Online Resource

Journal: Familial Cancer

Title: High-resolution melting (HRM) re-analysis of a polyposis patients cohort reveals previously undetected heterozygous and mosaic APC gene mutations

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Supplementary tables

Supp. Table S1 Primers and probes

Amplicon	F/R/Probe	Primer/Probe [a]	Bp [b]	GC% [b]	Annealing (°C)	Domains [c]
	M13 F [b]	TGTAAAACGACGGCCAGT …				
	M13 R [b]	CAGGAAACAGCTATGACC …				
1	F	… AAAAACAAGCAGCCACTAAAT	281	35	65	1
	R	… CAAGTTTACAAGAGGGAATACTGAATA				
2	F	TCTCAGCATACTTAAATGTCAAGAAATAC	239	29	61	1
	R	CTACACACCTAAAGATGACAATTTG				
3.1	F	TTTAGACTGCTTAAAGCAATTGT	197	40	61	2 [c]
	R	CTCTTCTTGGAAATGAACCCATAG				
3.2	F	GCCGGGAAGGATCTGTAT	242	36	65	1
	R	GCTCTAAGTGTTAGCTATCACC				
4	F	TATTGCTCTTCTGCAGTCTTTATTAG	268	29	65	1
	R	ATTCAGTTATCCTGAATTTTAATGGATTAC				
	probe	*GTTTAAACGTACCTTTTTTTAAAAAAAAAAAAAATAGGTC* [d]				
5	F	CATGCACCATGACTGACGTA	393	32	61	2
	R	GTTGCTCAGCAGCCATGATA				
	probe	*CTTTTTTATTATTTGTGGTTTTAGTTTTCCTTACAAACAG*				
6	F	AAGTGGTAGCCATAGTATGATTATTTC	216	32	65	2
	R	CAGAATAACTACCTATTTTTATACCCACA				
7	F	CCTTGGGCTAAGAAAGCCT	246	37	65	2 [c]
	R	ATCATTCTTAGAACCATCTTGCTTCATA				
8	F	GAGTACCTTAACATGATGTTATCTGTATT	224	39	63	2 [c]
	R	TAGCAAAGTAGTCATGGCATTAG				
9.1	F	TAAATATCCCATTCATCACTTAATTGGTT	172	37	63	1
	R	GGAGCTAGACATAGCTAGCAA				
9.2	F	GTCAATGCTTGGTACTCATGAT	226	48	63	1
	R	CTGTGAGTGAATGATGTTGTGG				
9.3	F	CAGTAAAGAGGCTCGGG	229	49	63	1
	R	CGCCCGCCGC-CTTTGAAACATGCACTACGAT				
10	F	TTTAAAGTACAATAAACATCATTGCTCTTC	212	33	65	1
	R	CCACCAGTAATTGTCTATGTCA				
11	F	AAGTTACCAACTTGGTACCAG	271	35	63	2
	R	TGAAAGTAAATTAACTCATACCTGAGCTA				
	probe	*CTGTGAAATGTACGGGCTTACTAATGACCAC*				
12	F	AAAACTGAATTAGACATTTAGTAGCCA	255	38	65	2
	R	AACTGCCCTCTAAGCCT				
13	F	AAACAAAAAAGCAACTAGTATGATTTTATG	294	31	65	1
	R	ATAGTACTGAATAATACACAGGTAAGAAA				
13.1	F	CGGCTAGCCAGAATTTCTTT	190	32	63	1
	R	CCAACTTCTCGCAACGTCTT				
	probe	*GCAGGTTATTGCGAGTGTTTTGAGG*				
13.2	F	AGTGTTTTGAGGAATTTGTCTTG	217	34	63	1
	R	CTGAATAATACACAGGTAAGAAATTAGGAAA				
14.1	F	CAATATCAGTAACATAGAAGTTAATGAGAG	192	38	65	1
	R	GAGTGCCAACCAAAAATGC				
14.2	F	TTGCACTGAGAATAAAGCTGATATATG	249	36	65	2 [c]
	R	CGCCCGC-ATTATATAAAACATTGCTTACAATTAGGTC				
15.1	F	TGTTACTGCATACACATTGTGACC	202	35	65	1
	R	CCCATGTCCCATAATGCTTC				
15.2	F	GTGGAATCTCTCAGCAAGAAAT	238	44	60	1
	R	TTCTGCTTCTAGGGCTTTTTGTT				
15.3	F	GGATGCCAATATTATGTCTCCTG	238	39	60	1
	R	GCCAGTATTAAAATTGTCTGACCTA				
15.4	F	CAAGCAAAGTCTCTATGGTGATTA	239	40	60	1
	R	CTGGATTTTCTGTTGCTGGATG				

15.5	F	TCGTTCTGAAAAAGATAGAAGTTTGG	230	45	65	1
	R	CATTTCTCTCATCTGTCACACAA				
15.6	F	GCCATTCATACCTCTCAGGA	230	38	65	1
	R	CCATCACTACTACTGACACTATTTAAAC				
15.7	F	GACATGTTCTATGCCTTATGCCA	238	36	65	1
	R	TGGTGTATCTAGTTCTCCATCATTAT				
15.8	F	CAATACCCAGCCGACCTA	233	36	65	1
	R	GGATAAGTTGTACTTTGATTCCTTGA				
15.9	F	GCAAGACCCAAACACATAATAGA	232	39	65	1
	R	GGCTTACATTTTGATTAATTCCATGAT				
15.10	F	ATACAGGTCACGGGGAG	225	38	65	1
	R	GGCTGATCCACATGACG				
15.11	F	ATGAAGAAGAAGAGAGACCAACA	240	37	65	1
	R	GATGGAGCTGATTCTGCCT				
15.12	F	ACATATGTCTTCAAGCAGTGAG	222	40	65	2
	R	GCTGATGACAAAGATGATAATGAAC				
15.13	F	CACTTGCAAAGTTTCTTCTAT	207	35	65	1
	R	GCTGACCTAGTTCCAATC				
15.14	F	GACACAGGAAGCAGATTCTGCTAATA	230	46	65	1
	R	GTGTCTGAGCACCACTTT				
15.15	F	AATCAGCCAGGCACAAAGCTG	241	47	65	1
	R	TCTGGAAGATCACTGGGGCTTA				
15.16	F	CAGCTCCGTTCAGAGTGA	230	48	65	1
	R	CCCTCTGAACTGCAGCA				
15.17	F	AATAAAGCACCTACTGCTGAAA	116	44	65	1
	R	GTGGCAAAATGTAATAAAGT				
15.18	F	AGAGGGTCCAGGTTCTTC	126	46	65	1
	R	CTTTCTGTATAAATGGCTCATCG				
	probe	*ATGCTGATACTTTATTACATTTTGCCACGGAAAGTAC*				
	probe	*TCCAGATGGATTTTCTTGTTCAT*				
15.19	F	TCCAGATGGATTTTCTTGTTCA	233	37	65	1
	R	TTTCAATATCATCATCATCTGAATCATCTA				
15.20	F	ATGAAAACCAAGAGAAAGAGGC	237	39	65	2
	R	GCAACCTGTTTTGTGATGG				
15.21	F	TCCACCTGTGGCAAGGAAA	227	46	65	1
	R	CTCCTCTAACTCCTTCTCCAG				
15.22	F	CCTATAAACTTTTCCACAGCTACAT	229	43	61	1
	R	GAATATCACCTTCCTCTGCTTTATTG				
	probe	*GTTAGAGGAGGAGCACAGTCAGGTGAATTTG*				
15.23	F	CTCAAGGAGGAAAAACCTCAT	238	41	65	1
	R	ATAGGTTTTACTGGTGAAGTTGG				
	probe	*CAAGCATCTGCGTCGTCTTCTGCACCCAA*				
15.24	F	CTTCTGCACCCAACAAAAATC	293	39	65	1
	R	GTAAGGAGTTCCTTCAATAGGCG				
	probe	*ATAATTCCAAGGACTTCAATGATAAGCTCCCA*				
15.25	F	ATGAAGATAGAGTCAGAGGAAGT	241	38	61	1
	R	GTCTTATTAGCTGATTGTTGGTTG				
15.26	F	AAAAGAAAATAAGGAATCAGAGGC	228	40	65	1
	R	GGAGTATTTTCAATAGCAAAATTCTGTAAC				
15.27	F	TTTTCCCCAGTCATCCAAAGAC	274	40	65	1
	R	TCTTGAGAAACAAACTGGGGT				
	probe	*GAAAATACTCCAGTTTGCTTTTCTCATAATTCCTC*				
15.28	F	AAACCTCAAGCATCAGGC	246	39	61	1
	R	ATCTTTCAAATCAAGTGTCAGAT				
15.29	F	GACTCAAGGGTGATAATGAAAAAC	249	39	65	1
	R	GGGAAAGGATGGAATCTGAAT				
15.30	F	GCAAATTCCATAGTAAGTAGTTTACATCAA	249	38	65	1
	R	TCCTTTGATTCCTTTACTTTCAGATTCTA				
15.31	F	GCCCACGAATTCTAAAACCAG	244	38	65	2
	R	GGACTTGTACTTGAGGAGCTA				
15.32	F	CCTTCAATCTCTCGAGGCAG	247	47	65	1

	R	ATCCTGATCTAGAAGGTGCTTT				
15.33	F	AGAATTAAGCCCTGTTGCC	247	46	65	1
	R	TCCAGAACCTGAGGACTTAGT				
15.34	F	GTCCTCCTAACAAATTATCTCAACTT	250	40	65	1
	R	GACATTCTAGAAAGTTCTACCTTTTTATTG				
15.35	F	AGTGAGTCTGCCTCCAAAG	249	39	65	1
	R	CCTAGTGGGAGAAGCTGG				
15.36	F	AGAAAATTGGAGGAATCTGCT	247	45	65	1
	R	GACTTTCAGAATGAGACCGT				
15.37	F	CTAATCTCAGTCCCACTATAGAGTATAA	217	43	65	1
	R	CTGGATTCTGATGAAGCAGAAAG				
15.38	F	ATCCCTTCCTCGAGTAAGC	246	38	65	1
	R	GCACCATTTGTAGCACCT				
15.39	F	GAACATGGAGAAAAATAAAAGAAAATGAAT	250	40	65	1
	R	TTTGGATTTGCCTTTTCTGAAAC				
15.40	F	TCCTAGATCTGGAAGATCTCCC	248	43	65	1
	R	TCTCTGATACAGGGACAGGAT				
15.41	F	TGGATGCCCCTGACCAAA	243	47	65	1
	R	CTGGAGTTGGGATCTGAGATG				
15.42	F	TTACAACCCAAGCCCTAGGA	245	42	65	2 [c]
	R	GAAACAAAATGTCTATATAGCAGTTGTAAT				

a GC clamps are underlined

b To all primers (not probes) M13 tails were attached. Primer sequences, bp and GC% are shown exclusive M13 tails

c If two melting domains and marked with "c", it was possible to analyze each melting domain separately, as explained in online resource Supp. Fig. S1

d This probe was not used for scanning of the patient group

Supp. Table S2 All variants found in the 171 patients [a]

Amplicon	Variant DNA	Variant protein	Times found (MAF)	Earlier method [b]	Reported [c]	dbSNP MAF [d]
Pathogenic						
6	c.679del	p.Asp227Thrfs*66	1	NA	-	
9.3	c.1180C>T	p.Gln394*	3	S- D-	L	
9.3	c.1248C>A	p.Tyr416*	1	S-	HGMD	
11	c.1548+1G>A	Splice donor site	1	S-	L	
14.2	c.1958+1_1958+2dup	Splice donor site	1	D-	-	
15.1	c.1972_1975del [e]	p.Glu658Thrfs*11	1	P-	References [c]	
15.1	c.2003del	p.His668Profs*2	1	P-	-	
15.2	c.2222dup	p.Asn741Lysfs*15	1	D- P-	-	
Mosaic						
15.3	c.2269C>T (5%)	p.Gln757*	1	D- P-	C L	
15.17	c.4393_4394dup (15%)	p.Ser1465Argfs*9	1	P- SEQ- [f]	L	
Polyp tissue only						
15.14	c.4057G>T	p.Glu1353*	1		C U	
Polyp tissue only (outside study group) [a]						
15.19/20	c.4666dup	p.Thr1556Asnfs*3	1		C L U	
Rare polymorphisms / VUS						
3.1	c.295C>T [e]	p.Arg99Trp	1 (0.003)	SEQ+	L U rs139196838	0.001
5	c.645+33G>A	p.?	2 (0.006)	SEQ+	rs200162147	0.001
6	c.705A>G	p.= (p.Leu235Leu)	1 (0.003)	D+, SEQ+	L U rs147036141	0.003
14.2	c.1958+8T>C	p.?	1 (0.003)	S- D-	L rs62626346	0.002
15.4/5	c.2569G>A	p.Gly857Arg	1 (0.003)	(P)	-	
15.8/9	c.3173A>G	p.Asp1058Gly	1 (0.003)	(SB)	L rs148725540	0.001
15.10	c.3386T>C	p.Leu1129Ser	1 (0.003)	(P)	C L rs143638171	0.003-0.007
15.13/14	c.3920T>A	p.Ile1307Lys	1 (0.003)	SEQ+	C L rs1801155	0.002-0.007
15.22/23	c.5140G>A	p.Asp1714Asn	1 (0.003)	(P)	rs148275069	0.0001
15.24	c.5348C>G	p.Thr1783Ser	1 (0.003)	(P)	-	
15.24	c.5501_5506del	p.Val1834_Arg1835del	1 (0.003)	(P)	-	
15.29/30	c.6363_6365dup	p.Ala2122dup	1 (0.003)	(P)	-	
15.33	c.6921G>A	p.= (p.Ser2307Ser)	1 (0.003)	NA	L rs2229993	0.002-0.008
15.34	c.7098T>C	p.= (p.Tyr2366Tyr)	1 (0.003)	(P)	-	
15.38	c.7786T>G	p.Ser2596Ala	1 (0.003)	(P)	rs138137162	0.0001
15.38	c.7862C>G	p.Ser2621Cys	1 (0.003)	SEQ+	L U rs72541816	0.005-0.03
Common polymorphisms			*het/hom*			
2	c.136-53T>C	p.?	12/0 (0.035)		rs2304793	0.009-0.048
5	c.645+32C>T	p.?	36/1 (0.111)		L rs2909961	0.083-0.093
11	c.1458T>C	p.= (p.Tyr486Tyr)	85/60 (0.600)		L rs2229992	0.575-0.603
13.1	c.1635G>A	p.= (p.Ala545Ala)	74/68 (0.614)		L rs351771	0.584-0.642
13.2	c.1743+78A>G	p.?	8/0 (0.023)		L rs62364023	0.033
15.16	c.4326T>A	p.= (p.Pro1442Pro)	3/0 (0.009)		C L U rs67622085	0.007-0.017
15.18	c.4479G>A	p.= (p.Thr1493Thr)	75/68 (0.617)		L U rs41115	0.586-0.642
15.22	c.5034G>A	p.= (p.Gly1678Gly)	73/70 (0.623)		L rs42427	0.592-0.642
15.23	c.5268T>G	p.= (p.Ser1756Ser)	77/68 (0.623)		rs866006	0.592-0.634
15.24	c.5465T>A	p.Asp1822Val	62/102 (0.780)		L U rs459552	0.765-0.796
15.27	c.5880G>A	p.= (p.Pro1960Pro)	72/67 (0.602)		L rs465899	0.592-0.642
15.34/35	c.7201C>T	p.= (p.Leu2401Leu)	5/0 (0.015)		L U rs2229994	0.008-0.021
15.36	c.7504G>A	p.Gly2502Ser	7/0 (0.205)		L rs2229995	0.008-0.097

a Of two patients DNA isolated from polyps was available for HRM of *APC*. Patient 13 was part of the study group of 171 patients referred between 1995 and 2007 and patient 14 was referred after this interval in 2008

b Earlier tested by “S” (SSCP), “D” (DGGE), “P” (PTT), “SEQ” (Sanger sequencing), “SB” (Southern blot). “NA”: not analyzed for this particular region. “+”: detectable and “-“: not detectable, by the earlier method. The method was shown between “()” it was not possible to detect the variant by this particular technique

c New variants, not reported elsewhere, are marked with “-“. Variants reported elsewhere, in independent patients, are marked with “C” (COSMIC database), the “rs” number (dbSNP), “L” (LOVD), “U” (UMD), or references [20,22]

d dbSNP build 138 allele frequency in Caucasian populations (http://www.ncbi.nlm.nih.gov/SNP/, [23]

e c.295C>T (VUS) and c.1972_1975del (pathogenic) were both detected in one patient (patient 8 in Table 2)

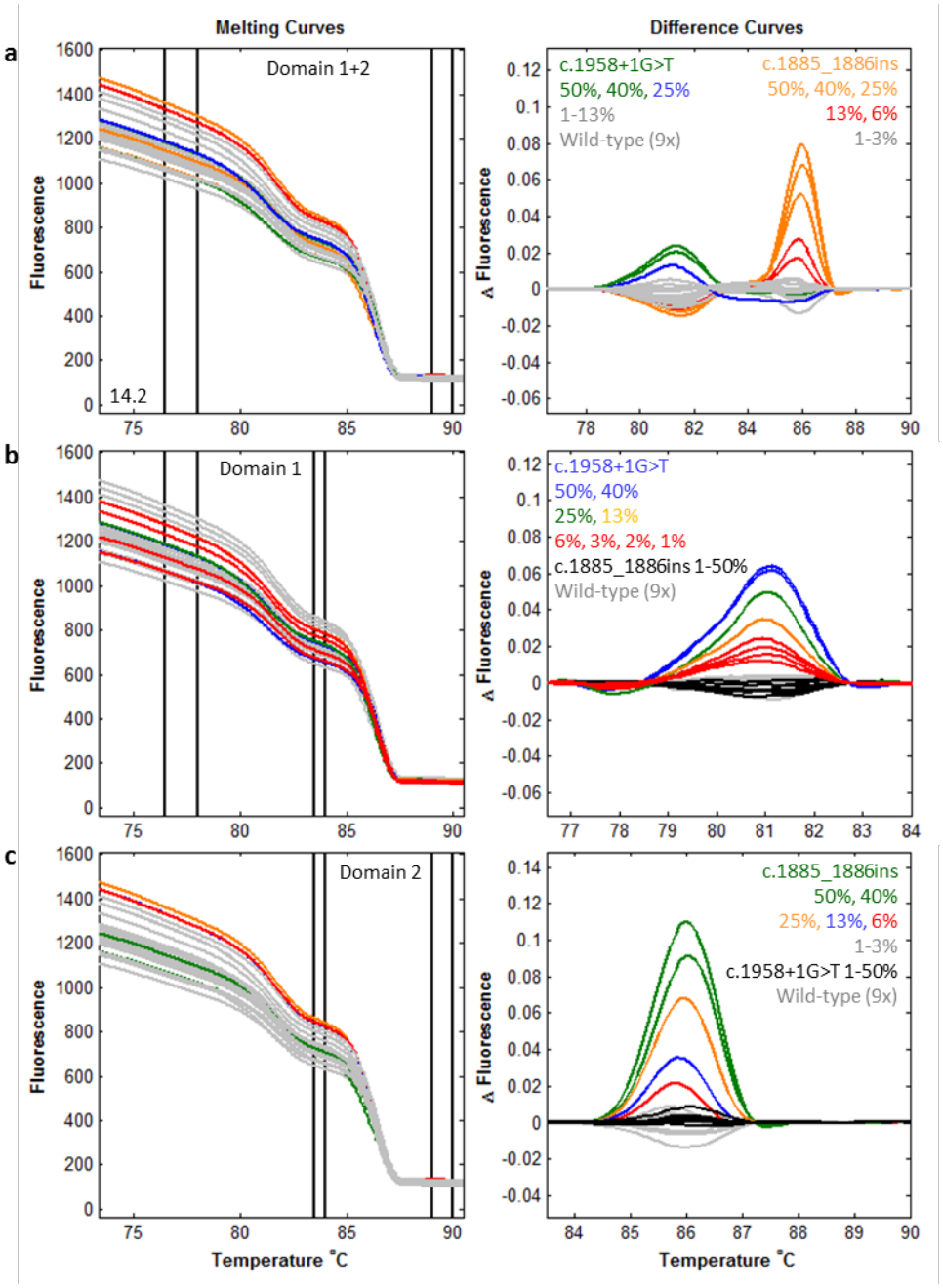
f Visible after revision of the sequence after the positive melting curve was found (Fig. 1)

Supp. Table S3 Validated variant types and allelic fraction detection limits [a]

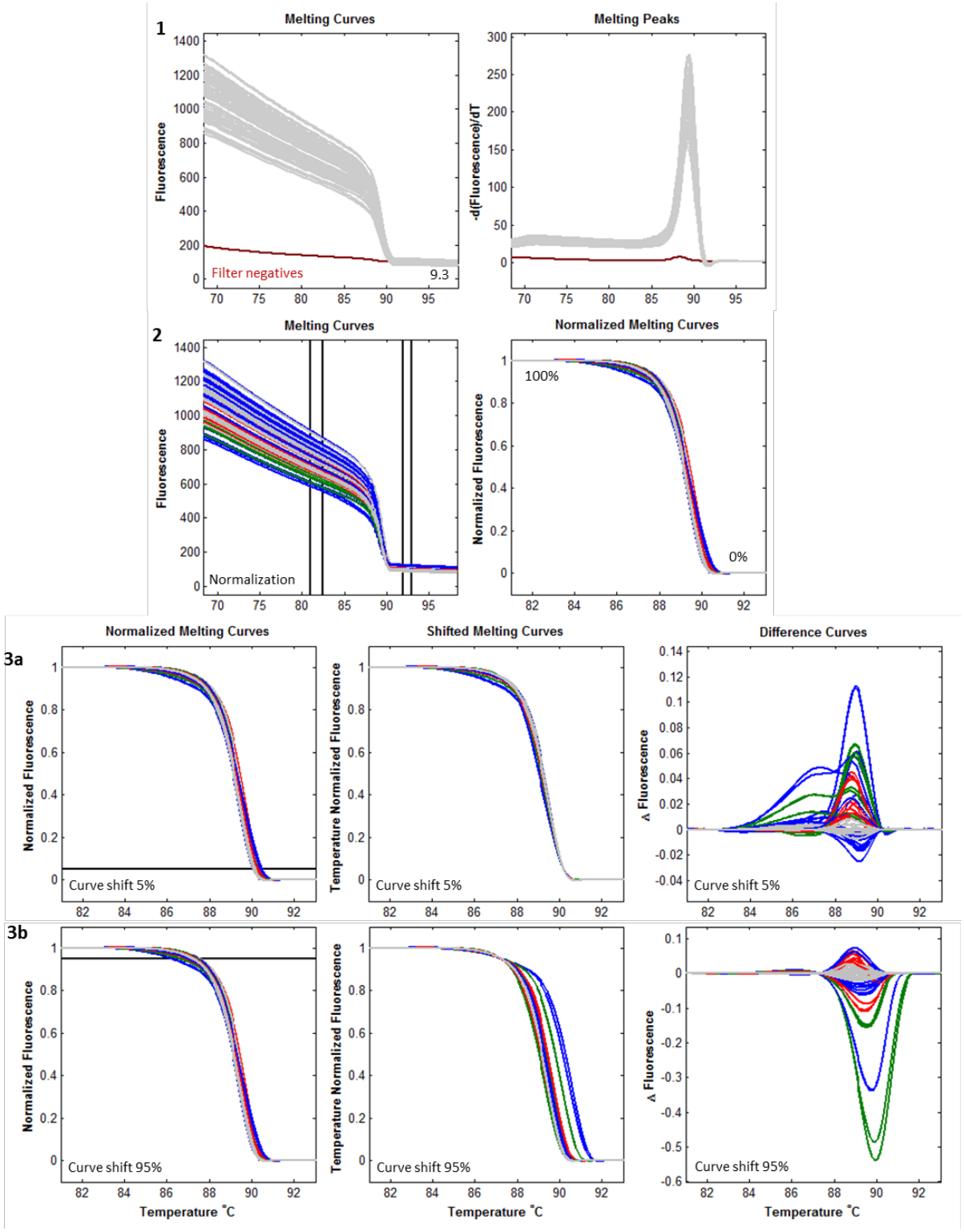
		Detectable down to			
Type	#	2%	3%	6%	13%
A<>C or G<>T	10	1 (10%)	6 (60%)	3 (30%)	
A<>G or C<>T	40	3 (8%)	6 (15%)	30 (75 %)	1 (3%)
A<>T	7		1 (14%)	4 (57%)	2 (29%)
C<>G	3			3 (100%)	
del 1 bp	16	1 (6%)	3 (19%)	9 (56%)	3 (19%)
del 2-5 bp	18		3 (17%)	15 (83%)	
del >5 bp	2			2 (100%)	
ins 1 bp	14		4 (29%)	5 (36%)	5 (36%)
ins 2-5 bp	3	1 (33%)	1 (33%)	1 (33%)	
ins >5 bp	3		2 (67%)	1 (33%)	
All	116	6 (5%)	26 (22%)	73 (63%)	11 (9%)
cumulative %		5%	28%	91%	100%

a Variants were grouped for type of base-pair change or according to number of deleted or inserted nucleotides. Per type of variant the total number of tested unique variants and is shown and the portions of variants detectable down to each tested allelic fraction detection limit

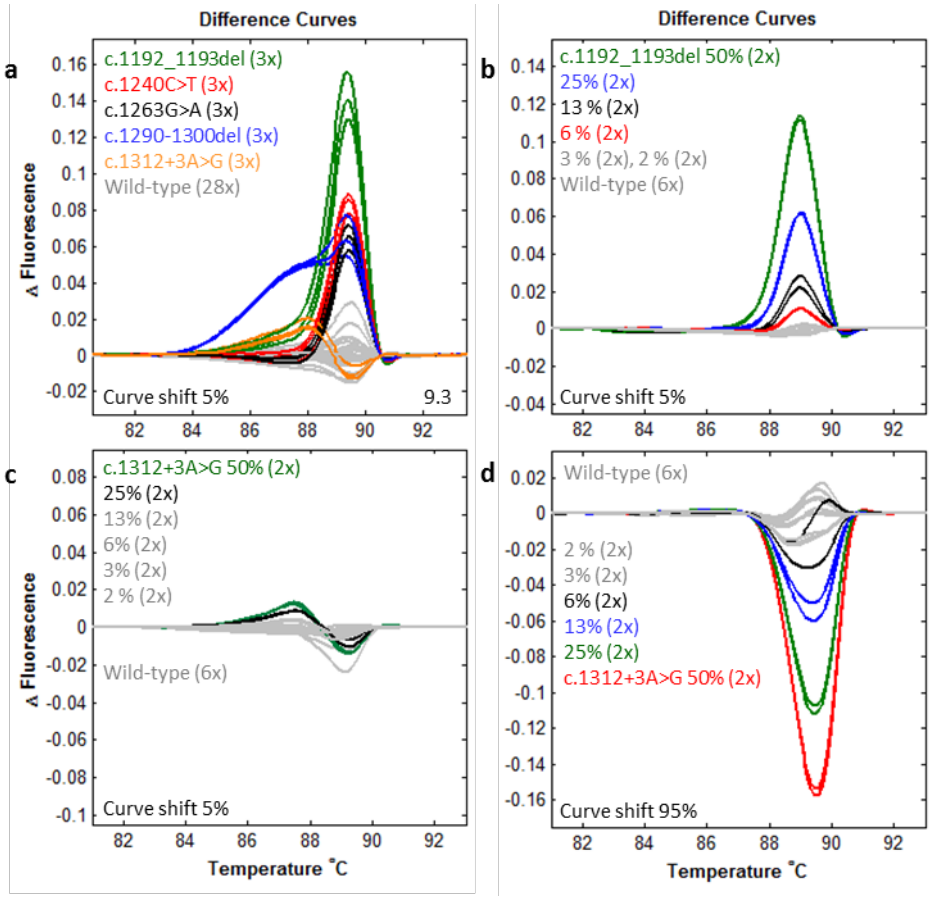
Supplementary figures



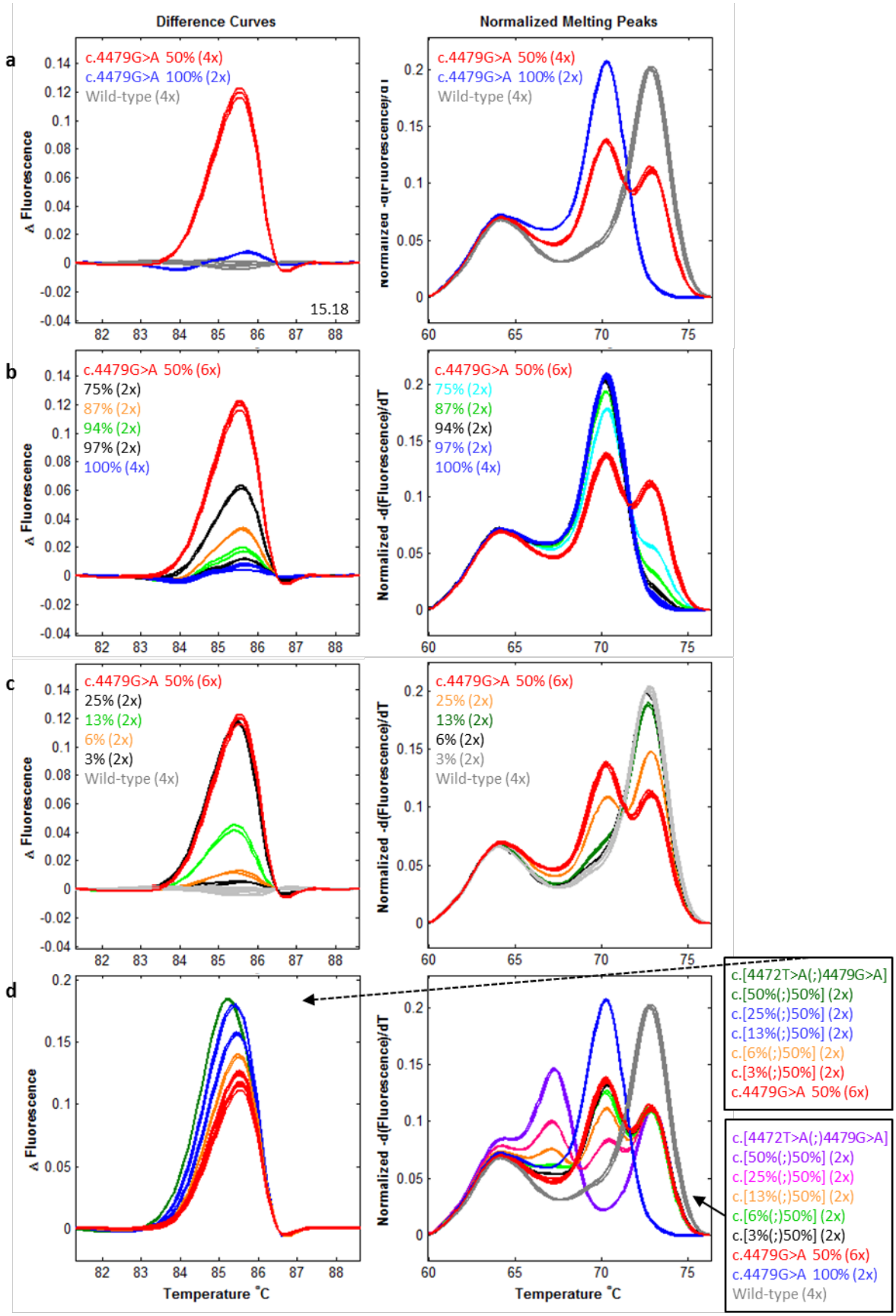
Supp. Fig. S1 Example of melting curves of an amplicon with two melting domains (amplicon 14.2). a. detection levels when normalized at the start of the melting curve (near 100% fluorescence) and at the end of the melting curve (near 0% fluorescence), analyzing domains 1 and 2 together. b. normalization directed at domain 1. c. normalization directed at domain 2. It must be noted that application of such settings did not work as well for all of the amplicons with two melting domains. Some amplicons needed to be redesigned or split in two (data not shown). Melting curves with more than two melting domains are not suitable to analyze (data not shown)



Supp. Fig. S2 Analysis steps and settings in Lightscanner melting curve analysis: 1. Filtering out negatives, 2. Normalizing curves at 100% and 0% fluorescences for fluorescence intensity differences between samples, 3. Temperature shifting correction for temperature differences across the PCR plate (a. at 5% fluorescence, b. at 95% fluorescence). Difference plot of melting speed were used for final analysis of melting curve differences (an analysis called “expert scanning” in the Lightscanner software)



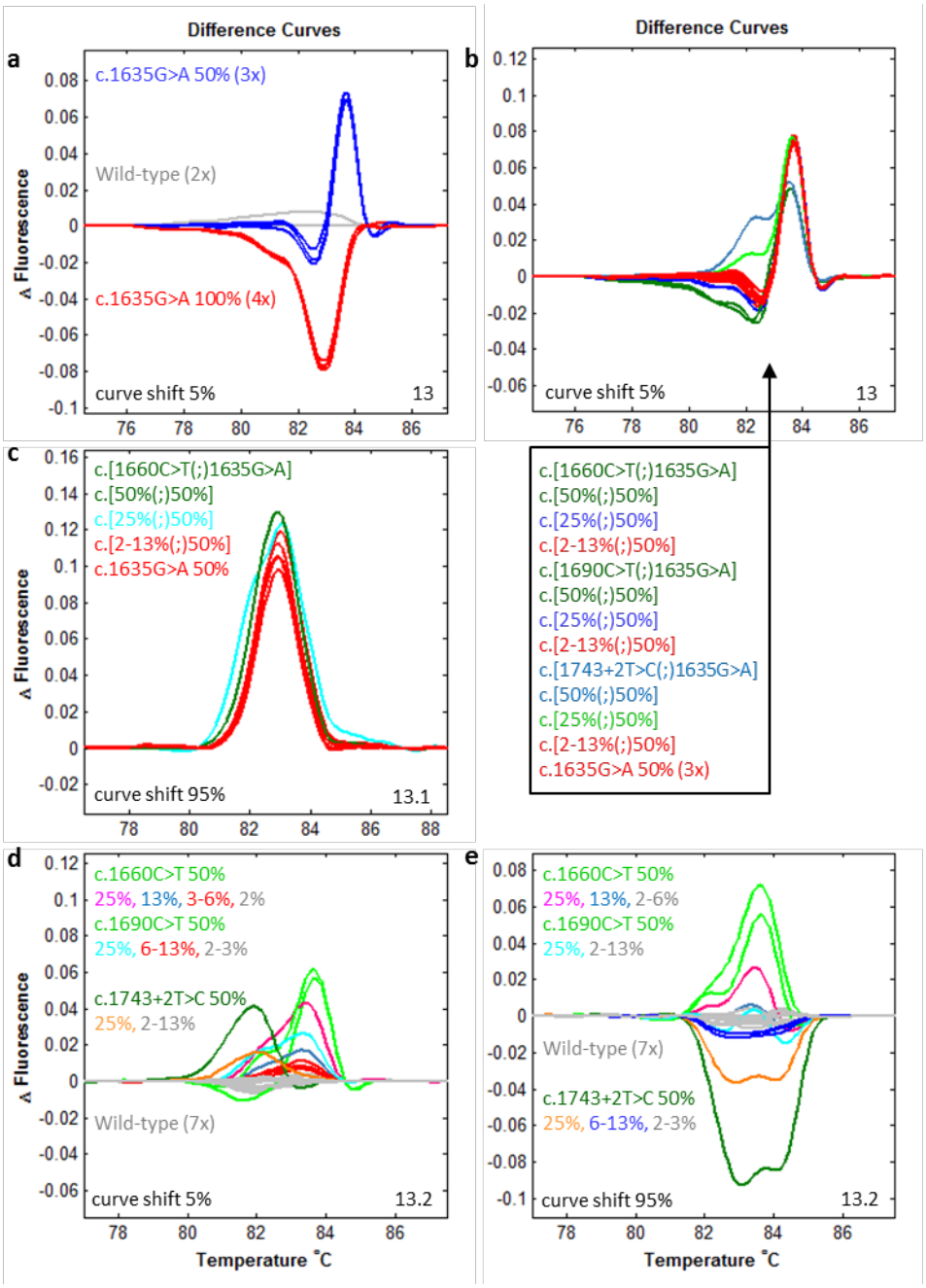
Supp. Fig. S3 Differences in detectability between different heterozygous variants and their serial dilutions. a. Different heterozygous variants in amplicon 9.3. Heterozygous variants with a difference curve with a low amplitude were generally detectable down to higher minimal allelic fractions compared to variants with high amplitude (e.g. c.1192_1193del versus c.1312+3A>G, shown in b, c). d. using curve shift setting 95%, improved results for variant c.1312+3A>G, from 25% to 13% detected dilution



Supp. Fig. S4 Detectability of low allelic fractions by probe genotyping and expert scanning, including detection of two variants in one sample. a. Expert scanning and unlabeled probe genotyping of control samples with homozygous, heterozygous and wild-type genotypes of the common SNP c.4479G>A in the 126 bp amplicon 15.18. b-c. Serial dilutions of

a c.4479G>A heterozygous sample, with a homozygous sample and with a wild-type sample, respectively. Expert scanning shows more sensitive detection compared to probe genotyping for small allelic fraction differences. d. Detection of multiple variants in one sample. The sample contained the common SNP and the variant c.4472T>A, both located under the same probe*. In serial dilutions (with DNA heterozygous for the common SNP), c.4472T>A was detectable down to 6% by expert scanning and probe genotyping. The 3% curve in probe genotyping is shown in black, but was hardly distinguishable from samples with only the common SNP.

*For amplicon 15.18 two adjacent probes were designed, covering 60 bp in total. One probe covered the common SNP and one probe covered the area next to the SNP, to add possible sensitivity. However no variants were found in the area of the second probe (online resource Supp. Table S1)



Supp. Fig. S5 Detectability of multiple variants per sample in one amplicon. a. Control samples with homozygous, heterozygous and wild-type genotypes of the common SNP c.1635G>A in the 294 bp exon 13 amplicon. b. Three samples, each heterozygous for c.1635G>A and each with a unique additional variant in exon 13 (c.1660C>T, c.1690C>T and c.1743+2T>C). In serial dilutions (with DNA heterozygous for the common SNP) the lowest detectable allelic fraction for all three variants was 25%. c-e. Detection of the same three variants in exon 13 after splitting the amplicon into two amplicons, one with (13.1, 190 bp) and one without the common SNP (13.2, 217 bp). In amplicon 13.2 the three rare variants were detectable down to 6.3%. In amplicon 13.1 one rare variant was located and detectable down to 25%, like in the larger exon 13 amplicon