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ProductInformation

GeneSnare™ Differential Expression Kit

Product Number **GS0010**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

The GeneSnare Differential Expression Kit provides an effective tool for RT-PCR based mRNA differential display using a proprietary ACP technology (**A**nnealing **C**ontrol **P**rimer) for screening differentially expressed genes. The major applications of the product fall in the following research areas: gene discovery by display genes differentially expressed in different tissues, treatments or environments; expression profiling; and pattern-specific mutant screening. All the ACP primers have been validated by differential display analysis for *Arabidopsis*, corn, soybean, as well as cultured CHO and HeLa cells, and is suitable for many organisms.

How ACP Technology Works

The core of ACP technology is a suite of annealing control primers or ACP primers. As shown below, ACP primers comprise a tripartite structure with a polydeoxyinosine linker (part 3) between the 3' end target core sequence (part 1) and the 5' end non-target universal sequence (part 2). This unique structure prevents annealing of the 5' end non-target sequence to the template and thus facilitates primer hybridization at the 3' end to the target sequence at specific temperatures, resulting in a dramatic improvement in annealing specificity. There are two types of ACP primers: 1) ACP anchor primer containing oligo dT as core sequence for first-strand cDNA amplification using total RNA as template; and 2) ACP arbitrary primers with arbitrary 10-mer as core sequence.

2
3 1

A unique two-step RT-PCR procedure is used for differential expression analysis of the gene of interest. The first-strand cDNA is synthesized in a reverse transcription reaction (Step 1) at 42 °C using total RNA as the template with an ACP oligo dT anchor primer. Both ACP anchor primer and ACP arbitrary primer are applied for down-stream PCR amplification (Steps 2 and 3). The first cycle of PCR is conducted with an annealing temperature of 50 °C (Step 2), followed by 30 cycles of PCR reaction with an annealing temperature of 65 °C (Step 3). Only perfectly matched DNA fragments can be amplified under these conditions. Subsequently, the PCR products can be resolved on agarose gel for band separation and excision (Step 4).

Step 1

dT- ACP1

First-strand cDNA synthesis at 42°C

Arbitrary ACP

Step 2

First-stage PCR for second-strand cDNA synthesis at 50°C

Step 3

Second-stage PCR for amplifying the second-strand cDNA at 65°C

1 2 3 4 5
AB AB AB AB AB

Step 4

Reagents provided

Components	Product Code	Amount for 50 Rxn	Amount for 250 Rxn
ACP arbitrary primers, 10μM/primer	GS0040	1 plate (see appendix)	1 plate (see appendix)
ACP anchor primer, 20μM	GS0020	100 μl	2 x 100 μl
Universal primer-forward, 20 μM	GS0020	100 μl	100 μl
Universal primer-reverse, 20 μM	GS0020	100 μl	100 μl
dNTP Mix, 10 mM	D 7295	50 μl	250 μl
RNase inhibitor, 20 U/μl	R 1274	25 μl	25μl
M-MLV RT, 200 U/μl	M 1427	60 μl	60μl
10X buffer for RT	B 8559	1.5 ml	1.5 ml
JumpStart™ REDAccuTaq™, 1 U/μl	D 1938	50 μl	250μl
10x buffer for AccuTaq™	B 0174	0.5 ml	2 x 0.5ml
25 mM $MgCl_2$	M 8787	1.5 ml	1.5 ml
Water, PCR Reagent	W 1754	1.5 ml	1.5 ml

Materials required but not provided

For RNA Preparation

- GenElute™ Mammalian Total RNA Miniprep Kit, Product Codes RTN10, RTN70, or RTN350
- TRI Reagent®, Product Code T 9424
- On-Column DNase I Digestion Set, Product Code DNASE10 or DNASE70 **OR**
- DNase I, Amplification Grade ,Product Code AMP-D1

For RT-PCR Amplification

- Dedicated pipettes
- Aerosol resistant pipette tips
- PCR tubes/plates
- Thermal cycler

For Agarose Gel Electrophoresis

- Agarose
- TBE/SB buffer
- Power supply
- Electrophoresis apparatus

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.

Storage/Stability

Store primers and RT-PCR reagents at –20 °C. The reconstituted primers 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 differential display 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. Sigma-Aldrich provides high-quality kits for total RNA isolation and downstream genomic DNA removal (see the Section for **Materials required but not provided**). RNA integrity should be tested by agarose gel electrophoresis and OD_{260}/OD_{280} measurements. Good quality RNA should show two sharp ribosomal bands and have an OD_{260}/OD_{280} ratio >1.8.

Concentration of RNA Template

To ensure that cDNA population is representative, enough total RNA needs to be provided for the reverse transcription reaction. We have tested the effect of total RNA concentration on RT-PCR amplification for differential display and recommend using 4-8 μg of total RNA for every 50 μl RT reaction.

Comparable Samples

When using this kit to display differentially expressed gene(s) among comparable samples, the only variable component should be RNA from comparable biological materials. Thus, the rest of the components for - RT-PCR should come from the same source(s) at identical concentrations. To minimize sample-to-sample variation, we suggest applying a premixed master reaction solution to each RT and PCR reaction, respectively.

Procedure

Preparation of primers

ACP Arbitrary primers are lyophilized and packaged in a 96-well plate format. Each of 24 primers is located in a defined well in the first three columns of a 96-well plate (See Appendix for the list and position of the primers). The rest of the reagents are stored in individual vials. The RT-PCR reagents included in the kit are enough for 50 or 250 RT-PCR reactions (20 µl PCR reaction volume).

The arbitrary primer (0.5 nmole for each primer) in each well is lyophilized. Before use, dissolve the primers in 50 µl TE buffer (10 mM Tris, pH 8.0, 0.001 mM EDTA) You may want to aliquot primer sets into 2 to 3 plates, and use only one plate repeatedly until the depletion before switching to a new aliquot plate to avoid cross-contamination and frequent freeze/thaw cycles.

ACP anchor primer and a pair of universal primers are supplied at 20 µM concentration in TE buffer (10 mM Tris, pH 8.0, 0.001 mM EDTA) in individual tubes.

Procedures used for differential display

The ACP primers have been tested for differential display in *Arabidopsis*, corn, and HeLa cells. The procedures listed below were developed using Sigma's reagents and kits. These procedures can be used as a guideline for other studies.

First-strand cDNA synthesis by reverse transcription

This procedure is based on the 50 µl reverse transcription (RT) reaction volume provided in this kit. The total RT volume can be scaled up depending on how many µl of first-strand template are needed for the second-strand PCR amplification.

1. Add the following reagents to a thin-walled 200 or 500 µl PCR microcentrifuge tube on ice:

Volume/ reaction	Reagent
X µl	Water
Y µl (4-8 µg)	Total RNA template
2 µl	20 µM ACP Anchor Primer

20 µl total volume

2. Mix gently and briefly centrifuge to collect all components to the bottom of the tube.
3. Place tube in a thermal cycler at 70 °C for 10 minutes.
4. Remove tube, place on ice for 1 min., centrifuge briefly, and add the following components to start the reverse transcription reaction:

Volume/ reaction	Reagent	Final Concentration
21 µl	Water, PCR Reagent	-------
5 µl	10X buffer for MMLV-RT	1X
2 µl	Deoxynucleotide Mix	400 µM each dNTP
1 µl	RNase inhibitor	0.4 U/µl
1 µl	M-MLV- RT	4 U/µl

50 µl Total Volume

5. Mix well and incubate at 42 °C for 50 minutes. (Make a PCR master mix during this step)
6. Heat the reaction at 94 °C for 3 min and then chill on ice immediately to inactivate the MMLV-RT and denature the RNA/DNA hybrid.

PCR amplification

Make a PCR master mix according to following table (make enough master mix for 50 reactions for running 24 PCR reactions for each control and treated sample).

Volume/ reaction	Reagent	Final Concentration
13 µl	Water	-------
2 µl	10X PCR buffer	1X
1 µl	25 mM $MgCl_2$	1.25 mM
0.5 µl	10 mM Deoxynucleotide Mix	250 µM each dNTP
0.5 µl	20 µM ACP Anchor primer	500 nM
1 µl	JumpStart Red AccuTaq DNA polymerase	0.05 U/µl

Total volume: 18 µl/reaction

1. Mix the master mix well and place on ice.
2. Divide master mix equally in two parts, 25 Rxn each. Add 25 µl RT reaction for control sample to one part of master mix, and 25 µl RT reaction for treated sample to the second part of master mix. Mix well.
3. Add 1 µl of the ACP arbitrary primer to a 0.2 ml PCR tube or plate.
4. Add 19 µl of the PCR master mix to each tube, mix well, and start PCR amplification using the program in Table 4 or a customized program.

Table 4. Cycling program for PCR

Step	Temperature	Time
Denaturation Annealing Extension	94 °C 50 °C 72 °C	3 min 3 min 1 min
PCR cycling (25-30 cycles*) Denaturation Annealing Extension	 94 °C 65 °C 72 °C	 15 sec 30 sec 1 min
Final Extension	72 °C	10 min

*Depends on abundance of the fragments amplified

Evaluate the PCR product by directly loading 5 µl of the RT-PCR reaction on 1.2% agarose gel containing 0.5 µg/ml ethidium bromide dye.

Downstream procedures after differential display
After differential display, downstream band isolation, purification and DNA sequences are required for gene discovery. Below are some procedures we recommend for downstream analysis. The reagents included in these procedures are not provided in the GeneSnare kit unless indicated specifically.

Isolate DNA bands from agarose gel
Slice the band(s) of interest as thin as possible from agarose gel and spin down DNA with GenElute Agarose Spin Column (Product Code 5-6500) following the product's procedure for band re-amplification. Otherwise, DNA sequencing-compatible procedures should be applied for band purification/clean-up.

If more DNA is needed for band isolation, one can re-run the agarose gel with remaining PCR samples for the bands of interest.

Re-amplification using universal primers
Band re-amplification is only needed when recovery is low or the bands are too faint. The GeneSnare kit provides a pair of universal primers that can specifically re-amplify bands generated from ACP-based differential display.

Mix the reaction in the proportions given below:

Component	Volume
JumpStart 2x ReadyMix™	25
Universal primer-F	1
Universal Primer-R	1
Purified DNA	1
H_2O	22
Total	50

Run PCR reaction:

Step	Temperature	Time
Denaturation	94 °C	3 min
PCR cycling (30 cycles) Denaturation Annealing Extension	 94 °C 65 °C 72 °C	 15 sec 30 sec 1 min
Final Extension Hold	72 °C 4 °C	10 min Indefinite

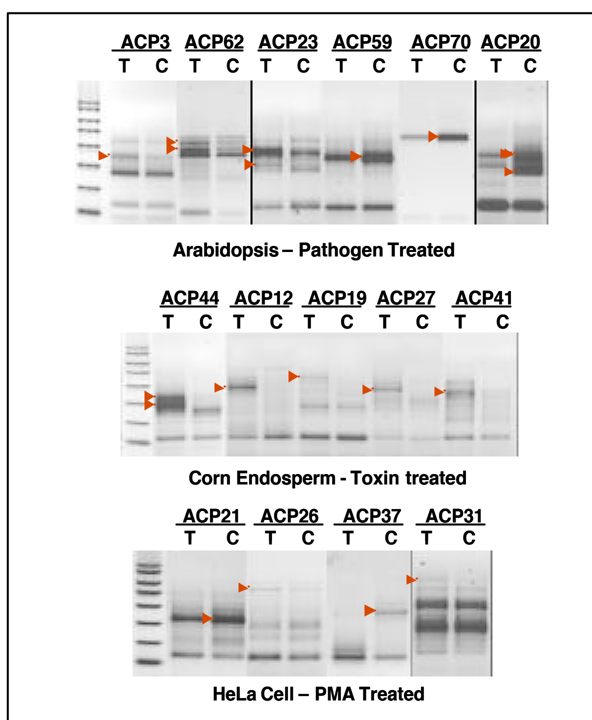
DNA sequencing and sequence analysis
DNA sequences can be performed using universal primers provided in the GeneSnare kit. Sequencing analysis tools are available in various public databases.

Performance Data
Validation of ACP primers in different organisms
All ACP arbitrary primers were validated in RT-PCR reaction using total RNA isolated from multiple species, including *Arabidopsis* leaves, corn endosperm and leaves, soybean leaves, as well as human (HeLa) and animal (CHO) cultured cells. All ACP primers can efficiently produce amplicons in a RT-PCR reaction with tested species/samples.

Differential Display

Differential display was performed on pathogen-infected *Arabidopsis*, PMA-treated HeLa cell, and toxin-treated corn endosperm. The following figure shows an agarose gel image displaying banding patterns with selected ACP arbitrary primers. As indicated by arrowheads, bands with changed intensity between treated (T) and control (C) samples reflect differentially expressed genes upon the given treatment.

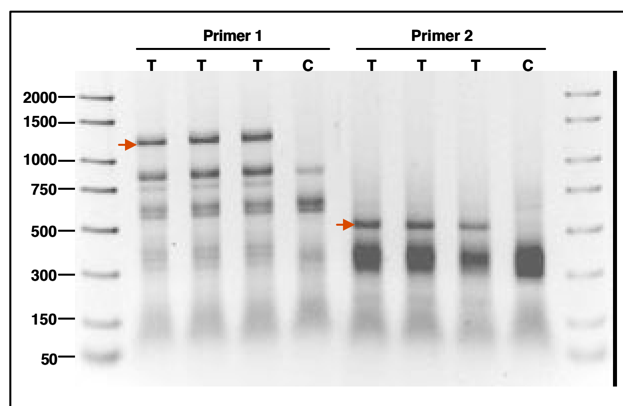
Figure 1. Agarose gel image of differentially displayed bands in treated *Arabidopsis*, corn and HeLa cells



Reproducibility

Reproducibility of banding patterns for a given biological material with a specific ACP arbitrary primer was tested in *Arabidopsis* pathogen-treated samples. RT-PCR reactions were repeated three times on treated sample (T) using two selected ACP arbitrary primers. As shown in Figure 2, the banding patterns are highly reproducible.

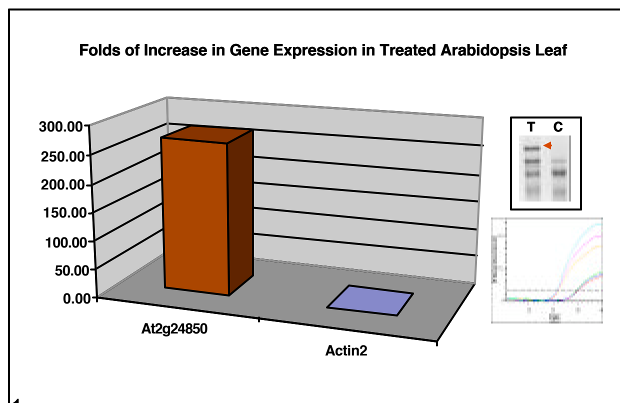
Figure 2. Test for reproducibility



Specificity

Differentially displayed bands (indicated by arrows in Figure 2) were extracted, purified and sequenced. DNA sequences revealed a 100% match for the two Arabidopsis genes. To confirm the specificity of the GeneSnare Differential Expression system, we designed and synthesized a pair of gene specific primers for one of the genes, At2g24850, and performed real-time RT-PCR. Figure 4 shows the real-time RT-PCR result with a more than 250 fold increase in gene expression in treated samples (T) for At2g24850.

Figure 3. Real-time RT-PCR confirms result from differential display



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Related Products

GenElute™ Mammalian Total RNA Miniprep Kit, Product Codes RTN 10, RTN 70, and RTN 350
On-Column DNase I Digestion Set, Product Codes DNASE10, and DNASE70
JumpStart™ RedTaq™ ReadyMix™, Product Code P 1107
GeneSnare Differential Expression Primer Kit, Product Code GS0020
GeneSnare Arbitrary Primer, Set 1, Product Code GS0040
GeneSnare Arbitrary Primer, Set 2, Product Code GS0050
GeneSnare Arbitrary Primer, Set 3, Product Code GS0060
GeneSnare RedAccuTaq™ RT-PCR kit, Product Code GS0030

References

1. Hwang, I., et al., Annealing control primer system for improving specificity of PCR amplification, BioTechniques, **35**, 1-5 (2003).
2. Pohjanvirta, R., Comparison of several Hot-start Taq DNA polymerases for detection of differentially expressed genes by GeneFishing, Biochemica, **2**, 17-18 (2004)
3. Bustin, S. A., Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems, J. Mol. Endocrinol., **29,** 23-29 (2002).
4. Sambrook, J., and Russell, D. W. Molecular Clonging, A Laboratory Manual (Cold Spring Harbor Lab. Press, Cold Spring Harbor, New York, 2001).

JumpStart, GenElute, RedTaq, ReadyMix and GeneSnare are trademarks of Sigma-Aldrich, Co.
TRI Reagent is a registered trademark of Molecular Research Center, Inc.

Appendix

List of arbitrary ACP primers in the primer plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A9	A17									
B	A2	A10	A18									
C	A3	A11	A19									
D	A4	A12	A20									
E	A5	A13	A21									
F	A6	A14	A22									
G	A7	A15	A23									
H	A8	A16	A24									

Troubleshooting Guide

Problem	Possible Causes	Comments
Little or no PCR product	RNA degraded	Check the RNA by a denaturing agarose gel electrophoresis. Poly A^+ RNA should appear as a smear between 0.5 kb and 2 kb. Total RNA should have two sharp ribosomal RNA bands without notable degradation. For purifying RNA, use Sigma's Tri Reagent (T 9424, T 3809, or T 3934) or RNA isolation kits (RTN-10, RTN-70, RTN-350).
	Incomplete removal of guanidinium during RNA isolation.	For any procedure using guanidinium based lysis buffer, remove as much residue liquid as possible after the first precipitation and then wash once with 70% alcohol.
	M-MLV Reverse Transcriptase thermally inactivated.	Add the M-MLV Reverse Transcriptase to the reaction mix after the initial primer-template denaturation /annealing step.
	A PCR component may be missing or degraded.	Run a positive control (e.g. human β-actin gene) along with the test sample. A checklist is also recommended when assembling reactions.
	There may be too few cycles performed.	Increase the number of cycles – 3-5 additional cycles at a time
	The annealing temperature may be too high.	Decrease the annealing temperature in 2-4 °C increments.
	There may not be enough template.	After increasing the number of cycles has shown no success, repeat the reaction with a higher concentration of template.
	The extension time may be too short.	Increase the extension time in 2-minute increments, especially for long templates.
	Mg^{++} levels may be too low	Increase magnesium concentration in 0.5 mM increments.
	Deoxynucleotides are degraded.	Try to avoid multiple freeze /thaw cycles by aliquoting deoxynucleotides into smaller volumes. Once thawed keep deoxynucleotides on ice.
	Target template is difficult.	In most cases, inherently difficult targets are due to unusually high GC content and/or secondary structure. 2-Betaine has been reported to help amplification of high GC content templates at a concentration of 0.8-1.3 M. In some cases, the addition of 1-4% DMSO may help.

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