A new version of the Primer Designer on-line search tool for PCR-assays for clinical Sanger sequencing

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Abstract

We evaluate the latest version of an on-line search tool called 'Primer Designer Tool' giving access to a database of > 300,000 pre-designed, in-silico validated PCR-assays for Sanger re-sequencing of exons in the human genome. The online tool allows the user to easily identify and order PCR-primers for confirmation sequencing of clinically relevant targets.
The interface provides the ability to search for multiple targets, for example by chromosome location, in a single search. Other search terms include Gene name, dbSNP ID and RefSeq accession number. File input in VCF variant file format provides rapid conversion of variant information obtained on microarray and NGS platforms to PCR-assays for clinical Sanger sequencing.
Wet-lab validation on a set of 192 PCR-assays selected from the PrimerDesigner database showed a better than 98% success rate for amplification and sequencing reactions. The performance of the PCR assays for Sanger sequencing is shown for reference human genomic DNA with known mutations and for eight examples of clinical samples of FFPE-extracted DNA where Sanger sequencing was able to resolve low-quality, ambiguous base calls by NGS sequencing.

Introduction

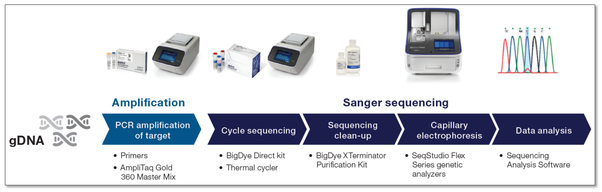


Figure 1. PCR-sequencing workflow

Wet-lab validation of the PCR assays in the Primer Designer database was originally (2013) performed on a representative set of 192 PCR Assays [1]. It showed a better than 98% success rate for amplification and sequencing reactions. All PCR-assays were tested with and without universal M13-tails as well as with standard desalting and HPLC purification methods.
Each version of the assay and sequencing chemistry generated substantially the same Sanger sequencing result.

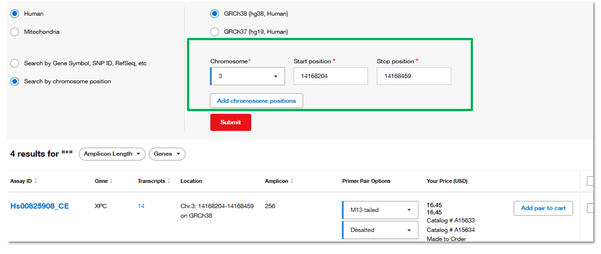


Figure 2. Primer Designer™ Tool User Interface

Detailed information on the PCR assay (Chromosome location, Gene and SNP details) and a genomic map view is available by clicking on the "Details" (Fig.3) and "Map" (Fig.4) buttons beneath the PCR-primer pair of interest.

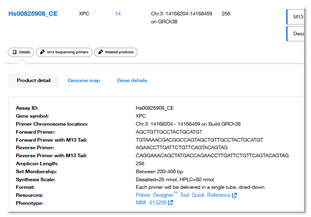


Figure 3. PCR primer sequences and mol. bio. information on the PCR assay

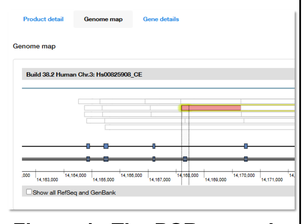


Figure 4. The PCR assay is mapped to the genomic exon-intron structure at the location of the assay

Results

Study 1: Assay Validation using human genomic Reference DNA

Commercial Oncology Reference standards (e.g. Horizon Discovery, Acrometrix) with known mutations, when available, provide a way to to validate individual PrimerDesigner assays for their ability to detect genetic variants.
We validated two PrimerDesigner assays targeting the KRAS hotspot mutation sites analyzing a sample generated using Horizon Discovery Reference DNAs. Example electropherograms are shown in Fig. 5

Exon	Genotype	Amino Acid Variant	ThermoFisher Assay#	Horizon Discovery DNA Cat.#
2	G12D	Gly12Asp	Hs00679698_CE	HD272
2	G12V	Gly12Val	Hs00679698_CE	HD289
2	G12C	Gly12Cys	Hs00679698_CE	HD269
2	G12S	Gly12Ser	Hs00679698_CE	HD288
2	G12A	Gly12Ala	Hs00679698_CE	HD265
2	G12R	Gly12Arg	Hs00679698_CE	HD287
2	G13D	Gly13Asp	Hs00679698_CE	HD290
3	Q61L	Gln61Leu	Hs00532900_CE	HD140
	WT			HD710

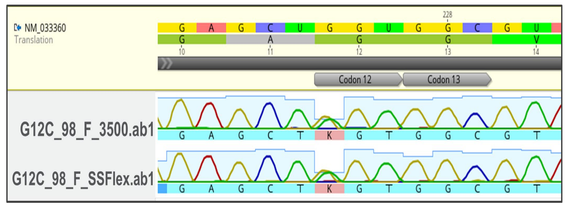


Figure 5. Sanger Electropherograms detecting the KRAS G12C variant on the 3500 Genetic Analyzer (top trace) and the SeqStudio Flex (bottom trace) CE Instruments

Study 2: Confirmation of NGS Genotyping Results

24 PrimerDesigner assays were selected to compared genotyping results obtained by NGS and Sanger sequencing (Table1). In some instances, highlighted in yellow, a definitive sequencing answer is found in only one sequencing direction due to additional sequence content such as homopolymers or heterozygous indels obscuring the sequencing results of the opposite strand.

Gene	Assay ID	REF	NGS Genotype	CE Genotype	NGS/CE confirmed
NOTCH2	247286	A	A/T	A/T	YES
PDE4DIP	247326	C	C/T	C/T	YES
PDE4DIP	247326	G	G/A	G/A	YES
EXT2	328600	T	T/C	T/C	YES
CCND2	115094	C	C/G	C/G	YES
ARID2	336029	T	T/C	T/C	YES
ALK	192315	A	A/T	A/T	YES
MSH6	359011	A	A/G	A/G	YES
MSH6	359024	T	C	C	YES
FN1	205929	T	C/T	C/T	YES
BCR	362193	G	G/A	G/A-fwd	YES
BCR	362194	C	C/T	C/T	YES
BCR	362197	G	G/A	G/A	YES
MN1	221970	A	A/G	A/A	NO
CYP2D6	225801	G	G>T	G>T	YES
CYP2D6	225801	G	G>A	G>A	YES
CYP2D6	225801	T	T>G	T>G	YES
CYP2D6	225801	G	G>C	G>C	YES
FANCD2	363573	G	G/T	G/T	YES
XPC	227180	G	G/A	G/A	YES
FOXP1	235541	A	A/T	A/T	YES
EPHB1	240070	G	G/A	G/A	YES
FBXW7	254918	G	G/A	G/A	YES
MTRR	257547	A	A/G	A/G-rev	YES
TNFAIP3	281646	C	C/T	C-f/indel-r	YES
TNFAIP3	281648	A	A/C	A/C	YES
SYNE1	282846	A	A/G	A/G	YES
SYNE1	282961	T	T/C	T/C	YES

Table 1. Comparison of Sanger sequencing vs. NGS genotype calls

Study 3 : Confirmation sequencing of 8 non-concordant NGS-variant calls on FFPE-extracted DNA samples

A set of eight conflicting variant calls in two different NGS platforms (Table 2) was used to show that Sanger sequencing using the PrimerDesigner assays is able to resolve ambiguous NGS variant assignments.

Gene	PrimerDesigner Assay ID	CHROM POS (GRCh37)	REF (NGS)	ALT (NGS)	NGS #1, Variant Present/Absent	NGS #2, Variant Present/Absent	Sanger, Variant Present/Absent	Sanger Result	NGS/CE concordance on Variant, if Present
PTEN	00684986	10:89692789	AGACCATAAC CCACCACAGC TAGAACTTATC AA	AG	A	P	P	Large, 31 bp deletion	Yes
NF1	00519571	17:29553483	CCG	C	A	P	P	SNV detected, Mixed base C&T	No
TP63 *	00766420	3:189456563	G	A	A	P	A	No variant	NA
MAP3K4	00678934	6:161519353	GCTGCTGCTG CTGCTGCTGC TGCTGCTGCT GTT	GCTGCTGCT GCTGCTGCT GCTGCTGCT GCTT	A	P	P	3 bp deletion	Yes
RECQL4 *	00521976	8:145741701	GC	G	A	P	P	SNV, G>C , ClinVarID 94898	No
TERT	00257248	5:1295250	G	A	P	A	P	SNV, G>A, ClinVarID 2443072	Yes
SLX4*	00827565	16:3639974	AGCGC	AGCA	P	A	P	SNV G>A ClinVar ID 262048	No
RAD51D	00764867	17:33446593	C	A	P	A	A	No variant	NA

Table 2. Comparison of non-concordant variant calls on two different NGS platforms vs. Sanger sequencing

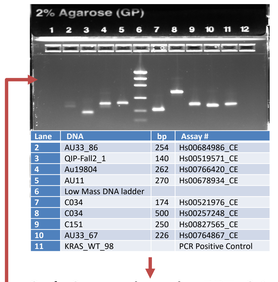
PCR reaction setup

Reagent	25uL rxn
FFPE-extracted DNA (4ng/µl)	1 µl
PCR assay (0.8µM ea primer)	7.5 µl
AmpliTaq™ Gold 360 Master mix	12.5 µl
dH2O	4 µl

PCR Thermocycling Protocol

Cycle	Temp	Time
1x	95	10-min
40x	96	30-s
	58	30-s
	70	45-s
1x	72	7-min
1x	4	hold

Exo-Sap™ It PCR Reaction clean up followed by agarose electrophoresis and Q-check using e-Gel™ (Invitrogen)



Lane	DNA	bp	Assay #
2	AU33_86	254	Hs00684986_CE
3	CIP-Fall2_1	140	Hs00519571_CE
4	Au19804	262	Hs00766420_CE
5	AU11	270	Hs00678934_CE
6	Low Mass DNA ladder		
7	C034	174	Hs00521976_CE
8	C034	500	Hs00257248_CE
9	C151	250	Hs00827565_CE
10	AU33_67	226	Hs00764867_CE
11	KRAS_WT_98		PCR Positive Control

Cycle Sequencing using BDT v3.1 sequencing kit and RapidSeq sequencing protocol SeqStudio Flex CE Instrument, 50cm array, POP-7 polymer
Data Analysis using Geneious Prime™ software

Figure 6. PCR reaction setup, T-cycling, ExoSap_It treatment and QC of the PCR-amplicons prior to Sanger Sequencing

NGS called: chr3|189456563|G|A; G→A Trp108Ter
Sanger: not called

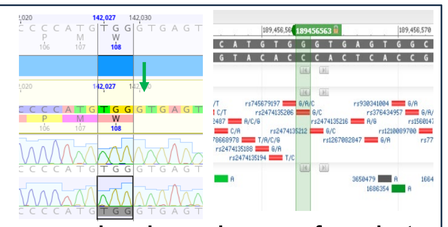


Figure 7. Sanger sequencing shows absence of a variant in TP63

NGS called: chr8|145741701|GC|G deletion, frameshift mutation
Sanger called: C>G: Glu (E) → Asp (D), SNV, ClinVar ID: 94898

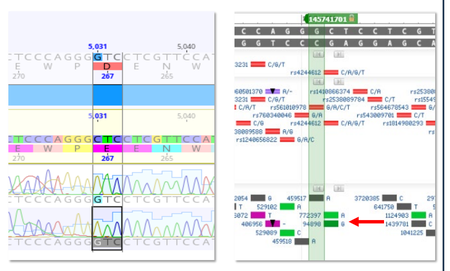


Figure 8. Sanger sequencing shows the presence of ClinVar ID 94898(benign), SNV C>G (red arrow) in RECQL4

NGS called: chr16|3639974|AGCGC|AGCA, GC deletion complex
Sanger called: G>A: SNV, ClinVar ID:262048

Figure 9. Sanger sequencing shows presence of ClinVar ID 262048 (benign), SNV G>A (red arrow) in SLX4

Conclusions

- The PrimerDesigner online tool offers convenient access to > 300,000 pre-designed, *in-silico* validated PCR assays for Sanger confirmation sequencing of NGS results.
- Wet-lab validation of a representative set of 192 PCR assays for Sanger sequencing resulted in 98% success rate (high-quality, target-specific PCR amplicons and Sanger sequencing).
- Individual PrimerDesigner assays can be validated by using commercially available Reference DNA with known mutations status.
- Confirmation of genotyping was shown on a set of 28 NGS SNP-genotyping results using 24 PrimerDesigner PCR amplicons (genes).
- Confirmation sequencing using the Primer Designer PCR assays for the detection of variants in oncology was demonstrated on a set of 8 conflicting variant calls on two different NGS platforms.

Reference

[1] Primer Designer Tool, Application Note , 2013, https://documents.thermofisher.com/ TFS-Assets/LSG/brochures/primer-designer-tool-app-note.pdf

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