

Product Information

SpyLine™ Plant mRNA Capture kit for Real Time RT-PCR

Product Code: **SL0020**

Storage Temperature: –20 °C

TECHNICAL BULLETIN

Product Description

The SpyLine Plant mRNA Capture kit for Real Time RT-PCR offers a simple, rapid, and cost-effective method for plant gene expression analysis by real time quantitative RT-PCR. This integrated system has three main features:

1. A lysis buffer that efficiently releases RNA from partially homogenized plant tissues that are processed without using liquid nitrogen,
2. A high density oligo-dT coated PCR plate for efficient capture of Poly A⁺ RNA in the presence of lysis buffer; and
3. Optimized SYBR[®] Green RT-PCR reagents for real time RT-PCR.

The kit has been extensively tested on multiple plant species and multiple genes. Therefore, it should be suitable for most plants and tissues.

Using this kit, plant tissues are processed in 1.5 ml microcentrifuge tubes in the presence of lysis buffer at room temperature. No liquid nitrogen is needed for grinding. After homogenization, samples are incubated at room temperature for 5 min. and centrifuged in a microcentrifuge for 10 min. The crude lysate is then transferred to the Poly A⁺ RNA Capture Plate and incubated for 30 minutes at room temperature. Following a brief wash procedure, RT-PCR reagents are added directly to the capture plate for subsequent RT-PCR amplification and analysis.

The captured mRNA can be used in conventional RT-PCR or in real time quantitative RT-PCR. The Capture Plate is compatible with most Thermocyclers, such as the ABI 9700 & 7700, Bio-Rad[®] iCycler[®], and Stratagene[®] Mx3000P. If desired, an optional elution step may be performed to release the mRNA for downstream uses.

Reagents and equipment provided*	Product Code	Quantity
Pellet pestle	P6748	10
SpyLine Poly A ⁺ RNA Capture Plate	P3993	1X 96-well plate
Lysis Solution	L7792	25 ml
Wash Solution	W4640	2X 40 ml
Elution Solution, RNase-free	E8026	10 ml
Enhanced AMV Reverse Transcriptase, 20 units/μl	A4714	500 units
SYBR Green Taq Ready Mix	D5191	2x 50 rxn
25 mM MgCl ₂	M8787	1.5 ml

*One bag of PCR caps (12 strips) and 2 pieces of AlumaSeal II™ film for the multiwell plate are also included.

Equipment and reagents required, but not provided

- 2-Mercaptoethanol (M3148)
- RNase-free tubes
- Thermocycler for real time PCR
- Gene-specific primers
- DNase/RNase – free water (W 4502)
- Dedicated pipettes
- Aerosol resistant pipette tips

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

RNases are ubiquitous and very stable proteins, which is a concern for any researcher attempting to isolate mRNA. Care must be taken not to introduce RNase, especially if performing the optional elution. Use RNase-free pipette tips, preferably those having an aerosol barrier. Wear latex or vinyl gloves and change them frequently. Keep bottles and tubes closed when not adding or removing their contents.

Storage and stability

Store the RT-PCR components at $-20\text{ }^{\circ}\text{C}$ and the rest at $2-8\text{ }^{\circ}\text{C}$. The kit components should be stable at these storage temperatures for at least 6 months upon receiving.

Preparation of reagents

1. Allow kit components for mRNA isolation to warm to room temperature before use.
2. Thoroughly mix reagents. Examine reagents for precipitation. If any reagent forms a precipitate, warm at $37\text{ }^{\circ}\text{C}$ until the precipitate dissolves and allow to cool to room temperature before use.
3. Prepare the working lysis solution by adding 2-mercaptoethanol (2-ME) at a final concentration of 4% (e.g. $40\text{ }\mu\text{l}$ of 2-ME to $960\text{ }\mu\text{l}$ of Lysis Solution). The working lysis solution should be freshly made and used the same day. 2-ME is required to fully inactivate RNases.

Preparation of plant tissues

Prepare a bucket containing dry ice. Harvest plant tissues in 1.5 ml microfuge tubes ($30 - 70\text{ mg/tube}$) and immediately place the tubes on dry ice for 5 minutes or longer to freeze the tissue. If using leaf tissues, the lid of a 1.5 ml microcentrifuge tube can be used to clip leaf discs directly. Normally, 4 - 6 leaf discs are used per RNA isolation; however, it is better to determine how many discs are needed so that the total tissue/tube is within $30 - 70\text{ mg}$. Sample harvested may be used for RNA isolation immediately or stored at $-70\text{ }^{\circ}\text{C}$. Alternatively, frozen samples stored at $-70\text{ }^{\circ}\text{C}$ can be crushed on dry ice and then aliquoted to 1.5 ml microfuge tubes at the $30 - 70\text{ mg/tube}$ ratio.

Procedure

RNA isolation:

1. Gently crush tissue on dry ice using a pipette tip or a pellet pestle (provided in the kit). This step is critical for efficient lysis.

2. Add $200\text{ }\mu\text{l}$ of the working lysis buffer (or $3\text{ }\mu\text{l}$ lysis buffer for 1 mg tissue) into each tube and grind the tissue in the tube for $20 - 30$ seconds using a pellet pestle. The amount of lysis solution can be scaled up or down with a range of $50 - 400\text{ }\mu\text{l}$.

For tough tissues, longer grinding time may be needed and addition of $20 - 50\text{ mg}$ sea sand or $0.1\text{ }\mu\text{m}$ glass bead per tube may be helpful for efficient grinding.

3. Incubate sample at room temperature for 5 - 10 minutes to lyse the cells.
4. Centrifuge sample in a microcentrifuge at maximum speed ($12,000 - 16,000\text{ xg}$) for 10 minutes or longer to spin down debris.
5. Warm up the Capture Plate at room temperature. If not using the whole plate, one can cut off the appropriate number of wells (e.g. 12 or 24 wells) and then cover the rest of the plate with the AlumaSeal II film provided and place it back in the original bag for future use.
6. Transfer $50\text{ }\mu\text{l}$ of supernatant (containing RNA) into each well of the Capture Plate. Pipette carefully to avoid formation of bubbles, especially in the bottom of wells, or excessive foaming of the cell lysate. If necessary, unused lysate can be stored at $-70\text{ }^{\circ}\text{C}$ for future use.
7. Cover the Capture Plate with the AlumaSeal II Film provided and incubate the plate at room temperature for 30 - 90 minutes. *Usually, 30 minutes is sufficient for detection of most mRNAs, but rare mRNAs may require longer incubation times for adequate detection.*
8. Pour off the lysate from the capture plate.
9. Wash the wells 4 times with Wash Solution ($200\text{ }\mu\text{l}$ /wash).
10. Dry the plate on blotting paper and make sure there is no residue liquid in wells. **The Poly A⁺ RNA captured on the plate is ready for real time RT-PCR.**
11. Optional elution of mRNA: Add $50\text{ }\mu\text{l}$ Elution Solution to each well. Seal the wells with a piece of the AlumaSeal II™ Film. Incubate the plate at $65\text{ }^{\circ}\text{C}$ for 10 minutes. Transfer the eluted mRNA solution to RNase-free tubes or plates, and store the RNA at $-70\text{ }^{\circ}\text{C}$.

General considerations for RT-PCR

1. The amount of template RNA required for real time RT-PCR depends on abundance of the target transcript in a RNA sample. In general, picogram and nanogram levels of total or Poly A⁺ RNA should be sufficient for RT-PCR. Although our mRNA isolation kit is designed for single RT-PCR use by performing RT-PCR directly in the Capture Plate, 5-10 µl (1/10 – 1/5) of the eluted RNA would be sufficient for expression analysis of most target genes.
2. Optimal design for gene-specific primers is very important for sensitivity and specificity of target gene amplification. We recommend using primer design software and following the general rules listed below:

Primer length:	20 – 30 bp
T _m value:	60 °C – 70 °C
T _m difference between forward & reverse primers:	2-3 °C
Maximum length of dimer:	3 bp
Maximum hairpin stem:	3 bp
GC content:	40-60%

For more accurate RNA quantitation, design primers so that the PCR product is between 150 – 300 bp to minimize bias from PCR amplification. Also, the amplicon should be located in the middle or near the 3' end of the target transcript to ensure efficient reverse transcription. In addition, selection of primers that span an intron will greatly reduce the possibility of amplifying from the genomic DNA. This will also allow genomic amplification products to be identified by their larger size.

Real Time RT-PCR Procedure

The following protocol serves as a guideline. Although this procedure has been optimized on multiple plant species and genes, modifications in PCR cycling parameters might be needed for specific target genes. The real time RT-PCR reagents provided in the kit is enough for 96 x 50 µl reactions; however, the RT-PCR reaction can be scaled down to 25 µl without compromising the performance.

1. Set up the thermal cycler according to the program outlined in Table 2 or your own program.
2. Place eAMV-RT enzyme and the SYBR Green Taq Ready Mix on ice. Thaw the PCR water and gene-specific primers at room temperature and then place them on ice.

3. Dilute the eAMV-RT 1:10 with water (e.g. 1 µl eAMV-RT solution + 9 µl water).
4. Make a master mix according to Table 1 (make enough master mix for 10 or more reactions at a time).

Table 1. Real time RT-PCR master mix

For direct real time RT-PCR in the capture plate:

Reagent	Volume/ 25 µl reaction	Volume/ 50 µl reaction
Water	8.5 µl	17 µl
SYBR Green Taq Ready Mix	12.5 µl	25 µl
25 mM MgCl ₂	2.5 µl	5 µl
Enhanced avian RT, 1:10 dilution	0.5 µl	1 µl
Mixture of forward & reverse gene-specific primers (10 uM each)	1 µl	2 µl
Total volume	25 µl	50 µl

For real time RT-PCR using eluted RNA:

Reagent	Volume/ 25 µl reaction	Volume/ 50 µl reaction
Water	3.5 µl	7 µl
SYBR Green Taq Ready Mix	12.5 µl	25 µl
25 mM MgCl ₂	2.5 µl	5 µl
Enhanced avian RT, 1:10 dilution	0.5 µl	1 µl
Mixture of forward & reverse gene-specific primers (10 uM each)	1 µl	2 µl
RNA eluted from the capture plate	5 µl	10 µl
Total volume	25 µl	50 µl

5. Conduct real time RT-PCR according to the program listed in Table 2. The following cycling parameters are recommended for use with Stratagene Mx3000P PCR machine. Amplification parameters may vary depending on gene-specific primers and the thermal cycler used.

Table 2. Real time RT-PCR cycling protocol

Step	Temperature	Time
First Strand Synthesis	50 °C	30 min.
Denature/RT Inactivation	94 °C	3 min.
For Cycles 1-40:		
Denaturation	94 °C	15 sec.
Annealing	65 °C	45 sec.
Extension	72 °C	1 min.
Plate Read	85 °C	10 sec.
Melting Curve	55 °C to 95 °C, read every 0.2 °C	Hold 30 sec.

Data Analysis for Real Time RT-PCR

Follow the recommendations of the real time instrument used to perform quantitative RT-PCR. The following may help new instrument users. Generally the number of cycles is plotted against the fluorescence. Threshold cycles (C_T) or crossing points are used to determine the template amount in each sample. Threshold cycle or crossing point is the first cycle that shows a detectable increase in fluorescence due to the formation of RT-PCR products. The cycles before the crossing point are the baseline cycles. The threshold used to determine when the first detectable increase in fluorescence occurs may also be adjusted manually. The threshold should always be determined with a logarithmic amplification plot. In a logarithmic amplification plot the threshold should be set in the exponential phase and not the plateau phase.

Control reaction:

For customers who wish to run a control reaction along with their test, a positive control reaction can be run using control primers for house-keeping genes of Arabidopsis (product code C 3615 and C 3115) with Arabidopsis young leaf tissue.

References

1. Lovatt, A., et al., Validation of quantitative PCR assays, *BioPharm.*, **15**, 22-32 (2002).
2. Bustin, S. A., Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems, *J. Mol. Endocrinol.* **29**, 23-29 (2002).

3. Ginzinger D. G., Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream, *Exp Hematol.* **306**, 503-512, (2002).
4. Morrison, T. B., et al., Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification., *BioTechniques*, **23**, 954-962 (1998).
5. Sambrook, J., and Russell, D. W., *Molecular Cloning, A Laboratory Manual* (Cold Spring Harbor Lab. Press, Cold Spring Harbor, New York, 2001).

Related Products

RNAseZAP[®]: a cleaning agent for removing RNase from laboratory surfaces: R 2020
 SpyLine[™] Plant mRNA Capture Kit: SL 0010
 SpyLine[™] Plant mRNA Capture RT-PCR Kit: SL 0030
 One-step RedTaq[™] RT-PCR Kit: R 1529
 SYBR Green Quantitative RT-PCR Kit: QR 0100

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Troubleshooting Guide

Problem	Cause	Solution
Insufficient Lysis	Lysis time was too short	Allow the Working Lysis Solution to lyse the cells for at least 5 minutes before centrifugation.
	Lysis solution was not at Room Temperature	Allow the Lysis Solution to reach room temperature before adding to the tissue.
	Failed to crush frozen tissue before adding lysis solution	Make sure to break up tissues in small pieces before adding lysis solution and check the homogenate to make sure there are no big pieces after grinding.
Weak RT-PCR signal	Plant tissues were not sufficiently lysed	See problem "Insufficient Lysis"
	mRNA was degraded	Make sure 4% 2-mercaptoethanol was added to the Lysis Solution before lysing the tissues
	Insufficient wash	Be sure to wash the wells four times. Components in lysis buffer and inhibitors from plant tissue may affect RT-PCR performance.
	Primers are for a rare mRNA	If the target mRNA is rare, the incubation time for the hybridization of the Poly A ⁺ RNA to the Capture Plate should be increased to 60 - 90 minutes.
	The annealing temperature may be too high.	Decrease the annealing temperature in 2-4 °C increments.
	Design of gene-specific primers not optimal.	Verify proper primer design (see the primer design section in this technical bulletin).

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