Gene Expression Application Note No. 2

Easy and Reliable qRT-PCR Analysis of Total RNA Isolated from Fresh Frozen and FFPE Tissue Samples

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1 Introduction

The use of tissue sections for molecular analysis of pathogenic states in mammalian tissue has become an indispensable approach for understanding of molecular mechanisms in etiology and disease progression.

With the identification of each new gene expression profile, one step forward is achieved, to develop therapeutic treatments tailored to a given individual.

In the biomedical research routine, a variety of different sample materials exist with potential interest in gene analysis. Especially tumor genesis, as a very local process, requires individual sample preparation and conservation. Most commonly, dissected tissue samples are prepared as fresh frozen or even more often as formalin-fixed paraffin embedded (FFPE) samples. Both techniques show both advantages and disadvantages.

Especially degradation of RNA in FFPE samples is the most severe drawback for this particular technique. However, the worldwide well established FFPE tissue banks have become an invaluable source for conserved tissue material.

On the other hand, despite the fact that fresh frozen sample material better reflects the pathological state of a tissue, the challenge to economically organize the required logistical chain necessary for fresh frozen tissue samples have not been satisfactory. (1, 2, 3).

Here, we present data for two independent workflows in gene expression analysis using Roche's LightCycler® 480 Instrument. RNA samples were isolated from HeLa xenograft tissue, FFPE or fresh frozen, using either the High Pure FFPE RNA Micro Kit or the MagNAPure LC RNA Isolation Kit III (Tissue) on the MPLC 2.0 Instrument, together with the MagNA Lyser Instrument.

Isolated RNA was subjected to cDNA synthesis using the Transcriptor First Strand cDNA Synthesis Kit. Employing RT-PCR using Roche's LightCycler® 480 Instrument together with SYBR Green and the **Universal ProbeLibrary** formats, we were able to generate excellent data sets suitable for gene expression analysis.

2 Methods

Step 1. RNA isolation

A) RNA isolation from fresh frozen tissue

Approx. 10 mg fresh frozen sample from HeLa xenograft tissue, were prepared using a scalpel and transferred into a tube of MagNA Lyser Green Beads pre-cooled on dry ice. Immediately before starting tissue homogenization, using a MagNA Lyser Instrument, 800 µl Lysis Buffer from MagNA Pure LC RNA Isolation Kit III (Tissue) were added. The MagNA Lyser Instrument was used with the following protocol: Tubes were placed in the MagNA Lyser Instrument and processed four times for 30 seconds at 6500 rpm. Between two processing steps, samples were cooled in a pre-cooled sample rack for one minute.
Subsequently samples were incubated for 30 minutes on a Roller Mixer at +15 to +25°C.
After centrifugation for two minutes at 8000 x g, 350 µl lysate each was used for the carrying out of two RNA isolations, following the MPLC protocol for the MagNA Pure LC RNA Isolation Kit III (Tissue) on MagNA Pure LC 2.0 system.

B) RNA isolation from FFPE material

Ten micrometer thick slices from a FFPE block prepared from HeLa xenograft tissue were cut using a microtome. Each slice was deparaffinized by adding 800 µl Xylene and following the protocol given in the pack insert of the High Pure FFPE RNA Micro Kit. The air dried deparaffinized section was used for RNA isolation following the protocol given in the pack insert of the High Pure FFPE RNA Micro Kit.

Step 2. Reverse transcription of the isolated RNA

Reverse transcription reactions were performed according to procedure A in the pack insert of the Transcriptor First Strand cDNA Synthesis Kit. One microgram of total RNA and anchored oligo(dT) primers was used for each reaction.

Step 3. cDNA quality check

In order to check the quality of cDNA generated from either fresh frozen tissue or FFPE material, a qPCR using SYBR-Green I detection format was performed on the LightCycler® 480 Instrument. The following protocol was used: The real-time PCR mixture contained 10 µl 2x LightCycler® 480 SYBR Green I Master, 0.4 µmol/L of each primer and 2 µl of cDNA. The cycling conditions included initial incubation step at 95°C for 5 minutes followed by a 45 cycles of amplification with 10 seconds at 95°C, 10 seconds at 60°C, and 10 seconds at 72°C (single acquisition). The melting analysis consisted of 95°C for 30 seconds followed by cooling to 65°C for 30 seconds before the temperature was raised to 95°C at a rate of 0.1°C/s with continuous fluorescence acquisition. The final cooling step was 40°C for 30 seconds. Two housekeeping genes, β2 Microglobulin and GAPDH were used to check cDNA from fresh frozen tissue and FFPE material, respectively.

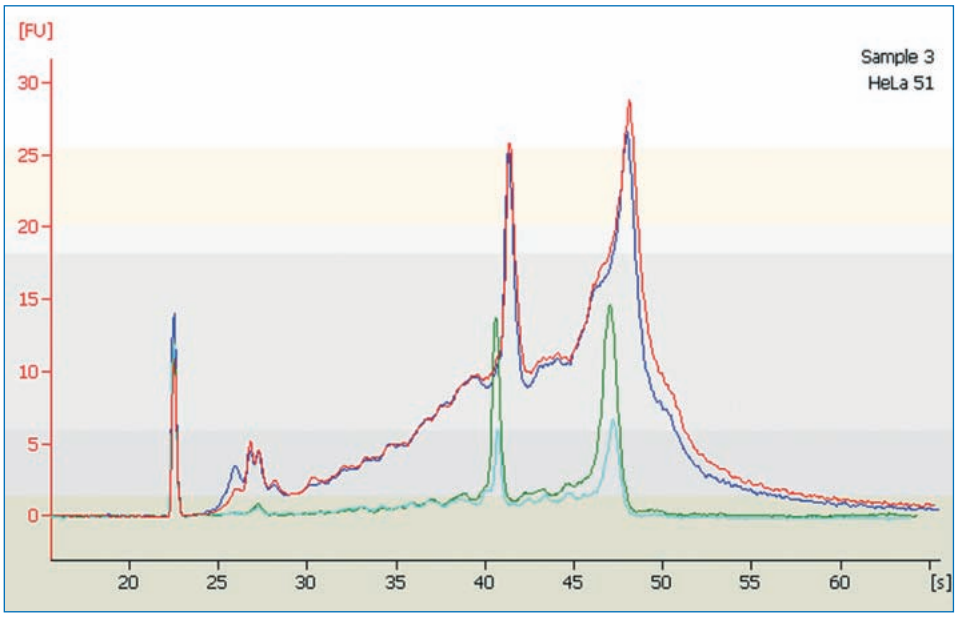
Step 4. qRT-PCR analysis

A qPCR using the Universal ProbeLibrary detection format was performed on the LightCycler® 480 Instrument to quantify gene expression in both tissues. Two sets of genes, including one gene of interest and one housekeeping gene, were quantified. The PCR protocol is as follows: The real-time PCR mixture contained 10 µl 2x LightCycler® 480 Probes Master, 0.4 µmol/L of each primer, 0.1 µmol/L of UPL probe and 2 µl of cDNA. The cycling conditions included initial incubation step at 95°C for 10 minutes, followed by a 45 cycles of amplification with 10 seconds at 95°C, 30 seconds at 60°C (single acquisition), and 1 seconds at 72°C. The final cooling step was 40°C for 30 seconds. Two housekeeping/target gene sets, β2 microglobulin /c-myc for FFPE material and GAPDH/BAD for fresh frozen tissue were measured, respectively.

3 Results

A. RNA quality check:

For the demonstration of two possible workflows starting with either FFPE or fresh frozen sample material, xenografted HeLa tissue either fresh frozen or FFPE material was used.
Total RNA was isolated using the MagNA Pure LC RNA Isolation Kit III (Tissue) together with the MPLC 2.0 Instrument for the fresh frozen tissue and the High Pure FFPE RNA Micro Kit for the FFPE material. As the quality of the RNA preparation is a crucial criterion for subsequent gene expression analysis, RNA was analyzed with a Nano Drop Instrument (Nano Drop Technologies) and a Bio-analyzer (Agilent Technologies). Both RNA preparation methods were of high quality. Ratio A260/A280 and the RNA integrity factor (RIN), which also considers the RNA fragment length distribution are summarized in Table 1.



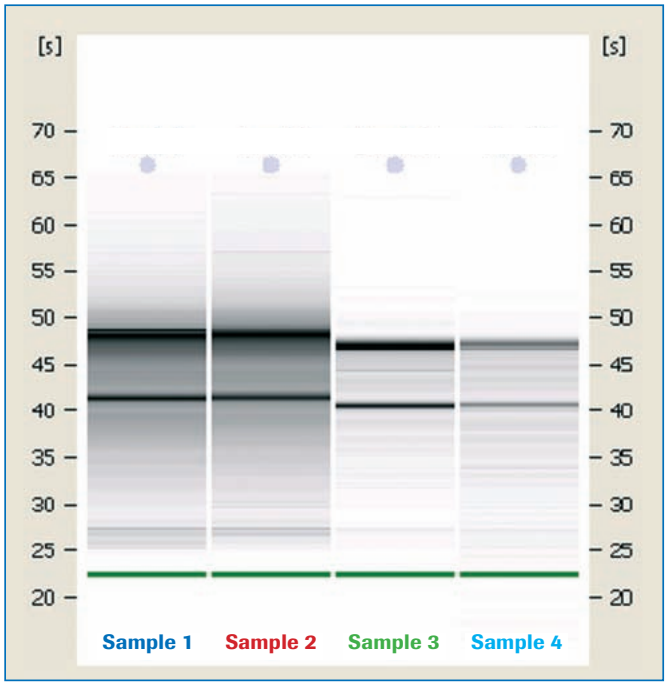


Figure 1: Electropherogram and Gel representation of total RNA from FFPE material (sample 1 and sample 2) and fresh frozen material (sample 3 and sample 4).

	Tissue	A_{260}/A_{280}	RIN
Sample 1	FFPE	1.96	7.1
Sample 2	FFPE	1.81	7.3
Sample 3	FF	2.12	8.9
Sample 4	FF	1.95	7.4

Table 1: Quality of RNA

B. cDNA quality check:

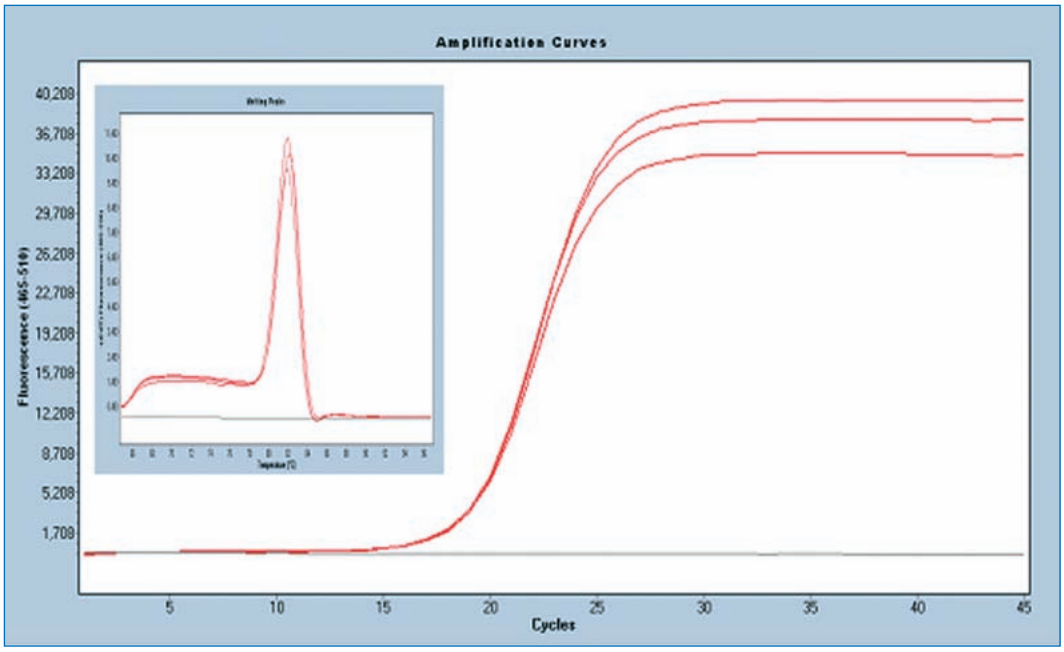


Figure 2: Quality check of cDNA generated from fresh frozen tissue.
LightCycler® real-time PCR was performed with primers specific for human β2 microglobulin, on 5% of cDNA generated from 1 µg of total RNA.

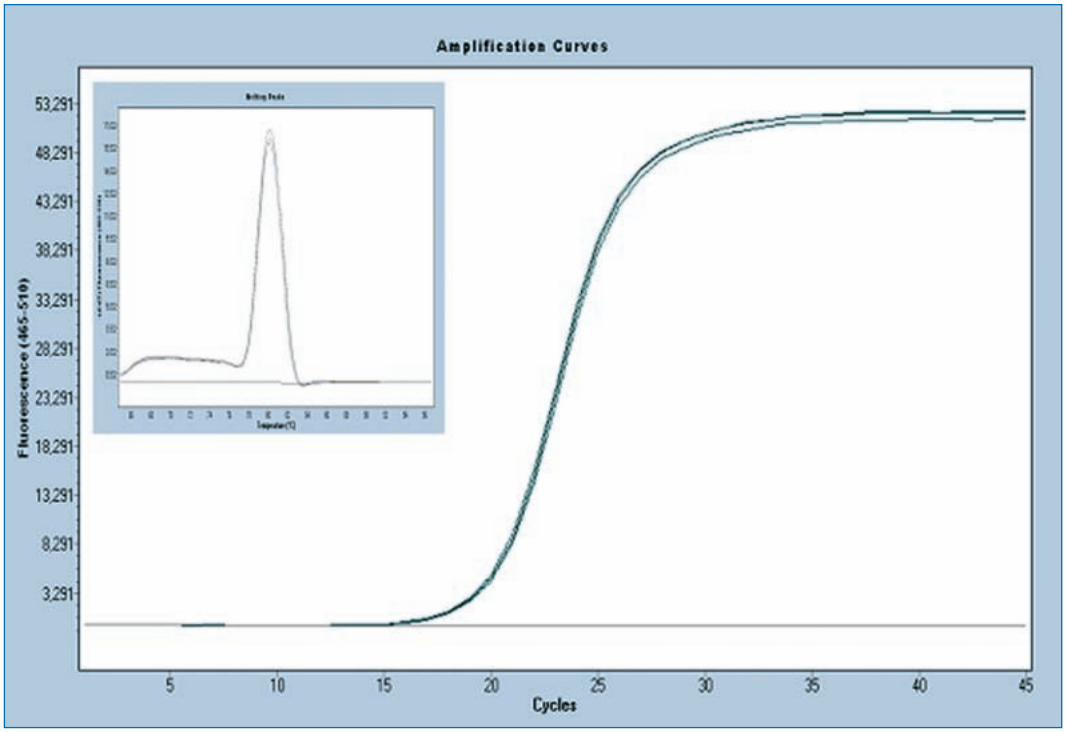


Figure 3: Quality check of cDNA generated from FFPE tissue.
LightCycler® real-time PCR was performed with primers specific for human GAPDH, on 5% of cDNA generated from 1 µg of total RNA.

In both cases, a clear, single peak in the melting curve indicates the purity and specificity of the amplified PCR fragment.

C. qRT-PCR analysis:

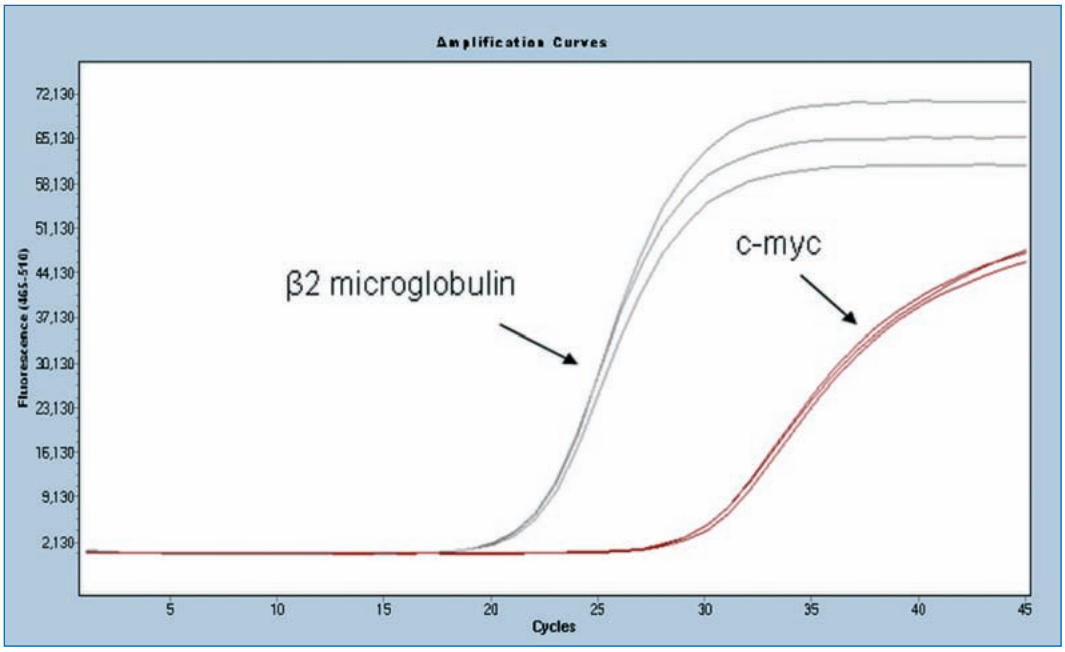


Figure 4: Amplification curve of the ß2 microglobulin and c-myc gene from FFPE.
When using RNA derived from FFPE tissue samples, the mean crossing point (CP) value (3 independent amplifications) of β2 microglobulin is 21.88 with a standard deviation of 0.10 (grey curve), while the mean CP of c-myc is 29.85 with a standard deviation of 0.15 (brown curve).

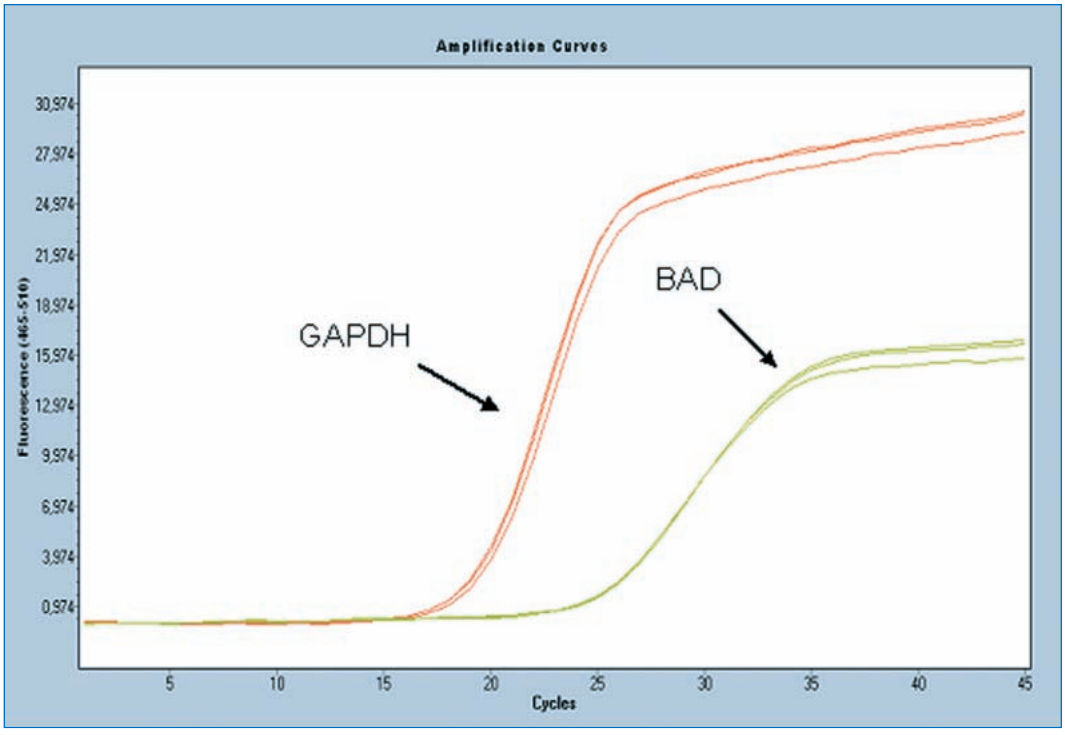


Figure 5: Amplification curve of the GAPDH and BAD gene from fresh frozen tissue.
When using RNA derived from fresh frozen tissue samples the mean crossing point (CP) value (3 independent amplifications) of GAPDH is 19.27 with a standard deviation of 0.18 (orange curve) while the mean CP of BAD is 25.53 with a standard deviation of 0.08 (yellow curve).

We deliberately chose two low-copy genes (c-myc and BAD) as target in order to see if they can be properly amplified. The results showed that all four genes are well amplified. The small standard deviations of the CP values indicate that the results are reproducible.

3 Conclusion

The most crucial point for the performance of qRT-PCR analysis of archived tissue samples is the quality of the RNA preparation used as a template. In other words, high quality RNA samples which reveal a high integrity are necessary for these analyses.

The quality characteristics of RNA preparations are strongly dependent on the quality of the tissue sample and the selected technique and good manufacturing practice applied for their preservation. Formalin fixed paraffin embedded tissue is certainly one of the most challenging sample materials available.

Within recent years, due to the development of new protocols and optimization of existing techniques, it is now possible to perform valuable gene expression analysis irrespective of the starting material as long as it is in general of the highest possible quality.

Here, we show two optimized workflows for gene expression analysis from both FFPE and fresh frozen sample material.

In both workflows, easy to establish procedures were linked together to achieve reproducible, highly reliable results.

Figure 6: Schematic overview of the applied workflows.

We have decided to use an automated workflow wherever possible to avoid handling issues. However, if a low number of samples is to be processed and the necessary precautions are taken into account, the manual workflow, using the High Pure RNA Tissue Kit for fresh frozen tissue will provide comparable results (data not shown).

For our experiments, we have selected a rather recent sample material from xenografted HeLa tissue (09/2008) prepared in our local routine lab for tissue sample preparation. Results with FFPE samples may vary in addition to the already mentioned factors also depending on the time of storage.

Both starting materials resulted in the highly reproducible and reliable detection of two low copy genes in comparison to a housekeeping gene. We have selected two different gene sets for the analysis of the RNA on purpose. It might be quite tempting to compare the expression rate in both sample preparations. This can certainly be achieved, if the processes are strongly validated and placed on a solid statistical basis. However, as our main goal was to show that it is technically possible to get highly reliable data, we did not focus on creating the statistics for such a comparison.

If the research focus is positioned on the direct comparison of expression rates in fresh frozen versus FFPE material with respect to a specific gene set, a statistical validation is mandatory and we strongly recommend using matched samples and respective controls, *e.g.,* healthy versus diseased or treated versus untreated tissue material.

References

1. A High Frequency of Sequence Alterations Is Due to Formalin Fixation of Archival Specimens. C. Williams et. al, American Journal of Pathology, Vol. 155: 14767–1471, 1999
2. Effect of Fixatives and Tissue Processing on the Content and Integrity of Nucleic Acids. M. Srinivasan et. al, American Journal of Pathology, Vol. 161: 1961–1971, 2002
3. Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. N. Masuda et. al, Nucleic Acids Res, Vol. 27: 4436–4443, 1999

Ordering Information

Product	Cat. No.	Pack Size
High Pure FFPE RNA Micro Kit	04 823 125 001	50 isolations
MagNA Lyser Instrument, 110V **MagNA Lyser Instrument, 220V**	03 358 968 001 03 358 976 001	1 instrument
MagNA Lyser Green Beads	03 358 941 001	100 tubes
MagNa Pure LC 2.0 Instrument	05 197 686 001	1 instrument
MagNA Pure LC RNA Isolation Kit III (Tissue)	03 330 591 001	192 isolations
Transcriptor First Strand cDNA Synthesis Kit	04 379 012 001 04 896 866 001 04 897 030 001	50 reactions 100 reactions 200 reactions
LightCycler® 480 II Instrument, 96 well	05 015 278 001	1 instrument
LightCycler® 480 SYBR Green I Master	04 707 516 001	Up to 500 reactions, 20 µl volume
Related Product Information		
High Pure RNA Paraffin Kit	03 270 289 001	50 isolations
DNase I recombinant, RNase-free solution	04 716 728 001	10 KU
Transcriptor HiFi cDNA Synthesis Kit	05 081 955 001 05 091 284 001 05 081 963 001	50 reactions 100 reactions 200 reactions

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Roche Diagnostics GmbH
Roche Applied Science
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