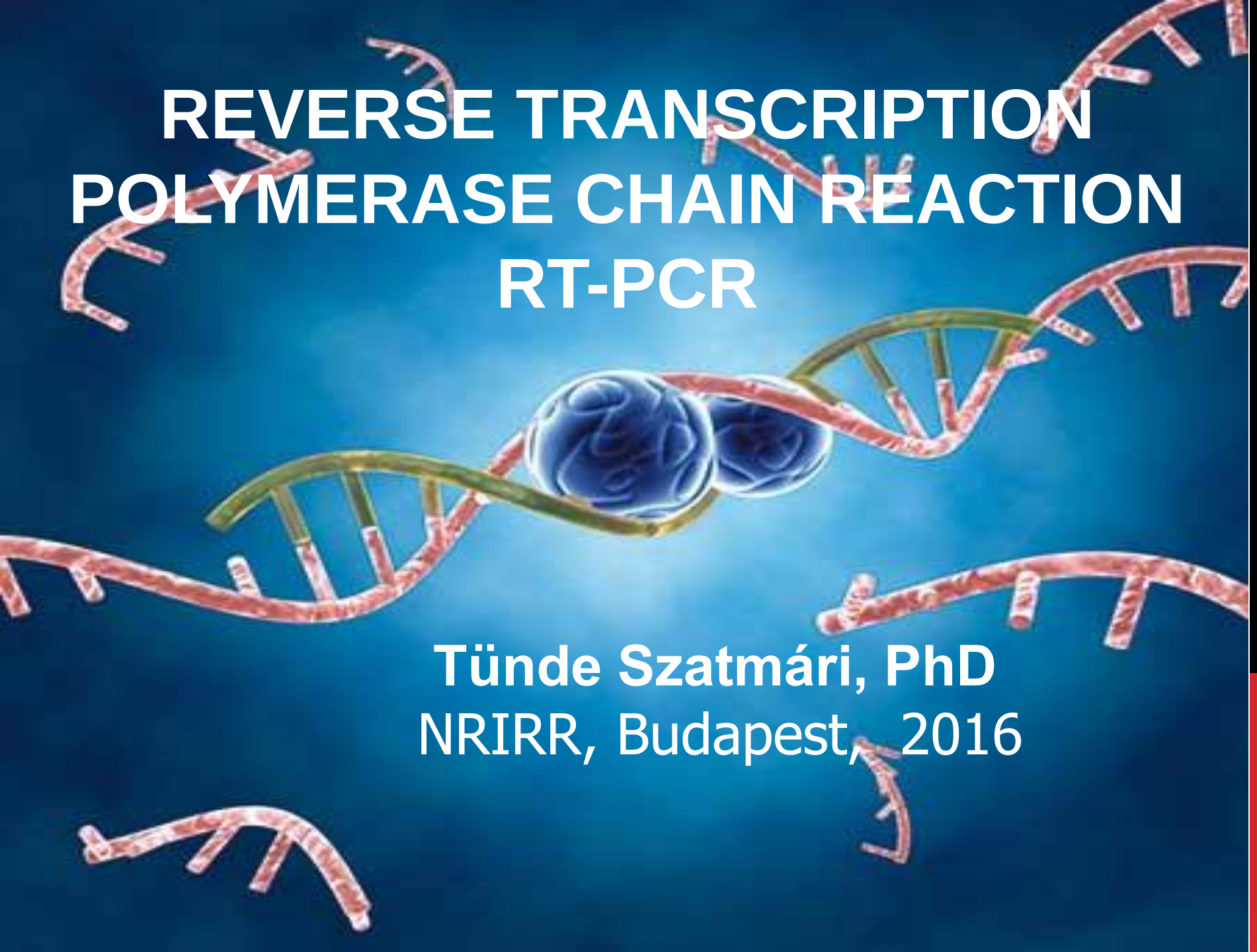
REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION RT-PCR



Tünde Szatmári, PhD
NRIRR, Budapest, 2016

RT-PCR AND TRADITIONAL PCR - DIFFERENCES

Both produce multiple copies of DNA through amplification

BUT

PCR amplifies target DNA sequences

RT-PCR reverse transcribes mRNA to cDNA and THEN amplifies this using traditional PCR.

WHY RT-PCR?

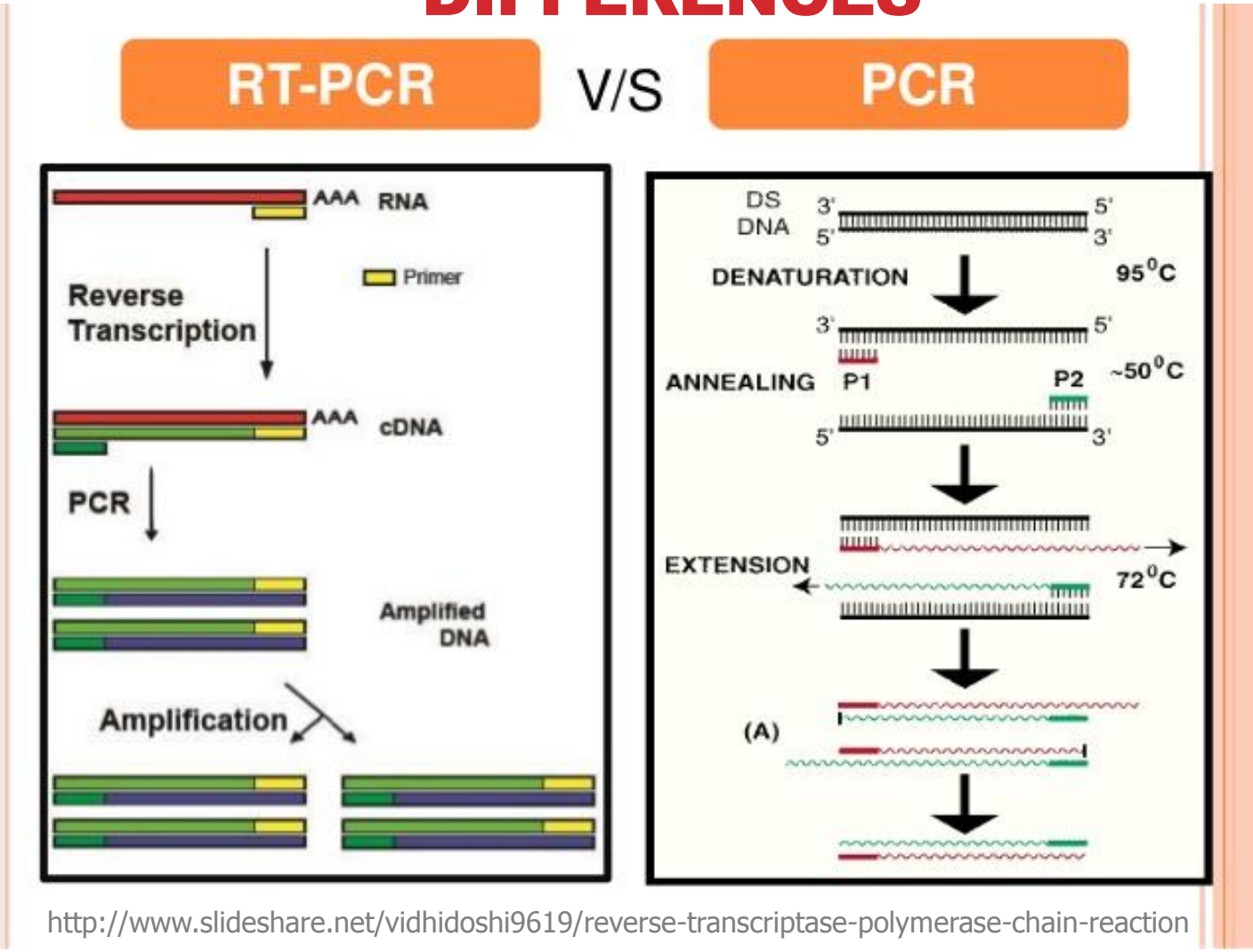
- **mRNA is the message sent for translation – gene expression**
- **DNA polymerase cannot read RNA**

DNA = exons + introns

cDNA = exons

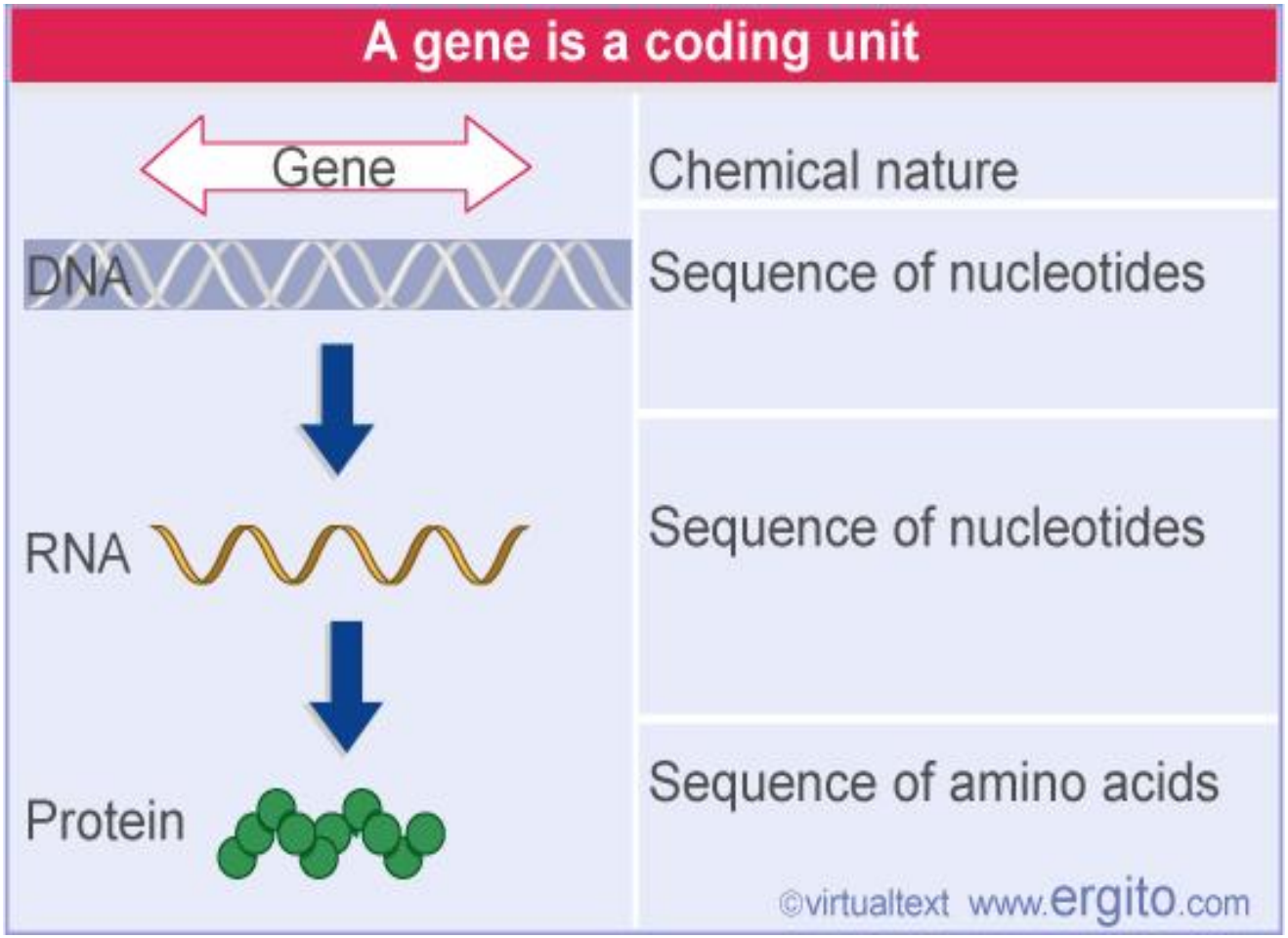
- **mature mRNA contains no introns or regulatory regions**

RT-PCR AND TRADITIONAL PCR - DIFFERENCES



http://www.slideshare.net/vidhidoshi9619/reverse-transcriptase-polymerase-chain-reaction

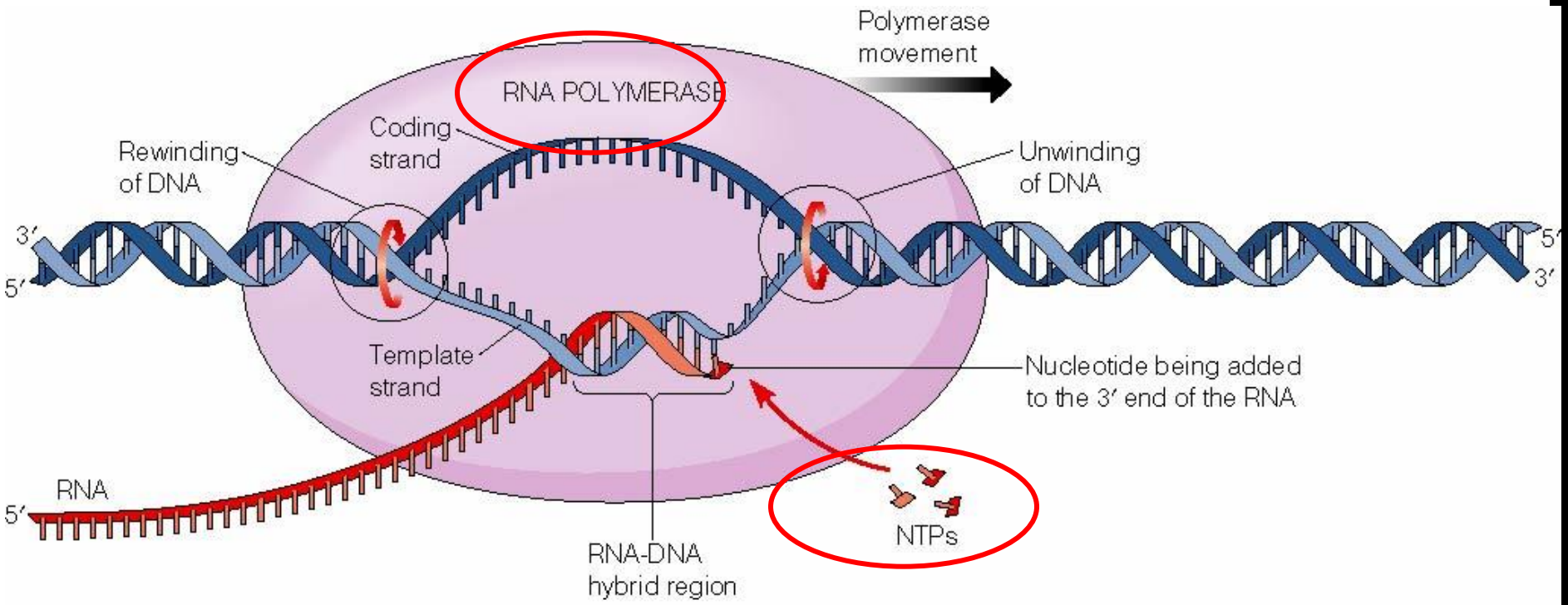
CENTRAL DOGMA



TRANSCRIPTION

TRANSLATION

TRANSCRIPTION
SYNTHESIS OF mRNA FROM DNA



A = T
C ≡ G

REVERSE TRANSCRIPTION
SYNTHESIS OF cDNA FROM RNA

- **Technique used in molecular biology to detect RNA expression by generation of complementary DNA (cDNA) transcripts from single stranded RNA**

- **Transcription: synthesis of RNA from DNA**

- **Reverse transcription: transcription of single stranded RNA into cDNA with the help of the enzyme Reverse Transcriptase.**

REVERSE TRANSCRIPTASE

- **also known as RNA directed DNA Polymerase**
- **was discovered by Howard Temin and David Baltimore in 1970 independently ; they shared Nobel Prize in Physiology or Medicine in 1975 for their discovery.**
- **are common in Retroviruses - copy the viral RNA genome into DNA prior to their integration in the host cell**

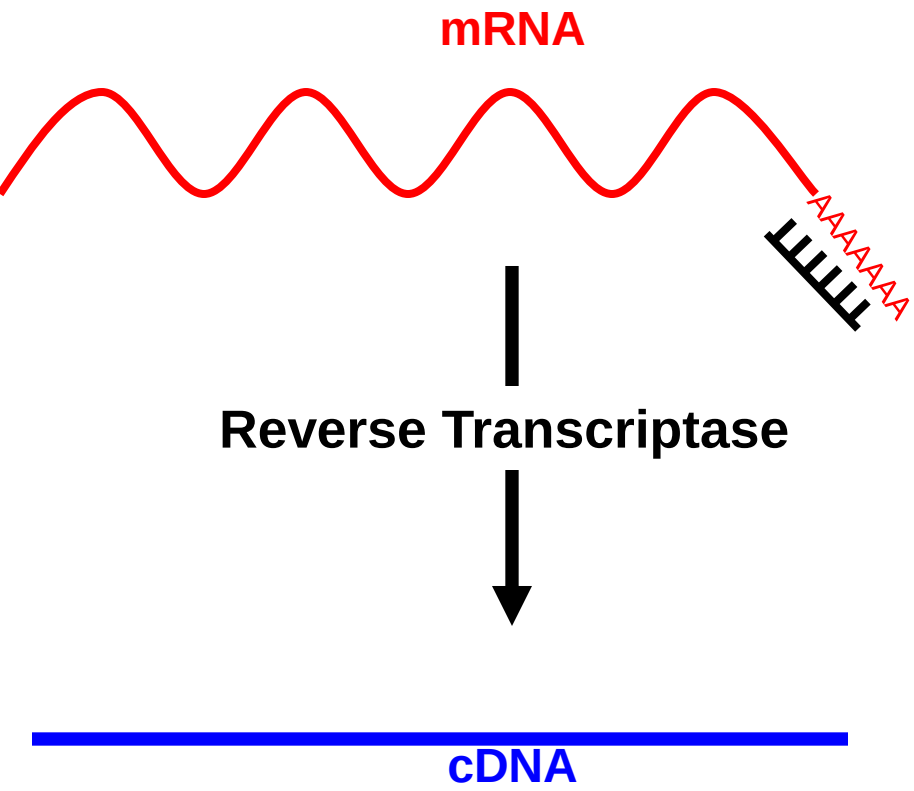
  HIV

  M-MLV (Moloney Murine Leukemia Virus)

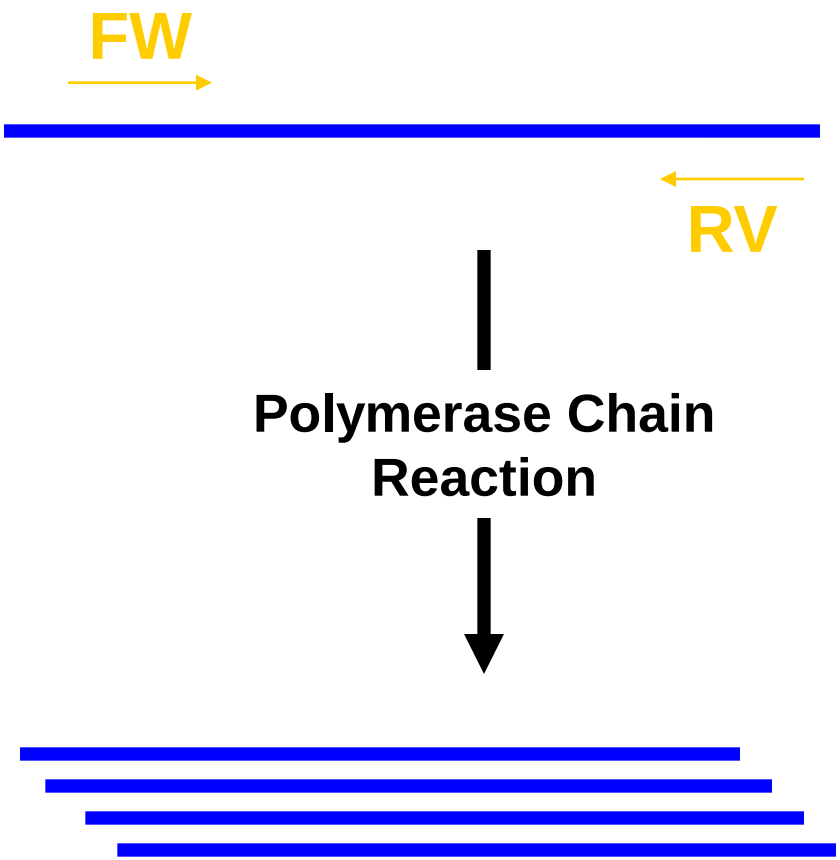
  AMV (Avian Myeloblastosis Virus)
- **RT enzymes derive from M-MLV or AMV by purification of the virus or expression in E.coli**
- **RT enzyme has two activity: DNA polymerase and RNase H**

STEPS OF RT-PCR

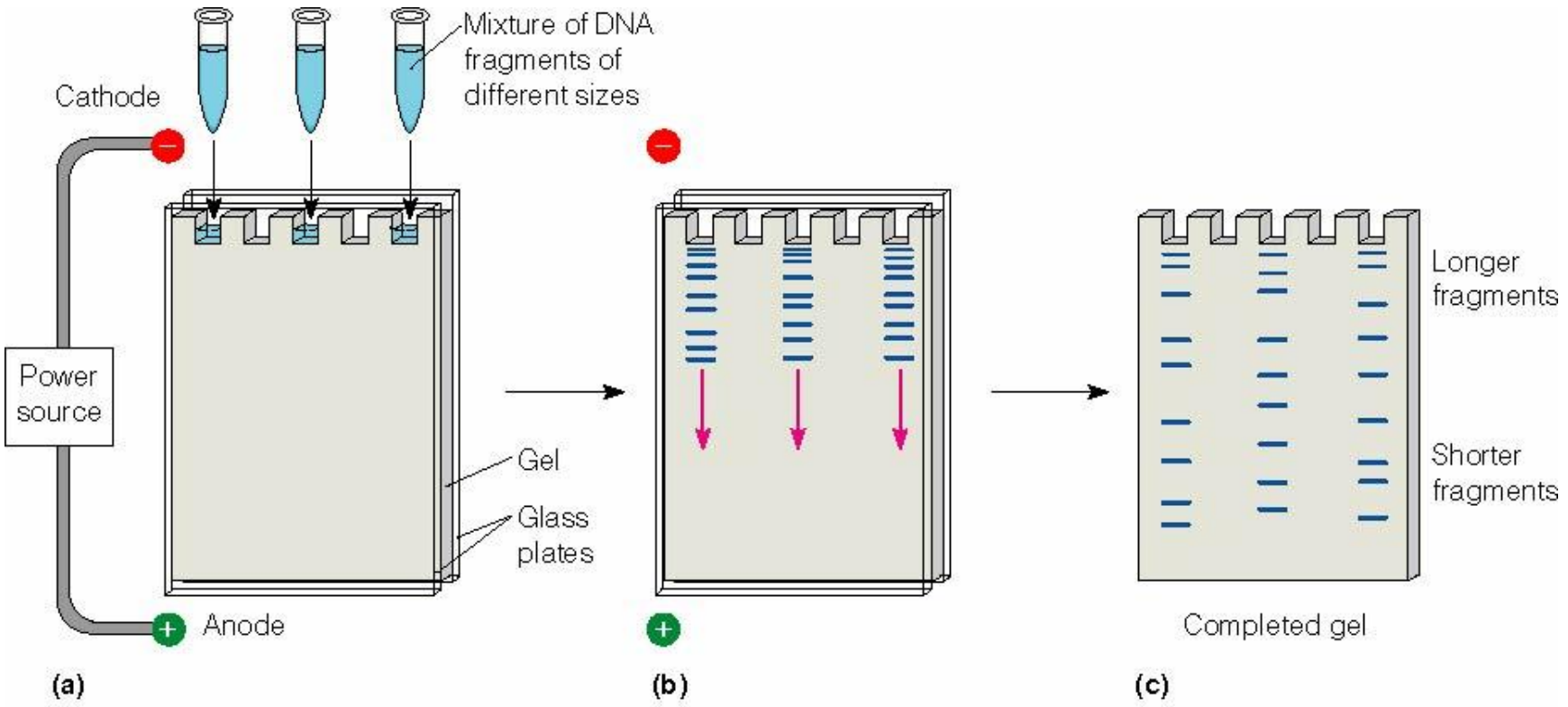
1.
Synthesize cDNA by RT



2.
Amplify cDNA by DNA Polymerase

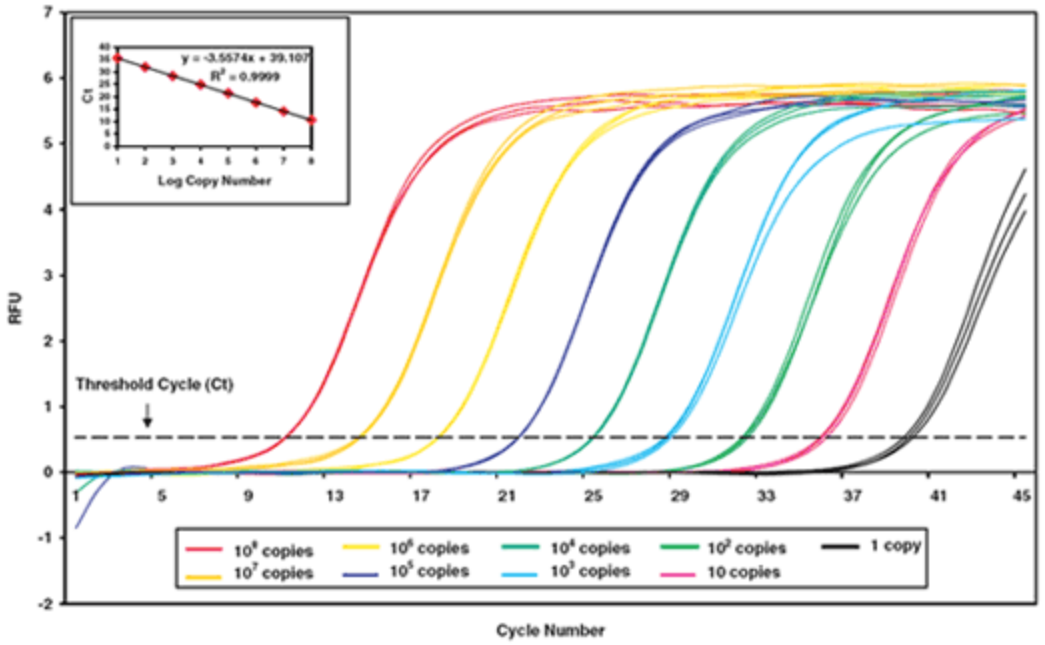


3. VISUALIZE WITH GEL ELECTROPHORESIS

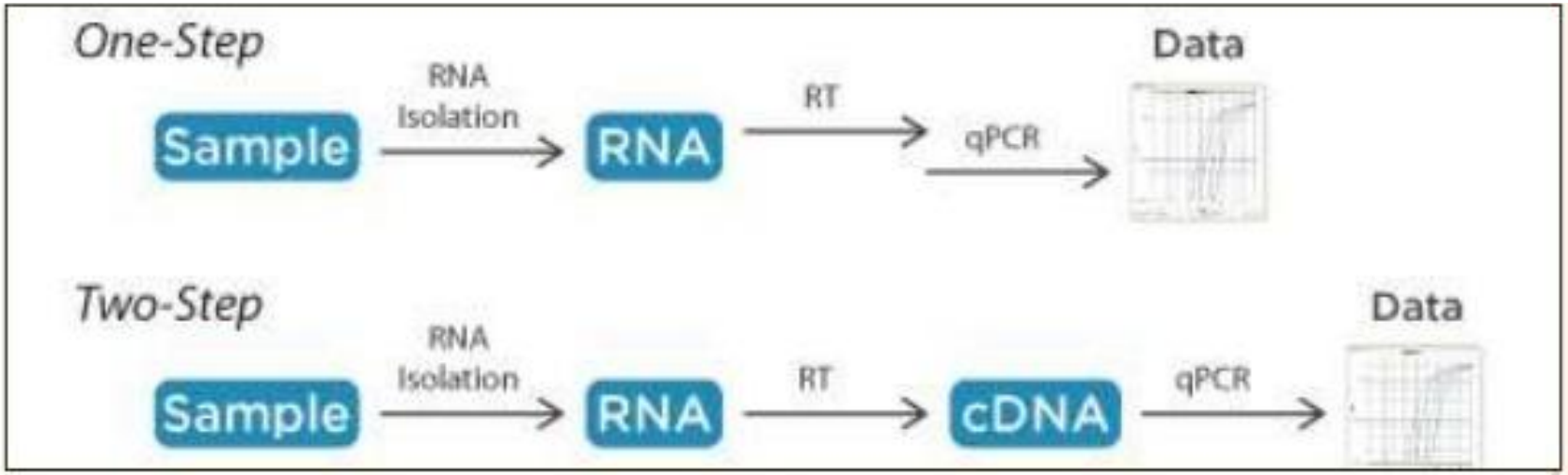


3. QUANTIFY BY qPCR

Fig. 2. Real-time PCR Amplification using HotStart-IT™ Probe qPCR Master Mix with UDG (PN 75764).



ONE STEP V/S TWO STEP RT-PCR PROCEDURES



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COMPARISON OF ONE-STEP & TWO-STEP RT-PCR PROCEDURES

	Two-Step Procedure	One-Step Procedure
Prime first-strand cDNA with:	• Oligo(dT) primer • Random hexamers • Gene-specific primers	• Gene-specific primers
Provides	**Flexibility** • Choice of primer • Choice of amplification system • Ability to save some RNA sample for later use • Ability to optimize for difficult RT-PCR (combine with Platinum® enzymes for higher specificity or combine with Platinum® Pfx for greater fidelity)	**Convenience** • Amplifcation enzymes premixed with reverse transcriptase • Fewer pipetting steps and reduced chances of contamination • High sensitivity
Recommended uses:	• Ideal for detection or quantifying several messages from, a single sample	• Ideal for analysis of large numbers of samples • Ideal for real-time quantitative

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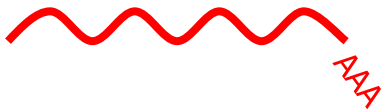
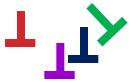
Two step PCR
Reverse Transcription

Components:

Buffer 

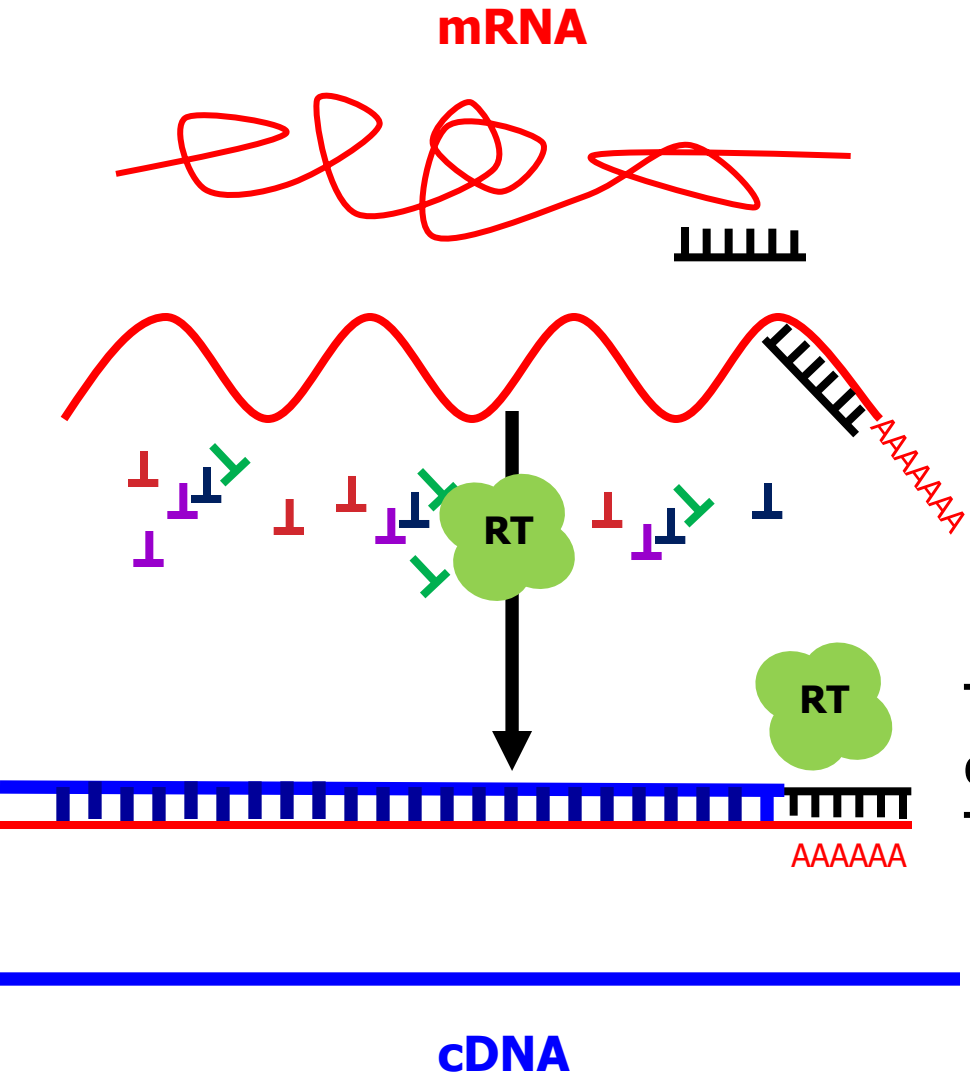
mRNA 

Primer 

dNTPS 

Enzyme- reverse transcriptase 

Two step PCR
First strand cDNA synthesis using RT



RNA incubated (with primer) to denature secondary structures –at 70°C

chilled quickly on ice to let the primer anneal

The other components added – extension by enzyme at 37-42 °C – TRANSCRIPTION

TERMINATE REACTION: 70 °C TO INACTIVATE THE ENZYME

Optional : RNASE H added

RT Buffer

TrisHCl: for maintaining the pH

MgCl2 (MnCl2), KCl salt: cofactor.

- the polymerase uses it in the catalytic area to balance the negativley charged phosphate groups of RNA template backbone.
- stabilizes duplex's structure because the negative charges would otherwise repel one another in the DNA strands
- forms soluble complex with dNTPs

DTT:

loosen the secondary structure of RNA,
breaks disulfide bonds - reduces thermostability of the bonds

Choosing the RT ENZYME:

AMV-RT thermostable = less sensitive to inhibition by strong RNA secondary structure
high RNASE H activity - reduced total cDNA yield
⟶ RNAs longer than ~5kb cannot be processed

M-MLV less thermostable: not suitable if - RNA secondary structures
-high GC content

low RNASE H activity

A = T
C ≡ G

ENGINEERED ENZYMES

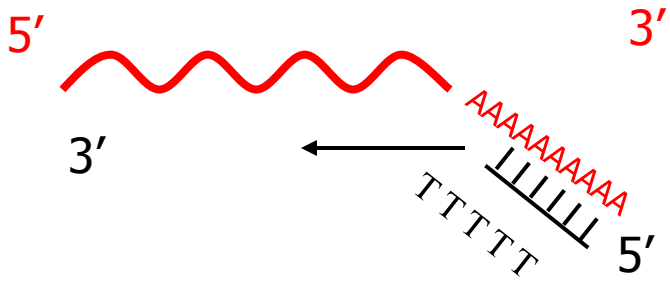
Superscript II and III (SSC III)
Superscript VILO
GoSCRIPT

What has to be considered:

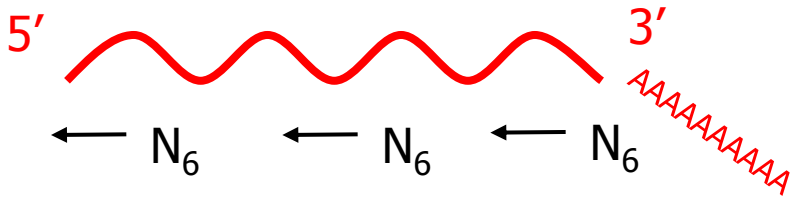
- **basic enzymatic properties**
- **enzyme's level of RNase H activity**
- **the length of the target RNA**
- **presence of complex RNA secondary structure**
- **downstream application**

Choice of PRIMER:

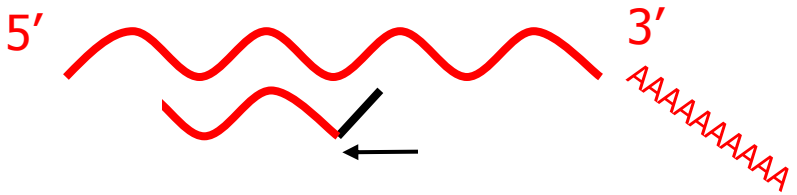
Oligo dT: eucaryote mRNA –3′ polyA tail
Problems: procaryotic genes
degraded samples

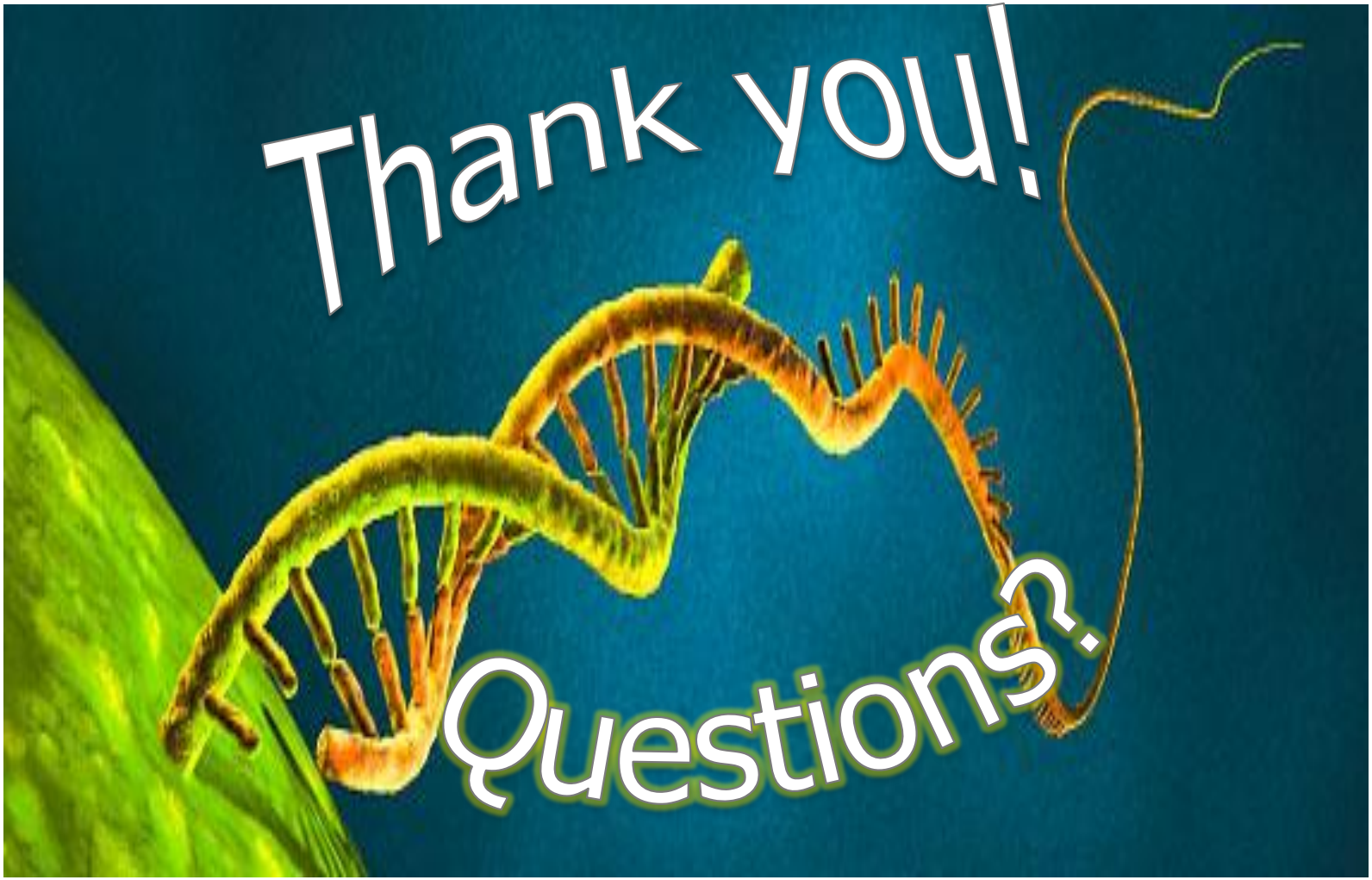


Random hexamers: random nucleotide sequences - bind all along mRNA
Pieces of cDNA, not full-length: all regions of the gene may not be equally represented



Gene specific primers: similar to conventional PCR
cDNA for one specific target transcript
Used for one-step RT-PCR




Thank you!
Questions?