Int J Clin Exp Med 2017;10(7):10411-10421
www.ijcem.com /ISSN:1940-5901/IJCEM0049458

Original Article

A novel asymmetric and competitive allele specific real-time fluorescent PCR with MGB universal probes for detection of SNPs and point mutations

Wei-Qin Jiang[1*], Cong Yan[2*], Yi Zheng[1], Yong-Feng Ding[1], Zhen-Ya Lu[3], Peng Zhao[1,4], Lu-Lu Liu[1], Zhou Tong[1], Wei-Jia Fang[1,4]

[1]Cancer Biotherapy Center, The First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310003, Zhejiang Province, China; [2]Zhejiang University, Zhejiang Province, China; [3]Department of Internal Medicine, The First Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou 310009, Zhejiang Province, China; [4]Key Laboratory of Precision Diagnosis & Treatment for Hepatobiliary & Pancreatic Tumor, The First Affiliated Hospital, College of Medicine, Zhejiang University, Zhejiang Province, China. [*]Equal contributors.

Received January 22, 2017; Accepted May 2, 2017; Epub July 15, 2017; Published July 30, 2017

Abstract: Background: Current technologies for detecting gene mutations have some disadvantages in terms of equipment requirements, flexibility, cost, technical difficulties and assay performance problems including sensitivity, reliability, reproducibility and accuracy. In this study, we developed an asymmetric and competitive allele specific PCR system with minor groove binder (MGB) universal probes (F-CAUM PCR) for detection of single nucleotide polymorphisms (SNP) and point mutations. Methods: By combining characteristics of competitive allele specific PCR (Kompetitive Allele Specific PCR, KASP) and asymmetric PCR, there are three essential components contributing to the performance of this F-CAUM PCR assay, including the unique composition of the forward primers, the ratio between the forward and the reverse primers, and the structure of MGB-labeled universal probes. The 3' ends of the two forward primers are designed complementary to the target sequences of the wild type and the mutant type respectively; each forward primer is comprised of target sequence specific region, universal tag region and the polymerase-binding region. Furthermore, the quantity of forward primers is less than the reverse primer, which minimizes the interference caused by its competitive binding to the universal probe. Lastly, MGB is attached to the quenching molecules, which further improves the assay specificity. We assessed the specificity, sensitivity and reproducibility of F-CAUM PCR. Results: In specificity assay, F-CAUM PCR could identify KRAS G12D mutation with high specificity, without interference by other KRAS mutations at the same locus. In sensitivity assay, the lowest detectable concentration by F-CAUM PCR was 10-1 copies/μl for brain derived neurotrophic factor gene (BDNF) rs6265 and KRAS G12D mutation. The lowest detectable percentage of the KRAS G12D mutation was 1% for KRAS G12D mutation. In reproducibility assay, for detecting the BDNF human genomic DNA standards and the KRAS plasmid mixture, the Ct values and ΔCt values among six replicates showed minimal variance, with standard deviation (SD) ranging from 0.15 to 0.48, and coefficient of variation (CV) ranging from 3.22% to 6.15%. For clinical samples, the genotyping results of BDNF rs6265 in 30 oral epithelium mucosa specimens and KRAS mutation detection in colorectal cancer patients by the F-CAUM assay were consistent with those from Sanger sequencing. Conclusions: Our results confirmed that F-CAUM PCR was highly specific, sensitive and reproducible for SNPs and gene mutation detection. The results of clinical samples by F-CAUM PCR were consistent with those from Sanger sequencing.

Keywords: PCR, SNP, DNA, asymmetric PCR, point mutation, universal probe

Introduction

Gene mutation is the abnormal alteration of genetic material in the genome, including the substitution, insertion and deletion of nucleotide bases in DNA molecules [1-5]. Single nucleotide polymorphism (SNP) is a variation in a single nucleotide, which occurs at high frequency in the human genome [6, 7]. The rapid and accurate identification of gene mutations and SNPs have great applications in human genetics, drug discovery, molecular diagnostics, disease prognosis and personalized medicine [8-10].

Current technologies for detecting gene mutations include Sanger sequencing, Taqman probe method [11], Allele-specific polymerase chain reaction method (AS2-PCR) [12], Universal probe-based real-time PCR and competitive allele specific PCR (Kompetitive Allele Specific PCR, KASP). However, these technologies have certain issues in terms of equipment requirements, flexibility, cost, technical difficulties and assay performance problems including sensitivity, reliability, reproducibility and accuracy [13-15]. Among these technologies, KASP has been established for more than ten years and widely used in gene mutation detection [16, 17]. KASP is a homogenous, fluorescence-based gene mutation detection technology which is based on allele-specific oligo extension and fluorescence resonance energy transfer for signal generation. In brief, in the first round of KASP PCR, two allele-specific forward primers and the single reverse primer are used to amplify PCR product containing the target sequence of interest. Next, the fluorescently labeled universal primer (probe) found in the PCR reaction allows further amplification of PCR product containing both of the target and the universal tag sequence. In the KASP system, the fluorescently labeled primer (probe) is used as a probe as well as a primer. The mutation is thus detected through fluorescent signal emitted by the universal primer (probe). The 3' end of both forward primers are complimentary to the wild type and mutant type respectively, thus the KASP method has relatively high specificity. Apart from these, the KASP system utilizes a universal probe, therefore only primers need to be replaced for detections of other genes. This is not only convenient and fast, but also reduces the assay cost [17]. However, given that the KASP system is a continuous relay reaction, it is inevitable that two PCR systems mutually interfere with each other's amplifications, which eventually affect the performance of the KASP assay [18, 19].

To address these problems, we sought to develop an asymmetry and competitive allele specific PCR system using MGB probes by combining the KASP method and characteristics of asymmetric PCRs [20-23], which is named as F-CAUM PCR herein. The novel F-CAUM PCR reported here is fast, simple, with low cost, and offers high specificity and high sensitivity. In the current study, we further validated the specificity, sensitivity and reproducibility of F-CAUM PCR method, as well as its application in clinical specimens.

Materials and methods

DNA samples

DNA standards were human genomic DNA standards of BDNF rs6265, KRAS wild-type (12G) plasmid and six types of KRAS mutant plasmids (G12D, G12A, G12V, G12S, G12R, G12C), provided by Department of molecular biotechnology, School of medicine, Zhejiang University. The test samples used for BNDF genotyping were oral epithelium mucosa specimens obtained from 30 volunteers. The clinical samples for KRAS mutation detection were 30 paraffin-embedded specimens obtained from pathologically confirmed colorectal cancer patients with consents (First affiliated hospital of Zhejiang University).

Primers and probes

All primers and probes were designed using Primer Premier 5 (**Table 1**) and synthesized by Shanghai Bioligo Co., Ltd (China). For BDNF gene, two forward primers were against different rs6265 genotypes respectively; for KRAS gene, the forward primer of the mutant was against KRAS G12D mutation; the forward primer for the wild type was against the upstream conserved sequence.

PCR and result interpretations

All PCR conditions are shown in the Supplementary 1. The pre-amplifications products were then used as DNA templates of F-CAUM PCR amplification. The fluorescent signals were analyzed through real-time PCR software, and ΔCt value was calculated (ΔCt=Ct2-Ct1) for result interpretation. The interpretation standards are shown in Supplementary Table 1.

Specificity assay

We assessed whether the existence of other KRAS mutations will interfere with the identification of the KRAS G12D mutation in F-CAUM PCR. The KRAS G12D mutant-type plasmid (10^6 copies/μl) was mixed with the KRAS wild-type plasmid and other five KRAS mutant-type plasmids (KRAS G12D, G12A, G12V, G12S,

Table 1. Summary of primers and probes

Purpose of primers	Name	Sequence (5'-3')
F-CAUM PCR universal probes	QFAM-MGB	FAM-CACTCCTCTGGTCCTGC-MGB
	QVIC-MGB	VIC-GTGCTCTCCAGGGACTC-MGB
BDNF gene specific primers for pre-amplification	BDNF-F	AAACATCCGAGGACAAGGTG
	BDNF-R	AGAAGAGGAGGCTCCAAAGG
KRAS gene specific primers for pre-amplification	KRAS-F	AATGACTGAATATAAACTTG
	KRAS-R	TGAATTAGCTGTATCGTCAAGGC
BDNF gene specific primers for F-CAUM PCR	BDNF-QW (wild-type) (Channel 1, FAM)	ACACCCACTT-CACTCCTCTGGTCCTGC-TTGGCT-GACACTTTCGAACATG
	BDNF-QM (mutant-type) (Channel 2, VIC)	ACACCCACTT-GTGCTCTCCAGGGACTC-TTGGCT-GACACTTTCGAACATA
	BDNF-QR	GGTCCTCATCCAACAGCTCT
KRAS gene specific primers for F-CAUM PCR	KRAS-QW (wild-type) (Channel 1, FAM)	ACACCCACTT-CACTCCTCTGGTCCTGC-ACTTGTGG-TAGTTGGAGCTA-G
	KRAS-QM (wild-type) (Channel 2, VIC)	ACACCCACTT-GTGCTCTCCAGGGACTC-ACTTGTGG-TAGTTGGAGCTA-T
	KRAS-QR	TGAATTAGCTGTATCGTCAAGGC (same as the reverse primer for KRAS pre-amplification)

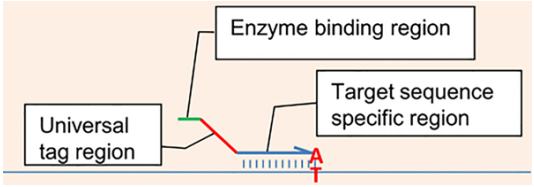

Figure 1. The composition of forward primers in F-CAUM PCR. Each forward primer comprises three regions: target sequence specific region (blue region), universal tag region (red region) and polymerase binding region (green region) from 3' end to 5' end.

G12R, G12C) individually, and each was used as the DNA template for KRAS G12D mutation detections. The KRAS wild-type plasmid was used as the negative control, and the KRAS G12D mutant-type plasmid was used as the positive control.

Sensitivity assay

Human genomic DNA standards for heterozygous BDNF rs6265 were diluted at various concentrations (10^3, 10^2, 10^1, 10^0, 10^{-1}, 10^{-2} copies/μl) for rs6265 genotyping; KRAS wild-type plasmid and mutant-type plasmids (G12D, G12A, G12V, G12S, G12R, G12C) were diluted in the same way for KRAS G12D mutation detection. Next, KRAS wild-type plasmid and the mutant-type G12D were mixed at various ratios used as DNA templates for F-CAUM PCR, with the final percentage of KRAS G12D plasmid at 0.1%, 1%, 3%, 6%, 10%, 15%, 50% and 100%. The assays were repeated three times for each DNA template. Then all replicates per concentration were analyzed by probit regression.

Reproducibility assay

We used the BDNF human genomic DNA standards at 10^3 copies/μl and 10^4 copies/μl respectively, as well as the KRAS plasmid mixture of equal wild-type copies/μl and G12D mutant-type plasmid at 10^4 copies/μl as the DNA templates. The assays were repeated six times for each DNA template. The interassay variation was assessed.

Clinical application and agreement with regular method

We detected BDNF rs6265 in 30 oral epithelium mucosa specimens and KRAS mutation in 30 colorectal cancer patients by F-CAUM assay. Meanwhile, we detected pre-amplification PCR product of all the 60 samples by Sanger sequencing. Then, agreement of two methods was analyzed.

Sanger sequencing

The pre-amplification product were purified using the PureLink Quick Gel Extraction Kit (Invitrogen, USA) and sequencing reactions were performed in the automated sequencer ABI PRISM 3730XL (Applied Biosystems, USA) using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The Sanger sequencing results were analyzed by BLAST (Basic Local Alignment Search Tool) (http://

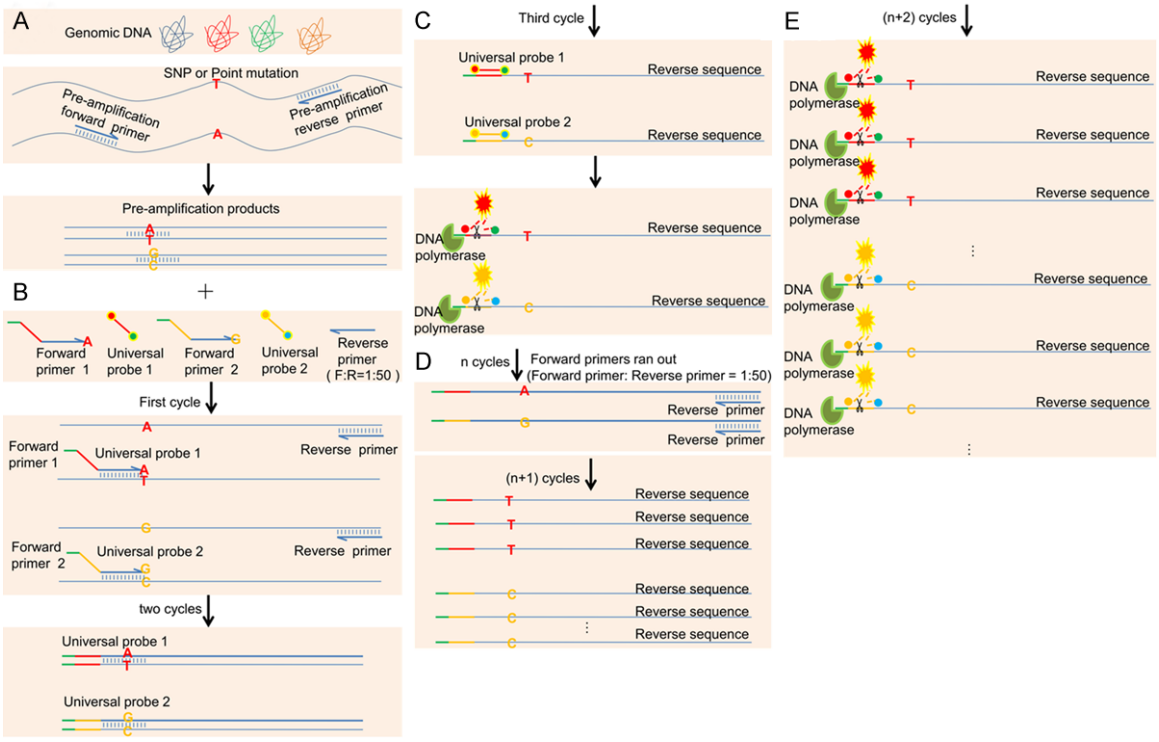

Figure 2. Principle of F-CAUM PCR. A: Pre-amplification. B: PCR product withsequence tags (red region or yellow region) complementary to universal probes and sequence tag (green region) complementary to polymerase was made after two cycles. C: Fluorescent signal was produced for the first time. D: Large quantity of reverse F-CAUM PCR product was made in the late cycles. E: Large quantity of fluorescent signal was produced in the later cycles.

blast.ncbi.nlm.nih.gov/Blast.cgi) and manual review of chromatograms.

Statistical analysis

Statistical analyses were performed with the IBM SPSS Statistics 19.0. Probit regression was performed to determine the limit of detection. Differences between groups were assessed using the U-test. Correlation and linear regression were calculated by Pearson t-test with 95% CI. The significance level was set at $P<0.05$.

Results

Principle of F-CAUM PCR

Each forward primer comprises three regions, including target sequence specific region, universal tag region and polymerase binding region (**Figure 1**). The target sequence specific region is near the 3' end and complementary to the target sequence (wild type and mutant type respectively) with a length of 18-28 base pairs (bp); the universal tag region in the middle has a length of 15-25 bp and contains the same sequence as that of universal probe in order to generate a universal probe binding sequence in the asymmetric PCR amplification product; the polymerase binding region is near the 5' end with a length of 5-25 bp to generate polymerase binding site for breaking down quenching molecules downstream and emitting fluorescent signal.

For F-CAUM PCR, firstly pre-amplification was carried out to generate short PCR product containing the target sequence (SNP or point mutation) (**Figure 2A**). The pre-amplification product were then used as the DNA template for F-CAUM PCR, with two gene specific forward primers, one reverse primer, two universal probes labeled with different fluorophores and polymerase with exonuclease activity added to the reaction. Sequence tags complementary to universal probes and sequence tag complementary to polymerase were added to the sequence of new PCR product after two cycles (**Figure 2B**). The universal tag region in the 5' end of the reverse sequence of new PCR prod-

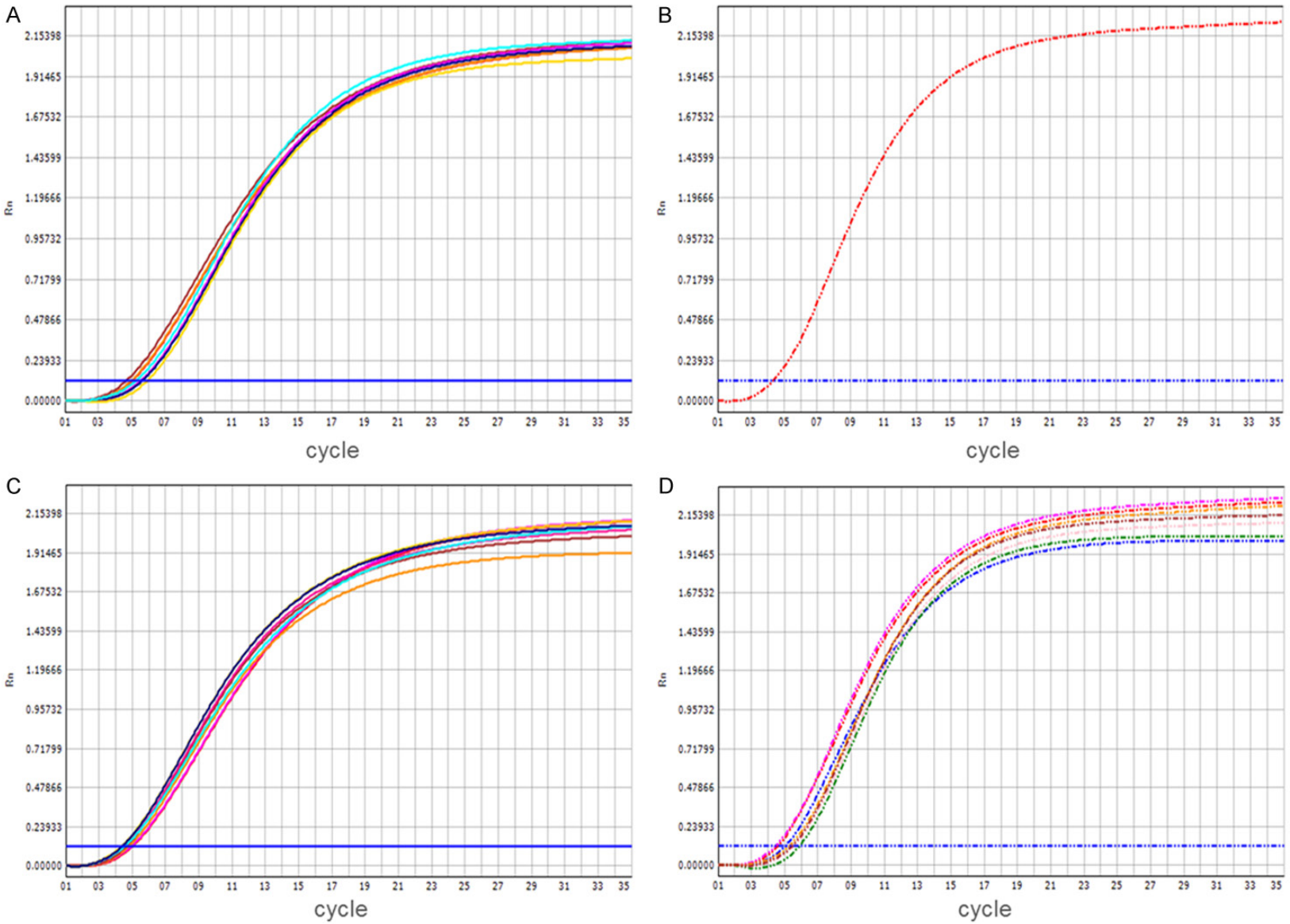

Figure 3. Specificity assessment of F-CAUM PCR for detecting KRAS G12D mutation. The fluorescence curves of F-CAUM PCR were detected for KRAS G12D mutation in 7 kinds of individual KRAS recombinant plasmids in reference gene channel (A) and KRAS gene channel (B). The fluorescence curves of F-CAUM PCR were detected for KRAS G12D mutation in analog hybrid plasmid in reference gene channel (C) and KRAS gene channel (D).

Table 2. Sensitivity assessment of F-CAUM PCR for detecting BDNF rs6265 and KRAS G12D mutation

Gene		Concentration of series dilution								LOD
BDNF genomic standards	Concentration (copies/ul)	10^3	10^2	10^1	10^0	10^{-1}	10^{-2}			10^{-1}
	PCR result	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Homozygotes			
Mutant KRAS 12Asp (D) plasmid	Concentration (copies/ul)	10^3	10^2	10^1	10^0	10^{-1}	10^{-2}			10^{-1}
	PCR result	Mutant	Mutant	Mutant	Mutant	Mutant	Wild-type			
Wild-type KRAS 12Gly (G) and mutant KRAS 12Asp (D) plasmid mixture	Mutation ratio (Concentratios are 10^6 copies/ul)	100%	50%	15%	10%	6%	3%	1%	0.1%	1%
	PCR result	Mutant	Mutant	Mutant	Mutant	Mutant	Mutant	Mutant	Wild-type	

LOD, limit of detection.

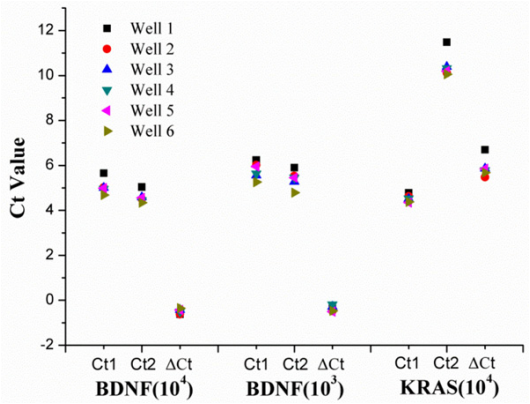

Figure 4. Data distribution of reproducibility assessment on F-CAUM PCR for detecting BDNF and KRAS genes. For detecting the BDNF human genomic DNA standards at 10^3 copies/μl and 10^4 copies/μl respectively, as well as the KRAS plasmid mixture at 10^4 copies/μl six times, the Ct values of Channel 1 and Channel 2 and ΔCt values of Channel 1 and Channel 2 among six replicates showed minimal variance.

uct can identify the fluorescently labeled universal probe; DNA polymerase binds to its upstream to execute its exonuclease activity, enabling the emission of the fluorescent signals (**Figure 2C**). The ratio of the forward primers and the reverse primers is 1:50. At earlier PCR phase, both forward and reverse primers are still abundant, the intensity of the fluorescent signal increases exponentially. Then, gradually the forward primers will ran out and there are still plenty of reverse primers in the later phase of PCR, which will amplify large quantity of PCR amplicons containing the reverse sequence (**Figure 2D**). The fluorescently labeled universal probe will identify and bind the reverse sequence, with DNA polymerase bound upstream to execute its exonuclease activity, enabling emission of large quantity of fluorescent signals (**Figure 2E**). As only PCR amplicons containing the reverse sequence will increase, accordingly it leads to a linear increase of the fluorescent signals.

Specificity assay

In assay of assessing the specificity of F-CAUM PCR, only plasmids with KRAS G12D mutation showed standard fluorescent curve in the assay, indicating that F-CAUM PCR method could identify KRAS G12D mutation with high specificity (**Figure 3A** and **3B**). In the assay of assessing other KRAS mutations interference, it showed strong fluorescent signals for all six DNA templates, but no signal for the negative control, indicating that the detection KRAS G12D mutation by the F-CAUM assay was with high specificity, without interference by other KRAS mutations at the same locus (**Figure 3C** and **3D**).

Sensitivity assay

For detecting BDNF rs6265 and KRAS G12D mutation, the fluorescent signals were detectable when the DNA template concentration was at 10^{-1} copies/μl but not at 10^{-2} copies/μl. So, the lowest detectable concentration by F-CAUM PCR was 10^{-1} copies/μl. The fluorescent signals were detectable when the KRAS G12D percentage was at 1% but not at 0.1%. So, the lowest detectable percentage of the KRAS G12D mutation was 1% for determining KRAS G12D mutation status using F-CAUM PCR (**Table 2**). Thus, FQ-CAUM PCR has high sensitivity and can be used for detection of SNPs and point mutations.

Reproducibility assay

For detecting the BDNF human genomic DNA standards at 10^3 copies/μl and 10^4 copies/μl respectively, as well as the KRAS plasmid mixture (mixed with equal amount of the wild-type and G12D mutant-type plasmid at 10^4 copies/μl) six times, the Ct values and ΔCt values among six replicates showed minimal variance (**Figure 4**), with standard deviation (SD) ranging from 0.15 to 0.48, and coefficient of variation (CV) ranging from 3.22% to 6.15% (**Table 3**). Our results suggest that F-CAUM PCR assays are highly reproducible.

SNP genotyping and KRAS mutation detection in clinical samples

The genotyping results of BDNF rs6265 in 30 oral epithelium mucosa specimens by the F-CAUM assay were consistent with those from Sanger sequencing (**Table 4**). For KRAS mutation detection in colorectal cancer patients, 6 out of 30 specimens were found to be KRAS G12D by F-CAUM PCR, in good agreement with the results from Sanger sequencing. However, among the 24 KRAS wild-type specimens identified by F-CAUM PCR, only 19 were validated by Sanger sequencing. Moreover, Sanger sequencing detected additional five KRAS mutations in

Table 3. Data analysis of reproducibility assessment on F-CAUM PCR for detecting BDNF and RAS genes

DNA template	Channel	Mean Ct	SD*	CV* (%)	Results
BDNF genomic standards (10^4 copies/μl)	1	5.05	0.29	5.81	Heterozygous
	2	4.58	0.22	4.81	
	Ct1-Ct2	-0.49			
BDNF genomic standards (10^3 copies/μl)	1	5.78	0.33	5.64	Heterozygous
	2	5.40	0.33	6.15	
	Ct1-Ct2	-0.38			
Equivalent wild-type KRAS 12Gly (G) and mutant KRAS 12Asp (D) plasmid mixture (10^4 copies/ul)	1	4.53	0.15	3.22	Mutant
	2	10.43	0.48	4.65	
	Ct1-Ct2	5.90			

*SD, standard deviation; CV, coefficient of variation.

Table 4. Summary results of BDNF rs6265 genotyping in 30 oral epithelium mucosa specimens by F-CAUM PCR

Technology/Results		F-CAUM PCR			Total (n)
		Wild-type	Heterozygous mutant-type	Mutant-type	
Sanger sequencing	Wild-type	22	0	0	22
	Heterozygous mutant-type	0	6	0	6
	Mutant-type	0	0	2	2
Total (n)		22	6	2	30

Table 5. Summary results of KRAS G12D detecting in 30 colorectal cancer specimens by F-CAUM PCR

Technology/Results		F-CAUM PCR		Total (n)
		Wild-type	G12D mutation	
Sanger sequencing	Wild-type	19	0	19
	G12D mutation	0	6	6
	Other mutation	5	0	5
Total (n)		24	6	30

exon 12, including four specimens with KRAS G12V and one with KRAS G12S (**Table 5**). This demonstrates that F-CAUM PCR not only has the same accuracy as Sanger sequencing but also identify point mutations with high specificity, and will not produce false positive results due to other types of point mutations.

Discussion

In this study, we developed an asymmetric and competitive allele specific PCR system with MGB probes by combining the KASP method and characteristics of asymmetric PCRs. This novel F-CAUM PCR is fast, simple, and offers high specificity and high sensitivity. There are three essential components contributing to the performance of F-CAUM PCR reported in this study. These include the unique composition of the forward primer, the ratio between the forward and the reverse primer, and the structure of MGB-labeled universal probes. These particular designs greatly improved the specificity and sensitivity of F-CAUM PCR, which can be applied to real-time, accurate identification of SNPs and point mutations.

First of all, each forward primer in F-CAUM PCR comprises three regions, target sequence specific region, universal tag region, and a polymerase binding region. The KASP and other traditional probe-based real-time PCR system only contain target sequence specific region and universal probe region. Furthermore, the target sequence specific region in the forward primer is complementary to that of the probe in these systems, thus the fluorescently labeled universal probe binds to the 5' end of the forward primer and is broken down by the DNA polymerase carried by the reverse primer. This leads to non-specific exonuclease activity on the universal probe by the reverse primers and generate large amount of non-specific signal. In contrast, the unique composition of the forward primer in F-CAUM PCR can avoid these non-specific activities and greatly improves the assay specificity. In brief, the forward primer of

F-CAUM PCR contains a universal tag region that generates a universal probe binding site in the reverse sequence of new PCR product. Moreover, a polymerase-binding region is added to the 5' end of the forward primer, upstream of the universal probe tag, generating a DNA polymerase binding site in the reverse sequence of new PCR product. Thus, fluorescently labeled universal probes will bind to the 5' end of the reverse sequence of new PCR product only, and DNA polymerase will bind to the polymerase-binding site upstream. Then the fluorescently labeled universal probes can be broken down specifically by the 5' to 3' exonuclease activity of the DNA polymerase, allowing unquenched emission of fluorescence.

Secondly, in F-CAUM PCR, the molar ratio of the forward and the reverse primers ranges from 1:10 to 1:1000 with an optimal ratio at 1:50. Therefore, the forward primers will be exhausted at the later phase of PCR, and the reverse primers will continue amplify large quantities of PCR amplicons containing the reverse sequence. As such, there are only limited amount of PCR product with forward sequence in the reactions, which will minimize the interference due to competitive binding to the fluorescent probe by the forward primers and PCR amplicons with the forward sequence. Therefore, the fluorescently labeled universal probes could bind to the PCR amplicons containing reverse sequence with high specificity. This design successfully minimizes the interference caused by competitive binding with other primers and amplicons as seen in the traditional methods and greatly enhanced the sensitivity of probe quenching.

Lastly, the structure of MGB-labeled universal probes was further optimized in order to achieve high specificity. The 15-25 bp oligonucleotide probes were labeled with double fluorescence groups, with fluorophores such as FAM/VIC/ROX/CY5 at one end, and MGB attached to the quencher molecule at the other end. A fluorescent signal is emitted when the oligonucleotide is broken down by the polymerase binding to its 5' end upstream. In the KASP system, the fluorescently labeled universal probes and the quenching probe are two kinds of complementary oligonucleotide chains, and the universal probe functions as a primer as well. The quenching probe can only be broken down in the second round of PCR that uses the universal probe as a primer. However, KASP is a continuous relay PCR, and the mutual interference of two PCR systems is inevitable affecting the performance of both systems. One solution is to perform one-step PCR with two sets of primers. However, this is technically challenging, as primer concentrations need to be optimized and the assay has high failure and error rates. The other approach is to carry out the assay step by step; amplification product of the first round PCR are purified before the second round PCR. However, this is very tedious, and the primers may bind to the fluorescent probe and lead to non-specific signals in the earlier phase of the assay. The unique structure of MGB-labeled probes in F-CAUM PCR enables a single amplification system that has less interferences and ensures high assay accuracy. Meanwhile, the quenching molecule is labeled with MGB, which offers the advantages of low fluorescence background, high resolution and specificity [24, 25], further improves the specificity of F-CAUM PCR.

Compared with other mutation detection technologies, F-CAUM PCR offers the following advantages: 1) Compared with the capillary electrophoresis sequencing, there are no post-PCR and pre-electrophoresis purification steps required for F-CAUM PCR. Assay was performed and mutation detected in real-time quantitative PCR instruments, which is simple and saves time significantly. Furthermore, there is no need to buy fluorescently labeled ddNTPs for capillary electrophoresis sequencing, which further saves cost. 2) Compared with the Taqman probe method, F-CAUM PCR uses two target sequence specific forward primers to identify two genotypes respectively, which enables point-to-point recognition. This ensures the high specificity and sensitivity of F-CAUM PCR. Apart from these, F-CAUM PCR utilizes universal probe. Therefore, there is no need to synthesize and screen new probes for detecting other gene mutations, which saves time and cost. 3) Compared with AS2-PCR, F-CAUM PCR does not need to be assayed in two different tubes. Its simple procedure reduces false positivity and increases assay accuracy. 4) Compared with probe-based real-time fluorescent PCR, the signal detected by F-CAUM PCR are directly correlated with the quantity of specific PCR product. There was minimal interference from non-specific signals, thus the assay is highly accurate. 5) Compared with the KASP

assay, F-CAUM PCR reaction is a single PCR system rather than a complicated relay PCR. Therefore, the signals detected by F-CAUM PCR are only associated with the specific PCR product, without mutual interference due to relay PCR amplifications. Meanwhile, F-CAUM PCR is easily optimized since the ratio of the forward primers and the reverse primers are fixed.

Our results in the clinical samples further demonstrated that F-CAUM PCR was highly specific, sensitive and accurate, thus can be used for SNP genotyping and point mutation detection. In addition, F-CAUM PCR has advantages including high accuracy, short detection period, simple assay procedures and low cost. However, range of the ΔCt values need to determined based on different primers and further optimized for identification of other SNPs and mutations.

Conclusion

F-CAUM PCR system reported here greatly improves the specificity and sensitivity of gene mutation detection, and has advantages including high accuracy, short detection period, simple assay procedures and low cost. Therefore, this novel assay can be further applied to detection of SNPs and point mutations.

Acknowledgements

This work was supported in whole by Natural Science Foundation of Zhejiang Province Y15-H160049. We thank Prof. Lin-Fu Zhou for support of experiment apparatus used in this work. This work was supported by the Natural Science Foundation of Zhejiang Province Y15H160049; the National Natural Science Foundation of China (81472210); the Zhejiang Key Subject of Clinical Medical Engineering (G3221); and the Major Scientific Project of Zhejiang (2016C0-3G1121060).

We have obtained consent to publish from all volunteers and patients to report data from individual volunteer and patient.

Disclosure of conflict of interest

None.

Authors' contribution

Wei-Qin Jiang and Cong Yan designed all primers and probes, conducted most of the experiments, analyzed the results, and wrote most of the paper. Yi Zheng and Yong-Feng Ding conducted Sanger sequencing and searching assay sensitivity Peng Zhao, Lu-Lu Liu and Zhou Tong extracted DNA from samples and plasmids. Wei-Jia Fang conceived the idea for the project, analyzed the results and wrote the paper with Wei-Qin Jiang and Cong Yan.

Abbreviations

PCR, polymerase-chain reaction; BNDF, brain derived neurotrophic factor; MGB, minor groove binder; AS2-PCR, allele-specific polymerase chain reaction; KASP, kompetitive allele specific PCR; KRAS, kirsten rat sarcoma viral oncogene homolog; SD, standard deviation; CV, coefficient of variation.

Address correspondence to: Wei-Jia Fang, Cancer Biotherapy Center, The First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310003, Zhejiang Province, China; Key Laboratory of Precision Diagnosis & Treatment for Hepatobiliary & Pancreatic Tumor, The First Affiliated Hospital, College of Medicine, Zhejiang University, Zhejiang Province, China. Tel: +86-571-87236858; E-mail: fangweijia_tgzy@163.com

References

[1] Ross KA. Coherent somatic mutation in autoimmune disease. PLoS One 2014; 9: e101093.
[2] Poduri A, Evrony GD, Cai X and Walsh CA. Somatic mutation, genomic variation, and neurological disease. Science 2013; 341: 1237758.
[3] Nikiforov YE. Molecular diagnostics of thyroid tumors. Arch Pathol Lab Med 2011; 135: 569-577.
[4] Erickson RP. Somatic gene mutation and human disease other than cancer: an update. Mutat Res 2010; 705: 96-106.
[5] Thomas P and Fenech M. A review of genome mutation and alzheimer's disease. Mutagenesis 2007; 22: 15-33.
[6] Sachidanandam R, Weissman D, Schmidt SC, Kakol JM, Stein LD, Marth G, Sherry S, Mullikin JC, Mortimore BJ, Willey DL, Hunt SE, Cole CG, Coggill PC, Rice CM, Ning ZM, Rogers J, Bentley DR, Kwok PY, Mardis ER, Yeh RT, Schultz B, Cook L, Davenport R, Dante M, Fulton L, Hillier L, Waterston RH, McPherson JD, Gilman B, Schaffner S, Van Etten WJ, Reich D, Higgins J, Daly MJ, Blumenstiel B, Baldwin J, Stange-Thomann NS, Zody MC, Linton L, Lander ES, Altshuler D; International SNP Map Working Group. A map of human genome sequence variation containing 1.42 million single nucleo-

tide polymorphisms. Nature 2001; 409: 928-933.
[7] Sherry ST, Ward MH, Kholodov M, Baker J, Phan L, Smigielski EM and Sirotkin K. dbSNP: the NCBI database of genetic variation. Nucleic Acids Res 2001; 29: 308-311.
[8] Chaudhry AS, Prasad B, Shirasaka Y, Fohner A, Finkelstein D, Fan YP, Wang SG, Wu G, Aklillu E, Sim SC, Thummel KE and Schuetz EG. The CYP2C19 intron 2 branch point SNP is the ancestral polymorphism contributing to the poor metabolizer phenotype in livers with CYP2C19*35 and CYP2C19*2 alleles. Drug Metab Dispos 2015; 43: 1226-1235.
[9] Di Stefano AL, Labussiere M, Lombardi G, Eoli M, Bianchessi D, Pasqualetti F, Farina P, Cuzzubbo S, Gallego-Perez-Larraya J, Boisselier B, Ducray F, Cheneau C, Moglia A, Finocchiaro G, Marie Y, Rahimian A, Hoang-Xuan K, Delattre JY, Mokhtari K and Sanson M. VEGFA SNP rs2010963 is associated with vascular toxicity in recurrent glioblastomas and longer response to bevacizumab. J Neurooncol 2015; 121: 499-504.
[10] Arfaoui A, Douik H, Kablouti G, Ben Chaaben A, Handiri N, Zid Z, Ouni N, Zouiouch F, Ayari F, Mamoughli T, Bouassida J, Abazza H, Harzallaha L and Guemira F. Role of p53 codon72 SNP in breast cancer risk and anthracycline resistance. Anticancer Res 2015; 35: 1763-1769.
[11] Orue A and Rieber M. Optimized multiplex detection of 7 KRAS mutations by taqman allele-specific qPCR. PLoS One 2016; 11: e0163070.
[12] Staiger AM, Ott MM, Parmentier S, Rosenwald A, Ott G, Horn H and Griese EU. Allele-specific PCR is a powerful tool for the detection of the MYD88 L265P mutation in diffuse large B cell lymphoma and decalcified bone marrow samples. Br J Haematol 2015; 171: 145-148.
[13] Sevall JS. Rapid allelic discrimination from real-time DNA amplification. Methods 2001; 25: 452-455.
[14] Livak KJ, Flood SJ, Marmaro J, Giusti W and Deetz K. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic-acid hybridization. PCR Methods Appl 1995; 4: 357-362.
[15] Newton CR, Graham A, Heptinstall LE, Powell SJ, Summers C, Kalsheker N, Smith JC and Markham AF. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). Nucleic Acids Res 1989; 17: 2503-2516.
[16] He C, Holme J and Anthony J. SNP genotyping: the KASP assay. Methods Mol Biol 2014; 1145: 75-86.
[17] Barbano R, Pasculli B, Coco M, Fontana A, Copetti M, Rendina M, Valori VM, Graziano P, Maiello E, Fazio VM and Parrella P. Competitive allele-specific TaqMan PCR (Cast-PCR) is a sensitive, specific and fast method for BRAF V600 mutation detection in melanoma patients. Sci Rep 2015; 5: 18592.
[18] Ertiro BT, Ogugo V, Worku M, Das B, Olsen M, Labuschagne M and Semagn K. Comparison of kompetitive allele specific PCR (KASP) and genotyping by sequencing (GBS) for quality control analysis in maize. BMC Genomics 2015; 16: 908.
[19] Hubacek JA, Pikhart H, Peasey A, Kubinova R and Bobak M. Nobody is perfect: comparison of the accuracy of PCR-RFLP and KASP (TM) method for genotyping. ADH1B and FTO polymorphisms as examples. Folia Biol 2015; 61: 156-160.
[20] Wu L, Di DW, Zhang D, Song B, Luo P and Guo GQ. Frequent problems and their resolutions by using thermal asymmetric interlaced PCR (TAIL-PCR) to clone genes in arabidopsis T-DNA tagged mutants. Biotechnol Biotechnol Equip 2015; 29: 260-267.
[21] Botezatu IV, Nechaeva IO, Stroganova AM, Senderovich AI, Kondratova VN, Shelepov VP and Lichtenstein AV. Asymmetric real-time PCR and multiplex melting curve analysis with TaqMan probes for detecting PIK3CA mutations. Data Brief 2015; 5: 913-917.
[22] Oh JE, Lim HS, An CH, Jeong EG, Han JY, Lee SH and Yoo NJ. Detection of low-level KRAS mutations using PNA-mediated asymmetric PCR clamping and melting curve analysis with unlabeled probes. J Mol Diagn 2010; 12: 418-424.
[23] Gorelov VN, Roher HD and Goretzki PE. A method to increase the sensitivity of mutation specific oligonucleotide hybridization using asymmetric polymerase chain-reaction (PCR). Biochem Biophys Res Communs 1994; 200: 365-369.
[24] Afonina IA, Reed MW, Lusby E, Shishkina IG and Belousov YS. Minor groove binder-conjugated DNA probes for quantitative DNA detection by hybridization-triggered fluorescence. Biotechniques 2002; 32: 940.
[25] Itabashi T, Maesawa C, Uchiyama M, Higuchi T and Masuda T. Quantitative detection of mutant alleles of the K-ras gene with minor groove binder-conjugated fluorogenic DNA probes. Int J Oncol 2004; 24: 687-696.

Supplementary 1. PCR conditions.

All pre-amplification PCR were carried out in a reaction volume of 15 μl containing 1 × rTaq buffer, 0.2 mmol/ul of dNTP mix, 1 U of rTaq polymerase, 5 pmol of each forward and reverse primer, 20 μg of DNA template. Thermocycling conditions for pre-amplifications were as follows: initial denaturalization at 95°C for 5 min, followed by 35 cycles of 95°C for 45 sec, 65°C (-1/cycle) for 45 sec, and 72°C for 45 sec, and a final extension at 72°C for 5 min, 16°C forever. All pre-amplifications were performed in GeneAmp PCR System 9700 (ABI, USA). The PCR reagents including rTaq DNA polymerase, 10 × rTaq buffer and dNTPs were obtained from TaKaRa, Japan.

F-CAUM PCR amplifications were carried out in a 15 μl reaction volume containing 1 × rTaq buffer, 0.2 mmol/ul of dNTP mix, 1 U rTaq polymerase, 1 μl primer mixture of two 0.1 μM forward primers and one 0.5 μM reverse primer, 2 μl pre-amplification product, 1 pmol MGB-FAM Probe and MGB-VIC Probe each. Thermocycling conditions were as follows: initial denaturalization at 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec, 50°C for 30 sec, and 68°C for 30 sec, and a final extension at 68°C for 5 min, and 16°C forever. They were carried out in the SLAN-96P real-time fluorescent PCR machine (Shanghai Hongshi Medical Technology Co., Ltd, China).

Supplementary Table 1. F-CAUM PCR result interpretation standard

SNP/Mutation	Standard			Result
	Channel 1 (wild-type)	Channel 2 (mutant)	ΔCt*	
KRAS G12D point mutation	+	- or +	ΔCt>10	Wild-type
	+	+	ΔCt≤7	Mutant
	+	+	7<ΔCt≤10	Potential mutant
	-	\	\	Repeat DNA extraction
BDNF rs6265 polymorphism	\	\	ΔCt<-2	Mutant
	\	\	ΔCt=-2~2	Heterozygous
	\	\	ΔCt>2	Wild-type

*ΔCt=Ct2-Ct1.