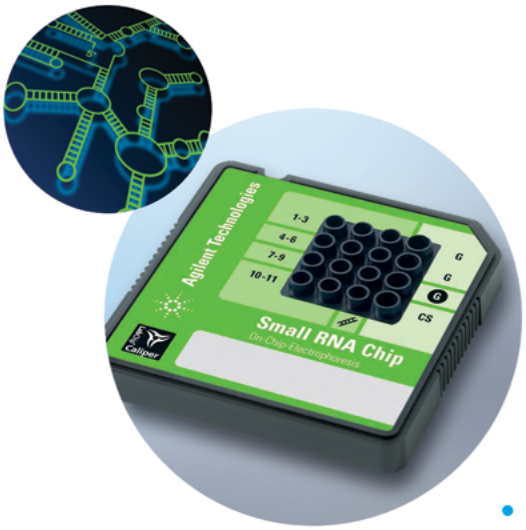

Agilent Small RNA

Kit Guide

Notices

Manual Part Number

G2938-90093 Rev. B

Edition

11/2013

Printed in Germany

Agilent Technologies
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Resarch Use Only

Not for use in Diagnostic Procedures.

Software Revision

This guide is valid for 2100 expert B.02.05 revision and higher of the Agilent expert software, where 05 refers to minor revisions of the software that do not affect the technical accuracy of this guide.

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CAUTION

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WARNING

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Contents

Agilent Small RNA Kit

Table 1 Agilent Small RNA Kit (reorder number 5067-1548)

Agilent Small RNA Chips
25 Small RNA Chips
3 Electrode Cleaners
Agilent Small RNA Reagents (reorder number 5067-1549)
(blue) Small RNA Dye Concentrate[1]
(green) Small RNA Marker (4 vials)
(white) Small RNA Conditioning Solution
(red) Small RNA Gel Matrix (2 vials)
(yellow) Small RNA Ladder (reorder number 5067-1550).
2 Spin Filters
Tubes for Gel-Dye Mix
30 Safe-Lock Eppendorf Tubes PCR clean (DNase/RNase free) for gel-dye mix
Syringe Kit
1 Syringe

1 "This product is provided under a license by Life Technologies Corporation to Agilent Technologies. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product only as described in accompanying product literature. The sale of this product is expressly conditioned on the buyer not using the product or its components (1) in manufacturing; (2) to provide a service, information, or data to an unaffiliated third party for payment; (3) for therapeutic, diagnostic or prophylactic purposes; (4) to resell, sell or otherwise transfer this product or its components to any third party, or use for any use other than use in the subfields of research and development, quality control, forensics, environmental analysis, biodefense or food safety testing. For information on purchasing a license to this product for purposes other than described above contact Life Technologies Corporation, Cell Analysis Business Unit, Business Development, 29851 Willow Creek Road, Eugene, OR 97402, Tel: (541) 465-8300. Fax: (541) 335-0354"

Table 2 Physical Specifications

Type	Specification
Analysis run time	30 minutes
Number of samples	11 samples/chip
Sample volume	1 µl
Assay kit stability	4 months (Storage temperature see individual box!)

Table 3 Analytical Specifications

Specification	Small RNA Assay
Analysis range	6-150 nt
Quantitation reproducibility	25 % CV (for ladder as an example)
Quantitative Range	50-2000 pg/µl for purified miRNA (in water)
Max. Buffer concentration*	10 mM Tris and 0.1 mM EDTA
Sensitivity (Signal/Noise > 3)	50 pg/µl (for 40 nt fragment of diluted ladder)

*) Due to the high sensitivity of the assay, different ions and higher salt concentrations might influence the performance of the assay.

Table 4 Recommended Concentration

Sample type	Concentration
Total RNA	1-100 ng/µl
Enriched Small RNA*	1-20 ng/µl
Oligonucleotides	100 to 2000 pg/µl

*) For example by commercial purification kit or preparative gel extraction.

Required Equipment for Small RNA Assay

Equipment Supplied with the Agilent 2100 Bioanalyzer

- Chip priming station (reorder number 5065-4401)
- IKA vortex mixer

Additional Material Required (Not Supplied)

- RNaseZAP® recommended for electrode decontamination (Ambion, Inc. cat. no. 9780)
- RNase-free water
- Pipettes (10 µL and 1000 µL) with compatible tips (RNase-free, no filter tips, no autoclaved tips)
- 0.5 mL microcentrifuge tubes (RNase-free).Eppendorf Safe-lock PCR clean or Eppendorf DNA LoBind microcentrifuge tubes are highly recommended.
- Microcentrifuge (≥13000 g)
- Heating block or water bath for ladder/sample preparation
- Mandatory: bayonet electrode cartridge (reorder number 5065-4413)

Check the Agilent Lab-on-a-Chip webpage for details on assays: www.agilent.com/chem/labonachip.

Setting up the Assay Equipment and Bioanalyzer

Setting up the Assay Equipment and Bioanalyzer

Before beginning the chip preparation protocol, ensure that the chip priming station and the bioanalyzer are set up and ready to use.

You have to

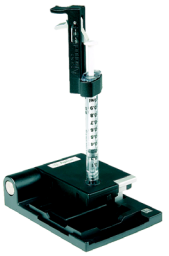
- replace the syringe at the chip priming station with each new kit
- adjust the base plate of the chip priming station
- adjust the syringe clip at the chip priming station
- adjust the bioanalyzer's chip selector
- set up the vortex mixer
- finally, make sure that you start the software before you load the chip.

NOTE The Small RNA assay is a high sensitivity assay. Please read this guide carefully and strictly follow all instructions to guarantee satisfactory results.

Setting up the Chip Priming Station

NOTE Replace the syringe with each new Reagent Kit.

1 Replace the syringe:



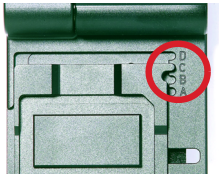
   a Unscrew the old syringe from the lid of the chip priming station.

   b Release the old syringe from the clip. Discard the old syringe.

   c Remove the plastic cap of the new syringe and insert it into the clip.

   d Slide it into the hole of the luer lock adapter and screw it tightly to the chip priming station.

2 Adjust the base plate:



   a Open the chip priming station by pulling the latch.

   b Using a screwdriver, open the screw at the underside of the base plate.

   c Lift the base plate and insert it again in position C. Retighten the screw.

3 Adjust the syringe clip:

   a Release the lever of the clip and slide it down to the lowest position.

Setting up the Bioanalyzer

Adjust the chip selector:

1 Open the lid of the bioanalyzer and make sure that the electrode cartridge is inserted in the instrument. If not, open the latch, remove the pressure cartridge and insert the electrode cartridge.

2 Remove any remaining chip and adjust the chip selector to position (1).

Vortex Mixer

IKA - Model MS3

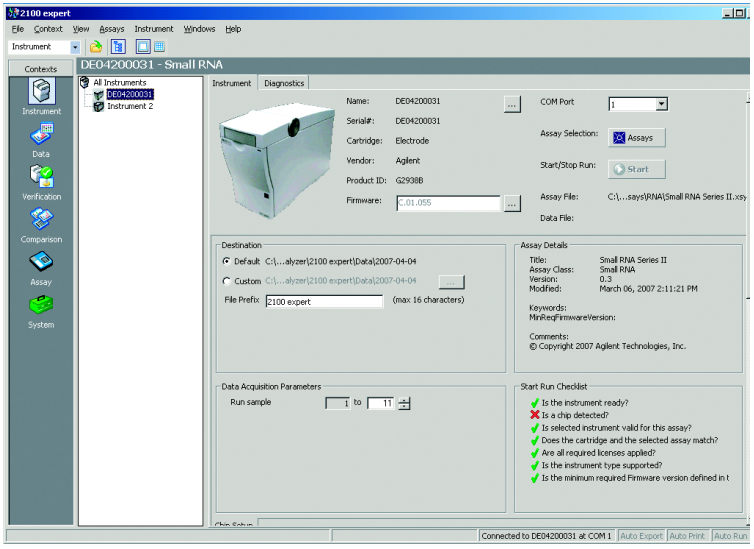
1 To set up the vortex mixer, adjust the speed knob to 2400 rpm.

Starting the 2100 Expert Software

To start the software:

1 Go to your desktop and double-click the following icon.

The screen of the software appears in the *Instrument* context. The icon in the upper part of the screen represents the current instrument/PC communication status:



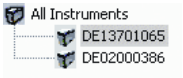
Lid closed, no chip or chip empty

Lid open

Dimmed icon: no communication

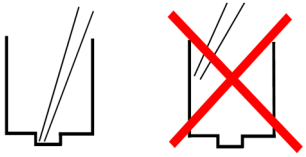
Lid closed, chip inserted, RNA or demo assay selected

2 If more than one instrument is connected to your PC, select the instrument you want to use in the tree view.



Essential Measurement Practices

- Handle and store all reagents according to the instructions on the label of the individual box.
- Avoid sources of dust or other contaminants. Foreign matter in reagents and samples or in the wells of the chip will interfere with assay results.
- Keep all reagent and reagent mixes refrigerated at 4 °C when not in use.
- Allow all reagents to equilibrate to room temperature for 30 minutes before use. Thaw the sample on ice.
- Protect marker solution, dye and dye mixtures from light. Remove light covers only when pipetting. The dye decomposes when exposed to light and this reduces the signal intensity.
- Always insert the pipette tip to the bottom of the well when dispensing the liquid. Placing the pipette at the edge of the well may lead to poor results.



- Always wear gloves when handling RNA and use RNase-free tips, microfuge tubes and water.
- The gel matrix is very viscous! Proper pipetting and mixing requires special attention.
- It is recommended to heat denature all RNA samples and RNA ladder before use for 2 minutes at 70 °C (once) and keep them on ice.
- Do not touch the Agilent 2100 bioanalyzer during analysis and never place it on a vibrating surface.
- Always vortex the dye concentrate for 10 seconds before preparing the gel-dye mix and spin down afterwards.

- Use a new syringe and electrode cleaners with each new kit.
- Use loaded chips within 5 minutes after preparation. Reagents might evaporate, leading to poor results.
- To prevent contamination problems, it is strongly recommended to use a dedicated electrode cartridge for RNA assays. For running the Small RNA assay, the 16 pin bayonet cartridge is mandatory.

Preparing the RNA Ladder after Arrival

For proper handling of the ladder, following steps are necessary:

CAUTION

To avoid RNase contamination and repetitive freeze/thaw cycles, the RNA ladder must be aliquoted in RNase-free vials.

➔ Tests have shown that some plastic vial types can bind RNA on their surface. This can have an effect on RNA ladder concentration which affects the ladder identification and the quantitation of the sample. Eppendorf Safe-Lock PCR clean and Eppendorf DNA LoBind 0.5ml Microcentrifuge tubes were successfully tested for RNA ladder aliquoting.

1 After reagent kit arrival, spin ladder down. The ladder can be ordered separately (reorder number 5067-1550).

NOTE

In case that the ladder vial type is not compatible with your heating block, transfer the entire volume (35 µl) to a recommended RNase-free vial.

2 Heat denature the ladder for 2 min at 70 °C.

3 Immediately cool the vial on ice.

4 Prepare aliquots in recommended 0.5 mL RNase-free vials with the required amount for typical daily use.

5 Store aliquots at -70 °C.

6 Before use, thaw ladder aliquots and keep them on ice (avoid extensive warming upon thawing process).

Agilent Small RNA Protocol Assay

After completing the initial steps in "Setting up the Assay Equipment and Bioanalyzer" on page 7, you can prepare the assay, load the chip, and run the assay, as described in the following procedures.

NOTE If you use the Small RNA kit for the first time, you must read these detailed instructions. If you have some experience, you might want to use the *Agilent Small RNA Quick Start Guide*.

Cleaning the Electrodes before Running Assays

To avoid decomposition of your RNA sample, follow this cleaning procedure on a daily basis before running any Small RNA assays.

NOTE The bayonet electrodes can be suspected to be contaminated with RNase. In this case, perform the decontamination procedure "Decontaminating the Electrodes" on page 24. To prevent contamination problems, it is strongly recommended to use a dedicated electrode cartridge for RNA assays.

1. Slowly fill one of the wells of the electrode cleaner with 350 µl of fresh RNase-free water.
2. Open the lid and place the electrode cleaner in the Agilent 2100 bioanalyzer.
3. Close the lid and leave it closed for about 5 min.
4. Open the lid and remove the electrode cleaner. Label the electrode cleaner and keep it for future use.
5. Wait another 30 seconds to allow the water on the electrodes to evaporate before closing the lid.

Preparing the Gel

1 Allow all reagents to equilibrate to room temperature for 30 minutes before use.

2 Transfer complete volume (approx. 650 µl) of Small RNA gel matrix (red ●) into the top receptacle of a spin filter.

NOTE Always use RNase-free microfuge tubes, pipette tips and water.

3 Place the spin filter in a microcentrifuge and spin for 15 minutes at 10000 g ± 20%.

4 Remove the filter.

5 Label the filtered gel with the filtration date.

6 Store the filtered gel at 4 °C and use it within one month of preparation.

Preparing the Gel-Dye Mix

WARNING

Handling DMSO

Kit components contain DMSO. Because the dye binds to nucleic acids, it should be treated as a potential mutagen and used with appropriate care.

➔ Wear hand and eye protection and follow good laboratory practices when preparing and handling reagents and samples.

➔ Handle solutions with particular caution as DMSO is known to facilitate the entry of organic molecules into tissues.

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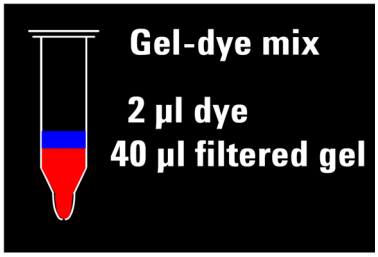
1 Allow all reagents to equilibrate to room temperature for 30 minutes before use. Protect the dye concentrate from light while bringing it to room temperature.

2 Vortex Small RNA dye concentrate (blue ●) for 10 seconds and spin down.

3 Pipette 2 µl of dye into 0.5 ml RNase free microtubes (provided with the kit).



CAUTION

The gel matrix is very viscous!

Proper pipetting and mixing with the dye requires special attention.

➔ When pipetting the gel it's important to leave the pipette tip in the matrix liquid for some time (at least 10 seconds) after releasing the pipette plunger (drawing the gel matrix in the tip). Visually check that the liquid level within the tip is not increasing anymore. Pipetting times might differ depending on the type of pipette and tips.

➔ Mixing of the dye with the gel is not possible using the vortexer!

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4 Add 40 µl of filtered gel (gel is very viscous, careful pipetting is highly recommended).

5 Mix solution by pipetting, or by flipping over and flicking the vial several times until the dye is distributed equally. Visually inspect that the mix is homogeneous.

6 Spin tube at 13000 g for 10 minutes at room temperature.

NOTE

A larger volume of gel-dye mix can be prepared in multiples of the 40+2 ratio, if more than one chip will be used within one day. Always re-spin the gel-dye mix at 13000 g for 10 minutes before each use.

Loading the Gel-Dye Mix

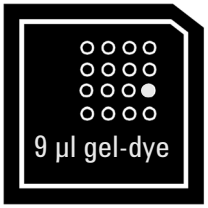
NOTE Before loading the gel-dye mix, make sure that the base plate of the chip priming station is in position (C) and the adjustable clip is set to the lowest position. Refer to "Setting up the Chip Priming Station" on page 8 for details.

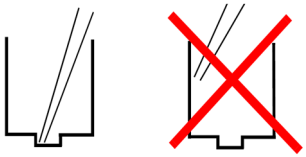
1 Allow the gel-dye mix to equilibrate to room temperature for 30 minutes before use and protect the gel-dye mix from light during this time.

2 Take a new Small RNA chip out of its sealed bag.

3 Place the chip on the chip priming station.

4 Slowly pipette 9.0 µl of the gel-dye mix at the bottom of the well marked G and dispense the gel-dye mix.





NOTE When pipetting the gel-dye mix, make sure not to draw up particles that may sit at the bottom of the gel-dye mix vial. Insert the tip of the pipette to the bottom of the chip well when dispensing. This prevents a large air bubble forming under the gel-dye mix. Placing the pipette at the edge of the well may lead to poor results.

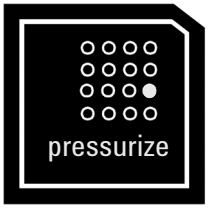
NOTE The gel is very viscous. Slowly pipetting is highly recommended.

5 Set the timer to 60 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 correctly.

6 Press the plunger of the syringe down until it is held by the clip.

7 Wait for exactly 60 seconds and then release the plunger with the clip release mechanism.

8 Visually inspect that the plunger moves back at least to the 0.3 ml mark.



9 Wait for 5 seconds, then slowly pull back the plunger to the 1 ml position.

10 Open the chip priming station.

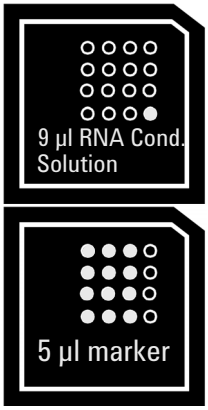
11 Pipette 9.0 µl of the gel-dye mix in each of the wells marked G

NOTE Please discard the remaining vial with gel-dye mix.

Loading the Small RNA Conditioning Solution and Marker

1 Pipette 9 µl of the Small RNA conditioning solution (white ○) into the well marked CS.

2 Pipette 5 µL of the Small RNA marker (green ●) into the well marked with a ladder (symbol ⋕) and each of the 11 sample wells.



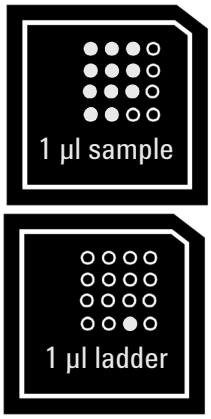
NOTE Do not leave any wells empty or the chip will not run properly. Add 5 µL of the RNA marker (green ●) plus 1 µL of deionized water to each unused sample well.

NOTE Protect Marker solution from light; it contains a fluorescent dye.

Loading the Ladder and Samples

NOTE Always use RNase-free microfuge tubes, pipette tips and water.

1. To minimize secondary structure, you may heat denature (70 °C, 2 minutes) the samples before loading on the chip.
2. Before use, thaw ladder aliquots and keep them on ice (avoid extensive warming upon thawing process).
3. Pipette 1 µl of the Small RNA ladder into the well marked with the ladder symbol.
4. Pipette 1 µl of each sample into each of the 11 sample wells.
5. Place the chip horizontally in the adapter of the IKA vortex mixer and make sure not to damage the buldge that fixes the chip during vortexing.



   If there is liquid spill at the top of the chip, carefully remove it with a tissue.
6. Vortex for 60 seconds at the indicated setting (2400 rpm).
7. Refer to the next topic on how to insert the chip in the Agilent 2100 bioanalyzer. Make sure that the run is started within 5 minutes.

NOTE Depending on the RNA isolation protocol, varying results can be expected. Known dependencies include: salt content, cell fixation method and tissue stain. Best results are achieved for RNA samples which are dissolved in deionized and RNase-free water. Avoid genomic DNA contamination by including DNase treatment in the preparation protocol.

Inserting a Chip in the Agilent 2100 Bioanalyzer

1 Open the lid of the Agilent 2100 bioanalyzer.

2 Check that the electrode cartridge is inserted properly and the chip selector is in position (1). Refer to "Setting up the Bioanalyzer" on page 9 for details.

3 Place the chip carefully into the receptacle. The chip fits only one way.

CAUTION

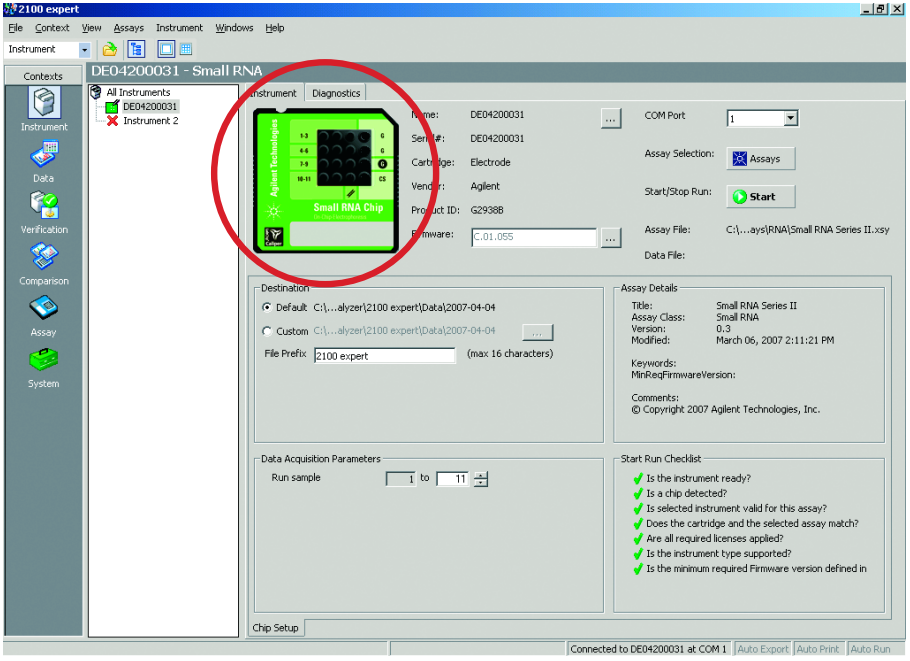
Sensitive electrodes and liquid spills

Forced closing of the lid may damage the electrodes and dropping the lid may cause liquid spills resulting in bad results.

➔ Do not use force to close the lid and do not drop the lid onto the inserted chip.

4 Carefully close the lid. The electrodes in the cartridge fit into the wells of the chip.

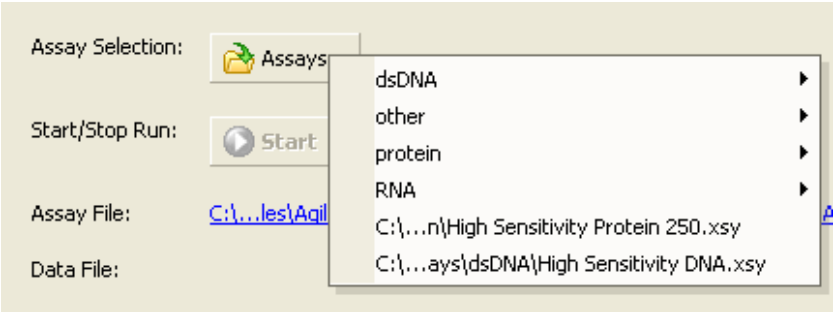
5 The 2100 expert software screen shows that you have inserted a chip and closed the lid by displaying the chip icon at the top left of the *Instrument* context.



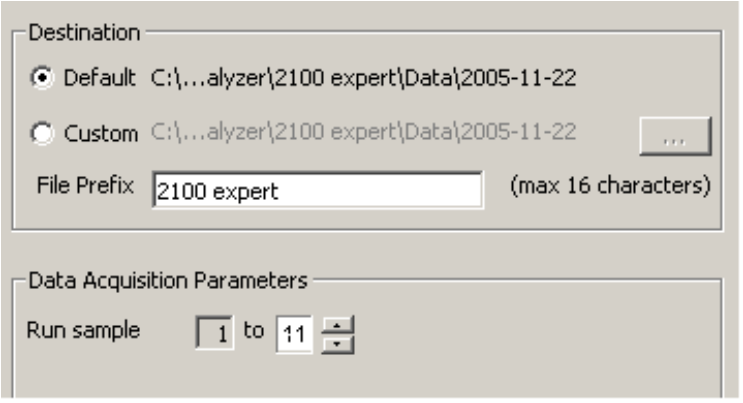
Starting the Chip Run

NOTE Please note that the order of executing the chip run may change if the Agilent Security Pack software (only applicable for Agilent 2100 expert software Revision B.02.04 and higher) is installed. For more details please read the 'User's Guide' which is part of the Online Help of your 2100 expert software.

1 In the **Instrument** context, select the appropriate assay from the Assay menu.



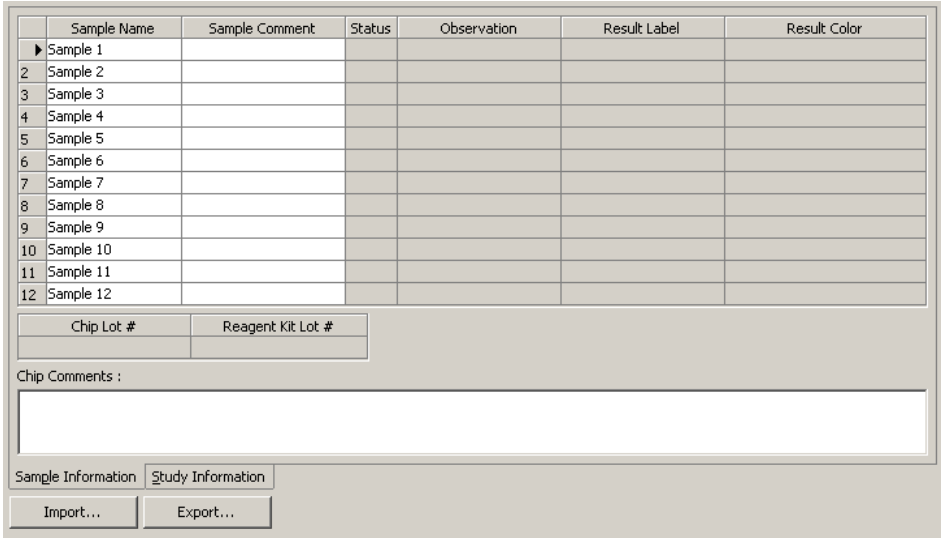
2 Accept the current **File Prefix** or modify it.



Data will be saved automatically to a file with a name using the prefix you have just entered. At this time you can also customize the file storage location and the number of samples that will be analyzed.

3 Click the *Start* button in the upper right of the window to start the chip run. The incoming raw signals are displayed in the *Instrument* context.

4 To enter sample information like sample names and comments, select the *Data File* link that is highlighted in blue or go to the *Assay* context and select the *Chip Summary* tab. Complete the sample name table.



5 To review the raw signal trace, return to the *Instrument* context.

CAUTION

Contamination of electrodes

Leaving the chip for a period longer than 1 hour (e.g. over night) in the Bioanalyzer may cause contamination of the electrodes.

→ Immediately remove the chip after a run.

6 After the chip run is finished, remove the chip from the receptacle of the bioanalyzer and dispose it according to good laboratory practices.

Cleaning Electrodes after a Small RNA Chip Run

When the assay is complete, *immediately* remove the used chip from the Agilent 2100 bioanalyzer and dispose it according to good laboratory practice. After a chip run, perform the following procedure to ensure that the electrodes are clean (i.e. no residues are left over from the previous assay).

NOTE Use a new electrode cleaner with each new kit.

CAUTION Leak currents between electrodes

Liquid spill may cause leak currents between the electrodes.

➔ Never fill too much water in the electrode cleaner.

1. Slowly fill one of the wells of the electrode cleaner with 350 µl of fresh RNase-free water.
2. Open the lid and place the electrode cleaner in the Agilent 2100 bioanalyzer.
3. Close the lid and leave it closed for 30 s.
4. Open the lid and remove the electrode cleaner.
5. Wait another 30 seconds to allow the water on the electrodes to evaporate before closing the lid.

NOTE Replace the water in the cleaning chip after each use. Use a new cleaning chip after 12-13 electrode cleaning procedures and with new kit.

Decontaminating the Electrodes

When you suspect the bayonet electrodes to be contaminated with RNase, perform the following decontamination procedure:

1. Remove the bayonet electrodes from the cartridge.
2. Spray RNaseZap® onto electrodes and soak for 1 minute.
3. Gently brush the electrodes.
4. Rinse electrodes thoroughly with RNase-free water.

NOTE The rinsing step is very important and needs special attention as residual RNaseZap may alter the results creating ghost peaks.

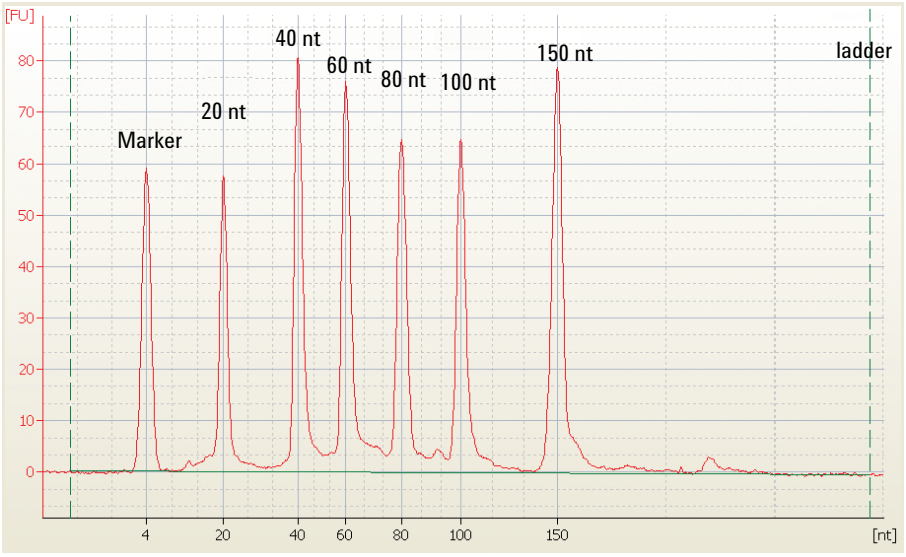
5. Dry the electrodes by using oil-free compressed air.
6. Reassemble the electrode cartridge and put it back into the instrument.
7. To verify that the electrodes are completely dry, perform the Short Circuit Test that you can find under the Diagnostics tab in the *Instrument* context.

NOTE If the short circuit test fails, the cartridge may still be wet. Repeat the drying procedure.

Checking Your Agilent Small RNA Assay Results

Small RNA Ladder Well Results

To check the results of your run, select the Gel or Electropherogram tab in the *Data* context. The electropherogram of the ladder well window should resemble those as shown below.



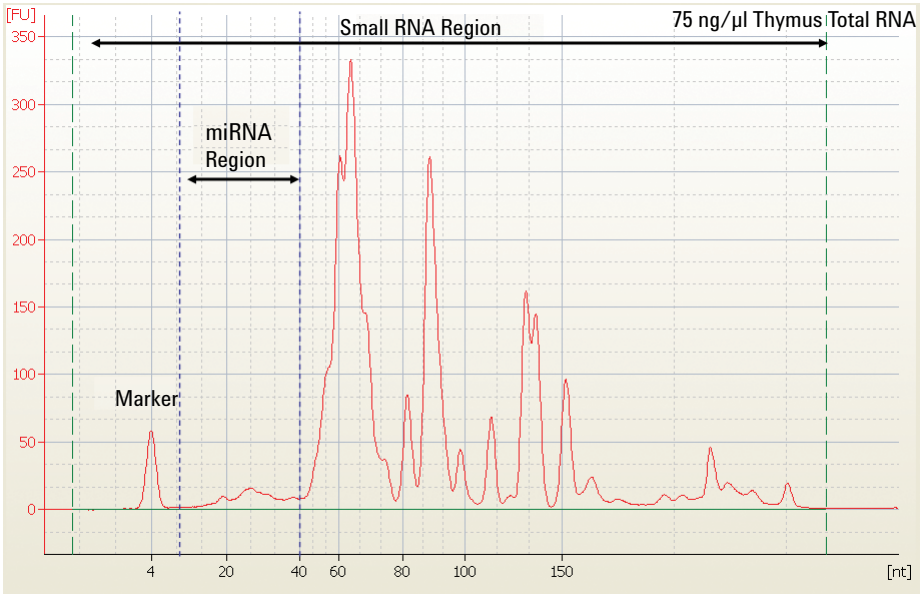
Major features of a successful ladder run are:

- 1 marker peak
- 6 RNA peaks
- All 7 peaks are well resolved
- Correct peak size assignment in electropherogram

If the electropherogram of the ladder well window does not resemble the one shown above, refer to the *2100 Expert Maintenance and Troubleshooting Guide* for assistance.

Small RNA Sample Well Results

To review the results of a specific sample, select the sample name in the tree view and highlight the *Results* sub-tab. The electropherogram of the sample well window for total RNA (eukaryotic) should resemble the one shown here.



Major features for a successful total RNA run are:

- 1 marker peak

Two distinct regions are defined arbitrarily. The Small RNA region from 0 to 150 nt, and the micro RNA region (mi RNA) from the 10 to 40 nt. These regions can be modified by selecting the Region table or by sliding borders in the electropherogram, implemented with the 2100 expert software version B02.04.

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In this book

you find the procedures to analyze RNA samples with the Agilent Small RNA reagent kit and the Agilent 2100 Bioanalyzer instrument.

- Agilent Small RNA Kit
- Required Equipment for Small RNA Assay
- Setting up the Assay Equipment and Bioanalyzer
- Essential Measurement Practices
- Preparing the RNA Ladder after Arrival
- Agilent Small RNA Protocol Assay
- Checking Your Agilent Small RNA Assay Results

Printed in Germany
11/2013

G2938-90093
Rev. B

Agilent Technologies