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Exploring zinc transporter gene expression in human saliva

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Introduction

Saliva is a complex diagnostic material which comprises of biomolecules from multiple origins such as blood, salivary glands, buccal mucosal cells and microbiota from the oral cavity. There is a growing interest in utilising non---invasive samples, such as saliva, in human nutritional studies. Transcriptomic analysis of cell---free saliva has initially been developed for detection of oral cancers and other systemic diseases [1]. Hence RNA profiling of saliva has been mainly focussed on cell---free fraction of saliva where the transcriptome measured is representative of secreted gene transcripts originating from the salivary gland or distant sources [2].

Gene expression analysis of whole saliva has not been well documented in the literature as compared to the cell---free fraction of saliva. However, with new collection methods such as Oragene RNA collection kits, it has become more feasible to access saliva samples. The Oragene RNA collection kit allows the collection of unstimulated human saliva into the Oragene RNA solution which releases cellular RNA and inactivates endogenous ribonucleases present in saliva. The inclusion of cellular phase of saliva may also assist in ensuring high yield of human RNA and better reflect nutritional status of the individual.

To our knowledge, analysis of salivary gene expression in relation to nutritional status in humans has not been investigated before. This report describes attempted optimisation in measuring gene expression of previously validated zinc---related transcripts in whole saliva and cell---free saliva.

Methods

<u>Whole saliva collection and RNA isolation</u>

Saliva samples were collected according to the instructions of Oragene RNA kits. After collection, the samples and Oragene RNA solution were mixed vigorously within the collection vessel and incubated in their original vial at 50°C for 1 hour. Samples were processed immediately or stored at ---20°C until further analysis.

RNA was isolated as per Oragene RNA purification protocol using Qiagen RNeasy Micro kit. In brief, 500uL aliquots of the saliva samples were incubated at 90°C for 15 minutes. 1/25th volume of Oragene Neutralizer solution was added and mixed by vortex before a 10 minute incubation on ice. Samples were then centrifuged for 3 minutes at maximum speed and the clear supernatant is isolated and to which 2 volumes of 95% ethanol were added and mixed thoroughly. The samples were then incubated at ---20°C for at least 30 minutes. The pellet from samples were then retrieved and processed as per instructions for purification of total RNA from animal and human cells in RNeasy Micro Handbook.

Cell---free saliva and RNA isolation

5mL of unstimulated saliva samples were also collected in 50mL falcon tubes and centrifuged at 2600xg for 15 minutes at 4°C. Supernatant, which represented cell---free saliva fraction, was processed to RNA extraction by TRI reagent. ~3mL of saliva supernatant was mixed vigorously with 3 volumes of TRI reagent (9mL). After incubation at room temperature for 10 minutes, 1.6mL of chloroform was added and mixed by vortex. The clear supernatant was retrieved and an standard ethanol precipitation was performed with the addition of 2 volumes of 100% ethanol, sodium acetate (0.03M final concentration) and glycogen before an overnight incubation at ---20°C. The RNA pellet was isolated the next day and washed twice with 70% ethanol before resolubilisation with RNase---free water.

RNA yield and quality check

Yield and quality of RNA was assessed by UV nanospectrophotometer, reading over the range of 220nm to 350nm. A well defined peak absorbance at 260nm, in addition to A260:A280 and A260:A230 ratios being greater than 1.9 were considered acceptable RNA quality. Integrity of RNA samples were checked against previously extracted RNA from MEL cells with denaturing agarose gel electrophoresis, stained with ethidium bromide.

cDNA synthesis and real---time quantitative PCR

Total RNA (100ng---500ng) were reverse transcribed into cDNA using the Superscript VILO cDNA synthesis kit (random hexamer and oligo---dT primed) or the First Strand cDNA synthesis kit (oligo---dT primed), following the instructions of the manufacturers. Primers for genes encoding zinc---related transcripts (Zip1, Zip7, Zip10, MT---2A) and housekeeping genes (GAPDH, β---actin, 18S) were measured and validated previously in PBMCs. Real time quantitative---PCR was done with Taqman Gene Expression Master Mix and associated primers. 16S transcripts were detected by SybrGreen system. Amplifications and relative quantification over a range of cDNA input were performed using the StepOne real---time PCR system. CTs below 37 were considered to be within the detectable range.

Figure 1 summarises the protocols described.

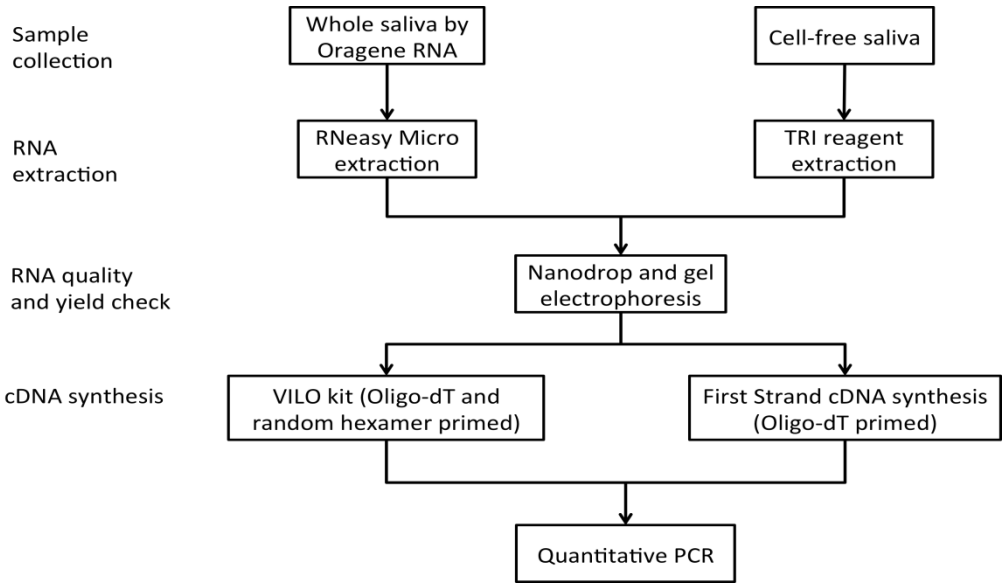

Figure 1 Summary of protocols for gene expression analysis of whole saliva and cell---free saliva

Results

RNA yield and quality

RNA obtained from the Oragene collection method were of high quality and yield, with an average of 7.5ug of RNA per saliva sample. RNA as analysed on spectrometer showed the sample was of good quality, with both 260/280 and 260/230 ratios being greater than 2.0. A typical spectrometer reading is depicted in **Figure 2**.

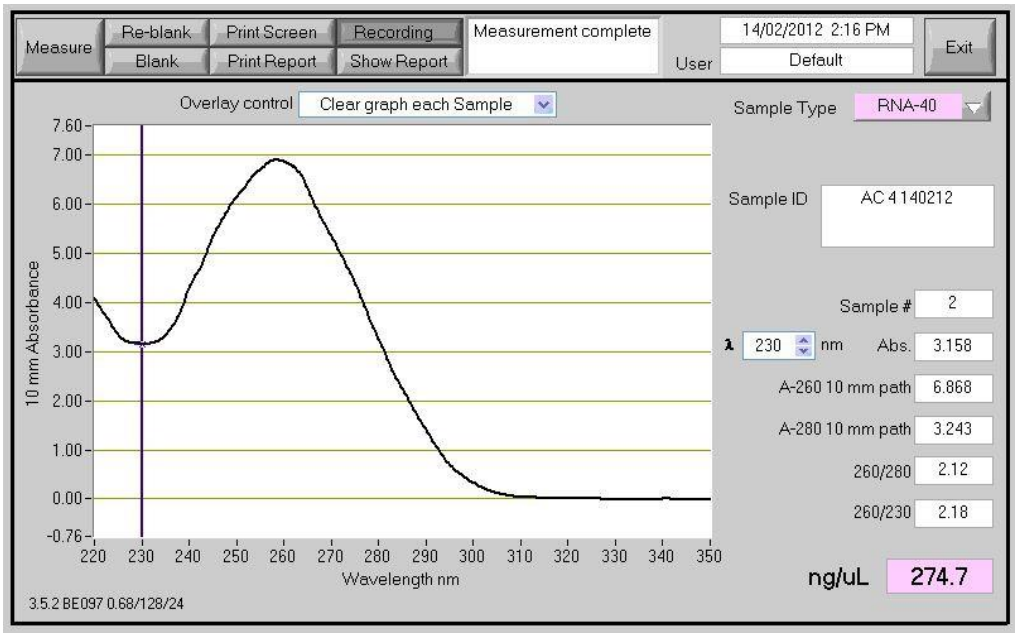

Figure 2 Typical spectrometer reading of RNA isolated from Oragene collection method

RNA extraction from the cell---free phase of saliva was challenging. Initial results indicate that there were isolation of RNA from saliva supernatant did not yield significant RNA. Further optimisation using RNeasy Micro kit RNA extraction method instead of TRI reagent did not improve RNA quality or yield. A typical spectrometer reading (**Figure 3**) shows an absence of a 260nm peak which is indicative of poor quality RNA extracted from the sample.

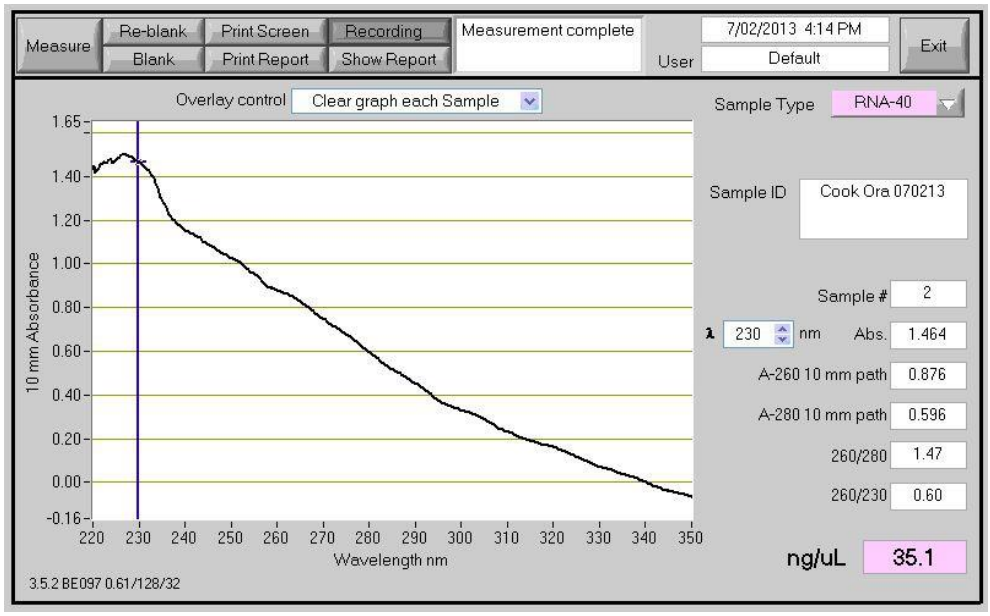

Figure 3 Typical spectrometer reading for RNA extracted from cell---free saliva

Gel electrophoresis of RNA samples were conducted to assess for possible RNA degradation in the extraction process. **Figure 4** shows the visualisation of the ribosomal RNA bands of whole saliva samples, control RNA and a commercial RNA ladder. Distinct downward shifts of the two bands are observed in the saliva RNA samples when compared to control RNA. This suggests presence of contaminants such as salts or other co---extracted substance in the RNA extraction process.

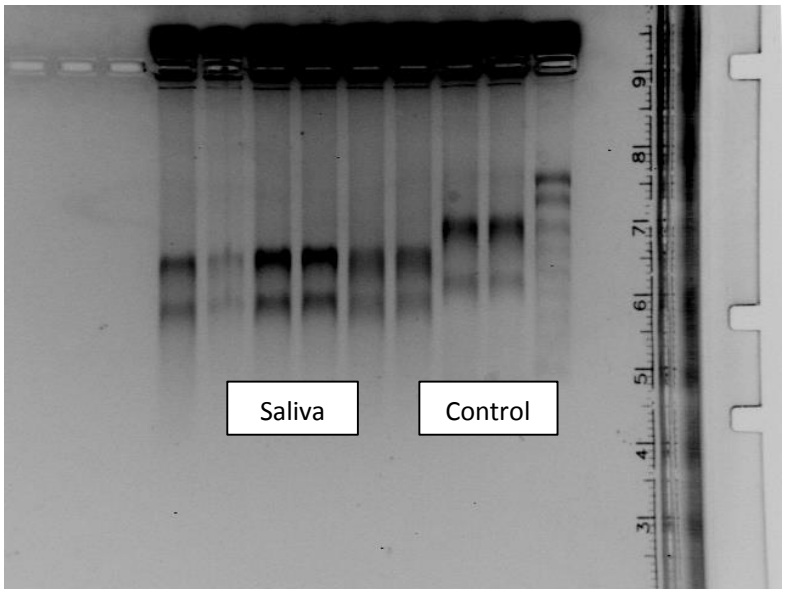

Figure 4 Visualisation of denaturing agarose gel with saliva RNA samples visualised. Lanes 1---6: Whole saliva RNA, Lanes 6---8 control RNA, Lane 9: RNA ladder

Quantitative PCR

Initial results in quantifying zinc related targets showed low abundance of targeted transcripts in whole saliva RNA sample. Average CTs for 18S, Zip1 and MT---2A with 10ng cDNA input were 18.09, 31.03 and 31.32 respectively. Standard curves for Zip1 (**Figure 5**) and MT---2A (**Figure 6**) showed PCR efficiencies of 120% and 140% which are above the recommended range of 90%---110%. The 18S standard curve (**Figure 7**) did not show a linear relationship between cDNA input and transcript abundance as measured by mean CT. This could be indicative of the presence of an inhibitor in the quantitative PCR process. Quantitative PCR with saliva supernatant for the 18S transcripts also showed similar result.

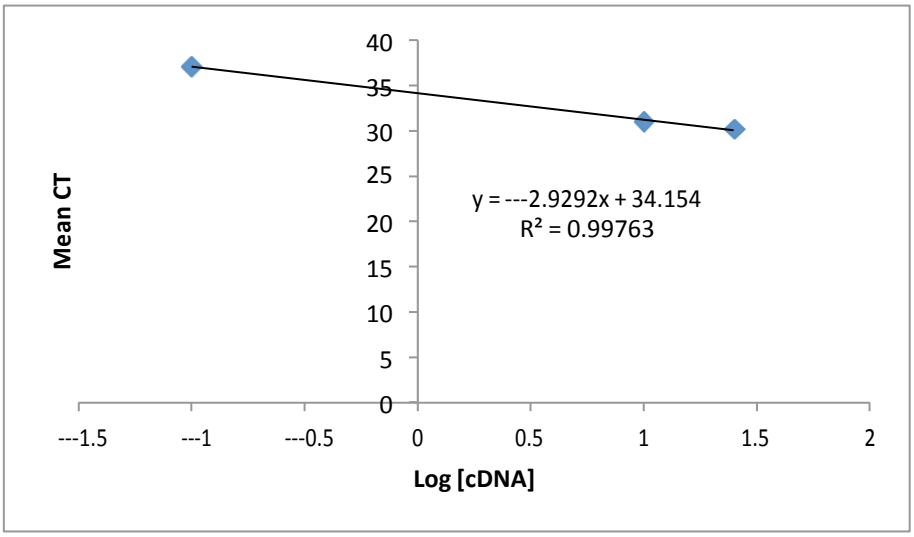

Figure 5 Standard curve for Zip1 with cDNA input of 0.1ng -- 100ng

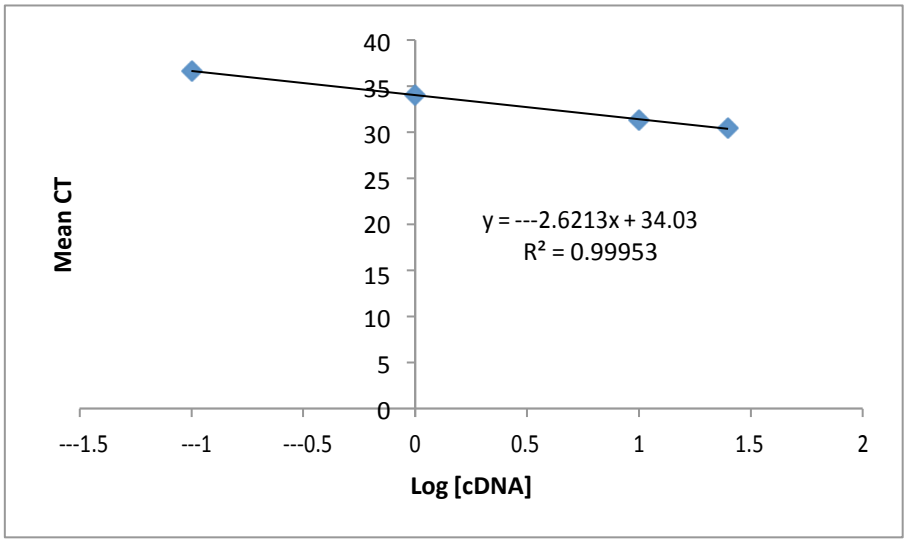

Figure 6 Standard curve for MT---2A with cDNA input of 0.1ng---100ng

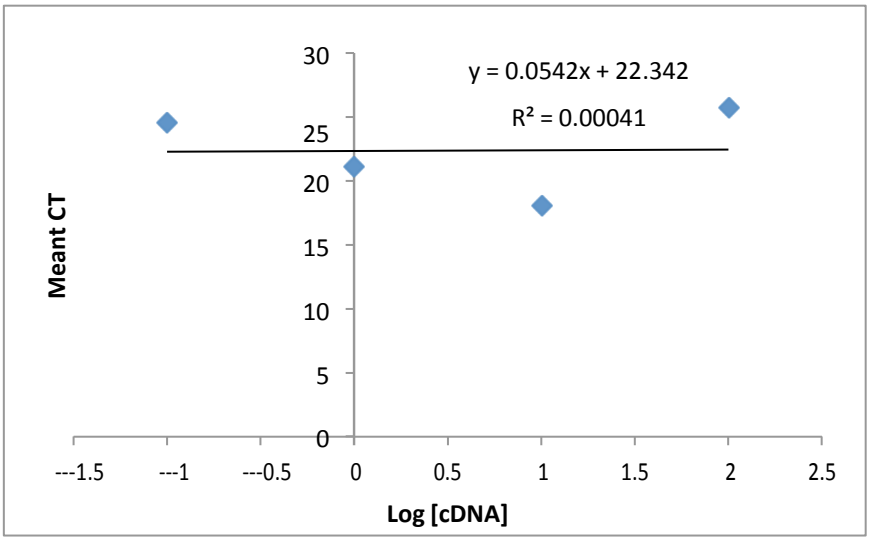

Figure 7 Standard curve for 18S with cDNA input of 0.1ng---100ng

In addition to the standard curve analysis, spiking of saliva RNA with previously extracted RNA from human peripheral blood mononuclear cells (PBMCs) were performed to confirm the presence of PCR inhibitors. Ratios of 50:50, 90:10 and 10:90 cDNA solutions made from PBMC RNA and whole saliva RNA and were analysed by quantitative PCR. CT values were correlated with PBMC cDNA input (**Figure 8**), but not with total cDNA or saliva cDNA. This suggests limited PCR inhibition reaction and saliva cDNA were not contributing significantly to the detectable target transcripts.

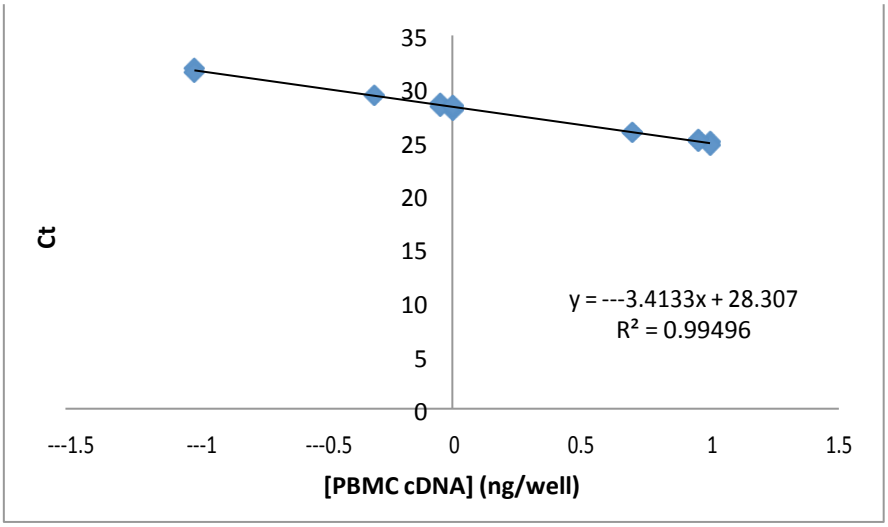

Figure 8 Standard curve for Zip10 with cDNA made from PBMC and whole saliva

Further analysis of saliva RNA with quantitative PCR targeting 16S (bacterial ribosomal RNA) showed substantial amount of bacterial RNA detected. Using the SybrGreen quantitative PCR system, 10ng of saliva cDNA input elicited a mean CT value of 17.48. When compared to the CT values of 18S, this suggests almost 50% of bacterial RNA was co---extracted with saliva RNA within samples, which was detected by spectrometer as RNA isolated. Hence, adjustments are required to ensure correct input of eukaryotic RNA into cDNA synthesis to provide appropriate detection in quantitative PCR.

To avoid amplification of bacterial RNA, we used an alternative cDNA synthesis kit which uses oligo---dT primers and hence only synthesis cDNA from mRNA with poly---A tails. PCR efficiencies of GAPDH (**Figure 9**) and β---actin (**Figure 10**) were poor at 68% and 58% respectively. Standard curve for the zinc transporter Zip7 showed good PCR efficiency at 94% (**Figure 11**).

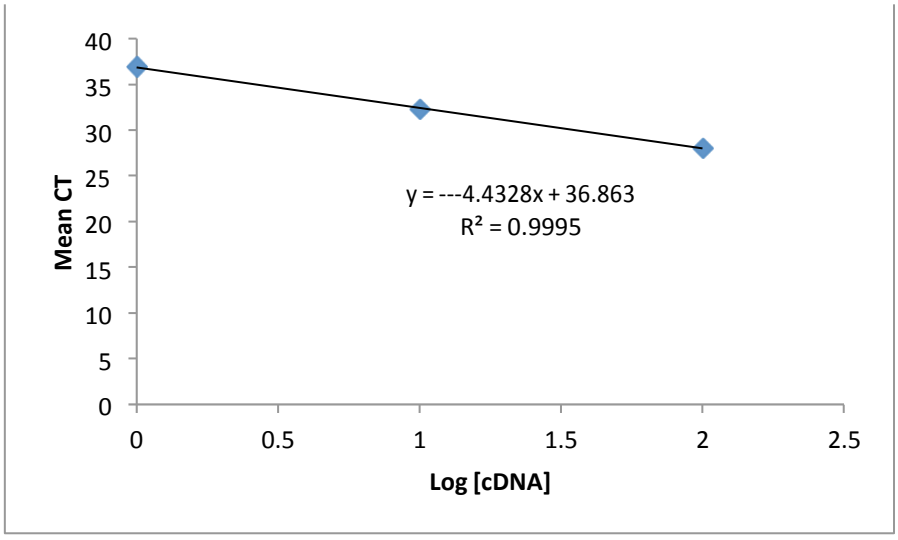

Figure 9 Standard curve for GAPDH with saliva cDNA primed from Oligo---dT (1ng---100ng)

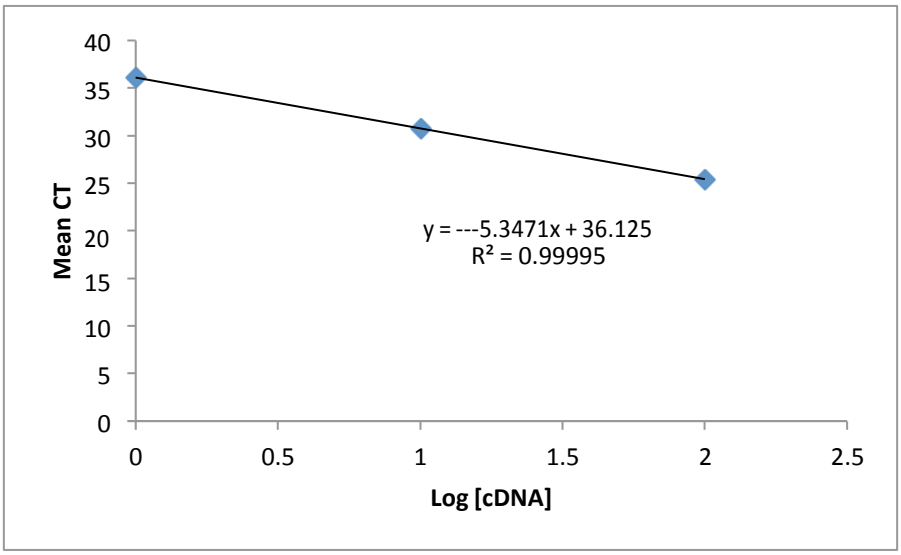

Figure 10 Standard curve for B---actin with saliva cDNA primed fom Oligo---dT (1ng---100ng)

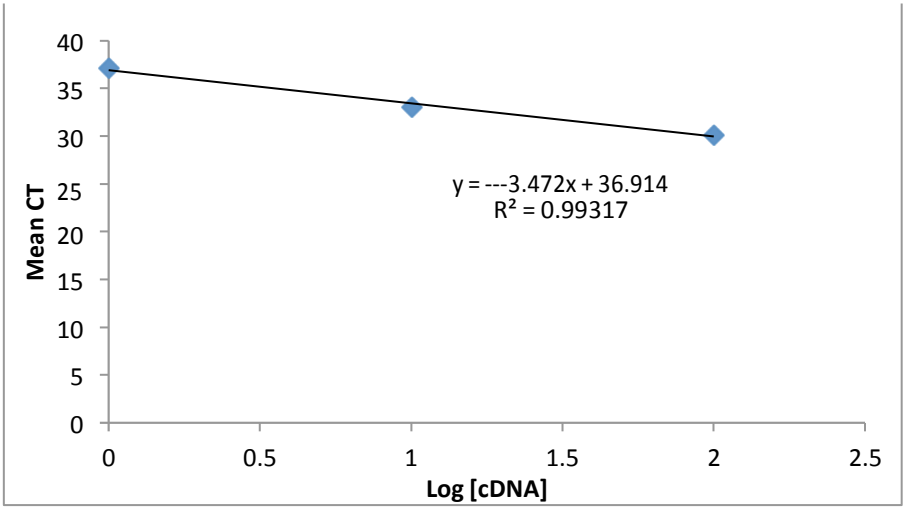

Figure 11 Standard curve for Zip7 with saliva cDNA primed from Oligo---dT (1ng---100ng)

A number of optimisation attempts, such as reducing RNA load into cDNA synthesis or using different RNA extraction method, failed to solve the issues in validating analysis of gene expression in saliva.

Discussion

Results presented in this report showed our failed attempts in validating gene expression analysis in saliva. Despite multiple papers have used RNA extracted from whole saliva and cell---free saliva to

describe the saliva transcriptome in diagnostic applications, validity of measured results remains uncertain [3].

Bacterial contamination

As saliva contain RNA originating from a number of sources, including buccal epithelial cells, oral microbes, it is important to be able to distinguish between RNA transcripts from human or bacterial origin. Although no data is available for the amount of non---human RNAs in whole saliva, an estimate of 32% of salivary DNA is predicted to be from the oral microbiota [4]. Higher RNA yield from saliva has been associated with higher abundance of 16S rRNA in the sample [5]. A downward shift of ribosomal RNA bands in the salivary RNA when compared to the control and detection of 16S by quantitative PCR confirms the presence of significant bacterial RNA co---extracted from saliva. Hence to ensure detection of targeted RNA transcripts from human origin, we used a sensitive quantitative PCR kit, Taqman Gene Expression Assay and primers targeted at human transcripts. In addition, we elected to use a cDNA synthesis kit which uses oligo---dT primers to ensure only mRNA from human origin (those with poly---A tails) will be synthesised into cDNA.

Endogenous ribonucleases

The presence of endogenous ribonucleases in saliva affects the length and stability of salivary RNA. Although there are mechanisms identified which contribute to stabilisation of salivary RNA, it has been reported that the average salivary mRNA retains only 42% of its original length. Therefore it was important for salivary ribonucleases to be inhibited before processing the samples. By using a commercial salivary collection kit with RNase inhibition activity, this minimises further degradation of salivary RNA after sample collection. The presence of partially degraded mRNA in saliva may also sequester available primers which would inhibit the detection of target genes within the sample, thereby affecting PCR efficiency.

PCR inefficiency

In previously study in gene expression of PBMCs, primers for zinc---related transcripts were validated using standard curve method in quantitative PCR. Efficiencies of references genes (18S) and target genes were within the manufacturer's recommended range of 90---110%. This validation was imperative in relative quantification using the comparative CT method (ΔΔCT) to ensure accurate gene expression analysis when normalising to the reference genes. Despite multiple attempts at optimising the quantitative PCR protocol and RNA extraction, we failed to achieve the recommended PCR efficiencies and hence could not truthfully quantify gene expression of targets.

Multiple explanations for failure in optimisation could be valid. Saliva, being a crude biopsy sample with RNA sourced from multiple origins, poses as problems in isolated detection of the targeted transcripts. Although we have attempted to selectively amplify and quantify zinc---related targets, the mere presence of bacterial contamination within the saliva sample has been suggested to be an inhibiting factor in downstream applications (cDNA synthesis and/or quantitation of targets). In an extensive validation of salivary RNA in microarray studies, Kumar et al [3] concluded signal arising from RT---PCR may be non---specific and originating from genomic DNA. The authors' also commented on the poor validation methods in previous gene expression studies in saliva and cautioned against using saliva for RNA sampling within proper validation. A recent paper [6] has illustrated an in---house method for RNA extraction in saliva and subsequent validation of reference gene expression in the sample by melting curves, however validation from standard curves were not performed.

Conclusion

Although studies have reported successful quantification of target mRNA transcripts by saliva samples, we have been unable to replicate these results with appropriate validation. The difficulties in saliva samples, such as the presence of endogenous endonucleases and bacterial RNA, made the accurate detection of the target transcripts difficult. Further optimisation and appropriate validation

would need to be performed before the use of saliva sample as a biopsy sample for gene expression assays.

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Exploring novel biomarkers of zinc status in humans with non---invasive sampling methods:

Analysis of zinc transporter gene expression in buccal mucosa

Introduction

Zinc is involved in numerous biochemical processes in the body and is essential for activities of some 300 metalloenzymes. Without a well---defined body store of zinc, accurate assessment of zinc status has been difficult to achieve in humans. Serum zinc, the most commonly reported biomarker of zinc status, represents less than 1% of total metabolic pool of zinc. Consequently, serum zinc provides limited information regarding the zinc status of an individual.

Discoveries of cellular zinc transporters in mammalian cells have led to research of their association with zinc status in humans. Two families of zinc transporters have been described to regulate the concentration of zinc in the cytosol; the ZnT transporters are responsible for export of zinc from the cell while Zip transporters promote cellular zinc import. Expression of cellular zinc transporters have been successfully measured in a number of human tissues such as peripheral blood mononuclear cells (PBMCs) and adipocytes [1, 2].

In recent years, advancement of molecular biology techniques and the attractiveness of non---invasive sampling methods have sparked interest in measuring zinc transporter expressions in novel cell types such as buccal epithelial cells. Two studies had previously reported quantitative measures of zinc transporters mRNA expression in buccal cells. Michalczyk et al [3] used buccal cells collected from mouthwashes after vigorous brushing of the oral mucosa. Reverse transcription PCR with human ZnT4 primers was carried out from isolated RNA and the products were visualised on agarose gel electrophoresis. In a dietary zinc depletion---repletion study, Ryu et al [4] measured 14 zinc---related transcripts in buccal RNA, of which only metallothioein and ZnT1 were detected by Taqman

quantitative PCR. In this study, buccal cell samples were applied on FTA cards which trap nucleic acids and minimise activities of ribonucleases.

This report describes our attempts to measure zinc transporter gene expressions under validated experimental conditions, using previously reported buccal cell collection methods.

Methods

FTA card buccal cell collection (Ryu)

Buccal cells were collected via Whatman Easicollect kit. Duplicate samples were taken from the subject within 15 minutes. In brief, a foam applicator was used to swab the oral mucosa on the inside of each cheek for 15 seconds. Then the Easicollect device folded to allow contact between the foam head and FTA card for 10 seconds. The FTA card was then allowed to dry for 2---3 hours under the fume hood. The samples were then processed for RNA extraction.

Half of a FTA card circle (cut into 5---6 small strips) was placed in 750uL of TRI reagent and incubated at room temperature for 15 minutes. Samples were agitated every 5 minutes during the incubation period. After centrifugation at 16000xg for 30 minutes at 4°C, the lysate was separated with 200uL of chloroform added and mixed by vigorous vortex for 2 minutes. Samples were centrifuged for 10 minutes at 16000xg and supernatant was transferred for ethanol precipitation. 1uL of glycogen, NaOAc (300mM final concentration) and 2 volume of cold 100% ethanol were added before overnight incubation at ---20°C. Samples were then centrifuged at 16000xg for 10 minutes at 4°C and supernatant was removed. A second treatment of 750uL TRI reagent was applied to the pellet and the phenol---chloroform extraction/ethanol precipitation step were repeated. After second overnight incubation, the RNA pellet was solubilised in 20uL of RNase---free water. 4 reaction runs (total 80uL of RNA solution) were collated and analysed for RNA yield and quality.

Mouthwash buccal cell collection (Michalczyk)

Subjects rinsed their mouth with 20mL Listerine solution for 10 seconds prior to buccal cell collection. The inside of the donor's oral cavity was brushed using a sterile toothbrush for 30 seconds on each cheek. Cells were recovered by rinsing the mouth and toothbrush with 15mL and 10mL of deionized water respectively.

Buccal cell samples were centrifuged at 3000xg for 15 minutes at 4°C. Supernatant was removed and pellet was resuspended in 2mL phosphate---buffered saline (PBS). The samples were further centrifuged at 3000xg for 15 minutes and supernatant was removed. After two PBS washes, the cell pellet was solubilised in 250uL of TE buffer [10mM Tris---HCl, pH8.0, 10mM EDTA], containing 200mM NaOH and 1% sodium dodecyl sulphate and incubated at room temperature for 5 minutes. 250uL of 3M potassium acetate, pH 5.5 was added to the samples and incubated on ice for 5 minutes. Samples were centrifuged at 16000xg for 10 minutes. Supernatant was collected and processed according to RNA extraction protocol for animal cells as outlined in the RNeasy Mini Kit.

RNA yield and quality check

Yield and quality of RNA was assessed by UV nanospectrophotometer, reading over the range of 220nm to 350nm. A well defined peak absorbance at 260nm in addition to A260:A280 and A260:A230 ratios being greater than 1.9 were considered acceptable RNA quality. Integrity of RNA samples were checked against previously extracted RNA from MEL cells on a denaturing agarose gel electrophoresis, stained with ethidium bromide.

cDNA synthesis and real---time quantitative PCR

Total RNA (500ng---2000ng) were reverse transcribed into cDNA using the Superscript VILO cDNA synthesis kit, following the instructions of the manufacturers. Primers for genes encoding zinc---related transcripts (ZnT1, Zip1, Zip3, Zip7, Zip10, MT---1A, MT---2A) and housekeeping genes (GAPDH,

B---actin, 18S) were measured and validated previously in PBMCs. Real time quantitative---PCR was done with Taqman Gene Expression Master Mix and associated primers. Amplifications and relative quantification of a range of cDNA input were performed with the StepOne real---time PCR system. CTs below 37 were considered to be within the detectable range.

Figure 1 summarises the workflow of the two methods.

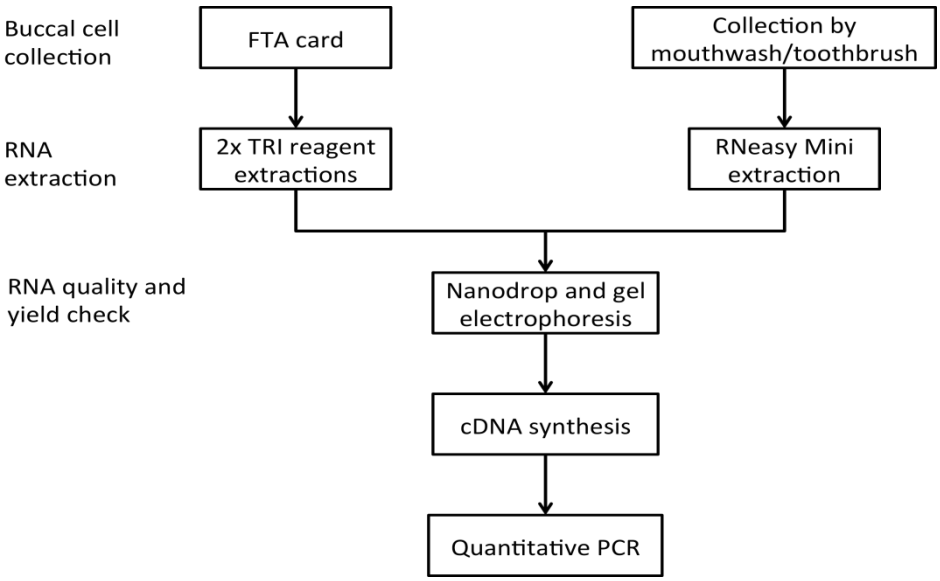

Figure 1 Summary of methods used in determination of zinc---related mRNA transcripts in buccal RNA

Results

RNA yield and quality

The mouthwash buccal cell extraction method with RNeasy Mini extraction kit provided high quality and high yield RNA. Up to 44ug of RNA per sample were extracted from this method with excellent A260/A280 and A260/A230 ratios (both >2). A typical spectrophotometer reading is shown in **Figure 2**.

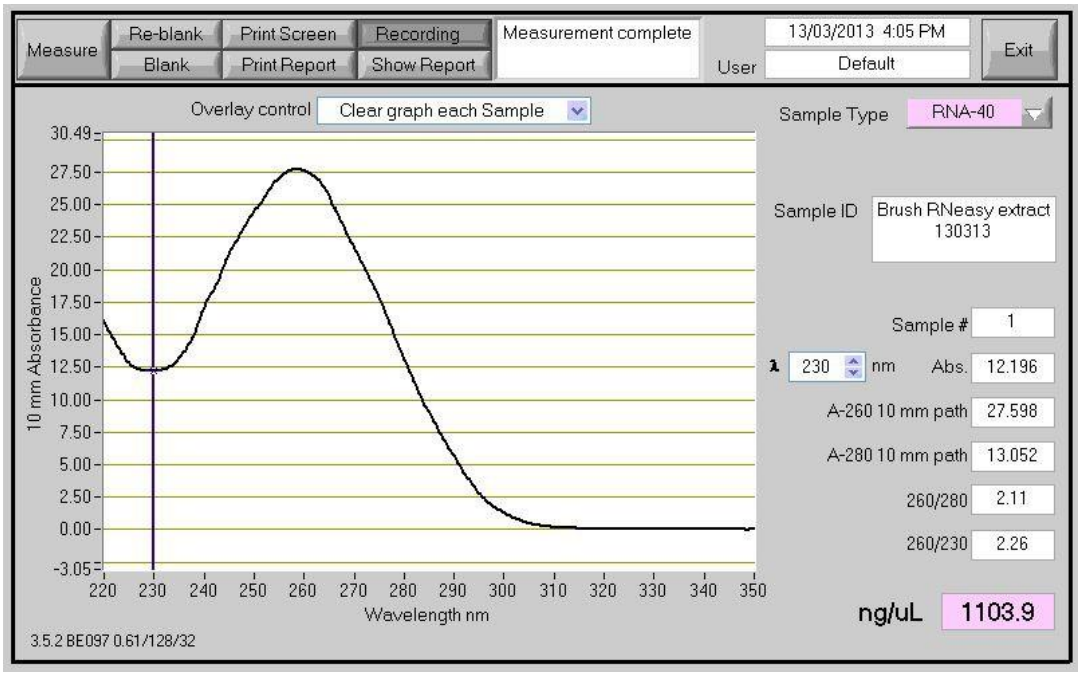

Figure 2 Typical spectrophotometer reading for RNA extracted by mouthwash buccal cell collection

In contrast, there were difficulties in obtaining RNA of significant yield and acceptable quality from elution of FTA cards as described in Ryu's method. Typical yield from one buccal cell sample collection was 1284---1784ng RNA of poor purity as indicated by low 260/230 ratios (0.08---0.19) and marginal 260/280 ratios (1.49---1.51). A typical reading of RNA extracted from FTA cards is shown in **Figure 3**. Peak absorbance at 230nm and 270nm were commonly observed.

Multiple attempts to improve the yield and quality of buccal RNA obtained from FTA card were conducted. However none were particularly successfully in achieving better results. In brief, although an extra overnight ethanol precipitation after extraction described in the method did improve A260/A230 ratio from 0.09 to 0.68 and A260/A280ratio from 1.49 to 1.65, the resultant yield was reduce to 132ng per buccal swab sample. Similar results were also seen when the second TRI reagent---chloroform extraction step was replaced with a RNA clean up protocol from the RNeasy Mini Kit.

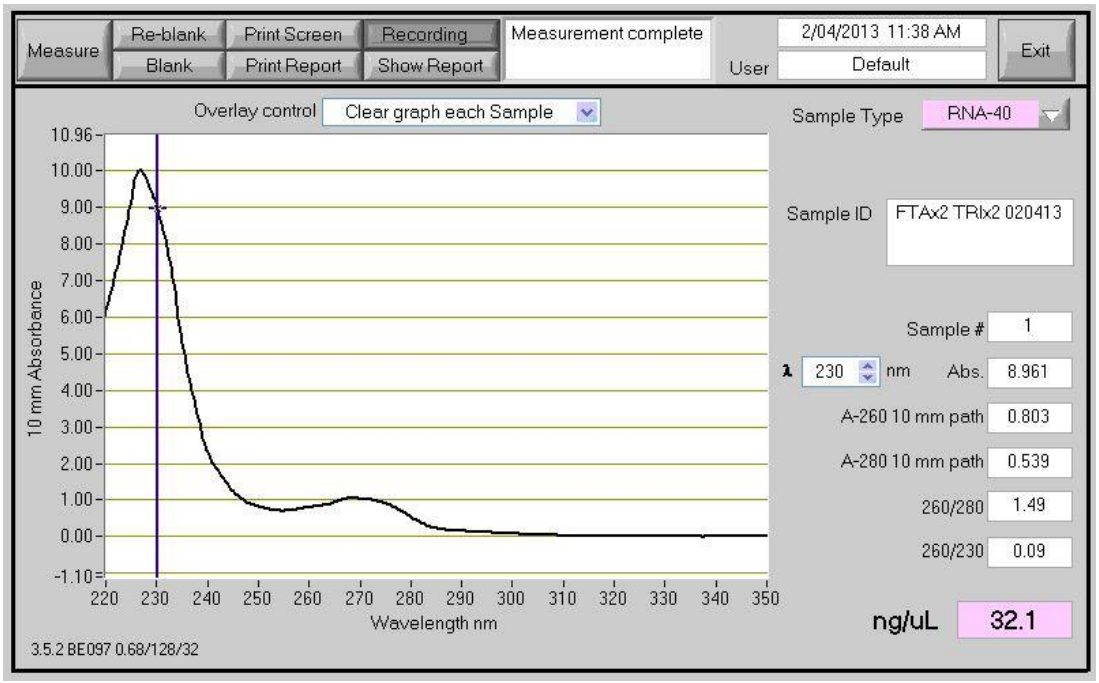

Figure 3 Typical spectrophotometer reading for RNA extracted from FTA card

Denaturing agarose gel electrophoresis was used to determine the integrity of RNA samples. Due to insufficient RNA yield from the FTA card collection method, only RNA samples from the mouthwash collection could be visualised after ethidium bromide staining. **Figure 4** shows the visualisation of control (MEL cell RNA) and buccal RNA extracted. A downward shift of the 2 ribosomal RNA bands was seen in the buccal RNA extracted from mouthwash method when compared to the MEL cell RNA, which could be indicat8ive of the presence of contaminants.

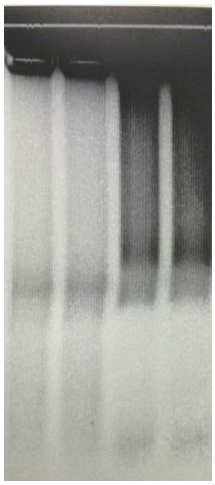

Figure 4 Denaturing agarose gel. From left to right: lanes 1---2 buccal RNA extracted from mouthwash method, lanes 3---4 control (RNA from MEL cells)

Quantitative PCR

RNA obtained from both collection methods were measured using two---step quantitative PCR. Amplification efficiency of each assay was determined using the standard curve method over the range of 0.1---100ng cDNA made from buccal RNA isolated from mouthwash. Zinc transporter transcripts (ZnT1, Zip1, Zip3 and MT---2A) were undetectable at lower range of standard curves (0.1---1ng) and therefore efficiencies for these PCR amplifications could not be determined. B---actin standard curve provided acceptable amplification efficiency at 101% over the range of 1---100ng cDNA (**Figure 5**). The amplification efficiencies for GAPDH (**Figure 6**) and 18S (**Figure 7**) were outside of the acceptable range for quantitative PCR detection (139% and 78% respectively). To test the repeatability of assay, a different buccal cell sample collected was assayed and achieved an amplification efficiency of 111% for the 18S target, showing high variability in measurement.

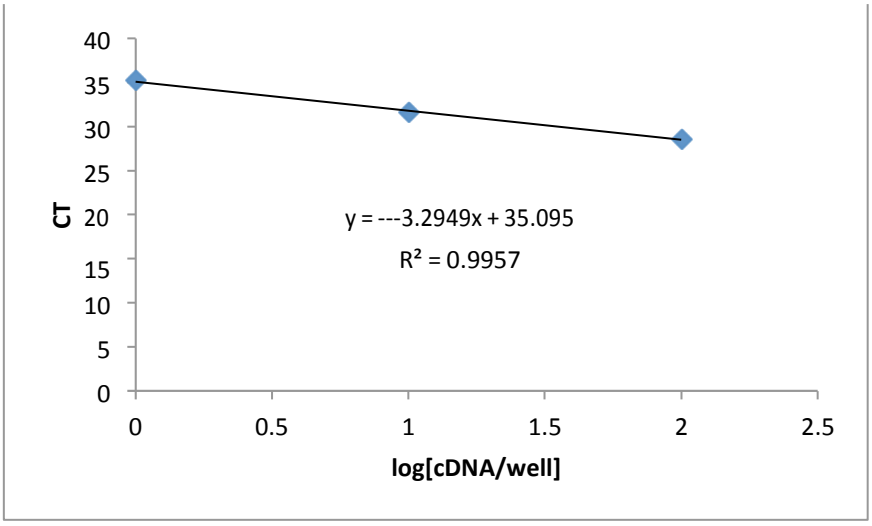

Figure 5 Standard curve for B---actin in buccal RNA isolated from mouthwash (1---100ng cDNA/well)

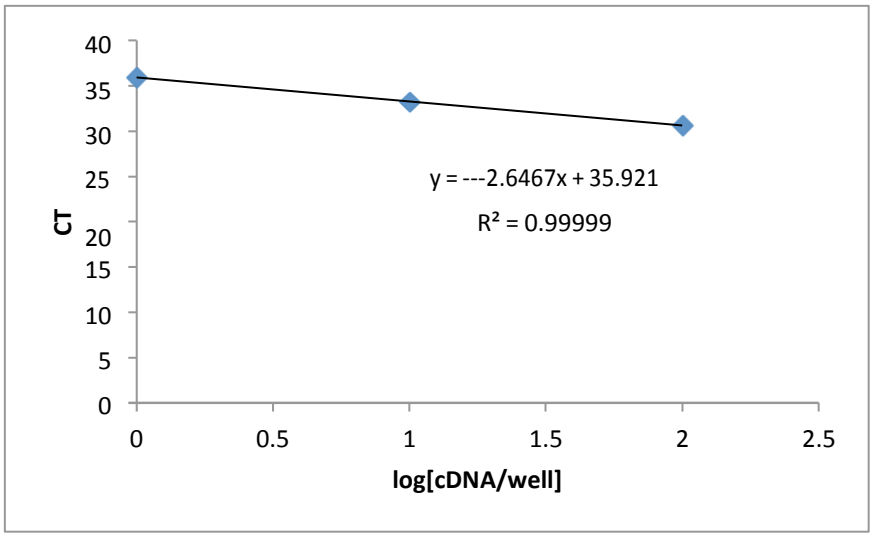

Figure 6 Standard curve for GAPDH in buccal RNA collected from mouthwash (1---100ng cDNA/well)

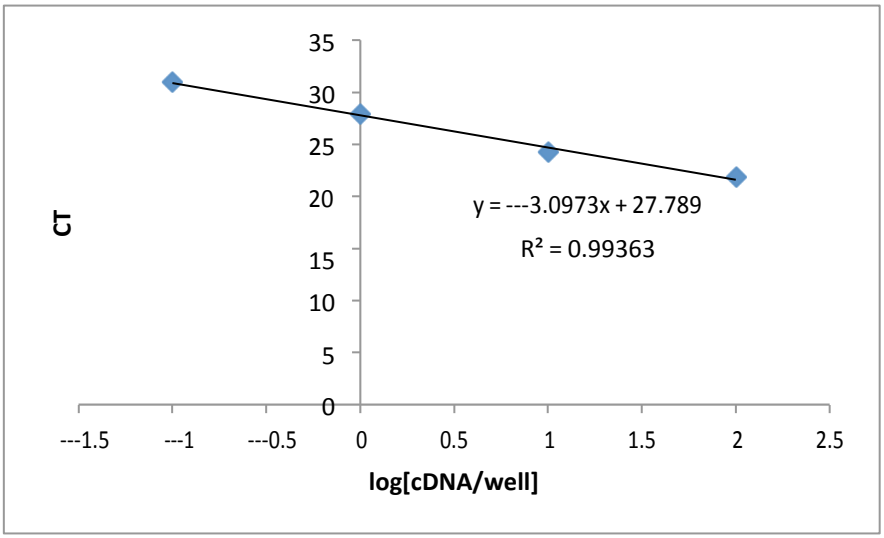

Figure 7 Standard curve for 18S for buccal RNA collected from mouthwash (0.1---100ng cDNA/well)

FTA card isolated RNA was of insufficient yield and hence only the standard cDNA input (10ng/well) was assayed in quantitative PCR. All 6 zinc related transcripts tested (ZnT1, Zip1, Zip7, Zip10, MT---1A and MT---2A) were able to be detected below CT of 37. **Table 1** shows the average CT values for each target tested.

Table 1 Average CTs of tested targets in buccal RNA isolated from FTA cards

Targets	Average CT
ZnT1	34.62
Zip1	35.84
Zip7	36.75
Zip10	36.83
MT-1A	32.88
MT-2A	28.89
GAPDH	32.89
B-actin	31.99
18S	20.61

Discussion

This report describes our attempts in measuring gene expression of zinc---related transcripts from buccal mucosa cells in humans. Although there has been studies reporting the use of buccal cells as a source of RNA, we experienced a number of difficulties in obtaining good quality and yield RNA from similar collection methods. The entrapment of buccal cell nucleic acid on the FTA card provides protection from endogenous and foreign ribonucleases, however pure RNA of high yield were difficulty to be isolated. The presence of peak absorbances at 230nm and 270nm were commonly observed and could be attributed to contamination of FTA paper itself. Using similar method, Ryu reported a similar RNA yield of 989 ± 492 ng per oral swab after 2 TRI reagent---chloroform extractions, however RNA quality was not reported in this paper. Additional processing steps such as reprecipitation or TRI reagent---chloroform extraction did improve RNA purity, however total RNA yield suffered. After reprecipitation, only around 20% of initial RNA remained which made downstream applications difficult. The use of TRI reagent---chloroform extractions and overnight ethanol precipitations also made this method of buccal RNA extraction more time---consuming than other extraction methods.

Buccal cell sampling from mouthwash has been described in a number of nutritional studies [5, 6] due to the ease of collection. RNA obtained from buccal mucosa was first described as extractions from scrapings with the serrated edge of the micropipette tip [7]. Spira et al reported isolation of

300---1500ng RNA from each subject and some evidence of partial RNA degradation when observed on denaturing agarose gel. However, the ribosomal RNA bands extracted from buccal cell scrapings were in line with RNA extracted from epithelial cell lines. This was different from the distinctive shifts in ribosomal RNA observed in our buccal cell samples when compared to control. A number of explanations for ribosomal RNA shifts could be valid including bacterial contamination and differences in ion concentrations in solution. Although a Listerine wash prior to sampling was included in the protocol, the possibility of bacterial presence could not be completely eliminated. In a similar analysis of salivary RNA [8], it was noted that higher RNA concentration extracted from salivary samples were correlated with high abundance of bacterial 16S ribosomal RNA as determined by quantitative PCR. The probability of bacterial RNA contamination also adds uncertainty to the accuracy of buccal RNA yield as measured by spectrophotometer.

In a recent study of gene expression in buccal mocosa of subjects with asthma [9], analysis of RNA quality showed samples were of average quality as determined by ribosomal RNA integrity. However, the authors subsequently performed GAPDH 3':5' assays to determine the adequacy of successful reverse transcription across the GAPDH gene. Acceptable GAPDH 3':5' ratio of less than 5 was observed and hence the quality of RNA isolated from buccal mucosa were considered acceptable for RT---PCR applications.

Another obstacle to RNA extraction in buccal cell samples may be the low cell viability when compared to other cell types such as PBMCs. While PBMC samples reported to have around 92% of viable cells [10], buccal cells collected from mouthwash have a much lower cell viability of 7.2 ± 1.6% [3]. As the majority of buccal cells within a mouthwash sample are not viable, it is highly probable that minimal amount of RNA may be present in the dying cells due to the process of cellular apoptosis and senescence.

When using comparative CT method (ΔΔCT) to compare differences in gene expression normalised to an internal control, it is imperative for the target gene and internal control to have similar amplification efficiencies. This will ensure the amount of primed RNA transcript to be the limiting factor in the assay. As described in our results, we failed to obtain reliable amplification efficiencies of internal controls between the target range of 90---110% as recommended by the manufacturers. We were unable to attain amplification efficiencies for our target genes due to their low abundance in the extracted buccal RNA. When abundances of target genes and internal controls in buccal cells were compared to previously validated cell types, there appears to be lower abundances of transcripts when expressed as amount of cDNA input in quantitative PCR. For example, a typical CT reading of 9---11 was found in 18S transcripts isolated from PBMCs, which when compared to the 18S CT of 20 in buccal cell samples, suggests a 10^3 difference in 18S abundance between the two cell types. The apparently low abundance of target genes from buccal cell samples and poor amplification efficiencies in quantitative PCR could be explained by the presence of bacterial contamination as a possible inhibitor of downstream applications (reverse transcription or quantification of target transcripts).

High CT values at the lower range of cDNA input isolated from buccal cells for the zinc---related transcripts pose a problem in obtaining amplification efficiencies of the PCR. Given RNA yield and quality are problematic, another technique which may be useful is additional pre---amplification step prior to quantification of target cDNA transcripts [9]. This will increase the amount of target cDNA transcripts and hence could decrease CT values of target genes to the detectable range.

Conclusion

Although studies have reported successful quantification of target mRNA transcripts by normalisation to internal controls in quantitative PCR, we have been unable to replicate these results. Low abundance of the targeted zinc---related transcripts and poor amplification efficiencies

hinder the feasibility of accurate assessment of gene expression. This may be due to the presence of bacterial contamination which acted as an inhibitor to downstream applications and/or genuine low RNA expression in the buccal sample of largely apoptosed cells. Further optimisation steps such as additional pre---amplification prior to quantitative PCR may be required to utilise buccal cell as a RNA sampling source for zinc transporter transcripts.

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