Biosensors and Bioelectronics 267 (2025) 116813

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

A novel platform for mutation detection in colorectal cancer using a PNA-LNA molecular switch

Md Sajedul Islam [a], Sharmin Aktar [a], Neda Moetamedirad [a], Nan Xie [a], Cu Tai Lu [a,b], Vinod Gopalan [a,**], Alfred K. Lam [a,c,***], Muhammad J.A. Shiddiky [d,*]

[a] School of Medicine & Dentistry, Griffith University, Gold Coast Campus, Southport, QLD 4222, Australia
[b] Department of Surgery, Gold Coast University Hospital, Southport, QLD, 4215, Australia
[c] Pathology Queensland, Gold Coast University Hospital, Southport, QLD, 4215, Australia
[d] Rural Health Research Institute, Charles Sturt University, Orange, NSW, 2800, Australia

ARTICLE INFO

Keywords:
Colorectal cancer
KRAS
Isothermal amplification
PNA
LNA

ABSTRACT

Detection of *KRAS* mutation in colorectal cancer (CRC) is important in the prediction of response to target therapy. The study aims to develop a novel mutation detection platform called the "PNA-LNA molecular switch" for the detection of *KRAS* mutation in CRC. We employed the enhanced binding specificity of peptide nucleic acid (PNA) and locked nucleic acid (LNA) in conjunction with a loop-mediated isothermal amplification (LAMP) approach to identify the mutation status of *KRAS* oncogene codon 12 (c.35G>T/G12V and c.35G>A/G12D) using synthetic oligonucleotides and colon cancer cell lines (Caco-2 and SW480). This method specifically blocked the amplification of the wild-type sequences while substantially amplifying the mutated ones, which was visualized by both colorimetric and fluorescence assays. We then checked the mutation profile of *KRAS* codon 12 in the DNA derived from tumor tissue samples (number of samples, $n = 30$) and circulating tumor cells ($n = 24$) from CRC patients. Finally, we validated the results by comparing them with the data obtained from DNA sequencing of colon tumors ($n = 21$) of the same CRC patients. This method showed excellent sensitivity (1 DNA copy/μl), reproducibility [relative standard deviation (%RSD) < 5%, for n = 3], and linear dynamic range (1 ag/μl-10 pg/μl, $R^2 = 0.94$). This platform is significantly faster, relatively cheaper, has superior sensitivity and specificity, and does not require any high-end equipment. To conclude, this method has the potential to be translated into clinical settings for the detection of mutations in diverse diseases and conditions.

1. Introduction

According to the World Health Organization (WHO), colorectal cancer (CRC) is the third most diagnosed cancer and second in terms of mortality. In terms of financial burden, CRC is second to only breast cancer, accounting for 12.6% of all cancer treatment costs (Mariotto et al., 2020). Complications associated with CRC surface at the advanced stages, which quite often leads to fatality. The overall 5-year survival rates of colon cancer and rectal cancer are 64% and 67%, respectively. Depending on the stage and the spread of the cancer, the 5-year survival rate can drastically decrease from 91% to 14% (Shin et al., 2023).

KRAS is a Kirsten Ras oncogene homolog from the mammalian *RAS* gene family that encodes a protein belonging to the GTPase superfamily. KRAS protein conveys the signal from the ligand-bound epidermal growth factor receptor (EGFR) to the nucleus. Wild-type KRAS, when activated, binds to GTP and activates different downstream effectors, including BRAF, mTOR, MEK, ERK, AKT, and PIK3CA. This leads to apoptosis prevention, cell proliferation, promotion of cellular growth, angiogenesis, migration, and differentiation (Malumbres and Barbacid, 2003; Schubbert et al., 2007). *KRAS* is one of the most frequently altered genes with mutations accounting for 17–25% of all cancers. Moreover, about 35–50% of the patients with CRC have mutations in the *KRAS* oncogene (Formica et al., 2022; Dai et al., 2020). Nearly 85% of *KRAS* mutations occur in one of the three mutation hotspots in codons 12, 13, or 61. Among them, mutation at codon 12 is the most prevalent (65%), and the two most frequent subtypes in CRC are G12V (c.35G>T), and

* Corresponding author.
** Corresponding author.
*** Corresponding author. School of Medicine & Dentistry, Griffith University, Gold Coast Campus, Southport, QLD 4222, Australia.
E-mail addresses: v.gopalan@griffith.edu.au (V. Gopalan), a.lam@griffith.edu.au (A.K. Lam), mshiddiky@csu.edu.au (M.J.A. Shiddiky).

https://doi.org/10.1016/j.bios.2024.116813
Received 7 August 2024; Received in revised form 18 September 2024; Accepted 23 September 2024

G12D (c.35G>A) alterations (Haigis, 2017). Mutations in the *KRAS* oncogene hamper the efficacy of anti-EGFR therapy at the downstream pathway (Liu et al., 2019; Ung et al., 2014). *KRAS* point mutations can be a good predictor for the therapeutic effect of cetuximab (inhibitor of EGFR), which can be measured by overall survival and progression-free survival (Karapetis et al., 2008). Thus, knowing if a CRC contains the *KRAS* mutations is crucial to predicting the response to anti-EGFR therapy.

Various methods, such as polymerase chain reaction (PCR), direct sequencing, amplification refractory mutation systems, and surface-enhanced Raman spectroscopy, have widely been utilized for the detection of single nucleotide polymorphisms (SNPs). However, these methods often face challenges such as amplification error rates, issues with inhibitors, prolonged analysis times, limitations in detecting low allelic frequencies, or reliance on expensive and sophisticated instrumentations that may necessitate expert personnel (Islam et al., 2023). Among these techniques, direct sequencing stands out as the most reliable and widely used method for identifying genetic variations, specifically point mutations, within a gene sequence. However, direct sequencing has its limitations, particularly in clinical settings. It may lack the sensitivity required to detect specific point mutations with low allelic frequencies within a heterogeneous mixture of sequences (Anderson, 2011). Additionally, implementing direct sequencing in point-of-care (POC) or on-site clinical settings can be complex and time-consuming. To address some of these challenges, we have used a loop-mediated isothermal amplification (LAMP)-based detection method that incorporates peptide nucleic acid (PNA)-locked nucleic acid (LNA) molecular switch technology. This method can detect specific point mutations with superior sensitivity and specificity in a very short amount of time compared to traditional methods.

PNA is a synthetic DNA analog that contains a 2-aminoethylglycine chain replacing the usual phosphodiester backbone (Menchise et al., 2003). The melting temperature (T_m) of a PNA-DNA duplex is considerably higher and a single base-pair mismatch can lower the T_m up to 10–18°C. This large T_m difference makes it a good sensor for detecting point mutations. It can resist the annealing and exonuclease activities of polymerase, blocking the amplification of the targeted sequence (Sun et al., 2002). On the contrary, LNA contains a methylene bridge between the 2′-oxygen and the 4′-carbon atoms of the ribose, making the structure more stable (Obika et al., 1997). It can significantly enhance the amplification of the targeted sequence due to its superior structural stability. By leveraging the alternating characteristics of PNA and LNA, we can accurately target specific mutations by blocking the amplification of the wild-type sequence while selectively amplifying the mutated one. This strategy has been applied in several studies. For instance, Nagai et al. demonstrated the genetic heterogeneity of the EGFR gene in non-small cell lung cancer using a combination of PNA and LNA (Nagai et al., 2005). Similarly, Itonaga and colleagues employed PNA-LNA-mediated LAMP to detect *KRAS* mutations in pancreatic cancer cells using complementary DNA (cDNA) (Itonaga et al., 2016). In 2018, Guojun Cao et al. utilized PNA and LNA with a microfluidic platform to distinguish between Calreticulin type 1 and type 2 mutations in myeloproliferative neoplasms (Cao et al., 2018). Additionally, N. Sharma et al. developed a PNA-LNA-LAMP assay to identify an SNP linked to resistance against QoI fungicides in *Erysiphe necator* (Sharma et al., 2023). More recently, Kristian A. Choate and colleagues used a LNA-mediated isothermal amplification technique to design a genotyping panel capable of discriminating among five variants of the *IDH1*-R132 mutation (Choate et al., 2024).

In this study, we developed an isothermal amplification-based method with a PNA probe and LNA primers to detect the most prominent mutations in codon 12 (G12V and G12D) in *KRAS* oncogene using synthetic oligonucleotides and colon cancer cell lines. We then identified the mutation profile of *KRAS* codon 12 in the DNA extracted from cancer tissue samples and circulating tumor cells (CTCs) derived from CRC of the adenocarcinoma subtype. Lastly, we validated the findings of this study by comparing them with the High-Resolution Melt (HRM) curve and Sanger sequencing data. While several previous studies have utilized PNA and LNA along with isothermal amplification techniques, this study is the first to apply these methods to genomic DNA isolated from CTCs and primary tissues of colorectal cancer patients. What distinguishes this research from similar studies is its combination of synthetic oligonucleotides with clinical samples, followed by validation using HRM analysis and Sanger sequencing. This comprehensive approach enhances the study's reliability and sets it apart from earlier work in the field.

2. Material and methods

All reagents used in this study were of molecular biology grade and were utilized without additional purification. A specific set of four primary LAMP primers targeting the *KRAS* gene, along with a clamping PNA probe against the wild-type sequence, were employed. Additionally, two LNA primers were developed to selectively detect the G12V and G12D mutations (Supplementary Table S1). For cell line experiments, two colon cancer cell lines were used: Caco-2 (wild-type *KRAS*) and SW480 (*KRAS* G12V). Peripheral blood and colon tumor tissues were collected from CRC patients, from which genomic DNA was extracted, both from the tumor tissues and circulating tumor cells (CTCs). Both colorimetric and fluorescent LAMP assays were performed simultaneously in the same PCR tube for each experiment. DNA extracted from CTC-positive CRC tissue samples was further analyzed using HRM analysis, followed by Sanger sequencing for mutation validation. Chromatograms were examined, and sequences were compared to the reference *KRAS* gene to confirm the presence of mutations. Detailed experimental procedures are provided in **Supplementary Information**.

3. Results

3.1. Principle of PNA-LNA molecular switch

Fig. 1 depicts the general principle of the method. In brief, we designed a clamping PNA probe targeting the wild-type sequence of the *KRAS* gene, while two LNA primers were designed to target the two variants of *KRAS* codon 12 mutations (c.35G>T/G12V and c.35G>A/G12D). The PNA probe, with its 2-aminoethylglycine backbone, binds to the template in the presence of the wild-type sequence during the LAMP reaction, inhibiting polymerase activity and blocking target amplification. Conversely, the rigid structure of the LNA, due to its locked atoms, allows LNA primers to compete with and prevent the binding of the PNA in the presence of mutant sequences (G12V/G12D), leading to enhanced amplification of the target sequences over time. Detection of sequence amplification is achieved by measuring the RFU over the threshold limit. Mutations can also be detected through colorimetric LAMP assay. We utilized the WarmStart® Colorimetric LAMP 2x master mix (NEB), which includes a pH-sensitive phenol red. During the LAMP amplification process, as nucleotides are incorporated into the growing strands, the pH of the reaction solution decreases, indicating a distinct color change from bright pink to yellow. Conversely, unamplified reactions retain their initial bright pink color. Thus, the appearance of a yellow color indicates successful target amplification, whereas the persistent bright pink color indicates a lack of target amplification.

3.2. Assay optimization

To optimize the assay, we serially diluted the synthetic oligonucleotides from 10 pg/μl (10^7 DNA copies/μl) to 1 ag/μl (1 DNA copy/μl). We observed the amplification of the target gene from all the samples with varied concentrations (Fig. 2a). We considered 1 fg/μl (10^3 DNA copies/μl) to be the optimum concentration of the synthetic oligonucleotides for further analyses/optimization, as the threshold time for this

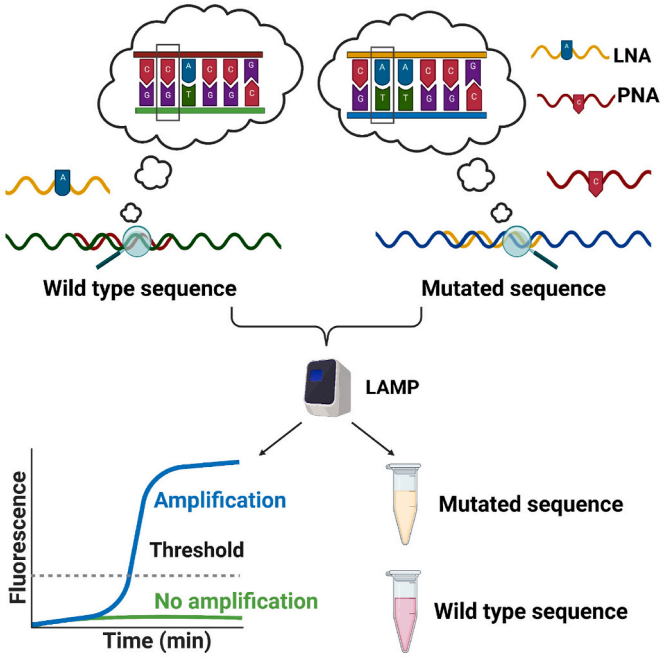

Fig. 1. Mechanism of action of PNA-LNA molecular switch. PNA probe (Red) binds to the wild-type sequence (Green) and blocks its amplification. LNA primer (Golden) prevents the binding of PNA to the mutated sequence (Blue) and facilitates sequence amplification. Amplification can also be determined by visualizing color change from characteristic pink to yellow in colorimetric assays.

sample was below 40 min. For optimizing the concentration of the clamping PNA probe, we used different concentrations ranging from 0.5 μM to 2 μM and observed that the probe was able to block the amplification even at 0.5 μM concentration (Fig. 2b). This verified that the clamping PNA probe was also functional at lower concentrations. We then used varied concentrations of the LNA from 0.1 μM to 1.5 μM and observed that LNA concentration of at least 0.5 μM was required to amplify the target gene (Fig. 2c). However, due to the larger nucleotide content in the chromosomes, we considered the LNA concentration of 1 μM for subsequent experiments. In each case, the synthetic wild-type sequence was used to assess the functionality of the PNA, while both mutant synthetic oligonucleotides were used to check the efficiency of the LNAs. Finally, to consider the clinical samples, we conducted the LAMP experiment using genomic DNA extracted from the colon cancer cell lines (Caco-2 and SW480) with concentrations ranging from 100 pg/μl to 10 ng/μl. SW480 contained the corresponding mutated G12V *KRAS* sequence, whereas Caco-2 contained the wild-type *KRAS* sequence. We observed the amplified genomic DNA from SW480 in all but one sample containing 100 pg/μl DNA (Fig. 2d). Thus, we optimized the genomic DNA concentration to 1 ng/μl, corresponding to 10^3 DNA copies/μl. One "No PNA Control" (NPC) containing no clamping PNA probe and one "No Template Control" (NTC) containing no DNA template were used as positive and negative controls, respectively.

3.3. Assay specificity testing

3.3.1. Specificity testing with synthetic oligonucleotides

To assess the specificity of the PNA-LNA molecular switch, we used three optimized synthetic oligonucleotide sequences of the *KRAS* gene (1 fg/μl), one of which was the wild-type sequence, while the other two had corresponding G12V and G12D mutations and performed LAMP reactions (Fig. 3a). Following LAMP reaction, we observed no significant

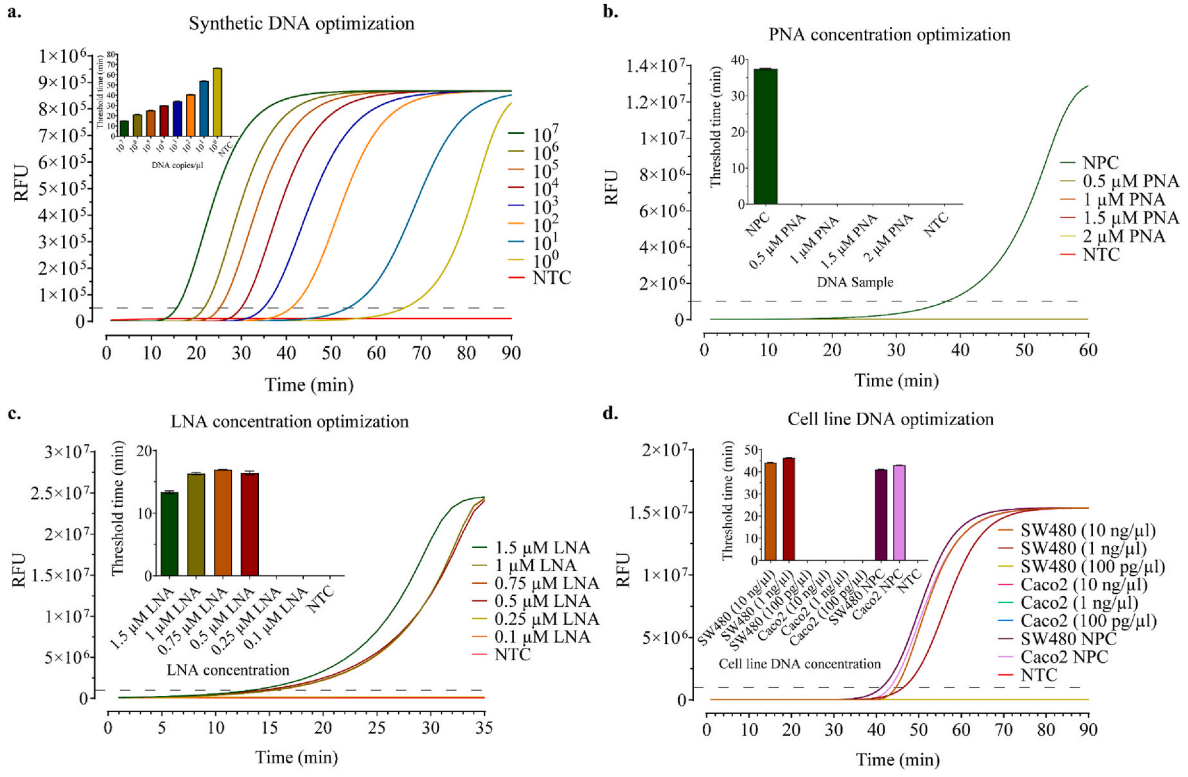

Fig. 2. Optimization of the reagents and templates. a. Optimization of the synthetic oligonucleotide concentration. b. Optimization of the clamping PNA probe concentration. c. Optimization of the LNA concentration. d. Optimization of the colon cancer cell line DNA concentration. The inset shows the corresponding amplification threshold time bar diagram for each of the optimization experiments. Error bars in each column represent the variability of the amplification threshold time for three repetitive experiments (biological replications). NPC - No PNA control. NTC – No template control.

amplification of the wild-type sequence and the NTC, whereas both the mutated sequences and the NPC were amplified. These findings imply that the assay was able to specifically block the amplification of the wild-type sequences, while the mutated sequences were preferentially amplified. This proved the specificity of the PNA-LNA molecular switch for the synthetic oligonucleotides.

However, *in vivo* the tumors are usually heterogenous, and the wild-type and mutated sequences may persist simultaneously. To mimic the clinical setting, we mixed both the wild-type and mutated synthetic oligonucleotides and repeated the LAMP reaction. We observed the amplification of the templates where the mutated sequences were present, but the wild-type sequence alone was not amplified (Fig. 3b). This result verified that the assay could amplify the mutant DNA from a heterogeneous sample, concurrently blocking the amplification of the wild-type sequences. Results from agarose gel electrophoresis and colorimetric assays also corresponded similarly. Taken together, the PNA-LNA molecular switch can be considered for picking up the mutated sequences from a heterogeneous synthetic oligonucleotide sample.

3.3.2. Specificity testing with cancer cell lines

To confirm whether the method is also suitable for viable cancer cells, we used the genomic DNA extracted from SW480 and Caco-2, then performed LAMP with the PNA probe and the LNA primer for the corresponding G12V mutation. Here, the concentration of the cell line DNA was 1 ng/μl, as optimized previously. Following LAMP experiments, we observed the amplification of the DNA from SW480 but didn't observe any significant amplification of the DNA from Caco-2 (Fig. 4a). This confirmed that the PNA-LNA molecular switch was also applicable to the cancer cell lines. After that, to further mimic the clinical setting, we mixed the DNA from SW480 with that of Caco-2 in varied concentrations and noted that the method was able to amplify the mutated sequence from even a mixture of 25% mutant to wild-type heterogeneous sample (Fig. 4b). This consequently validated the effectiveness of the method for the cancer cells.

3.3.3. Specificity testing with non-specific targets

To further assess the specificity of the method and prevent non-specific amplification, we also used a few non-specific targets. Along with the target synthetic oligonucleotides, we ran the experiment with another *KRAS* synthetic DNA that contained a corresponding G13D (c.38G>A) mutation and a synthetic *BRAF* V600E (c.1799T>A) sequence, which acted as a non-specific target and a non-target, respectively. After performing LAMP, we observed that the target *KRAS* G12V synthetic DNA was amplified at ~30 min, whereas the G13D synthetic DNA was amplified after 70 min (Fig. 5a). Moreover, we didn't observe any amplification of the *BRAF* V600E synthetic DNA sequence. Although the non-specific target G13D showed some amplification, it took more than double its time to amplify the specific target.

To reconfirm the specificity of the method with the cell lines, we ran a similar experiment with the DNA extracted from the colon cancer cell line HCT-116 along with SW480 and Caco-2. As in the previous experiment, HCT-116 contains a *KRAS* sequence corresponding to the G13D mutation, which can act as a nonspecific target. Following LAMP, we observed the amplification of *KRAS* in SW480 within 40 min, while there was no significant amplification of the DNA from Caco-2 and HCT-116 (Fig. 5b). These experiments concluded that the proposed method is particular to its target sequence and is devoid of any non-specific amplification within the time limit of the experiment. Furthermore, when performing LAMP without the LNA, the mutated sequences didn't amplify within the limit of the experiment time (data not shown). This consequently bolsters the significance of the LNA in enhancing the amplification rate of the mutated sequences in the presence of PNA.

3.3.4. Specificity testing with KRAS mutant subtypes

To differentiate between the *KRAS* mutation subtypes G12V and

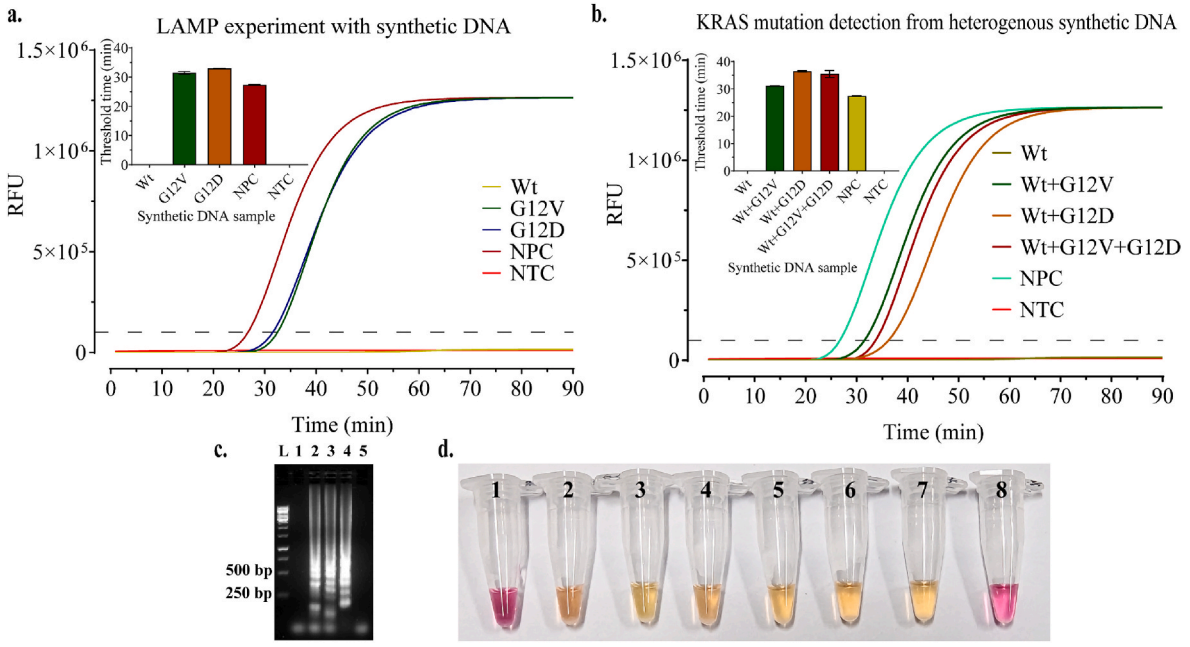

Fig. 3. Specificity testing with synthetic oligonucleotides. a. LAMP reaction with synthetic wild-type (Synth. Wt) and mutated (synth. G12V and synth. G12D) sequences. b. LAMP reaction with a heterogeneous mixture of synthetic wild-type and mutated sequences. The inset shows the corresponding amplification threshold time bar diagram for each of the experiments. Error bars in each column represent the variability of the amplification threshold time for three repetitive experiments (biological replications). c. Agarose gel electrophoresis with synthetic wild-type, mutated oligonucleotides, and a heterogenous mixture of synthetic DNA sequences. L = 100 kb DNA ladder, 1 = Synthetic wild-type sequence, 2 = Synthetic G12V sequence, 3 = Synthetic G12D sequence, 4 = Synthetic wild-type, G12V, and G12D sequences, 5 = NTC. d. Colorimetric assay with synthetic wild-type, mutated oligonucleotides, and a heterogenous mixture of synthetic DNA sequences. Tube 1 = Synthetic wild-type sequence, tube 2 = Synthetic G12V sequence, tube 3 = Synthetic G12D sequence, tube 4 = Synthetic wild-type and G12V sequence, tube 5 = Synthetic wild-type and G12D sequence, tube 6 = Synthetic wild-type, G12V, and G12D sequences, tube 7 = NPC, tube 8 = NTC.

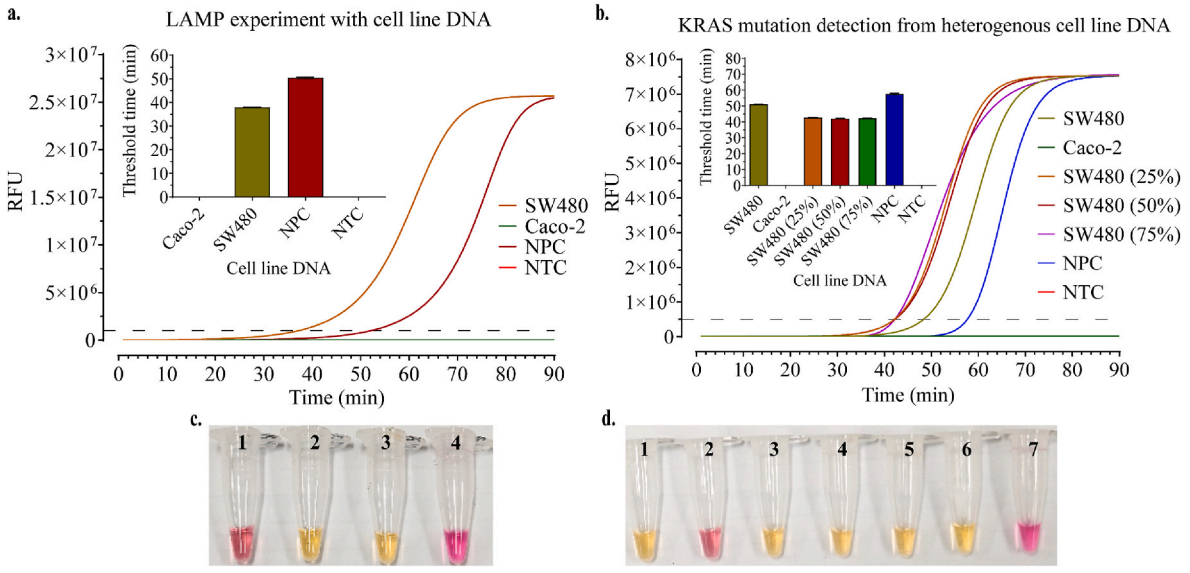

Fig. 4. Specificity testing with cell line DNA. a. LAMP reaction with the DNA from the cell lines Caco-2 and SW480. b. LAMP reaction with a heterogeneous mixture of the DNA from Caco-2 and SW480. The inset shows the corresponding amplification threshold time bar diagram for each experiment. Error bars in each column represent the variability of the amplification threshold time for three repetitive experiments (biological replications). c. Colorimetric LAMP assay with cell line DNA. Tube 1 = Caco-2, tube 2 = SW480, tube 3 = NPC, tube 4 = NTC. d. Colorimetric LAMP assay with a heterogeneous mixture of cell line DNA. Tube 1 = SW480, tube 2 = Caco-2, tube 3 = SW480 (25%), tube 4 = SW480 (50%), tube 5 = SW480 (75%), tube 6 = NPC, tube 7 = NTC.

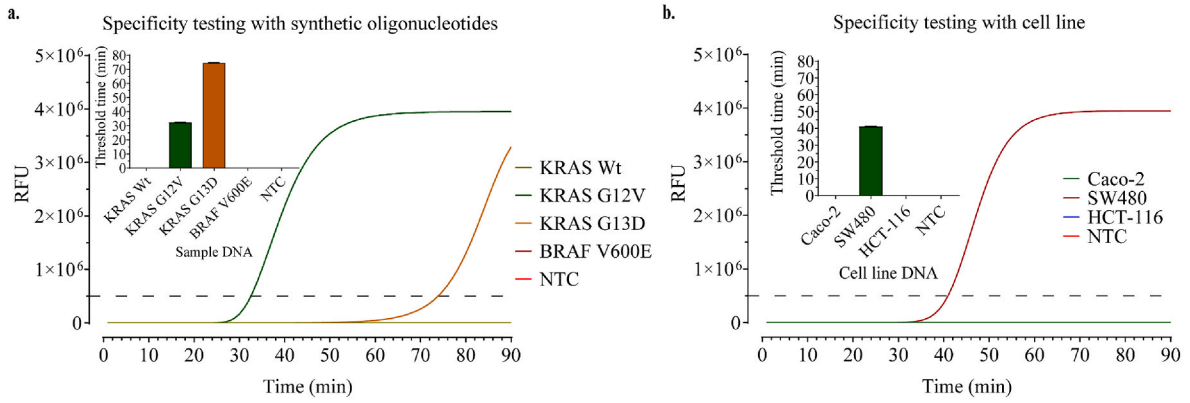

Fig. 5. Specificity testing with non-specific targets. a. LAMP reaction with synthetic *KRAS* wild-type oligonucleotides, G12V, G13D, and *BRAF* V600E. b. LAMP reaction with DNA from colon cancer cell lines Caco-2, SW480, and HCT-116. The inset shows the corresponding amplification threshold time bar diagram for each of the experiments. Error bars in each column represent the variability of the amplification threshold time for three repetitive experiments (biological replications).

G12D, we used alternating LNA primers. Each reaction panel was designed to contain either the G12V or G12D-specific LNA, and both were tested using synthetic oligonucleotides representing each mutant. Following the LAMP reaction, we observed that the G12V mutant template, when paired with the G12V-specific LNA, amplified within 30 minutes. In contrast, amplification with the G12D-specific LNA took place after 40 minutes. A similar pattern was observed with the G12D mutant synthetic oligonucleotide, further confirming the method's efficacy (Supplementary Fig. S1). These findings demonstrate that our proposed method can effectively distinguish between the *KRAS* G12V and G12D mutations with a resolution time of approximately 10 min. However, due to resource limitations, we were unable to extend the specificity testing to include cell line DNA in this experiment.

3.4. Assay sensitivity testing

To evaluate the sensitivity of the assay, we performed the LAMP reaction with serially diluted synthetic oligonucleotides with 10-fold dilutions from 10 pg/μl to 1 ag/μl that corresponded to 10^7 DNA copies/μl to a single DNA copy/μl, respectively. The result showed that this assay followed linearity, and the amplification signal was directly proportional to the amount of input DNA (Fig. 2a, Supplementary Fig. S2a). The assay was consistent throughout the dynamic range of the target synthetic oligonucleotides and the efficiency of the assay was also excellent, which was indicated by the correlation coefficient, R^2 (0.94). Although theoretically, the limit of detection (LOD) of the assay was measured to be 0.00009, the lowest practical detectable synthetic target concentration for this assay was 1 ag/μl (1 DNA copy/μl), highlighting its extraordinary sensitivity. Furthermore, the results of colorimetric LAMP corresponded to those from fluorescent LAMP. A positive reaction demonstrated by color change from pink to yellow in all the serially diluted DNA samples was observed, whereas the pink color was retained by the NTC (Supplementary Fig. S2b). Utilizing the standard curve generated from the sensitivity testing of this study, we can quantify the amount of DNA from a sample as well.

3.5. LAMP experiments with clinical samples and validation

To evaluate the clinical application of the PNA-LNA molecular switch, we proceeded with profiling the mutation status in *KRAS* codon 12 with the genomic DNA extracted from the resected colon tumors (adenocarcinoma) (n = 30) and CTCs (n = 24) from patients with CRC using both the LNAs targeting G12V and G12D mutations. Of the 30 tumor tissue samples, we observed the specific *KRAS* mutations in 13 patients (43%), whereas out of 24 CTCs, we detected the specific mutation in 9 cases (37.5%) (Supplementary Fig. S3). Colorimetric LAMP assay also provided consistent results as fluorescent LAMP in all cases. Next, to validate the findings of the assay, we performed HRM analysis of the colon tumor tissue samples (n = 21) followed by Sanger sequencing, where we observed the mutation in 9 patients (43%). All the 21 selected cases correlated with the findings of the LAMP experiments from tissue samples. The accuracy of the wild-type and mutant detection using the PNA-LNA molecular switch was 100% compared to the sequencing methods. Supplementary Table S2 shows the mutation status of the target gene in individual patients using colon tissues, CTCs, and Sanger sequencing. Fig. 6 provides a snapshot of representative wild-type (Patient 7) and mutated (Patient 10) LAMP experiments and the corresponding results from HRM analysis and Sanger sequencing. As the results from the LAMP reaction with PNA/LNA and those of the HRM and Sanger sequencing analyses matched perfectly, we can confirm the performance of the PNA-LNA molecular switch to be comparable to the conventional methods.

4. Discussion

In this study, we have developed a novel method, "PNA-LNA molecular switch", using the synthetic DNA analogs PNA and LNA along with an isothermal amplification-based technique, LAMP to detect the mutations at *KRAS* codon 12. In this method, we used synthetic oligonucleotides and colon cancer cell lines and later identified the mutation status in tumor tissues and CTCs derived from patients with CRC. Previously, the LAMP method was used to detect gene mutations in mosquitos and herbicide-resistant weeds only (Badolo et al., 2012; Pan et al., 2015). Itonaga et al.[17] also utilized PNA and LNA probes with LAMP to detect mutations in *KRAS*, derived from cDNA obtained from a pancreatic cell line model. However, they did not test the method with clinical samples or validate it with gold-standard methodologies. In our study, we employed both fluorescent and colorimetric LAMP simultaneously to observe the results from the same experiment. Initially, we developed the method using synthetic oligonucleotides and colon cancer cell line models. Subsequently, we tested it with a cohort of clinical samples obtained from CRC patients and validated its analytical performance using HRM analysis and Sanger sequencing. Nonetheless, the use of colorimetric LAMP offers additional advantages. It allows for result observation with the naked eye, eliminating the need for a thermal cycler. A typical heat block capable of generating a constant temperature of 65 °C is sufficient to perform the experiments. Finally, we utilized the genomic DNA of the *KRAS* gene as the template, whereas Itonaga et al.[17] employed cDNA. We believe this approach reduces the operational time of the experiment and minimizes redundant steps.

This method was able to selectively block the amplification of the wild-type sequences of *KRAS*, while specifically amplifying the mutated codon 12 *KRAS* sequence. We have also demonstrated that this method can amplify a mutated sequence from a heterogeneous mixture of samples, which is the usual setting for the tumors *in vivo*. More importantly, this method prevents non-specific amplification while effectively amplifying the target sequences. Additionally, this method was able to distinguish between *KRAS* mutant subtypes that can prove effective for devising therapeutic strategies. Furthermore, in this study, we used only three locked nucleotides in the LNA as opposed to the previous studies where all the nucleotides were locked and obtained similar results in terms of specificity, sensitivity, and amplification time with significantly reduced cost. This proves beneficial for the researchers with limited resources as well as makes it feasible in clinical application.

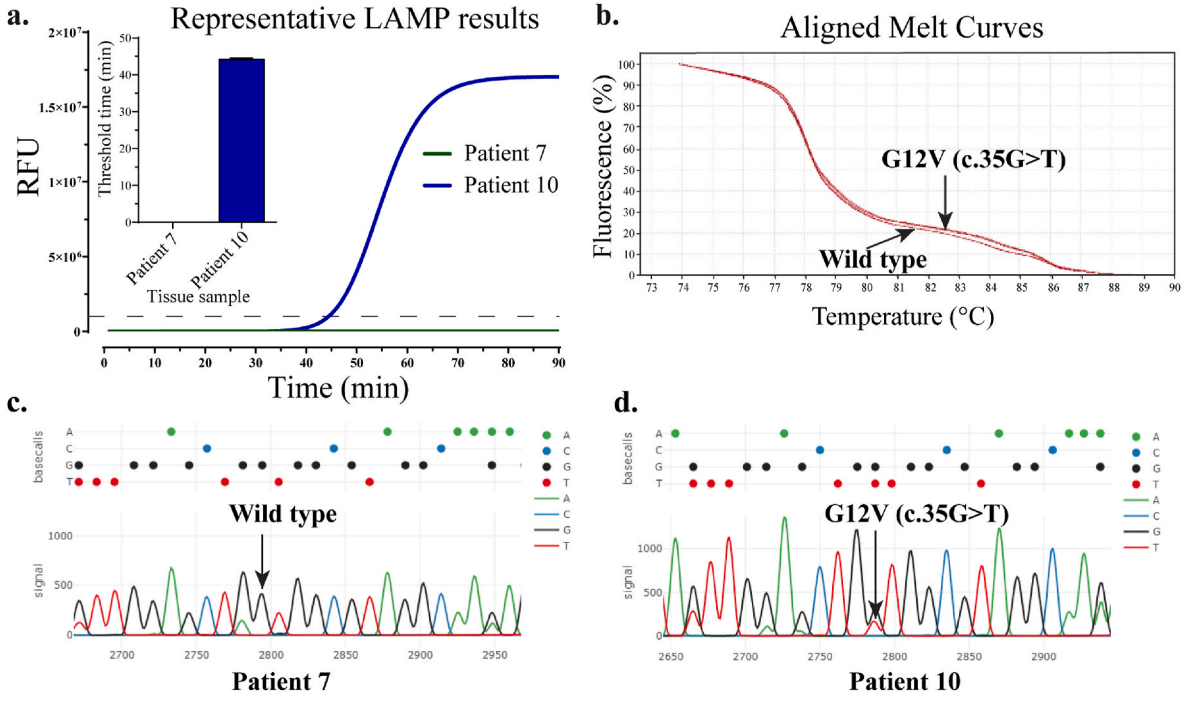

Fig. 6. Representative chromatograms for LAMP, HRM, and Sanger sequencing results. a. LAMP results with representative *KRAS* wild-type (Patient 7) and *KRAS* mutant (Patient 10) tissue samples. The inset shows the corresponding amplification threshold time bar diagram for each of the experiments. Error bar represents the variability of the amplification threshold time for three repetitive experiments (biological replications). b. Representative wild-type and mutated (G12V/c.35G>T) sequence variants in HRM. c. Sanger sequencing chromatogram representing *KRAS* wild-type sample (Patient 7). d. Sanger sequencing chromatogram representing *KRAS* heterozygous mutant sample (Patient 10).

The proposed method provides a significantly faster approach to detecting mutations compared to traditional methods, such as direct sequencing. For clinical samples, the total experimental time is approximately 40 min. Colorimetric detection occurs instantly, while fluorescent detection requires only about 5 min, making this method significantly faster than most other mutation detection methods, which often take much longer–sometimes even day(s)-for both experimentation and analysis. This rapid turnaround can be crucial in the timely management of CRC patients, allowing for quicker decision-making regarding treatment options. In addition to its speed, this method is cost-effective. The average cost per experiment with PNA and LNA is approximately AUD$7.5, which is considerably cheaper than the AUD $15-AU$20 typically charged by commercial sequencing facilities such as AGRF. Moreover, these commercial methods often require additional steps, like PCR amplification and purification, which add an extra cost of AU$10-AU$15 per experiment (Qiagen). This affordability of the method makes it more accessible to laboratories and healthcare facilities with limited resources, thereby widening its potential impact in clinical and research environments.

Another significant advantage of the PNA-LNA method is its high specificity for target sequences. It not only amplifies specific target sequences within a short timeframe but also effectively prevents the amplification of non-specific or non-target sequences during the experimental process. This precision reduces the likelihood of false-positive results, thereby improving the accuracy of mutation detection. Moreover, the method demonstrates sensitivity and specificity that are comparable to contemporary mutation detection techniques. For instance, when detecting mutations in the *KRAS* codon 12, it shows sensitivity on par with allele-specific qRT-PCR assays, which typically detect mutations ranging from 2.97 to 27.43 DNA copies per reaction (Li et al., 2022). Similarly, it performs at a level comparable to surface-enhanced Raman assays, which detects approximately 100 DNA copies per reaction (Liu et al., 2020). In our method, the sensitivity was estimated at just 1 DNA copy/μl, positioning it as one of the more sensitive options available for mutation detection. When it comes to detecting low allelic frequencies, the PNA-LNA method is highly effective. It successfully identified mutated sequences in samples with at least 25% mutant DNA, which is in line with other mutation detection techniques. For example, Sanger sequencing has a detection range of 7.7–29% (Qu et al., 2013; Lade-Keller et al., 2013), pyrosequencing detects mutations within a 20.6–49% range (Mourah et al., 2015), and qPCR combined with a mutation-specific primer and a non-productive oligonucleotide can identify mutations within a 5–10% range (Alvarez-Garcia et al., 2018). The ability to detect low-frequency mutations is vital for assessing the genetic landscape of tumors, guiding personalized treatment plans, and avoiding unnecessary interventions. Furthermore, this method is reproducible, as evidenced by the low standard deviation (%RSD <5%) of the amplification time calculated from the replication experiments. The proposed method has a linear dynamic range of 10 pg/μl-1 ag/μl ($R^2 = 0.94$) as well which makes it suitable for a broader range of sample amounts. Supplementary Table S3 provides a summarized comparison of various parameters between our proposed method and contemporary standard mutation detection techniques.

Successful application of the PNA-LNA molecular switch to clinical samples from CRC patients, followed by subsequent validation with Sanger sequencing provided the viability of the assay for a clinical setting. However, to effectively implement this assay in a clinical setting, we need to apply this method in a larger cohort of clinical samples. Nonetheless, this assay has tremendous potential in earlier detection of specific SNPs, thereby prescribing personalized treatment regimens to patients with chronic diseases. Furthermore, the clinical significance is broad as it can be implemented for diverse diseases and disorders as well as multidisciplinary fields. A combination of PNA/LNA with LAMP could allow the detection of SNPs with better sensitivity and accuracy, not to mention lowering the time and cost significantly. While this method offers significant advantages, it does have certain limitations. One drawback is its capacity to identify only known SNPs, limiting its ability to detect novel or unknown mutations. Additionally, each experiment is restricted to targeting a single genetic region, which can reduce efficiency when analyzing multiple mutations simultaneously. Furthermore, the method relies on commercially available synthetic nucleotides (PNA/LNA), which may add to the overall cost and dependency on external suppliers. These factors present challenges that could impact its broader application in some research and clinical settings.

To the best of our knowledge, this assay is the first isothermal amplification-based method employing PNA/LNA to detect mutation in the genomic DNA from CRC. The superior specificity, sensitivity, accuracy, faster readout, and relatively inexpensive nature of this assay potentially make it a better tool for detecting SNPs compared to contemporary methods. Further testing with a large cohort of clinical samples is required to develop this method into a commercial venture, which is currently underway.

5. Conclusion

To develop a novel platform for detecting CRC-associated genetic mutations, we utilized the structural superiority of PNA and LNA with the isothermal amplification method, LAMP. This novel "PNA-LNA molecular switch" method showed significantly lower analytical time, is cheap, and has superior sensitivity compared to the contemporary mutation detection methods. The simpler workflow of the assay could facilitate the integration of it into POC device-based biosensing applications as well.

Funding

This research received no external funding.

CRediT authorship contribution statement

Md Sajedul Islam: Writing – original draft, Validation, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sharmin Aktar:** Validation, Investigation, Formal analysis, Data curation. **Neda Moetamedirad:** Investigation, Data curation. **Nan Xie:** Investigation, Data curation. **Cu Tai Lu:** Resources. **Vinod Gopalan:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization. **Alfred K. Lam:** Writing – review & editing, Supervision, Resources. **Muhammad J.A. Shiddiky:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization.

Declaration of competing interest

The authors declared no conflict of interest.

Data availability

Data will be made available on request.

Acknowledgment

M.S.I. acknowledges the support from Griffith University Postgraduate Research Scholarship. M.S.I. conveys his gratitude to Tanzena Tanny for sharing Biorender.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2024.116813.

References

Alvarez-Garcia, V., Bartos, C., Keraite, I., Trivedi, U., Brennan, P.M., Kersaudy-Kerhoas, M., Gharbi, K., Oikonomidou, O., Leslie, N.R., 2018. A simple and robust real-time qPCR method for the detection of PIK3CA mutations. Sci. Rep. 8, 4290. https://doi.org/10.1038/s41598-018-22473-9.

Anderson, S.M., 2011. Laboratory methods for KRAS mutation analysis. Expert Rev. Mol. Diagn. 11, 635–642. https://doi.org/10.1586/erm.11.42.

Badolo, A., Okado, K., Guelbeogo, W.M., Aonuma, H., Bando, H., Fukumoto, S., Sagnon, N.F., Kanuka, H., 2012. Development of an allele-specific, loop-mediated, isothermal amplification method (AS-LAMP) to detect the L1014F kdr-w mutation in Anopheles gambiae s. l. Malar. J. 11, 227. https://doi.org/10.1186/1475-2875-11-227.

Cao, G., Kong, J., Xing, Z., Tang, Y., Zhang, X., Xu, X., Kang, Z., Fang, X., Guan, M., 2018. Rapid detection of CALR type 1 and type 2 mutations using PNA-LNA clamping loop-mediated isothermal amplification on a CD-like microfluidic chip. Anal. Chim. Acta 1024, 123–135. https://doi.org/10.1016/j.aca.2018.04.022.

Choate, K.A., Raack, E.J., Mann, P.B., Jones, E.A., Winn, R.J., Jennings, M.J., 2024. Rapid IDH1-R132 genotyping panel utilizing locked nucleic acid loop-mediated isothermal amplification. Biol. Methods Protoc. 9. https://doi.org/10.1093/biomethods/bpae012.

Dai, D., Wang, Y., Zhu, L., Jin, H., Wang, X., 2020. Prognostic value of KRAS mutation status in colorectal cancer patients: a population-based competing risk analysis. PeerJ 8, e9149. https://doi.org/10.7717/peerj.9149.

Formica, V., Sera, F., Cremolini, C., Riondino, S., Morelli, C., Arkenau, H.T., Roselli, M., 2022. KRAS and BRAF mutations in stage II and III colon cancer: a Systematic review and meta-analysis. J. Natl. Cancer Inst. 114, 517–527. https://doi.org/10.1093/jnci/djab190.

Haigis, K.M., 2017. KRAS alleles: the devil is in the detail. Trends Cancer 3, 686–697. https://doi.org/10.1016/j.trecan.2017.08.006.

Islam, M.S., Gopalan, V., Lam, A.K., Shiddiky, M.J.A., 2023. Current advances in detecting genetic and epigenetic biomarkers of colorectal cancer. Biosens. Bioelectron. 239, 115611. https://doi.org/10.1016/j.bios.2023.115611.

Itonaga, M., Matsuzaki, I., Warigaya, K., Tamura, T., Shimizu, Y., Fujimoto, M., Kojima, F., Ichinose, M., Murata, S., 2016. Novel methodology for rapid detection of KRAS mutation using PNA-LNA mediated loop-mediated isothermal amplification. PLoS One 11, e0151654. https://doi.org/10.1371/journal.pone.0151654.

Karapetis, C.S., Khambata-Ford, S., Jonker, D.J., O'Callaghan, C.J., Tu, D., Tebbutt, N.C., Simes, R.J., Chalchal, H., Shapiro, J.D., Robitaille, S., Price, T.J., Shepherd, L., Au, H.-J., Langer, C., Moore, M.J., Zalcberg, J.R., 2008. K-Ras mutations and benefit from cetuximab in advanced colorectal cancer. New Eng. J. Med. 359, 1757–1765. https://doi.org/10.1056/NEJMoa0804385.

Lade-Keller, J., Rømer, K.M., Guldberg, P., Riber-Hansen, R., Hansen, L.L., Steiniche, T., Hager, H., Kristensen, L.S., 2013. Evaluation of BRAF mutation testing methodologies in formalin-fixed, paraffin-embedded cutaneous melanomas. J. Mol. Diagn. 15, 70–80. https://doi.org/10.1016/j.jmoldx.2012.08.003.

Li, J., Gao, Z., Chen, J., Cheng, R., Niu, J., Zhang, J., Yang, Y., Yuan, X., Xia, J., Mao, G., Liu, H., Dong, Y., Wu, C., 2022. Development of a panel of three multiplex allele-specific qRT-PCR assays for quick differentiation of recombinant variants and Omicron subvariants of SARS-CoV-2. Front. Cell. Infect. Microbiol. 12, 953027. https://doi.org/10.3389/fcimb.2022.953027.

Liu, P., Wang, Y., Li, X., 2019. Targeting the untargetable KRAS in cancer therapy. Acta Pharm. Sin. B 9, 871–879. https://doi.org/10.1016/j.apsb.2019.03.002.

Liu, Y., Lyu, N., Rajendran, V.K., Piper, J., Rodger, A., Wang, Y., 2020. Sensitive and direct DNA mutation detection by surface-enhanced Raman spectroscopy using rational designed and tunable plasmonic nanostructures. Anal. Chem. 92, 5708–5716. https://doi.org/10.1021/acs.analchem.9b04183.

Malumbres, M., Barbacid, M., 2003. RAS oncogenes: the first 30 years. Nat. Rev. Cancer 3, 459–465. https://doi.org/10.1038/nrc1097.

Mariotto, A.B., Enewold, L., Zhao, J., Zeruto, C.A., Yabroff, K.R., 2020. Medical care costs associated with cancer survivorship in the United States. Cancer Epidemiol. Biomark. Prev. 29, 1304–1312. https://doi.org/10.1158/1055-9965.Epi-19-1534.

Menchise, V., De Simone, G., Tedeschi, T., Corradini, R., Sforza, S., Marchelli, R., Capasso, D., Saviano, M., Pedone, C., 2003. Insights into peptide nucleic acid (PNA) structural features: the crystal structure of a d-lysine-based chiral PNA–DNA duplex. Proc. Natl. Acad. Sci. U.S.A. 100, 12021–12026. https://doi.org/10.1073/pnas.2034746100.

Mourah, S., Denis, M.G., Narducci, F.E., Solassol, J., Merlin, J.-L., Sabourin, J.-C., Scoazec, J.-Y., Ouafik, L.H., Emile, J.-F., Heller, R., Souvignet, C., Bergougnoux, L., Merlio, J.-P., 2015. Detection of BRAF V600 mutations in melanoma: evaluation of concordance between the Cobas® 4800 BRAF V600 mutation test and the methods used in French national cancer institute (INCa) platforms in a real-life setting. PLoS One 10, e0120232. https://doi.org/10.1371/journal.pone.0120232.

Nagai, Y., Miyazawa, H., Huqun, Tanaka, T., Udagawa, K., Kato, M., Fukuyama, S., Yokote, A., Kobayashi, K., Kanazawa, M., Hagiwara, K., 2005. Genetic heterogeneity of the epidermal growth factor receptor in non-small cell lung cancer cell lines revealed by a rapid and sensitive detection system, the peptide nucleic acid-locked nucleic acid PCR clamp. Cancer Res. 65, 7276–7282. https://doi.org/10.1158/0008-5472.Can-05-0331.

Obika, S., Nanbu, D., Hari, Y., Morio, K.-i., In, Y., Ishida, T., Imanishi, T., 1997. Synthesis of 2′-O,4′-C-methyleneuridine and -cytidine. Novel bicyclic nucleosides having a fixed C3, -endo sugar puckering. Tetrahedron Lett. 38, 8735–8738. https://doi.org/10.1016/S0040-4039(97)10322-7.

Pan, L., Li, J., Zhang, W.N., Dong, L., 2015. Detection of the I1781L mutation in fenoxaprop-p-ethyl-resistant American sloughgrass (Beckmannia syzigachne Steud.), based on the loop-mediated isothermal amplification method. Pest Manag. Sci. 71, 123–130. https://doi.org/10.1002/ps.3777.

Qu, K., Pan, Q., Zhang, X., Rodriguez, L., Zhang, K., Li, H., Ho, A., Sanders, H., Sferruzza, A., Cheng, S.-M., Nguyen, D., Jones, D., Waldman, F., 2013. Detection of BRAF V600 mutations in metastatic melanoma: comparison of the cobas 4800 and sanger sequencing assays. J. Mol. Diagn. 15, 790–795. https://doi.org/10.1016/j.jmoldx.2013.07.003.

Schubbert, S., Shannon, K., Bollag, G., 2007. Hyperactive Ras in developmental disorders and cancer. Nat. Rev. Cancer 7, 295–308. https://doi.org/10.1038/nrc2109.

Sharma, N., Neill, T., Yang, H.-C., Oliver, C.L., Mahaffee, W.F., Naegele, R., Moyer, M.M., Miles, T.D., 2023. Development of a PNA-LNA-LAMP assay to detect an SNP associated with QoI resistance in Erysiphe necator. Plant Dis. 107, 3238–3247. https://doi.org/10.1094/pdis-09-22-2027-re.

Shin, A.E., Giancotti, F.G., Rustgi, A.K., 2023. Metastatic colorectal cancer: mechanisms and emerging therapeutics. Trends Pharmacol. Sci. 44, 222–236. https://doi.org/10.1016/j.tips.2023.01.003.

Sun, X., Hung, K., Wu, L., Sidransky, D., Guo, B., 2002. Detection of tumor mutations in the presence of excess amounts of normal DNA. Nat. Biotechnol. 20, 186–189. https://doi.org/10.1038/nbt0202-186.

Ung, L., Lam, A.K., Morris, D.L., Chua, T.C., 2014. Tissue-based biomarkers predicting outcomes in metastatic colorectal cancer: a review. Clin. Transl. Oncol. 16, 425–435. https://doi.org/10.1007/s12094-013-1154-6.