

Successful measurement of gene expression by quantitative PCR and DNA chip analysis with RNA derived of FFPE material

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Contents of talk

- **Material**
 - kryo-preserved
 - FFPE material
 - 4x 25 µm thick sections
 - 5-10x 10 µm thick sections
- **Methods**
 - RNA Isolation
 - "own" protocol
 - homogenize in lysis buffer
 - digest proteinase K
 - De-modify chemical modifications induced by formalin
 - cDNA synthesis
 - random primer vs. gene-specific primers
 - QPCR
 - short amplicons
 - MGB assays or LNA assay
 - DNA chip experiments
 - Arrays
 - "own" primers for cDNA synthesis
 - CombiMatrix 12k
 - Agilent 44k
- **Results**
 - QPCR
 - single genes
 - profiles (expression of multiple genes)
 - DNA chips
 - kryo vs. FFPE
 - single genes
 - profiles

Procedure/major steps for QPCR-based analyses

- Identification of marker genes from microarray studies, literature etc.
- Reduce number of test genes to minimum
- Select appropriate control genes
- Isolate RNA from FFPE sections
- Establish robust real-time assays which give reproducible results even with fragmented RNA
- Evaluate results on the basis of single genes
- Develop model (= scoring system) involving groups of genes, we used

Estrogen score
HER2 score
Proliferation score } **Prognostic score**

e.g. Oncotype DX

⇒ 16 test genes & 5 control genes

⇒ Recurrence Score (RS)
→ prognostic for patients with primary breast cancer (stage I or II, N0, ER+)
commercial test is available
(~3'000\$ per test)

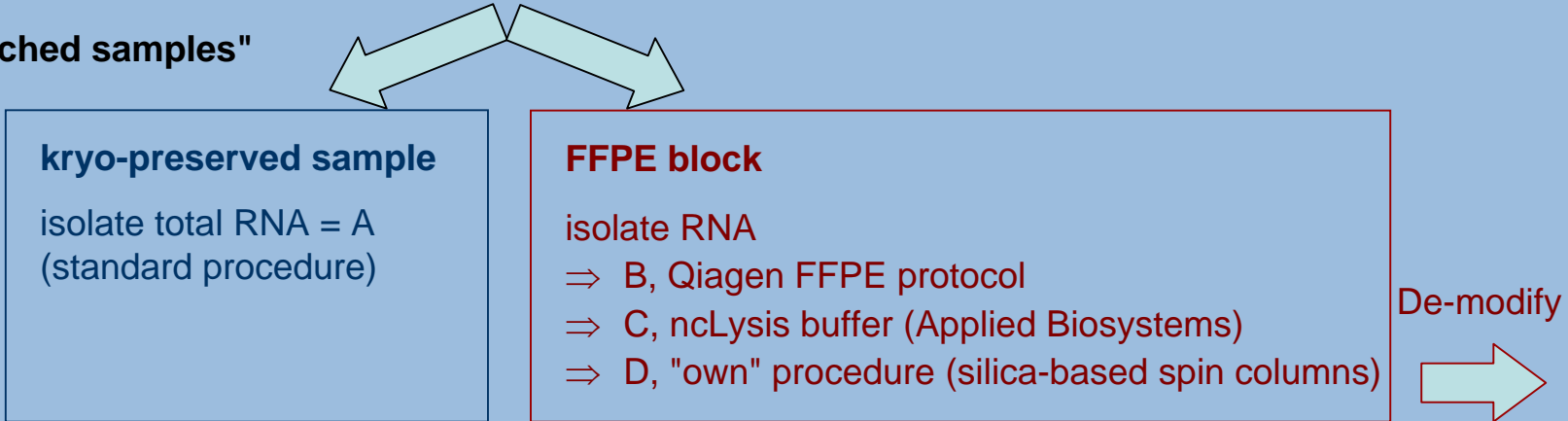
⇒ RS is also predictive for response to chemotherapy

Cronin et al. Am J Pathol 164 (2), 35-42, 2002. Measurement of Gene Expression in Archival Paraffin-Embedded Tissues.

Experimental design

14 breast cancers

"matched samples"

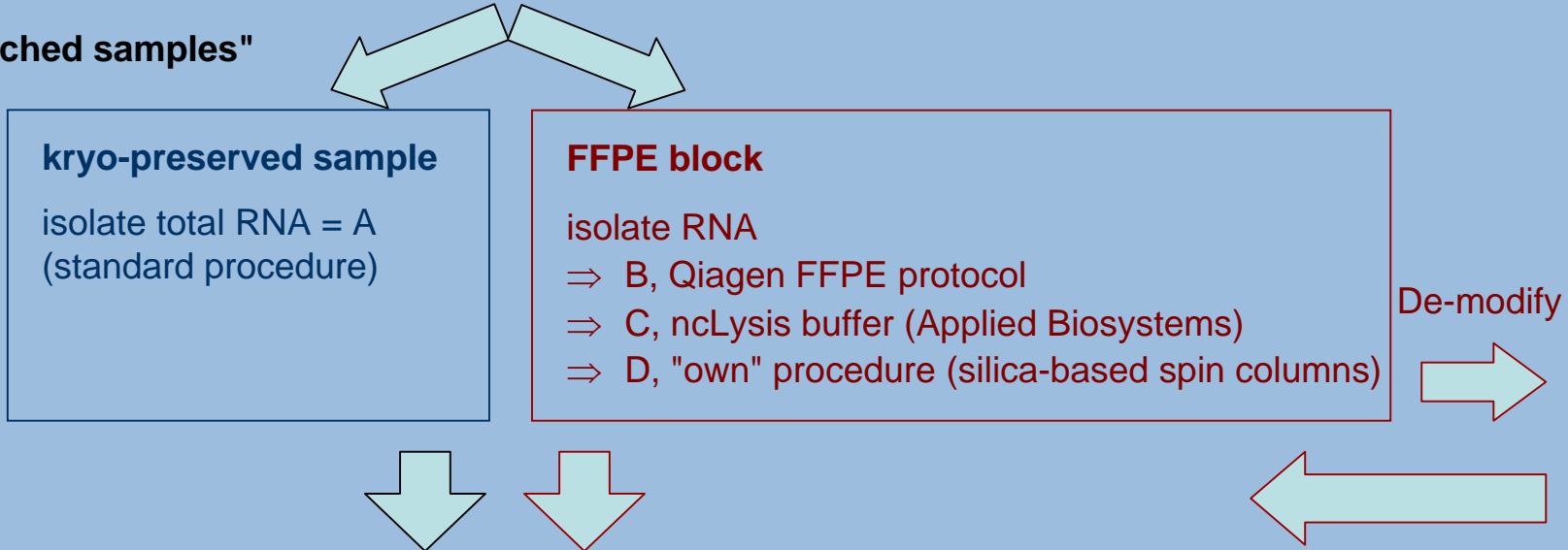


make gene-specific cDNA
TaqMan PCR (MGB assays or LNA probes)
Evaluation:
 Expression analysis of individual genes
 Normalization
 Develop models (= scoring system) of multiple genes
 Estrogen-response score
 Proliferation score
 Prognostic score

Experimental design

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Evaluation:

Expression analysis of individual genes

Normalization

Develop models (= scoring system) of multiple genes

Estrogen-response score

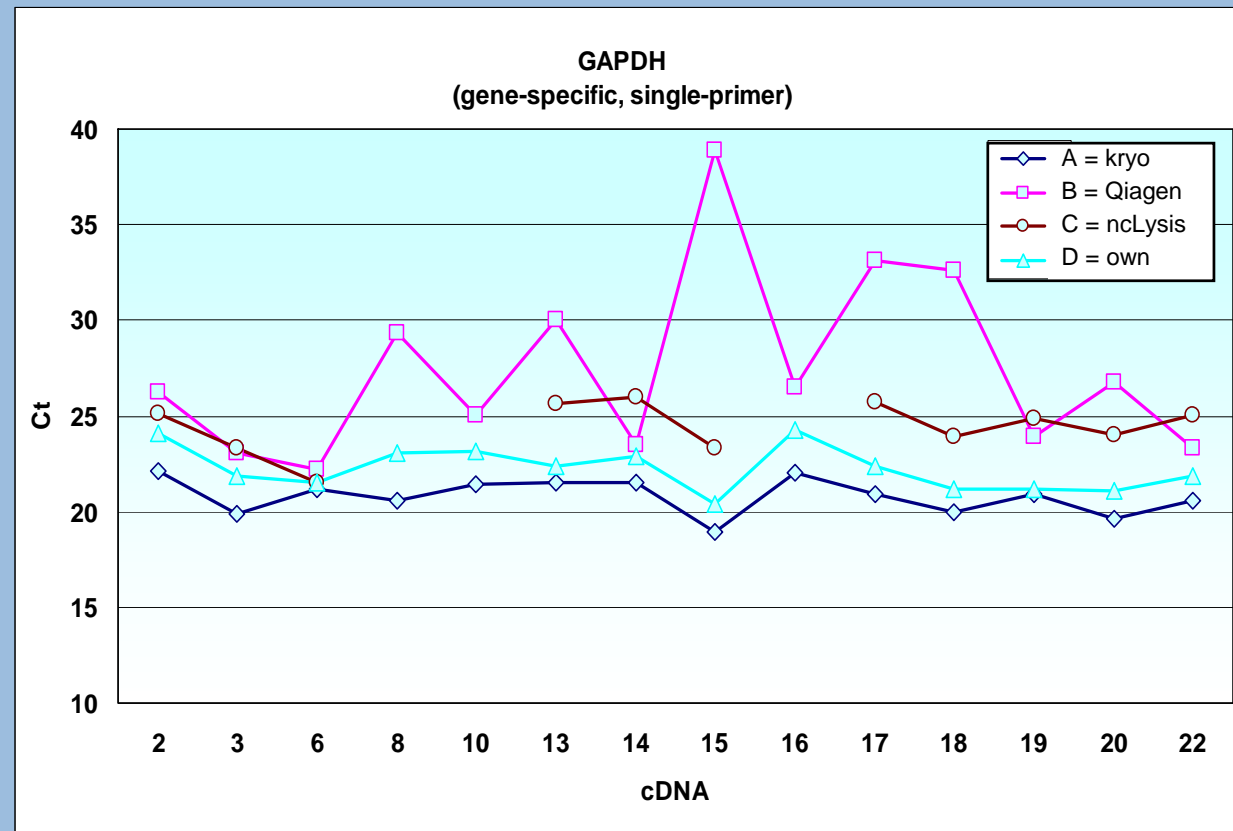
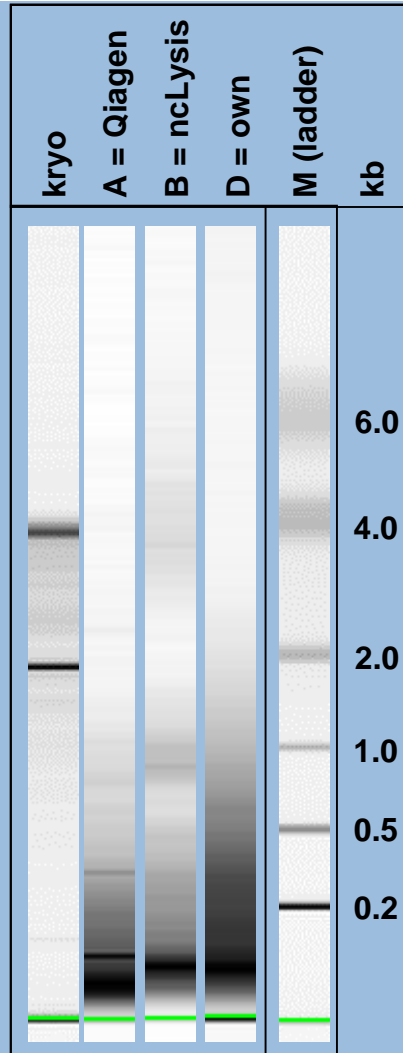
Proliferation score

Prognostic score

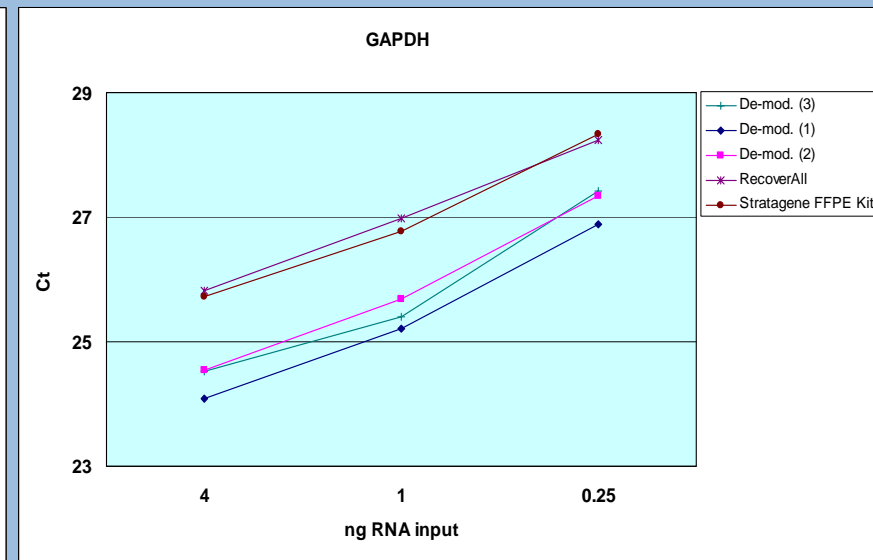
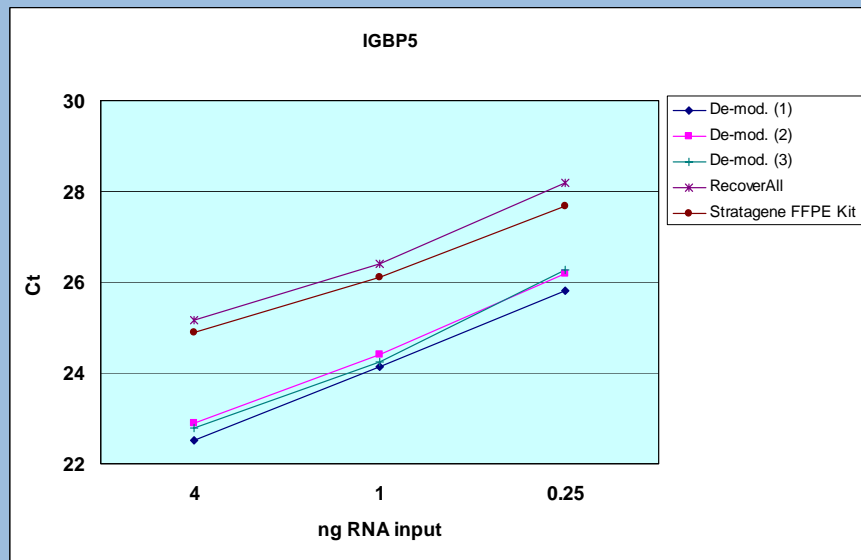
QPCR

single gene data

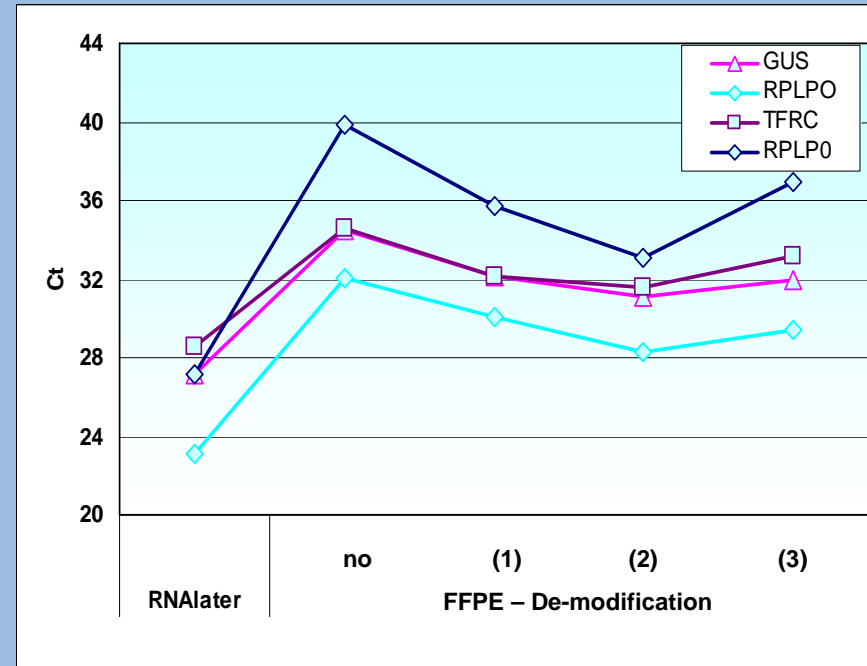
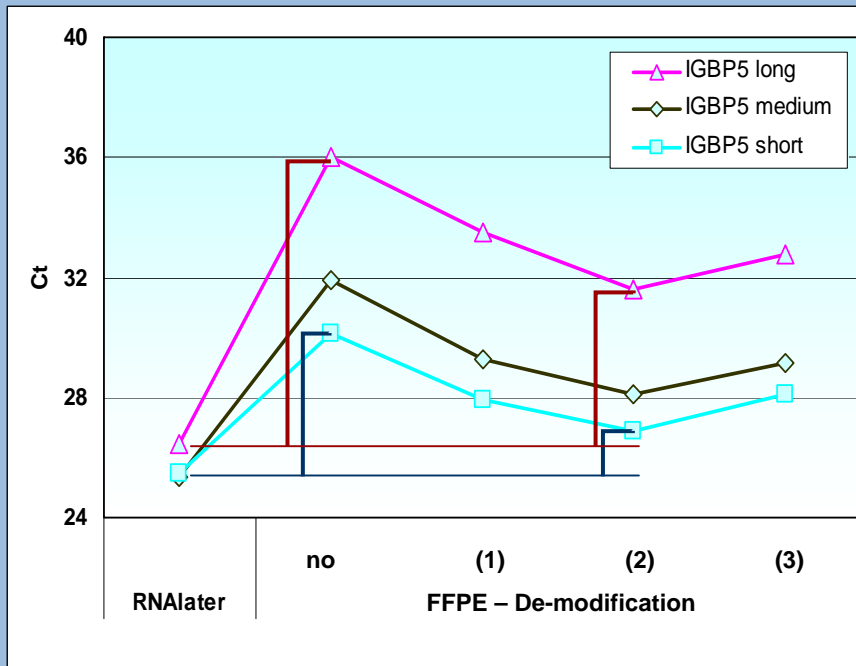
RNA quality is critical: comparison of different protocols



Comparison of own protocol with different commercial kits Stratagene, RecoverAll (Ambion)

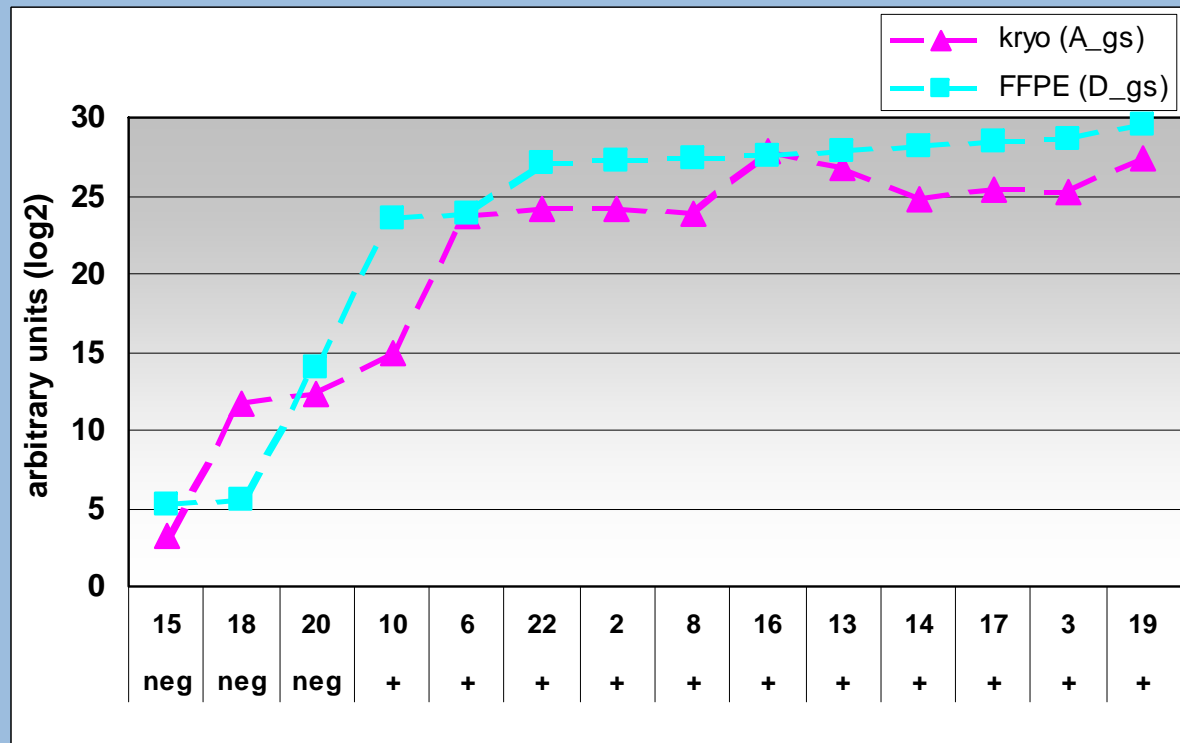


Comparison of results with different de-modification protocols (1)



Comparison of estrogen receptor expression and results based on immunohistochemistry

ESR1 expression



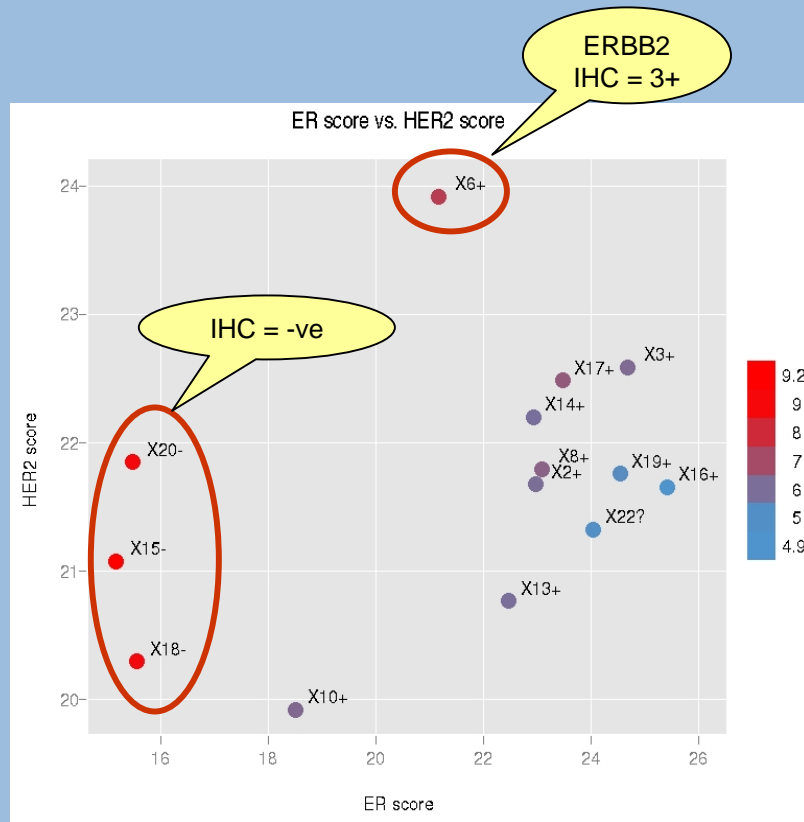
AB: MGB assays

QPCR

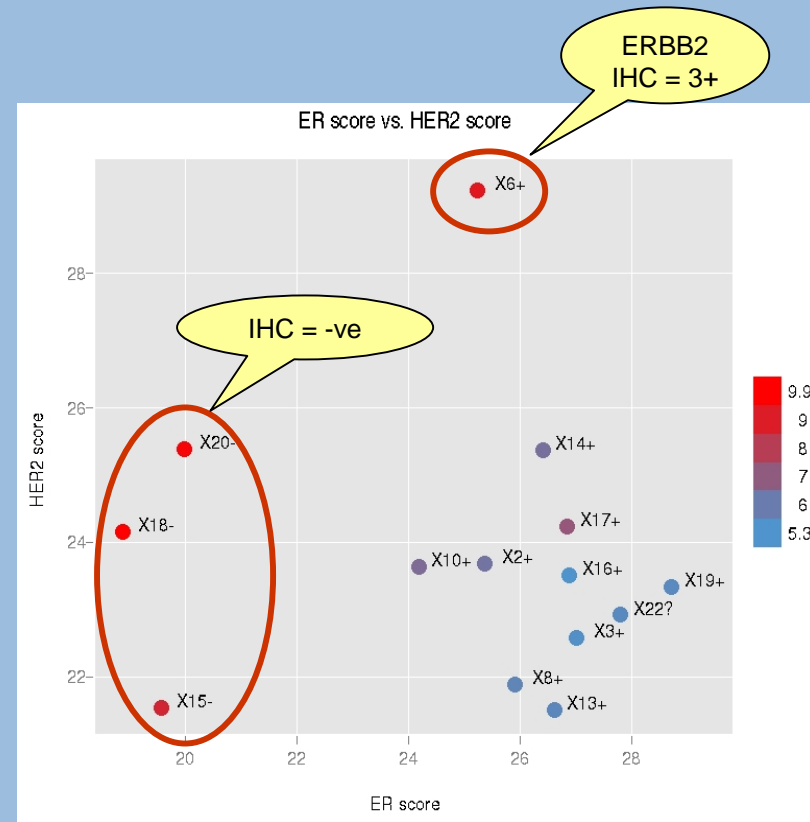
Multiple genes \Rightarrow Scores

Proliferation score: results from 14 tumors

kryo-preserved tumor material



FFPE-derived material

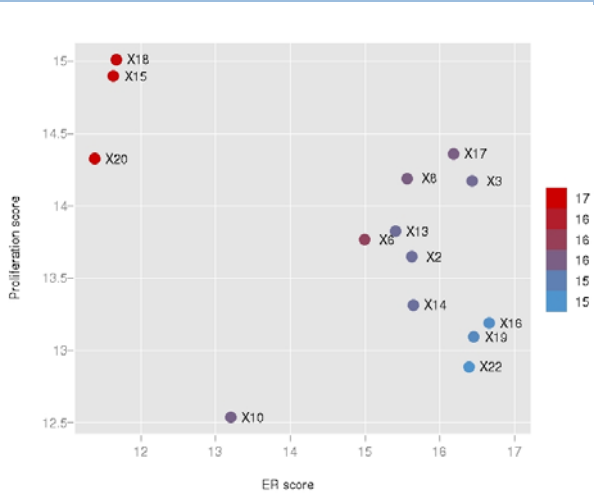


Test	Rank corr.	p-value
A_gs vs D_gs	0.95	< 0.001

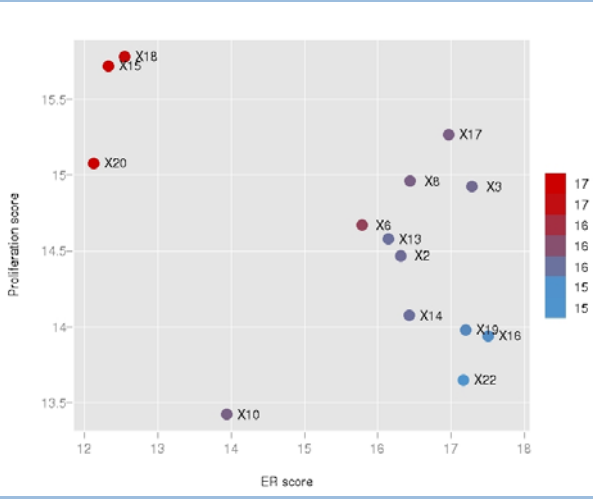
Normalization based on reference genes



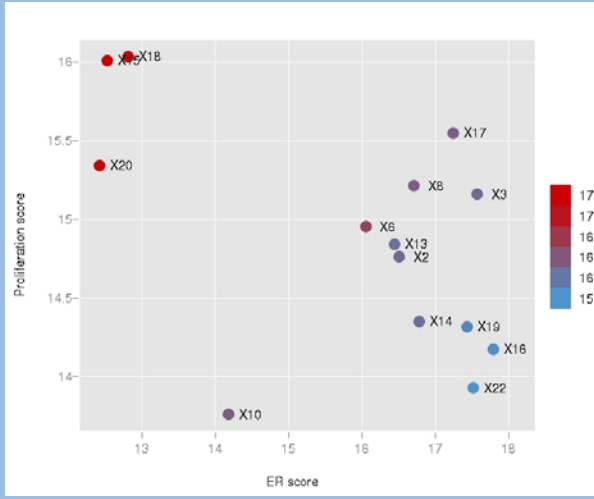
**ER score
(based on mean)**



**ER score
(based on median)**



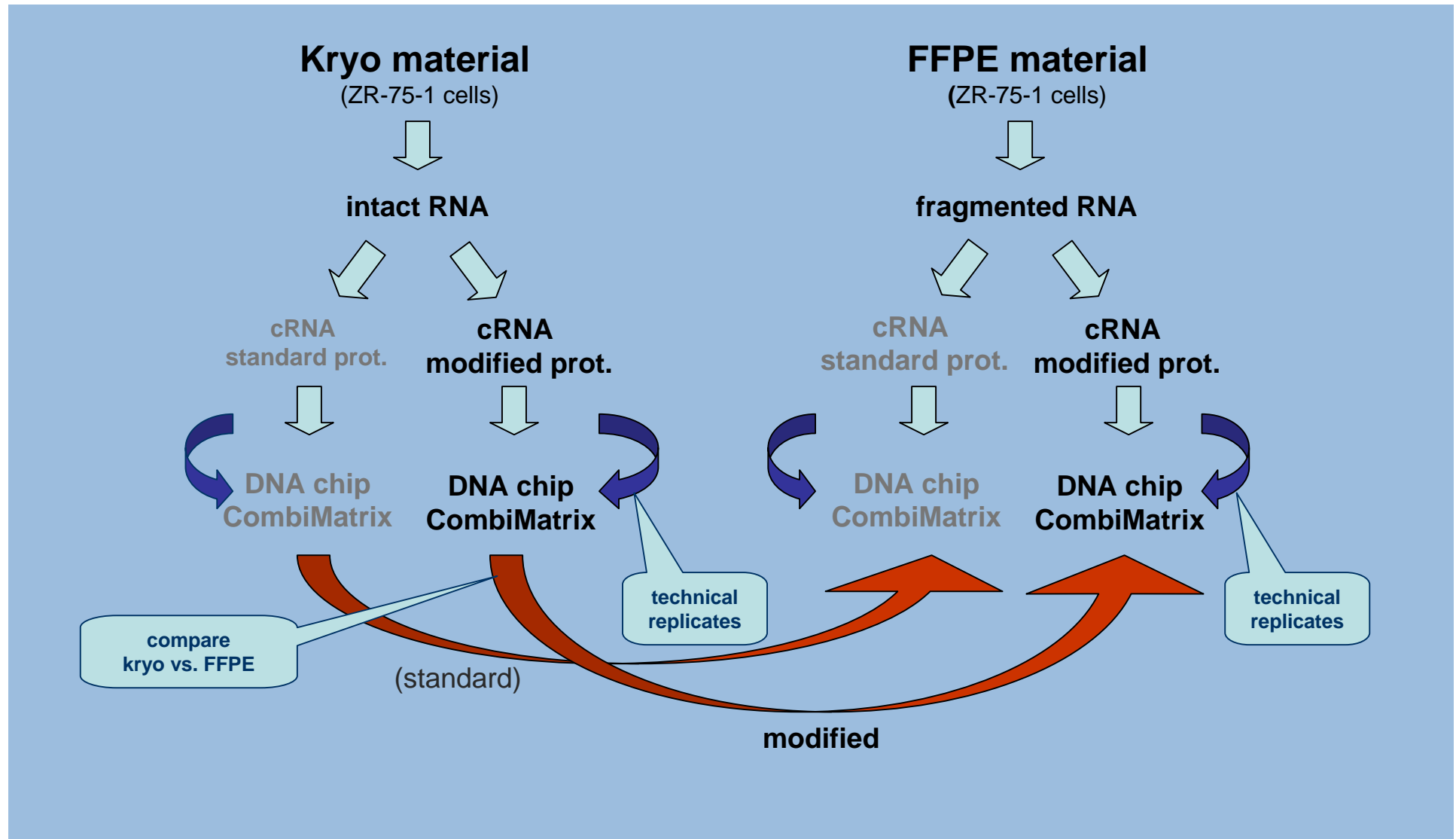
**ER score
(based on geometric mean)**



Array data

- Limited number of features/experiment
- + High sensitivity of method
- + Works with degraded RNA (used with optimized reverse transcription and qPCR assays)
- + Large number of samples potentially available from clinical trials
- No standards for normalization
- + Large number of features/array
- + Method depends on good quality RNA (e.g. kryo)
- + Established procedures for normalization
- Samples are often heterogeneous
- Limited number of samples per study
- Various treatment regimens
- Outcomes sometimes unknown

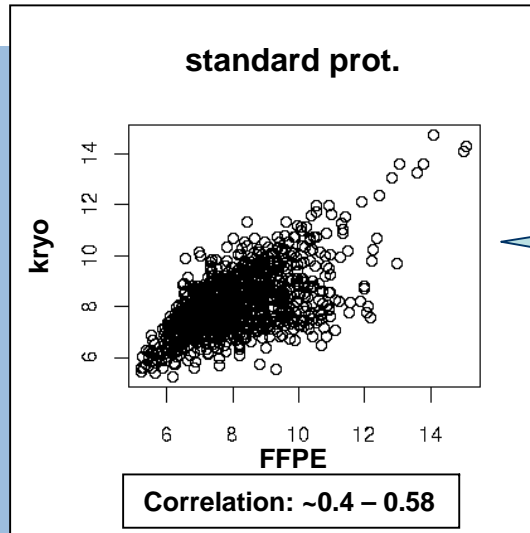
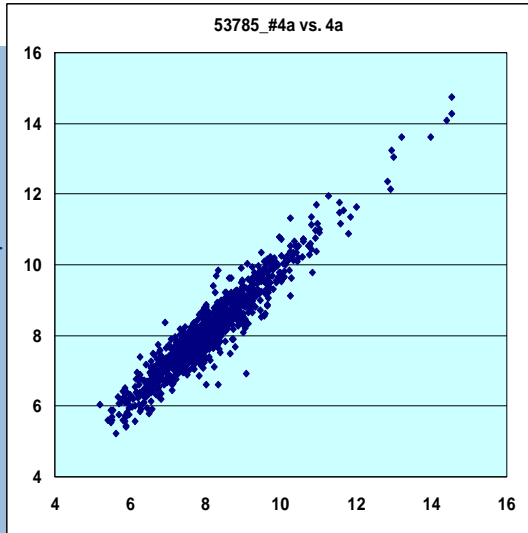
Application of FFPE-derived RNA to DNA chips



CombiMatrix 12k chips: Technical replicates

standard protocol

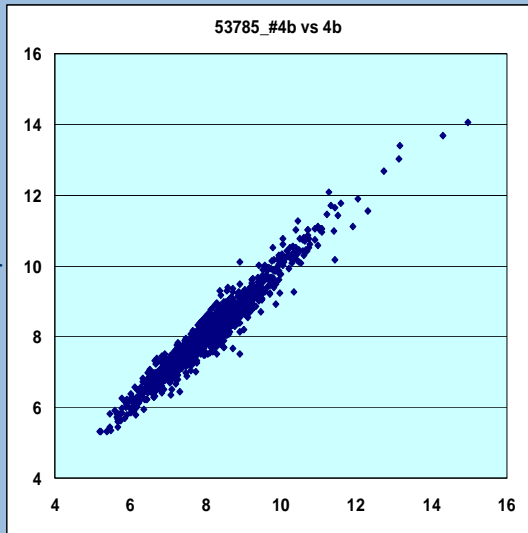
technical replicates



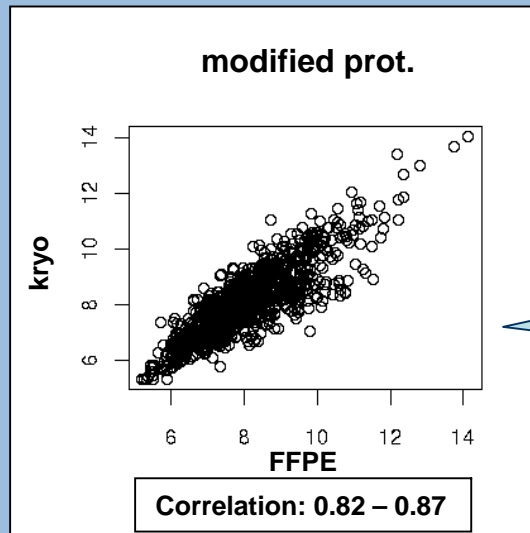
compare kryo vs. FFPE

modified protocol

technical replicates



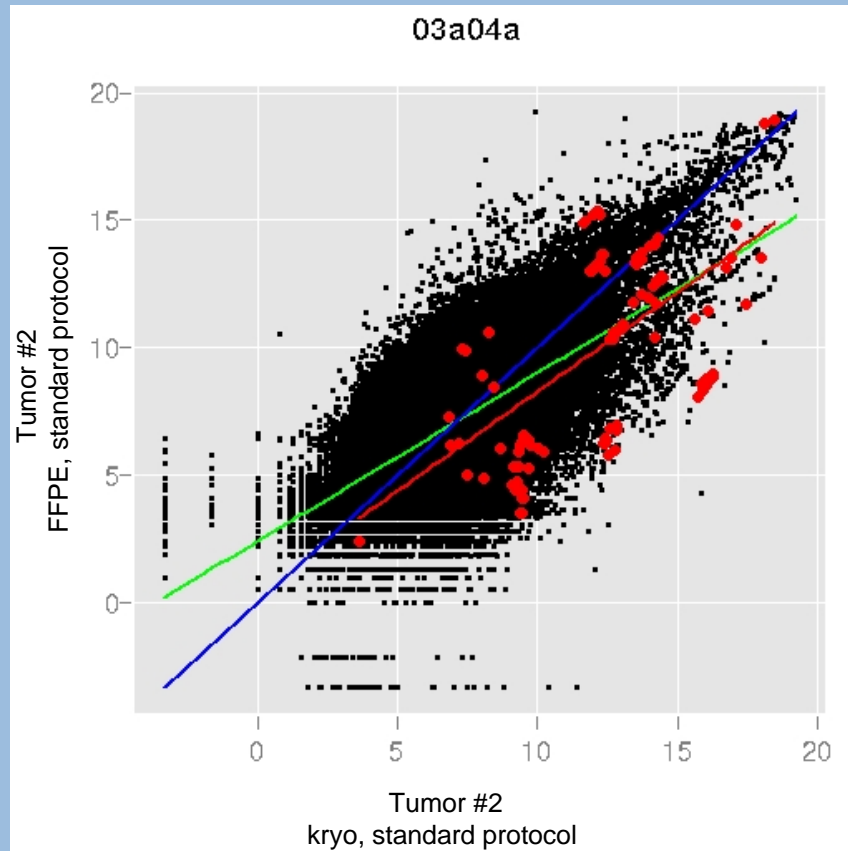
Correlation: ~0.98



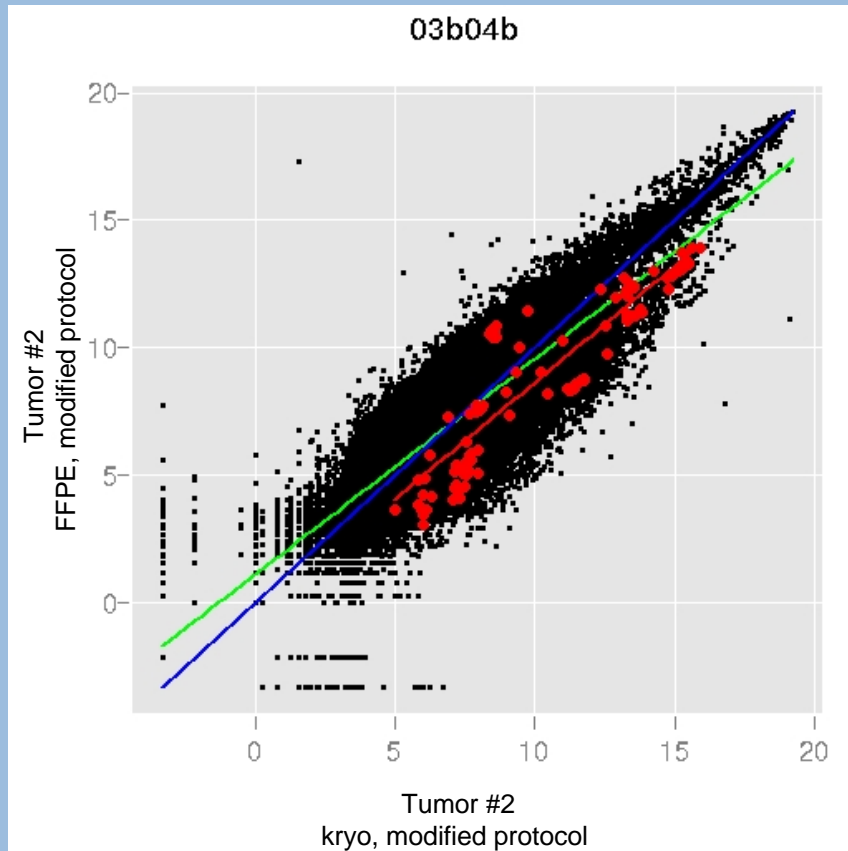
compare kryo vs. FFPE

Agilent chips: kryo vs. FFPE tumor RNA (Scatterplot)

Tumor #2
kryo vs. FFPE, standard protocol



Tumor #2
kryo vs. FFPE, modified protocol

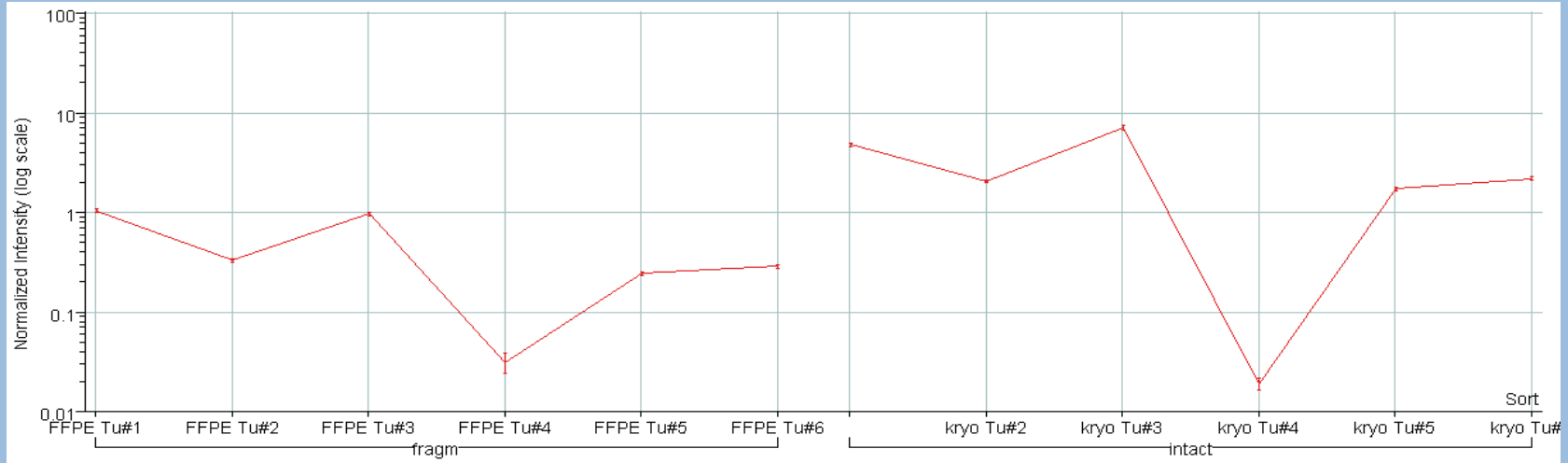


ER Expression in 6 tumors

DNA chip

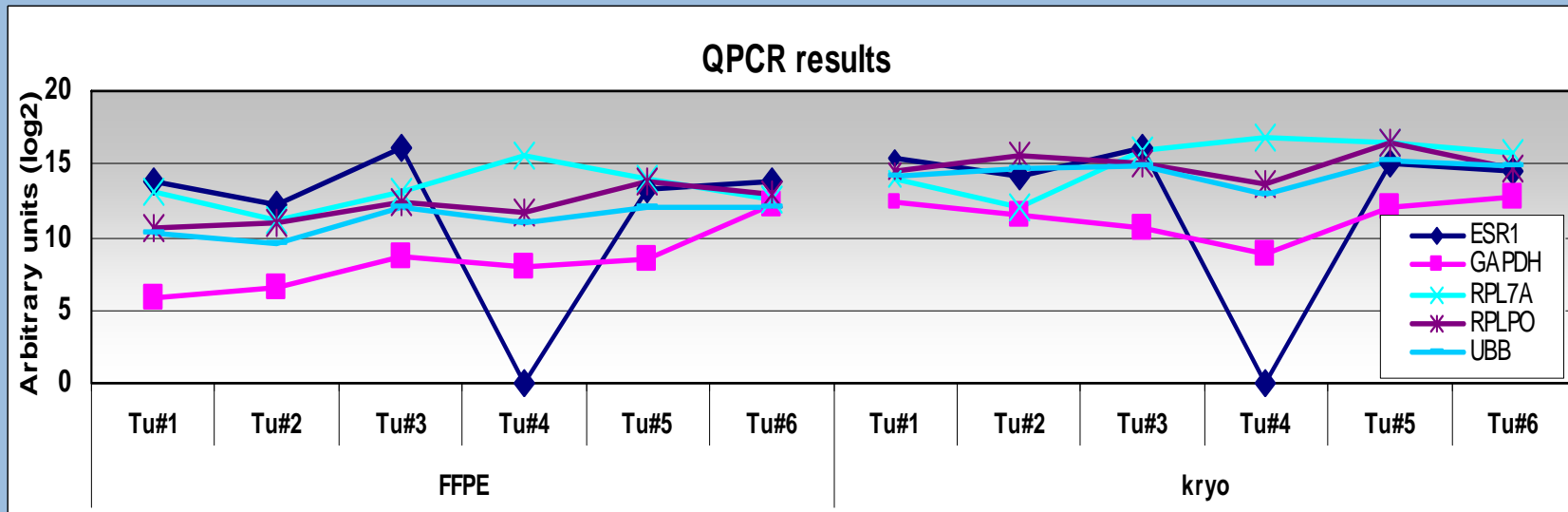
FFPE

kryo/RNAlater



ER Status

+ + + - + + + + - + +



Summarization of results: correlations



| 41'000 genes | intact vs. fragmented RNA fom cells | | kryo vs. FFPE tumor RNA from breast cancer | | | | | | | |
|------------------------------|-------------------------------------|------------------|--|----------------|----------------|----------------|----------------|----------------|--------------|--------------|
| | ZR-75-1 standard | ZR-75-1 modified | Tu #1 standard | Tu #1 modified | Tu #2 standard | Tu #2 modified | Tu #3 modified | Tu #4 modified | Tu #5 modif. | Tu #6 modif. |
| Pearson (all genes) | 0.932 | 0.908 | 0.661 | 0.845 | 0.622 | 0.838 | 0.829 | 0.862 | 0.857 | 0.862 |
| Spearman (ranks) (all genes) | 0.935 | 0.905 | 0.585 | 0.817 | 0.563 | 0.814 | 0.793 | 0.837 | 0.823 | 0.835 |
| 21 Paik genes | ZR-75-1 standard | ZR-75-1 modified | Tu #1 standard | Tu #1 modified | Tu #2 standard | Tu #2 modified | Tu #3 modified | Tu #4 modified | Tu #5 modif. | Tu #6 modif. |
| Pearson (116) | 0.923 | 0.962 | 0.581 | 0.897 | 0.459 | 0.889 | 0.92 | 0.936 | 0.918 | 0.93 |
| Spearman (ranks) (116) | 0.779 | 0.941 | 0.468 | 0.947 | 0.451 | 0.893 | 0.944 | 0.924 | 0.914 | 0.912 |

Agilent 44k chips 60-mer oligos
 all genes 41'000
 21 Paik genes represented by 116 spots on the chip

- Material

- | | | |
|-----------------|------------------------|--|
| - kryo material | 4x 25µm thick sections | QPCR/DNA chip
feasible |
| - FFPE Material | 5-10x 10µm sections | QPCR
technically demanding
DNA chip
feasible
specialized protocols |

- Technology

- | | |
|------------------|---|
| - RNA Isolation | Homogenization
Proteinase K digestion
De-modification |
| - cDNA synthesis | Gene-specific primers during RT |
| - QPCR | short amplicons
MGB assays or LNA assays |

Collaborators

- Janine Antonov
Sybille Matthey
Andrea Oberli
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- Hans Jörg Altermatt, MD Pathology Länggasse, CH-3012 Bern

- Achim Fleischmann, MD



- Vlad Popovic, PhD
Mauro Delorenzi, PhD



tumorbank bern

- Support:
Bernese Cancer League (BKL)
Swiss Cancer League (SKL)
International Breast Cancer Study Group (IBCSG)
Applied Biosystems, Rotkreuz, Switzerland