Current issues in *Aspergillus* quantitative real time PCR (RTqPCR) standardization

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IAAM
INVASIVE ASPERGILLOSIS ANIMAL MODELS

S ◆ A ◆ C ◆ M ◆ M
San Antonio Center for Medical Mycology

Background

- Ubiquitous nature makes exposure difficult to avoid.
- IA life- threatening nature makes accurate diagnosis and early detection crucial.
- Current methods need improved sensitivity
- RTqPCR offers a rapid and sensitive method that needs to be calibrated in order to be tested between institutions.
- Lack of validated RTqPCR data remains a limiting factor for standardization and calibration between laboratories

RTqPCR Assay Variables

Instrumentation
- Various instruments different thresholds settings.
- Need to standardize settings between laboratories.

Reagents
- Taq polymerases, buffers probes, primers

Target
- multi copy rDNA
- FKS1 (1 copy)
- Conservation (primer design)
- amplification efficiency

Template preparation
- specimen type
- tissue vs. fluid
- DNA isolation methodology
  - yield
  - purity

Cycling parameters
- Ct values for positive samples should be validated by a standard curve. Limiting threshold should be established.

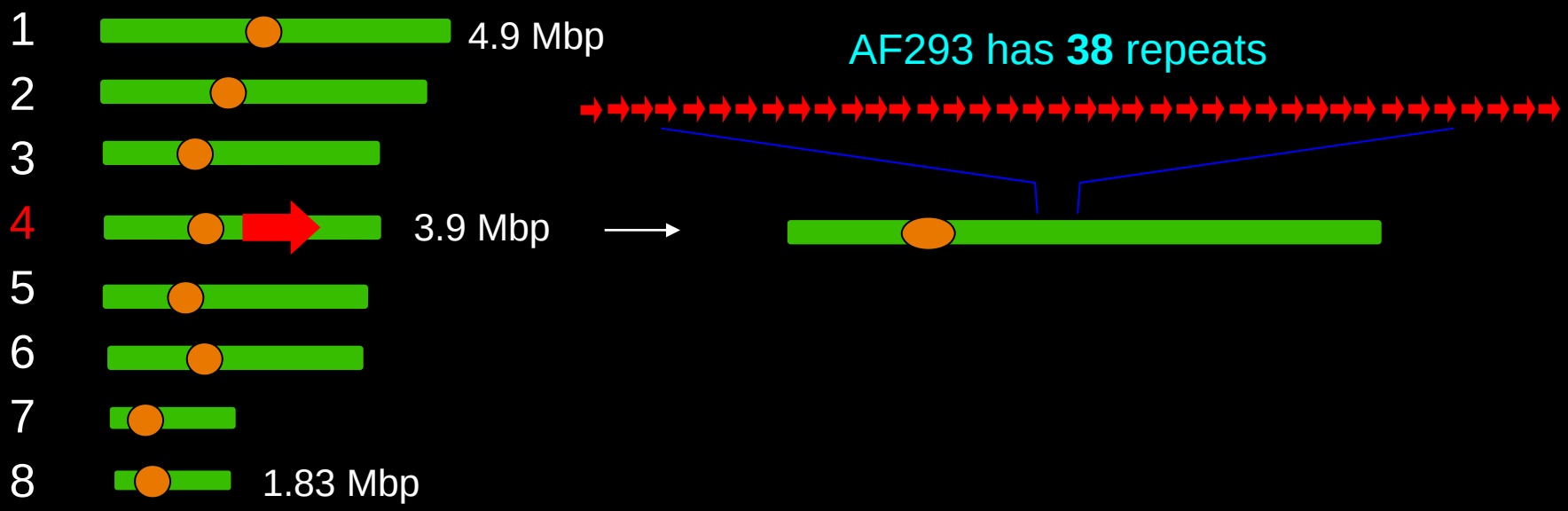
Data interpretation
- Allowable limit of detection Ct

#of replicates to detect interference effect with 95% confidence power

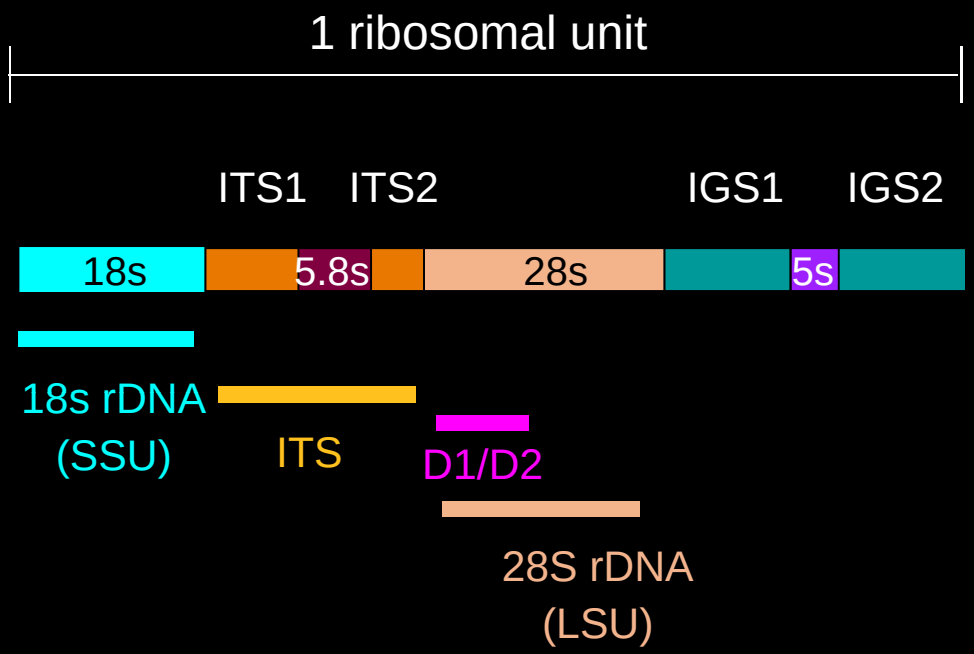
RTqPCR Targets: Multicopy

rDNA Organization of the *Aspergillus fumigatus* Genome

29.4 Megabases
9,926 ORFs



Significance of rDNA in Fungal Detection and Identification



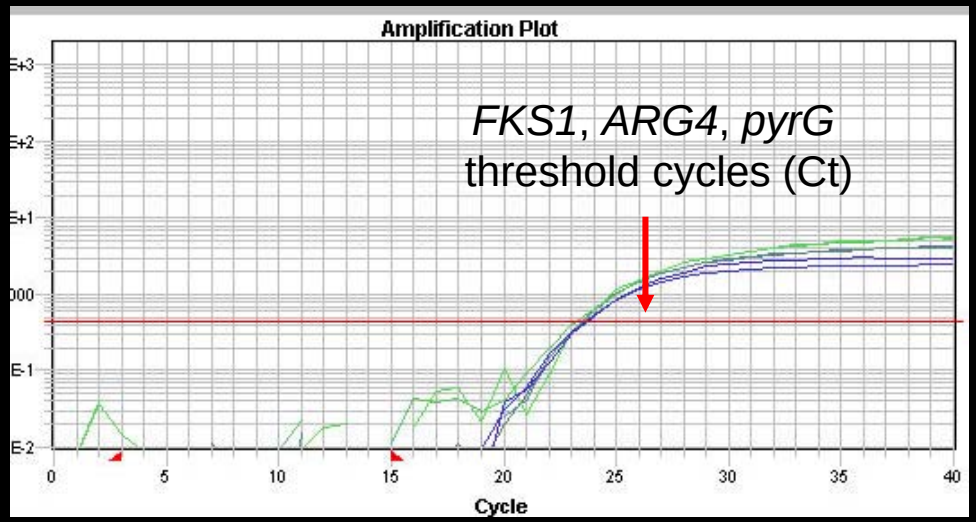
1) rDNA subunits are highly <u>conserved</u>

- ✓ *ITS, and D1/D2 regions are species-specific – variable region*
- ✓ *Allows universal PCR primer or probe sites in 18s and 28s regions*

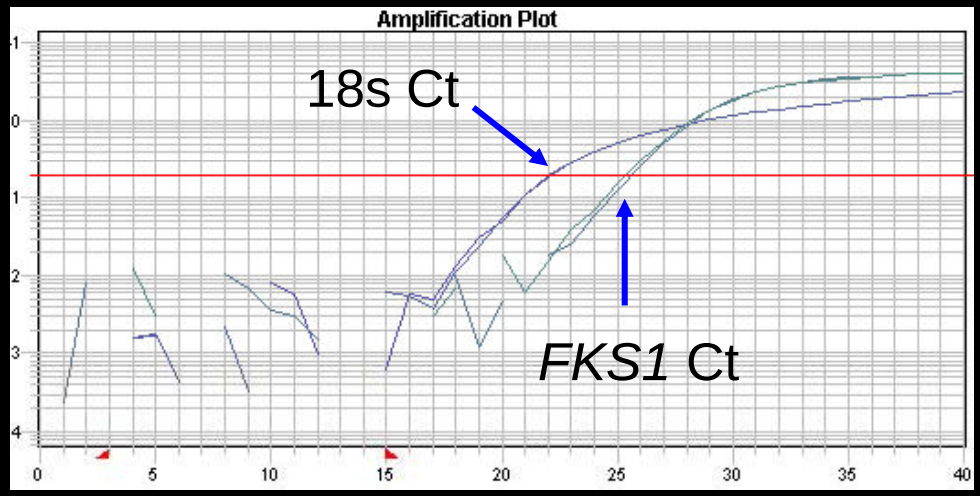
2) <u>Multicopy</u> nature enhances PCR sensitivity 38-100x

RTqPCR Targets: Single copy vs. multiple copies

Af293
FKS1 vs. *ARG4* and *pyrG*



Af293
18s rDNA vs *FKS1*



A. fumigatus multicopy rDNA Genes Vary in Copy Number

STRAIN	18s rDNA copy #
Af293	38 +/- 0.01
WSA-450	42 +/- 0.07
WSA-172	46 +/- 0.03
WSA-446	47 +/- 0.01
WSA-445	49 +/- 0.06
WSA-271	49 +/- 0.05
WSA-270	53 +/- 0.01
WSA-621	70 +/- 0.03
WSA-419	91 +/- 0.03

Range: 38-91, Avg: 54

Af293 genome=35, QRT-PCR=38

Aspergillus DNA isolation/ quantitation for standards construction

Culture for 5-7days

↓

24hr broth culture

↓

Harvest, grind frozen cells

↓

Lysis buffer, proteinase K

↓

Neutralize, precipitate

Rnase

↓

Extract

↓

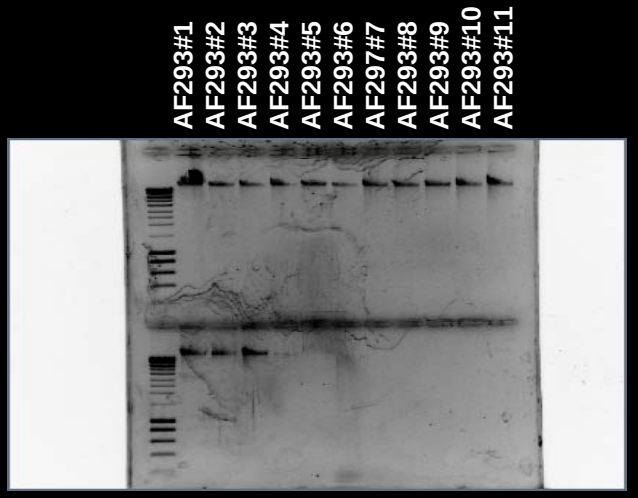
Precipitate

↓

Gel purification

↓

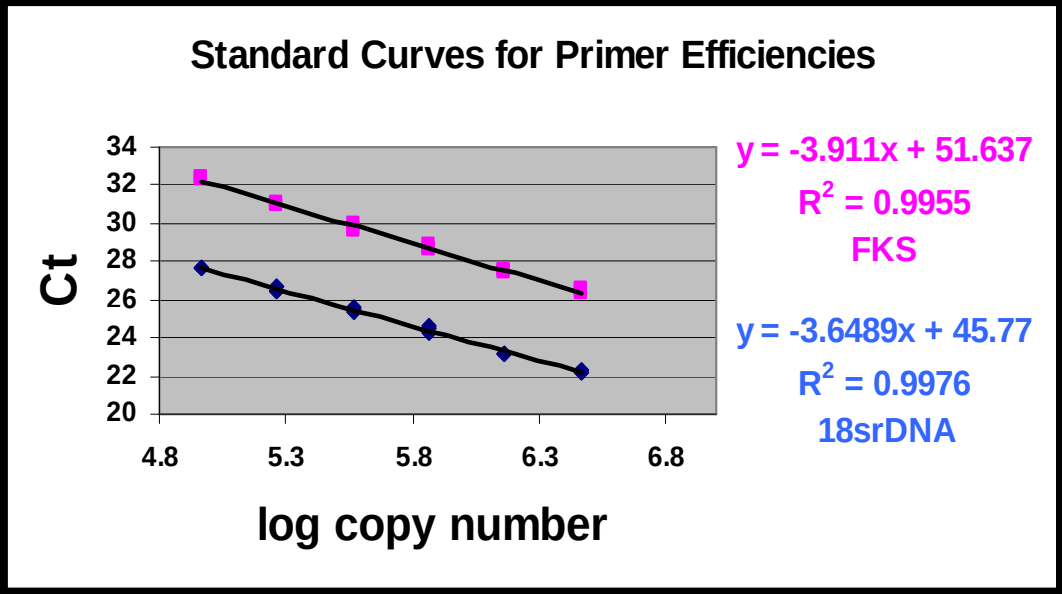
Quantified by gel



A. Fumigatus DNA quantification gel vs. nanodrop

DNA Concentration by gel quantification		
Sample #	gel ng/ul	nanodrop ng/ul
AF293#1	9.2	447
AF293#2	9.8	656
AF293#3	10.8	422
AF293#4	8.2	364
AF293#5	10	1723
AF293#6	12	2861
AF293#7	11.2	1496
	AVG 50.86	AVG 1138

Primer Efficiencies for FKS1 and 18srDNA genes

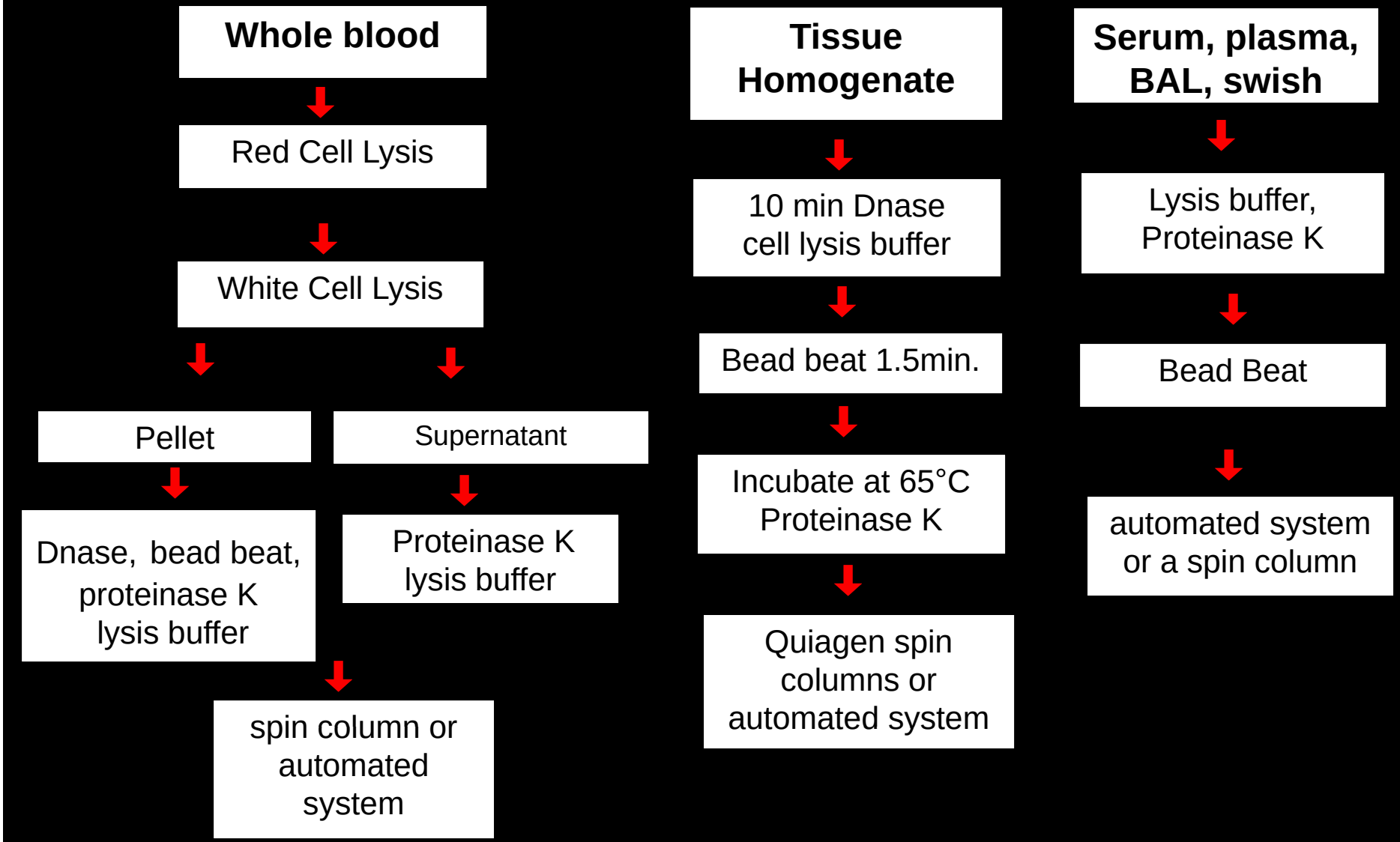


Primer Efficiencies

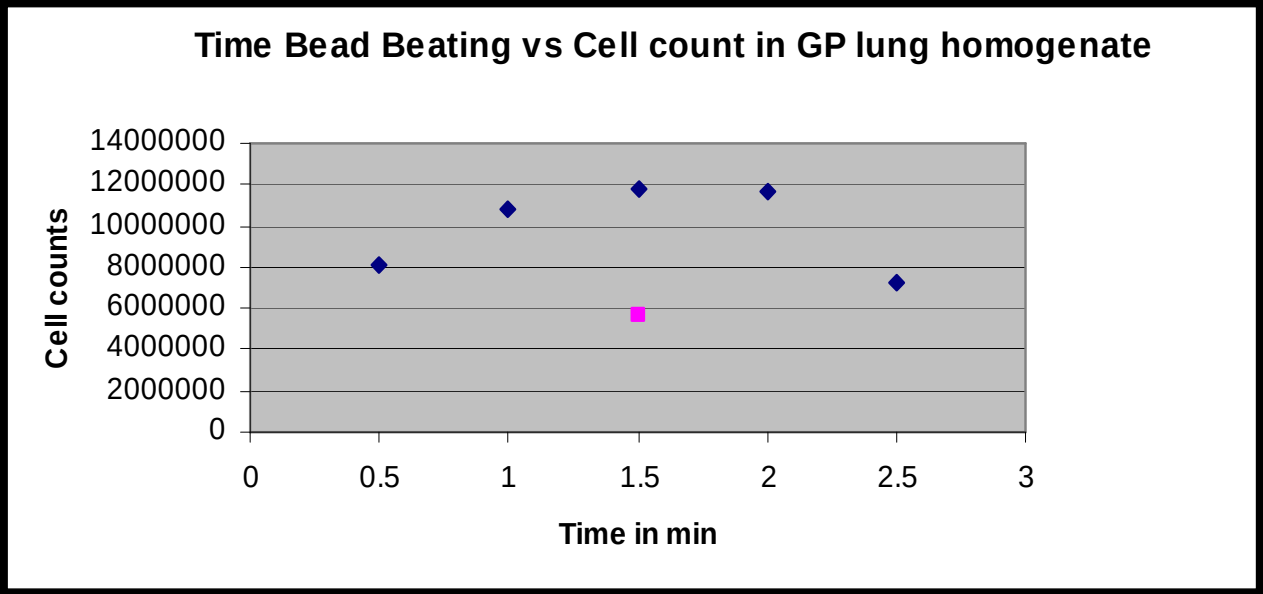
FKS1	18srDNA
0.87955	0.85172

Primer efficiencies are important because they are a measure of the sensitivity and efficiency of our assay.

Aspergillus DNA isolation from Biological fluids, tissue

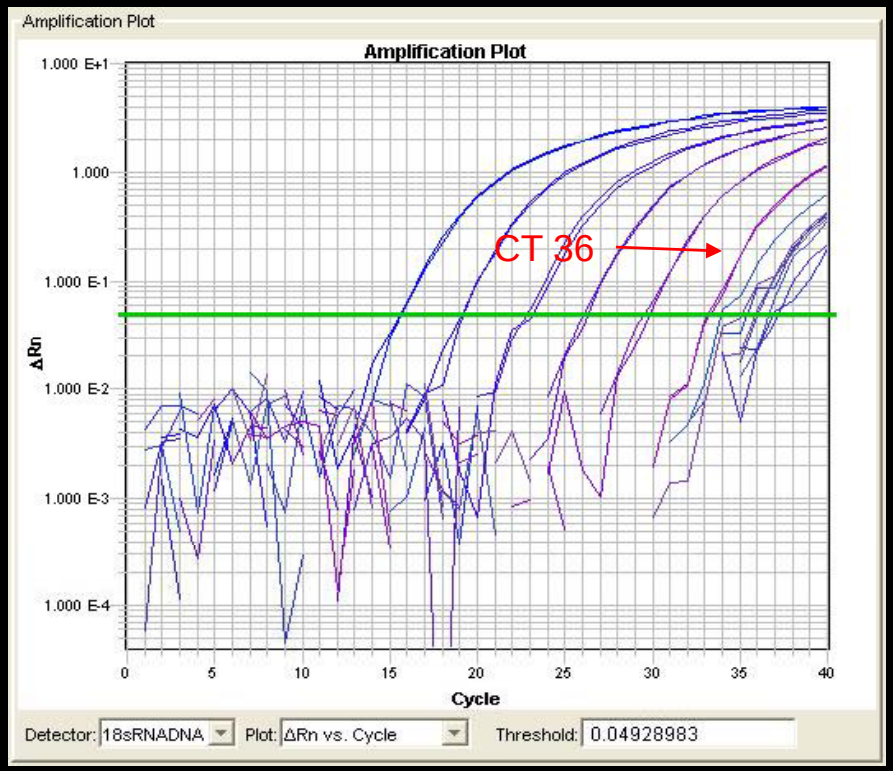


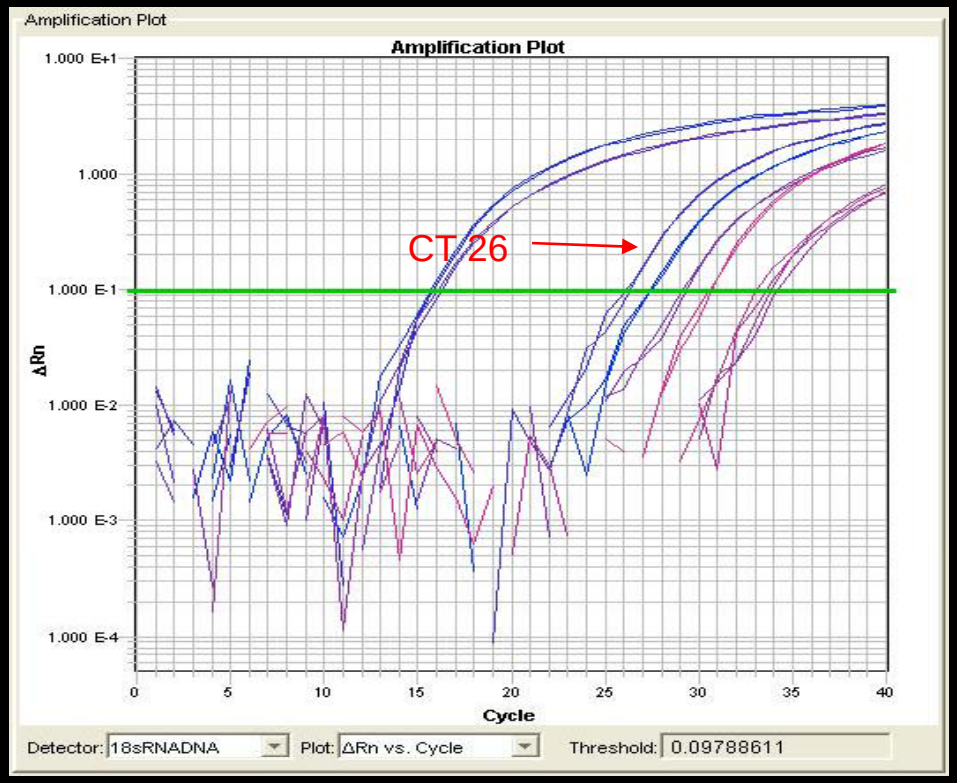
Sample processing for fungal tissue DNA extraction



RTqPCR Results of GP infected aspergillus lung homogenate during bead beating at different time intervals

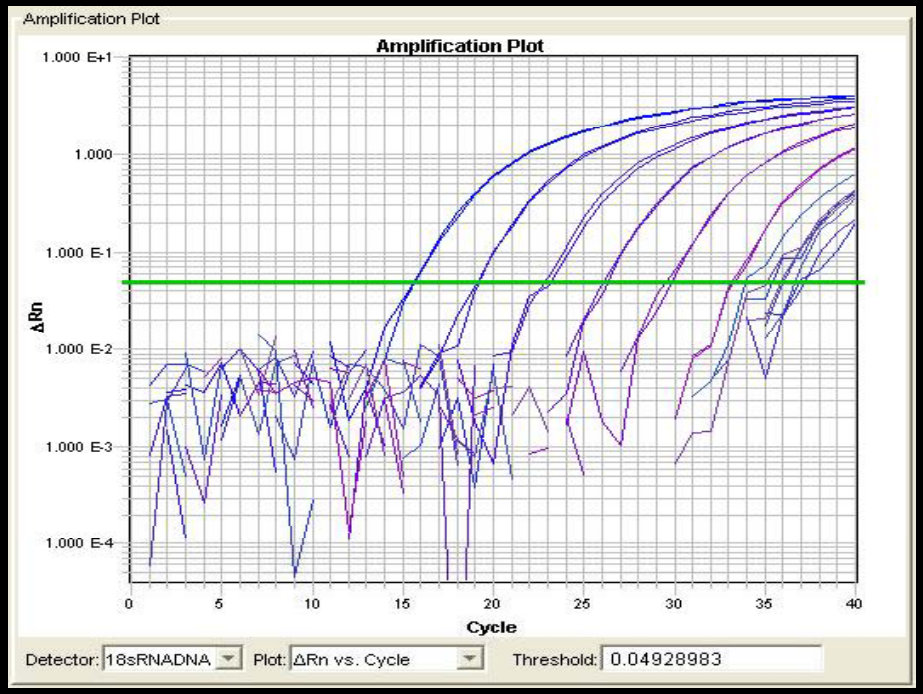
Sample processing for fungal blood DNA extraction

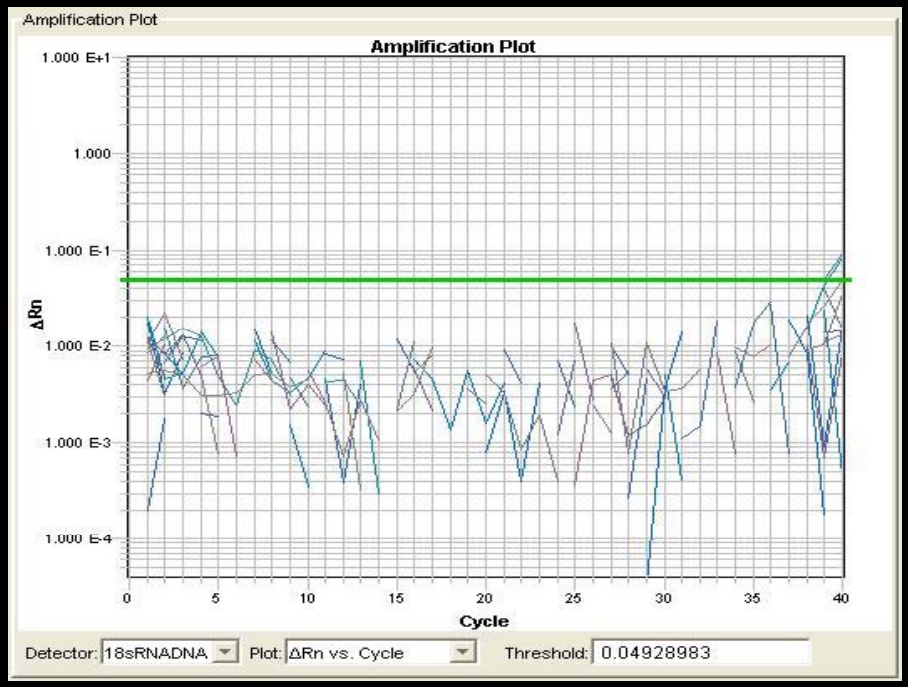




RTqPCR results of GP infected aspergillus blood samples processed through a column vs. automated protocol

Sample analysis in RTqPCR of fungal samples





A standard curve is fundamental to establish valid thresholds for calling a sample a true positive or negative.

Summary and Conclusions

- Calibration of RT-qPCR using standard curves enhances accuracy.
  - ✓Standard curves can identify technical errors
  - ✓Standard curves are essential for interlaboratory agreement
- Template extraction methods greatly affect results.
  - ✓DNA standards should be high purity
  - ✓Automated extraction may enhance sensitivity
- Instrumentation parameters also greatly affect results.
  - ✓Ct values can be arbitrarily adjusted

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