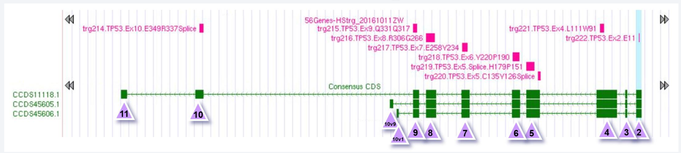
Automated Primer Design for Single-Tube Multiplex PCR for Tiled Amplicon Resequencing – TP53 Assay Design and Characterization as a Pilot Study

Martin Zillmann, Yue Ke, Nicholas Lodato, Melanie Prasol, Zhaohui Wang - Pillar Biosciences, 12 Michigan Drive, Natick, MA 01760

BACKGROUND

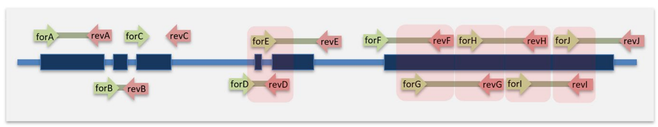
Target enrichment by PCR is widely used to prepare libraries for NGS, especially for FFPE specimens, where DNA amount may be low and quality compromised. Very often, amplicon tiling (overlap) is needed to cover continuous sequences of Regions of Interest (ROI). In this work, we use a customized primer design tool for rapid primer design to the TP53 oncogene and evaluate the performance of the resulting assay.

Structure, Transcripts, and of Key Somatic Mutations for the TP53 Oncogene



- TP53 transcript variants (green) contain 12 possible exons and 11 coding exons that include key somatic mutations (pink)
- All 11 coding exons were targeted for amplification using overlapping amplicon “tiles” to span longer exonic regions (e.g. exon 10) or exons in close proximity (e.g. exons 2-4)

Dilemma for Overlapping Amplicons in Single Tube PCR Reaction

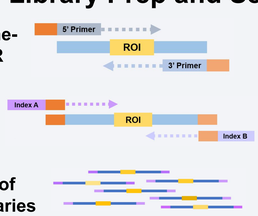


- To obtain full sequence coverage for longer Regions of Interest (ROI - blue), amplicons (brown lines) must be overlapped
- Multiplexing primers for such ROIs in a single reaction will produce the desired amplicons (green lines), but will also produce short “parasitic” amplicons for proximal primers (red highlight)
- Pillar Bioscience’s SLIMamp technology suppresses the formation of undesired amplicons (red highlights), allowing PCR a single pool

MATERIALS & METHODS – LIBRARY PREP

Workflow for Library Prep and Sequencing

1. **One-Pot Gene-Specific PCR**
2. **Indexing PCR**
3. **Pooling and Sequencing of Sample Libraries**

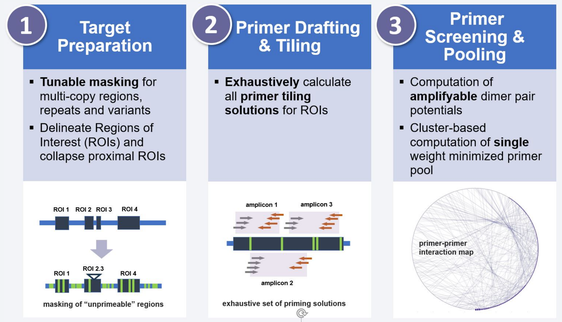


- Only one amplicon in pool is shown for clarity
- Orange regions denote matched SLIMamp tags
- Reactions are purified using AMPure beads
- After normalization and pooling, samples were loaded onto an Illumina MiSeq v2 or v3 flow-cell and sequenced (2 x150 cycles)

MATERIALS & METHODS

An automation pipeline built in Python was used for TP53 primer design beginning with variant and repeat masked ROIs and flanking sequences.

ampPD Workflow for Automated Primer Design

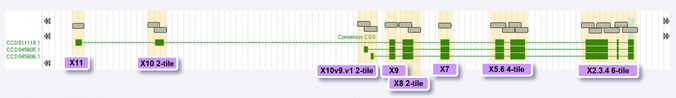


- Nominal amplicon length, amplicon overlap, and primer parameters (%GC, Tm) are user specified
- In the candidate primer generation step, all primers with properties within design constraints were created forming a large number of combinatorial possibilities for each ROI
- In the primer selection and pooling stage, all primer-primer interactions were considered, and an interaction minimized pool with full coverage of the ROI regions was selected. SLIMamp tags were added to the primers in this pool

RESULTS – TP53 PANEL DESIGN OUTPUTS

Automated TP53 primer design generated an initial pool of 627 compatible primers that was reduced to an optimized pool of 19 amplicons varying between 138 and 180 bp with 16 overlapping amplicons. The design process took roughly 10 seconds to complete

TP53 Primer Pool Design Output



- Ten FFPE samples were analyzed at 3 different input amounts (1, 5, 10 ng) as determined by DNA fluorescence on Qubit analyzer
- Variant detection was highly reproducible and 100% concordant with known outcome at 5 and10 ng. At 1 ng DNA input all positive variants were still detected but with increased variant noises at allele frequencies of 1-3%
- The median on-target rate was 87% for 5 ng and 10 ng samples and 31% for the 1 ng samples.
- The assay displayed high coverage uniformity with 100% of targeted based covered above 0.2X mean coverage for all samples.

Summary Information	
Sample Type	DNA, FFPE DNA
Input Amount	5-50 ng
Pool Number	single
Amplicon Size	138-180

Panel Performance	
Mapping Rate	87% @ 1-10 ng *
On-Target Rate	73%@ 1-10 ng *
Coverage Uniformity	100% @ 0.20x of mean 100% @ 0.15x of mean
MAF Sensitivity	2% at 5 ng
PPA	100%
NPA	100%
Multiplex Level	Limited by indices

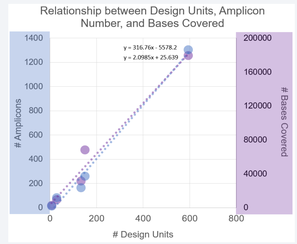
* low mapping and on-target rates likely due to low amount and high fragmentation of FFPE input DNA

RESULTS – DESIGN PIPELINE PERFORMANCE

To characterize the primer design pipeline further and test its limitations, design was performed for several additional panels of varying complexity. Results are summarized below. As expected, a linear relationship exists between Design Units, Amplicons, and Bases Covered.

Primer Design Pipeline Preformance

Summary Information								
Name	Sample Type	# Design Units (DU)	Ave DU Flank %GC	Min - Max Amplicon (Amp) %GC	# Amps	Ave Amp Length	Total Bases Covered	Longest Tiled Region
TP53	FFPE DNA	7	52	32 - 62	19	149	2.1 kbp	0.4 kbp
NOTCH3	DNA	28	59	56 - 82	66	231	11.9 kbp	1.1 kbp
CH Multi-Gene	FFPE DNA	133	48	28 - 77	221	153	23.6 kbp	1.4 kbp
DFB Multi-Gene	FFPE DNA	150	41	28 - 81	533	152	37.1 kbp	6.6 kbp
ZOHB Multi-Gene	DNA	594	46	27 - 79	1257	224	18.6 kbp	4.2 kbp



Design Success Metrics			
Name	In Silico Success *	Unoptimized Lab Success **	Overall Success
TP53	100%	100%	100%
NOTCH3	100%	100%	100%
CH Multi-Gene	99%	~99%	~98%
DFB Multi-Gene	100%	~98%	~98%
ZOHB Multi-Gene	97%	~98%	~95%

* In Silico Success = number of amplicons produced / total number of amplicons attempted

** Unoptimized Lab Success = 1 - (number of amplicons dropped / total number of amplicons)

CONCLUSIONS

We have demonstrated a rapid and robust primer design pipeline using the TP53 oncogene as a model system. The resulting primers were shown to provide uniform coverage across TP53 and sensitive variant detection at FFPE-derived DNA inputs greater than 1 ng. In addition, the platform has been shown to be extensible to very large primer pools.

Future work will focus on:

- Optimization of the underlying algorithms and parameter settings for performance and overall design success
- Continuing to push the limits of the automated primer design platform with larger panels

ACKNOWLEDGEMENTS

The authors wish to acknowledge the generous gift of ten FFPE samples used for performance assesment from Matthew D. Stachler, MD,PhD, Brigham and Women's Hospital, Pathology