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RESEARCH

Setting Up a Probe Based, Closed Tube Real-Time PCR Assay for Focused Detection of Variable Sequence Alterations

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Abstract During diagnostic workflow when detecting sequence alterations, sometimes it is important to design an algorithm that includes screening and direct tests in combination. Normally the use of direct test, which is mainly sequencing, is limited. There is an increased need for effective screening tests, with "closed tube" during the whole process and therefore decreasing the risk of PCR product contamination. The aim of this study was to design such a closed tube, detection probe based screening assay to detect different kind of sequence alterations in the exon 11 of the human c-kit gene region. Inside this region there are variable possible deletions and single nucleotide changes. During assay setup, more probe chemistry formats were screened and tested. After some optimization steps the taqman probe format was selected.

Keywords qPCR · Taqman probe · Simple probe · c-Kit · Mutations · DNA

Introduction

The aim of this study was to set up a closed tube, probe based real-time PCR assay to create a more focused screening test before applying direct sequencing. In the diagnostic workflow it is important to decrease the manual steps and minimize the opening of tubes or wells with amplified PCR product inside. During experiments different oligonucleotide probe based labeling technique were tested using real time PCR method to screen variant sequences more effectively. The target region was the exon 11 of human c-kit gene (4q11–q12). This tyrosine-kinase receptor is frequently altered in various human tumors, especially in GIST (Gastrointestinal Stromal Tumor) [1] Fig. 1. In exon 11 there can be found a broad range of sequence changes. For effective testing the main type of variants was generated using long PCR primers. For detection of sequence changes three different probe formats were tested, such as simple probes, unlabeled probes and taqman probes.

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Materials and Methods

For this purpose the fragment was generated with PCR where the long primers are extended through the overlapping region. The longer primer has the variant region and the shorter one makes the constant end. The controls represent various deletions in different size and combination (Table 2).

Positive Control Design

For the assay optimization there is a need to create a set of positive controls which have different motifs. Analyzing the main alterations of human c-kit, exon 11 region the representative deleted and altered sequences were created (Table 1). The exon 11 sequence between primers was separated into four sub-regions. In any sub region 1 triplet was deleted or the whole sub region was missing. The following combination

Fig. 1 Target sequence of the exon 11 of the c-kit oncogene in the Human genome, chromosome: 4; Location: 4q11-q12

>ref|NC_000004.10|NC_000004:55288339-55288465 Homo sapiens chromosome 4, reference assembly, complete sequence, exon 11 is 127bp long
AAA CCC ATG TAT GAA GTA CAG TGG AAG GTT GTT GAG GAG ATA AAT GGA AAC AAT TAT GTT TAC ATA GAC CCA ACA CAA CTT CCT TAT GAT CAC AAA TGG GAG TTT CCC AGA AAC AGG CTG AGT TTT G

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1741 ACAGAAACCCATGTATGAAGTACAGTGGAAGGTTGTTGAGGAGATAAATGGAAACAATTA
 548 --Q--K--P--M--Y--E--V--Q--W--K--V--V--E--E--I--N--G--N--N--Y

1801 TGTTTACATAGACCCAACACAACTTCCTTATGATCACAAATGGGAGTTTCCCAGAAACAG
 568 --V--Y--I--D--P--T--Q--L--P--Y--D--H--K--W--E--F--P--R--N--R

1861 GCTGAGTTTTGGGAAAACCCTGGGTGCTGGAGCTTTCGGGAAGGTTGTTGAGGCAACTGC
 588 --L--S--F--G--K--T--L--G--A--G--A--F--G--K--V--V--E--A--T--A
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was generated by long PCR: Deletion of the first, the second, the third and the forth sub region individually, deletion of the first and the second sub region together, deletion of the first, second and third sub-region in one, and deletion of all the four sub-regions. Additionally triplet deletions were generated in any sub-region individually and also in combination of 2, 3 and 4 triplet deletions (Fig. 2).

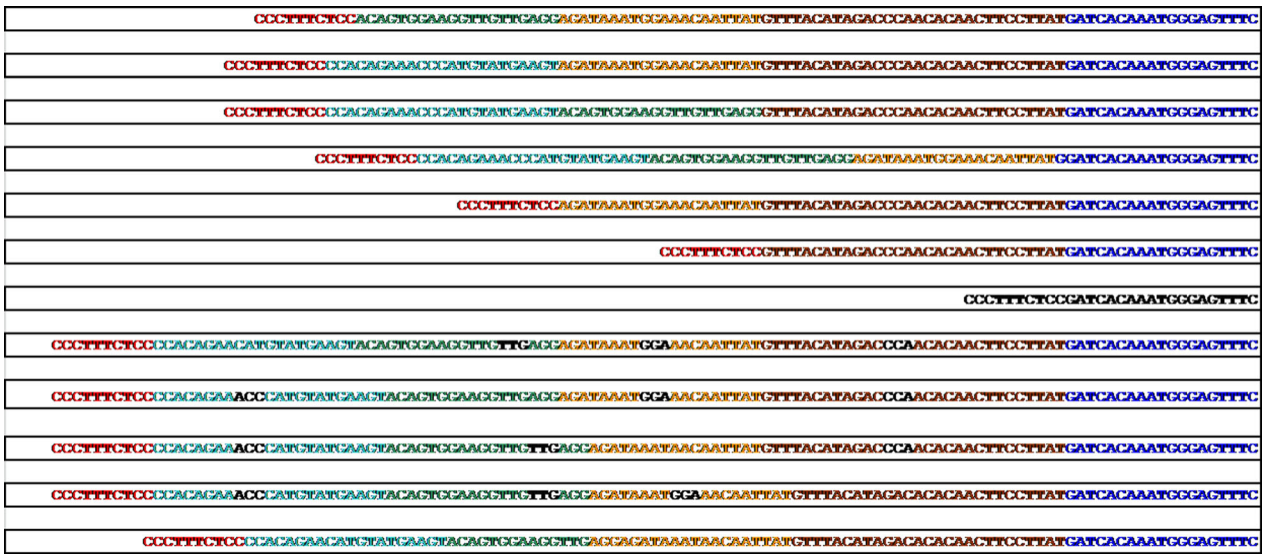
Using this control fragments, three different strategies were used. The primers and the probe sequences were constant. The first set was FAM labeled simple probes [2–5] with different melting temperatures, the second set was FAM-BHQ labeled hydrolysis probes [6, 7] with three different fluorophores and the last two sets were unlabeled probes [8] with the same and also with shorter probes. The four probe sets were the following (Table 2).

The orientations of probe sets on the target sequence were aligned in Table 3.

Real-Time PCR Experiments

The optimized protocol for different detection strategies were the following. For simple probes the Light Cycler 480 genotyping master (Roche diagnostic GmbH, Mannheim) was used with asymmetric primer concentrations. For unlabeled probes the light Cycler HRM master (Roche diagnostic GmbH, Mannheim) was used also with primer asymmetry [9, 10]. Light Cycler Probe Master (Roche diagnostic GmbH, Mannheim) was applied for hydrolysis probe assay. The final reaction volumes were 30 μl, the concentrations of asymmetric primers (Metabion GmbH, Martinsried) were 5 μMol forward and 10 μMol for reverse, in hydrolysis reaction 5 μMol each (Fig. 3). The probes (Metabion GmbH, Martinsried) were applied in 5 μmol concentrations individually in unlabeled and simple probe reactions and 5 μMol each in hydrolysis probe assay, which was a multicolor experiment.

Table 1 c-kit exon 11 variant controls without the constant end. The names are, WT, DEL1, DEL2, DEL3, DEL4, DEL12, DEL123, DEL1234, 1ACC, 2TTG, 3GGA, 4CCA, 1234ACCTTGGGACCA



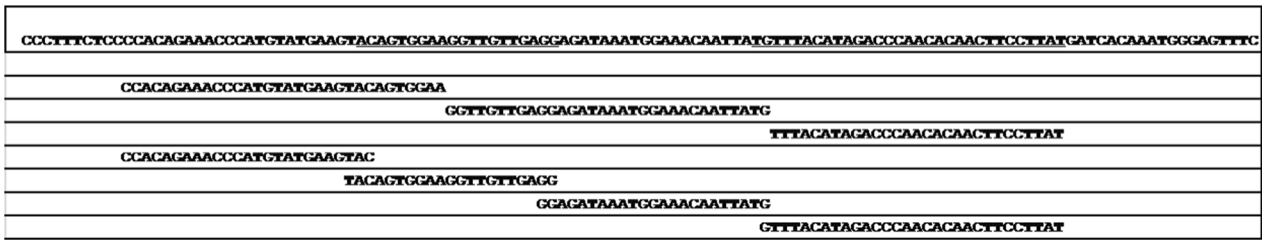
5'-CCCTTTCTCC-CCACAGAAACCCATGTATGAAGT-ACAGTGGAAGGTTGTTGAGG-AGATAAATGGAAACAATTAT-
GTTTACATAGACCCAACACAACTTCCTTAT-GATCACAAATGGGAGTTTC-3'

Fig. 2 Positions of the sub regions and triplet deletions

Table 2 Names, nucleic acid sequences, melting temperatures and detection probes positions in amplicons

Simp1xy	Fluorescein-SPC-CCACAGAAACCCATGTATGAAGTACAGTGGAA-Phosphate	67.2	81-103	(32)
Simp2x	Fluorescein-SPC-GGTTGTTGAGGAGATAAATGGAAACAATTATG-Phosphate	63.9	93-124	(32)
Simp3y	Fluorescein-SPC-TTTACATAGACCCAACACAACTTCCTTAT-Phosphate	63.3	125-153	(29)
tm1x	FAM-CCACAGAAACCCATGTATGAAGTACAGTGGAA-BHQ	67.2	81-103	(32)
tm2x	LC610-GGTTGTTGAGGAGATAAATGGAAACAATTATG-BHQ		63.9	93-124 (32)
tm3x	LC640-TTTACATAGACCCAACACAACTTCCTTAT-BHQ	63.3	125-153	(29)
Up1axy	CCACAGAAACCCATGTATGAAGTACAGTGGAA-Phosphate		67.2	81-103 (32)
Up2ax	GGTTGTTGAGGAGATAAATGGAAACAATTATG-Phosphate		63.9	93-124 (32)
Up3ay	TTTACATAGACCCAACACAACTTCCTTAT-Phosphate	63.3	125-153	(29)
Up1bx	CCACAGAAACCCATGTATGAAGTAC-Phosphate	62.2	61-85	(25)
Up2by	TACAGTGGAAGGTTGTTGAGG-Phosphate	60.8	83-103	(21)
Up3bx	GGAGATAAATGGAAACAATTATG-Phosphate	55.5	102-124	(23)
Up4by	GTTTACATAGACCCAACACAACTTCCTTAT-Phosphate	64.4	124-153	(30)

Table 3 Orientations of detection probes and sub regions. The first row represents the wild type sequence, with marked sub regions



The MgCl2 concentrations were 3 mmol in simple probe and unlabeled probe formats and no additional MgCl2 was given to the probe master. The PCR was generated by Light Cycler 480 system (Roche Diagnostic GmbH, Mannheim) by touchdown protocol [11]. Table 4 shows the thermocycling conditions and detection programs for real-time PCR experiments.

Results

The unlabeled probe based method and the simple probe assay were optimized for lower annealing, because the Tm calling analysis was applied for evaluation. After amplification the melting profile of probes were identified according to the resulted data (Tables 5 and 6 and Fig. 4). Both methods can be used for wild type identification and detection of middle and short deletions with melting temperature shift of probes. The reactions were monocolor for simple probe assays. The unlabeled probes were not able to effectively differentiate between melting temperatures, because the high level of background fluorescence caused by complementarity target re- and denaturation. The simple probes achieved higher signal levels and more effective differentiation ability.

The hydrolysis probe assay was optimized for stringent annealing criteria. Under these circumstances, it was able to identify all kind of produced alterations in the distinct sub-region. For every sub-region a yes or no answer was generated during amplification on a multiplex way, or graphical representation via end point genotyping.

	5' - 3' sequence	Tm (°C)
Forw	GAGTGCTCTAATGACTGA	55
Rew	GAAACTCCCATTTGTGA	53

Fig. 3 Primer sequences for real-time PCR reactions

Table 4 PCR conditions (signal acquisitions are marked with diagonal stripes)

	cycles	denaturation (°C)	t (sec)	extension (°C)	t (sec)	annealing (°C)	t (sec)
simple probe	10	95	10	72	10	68	10
	50					55	
unlabelled probes	10	95	10	72	10	58	10
	50					55	
taqman probes	10	95	10	63			20
	50			62			

Table 5 Amplification and melting results of detection probes (unlabeled probes = UP, simple probes = SIMP, taqman probes = TM). The dark cells represent the altered sequences with modified melting peaks or with lack of amplifications and fluorescence accumulation

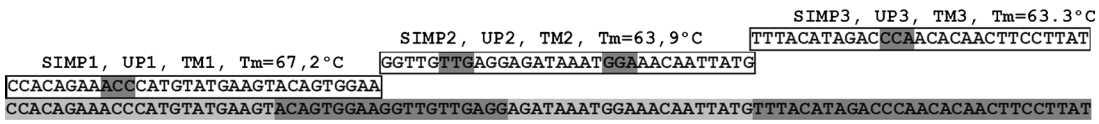
	UP1	SIMP1	TM1	UP2	SIMP2	TM2	UP3	SIMP3	TM3
WT	67,2			63,9			63,3		
DEL1	no amplification signal, no melting peek			63,9			63,3		
DEL2	no amplification signal, no melting peek						63,3		
DEL3	67,2			no amplification signal, no melting peek			63,3		
DEL4	67,2			63,9			no amplification signal, no melting peek		
DEL12	no amplification signal, no melting peek						63,3		
DEL123	no amplification signal, no melting peek						63,3		
DEL1234	no amplification signal, no melting peek								
1ACC	Tm shift 65,4		no amp.	63,9			63,3		
2TTG	67,2			Tm shift 61,4		no amp.	63,3		
3GGA	67,2			Tm shift 60,8		no amp.	63,3		
4CCA	67,2			63,9			Tm shift 61,5		no amp.
1234ACCTTGGGACCA	Tm shift 65,4		no amp.	Tm shift 58,4		no amp.	Tm shift 61,5		no amp.

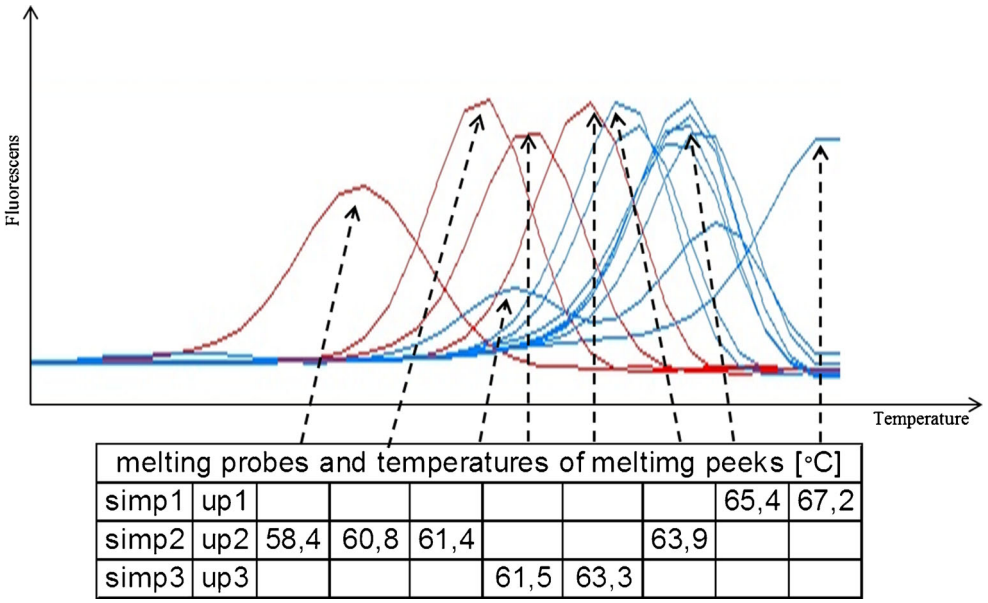
With the use of end point genotyping analysis module the emission signals of 510 nm versus 610 nm and the 510 nm versus 645 nm were evaluated (Fig. 5a, b).

Discussion

For the detection of the above detailed experimentally prepared alterations (short, longer and triplet deletions and also their combinations), the most useful assay was the multicolor hydrolysis probe based assay. This assay is a one tube multiplex solution and there's no need to run more reactions per sample. The needed amount of sample DNA is low and in end point genotyping analysis mode the evaluation process is simple and fast. With this method the type and position of alterations can easily be identified. With melting probes there is a chance to detect qualitatively the lack of single or triple nucleotides specifically, but the evaluation is not robust

Table 6 Probes are on target with triplet positions





melting probes and temperatures of meltimg peeks [°C]									
simp1	up1							65,4	67,2
simp2	up2	58,4	60,8	61,4			63,9		
simp3	up3				61,5	63,3			

Fig. 4 Melting peaks of simple probes in monocolor, monoplex experiment

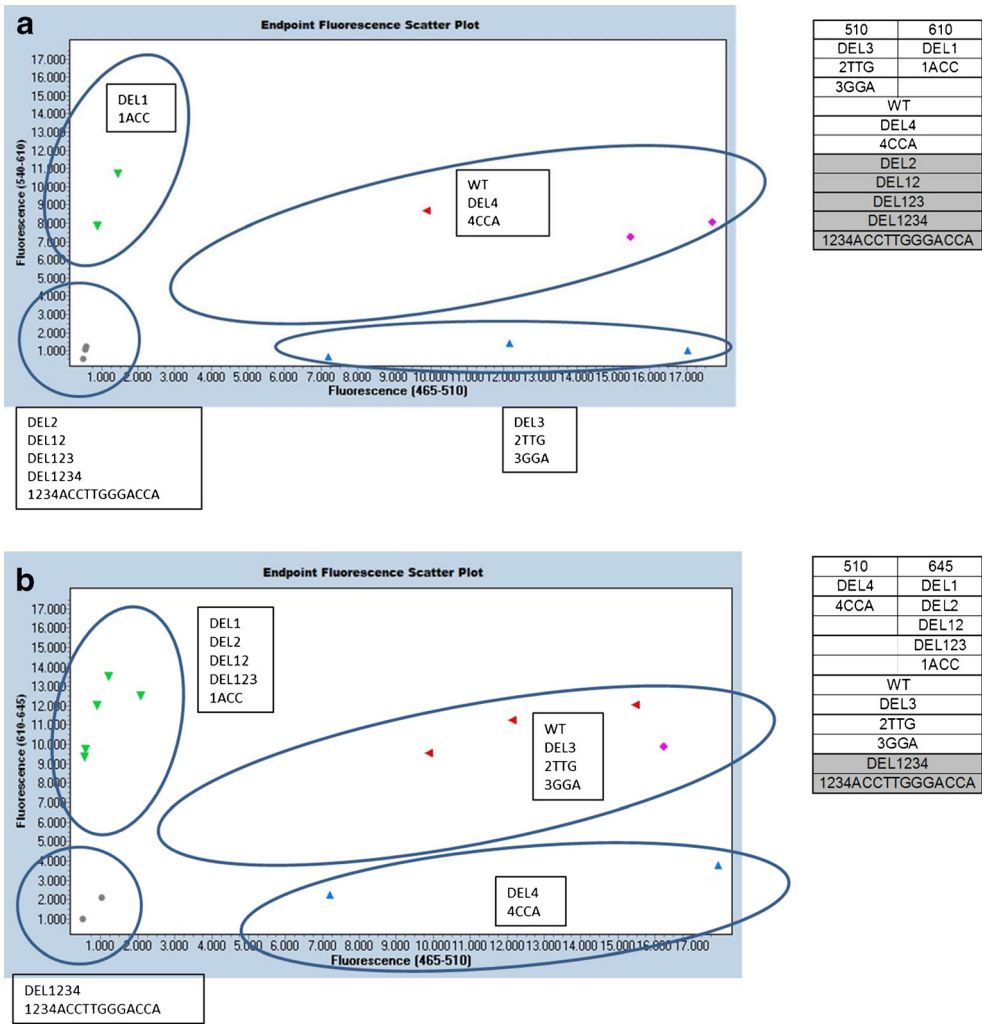


Fig. 5 Evaluation of the taqman probe based detection system with the end point genotyping module of the Light Cycler 480 software. **a** show 510–610 nm results and **b** show 510–645 nm results

because of the possibility of the same or slightly different melting points. The unlabeled probes cannot be multiplexed due to the general labeling strategy using saturated dye. The hydrolysis probe format is not suitable for genotyping itself but with stringent, well optimized amplification it is very reliable and confident.

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