

Agilent DNF-471 (15 nt) RNA Kit

- IVT mRNA

Quick Guide

For Research Use Only. Not for use in diagnostic procedures

This Quick Guide is intended for use with the Agilent 5200, 5300, and 5400 Fragment Analyzer systems only. The DNF-471 RNA kit is designed for analysis of IVT mRNA in the range of 20 to 100 ng/μL input sample concentration as well as Total RNA. This quick guide covers methods for IVT mRNA less than or equal to 6,000 nt and an extended method for samples up to 9,000 nt. A separate quick guide (SD-AT000131) covers Total RNA methods.

Specifications

Analytical Specifications ¹	IVT mRNA Analysis ¹
Sizing Range	200 nt – 6,000 nt 500 nt – 9,000 nt (with Lonza RNA Marker)
Sizing Accuracy ¹	± 10%
Sizing Precision ¹	5% CV
Limit of Detection (S/N > 3)	1 ng/μL
Qualitative Range (per smear)	1 ng/μL – 100 ng/μL
Quantitative Range (per smear)	20 ng/μL – 100 ng/μL
Quantification Accuracy ¹	± 20%
Quantification Precision ¹	10% CV

Physical Specifications

Total Electrophoresis Run Time	33cm: 40 minutes (DNF-47133) 33cm: 90 minutes (DNF-471E33 Extended IVT mRNA method)
Samples Per Run	12, 48 or 96; depending on the instrument type
Sample Volume Required	2 μL
Kit Stability	4 months

¹ Results using 900 nt, 6,000 nt, and 9,000 nt IVT mRNA samples with no modifications on the Fragment Analyzer instruments with a FA Capillary Array Short 33cm array..

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Kit Components – 500 Sample Kit – Refer to product label for proper storage conditions

Kit Component Number	Part Number (Re-order Number)	Description	Quantity Per Kit
5191-6572*		RNA (15 nt), 500, 4°C	
	DNF-265-0240	RNA Separation Gel, 240 mL	1
	DNF-300-0008	BF-25 Blank Solution, 8 mL	1
	DNF-355-0125	5x 930 dsDNA Inlet Buffer, 125 mL	1
	DNF-497-0125	0.25x TE Rinse Buffer, 125 mL	1
DNF-471-FR*		RNA (15 nt), FR	
	DNF-600-U030	Intercalating Dye, 30 µL	1
	DNF-369-0004	RNA Diluent Marker (15 nt), 4 mL	3
	DNF-382-U020	RNA Ladder, 20 µL	5
DNF-475-0050	DNF-475-0050	5x Capillary Conditioning Solution, 50 mL	1

Kit Components – 1000 Sample Kit – Refer to product label for proper storage conditions

Kit Component Number	Part Number (Re-order Number)	Description	Quantity Per Kit
5191-6573*		RNA (15nt), 1000, 4°C	
	DNF-265-0500	RNA Separation Gel, 500 mL	1
	DNF-300-0008	BF-25 Blank Solution, 8 mL	1
	DNF-355-0300	5x 930 dsDNA Inlet Buffer, 300 mL	1
	DNF-497-0125	0.25x TE Rinse Buffer, 125 mL	1
DNF-471-FR*		RNA (15 nt), FR	
	DNF-600-U030	Intercalating Dye, 30 µL	2
	DNF-369-0004	RNA Diluent Marker (15 nt), 4 mL	6
	DNF-382-U020	RNA Ladder, 20 µL	10
DNF-475-0100	DNF-475-0100	5x Capillary Conditioning Solution, 100 mL	1

*Not orderable.

Additional Material Required for Analysis with Fragment Analyzer Systems (not supplied)

Instrument	Compatible Arrays	Part Number
5200 Fragment Analyzer	FA 12 Capillary Array Short	A2300-1250-3355
5300 Fragment Analyzer	FA 48 Capillary Array Short FA/ZAG 96 Capillary Array Short	A2300-4850-3355 A2300-9650-3355
5400 Fragment Analyzer	FA/ZAG 96 Capillary Array Short	A2300-9650-3355

Software

- Fragment Analyzer controller software
- ProSize data analysis software

Reagents

- Capillary Storage Solution (GP-440-0100)

Additional equipment required (not supplied)

- 96-well PCR sample plates (*Refer to Appendix in Fragment Analyzer User Manual*)
- Multichannel pipettor and/or liquid handling device capable of dispensing 1-100 μ L (sample plates) and 1,000 μ L (inlet buffer plate)
- Pipette tips
- 96-well plate centrifuge
- Adhesive PCR plate seals
- Sub-micron filtered DI water system: for dilutions.
- 96-deepwell 1 mL plate: inlet buffer and/or waste plate (Agilent #P60-20 or Fisher Scientific #12-566-120)
- Reagent reservoir 50 mL: for use in pipetting inlet buffer plates (VWR #89094-680, or similar)
- Conical centrifuge tubes for prepared separation gel+dye mixture and/or 1x Capillary Conditioning Solution
 - 50 mL for 5200 Fragment Analyzer system (BD Falcon #352070, Fisher Scientific #14-432-22 or VWR #21008-940)
 - 250 mL for 5300 and 5400 Fragment Analyzer systems (Corning #430776, Fisher Scientific #05-538-53 or VWR #21008-771)
- Vortexer (VWR, part number 102093-352)
- RNase-free/nuclease free water
- RNaseZap (Ambion #AM9782 or equivalent product)
- Thermal cycler
- Lonza RNA Marker when running extended IVT mRNA method (Lonza p/n 50705)
- Eppendorf DNA LoBind® tubes for aliquoting RNA Ladder (Eppendorf p/n 022431021)

WARNING**Working with Chemicals**

- Refer to product safety data sheets for further information
- When working with the Fragment Analyzer kit components follow the appropriate safety procedures such as wearing personal protective equipment (PPE).

Essential Measurement Practices

Environmental conditions	<ul style="list-style-type: none"> • Ambient operating temperature: 19 – 25 °C (66 – 77 °F) • Keep instrument reagents at room temperature during sample preparation.
Sample Input Concentration	<ul style="list-style-type: none"> • Ensure sample input concentrations lie within kit specifications. • Sample signal should not exceed 60,000 RFU.
Working with RNA Samples	<ul style="list-style-type: none"> • Ensure all working areas, reagents and plastic ware are RNase free. • Handle RNA samples with care. • Wear gloves at all times. • Thaw RNA samples on ice. • Vortex and centrifuge all samples before use. • Store RNA samples on ice throughout the preparation and analysis procedure.
Steps before sample preparation	<ul style="list-style-type: none"> • Allow instrument reagents to equilibrate at room temperature for 30 min prior to use. • Thaw RNA ladder on ice prior to use. • Keep RNA Diluent Marker on ice before use. Vortex the tube briefly to mix the contents.
Pipetting practice	<ul style="list-style-type: none"> • Pipette reagents against the side of the 96-well sample plate or sample tube. • Ensure no sample or Diluent Marker remains within or on the outside of the tip.
RNA Ladder Preparation	<ul style="list-style-type: none"> • Upon arrival of the ladder, it is recommended to divide the ladder into aliquots with working volume typical for one day use or one sample plate. Store aliquots in Eppendorf 0.5 mL LoBind tubes (Eppendorf p/n 022431021) at -70°C or below. • Thaw RNA ladder aliquot in PCR tube on ice. • Spin down the contents and mix by pipetting the solution up and down with a pipette tip. Transfer the Ladder to a RNase-free PCR tube. Heat denature the ladder at 70°C for 2 minutes, immediately cool to 4°C and keep on ice. • The RNA ladder must be run in parallel with the samples for each experiment to ensure accurate sizing.
Extended Method Lonza RNA Marker (Lonza ladder) Preparation	<ul style="list-style-type: none"> • When using the Extended Method for analysis of IVT mRNA $\geq 6,000$ nt this was developed in conjunction with the Lonza RNA Marker (Lonza ladder), diluted in nuclease free water to a concentration of 96 ng/μL. • Upon arrival of the Lonza ladder dilute to 96 ng/μL with nuclease free water and divide the ladder into 3 μL aliquots. Store aliquots in the provided Eppendorf 0.5 mL LoBind tubes at -70°C or below. • Thaw a 3 μL 96 ng/μL Lonza ladder aliquot on ice. • Spin down the contents and mix by pipetting the solution up and down with a pipette tip set to a 2 μL volume. <ol style="list-style-type: none"> 1. Transfer 2 μL of the 96 ng/μL Lonza ladder to a fresh Eppendorf LoBind 0.5 mL tube. 2. Heat denature the Lonza ladder at 70°C for 2 minutes, immediately cool to 4°C and keep on ice. • The 96 ng/μL working concentration Lonza ladder must be run in parallel with the samples for each experiment to ensure accurate sizing.
IVT mRNA Sample Preparation	<ul style="list-style-type: none"> • When using this kit for analysis of IVT mRNA, the sample must be within a concentration range of 1 ng/μL – 100 ng/μL for optimal results. If the concentration of the sample is above this range, dilute with RNase-free water. • When running IVT mRNA $\geq 6,000$ nt in size it is recommended to extend the separation time by 5 minutes to ensure the entire sample is separated out. • When running IVT mRNA $\geq 6,000$ nt in size it is recommended to use the DNF-471E33 – SS Total RNA 15 nt Extended run method. This was developed in conjunction with the Lonza RNA Marker (ladder), diluted in nuclease free water to a working concentration of 96 ng/μL. This method is only applicable for the 'Short' 33 cm arrays. • For further information on IVT mRNA analysis on the Fragment Analyzer system please refer to the application notes "Benefits of Quality Control in the IVT RNA Workflow Using the Agilent 5200 Fragment Analyzer System" (Document number 5994-0512EN), "Assessment of Long IVT RNA Fragments with the Agilent 5200

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Fragment Analyzer system" (Document number 5994-0878EN), and "Best Practices for Analysis of In Vitro Transcribed (IVT) mRNA" (Document number 5994-5927EN) on Agilent's website.

Sample Plate Preparation

- Using a fresh RNase-free 96-well sample plate, pipette 22 μ L of the RNA Diluent Marker (DM) solution to each well in a row that is to contain sample or RNA Ladder.
- Fill any unused wells within the row of the sample plate with 24 μ L blank solution.
- Pipette 2 μ L of each denatured RNA sample into the 22 μ L of DM solution in the respective wells of the sample plate; mix well.
- Pipette 2 μ L of denatured RNA Ladder into the 22 μ L of DM solution in the designated ladder well.

Mixing and centrifugation recommendations

- When mixing sample with Diluent Marker (DM), mix the contents of the well thoroughly. It is suggested to perform **one** of the following methods to ensure complete mixing.
 - After adding 2 μ L of sample or ladder to the 22 μ L of DM, place a plate seal on the sample plate and vortex the sample plate at 3,000 rpm for 2 min. Avoid intense vortexing that causes splashing. The plate should be spun via a centrifuge after vortexing to ensure there are no trapped air bubbles in the wells.
 - After adding 2 μ L of sample or ladder to the 22 μ L of DM, use a separate pipette tip set to a larger 20 μ L volume, and pipette each well up/down to further mix.
 - Use an electronic pipettor capable of mixing a 10 μ L volume in the tip after dispensing the 2 μ L sample or ladder volume.
- After mixing, centrifuge the plate to remove any air bubbles.
- Run samples immediately after preparation, or within a day. If not using right away, cover and keep at 4°C, warm to RT and centrifuge before running plate.

Gel Preparation

Centrifuge dye prior to opening the vial to reduce risk of leaking. Ensure the gel + dye is mixed without generating bubbles, gently invert tube 5-10 times.

Number of Samples	Intercalating Dye Volume (μ L)	Separation Gel Volume (mL)
12	1	10
24	1.5	15
48	2.5	25
96	4.5	45
192	8	80
384	16	160

Conditioning Solution

The provided 5X Conditioning Solution must be diluted to 1X using submicron DI water prior to use. Gently invert to mix.

Number of Samples	Volume of 1X Conditioning Solution (mL)
12	10
24	15
48	25
96	45
192	80
384	160

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Agilent RNA DNF-471 Kit Operating Procedure


1. Mix fresh gel and dye according to the volumes in the preparation table. Update solution level in controller software.
2. Refill 1X Capillary Conditioning Solution as needed. Update solution level in controller software.
3. Inspect and empty, if necessary, waste plate located in drawer 'W'.
4. Place a fresh 1X Inlet Buffer, 1 mL/well, in drawer "B". Replace daily.
 - 5200 – row A
 - 5300 – 48 capillary, rows A-D
 - 5300/5400 – 96 capillary, all rows

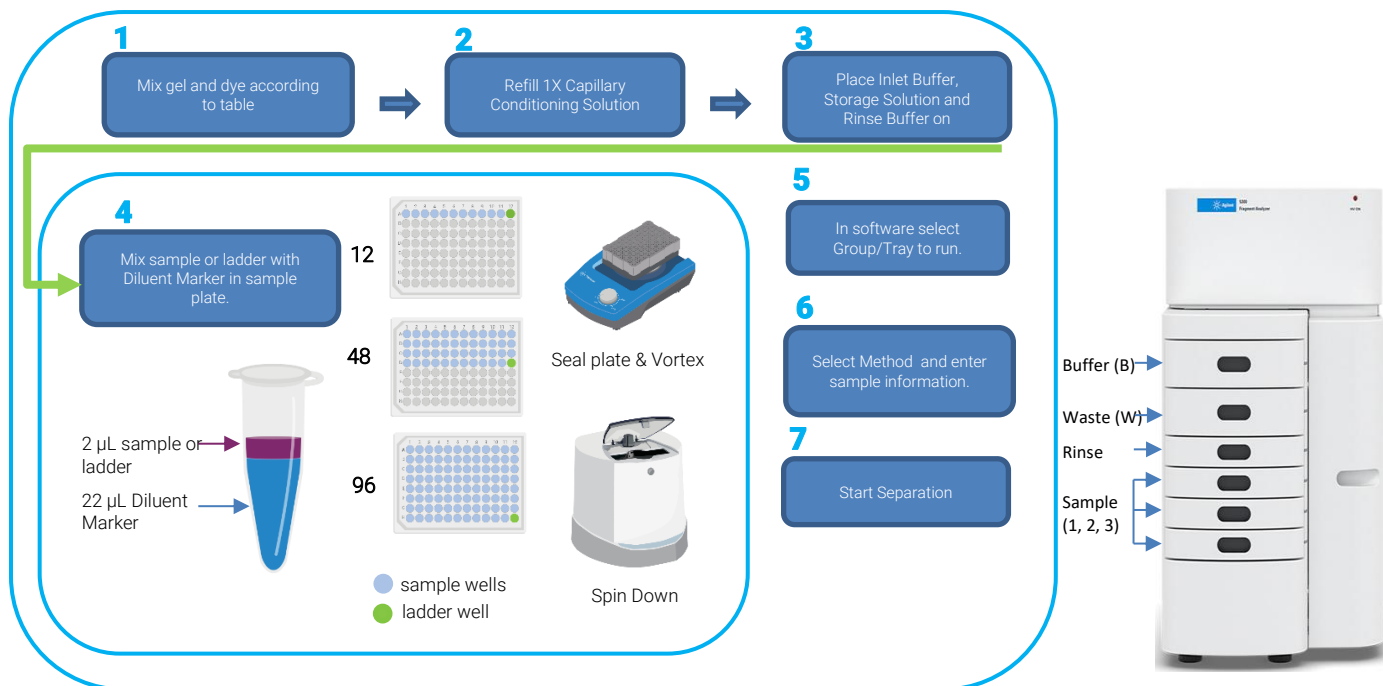
Prepare Capillary Storage Solution plate. Replace every 2 weeks for optimal results.

 - 5200 – row H, 1 mL/well, drawer B
 - 5300 – 48 capillary, rows A-D, 100 μ L/well, drawer 3
 - 5300/5400 – 96 capillary, all rows, 100 μ L/well, drawer 3

Place 0.25x TE Rinse Buffer plate, 200 μ L/well, in drawer "M". Replace daily.

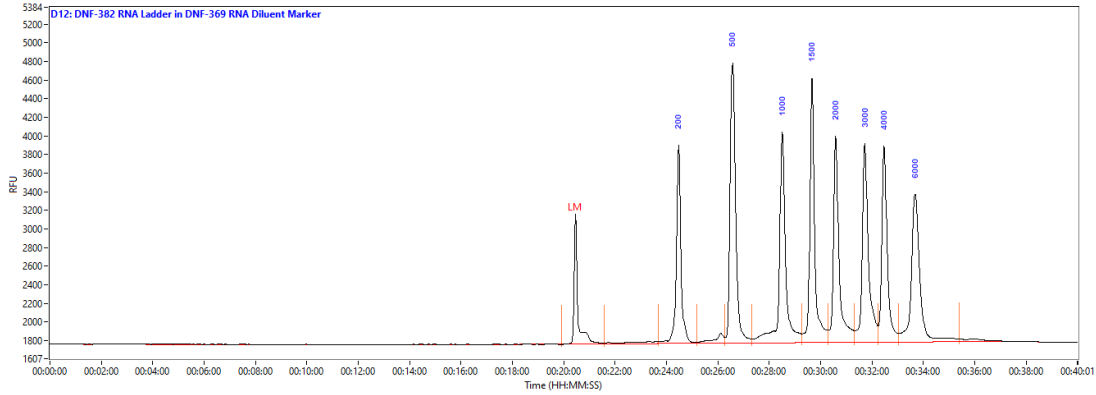
 - 5200 – Row A
 - 5300 – 48 capillary, rows A-D
 - 5300/5400 – 96 capillary, all rows
5. Mix samples or ladder with diluent marker in sample plate, add 24 μ L of Blank Solution to unused wells. Place ladder in corresponding well (see sample plate image below), depending on capillary array used. Use Lonza RNA Marker for Extended Methods.
6. Select Row/Group/Tray to run. Enter sample ID and Tray ID, if desired.
7. Add to queue, from the dropdown select the corresponding method;
 - DNF-471-33
 - DNF-471E33 (Extended IVT mRNA method)

Enter Tray Name, Folder Prefix and Notes, if desired.
8. Add method to the queue by selecting "OK", press play  to start the separation.



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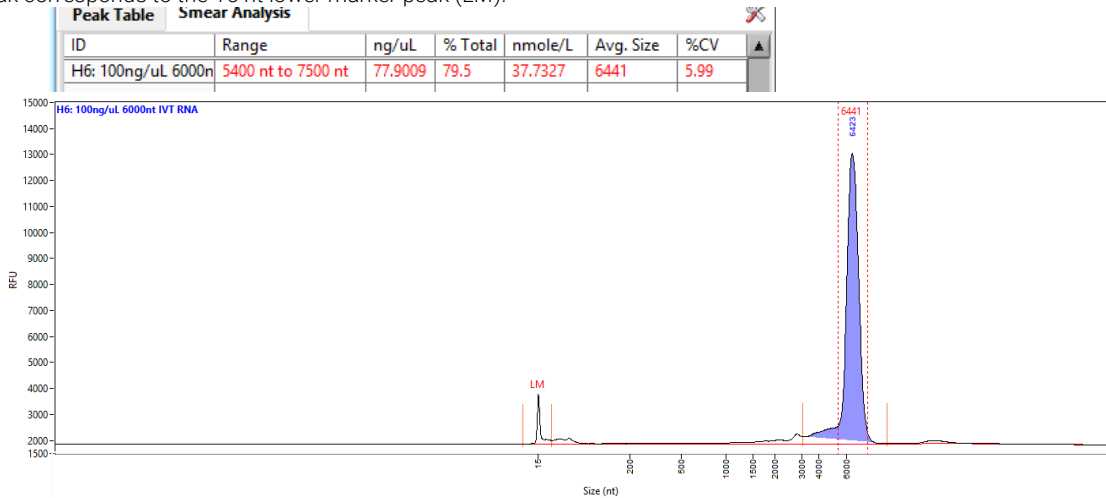
RNA Ladder result



Representative RNA Ladder result using the Fragment Analyzer system with the DNF-471 RNA kit (15 nt). Peaks annotated by size (nt) Method: DNF-471-33. RFU values may differ between instruments.

IVT mRNA sample:

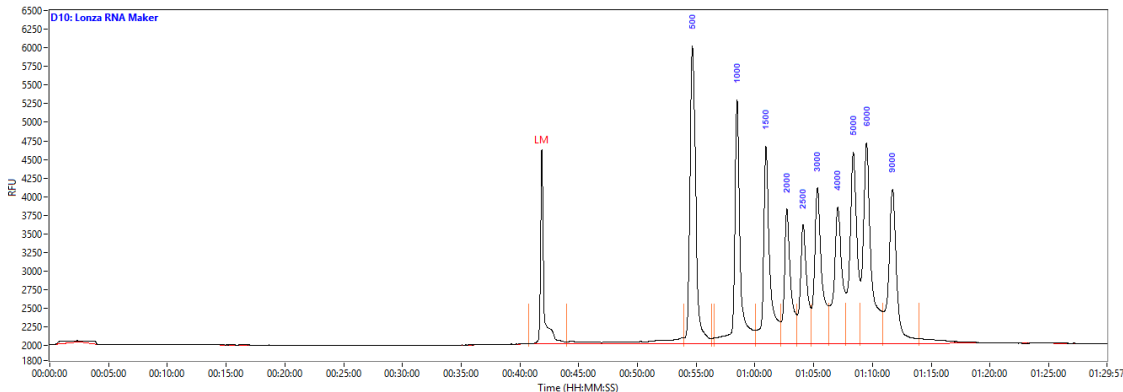
Typical result for a ~6000 nt transcript at 100 ng/μL total input concentration. A Smear Analysis has been performed, as indicated by the dashed lines, between 5,400 and 7,500 nt. This allows the user to determine the percentage and concentration of their peak of interest. The first peak corresponds to the 15 nt lower marker peak (LM).



~6000 nt IVT mRNA sample result using the Fragment Analyzer system with the DNF-471 RNA kit (15 nt). Peaks annotated by size (nt). Method: DNF-471-33

IVT mRNA extended separation method with Lonza RNA Marker

Typical result for the Lonza RNA Marker at the recommended dilution of 96 ng/μL. A total of 11 peaks should be observed with the sizes annotated in the figure below. The first peak corresponds to the 15 nt lower marker peak (LM).

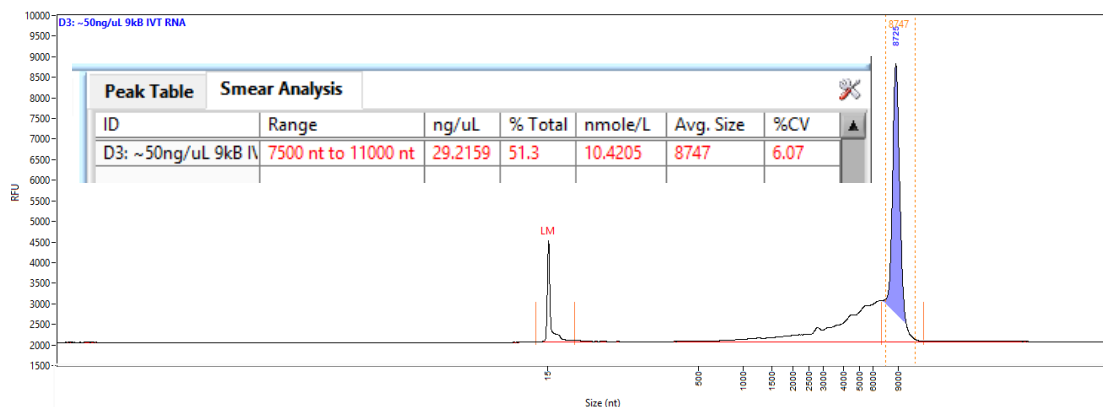


Representative result for the Lonza RNA Marker diluted to 96 ng/μL using the Fragment Analyzer system with the DNF-471 RNA kit (15 nt). Peaks annotated by size (nt). Method: DNF-471E33 – SS Total RNA 15 nt Extended run method

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IVT mRNA sample: extended separation method

The figure below shows a typical result for a ~9,000 nt transcript at 50 ng/μL total concentration, using the Lonza RNA Marker. A Smear Analysis has been performed, as indicated by the dashed lines, between 7,500 and 11,000 nt. This allows the user to determine the percentage and concentration of their peak of interest. The first peak corresponds to the 15 nt lower marker peak (LM).



~9,000 nt IVT mRNA sample result using the Fragment Analyzer system with the DNF-471 RNA kit (15 nt). Peaks annotated by size (nt). Method: DNF-471E33- SS Total RNA 15 nt Extended run method

Troubleshooting

The following table lists several potential kit specific issues which may be encountered when using the DNF-471 RNA kit (15 nt) and suggested remedies. Contact Agilent technical support if you have any additional troubleshooting or maintenance questions.

Issue	Cause	Corrective Action
Sample and/or ladder signal too weak.	1 Sample and/or ladder degraded.	1 Make sure to store ladder at -70°C and keep on ice before use. Use fresh sample and/or ladder.
	2 Diluent marker degraded.	2 Make sure the diluent marker is stored at -20°C and keep on ice before use. Use a new vial of diluent marker.
	3 Sample, ladder and/or diluent marker are contaminated.	3 Clean working area and equipment with RNaseZap. Always wear gloves when preparing sample/ladder. Use new sample, ladder aliquot, and diluent marker.
	4 Sample concentration is too low and out of range.	4 Verify sample was within concentration range specified for the DNF-471 RNA kit (15 nt). Prepare sample at higher concentration; OR Analyze sample using High Sensitivity RNA kit DNF-472.
	5 Sample not added to Diluent Marker solution or not mixed well.	5 Verify sample was correctly added and mixed to sample well.
Split RNA peak.	1 Sample's salt concentration was too high.	1 Take steps to lower the salt content in the sample and repeat experiment.
	2 Sample was not heat denatured.	2 Heat denature sample/ladder plate at 70°C for 2 minutes.

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Peak too broad, signal too low and/or migration time too long.	1 Capillary array needs to be reconditioned.	1 Flush array with 0.5 N NaOH solution and repeat experiment. (See Appendix – Capillary Array Cleaning of the Fragment Analyzer User Manual for details).
	2 Capillary array vent valve is clogged.	2 Clean vent valve with deionized water (See Fragment Analyzer User Manual for details).
Peak too broad, signal too low and/or migration time too long.	1 Capillary array needs to be reconditioned.	1 Flush array with 0.5 N NaOH solution and repeat experiment. (See Appendix – Capillary Array Cleaning of the Fragment Analyzer User Manual for details).
	2 Capillary array vent valve is clogged.	2 Clean vent valve with deionized water (See Fragment Analyzer User Manual for details).
No sample peak or marker peak observed for individual sample.	1 Air trapped at the bottom of the sample plate well, or bubbles present in sample well.	1 Check sample plate wells for trapped air bubbles. Centrifuge plate.
	2 Insufficient sample volume. A minimum of 20 µL is required.	2 Verify proper volume of solution was added to sample well.
	3 Capillary is plugged.	3 Check waste plate for liquid in the capillary well using 96-deepwell plate. If no liquid is observed, follow the steps outlined in the System Manual for unclogging a capillary array.

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Technical Support and Further Information

For technical support please visit www.agilent.com which offers useful information and support regarding the products and technology.

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