

# PyroScript™ RT-PCR 2X Master Mix Kit

**IMPORTANT!**  
**-20°C Storage Required**  
**Immediately Upon Receipt**

**Lucigen® Corporation**  
*Advanced Products for Molecular Biology*

2120 W. Greenview Drive  
Middleton, WI 53562  
Toll Free (888) 575-9695  
Phone (608) 831-9011  
FAX (608) 831-9012  
lucigen@lucigen.com  
www.lucigen.com

## Technical Support

Lucigen is dedicated to the success and satisfaction of our customers. Our products are tested to assure they perform as specified when used according to our recommendations. It is imperative that the reagents supplied by the user, especially the RNA specimens to be amplified, are of the highest quality. Please follow the manual carefully and contact our technical service representatives if additional information is necessary. We encourage you to contact us with your comments regarding the performance of our products in your applications. Thank you.

## Product Designations

### PyroScript RT-PCR 2X Master Mix Kit

Product	Size	Catalog number
PyroScript RT-PCR 2X Master Mix Kit	50 rxn	30055-0
	100 rxn	30055-1
	500 rxn (5 x 100 rxn)	30055-2

## Components and Storage

### Store at -20°C

Component	50 rxn	100 rxn	500 rxn
PyroScript RT-PCR 2X Master Mix	1 x 1.25 ml	2 x 1.25 ml	5 x 100 rxn
Magnesium Sulfate, 100 mM	1 x 1.0 ml	1 x 1.0 ml	
Nuclease-Free Water	1 x 1.25 ml	2 x 1.25 ml	
RNA Control I	1 x 20 µl	1 x 20 µl	
Primer Set, RNA Control I	1 x 40 µl	1 x 40 µl	

## Product Description

The PyroScript RT-PCR 2X Master Mix incorporates a thermostable PCR enzyme discovered at Lucigen that also has efficient reverse transcription activity. PyroScript RT-PCR 2X Master Mix simplifies RNA detection by allowing accurate single-tube, single-enzyme reverse transcription PCR (RT-PCR). In fact, the initial low temperature reverse transcription step needed by traditional reverse transcriptases is not required when using PyroScript RT-PCR 2X Master Mix. Low background fluorescence and enzyme compatibility with commonly used fluorescent stains make PyroScript RT-PCR 2X Master Mix excellent for qPCR. PyroScript RT-PCR 2X Master Mix is especially useful for RT-PCR detection and quantification of viral as well as transcript RNA.

PyroScript RT-PCR 2X Master Mix contains all the components needed for RT-PCR or standard PCR except primers and target. Also supplied are Nuclease-Free water and Magnesium Sulfate solution.

An RNA Positive Control Template and Primers are included to verify performance of the PyroScript RT-PCR 2X Master Mix reagent. The RNA Positive Control I is an intact MS2 RNA bacteriophage. Proper care should be exercised to separate the reagent from areas where RT-PCR is set-up and from cultures of *E. coli*. The RNA Positive Control I can be used directly in RT-PCR or extracted by standard methods as a process control.

The PyroScript RT-PCR 2X Master Mix is capable of PCR amplification of fragments greater than 1 kb. However, we have found that RT-PCR amplification of fragments greater than 400 bp in length is poor and is not recommended. The products amplified by PyroScript RT-PCR 2X Master Mix contain a single 3'-A or G overhang, like PCR products amplified by Taq polymerase, allowing direct cloning into any vector with complementary 3' overhangs.

As with most PCRs, amplification is highly dependent on primers and targets. Particular primer/template sets can result in undesired reaction products. Such variability may be reduced by the use of alternative primer sets.

Cycling conditions should be optimized using conditions under which the PyroScript polymerase performs best. Specificity is improved with PyroScript 2X RT-PCR Master Mix by eliminating a low temperature RT incubation step prior to denaturation and PCR, and by increasing annealing temperatures. Cold reaction set-up and use of 2 step PCR cycling parameters with a combined annealing/extension step at a temperature of 72 °C greatly shortens cycling time and is recommended for best results. See Amplification Guidelines for PyroScript RT-PCR 2X Master Mix for more information.

## Related products available separately

Product	Size	Catalog number
Gel-Ready™ 100bp DNA Ladder	100 gel lanes	50120-1
Gel-Ready™ 1kb DNA Ladder	100 gel lanes	50010-1
2.5 mM dNTP Mix, PCR Grade	2 ml (2 X 1 ml)	30030-1
	10 ml (10 X 1 ml)	30030-2
	20 ml (2 X 1 ml)	30030-3
10 mM dNTP Set, PCR Grade	750 µl of each dNTP	30029-1
	5 X 750 µl of each dNTP	30029-2
EconoTaq PLUS GREEN 2X Master Mix	Trial size (10 rxns)	30033-0
	500 rxns	30033-1
	1,000 rxns	30033-2
EconoTaq PLUS 2X Master Mix	500 rxns	30035-1
	1,000 rxns	30035-2

## PyroScript RT-PCR 2X Master Mix for Reverse Transcription PCR

PyroScript RT-PCR Master Mix for reverse transcription and subsequent PCR in a single tube.

### Reverse Transcription PCR Reaction - Assemble on ice

1. Thaw the reaction components and place on ice.
2. Vortex briefly and collect by centrifugation before reaction set up. Take special care to ensure that PyroScript RT-PCR 2X Master Mix is completely mixed prior to use.
3. Set up reactions using components in the order listed in a nuclease-free microfuge tube on ice.

#### Reaction Set-Up

X µl	Nuclease-Free Water
25.0 µl	PyroScript RT-PCR 2X Master Mix
1.0 µl	Primer 1 (10 µM, 200 nM final)*
1.0 µl	Primer 2 (10 µM, 200 nM final)*
0.01 to 100 ng	Total RNA
50.0 µl	Total

\*See Amplification Guidelines, G.

#### Recommended Cycling Conditions

Step	Two Step Cycling		# of Cycles
	Temp.	Time	
Initial Denature	94 °C	2 mins	1
Denature	94 °C	15 secs	25-40
Anneal*	72 °C	1 min/kb	
Extend			
Hold	4 °C	Indefinitely	1

\*Annealing at 72 °C is strongly recommended. See Amplification Guidelines, D, E.

4. Mix reactions and collect by centrifugation prior to cycling in a thermal cycler pre-heated to 94 °C.

### Control RT-PCR

A Control Primer set and an RNA Control template are supplied to allow functional testing of PyroScript RT-PCR 2X Master Mix in RT-PCR. RT-PCR amplification using this primer/template set results in a 160-bp amplicon of the RNA phage genome. The RNA Control is supplied in the form of intact RNA bacteriophage particles.

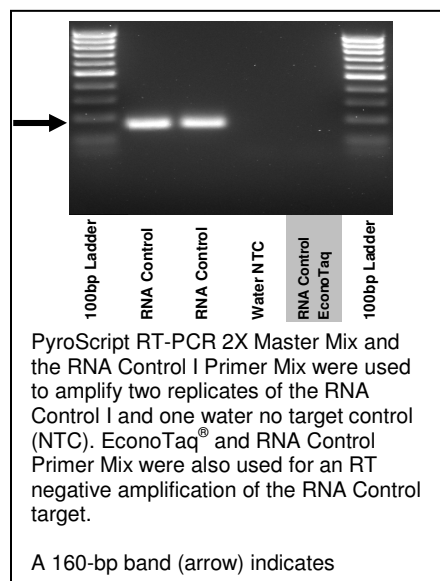
#### Control RT-PCR Reaction

21 µl	Nuclease-Free Water
25 µl	PyroScript RT-PCR 2X Master Mix
2 µl	25X Primer Set, RNA Control I
2 µl	RNA Control I
50 µl	Total volume

#### Cycling Conditions:

Pre-heat thermal cycler to 94 °C.

Denature	2 min. at 94 °C	X 1 cycle
Denature	15 sec. at 94 °C	X 35 cycles
Anneal/Extend	30 sec at 72 °C	
Hold	Indefinitely at 4 °C	



Analyze 10 µl by electrophoresis on a 2% agarose gel. A distinct band of 160 bp should be visible.

## Amplification Guidelines for PyroScript RT-PCR 2X Master Mix

### A. Avoid Ribonuclease (RNase) Contamination

Major sources of RNase contamination in a typical laboratory include solutions and reagents, environmental exposure and contact with human hands and skin. Avoid introducing RNases, rather than trying to remove them. Some basic precautions must be taken to work successfully with RNA.

- Always wear gloves to prevent introducing RNase contamination from human hands.
- Change gloves frequently especially after touching skin, door knobs, and common surfaces.
- Use a set of pipettors dedicated solely for RNA work.
- Use RNase-free plasticware and reagents.
- Designate an RNase-free area of the lab.

### B. Cold Reaction Set-Up

The PyroScript RT-PCR 2X Master Mix has residual activity at temperatures above 4°C that can cause non-specific background amplification.

- All reactions using PyroScript RT-PCR 2X Master Mix must be set up on ice and maintained at 4°C prior to amplification.
- Primers should be added just prior to target addition and incubation/cycling.

### C. Denaturation

A 2 minute initial denaturation step at 94°C is recommended, although as little as 15 seconds has proven sufficient. Subsequent denaturation steps should be 5 to 15 seconds.

### D. Annealing

Optimize the reaction conditions by performing the reaction starting with an annealing temperature 2°C below the calculated melting temperature of the primers. Vary the annealing temperature to optimize for the primer/template combination. Reducing the annealing temperature improves yield and reaction efficiency, while increasing annealing temperature improves specificity. The annealing step is typically 30 to 60 seconds for two-step cycling and 10 to 15 seconds for three-step cycling. Use of two-step cycling can improve RT-PCR specificity and reaction performance since the PyroScript polymerase enzyme is optimal at 72°C.

### E. Extension

The optimal extension temperature is 72°C. Allow 1 minute for every 1 kb to be amplified. A final extension of 5 to 10 minutes at 72°C may increase PCR product yield.

### F. Cycle Number

Between 25 to 30 cycles of PCR is usually optimal for amplification of more concentrated templates. Up to 40 cycles allows detection of low-copy targets. Some loss of fidelity is associated with higher cycle numbers.

### G. Primers

#### Guidelines for primer selection:

The best PCR primer annealing temperatures is 72°C. Longer primers up to 40 nucleotides in length provide higher specificity and allow for two-step cycling recommended for best product performance.

Best amplification efficiency results from RT-PCR of target lengths of between 100 and 400 bp.

Typical PCR primer design guidelines should be followed. Preferred GC content is 40-60%, distributed uniformly throughout the primer. Avoid the use of multiple G or C nucleotides at the 3'-end of the primer, as these may promote nonspecific priming. The primer should be free of inverted repeats that promote the formation of hairpins. To avoid primer self-annealing, the 3' end of the primer should not be complementary to itself or to any other primer in the reaction mixture. The melting temperature of the primers should not differ by more than 5°C. If degenerate primers are used, at least 3 conservative

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nucleotides must be located at the 3' end of the primer. Both the PrimerBLAST webtool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi>) or the Primer3 program (<http://frodo.wi.mit.edu/>) have been successfully used to design primers for use with PyroScript RT-PCR 2X Master Mix.

### **Estimation of the melting and annealing temperatures of primer:**

If the primer is shorter than 25 nucleotides, the melting temperature ( $T_m$ ) can be approximated using the following formula:  $T_m$  (°C) = 4 (G + C) + 2 (A + T), where G, C, A, T = number of specified nucleotides in the primer. Initial annealing temperature should be 2°C lower than the melting temperature. More accurate melting temperature predictions may be obtained using specialized computer programs or web-based tools (e.g. Primer3, above) where the interactions of adjacent bases, the influence of salt concentration, etc. can be evaluated.

### **Primer Concentration**

Primer concentrations of between 200 and 500 nM are recommended for RT-PCR. Primer concentrations of between 200 nM and 1000 nM are recommended for PCR.

### **H. Template**

Total RNA template should be used at concentrations from 5 fg to 100 ng depending on the abundance of the target RNA.

Concentrations of 0.01 to 10 ng of DNA plasmid or phage or 10 to 100 ng of genomic DNA is recommended in a typical reaction volume of 50 µl.

If fidelity is crucial, higher initial template concentrations with a limited number of PCR cycles are recommended. Extremely high concentrations of template can inhibit both RT and PCR activity and may lead to generation of undesired products.

Most routine methods of template purification are sufficient (e.g. phenol/chloroform or guanidine/silica-based methods). However, trace amounts of purification agents (phenol, EDTA, Proteinase K, ethanol, etc.) may inhibit RT-PCR. It is recommended that the template be dissolved in water or EDTA-free buffer rather than TE following purification.

### **I. Reaction Overlay**

A thermal cycler with a heated lid should be used to prevent evaporation of the RT-PCR reaction mix. If no such lid is available, the reaction mixture can be overlaid with one-half reaction volume of PCR-grade mineral oil or paraffin (melting temperature 50-60°C).

### **J. Magnesium**

Magnesium is contained in the PyroScript RT-PCR Master Mix at 2.0 mM final 1X concentration. If the DNA samples contain EDTA or other chelators, additional magnesium sulfate can be added. It is best to avoid the use of EDTA by dissolving the template in water rather than TE. Some primer templates may exhibit better sensitivity and performance with addition of 1 to 2 mM additional magnesium sulfate.

### **K. dNTPs**

The dNTPs are included in the PyroScript RT-PCR 2X Master Mix at a concentration of 0.2 mM each (0.1 mM final concentration). PCR Grade dNTPs are available from Lucigen, please see page 3. Addition of high concentrations of dNTPs chelates available magnesium in the supplied buffer, so additional magnesium sulfate should be supplemented when dNTPs are used at elevated concentrations.

### **L. Taq Polymerase RT Negative Control**

A Taq polymerase-only amplification control is used to test whether an RNA sample has DNA contamination. Lucigen EconoTaq® PLUS or EconoTaq PLUS Green reagents are recommended.

## PyroScript RT-PCR 2X Master Mix and Quality Control Assays

**PyroScript RT-PCR 2X Master Mix:** Polymerase enzyme and 2X buffer including stabilizers, 40 mM monovalent salts, 0.2 mM each dNTP (N=A,C,G,T), and 4 mM MgSO<sub>4</sub>.

### Activity Assay

Polymerase activity is assayed at 70 °C with 0.2 mM each of dATP, dGTP, dTTP, dCTP (mix of unlabeled and [<sup>33</sup>P] dCTP); 10 µg activated calf thymus DNA, and 0.1 mg/ml BSA in a final volume of 50 µl.

### Absence of Endonuclease

Polymerase is determined to be free of detectable endonuclease or nicking activity. One µg of supercoiled plasmid DNA is incubated with enzyme for 16 hours at 70 °C. Agarose gel electrophoresis shows no alteration in mobility consistent with endonuclease or nicking activity.

### Absence of Exonuclease

Polymerase is tested to be free of contaminating exonuclease activity by incubating 1 µg of Hind III-digested lambda DNA with enzyme at 70 °C for 16 hours. Agarose gel electrophoresis shows no alteration in mobility consistent with exonuclease activity.

### Absence of Ribonuclease

Polymerase and PyroScript RT-PCR 2X Master Mix are tested to be free of contaminating RNase activity by incubating with a fluorogenic RNase substrate for 1 hour at 37 °C. No increase in assay fluorescence above background is detected.

### Functional Assays

PyroScript RT-PCR 2X Master Mix is tested for performance in PCR by amplification of a 2 kb region of the *endA* gene from 2 X 10<sup>6</sup> molecules (10 ng) of *E. coli* gDNA. The resulting PCR product is visualized on an ethidium bromide-stained agarose gel.

PyroScript RT-PCR 2X Master Mix is tested for performance in RT-PCR with Control Primer Set I to amplify a 160 bp region of the RNA Control I. The RT-PCR is tested for linearity by RT-qPCR and amplification products are visualized on an ethidium bromide-stained 2% agarose gel.

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