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NUCLEIC ACID AMPLIFICATION TECHNIQUES IN THE DETECTION OF INFLUENZA VIRUS: FOR BETTER UNDERSTANDING IN DEVELOPING COUNTRIES

*[1, 2] Anjorin, AA, [2]Oke, BO, [2,3]Obi, RK, [2]Salu, OB, [1]Oyefolu, AOB, [2]Omilabu, SA

[1]Department of Microbiology, Lagos State University, Ojo, Lagos, Nigeria
[2]Virology Research Laboratory, Department of Medical Microbiology and Parasitology, College of Medicine, University of Lagos, Nigeria
[3]Department of Microbiology, Federal University of Technology, Owerri, Imo, Nigeria

*Correspondence: Anjorin, A. A.: abdul-azeez.anjorin@lasu.edu.ng
+2348020956357

OUTLINES

- *Introduction*
- *Classical methods and types*
- *Principle and techniques involved*
- *Limitations*
- *Conclusion*
- *References*

INTRODUCTION

BACKGROUND INFORMATION

- Advanced laboratory detection and surveillance of influenza virus dates back to 1930.
  - In Africa, they were not mentioned in literature until later in the 19th century (Yazdanbakhsh and Kremsner, 2009).
- Viral activities persisted but there was poor surveillance system due to
  - inadequate information on current techniques and research funding.
- Despite the use of molecular techniques for over 3 decades (Schoub *et al*., 2002).

BACKGROUND INFORMATION..2

- Tissue culture can generate a large quantity of IV but requires up to 10 days (Vemula *et al*., 2016).
- Serological assays can provide information on surveillance but in retrospective.
  - Not suitable for novel subtypes.
- Rapid influenza diagnostic tests (RIDTs) can be used in 10 minutes.
  - Not so sensitive (Kim and Poudel, 2013).

PROBLEM STATEMENTS

- Most studies on IV in Nigeria were carried out using traditional assays.
- These methods are time wasting and are not suitable for Influenza virus subtyping.
- Subtypes are important for WHO IV preparedness: in vaccine development.

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AIM

- *To review the underlining principles and processes of NAAT in the detection and epidemiological surveillance of influenza virus.*

SAMPLE COLLECTION

- At the peak of viral shedding, before antiviral (To *et al*., 2010a).
  - From trachea or cloaca (Hoffmann *et al*., 2001).
- Samples from 2 main parts:
  - Nasal and throat wash or swab (Ngaosuwankul *et al*., 2010).
  - Sputum and endotracheal aspirate (Blyth *et al*., 2009).

MOLECULAR METHODS

- Generally, there are about 30 molecular tests:
  - Some use protein, others, NA (Prusti, 2015).
- Viral molecular assays are based on the detection of their core NA molecule.
- Simply grouped into 2:
  - Non and Amplification molecular techniques.
- Major challenge is to compare how:
  - Sensitive, specific and the cost (Shojaei, 2015).

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AMPLIFICATION MOLECULAR TECHNIQUES

- Amplification of NA is a gold standard for small amount of nucleotides.
- Very sensitive: Can detect <10 NA copies:
  - Nuclear DNA, cytosolic DNA, mRNA, and non-coding RNAs (ncRNAs) (Chang *et al.*, 2012; Goda *et al.*, 2015).

AMPLIFICATION MOLECULAR TECHNIQUES

In NAAT, there are PCR and Non-PCR based methods/ broadly divided into 3 based on mechanism

Table 1: Nucleic acid amplification methods based on 3 main mechanisms:

Technique	Type
AMPLIFICATION METHODS	
Target Amplification	PCR techniques
	NASBA
	LAMP
Signal Amplification	bDNA assays
	Hybrid capture assays
Probe Amplification	Ligase chain reaction
	Cycling probe technology
	Cleavase-invader technology
Nucleic Acid sequencing	Sanger sequencing
	Pyrosequencing
Others: Microarrays	DNA microarrays
	Multiplexed microsphere-based array

Source: Adapted from Cobo, 2012.

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PCR-BASED METHODS

PCR-BASED METHODS

- PCR is a NA amplification method that transformed biological science
  - by specific detection/ production of large amounts of DNA for the first time (Mullis, 1990; Garibyan and Avashia, 2013).
- Derived its name from the word "polymerase"- the only enzyme involved: DNA polymerase.
  - Called "chain" because the products of one reaction become substrates of the next (Olaleye, 2013; Saeed and Ahmad, 2013).

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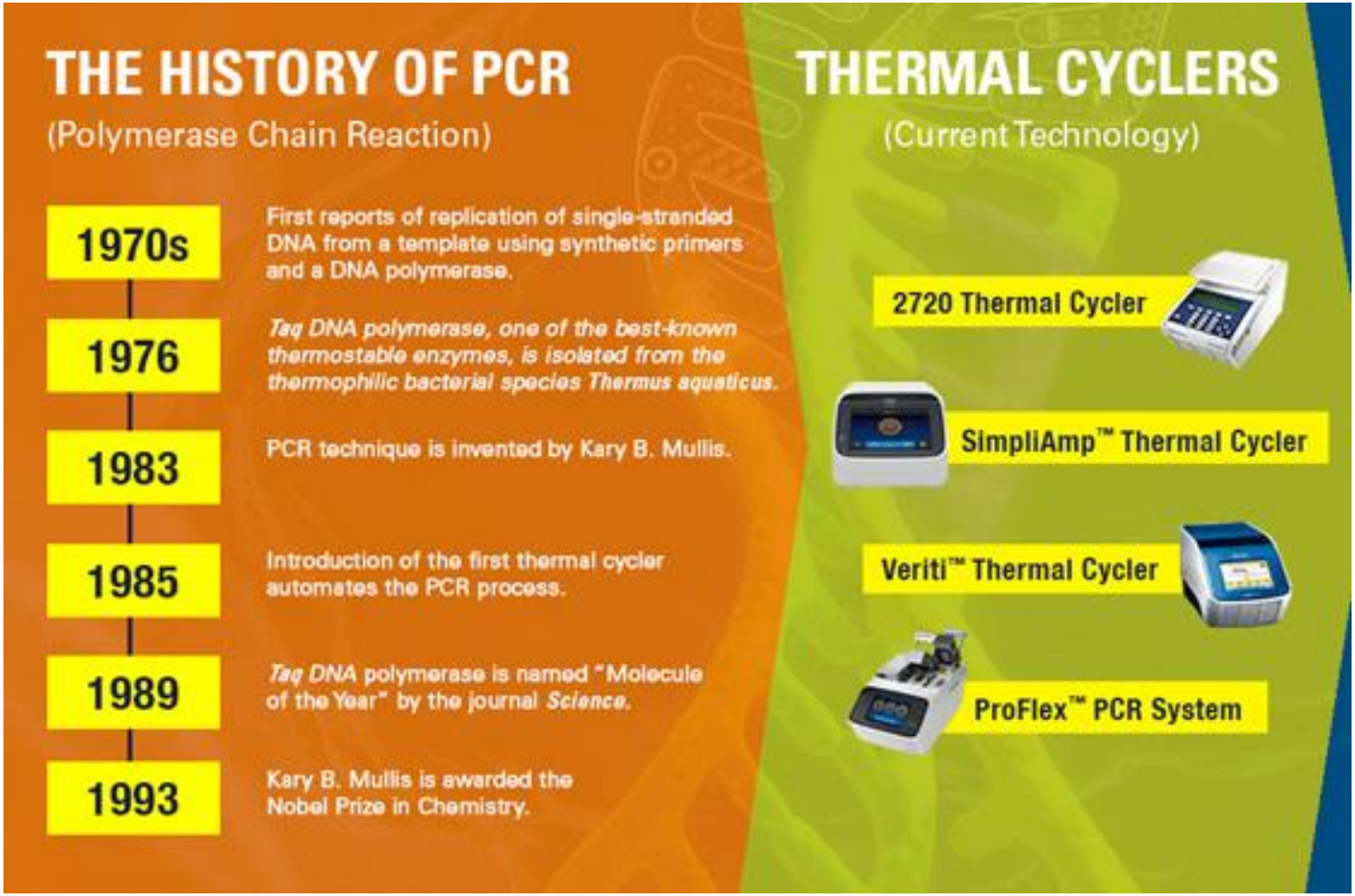

Figure 1a: Conventional PCR/ Reverse Transcriptase-PCR.

Source: Applied Biosystems

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CONVENTIONAL PCR/ REVERSE TRANSCRIPTASE-PCR

- PCR uses primers flanking target region of the gene for amplification e.g. IV NP and matrix (Gatica-Wilcox, 2014).
- Principle is based on nucleotide complementarity/ enzymatic exponential synthesis under thermal cycling.
  - Kary Mullis, the father of PCR elucidated that "it allows you to pick the piece of DNA you're interested in and have as much of it as you want".

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CONVENTIONAL PCR/ REVERSE TRANSCRIPTASE-PCR…2

- First PCR-based assay for IV was described by Zhang and Evans in 1991.
- It involves conversion of viral RNA into complementary (c) DNA.
- There are 2 methods of performing RT-PCR:
  - One-step: RT in a combined reaction +PCR
  - Two-step: RT, prior to the actual PCR.

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PCR AMPLIFICATION STEPS

- Under repeated cycles in 3 basic steps:
- In denaturation: cDNA strands are separated by heating at 95°C to break the bond.
- Annealing: hybridisation process at 40-60°C for primer binding.
- Elongation: Taq polymerase synthesis with nucleotides addition.
  - (Garibyan and Avashia, 2013; Saeed and Ahmad, 2013).

PCR AMPLIFICATION STEPS…2

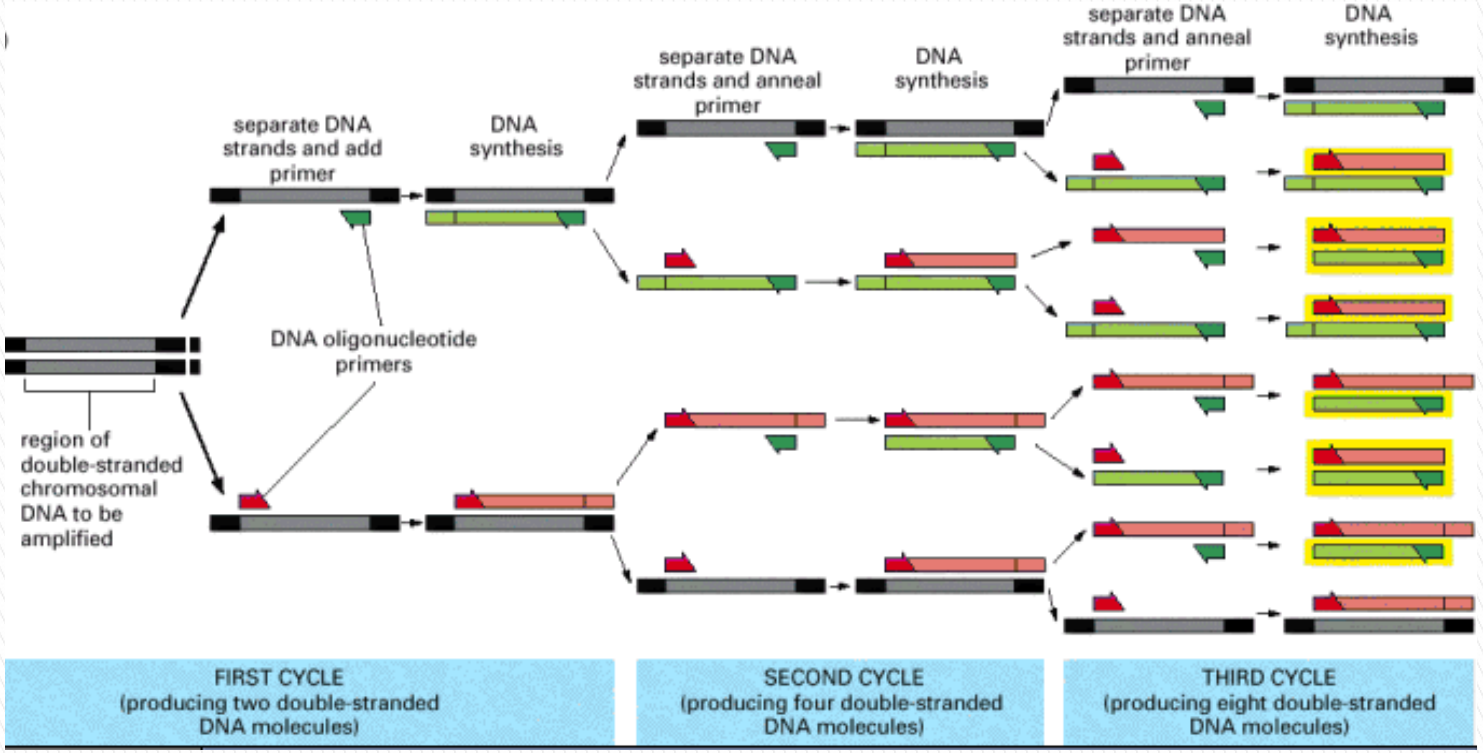

Figure 1b: Illustration of the steps involved in Polymerase Chain Reaction.

Source: Prusti, 2015.

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REAL-TIME REVERSE TRANSCRIPTASE-PCR

- Real-time/q RT-PCR (rT/qRTPCR) is a type of PCR for both detection and quantification.
- Recommended by CDC, WHO and OIA for IV due to high sensitivity/high-through put.
- Fundamental principle is the ability to monitor amplification with a good detection system
  - and quantify the amplicons as they are synthesized (Saeed and Ahmad, 2013).

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REAL-TIME REVERSE TRANSCRIPTASE-PCR…3

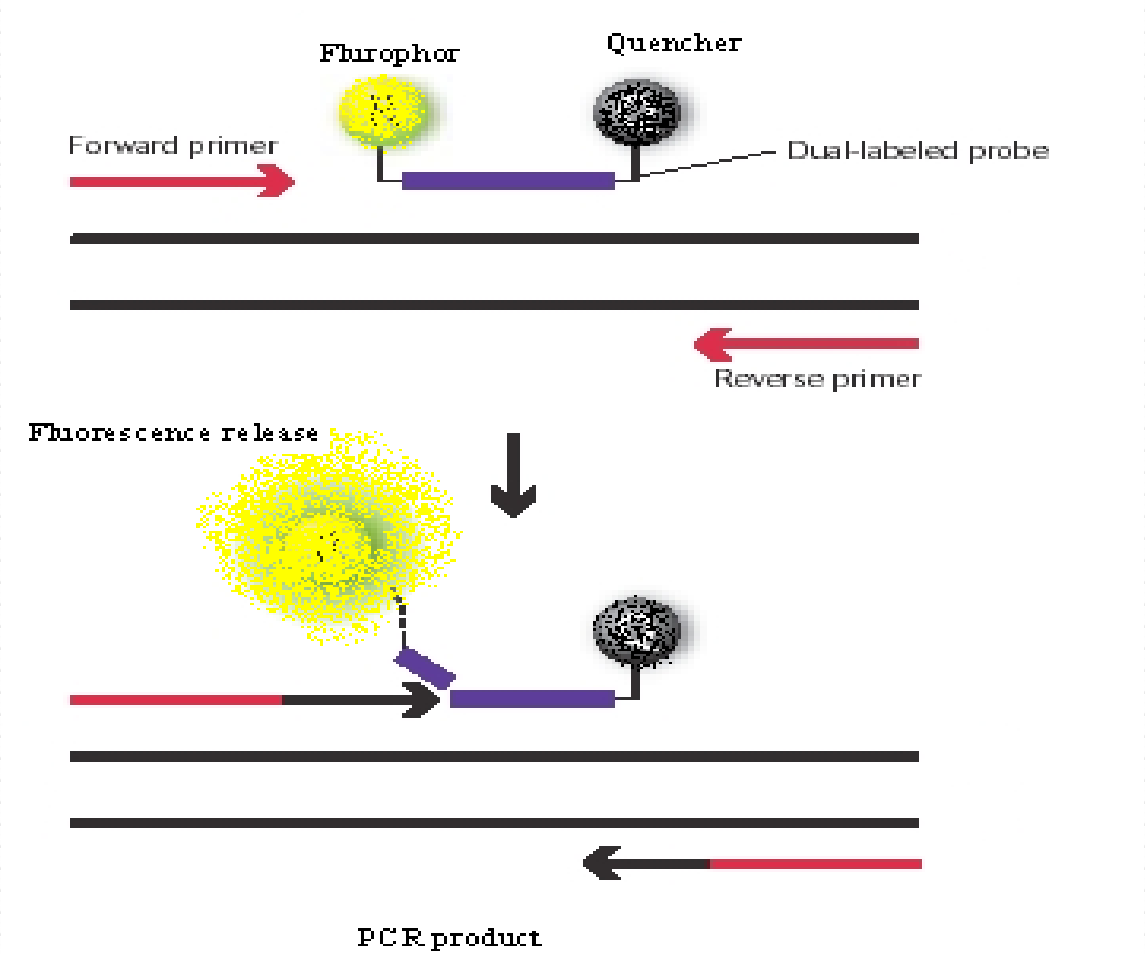

Figure 2: Principle of Real time PCR using Taqman probe.

Source: Saeed and Ahmad, 2013. Nucleic Acid Amplification Techniques in the Detection of Influenza virus by ANJORIN et al., 2017

REAL-TIME REVERSE TRANSCRIPTASE-PCR…4

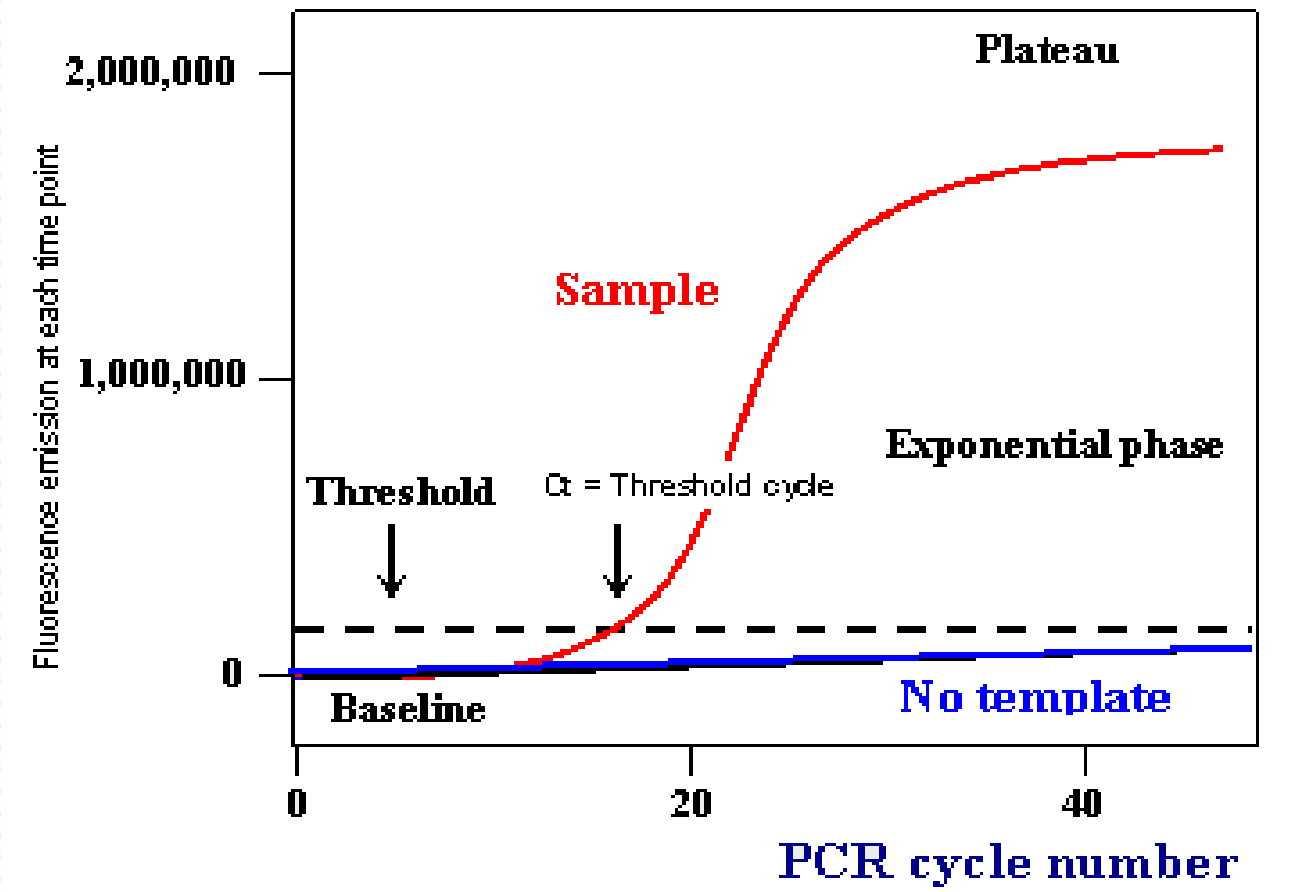

Figure 3: Fluorescence signal versus cycle number: Amount of fluorescence released is measured after each cycle by plotting the intensities.

Source: Saeed and Ahmad, 2013.

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VARIANT PCR

- Other types of PCR for IV detection include:
- Nested PCR: Uses 2 sets of primers: internal to the other.
- Duplex, Triplex and Multiplex PCR: Combine 2, 3 or more sets of primers for different targets simultaneously
  - for differential detection and discrimination of multiple amplicons in a single tube (Auewarakul *et al*., 2007).
- Also, multiplex PCR can be combined with RT-PCR or rT-PCR in the detection/subtyping of IVs.

OTHER AMPLIFICATION-BASED METHODS

LIGASE CHAIN REACTION

- Ligase Chain Reaction (LCR) is a probe amplification technique that uses 2 enzymes:
  - DNA polymerase and DNA ligase (Auwal, 2014).
- Principle is based on the ability of DNA ligase to join 2 complementary probes only when they are hybridized to their target (Wiedmann *et al*., 1994; Fakruddin *et al*., 2013).
- Ligated probes serve as template for further annealing
  - Each cycle yields double result of the target NA (Wiedmann *et al*., 1994).

PYROSEQUENCING

- Pyrosequencing is an amplification assay that permits sequencing of smaller <100 bp amplicons (Pabbaraju *et al*., 2011; Tamura *et al*., 2015).
- It was developed by Ronaghi *et al* in 1996, based on
  - Enzymatic luminometric inorganic pyrophosphate detection assay (ELIDA) established by Nyren in 1987.
- It operates on a DNA sequencing-by-synthesis principle based on real-time measurement of pyrophosphate
  - (Deydea and Gubareva, 2009; Wang and Taubenberger, 2010).
- Deng *et al* (2011) subtyped IAVs with pyrosequencing.
  - It is sensitive, rapid and cheaper than conventional sequencing method (Gatica-Wilcox, 2014; Tamura *et al*., 2015).

PYROSEQUENCING…3

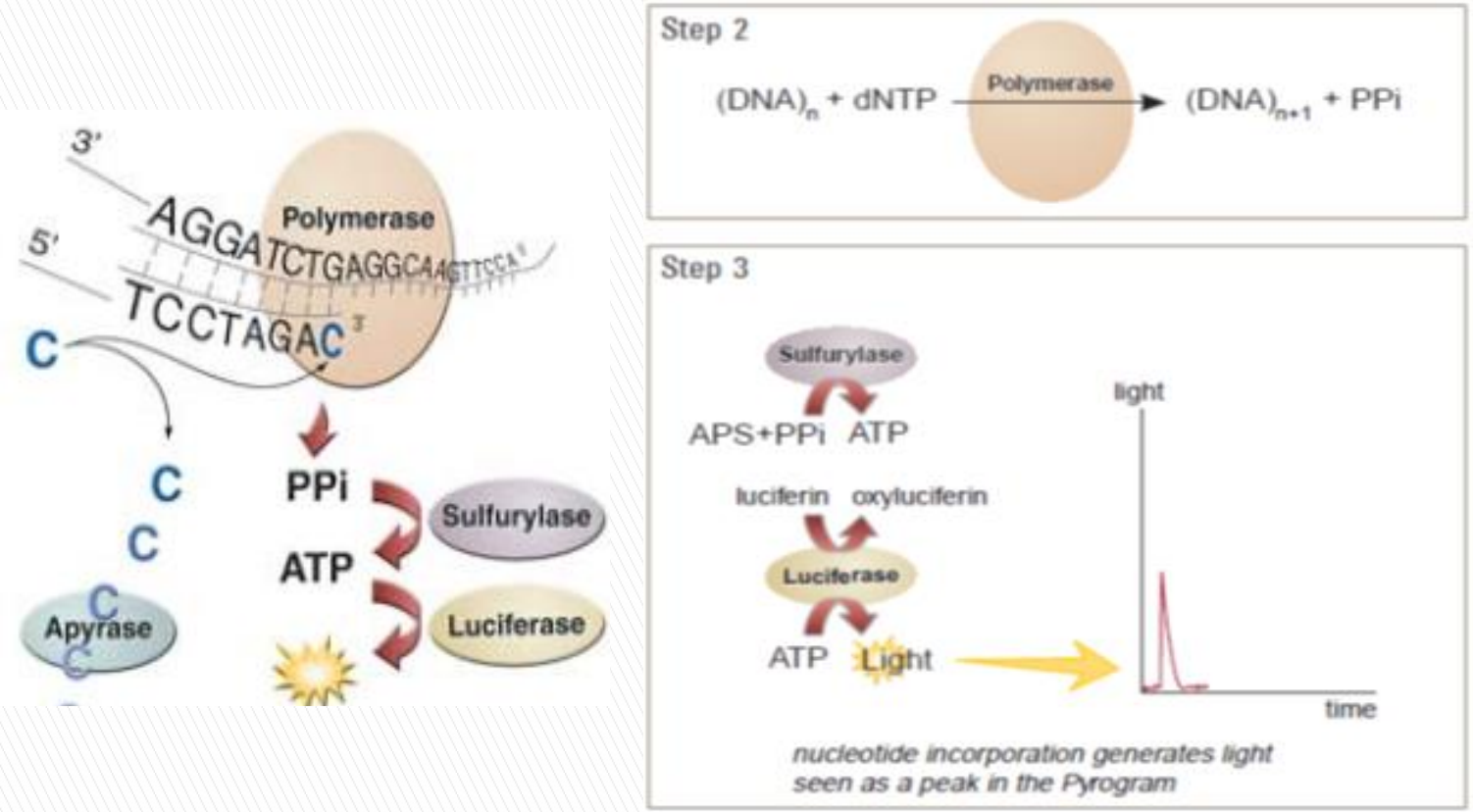

Figure 4: Pyrosequencing processes: 1. Selection of oligonucleotides/ incubation with 4 enzymes and dNTP. 2. Nucleotide insertion for use by the DNA polymerase. 3. This generates bioluminescent enzymatic cascade if N is complementary. Light signal peaks are generated according to the number of nucleotides incorporated in each sequence as Pyrogram.

Sources: Adapted from Fakhrai-Rad *et al.*, 2002; Biotage, 2004; CADTH, 2013.

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NUCLEIC ACID SEQUENCING-BASED AMPLIFICATION METHOD

- Nucleic acid sequencing-based amplification (NASBA) is an alternative, rapid and enzyme-based technology
  - Developed by Compton, J in 1991 from transcription-based amplification system (Romano *et al*., 1996; Pasick, 2008) first described by Kwoh *et al*. (1989).
- It uses the principle of continuous isothermal reaction.

NUCLEIC ACID SEQUENCING-BASED AMPLIFICATION METHOD…2

- It occurs in 2 stages:
  - Initial stage of 65°C (for RNA) denaturation/primer annealing and
  - a cyclic process for target amplification at a constant temperature of 41°C.
- NASBA uses 3 enzymes: reverse transcriptase (AMV-RT), ribonuclease-H (RNase H) and RNA polymerase (T7 RNA polymerase).
- And 2 primers: reverse DNA primer P1 containing T7 promoter region and forward DNA primer P2 (Lau *et al*., 2006; Tröger *et al*., 2015).

NUCLEIC ACID SEQUENCING-BASED AMPLIFICATION METHOD…3

- Briefly, the initial procedure include: Binding of Primer P1 to the sample RNA.
  - P1 is extended by the reverse transcriptase that synthesizes the cDNA.
  - P2 then binds to the DNA strand (figure 5).
- T7 RNA polymerase generates complementary RNA strand.

NUCLEIC ACID SEQUENCING-BASED AMPLIFICATION METHOD…4

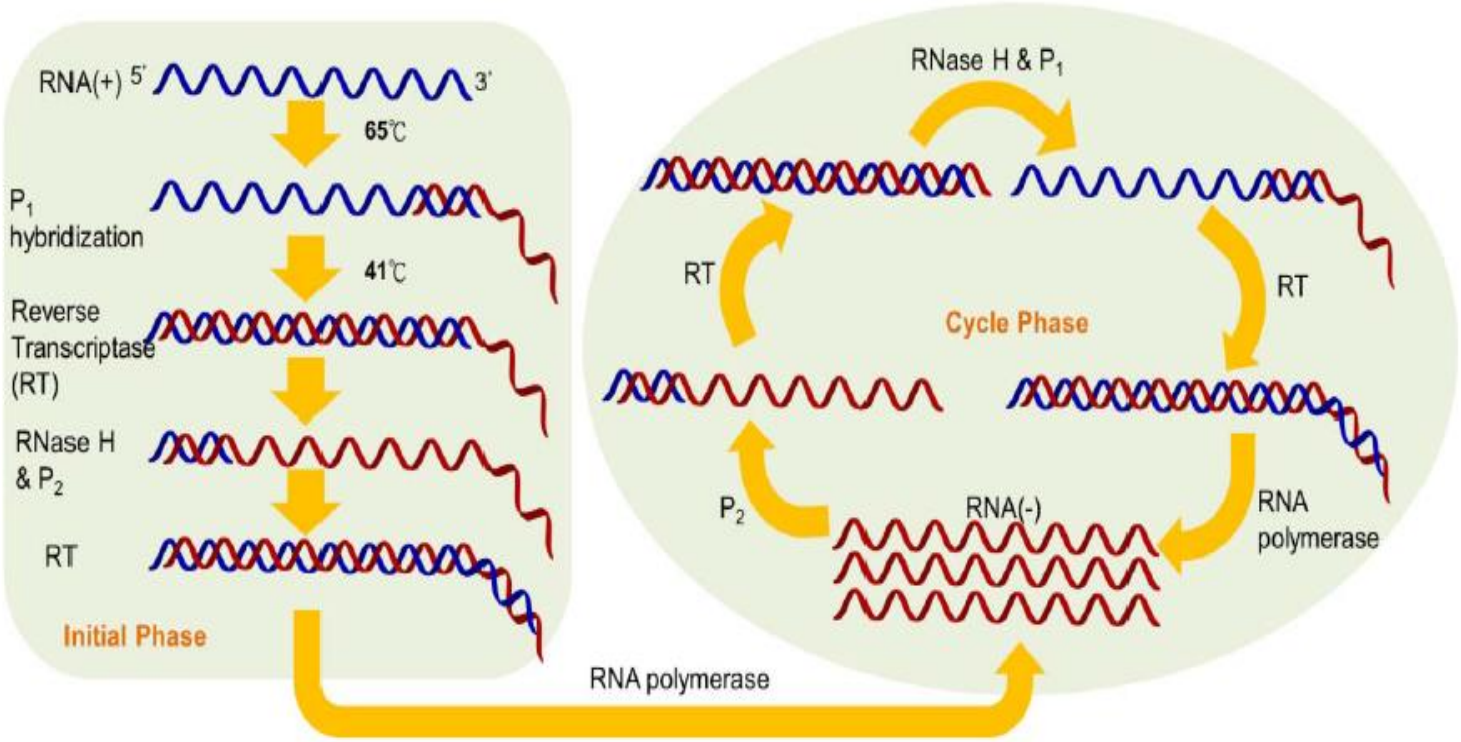

Figure 5: NASBA. Binding of P2 is followed by elongation process by the AMV-RT in order to produce a double-stranded DNA molecule. Usually, primer P1 is designed in a way that immediately a double-stranded DNA is synthesised, it codes for a T7 RNA polymerase promoter site that can generate an antisense RNA copies using a DNA template. This results in the formation of new copies of DNA from the generated RNA copies. The process is repeated for anti-sense strand but primer P2 will bind first.

Source: Chang *et al.*, 2012.

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LOOP-MEDIATED ISOTHERMAL AMPLIFICATION

- Loop-mediated isothermal amplification (LAMP) is a specific and rapid one-step method first described by Notomi *et al*. (2000).

- Uses principle of strand displacement reaction, similar to PCR but the target DNA amplification process occurs under an isothermal condition.

  - (Notomi *et al*., 2000; Wang and Taubenberger, 2010).

EMERGING DIAGNOSTIC ASSAYS

DNA MICROARRAY

- Microarray is simply the arrangement of biomolecules on a solid surface in order to generate qualitative and
  - quantitative information (Miller and Tang, 2009; Mukherjee and Chakrabarti, 2012).
- It is a technology used to identify labelled targets by hybridizing them with specific probes and
  - hybridization signals are mapped within the array.

DNA MICROARRAY…2

- The principle relies on the fact that targets like DNA/ RNA can be detected on the basis of
  - complementarity with spotted probes (Miller and Tang, 2009).
- Hybridization between the immobilized probe and labelled target will generate fluorescence on
  - background which can be measured with a fluorescent scanner (Miller, 2009) for data analysis (figure 6).

DNA MICROARRAY…3

- Briefly, the process involves: attachment of probes to a solid support: Glass, nylon/ silicon
- RNA conversion into cDNA and fluorescent dye labelling using RT-PCR before
  - adding the labelled targets onto known probes for hybridization (Shojaei, 2015).
- Hybridized probes can be detected by a sensitive detection system.

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DNA MICROARRAY…4

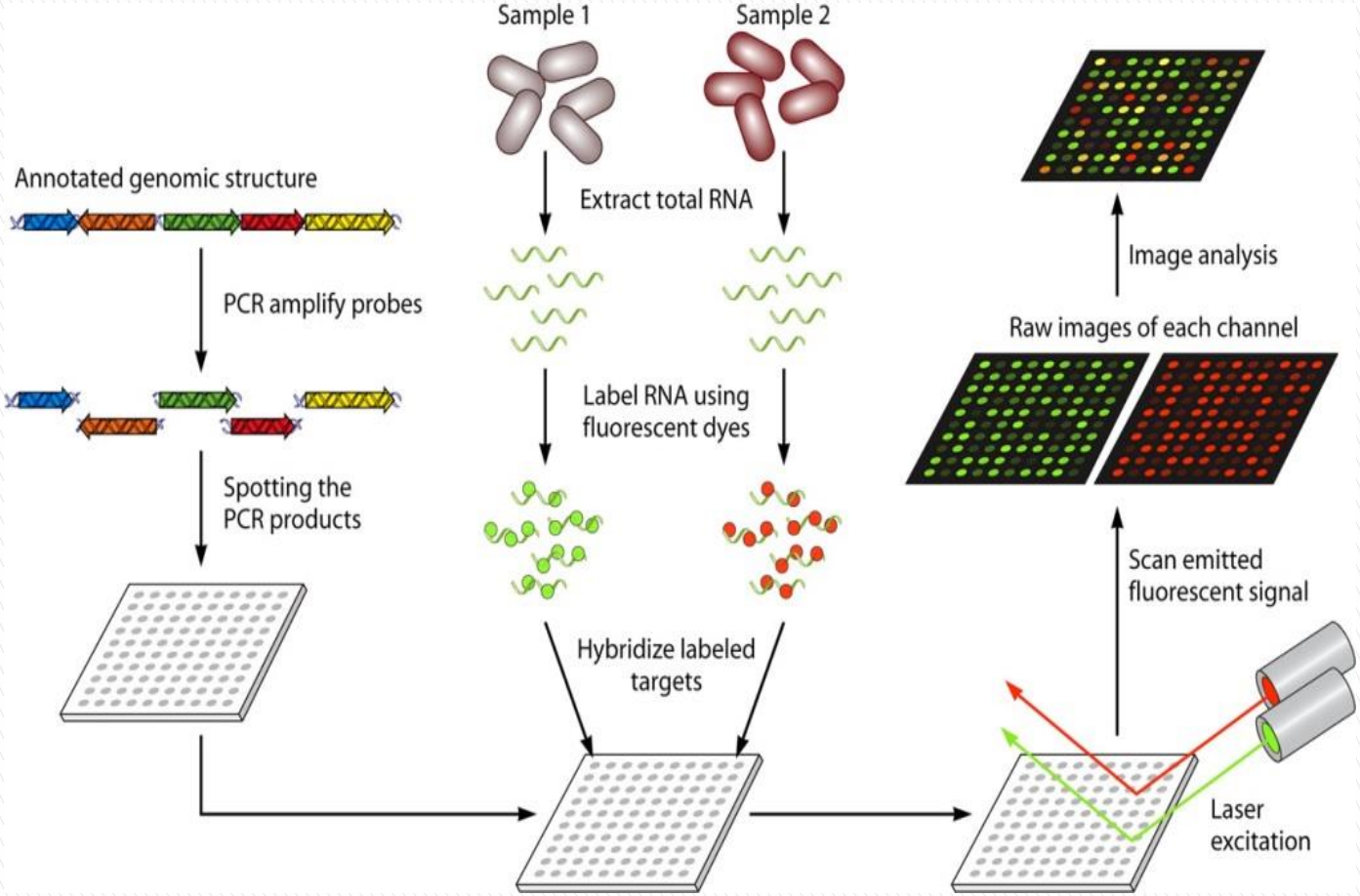

Figure 6: Workflow summary of printed microarray in 3 parts: Glass slide spotting of probes (PCR amplified or synthesized oligonucleotides); Sample extraction, fluorescent labeling and hybridization; Fluorescent scanning of labeled target nucleic acids hybridized to the probe array.

Source: Adapted from Ehrenreich, 2006.

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DNA MICROARRAY…5

- It can detect thousands of targets at the same time;
  - Can be used to prove phylogenetic relationships between isolates (Gall, 2009).
- IVs are ideal for evaluation by microarrays because of their genetic and host diversity
  - and availability of an extensive sequence database (Macken *et al*., 2001).
- It is a technique for identifying and subtyping IAV during surveillance (Dawson *et al*., 2006; Townsend *et al*., 2006).

OTHER EMERGING MOLECULAR SYSTEMS

- Other emerging molecular systems have generated promising models for the detection of IVs including:
  - Proximity ligation assay (PLA),
  - Biosensor-based methods,
  - Fo¨rster/fluorescence resonance energy transfer (FRET)-based methods and
  - Nanoparticle-based techniques (Shojaei, 2015).
  - Nucleic Acid sequencing: Next-Generation Sequencing (Vemula, 2016).

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Table 2: Comparison of Nucleic acid amplification methods

TECHNIQUE	MERIT	DEMERIT	COMMENT
Conventional PCR/ RT-PCR	Relatively sensitive and specific allows further sequence analysis	Not early quantitative; not ideal for high-throughput	Time-consuming for large screens
Multiplex PCR	Relatively sensitive and specific can test multiple targets in one assay; downstream sequence analysis is available; time and cost savings by running in single tube	Multiple primers easily cause nonspecific amplification	Most common method used for surveillance detection for identifying subtype using different segments, especially combined with real-time PCR
Probe-based real-time PCR	High sensitivity and specify, ideal for quantitative and multiplex detections; can be high-throughput	High-cost probe and special equipment required; further sequence analysis is not generally possible	Sensitive and reliable method for clinical laboratories and core facilities
NASBA	Relatively sensitive, specific and quantitative	Requires optimization or primer selection	Does not require expensive instruments so is a good choice for field applications especially when surveillance targets are well identified
LAMP	Simple, fast and cost-saving	Requires extensive optimization of primers design to achieve high sensitivity	A regular laboratory water bath or heat block is needed for reaction so it is an ideal detection
Pyrosequencing	Accurate and sensitive; can be high throughput	Time-consuming sequencing length limitation	Very useful for detection of molecular markers of drug resistance
Microarray	Sensitive, specific and large amount of targets in single assay, very high-throughput	Expensive equipment needed; downstream analysis complex	Good for surveillance in core facility or high-level laboratory

Source: Adapted from Wang and Taubenberger, 2010.

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CONCLUSION

- We hope that by understanding the techniques and basic principles of Nucleic acid amplification, we can develop less expensive, and more convenient protocols for influenza virus detection and surveillance.

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APPRECIATION

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