

Effects of *ex vivo* Duration and Host Organ Contamination on RNA Expression Profiles of Breast Cancer

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Overview of the Project

Nested study design to evaluate sources of variance that affect microarray-based gene expression

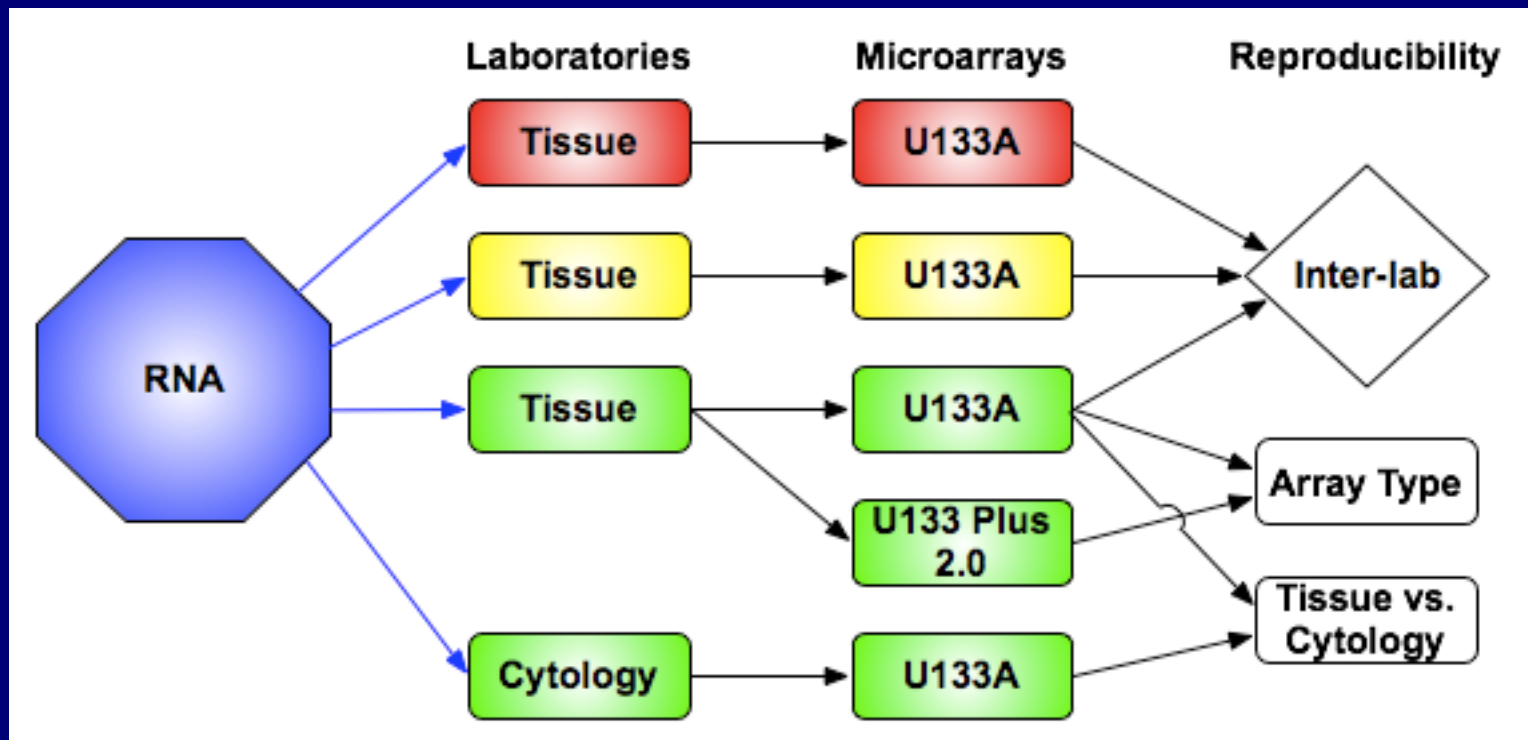
- **Pre-analytical variance in the context of biological and analytical variance**
 - **Inter-laboratory variance**
 - **Inter-sample type variance**
 - **Intra-tumoral variance**
 - **Analytical variance of key steps in the assay**
 - **Variance due to host organ dilution or contamination of a clinical sample**
 - **Duration of *ex vivo* delay until sample preservation**

Annotation of clinico-pathologic information relevant to sample

Evaluation of RNA quality metrics, single gene expression, multi-gene signatures relevant to breast cancer

Overview of This Project

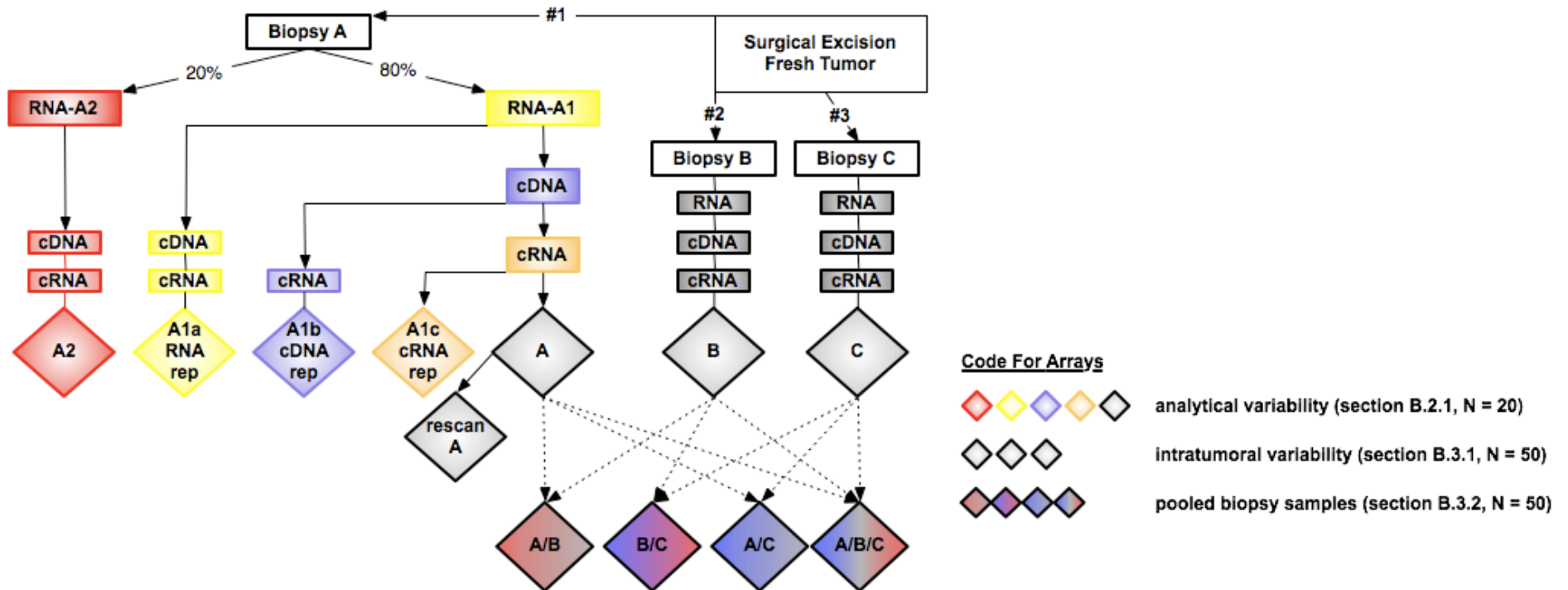
Inter-laboratory and Inter-sample Type Reproducibility (Years 1 – 3)



Collected 200 of desired 245 samples from 6 regional sites
Sample size for complete analysis = 125 tumors

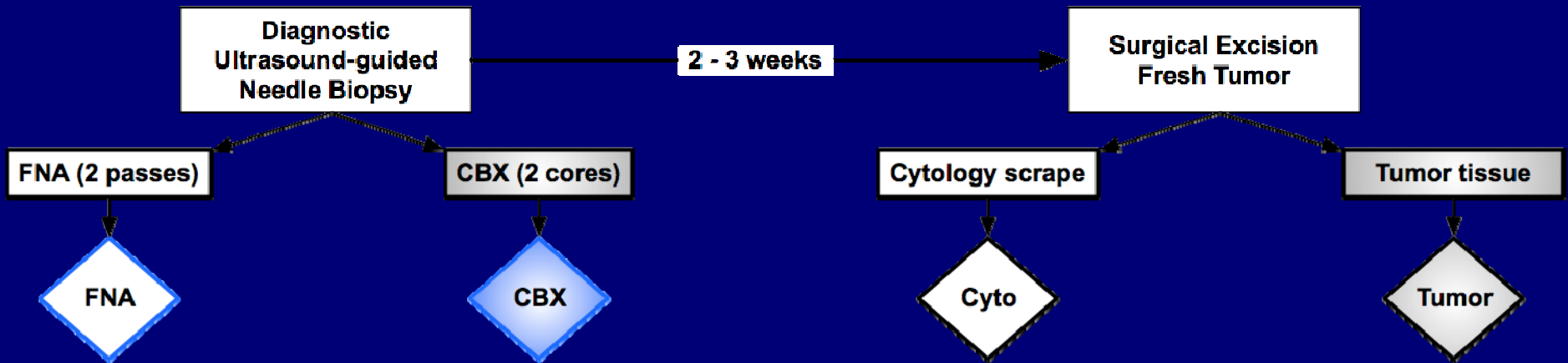
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Intratumoral heterogeneity in the context of analytical variance (Years 2 – 3)



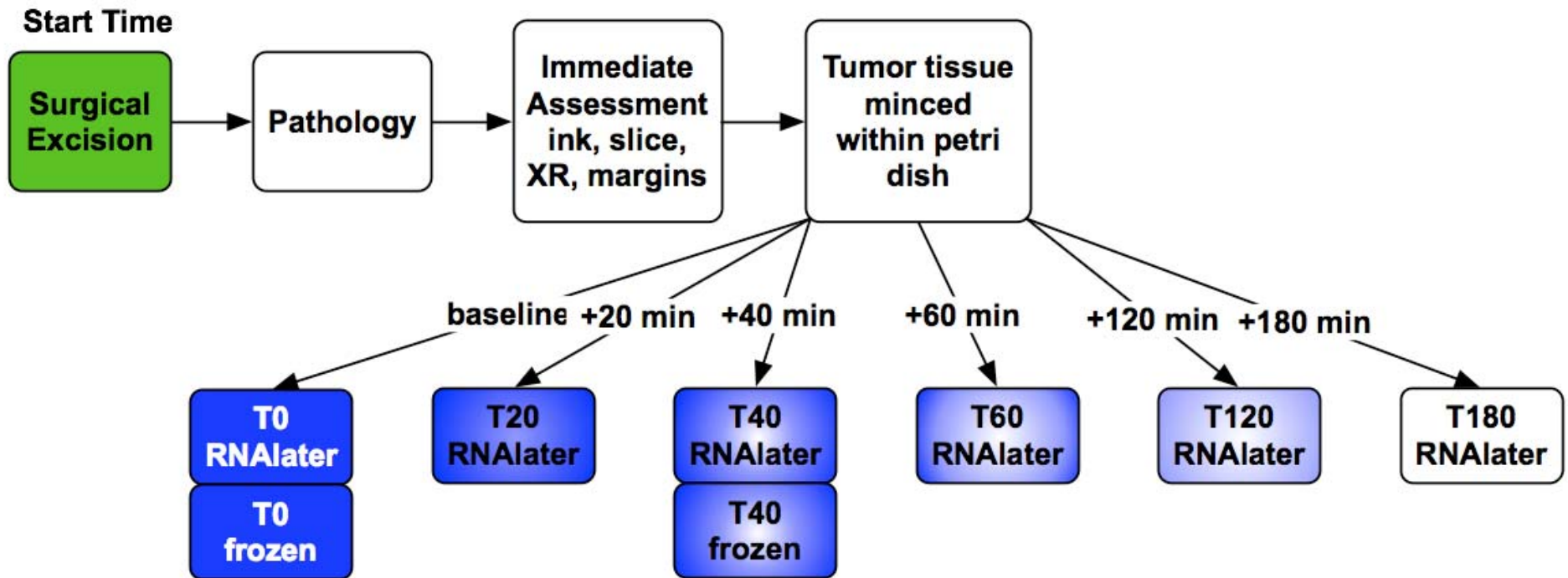
Overview of This Project

Clinical versus Surgical Sample Procurement (Year 3)



Duration of Sample Degradation

Year 1



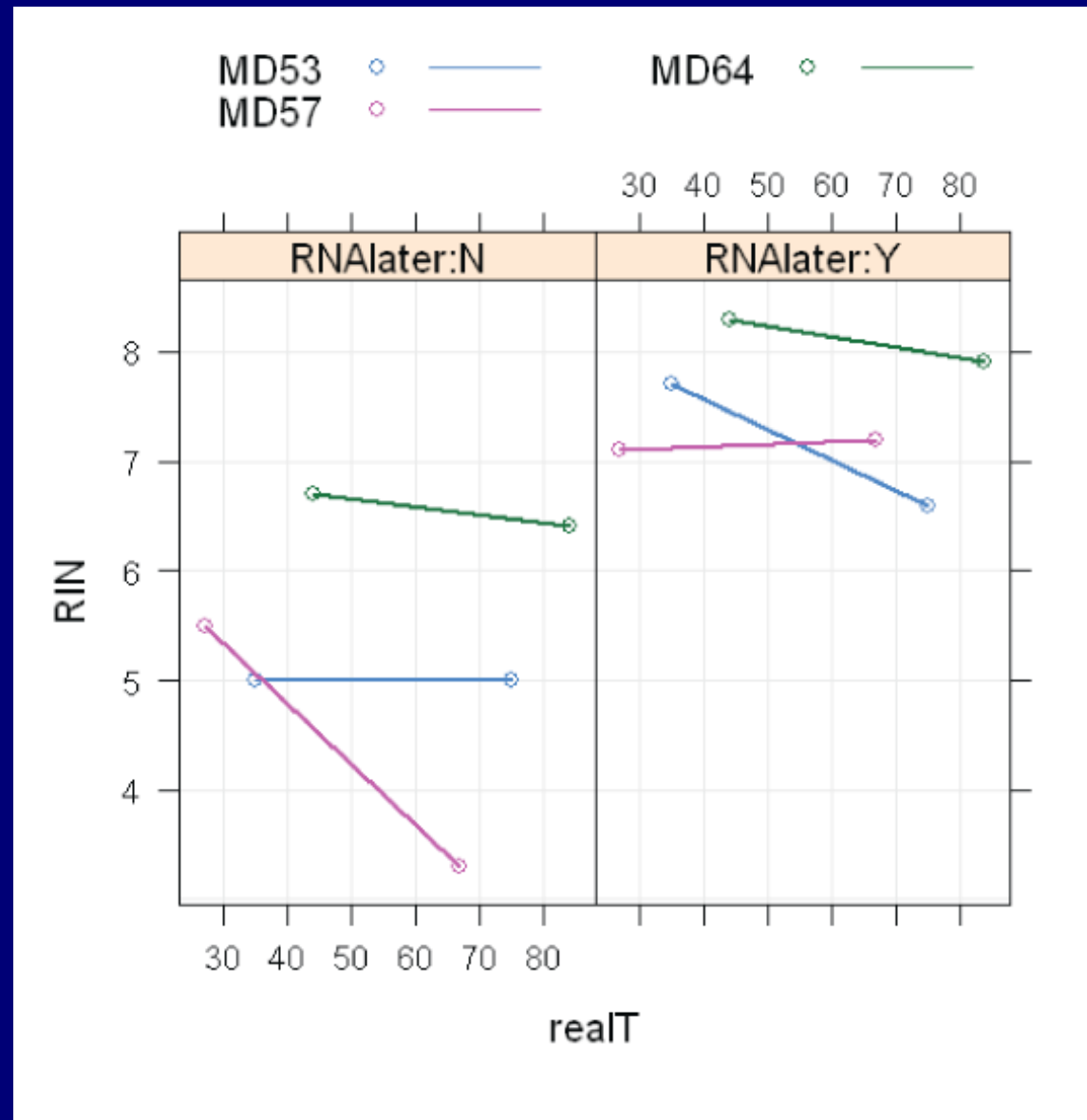
Assess RNA quality:

- RIN from bioanalyzer
- 3'-5' ratios of housekeeper genes
- Single gene expression values
- Multi-gene expression signatures

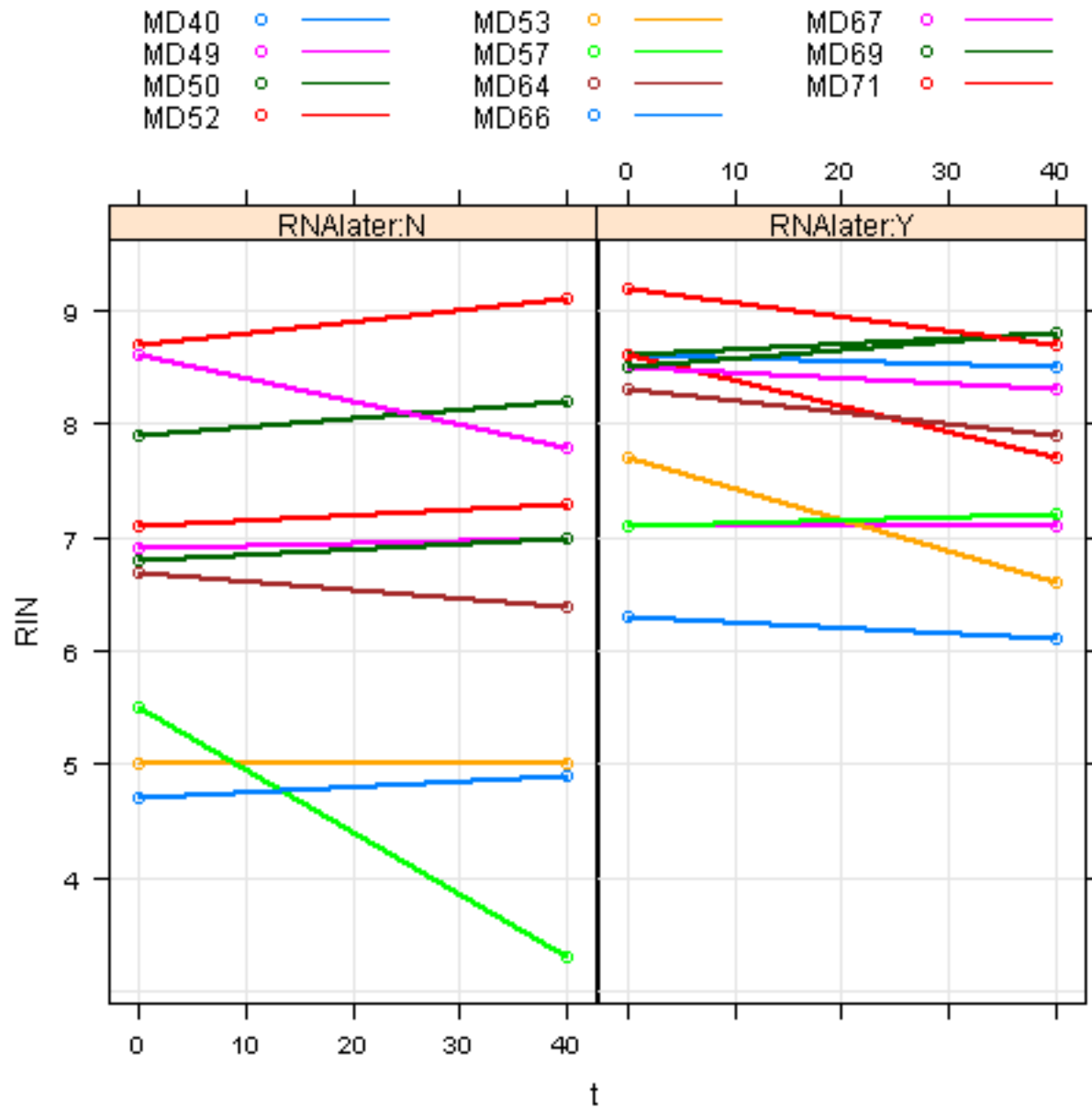
Tumors Included in Degradation Study

Sample	ER	PR	HER2	Grade
MD40	N	N	N	3
MD49	P	P	N	2
MD50	P	P	N	2
MD52	P	P	N	2
MD53	N	N	N	3
MD57	P	P	N	2
MD64	N	N	P	3
MD66	P	P	N	2
MD67	P	P	N	2
MD69	P	P	N	2
MD71	P	P	N	3

Initial Results: Snap Frozen vs. RNAlater



Comparison of Frozen vs. RNAlater



Description of Variables Analyzed

Variable	# probe sets	Description
RIN		RNA Integrity Number
3'5' actin	2	Housekeeper RNA 3' to 5' probe set ratio
3'5' GAPDH	2	Housekeeper RNA 3' to 5' probe set ratio
3'5' 18SrRNA	2	Housekeeper RNA 3' to 5' probe set ratio
ESR1	1	Estrogen receptor mRNA (ER α)
ERBB2	1	HER2 gene mRNA
Ki67	1	Proliferation gene mRNA
SET index	165	ESR1-related transcriptional signature
GGI index	96	Genomic grade index
Recurrence score	21	Microarray-based approximation of a commercial prognostic test (RT-PCR)
Intrinsic Subtype	43	Genomic classification signature

Analyses of Duration *ex vivo* Until RNA later First 3 hours (T0 to T0+180')

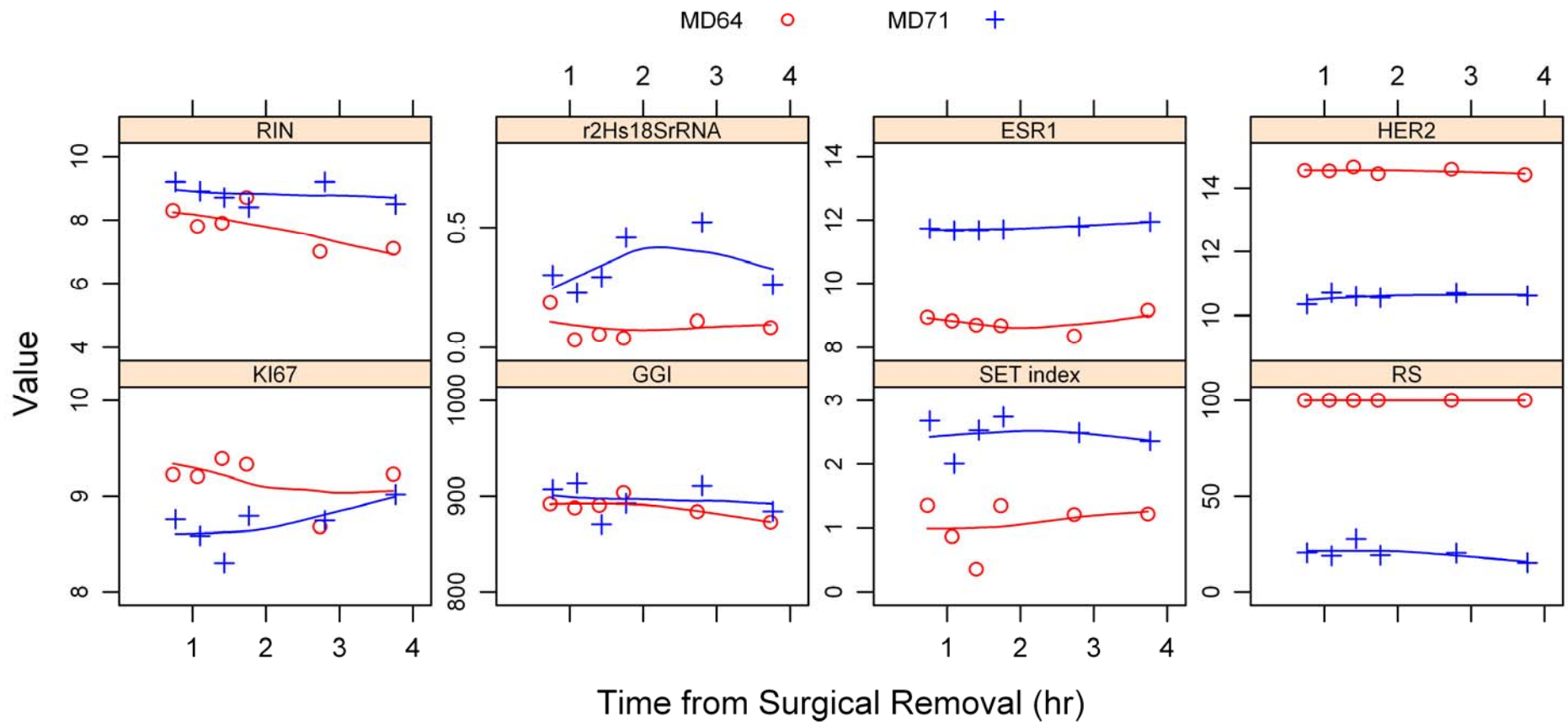
Variable	# tumors	# samples	t-value	p-value
RIN	10*	60	-2.22	0.03
3'5' actin	11	66	2.34	0.02
3'5' GAPDH	11	66	0.96	0.34
3'5' 18SrRNA	11	66	2.85	0.01
ESR1	11	66	-1.13	0.26
ERBB2	11	66	-1.63	0.11
Ki67	11	66	0.45	0.66
SET index	11	66	-1.78	0.08
GGI index	11	66	-2.75	0.01
Recurrence score	8	48	0.17	0.87
Intrinsic Subtype	11	66	0.96	0.34

**Negative t-values indicate degradation for RIN and gene expression
Positive t-values indicate degradation for 3'5' gene ratios**

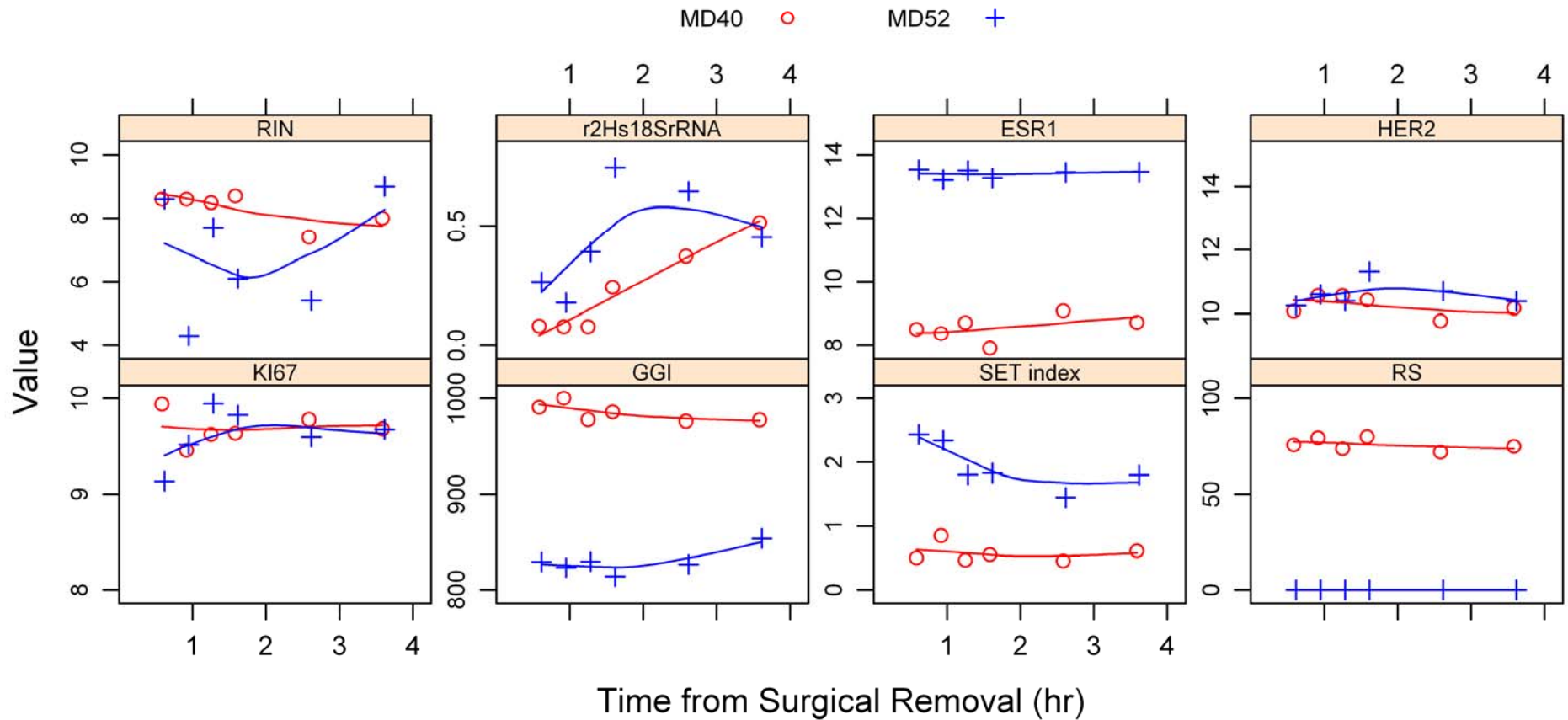
Intrinsic Subtype by *ex vivo* Duration

Sample	ER	PR	HER2	Grade	baseline	+20 min	+40 min	+60 min	+120	+180
MD40	N	N	N	3	Basal	Basal	Basal	Basal	Basal	Basal
MD49	P	P	N	2	Lum A	Lum A	Lum A	Lum A	Lum A	Lum A
MD50	P	P	N	2	Lum A	Lum A	Lum A	Lum A	Lum A	Lum A
MD52	P	P	N	2	Lum A	Lum A	Lum A	Lum A	Lum A	Lum A
MD53	N	N	N	3	Basal	Basal	Basal	Basal	Basal	Basal
MD57	P	P	N	2	Lum A	Lum A	Lum A	Lum A	Lum A	Lum A
MD64	N	N	P	3	HER2	HER2	HER2	HER2	HER2	HER2
MD66	P	P	N	2	Lum A	Lum A	Lum A	Lum A	Lum A	Lum A
MD67	P	P	N	2	Lum A	Lum A	Lum A	Lum A	Lum A	Lum A
MD69	P	P	N	2	Lum A	Lum A	Lum A	Lum A	Lum A	Lum A
MD71	P	P	N	3	Basal	Lum B	Lum A	HER2	HER2	LumA

Case Illustration of RNA Measurements ER-/HER2+ and ER+/HER2- Breast Cancer



Case Illustration of RNA Measurements ER-/HER2- and ER+/HER2- Breast Cancer



Analyses of Duration *ex vivo* Until RNA later First hour (T0 to T0+60')

Variable	# tumors	# samples	t-value	p-value
RIN	10	40	-1.79	0.08
3'5' actin	11	44	0.58	0.57
3'5' GAPDH	11	44	1.34	0.19
3'5' 18SrRNA	11	44	2.84	0.01
ESR1	11	44	-0.69	0.50
ERBB2	11	44	-1.11	0.28
Ki67	11	44	0.32	0.75
SET index	11	44	-1.98	0.06
GGI index	11	44	-2.92	0.01
Recurrence score	8	32	0.66	0.52
Intrinsic Subtype	11	44	1.14	0.27

Negative t-values indicate degradation for RIN and gene expression
Positive t-values indicate degradation for 3'5' gene ratios

Comments on RNA Degradation Study

The expression of mRNA species with known relevance to breast cancer are quite stable after prolonged duration of cold ischemia

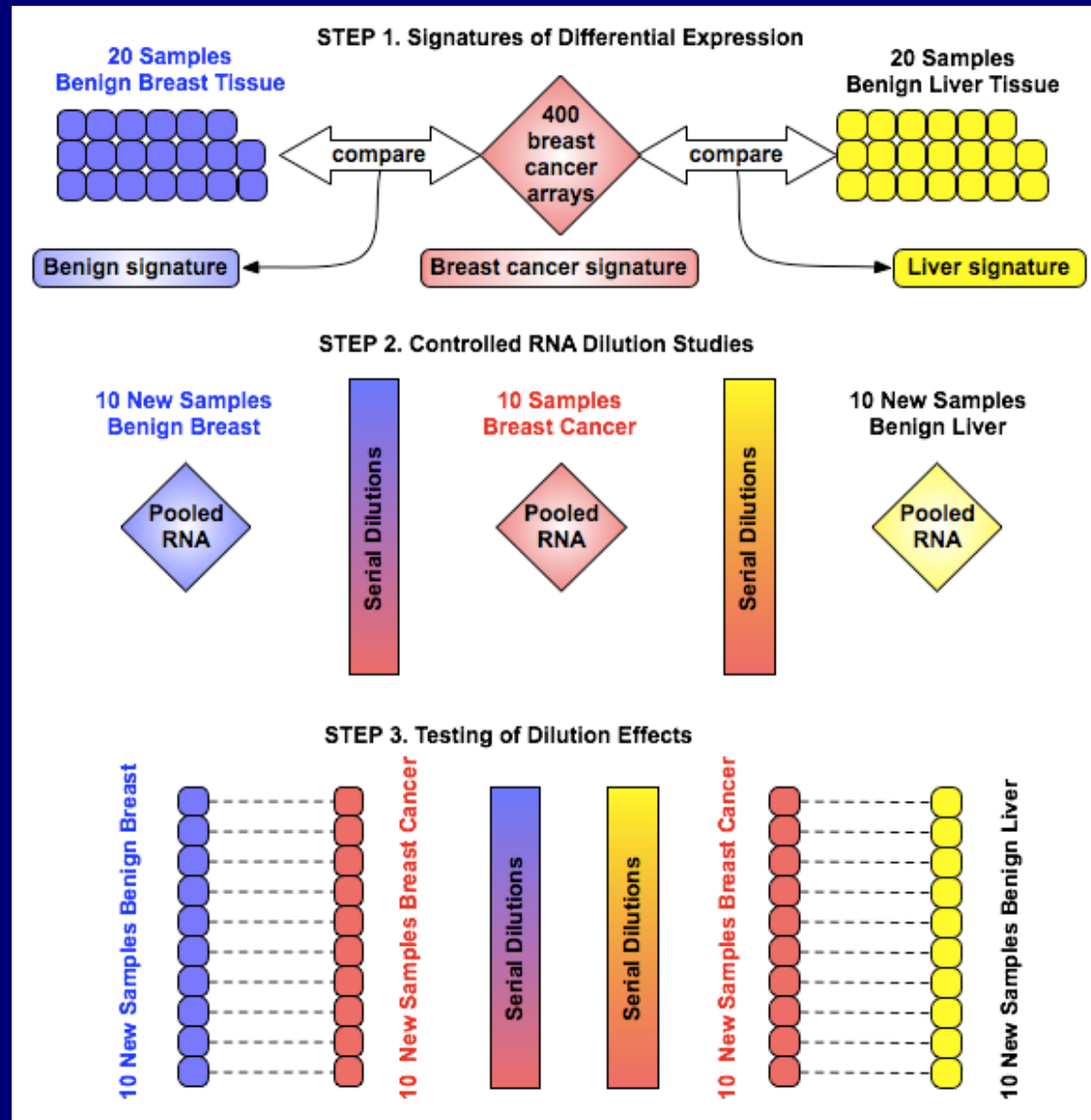
- RNAlater is a practical solution that provides good preservation of RNA after cold ischemia
- Difficulties with snap frozen preservation were partially overcome by stabilization of sample immediately after removal from the -70 freezer
- Anticipate that some mRNA species or signatures might behave differently during prolonged cold ischemia
- Different approaches to data normalization techniques should also be studied

Although statistically significant trends can be identified using RIN or 3':5' expression ratios for some housekeeper genes, these alone would not adequately inform a quality control process:

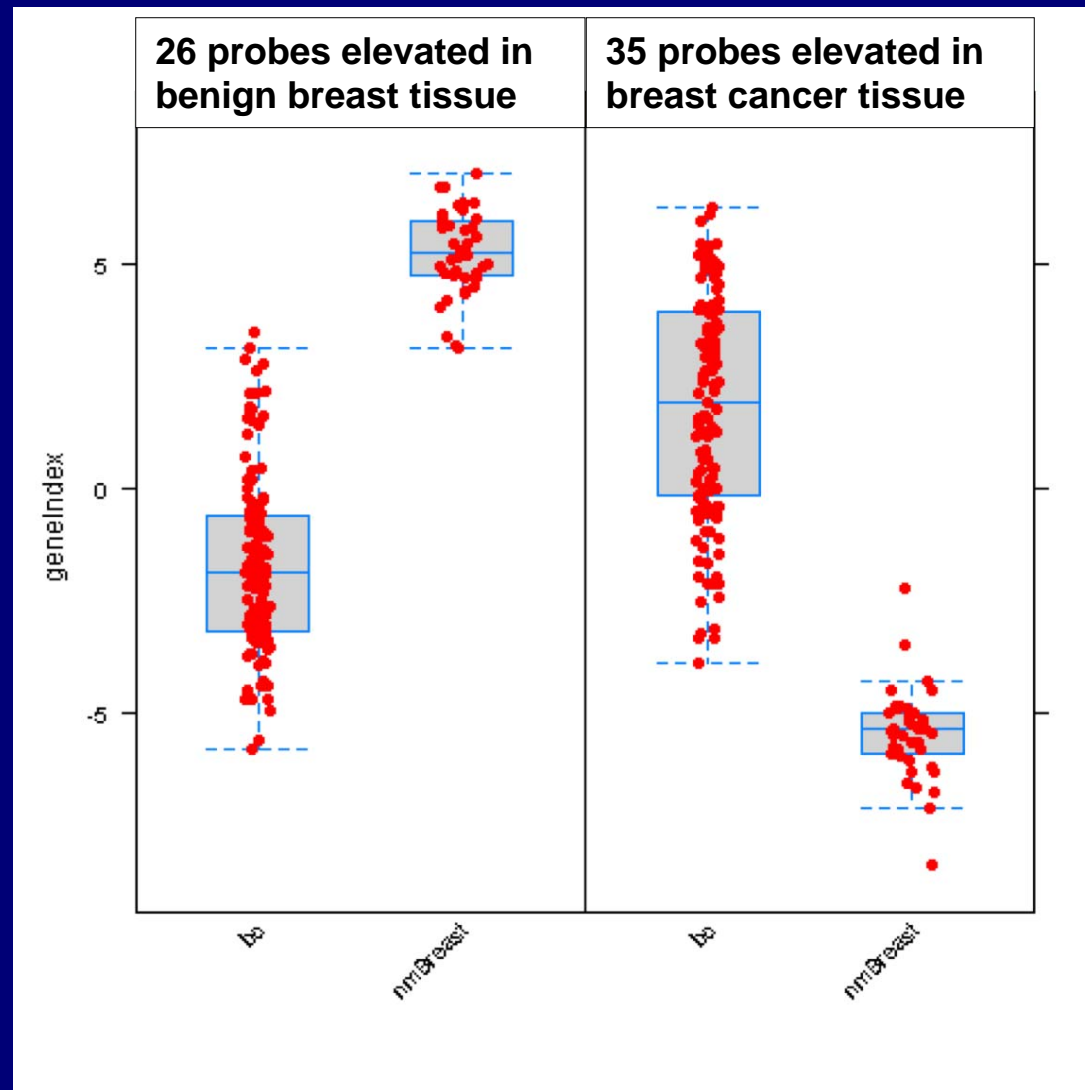
- The absolute effects are modest
- The effects are not consistent among different tumors

Effect of Host Organ Contamination

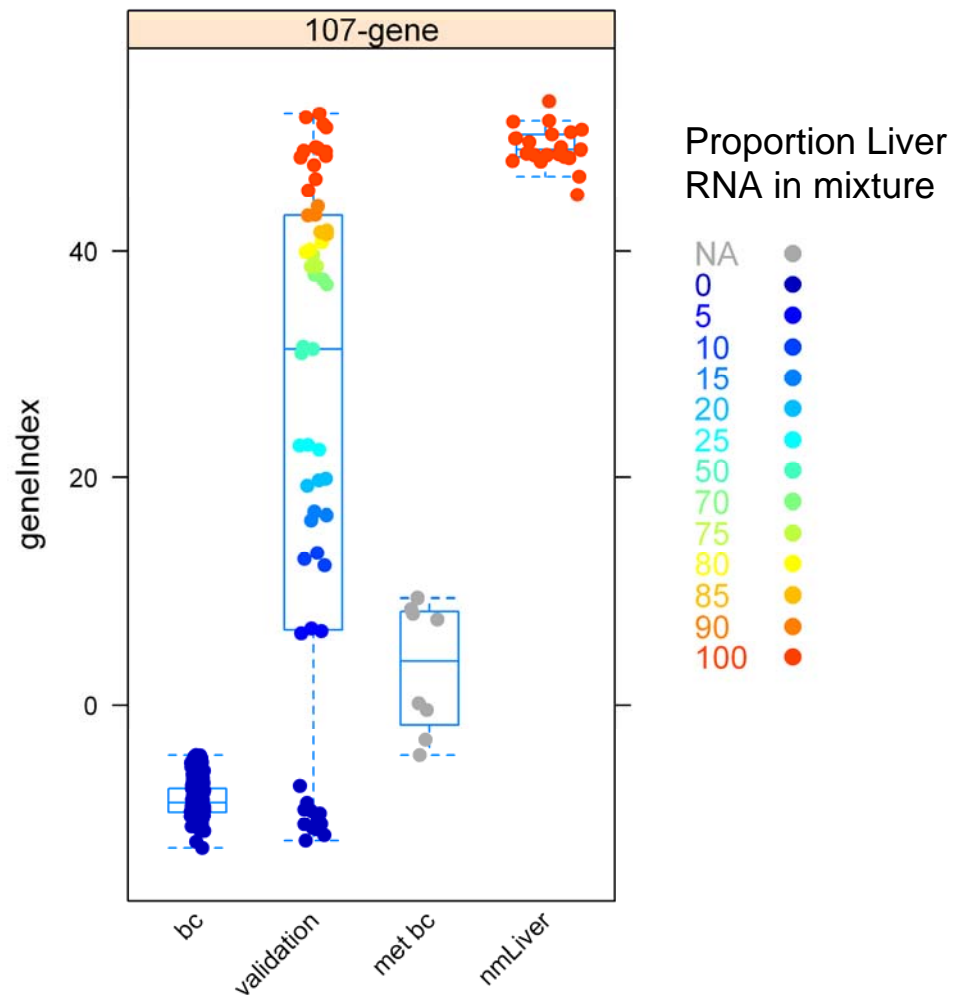
Years 1 - 2



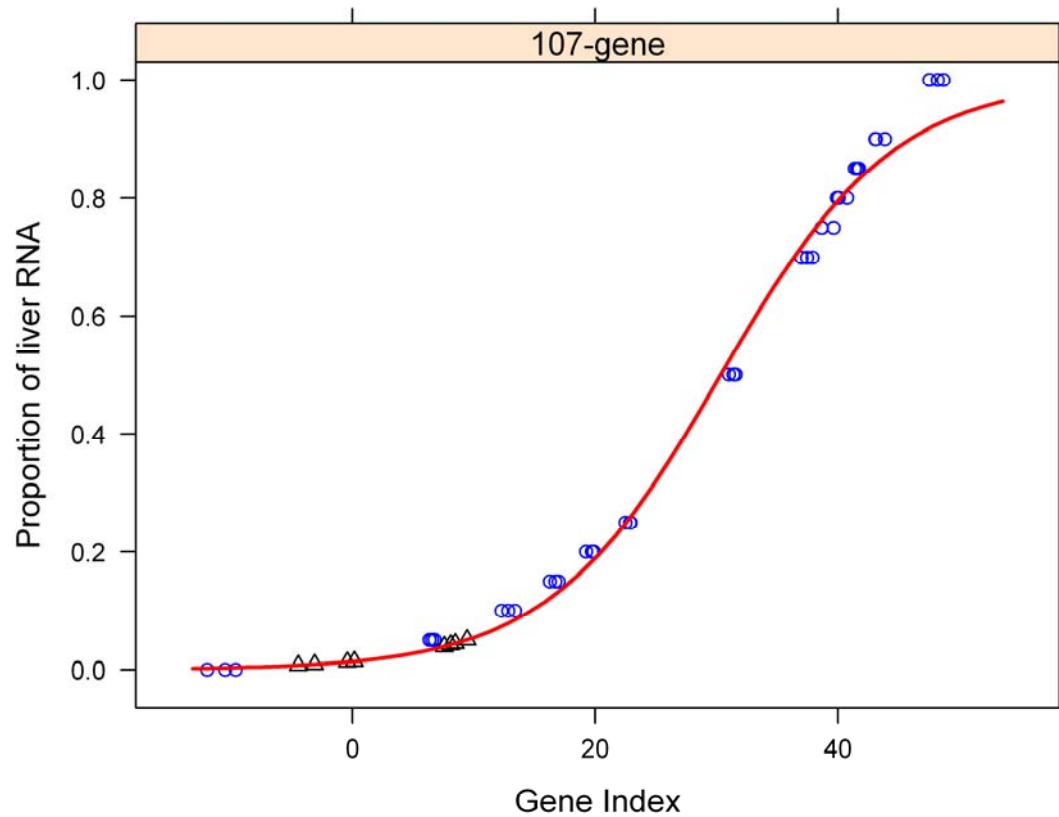
Development of a Genomic Index to Differentiate Breast Cancer From Benign Breast Tissue



Liver RNA Expression Signature



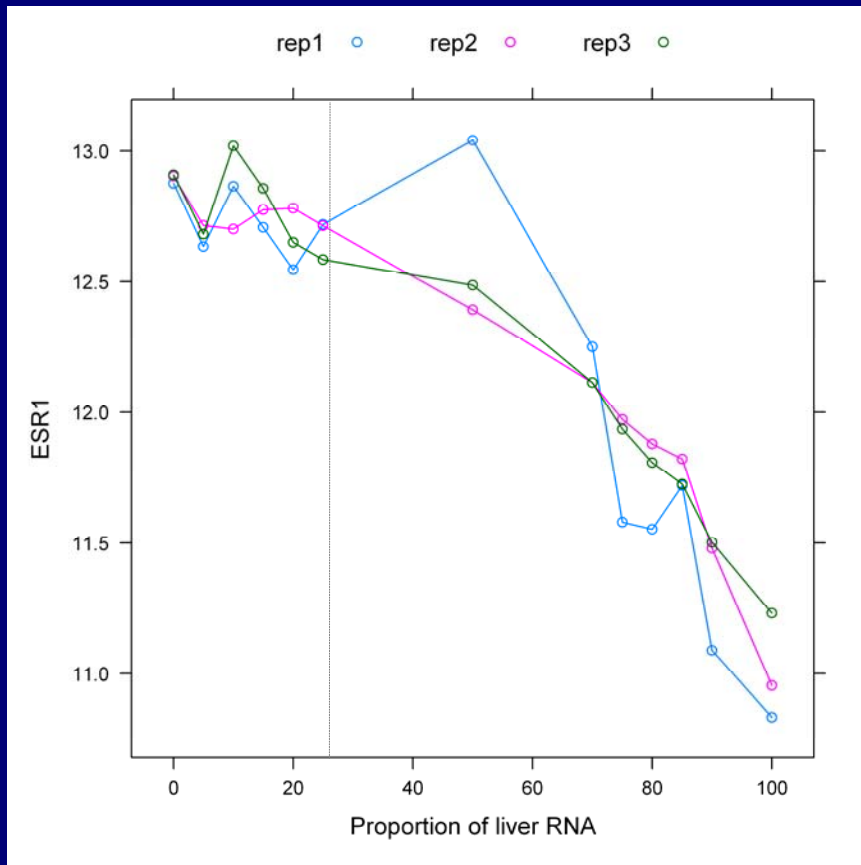
Dilution Curve for Liver vs. Breast Cancer Pooled RNA (triplicate)



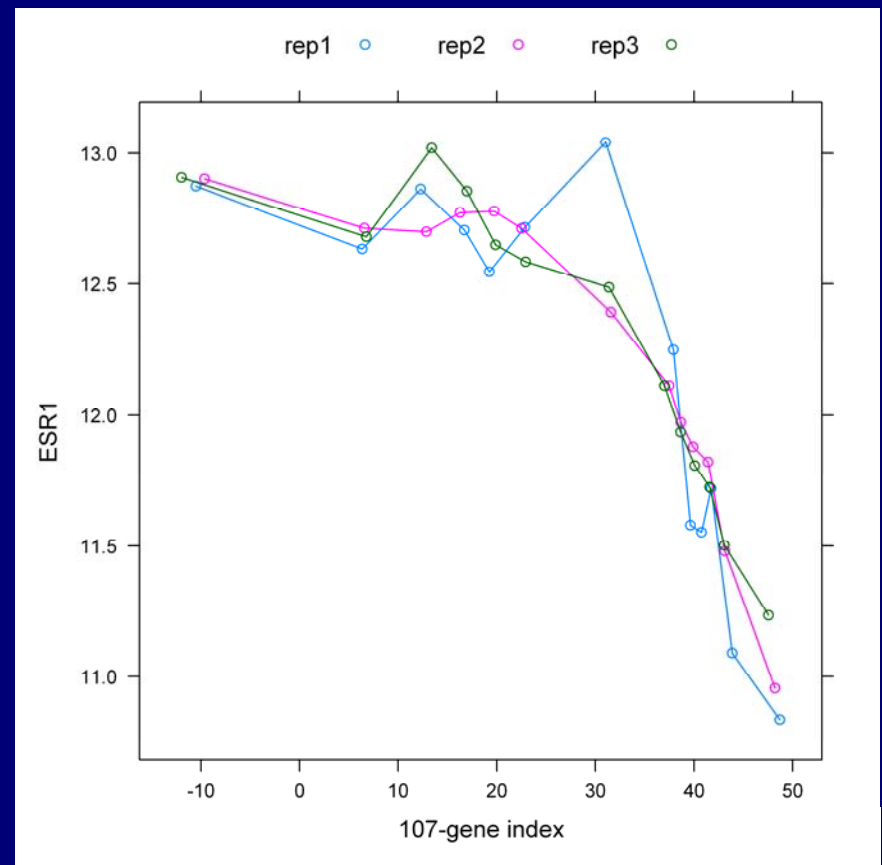
△ = radiologic-guided FNA biopsy of MBC in liver

Single Gene ESR1

ESR1 vs. % Liver RNA

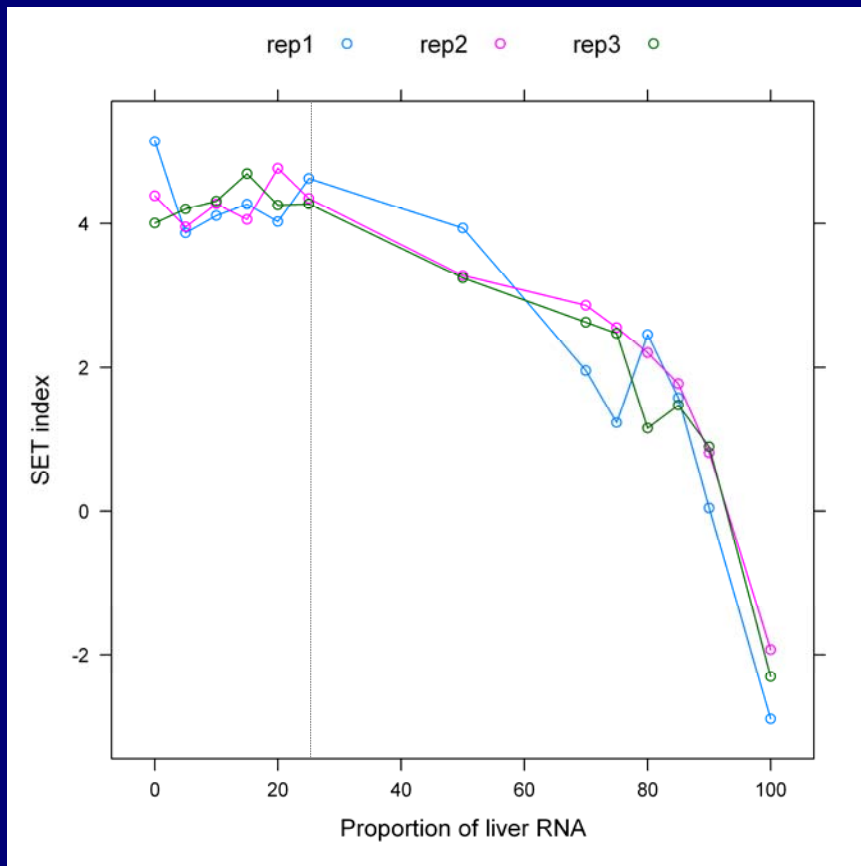


ESR1 vs. Liver Gene Index

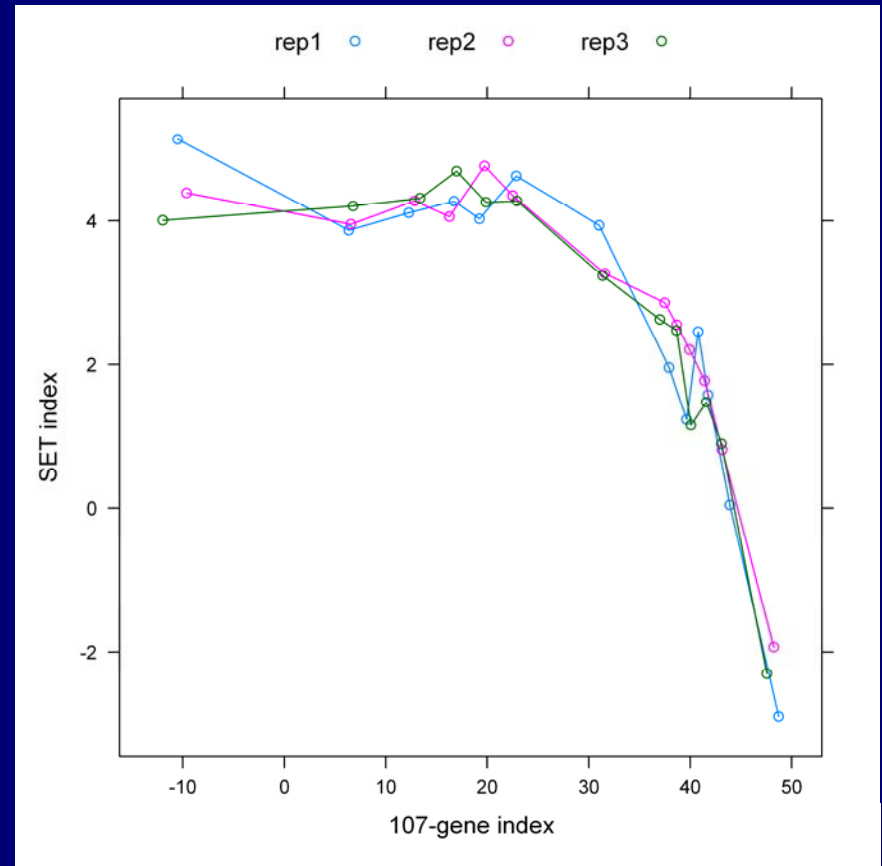


165-gene SET Index

SET vs. % Liver RNA



SET vs. Liver Gene Index



Conclusions

- Integrity and measurements of RNA expression were quite stable following cold ischemia when samples were collected and stored in RNAlater solution
- Contamination of sample with host organ RNA could be detected and estimated using bio-informatic tools and mathematical models. Our initial analyses suggest that:
 - Up to 25% liver RNA within a mixed sample might be tolerable for assessment of ESR1 and SET index
 - Actual clinical needle biopsies of metastatic breast cancer in the liver (10 patients) appear to have a relatively low proportion of contaminating liver RNA (estimated 5-10%)
- These are initial findings from work in progress, but are encouraging for integration of RNA profiling in clinical practice

Acknowledgements

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All of our patients who volunteer for studies!