

Absolute digital(d)PCR quantification of micro(mi)RNA molecules for the diagnostic screening of colon cancer in human stool: A methodological review

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Introduction

There is currently no validated micro(mi)RNA diagnostic stool test to screen for colon cancer (CC) on the market because of the complexity of fecal density, vulnerability of stool to daily changes, and the presence of three sources of miRNAs in stool (cell-free from fecal homogenates, exosomal miRNAs from fecal exosomes, and fecal colonocytes). By employing earlier on a microarray miRNA experiment, using Affymetrix GeneChip miRNA 2.0 Arrays, on immunocaptured and enriched stool colonocytes of 15 subjects [three healthy controls and twelve colon cancer patients [three TNM stage 0-1 (e.g., polyps ≥ 1 cm, villous or tubvillous, or with high grade dysplasia), three stage 2, three stage 3, and three stage 4] in triplicates, this allowed for selection of a smaller panel of 14 preferentially expressed mature miRNAs associated with colon cancer (12 Up-Regulated, miR-19a, miR-20a, miR-21, miR-31, miR-34a, miR-96, miR-106a, miR-133a, miR-135b, miR-206, miR-224 and miR-302; and 2 Down-Regulated, miR-143 and miR-145). Then carrying out an absolute quantitative digital PCR on these 15 stool samples from TNM stages 0-4 on total small RNA extracted by immunocapture, followed by RT that employed a Custom TaqMan[®] miRNA Reverse Transcription (RT) Kit and TaqMan RT Primer Pool, and absolute quantification of miRNAs, in copies/ μ l, measured using a chip-based Absolute QuantStudio 3D Digital PCR analysis, allowed for validating the microarray results. To ensure that human and not bacterial small total RNA was chosen, coextraction protocols with *E. coli* K1 strain RS18 was carried out, followed by comparing Agilent electrophoretic patterns with human and bacterial electrophoretic patterns, and also random samples were sequenced using mRNA/miRNA sequencing, to ensure that human and not bacterial mRNA was chosen.

Quantitative dPCR miRNA data presented in herein, show that the quantitative changes in the expression of a few mature miRNA genes in stool, which are associated with right and left colon cancer, would provide for a more convenient, sensitive and specific diagnostic screening molecular markers, more useful than markers currently available on the market, such as the low-sensitivity (<15%) fecal occult blood test (FOBT); result in better compliance; and is more economical than the invasive and expensive colon cancer colonoscopy exam, resulting in a higher probability of curing that cancer, if detected at the early TNM stages, and which becomes incurable and deadly if not diagnosed before metastasis.

Advantages of using a MiRNA diagnostic colon cancer screening test

The expression of individual genes may be altered by mutations in the DNA, or by a change in their regulation at the RNA or protein levels [1]. Epigenetic silencing is an important mechanism that contributes to gene inactivation in colorectal cancer (CRC) [2]. Analysis of promoter methylation of hypermethylated in cancer 1 (HIC1) gene in human stool showed it to be highly specific (98%) for both colon adenoma and carcinoma [3], but the sensitivity was quite low (31% for adenoma & 42% for all cancer), suggesting that an epigenetic marker only is not adequate for an accurate diagnostic screening, but a combination of genetic and epigenetic markers would be required to reliably identify CRC at an early disease stage [4]. Working with the stable DNA has been relatively easy compared to working with the fragile RNA molecule [1].

A study by scientists at Exact Sciences Corp., Marlborough, MA, which markets a mutation-based DNA test "Cologuard", assessed a newer version of a fecal DNA test for CRC screening using a vimentin methylation marker and another mutation DY marker plus non degraded DNA in a limited sample of 44 CRC patients and 122 normal controls [5]. It cited a sensitivity of 88% and a specificity of 82%, only for advanced cancer, but not for the early adenoma stage. Besides, DNA mutation tests are not cost-effective, as screening for multiple mutations is expensive because these demanding mutation tests are not automated and are labor intensive. In addition, mutation detection in oncogenes and suppressor genes suffers from: a) the detection of mutations in these genes in fewer than half of large adenomas and carcinomas, b) the detection of gene mutations in non-neoplastic tissues, c) mutations found only in a portion of the tumor, and d) mutations often produce changes in the expression of many other genes [6,7].

Protein-based methods are currently not suited for screening and early diagnosis, either because proteins are not specific to one tumor

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or tissue type (e.g., CEA), their susceptibility to proteases, current lack of means to amplify proteins, no function is known for more than 75% of predicted proteins of multicellular organisms, there is not always a direct correlation between protein abundance and activity, and most importantly because detection of these markers exfoliates often signifies the presence of an advanced tumor stage. The dynamic range of protein expression in minimally-invasive body fluids (e.g., blood) is as large as 10^{10} . Moreover, mRNA levels do not necessarily correlate with protein expressions. Protein microarray studies revealed that protein expression vastly exceeds RNA levels, and only posttranslationally modified proteins are involved in signal transduction pathways leading to tumorigenesis. There is no well-documented protein test that has been shown in clinical trials to be a sensitive and a specific indicator of colon neoplasia, especially in early stages [7]. A serum proteomic study employing liquid chromatography (LC)-mass spectrometry (MS) carried out in a non-biased fashion failed to differentiate between individuals with large adenoma (≤ 1 cm) and normal individuals [9]. Compared to nucleic acids, proteomic research is a newer discipline; therefore, it will take considerable time to identify and validate proteins suitable for use as clinical markers, and resolve issues of bias and validations [10].

On the other hand, a transcriptomic mRNA approach, has been shown to detect both adenomas and colon carcinomas with high sensitivity and specificity in preliminary studies [1], but no randomized, standardized, blinded prospective clinical studies have been carried out to validate the superiority of the mRNA approach. A study indicated that a combination of a transcriptomic mRNA and miRNA expression signatures improves biomolecular classification of CRC [11]. Furthermore, not only does miRNAs regulate mRNA, but they also regulate protein expression. Two studies have shown that a single miRNA act as a rheostat to fine tune the expression of hundreds of proteins [12,13]. Hence, for CRC screening, miRNA markers are much more comprehensive and preferable to a DNA-, epigenetic-, mRNA- or a protein-based markers [14-18]. An added advantage for the use of the stable, non-degradable miRNAs by PCR expression, by chip-based methods, is its being automatable, making them much more economical and more easily acceptable by laboratory personnel performing these assays [4].

The discovery of small non-coding protein sequences, 17-27 nucleotides long RNAs (microRNAs), has opened new opportunities for developing a non-invasive screening test for early diagnosis of many cancers. The latest *miRBase* release 22 on, March 12, 2018 [<http://www.mirbase.org>] indicates the total number of miRNAs labeled "high confidence" has increased by 168, to 1996, than in the previous release [19]. MiRNA functions seem regulate development [20], apoptosis [21], and specific miRNAs are essential in oncogenesis [22,23], effective in classifying solid [24-26], and liquid tumors [27,28], and could serve as oncogenes or suppressor genes [30]. MiRNA genes are frequently found at fragile sites, as well as minimal regions of loss of heterozygosity, or amplification of common break-point regions [30], implying their involvement in carcinogenesis. MiRNAs have potential to serve as biomarkers for cancer diagnosis, prognosis and/or response to therapy [31,32]. Profiles of miRNA expression differ between normal and tumor tissues [33,34], suggesting that their expression profiles cluster similar tumor types together more accurately than expression profiles of protein-coding mRNA genes [35,36].

A study that examined global expression of 735 miRNAs in 315 samples of normal colonic mucosa, tubulovillus adenomas, adenocarcinomas proficient in DNA mismatch repair (pMMR), and

defective in DNA mismatch repair (dMMR) representing sporadic and inherited CRC stages I-IV suggest involvement of common biologic pathways in pMMR and dMMR tumors in spite of the presence of numerous molecular differences between them, including differences at the miRNA level; indicating the need to pay attention to mismatch DNA repair (MMR) [36].

Unlike screening for large numbers of messenger (m)RNA (1), a modest number of miRNAs is used to differentiate cancer from normal [37], and unlike mRNA, miRNAs in stool remain largely intact and stable for detection [38], therefore, leading to conclude that miRNA molecules are better markers to use for developing a reliable noninvasive diagnostic marker screen for colon cancer [14-18], since: a) the presence of the bacterium *Escherichia coli* does not hinder detection of miRNA by a sensitive technique such as dPCR [38], and b) the miRNA expression patterns are the same in primary tumor, or diseased tissue, as in stool samples [37-40]. The gold standard, to which the miRNA test is compared to, has been "colonoscopy", obtained from patients' medical records [41]. However, because the low sensitivity guaiac FOBT is still the most commonly used screen in annual checkups (www.cancer.org) [42], this test should also be included for comparison with the proposed dPCR diagnostic miRNA screening approach in human stool.

Advantages of stool over other testing media

Stool testing has several advantages over other colon cancer screening methods as it is truly noninvasive and requires no unpleasant cathartic preparation, formal health care visits, or time away from work or routine activities [43-46]. Unlike sigmoidoscopy, it reflects the full length of the colorectum and samples can be taken in a way that represents the right and left side of the colon. It is also believed that colonocytes are released continuously and abundantly into the fecal stream, contrary to blood that is released intermittently as in guaiac fecal occult blood test (FOBT) [42]; therefore, this natural enrichment phenomenon partially obviates the need to use a laboratory-enrichment technique to enrich for tumorigenic colonocytes, as for example when blood is used for screen testing [47]. Furthermore, because testing can be performed on mail-in-specimens, geographic access to stool screening is essentially unimpeded [4]. The American Cancer Society (ACS) has recognized stool-based molecular testing as a promising screening technology for CRC (www.cancer.org).

Isolation of colonocytes from stool, and comparing the Agilent electrophoretic (18S and 28S) patterns to those obtained from total RNA extracted from whole stool [48-50], and differential lysis of colonocytes by RT lysis buffer (Quagen), could be construed as a validation that the electrophoretic pattern observed in stool (18S and 28S) is truly due to the presence of human colonocytes, and not due to stool contamination with *Escherichia coli* (16S and 23S). Taking into account that some exosomal RNA will be released from purified colonocytes into stool, attempts must be made to correct for exosomal RNA effect [51].

MiRNA dPCR study design

To test miRNAs as reliable, quantitative, sensitive and specific diagnostic biomarkers, for early non-invasive screening of colon cancer, using absolute dPCR test, preliminary work must be validated in a study using a nested case control epidemiology design and employing a prospective specimen collection, retrospective blind evaluation (PRoBE) of control subjects and test colon cancer patients, as specifically delineated by the National Cancer Institute's

Early Detection Research Network <http://edrn.nci.nih.gov> for cancer biomarker discovery studies.

Selection of 14 miRNAs, 12 of them showed increased expression and 2 showed decreased expression for analysis of absolute miRNAs expression by a chip-based digital (d) PCR test is presented in Tables 1 and 2, and Figure 1.

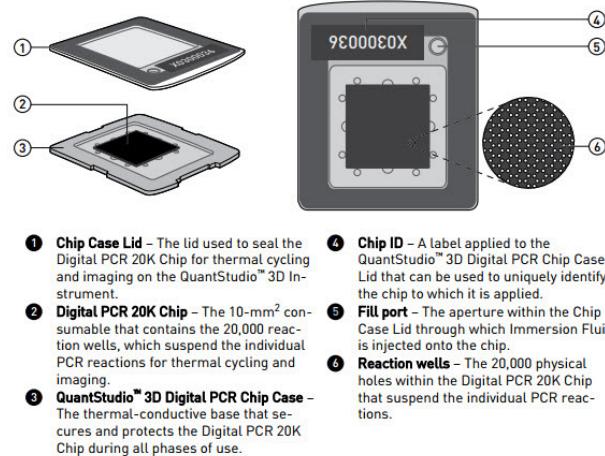


Figure 1. Diagram illustrating QuantStudio™ 3D Digital PCR System Chip; ChipCase Lid (1);Digital PCR 20K 10 mm² nanofluidic v2 chip (2), which contains 20,000 reaction wells; QuantStudio™ 3D Digital PCR Chip Case (3); Chip ID (4); Fill port (5); and Reaction wells, the 20,000 physical holes that suspend individual PCR reactions

The FAM™, VIC®, and ROX™ dyes, available from Life Technologies [49]

Table 1. Characteristics of fourteen up- or down-regulated miRNAs in human stool.

MiRNA	Up-Regulated	Down-Regulated	Chromosome Location	Known Putative Cancer Target Gene(s)
MiR-19a	Yes	No	13q31.3	Undetermined
MiR-20a	Yes	No	13q31.3	PTEN, TMP1
MiR-21	Yes	No	17q23.1	PTEN,BCL2,PDCD4,TIMP3,SPRY2,REC,T1AM1
MiR-31	Yes	No	9p21.3	T1AM1,AX1N1,FOXC2,FOXP3,H1F1AN
MiR-34a	Yes	No	1p36.22	BCL2,TP53,E2F3,NOTCH1,E2F1,SIRT
MiR-96	Yes	No	17q32.2	KRAS
MiR-106a	Yes	No	Xq26.2	PTEN,E2F1,RB1
MiR-133a	Yes	No	18q11.2/20q13.33	BAX,KRAS
MiR-135b	Yes	No	1q32.1	MSH2
MiR-200c	Yes	No	12p13.31	ZEB1
MiR-224	Yes	No	Xp23	Undetermined
MiR-30a	No	Yes	6q13	RASA1,ERG,SEMA6D,SEMA3A
MiR-143	No	Yes	5q32	KRAS,MAPK7,DNMT3A
MiR-145	No	Yes	5q32	TGFBR2,APC,IRS1,STAT1,YES1,FLI1

Table 2. Absolute Quantification of Up-/Down- Regulated miRNAs in Stool by QuantStudio™ 3D Chip-Based Digital PCR.

Type	miR-19a	miR-20a	miR-21	miR-31	miR-34a	miR-96	miR-106a	miR-133a	miR-135b	miR-200c	miR-224	miR-30a	miR-143	miR-145
control	9964.23	9724.14	9699.68	9591.16	9580.92	9590.59	9464.64	9574.13	9568.15	9556.85	9631.73	9401.81	9585.54	9683.18
control	9984.55	9890.38	9795.44	9588.24	9602.9	9587.82	9592.68	9680.24	9515.46	9511.29	9592.62	9580.92	9504.61	9506.12
control	9950.19	9898.88	9938.74	9791.83	9894.82	9862.24	9875.88	9800.08	9824.18	9843.18	9810.2	9780.74	9699.52	9823.54
stage01	7998.16	8011.92	7949.68	7864.18	7880.18	7790.44	7682.74	7687.88	7561.64	7402.8	6994.24	6892.54	1995.92	1884.54
stage01	7814.22	7901.24	7890.32	7798.92	7780.28	6849.68	6999.68	6742.6	6640.16	6616.1	6872.54	6640.24	1879.04	1764.92
stage01	7764.5	7745.38	7690.32	7549.28	7610.32	6787.62	6870.96	6739.42	6690.82	6584.74	6477.52	6454.44	1799.92	1668.19
stage2	7414.42	7569.16	7529.9	7492.68	7384.82	7189.64	6794.88	6690.98	6504.2	5702.16	5464.16	4870.22	1346.48	1040.26
stage2	7390.84	7490.96	7501.62	7379.04	7202.28	7102.28	6472.48	6598.24	6242.82	4387.76	5414.08	4189.42	988.14	862.08
stage2	7208.16	7378.74	7402.68	7299.76	7124.56	7098.04	6402.18	6401.16	6218.92	4123.18	4098.78	3894.9	872.4	763.14
stage3	6850.14	6936.16	6902.04	6890.14	7092.18	6586.18	6319.08	5898.36	5386.66	3821.22	3679.62	3601.4	365.42	256.28
stage3	6792.75	6790.29	6776.26	6658.78	6674.54	6560.68	6116.84	5602.16	4999.16	3715.22	3686.92	3570.92	260.14	154.02
stage3	6622.84	6662.9	6694.28	6558.84	6554.28	6510.27	6039.84	5404.68	5498.82	3421.22	3614.62	3120.18	194.84	133.37
stage4	6506.92	6538.8	6419.02	6227.54	5978.48	5766.32	5686.36	5256.81	4973.28	3327.28	3479.52	2052.38	92.45	88.49
stage4	6468.22	6384.12	6397.92	6117.12	5856.66	5681.82	5259.84	4905.76	3840.86	3244.16	3276.42	1096.44	76.88	67.42
stage4	6488.38	6434.48	6346.06	5898.78	5466.16	5372.56	4896.36	4812.44	3784.56	3164.8	3186.14	678.56	56.82	49.26

(positive), while others do not (negative). A single molecule can be amplified a million-fold or more. During amplification, TaqMan chemistry with dye-labeled probes is used to detect sequence-specific targets. When no target sequence is present, no signal accumulates. Following PCR analysis, the fraction of negative reactions is used to generate an absolute count of the number of target molecules in the sample, without the need for standards or endogenous controls. In conventional qPCR, the signal from wild-type sequences dominates and obscures the signal from rare sequences. By minimizing the effect of competition between targets, dPCR overcomes the difficulties inherent to amplifying rare sequences and allows for sensitive & precise absolute quantification of the selected miRNAs.

Applied Biosystem QuantStudio™ 3D instrument used in this research study, only performs the imaging and primary analysis of the digital chips. The chips themselves must be cycled offline on a Dual Flat Block GeneAmp® 9700 PCR System, or the ProFlex™ 2x Flat PCR System. The QuantStudio™ 3D Digital PCR System can read the digital chip in less than 1 minute, following thermal cycling [48]. It allows for one sample per chip; although, duplexing allows for analysis of two targets per chip. Sample prep for digital PCR is no different than for real-time qPCR, when using the QuantStudio™ 3D Digital PCR System. To figure out the concentration of cDNA stock from results, if one includes all of the necessary dilution factors into the AnalysisSuite™ software, the software will give the copies/µL in the stock.

There are 2 dilutions that one needs to take into account: (a) The first is the dilution of the sample in the reaction, and (b) The second is the dilution of the stock that one makes before adding it to the digital PCR reaction. For example, if one wants to add 1 µL of a sample that has been diluted 1:10 from the stock. Thus, if one adds 1 µL of his/her sample to a 16 µL (final volume) reaction, the dilution factor of the sample is 1:16 or 1/16 = 0.0625. Since the stock has also been diluted 1:10 (0.1), one also needs to factor this in. The final dilution factor to enter into the software is $0.0625 \times 0.1 = 0.00625$ (1:160). One can use either annotation to indicate the dilution factor in the AnalysisSuite™ software. If one enters that value into the “Dilution” column, the software will give the copies/µL in the starting material (stock). The Poisson Plus algorithm for projects that contain QuantStudio™ 3D Chips with target, quantities >2000 copies/µL. The Poisson Plus algorithm corrects for well-to-well load volume variation, on a per Chip basis. This becomes important at higher target concentrations. There is also an option to export the Chip data as XML on the Export tab-thousands of discrete subunits prior to amplification by PCR, each ideally containing either zero or one (or at most, a few) template molecules [50].

Each partition behaves as an individual PCR reactions-as with real-time PCR-fluorescent FAM probes [or others, as VIC fluorescence]. Samples containing amplified products are considered positive (1, fluorescent), and those without product –with little or no fluorescence (i.e., are negative, 0). The ratio of positives to negatives in each sample is the basis of amplification. Unlike real-time qPCR, dPCR does not rely on the number of amplification cycles to determine the initial amount of template nucleic acid in each sample, but it relies on Poisson Statistics to determine the absolute template quantity. The unique sample partitioning step of dPCR, coupled with Poisson Statistics allows for higher precision than both traditional end point PCR and qPCR methods; thereby allowing for analysis of rare miRNA targets quantitatively and accurately [50,51].

The use of a nanofluidic chip, shown below, provides a convenient and straight forward mechanism to run thousands of PCR reactions in

parallel. Each well is loaded with a mixture of sample, master mix, and Applied Biosystems TaqMan Assay reagents, and individually analyzed to detect the presence (positive) or absence (negative) of an endpoint signal. To account for wells that may have received more than one molecule of the target sequence, a correction factor is applied using the Poisson model. It features a filter set that is optimized for the FAM™, VIC®, and ROX™ dyes, available from Life Technologies [49].

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A workflow of the dPCR procedure by the QuantStudio™ 3D Digital PCR System is presented in Figure 2.

Digital PCR, however, has several tips to follow: 1) A rough estimate of the concentration of miRNAs of interest has to be first carried out, in order to make appropriate dilutions, so that not too many partitions will get multiple copies that prevent accurate calculation of the copy number of miRNAs of interest; 2) Non-template controls and a RT negative control must be set up for each miRNA, when using a “primer pool method” for retro-transcription; 3) A chip-based dPCR method requires less pipetting steps, which reduces potential PCR contamination compared to another type of dPCR marketed by Bio-Rad Laboratories, thus called “Bio-Rad’s droplet digital PCR”, which requires multiple pipette transfers that potentially increase the risk of contamination [50], and 4) Quant Studio™ 3D chip has 20,000 fixed reaction wells, whereas Bio-Rad’s droplet PCR relies upon the generation of droplets; a step that could be extremely variable, as reported by Miotto et al. [11, 48].

Absolute dPCR data tabulated in Tables 1 and 2, and presented graphically in Figure 1 below, which show 14 preferentially expressed mature miRNAs associated with colon cancer (12 Up-Regulated, miR-19a, miR-20a, miR-21, miR-31, miR-34a, miR-96, miR-106a, miR-133a, miR-135b, miR-206, miR-224 and miR-302; and 2 Down-Regulated, miR-143 and miR-145) in stool samples from healthy controls, and stages 0-1 to 4 individuals with colon cancer.

Standard deviations (sd) obtained from the one way ANOVA, using the 5 level factor Type (normal, stage01, stage2, stage3, stage4)

were calculated. The adjusted R-squared values representing the proportion of variation explained by Type are also reported. Type was statistically significant for every gene; all p-values were less than 0.000001 (no adjustments for multiple comparisons). These data are tabulated in Table 3, and shown graphically in Figure 1.

For each gene on the graph in Figure 1, the min and max have been shown, in order to make the presentation clearer. At top left is high expression Value of 9985, which is the maximum value for that gene, at the bottom one finds the value for the minimum. The colors range from dark blue (control) to orange (stage 4). The groups are also distinguished by line type: control (solid), stage 0-1 (long dash), stage 2 (dash), stage 3 (dot), stage 4 (dash nd dot). The figure is a parallel coordinate plot made in R, using the package MASS. For statistical analysis.

Innovation and clinical significance of the dPCR-miRNA diagnostic stool screening approach

Innovation lies in the collective use of many methods, such as: immunoparamagnetic beads to capture colonocytes from the harsh, noninvasive stool environment, whose extracted fragile total small RNA is stabilized shortly after stool excretion by commercial kits so it does not ever fragment, followed by standardized analytical quantitative miRNA dPCR-chip profiling in stool samples, which are neither labor intensive, nor require extensive sample preparation, to develop a panel of few stable miRNAs for absolute quantitative diagnostic screening of *early* sporadic colon cancer (stage 0-1), cheaper, with higher sensitivity and specificity than any other colon cancer screening test on the

Table 3. Representation of SDs and R^2 for miRNAs tested by absolute digital PCR.

Type	M-19a	miR-20a	miR-21	miR-31	miR-34a	miR-96	miR-106a
sd	92.239	111.1033	99.76355	146.641	209.0491	278.4756	301.8764
r2	99.4831	99.18486	99.34603	98.65141	97.63002	96.13899	96.19772
Type	miR-133a	miR-135b	miR-200c	miR-224	miR-30a	miR-143	miR-145
sd	300.0619	409.6717	449.8674	376.8437	424.9972	132.7633	110.8927
r2	96.85741	95.49454	96.70427	97.61795	97.95389	99.87075	99.91289

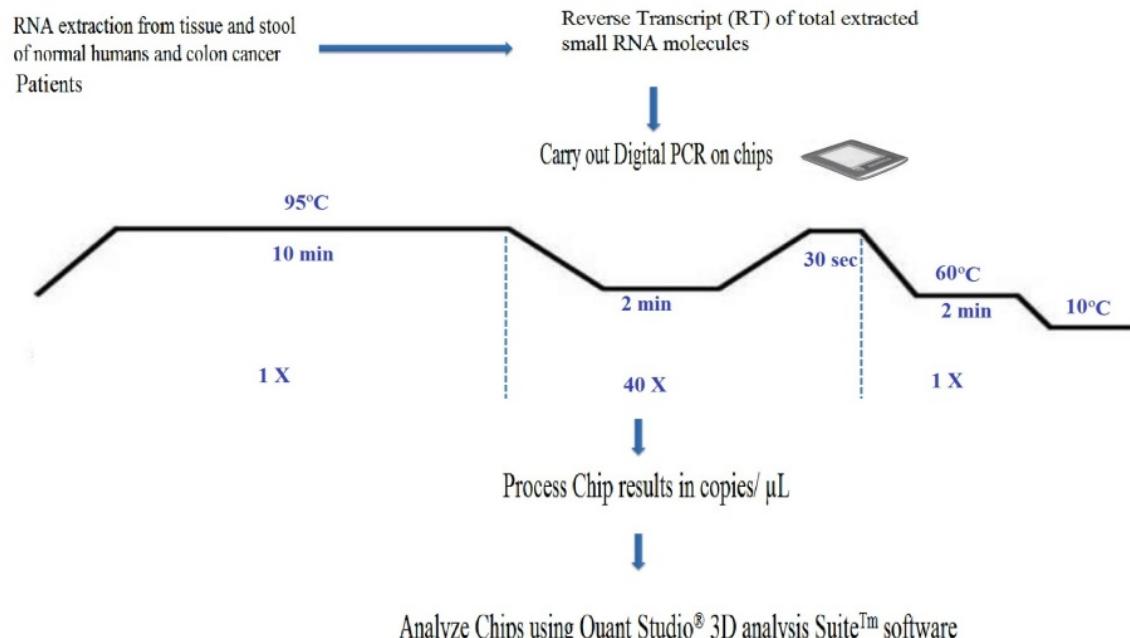


Figure 2. Workflow of a digital miRNAs PCR for colon cancer profiling in human colon tissue or stool samples

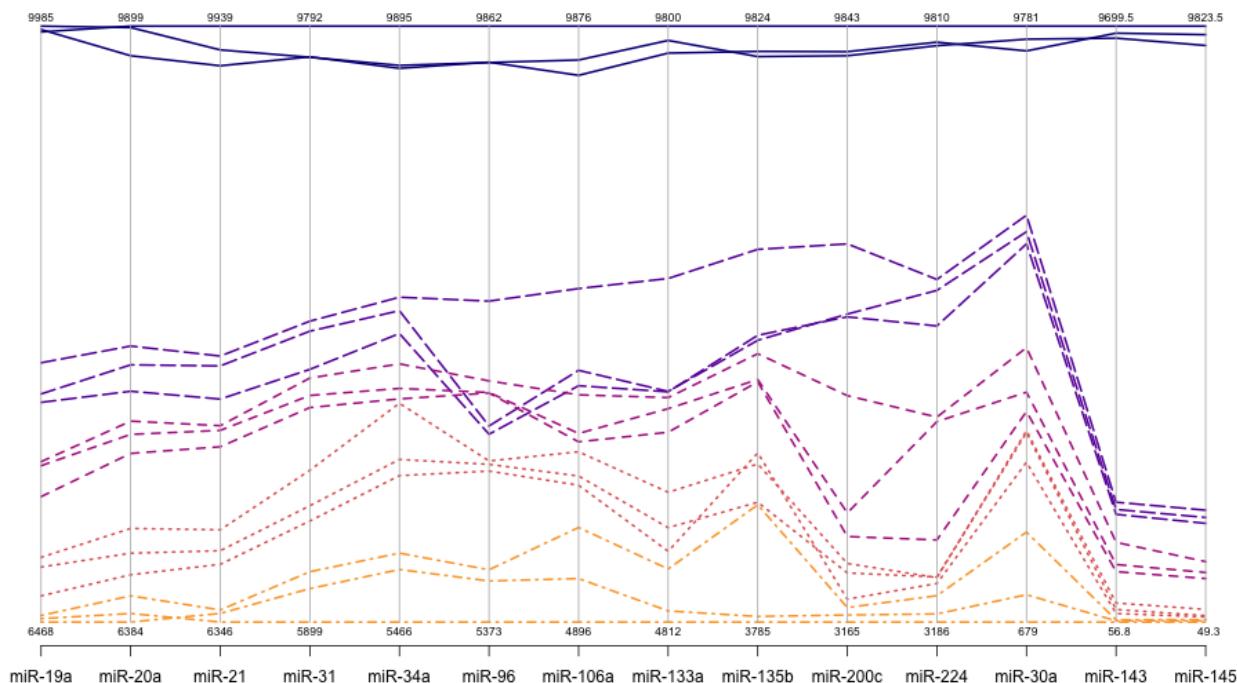


Figure 3. Absolute quantification of up- or down-regulated miRNAs in human stool by QuantStudio™ 3D digital PCR chip system

market. Isolation of colonocytes from stool samples is needed to provide a quantitative estimate of how our proposed miRNA method performs. Although we may miss exosomal RNA, a parallel test could also be carried out on miRNAs obtained from stool samples to compare the extent of loss when colonocytes are only used, and an appropriate corrections for exosomal loss can be made [51] (Figure 3).

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