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A Rapid Colorimetric Peptide Nucleic Acid Loop-Mediated Isothermal Amplification Assay for the Detection of the IDH1 Mutation in Glioblastoma

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A RAPID COLORIMETRIC PEPTIDE NUCLEIC ACID LOOP-MEDIATED ISOTHERMAL AMPLIFICATION ASSAY FOR THE DETECTION OF THE *IDH1* MUTATION IN GLIOBLASTOMA

By

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A RAPID COLORIMETRIC PEPTIDE NUCLEIC ACID LOOP-MEDIATED ISOTHERMAL AMPLIFICATION ASSAY FOR THE DETECTION OF THE *IDH1* MUTATION IN GLIOBLASTOMA

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ABSTRACT

A RAPID COLORIMETRIC PEPTIDE NUCLEIC ACID LOOP-MEDIATED ISOTHERMAL AMPLIFICATION ASSAY FOR THE DETECTION OF THE *IDH1* MUTATION IN GLIOBLASTOMA

By
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The *IDH1* mutation is an important diagnostic and prognostic biomarker used to characterize glioblastoma (GBM). Patients harboring the *IDH1* mutation have improved overall survival following maximal resection. Knowledge of the *IDH1* mutation status allows the surgeon to modify the surgical plan; however, no existing molecular test can provide this information intraoperatively. We designed a novel colorimetric peptide nucleic acid loop-mediated isothermal amplification (PNA-LAMP) method that rapidly detects the *IDH1* R132H mutation in GBM. PNA-LAMP amplifies target DNA under isothermal conditions with high specificity and speed. The PNA prevents amplification of wild-type *IDH1* DNA, while allowing amplification of the R132H variant if present. We used a pH-sensitive colorimetric detection method for visual determination of amplification in under one hour. Characterization of the assay was performed with plasmid DNA containing the *IDH1* wild-type and R132H variant sequences. Amplification was confirmed using gel electrophoresis, and this analysis suggests that the assay is more sensitive than Sanger sequencing – the gold standard for *IDH1* mutation identification. This study is the first to attempt to develop a colorimetric LAMP assay for GBM tumor characterization, and only the third application of the PNA-LAMP method to detect acquired mutations in cancer. This novel molecular assay is a simple, specific, and rapid way to identify the presence of the *IDH1* R132H variant associated with GBM.

TABLE OF CONTENTS

List of Tables……...v

List of Figures……vii

Chapter One: Introduction and Literature Review……..1

Chapter Two: Materials and Methods…….8

Primer Design……..8

Genomic DNA Extraction and Purification……...10

Preparation of Synthetic *IDH1* WT and R132H Sequences……..10

Synthetic Plasmid DNA Copy Number Determination…….12

Colorimetric LAMP Methods……....12

Agarose Gel Electrophoresis……..13

Chapter Three: Results……..14

AS-LAMP (B3 Mutation Site) Method…….14

Primer Design……14

Analysis of WT B3 AS-LAMP Primer Set……....15

AS-LAMP (B2 Mutation Site) Method…….16

Primer Design……....16

Initial Analysis of WT B2 AS-LAMP Primer Set…….16

Reaction Temperature Optimization……..17

1M Betaine Enhanced Amplification……....18

0.8μM F3/B3 Improved Reaction Time……19

Evaluation of B2 AS-LAMP Assay……..20

SNP-LAMP Method……..21

Primer Design…….…...21

Evaluation of SNP-LAMP Assay…….….22

PNA-LAMP Method…….23

Primer Design……23

PNA Blocked Amplification of WT gDNA……..24

Loop Primers Significantly Improved Reaction Time……...25

Effect of R132H "Self-Annealed" Loop Primers

on PNA-LAMP…….…….26

Reaction Temperature Optimization with PNA…….28

Limit-of-Detection (LOD)…….39

LAMP Can Be Applied to Whole Cell Lysates…….31

PNA-LAMP Assay Detected R132H Sequence

Diluted in Whole Cell Lysates……...32

Chapter Four: Discussion……...33

References……..40

Appendix A……46

LIST OF TABLES

Table 1: Primer Sequences

Table 2: Synthetic Sequences

Table 3: B2 AS-LAMP Reaction Components

Table 4: PNA Effect on WT gDNA Reaction Components

Table 5: Effect of Loop Primers Reaction Components

Table 6: PNA-LAMP LOD on Straight R132H DNA Reaction Components

Table 7: PNA-LAMP LOD on R132H DNA Diluted in gDNA Reaction Components

Table 8: LAMP on Whole Cell Lysates Reaction Components

Table 9: PNA-LAMP on R132H DNA Diluted in Whole Cell Lysates Reaction Components

LIST OF FIGURES

Figure 1: Mechanisms Affected by the *IDH1* Mutation in GBM

Figure 2: Pyrophosphate and Hydrogen Ions Are Byproducts of DNA Synthesis

Figure 3: Conventional LAMP Process

Figure 4: Structure of a PNA compared to DNA

Figure 5: Custom pUCIDT Gene Map

Figure 6: LAMP Colorimetric Indicator

Figure 7: B3 AS-LAMP Primer Design

Figure 8: B3 AS-LAMP Reaction Scheme

Figure 9: WT B3 AS-LAMP Initial Color Change Result

Figure 10: B2 AS-LAMP Primer Design

Figure 11: B2 AS-LAMP Reaction Scheme

Figure 12: WT B2 AS-LAMP Initial Color Change Result

Figure 13: B2 AS-LAMP Temperature Gradient from 56°C-68°C

Figure 14: B2 AS-LAMP Temperature Gradient from 61°C-68°C

Figure 15: Addition of 1M Betaine Enhanced the LAMP Reaction

Figure 16: 0.8μM F3/B3 Improved Reaction Time

Figure 17: B2 AS-LAMP Assay Color Change Results

Figure 18: SNP-LAMP Primer Design

Figure 19: SNP-LAMP Reaction Scheme

Figure 20: Evaluation of the SNP-LAMP Assay

Figure 21: PNA-LAMP Primer Design

Figure 22: PNA and “Self-annealed” Loop Primer Mechanisms

Figure 23: PNA Blocked Amplification of WT gDNA

Figure 24: Loop Primers Reduced Reaction Time

Figure 25: Effect of R132H “Self-annealed” Loop Primers on PNA-LAMP

Figure 26: PNA-LAMP Reaction Temperature Optimization

Figure 27: PNA-LAMP LOD on R132H DNA Diluted in Sterile H_2O

Figure 28: PNA-LAMP LOD on R132H DNA Diluted in gDNA

Figure 29: LAMP of Whole Cell Lysates Without DNA Extraction

Figure 30: Detection of R132H Sequence Diluted into Whole Cell Lysates

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

Glioblastoma (GBM) is the most common form of malignant brain cancer, with a 5-year survival rate of 5.5% (Ostrom et al., 2013). There are two types of GBM: primary and secondary. Primary GBM arises *de novo* without lower grade precursors, presents as advanced cancer at diagnosis, and is found in adults with a median age of 60 (Cohen, Holmen, & Colman, 2013). Secondary GBM progresses from lower-grade tumors and is generally found in younger patients with a median age of 45 (Cohen et al., 2013). There has been considerable research on prognostic biomarkers, such as the isocitrate dehydrogenase 1 (*IDH1*) mutation, which is now recommended by the World Health Organization for diagnostic and prognostic analysis of glioma (Louis et al., 2016). *IDH1* mutations are commonly found in secondary GBMs and low-grade gliomas (Liu, Hou, Chen, Zong, & Zong, 2016). Currently, treatments for patients with GBM include surgical resection, radiation therapy, and chemotherapy. Recent studies have shown that GBMs harboring the *IDH1* mutation offer a better prognosis with median overall survival of 3.8 years compared to 1.1 years for patients with wild-type *IDH1* (Parsons et al., 2008). The difference in survival benefit is associated with the maximal surgical resection of tumors with the *IDH1* mutation (Beiko et al., 2014). This suggests that surgeons may alter their surgical plans intraoperatively and resect tumor tissue in areas where tumor is often left behind as to maximize the benefit of harboring the *IDH1* mutation. The *IDH1* genotype can be used to assist physicians in the surgical strategy, but only if the information is reported to the operating room before the surgery concludes.

IDH1 is an enzyme that catalyzes the oxidative decarboxylation of isocitrate, using NADP+ as an electron acceptor, to produce alpha-ketoglutarate and NADPH (Figure 1). These intermediate metabolic byproducts are essential in the Kreb's cycle and limit reactive oxygen species (ROS) (Liu et al., 2016). A heterozygous point mutation (guanine to adenine) in the coding region of the active site of *IDH1* results in a change of the amino acid residue from arginine to histidine (R132H). This causes reduced affinity for isocitrate while also increasing affinity for NADPH and alpha-ketoglutarate, resulting in gain-of-function activity and the conversion of alpha-ketoglutarate to the oncometabolite, 2-hydroxyglutarate (Dang et al., 2009). Because of this abnormal accumulation of 2-hydroxyglutarate, alpha-ketoglutarate-dependent dioxygenases are inhibited, which alters numerous downstream mechanisms such as increasing global DNA hypermethylation, histone tail methylation and hypoxia-induced factors, while decreasing collagen hydroxylation; these alterations are associated with tumorigenesis (Liu et al., 2016). Ninety percent of *IDH1* mutations found in GBMs involve this point mutation at codon 132 in exon four, which converts an arginine to a histidine (Liu et al., 2016). *IDH1* alterations promote tumorigenesis and because this mutation is common and predictable, it has been used as a target for molecular diagnostic applications.

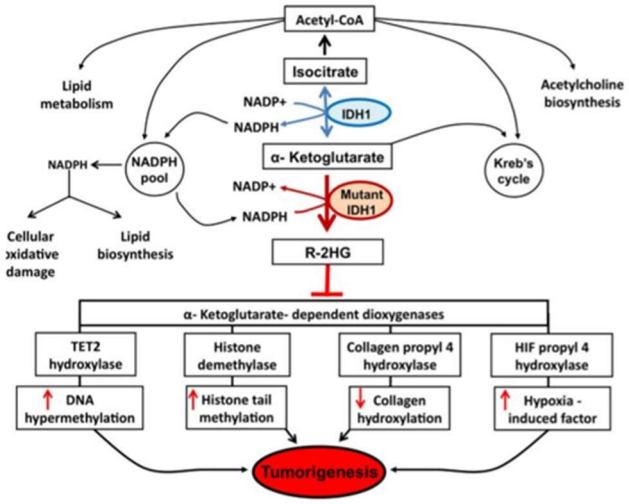

Figure 1: Mechanisms Affected by the *IDH1* Mutation in GBM (Liu et al., 2016)

Traditionally, the *IDH1* mutation has been identified through standard Sanger sequencing and PCR (Liu et al., 2016). These technologies are highly complex, time-consuming, expensive, and must be performed in a laboratory with trained professionals; therefore, the usefulness of these assays during intraoperative procedures is limited because of the resources required to perform them. Considerable efforts have been made to develop molecular diagnostics that both rapidly and accurately detect the *IDH1* mutation in GBM. A combination of real-time PCR and post-PCR fluorescent melting curve analysis has shown promise, with a detection rate of as little as 10% mutant DNA and a turnaround time of 80 minutes (Boisselier et al., 2010). This method still requires expensive equipment and extensive preparation that includes a nucleic acid extraction step. To date, no molecular diagnostic technique can be used in an intraoperative setting to rapidly detect the *IDH1* genotype in GBM in less than an hour.

Loop-mediated isothermal amplification (LAMP) is a technique that uses isothermal strand-displacement synthesis to amplify and detect target DNA sequences (Notomi et al., 2000). The three hallmarks of this method include its isothermal conditions, high specificity, and high amplification efficiency. LAMP utilizes the *Bst* DNA polymerase, which has strand displacement activity and lacks 5'→3' exonuclease activity. This eliminates the need for a

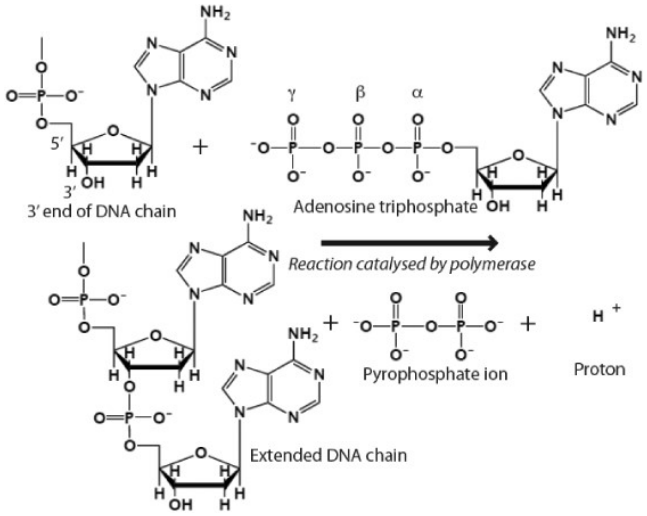

Figure 2: Pyrophosphate and Hydrogen Ions Are Byproducts of DNA Synthesis (Lindsay, 2012)

thermocycler, and the method can be performed with any piece of equipment that maintains a constant temperature, such as a water bath or heat block. Amplification can be detected through fluorescence, turbidity, or pH. When the *Bst* DNA polymerase incorporates a dNTP during DNA synthesis, a pyrophosphate and hydrogen ion are released as byproducts (Figure 2) (Tanner, Zhang, & Evans, 2015). Phenol red, a pH indicator dye, can be incorporated into the reaction tube to visualize the change in pH due to the accumulation of hydrogen ions. This provides the added benefit of not requiring extensive personnel training or data analysis. Furthermore, LAMP can be performed using crude lysates, eliminating the need for a DNA extraction step (Geojith, Dhanasekaran, Chandran, & Kenneth, 2011).

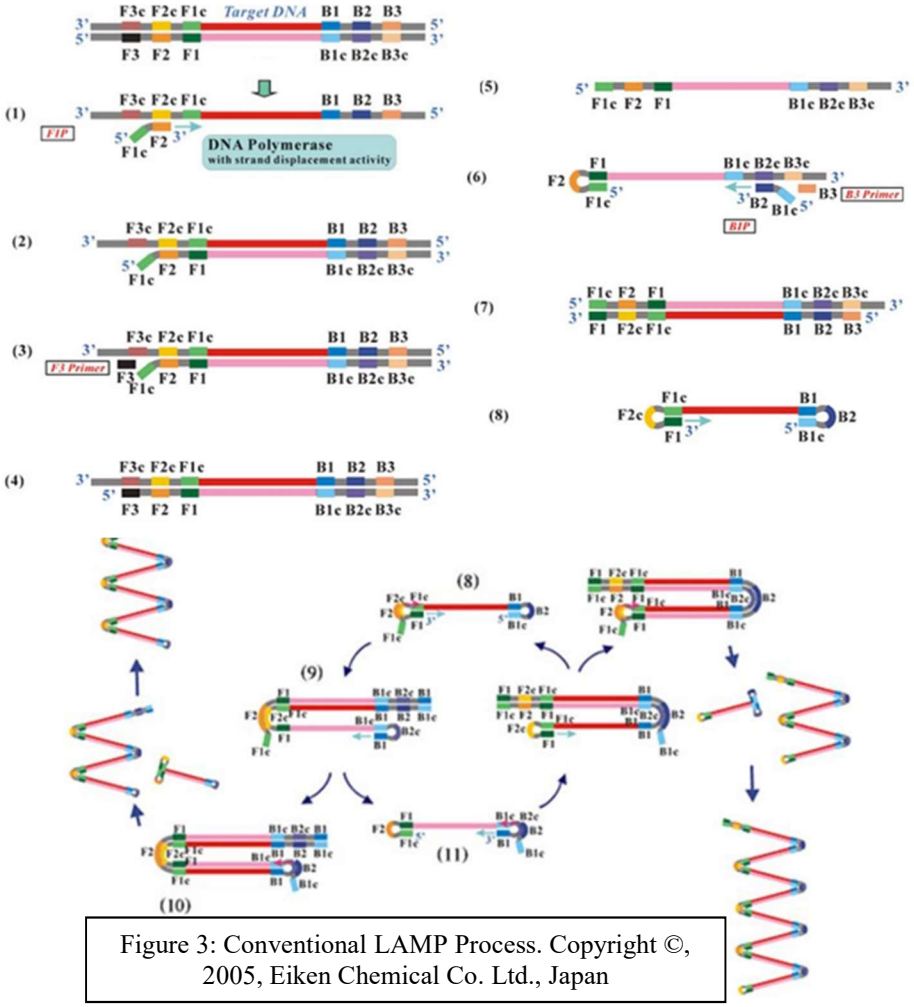

Figure 3: Conventional LAMP Process. Copyright ©, 2005, Eiken Chemical Co. Ltd., Japan

The high specificity of LAMP as compared to other amplification methods is due to the use of four primers designed to recognize a total of six distinct sequences on the target (Fu et al., 2011). The F3 and B3 primers are the outer primers that flank the region of interest. The FIP (F2 and F1c) and BIP (B2 and B1c) stand for forward inner primer and backward inner primer, respectively, and are unique in that they each recognize two different regions on the target DNA. After initial strand displacement by the *Bst* DNA polymerase, the F2 region of the FIP binds to its complementary sequence and gets extended (Figure 3). The F3 primer then binds upstream from the FIP and displaces the newly synthesized strand resulting in two products: a double stranded DNA and single stranded DNA. The single stranded DNA is used as a template for the reverse reaction primed by the BIP and B3 primers. The resulting product is a single stranded dumbbell structure that is the true starting template for the LAMP process. The FIP and BIP serve a dual purpose: they created the starting template and prime the rest of the amplification process by annealing to complementary sequences on the newly synthesized loops. The process is highly efficient and can produce 10^9 copies of target in less than an hour (Fu et al., 2011). An additional two primers, called loop primers, can also be incorporated into the LAMP process, which can cut the reaction time by as much as half compared to the original method. Loop primers are designed to be complementary to the regions between F1c and F2 or B1c and B2 on the single stranded dumbbell structures.

Variations of the LAMP method can be applied to discriminate single nucleotide polymorphisms (SNPs). One study developed an allele specific LAMP (AS-LAMP) method to identify the West-African *kdr* mutation that is responsible for resistance to insecticides in certain mosquito populations (Badolo et al., 2012). Another group utilized

primers designed to amplify the wild-type target, while preventing amplification when a SNP is present, and vice versa (Iwasaki et al., 2003). LAMP has also been applied to detect CYP2C19 polymorphisms in humans in less than 30 minutes with high sensitivity and specificity (Zhang et al., 2016).

Peptide nucleic acids (PNAs) have been commonly used in PCR-clamping applications as blocking agents for wild-type DNA sequences (Orum et al., 1993). PNAs are synthetic oligonucleotide analogs which contain a neutral backbone as opposed to the negatively charged phosphodiester backbone present in DNA (Itonaga et al., 2016). Due to the change in charge, a PNA-DNA duplex is much more stable than a DNA-DNA duplex. Furthermore, the lack of a 3'-hydroxyl group on the PNA prevents the DNA polymerase from extending the sequence once it is bound to its target. When the PNA binds to its complementary sequence, the melting temperature of the PNA-DNA duplex is much higher than a perfectly matched DNA-DNA duplex. A variation of the LAMP procedure was recently developed and utilized a PNA that blocks amplification of the wild-type allele (Minnucci et al., 2012). In addition, the study used a "self-annealed" loop primer that is complementary to the sequence containing the mutant allele. If there is a single mismatch in the target DNA, it prevents the PNA from annealing due to a significantly reduced melting temperature compared to a perfectly matched PNA-DNA duplex. This allows the "self-annealed" loop primer to bind and amplify the mutant sequence. The loop primers are also designed to have self-complementary ends in case the PNA is not 100% efficient. This should prevent amplification from proceeding because the melting temperature of the self-complementary hairpin structure of the loop

primers is higher than the primer-target hybridization temperature if there is WT sequence present (Minnucci et al., 2012)

The primary objective of this thesis project is to show proof of principle that a colorimetric PNA-LAMP method can rapidly and accurately detect the *IDH1* mutation in GBM. The ultimate goal is to develop an assay that provides surgeons with the *IDH1* mutation status and allows them to alter their surgical plan intraoperatively to maximize the survival benefit for GBM patients.

CHAPTER TWO: MATERIALS AND METHODS

Primer Design

Table 1: Primer Sequences

Method	Sequence (5'→3') with mutation site in bold		T_m (°C)
AS-LAMP	B2	F3: GCGTCAAATGTGCCACTA	57.11
		B3: ACATTATTGCCAACATGACTT	55.74
		FIP: GGTGCCATTTGGTGATTTCCA-CCTGATGAGAAGAGGGTTG	66.30
		BIP(WT): AAGCCATTATCTGCAAAAATATCCC-TGATCCCCATAAGCATGA**C**	64.80
		BIP(R132H): AAGCCATTATCTGCAAAAATATCCC-TGATCCCCATAAGCATGA**T**	64.70
	B3	F3: GCGTCAAATGTGCCACTA	57.11
		B3(WT): CTTGATCCCCATAAGCATGA**C**	58.07
		B3(R132H): CTTGATCCCCATAAGCATGA**T**	57.26
		FIP: TGCCATTTGGTGATTTCCACAT-CTCCTGATGAGAAGAGGGT	66.50
		BIP: CACGGTCTTCAGAGAAGCCA-CCTATGATGATAGGTTTTACCCA	65.60
SNP-LAMP	F3: GCACCATACGAAATATTCTGG		56.24
	B3: CATACAAGTTGGAAATTTCTGG		55.14
	FIP(WT): **C**GACCTATGATGATAGGTTTTACCC-ACGGTCTTCAGAGAAGCC		65.30
	BIP(WT): **G**TCATGCTTATGGGGATCAAGTAAG-GCCATGAAAAAAAAAACATGCA		65.30
	FIP(R132H): **T**GACCTATGATGATAGGTTTTACCC-ACGGTCTTCAGAGAAGCC		65.20
	BIP(R132H): **A**TCATGCTTATGGGGATCAAGTAAG-GCCATGAAAAAAAAAACATGCA		65.20
PNA-LAMP	F3: TGTGGAAATCACCAAATGG		55.04
	B3: GCCATGAAAAAAAAAACATGC		55.30
	FIP: CGGGGGATATTTTTGCAGATAATGG-CACCATACGAAATATTCTGGGT		65.70
	BIP: GTGGATGGGTAAAACCTATCATCATAG-CACATTATTGCCAACATGACT		64.80
	PNA (WT): GTC**G**TCATGCTTATGG		72.20
	R132H-1: CCCATAGTC**A**TCATGCTTATGGG		55.30
	R132H-2: ATCCCCATGTC**A**TCATGCTTATGGGGA		60.00
	R132H-3: TACTTGAGTC**A**TCATGCTTATGGGGATCAAGTA		64.00

All LAMP primers (Integrated DNA Technologies) were designed using Eiken's PrimerExplorer V5. Careful consideration was taken regarding the delta G (ΔG) scores of the LAMP primers. They were designed so that the ΔG value of each end was ≤ -4.0 kcal/mole. A lower ΔG score indicates primer stability and lowers the likelihood of homodimerization. The F3 and B3 primers' melting temperatures (T_m) were designed to be lower than the FIP and BIP, so that they would anneal and begin synthesis earlier. The T_m's of the FIP (F1c and F2) and BIP (B1c and B2) were designed to be within the optimal range of *Bst* polymerase, 60-70°C. The F2 and B2 regions of the FIP and BIP, respectively, had a slightly lower T_m than the F1c and B1c regions. This allows the F2 and B2 primer sequence to anneal to their complement on the target prior to the F1c and B1c, which is essential in creating the loop structure of the starting template (Notomi et al., 2000).

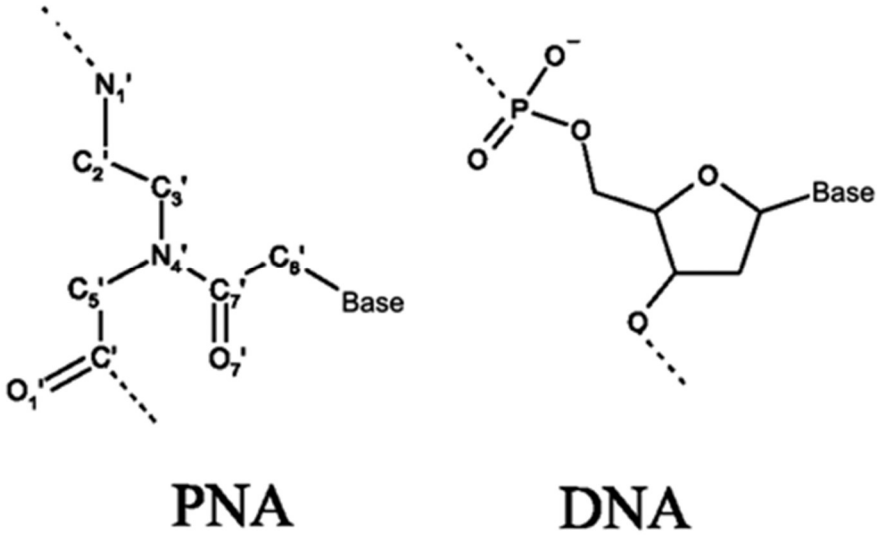

Figure 4: Structure of a PNA Compared to DNA
(Menchise et al., 2003)

The PNA-clamping oligonucleotide was designed to be complementary to the WT allele (Panagene). An ideal PNA is 12-18 bases long with a purine content of under 60%

and no self-complementary sequences. The PNA used in this study is 16-mer and contains one lysine residue on each end to enhance solubility.

Genomic DNA Extraction and Purification

Genomic DNA (gDNA) was extracted and purified from buccal swabs using the QIAamp DNA Mini Kit protocol (Qiagen). Briefly, a buccal swab was placed in a microcentrifuge tube containing 400µL of AL Buffer and 20µL of Proteinase K. The mixture was incubated at 56°C for 10 minutes and then 400µL of ethanol was added. The solution was added to a QIAamp Spin Column and centrifuged at 6000 x *g* for one minute. 500µL of AW1 Buffer was added to the column and centrifuged for one minute. 500µL of AW2 Buffer was added and the column was centrifuged at 20000 x *g* for three minutes. The spin column was placed in a 1.5mL microcentrifuge tube, and 150µL of sterile H_2O was added. The spin column and microcentrifuge tube was centrifuged at 6000 x *g* for one minute to elute the gDNA. The gDNA was stored at -80°C.

Preparation of Synthetic *IDH1* WT and R132H Sequences

Table 2: Synthetic Sequences (*exon four is demarcated by all caps)*

Genotype	Sequence (5'→3')
WT/R132H	CTATGATTTAGGCATAGAGAATCGTGATGCCACCAACGACC AAGTCACCAAGGATGCTGCAGAAGCTATAAAGAAGCATAA TGTTGGCGTCAAATGTGCCACTATCACTCCTGATGAGAAGA GGGTTGAGGAGTTCAAGTTGAAACAATGTGGAAATCACCA AATGGCACCATACGAAATATTCTGGGTGGCACGGTCTTCAG AGAAGCCATTATCTGCAAAAATATCCCCCGGCTTGTGAGTG GATGGGTAAAACCTATCATCATAGGT**C[G/A]T**CATGCTTAT GGGGATCAAgtaagtcatgttggcaataatgtgattttgcatgttttttttttcatggcccagaa atttccaacttgtatgtgttttattcttatcttttggtatctacacccattaagcaaggtatgaaattgagaa atgcatatatgtataactgtatatttacacacatttagctaaaggcaaatacaaataaacttacaaatag gcgtccatctcaacacatttttttttaaacatgctgtttttttttcctttatccttttattcagttataccatatg

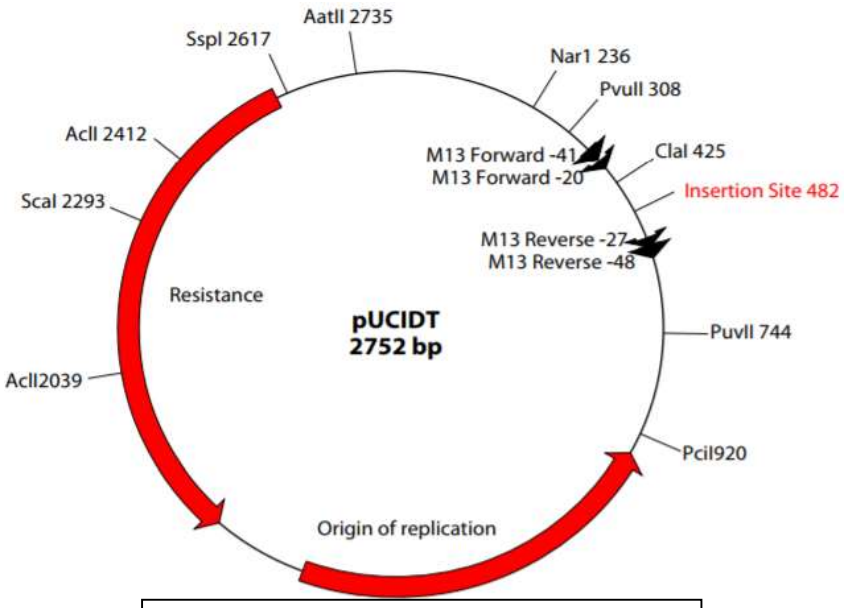

Figure 5: Custom pUCIDT Gene Map
("IDT DNA Custom Gene Synthesis," n.d.)

Custom plasmid vectors with the *IDH1* WT and R132H sequences (Integrated DNA Technologies) were transformed into competent *E. coli* using standard methods (Figure 5). Briefly, 1µL from working stocks (0.1µg/µL) of each custom plasmid vector was added to prechilled sterile tubes containing 40µL of cells and incubated on ice for 30 minutes. The cells were then heat shocked at 42°C for 45 seconds and returned to ice for two minutes. 460µL of SOC Medium was added to the cells and placed in an incubator for one hour at 37°C. After incubation, 400µL, 100µL, and 10µL of each transformation was plated on agar plates containing the appropriate antibiotic (*IDH1* WT vectors contained an Ampicillin resistance gene and R132H vectors contained a Kanamycin resistance gene). Agar plates were incubated overnight at 37°C. After overnight incubation, single colonies containing each sequence were selected and placed in a 5mL solution of LB and the appropriate antibiotic. These tubes were incubated overnight at 37°C. After overnight incubation, the cell cultures were re-streaked on agar plates and set aside for glycerol stock cryopreservation. The remaining 5mL solution was pelleted and

the plasmid DNA was extracted and purified using the GF-1 Plasmid DNA Extraction Kit (Vivantis) and stored at -80°C.

Synthetic Plasmid DNA Copy Number Determination

Copy numbers for the WT and R132H plasmid DNA were determined using the "Creating Standard Curves with Genomic DNA or Plasmid DNA Templates for Use in Quantitative PCR" protocol ("Applied Biosystems," n.d.). In brief, the mass of human gDNA that contains one copy of the *IDH1* gene was calculated and used to calculate the mass of gDNA containing the copy numbers of interest. Then, the mass of gDNA needed to obtain these copy numbers was divided by the amount of sample pipetted into each LAMP reaction. The mass of plasmid DNA was calculated using this same process. Highly concentrated *IDH1* WT and R132H plasmid DNA stocks were diluted down to a workable concentration and serial dilutions were prepared based on the corresponding mass of human gDNA needed to obtain the copy numbers of interest (Appendix A).

Colorimetric LAMP Methods

All methods used the 1X WarmStart Colorimetric LAMP 2X Master Mix (New England Biolabs), LAMP primers, and 1M betaine. This master mix contains the phenol red pH indicator that provides visual detection of the accumulation of hydrogen ions during DNA synthesis. A positive reaction is defined as a color change from pink to orange-yellow (Figure 6). All LAMP reactions were performed in a PCR tube in a 25µL reaction volume. The AS-LAMP and SNP-LAMP primer concentrations were 0.8µM F3, 0.8µM B3, 1.6µM FIP, and 1.6µM BIP. These reactions

Figure 6: LAMP Colorimetric Indicator.
Pink = Negative Reaction
Yellow = Positive Reaction
(New England Biolabs)

were conducted at a constant temperature of 65°C for 60 minutes and then 80°C for five minutes to terminate the reaction. The PNA-LAMP primer concentrations were 0.2µM F3, 0.2µM B3, 1.6µM FIP, 1.6µM BIP, 0.8µM R132H-1, and 0.25µM PNA, and the reaction was conducted at a constant temperature of 63°C for 60 minutes and then 80°C for five minutes.

Agarose Gel Electrophoresis

Amplification was confirmed via 4% agarose gel electrophoresis. Agarose was combined with 1X TBE, heated in a microwave, and allowed to cool. Ethidium bromide was then added to the solution, and the solution was transferred to a casting tray. All gels were run for at least one hour, and the products were visualized with UV light. Each run included a 20bp DNA Ladder (Lonza) to approximate the size of the LAMP products.

CHAPTER THREE: RESULTS

AS-LAMP (B3 Mutation Site) Method

Primer Design

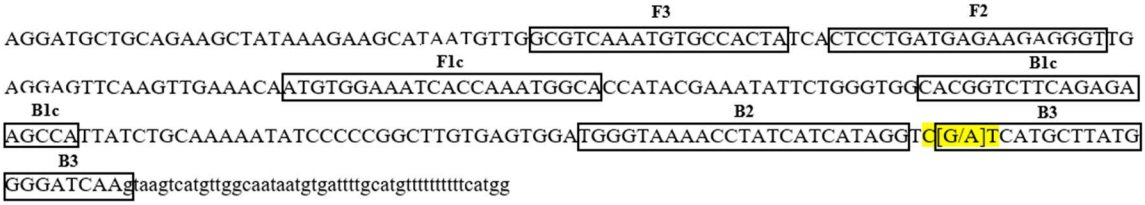

Figure 7: B3 AS-LAMP Primer Design

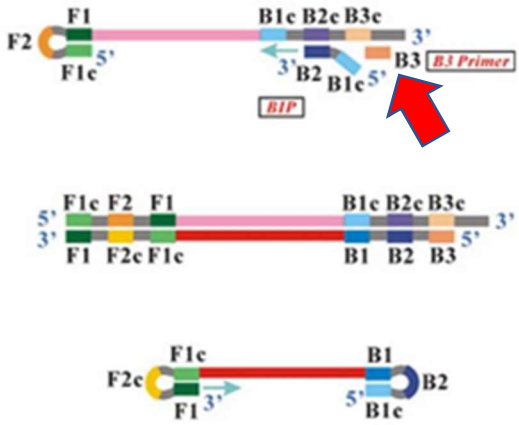

Figure 8: B3 AS-LAMP Reaction Scheme
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The initial purpose for designing these primers was to determine if the LAMP method could be used to detect the region containing the R132H SNP in the *IDH1* gene. Since the mutation is located in the 3'-distal region of exon four, allele-specific primers were designed to avoid the exon-intron junction by incorporating the allele at the 3'-end of the B3 outer primer (Figure 7 and 8). Avoiding intronic regions is ideal in primer design because it reduces the likelihood of non-specific priming, as well as troublesome long stretches of repeats. This modification is derived from the same principle as allele-specific PCR, which is a common technique used to discriminate SNPs via a 3' mismatch on one of the primers (Staiger et al., 2015). The additional advantage of having the allele located on the B3 primer was that it should hypothetically stop the LAMP process prior to the formation of the starting template.

Analysis of WT B3 AS-LAMP Primer Set

10ng gDNA was applied to a tube containing WT primers (Figure 9). After 60 minutes, there was a weak color change observed (from pink to light pink) as compared to the results seen in the LAMP Colorimetric Indicator (Figure 6). This suggested that the assay as designed did not efficiently amplify the region of interest, but that the *IDH1* gene could be targeted using LAMP.

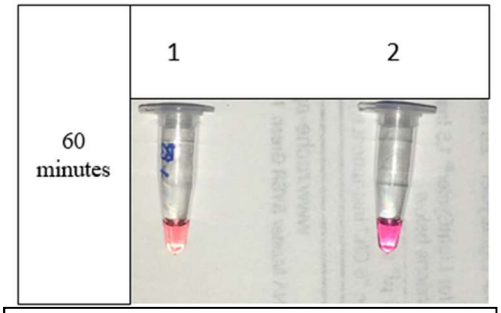


Figure 9: WT B3 AS-LAMP Initial Color Change Result. 1 – WT primers + 10ng gDNA; 2 – Negative control

AS-LAMP (B2 Mutation Site) Method

Primer Design

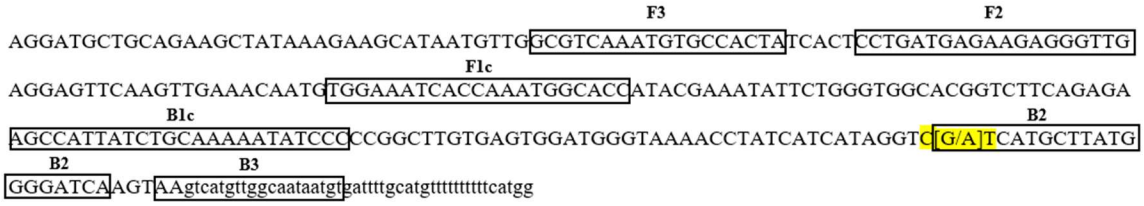


Figure 10: B2 AS-LAMP Primer Design

Since the B3 AS-LAMP method was unsuccessful for our purposes, we sought to target the mutation with a different LAMP primer set to improve the assay. Other studies have shown that targeting a mutation using the BIP primer was feasible (Badolo et al., 2012). The next sets of primers were designed so that the mutation site was located on the 3'-end of the B2 region of the BIP (Figures 10 and 11). Hypothetically, this will still stop the LAMP reaction prior to the formation of the single stranded dumbbell starting template. A concern of this design was that a large portion of the B3 primer was complementary to a sequence in the downstream intron.

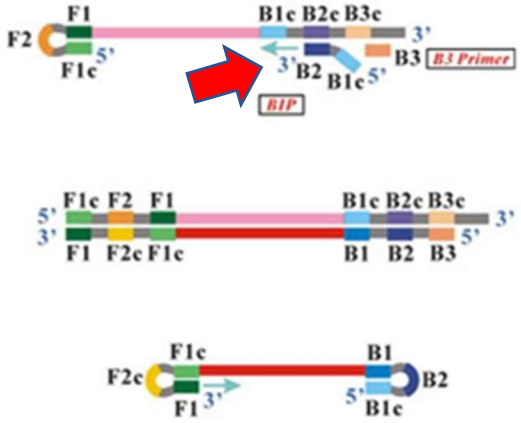


Figure 11: B2 AS-LAMP Reaction Scheme. Copyright ©, 2005, Eiken Chemical Co. Ltd., Japan

Initial Analysis of WT B2 AS-LAMP Primer Set

10ng gDNA was added to a tube containing the new WT primers (Figure 12). There was a color change observed (from pink to yellow) that was much more evident compared to the previous method (Figure 9). This data indicated that amplification occurred

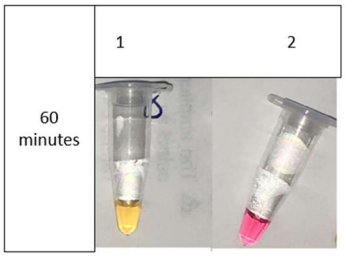


Figure 12: WT B2 AS-LAMP Initial Color Change Result. 1 – WT primers + 10ng gDNA; 2 – Negative control

sufficient to lower the pH of the solution, suggesting that these primers successfully targeted the IDH1 gene. As a result, the B2 AS-LAMP primer sets were chosen for further optimization of the LAMP method.

Reaction Temperature Optimization

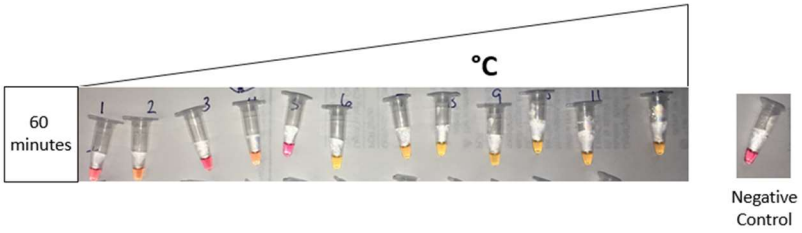


Figure 13: B2 AS-LAMP Temperature Gradient From 56°C-68°C

Next, we sought to determine the optimal reaction temperature by running the assay at temperatures ranging from 56°C – 68°C (Figure 13). The assay was performed with 10ng gDNA, and the reaction was run for 60 minutes. A more pronounced color change was observed (from pink to yellow) in tubes incubated at 61°C and higher. An additional experiment was conducted to further evaluate the optimal reaction temperature from 61°C – 68°C (Figure 14). There was a strong visible color change (pink to yellow) observed starting in the tube incubated at 65°C and higher. Based on these results, a reaction temperature of 65°C was selected.

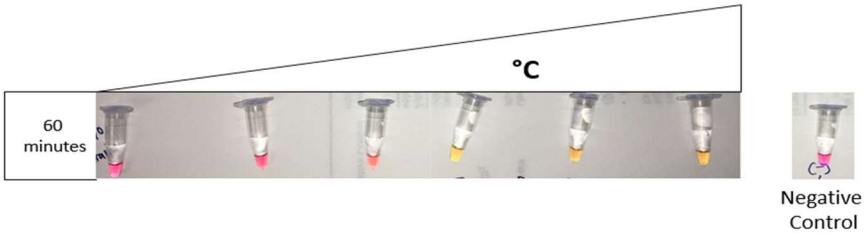


Figure 14: B2 AS-LAMP Temperature Gradient From 61°C-68°C

1M Betaine Enhanced Amplification

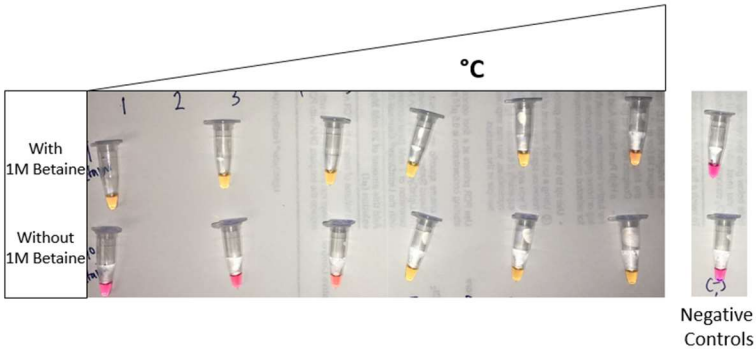


Figure 15: Addition of 1M Betaine Enhanced the LAMP Reaction

In the development of the original LAMP method, it was found that DNA helix-destabilizing chemicals such as 1M betaine enhanced the rate of reaction, while reducing non-specificity (Notomi et al., 2000). To determine the effect of betaine on this LAMP assay, the experiment was run with and without 1M betaine at temperatures ranging from 61°C – 68°C (Figure 15). After 10ng gDNA was added to the tubes, the reaction was observed after 60 minutes for the presence of a color change. There was a color change observed (from pink to yellow) in all the tubes containing 1M betaine and gDNA, while there was no observable color change in the tubes incubated below 65°C and lacking 1M betaine. These results suggested that 1M betaine and incubation at 65°C for this B2 AS-LAMP assay are the optimal conditions for this assay. Subsequent reactions were all performed with 1M betaine at 65°C.

0.8µM F3/B3 Improved Reaction Time

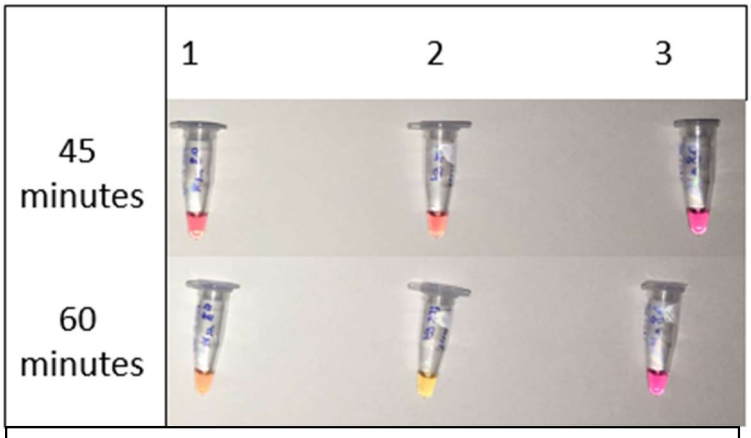


Figure 16: 0.8µM F3/B3 Improved Reaction Time: 1 – 100ng gDNA; 2 –10ng gDNA; 3 – Negative control

Once the optimal reaction temperature was established, the ideal F3/B3 primer concentrations were assessed by titrating them at 0.4µM, 0.8µM, 1.0µM, and 1.2µM. As the F3/B3 primer concentrations are increased, there should hypothetically be more starting template for the FIP and BIP to anneal and reduce the reaction time. 100ng and 10ng of gDNA were added to the tubes in lanes 1 and 2, respectively. These amounts of gDNA were used because previous studies have successfully run LAMP experiments with similar amounts of starting material (Notomi et al., 2000). After 45 minutes, there was a weak color change observed (light pink) in tubes containing gDNA and F3/B3 concentrations of 0.8µM (Figure 16). Tubes with other F3/B3 concentrations did not have an observable color change at the 45-minute time point *(data not shown)*. After 60 minutes, there was a color change (light pink to yellow) in tubes in lanes 1 and 2 (Figure 16). Due to these results, it was determined that the optimal F3/B3 concentrations for this assay design was 0.8µM.

Evaluation of B2 AS-LAMP Assay

Table 3: B2 AS-LAMP Reaction Components						
	1	2	3	4	5	6
gDNA (ng)	135	13.5	-	135	13.5	-
WT B2 primers	+	+	+	-	-	-
R132H B2 primers	-	-	-	+	+	+

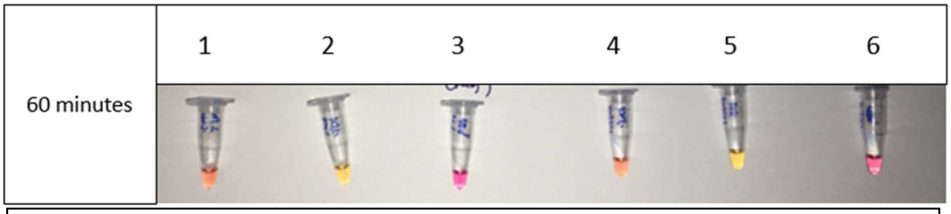


Figure 17: B2 AS-LAMP Assay Color Change Results

gDNA extracted from GBM tumors will be heterozygous for the *IDH1* mutation. As a result, clinical samples will all contain the WT allele and may contain the R132H variant if the specific mutation has occurred. Therefore, detecting amplification with the WT primers can serve as a positive amplification control in this assay, while the reaction with R132H primers should only result in a color change if the mutant allele is present. After 60 minutes, there was an observable color change in tubes containing gDNA (135ng and 13.5ng, respectively) using both primer sets (Figure 17). Analysis via gel electrophoresis confirmed the presence of LAMP products in the tubes that changed color *(data not shown)*. This indicated that the R132H AS-LAMP primer set was not allele-specific. However, the results showed that a colorimetric assay could be used to detect amplification of the *IDH1* gene.

SNP-LAMP Method

Primer Design

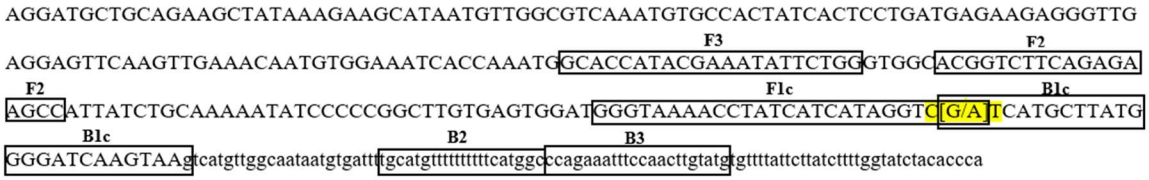


Figure 18: SNP-LAMP Primer Design

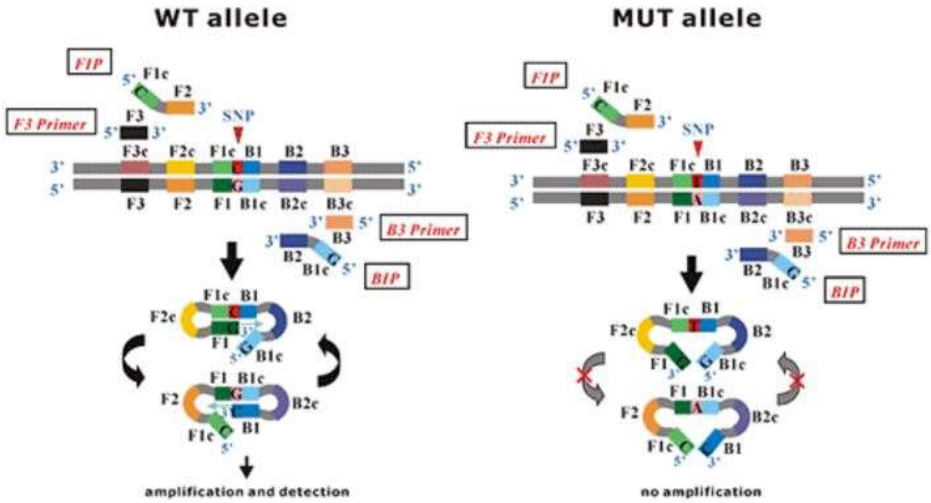


Figure 19: SNP-LAMP Reaction Scheme. Copyright ©, 2005, Eiken Chemical Co. Ltd., Japan

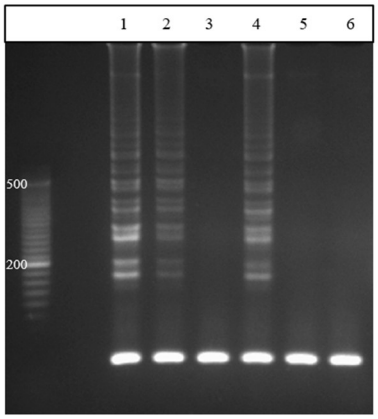
Since multiple attempts at developing an AS-LAMP method were unsuccessful, we sought to develop an alternative method with higher discriminatory power. This design was based on a previous method to detect a SNP using LAMP (Iwasaki et al., 2003). In this method, the primers are designed so that the mutation site is located on the 5'-ends of the F1c and B1c regions of the FIP and BIP primers, respectively (Figure 18). If the target contains the WT allele, the dumbbell structures of the starting template will form when using WT primers, and amplification will proceed. However, if the target DNA contains the mutant allele, the formation of the starting template will be prevented, and amplification will not occur (Figure 19).

Evaluation of SNP-LAMP Assay

Reaction components						
	1	2	3	4	5	6
gDNA (ng)	135	13.5	-	135	13.5	-
WT primers	+	+	+	-	-	-
R132H primers	-	-	-	+	+	+

A

B



C

Figure 20: Evaluation of the SNP-LAMP Assay. A) Reaction components B) Colorimetric result C) Gel electrophoresis

Assays using WT and R132H SNP-LAMP primer sets were run in parallel. The tubes containing WT primers serve as a positive control and to confirm that the LAMP assay was successful. The tubes containing R132H primers should only amplify DNA containing the mutant allele. gDNA was added to the tubes (135ng and 13.5ng, respectively), and the reactions were observed after 60 minutes. There was no color change observed in any tube (Figure 20B); however, analysis of the gel electrophoresis showed banding patterns in lanes 1, 2 and 4 – which are typical of LAMP amplification products (Figure 20C). Since a banding pattern was seen with the R132H primer set on WT DNA, this indicated that the assay did not discriminate from WT sequence.

Since both the AS-LAMP and SNP-LAMP methods had false positive results, this suggested that these assay designs were not ideal for our purposes. Although there was evidence to suggest that these primer sets could be optimized for use, the end goal for this assay is to be applied on GBM tumors containing unknown concentrations of gDNA. Therefore, it was determined that an assay needed to be developed that only amplified R132H DNA and not WT.

PNA-LAMP Method

Primer Design

AGGATGCTGCAGAAGCTATAAAGAAGCATAATGTTGGCGTCAAATGTGCCACTATCACTCCTGATGAGAAGAGGGTTG

F3 F2

AGGAGTTCAAGTTGAAACAATGTGGAAATCACCAAATGGCACCATACGAAATATTCTGGGTGGCACGGTCTTCAGAGA

F1c B1c

AGCCATTATCTGCAAAAATATCCCCCGGCTTGTGAGTGGATGGGTAAAACCTATCATCATAGGTC[G/A]TCATGCTTAT

B2 B3

GGGGATCAAGTAAgtcatgttggcaataatgtgattttgcatgttttttttttcatggccagaaatttccaacttgtatgtgtttattcttatctttggtatctacaccca

PNA(WT): GTCGTCATGCTTATGG
R132H-1: CCCATAGTCATCATGCTTATGGG
R132H-2: ATCCCCATGTCATCATGCTTATGGGGAT
R132H-3: TACTTGAGTCATCATGCTTATGGGGATCAAGTA

Figure 21: PNA-LAMP Primer Design

Since neither the AS-LAMP method nor the SNP-LAMP method were successful, additional methods to detect SNPs using LAMP were investigated. A recent study utilized a modification of the LAMP method, called PNA-LAMP, to detect the *JAK2V617F* mutation in chronic myeloproliferative neoplasms (Minnucci et al., 2012). To use this technique, the mutation site must be between F1c and F2 or B1c and B2 of the FIP and BIP, respectively (Figures 21 and 22). The PNA was designed to be complementary to the WT sequence to prevent amplification of the WT allele. The "self-annealed" loop primers (R132H-1, R123H-2, or R132H-3) were designed to be specific for the R132H variant. Due to the location of the R132H variant at the 3'-end of exon four, PNA-LAMP primers were designed so that the mutation site was located between the B1c and B2 regions. The two major advantages of this design were that only one set of LAMP

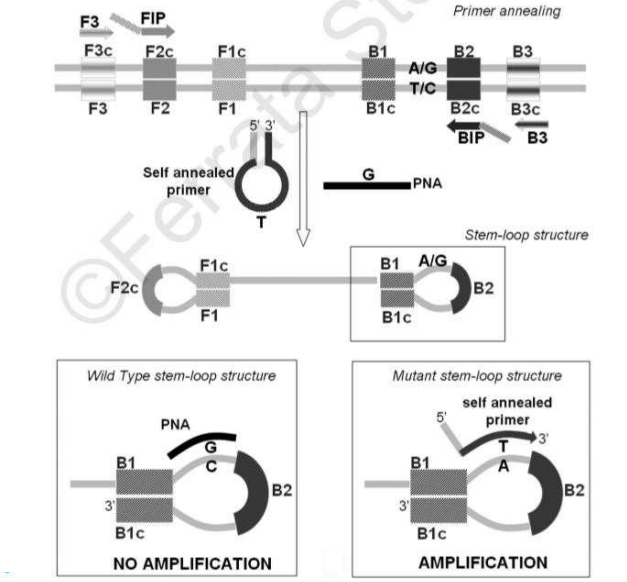


Figure 22: PNA and "Self-annealed" Loop Primer Mechanisms (Minnucci et al., 2012)

primers was required and a backward loop primer was incorporated, which should reduce the reaction time.

PNA Blocked Amplification of WT gDNA

We sought to determine if the PNA could block amplification of WT gDNA in the presence of a loop primer specific to the R132H mutation (Figure 23). After 10ng gDNA was added, there was an observable color change in one tube (lane 5). All reactions with the PNA did not have any LAMP banding patterns (lanes 3 and 7). Banding patterns were observed in lanes without the PNA (lanes 1 and 5), and the pattern in lane 5 (with loop primer) was more pronounced than in lane 1 (without loop primer). These data indicated that the PNA could block amplification of WT sequence and suggested that the R132H loop primer enhanced the LAMP reaction even on WT sequence. This meant that the ability of the PNA to block WT amplification was essential. This result suggested the feasibility of the PNA-LAMP method.

Table 4: PNA Effect on WT gDNA Reaction Components

	1	2	3	4	5	6	7	8
gDNA (ng)	10	-	10	-	10	-	10	-
PNA	-	-	+	+	-	-	+	+
R132H loop primer	-	-	-	-	+	+	+	+

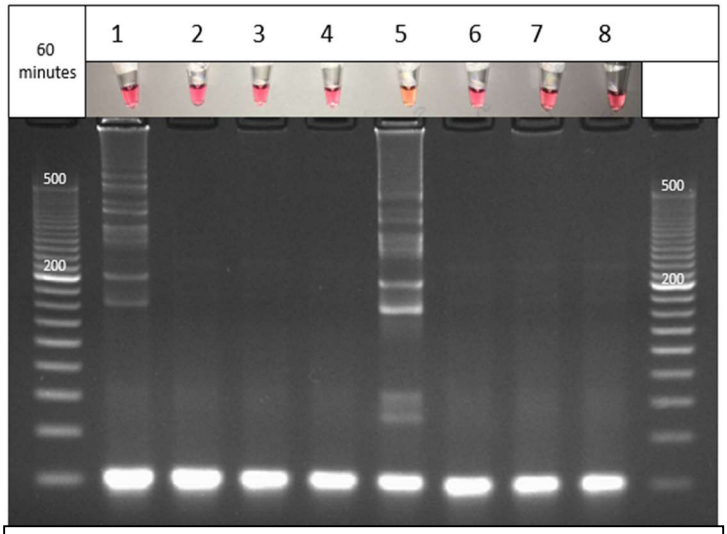


Figure 23: PNA Blocked Amplification of WT gDNA

Loop Primers Significantly Improved Reaction Time

Table 5: Effect of Loop Primers Reaction Components												
	1	2	3	4	5	6	7	8	9	10	11	12
gDNA (ng)	135	13.5	-	135	13.5	-	135	13.5	-	135	13.5	-
Forward loop primer	-	-	-	+	+	+	-	-	-	+	+	+
Backward loop primer	-	-	-	-	-	-	+	+	+	+	+	+

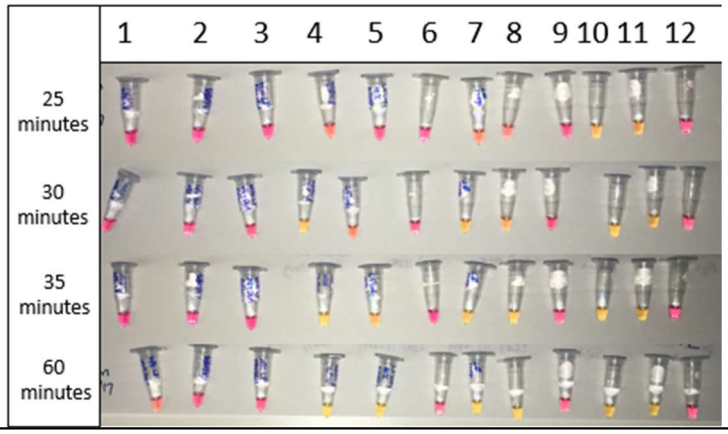


Figure 24: Loop Primers Reduced Reaction Time

Since the PNA successfully blocked amplification of WT gDNA, and the incorporation of a loop primer improved the amount of amplification, we sought to determine the effect of loop primers on the reaction (Figure 24). Previous studies have shown that loop primers can reduce the reaction time of LAMP down to less than 30 minutes (Nagamine, Hase, & Notomi, 2002). WT loop primers *(sequences not shown)* were added to the PNA-LAMP assay to amplify gDNA to evaluate their impact on the reaction. The addition of loop primers reduced the time to observable color change by 35 minutes in the tubes with both loop primers (columns 10 and 11) compared to the tubes without either loop primer (columns 1 and 2). The products were analyzed via gel electrophoresis, and the results confirmed that amplification occurred in the tubes that changed color *(data not shown)*. These results further indicated that the addition of a loop primer improved the assay.

Effect of R132H "Self-Annealed" Loop Primers on PNA-LAMP Assay

Sign	Color	Interpretation
-	pink	no amplification
+	light pink	weak amplification
++	orange	amplification
+++	yellow	strong amplification

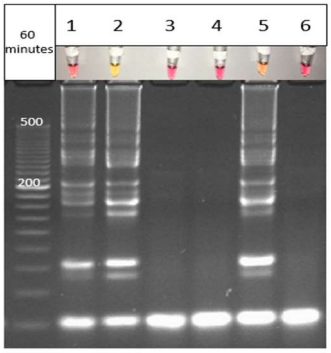
A

Reaction components						
	1	2	3	4	5	6
PNA	-	-	-	+	+	+
WT DNA (copy #)	$6.0x10^3$	-	-	$6.0x10^3$	-	-
R132H DNA (copy #)	-	$6.0x10^3$	-	-	$6.0x10^3$	-

B

Reaction Time (min)	1	2	3	4	5	6
20	-	-	-	-	-	-
25	-	-	-	-	-	-
30	-	-	-	-	-	-
35	-	-	-	-	-	-
40	-	+	-	-	-	-
45	-	+++	-	-	-	-
50	-	+++	-	-	-	-
55	-	+++	-	-	+	-
60	+	+++	-	-	+++	-

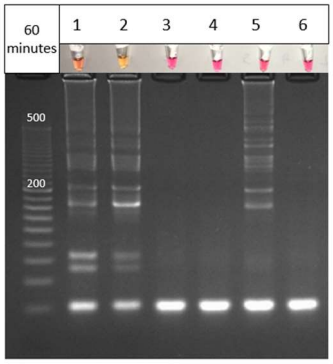
C



D

Reaction Time (min)	1	2	3	4	5	6
20	-	-	-	-	-	-
25	-	-	-	-	-	-
30	-	-	-	-	-	-
35	-	-	-	-	-	-
40	-	-	-	-	-	-
45	-	+	-	-	-	-
50	-	+++	-	-	-	-
55	-	+++	-	-	-	-
60	+	+++	-	-	-	-

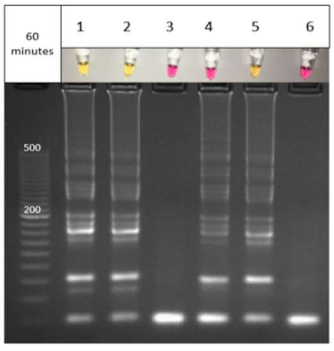
E



F

Reaction Time (min)	1	2	3	4	5	6
20	-	-	-	-	-	-
25	-	-	-	-	-	-
30	+	+++	-	-	-	-
35	+++	+++	-	-	+	-
40	+++	+++	-	-	+++	-
45	+++	+++	-	-	+++	-
50	+++	+++	-	-	+++	-
55	+++	+++	-	-	+++	-
60	+++	+++	-	+	+++	-

G



H

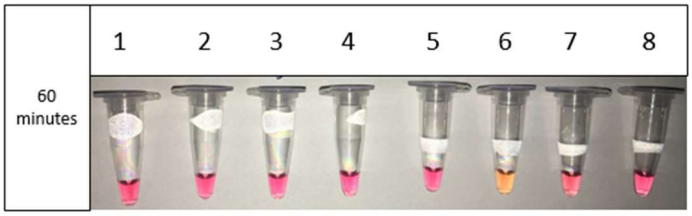
Figure 25: Effect of R132H "Self-annealed" Loop Primers on PNA-LAMP. A) Color interpretation key B) Reaction components C) Time to color change with R132H-1 loop primer D) Gel analysis results with R132H-1 loop primer E) Time to color change with R132H-2 loop primer F) Gel analysis results with R132H-2 loop primer G) Time to color change with R132H-3 loop primer H) Gel analysis results with R132H-3 loop primer

The principle behind the PNA-LAMP method is that the PNA competitively inhibits the loop primer from amplifying the WT sequence. Multiple "self-annealed" loop primers (R132H-1, R132H-2, and R132H-3) of varying lengths were designed to determine which was optimal for use with this PNA. Three experiments were run with identical primer concentrations and equal amounts of WT or R132H DNA (Figure 25). The reaction tubes were observed every five minutes starting at the 20-minute mark through 60 minutes. The interpretations of the color change were recorded, and the LAMP products were analyzed via agarose gel electrophoresis. After 60 minutes, the PNA-LAMP assay with R132H-1 blocked WT amplification and amplified R132H DNA (Figures 25C and 25D). There was no observable color change when R132H DNA was added to the PNA-LAMP assay with R132H-2; however, the gel analysis results showed a banding pattern indicative of amplification in lane 5 (Figures 25E and 25F). Finally, the PNA-LAMP assay with R132H-3 did not block WT amplification in lane 4 (Figures 25G and 25H). Collectively, these results suggested that the R132H-1 loop primer worked best in the PNA-LAMP assay; therefore, the R132H-1 loop primer was selected.

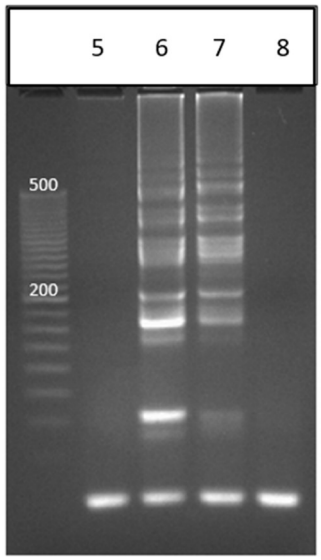
Reaction Temperature Optimization with PNA

Reaction Components								
	61°C				63°C			
	1	2	3	4	5	6	7	8
PNA	+	+	+	+	+	+	+	+
WT DNA	$5.0x10^5$	-	-	-	$5.0x10^5$	-	-	-
R132H DNA	-	$5.0x10^5$	$5.0x10^3$	-	-	$5.0x10^5$	$5.0x10^3$	-

A



B



C

Figure 26: PNA-LAMP Reaction Temperature Optimization. A) Reaction components B) PNA-LAMP assay with R132H-1 at 61°C (lanes 1-4) and 63°C (lanes 5-8) C) Gel electrophoresis

To maximize the effectiveness of the PNA in combination with the R132H-1 loop primer, we sought to determine the lowest reaction temperature the PNA-LAMP assay could function without hindering the visual detection. It has already been shown that the PNA-LAMP assay was functional at 65°C, but performing the assay at a lower reaction temperature may improve the ability of the PNA to suppress amplification of the WT allele. To determine the optimal reaction temperature, PNA-LAMP with R132H-1 was run at two temperatures: 61°C and 63°C (Figure 26). Preliminary research had shown that LAMP did not function as efficiently at temperatures lower than 61°C *(data not shown)*. There was no visible color change at 61°C, but there was a color indicative of a positive reaction at 63°C (Figure 26B, lane 6). The products from the tubes incubated at 63°C were analyzed by gel electrophoresis, and the results were indicative of amplification when R132H DNA was present (Figure 26C, lanes 6 and 7). Lane 5 also showed that

amplification of WT was blocked (Figure 26C). These data suggested that the PNA-LAMP assay with R132H-1 functioned as desired at 63°C. Therefore, subsequent experiments were run at this temperature.

Limit-of-Detection (LOD)

Table 6: PNA-LAMP LOD on Straight R132H DNA Reaction Components												
	1	2	3	4	5	6	7	8	9	10	11	12
PNA	-	+	+	+	+	+	+	+	+	+	+	-
WT DNA (copy #)	$5.0x10^5$	$5.0x10^5$	-	-	-	-	-	-	-	-	-	-
R132H DNA (copy #)	-	-	$5.0x10^5$	$5.0x10^4$	$4.0x10^4$	$3.0x10^4$	$2.0x10^4$	$1.0x10^4$	$5.0x10^3$	-	-	-
H_2O (blank)	-	-	-	-	-	-	-	-	-	+	-	-
E. coli DNA (negative control)	-	-	-	-	-	-	-	-	-	-	+	+

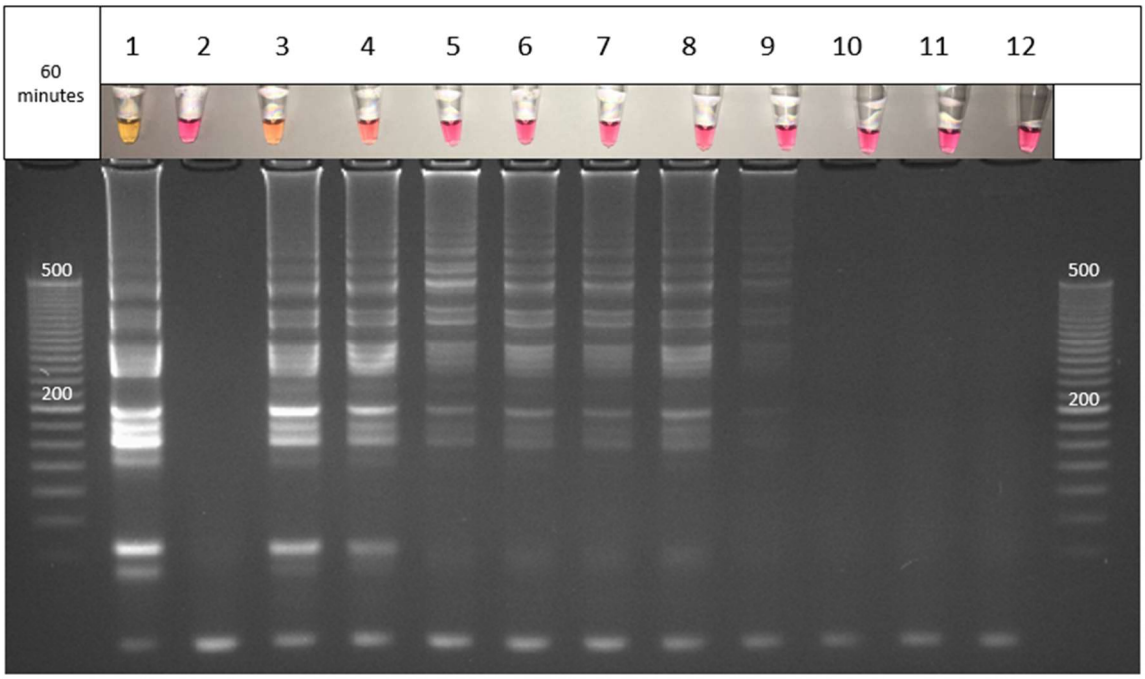


Figure 27: PNA-LAMP LOD on R132H DNA Diluted in Sterile H_2O

The LOD, also known as analytical sensitivity, is defined as the lowest amount of target sequence that the assay can detect. Characterization of the LOD of the PNA-LAMP assay was conducted a total of three times with synthetic R132H DNA diluted into sterile

H_2O at the following copy numbers: $5.0x10^5$, $5.0x10^4$, $4.0x10^4$, $3.0x10^4$, $2.0x10^4$, $1.0x10^4$, and $5.0x10^3$ (Figure 27). A color change was observed down to $5.0x10^4$ copies of R132H DNA. Additionally, gel analysis showed LAMP banding patterns in all samples at $5.0x10^3$ copies R132H DNA or higher.

Table 7: PNA-LAMP LOD on R132H DNA Diluted in gDNA Reaction Components

	1	2	3	4	5	6	7	8	9	10	11	12	13
PNA	-	+	+	+	+	+	+	+	+	+	+	+	-
WT gDNA (copy #)	$3.0x10^4$	$3.0x10^4$	$3.0x10^4$	$3.0x10^4$	$3.0x10^4$	$3.0x10^4$	$3.0x10^4$	$3.0x10^4$	$3.0x10^4$	$3.0x10^4$	-	-	-
R132H DNA (copy #)	-	-	$5.0x10^5$	$5.0x10^4$	$4.0x10^4$	$3.0x10^4$	$2.0x10^4$	$1.0x10^4$	$5.0x10^3$	$5.0x10^2$	-	-	-
H_2O (blank)	-	-	-	-	-	-	-	-	-	-	+	-	-
E. coli DNA (negative control)	-	-	-	-	-	-	-	-	-	-	-	+	+

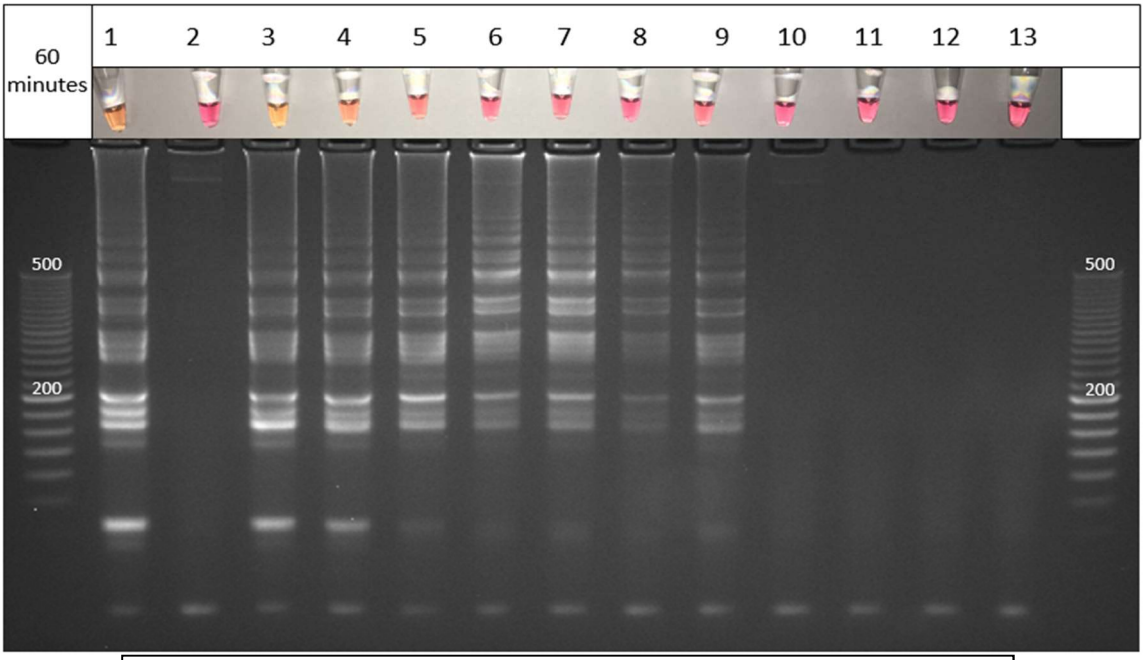


Figure 28: PNA-LAMP LOD on R132H DNA Diluted in gDNA

Next, we sought to determine the assay's ability to detect R132H DNA in a background of WT gDNA. The PNA-LAMP assay was run a total of three separate times on dilutions of synthetic R132H DNA spiked into 100ng gDNA (approximately $3.0x10^4$ copies of the *IDH1* gene) at the following copy numbers: $5.0x10^5$, $5.0x10^4$, $4.0x10^4$, $3.0x10^4$, $2.0x10^4$, $1.0x10^4$, $5.0x10^3$, and $5.0x10^2$ (Figure 28). As previously observed, the

visual detection limit was determined to be $5.0x10^4$ copies, and gel analysis results showed a banding pattern down to $5.0x10^3$ copies. These data showed that the higher complexity of this LOD study did not negatively impact the analytical sensitivity of the assay, and the assay detected $5.0x10^3$ copies of *IDH1* R132H in a background of $3.0x10^4$ copies *IDH1* WT (16.67%). Since analysis of the gel electrophoresis results indicated a tenfold greater sensitivity compared to the colorimetric analysis, the colorimetric component of the assay should be further optimized to take advantage of the analytical sensitivity of the method.

LAMP Can Be Applied to Whole Cell Lysates

Table 8: LAMP on Whole Cell Lysates Reaction Components

	1	2	3	4	5	6
Cell lysates (frozen)	+	-	-	-	-	-
Cell lysates (heated)	-	+	-	-	-	-
gDNA (ng)	-	-	135	13.5	-	-
E. coli DNA (negative control)	-	-	-	-	+	-
H_2O (blank)	-	-	-	-	-	+

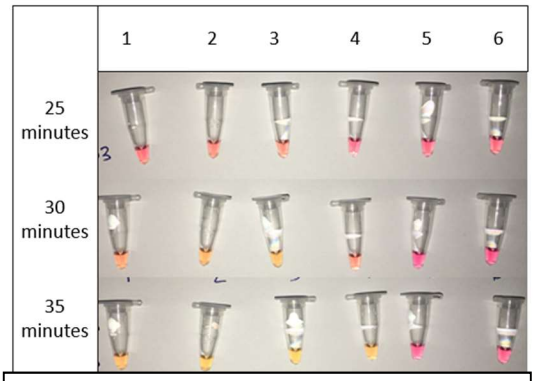


Figure 29: LAMP of Whole Cell Lysates Without DNA Extraction

One of the benefits of the LAMP method is that it can be applied to whole cell lysates, eliminating the need for a DNA extraction step (Geojith et al., 2011). The LAMP method, with WT loop primers *(sequences not shown)* and without the PNA, was run at 65°C on lysates from heated or frozen buccal cells for 60 minutes (Figure 29). There was a significant color change in the tubes containing cell lysates after 35 minutes, and gel analysis confirmed the presence of LAMP products in the tubes that changed color *(data not shown)*. The results indicated that IDH1 gene could be detected via LAMP on cell lysates and suggested that the *IDH1* mutation status could be identified via LAMP on whole GBM tumor specimens without DNA extraction.

PNA-LAMP Assay Detected R132H Sequence Diluted in Whole Cell Lysates

Table 9: PNA-LAMP on R132H DNA Diluted in Whole Cell Lysates Reaction Components							
	1	2	3	4	5	6	7
PNA	-	+	+	-	-	+	+
Cell lysates	+	+	+	-	-	-	-
R132H DNA (copy #)	-	-	$5.0x10^5$	-	-	-	-
H_2O (blank)	-	-	-	+	-	+	-
E. coli DNA (negative control)	-	-	-	-	+	-	+

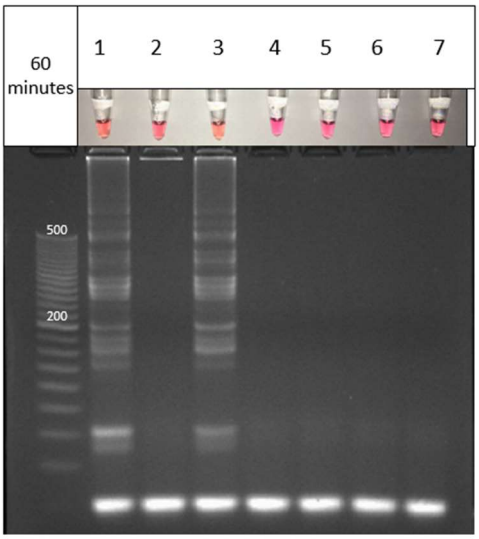


Figure 30: Detection of R132H Sequence Diluted into Whole Cell Lysates

Since it was shown that the assay could detect R132H DNA in the presence of gDNA, and the LAMP method could be applied on cell lysates, the PNA-LAMP assay's ability to detect the R132H DNA in the presence of cell lysates was evaluated (Figure 30). Cell lysates were added to tubes with and without PNA (lanes 1 and 2, respectively), while R132H DNA diluted in cell lysates was applied to a tube with PNA (lane 3). After 60 minutes, there was a minor color change observed in lanes 1 and 3, and the banding patterns visible from the gel analysis confirmed the presence of LAMP products in these lanes. Furthermore, no banding pattern was visible in lane 2. These data suggested that the assay can detect the R132H sequence, while preventing amplification of the gDNA from the lysed cells. This experiment showed the potential of the PNA-LAMP method to be used on whole tumor specimens in an intraoperative, time-sensitive setting where DNA extraction may not be feasible.

CHAPTER FOUR: DISCUSSION

This study was the first to attempt to develop a colorimetric LAMP-based assay to detect the *IDH1* R132H mutation in GBM, and it is only the third time a PNA-LAMP design has been used for biomarker identification in cancer. The results of this study show proof of principle that a colorimetric PNA-LAMP method can detect the *IDH1* R132H variant in GBM with high sensitivity and speed. We have also provided evidence that this assay design can be adapted for use on whole tumor specimens in a time-sensitive, intraoperative setting.

The work performed has an impact on patient outcomes because there is no current method that provides the *IDH1* mutation status in a time frame acceptable for an intraoperative setting. Sanger sequencing is the gold standard for the identification of the *IDH1* mutation status, but the process is time-consuming, and the sensitivity is reported at approximately 20% mutant alleles in a background of normal alleles (Horbinski, Kofler, Kelly, Murdoch, & Nikiforova, 2009). We have presented evidence that the PNA-LAMP assay has a sensitivity of 16.67% mutant alleles in a background of WT alleles. Another group developed a pyrosequencing-based platform with improved sensitivity, but the set-up time, run time, and data analysis take multiple hours (Felsberg et al., 2010). Previous studies have also developed real-time PCR designs such as real-time reverse transcription-PCR with fluorescence melting curve analysis (Horbinski, Kelly, Nikiforov, Durso, & Nikiforova, 2010), qPCR with Amplification Refractory Mutation System (Catteau et al., 2014), and co-amplification at lower temperature-PCR with high-resolution melting (Boisselier et al., 2010). Interestingly, the qPCR with Amplification

Refractory Mutation System is capable of identifying six *IDH1* mutations, including the R132H variant, in glioma by utilizing a PCR-clamping probe design similar to the one used in our study (Catteau et al., 2014). Although this technique is rapid and highly sensitive, it can only be performed with extracted and purified DNA samples, which limits its usefulness in intraoperative settings. We provided evidence that our assay can be adapted for use on whole cell lysates. By eliminating the DNA extraction step, our assay not only saves time, but does not require trained laboratory personnel to evaluate the qualitative results.

All the platforms mentioned above also require expensive equipment that is not commonly available in hospital labs. The colorimetric design of our assay is desirable because a color change visible to the naked eye is indicative of a positive reaction for the *IDH1* R132H variant. Our study focused on this modification and demonstrated that it can be applied to *IDH1* mutation characterization, but there are also other amplification monitoring systems that can be used with LAMP. In particular, turbidity of magnesium pyrophosphate can be visually observed due to the presence of a white precipitate if amplification occurred (Mori, Nagamine, Tomita, & Notomi, 2001). Although accurate assessment of turbidity would require a turbidimeter this could potentially alleviate the difference between the visual detection limit and the LOD of the gel analysis discovered in our study. Another benefit of using a turbidimeter is the capability of analyzing amplification in real-time, which may further reduce the time to an observable positive reaction. Incorporating other detection reagents such as ethidium bromide, SYBR Green I, calcein, and most recently, malachite green, into LAMP reactions has also shown potential. The use of malachite green is advantageous because it does not require UV

illumination or fluorescence analysis for LAMP product discrimination (Lucchi, Ljolje, Silva-Flannery, & Udhayakumar, 2016). If amplification occurs, the solution turns blue/green, but remains colorless if the reaction is negative. This is another modification worth examining because malachite green provides a visual indication of amplification similar to the one used in our study (phenol red).

Since a colorimetric LAMP method had never been applied to GBM characterization, we developed several methods to determine if it was feasible. The location of the allele of interest played a significant role in method development. As previously mentioned, the codon is located at the 3'-distal region of exon four in the *IDH1* gene, so we hypothesized that an AS-LAMP method with the 3'-end of the B3 primer targeting that region would be ideal because it avoided the downstream intron. We were not able to detect a visual color change with the primers specific for WT gDNA, so we determined that it would not work for our purposes. A recent study employed another AS-LAMP technique that utilized the BIP to target the *kdr* mutation responsible for insecticide resistance in a population of mosquitos (Badolo et al., 2012). In our study, this design failed to differentiate between WT and R132H DNA. Although the mismatch should have prevented amplification through the BIP, it is possible that the other LAMP primers contributed to amplification of target DNA sufficient to detect a color change. Furthermore, the study by Badolo et al. (2012) incorporated an additional mismatched nucleotide alongside the mismatch specific for the mutation of interest. This could explain why they achieved allele specificity, while our design did not. Since a color change was visible at the 45-minute mark when these primers were used to amplify WT

gDNA *(data not shown)*, developing new B2 AS-LAMP primers with the additional mismatch is something that should be considered.

To our knowledge, previous attempts to apply the LAMP method on the human genome have focused on inherited diseases rather than acquired point mutations (Zhang et al., 2016). This may explain their success in developing AS-LAMP and SNP-LAMP methods as inherited mutations are present in a higher abundance throughout the genome. However, the findings from two previous studies suggested that the PNA-LAMP design could be applied to detect acquired point mutations in cancer (Itonaga et al., 2016; Minnucci et al., 2012). Based on this evidence, we determined that PNA-LAMP was the most effective way to specifically detect the *IDH1* R132H mutation. After successfully showing that the PNA could block WT gDNA, we sought to determine what the best "self-annealed" loop primer was in relation to the PNA by testing the assay with loop primers of various lengths (R132H-1, R132H-2, and R132H-3). Upon analysis, it was determined that the R132H-1 loop primer was the most effective. The R132H-2 loop primer with the PNA did not amplify R132H DNA sufficient to detect an observable color change, which may have been due to the four G/C repeats located in the self-complementary regions of the loop primer preventing hybridization to the target DNA. The R132H-3 loop primer was complementary to the entire sequence between the B1c and B2 region on the dumbbell structure of the starting template of LAMP. The high specificity of this loop primer for the target region may have played a role in outcompeting the PNA in this assay design. This evidence suggests that additional PNA designs need to be assessed. The PNA used in this study was 16-mer and had a T_m of 72.2°C. The T_m can be raised to 76.0°C by incorporating an additional nucleotide on the

3’-end. This could improve the assay because it would reduce the likelihood of the PNA melting off its target at the optimized reaction temperature. It should be noted that the PNA cannot be extended any further on the 3’-end because of four consecutive guanines on the target sequence. This is known as a guanine quadruplex (G-quadruplex) and is problematic in amplification reactions and oligonucleotide synthesis. G-quadruplexes negatively affect amplification reactions similar to hairpin structures by preventing the primer from annealing to the target. These structures also make it difficult to manufacture the oligonucleotides and result in lower yield and purity (“IDT DNA DECODED Newsletter,” n.d.). Additional nucleotides can be added to the 5’-end of the PNA, but it would enter into the target sequence that the B2 region of the BIP anneals to. Hypothetically, this could also improve the assay by preventing the BIP from annealing in the presence of the PNA.

The two previous studies that applied the PNA-LAMP method to biomarker identification did not determine the amount of WT DNA in which their PNA could suppress amplification. This is important data to collect during future characterization of the assay described in this study because it will eventually be applied to samples with unknown concentrations of WT DNA. In our study, the data suggests that 0.25µM PNA can block at least $5.0x10^5$ copies of *IDH1* WT DNA. The LOD studies revealed a large discrepancy between the number of copies that can be visually detected ($5.0x10^4$ copies R132H DNA) and the number of copies detected through gel electrophoresis analysis ($5.0x10^3$ copies R132H DNA). This suggests that the visual detection of the assay may be improved tenfold. To remedy this difference, an in-house colorimetric mix can be developed with a lower buffering capacity to allow for the visual detection of lower

amounts of amplification. Furthermore, upgrading to the more efficient *Bst* 3.0 enzyme could improve the time to observable color change of the assay as well as lower the limit of detection. According to New England Biolabs, this enzyme reaches threshold time 10-15 minutes faster than the *Bst* 2.0 enzyme used in our study ("NEB Newsletters - Bst 3.0 Rapid LAMP-validated Detection for MDx Application," n.d.).

Currently, we are working on improving the assay. As the primers used in our study were suspended in IDTE buffer (1X TE solution, 10mM Tris, 0.1mM EDTA), this may have provided additional buffering capacity and reduced the effectiveness of the colorimetric master mix. Furthermore, the group that detected the *KRAS* mutation in colorectal and pancreatic cancers utilized locked nucleic acids (LNAs) rather than "self-annealed" loop primers (Itonaga et al., 2016). The advantage of using LNAs is the ability to detect mutant subtypes other than the R132H variant, and we are considering their use in our assay. To take this a step further, multiple PNAs can be developed to block amplification of the different *IDH1* mutant subtypes, which would hypothetically provide a more accurate characterization of the GBM mutation status. There are also other variations of LAMP that can be assessed such as reverse transcription LAMP (RT-LAMP) and methylation specific LAMP (MS-LAMP). RT-LAMP could alleviate the problems that the location of the allele poses and potentially improve the sensitivity of the assay. A recent study developed a RT-LAMP assay to detect the tomato torrado virus and reported a sensitivity tenfold higher compared to reverse transcription PCR (Budziszewska, Wieczorek, & Obrępalska-Stęplowska, 2016). Another group developed a RT-LAMP assay that could detect a single copy of ZIKV RNA in human whole blood, eliminating the need for RNA extraction (Lee et al., 2016). MS-LAMP could provide

valuable information for GBM patients because the methylation status of the MGMT promoter is another prognostic biomarker and indicator of the patient's response to temozolomide treatment (Hegi et al., 2005). A group reported a MS-LAMP assay sensitivity of 0.1% methylated DNA in the presence of large quantities of unmethylated DNA (Wen et al., 2016). Finally, we plan to continue to develop the colorimetric PNA-LAMP assay for use on gDNA extracted from established GBM cell lines, then on crude extracts and clinical samples, and eventually validate it for use in an intraoperative setting.

Overall, this study showed proof of principle that a colorimetric LAMP assay can be used to rapidly detect acquired point mutations in cancer. The work described here has a direct impact on patient outcomes because of the potential survival benefit associated with knowing the *IDH1* mutation status of GBM patients prior to surgery. Furthermore, our evidence suggests the PNA-LAMP assay has a greater sensitivity compared to the current gold standard. Although we focused on the *IDH1* mutation, this technology is not limited to only GBM, but can be adapted for other common diagnostic and prognostic biomarkers.

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Appendix A

Table of Calculations for Synthetic Plasmid DNA Copy Numbers

Mass of gDNA per one copy of IDH1 (ng)
3.30E-03

Size of plasmid vector (bp)	Mass of DNA per base pair (ng/bp)	Mass of one pDNA molecule (ng)
3.30E+03	1.096E-12	3.62E-09

Copy #	mass of gDNA (ng)	Final Conc of gDNA (ng/uL)
5000000	1.65E+04	3.30E+03
500000	1.65E+03	3.30E+02
50000	1.65E+02	3.30E+01
40000	1.32E+02	2.64E+01
30000	9.90E+01	1.98E+01
20000	6.60E+01	1.32E+01
10000	3.30E+01	6.60E+00
5000	1.65E+01	3.30E+00
500	1.65E+00	3.30E-01
50	1.65E-01	3.30E-02
5	1.65E-02	3.30E-03

Copy #	mass of plasmid (ng)	Final Conc of pDNA (ng/uL)
500000000	1.81E+00	3.62E-01
50000000	1.81E-01	3.62E-02
5000000	1.81E-02	3.62E-03
500000	1.81E-03	3.62E-04
50000	1.81E-04	3.62E-05
40000	1.45E-04	2.89E-05
30000	1.09E-04	2.17E-05
20000	7.23E-05	1.45E-05
10000	3.62E-05	7.23E-06
5000	1.81E-05	3.62E-06
500	1.81E-06	3.62E-07
50	1.81E-07	3.62E-08
5	1.81E-08	3.62E-09

Dilution #	Source of pDNA for Dilution	C1 = Initial Conc. (ng/uL)	V1 = Volume of pDNA (uL)	Volume of Diluent (uL)	V2 = Final Volume (uL)	C2 = Final Conc. In (ng/uL)	Resulting Copy # of IDH1 Sequence / 5uL
500M	Stock	2.00E+00	**27.1**	**122.9**	150	3.62E-01	500000000
50M	500M	3.62E-01	**15.0**	**135.0**	150	3.62E-02	50000000
5M	50M	3.62E-02	**15.0**	**135.0**	150	3.62E-03	5000000
500k	5M	3.62E-03	**5.0**	**45.0**	50	3.62E-04	500000
50k	500k	3.62E-04	**5.0**	**45.0**	50	3.62E-05	50000
40k	500k	3.62E-04	**4.0**	**46.0**	50	2.89E-05	40000
30k	500k	3.62E-04	**3.0**	**47.0**	50	2.17E-05	30000
20k	500k	3.62E-04	**2.0**	**48.0**	50	1.45E-05	20000
10k	50k	3.62E-05	**10.0**	**40.0**	50	7.23E-06	10000
5k	50k	3.62E-05	**5.0**	**45.0**	50	3.62E-06	5000
500	5k	3.62E-06	**5.0**	**45.0**	50	3.62E-07	500