The Agilent 2100 Bioanalyzer

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

qPCR Symposium
Leipzig, 2005

Marc Valer
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 quantify 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
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
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

1. Data Preparation
2. Feature Generation
3. Feature Selection
4. Adaptive Learning
5. Best Model Selection
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
RIN Visualization

Sample 1:
- RNA Area: 61.7
- RNA Concentration: 102 ng/µl
- rRNA Ratio [28s / 18s]: 1.2
- RNA Integrity Number (RIN): 7.8

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

**RNA Area:** 26.3
**RNA Concentration:** 75 ng/μl
**rRNA Ratio [28s / 18s]:** 1.9

**RNA Integrity Number (RIN):** 9.6

Anomaly threshold settings

<table>
<thead>
<tr>
<th>RNA Integrity Number</th>
<th>Value</th>
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<tbody>
<tr>
<td>Pre Region Anomaly Threshold</td>
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<tr>
<td>5S Region Anomaly Threshold</td>
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<tr>
<td>Fast Region Anomaly Threshold</td>
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<td>Inter Region Anomaly Threshold</td>
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<td>Post Region Anomaly Threshold</td>
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<td>Ribosomal Ratio Anomaly</td>
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<td>Unknown Sample Type Threshold</td>
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<td>Marker Anomaly Threshold</td>
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</table>

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Results
RIN Application – Assessment of RNA Integrity

Intact RNA: RIN 10

Partially degraded RNA: RIN 5

Strongly Degraded RNA: RIN 3
RIN Application – Assessment of RNA Integrity

Intact RNA: RIN 10

ribosomal ratio: 1.9

Intact RNA: RIN 10

ribosomal ratio: 1.4
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)

<table>
<thead>
<tr>
<th>integrity class</th>
<th>% labeled BLACK</th>
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<tbody>
<tr>
<td>10</td>
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<td>100</td>
</tr>
</tbody>
</table>

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

Cells / Culture

RNA isolation

Total RNA

RNA QC via Agilent 2100 bioanalyzer

RIN

RIN above threshold

Continue with downstream experiment (microarray, real-time PCR, etc.)

Start again with sample isolation
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) analyze z 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.

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.
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

• Application
  – Samar Lightfoot
  – Ruediger Salowsky
  – Marcus Gassmann
  – Christine Miller*
  – Rainer Wittig+

We would like extend special thanks to our collaboration partners Ambion Inc. and the German cancer research center as well as to Quantiom Bioinformatics. We would also like to thank all researchers who beta-tested the RIN and provided valuable inputs.

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