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Determination of the Feasibility of Using Cell-Free DNA (cfDNA) in the Q-Clamp KRAS Codon Specific Mutation Test

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Determination of the Feasibility of Using Cell-Free DNA (cfDNA) in the Q-Clamp® KRAS Codon Specific Mutation Test

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By
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ABBREVIATIONS

µL – microliter

CC – Clamping Control/Negative Control – Wild Type DNA

CE/IVD – CE-Marking, In Vitro Diagnostics

Ct – Cycle Threshold

DNA – Deoxyribonucleic acid

FFPE – Formalin Fixed Paraffin Embedded

MAPK – Mitogen-Activated Protein Kinase

MAPKK – Mitogen-Activated Protein Kinase Kinase

MAPKKK – Mitogen-Activated Protein Kinase Kinase Kinase

ng – nanogram

NTC – No-Template Control – water

PC – Positive Control

PCR – Polymerase Chain Reaction

qPCR – Quantitative Polymerase Chain Reaction

S – Sample

XNA – Xenonucleic acid

ABSTRACT

Context

KRAS is a proto-oncogene which was first identified in Kirsten rat sarcoma virus. The normal KRAS protein performs an essential function in normal tissue signaling and a mutation of a KRAS gene is an essential step in the development of many cancers. Q-Clamp® KRAS codon specific mutation test is an in vitro real-time qualitative PCR assay for the detection of mutations in the targeted regions and KRAS mutation often serves as a useful marker in drug response. The Q-Clamp® KRAS codon specific mutation test experiment was performed using cell-free extracted DNA (cfDNA) instead of FFPE extracted DNA. The results of the experiment showed a clear difference in the Ct's between the mutant and internal control. Since the delta Ct's can be discriminated, the assay showed a positive feasibility of using a cfDNA in the Q-Clamp® KRAS codon specific mutation test.

Objectives

1. Describe KRAS, the specific codons it mutates, and its pathways.
2. Describe the mechanics of the assay, how it works, and its intended use.
3. Describe cell-free DNA (cfDNA) and circulating tumor DNA (ctDNA).
4. Determine the feasibility of using the Q-Clamp® KRAS Mutation Test for cfDNA testing.

Data Sources

This review is based on published, peer-reviewed literature; available information from medical organizations (eg, National Comprehensive Cancer Network, American Society of Clinical Oncology, College of American Pathologists); and information from clinical laboratories conducting KRAS mutation.

ACKNOWLEDGMENT

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But on top of all these, to the Great Almighty, the author of knowledge and wisdom, for His countless love.

Thank you.

Manuel Mina Garcia

I. INTRODUCTION

A. KRAS

KRAS is a proto-oncogene which was first identified in Kirsten rat sarcoma virus (Tsuchida, et al., 1982). The KRAS gene provides instructions for making a protein called K-Ras that is part of a signaling pathway known as the RAS/MAPK pathway (http://www.diacarta.com/products/gene-mutation-detection-kits-2/kras-mutation-test/). The normal KRAS protein performs an essential function in normal tissue signaling and the mutation of a KRAS gene is an essential step in the development of many cancers, according to Kranenburg (2005), including colorectal, lung, thyroid and pancreatic cancers, and colangiocarcinomas. The KRAS gene is in the Ras family of oncogenes, which also includes two other genes: HRAS and NRAS. These proteins play important roles in cell division, cell differentiation, and the self-destruction of cells (apoptosis) (http://www.diacarta.com/products/gene-mutation-detection-kits-2/kras-mutation-test/).

The codons where KRAS clinically relevant mutations are usually observed are within codons 12 and 13 of exon 2, codons 59 and 61 in exon 3, and codons 117 and 146 in exon 4. KRAS mutation will lead to abnormal growth signaling and the alterations in cell growth and division may trigger cancer development because of excessive cell growth and division due to excessive signaling. A KRAS mutation can also serve as a useful prognostic marker response to tyrosine kinase inhibitors and in certain colorectal cancer drugs (Q-Clamp® KRAS codon specific mutation test instruction manual).

B. MAP Kinase (Mitogen-Activated Protein Kinase) Pathway

The RAS proteins such as KRAS, HRAS, and NRAS are small guanosine triphosphatases (GTPases) associated with a number of signal transduction pathways involved in cell cycle progression, cell motility, apoptosis, senescence and other vital functions.

Numerous cell surface molecules activate RAS proteins, which in turn activate MAPK cascades, including RAF, MEK and ERK, a major effector pathway of RAS with a well-described role in cancer. Signaling events initiated by RAS that are transduced through RAF, MEK, and ERK 1 & 2 are transmitted to the nucleus where they regulate transcription factors to modulate gene expression required for cellular proliferation, growth differentiation, transformation, and survival. Dysregulation of key mediators of the MAPK pathway have been implicated as drivers of cancer development. RAS mutations have been identified in both hematologic and solid tumors. KRAS is frequently mutated in cancer of the colon and lungs.

MAPK pathway activation begins at the cell membrane, where small GTPases and various protein kinases phosphorylate and activate MAPKKKs. Subsequently, MAPKKKs directly phosphorylate MAPKKs which, once activated, phosphorylate MAPKs. Activated MAPKs interact with and phosphorylate other cytoplasmic substrates and ultimately modulate transcription factors that drive gene expression. This is turn results in various biological responses like osmotic shock, cell cycle progression, or induction of interferon production (Lifetechnologies.com/mapk).

Figure 1 presents the KRAS mutation wherein the transmembrane receptor which is activated by ligand binding triggers the intracellular signaling cascade resulting in the impact of the gene expression which leads to the cell proliferation that eventually dysregulate the pathway, thus leads to tumor progression.

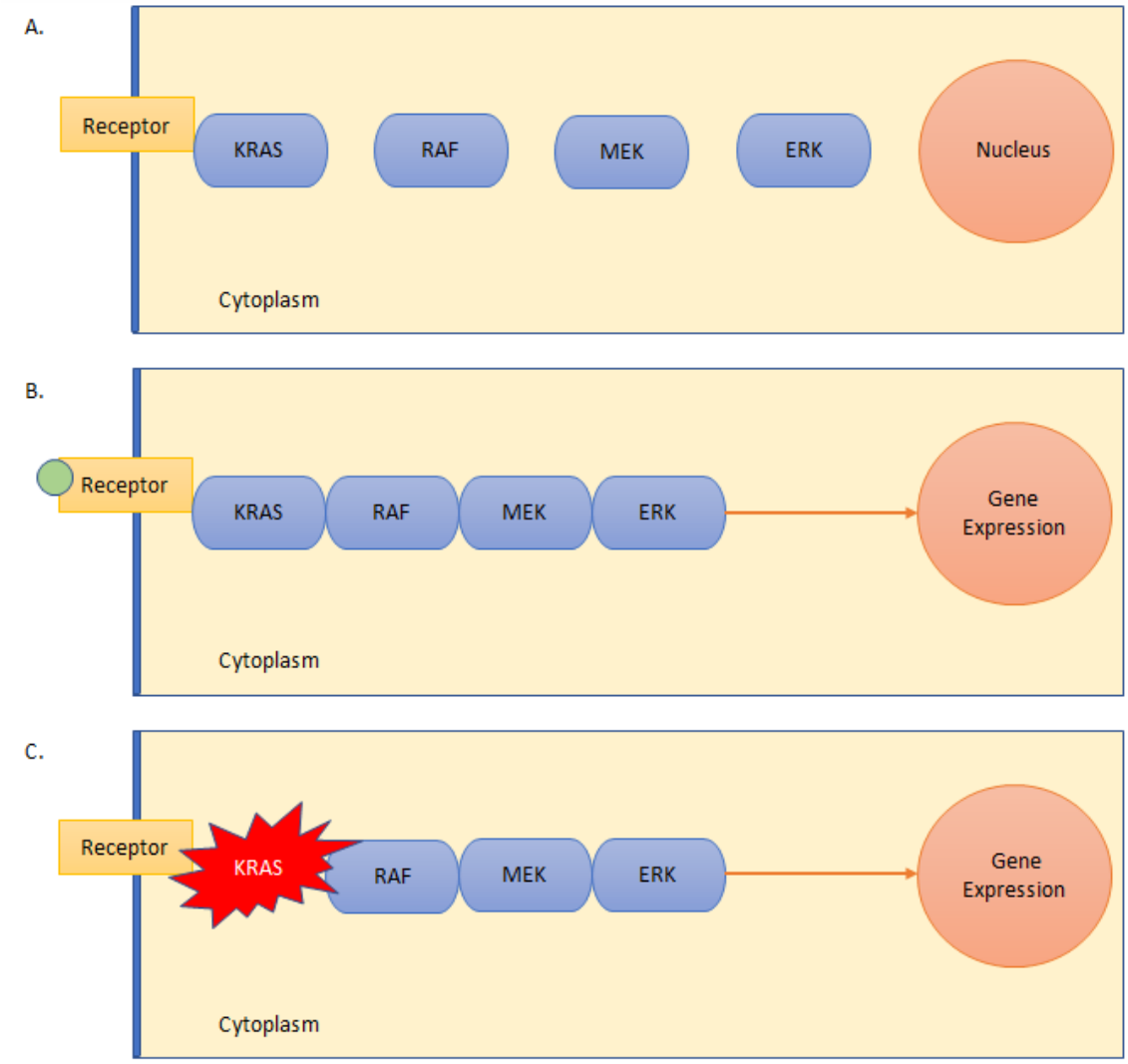

Figure 1. KRAS Mutation. A) In a cell where no mutation is present, the transduction/signaling pathway is shut off. **B)** When growth factors (shown in green) are present and bind to their corresponding receptor, KRAS protein is activated; downstream proteins carry the signal to the nucleus and activate gene transcription. **C)** In a cell where KRAS is mutated, the transduction pathway is active even without the presence of growth factor.

C. Q-Clamp® KRAS Codon Specific Mutation Test

The Q-Clamp® KRAS Codon Specific Mutation Test (DiaCarta, Inc, Richmond,CA) is a CE marked in vitro diagnostic real-time qualitative PCR test that will detect genetic mutations in codons 12 and 13 in exon 2, codons 59 and 61 in exon 3, and codons 117 and 146 in exon 4 in the human KRAS gene. According to the assay manual, sample sources for the test can be from extracted and purified DNA from formalin fixed paraffin embedded (FFPE), plasma, cells, or tissue. The version of the assay (not an FDA-approved assay) was developed with FFPE extracted DNA and reference standards. Because the mutation sensitivity is at 1% or below, it has the capability of being used for plasma samples and cfDNA. The test detects the presence or absence of KRAS mutations in the targeted regions but does not specify the exact nature of the mutation (http://www.diacarta.com/products/gene-mutation-detection-kits-2/kras-mutation-test).

The validated instruments and product specification are shown in Table 1.

Specification	QClamp KRAS Codon Specific Mutation Test
Intended Use	For In Vitro Diagnostic Use (CE/IVD) or For Research Use
Sample Type	Extracted (DNA) at 2.5ng/µL concentration
Pack Size	10 Samples, 30 Samples, and 60 Samples
Validated Instruments	Roche LC 96, LC 480 II, Bio-Rad CFX384 and ABI Quant Studio 5
Detection Channel	FAM; HEX
Turnaround Time	<4 hours
Stability	Stable for 12 months at -25°C to -15°C

Table 1. Instruments and Product Specification.

D. Q-Clamp® KRAS Codon Specific Mutation Test Principle

The Q-Clamp® KRAS Codon Specific Mutation Test is based on xenonucleic acid (XNA) mediated Polymerase Chain Reaction (PCR) clamping technology. XNA is a synthetic DNA analog in which the phosphodiester backbone has been replaced by a repeat formed by units of (2-aminoethyl)-glycine. If there is a complete match of DNA target sequence, the XNA will hybridize tightly to that matching complementary DNA target sequences and this binding will block the elongation of the DNA strand by DNA polymerase, hence preventing its amplification. When there is a mismatch in the target site, in the case of a mutation, the XNA will not be able to hybridize tightly to the target sequence making the XNA:DNA duplex unstable. If the XNA:DNA duplex is unstable, the DNA polymerase will be able to elongate the DNA strand. Thus, the addition of XNA, with a sequence completely matching the wild-type of DNA sequence, in a PCR reaction, will selectively prevent the wild type DNA from being amplified allowing the mutated DNA to amplify. Furthermore, the DNA polymerase will not recognize XNA oligomers and therefore it cannot be used as primers in the following real-time PCR reactions (http://www.kleegmbh.ch/diagnostics/mutation-detection.html).

Figure 2 is the schematic representation of the molecular approach used for mutation and translocation detection. Samples are amplified in reactions with and without the XNA clamp. The sample is considered positive when the threshold cycle (C_T) difference in reactions with and without the XNA clamp was less than a validated number of Ct's - usually 5 C_Ts.

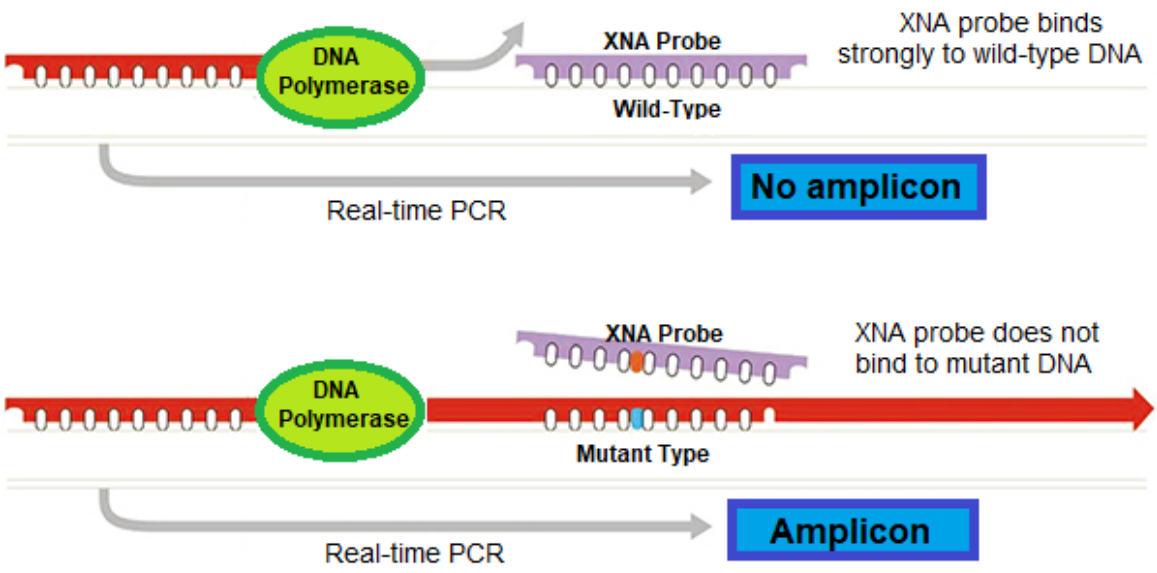

Figure 2. Q-Clamp® PCR Principle Diagram. To reduce the wild-type background and improve sensitivity, a molecular clamp has been designed to hybridize selectively to wild-type template DNA and block its amplification. This molecular clamp consists of a synthetic, sequence-specific Xeno-nucleic acid (XNA) probe. In the presence of a mutation such as a single nucleotide polymorphism (SNP) gene deletion, insertion, or rearrangement in the region of the XNA probe sequence, the XNA probe molecule melts off the mutant template DNA during the PCR cycling process, and only mutant templates are amplified efficiently (Q-Clamp® Kras codon specific mutation test instruction manual).

E. Cell-Free DNA

Cell-free DNA or cfDNA refers to all non-encapsulated DNA in the blood stream. A portion of that cell-free DNA originates from a tumor clone and is called circulating tumor DNA or ctDNA. cfDNA are nucleic acid fragments that enter the bloodstream during apoptosis or necrosis. Normally, these fragments are cleaned up by macrophages, but the overproduction of cells in cancer leaves more of the cfDNA behind. These fragments average around 170 base pairs in length, have a half-life of about two hours and are present in both early and late stage disease in many common tumors including non-small cell lung and breast cancers (https ://www. thermofisher.com/

blog/behindthebench/what-is-cell-free-dna-cfdna-seq-it-out-19). ctDNA should not be confused with cell-free DNA (cfDNA), a broader term which describes DNA that is freely circulating in the bloodstream, but is not necessarily of tumor origin. ctDNA originates directly from the tumor or from circulating tumor cells (CTCs), which describes viable, intact tumor cells that shed from primary tumors and enter the bloodstream or lymphatic system. In healthy tissue, infiltrating phagocytes are responsible for clearance of apoptotic or necrotic cellular debris, which includes cfDNA. Levels of cfDNA in healthy patients is only present at low levels but higher levels of ctDNA in cancer patients can be detected. This possibly occurs due to inefficient immune cell infiltration to tumor sites, which reduces effective clearance of ctDNA from the bloodstream (https://en.wikipedia.org/wiki/Circulating_tumor_DNA).

II. MATERIALS AND METHODS

Materials:

- KRAS Q-Clamp® Kit
- cfDNA derived from human plasma
- Horizon Diagnostics cfDNA Reference Standard (Horizon Discovery, www.horizondiscovery.com)
- Thermal Cyclers (eBioRad CFX 384)
- 384-well plate

Purpose

This experiment was performed to test if the limit of detection could go low enough using cfDNA in an assay kit designed and optimized using FFPE extracted DNA and genomic DNA references.

Q-Clamp® Workflow:

The assay workflow consists of four (4) major steps:

1. DNA isolation;
2. Set up qPCR;
3. Enter amplification parameters; and
4. Data Analysis

A. DNA Isolation

Acquisition of cfDNA or ctDNA typically requires collection of approximately 3 ml of blood into EDTA-coated tubes. The plasma fraction of blood is separated through centrifugation. Plasma is then processed again by another centrifugation to remove residual intact blood cells. The supernatant is used for DNA extraction which can be performed using commercially available kits (https://en.wikipedia.org/wiki/circulating_tumor_DNA).

B. Set Up qPCR

Samples used are Reference Standard (mutant DNA), Diluent (wild type DNA); both are cell-free DNA.

The assay was set up by serially diluting 5 ng (2uL) of the 5% mutant cfDNA standard reference with 5 ng of the wild type cfDNA (2uL) at 0.5, 0.25, 0.125 and 0.0625 dilutions. Two µL of this mixture from these dilutions was added to the reaction plate. Each sample and control was run in duplicate.

The PCR reactions are set up in a total volume of 10 µL/reaction as shown in Table 2.

Components	Volume/Reaction
2x PCR Master mix	5µL
Primer / Probe mix	1µL
XNA	1µL
Nuclease Free water	1µL
DNA Template (Samples and Controls)	2µL → to be added last in the plate
Total Volume	**10µL**

Table 2. Q-Clamp® Assay Components and Reaction Volume.

A set of positive controls must also be run with each reaction mix, every time the assay is run. The Positive Control contains one mutant template for each reaction mix. Negative Controls use wild-type DNA as the template. Wild type DNA should have no mutations. A set of no template control (tube NTC) is run with each reaction mix every time the assay is run. Nuclease-Free Water is used in the place of template. The NTC serves as a negative control and assesses potential contamination during assay set-up.

C. Enter Amplification Parameters

Eight uL of the mixture (PCR master mix, PPM, XNA, and water) was transferred to each well in a 384-well plate using a multichannel pipette as reflected in Table 3.

					Dilution			
	Codon Reaction designation (Not samples)				**0.5**	**0.25**	**0.125**	**0.0625**
A	C12	PC	CC	NTC	S	S	S	S
B	C12	PC	CC	NTC	S	S	S	S
C								
D	C13	PC	CC	NTC	S	S	S	S
E	C13	PC	CC	NTC	S	S	S	S
F								
G	C59	PC	CC	NTC	S	S	S	S
H	C59	PC	CC	NTC	S	S	S	S
I								
J	C61	PC	CC	NTC	S	S	S	S
K	C61	PC	CC	NTC	S	S	S	S
L								
M	C146	PC	CC	NTC	S	S	S	S
N	C146	PC	CC	NTC	S	S	S	S

Table 3. Plate Map BioRad CFX 384. PC – Positive Control; **CC** – Clamping Control/Negative Control; **NTC** – No-Template Control; **S** – Sample

D. Data Analysis

Each sample tube is a multiplex PCR consisting of amplification primers and probes for an internal amplification control (B-actin gene) and the specific KRAS codon. During PCR, the accumulating amplicons for both the Internal control gene (B-actin) and the KRAS codon are measured by means of fluorescent tagged qPCR probes (HEX for B-actin and FAM for KRAS).

Analysis is performed using both FAM and HEX channel readings. The Ct values from these files is used to evaluate the presence or absence of mutations using the Q-Clamp® KRAS codon specific mutation test user manual.

A cycle threshold (Ct) value for each analyzed sample is generated by the PCR instrument. The Ct is the cycle number at which a signal is detected above the set threshold for fluorescence. The higher the cycle number at which signal is detected, the weaker is the PCR reaction. On the other hand, the lower the cycle number at which signal is detected, the stronger the PCR reaction it represents.

Controls Analysis:

Wild type DNA is used as the template for the mutation negative controls (Clamping Controls). This wild type DNA does not contain the targeted mutations, so the XNA probes will strongly bind to it inhibiting the KRAS amplification. The internal control should amplify to specified amounts though. This will monitor the performance of the primers, polymerase and sample DNA quality and quantity.

The positive control template contains mutations, so the XNA probe will not bind to the template allowing the KRAS amplification. Positive controls must show the appropriate value in both HEX and FAM channels to make the run valid.

The no-template controls (NTC) should have no amplification for any of the reactions. Cycle threshold (Ct) should be undetermined.

Mutation Analysis:

The presence or absence of a mutation is determined by the difference in the Ct values in the B-actin (internal control) and the KRAS (mutation) amplifications reactions. The difference in Ct value between the Mutation assay and the Internal Control assay (HEX) as follows:

Ct difference (ΔCt) = Mutation Assay Ct – Internal Control assay Ct

III. RESULTS

Codon	Channel	PC	NC	NTC	Dilution			
					0.5	0.25	0.125	0.625
C12	FAM	32.42	42.94	45	34.08	35.88	39.74	36.25
	HEX	26.45	26.80	45	30.51	30.78	30.56	30.60
C13	FAM	32.83	42.38	45	34.94	37.83	36.06	41.66
	HEX	26.75	26.38	45	30.68	31.07	30.34	30.43
C59	FAM	34.29	39.79	45	35.82	35.47	35.24	36.46
	HEX	26.80	26.52	45	30.58	31.10	30.85	30.45
C61	FAM	36.83	43.01	45	*	38.81	*	40.87
	HEX	26.25	26.06	45	32.02	30.59	*	30.36
C146	FAM	35.96	43.08	45	44.95	38.36	38.22	*
	HEX	26.53	26.17	45	30.29	31.01	30.03	*

Table 4. Results for Run in BioRad CFX 384 (Average Cts of FAM and HEX). PC = Positive Control; **NC =** Clamping Control - Negative Mutation Control; **NTC =** No Template Control; ∗ = No Amplification

Codon	PC	NC	NTC	0.5	0.25	0.125	0.0625
C12	5.975	16.775	0	3.565	5.095	9.185	5.64
C13	6.08	15.99	0	4.26	6.76	5.72	11.23
C59	7.49	13.27	0	5.24	4.365	4.39	6.01
C61	10.58	16.95	0	*	8.23	*	10.51
C146	9.425	16.9	0	14.66	7.35	8.19	*

Table 5. Data Analysis for BioRad CFX 384 (Average ∆ CT). PC = Positive Control; **NC =** Clamping **Control** – Negative Mutation Control; **NTC =** No Template Control; * = No Amplification

Codon	(Positive) Control Acceptable Values	Clamping Control (Negative) Acceptable Values
C12	Δ CT ≤ 7	Δ CT > 10.5
C13	Δ CT ≤ 7	Δ CT > 10.5
C59	Δ CT ≤ 8.4	Δ CT > 10.5
C61	Δ CT ≤ 11	Δ CT > 13.5
C146	Δ CT ≤ 11	Δ CT > 13

Table 6. Acceptable Values for Positive Controls and Clamping Controls for BioRad CFX 384.

	KRAS Target Codon				
Call	C12	C13	C59	C61	C146
Positive	≤ 10.5	≤ 10.7	≤ 10.6	≤ 13.65	≤ 13
Negative	>10.5	>10.7	>10.6	>13.65	>13

Table 7. Scoring Mutational Status for BioRad CFX 384.

	Copy Number of Mutant DNA (5ng input DNA)			
% Mutant DNA	2.5 %	1.25 %	0.625 %	0.3125 %
Copies of Mutant DNA/rxn	38	19	9	5

Table 8. Gene Concentrations (copies/µL) of Input DNA (ng).

Calculation (Copies of Mutant DNA):

3.3ng = 1,000 copies

5ng = x

$$\frac{3.3}{1{,}000} = \frac{5}{x}$$

$$x = \frac{5{,}000}{3.3} \quad x \ \%\ \text{mutation} = \text{copy number of mutant DNA}$$

IV. CONCLUSION

In this experiment, a cell-free DNA reference standard containing all the mutations diluted into cell-free DNA was used as the sample instead of FFPE extracted genomic DNA. The experiment went well as evidenced by the results of the control values passing the criteria stated in the instruction manual. The average CTs of the controls (Positive Control, Negative Control and the No-template Control) of both the FAM and HEX channels of all the codons tested were all within the specified limits in the instruction manual.

As for scoring the mutational status of the codons tested, codon C12 is positive for mutation at 0.5, 0.25, 0.125 and 0.0625 dilutions which means that detection is observed up to 0.3%. Codon C13 is positive for mutation at 0.5, 0.25 and 0.125 dilutions which indicates that the limit of detection with this particular codon is at 0.6%. Codon C59 is positive for mutation at 0.5, 0.25, 0.125 and 0.0625 dilutions so detection is up to 0.3% and with codon C61 the assay was able to detect mutation at 0.25 and 0.0625 dilutions. Codon C146 had mutation detected at 1.25 and 0.625 dilutions.

With these results, it is observed that three of the five codons (C12, C59 and C61) tested can be detected up to 0.3%. The limit of detection for codon C13 is at 0.6% and C146 at 0.6%. There was no attempt to proceed further dilutions to see how far can possibly detect mutation for C12, C59 and C61 because of unavailability of resources.

The corresponding copy number of mutant DNA is approximately 38 copies for the 2.5% mutation, 19 copies for the 1.25% mutation, 9 copies for the 0.6% mutation and 5 copies for the 0.3% mutation.

The internal control values for the samples were higher than expected which indicates not enough amplifiable DNA was in the reactions or there may have been some inhibition in the sample resulting to sub-optimal amplification. Again because of limited materials, no further testing was done. Future work would include repeating this experiment to see if there was a problem with just this run or with cfDNA in general, looking at different cell-free extracts with reference standards in them and when optimized, looking at different clinical samples. Based on the Ct value specification for the internal control, for clinical samples in a validated test, the mutation scoring would have not proceeded. However, since a clear difference could be distinguished in the delta Ct's between mutation positive (the dilution series) and mutation negative (clamping control/negative control) reactions, it provides the basis for moving forward with continued experiments to further prove the feasibility and optimize the assay for cfDNA use.

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DiaCarta Instruction Manual: Q-Clamp® KRAS Codon Specific Mutation Test in Codons 12, 13, 59, 61, 117, and 146 Instruction Manual (CE/IVD).

APPENDIX

CERTIFICATION OF APPROVAL
Research Project

I certify that I have reviewed “Determination of the Feasibility of Using cfDNA in the Q-Clamp® KRAS Codon Specific Mutation Test” by Manuel Mina Garcia, and I approve this completed project to be submitted in partial fulfilment of the requirements for the CLS 5700 Course Master’s Research Project.

(Optional) Additional Project Supervisor’s Comments:

_____

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