# The Agilent 2100 Bioanalyzer

## RNA Integrity Number (RIN)

## A standardized approach for RNA integrity assessment

**qPCR 2005**
**Freising**
**Marc Valer**

# Acknowledgment


## Software

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


## Application

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

## Array Correlation work

- Kohei Ando

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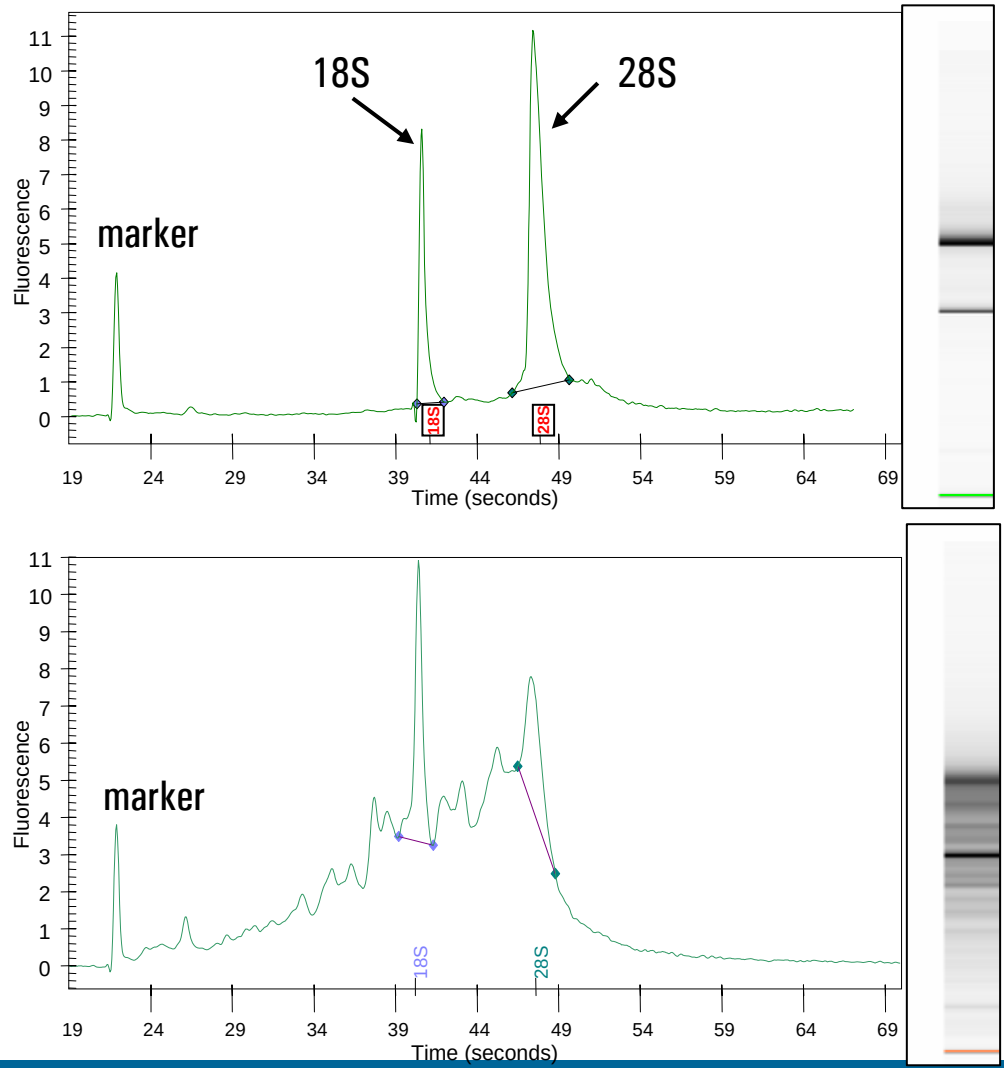
*Johns Hopkins Medical Institute
+ German Cancer Research Center (DKFZ)

# 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

# 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

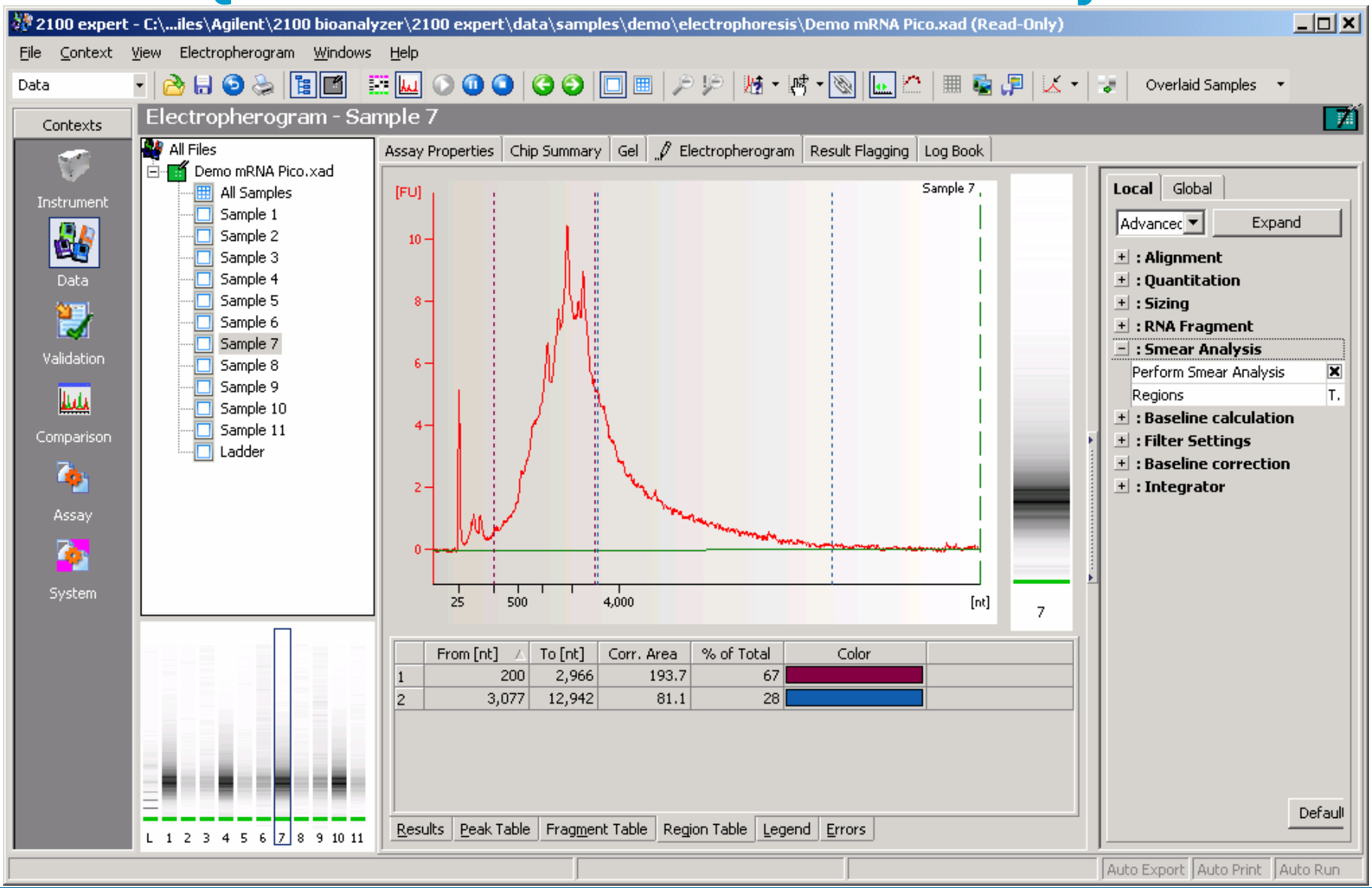
RNA degradation is a gradual process.

Results have to be interpreted by visual inspection.

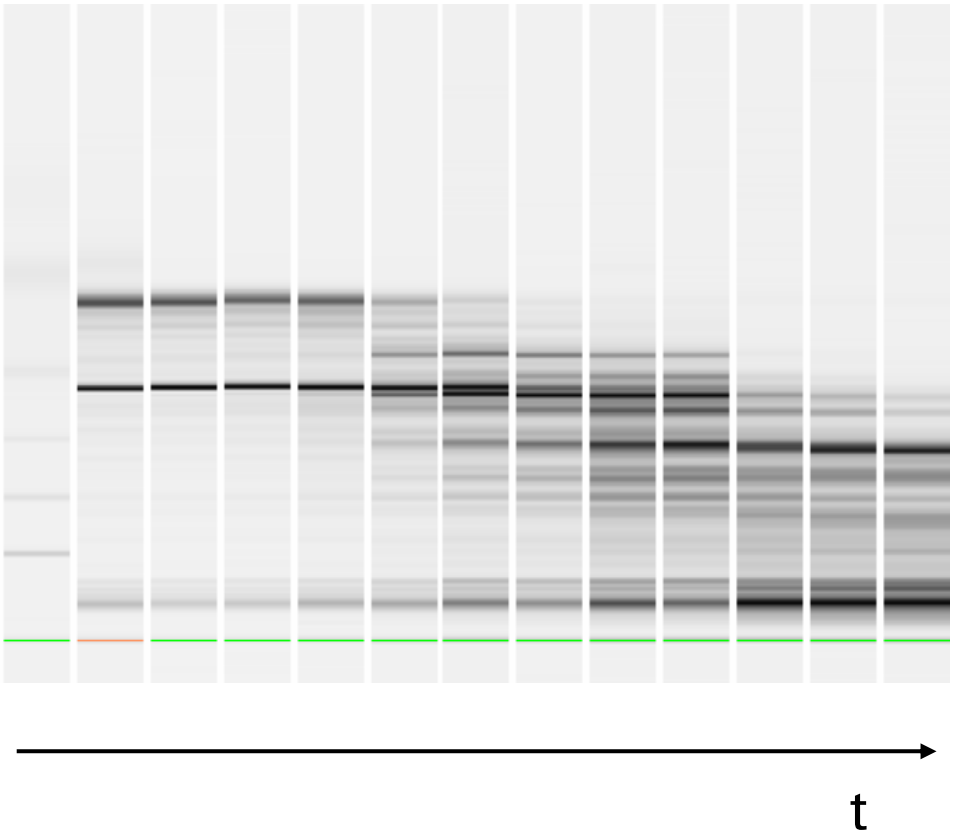
Overlay of electropherograms only works well for samples with the same concentration.

Instrument dependency in signal height

# mRNA QC is addressed with smear analysis tools



# 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

# The RNA Integrity Number (RIN)

The RIN is a software algorithm that allows classification of RNA integrity

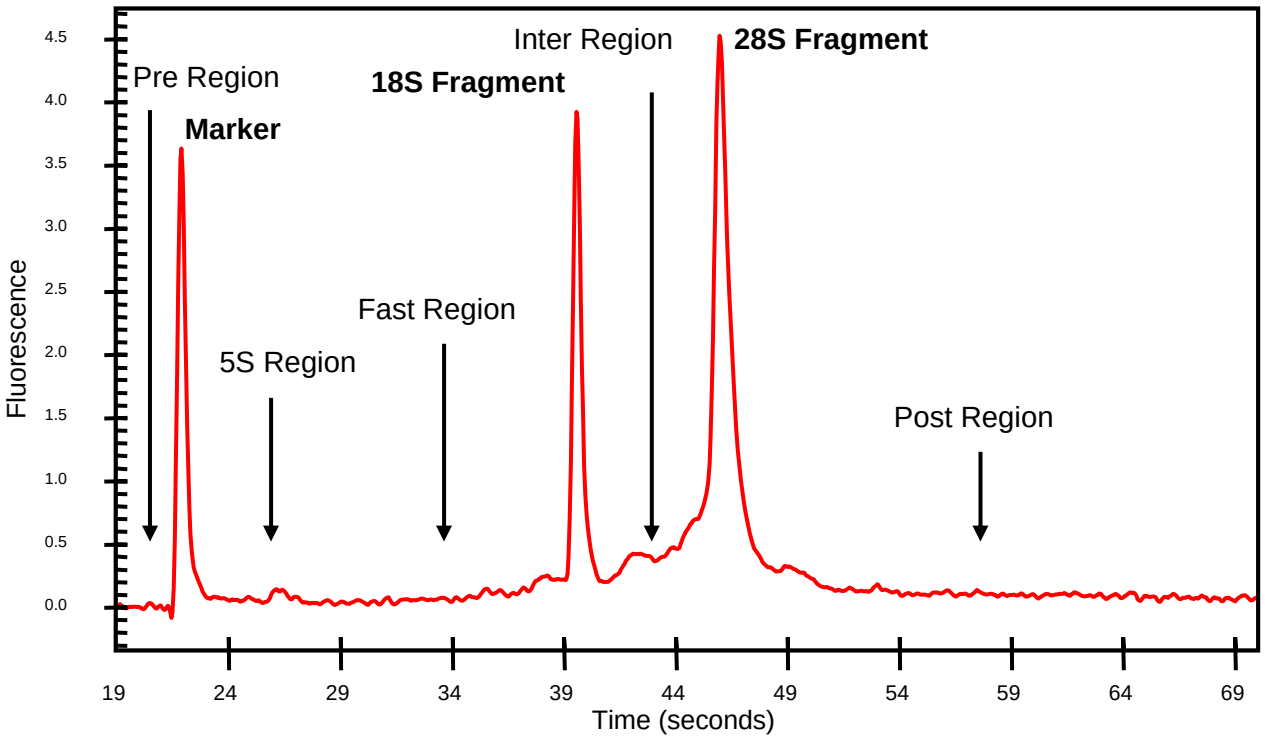
It extracts a number of characteristic features from the bioanalyzer electropherogram

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.

# Feature Generation

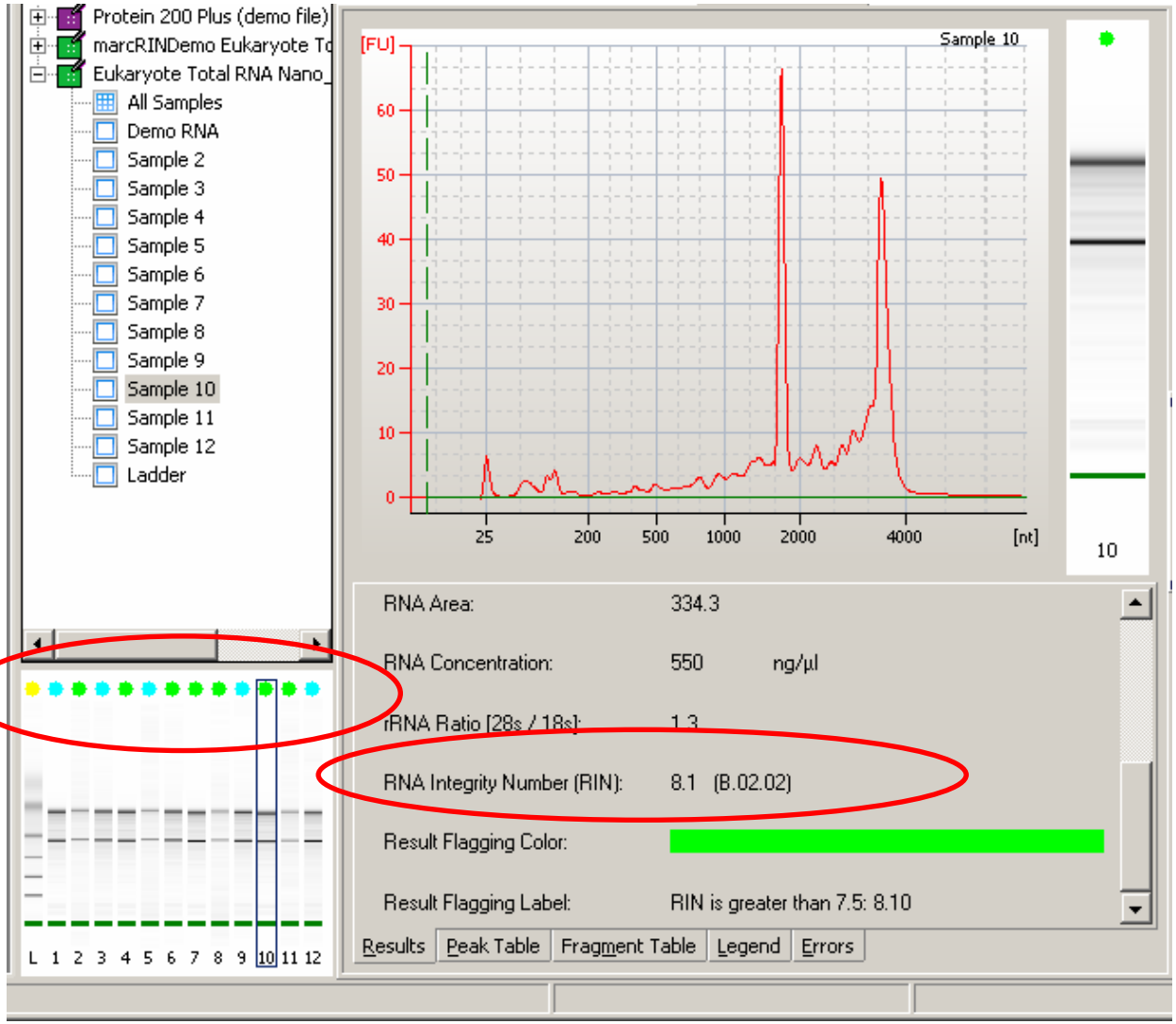
Approach: Training of regression models for peak positions and Intensities based on peak labels




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

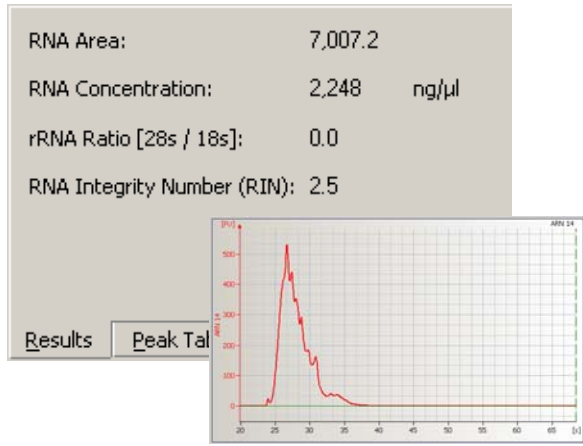
**Selection of the best feature combination**

# 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 |



# RIN - Limitations

*What the RIN can do:*

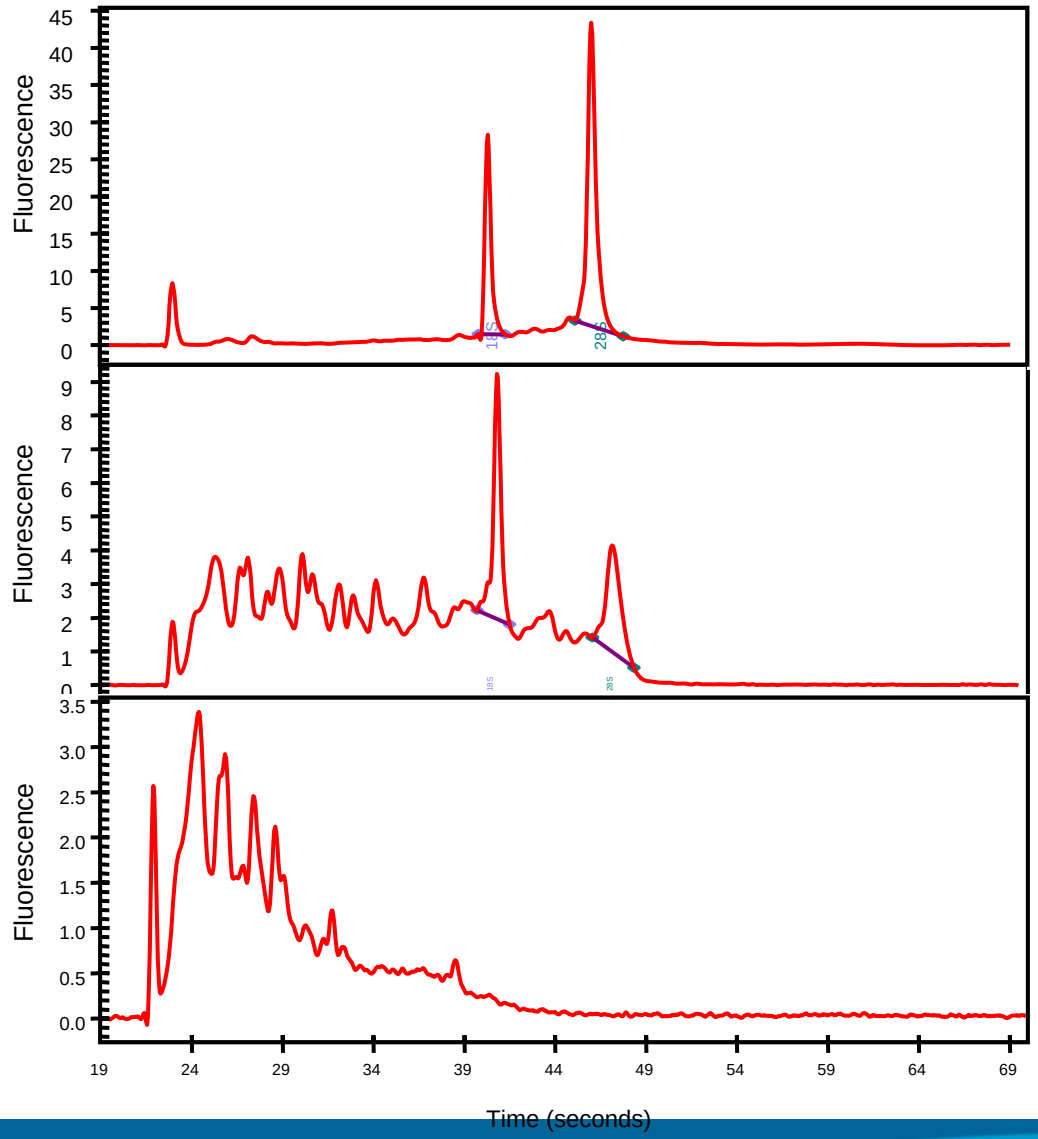
- **Obtain an assessment of RNA integrity.**
- **Directly compare RNA samples**
- **Ensure repeatability of experiments**

*What it CANNOT do:*

- **Predict the outcome of an experiment if no prior validation was done**

# Results

# RIN Application – Assessment of RNA Integrity



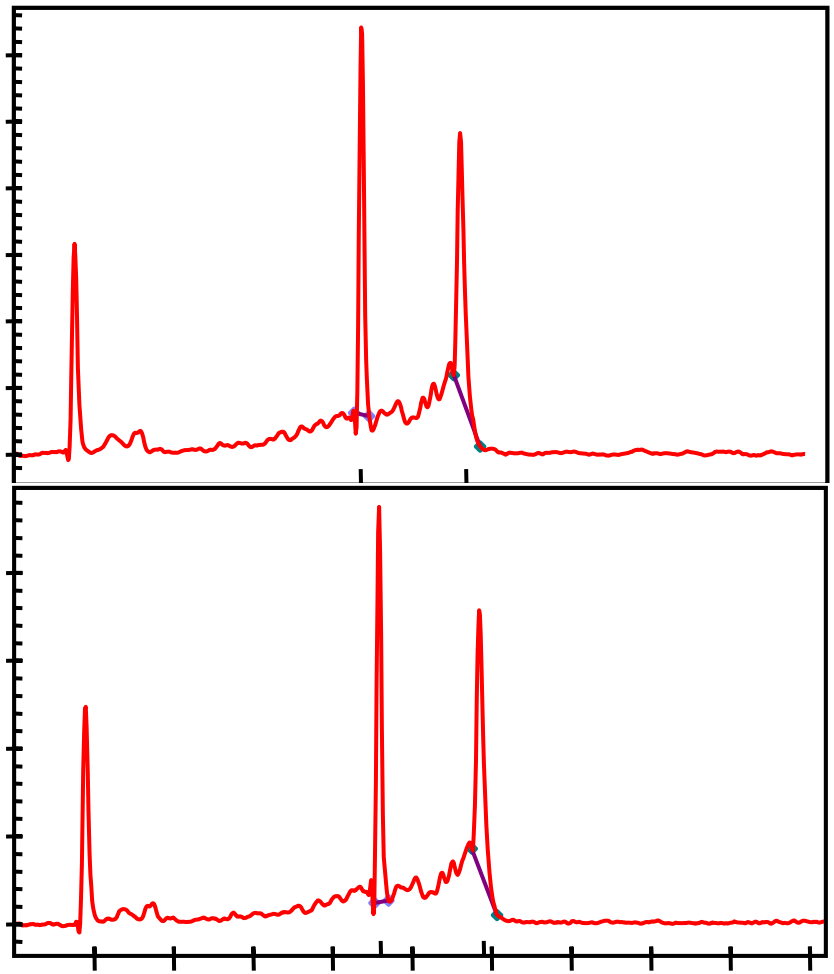

Intact RNA: RIN 10

Partially degraded RNA: RIN 5

Strongly Degraded RNA: RIN 3

# RIN Application – Directly Compare Samples

## same sample on different instruments

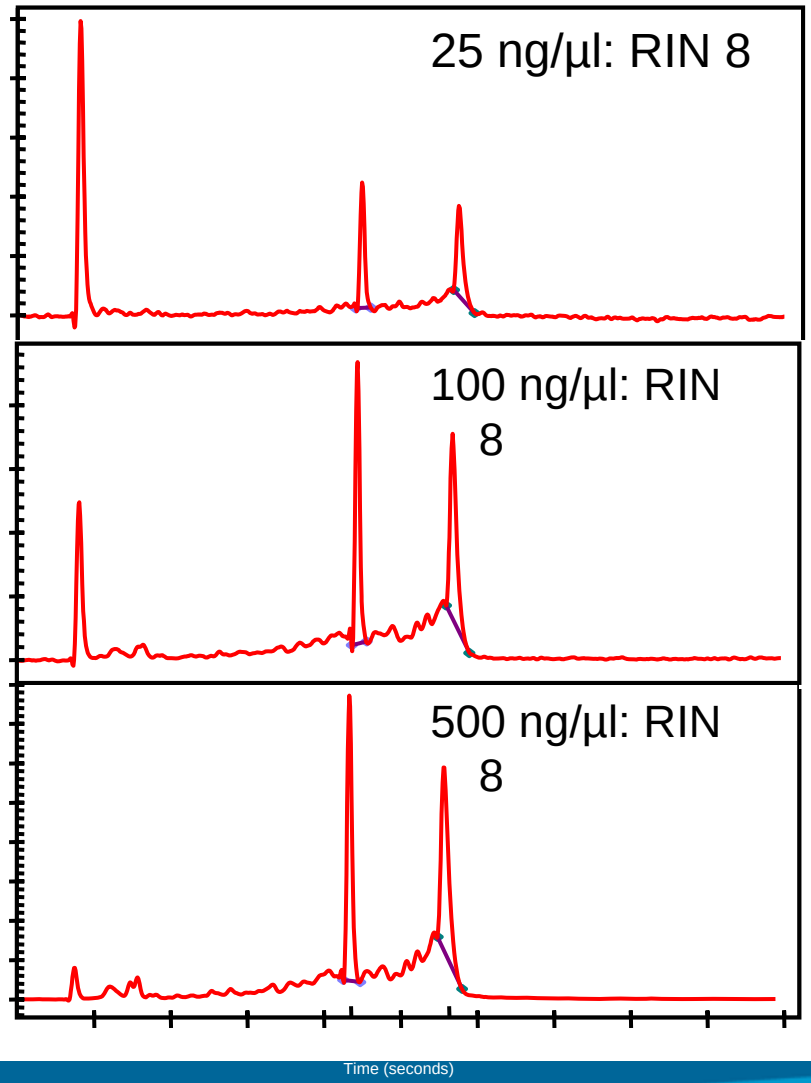


**When testing an identical RNA sample on various instruments, identical RINs are obtained – within narrow limits**

**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

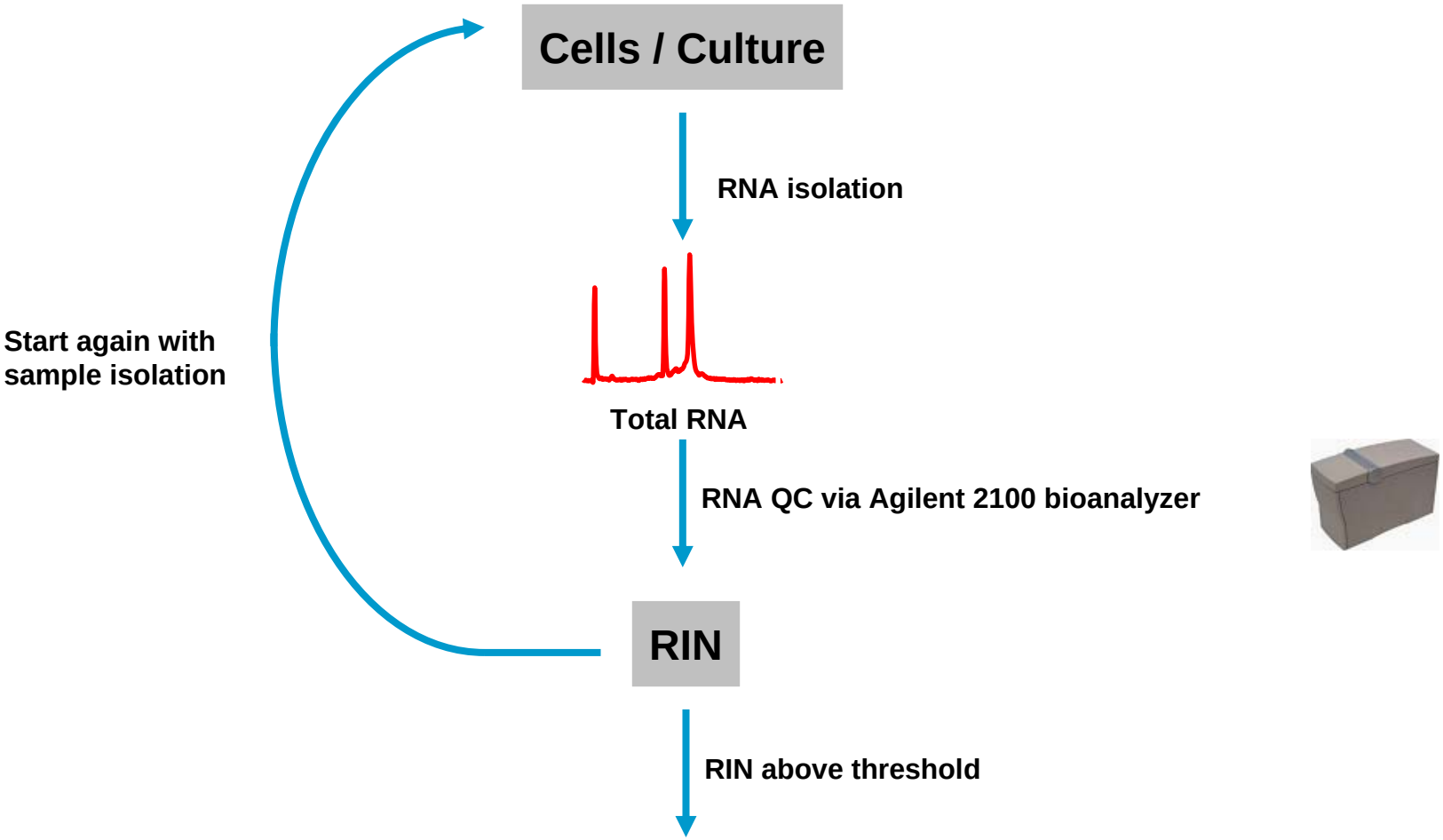
When applying the degradometer tool to a random data set, a significant number of samples are “unclassified” (BLACK CODING)

| 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 samples**

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.

# RNA QC in Routine Gene Expression Workflow



# Experimental: Correlation of RIN to Microarray

**Materials and Methods**

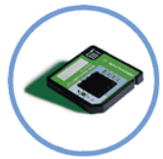
- **Preparation of Total RNA Degradation**
- **Start Total RNA Samples**
- **Design of Experiments**

**Results**

- **Labeled cRNA Quality Check by BioAnalyzer**
- **Scatter Plots : self to self**
- **Scatter Plots : Intact vs. Degradation**
- **Reappearance : Compare Plots**

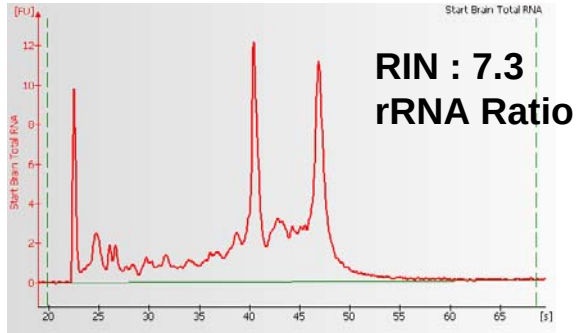
**Conclusions**

# Material and Method

| Steps | Material & Reagent | Conditions |
|---|---|---|
| Preparation of Total RNA | Biochain<br>Human Brain Total RNA | Human Brain Intact Total RNA<br>Brain Partial Degradation<br>Brain Complete Degradation |
| Labeling & cRNA check | Agilent<br>Low RNA Input<br>Linear & Amplification Kit<br>(5184-3523)  | Total RNA 500 ng start<br>Agilent Standard Protocol<br><br>Agilent 2100 BioAnalyzer<br>(RNA 6000 Nano LabChip kit)<br>NanoDrop  |
| Microarray | Agilent Human 1A (ver. 2)<br>(G4110B)  | |
| Hybridization & Scanning | In Situ<br>Hybridization Kit Pls<br>(3184-3568)<br>SureHyb Chamber<br>(G2534A)  <br>Agilent Scanner<br>(G2565BA) |  <br>SSC Wash<br>Ozone Free |

# Preparation of Total RNA Degradation

Products from BIOCHAIN Co.
Human Brain Total RNA
**Intact**

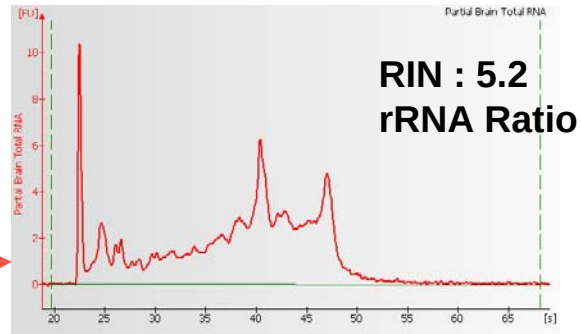



**RIN : 7.3**
**rRNA Ratio : 1.3**

60 °C
8 hours

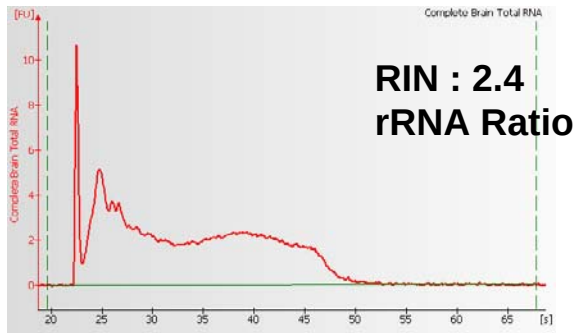
60 °C
2 hours

**Partial Degradation**



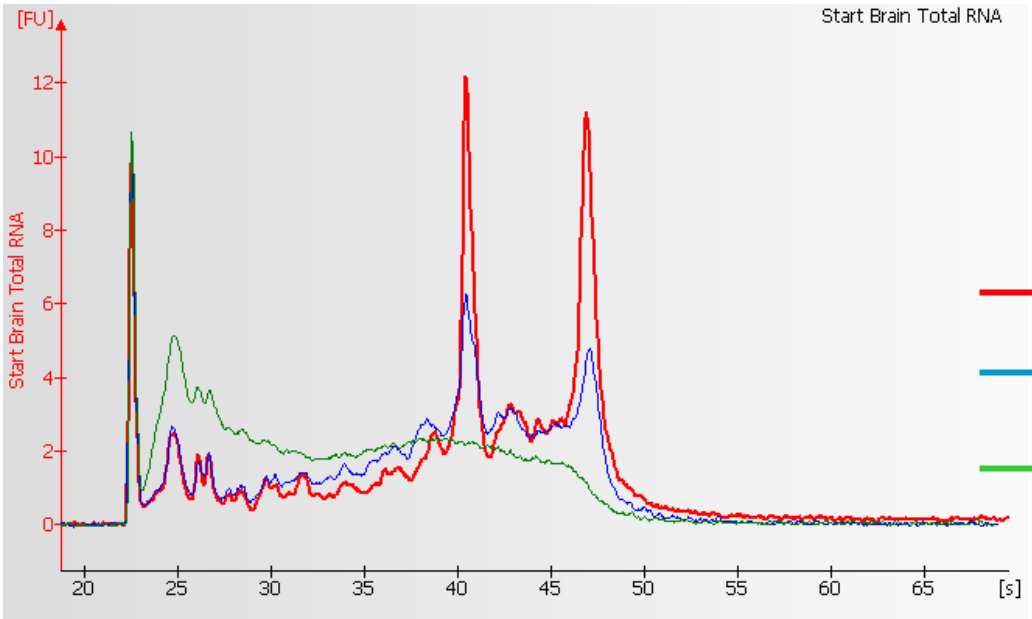

**RIN : 5.2**
**rRNA Ratio : 0.5**

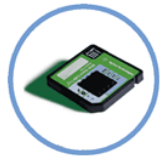
**Complete Degradation**




**RIN : 2.4**
**rRNA Ratio : 0.0**

# **Start Total RNA Samples :** Intact, Partial Degradation and Complete Degradation



**Intact**

**Partial Degradation (60C 2 hours)**

**Complete Degradation (60C 8 hours)**

| | 260/230 | 260/230 | RIN | rRNA Ratio |
|---|---|---|---|---|
| **Intact** | **2.14** | **1.94** | **7.3** | **1.3** |
| **Partial Degradation** | **2.11** | **1.85** | **5.2** | **0.5** |
| **Complete Degradation** | **2.13** | **1.78** | **2.4** | **0.0** |

# Design of Experiments

Samples : Human Brain Total RNA (from BIOCHAIN Co.)

- Intact
- Partial Degradation
- Complete Degradation

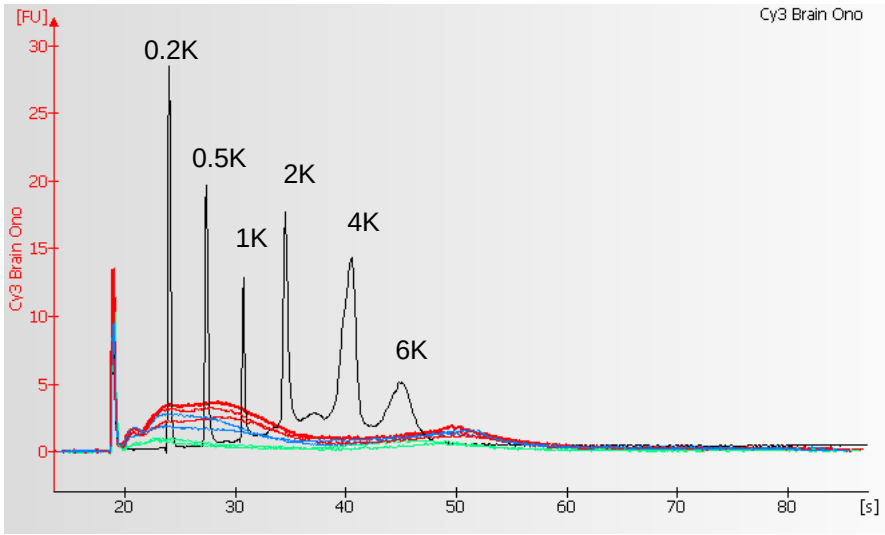
| No. | Barcode | Cyanine 3 | Cyanine 5 | Comment |
|---|---|---|---|---|
| 1 | 251209734174 | Intact | Intact | Self |
| 2 | 251209735151 | Partial Degradation | Partial Degradation | Self |
| 3 | 251209735054 | Complete Degradation | Complete Degradation | Self |
| 4 | 251209735149 | Intact | Partial Degradation | Differential |
| 5 | 251209735150 | Partial Degradation | Intact | Differential |
| 6 | 251209735053 | Intact | Complete Degradation | Differential |
| 7 | 251209735055 | Complete Degradation | Intact | Differential |

Dye Swap

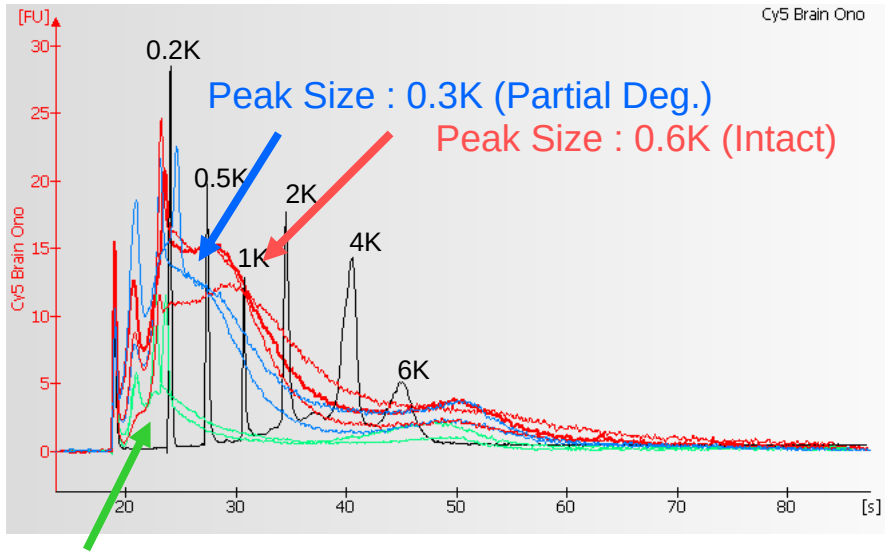
Dye Swap

# Labeled cRNA Quality Check by BioAnalyzer

## Cy3 labeled cRNA



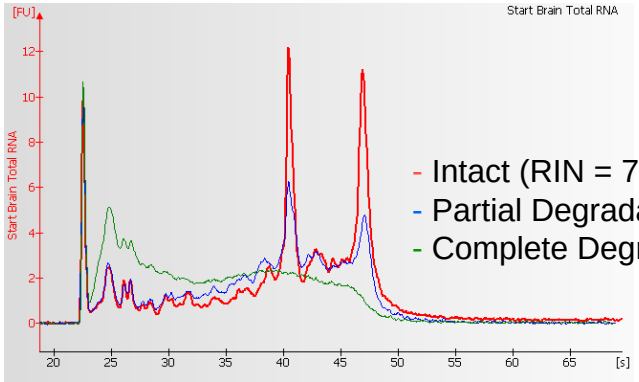

## Cy5 labeled cRNA




Peak Size : 0.1K (Complete Deg.)

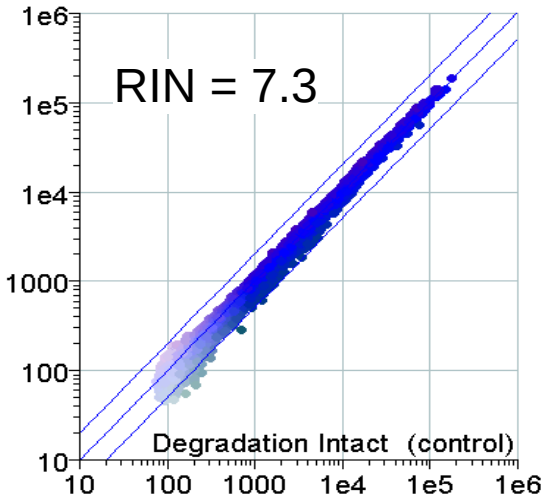
- Ladder
- Intact (RIN = 7.3)
- Partial Degradation (RIN = 5.2)
- Complete Degradation (RIN = 2.4)

# Scatter Plots : Self to Self

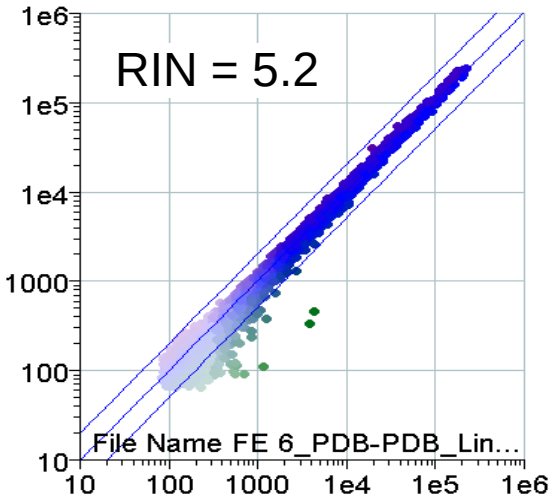



- Intact (RIN = 7.3)
- Partial Degradation (RIN = 5.2)
- Complete Degradation (RIN = 2.4)

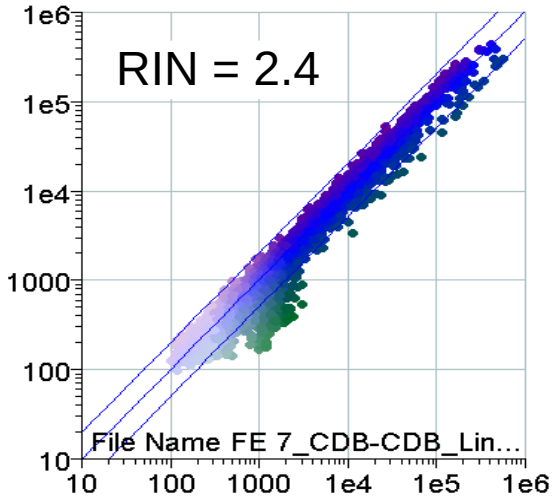
## Intact



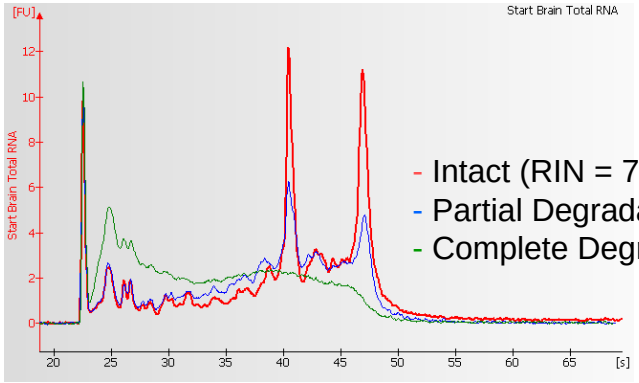

## Partial Degradation



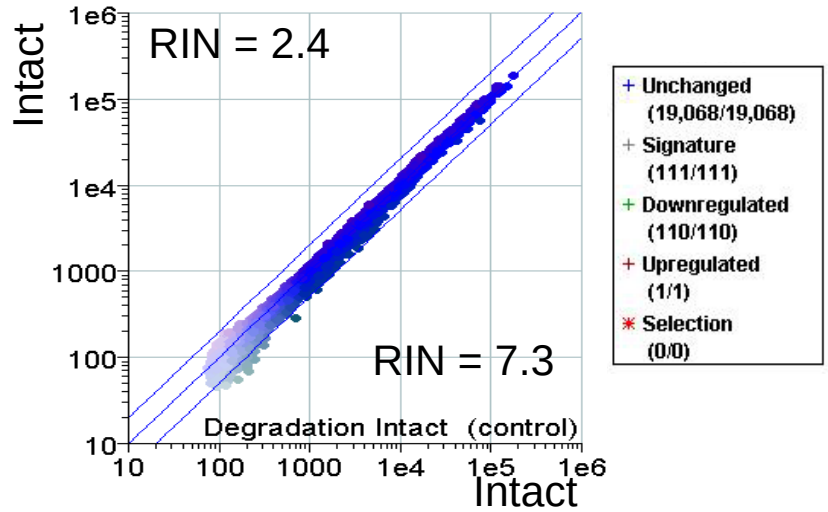

## Complete Degradation



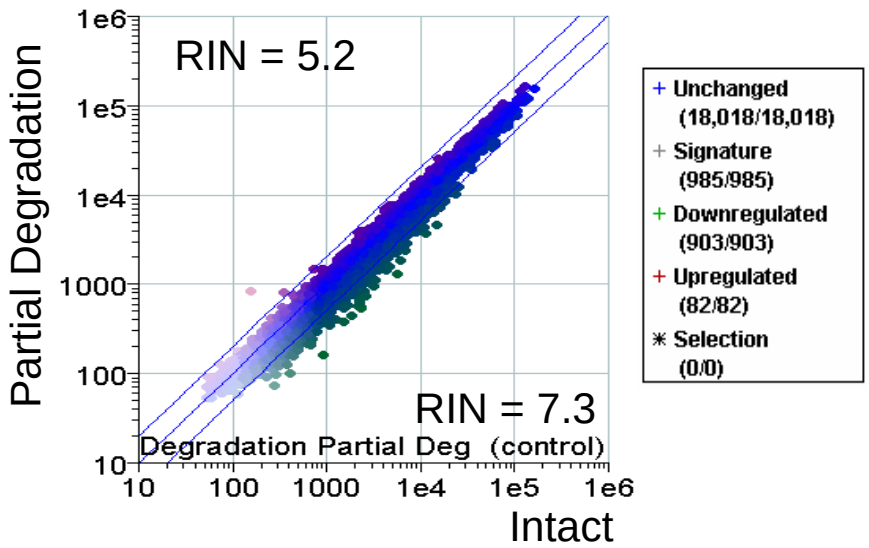
# Scatter Plots : Intact vs. Degradation



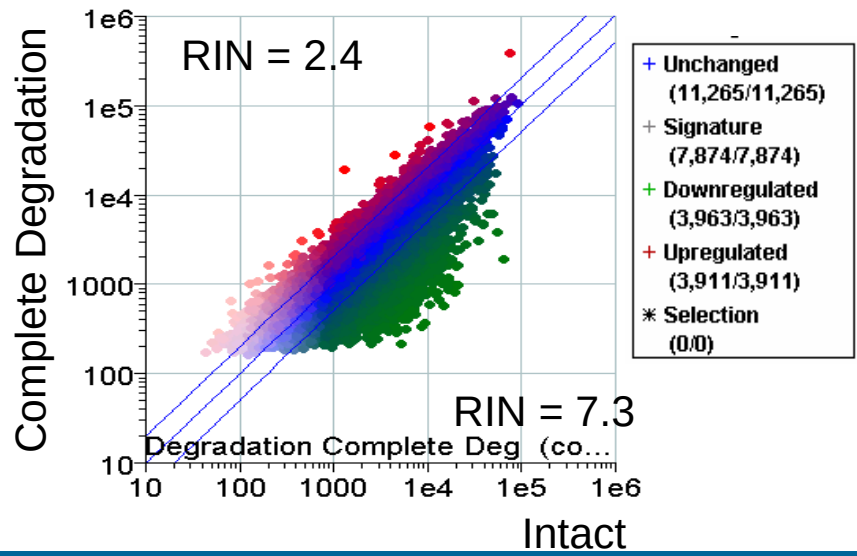

## Intact (self to self)




## Intact vs. Partial Degradation




## Intact vs. Complete Degradation



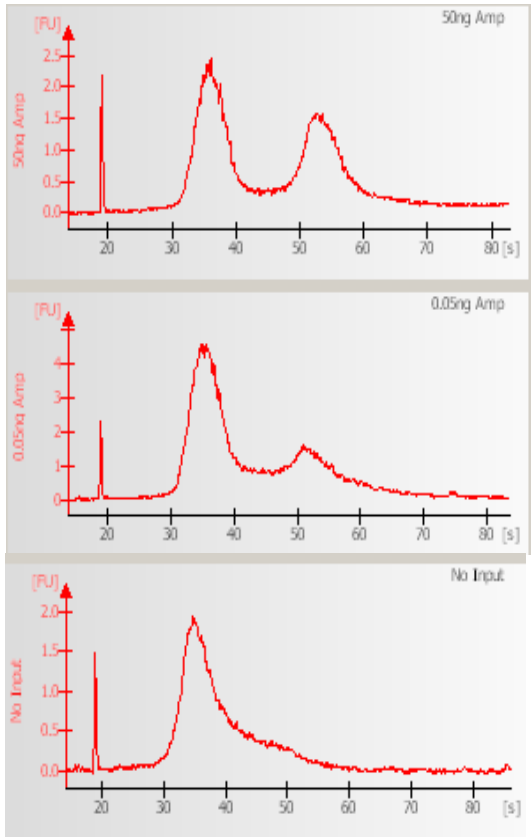
# Other developments

The principles applies for the RNA integrity number are valid for any kind of curve where differences, even if slight , are highly reproducible. The algorithm is ideal for sample classification where categories can be predefined and are pure.

A new algorithm which is able to automatically select the most informative features has been in the meantime developed. It proved already a good correlation with the expert selected RIN features. It will now be applied for other classification tasks

# RIN to DIN? aCGH sample QC amplification

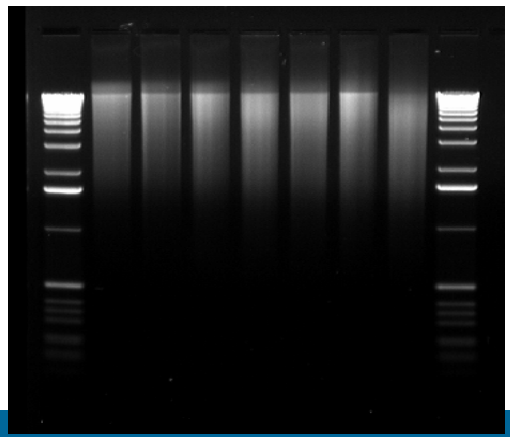
**Genomic DNA amplification with phi29**
**titration series (50ng, 0.05ng, 0.005ng & no input) analysez with the RNA 6000 Nano kit.**
**The increasing non-specific peaks can be seen in the 30-40 second range and the decreasing specific peaks can be seen migrating in the 50-60 second range.**




**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.**

# Summary

- **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**
- **Results indicate that the software measurements are independent from the instrument variability and can ensure repeatability of results (e.g. RT-PCR or microarray)**
- **Degradation level of starting RNA material seriously influences microarray results.**
- **Labeled cRNA from different levels of RNA degradation results in low Cy Dye incorporation and low yield of cRNA**

# Thank you for you attention!!!

Q&A…all along

Live Demo:

-Kit preparation

-Instrument setup

-Sample: RNA Ladder, Ambion Human Liver totalRNA

-Start Run

-See results