3050 Spruce Street
Saint Louis, Missouri 63103 USA
Telephone 800-325-5832 • (314) 771-5765
Fax (314) 286-7828
email: techserv@sial.com
sigma-aldrich.com

ProductInformation

Primer Library for Arabidopsis Pathogen-inducible Genes

Product Number **PR0100**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

The Primer Library for Arabidopsis Pathogen-inducible Genes is designed for expression analysis of Arabidopsis defense response genes by quantitative RT-PCR (Q-RT-PCR). This primer library contains 96 sets of gene-specific primers that can be used for expression profiling of 96 genes, of which 93 are pathogen-inducible genes and 3 are housekeeping genes. The pathogen-inducible genes are selected based on a search of an Arabidopsis database and microarray analysis. The housekeeping genes, Actin-2, CBP20, and Ubiquitin, are selected based on their expression patterns. The Actin-2 gene is designed for high abundant genes, whereas CBP20 and Ubiquitin genes are designed for medium to low abundant genes. All of the control genes are constitutively expressed in different tissues.

All primers in the library are suitable for one-step or two-step RT-PCR and for both real time and non-real time RT-PCR analysis. In addition, the specificity and amplification efficiency of all primers have been validated using pathogen-infected Arabidopsis leaf samples. The primer library, combined with Sigma's robust RT-PCR reagents, provides cost effective, accurate, and non-chip based assays for expression profiling of a set of pathogen-inducible genes in Arabidopsis. Some of the genes included in the library can also be used as signature genes to characterize disease-resistance mutants.

Primers are packaged in a 96-well plate format. Each well contains a lyophilized set of forward and reverse primers for amplification of a specific gene. Each primer set has enough quantity for 200 RT-PCR reactions (20-25 µl reaction volume).

Reagents

- Primer Library for Q-RT-PCR of Arabidopsis Genes, Product Code P 5621
  A 96-well plate containing 96 pairs of gene specific primers as lyophilized powder. See Appendix for the list of primer names.
- A set of 4 PCR plates for aliquoting primers, Product Code P 2068.
- A set of 10 sealing films, Product Code F 2022.

Materials required but not provided

For RNA Preparation

- GenElute™ Total RNA Purification Kit (Product Code RTN10, RTN70, or RTN350
- TRI Reagent® (Product code T 9424)
- On-column DNase Set (Product Code DNASE10 or DNASE70) **OR**
- Amplification Grade DNase I (Product code AMP-D1)

For One-Step or Two-Step RT-PCR

- SYBR® Green Quantitative RT-PCR Kit (Product Code QR0100) **OR**
- JumpStart™ RED HT RT-PCR Kit (Product Code J 3520)
- Dedicated pipets
- Aerosol resistant pipet tips
- PCR tubes/plates
- Thermal cycler

Precautions and Disclaimer

This product is for R&D use only. Not for drug, household or other uses. Consult the MSDS for information concerning hazards and safe handling practices.

Procedure

Preparation of primers

The primer set (2 nmole for each primer) in each well is lyophilized. Before use, dissolve the primers in 200 µl TE buffer (10 mM Tris, pH 8.0, 0.001 mM EDTA) and transfer into individual PCR plate provided, 50µl/well. Seal each plate carefully with provided sealing film.

Storage/Stability

Store primers at –20 °C. The reconstituted primer library should be stored at –20 °C The reconstituted primers are stable at –20 °C for at least 1 year and can be frozen and thawed at least 10 times without compromising performance.

Primary considerations

RNA Preparation

One of most important steps in assuring success with Q-RT-PCR is high quality RNA preparation. Integrity and purity of RNA template is essential. Either total or poly(A)$^+$ RNA can be used as template for the reverse transcription reaction. All RNA preparations should be DNase-treated to minimize contamination from genomic DNA.

One-step RT-PCR vs. two-step PCR

In general, the one-step RT-PCR procedure is recommended for expression profiling because it minimizes hands-on work and is commonly used for real time RT-PCR. However, when the test RNA is difficult to obtain and the amounts of the samples are small, the two-step RT-PCR may be the preferred procedure. Using the one-step RT-PCR procedure, the expression of only a single gene from one RT reaction can be examined, whereas using the two-step RT-PCR procedure, multiple genes can be examined from a single RT reaction, thus saving precious RNA samples. In addition, the two-step the RT-PCR procedure may give cleaner PCR products.

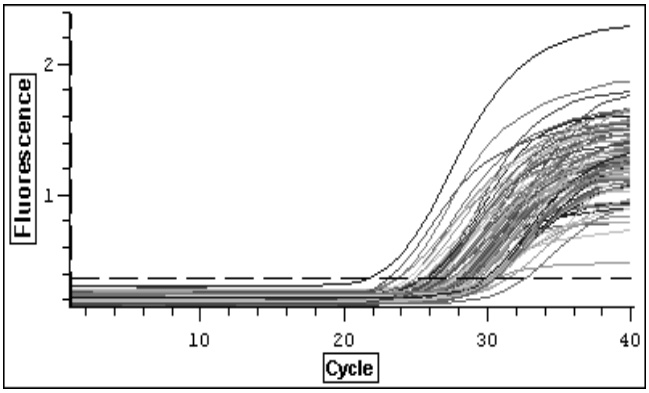
Controls

Three housekeeping genes, CBP20, Actin-2, and UBC, are included in the library as internal controls for normalizing quantitative RT-PCR experiments. Although all three control genes have been demonstrated to be constitutively expressed in different Arabidopsis tissues, the expression pattern of an individual control gene might vary depending on specific tissue type or treatment. Therefore, it is important to examine expression of all three genes and incorporate the information in data analysis.

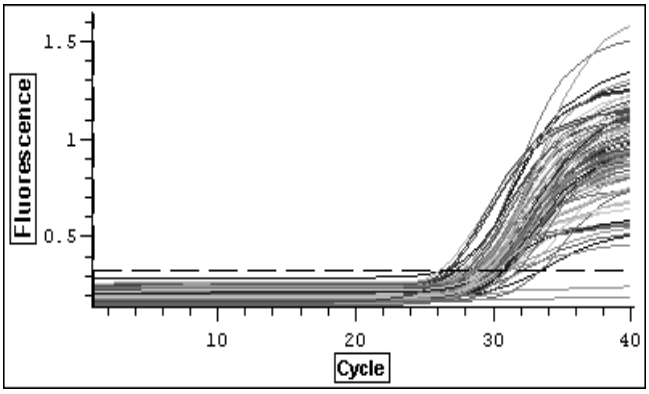
Performance Data

Expression profiling

The primer library has been tested with RNA from at least four sets of pathogen-treated or wounding-treated samples and their correspondent controls. Equal amount of total RNA isolated from treated and control samples were tested against the primer library by real time RT-PCR. The following two graphs show the amplification patterns for all of the 96 amplicons in both pathogen-treated and control tissues.



Pathogen-infected, 3hr



Uninfected control, 3hr

Dramatic decreases in C_t (threshold cycle) value are observed for many RT-PCR products in pathogen-treated tissue, indicating that many genes were induced upon the pathogen infection. The C_t values for most genes in the treated sample range from 22 to 31 cycles, whereas the C_t values for the same genes in the untreated sample range from 26 to 33.

Signature genes

The following table shows the amounts of inducible expression for selected genes in different treated samples relative to each of their untreated controls,

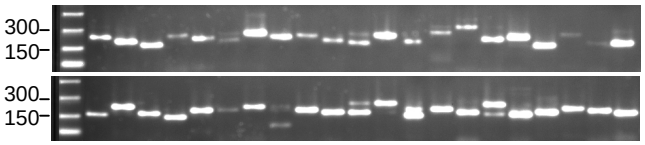
derived from real-time Q-RT-PCR data. Primer UBC is the internal control for normalizing the RT-PCR reaction and RNA concentration. In the following table, T1 and T2 are from pathogen treated samples. W4 is a wound-treated sample. Induced genes (more than 2 fold increases) are highlighted. Most of these genes only respond to pathogen infection, not to wounding treatment. These genes could be used as signature genes in defense-related research.

	T1-3hr	T2-3hr	T1-8hr	W1-3hr
At3g56710	12.26	4.41	1.97	2.33
At4g02380	14.15	25.42	14.56	1.07
At1g18890	4.07	3.86	1.10	0.60
At3g16530	13.05	24.95	7.77	2.86
At4g12720	39.48	21.10	1.39	1.00
At3g04720	1.04	0.99	49.01	1.74
At5g18470	3.05	6.97	2.24	1.24
At2g31880	4.18	4.85	2.53	0.59
At3g59700	4.97	2.19	2.26	0.22
At5g45110	5.38	4.15	0.36	0.75
At2g32200	27.36	6.68	0.82	0.71
At5g26920	21.60	9.53	3.79	0.59
At4g17500	47.37	12.74	3.01	0.43
At5g47200	2.94	4.07	1.02	0.33
At3g26820	37.30	10.34	71.26	0.21
At2g38470	66.12	26.95	3.86	1.72
At4g34390	24.40	26.23	2.39	1.11
At5g47910	36.63	9.47	1.94	0.74
At4g39670	4.49	22.99	6.35	1.48
At4g24340	1.12	1.57	31.78	1.28
UBC	**1.00**	**1.00**	**1.00**	**1.00**

Specificity

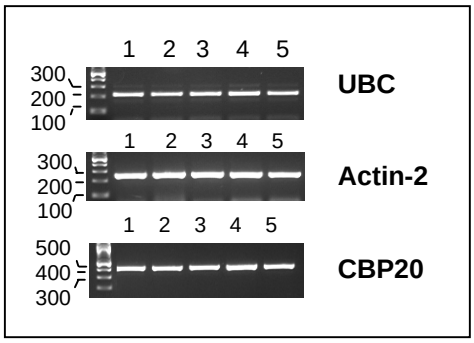
All the primer sets are designed for the generation of gene specific RT-PCR products. Each primer set has been demonstrated to generate a gene-specific RT-PCR product or major amplicon using various RNA samples.

The following gel image compares RT-PCR amplification of randomly selected primer sets with RNA from pathogen-infected Arabidopsis leaf. For each one-step RT-PCR reaction, a total of 200 ng of RNA was used. Thirty cycles of PCR amplification were conducted after 30-minute reverse transcription. The first lane is molecular weight standard.



Expression of control genes

Internal controls are critical for expression profiling of different RNA samples. Three control primers sets are included in the primer library and have been tested extensively on various tissue types. The following figure shows that all three housekeeping genes are expressed at the same level in five different Arabidopsis tissues, leaf (1), root (2), stem (3), flower (4), and siliques (5).



Procedures used for expression profiling study

The primer library has been tested for both real time and non-real time RT-PCR. The procedures listed below were developed using Sigma's reagents and kits. These procedures can be used as references for other studies.

Total RNA isolation from Arabidopsis tissues

This procedure is based on the GenElute Total RNA Isolation Kit (Product Code RTN70). Components are from the kit unless otherwise noted.

1. Homogenize 50 to 100 mg of fresh or frozen Arabidopsis tissues in 500 µl lysis buffer.

2. Transfer lysate to blue Filtration Column, centrifuge for 2 minutes.

3. Add equal volume of 70% ethanol to filtrate, mix thoroughly. Transfer the mixture to clean Binding Column.

4. Add 250 µl wash solution 1 to column, spin 15 sec. Transfer column to new Collection Tube.

5. For each preparation, mix 10 µl of DNase I (Product Code AMP-D1) with 70 µl of DNase Digestion Buffer (Product Codes NA6000 and NA0610), apply 80 µl of the digestion buffer-DNase I mixture directly onto the filter in the binding column. Incubate at room temperature for 15 min.

6. Add 250 µl of Wash Solution 1, centrifuge 15 sec. Transfer the Binding Column to a fresh 2.0 ml Collection Tube.

7. Add 500 µl Wash Solution 2 to column, centrifuge 15 sec., and discard wash solution. Repeat wash step one more time with Wash Solution 2, centrifuge 2 minutes to remove ethanol.

8. Transfer Binding Column to new Collection Tube, add 50 µl Elution Solution warmed at 65 °C to the column, spin for 1 minutes.

9. Check RNA by A_{260} and A_{280} measurements and by agarose gel electrophoresis.

Real time Q-RT-PCR for 96-well plate reaction (20 µl reaction volume)
This procedure is based on SYBR Green Quantitative RT-PCR kit (Product Code QR0100). Components are from the kit unless otherwise noted.

1. Make a master mix (for 96 reactions) on ice with following reagents except primer pairs, which will be added to each well separately.

Volume	Reagent	Final Concentration
X µl	Water	q.s. to 1900 µl
1000 µl	SYBR Green Taq Ready Mix	1x
200 µl	25mM $MgCl_2$	3.5 mM
Y µl	Total RNA	20 ug
2 µl	eAMV-RT (20 unit/µl)	50x dilution
Total volume = 1900 µl		

2. Add 1 µl primer mix to each well of 96-well PCR-reaction plate and double check the presence of primers. Then add 19 µl master mix to each well.

3. The following cycling parameters are recommended for use with the DNA Opticon MJ Research, Inc. Other instruments may require optimization of amplification parameters.

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	80 °C	1 sec.
Melting Curve	65 °C to 99 °C, read every 0.2 °C	Hold 1 sec.
Incubate	72 °C	10 min

Note: Temperature for plate reading is suggested at 80 °C, based on the result of melting curve test.

Non-real time RT-PCR
This procedure is based on JumpStart RED HT RT-PCR kit (Product Code J 3520). Components are from the kit unless otherwise noted.

1. Set up the thermal cycler according to the program outlined in Table 2 or your own program.

2. Thaw at room temperature the RNA template, gene specific primers, dNTP mix, $MgCl_2$, water, and PCR buffer. Mix well and spin down briefly. Place all RT-PCR components on ice.

3. Dilute the eAMV-RT 1:10 with 1x PCR buffer. (e.g. 1 µl eAMV-RT solution + 1 µl 10x PCR buffer + 8 µl water).

4. Make a master mix according to the following table (make enough master mix for 5 or more reactions at a time).

Volume	Reagent	Final Concentration
14.25 µl	Water	-------
2.5 µl	10x PCR buffer	1x
3.5 µl	25 mM $MgCl_2$	3.5 mM
0.5 µl	10 mM Deoxy-nucleotide mix	200 µM each dNTP
0.5 µl	RNase inhibitor	0.4 U/µl
0.5 µl	eAMV-RT, 1:10 dilution	0.04 U/µl
1.25 µl	JumpStart RedTaq DNA polymerase	0.05 U/µl
1 µl	Primer mix	200 nM each
1 µl	Template RNA[a]	50 pg – 500 ng

Total volume: 25 µl[b]

Notes:
a. Add template RNA and primers separately if multiple genes and/or multiple RNA samples need to be tested.
b. Total reaction volume can be scaled up to 50 ul without compromising the performance.

5. Conduct one-step RT-PCR according to the program listed below. The program has been optimized on the PE9700 thermal cycler.

Reverse transcription	50 °C	30 min.
Denaturation/ RT Inactivation	94 °C	3 min.
PCR cycling	30 - 40 cycles[1]	
Denaturation	94 °C	15 sec.
Annealing	65 °C	30 sec.
Extension	72 °C	1 min.
Final Extension	72 °C	10 min

Note: 1) For quantitative analysis, stop (pause) the PCR process at 25-30 cycle and remove a small aliquot (5 µl) and store at 2-8 °C for future analysis and then resume the PCR process to completion (35-40 cycles).

6. Evaluate the PCR product by directly loading 5 µl of the RT-PCR reaction on 1.5 -2% agarose gel containing 0.5 ug/ml ethidium bromide dye.

Data Analysis for Real Time Q-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.

References

1. Lovatt, A., et al. Validation of quantitative PCR assays, BioPham, **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 Clonging, A Laboratory Manual* (Cold Spring Harbor Lab. Press, Cold Spring Harbor, New York, 2001).

Related Products
GenElute™ Total RNA Kit, product codes RTN10, RTN70, and RTN350
GenElute™ Total RNA Purification Kit, product codes NA6000 & NA0610
Enhances Avian HS RT-PCR Kit, product codes HSRT-20 & HSRT-100
SYBR Green Quantitative RT-PCR kit, product code QR0100
JumpStart RED HT RT-PCR kit, product code J3520

JWM 5/29/03

Appendix
List of primer sets in the primer library.

Well #	Primer name	Amplicon Length (bp)
A1	At2g32680	210
A2	At3g56710	150
A3	At4g02380	150
A4	At5g60900	164
A5	At1g18890	242
A6	At3g16530	156
A7	At5g44420	152
A8	At4g04490	158
A9	At4g11830	151
A10	At2g44790	186
A11	At1g10140	213
A12	At4g12720	160
B1	At3g04720	228
B2	At5g18470	223
B3	At2g28210	184
B4	At2g31880	159
B5	At3g59700	157
B6	At4g16650	229
B7	At5g60900	194
B8	At4g23180	238
B9	At5g45110	175
B10	AT2g14560	150
B11	At4g36950	195
B12	At4g14400	161
C1	At3g56400	216
C2	AT2g31180	236
C3	AT4g25390	182
C4	At1g47510	179
C5	At2g22270	196
C6	At3g04640	217
C7	At2g32200	161
C8	At1g05430	166

Well #	Primer name	Amplicon Length (bp)
C9	At1g03220	245
C10	At1g21250	179
C11	At5g26920	203
C12	At4g17500	156
D1	At5g47200	249
D2	At4g17490	175
D3	At3g50770	179
D4	At3g26820	199
D5	At4g23150	175
D6	At1g65970	225
D7	At4g24340	199
D8	At1g28480	197
D9	At1g73800	151
D10	At2g47130	175
D11	At4g37520	228
D12	At2g35980	163
E1	At3g57260	221
E2	At3g57240	200
E3	At2g14610	166
E4	At4g11890	177
E5	At4g23130	176
E6	At4g16260	240
E7	At3g16530	156
E8	At3g46090	216
E9	At4g03450	197
E10	At1g13470	245
E11	At5g10760	247
E12	At5g27600	171
F1	At1g02920	213
F2	At2g14560	151
F3	At4g04490	160
F4	At1g24140	193

Well #	Primer name	Amplicon Length (bp)
F5	At2g40000	177
F6	At5g20230	210
F7	At2g02930	164
F8	At4g38540	189
F9	At2g45680	217
F10	At2g40140	159
F11	At4g39890	190
F12	At4g26070	226
G1	At2g13790	225
G2	At2g32210	169
G3	At4g11370	213
G4	At4g34390	189
G5	At5g47910	207
G6	At4g39670	174
G7	At5g59820	170
G8	At2g35660	223
G9	At2g38470	250
G10	At4g11280	228
G11	At3g28210	152
G12	At4g37260	163
H1	At3g52430	172
H2	At2g39400	169
H3	At5g33340	266
H4	At5g33350	361
H5	At1g67980	217
H6	At3g25882	216
H7	At2g45220	192
H8	At1g05100	164
H9	At1g66700	245
H10	CBP20?(locus#)	409
H11	Actin2?(locus#)	257
H12	UBC?(locus#)	217

Note: Primer name equals gene locus name.