



Product Information

Automated Protocol for Quantitative PCR Kits Using the Sciclone ALH 3000 Workstation (Caliper Life Sciences)

Quantitative PCR Product Codes S4438, S5193, S9194, QR0100, D6442, D7440, and D9191

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Automation Guide

I. Description

Sigma-Aldrich offers a full range of automation-compatible, high-throughput Quantitative PCR (QPCR) products for use with either probe-based or SYBR[®] Green based applications to provide accurate real-time quantification of DNA or RNA templates. Each product is conveniently packaged as a 2x ReadyMix[™] to include everything necessary for QPCR, leaving only the addition of primers, template, and in the case of probe-specific mixes, fluorescent detection chemistry. Each ReadyMix contains Sigma's antibody mediated hot start mechanism, JumpStart[™] Taq polymerase, for highly specific amplification.

Sigma's QPCR products include:

- JumpStart Taq ReadyMix Kits (D6442, D7440, and D9191) are formulated for use in probe-based Quantitative PCR applications.
- SYBR Green JumpStart Taq ReadyMix Kits (S9194, S4438, and S5193) combine the performance enhancements of JumpStart Taq antibody for hot start PCR with SYBR Green I Dye.
- SYBR Green Taq ReadyMix Kit for Quantitative RT-PCR (QR0100) includes the Enhanced Avian Reverse Transcriptase (eAMV[™] RT) and JumpStart Taq.

An automated method has been created and validated for use with Sigma's Quantitative PCR ReadyMixes utilizing the Sciclone ALH 3000 Liquid Handling Workstation from Caliper Life Sciences. In 15 minutes, the Sciclone ALH 3000 can complete QPCR reaction setup for 96 samples.

II. Product Components

Product Code	JumpStart Taq ReadyMix Kits For Quantitative PCR D6442, D7440 and D9191	SYBR Green JumpStart Taq ReadyMix Kits S9194, S4438 and S5193	SYBR Green Taq ReadyMix Kits for Quantitative RT-PCR QR0100
Applications	Probe-based applications	SYBR Green based applications	SYBR Green based RT-PCR applications
Package Size	200 or 800 PCR Reactions	200 or 1,000 PCR Reactions	200 RT-PCR Reactions
Reaction Volume	20 µl	20 µl	20 µl
Reagents Provided	JumpStart Taq ReadyMix Reagent	SYBR Green JumpStart Taq ReadyMix Reagent	SYBR Green Taq ReadyMix Reagent for Quantitative RT-PCR
	Magnesium Chloride	Magnesium Chloride (part of S5193 only)	Magnesium Chloride
	Internal Reference Dye (part of D7440 and D9191)	Internal Reference Dye (part of S4438 and S5193)	Internal Reference Dye
			eAMV RT
			10x PCR Buffer

III. Storage

Store JumpStart Taq ReadyMix reagent at 2–8 °C for up to 6 months. For long-term storage (up to 18 months), store at –20 °C. Store the Reference dye for quantitative PCR and magnesium chloride solution at 2–8 °C.

Store SYBR Green JumpStart Taq ReadyMix reagent at 2–8 °C for up to 3 months. For long-term storage (up to one year), store at –20 °C.

Store SYBR Green Taq ReadyMix reagent for RT-PCR at –20 °C. Protect from light.

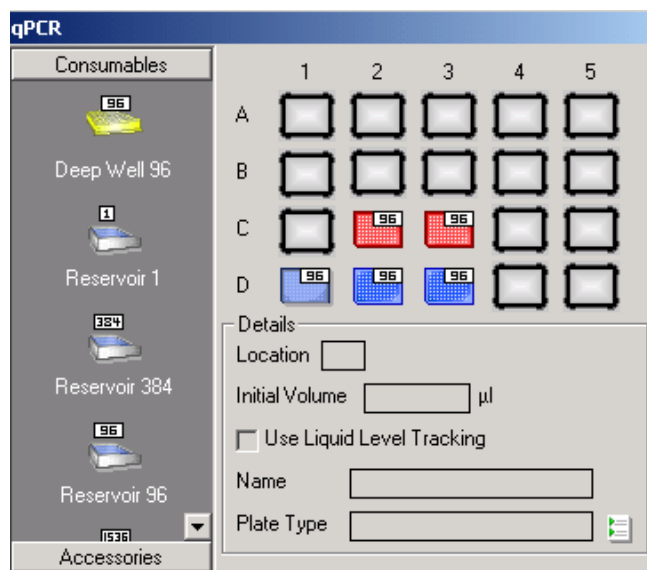
IV. Materials to Be Supplied by the User

1. Primers for genes of interest
2. Dual-labeled fluorescent probes for use with D6442, D7440 and D9191 (Sigma-Genosys)
3. DNA or RNA template
4. Water, molecular biology reagent (Sigma, W4502 or W1754)
5. (Optional) Deoxyribonuclease I, Amplification Grade Kit (Sigma, AMPD1) for use with QR0100
6. Dedicated pipettes
7. Aerosol resistant pipette tips
8. 96-well PCR plates, with half skirt (ABgene, AB-1100)
9. Ultra clear cap strip (ABgene, AB-0866)
10. Corning plate holder (Corning, 6525)
11. 12-column reagent reservoir with low profile (Innovative Microplates, S30028)
12. (Optional) 12-column reagent reservoir with high profile (Innovative Microplates, S30019)
13. Thermal cycler for quantitative PCR

V. Instrument Requirements for the Sciclone ALH 3000 Workstation

Part Description	Qty	Ordering Information
Z8™ Pipettor	1	Contact Caliper
Deck Locator	3	Contact Caliper
Tip Box Locator	2	# 76523 (Caliper)
80 µl Barrier Tip Box	2	# 68759 (Caliper)

VI. Deck Layout



Deck Position	Equipment
C2	80 µl Barrier Tip box
C3	80 µl Barrier Tip box
D1	12-column reservoir for PCR master mix
D2	96-well PCR plate with half skirt seated in a plate holder (PCR amplification plate)
D3	96-well PCR plate with half skirt seated into a plate holder (containing samples for quantification)

VII. Automated Method Description

A. Methods

Three methods have been created for this application:

MAIN_qPCR: Performs all steps necessary to set up 96 quantitative PCR reactions including transferring the master mix and DNA or RNA template into the PCR amplification plate. This method should be used with SYBR Green JumpStart Taq ReadyMix kits (S9194, S4438 and S5193), SYBR Green Taq ReadyMix kits for Quantitative RT-PCR (QR0100), and JumpStart Taq ReadyMix kits (D6442, D7440 and D9191).

SUB_Tip_Touch_12 Column Reservoir: This sub-routine is called up in the main method to perform the tip touching steps after aspirating the PCR master mix from the 12-column reagent trough. This method must be modified if using a different trough for the PCR master mix.

SUB_Tip_Touch_ABgene Plate: This sub-routine is called up in the main method to perform the tip touching steps after aspirating the DNA or RNA controls from the ABgene plate. This sub-routine is also called up to perform tip touching steps after dispensing the DNA or RNA samples into the PCR amplification plate. This method must be modified if using a different plate for the PCR amplification.

B. Getting Started

1. Set up deck layout: place the tip boxes, plates, and reservoirs at the appropriate positions on the deck as described in Section VI.
2. Add reagents to the appropriate reservoirs as described in Sections VII-X.
3. Run the method using Sciclone Software Version 3.2.
4. At the completion of the method, place the cap strips onto the PCR plate, vortex to mix the solution and briefly centrifuge.
5. The PCR plate is now ready for placement into the thermal cyclers.

C. Method Overview

Below is a summary of the steps of the *MAIN_qPCR* method. For complete program details, download the automation program from www.sigmaldrich.com/automation.

1. The PCR master mix (18 μ l) is dispensed into a third of the PCR plate with the Z8 head. This is repeated until the PCR master mix is added to all 96 wells of the PCR plate.
2. A loop has been created in the program, that allows for all DNA or RNA samples (2 μ l) to be dispensed into the PCR plate with the Z8 head.

VIII. JumpStart Taq ReadyMix Kits

A. Reagent Preparation

1. ***PCR Master Mix Preparation:*** The JumpStart Taq ReadyMix reagent for Quantitative PCR is a 2x reaction mixture containing buffer, salts, gelatin, dNTPs, stabilizers, Taq DNA polymerase, and JumpStart Taq antibody. To prepare a Master mix add primers, dual-labeled fluorescent probe, reference dye, and water to the JumpStart Taq ReadyMix reagent as described in the table below.

Note: A reference dye is needed when running the samples on the ABI PRISM® 7700 Detection System. The reference dye is already added to the JumpStart Taq ReadyMix if using Product Code D6442.

Stock	Water	PCR Mix (P2893)	Magnesium Chloride (M8787)	Forward Primer (100 µM)	Reverse Primer (100 µM)	Fluorescent Probe (100 µM)	Reference Dye (100X)
PCR Master Mix (2.70 ml)	915 µl	1.5 ml	240 µl	6 µl	6 µl	3 µl	30 µl

To set up one 96-well plate of 20 µl PCR reactions, a total of 2.70 ml PCR master mix needs to be added to the first column of reservoir located at position D1. If setting up more than 3 plates of samples for PCR, it will be necessary to use reservoir S30019.

2. ***DNA Master Plate Preparation:*** To prepare a DNA master plate, add 30 µl of template DNA to each desired well.
3. ***No-template Control (optional):*** Add water into the desired wells of the 96-well plate containing DNA samples located in position D3.
4. ***DNA Controls (optional):*** Prepare genomic DNA controls for quantification of the DNA. These controls should be placed in the 96-well plate containing DNA samples located in position D3.

B. Recommended Parameters for PCR Amplification

Step	Temperature	Time	Cycles
Initial Denaturation	94–96 °C	2 minutes	1
Denaturation	94–96 °C	15 seconds	40
Annealing	45–68 °C	1 minutes	
Extension	72 °C	1–2 minutes (~1 kb/min)	
Final Extension (Detection)	80 °C	25 seconds	
Hold	4 °C	Indefinitely	

C. Performance Characteristics

Amplification of Human Genomic DNA Samples

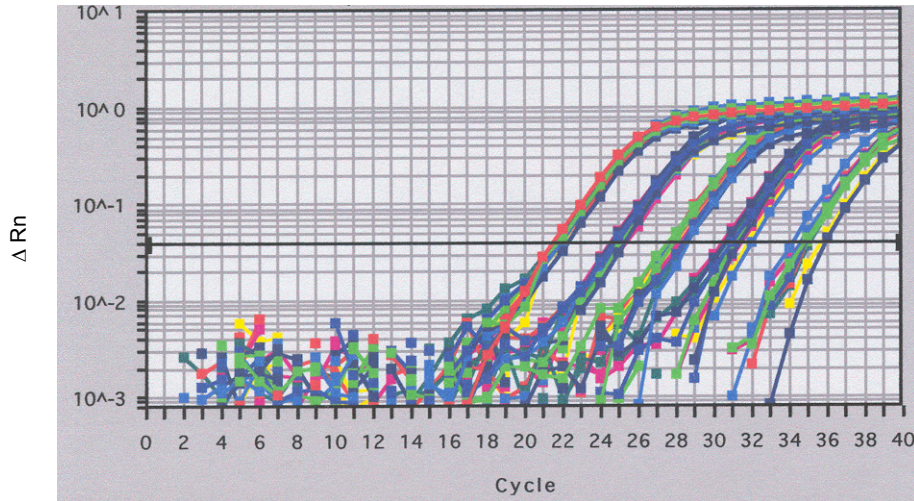


Figure 1. A PCR master mix containing JumpStart Taq ReadyMix reagent, GAPDH forward and reverse primers, and probe set was added to dilutions of human genomic DNA using the automated method for the Sciclone ALH 3000 Liquid Handling Workstation. GAPDH specific primers were designed to produce a 284 bp amplicon. The dual labeled probe was designed with a FAM reporter and a dark hole quencher. Initial template copy number was 9,000 and diluted 10-fold in subsequent wells. Amplification was carried out for the following concentrations of DNA in 12 replicates: 15 ng/ μ l, 1.5 ng/ μ l, 150 pg/ μ l, 15 pg/ μ l, and no template control, as pictured here from left to right. Reactions were carried out on the ABI PRISM 7700 Sequence Detection System.

Linearity of Human Genomic DNA

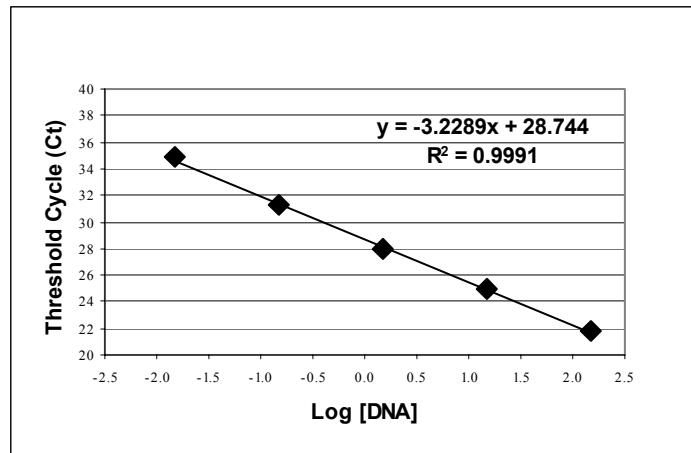


Figure 2. The average Ct values calculated were plotted versus the log of the concentration of genomic DNA. The slope and R^2 value represent linearity over 5 logs.

Cross-Contamination Analysis

	1	2	3	4	5	6	7	8	9	10	11	12
A	28.6	40	28.8	40	28.3	40	28.1	40	28.7	40	28.4	40
B	40	28.3	40	28.7	40	28.5	40	28.6	40	28.7	40	28.2
C	28.3	40	28.5	40	28.7	40	28.4	40	28.3	40	28.4	40
D	40	28.1	40	28.3	40	28.7	40	28.6	40	28.2	40	28.4
E	27.9	40	27.9	40	28.6	40	28.6	40	28.5	40	28.2	40
F	40	28.2	40	28.1	40	28.7	40	28.3	40	28.5	40	28.1
G	27.7	40	28.1	40	28.2	40	28.5	40	28.4	40	28.4	40
H	40	28.1	40	28.0	40	28.1	40	28.2	40	28.2	40	28.0

Figure 3. To test for cross contamination, a method was developed in which two different master mixes were dispensed across a 96-well plate to alternating wells. The first master mix contained the JumpStart Taq ReadyMix reagent, human genomic DNA (1.5 ng/ μ l), GAPDH primers, and probe set. The second mix included everything in the first mix except the genomic DNA. All samples were subjected to amplification on the ABI PRISM 7700 Sequence Detection System. The numbers shown in above table indicate the values of Cycle Threshold (Ct) for each well. The wells without genomic DNA (in yellow) showed no evidence of amplified PCR products, demonstrating that no cross contamination is present.

IX. SYBR Green JumpStart Taq ReadyMix Kits

A. Reagent Preparation

1. ***PCR Master Mix Preparation:*** The SYBR Green JumpStart Taq ReadyMix reagent is a 2X reaction mixture containing buffer, salts, dNTPs, stabilizers, Taq DNA polymerase, JumpStart Taq antibody and SYBR Green I dye. To prepare a master mix add primers, reference dye, and water to the SYBR Green Taq ReadyMix reagent as described in the table below.

Note: A reference dye is needed when running the samples on the ABI PRISM 7700 Detection System. The reference dye is already added to the SYBR Green JumpStart Taq ReadyMix if using Product Code D9194.

Stock	Water	PCR Mix (S9939)	Forward Primer (20 µM)	Reverse Primer (20 µM)	Reference Dye (100x)
PCR Master Mix (2.70 ml)	1.125 ml	1.5 ml	30 µl	30 µl	15 µl

To set up one 96-well plate of 20 µl PCR reactions, a total of 2.70 ml PCR master mix needs to be added to the first column of the reservoir located at position D1. If setting up more than 3 plates of samples for PCR, it will be necessary to use the high profile reagent reservoir (S30019).

2. ***DNA Master Plate Preparation:*** To prepare a DNA master plate, add 30 µl of template DNA to each desired well.
3. ***No-template Control (optional):*** Add water into the desired wells of the 96-well plate containing DNA samples located in position D3.
4. ***DNA Controls (optional):*** Prepare genomic DNA controls for quantification of the DNA. These controls should be placed in the 96-well plate containing DNA samples located in position D3.

B. Recommended Parameters for PCR Amplification

Step	Temperature	Time	Cycles
Initial Denaturation	94–96 °C	2 minutes	1
Denaturation	94–96 °C	15 seconds	40
Annealing	45–68 °C	1 minute	
Extension	72 °C	1–2 minutes (~1 kb/min)	
Final Extension (Detection)	80 °C	25 seconds	
Hold	4 °C	Indefinitely	

C. Performance Characteristics

Amplification of Human Genomic DNA Samples

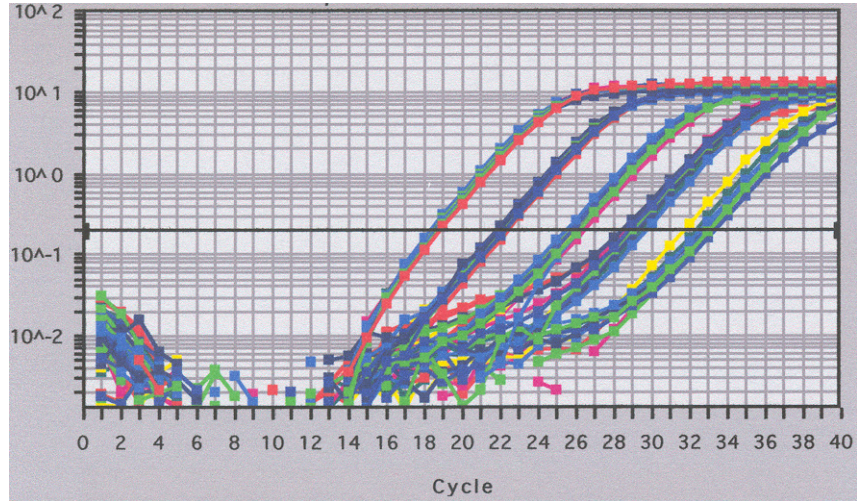


Figure 4. A PCR master mix containing SYBR Green JumpStart Taq ReadyMix reagent and GAPDH forward and reverse primers was added to dilutions of human genomic DNA template using the automated method for the Sciclone ALH 3000 Liquid Handling Workstation. GAPDH specific primers were designed to produce a 284 bp amplicon. Initial template copy number was 9,000 and diluted 10-fold in subsequent wells. Amplification was carried out for the following concentrations of DNA in 12 replicates: 15 ng/ μ l, 1.5 ng/ μ l, 150 pg/ μ l, 15 pg/ μ l, 1.5 pg/ μ l, and no template control, as pictured here from left to right. Reactions were carried out on the ABI PRISM 7700 Sequence Detection System.

Linearity of Human Genomic DNA

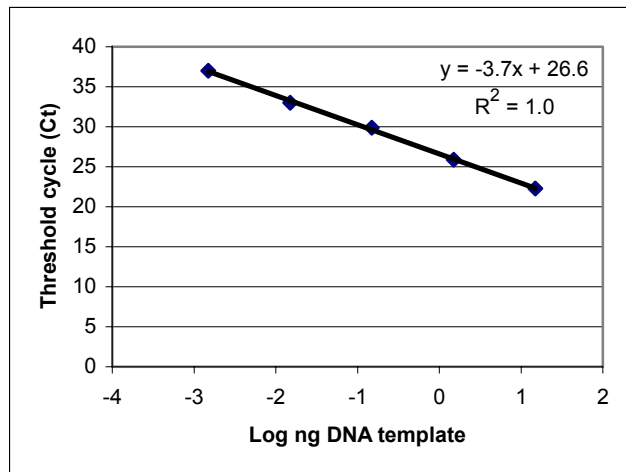


Figure 5. The average Ct values were calculated and plotted versus the log of the concentration of genomic DNA. The slope and R^2 value are linear over 5 logs.

X. SYBR Green Taq ReadyMix Kits for Quantitative RT-PCR

A. RNA Template Preparation

RNA template must be treated with DNase prior to qRT-PCR. Below is a general protocol for DNase treatment. Please consult the Deoxyribonuclease I Amplification Grade Technical Bulletin (AMPD1) for more details.

1. Prepare working solution as indicated in the table below

	Water	Total RNA (1 µg/µl)	Reaction Buffer, 10x (R6273)	DNase I (D5307)
Working (250 µl)	175 µl	25 µl	25 µl	25 µl

2. Incubate the reaction at room temperature for 15 minutes.
3. Add 25 µl of the Stop Solution (S4809).
4. Heat the DNase-treated RNA solution at 70 °C for 10 minutes.
5. Store the RNA solution on ice until use.
6. Make a serial dilution of 50 ng/µl, 5 ng/µl, 0.5 ng/µl, 50 pg/µl, and 5 pg/µl.
7. Aliquot 30 µl of each diluted RNA into each well of a 96-well plate in 12 replicates.

B. Reagent Preparation

1. **PCR Master Mix Preparation:** The SYBR Green Taq ReadyMix reagent for Quantitative PCR is a 2x reaction mixture containing buffer, salts, dNTPs, stabilizers, glass passivator, RNase Inhibitor, Taq DNA polymerase, JumpStart Taq antibody and SYBR Green I dye. To prepare a master mix for one-step RT-PCR reactions, add magnesium chloride, primers, reference dye, eAMV, and water to the SYBR Green Taq ReadyMix reagent as described in table below.

Stock	Water	PCR Mix (D5191)	Magnesium Chloride (M8787)	Forward Primer (100 µM)	Reverse Primer (100 µM)	eAMV RT (A4714)	Reference Dye (100x)
RT-PCR Master Mix (2.7 ml)	910 µl	1.5 ml	240 µl	6 µl	6 µl	7.5 µl	30 µl

To set up one 96-well plate of 20 µl PCR reactions, a total of 2.7 ml RT-PCR master mix needs to be added to the first column of reservoir located at position D1. If setting up more than 3 plates of samples for RT-PCR, it will be necessary to use the high profile reagent reservoir (S30019).

2. **BSA-1x TE Solution:** Prepare a BSA-1x TE Solution for use in diluting the RNA for the controls, according to the following table.

	Water	Tris-EDTA Buffer, 10x (T9285)	BSA (B8667)
Working (5.0 ml)	4.825 ml	50 µl	125 µl

3. RNA Controls (optional): Prepare DNase-Treated RNA controls for quantification of cDNA. DNase-Treated RNA was diluted with the BSA-1x TE Solution and placed in rows of the 96-well plate located in position D3.
4. No-template Control (optional): Add BSA-1x TE solution into the desired wells of the 96-well plate containing RNA samples located in position D3.
5. No RT Control: Prepare the PCR Master Mix without adding the eAMV Reverse Transcriptase and place in the 12-column reservoir located at position D1.

C. Recommended Parameters for PCR Amplification

Step	Temperature	Time	Cycles
First Strand Synthesis	48–50 °C	30 minutes	1
Denaturation/RT Inactivation	94–96 °C	2 minutes	1
Denaturation	94–96 °C	15 seconds	40
Annealing	45–68 °C	30 seconds	
Extension	72 °C	1–2 minutes (~1 kb/min)	
Final Extension (Detection)	81 °C	25 seconds	
Hold	4 °C	Indefinitely	

D. Performance Characteristics

Amplification of Human Total RNA Samples

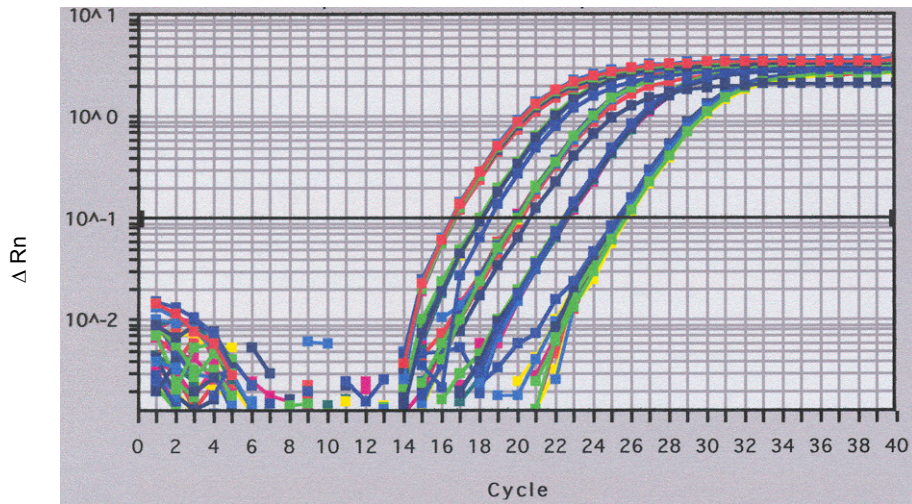


Figure 6. A PCR master mix containing SYBR Green Taq ReadyMix reagent for qRT-PCR, GAPDH forward and reverse primers was added to dilutions of human total RNA template using the automated method for the Sciclone ALH 3000 Liquid Handling Workstation. GAPDH specific primers were designed to produce a 284 bp amplicon. Amplification was carried out for the following concentrations of total RNA in 12 replicates: 50 ng/ μ l, 5 ng/ μ l, 500 pg/ μ l, 50 pg/ μ l, and 5 pg/ μ l, as pictured here from left to right. Reactions were carried out on the ABI PRISM 7700 Sequence Detection System.

Linearity of Human Total RNA Dilutions

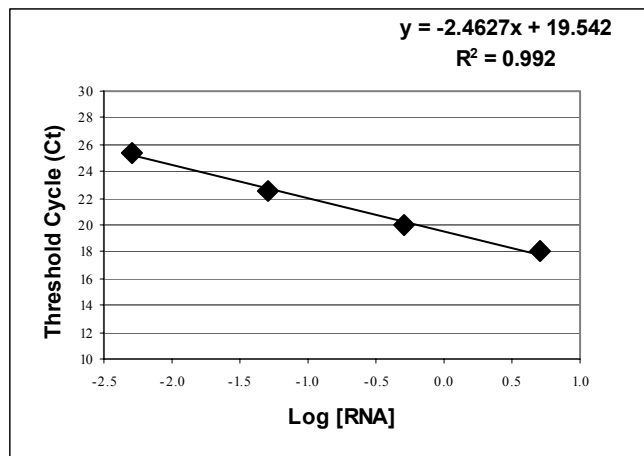


Figure 7. The average Ct values calculated were plotted against the log of the concentration of genomic DNA. The slope and R^2 value represent linearity over 4 logs.

XI. Method Customization

A. Use of a different PCR plate

The automated method was created using the 96-well PCR plates with half skirt from ABgene. Other PCR plates may be used in this method, but may require the creation of a new labware in the Sciclone software. If a different PCR plate is used, adjustments will need to be made to the tip touch sub-routines: SUB_Tip_Touch_12 Column Reservoir and SUB_Tip-Touch_ABgene Plate. Tip touching is necessary to achieve accuracy and precision during low volume dispensing steps such as the addition of DNA or RNA to the reaction mixture.

B. PCR setup using multiple primer sets

If it is desired to use different primer sets across a 96-well plate of PCR reactions, primers can be added to a new column in the 12-column reservoir for the PCR master mix. A step will need to be added to the program to account for the addition of primer to the PCR amplification plate.

C. PCR setup using No Reverse Transcriptase

If it is desired to use a negative control for qRT-PCR reactions, a new master mix with no eAMV RT can be added to a new column in the 12-column reservoir.

D. Use of the multichannel head

The automated method was created using the Z8 Pipettor, in order to conserve PCR master mix. If it is desired to pipette all 96-wells simultaneously, the Sciclone needs to be configured with the Low Volume Dispense Module from Caliper. Instead of using a reagent reservoir, PCR master mix will need to be aliquoted to a 96-well plate.

E. Set up of 384 PCR reactions

The Sciclone ALH 3000 Liquid Handling Workstation can access 384-well plates with a 96-well head. The instrument will access all of the wells by dividing the plate into four "quadrants". Each grouping of four wells on a 384-well plate is equivalent to the size of one well on a 96-well plate.

If transfer to 384-well plates is desired simultaneously, Caliper offers the 384-Channel Low Volume EZ-Swap Sciclone Head.

XII. Troubleshooting

A. General qPCR

Problem	Cause	Solution
Little or no PCR product is detected.	A PCR component is missing or degraded.	Run a positive control to ensure components are functioning.
	Too few cycles are performed.	Increase the number of cycles (5–10 additional cycles at a time).
	The annealing temperature is too high.	Decrease the annealing temperature in 2–4 °C increments.
	The primers are degraded.	Check for primer degradation on polyacrylamide gel. If the primers are degraded, new primers need to be ordered.
	The primers are not designed optimally.	Confirm the accuracy of the sequence information. If the primers are less than 27 nucleotides long, try to lengthen the primer to 27–33 nucleotides. If the primer has a GC content of less than 45%, try to redesign the primer with a GC content of 45–60%.
	There is not enough template.	After increasing the number of cycles has shown no success, repeat the reaction with 10-fold higher concentration of the template.
	The template is of poor quality.	Evaluate the template integrity by agarose gel electrophoresis. It may be necessary to repurify template using methods that minimize shearing and nicking.
	The target template is difficult.	In most cases, inherently difficult targets are due to unusually high GC content and/or secondary structure. Betaine has been reported to help amplification of high GC content templates at a concentration of 0.8–1.3 M. [Rees, W. <i>et al.</i> , <i>Biochemistry</i> , 32 , 137-144 (1993)]
	The PCR product is too long.	The best results are obtained when the PCR product is 100–150 bp long and does not exceed 800 bp.
	The Mg ²⁺ concentration is not optimal.	Start with the magnesium concentration provided in SYBR Green Taq ReadyMix. A 25 mM vial of magnesium chloride is provided if increased magnesium concentration is needed for optimal results.
Others	Refer to the Technical Bulletin of the Quantitative PCR Kits.	

Signal is independent of template dilution.	The annealing temperature is too low.	Increase the annealing temperature in increments of 2–3 °C.
	The primers are not designed optimally.	Confirm the accuracy of the sequence information. If the primers are less than 27 nucleotides long, try to lengthen the primers to 27–33 nucleotides. If the primer has a GC content of less than 45%, try to redesign the primers with a GC content of 45–60%.
	The template concentration is too high.	Reduce the concentration of the template in the PCR reaction.
	The primer concentration is too high.	Reduce the primer concentrations in a series of two-fold dilutions (i.e. 0.1 μM, 0.05 μM, 0.025 μM and 0.0125 μM) and subject these trial reactions to PCR.
Large variability within samples and/or duplicates.	Reactions not mixed well.	Gently vortex and centrifuge reactions.
	Wells not tightly capped or covered.	Tightly cap or cover all wells, even the empty ones.
	Initial denaturation is too long.	Decrease the initial denaturation to not exceed two minutes.

B. Instrument Specific

Problem	Instrument	Cause	Solution
Little or no PCR product (signal) is observed.	Sciclone ALH 3000	Bad performance of the Liquid Handler	Purge the Z8 module with system fluid to get rid of air bubbles in the system. Test the pipetting accuracy and precision of Z8 module and contact Caliper Technical Support to fix or replace the identified bad pipettor.
	ABI Sequence Detection Systems	Detection was not activated.	Activate SYBR fluorescence detection in the cycling program.
		Wrong dye layer chosen.	Ensure the reporter being used is activated in the setup view of the Sequence Detection Software.
		Incorrect values on Y-axis.	Change the values on the y-axis. By double clicking on ΔR_n , the values of the y-axis can be changed.
Varying fluorescent intensity	ABI Sequence Detection Systems	Wavy amplification curves at high temperature amounts	Reduce the number of cycles used for the baseline calculation.
		Improper exposure time.	Change the exposure time appropriately if using caps (25) or optical adhesive covers (10).
		Wrong quencher activated.	Ensure the proper quencher is activated in the setup view.

C. Quantitative RT-PCR Specific

Problem	Cause	Solution
No RT-PCR product (signal) is observed, RT-PCR product is detected late in PCR, or only primer-dimers are detected	Reverse Transcriptase is not active.	Switch to a new lot of the enzyme.
	RNA template is degraded.	Isolate the RNA samples with precaution to ensure no contamination of RNase.
	The denaturation time is too long or too short.	Optimize the denaturation time by increasing or decreasing it in 10-second increments.
	Magnesium concentration is not optimal.	Start with the magnesium concentration provided in SYBR Green Taq ReadyMix. A 25 mM vial of magnesium chloride is provided if increased magnesium concentration is needed for optimal results.
Multiple RT-PCR products.	Reactions set up at room temperature.	Set up RT-PCR reactions on ice to avoid premature cDNA synthesis from nonspecific primer annealing.
	JumpStart Taq activated too early.	Complete the reverse transcription reaction before the denaturation step, which inactivates the JumpStart antibody.
	Genomic DNA is contaminating the RNA template in the reverse transcriptase reaction.	Digest the RNA with RNase-free DNase (Product Code AMPD1). When possible, use primers that span an intron so that amplification from genomic DNA is minimized.
	Primer-dimers are co-amplified.	Include an additional detection step in the cycling program to avoid detection of primer-dimers.
	Reverse transcription reaction temperature is too low.	Start out a reaction temperature between 42–50 °C. The reverse transcription reaction temperature may be increased if mispriming is detected.
No linearity in ratio of C_T value to log of the template amount.	Genomic DNA is contaminating the RNA template in the reverse transcriptase reaction	Digest the RNA with RNase-free DNase (Product Code AMPD1).

XIII. Contact Information

Technical Service
(800) 325-5832
Email: techserv@sial.com

Customer Service
(800) 325-3010
(800) 588-9160
www.sigma-aldrich.com/order

Purchase of this product is accompanied by a limited license for use in the Polymerase Chain Reaction (PCR) process for research purposes only and in conjunction with a thermal cycler whose use in the automated performance of the PCR process is covered by an up-front license fee, either by payment to Applied Biosystems or as purchased, i.e., and authorized thermal cycler. Antibody licensed for *in vitro* research use under U.S. Patent No. 5,338,671 and 5,587,287, and corresponding patents in other countries. SYBR Green nucleic acid stain is licensed from Molecular Probes, Inc. under US Patent No. 5,436,134 for use in Real-Time PCR by end-users for research and development only. SYBR is a registered trademark of Molecular Probes, Inc. The addition of betaine has been reported to enhance the specificity of the polymerase chain reaction by eliminating the base pair composition dependence of DNA melting (Rees, WA et al).

The purchase of this product does not include a license to practice the claims of Patent Nos. US 5,545,539, US 6,270,962, DE4411588, or DE4411594. The practice of the claims of these patents may require a license from the patent owners.

ABI PRISM is a registered trademark of Applied Biosystems.

Z8 is a trademark of Caliper Life Sciences, Inc.

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