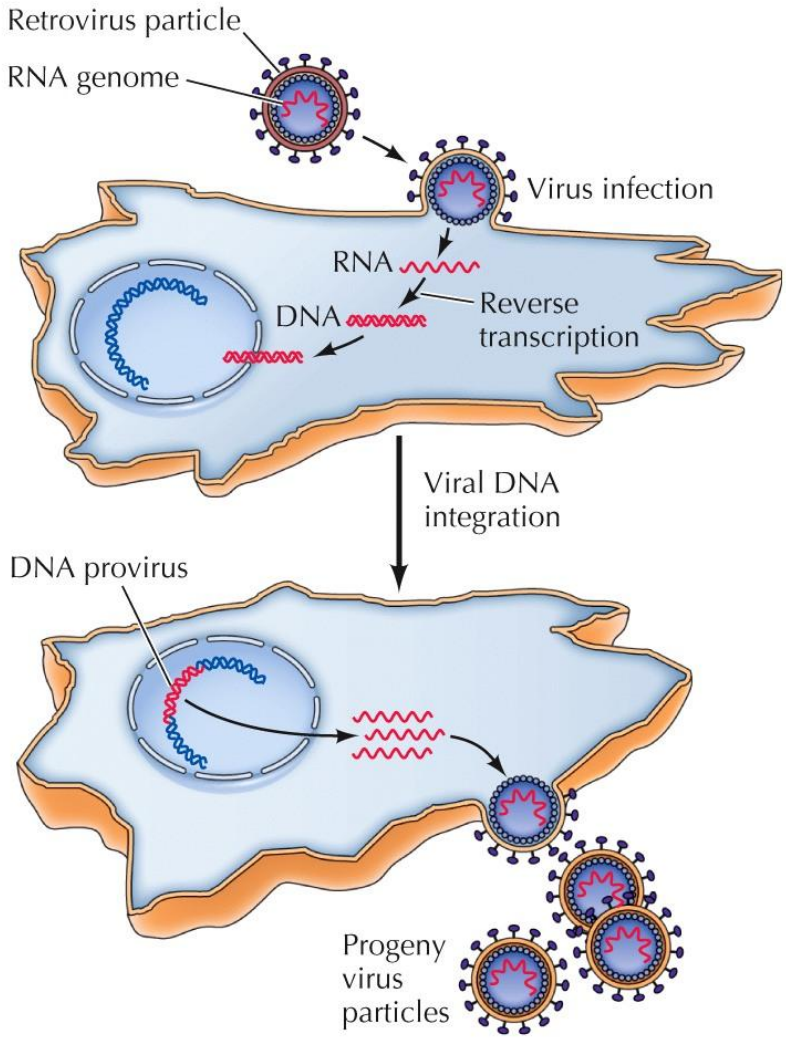


Central Dogma of Molecular Biology



When does reverse transcription occur in nature?

Retroviruses and Reverse Transcription

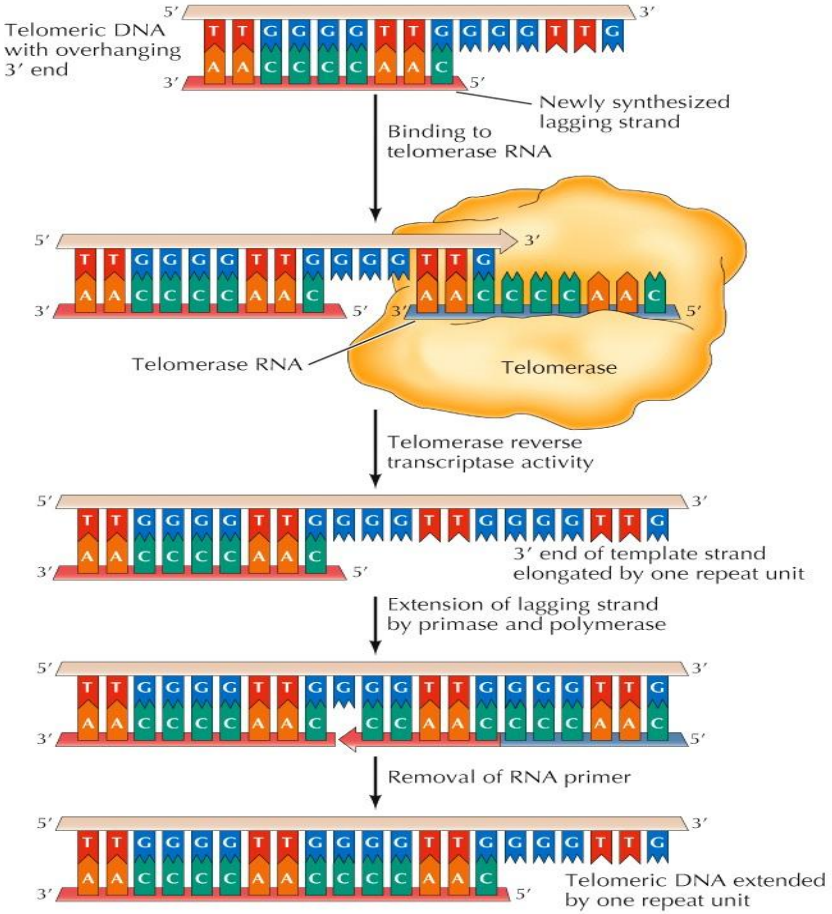


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REGULATION OF SENESCENCE AND APOPTOSIS

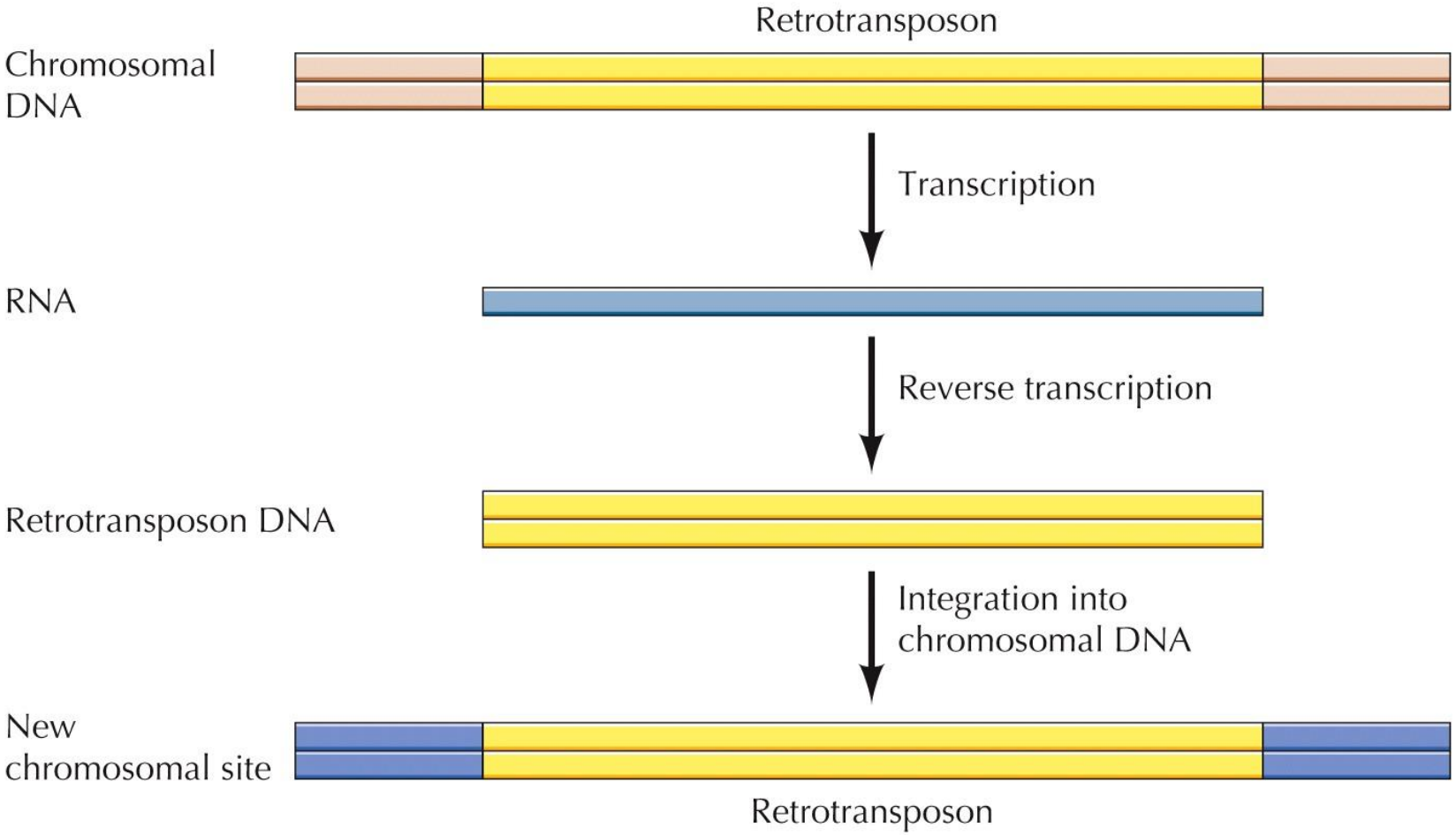
TELOMERES



***THE CELL: A MOLECULAR APPROACH 6e*, Figure 6.16**

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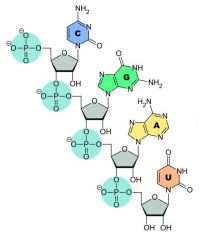
Retrotransposons and Reverse Transcription



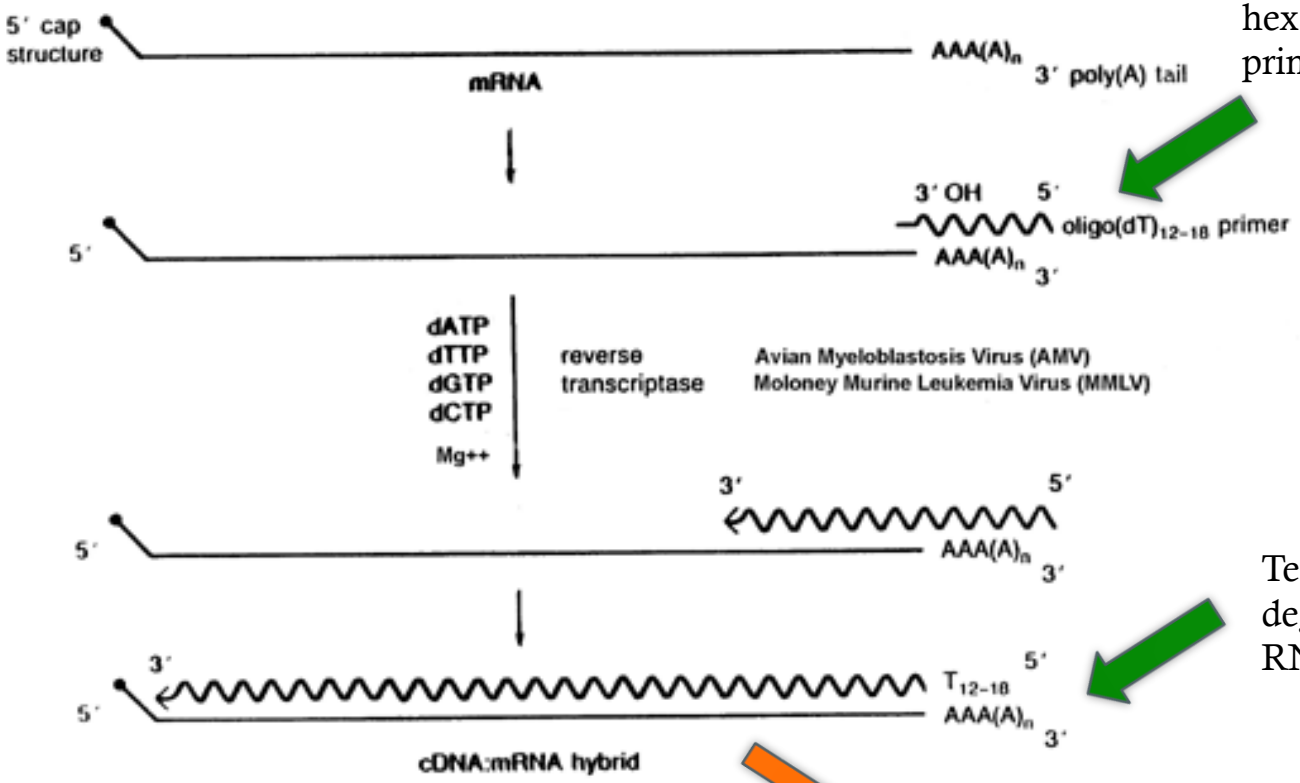
***THE CELL: A MOLECULAR APPROACH 6e*, Figure 5.7**

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Step 2: Convert mRNA to cDNA



Traditional cDNA synthesis



Alternatively, random hexamer or specific primers

Template mRNA degradation by RNase

cDNA used for qPCR

cDNA synthesis for qRT-PCR

Goal: Convert isolated mRNA into cDNA for further processing with qPCR

Procedure: Use of Oligo$(dT)_{15}$ primer, M-MuLV Rnase H^+ reverse transcriptase and other components of the DyNAmo cDNA Synthesis Kit

Outcome: Sample of cDNA from both control and experimental cells of known concentration and purity

Materials

Table 2. Reaction setup.

We'll be using oligo-dT

Components	Stock	20 µl reaction	Comments
RT buffer	2x	10 µl	RT buffer includes dNTPs and $MgCl_2$.
Random hexamer primer set	300 ng/µl	1 µl	Alternatively oligo(dT) primer or a specific primer can be used. See Chapter 2.
Template RNA		X µl	Max 1 µg
M-MuLV RNase H^+ reverse transcriptase		2 µl	Includes RNase inhibitor.
RNase-free H_2O		X µl	Add water to fill up to the final reaction volume.
Total volume		20 µl	

How do we know the concentration of our RNA?

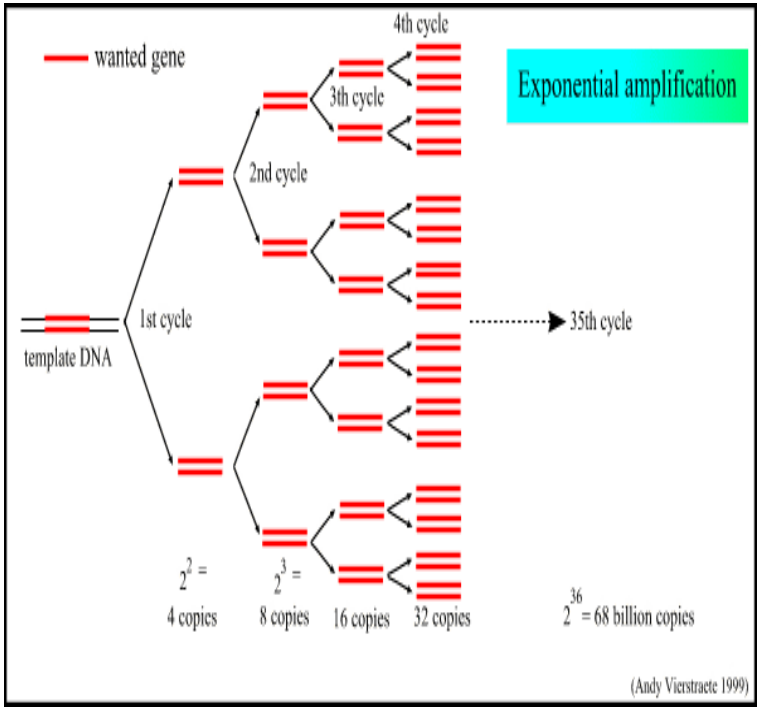
Step 3: amplify and analyze with qPCR

Review of PCR

http://www.sumanasinc.com/webcontent/animations/content/pcr.html

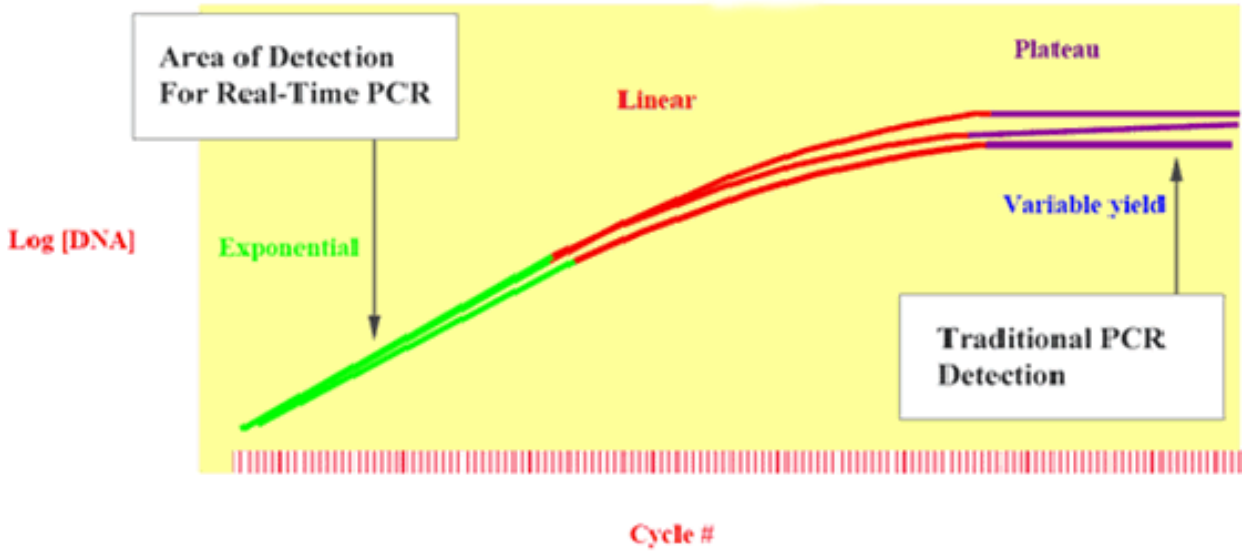
http://learn.genetics.utah.edu/content/labs/pcr/

Step	Temperature (°C)	Time	No. of Cycle
Initial Denature	98	5 min	1
Denature	98	10 sec	31
Annealing	55	20 sec	
Extension	72	40 sec	
Final Extension	72	5 min	1



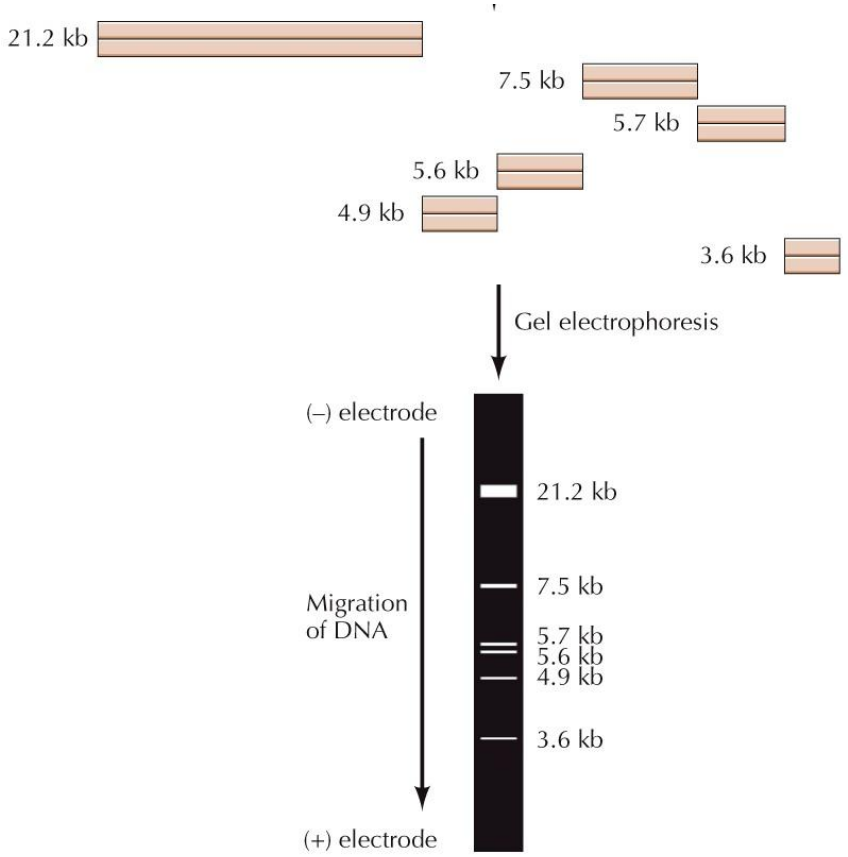
Step 3: amplify and analyze with qPCR

Review of PCR



Step 3: amplify and analyze with qPCR

Review of PCR



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Step 3: amplify and analyze with qPCR

PCR vs. qPCR

	qPCR	PCR
Overview	Measures PCR amplification as it occurs	Measures the amount of accumulated PCR product at the end of the PCR cycles
Quantitative?	Yes. Data is collected during exponential growth when fluorescence is directly proportional to product	No. Although comparing the relative intensity of the band on a gel to a standard of known concentration can be semi-quantitative
Applications	•Quantify gene expression •Pathogen detection •Quality control •Assay validation •Microarray verification •Other	•Amplification of DNA for sequencing, genotyping or cloning

Step 3: amplify and analyze with qPCR

PCR Thoughts:

Q: Why do we include a control in PCR that replaces your DNA with water?

Q: Why do we generally anneal at 50-60°c?

Q: Why are primers generally at least 20bp long?

Q: What is the rate of DNA elongation by Taq polymerase at 70°c?

Q: What is the "plateau phase" in PCR amplification and why does it occur?

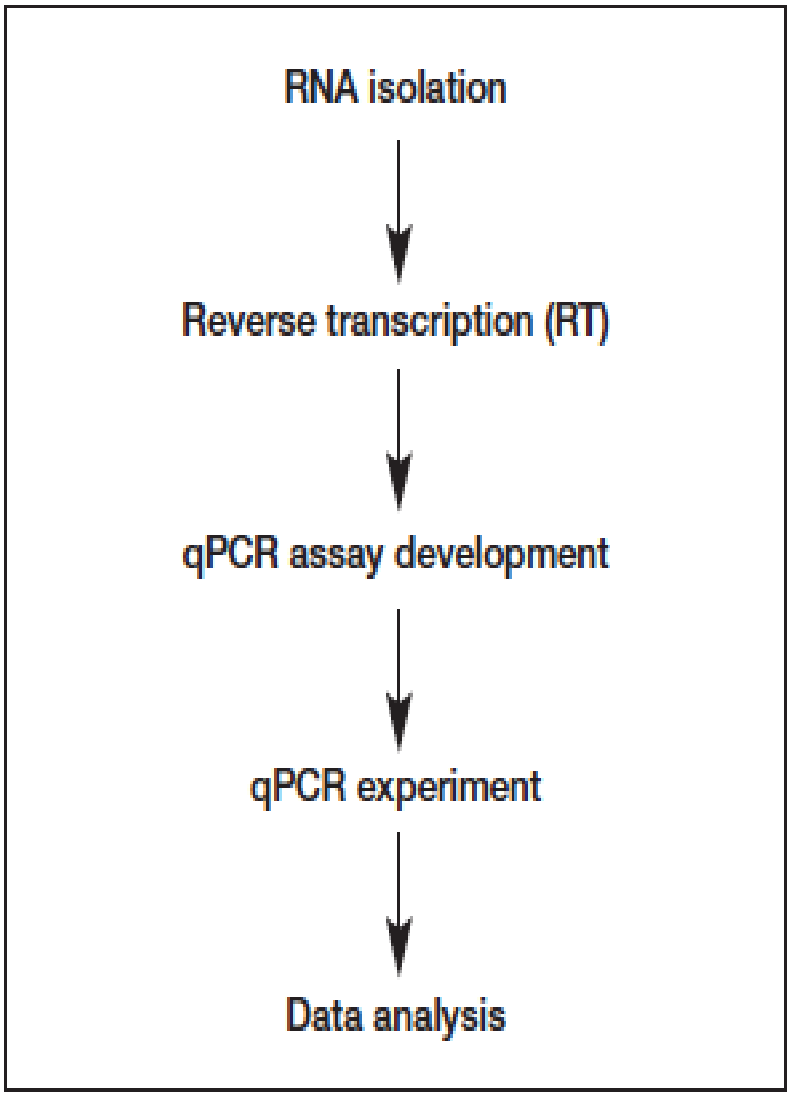
Step 3: amplify and analyze with qPCR

What makes qPCR different??

1. Each copy of the DNA segment is tagged with a fluorescent probe. The intensity of fluorescence can then be used as a measure of the number of DNA copies present after each cycle **during the exponential growth phase**.

2. With a little math, this allows for a very precise method to quantify the concentration of the DNA segment of interest in the original sample (prior to amplification)… not just the approximate concentration at the end as in PCR.

3. No need to run an agarose gel to detect the DNA segment, although this can be done if needed for further experiments.



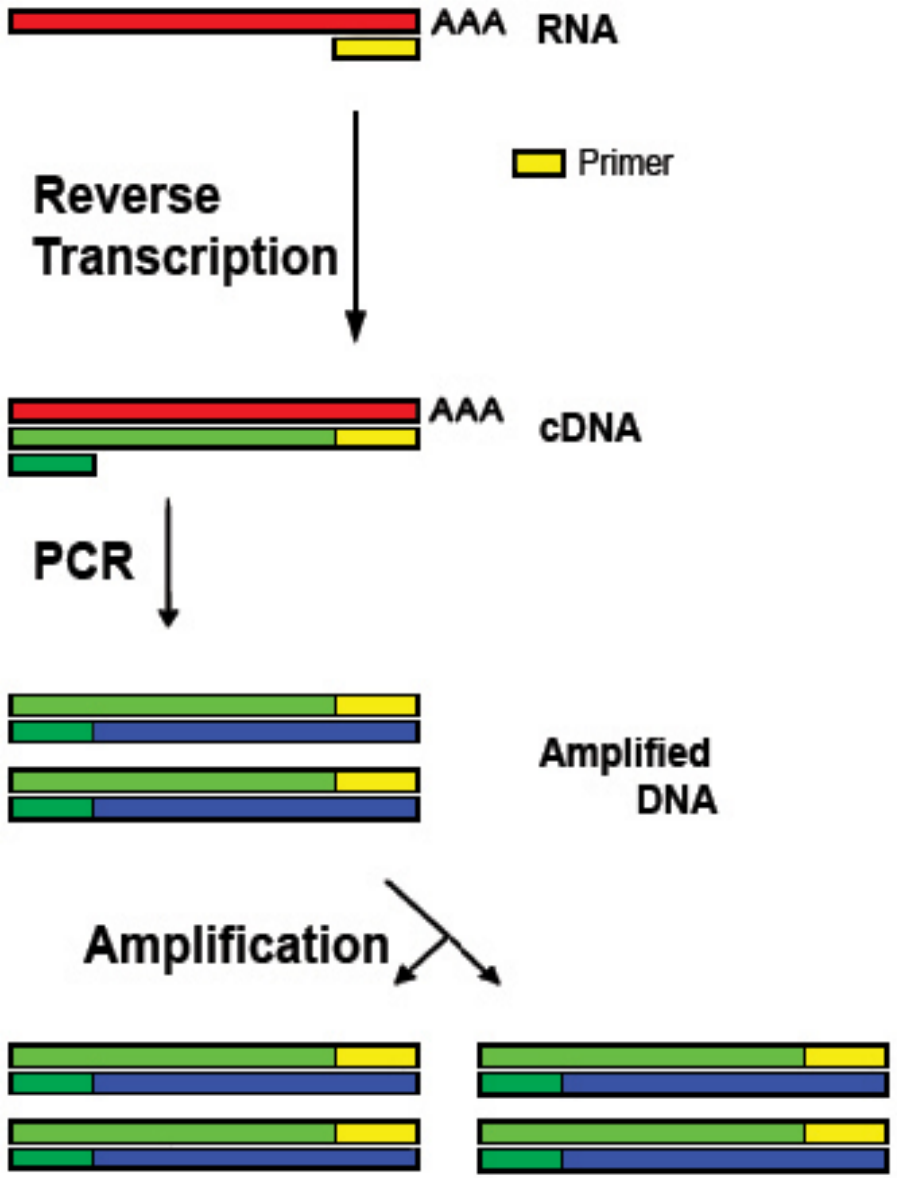
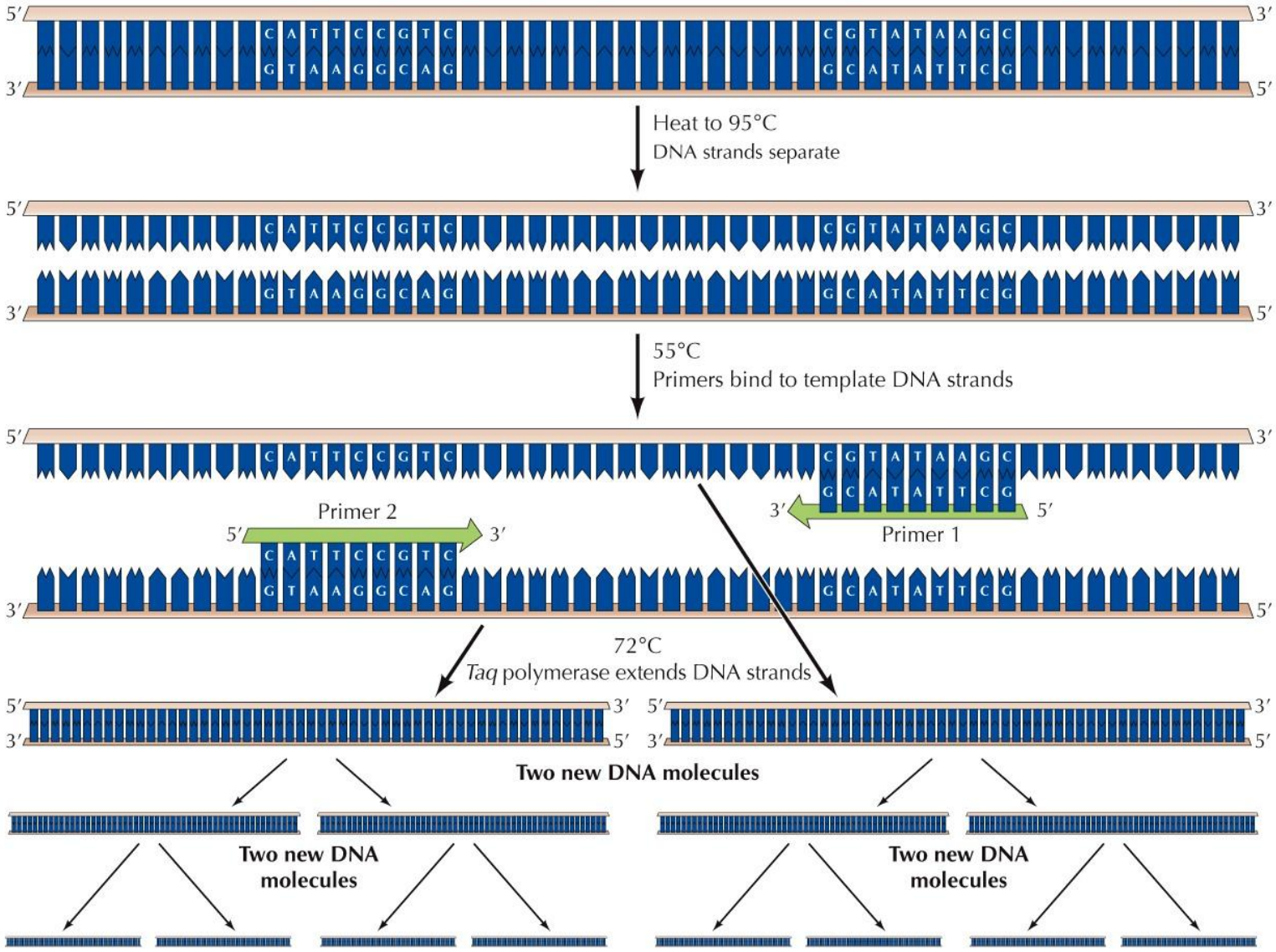


Fig. 5.1. Gene expression analysis workflow.



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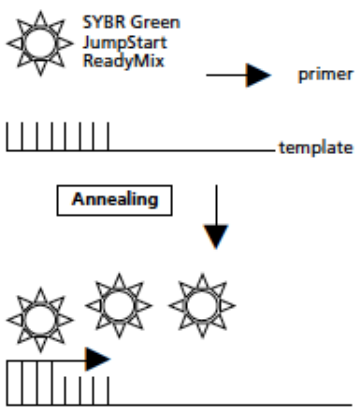
SYBR Green based assay

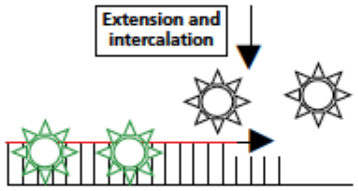
Figure 3. Dye-Based qPCR using SYBR Green I Binding Dye

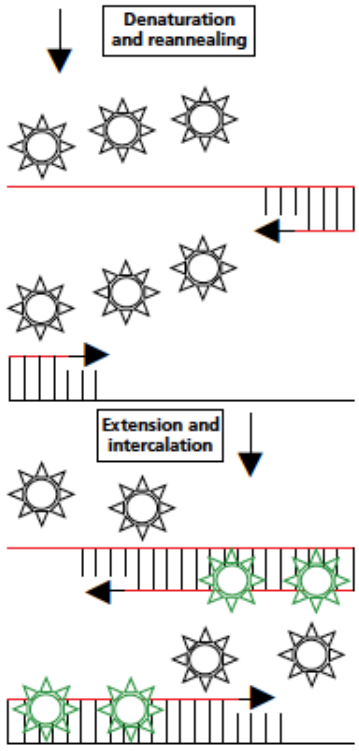
1) The PCR reaction contains enzyme, dNTPs, buffer and SYBR Green I, primers and template. SYBR Green I has no significant fluorescence in the presence of single-stranded DNA.

2) As the reaction progresses, double-stranded products are generated. The SYBR Green I intercalcates into these products and begins to fluoresce.

3) When enough products have accumulated the fluorescence rises above background. This is called the threshold cycle of Ct. The Ct value is used to quantitate the starting amount of template.







TaqMan based assay

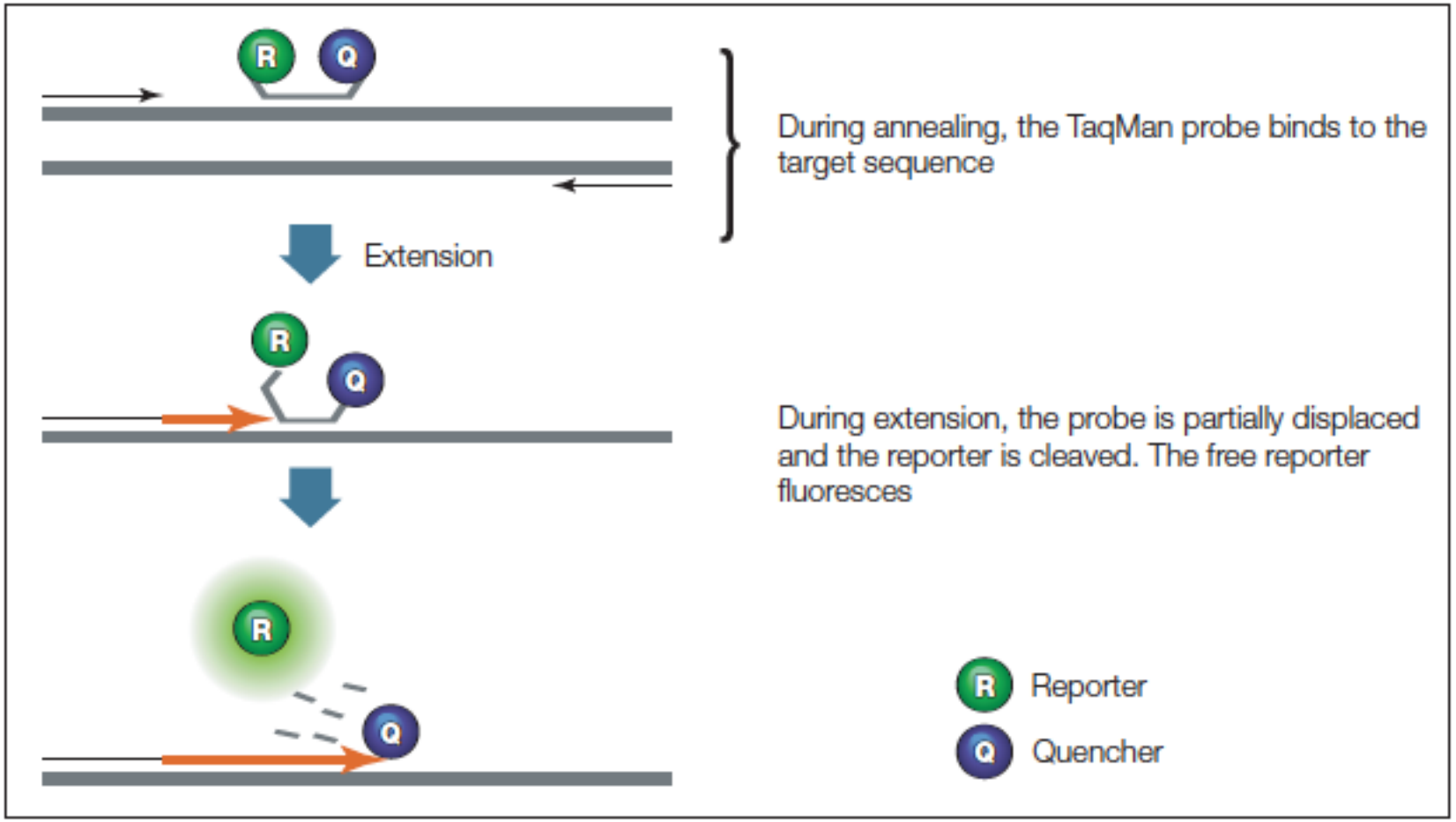
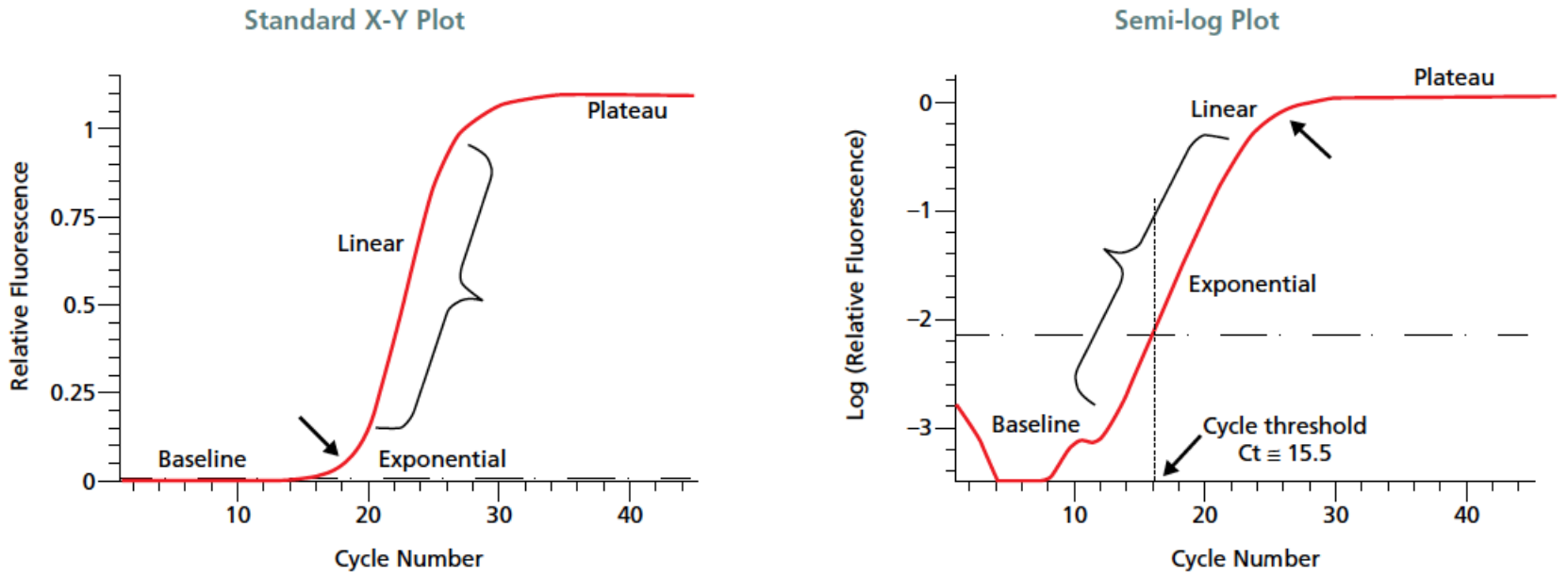


Fig. 2.3. TaqMan assay.

$\log_{10} x = y \quad 10^y = x$

Figure 1. PCR Amplification Plots



Standard: 1 → 2 → 4 → 8 → 16 → 32

$\log_{10}$: 0 → 0.3 → 0.6 →0.9 → 1.2 → 1.5

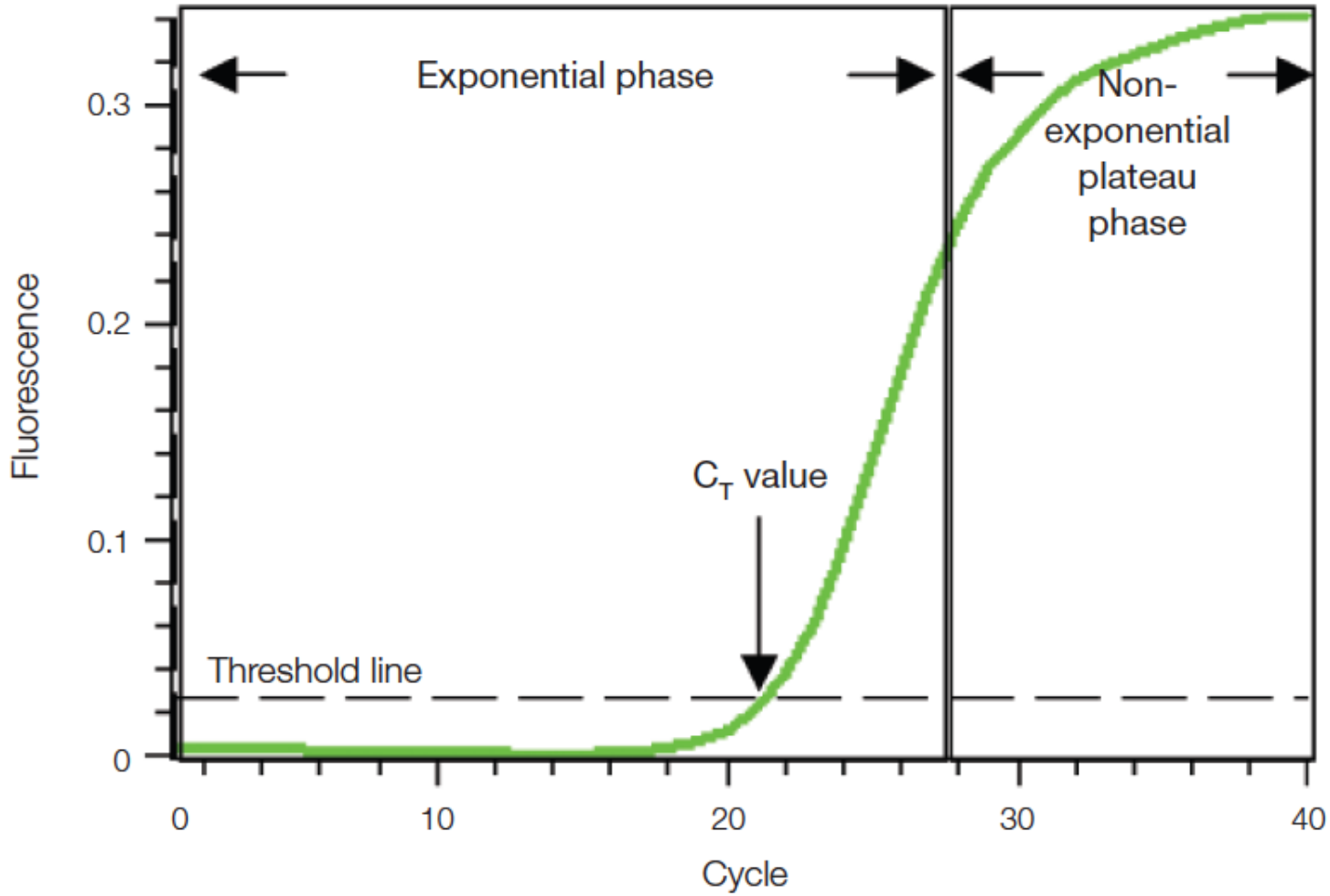
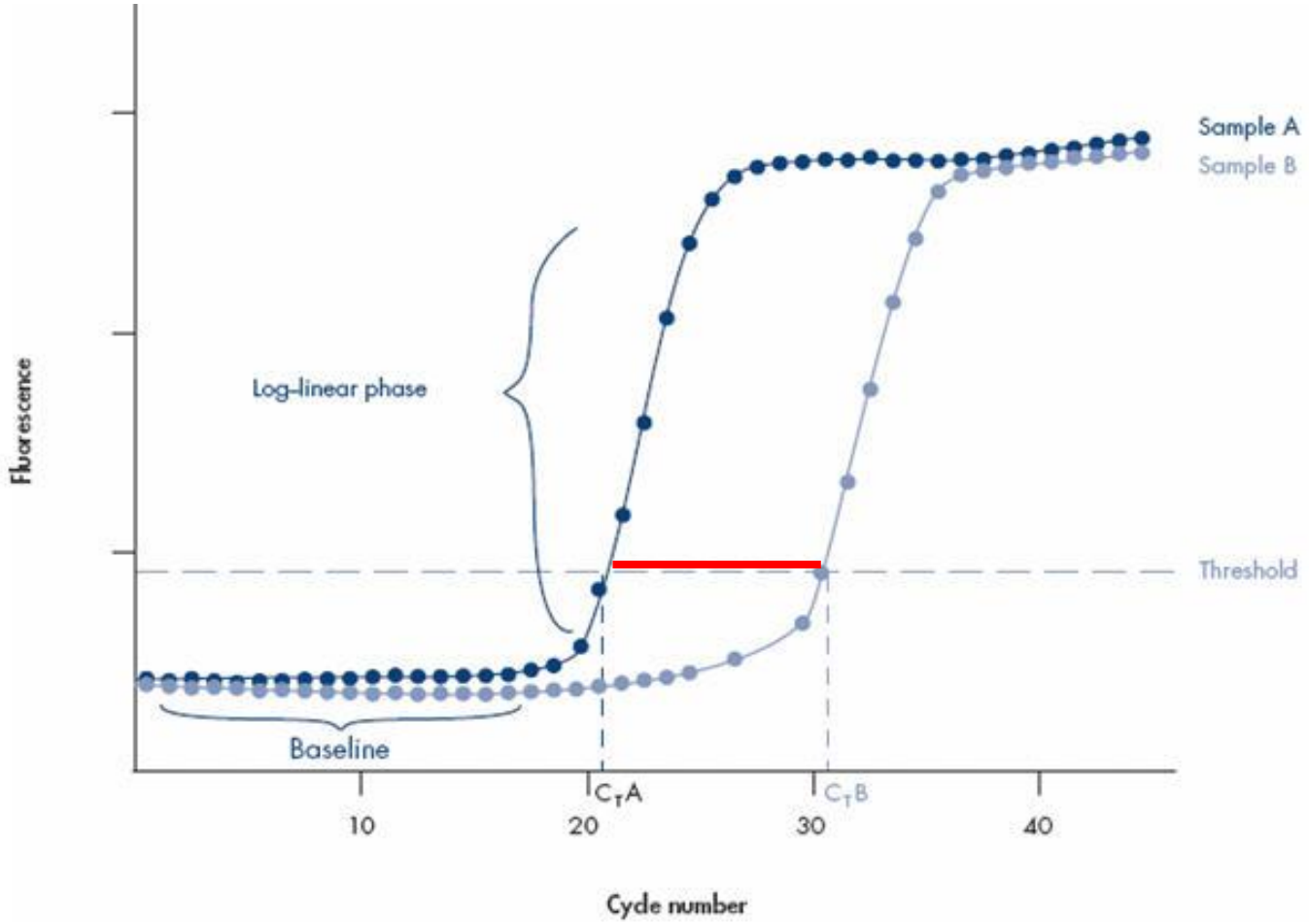


Fig. 1.1. Amplification plot. Baseline-subtracted fluorescence is shown.



The amount of starting material is extrapolated back from the reliable fluorescence signal (above the Ct).

$2^{(CtB-CtA)}$ = fold difference in starting material

END Wk11