miRNA Purification from Plant Tissues: Corn, Soybean and Arabidopsis

A Maxwell® RSC miRNA Tissue Kit Application Note

Materials Required:

- Maxwell® RSC miRNA Tissue Kit (Cat.# AS1480)
- QuantiFluor® RNA System (Cat.# E3310)
- TaqMan® microRNA Reverse Transcription Kit (Life Technologies #4366597)
- MultiScribe™ Reverse Transcriptase (Life Technologies #4311235)
- GoTaq® Probe qPCR Master Mix (Cat.# A6101)
- GoTaq® Probe 1-Step RT-qPCR System (Cat.# A6120)

Instrument Requirements:

- Maxwell® RSC Instrument (Cat.# AS4500) (Method: microRNA Tissue Kit v0.9.0)
- NanoDrop® 1000 Spectrophotometer
- Quantus™ Fluorometer (Cat.# E6150)
- BioRad CFX 96 Real-Time PCR Detection System
- Tissue Tearor (BioSpec)

The results presented here illustrate successful purification of high-quality RNA, including miRNA, from each plant tissue tested.

Introduction

The Maxwell® Rapid Sample Concentrator (RSC) provides automated purification of DNA, RNA or total nucleic acids from up to 16 samples in a single run. Used with the prefilled reagent cartridges supplied in the Maxwell® purification kits, the Maxwell® RSC Instrument can purify DNA or RNA from a wide range of sample types. The intuitive graphical user interface makes the instrument easy to use, and the integrated Quantus™ fluorometer lets you collect purification and quantification data in one report.

The Maxwell® RSC miRNA Tissue Kit provides a simple method for purifying total RNA, including miRNA, from tissue samples. Here we present results illustrating performance of the Maxwell® Instrument and Maxwell® RSC miRNA Tissue Kit for extraction of miRNA from plant leaf samples using corn, soybean and arabidopsis as examples.

Methods

Corn and Soybean miRNA Purification

Corn and soybean leaf tissue was ground in liquid nitrogen using a mortar and pestle, then processed as follows:

- 600µl Homogenization Solution + 2% 1-thioglycerol was added to 50mg of plant tissue and the tissue homogenized using a Tissue-Tearor.
- 200µl Lysis Buffer and 15µl Proteinase K were added to 400µl homogenate, mixed by vortex, and incubated at room temperature for 10 minutes.
- Samples were loaded into well #1 of the Maxwell® RSC cartridge, and 10µl DNaseI was added to well #4.
- RNA was eluted in 60µl of Nuclease-Free Water and stored at –80°C until use.

Arabidopsis miRNA Purification

Arabidopsis leaf tissue was ground in liquid nitrogen using a mortar and pestle, then processed as follows:

- 600µl of Homogenization Solution + 2% 1-thioglycerol was added to 50mg of plant tissue and the tissue homogenized using a Tissue-Tearor.
- 200µl Lysis Buffer and 15µl Proteinase K were added to 400µl homogenate, mixed by vortex, and incubated at room temperature for 10 minutes.
- Samples were loaded into well #1 of the Maxwell® RSC cartridge, and 5µl of DNaseI was added to well #4.
- RNA was eluted in 50µl of Nuclease-Free Water and stored at –80°C until use.

RNA Concentration and Yield and Purity

Concentration, yield, and purity of the extracted RNA were determined using absorbance (NanoDrop®-1000)- and fluorescence (QuantiFluor®/Quantus™ Fluorometer)-based methods.

Results

Concentration, Yield and Quality of Purified RNA

Concentration, yield, and purity ratios for all samples are shown in Table 1 and Figures 1–3. Average yields of RNA from corn and soybean (14.4µg and 15.1µg, respectively) were higher than from arabidopsis (4.2µg). Average concentrations of RNA from corn and soybean (337.8ng/µl and 350.8ng/µl, respectively) were also higher than those from arabidopsis (135.6ng/µl), even though a lower elution volume was used for arabidopsis samples (50µl vs 60µl for corn and soybean). Corn RNA samples had higher purity ratios than RNA isolated from soybean and arabidopsis. On average, A_{260}/A_{280} values for corn were ≥2.20 and A_{260}/A_{230} values were ≥2.01. Soybean and arabidopsis RNA purity ratios to each other. A_{260}/A_{280} ratios ranged from 2.02–2.08 and A_{260}/A_{230} values ranged from 1.30–1.67.

Table 1. Concentration, Yield and Purity Ratios of RNA Samples.

		NanoDrop® Spectrophotometer				QuantiFluor®/Quantus™ Fluorometer	
Plant	Volume (µl)	ng/µl	A_{260}/A_{280}	A_{260}/A_{230}	Yield (µg)	ng/µl	Yield (µg)
Corn	42	352.1	2.18	1.99	14.8	404	17.0
	42	335.2	2.20	2.00	14.1	405	17.0
	43	338.0	2.21	1.98	14.5	402	17.3
	43	326.0	2.22	2.05	14.0	386	16.6
Average		337.8	2.20	2.01	14.4	399.3	17.0
SD		10.8	0.02	0.03	0.4	8.9	0.3
Soybean	43	327.3	2.08	1.42	14.1	351	15.1
	43	350.7	2.02	1.30	15.1	386	16.6
	43	367.3	2.06	1.40	15.8	381	16.4
	43	357.9	2.04	1.40	15.4	393	16.9
Average		350.8	2.05	1.38	15.1	377.8	16.2
SD		17.1	0.03	0.05	0.7	18.5	0.8
Arabidopsis	32	132.9	2.03	1.52	4.3	127	4.1
	31	124.4	2.04	1.67	3.9	129	4.0
	31	128.2	2.02	1.54	4.0	124	3.8
Average	30	156.8	2.02	1.54	4.7	148	4.4
SD		14.6	0.01	0.07	0.4	10.9	0.3

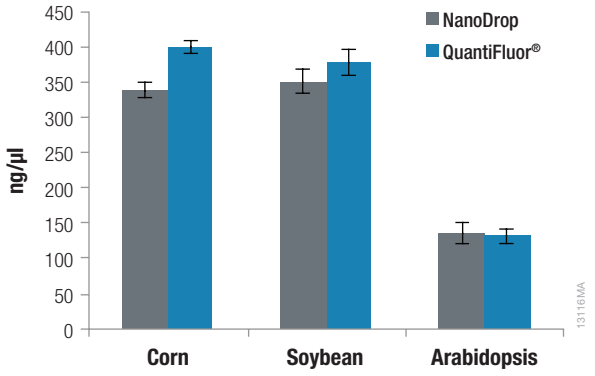

Figure 1. Concentration of purified RNA. Concentration was determined using NanoDrop®-1000 and QuantiFluor® methods. Results show the mean of n = 4 purifications from each sample type.

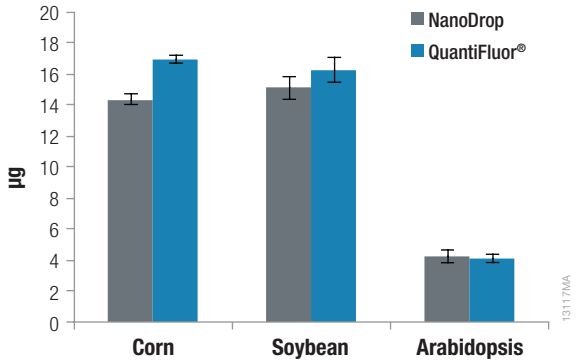

Figure 2. Yield of RNA from each sample type. Results show the mean yield values for n = 4 purifications from each sample type.

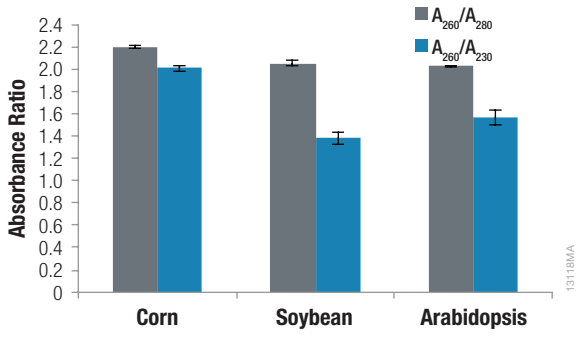

Figure 3. RNA Purity. A_{260}/A_{280} and A_{260}/A_{230} ratios for RNA purfied from corn, soybean and arabidopsis leaf samples are shown (n = 4).

TaqMan® MicroRNA Assays

RNA samples were diluted to 2ng/µl in Nuclease-Free Water, and TaqMan® reverse transcription (RT) reactions set up as follows:

Reverse Transcription Master Mix

Component	Volume Per Reaction
dNTPs (100mM)	0.15µl
Multiscribe™ RT (50 U/µl)	1µl
10X RT Buffer	1.5µl
RNase Inhibitor (20 U/µl)	0.19µl
Nuclease-Free Water	4.16µl
5X RT Primer	3µl
Total	10µl

Ten microliters of the reverse transcription master mix was added to 5µl of RNA template. (For no-template control reactions, 5µl of Nuclease-Free Water was used) Samples were run on a BioRad CFX 96 Real-Time PCR System using the following parameters:

Step	Time	Temperature
HOLD	30 minutes	16°C
HOLD	30 minutes	42°C
HOLD	5 minutes	85°C
HOLD	infinity	4°C

Following reverse transcription, qPCR was performed using TaqMan® miRNA-specific primers for each target. 1.3µl of the RT reaction (in duplicate) was added to 18.7µl of GoTaq® Probe qPCR Master Mix prepared as shown below.

qPCR Master Mix

Component	Volume Per Reaction
20X PCR Primer	1µl
GoTaq® 2X Master Mix	10µl
Nuclease-Free Waterr	7.7µl

qPCR was performed on a BioRad CFX 96 Real-Time PCR System using the following parameters:

2 minutes at 95°C	1 cycle
15 seconds at 95°C, 60 seconds at 60°C	40 cycles

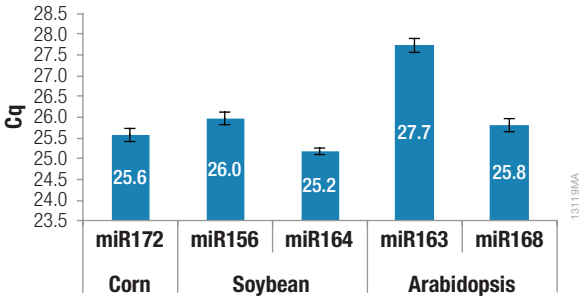

Figure 4. Performance in RT-qPCR. RT-qPCR was performed using RNA purified from each plant sample type and TaqMan® miRNA specific primers. Results show the mean of n = 4 reactions for all samples except miR156, where n = 6.

mRNA Transcript Analysis

For transcript analysis, real-time RT-qPCR was performed using primers for the housekeeping genes shown in Table 2 and the GoTaq® Probe 1-Step RT-qPCR System. RNA samples were diluted to 2ng/µl in Nuclease-Free Water and 10ng used in each reaction as shown below:

Reverse Transcription Reaction Setup

Component	+RT Reaction	–RT Reaction
GoTaq® 2X Master Mix	10µl	10µl
Forward Primer (10µM)	1µl	1µl
Reverse Primer (10µM)	1µl	1µl
GoScript® Reverse Transcriptase	0.4µl	—
Nuclease-Free Water	2.6µl	3µl
RNA Template	5µl	5µl
Total	20µl	20µl

Samples were run on a BioRad CFX 96 Real-Time PCR System using the following parameters:

Step	Time	Temperature
1 cycle	15 minutes	37°C
1 cycle	10 minutes	95°C
40 cycles	10 seconds	95°C
	30 seconds	60°C
	30 seconds	72°C
Dissociation		60–72°C

mRNA transcript analysis results are shown in Figure 5 and Table 3. Reference genes were successfully amplified for all plant samples. Table 3 shows the Cq values for all samples and no-RT controls. For corn and arabidopsis, the no-RT control Cq values were significantly higher than those of the reactions containing plant RNA samples. For soybean, the no-RT control Cq values were similar to those for the plant samples, suggesting that the primers used for soybean samples were not RNA-specific. Sequences for the reference genes were obtained from the literature (1–3).

Table 2. Housekeeping Genes Used in RT-qPCR.

Plant	Gene*	Sequence
Corn	Elongation factor 1-alpha (EF1a; 2)	F (5′-3′): TGGGCCTACTGGTCTTACTACTGA R (5′-3′): ACATACCCACGCTTCAGATCCT
	B-tubulin (βTUB; 2)	F (5′-3′): CTACCTCACGGCATCTGCTATGT R (5′-3′): GTCACACACACTCGACTTCACG
Soybean	ATP-binding cassette transporter (cons4 (ATP); 1)	F (5′-3′): GATCAGCAATTATGCACAACG R (5′-3′): CCGCCACCATTCAGATTATGT
	F-box protein (cons6 (F-box); 1)	F (5′-3′): AGATAGGGAAATGGTGCAGGT R (5′-3′): CTAATGGCAATTGCAGCTCTC
Arabidopsis	UBQ10 (3)	F (5′-3′): GGCCTTGTATAATCCCTGATGAATAAG R (5′-3′): AAAGAGATAACAGGAACGGAAACATAGT
	Elongation factor 1-alpha (EF1a; 3)	F (5′-3′): TGAGCACGCTCTTCTTGCTTTCA R (5′-3′): GGTGGTGGCATCCATCTTGTTACA

*Primers were obtained from IDT Technologies.

Table 3. RT-qPCR of 10ng of purified RNA using primers for various reference genes.

Plant	Gene	Cq	SD*	No-RT Control Cq	No-RT Control SD*
Corn	EF1a	24.3	0.1	33.0	0.3
	βTUB	25.3	0.1	33.7	0.4
Soybean	ATP	28.5	0.1	29.5	0.4
	F-box	26.2	0.1	28.7	0.1
Arabidopsis	UBQ10	21.1	0.2	29.3	0.1
	EF1a	21.7	0.2	NA	NA

*N = 4. No-RT indicates control samples without reverse transcriptase.

Solaris™ RNA Spike Control Test for Inhibition

The presence of inhibitors in the purified RNA was analyzed using the Solaris™ RNA Spike Control Kit. Purified RNA or Nuclease-Free Water (NIC–No Inhibition Control) was added to a GoTaq® Probe 1-Step RT-qPCR reaction containing the RNA spike control template and primers as shown below:

RNA Spike Control Test Reaction Setup

Component	Volume Per Reaction	No-Inhibition Control
2X GoTaq® Probe + CXR Ref Dye	10µl	10µl
50X GoScript RT Mix	0.4µl	0.4µl
100X Solaris™ RNA Spike Control (Thermo Scientific Cat.# AX-002200-00-100)	0.2µl	0.2µl
20X Solaris qPCR RNA Spike Assay (Thermo Scientific Cat.# AX-002200-00-100)	1µl	1µl
Nuclease-Free Water	6.4µl	8.4µ
RNA Sample	2µl	–
Total	20µl	20µl

To calculate inhibition, the average Cq values of the no-inhibition control samples were subtracted from the average Cq values of the samples containing purified RNA. The results are shown in Table 4. Little to no inhibition was evident in the RNA samples purified from corn, soybean and arabidopsis.

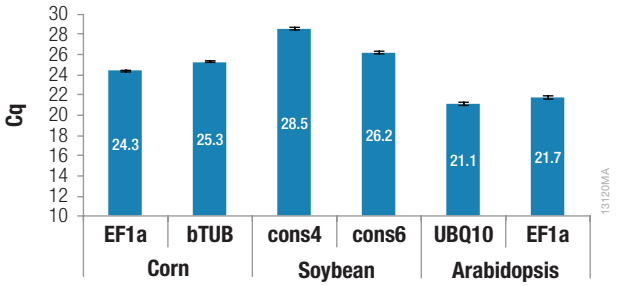

Figure 5. Amplification of reference genes from purified RNA. Cq values from RT-qPCR using 10ng of purified RNA and primers for the reference genes shown in Table 2. Results represent the mean of n = 4 reactions.

Table 4. RNA inhibition based on the Solaris™ RNA Spike Control Kit test.

Plant	Average Cq	Mean Delta Cq
Corn	28.62	0.05
Soybean	28.57	0.10
Arabidopsis	28.27	0.40
No-Inhibition Control	28.67	NA

Conclusions

The results presented here illustrate successful purification of high-quality RNA, including miRNA, from each plant tissue tested. Performance was determined by RT-qPCR with various plant-specific miRNA targets.

Transcripts of reference genes from each plant were successfully amplified by RT-qPCR, and no inhibition was evident in reactions containing RNA purified from any of the three plants tested, based on the Solaris™ RNA Spike Control Kit Test.

References

1. Libault M, *et al.* (2008) Identification of four soybean reference genes for gene expression normalization. *Plant Genome* **1**, 44-54.
2. Lin Y, *et al.* (2014) Validation of potential reference genes for qPCR in maize across abiotic stresses, hormone treatments, and tissue types. *PLOS ONE* **9**(5), e95445.
3. Wang H. (2014) Reference gene primer sequences in Arabidopsis. *Scientific Reports* **4**, 6781.

Ordering Information

Product	Cat.#
Maxwell® RSC miRNA Tissue Kit	AS1480
Maxwell® RSC Instrument	AS4500
QuantiFluor® RNA System	E3310
GoTaq® Probe 1-Step RT-qPCR System	A6120
GoTaq® Probe qPCR Master Mix	A6101

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