

Leptospirosis laboratory diagnosis & optimal sampling time for serology/PCR

Cyrille GOARANT
Leptospirosis Unit
Institut Pasteur in New Caledonia

Leptospirosis biological diagnosis

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

Laboratory criteria

Presumptive diagnosis:

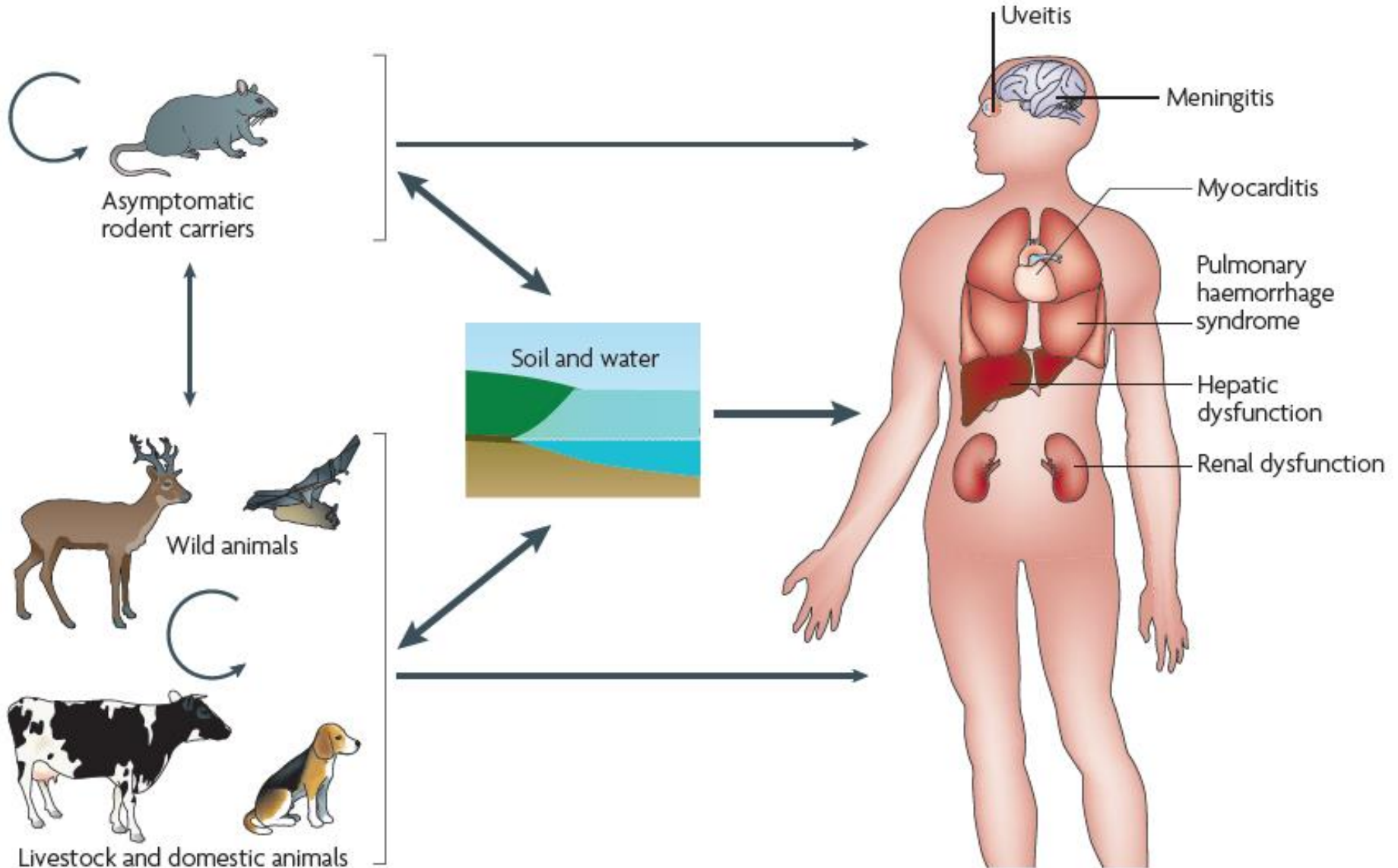
- A positive result of a rapid screening test such as IgM ELISA, latex agglutination test, lateral flow, dipstick etc.

Confirmatory diagnosis:

- Isolation from blood or other clinical materials through culture of pathogenic leptospire.
- 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

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

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

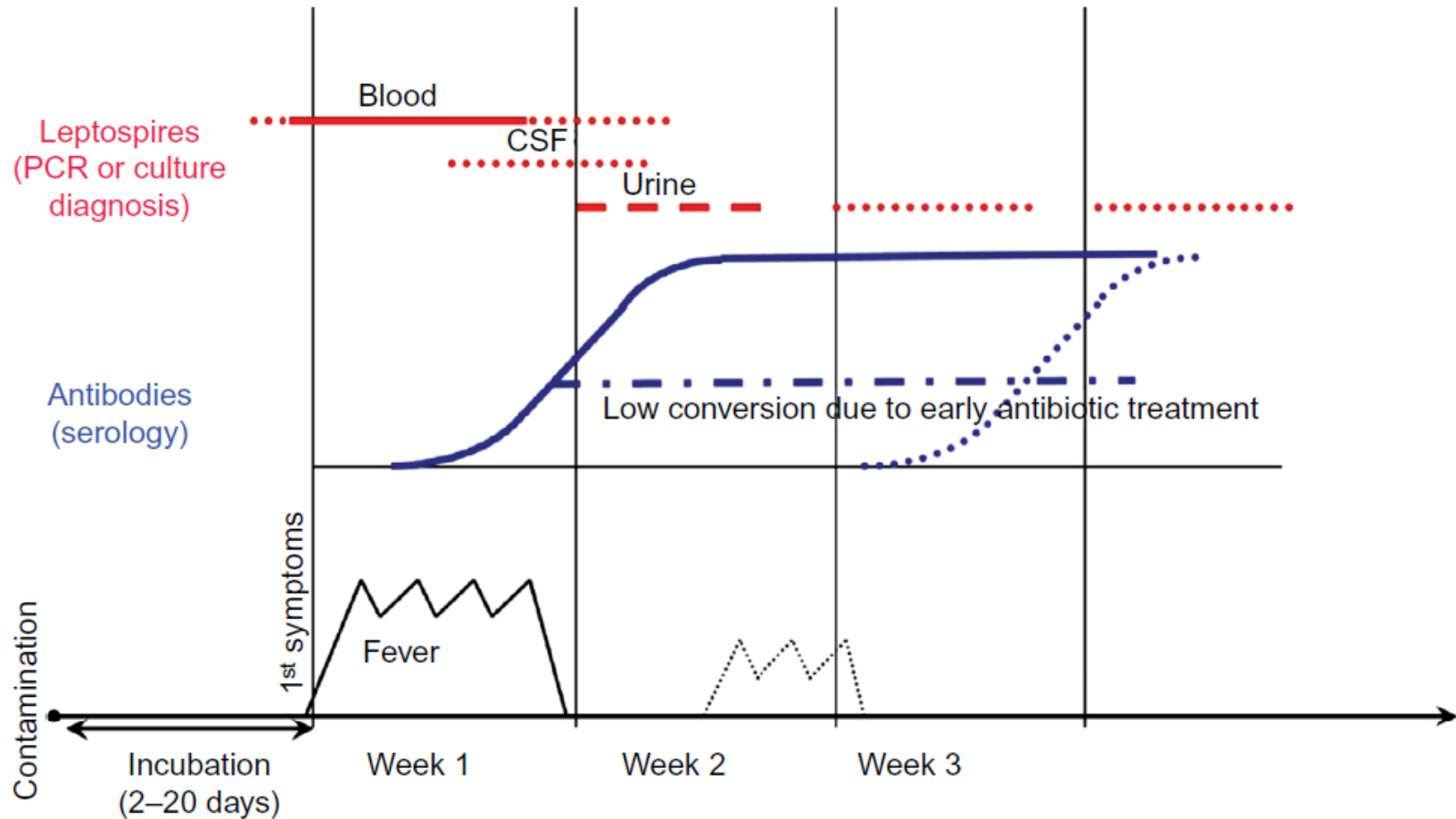


Figure 3 Basic principles underlying the biological diagnosis of leptospirosis.

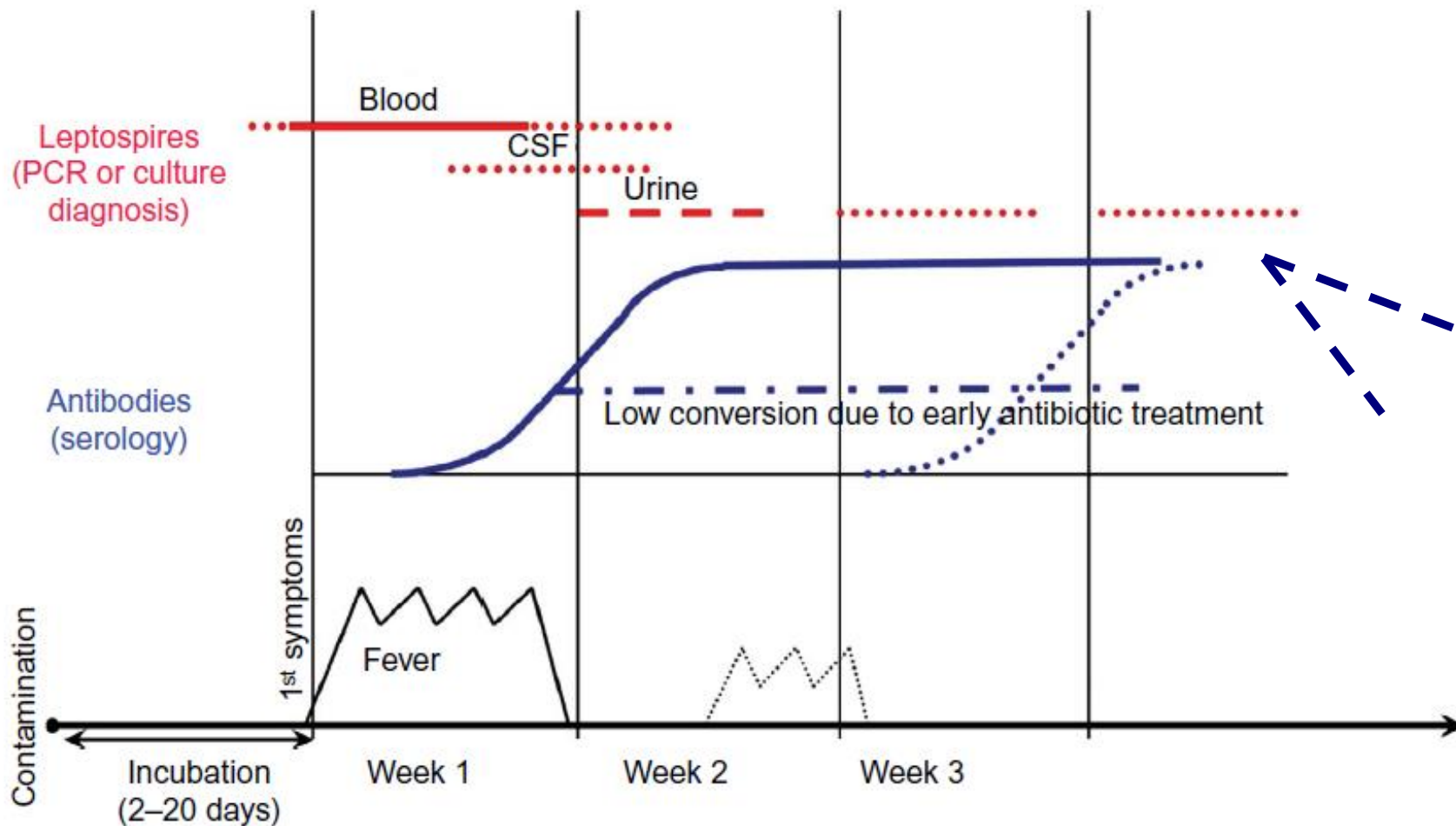
Notes: Adapted from Turner. Leptospirosis I. *Trans R Soc Trop Med Hyg.* 1967;61(6):842–855,¹²⁴ by pe1 Leptospirosis: risk factors and management

Abbreviations: PCR, polymerase chain reaction; CSF, cerebrospinal fluid.

challenges in developing countries

Applications to biological diagnosis

- ▣ **“Age of disease” needs to be known**
 - Dn = Date of sample collection - Date of onset of symptoms
- ▣ **Look for leptospire (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**



Detect leptospires (or their products)

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

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.

	Onse 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

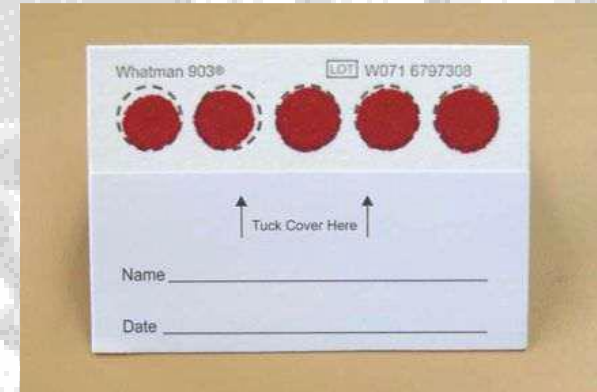
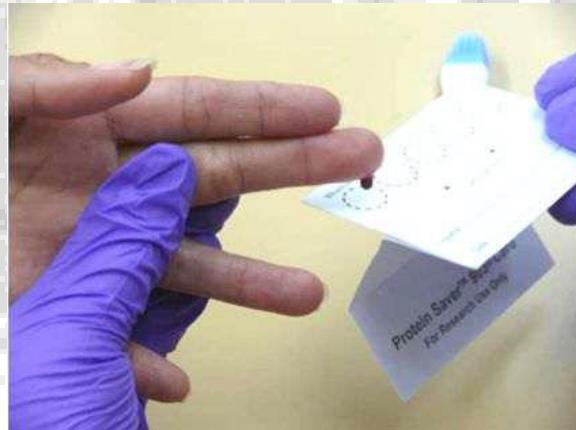
- Crude serum sample: needs to be kept frozen
 - ⇒ Dry ice for shipment
 - ⇒ IATA “Biological substance”
- Dried on filter paper
 - ⇒ Ambient temperature
 - ⇒ Not a biological substance for IATA

Serum transportation for serology



Whatman 903 Protein saver card

Blood or serum



Drying time: 1-3 hours



Once dry

1. Plastic pouch

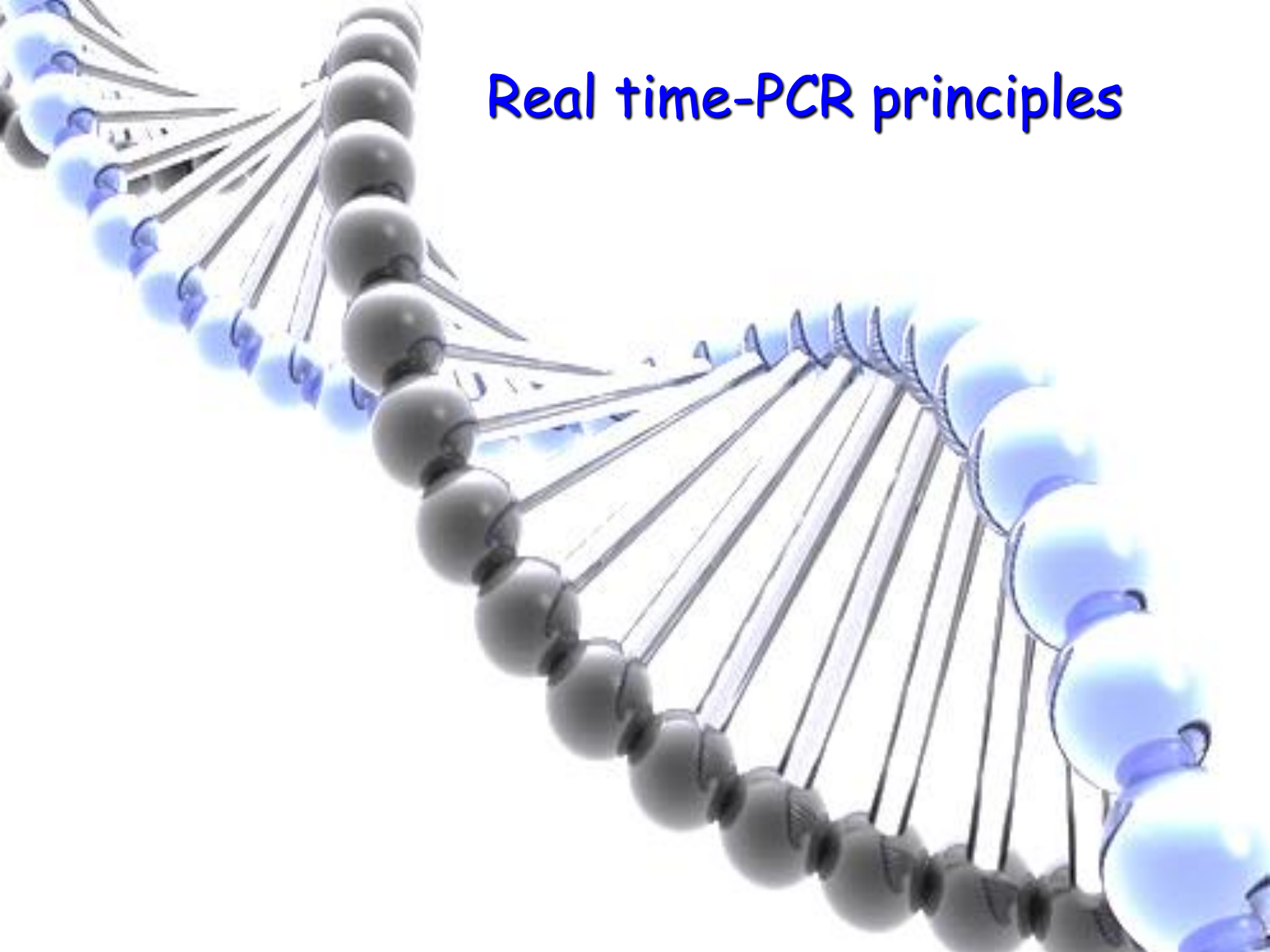


2. Storage at 4°C



3. Shipment at ambient temperature

Real time-PCR principles





ECOMORE II

- 1953 :
- Watson, Crick, Wilkins
Nobel 1962
+ Rosalind Franklin



No. 436 April 25, 1953

NATURE

MOLECULAR STRUCTURE OF NUCLEIC ACIDS A Structure for Deoxyribose Nucleic Acid

We wish to suggest a structure for the salt of deoxyribose nucleic acid (DNA). This structure has novel features which are of considerable biological interest.

A structure for nucleic acid has already been proposed by Pauling and Corey. They study nucleic acid from a non-biological point of view, with the phosphate and the ribose and the bases on the outside. In our opinion, this structure is unsatisfactory for two reasons:

(1) We believe that the model which gives the X-ray diagrams is the salt, not the free acid. Without the acidic hydrogen atom it is not clear what force would hold the structure together, especially as the negatively charged phosphates near the axis would repel each other. (2) Some of the van der Waals distances appear to be too small.

Another three-chain structure has also been suggested by Dreyer (in the press). It has modelled the phosphates on the outside and the bases on the inside, linked together by hydrogen bonds. This structure is described, rather ill-defined, and for this reason we shall not comment on it.

We wish to put forward a radically different structure for the salt of deoxyribose nucleic acid. This structure has two helical chains each coiled round the same axis (see diagram). We have made the usual chemical assumptions, namely, that each chain consists of phosphate ester groups joining $3', 5'$ deoxyribose residues with $2', 3'$ linkages. The two chains for each chain are linked by a cross perpendicular to the line between the chains, following a standard helical twist. The separation of the atoms in the two chains may be regarded as direct. Each chain loosely resembles Pauling's model No. 1; that is, the bases are on the inside of the helix and the phosphate on the outside. The configuration of the sugar and the atoms near it is close to Pauling's standard conformation, the sugar being roughly perpendicular to the attached base. There is a rotation on each chain every 3.4 Å. In the construction, we have assumed an angle of 36° between adjacent residues in the same chain, so that the structures repeat after 10 residues on each chain; that is, after 34 Å. The distance to a phosphate group from the base axis is 10 Å. As the phosphates are on the outside, curves have some access to them.

The structure is an open one, and its water content is rather high. At lower water content we would expect the bases to lie so that the structure would become more compact.

The novel feature of the structure is the manner in which the two chains are held together by the phosphate and nitrogenous bases. The planes of the bases are perpendicular to the lines joining the phosphate groups. A single base from one chain being hydrogen-bonded to a single base from the other chain, so

that the two lie side by side with stacked π -electrons. One of the π electrons on a purine and the other π electron from bonding to oxygen. The hydrogen bonds are made as follows: guanine pairs with a pyrimidine position 1, guanine position 6 to pyrimidine position 6.

It is assumed that the bases only occur in the structure in the most plausible tautomeric forms (that is, with the keto rather than the enol configuration). It is found that only special pairs of bases can bond together. These pairs are adenine (purine) with thymine (pyrimidine), and guanine (purine) with cytosine (pyrimidine).

In other words, if an adenine forms one member of a pair, no other chain than one of these conformational other members that the hydrogen atoms are for guanine and cytosine. The sequence of bases on a single chain does not appear to be restricted in any way. However, if only specific pairs of bases can be formed, it follows that if the sequence of bases on one chain is given, then the sequence on the other chain is automatically determined.

It has been found experimentally^{1, 2} that the ratio of the amounts of adenine to thymine, and the ratio of guanine to cytosine, are always very close to unity in deoxyribose nucleic acid. This is probably explicable in this structure as with a ribose sugar instead of the deoxyribose, as the other oxygen atom would make one chain a rather wide spiral.

The previously published X-ray data³ for deoxyribose nucleic acid are insufficient for a rigorous test of our structure. So far as we can tell, it is roughly compatible with the experimental data, and it is not regarded as unproved until it has been checked against more exact results. Some of these are given in this following communication. We were far from sure of the details of the model proposed here when we carried out the structure, which was nearly brought out entirely on published experimental data and stereo-chemical arguments.

It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material.

Full details of the structure, including the conditions assumed in building it, together with a set of stereoisomers for the model, will be published elsewhere.

We are much indebted to Dr. Jerry Donohue for generous advice and criticism, especially on intramolecular effects. We have also been stimulated by a knowledge of the general nature of the unpublished experimental results and ideas of Dr. M. H. Perkin, Dr. R. D. Briston and their co-workers at King's College, London. One of us (J.D.W.) has been aided by a fellowship from the National Foundation for Infectious Diseases.

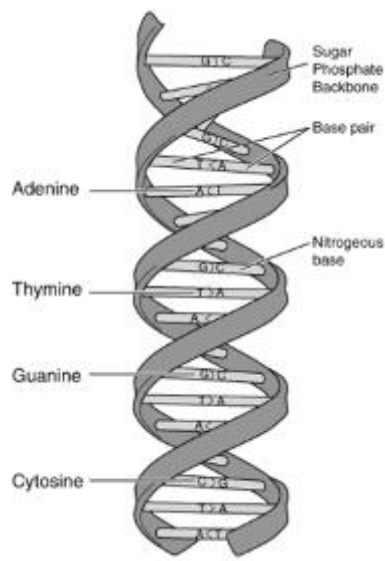
J. D. WATSON
F. C. CRICK

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

Received 14th March 1953; revised 25th March 1953; accepted 2nd April 1953.
¹Franklin, K., and Crick, F. H. C., *Nature*, **191**, 39 (1953).
²Franklin, K., and Crick, F. H. C., *Nature*, **191**, 39 (1953).
³Franklin, K., and Crick, F. H. C., *Nature*, **191**, 39 (1953).

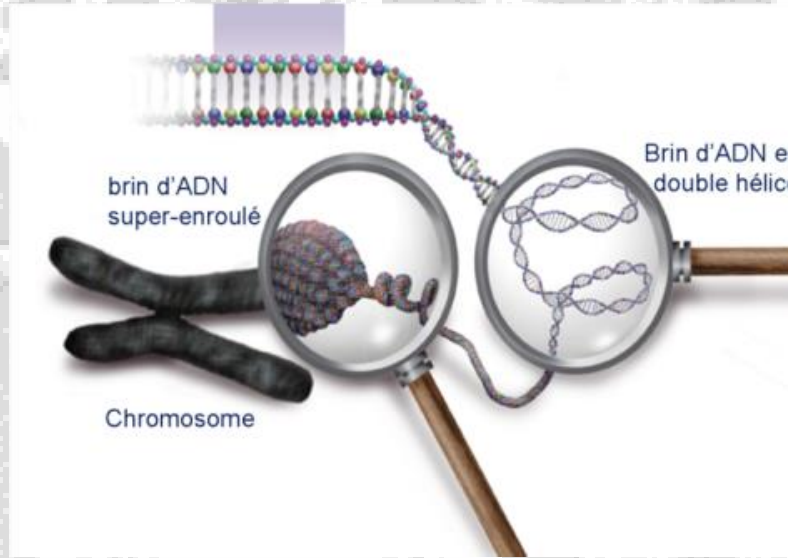
As in the previous communication, the $3', 5'$ deoxyribose residues are linked together by phosphate ester groups joining $3', 5'$ deoxyribose residues with $2', 3'$ linkages. The two chains for each chain are linked by a cross perpendicular to the line between the chains, following a standard helical twist.

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



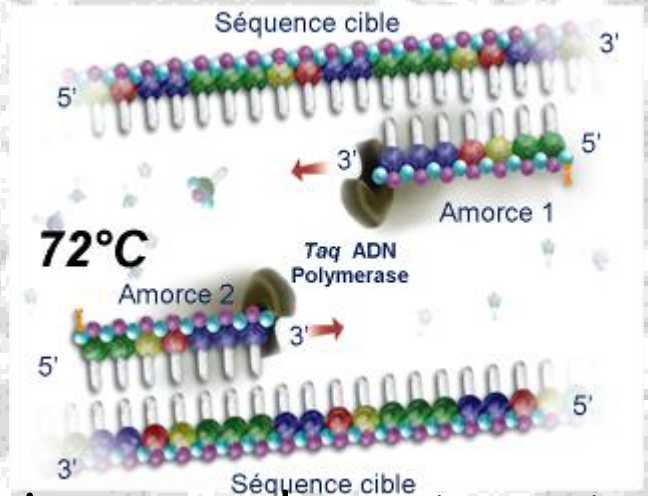
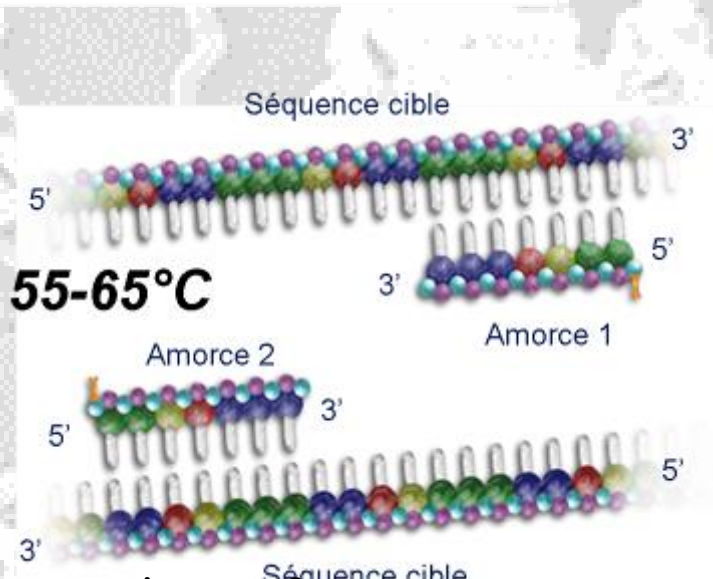
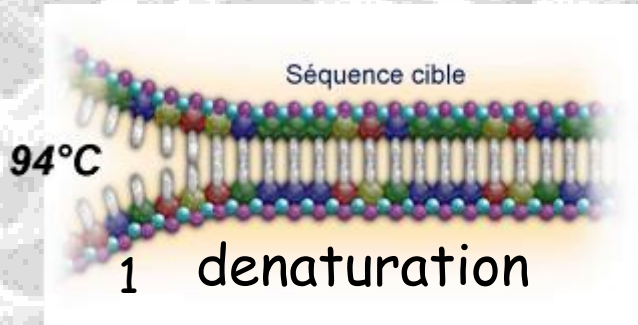


DNA extraction



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

PCR amplifies a target DNA sequence... ... in 3 steps





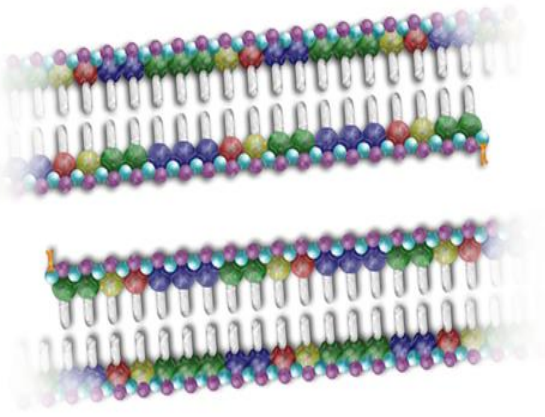
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Cycling

3 cycles :

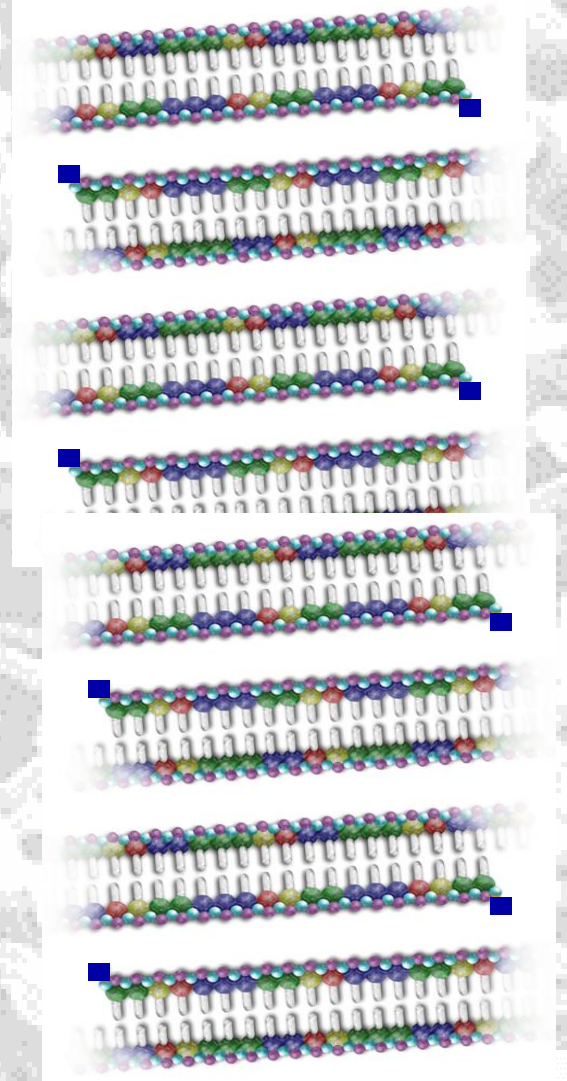
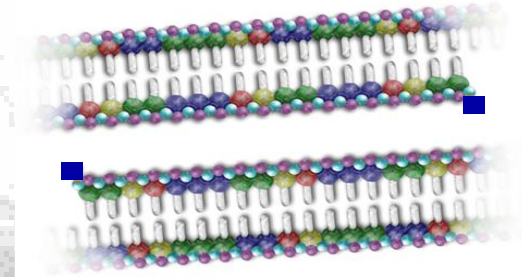
8 target sequences

2 target sequences



2 cycles :

4 target sequences




30 Cycles :

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

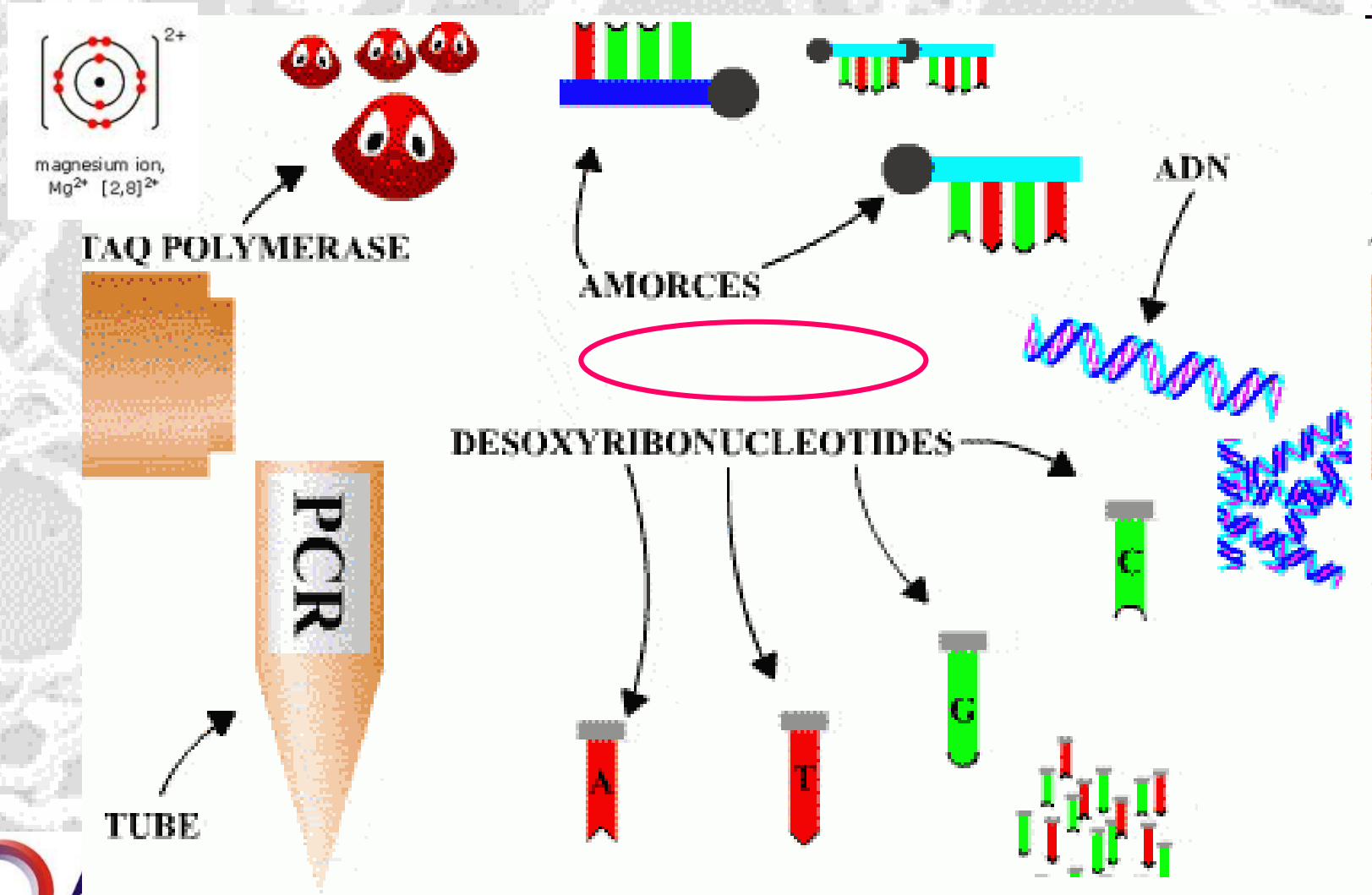


50 Cycles : 2^{50} copies =
1 125 899 906 842 620 000 copies !

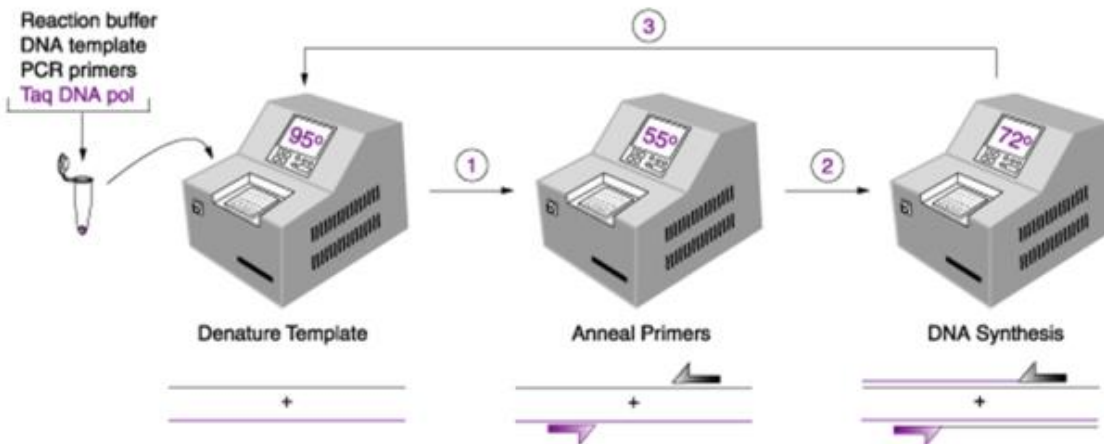
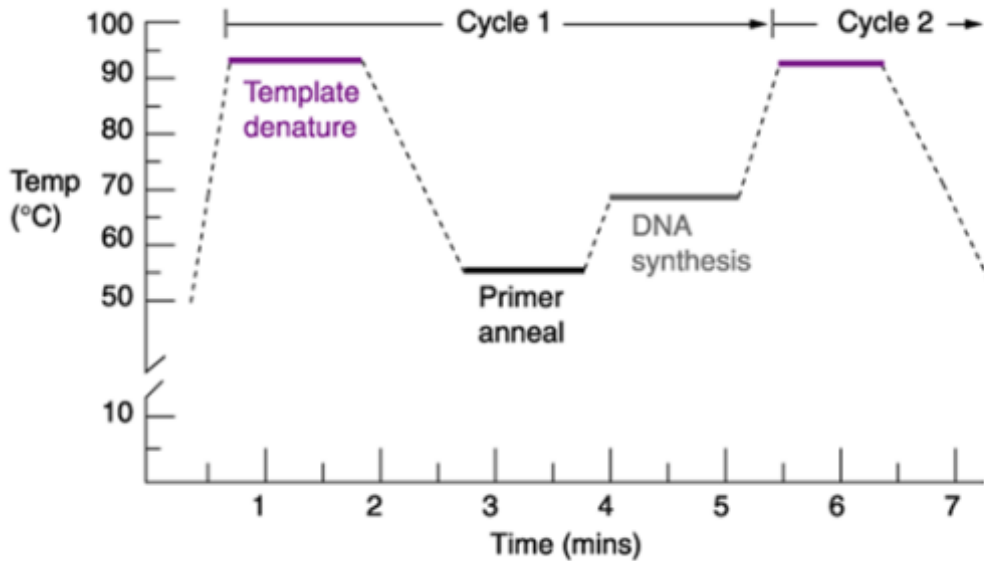


Risk of contamination +++
1 qPCR drop in an olympic swimming pool
would correspond to > 450 000 copies per ml !

PCR: Replication of DNA *in vitro*



Traditional PCR



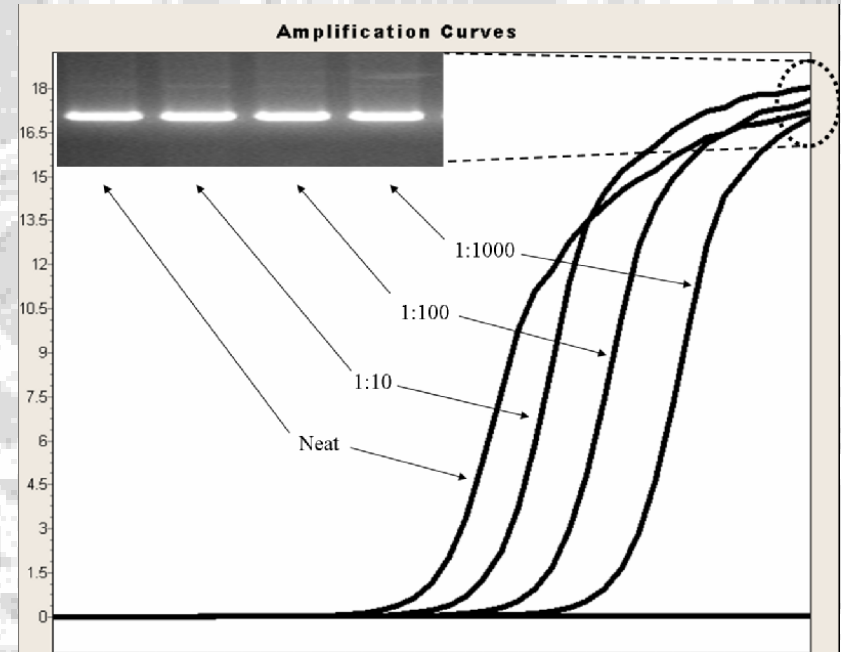
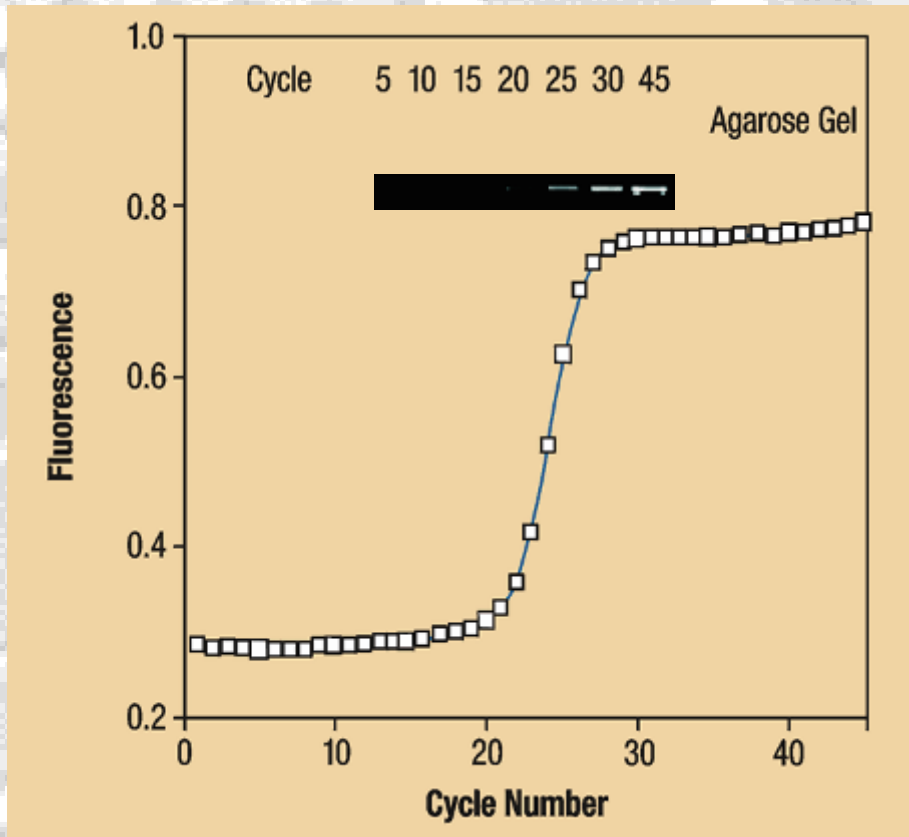
Real-time PCR



- ▣ **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!

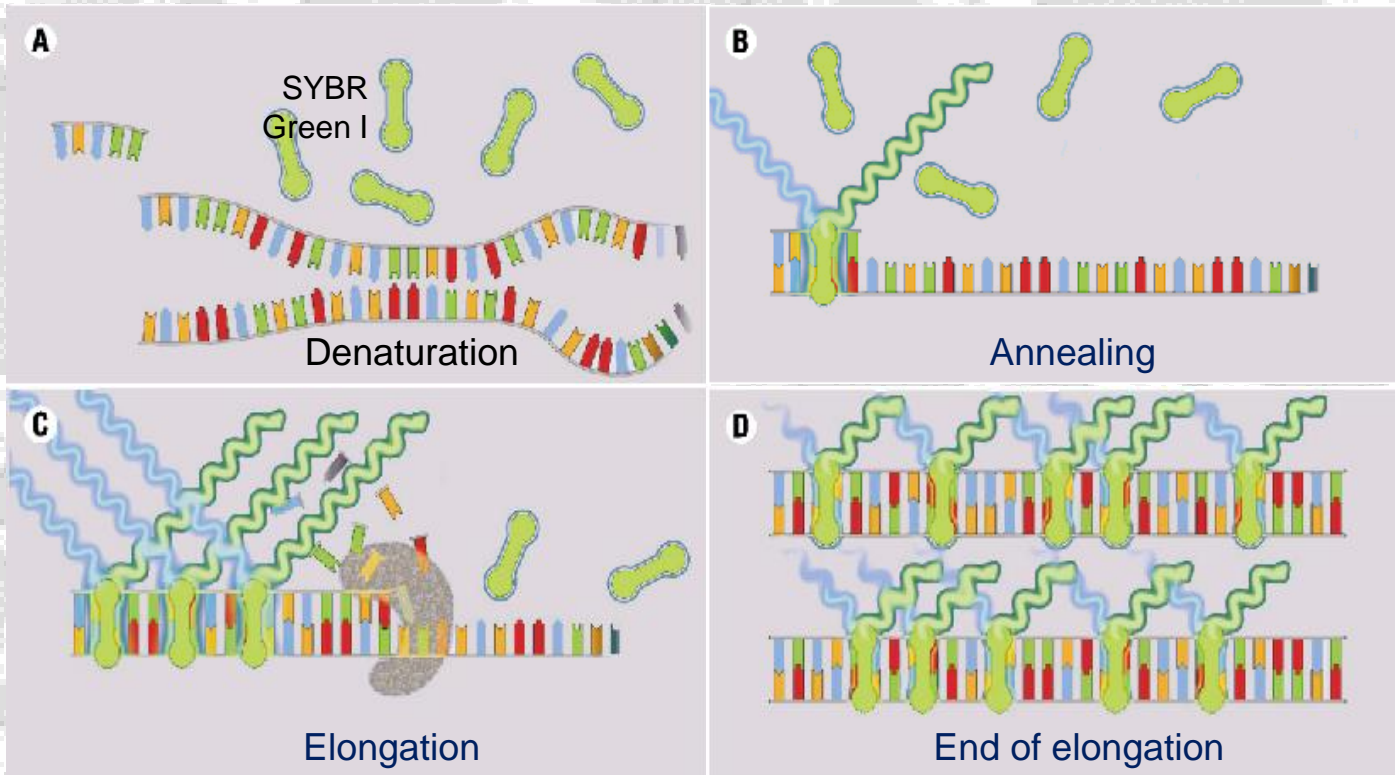
Detection in real time

At each cycle, direct fluorescence reading



SYBR Green I

Fluorescence of all double-stranded DNA

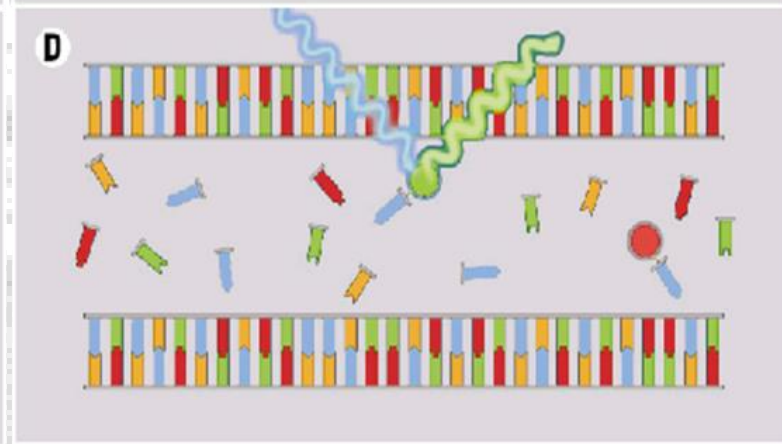
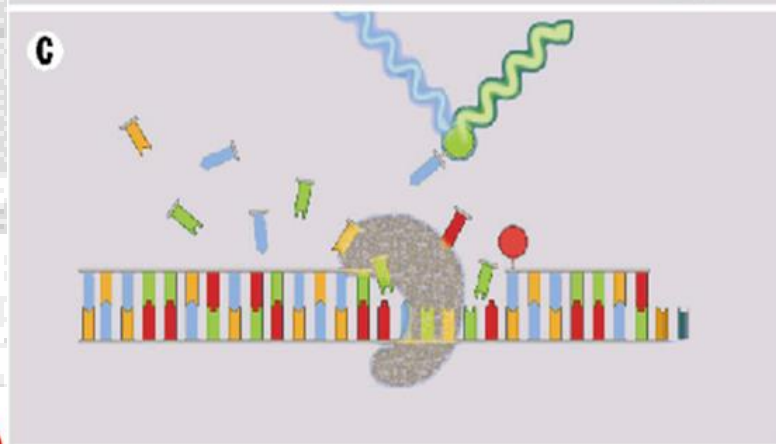
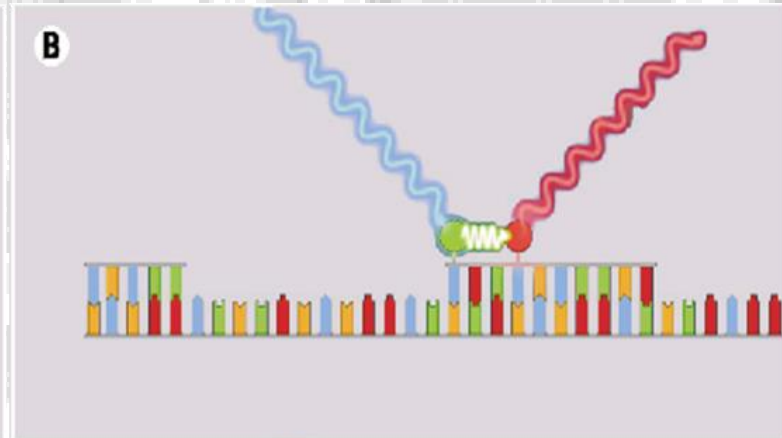
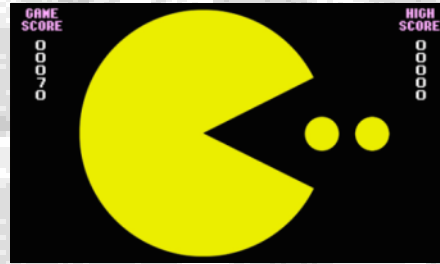


Other fluorescence detection formats in real-time PCR: Probes

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

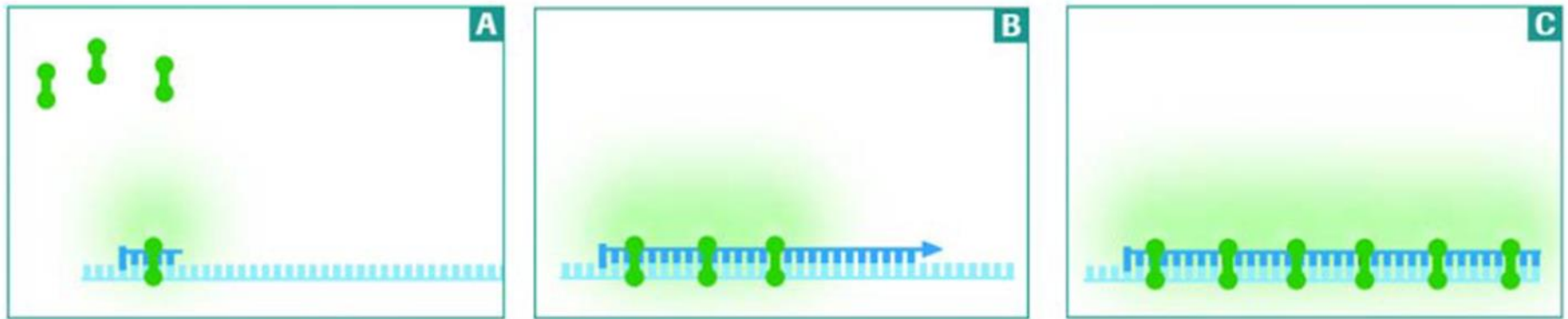
- → additional proof of specific detection of the target product amplified

TaqMan (Hydrolysis) probes

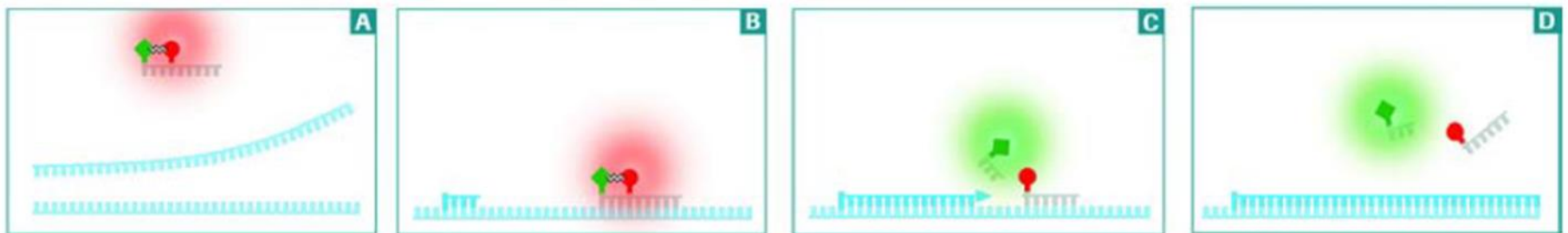


Summary

SYBR Green I



TaqMan Probe

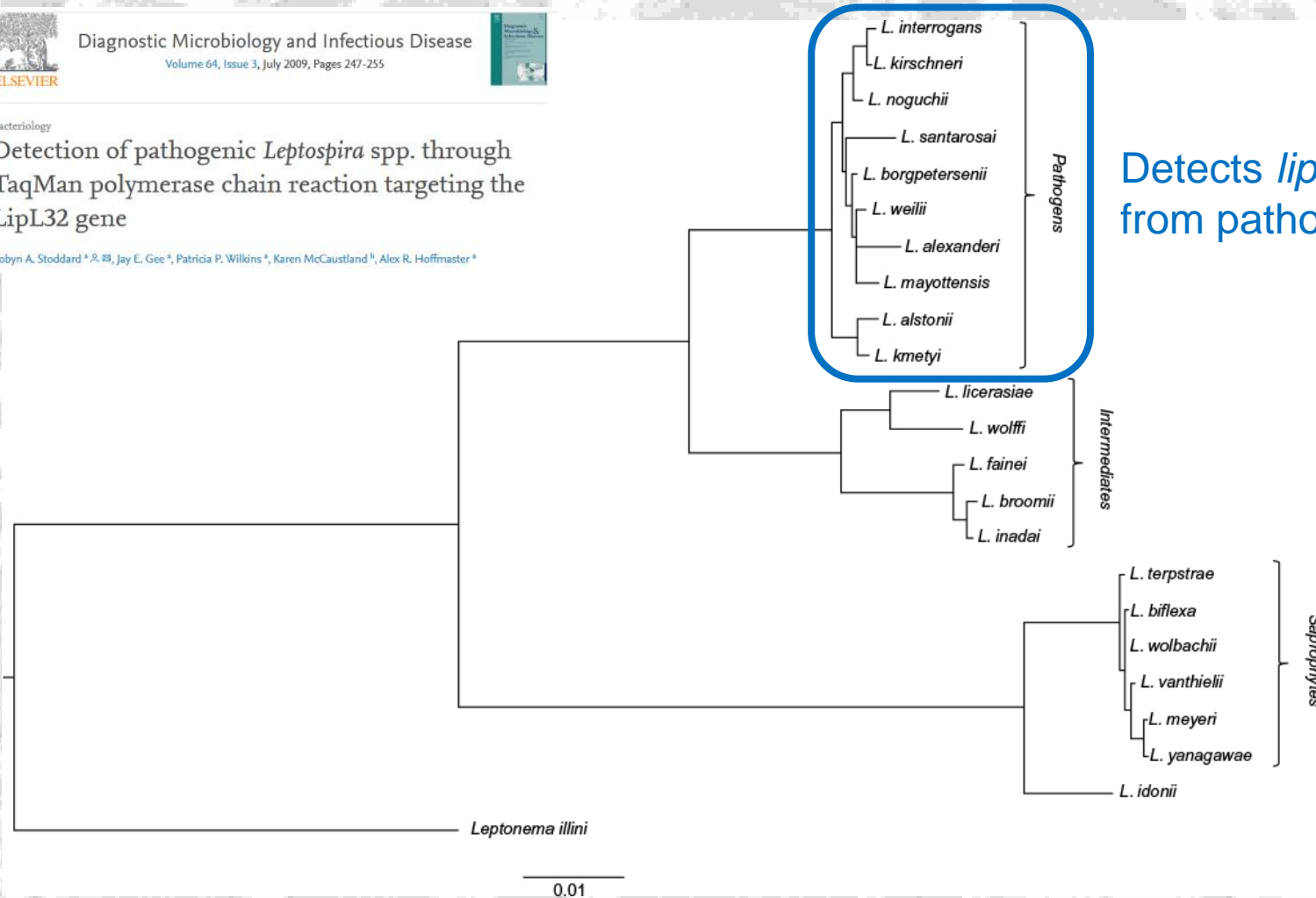


Leptospira qPCR used at NHL



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

Robyn A. Stoddard^{a, B}, Jay E. Gee^a, Patricia P. Wilkins^a, Karen McCaustland^b, Alex R. Hoffmaster^a



Detects *lipL32* gene from pathogenic species



ECOMORE II

Thank you!

Questions before we move to the bench?