SUPPLEMENTARY APPENDIX

Monitoring multiple myeloma by quantification of recurrent mutations in serum

Even Holth Rustad,[1] Eivind Coward,[1,2] Emilie R Skytøen,[1] Kristine Misund,[1] Toril Holien,[1] Therese Standal,[1,3] Magne Børset,[1] Vidar Beisvag,[1] Ola Myklebost,[2,4,5] Leonardo A Meza-Zepeda,[2,5] Hong Yan Dai,[6] Anders Sundan[1,3] and Anders Waage[1,2,7]

[1]Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, NTNU, Trondheim; [2]Norwegian Cancer Genomics Consortium; [3]CEMIR – Center for Molecular Inflammation Research, Norwegian University of Science and Technology, NTNU, Trondheim; [4]Institute for Clinical Science, University of Bergen; [5]Institute for Cancer Research, Oslo University Hospital; [6]Department of Pathology and Medical Genetics, St. Olav's University Hospital, Trondheim and [7]Department of Hematology, St. Olav's University Hospital, Trondheim, Norway

 doi:10.3324/haematol.2016.160564

Received: November 28, 2016.

Accepted: March 31, 2017.

Pre-published: April 6, 2017.

Correspondence: anders.waage@ntnu.no

Monitoring of Multiple Myeloma by quantification of recurrent mutations in serum

Even H Rustad[1]; Eivind Coward[1, 2]; Emilie R Skytøen[1]; Kristine Misund[1]; Toril Holien[1]; Therese Standal[1, 3]; Magne Børset[1]; Vidar Beisvag[1]; Ola Myklebost[2, 4, 5];Leonardo A Meza-Zepeda[2, 5]; Hong Yan Dai[6]; Anders Sundan[1, 3]; Anders Waage[1, 7]

Affiliations:

1. Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, NTNU, Trondheim, Norway
2. Norwegian Cancer Genomics Consortium (http://cancergenomics.no)
3. CEMIR – Center for Molecular Inflammation Research, Norwegian University of Science and Technology, NTNU, Trondheim, Norway
4. Institute for Clinical Science, University of Bergen, Norway
5. Institute for Cancer Research, Oslo University Hospital, Norway.
6. Department of Pathology and Medical Genetics, St. Olavs University Hospital, Trondheim, Norway
7. Department of Hematology, St. Olavs University Hospital, Trondheim, Norway

Corresponding author: Anders Waage, Department of Hematology, St Olavs Hospital, Postbox 3250 Sluppen, 7006 Trondheim, Telephone +47 72825100, Fax +47 72825733, e-mail anders.waage@ntnu.no

Contents

Supplemental methods

Blood sample collection and handling

Serum (n=249) and citrate-plasma (n=2) samples were collected at the Department of Hematology, St Olavs Hospital and stored in the Norwegian myeloma biobank. Blood samples were centrifuged at room temperature at 2200*g* for 10 minutes within 1.5 hours after sampling and stored at -80 ^{0}C. For preparation of serum, blood samples were left to coagulate for at least 30 minutes prior to centrifugation. Median storage time before DNA extraction was 5 years (range 0-11).

Serum DNA extraction

Serum samples were thawed and centrifuged at room temperature at 4000*g* for 10 minutes. The supernatants (median volume 1.8 ml, range 0.4-3 ml) were transferred to a clean tube for DNA extraction by QiaAmp Circulating Nucleic Acid kit (Qiagen Hilden, Germany) using a QiaVac 24 plus vacuum system (Qiagen). Elution volumes varied from 30 to 75 µl depending on the analyses planned for each sample. DNA quantification was performed by Qubit dsDNA High Sensitivity assay kit on a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA). Median DNA yield was 27 ng/ml of input sample (range 3.8-671.7 ng/ml). There was no relationship between DNA yield and sample storage time (r=-0.077, n=250, p=0.225).

Detection of mutations in serum by digital droplet PCR

Digital PCR allows sensitive detection and absolute quantitation of specific mutations and has previously been described in detail[1]. We used the droplet-based digital PCR (ddPCR) system QX100/200 from Bio-Rad Laboratories (Hercules, CA, USA) to detect the mutant and wild-type alleles of 13 unique point mutations. Commercially available validated primer/probe sets were purchased when available and otherwise designed by the manufacturer on demand (PrimePCR ddPCR Mutation Assays, Bio-Rad). Detailed assay information is provided in table S1. Each 20 µl PCR reaction mix consisted of 10 µl ddPCR Supermix for Probes (No dUTP, Bio-Rad), 1 µl of each target and reference primer/probe mix at a final concentration of 450 and 250 nmol/L, respectively, and 8 µl of DNA or water. The reaction mix was then partitioned into droplets by a QX100 Droplet Generator (Bio-Rad). Thermal cycling was performed with an initial activating step at 95 ^{0}C for 10 minutes before 40 cycles of denaturation at 94 ^{0}C for 30 seconds and annealing for 1 minute at a validated optimum temperature for each assay (shown in Table S1), and finally a 10 min stabilization step at 98 ^{0}C. Mutant and wild type fluorescence amplitudes for each droplet were registered by a QX200 Droplet Reader (Bio-Rad). Patient samples were analyzed in duplicate wells.

Digital droplet PCR data analysis and limit of detection

Raw droplet data were analyzed in QuantaSoft software v 1.7 (Bio-Rad) and then processed using in-house scripts and exported to Microsoft Excel v.2010 for further computation and analysis.

Based on the control experiments, we determined assay-specific gating thresholds for mutant and wild type-positive droplets. Examples of raw droplet data and gating are shown for the *BRAF* V600E mutation assay (Fig. S2). All ddPCR assays performed well in dilution series of mutant DNA in a wild-type background and the linearity was maintained as long as mutated alleles were present (Table S2, Fig. S3). The false positive rate of each assay was determined in a series of negative control experiments. Patient samples were considered to be mutation-positive if the mutant concentration in the sample was higher than the 95 % confidence interval of the assay-specific false positive rate (Table S2, Fig. S3). The estimated number of mutant copies required in a sample to be considered mutation positive, ranged from 0.84 to 2.96 copies of mutated DNA (median 1.4). The quantity of mutated DNA in positive samples was reported in copies per ml of serum.

Despite the very high technical sensitivity of ddPCR as outlined above, the ability of ddPCR to detect rare mutations is limited by the number of alleles screened[1]. This is dependent on the patient sample volume and the concentration and quality of DNA in the sample. If the true concentration of mutated alleles in a sample is 1 copy per 10 000 wild-type alleles, approximately three times as many alleles (30 000) must be screened to reach 95 % certainty that at least 1 mutated allele is present in the reaction mix. The median number of alleles screened per sample in this study was 3 400, yielding a limit of detection of approximately 1 mutated allele per 1 000 alleles (0.1 %).

Whole exome sequencing

Whole exome sequencing of purified plasma cells and matched germline controls were performed as previously described[2]. The target coverage of >100x was achieved for 85 % of exonic target regions. Mutated allele fractions were reported after correcting for the admixture of DNA from normal cells in the sample by the program ASCAT[3]. The lower limit of detection of WES was a mutated allele fraction of 2-4 % in the bone marrow sample. To validate the findings of recurrent mutations by WES, we performed ddPCR in all samples where we had sufficient available material. For this ddPCR analysis, the median limit of detection was 0.01 %.

References

1. Huggett JF, Cowen S, Foy CA. Considerations for digital PCR as an accurate molecular diagnostic tool. Clin Chem. 2015;61(1):79-88.
2. Rustad EH, Dai HY, Hov H, et al. BRAF V600E mutation in early-stage multiple myeloma: good response to broad acting drugs and no relation to prognosis. Blood Cancer J. 2015;5(e299.
3. Van Loo P, Nordgard SH, Lingjaerde OC, et al. Allele-specific copy number analysis of tumors. Proc Natl Acad Sci U S A. 2010;107(39):16910-16915.

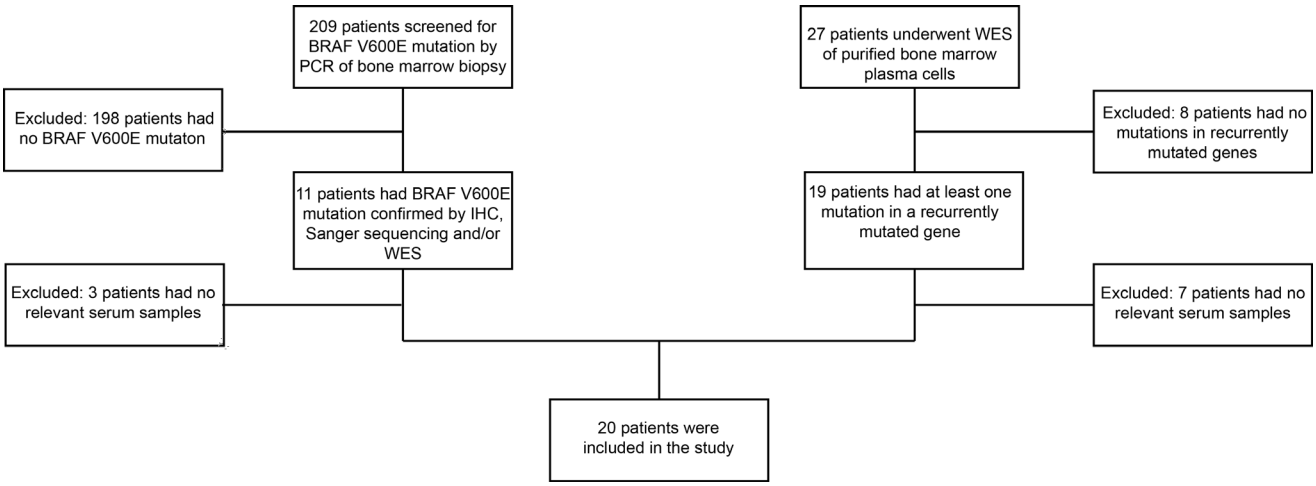
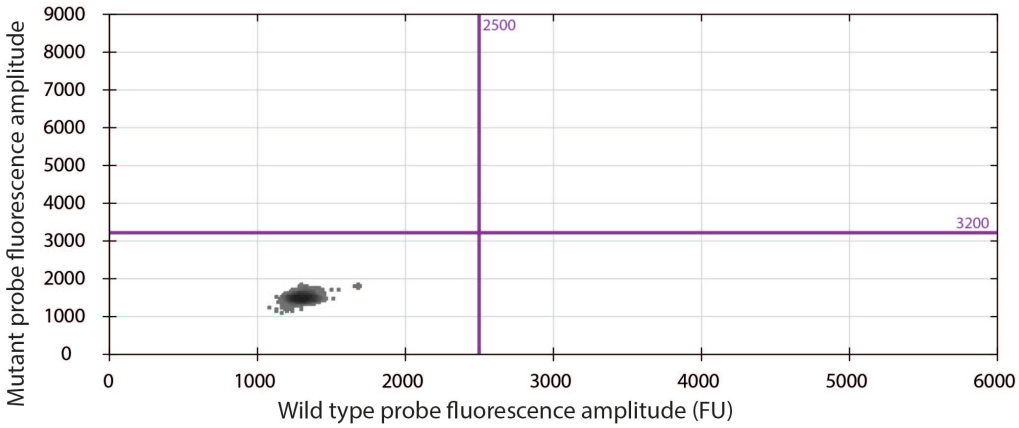


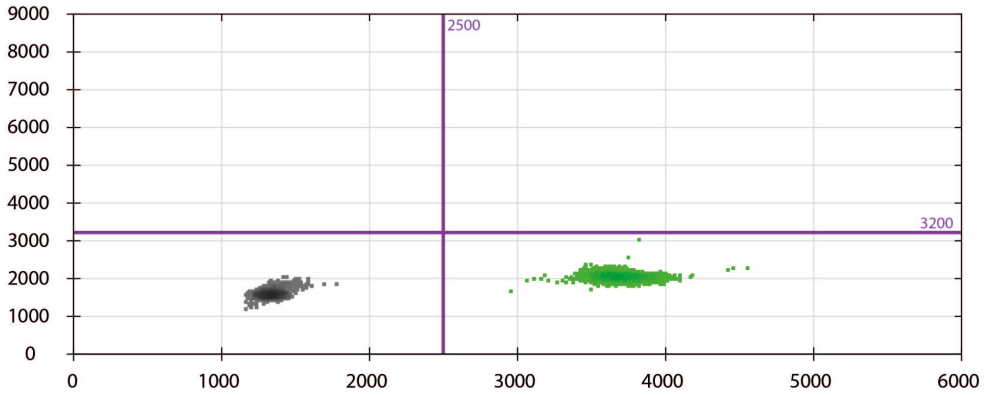
Figure S1: Patient inclusion flowchart.

Patients were considered to have relevant serum samples if fulfilling at least one of the following criteria: 1) sequential samples spanning at least two periods of disease activity or 2) paired samples from serum and bone marrow taken within 10 days. In the final study population of 20 patients, two had only sequential serum samples, nine had only paired serum and bone marrow samples and nine had both sequential and paired samples.

A: Non-template control



B: BRAF V600 Wild type control



C: BRAF V600E mutant control

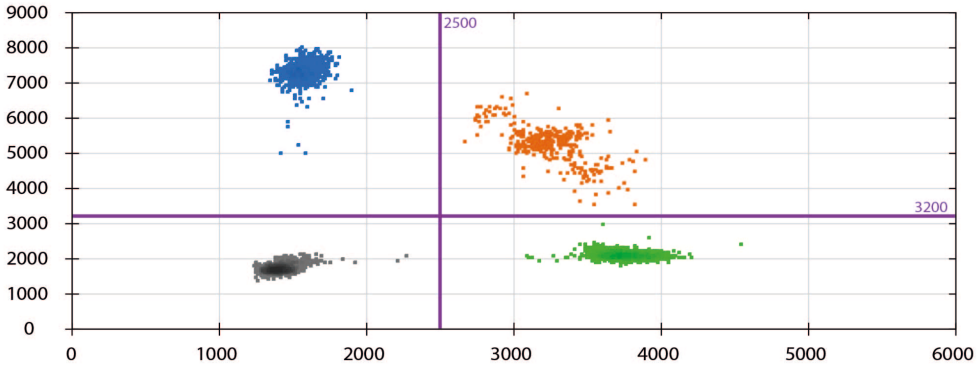


Figure S2: Raw data from BRAF V600E non-template, wild type and mutant controls. Each droplet (dot) is placed in a two-dimensional plot based on its mutant- and wild type probe fluorescence amplitude. Fluorescence is measured in arbitrary "fluorescence units" (FU). The purple cross drawn at 3200 mutant FU and 2500 wild type FU represents the manual gating threshold set to separate droplets into color-coded clusters. Gray droplets contain no amplifiable DNA (i.e. the BRAF V600 locus is not present), green droplets contain only the wild type sequence (BRAF V600), blue droplets contain only the mutant sequence (BRAF V600E), whereas orange droplets contain both the wild type and mutant sequences.

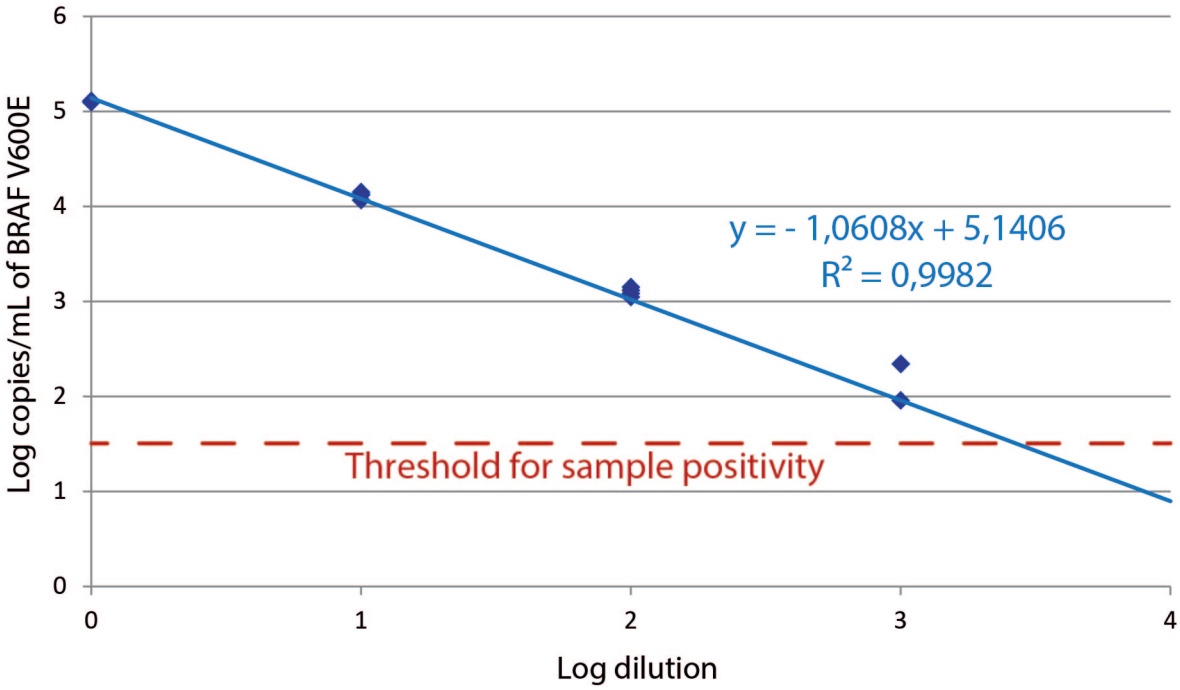
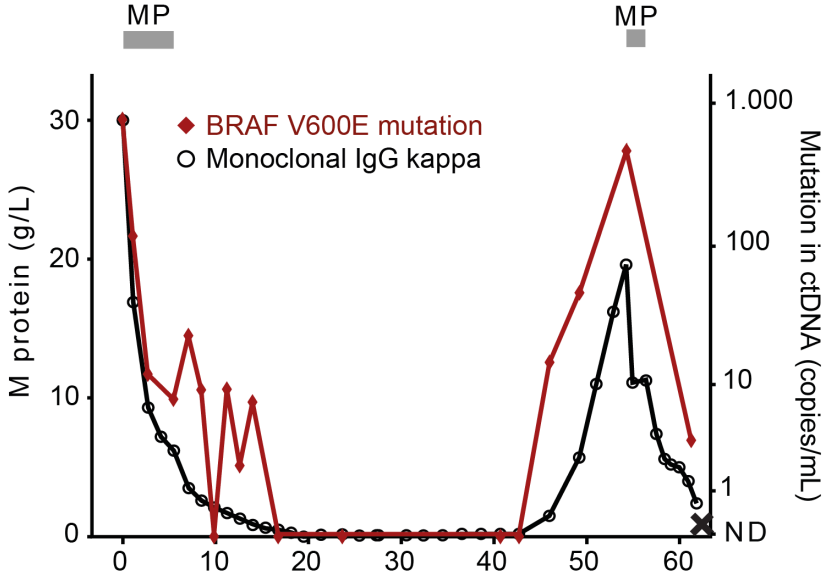
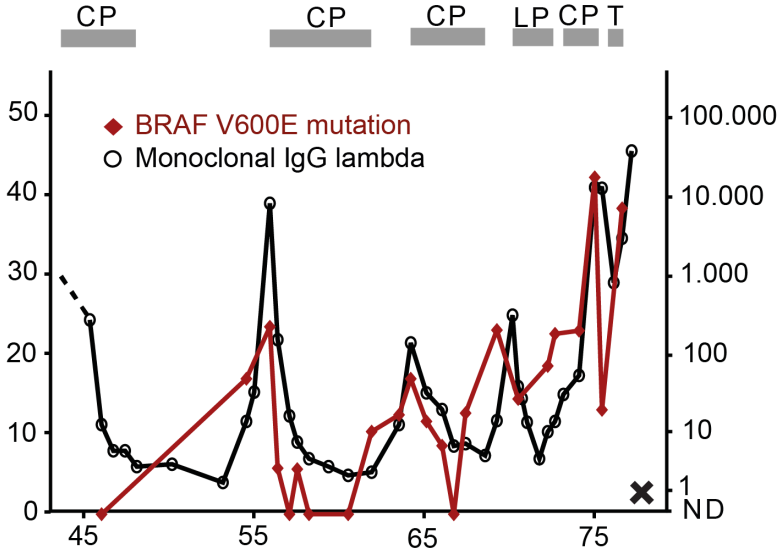


Figure S3: Dilution curve and threshold for sample positivity of BRAF V600E mutation assay
DNA from colon carcinoma cell line HT29, harboring a heterozygous BRAF V600E mutation, was serially diluted 1:10 from a starting quantity of 24 ng per reaction, in a constant background of 24 ng DNA from healthy donors´ peripheral blood leukocytes. Each blue square represents a single replicate well of the dilution experiment, whereas the regression line in blue is based on the average concentration at each dilution. The dotted red line represents the threshold for considering a sample mutation-positive, which is set to the upper 95 % CI of the assay false positive rate. The false positive rate was estimated based on 14 negative control wells.

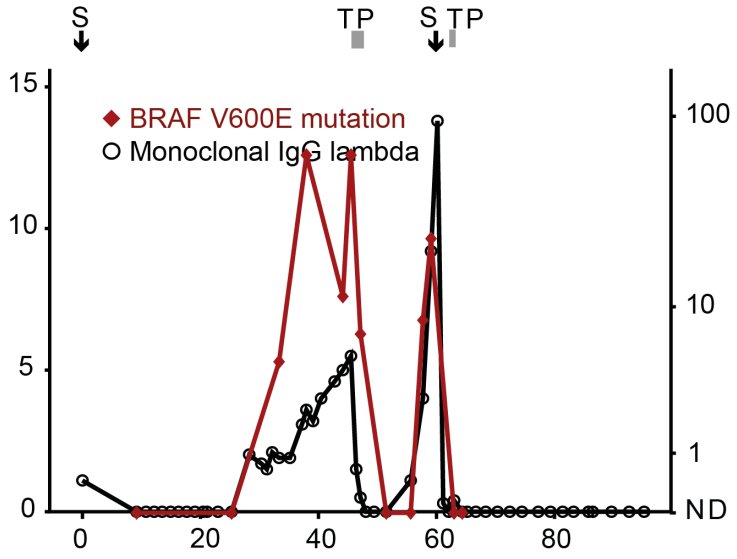
A: Patient 6



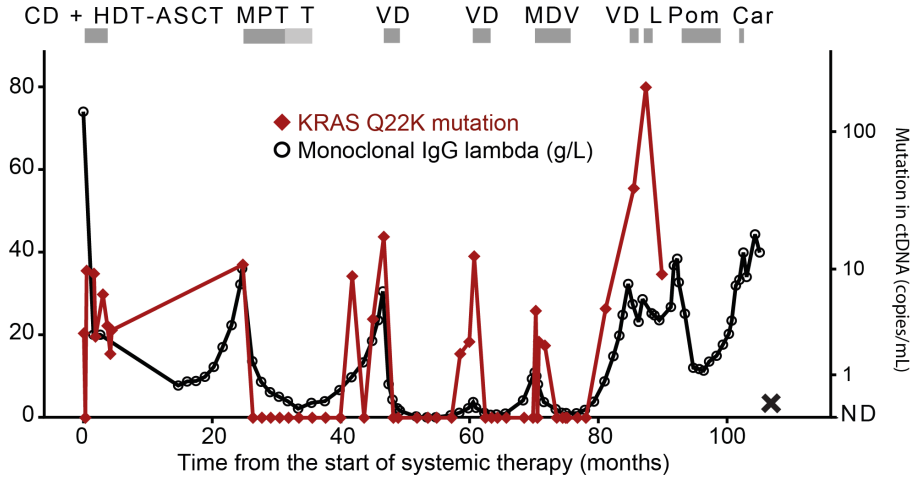
B: Patient 7



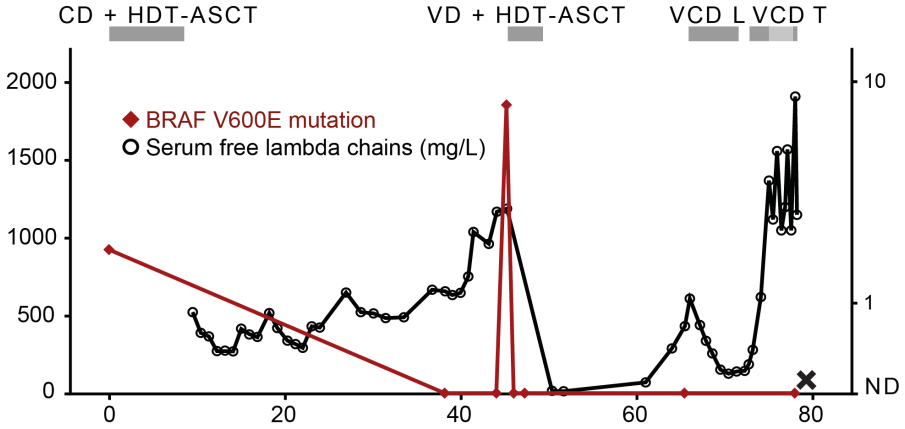
C: Patient 8



D: Patient 9



E: Patient 10



F: Patient 11

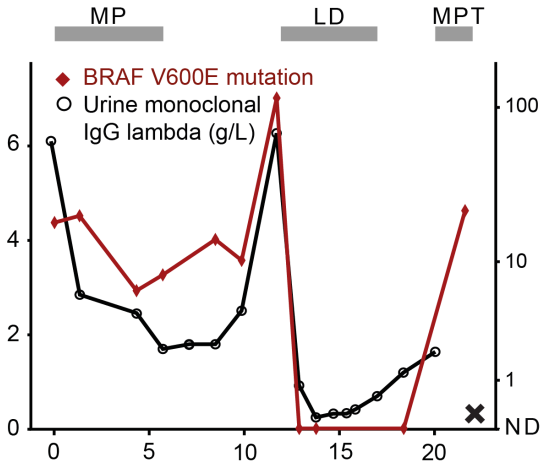


Figure S4: Sequential levels of M-protein and ctDNA

Patients with sequential ctDNA samples not shown in the article itself are presented here. Axis legends are as in panel A. Treatments are: M, melphalan; P, prednisone; C, cyclophosphamide; L, lenalidomide; T, thalidomide; S, surgery; D, dexamethasone; HDT-ASCT, high-dose melphalan therapy with autologous stem-cell transplant; V, bortezomib; Pom, pomalidomide; Car, carfilzomib.

Table S1: Digital droplet PCR assay information

Gene	BRAF	NRAS	KRAS	NRAS	KRAS	NRAS	KRAS	NRAS	KRAS	IRF4	FAM46C	DIS3	TP53
Protein change	p.V600E	p.Q61K	p.Q22K	p.G12D	p.A146P	p.G12A	p.Q61R	p.Q61R	p.Q61H	p.K123R	p.S272Y	p.H788R	p.Y236N
Nucleotide change	c.1799T>A	c.181C>A	c.64C>A	c.35G>A	c.436G>C	c.35G>C	c.182A>G	c.182A>G	c.183A>C	c.368A>G	c.815C>A	c.2399A>	c.706T>A
Manufacturer validation	Wet-lab	Wet-lab	In silico	Wet-lab	In silico, custom	In silico, custom	Wet-lab	Wet-lab	Wet-lab	In silico, custom	In silico, custom	In silico, custom	In silico
Positive controll	HT29	OH2	gBlocks	INA6	gBlocks	gBlocks	gBlocks	gBlocks	KJON	gBlocks	gBlocks	gBlocks	gBlocks
Annealing temperature (°C)	55	55	55	53,8	53,8	53,8	55	55	55	53,8	53	53,8	56,4
Unique assay ID (mut, wt)	dHsaCP2000027, dHsaCP2000028	dHsaCP2000067, dHsaCP2000068	dHsaIS2506108, dHsaIS2506109	dHsaCP2000095, dHsaCP2000096	Not available	Not available	dHsaCP2000135, dHsaCP2000136	dHsaCP2000071, dHsaCP2000072	dHsaCP2000133, dHsaCP2000134	Not available	Not available	Not available	dHsaIS2501200, dHsaIS2501201
Assay context sequence (chromosome location)	CACTCCATCGAGATTTCACTGTAGCTAGACCAAAATCACCTATTTTTACTGTGAGGTCTTCATGAAGAAATATATCTGAGGTGTAGTAAGTAAAGGAAAACAGTAGATCTCATTTTCCT	GTCTCTCATGGCACTGTACTCTTCTTGTCCAGCTGTATCCAGTATGTCCAACAAACAGGTTTCACCATCTATAACCACTTGTTTTCTGTAAGAATCCTGGGGGTGTGGAGGGTAAGGG	12:25398194-25398316	ATATTCATCTACAAAGTGGTTCTGGATTAGCTGGATTGTCAGTGCGCTTTTCCCAACACCACCTGCTCCAACCACCACCAGTTTGTACTCAGTCATTTCACACCAGCAAGAACCTGTTG			TACACAAAGAAAGCCCTCCCCAGTCCTCATGTACTGGTCCCTCATTGCACTGTACTCCTCTTGACCTGCTGTGTCGAGAATATCCAAGAGACAGGTTTCTCCATCAATTACTACTT	TGGTCTCTCATGGCACTGTACTCTTCTTGTCAGCTGTATCCAGTATGTCCAACATGTCCAACAAACAGGTTTCACCATCTATAACCACTTGTTTTCTGTAAGAATCCTGGGGGTGTGGAGGGTAA	ATACACAAAGAAAGCCCTCCCCAGTCCTCATGTACTGGTCCCTCATTGCACTGTACTCCTCTTGACCTGCTGTGTCGAGAATATCCAAGAGACAGGTTTCTCCATCAATTACTACTT				17:7577514-7577636
Amplicon length (nt)	91	65	93	70	80	65	61	65	61	64	70	80	68
Forward primer	Not available	Not available	Not available	Not available	TGTATTTATTTCAGTGTTACTTACC	CTGGATTGTCAGTGCG	Not available	Not available	Not available	AGCCAGCTGGACATC	ACCAGGAAGAAATCAAAACT	CAGCCCCAATAGCCAC	Not available
Reverse primer	Not available	Not available	Not available	Not available	ACTTAGCAAGAAGTTATGGAA	TGACTGAGTACAAACTGGT	Not available	Not available	Not available	CTTTTTTGGCTCCCTCAG	AGGATGTCCGGGAAGT	ACCATGGTTCTTATTTTTCC	Not available
Mutant probe (FAM)	Not available	Not available	Not available	Not available	TTGTCTTTGGTGATGTTTCA	ACACCAGCTGCTCCA	Not available	Not available	Not available	CCGTACAGAGTGTACAGG	ATGAAGAACCTGTAGCACA	TCATTGTTCGTCGGCTT	Not available
WT probe (HEX)	Not available	Not available	Not available	Not available	TTGTCTTTGCTGATGTTTCA	ACACCACCTGCTCCA	Not available	Not available	Not available	CCGTACAAAGTGTACAGGA	AGAACCTGGAGCACATG	TCATTGTTCATCGGCTTTT	Not available
COSMIC ID	COSM476	COSM580	COSM543	COSM564	COSM19905	COSM565	COSM552	COSM584	COSM554	COSM329431	NA	NA	COSM43826

Table S2. Performance data for all ddPCR assays.

R^2, correlation coefficient of dilution series based on average values of replicate wells; False positive rate, average concentration of mutated copies falsely detected in a series of wild type control wells; Threshold, threshold for considering a sample mutation-positive, here set to the upper 95 % CI of the false positive rate. The threshold is reported in copies/µl of reaction, and as the estimated number of mutant copies required present in a sample for it to be considered mutation-positive, assuming the sample is run in duplicate (2x20 ul reactions).

Assay	R^2	False positive rate (copies/µl)	Threshold (copies/µl)	Threshold (copies/sample)
BRAF V600E	0.9982	0.006	0.031	1.24
DIS3 H788R	0.9922	0.036	0.074	2.96
FAM46C S272Y	0.9992	0.019	0.049	1.96
IRF4 K123R	0.9892	0.006	0.028	1.12
KRAS A146P	0.9935	0	0.022	0.88
KRAS Q22K	0.9954	0.009	0.045	1.8
KRAS Q61H	0.9999	0	0.018	0.72
KRAS Q61R	0.9992	0.011	0.035	1.4
NRAS G12A	0.9985	0.013	0.04	1.6
NRAS G12D	0.9981	0.034	0.07	2.8
NRAS Q61K	0.9998	0	0.021	0.84
NRAS Q61R	0.9999	0.016	0.042	1.68
TP53 Y236N	0.9972	0.011	0.035	1.4

Table S3: Correlation of MAPK-mutation levels in ctDNA and normalized M-protein

Patient	Spearman correlation
1	r= .835, n=41, p < .0001
2	r= .651, n=13, p = .016
3	r= .985, n=6, p < .0001
4	r= .790, n=31, p < .0001
5	r= .800, n=4, p = .200
6	r= .846, n=18, p < .0001
7	r= .603, n=23, p = .002
8	r= .877, n=13, p < .0001
9	r= .649, n=46, p < .0001
10	r= .354, n=5, p = .559
11	r= .914, n=10, p < .0001
Combined	r= .631, n=210, p < .0001