Comparison of qNPA vs qPCR on FFPE Tissue

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INTRODUCTION

Accurate measurements of gene expression fixed tissue and cells are required for clinical diagnostic tests and retrospective analysis of archived samples. We evaluated the measurement of gene expression in FFPE tissues or cells prepped for flow cytometry using HTG Molecular's qNPA™ assay and/or qPCR. qNPA uses a lysis-only protocol and does not require extraction or amplification of sample RNA. The results show that HTG's qNPA provides a sensitive and accurate method for measuring gene expression in fixed tissue samples.

First, performance specs for qNPA were established across different fixative and ischemic times. Time courses demonstrated that fixation times up to 72 hours, or ischemic times of up to 8 hours, do not significantly affect signal profile or intensities. This is important, since most clinical or retrospective studies include samples collected from a range of laboratories where the fixation and ischemic times are likely to differ. A collation of service metrics from 1,625 FFPE cancer samples (run in triplicate), shows that the failure rate for qNPA is low (0 to 2%), and the reproducibility is good (8% to 14% CV). Together, these studies demonstrate that qNPA is a robust method that works well on fixed tissue, even when the fixation and ischemic times are varied.

Second, a comparison study between qPCR and qNPA was performed using mirrored frozen and fixed pancreas tissue. Both qPCR and qNPA performed well on RNA from the frozen tissue. However, qPCR performed poorly on RNA extracted from FFPE (RIN = 1.1), with only 6 of the 35 genes called as "present". These 6 genes had acceptable correlation to their levels in frozen tissue (R=0.87). In contrast, qNPA returned a "present call" of 32 of the 35 genes in FFPE lysate, with good correlation to the frozen tissue RNA (R=0.97). Further, qNPA required approximately 25 times less fixed tissue than qPCR to achieve these results. The results highlight the advantages of the extraction-free qNPA method: small sample size, excellent correlation to frozen tissue samples, and the ability to work with low-quality RNA without significant dropout.

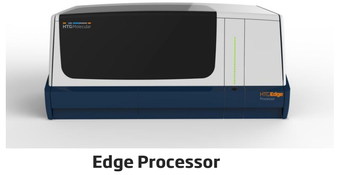
Finally, gene expression in murine islet cells was measured either before or after dissociation, antibody staining, and fixation (in preparation for flow cytometry), using qNPA or qPCR. Both qPCR and qNPA performed well on fresh tissues (dissociated or whole islet cells), with R^2 values of 0.95 or 0.98, respectively. However, once cells were prepared and fixed for flow cytometry, qPCR was less informative, with no significant correlation seen between the fixed and unfixed samples. Conversely, qNPA was able to successfully measure all 16 genes from the fixed and antibody-stained cells, with correlation between fixed and unfixed samples of R^2=0.98. qNPA measurements from islet cells were used to identify induced expression of embryonic gene MafB associated with pregnancy, a result that was confirmed at the protein level using IHC[1]. Overall, these results show that qNPA is an excellent technology for fixed tissues, demonstrating good preservation of gene expression information following fixation.

qNPA™ ASSAY PROCESS

HTG Edge

Automation

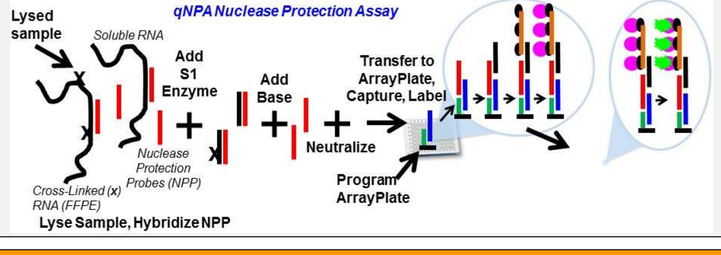
Sample Prep Manual



Edge Processor

Edge Reader

Chemistry



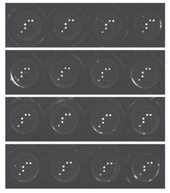
EDGE AUTOMATED qNPA ASSAY: NEW IN 2013

Your samples: cells, RNA, FFPE, fixed/stained/sorted cells
- All reagents provided in Sample Prep Kit

Walk-Away qNPA Assay on the Edge Processor: runs assay with minimal user intervention
- Up to four (4) unique RUO protocols, up to 47 genes per sample or 4,512 data points per plate
- All reagents and consumables provided

Analysis on the Edge Reader
- Transfer the ArrayPlate to the Edge Reader and add provided reagents.
- Edge Reader provides analytical data based on captured images, view and export data, print quality and sample run reports

FIXATION AND ISCHEMIC TIME IMPACT

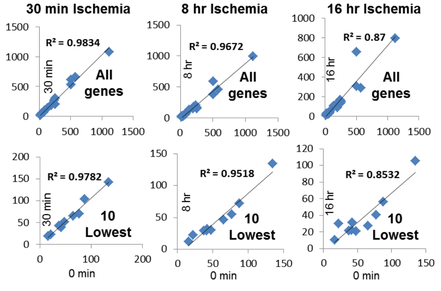


Fixative time:

- Same tissue divided and fixed for indicated times in neutral-buffered formalin. Samples lysed.
- qNPA housekeeper assay run in quadruplicate (shown)
- Fixation times did not significantly affect the qNPA results

Ischemic Time:

- Murine lung tissue divided and placed into fixative at various times after dissection.
- Correlation plots shown of 0 (X-axis) versus up to 16 hr (Y-axis) ischemic time of all 19 housekeeper, or 10 lowest expressed
  - $R^2 \geq 0.95$ up to 8 hr ischemia
  - $R^2 \leq 0.87$ after 16 hr ischemia



Performance on Client Samples (FFPE):

Tissue Type	# of Samples	Area/Well (cm^2)	S1 Failure Rate (%)	Average CV (%)
Breast	300	0.5	1	10.5
H&N	280	0.15	2	14
Lung	700	0.25	0.5	9.5
Lymphoma	300	0.25	0.5	8
Ovary	15	0.13	0	10
Prostate	15	0.25	0	11
Colon	15	0.25	0	9

CONCLUSIONS

- qNPA results are not significantly impacted by fixation times ranging from 4 to 72 hours
- qNPA results are not significantly impacted by ischemic times up to 8 hours for lung tissue. Different tissues are expected to have varied acceptable times.

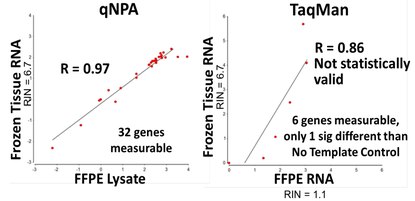
FFPE PERFORMANCE

Gene expression in FFPE: qNPA vs TaqMan

- Matched samples of FFPE and frozen pancreas tissue purchased from Asterand. 35 genes measured by a single well qNPA assay or by a multi-well TaqMan real time qPCR assay.
- RNA extraction and qPCR run by Asuragen.
- Sample lysis and qNPA run by HTG.

Experimental overview:

	qNPA				RT-qPCR			
Source Material	Frozen Tissue		FFPE		Frozen Tissue		FFPE	
Sample type	Purified RNA		Lysate		Purified RNA		Purified RNA	
Amount used	1000ng		1.25 mm		1050ng		1050ng from 32.44 mm	
Genes Detected (3 SD over bckgr or Ct >40)	35	35	31	32	35	35	21	6
% Success	100%	100%	89%	91%	100%	100%	60%	17%



CONCLUSIONS

- qNPA and qPCR performed well on RNA from frozen tissues.
- qNPA on FFPE samples was relatively insensitive to RNA degradation, and had a 91% present call percentage.
- Multiplexed qNPA used far less fixed tissue than the corresponding TaqMan assays.
- TaqMan performed poorly on RNA isolated from fixed tissue.

Learn more about the HTG Edge System

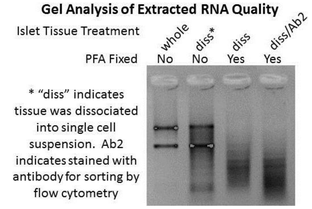
HTG Molecular
3430 E. Global Loop | Tucson, AZ 85706
(877) 289-2615 | htgmolecular.com

FIXED/STAINED TISSUE for SORTING

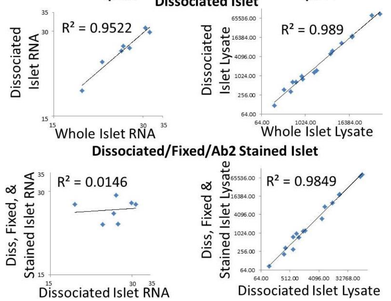
Measurement of mRNA from Cells Prepared for Flow Sorting

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

Gel Analysis of Extracted RNA Quality



Correlation: Unfixed vs Fixed Islet Samples



- qPCR and qNPA measurements from whole or dissociated islet RNA are identical (R^2=0.95 and 0.99, respectively)
- Fixing, or fixing and staining, correlated to whole islet:
  - qNPA R^2 = 0.98, qPCR data (R^2 = 0.01)

CONCLUSIONS

- qPCR did not provide useful data on fixed/stained cells.
- qNPA enables accurate & robust measurement of gene expression from fixed and stained samples prepared for flow cytometry
  - Unfixed tissue correlated to dissociated/fixed/stained, R^2 = 0.98

[1]Pechhold et al, *Nature Biotechnology*, published online 18 October 2009; doi:10.1038/nbt.1579