Supplemental Information

Use of the GeneReader NGS System in a Clinical Pathology Laboratory: a Comparative Study

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Supplemental Material and Methods

In-house target enrichment and Illumina MiSeq sequencing

For multiplex PCR-based target enrichment, GeneRead DNAseq customized V2 panels from Qiagen (Hilden , Germany) were used, either targeting lung cancer relevant gene loci (lung cancer panel) or melanoma and gastrointestinal cancer relevant ones. These three panels comprise a subset of cancer-related genes including among others *KRAS, NRAS, KIT, BRAF, PDGFRA, ALK, EGFR, ERBB2* and *PIK3CA*. Multiplex PCR, using the GeneRead DNAseq Panel PCR Kit V2, was performed in four separate multiplex PCR reactions per sample according to the GeneRead DNASeq Gene Panel Handbook (Qiagen). After 23 cycles, amplicons of each sample were pooled and purified by means of Agencourt® AMPure® XP magnetic beads and a Biomek® FXp workstation (Beckman Coulter Inc, Fullerton, USA). Libraries were constructed using the Gene Read DNA Library I Core Kit and the Gene Read DNA I Amp Kit (Qiagen). After End-Repair and adenylation, NEXTflex DNA Barcodes were ligated (Bio Scientific, Austin, TX, USA). Following Agencourt® AMPure® XP magnetic bead purification and size selection, barcoded libraries were amplified by 9 PCR cycles. Library products were quantified with Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) on the Qubit 2.0 Fluorometer (Thermo Fisher Scientific), diluted and pooled in equal amounts. Finally, 12 pM of the constructed libraries were sequenced on a MiSeq instrument (Illumina, Inc.) with a MiSeq reagent Kit V2 (300-cycles) (Illumina, Inc.) following the manufacturer's recommendations.

In-house Data analysis

Fastq files were generated by the MiSeq Reporter Software (Illumina, Inc.) and analysed by an in-house validated bioinformatics pipeline based on our general cancer genome analysis algorithm [1] which was further optimized for the diagnostic workflow as previously described [2, 3]. All variants were listed but only non-synonymous variants with an allelic frequency above 5% and a coverage above 200x were reported directly after further evaluation by the Integrative Genome Viewer (IGV,

Broad Institute, Cambridge, MA, USA). In addition, therapy relevant activation mutations with an allele frequency below 5 % of samples with a tumor cell content of 10 – 20% were also reported.

Sanger sequencing

In the reference samples, *KIT* mutations have been detected by Sanger sequencing according to Heydt et al. [4]. *PDGFRA* mutations were also identified by conventional Sanger sequencing as described by Kuenstlinger et al. [5, 6].

Furthermore, for validation of the NGS results, conventional Sanger sequencing was carried out. Sanger sequencing was performed using the Big Dye Terminator Technology and an ABI 3500 sequencer (Applied Biosystems). Primer sets, used for Sanger sequencing are listed in Supplemental Table 1.

References of the Supplemental Material and Methods

1. Peifer M, Fernandez-Cuesta L, Sos ML, et al. Integrative genome analyses identify key somatic driver mutations of small-cell lung cancer. *Nat Genet* 2012; 44: 1104-1110.
2. Konig K, Peifer M, Fassunke J, et al. Implementation of Amplicon Parallel Sequencing Leads to Improvement of Diagnosis and Therapy of Lung Cancer Patients. *J Thorac Oncol* 2015; 10: 1049-57.
3. Vollbrecht C, Mairinger FD, Koitzsch U, et al. Comprehensive Analysis of Disease-Related Genes in Chronic Lymphocytic Leukemia by Multiplex PCR-Based Next Generation Sequencing. *PLoS One* 2015; 10: e0129544.
4. Heydt C, Kumm N, Fassunke J, et al. Massively parallel sequencing fails to detect minor resistant subclones in tissue samples prior to tyrosine kinase inhibitor therapy. *BMC Cancer 2015*; 15: 291.

5. Kunstlinger H, Binot E, Merkelbach-Bruse S, et al. High-resolution melting analysis is a sensitive diagnostic tool to detect imatinib-resistant and imatinib-sensitive PDGFRA exon 18 mutations in gastrointestinal stromal tumors. *Hum Pathol* 2014; 45: 573-82.

6. Ney JT, Froehner S, Roesler A, et al. High-resolution melting analysis as a sensitive prescreening diagnostic tool to detect KRAS, BRAF, PIK3CA, and AKT1 mutations in formalin-fixed, paraffin-embedded tissues. *Arch Pathol Lab Med* 2012; 136: 983-92.

Supplementary Tables

Supplemental Table 1: Primer sets used for Sanger sequencing

Primer	Sequence	Annealing T°	Amplicon size
KIT E 8-F	GAA GTG AAT GTT GCT GAG G	57°C	234 bp
KIT E 8-R	GTG AAT TGC AGT CCT TCC		
KIT E 9-F	GCC ACA TCC CAA GTG TTT TAT G	60°C	310 bp
KIT E 9-R	GAG CCT AAA CAT CCC CTT AAA TTG		
KIT E 11-F	GTG CTC TAA TGA CTG AGA C	57°C	232 bp
KIT E 11-R	TAC CCA AAA AGG TGA CAT GG		
KIT E 13-F	CAT CAG TTT GCC AGT TGT GC	57°C	246 bp
KIT E 13-R	AAT CTA GCA TTG CCA AAA TCA		
KIT E 14-F	TGG GAG GCA GAA TTA ATC	57°C	261 bp
KIT E 14-R	CCC ATG AAC TGC CTG TC		
KIT E 15-F	GAG TGC CCT TCT ACA TGT C	57°C	243 bp
KIT E 15-R	CAT TGC TAC TGG GAA TGA TG		
KIT E 17-F	AAA AAG TTA GTT TTC ACT CTT TAC AA	57°C	272 bp
KIT E 17-R	TCA CAG GAA ACA ATT TTT ATC GAA		
KIT E 18-F	TGT TCA ATT TTG TTG AGC TTC TG	57°C	250 bp
KIT E 18-R	AAG GAA GCA GGA CAC CAA TG		
EGFR E 18F	TCC AGC ATG GTG AGG GCT GAG	62°C	223 bp
EGFR E18R	GGC TCC CCA CCA GAC CAT G		

EGFR E 19F	TGG GCA GCA TGT GGC ACC ATC	60°C	217 bp
EGFR E 19R	AGG TGG GCC TGA GGT TCA G		
EGFR E 20F	CCT CCT TCT GGC CAC CAT GCG	64°C	296 bp
EGFR E 20R	CAT GTG AGG ATC CTG GCT CC		
EGFR E21F	CGG ATG CAG AGC TTC TTC CC	60°C	275 bp
EGFR E 21R	AGG CAG CCT GGT CCC TGG TG		
HER E19	GGT GAA GGA TGT TTG GAG GA	60°C	278 bp
HER E19	CCC CAA TGA AGA GAG ACC AG		
HER2-20-F	GTT TGG GGG TGT GTG GTC T	60°C	289 bp
HER2-20-R	GTG GAC ATA GGG GTT TGC TC		
HER2 E 21 F	AGA AGG TCT ACA TGG GTG CTT C	60°C	258 bp
HER2 E 21 R	CAC TCA GAG TTC TCC CAT GGG		
K-RAS-12,13-F	GGT GAG TTT GTA TTA AAA GGT ACT GG	60°C	265 bp
K-RAS-12,13-R	GGT CCT GCA CCA GTA ATA TGC		
BRAF HRM FP3	ATG CTT GCT CTG ATA GGA AAATGA	60°C	163 bp
BRAF HRM RP3	ATC CAG ACA ACT GTT CAA ACT		
PIK3CA 9 For	TGA CAA AGA ACA GCT CAA AGC AA	60°C	100 bp
PIK3CA 9 Rev	TTT TAG CAC TTA CCT GTG ACT CCA		
PIK3CA 20 FP3	GCA AGA GGC TTT GGA GTA TTT CA	60°C	100 bp
PIK3CA 20 RP3	ATG CTG TTT AAT TGT GTG GAA GAT C		
PDGFRA-10-F	CAC TCA TTG CCA TGA CTC TC	60°C	295 bp
PDGFRA-10-R	TGC GGC TCA GCT GAT GAG		
PDGFRA-12-F	GTG AAG CTC TGG TGC ACT G	57°C	251 bp
PDGFRA-12-R	GTA AAG TTG TGT GCA AGG G		
PDGFRA-14-F	TGA GAA CAG GAA GTT GGT AGC TC	60°C	260 bp
PDGFRA-14-R	GGG ATG GAG AGT GGA GGA TT		
PDGFRA-18-F	CAG CTA CAG ATG GCT TGA TC	60°C	216 bp
PDGFRA-18-R	GAA GGA GGA TGA GCC TGA C		
PDGFRA18 HRM For	GCA CAA GGA AAA ATT GTG AAG AT	60°C	102 bp
PDGFRA18 RM Rev2	AGG GAA GTG AGG ACG TAC ACT G		

Supplemental Table 2:

List of samples under investigation with indication of tumor portion, DNA concentration, and allele frequencies of clincical variants as determined on the GeneReader, the Miseq platform or by Sanger sequencing.

Sample no.	Diagnosis	Tumor Portion	DNA[#] (ng/µl)	Gene	AA change	Variant allele fraction (%)		
						GeneReader	MiSeq	Sanger
Q 01	CRC	60%	5,8	KRAS	p.G12S	89.0	86.6	
Q 02	CRC	90%	13,6	KRAS	p.Q61K	22.8	31.8	
Q 03	CRC	70%	4,9	KRAS	p.G12V	59.5	59.8	
				PIK3CA	p.E545G	51.8	47.0	
Q 04	CRC	90%	0,9	KRAS	p.G12D	35.1	35.7	
				PIK3CA	p.Q546K	17.3	16.4	
Q 05	CRC	80%	0,3	KRAS	p.G12V	47.6	40.3	
Q 06	CRC	40%	12,8	KRAS	p.G12D	5.3	6.4	
				NRAS	p.G12C	54.8	59.3	
Q 07	CRC	90%	20,5	NRAS	p.Q61K	29.2	32.5	
Q 08	CRC	80%	20,5	NRAS	p.G12D	25.1	29.3	
Q 09	CRC	60%	10,7	NRAS	p.Q61L	50.8	51.2	
Q 10	CRC	40%	21,2	NRAS	p.Q61K	28.9	33.7	
Q 11	CRC	50%	7,0	PIK3CA	p.E542K	2.8	2.8	
Q 12	CRC	30%	1,9	PIK3CA	p.Y985N	16.7	12.5	
Q 13	CRC	80%	23,9	BRAF	p.V600E	52.5	54.1	
Q 14	CRC	80%	9,5	BRAF	p.V600E	25.2	31.6	
Q 15	CRC	50%	5,7	BRAF	p.V600E	7.9	9.5	
Q16	NSCLC	30%	3,0	ALK	p.S1136F	NOT in ROI	15.4	
Q17	NSCLC	20%	3,8	WT				
Q18	NSCLC	30%	13,8	ALK	p.P1139fs*11	Not in ROI	9.3	
Q 19	NSCLC	30%	3,2	ALK	p.F1271L	8.1	8.5	
				KRAS	p.G12V	38.0	38.8	
Q 20	NSCLC	70%	3,6	PIK3CA	p.E545	29.2	17.5	
Q 21	NSCLC	30%	12,9	EGFR	p.L747-T751delinsP	61.6	50.4	
Q 22	NSCLC	60%	11,8	EGFR	p.L858R	31.0	26.4	
Q 23	NSCLC	20%	0,5	EGFR	p.E746-S752delinsV	6.5	8.1	
Q 24	NSCLC	20%	5,3	EGFR	p.D770delinsGY	17.5	17.1	

#: The concentration of samples´ DNA was determined by qPCR.

Supplemental Table 2 Continuation:

Sample no.	Diagnosis	Tumor Portion	DNA# (ng/µl)	Gene	AA change	Variant allele fraction (%)		
						GeneReader	MiSeq	Sanger
Q 25	NSCLC	30%	3,8	ERBB2	p.A775-G776insYVMA*	22.3	19.6	
Q 26	NSCLC	20%	4,8	ERBB2	p.G776delinsVC	87.7	89.0	
Q 27	NSCLC	60%	17,4	ERBB2	p.P780-Y781 insGSP	44.4	40.8	
Q 28	NSCLC	30%	0,9	ERBB2	p.G776delinsVC	36.9	40.5	
Q 29	NSCLC	30%	5,1	ERBB2	p.A775-G776insYVMA*	12.7	12.8	
Q 30	NSCLC	25%	2,4	ERBB2	p.A775-G776insYVMA*	43.3	37.0	
Q 31	Melanoma	70%	3,3	BRAF	p.V600E/p.V207E	31.2	36.6	
Q 32	Melanoma	40%	9,7	BRAF	p.V600E/p.V207E	37.6	33.1	
Q 33*	Melanoma	40%	1,3	PIK3CA	p.E542K	49.4	54.3	
Q 34	Melanoma	50%	12,9	BRAF	p.V600E/p.V207E	54.3	62.3	
Q 35	Melanoma	50%	8,5	BRAF	p.V600E/p.V207E	34.8	31.8	
				ERBB2	p.V842I	31.0	29.4	
Q 36	Melanoma	90%	20,1	BRAF	p.V600E/p.V207E	71.9	68.1	
Q 37	Melanoma	30%	3,4	BRAF	p.V600E/p.V207E	21.8	19.2	
Q 38	Melanoma	50%	11,0	BRAF	p.V600E/p.V207E	33.5	31.0	
Q 39	GIST	100%	146,00	PDGFRA	p.D842V	37.2		20-50
Q 40	GIST	90%	8,2	KIT	p.V560E	43.3		20-50
Q 41	GIST	60%	6,3	KIT	p.W557-K558del	8.6		20-50
Q 42	GIST	80%	16,3	KIT	p.A502-Y503dup/ p.Y503-F504insAY	40.8		20-50
Q 43	GIST	90%	22,8	KIT	p.A502-Y503dup/ p.Y503-F504insAY	39.7		20-50
Q 44	GIST	90%	14,6	KIT	p.K642E	48.8		20-50
Q 45	GIST	100%	19,5	KIT	p.N822K	38.1		20-50
Q 46	GIST	70%	7,7	KIT	p.K642E	61.4		20-50
				KIT	p.D816E	30.6		20-50
Q 47	GIST	90%	21,9	PDGFRA	p.D842V	35.7	32.2	
Q 48	Stomach	70%	1,35	PIK3CA	pE545K	37.1	42.8	
Q 49	Stomach	60%	11	KRAS	p.G12D	41.1	44.4	
				PIK3CA	pE542K	15.1	11.6	
Q 50	NSCLC	60%	3,01	PIK3CA	p.H1047R	36.0	36.9	

* alternative nomenclature of the ERBB2 p.A775-G776insYVMA mutant: p.A745-G746insYVMA or p.Y772-A775dup

#: The concentration of samples´ DNA was determined by qPCR.