ExpressArt® FFPE Clear RNAready kit
Features and Example Results

AmpTec
Amplification Technologies

General problems with FFPE samples

Formalin-fixation of tissues results in severe RNA fragmentation, as well as in RNA–RNA, RNA-DNA and RNA–protein cross-linking, which impairs RNA solubilisation and template activity in reverse transcription and subsequent downstream assays.

The FFPE Clear RNAready kit

- **is outstanding** in its consistency of providing high quality FFPE RNA, as determined by RNA yields, bioanalyzer RNA profiles and qRT-PCR data.
- is optimised for isolation of total RNA from archival, formaldehyde or paraformaldehyde-fixed, paraffin-embedded (FFPE) tissues and results in a concentrated RNA product that is ready-to-use (no RNA precipitation required).
- requires no overnight lysis step - deparaffinise in the morning and perform qRT-PCR in the afternoon.
- works with
  LARGE SAMPLES: up to five sections (10 to 20 µm thick), or up to 30 mg per reaction.
  ➔ results in typical yields of ~ 2 µg RNA per slide (10 µm thick, ~ 1 cm^2 tissue area).
- *small samples*: like Laser microdissected FFPE samples
  ➔ results in yields of ~ 2 to 7 pg/cell (15 to 50 ng RNA from 7500 microdissected cells).
- results in RNAs suitable for qRT-PCR, mRNA amplification, microarray analyses & Next-Generation Sequencing.

This kit provides a further development of a previously published procedure. Its outstanding performance is documented in multiple publications:

[1] Oberli et al. (2008) *Expression profiling with RNA from formalin-fixed, paraffin-embedded material.* BMC Medical Genomics 1:9.

[2] Schobesberger et al. (2008) *Gene expression variation between distinct areas of breast cancer measured from paraffin-embedded tissue cores.* BMC Cancer 8:343.

[3] Antonov et al. (2010) *Molecular risk assessment of BIG 1-98 participants by expression profiling using RNA from archival tissue.* BMC Cancer 10:37.

[4] Wilkens et al. (2011) *The homeobox gene HLXB9 is upregulated in a morphological subset of poorly differentiated hepatocellular carcinoma*. Virchows Arch 458: 697–708.

[5] Jaggi et al. (2011) *A Case Study: Molecular Profiling of Breast Cancer from Formalin-Fixed, Archival Material - Gene Expression Profiles from FFPE Samples with Improved RNA Decrosslinking Technology.* J Biomol Tech. 22: Supplement S45.

[6] Cathomas et al. (2012) *Efficacy of cetuximab in metastatic castration-resistant prostate cancer might depend on EGFR and PTEN expression: results from a phase II trial (SAKK 08/07).* Clin Cancer Res 18:6049-57.

[7] Masqué-Soler et al. (2013) *Molecular classification of mature aggressive B-cell lymphoma using digital multiplexed gene expression on formalin-fixed paraffin-embedded biopsy specimens.* Blood 122:1985-1986.

[8] Gautschi et al. (2014) *Thymidylate synthetase (tyms) expression is not predictive in patients with metastatic NSCLC treated with pemetrexed, cisplatin and bevacizumab in the SAKK19/09 trial.* J Thoracic Oncology 9(4 S1) 101P.

[9] Hedegaard et al. (2014) *Next-Generation Sequencing of RNA and DNA Isolated from Paired Fresh-Frozen and Formalin-Fixed Paraffin-Embedded Samples of Human Cancer and Normal Tissue.* PLoS ONE 9(5): e98187. doi:10.1371/journal.pone.0098187

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Principle of the FFPE Clear RNAready kit

The **FFPE Clear RNAready Kit** provides special lysis and incubation conditions for optimal RNA recovery and improved template activity.

Deparaffinisation is not required any more: simply add our proprietary FFPE Clear solvent mix, heat at 80°C, followed by homogenisation and lysis by incubation with proteinase K and detergents. The lysis buffer is supplemented with the nucleic acid analogue **NucleoGuard**, which acts as universal inhibitor of RNases and displaces tightly bound RNA from high-molecular-weight aggregates, resulting in efficient release of RNA and preservation of the (already limited) RNA quality.

RNA solubilisation is followed by an **innovative demodification step** (patent EP09733523) to reverse formalin-induced cross-links and this enables the purified RNA to deliver maximal performance as template in reverse transcription reactions and subsequent downstream assays.

Then, RNAs are isolated via spin column purification: RNA (and DNA) bind to the column matrix, while contaminating substances (salts, proteins, and other tissue contaminants) are washed away. DNA is digested on the column with DNase I, and the DNA fragments and the DNase enzyme are removed in a series of wash steps. Finally, the purified RNA is eluted from the column in low-salt buffer (or water).

Experimental Flow Chart

Place up to 5 FFPE sections in a reaction tube

No Deparaffinisation: Add FFPE Clear
Time required: 0.1 h

Lysis
Time required: 1 h

Demodification
Time required: 0.4 h

Spin column purification, including DNA digestion
Time required: Approximately 0.75 h

Purified RNA in ~50 µl eluate
Expected yield: up to 2 µg per slide
Total time required: **Approximately 2.25 h**

Example Results

1. Size distribution of FFPE RNAs

Serial microtome slides (10 µm thick) were prepared from individual FFPE breast cancer tissue blocks. Five slides were combined per reaction tube, and FFPE RNAs were prepared with three different commercial kits.

As expected, the quality of all FFPE RNA samples is low and indicates severe RNA degradation. The comparison indicates that a higher fraction of larger RNAs is recovered and preserved with the FFPE RNAready kit with a typical size range from 50 and up to 4,000 nucleotides.

Please note that several factors will affect the yield and quality of the isolated FFPE RNAs, like the age of the samples, thickness of the original tissue sample (slow penetration by fixative), thickness of the microtome slides, tissue type and the applied fixation protocol

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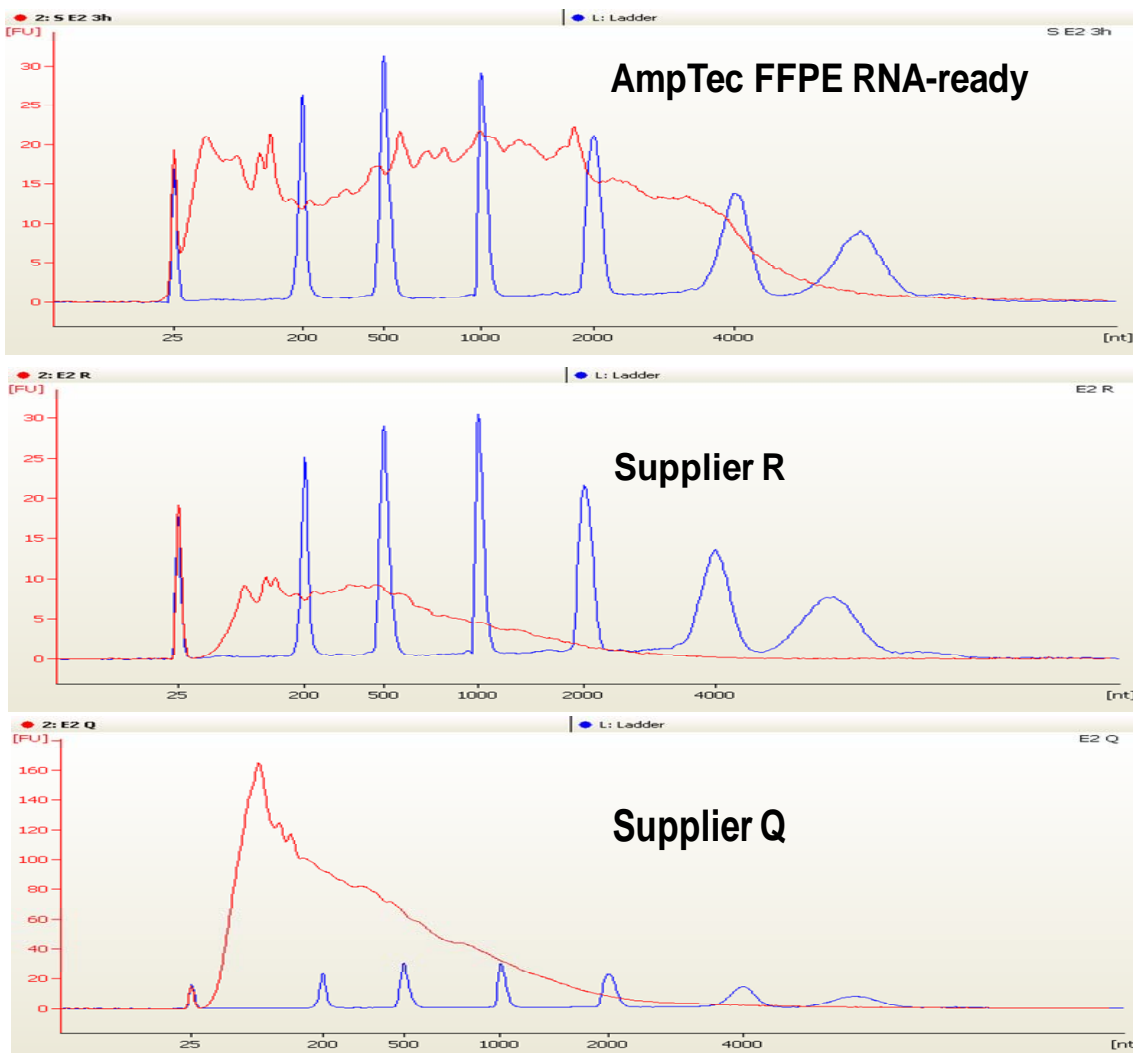
2. Comparison of RNAs isolated from fresh-frozen and FFPE samples

FFPE RNAs were isolated from a series of FFPE breast cancer tissue blocks and analysed with a set of RT-qPCR assays for house-keeping genes.

Conclusion: As expected, RNAs isolated from matched fresh-frozen samples resulted in lower C_T values with less variability, but only moderate increases were observed for both values, if the FFPE RNAs were isolated with the FFPE RNA-ready protocol.
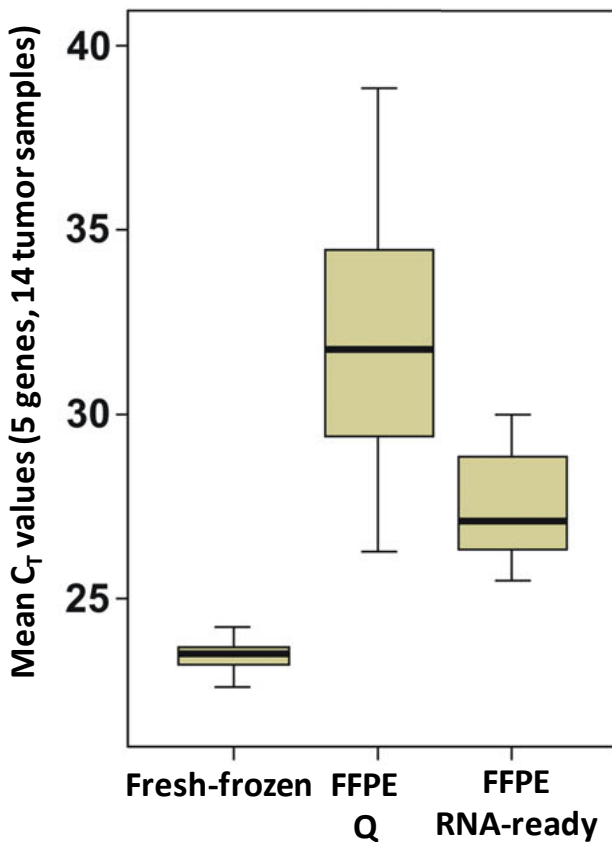
Mean C_T values for five reference genes (GAPDH, GUSB, RPLP0, TFRC, UBB) were determined with 14 individual breast tumor samples, comparing RNA samples from fresh-frozen and from matched FFPE material, either using the FFPE RNA isolation protocol of Supplier Q or using the ExpressArt FFPE RNA-ready protocol.

3. Results with Challenging samples

Data were generously provided by M. Szczepanowski, N. Masque-Soler & W. Klapper, Section Hematopathology and LKR, UKSH Campus Kiel, Germany.

Results were published in: Masqué-Soler et al. (2013) Molecular classification of mature aggressive B-cell lymphoma using digital multiplexed gene expression on formalin-fixed paraffin-embedded biopsy specimens. Blood 122: 1985-1986

3.1. Size distribution and yield

Serial microtome slides (10 µm thick) were prepared from individual FFPE tissue blocks. FFPE RNA extraction with the kit from supplier A frequently failed. RNA yields and quality were dramatically improved with the AmpTec kit, even a RIN value of 6.0 could be observed..

FFPE RNA with kit from supplier A
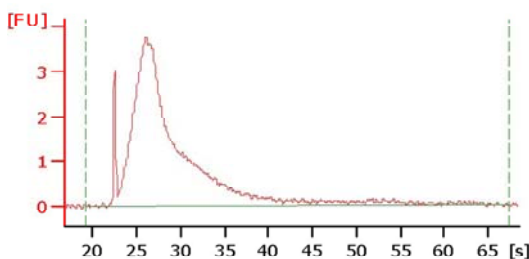
Overall Results
RNA Concentration: 70 ng/µl
RNA Integrity Number (RIN): 2.4 (B.02.03)
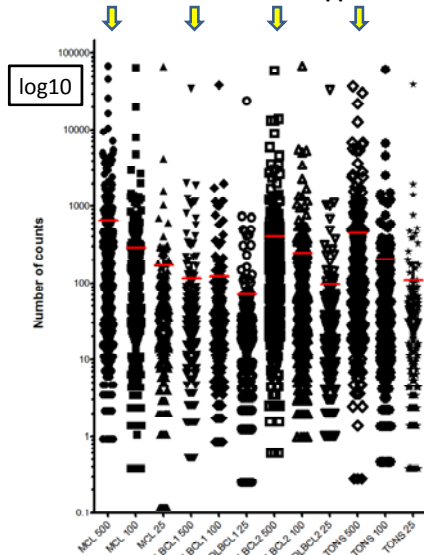
FFPE RNA with FFPE Clear RNAready kit
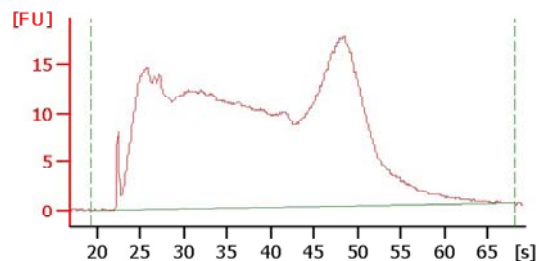
Overall Results
RNA Concentration: 634 ng/µl
RNA Integrity Number (RIN): 6.0 (B.02.03)

3.2. Application: nCounter (NanoString Inc) technology for quantitative gene expression profiling

FFPE RNAs were extracted from a series of paraffin samples (different tumor entities, different sample ages) and analysed with the amplification-free nCounter technology (NanoString).

The majority of RNA samples with the kit from supplier A gave very low yields and proper analysis was impossible. Consistently high yields and qualities obtained with the AmpTec kit allowed the analysis of all samples with a maximum of 500 ng FPPE RNA. For a few samples, comparison of absolute counts was possible as illustrated below. Arrows indicate data obtained with 500 ng FFPE RNA samples: with the AmpTec kit, absolute counts were **2.5- to >4-fold higher.**

FFPE RNA with kit from supplier A   **FFPE RNA with FFPE Clear RNAready kit**