High-Throughput Gene Expression Research

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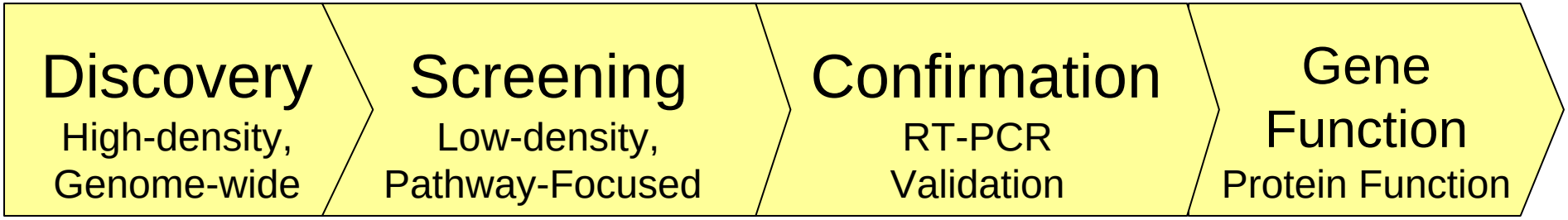
Pathway-Centric Tools and Technology™

SuperArray Bioscience Corporation

TRI Biotech

4 Stage Segmentation for Gene Expression Research



Customers in gene expression research fit into one or more of these segments.

Each segment has its own needs.

SuperArray offers products to meet the customer's need in each segment.

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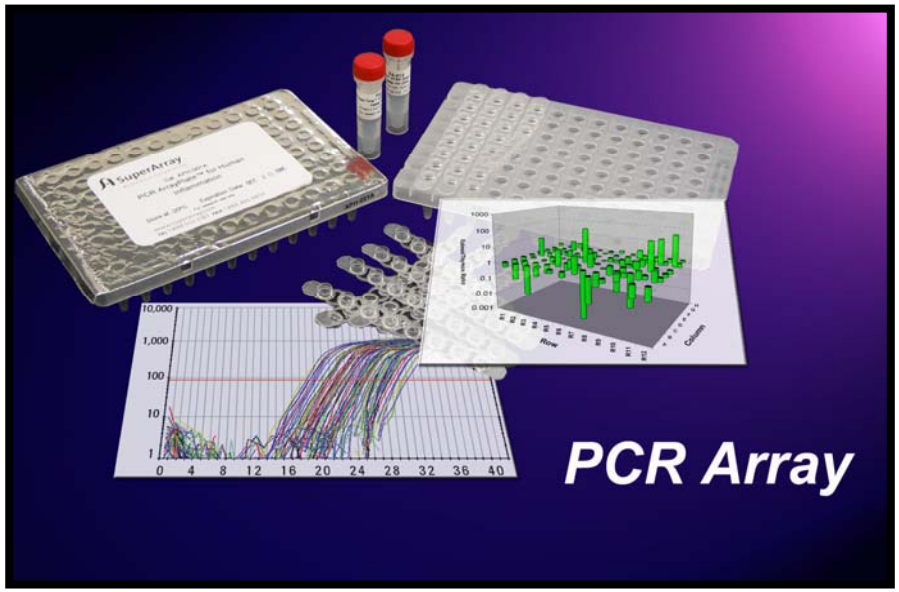
4 Stage Segmentation for a typical Gene Expression Research project.

- **1 - Discovery – High density genome wide**
  - Low sample #, High gene content
  - Affymetrix Services – Agilent?
- **2 – Screening – Low Density Pathway focused**
  - High sample number, lower gene number
  - SuperArray Oligo, RT PCR, cDNA Arrays
- **3 – Confirmation – RT PCR**
  - Very low gene number, high value genes
  - Superarray PCR products
- **4- Confirmation – Translation level**
  - Functional Biology
  - CASE Kits
  - siRNA

RT²*Profiler*™ PCR Array: Data Analysis & Tech Support



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HOW THE PCR ARRAY WORKS:

Control & Experimental Cells or Tissue

↓

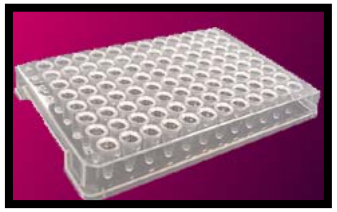
Isolate total RNA

↓

ReactionReady First Strand Synthesis Kit (C-01)

↓

Generate cDNA & Master Mix Cocktail. Aliquot into PCR Array.



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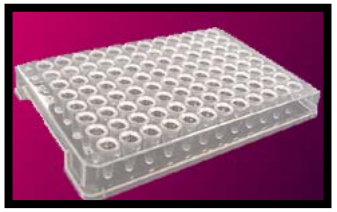
PCR (40 cycles)



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Data Analysis: Determine Fold Changes ($\Delta\Delta$ C_t Method)

~ 4 h

1.5 h

2.5 h

30 m

HOW THE PCR ARRAY SYSTEM 2.0 WORKS:

Control & Experimental Cells or Tissue

↓

Isolate total RNA → RT² PCR Array RNA QC Plate

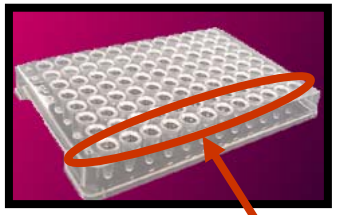
External RNA Control Spiked-In →

↓

RT² PCR Array First Strand Synthesis Kit (C-02)

↓

Generate cDNA & Master Mix Cocktail. Aliquot into PCR Array.



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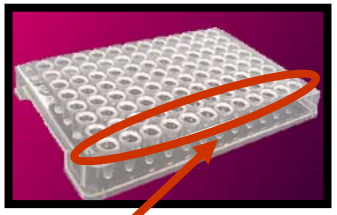
PCR (40 cycles)



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Data Analysis: Determine Fold Changes ($\Delta\Delta C_t$ Method)

New, More Useful Controls

Post-Run: Baseline and Threshold

- **Manual Setting**
- **Baseline**
  - Use Linear View
  - Set to Cycle #2 up to 1 or 2 cycle values before earliest amplification
- **Threshold Value**
  - Use Log View
  - Place in
    1) Linear phase of amplification curve
    2) Above background signal, but within lower third of curve
- **Human 18S rRNA Check**
  - Omit if C_t value <10
- **Export C_t values to blank spread sheet (Excel).**

- **<u>Baseline/Threshold Must Be Same Between Runs</u>**

Baseline and Threshold: Definitions

Baseline Value

During PCR, changing reaction conditions and environment can influence fluorescence. The background signal is most evident during the initial cycles of PCR prior to significant accumulation of the target amplicon. During these early PCR cycles, background signal in all wells is used to determine the “baseline fluorescence” across the entire reaction plate. The goal of data analysis is to determine when target amplification is sufficiently above the background signal, facilitating more accurate measurement of fluorescence.

Threshold

The threshold is the numerical value assigned for each run, statistically significant point above the calculated baseline.

Ct Value

The Threshold Cycle (Ct) reflects the cycle number at which the fluorescence generated within a reaction crosses the threshold.

From AppliedBiosystems Technical Note

iCycler

Services Help

Library

Workshop

Run-Time Central

Data Analysis

View/Save Data | PCR Quantification | PCR Standard Curve | Melt Curve | Allelic Discrimination N/A

Data File: APH_024_UniversailcDNA.opd

Select analysis mode: PCR Base Line Subtracted Curve Fit

Select Wells

Reports

SYBR-490

Select a Reporter

SYBR-490

Threshold Cycle Calculation

Baseline Cycles: 2 through 15 — Auto Calculated / User Defined

Threshold Position: 22.6 — Auto Calculated / User Defined

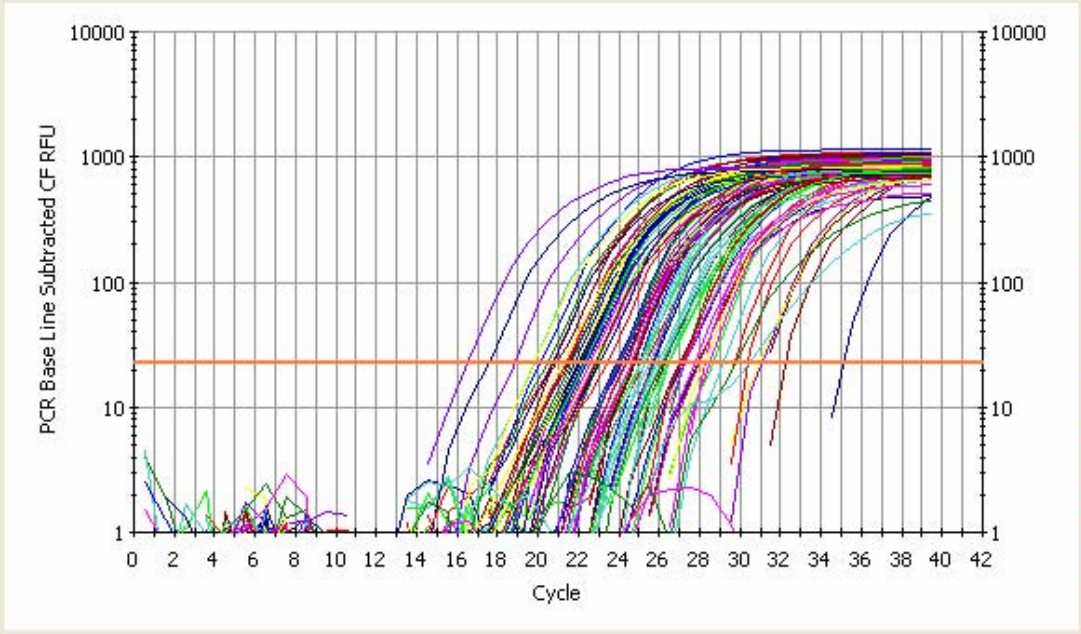
Recalculate Threshold Cycles

Normal View

Save for X-axis Allelic Analysis

Save for Y-axis Allelic Analysis

Save data from current dye layer for allelic analysis (Y-axis).



A1 A2 A3 A4 A5 A6 A7 A8 A9 A10 A11 A12 B1

	Ct
A1	19.8
A2	26.5
A3	23.8
A4	25.3
A5	26.4
A6	21.7
A7	31.0
A8	27.0
A9	23.5
A10	21.4
A11	23.8
A12	23.9
B1	26.7
B2	25.1
B3	27.1
B4	27.0
B5	25.6

Running: Instrument not in Remote | 9/15/2006 | 11:28 AM

start | Inbox - Microsof... | Outlook Today -... | TroubleShooting | 2 Internet Exp... | iCycler | Microsoft Power... | 11:28 AM

Fold Change Calculation

Due to the inverse proportional relationship between threshold cycle and the original gene expression level, and the doubling of the amount of product of every cycle, the original expression level (L) for each gene of interest is expressed as:

$$L = 2^{-C_t}$$

To normalize the expression level of a gene of interest (GOI) to a housekeeping gene (HKG), the expression levels of the two genes are divided:

$$\frac{2^{-C_t\,(GOI)}}{2^{-C_t\,(HKG)}} = 2^{-[C_t\,(GOI) - C_t\,(HK)]} = 2^{-\Delta C_t}$$

To determine fold change in gene expression, the normalized expression of the GOI in the experimental sample is divided by the normalized expression of the same GOI in the control sample:

$$\frac{2^{-\Delta C_t\ expt}}{2^{-\Delta C_t\ control}} = 2^{-\Delta\Delta C_t}$$

Where $\Delta\Delta C_t$ is equal to ΔC_t expt – ΔC_t control

The complete calculation is as follows:

$$\frac{\frac{2^{-C_t\,(GOI)\ expt}}{2^{-C_t\,(HKG)\ expt}}}{\frac{2^{-C_t\,(GOI)\ control}}{2^{-C_t\,(HKG)\ control}}} = \frac{2^{-[C_t\,(GOI) - C_t\,(HK)]\ expt}}{2^{-[C_t\,(GOI) - C_t\,(HK)]\ control}} = \frac{2^{-\Delta C_t\ expt}}{2^{-\Delta C_t\ control}} = 2^{-\Delta\Delta C_t}$$

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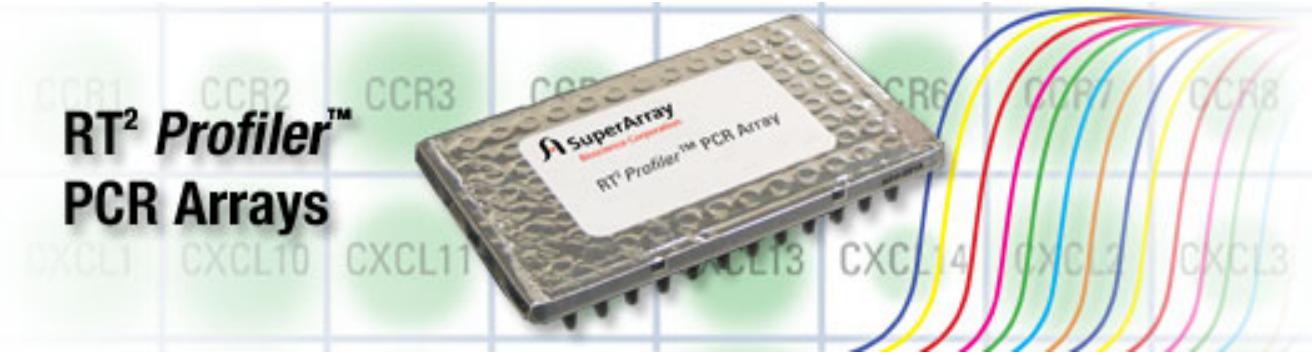
PCR Array System 2.0



Technical Sales Training

RT²*Profiler* PCR Array

- **Pathway Focused**

  Profile the expression of a panel of genes relevant to a pathway or disease state.

- **Simple and Accurate**

  Simple real-time PCR method provides high sensitivity and wider dynamic range.

- **Designed for Routine Use**

  Brings expression profiling to almost any lab with a real-time PCR instrument.

- Continued successful marketing message
- Today: 2.0 = higher-level customer satisfaction

ISSUES AND PROBLEMS

- **RNA Quality Control**
  - Purity and Integrity, Genomic DNA Contamination
- **Sensitivity**
  - Lower (ng) RNA amounts
  - Lower C_t values, higher positive calls
- **Convenience**
  - Too many cocktails and dilutions, esp. for 384-well
- **PCR Efficiency and Consistency**
  - Internal PCR control, Replicates of Controls

SOLUTION = PCR ARRAY SYSTEM 2.0

- **RT²*Profiler* PCR Array**
  - **With NEW Control Features & Simpler Protocol**

Internal & External Control, gDNA Detection, Only One Cocktail

- **RT² Real-Time SYBR Green PCR Master Mixes**

- **NEW: RT² PCR Array First Stand Synthesis Kit**

Improves and Controls for RT Efficiency

- **NEW: RT² PCR Array RNA QC Plate**

Tests for genomic DNA and inhibitors before running PCR Array

HOW THE PCR ARRAY WORKS:

Control & Experimental Cells or Tissue

↓

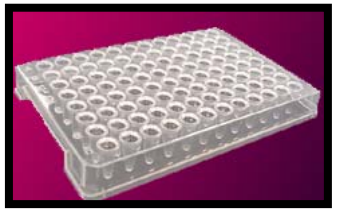
Isolate total RNA

↓

ReactionReady First Strand Synthesis Kit (C-01)

↓

Generate cDNA & Master Mix Cocktail. Aliquot into PCR Array.



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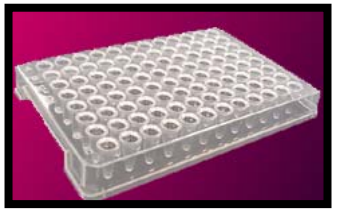
PCR (40 cycles)



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Data Analysis: Determine Fold Changes ($\Delta\Delta$ C_t Method)

HOW THE PCR ARRAY SYSTEM 2.0 WORKS:

Control & Experimental Cells or Tissue

↓

Isolate total RNA → RT² PCR Array RNA QC Plate

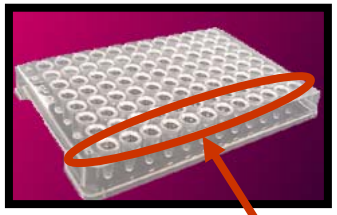
External RNA Control Spiked-In →

↓

RT² PCR Array First Strand Synthesis Kit (C-02)

↓

Generate cDNA & Master Mix Cocktail. Aliquot into PCR Array.



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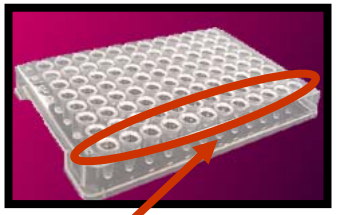

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Data Analysis: Determine Fold Changes ($\Delta\Delta C_t$ Method)

New, More Useful Controls

NEW PCR ARRAY AVAILABILITY

- **40 Pathways available for Human & Mouse**
  - cDNA GEArray and MultiGene-12 conversions
- **12 Pathways for Rat**
- **96-well (A,B,C,D) and 384-well (E) formats**
- **Customization**

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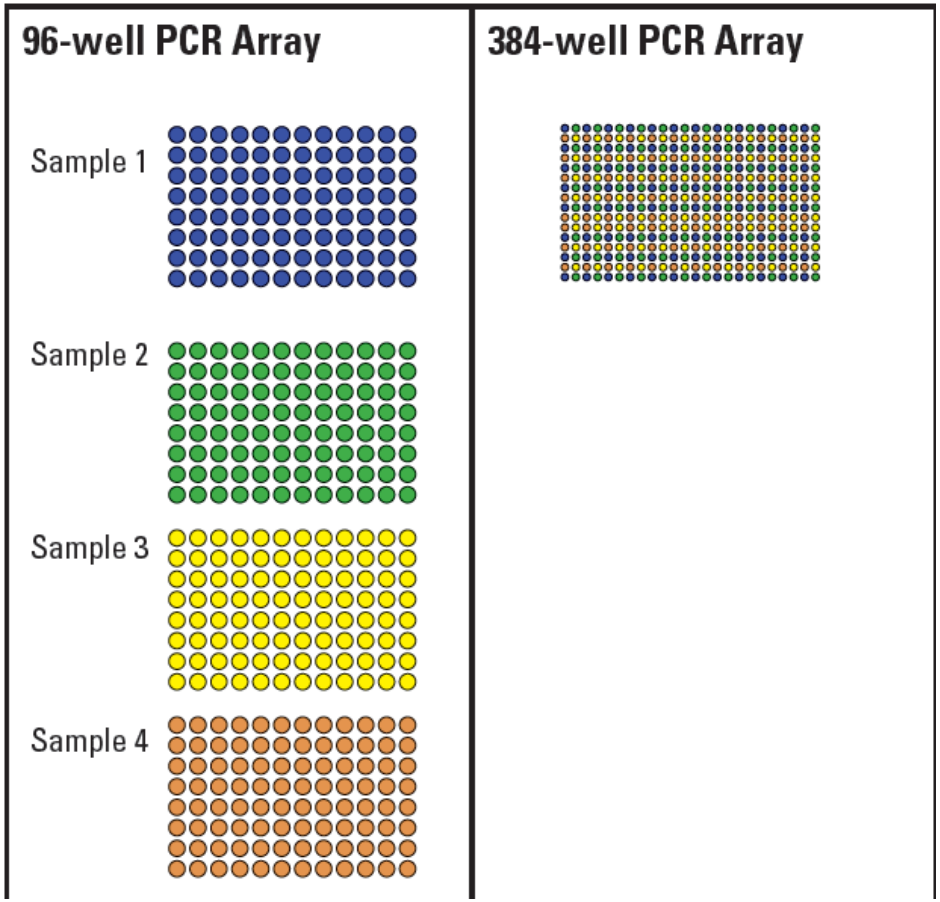
New Format: 384-well PCR Array (Type E)

- **Specific for customers using ABI 7900HT with 384 well block**
- **Sold in sets of 4 plates**



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RT² PCR Array RNA QC Plate LAYOUT

HK1	HK1	HK1	HK1	HK1	HK1	HK1	HK1	HK1	HK1	HK1	HK1
HK2	HK2	HK2	HK2	HK2	HK2	HK2	HK2	HK2	HK2	HK2	HK2
IVT	IVT	IVT	IVT	IVT	IVT	IVT	IVT	IVT	IVT	IVT	IVT
POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
gD	gD	gD	gD	gD	gD	gD	gD	gD	gD	gD	gD
NRT	NRT	NRT	NRT	NRT	NRT	NRT	NRT	NRT	NRT	NRT	NRT
POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC

- HK1, HK2 — High and Low Level Housekeeping Genes
- IVT — RNA Control spiked-in from C-02
- POS — Positive PCR Control ± Template
- gD — Detects Genomic DNA Contamination
- NRT — No Reverse Transcription Control
- NTC — No Template Control

Supporting Existing Customers

- **All new customers: PCR Array System 2.0**
- **Existing Customers:**
  - New features more useful than old
  - New features help with technical support issues
- **If old features preferred,**
  - Cannot compare results with System 2.0
  - Finish project in 6 months – discontinuation of

    System = C-01, master mix, PCR Array 1.0 (APM,APM,APR)

Typical Customer Features

- Interested in real-time PCR gene expression profiling
- Focusing on a pathway or disease state
- Limited time resources necessary for primer design and optimization

- **NEW FEATURES:**
- High-volume core facilities needing consistency controls
- 384-well: Industrial accounts
- Naïve users needing help with RNA QC

CASE™ Kits

Cellular Activation Signaling ELISA

SuperArray Bioscience Corporation

Heather Fox-Brashears

Technical Support

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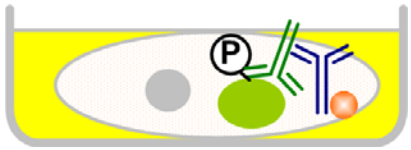
Where is CASE™ used in Gene Expression Research?

Discovery	Screening	Confirmation	**Gene Function Assay**
High-density, Genome-wide	Low-density, Pathway-Focused	RT-PCR Validation	Activation



ELISA-based assays for *in cell* Detection of Protein Activation

What is CASE™?

- **CASE**™ = **C**ellular **A**ctivation of **S**ignaling **E**LISA
- A cell-based assay designed to quantify the level of phosphorylation of a specific protein
- Includes complete antibody-based detection system
- Monitors activation of signal transduction pathways
- Determine whether pathway or protein is activated or inhibited by experimental conditions

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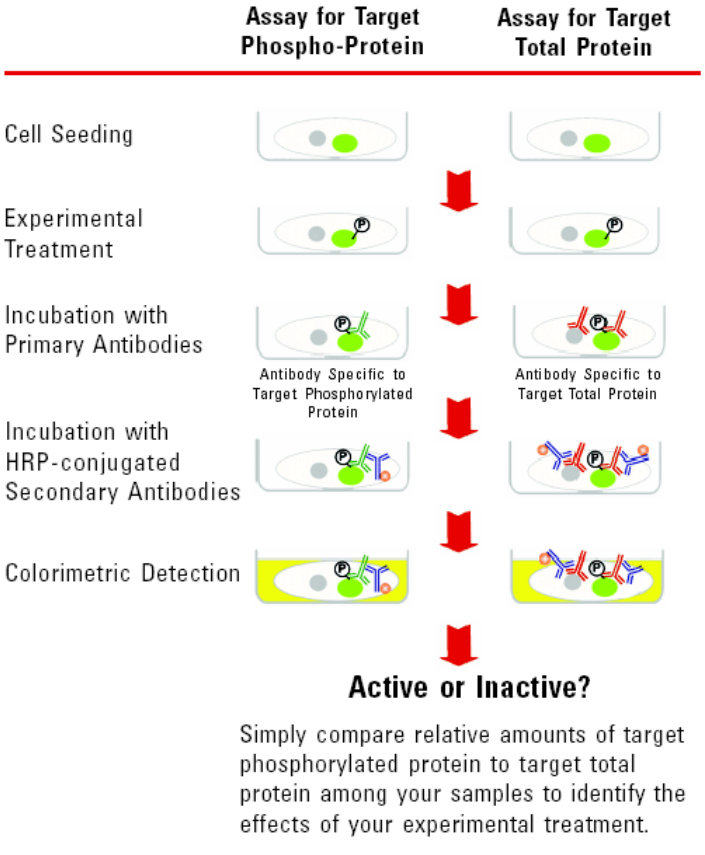
How It Works

All experimental steps take place in a single 96 well cell culture plate.



Simply compare relative amounts of target phosphorylated protein to target total protein among your samples to identify the effects of your experimental treatment.

How Does the CASE™ Kit Work?

Benefits of CASE Kits

Quantitative:

Cell-based ELISA determines level of total and phosphorylated form of a protein at same time

Simple:

Easy, quantitative, and non-radioactive protocol with minimal hands-on time

No Extractions:

Directly measure protein phosphorylation state on cells fixed in a 96-well culture plate

Protein Activation: Old Techniques

- Western blotting
  - ☹ requires cell lysate preparation, immunoprecipitation, and can lead to loss of activated protein
  - ☹ laborious multi-step, time consuming process (low throughput)
  - ☹ separate blots required for total / phosphorylated forms of protein
- *In vitro* kinase assays
  - ☹ requires protein extraction
  - ☹ requires lots of material
  - ☹ sometimes requires radio-labeling

Protein Activation: New CASE Kits

- CASE Benefits:
  - ☺ No protein extraction or lysate preps – Cell-based (maintains native activated protein levels)
  - ☺ 96-well plate format – high-throughput compatible
  - ☺ Simple ELISA-based procedure, non-radioactive
  - ☺ Simultaneous detection of total & phosphorylated forms of protein
  - ☺ Little material required – minimal hands-on time
  - ☺ Quantitative results

CASE Kits Currently Available

Protein	Site	Species	Catalog #	Inhibitor
AKT	S473	human, mouse	FE-001	FA-002
ATF2	T69/T71	mouse	FE-020	N/A
BAD	S112	human, mouse	FE-021	N/A
BCR	Y177	mouse	FE-019	N/A
EGFR	Y845	human, mouse	FE-013	N/A
ErbB2	Y877	human, mouse	FE-012	N/A
ERK1/2	T202/Y204	human, mouse	FE-002	FA-003
IkBa	S32	human	FE-008	FA-006
JNK	T183/Y185	human, mouse	FE-004	FA-005
JUN	S73	human, mouse	FE-009	FA-005

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CASE Kits Currently Available

Protein	Site	Species	Catalog #	Inhibitor
NFkB p65	S276	human	FE-007	FA-006
NFkB p65	S468	human	FE-006	FA-006
NFkB p65	S536	human	FE-005	FA-006
p38	T180/Y182	human, mouse	FE-003	FA-004
p53	S9	human	FE-014	N/A
p53	***S15***	***human***	***FE-015***	***N/A***
p53	S37	human	FE-014	N/A
PIK3R1	***YxxM***	***human, mouse***	***FE-010***	***N/A***
SRC	Y418	human, mouse	FE-011	N/A
STAT3	Y705	human, mouse	FE-018	N/A
STAT3	S727	human, mouse	FE-017	N/A

Inhibitors Currently Available

Catalog #	Product	Inhibits
FA-002	LY294002	PI3K
FA-003	U0126	MEK1, 2
FA-004	SB202190	p38 MAPK
FA-005	SP600125	JNK1, 2, 3
FA-006	Bay11-7085	IKBKA/B

Each stock inhibitor will serve > 200 assays

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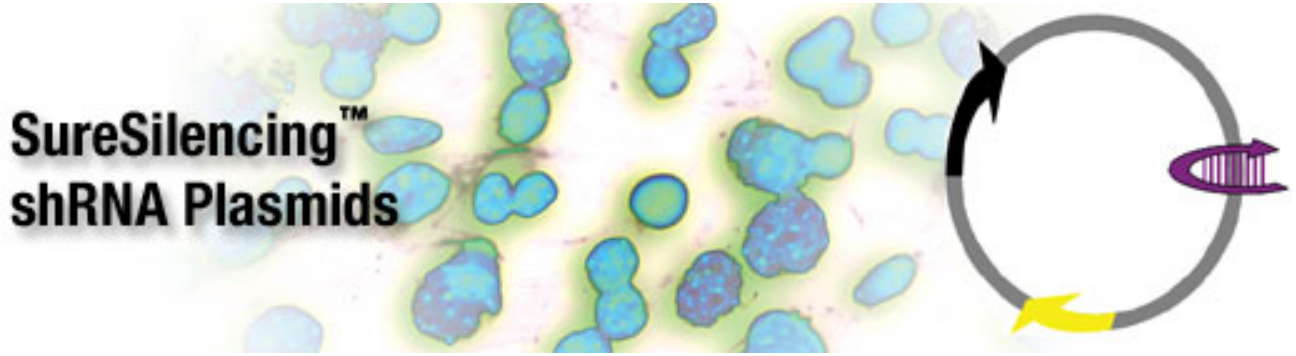
SureSilencing™ shRNA

Genome-Wide, Plasmid-Based RNA Interference For Human, Mouse, and Rat



Technical Sales Training

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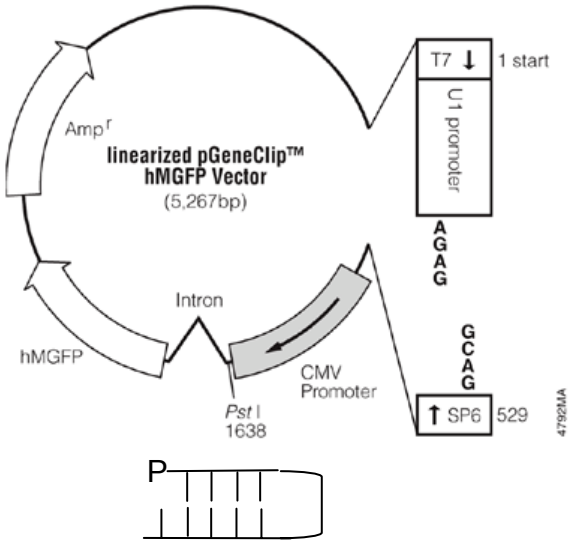
SureSilencing™ shRNA Plasmids

Discovery	Screening	Confirmation	Gene Function Assay
High-density, Genome-wide	Low-density, Pathway-Focused	RT-PCR Validation	RNA Interference



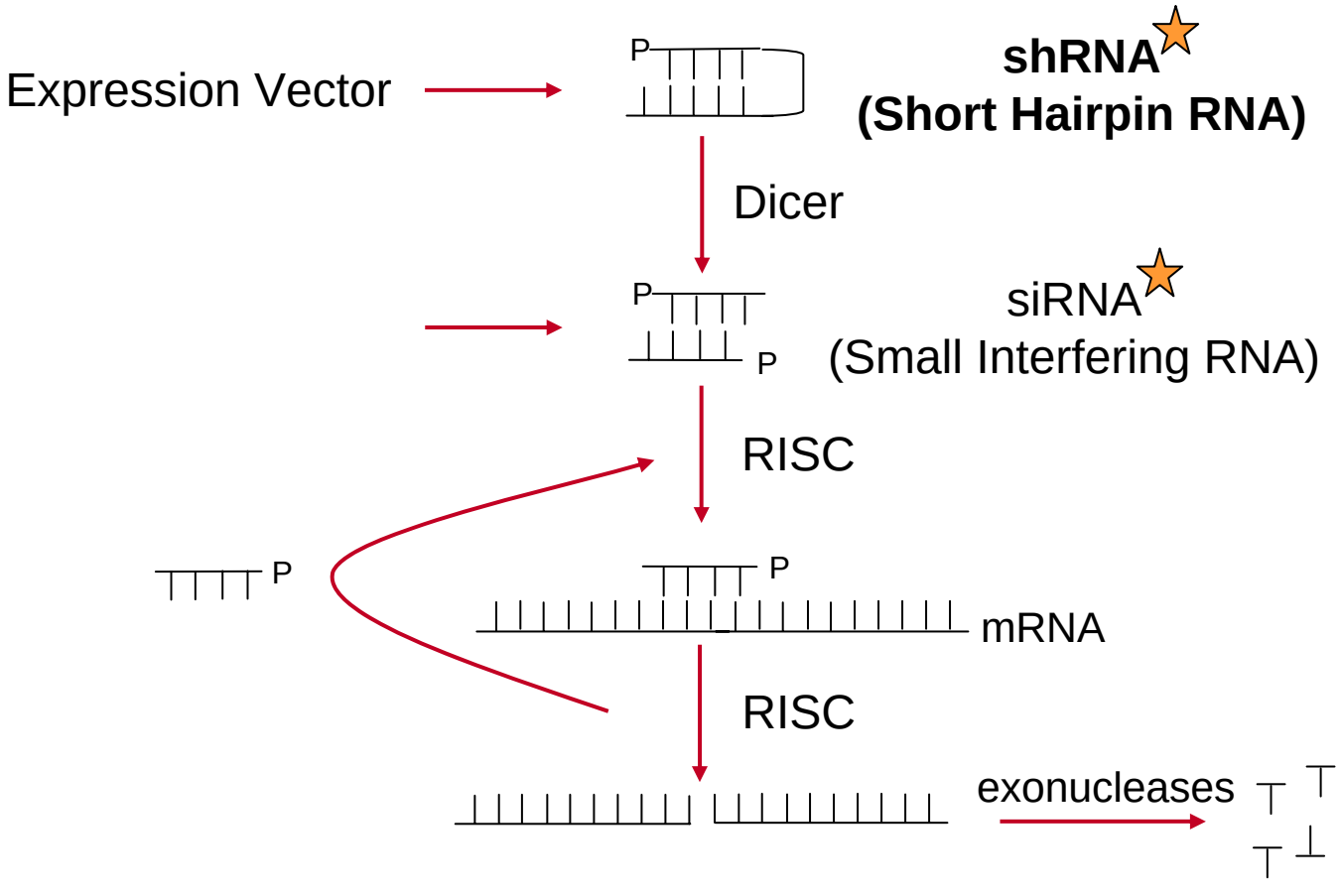
- Genome-Wide Availability
- Still new and emerging field **but …**
- Potential Success Story
- Sales Tools
- Important Technical Support Tips

SureSilencing™ shRNA Introduction

- Knock down gene expression to determine gene function
- Pre-designed shRNA specific for a given target gene
- Unique and Powerful Combination of:
  - Promega GeneClip™ U1 Hairpin Cloning System
  - SuperArray's experimentally verified design algorithm
- For every **human, mouse, or rat gene**, four (4) pre-designed shRNA are provided on separate plasmids.
- All genes also include one negative control shRNA, a scrambled artificial sequence with no sequence identity to either genome.

How RNA Interference Works

Experimentally in Mammalian Cells



Benefits of SureSilencing™ shRNA

Select or Enrich:

☺ Plasmids available with either Neomycin Resistance or GFP: Enables selection for stable transfectants and the study of the long-term effects of gene suppression or enrichment of transient transfectants for short term studies

GUARANTEED:

☺ Knock down expression of any targeted by at least 70 percent!

Convenient & Cost-Effective:

☺ Use standard plasmid-based and lipid-mediated transfection methods. Plasmids provide a renewable source of RNA Interference.

SuperArray Bioscience Corporation

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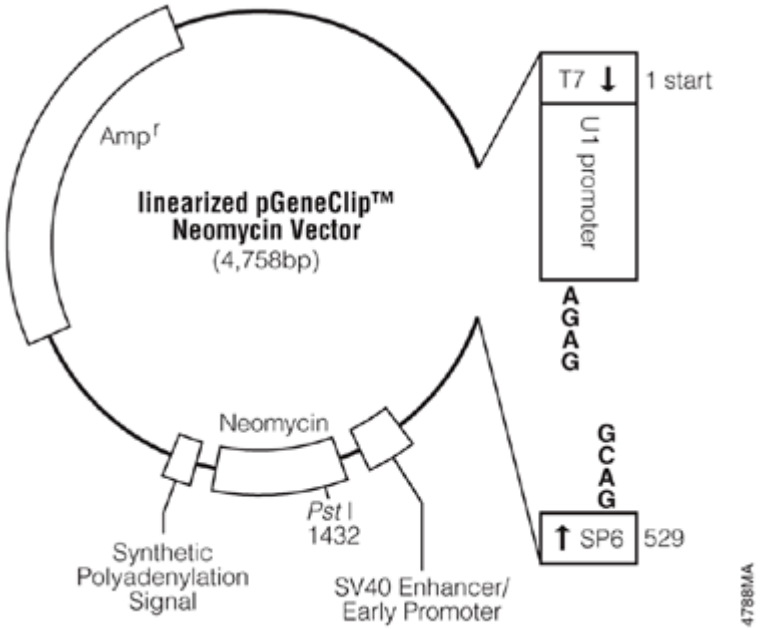
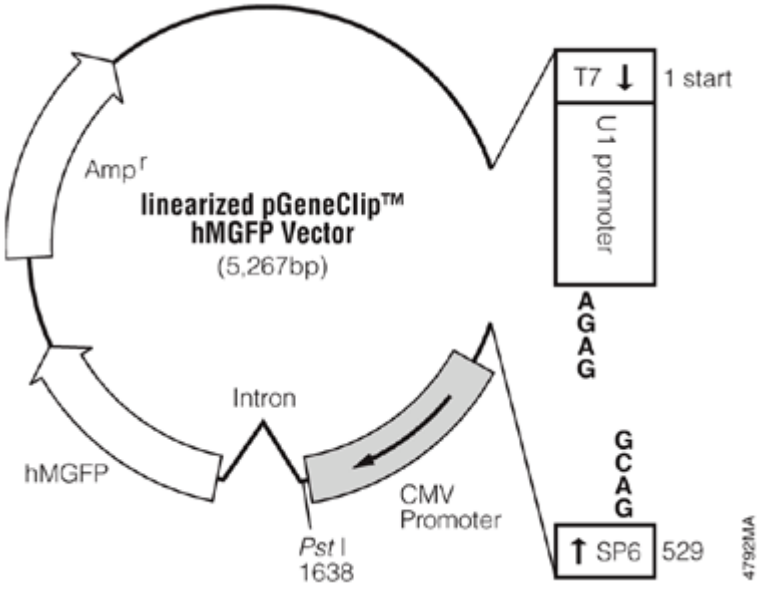
Promega Corporation's GeneClip™ U1 Hairpin Cloning System—hMGFP GeneClip™ U1 Hairpin Cloning System—Neomycin



Promega Catalog Number C8780



Promega Catalog Number C8790

SureSilencing™ shRNA Features

- **Promega GeneClip™ Plasmid Backbone**
  - Bacterial origin of replication and ampicillin-resistance marker
  - Choice of one of two mammalian markers is available and included on all plasmids per gene.
    - Neomycin for stable transfections
    - GFP for transient transfections
  - U1 promoter, transcribed by RNA Polymerase II
    - Normally transcribes mRNA
    - Provides moderate shRNA expression level
    - Minimizes non-specific off-target and toxic side effects

SureSilencing™ shRNA Features

- **SuperArray's shRNA Design Algorithm**
  - Experimentally verified computer algorithm insures gene-specificity and efficacy.
  - Minimizes off-target effect with an advanced specificity search in addition to BLAST.
  - Includes filters for many of the chemical and sequence properties of shRNA known to be important for activity.
  - Effective (>70%) knock down determined by rigorous real-time RT-PCR assay.
  - Pre-design performed for every human, mouse, and rat gene based on the latest annotation from the NCBI.

TYPICAL CUSTOMER FEATURES

- **Users tired of repeated siRNA purchases**
- **Using stable (transformed, 2ndary) but …**

  More-difficult-to-transfect cell lines
  - Not using primary or macrophages or doing animal work
  - Recommend viral delivery system instead
- **Stable OR Transient transfection applications (50:50)**
- **Cell biologists with less molecular experience**
  - Purchase GFP and Validate by Western
  - Difficult to convince of need for real-time PCR