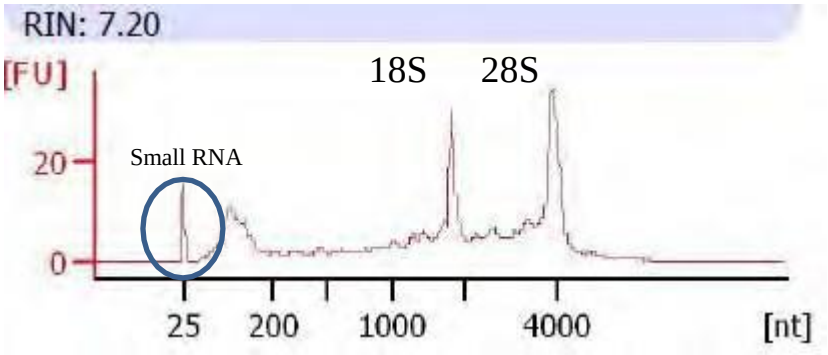
# Standard Operating Procedure (SOP) for RNA Nanoassay

## I. SCOPE AND PURPOSE

RNA samples frequently contain low levels of degradation that are difficult to detect by standard gel electrophoresis methods, but which may hinder downstream applications. Such degradation can be detected and analyzed by the Agilent Bioanalyzer. This instrument estimates the concentration of RNA and calculates the amount of 18S and 28S ribosomal RNA in each sample. In addition, the Bioanalyzer calculates the RNA integrity number (RIN), from the electrophoretic trace of the RNA sample, which includes the presence of degradation products. The assigned RIN is independent of sample concentration, instrument, and analyst, therefore becoming a de facto standard for measuring RNA integrity. Small RNAs are apparent when using this kit, see diagram below. This assay should be used to determine the overall integrity of RNA following nucleic acid extraction.




Any deviation from this Standard Operating Procedure will be noted on the Agilent printout; the number of the samples affected by the deviation will be noted as well.

## II. PROCEDURE

### A. Safety Precautions

1. Use Standard Precautions when handling all body fluids, tissues and cell cultures. Refer to the Specimen Collection and Handling procedure, GEN-1, for guidelines specific for the Molecular Genetics Laboratory and samples.
2. The RNA Nano dye binds to RNA and contains dimethysulfoxide (DMSO), which is known to facilitate the entry of organic molecules into tissues. The dye should be treated as a potential mutagen and used with appropriate care. Wear nitile gloves (latex gloves may not be substituted) and follow good laboratory practices when preparing and handling reagents and samples.
3. RNA is extremely susceptible to degradation by ribonucleases that are ubiquitous in the environment. To ensure preservation of target RNA, special precautions are needed. Records are maintained to show that RNase-free conditions (i.e. wiping the lab areas with RNaseZAP) are met, with corrective action if conditions are not met.

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**a.** In addition, the Agilent RNA Nano Assay uses pre-made aliquots that are disposed after use. The use of these pre-made aliquots prevents the need to thaw the stock RNA vial for RIN testing (in most instances)**.**

## B. Equipment and materials

1. Agilent RNA 6000 NanoChip kit (Agilent, 5067-1511)
   a. 25 RNA Nano Chips
   b. 2 electrode cleaners
   c. 1 syringe
   d. RNA Nano Dye Concentrate
   e. RNA Nano Marker
   f. RNA 6000 Nano Gel Matrix
   g. Agilent RNA Nano 6000 molecular weight ladder
   h. 4 spin filters
2. 0.5 mL RNase-free microcentrifuge tubes
3. Sterile, nuclease free water (Fisher, BP-2484-50)
4. RNase Zap (Ambion, AM9780)
5. Thermocycler or dry heat block
6. Microcentrifuge
7. Pipette man
8. Pipette tips, assorted sizes
9. Wet ice
10. Vortex mixer
11. Agilent 2100 Bioanalyzer
12. Agilent IKA vortex mixer
13. Universal Human Reference RNA (Agilent, 740000)

*It is possible to substitute disposable materials and certain equipment from other vendors, as long as they are the equivalent of the items described above.*

*Products and disposable materials used need to be RNAse-free and handled only with gloved hands in order to prevent contamination with RNAses.*

*All reagents must be made with RNAse-free materials and chemicals, and containers and tubes for sample handling must be covered whenever possible during the entire procedure to ensure that they remain dust- and RNAse-free. Before beginning the procedure wipe down the bench with RNase Zap.*

*In the event that a reagent or disposable item either becomes contaminated or is suspected of being contaminated, it must be discarded.*

## C. Quality Control

1. This quantitative assay includes a reportable range of RIN values between 0 and 10.

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2. All chips must include a reference control RNA with a known average RIN to monitor assay performance on each run. This control sample should have a RIN of ± 0.5 of its average RIN. For example, control AV-AB03-20A-01R has an average RIN of 9.3, so it's acceptable range would be 8.8 to 9.8. If control RNA produces a RIN outside of this range, the entire chip will be repeated.

3. Some common situations that may cause analytically inaccurate results would be improperly loaded gel matrix, RNase contamination, and/or bubbles. Control RIN values will be monitored monthly to document and follow up on outliers, trends or omissions. This monthly review must include calculations for standard deviation (SD) and coefficient of variability (CV).
4. This SOP includes a process for automatic import of Bioanalyzer test data into the LIMS system. Imported data is cross checked against printed Agilent reports in an effort to provide an additional level of control over data integrity.
5. All new lots of reagents are tested in parallel with the one in current use before being put into use. Results are recorded in QC log. All kit components must be quality control tested and used together thereafter. All reagents supplied in a kit must be used only with other reagents in the same kit lot number; reagents with identical lot numbers cannot be interchanged between kit lot numbers.

**D. Preparing the RNA ladder**

1. Upon receipt, thaw ladder on wet ice. Heat denature the ladder at 70°C for 2 minutes. Immediately cool the vial on wet ice and aliquot 5 µL of the RNA ladder into 0.5 mL RNase-free microcentrifuge tubes (provided by the Agilent kit) on wet ice. Store the aliquots in a -80°C freezer.
2. Before use, thaw an aliquot of the ladder on wet ice (avoid extensive warming of the ladder during the thawing process).

**E. Preparing the gel**

1. The Agilent LabChip kit reagents are stored refrigerated and used before the manufacturer's expiration date (the physical ships are stored at room temperature). Allow the RNA gel matrix to equilibrate to room temperature for 30 minutes.
2. Pipette 550 µL of RNA gel matrix to the spin filter (provided).
3. Place the spin filter in a microcentrifuge and spin for 10 minutes at room temperature at 1,500 x g.
4. Aliquot 65 µL of the filtered gel into 0.5 mL RNase-free microcentrifuge tubes (provided). Label the tube with the date of preparation, lot number, and date of expiration. Store the filtered gel in a refrigerator and use within 4 weeks of preparation.

**F. Preparing the gel/dye mix**

1. Allow the RNA dye concentrate (blue-capped tube) and RNA filtered gel to equilibrate to room temperature for 30 minutes. Protect the dye concentrate from light at all times. (Reference the kit instructions.)
2. Vortex the RNA dye concentrate for 10 seconds and centrifuge briefly.
3. Pipette 1 µL of the dye concentrate into a RNA filtered gel tube.

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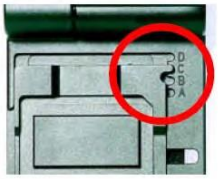
4. Cap the tube, vortex for 10 seconds, then centrifuge at 13,000 x g for 10min at room temperature. Visually inspect for homogenous mixing of gel and dye.
5. The prepared gel/dye mix can be stored for up to 8 hours in a refrigerator, and then must be discarded.

**G. Preparing the priming station**

1. Make sure the base plate is in position C as shown below. Using a screwdriver, open the screw at the underside of the base plate.




2. Make sure the syringe clip is at the top position by releasing the lever of the clip and sliding it up.

**H. Heat denaturation of the RNA**

1. Thaw the Agilent aliquot (test samples and controls) and a previously prepared aliquot of ladder on ice.
2. Place the tubes containing the ladder and each of the test samples and control in a thermocycler or heat block set to 70°C.
3. Heat the samples for 2 minutes while preparing the gel and chip.

**I. Loading the gel/dye mix**

1. Allow the gel/dye mix to equilibrate to room temperature for 30 minutes before use. Protect the gel/dye mix from light during this time. If the gel/dye mix is freshly prepared and has not been placed in the refrigerator, this step may be skipped.
2. Remove the RNA chip from its sealed bag.
3. Pipette 9.0 µL of the gel/dye mix at the bottom of the well marked G. Do not introduce air bubbles into the well when dispensing.
4. Place the RNA chip on the chip priming station. Set the timer for 30 seconds, make sure that the plunger is positioned at 1 mL, and then close the chip priming station. The lock of the latch will click when the chip priming station is closed properly.
5. Press the plunger of the syringe down until it is held by the clip.
6. Wait for exactly 30 seconds and then release the plunger with the clip release mechanism.
7. Visually inspect that the plunger moves back to at least the 0.8 mL mark.
8. Wait for 5 seconds and then slowly pull back the plunger to the 1 mL position.
9. Open the chip priming station.

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10. Pipette 9.0 µL of the gel/dye mix in each of the wells marked G.
11. Protect the gel/dye mix from light and store in the refrigerator when not in use for more than one hour. The gel/dye mix has a maximum shelf life of 8 hours.

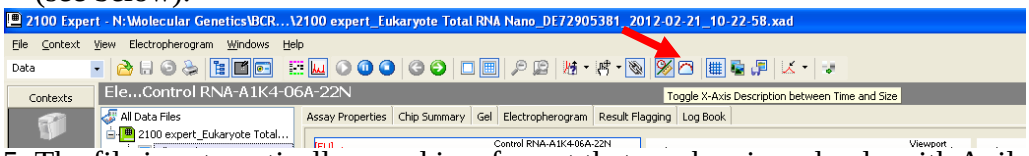
**J. Loading the Marker** - Pipette 5 µL of the RNA 6000 Nano Marker into the well marked with the ladder symbol and into each of the 12 sample wells. If not running a full chip (12 samples), pipette 6ul of the RNA 6000 Nano Marker into any unused sample wells. Avoid introducing air bubbles into the wells when dispensing.

**K. Loading the Ladder and Samples**

1. Pipette 1 µL of the heat-denatured RNA ladder into the well marked with the ladder symbol. Avoid introducing air bubbles into the wells when dispensing.
2. Add 1 µL of heat-denatured test sample to the appropriate well. Avoid introducing air bubbles into the wells when dispensing.
3. Place the chip horizontally in the adapter of the IKA vortex mixer.
4. Vortex for 60 seconds at 2,400 rpm. NOTE: If the vortexing speed is too high, liquid may spill out of the channels and interfere with the analysis.
5. Run the chip in the Agilent 2100 Bioanalyzer within 5 minutes of loading.
6. Record the number of samples run on each machine on the equipment log.

**L. Running the chip**

1. Turn the Bioanalyzer unit on (switch is located on the back of the instrument).
2. Clean the Bioanalyzer probes (see section J) with RNase ZAP and Nuclease free water.
3. Place the chip in the Bioanalyzer. Close lid.
4. Start the software "2100 Expert".
5. Select Assay->RNA->"Eukaryote Total RNA Nano Series II"
6. Verify that the chip image displayed matches the chip being used.
7. Enter sample names on the instrument tab under the chip summary section.
8. Chip lot number and Reagent Kit lot number should also be recorded in this section.
9. Save file with the default date and time only.
10. Select the number of wells to be analyzed (e.g. 1-12).
11. Press "Run".
12. Select the pull down window labeled "Instrument" and select "Data".
13. After the run is complete, make sure the x-axis on the electropherogram is labeled with nt (nucleotide) units. If the x-axis is labeled with sec (seconds) click the Axis button at the top of the screen to change it back to nt.
14. If running the RNA Nano Assay for FFPE RNA samples, perform Smear Analyis (see below).




15. The file is automatically saved in a format that can be viewed only with Agilent software. To make a PDF copy, select file->print. Select "PDF", choose the correct path (e.g., external USB drive), verify that the following are checked: electropherogram, gel like, result tables, and include ladder, then press "save".

16. Save the file as an XML. Select File. Click on Export. Check the box that says "Export to XML". Under Export Directory, highlight "Custom". Select the "Export RESLABVANTAGE" drive. Click Export.
17. Print a copy of the results: Select File, Print. Verify that the following options are checked: run summary, result tables, and include ladder. Click Print.
18. Refer to A005: LabVantage Manual section II.H.10 for importing results into LabVantage.

**M. FFPE Smear Analysis**

1. Highlight sample(s) to be analyzed.
2. At the right border of the software window, there are two tabs for additional options: Local and Global. Local is used for the individual sample selected. Global is used to apply the option to all samples.
3. Use the drop down menu to select "Advanced Settings."
4. Scroll down to "Smear Analysis" and check the box for "Smear Analysis."
5. Double click on row "Regions" to add table.
6. Add the following four smear reasons:
   Low: base pair 35 – 235
   Medium: base pair 500 – 1280
   High: base pair 1800 – 3440
   DV200: base pair 200 to max
7. Click "OK" to initiate calculation. The smear analysis will provide the total peak heights in the defined base pair ranges.

## III. REFERENCES - None

## IV. COMPREHENSIVE REVISION HISTORY

**A.** Changes made in Version 3, Effective Date 6/6/2016

1. Made title not all capitalized
2. Updated safety information section.
3. Quality Control - removed RIN Control table. Any chip with failed control will be fully repeated. Imported data checked against printed agilent report. All kits are QC'd together and used together as kits.
4. Ladder Prep - thawed and held on wet ice while prepared.
5. Preparing gel - reagents are stored refrigerated, used by mfg exp date.
6. Preparing gel/dye mix - protect from light, mix dye before use, gel/dye should be mixed to homogenous.
7. NEW - FFPE Smear Analysis (how to perform, what is recovered).

**B.** Changes made in Version 2, Effective Date **9/11/2014**

1. New format used
2. Added flow chart for repeating samples
3. Added section of preparing the priming station
4. Updated materials by removing PCR strip tubes and adding 0.5mL tubes

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5. Updated disclaimer
6. Deleted section for cleaning electrodes before use/after use (this is part of equipment maintenance), as this information is found in MGL-EQP-9, "Agilent 2100 Bioanalyzer"

**C.** Version 1, Effective Date 9/14/2012 - New

## Signatures

**Approved By:** **Signature on file** **Date:** **Date on file**

**Julie Gastier-Foster, PhD, FACMG**
**Principal Investigator**