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Gut neuroendocrine tumor blood qPCR fingerprint assay: characteristics and reproducibility

Abstract

Background: We have developed a PCR-based tool that measures a 51-gene panel for identification of gastroenteropancreatic (GEP) neuroendocrine neoplasms (NENs) in peripheral blood. This manuscript assesses the robustness (performance metrics) of this tool with a specific focus on the effects of individual parameters including collection, storage, acid suppressive medication [proton pump inhibitor (PPI)], age, sex, race and food on accuracy.

Methods: Performance metrics were evaluated using a gold standard (mRNA derived from three individual human neuroendocrine tumor cell lines) and clinical samples using qPCR.

Results: One hundred percent of the 51 transcripts were amplified in the gold standard (NEN cell line-derived mRNA) (C_Q<35, average efficiency 1.94). The inter- and intra-assay variations were 1%–2%. In clinical samples, 50 of 51 targets (98%) were amplified. The inter- and intra-assay reproducibility ranged between 0.4% and 1.2%. The coefficient of variation (CV) was 5.3%. Expression of the reference gene, *ALG9*, was robust (low variation, low M-value, PCR efficiency) and unaffected by sample processing. Test meals, long-term PPI use (>1 year), age, sex and ethnicity had no effect on the signature. Expression of two genes, *ALP2* and *CD59* correlated strongly with RNA integrity (R=0.72, p<0.001) and could be used to assess storage and processing.

Conclusions: The 51 marker gene signature was robust and reproducible, exhibiting acceptable inter- and intra-assay metrics (<5%). Feeding, PPI intake, age, sex and ethnicity do not affect the signature. Expression levels of *APLP2* and *CD59* are effective surrogate markers of proper sample collection and processing.

Keywords: blood; gene marker; MIQE; neuroendocrine tumor; qPCR; reproducibility; score; variability.

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Gene names

ALG9 – ALG9, α-1,2-mannosyltransferase; *APLP2* – amyloid β (A4) precursor-like protein 2; *CD59* – CD59 molecule, complement regulatory protein; *HSF2* – heat shock transcription factor 2; *MAP3K7CL* – (C21ORF7) MAP3K7 C-terminal like; *NOL3* – nucleolar protein 3 (apoptosis repressor with CARD domain); *NUDT3* – nudix (nucleoside diphosphate-linked moiety X)-type motif 3; *PKD1* – polycystic kidney disease 1 (autosomal dominant); *SSTR1* – somatostatin receptor 1; *SSTR5* – somatostatin receptor 5; *TRMT112* – tRNA methyltransferase 11-2 homolog (S. cerevisiae).

Introduction

Real time PCR (qPCR) is the gold standard for nucleic acid quantification. Its strengths embrace a number of parameters including: dynamic quantitative range (5–8 log orders of magnitude); increased sensitivity (e.g., ≥1000× more sensitive than dot blots); and ability to detect single transcript copies and reliably differentiate small differences in gene expression (approx. 25% changes). Quantitative PCR has become standard for the detection of BCL-ABL alterations in leukemia [1], determining prognosis of breast cancer [2–4] and identification of metastasis, recurrence, and prediction of responses to radio- and chemotherapy in colon cancer [5, 6]. Despite the clinical significance, translation has proven challenging, e.g., peripheral blood screens for colorectal cancer [7], because of a number of variables including expense, low availability and the high sensitivity of the technique (erroneous amplification).

The exponential nature of the amplification protocol combined with small quantities of target may result in substantial differences in final yield if there are minor variations in reaction components and thermal cycling conditions and/or mispriming events [8–12]. These considerations have led to rigorous assessments of assay utility [13], particularly in the routine setting [14, 15]. Stringent quality control, standardization of sample acquisition and processing are therefore a prerequisite in the development and application of molecular tools [16].

This text describes our development of a hydrolysis probe-based molecular signature for gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs) based on a 51 marker gene signature we derived [17]. Peripheral blood expression of these markers is included in four gene-based classifiers that are used to derive a quotient, the "NEN score". This differentiates GEP-NENs and controls with a high PPV and NPV (>90%) [17]. Typically, a consistent protocol for RNA isolation, cDNA synthesis and qPCR should provide a stable platform for target and reference gene analyses [8, 18–21]. We assessed the robustness of this approach and examined potential sources of variation including meals and proton pump inhibitor (PPI) usage, both known to significantly alter peripheral blood biomarker measurements in GEP-NENs [22]. Additionally, we describe the utility of a specific subset of the panel as a surrogate tool to assess suitability of sample processing and preparation.

Materials and methods

Studies were undertaken to assess the effect of a number of variables on assay performance and NEN score (detailed descriptions in the Supplemental Data, which accompanies the article at http://www.degruyter.com/view/j/cclm.2014.52.issue-3/issue-files/cclm.2014.52.issue-3.xml). These included: the limit of detection and quantification of the assay, the inter- and intra-assay specifications, reproducibility in individual samples, storage (blood manipulation prior to analysis), long-term (>1 year) PPI usage, feeding and daily variation. These parameters were evaluated using a gold standard (mRNA derived from three individual human neuroendocrine tumor cell lines) and in clinical samples (Figure S1).

All samples were collected and analyzed according to a standard IRB protocol (Yale University: 6/5/2012) in accordance with the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects [17]. Details of the cases and controls are included (Supplemental Data, Table S1). Blood samples (5 mL) were collected in 9 mg K_2EDTA tubes (BD Vacutainer Venous Blood Collection Tubes, BD Diagnostics, Franklin, NJ, USA). Aliquots of whole blood were stored at –80°C within 2 h of collection (samples immediately stored on ice/4°C after sampling) per standard molecular diagnostics protocols [23]. For timed experiments and analyses of RNA degradation, samples were collected and stored at 4°C for 30 min–48 h prior to freezing at –80°C and then processed.

A two-step protocol (RNA isolation with cDNA production and qPCR) was undertaken using a manual technique in the authors' laboratory. Transcripts (mRNA) were isolated from 1 mL EDTA-collected blood samples using the mini blood kit (Qiagen, Valencia, CA, USA). The RNA quantity was 50 µL, the quality was >1.8 ($A_{260:280}$ ratio); analysis of the RNA pattern on electrophoresis (Agilent Technologies) RIN>5.0 [24]. The standard Qiagen isolation protocol (heme/gDNA contamination not detected) with no modifications was used. cDNA was produced from 50 µL RNA using a High Capacity Reverse transcriptase kit (Life Technologies, Grand Island, NY, USA: cDNA production 2000–2500 ng/µL) and stored at –80°C. qPCR was performed (384-well plate, HT-7900) with the cDNA (200 ng/µL) and 16 µL of reagents/well (Universal Master Mix II with UNG, Life Technologies, triplicate wells) (50°C 2 min, 95°C 10 min, then 95°C 15 s, 60°C, 60 s for 40 cycles). The majority of primers (Table S2) were exon spanning (82%, the remainder spanned one exon); all were <160 bprs.

Sample sets

A schematic demonstrating our approach is included in Figure S1. Briefly, we examined qPCR efficiency, limit of detection (LOD), limit of quantification (LOQ), inter- and intra-assay variation. In addition we assessed the daily stability of the test, the effects of feeding, age, sex, ethnicity (Table S3) and long-term PPIs on the molecular fingerprint (Table S4). We further assessed sample stability and the adequacy of sample preparation, by measuring the effects of refrigeration (4°C).

Data analysis

Raw C_Q values as well as normalized values (using *ALG9* and the $\Delta\Delta C_Q$ method [17, 25]) were calculated (Microsoft Excel, Redmond, WA, USA). Non-parametric Mann-Whitney and Spearman's correlations were used to compare samples and the Fisher's test was used for binary comparison (GraphPad Prism 5, La Jolla, CA, USA). For generation of the NEN score, we used a MATLAB (R2011a, Mathworks, Natick, MA, USA) implementation of prediction approaches (Supplemental Data and Table S5) [17].

Results

qPCR efficiency and limits of detection and quantification

Using the NEN standard, the average C_Q for the 51 marker genes and the reference gene *ALG9* (detailed discovery in Supplemental Data, Figure S2-7) was 28.86±0.62 and ranged from 25.14 (*TRMT112*) to 35.14 (*C21ORF7*) (Figure 1A). The overall qPCR efficiency for the genes was 1.94±0.11 and ranged from 1.70 (*NUDT3*) to 2.34 (*NOL3*) (Figure 1B). The efficiency for *ALG9* was 1.995. qPCR reactions are regarded as not inhibited when the qPCR efficiency ranges 75%–120% [26].

To assess the limit of the blank (LOB), we measured transcripts in six runs replacing cDNA with water. Single wells (one of three) were identified as positive for three genes, *SSTR1* (C_Q=36.95), *SSTR5* (C_Q=37.01) and *TRMT112* (C_Q=37.02) in one of the six runs. Using a cut-off of ≥2 positive wells as a measure of positivity, each of these three genes was considered not amplified. The calculated LOB therefore was 0.

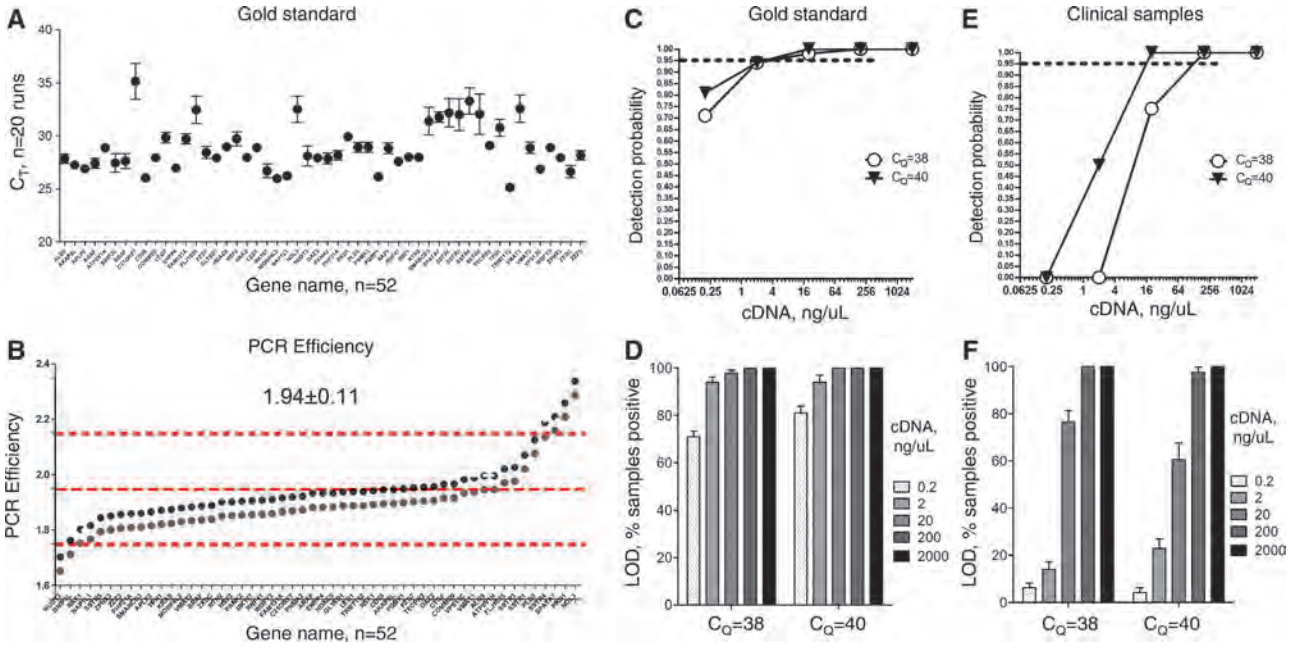

Figure 1 qPCR characteristics and detection analysis of the neuroendocrine tumor cell lines (gold standard) and clinical samples. (A) Mean C_Q values for each gene (2, 20, 200 ng/µL cDNA) demonstrate that 50/51 (98%) of genes are amplified at <35 cycles. (B) A waterfall plot of the qPCR efficiencies demonstrate that 45/51 (88%) of genes range between 1.8 and 2.2. The average efficiency was 1.94. (C) Detection probability curve for the gold standard (NET cell lines) demonstrating the 95% efficiency was 2 ng/µL. (D) >95% of genes were amplified using >2 ng/µL. (E) Detection probability curve for the clinical sample mix demonstrated that the 95% efficiency ranged between 8 and 64 ng/µL cDNA. (F) Analysis of all targets identified that the majority >95% could be amplified using 100 ng/µL at C_Q=40. Mean±SD, n=8.

The LOD in the gold standard was 2 ng/µL (Figure 1C). The percentage of targets that were positive at each of the cDNA concentrations ranged from 71% to 81% (0.2 ng/mL) to 100% (≥200 ng/mL) (Figure 1D). The LOD calculated from the regression curve was 0.21 ng/µL and the LOQ was calculated as 0.63 ng/µL. The dynamic range was 1000.

In the clinical sample mix, the LOD ranged from 8 to 100 ng/µL (Figure 1E). The percentage of targets that were positive at each cDNA concentration ranged from 4.25% to 6.25% (0.2 ng/µL) to 98%–100% (≥200 ng/µL) (Figure 1F). For the clinical mix, increasing the number of cycles to 45–50 cycles identified positive expression in <1% of target samples; the false-negative rate (at C_Q=40) was calculated to be 0.8%. The LOD calculated from the regression curve was 0.43 ng/µL and the LOQ was 1.32 ng/µL. The dynamic range was determined to be 100.

In additional studies, we evaluated the effect of the different cDNA dilutions on the MATLAB-derived "NEN score" (Supplemental Data and Table S5) to assess whether this was altered. This was analyzed in both the gold standard and in six clinical samples at concentrations ranging between 2 and 2000 ng/µL (n=6 samples/dilution). For the gold standard, all samples were classified at all dilutions; the NEN scores at 2 ng/µL were lower (2.5±0.25) than at 20–2000 ng/µL (all scores 3.6±0.25, no difference between scores). For the clinical samples, a classification could not be performed at 2 ng/µL (below detection level), 3/6 (50%) samples were classified at 20 ng/µL, while all six samples were classified at 200 and 2000 ng/µL. Sample classifications were not different at the 200 and 2000 ng/µL dilution (majority vote score was 3.3±0.2).

These results demonstrate that clinical samples ≥200 ng/µL can be effectively and reproducibly analyzed. All further analyses were thereafter undertaken with a concentration of 200 ng/µL and a cut-off of 40 cycles.

Inter- and intra-assay variability

To determine the inter- and intra-assay reproducibility, we assessed both the gold standard and the expression in the same clinical sample processed on separate days (n=3, SI NEN samples). For the gold standard, variability was assessed in 20 different qPCR runs. The inter-assay variability was 2.14%±1.14% and ranged from 0.91% (*PKD1*) to 5.6% (*SSTR5*) (Figure 2A). The intra-assay (5 runs) was 1.02%±0.74% and ranged from 0.12 (*HSF2*) to 2.31 (*SSTR5*) (Figure 2B). Assay precision is summarized in Figure 2C. For the clinical samples, the inter-assay variability was

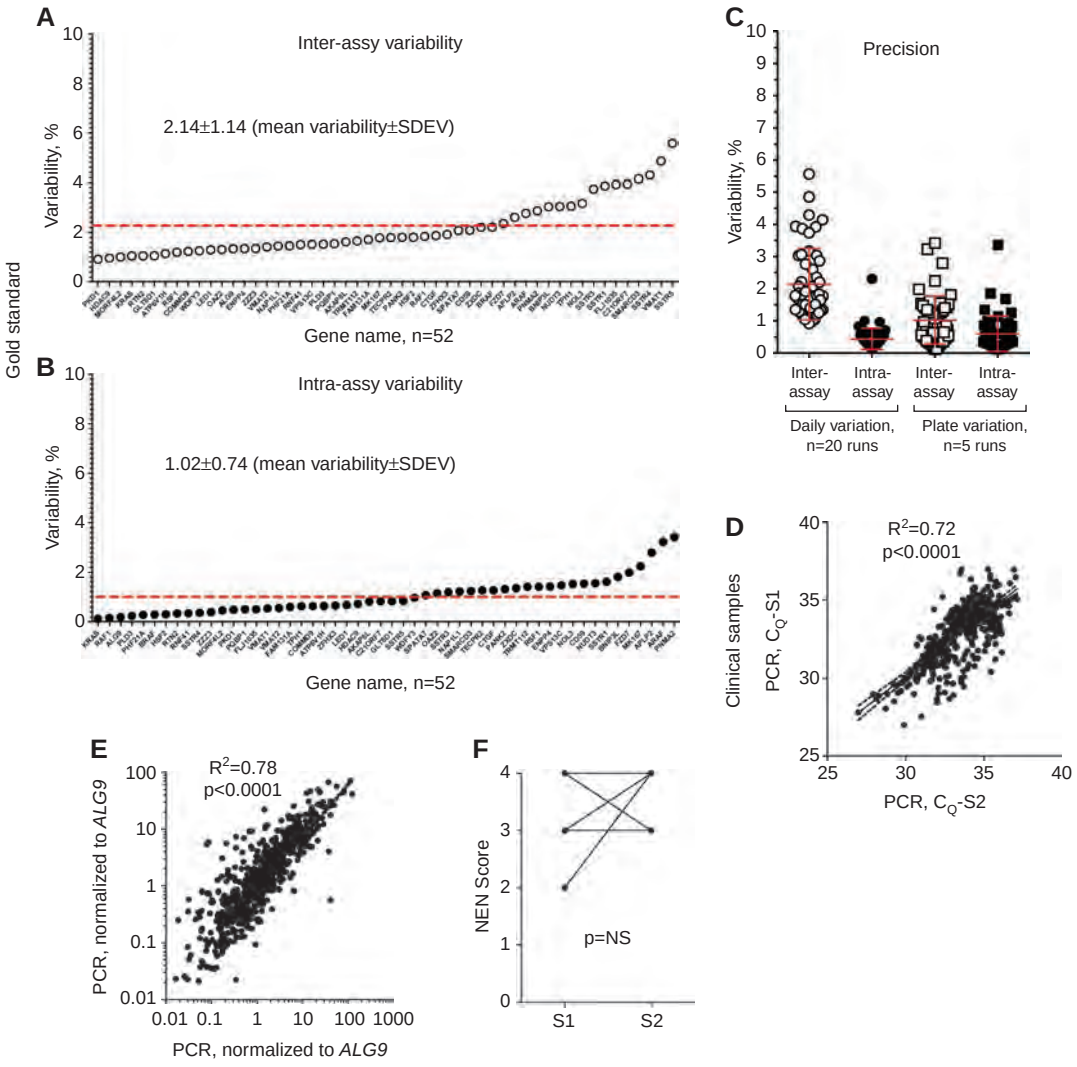

Figure 2 Reproducibility of the qPCR-based fingerprint in the neuroendocrine tumor cell lines (gold standard) and clinical samples. (A) Waterfall plot of inter-assay variability (n=20 runs) for each of the 51 genes and ALG9. The mean variability (for all genes) was 2.14%. (B) Waterfall plot of the intra-assay variability (n=5 runs); the mean variability was 1.02%. (C) Precision of the qPCR demonstrating inter- and intra-assay variability for each of the 51 marker genes and the 1 reference gene (52 genes) was <6%, with averages <2%. (D) Analysis of the 14 clinical samples identified the correlation for C_Q values as 0.72 (Spearman's, p<0.0001). (E) Following normalization to *ALG9*, the Spearman's correlation was 0.784 (p<0.0001). (F) Individual NEN scores were not different (p=0.87). Mean±SD. Data is log scaled in (B).

0.5%–1.2% while the intra-assay reproducibility was 0.4%–1%.

In a second analysis, blood from 14 individual patient samples (divided into two aliquots prior to processing) demonstrated that, as a group, the Spearman's correlation was 0.72 (p<0.0001). Samples were therefore significantly correlated at a C_Q level (Figure 2D). The CV was 5.33%. Samples were also normalized to *ALG9* per protocol; the Spearman's correlations for each of the normalized genes ranged between 0.66 and 0.9, (p<0.0001) with a median of 0.81. As a group, the overall correlation was 0.78 (p<0.0001) (Figure 2E). An analysis of the NEN scores identified that the samples exhibited similar scores [p=0.87 (not different), CV=0.9] (Figure 2F). The qPCR test is therefore highly reproducible at three different levels: C_Q value, normalized expression and the NEN score.

Day-to-day variability

A consecutive daily analysis of eight small intestinal (SI) NEN patients, identified that the Spearman's correlation ranged between 0.8 and 0.92 (p<0.0001) with a median

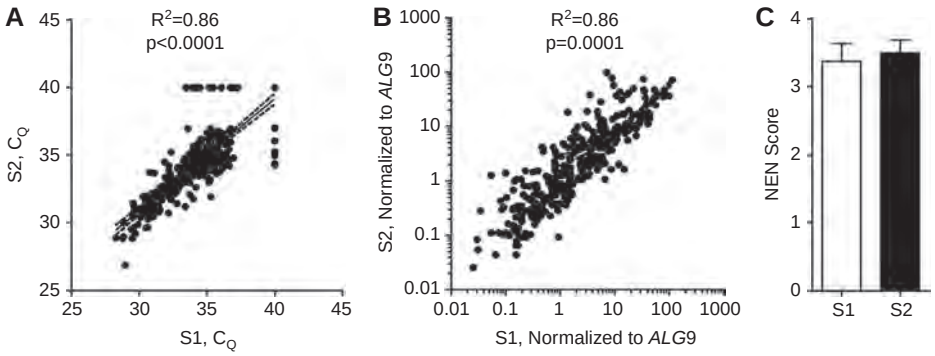

Figure 3 Day-by-day variability in the qPCR-fingerprint in 8 small intestine (SI) NEN samples.
(A) The Spearman's correlation for C_Q values was 0.86 ($p<0.0001$). (B). Following normalization to *ALG9*, the Spearman's correlation was 0.86 ($p<0.0001$). (C) Individual NEN scores were not different ($p=1$). Mean±SD. Data is log scaled in (B).

of 0.86. As a group, the overall correlation was 0.86 ($p<0.0001$) (Figure 3A). The overall CV was 7.7%. Samples normalized to *ALG9* exhibited Spearman's correlations ranging between 0.78 and 0.93, ($p<0.0001$) with a median of 0.86 (Figure 3B). The samples exhibited similar NEN scores ($p=1.0$) which were closely correlated ($Rs=0.96$, $p<0.001$, $CV=0.95$) (Figure 3C). These data indicate that the day-to-day variability in the qPCR test is low and the test is highly reproducible.

Assessment of feeding on the molecular fingerprint

We evaluated the effects of feeding on the qPCR fingerprint in five SI NENs. Unsupervised hierarchical clustering based on marker gene expression values did not identify intrinsic relationships between feeding and gene expression (Figure 4A) or the NEN score (Figure 4B) over a 4-h experimental period. No differences were noted in reference gene expression (Figure S8B). The fingerprint is therefore robust and is not affected by food intake.

Relationship between age, sex, ethnicity and PPI usage on molecular fingerprint

No relationship was noted between age, sex, ethnicity and the NEN score in an analysis of 61 individuals with NENs (Supplemental Data, Table S3, Figure S9). Eleven individuals with gastroesophageal reflux disease (GERD) on long-term (>1 year PPI) exhibited scores of 0–1 (normal range) despite elevated plasma CgA levels (mean 32.6 U/L, range: 19–94) in 10 of the 11 (91%) individuals (Supplemental Data, Table S4, Figure 5).

Assessment of storage conditions on the qPCR fingerprint

We analyzed 51 marker genes in six patients with samples stored at time 0, 0.5, 1, 2, 4, 24 and 48 h at 4°C prior to processing. A time-dependent decrease in average expression ~~over time~~ prior to freezing (1.4–2.5-fold decrease, Figure 6A) was evident. This variability resulted in a reduction in the NEN score at times >2 h (Figure 6B). After 2 h, 15 of 42 (36%) of samples had an abnormal score (>"1" difference from the index time point of T=0 h storage). These samples all exhibited a RIN <3, consistent with RNA degradation. Four of 6 (67%) patients exhibited an abnormal score. This was evident at 4 h (2 patients) and 24 h (2 patients). At this time point such samples would have been identified as "Normal", i.e., scores 0–1 (Figure 6C). The GEP-NEN molecular signature can be considered stable for approximately hours at 4°C. Beyond this gene expression decreases as a function of storage and RNA degradation.

Derivation of a qPCR fingerprint that identifies adequacy of sample preparation

An analysis of marker genes in the previous section (*Assessment of storage conditions on the qPCR fingerprint)* identified expression of a subset that could be used to define appropriate sample storage and preparation. Unsupervised hierarchical clustering of the transcript data (from Figure 6A) identified three sample populations: *first*, characterized by samples stored for shorter periods (brown and yellow clusters), *second*, samples stored for intermediate periods of time (green and turquoise clusters), and *third*, samples stored for long periods of time (blue cluster). Clusters were not characterized by homogeneous patient

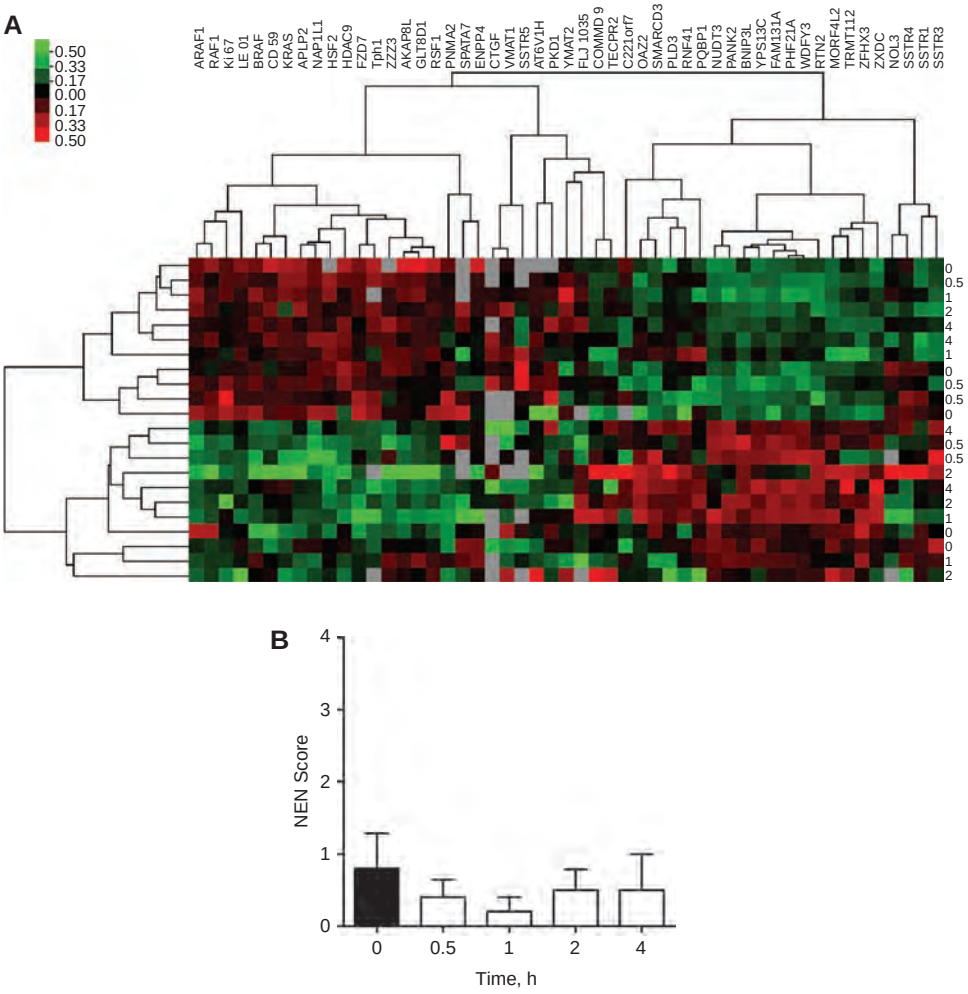

Figure 4 Effects of feeding on gene expression measured over a 4-h period.
(A) Hierarchical cluster analysis demonstrated no effect of feeding on gene expression. (B) No significant alterations were noted in NEN scores over the 4-h period. (n=5). Mean±SD, n=5 SI NEN patients. Black bar = index case (time = T0).

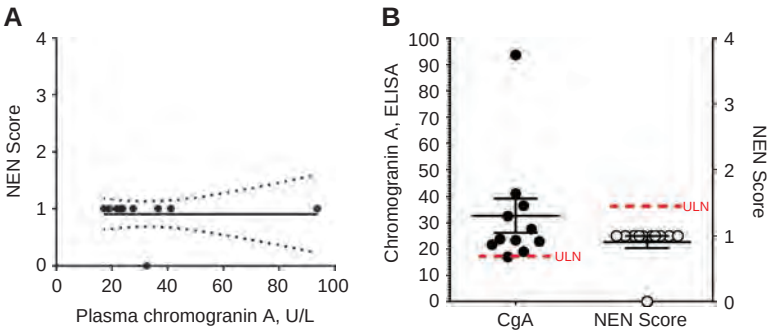

Figure 5 Assessment of relationship between PPI usage, elevated chromogranin A and the NEN score.
(A) No correlation was noted between plasma CgA levels and score (line = linear correlation curve, dotted lines = 95% CI). (B) Ten of 11 patients had elevated CgA (>19 U/L); none of the 11 patients had a NEN score >1. Mean±SD. ULN, upper limit of normal.

groups (i.e., consisting of samples collected from the same individual) but were grouped according to the time delay prior to RNA extraction (Figure 7A). Differential expression analysis revealed that *APLP2* (long vs. short: log fold change=–1.3, adjusted p-value=1.1×10^{-6}, medium vs. short: log fold change=–0.9, adjusted p-value=6.4×10^{-5}) and *CD59* (long vs. short: log fold change=–1.2, adjusted p-value=1.4×10^{-6}, medium vs. short: log fold change=–0.8,

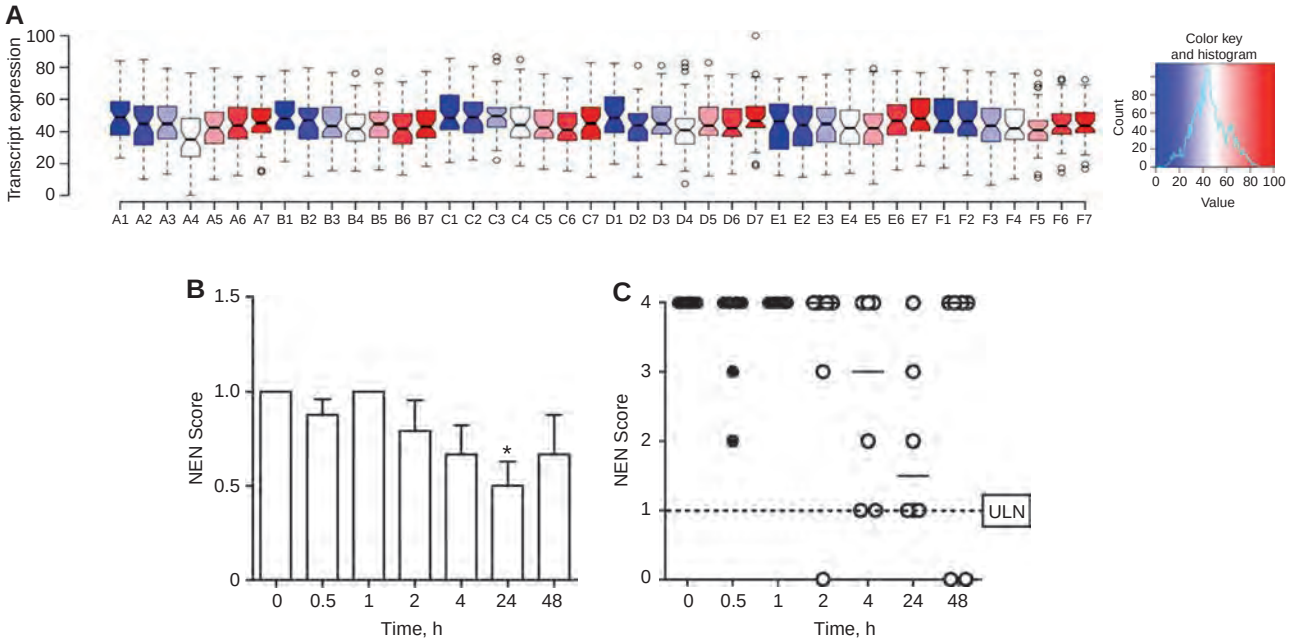

Figure 6 Variability in gene expression and NEN scores as a function of storage at 4°C prior to −80°C storage.
(A) Average gene expression of 51 marker gene expression in each of 5 SI NEN samples at each of the time points (labeled 1–7: T1=0 h prior to storage, T7=48 h prior to storage). Dark blue is the index case (T=0), red=last time point (T=48 h). Variability (loss) in expression is evident with increasing time at 4°C (in refrigerator) prior to storage (−80°C). (B) Average classification scores as a function of time. Raw scores were significantly decreased at 24 h compared to T0. (C) Two patients would have been classified as "Normal" at T=4, 24 and 48 h. Two thirds of the patients would have incorrect classification scores at T>2 h. *p<0.05 vs. T0 (2-tailed, matched test). Mean±SD.

adjusted p-value=1.1×10^{-4}) were the most significantly down-regulated genes compared to short time delay (<2 h before freezing) samples. Raw expression of these transcripts correlated with RNA degradation. We prospectively validated this in clinical samples collected over a 4 month period (n=56) and compared the gene signature directly with RINs from the samples. Firstly, expression of each of these genes was significantly decreased in medium- and long-term delayed samples compared to short-term samples [*APLP2*: 1.64 vs. 1.08 (medium) and 0.52 (long), p<0.05; CD59: 3.14 vs. 1.29 and 0.32, p<0.05] (Figure 7B and C). Secondly, the RIN score was directly related to the predicted processing times (Figure 7D). Significant correlations with RNA degradation measurements were identified between *APLP2* (R^2=0.19, p<0.001, Figure 7E) and *CD59* (R^2=0.72, p<0.0001, Figure 7F). These results demonstrate that down-regulation of both *APLP2* and *CD59* can be used to identify samples that have been inadequately collected and/or stored (Figure S10A–E).

Discussion

Identification in the peripheral blood of minute traces of tumor activity is a key goal for early detection of tumor growth/spread and assessing the therapeutic efficacy. It is therefore of considerable relevance in the diagnosis and management of neoplasia. qPCR is widely considered as the gold standard for nucleic acid quantification and is commonly used for microarray result validation [19]. Of particular significance is that this methodology is most useful when the starting materials (e.g., CTCs or mRNA) are limited, high sensitivity is required [27] and high accuracy critical [28].

qPCR-based protocols are more sensitive for identification of CTCs than CTC collection per se in the detection of melanomas [29] and breast cancer [30]. However, this sensitivity represents a critical inflection point [31]. Thus, certain qPCR-based protocols are characterized both by significant variation and lack of reproducibility [8–12]. Variations in reaction components, cycling conditions and mispriming events combined with the efficiency of enzymatic amplification have been described as substantially affecting the yield of the amplified target(s) with a consequent alteration in target gene quantification (and gene scores based on expression) [8]. In clinical settings characterized by a low burden of detectable disease, e.g., micrometastases, this could be problematic [9–11]. The development and usage of any qPCR-based test therefore requires stringent quality control and standardizations

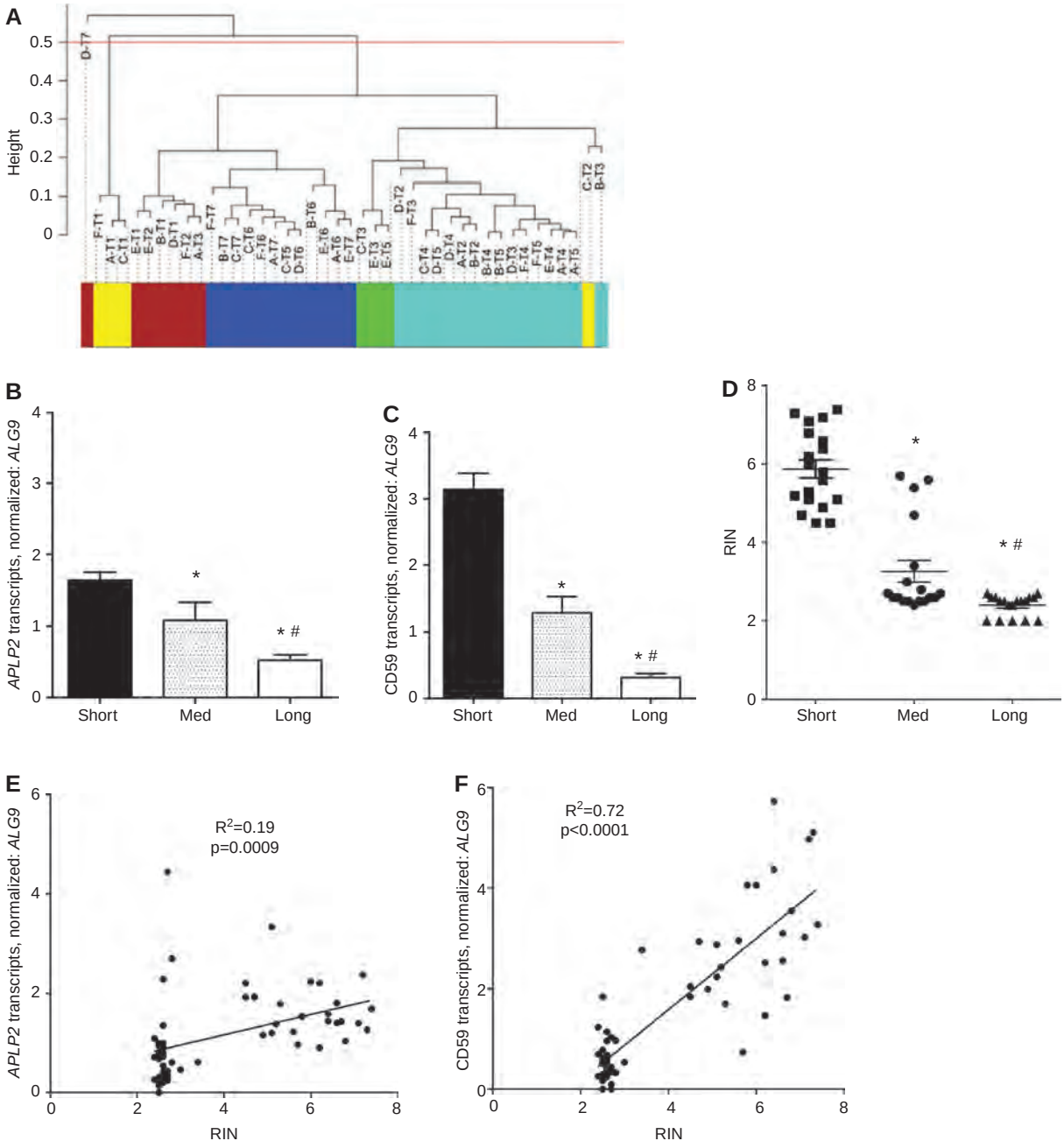

Figure 7 Development of a fingerprint to identify sample stability.
(A) Unsupervised hierarchical clustering of 42 blood samples at seven time points using expression profiles of 51 marker genes. Colors on the x-axis indicate group assignments obtained by cutting the hierarchical tree at 0.5 (red line). Samples with similar marker gene expression are assigned into the same cluster. (B) *CD59* transcripts and processing demonstrating a decrease with increasing processing times (short, medium, long). (C) Relationship between *APLP2* transcripts and processing times. (D) Relationship between RINs and processing times. (E) Significant correlation (R^2=0.72) between *CD59* transcripts (normalized to *ALG9*) and RNA integrity number (RIN). (F) Significant correlation (R^2=0.19) between normalized *APLP2* transcripts and RIN. SHORT = short (≤2 h), MED = medium (4–24 h), Long = long (>24 h). *p<0.05 vs. SHRT, #p<0.05 vs. MED. Mean±SD.

of sample acquisition and processing to ensure detection limits are sensitive, predictable and reproducible (summarized in detail in MIQE criteria [16]).

Our strategy describes a two-step protocol (RNA isolation with cDNA production and qPCR) for the detection of circulating neuroendocrine tumor transcripts. In preliminary studies we identified that the most effective method for mRNA isolation from blood samples was the Qiagen mini blood kit [24] with cDNA produced using the Applied Biosystems High Capacity Reverse transcriptase kit. We

used a HT-7900 machine with 384-well plates and 16 μL of reagents/well and include primers (<160 bprs) that are exon spanning to minimize genomic DNA amplification. These consistent parameters for RNA isolation, cDNA synthesis and qPCR were selected to ensure a stable platform for target and reference gene analysis [16]. Our reference gene selection (Supplemental Data, Figures S2–8) identified a robust gene (*ALG9*) that could be effectively used in our normalization protocol. Examination of the assay characteristics and reproducibility included analyses of two sample sets a NEN gold standard comprising transcripts from three individual neuroendocrine tumor cell lines and clinical samples from 107 individual SI NENs.

The LOB for the assay was 0. The LOD ranged from 0.21–0.43 ng/μL (gold standard-clinical samples) and the LOQ ranged from 0.63–1.32 ng/μL. The dynamic range of the assay was 100–1000 ng/μL. These results demonstrated that samples ≥200 ng/μL could be effectively and reproducibly analyzed at a C_Q cut-off of 40 cycles. False negatives were calculated to be <1%. Further observations from subsequent individual patient studies (n~400 samples) [17] using the LOD as 40 cycles and 200 ng/μL cDNA identified target amplification in 95.3%±0.2% of genes examined. These parameters compare well with similar effective laboratory tests. For example, a clinical laboratory qPCR-based protocol for the detection of BCR-ABL fusion genes in CML can amplifies the gene or its fusion in 90%–94% [32].

The inter-assay variability was 0.5%–2.1% while the intra-assay variability ranged from 0.4% to 1.02%. Using hydrolysis probe assays, the variability between duplicates and triplicates within the same run, and between different runs, was between 0% and 5% [21]. In the case of multiplex approaches this may range as high as 13% [33]. Our protocol results are therefore consistent with other hydrolysis probe-based studies [8, 18–21].

In 14 patients, the CV for the same sample analyzed on two different days was 5.3% demonstrating the effectiveness of this approach. A CV of 4%–24% can routinely be achieved and is generally reported and accepted [34, 35]. While C_Qs are generally used as measures for reproducibility, they are logarithmic units and may misrepresent true variability [34]. A number of other groups have generated scores (usually a summation) from normalized qPCR values, e.g., for interferon gene expression [36] or T-cell function [37] (both in rheumatoid arthritis [RA]). Although these demonstrate efficacy in differentiating RA subtypes, there exists limited information in the literature validating the robustness of summation-based scores. Our final output is a MATLAB-generated score from the normalized expression values (the NEN score). Assessment of scores identified these to be significantly correlated and was not different between the two assay times (CV=0.9–0.95). This indicates this additional parameter was highly stable. By way of comparison, the correlation coefficients for gene expression in leukemia protocols can range between 0.62 and 0.79 [1]. This demonstrates that the NEN score is as robust as clinically-based PCR assays. It is also significantly better than other NEN assays, e.g., the currently used peripheral blood biomarker chromogranin A (CgA). The CgA assay specifics vary broadly with median sensitivities of 63% and CV of approximately 0.6 between test platforms [22] compared to the NEN score which exhibits sensitivities of >90% and CV >0.9 [17].

Circadian alteration in CTCs appears in a minority of patients (<5%) [38]. However, data on day-to-day variation is not available. We sought to evaluate whether a molecular signature was stable or exhibited significant daily variability. Samples did not exhibit significant day-to-day variation which indicates that variability in the qPCR test is low, an important requisite for longitudinal evaluation of clinical status.

Food intake significantly alters the level of GEP NEN biomarkers thus patient fasting is an important prerequisite for diagnostic accuracy. The assessment of fasting samples is therefore a necessity for numerous GEP-NEN peripheral blood biomarkers including CgA, gastrin, pancreatic polypeptide, insulin, serotonin, VIP and somatostatin [22]. In assessment of the molecular footprint, unsupervised hierarchical clustering failed to identify intrinsic relationships between feeding and gene expression, and the NEN score was not altered over a 4-h period following a test meal. We therefore conclude that the fingerprint is robust and is not affected by food intake.

Other factors that may influence the score include sex, age, ethnicity and medication. The latter is particularly influential for the CgA assay which is elevated by PPI usage [39]. We could identify no relationship between age, sex and ethnicity and the NEN score in an analysis of 61 patients. No elevation in NEN score (0–1) was noted in 11 GERD patients (10 with elevated CgA) treated with PPIs >1 year.

The GEP-NEN molecular signature was stable for approximately 2 h at 4°C; gene expression, however, decreased as a function of storage and RNA degradation. To ensure optimal and reproducible data, samples should be stored at −80°C within 2 h of collection. A critical feature of any test development is the ability to confirm the integrity/suitability of the sample under evaluation. Typically, the complete RNA pattern on electrophoresis (RIN score) [40] is used to assess sample degradation in qPCR-based protocols. We evaluated the efficacy of storage time

(at 4°C) on the RIN score and determined prospectively whether any set of genes could be used as a predictor of sample integrity. Our initial analyses identified a variable degradation in RNA with storage but also identified the expression of two candidate genes, *APLP2* and *CD59*, as accurate markers of degradation. In 56 prospectively collected samples, we identified significant correlations between normalized gene expression, the predicted processing times and the RIN scores. This confirmed that the expression of both these genes could be used as surrogate markers of adequate sample collection and processing.

In this manuscript, we have examined the assay characteristics and reproducibility of a peripheral blood qPCR-based fingerprint ("NEN score") for GEP-NENs to assess the robustness of the process. Our data demonstrates that the 51 gene transcript signature was robust and reproducible, exhibiting suitable inter- and intra-assay metrics (<5%). The score was stable and unaffected by feeding, age, sex, ethnicity or long-term PPI usage. These parameters indicate this may provide an accurate and sensitive multi transcript molecular tool to identify NENs and assess disease progress using peripheral blood samples.

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Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article. Research funding played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.
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