Infectious disease diagnostic research in the developing world; the role of real time PCR.

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- **Background to The Center for Infectious Diseases**
- **Molecular diagnosis of infectious disease**
- **Some examples**

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Research at the Center for infectious diseases

- Clinical trials (Drugs, Vaccine, Diagnostic tests)
- Epidemiological studies
- Basic science research

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International Collaborator Location



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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)

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- 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**

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

*Chifumbe Chintu, Victor Mudenda, Sebastian Lucas, Andrew Nunn, Kennedy Lishimpi, Daniel Maswahu, Francis Kasolo, Peter Mwaba, Ganapati Bhat, Hiroshi Terunuma, Alimuddin Zumla, for the UNZA-UCLMS Project Paediatric Post-mortem Study Group**

Summary

Background Accurate information about specific causes of death in African children dying of respiratory illnesses is scarce, and can only be obtained by autopsy. We undertook a study of children who died from respiratory diseases at University Teaching Hospital, Lusaka, Zambia.

Methods 137 boys (93 HIV-1-positive, 44 HIV-1-negative), and 127 girls (87 HIV-1-positive, 40 HIV-1-negative) aged

Interpretation Most children dying from respiratory diseases have preventable or treatable infectious illnesses. The presence of multiple diseases might make diagnosis difficult. WHO recommendations should therefore be updated with mention of HIV-1-positive children. Improved diagnostic tests for bacterial pathogens, tuberculosis, and *P carinii* pneumonia are urgently needed.

Lancet 2002; **360:** 985–90. Published online September 3, 2002
http://image.thelancet.com/extras/01art12073web.pdf

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	Total*
Diagnosis	
Acute pyogenic pneumonia	116 (44%)
PCP	58 (22%)
Tuberculosis	54 (20%)
CMV	43 (16%)
Interstitial pneumonitis	30 (11%)
Shock lung	27 (10%)
Pulmonary oedema	19 (7%)
Lymphocytic interstitial pneumonitis	10 (4%)

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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

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Top ten biotechnologies for improving health in developing countries

Abdallah S. Daar[1–4], Halla Thorsteinsdóttir[1,2], Douglas K. Martin[2,5,6], Alyna C. Smith[1,2], Shauna Nast[1,2] & Peter A. Singer[2,4,7]

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.

/www.nature.com/naturegenetics

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

Final ranking	Biotechnology	Final score
1	Modified molecular technologies for affordable, simple diagnosis of infectious diseases	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

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Diagnosis

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

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A diagnostic test can:

- Detect a host marker
- Detect the pathogen

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The Journal of Immunology

Healthy Individuals That Control a Latent Infection with *Mycobacterium tuberculosis* Express High Levels of Th1 Cytokines and the IL-4 Antagonist IL-4δ2[1]

Abebech Demissie,* Markos Abebe,* Abraham Aseffa,* Graham Rook,‡ Helen Fletcher,‡ Alimuddin Zumla,‡ Karin Weldingh,† Inger Brock,† Peter Andersen,† T. Mark Doherty,[2†] and the VACSEL Study Group[§]

The majority of healthy individuals exposed to *Mycobacterium tuberculosis* will not develop disease and identifying what constitutes "protective immunity" is one of the holy grails of *M. tuberculosis* immunology. It is known that IFN-γ is essential for protection, but it is also apparent that IFN-γ levels alone do not explain the immunity/susceptibility dichotomy. The controversy regarding correlates of immunity persists because identifying infected but healthy individuals (those who are immune) has been problematic. We have therefore used recognition of the *M. tuberculosis* virulence factor early secretory antigenic target 6 to identify healthy, but infected individuals from tuberculosis (TB)-endemic and nonendemic regions (Ethiopia and Denmark) and have compared signals for cytokines expressed directly ex vivo with the pattern found in TB patients. We find that TB patients are characterized by decreased levels of Th1 cytokines and increased levels of IL-10 compared with the healthy infected and noninfected community controls. Interestingly, the healthy infected subjects exhibited a selective increase of message for the IL-4 antagonist, IL-4δ2, compared with both TB patients or noninfected individuals. These data suggest that long-term control of *M. tuberculosis* infection is associated not just with elevated Th1 responses but also with inhibition of the Th2 response. *The Journal of Immunology,* 2004, 172: 6938–6943.

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Notes & Tips

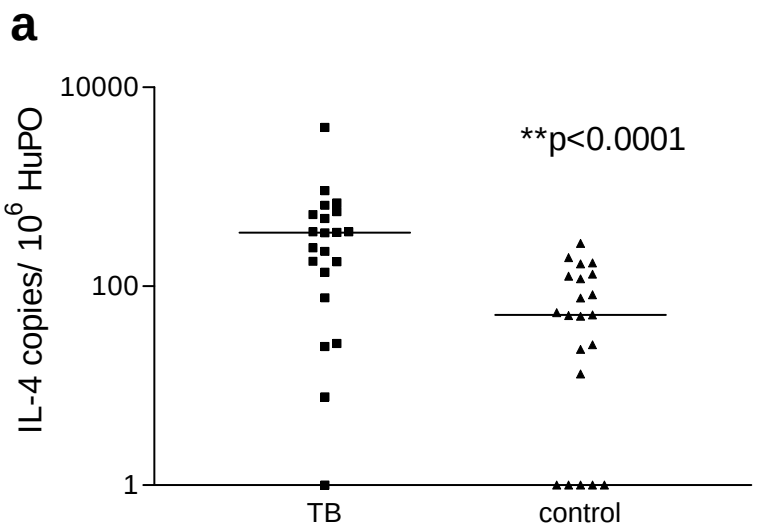
The implications of using an inappropriate reference gene for real-time reverse transcription PCR data normalization

K. Dheda [a,b], J.F. Huggett [a,*], J.S. Chang [a], L.U. Kim [a], S.A. Bustin [c], M.A. Johnson [b], G.A.W. Rook [a], A. Zumla [a]

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[b] *Department of Thoracic and HIV Medicine, Royal Free Hospital, London NW3 2QG, UK*
[c] *Centre for Academic Surgery, Barts and the London, Queen Mary's School of Medicine and Dentistry, London E1 2AD, UK*

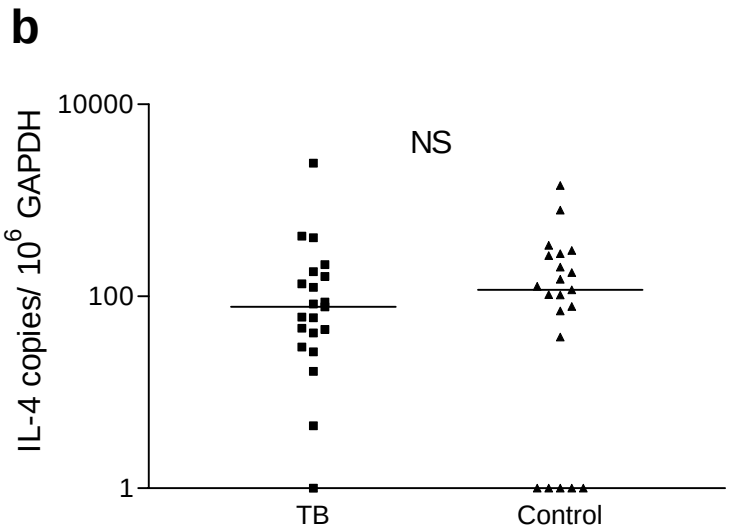
Received 3 March 2005
Available online 8 June 2005

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Normalised to 10^6 copies HuPO

Normalised to 10^6 copies GAPDH



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A diagnostic test can:

- Detect a host marker
- **Detect the pathogen**

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Detect the pathogen

- **Molecular diagnostic tests for many diseases are not trusted by the medical profession**
- **Often used to confirm an empirical opinion**
- **Specialist requirement (drug resistance)**
- **Real time RT-PCR can be the gold standard (HIV, HCV)**

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Detect the pathogen

- **Often assays are poorly:**
  - **designed**
  - **optimised**
  - **standardised**

All PCR assays are NOT the same

- **Confounded by the view that PCR is a single entity**

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Reliability of Nucleic Acid Amplification for Detection of *Mycobacterium tuberculosis*: an International Collaborative Quality Control Study among 30 Laboratories

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Nucleic acid amplification to detect *Mycobacterium tuberculosis* in clinical specimens is increasingly used as a laboratory tool for the diagnosis of tuberculosis. However, the specificity and sensitivity of these tests may be questioned, and no standardized reagents for quality control assessment are available. To estimate the performance of amplification tests for routine diagnosis, we initiated an interlaboratory study involving 30 laboratories in 18 countries. We prepared blinded panels of 20 sputum samples containing no, 100, or 1,000 mycobacterial cells. Each laboratory was asked to detect *M. tuberculosis* by their routine method of nucleic acid amplification. Only five laboratories correctly identified the presence or absence of mycobacterial DNA in all 20 samples. Seven laboratories detected mycobacterial DNA in all positive samples, and 15 laboratories correctly reported the absence of DNA in the negative samples. Lack of specificity was more of a problem than lack of sensitivity. Reliability was not found to be associated with the use of any particular method. Reliable detection of *M. tuberculosis* in clinical samples by nucleic acid amplification techniques is possible, but many laboratories do not use adequate quality controls. This study underlines the need for good laboratory practice and reference reagents to monitor the performance of the whole assay, including pretreatment of clinical samples.

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Example 1

- **Design**

Pneumocystis Pneumonia (PCP)

Caused by an opportunistic fungal pathogen (*Pneumocystis jiroveci*) that causes pneumonia in immunocompromised individuals.

Current diagnosis by microscopy using BAL sample

Molecular test could significantly improve diagnosis

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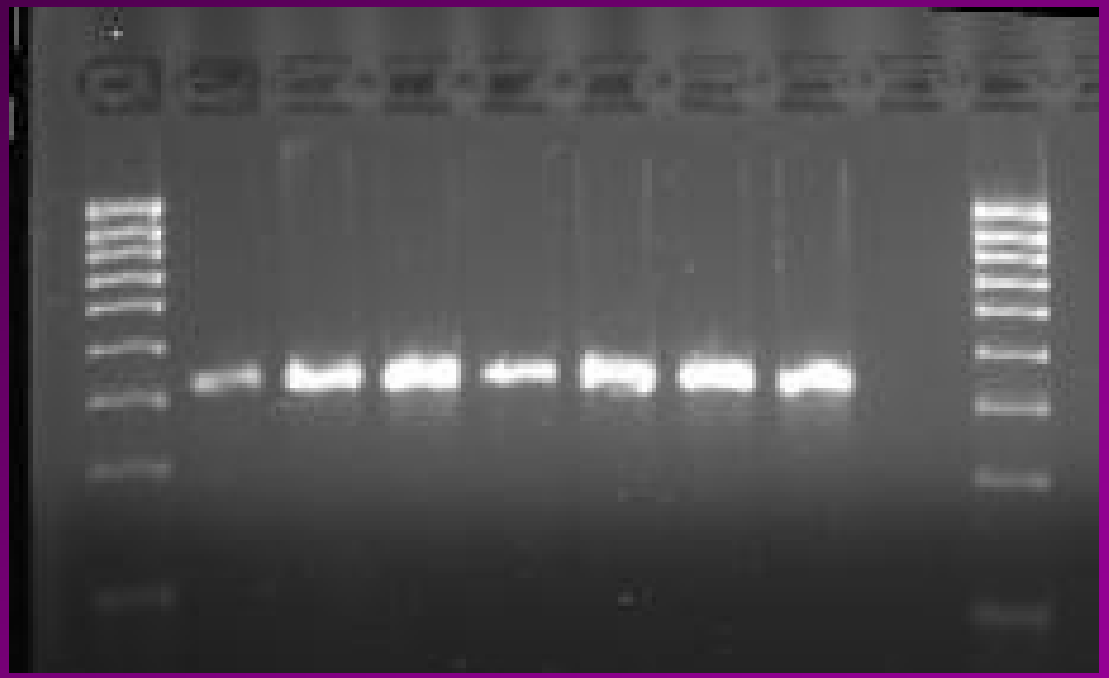
Molecular diagnosis of PCP

Detection of *Pneumocystis carinii* with DNA amplification

The Lancet
Volume 336, Issue 8713
1990

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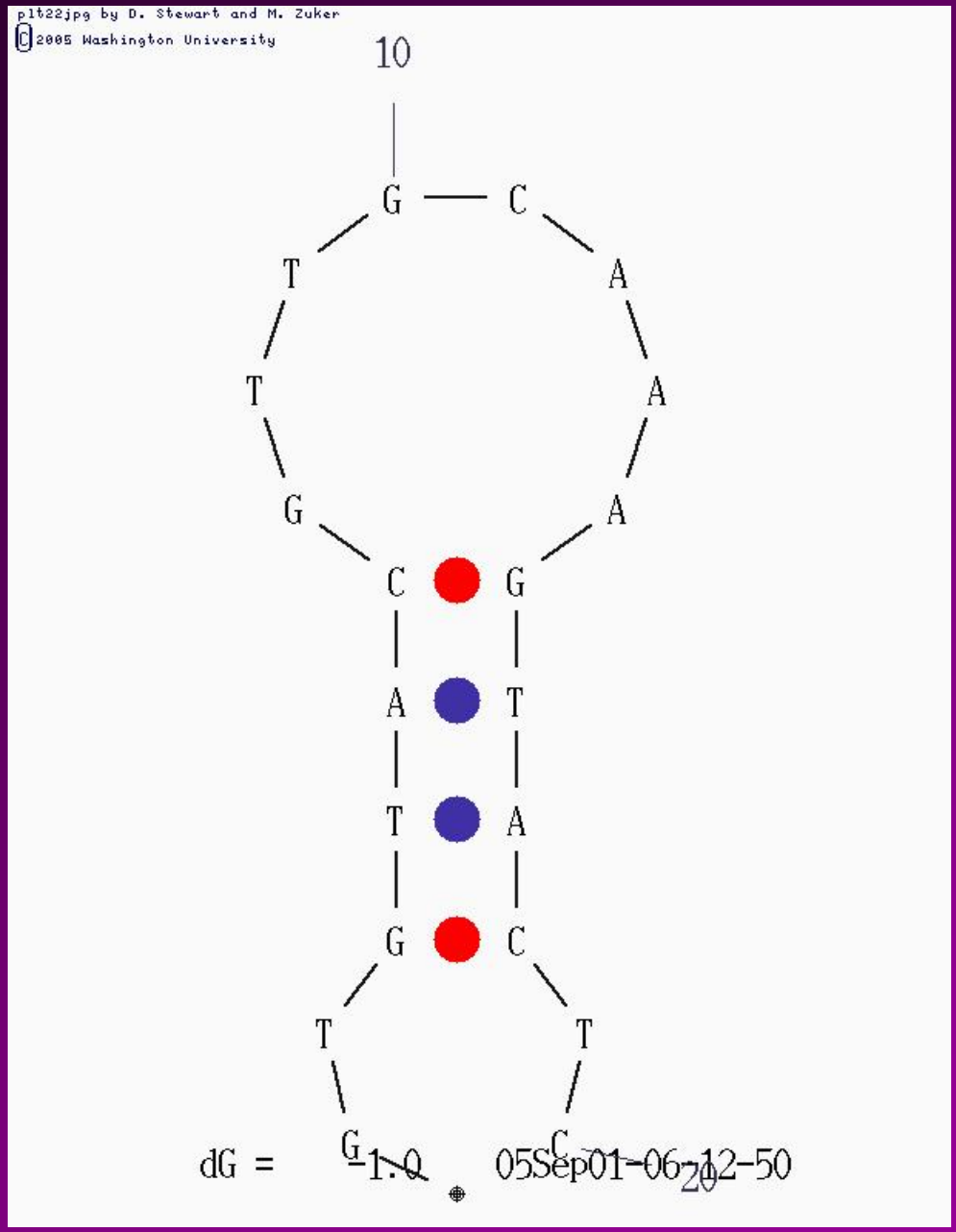
PCR designed to amplify part of the Large subunit of the mitochondrial rRNA gene



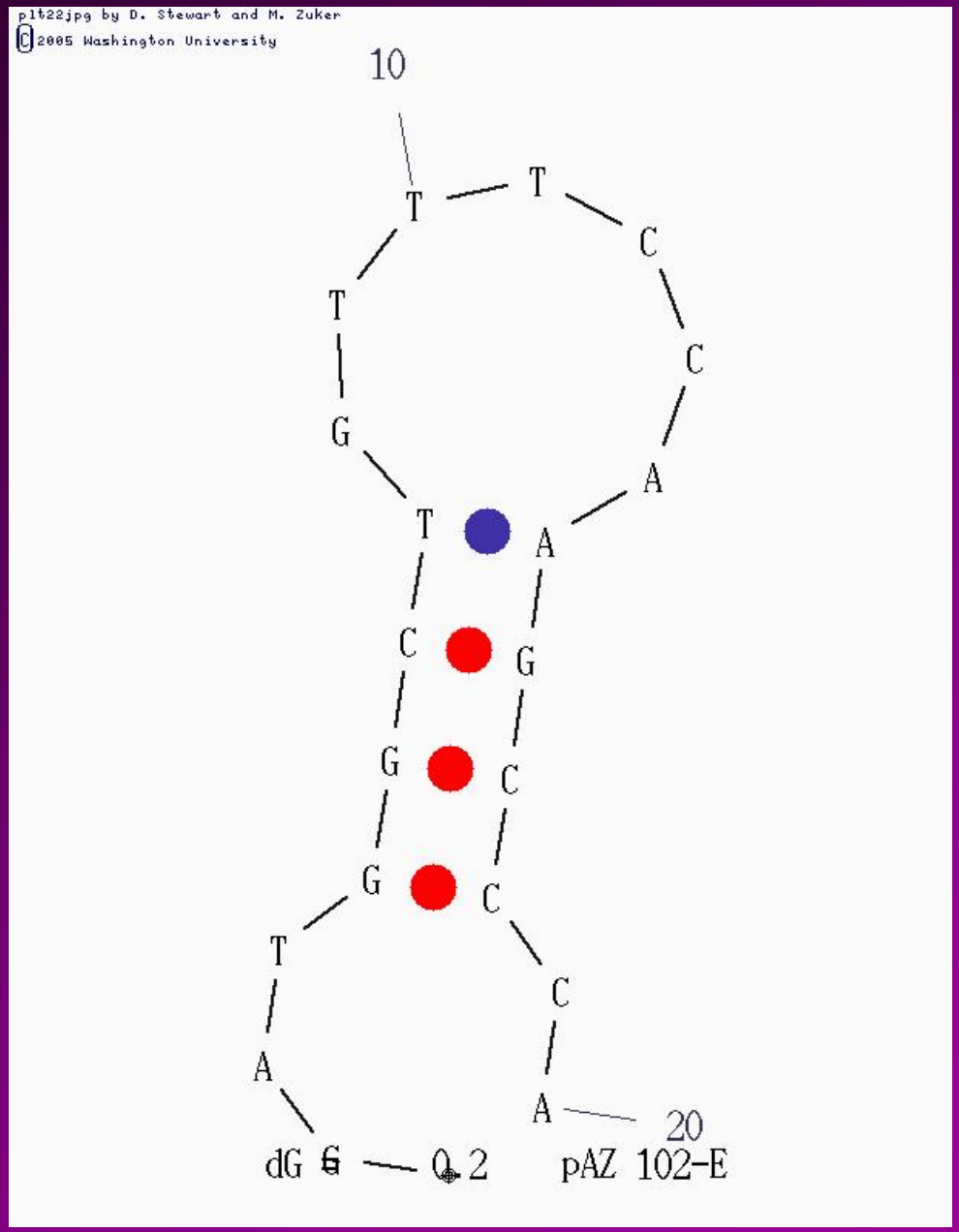
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TM difference 5-15.3 °C

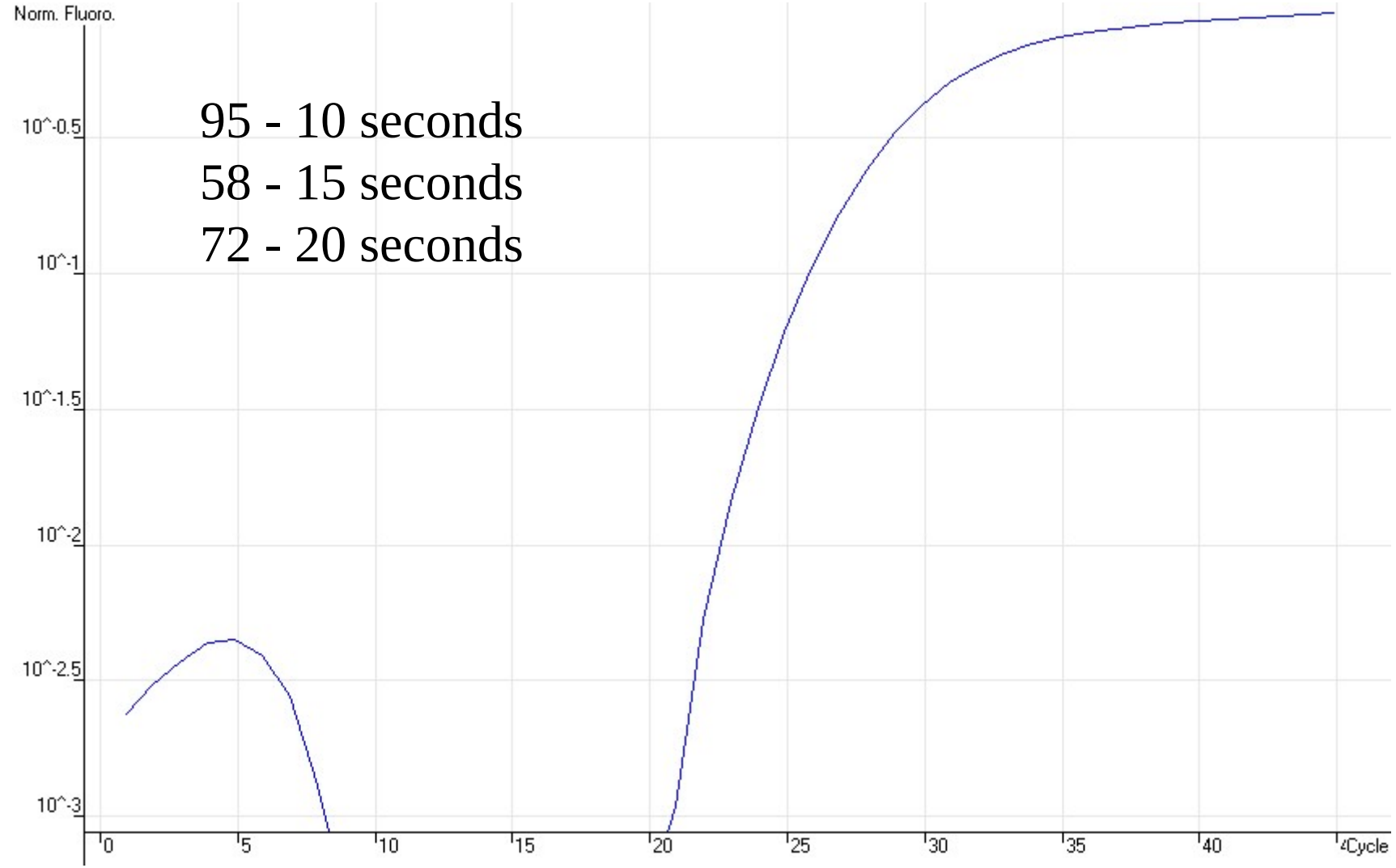
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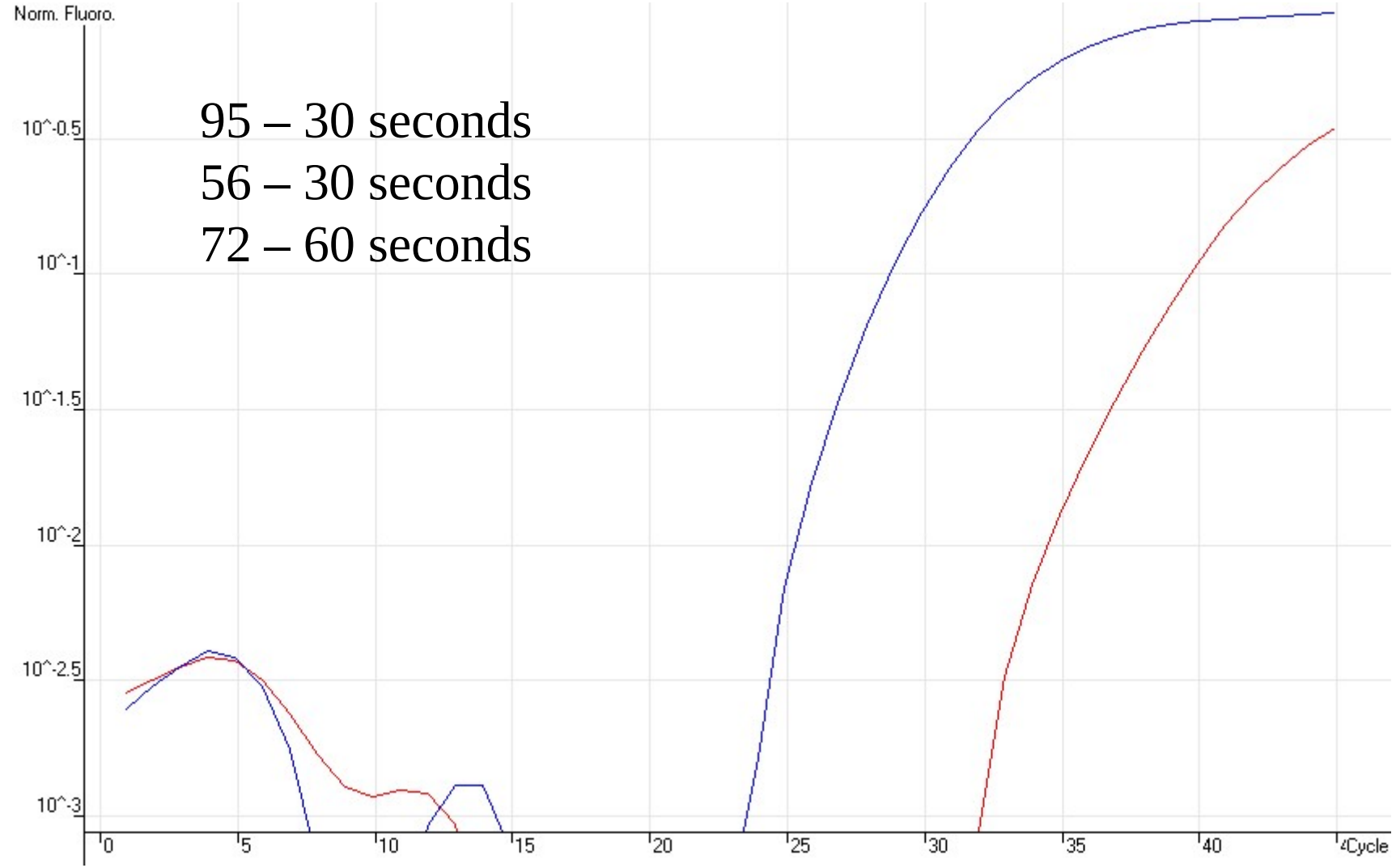
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New primers

1990

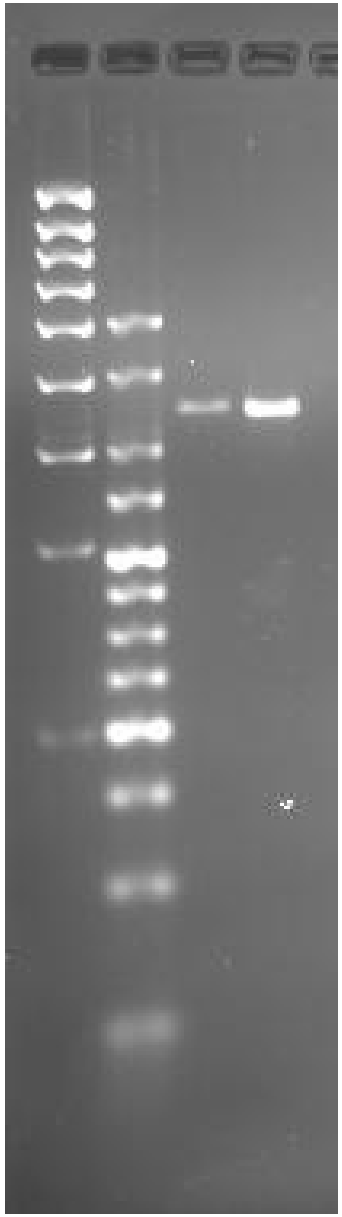
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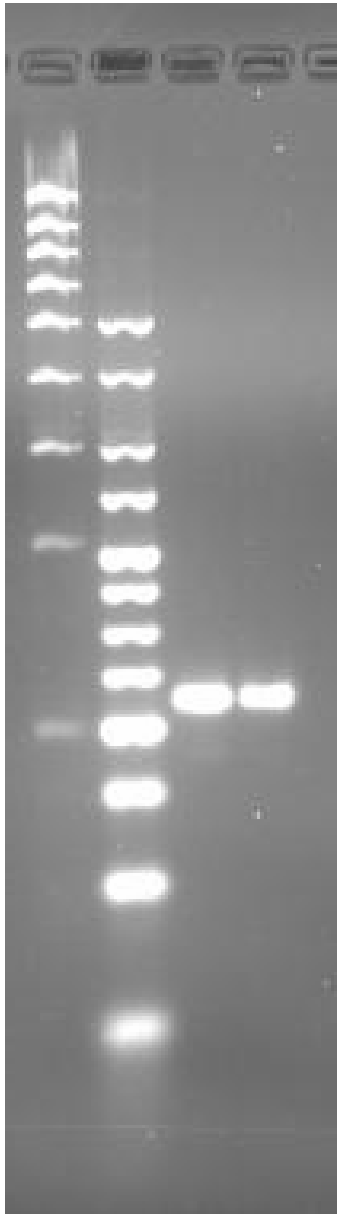
New primers

1990

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1990



New primers

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

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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.

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

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Example 2

- PCR as a single entity

Tuberculosis

~ 1/3 of the worlds population infected

~ 8 million new active cases a year

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TB diagnosis

X-Ray (sensitive, not very specific)

Microscopy (specific, not very sensitive)

Culture (sensitive, specific but can take 6 weeks)

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PERGAMON

The International Journal of Biochemistry & Cell Biology 35 (2003) 1407–1412

IJBCB

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Medicine in focus

Tuberculosis: amplification-based clinical diagnostic techniques

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Abstract

Tuberculosis (TB) is one of the major infectious causes of morbidity and mortality worldwide. TB is difficult to control due to the time taken for the microbiological diagnosis; typically culture on solid media takes 6–8 weeks. There are number of rapid molecular methods that have been developed to diagnose new cases of tuberculosis, detect drug resistance and identify the type of mycobacteria. These assays are based on recognition of mycobacterial DNA sequences and the subsequent amplification of nucleic acid sequences to facilitate detection. This review will describe some of the molecular assays that are in use for TB diagnosis and the considerations in designing and performing such assays. Early diagnosis of tuberculosis is critical for the successful management of patients allowing informed use of chemotherapy ensuring that the right patients are treated with the right antimicrobials.

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Keywords: Tuberculosis; Molecular diagnosis; PCR

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Urine as a diagnostic sample for Tuberculosis

Aceti,A., Zanetti,S., Mura,M.S., Sechi,L.A., Turrini,F., Saba,F., Babudieri,S., Mannu,F. and Fadda,G. (1999) Identification of HIV patients with active pulmonary tuberculosis using urine based polymerase chain reaction assay. *Thorax*, **54, 145-146.**

Kafwabulula,M., Ahmed,K., Nagatake,T., Gotoh,J., Mitarai,S., Oizumi,K. and Zumla,A. (2002) Evaluation of PCR-based methods for the diagnosis of tuberculosis by identification of mycobacterial DNA in urine samples. *Int.J.Tuberc.Lung Dis.*, **6, 732-737.**

Sechi,L.A., Pinna,M.P., Sanna,A., Pirina,P., Ginesu,F., Saba,F., Aceti,A., Turrini,F., Zanetti,S. and Fadda,G. (1997) Detection of *Mycobacterium tuberculosis* by PCR analysis of urine and other clinical samples from AIDS and non-HIV-infected patients. *Mol.Cell Probes*, **11, 281-285.**

Torrea,G., Van de,P.P., Ouedraogo,M., Zougba,A., Sawadogo,A., Dingtoumda,B., Diallo,B., Defer,M.C., Sombie,I., Zanetti,S. *et al.* (2005) PCR-based detection of the *Mycobacterium tuberculosis* complex in urine of HIV-infected and uninfected pulmonary and extrapulmonary tuberculosis patients in Burkina Faso. *J.Med Microbiol.*, **54, 39-44.**

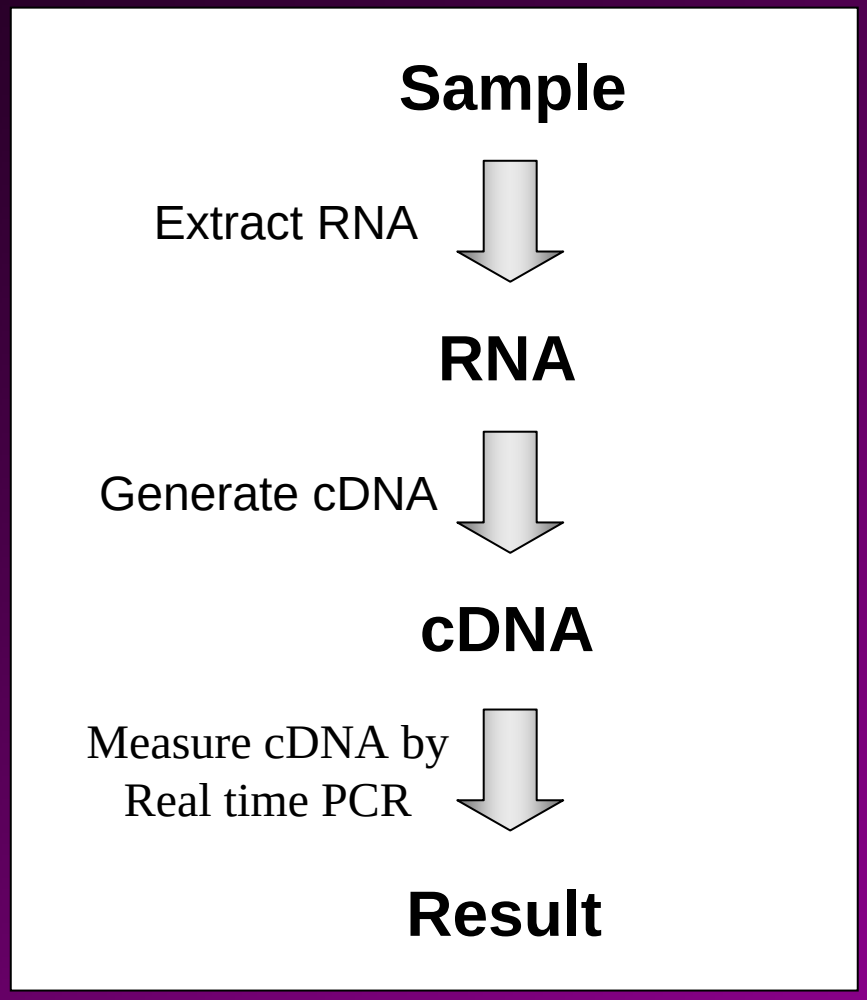
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sensitivity ranges from 27-100%,

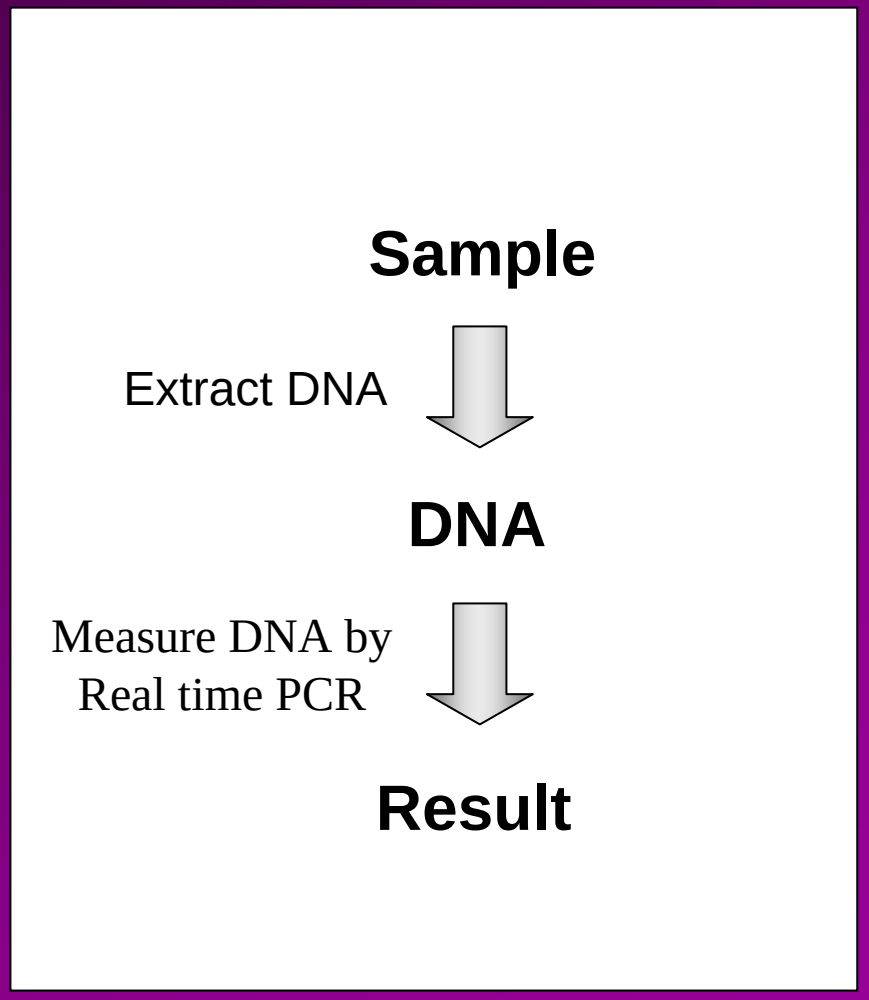
specificity was ~98%

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real time RT-PCR

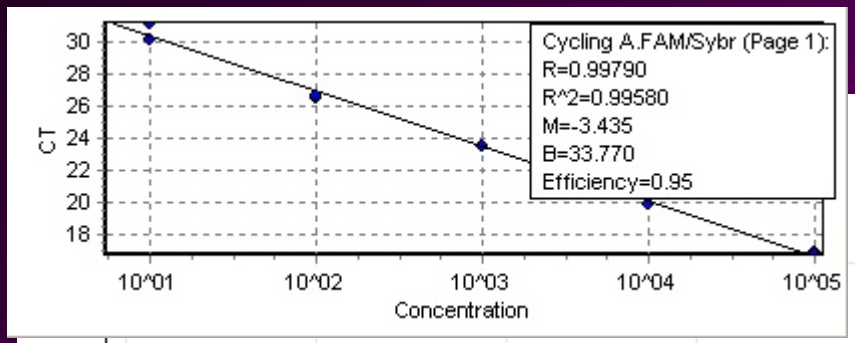


real time PCR



Huggett, J., K. Dheda, S. Bustin, and A. Zumla. 2005. Real-time RT-PCR normalisation; strategies and considerations. Genes Immun 6:279-284.

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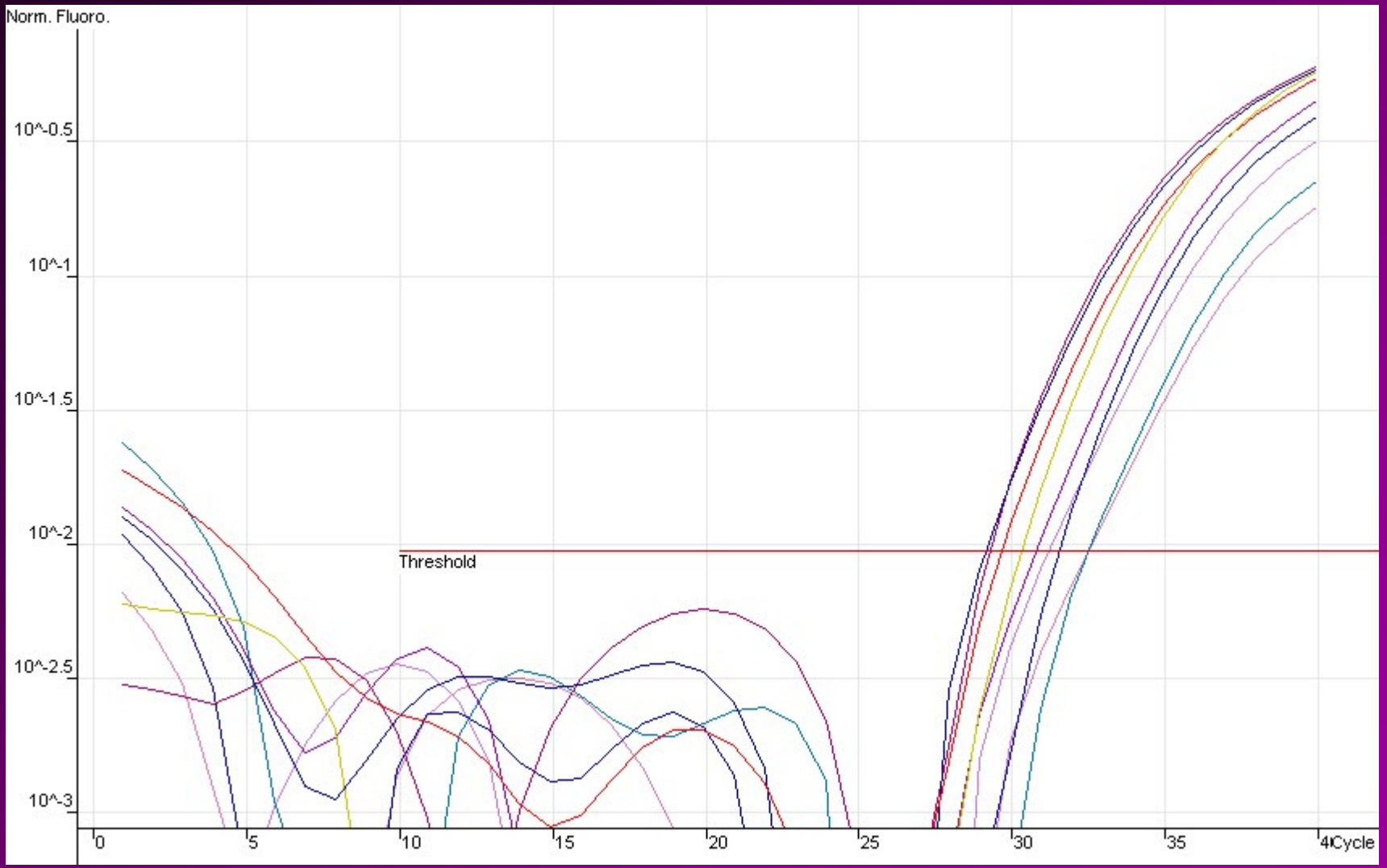


Mycobacterium specific PCR

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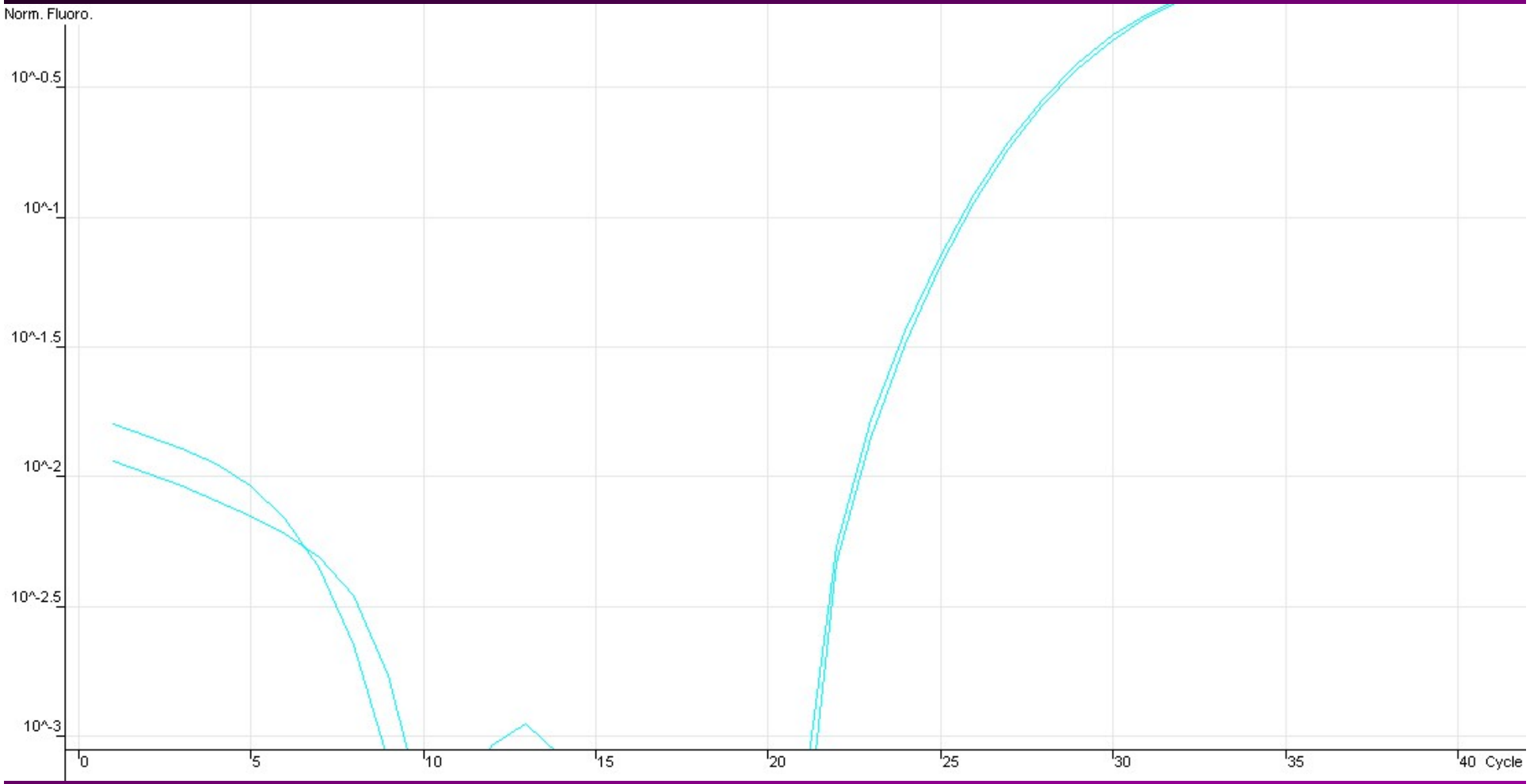
- **20 urine samples (some from TB patients)**
- **DNA extracted from 750 ul using Qiamp Ultrasens (Qiagen)**

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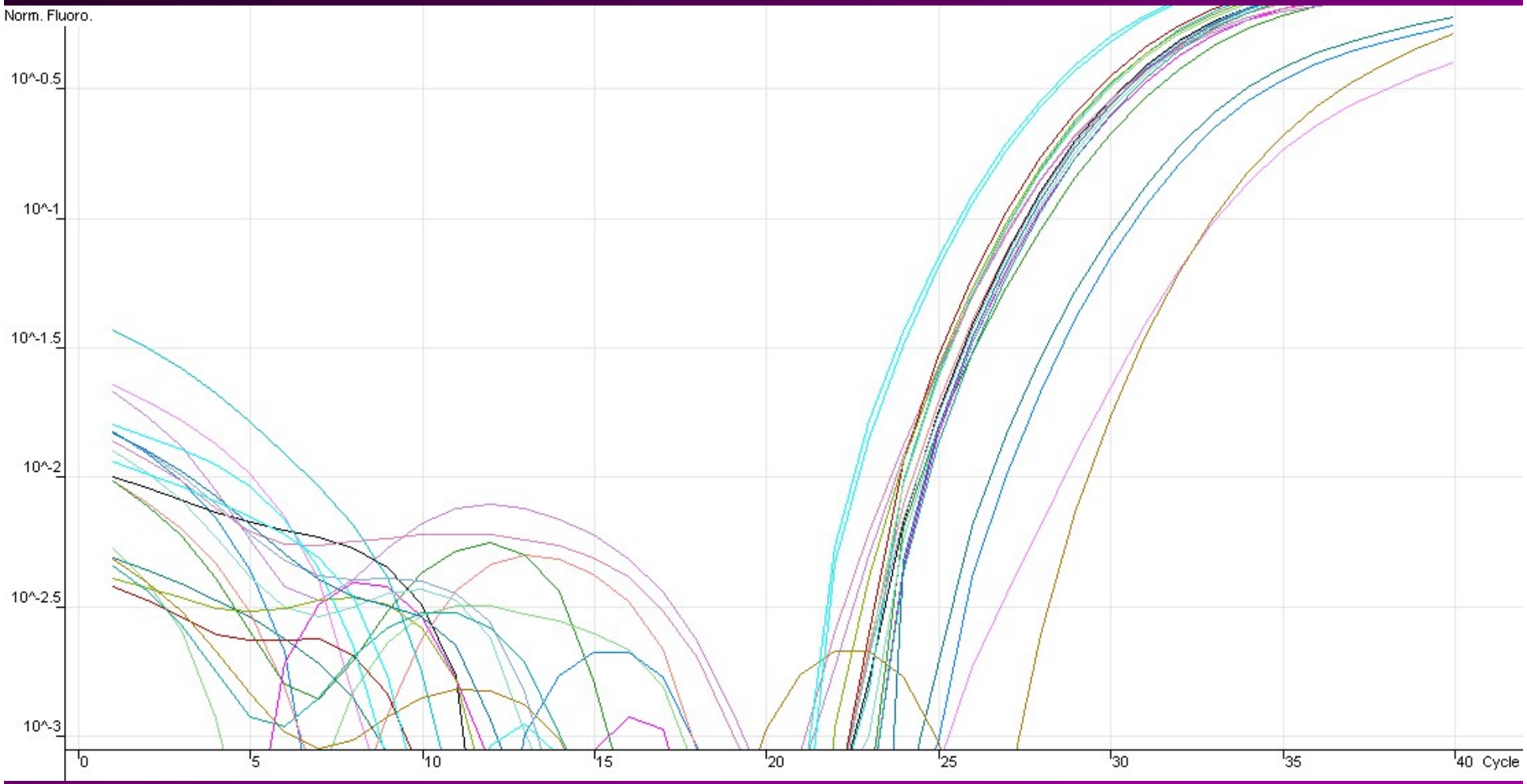
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Spike

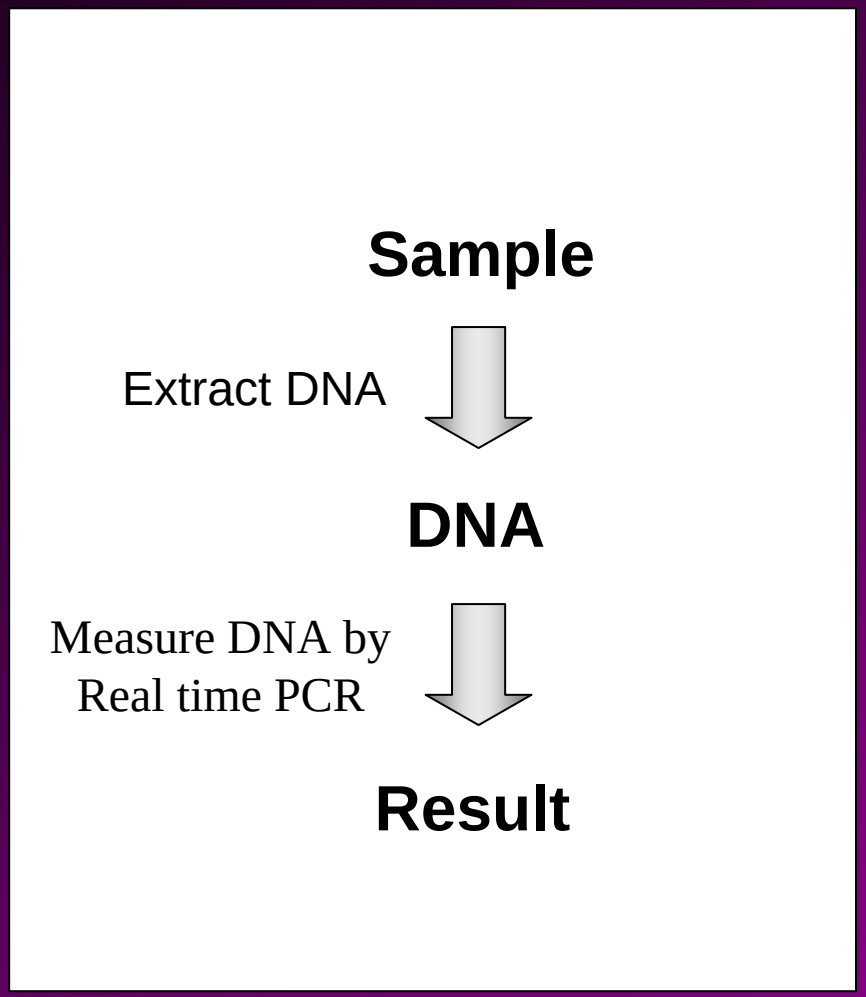


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Spike



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Richard Tedder:
"you are starting to realise that these procedures have a beginning, a middle and an end"

To assess a procedures diagnostic efficacy its individual components must be considered individually

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Final thought

Role of real time PCR in infectious disease diagnosis in the developing world

Research letters

Whole blood versus plasma spots for measurement of HIV-1 viral load in HIV-infected African patients

Peter Mwaba, Sharon Cassol, Andrew Nunn, Rick Pilon, Chifumbe Chintu, Michelle Janes, Alimuddin Zumla

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Voahangy Rasolofo

•Sigma-Genosys
Natalie Simpson
Tania Nolan

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Thank you



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