Automated Measurement of Gene Expression from FFPE: qNPA vs. qPCR

Presented at Molecular Med Tri-Con | San Francisco, CA | February 2013

Matt Rounseville[1], Klaus Pechhold[2], Debrah Thompson[1], Mark Schwartz[1], Bruce Seligmann[3]
[1]HTG Molecular Diagnostics, Tucson, AZ, [2]University of Massachusetts Medical School, Worcester, MA, , [3]MDDx, Tucson, AZ

INTRODUCTION

Accurate, sensitive and robust multiplexed measurements of gene expression from formalin fixed paraffin embedded (FFPE) tissue or paraformaldehyde fixed and stained samples sorted by flow cytometry (FCS) are ideally required for clinical diagnostic tests and retrospective analysis of archived samples. We evaluated the measurement of gene expression from FFPE tissues using HTG Molecular's qNPA™ assay and made comparisons to qPCR. The results show that qNPA provides a highly sensitive, accurate, quantitative, and robust multiplexed assay of gene expression from fixed samples on the fully automated EDGE System. qNPA uses a lysis-only, extraction-free protocol, in contrast to qPCR which requires RNA extraction/ reverse transcription. Accuracy was determined by correlating measurements from fresh frozen versus FFPE.

qNPA measurements provided results from FFPE which correlated to matched frozen, R=0.97, and provided a 91% "Present Call" rate using just 0.25 cm^2 area of a 5 μm section. qNPA measurements provided results from paraformaldehyde fixed and stained cells prepared for FCS which correlated to unfixed, unstained, R^2=0.98.

In contrast, qPCR measurements provided results from FFPE which correlated poorly to matched frozen, R=0.86 (not statistically valid), and provided only a 17% "Present Call" rate, using 6.4 cm^2 area of a 5 μm section. qPCR measurements did not provide any useful data from paraformaldehyde fixed and stained cells prepared for FCS.

Thus, qNPA provides data on gene expression from multiple types of clinical specimens which qPCR cannot.

MATERIALS & METHOD

Sample Preparation Kit and Protocol for Fixed Tissue on EDGE System

- Select samples
  - FFPE, Paraffin embedded FFPE
  - Paraformaldehyde fixed, non-HIERT antibody stained, and FCS cells
- Add fixed tissue, lysis buffer & denaturation oil from sample prep kit
  - Incubate at 95°C for 10 min
- Add proteinase K from sample prep kit
  - Incubate at 50°C for 60 min
- Transfer lysates to 96 well sample plate

Walk-Away qNPA Assay on the EDGE Processor

- Flexible format, for up to four (4) unique RUO assays simultaneously on the same arrayplate
  - Select plate format and sample layout
  - Multiplex up to 47 genes per sample
  - Up to 4,512 data points per plate
- Select assay protocol and sample layout
- Load reagents and consumables
- Load sample plate prepared above
- Press the Start Button
  - EDGE Processor performs the qNPA assay, generates an ArrayPlate ready for imaging and data analysis

Analysis on the EDGE Reader

- Transfer the ArrayPlate from the EDGE Processor to the EDGE Reader and add Reader Reagent Tray
- Press the Start Button
  - EDGE Reader provides analytical data based on captured images
    - Level of each gene, each sample
    - Averaged (between replicates) and normalized level of each gene (based on selected housekeeper genes)
    - Reproducibility (standard deviation and %CV)
- View and export data for further analysis
- Print quality control and sample run reports

EDGE SYSTEM

Processor **Reader**

FFPE STUDY

Measurements from FFPE by qNPA vs TaqMan

Matched samples of FFPE and fresh frozen pancreas tissue purchased from Asterand, 35 genes measured by a single well qNPA assay Kit or by a multi-well TaqMan assay

RNA extracted from fresh frozen tissue by Asuragen and used as a reference sample

- RNA Integrity Score (RIN) of 6.7 (minimally degraded)
- qNPA performed by HTG
  - Negative control ANT plant gene run as component of each array, each sample tested
  - 35 genes gave measurable and quantifiable signal above the negative control
- qRT-PCR performed by Asuragen, catalog TaqMan primers selected producing the shortest amplicons, avg. 73 bases
  - 30 ng/sample,
  - No Template Control (NTC) and placenta RNA positive control run as separate samples
  - 32 genes gave measurable values, but only 29 were significantly expressed above NTC

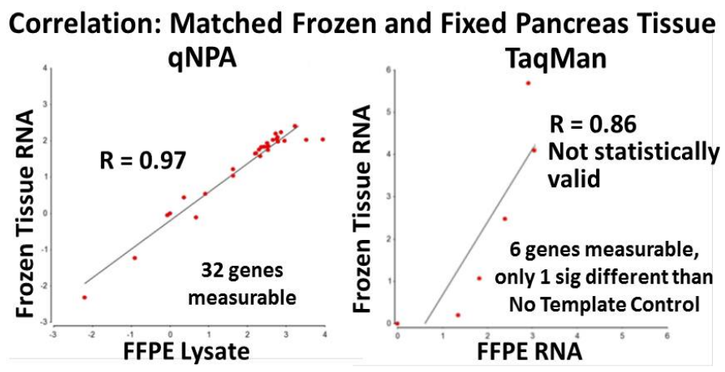
RNA extracted from FFPE by Asuragen for TaqMan Assay measurement

- 32 μm thick by 1 cm^2 section required to measure 35 genes by TaqMan (or 6.4 cm^2 area of a 5 μm thick section)
- RIN of 1.1 (highly degraded)
- 6 genes measurable
  - Present Call rate of 17%, based on these 6 genes having measurable expression
  - Ct's for FFPE RNA for the 6 genes were 11 to 13 Ct units higher than measured from RNA extracted from frozen
  - Only 1 of the 6 genes was significantly expressed above NTC
- R^2 correlation, fresh frozen to FFPE, of 0.86 for the six genes, not statistically valid

FFPE lysed for qNPA measurement

- 0.25 cm^2 area of a 5 μm section required, per sample, to measure all 35 genes
  - Same amount of sample required to measure up to 47 genes
- 32 genes gave measurable and quantifiable signal above the negative control
  - Present Call rate of 91%
- 0.97 R^2 correlation of fresh frozen to FFPE for the 32 genes

FFPE DATA



- qNPA: 32 genes measured from FFPE as significantly expressed compared to negative control.
- TaqMan: R value not statistically valid, of 6 genes measured from FFPE, only one had significant expression compared to NTC

TaqMan Data Analysis

Gene	Frozen	FFPE	Gene	Frozen	FFPE
ACTB	36.66	ND	MAST2	29.69	ND
ANUBL1	31.56	ND	NEIL1	31.68	ND
B2M	23.41	37.59	PMM2	26.87	ND
BATF	34.31	ND	RPLP0	29.62	ND
BLNK	29.83	ND	SEL1L3	25.8	ND
BSPRY	27.31	39.28	SEPX1	28.74	ND
C13Orf18	33.31	ND	SLC38A5	25.85	ND
CYB5R2	33.18	ND	SPINK2	34.96	ND
FAM46C	26.36	ND	SSR3	24.28	38.06
FOXP1	34.92	ND	STAG3	37.23	ND
GAPDH	23.21	36.24	TARS	26.03	ND
HSP90B1	27.11	ND	TEX9	30.82	ND
KCNH8	32.01	ND	TFRC	28.28	ND
LMAN1	25.69	38.68	TOX2	39.42	ND
LRMP	28.9	39.15	TRAM2	28.4	ND
MARCKSL1	27.98	ND	ABTN32	39.14	ND

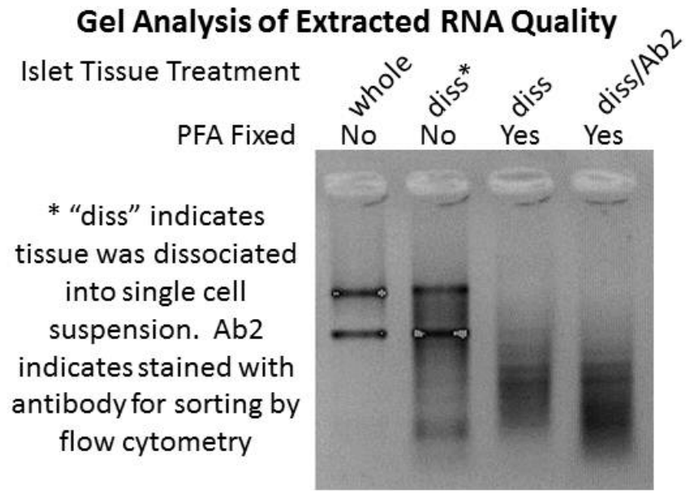
Ct values for 3 genes measured from fresh frozen pancreas RNA were not significant (within 3 SD of the NTC value, yellow highlighted). Ct values for 5 of 6 genes measured from FFPE RNA were not significant. FFPE Ct's were ~11 to ~13 Ct higher than the Ct measured from frozen sample RNA (red values).

FIXED/STAINED CELLS STUDY

Measurement of mRNA from Tissue Cells Prepared for Flow Sorting

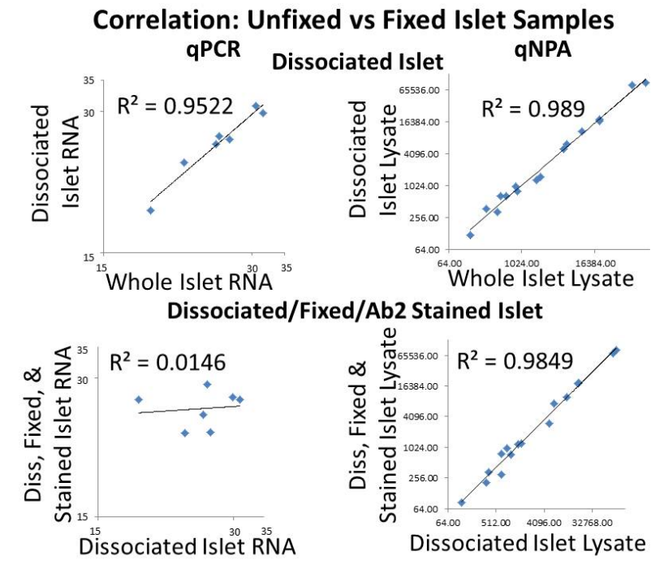
- Islet tissue was prepared whole as reference control sample
  - Lysed and measured using qNPA
  - RNA extracted and measured by qPCR
- Islet tissue was dissociated into single cell suspension, fixed in paraformaldehyde (PFA), stained, ready for FCS
  - Lysed and measured using qNPA
  - RNA extracted and measured by qPCR

Gel Analysis of Extracted RNA Quality



* "diss" indicates tissue was dissociated into single cell suspension. Ab2 indicates stained with antibody for sorting by flow cytometry

FIXED STAINED/CELLS DATA



- qPCR and qNPA measurements from whole islet and dissociated islet RNA are identical (R^2=0.95 and 0.99, respectively). Fixing leads to degradation of the RNA extracted from these samples, and useless qPCR data (R^2 = 0.01).
- qNPA measurements from fixed and stained islet cells give identical results as from whole islet or dissociated islet (R^2 = 0.98).

CONCLUSIONS

qNPA enables accurate and robust measure of gene expression from fixed samples

- Frozen control correlated to FFPE, R = 0.97
- qNPA Present Call rate of 91%
- Relatively insensitive to RNA degradation
  - RIN of 6.7 for RNA extracted from frozen tissue compared to RIN of 1.1 for RNA extracted from FFPE, indicating significant degradation
  - TaqMan did not provide significant data from FFPE
- Fixed and stained samples prepared for FCS
  - Whole unfixed tissue measurements correlated to dissociated/fixed/stained for FCS, R^2 = 0.98
  - RNA extracted from fixed and stained samples prepared for FCS was degraded and did not provide useful qPCR data

qNPA enables the sensitive measurement of gene expression from FFPE tissue

- 0.25 cm^2 area of a 5 μm section per sample to measure expression levels of up to 47 genes
  - Taqman required 26 times more sample to measure just 35 genes

Sample preparation of FFPE tissue for qNPA assay is simple, just add reagents and incubate

qNPA provides standardized measurement from FFPE tissue using the automated EDGE System

3430 E. Global Loop | Tucson, AZ 85706 | (877) 289-2615 | htgmolecular.com