Agilent DNF-471 RNA Kit (15 nt) Quick Guide for Fragment Analyzer Systems

The Agilent Fragment Analyzer systems are automated capillary electrophoresis platforms for scalable, flexible, fast, and reliable electrophoresis of nucleic acids.

This Quick Guide is intended for use with the Agilent 5200, 5300, and 5400 Fragment Analyzer systems only. This kit is designed to detect Total RNA within the range of 5 ng/µL to 500 ng/µL input sample concentration and IVT RNA in the range of 1 ng/ µL to 100ng/ µL

Specifications

Analytical specifications[1,2,3]	RNA kit (15nt)
Sizing Range	200 nt – 6,000 nt
Sizing Accuracy[1]	± 5%
Sizing Precision[1]	5% CV
Limit of Detection (S/N > 3)	5 ng/µL
Qualitative Range (per smear)	5 ng/µL – 500 ng/µL
Quantitative Range (per smear)	25 ng/µL – 500 ng/µL
Quantification Accuracy[1]	± 20%
Quantification Precision[1]	10% CV
Physical Specifications[3]	
Total electrophoresis run time	33cm: 40 minutes, 55cm: 70 minutes
Samples per run	12, 48 or 96; depending on the instrument type
Sample volume required	2 µL
Kit stability	4 months

1 Results using RNA Ladder as sample.

Kit Components – 500 Sample Kit

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, 8mL	1
	DNF-355-0125	5x 930 dsDNA Inlet Buffer, 125 mL • Dilute with sub-micron filtered water prior to use	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 Soln, RT • Dilute with sub-micron filtered water prior to use	1

*Not orderable.

WARNING

- 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 goggles, safety gloves and protective clothing.

Kit Components – 1000 Sample Kit

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, 8mL	1
	DNF-355-0300	5x 930 dsDNA Inlet Buffer, 300 mL • Dilute with sub-micron filtered water prior to use	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 Soln, RT • Dilute with sub-micron filtered water prior to use	1

*Not orderable.

WARNING

- 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 goggles, safety gloves and protective clothing.

Additional Material Required for Analysis with the Fragment Analyzer Systems

- Fragment Analyzer systems with LED fluorescence detection:
- 5200 Fragment Analyzer system (p/n M5310AA)
    - FA 12-Capillary Array Ultrashort, 22 cm (p/n A2300-1250-2247) OR
    - FA 12-Capillary Array Short, 33 cm (p/n A2300-1250-3355) OR
    - FA 12-Capillary Array Long, 55 cm (p/n A2300-1250-5580)
- 5300 Fragment Analyzer system (p/n M5311AA)
    - FA 48-Capillary Array Short, 33 cm (p/n A2300-4850-3355) OR
    - FA/ZAG 96-Capillary Array Short, 33 cm (p/n A2300-9650-3355) OR
    - FA/ZAG 96-Capillary Array Long, 55 cm (p/n A2300-9650-5580)
- 5400 Fragment Analyzer system (p/n M5312AA)
    - FA 48-Capillary Array Short, 33 cm (p/n A2300-4850-3355) OR
    - FA/ZAG 96-Capillary Array Short, 33 cm (p/n A2300-9650-3355) OR
    - FA/ZAG 96-Capillary Array Long, 55 cm (p/n A2300-9650-5580):
- Agilent Fragment Analyzer controller software (Version 1.1.0.11 or higher)
- Agilent ProSize data analysis software (Version 2.0.0.61 or higher)

Additional equipment/reagents required (not supplied)

- 96-well PCR sample plates. Please refer to Appendix – Fragment Analyzer Compatible Plates and Tubes in the Fragment Analyzer System User Manual for a complete approved sample plate list
- Multichannel pipettor(s) and/or liquid handling device capable of dispensing 1 – 100 µL volumes (sample plates) and 1,000 µL volumes (inlet buffer plate)
- Pipette tips
- 96-well plate centrifuge (for spinning down bubbles from sample plates)
- Sub-micron filtered DI water system (for diluting the 5x 930 dsDNA Inlet Buffer and 5x Capillary Conditioning Solution)
- 96-deepwell 1mL plate: Fisher Scientific #12-566-120 (inlet buffer and/or waste plate)
- Reagent reservoir, 50 mL (VWR #89094-680 or similar) (for use in pipetting inlet buffer plates/sample trays)
- Conical centrifuge tubes for prepared separation gel/dye mixture and/or 1x Capillary Conditioning Solution
    - 50 mL (for 5200 Fragment Analyzer system or 50 mL volumes): BD Falcon #352070, available from Fisher Scientific #14-432-22 or VWR #21008-940
- 250 mL (for 5300 and 5400 Fragment Analyzer systems or larger volumes): Corning #430776, available from Fisher Scientific #05-538-53 or VWR #21008-771
- Vortexer (for mixing of samples, ladders, and/or markers in tubes and/or plates)
- Capillary Storage Solution (p/n GP-440-0100)

Essential Measurement Practices

Environmental conditions	• Ambient operating temperature: 19 – 25 °C (66 – 77 °F) • Keep reagents during sample preparation at room temperature
Steps before sample preparation	• Allow reagents to equilibrate at room temperature for 30 min prior to use
Pipetting practice	• Pipette reagents carefully against the side of the 96-well sample plate or sample tube • Ensure that no sample or Diluent Marker remains within or on the outside of the tip

RNA Ladder Preparation

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 at -70°C or below.

1. Thaw RNA Ladder aliquot in PCR tube on ice.

2. Mix by pipetting the solution up and down with a pipette tip and spin down. Transfer the ladder to a RNase-free PCR tube. Heat-denature the ladder at 70°C for 2 min, immediately cool to 4°C and keep on ice.

RNA Sample Preparation

1. Heat-denature all total RNA samples at 70°C for 2 min if needed and immediately cool to 4°C and keep on ice before use.

2. The total RNA input sample must be within a total concentration range of
5 ng/µL to 500 ng/µL or 1 ng/µL to 100 ng/µL for IVT mRNA for optimal assay results. If the concentration of the sample is above this range, dilute with RNase-free water.

Sample Plate Preparation

1. Using a fresh RNase-free 96-well sample plate, pipette 22 µL of the RNA Diluent Marker (15 nt) (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 µL/well of BF-25 Blank Solution.

2. Pipette 2 µL of each denatured RNA sample into the respective wells of the sample; mix the contents of the well using the pipette by aspiration/expulsion in the pipette tip.

3. RNA Ladder: The RNA Ladder must be run in parallel with the samples for each experiment to ensure the accurate quantification. Pipette 2 µL of denatured RNA Ladder into the 22 µL of Diluent Marker (15 nt) (DM) Solution in Well 12 of each row to be analyzed (12-capillary system) or Well H12 (96-capillary system). Mix the contents of the well using the pipette by aspiration/expulsion in the pipette tip.

4. After mixing sample/RNA Ladder and Diluent Marker (15 nt) Solution in each well, centrifuge the plate to remove any air bubbles. Check the wells of the sample plate to ensure there are no air bubbles trapped in the bottom of the wells. The presence of trapped air bubbles can lead to injection failures.

5. For best results, run the plate as soon as possible. If the sample plate will not be used immediately, cover the sample plate with RNase-free cover film, store at 4°C and use within 24 hours. Remove the cover film before placing the plate into the instrument.

6. To run the samples, place the plate in one of the three sample plate trays (Drawers 4-6 from the top) of the Fragment Analyzer instrument. Load or create the experimental method.

Important Sample Mixing Information:

When mixing sample with diluent Marker solution, it is important to mix the contents of the well thoroughly to achieve the most accurate quantification. It is highly suggested to perform one of the following methods to ensure complete mixing:

- When adding 2 µL of sample or ladder to the 22 µL of diluent marker, swirl the pipette tip while pipetting up/down to further mix.
- After adding 2 µL of sample or ladder to the 22 µL of diluent marker, place a plate seal on the sample plate and vortex the sample plate at 3000 rpm for 2 min. Any suitable benchtop plate vortexer can be used. Ensure that there is no well-to-well transfer of samples when vortexing. The plate should be spun via a centrifuge after vortexing to ensure there are no trapped air bubbles in the wells.
- After adding 2 µL of sample or ladder to the 22 µL of diluent marker, use a separate pipette tip set to a larger 20 µL volume, and pipette each well up/down to further mix.
- Use an electronic pipettor capable of mixing a 10 µL volume in the tip after dispensing the 2 µL sample volume. Some models enable using the pipette tip for both adding and mixing.

In Vitro Transcribed (IVT) messenger RNA (mRNA)

This section describes how to best run IVT mRNA using the RNA kit (p/n DNF-471 0500 or -1000). If you would like more information on IVT mRNA, please see the application notes "Benefits of Quality Control in the IVT RNA Workflow Using the Agilent 5200 Fragment Analyzer System" (5994-0512EN) and "Assessment of Long IVT RNA Fragments with the Agilent 5200 Fragment Analyzer system" (5994-0878EN).

Type	Specifications
Sample Volume Required	2 µL
Number of Samples per Run	12-capillary: 11 (+ 1 well RNA Ladder) 48-capillary: 47 (+ 1 well RNA Ladder) 96-capillary: 95 (+ 1 well RNA Ladder)
Total Electrophoresis Run Time	45 min (short capillary arrays, 33 cm) 90 min (short capillary arrays, 33 cm for fragments >6000 nt)[1]

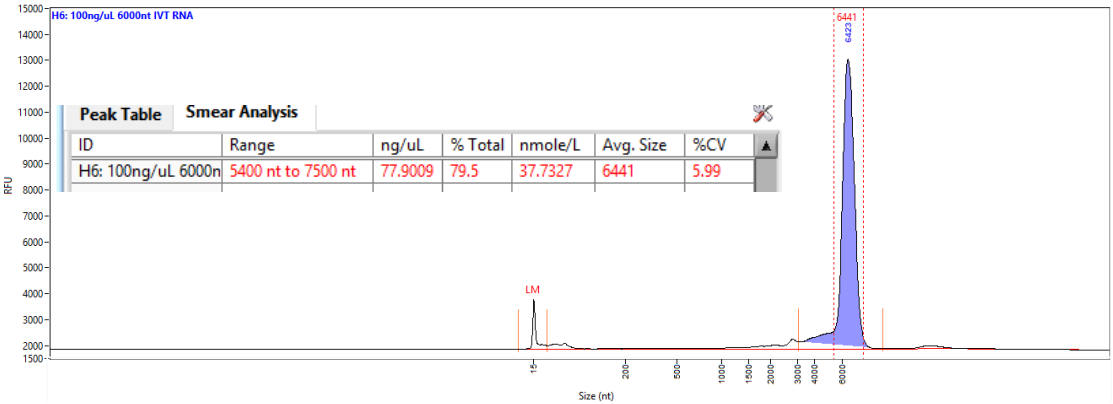
Type	Specifications
RNA Sizing Range	200 – 6,000 nt **OR** 200 – 9,000 nt (with Lonza RNA Marker instead of Agilent's RNA Ladder)[1]
Sizing Accuracy[2]	± 10%
Sizing Precision[2]	5% CV
Limit of Detection (S/N > 3)[2]	1 ng/µL
Qualitative Range	1 ng/µL - 100 ng/µL (IVT mRNA)
Quantitative Range	20 ng/µL - 100 ng/µL (IVT mRNA)
Quantification Accuracy[2]	± 20%
Quantification Precision[2]	10% CV

[1] For use with short capillary arrays, 33 cm, only. [2] Results using 900, 6,000, and 9,000 nt IVT mRNA samples.

IVT mRNA sample: standard separation method

When running IVT mRNA <6,000 nt in size, it's recommended to extend the separation time by 5 minutes in order to ensure the entire sample is separated out. This can be done by clicking **Edit Method**, then increasing the separation **Time** to 45 minutes.

The figure below shows the typical result for a ~6000 nt transcript at 100 ng/µL total. 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).

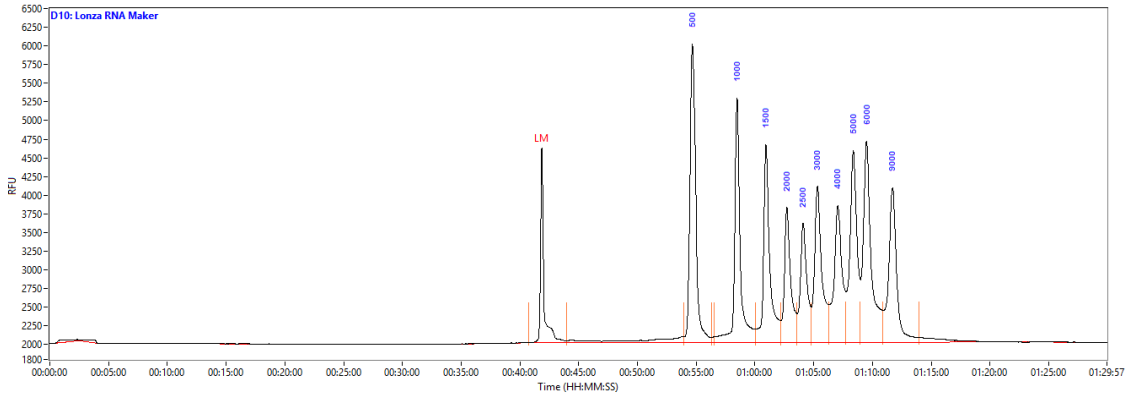


~6000 nt IVT mRNA sample result using the Fragment Analyzer systems with the DNF-471 RNA kit (15 nt). Peaks annotated by size (nt). Method: DNF-471-33.

IVT mRNA extended separation method with Lonza RNA Marker

When running IVT mRNA >6,000 nt in size, it's recommended to use the **DNF-471E33 – SS Total RNA 15nt Extended** run method in order to achieve best resolution for these larger fragments. This method was developed in conjunction with **Lonza RNA Marker** (#50575) diluted in nuclease-free water to a working concentration of 96 ng/µL. Note that this method is only for short 33-cm arrays. This method and its corresponding configuration file can be found on Agilent's website and must be downloaded and placed in the appropriate folders.

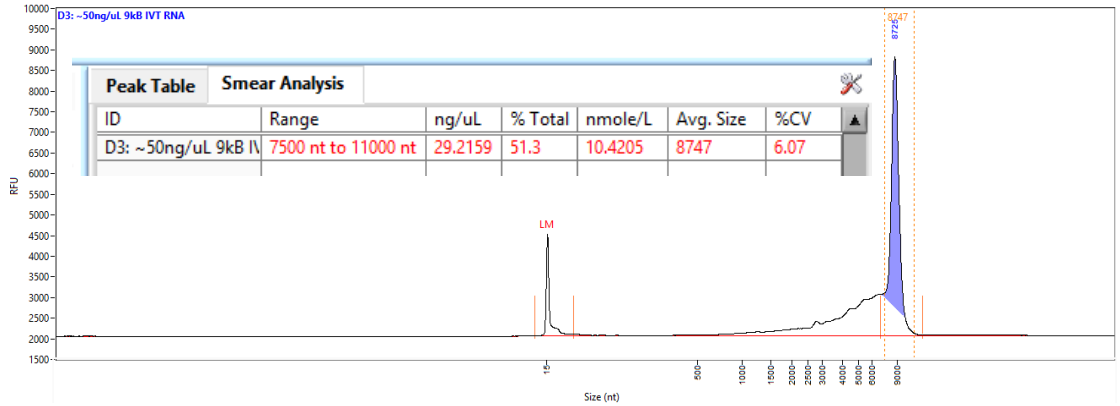
The figure below shows the typical result for the Lonza RNA Marker at the recommended dilution of 96 ng/µL. A total of 11 peaks should be observed with the sizes annotated as in Figure 13. The first peak corresponds to the 15 nt lower marker peak (LM).



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

IVT mRNA sample: extended separation method

The below figure shows the typical sizing and quantitation 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 systems with the DNF-471 RNA kit (15 nt). Peaks annotated by size (nt). Method: DNF-471E33.

Gel preparation

Prepare gel/dye mixture for 5200, 5300, and 5400 Fragment Analyzer Systems. To ensure the gel/dye mixture is mixed homogeneously without generating bubbles, gently invert the centrifuge tube 5 to 10 times, depending on the volume of the mixture.

5200 Fragment Analyzer system volume specifications

# of Samples to be Analyzed[1]	Volume of Intercalating Dye	Volume of Separation Gel[2]	Volume of 1x Conditioning Solution[2]
12	1.0 µL	10 mL	10 mL
24	1.5 µL	15 mL	15 mL
36	2.0 µL	20 mL	20 mL
48	2.5 µL	25 mL	25 mL
96	4.5 µL	45 mL	45 mL

[1] One sample well per separation is dedicated to the ladder.
[2] A 5 mL minimum volume in the tube is included.

5300 Fragment Analyzer system volume specifications with 48-capillary array

# of Samples to be Analyzed[1]	Volume of Intercalating Dye	Volume of Separation Gel[2]	Volume of 1x Conditioning Solution[2]
48	2.5 µL	25 mL	25 mL
96	4.0 µL	40 mL	40 mL
144	5.5 µL	55 mL	55 mL
192	7.0 µL	70 mL	70 mL
240	8.5 µL	85 mL	85 mL
288	10.0 µL	100 mL	100 mL

[1] One sample well per separation is dedicated to the ladder.
[2] A 5 mL minimum volume in the tube is included.

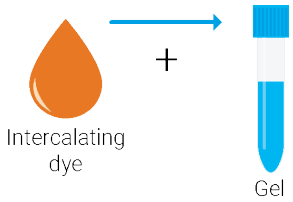
5300 and 5400 Fragment Analyzer systems volume specifications with 96-capillary arrays

# of Samples to be Analyzed[1]	Volume of Intercalating Dye	Volume of Separation Gel[2]	Volume of 1x Conditioning Solution[2]
96	4.0 µL	40 mL	40 mL
192	8.0 µL	80 mL	80 mL
288	12.0 µL	120 mL	120 mL
384	16.0 µL	160 mL	160 mL
480	20.0 µL	200 mL	200 mL

[1] One sample well per separation is dedicated to the ladder.
[2] A 5 mL minimum volume in the tube is included.

Agilent RNA (15nt) DNF-471 assay operating procedure

1. Mix fresh gel and dye according to the volumes in the Gel preparation tables. Refill 1x Capillary Conditioning Solution as needed.



2. Place a fresh 1x 930 dsDNA Inlet Buffer in drawer 'B' on the system, 1.0 mL/well. Replace daily.

    2.1. 5200 system; Fill row A of buffer plate

    2.2. 5300 system - 48 capillary; Fill rows A-D of buffer plate

    2.3. 5300/5400 system - 96 capillary; Fill all rows of buffer plate

3. Prepare Capillary Storage Solution plate. Replace every 2-4 weeks for optimal results.

    3.1. 5200 system; Fill row H of buffer plate with 1.0mL/well, place in drawer "B "

    3.2. 5300 system - 48 capillary; Fill rows A-D of a sample plate with 100 µL/well, place in drawer '3'

    3.3. 5300/5400 system - 96 capillary; Fill all rows of a sample plate with 100 µL/well, place in drawer '3'

        3.3.1. 5400 system; place in drawer "S"

4. Place 0.25x TE Rinse Buffer plate in drawer 'M' on the system, 200 µL/well. Replace daily.

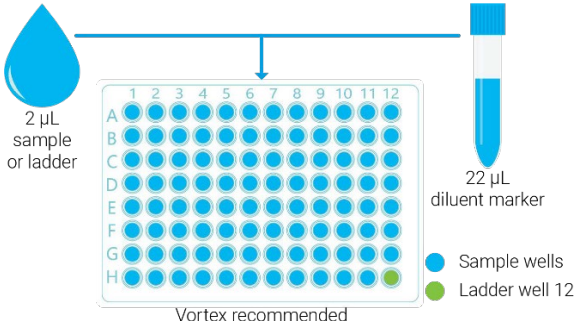
    4.1. 5200 system; Fill row A of sample plate

    4.2. 5300 system - 48 capillary; Fill rows A-D of sample plate

    4.3. 5300/5400 system - 96 capillary; Fill all rows of sample plate

5. Heat denature samples and RNA Ladder at 70°C for 2 minutes, immediately cool to 4°C and keep on ice before use.

6. Mix samples or Ladder with Diluent Marker in sample plate, add 24 µL of BF-25 Blank Solution to unused wells. Place ladder in corresponding well dependent on the capillary size.



5200 system; Ladder – well 12, depending on which row is chosen

5300 system - 48 capillary; Ladder – well D12 or H12, depending on which group is chosen

5300/5400 system - 96 capillary; Ladder – well H12

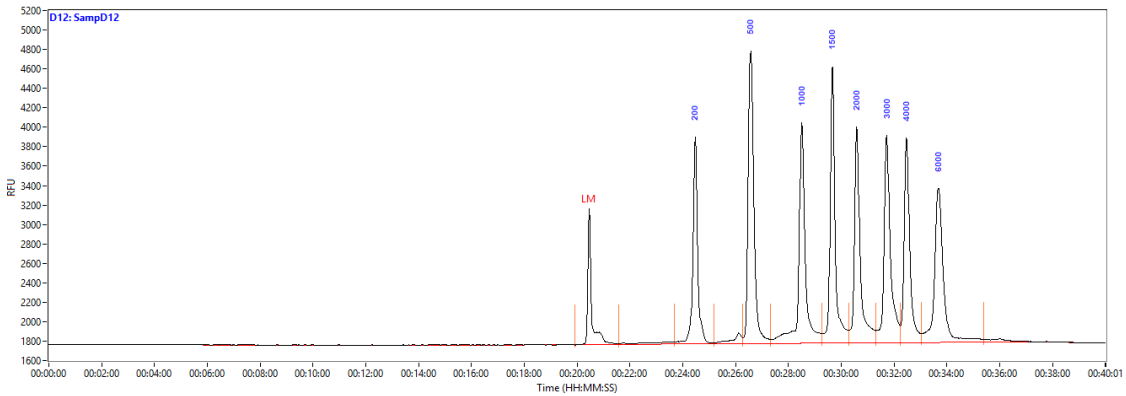
WARNING Working with Chemicals
The handling of reagents and chemicals might hold health risks.

- Refer to product material safety datasheets for further chemical and biological safety information.
- Follow the appropriate safety procedures such as wearing goggles, safety gloves and protective clothing.

Agilent Fragment Analyzer software operating procedure

1. Select Row, Group or Tray to run.
2. Enter **sample ID** and **Tray ID** (optional).
3. Select **Add to Queue**, from the dropdown menus select the corresponding method based on your capillary length;
   3.1 DNF-471-33 – SS Total RNA 15nt
   3.2 DNF-471-55 – SS Total RNA 15nt
4. Enter **Tray Name**, **Folder Prefix**, and **Notes** (optional).
5. Select **OK** to add method to the queue.
6. Select to start the separation.

RNA Ladder result



Representative RNA Ladder result using the Fragment Analyzer system with the DNF-471 RNA kit (15 nt).

Troubleshooting

The following table lists several potential assay specific issues which may be encountered when using the HS NGS Fragment kit (1-6000 bp) (Part #DNF-474) 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 the ladder at -80°C and keep on ice before use. Use fresh sample and/or ladder.
	2 Diluent marker degraded.	2 Make sure to store the diluent marker at -20°C and keep on ice before use. Use a new vial of the 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 RNA kit (15 nt). Prepare sample at higher concentration; OR Repeat experiment using increased injection time and/or injection voltage; OR Analyze sample using High Sensitivity RNA kit (15 nt), (Part # 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.
Missing 25S or 28S ribosomal peak.	1 Sample concentration too high and out of range.	1 Verify sample was within concentration range specified for the RNA kit (15 nt).
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.
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 1 Air trapped at the bottom of sample plate well, or bubbles present in sample well. 2 2 Insufficient sample volume. A minimum of 20 µL is required. 3 Capillary is plugged.	1 1 Check sample plate wells for trapped air bubbles. Centrifuge plate. 2 2 Verify proper volume of solution was added to sample well. 3 Check waste plate for liquid in the capillary well. If no liquid is observed, follow the steps outlined in Appendix – Capillary Array Cleaning of the Fragment Analyzer System User Manual for unclogging a capillary array.
Sizing of IVT mRNA is outside the kit specifications.	1 A modification was made to the IVT mRNA such as additions of chemical groups or nucleotides	1 Sizing accuracy cannot be guaranteed when IVT mRNA has been modified as this can alter migration of the sample within the capillary. Results noted in this manual and for kit specs were form unmodified IVT mRNA.

Technical Support and Further Information

For technical support, please visit www.agilent.com. It offers useful information, support and current developments about the products and technology.

www.agilent.com

Edition 10/20

SD-AT000131