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MicroRNA Molecular Profiling Reveals Increase Expression of hsa-miR-186-5p, hsa-miR-320a-3p, and hsa-miR-486-5p With Storage Time in Packed Blood Cells

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Abstract:

BACKGROUND: Compared to other blood cells, adult red blood cells have a higher concentration of microRNAs (miRNAs). The effectiveness of preserved blood cells following transfusion is affected by a variety of factors, like changes in miRNA levels. One day, these small RNAs might help determine the efficacy and safety of blood products.

AIMS: This study sought to identify the miRNA present in both fresh blood and stored blood because there is growing evidence that these cells are enriched with miRNAs.

MATERIALS AND METHODS: Blood samples were taken from three healthy donors to detect the expression of miRNAs using illumina platform for RNA sequencing. Total RNA was isolated from stored units at 0, 14, 21, and 28 days, respectively. Bioinformatics analysis has been carried out to analyze the miRNAs.

RESULTS: The majority of miRNA expression was decreased in time-dependent manner, particularly after day 14 of packed blood cells (PBCs) storage such as hsa-miR-20a-5p, hsa-miR-17-5p, and hsa-miR-423-3p. While other miRNAs such as hsa-miR-320a-3p, hsa-miR-186-5p, and hsa-miR-486-5p, showed a significant re-upregulated after day 21.

CONCLUSION: In summary, the PBCs at days 14 and 21 had the lowest levels of miRNAs, which may indicate less of a relationship with storage lesions. However, older PBCs displayed significant levels of miRNAs, which could indicate storage lesions or cause a number of clinical issues for the recipients.

Keywords:

Microrna, packed blood cells, RNA sequencing, storage lesion

Introduction

Transferring blood cells or other blood components from a donor into the circulatory system of another person is known as a blood transfusion. Approximately 16 million and 2.6 million blood components are transfused annually in the US and UK, respectively. In addition, approximately 10% of all hospitalized patients receive transfusions.[1] In Jordan, a total of 24,770 blood units were collected in 2019, according to the Jordan Ministry of Health/Blood Bank.

For patients with anemia or hemoglobin levels under 8 g/dL, or with active or acute hemorrhage and anemia-related symptoms

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including weakness and tachycardia transfusion may be necessary[2] according to the American Association of Blood Bank.[3-5] Blood transfusions are the foundation of modern medicine, and since they were first routinely used in clinical settings, transfusion therapy has made significant strides. Due to advancements in preservation technology, blood bags can now be kept for up to 42 days at 2°C–5°C. Even in ideal storage circumstances, the components in blood bags might alter or deteriorate. The useable lifespan and quality of products created from preserved blood are affected by these modifications, which are referred to as storage lesions and include biochemical changes, gene expression, microRNA (miRNA) levels, platelet activation, and the expression of platelet surface receptors.[6-9]

Small RNAs called miRNAs are about 22 nucleotides long that control the pleotropic of cellular genes which are in charge of a number of essential cellular functions. By attaching to mRNA at its 3′ untranslated RNA region (3-UTR), miRNAs control the expression of these genes by either stopping mRNA translation or suppressing it.[10,11]

To produce highly specialized red blood cells (RBCs), erythroid cells undergo considerable mRNA reduction and shed a number of features found in conventional mammalian cells, such as the nucleus and endoplasmic reticulum.[12] However, RBCs continue to have a diverse population of miRNAs at levels comparable to nucleated cells.[13] Providing that, miRNAs and mRNAs are found in preserved blood cells and that these cells do experience morphological and molecular alterations during storage (storage lesions). It is fair to hypothesize that miRNAs regulate the translation of these cells' mRNAs. These decision-making processes within the stored cell will ultimately decide whether it will survive during storage by fending off the onset of physiological alterations (storage lesions), or whether it will eventually die. If this theory is accurate, then both the expression profiles of miRNA and mRNA in RBCs and platelets should change during storage.

Previous bioinformatics research found that a number of miRNAs, including hsa-miR-16-5p, hsa-miR-320a, hsa-miR-320b, hsa-miR-106a-5p, hsa-miR-15a-5p, and hsa-miR-15b-5p, affected the expression of proteins linked to platelet activation.[14] Another study by Kannan *et al*. focused on 52 platelet miRNAs that were associated with apoptosis.[15] They discovered that different stages of platelet preservation resulted in varied expression patterns for several of these miRNAs. Suggest using these miRNAs as potential biomarkers to assess the activity of stored platelets. Recently, miRNAs have been validated for the detection of autologous blood doping in sports. 28 miRNA signatures were found to be altered by the researcher; about six of them were altered during blood storage and the rest during processing.[16] The majority of reports said that starting on day 21, the biochemical, cytokine, and exosome levels in older blood bags showed a considerable improvement.[17,18]

One factor reducing the effectiveness of preserved blood cells following transfusion is storage lesions. *Helicobacter pylori* induces miRNA155 expression in T cells.[19] The use of plasma miRNA-122 levels as a biomarker of hepatic diseases,[20] and the observed link between miRNA expression levels and clinical parameters associated with chronic hepatitis C viral infection.[21] In humans, there are few examples from other fields of research that suggest that if changes in miRNA profiles are proven to be consistent with the cellular status of stored blood cells, hence, these small RNA molecules could be responsible for the storage lesion.

While these recent studies demonstrated the potential advantages of profiling miRNAs in stored blood cells, these findings only represent a promising first step toward conducting a deep miRNA profiling to find a correlative trend of miRNAs that could act as a replacement for quantifiable quality traits for these cells to predict storage lesion. In addition, to validate the practice effectiveness of the old blood bags compared to the blood bag that has been in use for <21 days.

Materials and Methods

Study design

This is prospective study tracked the changes in miRNA in blood bags over the course of 1 month. The study was approved by the review ethical committee of scientific research and innovation, Al-Balqa Applied University, Jordan.

Donor

Blood was collected from three different healthy donor males using the same strategy as used in the blood bank according to practice. All donors were nonsmoker, had normal blood pressure, were aged <40 years, and normal weight with no diseases. Verbal consent was obtained from all the donors before drawing blood and informed them the blood will be used for research purposes. Then, blood was collected from blood bags at days; 0, 14, 21, and 28 of blood donation. The 450 ml of blood was separated into packed RBC, platelets, and fresh frozen plasma (FFP). Platelets and FFP were excluded. Packed RBCs were stored in blood bank at 4°C according to practice. All bags were tested for HIV, hepatitis C virus, hepatitis B virus, and all were negative.

Total RNA extraction

Using Transozol reagent (Trans, China) and Direct-zol RNA Miniprep (Zymo, USA), total RNA was extracted

from the packed blood cells (PBCs) for each relevant period of storage. In short, the samples were 3-1 homogenized with transozol reagent. The samples were then mixed and incubated for 10 min. An equal volume of 95% ethanol was added to the samples after centrifugation. The mixes were then put into the collection tubes and spun in a centrifuge. Then, 400 µL of prewashed buffer and 700 µL of RNA washing buffer were added. Using a Nabi UV/Vis Nano Spectrophotometer, the total extracted RNA was quantified and its quality was estimated. The RNA concentration was changed to 0.5 g/L.

MicroRNA libraries preparation

To create miRNA libraries, tiny RNA (17–25 bps) was size-selected from total RNA using the flashPAGETM Tiny Nucleic Acid Purification System (Life Technologies, USA). The short RNA was immediately ligated with TrueQuant Adapters, essentially as described by Hafner *et al*.[22] On a Hiseq2000 device, the miRNA population with flanking p5 and p7 adapters was sequenced. GenXPro GmbH, a German company, carried out the miRNA library preparation.

Quantification of microRNA expression

OmiRas was used to analyze small RNA-Sequencing libraries (a comprehensive procedure is shown at http://tools.genxpro.net/omiras).[23] The 3′ adaptor clipping step of data processing for each tiny RNA-Seq library was followed by a local alignment of the adaptor sequence to each read. The designated quality zone by Illumina was then deleted, and the reads were summarized to UniTags.

After singletons were removed from the data set,[19] the remaining tags were mapped to the human genome (hg38) using a bowtie. The mapped tags were annotated using multiple coding and noncoding RNA models that were retrieved using the UCSC table browser. The examination of tags that correspond to exonic regions of coding genes was ended. Each library's noncoding RNA concentrations were measured. The amount of reads corresponding to a tag was divided by the number of mapping loci for tags that map to multiple genomic loci.

Statistical analysis

Statistical analysis was performed using SPSS 18.0 (IBM SPSS, Inc., Chicago, IL, USA) and Sigma Plot 10.0 using the Student's paired test and one-way ANOVA. P * as < 0.05,** for < 0.01 and ***<0.001 were considered statistically significant.

Results

RNA quality

After RNA extraction from blood cells, the quantity and quality of RNA was assessed by Nabi, UV/Vis Nano Spectrophotometer. The ratio between 260/280 was around 2.0. Furthermore, Using the Bioanalyzer 2100 (Agilent Technologies, USA), RNA quality and RIN values were assessed. All samples had integer total RNA and mean RIN values of 18.68 ± 0.84 in all cases [Figure 1]. The Bioanalyzer 2100 analysis program calculated the miRNA content of total RNA to be 18.68% ± 2.54% [Supplementary Figures].

Phred score

A quality value called the Phred score, which assesses sequence correctness, was calculated for each sample. A mean Phred score of >30 denotes a 99.9% likelihood of selecting the correct base.[14] Each sample received an average Phred score of 35. An illustration of a Phred score is shown in [Figure 2a].

Most significant upregulated microRNA during packed blood cell storage time

For patients who require erythrocytes to raise their hemoglobin levels, the majority of blood banks prepare PBCs for transfusion. Numerous RBCS bags contain various types of miRNA, according to a recent study.[20,21,24] These miRNA may have an impact on storage lesions. To do that, we examined the degree of miRNA expression during blood preservation. The most upregulated and downregulated miRNAs among the 520 miRNAs that were evaluated are shown in Figure 2b and c at day 0, 14, 21, and 28 of storage. The blue hue denotes a low expression level of miRNA in transcription per million (TPM), and the red color denotes a high TPM read. According to the Heatmap graphic, the majority of miRNA expression declined between days 14 and 21 of storage and increased in some cases on day 28.

Interestingly, at day 28 of PBC storage, Table 1 lists the miRNAs that were most upregulated: Hsa-miR-486-5p, hsa-miR-92a-3p, hsa-miR-320a-3p, hsa-miR-423-5p, hsa-miR-3184-3p, and hsa-miR-186-5p. Hsa-miR-486-5p = 48520.41 TPM had the greatest expression of miRNA. A list of the downregulated miRNAs with storage time may be seen in Table 2. Hsa-miR-223-3p, Hsa-miR-126-3p, and Hsa-miR-20b-5p had the lowest expression levels for miRNA. The fold change of the miRNA with the highest level of expression is also displayed in [Figure 2d]. Hsa-miR-186-5p and

Table 1: The most upregulated microRNA during storage of packed blood cells

miRNA	Day 0 (TPM)	Day 14 (TPM)	Day 21 (TPM)	Day 28 (TPM)
hsa-miR-486-5p	40,613.54	6200.22	7830.52	48,520.41
hsa-miR-92a-3p	7062.22	538.44	3347.37	8325.58
hsa-miR-320a-3p	2403.77	972.25	2096.78	3611.60
hsa-miR-423-5p	1025.13	358.31	801.61	1100.21
hsa-miR-3184-3p	893.05	351.15	707.72	1014.95
hsa-miR-186-5p	252.82	26.52	220.34	374.22

TPM=Transcription per million, miRNA=microRNA

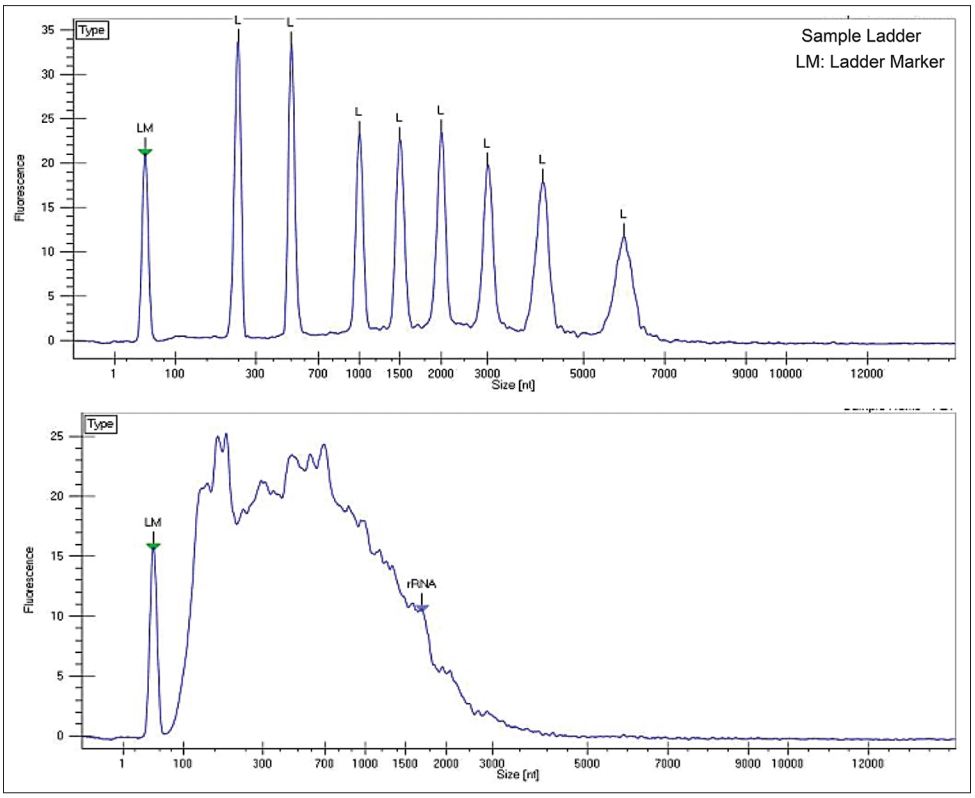

Figure 1: The purity of the RNA was assessed using the Bioanalyzer. An example electropherogram of human blood cells total RNA. This illustration displays a high-quality sample. Nucleotide number; Fluorescence. The top image is the control, and the lower image is a sample

Table 2: The most downregulated microRNA during storage of packed blood cells

miRNA	Day 0 (TPM)	Day 14 (TPM)	Day 21 (TPM)	Day 28 (TPM)
hsa-miR-20a-5p	417.11	5.04	148.93	231.62
hsa-miR-17-5p	373.48	15.84	144.68	234.34
hsa-miR-423-3p	256.15	56.90	86.92	208.68
hsa-miR-22-3p	400.31	71.45	75.05	181.45
hsa-miR-26a-5p	287.13	30.10	110.24	208.18
hsa-miR-484	319.86	48.05	98.21	186.66
hsa-miR-181a-5p	296.19	98.09	88.85	207.01
hsa-miR-30d-5p	214.46	17.15	73.38	174.53
hsa-miR-150-5p	251.53	171.65	96.24	165.88
hsa-miR-425-5p	248.20	53.87	84.40	126.17
hsa-miR-19b-3p	184.87	13.54	85.87	73.25
hsa-miR-21-5p	133.16	8.29	44.78	98.84
hsa-miR-324-5p	139.75	56.29	60.66	121.26
hsa-miR-142-3p	88.51	8.54	42.16	43.97
hsa-miR-30b-5p	119.63	18.52	68.24	54.70
hsa-miR-106a-5p	95.41	10.22	34.92	68.30
hsa-miR-182-5p	137.34	24.92	38.97	67.19
hsa-miR-15a-5p	95.25	4.02	40.56	57.98
hsa-miR-20b-5p	85.23	3.00	30.82	39.46
hsa-miR-363-3p	94.62	12.68	32.78	44.27
hsa-miR-126-3p	60.02	4.58	15.95	25.05
hsa-miR-362-5p	34.00	8.16	10.55	29.20
hsa-miR-374a-5p	36.25	0.67	14.81	25.46
hsa-miR-532-5p	44.56	6.14	10.37	28.42
hsa-miR-223-3p	51.42	11.62	13.29	13.34

TPM=Transcription per million, miRNA=microRNA

Hsa-miR-320a-3p had the greatest fold changes, 1.5 fold, from zero time, of any miRNAs. The expressiveness of the other is also 1.2 times higher.

Discussion

RBCs in preserved PBC units may undergo a variety of changes, including reduced levels of ATP, 2, 3-DPG, NADH, pH, and oxidative stress.[25,26] Furthermore, it has been observed that as preservation progresses, the concentration of released extracellular vesicles increases, which could be unfavorable to receivers (storage lesion).[25] In contrast to their progenitors, the reticulocytes, erythrocytes have long been believed to be genetically deficient. Since a decade ago, there has been mounting proof that erythrocytes contain many miRNAs, even more than other blood fractions.[13,27]

When energy and antioxidant levels decrease, when blood is stored in a basic solution or a solution with low salt concentration, or when blood is stored at low temperatures, EVs are secreted from the stored RBC in the blood unit. Exosomes released from RBCs have been found to contain several forms of miRNA.[28] These miRNA may have an impact on how immune cells perform. A previous study demonstrated that[28] up to 21 days of storage, the expression of abundant types of miRNA was not influenced by storage time; however,

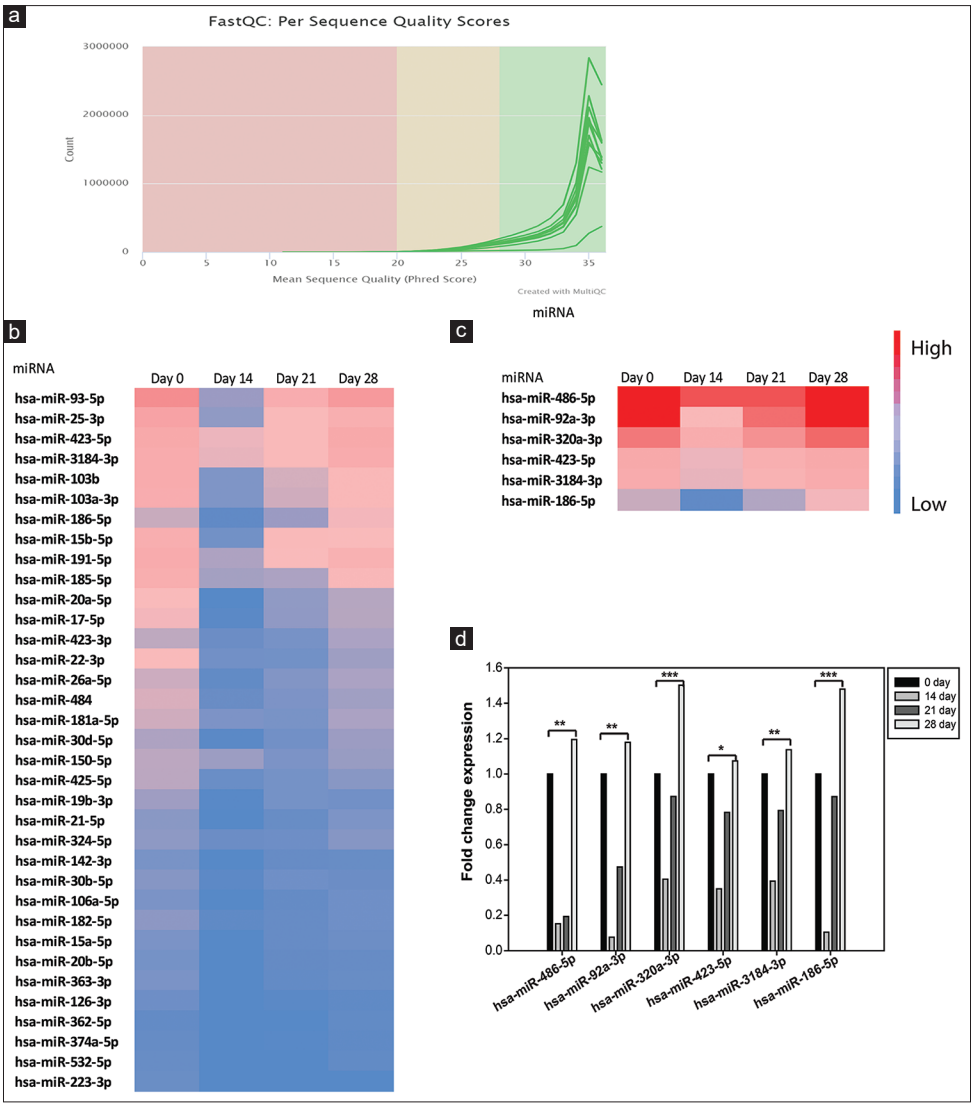

Figure 2: Expression and purity of miRNAs in packed blood cells (PBCs). (a) Phred score for each sample which indicates the sequence accuracy. (b and c) Excel was used to create the heat map. The red bar represents a high expression level, whereas the blue bar shows a reduction in expression. (b) The low expressed miRNA time-dependent manner in PBC. (c) The most upregulated miRNA after day 21 of storage. (d) Expression profile of the most highly effected miRNAs in fold change to day 0 of storage (hsa-miR-320a-3p, hsa-miR-186-5p and hsa-miR-486-5p, hsa-miR-92a. 3p, hsa-miR-423-5p, hsa-miR-3184-3p). miRNA: MicroRNA

after 21 days, the level of expression increased and reached its peak at 35 days for miR-125b-5p; miR4454 started to increase after 14 days; and miR451a did not correlate with storage time. Another study revealed that the expression of miRNAs is effected by the level of oxygen. The miRNA expressions showed to be different in hypoxia conditions compare with low hypoxia environments.[29]

As shown in this study, several miRNAs have been upregulated with storage time, for example, hsa-miR-486-5p, hsa-miR-92a-3p, hsa-miR-320a-3p. The expression of these miRNAs was decreased in the 2nd week; day 14 of storage and start to increase in 3rd week; day 21. This increase in expression was not expected as normal degradation for miRNA over storage time. A number of hypotheses exist to explain this finding; The number of detected miRNAs, however, is likely to have increased which may be due to increase cleavage of the miRNA precursors (pre-miRNAs) of mature miRNAs that control senescence after the 21 days of storage by RNA editing enzymes such RNase and RNA helicases.[30] According to previous research, overexpression of miRNA may also be caused by the breakdown of RNA-carrying exosomes in RBCs.[28] It was suggested that this phenomenon may be influenced by exosome degradation. Another possibility that could account for the increase in the number of miRNAs from 21 to 28 days is the assumption that RNA precursors can be broken up into small RNA fragments that have the ability to regulate protein translation in response to stress. Alternatively, miRNAs are attached to the AGO2 protein that is found within the plasma which protects them from degradation.[31]

In consistence with a previous study[32] which suggests the use of the miRNAs miR127 and miR320a as biomarkers to assess the "validity period" of PBC bags stored in blood banks for more than 5 days. Hsa-miR-320a-3p, hsa-miR-92a-3p, and hsa-miR-423-5p were shown to be increased with storage time also in our study particularly after 21 days. The other upregulated miRNAs have not been reported in association with the storage of PBCs.

The clinical importance of miRNA 486-5p has been reported previously. Several cancers, including myeloid leukemia,[33,34] gastric adenocarcinoma,[35] and lung cancer,[36] have been shown to express miR-486-5p abnormally. In cervical cancer patients, the expression level of miRNA 486-5p has been shown to be increased in serum samples as well as tissue biopsies,[37] Hence, it is known that one therapeutic strategy for cancer patients is PBC transfusion which would in this case make more alternations for this miRNA.

More significantly, studies have demonstrated that patients who have diabetes and coronary artery disease have higher expression levels of hsa-miR-320a-3p.[38] Recent research revealed that nuclear miR-320a caused lipotoxicity in the heart by causing fatty acid metabolic gene transcription to be activated, which in turn caused diabetes to produce cardiac dysfunction.[39]

Conclusion

In this study, we conclude that the expression of miRNA in PBC dramatically changed over their storage time. The majority of miRNAs showed a decrease in expression after day 14 of the storage; however, some of those that have been studied showed a considerable rise in expression after day 21 of storage. These miRNAs are associated with several clinical disorders such as diabetes and cancers. Studying the correlation between the expressions of these miRNAs with the clinical outcomes of these patients is recommended. Finally, the results also suggest that the expression of these miRNAs could be used to evaluate the changes during the storage time of PBC units.

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Conflicts of interest
There are no conflicts of interest.

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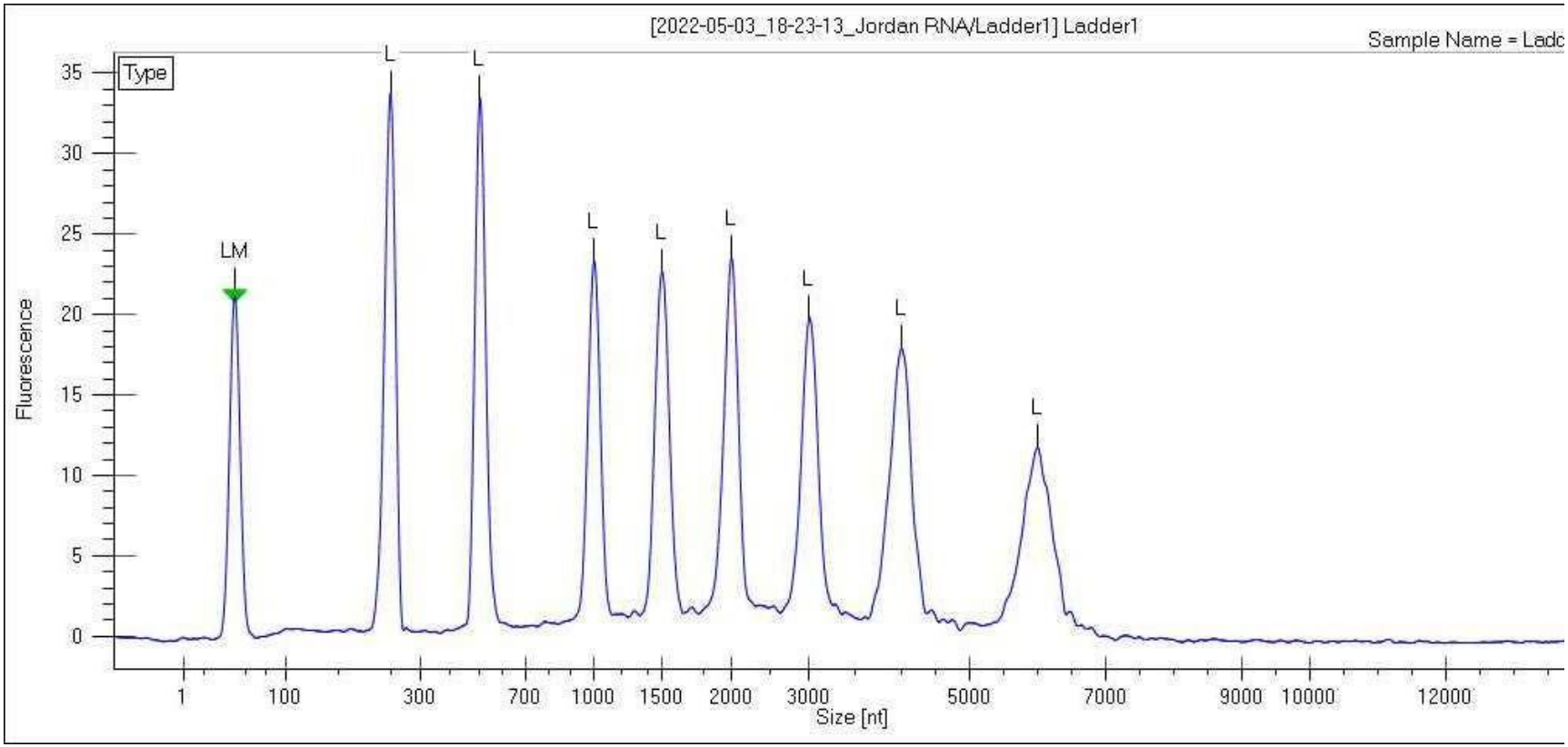
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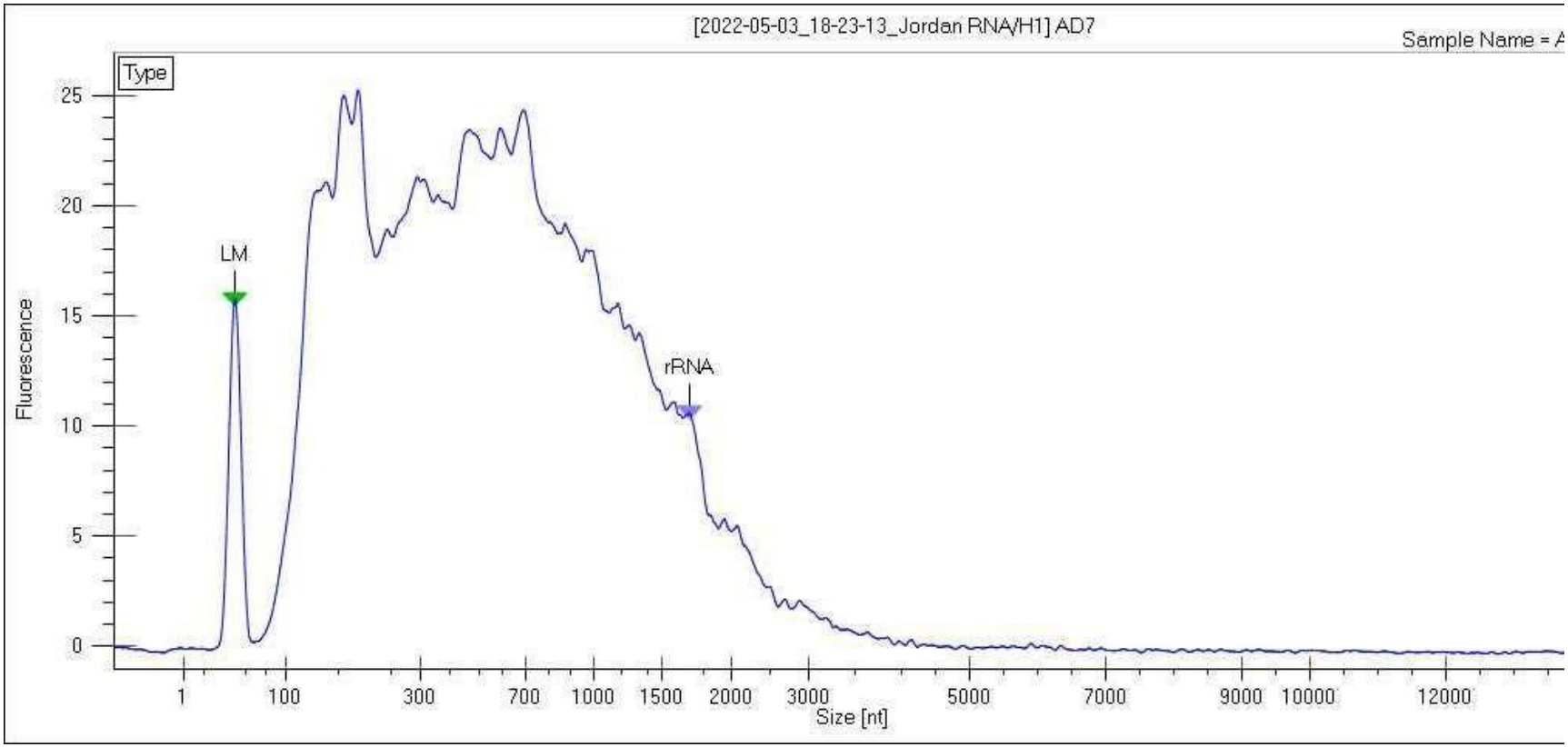
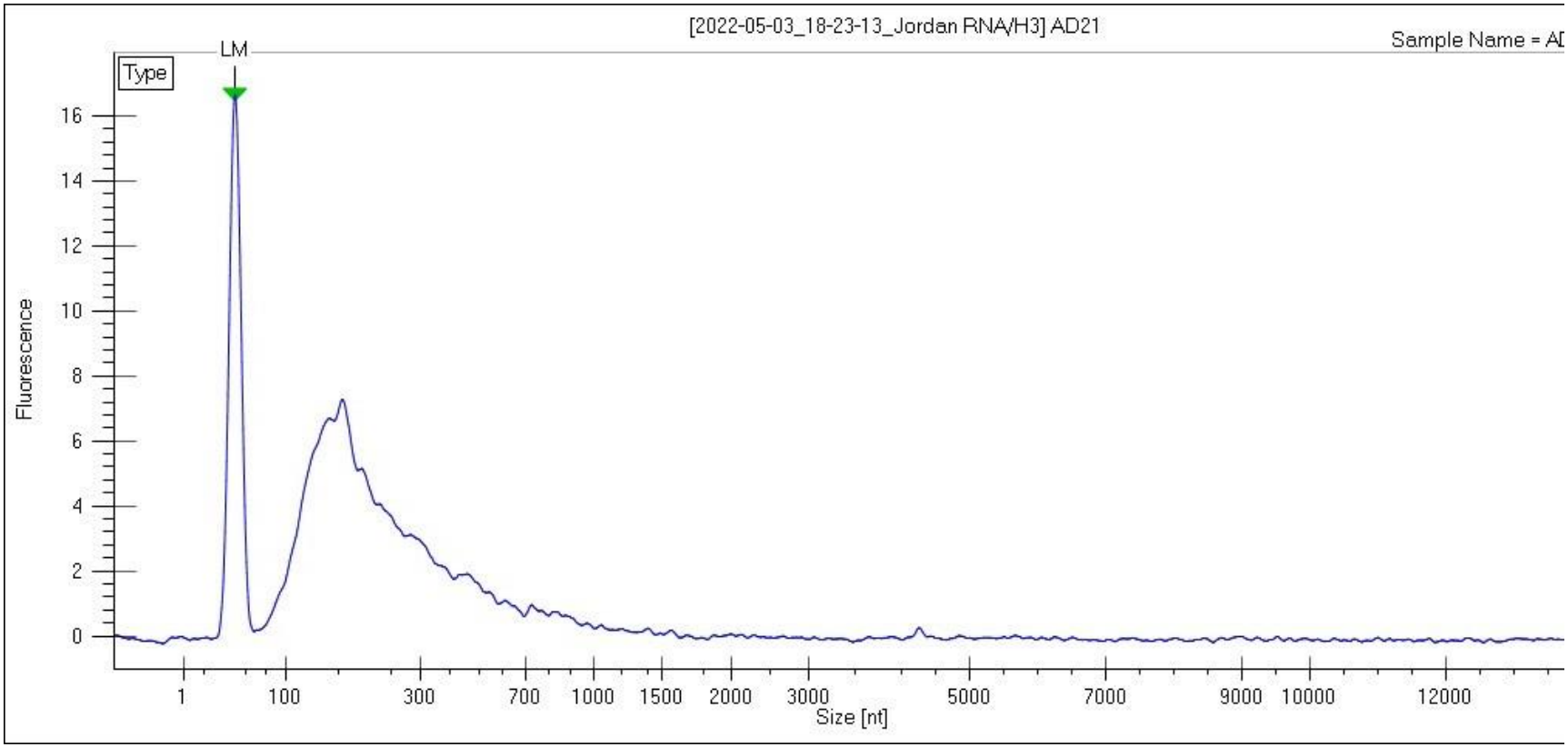


Datafile Created 03.05.2022 18:23:13 with S/W Version: 1.9.1010.0
Analysis Version: 0
Chip Y646B-0564N-05 (Expiry: 20.01.2022)
2022-05-03_18-23-13_Jordanien RNA.gxd

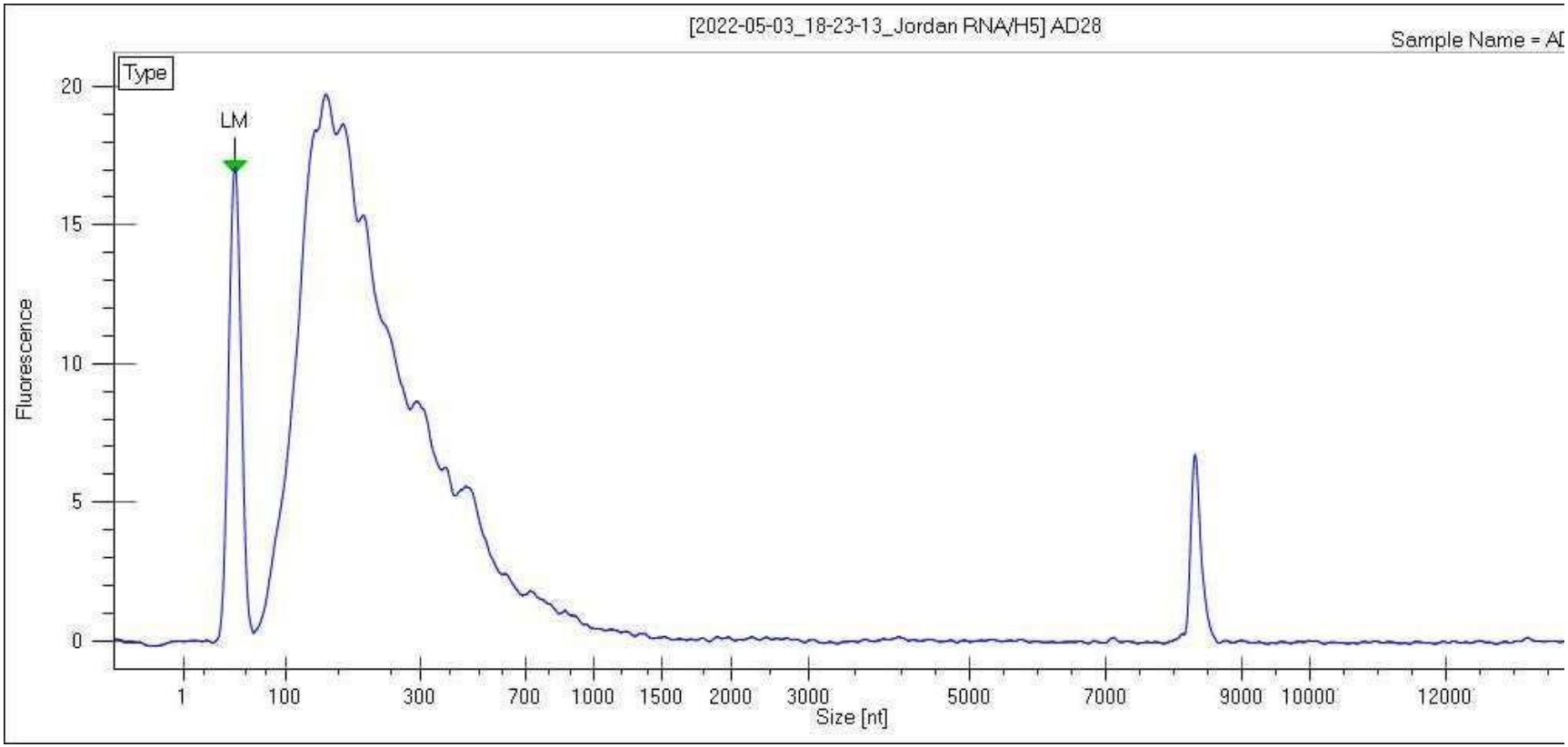
Data collected: 03.05.2022 18:23:13
Printed: 03.05.2022 19
Firmware Version: 1.02.0.3727
Instrument: LABCHIP (S/N GT1611N
Operat



LabChip GX Software Version 5.2.2009.0

Datafile Created 03.05.2022 18:23:13 with S/W Version: 1.9.1010.0
Analysis Version: 0
Chip Y646B-0564N-05 (Expiry: 20.01.2022)
2022-05-03_18-23-13_Jordanien RNA.gxd

Data collected: 03.05.2022 18:23:13
Printed: 03.05.2022 19
Firmware Version: 1.02.0.3727
Instrument: LABCHIP (S/N GT1611N
Operat



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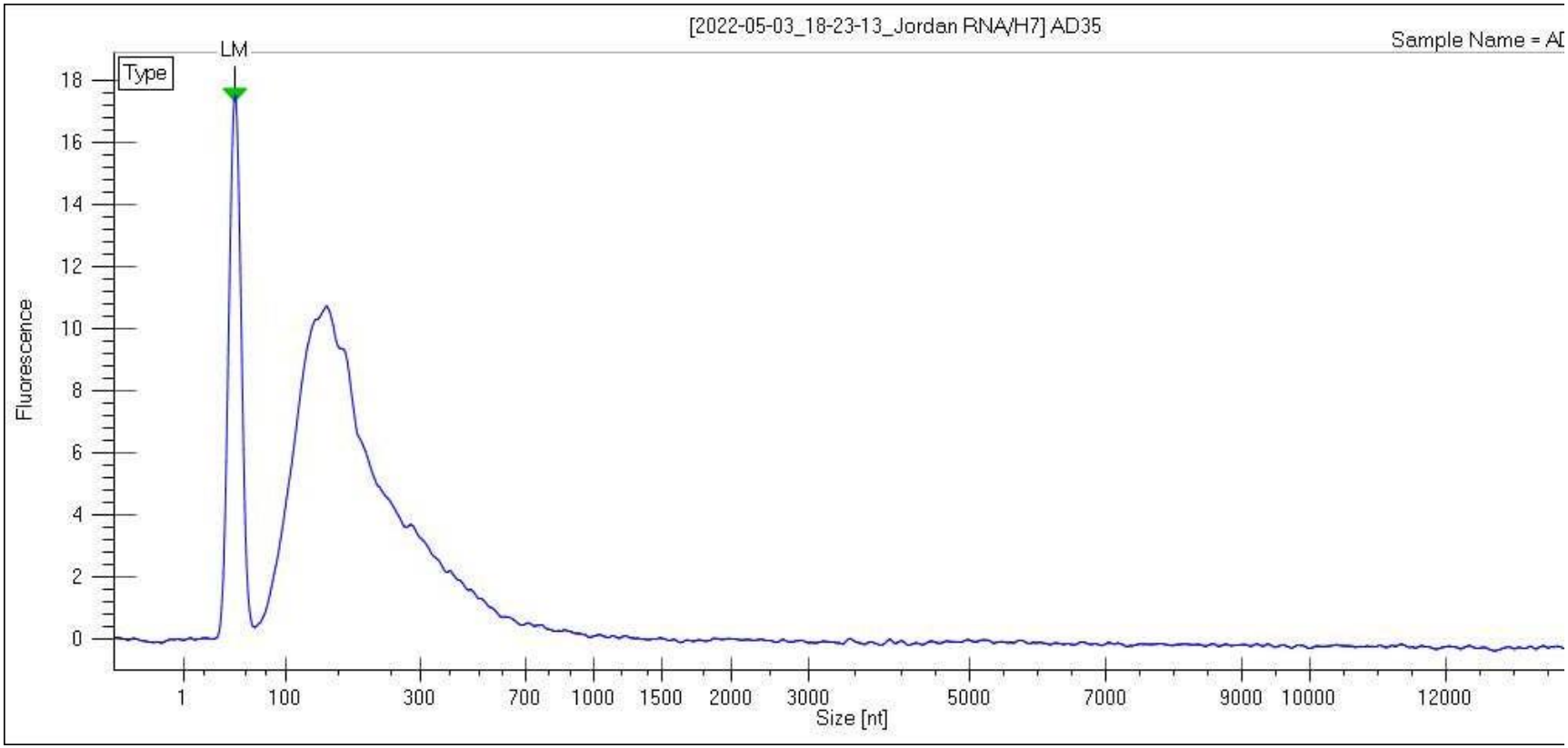
Chip Y646B-0564N-05 (Expiry: 20.01.2022)
2022-05-03_18-23-13_Jordanien RNA.gxd

Data collected: 03.05.2022 18:23:13
Printed: 03.05.2022 19
Firmware Version: 1.02.0.3727
Instrument: LABCHIP (S/N GT1611N
Operat



LabChip GX Software Version 5.2.2009.0

Datafile Created 03.05.2022 18:23:13 with S/W Version: 1.9.1010.0
Analysis Version: 0
Chip Y646B-0564N-05 (Expiry: 20.01.2022)
2022-05-03_18-23-13_Jordanien RNA.gxd

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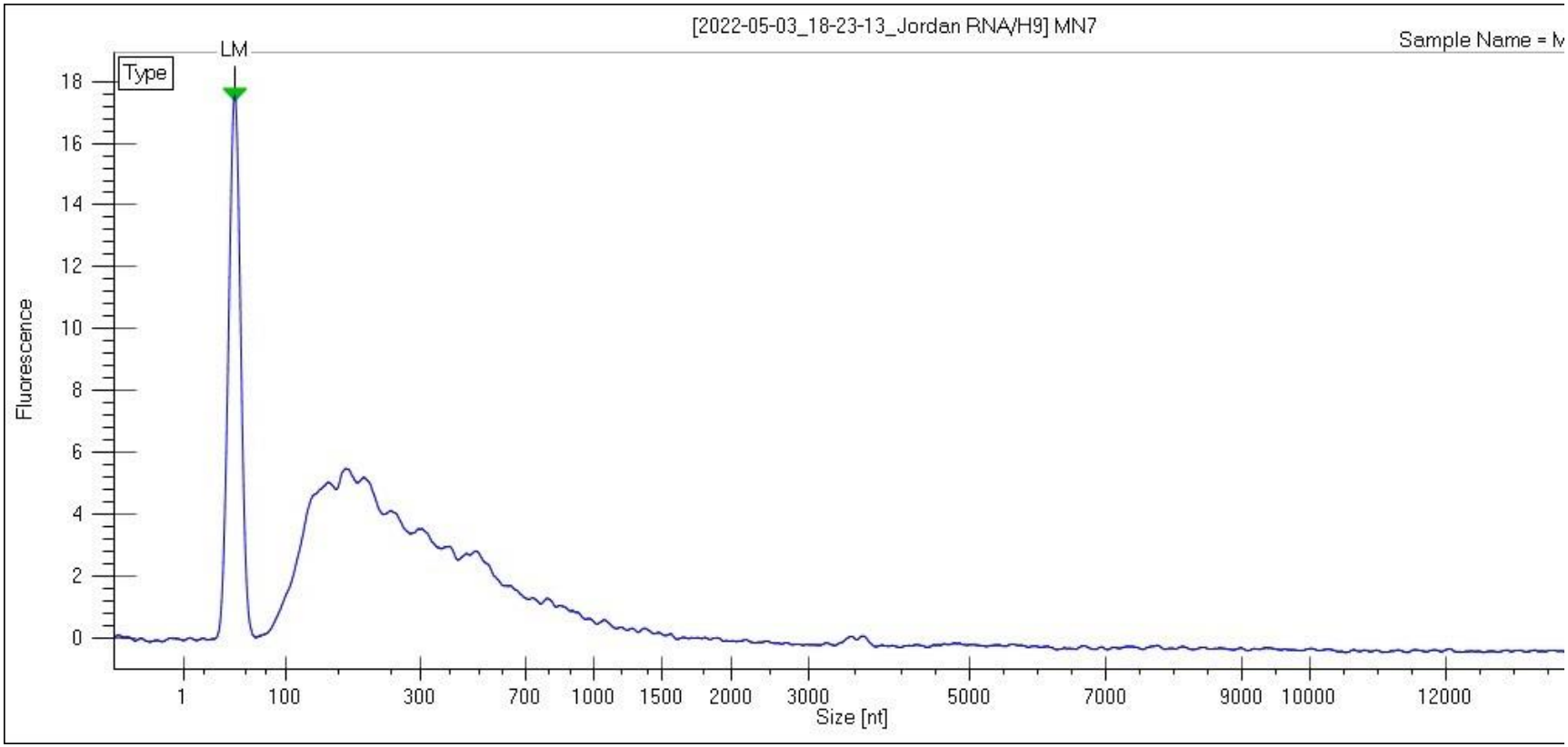
Instrument: LABCHIP (S/N GT1611N
Operat



LabChip GX Software Version 5.2.2009.0

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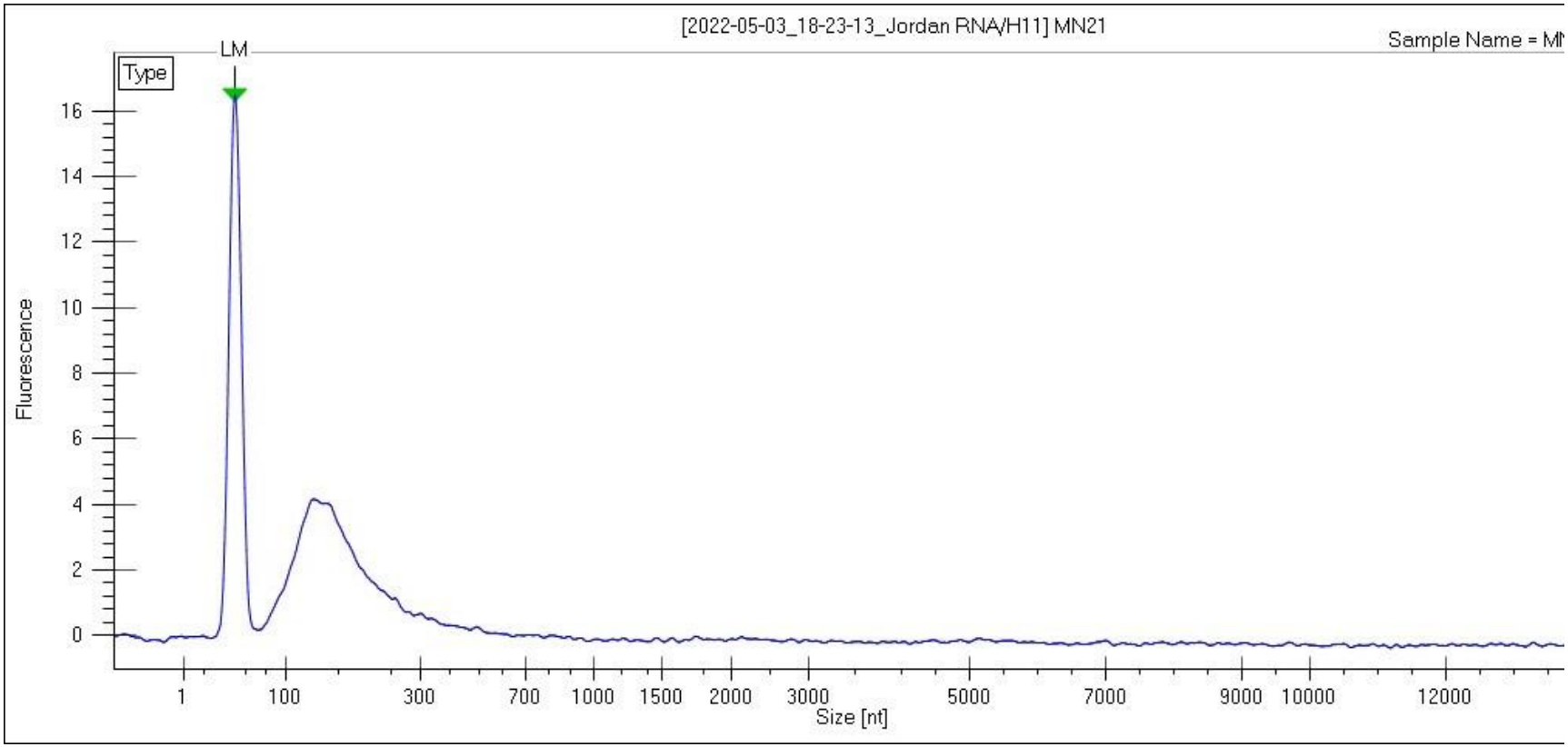
Data collected: 03.05.2022 18:23:13
Printed: 03.05.2022 19
Firmware Version: 1.02.0.3727
Instrument: LABCHIP (S/N GT1611M
Operat



LabChip GX Software Version 5.2.2009.0

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Chip Y646B-0564N-05 (Expiry: 20.01.2022)
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Data collected: 03.05.2022 18:23:13
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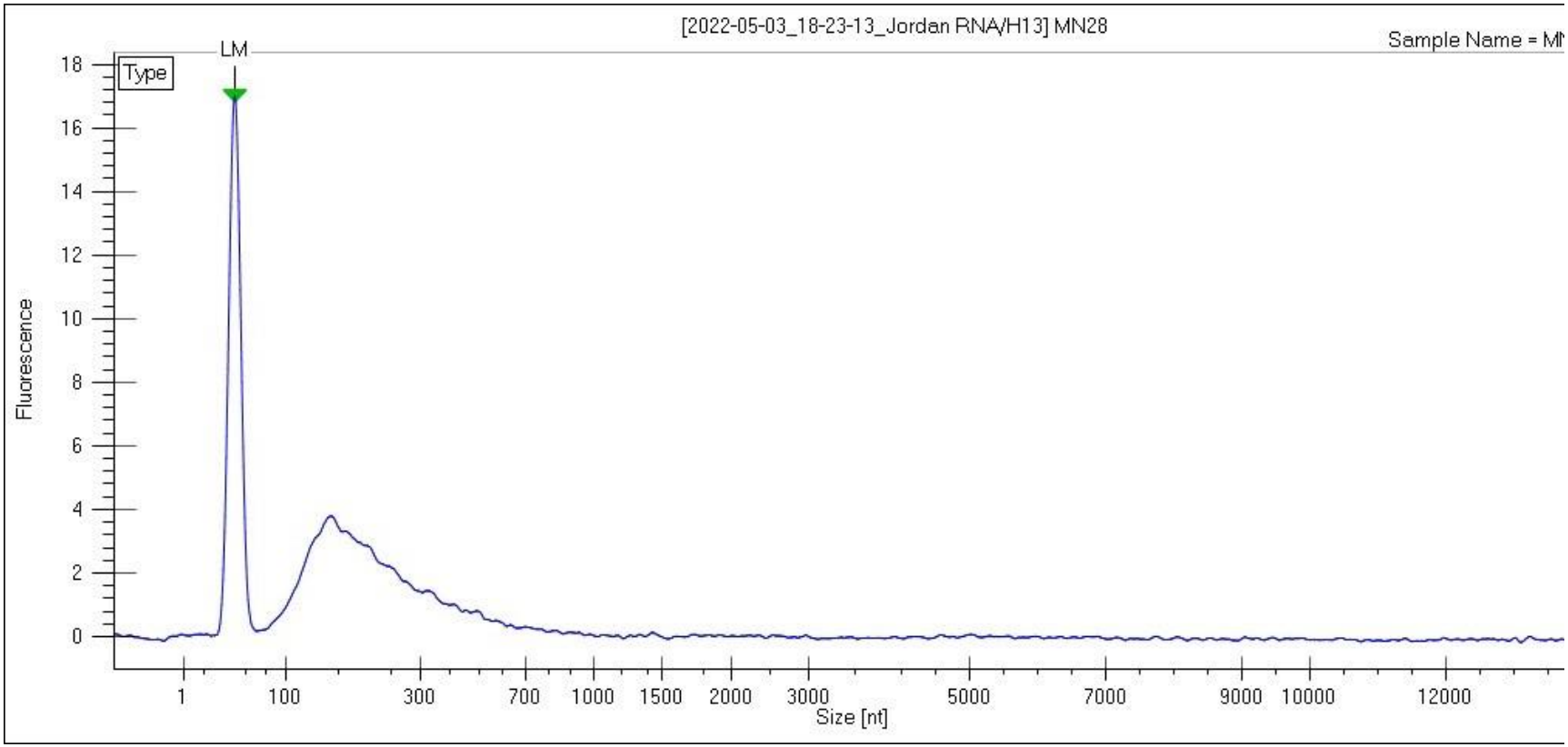
Firmware Version: 1.02.0.3727
Instrument: LABCHIP (S/N GT1611N
Operat



LabChip GX Software Version 5.2.2009.0

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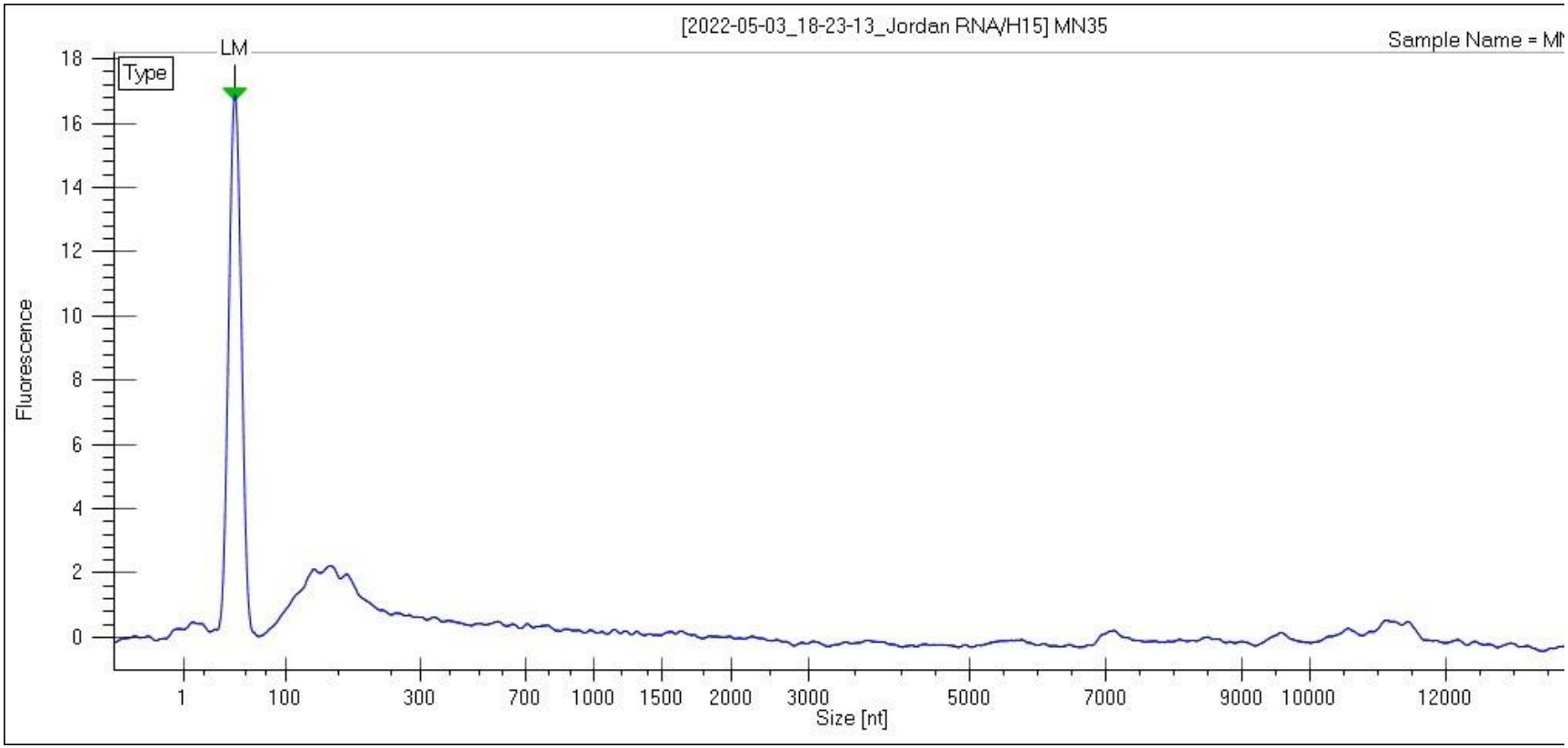
Analysis Version: 0
Chip Y646B-0564N-05 (Expiry: 20.01.2022)
2022-05-03_18-23-13_Jordanien RNA.gxd

Data collected: 03.05.2022 18:23:13
Printed: 03.05.2022 19
Firmware Version: 1.02.0.3727
Instrument: LABCHIP (S/N GT1611M
Operat



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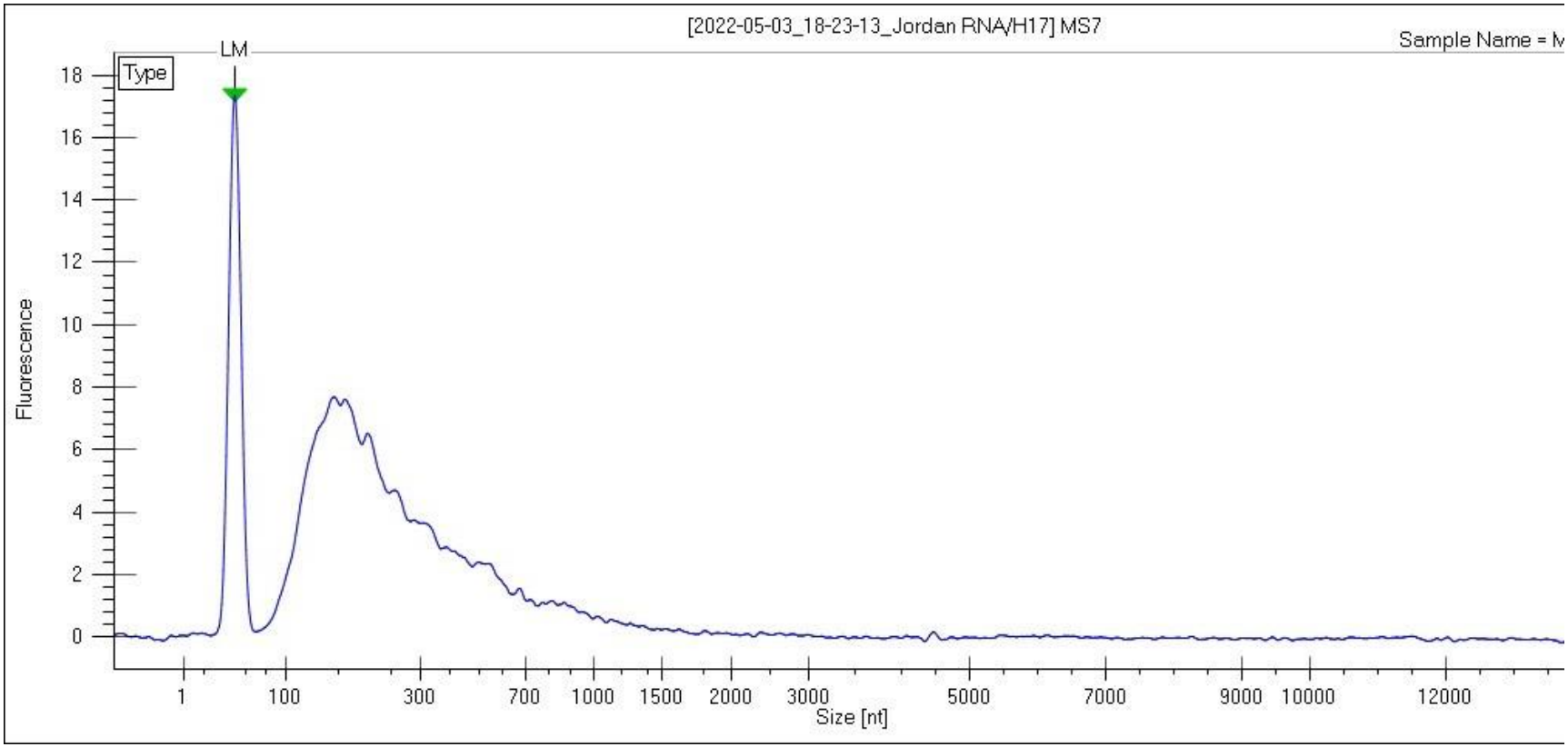
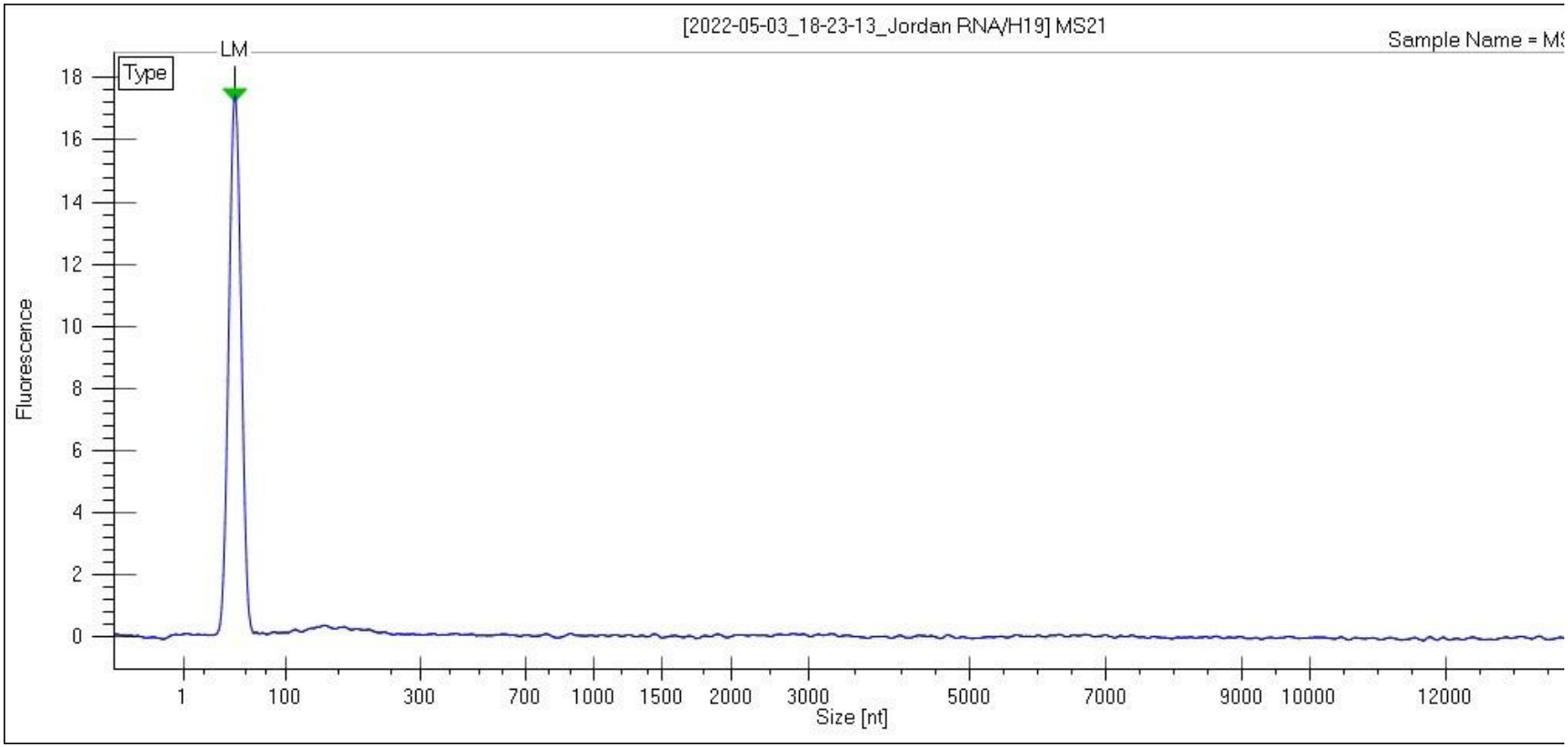
Analysis Version: 0
Chip Y646B-0564N-05 (Expiry: 20.01.2022)
2022-05-03_18-23-13_Jordanien RNA.gxd

Data collected: 03.05.2022 18:23:13
Printed: 03.05.2022 19
Firmware Version: 1.02.0.3727
Instrument: LABCHIP (S/N GT1611M
Operat

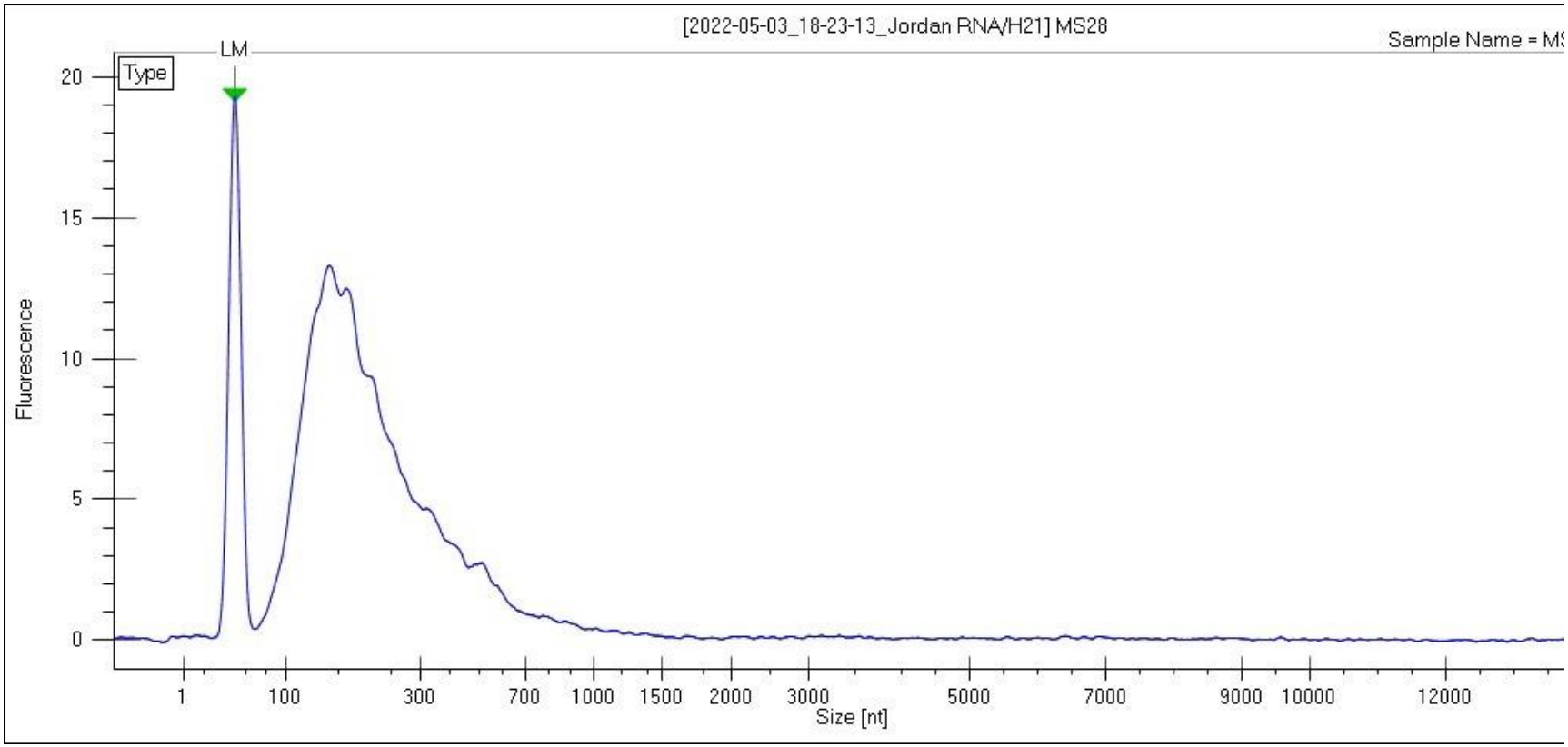

[2022-05-03_18-23-13_Jordan RNA/H17] MS7
Sample Name = M
LM
Type
Fluorescence
18
16
14
12
10
8
6
4
2
0
1
100
300
700
1000
1500
2000
3000
5000
7000
9000
10000
12000
Size [nt]

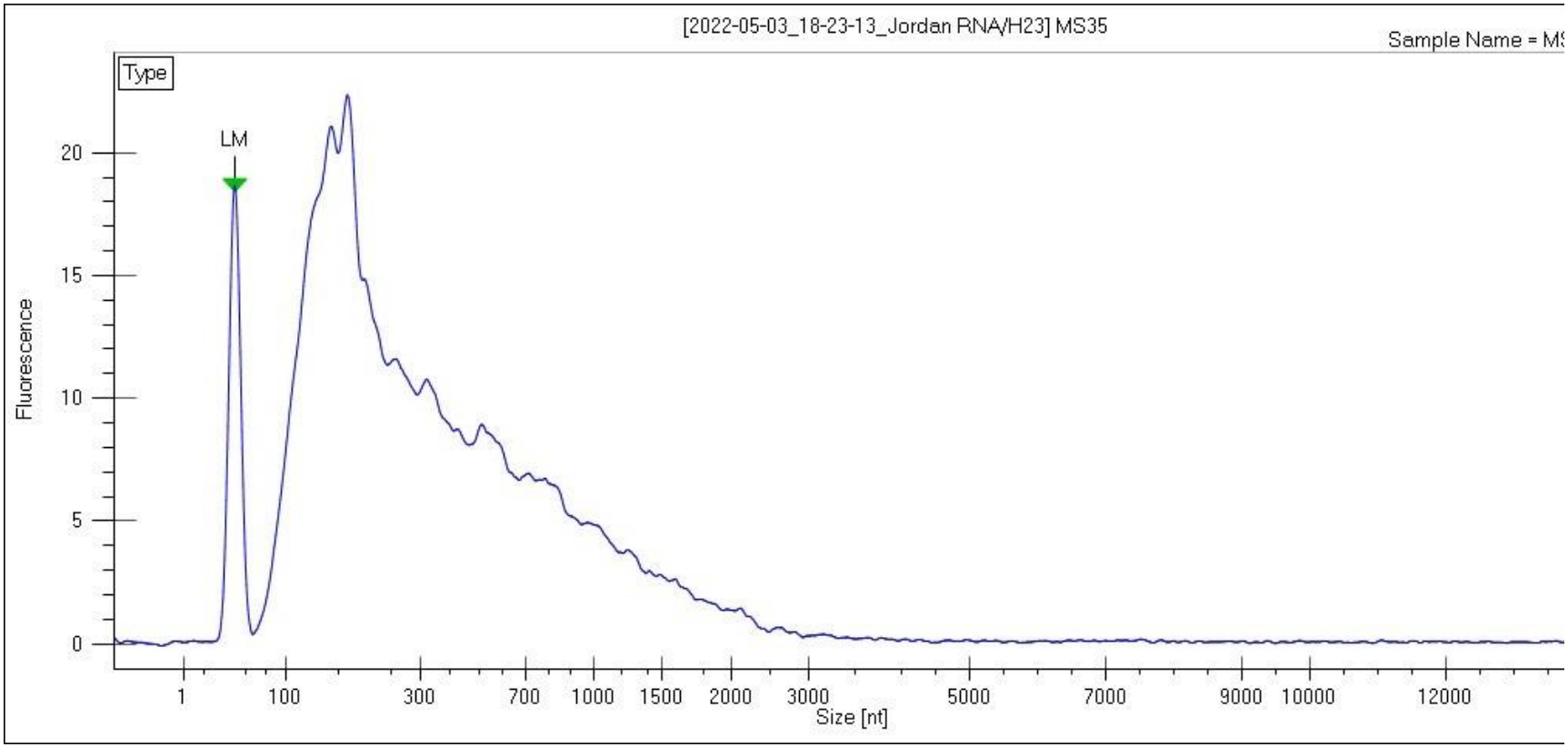


LabChip GX Software Version 5.2.2009.0

Datafile Created 03.05.2022 18:23:13 with S/W Version: 1.9.1010.0
Analysis Version: 0
Chip Y646B-0564N-05 (Expiry: 20.01.2022)
2022-05-03_18-23-13_Jordanien RNA.gxd

Data collected: 03.05.2022 18:23:13
Printed: 03.05.2022 19
Firmware Version: 1.02.0.3727
Instrument: LABCHIP (S/N GT1611N
Operat


[2022-05-03_18-23-13_Jordan RNA/H21] MS28
Sample Name = MS
LM
Type
Fluorescence
20
15
10
5
0
1
100
300
700
1000
1500
2000
3000
5000
7000
9000
10000
12000
Size [nt]


[2022-05-03_18-23-13_Jordan RNA/H23] MS35
Sample Name = MS
Type
LM
Fluorescence
20
15
10
5
0
1
100
300
700
1000
1500
2000
3000
5000
7000
9000
10000
12000
Size [nt]