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Optimization of BRAF V600 Assay as A 2-Step Real-time PCR Protocol

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Optimization of BRAF V600 Assay as A 2-Step Real-time PCR Protocol

By

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Abstract

The QClamp® BRAF Codon Specific Mutation Detection Kit is a real-time PCR assay for the detection of somatic mutations in codon 600 Valine at exon 15 in the BRAF gene which encodes the serine/threonine protein kinase, using purified DNA. The V600E mutation is the most common BRAF gene mutation found in human cancers. This mutation leads to production of a BRAF protein that is abnormally active, which disrupts regulation of cell growth and division. Mutations in this gene have been found in cancers, including non-Hodgkin lymphoma, colorectal cancer, malignant melanoma, papillary thyroid carcinoma, non-small-cell lung carcinoma, gastric cancer, and even prostate cancer. Currently, the established qPCR protocol for the QClamp® BRAF Mutation Detection Assay is comprised of a 4-step procedure: *Denaturation, XNA Annealing, Primer Annealing and Extension*. The purpose of this experiment was to test the feasibility of optimizing this assay to a more efficient and faster 2-step Real-time PCR which has just the Denaturation and the Primer Annealing/Extension steps. Optimization was attempted on both the ABI-QS5 and LC480 thermocycling instruments using parallel testing. The newly established 2-step thermocycling parameters were successfully tested and validated on the ABI-QS5 instrument. For the LC480, however, the experiment was not successful. This result might be due to the different platforms and technologies of the two instruments. Further research is needed to develop the mutational status scoring and acceptance criteria for clinical samples on the ABI-QS5, and to complete the development of the 2-step qPCR protocol individually on the LC480, and also, to study the effects of factors such as temperature, ramp rates, PCR enzymes/master mix, primer/probes and XNA concentrations for both ABI-QS5, and the LC480.

KEYWORDS: 2-step PCR, 2-step Real-time PCR, 2-step qPCR protocol, combined annealing/extension protocol, XNA oligomers, BRAF c600 mutation, The QClamp® BRAF Codon Specific Mutation Detection Kit.

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Table of Contents

List of Tables

List of Figures

Introduction

BRAF is a human gene that encodes a protein called B-Raf formally known as serine/threonine-protein kinase B-Raf. This protein is part of a signaling pathway known as the RAS/MAPK pathway which helps transmit chemical signals from outside the cell to the cell's nucleus and regulates cell growth and proliferation. The BRAF protein is also known as 94 kDa B-raf protein; p94; B-raf 1; BRAF1; BRAF1_HUMAN; B-Raf proto-oncogene serine/threonine-protein kinase. The gene, also known as murine sarcoma viral (v-raf) oncogene homolog B1; RAFB1; and v-raf murine sarcoma viral oncogene homolog B, belongs to a class of genes known as oncogenes which have the potential to cause normal cells to become cancerous when mutated. The chromosomal location/cytogenetic location is: 7q34, which is the long (q) arm of chromosome 7 at position 34. The molecular location is: base pairs 140,719,331 to 140,924,764 on chromosome 7 (Genetics Home Reference):

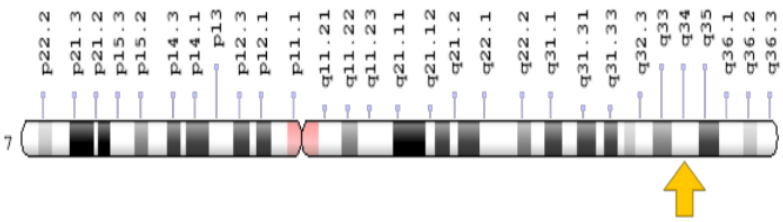


Figure 1: Location of BRAF gene

BRAF V600 is a specific location in the BRAF gene which causes a change in the B-RAF protein. This mutation causes the BRAF protein to be abnormally

active, which disrupts regulation of cell growth and division; therefore, leads to the increase in growth and spread of cancer cells. The V600E mutation is the most common BRAF gene mutation. Approximately 80–90% of BRAF V600 mutations are V600E, which is the result of a single amino acid substitution from valine (V) to a glutamic acid (E) at codon 600 in BRAF gene (My Cancer Genome). This specific mutation along with several other somatic mutations in the BRAF gene are found in different types of human cancers including non-Hodgkin lymphoma, colorectal cancer, malignant melanoma, papillary thyroid carcinoma, non-small- cell lung carcinoma, gastric cancer, and even prostate cancer. Targeting BRAF mutations in tumor tissue using real-time PCR aides in the cancer detection and treatment (Cancer Genetics Web).

Background

QClamp® Technology for Mutation Detection -

The QClamp® BRAF Codon 600 Specific Mutation Detection Kit is a real-time PCR assay for the detection of somatic mutations in codon 600 (Valine) at exon 15 of the BRAF gene using purified DNA. This kit identifies the presence or absence of mutations in the targeted region using the The QClamp® or the xenonucleic acid (XNA) mediated PCR clamping technology but does not specify the exact nature of the mutation. XNA is a synthetic DNA analog with phosphodiester backbone replaced by a novel synthetic backbone chemistry (DiaCarta's proprietary novel uncharged backbone chemistry). XNAs are

designed to hybridize tightly to complementary DNA target sequences with a complete match to wild-type DNA only. XNA oligomers are not recognized by DNA polymerases and cannot be utilized as primers in subsequent real-time PCR reactions. Therefore, specific-target binding of XNA blocks strand elongation by DNA polymerase so when there is a mutation in the target site, hence a mismatch in the sequence, the XNA:DNA binding is unstable causing the XNA probes to fall off allowing amplicon extension by DNA polymerase. Thus, addition of an XNA to a PCR reaction, blocks amplification of wild-type DNA allowing selective amplification of mutant DNA (DiaCarta Inc., QClamp® BRAF Codon Specific Mutation Test in Codon 600 Instruction Manual – Research Use Only). See illustration below:

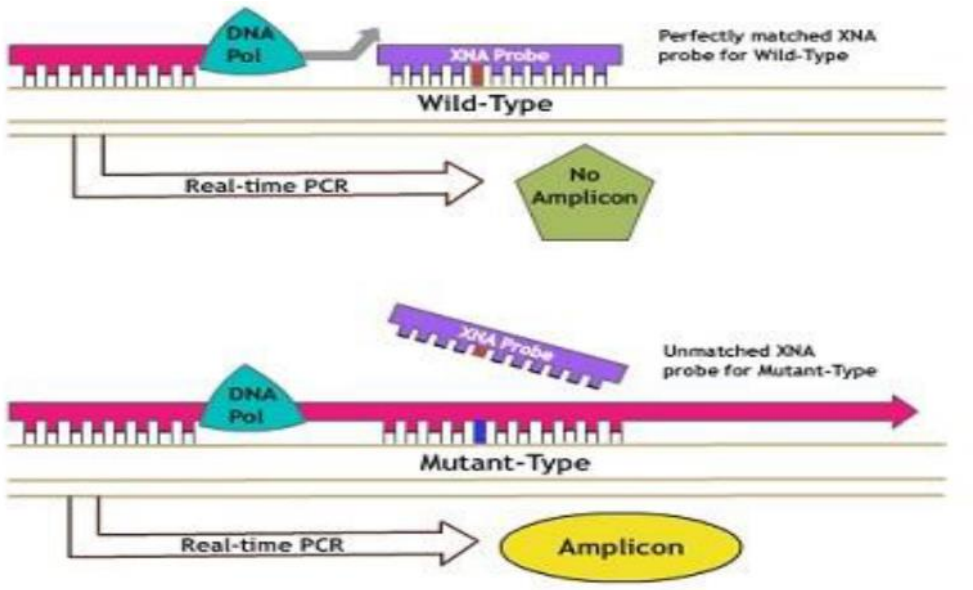


Figure 2: Principle of The QClamp® Technology. The detection kits are designed to detect any mutation at or near the stated codon site without specifying the exact nucleotide change.

(From the *QClamp® BRAF Codon Specific Mutation Test in Codon 600 - Instruction Manual (RUO) MAN.0006 Rev.*

Purpose

The QClamp® BRAF Codon Specific Mutation Test in Codon 600 Real-time PCR (qPCR) assay was developed and validated on several of the ABI instruments and the Roche Light-Cycler platforms using traditional cycling steps: *Denaturation, XNA Annealing, Primer Annealing and Extension*. In this experiment, the focus was to maximize throughput and to optimize this assay to a faster PCR test by using the 2-Step Real-time PCR (qPCR) protocol with just *Denaturation, and Annealing/Extension parameters*.

Materials

The BRAF Codon Specific Mutation Test in Codon 600 Kit reagents were used in this experiment. The assay detects specific changes in Codon 600 as depicted in the Table-1:

Exon	Amino Acid Change	Nucleotide change
15	V600>E	c1799T>A
	V600>K	c1798_1799GT>AA
	V600>D	c1799_1800TG>AT
	V600>R	c1798_1799GT>AG
	V600>M	c1798G>A

Table 1: Listed specific mutational changes in Codon 600 at Exon 15 of BRAF Gene. QClamp® BRAF Codon Specific Mutation Test.

The following tables list all the materials (samples, controls, reagents) in the kit, and the instruments used in the experiment:

Company	Instrument Model
Roche	Light cycler 96
Roche	Light cycler 480 II
ABI	QS5

Table 2: LC96 was used for the Temperature Gradient Analysis. Optimization was performed on both the LC480 and the ABI-QS5.

Assay Components	Volume/Reaction
2X PCR Master mix	5 µl
Primer and probe Mix	2 µl
V600 XNA	1 µl
DNA sample or Controls	2 µl
Total volume	**10 µl**

Table 3: Samples and Reagents used per 10µl total assay volume with 5ng DNA input, and as low as 0.5% mutant allelic frequency.

Reagents	Description	Starting Conc.	Vol. in a 10ul assay	Final Conc.	Test Samples	Description	Starting Conc.	Vol. in a 10ul assay	Final Conc.
2X PCR Master Mix	PCR Master Mix	2x	5ul	1x	DNA template	Undiluted Positive template	2.5ng/ul of DNA	2ul	5ng of DNA input
5X BRAF c600 Primer/ Probe Mix	BRAF V600 Primers and Probes	5x	2ul	1x	DNA template	0.5 % *mutant allele frequency Positive template*	2.5ng/ul of DNA	2ul	5ng DNA input
10X BRAF c600 XNA	BRAF V600 XNA	10x	1ul	1x	CAP samples	Undiluted	2.5ng/ul of DNA	2ul	5ng of DNA input
Controls			Description			Starting Conc.	Vol. in a 10ul assay		Final Conc.
Negative Control (Clamping Control – CC)			Wild-Type DNA Templates			2.5ng/ul of DNA	2ul		5ng of DNA input
Mixed Positive Control (PC)			BRAF c600 Mutant Templates			2.5ng/ul of DNA	2ul		5ng of DNA input
Non-template control (NTC)			Nuclease-Free Water			-	2ul		-

Table 4: Details of specific volume and concentration of reagents and samples used in experiment.

For the purpose of this experiment, all samples including the College of American Pathologist (CAP) proficiency test samples were purified genomic DNA, so the extraction step was not necessary. The BRAF c600 Mutant Templates, which is

the Positive Control (PC) of the assay kit, is pre-made with a 5% mutant allele frequency. The final DNA concentration for each sample used in this experiment was of 5ng DNA input. For the Limit of Detection (LOD) study, a positive sample was prepared with mutant allelic frequency of 0.5%. This was done by diluting the 5% mutant DNA Positive Control (PC) into the Wild-Type (WT) DNA Templates. WT-DNA Templates, also known as the Negative Control or Clamping Control (CC), PCR Master Mix, 5X BRAF c600 Primer/Probe and the 10X BRAF c600 XNA were all obtained directly from the assay kit.

Methods

The current 4-Step with Melt Curve protocol is set as listed in Table – 5 for each assay run on the ABI instruments. Meanwhile, Table – 6 lists the thermocycling parameters set for the Light-Cycler platforms with specific ramp rates:

Steps	Temperature (°C)	Time (Seconds)	Cycles
Preincubation	95	300	1
Denaturation	95	20	X50
XNA Annealing	70	40	
Primer Annealing	66	30	
Extension	72	30	
Melt Curve	Default	-	-

Table 5: Current Applied Biosystems Platforms Thermocycling Protocol.

Steps	Temp (°C)	Time (Sec)	Cycles	Ramp Rate
Preincubation	95	300	1	4.4
Denaturation	95	20	X50	2.2
XNA Annealing	70	40		2.2
Primer Annealing	64	30		2.2
Extension	72	30		1.0
Melting	95	10		4.4
Melting	65	60	1	2.2
	97	1		0.2
Cooling	37	30	1	2.2

Table 6: Current Thermocycling Protocol for the LC96 and LC480 Platforms.

To optimize this assay by reducing the run time for a faster real-time PCR test, a 2-step qPCR protocol (combining annealing and extension into one step) was considered. In general, when using primers with annealing temperatures between 70-72°C such as the ones seen here for XNA, a 2-step thermocycling protocol is possible. Since most polymerases are highly active at the typical primer annealing temperature range of 55-70ºC (Bio-Rad), a single run of the Temperature Gradient Analysis would show the optimal annealing temperature that can be used for this combined protocol. The followings were steps taken to design and validate this 2-step qPCR assay:

Optimization and Validation Steps

1). Temperature Gradient Analysis performed to find the optimum annealing/extension temperature for the assay:

For the BRAF V600 – Gradient Analysis, using the LC96 instrument and the white 96-well PCR plate, a set of samples: one Negative or Clamping Control (CC), and one Positive Control (PC) - BRAF V600 Mutant DNA Templates, were plated from column 1-11 along with a set of two Non-Template Controls (NTC) -

Nuclease-Free Water, plated at the end at column 12 were run. The temperature gradient range was set from 61ºC to 68ºC/30sec.

2). Optimization Experiments performed on both the ABI QS5 and LC480 instruments by running parallel tests:

From the results obtained in the Temperature Gradient Analysis, the optimal temperature selected was 63ºC. The previous 4-step PCR protocol was reduced to a 2-step qPCR protocol as listed in the following Table – 7:

Steps	Temperature (°C)	Time (Seconds)	Cycles
Preincubation	95	300	1
Denaturation (1)	95	30	X50
XNA Annealing *Primer Annealing* *Extension (2)*	63	40	
Melt Curve	Default	-	-

Table 7: Newly established 2-Step qPCR Protocol for the ABI-QS5.

Optimization of PCR was attempted for both the ABI-QS5 and LC480 cyclers simultaneously with same set of CC, PC and NTC samples, all tested in triplicates.

3). Evaluation of Reproducibility, and Assay Precision:

To assess the Intra-Assay Precision, replicate results of the same sample in the Limit of Detection Study were calculated for within-run reproducibility. Inter-Assay Precision for between-runs study was done by observing the replicate

results of the same set of PC, and CC samples tested in separate runs, and on different days using the same instrument, the ABI-QS5. Data were calculated; standard deviation (SD) and coefficient of variation (%CV) values were produced to evaluate assay reproducibility/precision.

4). Limit of Detection (LOD) Test:

Reference Mutant DNA Templates was diluted with the WT DNA Templates (CC) to obtain a Positive Mutant DNA sample of 0.5% mutant allelic frequency at 5ng DNA input. This diluted sample was then run along with a Clamping Control (CC), repeatedly 10 times each for the study of LOD.

5). Accuracy study using CAP samples:

To evaluate the accuracy of our method on the ABI-QS5, a panel of 4 previously tested CAP proficiency testing samples with known results were tested along with the Negative Clamping (CC) and Positive Control (PC), all in duplicates plus 1 Non-Template Control (NTC) using the newly established 2-step qPCR protocol. Results were observed and compared with known values.

Results

In this experiment, the assay generates a cycle threshold (Ct) value for each sample at both the FAM and HEX (the 2 dyes used by the thermal cyclers) channels. FAM dye is used to detect the BRAF amplification of WT and Mutant

DNA whereas the HEX dye is used to detect the Beta Actin gene amplification, which acts as the Internal Control (IC). When assessed using the HEX channel, internal control should make amplicons efficiently for all samples and controls except NTC, providing a way to monitor performance of the primers, probes, polymerase, and sample DNA quality/quantity. Ct is the cycle number at which a signal is detected above the set threshold for fluorescence. The lower the Ct, the stronger the PCR reaction it represents. Since this is a newly developed 2-step qPCR protocol using the QClamp® BRAF Codon 600 Specific Mutation Detection Kit, no set criteria have been established for result interpretation, therefore, the following guidelines were set for the validity of the experiment. These acceptance criteria were derived from previous in-house experiments and studies with this kit and similar assays using the same testing platforms.

Data Analysis

Acceptance Guidelines:

The Ct of the Clamping Control (CC) with each of the mutation reaction mixes should be at least 5 Ct greater than the Ct of the Positive Control (PC) with the same reaction mix, observed in the FAM channel:

$CC_{Ct} – PC_{Ct}$ must be >5.00

This ΔCt value of >5.00 was chosen so that enough variation gap is allowed to distinguish between Negative and Positive samples. In addition, the validity of the assay is assured with the use of the Ct value from the Internal Control (IC) mix. The Ct values for the Internal Control (observed in the HEX channel) should

be in the range of 25< Ct <31 for all samples:

IC_{Ct} should be = 25 < Ct < 31

If these criteria are not met, the PCR assay did not work, and the results are invalid.

Experimental Results

The Temperature Gradient Analysis was successful with a single run on the LC96, and the optimal annealing-extension temperature was established at 63^{o}C based on the highest ΔCt (threshold cycle) value calculated from the BRAF V600 WT, and the BRAF V600 PC results at column 4 (position A4 and B4). Hence the optimal temperature obtained was of the highest ΔCt value **6.37** (Figure – 3 and Table – 8).

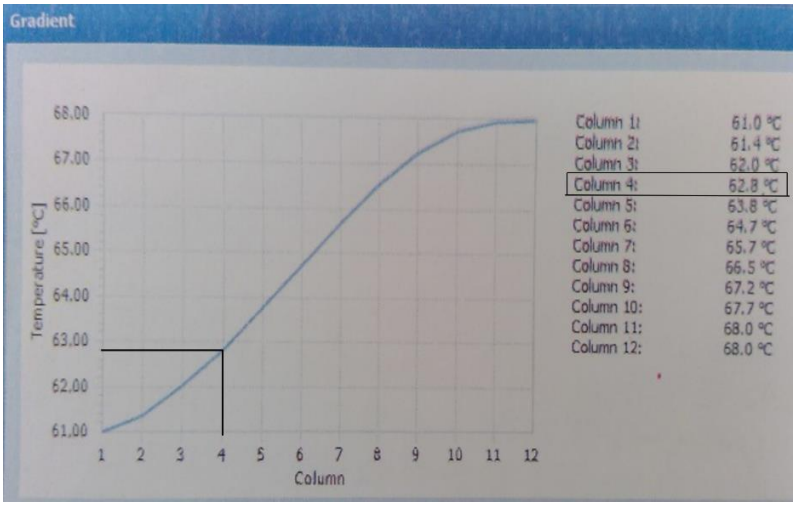


Figure 3: Temperature Gradient Analysis performed on the LC96. Same samples: 1 Positive and 1 Negative Control run repeatedly from column 1 to 11 with temperature range set at 61^{o}C –68^{o}C. Non-Template Control run at column 12). Column 4 at 62.8^{o}C is where highest ΔCt value was detected.

Position	Sample Name	Ct	Dye	Position	Sample Name	Ct	Dye
A1	BRAF V600 WT	36.6	FAM	B1	BRAF V600 PC	31.4	FAM
A1	BRAF V600 WT	25.2	Hex	B1	BRAF V600 PC	26.17	Hex
A2	BRAF V600 WT	35.71	FAM	B2	BRAF V600 PC	30.22	FAM
A2	BRAF V600 WT	25.27	Hex	B2	BRAF V600 PC	26.06	Hex
A3	BRAF V600 WT	36.11	FAM	B3	BRAF V600 PC	31.14	FAM
A3	BRAF V600 WT	25.4	Hex	B3	BRAF V600 PC	26.18	Hex
A4	BRAF V600 WT	37.28	FAM	B4	BRAF V600 PC	30.91	FAM
A4	BRAF V600 WT	25.44	Hex	B4	BRAF V600 PC	25.97	Hex
A5	BRAF V600 WT	35.66	FAM	B5	BRAF V600 PC	29.31	FAM
A5	BRAF V600 WT	25.63	Hex	B5	BRAF V600 PC	25.78	Hex
A6	BRAF V600 WT	35.38	FAM	B6	BRAF V600 PC	30.64	FAM
A6	BRAF V600 WT	25.55	Hex	B6	BRAF V600 PC	25.99	Hex
A7	BRAF V600 WT	35.19	FAM	B7	BRAF V600 PC	30.76	FAM
A7	BRAF V600 WT	25.84	Hex	B7	BRAF V600 PC	26.16	Hex
A8	BRAF V600 WT	37.69	FAM	B8	BRAF V600 PC	31.13	FAM
A8	BRAF V600 WT	26.21	Hex	B8	BRAF V600 PC	26.25	Hex
A9	BRAF V600 WT	39.81	FAM	B9	BRAF V600 PC	30.96	FAM
A9	BRAF V600 WT	26.69	Hex	B9	BRAF V600 PC	26.68	Hex
A10	BRAF V600 WT	-	FAM	B10	BRAF V600 PC	32.07	FAM
A10	BRAF V600 WT	27.2	Hex	B10	BRAF V600 PC	27.16	Hex
A11	BRAF V600 WT	-	FAM	B11	BRAF V600 PC	38.41	FAM
A11	BRAF V600 WT	28.5	Hex	B11	BRAF V600 PC	28.43	Hex
A12	Sample 12	-	FAM	B12	Sample 24	-	FAM
A12	Sample 12	-	Hex	B12	Sample 24	-	Hex

Table - 8: ***TEMPERATURE GRADIENT ANALYSIS DATA*** *- BRAF V600 WT or the Clamping Control (CC) along with Mutant DNA templates (PC) were used. Optimal annealing/extension temperature were obtained from calculating the ΔCt value at each column and selecting the highest value:* ***(A4) CC_{Ct} – (B4) PC_{Ct} = 37.28 – 30.91= 6.37.*** *The internal control (IC) Ct values shown in the Hex channel are =* ***25 < Ct < 31*** *which also serve as a reference for the assay validity for the detection of mutations.*

The optimization experiment was carried out on both the ABI-QS5 and LC480 at first using the 63°C/30sec for annealing and extension parameter. Test results were acceptable but *not* optimal for the ABI-QS5 (ΔCt values were around 5), and there was no DNA amplification observed at all on the LC480.

Since previously tested and validated data obtained in-house shown that usually, the LC480 runs optimally at 2°C lower than the ABI-QS5, the annealing/extension temperature was modified to 61°C for the LC480 on subsequent runs, and the

annealing-extension time was extended to 40 seconds as well. ABI-QS5 was still set at 63^{o}C but with 40 sec annealing-extension time also to optimize assay performance. Different ramp rates and concentrations of BRAF 600 XNA (0.8x and 1.3x) beside the original 1x were also tested in the optimization study. The results showed both the 0.8 and the original 1x concentrations for XNA primers worked comparably well with the new 2-step qPCR protocol on the ABI-QS5 cycler. However, reactions with 1.3x concentration of XNA did not amplify. The revised protocol of 61^{o}C/40sec on the LC480 also resulted in poor amplification even with different ramp rates selected. More time and further research are needed to individually study and optimize the diagnostic performance of the 2-step qPCR protocol on the LC480. The optimization experiment for this thermal cycler was, therefore, unsuccessful. The plan for this experiment was to perform the optimization assay on both the ABI-QS5 and the LC480 simultaneously for Comparison Study. However, optimization on the LC480 could not be completed with the newly established 2-step qPCR protocol. As a result, validation proceeded on the ABI-QS5 only and the results were as followed:

1. Precision:

The acceptance criteria for precision was that ΔCt values obtained satisfied the cut-off value for distinguishing positive from negative mutations, and the range of ΔCt results is acceptable and within ± 2SD (standard deviation) of the mean. Although the %CV is slightly high due to the small set of data, the tested range of

ΔCt values is well within the 95% of Normal Distribution curve as seen in Fig-2 below:

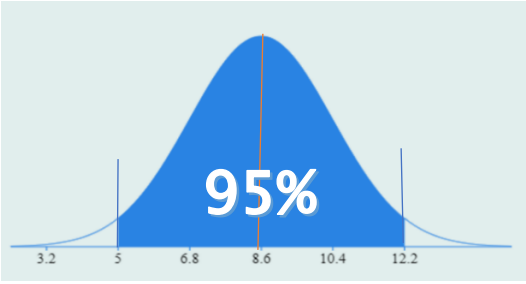


Figure 4: Normal Distribution Curve of ΔCt values between ±2SD. Area (probability) = 0.9545.

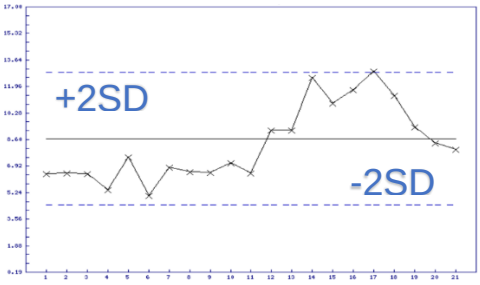


Figure 5: Plot of ΔCt precision data. X-axis = Sample Replicates, Y-axis = ΔCt values (from Table-9). Horizontal central line indicates the mean of the ΔCt values.

PRECISION STUDY	Within-Run	Between-Runs		
Replicates	(ΔCt)	Run #1 (ΔCt)	Run #2 (ΔCt)	Run #3 (ΔCt)
1	6.42	6.46	12.05	8.36
2	6.43	9.16	10.88	7.97
3	6.40	9.15	11.74	–
4	5.41	–	12.90	–
5	7.47	–	11.34	–
6	5.01	–	9.36	–
7	6.80	–	–	–
8	6.54	–	–	–
9	6.48	–	–	–
10	7.10	–	–	–
Test Count:	10	3	6	2
Average:	6.41	8.26	11.38	8.17
SD:	0.73	1.56	1.20	0.28
Total Average = 9.27	Total SD = 1.83		%CV = CV x 100 19.72	

Table 9: List of Precision Study Data. Data and the following formulas were used to calculate Between-Runs Total Mean of ΔCt, Total Standard Deviation (SD), and the Coefficient of Variation (CV) for the Precision Study:

The formula to find the sample mean

$$\mu = \frac{\sum x}{n}$$

Formula to calculate sample standard deviation

$$\sigma = \sqrt{\frac{\sum (x - \mu)^2}{n - 1}}$$

Formula to calculate **coefficient of variation**

$$CV = \frac{\sigma}{\mu}$$

2. Limit of Detection (LOD):

For the evaluation of LOD, reference DNA samples of 0.5% mutant allelic frequency with 5ng DNA input was utilized and run a total of 10 times along with the Clamping Control (CC). All 10 replicates of the diluted reference Mutant DNA

sample were tested positive using the 2-Step qPCR protocol on the ABI-QS5. Therefore, 0.5% mutation frequency can be readily detected at 5 ng DNA input. Further studies will be needed to test if the assay can detect mutations at lower allelic frequencies than 0.5%.

3. Accuracy:

Four CAP specimens, Sample 1: 15A002, Sample 2: 16A054, Sample 3: 16A055 and Sample 4: 16A056 were run in duplicates each, along with a CC, a PC and 1 NTC control samples for accuracy study and clinical evaluation. All IC Ct values in the Hex channel = 25 < Ct < 31, which met the acceptance criteria and validated the assay. CAP results were in concordance with the expected values: S1 and S4 were negative, and S2 and S3 tested positive for BRAF c600 mutant gene.

Based on the "Gaussian Distribution" model and the formula: **Mean ± (1.96 x SD)**, and with the calculated total SD and the ΔCt total average of 9.27 (Table – 9), the mutational status score was calculated as **9.27 - (1.96 x 1.83) = 5.68.** Therefore, the mutational status can be determined as followed:

ΔCt < 5.68 = Positive, and ΔCt ≥ 5.68 = Negative.

From the given data, the average ΔCt value for Positive CAP samples was calculated as 0.29 for S2, and 2.93 for S3 with IC Ct values ranged from 27.30 – 27.84. The ΔCt values total average for Negative CAP samples S1 and S4 were 8.40 and 12.98 respectively. All results met the acceptance criteria set for this experiment (Table – 10).

Sample ID	FAM Channel Ct Values			HEX Channel Ct Values			ΔCt (FAM – HEX)
	Replicate 1	Replicate 2	Average	Replicate 1	Replicate 2	Average	
S1	36.78	38.29	37.54	28.35	29.93	29.14	8.40
S2	28.42	27.44	27.93	27.84	27.44	27.64	0.29
S3	30.32	30.58	30.45	27.73	27.30	27.52	2.93
S4	41.01	40.87	40.94	27.80	28.11	27.96	12.98
CC	38.44	38.25	38.35	27.44	27.52	27.48	10.87
PC	30.08	30.28	30.18	26.99	27.07	27.03	3.15

Table 10: Accuracy Study Results - CAP samples S1, S2, S3, S4, Clamping Control, and Positive Control were all run in duplicates for Accuracy test. The ΔCt values (FAM – HEX) for ***Positive CAP samples (S2, S3) were < 5.68****; for* ***Negative CAP samples (S1, S4) were > 5.68****; and Hex channel shows* ***IC Ct values = 25 < Ct < 31****, all of which met the acceptance criteria set for this experiment.*

Discussion and Conclusion

From this experiment, we can see that the "Proof of Concept" (PoC) study confirmed the feasibility of a 2-Step qPCR program for the ABI-QS5 cycler. The newly established protocol tested and validated successfully on the ABI-QS5 instrument. Accuracy and Sensitivity of the assay were not affected by the changes of the thermocycling parameters. For the LC 480, however, optimization of the 2-Step qPCR protocol did not work even with several modifications of the program including modification in the ramp rates and the annealing/extension time/temperatures.

In conclusion, the new and improved thermocycling parameters can be used as a starting point for further feasibility work for the BRAF Codon 600 Specific Mutation Detection Assay on the ABI-QS5. Although, clinical evaluation with more clinical samples is still needed to develop a more definitive mutational status acceptance criteria for the ABI-QS5. Nevertheless, based on the obtained

results and due to the different platforms and technology between the tested instruments, further research and future experiments are needed to conclude the comparison and optimization of the 2-step real-time PCR protocol on the LC 480. Additionally, a better understanding of the effects of factors such as temperature, ramp rates, PCR enzymes/master mix, primer/probes and XNA concentrations can be further studied and tested out more extensively in future research for the optimization of the 2-step qPCR protocol in general, and for the optimization of the Light-Cycler platforms specifically, since cycling protocol that works on one brand of instrument may not necessarily work on another.

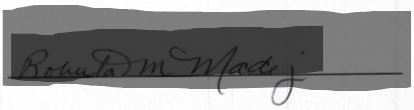
Appendix

CERTIFICATION OF APPROVAL

Research Project

I certify that I have reviewed "OPTIMIZATION OF BRAF V600 ASSAY AS A 2-STEP REAL-TIME PCR PROTOCOL" by Mimi Phuong, and I approve this completed project to be submitted in partial fulfillment of the requirements for the CLS 5700 Course Master's Research Project.

Additional Project Supervisor's Comments:



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