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Streamlining the process of Sanger sequencing in a diagnostic laboratory by HotStart TouchDown PCR

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INTRODUCTION

DNA amplification via polymerase chain reaction (PCR) is a critical step for Sanger based DNA sequencing but the amplification of GC rich sequences are often problematic.

In our laboratory, primers for GC-rich and non-GC-rich amplicons were processed separately as GC-rich amplicons were optimised to work at higher annealing temperatures and required additives that enable efficient denaturation.

For a diagnostic laboratory, having two different processing protocols adds delay to the result and creates a bottle neck in processing the samples.

TouchDown PCR has been shown to increase specificity and sensitivity of PCR amplification1. Combining HotStart polymerase and TouchDown PCR (HTD-PCR) we have developed a process for specific PCR amplification of any amplicon independent of GC content in order to streamline the sequencing of different genes in a diagnostic laboratory.

OBJECTIVE

Validation of HTD-PCR for diagnostic laboratory.

MATERIAL AND METHODS

HTD-PCR protocol was validated following the recommended principles and practices for validating clinical molecular pathology tests[2].

Control samples were chosen by different scientist from patients that give their consent. Normal controls were used to amplify a total of 633 amplicons from 79 genes (table 1) of which 574 were not repeated. The result was evaluated as positive or negative for pathogenic variants. For the estimation of the sensitivity, we have also tested 136 different positive controls (known variants) from 50 genes (table 2). Multiple examples of the same variant in the same amplicon will not increase the power of the study to determine sensitivity[2].

Only tested with Normal Control					
% GC	Gene	% GC	Gene	% GC	Gene
34.44	FAT4	40.29	ACTA2	51.23	ARAF
35.08	SHOC2	40.71	KAT6B	51.55	KCNQ1
35.64	VEGFC	42.03	TGFB2	54.88	NOTCH3
35.77	RASA1	42.20	CBL	57.42	MAP2K2
36.35	RASA2	42.69	RAF1	59.05	RRAS
36.37	KRAS	42.80	CCBE1	59.57	KCNH2
37.62	NF1	43.16	PTPN11	60.24	FLT4
37.80	SOS1	43.29	NSUN2	60.45	GATA2
38.00	BRAF	44.09	BRCA1	62.35	GJC2
38.11	BRCA2	44.23	A2ML1	67.48	CDKN1C
38.66	NRAS	44.72	MAP2K1		

Tested with both Normal and Positive Control					
% GC	Gene	% GC	Gene	% GC	
35.10	DYNC2H1	40.00	SPAST	49.96	DCTN1
35.80	DSG1	40.42	ERCC6	50.03	DHX30
35.91	PTEN	40.62	GRIN2B	51.36	BICD2
36.07	ZBTB20	40.81	TGFBR2	53.43	DNMT3A
36.19	ASXL3	41.35	CSNK2A1	54.26	DNM1
38.68	SON	42.17	ASXL1	54.43	AARS2
38.89	KAT6A	42.17	TLK2	54.77	EPHB4
38.92	FBN1	42.34	NSD1	55.28	NFIX
39.13	SETD5	42.48	DIS3L2	55.75	BAP1
39.21	EZH2	43.58	TARDBP	56.44	SLC6A9
39.27	GRIA3	43.80	RIT1	58.39	LZTR1
39.49	NEXMIF	46.19	ARID1A	60.2	KCTD17
39.66	GPC3	48.33	SMAD3	61.99	PIEZO1
39.79	DNAJC3	48.8	OTOG	63.17	FOXC2
39.90	CTNNB1	48.86	TP53	69.01	HRAS
39.99	CHD7	49.02	CHD4		

Table 1: only normal control available for testing; table 2: both normal and positive control tested.

Results were evaluated as positive or negative detection. Positive controls were chosen to represent as broad a range of results as possible including indels, missense, nonsense, and synonymous variants in either exonic or intronic regions. Pathogenic and benign variants were included in the positive group as well as polymorphisms.

Automated Multigene PCR amplification and Sanger sequencing were done in 96 well format using the Biomek Span 8 robot for aliquoting mastermix, primers and DNA. PCR set up and HTD-PCR program are shown in table 3 and table 4 respectively. Then, PCR products were cleaned up using ExoSAP and then sequenced in two directions using oligonucleotide M13 Primers (F

Afterwards, products were cleaned up using the Agencourt CleanSEQ (Beckman Coulter) bead method on the Beckman NXp 96 Multichannel Robot. Sequencing products were eluted into 0.2mM EDTA and loaded onto the ABI3730 for Sanger sequencing.

PCR	Conc.	Vol/tube (uL)
Water		1.5
FastStart mix	2x	7.5
Total		9.0
Primers F+R	5uM	3.0
DNA	30ng/uL	3.0
Total		15.0

Touchdown		
Temp	Time	
95°C	4mins	
95°C	30sec	x 22 Reduce 0.5°C each cycle
65°C	40sec	
72°C	40sec	
95°C	30sec	x 13 cycles
55°C	40sec	
72°C	40sec	
72°C	10mins	
10°C	∞	

Table 3: PCR settings; table 4: HTD-PCR temperature program.

Molecular techniques are highly sensitive with experimental sensitivity often yield 100%. However, it is likely that both false negatives and false positives are found when a number of samples (n) enough big is used. Therefore, we calculate the expected sensibility and sensitivity using Hanley´s simple rule of 3. This rule states that if none of n results shows the event about which we are concerned then we can be 95% confident that the chance of this event is at 3/n. This rule is only applied for n>30[4].

RESULTS

Experimental sensitivity and specificity were 100%. The probability of false positive (FP) was 0.53% and the expected sensitivity >99.5% (95% CI). The probability of false negative (FN) was 2.38% and the expected sensitivity > 97.6% (95% CI) - table 5.

Specificity		Sensitivity	
(n)	568	(n)	126
Experimental	100%	Experimental	100%
3/n	0.0053	3/n	0.0238
FN	0.53%	FP	2.38%
Expected (95% CI)	99.50%	Expected (95% CI)	97.60%

Table 5: results

CONCLUSION

There is no expected significant difference in sensitivity and specificity with the previous method.

Therefore, HTD-PCR is a reliable method in a diagnostic setting that streamline the process of Sanger sequencing saving time thus, money.

REFERENCES

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