World Journal of Clinical Pathology

Commentary
Published: 30 Jan, 2018

Use of Melt Curves for Analysis of Nutrigenomic MicroRNA Human RT-q PCR Data

Farid E. Ahmed and Nancy C. Ahmed*

GEM Tox Labs, Institute for Research in Biotechnology, 2905 South Memorial Drive, Greenville, NC 27834, USA

Commentary

Several gene expression profiling methods at the RNA level have emerged during past years, and have been successfully applied to cancer research profiling by micro arrays. Control of gene expression has been studied by miRNA molecules, a small non-coding RNA molecules (18–24 nt long), involved in transcriptional and post-transcriptional regulation of gene expression by inhibiting gene translation. MiRNAs silence gene expression through inhibiting mRNA translation to protein, or by enhancing the degradation of mRNA. The latest miRBase release (v20) contains 24,521 miRNA loci from 206 species, processed to produce 30,424 mature miRNA products, and each miRNA may control multiple genes, and one or more miRNAs regulate a large proportion of human protein-coding genes, whereas each single gene may be regulated by multiple miRNAs. MiRNAs inhibit gene expression through interaction with 3-untranslated regions (3 UTRs) of target mRNAs carrying complementary sequences.

Effect of antioxidant polyphenols-abundant in Mediterranean diets- on gene expression unraveled by the availability of molecular biology techniques, reveals our adaptation to environmental changes. Efforts to study the human transcriptome have collectively been applied to tissue, blood, and urine (i.e., normally sterile materials), as well as stool (a non-sterile medium). Extraction protocols that employ commercial reagents to obtain high-yield, reverse-transcribable (RT) RNA from human stool in studies performed on colon cancer have been reported.

MiRNAs have been used as biomarkers for assessing the effect of intake of PP-rich or fermented foods on the expression of 88 miRNA genes known to influence cancer.

Pomegranate juice (PGJ) and derived products are considered the richest sources of polyphenolic compounds, with positive implication plasma lipid profile, cholesterol and bile acid absorption and re-absorption. Colonic microbiota is a central site for the metabolism of dietary polyphenol (PP)-rich, functional fermented foods rich in lactobacilli (FS), or their combination, and colonization of probiotic bacteria. The interaction of PP with the gut microbiota influences the expression of some human genes.

Stool was obtained from the 25 healthy adults, twice at day 0 and three weeks after the dietary intervention using the RN easy isolation Kit®, Qiagen, Valencia, CA, USA, and total RNA was determined spectrophometrically by an Agilent 2100 Bio analyzer.

The RT2 miRNA First Strand Kit® from sabio sciences Corporation (Frederick, MD, USA) was employed for making a copy of ss-DNA.

We used a SABiosciences RT2 miRNA qPCR Array Plate System for Human (Qiagen) that employs a SYBR Green real-time PCR detection system to analyze miRNA expression using real-time.

We employed a Roche Light Cycler 480® 96-well block PCR Machine (Roche, Mannheim, Germany) to carry out quantitative real-time miRNA expressions.

Data were analyzed using the 2-ΔΔCt method. Resulting threshold cycle values for all wells were exported to a blank Excel sheet for analysis. We also ran a Dissociation (Melt) Curve Program after the cycling program, and generated a first derivative dissociation curve for each well in the plate, using the LC (Light cycler's®) software.

Gene expressions were standardized by dividing the SNORD48 value while raw melting temperatures were used. Analysis were done using the software R (version 3.1.3), with the package MASS. For standardized gene and each melting temperature, a one way ANOVA was used to

OPEN ACCESS

Correspondence:
Farid E. Ahmed, GEM Tox Labs, Institute for Research in Biotechnology, 2905 South Memorial Dr, Greenville, NC 27834, USA, Tel: 2523759656; E-mail: gemtoxconsultants@yahoo.com

Received Date: *07 Dec 2017*
Accepted Date: *23 Jan 2018*
Published Date: *30 Jan 2018*

Citation:
Ahmed FE, Ahmed NC. Use of Melt Curves for Analysis of Nutrigenomic MicroRNA Human RT-q PCR Data. World J Clin Pathol. 2018; 1(1): 1001.

obtain a p-value. There were four levels of the explanatory variable: Control, Sobya, Pom, and Both. Parallel coordinate plots (parcoord command in R) were used to visualize the data for each gene and each melting temperature. Coordinates were ordered using the magnitude of the p-value. The two sample t-test was used on gene expression to compare control to sobya and control to Both (t test command in R with var. equal=FALSE). P-values were adjusted to control for false discovery rate. We have bio informatically correlated the 2-7 or 2-8 complement nucleotide bases in the mature miRNAs with the un translated 3' region of target mRNA (3' UTR) of a message using a basic algorithm such as Broad's Institute's Target Scan.

Five miRNA genes (miR-184, miR-203, miR-124, miR-96 and miR-378) show clear separation. Gene miR-184 has the highest separation from the control gene. MiR-203 gene is hardly amplified in Sobya, while it is highly expressed in Pomegranate. For miR-373 gene, the control group is different from the other three treatment groups. For genes miR-124, miR-96 and miR-378, Pomegranate is well separated from other three groups.

Bioinformatics analysis using the Target Scan algorithm yielded 21 mRNA genes encoding different cell regulatory functions. The first 12 of these mRNAs were found with the DAVID program to be active in the nucleus and related to transcriptional control of gene regulation. For down regulated miRNAs, the DAVID algorithm found the first four of these mRNAs to be clustered in cell cycle regulation categories.

The existence of polysaccharides in stool could decrease the capacity to re suspend precipitated RNA, or disrupt the enzymatic reaction by mimicking the structure of nucleic acid. The DNA template of the PCR, as well as primers binding to DNA template can be inhibited by nucleases and other inhibitors. Remedial strategies for removal of inhibitors in stool, such as additional extraction steps, sephadex G-200 chromatography, heat treatment before the PCR, chloroform extraction, treatment with activated carbon, adding BSA, or dilution of sample, have been suggested. We found the dilution method, in which the extracted ribonucleic acid (RNA) is diluted in the reaction mixture with distilled water or an isotonic buffer, to be the most practical method for preventing PCR inhibition using a commercially available diluent.

MiRNA functions were shown to regulate development and apoptosis, and dysregulation of miRNAs has been associated with many diseases such as various cancers, heart diseases, kidney diseases, nervous system diseases, alcoholism, obesity, auditory diseases, eye diseases, skeletal growth defects, as well as key role in host–virus pathogenesis of viral diseases. A negative correlation was found between tissue specificity of interactions and miRNA in a number of diseases, and an association between miRNA conservation and disease, and predefined miRNA groups allow for identification of novel disease biomarkers at the miRNA level. Specific miRNAs are crucial in oncogenesis, effective in classifying solid and liquid tumors, and function as oncogenes or tumor suppressor genes. MiRNA genes are often located at fragile sites, as well as minimal regions of loss of heterozygosity, or amplification of common breakpoints regions, suggesting their involvement in carcinogenesis. MiRNAs have shown to serve as biomarkers for cancer diagnosis, prognosis and/or response to therapy. Profiles of miRNA expression differ between normal tissues and tumor types, and evidence suggests that miRNA expression profiles can cluster similar tumor types together more accurately than expression profiles of protein-coding messenger (m) RNA genes. Besides, small miRNAs (~18-22 nt long) are stable molecules than the fragile mRNA.

MCA is an assessment of dissociation characteristics of ds DNA during heating, leading to rise in absorbance, intensity and hyperchromicity. The temperature at which 50% of DNA is denatured is referred to as melting point, Tm.

Gathered information can be used to infer the presence of single nucleotide polymorphism (SNP), as well as clues to molecule's mode of interaction with DNA, such as intercalator slots in between base pairs through pi stacking and increasing salt concentration, leading to rise in melt temperature, whereas pH can affect DNA's stability, leading to lowering of its melting temperature. Originally, strand dissociation was measured using UV absorbency, but now techniques based on fluorescence measurements using DNA intercalating fluorophores such as SYBR Green I, Eva Green, or Fluorophore-labelled DNA probes (FRET probes) when they are bound to ds DNA are now common. Specialized thermal cyclers that run the qPCR, such as Roche Light Cycler (LC) 480® used, is programmed to produce the melt curve after the amplification cycles are completed. As the temperature increases, ds DNA denatures becoming ss and the dye dissociates, resulting in decrease in fluorescence. The graph of the negative first derivative of the melting-curve (-df/dt) represents the rate of change of fluorescence in the amplification reaction, and allows pin-pointing the temperature of dissociation (50% dissociation) using formed peaks to obviate or complement sequencing efforts.

The melting temperature (Tm) of each product is defined as the temperature at which the corresponding peak maximum occurs. The MCA confirms the specificity of the chosen primers, as well as reveals the presence of primer-dimers, which usually melt at lower temperatures than the desired product, because of their small size, and their presence severely reduce the amplification efficiency of the target gene as they compete for reaction components during amplification, and ultimately the accuracy of the data. The greatest effect is observed at the lowest concentrations of DNA, which ultimately compromises the dynamic range. Moreover, nonspecific amplifications may result in PCR products that melt at temperatures above or below that of the desired product. Optimizing reaction components ($Mg2^+$, detergents, SYBR Green I concentration) and annealing temperatures aid in decreasing nonspecific product formation. Adequate product design, however, is considered to be the best method to avoid nonspecific products' formation. Including a negative control will determine if there is a co-amplified genomic DNA [57,58]. The formula for Tm calculation is shown by the equation:

$$Tm = \frac{\sum \Delta H^\circ_{n\text{-}n}}{\Delta S^\circ} - 273.15$$

where, thermodynamic parameter ΔH° is Enthalpy changes, ΔS° parameter

$$\sum \Delta S^\circ_{n\text{-}n} + RL_n C_T$$

is Entropy changes, and CT is total strand concentration; these free-energy parameters predict Tm of most oligonucleotide duplexes to within 5°C; and permit prediction of DNA, as well as RNA duplex stabilities. It should be noted that Tm depends on the conditions of the experiment, such as oligonucleotide concentration, salts' concentration, mismatches and single nucleotide polymorphisms (SNPs). Oligo Analyzer® Tool [www.idtdna.com/analyzer/Applications/Oligoanalyzer] allows for calculating the Tm of employed nucleotides.

MCA has been an effective and economical way for identification of virus stains, genes, bacterial strains, insect species, temperature validation of PCR cyclers, detection of translocations in lymphomas and RNA interference/gene silencing. Thus, the presence of double peaks during MCA, is not always indicative of non-specific amplification, and other methods such as agarose gel electrophoresis, and use of melt curve prediction software are also needed in order to determine the purity of an amplicon. It should be emphasized that intercalating dyes used in qPCR, such as SYBR Green, will fluoresce only when the dye is bound to ds DNA, but not in the presence of a ssDNA, or when the DNA is free in solution. After the amplification cycle in qPCR, the instrument starts at a preset temperature above the primer Tm, and as the temperature increases dsDNA denatures becoming ssDNA and the dye therefore dissociates from the ssDNA. The change in slope of this curve when blotted as a function of temperature to obtain a melt curve. However, if we allow for the possibility that DNA my assume an intermediate state that is neither dsDNA nor ssDNA. This could happen when there are regions of the amplicon that are more stable (e.g. G/C rich), which do not melt immediately, but maintain their ds configuration until the temperature becomes sufficiently high to melt it, which results in two phases. Additional sequence factors, such as amplicon misalignment in A/T rich regions, and designs that have secondary structure in the amplicon region, can also produce products that melt in multiple phases.

We found the melt curve analysis to be a useful and an informative method because after the statistical analysis carried on our miRNA expression samples showed no preferential expression of any of the 88 miRNA genes, a melt curve analysis on the same samples found that we could distinguish 7 miRNA (miR-184, miR-203, miR-373, miR-124, miR-96, miR-373 and miR-301a), due to different separation melting profiles. Thus, we believe that it is imperatives for investigators to run this kind of analysis on samples that particularly may not show expression differences in their mRNA or miRNA studied genes, such as nutritional samples.

Bioinformatic methods showed 21 up regulated mRNA genes encoding different cell regulatory functions, and 12 of these mRNAs were found to be active in the nucleus and related to transcriptional control of gene regulation. For down-regulated miRNAs, four of the mRNAs appeared to be clustered in cell cycle regulation categories.

The clinical significance of using melting temperatures for analyzing nutrient-gene data is a promising new approach for identifying key regulatory miRNA genes related to metabolites rich in polyphenols, probiotic lactobacilli, or combinations of the two metabolites. Melt curve analysis is a powerful novel approach because after the statistical analysis carried on our miRNA samples produced negative gene expression (Cq) results, running melt curve analysis on the same samples identified 7 of the 88 miRNA genes imprinted on the highly sensitive focused PCR arrays (~8% of the genes), and using parallel coordinates plots showed noticeable separation of melt curve profiles. Thus, we believe that it is imperatives for investigators to run this kind of MCA on nutrition samples that are mild in nature, and many not always show significant differences in the expression of studied miRNA genes. The same analysis can also be envisioned for messenger mRNA amplifications, using mRNA arrays, and then using bioinformatics resources to correlate mRNA with miRNA data.

Melt curve analysis (MCA) in essential in interpretation of mild nutrogenomic miRNA, which initially appear to show no significant expressions expression data as markers for measuring the magnitude of the expression of key miRNA molecules in stool of healthy human adults following the intake of Pomegranate juice (PGJ), functional fermented sobya (FS), rich in potential probiotic lactobacilli, or their combination. before and Following a three week dietary intervention trial, the expression of 88 miRNA genes was evaluated using Qiagen's 96 well plate RT2 miRNA qPCR arrays, of total small RNA isolated from stool of 25 normal healthy volunteers, and employing parallel coordinates plots. Although there was no observed significant separation for the gene expression (Cq) values, using Roche 480® PCR Light Cycler instrument, and none of the miRNAs showed significant statistical expression after controlling for the false discovery rate, melting temperature profiles produced during PCR amplification run-on the other hand-found seven significant genes (miR-184, miR-203, miR-373, miR-124, miR-96, miR-373 and miR-301a), which separated candidate miRNAs that could function as novel molecular markers of relevance to oxidative stress and immunoglobulin function, for the intake of PP-rich, functional fermented foods rich in lactobacilli, or their combination. Therefore, melt curves are essential for analyzing nutigenomic miRNA expression data, which initially appear to show no significant expressions, are essential for a comprehensive understanding of the role of MCA data.