Deep Amplicon Sequencing of *PIK3CA* Gene Using **fastGEN** Technology

Jana Stránská[1,2], Rastislav Slavkovský[1], Gabriela Kořínková[3], Barbora Líznerová[1],
Jiří Drábek[1,2], Martina Onderková[4], Veronika Chladová[4], Kateřina Bezděková[4], Petr Brož [5,6], Hajdúch Marián[1,2,7]

[1] Institute of Molecular and Translational Medicine, Palacký University Olomouc, Olomouc, Czech Republic; [2] Laboratory of Experimental Medicine, University Hospital Olomouc, Olomouc, Czech Republic;
[3] Department of Clinical and Molecular Pathology, Olomouc University Hospital and Palacký University Olomouc, Olomouc, the Czech Republic;
[4] BioVendor Group, Research & Diagnostic Products, Brno, the Czech Republic; [5] BIOXSYS s.r.o., Ústí nad Labem, the Czech Republic; [6] Department of Biology and Medical Genetics, 2nd Faculty of Medicine, Charles University Prague and Faculty Hospital Motol, Prague, Czech Republic; [7] Czech Advanced Technology and Research Institute, Palacký University Olomouc, Olomouc, Czech Republic

Introduction and aims

The ***PIK3CA* gene** encodes Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit *alpha*, also called p110α protein. PI3K enzymes are part of the **PI3K/AKT/mTOR pathway regulating cell growth and survival**. *PIK3CA* is the most recurrently **mutated gene in breast cancer** (up to 36 %) and is also detected in other cancer types. It acts as a molecular target for treatment with **PIK3CA inhibitor alpelisib** (or alpelisib/fulvestrant), which has a benefit for **hormone receptor-positive and HER2-negative metastatic breast cancer patients**. **Testing most *PIK3CA* mutations**, not only two main hotspots, 542/545 and 1047, is still challenging for laboratories (Fig. 1).

Material and methods

Deep amplicon sequencing (DAS) has a high potential to be a suitable method for the simultaneous detection of somatic mutations within **hotspot regions** with a defined **detection limit** down to **1 % minor allelic frequency** (MAF). We have developed and validated a unique **fast, sensitive, and robust method** called **fastGEN** (Fig. 2) using Illumina platforms. **Formalin-fixed paraffin-embedded breast tumors** were genotyped for more than 25 clinically relevant mutations of the **PIK3CA** exonuclease domain (Fig. 3) and sequenced on MiSeq (Illumina).

Results and discussion

First, we have prepared ***v1* of *PIK3CA* test**, including **14+ mutations in 542/545 and 1047 hotspots**. We tested 24 samples with mutations detected in 9 out of 24 samples (37.5%). The test was **highly reproducible** (n=11, PIK3CA p.E545K, MAF = 40.5% ± 1.1%; n=8, PIK3CA p.H1047R, MAF = 26% ± 1.4%).

Then, we continued with ***v2* of *PIK3CA* test**, including **25+ mutations in exons 2, 3, 5, 7, 8, 10, 14,** and **21**. Larger somatic NGS panels (Qiagen QIAseq TMB Panel or QIAseq Custom Panel) were used for validation of fastGEN results confirming mutations in all exons. Using samples, where results of both methods were available (Tab. 1), we observed a **concordance with 100 % specificity and sensitivity**. The test was **highly reproducible** (n=6, PIK3CA p.H1047R, MAF = 47.2% ± 1.5%; n=4, PIK3CA p.V344G, MAF = 6% ± 0.4%) and **sensitive** (Tab. 2; n = 4, input = 1 ng DNA). A **minimum turn-around time** (from sample receive to final report) was **less than 24 hours**. **fastGEN** technology is routinely performed for tumor testing of ***POLE, RAS, BRAF, EGFR, IDH1/2, TERT*** and **TP53** in our lab; other genes are under development. The technology was licensed by the partner BioVendor Group.

Conclusion

Detection of ***PIK3CA* somatic mutations** by **fastGEN technology** is really **fast and easy** to perform with a **high success rate**, including samples with **low amount and low quality DNA**. With other often requested predictive biomarkers, laboratories with **Illumina sequencers** can easily implement fastGEN kits. A user-friendly and robust **bioinformatic pipeline** is based on **Genovesa fastGEN platform**. We have shown that using the fastGEN kit could be a suitable method for routine diagnostics.

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a)

Exon	Mutation	COSMIC* ID	Base change	LoD (% MAF)
7	C420R	757	1258T>C	2.41†
9	E542K	760	1624G>A	5.47‡
	E545A	12458	1634A>C	3.54†
	E545D	765	1635G>T	2.69‡
	E545G	764	1634A>G	4.98‡
	E545K	763	1633G>A	4.13‡
	Q546E	6147	1636C>G	4.50†
	Q546R	12459	1637A>G	6.08‡
20	H1047L	776	3140A>T	2.56‡
	H1047R	775	3140A>G	3.13‡
	H1047Y	774	3139C>T	14.04†

b)

PIK3CA Exon Number	PIK3CA Mutation
2	R88Q
5	N345K
8	C420R
10	E542K, E545A, E545D, E545G, E545K, Q546E, Q546K, Q546L, Q546R
21	H1047L, H1047R, H1047Y, G1049R, M1043I

Fig. 1: Detection of *PIK3CA* mutations. Available kits on the market in beginnings of our *PIK3CA* testing were a) therascreen PIK3CA RGQ PCR Kit (Qiagen) detecting 11 variants, and b) cobas® PIK3CA Mutation Test (Roche Diagnostics) detecting 17 variants.

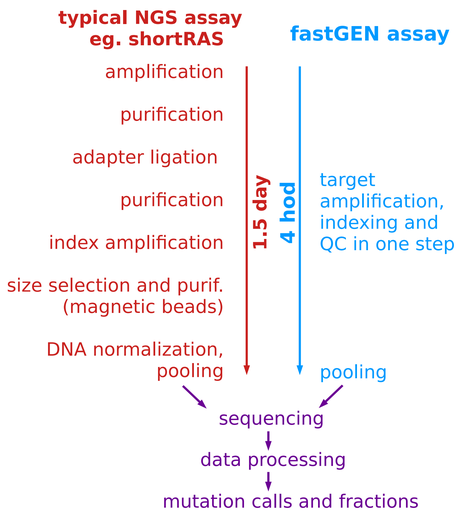

Fig. 2: Scheme of procedures in **typical** and **fastGEN** NGS assays. (typical assay is described for example in Slavkovsky 2022, Neoplasma Vol.69)

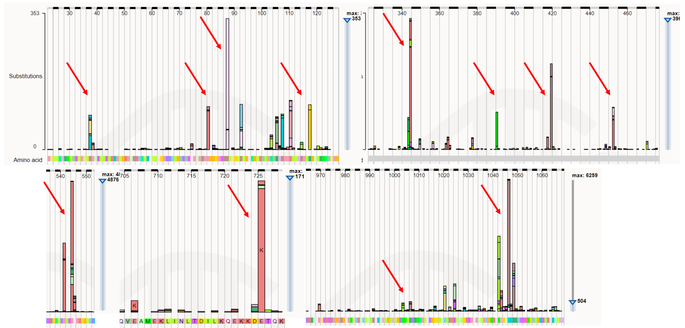

Fig. 3: *PIK3CA* mutation survey. We searched for *PIK3CA* variants suitable for testing ↘ in the Catalogue Of Somatic Mutations In Cancer. As a result, fastGEN *PIK3CA* test *v2* covers more than 25 clinically relevant variants (c; zoomed view).

Sample ID	Method	Result	MAF	Sequencing Depth (x)
15635	fastGEN *PIK3CA test v2*	wt	0 %	>1000*
	QIAseq Tumor Mutational Burden Panel	wt	0 %	>1000*
15636	fastGEN *PIK3CA test v2*	wt	0 %	>1000*
	QIAseq Tumor Mutational Burden Panel	wt	0 %	>1000*
15642	fastGEN *PIK3CA test v2*	wt	0 %	>1000*
	QIAseq Tumor Mutational Burden Panel	wt	0 %	>1000*
13727	fastGEN *PIK3CA test v2*	wt	0 %	>1000*
	QIAseq Custom Small Cancer Panel – 41 genes	wt	0 %	>1000*
15662	fastGEN *PIK3CA test v2*	wt	0 %	>1000*
	QIAseq Tumor Mutational Burden Panel	wt	0 %	>1000*
14446	fastGEN *PIK3CA test v2*	E542K	9 %	895
	The Cell3™ Target: Pan-Cancer Panel	E542K	8 %	366
15406	fastGEN *PIK3CA test v2*	L1006F	6 %	6390
	QIAseq Tumor Mutational Burden Panel	L1006F	13 %	n/a
14436	fastGEN *PIK3CA test v2*	G118D	11 %	6010
	QIAseq Tumor Mutational Burden Panel	G118D	13 %	n/a
13580	fastGEN *PIK3CA test v2*	R38S	14 %	420
		M1004I	6 %	3470
		M1043I	9 %	3081
		D1045Y	9 %	3091
	QIAseq Tumor Mutational Burden Panel	R38S	16 %	n/a
		M1004I	7 %	
		M1043I	17 %	
		D1045Y	15 %	
14800	fastGEN *PIK3CA test v2*	V344G	6 %	42122
	QIAseq Tumor Mutational Burden Panel	V344G	6 %	n/a
15081	fastGEN *PIK3CA test v2*	H1047R	39 %	2042
		C420R	36 %	5670
	QIAseq Tumor Mutational Burden Panel	H1047R	45 %	n/a
		C420R	34 %	
15734	fastGEN *PIK3CA test v2*	E545K	57 %	6192
	QIAseq Tumor Mutational Burden Panel	E545K	60 %	n/a
HD701	fastGEN *PIK3CA test v2*	H1047R	19 %	2317
		E545K	10 %	6691
	declared by manufacturer	H1047R	17.5 %	n/a
		E545K	9 %	

Tab. 1: Validation of fastGEN *PIK3CA* test *v2* results - testing of diagnostic parameters – a concordance of diagnostic sensitivity and specificity was 100%. * values for sequencing depth for all the studied regions, wt – wild-type genotype (negative), n/a – not available.

Sample ID	DNA input [ng]	Ct	Result	MAF	Sequencing Depth (x)
15706	10	29.1	E542K	11 %	>2000
	5	31.62	E542K	9 %	>6000
	1	34.05	E542K	8 %	>1700
14800	20	26.28	V334G	6 %	>10000
	5	28.49	V334G	6.5 %	>7000
	1	30.12	V334G	6 %	>2000
14983	20	29.9	H1047R	48.5 %	>1500
	5	30.46	H1047R	47 %	>2000
	1	32.04	H1047R	21 %	>2400
14069	10	28.91	wt	0 %	>7500*
	5	30.17	wt	0 %	>9000*
	1	31.01	wt	0 %	>2500*

Tab. 2: Detection limit of fastGEN *PIK3CA* test *v2* was set to 1 ng of DNA input. Ct, cycle treshold, * values for sequencing depth for all the studied regions, wt – wild-type genotype (negative).