qPCR in Academics, Research and Health Science

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History of PCR

Molecular photocopying

1983→Invented; Kary Mullis: Technician at the Cetus Corporation

1985→ 1st publication Cetus Corporation

1986→ Taq Polymerase purified and used

1988→ Automated Thermal Cycler

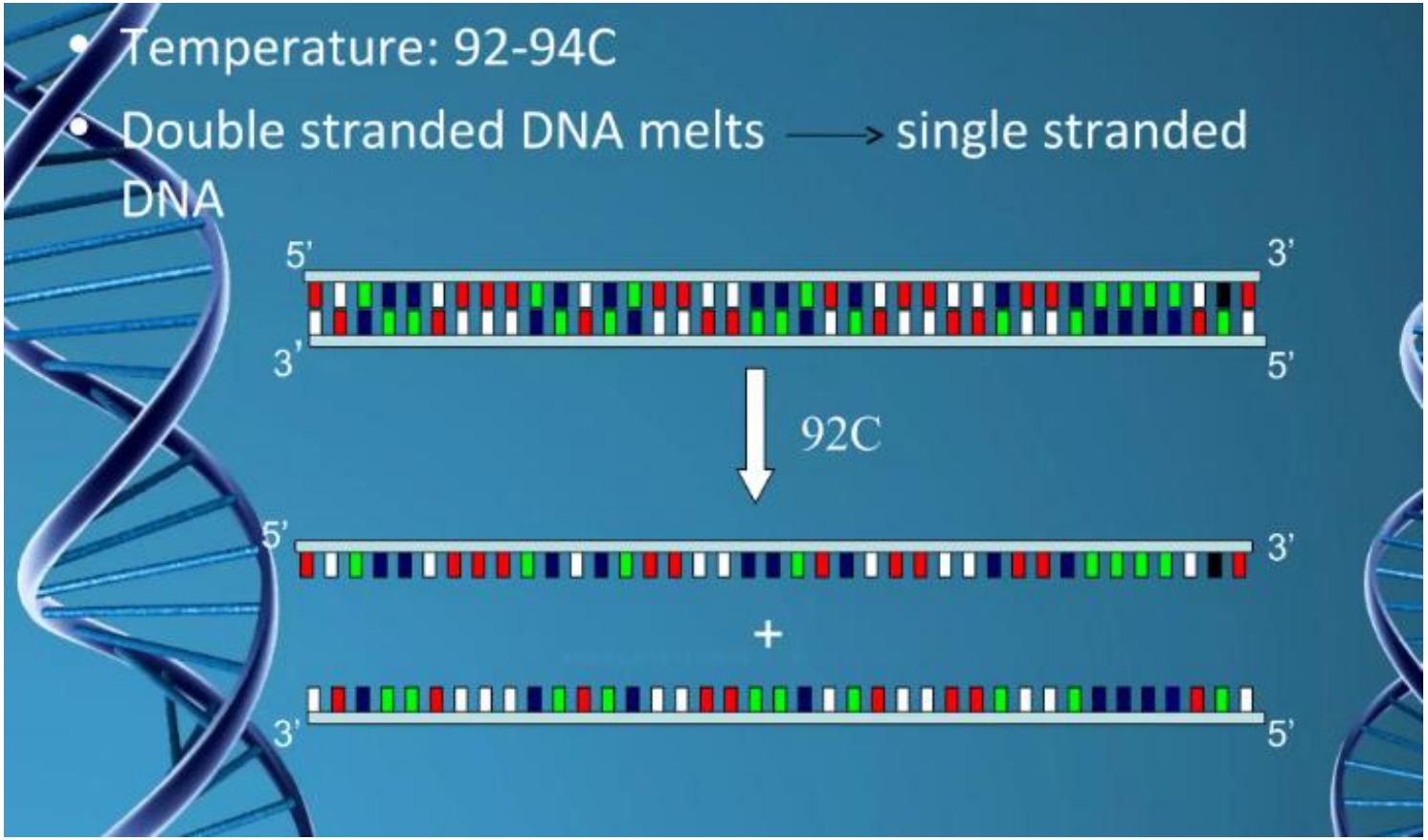
1989→ Taq Polymerase:- Molecule of the year By Science Megazine

1990→ Foundation of Real Time PCR by using fluorescent dye

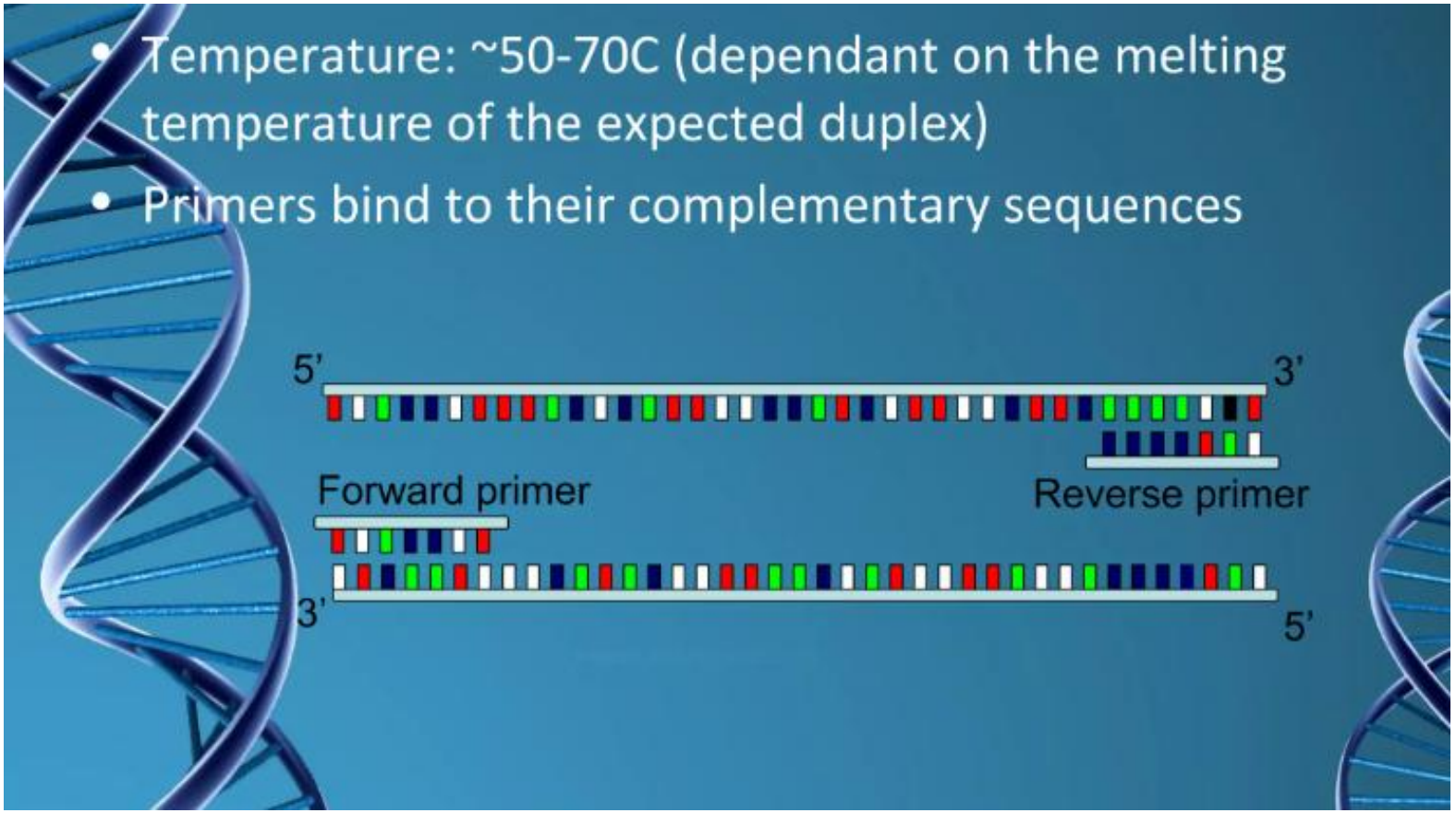
1991→ Diagnosis-RNA viruses

1993→ Nobel for Kary Mullis for his PCR Tech invention

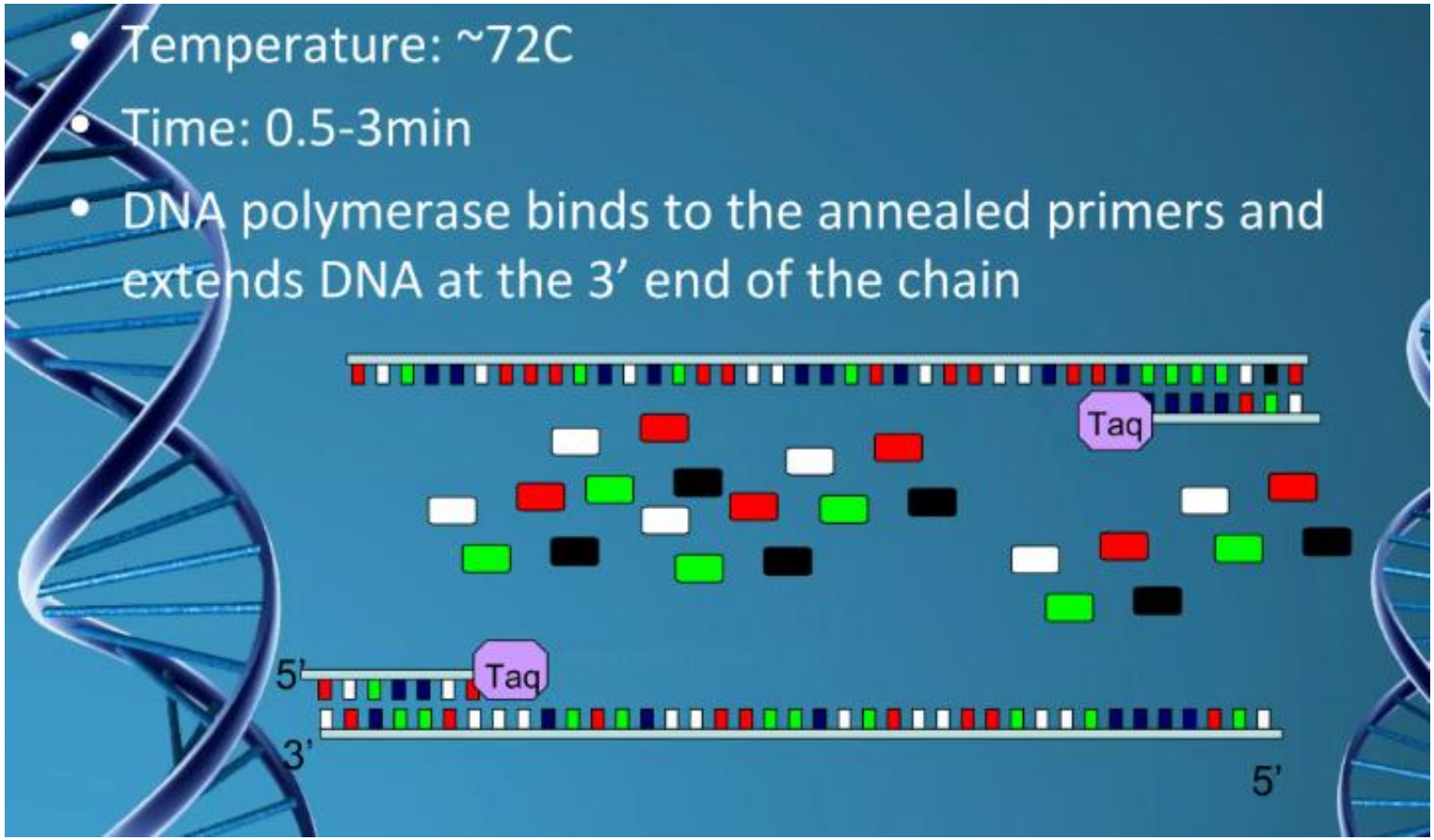
Denaturation



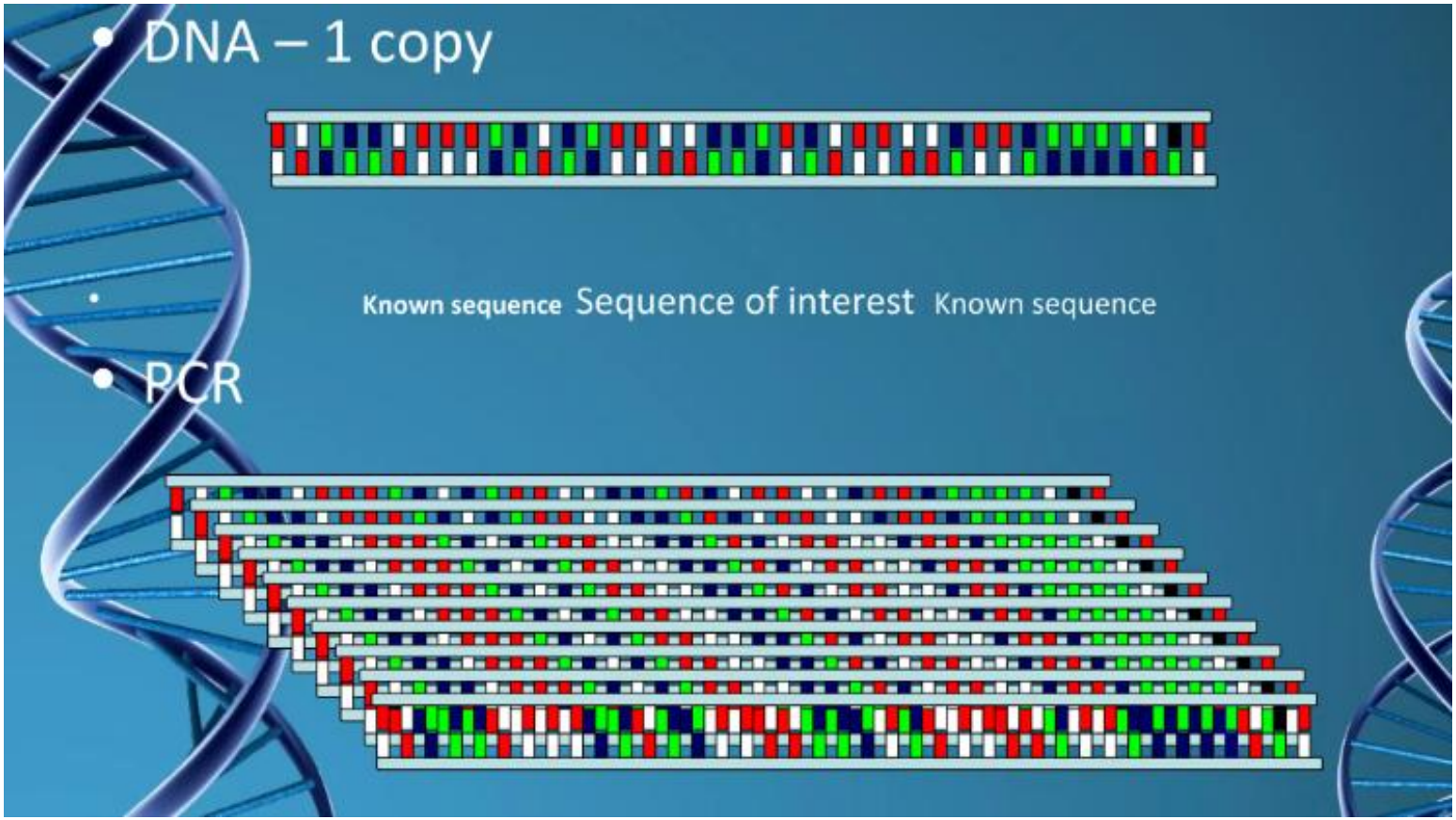
Annealing


• Temperature: ~50-70C (dependant on the melting temperature of the expected duplex)
• Primers bind to their complementary sequences
5'
3'
Forward primer
Reverse primer
3'
5'

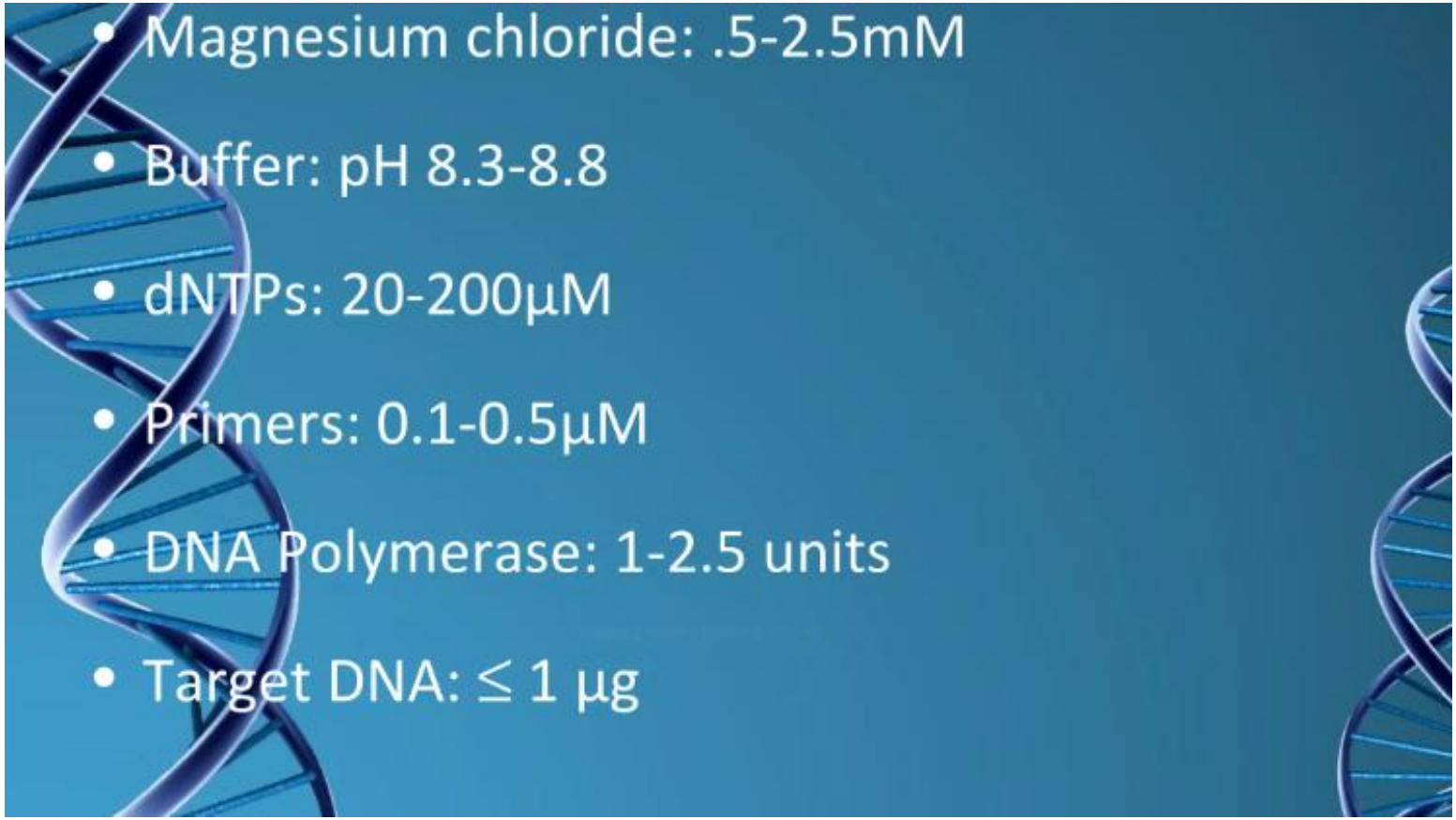
Extension


Temperature: ~72C
Time: 0.5-3min
DNA polymerase binds to the annealed primers and extends DNA at the 3’ end of the chain
Taq
Taq
5’
3’
5’

Overall Principle of PCR



Chemical Components



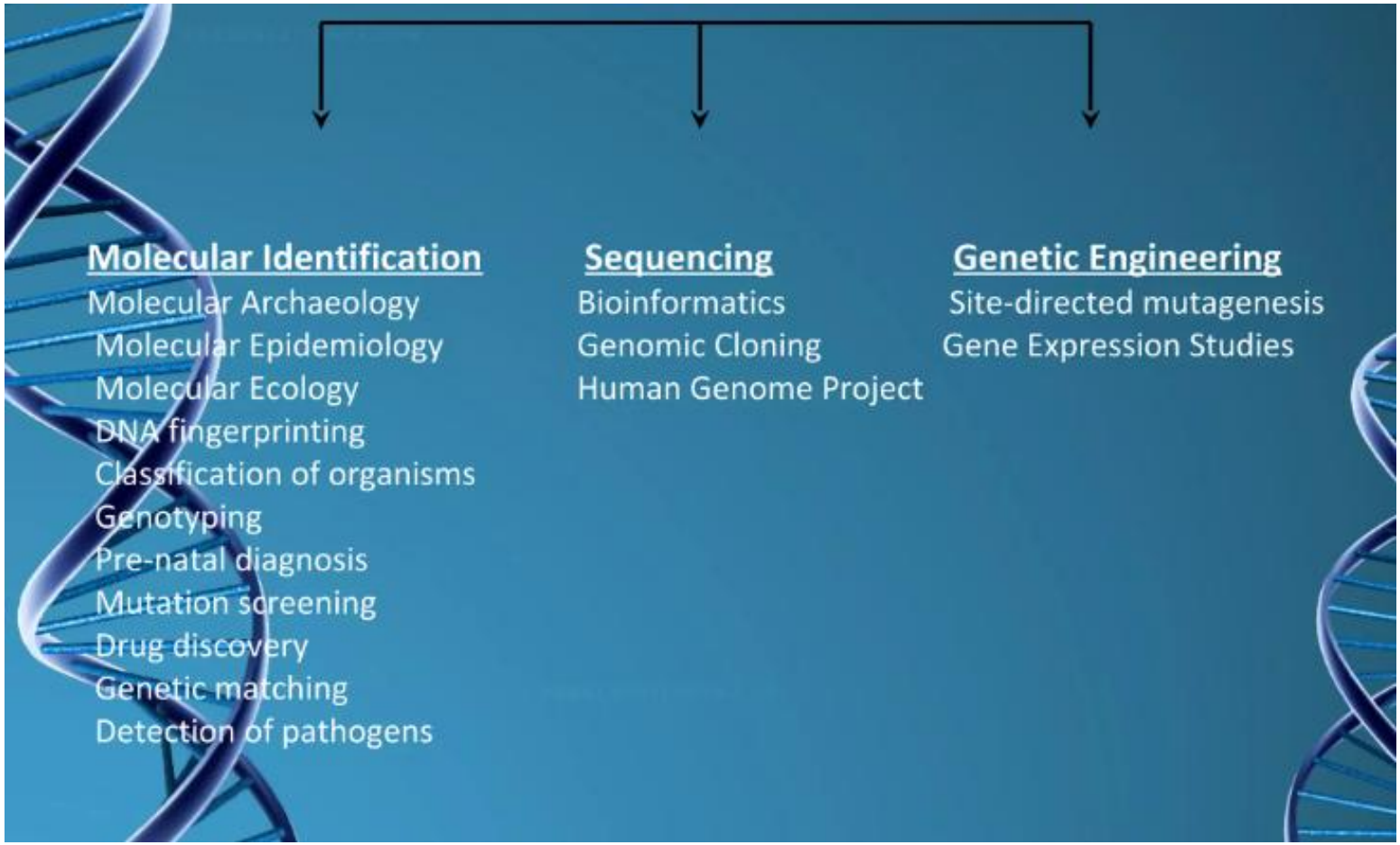
Three Aspects of PCR

- Specificity
- Efficiency
- Fidelity

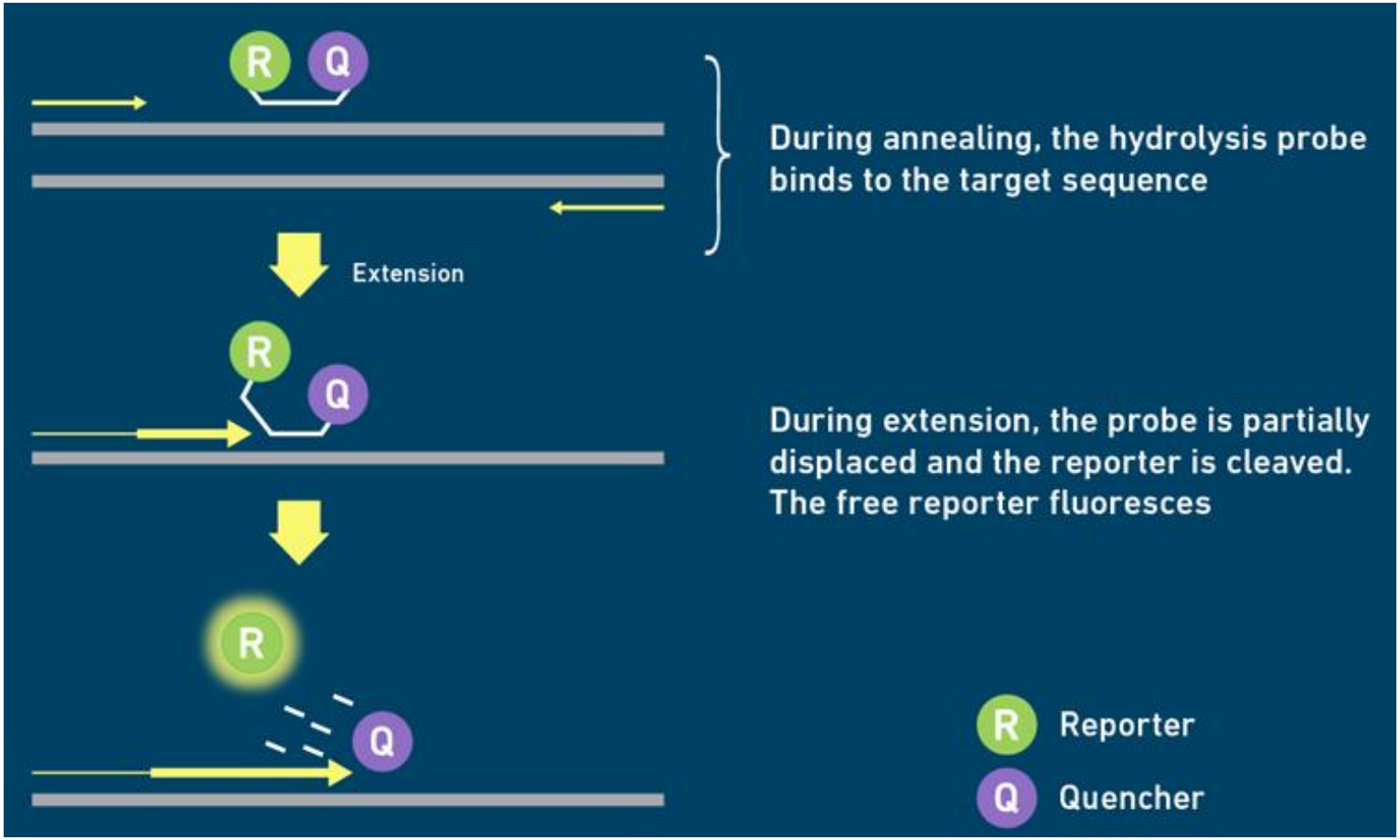
Applications of PCR

- HLA-Typing
- HIV-1
- Factor V Leiden
- *Chlamydia trachomatis*
- *Neisseria gonorrhea*
- Forensic testing and many others

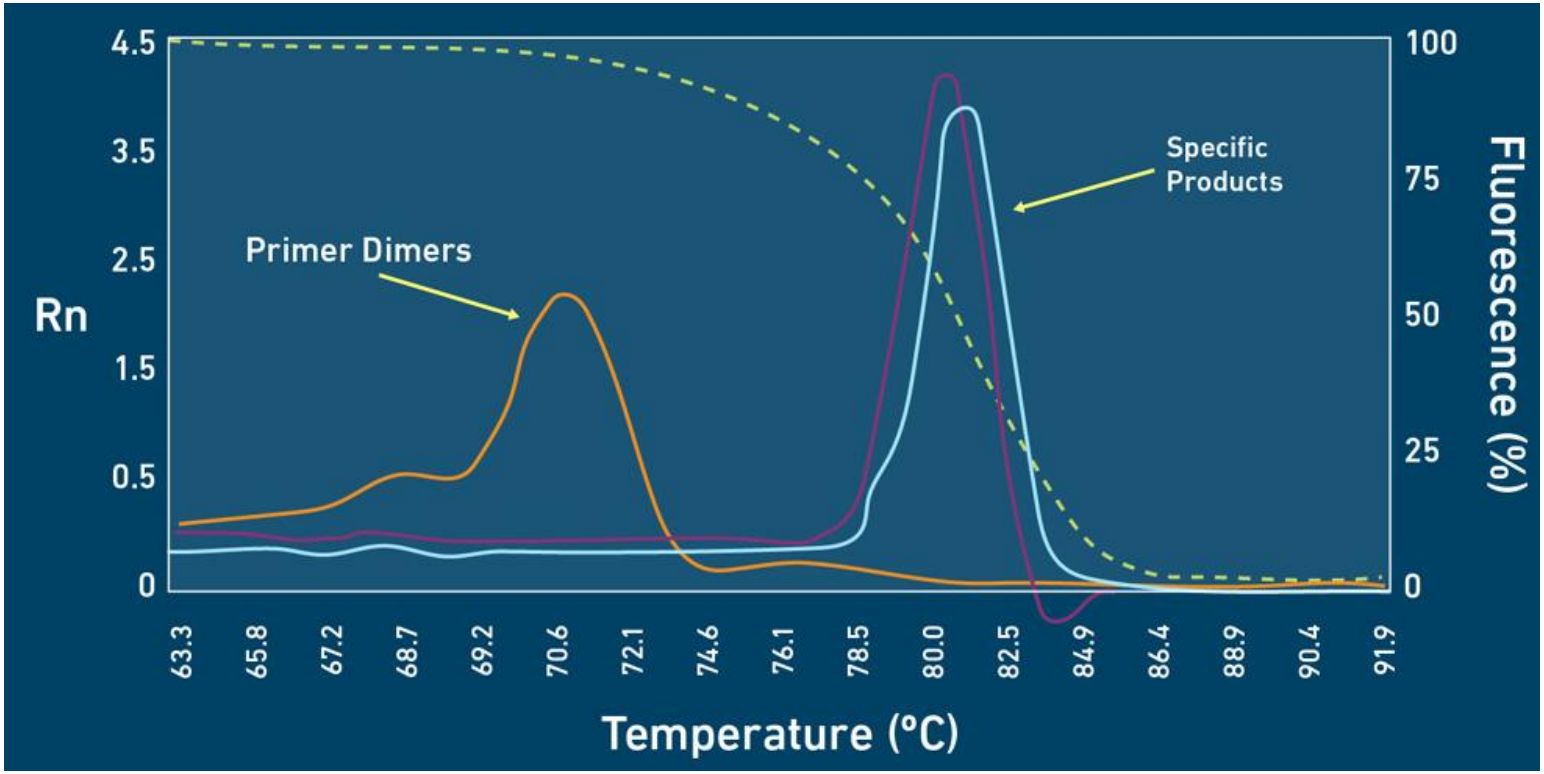
Applications of PCR



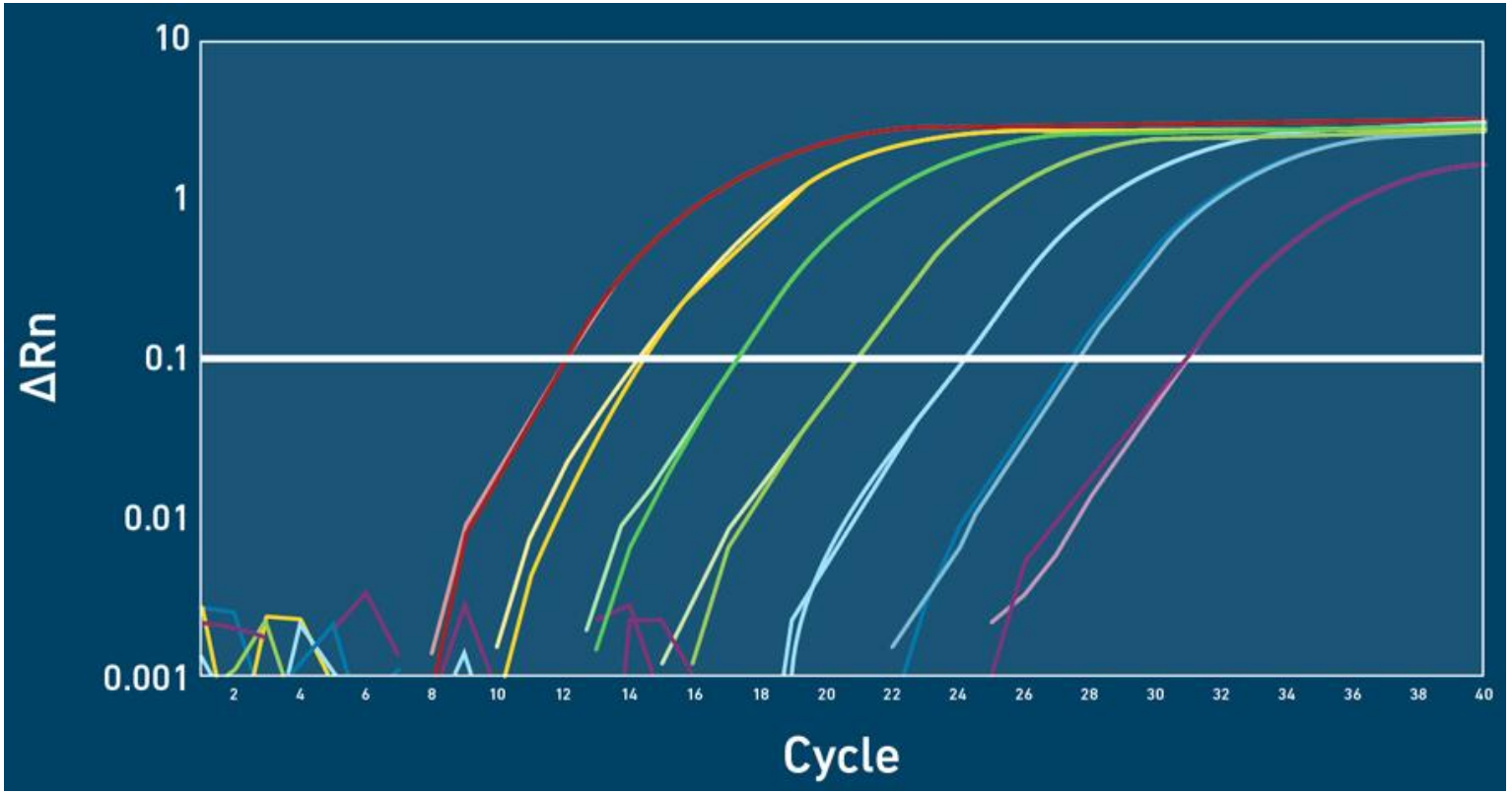
Detection in Real Time PCR



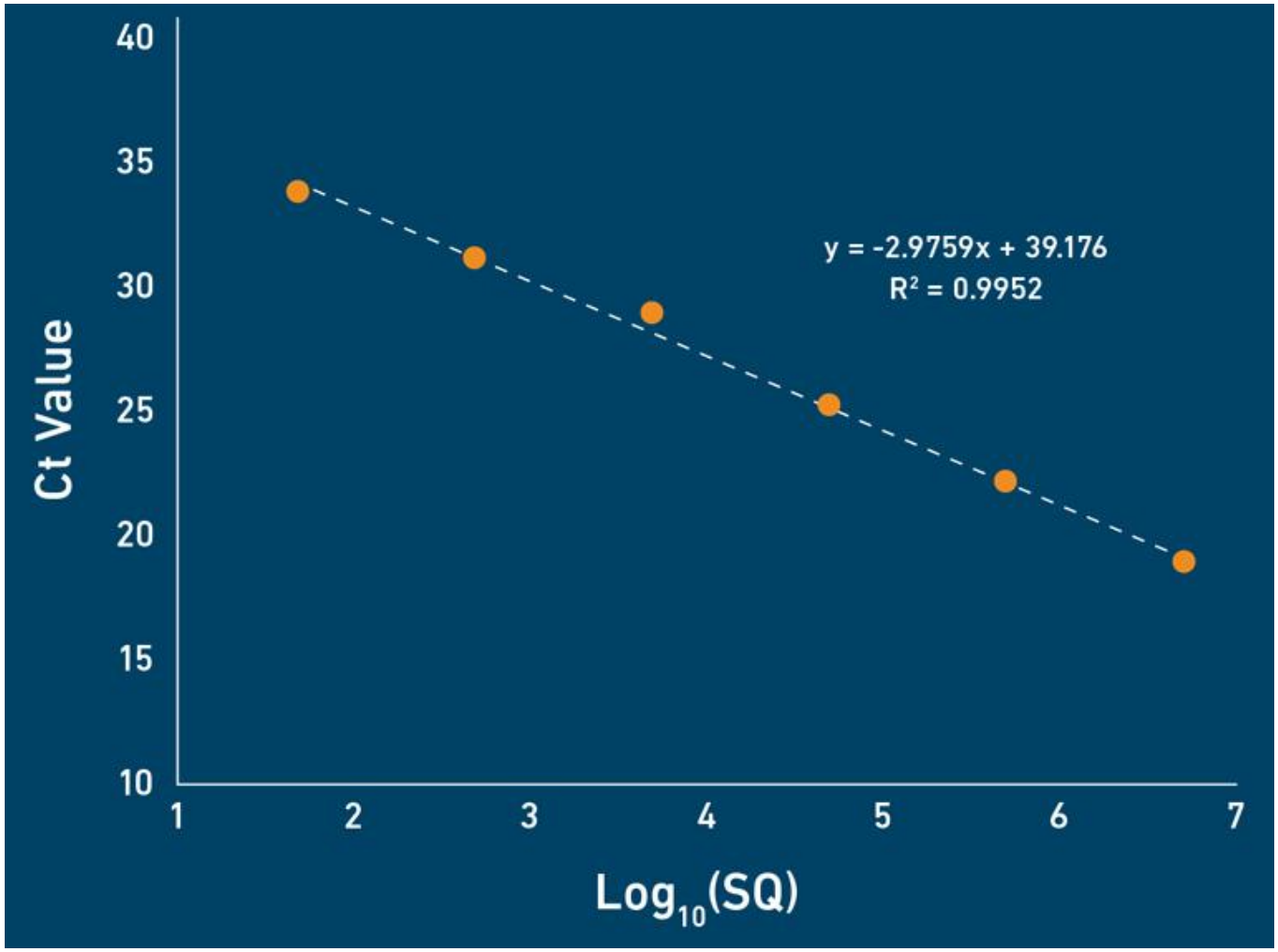
Primer Dimer Visualization



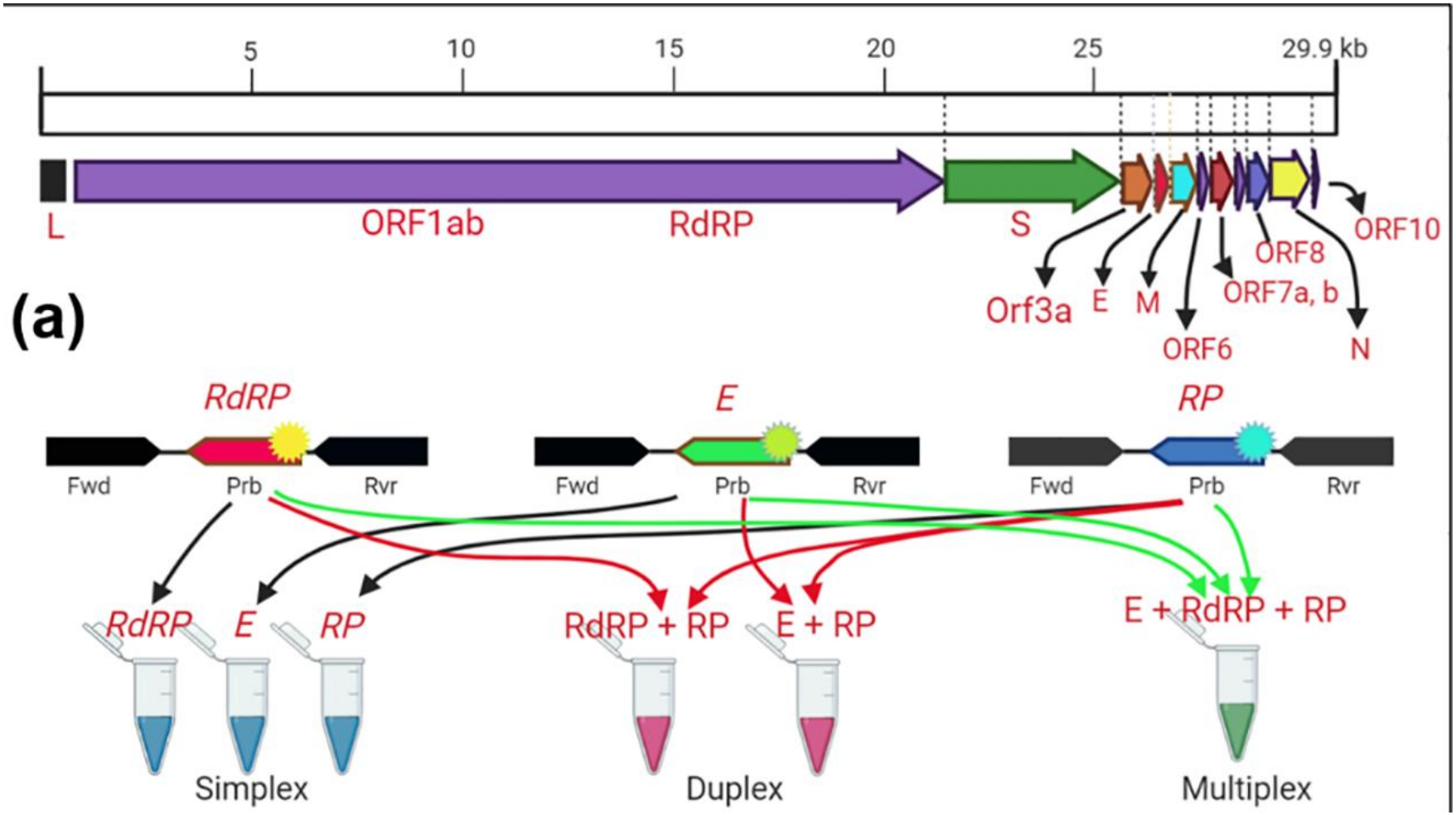
Amplification Plots



Standard Curve



SARS-CoV2



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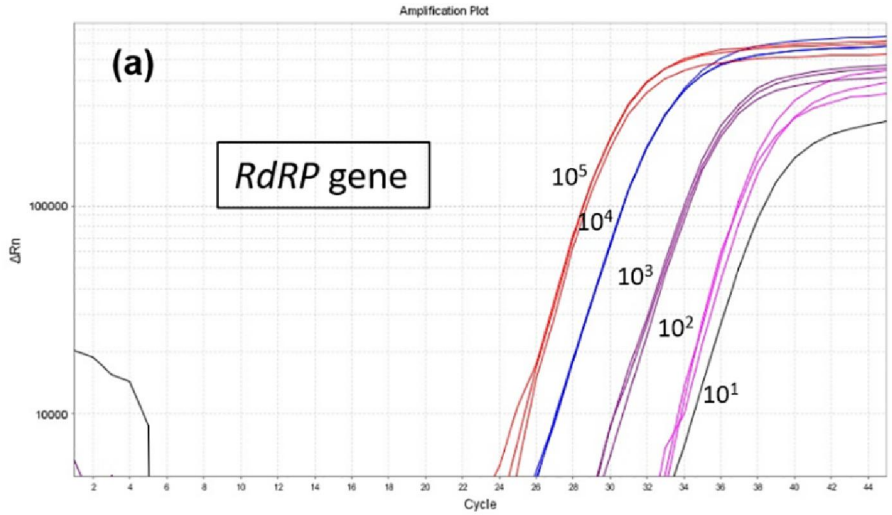
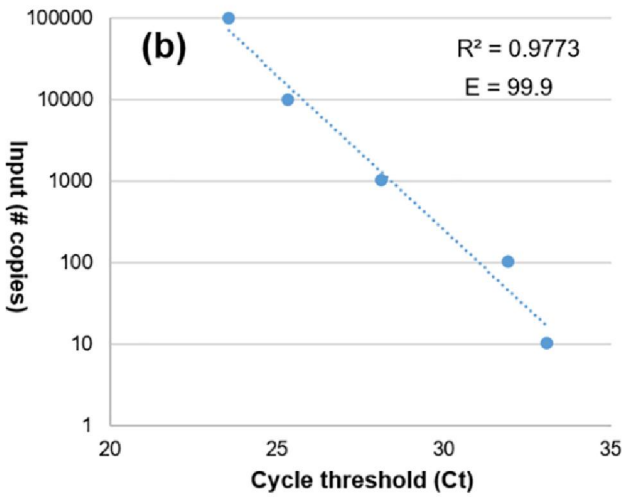
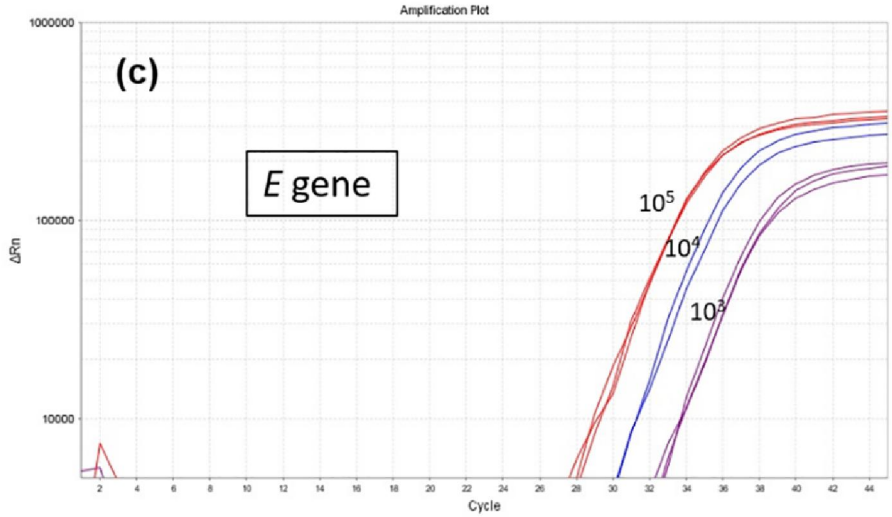
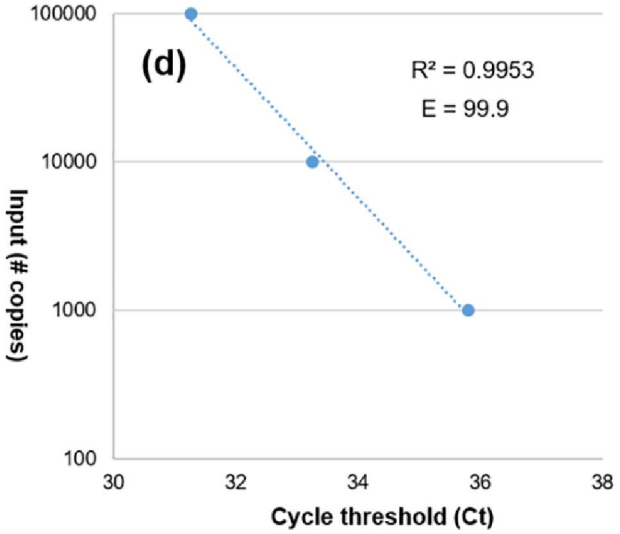
Quantification of Viral Load









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THANK YOU

MITS School of Biotechnology,

2(P), Infocity, Patia, Bhubaneswar-751024

Workshop on RT PCR

for faculty members of Life Sciences

16th February, 2023

Agenda:

10:00-10:30 | Registration

10:30-11:00 | Inaugural Session

11:00-11:15 | Hi-Tea

11:15-11:30 | Tech Lect: qPCR in academics, research and health science

11:30-01:00| Tech Lab

01:00-01:30 | Lunch Break

01:35-04:35 | Tech Lab

- **RNA extraction and cDNA preparation**
- **PCR and graphical analysis**

04:45-05:15 | Valedictory session

For Registration (visit msb.ac.in/Events/WoRTPCR/Registration)

For personal enquiry (Please contact Dr. RR Mishra, 7978528311)

LECTURE AND LAB MANUAL

1. **Tech Lec: QPCR in academics, Research and Health Science**
   a. Role of QPCR during Covid Pandemic
   b. Role of QPCR for analyzing gene expression of new variants
   c. To understand the basics of gene and genomes
   d. Application of QPCR in genomics

2. **TECHNICAL SESION (LAB WORK)**

2.1 Lab-1 : RNA extraction from Cell pellet

Key points:

Collection of Tissue/Pellet, Homogenization / Lysis of Tusse/Cells, Centrifugation, Phenol chloroform of RNA Precipitation, Purification using ethanol, Quantification and Quality Evaluation

Protocol: TRIzol method

- Transfer 50-100 mg of frozen tissue in a 15 ml tube with 1 ml TRIzol (GIBCO BRL)
- Homogenize for 60 sec in the polytron
- Add 200 µl chloroform
- Mix by inverting the tube for 15 sec
- Incubate for 3 min at room temperature
- Centrifuge at 12.000 g for 15 min
- Transfer the aqueous phase into a fresh Eppendorf tube (≈200µl)
- Add 500 µl isopropanol (Mix by inverting the tube)
- Centrifuge at max. 12.000 g for 10 min in the cold room
- Wash the pellet with 500 µl 70 % ethanol
- Centrifuge at max. 7.500 g for 5 min at 4^0C
- Dry the pellet on air for 10 min

2.2 Lab-2: cDNA preparation using RNA extraction (Superscript II, Invitrogen)

Oligos/Random primers :1µl
mRNA:Xµl
H2O:10-Xµl
Total: 12µl (Mix it gently) 700C 7minutes

Chilled it in ice for 2 minutes
5X FS buffer: 10µl
0.1M DTT : 2.5µl
dNTP: 2.5µl
RNAse out: 1µl
Mix gently incubate at 42^0C for 2minutes

SSII (RT): 1µl
21µl Mix it gently at 420C for 50 minutes
70^0C for 15minutes
cDNA is ready for the experiments

3. qPCR overview

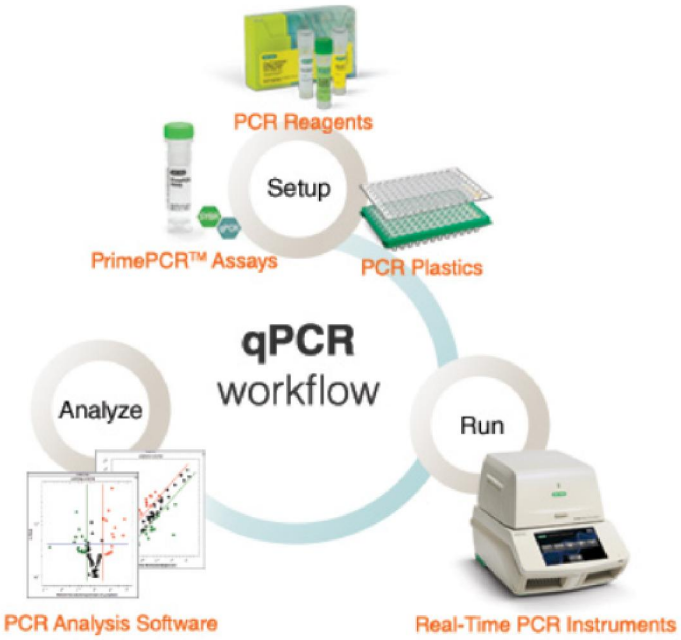

Fig.1 work flow showing PCR setup, instrumentation and analysis

4. Lab-3: Preparation of Master mixture for PCR: Components of master mixture, Functions of individual function, Concentration calculation and methodologies, How to preserve the master mixture
   Lab-4 Running and configuring experiments using Software
   4.1 Protocol setup: the denaturation, annealing, and extension parameters, the number of repeated cycles, and the steps at which data are to be collected are specified.

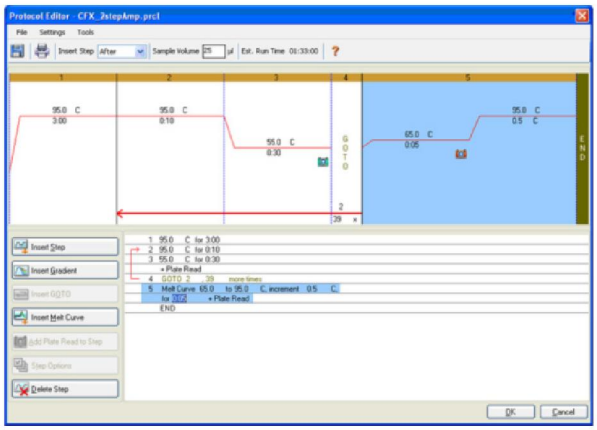

Fig. 2. Protocol Editor window in CFX Manager Software**.** This protocol consists of amplification followed by a melt curve to verify that a specific product has been produced.

4.2 Set up the plate — indicate position of unknown, no template controls (NTCs), and standard curve samples on plate (Figure 10). This can be done before, during, or after a run.

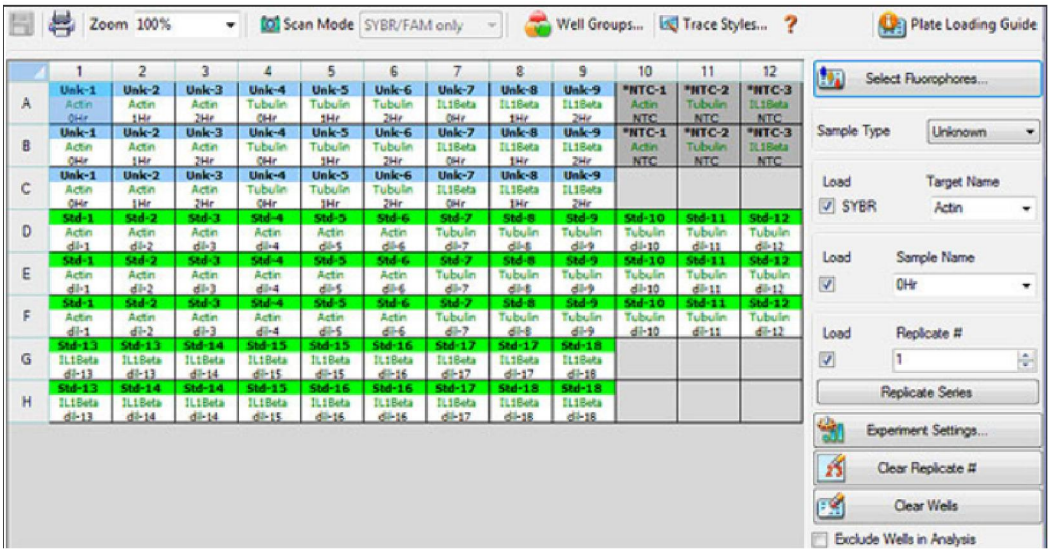

Fig. 3. Run Details window in CFX Manager software. The intensity of FAM fluorescence for all wells of a 96-well plate is shown up to cycle 16.

Lab-5 Data Collection and Analysis

5.1. Amplification plot

To understand how real-time PCR works, we illustrate a qPCR analysis using a typical amplification plot. In this plot, the number of PCR cycles is shown on the x-axis, and the fluorescence from the amplification reaction, which is proportional to the amount of amplified product in the tube, is shown on the y-axis.

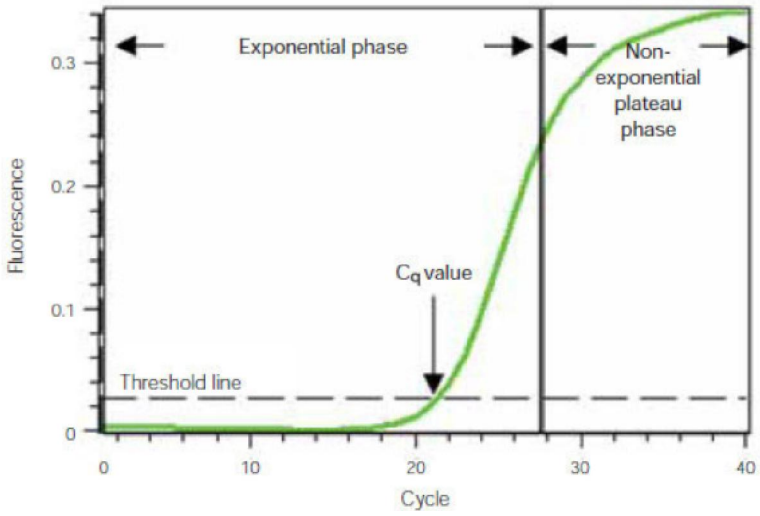

Fig.4 . Amplification plot. Baseline-subtracted fluorescence versus number of PCR cycles. The amplification plot shows two phases, an exponential phase followed by a non-exponential plateau phase. During the exponential phase, the amount of PCR product approximately doubles in each cycle. As the reaction proceeds, however, reaction components are consumed, and ultimately one or more of the components becomes limiting. At this point, the reaction slows and enters the plateau phase (cycles 28–40 in Fig).

5.2 Data collection: The fluorescence intensity of the reaction mixture in each well is measured and plotted versus the reaction cycle. If a target of interest is present, its amplification can be monitored in real time.

5.3 Analysis of the data — a data file is automatically generated after a run with the gene expression module. Easily view up to six different charts or tables, such as the amplification plot, standard curve, gene expression chart, plate layout, or melt peak with the Custom Data View tab (Figure 5). Check the efficiency and R^2 of the standard curve. The efficiency should be within 90–110% and the R^2 should be >0.990. If these values are out of range, you will need to troubleshoot your experiment.