

MicroRNA-451a in Colorectal Cancer Detection: Analytical and Preliminary Clinical Validation of a PCR-Free Chemiluminescent Assay

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Article

Keywords: microRNA, miR-451a, colorectal cancer detection, Dynamic Chemistry Labelling, PCR-free detection, chemiluminescent assay

Posted Date: August 7th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10190555/v1>

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Additional Declarations: Competing interest reported. BS, MTC and JJGM are affiliated with DESTINA Genomica S.L., which develops Dynamic Chemistry Labelling technologies. SP and JJDM are shareholders of DESTINA Genomica S.L. The remaining authors declare that they have no competing interests.

Abstract

Faecal microRNAs are promising molecular biomarkers for non-invasive colorectal cancer detection because of their stability in stool and disease-associated expression profiles. miR-451a is highly enriched in red blood cells and is therefore linked to erythrocyte-derived material associated with lesion-related bleeding. Here, we developed a direct chemiluminescent Dynamic Chemistry Labelling assay for miR-451a detection in a non-invasive colorectal cancer testing workflow. A chemiluminescent Dynamic Chemistry Labelling assay was developed for direct analysis of clarified stool lysates without RNA extraction, reverse transcription or enzymatic amplification. Analytical performance was evaluated using synthetic canonical miR-451a, abundant miR-451a isomiR variants and red blood cell spike-ins. Clinical performance was assessed in stool samples from 90 colonoscopy-characterised individuals, including 44 colorectal cancer cases and 46 colonoscopy-negative controls. Small RNA sequencing was used as an orthogonal comparison method. Group differences were assessed using non-parametric tests, agreement with sequencing data using Spearman correlation, and diagnostic performance using colonoscopy as the reference standard. The assay achieved a limit of detection of 4.25 pg/mL and a lower limit of quantification of 13 pg/mL. It detected canonical miR-451a and its abundant isomiR variants with sequence specificity. Red blood cell spike-in experiments confirmed detection of erythrocyte-derived miR-451a within the stool matrix, with the lowest quantifiable input corresponding to 1,689 red blood cells/ μ L. Using a predefined positivity threshold of 26 pg/mL, 36 of 44 colorectal cancer cases were miR-451a-positive and 36 of 46 colonoscopy-negative controls were miR-451a-negative, corresponding to 81.8% sensitivity and 78.3% specificity. Ten colonoscopy-negative controls were miR-451a-positive, whereas eight colorectal cancer cases were below the threshold. Dynamic Chemistry Labelling showed a positive association with small RNA sequencing and detected miR-451a in selected colorectal cancer samples with sequencing non-detection. This study establishes a direct PCR-free strategy for interrogating microRNAs in stool samples. miR-451a provides a sequence-defined molecular readout of erythrocyte-derived material and a CRC-relevant target suitable for stool-based nucleic acid testing. This approach provides a translational route toward future multiplexed faecal microRNA panels for colorectal cancer detection.

1. Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related morbidity and mortality worldwide, accounting for over 1.9 million new cases and 900,000 deaths each year [1]. Although advances in screening and therapy have improved clinical outcomes, early detection remains essential, as CRC often develops asymptotically and is frequently diagnosed only at advanced stages [2]. Population-based screening programmes reduce CRC incidence and mortality by identifying precancerous lesions and early-stage malignancies [3]. While colonoscopy is the diagnosis gold standard, its invasive nature, high cost, and resource requirements limit its clinical utility as a primary population-wide screening tool [4]. These limitations support the development of non-invasive, stool-based approaches.

Historically, stool-based CRC screening relied on guaiac-based faecal occult blood testing (gFOBT), which detects the peroxidase activity of haem in stool but is hampered by limited specificity and the need for dietary restriction. This approach has been largely superseded in organised screening programmes by the faecal immunochemical test (FIT), which detects human haemoglobin with greater specificity and provides quantitative results [5–7]. Although FIT is now a highly validated population-screening tool, being a protein assay, its performance depends on the preservation and immunorecognition of haemoglobin, which may be affected by protein degradation, conformational instability and stool matrix effects during gastrointestinal transit and sample handling [8]. While FIT performs well for CRC detection it shows more limited sensitivity for precancerous lesions. Therefore, complementary molecular strategies are valuable to retain the biological relevance of occult bleeding while shifting the analytical readout toward more stable and sequence-defined targets.

Molecular stool tests have expanded the landscape of non-invasive CRC screening beyond haemoglobin-based assays. Multi-target stool DNA assays, which combine mutation, methylation and haemoglobin-based markers, have demonstrated the clinical utility of stool-based molecular diagnostics [9]. More recently, multi-target stool RNA (mt-sRNA) testing has further highlighted the potential of stool-derived RNA biomarkers for CRC screening [10]. The FDA-approved mt-sRNA test ColoSense combines stool RNA markers, occult haemoglobin detection and clinical variables, with clinical validation studies reporting high sensitivity for CRC detection, approximately 93–94%, and moderate sensitivity for advanced adenomas, approximately 45–46% [11].

Despite these advances, FIT remains the most widely implemented first-line stool screening test in population programmes because of its low cost, simplicity, scalability and extensive clinical validation. By contrast, molecular stool tests provide higher-content biomarker information, but generally require specialised processing, nucleic acid extraction and/or amplification-based workflows, dedicated infrastructure and higher costs [12].

This highlights the need for complementary molecular screening technologies that combine nucleic acid specificity with operational simplicity, scalability and cost-effective implementation. In this context, PCR-free and extraction-free approaches such as DCL may offer a simplified route for direct stool miRNA interrogation, while remaining distinct from both conventional FIT and current multi-target molecular stool tests.

MicroRNAs (miRNAs), discovered in the early 2000s, represent promising stool biomarkers because of their small size, stability in complex biological matrices, and disease-associated expression profiles [13–15]. Unlike protein markers, miRNAs are sequence-defined nucleic acid targets that can be interrogated with high molecular specificity. Among candidate stool miRNAs, miR-451a is of particular interest because it is highly enriched in erythrocytes, with expression levels several orders of magnitude higher than in other circulating cell types [16]. Because colorectal lesions can release blood into the intestinal lumen [17], faecal miR-451a can serve as a molecular surrogate for occult bleeding. In this respect, miR-451a maintains the biological link to lesion-associated bleeding in a manner similar to FIT but shifts the

analyte from a labile protein to a highly stable nucleic acid. Additionally, because the miR-451 family has also been reported to have diagnostic and prognostic relevance in CRC [18], miR-451a represents an analytically robust candidate for inclusion in multiplexed stool panels that combine bleeding indicators with tumour-specific miRNA biomarkers.

Despite their advantages, stool miRNAs have not yet been widely adopted for routine screening. Standard analytical methods, such as RT-qPCR and next-generation sequencing (NGS), require multi-step RNA extraction, reverse transcription, enzymatic amplification and/or library preparation [19]. These steps increase costs, turnaround times and technical complexity, and introduce quantitative bias through extraction losses, ligation inefficiencies and amplification artefacts. These limitations are particularly relevant for stool samples, where low-abundance or fragmented nucleic acid targets must be detected within a complex biological matrix [20].

To overcome these barriers, we developed a PCR-free platform based on Dynamic Chemistry Labelling (DCL) [21–26]. DCL is a chemistry-driven approach that analyses nucleic acids, particularly miRNAs, directly via sequence-specific hybridisation and template-directed chemical labelling, eliminating the need for RNA extraction, reverse transcription, or enzymatic target amplification [27, 28]. By recognising target sequences through highly specific chemical-driven reactions, DCL simplifies the analytical workflow and reduces dependence on conventional nucleic acid processing (see Supplementary Figure S1). This makes the platform particularly suited to stool-based testing, where preserving low-abundance targets and minimising pre-analytical manipulation are critical [29–31].

In this study, we developed and implemented a chemiluminescent DCL assay for direct detection of stool miR-451a in colonoscopy-characterised subjects. We evaluated its analytical sensitivity using synthetic mimics of canonical miR-451a, its most abundant isomiR isoforms, and red blood cell (RBC) spike-ins. We then performed a preliminary clinical evaluation using stool samples from 90 individuals undergoing diagnostic colonoscopy. Small RNA sequencing was used as an orthogonal comparison method.

To our knowledge, this is the first time that an extraction- and PCR-free system has been used to directly analyse total miR-451a in stool samples. This work demonstrates the feasibility of DCL as a simplified analytical strategy for the molecular detection of miRNAs in stool samples and supports its future development toward multiplexed stool biomarker panels.

By translating a chemistry-driven detection principle into a workflow that operates directly on a complex clinical matrix, this study addresses a recognised translational gap between molecular biomarker discovery and deployable, low-complexity diagnostic assays, an objective central to improving access to non-invasive colorectal cancer testing.

2. Methods

Clinical Samples

Human stool samples were retrieved from a cohort of subjects participating in a previous Italian study and recruited at Clinica Santa Rita Hospital in Vercelli, Italy (full description of the study population is reported by Birkeland et al. [32]). Participants were referred for a colonoscopy based on clinical indications, including gastrointestinal symptoms that suggested colorectal pathology, or a family history of CRC.

The cohort comprised 90 individuals characterised by colonoscopy: 44 cases with a confirmed CRC diagnosis and 46 colonoscopy-negative controls. All participants were adults, with a mean age of 62.7 ± 14.3 years and an age range of 22–88 years. Written informed consent was obtained prior to enrolment. The study population included both male and female participants, as well as smokers and non-smokers.

Additional demographic and clinicopathological information, including tumour staging, anatomical localisation, and relevant clinical variables, can be found in the Supplementary Information, Table S1. Stool samples were collected in nucleic acid stabilisation tubes containing RNA preservation buffer (Norgen Biotek Corp., catalogue number 45660) and stored at -80°C until analysis.

The study was approved by the local ethics committee of Azienda Ospedaliera Nazionale SS. Antonio e Biagio e Cesare Arrigo of Alessandria (Italy) under the protocol *Colorectal miRNA CEC2014*.

Reagents, Synthetic Oligonucleotides, DGL Capture Probes and SMART-C-Biotin

Synthetic mimic sequences of the human miR-451a (ssDNA-451a) and miR-451b (ssDNA-451b) were purchased from IDT as single-stranded DNA oligonucleotides (ssDNA), which were used in their desalted form without further purification.

The sequences of the oligonucleotides used in this study can be found in Table 1 below. Paramagnetic Dynabeads M-270 with a diameter of $2.8\text{ }\mu\text{m}$ and carboxylate functionalisation (catalogue number 14305D) were purchased from Thermo Fisher Scientific.

Synthesis of the abasic PNA probe, referred to as the DESTINA Genomica Labelling (DGL) capture probe (DGL-miR451a), was carried out by DESTINA Genomica S.L. in Granada, Spain.

The 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) used for bead activation was obtained from Sigma-Aldrich.

Pierce high-sensitivity streptavidin–HRP (horseradish peroxidase) conjugate was purchased from Thermo Fisher Scientific (Pierce™ High Sensitivity Streptavidin-HRP, catalogue number 21130). The aldehyde-modified biotinylated nucleobase (SMART-C-Biotin) and the lysis buffer were supplied by DESTINA Genomica S.L. and synthesised as described elsewhere [33]. The reaction buffer consisted of 2 x Saline Sodium Citrate (SSC) containing 0.1% Sodium Dodecyl Sulphate (SDS), with the pH adjusted to 6.0 using HCl.

Table 1. Sequences of miR-451a, oligomers and DGL capture probe used in the study

	Name	Sequence	Length (bases)
1	miR-451a	<u>AAACCGUUACCAUUACUGAGUU</u>	22
2	ssDNA-451a	<u>AAACCGTTACCATTACTGAGTT</u>	22
3	ssDNA-451b	TAGCAAGAGAACCATTACCATT	22
4	DGL-miR451a	xx AGTgluAATGGTgluAA*L-Ser*GGTgluTT	17

Note: **1:** Canonical sequence of miR-451a; **2:** ssDNA oligomer mimicking the miR-451a canonical sequence; **3:** ssDNA oligomer mimicking the miR-451b canonical sequence; **4:** Sequence of DGL capture probe. All sequences are reported in 5'-3' orientation, except for the DGL-miR451a, which is reported in N-C terminal orientation. x = amino-PEG linker. Tglu = thymidine bearing a propanoic acid side chain at the γ-position. L-Ser = abasic monomer. Regions complementary to the DGL-miR451a are underlined and the G that templates the incorporation of SMART-C-Biotin is shown in bold.

DGL-miR451a Synthesis

The DGL-miR451a probe was designed as a 17-mer sequence complementary to a portion of the mature canonical sequence of miR-451a (underlined in Table 1). The probe contained an internal abasic position represented by the L-Ser monomer, positioned in the central region of the probe to allow templated incorporation of the SMART-C-Biotin during the DCL reaction as described in Supplementary Figure S1. The probe sequence was synthesised using standard solid-phase PNA synthesis on Tentagel resin (Polymer Laboratories, UK) with an Intavis Bioanalytical MultiPrep CF synthesiser (Intavis AG GmbH, Germany), as previously reported [34].

This process involved repeated cycles of coupling activated PNA monomers, followed by the removal of the protecting group from the terminal amino group. The resulting DGL-miR451a was purified by high-performance liquid chromatography (HPLC) and characterised by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-ToF) mass spectrometry. As previously reported [35], SMART-C-Biotin was synthesised, purified by HPLC, and characterised by mass spectrometry.

Coupling of DGL-miR451a to Magnetic Beads

Dynabeads were functionalised with DGL-miR451a to generate DGL-451a capture beads.

Briefly, 100 µL of Dynabeads (2 x 10⁹ beads/mL) were washed in 100 µL of 0.01 mol/L NaOH and rinsed with 100 µL of distilled water. The beads were then activated using 150 µL of freshly prepared EDC solution (50 mg/mL), after which they were incubated with slow tilt rotation at room temperature for 30 minutes. Following activation, the beads were washed with cold water and MES buffer (50 mM, pH 5.0).

A 100 µL solution containing 2100 pmol of DGL-miR451a in 0.125 M NaHCO₃ / 0.1% Tween-20 (pH 9.84) was then added to the activated beads. The coupling reaction was performed for three hours at room temperature with slow tilt rotation. The remaining activated carboxyl groups were then quenched by adding 100 µL of 50 mM ethanolamine in PBS (pH 8) and incubating for 1 hour. The beads were then washed with 100 µL of 10% PEG10K and 0.1% Tween-20 in PBS. The functionalised beads were then stored at 4 °C in PBS containing 0.1% Tween-20 until required.

Calibration Curves for miR-451a and miR-451b Spike-In

Calibration curves were generated using ssDNA-451a diluted in lysis buffer at the following concentrations: 9114, 3038, 1013, 338, 112, 38, and 13 pg/mL. A blank control containing lysis buffer without ssDNA-451a was included as the zero standard to establish the assay background. Analytical specificity was assessed in parallel using an equivalent calibration series prepared with ssDNA-451b, diluted in lysis buffer at the same concentrations: 9114, 3038, 1013, 338, 112, 38, and 13 pg/mL, together with a blank control containing no ssDNA-451b target.

This parallel calibration was employed to assess the DCL assay's capacity to distinguish between the miR-451a and miR-451b sequences. All measurements were performed in triplicate, as detailed in Supplementary Section S2. Calibration curves were generated under the assay conditions described in Section 2.7. The signal-response data were fitted using a five-parameter logistic (5PL) regression model to enable interpolation of unknown concentrations and estimation of analytical sensitivity parameters.

Interrogation of miR-451 IsomiR Variants

Six ssDNA oligomers, each mimicking one of the most abundant isomiR variant sequences of miR-451a (namely +1, -1, -2, -3, -4 and -5), together with the canonical mimic miR-451a sequence (see Table 2), were spiked into lysis buffer prior to analysis. The sequences were selected using the Stem-loop hsa-mir-451a miRNABase database (MI0001729).

Calibration curves were generated for each isomiR using serial dilutions in lysis buffer at final concentrations of 9114, 3038, 1013, 338, 112, 38, and 13 pg/mL, together with a blank control (0 pg/mL) containing no ssDNA-451a.

All measurements were performed in triplicate, as detailed in Supplementary Section S3. The calibration curves were generated under the assay conditions described in Section 2.7.

Table 2. Sequences of the canonical miR-451a and its most abundant isomiR variant sequences

Name	Sequence
miR-451a	<u>AAACCGUUACCAUUACUGAGUU</u>
miR-451a (+1)	<u>AAACCGUUACCAUUACUGAGUUU</u>
miR-451a (-1)	<u>AAACCGUUACCAUUACUGAGU</u>
miR-451a (-2)	<u>AAACCGUUACCAUUACUGAG</u>
miR-451a (-3)	<u>AAACCGUUACCAUUACUGA</u>
miR-451a (-4)	<u>AAACCGUUACCAUUACUG</u>
miR-451a (-5)	<u>AAACCGUUACCAUUACU</u>

Note: The regions complementary to the DGL-miR451a are underlined, and the G that templates the incorporation of SMART-C-Biotin is shown in bold. Sequences of miR-451a and selected isomiR variants extrapolated from the miRBase reference sequence.

DCL Assay Workflow and Chemiluminescent Detection

The DCL assay comprises three main steps, as follows: (a) Target capture on DGL-451a capture beads; (b) dynamic chemical labelling reaction; and (c) chemiluminescent detection.

In brief, 60 μL of the test solution (either a stool sample diluted in RNA preservation buffer, or synthetic mimic oligomers prepared in lysate buffer) was mixed with 40 μL of lysis buffer containing 1.5×10^5 DGL-451a capture beads per reaction to make a total volume of 100 μL . Target capture was then carried out in 96-well plates under orbital shaking at 800 rpm for two hours at 30 °C. After capture, the beads were separated using a magnet and washed three times with 200 μL of PBS containing 0.1% Tween-20.

DCL was then performed by adding 50 μL of a master mix containing 2 x SSC, 0.1% SDS, 2 μM SMART-C-Biotin and 4.5 mM sodium cyanoborohydride. The plates were then incubated at 40 °C for 1 hour under orbital shaking at 800 rpm. Following labelling, the beads were separated using a magnet and washed three times with 200 μL of PBS containing 0.1% Tween-20. To generate the signal, 100 μL of a 1:80000 diluted solution of streptavidin-HRP was added and the plate was incubated for 30 minutes at 30 °C with shaking.

After a final washing step, the beads were resuspended and transferred to white 96-well plates. Chemiluminescent detection was performed using SuperSignal™ ELISA Femto Substrate, which is based on luminol/hydrogen peroxide chemistry. Luminescence signals were measured using a FLUOstar Omega microplate reader with top optics, a start time of 0 s, an interval of 0.02 s, and a gain setting of 2000.

Apparatus

Luminescence signals were measured using a FLUOstar Omega microplate reader (BMG Labtech). The instrument was operated using the Omega software package, and Belysa® Immunoassay Curve Fitting Software (Merck KGaA) was employed for calibration curve generation and quantitative analysis. All graphs were generated using GraphPad Prism software (version 8.0.2.263). Microplate incubations were performed using a thermoshaker under controlled temperature and orbital shaking conditions. Magnetic separations were carried out using a magnetic rack (LifeSep™ 96F Separation Block), which is compatible with 96-well microplates.

Red Blood Cell (RBC) Spike-In Experiments

Whole blood from a healthy donor, collected with informed consent, was used as the source of erythrocytes. Collection and analysis of this blood sample were covered by the same single ethics approval as the stool sample collection described in Section 2.1, namely protocol Colorectal miRNA CEC2014 issued by the local Ethics Committee of Azienda Ospedaliera Nazionale SS. Antonio e Biagio e Cesare Arrigo of Alessandria, Italy; both stool and blood sample collection were approved under this same protocol. This sample was known to contain 5.54×10^6 RBC per μL . Defined RBC inputs were prepared and added to approximately 2 mg of stool matrix diluted in FIT buffer to generate a stool-compatible spike-in series. These spike-in preparations contained decreasing total RBC inputs ranging from 1.35×10^7 to 82 RBCs per analysed preparation. No-spike controls consisting of stool/FIT buffer matrix and FIT buffer alone were included in parallel. For each condition, 100 μL of the RBC-containing preparation or control was mixed with 100 μL of lysis buffer to release intracellular miR-451a.

The haemolytic activity of lysis buffer was verified by Neubauer chamber microscopy using blood-containing control samples. Samples incubated with lysis buffer showed effective erythrocyte lysis, whereas blood incubated in PBS, used as a negative control, remained non-haemolysed (see Supplementary Section S4 and Figure S2). Each RBC input was analysed in triplicate. Chemiluminescence signals were recorded as RLU slope values, with mean RLU, standard deviation and coefficient of variation calculated for each condition. The full RBC spike-in dataset is provided in Supplementary Section S5.

RNA Extraction and Small RNA Sequencing

Total RNA was extracted from stool samples using the stool total RNA purification kit (Norgen Biotek Corp) as described by Birkeland et al. [32]. Small RNA sequencing libraries were prepared using the NEBNext Multiplex Small RNA Library Prep kit (protocol E7330, New England Biolabs) according to the manufacturer's protocol. For each sample, 250 ng of RNA was used as starting material to prepare

libraries. Multiplex adapter ligations, reverse transcription primer hybridization, reverse transcription reactions, and PCR amplification were performed as described elsewhere [32].

The obtained libraries were subjected to the Illumina sequencing pipeline, passing through clonal cluster generation on a single-read flow cell by bridge amplification on a cBot (TruSeq SR cluster kit, v3-cBOT-HS) and 50 cycles of sequencing by synthesis using a HiSeq 2000 sequencer (in collaboration with Genecore Facility at EMBL, Heidelberg, Germany).

Bioinformatic and Statistical Analysis

Sequencing data were analysed using a previously published Docker-based small RNA-seq pipeline to ensure computational reproducibility [36]. Trimmed reads were aligned to a curated reference of human miRNAs based on miRBase v22.1 using the BWA algorithm. miRNAs were considered detected when supported by at least 20 DESeq2-normalised reads. In the present study, DESeq2-normalised hsa-miR-451a counts were used for comparison with DCL-derived miR-451a concentrations, rather than for a full differential expression analysis. A previous analysis of the same cohort reported that miR-451a was altered in CRC subjects, although it was not among the strongest differentially represented miRNAs. Statistical analyses were performed using GraphPad Prism software (version 8.0.2.263). Group differences were assessed using Wilcoxon rank-sum tests, Kruskal-Wallis tests, or chi-square tests, where appropriate. Correlation between DCL-derived miR-451a concentrations and DESeq2-normalised small RNA sequencing counts was assessed using Spearman correlation. Diagnostic performance was calculated using colonoscopy status as the reference classification and a predefined positivity threshold of 26 pg/mL, corresponding to $2 \times \text{LLOQ}$. All statistical tests were two-sided, and $p < 0.05$ was considered statistically significant.

Stool Study Cohort and Sample Processing for the DCL Assay

The DCL assay was applied to the same 90 stool samples analysed by small RNA sequencing, comprising 44 colonoscopy-confirmed CRC-positive samples and 46 colonoscopy-negative control samples. Prior to DCL analysis, stool samples underwent a lysis step to release erythrocyte-associated miR-451a, if present.

Briefly, 100 μL of stool sample diluted in RNA preservation buffer was mixed with 100 μL of lysis buffer at a 1:1 v/v ratio. The mixture was incubated for 20 min at room temperature and then centrifuged at $12100 \times g$ for 10 min at 4°C to obtain a clarified supernatant containing released miR-451a. The resulting clarified supernatants were subsequently analysed using the DCL assay workflow and chemiluminescent detection protocol described above in Section 2.7.

3. Results

Platform Assembly and Strategy for Direct Stool Interrogation

A chemiluminescent DCL assay workflow was established for the direct detection of stool-derived miR-451a. Stool samples were treated with lysis buffer to promote sample clearing and release of erythrocyte-associated miR-451a, without prior RNA extraction, reverse transcription, or target amplification. The released target miRNA was captured on magnetic beads functionalised with DGL-451a probes. Upon hybridisation, the target guanine residue in miR-451a is positioned opposite the abasic site of the DGL-451a probe, enabling template-directed incorporation of SMART-C-Biotin at this position. Further chemical details of the DCL assay are reported in Supplementary Section S1. The resulting biotin-labelled DGL-451a/miR-451a duplexes were detected through streptavidin-HRP binding, followed by luminol/peroxide-mediated chemiluminescent signal generation and readout using a standard microplate reader (Figure 1).

Quantitative Calibration Curve and Sequence Specificity

Analytical evaluation using synthetic target standards across a seven-point concentration range produced a concentration-dependent chemiluminescent response, which was fitted using a five-parameter logistic regression model (Figure 2A). The assay achieved a calculated limit of detection (LoD) of 4.25 pg/mL (0.70 pM) and a lower limit of quantification (LLOQ) of 13 pg/mL (2.06 pM). Parallel specificity assessment using the non-target control sequence ssDNA-451b, tested across the same concentration range, generated background-level signals across the entire range, supporting sequence-specific detection of ssDNA-451a (Figure 2B).

Quantitative Detection of Erythrocyte-Derived miR-451a in Stool via RBC Spike-In

To establish the biological sensitivity of the platform in a stool-compatible matrix, decreasing amounts of whole blood cells were spiked into stool matrix and analysed using an amount-response titration model. Following buffer-mediated lysis, the chemiluminescent signal output increased progressively with the number of RBCs added (Figure 3).

Concentration interpolation against the standard curve demonstrated that the lowest RBC input generating a quantifiable concentration of total miR-451a (comprising both the canonical sequence and its isomiR variants) above the LLOQ was 337,805 total RBCs in the analysed preparation, defining an operational biological limitation of 1689 RBC/ μ L (interpolated concentration: 22 pg/mL; Supplementary Section S5). A lower RBC input of 112,600 total RBCs (563 RBC/ μ L) generated a measurable chemiluminescent signal tracking an interpolated target value of 11 pg/mL, remaining detectable but resting strictly below the verified LLOQ border line.

Equitable Detection of miR-451a IsomiR Variants by the DCL Assay

Evaluating the six most abundant naturally occurring endogenous miR-451a terminal isoforms (+1, -1, -2, -3, -4, and -5) generated parallel, concentration-dependent chemiluminescent responses under identical DCL assay parameters (13 - 9114 pg/mL; Figure 4).

The overlapping standard response profiles verified that sequence modifications resulting from alternative trimming or template extension did not disturb hybridization to the central conserved probe region or suppress template-directed signalling nucleobase lock-up (Supplementary Section S3).

Comparison between DCL Detection and Small RNA Sequencing Outcomes

DCL-derived miR-451a concentrations obtained from the extraction-free clinical stool workflow were benchmarked against DESeq2-normalised counts generated by small RNA sequencing. Across the 36 CRC-positive specimens scoring above the predetermined technical evaluation threshold of $2 \times \text{LLOQ}$ (26 pg/mL), a statistically significant positive correlation was confirmed between the two independent modalities (Figure 5). Notably, seven histologically confirmed CRC-positive samples that presented zero read counts during small RNA sequencing analysis were captured and quantified by direct chemiluminescent DCL interrogation (mapped to the left of the x-axis break in Figure 5; Supplementary Table S5 highlighted in yellow).

Clinical Performance Evaluation and Cohort Quadrant Stratification

Stratification of the full 90-sample clinical cohort was conducted using the verified 26 pg/mL positivity threshold. For the primary group comparison, a balanced, concordant validation set of 72 samples was analysed: 36 CRC-positive samples tracking above the threshold, and 36 colonoscopy-negative control samples resting below it (see Supplementary Tables S5 and S6.2). Stool miR-451a levels were significantly higher in CRC-positive samples than in colonoscopy-negative controls in this concordant comparison set (Figure 6A).

The remaining 18 clinical specimens exhibited divergent profiles and were stratified into two distinct subgroups to track matrix-associated target variations: a) colonoscopy-negative controls / DCL-positive group (n = 10): these 10 samples derived from colonoscopy-negative subjects and displayed analytical values above the 26 pg/mL threshold, with supporting small RNA sequencing reads and clinical annotation consistent with non-neoplastic benign lower gastrointestinal or anorectal bleeding events (Figure 6B; Supplementary Table S6.3); b) CRC cases / DCL-negative group (n = 8): these 8 specimens

derived from histologically verified cancer patients and presented target mass values below the 26 pg/mL clinical boundary, with sequencing read dropouts and clinical annotation consistent with non-bleeding or intermittently bleeding CRC lesions (Figure 6B; Supplementary Table S6.4).

Integrating colonoscopy outcomes with the predefined miR-451a positivity threshold resolved the 90-sample cohort into four groups: CRC cases /miR-451a-positive ($n = 36$), CRC cases /miR-451a-negative ($n = 8$), colonoscopy negative/miR-451a-positive ($n = 10$) and colonoscopy negative/miR-451a-negative ($n = 36$) (Figure 6B). Using colonoscopy as the reference classification, these groups corresponded to 36 true positives, 8 false negatives, 10 false positives and 36 true negatives. Based on these quadrant distributions, the stool miR-451a DCL assay, using 26 pg/mL as threshold, achieved a clinical sensitivity of 81.8% (36/44) and a specificity of 78.3% (36/46) using colonoscopy as the reference standard.

4. Discussion

In 2018, we first described a fluorescence-based DCL assay for the detection of total miR-451a, establishing the probe architecture and template-directed chemical labelling strategy using haemolysed blood as a model system for erythrocyte-derived miR-451a [35]. In the present study, we report a further development of this technology through the implementation of a highly sensitive chemiluminescent DCL format and its application to the direct analysis of stool samples. This represents a significant technological advance over the original fluorescence-based platform, extending DCL from a proof-of-concept assay to a clinically relevant workflow compatible with a complex biological matrix. By replacing the fluorescence readout with an HRP-mediated chemiluminescent signal generation system [22], the assay achieved an approximately 29-fold improvement in analytical sensitivity. Importantly, this gain in performance was achieved at the level of signal transduction, while preserving the core characteristics of DCL, meaning direct sequence-specific target interrogation without RNA extraction, reverse transcription, or enzymatic amplification.

This chemically driven labelling mechanism is particularly advantageous for human stool specimens. Faeces represent a highly challenging biological matrix containing polysaccharides, bile salts, lipids, humic acids and other components known to interfere with conventional enzymatic nucleic acid assays [37]. Methods such as RT-qPCR and NGS are vulnerable to these matrix-associated effects and may introduce quantitative biases during RNA extraction, adapter ligation and amplification steps [20]. By operating directly on clarified stool lysates and avoiding enzymatic target amplification, DCL minimises these sources of analytical variability while preserving a simplified workflow.

The high sequence specificity of the DCL assay, demonstrated by the clear discrimination between the target mimic and the single-base mismatched non-target control, underscores the molecular robustness of DCL chemistry [38]. Signal generation relies on two separate molecular recognition steps: sequence-specific hybridization between the abasic PNA probe and its target, governed by Watson-Crick base-pairing rules, and chemical labelling through a template-driven dynamic covalent reaction [39]. Furthermore, the probe capability to uniformly capture and process the major biological isomiRNA

variants highlights its utility for heterogeneous clinical specimens [40], where terminal trimming or extension variations occur naturally as part of endogenous miRNA biogenesis.

The RBC spike-in experiments provide a quantitative biological framework for interpreting the assay signal. Because total miR-451a represents a major component of the small RNA content of mature red blood cells, tracking its concentration provides a molecular estimate of blood-associated material within the stool matrix [41]. It is therefore important to emphasize that miR-451a should not be interpreted as an autonomous, tumour-specific biomarker that sheds independently of vascular disruption. Rather, in this diagnostic framework, it serves as a robust, sequence-defined nucleic acid surrogate for lesion-associated bleeding.

This distinction is crucial when positioning this technology alongside current clinical standards of care, particularly FIT. This assay is not designed to replace or diminish FIT, which remains a benchmark, non-invasive population screening strategy supported by extensive clinical validation. Instead, the added value of this approach lies in an analytical shift from a labile haemoglobin protein complex to a sequence-defined, stable miRNA target. Haemoglobin is prone to environmental degradation and conformational changes during gastrointestinal transit and sample transport. Shifting the target to total miR-451a addresses these pre-analytical liabilities while providing a molecular readout compatible with future interrogation of emerging miRNA biomarkers in stool.

The comparison with small RNA sequencing further shows the practical value of direct chemical interrogation. The detection of seven confirmed CRC-positive specimens that generated zero read counts during sequencing underscores the technical vulnerabilities of multi-step genomic discovery pipelines. Sequencing workflows require column extraction, sequential ligations, reverse transcription and PCR library preparation, each of which can expose low-abundance small RNAs to representation biases and target loss. By eliminating these enzymatic manipulation steps, the DCL assay can preserve readable molecular target signals in degraded or low-abundance samples that may otherwise result in sequencing non-detection.

The clinical analysis confirmed the ability of DCL-measured stool total miR-451a to discriminate between CRC cases and colonoscopy-negative controls at the group level, as shown by the significant difference observed in the concordant comparison set. At the same time, the distribution of the full clinical cohort across four quadrants provides a nuanced biological interpretation of the assay. The 10 colonoscopy-negative but total miR-451a-positive samples are consistent with non-malignant, benign sources of lower gastrointestinal or anorectal bleeding, such as active haemorrhoidal disease or diverticular irritation, where the presence of an erythrocyte-associated marker was supported by small RNA sequencing reads. Conversely, the eight colonoscopy-positive but DCL-negative CRC samples highlight the well-known biological phenomenon of intermittent tumour bleeding, where localised malignant lesions may not shed blood during the specific stool collection window. This clinical distribution pattern reinforces the interpretation that total miR-451a tracks vascular micro-bleeding events rather than isolated tumour cell sloughing.

A key future opportunity is multiplexing. In its current single-marker configuration, miR-451a remains biologically linked to bleeding, similarly to FIT. However, the DCL architecture can be adapted to detect additional miRNA targets using different sequence-specific capture probes. In this context, miR-451a could be combined with additional stool miRNAs and protein markers associated with CRC biology, adenoma progression or the tumour microenvironment, improving molecular discrimination beyond bleeding alone [27]. The present study represents a preliminary clinical evaluation in a single-centre Italian cohort; future work will address validation in larger, geographically diverse populations, assessment of performance for advanced adenoma detection, and compatibility with standard stool collection workflows used in organised screening programmes.

5. Conclusions

This study establishes a PCR-free and extraction-free chemiluminescent DCL assay for direct quantification of stool total miR-451a in clarified stool lysates. By bypassing total RNA extraction, reverse transcription and enzymatic amplification, the platform reduces workflow complexity and avoids key sources of pre-analytical target loss and amplification-related bias.

In its current configuration, the assay should be interpreted as a sequence-specific molecular readout of blood-associated material in stool, rather than as a replacement for FIT. Its value lies in shifting the analytical target from haemoglobin protein to a stable miRNA sequence, while preserving compatibility with practical, microplate-based screening workflows.

The broader significance of this work is the demonstration that DCL can interrogate nucleic acids using an immunoassay-like format. This creates a direct route toward multiplexed stool biomarker testing, in which miR-451a can be combined with additional disease-associated miRNAs. In particular, the multi-faecal miRNA molecular signature recently identified by our group [36] provides a strong basis for developing multiplex DCL assays aimed at detecting CRC-associated miRNA patterns directly in stool.

Because DCL is compatible with bead-based platforms such as Luminex, and because simultaneous miRNA and protein biomarker detection has already been demonstrated in the context of liver injury [26], this approach could also support combined miRNA–protein panels. Overall, the present work provides a foundation for next-generation non-invasive stool assays that integrate blood-associated markers, disease-specific miRNA signatures and complementary protein biomarkers to improve early CRC detection and discrimination between CRC and advanced adenomas.

Abbreviations

5PL, five-parameter logistic regression;

BWA, Burrows–Wheeler Aligner;

CRC, colorectal cancer;

CV, coefficient of variation;

DCL, Dynamic Chemistry Labelling;

DESeq2, differential expression analysis for sequence count data 2;

DGL, DESTINA Genomica Labelling;

DNA, deoxyribonucleic acid;

EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide;

FDA, Food and Drug Administration;

FIT, faecal immunochemical test;

gFOBT, guaiac-based faecal occult blood test;

HPLC, high-performance liquid chromatography;

HRP, horseradish peroxidase;

isomiR, microRNA isoform;

LLOQ, lower limit of quantification;

LoD, limit of detection;

MALDI-ToF, matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry;

MES, 2-(N-morpholino)ethanesulfonic acid;

miRNA, microRNA;

mt-sRNA, multi-target stool RNA;

NGS, next-generation sequencing;

PBS, phosphate-buffered saline;

PCR, polymerase chain reaction;

PEG, polyethylene glycol;

PNA, peptide nucleic acid;

RBC, red blood cell;

RLU, relative light units;

RNA, ribonucleic acid;

RT-qPCR, reverse transcription quantitative polymerase chain reaction;

SD, standard deviation;

SDS, sodium dodecyl sulphate;

SMART-C-Biotin, aldehyde-modified biotinylated cytosine SMART base;

SSC, saline sodium citrate;

ssDNA, single-stranded DNA.

Declarations

Ethics approval and consent to participate: The study was conducted in accordance with the Declaration of Helsinki. Both the clinical study with stool sample collection and the healthy donor blood sample collection used for the red blood cell spike-in experiments (Section 2.9) were approved under the same single ethics approval, protocol Colorectal miRNA CEC2014, issued by the local Ethics Committee of Azienda Ospedaliera Nazionale SS. Antonio e Biagio e Cesare Arrigo of Alessandria, Italy. All participants provided written informed consent prior to enrolment. Stool samples were retrieved from a previously described Italian cohort recruited at Clinica Santa Rita Hospital, Vercelli, Italy.

Consent for publication: Not applicable. The manuscript does not contain identifiable individual person's data, images, or clinical details. All clinical information is reported in anonymised and aggregate form.

Data availability: The datasets generated and/or analysed during the current study are available in the Gene Expression Omnibus (GEO) repository, accession number GSE337958 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE337958>; reviewer access token: cjglwwwkdjwblp). All other data generated or analysed during this study are included in this article and its supplementary information files.

Competing interests: BS, MTC and JJGM are affiliated with DESTINA Genomica S.L., which develops Dynamic Chemistry Labelling technologies. SP and JJDM are shareholders of DESTINA Genomica S.L. The remaining authors declare that they have no competing interests.

Funding: This work was undertaken within the framework of ColoMARK, funded by the European Union's Horizon Europe research and innovation programme under the Marie Skłodowska-Curie Doctoral Network Grant Agreement No. 101072448 (DOI: 10.3030/101072448), within the programme HORIZON.1.2 – Marie Skłodowska-Curie Actions (MSCA), call HORIZON-MSCA-2021-DN-01-01 – MSCA Doctoral Networks 2021. ColoMARK provided doctoral fellowship support for BS's PhD project, with

DESTINA Genómica S.L. acting as the beneficiary organisation. Additional support was provided by the European Union's Horizon 2020 research and innovation programme under grant agreement No. 825410 (ONCOBIOME project, to AN and BP). The research leading to these results also received funding from AIRC under IG 2020 – ID: 24882, P.I. AN. The funders had no role in the design of the present study, data analysis, interpretation of results, writing of the manuscript, or decision to submit the work for publication.

Author contributions: SP and JJD conceived the study and oversaw the development of the DCL assay. BS performed the experimental work, including assay implementation, analytical evaluation, and stool sample analysis, with support from MTC. RMSM contributed to the chemical methodology. JJGM contributed to the chemical component of the manuscript. VT and DG contributed to the translational and clinical interpretation of the study and reviewed the manuscript. BP and AN provided access to the clinical cohort, clinical metadata, and small RNA sequencing datasets, and contributed to the clinical interpretation of the results. GF performed the bioinformatic analysis and supported the comparison between DCL data and small RNA sequencing results. BS, SP, BP, AN and GF contributed to the analysis and interpretation of the data. BS and SP coordinated preparation of the manuscript with input from BP, AN, GF, JJGM and JJD. All authors read and approved the final manuscript.

Acknowledgements: The authors gratefully acknowledge the scientists of the ColoMARK consortium for their valuable input and constructive discussions during consortium meetings. In particular, the authors thank Dr Meritxell Gironella Cos from IDIBAPS-CIBERehd-Clinic Barcelona, Spain, for her scientific feedback, and Dr Ceres Fernández Rozadilla from the Fundación Pública Galega de Medicina Xenómica/IDIS, Santiago de Compostela, Spain, for coordinating the ColoMARK consortium and leading the successful preparation of the proposal that secured ColoMARK funding. The authors also thank all scientists and technical staff at DESTINA Genómica S.L. for their valuable input and support during the development of the DCL platform and implementation of the stool miRNA assay.

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Figures

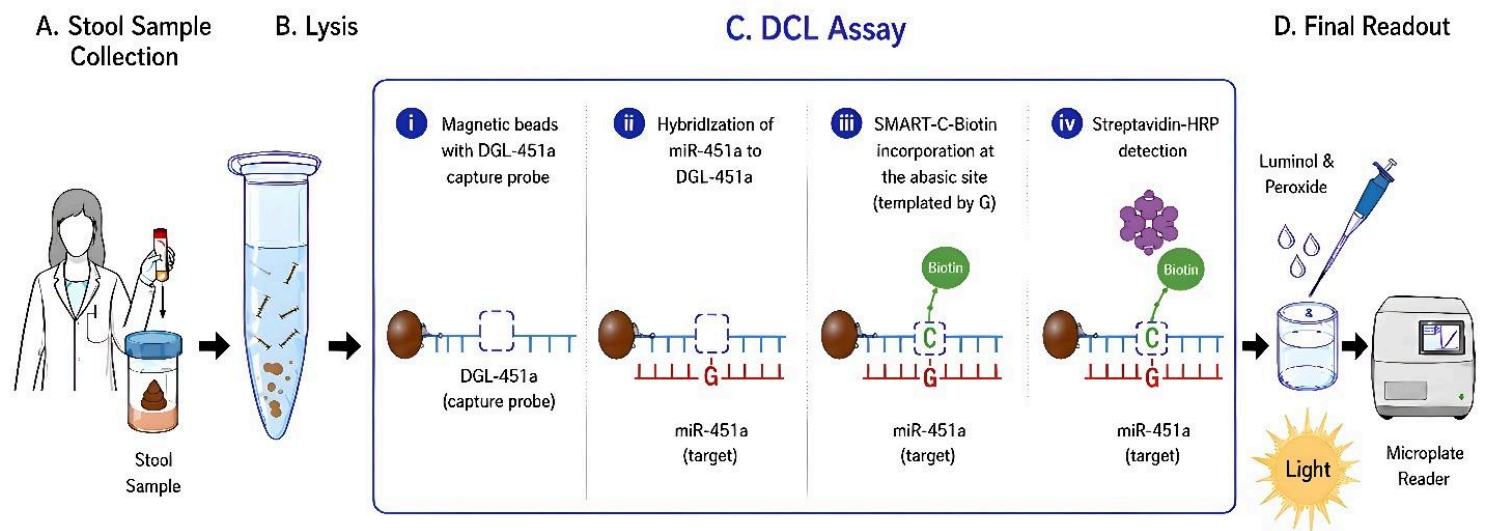


Figure 1

Chemiluminescent DCL workflow for direct detection of stool-derived miR-451a. Stool samples are processed using lysis buffer to release stool-associated miR-451a. The DCL assay uses bead-immobilised DGL-451a capture probes to hybridise free miR-451a target molecules, positioning the templating guanine of miR-451a opposite the abasic site of the probe. SMART-C-Biotin incorporation generates a biotin-labelled DGL-451a/miR-451a duplex, which is subsequently detected through streptavidin-HRP binding. Chemiluminescent signal is generated using luminol/peroxide and measured with a standard microplate reader. The timed workflow is completed in approximately 4 h.

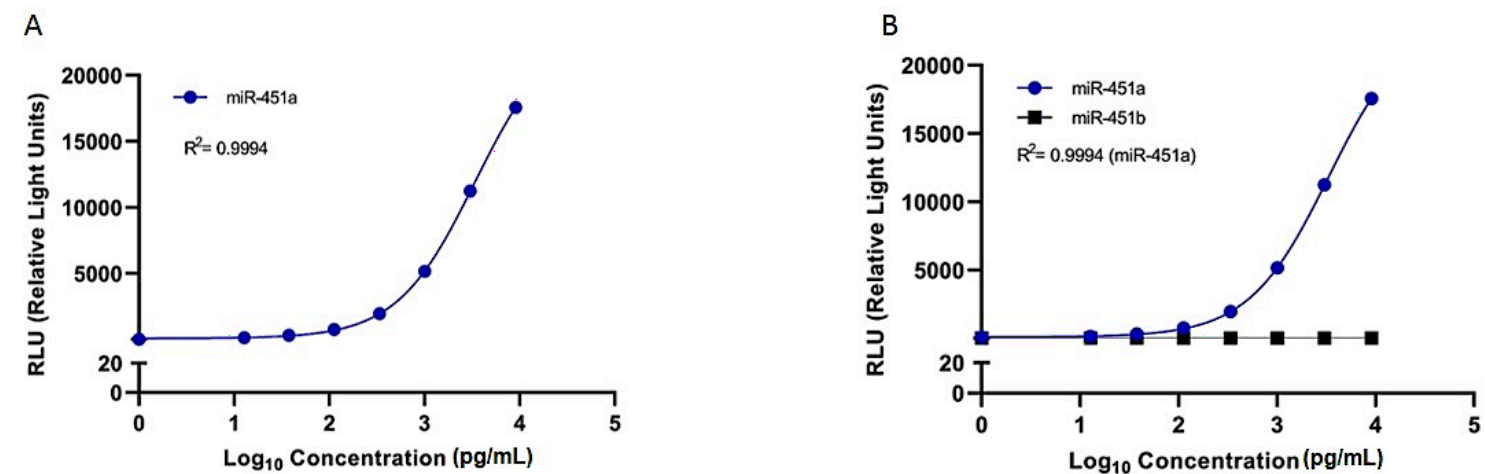


Figure 2

Calibration and analytical specificity of the chemiluminescent DCL assay. (A) Calibration curve generated using synthetic ssDNA-451a mimic diluted in lysis buffer across a seven-point concentration

range from 13 to 9114 pg/mL, plus a no-spike control. Data were fitted using a five-parameter logistic regression model. Mass-to-molar conversions were calculated using the molecular weight of the synthetic ssDNA-451a target, MW = 6076 g/mol. (B) Specificity assessment using synthetic ssDNA-451a and ssDNA-451b mimics under identical DCL conditions. Data are shown as mean values from technical triplicates, with error bars indicating standard deviation. The chemiluminescent signal is reported as relative light units. Raw data are provided in Supplementary Section S2.

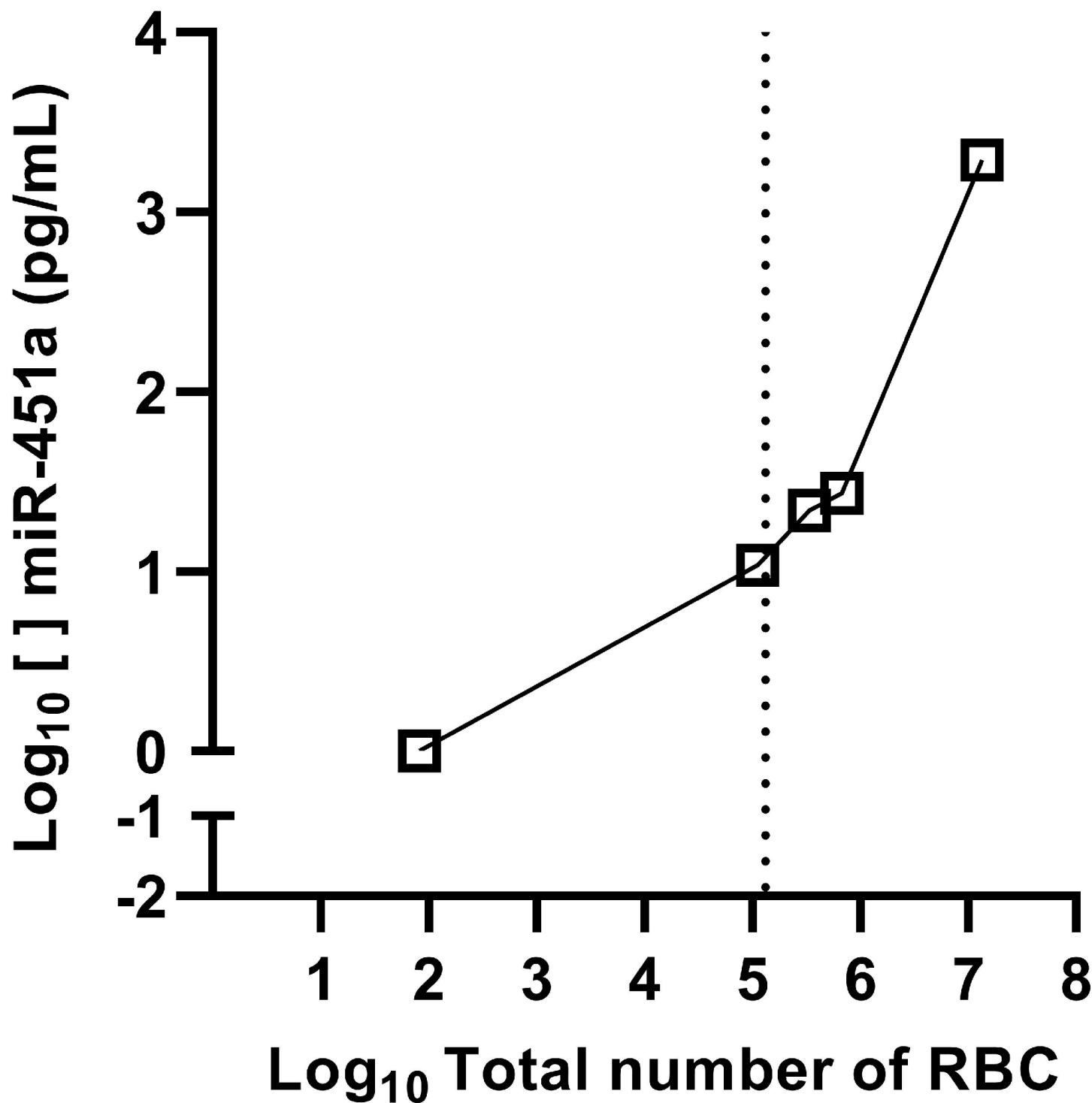


Figure 3

RBC spike-in model for DCL detection of erythrocyte-derived miR-451a in stool matrix. The x-axis shows the $\log_{10}$ -transformed total RBC input, while the y-axis reports the interpolated miR-451a concentration as $\log_{10}$ pg/mL, calculated from the miR-451a calibration curve. The vertical dotted line indicates the lowest RBC input producing a quantifiable miR-451a concentration above the LLOQ, corresponding to 337805 total RBCs, equivalent to 1689 RBC/ μ L. Measurements represent mean values from technical triplicates. Raw data are provided in Supplementary Section S5.

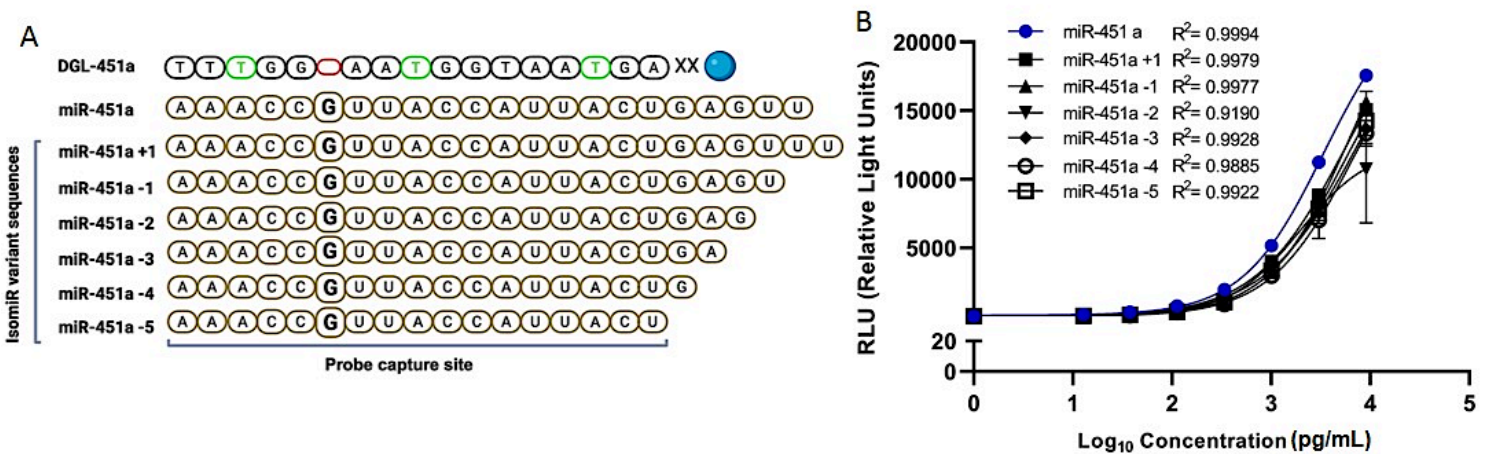


Figure 4

Interrogation of miR-451a isomiR variants by the chemiluminescent DCL assay. (A) Sequence alignment of the tested miR-451a isomiR variants, showing the position of the DGL-451a capture probe relative to the target sequences. The templating guanine residue required for SMART-C-Biotin incorporation is shown in bold. In the DGL-451a probe, Tglu residues, thymidines bearing a propanoic acid side chain at the γ -position, are indicated in green circles, while the abasic L-Ser monomer is highlighted in red. Dynabeads are represented as blue spheres. x = amino-PEG linker. (B) Overlaid chemiluminescent response curves generated using synthetic mimics of the canonical miR-451a sequence and the +1, -1, -2, -3, -4 and -5 isomiR variants. Data are shown as mean relative light units, RLU, from technical triplicates with standard deviation. Raw data are provided in Supplementary Section S3.

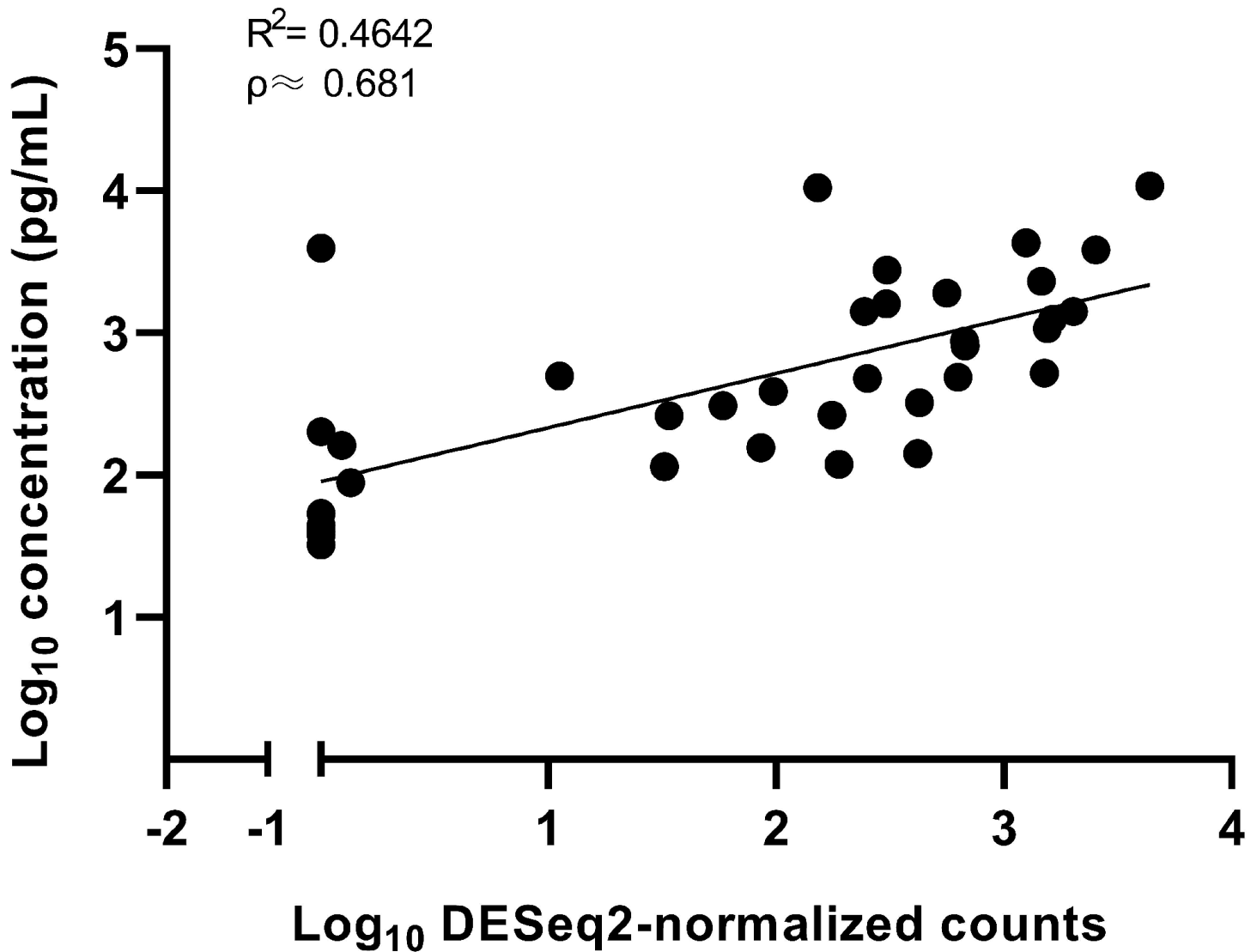


Figure 5

Correlation between DCL and small RNA sequencing. Scatter plot showing the relationship between total miR-451a concentration measured by the chemiluminescent DCL assay and miR-451a abundance obtained by small RNA sequencing. The y-axis reports DCL-derived total miR-451a concentration as log_{10} (pg/mL), while the x-axis reports log_{10} -transformed DESeq2-normalised total miR-451a counts. The association between the two methods was assessed by Spearman correlation; the correlation coefficient and p-value are reported in the graph. The linear regression line is shown for visualisation, with the corresponding R^2 reported in the graph. The x-axis break indicates CRC-positive samples with zero sequencing counts but detectable DCL signal.

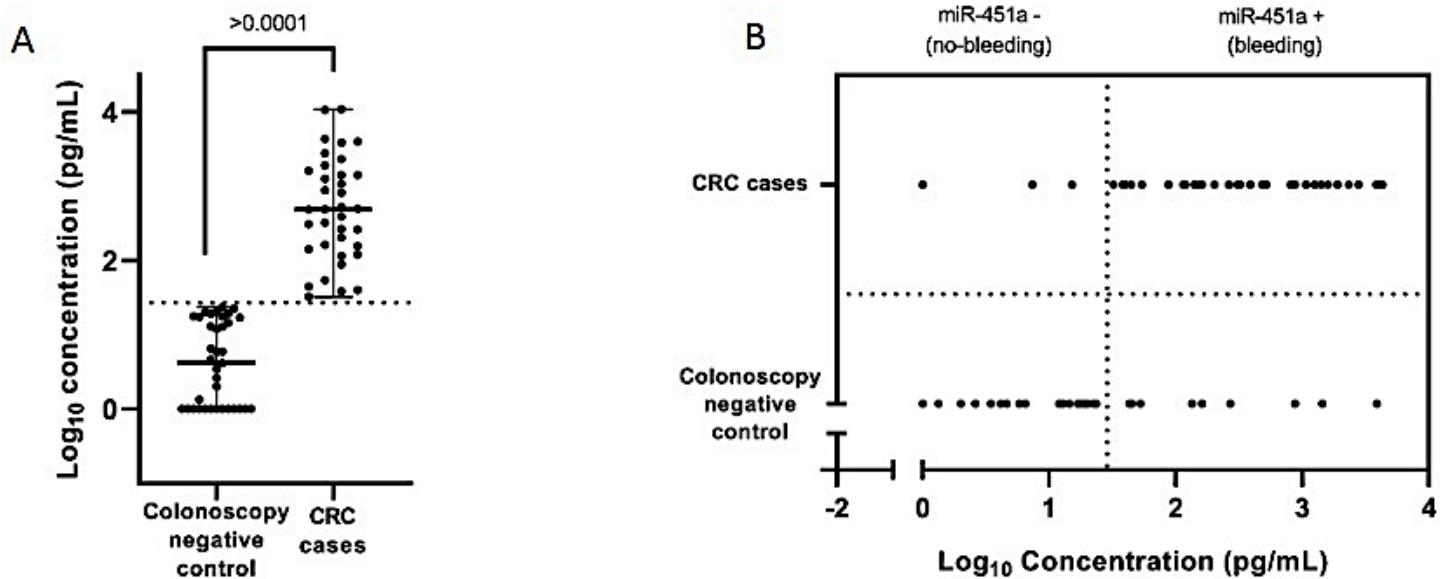


Figure 6

Clinical distribution and classification of stool miR-451a, as measured by the DCL assay. (A)

Distribution of log-transformed stool miR-451a concentrations measured by the chemiluminescent DCL assay in the 72-sample concordant comparison set. This set included 36 CRC-positive samples with concentrations above the predefined threshold of $2 \times \text{LLOQ}$ (26 pg/mL) and 36 colonoscopy-negative samples with concentrations below this threshold. Each dot represents one stool sample. Statistical significance between groups is indicated above the plot. (B) Classification of the full cohort of 90 colonoscopy-characterised stool samples according to colonoscopy status and total miR-451a detection profile. Samples were stratified into four groups using the 26 pg/mL threshold, represented by the vertical dashed line. The horizontal line separates CRC cases from colonoscopy-negative controls.

Supplementary Files

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