Supplementary Material

Reporter emission multiplexing in digital PCR (REM-dPCR): Direct quantification of multiple target sequences per detection channel by population specific reporter

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SUPPLEMENTAL TABLE S1: TARGET PANEL.

Table S1 In the target region mutations are highlighted in red, complementary probe binding regions are highlighted in yellow and red.

Target	Gene	Name	Codon	Target region (5´ → 3´)									Change of nucleotide	Change of amino acid	Library number (NCBI)	Chromosome / position	Detection channel	PSR-Type
I	*BRAF*	WT	600	*T*	*T*	*T*	*C*	*A*	*C*	*T*	*G*	*T*	WT				blue	08
II		V600E	600	T	T	T	C	T	C	T	G	T	c.1799T>A	p.Val600Glu	rs113488022	GRch38 Chr7:140753336	blue	07
III	*KRAS*	WT	12	*G*	*C*	*T*	*G*	*G*	*T*	*G*	*G*	*C*	WT				green	02
																	infra-red	06
IV		G12A	12	G	C	T	G	C	T	G	G	C	c.35G>A	p.Gly12Asp	rs121913529	GRch38 Chr12:25245350	green	04
V		G12D	12	G	C	T	G	A	T	G	G	C	c.35G>C	p.Gly12Ala	rs121913529	GRch38 Chr12:25245350	red	05
VI		G12V	12	G	C	T	G	T	T	G	G	C	c.35G>T	p.Gly12Val	rs121913529	GRch38 Chr12:25245350	red	06
																	infra-red	01

SUPPLEMENTAL TABLE S2: OLIGONUCLEOTIDE SEQUENCES

Table S2 Sequences of primer, mediator probes, universal reporters and synthetic templates. Point mutations are highlighted in red.

Oligo-type	Name	Sequence (5´- 3´)	Modifications		
			5'- end	3'- end	Internal (2/7/8)
Primer	KRAS_fwd [1]	GGCCTGCTGAAAATGACT			
	KRAS_rev [1]	ACAAAATGATTCTGAATTAGCTGTA			
	BRAF_fwd [2]	GACCCACTCCATCGAGATTTC			
	BRAF_rev [2]	GCTTGCTCTGATAGGAAAATGAG			
Mediator probe	MP_PSR01_KRAS_G12V	TCGTTCTGGGCTTGGCGTAGGCAAGAGTGCCTTGACGAT		MP-block	
	MP_PSR04_KRAS_G12A [2]	GATACAGGGTCCACTGGCGTAGGCAAGAGTGCCTTGACG		MP-block	
	MP_PSR05_KRAS_G12D [1]	ATGTCCCAGGTGCATGGCGTAGGCAAGAGTGCCTTGACGAT		MP-block	
	MP_PSR06_KRAS_G12V [1]	AGGTAGGCTCACTTGGCGTAGGCAAGAGTGCCTTGACGAT		MP-block	
	MP_PSR06_KRAS_WT	TAGGCTCACTGAACCAGCTCCAACTACCACAAGTTTATATTCAG		MP-block	
	MP_PSR07_BRAF_V600E	ACATGCTCATGTTGTGTCTGTAGCTAGACCAAAATCACCTATTTTTACTGTGAG		MP-block	
	MP_PSR08_BRAF_WT	GTGTTCCTCACATGCTACTGTAGCTAGACCAAAATCACCTATTTTTACTGTGAG		MP-block	
Population Specific Reporter	PSR01	GACCGGCCAAGACGCGCCGGT7TGTTGGTCGTAGAGCCCAGAACGA	BHQ-2	UR-block	dC-Cy5.5
	PSR02	GACCGGCCAAGACGCGCCGGT7TGTTCACTGACCGAACTGGAGCA	BMN-Q1	UR-block	dC-DY-530
	PSR04	GACCGCACTAGTAGATGCGGT7TGTCGTGGACCCTGTATCGAGCA	BHQ-1	UR-block	dC-BMN536
	PSR05	GACCGGCTAAGACGCGCCGGT7TGTTGCACCTGGGACATCGACTAT	BHQ-2	UR-block	dC-Cy5
	PSR06	GACGCGTAGTACAGAACGCGT7TGTTCAGTGAGCCTACCTGCCTTC	BHQ-2	UR-block	dC-Atto 647N
	PSR06	GACGCGTAGTACAGAACGCGT2TGTTCAGTGAGCCTACCTGCCTTC	BHQ-2	UR-block	dC-Atto-680
	PSR07	ATGCCGATTAGATGCGGCA8TCGATCACACAACATGAGCATGTGTAC	BHQ-1	UR-block	dT-FAM
	PSR08	ATCCGCCAAGACGCGCGGA8TAGCATGTGAGGAACACGATGACAC	BMN-Q1	UR-block	dT-Atto 488

Oligo-type	Name	Sequence (5´- 3´)
Synthetic template	KRAS_WT	CATTTTCATTATTTTTATTATAAGGCCTGCTGAAAATGACTGAATATAAACTTGTGGTAGTTGGAGCTGGTGGCGTAGGCAAGAGTGCCTTGACGATACAGCTAATTCAGAATCATTTTGTGGACGAATAT
	KRAS_G12A	CATTTTCATTATTTTTATTATAAGGCCTGCTGAAAATGACTGAATATAAACTTGTGGTAGTTGGAGCTGCTGGCGTAGGCAAGAGTGCCTTGACGATACAGCTAATTCAGAATCATTTTGTGGACGAATAT
	KRAS_G12D	CATTTTCATTATTTTTATTATAAGGCCTGCTGAAAATGACTGAATATAAACTTGTGGTAGTTGGAGCTGATGGCGTAGGCAAGAGTGCCTTGACGATACAGCTAATTCAGAATCATTTTGTGGACGAATAT
	KRAS_G12V	CATTTTCATTATTTTTATTATAAGGCCTGCTGAAAATGACTGAATATAAACTTGTGGTAGTTGGAGCTGTTGGCGTAGGCAAGAGTGCCTTGACGATACAGCTAATTCAGAATCATTTTGTGGACGAATAT
	BRAF_WT	CAAACTGATGGGACCCACTCCATCGAGATTTCACTGTAGCTAGACCAAAATCACCTATTTTTACTGTGAGGTCTTCATGAAGAAATATATCTGAGGTGTAGTAAGTAAAGGAAAACAGTAGATCTCATTTTCCTATCAGAGCAAGCATTATGAAGAGTTT
	BRAF_V600E	CAAACTGATGGGACCCACTCCATCGAGATTTCTCTGTAGCTAGACCAAAATCACCTATTTTTACTGTGAGGTCTTCATGAAGAAATATATCTGAGGTGTAGTAAGTAAAGGAAAACAGTAGATCTCATTTTCCTATCAGAGCAAGCATTATGAAGAGTTT

SUPPLEMENTAL TABLE S3: SIGNAL TO NOISE RATIOS (SNR) OF DESIGNED UNIVERSAL REPORTERS (PSR) USED IN THE NAICA PRISM III

Table S3: SNR ± STD were determined in a mediator extension assay [3] using following conditions: 150 nM mediator; 100 nM PSR. Mediator extension assay was performed in a qPCR cycler (RotorGeneQ from Qiagen) with following PCR setting: 300 s at 95°C initial denaturation, followed by 45 cycles 95°C 5 s and 60°C 30 s. Signals were acquired at 28°C; values were taken from a melting curve measurement. Both signal and noise represent values of technical triplicates (n=3).

PSR:	PSR07	PSR08	PSR02	PSR04	PSR05	PSR06
Fluorophore	FAM	Atto 488	DY-530	BMN536	CY5	Atto 647N
Quencher	BHQ-1	BMN-Q1	BMN-Q1	BHQ-1	BHQ-2	BHQ-2
SNR:	41.4 ± 6.5	15.5 ± 1.0	26.8 ± 2.3	16.0 ± 1.1	82.6 ± 1.6	63.3 ± 2.5

SUPPLEMENTAL TABLE S4: VMR RATIOS

Table S4 Variance to mean ratios (VMR) of leading duplex oligonucleotide titration conditions.

Primer		MP [nM]	PSR [nM]	No. of positive droplet population	Mean	Variance	VMR ($\times 10^2$)	Significance test	Condition
forward [nM]	reverse [nM]								
500	500	1200	400	2,417	1.14	0.15	12.89	46,097	Primer titration (Fig. S2)
750	500	1200	400	2,683	1.15	0.17	14.92	42,521	
1000	500	1200	400	2,598	1.16	0.18	15.38	50,693	
1250	500	1200	400	2,646	1.17	0.20	17.18	54,715	
500	1200	1400	600	2,918	1.15	0.18	15.46	30,057	MP:PSR titration (Fig. S3)
500	1200	1200	600	3,050	1.19	0.23	19.07	32,772	
500	1200	1000	600	2,952	1.14	0.16	13.62	32,290	
500	1200	800	600	3,036	1,18	0.21	18.12	25,705	
1000	500	1200	600	2,967	1.23	0.27	22.12	58,628	Combination of primer and MP:PSR titration (Fig. S4)
1000	500	800	400	3,009	1.22	0.27	22.10	35,318	
1250	500	1200	600	3,183	1.22	0.27	22.07	39,323	
1250	500	600	400	3,112	1.20	0.21	20.57	39,323	

Primer		MP [nM]	PSR [nM]	No. of positive droplet population	Mean	Variance	VMR ($\times 10^2$)	Significance test	Condition		
forward [nM]	reverse [nM]										
1,000	1,000	1,000	250	2,966	1,34	0,38	28.64	29,456	Blue		
1,000	1,000	1,000	250	2,254	1.21	0.24	20.11	26,768	Green	C1	Two-plex REM Fig. 2
1,000	1,000	1,000	250	4,718	1.31	0.28	28.13	22,717	Red		
1,000	500	1,200	600	4,978	1.68	1,03	61.09	25,851	Blue		
1,000	500	1,200	600	4,795	1.17	0.19	16.07	53,195	Green	C2	
1,000	500	1,200	600	2,902	1.22	0.26	20.93	35,283	Red		

SUPPLEMENTAL TABLE S5: ABSORPTION AND EMISSION MAXIMA OF FLUOROPHORES USED IN UNIVERSAL REPORTER (PSR) STRUCTURES

Table S5: Maxima absorption and emission wavelengths in nm according to the website of the PSR manufacturer.

Fluorophore	**FAM**	**Atto 488**	**BMN536**	**DY-530**	**Atto 647N**	**Cy5**	**Cy5.5**	**Atto-680**
Maximum absorption [nm]	494	501	536	539	646	649	675	680
Maximum emission [nm]	520	523	553	561	664	670	694	700

SUPPLEMENTAL FIG. S1: ANNEALING TEMPERATURE GRADIENT

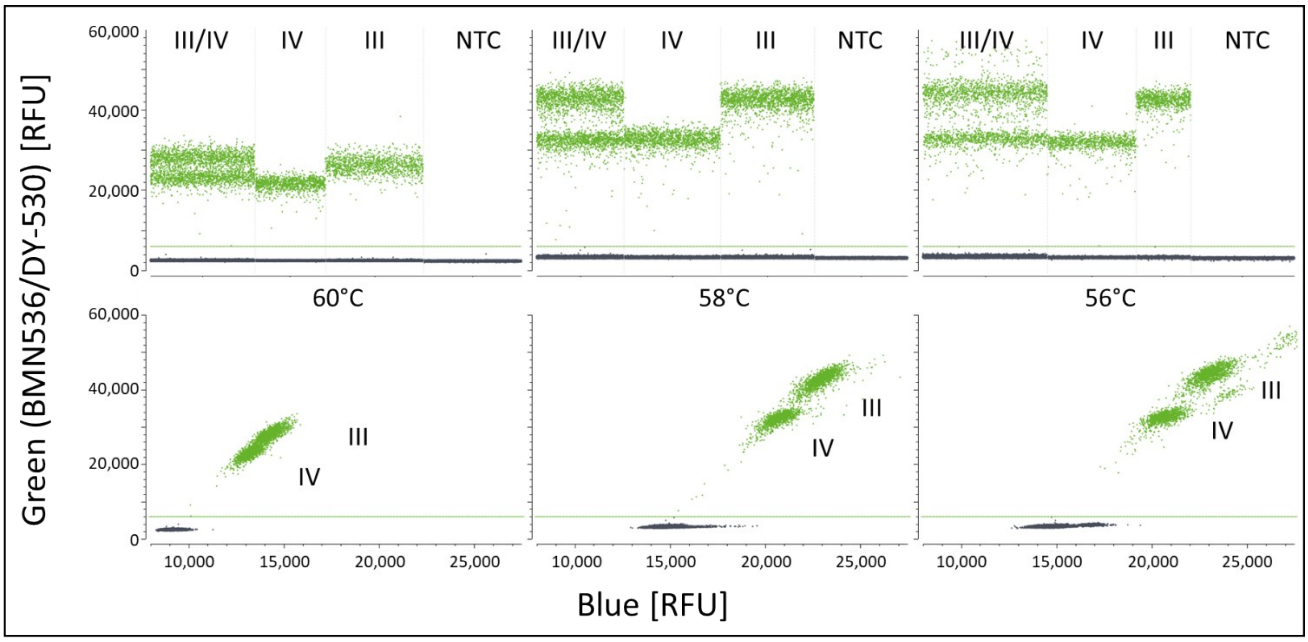

Fig. S1 1D and corresponding 2D scatterplots of two-plex REM-dPCR with changing annealing and extension temperatures targeting KRAS WT (III), KRAS G12A (IV). Threshold was set at same intensities (6,000 RFU) in all conditions, indicated by green line. Green populations were positive, negative droplets are indicated in dark grey.

SUPPLEMENTAL FIG. S2: TWO-PLEX PRIMER TITRATION

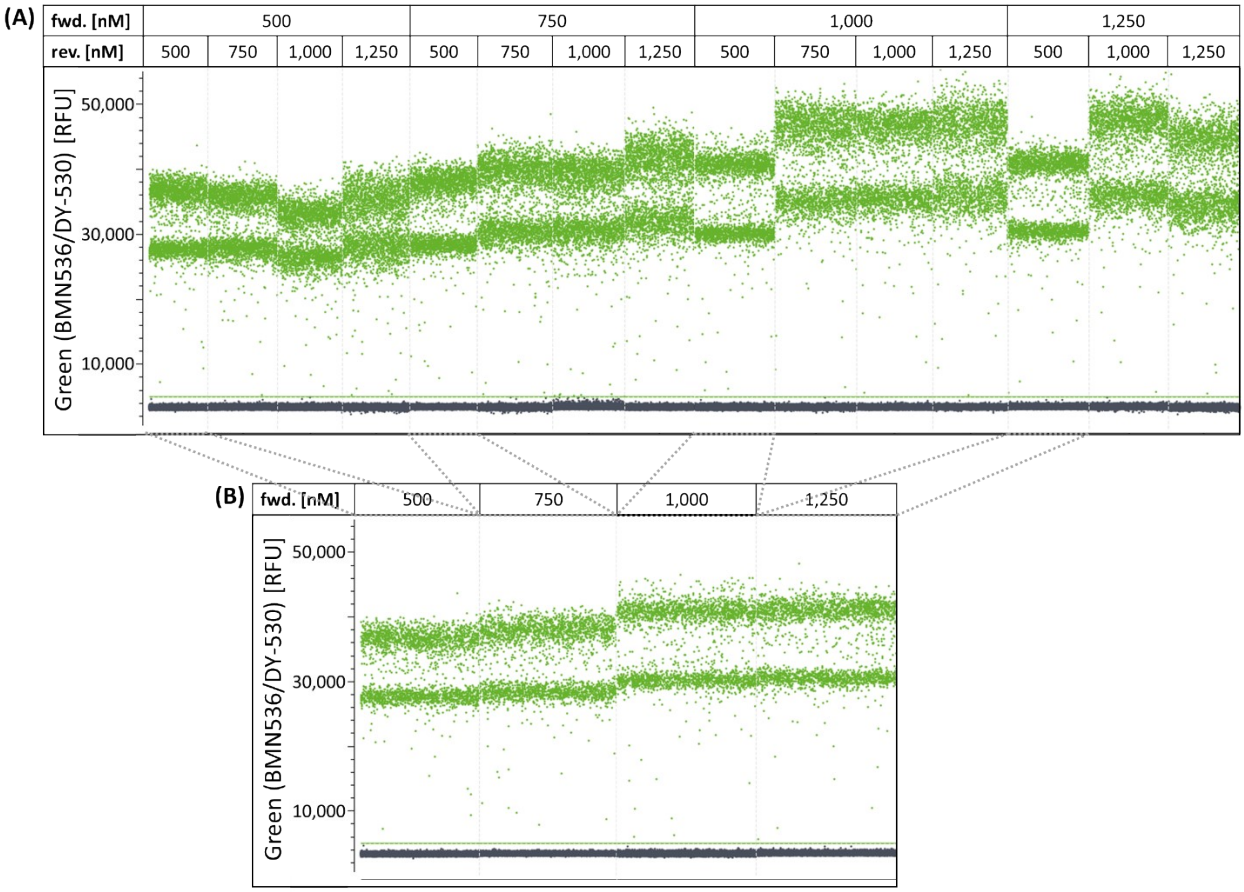

Fig. S2. 1D scatterplots of the two-plex REM-dPCR primer titration targeting KRAS WT (III, upper population), KRAS G12A (IV, lower population) **(A)**. Negative populations are highlighted in dark grey and positive populations in green. **(B)** Leading conditions of the primer titration. All conditions had 500 nM reverse primer with the different forward primer concentrations indicated at top of the graph. Threshold are indicated with a dashed green line. See Table S4 for VMR analysis.

SUPPLEMENTAL FIG. S3: TWO-PLEX MP:PSR TITRATION

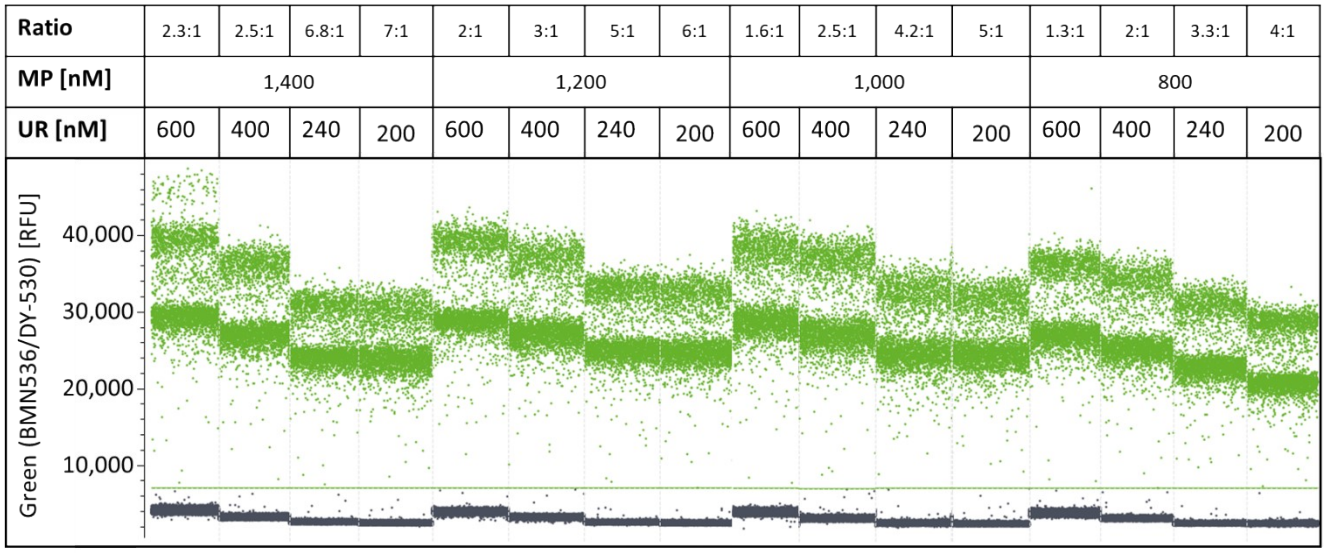

Fig. S3 1D scatterplots of the two-plex REM-dPCR MP:PSR titration targeting KRAS WT (III, upper population), KRAS G12A (IV, lower population). Threshold is displayed as a green dashed line, negative populations are highlighted in dark grey and positive populations in green. See Table S4 for VMR analysis for leading conditions with 600 nM PSR.

SUPPLEMENTAL FIG. S4. COMBINATION OF LEADING CONDITIONS

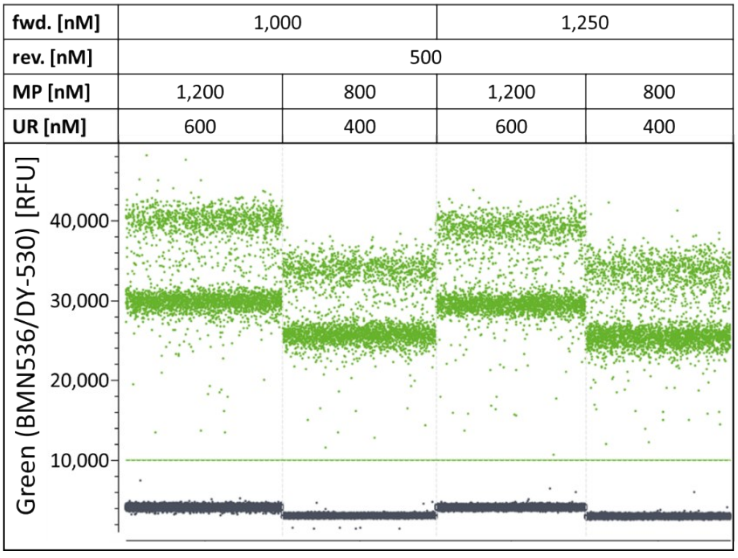

Fig. S4 Two-plex REM-dPCR comparing leading conditions of the MP:PSR and primer titration targeting KRAS WT (III, upper population), KRAS G12A (IV, lower population). The threshold was indicated with a green dashed line. The reactions were separated by perpendicular dashed light grey lines. See Table S4 for VMR analysis.

SUPPLEMENTAL FIG. S5: KRAS FOUR-PLEX REM-dPCR IN CONDITION C2

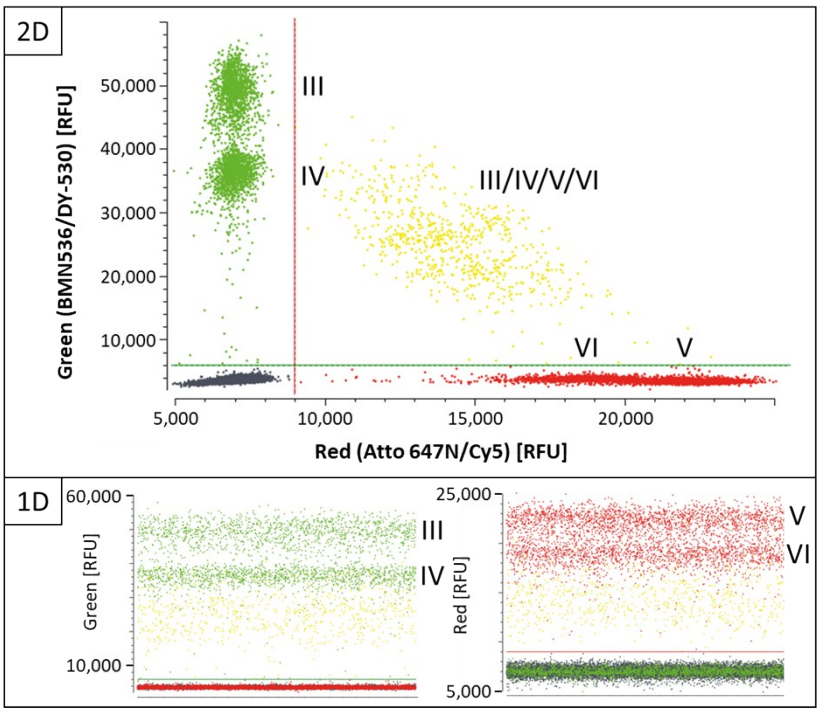

Fig. S5 Four-plex REM-dPCR scatterplots with condition C2 assay parameters targeting KRAS WT (III), KRAS G12A (IV) in the green channel and KRAS G12D (V) and KRAS G12V (VI) in the red channel. Double positive are indicated in yellow. Thresholds are indicated as a green and red line in the figure. Results are shown in 1D and 2D for the respective channels and channel combinations.

SUPPLEMENTAL FIG. S6: KRAS FOUR-PLEX TITRATION

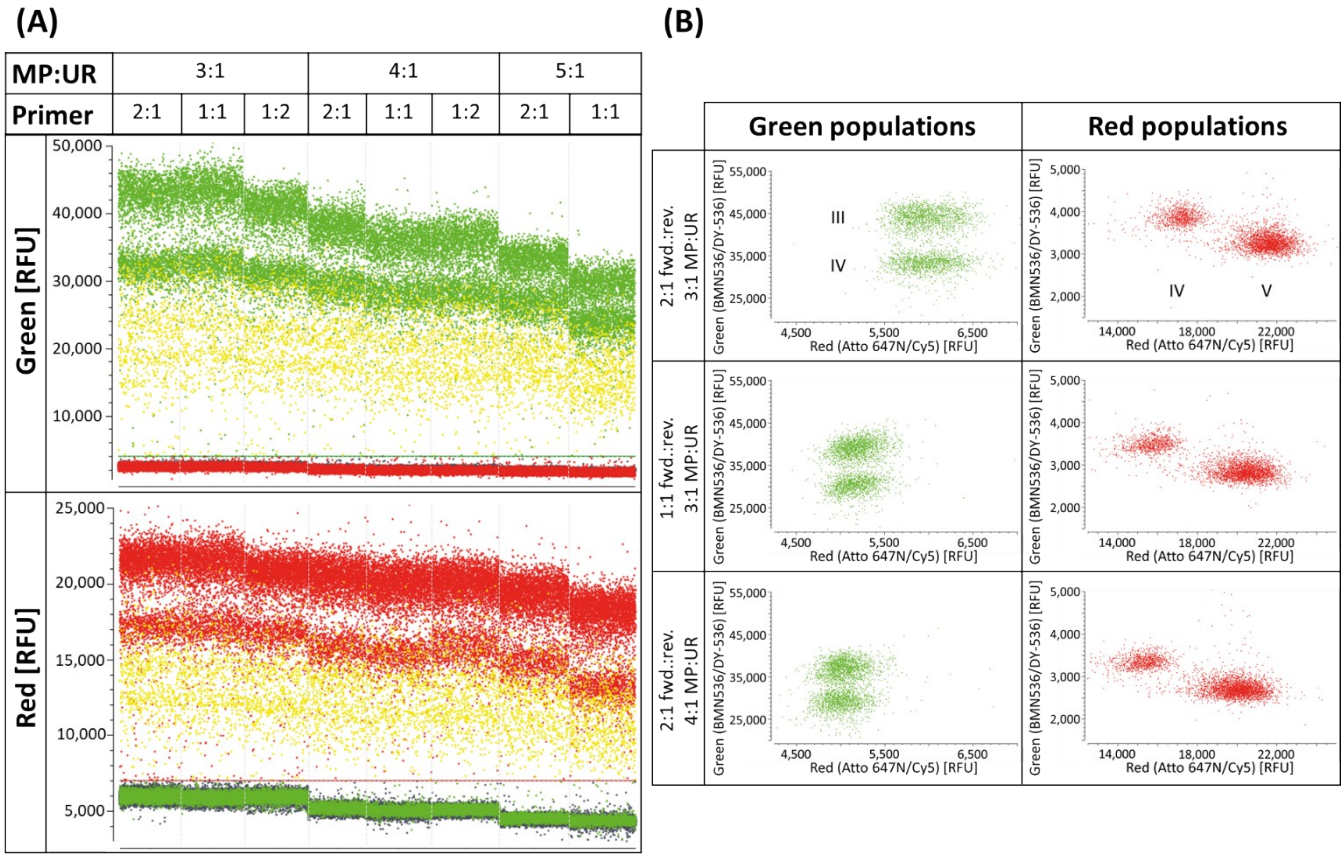

Fig. S6 Four-plex REM-dPCR scatterplots of MP, PSR and primer titrations targeting KRAS WT (III), KRAS G12A (IV) in the green channel and KRAS G12D (V) and KRAS G12V (VI) in the red channel **(A)**. Double positive are indicated in yellow. Thresholds are indicated as a green and red line in the figure. **(B)** Zoom in of leading conditions of the titrations showing 2D plots in the green and red channel.

REFERENCES

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