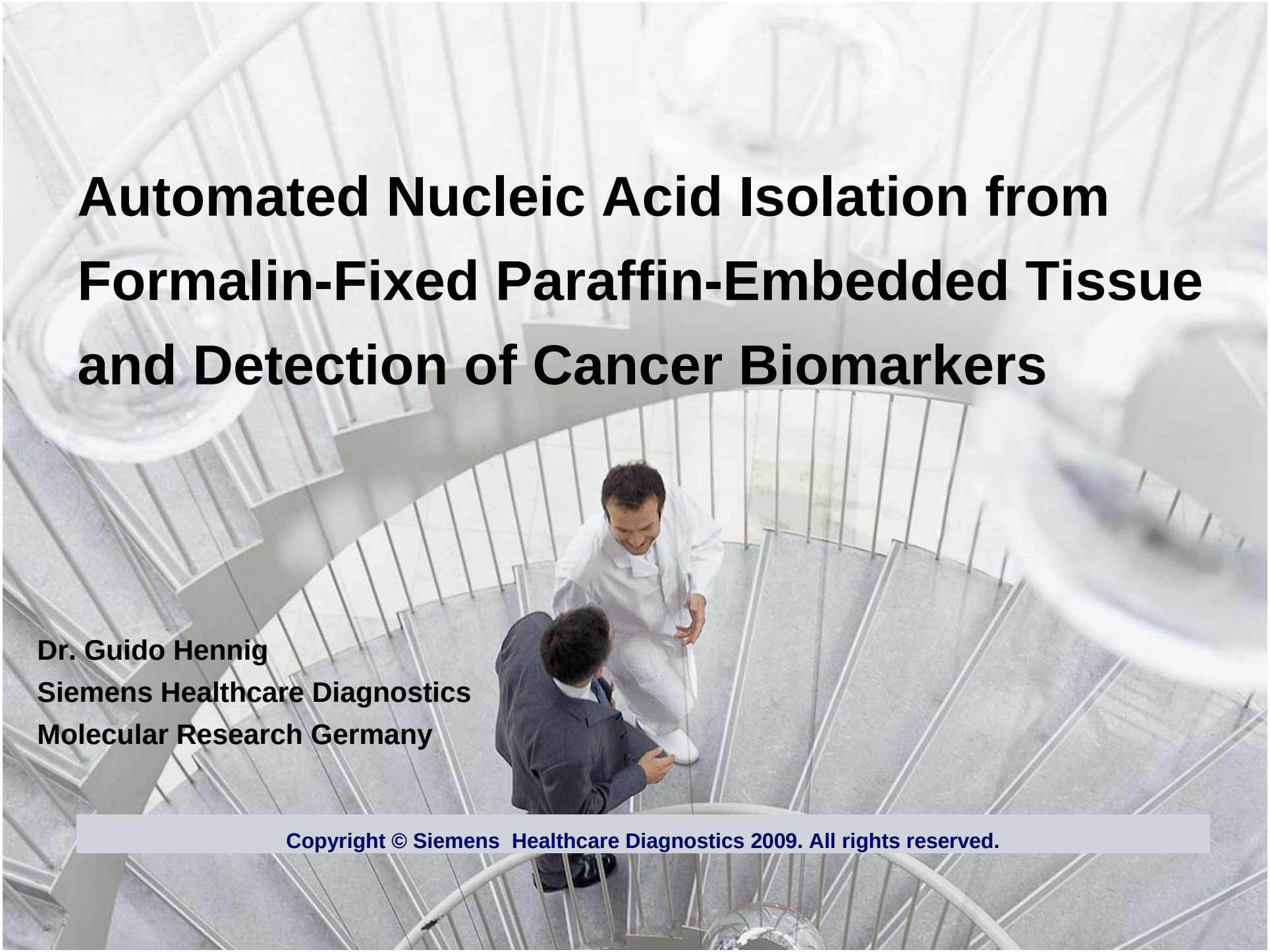
Automated Nucleic Acid Isolation from Formalin-Fixed Paraffin-Embedded Tissue and Detection of Cancer Biomarkers



Dr. Guido Hennig
Siemens Healthcare Diagnostics
Molecular Research Germany

Siemens Healthcare Diagnostics

Molecular Research Germany

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Location

- BioCampus Cologne

Team of 35 people

- Biologists, chemists, physicians, mathematicians, computer scientists, technicians

Research Focus

- Molecular (RNA+DNA) diagnostics in cancer
- Nucleic Acid Isolation Technology

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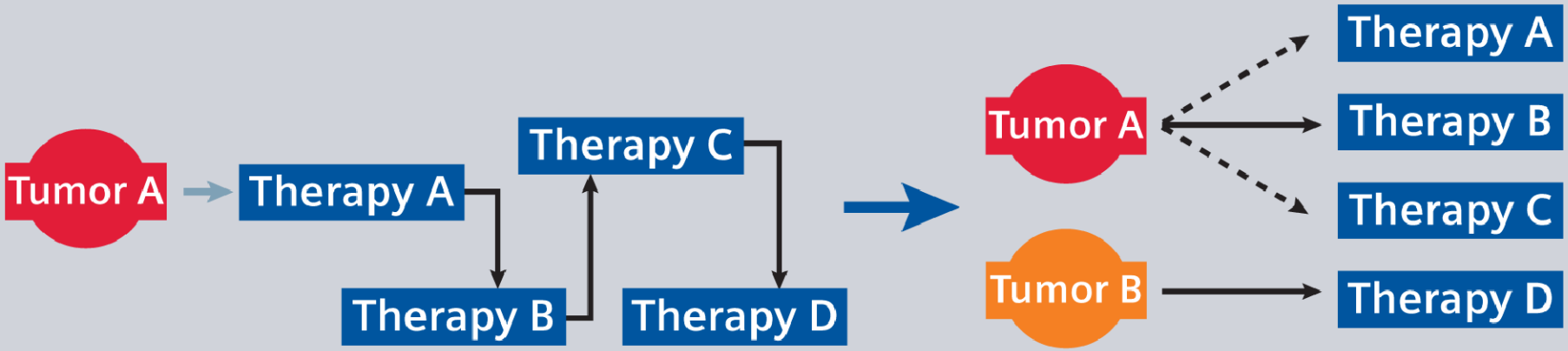
In-Vitro Diagnostic to Guide Breast Cancer Therapy

Today: Therapy guidelines based on histopathological parameters leaving still numerous therapy options
Some molecular testing recently included into guidelines

Future: Develop and validate more/better molecular tests to pick optimal therapy guided by molecular profiling of tumor



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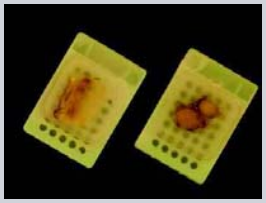
Why FFPE Tissue for Cancer Diagnostics?



Two principle sources of tumor material exist:

- fresh frozen tissue
- formalin-fixed paraffin-embedded (FFPE) tissue

- Access to pathological FFPE archives with linked clinical records allows high-throughput retrospective finding + validation studies

- FFPE tissue is the routine material after tumor surgery and key for successful implementation and commercialization of a diagnostic kit (requires no change of clinical practice)

Prognostic / Predictive Markers in Breast Cancer

Research Strategy of Siemens Diagnostics

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Fresh-frozen cohort

Marker Transfer

FFPE cohort

further FFPE cohorts

diagnostic test

Expression profiling in fresh-frozen tissue (Affymetrix)

Generation of algorithm with <20 genes

Transfer of algorithm to FFPE tissue and kPCR and verification

Validation of algorithm in independent retrospective and prospective randomized trials

Development and commercialization of *in vitro* diagnostic test

VERSANT™ kPCR Molecular System*

*** Not yet available in the US**

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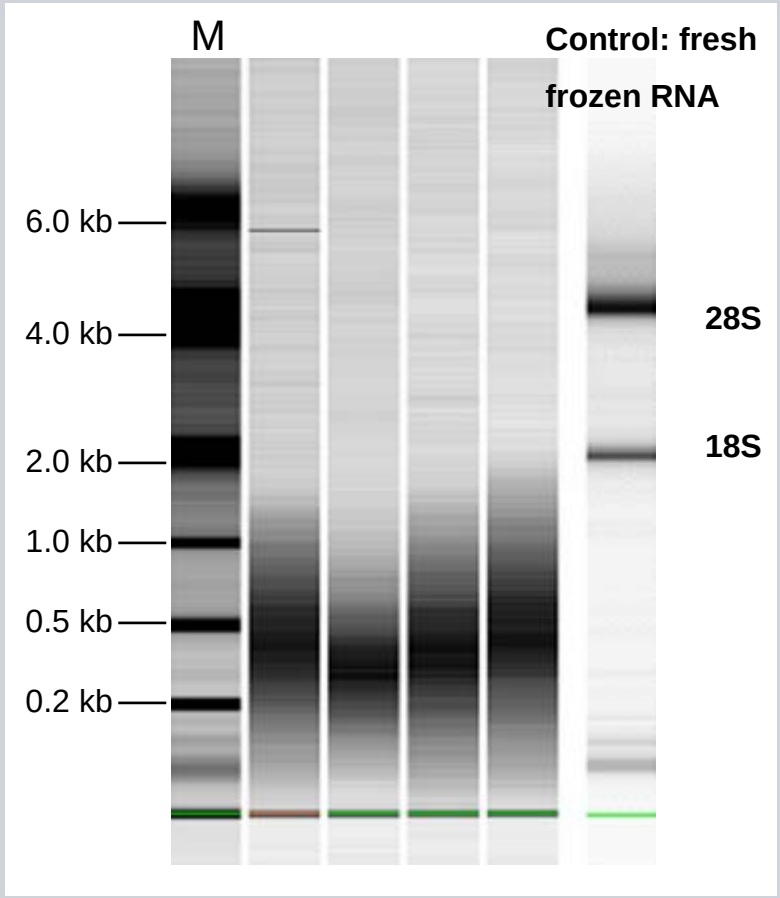
Issues with Total RNA from FFPE Tissue



- RNA from fixed tissue on Bioanalyzer
- RNA is chemically modified and crosslinked to other nucleic acids and protein
- RNA is fragmented

Mean size
~ 200-500 bp

Fragmentation over storage time

Open Questions for Accurate Expression Profiling in FFPE Tissue

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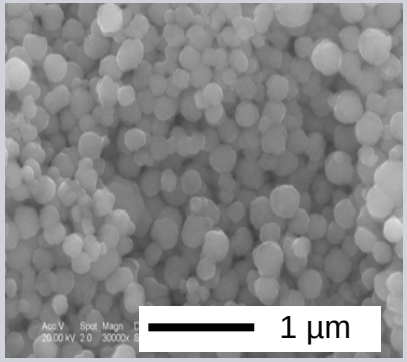
- No Standardized Isolation Technology for Nucleic Acids
- Different Fixation Conditions (Time in Fix / Time to Fix)
- Variability of Tumor Cell Content
- Block to Block Variability (Intra-tumor Heterogeneity)
- Correlation to Immunhistochemistry

Siemens´ „New“ Magnetic Particles

- „One particle chemistry“ for all applications in the field of nucleic acid isolation
- Coating of iron oxides with nanolayer of silica
- No change in morphology (spherical, evenly sized, superior active surface / volume ratio)



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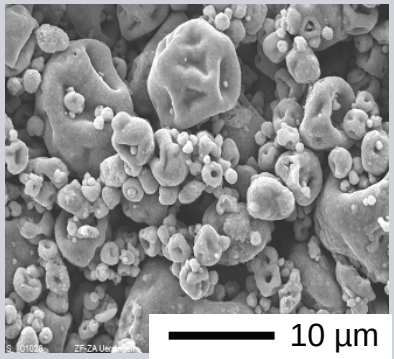

Others

Siemens´ Isolation Workflow for RNA (and DNA) from FFPE Tissue

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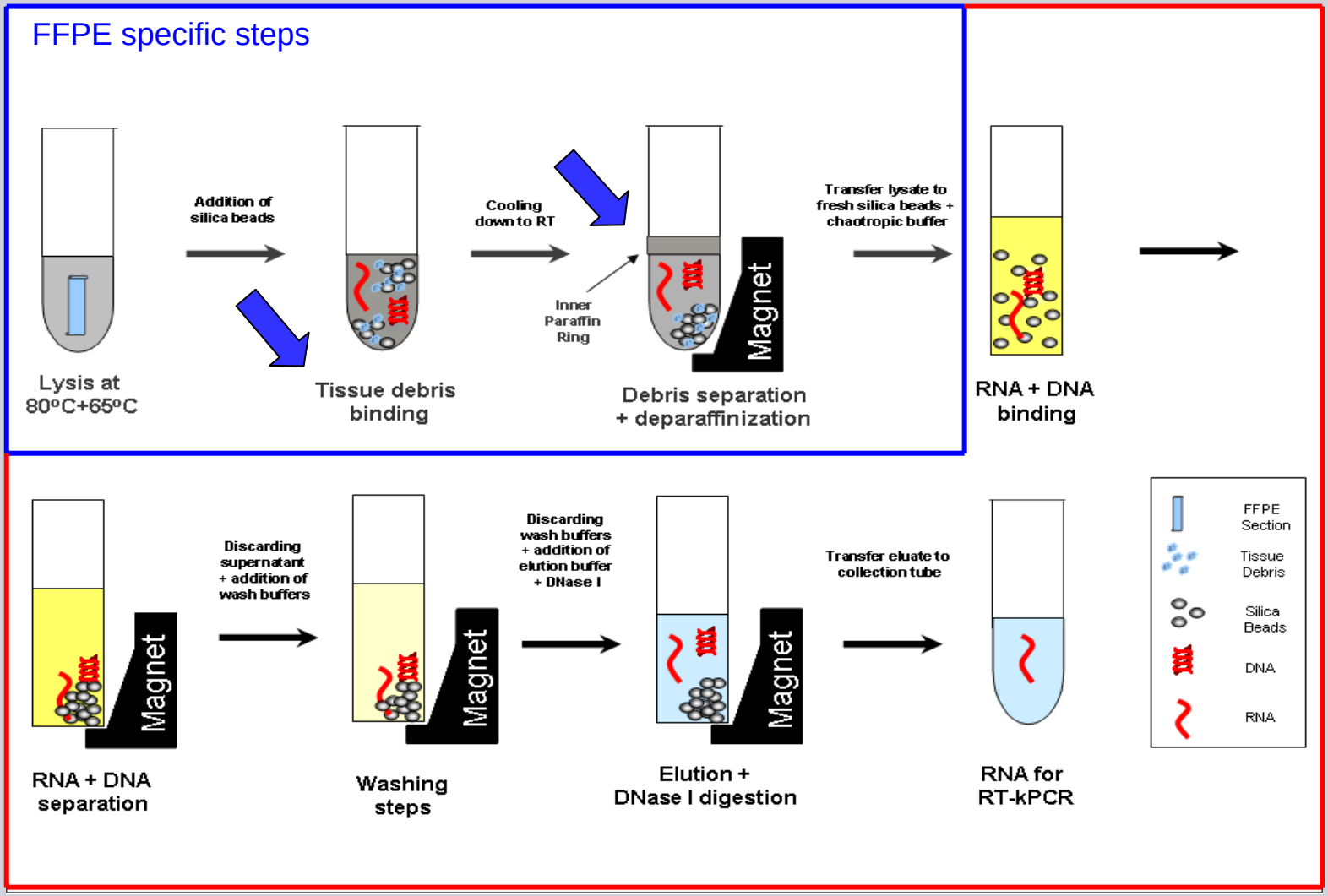

Müller et al, 2009 (submitted)

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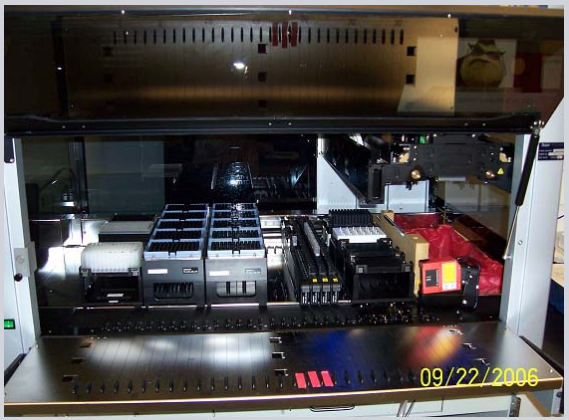
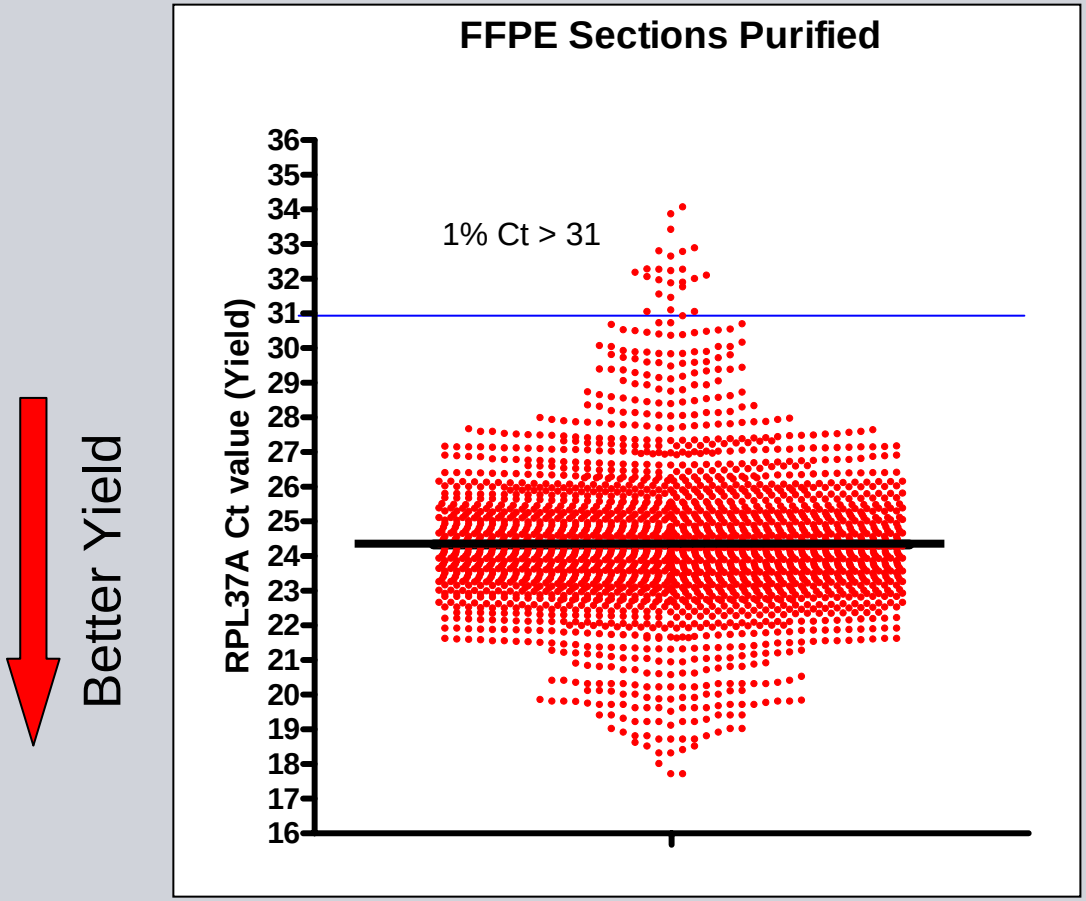
Automation of DNA/RNA FFPE Isolation



Adapted to VERSANT kPCR™ Platform

- Fully automated and high throughput (48 sections in 4 hours)
- 10 µm sections are loaded in plastic tubes on robot
- No manual deparaffinization with xylene/ethanol and centrifugation
- Standardization of lysis conditions by negative selection step for “tissue debris”
- Currently in development

Performance (Yield of Total RNA from FFPE Sections)



Breast Cancer Tumor Blocks:

3 month to 21 years of age

n ≥ 2314

Median RPL37A Ct = 24.5
~ 1 µg / 10 µm section

Success Rate: 100%

High Yield Rate: 99%

Sample requirement for RT-kPCR: 2 ng/well ⇨ ~ 500 PCRs per slide

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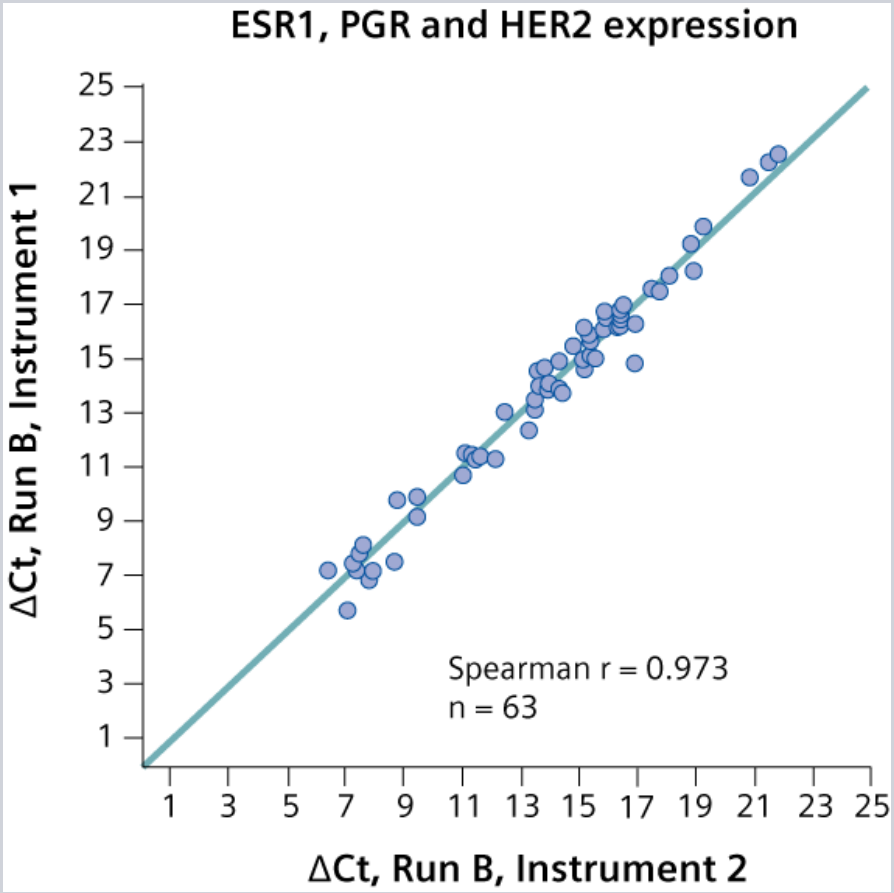
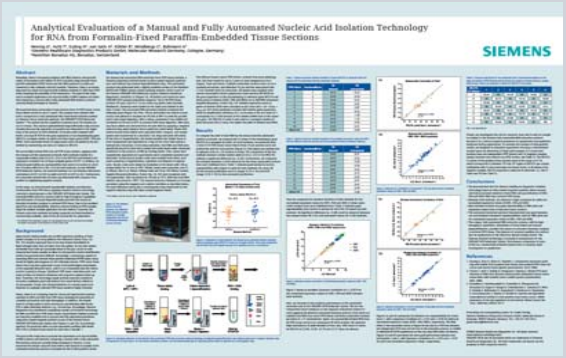
Between Instruments Reproducibility of mRNA Expression





Poster AMP 2008

Robust and reproducible method without Carry Over of target RNA from high positive to neighbouring negative sections

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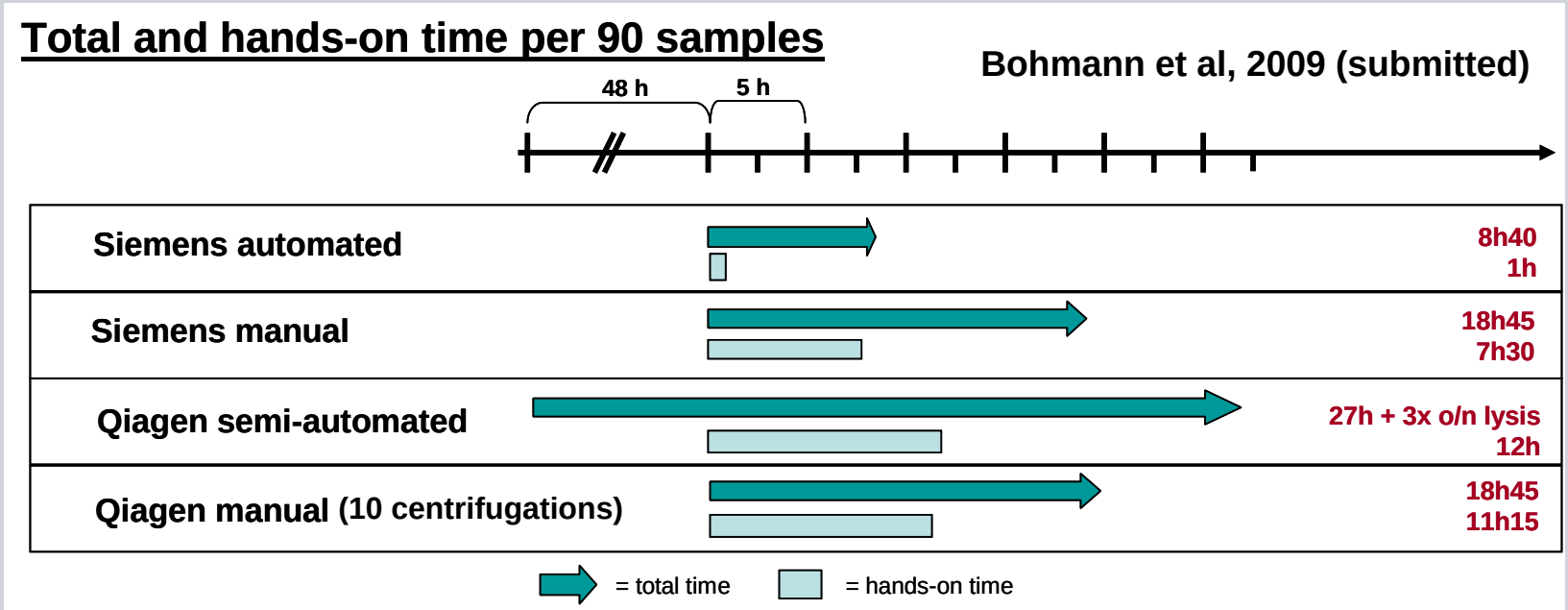
Benchmark to State-of-the-art Qiagen Kit´s



- Automated protocol superior with regards to total time and hands-on-time
- Automated protocol showed best section to section reproducibility

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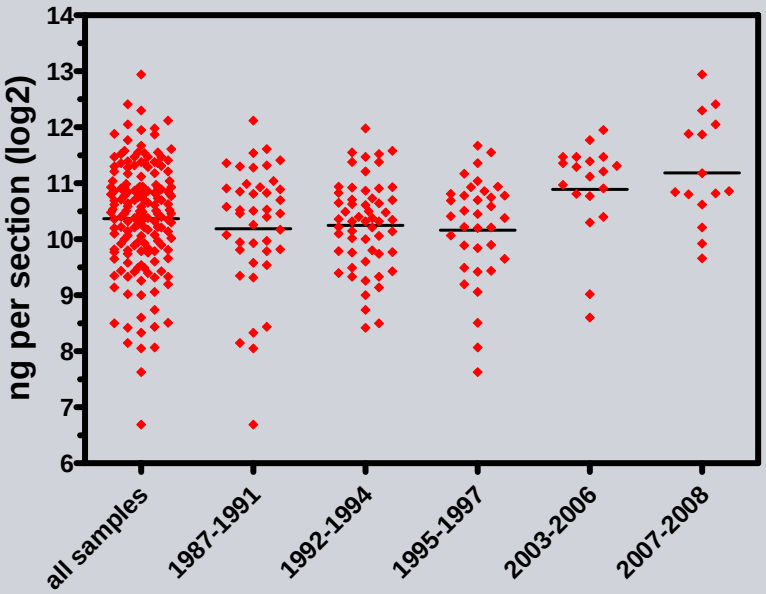
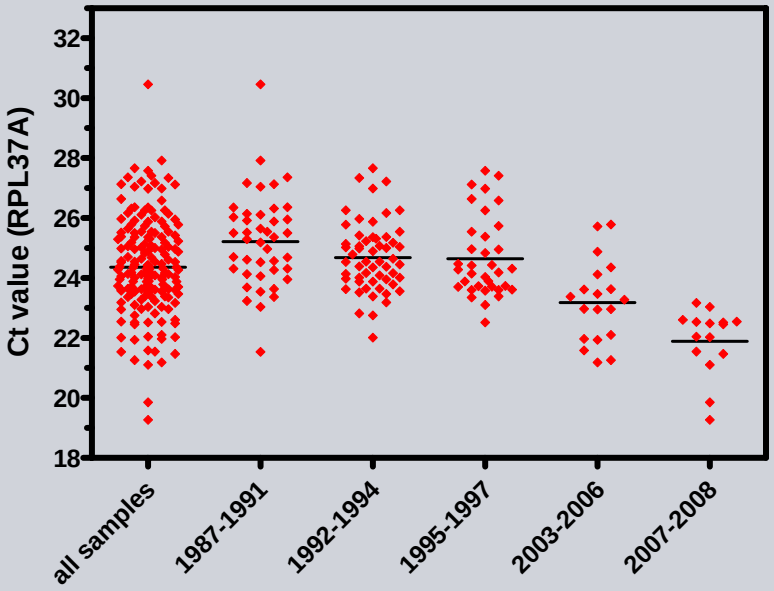
Influence of Storage Time on Yield

Müller et al, 2009, submitted (cooperation with Institute of Pathology, Charite Berlin)
Extraction of RNA from 167 breast carcinomas stored 2 months up to 21 years





Yield of total RNA by Ribogreen or RPL37A Ct value
significantly decreases with storage time

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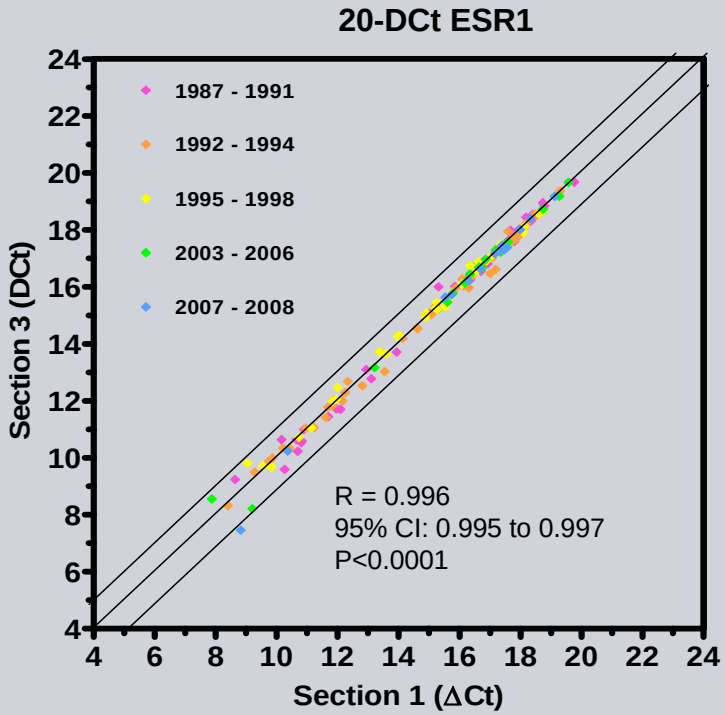
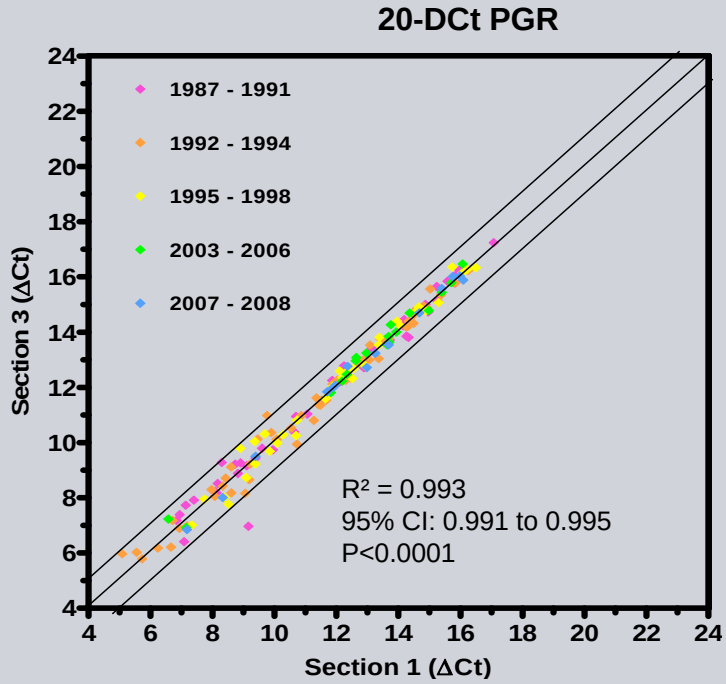
Section to Section Reproducibility of Normalized Expression

A



B



High reproducibility of quantitative RNA expression values (ΔCt for ESR, PGR and ERBB2) between consecutive sections

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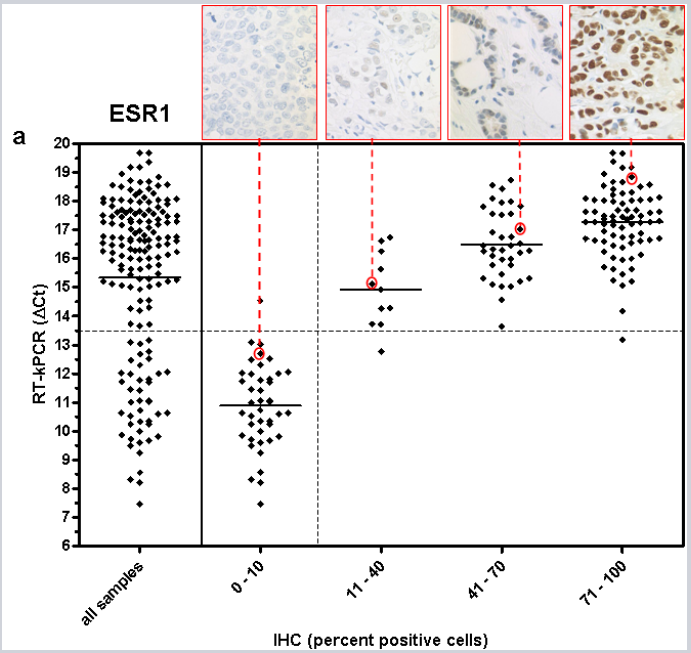
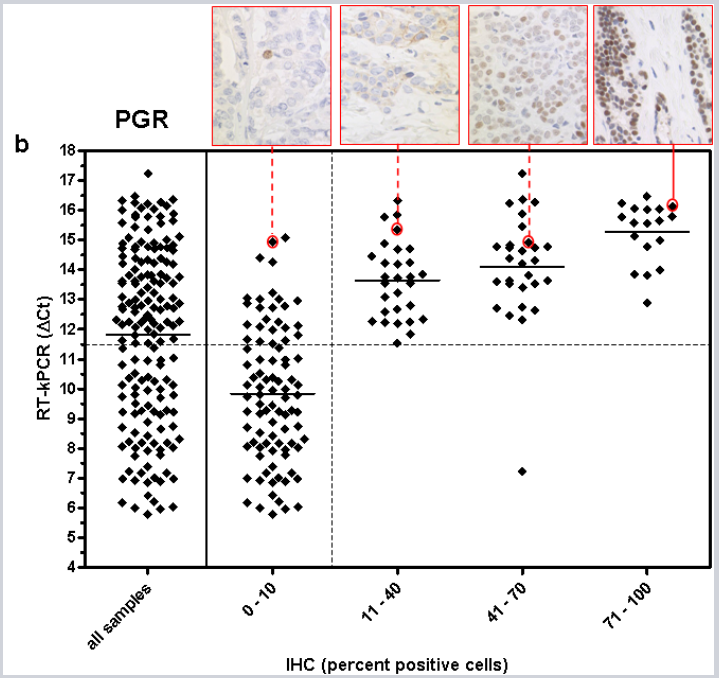
High Concordance between Potein and RNA level





RT-PCR Cut-off

Overall Agreement	ESR1	98,2%	(Pos 98,4% / Neg 97,6%)
	PGR	84,4%	(Pos 98,7% / Neg 72,5%)

High concordance between quantitative RNA expression and semiquantitative immunhistochemistry data for ESR1 and PGR

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Different Time in Fixation Study

Cooperation with Prof. Poremba,

Center for Histology, Cytology and Molecular Diagnostics, Trier

1 Breast Cancer Tumor

Portion 1: TIF 1h in Neutrally Bufferd Formalin (NBF)

Portion 2: TIF 20h in NBF (“routine”)

Portion 3: TIF 2 days in NBF

Portion 4: TIF 5 days in NBF

Portion 5: TIF 10 days in NBF

Portion 6: Fresh frozen within a few minutes (control)

Duplicates from each portion (independent isolations + detections)

10 genes detected with RT-kPCR and normalized to RPL37A

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Time in Fixation Normalized Expression Data

Even extreme fixation times allow accurate expression profiling in RNA from FFPE tissue

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Different Time to Fixation Study

1 Breast Cancer Tumor

Portion 1: TTF 10 min at 20°C (“routine”)

Portion 2: TTF 10 min at 37°C
Portion 3: TTF 1h at 4°C
Portion 4: TTF 1h at 37°C
Portion 5: TTF 12h at 4°C
Portion 6: TTF 12h at 20°C
Portion 7: TTF 12h at 37°C
Portion 8: TTF 24h at 20°C
Portion 9: TTF 24h at 37°C
Portion 10: FF within a few minutes (control)

Portion 1-9 were fixed with neutrally buffered formalin (NBF) for 16-20h

Duplicates from each portion (independent isolations + detections)
10 genes detected with RT-kPCR and normalized to RPL37A

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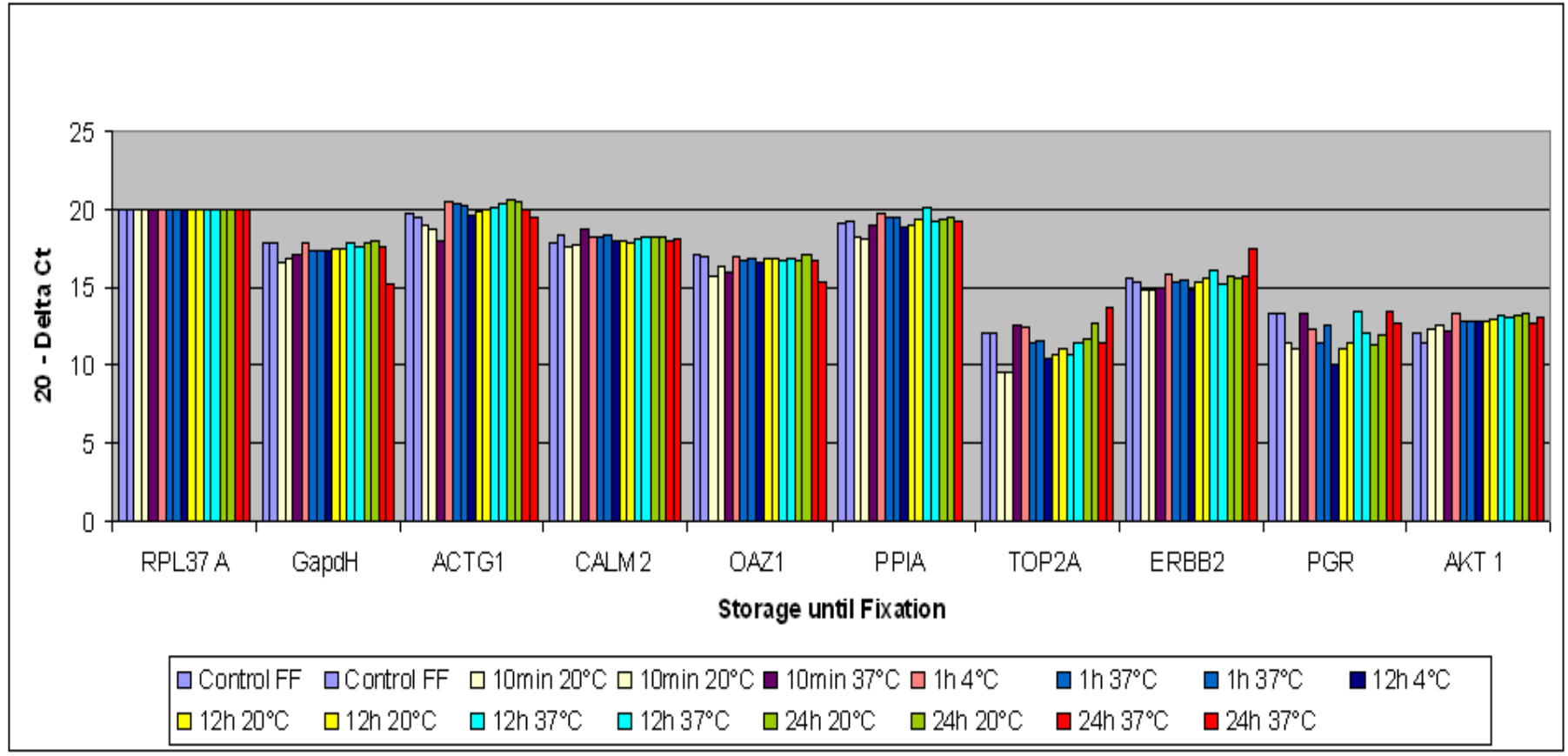
Different Time to Fixation Normalized Expression Data



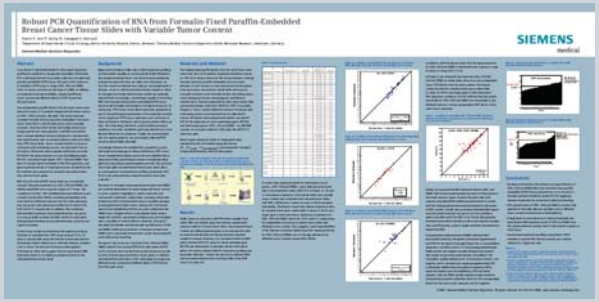
Even extreme time intervals from surgery to the fixative allow accurate expression profiling in RNA from FFPE tissue

Tumor Content + Heterogeneity Study

Cooperation with T. Tramm + J. Overgaard,
Department of Experimental Clinical Oncology, Aarhus University Hospital, Denmark

- Paraffinblocks from 22 pts. diagnozed with breast cancer 1991-1993
- Two independent FFPE blocks from one tumor
- 1 HE-section from each block
- H: whole section = 30 - 100 % tumor
- T+: trimmed section (invasive component) = 90 - 100 % tumor
- Automated isolation with Siemens´ FFPE RNA method
- Genes detected with RT-kPCR: RPL37A, ESR, PGR, ERBB2

ASCO 2007



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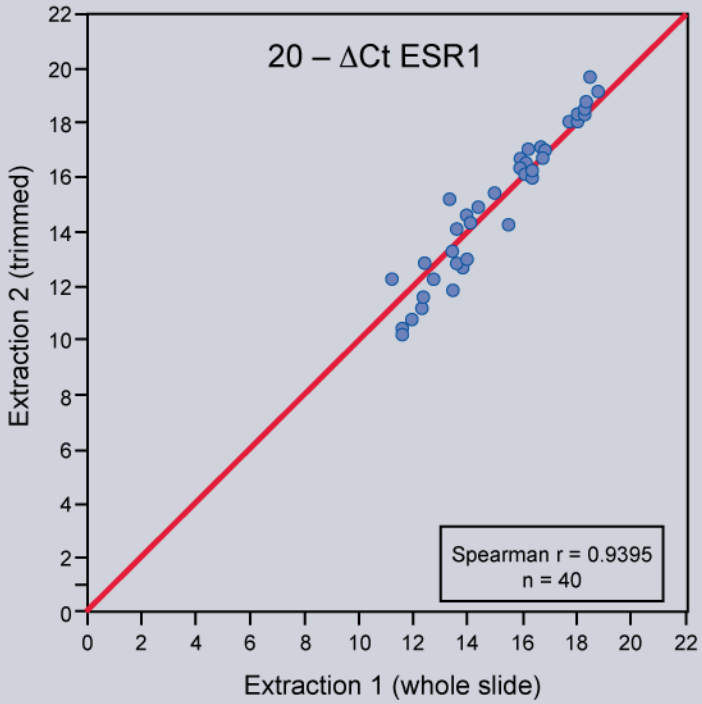
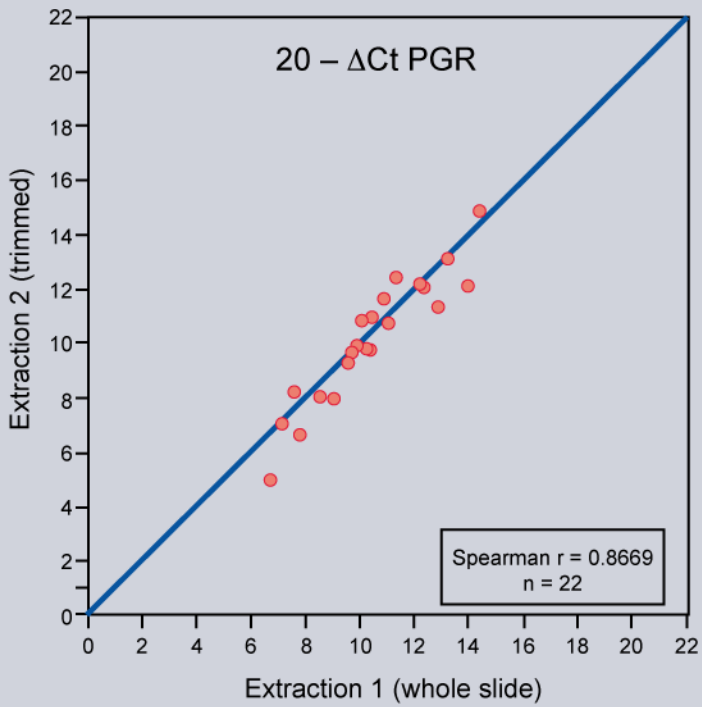
Different Tumor Content Study





High reproducibility between independent isolations and different tumor content for ESR, PGR and ERBB2 RNA expression

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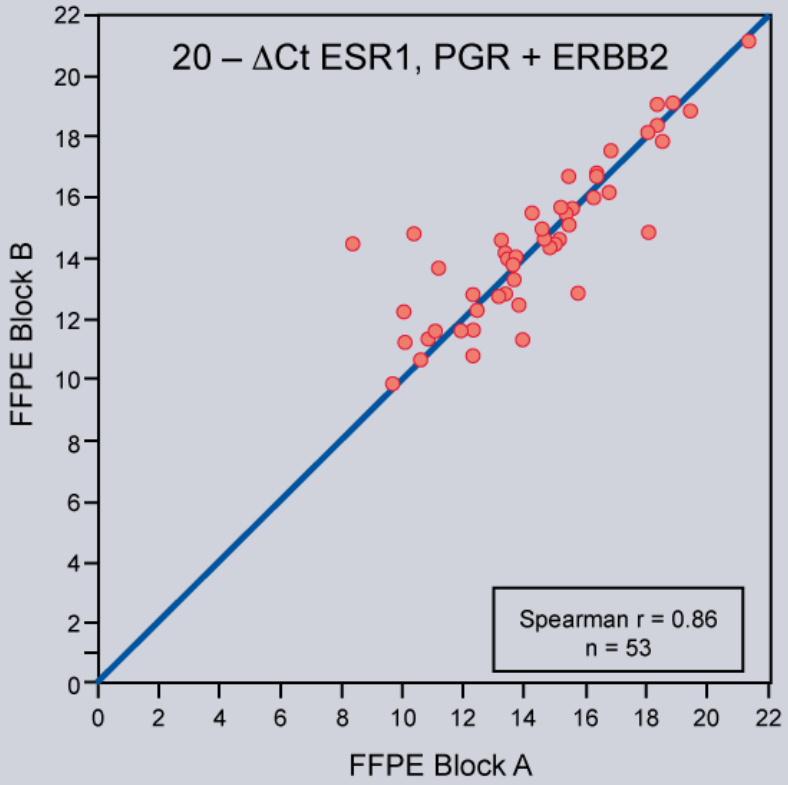
Intra-Tumor Heterogeneity



Good correlation between independent FFPE blocks
from one patient for ESR, PGR and ERBB2 RNA expression

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Summary

- Siemens developed a unique nucleic acid isolation technology for RNA (and DNA) from FFPE tissue based on silica magnetic beads
  - High yield + Carry Over free
  - Fully automated (No deparaffinization / High throughput)
  - Reproducible and robust (under different clinical conditions)
- Technology is key for detection and quantification of molecular markers by expression profiling (PCR or arrays), sequencing and genotyping
- Contribution for implementation of robust and high-throughput testing of biomarkers in clinical routine and retrospective + prospective research studies

Acknowledgements

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University Hospital Aarhus

T. Tramm and J. Overgaard
Department of Experimental Clinical Oncology; Aarhus

C. Denkert and B. Müller
Institute of Pathology, Charite Berlin

K-L Schäfer, Uwe Rogel
Institute of Pathology, Düsseldorf

Center for Histology, Cytology and Molecular Diagnostics

Ch. Poremba, Trier