Leptospirosis laboratory diagnosis & optimal sampling time for serology/PCR

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Institut Pasteur in New Caledonia

Leptospirosis biological diagnosis

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Excerpt from "*WHO recommended standards and strategies for surveillance, prevention and control of communicable diseases*"

Laboratory criteria

Presumptive diagnosis:

- A positive result of a rapid screening test such as IgM ELISA, latex agglutination test, lateral flow, dipstick etc.

Confirmatory diagnosis:

- Isolation from blood or other clinical materials through culture of pathogenic leptospires.
- A positive PCR result using a validated method (primarily for blood and serum in the early stages of infection).
- Fourfold or greater rise in titre or seroconversion in microscopic agglutination test (MAT) on paired samples obtained at least 2 weeks apart. A battery of *Leptospira* reference strains representative of local strains to be used as antigens in MAT.

- **A positive culture**
- **A positive PCR**
- **A serological conversion**

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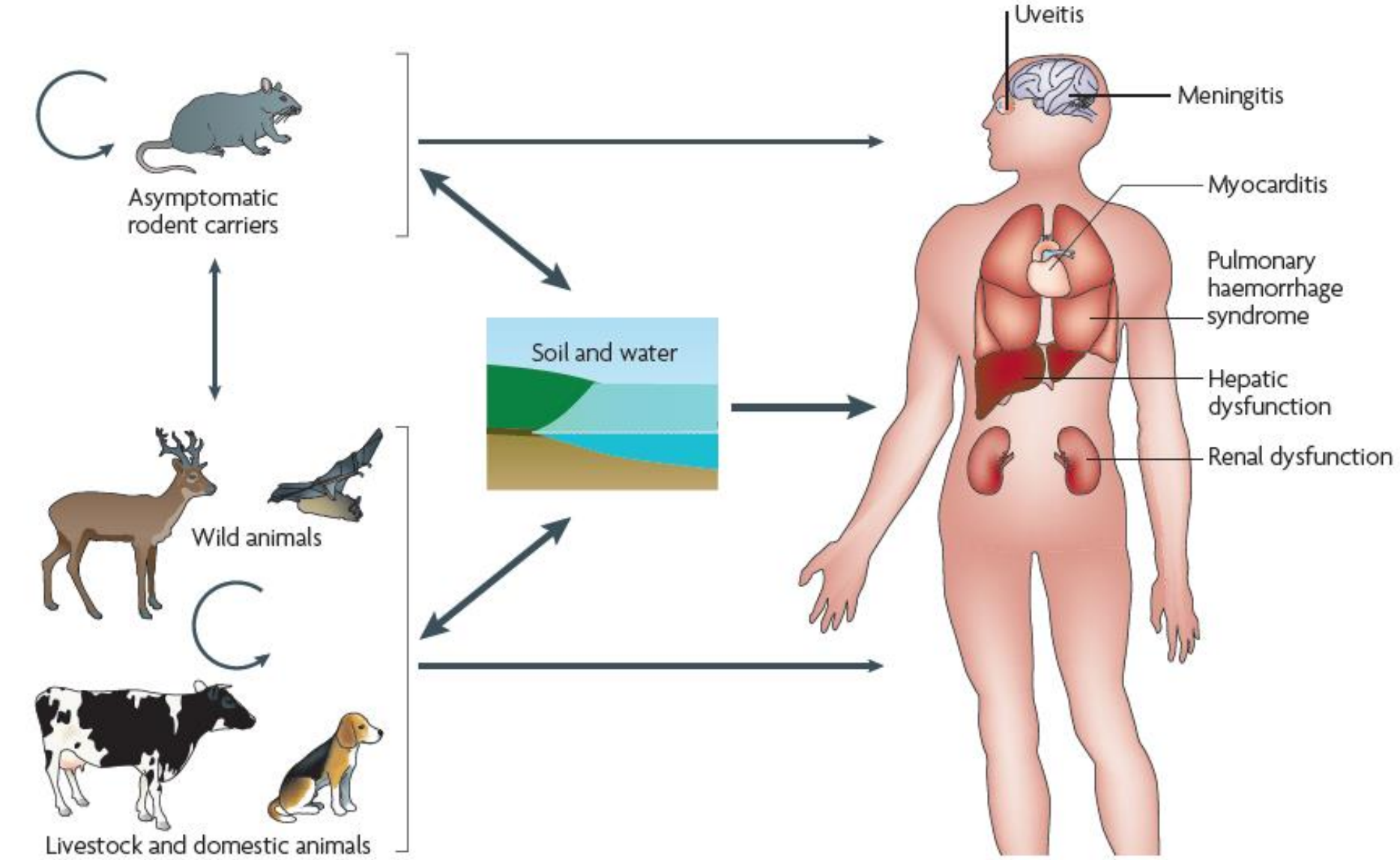
Where it all starts



Different phases
Infection to disease
INCUBATION

- The first step is penetration of **tissue barriers** to enter the body: skin breaches, cuts or abrasions, conjunctivae, oral mucosae

≠ Lyme *Borrelia* spp. or *Treponema pallidum*, no lesion at entry point

- The second step is (rapid) hematogenous spread and dissemination

→ bacteremic phase, initially symptom-free. Bacteremia usually low ($\leq 10^4$ / mL)

- Then only, symptoms appear: usually a high fever of rapid onset.

3-30 days, frequently 10-12

Different phases
Disease progression
Signs & Symptoms

- Bloodstream *Leptospira* reach target organs
  - Liver → high blood level of direct bilirubin (+ gastro-intestinal symptoms)
  - Lung → hemorrhages, of various severity: from small petechiae (non-productive cough) to severe pulmonary hemorrhage
  - Kidney → lower Sodium reabsorption: hyponatremia & hypokalemia and interstitial nephritis
  - Brain → "aseptic meningitis" headache to altered mental status

A few days after onset of fever

Different phases
Disease aggravation
Severe Leptospirosis

- Severe Pulmonary Hemorrhage Syndrome (SPHS)
- Severe renal failure
- Multi-organ failure (a result of a cytokine storm?)

As soon as 3-5 days after disease onset

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Different phases
Disease aggravation
Disease resolution

- Self-resolving (most frequent) after the rise of antibody titers
- **After antibiotic treatment (given early)**
- After intensive support of severe forms

Different phases
Disease aggravation
Post-disease sequelae

- Renal, hepatic and respiratory functions recover (though insufficiently studied)
- **Recurrent or chronic uveitis can occur** (at least partly auto-immune)
- **Guillain-Barré Syndrome**
- **Persistent fatigue, myalgia, malaise, headache, weakness (>24 months)**
- **Depression or other neuro-psychiatric disorders**

Diagnostic implications

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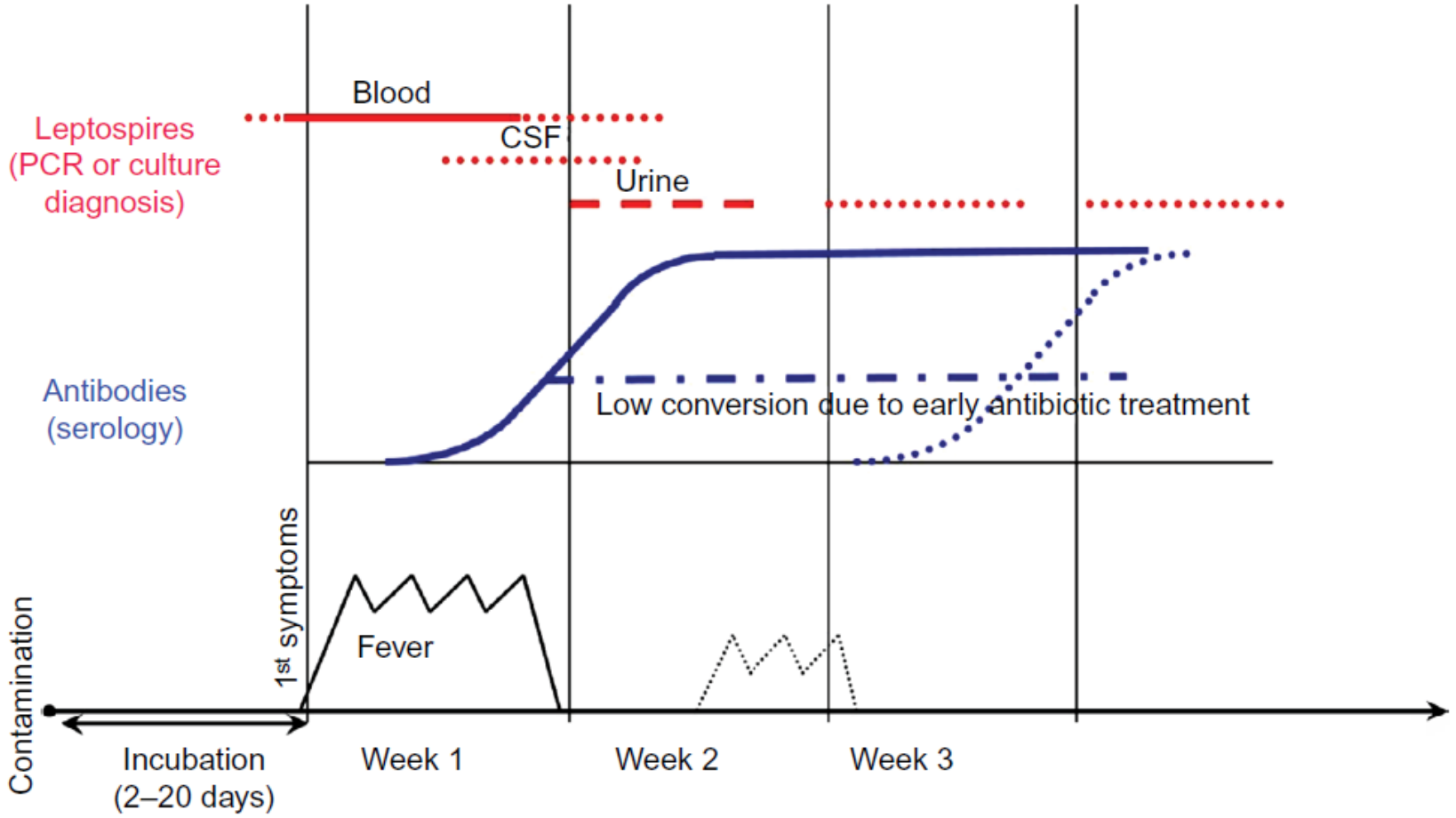
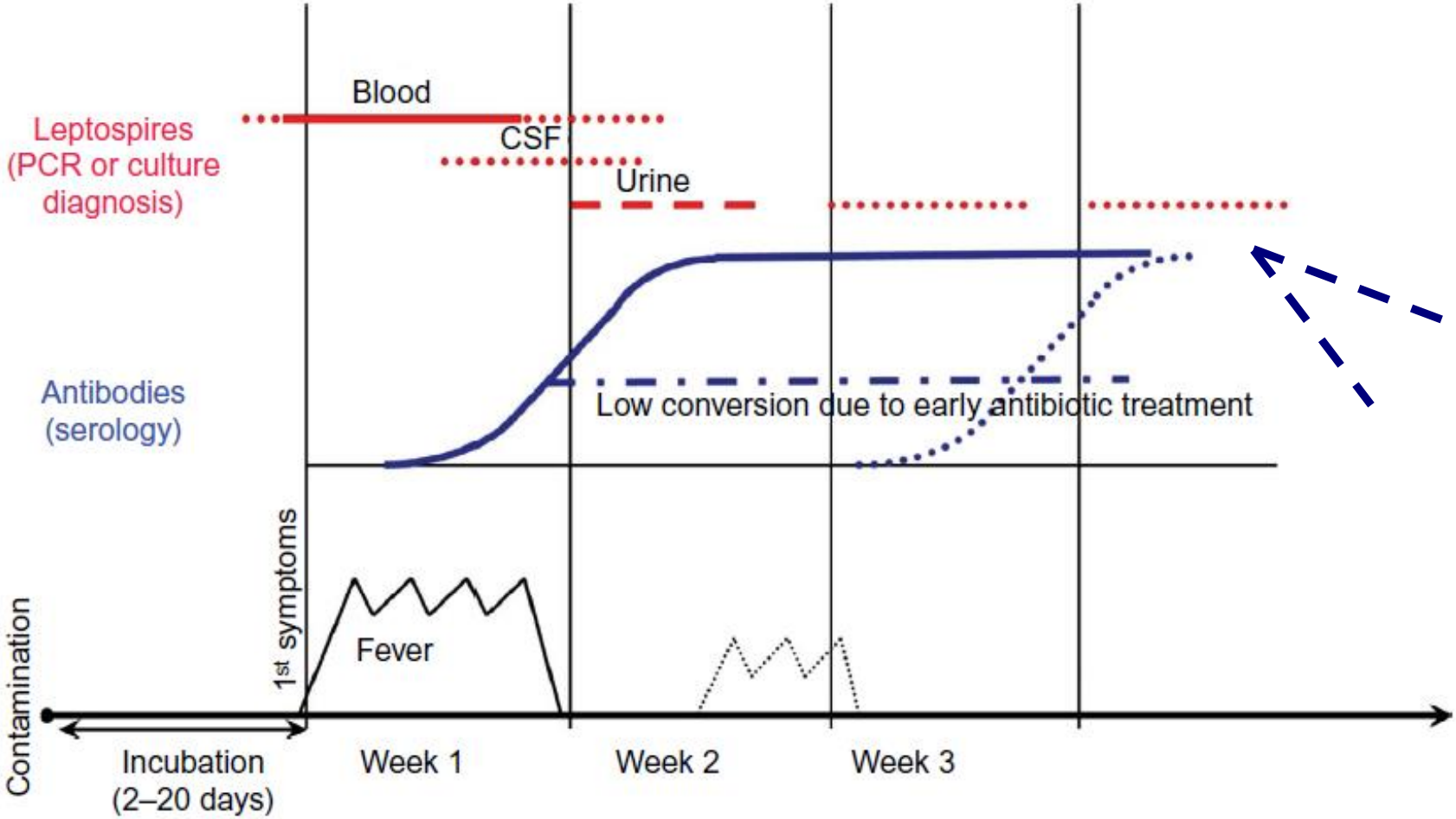


Figure 3 Basic principles underlying the biological diagnosis of leptospirosis.
Notes: Adapted from Turner. Leptospirosis I. *Trans R Soc Trop Med Hyg.* 1967;61(6):842–855,[124] by per
Abbreviations: PCR, polymerase chain reaction; CSF, cerebrospinal fluid.

Research and Reports in Tropical Medicine
Dovepress
REVIEW
Leptospirosis: risk factors and management challenges in developing countries

Applications to biological diagnosis

- "Age of disease" **needs to be known**
  - Dn = Date of sample collection - Date of onset of symtoms
- Look for leptospires (or their DNA or products) in blood early, then useless (except in urine if fresh)
- Serology (RDT, ELISA, MAT) useless if patient visits early, only valid after 5-7 days


Blood
Leptospires
(PCR or culture
diagnosis)
CSF
Urine
Antibodies
(serology)
Low conversion due to early antibiotic treatment
1st symptoms
Fever
Contamination
Incubation
(2–20 days)
Week 1
Week 2
Week 3

Detect leptospires (or their products)

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Where?

- From blood (~1 week after onset)
- In urines (2nd week after onset)
- In CSF, aqueous humor

Limitations?

- Low (and rapidly declining) bacteremia
- Intermittent excretion, short survival time of *Leptospira* in urine
- Rare specimens, clinical signs without *Leptospira* detection

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Detect leptospires (or their products)

How to increase success rate?

- Culture: before antibiotics are given to the patient!
  - Slow growing organisms (up to 12+ weeks!)
  - Very specific culture media
  - Highly sensitive to a number of stress (chemicals, UV, low temperatures…)
  - ➢ Late response, useless for individual diagnosis

- q-PCR
  - Highly sensitive & specific
  - Very rapid turnaround time
  - ➢ Also a rapid diagnosis (but not yet bedside)

- Evidence of circulating *Leptospira* antigens
  - An ongoing challenge (low bacteremia)

Detect the immune response

- Highly specific / reference technique: MAT
- More sensitive / easier: IgM ELISA, but less specific (IgM) → confirm with MAT
- Bedside presumptive, outbreak situation: IgM RDT
- After the rise of an immune response: from Day 5 on (ELISA) or 7 (MAT)

DOS-based biological diagnosis

- **DoS (number of Days since Onset of Symptoms) of prime importance and should be informed.**

	Onset t=D0	D1	D2	D3	D4	D5	D6	D7	D8+
qPCR / culture	from blood								
						from urine			
ELISA / RDT	expected negative (or former infection). Usable as baseline for conversion					valid			To be compared to earlier if available
MAT	expected negative (or former infection). Usable as baseline for conversion							valid	

Serum transportation for serology

- Crude serum sample: needs to be kept frozen

→Dry ice for shipment

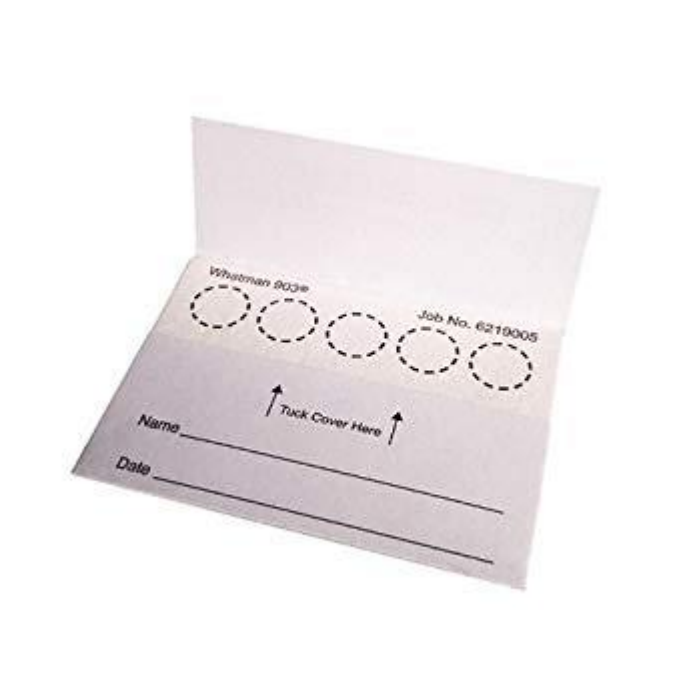
→IATA “Biological substance”

- Dried on filter paper

→Ambient temperature

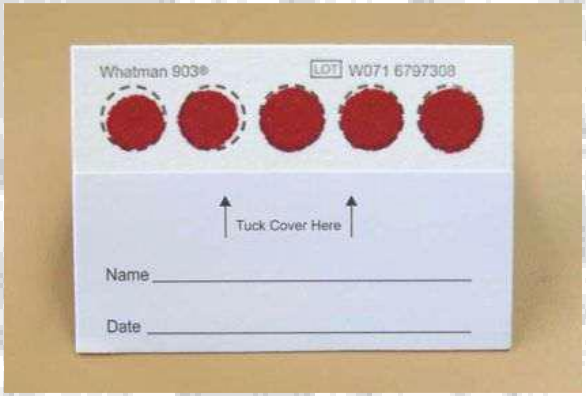
→Not a biological substance for IATA

Serum transportation for serology



Whatman 903 Protein saver card

Blood or serum



Drying time: 1-3 hours

Once dry

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1. Plastic pouch

2. Storage at 4°C

3. Shipment at ambient temperature

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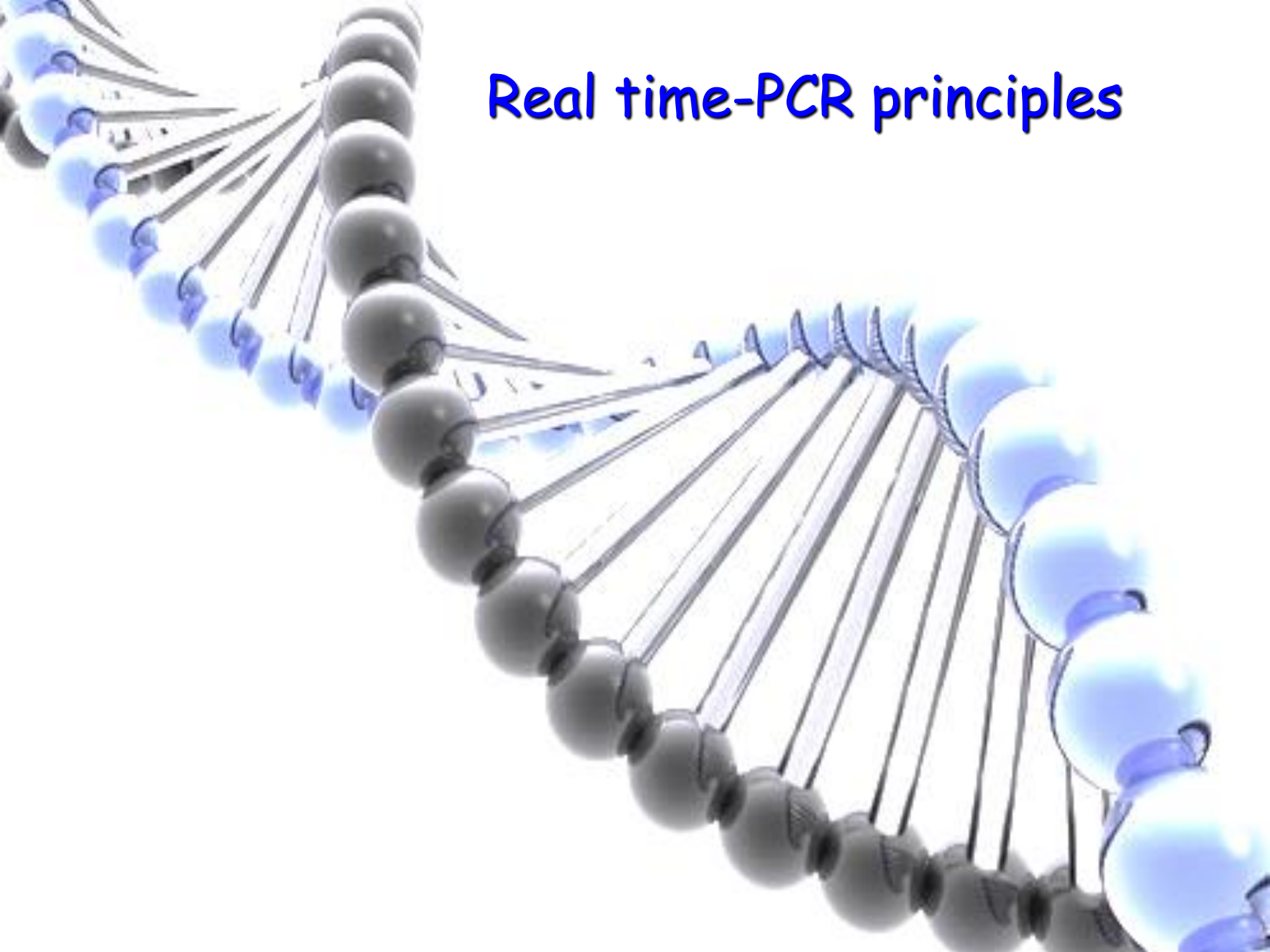
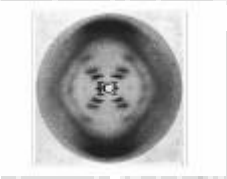
Real time-PCR principles

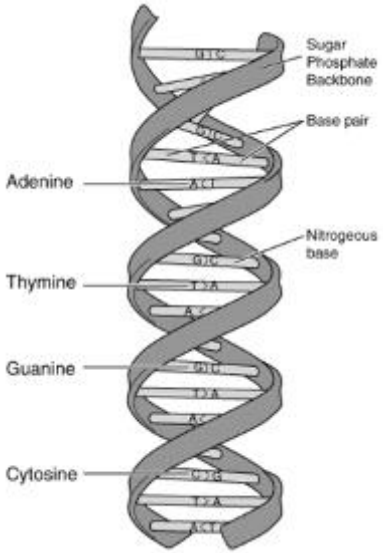


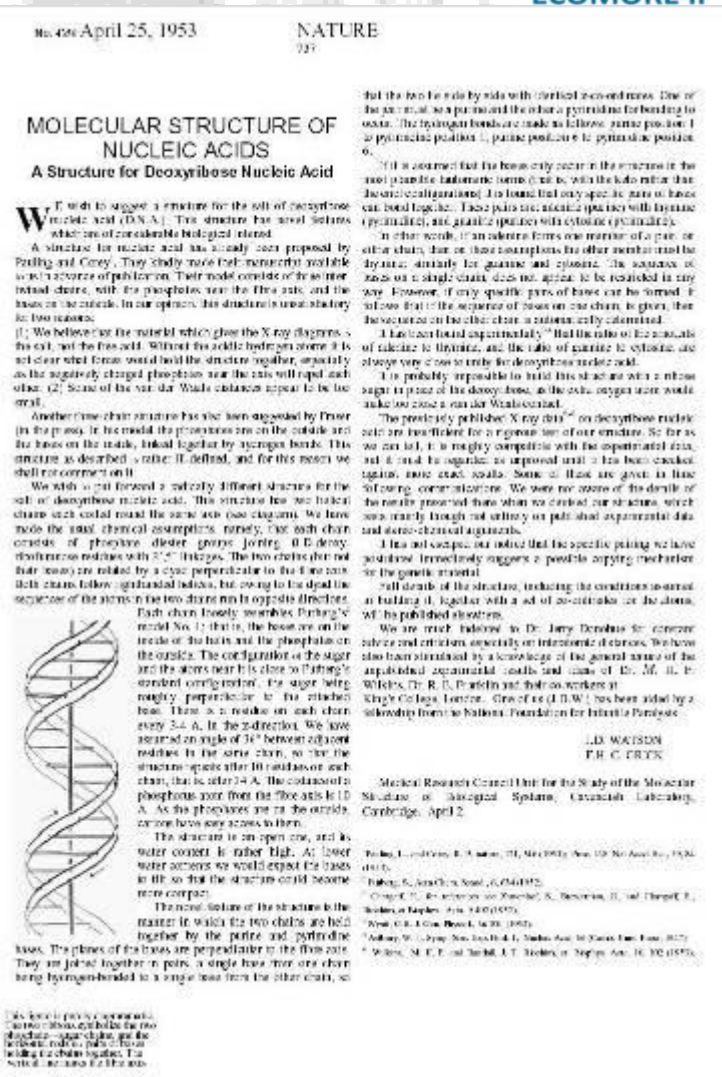
- 1953 :
- Watson, Crick, Wilkins Nobel 1962

  + Rosalind Franklin



- **1983 : Polymerase Chain Reaction (PCR) imagined (Kary Mullis)**
- **1986 : PCR published Prix Nobel 1993**
- **1988 : *Taq* polymerase**





No. 4356 April 25, 1953 NATURE 737

MOLECULAR STRUCTURE OF NUCLEIC ACIDS

A Structure for Deoxyribose Nucleic Acid

J.D. WATSON
F.H.C. CRICK

Medical Research Council Unit for the Study of the Molecular Structure of Biological Systems, Cavendish Laboratory, Cambridge. April 2

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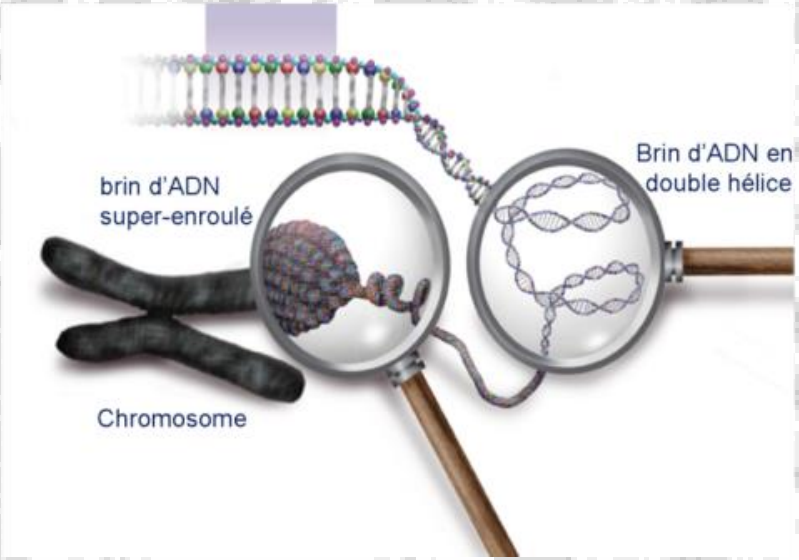
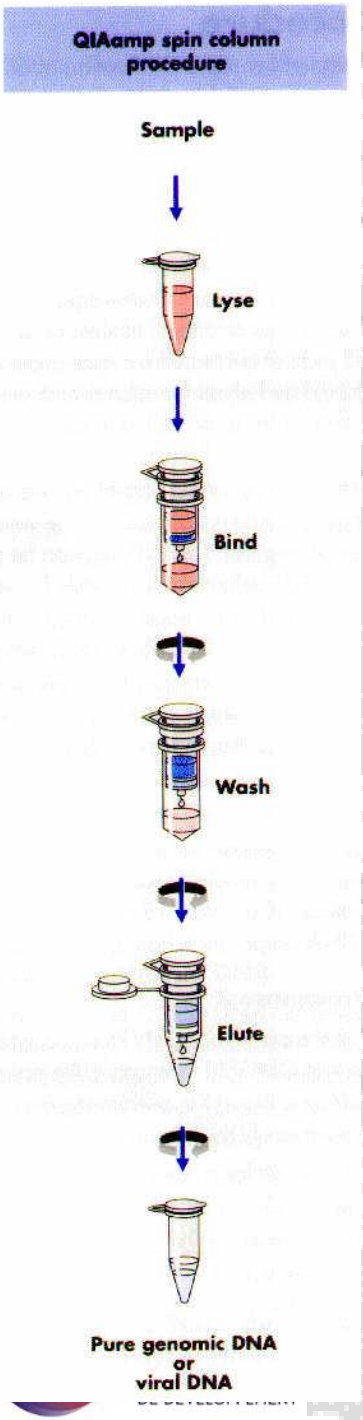
DNA extraction



- Lysis + Digestion of proteins (proteinase K)
- DNA precipitation (ethanol)
- Adsorption to silica (column)
- Washes
- Elution = desorption from silica



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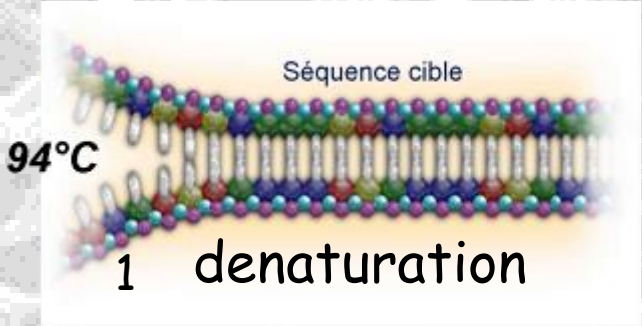
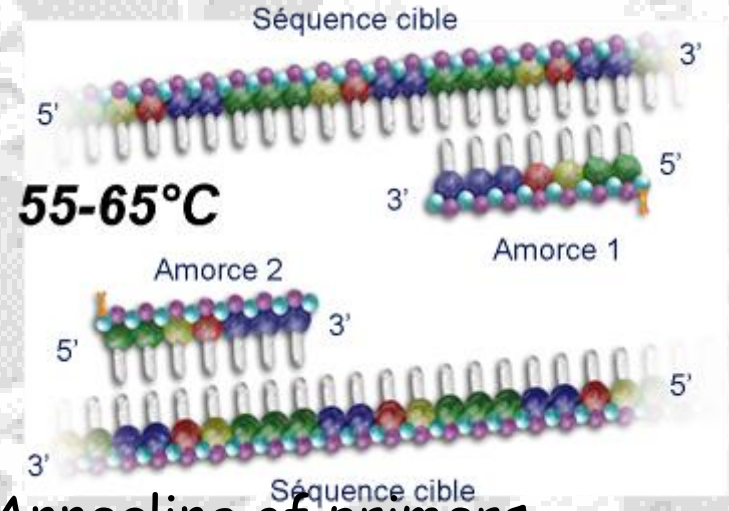
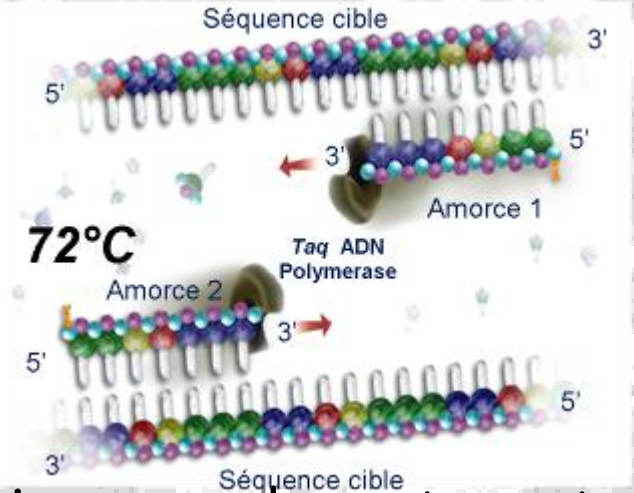
PCR amplifies a target DNA sequence…
… in 3 steps



1 denaturation



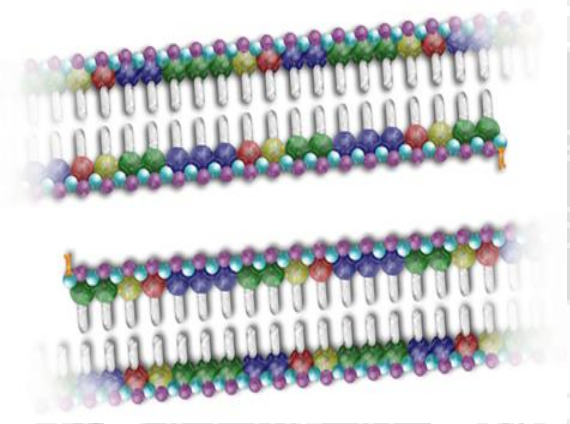
2 Annealing of primers (specificity)



3 Extension : complementary strand

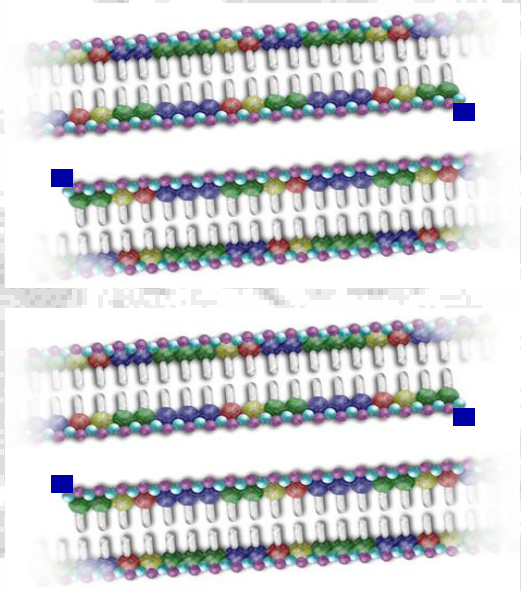
Cycling

2 target sequences



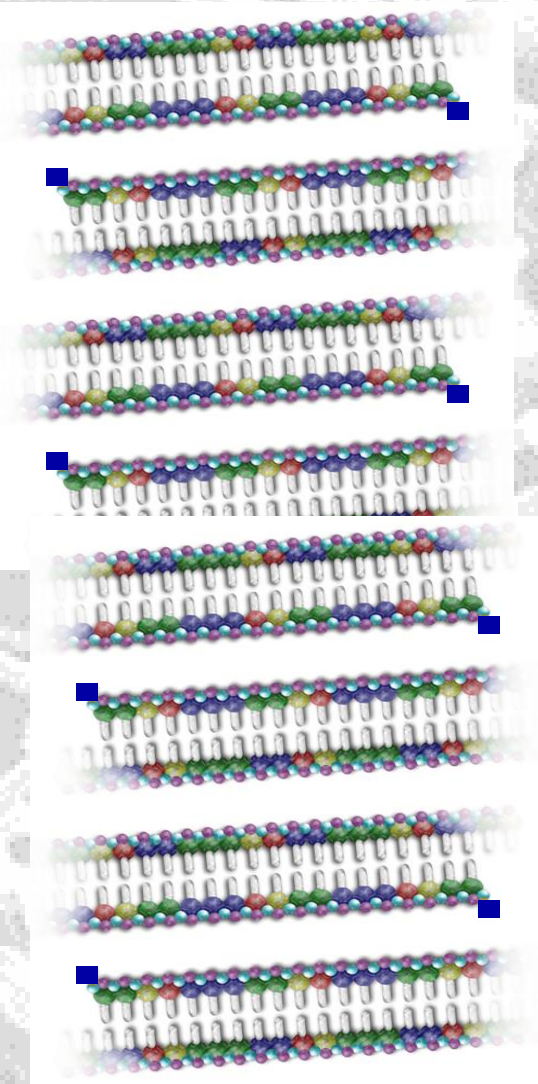
2 cycles :

4 target sequences



3 cycles :

8 target sequences



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30 Cycles :

2^{30} copies = 1 073 741 824 copies !

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50 Cycles : 2^{50} copies =
1 125 899 906 842 620 000 copies !

Risk of contamination +++
1 qPCR drop in an olympic swimming pool
would correspond to > 450 000 copies per ml !

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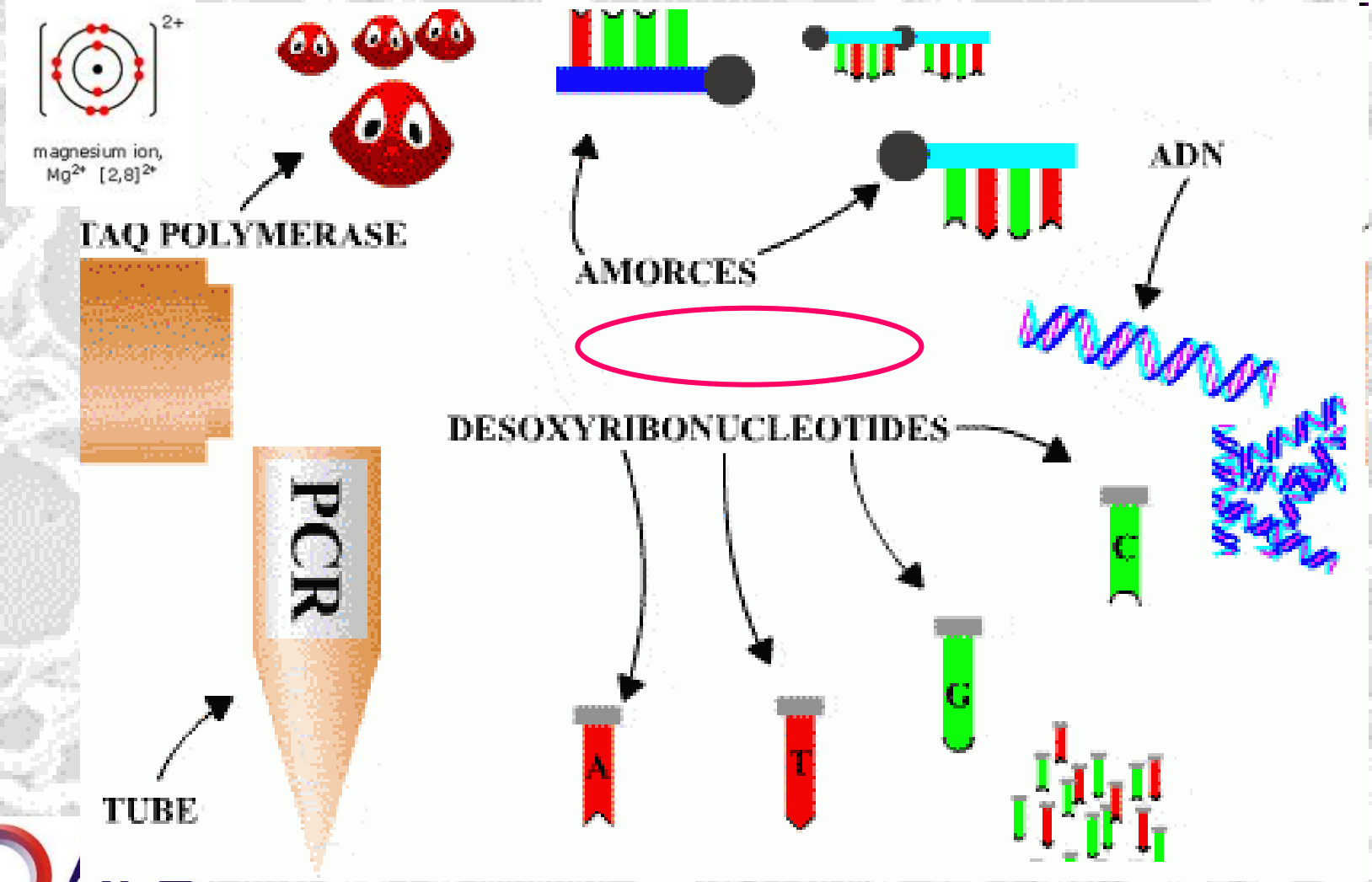
PCR:
Replication of DNA *in vitro*



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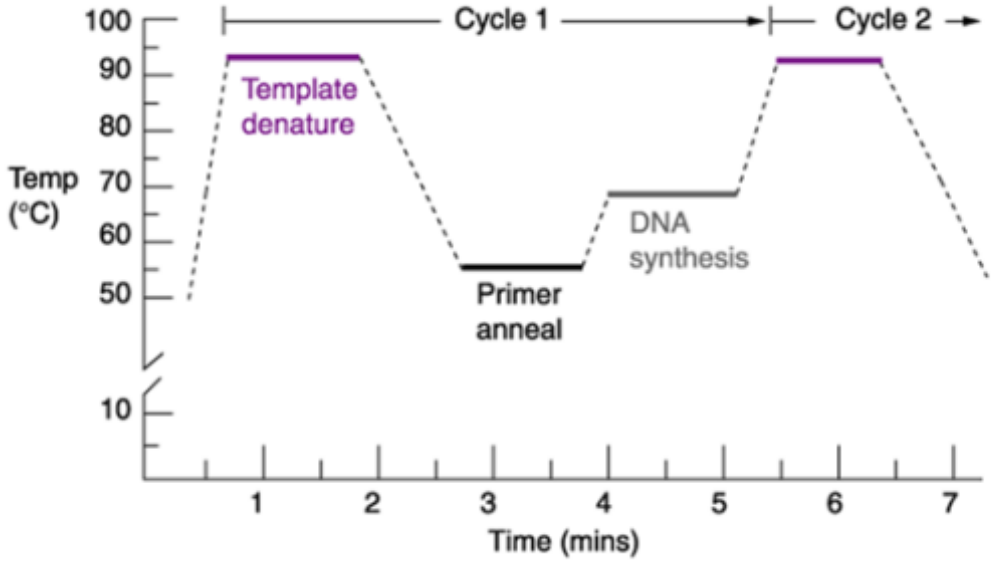
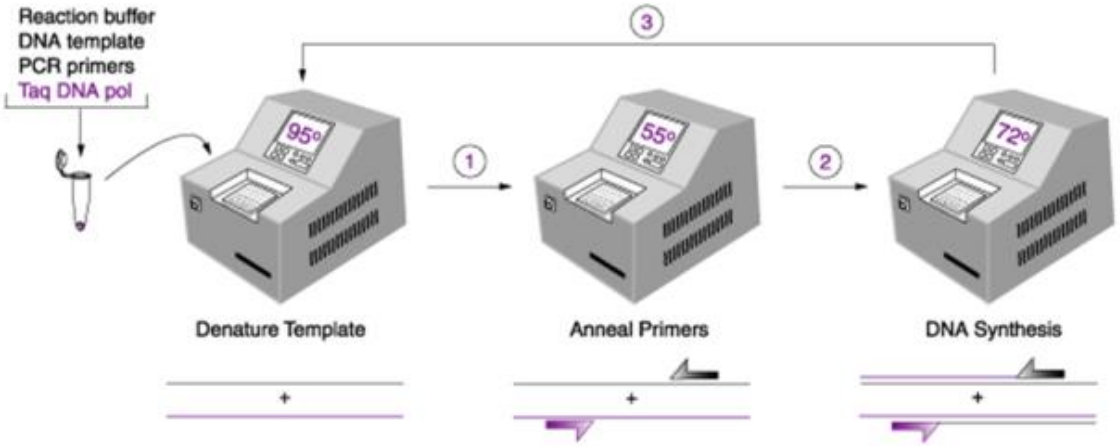
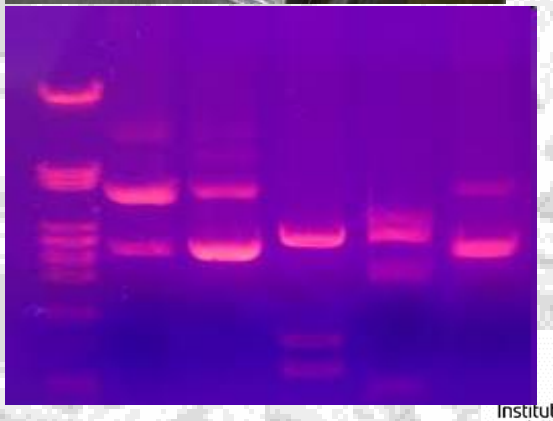
Traditional PCR







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Real-time PCR

- Detects the amount of target DNA at each cycle: "in real time"

- Faster and more sensitive

- Real-time reading
  - requires specific technologies (fluorescence)
  - allows quantification
  - + No need to manipulate amplified DNA!

Detection in real time

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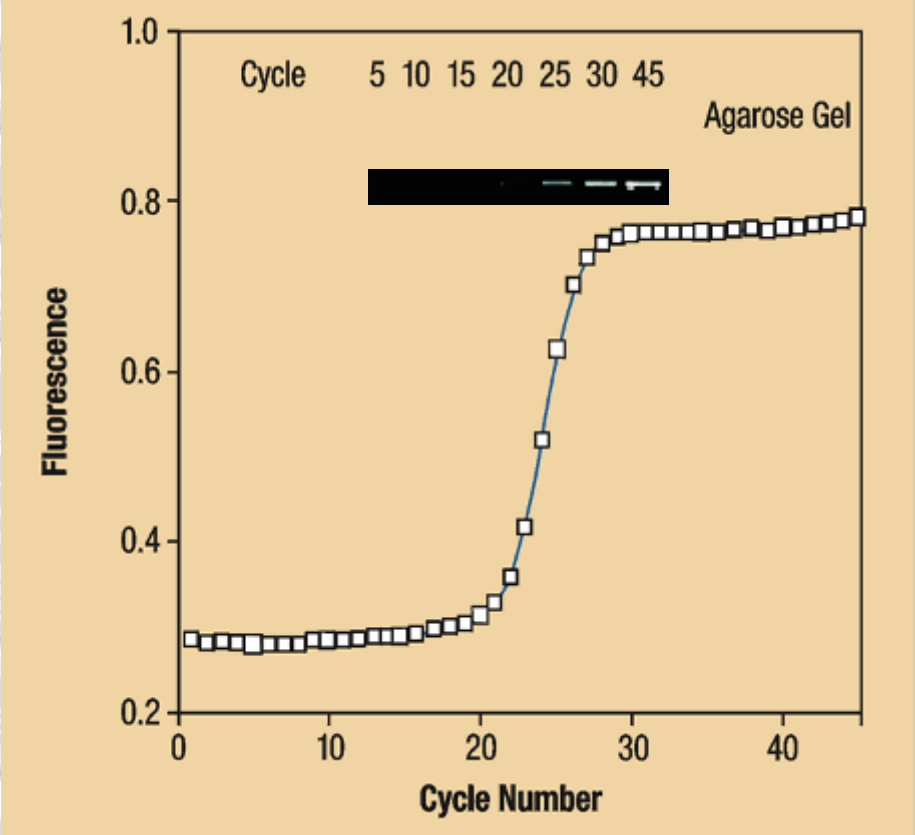
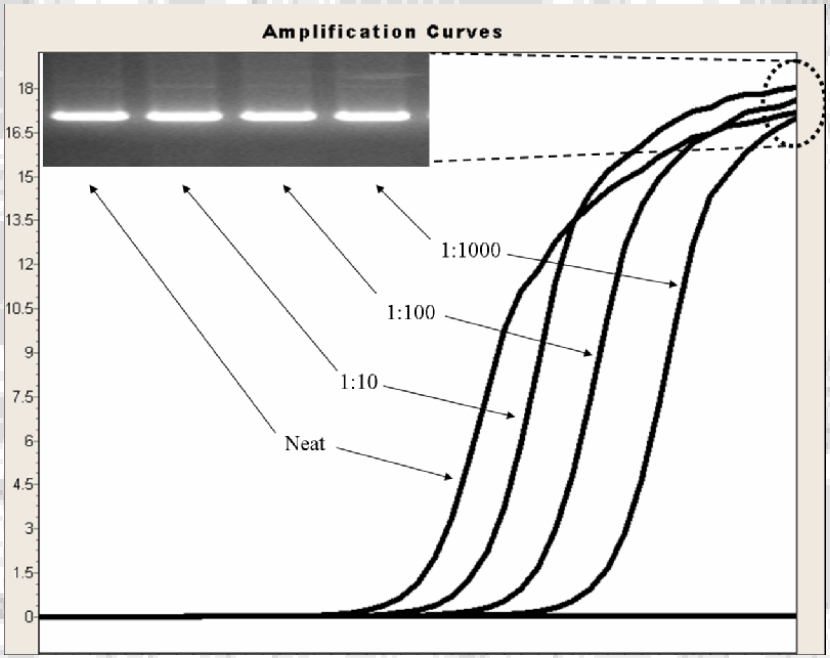
At each cycle, direct fluorescence reading





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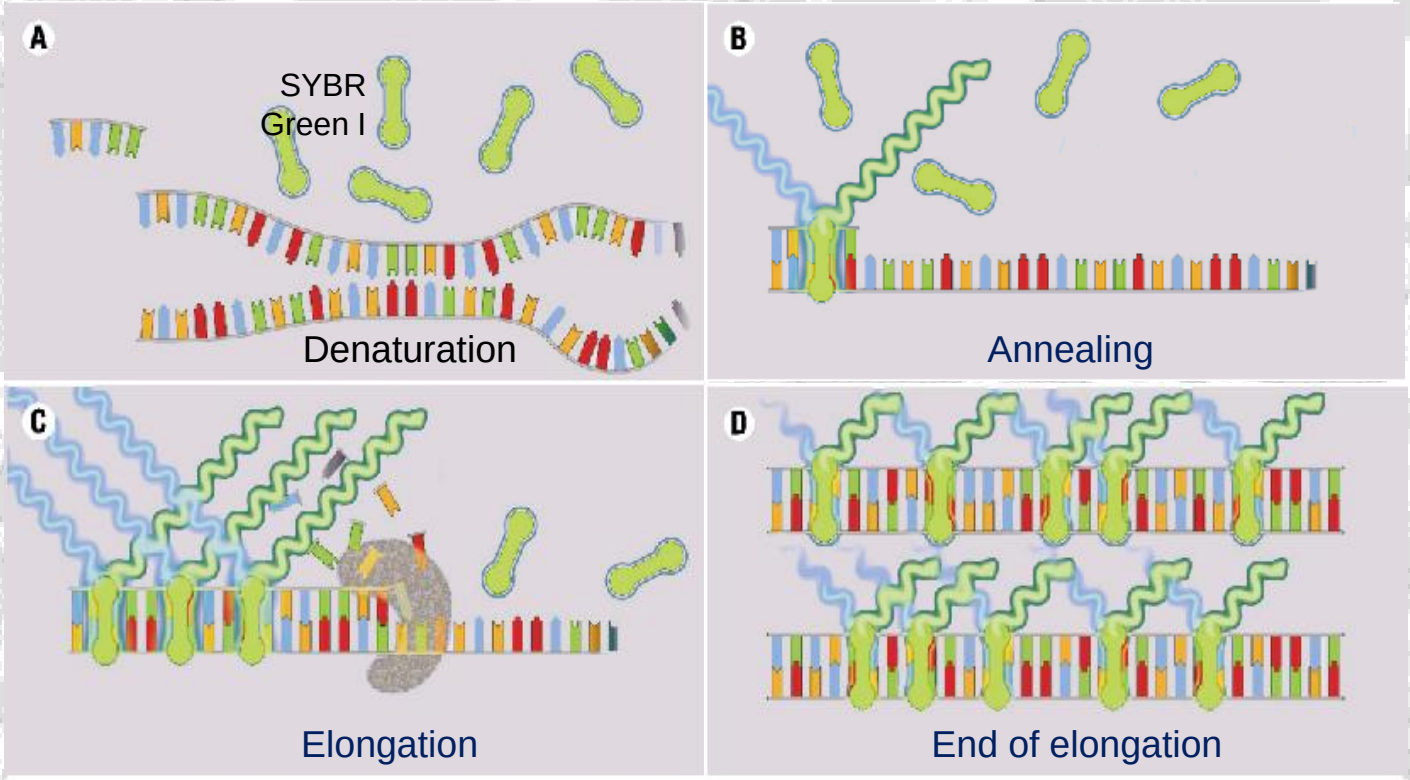
SYBR Green I

Fluorescence of all double-stranded DNA

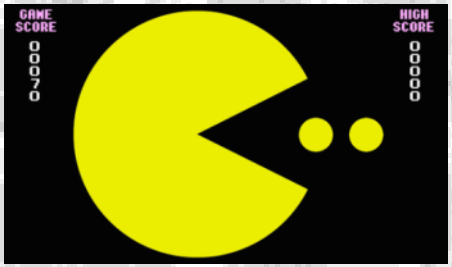


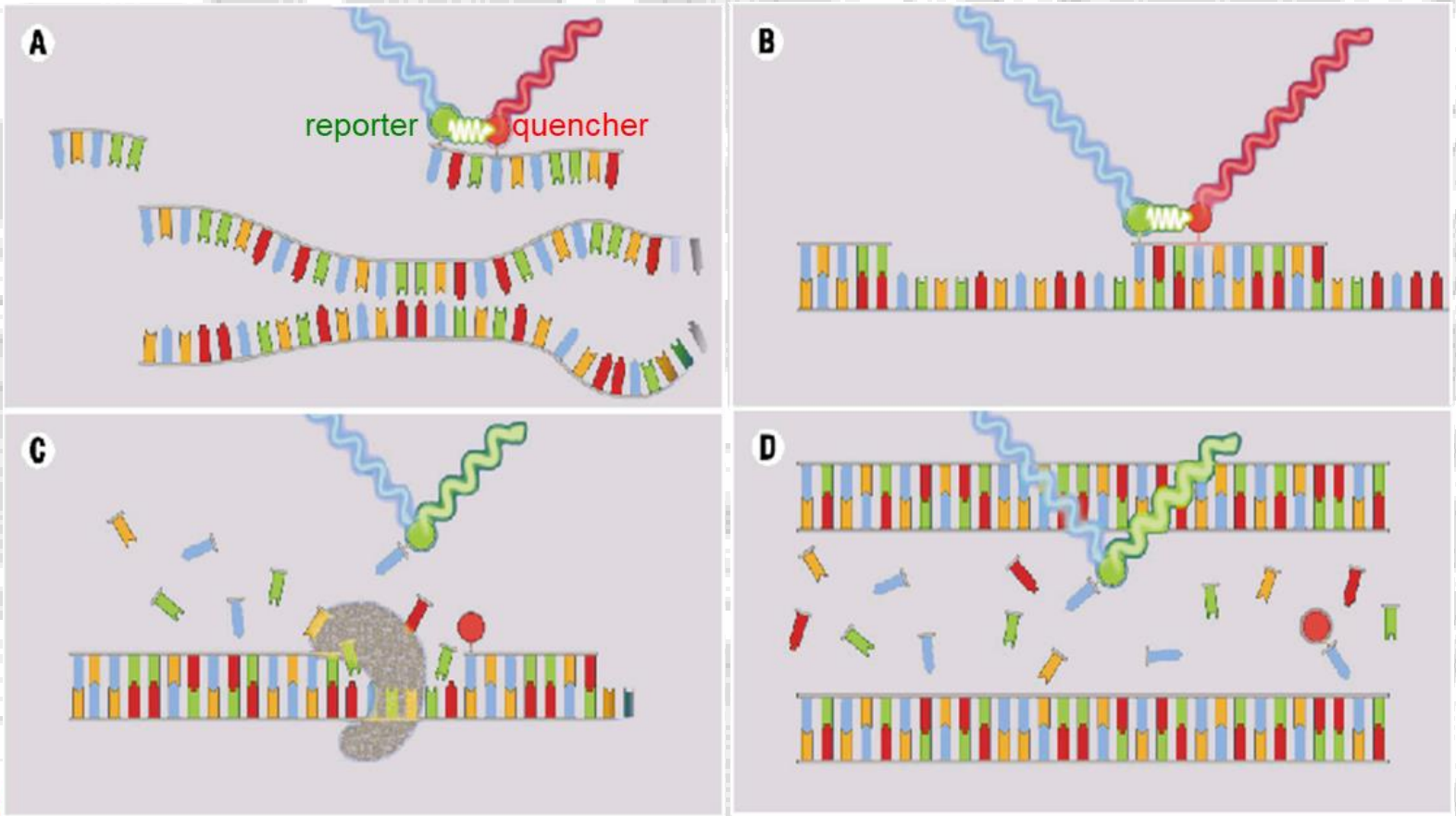
Other fluorescence detection formats in real-time PCR: Probes

- Other oligonucleotides…
- …hybridize during PCR
- and labelled with a fluorescent system

- → additional proof of specific detection of the target product amplified

TaqMan (Hydrolysis) probes





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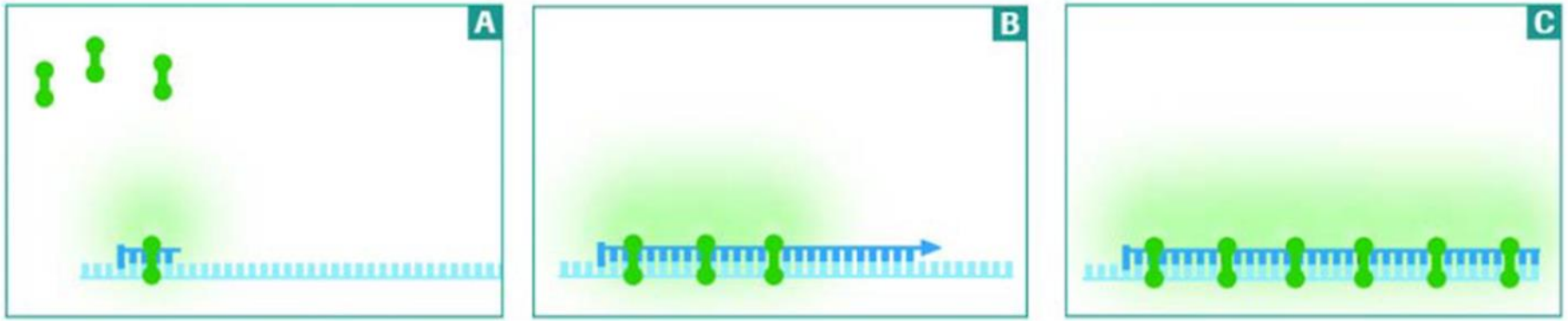
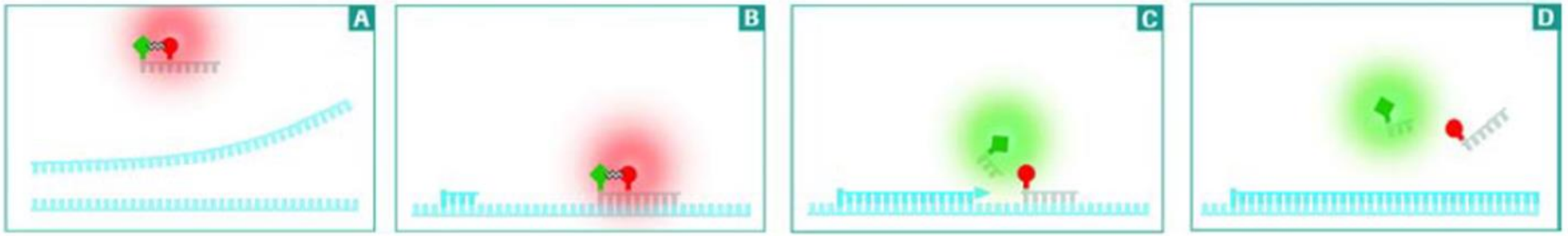
Summary

SYBR Green I



TaqMan Probe



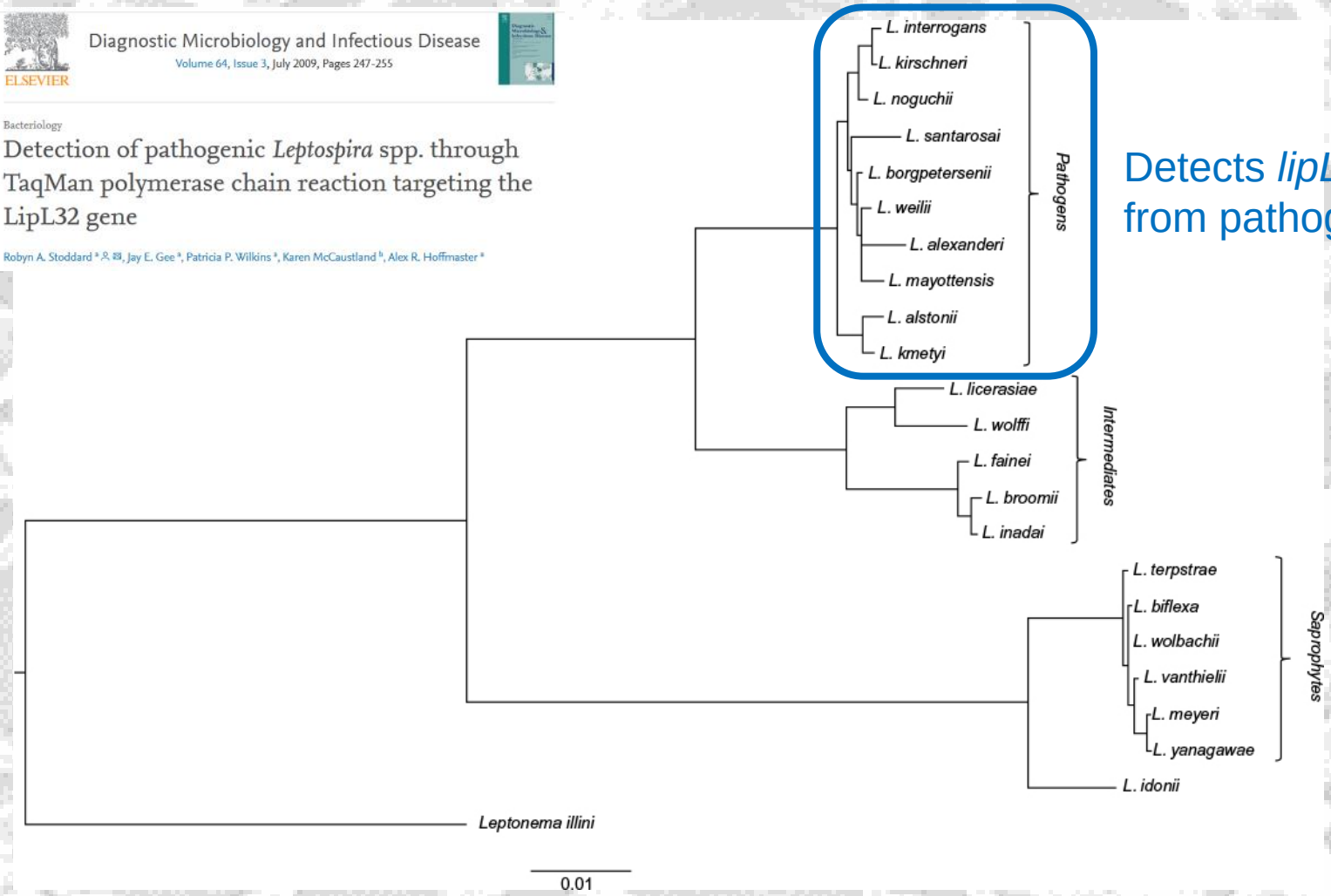
Leptospira qPCR used at NHL

Diagnostic Microbiology and Infectious Disease
Volume 64, Issue 3, July 2009, Pages 247-255

Bacteriology

Detection of pathogenic *Leptospira* spp. through TaqMan polymerase chain reaction targeting the LipL32 gene

Robyn A. Stoddard [a], Jay E. Gee [a], Patricia P. Wilkins [a], Karen McCaustland [b], Alex R. Hoffmaster [a]



Detects *lipL32* gene from pathogenic species

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Thank you!

Questions before we move to the bench?

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