A novel Next Generation Sequencing (NGS) panel to facilitate the diagnostic process of X-linked hypophosphataemia (XLH) and other genetic disorders of renal phosphate wasting

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Conclusion

- **In this study, we created a new NGS tool for an easy, fast, and reliable diagnostic process of XLH caused by inactivating mutations in the *PHEX* gene and including mosaicisms in *PHEX*.**
- **In addition, our approach further enables to reveal molecular changes in ten other candidate genes leading to related disorders of renal phosphate wasting as differential diagnosis to XLH.**
- **Molecular genetic proven diagnosis of a disorder of renal phosphate wasting allows not only a certain genetic counselling, but also to initiate the most promising therapeutic approach.**

Background

X-linked hypophosphataemia (XLH) is the most common genetic disorder of renal phosphate wasting. It is caused by inactivating mutations in *PHEX* (located at Xp22.1), encoding fibroblast growth factor 23 (FGF23)-cleavage enzyme, which regulates the phosphaturic secretion. Affected individuals present with a broad phenotypic spectrum, ranging from isolated hypophosphatemia up to severe symptoms, such as rickets with extreme lower limb deformities, bone pain, distinct tooth problems, pseudo fractures, and disproportionate short final height.

On one side, early treatment has a strong impact on the long-term outcome; but on the other side, the diagnosis of this rare disorder is often delayed. The clinical and laboratory based diagnosis in XLH is hampered by related disorders with an overlapping phenotype, but caused by other gene defects than in *PHEX.* Therefore, molecular confirmation of the diagnosis is strongly recommended (1).

The importance of molecular diagnosis has risen dramatically, since there is a new treatment with a selective licensure for XLH with an FGF23 antibody as alternative to the conventional therapy.

In the past, genetic testing of *PHEX* (composed of 22 exons) has been done by Sanger sequencing, being both expensive and time consuming, followed by MLPA analysis. Our goal was to develop an easy, fast, and reliable tool for XLH and related disorders.

Patients and Methods

First, a NGS panel was developed *in silico* (see table 1 for technical details) including *PHEX* and the ten other genes: *FGF23, DMP1, ENPP1, SLC34A3, CLCN5, SLC34A1, SLC9A3R1, FAM20C, FGFR1,* and *KL.*

Technical Panel Data	Values
Number of genes	11
Panel size	45,98 kb
Primer Pools	2
Total number of exons	136
Total number of amplicons	245
Amplicon lengths	ca. 125-275 bp
Covering	99,88%

Table 1: Technical details of the panel

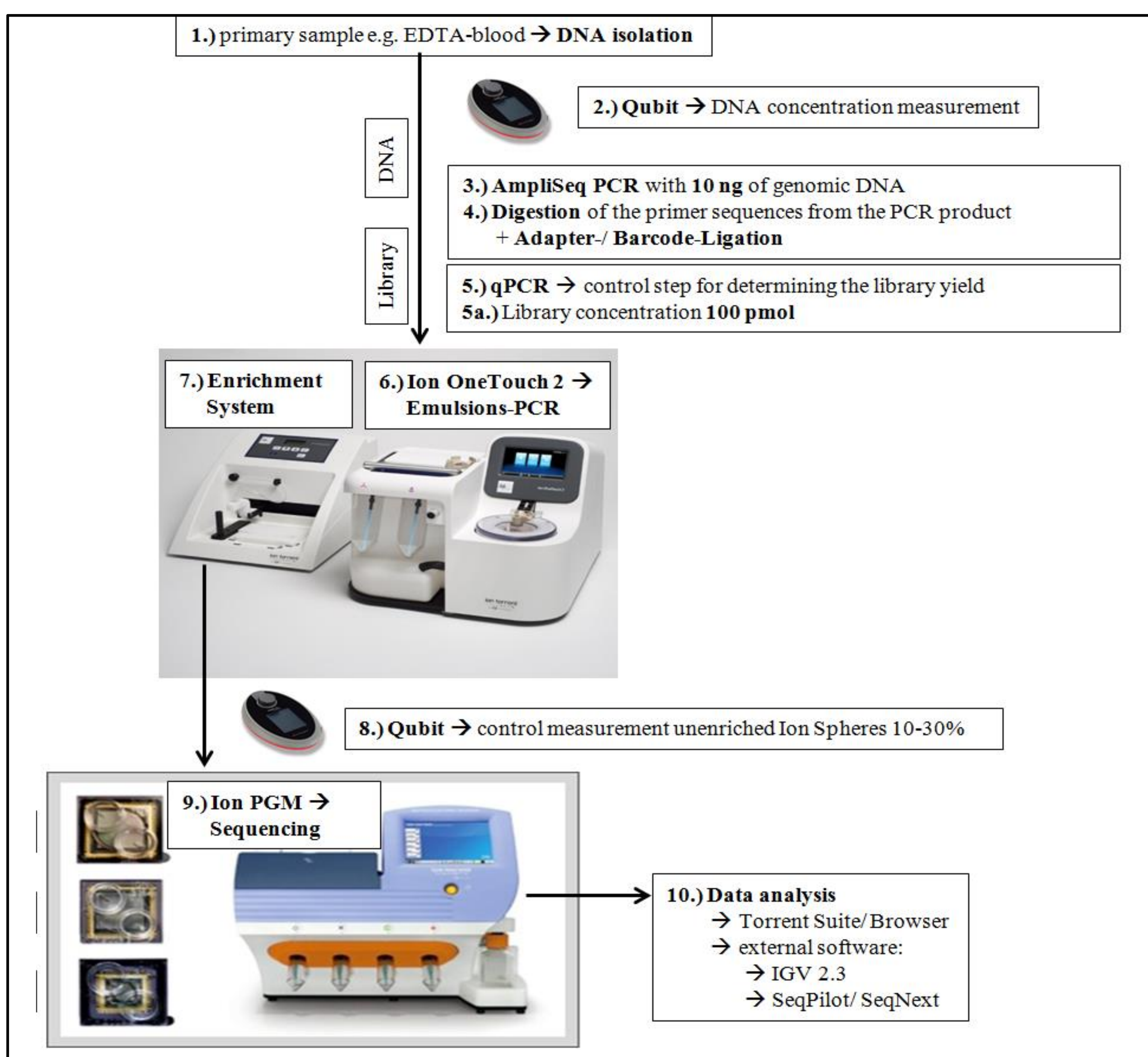

Figure 1: Methodical process of the molecular genetic analysis of the panel
DNA samples have been amplified using multiplex-PCR.
NGS of 11 genes up to 40 samples at once have been done using the Ion PGM.
For analysis of the sequencing data we used the software SeqPilot and for data analysis the Software GeneMarker 1.75.

For validation of the panel we analysed fifty-five DNA-samples from patients, which have been sent to our laboratory for molecular genetic testing under the suspicion of XLH, based on clinical and laboratory changes. In all (but five) we had detected a proven mutation in *PHEX* by Sanger sequencing, in two only a known polymorphism. In three samples (53-55), no mutation had been found in the past. Samples were sent anonymized and blinded to our partner company.

Results

In all 50 samples the known PHEX mutation and in two the known polymorphism have been detected by the panel. All together, 42 different mutations were found, including nonsense (n=16), missense (n=12), and splice site mutations (n=4); further small deletions (n=6), small insertions (n=2), small duplications (n=1), and a large duplication (n=1) (see table 2).

Gen	Exon/ Intron	Variante	Art der Mutation	PHEX-1	PHEX-2	PHEX-3	PHEX-4	PHEX-5	PHEX-6	PHEX-7	PHEX-8	PHEX-9	PHEX-10	PHEX-11	PHEX-12	PHEX-13	PHEX-14	PHEX-15	PHEX-16	PHEX-17	PHEX-18	PHEX-19	PHEX-20	PHEX-21	PHEX-22	PHEX-23	PHEX-24	PHEX-25	PHEX-26	PHEX-27	PHEX-28	PHEX-29	PHEX-30	PHEX-31	PHEX-32	PHEX-33	PHEX-34	PHEX-35	PHEX-36	PHEX-37	PHEX-38	PHEX-39	PHEX-40	PHEX-41	PHRX-42	PHEX-43	PHEX-44	PHEX-45	PHEX-46	PHEX-47	PHEX-48	PHEX-49	PHEX-50	PHEX-51	PHEX-52	PHEX-53	PHEX-54	PHEX-55
	Exon 1	c.1A>G (p.Met1Val)	missense								hemi																																															
	Exon 1	c.58C>T (Arg20*)	nonsense																																								het															
	Exon 1 + 2	c.61dupA (p.Ile21Asnfs*30)	insertion					hemi																																																		
	Exon 1	c.118+2T>A	splicing																																													het										
	Exon 2	c.124C>T (p.Gln42*)	nonsense																								het																															
	Exon 2	c.162C>A (p.Cys54*)	nonsense																																			het																				
	Exon 2	c.181G>T (p.Glu61*)	nonsense																																												het				het							
	Exon 3	c.208_212delGTAAA	deletion													hemi																																										
	Exon 3	c.230G>A (p.Cys77Tyr)	missense																																																				hemi			
	Exon 4	c.409delA (p.Ile137Serfs*7)	deletion																						hemi																																	
	Exon 4	c.417T>A (p.Tyr139*)	nonsense																											het																												
	Exon 5	c.614G>C (p.Arg205Pro)	missense																														hemi																									
	Exon 5	c.659T>A (p.Leu220*)	nonsense																																		hemi																					
	Exon 8	c.852_854delAAT (p.Ile284del)	deletion																							het						het			hemi																							
	Exon 8	c.854delT (p.Met285Argfs*16)	deletion																															het																								
	Exon 8	c.871C>T (p.Arg291*)	nonsense		het													het																							hom																	
PHEX	Exon 9	c.958_960delAAG (p.Lys320del)	deletion																																					het																		
	Exon 9	c.1079+1G>A	splicing																			hemi																																					
	Exon 10	c.1152T>G (p.Tyr384*)	nonsense																																															het								
	Exon 10	c.1158G>A (p.Trp386*)	nonsense										het																																													
	Exon 11	c.1178delT (p.Ile393Thrfs*15)	deletion																									het																														
	Exon 12	Duplikation von Exon 12	duplication																			dup																																				
	Exon 12	c.1328G>A (p.Arg443His)	missense																																														het									
	Exon 15	c.1601C>T (p.Pro534Leu)	missense																																																	hemi						
	Exon 15	c.1645C>T (p.Arg549*)	nonsense																		het																																					
	Exon 15	c.1645+1G>A	splicing						het																																																	
	Exon 16	c.1699C>T (p.Arg567*)	nonsense																																																			het				
	Exon 16	c.1700G>C (p.Arg567Pro)	missense							hemi																																																
	Exon 18	c.1779_1782dupTGAT (p.Lys595*)	insertion																																											hemi												
	Exon 18	c.1832_1833delTT (p.Phe611*)	nonsense																																				het																			
	Exon 18	c.1850G>T (p.Cys617Phe)	missense																																																		het					
	Exon 18	c.1853T>G (p.Met618Arg)	missense	hemi																																																						
	Exon 18	c.1862A>C (p.Gln621Pro)	missense												het																																											
	Exon 20	c.1971C>G (p.Tyr657*)	nonsense																																	het																						
	Exon 20	c.2040C>A (p.Asn680Lys)	missense											het																																												
	Exon 20	c.2051T>G (p.Phe684Cys)	missense																				het																																			
	Exon 20	c.2059_2070dupAGTTATGCTCAT	duplication																												hemi																											
	Exon 20	c.2066C>T (p.Ala689Val)	missense														hemi																																							
	Exon 21	c.2079C>A (p.Cys693*)	nonsense																																									hemi														
	Exon 21	c.2104C>T (p.Arg702*)	nonsense																										het																het													
	Exon 21	c.2104C=/>T (p.Arg702*)	nonsense																																																							het
	Intron 21	c.2148-10C>A	splicing				het																																																			
	Exon 22	c.2239C>T (p.Arg747*)	nonsense																het	hemi																						hemi																

Table 2: Results of the sequencing of the NGS panel: The patient samples are numbered from PHEX1-55. On the left side the different mutations, the kind of mutation and the concerned exon (and intron 21) of *PHEX* is demonstrated. Since the males only have one X-chromosome, they appear to be homozygous for the mutation, while the females appear to be heterozygous (with one exception). The blue underlined sample has been investigated in addition by MLPA, in the green one we detected a mosaicism in *PHEX.* het: heterozygous, hom: homozygous

In sample 55 we detected a mosaicism mutation in *PHEX* with the NGS panel (see figure 2). Based on this result, this patient could be treated by the new FGF23 antibody Burosumab.

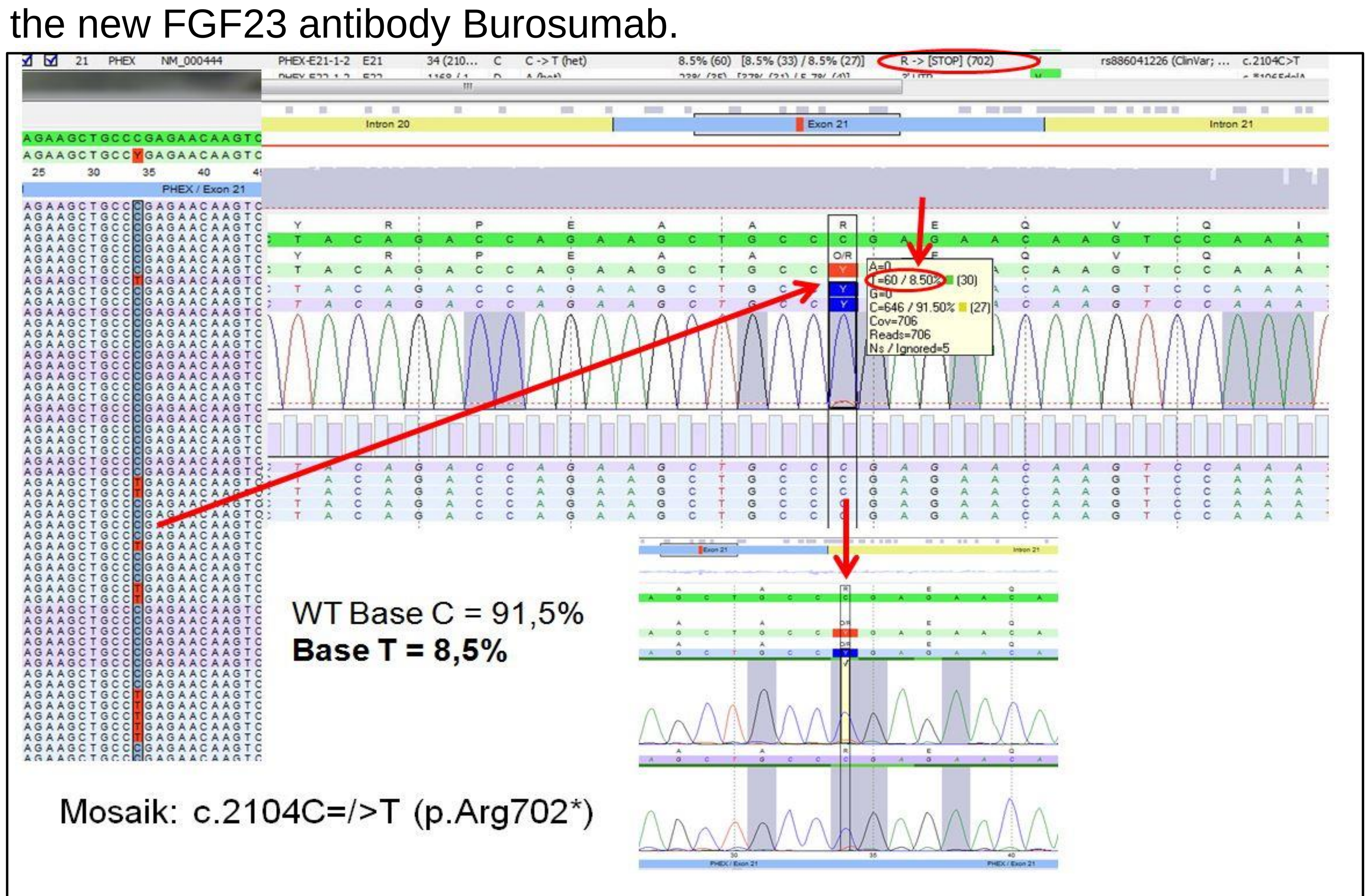

Figure 2: Mosaicism mutation in PHEX-55: NGS reads and confirmed result by Sanger Sequencing

In sample 53 we found two heterozygous mutations in the *ENPP1* gene and in sample 54 we revealed an homozygous mutation in *DMP1*.

Our results clearly demonstrate, that the panel is a reliable tool, not only for the molecular genetic diagnosis of XLH, but also for other disorders of renal phosphate wasting.

Reference

1)Clinical practice recommendations for the diagnosis and management of X-linked hypophosphataemia. Haffner et al. ***Nature Reviews Nephrology 2019***

2)FGF23 and its role in X-linked hypophosphaemia-related morbidity. Beck-Nielsen et al. ***Orphanet Journal of Rare Diseases 2019***