

STP Elective at LSHTM Investigating *Plasmodium malariae*

Harry Thynne and Dr Colin Sutherland

London School of Hygiene and Tropical Medicine

This Presentation

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



My Elective

- 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 ovale
curtisi

Plasmodium ovale
wallikeri

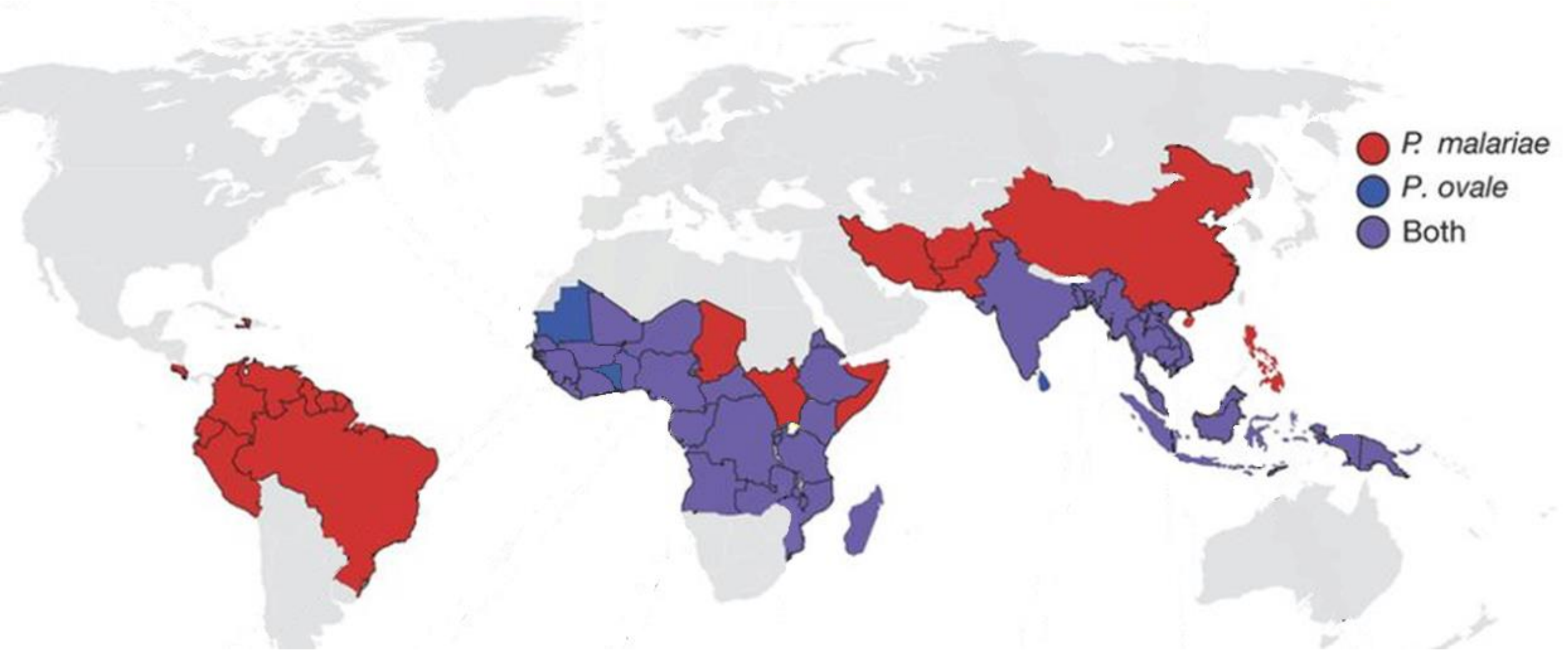
Plasmodium vivax

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)



Detection Methods

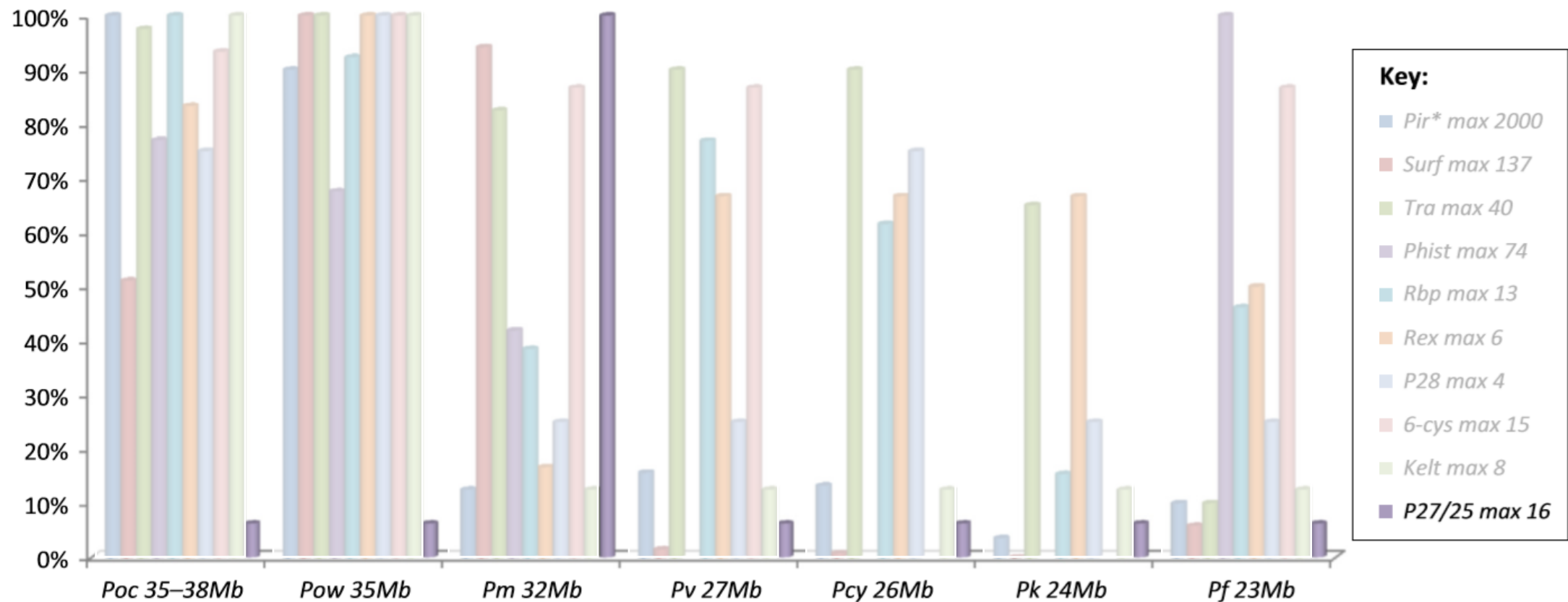
- Microscopy
- Rapid Diagnostics antigen detection
- PCR

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)
- *Gamete antigen 27/25*



Trends in Parasitology

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

```
6500:429 272 GTTACAATAAAA---ATGAAATAAAAAAGTTAAAAATGATTATGAAAAAGAAAAAGTATCAAATATTCACCGGAATGATCCATAATAATATAGTTACAGTTGGGAGC
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6700:620 377 GCAAAAATAAAT---CTGAAATTAAGGTTTCATATGTGTTTGGAAAAAGGTATAGTATAAACTTTTCAGAAAGATGATATACAATAATGTTACTACACTTGTGTCC
6800:384 329 GTAATGACGACG---ATGAAATAAAAAAGACTTTTATGTATTTTGTAGAAAAAGAAATAA-----
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7900:618 557 GCCAAAACGAAC---TTGCAATAAAAAAGTCTTATAAGCAAATTAGAAAGAGGTAGGACATATAA-----
8000:645 488 GTCAGAATGAAG---CTGAAATAAAAAACCTTTTAAGTGTTTTAGAAAGAAAAAGAGTATAAACTTCCAAACGGAATGATAGAAAATACTGTAAGTACGGTTGAAGAC
8100:783 527 GTAATGATTATG---ATGAAATGAAAAGAGTTATAAGTGTTTTATGAAAAAGGTACAGTGTTGAAATTAACAGATGATATAATTCATTTTACTCTAAGTTTAGCTAGAGCA
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8600:612 461 GTCGTAATGAAG---ATTTAATGAAAACAATTATAGGTGTTTCATGAAGAAGGTAGAGTATAAACTATCAGAAGATATTATTAATTATATTATACGTATGGCTAAGATG
PF: 654 458 TGAACGACAAGA---AATTGATCAGAATGTTATTTGACACCTATGAATATGTCAAGATGTCAAATTTACAGATGACCAATATAAGGACGCAGCAGCAAGAATAAGTCAA
PK: 1203 536 AATTAGCTGGCGAATATAGAATAGAAAGAGTGGACGAAATGTAGCAATTTTAAATGAAAACATAAATAGCATTTCATGAAATGCAAAGCCCACTTGACAGCTACAAAACC
Poc: 1080 431 AAAATATTGTAGAAAGATAGTCCAAA---TATTAATGAGAACATAGAA---GCAGTAGTGAAGAAGAAGACGTGAAGAGTCTGATTATTCGCTTGATTCCCTATAAGTCC
PV: 1179 512 AAATGAGTGGCGAATATATAGTACAAGAAGTGAACGATGATGTAATAGTTGTGCAGTAAAATATAAACAGCATTGACCAATTGGAAGATTCACTTGAATCCTATAGATCT
```

PmGam03

GAPDH Interspecies clustal alignment 9th Nov 18

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Pm:1437 1 ATGGCAGTAACAAAGATTGGTATTAATGGATTTCGGACGTATAGGACGTTTAGTTTTATAGAGCTGCATATGACAGGACTGATATT
Pf:1250 1 ATGGCAGTAACAAACCTTGAATTAATGGATTTCGGTTCGTATCGGACGTTTAGTATTTAGAGCAGCCTTTGGAAGGAAAGATATC
Pv:1382 1 ATGGCCGTAACAAAGCTTGAATTAATGGATTTCGGACGTATCGGACGTTTAGTTTTATAGGCTGCTTATGAAAGGAGTGACATC
Poc:1381 1 ATGGCAGTAACGAAAGTAGGAATTAATGGATTTCGGTTCGTATTCGGTCGTTTAGTGTTCAGGGCAGCCTACGAAAGGAGTGACATT
Pk:1372 1 ATGGCCGCAACAAAGCTTGAATTAATGGATTTCGGACGTATCGGACGTTTAGTGTTCAGATCGGCTTATGAAAGGAATGACGTC
```

Intron ↗ 100 bases

```
Pmi 1 -GTAAG--TTTGTATTTCGATAAAGAGAAAATGAAAATATGCTTGTTCCCACCTGTAGAGCATATTATATATGTATATTGTGAAGATTACAAATGTGTTTATATA
Pfi 1 -GTAAGTTTT-----AAAT-----ATTTAA-----ATATAAAAATATATGCATATA
Pvi 1 -GTAAGTTTTTTTTCATCGCGCGAGGGAGAAAATGAGCA-----ATGAAA-ATTGA-AAAATGAAAAATGTGAACATT-G
Poci 1 GTAAGTTTTTTTCACATTACGAGATGGAGAA-----A-AATGA-GAAATGAAAGAATGTGAAAATT-A
Pki 1 GTAAGTTTTTTTCACATTACGAGATGGAGAA-----A-AATGA-GAAATGAAAGAATGTGAAAATT-A
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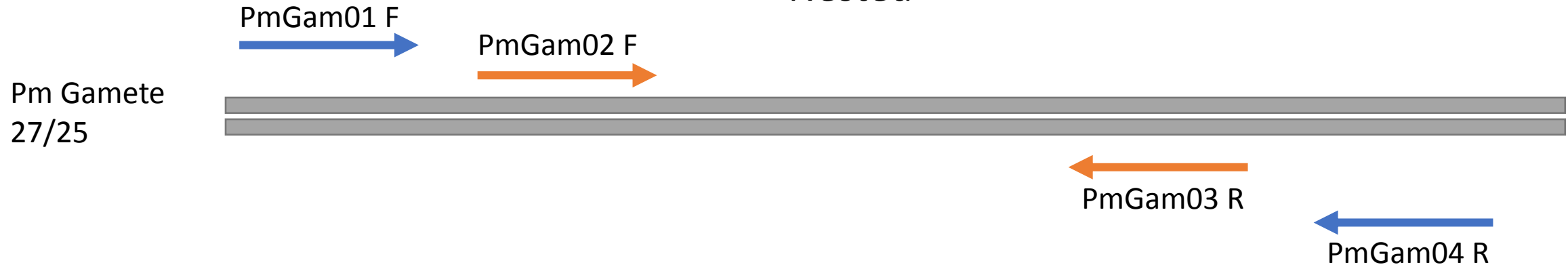
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Pmi 108 CCTATACAAATATATTCATATACGCATGTATTATATACATGTATGCGGGTATTTACTCCCATATCTGTGCGCAAATTTTATGCGGGCATATGACAGTAATAATTGTGAT
Pfi 41 TATGTAATATATTATGTTTATAAAAATTGATTATATA-----TATTTATGTTTC-----
Pvi 73 CATCATCCACATGATTGGTTCGCCGCGAAGTTATATGC-----GGCGGGCCGTGCTCAGCCTT--TGGCAAGCAGAGCGATGT-----TAC
Poci 60 CATCTTCTACATTATGACCTTGCCGAAATGTTATGATAT-----TTGCGCAGGCTGTGCTCAGCCTTTGGGGGAAGCAGAGCAATGC-----TAC
Pki 60 CATCTTCTACATTATGACCTTGCCGAAATGTTATGATAT-----TTGCGCAGGCTGTGCTCAGCCTTTGGGGGAAGCAGAGCAATGC-----TAC
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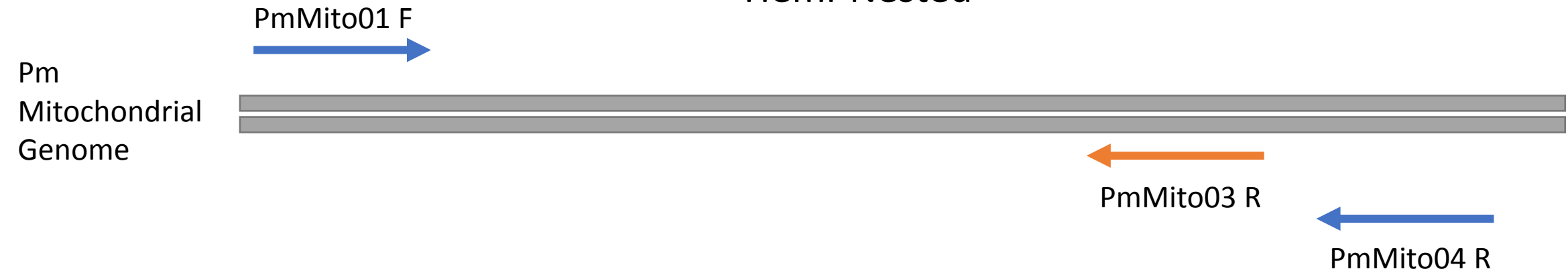
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Pmi 218 AGGAGTATGTTTCATGCAGTTGAGTATCTTTTCCTTTTATTATTTTTTACATAAATAACATTTGAATGGATGTATGTATAGTGAGAAACCGATGTATTTTTTTTCTTTATAA
Pfi 90 ---ATAATGTAAATATTATGAAGTAATATTGTAAATGCATAAATATACAAATATATAATATATATATATATATA---CATACATATATTTTTTTTTTTTTTTT
Pvi 152 ATGTGTACGTACACCATAATTGAATGTATTTTTTTTTTTCGCCAAGTTGATCCT-----GTTTTTTTTTTTTTTTCTCCCTGTTGATCTTTTTCTTTTT--TTTTTGTTT
Poci 145 GCGTGCAAGTACACCACCTTCAATGTATTTTATACTTTTGTCTGCCATATT-----CTCCTCCTC---TTTACACCATTTTGTACCCCCCCC-----TCAT
Pki 145 GCGTGCAAGTACACCACCTTCAATGTATTTTATACTTTTGTCTGCCATATT-----CTCCTCCTC---TTTACACCATTTTGTACCCCCCCC-----TCAT
```

Planned Nesting Arrangement

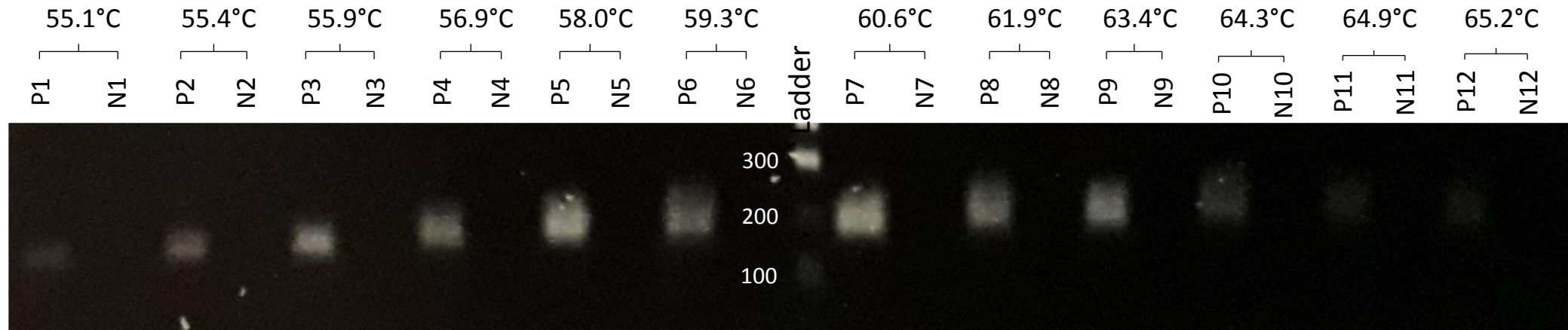
Nested



Hemi-Nested

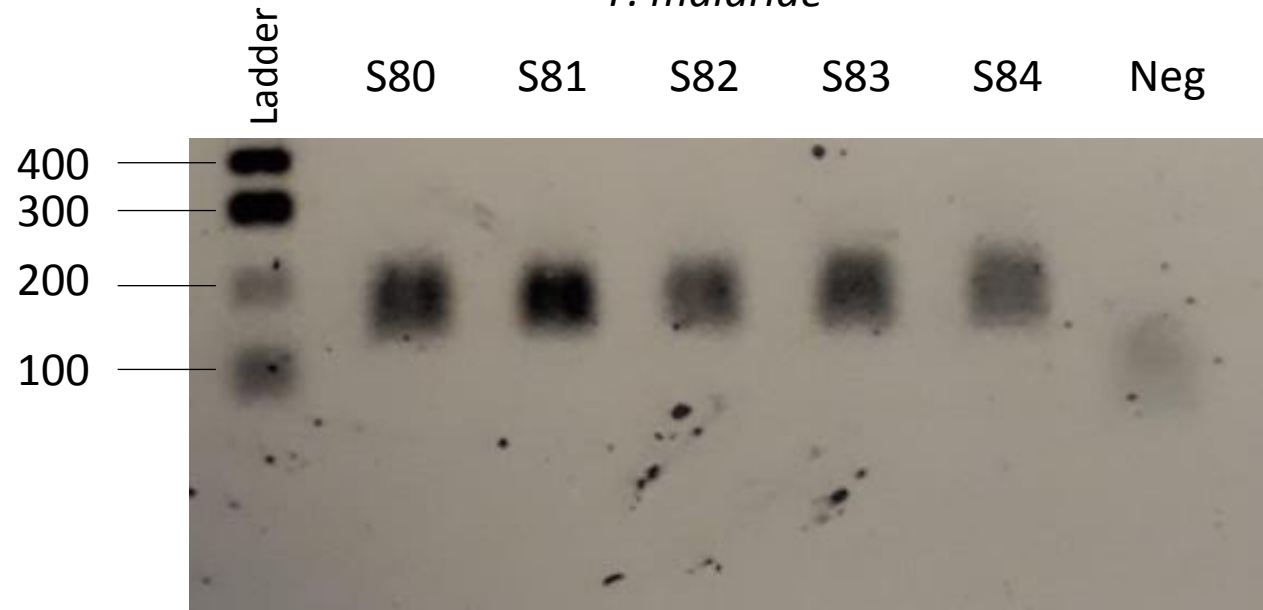


GAPDH Temperature Gradient

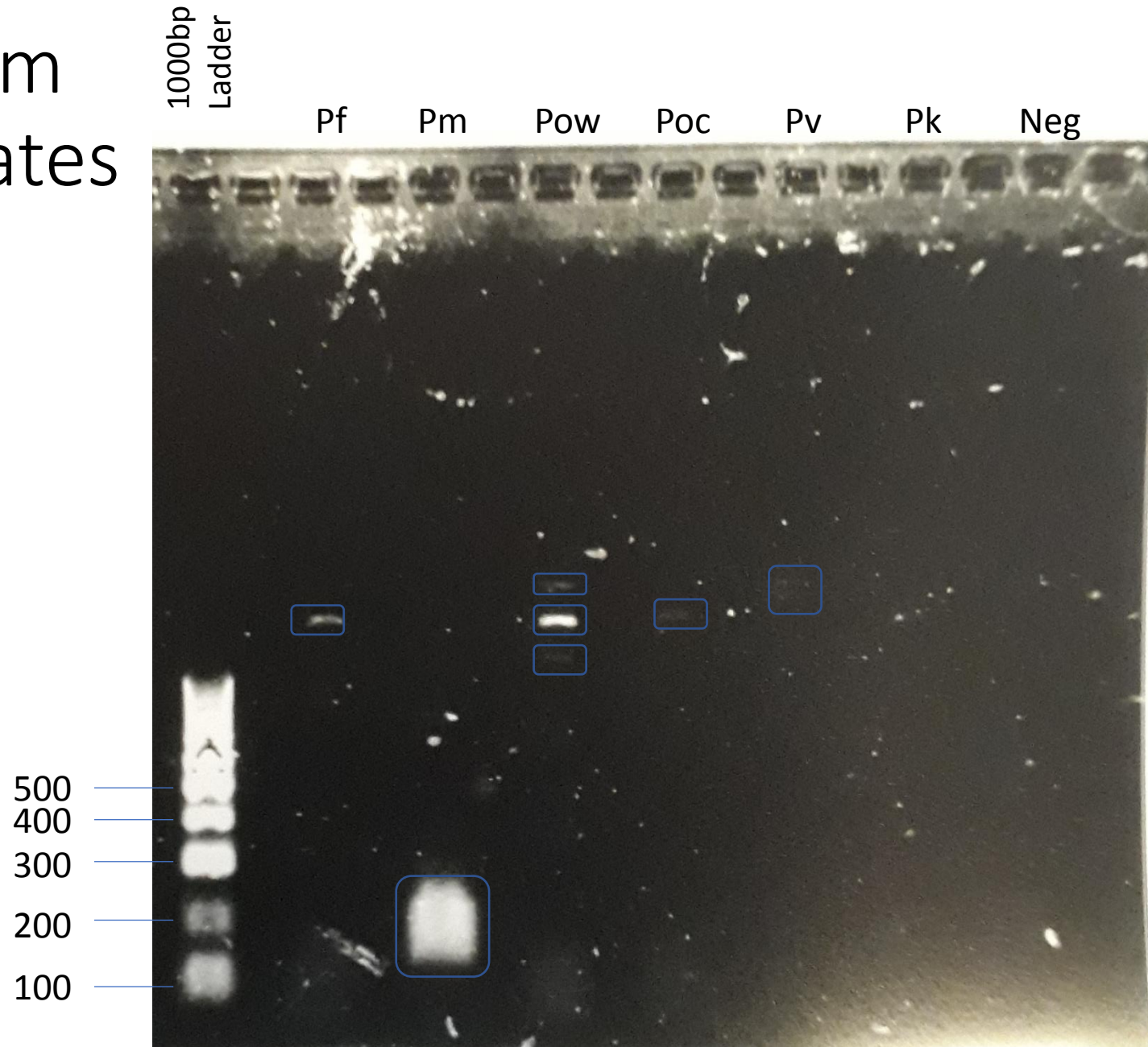


GAPDH for 5 P.m Isolates

P. malariae



GAPDH for Plasmodium Species Isolates



Limit of Detection

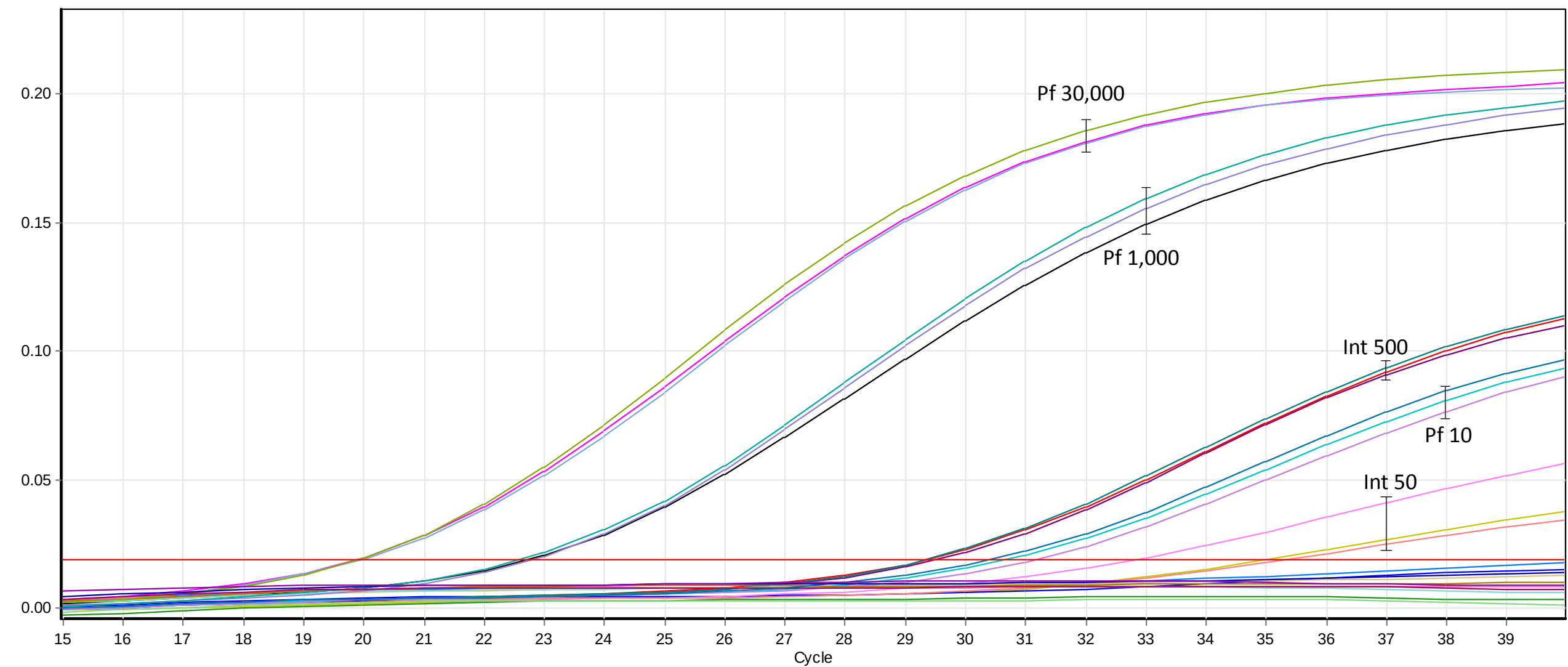
Problem

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

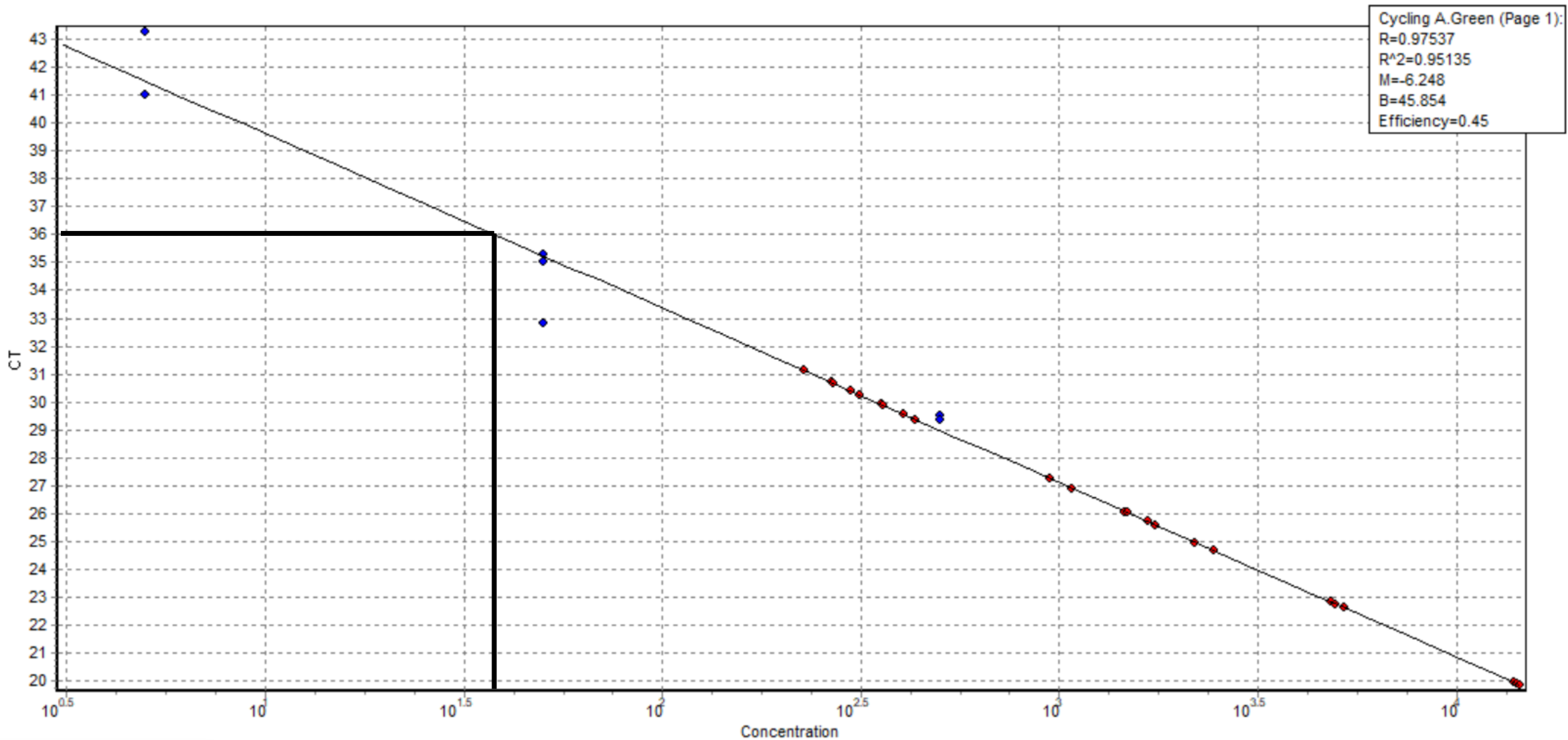
Solution

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.

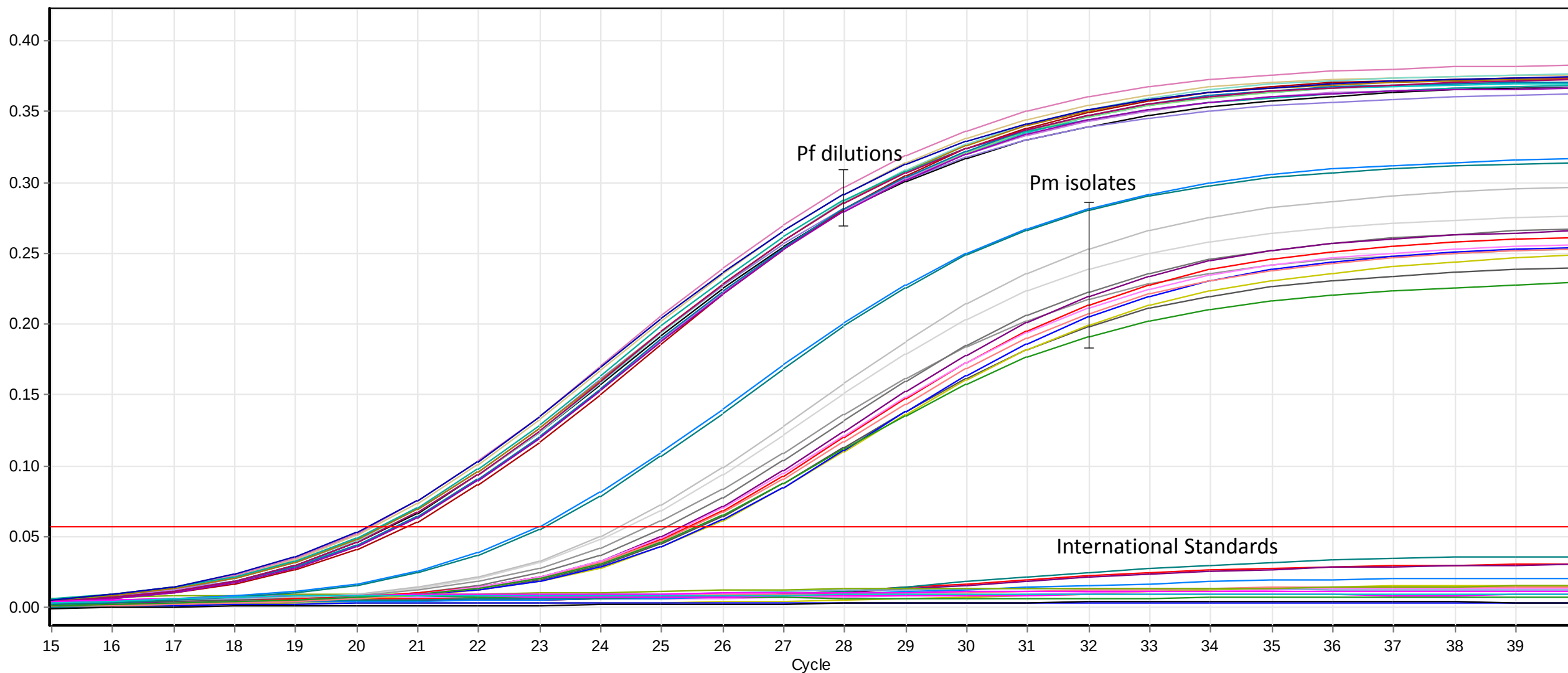
PgMET – Plasmodium Species Quantification



Standard Curve for PgMET Quantification



HumTuBB – Human β Tubulin Quantification

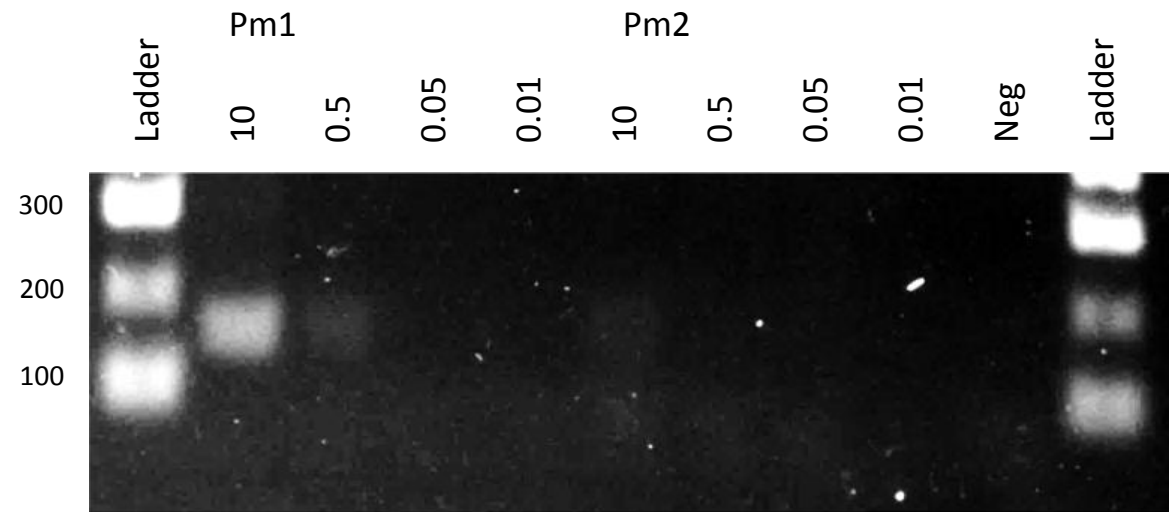
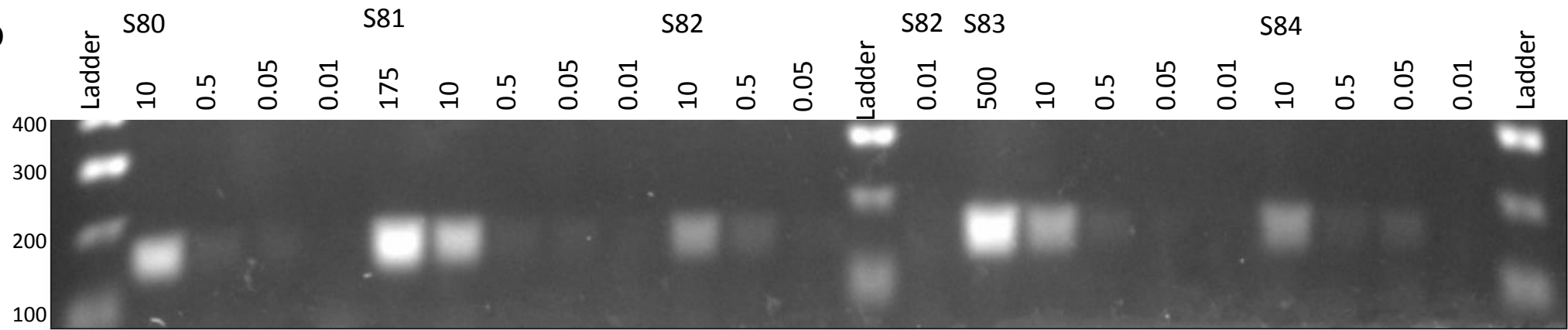


Parasite Density (Parasites/ μ L)

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

$\Delta\Delta$ CT Calculation

GAPDH LOD



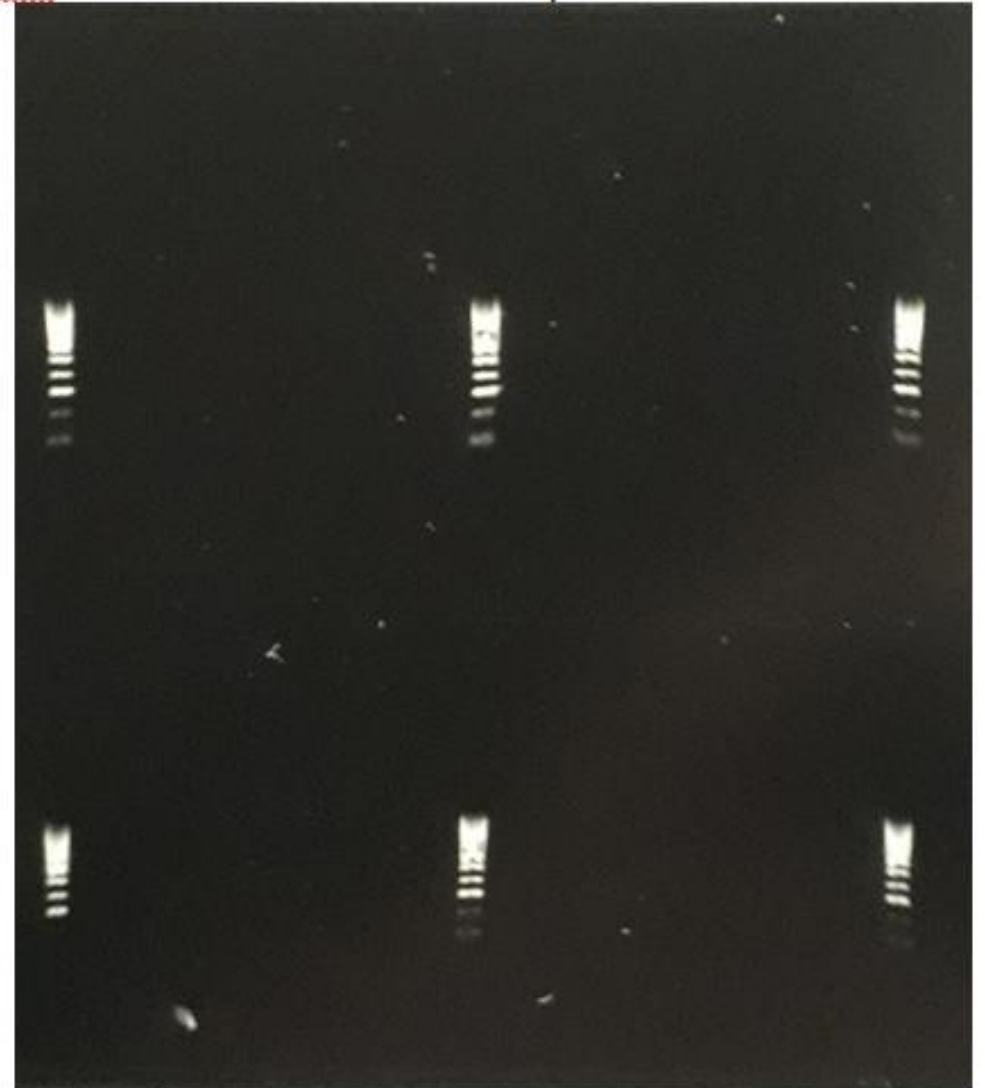
PmGamete and PmMito Temperature Gradients

- 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

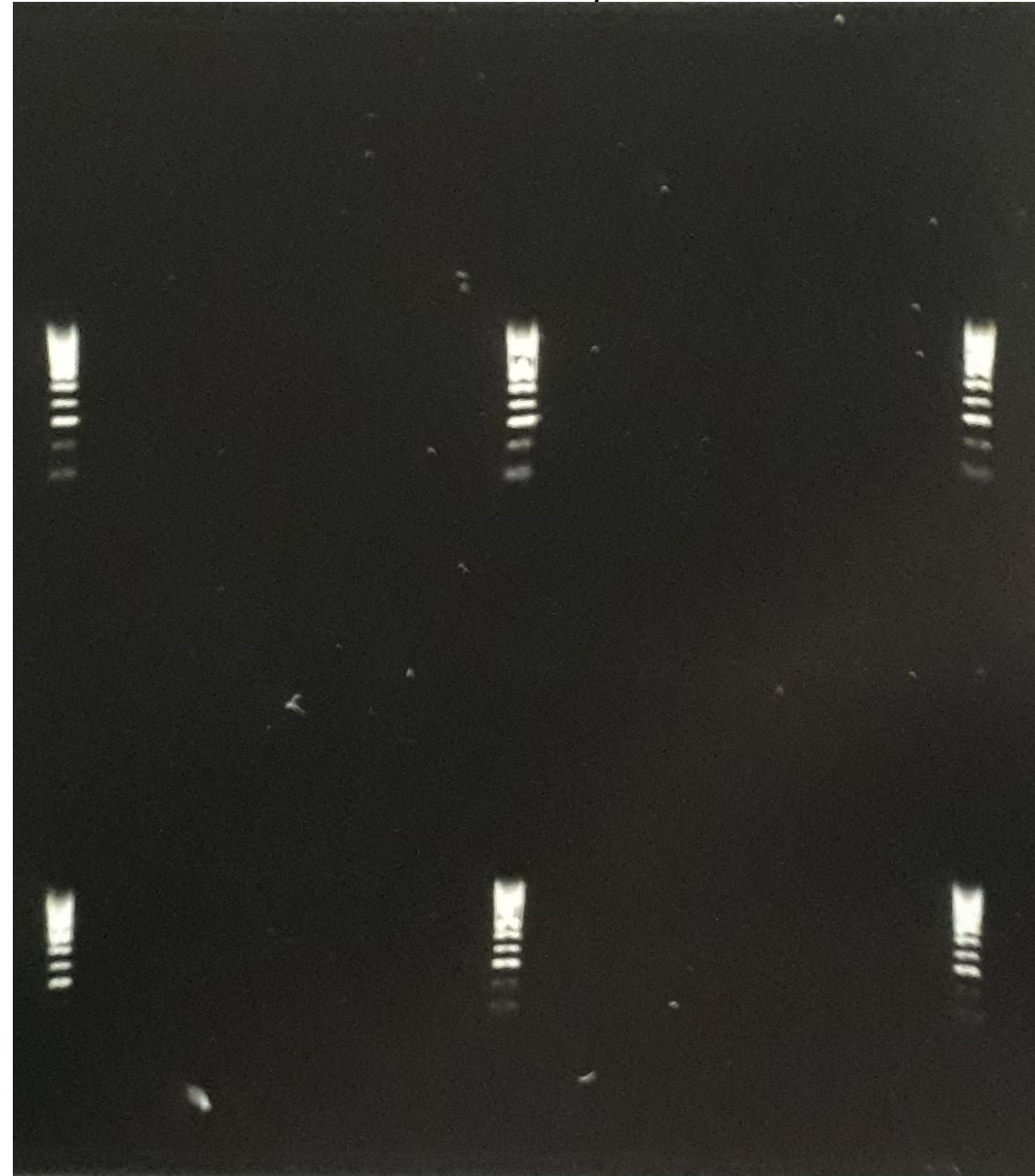
M2



PmMito Nest M1 and M2 Temperature Gradient

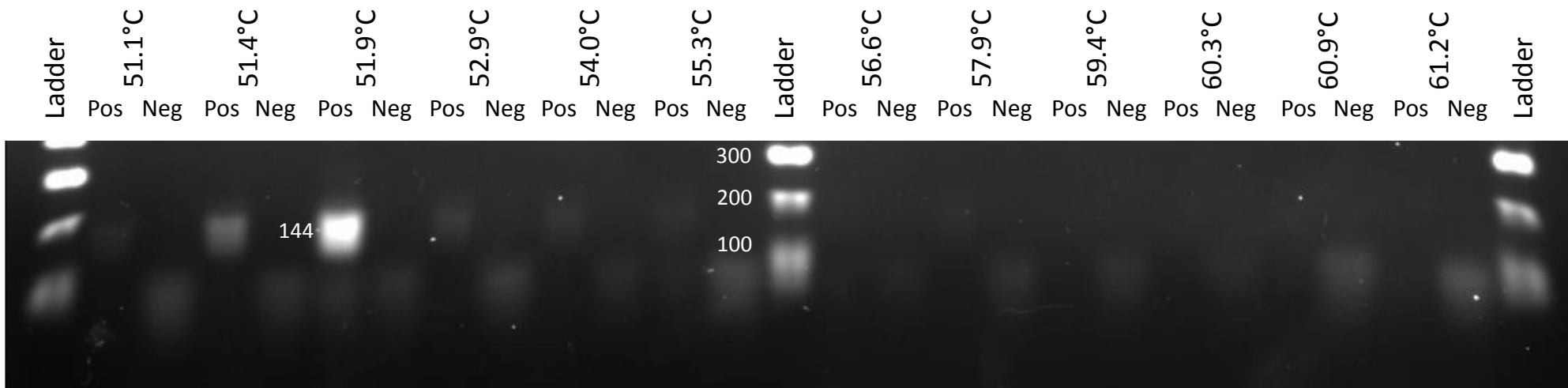
M1

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**

Nest 1

Nest 2

Neg

Pm1

Pm2

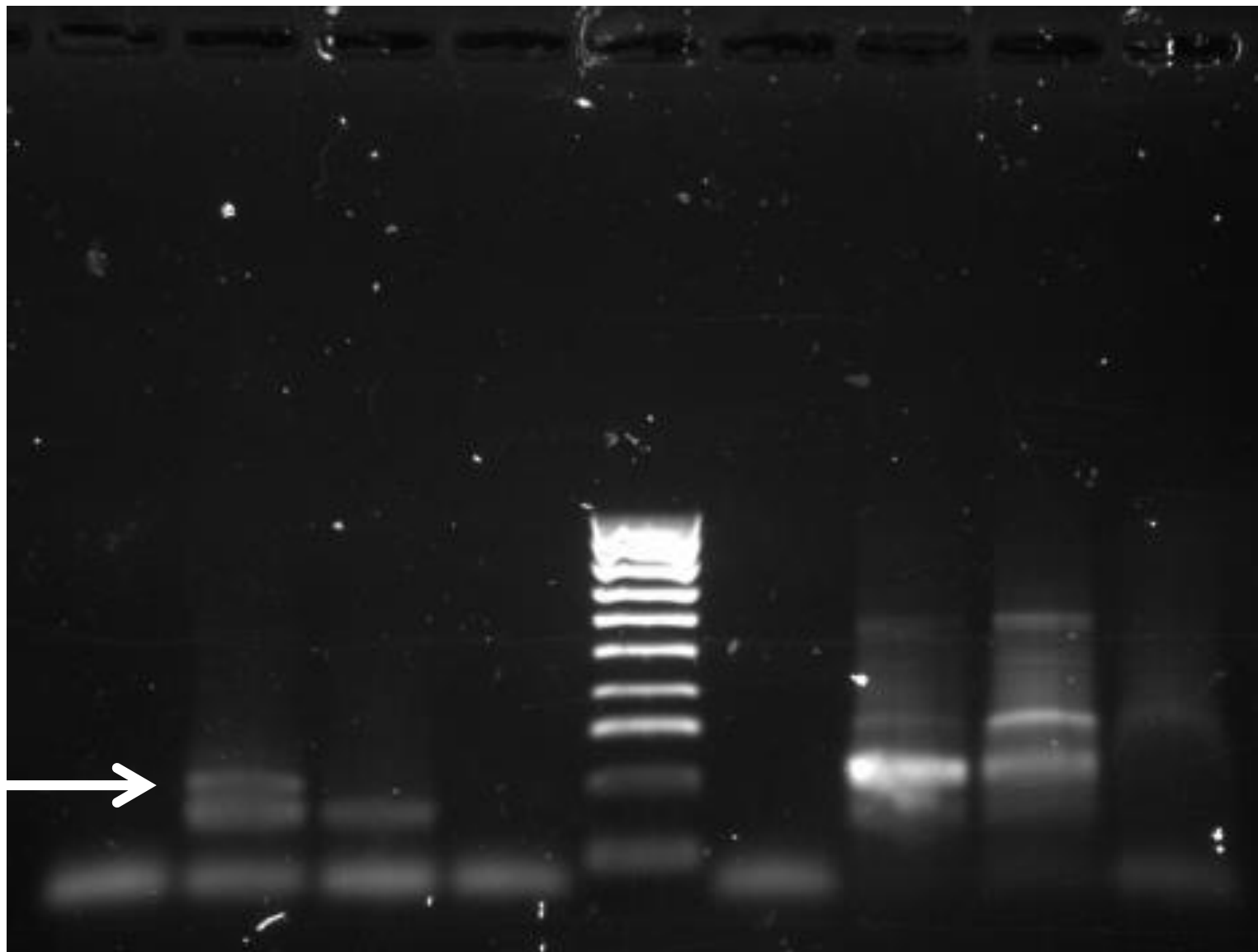
Pf

Neg

Pm1

Pm2

Pf



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.