

# qPCR analysis of molecular targets for developing world pathogen diagnosis;

## a multi-step approach to a multi-step problem

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# Why infectious diseases affecting the developing world?

## Tuberculosis, Malaria and HIV/AIDS

Kill > 5 million people per annum (0.1 % world population)

- Charities currently provide large amounts of antibiotics/anti viral treatment to the developing world
  - Recent pledges from Europe and the US to provide large amounts of aid for therapy
  - Large pharmaceutical companies agreeing to provide drugs at reduced prices for the developing world
- Hardly any of these consider diagnosis**



A. Zumla, J.M. Grange, Tuberculosis in: G.C. Cook. A. Zumla (Eds.), Mansons 21 edition, Saunders, 2002, pp. 997-1051

Ⓜ Lung diseases at necropsy in African children dying from respiratory illnesses: a descriptive necropsy study

		Total*	
<i>Chifumbe</i>	<b>Diagnosis</b>		
<i>Peter Mw</i>	Acute pyogenic pneumonia	116 (44%)	
<i>Study Grc</i>	PCP	58 (22%)	
<b>Summa</b>	Tuberculosis	54 (20%)	ises
<i>Backgrou</i>	CMTV	43 (16%)	The
<i>death in</i>	Interstitial pneumonitis	30 (11%)	cult.
<i>scarce, ar</i>	Shock lung	27 (10%)	with
<i>study of</i>	Pulmonary oedema	19 (7%)	ests
<i>University</i>	Lymphocytic interstitial pneumonitis	10 (4%)	onia
<b>Methods</b>			002
<i>and 127</i>			
<b>THE LANC</b>			985

Study performed at Zambia's main tertiary hospital

The therapy for many of the diseases was available

The lack of accurate diagnosis let these children down

# Diagnosis

- **The first stage in combating an infection**

# Top ten biotechnologies for improving health in developing countries

Abdallah S. Daar<sup>1-4</sup>, Halla Thorsteinsdóttir<sup>1,2</sup>, Douglas K. Martin<sup>2,5,6</sup>, Alyna C. Smith<sup>1,2</sup>, Shauna Nast<sup>1,2</sup> & Peter A. Singer<sup>2,4,7</sup>

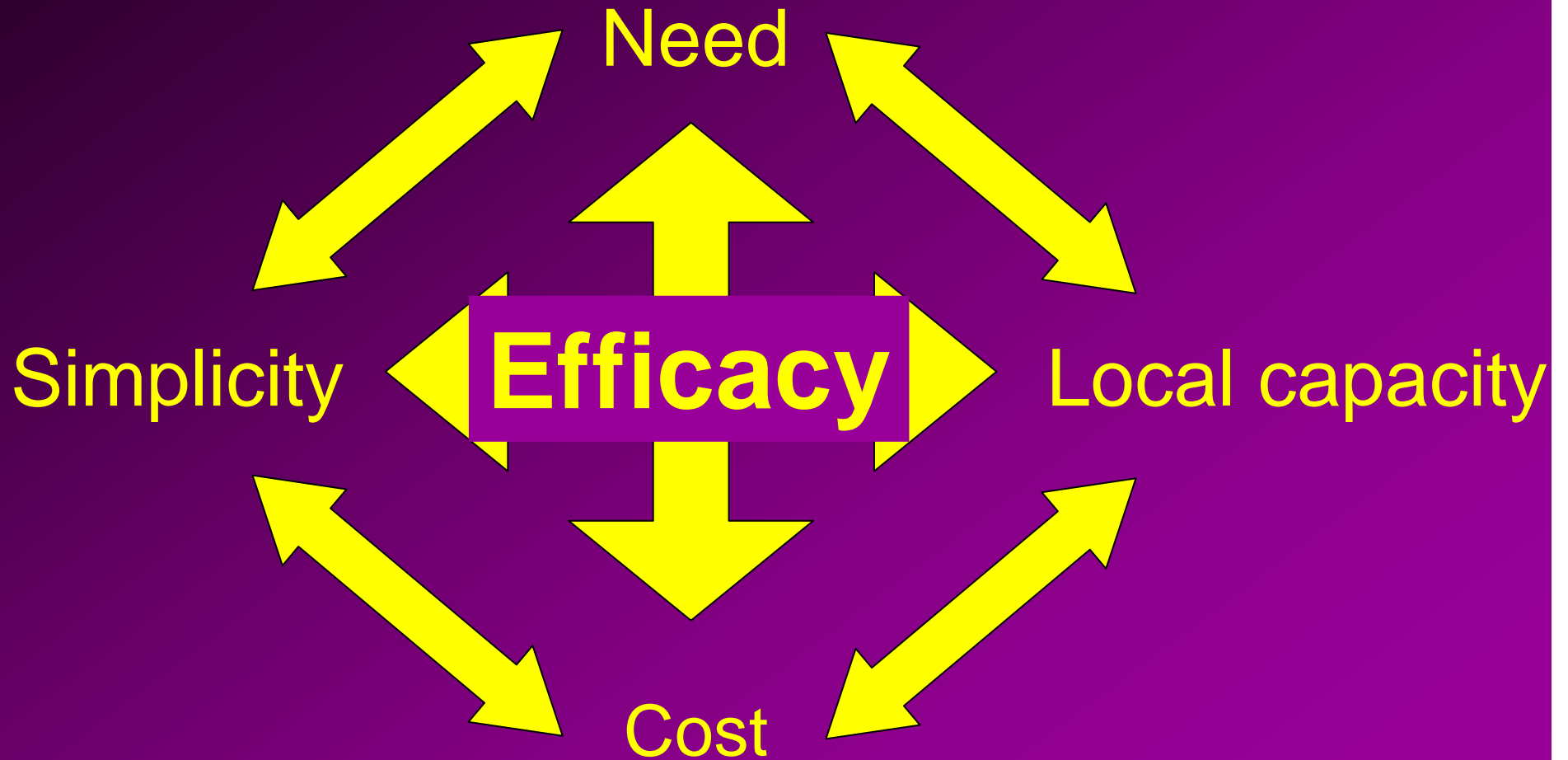
Most research into genomics and other related biotechnologies is concerned with the priorities of industrialized nations, and yet a limited number of projects have shown that these technologies could help improve health in developing countries. To encourage the successful application of biotechnology to global health, we carried out a study in which we asked an international group of eminent scientists with expertise in global health issues to identify the top ten biotechnologies for improving health in developing countries. The results offer concrete guidance to those in a position to influence the direction of research and development, and challenge common assumptions about the relevance and affordability of biotechnology for developing countries.

**Table 1 • The top ten biotechnologies with scores based on rankings of the expert panel**

Final ranking	Biotechnology	Final score
1	<u>Modified molecular technologies for affordable, simple diagnosis of infectious diseases</u>	288
2	Recombinant technologies to develop vaccines against infectious diseases	262
3	Technologies for more efficient drug and vaccine delivery systems	245
4	Technologies for environmental improvement (sanitation, clean water, bioremediation)	193
5	Sequencing pathogen genomes to understand their biology and to identify new antimicrobials	180
6	Female-controlled protection against sexually transmitted diseases, both with and without contraceptive effect	171
7	Bioinformatics to identify drug targets and to examine pathogen–host interactions	168
8	Genetically modified crops with increased nutrients to counter specific deficiencies	159
9	Recombinant technology to make therapeutic products (for example, insulin, interferons) more affordable	155
10	Combinatorial chemistry for drug discovery	129

## 1. Modified molecular technologies for affordable, simple diagnosis of infectious disease (Molecular Diagnostics)

# What is required for a new Diagnostic test to be used?



# Diagnostic test efficacy

- Sensitivity, Specificity
- New methods established through scientific research
- It is essential that the research is conducted correctly with appropriate controls
- The publication describing the result needs to be realistic in what can be concluded

# Pneumocustis Pneumonia (PCP)

Caused by the fungus *P. jirovecii* in immunocompromised

## Symptoms:

High fever, non-productive cough, shortness of breath (especially on exertion), weight loss and night sweats.

Definitive diagnosis by pathologic identification of the causative organism in induced sputum or bronchoalveolar lavage (BAL)

# Molecular diagnosis of PCP

## CLINICAL AND LABORATORY OBSERVATIONS

### Identification of *Pneumocystis carinii* DNA by polymerase chain reaction in necropsy lung samples from children dying of respiratory tract illnesses

*Francis Kasolo, PhD, Kennedy Lishimpi, MD, Chifumbe Chintu, MD, Peter Mwaba, MD, Victor Mudenda, MRCPATH, Daniel Maswabu, MD, Hiroshi Terunuma, PhD, Helen Fletcher, PhD, Andrew Nunn, MSc, Sebastian Lucas, FRCPath, and Alimuuddin Zumla, MD, PhD*

Polymerase chain reaction for *Pneumocystis carinii* DNA was performed on necropsy lung samples from children by means of *P carinii*-specific primers. *P carinii* DNA was identified in 22 of 22 (100%) samples with histologically proven *P carinii* pneumonia and 13 of 75 (17%) with non-*P carinii* pneumonia respiratory illness (sensitivity, 100%; specificity, 83%). The low specificity precludes the use of polymerase chain reaction as an alternative to histopathologic diagnosis. (J Pediatr 2002;140:367-9)

logic appearances can occur.<sup>2,3</sup> In these patients, results of conventional investigations for diagnosis of *P carinii* may be negative. The absence of the trophozoite(s) does not rule out presence of *P carinii* because identification is dependent on several factors, such as experience of the histopathologist, density of organism, site of infection, and sensitivi-

# Early Molecular diagnosis of PCP

Detection of *Pneumocystis carinii* with DNA amplification

Wakefield *et al*

The Lancet

Volume 336, Issue 8713

1990

**PCR designed to amplify part of the Large subunit of the mitochondrial rRNA gene**

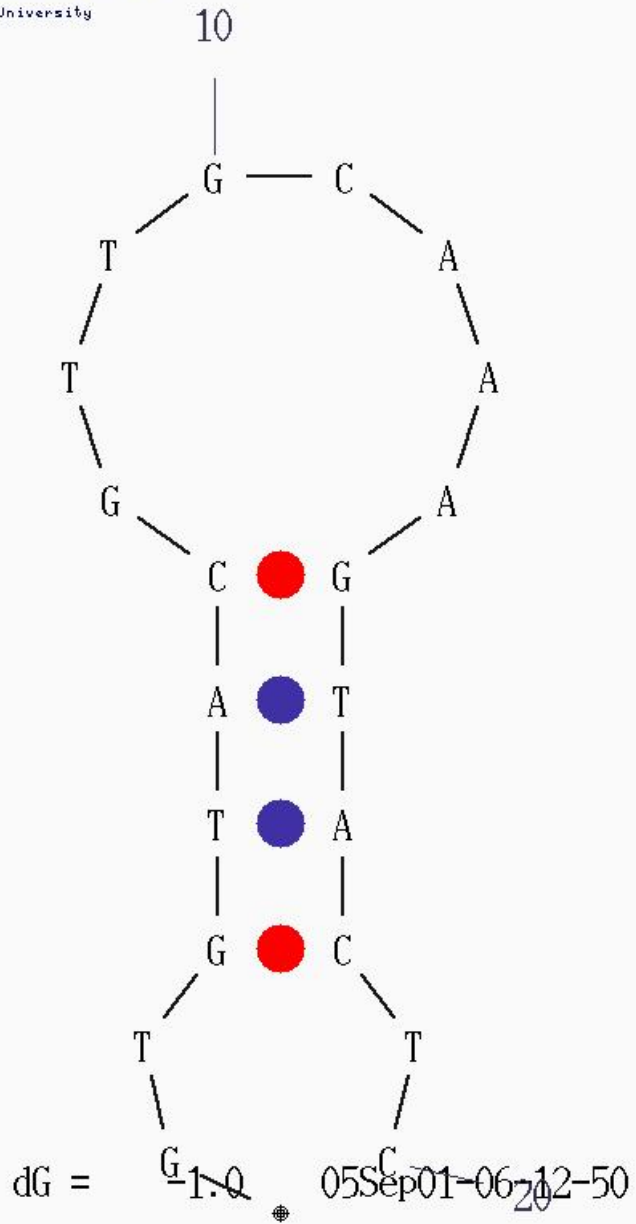
**Primers referred to as H & E**

**Ideal due mitochondrial multiplicity**

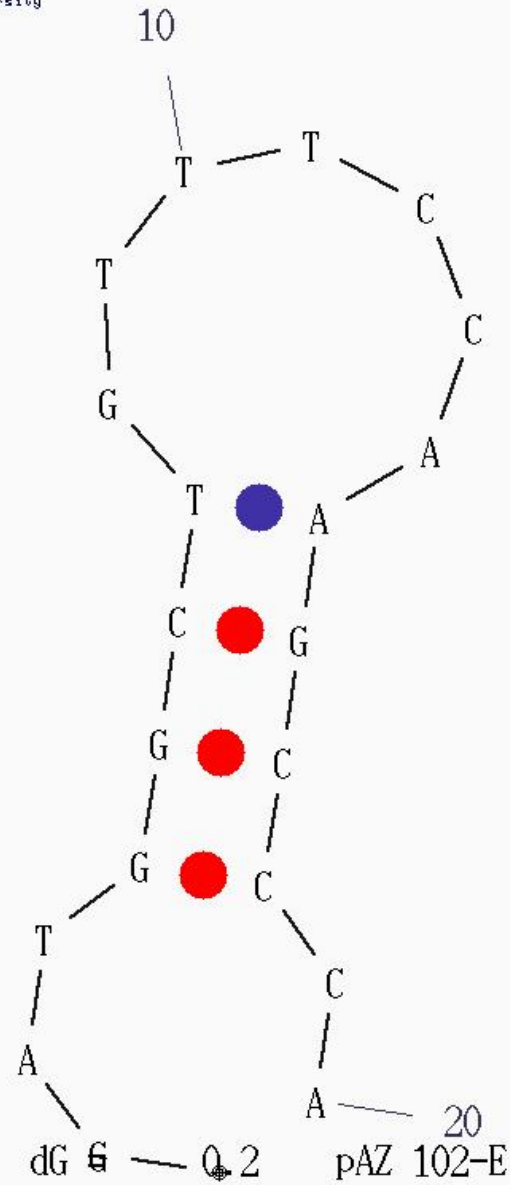


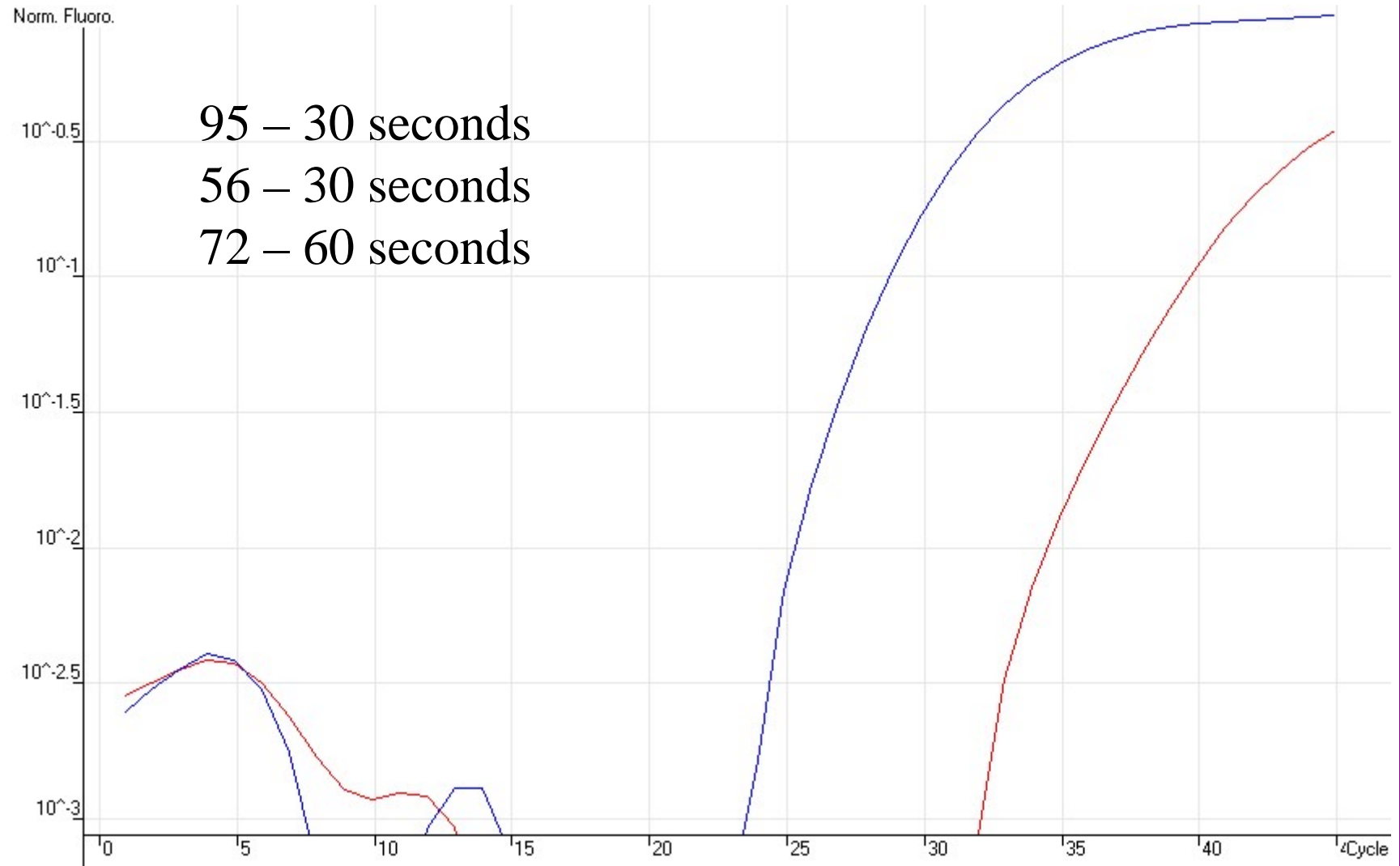
**TM difference 5-15.3 °C**

p1t22jpg by D. Stewart and M. Zuker  
© 2005 Washington University



p1t22.jpg by D. Stewart and M. Zuker  
© 2005 Washington University





— *PjHSP70a*  
— H & E

## Detection of *Pneumocystis carinii* DNA in Blood by PCR Is Not of Value for Diagnosis of *P. carinii* Pneumonia

ENRICA TAMBURRINI,<sup>1\*</sup> PAOLA MENCARINI,<sup>1</sup> ELENA VISCONTI,<sup>1</sup> MARIA ZOLFO,<sup>1</sup>  
ANDREA DE LUCA,<sup>1</sup> ALESSANDRA SIRACUSANO,<sup>2</sup> ELENA ORTONA,<sup>2</sup> AND ANN E. WAKEFIELD<sup>3</sup>

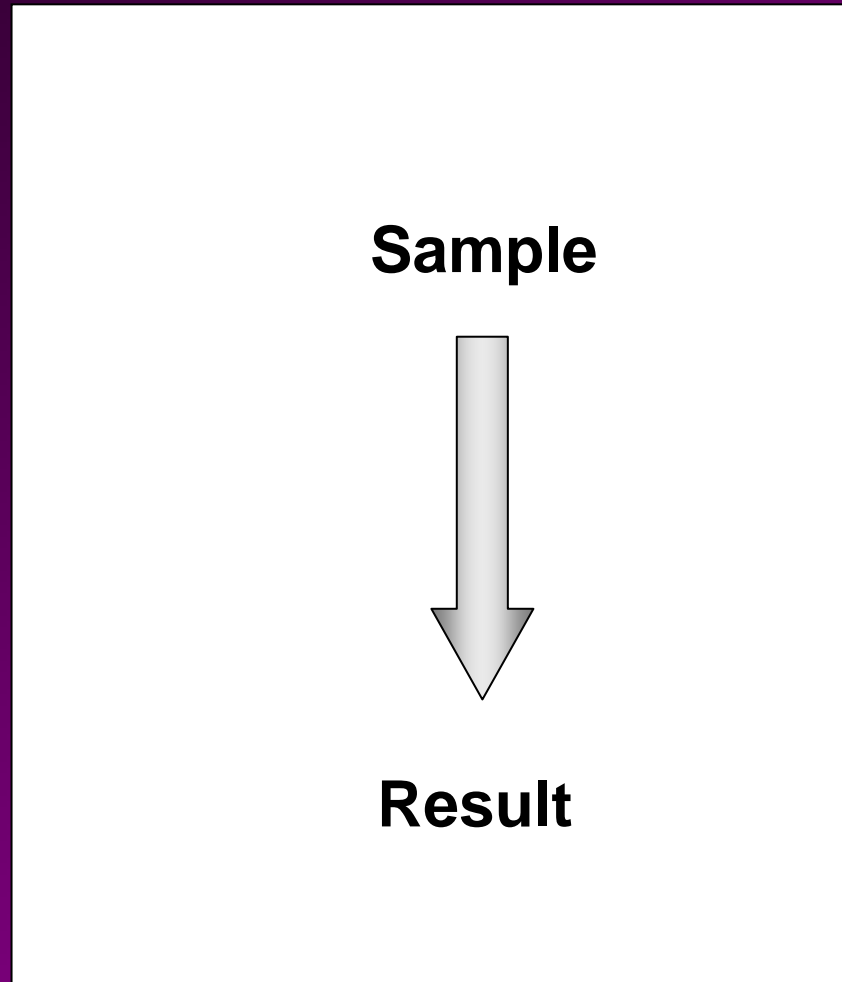
*Department of Infectious Diseases, Università Cattolica del S. Cuore,<sup>1</sup> and Department of Immunology, Istituto Superiore di Sanità,<sup>2</sup> Rome, Italy, and Molecular Infectious Diseases Group, Department of Pediatrics, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, United Kingdom<sup>3</sup>*

Received 29 November 1995/Returned for modification 16 January 1996/Accepted 16 March 1996

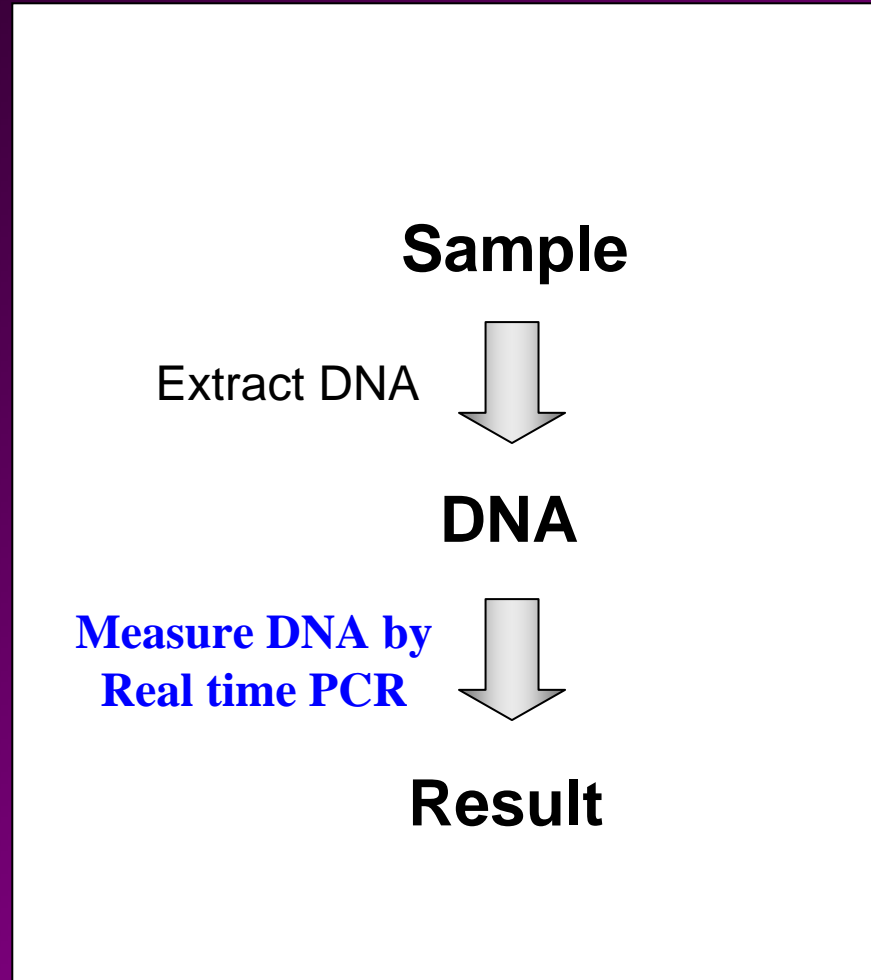
**A nested PCR which amplified a portion of the mitochondrial large-subunit rRNA gene of *Pneumocystis carinii* was used to detect *P. carinii* DNA in blood from patients with *P. carinii* pneumonia. *P. carinii* DNA was not detected in serum and was detected at low levels of blood cells.**

**Sweeping statements about the role of molecular diagnosis based on a single assay should be approached with caution.**

# PCR in a multi-step approach

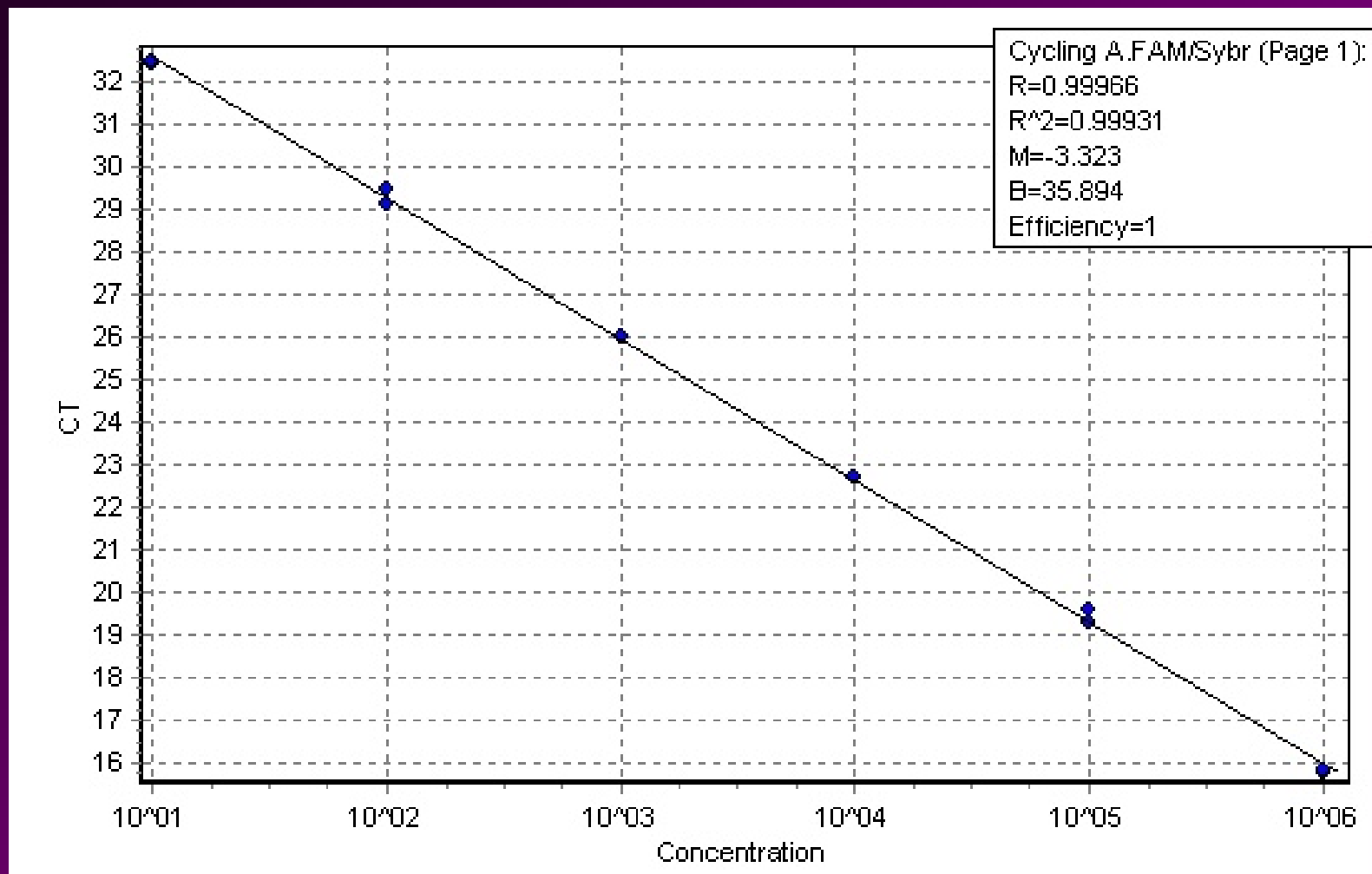


# New PCP assay beginning



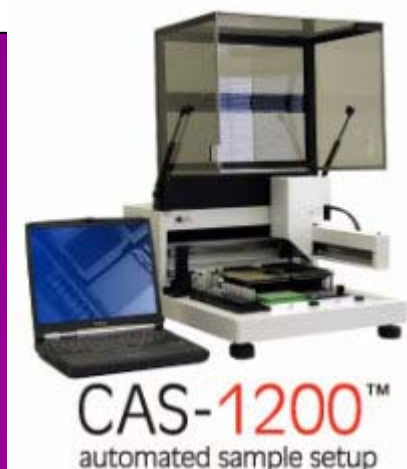
*PjHSP70a*

## Assay characteristics *PjHSP70a*



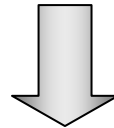
## Assay characteristics *Pj*HSP70a

~ Copy Number	500,000	500	50	5
Average readout (n =24)	~597,247	~439	~35	~2
Coefficient of Variation	19 %	24 %	67 %	59 %



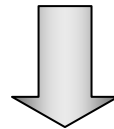
**Sample**

**Extract DNA**



**DNA**

Measure DNA by  
Real time PCR



**Result**

# Patients

Consecutive adult HIV-infected patients undergoing BAL for assessment of respiratory episodes (139 BAL from 132 patients)

n = 62 PCP defined by:

- Typical clinical/radiological presentation
- Grocott silver stain (+)
- Response to anti-*Pneumocystis* therapy

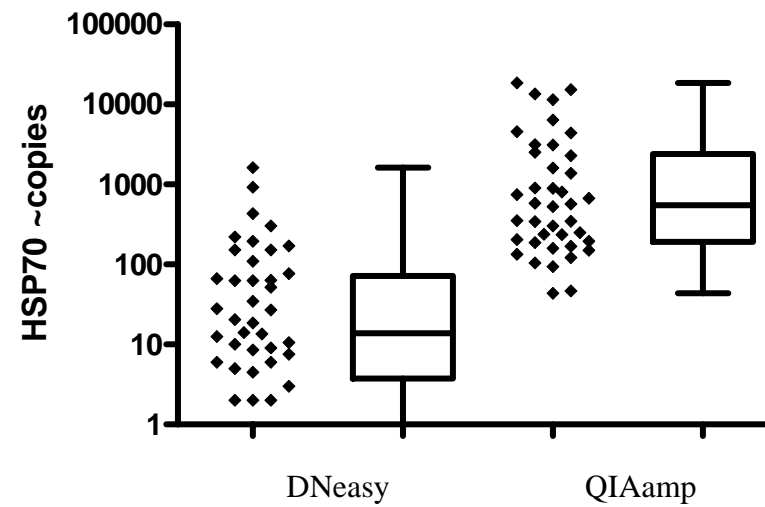
n = 75 Alternative diagnosis defined by:

- Atypical clinical/radiological presentation
- Grocott silver stain (-)
- No anti-*Pneumocystis* therapy given
- Confirmed alternative path/micro diagnosis

# DNA extraction

- **DNeasy tissue kit vs QIAamp UltraSens (Qiagen)**
- **Broncho alveolar lavage samples: 200  $\mu$ l vs 750  $\mu$ l**
- **UltraSens specially designed to extract nucleic acids from body fluid.**

## DNA extraction comparison PCP patients



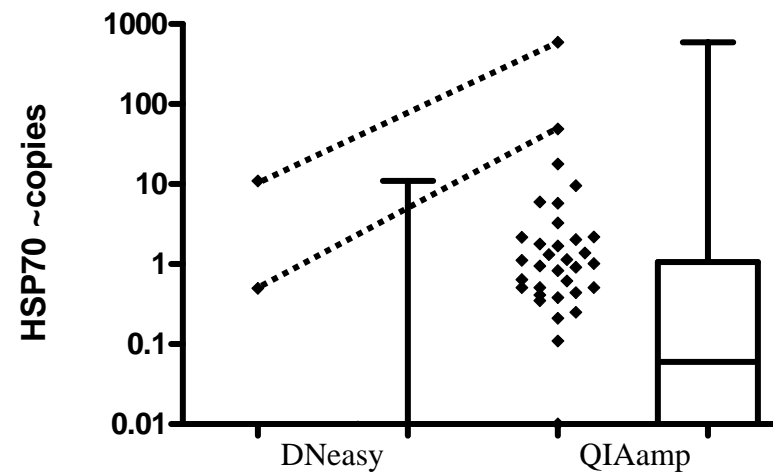
**RESPIRATORY INFECTIONS**

Asymptomatic carriage of *Pneumocystis jiroveci* in  
subjects undergoing bronchoscopy: a prospective study

N A Maskell, D J Waine, A Lindley, J C T Pepperell, A E Wakefield, R F Miller,  
R J O Davies

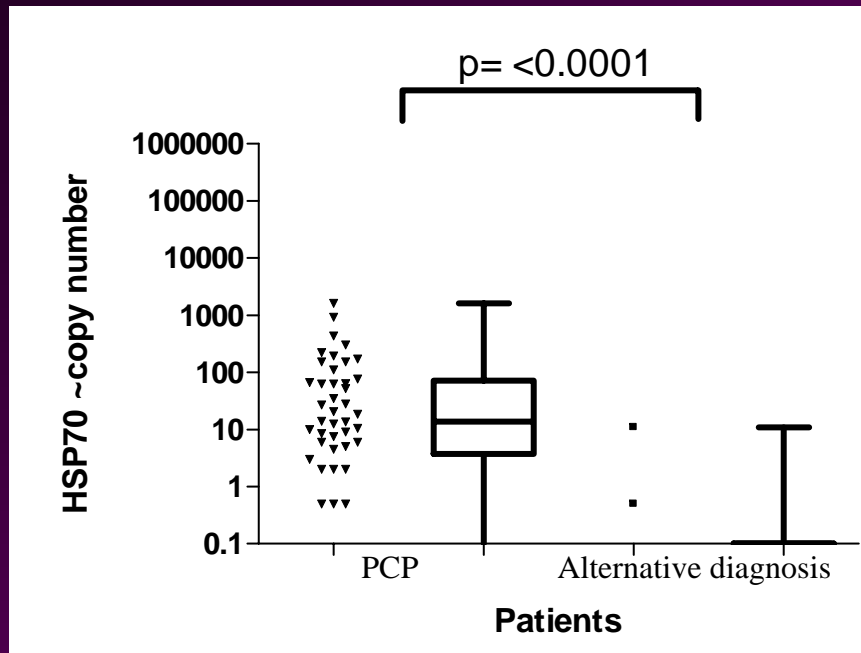
.....  
*Thorax* 2003;**58**:594–597

## DNA extraction comparison controls



# Implications for a molecular diagnostic test ?

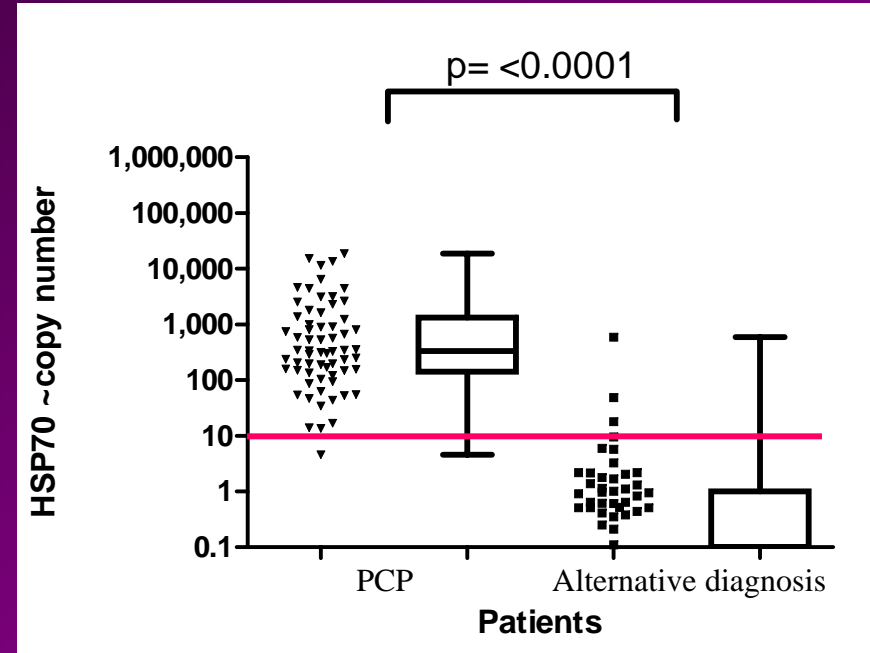
## DNeasy



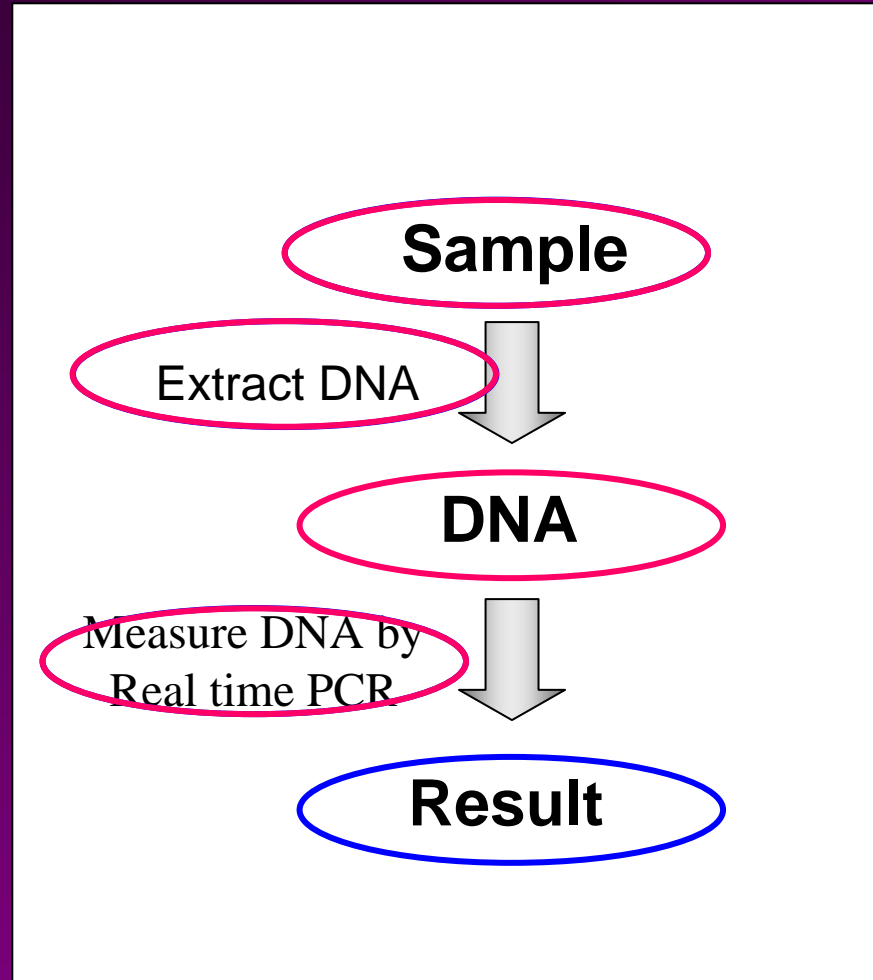
**Sensitivity = 93 %**  
**Specificity = 96.9 %**

**QIAamp H & E**  
**Sensitivity = 96.8 %**  
**Specificity = 68 %**

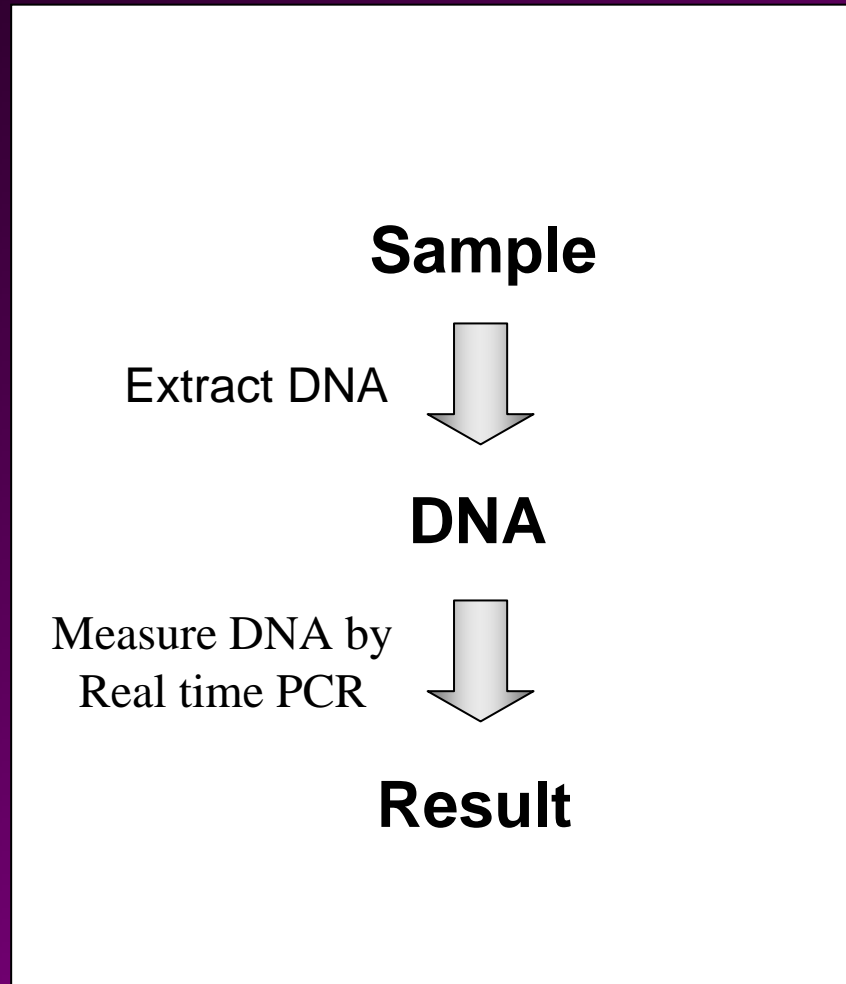
## QIAamp



**Sensitivity = 98.4 %**  
**Specificity = 95.9 %**  
**>10 copy cut-off**



**To assess a procedure's diagnostic efficacy its individual components must be considered individually**



**Prof. Richard Tedder:**  
**“you are starting to realise that these procedures have a beginning, a middle and an end”**

## **Last thought**

- **Molecular diagnostic research using real time PCR may never be applicable in a regional clinic in the developing world**
- **But real time PCR currently represents the most versatile and accurate quantitative molecular method we have.**
- **This type of research is essential as newer simpler molecular technologies are developed**

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