SUPPLEMENTAL DATA to:

Highly sensitive and absolutely specific detection of human tumor cells by amplicon-fusion-site polymerase-chain-reaction (AFS-PCR)

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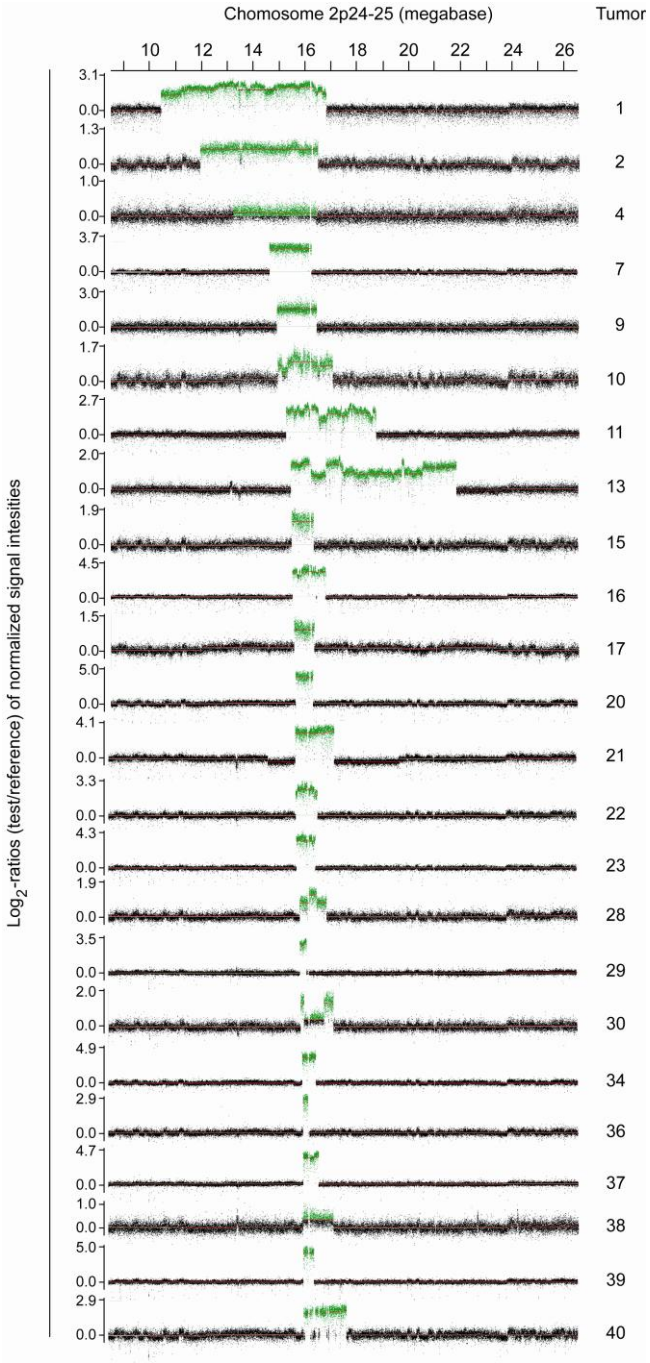
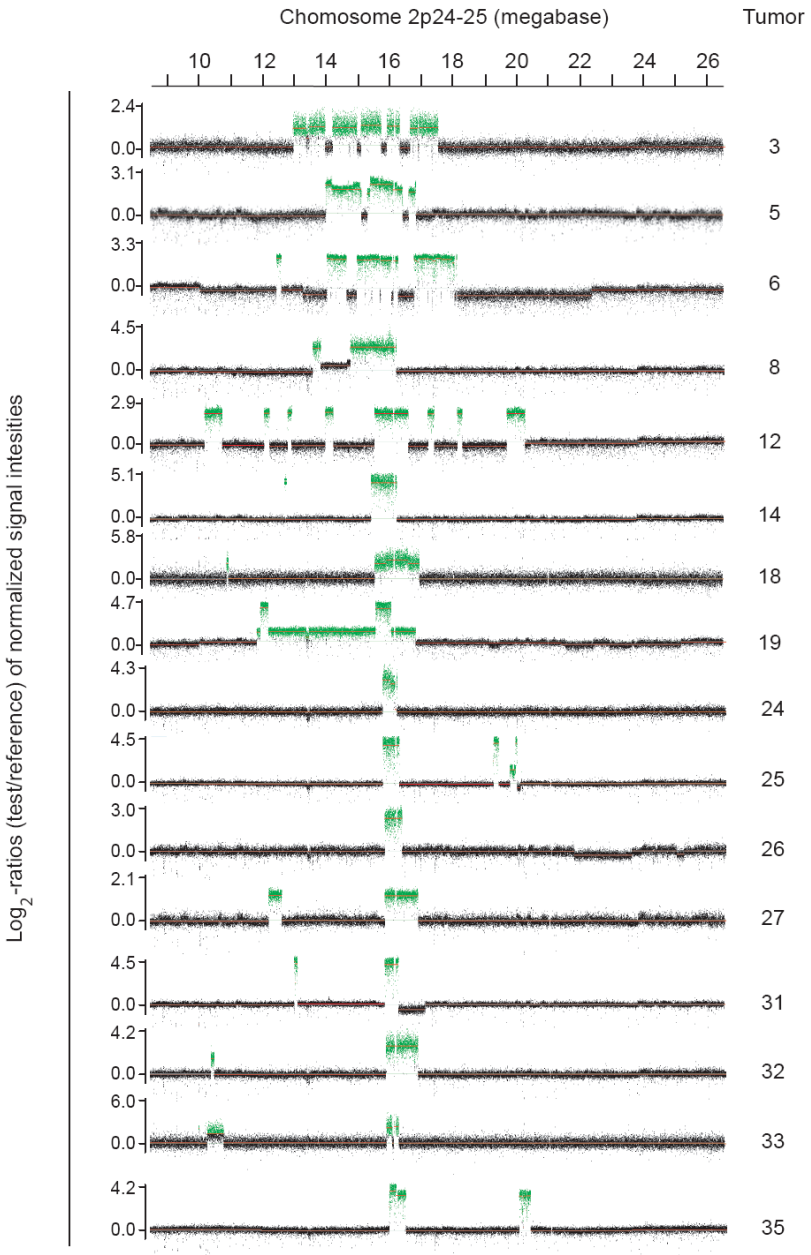
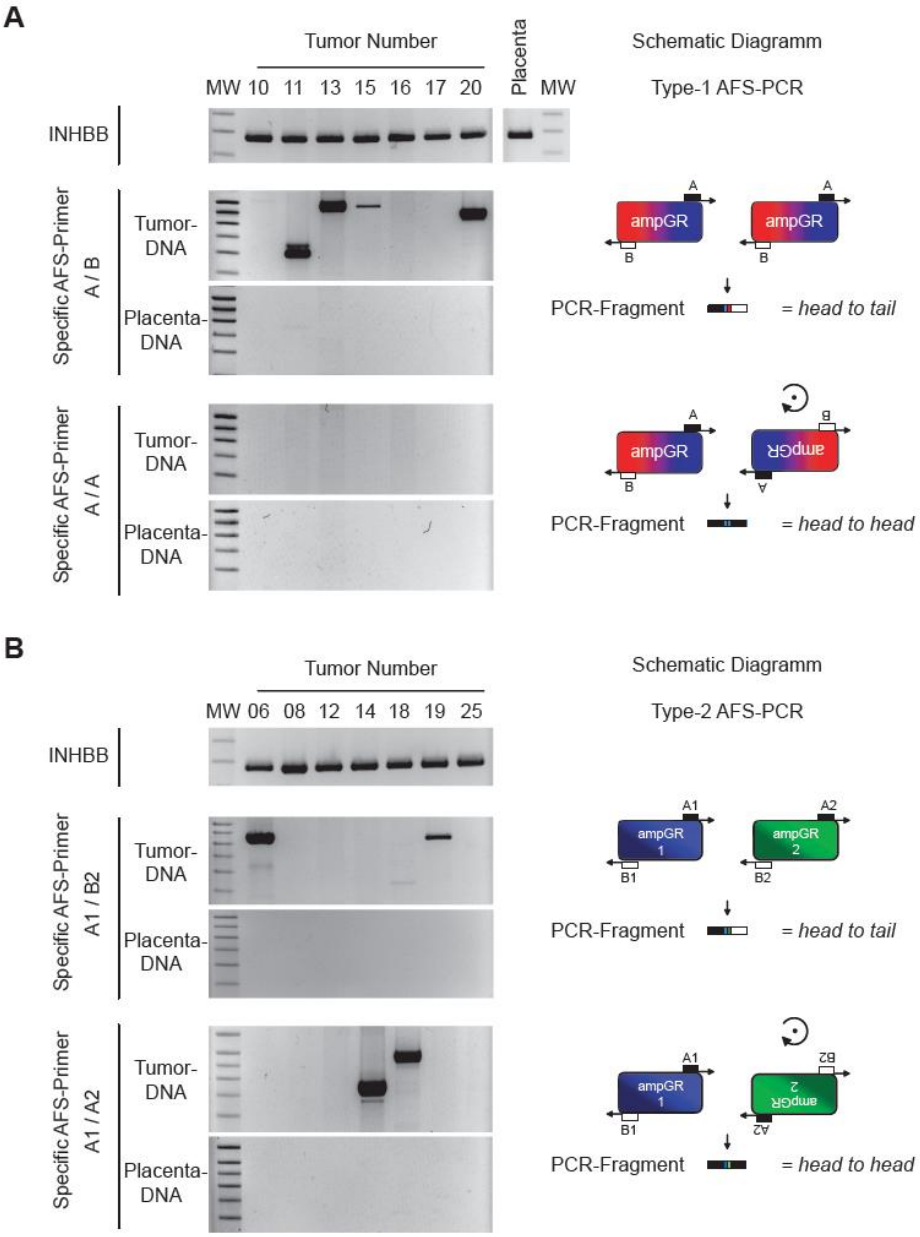
Keywords: *MYCN*, MRD, Amplicon, neuroblastoma



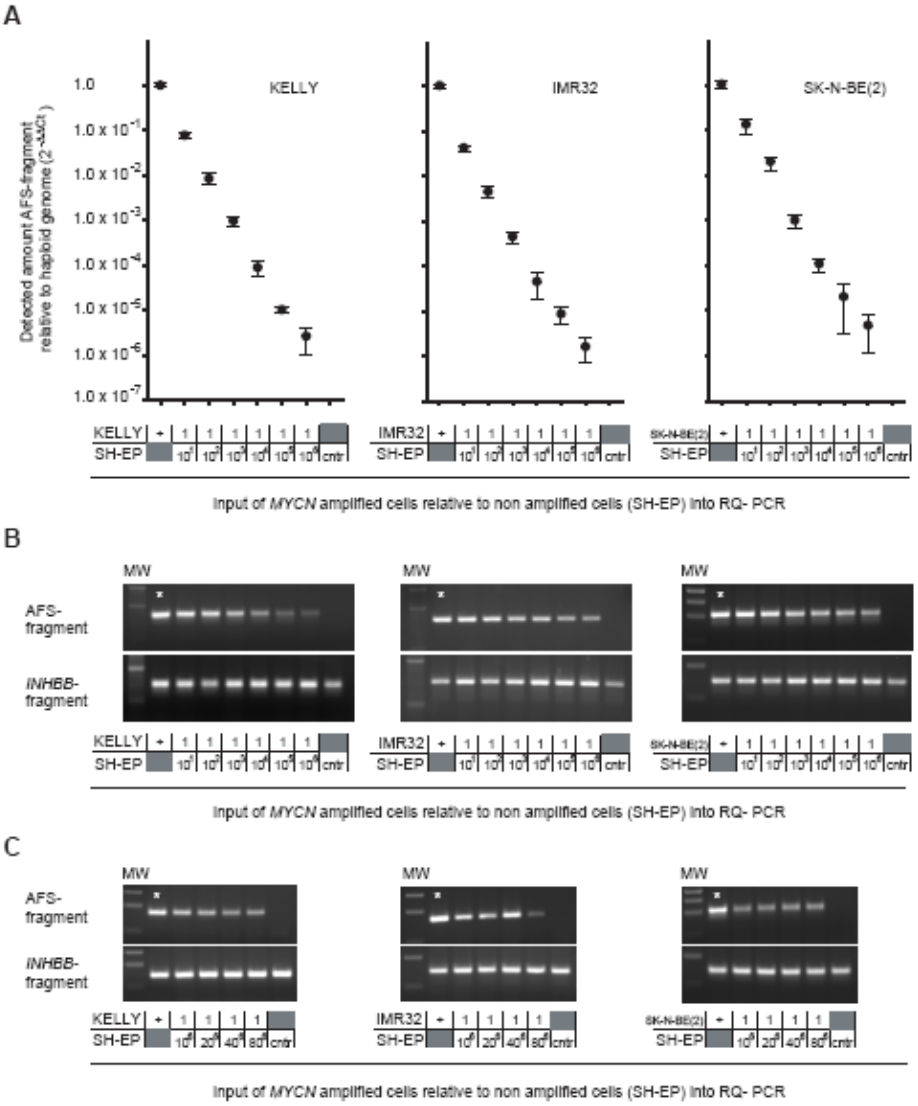
Supplemental Figure 1:
Amplified genomic regions (ampGR) around the *MYCN* gene on human chromosome 2 of the 24 *MYCN* amplified, primary neuroblastomas investigated with simple (type-1) amplicons. Signal-Map© Software (Vers. 1.9) was used for visualisation of the HR-TA data. The relative copy number for each printed oligonucleotide is presented as fluorescent intensity of the Cy5 labelled test-DNA (cell lines) normalized to the Cy3 labelled reference-DNA (healthy human female) (Log_2-ratios). The mean signal intensity values of the continuous genomic regions that were calculated by the Software are indicated by the red lines. The zero base line is indicated by the green line. AmpGR are highlighted green. Each single tumor harbors an individual amplicon composed of different copy numbers of one (Type-1) or more (Type-2) ampGR with unique telomeric and centromeric borders.



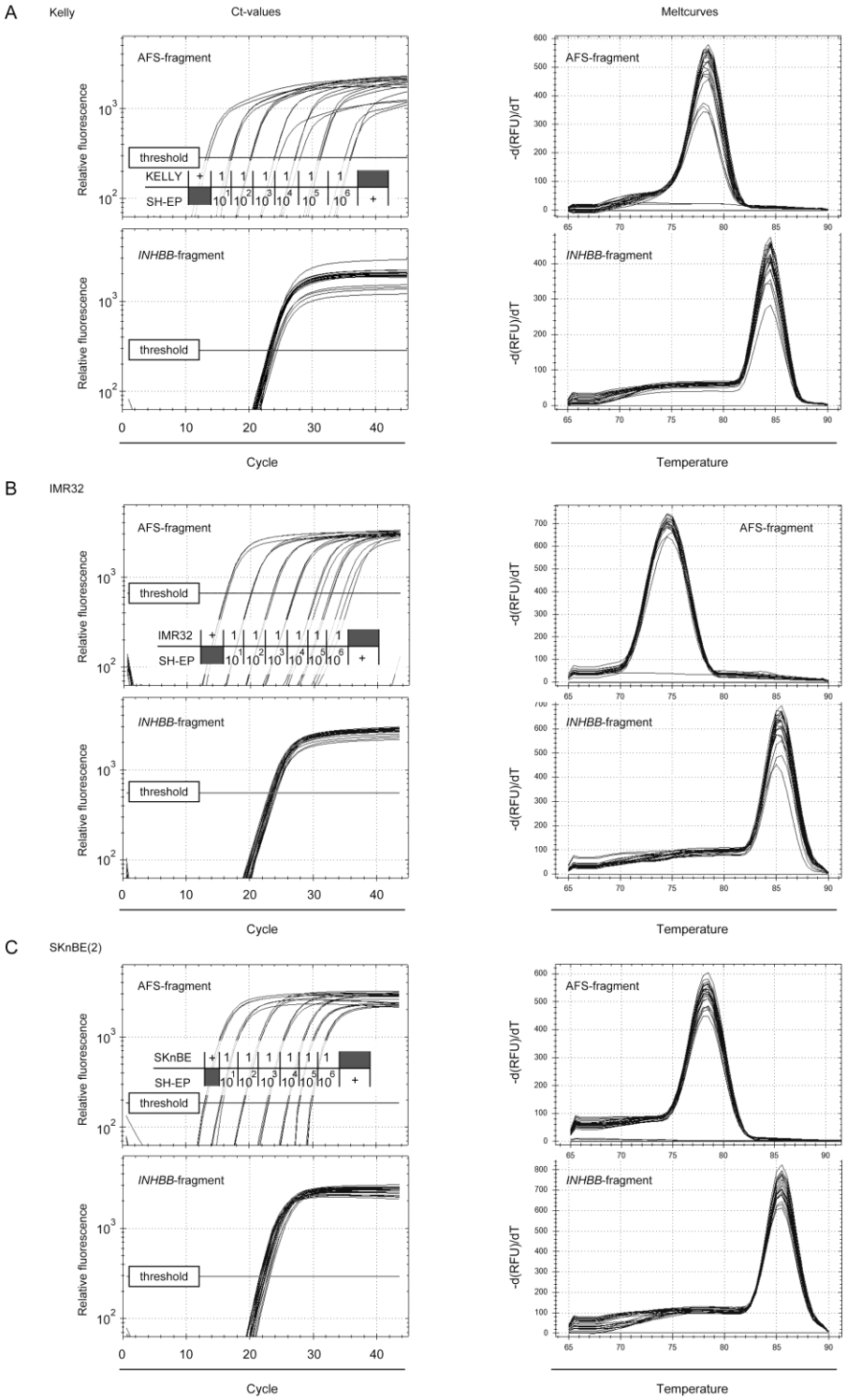
Supplemental Figure 2:
Amplified genomic regions (ampGR) around the *MYCN* gene on human chromosome 2 of the 16 *MYCN* amplified, primary neuroblastomas investigated with complex (type-2) amplicons. Signal-Map© Software (Vers. 1.9) was used for visualisation of the HR-TA data. The relative copy number for each printed oligonucleotide is presented as fluorescent intensity of the Cy5 labelled test-DNA (cell lines) normalized to the Cy3 labelled reference-DNA (healthy human female) (Log_2-ratios). The mean signal intensity values of the continuous genomic regions that were calculated by the Software are indicated by the red lines. The zero base line is indicated by the green line. AmpGR are highlighted green. Each single tumor harbors an individual amplicon composed of different copy numbers of one (Type-1) or more (Type-2) ampGR with unique telomeric and centromeric borders.



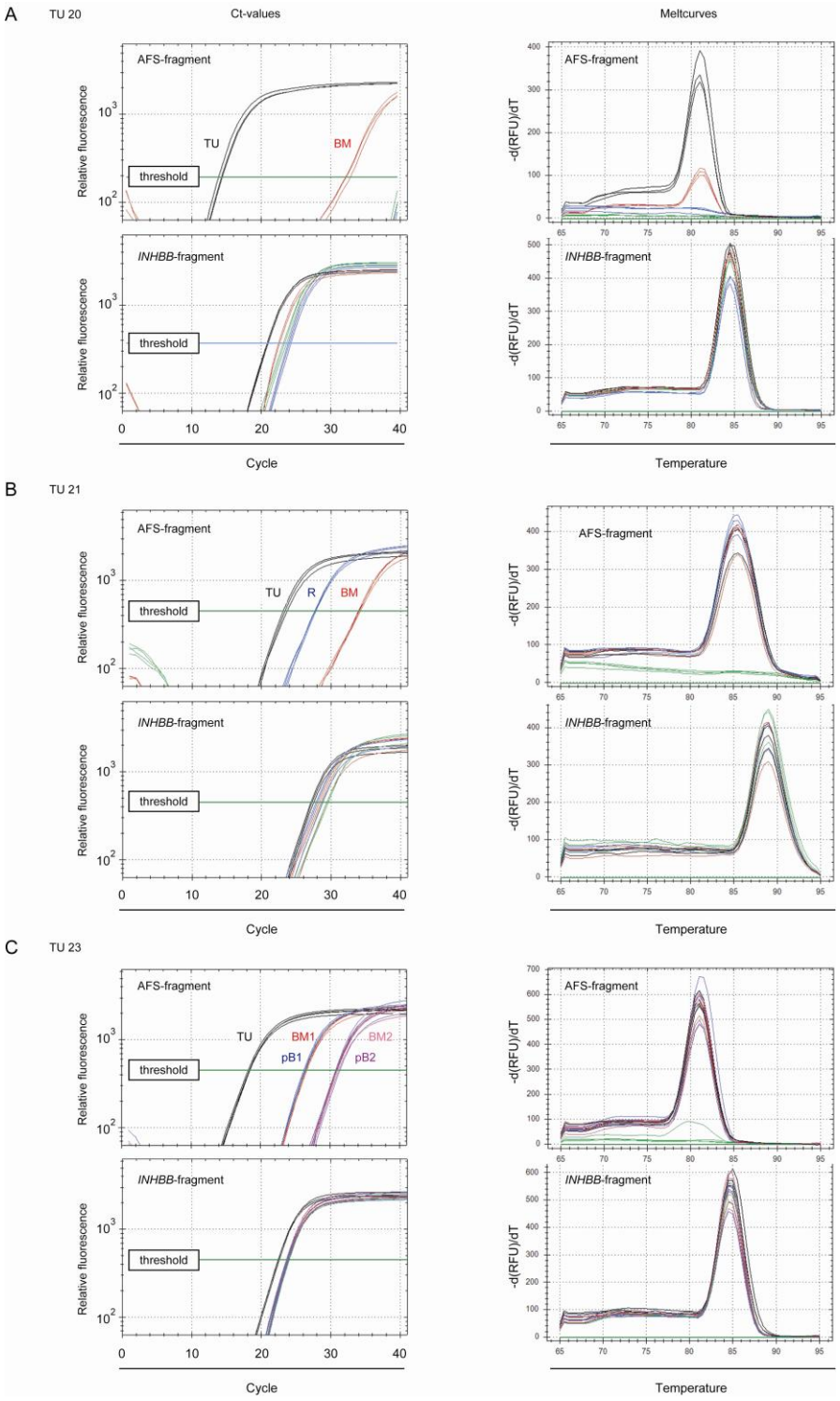
Supplemental Figure 3:
Representative PCR gels of different neuroblastomas with type-1 (A) and type-2 (B) amplicons and corresponding schematic diagrams. (**A**) Specific AFS-PCR products are only detected with the corresponding individual upper and lower primer in tumor DNA with type-1 amplicons. PCR were successful only with both primers present in the reaction. Thus, all type-1 amplicons were found to be orientated head to tail. (**B**) In neuroblastomas with type-2 amplicons the AFS between different ampGR were found orientated in head to tail but also in head to head orientation.



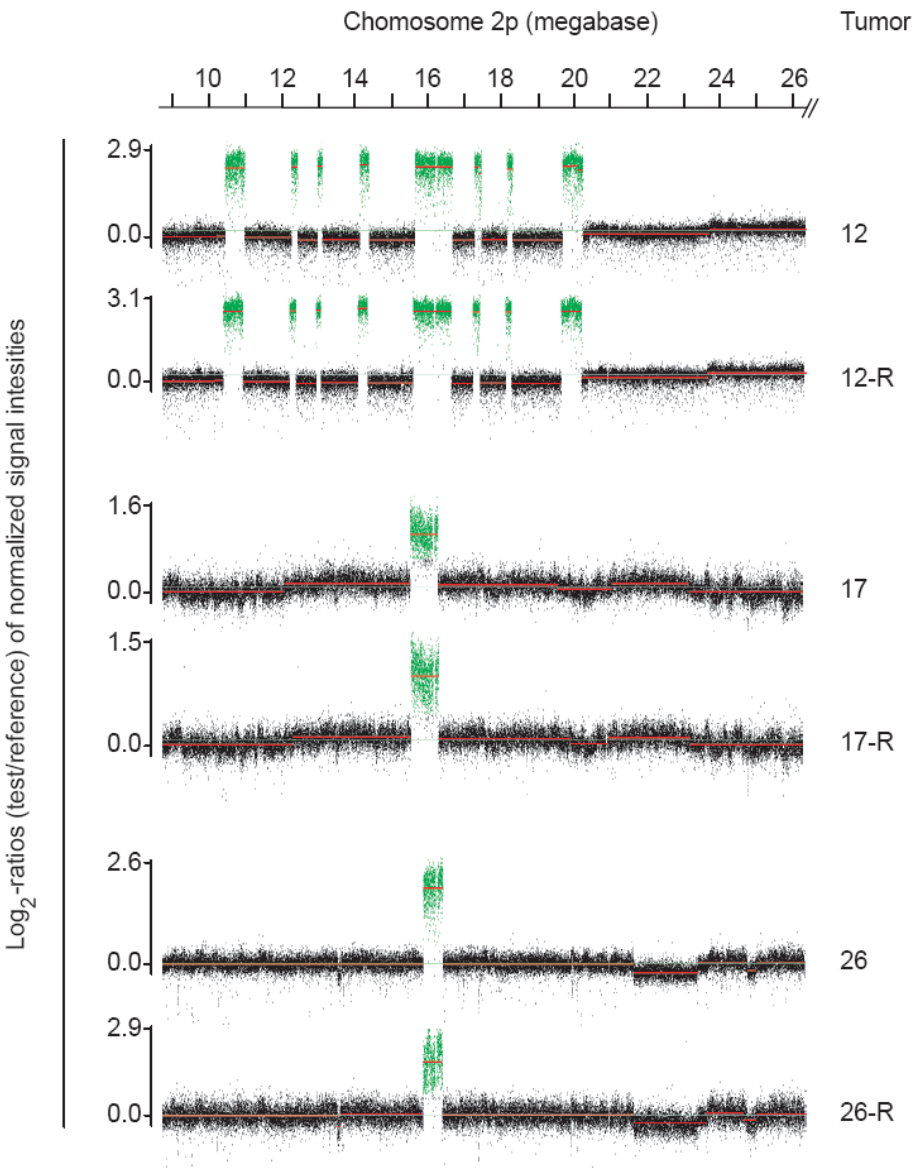
Supplemental Figure 4:
Sensitivity of the AFS-PCR. (**A**) A Log_{10} dilution series of suspended *MYCN* amplified cells (KELLY, IMR-32 and SK-N-BE(2)) with non *MYCN* amplified control cells (SH-EP) harboring an almost equal DNA content per cell was performed in triplicate prior to DNA isolation. Not diluted, *MYCN* amplified cell lines served as 100% tumor cell control. SH-EP cells served as negative control. 200 ng of DNA, isolated from 3 x 10^6 cells of each dilution step or control served as templates for RQ-PCR with either specific AFS primers or *INHBB* control primers. Each AFS or control RQ-PCR was performed in triplicate. Ct values of the specific AFS fragments were normalized to the corresponding *INHBB* Ct values. Each resulting ΔCt value was furthermore normalized to the ΔCt of the 100% tumor cell control according to the $2^{-\Delta\Delta Ct}$ calculation method. The results reflect almost exactly the corresponding dilution steps of the cell lines. Differences in accuracy are explainable partly by differences in fidelity of the individual AFS-PCRs. (**B**) AFS fragments and *INHBB* control fragments of the RQ-PCR separated in a 3% agarose gel by electrophoresis and visualized under UV-light after ethidium bromide staining. (**C**) Isolated DNA from the last cell line dilution step was further diluted with SH-EP DNA in a Log_2 dilution series. Specific AFS fragments are identified down to the last dilution step indicating DNA of one *MYCN* amplified cell per 8 x 10^6 control cells. Asterisk-marked lines of the 100% control in (B) and (C) are loaded with an amount of 50% of PCR product compared to the other lines.



Supplemental Figure 5:
Real time (RT) PCR traces and corresponding melt curves. (**A**) RT-PCR traces and melt curves of the specific AFS-PCR of the KELLY cell line dilution series (upper graph) and the *INHBB* control (lower graph). (**B**) RT-PCR traces and melt curves of the specific AFS-PCR of the IMR-32 cell line dilution series (upper graph) and the *INHBB* control (lower graph). (**C**) RT-PCR traces and melt curves of the specific AFS-PCR of the SKnBE(2) cell line dilution series (upper graph) and the *INHBB* control (lower graph).



Supplemental Figure 6:
Real time (RT) PCR traces and corresponding melt curves. (**A**) RT-PCR traces and melt curves of the specific AFS-PCR of neuroblastoma #TU-20 (upper graph) and the *INHBB* control (lower graph). (**B**) RT-PCR traces and melt curves of the specific AFS-PCR of neuroblastoma #TU-21 (upper graph) and the *INHBB* control (lower graph). (**C**) RT-PCR traces and melt curves of neuroblastoma #TU-23 (upper graph) and the *INHBB* control (lower graph). (Abbreviations: TU = initial tumor; BM = bone marrow; PB = peripheral blood; R = residual disease)



Supplemental Figure 7:
The amplicon structure is a stable attribute during therapy in primary human neuroblastomas. AmpGR are highlighted green. The ampGR identified by HR-TA of initial tissue probes of 3 primary neuroblastomas match exactly to the ampGR of the corresponding tissue probes of recurrent or residual diseases (R) during therapy. The secondary biopsies were taken after 38 months (tumor #12), 10 months (tumor #17), and 11 months (tumor #26), respectively.

Tumor number	Bp-coordinates on chromosome 2p (NCBI36/hg18)						Amplicon type	Bp-coordinates of sequenced AFS	
	telomeric satellite ampGR		core ampGR		centromeric satellite ampGR				
	Start	Stop	Start	Stop	Start	Stop		Start	Stop
TU 1			10.446.277	16.791.840			1	10.446.260	16.791.916
TU 2			11.927.721	16.414.997			1	11.927.709	16.414.884
TU 4			13.242.240	16.406.430			1	13.242.278	16.406.513
TU 7			14.643.061	16.262.660			1	14.643.133	16.262.318
TU 9			14.898.775	16.435.973			1	14.898.837	16.436.004
TU 10			14.936.419	17.057.712			1	14.936.368	17.057.806
TU 11			15.248.736	18.709.295			1	15.248.759	18.709.380
TU 13			15.452.885	21.821.063			1	15.452.866	21.821.250
TU 15			15.478.091	16.327.339			1	15.478.093	16.327.396
TU 16			15.528.114	16.810.758			1	15.528.132	16.810.404
TU 17			15.546.074	16.321.474			1	15.546.044	16.321.500
TU 20			15.649.442	16.316.110			1	15.649.412	16.316.149
TU 21			15.712.281	17.206.162			1	15.712.285	17.206.203
TU 22			15.722.215	16.556.579			1	15.722.227	16.556.630
TU 23			15.745.326	16.469.311			1	15.745.343	16.469.346
TU 28			15.865.487	16.896.500			1	15.865.489	16.896.567
TU 29			15.873.844	16.133.427			1	15.873.815	16.133.563
TU 30			15.877.357	17.137.159			1	15.877.241	17.137.209
TU 34			15.962.641	16.484.931			1	15.962.624	16.484.992
TU 36			15.972.481	16.166.944			1	15.972.309	16.166.996
TU 37			15.976.763	16.567.896			1	15.976.755	16.567.937
TU 38			15.977.993	17.136.454			1		
TU 39			15.989.251	16.399.851			1	15.989.266	16.399.907
TU 40			15.994.874	17.632.724			1	15.994.798	17.632.780
TU 3	13.015.040 13.523.871 14.255.406 15.139.087	13.410.245 14.021.167 15.017.750 15.774.495	15.958.148	16.356.914	16.685.565	17.567.346	2		
TU 5	**14.046.572**	15.168.558	15.359.708	16.460.642	16.654.099	**16.875.589**	2	14.046.582	16.875.644
TU 6	12.491.521 **14.074.464** 15.010.982 15.274.793 15.459.730 15.512.413	**12.652.944** 14.687.947 15.270.772 15.455.998 15.580.117 15.733.492	15.761.446	16.101.766	16.156.893 16.805.211 17.254.815 17.664.656 18.128.905	16.300.914 17.245.188 17.659.710 18.073.478 18.159.054	2	12.652.995	14.074.477
TU 8	**13.616.118**	13.879.795	14.812.386	**16.246.658**			2	13.616.124	16.246.726
TU 12	10.304.906 12.152.775 12.889.426 14.061.769	10.852.176 12.325.002 13.021.458 14.321.347	15.600.284	16.659.440	17.264.301 18.175.682 19.724.910	17.455.964 18.334.541 20.292.429	2		
TU 14	12.775.088	12.790.135	**15.460.280**	**16.258.945**			2	15.460.303	16.259.015
TU 18	10.938.515	**10.999.316**	15.586.505	**16.996.301**			2	10.999.511	16.996.377
TU 19_a	**11.980.909**	12.243.363	**15.595.378**	16.092.881			2	11.980.911	15.595.233
TU 19_b			*11.875.703*	*16.861.560*			*1*	*11.875.742*	*16.861.602*
TU 24			15.790.962	16.170.252	16.227.025	16.250.631	2		
TU 25			15.797.033	**16.304.076**	19.245.153 19.749.286	**19.406.916** 19.978.480	2	16.304.122	19.406.958
TU 26			15.829.164	**16.368.999**	**63.352.423**	63.921.178	2	16.369.032	63.352.444
TU 27	12.217.508	**12.623.519**	**15.848.132**	16.890.932			2	12.623.559	15.848.132
TU 31	13.059.252	**13.158.546**	**15.888.484**	16.318.265			2	13.158.602	15.888.483
TU 32	10.429.644	10.549.042	15.902.203	16.906.189			2		
TU 33	10.068.112 10.290.727 10.334.740	10.073.862 10.291.637 10.841.575	15.927.961	16.106.040	16.164.548 16.439.283 20.227.919	16.316.475 16.440.713 20.229.294	2		
TU 35			**15.965.288**	16.479.166	**20.020.608**	20.369.490	2	15.965.300	20.020.589
Cell lines									
IMR-32			**14.657.079**	16.041.166	**17.219.733** 66.576.591 68.987.820	17.233.090 67.559.258 69.255.573	2	14.657.089	17.219.746
KELLY			15.751.648	16.704.281			1	15.751.592	16.704.345
SKnBE(2)			15.993.854	16.466.158			1	15.993.851	16.466.222

Supplemental Table 1:
Coordinates of the ampGR borders. Borders estimated from the array data in the cells with blue (cor ampGR) and purple (satellite ampGR) backround. Coordinates in red indicate tumors with unsuccessful AFS-PCR validation. Validated coordinates in the type-2 amplicons are indicated in bold numbers. Coordinates based on the sequencing of the AFS-PCR product are in the right columns with yellow backround.

Tumor number	Amplicon type	Amplicon copy number (*Lit. 16*)	Clinical Data			
			Age at diagnosis (months)	Death	Survival (months)	Tumor stage
TU 01	1	141	36,7	1	17,1	4
TU 02	1	44	25,9	0	84,6	3
TU 04	1	21	30,4	0	87,6	4
TU 07	1	71	33,5	0	21,0	3
TU 09	1	104	2,7	1	7,4	4
TU 10	1	45	4,1	0	85,2	3
TU 11	1	96	15,2	1	7,7	3
TU 13	1	76	48,3	1	15,2	4
TU 15	1	55	12,4	0	68,3	3
TU 16	1	146	12,5	0	30,8	4
TU 17	1	161	15,0	0	27,5	1
TU 20	1	109	19,7	0	60,1	4
TU 21	1	79	19,0	1	18,4	4
TU 22	1	122	52,0	1	18,3	3
TU 23	1	142	7,2	0	22,6	5
TU 28	1	42	57,6	1	12,6	4
TU 29	1	90	0,0	0	44,4	5
TU 30	1	3	44,5	1	19,5	4
TU 34	1	108	37,9	0	56,4	3
TU 36	1	90	25,8	0	70,3	4
TU 37	1	103	9,8	0	42,2	3
TU 38	1	21	22,4	1	20,6	4
TU 39	1	125	16,2	1	2,3	4
TU 40	1	101	37,0	0	70,9	3
TU 03	2	40	10,7	1	13,2	4
TU 05	2	117	18,9	0	28,3	3
TU 06	2	209	2,6	0	33,1	1
TU 08	2	99	78,6	0	36,7	4
TU 12	2	141	3,7	1	66,2	5
TU 14	2	161	32,7	0	65,4	3
TU 18	2	116	8,0	1	11,7	4
TU 19	2	136	62,2	1	7,1	4
TU 24	2	110	42,6	1	6,0	4
TU 25	2	121	35,5	1	2,1	3
TU 26	2	77	4,6	1	7,7	5
TU 27	2	96	4,5	1	3,0	4
TU 31	2	142	6,6	0	66,5	1
TU 32	2	114	3,1	1	0,4	4
TU 33	2	71	36,4	1	11,5	3
TU 35	2	157	12,0	1	4,7	4

Supplemental Table 2:
Amplicon Copy number and clinical Data of the neuroblastoma patients investigated.

Tumor Cell content	TU	100%	80%	60%	40%
		mean $2^{-\Delta\Delta Ct}$ (= calculated tumor cell number)			
TU #20	TU	**1,025E+00**	8,204E-01	6,153E-01	4,102E-01
	BM	1,364E-05	1,091E-05	8,183E-06	5,455E-06
	PB	0	0	0	0
	cntr.	0	0	0	0
TU #21	TU	**1,103E+00**	8,827E-01	6,620E-01	4,414E-01
	BM	2,569E-03	2,055E-03	1,542E-03	1,028E-03
	R	5,396E-02	4,317E-02	3,237E-02	2,158E-02
	cntr.	0	0	0	0
TU #23	TU	**1,003E+00**	8,022E-01	6,017E-01	4,011E-01
	BM1	7,452E-03	5,962E-03	4,471E-03	2,981E-03
	PB	1,554E-02	1,243E-02	9,323E-03	6,215E-03
	BM2	5,415E-04	4,332E-04	3,249E-04	2,166E-04
	BM3	4,833E-04	3,866E-04	2,900E-04	1,933E-04
	cntr.	0	0	0	0

abbreviations:

TU	primary tumor
BM	bone marrow
PB	peripheral blood
R	recurrent disease
cntr.	control DNA

Supplemental Table 3:
Overview of calculated tumor cell numbers of the subsequent samples of the neuroblastoma patients investigated (# TU-20, T-21 and TU-23) dependent on the tumor cell content of the initial tumor sample. The information of the tumor cell content of the initial samples were not accessible. Therefore, we theoretically set the tumor cell content to 100%, 80%, 60% and 40% and calculated the corresponding tumor cell numbers in the subsequent samples as described in the **Material and Methods** section in the manuscript.

Primer for AFS identification (cell lines)		
Cell line		Sequence (5´à3´)
IMR32	F	GGTTGTTGAGAGGATAATGGA
	R	TAGGAAAGGCTTTGGGTTAT
Kelly	F	TCTACCCAAGACACATGCTAA
	R	GCTGTCTGGTGCTACTCAA
SknBE2	F	TATAAAATACCCAACAATAAC
	R	GAAACCCTGTCTCTACAA

Primer for AFS-RQ-PCR (cell lines)		
Cell line		Sequence (5´à3´)
IMR32	F	CCCAGGATTAGACGGAAAAT
	R	CTACTGGTGATGCTTGACTC
Kelly	F	TCTGCCAAGCTGGGGTAAG
	R	TGTAAGTGGGCAGCGTGAC
SknBE2	F	TGAGCTACTGATCAGTTTAGAC
	R	CCTCCCTCAAAATGTGTAC

Primer for AFS identification (tumors)		
Tumor		Sequence (5´à3´)
1	F	AGCCTGAACTCTGCATTGAT
	R	AGATGATTGCCACGGAAAG
2	F	TTTGATACCAATTAGGGCATTT
	R	TCCCTCATTGCTAACCCA
3	F	Np
	R	Np
4	F	AACTGGCTATTCATGCAA
	R	TTGGCTCTTCTATAAGGT
5	F	CAAGGAAGTCTATGTTTCGTTC
	R	AAGCCCCTGTTTATCAGC
6	F	CCTTTGGGTCTTCGTCAGA
	R	TGCCAAGCCTTAGGGACTTAG
7	F	TCTGCCCAGAGTTAGCTG
	R	GCCCACACAGATAATCTAG
8	F	GGTCTGTTCCGCAGTCATC
	R	TTTGCCATGTGAAGTGCCTACT
9	F	GTCCTCAGCACAAACCCATTC
	R	GTGAAGTGGGATTCCTGTAGGC
10	F	TGAGTGCTGCACAGAGTAGGT
	R	GCCAAAATGTTCTTCAGTAGGT
11	F	GTGACATAAACCGTGTGCCTAA
	R	CTACAAAATGGCTGTGGGCTAT
12	F	Np
	R	Np
13	F	TCTGTGCGCCGAGACCTG
	R	CAGAATGTTTTTGTGCACCGTA
14	F	AATGCCCTTGTTTGGTAGCTTC
	R	GTGGATCTGGATGAGTACC
15	F	GCAAGGGCAGAAGTAGACAAG
	R	CTAGGTCTTTGAAGGGTTGTGA
16	F	CAGGTCCTTTTGTAGTCATGAA
	R	TTTTAGTAGAGACGGGGGTTC
17	F	TCTGCCACTTGCCTCGTA
	R	TGCCCTGACCTTATGATT
18	F	AAAGCGTATGTGTTCAGTTACA
	R	ATGCGTGCACTGTACCTGAT
19_2	F	CACTGCCTTCTCTCCATTACC
	R	GCTGTATTGCCTGTCCTATCA
19_1	F	CCAGGAGTTCCAGATTGATAG
	R	TCAATTTGGACTAGCCGTGTA
20	F	AAGAGGTCTAGAGGGGTTGAGA
	R	GTCACTGCCACGTTAAGAGG

Primer for AFS identification (tumors)		
Tumor		Sequence (5´à3´)
21	F	CAGGAGAGCTGCTCATCAATCT
	R	GATCTATCCTGGCACTGACGA
22	F	TCTGTTTTTGCTCCCTGATCCC
	R	GGCCTTTAATGTGGTGCAATGG
23	F	GAAAGCAAGAGGGGGAATCA
	R	TCACACCCCAGCTTGACCAG
24	F	Np
	R	Np
25	F	TAATGTAGATGACGGTTGATG
	R	TTGGGGGTGATAGGTATGTTT
26	F	CCTGTACACACCTGCATAC
	R	CGTTTCTCTGTGCCTAGT
27	F	CACCAGTAGAATAAAGGCTAA
	R	CTAGAAAGACACAAATTATCCC
28	F	GCAACCATCAGAAGCTAGTG
	R	GTGAGGTTGGGTTTATGGACT
29	F	CCACCAACGGCATTCCTT
	R	TCACTCCCAAAGATCCTATCC
30	F	ATGGCCGAATATTAAGCTAAA
	R	CGAGTGCGCTACATACCT
31	F	TGGGCCTAAAGGATAGAGCA
	R	ACATTTGTAGGCCATCATTGTC
32	F	Np
	R	Np
33	F	Np
	R	Np
34	F	CCTGCCCAACCAATCAAG
	R	TGAAGAGCAAAGGCAAGGTTA
35	F	GGCTGTGTTCTTGTCCTA
	R	GGATTACAGGCATGAACCAT
36	F	AGTATGAAATTATGCGGCACT
	R	CAAAGTACCGGGATCATAGG
37	F	TTAGAGAGCTGGGCCTCGTC
	R	TTGTTTATGCCAGCCCTAGGA
38	F	Np
	R	Np
39	F	GCAGGGTTCAAGCGATTCTC
	R	AGGGCAGTGGGCTTAGATTCA
40	F	GATGAGAGATGAGCGAAACTC
	R	ATATGGGTGTTCTCGGAAGT

Primer for AFS-RQ-PCR (tumors)		
Tumor		Sequence (5´à3´)
20	F	TTAAGCAATGGGTTTATGG
	R	CCCCAGAGTTTCCACA
21	F	AAAGCAAGCTTCAAACCTC
	R	GTTCCAGCAGAGTTATGTG
23	F	GTAAACAACAAGGTCTGGT
	R	CTGCTTCTCCTAAAGTCA

Primer for *INHBB* control		
		Sequence (5´à3´)
INHBB	F	AGTGTGTTTCCCCCATTGCCT
	R	TCACACTGCACGTCTAGGTT
INHBB-RQ	F	AACTCCTGCCCTACGTC
	R	CGTGAGTGGGAAGGTATG

Np not possible

Supplemental Table 4:
Primers used for AFS-PCRs and for AFS sequencing based on the HR-TA data and for the RQ -AFS-PCRs of the neuroblastoma cell lines and primary tumors.