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Characterization of Recombinase Polymerase Amplification Primers Targeting *EGFR* Exon 19 Deletions for a Proposed Isothermal Lung Cancer Diagnostic Workflow

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Abstract

The analysis of circulating tumor DNA (ctDNA) holds significant promise for early lung cancer detection. However, current genomic screening modalities remain limited by laboratory costs and resource access. This study presents the preliminary design and experimental characterization of Recombinase Polymerase Amplification (RPA) primers designed to target the *EGFR* exon 19 deletion (ex19del) biomarker using synthetic control templates. This primer framework is evaluated as the foundational core for a proposed downstream workflow intended to eventually incorporate CRISPR-Cas9 specificity processing and Lateral Flow Assay (LFA) readouts. In vitro testing demonstrated successful amplification of the target sequence, with a 5 bp overlap forward primer yielding prominent amplification. Concurrently, persistent non-specific amplification was observed, establishing the necessity for subsequent enzymatic filtration. While the complete LFA readout and CRISPR-Cas9 components represent future development steps that require clinical verification in plasma samples, this work validates the operational range of the foundational amplification primers.

Keywords

Non-Small Cell Lung Cancer, CRISPR-Cas9,
Recombinase Polymerase Amplification, Lateral Flow Assay,
Molecular Diagnostics, Point-of-Care Testing

1. Introduction

Non-small cell lung cancer (NSCLC) is the most common form of lung cancer,

including subtypes such as adenocarcinoma, squamous cell carcinoma, and large cell carcinoma, and is defined as any type of epithelial lung cancer other than small cell lung cancer [1]. NSCLC accounts for 85% of all lung cancer cases [2] and is a leading cause of cancer deaths worldwide with approximately 2.5 million new cases of lung cancer diagnosed globally in 2022 [3]. In 2022, an estimated 1.8 million deaths were attributed to lung cancer, which was more common in highly industrialised regions such as: Eastern Asia, North America, and Europe [3].

The pathogenesis of non-small cell lung cancer (NSCLC) is fundamentally driven by the amplification of oncogenes and the functional inactivation of tumour suppressor genes. During the initiation and promotion phases of carcinogenesis, exposure to environmental carcinogens induces critical genetic mutations and cellular alterations. These mutations transform proto-oncogenes into active oncogenes, facilitating rapid, dysregulated, and uncontrolled cellular proliferation. Key molecular drivers frequently identified in clinical settings include [1]:

1) Epidermal Growth Factor Receptor (EGFR), which is prevalent in more than 32% of cases of NSCLC.

2) Anaplastic Lymphoma Kinase (ALK).

3) Kirsten rat Sarcoma Virus (KRAS).

Persistent disruption of these regulatory pathways leads to advanced disease progression, as compromised tumour suppressor genes fail to execute apoptosis. Currently, diagnostic protocols for NSCLC primarily involve invasive and high-cost procedures [4], such as:

1) Biopsies and bronchoscopies.

2) Endobronchial ultrasound (EBUS).

3) Radiographic imaging for metastatic staging.

Although polymerase chain reaction (PCR) testing offers a less invasive molecular alternative, its clinical utility is often limited by significant costs and the requirement for specialised laboratory infrastructure.

To address these limitations, isothermal readout technologies present a viable solution for rapid, point-of-care diagnostics without the prohibitive overhead of traditional methods [4]. Such advancements could significantly enhance the global accessibility of cancer screening and diagnostic services. Furthermore, the integration of patient data from these diagnostics facilitates the development of risk-screening algorithms, enabling more precise patient stratification and the optimized allocation of medical resources.

To overcome infrastructure constraints, isothermal molecular amplification techniques like RPA offer an adaptable decentralized approach [5]. In this study, we experimentally validate the performance and constraints of multiple RPA primer structures tested against synthetic *EGFR* control DNA templates. We characterize their amplification thresholds and investigate non-specific artifact generation. The results presented here serve as a necessary baseline validation. The integration of these elements into a functional Lateral Flow Assay (LFA) and the addition of CRISPR-Cas9 specificity enzymes represent conceptual next steps aimed

at generating a portable screening pipeline, the eventual real-world application of which remains contingent on testing within clinical patient cohorts [6].

To overcome infrastructure constraints, isothermal molecular amplification techniques like RPA offer an adaptable decentralized approach. Epidermal Growth Factor Receptor (EGFR): Mutations can be detected in the bloodstream as circulating free tumour-derived DNA (ctDNA). This provides a non-invasive alternative to tissue biopsies, which carry a 27% - 31% comorbidity risk. However, because ctDNA is highly diluted, high-specificity methods are critical to isolate true malignant signals from background noise. Lateral Flow Assays (LFAs): These paper-based platforms leverage capillary action to deliver rapid, low-cost diagnostic lines within 30 minutes without laboratory infrastructure. Recombinase Polymerase Amplification (RPA): Operating isothermally below 42°C, RPA utilizes a recombinase-primer complex to achieve rapid exponential amplification in under 20 minutes. CRISPR-Cas9 Specificity: To compensate for downstream cross-reactivity, combining targeted guide RNA with Cas9 protein allows high-fidelity cleaving to gate true target amplicons

1.1. Epidermal Growth Factor Receptor (EGFR)

Epidermal growth factor receptor (EGFR) mutations can be detected non-invasively via circulating free tumor-derived DNA (ctDNA) in patients with advanced non-small cell lung cancer (NSCLC). This is highly valuable for tyrosine kinase inhibitor (TKI) therapy targeting resistance mutations (IASLC Lung Cancer News 2024). Standard tissue biopsies pose significant health risks to 27% - 31% of NSCLC patients due to comorbidities. While ctDNA testing is less accurate than a biopsy and cannot detect mutations in all positive patients, its non-invasive nature makes it an excellent preliminary diagnostic alternative, especially in resource-limited settings [7].

The EGFR gene was discovered in 1995, and its link to clinical drug responses (like gefitinib) via serum mutations was established in 2006. In EGFR-positive NSCLC cases, exon 19 deletions (ex19del) and L858R are the most prominent variants [7]. Other notable variants include exon 20 insertions (ex20ins) and the T790M point mutation. The remaining oncogenic kinase domain mutations account for 10% - 15% of cases (IASLC Lung Cancer News 2024).

Biologically, ctDNA is hypothesized to originate from cellular apoptosis, macrophage-phagocytosed necrotic cells, cellular breakdown, or direct active secretion by tumor cells. Meta-analyses validate ctDNA for diagnostic utility, but detecting EGFR mutations in plasma is fundamentally harder than in tumor tissue because ctDNA is highly diluted.

Advanced methods are required to capture mutant allele concentrations below 1% of total DNA [8]. While precise devices are necessary to separate true signals from background noise, extreme sensitivity can cause false positives. Consequently, clinicians often favor testing methods with higher specificity—even with a slight reduction in sensitivity—to avoid false positives and secure reliable, ac-

tionable results.

Diagnostic models include next-generation sequencing (NGS) and enhanced PCR techniques like droplet PCR (ddPCR) and BEAMing [8]. While all these profiling methods incur high costs and processing delays, ddPCR is recognized as the fastest turnaround option, whereas BEAMing is the most cost-effective [8].

1.2. Lateral Flow Assays (LFAs)

Lateral Flow Assays (LFAs) are cost-effective, paper-based diagnostic platforms designed for the rapid detection and quantification of analytes in complex mixtures within 30 minutes [9]. Utilised in common applications such as pregnancy and COVID-19 testing, these devices employ capillary action to transport a sample through porous polymeric zones for molecular binding. The process begins at the sample pad, where integrated buffer salts and surfactants optimize the specimen for the detection system, providing an efficient point-of-care solution that bypasses the need for extensive laboratory infrastructure.

In a lateral flow assay, the sample migrates through a conjugate pad to a detection zone, where antigens bind with antibodies to produce a visible response on test and control lines [9]. Depending on the analyte size, sandwich assays generate a line to signify a positive result via dual-antibody binding, whereas competitive assays indicate a positive result by the absence of a line.

1.3. Recombinase Polymerase Amplification (RPA)

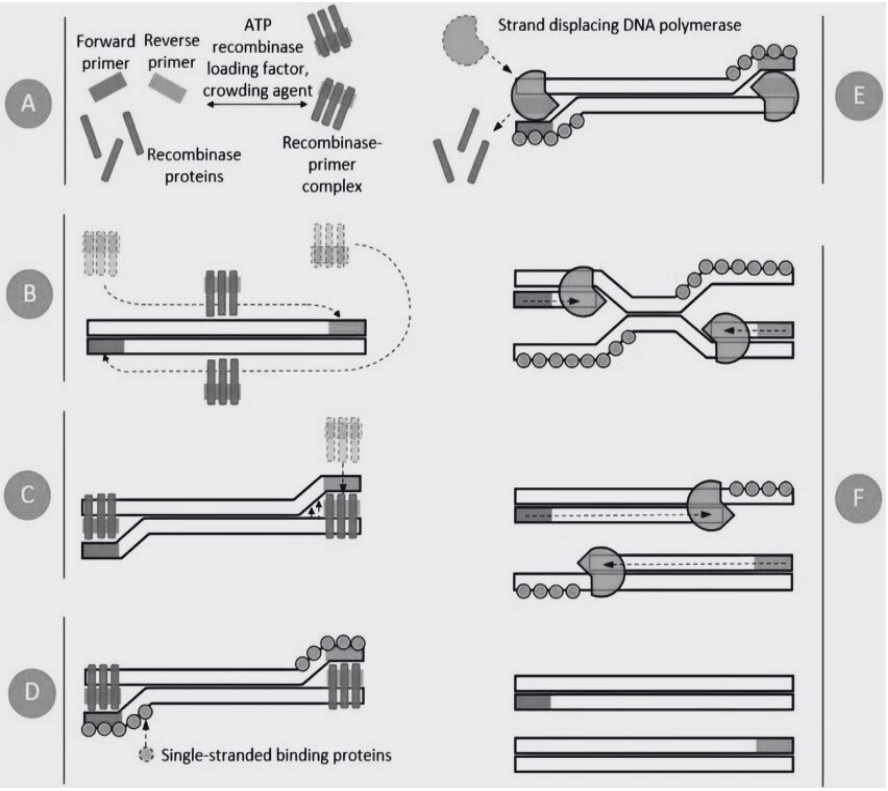

Figure 1. Recombinase polymerase amplification scheme. Adopted from Tan2022RPA.

Recombinase Polymerase Amplification (RPA) is a highly sensitive isothermal technique capable of amplifying a single DNA target copy in under 20 minutes [10]. The process initiates when a recombinase-primer complex facilitates strand invasion at homologous sequences, with single-stranded binding proteins stabilising the displaced DNA. Finally, the recombinase disassembles to allow strand-displacing DNA polymerase to elongate the primers, ensuring rapid exponential amplification without thermal cycling.

Exponential amplification is achieved through repetitive cycles using primers designed similarly to PCR, though modified oligonucleotides may be employed to prevent primer-dimer formation [10]. While crowding agents are essential to maintain strand stability, their high viscosity can impede diffusion and delay amplification at low target concentrations. Consequently, careful primer selection and agent balancing are critical to optimizing RPA performance and sensitivity—see **Figure 1**.

1.4. CRISPR-Cas9 (CRISPR-Associated Protein 9)

CRISPR-Cas9 is a gene-editing enzyme that uses guide RNA to cut specific, complementary DNA strands within living organisms [11]. The resulting break either inactivates the gene via natural cell repair or allows scientists to insert a modified sequence.

This research analyzes NSCLC epidemiology to identify a target mutation for a diagnostic tool. NSCLC growth is primarily driven by three mutations, EGFR, KRSV, and ALK, with the EGFR exon 19 deletion being the most common cause. Early diagnosis is vital to improve life expectancy by enabling targeted treatments.

Because early-stage patients have low plasma ctDNA concentrations, the diagnostic tool requires high sensitivity and specificity. Sensitivity is prioritized since secondary screening can confirm positive results, but avoiding cross-reactivity with common DNA remains crucial. Lateral Flow Assay (LFA) systems offer the most inexpensive, low-complexity solution for routine, on-site testing under standard office conditions. Screening at-risk individuals will increase early detection and save public health resources.

2. Methods

Here, we summarise only the key reaction conditions and emphasise the primer-design logic that underpins the assay's specificity.

2.1. Key Reaction Conditions

RPA reactions were performed under isothermal conditions (42˚C for ~20 min) using primer concentrations of 0.3 μM each, with MgCl2 added to initiate the reaction. Amplification products were assessed by agarose gel electrophoresis (1% - 2% agarose).

Where required for downstream verification (PCR and Sanger sequencing), amplicons were purified using silica-membrane spin columns. PCR was used as

an orthogonal verification step to assess whether primer pairs discriminated mutant from wildtype templates. Annealing-temperature stringency was explored using a short temperature gradient (61˚C - 72˚C) while keeping the primer pair fixed, and products were analysed by gel electrophoresis.

2.2. Design Rationale for Overlap Primers

The target is the EGFR exon 19 deletion (ex19del), a 23 bp deletion that creates a unique mutant junction sequence absent in the wildtype allele. Overlap (junction-spanning) primers were designed so that the 3' end interrogates the deletion junction: in the mutant template the primer's 3' nucleotides are perfectly paired, whereas in the wildtype template the same 3' region is forced into a mismatch/overhang configuration that suppresses polymerase extension.

2.3. Length-Gradient Selection Strategy

Because RPA can tolerate imperfect priming under low stringency, the overlap length at the junction was treated as a tunable specificity dial. A short over lap (e.g., 4 - 5 bp) maximises discrimination against wildtype but can reduce initiation efficiency and yield. A longer overlap (e.g., 6 - 8 bp) increases binding stability and yield but risks partial pairing on wildtype, increasing false-positive amplification. Therefore, a small length-gradient panel was evaluated to identify 3 the shortest overlap that still supports robust amplification while preserving junction selectivity.

2.4. Sequence Characteristics and Screening

Candidate primers were screened to minimise self-dimers and cross-dimers (espe cially 3' complementarity), avoid long homopolymer runs, and maintain balanced GC content to reduce secondary structure. Junction-spanning primers were additionally checked to ensure that the 3' end is anchored on the mutant-specific junction rather than within flanking sequence that is shared with wildtype.

3. Results

In four design cycles, the core amplification and post-reaction purification workflows were characterized. The resulting amplicons (see **Figure 2**) were evaluated via agarose gel electrophoresis alongside PCR validation and Sanger sequencing to confirm sequence integration.

In four design cycles, the core amplification and post-reaction purification workflows were characterized. Independent parallel primer evaluations revealed that the 5 bp overlap variant (BBa_25ZSP4AQ) yielded the highest absolute amplification performance. To support this conclusion objectively, a relative densitometric (grayscale) analysis of the target bands was conducted. Digital band-intensity quantification showed that the 5 bp overlap configuration achieved a 1.5-fold ± 0.12higher relative grayscale density compared to the 6 bp variant, and a 2.3 -fold ± 0.18increase over the 4 bp variant. This objective metric confirms the

5 bp overlap primer as the optimal design for maximum amplification yield—see Figure 3.

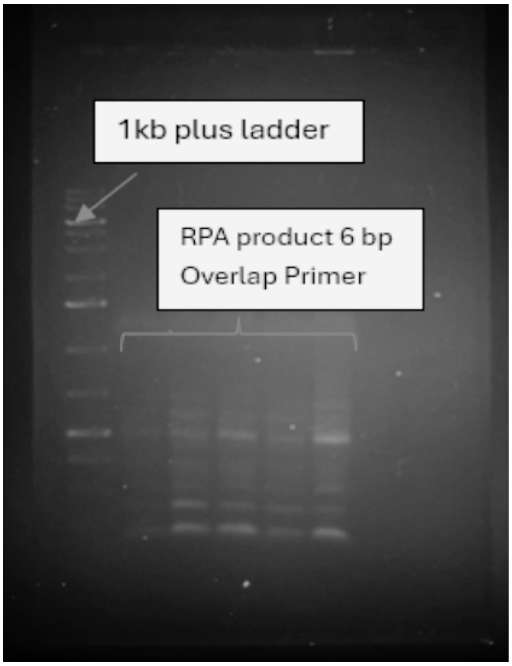

Figure 2. Gel Electrophoresis of RPA reactions displaying 5 identical trials utilizing the 6 bp Overlap Primer with 10 ng of synthetic mutation input template.

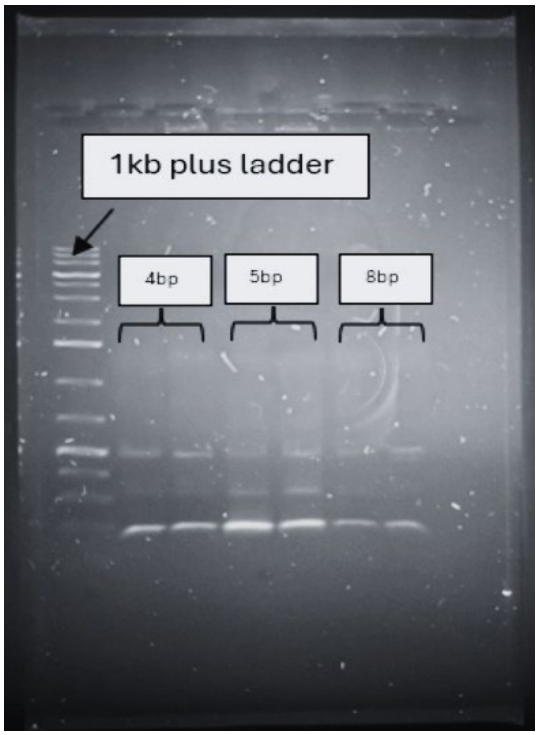

Figure 3. Gel Electrophoresis tracking RPA amplification profiles across an input template mass gradient. The specific lane layout matches lanes 1 through 8 from left to right as detailed above.

The order of the sample ran in the gel are as follows:

1) 100 - 300 bp DNA ladder.

2) RPA sample with 0 ng of mutated DNA template input.

3) RPA sample with 0.5 ng of mutated DNA template input.

4) RPA sample with 1 ng of mutated DNA template input.

5) RPA sample with 5 ng of mutated DNA template input.

6) DNA input (5bp Overlap Forward Primer, Reverse primer, mutated DNA template input).

7) 100 ng of wildtype DNA template.

8) 100 ng of mutated DNA template.

Gel Electrophoresis of RPA with wildtype, mutation input reactions and negative control Gel electrophoresis after PCR purification showing sharper bands.

To evaluate sensitivity boundaries, input concentrations were modified. The target band remains prominent within the fractional nanogram lanes, but persistent off-target products continue to form.

Figure 4 captures a representative 10-lane analytical PCR gel used to evaluate cross-reactivity properties across selected primer pairs. The results confirm that the tested primer configurations alone lack the necessary discriminatory resolution, as they simultaneously amplify both the mutant and wildtype sequences (Figure 4). This lack of isolated specificity demonstrates the clear necessity of incorporating a secondary enzymatic selection mechanism, such as a downstream CRISPR-Cas system, prior to adapting the assay for lateral flow devices [6] [8] [12] [13] [14].

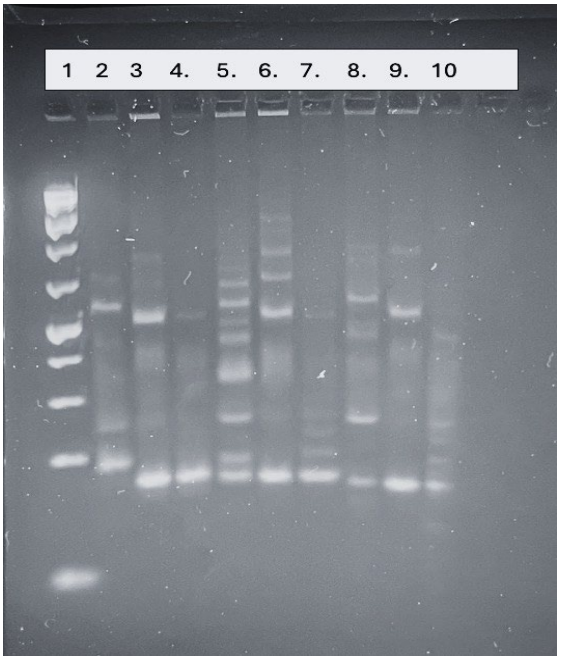

Figure 4. 10-lane analtytical Gel Electrophoresis of PCR to evaluate cross-reactivity properties across selected primer pairs.

The order run on the gel from left to right is always the same primer pair with wildtype template, mutation template, and then the negative control in groups. Then the variation between groups from left to right is as follows with the annealing temperature specified. Figure 5 shows the electrophoresis results of PureLink PCR Purification with input DNA material (template plus primer pairs).

1) Wildtype forward primer + reverse primer (72 degrees Celsius).
2) 4 bp overlap primer + reverse primer (72 degrees Celsius).
3) 5 bp overlap primer + reverse primer (71 degrees Celsius).
4) 6 bp overlap primer + reverse primer (70 degrees Celsius).
5) 8 bp overlap primer + reverse primer (72 degrees Celsius).
6) Forward template primer + reverse template primer (61 degrees).

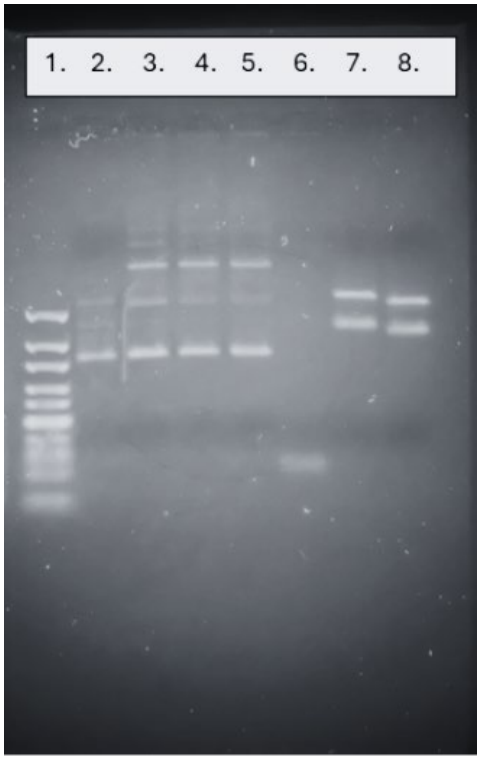

Figure 5. Gel Electrophoresis Results of Pure Link PCR Purification with input DNA material (template plus primer pairs).

4. Discussion

4.1. Mechanistic Basis for the 5 bp Overlap Primer

The superior performance of the 5 bp junction-overlap primer is most plausibly explained by a balance between (i) extension gating at the mutant-specific junction and (ii) sufficient duplex stability to support rapid initiation under RPA conditions. In a junction-spanning design, the decisive specificity comes from the 3' terminus: the mutant template presents a perfectly base-paired 3' end, whereas the wildtype forces a mismatch/overhang across the deletion boundary, which should strongly suppress polymerase extension. If the overlap is too short (e.g., 4 bp),

correct mutant pairing becomes highly contingent on a very small interaction surface, increasing stochastic initiation failures and lowering yield. If the overlap is too long (e.g., 6 - 8 bp), partial pairing on wildtype becomes thermodynamically more permissive, and RPA's lower-stringency strand-invasion dynamics can tolerate these near-matches, eroding discrimination. The 5 bp configuration appears to sit near an empirical optimum where the mutant junction provides a strong kinetic advantage while still sustaining robust amplification.

4.2. Origin of Non-Specific Products and a Path to Verification

Any mechanistic attribution of the ~180 bp and ~500 bp non-specific bands based only on thermodynamic ΔG predictions (e.g., primer-dimer formation or template self-folding) is an unverified conjecture. More broadly, the off-target bands observed across conditions could arise from primer-dimers (3' complementarity enabling short self-extended products), mis-priming at partially homologous sites within the synthetic templates, and RPA-specific artefacts such as recombinase-mediated initiation at transient micro-homologies; higher molecular-weight bands can also be consistent with concatemerisation or template switching during strand-displacing synthesis. To verify the origin of these products (and to distinguish true mutant amplicons from artefacts), a staged workflow is required: (1) confirm reproducibility across technical replicates and the complete absence of the band in no-template con trols; (2) excise bands at the expected and off-target sizes and perform Sanger sequencing to identify the underlying sequence; (3) run restriction/guide-based diagnostic cuts (if a unique junction site is present) to confirm identity; and (4) repeat using fragmented background DNA to test whether the same non-specific products persist under a more realistic complexity load.

4.3. Limitations of the Current Synthetic Template System

The present evaluation relies on synthetic templates that do not fully represent plasma ctDNA. Synthetic inputs can be orders of magnitude less fragmented and less sequence-complex than clinical material, lack the broad wildtype background found in patient plasma, and can overstate primer specificity by presenting an unrealistically clean junction context. They also do not reproduce the very low mutant allele fraction typical of early-stage disease, where even small levels of wildtype cross-amplification become clinically unacceptable. Consequently, performance metrics inferred from the template system should be interpreted as proof-of-principle for primer logic rather than as a direct proxy for clinical sensitivity/specificity.

4.4. Why a Downstream CRISPR Filtration Step Is Necessary

The remaining wildtype amplification and non-specific products motivate a downstream sequence-filtration layer. A CRISPR-based step can be engineered to selectively cleave (or block) wildtype amplicons using guide RNAs that span the

wildtype junction context, while sparing the mutant junction product. This converts the assay from a single-stage "primer-only" discrimination problem into a two-stage selectivity cascade: RPA enriches the region rapidly, and CRISPR then imposes high-fidelity sequence recognition to suppress false positives. In the context of low-abundance ctDNA, this filtration is essential to maintain diagnostic specificity while preserving the sensitivity benefits of isothermal amplification.

Figure 6 below shows the gel electrophoresis results of PCR for all primers with mutated, wildtype inputs, and negative control using water instead of input.

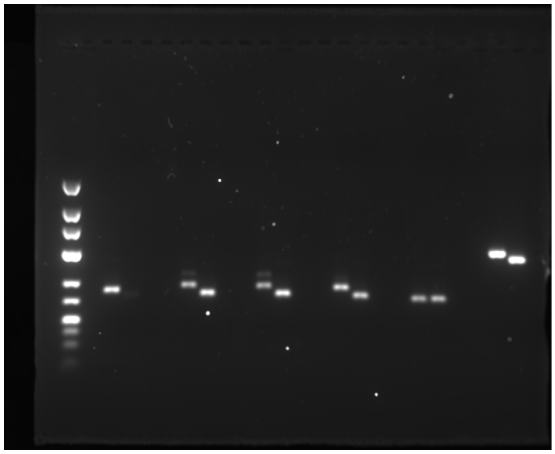

Figure 6. Gel Electrophoresis of PCR for all primers with mutated, wildtype inputs, and negative control using water instead of input.

5. Conclusion

This investigation successfully characterizes a preliminary isothermal RPA framework capable of amplifying target sequences modeled after the EGFR ex19del mutation. Gel electrophoresis results validate that a 5 bp overlap forward primer configuration optimizes diagnostic amplification yields under standard 42˚C reaction configurations. However, because the primer pairs exhibit baseline cross-reactivity with wild-type sequences, the current diagnostic framework cannot establish isolated clinical specificity independently. Future perspectives look to integrate downstream CRISPR-Cas9 enzymatic filters to potentially suppress off-target pathways. Long-term transitions toward "one-pot" microfluidic platforms, paper-fluidic LFA readouts, or automated screening risk-algorithms remain purely theoretical hypotheses until this base amplification chemistry is validated within clinical human plasma cohorts.

Registered Parts

1) EGFR exon 19 analyte wildtype: See iGEM Registry entry igem$_b$*ba*25*f c*1*yhp.*
   a) BBa_25FC1YHP
2) EGFR exon 19 analyte mutation: See the iGEM Registry en-try igem$_b$*ba*25*ok*44*kf*
   b) BBa_25OK44KF

3) RPA ForwardPrimer for EGFR exon 19 23 bp deletion mutation: See iGEM Registry entry igem$_b$*ba*25*zsp*4*aq.*

c) This was referred to as 5bp Overlap Primer in documentation

d) BBa_25ZSP4AQ

4) Reverse primer: See iGEM Registry entry igem$_b$*ba*250*cqdqm.*

e) BBa_250CQDQM

5) Forward Template primer: See iGEM Registry entry igem$_b$*ba*25*wp*03*jr.*

f) BBa_25WP03JR

6) Reverse Template primer: See iGEM Registry entry igem$_b$*ba*25*rz*50*sr.*

g) BBa_25RZ50SR

7) RPA forward primer EGFR exon 19: See iGEM Registry en-try igem$_b$*ba*25*kpuu*24*.*

h) BBa_25KPUU24

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Author Contributions

O.G. and C.T. conducted the experiments and wrote the main paper, T.K. supervised the research and wrote the paper and did the administration work.

Conflicts of Interest

The authors declare that no conflict of interest was associated with this research and also that no funding was associated with it.

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