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Comparison of quantitative real-time polymerase chain reaction with NanoString® methodology using adipose and liver tissues from rats fed seaweed

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Experimental methods are constantly being improved by new technology. Recently a new technology, NanoString®, has been introduced to the market for the analysis of gene expression. Our experiments used adipose and liver samples collected from a rat feeding trial to explore gene expression changes resulting from a diet of 7.5% seaweed. Both quantitative real-time polymerase chain reaction (qPCR) and NanoString methods were employed to look at expression of genes related to fat and glucose metabolism and this paper compares results from both methods. We conclude that NanoString offers a valuable alternative to qPCR and our data suggest that results are more accurate because of the reduced sample handling and direct quantification of gene copy number without the need for enzymatic amplification. However, we have highlighted a potential challenge for both methods, which needs to be addressed when designing primers or probes. We suggest a literature search for known splice variants of a particular gene to be completed so that primers or probes can be designed that do not span exons which may be affected by alternative gene sequences.

Introduction

Q3 Quantitative real-time polymerase chain reaction (qPCR) involves the amplification of DNA which is detected in real time as the reaction progresses. Fluorescent dyes are used to detect the DNA products. These can be non-specific fluorescent dyes that bind to any double-stranded DNA, or DNA probes labelled with a fluorescent signal that binds a specific sequence of DNA. The latter is a more expensive method, but ensures that only the DNA of interest (i.e. a target gene) is detected. To quantify a gene, a reference gene that is stably produced by the cell or tissue of interest is required. This produces a relative expression level that can be used to compare treatments within an experiment. Despite qPCR being a common laboratory technique for quantifying gene expression, its accuracy can be affected by non-uniform enzymatic reactions, pipetting errors and reference gene instability, affecting sensitivity, reproducibility and specificity [1]. Recently, a new method known as NanoString®, has become readily available for quantifying gene expression [2]. This method uses a multiplexed chip-based technique, where barcoded probes specific to the gene(s) of interest bind to samples and individual copies are detected digitally using an nCounter® Analysis System [3]. NanoString does not require amplification, and up to 800 genes can be detected in a single sample, which reduces sample-handling errors and removes enzymatic reaction errors, although reference gene stability can be a challenge as with qPCR, because of differences in reference gene expression in different tissues [4]. However, the ease of multiplexing genes in a single sample means that it is easier to analyse more reference genes for normalization of results, so that genes which are not stable can be excluded from the analysis. Therefore, NanoString methodologies should theoretically produce more accurate results.

Our experiments used adipose and liver samples collected from a rat feeding trial to explore gene expression changes resulting from a diet of seaweed. Seaweed has been linked to multiple health

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benefits, such as anti-obesity and anti-diabetic properties [5,6]. Both qPCR and NanoString methods were employed to look at expression of genes related to fat and glucose metabolism, and this article compares results from both methods.

Materials and methods

Rat diets

The animal feeding study was approved by the AgResearch Grasslands Animal Ethics Committee, New Zealand (Application 13189). Rats were raised from weaning on standard rat pellets to 150 ± 20 g (3 weeks post-weaning) and placed individually in hanging cages for the 4-week feeding trial. The experimental diets were fed ad libitum and comprised 7.5% freeze-dried seaweed (*Undaria pinnatifida*) or a control diet matched for equivalent starch content (g), with nine rats per treatment (full diet composition is shown in Table 1). At 28 days, rats were euthanized by CO_2 overdose and blood withdrawn with cardiac puncture. Epididymal fat pads were removed, along with the liver and snap frozen at −80°C for 5 months.

RNA extraction from liver and adipose tissue

Snap frozen liver and adipose tissue (30–50 mg), from rats fed the control and seaweed diets, were placed into 500 μL Trizol® Reagent (Ambion®, Life Technologies, New Zealand). The samples were homogenized at full speed for 20 s with an OMNI THQ homogenizer (OMNI International™, USA) and incubated for 5 min at room temperature. $CHCl_3$ (150 μL) was added to the homogenates and then vortexed for 15 s, followed by an incubation for 3 min at room temperature. The samples were then centrifuged at 17,000 × *g* for 5 min and the aqueous phase was transferred to a new tube. An equal volume of 70% reagent grade ethanol was added to the aqueous phase, and then centrifuged at 8000 × *g* for 1 min to pellet fat. The supernatant with no fat was transferred to a Qiagen RNeasy MINI column (Qiagen, Germany) and the manufacturer's protocol was followed to extract RNA into 15 μL of water for gene expression of 12 genes (Table 2) by the nCounter® Analysis System (NanoString Technologies®, USA). A second column wash was also completed using 45 μL of water for qPCR of 12 genes (Table 3). RNA quality and quantity were assessed by NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Inc., USA) and Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies, New Zealand). Samples analysed by the nCounter Analysis System were also analysed by Agilent 2100 Bioanalyzer (Agilent Technologies, USA) to assess RNA integrity.

TABLE 1

Rat diet composition (g/kg) fed for 28 days to compare the effects of a seaweed (*Undria*) diet with a control diet

Food	Seaweed diet (g/kg)	Control diet (g/kg)
Lactic casein	100	100
Corn oil	50	50
Anhydrous milk fat	150	150
Vitamin mix	100	100
Mineral mix	100	100
Basal fibre mix	50	50
Starch	350	450
Seaweed	100	0
Total	1000	1000

Gene expression analysis – NanoString nCounter Analysis System

Total RNA (minimum concentration 20 nM) was shipped on dry ice to Otago Genomics & Bioinformatics Facility (Otago, New Zealand) for gene expression analysis using a custom CodeSet (Table 2) along with a nCounter Analysis System. One hundred ng of RNA sample was analysed in sets of 12. Samples were spiked with six different positive internal control probes at concentrations ranging from 128 fM to 0.125 fM in fourfold dilution steps. Positive controls were used to determine the linearity of the assays and were used for normalization. In addition, eight negative probes were used to control for carryover of reporter probes, since no RNA target was included for these probes. RNA samples were incubated for 20 hours at 65°C in hybridization buffer containing the CodeSet, which consisted of reporter and capture probes and together with the target RNA formed a tripartite complex. Each reporter probe had a 5′-colour code signal which was unique for each target RNA analysed within the CodeSet. After hybridization, the complex was bound by its biotin-labelled capture probe on a streptavidin-coated glass slide and was stretched within an electric field. Hybridized samples were processed using the robotic Prep Station (High Sensitivity Protocol, 3 hours per 12-sample cartridge). Data acquisition was performed by using the GEN2 Digital Analyzer, with the 'Max' Field of View setting (555 images per sample; 5-hour scan per cartridge). Raw counts were normalized using the positive controls, and target genes were normalized to the internal reference genes: beta-2 microglobulin (B2M), peptidylprolyl isomerase A (CYCA), hypoxanthine phosphoribosyltransferase 1 (HPRT1), TATA box binding protein (TBP) and ribosomal protein S29 (WAT) in liver samples, whilst B2M was excluded from adipose tissue normalization since it did not correlate with other reference genes (Table 4).

Gene expression analysis – qPCR

Total RNA (100 ng) was reverse transcribed using Transcriptor First Strand cDNA Synthesis kit (Roche, Auckland, New Zealand) according to the manufacturer's instructions for oligo-dT primed reactions. Briefly, 2.5 μM oligo-dT primers were added to each RNA sample and heated at 65°C for 10 min on a thermal block cycler and cooled on ice whilst adding RNase inhibitor (20 U), DNTPs (1 mM) and transcriptor RT (10 U) to each reaction. Samples were then incubated at 55°C for 30 min, followed 85°C for 5 min and then rapid cooling to 4°C. The cDNA was stored at −20°C. Primers and primer-probe combinations (Table 3) were designed online using the Universal probe library system assay design centre (Roche Applied Science). After primer design, all primers were run through the National Center for Biotechnology Information (NCBI) Blast database to check for specificity. Dual-hybridization probes from the Universal Probe Library (Roche Diagnostics, Mannheim, Germany) were paired with unmodified, desalted primers (Invitrogen, Life Technologies, New Zealand). A manually set up, 96-well format plate (Roche Diagnostics, Mannheim, Germany; catalogue number 04729692001), reverse-transcription qPCR assay was performed using the LightCycler® 480 system (Roche Diagnostics, Mannheim, Germany), with three reactions (technical replicates) for each sample. Each reaction contained 5 μL of

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TABLE 2

Gene target sequences and probe combinations used for NanoString® experiments to test rat liver and adipose samples following a seaweed or control diet

Gene name	GenBank accession number	Target region	Target sequence
Adiponectin C1Q (ADIPOQ)	NM_144744.3	741–840	GGGACAACAATGGACTCTATGCAGATAATGTCAATGACTCTACATTTACAGGCTTCCTTCTCTACCATGATACCAACTGACTGCCACTAATTCAGAGCAG
Adiponectin receptor 1 (AR1)	NM_207587.1	1689–1788	TTACCCTCCTCCTTACTTTGTAACCTGGCGGATGACAGGCCATCCATTTTTGTAGCACACTTTTCAGAAACAAGTATAACCTGGTCCCATCTTTCTAGGA
Adiponectin receptor 2 (AR2)	NM_001037979.1	271–370	CTATGGAACGAATGGAAGAGTTTGTTTGTAAGGTGTGGGAAGGTCGATGGCGAGTGATCCCTCATGATGTGCTGCCAGATTGGCTTAAGGACAATGACTT
Beta-2 microglobulin (B2M)	NM_012512.1	286–385	CGAGACCGATGTATATGCTTGCAGAGTTAAACACGTCACTCTGAAGGAGCCCAAAACCGTCACCTGGGACCGAGACATGTAATCAAGCTCTATGGAGCTC
Carnitine palmitoyltransferase 1a (CPT1A)	L07736.1	2246–2345	TTGCCGATGACGGCTATGGTGTCTCCTACATTATAGTGGGAGAGAATTTCATCCACTTCCATATTTCTTCCAAGTTCTCTAGCCCTGAGACAGACTCACA
Peptidylprolyl isomerase A (CYCA)	M19533.1	61–160	TTCTTCGACATCACGGCTGATGGCGAGCCCTTGGGTCGCGTCTGCTTCGAGCTGTTTGCAGACAAAGTTCCAAAGACAGCAGAAAACTTTCGTGCTCTGA
Fatty acid binding protein 1 (FABP1)	NM_012556.1	111–210	GCCTGAGGACCTCATCCAGAAAGGGAAGGACATCAAGGGGGTGTCAGAAATCGTGCATGAAGGGAAGAAAGTCAAACTCACCATCACCTATGGGTCCAAG
Fatty acid binding protein 4 (FABP4)	NM_053365.1	25–124	TTCGGCACGAGTCCTTGAAAGCTTACAAAATGTGCGACGCCTTTGTGGGGACCTGGAAACTCGTCTCCAGTGAGAACTTCGATGATTACATGAAAGAAGT
3-Hydroxy-3-methylglutaryl-CoA reductase (HMGCR)	NM_013134.2	1571–1670	TCTGCAGTACCTGCCTTACAGAGACTATAATTACTCCTTGGTGATGGGAGCTTGCTGTGAGAACGTGATCGGATATATGCCCATCCCTGTTGGAGTGGCA
Hypoxanthine phosphoribosyltransferase 1 (HPRT1)	NM_012583.2	21–120	AGCTTCCTCCTCAGACCGCTTTTCCCGCGAGCCGACCGGTTCTGTCATGTCGACCCTCAGTCCCAGCGTCGTGATTAGTGATGATGAACCAGGTTATGAC
Leptin	NM_013076.3	2265–2364	ATACCTTTCAGGGCACATTAGCATCCACAGCTATCGTAGCACACTGTTGACACAGGATAACTGGGATCATAAAAATAAGAAAATACAGGTTGACTGTCCC
Peroxisome proliferator-activated receptor gamma (PPARγ)	NM_013124.1	1291–1390	TTTATAGCTGTCATTATTCTCAGTGGAGACCGCCCAGGCTTGCTGAACGTGAAGCCCATCGAGGACATCCAAGACAACCTGCTGCAGGCCCTGGAACTCC
Stearoyl AoA desaturade-1 (SCD-1)	AF509569.1	1436–1535	CTGTTTTGATGCTAGGGCAAGCATGGTGCTCTTTCCCTGTTTGCACTGCATCCAGCTTCGTTCCTTCCTCTGTCATCACCATCTTCAGGCAAATAGCTGA
Sterol regulatory element binding transcription factor 1 (SREBP1)	XM_213329.6	2869–2968	GTGAGAAGGCCAGTGGGTACCTGCGGGACAGCTTAGCCTCTACATCAACTGCCAGTTCCATTGACAAGGCCATGCAGCTGCTCCTGTGTGATCTACTTCT
TATA box binding protein (TBP)	NM_001004198.1	151–250	TGTCCCCACATCACTGTTTCATGGTGCGTGACGATAACCCAGAAAGTCGAAGACGTTTCTAAGGAGATAAGAGGATGCTCTAGGAAAAATCTGAGTACCG
Uncoupling protein 1 (UCP-1)	NM_012682.2	415–514	GCCTGCTGGCATCCAGAGGCAAATCAGCTTTGCTTCCCTCAGGATTGGCCTCTACGATACGGTCCAAGAGTACTTCTCTTCAGGGAGAGAAACGCCTGCC
Ribosomal protein S29 (WAT)	X59051.1	46–145	CCAGCAGCTCTACTGGAGTCACCCGCGGAAGTTCGGCCAGGGTTCTCGCTCTTGCCGCGTCTGCTCTAACCGCCACGGTCTGATCCGTAAATACGGGCTG

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TABLE 3

Primer and probe combinations of genes used in qPCR experiments to test rat liver and adipose samples following a seaweed or control diet

Gene name	GenBank accession number	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Amplicon length	Rat probe number
ADIPOQ	NM_144744.3	TGGTCACAATGGGATACCG	CCCTTAGGACCAAGAACACCT	93	80
AR1	NM_207587.1	AGCACCGGCAGACAAGAG	GGTGGGTACAACACCACTCA	62	95
AR2	NM_001037979.1	ATGTTTGCCACCCCTCAGT	GATTCCACTCAGACCCAAGC	69	15
CPT1A	L07736	GACCGTGAGGAACTCAAACC	GGATCCGGGAAGTATTGAAGA	90	26
FABP1	NM_012556.1	CTTCTCCGGCAAGTACCAAG	TTCCCTTTCTGGATGAGGTC	93	21
FABP4	NM_053365.1	AATGTGCGACGCCTTTGT	TGATGATCAAGTTGGGCTTG	131	85
HMGCR	NM_013134.2	GACCTTTCTAGAGCGAGTGCAT	GCTATATTCTCCCTTACTTCATCCTG	77	122
HPRT1	NM_012583.2	GACCGGTTCTGTCATGTCG	ACCTGGTTCATCATCACTAATCAC	61	95
Leptin	NM_013076.3	CCAGGATCAATGACATTTCACA	AATGAAGTCCAAACCGGTGA	71	13
PPARγ	NM_013124.1	GACAGGAAAGACAACAGACAAATC	GGGGTGATGTGTTTGAACTTG	76	7
SCD-1	AF509569	GAAGCGAGCAACCGACAG	GGTGGTCGTGTAGGAACTGG	71	125
SREBP1	NM_001276707.1	ACAAGATTGTGGAGCTCAAGG	TGCGCAAGACAGCAGATTTA	72	77
TBP	NM_001004198	CCCACCAGCAGTTCAGTAGC	CAATTCTGGGTTTGATCATTCTG	75	129
UCP-1	NM_012682.1	GCCTGCCTAGCAGACATCAT	TGGCCTTCACCTTGGATCT	74	130

cDNA template, primers (200 mmol/L), probes (100 mmol/L), and LightCycler 480 Probes Master (FastStart Taq DNA Polymerase, 6.4 mmol/L $MgCl_2$; Roche Diagnostics). Real-time PCR parameters were as follows: 10 min (0:10:00) pre-incubation at 95°C, 40 cycles of amplification from 95°C (0:00:10), to 58°C (0:00:20), to 72°C (0:00:01), followed by cooling at 40°C (0:00:10). No-template controls included in reverse-transcription reactions and RT-qPCR runs were negative for amplification products. Standard curves for each gene and tissue were generated on separate runs using up to seven serial dilutions (1/10–1/1000) of pooled cDNA samples. Cq for each sample was determined by 2nd derivative maximum within Lightcycler 480 Software 1.5.0 Version 1.5.0.39. Reference genes aldolase A, HPRT and TBP were analysis with NormFinder Excel Add-In (MS Excel 2003) v0.953. NormFinder identified TBP and HPRT as the best combination of reference genes with stability values of 0.005 for adipose tissue and 0.002 for liver tissue. All target genes were normalized with the geometric mean of reference genes TBP and HPRT1 and standard curves were analysed again using the Lightcycler® 480 Software. Outliers were determined with residual plots using GenStat 14th edition (VSN International, Hemel Hempstead, UK).

Comparison of qPCR and NanoString methods

A direct comparison of NanoString and qPCR results is not possible since NanoString experiments return total counts for each transcript, whilst qPCR returns Cq values. A Cq value is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background amounts). Cq values are inversely proportional to the amount of the target nucleic acid in the sample. Direct graphical visualization will create negatively correlating plots compared with qPCR data because high expression levels will result in huge transcript numbers but small Cq values from qPCR [1]. Radke et al. [1] used an equation based on the fact that one DNA copy amplified with a perfect efficiency of 2.0 should reach Cq after about 35 PCR cycles, to enable statistical analysis and to facilitate the comparison of NanoString and qPCR results. Our NanoString data were transformed into Cq equivalents using a calculation adapted from Radke et al. [1] (Eqn 1). Differences in the binding efficiencies of the NanoString probes cannot be determined and are not accounted for in the calculation [1]. To improve accuracy, differences in amounts of input material were considered by comparing relative expression (normalized to reference genes: HPRT1 and TBP) in addition to non-normalized Cq data.

TABLE 4

Correlation (*R*) of each reference gene to each other for each rat tissue type (adipose: *n* = 18 and liver: *n* = 18), along with results (*P* value) of a two-sided test of correlations analysing the difference from 0.

Tissue	Reference gene	Correlation to reference gene (*R*)				Two-sided test of correlations (*P* value)			
		B2M	HPRT1	WAT	TBP	B2M	HPRT1	WAT	TBP
Adipose	HPRT1	0.29	–	–	–	0.245	–	–	–
	WAT	0.32	0.67	–	–	0.201	0.002	–	–
	TBP	0.34	0.84	0.78	–	0.162	<0.001	<0.001	–
	CYCA	0.63	0.65	0.60	0.76	0.005	0.003	0.009	<0.001
Liver	HPRT1	0.65	–	–	–	0.003	–	–	–
	WAT	0.66	0.81	–	–	0.003	<0.001	–	–
	TBP	0.70	0.81	0.82	–	0.001	<0.001	<0.001	–
	CYCA	0.76	0.79	0.92	0.82	<0.001	<0.001	<0.001	<0.001

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Bland–Altman plots compare the difference between two values of one sample (one from each of the methods) plotted as a function of their mean. The plots will highlight any bias between qPCR and NanoString data and were used to compare Cq values (qPCR) to their Cq equivalents (NanoString) (Fig. 1). In addition, Spearman's rank correlation coefficient was used to determine how well the two methods agreed on the relative ordering of the samples (e.g., high expression sample using qPCR is a high expression sample in NanoString experiments) (Table 5). In the attempt to remove slight variances in Cq data, Bland–Altman plots (Fig. 2) and Spearman's rank tests (Table 6) were also completed with relative expression data (normalized to reference genes HPRT1 and TBP) from both experimental methods. Tissue samples were analysed separately, and genes that had more than 1 sample with a qPCR Cq values of 40 (deemed as undetectable expression) were excluded from Bland–Altman and Spearman's rank analysis. In addition, PPARγ and leptin were removed from statistical analysis since they produced qPCR calibration curves with R^2 values of 0.70 (PPARγ) and 0.92 (leptin), which is below the recommended 0.98 or above, set out in the MIQE guidelines for qPCR [7].

Calculation for converting NanoString® counts into Cq equivalents adapted from Radke et al. [5]

TABLE 5

Spearman's Rank correlation (*R*) for each gene and each rat tissue type (adipose: *n* = 18 and liver: *n* = 18) using Cq and Cq equivalent data, along with exact *P* values

Tissue	Gene	Spearman's rank correlation (*R*)	Exact *P* value
Adipose	SCD-1	0.45	0.016
	ADIPOQ	0.53	0.006
	AR1	0.49	0.010
	AR2	0.42	0.020
	FABP4	0.37	0.033
	SREBP1	0.29	0.060
	HPRT1	0.23	0.087
	TBP	0.42	0.020
Liver	HMGCR	0.24	0.085
	SCD-1	0.51	0.008
	AR1	0.17	0.123
	AR2	0.05	0.212
	FABP1	0.23	0.086
	FABP4	0.36	0.034
	SREBP1	−0.09	0.181
	HPRT1	0.03	0.225
	TBP	−0.12	0.158

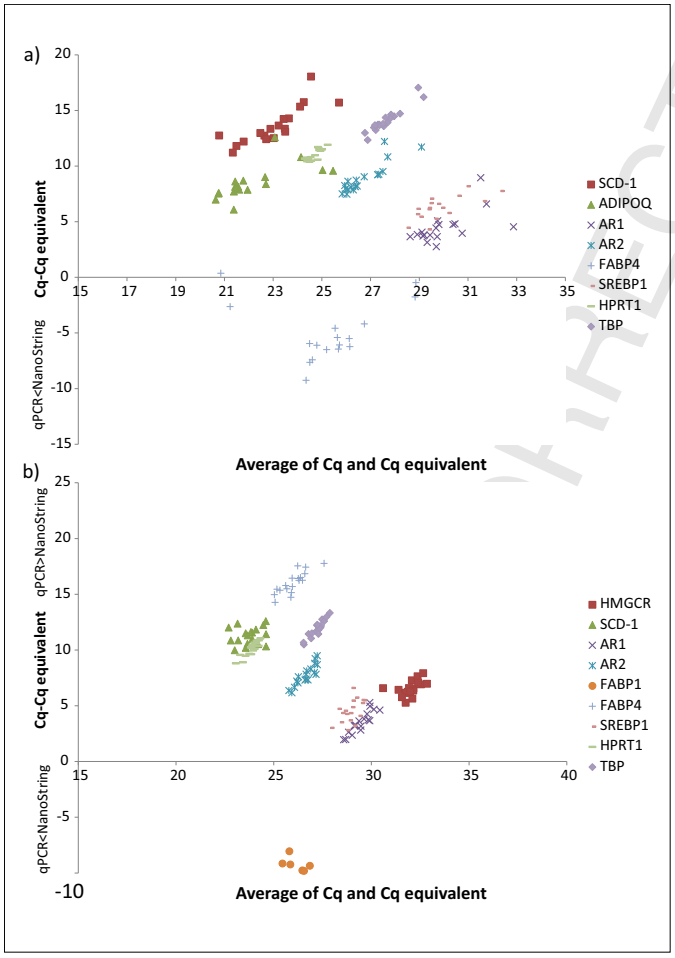

FIGURE 1

Bland–Altman plots of rat **(a)** adipose tissue samples (*n* = 18) and **(b)** liver tissue samples (*n* = 18) comparing Cq and Cq equivalents from qPCR and NanoString® methods respectively.

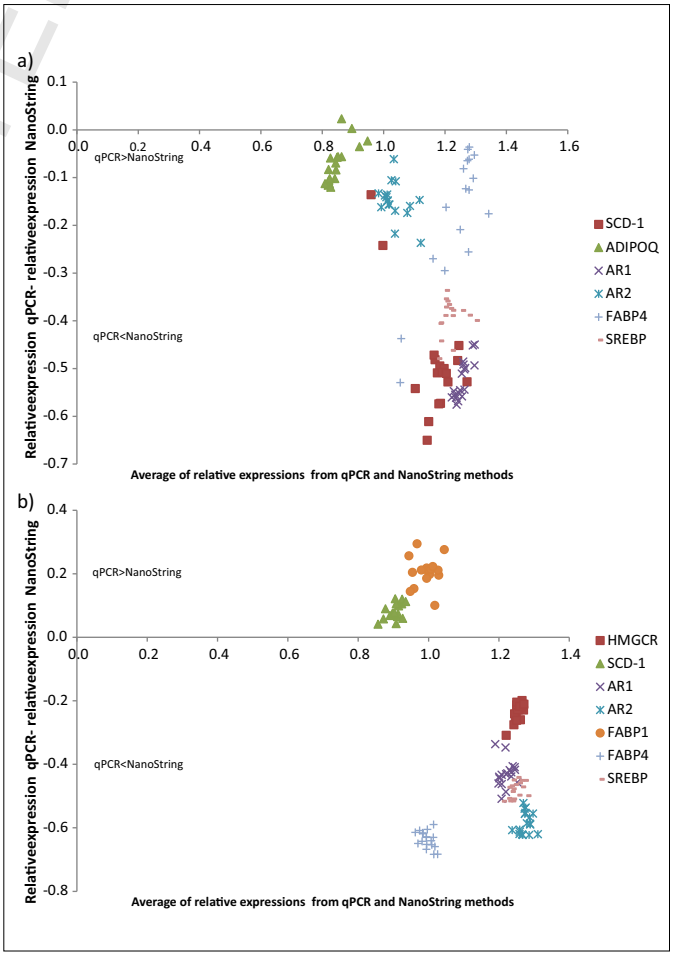

FIGURE 2

Bland–Altman plots of rat **(a)** adipose tissue samples (*n* = 18) and **(b)** liver tissue samples (*n* = 18) comparing relative expression of genes (normalized to HPRT1 and TBP) from qPCR and NanoString® methods respectively.

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TABLE 6

Spearman's rank correlation (*R*) for each gene and each rat tissue type (adipose: *n* = 18 and liver: *n* = 18) using relative expression data, along with exact *P* values

Tissue	Gene	Spearman's rank correlation (*R*)	Exact *P* value
Adipose	SCD-1	0.52	0.007
	ADIPOQ	0.46	0.014
	AR1	0.13	0.152
	AR2	0.52	0.007
	FABP4	0.45	0.025
	SREBP1	0.40	0.072
Liver	HMGCR	−0.26	0.072
	SCD-1	0.48	0.011
	AR1	0.07	0.196
	AR2	−0.12	0.155
	FABP1	0.64	0.001
	FABP4	0.17	0.124
	SREBP1	0.26	0.076

$$\text{Cq equivalent} = \log_{E}\left[\frac{2^{34}}{nrc}\right] \quad (1)$$

E is the efficiency of amplification in qPCR experiment and *nrc* is the positive control normalized raw counts.

Results and discussion

qPCR and NanoString data expressed as Cq were not comparable

NanoString Cq equivalents were commonly lower (higher gene expression) than qPCR Cq values, the exception being FABP4 in adipose tissue samples and FABP1 in liver tissue samples (Fig. 1). This could be the result of the assumptions in the Cq equivalent calculation: non-uniform enzymatic reactions or pipetting errors in the qPCR could mean the numbers used the calculation of Cq equivalents were inaccurate. For instance, if the qPCR had an efficiency of 1.8 and a sample had a Cq of 30, then the same amount of DNA counted by NanoString then put through the Cq-equivalent calculation, assuming an ideal efficiency of 2, would have a Cq-equivalent of 25.4. We attempted to remove the potential variance in qPCR data compared with NanoString by comparing relative expression data (normalized to HPRT1 and TBP) from both methods (Fig. 2). In contrast to the Cq data, NanoString with relative gene expression results tended to be higher than qPCR values. Radke et al. [1] showed that the majority of genes tested had very similar Cq values in both qPCR and NanoString methods. However, they showed that one reference gene (peptidylprolyl isomerase A) resulted in higher Cq values using a qPCR method similar to that used in our study. They speculated that the difference was the result of an underestimation of amplification efficiency, which is used in the Cq equivalent calculation.

qPCR and NanoString ranked data correlated for adipose samples but not liver samples

When completing an experiment to assess gene expression, a negative control treatment is commonly used. In our experiment, our samples came from two groups of rats fed different treatments (control or seaweed). This removes the necessity for Cq values being equal in both methods, as data are normalized to a reference gene and then compared with a control. Whilst our Bland–Altman plots did not compare with those of Radke and colleagues [1], a critical comparison between methods is the agreement of sample ranking from high to low expression, which cannot be determined in detail using our Bland–Altman plots. Cq and Cq equivalents were analysed using a Spearman's rank test to compare the correlation of ranked data (Table 5). The results suggest there was a significant positive correlation for genes in adipose samples the using qPCR and NanoString methods, whilst liver samples were predominately not correlated. Spearman's rank tests were also used to compare relative expression data from both methods (Table 6). This comparison supported a stronger correlation of genes in adipose tissue compared to liver. In addition, we were alerted to a negative correlation in relative expression of HMGCR in liver tissue samples ($P = 0.07$). This result is concerning, and implicates a critical error in one or both methods when dealing with liver samples. Interestingly, both sample types from an individual rat were run on the same plate or chip in qPCR and NanoString experiments respectively, removing the probability that the non-correlation of liver samples was due to experimental error. There is a lack of publications comparing liver tissue gene expression between methods and we have not been able to determine the cause of the difference in data. We can only speculate that the reason NanoString and qPCR data did not correlate when using liver samples, was there could have been an undetected contaminant introduced from the liver tissue itself, which altered the performance of either the qPCR or NanoString methods. Why liver and not adipose samples would alter gene expression results in one or both methods is perplexing. Interestingly, Prokopec et al. showed a similar lack of correlation with rat liver samples after the application of a quartile-normalization procedure [8]. This procedure ranks data similarly to Spearman's rank and on completion of the quartile method of normalization with our data, we obtained very similar data to those of Spearman's rank (data not shown).

HMGCR splice variants may explain a negative correlation between ranked liver data

We decided to explore the negative correlation of HMGCR relative expression data between methods, since this result stood out in the liver tissues samples ($R = -0.26$, $P = 0.07$). Stormo et al. [9] analysed splice variants in exon 1 of human HMGCR. Others have described splice variants in alternative exons of HMGCR [10,11]. It is thought that splice variants of HMGCR could be a mechanism for cholesterol homeostasis [12]. Interestingly, a splice variant of HMGCR lacking exon 13 decreased enzymatic activity. A decrease in HMGCR activity would lead to lower cellular cholesterol synthesis and subsequently a counter-regulatory increase of cholesterol uptake from the plasma via the LDL-receptor pathway, to maintain intracellular cholesterol homeostasis. We could not find any information regarding splice variants in rat HMGCR. However, our NanoString probe crossed exons 12 and 13, whilst our qPCR target sequence spanned exons 5 and 6. We decided to explore whether our data may support a potential problem with our NanoString probe, by comparing NanoString and qPCR Cq data from both dietary treatments. It was interesting to find that there were no differences between control and seaweed treatments using

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qPCR methodology (*t*-test *P* value = 0.24), whilst using NanoString methodology, seaweed treatments produced results significantly lower than the controls (*t*-test *P* value = 0.03). This accounted for the negative correlation of ranked data between NanoString and qPCR, and may be explained by an increase of HMGCR lacking exon 13 following seaweed treatment. If this could be verified, a seaweed diet could induce a beneficial splice variant of HMGCR. We are not the first to describe the potential effects of splice variants on the performance of the NanoString and qPCR methods. Prokopec and colleagues [8] found that varying performance of genes between methods could be the result of differences in probe placement or specificity, leading to distinct splice variants or other transcript isoforms being identified.

NanoString reference gene data had smaller sample variance than qPCR data

Lastly, to aid in the determination of method accuracy, we compared the spread of reference gene expression, using Cq and Cq equivalents from both the qPCR and NanoString methods. Assuming that reference gene expression should be stable across tissue samples, we expected that the more accurate method should have lower sample standard deviation (SD). Adipose tissue expression of HPRT1 in qPCR experiments was 3.5 times the SD of NanoString Cq, whilst TBP expression was 5.9 times the SD of NanoString Cq. In agreement, liver tissue expression in qPCR experiments was 4.2 times the SD of NanoString Cq, whilst TBP was 5.5 times the SD of NanoString Cq. These data support the hypothesis that the NanoString method is more accurate than qPCR, as predicted by the reduction in sample handling and removal of sample amplification steps that rely on enzymatic reactions.

Conclusion

NanoString offers a valuable alternative to qPCR and evidence suggests that data are more accurate because of the reduced sample handling and direct quantification of gene copy number without the need for enzymatic amplification. We noted a difference in data produced by different methods which was dependent on tissue type. Liver tissue data from both methods did not correlate and we were not able to determine the reason for the discrepancy. This is a critical concern for data published on liver tissues by one method only, and further research is essential. Despite this, we were able to progress our investigation into one liver gene, which was negatively correlated ($R = -0.26$, $P = 0.07$) between methods when comparing relative expression data. We have highlighted a potential issue for both methods, which needs to be addressed when designing primers or probes. We suggest a literature search for known splice variants of a particular gene to be completed, so that primers or probes can be designed that do not span exons which may be affected by alternative gene sequences. This could be very problematic for uncommon genes or species to be investigated, as information on splice variants may not be known.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.nbt.2016.01.002.

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GLOSSARY

Cq value Cq value is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background amount). Cq values are inversely proportional to the amount of target nucleic acid in the sample.

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