SUPPORTING INFORMATION

Multiplexed target detection using DNA-binding dye chemistry in droplet digital PCR.

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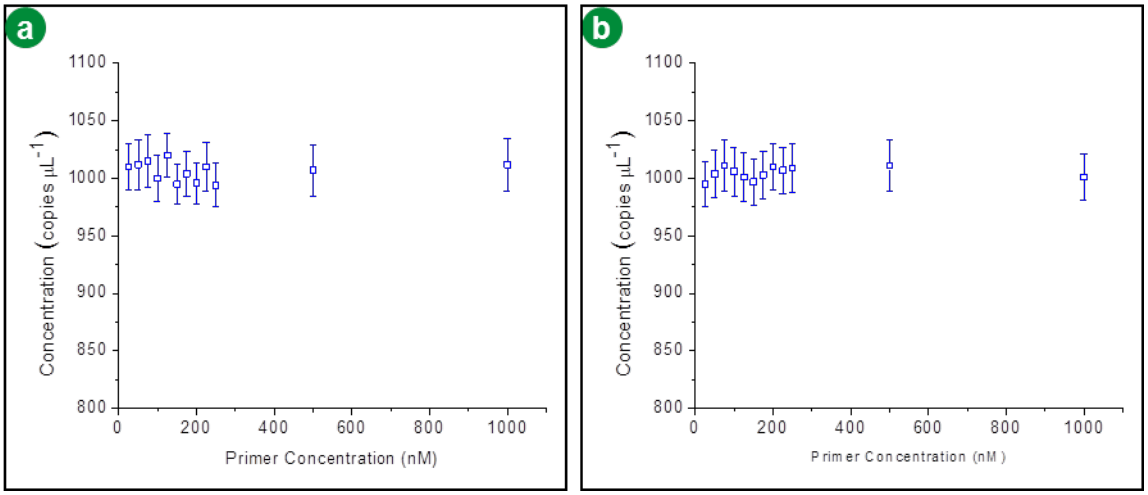

Figure S1. Measured concentrations of the *IL-4* target gene where the amount of primer was varied from 25 – 250 nM in 25 nM increments then 500 and 1000 nM in the presence of (a) 0.33 or (b) 3.33 ng μL^{-1} of purified human genomic DNA. Error bars denote the Poisson 95% confidence interval.

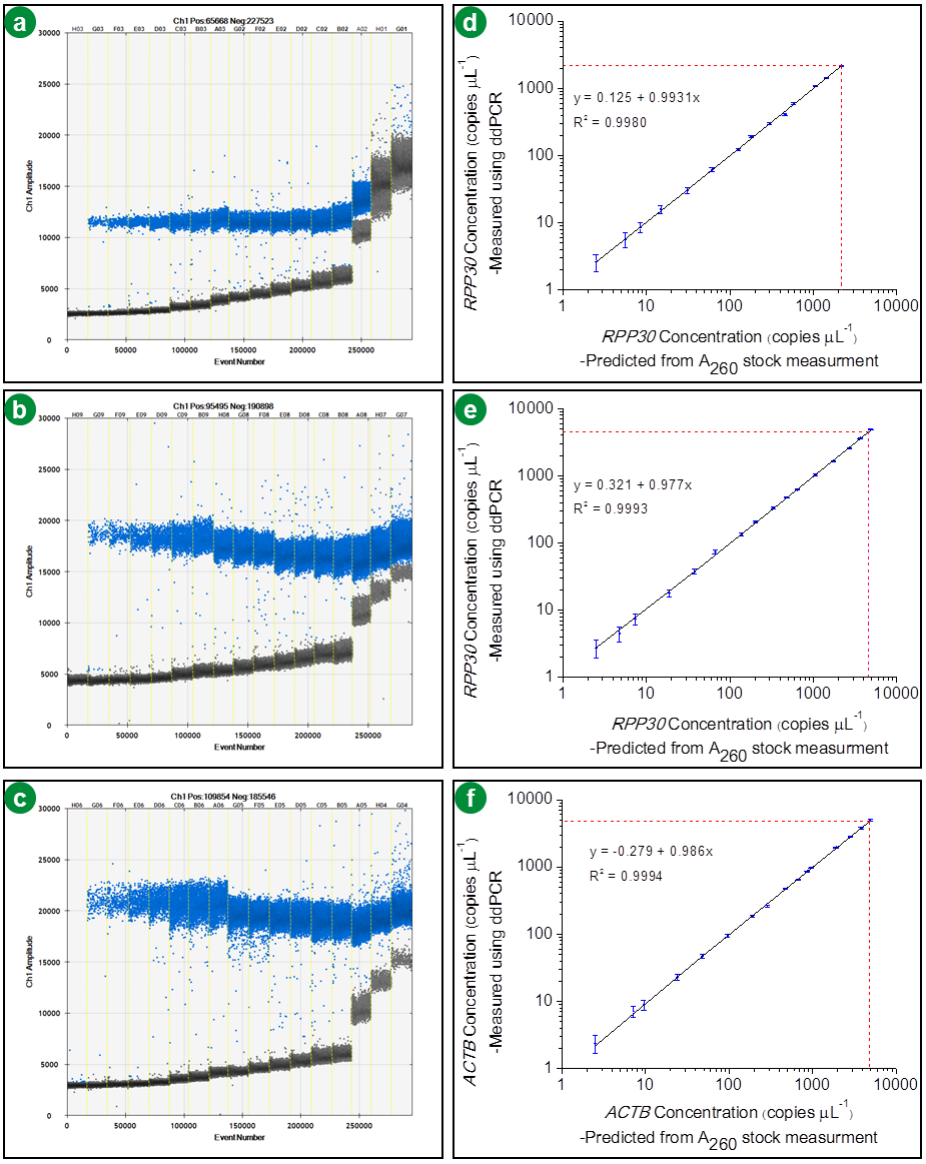

Figure S2. Measurement of the *RPP30* and *ACTB* genes across a dilution series of human genomic DNA (8.2 pg μL^{-1} to 16.5 ng μL^{-1}, corresponding to 2.5 – 5000 copies μL^{-1} of each target) using EvaGreen-based ddPCR. 1D Droplet Plots for the *RPP30* assay using a primer concentration of (a) 100 nM and (b) 200 nM (c) 1D Droplet Plots for the *ACTB* assay using a primer concentration of 100 nM. Linearity plots corresponding to the (e) *RPP30*, 100 nM primers (f) *RPP30*, 200 nM primers and (g) *ACTB* assays. Error bars denote the Poisson 95% confidence interval and red dotted lines indicate the highest concentration that was reliably measured (according to the ability to distinguish positive from negative droplets).

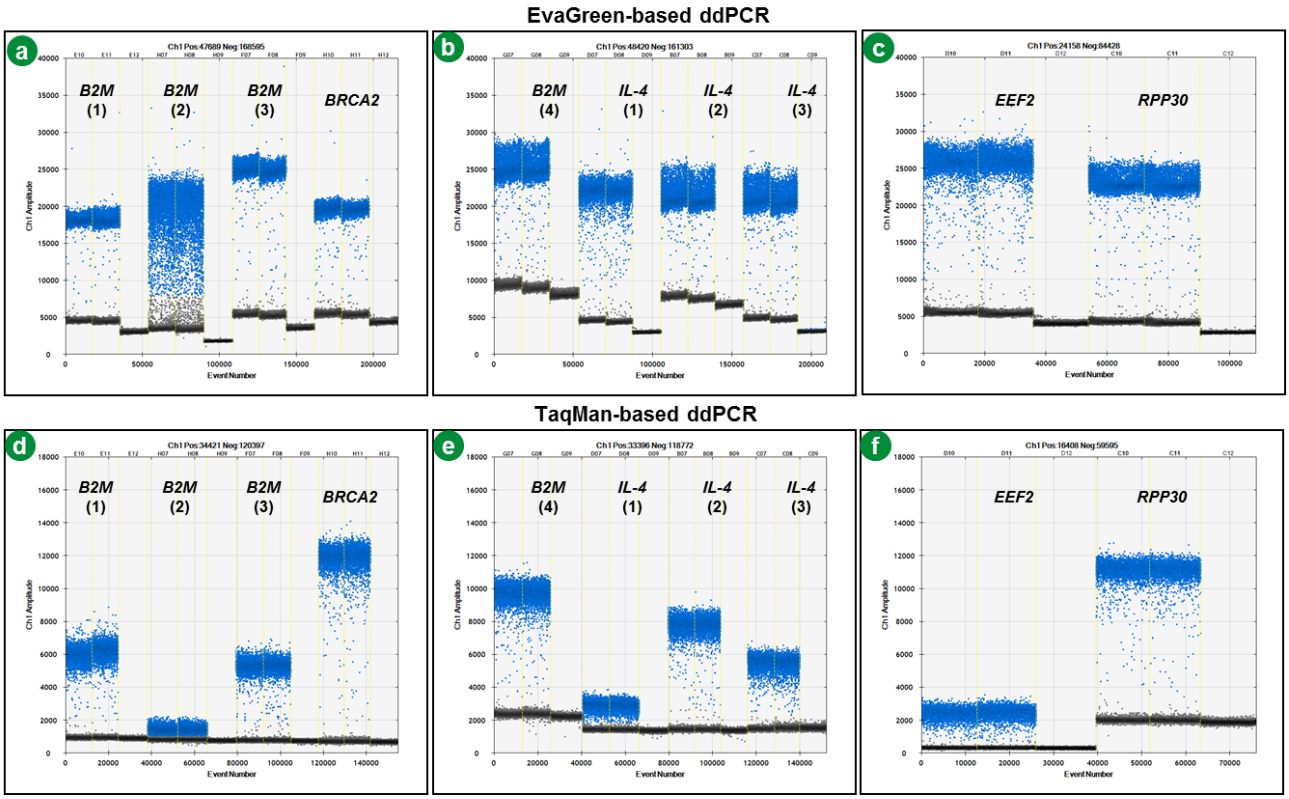

Figure S3. Detection of 10 target sequences on five separate genes using (a,b & c) EvaGreen and (d,e & f) TaqMan-based ddPCR. The amount of template DNA was kept constant for each reaction (1.65 ng μL^{-1}, with the exception of NTC wells). Results are arranged according to %GC content of resulting amplification products (see experimental section of the Supporting Information).

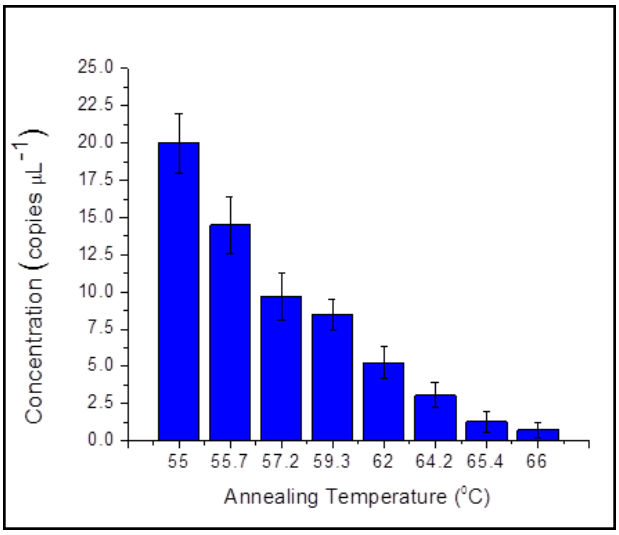

Figure S4. Concentration measurements of primer-dimer products obtained in the *NCoA-1* assays NTC wells. Temperature gradient thermal cycling conditions were employed. Error bars denote the Poisson 95% confidence interval.

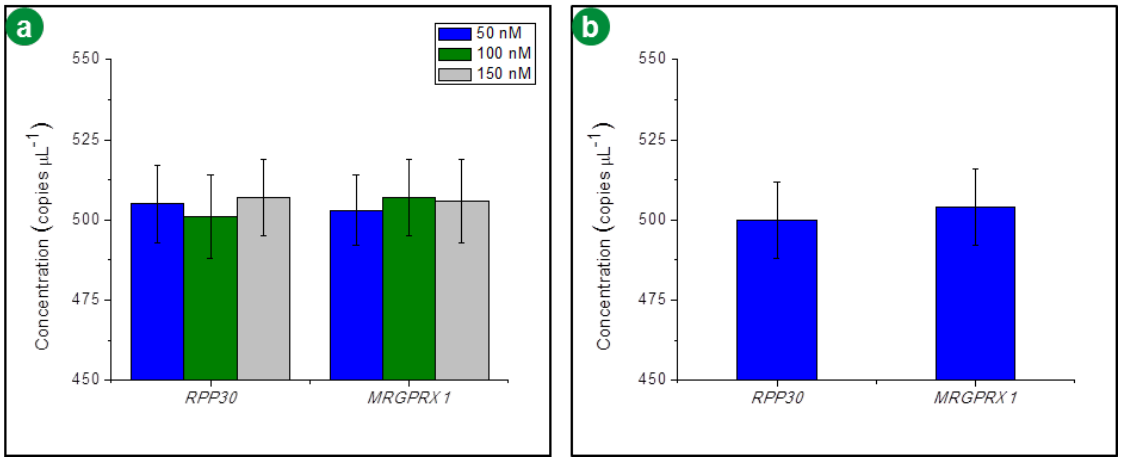

Figure S5. Multiplexed target detection (achieved by differing primer concentrations) using EvaGreen-based ddPCR. (a) Concentration measurements of the *RPP30* and *MRGPRX1* targets from singleplex reactions. Primer concentrations of 50,100 or 150 nM were employed. (b) Concentration measurements of the *RPP30* and *MRGPRX1* targets from a multiplex reaction where primer concentrations of 150 and 50 nM were used, respectively. Error bars in (a) and (b) indicate the Poisson 95% confidence interval.

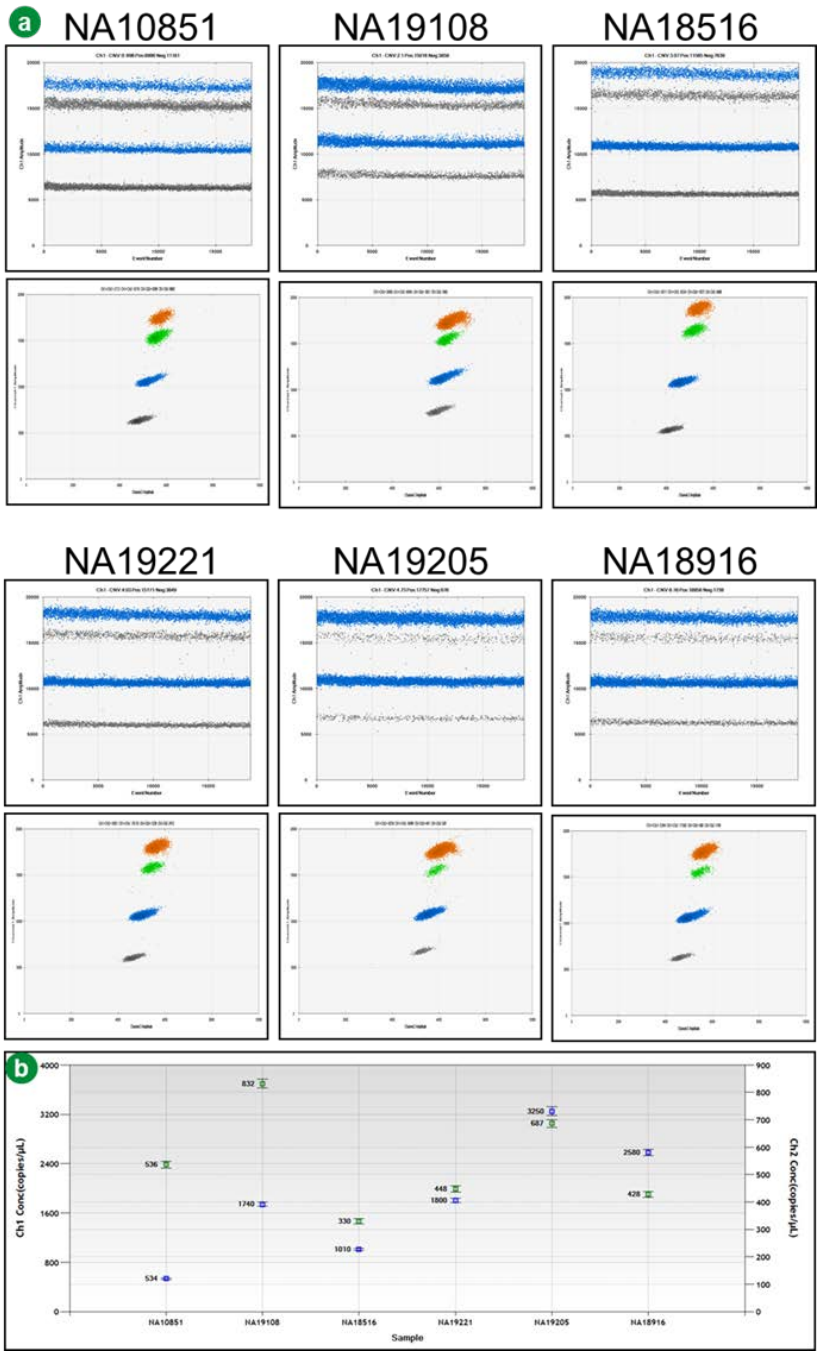

Figure S6. Measurements of *MRGPRX1* copy number states in HapMap samples using multiplexed EvaGreen-based ddPCR. (a) HapMap sample identifiers with associated 1D and 2D Droplet Plots. Manual thresholds were drawn in 2D Droplet Plots to assign clusters. (b) Concentration measurements of the *RPP30* (blue) and *MRGPRX1* (green) targets from each multiplex reaction. Error bars indicate the Poisson 95% confidence intervals.

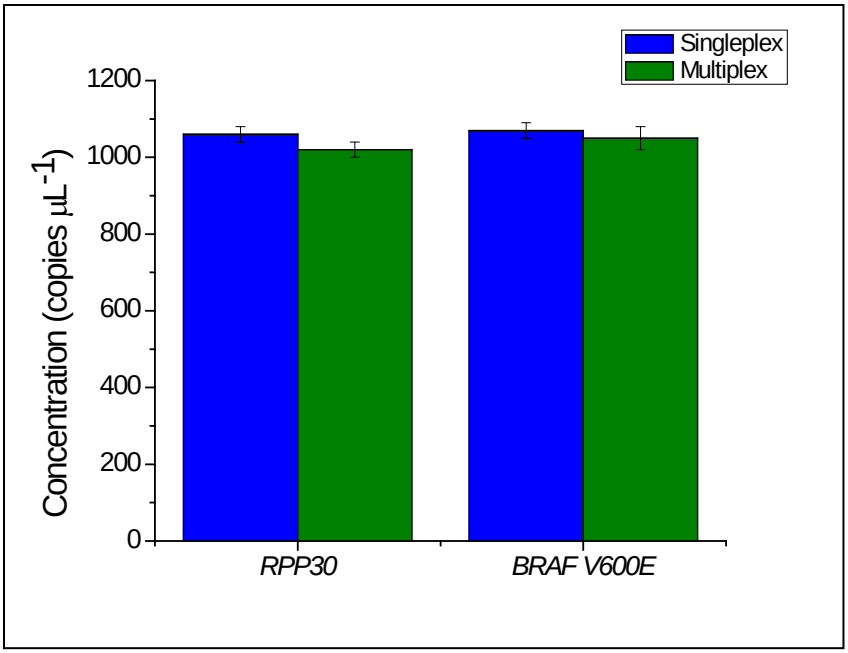

Figure S7. Multiplexed target detection using EvaGreen and TaqMan Probe based chemistry in a single reaction. Concentration measurements from: a singleplex *RPP30* assay which included 100 nM of a VIC-labeled probe sequence in an EvaGreen-based reaction mixture (blue); a singleplex *BRAF V600E* assay prepared in EvaGreen-based Supermix (blue); a multiplexed assay of both *RPP30* and *BRAF V600E* targets achieved by combining the two aforementioned assays into one reaction (green).

Table S1. Assay primer/probe sequences and resulting amplicon length/%GC content.

Assay		Primer Sequences	Probe Sequence	Amplicon	
				Length	%GC
***B2M* (1)**	**Forward**	5′-CCAAATTCTGCTTGCTTGCT-3′	5′-6FAM-CACTTACAC/ZEN/TTTATGCACAAAATG-IABkFQ-3′	99	30
	Reverse	5′-GATCATGTCCATGTTAACATTATTATAACCCTA-3′			
***B2M* (2)**	**Forward**	5′-CCAAATTCTGCTTGCTTGCT-3′	5′-6FAM-TCTGAGCAGGTTGCTCCACA-MGBNFQ-3′	458	34
	Reverse	5′-AACCACAACCATGCCTTACTTT-3′			
***B2M* (3)**	**Forward**	5′-CCAAATTCTGCTTGCTTGCT-3′	5′-6FAM-TCTGAGCAGGTTGCTCCACA-MGBNFQ-3′	184	37
	Reverse	5′-GCCAGCCCTCCTAGAGCTA-3′			
BRCA	**Forward**	5′-GTGAGGTAGATTGTAAAGTCAAAGG-3′	*5′-6FAM-AAcTGTTTtGTATTcACcAT-IABkFQ -3′	119	38
	Reverse	5′-ACCCTCATTTGCTACATGGT-3′			
***B2M* (4)**	**Forward**	5′-AGATTCAGGTTTACTCACGTCATCC-3′	5′-6FAM-TCACACGGCAGGCATACTCA-MGBNFQ-3′	263	42
	Reverse	5′-AACTATCTTGGGCTGTGACAAAGT-3′			
***IL-4* (1)**	**Forward**	5′-AACTGCTTCCCCCTCTGTTCTT-3′	5′-6FAM-TCCAACCGGACAGAGAAGTCC-MGBNFQ-3′	502	50
	Reverse	5′-GTTCAGACATTTGGGGATGGAT-3′			
***IL-4* (2)**	**Forward**	5′-CCTCAGTTGGAGGGAGTGAGAG-3′	5′-6FAM-TCCAACCGGACAGAGAAGTCC-MGBNFQ-3′	207	52
	Reverse	5′-GTTCAGACATTTGGGGATGGAT-3′			
***IL-4* (3)**	**Forward**	5′-GTTGGAACTGGTGGTTGGTG-3′	5′-6FAM-TCCAACCGGACAGAGAAGTCC-MGBNFQ-3′	294	53
	Reverse	5′-GTTCAGACATTTGGGGATGGAT-3′			
EEF2	**Forward**	5′-AAGAAGGAGGACCTCTACCTGAAG-3′	5′-6FAM-TGCTCCAAGTCTTGGGCTCC-MGBNFQ-3′	508	61
	Reverse	5′-CGTAGCGGCCCATCATCAA-3′			
RPP30	**Forward**	5′-CGGTGTTTGCAGATTTGGAC-3′	5′-6FAM-TCTGACCTGAAGGCTCTGCGCG-MGBNFQ-3′	241	62
	Reverse	5′-CGCTAGGAATCAGACCAACA-3′			

*Locked nucleic acid (LNA) nucleotides are denoted in lower case, DNA nucleotides are denoted in upper case.

EXPERIMENTAL

Droplet Digital PCR Reaction Mixtures. Unless otherwise stated, EvaGreen-based ddPCR reaction mixtures contained 1X ddPCR EvaGreen Supermix (Bio-Rad), 100 nM primers (Integrated DNA Technologies, Coralville, IA, USA) and human genomic DNA template (Raji gDNA, Lofstrand Labs Limited, Gaithersburg, MD, USA) in a final volume of 25 µL. For TaqMan-based ddPCR experiments, the reaction mixtures comprised of 1X Droplet PCR Supermix (Bio-Rad), primers/FAM-MGBNFQ probes (900/250 nM, Applied Biosystems, Foster City, CA, USA) and human genomic DNA template (Raji, Lofstrand Labs) in a final volume of 25 µL. No template control wells were created by replacing input DNA solutions with TE buffer (pH 8.0).

Primer Concentration. Primer titrations were performed by serially diluting a 100 µM stock solution of forward and reverse *IL-4* primers to the following concentrations: 25 – 250 nM in 25 nM increments along with 500 and 1000 nM. Three EvaGreen-based ddPCR mixtures were prepared, containing a final concentration of 0, 0.33, or 3.33 ng μL^{-1} of human genomic DNA (Raji, Lofstrand Labs). An aliquot of 6.25 µL was taken from each primer dilution and combined with 18.75 µL of reaction mixture for ddPCR analysis. *IL-4* assay sequences were (forward primer) 5′-GTTGGAACTGGTGGTTGGTG-3′, (reverse primer) 5′-GTTCAGACATTTGGGGATGGAT-3′.

Amplicon Length. Three EvaGreen-based ddPCR formulations were prepared for the *RPP30* target, each containing a final human genomic DNA (Raji, Lofstrand Labs) concentration of 0.5 ng μL^{-1}. Each assay contained the same *RPP30* forward primer sequence 5′-GATTTGGACCTGCGAGCG-3′. The reverse primer sequence was varied across the three

reactions: (1), 5′-GCGGCTGTCTCCACAAGT-3′; (2), 5′-GGTTGGCCAGGCGCGAAG-3′; (3), 5′- CATCAGCCTCCAGGGACATC-3′.

Dynamic Range. The concentration of human genomic DNA (Raji, Lofstrand Labs) stock solution was estimated to be 230 ng μL^{-1} by monitoring the visible absorbance at 260 nm of non-denatured dsDNA using UV absorption spectroscopy as previously described.[52] Human genomic DNA was digested using *Nla*III (New England BioLabs, Ipswich, MA, USA) for 1 h at 37 ºC to yield a final concentration of 115 ng μL^{-1} and then serially diluted from 52 down to 0.03 ng μL^{-1} into TE buffer (pH 8.0). For the *RPP30* target a second EvaGreen-based ddPCR mixture containing a final concentration of 200 nM primers was also formulated. An aliquot of 7.2 μL was taken from each DNA dilution and combined with 17.8 μL of reaction mixture for ddPCR analysis. *RPP30* assay sequences were (forward primer) 5′-GATTTGGACCTGCGAGCG-3′, (reverse primer) 5′-GCGGCTGTCTCCACAAGT-3′. *ACTB* assay sequences were (forward primer) 5′-CGCCGTTCCGAAAGTT-3′, (reverse primer) 5′-CGGCGGATCGGCAAA-3′.

Comparison of EvaGreen and TaqMan ddPCR. Both EvaGreen and TaqMan assays included human genomic DNA template at a final concentration of 1.65 ng μL^{-1}. The primer/probe sequences for each assay are located in the Supporting Information (Table S-1). Results (Figure S3) are displayed according to %GC content of the amplicon produced from each assay which ranged from: low, 30 – 38% (Figure S3a and d); medium, 42 – 52% (Figure S3b and e) and High, 60 – 62% (Figure S3c and f).

Non-Specific Amplification Products. For the *NCoA-1* primer-dimer assay, two EvaGreen-based ddPCR mixtures were formulated. The first contained 0.77 ng μL^{-1} of human genomic DNA (Raji, Lofstrand Labs) whilst no template was added to the second. *NCoA-1* assay sequences were (forward primer) 5′-ACCACTTTGTCTGTCGAGCCTGAT-3′ and (reverse primer) 5′-TCCAGGCTCAGGTTTGGAGTTGAT-3′. For the *GAPDH* assay that generated non-specific products at low annealing temperatures, two EvaGreen-based ddPCR mixtures were formulated. The first contained 1.32 ng μL^{-1} of human genomic DNA (Raji, Lofstrand Labs) whilst no template was added to the second. *GAPDH* assay sequences were (forward primer) 5′-CCACTAGGCGCTCACTGTTC-3′ and (reverse primer) 5′-TGACTCCGACCTTCACCTTC-3′. For the *ACTB* assay that also generated non-specific products at low annealing temperatures, two EvaGreen-based ddPCR mixtures were formulated. The first contained 1.47 ng μL^{-1} of human genomic DNA (Raji, Lofstrand Labs) whilst no template was added to the second. *ACTB* assay sequences were (forward primer) 5′-AGTGTGGTGTTGGGGGAGT-3′ and (reverse primer) 5′-GAGTGTGGTCCTGCGACTTC-3′. Gradient thermal cycling conditions for the *NCoA-1*, *GAPDH* and *ACTB* EvaGreen assays were: 95 ºC × 5 min (1 cycle), 95 ºC × 30 s and 66-55 ºC × 1 min (40 cycles), 4 ºC × 1 min (1 cycle), 95 ºC × 1 min (1 cycle) and a 4 ºC hold.

Single Color Multiplexing. To independently quantify the *RPP30* and *ACTB* genes, two EvaGreen-based ddPCR formulations were prepared. Each of these contained 100 nM of primers and 1.31 ng μL^{-1} of human genomic DNA (Raji) which had been digested using *Nla*III (NEB) for 1 h at 37 ºC. To achieve multiplexed quantification of *RPP30* and *ACTB* genes a third EvaGreen-based reaction mixture was prepared which also contained 1.32 ng μL^{-1} of *Nla*III digested human genomic DNA (Raji, Lofstrand Labs) along with the primer sequences for both

targets at a final concentration of 100 nM each. *RPP30* assay sequences were (forward primer) 5′-GATTTGGACCTGCGAGCG-3′, (reverse primer) 5′-GCGGCTGTCTCCACAAGT-3′. *ACTB* assay sequences were (forward primer) 5′-CGCCGTTCCGAAAGTT-3′, (reverse primer) 5′-CGGCGGATCGGCAAA-3′.

The *MRGPRX1* and the *RPP30* genes were quantified separately using three EvaGreen-based ddPCR formulations per target each containing 1.65 ng μL^{-1} of human genomic DNA (Raji, Lofstrand Labs) which was digested with *Hae*III (NEB) for 1 h at 37 ºC and either 50, 100 or 150 nM of primers. For the multiplexed detection of *MRGPRX1* and *RPP30* targets, an EvaGreen-based reaction mixture was prepared containing 50 and 150 nM primers respectively and 1.65 ng μL^{-1} of *Hae*III digested human genomic DNA (Raji, Lofstrand Labs). *MRGPRX1* assay sequences were (forward primer) 5′-TTAAGCTTCATCAGTATCCCCCA-3′, (reverse primer) 5′-CAAAGTAGGAAAACATCATCACAGGA-3′. *RPP30* assay sequences were (forward primer) 5′-GATTTGGACCTGCGAGCG-3′, (reverse primer) 5′-GCGGCTGTCTCCACAAGT-3′. For the determination of *MRGPRX1* copy number variation, each purified human genomic DNA sample (Coriell Institute for Medical Research, Camden, NJ, USA, Samples: NA10851, NA19108, NA18516, NA19221, NA19205 and NA18916) normalized by 260 nm spectroscopy,[52] digested with *Hae*III (NEB) in 50 µL for 1 h at 37 ºC to yield a final concentration of ~150 ng μL^{-1}. A 5 µL aliquot of digest DNA was diluted into 20 µL of reaction mixture which comprised of ddPCR EvaGreen Supermix along with *RPP30* and *MRGPRX1* primers. The primer concentrations and sequences used for *MRGPRX1* and *RPP30* targets were identical to those previously mentioned.

For the multiplexed detection of *BRAF V600E* and *RPP30* using dye and probe based chemistries, three separate reaction mixtures were prepared in EvaGreen Supermix, each containing 3.3 ng μL^{-1} of DNA (Corielle, NA19205) which had been digested with *Mse*I (NEB) in 50 μL for 1 h at 37 ºC. Each reaction mixture also contained 100 nM of *BRAF V600E* primers or 100 nM *RPP30* primers/VIC-MGBNFQ probe or a combination of both *BRAF V600E*/*RPP30* primers and primer/probes at 100 nM each. *RPP30* assay sequences were (forward primer) 5′-GATTTGGACCTGCGAGCG-3′, (reverse primer) 5′-GCGGCTGTCTCCACAAGT-3′ and (probe) VIC-CTGACCTGAAGGCTCTMGBNFQ. *BRAF V600E* assay sequences were (forward primer) 5′-GGAAAATAGCCTCAATTCTTACCA-3′, (reverse primer) 5′-TGATTTTGGTCTAGCTACTGTGA-3′. Thermal cycling conditions were: 95 ºC × 5 min (1 cycle), 95 ºC × 30 s and 59 ºC × 1 min (40 cycles), 4 ºC × 1 min (1 cycle), 95 ºC × 1 min (1 cycle) and a 4 ºC hold.

REFERENCES

52. Bhat, S.; Curach, N.; Mostyn, T.; Bains, G. S.; Griffiths, K. R.; Emslie, K. R.; *Anal. Chem.*, **2010**, 82, 7185-7192.