RESEARCH ARTICLE

Editorial Process: Submission:04/30/2025 Acceptance:07/24/2025

Optimization and Validation of a Folate Ligand-Targeted qPCR Method for Detection of Folate Receptor-Positive Circulating Tumor Cells in Gastric Cancer

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Abstract

Background: Gastric cancer represents a significant global health concern with poor prognosis and limited early detection methods. Circulating tumor cells (CTCs) that express folate receptors (FRs) have emerged as promising biomarkers for cancer monitoring, as folate receptor-alpha is overexpressed in cancer tissues while remaining absent in normal cells. **Objective:** This study aimed to develop and optimize a Folate-Ligand Targeted quantitative PCR (Folate-LT qPCR) method for the selective detection and quantification of FR+ CTCs in gastric cancer patients. **Methods:** The methodology involved designing a folate-oligonucleotide conjugate with a 5' folate moiety, triethylene glycol spacer, and phosphorothioate bond for stability. A specific stem-loop primer was developed for complementary DNA synthesis, followed by qPCR optimization using four candidate primers (F1, F1.1, F1.2, F1.3). Validation was conducted through spiking experiments with *HeLa* cells in healthy donor blood samples, followed by CTCs enrichment using negative depletion, labelling with the folate-conjugate, and two-step PCR amplification. Performance was assessed through amplification efficiency, melt curve analysis, and gel electrophoresis. **Results:** Among tested primers, F1.1 demonstrated superior performance with consistent amplification across all tested concentrations without non-specific binding. Optimal annealing temperature for stem-loop primers were established at 40-45°C. The standard curve showed strong linearity (R^2 = 0.9970) between cycle threshold values and logarithmic CTCs concentrations. The assay demonstrated 104% amplification efficiency with a regression equation of y = -3.231x+29.69, confirming reliable quantification capability across a wide dynamic range. **Conclusion:** The optimized Folate-LT qPCR method provides a sensitive and specific approach for detecting FR-positive CTCs in gastric cancer. This technique addresses limitations of existing CTC detection methods by combining negative depletion with specific ligand targeting, requiring minimal blood volume, and avoiding size-based or epithelial marker-dependent limitations, and shows promise for early cancer detection, disease progression monitoring, and treatment response assessment, potentially improving clinical outcomes for gastric cancer patients.

Keywords: Biomarker Detection- Treatment Monitoring- Liquid Biopsy- Stem-loop Primer

Asian Pac J Cancer Prev, **26 (7)**, 2687-2694

Introduction

Globally cancer-related deaths remain a significant health concern. Among which gastric cancer (GC) is the second leading cause of cancer-related deaths and fifth most common malignancy worldwide [1, 2]. Despite advancements in diagnostics and therapeutics, the prognosis for advanced GC remains poor, with a median survival of 8-10 months and a five-year overall survival rate below 30% [3]. This is because of the disease complexity, and multifactorial nature that involves both genetic and environmental factors, contributing to its pathogenesis and varied geographical distribution. This poor prognosis of the disease highlights the urgent need for improved strategies in early detection, precise staging, and personalized treatment approaches [4].

Recently, the folate receptors (FRs), particularly the alpha isoform (FRα), has emerged as a promising biomarker candidate in various solid malignancies, including gastric cancer. FRα, a glycosylphosphatidylinositol (GPI)-anchored membrane protein that mediates cellular folate uptake and is selectively overexpressed in numerous cancers while exhibiting minimal expression in normal tissues [5, 6]. In gastric cancer, approximately 82.76% of cancer tissues are found to be FRα positive, while it remains absent in normal gastric mucosa. This differential

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expression pattern highlights FRα's potential as a tumor-specific biomarker for GC [7].

Circulating tumor cells (CTCs) are the cells that serve as a seed for the metastasis of cancer which is the main cause of mortality among these patients. CTCs are extremely rare in the bloodstream and exhibit significant heterogeneity, complicating their detection and quantification. It has gained significant attention as a biomarker for early detection and real-time treatment monitoring in cancer. Folate receptor-positive CTCs (FR+ CTCs) have gained particular interest due to the overexpression of folate receptors on the surface of cancer cells [8]. The detection and enumeration of FR+ CTCs could provide valuable insights into disease progression, treatment response, and prognosis in gastric cancer patients [9].

The Folate-Ligand Targeted qPCR (Folate LT-PCR) method is a sensitive and specific technique designed to detect CTCs using cancer cell-specific ligand molecule. It has shown promise in detecting FR+ CTCs in various cancers, including breast, prostate, ovarian, and lung cancers [8, 10, 11]. The technique involves two main steps i.e. enrichment of CTCs by negative depletion of leukocytes and labelling of CTCs with folate ligand-oligonucleotide conjugate, followed by qPCR amplification of the bound oligonucleotide. This technique combines the sensitivity of qPCR with the specificity of ligand-target binding to allow highly selective enumeration of CTCs.

The present study aims to optimize and validate a Ligand-Targeted Polymerase Chain Reaction (LT-PCR) technique for enumerating FR+ CTCs in gastric cancer by employing folate-oligo, stem-loop and oligo primers specifically designed for detection of folate receptors. The optimization and validation processes will focus on critical elements of the assay, including folate oligo design, primer design, and PCR conditions,

Materials and Methods

Folate-oligo conjugation

The first step in the folate-oligo conjugation methodology was the design of a 30-nucleotide sequence. This sequence was carefully designed to ensure high specificity, efficient bonding, proper hybridization, and minimal off-target effects. The designed oligonucleotide was then conjugated to a 5' folate moiety which acts as a ligand for folate receptor. An iSp9 (Triethylene glycol PEG) spacer was incorporated between the folate ligand and oligonucleotide to provide flexibility and to reduce steric hindrance that allows optimal conformations for receptor binding. Additionally, phosphorothioate bond was added to oligo at 3' end to make the oligonucleotide more resistant to degradation by nucleases (Figure 1).

The conjugation of the folate ligand to the designed oligonucleotide was performed and purchased as a non-catalogue product from Integrated DNA Technologies (IDT), India using their standard conjugation chemistry protocols. During the process, folate ligand was covalently attached to the 5' end of the synthesized oligonucleotide sequence. Conjugated probe was purified using High-Performance Liquid Chromatography (HPLC) to ensure high purity and quality. The final folate-oligonucleotide conjugate acts as a highly specific detection probe for the FL-LT-qPCR method, that bind specifically to folate receptors on CTCs

Stem-Loop primer design

The stem-loop RT primer is a structure that consists of 5' linear sequence portion (overhung), a double-stranded stem region made by complementary base pairing, and a single-stranded loop region at the 3' end for specific target recognition (Figure 2). Sequence optimization involved designing the loop region for specific target recognition, stem-region with complementary sequence to from a stable hair-pin structure and single strand overhung to bind to the target. The RNAfold web server was utilized to calculate and adjust the Gibbs free energy (ΔG) value of the designed stem-loop structure to achieve thermodynamically stable structure with a ΔG value of approximately -23.40.

Functional verification was conducted through *in-silico* simulations to confirm the primer's ability to recognize and hybridize to its complementary sequence i.e. 8-base pair sequence at the 3' end of the folate oligo while maintaining stem structure stability. Experimental validation of the primer's performance was performed in a two-step process: In the first step, complementary DNA synthesis occurs when the 8-base pair sequence at the 3' folate-oligonucleotide binds to the overhung of the stem-loop primer, followed by real-time PCR amplification using both forward and reverse primers.

The working mechanism, as illustrated in the flowchart (Figure 3), shows how the stem-loop primer participates in a two-step process. In the first step, folate-oligonucleotide

/5'[Folate]/iSp9/*AAAGCGACCTTTGGATTGAAGGGAGCTCT*A

- *[Folate]- Folate ligand conjugated at 5' end*
- *iSp9 -Internal Triethylene glycol spacer*
- *"AAAGCGACCTTTGGATTGAAGGGAGCTCTA" is the oligonucleotide designed to bind to the target sequence. (*) represents a phosphorothioate bond*

Figure 1. Structure of the Probe

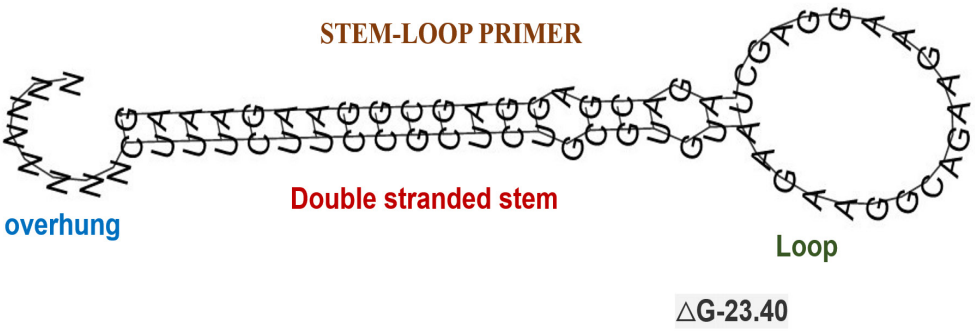

Figure 2. Structure of the Stem-Loop RT Primer. The diagram illustrates the key structural components: an overhung region (blue), a double-stranded stem section (red), and a single-stranded loop region (green). The structure has a calculated Gibbs free energy (ΔG) of -23.40 kcal/mol, indicating strong thermodynamic stability.

binds to 8-base pair overhung in stem-loop primer to generate complimentary DNA strands. This was followed by the second step involving PCR amplification, where both forward primer attaches to the target site in the folate oligo, and universal reverse primer to the stem-loop primer to facilitate exponential amplification with fluorescent detection. The entire system combines the targeting capability of folate with the high specificity of the stem-loop structure, creating a robust and sensitive detection method for the intended target sequence.

The optimal secondary structure with a minimum free energy of -23.40 kcal/mol.

qPCR primer design

The qPCR primers were specifically designed to amplify the target sequences. Four primer variants (F1, F1.1, F1.2, and F1.3) and universal reverse primer (Figure 4, 5) were designed to identify an optimal primers sequence for LT-qPCR. Primer optimization was conducted through qPCR assays using SYBR Green to evaluate the amplification efficiency and specificity of the four designed primers. Each primer was tested at three concentrations (0.1 pmol, 0.01 pmol, and 0.001 pmol per reaction) of folate oligo (Template), with No Template Control (NTC) included in each assay to identify non-specific amplification to the stem-loop primer or primer dimer formation. qPCR results were analysed to determine the optimal primer and concentration that minimized non-specific product formation while maximizing amplification efficiency.

After qPCR assay, the PCR products were subjected to agarose gel electrophoresis to further validate the primer optimization process. Agarose gel electrophoresis was performed using a 2% gel in 1X TAE buffer with ethidium bromide. Gels were run at 100V for approximately 60 minutes, visualized under gel documentation system

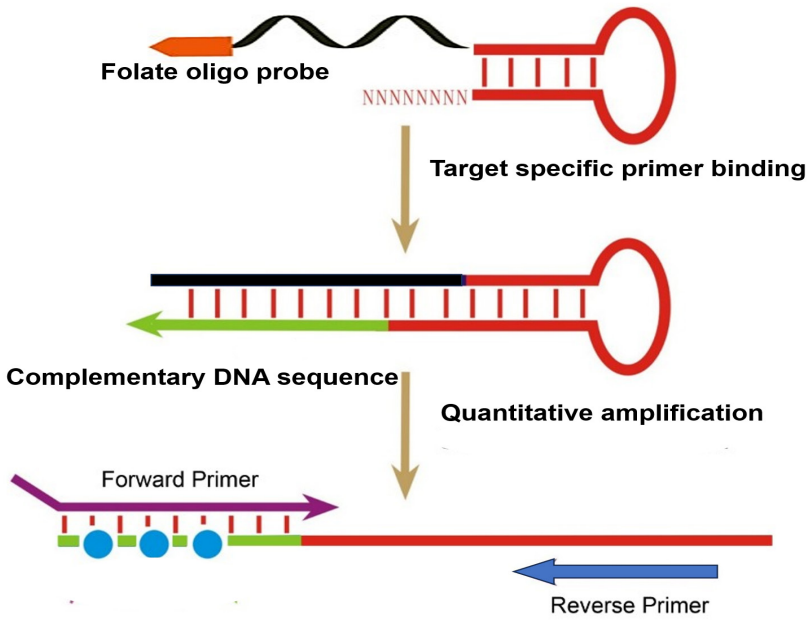

Figure 3. Working Flowchart of Stem-loop RT Primer. Schematic representation of folate-based oligonucleotide probe hybridization and quantitative PCR amplification process. The workflow illustrates three key steps: (1) Initial hybridization of the folate oligonucleotide probe to its target sequence; (2) Target-specific primer binding to the complementary DNA sequence; and (3) Quantitative amplification using forward and reverse primers to exponentially amplify the target DNA.

Primer F1: *AAGCGACCTTTGGATTGAAGG*
Primer F1.1*: AAGCGACCAAAGCGACCT*
Primer F1.2: *CGACCAAAGCGACCTTTGG*
Primer F1.3: *GACCAAAGCGACCTTTGGATTG*

Figure 4. The Forward Primers

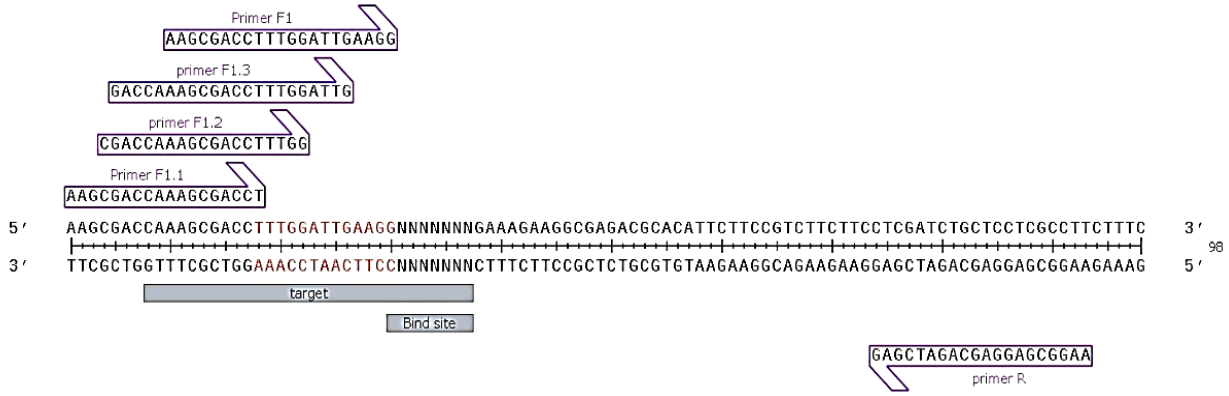

Figure 5. Primers with Folate Oligo and Stem-Loop Primer. The figure illustrates the design of primers for amplifying a target DNA sequence. Primer F1, F1.1, F1.2, and F1.3 are forward primers binding to different regions of the 5' end of the target sequence; primer R is a reverse primer binding to the complementary 3' end. The target region is highlighted (Gray)with its binding site marked.

(Biorad), and analysed for amplicon size, primer specificity, and potential non-specific amplification. qPCR (Applied Biosystems QuantStudio™ 5 Real-Time PCR) was used to optimize annealing temperature for both stem-loop primer (25°C to 50°C) and qPCR primer (35°C to 60°C), followed by melt curve analysis (60°C to 95°C with 0.5°C increments). The amplified PCR products were then subjected to gel electrophoresis for size-based separation and visualisation. The results of gel electrophoresis were compared with qPCR amplification curves and melt curve data to determine the optimal annealing temperature. Upon standardization of primers and annealing temperature, LT-qPCR was performed with a blood spiking protocol, following the manufacturer's instructions for RT-PCR using SYBR Green.

Selection of cell line

To construct the standard curve for LT-qPCR, two cell lines, AGS (gastric adenocarcinoma), and *HeLa* (cervical cancer) were selected based on their documented folate receptor expression levels. Additionally, Jurkat cells, HEK293T cells, and blood sample (healthy control) were included as controls to represent low or negligible *FOLR1* expression to ensure a dynamic range of folate expression levels for the accurate quantification in LT-qPCR experiments.

Real-Time PCR

RT-PCR was performed to evaluate the mRNA expression levels of *FOLR1* in both cell lines. Total RNA was extracted using a Direct-Zol RNA Miniprep (Zymo Research, Orange, CA, USA) and quantified spectrophotometrically. Reverse transcription was done and the resulting cDNA was amplified using qPCR primers.

Confocal microscopy

Confocal imaging was done to visualize the localization and relative abundance of *FOLR1* protein in the selected cell lines. Cells were seeded onto coverslips and fixed with paraformaldehyde. Immunofluorescence staining was performed using anti-*FOLR1* antibodies. DAPI staining was used to label nuclei. Imaging was performed using an Olympus Laser scanning confocal microscope system (Spectral version; model Olympus FV1000), capturing separate channels for *FOLR1* fluorescence (red), DAPI fluorescence (blue), and images were merged.

LT-qPCR using blood spiking protocol

Spiking experiments were essential for validating the performance of the FL-RT-qPCR method, for quantifying CTCs in complex biological samples.

Preparation of spiking material

Cell lines were purchased from National Centre for Cell Science (NCCS), Pune, India. Cells were cultured under appropriate conditions, harvested using trypsin-EDTA, and resuspended in culture medium. Cultured cells were counted using a haemocytometer to determine the concentration of the suspension.

Preparation of biological samples

Blood samples were collected from healthy donors

under an approved protocol using anticoagulant-treated tubes (e.g., EDTA) to prevent clotting. The samples were stored at 4°C and processed within 2 hours to maintain cell viability.

Spiking procedure

Serial dilutions of harvested cells were prepared to achieve a range of concentrations (e.g., tenfold dilutions from $10^{0.3010}$ to $10^{5.3010}$ per 3 mL equivalent to 2 to 2,00,000 cells). A known volume of diluted cell suspension was then added to blood samples to achieve desired final concentrations.

Enrichment

Negative Enrichment: The 3 mL spiked blood sample was transferred into a 5 mL polystyrene round-bottom tube (Catalog #38007). 50 µL/mL of EasySep™ Direct Human CTC Enrichment Cocktail was added to the blood sample, mixed thoroughly, and incubated at room temperature (RT) (15-25°C) for 5 minutes. The EasySep™ Direct RapidSpheres™ was vortexed for 30 seconds to ensure even dispersion, and 50 µL/mL of RapidSpheres™ was added to the blood sample. The sample was mixed thoroughly by gentle pipetting up and down.

The volume of the sample was doubled by adding the recommended medium, bringing the total volume to 6 mL. The sample was gently mixed by pipetting up and down 2-3 times. The tube, was placed into the EasySep™ Magnet and incubated at RT for 10 minutes. After incubation, the magnet and tube were carefully inverted in one continuous motion to pour the enriched cell suspension into a new tube without shaking or blotting any remaining drops. To enhance the enrichment process, an additional 50 µL/mL of RapidSpheres™ was added to and same step was repeated to collect the enriched cell suspension in new tube.

Labelling

This is a crucial step of LT-PCR. Following enrichment, 10 µl of detection probe in binding buffer (Tris buffer, 150 mM NaCl) was added to the cell suspension and incubated at RT for 60 minutes. This allows for probe diffusion and specific binding of folate molecules to the overexpressed folate receptors on cancer cell surface.

Washing and stripping

For washing, 1 ml of binding buffer was added to the labelled cell suspension and centrifuged at 300x g for 5 minutes at RT. The supernatant is carefully collected into the new tube. Washing was done three times to ensure thorough removal of unbound conjugates to aid in reducing background signal and improve the specificity of CTC detection in the downstream PCR amplification.

After washing unbound conjugates, stripping buffer (acid-based buffer) was then added to the sample at a volume ratio of 1:10 (sample: buffer). The mixture was then incubated at RT for 15 minutes with gentle shaking every 5 minutes. Following incubation, the sample was centrifuged at 1000xg for 5 minutes at RT, and supernatant containing stripped oligo was carefully collected to a new tube and neutralized with binding buffer.

Reverse transcription and qPCR

The bound oligonucleotides were subjected to two-step process for folate-targeted detection. Firstly, complimentary stand synthesis using a stem-loop primer followed by quantitative PCR (qPCR) for amplification and quantification of the target sequence using qPCR primer.

Step 1: Complementary DNA strand synthesis

This is a critical stage in the PCR protocol that consists one cycle with three temperature-controlled steps. An initial denaturation step at 95.0°C for 2 minutes followed by annealing step, the temperature then drops to 40.0°C for one minute, where the specially designed stem-loop primers, bind specifically to folate oligo, allowing DNA polymerase to efficiently synthesize new complementary DNA strands (target sequence) using the stem-loop primers in first step of qPCR.

Step 2: qPCR amplification

qPCR was performed using forward and reverse primers specifically designed to amplify the target sequence. The qPCR cycling parameters consisted of three main stages: an initial denaturation step at 95°C for 2 minutes, followed by 40 amplification cycles (denaturation at 95°C for 10 seconds, annealing at 60°C for 30 seconds), and a melt curve stage to confirm product specificity. SYBR Green was used for fluorescence detection during amplification.

Data analysis

Standard curve generation

The Ct values for each dilution of cells (2 to 200000 cells) were recorded, and a standard curve was plotted with Ct values on the y-axis and the $\log_{10}$ transformed cell concentrations on the x-axis. This curve serves as a reference to interpolate the concentration of the target in experimental samples.

qPCR efficiency and linearity assessment

The efficiency and linearity of the qPCR assay were evaluated using the slope and R^2 value of the standard curve. The slope (m) of the standard curve was calculated using the equation:

$$E=(10_{(-1/m)} -1) \times 100$$

Where E represents the amplification efficiency. An efficiency range of 90–110% (corresponding to a slope between -3.1 and -3.6) was considered acceptable. The linearity of the assay was assessed by calculating the R^2 value, which indicates how well the data points fit a straight line. An R^2 value close to 1 confirms high linearity and reliable quantification.

Results

Primer optimization

The optimization and validation of four primer variants (F1, F1.1, F1.2, and F1.3) for ligand-targeted PCR were

assessed using real-time PCR amplification efficiency, melt curve analysis, and gel electrophoresis. The first primer, F1 showed non-specific amplification in the No Template Control (NTC) equal to that of primers, because of its binding to the stem-loop primers, that can result in false-positivity (Supplementary Figure 1). Primers F1.1 demonstrated significant enhancements with early Ct values, effective amplification at all tested concentrations (0.1, 0.01, and 0.001 pmol), and clear concentration-dependent amplification with no bands in the NTC gel elctrophoresis (Supplementary Figure 2). Primer F1.2 performed with same efficiency as the primer F1.1, but showed light band in the NTC well, which signifies the partial binding of the primer to the stem-loop primer (Supplementary Figure 3). Primer F1.3 was not eligible for qPCR since there was a complete binding to the stem-loop primer rather than the folate oligo shown by amplification as that of product (early ct) sequence, and evident by the NTC gel image (Supplementary Figure 4). Overall, primers F1.1 and F1.2 exhibited the best performance among the tested variants with early Ct values, efficient amplification across all tested concentrations (0.1 pmol, 0.01 pmol, and 0.001 pmol), and no non-specific amplification observed in the NTC (Supplementary Figure 5).

Annealing temperature optimization for LT-PCR For Stem-Loop primers

Two candidate primers that were optimized (primer F1.1 and F1.2), were evaluated for their performance in the FL-LT-qPCR assay across a temperature gradient of 25°C to 50°C. Both primers demonstrated robust amplification efficiency and specificity throughout the tested range. Primer F1.1 exhibited consistent amplification across the entire temperature gradient (25-50°C). Ct values remained stable, with optimal efficiency observed at 40°C and 45°C, as evident by the lowest Ct values and single sharp peak in melt curve. Agarose gel electrophoresis corroborated these findings, showing a single band of the expected amplicon size across all temperatures, with maximum intensity at 40°C and 45°C (Supplementary Figure 6, 7). Primer F1.2 demonstrated performance characteristics similar to F1.1. Consistent amplification was observed across the temperature gradient, with optimal efficiency at 40°C and 45°C, as indicated by the earliest Ct values. Although it showed higher efficiency in terms of lower Ct values and more yield, still showed non-specificity in melt curve analysis and gel electrophoresis (Supplementary Figure 8, 9).

Annealing temperature (Ta) for primer F1.1 for qPCR

Next, the qPCR annealing was optimized across temperature ranges from 35°C to 60°C. Primer F1.1 demonstrated consistent amplification across temperatures, with optimal efficiency at 40-45°C, as evidenced by the lowest Ct values, highest band intensities and no band NTC in gel electrophoresis (Supplementary Figure 10, 11). Primer F1.2 exhibited optimal performance at higher temperatures, with the most efficient amplification observed at 55-60°C. However, a faint band was observed in the NTC well that indicates non-specificity (Supplementary Figure 12, 13). Both primers showed specific amplification across all tested temperatures, as confirmed by single sharp peaks in melt curve analyses and distinct bands of expected size in gel electrophoresis. These results establish 40-45°C as the optimal annealing temperature range for primer F1.1 and 55-60°C for primer F1.2.

Based on these extensive evaluations, primer F1.1 was selected for the LT-qPCR assay. This selection was made due to its consistent superior performance throughout the optimization process, demonstrating high specificity, good yield, and reliable results across various experimental conditions.

Cell line selection

qPCR analysis revealed significant differences in *FOLR1* mRNA expression across the selected cell lines and sample. Among the tested models, *HeLa* cells exhibited the highest levels of *FOLR1* mRNA expression, with normalized relative expression approximately four times greater than that observed in AGS cells (Supplementary Figure 14). AGS cells demonstrated moderate *FOLR1* expression, serving as a model for lower receptor abundance. Jurkat cells, HEK293T cells, and blood sample showed negligible or undetectable levels of *FOLR1* mRNA, confirming their suitability as negative controls in this study.

Confocal microscopy further validated these results by visualizing *FOLR1* protein localization and abundance in *HeLa* and AGS cells. In *HeLa* cells, intense red fluorescence corresponding to *FOLR1* was predominantly localized on the cell surface and cytoplasmic regions. Merged images demonstrated clear co-localization of *FOLR1* signals with DAPI-stained nuclei, confirming robust folate receptor expression (Supplementary Figure 15). AGS cells displayed weaker red fluorescence compared to *HeLa*, indicating moderate *FOLR1* protein levels. The merged images confirmed co-localization of *FOLR1* signals with cellular structures.

HeLa cell line was selected for the experiment for its high expression of *FOLR1* to optimize LT-PCR assays aimed at quantifying folate receptor-positive targets with high sensitivity and specificity.

Standard curve and linear regression analysis

The standard curve was generated by plotting the cycle threshold (Ct) values against the logarithm (Log10) of known concentrations of *HeLa* cells. The data points followed a strong linear relationship, providing a robust model for quantifying unknown CTC concentrations (Table 1).

The equation derived from the linear regression model:
$y=-3.231x+29.69$

Where:
y: Ct value
x: Log_{10} of the CTC concentration

Table 1. Quantitative PCR Data: CTCs Log_{10} vs. Ct values

CTCs Log_{10}	Ct value 1	Ct value 2	Average
0.30103	28.82	28.41	28.615
1.30103	25.651	25.105	25.378
2.30103	22.633	22.412	22.5225
3.30103	19.616	19.124	19.37
4.30103	15.003	15.451	15.227
5.30103	12.633	12.805	12.719

The Ct values are indicators of the amount of target nucleic acid present in a sample, with lower Ct values indicating higher concentrations. The data includes two replicate Ct values for each CTCs Log10 value, along with their average. This data can be used to analyse the efficiency and reliability of the PCR reaction across different concentrations of CTCs.

The slope of the standard curve was calculated as −3.231, corresponding to an amplification efficiency of approximately 104% [E= $(10_{-1/slope}-1) \times 100=104\%$] which falls within the acceptable range for qPCR assays (90–110%), confirming optimal primer performance (Supplementary Figure 16). The slope of −3.231 indicates that for every tenfold increase in CTC concentration, the Ct value decreases by approximately 3.231 cycles. The Y-intercept (29.69) represents the theoretical Ct value when the Log_{10} CTC concentration is zero (i.e., equivalent to 1 CTC). The R^2 value of 0.9970 indicates an excellent linear correlation between Ct values and Log_{10} CTC concentrations. This demonstrates that over 99% of the variability in Ct values is explained by changes in CTC concentration. The p-value of <0.0001confirm that the slope is significant, validating the reliability of the standard curve for qPCR analysis (Supplementary Figure 17). The standard curve analysis confirmed that the qPCR assay is highly efficient (104%), precise (R^2=0.9970), and reliable for quantifying CTCs across a wide dynamic range. The narrow confidence intervals and significant statistical validation further underscore its robustness, making it suitable for applications such as cancer diagnostics.

CTC units from a regression line and log_{10} *concentration*

The standard curve generated through qPCR amplification of known template concentrations ($10^{0.3010}$ to $10^{5.3010}$) established a linear relationship between Ct values and log_{10} concentrations. For determining CTC units, the measured Ct values were interpolated using the regression equation rearranged to solve for x: x = (y - b)/m. The resulting log_{10} concentration (x) was then transformed to absolute CTC units through the antilog calculation (10^x). This mathematical conversion provides the final CTC concentration in terms of folate units.

Discussion

The development and optimization of a Folate Ligand-Targeted qPCR (LT-PCR) method for detecting folate receptor-positive circulating tumor cells (FR+ CTCs) in gastric cancer represents a significant advancement in liquid biopsy technologies. This innovative approach is pivotal in GC, given that it remains a significant global health burden. Clinical utility of Folate LT-PCR is significant and could transform the management of gastric cancer. FR+ CTCs could provide valuable insights into disease pathogenesis, treatment response and prognosis in these patients. Early detection of FR+ CTCs provides stage-shift diagnosis before any conventional marker positivity or radiographic evidence appears [12]. Moreover, changes in CTC numbers between treatments could predict chemoresistance earlier than conventional imaging methods, thus allowing for timely modifications in the therapy strategies [13].

LT-PCR method provides several key advantages over the existing CTC detection methods. It is highly specific through dual targeting i.e combining negative depletion with folate ligand binding, thus reducing false positives from hematopoietic cells, a common challenge in CTC detection. LT-PCR technique offers a significant advantage over CellSearch system, which is a semi-automated FDA-approved system that uses immunomagnetic coated beads with anti-EpCAM antibody. This technique potentially misses out EpCAM negative CTCs that undergo epithelial to mesenchymal transition. FR-LTPCR demonstrates superior sensitivity of 72–81% in NSCLC vs. 23–27% for CellSearch and specificity of 82–93% [14]. It only requires minimal blood volume (3 mL) and avoids size-based limitations of methods like ISET (Isolation by SizE of Tumor cells), which may miss smaller CTCs less than 8 μm [15].

The traditional Reverse transcriptase polymerase chain reaction (RT-PCR) can detect CTCs with high sensitivity; however, it fails to capture cancer cells that undergo post-transcriptional regulation, and risk false positives from non-CTC nucleic acids [8, 16]. FR-LTPCR's ligand specificity reduces interference from normal cells as they are negligible in non-malignant blood cells except rare macrophages.

The Folate-Ligand Targeted qPCR (Folate LT-PCR) method has shown promise in detecting FR+ CTCs in various cancers, including breast, prostate, ovarian, and lung cancers. FR-targeted LT-PCR demonstrates high sensitivity and specificity in detecting CTCs across various cancer types. In non-small cell lung cancer (NSCLC), this method showed a sensitivity of 77.7% and specificity of 89.5% with a threshold of 8.7 CTC units [8]. For pancreatic cancer, when combined with CA19-9, it achieved a sensitivity of 97.8% and specificity of 83.3% [17]. LT-PCR allows for ultrasensitive analysis, enabling advanced disease stratification1. This high sensitivity is particularly valuable for detecting rare CTCs in early-stage cancers, with one study showing detection rates of 80% for stage I/II NSCLC [18]. The high sensitivity of LT-PCR allows for the detection of very low numbers of CTCs, potentially enabling earlier diagnosis and more accurate monitoring of disease progression. Some studies have reported detection limits as low as 1-5 CTCs per 7.5 mL of blood, which is comparable to or better than many existing CTC detection platforms [19]. These advantages of FR LTPCR suggest adaptability of this technique to other FR- positive malignancies.

While the LT-PCR technique shows great promise and clinical utility, it requires further refinement and

multicentre validation across gastric cancer subtypes. Future direction includes, correlation with emerging biomarkers, and integration of Next generation sequencing (NGS) for molecular profiling.

In conclusion, FR-targeted LT-qPCR offers a comprehensive approach to CTC detection, addressing limitations of traditional methods, particularly in identifying EMT and MET CTCs. Its high sensitivity, specificity, and versatility make it a promising tool for cancer diagnosis, prognosis, and treatment monitoring across various cancer types.

Author Contribution Statement

Anitha S, and Raveendran Ramasamy contributed equally to the conception, design, experimental work, data analysis, and manuscript writing. Biswajit Dubashi, and Rajesh Nachiappa Ganesh contributed to the patient/sample recruitment, clinical interpretation, and critical revision.

Acknowledgements

Ethical approval

The study was reviewed and approved by the Institutional Ethics Committee of Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry, India.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Conflict of interest

The authors declare no conflict of interest regarding the publication of this paper.

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