Comparison of RNA integrity from morphologic annotated tissues by varying preservations

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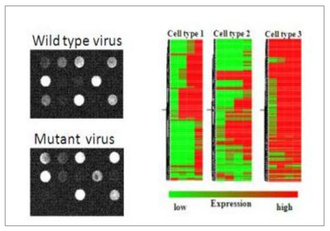
1. Van Andel Research Institute, 2. Translational Genomics Research Institute

Background:

Transcriptome & Functional Genomics ⟷ dynamic genome / epigenome ⟷ tumor heterogeneity and collection strategy for enriched cellular samples

A.

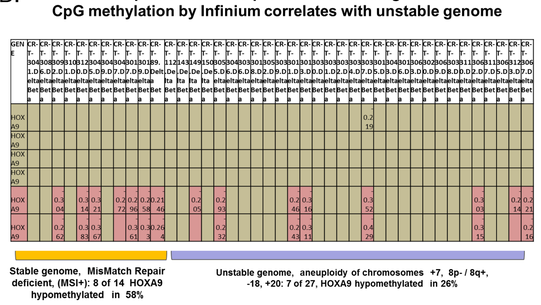


A. The dynamic transciptome by virus depending on human cell type infected. From the Liverpool Microarray Facility (www.liv.ac.uk/lmf/)

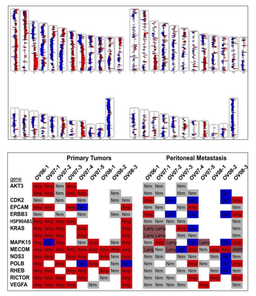
B. Example of gene centric (HOXA, 7p15.3) data overlay from high content assays of arrayCGH (244 K 60-mer oligos, Agilent, Inc.) and CpG methylation status by Infinium Methylation 27K Bead Chip (Illumina, Inc.) in colorectal tumors selected by unstable genome phenotype.

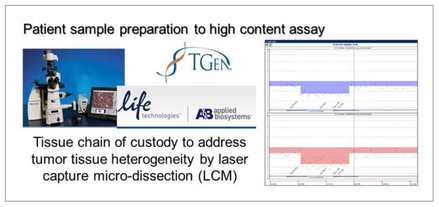
C. Reported data of disparate arrayCGH findings in ovarian carcinoma with peritoneal spread (adapted from reference 6) supports findings in our dosage profiling of 44 ovarian tumors. These findings support reports from public database of TCGA experiments and highlight the need for matching datasets from more labile molecules (RNA, protein).

B.



C.





D. Split sample experiment to assess gene dosage by degree of cellular enrichment; frozen tissue chip assessed with > 50% tumor (top) versus LCM. Same sized deletion of *PTEN*, but LCM aliquot with log 2 ratio of 1.8 vs. 1.2

Materials and Methods:

Transport / preservation medias evaluated:

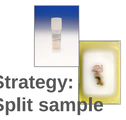
RNAlater, (tissue)
- RNAlater preserved without mirror image for morphology; murine liver harvests, stored RNAlater @-80 deg C ~ 1 month

PaxGene, Qiagen, Inc. (tissue)
- Tissues processed in formalin free processor (otherwise standard chemicals) and paraffin embedded

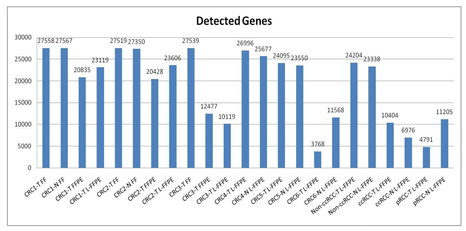
Fresh frozen (gold standard)
- Snap freeze LN, within 20 minutes post expiration

Use of RNAse inhibitors [LCM collected, RNAseq grade]
- Aqueous steps had 1x RNase inhibitors (Sigma, R7397)
- All aqueous solutions made with RNase and DNase free H2O, made fresh daily
- All tissue sections incubated in Neutral Red Stain (Sigma, N4638)

Evaluation by HCA platform:
informative but not cost effective:



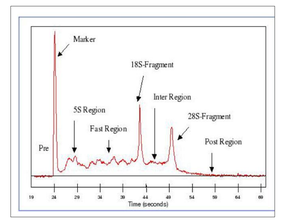
Strategy: Split sample frozen/FFPE Platform: Infinium Human Methylation27, RevB BeadChip

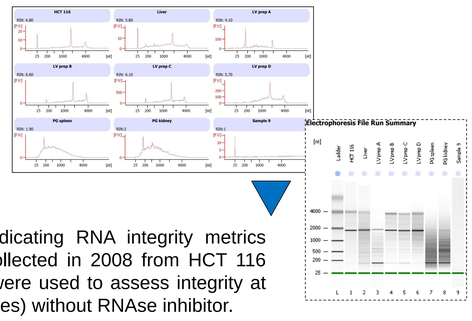


D. Assay performance by detected CpG sites s/p bisulfite treat DNA. HIGH variability FFPE ; 3,768 to 23,606 detected of total 27,000 probes.

Presented at ISBER 2010 annual meeting, Rotterdam, Netherlands, selected oral abstract; G. Hostetter, et.al.

Standardized measures of RNA integrity: Agilent 2100 Bioanalyzer, RNA Integrity Number (RIN) of 1-10



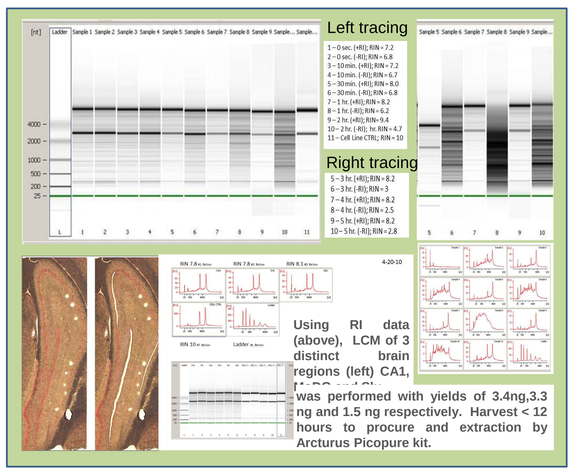


E. Above is annotated graph indicating RNA integrity metrics (see reference 1). Murine liver collected in 2008 from HCT 116 and SW 480 cell line xenografts were used to assess integrity at LCM harvest duration (15-20 minutes) without RNAse inhibitor.

ABSTRACT: The current pace of assay development and accompanying large datasets increases the necessity for optimal quality tissue samples and methods to assess macroanalyte integrity. Herein we show the integrity of RNA from four independent sample sets gathered from different laboratories at our respective institutions to highlight aspects of sample collection, transport and extraction with RNA integrity assessed by Agilent 2100 Bioanalyzer. Experimental data was gathered through an iterative process with fresh frozen aliquot stored at -86 deg. C as the comparative gold standard. Murine liver tissue stored for 4 years (RIN = 7.5) by whole tissue cryosections compared to laser capture microdissected (LCM) Histogene stained tissue with (RIN=6.8) and without (RIN=2.9) RNAase inhibitor ▼. The second tissue preparation set used RNALater® and H&E stained tissue for morphology evaluation with specialized processing step to reduce the associated high salt concentration. Similar to LCM, this approach provided a higher degree of confidence in the isolation of cellular components from heterogeneous tissue samples. The results gave better RIN scores (9.5) from specialized processed samples compared to the fresh frozen counterparts (RIN= 9.2) ● A third collection media assessed was the PaxGene assessment of RNA integrity in samples subject to automated tissue processing and paraffin embedding ▼. The fourth independent dataset demonstrated RNA stabilization from a neuronal subpopulation from the hippocampus utilizing LCM for downstream assay by RNA-seq. A direct comparison of split samples treated with and without Rnase inhibitors showed consistent improved RIN scores in time dependent manner from delta 0.5 (10 minutes) to 2.0 (1 hour) to 4.7 (2 hours) and RIN > 8 at 5 hours, to demonstrate need for RNAse inhibitor in any aqueous containing step ▲ In conclusion, we show our collaborative efforts in RNA analyte preparation for varied collection media and cellular harvests will ensure success in downstream transcriptomics to include excellent RNA-seq results.

Results: RNA integrity by varied preservation with tissue morphology VERIFIED

Cellular enrichment by LCM, fit for downstream application, RNAseq



▲ RNAse inhibitors incorporated into all aqueous steps provided excellent RIN scores in LCM harvested discrete cell populations.

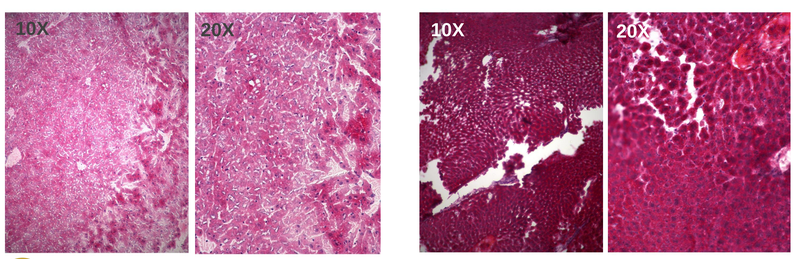
Very poor concordance, PaxGene fixed, paraffin processed; split samples, murine kidney

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	yield (ug)	RIN
1P	SK	2/9/2012	1:32 PM	78.37	1.959	0.928	2.11	2.15	4.7022	2.2
2P	SK	2/9/2012	1:33 PM	45.26	1.131	0.522	2.17	1.51	2.7156	2.7
3P	SK	2/9/2012	1:34 PM	57.55	1.439	0.693	2.08	1.85	3.453	2.5
1F	SK	2/9/2012	3:36 PM	600.72	15.018	7.199	2.09	2.28	30.036	9.5
2F	SK	2/9/2012	3:37 PM	255.46	6.387	3.07	2.08	1.69	12.773	9.8
3F	SK	2/9/2012	3:37 PM	531.05	13.276	6.368	2.08	2.21	26.5525	9.3

P from paxgene block (5x10 um) extracted with Ambion RecoverAll Total Nucleic Acid kit
F from fresh frozen extracted with Qiagen RNeasy mini kit

▼ Split sample of murine whole kidney was used to test effect of formalin free processing of PaxGene preserved and stabilized per commercial recommendations. Paraffin embedding offers comparable morphology (known) for formalin-free processing was tested to assess RNA integrity post PaxGene fixation and stabilization. Preliminary data would indicate unacceptable RINs and potential for degradation in processing process. A next approach would be adding RNAse inhibitors to all aqueous containing steps. Exposure to organics or chemical modification could be contributory and additional experiments are needed.

Adaptation of existing resource RNALater stored tissues, cryo-section / stain for morphology; higher RINs than frozen aliquot



● Murine liver harvests were collected to assess RNA integrity post collection and storage in RNALater in samples that lack morphologic annotation or from heterogeneous tissues (pancreatic cancer). This pilot study compared tissue morphology from OCT embedded fresh frozen (left) and RNALater sample (right) subjected to specialized wash steps and OCT embedded / cryosectioned and H & E stained at 100 and 200 magnification.

Summary / Future Directions

- Disease molecular characterization continues to be **technology-driven**
- **Disparate clinical samples** are increasingly utilized (need appropriate controls)
- **Purity of tissue sample** is critical for downstream applications such as RNA-seq
- Controlled studies to assess RNA integrity by available collection medias **require tissue morphology and fresh frozen reference**
- Critical need to **develop, validate and commercialize** ‘ universal’ preservation medias to provide simultaneous high quality DNA, RNA and protein
- **Determine factors contributing to** ◄ **VS.** ▲ **RNA sample**

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