The Agilent 2100 Bioanalyzer

RNA Integrity Number (RIN)
A standardized approach for RNA integrity assessment

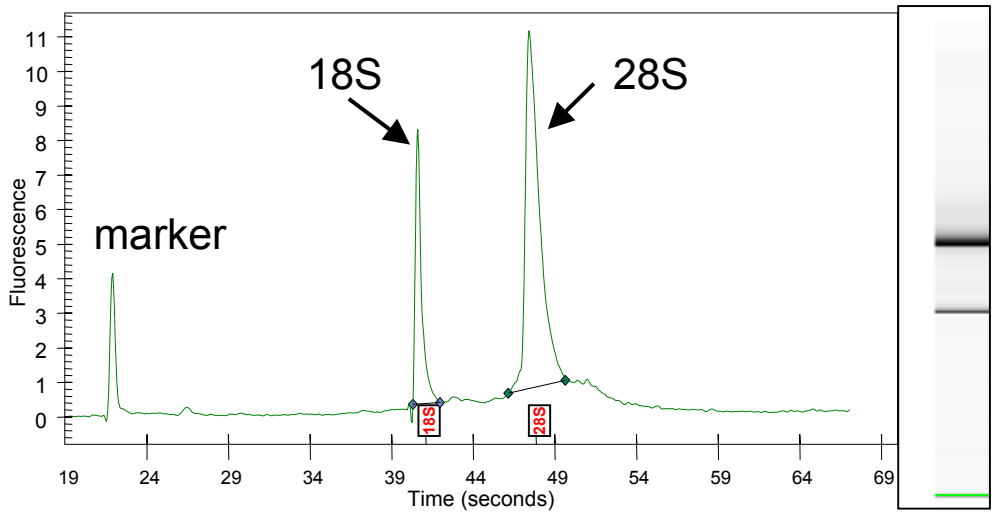
qPCR Symposium
Leipzig, 2005

Marc Valer

Agilent Technologies

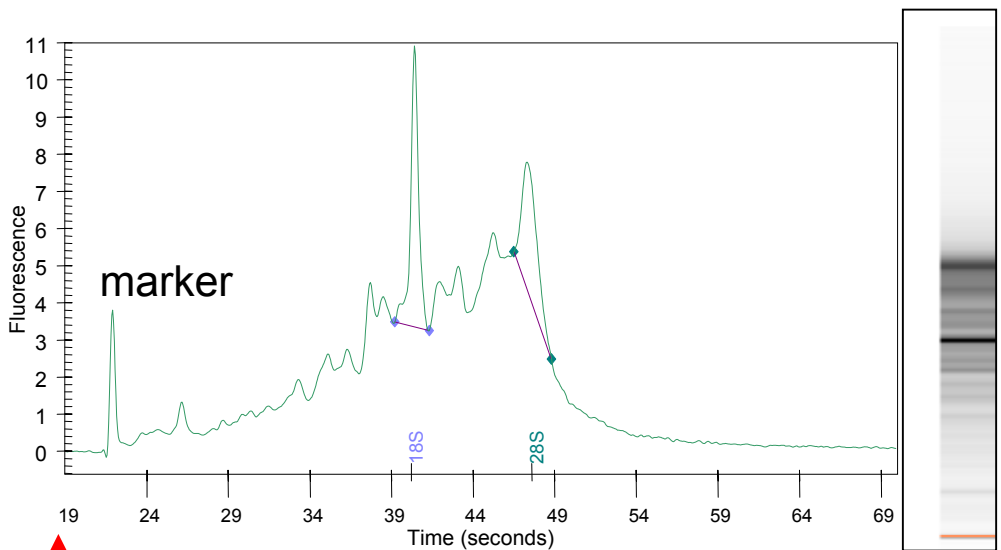
RNA LabChip kits

Analysis of Total RNA Integrity



Typical first QC step after RNA sample prep prior to microarrays or real-time PCR

High quality total RNA



Partially degraded total RNA

2100 bioanalyzer: single lane gel-like image

2100 bioanalyzer: electropherogram

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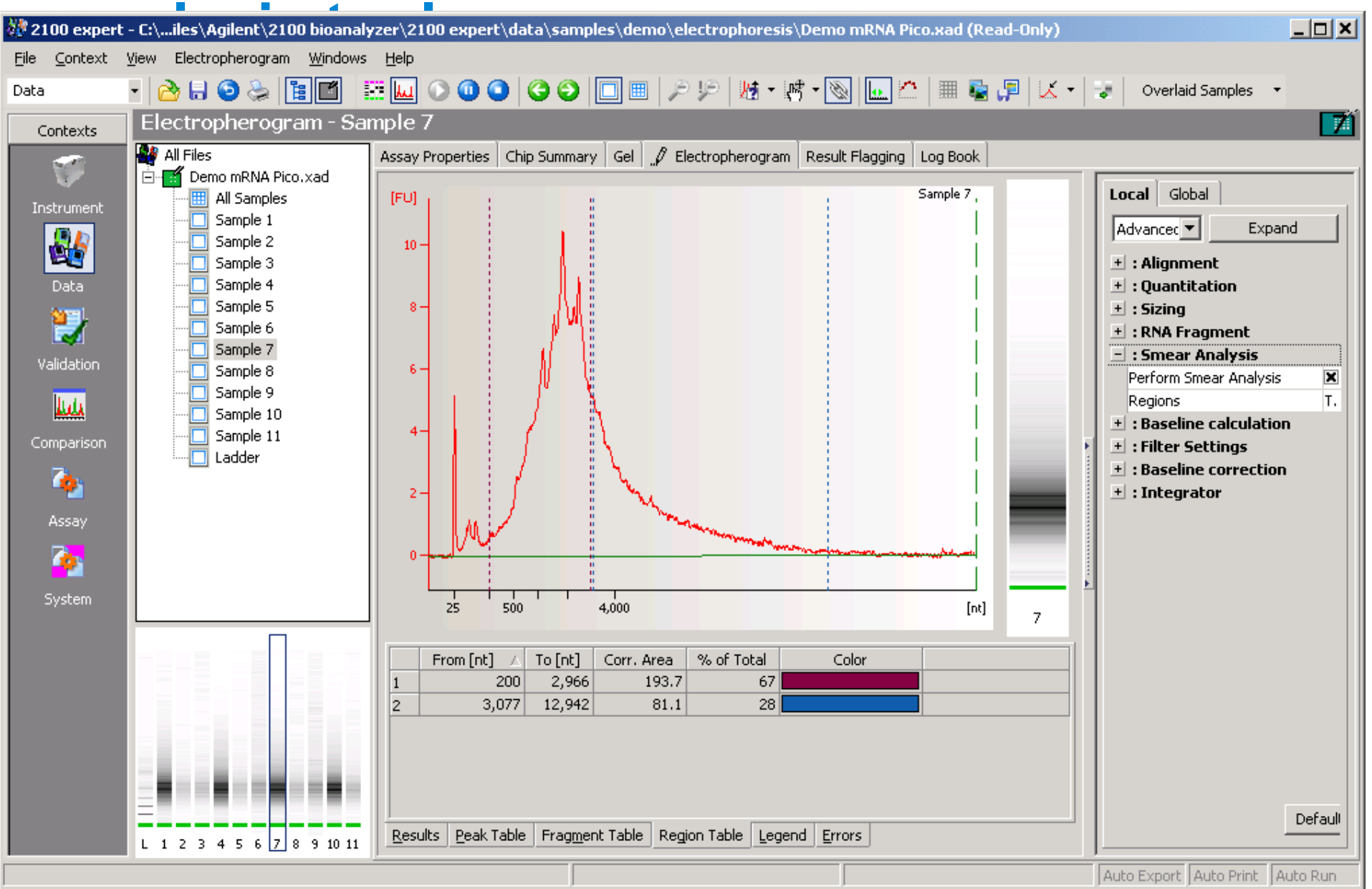
Current total RNA integrity measures

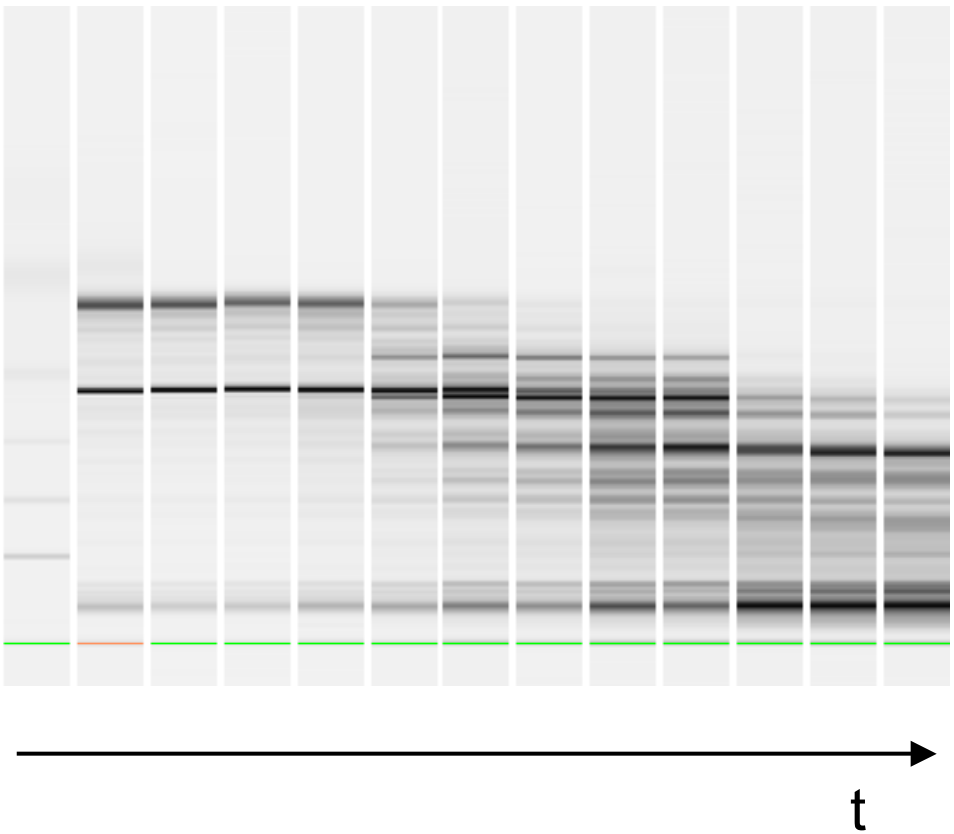
- UV offers no information on size distribution, and does “see” interferences of DNA, solvents and other contaminants. Sophisticated de-convolution is possible to individually quantity the components.
- Gels, have a limited linear range and ribosomal band intensity ratio had been used to differentiate good to bad samples. This measure is very subjective, dependent on the

- RNA degradation is a gradual process.
- Results have to be interpreted by visual inspection.
- Overlay of electropherograms only works well for samples with the same concentration.
- Instrument dependency in signal height

mRNA QC is addressed with smear



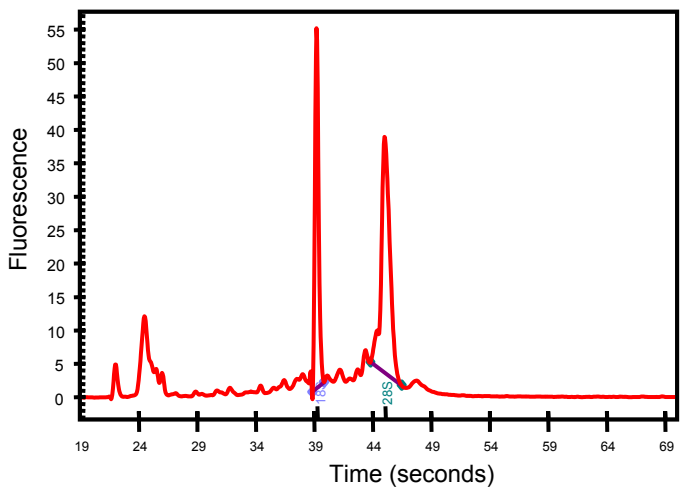
Problem Description



- The ratio of ribosomal bands is not sufficient to describe RNA integrity!
- RNA degradation is a gradual process.
- Results have to be interpreted by visual inspection.
- Overlay of electropherograms only works well for samples with the same concentration.
- Instrument dependency in signal height

Goal

- **Find a tool that describes the integrity of a RNA sample better than the ribosomal ratio alone could do.**
- **Have a bioanalyzer result that describes the sample independently of individual interpretation.**
- **Characterize a RNA sample reproducibly independent on the instrument is was analyzed on and independently of who is performing the analysis**
- **Allow comparison of validation results across different laboratories**



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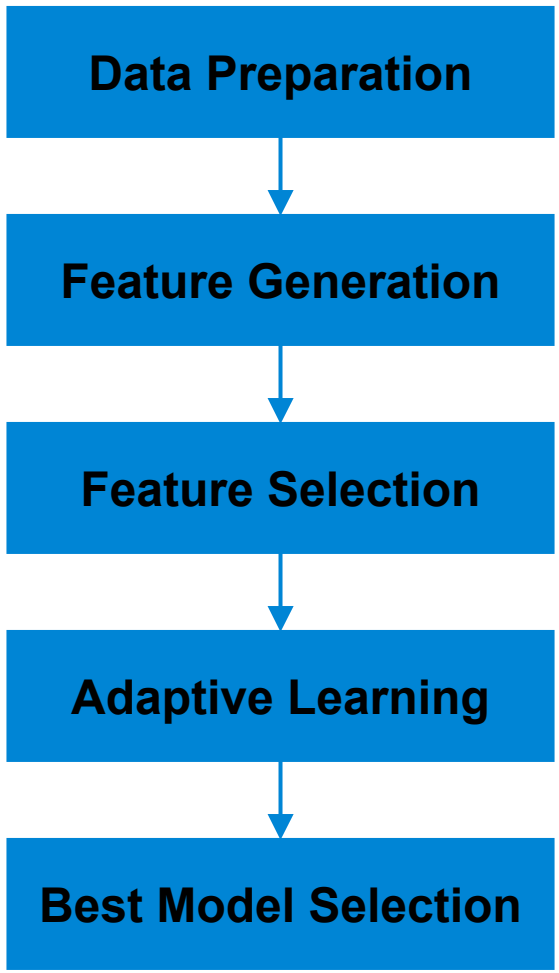
Introducing the RNA Integrity Number (RIN)

- **The RIN is a software algorithm that allows classification of RNA integrity**
- **It extracts a number of characteristic features from the bioanalyzer electropherogram**
- **An adaptive learning process is used to “teach” the algorithm about the relative importance of the extracted features (based on many example electropherograms).**
- **The RIN returns a value that is characteristic for the integrity of a specific sample.**

Approach – Flow chart

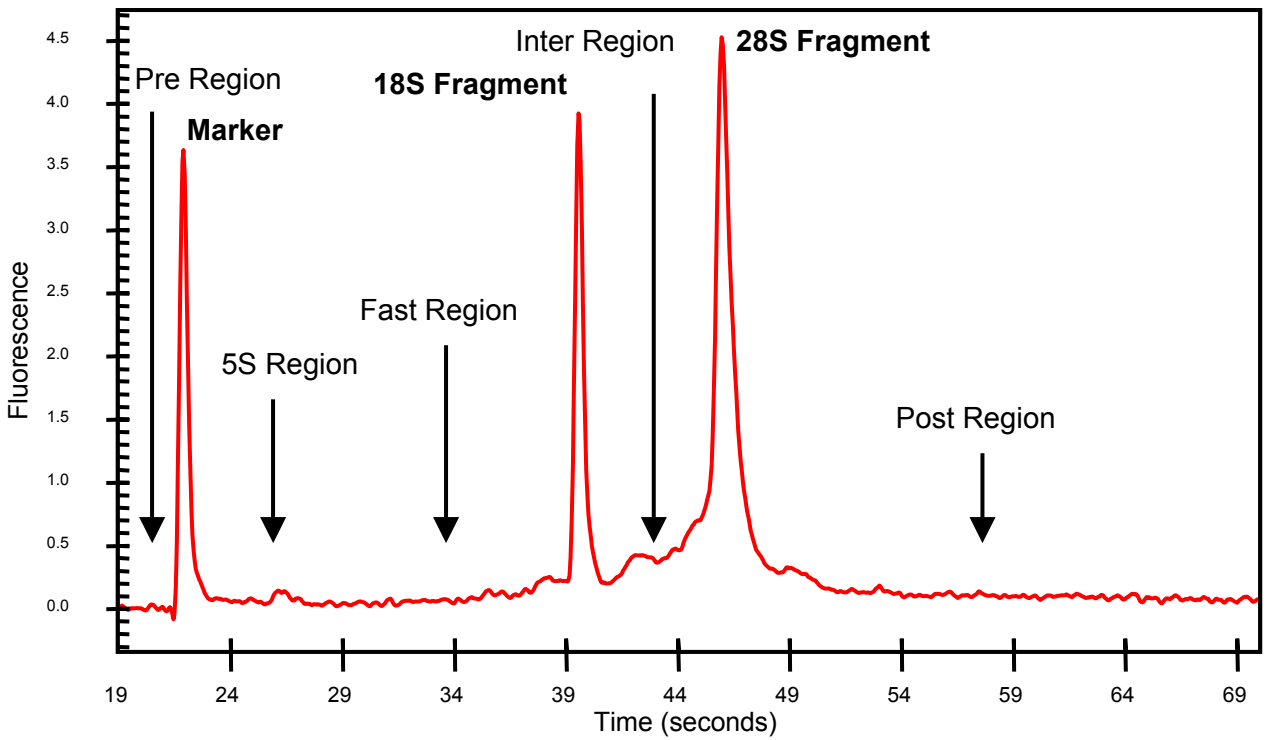


Data Preparation

- **Initially limited to Eukaryote Total RNA Nano Assay, expanded to Eukaryote RNA pico Assay**
- **Input data: Random selection of mostly human, mouse and rat total RNA**
- **>1400 samples analyzed and classified by application specialists, origin of samples: microarray service laboratory and Agilent Labs**
- **Manual generation of quality labels, *q* = targets for learning**
- **Manual generation of anomaly labels, *a* (Ghost Peaks, wavy Baseline)**
- **Quality is a continuous variable, no separate classes by nature → Introduction of 10 discrete classes (1 to 10 in ascending quality)**

Feature Generation

Approach: Training of regression models for peak positions and Intensities based on peak labels



Features

- **Peak height/position**
- **Areas/Area ratios**
- **S/N ratio**
- **Max, Min values**
- **Waviness of the curve**

…

Selection of the best feature combination

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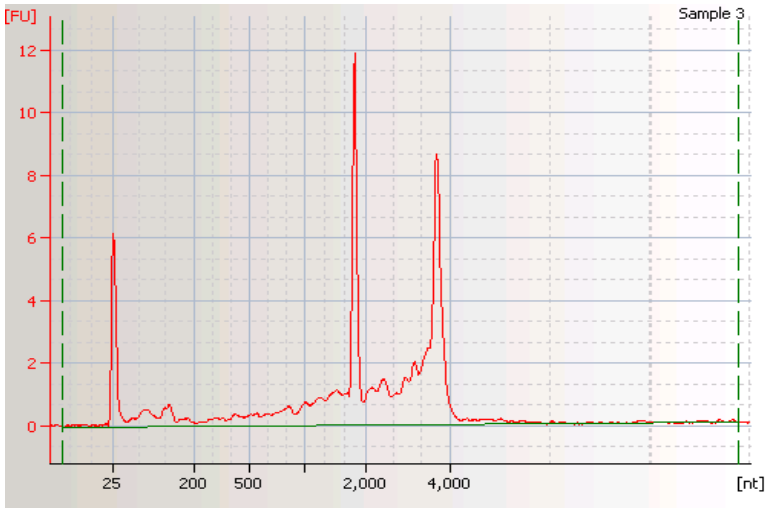
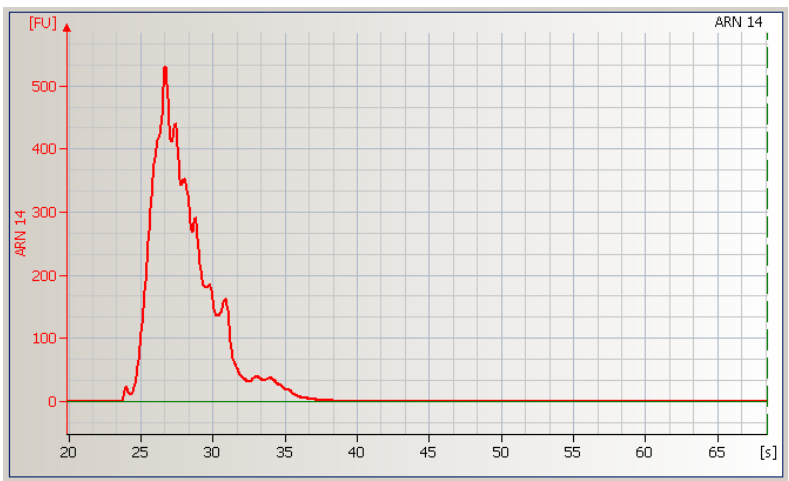
RIN Visualization



RNA Area:	61.7	
RNA Concentration:	102	ng/µl
rRNA Ratio [28s / 18s]:	1.2	
RNA Integrity Number (RIN):	7.8	

Results | Peak Table | Fragment Table | Legend | Errors



RNA Area:	7,007.2	
RNA Concentration:	2,248	ng/µl
rRNA Ratio [28s / 18s]:	0.0	
RNA Integrity Number (RIN):	2.5	

Results | Peak Table | Fragment Table | Legend | Errors

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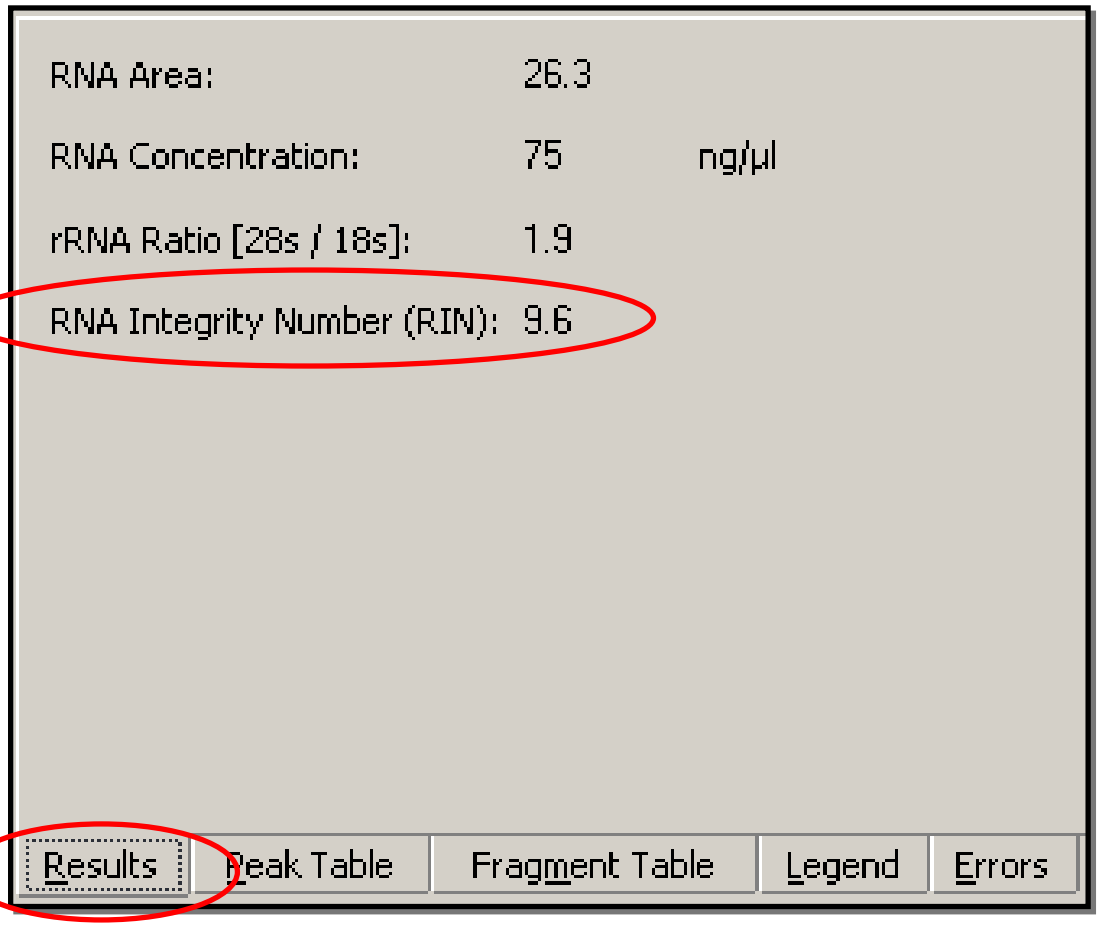
RIN - Limitations

- ***What the RIN can do:***
  - Obtain an assessment of RNA integrity.
  - Directly compare RNA samples
  - Ensure repeatability of experiments

- ***What it CANNOT do:***
  - Predict the outcome of an experiment if no prior validation was done

RIN Visualization



Anomaly threshold settings

: RNA Integrity Number	
Pre Region Anomaly Thres...	0.5
5S Region Anomaly Thresh...	0.3
Fast Region Anomaly Thre...	0.77
Inter Region Anomaly Thre...	0.21
Precursor Region Anomaly ...	0.3
Post Region Anomaly Thre...	0.29
Baseline Anomaly Threshold	0.56
Ribosomal Ratio Anomaly T...	0.89
Unknown Sample Type Thr...	0.83
Marker Anomaly Threshold	0.88

Results

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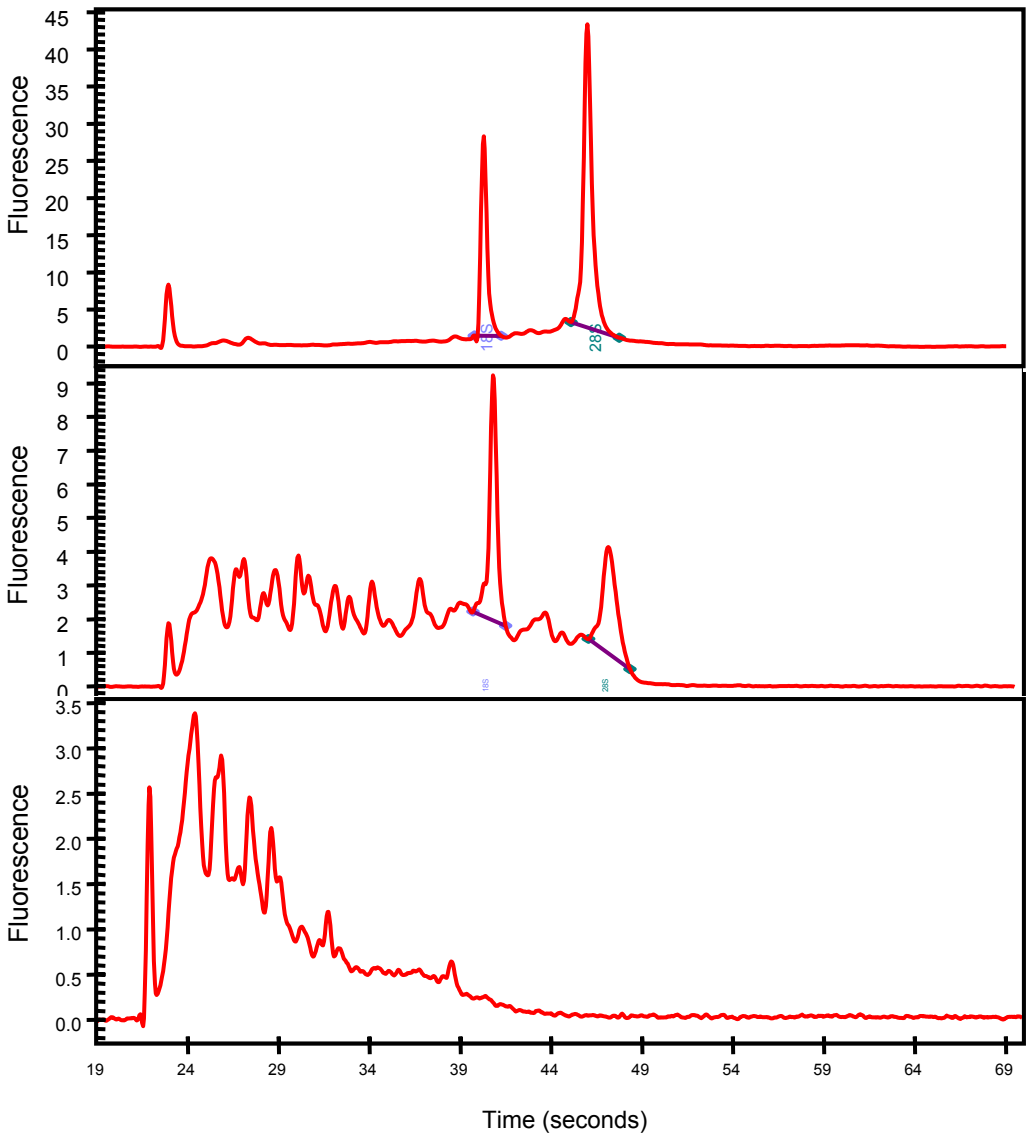
RIN Application – Assessment of RNA Integrity



Intact RNA: RIN 10

Partially degraded RNA: RIN 5

Strongly Degraded RNA: RIN 3

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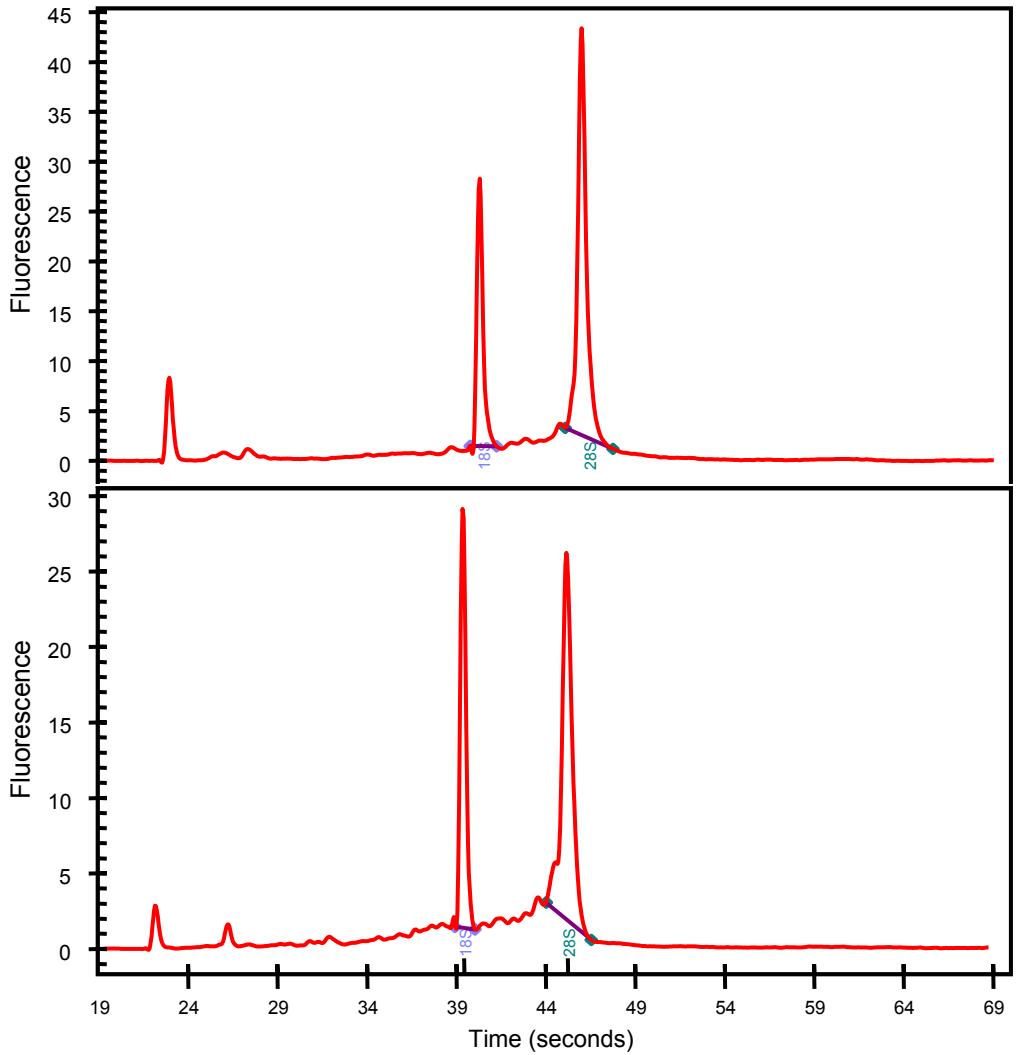
RIN Application – Assessment of RNA Integrity



Intact RNA: RIN 10

ribosomal ratio:

1.9

Intact RNA: RIN 10

ribosomal ratio:

1.4

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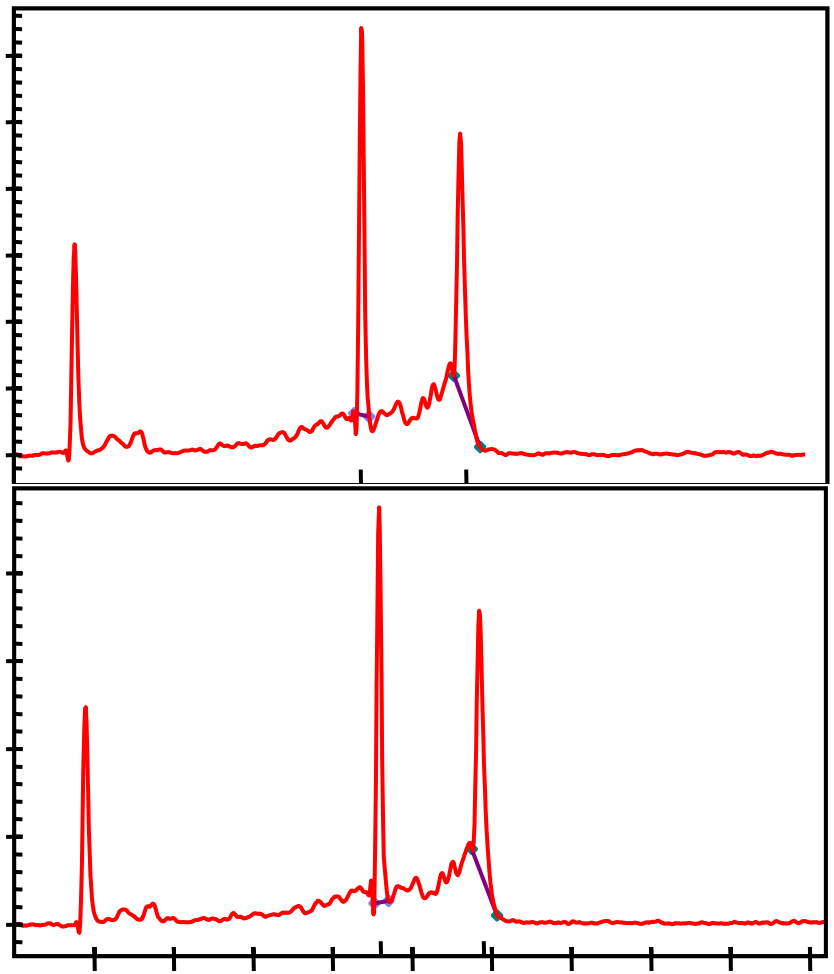
RIN Application – Directly Compare Samples

same sample on different instruments

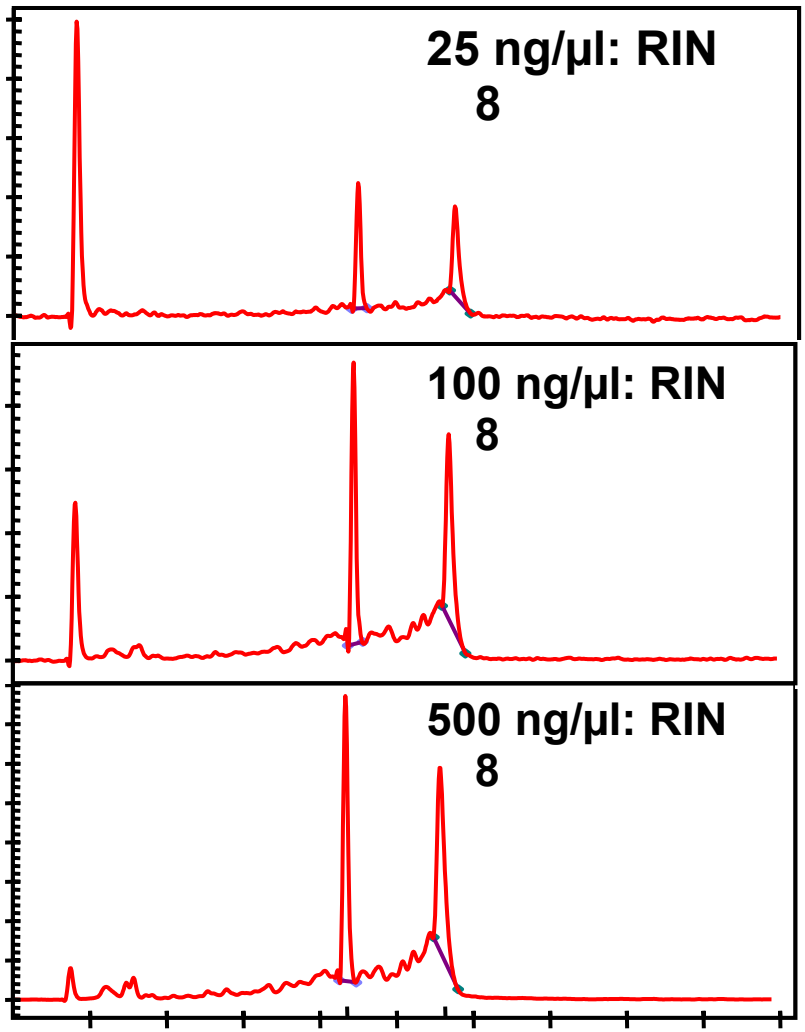


When testing an identical RNA sample on various instruments, identical RINs are obtained – within narrow limits

36 samples 3 instruments
CV RIN: 3 %
CV ribosomal ratio: 12 %

RIN Application – Directly Compare Samples

same sample in different dilutions



When testing an identical RNA sample in various dilutions, identical RINs are obtained – within narrow limits

108 samples 3 dilutions
CV RIN: 3 %
CV ribosomal ratio: 17 %

RIN Application – Comparison to alternative integrity measures

When applying the degradometer tool to a random data set, a significant number of samples are “unclassified” (BLACK CODING)

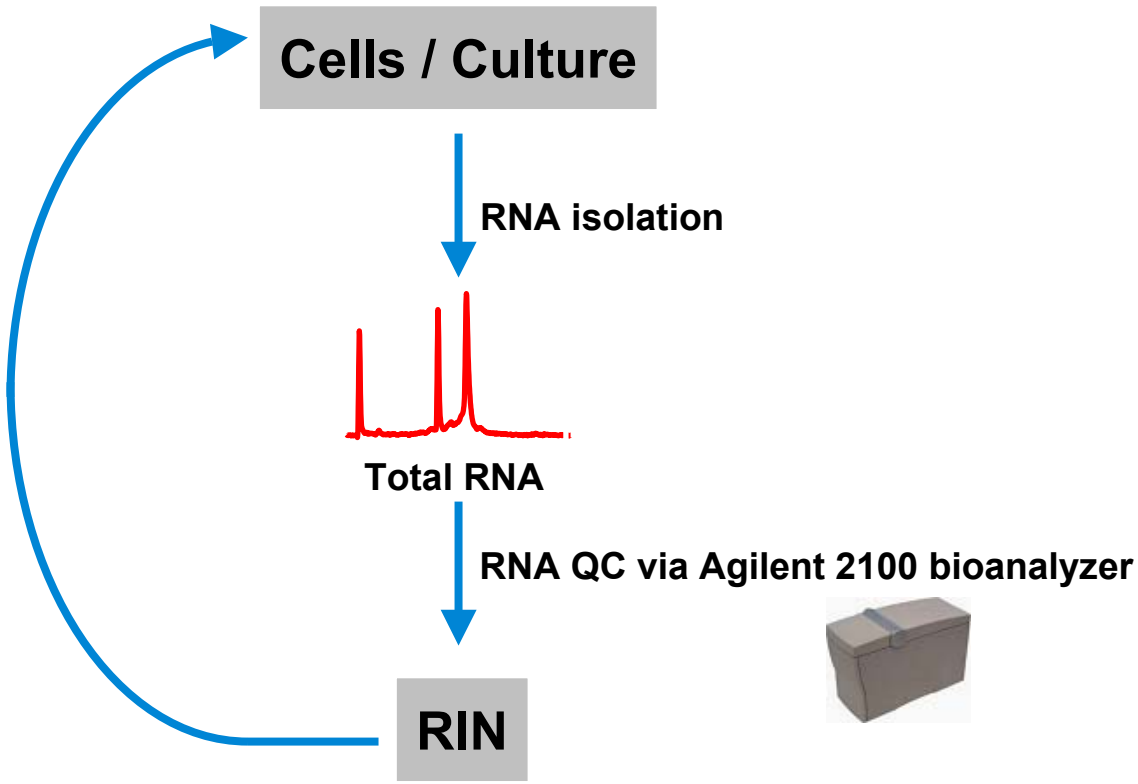
integrity class	% labeled BLACK
10	**21**
9	**30**
8	**27**
7	**35**
6	20
5	20
4	42
3	44
2	100
1	100

Unclassified sample

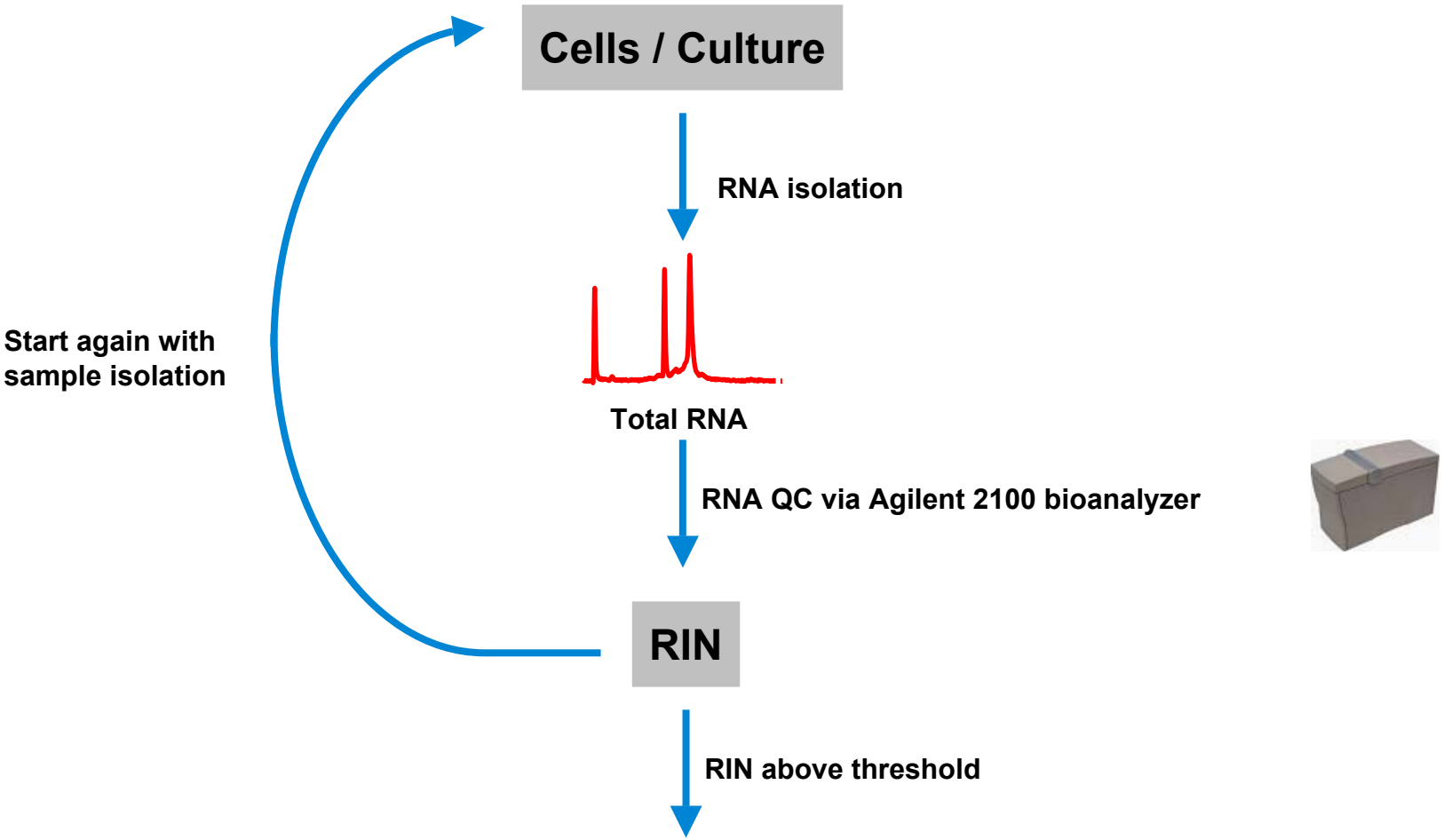
The degradometer is based on the ribosomal ratio and the area corresponding to our “fast region”.
The degradometer classifies samples in 4 categories, when RIN data is clustered in blocks the data correlates.

RIN Validation for Gene Expression Experiments

Correlate RIN with downstream experiment and determine threshold RIN for meaningful results
(iterative process)



RNA QC in Routine Gene Expression Workflow



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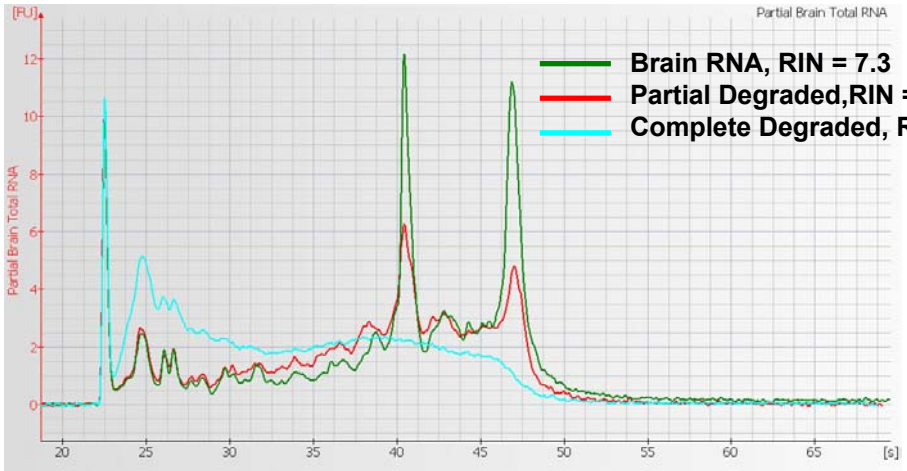
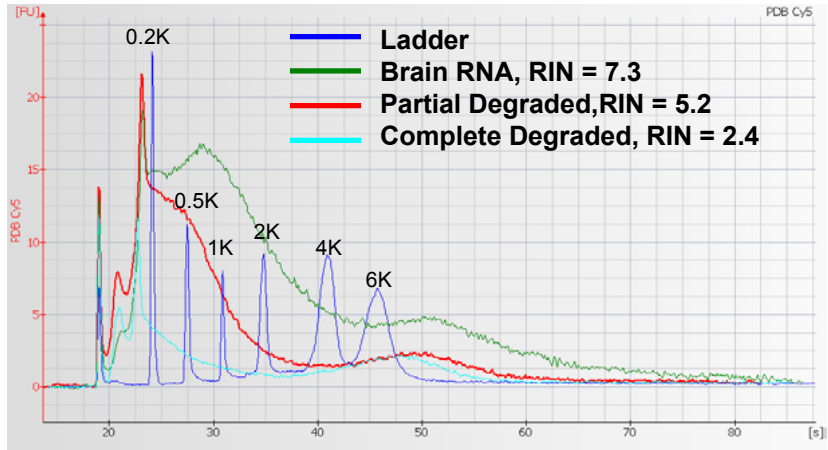
The effect of RNA degradation

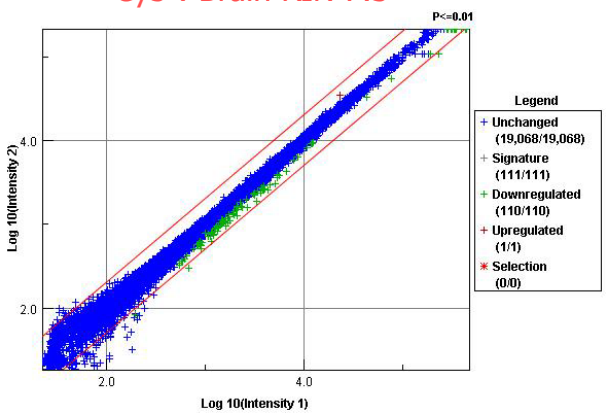
Total RNA profile



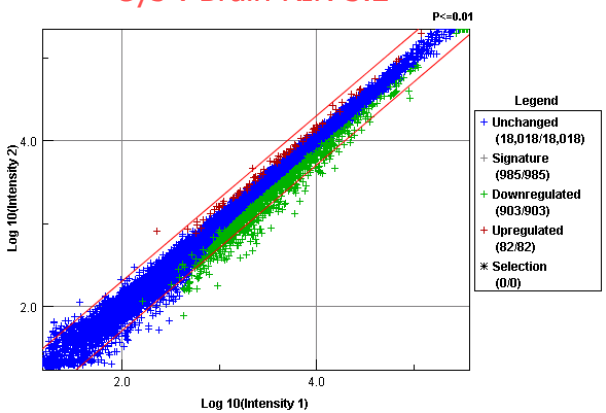
Labeled cRNA profile



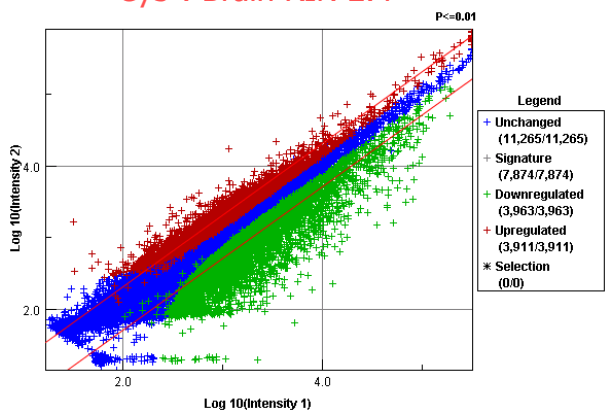
Cy3 : Brain RIN 7.3
Cy5 : Brain RIN 7.3



Cy3 : Brain RIN 7.3
Cy5 : Brain RIN 5.2



Cy3 : Brain RIN 7.3
Cy5 : Brain RIN 2.4



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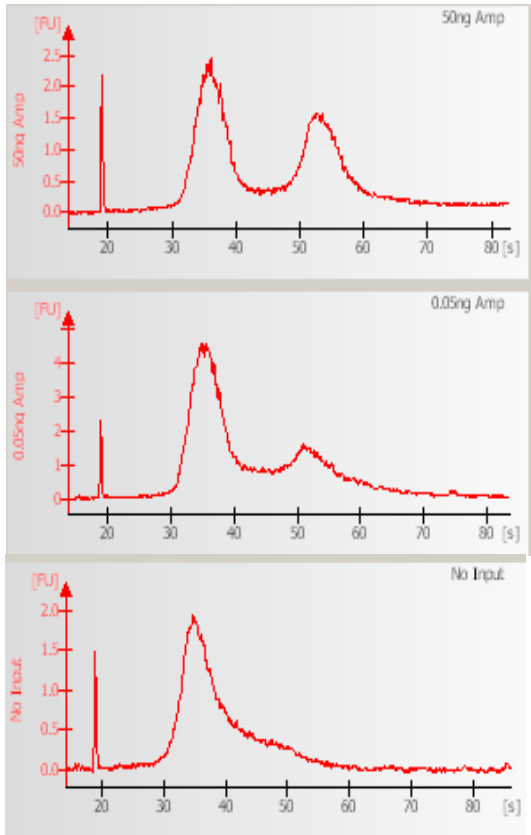
RIN to DIN? aCGH sample QC amplification

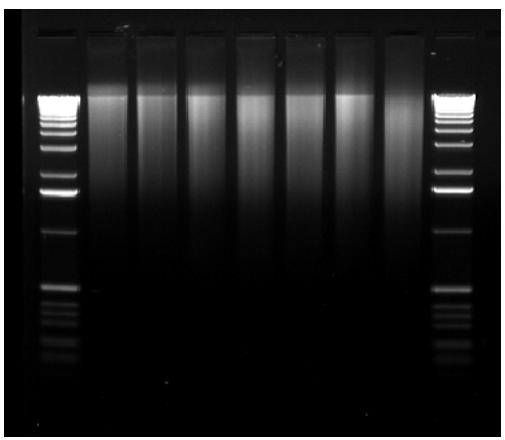
Genomic DNA amplification with phi29
titration series (50ng, 0.05ng, 0.005ng & no input) analysez with the RNA 6000 Nano kit.
The increasing non-specific peaks can be seen in the 30-40 second range and the decreasing specific peaks can be seen migrating in the 50-60 second range.



50ng genomic DNA

0.05ng

No Input



The same samples were analyzed on a 0.8% agarose gel and visualized with EtBr.
600ng of each sample was loaded
Lanes: 1-1kB marker, 2-50ng, 3-5ng, 4-0.5ng, 5-0.05ng, 6-0.005ng, 7-0.0005ng, 8-no input
Can not distinguish between the specific and non-specific amplification peaks.

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Conclusion

- A software algorithm was presented that allows standardization of RNA integrity assessment
- Ribosomal ratio is important parameter for RNA integrity but many other factors have to be taken into account
- Initial results indicate that the software algorithm is independent of instrumentation and can ensure repeatability of results (e.g. RT-PCR or microarray)

- Availability of RIN-tool:

  - included in beta-SW version: downloadable via Agilent Web-Page

  - included in next official SW release: May 2005

Acknowledgment

- **Software**
  - **Andreas Schroeder**
  - **Michael Leiber**
  - **Susanne Stocker**
  - **Thomas Ragg**

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  - **Samar Lightfoot**
  - **Ruediger Salowsky**
  - **Marcus Gassmann**
  - **Christine Miller***
  - **Rainer Wittig+**

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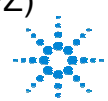
*Johns Hopkins Medical Institute
+ German Cancer Research Center (DKFZ)



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