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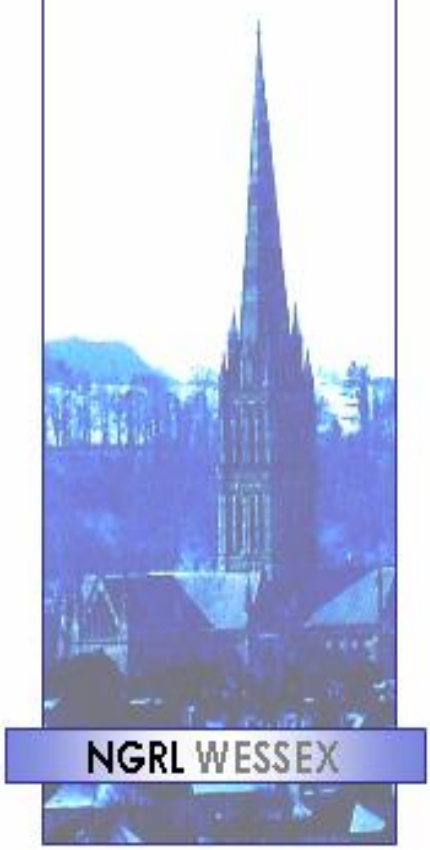
Reference Reagent Report



Plasmid based generic mutation detection reference reagents; production and performance indicator field trial

January 2006

Title	Plasmid based generic mutation detection reference reagents; production and performance indicator field trial
NGRL Ref	NGRLW_GenericRR_1.0
Publication Date	January 2006
Document Purpose	Dissemination of information about generic mutation detection control material
Target Audience	Laboratories performing mutation scanning and field trial participants
NGRL Funded by	

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Review and Approval

The document has been sent to all field trial participants for comments.

Conflicting Interest Statement

The authors declare that they have no conflicting financial interests

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SUMMARY

- To facilitate the evaluation of high throughput mutation detection strategies that are currently being introduced into UKGTN labs NGRL (Wessex) has developed a generic set of reference reagents which can be used to assess both new and existing mutation detection techniques.

- Plasmid controls have been produced which can be used to determine the sensitivity and specificity of these techniques by analysing factors that are of general importance for all technologies including: the type of base substitution, the GC content of the amplicon and the location of the mutation in the fragment.

- The controls can be used to amplify fragments ranging from 400-450bp with an average GC sequence content of 20%, 40%, 60% and 80%. The wild type sequence has been mutated to produce every possible heteroduplex (8 in total) at three positions within the amplicon

- Amplicons from 52 plasmid reagents were produced and sent to 16 laboratories for performance evaluation.

- 5 mutation detection techniques were performed; sequencing, dHPLC, TGCE, CSCE and MALDI-TOF.

- In general, each of the reagents performed satisfactorily however there were variations in the sensitivity and specificity of detection between techniques and also between laboratories using the same techniques.

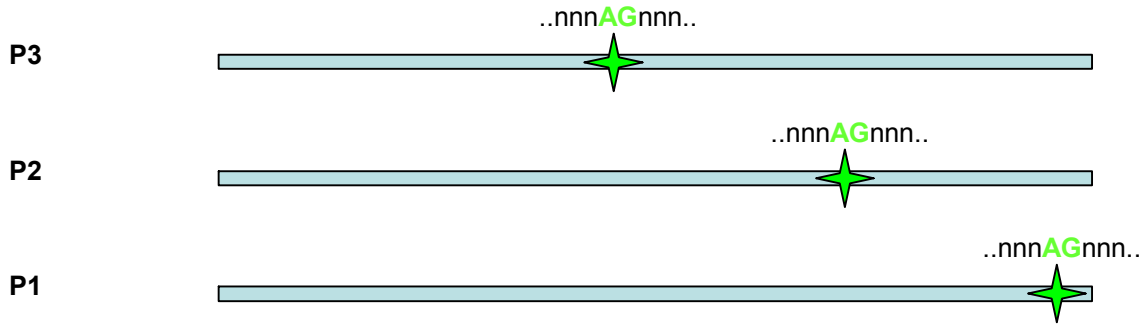
- The findings of this study indicate that this panel of reagents is a useful resource for monitoring the sensitivity and specificity of mutation detection technologies.

- Further work regarding the stability and final format of the reagents is ongoing and as a prelude to establishing these reagents as reference materials.

1. INTRODUCTION

To facilitate the evaluation of high throughput mutation detection strategies that are currently being introduced into UKGTN labs NGRL (Wessex) has developed a generic set of reference reagents which can be used to assess both new and existing mutation detection techniques. Plasmid controls have been produced which can be used to determine the sensitivity and specificity of these techniques by analysing factors that are of general importance for all mutation detection technologies including: the type of base substitution, the GC content of the amplicon and the location of the mutation in the fragment. A similar set of reagents has also been developed by Highsmith *et al.*, 1999.

Genomic DNA fragments ranging from 400-450bp with an average GC content of 20%, 40%, 60% and 80% have been cloned into pUC18 and the wild type sequences have been mutated to produce every possible heteroduplex (8 in total) at three positions within the amplicon as shown in figure 1. The resulting 4 wild type and 48 mutated plasmid constructs can be used to generate amplicons which can be used to investigate whether sequence context, mutation type and fragment length have an effect on the sensitivity and specificity of different mutation detection technologies.



Mutation created	Sequence generated	Heteroduplex produced
A > C	nnnCGnnn	C:T & G:A
A > T	nnnTGnnn	T:T & A:A
G > A	nnnAAnnn	A:C & G:T
G > C	nnnACnnn	C:C & G:G

Figure 1: Four wild type plasmids have been constructed which contain inserts with a 20%, 40%, 60% and 80% GC content. Each of these plasmids has been mutated at three positions within the amplicon (as shown above) to introduce the base changes listed in the table. When the mutated plasmids are mixed with the corresponding wild type plasmid the resulting 48 controls can be used to validate mutation detection techniques by analysing how effectively each of the possible heteroduplex configurations are detected at three different positions within amplicons of varying GC content.

NGRL (Wessex) have conducted a performance evaluation field trial of the reagents by sending samples to 16 laboratories. The reagents were analysed using five mutation detection technologies; sequencing, dHPLC, TGCE, CSCE and MALDI-TOF.

2. PRODUCTION OF GENERIC CONTROL REAGENTS

2.1 Amplification of genomic sequences

10ml peripheral blood was collected from 8 consenting healthy volunteers. DNA was extracted and pooled and genomic sequences with GC contents of 20%, 40%, 60% and 80% were amplified using the primers shown in table 1.

Amplicon	Oligo name	Sequence 5' to 3'
20% GC	MUT_20_F	TGATAAAATGAGTTGAGTATCTTTC
	MUT_20_R	ACTATCCTTTTGTTGTTAATACCTTA
40% GC	MUT_40_F	TGAGATGATGGGGTTTTCTA
	MUT_40_R	GGATGCAAGGCTGGTTC
60% GC	MUT_60_F	AATTTGGCCTCTGGGATGAA
	MUT_60_R	CCCTTTCTCCTTTGGCAATG
80% GC	MUT_80_F	CCGCCGCTCCGAGTGCT
	MUT_80_R	CCCCGCGCTCATCACCTG

Table 1: Sequences of primers used to amplify genomic DNA sequences with a GC content of 20, 40, 60 and 80%

50µl PCR reactions were set up using 100ng genomic DNA with the conditions shown in table 2.

	20% GC	40% GC	60% GC	80% GC
10X Buffer II (Applied Biosystems)	1X	1X	1X	1X
dNTPs (Promega)	0.2mM	0.2mM	0.2mM	0.2mM
AmpliTaq Gold (5U/µl, Applied Biosystems)	1U	1U	1U	1U
$MgCl_2$	2mM	2mM	2.5mM	2mM
Forward PCR primer	10pmol	10pmol	10pmol	10pmol
Reverse PCR primer	10pmol	10pmol	10pmol	10pmol
DMSO	-	-	-	5%
Betaine	-	-	10%	10%

Table 2: PCR conditions used to amplify amplify genomic DNA sequences with a GC content of 20, 40, 60 and 80%

Amplification was carried out using the following cycling conditions:

94°C 7min

94°C 30 sec
60°C 30 sec } 35 Cycles
72°C 1 min

72°C 7 min
15°C Soak

2.2 Cloning and Mutagenesis

PCR amplicons were sub-cloned into the non-proprietary vector pUC18. Site directed mutagenesis of the wild type plasmids was carried out using the QuikChange® Multi Site-Directed Mutagenesis Kit (Stratagene). Degenerate mutagenesis primers were used to mutate AG residues at each site (P1-3) as listed in table 4. Colonies obtained from the mutagenesis reactions were sequenced and clones containing the correct sequence variations were identified. Glycerol stocks of the 52 sequence verified plasmids have been stored at -80°C.

DNA from each plasmid was linearised by restriction enzyme digestion with *HindIII*, quantified and each mutant construct was mixed with an equal number of molecules of wild type plasmid DNA to generate 'heterozygous' samples for each mutation. Plasmids were then diluted to 10^5 copies/µl in 0.1XTE containing 50µg/ml tRNA as a carrier.

2.3 Verification of reagents by Sequencing, dHPLC and CSCE

Plasmid DNA from the 52 constructs was PCR amplified using the primers shown in table 1 with the amplification conditions shown in table 3. Amplicons were then verified by sequencing (appendix 1), dHPLC (appendix 2) and CSCE (appendix 3). dHPLC was performed using the following conditions produced using Navigator software (v 1.6.1):

20%GC: 53°C with timeshift -1
40%GC: 58°C with timeshift +1
60%GC: 62°C with timeshift -1
80%GC: 70°C with timeshift -1

	20% GC	**40% GC**	**60% GC**	**80% GC**
10X Buffer II (Applied Biosystems)	-	1X	1X	1X
10X PCR Buffer (Invitrogen)	1X	-	-	-
dNTPs (Promega)	0.2mM	0.2mM	0.2mM	0.2mM
AmpliTaq Gold (5U/µl, Applied Biosystems)	-	1U	1U	1U
Platinum Taq (5U/µl, Invitrogen)	1U	-	-	-
$MgCl_2$	2mM	2.5mM	1.5mM	1.5mM
Forward PCR primer	10pmol	10pmol	10pmol	10pmol
Reverse PCR primer	10pmol	10pmol	10pmol	10pmol
DMSO	-	-	-	10%
Plasmid DNA	10^5 copies	10^5 copies	10^5 copies	10^5 copies
Cycling parameters	94°C 5min 94°C 30 sec 60°C 30 sec } 40 Cycles 72°C 30sec 72°C 3 min 15°C Soak			

Table 3: PCR conditions and cycling parameters used to amplify PCR products with a GC content of 20, 40, 60 and 80% amplicons from plasmids reagents

Plasmid	Oligo name	Sequence 5' to 3'
20%GC P1	20.1YG	GTCAAATCGATATATAAT**Y**GGCCATGATCTCTATCCTC
20%GC P1	20.1AM	GTCAAATCGATATATAATA**M**GCCATGATCTCTATCCTC
20%GC P2	20.2YG	CTGGCACCCAA**Y**GAATATTTCAATAATGACC
20%GC P2	20.2AM	CTGGCACCCAAA**M**AATATTTCAATAATGACC
20%GC P3	20.3YG	CTTCAGAAATGAATTATATAATTTAAA**Y**GTTTGAACGAATGAAC
20%GC P3	20.3AM	CTTCAGAAATGAATTATATAATTTAAAA**M**TTTGAACGAATGAAC
40%GC P1	40.1GY	TTCCTCTTTTCCTAATTG**Y**ATACCCTTTATTTCCTTCTCCTG
40%GC P1	40.1MA	TTCCTCTTTTCCTAATT**M**AATACCCTTTATTTCCTTCTCCTG
40%GC P2	40.2YG	TATGTTGAATAGG**Y**GTGGTGAGAGAGGGC
40%GC P2	40.2AM	TATGTTGAATAGGA**M**TGGTGAGAGAGGGC
40%GC P3	40.3YG	CAGTTTTTGCCCATTC**Y**GTATGATATTGGCTGTGGG
40%GC P3	40.3AM	CAGTTTTTGCCCATTCA**M**TATGATATTGGCTGTGGG
60%GC P1	60.1YG	AGGTCAGAGTCTGC**Y**GCTTCTGACCATTTTC
60%GC P1	60.1AM	AGGTCAGAGTCTGCA**M**CTTCTGACCATTTTC
60%GC P2	60.2YG	ATAGACGGGGTG**Y**GTAGGTGCCTCTC
60%GC P2	60.2AM	ATAGACGGGGTGA**M**TAGGTGCCTCTC
60%GC P3	60.3YG	CCCTCACACCTAGAG**Y**GGCCCGCCCAC
60%GC P3	60.3AM	CCCTCACACCTAGAGA**M**GCCCGCCCAC
80%GC P1	80.1YG	AGGCCGGC**Y**GAGCCGGCAGGTG
80%GC P1	80.1AM	AGGCCGGCA**M**AGCCGGCAGGTG
80%GC P2	80.2YG	TACCGCTGGTG**Y**GGCGCGCGGC
80%GC P2	80.2AM	TACCGCTGGTGA**M**GCGCGCGGC
80%GC P3	80.3YG	GCTCAACATCCCCAA**Y**GTGCTGCTGCCC
80%GC P3	80.3AM	GCTCAACATCCCCAAA**M**TGCTGCTGCCC

Table 4: Sequences of oligonucleotides used for site-directed mutagenesis. Degenerate primers were used to generate all possible base changes at each position (P1-3) on the wild type plasmids. Mutations were introduced at AG dinucleotides sites in the amplicons. The adenine was mutated using primers with the Y(C/T) degeneracy to generate A to C and A to T mutations (C:T/G:A and A:A/T:T heteroduplexes) and the guanine was mutated using primers with the M (A/C) degeneracy to generate G to A and G to C mutations (A:C/G:T and C:C/G:G heteroduplexes).

3. FIELD TRIAL ORGANISATION

3.1 Aim of the Field Trial

The aim of the field trial was to establish if the panel of plasmids function as useful generic mutation detection reference reagents. It was not the intention of this trial to directly compare mutation detection techniques or laboratory performance.

3.2 Field Trial Participants and Methodologies

Invitations to participate in the field trial were sent out to 32 laboratories (diagnostic and research) on the 20th May 2005 and responses were requested by 17th June. Nineteen replies were received and 16 laboratories agreed to take part. Results were returned by 11 laboratories (appendix 4). Five mutations detection techniques were used; dHPLC (5 labs), CSCE (5 labs), sequencing (2 labs), TGCE (1 lab), MALDI-TOF (1 lab). Three laboratories tested the reagents using two methods.

One laboratory (CSCE) was unable to complete the analysis due to possible degradation of the reagents in transit.

3.3 Field Trial Design

Laboratories responding to the invitation to participate were asked to specify the mutation detection method used and to provide details of any primer modifications. Amplicons were produced with the following modifications :

Sequencing: Amplicons were produced with Uniseq tags and were cleaned up following PCR using Montage Seq_{96} sequencing reaction clean up kit (Millipore).

CSCE: Amplicons were FAM labelled but did not have M13 tags.

dHPLC/TGCE: Amplicons were unlabelled and did not have tags.

MALDI: Amplicons were produced with i)T7F tags plus stuffer sequence and ii) T7R tags and stuffer sequence.

Amplicons were prepared in bulk, pooled and then 20µl of each reagent was aliquoted into two 96 well plates as shown in figure 2. The mutated amplicons were plated out such that each amplicon was analysed once on each plate at a different well location. Amplicons were then air dried by placing the 96 well plates in a laminar flow cabinet overnight.

Amplicons were sent out to field trial participants at the beginning of August 2005. Results were requested to be returned by 9th September 2005 although data were in fact collected until the end of November 2005.

Participants were asked to analyse the amplicons using their usual mutation detection methodology. We provided dHPLC labs with our locally defined dHPLC conditions on a floppy disk along with the wild type amplicons sequences.

The field trial protocol forms are provided in appendix 5.

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Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	**20wt**	wt	P2(A to C)	P2(A to T)	P1(A to T)	P3(G to C)	wt	P3(A to T)	wt	P1(A to C)	P1(G to C)	P2(G to A)
B	**20wt**	wt	wt	P3(A to C)	wt	wt	wt	wt	P2(G to C)	wt	P1(G to A)	P3(G to A)
C	**40wt**	P2(G to C)	P3(A to T)	P3(A to C)	P1(G to C)	P3(G to A)	P2(A to T)	wt	P1(A to T)	wt	wt	wt
D	**40wt**	P1(A to C)	wt	P1(G to A)	wt	wt	P2(G to A)	wt	wt	wt	P3(G to C)	P2(A to C)
E	**60wt**	wt	wt	P2(G to C)	P1(G to A)	wt	wt	P3(G to A)	wt	P3(A to C)	P2(A to T)	wt
F	**60wt**	wt	P1(G to C)	P2(G to A)	wt	wt	P3(A to T)	wt	P1(A to T)	P3(G to C)	P2(A to C)	P1(A to C)
G	**80wt**	P1(A to T)	wt	P3(G to C)	P2(A to T)	wt	wt	P3(G to A)	P1(A to C)	wt	P3(A to C)	P2(G to C)
H	**80wt**	wt	wt	P1(G to A)	P2(A to C)	wt	P2(G to A)	wt	P1(G to C)	P3(A to T)	wt	wt

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	**20wt**	P1(G to A)	wt	P3(G to C)	P1(A to C)	wt	wt	wt	wt	wt	wt	P3(A to C)
B	**20wt**	P2(A to T)	P2(G to C)	P3(A to T)	P2(A to C)	wt	P3(G to A)	wt	P1(G to C)	wt	P2(G to A)	P1(A to T)
C	**40wt**	wt	P1(G to C)	wt	wt	P1(A to T)	P2(A to T)	P2(G to A)	wt	P1(G to A)	wt	wt
D	**40wt**	wt	wt	P3(A to C)	P3(A to T)	P3(G to A)	P2(A to C)	P2(G to C)	P1(A to C)	wt	P3(G to C)	wt
E	**60wt**	wt	wt	P3(A to C)	wt	wt	P3(G to C)	P1(G to A)	wt	P2(A to T)	wt	wt
F	**60wt**	wt	P3(A to T)	P2(A to C)	P1(A to C)	P2(G to C)	P1(A to T)	wt	P2(G to A)	P3(G to A)	P1(G to C)	wt
G	**80wt**	wt	wt	P1(G to A)	P1(G to C)	wt	wt	P3(A to C)	P2(A to T)	wt	P3(A to T)	P2(G to A)
H	**80wt**	P3(G to C)	P2(G to C)	wt	P1(A to C)	P1(A to T)	P3(G to A)	wt	wt	wt	wt	P2(A to C)

Figure 2: Location of mutated and wild type samples on the two 96 well plates sent out for analysis. Mutated samples were analysed once on each plate in different well locations. Forty eight mutated samples and 40 wild type samples were analysed on each plate. The %GC content for each row is specified by the wild type plasmids in column 1 (wild type reference). For example, Plate 1 well 5C contains the 40%GC plasmid with a G>C mutation at position 1.

4. FIELD TRIAL DATA

All data are summarized in appendix 6. The field trial data have been analysed such that a mutation is considered to be successfully detected if it was either detected on both plates or was detected successfully on one plate and called ambiguously on the other. False positive results (for wild type samples) are given for single results on each plate.

4.1 Performance evaluation of reagents by technique and amplicon type

4.1.1 20% GC rich amplicons

4.1.1.1 dHPLC (5 laboratories – untagged amplicons)

- All mutations were detected at positions 1, 2 and 3 with the exception P1 A>T, P2 A>T , P2 G>A ,P3 A>T and P3 G>A which were not detected by lab 8.
- Four false positive results were obtained for the wild type amplicons: 2 ambiguous calls from lab 7, an ambiguous call from lab 4 and a true false positive result from lab 8.
- All reagents performed successfully in at least 4 laboratories.

4.1.1.2 CSCE (4 laboratories – FAM labelled amplicons)

- All mutations were detected at positions 1, 2 and 3 with the exception of P1 A>C, P1 G>A or P2 G>A which were not detected by lab 5.
- Nine false positive results were obtained for the wild type amplicons: 1 ambiguous call from lab 9, 1 ambiguous call, 7 true false positive results and one failed reaction from lab 5.
- All reagents performed successfully in at least 3 laboratories

4.1.1.3 Sequencing (2 laboratories – UniSeq tagged amplicons)

The 20% amplicons sequenced poorly in both laboratories. Several reactions failed and the sequencing data produced was generally too poor to interpret although attempts were made to score mutations where they could be unambiguously detected in one sequencing reaction.

4.1.1.4 TGCE (1 laboratory- untagged amplicons)

- The lab which used TGCE (untagged amplicons) detected all mutations at positions 1, 2 and 3.
- One false positive result was obtained from a wild type amplicon.

4.1.1.5 MALDI-TOF (1 laboratory – T7 tagged amplicons)

- The lab which used MALDI-TOF detected all mutations at positions 1, 2 and 3 except P1 G>A. Mutation P2 G>C was detected on plate 1 but missed on plate 2.
- Ten reactions failed for the wild type amplicons.

4.1.2 40% GC rich amplicons

4.1.2.1 dHPLC (5 laboratories – untagged amplicons)

- All mutations were detected at positions 1 and 3. Detection of mutations at position 2 was more variable:

  P2 G>C: Detected by all labs, but three labs classed this as an ambiguous call.

  P2 A>C: Detected by 2 labs (1 and 4) but undetected by labs 7 and 8. Lab 3 detected the mutation (as an ambiguous call) on plate 2 only.

  P2 A>T: Detected by 2 labs (1 and 4) although lab 4 reported this as ambiguous for plates 1 & 2. Labs 3 and 7 detected the mutation (ambiguous call) on one plate only. Lab 8 did not detect the mutation on either plate.

  P2 G>A: Detected by 3 labs (1, 3 and 4) but undetected by 2 labs (7 and 8).

- Wild type amplicons were correctly assigned.

- Reagents performed successfully in at least 2 labs.

4.1.2.2 CSCE (4 laboratories – FAM labelled amplicons)

- All mutations were detected at positions 1, 2 and 3 with the exception of P1 A>C which was detected (ambiguous call) on plate 2 only by labs 5 and 10.

- Two ambiguous calls were obtained for wild type amplicons and four wild type reactions were assigned as failed.

- All reagents performed successfully in 4 labs

4.1.2.3 Sequencing (2 laboratories)

- All mutations were detected and correctly assigned for positions 1, 2 and 3. There were no false positive results.

4.1.2.4 TGCE (1 laboratory)

- All mutations were detected at positions 1, 2 and 3 although P1 A>T was not detected on plate 1.

- Nine false positive results were obtained for wild type amplicons: 5 ambiguous calls and 4 true false positives.

4.1.2.5 MALDI-TOF (1 laboratory)

- All mutations were detected at positions 2 and 3 although mutation P2 G>A was not detected on plate 1 and mutation P3 G>C was not detected on plate 2. For position 1:

  P1 G>C: Failed for plate 1 and was not detected on plate 2

  P1 A>C: Not detected

  P1 A>T: Failed for plate 1 but detected on plate 2

  P1 G>A: Not detected on plate 1 and ambiguous call on plate 2

- One ambiguous call was made for a wild type amplicon and two wild type reaction failed.

4.1.3 60% GC rich amplicons

4.1.3.1 dHPLC (5 laboratories – untagged amplicons)

- All mutations were detected for positions 1, 2 and 3 although lab 8 classed the detection as ambiguous in all cases (plate 2) and did not detect P3 A>C. Plate 1 failed completely for lab 8 (although wild types were successfully detected) and lab 7 reported failures for P2 G>C, P3 G>A and a wild type amplicon.
- Five false positive results were obtained for wild type amplicons (lab 3, plate 2).
- All reagents performed successfully in at least 2 labs.

4.1.3.2 CSCE (4 laboratories – FAM labelled amplicons)

- All mutations were detected for positions 1, 2 and 3.
- Seven false positive results were obtained for wild type amplicons: 4 ambiguous calls from lab 5 and 3 ambiguous calls from lab 9.
- All reagents performed successfully in 4 labs

4.1.3.3 Sequencing (2 laboratories)

- All mutations were detected and correctly assigned for positions 1, 2 and 3. There were no false positive results.

4.1.3.4 TGCE (1 laboratory)

- All mutations were detected at positions 1, 2 and 3 for plate 1. All reactions failed on plate 2.
- Ten false positive results were obtained for wild type amplicons: 6 true false positives and 3 ambiguous calls.

4.1.3.5 MALDI-TOF (1 laboratory)

- All mutations were detected at position 2 and 3 with the exception of P2 G>A. For position 1:

  P1 G>C: Ambiguous on plate 1 and detected on plate 2

  P1 A>C: Not detected on plate 1 but detected on plate 2

  P1 G>A: Detected on plates 1 and 2

- Two wild type reactions failed

4.1.4 80% GC rich amplicons

4.1.4.1 dHPLC (5 laboratories – untagged amplicons)

- All mutations were detected for positions 2 and 3 (all labs). Detection of mutations at position 1 were variable:

  P1 G>C: Detected by all labs although lab 7 classed this as an ambiguous call

  P1 A>C: Detected by 3 labs (1, 3 and 4). Not detected by lab 7. Detected in plate 1 only (ambiguous) by lab 8.

  P1 A>T: Detected by 3 labs (1, 3 and 4). Not detected by labs 7 and 8

  P1 G>A: Detected by 4 labs (1, 3, 4 and 8). Detected in plate 1 only (ambiguous) by lab 7.

- Two wild type amplicons failed.

- All reagents performed successfully in at least three labs

4.1.4.2 CSCE (4 laboratories – FAM labelled amplicons)

- All mutations were detected at positions 2 and 3 with the exception of P2 A>T and P3 G>C which were not detected by lab 9. Detection of mutations at position 1 were variable:

  P1 G>C: Detected by all labs. 3 labs classed this as an ambiguous call on at least one plate (labs 5, 9 and 10).

  P1 A>C: Detected by 3 labs (labs 4 and 10 ambiguous calls). Undetected by lab 9

  P1 A>T: Detected by 3 labs (ambiguous calls). Undetected by lab 9

  P1 G>A: Undetected by all labs although lab 5 reported an ambiguous call for plate 2.

- Eight false positive results were obtained for wild type amplicons: 3 ambiguous (lab 5), 1 true false positive (lab 9), 4 ambiguous (lab 10). Five wild type amplicon were scored as failed (lab 10).

4.1.4.3 Sequencing (2 laboratories)

- All mutations were detected and correctly assigned for positions 1, 2 and 3. There were no false positive results

4.1.4.4 TGCE (1 laboratory)

- All mutations were detected at positions 1, 2 and 3 although P1 A>C and P1 G>A were only detected on plate 1.

- Six false positive results were obtained for wild type amplicons: 2 true false positives and 4 ambiguous calls.

4.1.4.5 MALDI-TOF (1 laboratory)

- All mutations were detected at positions 2 and 3 although P2G>C was not detected on plate 1. The only mutation detected at position 1 was P1 A>T.

- 3 wild type reactions failed.

4.2 Evaluation of mutation detection techniques

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and efficiency of mutation detection for each technique and each laboratory is shown in table 5. The data has been analysed for each individual well on plates 1 and 2.

Lab ID	Method	TP	FP	FN	TN	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Efficiency (%)	Failure rate (%)
1	dHPLC	96	0	0	80	100	100	100	100	100	0
3	dHPLC	94	6	2	74	98	93	94	97	95	0
4	dHPLC	95	0	0	80	100	100	100	100	100	1
7	dHPLC	83	2	10	75	89	97	98	88	93	3
8	dHPLC	62	1	22	79	74	99	98	78	86	7
4	CSCE	94	0	2	80	98	100	100	98	99	0
5	CSCE	83	16	13	60	86	79	84	82	83	2
9	CSCE	89	6	7	73	93	92	94	91	93	1
10	CSCE	93	4	3	70	97	95	96	96	96	3
6	Seq	85	1	1	74	99	99	99	99	99	9
8	Seq	93	1	2	79	98	99	99	98	98	1
9	TGCE	83	26	5	54	94	68	76	92	82	5
2	MALDI	75	1	18	56	81	98	99	76	87	11

Table 5: Sensitivity and specificity of mutation detection for each laboratory. TP = true positive result (includes ambiguous calls), FP = false positive result (includes ambiguous calls), FN = false negative result, TN = true negative result. Sensitivity = TP/(TP+FN), Specificity = TN/(TN+FP), positive predictive value (PPV) = TP/(TP+FP), negative predictive value (NPV) = TN/(TN+FN), Efficiency = (TP+TN)/(TP+FP+FN+TN). The failure rate was calculated as the number of individual amplicon failures / total number of samples analysed (n=176). All percentages have been rounded to the nearest whole number.

4.3 Comments from field trial participants

4.3.1 Labs using unlabelled amplicons (dHPLC and TGCE)

40% and 60% GC reactions worked poorly for some labs (particularly 60% GC on plate 2) and some samples had denatured completely at the run temperatures recommended. Some labs commented that the concentration of the amplicons was too low for good dHPLC analysis (signal often <2mV) and that more product would have been useful to carry out temperature gradients.

4.3.2 Labs using FAM labelled amplicons (CSCE)

Some products were too strong for analysis and required diluting

4.3.3 Labs using UniSeq tagged amplicons (Sequencing)

20% amplicons were reported as too weak to analyse and sequenced poorly although other amplicons sequenced well. The data presented in appendix 6 has been scored where the mutations was unambiguously visible on one direction of sequencing.

4.3.4 Lab using T7 tagged amplicons (MALDI-TOF)

The PCR products supplied were produced using lower cycle number than the laboratory's normal protocol and so were lower than optimal concentration. The 20% GC amplicons failed quality thresholds due to deviation from the study protocol (plate 1 products were cleaned prior to MALDI analysis). Because this was a field trial, samples were put through the system once only, with no reworking of failures, and analysed manually which is not the case in diagnostic work.

4.3.5 General comments

Several labs commented that the trial was well organized and the protocols and reagents were easy to use. Labs suggested that the timing of the trial running over the summer break was not ideal. In general participants found the reagents useful for evaluating how well their mutation detection method was detecting different mutations. Labs evaluating two techniques pointed out the potential for 'cheating' as the randomization was uniform for all technologies!

5. DISCUSSION

In general, the reagents performed well in most laboratories and comments from participants suggest that the reagents are a useful resource for evaluating the sensitivity and specificity of laboratory mutation detection systems. Although this field trial was not designed to compare different mutation detection techniques or laboratory performance, it is notable that the sensitivity and specificity of mutation detection showed marked variation ranging from 75% - 100% and 68 – 100% respectively. However, it must be stressed that the reagents supplied were not optimal for all systems

Further work will need to be performed on the reagents to ensure consistent product quality. Although plates were prepared from a single stock several labs reported problems will specific amplicons, particularly the 20% amplicons supplied for sequencing and the 60% GC untagged amplicons on plate 2. It is possible that degradation of the amplicons had occurred in transit and oversea labs especially reported that the amplicons were weak. More work will be carried out to address the best format for reference material production *i.e*. whether the reagents should be supplied as air dried or liquid amplicons or as liquid plasmid stocks for labs to PCR 'in house'. Optimisation of the specific concentrations of amplicons required for the different techniques will also need to be clarified as many labs found dHPLC products to be too weak and CSCE products to be too strong. The reagents as also being adapted to enable shorter amplicons to be produced as these may be more optimal for some mutation detection technologies.

6. ACKNOWLEDGEMENTS

We would like to thank

- all field trial participants for their assistance with this project
- Ross Hawkins, Elaine Gray and Paul Metcalfe (NIBSC) for advice on field trial design.

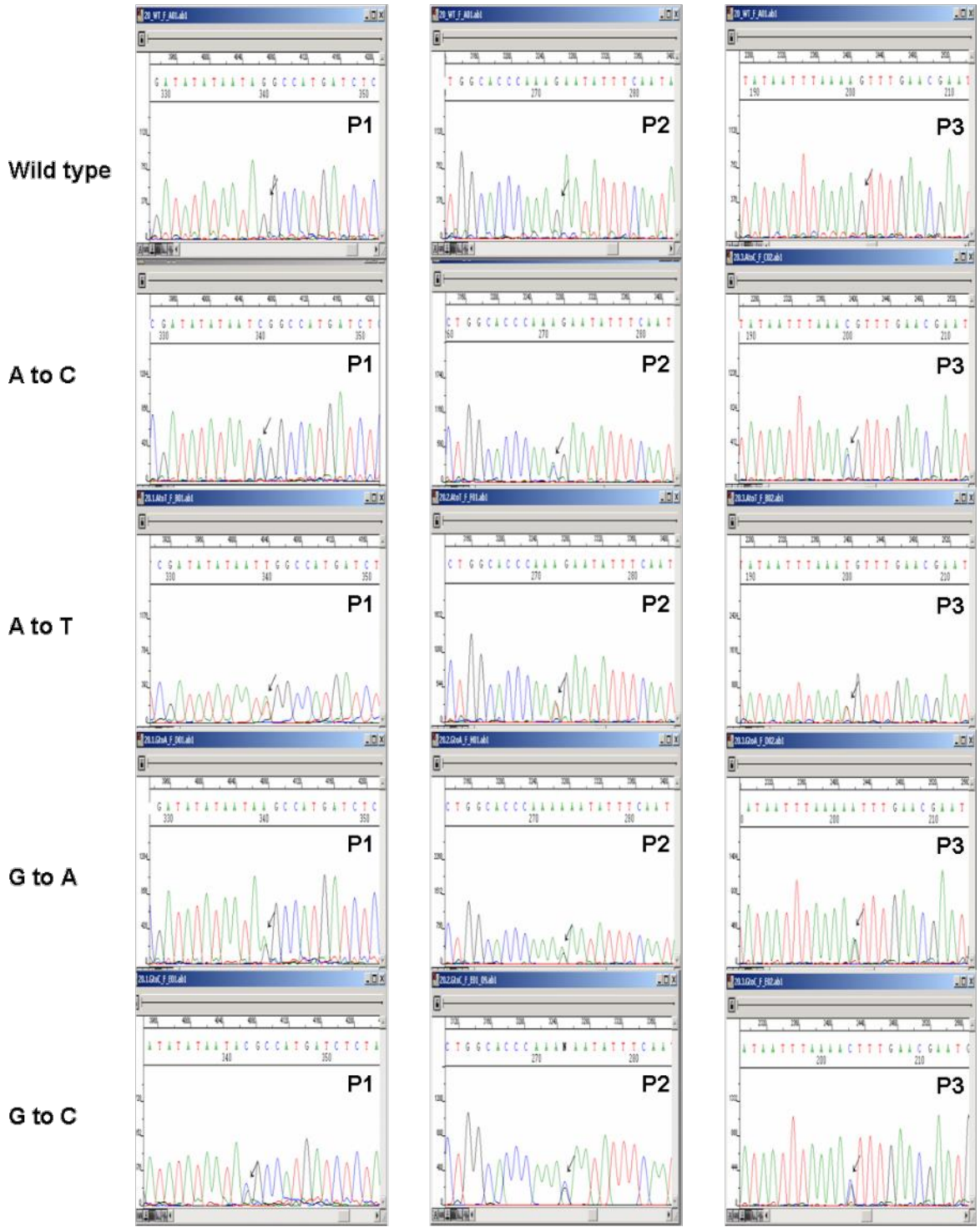
7. REFERENCES

Highsmith WE Jr, Jin Q, Nataraj AJ, O'Connor JM, Burland VD, Baubonis WR, Curtis FP, Kusukawa N, Garner MM. Use of a DNA toolbox for the characterization of mutation scanning methods. I: construction of the toolbox and evaluation of heteroduplex analysis. Electrophoresis. 1999 Jun;20(6):1186-94.

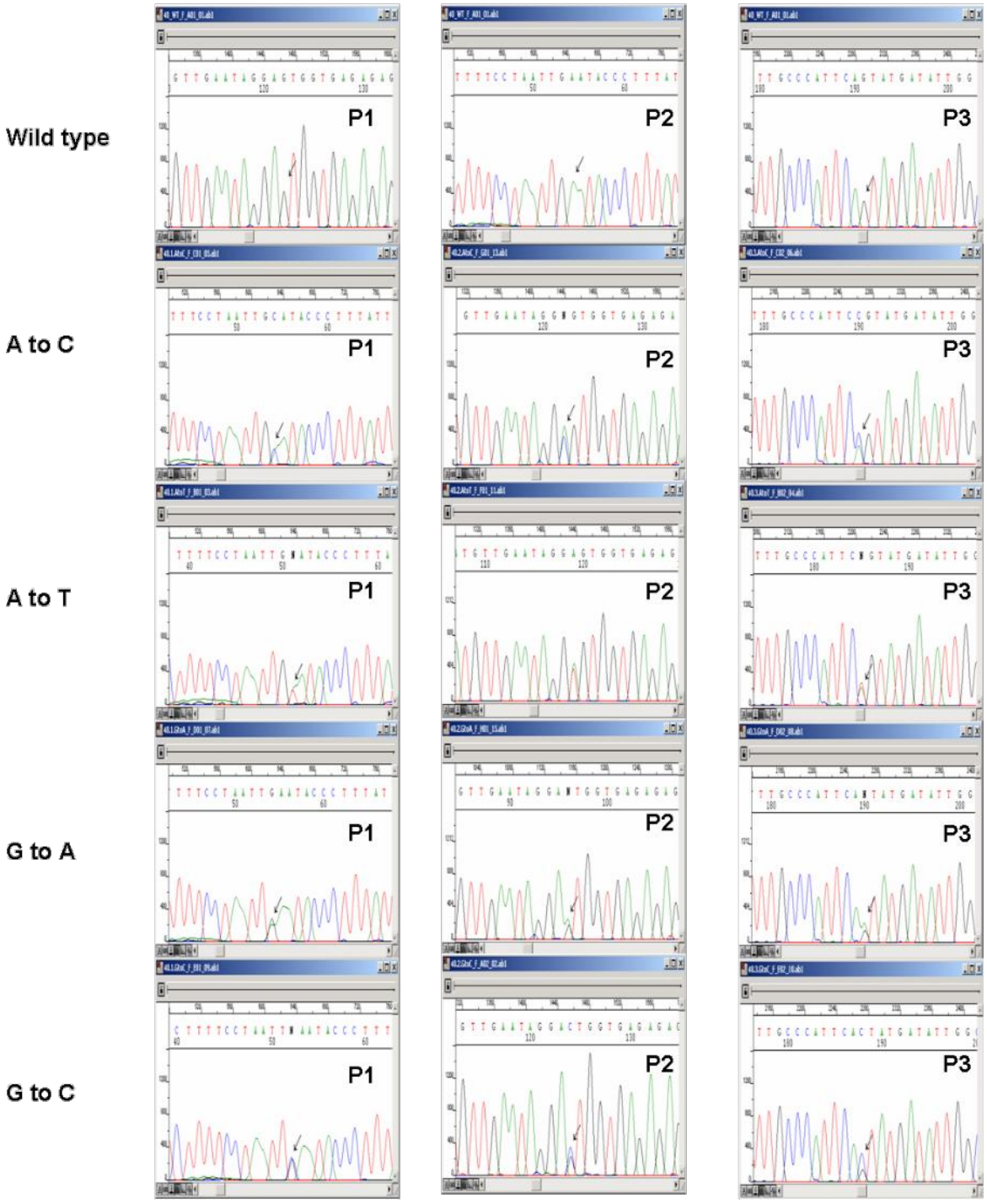
APPENDIX 1

SEQUENCING TRACES (REAGENT VALIDATION)

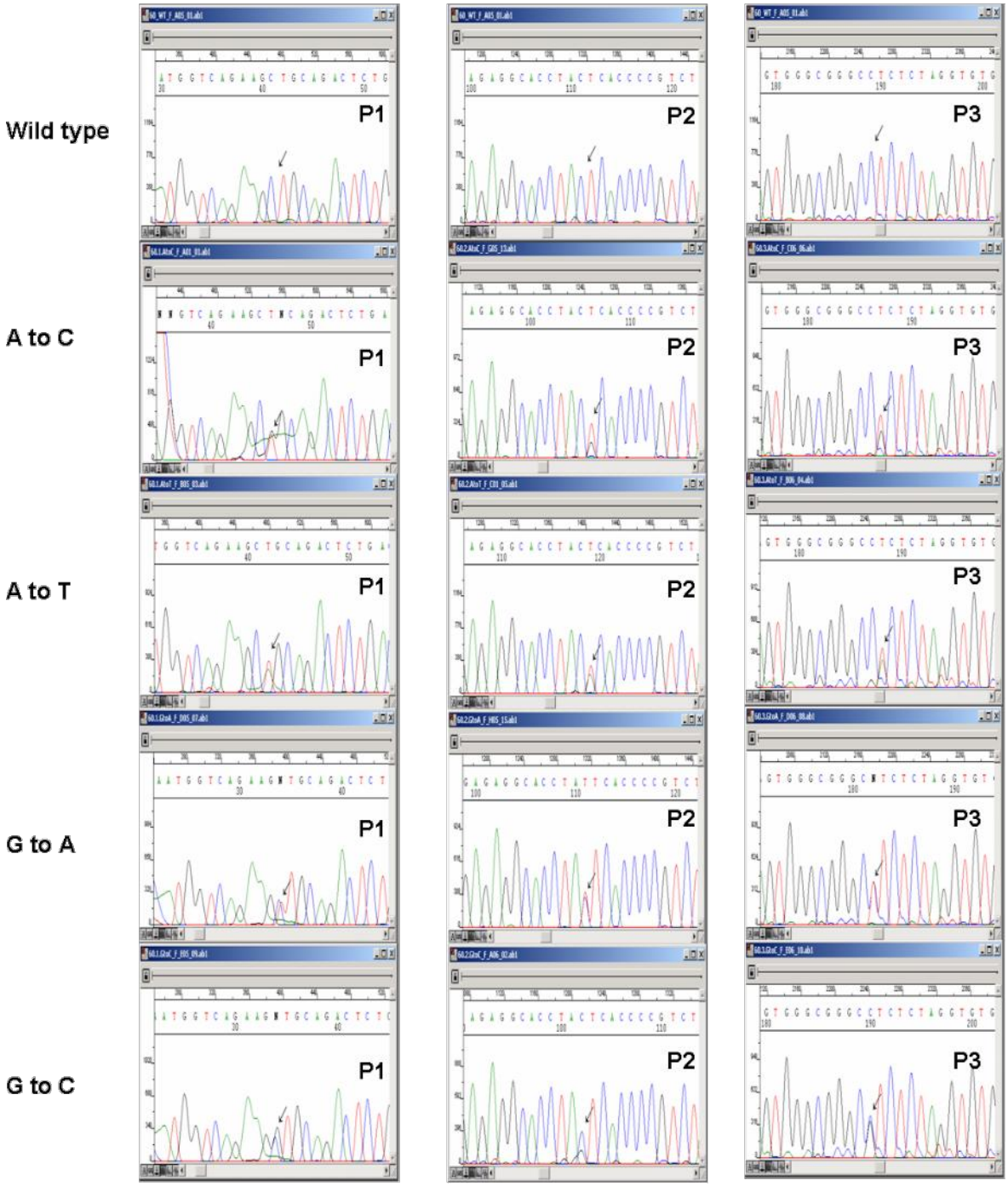
20% GC



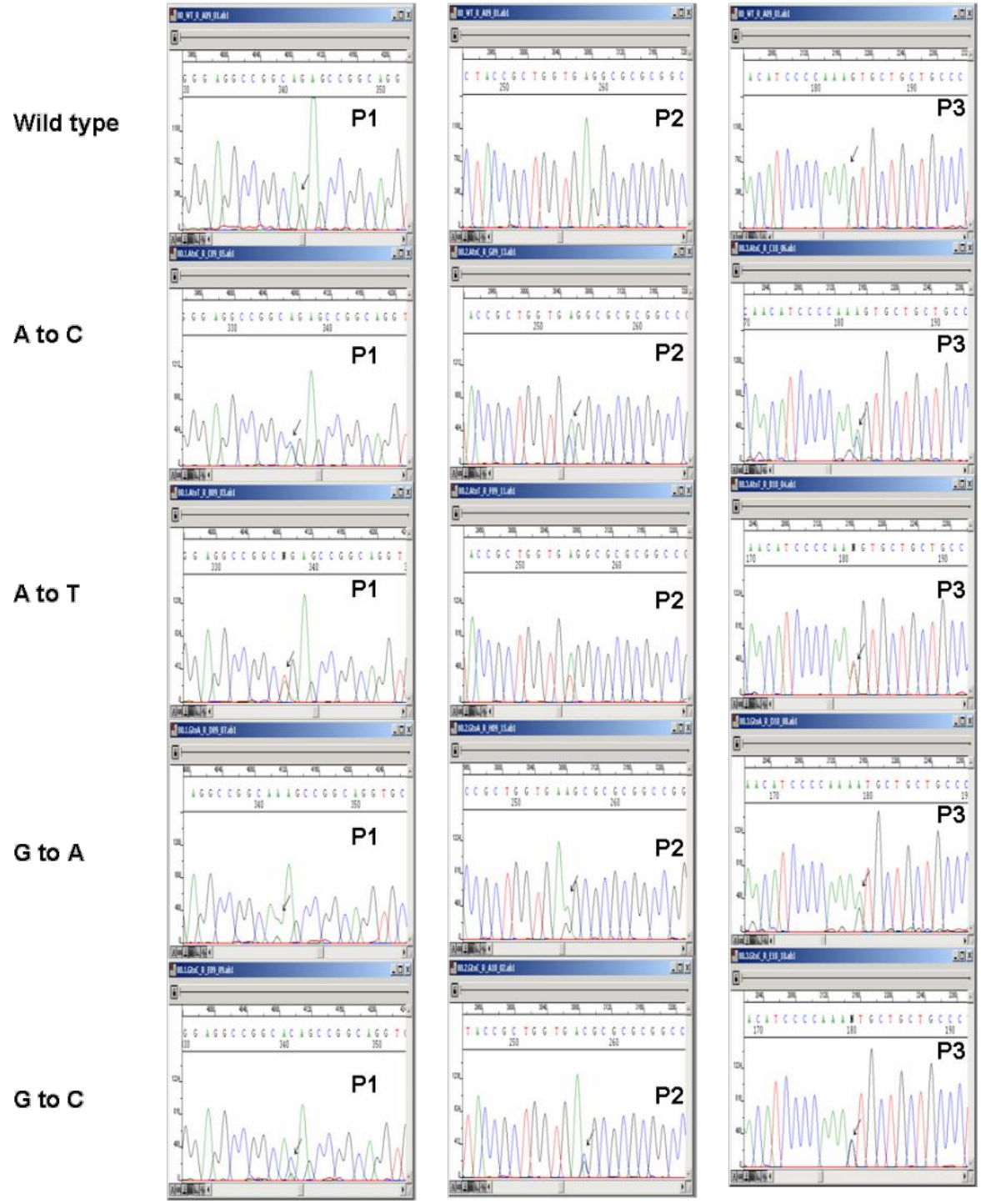
40% GC



60% GC



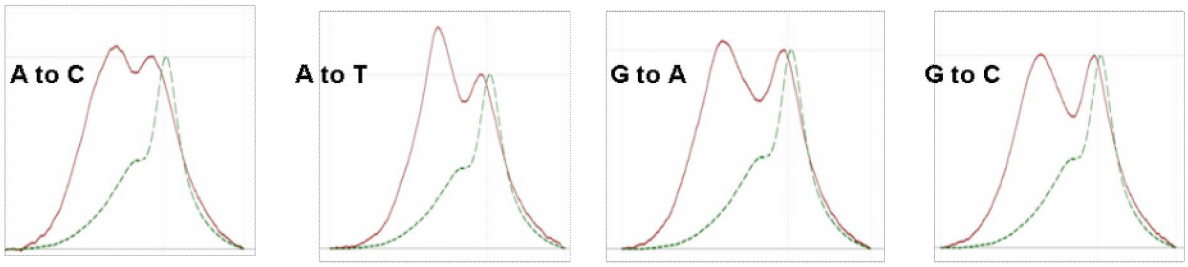
80% GC



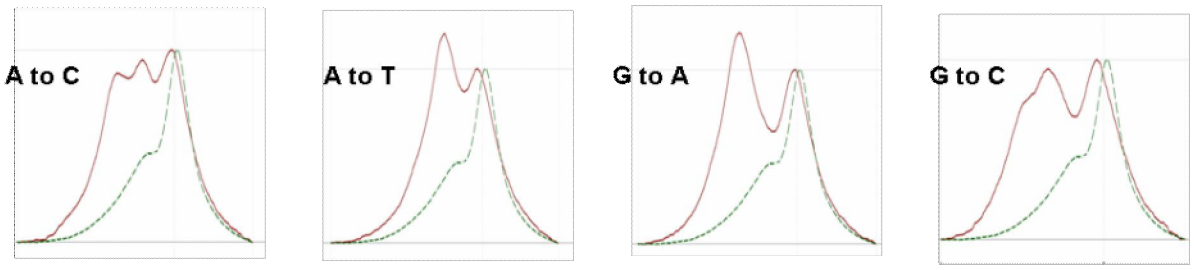
APPENDIX 2

DHPLC TRACES (REAGENT VALIDATION)

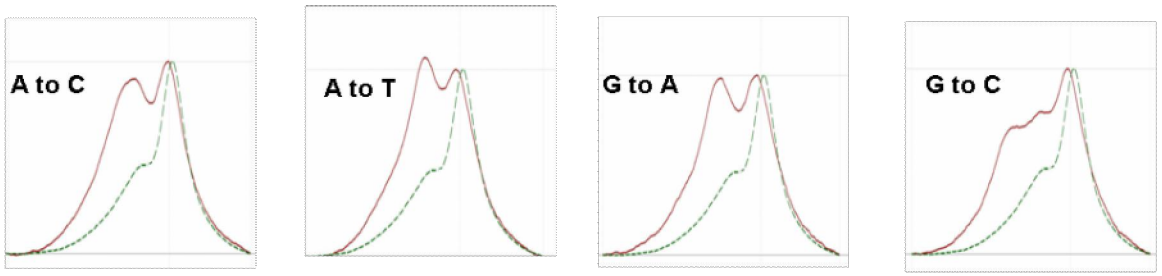
20% GC Position 1

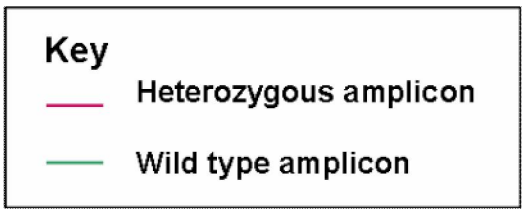


20% GC Position 2

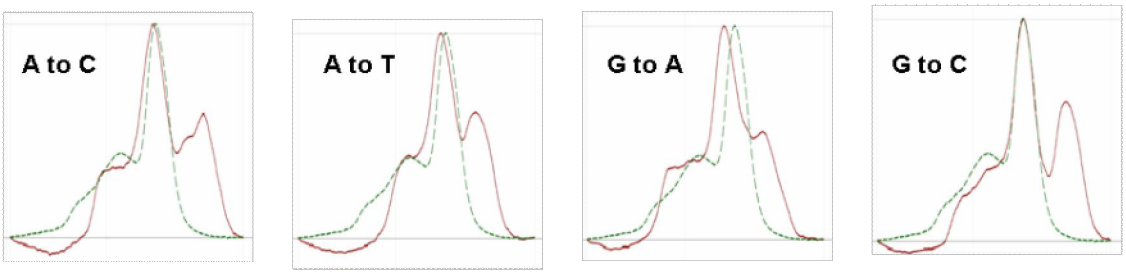


20% GC Position 3

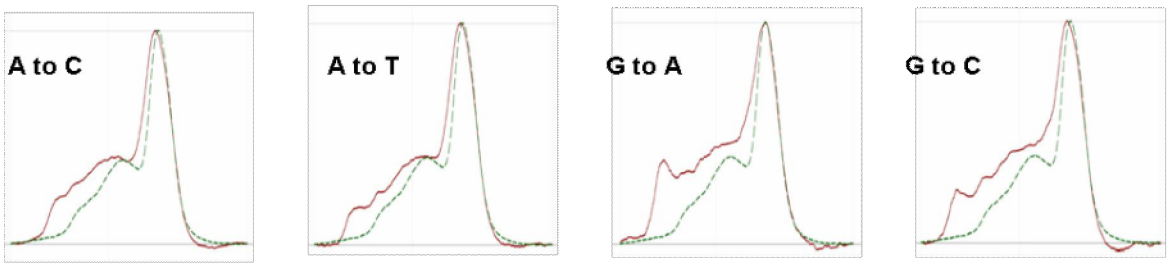




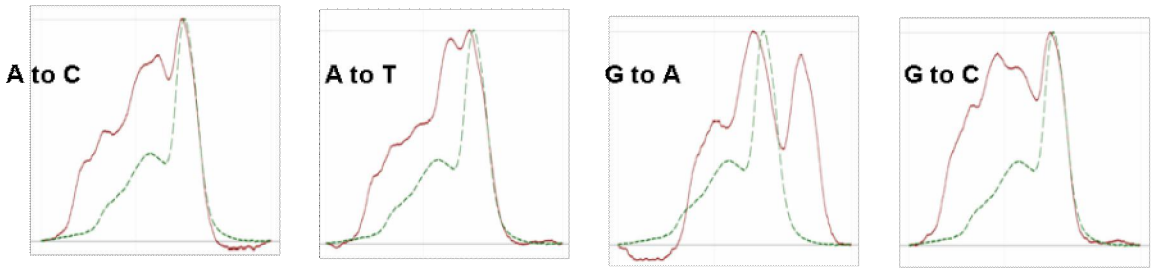
40% GC Position 1

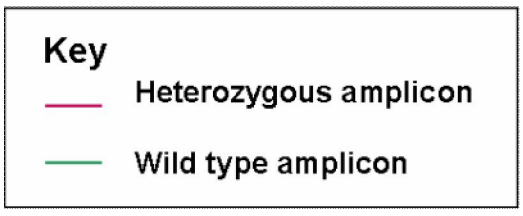


40% GC Position 2

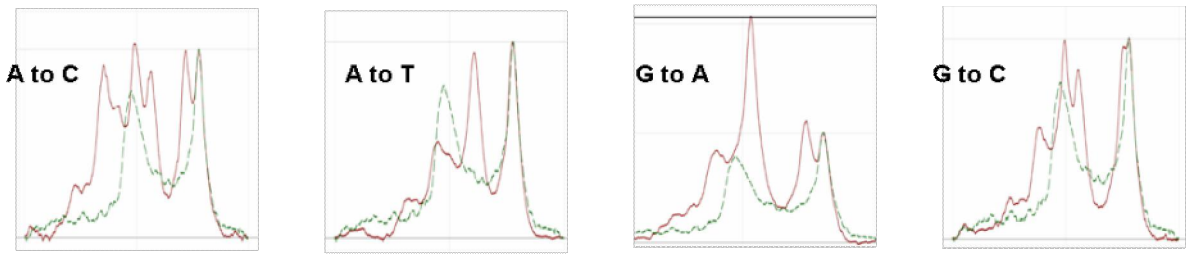


40% GC Position 3

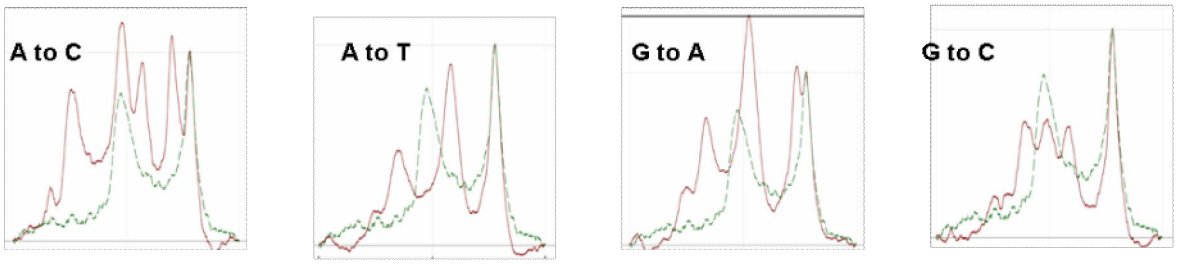




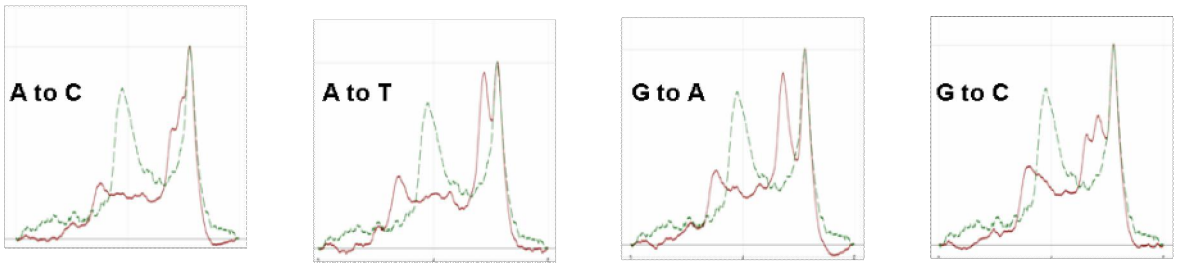
60% GC Position 1

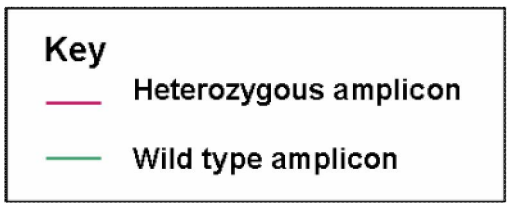


60% GC Position 2

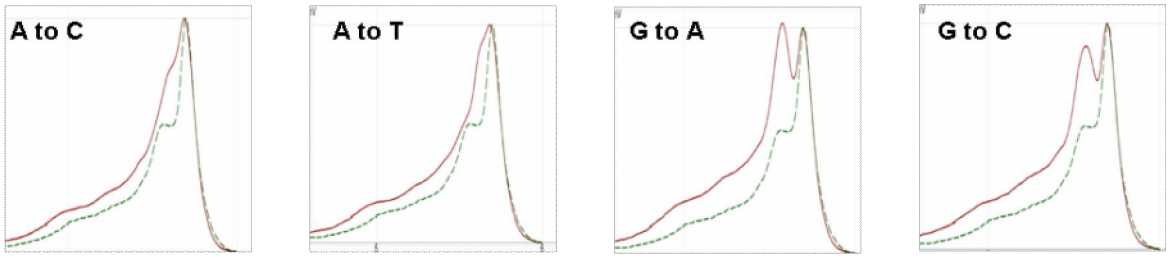


60% GC Position 3

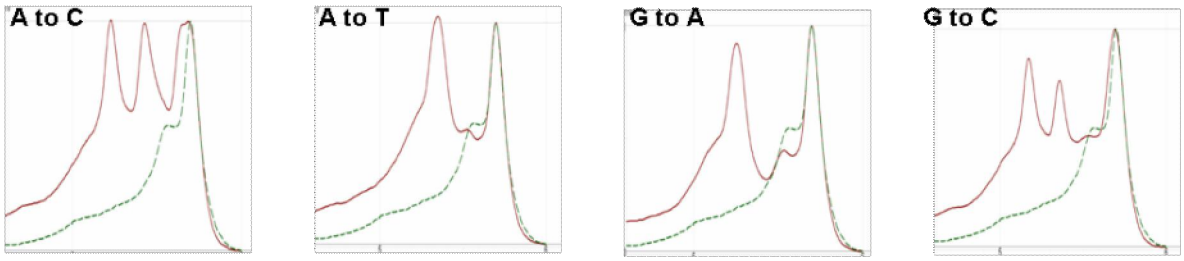




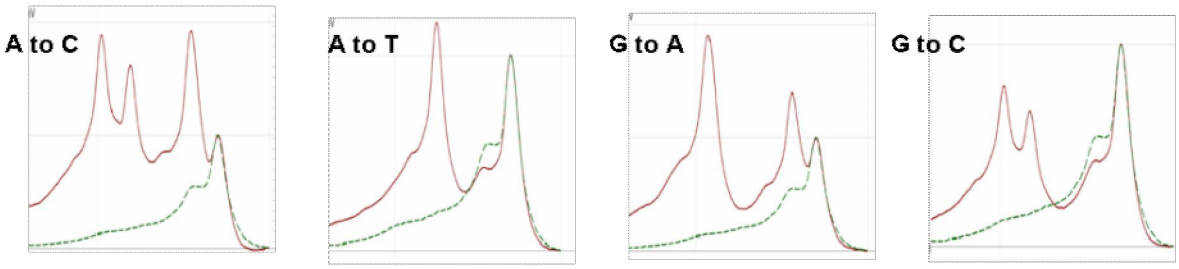
80% GC Position 1

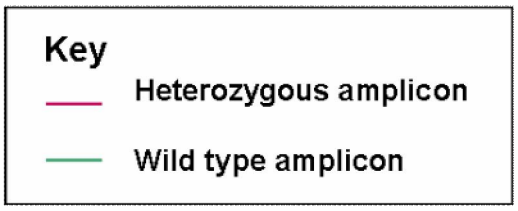


80% GC Position 2

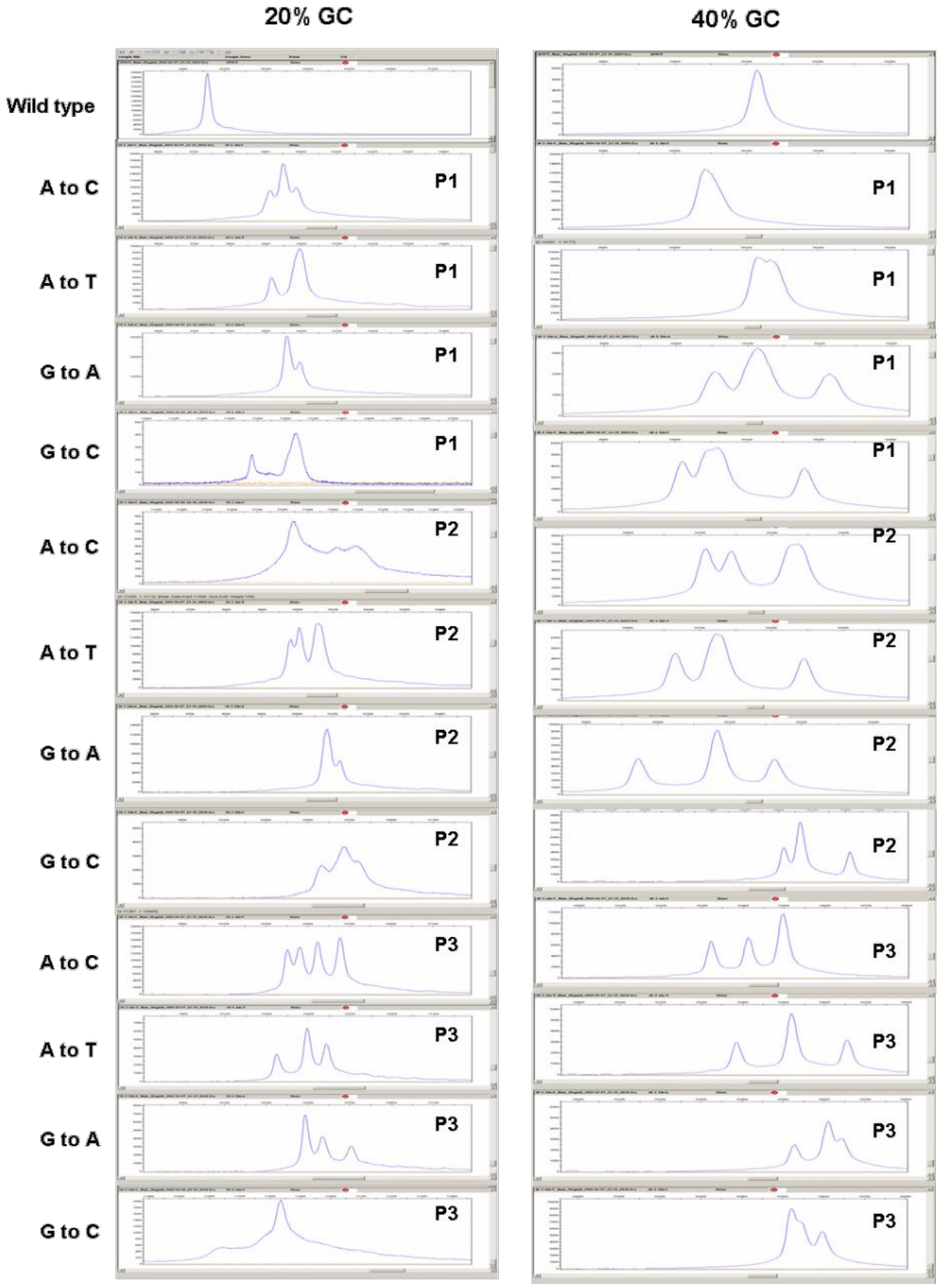


80% GC Position 3





APPENDIX 3
CSCE TRACES (REAGENT VALIDATION)


20% GC
40% GC
Wild type
A to C
A to T
G to A
G to C
A to C
A to T
G to A
G to C
A to C
A to T
G to A
G to C
P1
P1
P1
P1
P2
P2
P2
P2
P3
P3
P3
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P1
P1
P1
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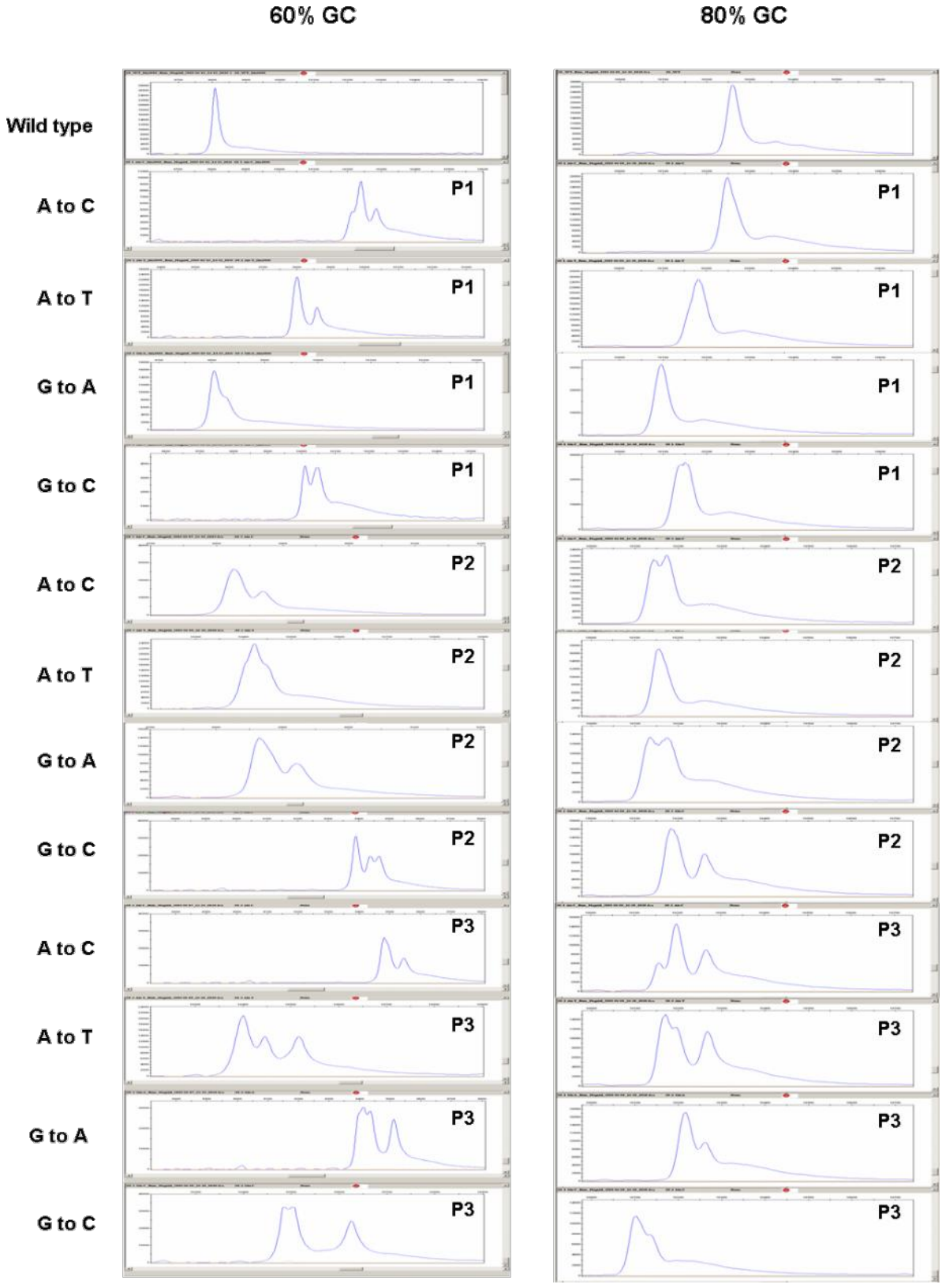
P2
P2
P2
P2
P3
P3
P3
P3


60% GC
80% GC
Wild type
A to C
A to T
G to A
G to C
A to C
A to T
G to A
G to C
A to C
A to T
G to A
G to C
P1
P1
P1
P1
P2
P2
P2
P2
P3
P3
P3
P3

APPENDIX 4

FIELD TRIAL PARTICIPANTS WHO RETURNED RESULTS (alphabetical list)

David Bunyan & Julie Sillibourne, Wessex Regional Genetics, Salisbury District Hospital, Salisbury, SP2 8BJ, UK.

Jeanne Buys, LGC, Queen's Road, Teddington, Middlesex, TW11 0LY, UK.

Joanna Campbell, Genetics Centre, 8th Floor Guy's Tower, Guy's Hospital, St Thomas Street, London SE1 9RT, UK.

Steve Edwards, Translational Cancer Genetics, The Institute of Cancer Research, Male Urological Centre, 15 Cotswold Road, Sutton, Surrey SM2 5NG, UK.

N Elanko, Medical Genetics Unit, St George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, UK.

Jo Field, Molecular Genetics, Centre for Medical Genetics, Nottingham City Hospital, Hucknall Rd, Nottingham NG5 1PB, UK.

Yogel Patel and Andrew Wallace, NGRL (Manchester), St Mary's Hospital, Hathersage Road, Manchester, M13 0JH, UK.

Els Schollen and Florence Le Calvez, Laboratory for Molecular Diagnosis, Center For Human Genetics, Herestraat 49, B-3000 Leuven, Belgium.

Erik Sistermans, Radboud University Nijmegen Medical Centre, Dept 120 Human Genetics, PO Box 9101, 6500 HB Nijmegen, The Netherlands.

Lisa Strain and Ruth Sutton, Institute of Human Genetics, International Centre for Life, Central Parkway, Newcastle-upon-Tyne, NE1 3BZ.

Spiros Tavandzis, P and R Lab s.r.o., Máchová 30, Nový Jičín 74101, Czech Republic.

APPENDIX 5

FIELD TRIAL PROTOCOL

NGRL (W) Field Trial of Generic Mutation Detection Reference Reagents August 2005

Study protocol and results forms

Aim of study

The aim of this field trial is to evaluate a panel of 52 plasmid based generic mutation detection reagents. The reagents can be used to amplify fragments ranging from 400-450bp with an average GC content of 20%, 40%, 60% and 80%. The four wild type sequences have been mutated to produce all possible base substitutions at three positions within the amplicon as shown in the attached diagram (figure 1). This field trial will analyse how effectively the 8 possible heteroduplex configurations are detected at three different positions within amplicons of varying GC content using as many different mutation detection techniques as possible.

Samples and reagents

The amplicons are provided in two 96-well microtitre plates using the plate format shown on page 2. Each well contains air dried PCR product. You should have received two coloured plates for each detection method that you requested labelled as follows:

Plate 1: CLEAR Plate 2: BLUE

Preparation and Storage of Plates

Immediately on receipt of the samples thoroughly resuspend the air dried amplicons in 20µl DNase free water. The 96 well plates should then be stored at -20°C until they are ready to be used (FAM labelled products should be stored in the dark). Try to avoid excessive freeze-thawing.

Mutation Detection

Please analyse the amplicons using your usual DNA mutation detection technique. Please read the following notes for the method(s) of analysis that you will be using:

Sequencing: Amplicons have Uniseq tags and have been cleaned up. They can be added directly to the sequencing reaction.

CSCE: Amplicons are FAM labelled but **DO NOT** have M13 tags. Please only use for CSCE

dHPLC/TGCE: Amplicons are unlabelled and do not have tags. Locally defined dHPLC conditions are provided on the floppy disk with the wild type amplicons sequences.

MALDI: Amplicons have T7 and stuffer sequence tags as indicated on package

Sequence information

Electronic copies of the wild type amplicon sequences are provided on the floppy disk for labs analysing by sequencing or dHPLC. These can also be obtained from Helen White: hew@soton.ac.uk.

Reporting of data

1) Please complete the result forms (enclosed) for Plates 1 and 2
2) Please provide a protocol for the mutation detection technique used
3) Please retain a copy of your raw data. We may request copies of data for some samples at a later date.

Please return the **3 results forms** (including your mutation detection technique protocol)

by **Friday 9th September 2005** to: Helen White
National Genetics Reference Laboratory (Wessex)
Salisbury District Hospital
Salisbury
Wiltshire
SP2 8BJ
UK
Fax: +44 1722 338095

96 well Plate format for Plates 1& 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	**20% wt**	20.01	20.02	20.03	20.04	20.05	20.06	20.07	20.08	20.09	20.10	20.11
B	**20% wt**	20.12	20.13	20.14	20.15	20.16	20.17	20.18	20.19	20.20	20.21	20.22
C	**40% wt**	40.01	40.02	40.03	40.04	40.05	40.06	40.07	40.08	40.09	40.10	40.11
D	**40% wt**	40.12	40.13	40.14	40.15	40.16	40.17	40.18	40.19	40.20	40.21	40.22
E	**60% wt**	60.01	60.02	60.03	60.04	60.5	60.06	60.07	60.08	60.09	60.10	60.11
F	**60% wt**	60.12	60.13	60.14	60.15	60.16	60.17	60.18	60.19	60.20	60.21	60.22
G	**80% wt**	80.01	80.02	80.03	80.04	80.05	80.06	80.07	80.08	80.09	80.10	80.11
H	**80% wt**	80.12	80.13	80.14	80.15	80.16	80.17	80.18	80.19	80.20	80.21	80.22

Rows A and B: Wells **A1** and **B1** should be used as 20% GC rich amplicon wild type reference samples.

Other wells contain 22 randomised samples (wt or heterozygous mutants) of the 20% GC rich amplicon (20.01 to 20.22)

Rows C and D: Well **C1** and **D1** should be used as 40% GC rich amplicon wild type reference samples.

Other wells contain 22 randomised samples (wt or heterozygous mutants) of the 40% GC rich amplicon (40.01 to 40.22)

Rows E and F: Well **E1** and **F1** should be used as 60% GC rich amplicon wild type reference samples.

Other wells contain 22 randomised samples (wt or heterozygous mutants) of the 60% GC rich amplicon (60.01 to 60.22)

Rows G and H: Well **G1** and **H1** should be used as 80% GC rich amplicon wild type reference samples.

Other wells contain 22 randomised samples (wt or heterozygous mutants) of the 80% GC rich amplicon (80.01 to 80.22)

NGRL (W) Field Trial of Generic Mutation Detection Reference Reagents August 2005

Result form 1: PLATE 1 (CLEAR)

Name:

Laboratory:

Method of Mutation Detection:

Date of Mutation Detection analysis:

Please mark the boxes with either **M** = mutation detected (if sequenced please give base substitution and position: P1, P2 or P3 ; see figure 1), **W** = wild type detected, **A** = ambiguous, **F** = Failed.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**20% wt**											
B	**20% wt**											
C	**40% wt**											
D	**40% wt**											
E	**60% wt**											
F	**60% wt**											
G	**80% wt**											
H	**80% wt**											

NGRL (W) Field Trial of Generic Mutation Detection Reference Reagents August 2005

Result form 2: PLATE 2 (BLUE)

Name:

Laboratory:

Method of Mutation Detection:

Date of Mutation Detection analysis:

Please mark the boxes with either **M** = mutation detected (if sequenced please give base substitution and position: P1, P2 or P3; see figure 1), **W** = wild type detected, **A** = ambiguous, **F** = Failed.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**20% wt**											
B	**20% wt**											
C	**40% wt**											
D	**40% wt**											
E	**60% wt**											
F	**60% wt**											
G	**80% wt**											
H	**80% wt**											

NGRL (W) Field Trial of Generic Mutation Detection Reference Reagents August 2005

Result form 3 (General Comments)

1. **If you were unable to report a result for any samples please give details**

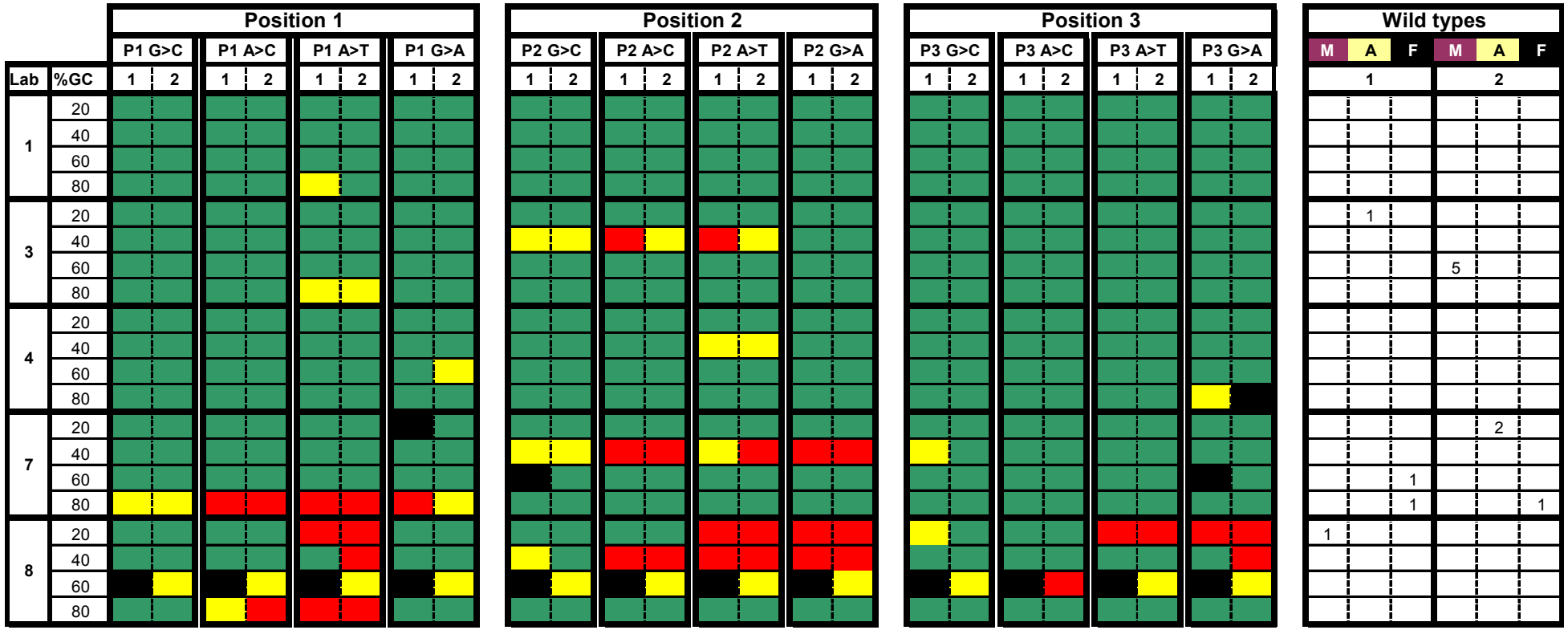
2. **Please give full details of the mutation detection technique used (provide this on another sheet of paper if necessary)**

3. Please make any comments about the materials supplied and the way that the trial was organised.

APPENDIX 6

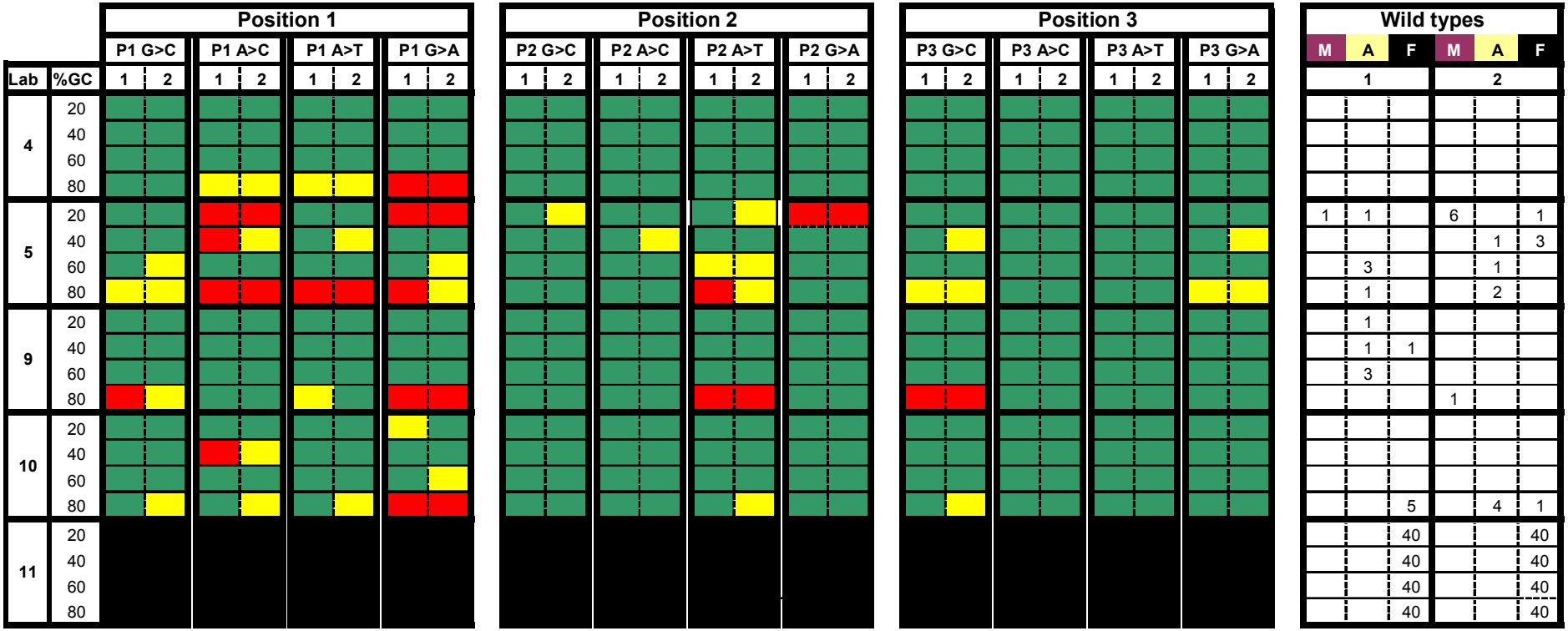
SUMMARY OF FIELD TRIAL DATA

DHPLC



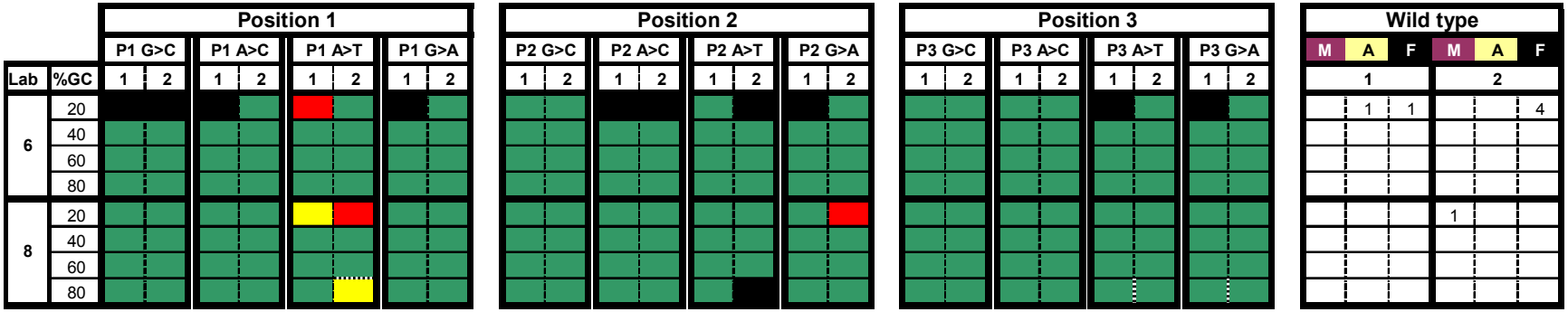
	Mutation detected
	Mutation undetected
	Ambigous call
	Fail
M	Wt assigned mutated (no.)
A	Wt assigned ambiguous (no.)
F	Wt assigned fail (no.)
1	Plate 1
2	Plate 2

CSCE

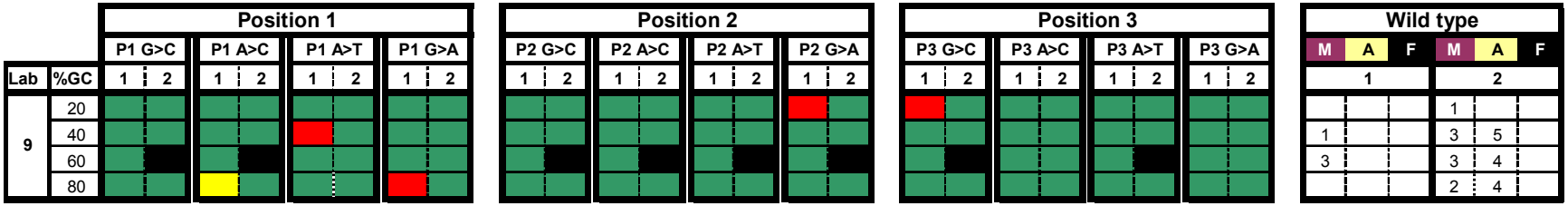


	Mutation detected
	Mutation undetected
	Ambigous call
	Fail
M	Wt assigned mutated (no.)
A	Wt assigned ambiguous (no.)
F	Wt assigned fail (no.)
1	Plate 1
2	Plate 2

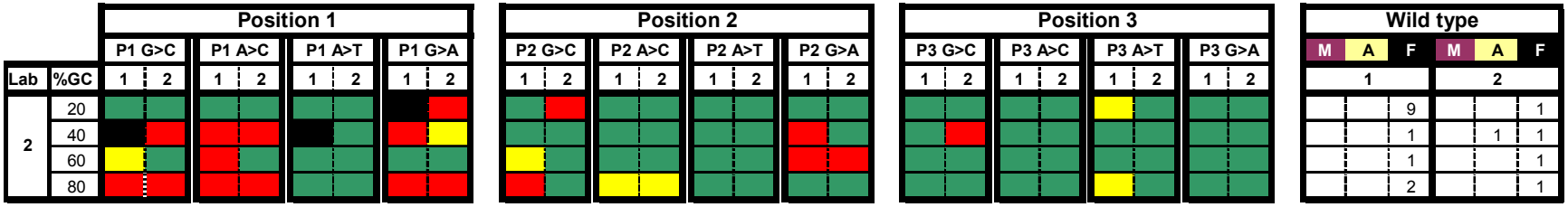
SEQUENCING



TGCE

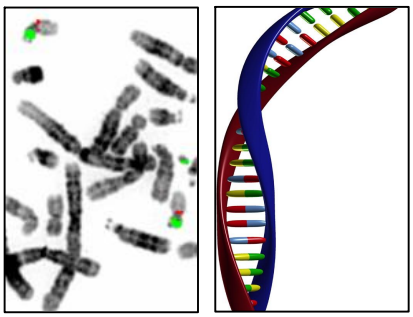


MALDI-TOF



	Mutation detected
	Mutation undetected
	Ambigous call
	Fail

M	Wt assigned mutated (no.)
A	Wt assigned ambiguous (no.)
F	Wt assigned fail (no.)
1	Plate 1
2	Plate 2



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