Stratagene QPCR Human Reference Total RNA

Instruction Manual

Catalog #750500

Revision B

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750500-12

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Stratagene QPCR Human Reference Total RNA

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Stratagene QPCR Human Reference Total RNA

MATERIALS PROVIDED

Catalog #750500

Materials provided	Quantity
Stratagene QPCR Human Reference Total RNA	25 µg (1 µg/µl in 0.1 mM EDTA/RNase-free H_2O)

STORAGE CONDITIONS

All Components: –80°C

ADDITIONAL MATERIALS REQUIRED

Spectrofluorometric thermal cycler
QRT-PCR reagents [e.g. Stratagene Brilliant II QRT-PCR Master Mix Kit, 1-Step (Catalog #600809)]
Nuclease-free PCR-grade water

Revision B

INTRODUCTION

Stratagene QPCR Human Reference Total RNA is a high-quality control for quantitative PCR gene-expression analysis. QPCR Human Reference Total RNA is composed of total RNA from 10 human cell lines (see the table below), with quantities of RNA from the individual cell lines optimized to maximize representation of gene transcripts present in low, medium, and high abundance. The reference RNA is carefully screened for contaminating genomic DNA, the presence of which can complicate interpretation of QRT-PCR assay data.

Stratagene QPCR Human Reference Total RNA Cell Line Derivations
Adenocarcinoma, mammary gland
Hepatoblastoma, liver
Adenocarcinoma, cervix
Embryonal carcinoma, testis
Glioblastoma, brain
Melanoma, skin
Liposarcoma
Histiocytic lymphoma; macrophage; histocyte
Lymphoblastic leukemia, T lymphoblast
Plasmacytoma; myeloma; B lymphocyte

Assay Optimization Applications

The Stratagene QPCR Human Reference Total RNA is ideally suited for optimizing QRT-PCR assays. Often only small amounts of experimental RNA template are available for setting up an expression profiling study. Using the extensive representation of specific mRNA species in the generic template, assays may be optimized for a variety of primer/probe systems. This eliminates the use of precious experimental RNA samples for assay optimization.

Protocols for RT-PCR assay optimization using the QPCR Human Reference Total RNA in conjunction with Stratagene's Brilliant II QRT-PCR master mix, 1-step (for TaqMan® or molecular beacons probes) are provided in the *Assay Optimization Protocols* section. These protocols and guidelines may be adapted for use with other reagent systems and platforms.

Guidelines for SYBR® Green dye assay optimization are also provided in *QRT-PCR Assay Optimization Considerations*. The Stratagene QPCR Human Reference Total RNA is especially useful in optimization of SYBR Green assays. SYBR Green I dye binds non-selectively to any double-stranded DNA, and thus detects primer-dimers as well as the specific amplicon. This problem is especially pronounced at low target concentrations. Very careful primer optimization is required to minimize the formation of non-specific amplification products. Use of the Stratagene QPCR Human Reference Total RNA allows extensive experimentation during assay development without depleting the experimental RNA.

Assay Standardization Applications

Using the QPCR Stratagene Human Reference Total RNA as a reference control allows data comparisons from multiple experiments, across platforms, and between laboratories. The reference RNA is produced in extremely large lots and subjected to stringent quality-control measures to ensure the availability of consistent reference RNA material over long-term experimental studies. See *Assay Standardization Considerations* for more information.

Sample Standard Curve Data Generated with the Stratagene QPCR Human Reference Total RNA

Results from an RT-PCR reaction monitored in real-time can be displayed as an amplification plot, which reflects the change in fluorescence during cycling. This information can be used to quantitate initial copy number based on the threshold cycle (Ct).[1] Ct is defined as the cycle at which fluorescence is determined to be statistically significant above background and is inversely proportional to the log of the initial copy number.[1] The more template that is initially present, the fewer the number of cycles it takes to reach the point where the fluorescence signal is detectable above background. The threshold cycle is based on measurements taken during the exponential phase of PCR amplification when PCR efficiency is not yet influenced by limiting reagents, small differences in reaction components, or cycling conditions. Ct values determined for a set of standard wells, containing known amounts of the template RNA, may be plotted to generate a standard curve that can be used to assess the quality of the QRT-PCR assay. Standard curve data may also be used to compare and assess differences in results obtained from the same assay system across experiments, platforms, or researchers.

Figure 1 shows standard curves for targets of high, medium and low abundance for serial dilutions of the Stratagene QPCR Human Reference Total RNA. The standard curve data were generated on the Mx4000 instrument for QRT-PCR reactions prepared with the Brilliant II QRT-PCR master mix, 1-step, according to the protocol provided in this manual. The table below shows the R^2 values and standard curve slopes calculated by the instrument for each target. The R^2 value (always between 0 and 1) is an indication of the quality of the fit of the standard curve to the standard data points plotted, with values closer to 1 indicating a better fit of the data to the line. The slope of the standard curve is directly related to the average efficiency of amplification throughout the cycling program and may be used to calculate the PCR efficiency for a given template in a given experiment. A reaction with 100% efficiency will produce a slope of –3.32.

Target	Target Abundance	R^2 Value	Slope	Efficiency (%)
cyclophilin	high	0.999	-3.40	96.8
GUS	medium	1.000	-3.37	98.0
IL-5	low	0.998	-3.29	101.3

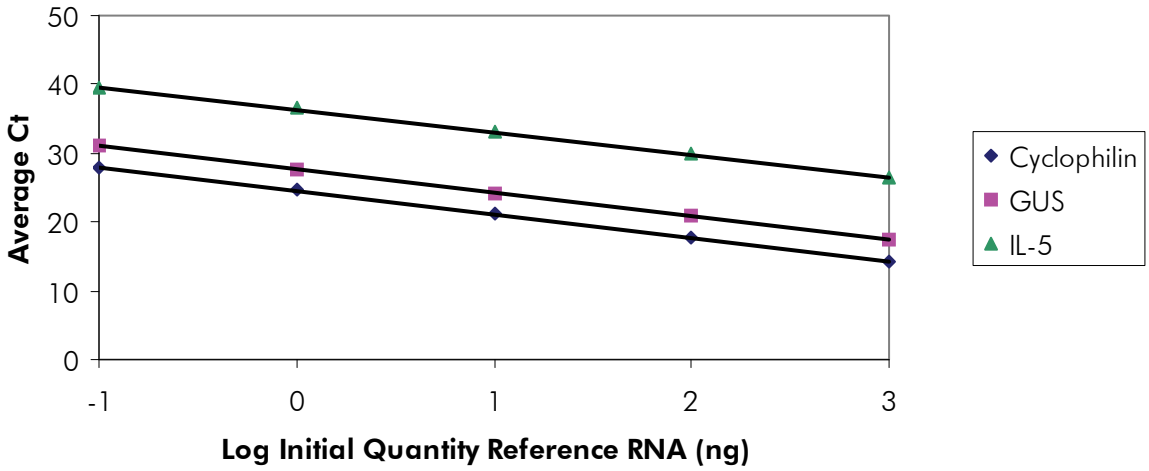

FIGURE 1 Standard curves for the high-abundance target cyclophilin (bottom curve; diamonds), the medium-abundance target GUS (middle curve; squares) and the low-abundance target IL-5 (top curve; triangles). The Stratagene QPCR Human Reference Total RNA was added as 10-fold serial dilutions from 1000 ng to 0.1 ng. QRT-PCR reactions were prepared using the Brilliant QRT-PCR master mix, 1-step, and TaqMan probes. The real-time fluorescence data were analyzed on the Mx4000 multiplex quantitative PCR instrument.

QRT-PCR ASSAY OPTIMIZATION CONSIDERATIONS

Summary of Primer and Probe Optimization Recommendations

Detection Chemistry	Primer Concentration Optimization Range (nM)	Probe Concentration Optimization Range (nM)
SYBR® Green I dye	50–300	—
TaqMan® probe	50–600	100–500
Molecular beacon	200–600	200–500

SYBR® Green Assays

Primer Optimization for SYBR® Green Assays

Detection with SYBR Green I dye requires very careful primer optimization. It is critical to minimize the formation of non-specific amplification products. This issue becomes more prominent at low target concentrations. To maximize the sensitivity of the assay, it is necessary to use the lowest concentration of primers possible without compromising the efficiency of PCR. The optimal concentration of the upstream and downstream PCR primers is the lowest concentration that results in the lowest Ct and an adequate fluorescence for a given target concentration, with minimal or no formation of primer-dimers. Titrate the concentration of each primer in the range of 50 to 300 nM. The best concentrations of the upstream and downstream primers are not always of equal molarity.

Use of Dissociation Profiles in Primer Optimization

Target quantification can be inaccurate due to the contributions of primer-dimers to the measured fluorescence intensity. Running a dissociation profile for determining the amount of primer-dimers is strongly recommended. The primer concentrations resulting in the lowest occurrence of primer-dimers and the lowest Ct should be chosen.

PCR Protocols

For SYBR Green I dye detection, three-step PCR cycling protocols are preferred over two-step PCR protocols. Set the QPCR instrument to acquire data at the annealing and the extension steps.

TaqMan® Assays

Primer Optimization for TaqMan® Assays

When developing a QRT-PCR assay, it is generally advantageous to optimize the primer concentration first, and then complete the probe optimization using the optimized primer concentrations. Titrate the concentration of each PCR primer in the range of 50 to 600 nM. The optimal concentration is the lowest concentration that results in the lowest Ct and an adequate fluorescence for a given target concentration. The best concentrations of the upstream and downstream primers are not always of equal molarity.

TaqMan® Probe Design and Optimization

Probes should have a melting temperature that is 7–10°C higher than the annealing temperature of the PCR primers. For additional considerations in designing TaqMan probes, refer to Primer Express® oligo design software from Applied Biosystems.

Resuspend lyophilized custom TaqMan probes in buffer containing 5 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA (low TE buffer).

The optimal TaqMan probe concentration should be determined empirically. The optimal concentration is the lowest concentration that results in the lowest Ct and an adequate fluorescence for a given target concentration. The TaqMan probe concentration can be optimized by varying the final concentration from 100 to 500 nM in increments of 100 nM.

PCR Protocols

Two-step PCR cycling protocols (including a denaturation step and an annealing/extension step) are generally recommended for TaqMan assays. Set the QPCR instrument to acquire data at the annealing/extension step.

Molecular Beacons Assays

Primer Optimization for Molecular Beacons Assays

When developing a QRT-PCR assay, it is generally advantageous to optimize the primer concentration first, and then complete the probe optimization using the optimized primer concentrations. Titrate the concentration of each PCR primer in the range of 200 to 600 nM. The optimal concentration is the lowest concentration that results in the lowest Ct and an adequate fluorescence for a given target concentration. The best concentrations of the upstream and downstream primers are not always of equal molarity.

Molecular Beacons Probe Design and Optimization

Probes should have a melting temperature that is 7–10°C higher than the annealing temperature of the PCR primers.

Resuspend lyophilized custom molecular beacons probes in buffer containing 5 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA (low TE buffer).

The optimal concentration should be determined empirically for each molecular beacon. The optimal concentration is the lowest concentration that results in the lowest Ct and an adequate fluorescence for a given target concentration. The molecular beacon concentration can be optimized by varying the final concentration from 200 to 500 nM in increments of 100 nM.

PCR Protocols

Three-step PCR cycling protocols (including separate denaturation, annealing and extension steps) are generally recommended for molecular beacons assays. Set the QPCR instrument to acquire data at the annealing and extension steps.

Use of a Passive Reference Dye

It is good practice to include a passive reference dye in QRT-PCR reactions to compensate for non-PCR related variations in fluorescence. Fluorescence from the passive reference dye does not change during the course of the PCR reaction but provides a stable baseline to which samples are normalized. In this way, the reference dye compensates for changes in fluorescence between wells caused by slight volume differences in reaction tubes. The reference dye is generally diluted before use in a QRT-PCR reaction, with the extent of dilution dependent on the correspondence of excitation and emission wavelengths of the reference dye compared to the excitation and detection properties of the instrument. Consult the reference dye manufacturer's protocol for dilution recommendations.

ASSAY STANDARDIZATION CONSIDERATIONS

Intra-laboratory and inter-laboratory standardization of assays is important to allow data comparisons across experiments, platforms, or researchers. Within a single laboratory, changes in the performance of the real-time reader and in the reagents used cannot be detected unless an independent standard is included in the runs. Including standard curve determinations using the Stratagene QPCR Human Reference Total RNA provides a means for comparing the results from different experiments. It is recommended that reference RNA standard curve reactions be included at regular intervals to confirm amplification and detection performance.

When two or more laboratories need to compare data, the Stratagene QPCR Human Reference Total RNA can be used as an inter-laboratory standard. The laboratories involved should run a defined set of reactions (generally standard curves) on their respective platforms. It is important that the laboratories use the same sets of reagents (shared primers and probes and the same supplier for kit reagents) and identical protocols. After the standard curves have been generated on the various platforms in the various laboratories statistical analysis will reveal the quality of each standard curve (the R^2-value, slope, y-axis intercept), the sensitivity of the assay/instrument system and the absolute quantification. Statistical analysis methods for comparing data generated in various laboratories is to date not standardized. One way of analyzing these data is utilizing the calculation of the Confidence Interval (CI) for the data points of the standard curve. Reference 2 provides a useful discussion of the calculation and use of confidence interval values in comparing data sets. With the CI calculated from the Stratagene QPCR Human Reference Total RNA standard curve the quality of the experimental sample data can be predicted and compared to experimental sample data generated on another platform.

QRT-PCR ASSAY OPTIMIZATION PROTOCOL

Preparing QRT-PCR Reactions

Note *The following assay optimization protocol uses the Stratagene Brilliant II QRT-PCR master mix kit, 1-step (catalog #600809), for TaqMan or molecular beacons assays analyzed on a Stratagene Mx3000P, Mx3005P, or Mx4000 instrument or the ABI PRISM® 7900HT instrument. Other single-tube or two-tube QRT-PCR reagents may be used (please follow the reagent manufacturer's instructions for preparing the reaction mixture). Other QPCR platforms may also be used (please consult the instrument manufacturer's instructions for reference dye preparation and RT-PCR program recommendations).*

Use the following reaction conditions to optimize the primer concentrations first, then the probe concentrations. See QRT-PCR Assay Optimization Considerations *for the concentration ranges recommended for each probe system.*

1. If the reference dye will be included in the reaction, (optional), dilute the dye solution provided **1:500 (for the Mx3000P, Mx3005P, and Mx4000 instruments)** or **1:50 (for the ABI Prism 7900HT instrument)** using nuclease-free PCR-grade H_2O. When used according to the protocol below, this will result in a final reference dye concentration of 30 nM for the Mx3000P, Mx3005P, and Mx4000 instruments and 300 nM for the ABI PRISM 7900HT instrument. **Keep all solutions containing the reference dye protected from light.**

   Note *If using a system other than the Mx4000, Mx3000P or Mx3005P instruments, the use of the reference dye may be required for optimal results.*

2. Thaw the 2× QRT-PCR master mix and store the solution on ice. Gently mix by inversion prior to pipetting.

3. Prepare the experimental reactions by combining the following components *in order*.

Reaction Mixture

Nuclease-free PCR-grade H_2O to adjust the final volume to 25 µl
- 12.5 µl of 2× QRT-PCR master mix
- *x* µl of experimental probe (variable while optimizing probe)
- *x* µl of upstream primer (variable while optimizing primers)
- *x* µl of downstream primer (variable while optimizing primers)
- 0.375 µl of the **diluted** reference dye (optional)
- 1 µl of RT/RNase block enzyme mixture
- 0.1 µl (100 ng) of Stratagene QPCR Human Reference Total RNA

4. Gently mix the reactions without creating bubbles (do not vortex), then centrifuge the reactions briefly.

Note *Bubbles interfere with fluorescence detection.*

RT-PCR Program

Note *The following protocol is to be used with the Stratagene Mx3000P, Mx3005P, and Mx4000 instruments and the ABI PRISM 7900HT instrument. If using any other QPCR platform, please consult the instrument manufacturer's instructions for reference dye preparation and RT-PCR program recommendations.*

5. Place the reactions in the QPCR instrument and run the appropriate PCR program below. Generally, TaqMan reactions utilize the 2-step program, and molecular beacons reactions utilize the 3-step program. These amplification protocols are recommended initially, but optimization may be necessary for some primer/template systems.

RT-PCR Program with Two-Step PCR (for TaqMan Probes)

Cycles	Duration of cycle	Temperature
1	30 minutes	50°C
1	10 minutes	95°C
40	15 seconds	95°C
	1 minute[a]	50–60°C[b]

[a] Set the temperature cycler to detect and report fluorescence during the annealing/extension step of each cycle.

[b] Choose an appropriate annealing temperature for the primer set used.

RT-PCR Program with Three-Step PCR (for Molecular Beacons Probes)

Cycles	Duration of cycle	Temperature
1	30 minutes	50°C
1	10 minutes	95°C
40	30 seconds	95°C
	1 minute[a]	50–60°C[b]
	30 seconds	72°C

[a] Set the temperature cycler to detect and report fluorescence during the annealing step of each cycle.

[b] Choose an appropriate annealing temperature for the primer set used.

6. Analyze the Ct values obtained to determine the lowest concentration of primer or probe that results in the lowest Ct and an adequate fluorescence.

QRT-PCR STANDARD CURVE PROTOCOL

Once the optimal primer and probe concentrations have been determined, it is advisable to assess the linearity and sensitivity of the assay by generating a standard curve using serial dilutions of the Stratagene QPCR Human Reference Total RNA. Additionally, standard curve data sets may be used for intra- or inter-laboratory comparisons of results for a given assay.

Generating a Standard Curve

Note *The following QRT-PCR standard curve protocol uses the Stratagene Brilliant II QRT-PCR master mix, 1-step (catalog #600809), for TaqMan or molecular beacons probes analyzed on a Stratagene Mx3000P, Mx3005P, or Mx4000 instrument or the ABI PRISM 7900HT instrument. Other single-tube or two-tube QRT-PCR reagents may be used (please follow the reagent manufacturer's instructions for preparing the reaction mixture). Other QPCR platforms may also be used (please consult the instrument manufacturer's instructions for reference dye preparation and RT-PCR program recommendations).*

1. If the reference dye will be included in the reaction, (optional), dilute the dye solution provided **1:500 (for the Mx3000P, Mx3005P, and Mx4000 instruments)** or **1:50 (for the ABI Prism 7900HT instrument)** using nuclease-free PCR-grade H_2O. When used according to the protocol below, this will result in a final reference dye concentration of 30 nM for the Mx4000 instrument and 300 nM for the ABI Prism 7900HT instrument. **Keep all solutions containing the reference dye protected from light.**

   Note *If using a system other than the Mx4000, Mx3000P or Mx3005P instruments, the use of the reference dye may be required for optimal results.*

2. Thaw the 2× QRT-PCR master mix and store the solution on ice. Gently mix by inversion prior to pipetting.

3. Prepare the appropriate dilutions of the Stratagene QPCR Human Reference Total RNA in RNase-free H_2O. We recommend the following dilution schemes, according to the abundance of the specific target. First, prepare a 40 ng/µl dilution of the reference RNA stock. Next, prepare four serial dilutions using the fold-dilution scheme indicated in the table below. When generating the standard curve, prepare reactions containing 2.5 µl of the 40 ng/µl dilution and four additional reactions containing each of the serial dilutions (in step 6 below). The amount of template may need to be adjusted for certain probe/primer systems.

Target mRNA abundance	Suggested fold dilution	Final amounts RNA template (ng) in standard curve reactions
High	10-fold	100, 10, 1, 0.1, 0.01
Medium	5-fold	100, 20, 4, 0.8, 0.16
Low	2-fold	100, 50, 25, 12.5, 6.25

4. Prepare the experimental reactions by combining the following components *in order*. Prepare a single reagent mixture for all of the target RNA dilutions tested (in duplicate), plus one reaction volume excess, using multiples of each component listed below.

Reagent Mixture

Nuclease-free PCR-grade H_2O to adjust the final volume to 25 µl (including 2.5 µl of the reference RNA dilution added in step 6)
12.5 µl of 2× QRT-PCR master mix
x µl of experimental probe (optimized concentration)
x µl of upstream primer (optimized concentration)
x µl of downstream primer (optimized concentration)
0.375 µl of the **diluted** reference dye (optional)
1 µl of RT/RNase block enzyme mixture

5. Gently mix the reactions without creating bubbles (do not vortex), then distribute the mixture to the experimental reaction tubes.

6. Add 2.5 µl of the appropriate dilution of Stratagene QPCR Human Reference Total RNA to each reaction.

7. Gently mix the reactions without creating bubbles (do not vortex), then centrifuge the reactions briefly.

Note *Bubbles interfere with fluorescence detection.*

RT-PCR Program

Note *The following protocol is for use with the Stratagene Mx3000P, Mx3005P, and Mx4000 instruments and the ABI PRISM 7900HT instrument. If using any other QPCR platform, please consult the instrument manufacturer's instructions for reference dye preparation and RT-PCR program recommendations.*

8. Place the reactions in the QPCR instrument and run the appropriate PCR program below. Generally, TaqMan reactions utilize the 2-step program, and molecular beacons reactions utilize the 3-step program. These amplification protocols are recommended initially, but optimization may be necessary for some primer/template systems.

RT-PCR Program with Two-Step PCR (for TaqMan Probes)

Cycles	Duration of cycle	Temperature
1	30 minutes	50°C
1	10 minutes	95°C
40	15 seconds	95°C
	1 minute[a]	50–60°C[b]

[a] Set the temperature cycler to detect and report fluorescence during the annealing/extension step of each cycle.

[b] Choose an appropriate annealing temperature for the primer set used.

RT-PCR Program with Three-Step PCR (for Molecular Beacons Probes)

Cycles	Duration of cycle	Temperature
1	30 minutes	50°C
1	10 minutes	95°C
40	30 seconds	95°C
	1 minute[a]	50–60°C[b]
	30 seconds	72°C

[a] Set the temperature cycler to detect and report fluorescence during the annealing step of each cycle.

[b] Choose an appropriate annealing temperature for the primer set used.

9. Using the software provided by the instrument, use the fluorescence values collected to generate a standard curve that relates Ct to the amount of template provided in the reaction. A good standard curve should have an R^2-value between 0.980 and 1.000 and a slope between –3.5 and –3.2.

TROUBLESHOOTING

Observation	Suggestion
Little or no increase in fluorescence with cycling	Primers may not be optimally designed. Design the primers so that the PCR product is <150 bp in length.
	If using TaqMan or molecular beacons probes, the probe may have a nonfunctioning fluorophore. For molecular beacons, fluorophore function is confirmed by an increase in fluorescence in the denaturation step of thermal cycling or at high temperatures in a melting curve analysis. For TaqMan probes, verify that the fluorophore functions by digesting the probe (100 nM probe in 25 µl 1× buffer with 10 U DNase or S1 nuclease) at room temperature for 30 minutes to confirm an increase in fluorescence following digestion. If increased fluorescence is not observed, resynthesize the probe or molecular beacon.
	Redesign the TaqMan or molecular beacon probe. The probe will not bind to its target efficiently if the annealing temperature is too high. Verify the calculated melting temperature using appropriate software.
	The RNA template may be degraded. Ensure that the template RNA is stored properly (at –20°C or –80°C) and is not subjected to multiple freeze-thaw cycles. Check the quality of the RNA in the sample by gel electrophoresis or using an automated RNA population analysis system such as the Agilent 2100 Bioanalyzer.
	If the target RNA contains extensive secondary structure, a higher temperature may be required for the first step of the RT-PCR program. Use a reverse transcriptase provided in a thermostable formulation, such as the Brilliant II QRT-PCR master mix, and increase the temperature for cDNA synthesis to up to 55°C.
	Verify that all reagents and supplies are RNase-free.
	Where possible, increase the amount of template RNA. (Do not exceed the recommended amount of template).
	The reaction is not optimized and no or insufficient product is formed. Verify formation of the specific product by gel electrophoresis.
	The reference RNA contains detectable amounts of the majority of targets expressed in wide range of cell types; however, it is possible that the target of interest is present in the reference RNA in such a low copy number that it cannot be detected.

REFERENCES

1. Higuchi, R., Fockler, C., Dollinger, G. and Watson, R. (1993) Biotechnology (N Y) 11(9):1026-30.
2. Zar, J. H. (1999). Biostatistical Analysis. Prentice Hall, Upper Saddle River, NJ.

ENDNOTES

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MSDS INFORMATION

The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on the web at *http://www.genomics.agilent.com*. MSDS documents are not included with product shipments.