Dear Members of the Geoffrey Ogram Memorial Research Grant Review Committee,

September 26, 2025

Dear Members of the Research Committee,

I am writing to submit my Letter of Intent for the 2025 Lung Cancer Canada Research Grant competition. The proposed project, *"Blood-Based Multiplex Lateral Flow Assay for Early Detection of Lung Cancer in Light Ex-Smokers and Never-Smokers,"* aims to address a critical gap in lung cancer detection by developing and validating a rapid, low-cost blood test designed for populations excluded from current LDCT screening guidelines.

Building on a prototype developed at McMaster University and recognized with the **Lung Ambition Award in 2023**, this project will conduct a one-year prospective case–control feasibility study involving twenty lung cancer patients (light ex-smokers or never-smokers) and twenty matched controls. The multiplex lateral flow assay will be benchmarked against qPCR to assess sensitivity, specificity, and clinical correlation with stage, histology, and driver mutations. The ultimate objective is to provide proof-of-concept evidence that this platform can expand early detection to underserved populations, reduce inequities in access, and improve survival outcomes for Canadian patients.

The proposed research aligns directly with Lung Cancer Canada's mandate to reduce the burden of lung cancer and optimize patient care. It will deliver a validated assay, pilot clinical data, and a translational framework for larger-scale studies that could inform future screening guidelines and national policy. The project will also generate community engagement and knowledge translation outputs, including participation in awareness events, contribution of a summary for Lung Cancer Canada's forums, and presentation of results at a national or regional lung cancer conference.

Thank you for considering this application. I look forward to the opportunity to contribute to Lung Cancer Canada's mission of advancing early detection and improving outcomes for all Canadians affected by lung cancer.

Sincerely,

Fei Geng, PhD

Associate Professor
School of Biomedical Engineering
McMaster University

Blood-Based Multiplex Lateral Flow Assay for Early Detection of Lung Cancer in Light Ex-Smokers and Never-Smokers

Rationale

Lung cancer is the leading cause of cancer-related mortality in Canada, with marked variation in incidence and outcomes across provinces and demographic groups [1,2]. The primary driver of mortality is late diagnosis[3]. Patients diagnosed at stage I can achieve five-year survival rates above 60%, while those diagnosed with advanced disease have survival rates below 20%[4,5].

Although low-dose CT (LDCT) screening has demonstrated mortality reductions in heavy smokers, it is limited by a high false-positive rate, radiation exposure from repeated scans, and narrow eligibility criteria. Current guidelines generally restrict access to individuals over 50 with a history of heavy smoking, excluding light ex-smokers and never-smokers[6]. Yet, these groups account for roughly one-quarter of lung cancers in Canada and include a disproportionate number of women and younger adults[7]. Because they fall outside the LDCT framework, such patients are most often diagnosed only when symptomatic, at which point curative options are far less effective.

Lung Cancer Canada and the Lung Ambition Alliance have identified this gap in screening as a national priority. Broadening access to early detection, particularly for underserved populations, is critical to reducing inequities in care and improving outcomes. Our proposal responds directly to this priority by developing and validating a blood-based multiplex lateral flow assay (LFA) capable of detecting lung cancer in light ex-smokers and never-smokers.

Impact Statement

This project targets a critical gap in Canadian lung cancer care by developing and validating a rapid, low-cost blood test for early detection in light ex-smokers and never-smokers, who represent up to one quarter of new cases but remain excluded from LDCT screening programs. By enabling diagnosis at earlier, curable stages, this assay has the potential to reduce mortality, improve patient outcomes, and expand equitable access to screening across underserved populations. Building on our award-winning prototype developed at McMaster University and recognized with the **Lung Ambition Award in 2023**, this work directly advances Lung Cancer Canada's mission to broaden early detection, reduce inequities, and optimize patient care.

Preliminary Work and Platform Development at McMaster

In 2023, Dr. Fei Geng, together with Dr. Rosalyn Juergens and Dr. Monsur Ali, received the Lung Ambition Award from Lung Cancer Canada, AstraZeneca Canada, and the Lung Ambition Alliance. This recognition supported the development of a prototype blood-based multiplex LFA at McMaster University. The prototype targets a validated panel of four microRNAs (miR-126, miR-145, miR-210, and miR-205-5p) that have demonstrated sensitivity greater than ninety percent and specificity greater than ninety-five percent for discriminating lung cancer from controls[8,9]. The technology combines ligation-PCR for probe specificity with a gold nanoparticle–based lateral flow strip for simple visual readout. Analytical testing has shown sensitivity down to 0.2 pM, reproducible detection in plasma-spiked samples, and minimal cross-reactivity between targets. The device delivers results within thirty minutes, is inexpensive to produce, and requires no specialized laboratory equipment. These features make it ideally suited for population-level screening and complementary use alongside LDCT.

Objectives

The overall objective of this project is to **clinically validate a blood-based multiplex lateral flow assay for early detection of lung cancer in light ex-smokers and never-smokers**. This will expand access to curative interventions for populations excluded from current LDCT guidelines. The specific aims are to confirm the assay's analytical sensitivity, specificity, and reproducibility in plasma samples compared to the qPCR gold standard; to conduct a pilot clinical study of patients with lung cancer and matched controls; to correlate miRNA signatures with stage, histology, and oncogenic drivers; and to define the assay's performance thresholds that would support future population-based screening initiatives.

Study Plan, Timeline, Milestones, and Deliverables

This one-year prospective case–control feasibility study will be conducted at McMaster University and Hamilton Health Sciences under the leadership of thoracic oncologist Dr. Rosalyn Juergens. A total of twenty patients with confirmed non-small cell lung cancer who are either light ex-smokers with fewer than twenty pack-years or never-smokers will be recruited, along with twenty age- and sex-matched controls without a history of lung cancer. Inclusion criteria will consist of histologically or cytologically confirmed NSCLC for the case group, no cancer diagnosis in the past five years for the control group, and a smoking history consistent with light ex-smoker or never-smoker status. Patients with heavy smoking histories, recent thoracic radiation or systemic therapy, or concurrent malignancies will be excluded.

Stage 1: In months #1–3, the project will focus on **assay optimization**. Plasma will be isolated from collected blood samples, ligation-PCR conditions refined, and reproducibility confirmed in spiked plasma. LFA strip reproducibility will be assessed in parallel. The deliverable for this stage will be a dataset confirming sensitivity, specificity, and reproducibility under controlled conditions.

Stage 2: In months #4–6, **analytical validation** will be carried out in **clinically relevant plasma conditions**. The multiplex LFA will be benchmarked directly against qPCR, and robustness will be tested against confounding factors such as hemolysis and RNase activity. The endpoint of this stage will be a validated assay ready for patient testing, with the milestone being a workflow demonstrating consistent performance in real biological samples.

Stage 3: In months #7–12, the **clinical feasibility study will be implemented**. Twenty patients and twenty matched controls will be enrolled through Hamilton Health Sciences. Ten-millilitre blood samples will be collected at diagnosis for cases and during routine visits for controls. Plasma will be isolated, miRNA extracted, and both ligation-PCR and LFA performed. Clinical data, including stage, histology, and driver mutation status (**EGFR, KRAS, ALK, ROS1**), will be recorded. LFA results will be compared directly with qPCR. The primary endpoint will be sensitivity and specificity. Secondary endpoints will include reproducibility, tolerance to plasma variability, and correlation of miRNA levels with stage and histology. Exploratory analyses will assess associations between miRNA patterns and molecular features such as EGFR, KRAS, and ALK mutations. Data analysis will calculate sensitivity, specificity, positive and negative predictive values, and receiver operating characteristic curves to assess diagnostic accuracy. Subgroup analyses will examine performance across stage, histology, and mutation status. The deliverable at this stage will be proof-of-concept clinical performance data demonstrating the assay's potential to detect lung cancer in populations excluded from LDCT screening.

Within three months after project completion, a **final report** will be submitted to Lung Cancer Canada summarizing analytical and clinical findings. The investigative team will also participate in a **community engagement event** to raise awareness of lung cancer and will present study results at a national or regional lung cancer conference such as the **Canadian Lung Cancer Conference** or the Ontario Thoracic Cancer Conference.

By the end of this one-year project, the team will have delivered **a validated multiplex LFA platform**, **proof-of-concept clinical performance data** in **light ex-smokers and never-smokers**, a final report to Lung Cancer Canada, a public-facing summary, community engagement activities, and dissemination of results at a peer-reviewed national conference.

Team

The investigative team brings together complementary expertise. Dr. Fei Geng has more than twenty years of experience in lung cancer biomarker discovery and is the scientific lead for the project. Dr. Rosalyn Juergens is a thoracic oncologist and national leader in lung cancer clinical trials who will direct the clinical aspects of the study. Dr. Monsur Ali has over twenty-five years of experience in biosensor engineering and lateral flow technology and will lead the technical development of the device. This combination of biomarker science, clinical oncology, and device engineering ensures feasibility, clinical relevance, and rapid translation to practice.

1. Yousefi, J. Geographical Disparities in Lung Cancer in Canada: A Review. *Curr Oncol Rep* **26**, 221–235 (2024).
2. Warkentin, M. T. *et al.* Age-specific lung cancer incidence trends in Canada from 1992 to 2022. *Cancer Epidemiol* **95**, 102774 (2025).
3. Candal-Pedreira, C. *et al.* Analysis of Diagnostic Delay and its Impact on Lung Cancer Survival: Results From the Spanish Thoracic Tumor Registry. *Arch Bronconeumol* **60**, S38–S45 (2024).
4. Wang, C. *et al.* Persistent increase and improved survival of stage I lung cancer based on a large-scale real-world sample of 26,226 cases. *Chin Med J (Engl)* **136**, 1937 (2023).
5. Woodard, G. A., Jones, K. D. & Jablons, D. M. Lung Cancer Staging and Prognosis. *Cancer Treat Res* **170**, 47–75 (2016).
6. Lam, S. *et al.* Management of screen-detected lung nodules: A Canadian partnership against cancer guidance document. *Canadian Journal of Respiratory, Critical Care, and Sleep Medicine* **4**, 236–265 (2020).
7. Linehan, V., Harris, S. & Bhatia, R. An Audit of Opportunistic Lung Cancer Screening in a Canadian Province. *J Prim Care Community Health* **12**, 21501327211051484 (2021).
8. Shen, J. & Jiang, F. Applications of MicroRNAs in the Diagnosis and Prognosis of Lung Cancer. *Expert Opin Med Diagn* **6**, 197 (2012).
9. Leng, Q. *et al.* A plasma miRNA signature for lung cancer early detection. *Oncotarget* **8**, 111902–111911 (2017).

Our pioneering project to develop a blood-based lateral flow device for the simultaneous detection of a microRNA (miRNA) panel is set to exert a sustained and transformative influence on lung cancer research. By addressing the unmet need for early detection in populations currently excluded from conventional screening criteria—such as light ex-smokers and non-smokers—this project has the potential to significantly reduce lung cancer incidence and mortality while improving patient quality of life.

Lung cancer remains the leading cause of global cancer-related deaths, largely due to the challenges of early detection. Most cases are diagnosed at an advanced stage, where treatment options are limited and prognoses are poor. Our project offers a new path forward by introducing a non-invasive, cost-effective, and accessible screening tool that targets individuals who are underserved by existing screening programs. This device will expand the reach of early-stage lung cancer screening, providing timely intervention to broader populations, which is critical to reducing mortality.

This innovative approach also promotes a major advancement in lung cancer research by accelerating the translation of scientific findings into real-world applications. Led by a team of researcher, oncologist and engineer, including Dr. Fei Geng, Dr. Rosalyn Juergens, and Dr. Monsur Ali, this endeavor is poised to exert a sustained, powerful influence on lung cancer research and significantly reshape the landscape of patient outcomes. By leveraging a novel miRNA panel, we aim to **bring laboratory discoveries into clinical settings**, where they can deliver tangible benefits to patients. In the short to medium term, our lateral flow device has the potential to become a powerful tool in routine screening, significantly **improving the chances of early diagnosis** and, consequently, **better treatment outcomes**.

Additionally, this project will foster a deeper understanding of **lung cancer etiology across different populations**, particularly those not covered by current screening methods. By focusing on these underserved groups, we aim to advance research into the diverse biological pathways of lung cancer, ultimately leading to more **tailored and effective screening strategies**.

Our project's key innovation lies not only in its **diagnostic capability** but also in its ability to **reshape early detection strategies**. By enabling earlier and broader detection, we anticipate a reduction in late-stage diagnoses, which in turn will facilitate more effective and less aggressive treatment options. This will optimize patient care, reduce the healthcare burden, and improve long-term survival and quality of life.

In the short term, we will focus on refining and validating the lateral flow device through rigorous testing, ensuring that it meets the high standards required for reliability and clinical use. As the project progresses, the knowledge gained will propel major advancements in lung cancer research, positioning the device for widespread implementation. In the medium term, we envision this lateral flow device becoming an integral component of lung cancer screening protocols, driving a more inclusive and effective approach to early detection.

Ultimately, this project has the potential to **revolutionize lung cancer screening**. By providing a **scalable** and **practical** solution for early detection, we aim to reduce lung cancer incidence and mortality while enhancing patient well-being. Through the accelerated translation of scientific knowledge into optimized patient care, this project is poised to make a lasting impact on the global fight against lung cancer.

Lung cancer remains one of the deadliest cancers worldwide, largely because it is often diagnosed at advanced stages when treatment options are limited and survival rates are low. Our project, led by a distinguished team of researchers, aims to achieve **early-stage lung cancer detection** using a microRNA (miRNA)-based lateral flow device. This innovative approach will significantly broaden access to early-stage lung cancer screening, particularly for individuals who do not meet the current criteria for traditional screening methods, such as light ex-smokers, non-smokers, and younger individuals.

Current screening methods, such as low-dose computed tomography (LDCT), are limited to high-risk groups (e.g., heavy smokers and older adults), leaving a large segment of the population without effective early detection tools. Our platform uses miRNAs—small molecules found in blood that can reflect disease states—as the basis for a new, non-invasive, and cost-effective approach to detecting lung cancer at its earliest stages. While miRNAs such as miRs-126, 145, 210, and 205-5p have already been shown in research to be associated with lung cancer, our goal is to develop a lateral flow assay that can efficiently and accurately detect these markers to facilitate earlier interventions.

This project directly supports two objectives: First, it advances **research into early detection methodologies** by creating a blood-based screening tool that is easy to use, rapid, and widely accessible. Second, it investigates the **etiology of lung cancer in diverse populations**, specifically focusing on light ex-smokers and non-smokers, who are often excluded from traditional screening criteria despite their potential risk of developing the disease.

Our project is structured into three phases. Initially, we will optimize the miRNA detection platform to ensure high sensitivity and specificity. Following this, the lateral flow assay components will be incorporated into an easy-to-use device similar to a home pregnancy test, offering a rapid, point-of-care solution. Finally, we will validate the performance of the device by conducting pilot tests using blood samples from lung cancer patients and healthy individuals to assess its clinical accuracy and feasibility.

The potential impact of this project is far-reaching. By developing this miRNA-based lateral flow platform, we are creating a practical solution that can be used in a variety of healthcare settings, including those with limited resources. It is not only designed to improve lung cancer detection among high-risk populations but also to extend early detection capabilities to those who do not meet traditional screening guidelines, such as younger individuals and non-smokers.

In the future, this platform could revolutionize lung cancer screening practices, leading to earlier diagnosis, more effective treatments, and improved survival rates. With strong proof-of-concept data, we plan to seek additional support, such as a CIHR research grant, to expand the testing of this platform across larger patient cohorts. Our ultimate vision is to make early lung cancer detection accessible to all, reducing lung cancer incidence and mortality while improving patient quality of life on a global scale.

Institution: **McMaster University**

DETAILED BUDGET		**FROM: 12/1/2025**	**TO: 11/30/2026**
PERSONNEL		**SALARY REQUESTED**	
NAME	**ROLE**	**Year 1**	**TOTALS**
Dr. Fei Geng	PI	N/A	N/A
Dr. Rosalyn Juergens	Co-PI	N/A	N/A
Dr. Monsur Ali	Co-PI	N/A	N/A
Paola Gonzalez Perez	M.A.Sc. Student	$12,500	$12,500
PERSONNEL TOTAL		$12,500	**$12,500**
SUPPLIES			
DNA-modified gold nanoparticles		$700	$700
Normal human serum		$500	$500
Synthetic miRNAs		$1,000	$1,000
Primer and probe synthesis		$800	$800
Conjugation reagents		$1,000	$1,000
Lateral flow strip supplies (nitrocellulose Membrane, sample pad, backing card, etc.)		$2,000	$2,000
			$6,000
FACILITY USAGE			
Lateral flow strip dispensing		$3,500	$3,500
Test strip cutting and assembly		$1,500	$1,500
			$5,000
OTHER EXPENSES			
Computer purchase		$1,500	$1,500
			$1,500
TOTAL DIRECT COSTS			**$25,000**
TOTAL COSTS			**$25,000**

PERSONNEL

The research team for the proposed study includes PI Dr. Fei Geng (Program Chair in Biotechnology at McMaster), Co-PI Dr. Rosalyn Juergens (Oncologist specializing in lung cancer), Co-PI Dr. Monsur Ali (Biomedical Engineer specializing in lateral flow-based system), and M.A.Sc. student Paola Gonzalez Perez.

Here's the information regarding the personnel required for the study:

Principal Investigator (PI): Dr. Fei Geng

Education: Dr. Geng received his M.D. degree from Jining Medical University (China) and his Ph.D. in Biochemistry from McMaster University.

Current Position: Associate Professor in School of Biomedical Engineering and Program Chair in Biotechnology at McMaster University.

Experience: Dr. Geng has been working on biomarker discovery and detection of lung cancer since 2004. He is a member of the School of Biomedical Engineering and Biointerfaces Institute at McMaster University.

Role: Dr. Geng will oversee the entire study, providing guidance and direction and supervise the graduate student in the development of miRNA detection module (**Phase 1**). Responsibilities include project planning, technical management, securing funding, managing the budget, and ensuring adherence to ethical guidelines.

Co-PI (Oncologist): Dr. Rosalyn Juergens

Education: Dr. Juergens received her M.D. degree from Georgetown University and Ph.D. in Clinical Investigation from The Johns Hopkins Bloomberg School of Public Health.

Experience: Dr. Juergens is an oncologist specializing in lung cancer at Juravinski Cancer Centre. She has clinical expertise in lungl cancer and has held various leadership positions in cancer research and patient advocacy.

Role: Dr. Juergens will contribute to the study as a Co-PI, leveraging her expertise in oncology and contribute to the patient recruitment process and clinical analysis. Dr. Juergens will provide the clinical insight and guide the development of miRNA lateral flow device for lung cancer early detection (**Phases 1 and 2**)

Co-PI (Biomedical Engineer specializing in lateral flow-based system): Dr. Monsur Ali

Education: Dr. Ali received his PhD in Pharmaceutical Sciences in Kyushu University, Japan. Dr. Ali has been the Scientist at University of California and McMaster University.

Current Position: Researcher at the Biointerfaces Institute, McMaster University.

Experience: Dr. Ali is one of the most cited researchers in the field of biosensor and lateral flow system. He is recognized for his work in developing disposable paper-based point-of-care diagnostic devices.

Role: Dr. Ali will be responsible for miRNA lateral flow device development (**Phase 2**) and will provide the supervision of graduate student in the establishment of lateral flow device setup and system optimization.

M.A.Sc. student: Ms. Paola Gonzalez Perez

Education: Ms. Paola Gonzalez Perez earned her Bachelor's degree in Biotechnology from McMaster University and is currently in the second year of her Master's studies in the School of Biomedical Engineering at McMaster University.

Role: The student will be responsible for conducting experiments, analyzing data and drafting the manuscript. She will work closely with the PI and Co-PIs to ensure the study is conducted rigorously and scientifically.

OTHER SOURCES OF FUNDING

Lung Ambition Award ($50,000) June 2024 to May 2025
Lung Ambition Award will be used to identify, characterize, and validate novel miRNA candidates for the panel focused on early detection of lung cancer. These activities are distinct and will not overlap with the research proposed for the Geoffrey Ogram Memorial Research Grant. The specific activities funded by Lung Ambition Award include:

- Leveraging existing datasets and computational tools to identify novel miRNA candidates that show promise for early lung cancer detection.
- Characterization of these identified miRNA candidates using cell lines and other biological models to assess their relevance and potential utility in early detection diagnostics.

McMaster University | Research Office for Administration, Development & Support

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September 26, 2025

Dear Lung Cancer Canada:

McMaster enthusiastically supports the proposed research project by Dr. Fei Geng, associate professor at McMaster University. The project, titled “Blood-Based Multiplex Lateral Flow Assay for Early Detection of Lung Cancer in Light Ex-Smokers and Never-Smokers” and submitted for consideration within the Geoffrey Ogram Memorial Research Grant competition, is a compelling and innovative endeavor that aligns seamlessly with our institution's research objectives and goals.

Having thoroughly reviewed the details of the proposed research, we are confident in its feasibility within our institution. Our institution possesses the necessary infrastructure, resources, and expertise to facilitate the successful execution of this project. Furthermore, we acknowledge Dr. Fei Geng's expertise and dedication to their work. Their proven track record and commitment to excellence make us confident in their ability to carry out this research successfully. We anticipate that the outcomes of this research will not only enhance the academic reputation of our institution but also contribute meaningfully to the broader scientific community.

McMaster University intends to provide support for this project in the areas of grant fund administration, data management consultations, and institutional administrative support. We look forward to the positive impact Dr. Fei Geng’s research will have on our institution and the broader academic community.

Sincerely,
Sherisse Webb

Director, Research Office for Administration, Development and Support

Appendix: Supporting Manuscript

This Appendix includes an unpublished manuscript presenting preliminary data, optimized protocols, and supplementary figures that directly reinforce the feasibility, rationale, and study plan outlined in the proposal “Blood-Based Multiplex Lateral Flow Assay for Early Detection of Lung Cancer in Light Ex-Smokers and Never-Smokers.”

Sensitive detection of lung cancer-associated microRNAs using ligation-PCR coupled with lateral flow assay

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Abstract

Early detection of lung cancer remains a major challenge due to the absence of reliable and accessible screening tools for populations outside high-risk groups. Circulating microRNAs (miRNAs) are promising biomarkers owing to their stability and disease-specific expression profiles. In this study, we present a ligation-PCR coupled with lateral flow assay (LFA) for the detection of miR-126 and miR-145 in serum samples from lung cancer patients. The workflow involves probe ligation, PCR amplification under optimized conditions, and visualization via gold nanoparticle–based LFA. Analytical sensitivity tests using synthetic miRNAs demonstrated detection down to 0.2 pM, with amplification controlled at 28 cycles to avoid non-specific products. Specificity experiments confirmed discrimination between miR-126 and miR-145. Spiking experiments in serum validated detection across serial dilutions, and application to patient and control serum samples confirmed clinical feasibility. This platform combines molecular specificity with visual readout, providing a cost-effective approach suitable for early lung cancer biomarker detection.

Keywords
Lateral flow assay; miRNA; lung cancer; ligation-PCR; biomarker detection

1. Introduction

Lung cancer remains as one of the leading causes of cancer-related deaths worldwide, with non-small cell lung cancer (NSCLC) accounting for 85% of the cases (1.1). The poor prognosis and survival rate of lung cancer is largely a result of it being more commonly diagnosed at late stages. Individuals diagnosed at an advanced stage have a five-year survival rate lower than 20% (1.2). Early-stage lung cancer detection significantly improves survival outcomes, with up to 62% of patients surviving for five years or more when the disease is identified at stage one. Existing screening methods, such as low dose computed tomography (LDCT), allows for an early detection of lung cancer cases. However, its accessibility is limited in scope and individuals aged 55 to 74 who smoked cigarettes every day for at least 20 years (1.3). This criterion excludes a large portion of the population regardless of the current rise of lung cancer cases in non-smokers.

Circulating microRNAs (miRNAs) have emerged as promising minimally invasive biomarkers. They are stable in body fluids, reflect underlying tumor biology, and have been associated with NSCLC progression. Among them, miR-126 and miR-145 have been consistently implicated in lung adenocarcinoma pathogenesis and prognosis… However, standard detection platforms such as qPCR, microarrays, and next-generation sequencing are costly and not readily deployable for point-of-care testing.

Here, we developed a detection platform integrating ligation-based probe recognition with PCR amplification and a lateral flow assay (LFA) readout. This strategy leverages the specificity of probe ligation, the sensitivity of PCR, and the simplicity of LFAs to enable rapid detection of lung cancer–associated miRNAs in patient serum samples, as schematically presented in Fig. 1.

2. Materials and Methods

2.1 Materials and reagents

Synthetic miRNA sequences, DNA probes, qPCR primers, thiolated oligonucleotide, and biotinylated DNA strands were custom ordered from Integrated DNA Technologies (IDT, Coralville, IA, USA). SplintR® Ligase and its 10X reaction buffer, the Luna qPCR Master Mix, and nuclease-free water were obtained from New England Biolabs (NEB, Ipswich, MA, USA). Agarose powder and sucrose were obtained from VWR (Radnor, PA, USA). GelRed nucleic acid stain, streptavidin, 10x phosphate-buffered saline (PBS), lyophilized Bovine Serum Albumin, and 30K molecular weight cutoff (MWCO) centrifugal filters (Amicon Ultra-0.5 mL) were purchased from Sigma-Aldrich (MilliporeSigma, Burlington, MA, USA). 40 nm standard gold nanoparticles and LFA cassettes were purchased from Cytodiagnostics (Burlington, ON, Canada). Tween-20 and NaCl were purchased from Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA). SDS was purchased from BioShop (BioShop Canada Inc., Burlington, ON, Canada. Lateral flow strip components, including nitrocellulose membrane (NCP), conjugate pads, sample pads, and absorbent pads were obtained from *standard suppliers*. Mili-Q water, 1X TAE buffer, NaCl, were used as standard laboratory reagents. GNP suspension buffer was prepared with PBS supplemented with 0.1% Tween-20 and 5% sucrose. Running buffer consisted of 2X PBS, 0.2% Tween-20, and 0.2% SDS.

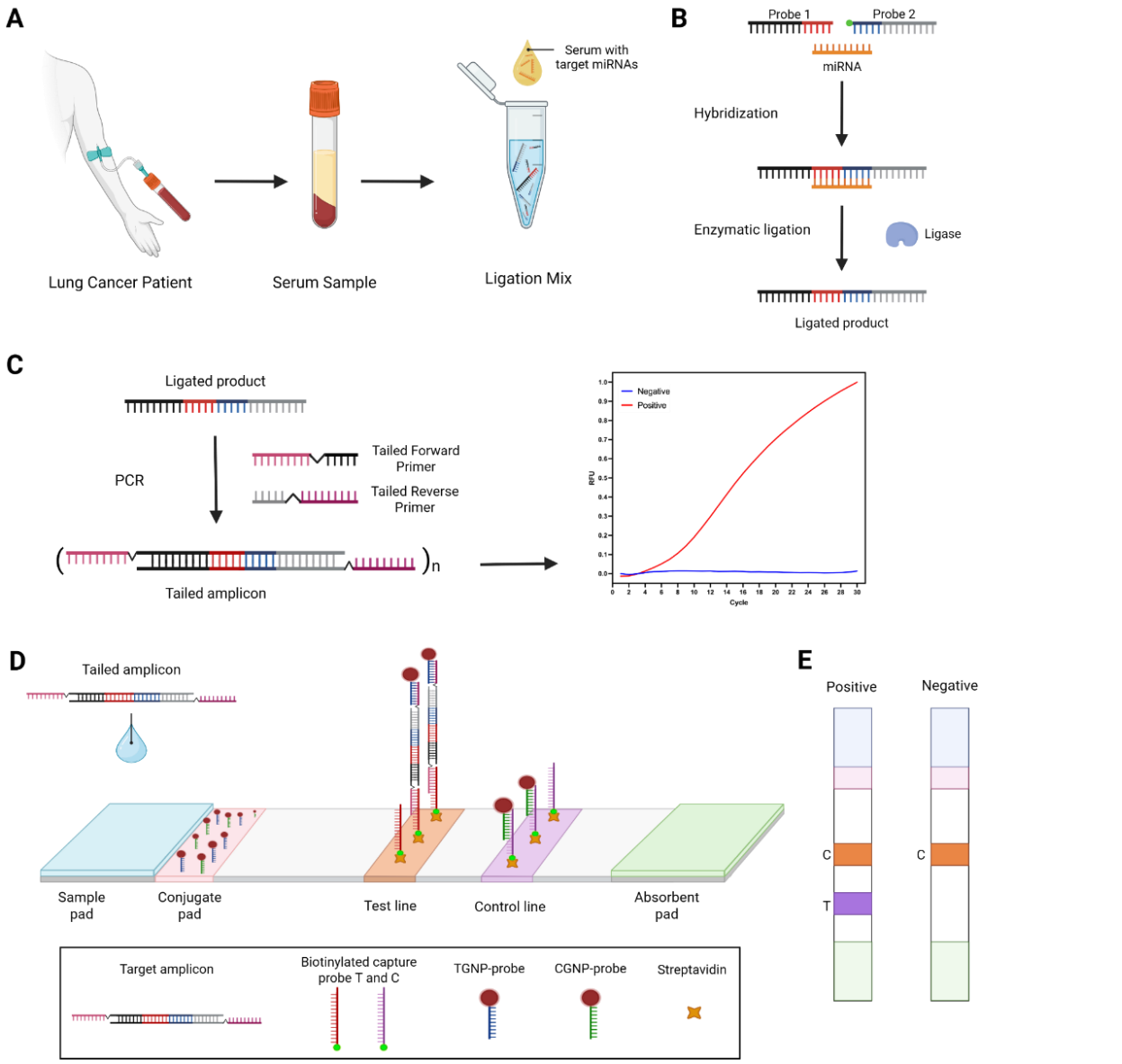

Figure 1. Lateral Flow Platform Based on Ligation-PCR; (A) Blood samples obtained and processed to extract serum to mix in the ligation mix (B) (C) (D) The Construction of Lateral Flow Strips; (E) Visual LFA Results Readout

2.2 Ligation and PCR amplification

Ligation reactions were performed using a modified protocol based on the SplintR® Ligase ligation assay from New England Biolabs. Synthetic miRNA and complementary DNA probes were first reconstituted in Milli-Q water to a stock concentration of 100 µM and then diluted to working concentrations of 1 µM. The ligation mixture consisted of 14 µL RNase-free water, 2 µL of 10X SplintR Ligase Reaction Buffer, 1 µL each of the 3′- and 5′-DNA probes (0.1 µM), and 1 µL of synthetic target miRNA at varying concentrations. The assembled reaction (18 µL total) was heated to 70 °C for 5 minutes and cooled gradually to room temperature over 15 minutes to facilitate hybridization. Following this, 2 µL of diluted SplintR ligase (1 µM, prepared from the 10.5 µM stock) was added to the reaction. The ligation was carried out at 25 °C for 30 minutes,

with shorter timepoints also tested to determine the minimum required ligation time. The enzyme was subsequently inactivated at 90 °C for 5 minutes. Samples were then either kept on ice or stored at −20 °C for later analysis.

Quantitative PCR (qPCR) was performed on a Quant Studio 3 from Applied Biosystems to assess ligation efficiency. Each 25 µL reaction contained 12.5 µL of 2X Luna qPCR Master Mix, 1 µL each of forward and reverse primers (5 µM), 10 µL of the ligated template, and 0.5 µL of RNase-free water. Thermal cycling was conducted using the following parameters: initial activation at 95 °C for 5 minutes, followed by 28 cycles of denaturation at 95 °C for 30 seconds, annealing at 54 °C for 30 seconds, and extension at 72 °C for 30 seconds. A melt curve analysis was performed at the end of the amplification to confirm specificity when lateral flow assays were not used.

2.2.1 Gel Electrophoresis

Agarose gel electrophoresis was used to verify the size of qPCR products. A 2% (w/v) agarose gel was prepared by dissolving 1 g of agarose in 50 mL of 1X TAE buffer. The solution was microwaved in short intervals until fully melted, cooled to 50 to 60°C, and stained with 5 µL of RedSafe. The gel was cast and allowed to solidify at room temperature for 15 to 20 minutes. DNA samples were mixed with loading buffer and loaded alongside a molecular weight ladder. Electrophoresis was carried out at 130 V until the tracking dye migrated approximately 75–80% down the gel, typically over 1 hour.

2.3 Lateral flow assay

2.3.1 Gold Nanoparticle DNA Conjugation

Gold nanoparticle conjugation was performed using a salt-aging method optimized for 40 nm citrate-stabilized gold nanoparticles. For each conjugation, 800 µL of GNPs (OD 10) was incubated with 125 µL of 100 µM thiolated TGNP-DNA or CGNP-DNA by gentle vertical rotation at room temperature overnight (~16 hours). To stabilize the conjugates, salt aging was performed by slowly adding 50 µL of 1 M NaCl with overnight rotation. Another 50 µL of 1 M NaCl were added followed by overnight rotation. The conjugates were then surface passivated with 100 µL of 10% BSA and incubated at room temperature for 2 hours. Following BSA blocking, the GNP-DNA conjugates were centrifuged at 5,000 rpm for 15 minutes, the supernatant discarded, and the pellet washed twice in ultrapure water. After final centrifugation, the conjugates were resuspended in 800 µL of GNP Suspension Buffer and stored at 4 °C in the dark.

2.3.2 Lateral Flow Strip Fabrication

To prepare the test and control lines, streptavidin was conjugated to biotinylated DNA (TL-DNA and CL-DNA). Each conjugation consisted of 300 µL of 100 µM DNA, 500 µL of 1 mg/mL streptavidin, and 200 µL PBS, incubated at room temperature for 30 minutes. The resulting conjugates were purified using Amicon Ultra 30K MWCO filters via a multi-step centrifugation and PBS washing protocol, followed by recovery and dilution to a final volume of 1mL. The TL- and CL-streptavidin conjugates were loaded into a BIODOT lateral flow printer and dispensed on nitrocellulose membranes at drop volumes of 20nL, with two passes per line. The test line was positioned approximately 15 mm below the membrane's top edge and the control line 20 mm below. Printed membranes were dried at 37 °C for 1 hour.

For conjugate pad preparation, TGNP-miR145 and CGNP-DNA conjugates were mixed at a 1:1 ratio, and 10 µL of the mixture was spotted onto one end of the pad, then dried at 37 °C for 1 hour. LFA strips were assembled by layering the sample pad, conjugate pad, printed nitrocellulose membrane, and absorbent pad onto a backing card with slight overlaps to facilitate capillary flow. The complete assembly was cut into 4 mm-wide strips and stored in a desiccator at room temperature.

2.3.3 Lateral Flow Assay

Lateral flow assays were conducted using the prepared strips to evaluate miRNA detection. After PCR amplification, 25 µL of test sample or negative control were mixed with 75 µL of 2X Running buffer. The 100 µL solution was applied to the sample pad. The strips were incubated for 30 minutes at room temperature to allow capillary flow. Results were interpreted visually: a positive result was indicated by both test and control lines, while a negative result showed only the control line. Absence of a control line was considered invalid. ImageJ was used to analyze and measure the intensity of the observed test and control bands.

2.4 Sensitivity and specificity testing

The specificity of the designed probes and primers were tested through by performing the ligation assay at different miRNA concentration, ranging from 5nM to 0.05pM of miRNA in the ligation reaction. Probe concentrations remained the same at different concentrations to assess the sensitivity of the probes across concentrations. Ligation of each sample was performed in duplicates. After ligation, each technical duplicates were pooled to perform the qPCR in triplicates. Primer concentration was kept the same across samples and runs. The input concentration range of the qPCR were 2nM to 0.02pM of ligated product, assuming 100% ligation efficiency (Table S1).

Specificity of the designed oligos was also tested for each set of probes and by cross-reacting them with a non-corresponding miRNA. Hence, miR-145 was added to the ligation mix with the probes for miR-126, and the qPCR with miR-126. The vice versa was performed, where the probes and primers for miR-145 were used with miR-126. A ligation NTC was included in both tests to assess the amplification of each sample and differentiate between background signals and true signals. Ligation assays were performed in duplicates while the qPCR was performed in triplicates.

2.5 Analysis of Plasma-Adulterated Targets

Pooled plasma aliquots from healthy donors were diluted to prepare 10% and 20% plasma samples. For spiking experiments, miR-145 was added to plasma at final concentrations of 1 nM, 10 nM, or 100 nM by mixing 5 µL of synthetic miRNA with 45 µL of plasma (1:10 dilution). Un-spiked plasma samples at each dilution were included as non-template controls (NTCs).

To evaluate the effect of plasma RNases, a heat pre-treatment was performed. Plasma samples were heated at 90 °C for 10 minutes and cooled on ice for 5 minutes prior to spiking. Treated and untreated plasma samples, both spiked and un-spiked, were subsequently processed in the ligation and qPCR assays as described above.

3. Results and Discussion

3.1 Design and optimization of Ligation-PCR reaction

The Probe5' and Probe3' were meticulously designed to enable an efficient SplintR ligase mediated ligation reaction targeting the miR-126 and miR-145 sequences (Table 1). Upon the presence of the target miRNA, the respective single-stranded DNA probes anneal to the complementary sequence of the RNA splint, where the 5'- phosphorylated "donor" probes (Probe3') is ligated to the unmodified "acceptor" probe (Probe5') by the SplintR ligase activity to forming a continuous ssDNA strand as the ligated product. Since the SplintR ligase activity required the presence of the RNA splint, the absence of the target miRNA results in no ligation reaction to take place. The ligated product serves as the template for qPCR amplification. Hence, the efficiency of the ligation to produce the ssDNA template from the miRNA target directly affects the proper amplification of the sample and the final concentration of amplicons available for the capture probes in the lateral flow strip.

Optimization of the ligation efficiency was crucial prior to ensuring high sensitivity of the assay. The ligase concentration, ligation incubation time, and probe concentration were tested to assess the optimal parameters to allow for a rapid ligation while still maintaining a high ligation efficiency and low background signal amplification. 50nM to 250nM of SplintR ligase were added to an NTC and a positive sample containing 5nM of miR-145 (Figure S1). Increasing ligase concentration improved the yield of ligated product as observed by the amplification signal. However, earlier amplification in the NTCs were also observed as the enzyme concentration was increased. Incubation times ranging from 10 to 60 minutes were tested using 200nM of ligase, revealing that 30 minutes provided the best balance between sensitivity of positive samples and suppression of NTC amplification (Figure S2). The optimized conditions of 200nM of ligase and a 30-minute incubation period were selected for all subsequent experiments.

The number of amplification cycles employed for the PCR were also optimized to avoid non-specific amplification which could result in false positive results. A positive sample along the NTC used to test x30 and x40 amplification cycles (Figure S3). In both cases, the CT of the NTC fell between 28 and 29 cycles, deeming 28 amplification cycles as the maximum number of cycles before causing background amplification.

3.2 Analytical sensitivity of designed ligation probes and PCR primers

Figure 1A shows the amplification curves of the miR-145 ligated product at concentrations ranging from 2nM to 0.002nM (5pM to 5nM of miRNA input), with a no-template control (NTC) over 35 cycles. Amplification followed the expected concentration-dependent trend, with higher concentrations amplifying earlier. The 2nM sample reached a CT of 14.7, whereas the 0.002nM sample amplified at 25 cycles. The NTC remained flat until after 29 cycles, indicating that amplification of the lowest concentration could be reliably distinguished up to 28 cycles without background interference.

Figure 1B presents the amplification of ligated miR-126 under the same PCR conditions. The amplification profiles generally followed the expected order, but anomalies were observed between the 0.2nM and 0.02nM dilutions, where curves overlapped and were partially inverted, with only 0.56 cycles difference. The highest concentration (2nM) amplified at 22.7 cycles, while the lowest (0.002nM) was detected at 29.24 cycles. The NTC showed no amplification within 35

cycles, suggesting minimal non-specific products. The irregular amplification trend of miR-126 may be attributed to mismatched primer annealing, as the primer set has a melting temperature ~5 °C higher than that of miR-145, potentially leading to suboptimal PCR performance under identical cycling conditions.

The standard curves in Figure 1C highlight the quantitative relationships between CT values and the logarithm of target concentration. For miR-145, the slope of −3.8 yielded an amplification efficiency (E) of ~83% according to the equation:

$$E = (10^{-1/slope} - 1) \times 100$$

The differences in CT values between consecutive dilutions varied from 1.6 to 4.8 cycles, reflecting variable efficiency across the range. Possible causes include inhibitory carryover from the ligation reaction or incomplete ligation, both of which would reduce effective template input. Nevertheless, a strong linear correlation was obtained (R^2 = 0.9778), indicating reliable quantification potential for miR-145. For miR-126, the slope of −2.78 corresponds to a markedly lower efficiency of ~67%. The poor separation of the 0.2nM and 0.02nM dilutions suggests inefficient ligation or reduced amplification consistency at intermediate concentrations. The regression analysis gave an R^2 of 0.8708, confirming weaker linearity compared to miR-145.

These results demonstrate that the designed probes and primers for miR-145 yield more robust amplification and quantification than those for miR-126. The reduced efficiency of miR-126 may be linked to both primer design constraints and ligation step variability. Importantly, the assay's overall analytical sensitivity is sufficient to detect ligated products down to 0.002nM, with no evidence of non-specific amplification in NTC reactions.

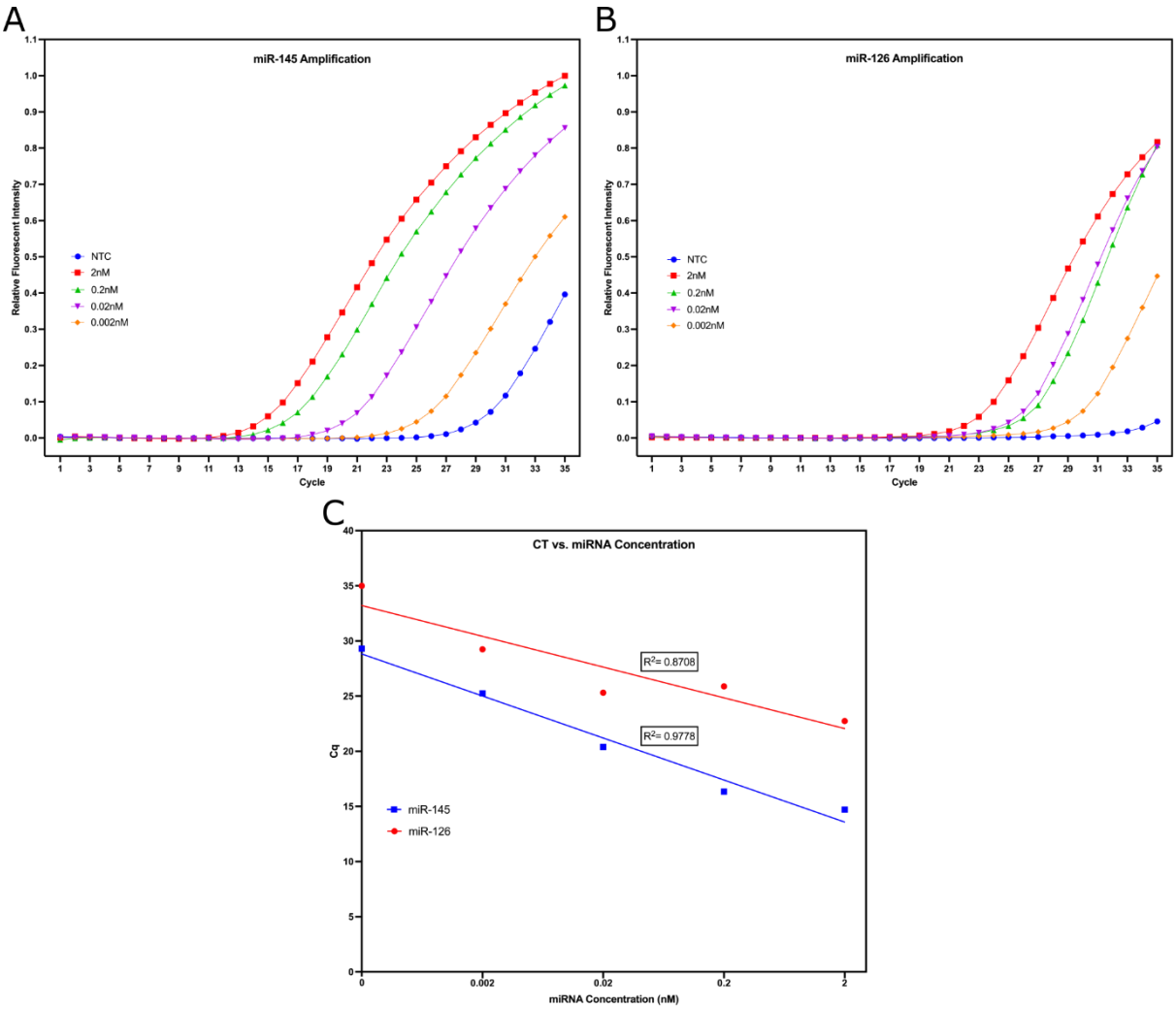

Figure 2. Analytical performance of designed ligation probes and PCR primers for miR-145 and miR-126. (A) Sensitivity of the miR-145 specific oligos displayed through the amplification curve of the ligated product with an initial miR-145 concentration from 0.005nM to 5nM. (B) Sensitivity of the miR-126 specific oligos displayed in the amplification curve of the ligated product with an initial miR-126 concentration from 0.005nM to 5nM. (C) Linear relationship between the CT values and the logarithm of the miRNA concentration for each target.

3.3 Analytical sensitivity of the lateral flow assay (LFA)

To evaluate the analytical sensitivity of the designed LFA, PCR-amplified products carrying engineered tails were hybridized to biotinylated capture probes and gold nanoparticle (AuNP)-conjugated reporter probes on nitrocellulose strips. The tails generated during amplification served as binding anchors, enabling specific hybridization and visual detection.

Figure 2A shows the 30-minute readout of the lateral flow strips corresponding to the miR-145 ligated products tested in Figure 1A, ranging from 0.005nM to 5nM, alongside an NTC. For all concentrations, strong control lines were observed with uniform Intensity, confirming consistent strip function and probe deposition. Test lines were visible across the concentration range, with the 5nM sample displaying the strongest signal and decreasing intensity with dilution. The

lowest concentration (0.005nM) produced a faint but discernible test line, indicating the lower detection limit of the assay. The NTC exhibited only a negligible background band, nearly indistinguishable from the membrane, validating assay specificity.

The signal profiles of the control and test lines were extracted using ImageJ (Figure 2B). As expected, the control line peak intensities remained stable across all concentrations, while test line intensities decreased proportionally with reduced input concentration. Quantitative analysis was performed by calculating the area under the curve (AUC) for each test line and plotting against the logarithm of the input concentration (Figure 2C). The calibration curve exhibited a sigmoidal response characteristic of saturation binding kinetics and was fitted with a four-parameter logistic regression model, yielding excellent correlation (R^2 = 1.0000). All test line values were background-corrected by subtracting the NTC signal.

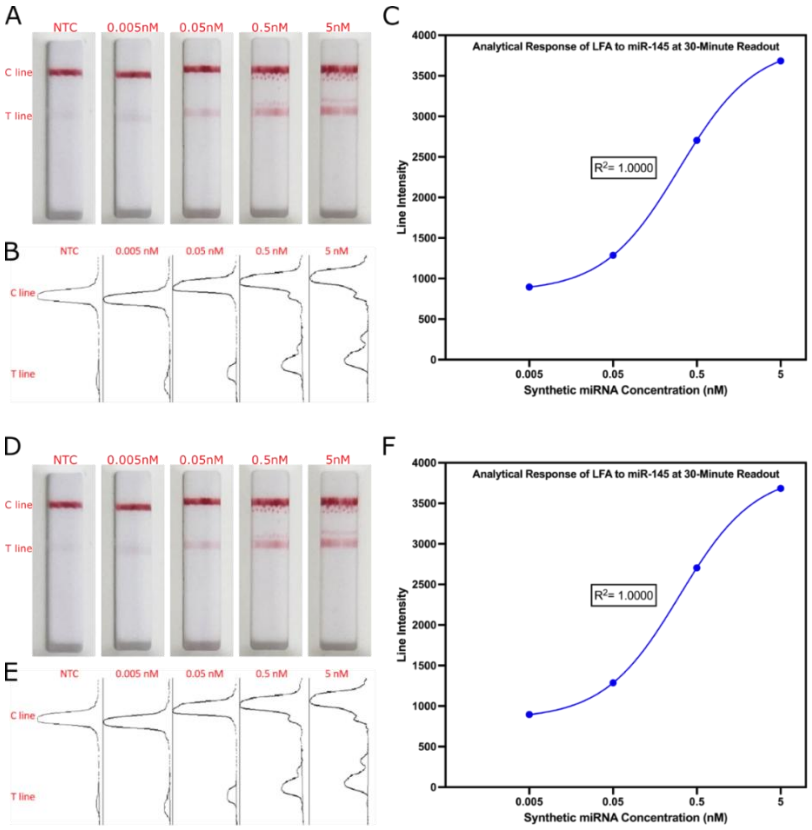

Figure 3. Analytical performance of the lateral flow assay (LFA) for miRNA detection. (A) Representative lateral flow strips for miR-145 ligated and amplified products at concentrations ranging from 0.005nM to 5nM and NTC, imaged at the 30-minute readout. C = control line, T = test line. (B) Line intensity profiles of the corresponding strips in (A), extracted using ImageJ. (C) Semi-log calibration curve of background-corrected test line intensities versus input miR-145 concentration, fitted with a four-parameter logistic regression model (R^2 = 1.0000).

3.5 Spiking in plasma optimization

To evaluate assay performance in the presence of plasma, miR-145 was spiked into 10% plasma at concentrations of 10nM and 100nM. A cross-reaction sample (100nM miR-126) and an un-spiked plasma control were included for comparison.

Figure 4A shows the amplification for the 10nM and 100nM miR-145 spiked plasma samples, with the 100nM sample yielding a Ct of 24 cycles. The 10nM sample showed delayed amplification, while the 100nM miR-126 cross-reaction control produced a stronger fluorescent signal (Ct = 18). Unexpectedly, the un-spiked plasma amplified earlier than all other samples, suggesting residual nucleic acid background.

In contrast, LFA readouts (Figure 4B) showed test line development only for the 10nM and 100nM miR-145 samples. Neither the un-spiked plasma nor the cross-reaction sample produced visible bands, even after 40 minutes. Intensity profiles extracted in ImageJ confirmed concentration-dependent band development (Figure 4C). A semi-log calibration curve fitted with a three-parameter logistic regression yielded a near-perfect correlation (R^2 = 1.000) across the tested range (Figure 4D).

Together, these results demonstrate that while plasma interferes with PCR by introducing background amplification, the LFA format preserves specificity and enables reliable detection of spiked miRNA in plasma at nanomolar concentrations.

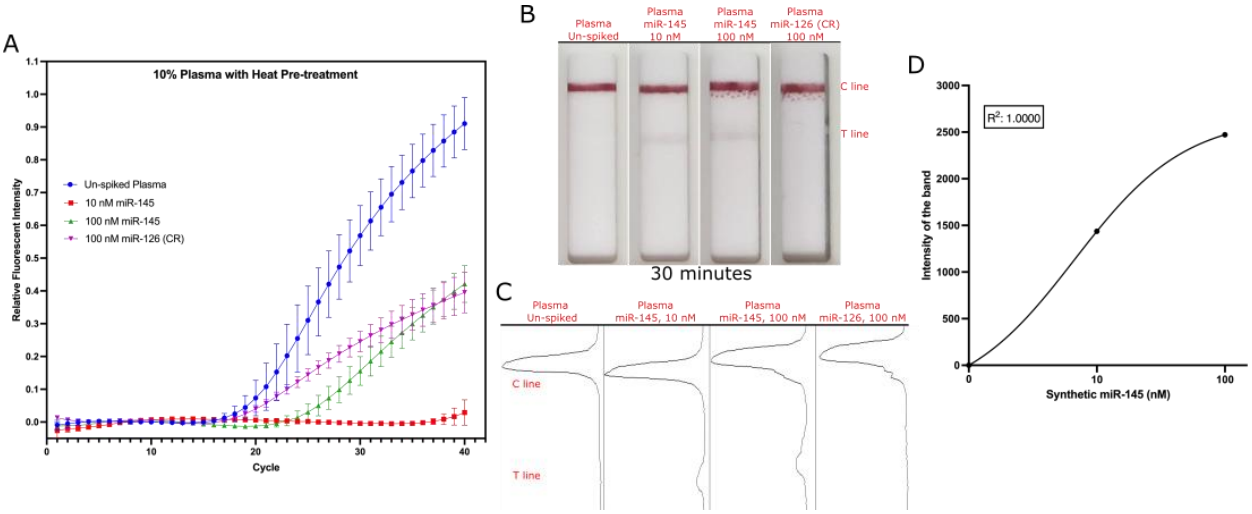

Figure 4. Analytical performance of 10% plasma-adulterated miR-145. (A) PCR amplification using the miR-145 system with 10% plasma spiked with 10nM of miR-145, 100nM of miR-145, 100nM of miR-126 (CR), and an un-spiked sample. (B) Lateral flow strips showing test and control lines for the 10% plasma spiked and un-spiked samples after PCR amplification. (C) Intensity profiles of test and control lines extracted using ImageJ, showing peak response at each concentration. (D) Semi-log calibration curve of test line intensity vs. miRNA concentration fit with a three-parameter logistic regression model (R^2 = 1.0000). Test line intensities were background-corrected by subtracting the signal of the 0nM (Plasma un-spiked) sample.

4. Conclusions

We developed a ligation-PCR-LFA platform for sensitive detection of lung cancer–associated miRNAs. The method achieves detection down to 0.2pM, demonstrates specificity between closely related miRNAs, and performs effectively in serum. This approach offers a cost-effective and portable tool for biomarker detection and may contribute to earlier lung cancer diagnosis in populations not covered by current screening guidelines.