All-in-One™ miRNA qRT-PCR Reagent Kits

For quantitative detection of mature miRNA

All-in-One™ miRNA First-Strand cDNA Synthesis Kit for miRNA qPCR array
AMRT-2020 (20 RT reactions), AMRT-2060 (60 RT reactions)

Used in combination with miProfile™ miRNA qPCR Arrays and All-In-One™ qPCR Mix

All-in-One™ miRNA qRT-PCR Detection Kit
AOMD-Q020 (20 RT and 200 qPCR reactions), AOMD-Q060 (60 RT and 600 qPCR reactions)

Used in combination with All-In-One™ miRNA qPCR Primers

User Manual

GeneCopoeia, Inc.
9620 Medical Center Drive, #101
Rockville, MD 20850
USA

301-762-0888
866-360-9531

inquiry@genecopoeia.com

www.genecopoeia.com

USER MANUAL

All-in-One™ miRNA qRT-PCR Reagent Kits

I. Introduction and Principle
II. Related Products
III. Contents and Storage
IV. Preparation
V. Procedure
VI. Examples
VII. Trouble Shooting Guide
VIII. Limited Use License and Warranty

I. Introduction and Principle

Small, non-coding miRNA are widely present in eukaryotes. They consist of about 22 nucleotides that control many important physiological processes in cell development and differentiation. Different miRNA express differently at different developmental stages and different tissues. Therefore, the quantitative assaying of miRNA is important in both basic and applied research.

The **All-in-One™ miRNA qRT-PCR Reagent Kits** use real-time PCR technology to quantitatively measure miRNAs. The experimental procedure includes three major steps (Figure 1)

1) **Adding poly-A tails** - Poly A polymerase is used to add poly-A tails to the 3' end of miRNAs
2) **cDNA Synthesis** - At the same time M-MLV RTase and a unique Oligo-dT Adaptor primer reverse transcribes the poly A miRNAs (The Universal Adaptor PCR primer in combination with a miRNA-specific primer allows detection of specific miRNA)
3) **qPCR** - The All-in-One qPCR Mix containing SYBR® Green specifically detects the reverse transcribed miRNA (The miRNA-specific forward primer is used with the Universal Adaptor primer).

When compared to traditional hybridization-based miRNA detection methods, such as Northern blot analysis, the method provided by the All-in-One miRNA RT-PCR Reagent Kits is faster, more specific and sensitive and uses less sample material.

Key advantages

- Provides efficient reverse transcription of miRNAs into cDNA in a single step
- Delivers a precise quantitative and accurate measurement of miRNA expression profiles
- Differentiates between mature and precursor miRNA
- Co-developed with validated primers, miRNA clones and other tools used for functional studies of miRNA

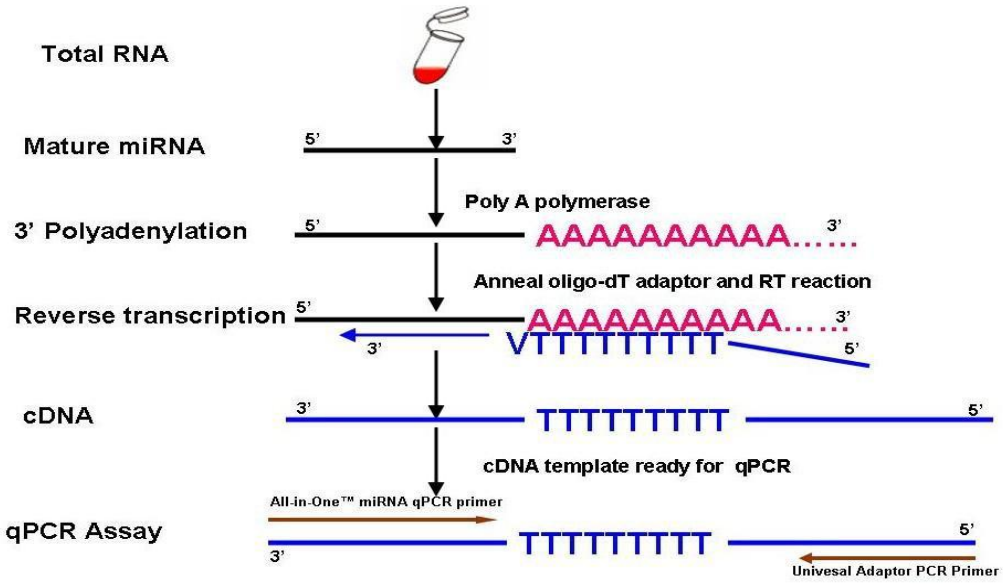

Figure 1. A graphic representation of the steps involved in the All-in-One miRNA qRT-PCR reagent kits.

II. Related Products

GeneCopoeia offers comprehensive solutions for studying human miRNAs. A careful process of co-development ensures that they work well together and provide robust and reproducible results.

Product	Description
All-in-One™ miRNA qPCR Primers	Validated for robust, reproducible and reliable quantitation of miRNA activity
miProfile™ miRNA qPCR Arrays	Genome-wide or disease-focused miRNA primer sets pre-deposited in 96- or 384-well plates for qPCR profiling of miRNA expression
miExpress™ Precursor miRNA Expression Clones	Study miRNA regulation on target genes and proteins
miTarget™ miRNA Target Validation Expression Clones	Cross validate data using luciferase reporter genes
OmicsLink™ Expression-Ready ORF cDNA Clones	Perform gain-of-function studies with expression-ready clones
All-in-One™ qPCR Mix	Used in combination with miProfile™ miRNA qPCR Arrays and All-in-One™ miRNA First-Strand cDNA Synthesis Kit to profile miRNA expression
RNAzol® RT RNA Isolation Reagent	Easy isolation of mRNA, microRNA or total RNA

III. Contents and Storage

All-in-One miRNA First-Strand cDNA Synthesis Kits for miRNA qPCR array(Cat. Nos. AMRT-2020 and AMRT-2060)

Contents	Quantity	Storage temperature/ conditions
2.5 U/µl Poly A Polymerase	20 µl 3 x 20 µl	–20°C (Stable for at least 12 months) Alternatively, the solution can also be stored at -80°C in aliquots. Avoid repeated freezing/ thawing.
RTase Mix	20 µl 3 x 20 µl	–20°C (Stable for at least 12 months) Alternatively, the solution can also be stored at -80°C in aliquots. Avoid repeated freezing/ thawing.
5X PAP/RT Buffer	100 µl 3 x 100 µl	–20°C (Stable for at least 12 months) Alternatively, the solution can also be stored at -80°C in aliquots. Avoid repeated freezing/ thawing.
Spike-in control (for use with miRNA qPCR arrays only)	20 µl 3 x 20 µl	–20°C (Stable for at least 12 months) Alternatively, the solution can also be stored at -80°C in aliquots. Avoid repeated freezing/ thawing.
dd H_2O (RNase and DNase free)	1 ml 3 x 1 ml	–20°C (Stable for at least 12 months) Alternatively, the solution can also be stored at -80 °C in aliquots. Avoid repeated freezing/ thawing.

All-in-One miRNA qRT-PCR Detection Kits (Cat. Nos. AOMD-Q020 and AOMD-Q060)

Contents	Quantity	Storage temperature/ conditions
2.5 U/µl Poly A Polymerase	20 µl 60 µl	–20°C (Stable for at least 12 months) Alternatively, the solution can also be stored at -80°C in aliquots. Avoid repeated freezing/ thawing.
RTase Mix	20 µl 60 µl	–20°C (Stable for at least 12 months) Alternatively, the solution can also be stored at -80°C in aliquots. Avoid repeated freezing/ thawing.
5X PAP/RT Buffer	100 µl 300 µl	–20°C (Stable for at least 12 months) Alternatively, the solution can also be stored at -80°C in aliquots. Avoid repeated freezing/ thawing.
dd H_2O (RNase and DNase free)	1 ml 3 x 1 ml	–20°C (Stable for at least 12 months) Alternatively, the solution can also be stored at -80 °C in aliquots. Avoid repeated freezing/ thawing.
2X All-in-One qPCR Mix	2 x 1 ml 6 x 1 ml	–20°C (Stable for at least 12 months) Alternatively, the solution can also be stored at -80 °C in aliquots. Avoid repeated freezing/ thawing.
ROX Reference Dye (30µM)	80 µl 240 µl	–20°C (Stable for at least 12 months) Alternatively, the solution can also be stored at -80 °C in aliquots. Avoid repeated freezing/ thawing.
50 µM Universal Adaptor PCR Primer T_m = 64.5;GC content = 50%	20 µl 60 µl	–20°C (Stable for at least 12 months) Alternatively, the solution can also be stored at -80 °C in aliquots. Avoid repeated freezing/ thawing.

IV. Preparation

RNA Sample Preparation

When working with RNA it is important to avoid RNases in your solutions, consumables and labware. When preparing your RNA samples, always wear a mask and disposable gloves in all procedures. Follow the described procedures you are using for RNA extraction carefully. Ready-to-use solutions that are RNase-free can be purchased. Alternatively treat solutions with diethyl pyrocarbonate (DEPC) and then autoclave. RNases on labware can also be inactivated by DEPC treatment or by baking at 250°C for 3 hours. Use DEPC to treat all microcentrifuge tubes, pipettes and pipette tips (if no RNase free) and then autoclave to deactivate RNases. RNase-free consumables are available for purchase from many commercial sources.

Primer Design

The reverse primer called "Universal Adaptor PCR Primer" (T_m = 64.5, GC% = 50%) are provided in the All-in-One miRNA qPCR Mix Kit.

You may wish to design and make specific forward primers for your miRNA of interest or order from GeneCopoeia. Please contact us for further information.

Since the length of miRNAs is generally between 18 ~ 24 nucleotides for some "easy" miRNAs, a forward primer may be designed directly according to the sequence of the miRNA. However, for some potentially "difficult" miRNAs (e.g. very high or very low T_m or highly homologous miRNAs) or miRNAs from specific tissues (e.g. tissues with high pre-miRNA/pri-miRNA) special primers may need to be designed to optimize the primer sequence in order to obtain specific amplification and avoid interference from pre- miRNA/pri-miRNA.

IMPORTANT NOTES:

1. Store kit at –20°C. Avoid storage or leaving reagents at 4°C or room temperature.
2. Mix reagents thoroughly by gently inverting tubes several times avoiding bubbles, and then briefly centrifuge before use.
3. Following the procedure carefully to avoid contamination with RNases which can rapidly degrade RNA and lead to inconclusive results.
4. Set up all reactions on ice to reduce risk of RNA degradation.

V. Procedure

Important note

Follow the miProfile miRNA qPCR array user manual for the complete instruction when using the All-in-One miRNA first-strand cDNA synthesis kit in combination with miProfile miRNA qPCR arrays and All-in-One qPCR mix.

1. Reverse transcription of miRNA

a. Thaw template RNA on ice. Thaw 5X PAP/RT Buffer and ddH2O (RNase/ DNase free) at room temperature (15–25°C).

b. Gently mix miRNA reverse transcription reagents by flicking to dissolve all reagents thoroughly. Briefly centrifuge to collect residual liquid from the sides of the tubes and then place on ice.

c. Prepare miRNA reverse transcriptase reaction solution.

Place RNase-free reaction tubes on ice and then add the following reagents to a final volume of 25 µl.

Reagent	Volume	Quantity
Total RNA or small-molecule RNA		2 μg† 100 ng
2.5 U/ μl Poly A Polymerase	1 μl	
RTase Mix	1 μl	
5X PAP/RT Buffer	5 μl	1X
Spike-in control (Only comes in All-in-One miRNA first-strand cDNA synthesis kits)	1 μl	
dd H_2O (RNase-/DNase- -free)	To final 25 μl	

* Total RNA must contain small-molecule RNA

† The amount of total RNA can be between 1 ng ~ 5 μg. If using purified small-molecule RNA, the amount can be between 0.1 ng ~ 1 μg.

d. Prepare reverse transcription reaction.

Mix the prepared reaction mix gently, but thoroughly. Incubate at 37°C for 60 minutes after a brief centrifugation.

Incubate at 85°C for 5 minutes to inactivate the enzyme.

The resulting reverse transcription reaction product should be diluted 5 ~ 50 times with sterile H_20 before using for the next qPCR experiment or it can be directly stored at –20°C.

2. Detection of miRNA with qPCR

a. Dissolve 2X All-in-One qPCR Mix by gently inverting. Briefly centrifuge and place on ice. If required, dissolve ROX Reference Dye.

b. Dilute the 50 μM Universal Adaptor PCR Primer to 2μM with sterile ddH_20 before using for the next qPCR experiment.

c. Prepare qRT-PCR solution on ice. See example.

Reagent	Volume	Final concentration
2X All-in-One qPCR Mix[i]	10 μl	1X
All-in-One miRNA qPCR Primer (2 μM) [ii]	2 μl	0.2 μM
Universal Adaptor PCR Primer (2 μM)	2 μl	0.2 μM
First-strand cDNA (diluted 1:5) [iii]	2 μl	
ROX Reference Dye[iV](30μM) if needed	0.4 -0.1μl	600nM-150nM
Water (double distilled)		
■ Not using ROX Reference Dye	4 μl	
■ Using ROX Reference Dye	3.6-3.9μl	
Final volume	**20 μl**	

Notes

i. Use the 2X All-in-One qPCR Mix as half of the total reaction volume and adjust other reagents accordingly. If the total reaction volume is changed, maintain each component in proper proportion.

ii. Primer concentration should be in the range of 0.2 to 0.4 μM. In general, a PCR reaction using 0.2 μM primers produces good results

iii. The first-strand cDNA should be diluted before using for the PCR reaction in order to avoid interference to the qPCR from the reverse transcription system.

iv. ROX Reference Dye is added only for qPCR instruments that require ROX for calibration. ROX Reference Dye provides an internal reference to which the reporter-dye signal can be normalized during data analysis. Normalization is necessary to correct for fluorescence fluctuations due to changes in concentration or volume. Adjust the ROX Reference Dye to optimal concentration according to different qPCR instruments.

Instrument	**ROX per 20μl PCR Reaction**	**Final Concentration**
BioRad iCycler，MyiQ，iQ5，CFX-96，CFX-384， Eppendorf Mastercycler realplex， Roche LightCycler 480，LightCycler 2.0	None	No ROX
ABI PRISM 7000/7300/7700/7900HT and 7900HTFast, ABI Step One, ABI Step One Plus	0.4 μl（0.2-0.4μl）	600 nM（300-600nM）
ABI 7500，7500 Fast, ABI Viia7，Stratagene Mx3000P，Mx3005P, Mx4000，	0.1 μl（0.02-0.1μl）	150 nM（30-150nM）

For other instruments which need calibration of ROX but have not been listed out in the table, please optimize the concentration of ROX according to the guide line of specific instrument.

d. Thoroughly mix the qPCR reaction solution, add to PCR tubes, and briefly centrifuge to make sure that all the reagents are in the bottom of the tubes.

e. The following standard 3-step method for the qPCR reaction is recommended (example adapted from the iQ5 real-time PCR detection system from Bio-Rad).

Cycles	Steps	Temperature	Time	Detection
1	Initial denaturation	95°C	10 min	No
40	Denaturation	95°C	10 sec	No
	Annealing	T_m –2°C	20 sec	No
	Extension	72°C	At least 10 sec	Yes

Notes

i. When using SYBR Green dye to monitor the qPCR reaction, a melting curve analysis should be performed immediately after qPCR cycling. For instructions, consult the documentation for your qPCR instrument. The following is an example adapted from the iQ5 real-time detection system from Bio-Rad Laboratories. The conditions for your instrument may differ:

Temperature Range	Heating Rate	Constant Temperature	Detection
65°C ～ 95°C	0.5°C/ time	6 sec/ time	Yes
30°C		30 sec	No

ii. The DNA polymerase used in the 2X All-in-One qPCR Mix is a chemically especially modified hot-start enzyme. Incubation for 10 minutes at 95°C will sufficiently activate the enzyme.

iii. Specific properties of a miRNA lead to special properties of the designed primer. Therefore the annealing temperature needs to be strictly controlled in order to avoid non-specific amplifications. For validated miRNA primers purchased from GeneCopoeia, please refer to the optimal conditions for the experiment.

iv. The Oligo-dT Adaptor primer for reverse transcription is 53 nucleotides, therefore the resulting PCR amplification fragment is about 75bp (assuming the sequence of miRNA is about 22 nucleotides), which requires at least about 10 seconds extension time. From the melting temperature of the products the T_m
value is generally determined to be between 75°C ～ 83°C. If the melting temperature exceeds this range, other assaying methods such as electrophoresis are suggested for the specific properties of the product.

v. The main conditions for the above reactions are for use with the iQ5 qPCR instrument from Bio-Rad. If a qPCR instrument from another commercial source is used, please reference the instrument manual and adjust the extension time and melting curve conditions accordingly.

VI. Examples

a) **Example 1: Specificity assay using the All-in-One miRNA qRT-PCR Detection Kit**

With 200 ng total RNA mixture from human brain and heart as template, the miRNA qRT-PCR Detection Kit and the All-in-One miRNA qPCR Primers were used to detect 31 miRNA and an internal reference snRNA U6. Results from qRT-PCR and electrophoresis showed neither non-specific amplification products nor primer-dimer formation.

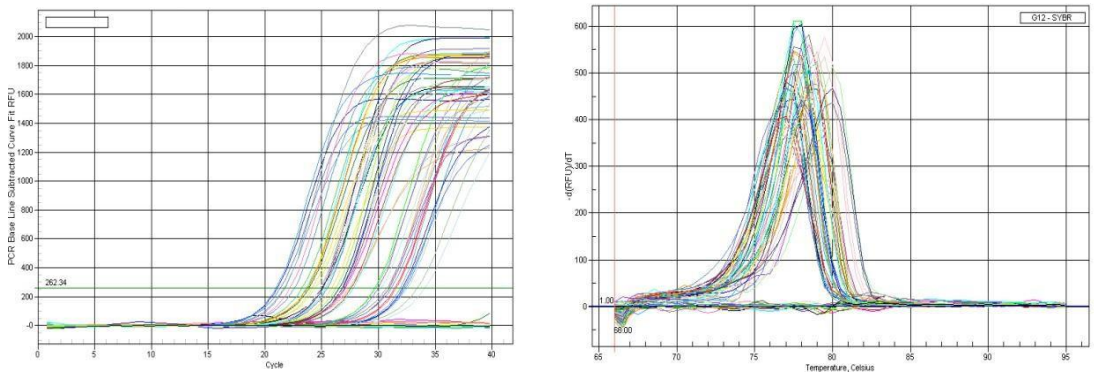

Figure 2. Amplification and melting curves of 31 miRNA and the internal reference snRNA U6, in which double channel detection was used for the positive control, and single channel detection was used for the NTC (No Template Control).

Figure 3. Agarose gel electrophoresis (3% agarose gel) of the amplification products of 31 miRNA and the internal reference snRNA U6, in which double channel detection was used for the positive control, and single channel detection was used for the NTC (No Template Control).

b) Example 2: Sensitivity assay using the All-in-One miRNA qRT-PCR Detection Kit

Starting with different amounts (5μg, 1μg, 200ng, 20ng, 2ng, 100pg) of human brain total RNA, the All-in-One miRNA qRT-PCR Detection Kit was used to detect the expression level of hsa-miR-124. The results showed that linear amplification can be detected between 5μg ~ 100pg of total RNA.

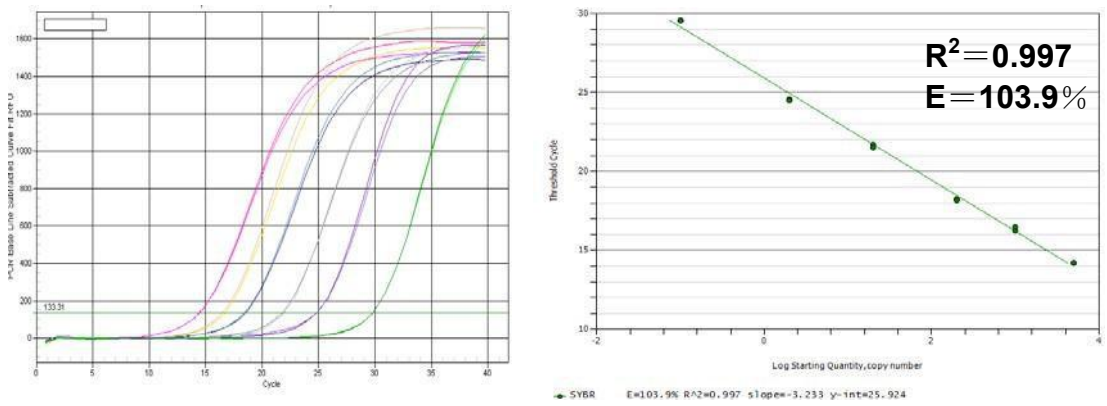

Figure 4. The amplification curve and standard curve generated from different amount of human brain total RNA as template, and using All-in-One miRNA qRT-PCR Detection Kit to detect hsa-miR-124 expression level.

VII. Trouble Shooting Guide

miRNA sequence homology problems	• Because the sequence of miRNA is short and some have a high degree of homology, the primer design can sometimes be tricky. Thus one needs to fully consider the specificity problems when designing the miRNA forward primers. Specifically for miRNAs that have a single nucleotide difference only, the demand for specificity is higher for designing and synthesizing primers, in addition to designing reaction conditions.
Confusion of amplification curves	• Make sure the initial denature time was set as 10min, sufficiently activating of the hot-start polymerase could avoid non-specific amplification and production of primer-dimers. • The fluorescence detection temperature may not be appropriate. Adjust accordingly. • The set up position for samples may not be right. Adjust accordingly. • Try to use 3.5% agarose gel electrophoresis to check the PCR products. Check the purity of the primers using electrophoresis or use PAGE- purified primers if the bands are diffused. One may also use phenol/chloroform extraction and ethanol precipitation methods to treat the primers before experiment.

Abnormal melting curves	• **Signals in blank (No Template Control) sample** • There may be contamination or positive samples in the PCR reaction system if the T_m of the melting curves of the blank control is the same as the positive control. Eliminate sample application error first. If the situation still persists, change PCR grade water, primers or use new 2X All-in-One ™ q-PCR Mix. • If the T_m of melting curves of blank control is lower than the positive control, the PCR reaction may have produced nonspecific amplification such as primer-dimers. Please prepare PCR reaction mix on ice and increase the temperature of fluorescence detection. If the C_t value of the negative control is >35 and the difference with the positive samples is more than 5 cycles, the PCR reaction system is up to the standard. On the other hand, if the $_{Ct}$ value cannot reach the aforementioned value, then redesign the primer or optimize the reaction conditions.
	• **Double peaks and multiple peaks in melting curves of positive control** • In the absence of other primers present in the reaction, double or multiple peaks in the positive control means that the PCR reaction produces nonspecific amplification fragments. Prepare the PCR reaction mix on ice; optimize the PCR reaction conditions such as by increasing the annealing temperature, decreasing the primer concentration or increasing the fluorescence detection temperature (no more than the T_m value of the expected product). If this does not work, optimize and redesign the forward primer. • **No peaks or abnormal peaks in the melting curve(or the amplification curves) of the positive control** • Adjust the ROX Dye to optimized concentration according to instrument.
No signal (Ct) or Ct value is too high	• Check if there are PCR products to exclude the possibility of instrument detector malfunction. • Not enough PCR cycles. For good sensitivity, one should generally set up more than 35 PCR cycles, but more than 45 cycles may result in too much background signals. • The amount of template may not be enough or the template may be degraded. Use the highest concentration of diluted template samples possible to set up PCR. At the same time, avoid freezing and thawing samples repeatedly. • Amplification efficiency is low and PCR reaction conditions are not optimal. Redesign primers and optimize reaction conditions.

VIII. Limited Use License and Warranty

Limited Use License

Following terms and conditions apply to use of all All-in-One™ miRNA qRT-PCR Reagent Kits (the Products). If the terms and conditions are not acceptable, the Product in its entirety must be returned to GeneCopoeia within 5 calendar days. A limited End-User license is granted to the purchaser of the Product. The Product shall be used by the purchaser for internal research purposes only. The Product is expressly not designed, intended, or warranted for use in humans or for therapeutic or diagnostic use. The Product must not be resold, repackaged or modified for resale, or used to manufacture commercial products without
prior written consent from GeneCopoeia. This Product should be used in accordance with the NIH guidelines developed for recombinant DNA and genetic research. Use of any part of the Product constitutes acceptance of the above terms.

Limited Warranty

GeneCopoeia warrants that the Product meets the specifications described in the accompanying Product Manual. If it is proven to the satisfaction of GeneCopoeia that the Product fails to meet these specifications, GeneCopoeia will replace the Product. In the event a replacement cannot be provided, GeneCopoeia will provide the purchaser with a refund. This limited warranty shall not extend to anyone other than the original purchaser of the Product. Notice of nonconforming products must be made to GeneCopoeia within 30 days of receipt of the Product. GeneCopoeia's liability is expressly limited to replacement of Product or a refund limited

to the actual purchase price. GeneCopoeia's liability does not extend to any damages arising from use or improper use of the Product, or losses associated with the use of additional materials or reagents. This limited warranty is the sole and exclusive warranty. GeneCopoeia does not provide any other warranties of any kind, expressed or implied, including the merchantability or fitness of the Product for a particular purpose.

GeneCopoeia is committed to providing our customers with high-quality products. If you should have any questions or concerns about any GeneCopoeia products, please contact us at 301-762-0888.

GeneCopoeia, Inc.
9620 Medical Center Drive
Rockville, MD 20850
Tel: 301-762-0888 Fax: 301-762-3888
Email: inquiry@genecopoeia.com
Web: www.genecopoeia.com