STP Elective at LSHTM Investigating *Plasmodium malariae*

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This Presentation

- My Elective
- Background of *P. malariae*
- What I Did and the Results
- Next Steps
- What I learned

My Elective

LONDON SCHOOL of HYGIENE &TROPICAL MEDICINE

- 6 Weeks at London School of Hygiene and Tropical Medicine
- First 2 weeks spent analysing whole genome sequence data and designing primers.
- Next 4 weeks spent testing primers against samples of malaria to establish optimal conditions, prove efficacy and find the limit of detection.
- Wrote up a draft lab report, gave a presentation to the lab team.
- Attended a range of meetings and lectures on the latest developments in the field of parasitology.

Plasmodium malariae

Plasmodium falciparum

Plasmodium vivax

Plasmodium ovale curtisi

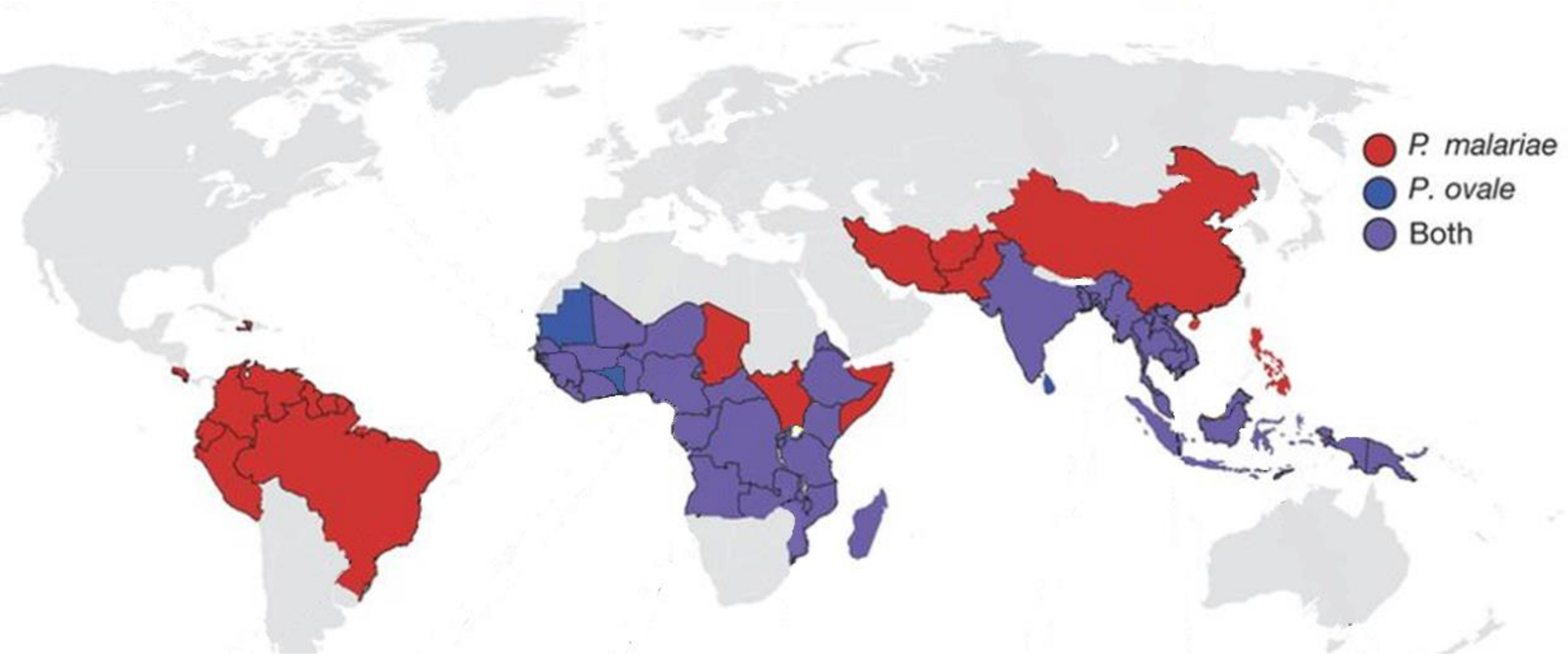
Plasmodium ovale wallikeri

Plasmodium malariae

Plasmodium knowlesi

- Limited data on prevalence
- Does cause life threatening disease
- Observed to recrudesce years post treatment

Countries with reported cases of locally acquired P.malariae (Red and Purple)



Rutledge, G.G., Böhme, U., Sanders, M., Reid, A.J., Cotton, J.A., Maiga-Ascofare, O., Djimdé, A.A., Apinjoh, T.O., Amenga-Etego, L., Manske, M. and Barnwell, J.W., 2017. Plasmodium malariae and P. ovale genomes provide insights into malaria parasite evolution. *Nature*, *542*(7639), p.101.

Detection Methods

- Microscopy
- Rapid Diagnostics antigen detection
- PCR

Methods

- Use gene alignment software to investigate the *P. malariae* genome for species specific sequences that could be viable PCR targets.
- Generate primers, establish working PCR protocols and test them against *Plasmodium* species.
- Investigate the limit of detection of the new assays.
- Prospectively investigate potential P. malariae isolates received to the PHE Malaria Reference Lab.

Gene Targets Selected

- Mitochondrial Genome
- Glyceraldehyde phosphate dehydrogenase (GAPDH)
- *G*amete antigen 27/25

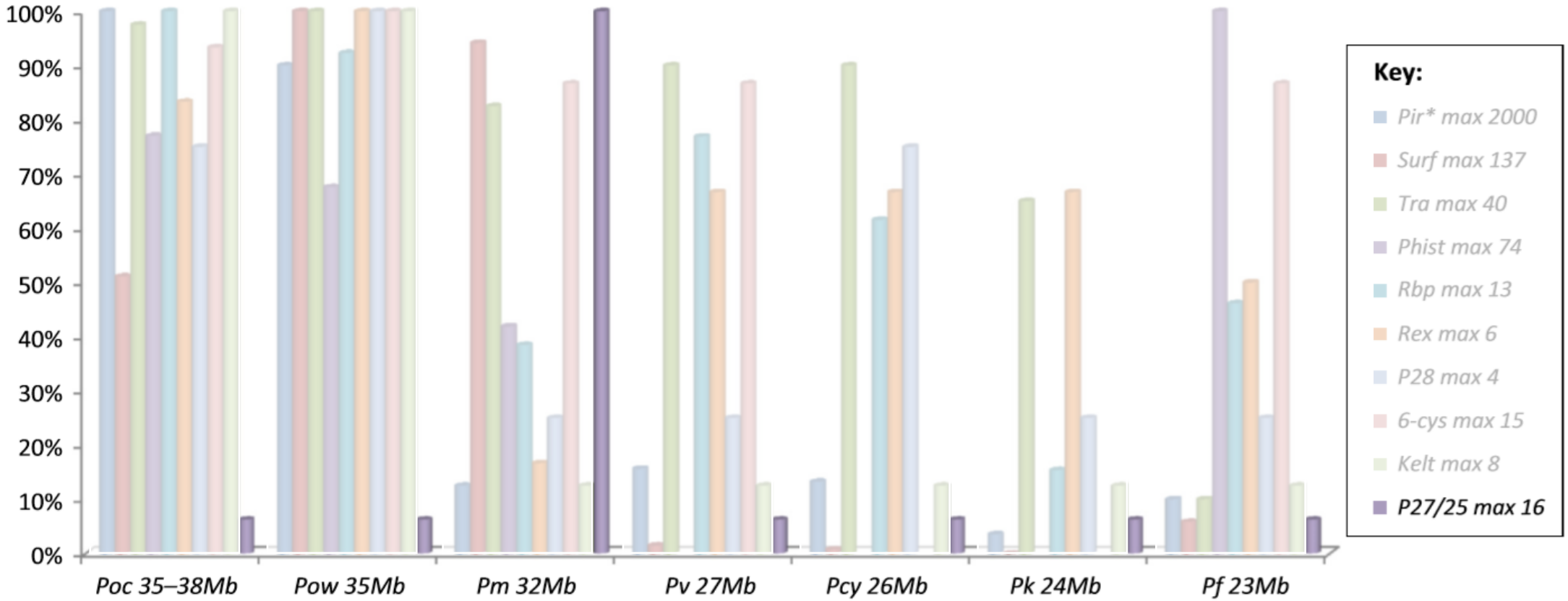
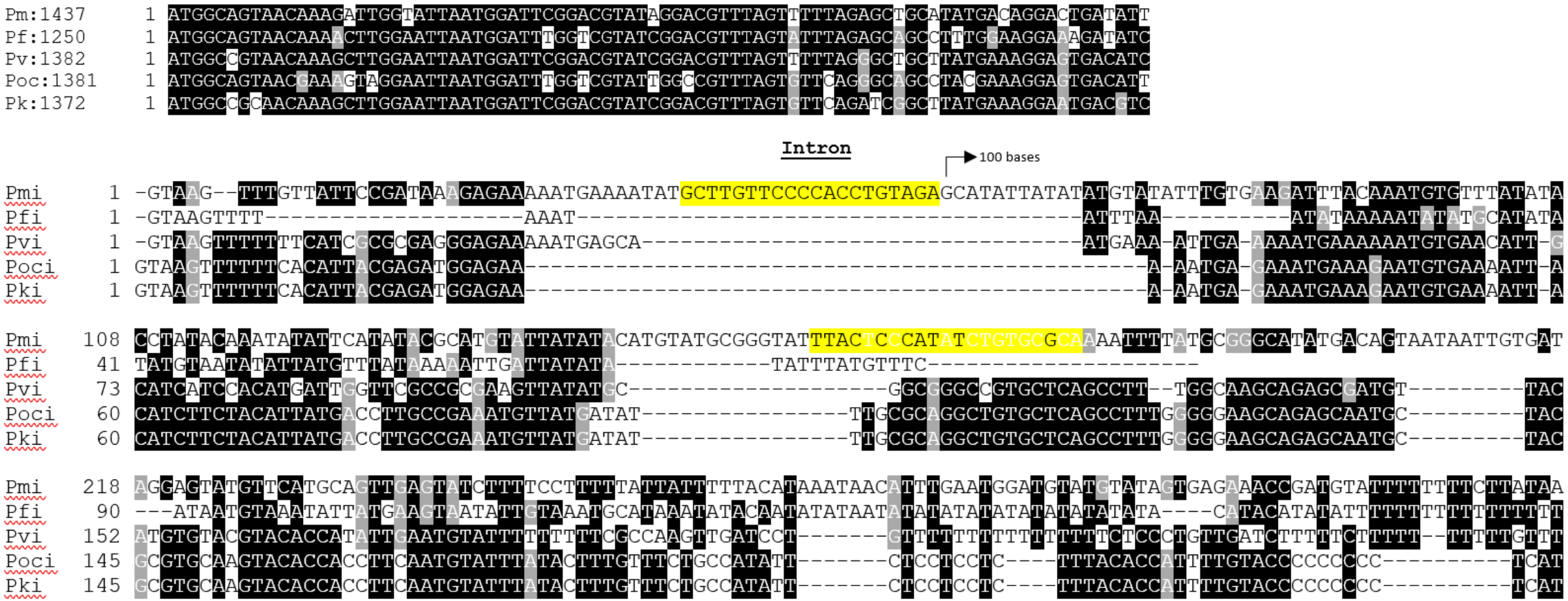


Figure 2. Relative Abundance of Gene-Family Members in the Genomes of Seven *Plasmodium* Parasites of Primates. Histograms represent the relative size of gene-family expansions expressed relative to the maximum expansion of that family (max) observed among the seven genomes depicted: *Plasmodium ovale curtisi* (*Poc*) [70]; *P. ovale wallikeri* (*Pow*) [70]; *P. malariae* (*Pm*) [70]; *P. vivax* (*Pv*) [82]; *P. cynomolgi* (*Pcy*) [71]; *P. knowlesi* (*Pk*) [83]; and *P. falciparum* (*Pf*) [84]. The genomes are arranged in decreasing order of total size in megabytes. Gene families shown encode: *Plasmodium* interspersed repeat proteins (*pir*) [85]; surface-associated interspersed proteins or SURFIN (*surf*) [72]; tryptophan-rich antigens (*tra*) [2]; *Plasmodium* helical interspersed subtelomeric PHIST proteins (*phist*) [74] (these have not been determined for *Pcy*, *Pk*, or *Pv*); reticulocyte-binding proteins (*rbp*) [2,70]; Maurer's cleft-associated ring-exported proteins 3 and 4 (*rex*) [86]; 28-kD ookinete surface protein P28 (*p28*) [87]; six-cysteine motif proteins (*6-cys*) [53] (these have not been determined for *Pcy* or *Pk*); the uncharacterised KELT proteins named for the C-terminal amino acid motif Lys-Glu-Leu-Thr (*kelt*) [70]; and the gamete antigen P27/25 (*p27/25*) [88]. Estimates of gene numbers for the two isolates each of *Poc* and *Pow* varied slightly for the *pir* and *surf* genes [70] and the average was used to generate this figure. Minimal estimates rather than true counts are given for *pir* genes in species where the repertoire is above 200 gene copies (*Poc*, *Pow*, *Pcy*, *Pv*). The newly identified *pm-fam-a* gene family is unique to *P. malariae* (>500 members) and so is not shown in this comparative plot.

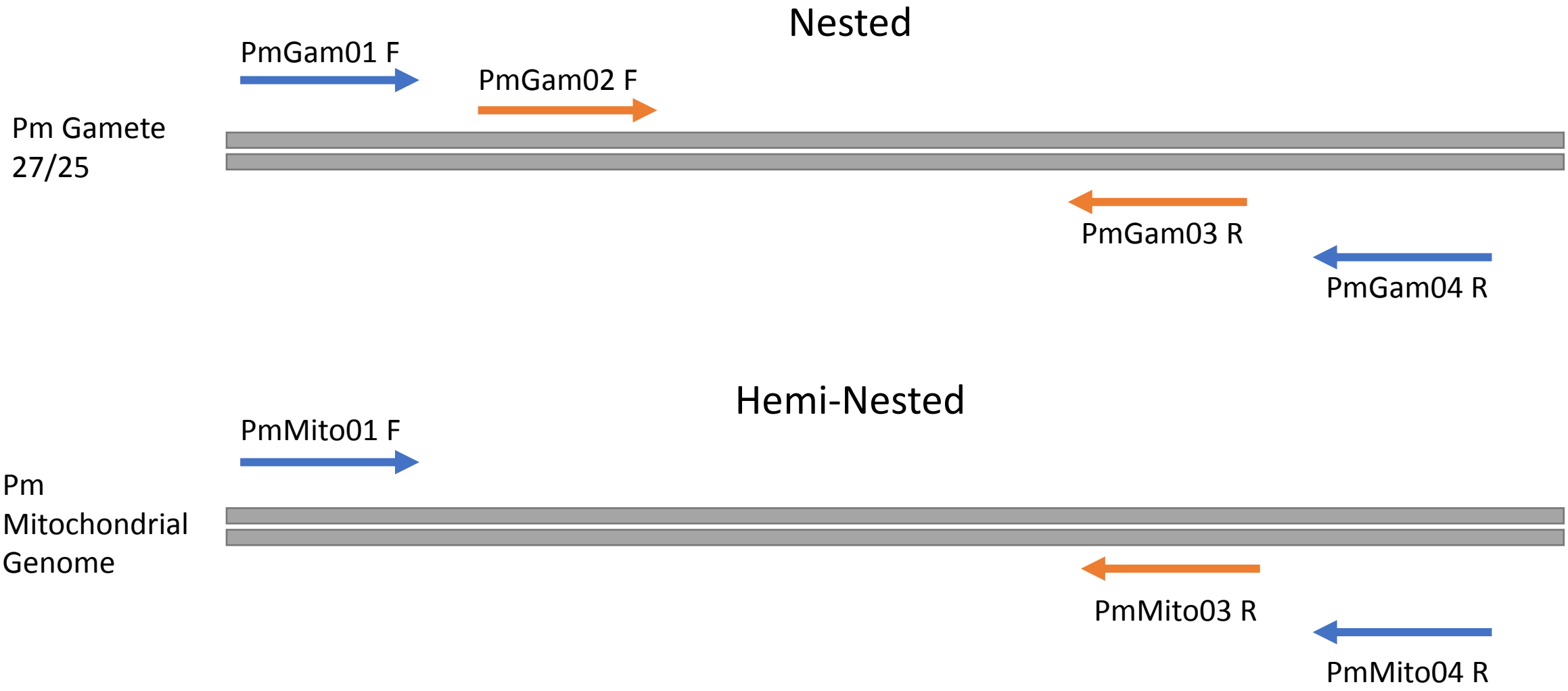
Sutherland, C.J., 2016. Persistent parasitism: the adaptive biology of malariae and ovale malaria. *Trends in parasitology*, *32*(10), pp.808-819.

Pm Gamete 27/25 and interspecies analogues clustal alignment 15th Nov 18

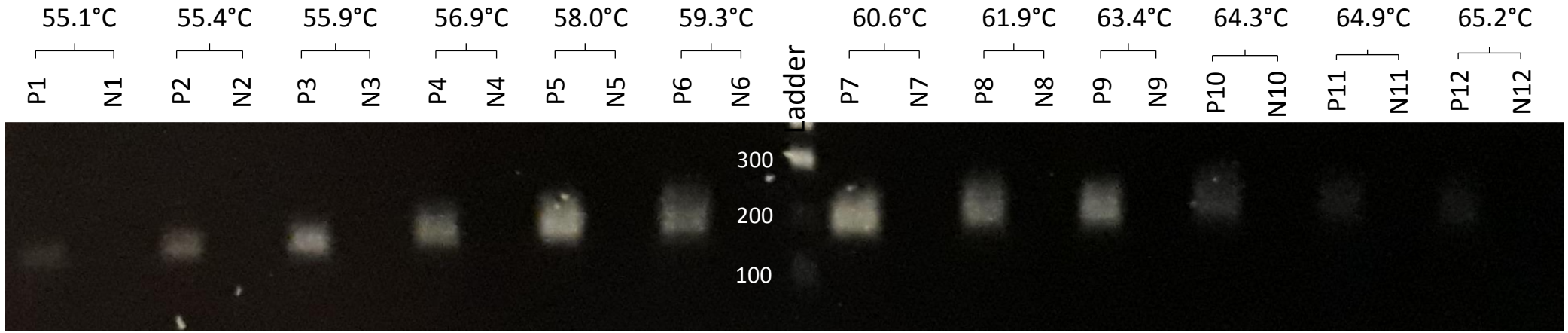
GAPDH Interspecies clustal alignment 9th Nov 18



Planned Nesting Arrangement

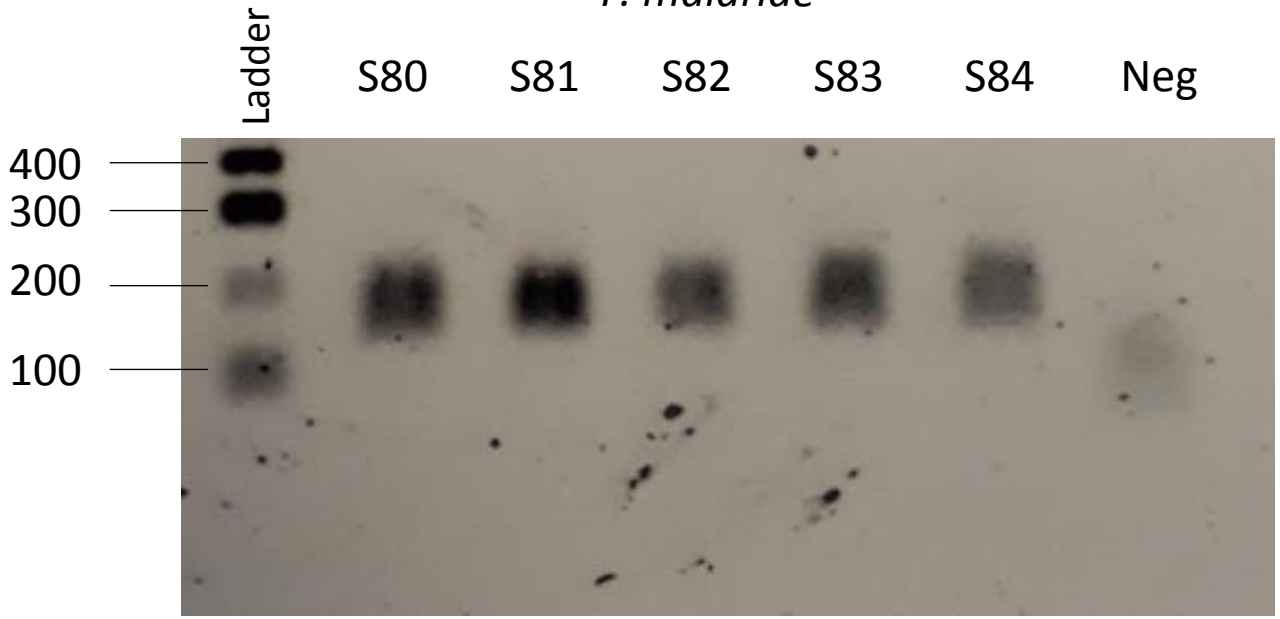


GAPDH Temperature Gradient

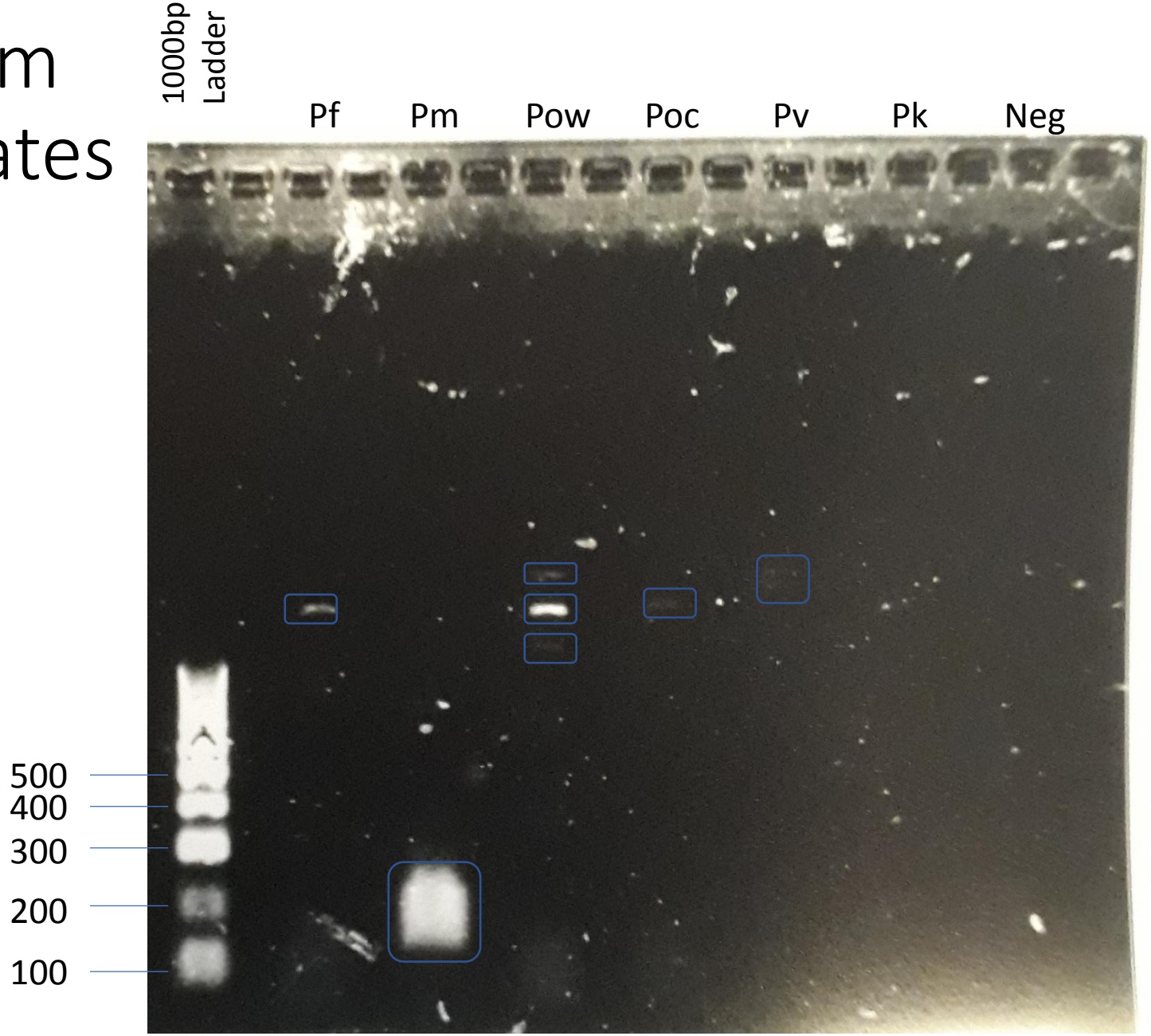


GAPDH for 5 P.m Isolates

P. malariae



GAPDH for Plasmodium Species Isolates



Fragments > 1000bp in size

Limit of Detection

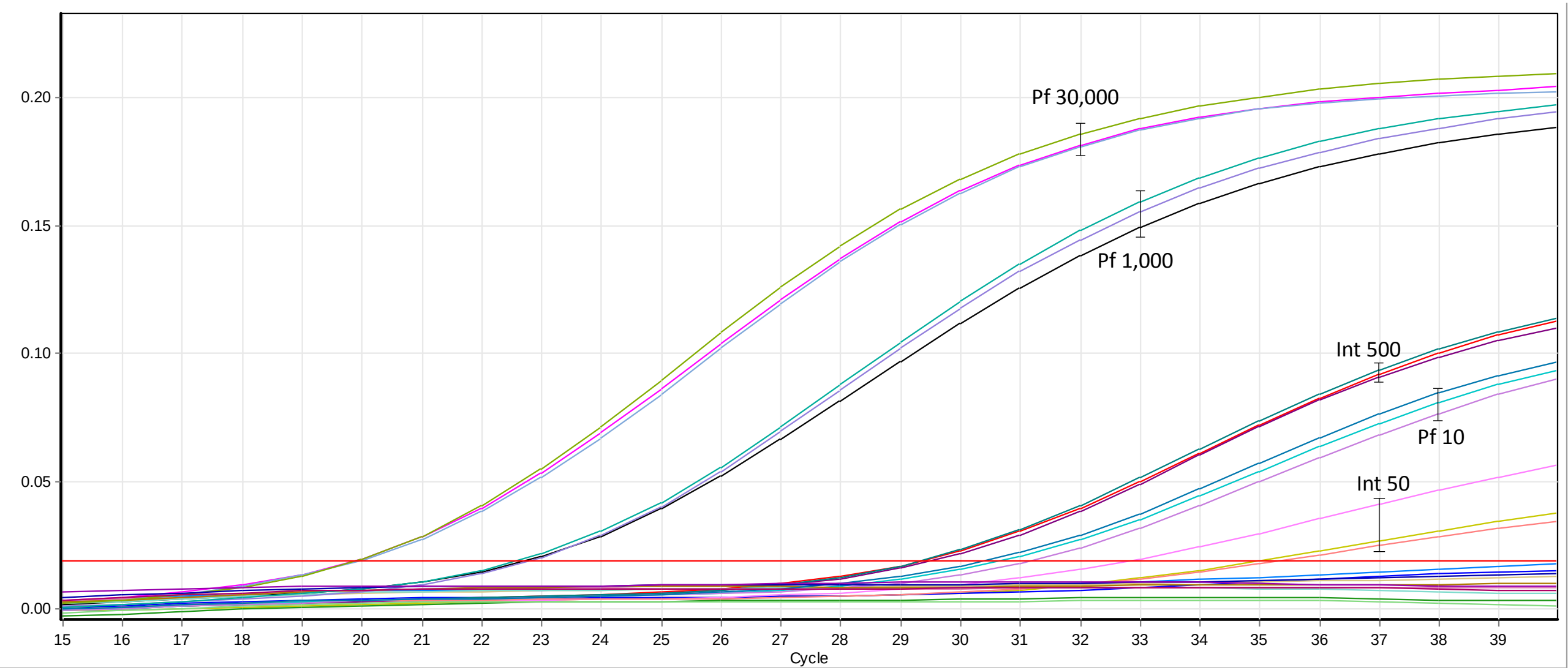
Problem

P. malariae is not routinely quantified in positive patient samples. Therefore the parasite density in the clinical samples used was not known.

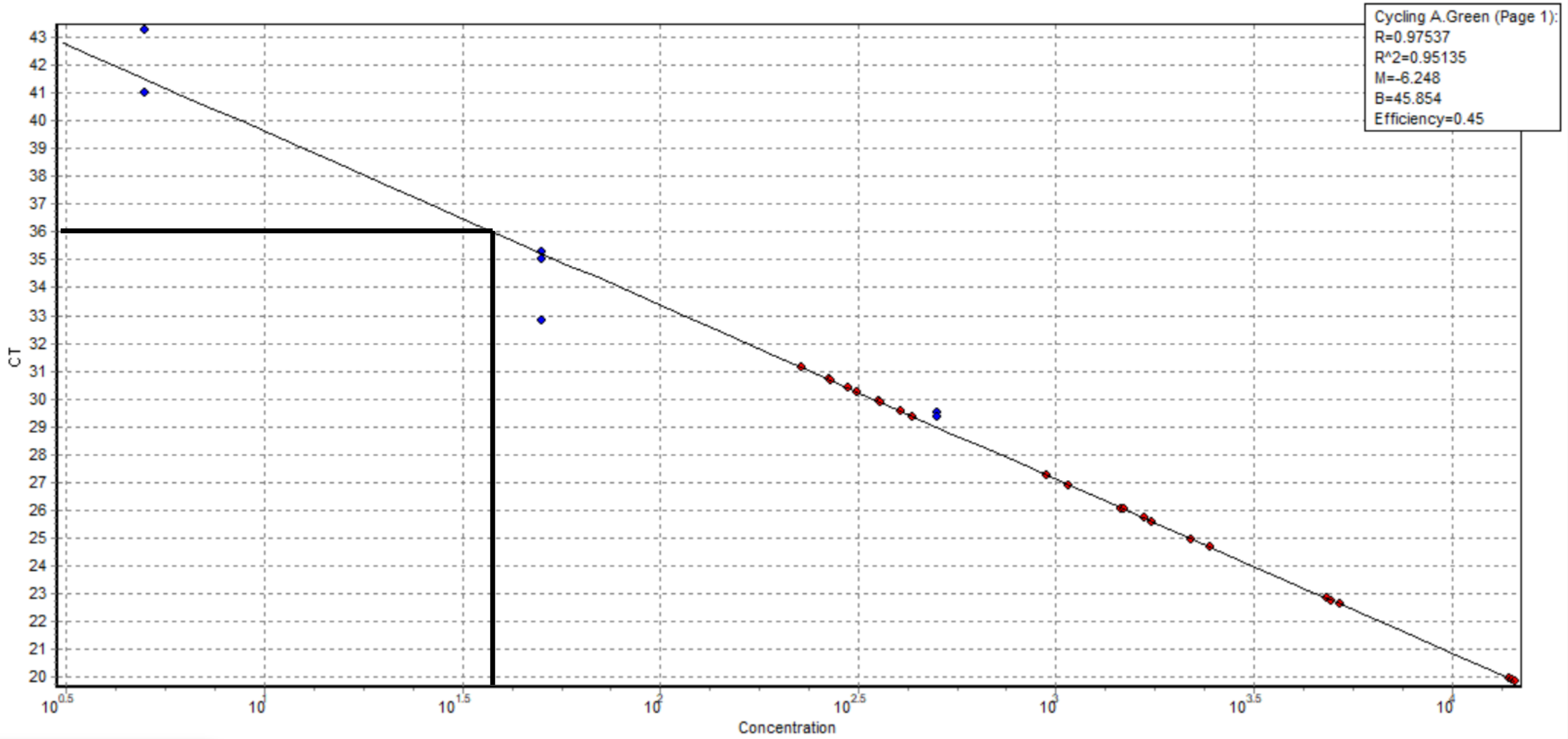
Solution

Generate a standard curve using known quantities of *P. falciparum* and compare to clinical samples using a *Plasmodium* specific primer. Normalise the results by Human Beta Tubulin content, indicative of human White Cell count.

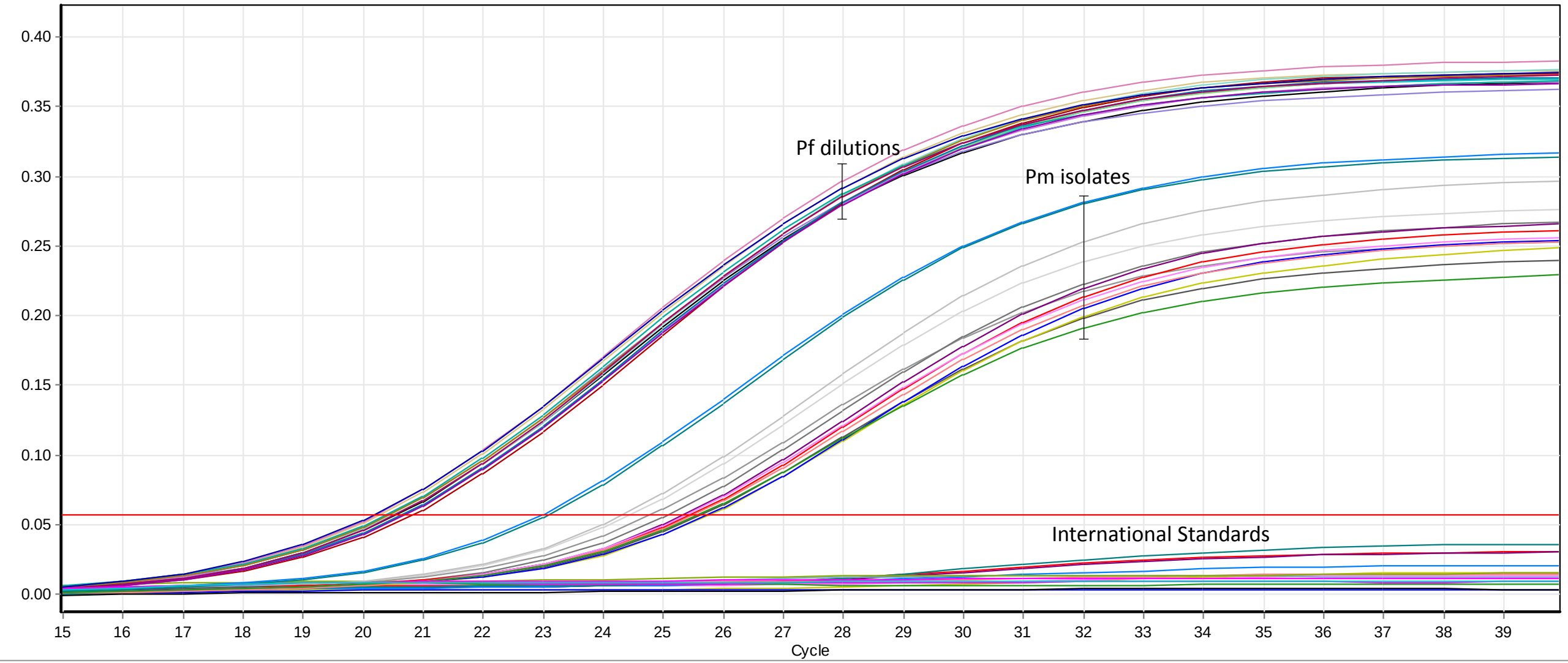
PgMET – Plasmodium Species Quantification



Standard Curve for PgMET Quantification



HumTuBB – Human β Tubulin Quantification

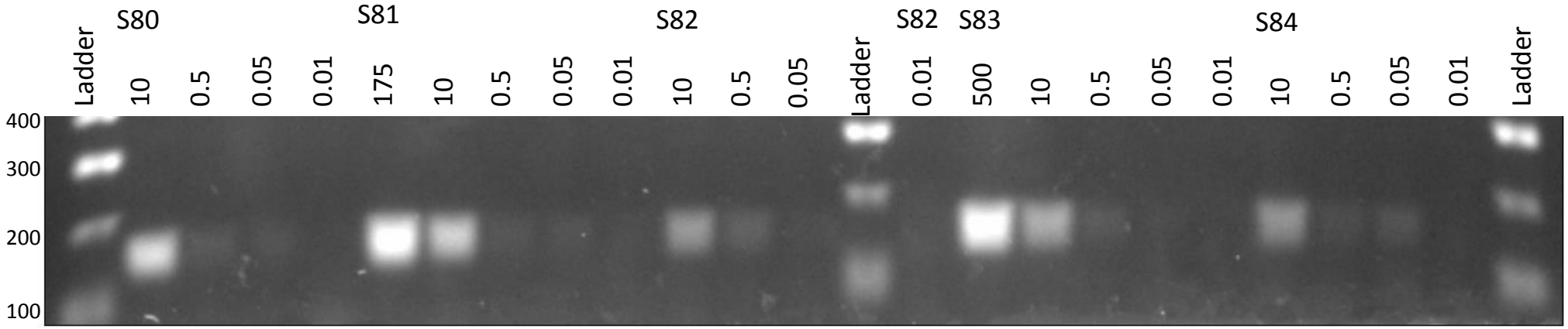


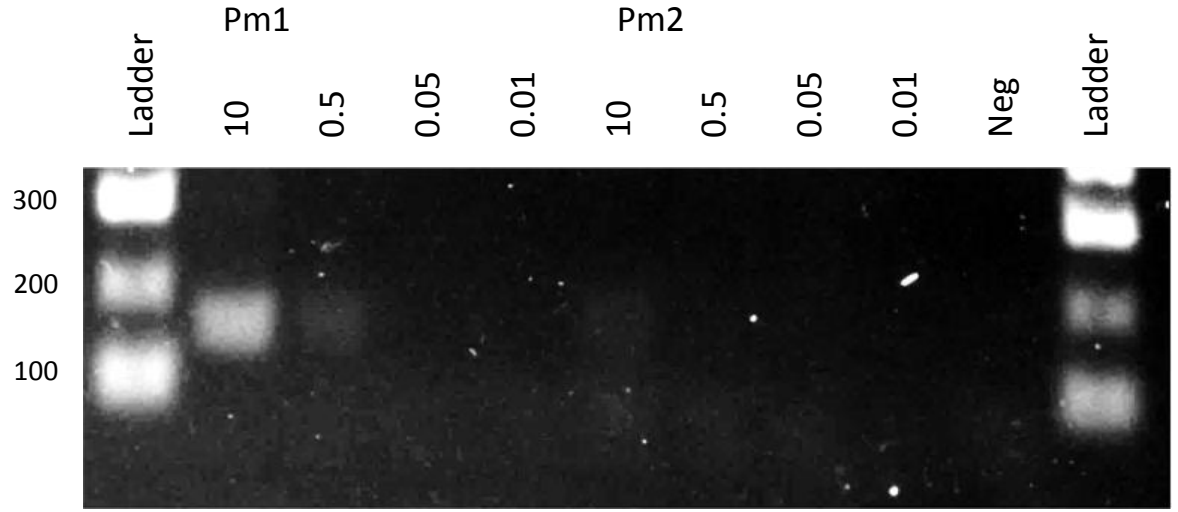
Parasite Density (Parasites/µL)

Sample	PD	SD
S80	1132	380.0
S81	6146	3679.5
S82	363	113.5
S83	4507	2842.0
S84	434	137.2
Pm1	1378	441.6
Pm2	260	79.0

ΔΔCT Calculation

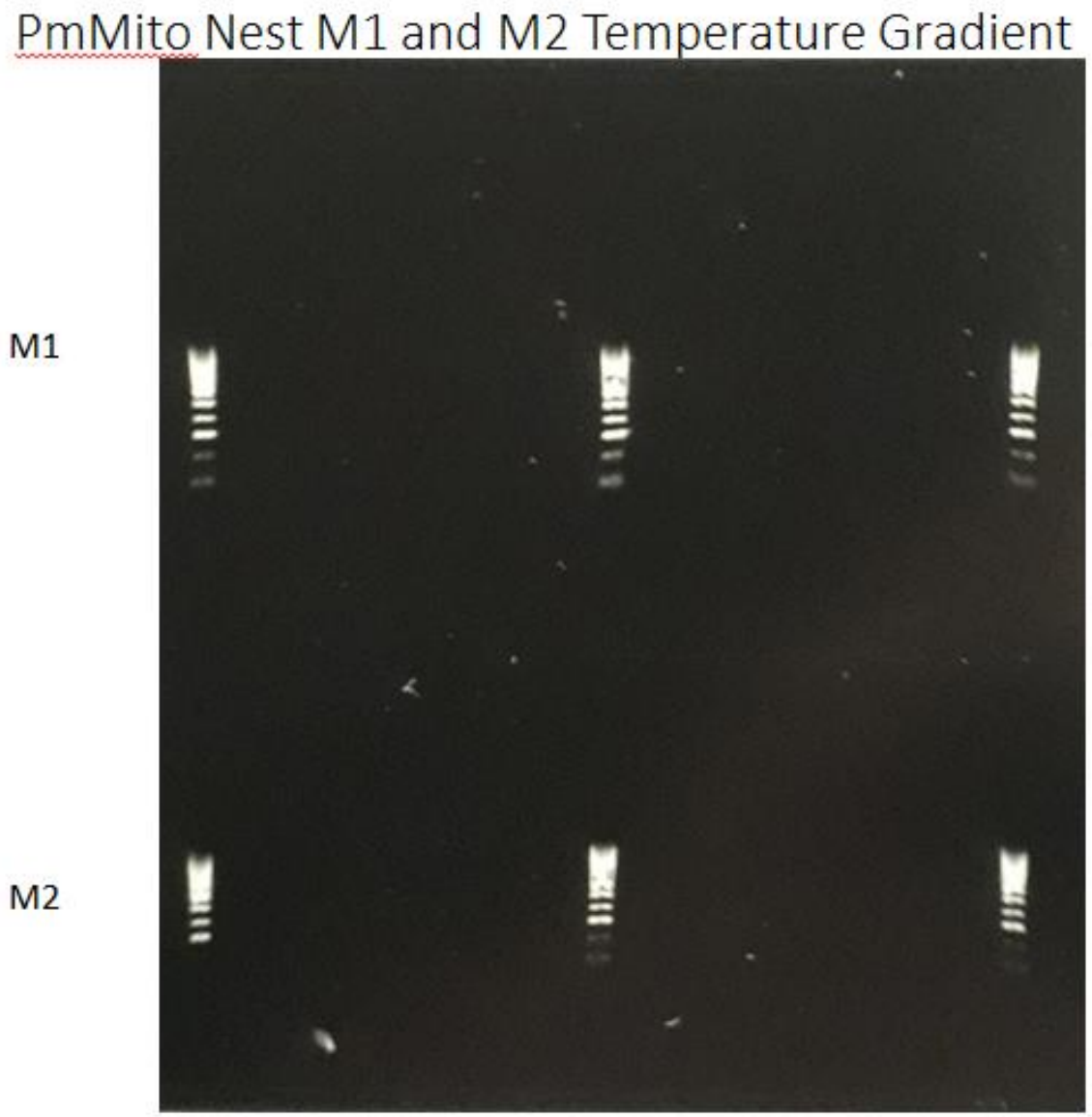
GAPDH LOD


Ladder
S80
10
0.5
0.05
0.01
S81
175
10
0.5
0.05
0.01
S82
10
0.5
0.05
Ladder
S82
0.01
S83
500
10
0.5
0.05
0.01
S84
10
0.5
0.05
0.01
Ladder
400
300
200
100


Ladder
Pm1
10
0.5
0.05
0.01
Pm2
10
0.5
0.05
0.01
Neg
Ladder
300
200
100

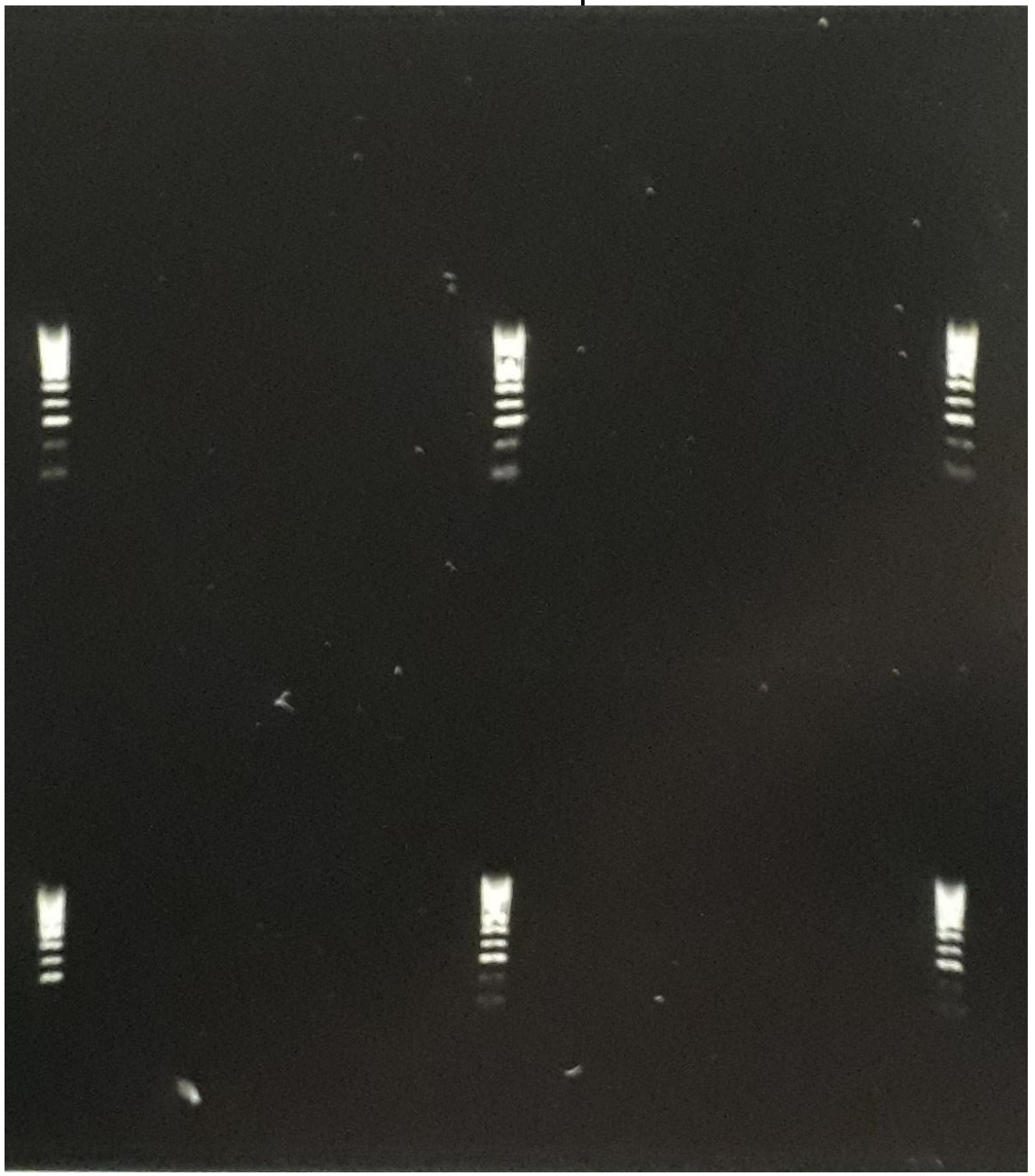
PmGamete and PmMito Temperature Gradients

- No bands were seen in the temperature gradient experiment
- This was due to fundamental failure of the primers



PmMito Nest M1 and M2 Temperature Gradient

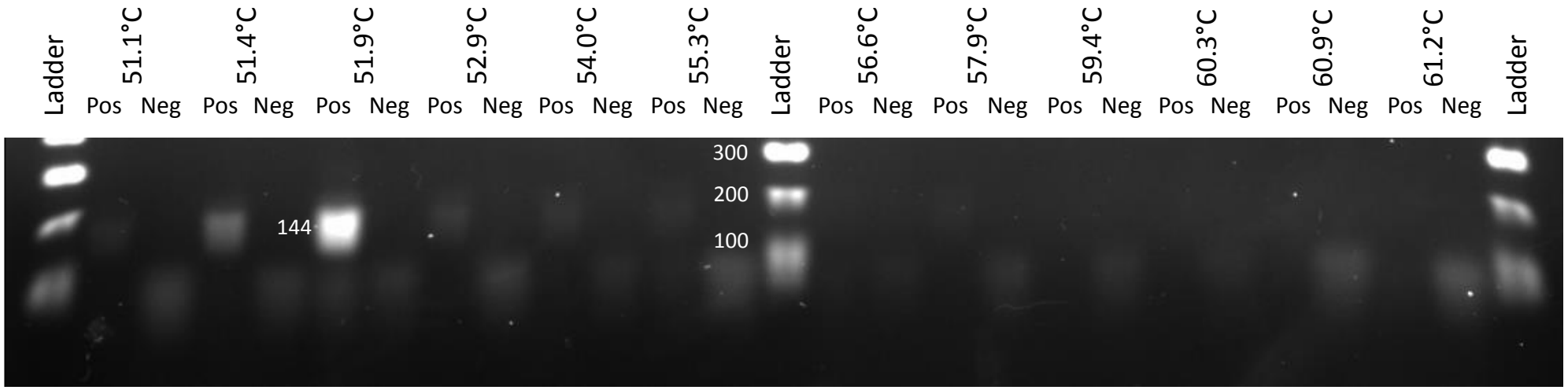
M1



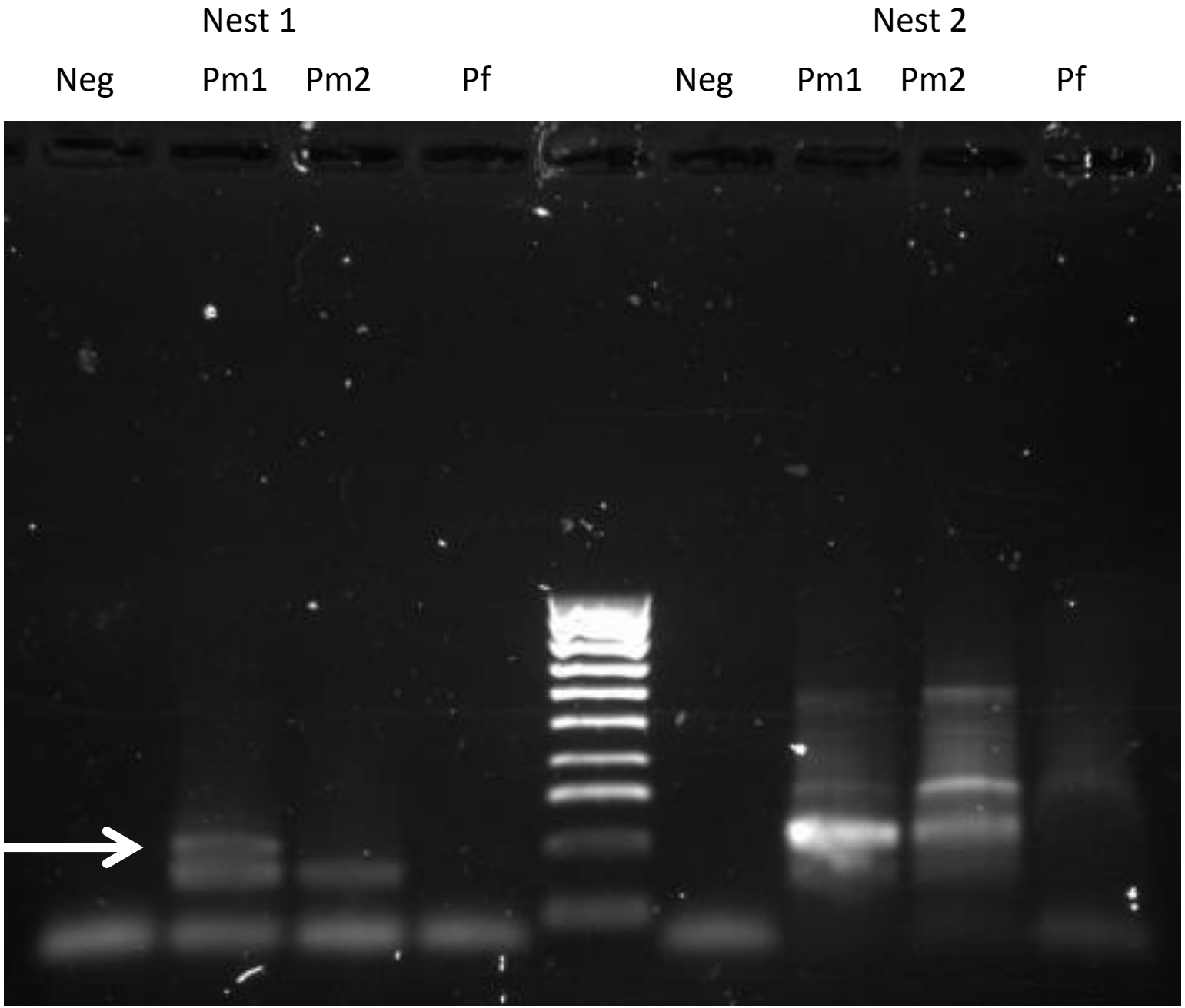
M2

	Nest 1		Nest 2	
Gene Copy	**PmGam05 F**	**PmGam08 R**	**PmGam06 F**	**PmGam07 A+B R**
PmUG01_14018600	X			x
PmUG01_14018400	X			x
PmUG01_14018500	X			x
PmUG01_14018100	X			
PmUG01_14018200	X			
PmUG01_14018300	X			
PmUG01_14017600	X		x	x
PmUG01_14017900		X	x	
PmUG01_14017500	X	X	x	x
PmUG01_14017700	X	X	x	x
PmUG01_14017400	X	X	x	x
PmUG01_14017800		X	x	x
PmUG01_14017100	*	X	x	*
PmUG01_14017000	X	X	*	*
PmUG01_14017200	X	X	*	*
PmUG01_14016700	X		x	x
PmUG01_14016500			x	
PmUG01_14017300		X		x
PmUG01_14016900		X	x	
PmUG01_14018000	*	X	*	
PmUG01_14016600	X	X	x	
PmUG01_14016800			*	

PmGam #2
Nest 2



Reworked Primers, Low temperature



Expected target: 197bp

Next Steps

- A stock of serially diluted clinical *P. malariae* DNA has been generated which will enable future limit of detection studies of potential *P. malariae* specific assays. If the Gamete or mitochondrial assays can be made to function then their LOD can be determined and compared to existing assays.
- Develop multiplex quantitative PCR assay – this will be more viable for use in a clinical setting.
- Conduct a prospective investigation of potential *P. malariae* isolates received by PHE MRL.

Summary

- How molecular PCR assays are designed from the gene sequence level through to an working test.
- How problems with a PCR assay can be identified and investigated.
- Got to experience the world of research that underpins the scientific developments that Clinical Scientists work to implement.