

## 1.0 INTRODUCTION

Historically, it has been noted that a significant amount of mis-priming and primer dimerization occurs prior to PCR during the initial ramping of the reaction from room temperature to the annealing temperature. Hot start PCR, an advanced PCR method to minimize mis-primers and primer dimers, requires the complete mixture of all reactants at a temperature that prevents primer annealing to non-target sequences. This involves withholding at least one essential reagent until the PCR reaction mixture has reached 60° to 80° C. HotStart Storage and Reaction Tubes provide a simple mechanism for synchronizing hot start amplifications without the need for manual intervention. HotStart Storage and Reaction Tubes do not require the use of mineral oil as a vapor barrier, eliminating the need for a chloroform extraction while providing the benefits of wax mediated hot start PCR.

HotStart Storage and Reaction Tubes are a unique combination of two essential components found in the wax mediated hot start reaction: 1) A premium, thin-walled reaction tube. 2) A specially formulated wax bead which has been precisely adhered to the inside surface of the reaction vessel. This not only facilitates the hot start PCR protocol, but also provides a novel approach to the storage of pre-aliquoted master mixes when performing routine amplifications. See section 4.0 for a detailed storage protocol.

Amplification using HotStart Storage and Reaction Tubes can be performed in four simple steps. At each step the necessary precautions to avoid contamination should be followed (3,4,5). See section 5.0 for further details on controlling contamination when using the HotStart Storage and Reaction Tubes for PCR. No special handling, shipping or storage conditions are required to maintain the integrity of the wax bead.

## 2.0 PROCEDURE

### IMPORTANT NOTES:

1) Do Not autoclave the HotStart Storage and Reaction Tube. The wax bead in the reaction tube has been positioned to optimize the hot start amplification process. HotStart Storage and Reaction Tubes are CERTIFIED RNase, DNase and DNA free and are ready-to-use.

2) When using thermostable polymerases that possess 3' to 5' exonuclease activity, the template DNA should be included in the lower layer components. This prevents degradation of the template by the enzyme in the absence of dNTPs.

### Step One: Assembling the lower layer reaction mixture.

Pipet the components of the lower layer into the HotStart Storage and Reaction Tube. Lower layer components usually include oligonucleotide primers, buffer, dNTPs and deionized water. The lower layer components should be mixed by gently pipetting up and down. For multiple reactions a "master mix" may be prepared. It is important that the final reaction volume is within the product's specified range (see table #1), so that the wax layer can form an effective barrier during the hot start process. The suggested starting volume for each HotStart Storage and Reaction Tube is listed in table 2 for your convenience.

### Step Two: Forming the wax barrier.

In order to ensure thorough melting of the wax bead, the HotStart Reaction Tube should be placed in a thermal cycler (See section 3.3 for machine compatibility), and maintained at 90° C for 30 seconds. After melting, the tube is brought to room temperature allowing the wax layer to harden over the lower layer reaction mix. Once sealed, these tubes allow safe storage of the lower layer reaction mixes at 4° C for four weeks. Please see the Storage Protocol (section 4.0) for additional information on this procedure. Please note that when using the HotStart Micro 20<sup>®</sup>, the wax will melt almost immediately at 85° C. This is due to the ultra-thin walls on the 0.2 ml reaction tube.

### Step Three: Assembling the upper layer reaction mixture.

Pipet the components of the upper layer on top of the wax barrier. Upper layer components will usually include DNA template, 10x PCR buffer with MgCl<sub>2</sub>, Thermostable DNA Polymerase\* and sterile, deionized water. If multiple reactions are being performed, a "master mix" may be prepared without the DNA target. See table #3 for suggested upper layer concentrations.

### Step Four: Beginning the hot start reaction.

Place the HotStart tubes into a thermal cycler and begin PCR. To ensure the thorough mixing of the reaction layers as well as the complete denaturation of your target, it is important that the first denaturation cycle be between one and five minutes. After completion of all the thermal cycles, the PCR product is removed from the HotStart Storage and Reaction Tube by penetrating the wax barrier with a pipet tip. It is strongly recommended that ART<sup>®</sup>, self-sealing barrier tips be used during the preparation of the lower and upper layer reaction mixes as well as any subsequent sample handling stages. See section 5.0 on avoiding contamination.

## 2.1 Suggestions

1) Exercise caution when withdrawing the sample. Vigorous penetration of the wax layer may result in a spray of the amplified product. Slowly puncture the center of the wax barrier (where thinnest), and rotate the pipettor in a circular motion to enlarge the hole.

2) It is useful to reheat the tube and let the wax re-solidify at a 45° angle to further reduce the wax thickness at the center of the tube.

3) When running low volume HotStart reactions, it is important that the volume of oil in the sensor tube approximates your PCR reaction volumes and that you are using a thin-walled reaction tube (such as the HotStart Storage and Reaction Tube) as your sensor tube.

To ensure sensor accuracy, dislodge a wax bead and use this tube as your sensor tube. It is important that the oil in your sensor tube matches your PCR reaction volume.

4) It is normal to see trace amounts of evaporation (less than 2 µl), this will not effect your PCR.

5) When using primers that anneal at temperatures higher than 60° C, you should use the next largest HotStart Storage and Reaction Tube. The reaction tubes for larger volumes contain greater amounts of wax, allowing a thicker barrier to form between the upper and lower layers. This will ensure that the reaction mix does not come together before your annealing temperature. For example, if using primers that anneal at a greater than 60° C with a reaction volume of 50 µl, use a HotStart 100® rather than a HotStart 50®.

6) You may save time and visualize the thermal mixing of your reaction by adding your loading dye to the upper layer reaction mixture.

### 3.0 REACTION LAYER PROPORTIONS

#### 3.1. Product Information

See Table 1

#### 3.2 Preparation of Lower Reaction Mix

See Table 2

#### 3.3 Preparation of Upper Reaction Mix

See Table 3

Suggested final concentration of the assembled reaction mixture is as follows: 10mM tris - HCl (pH 8.8); 50mM KCl; 1.5mM MgCl<sub>2</sub>; 200 µM dATP; 200 µM dCTP; 200 µM dGTP; 200 µM dTTP. 200 nM Primer 1; 200 nM Primer 2; 0.025 µl/µl Taq DNA polymerase; 102 to 106 copies of DNA template.

#### 3.4 Thermal Cycler Compatibility

Perkin-Elmer, Hybaid, Ericomp, Barnstead, Precision and MJ Research.

### 4.0 STORAGE PROTOCOL

When PCR reactions regularly require the use of the same primer sets, HotStart Storage and Reaction Tubes may be used as a convenient method to store pre-aliquoted "master mixes." The following procedure will save time, protect reagents from contamination, assure consistency, prevent oxidation, and allow for the improved sensitivity and accuracy that the hot start PCR protocol offers.

**Step One: Make the lower layer "master mix" per the HotStart Storage and Reaction Tube protocol (sections 2.0 to 3.3).**

**Step Two: Aliquot the master mix into the desired number of HotStart Storage and Reaction Tubes using an ART tip.**

**Step Three: Heat the reaction tubes in a thermal cycler or heat block for 30 seconds at 90° C such that an airtight wax barrier forms over the reagent mixture. Please note that when using HotStart Micro 20, the wax will melt almost immediately at 85° C.**

**Step Four: Master mixes can be stored for up to four weeks at 4° C in the original HotStart Storage and Reaction Tube unit box or other suitable tray.**

**Step Five: Prior to use, allow the HotStart Storage and Reaction Tube to come to room temperature for five to ten minutes and then vortex at low power to resuspend the MgCl<sub>2</sub>.**

**Step Six: Use tubes as needed, adding upper layer reaction mixture (see section 2.0 to 3.3). Verify that your initial denaturation cycle is between one and five minutes in duration to allow the thorough mixing of upper and lower layers. You may then proceed with your desired PCR profile.**

### 5.0 CONTROLLING CONTAMINATION

It is important to recognize that the elevated sensitivity gained through the hot start technique not only increases the PCR's detection of target DNA but non-target DNA as well. Therefore, special attention must be given to contamination control procedures. We suggest the following precautions:

- 1) Prepare upper and lower layer reaction mixtures under a laminar flow hood.
- 2) Wear and change gloves frequently. Wear a face mask and hair net.

It is possible to minimize the risks associated with carryover contamination by the meticulous handling of all laboratory supplies and experimental samples both prior to and after the PCR. The use of ART, self-sealing barrier tips, (also manufactured by MBP®) during all pre- and post-PCR phases will eliminate the cross contamination of samples by aerosol transfer from the pipettor to subsequent samples. These are the only tips that contain an interactive self-sealing barrier scientifically proven to eliminate aerosol contamination. ART tips are pre-sterilized and certified RNase, DNase and pyrogen free.

In order to prevent the transfer of DNA and PCR product from one surface to another, it is recommended that the thermal cycler sample block, counter space and the exterior of the pipettor mandrel be cleaned regularly with RNase AWAY®. When used as directed, this solution has been shown to degrade all DNA, RNA, DNase and RNase activity upon contact.

For catalog numbers and a copy of our Contamination Control Manual, call our Customer Service Representatives at:

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9880 Mesa Rim Road  
San Diego, CA 92121  
Toll-free: 800.995.2787  
Tel: 858.453.7551  
Fax: 858.453.4367  
e-mail: info@mbpinc.com  
www.mbpinc.com

TABLE 1

Product	HotStart Micro 20 Standard, Bulk & Strips	HotStart 50 / HotStart Micro 50 Standard, Bulk & Strips Micro 50 only)	HotStart 100 HotStart Micro 100 Standard & Bulk
Catalog Numbers	6008, 6308, 6208	6002, 6302/6010, 6310, 6210	6005, 6305, 6014
Reaction Range	15 µl-25 µl	20 µl-50 µl / 25 µl-50 µl	60 µl-100 µl

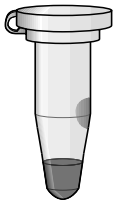

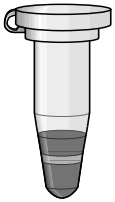
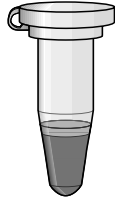
TABLE 2

Preparation of Lower Reaction Mix	For 20 µl Final Volume	For 50 µl Final Volume	For 100 µl Final Volume
Sterile Distilled Water	6.2 µl	15.5 µl	31.0 µl
10x PCR Buffer	1.0 µl	2.5 µl	5.0 µl
Primer 1 (10 µM)	0.4 µl	1.0 µl	2.0 µl
Primer 2 (10 µM)	0.4 µl	1.0 µl	2.0 µl
2.0-2.5 µM dNTP Mix	2.0 µl	5.0 µl	10.0 µl
Total Lower Layer Volume	10.0 µl	25.0 µl	50.0 µl

TABLE 3

Preparation of Upper Reaction Mix	For 20 µl Final Volume	For 50 µl Final Volume	For 100 µl Final Volume
Sterile Distilled Water	4.9 µl - 7.9 µl	12.25 µl - 21.25 µl	24.5 µl - 43.5 µl
10x PCR Buffer	1.0 µl	2.5 µl	5.0 µl
Taq DNA Polymerase (5 µ/µl)	0.10 µl	0.25 µl	0.5 µl
DNA Template	1 - 4 µl	1 - 10 µl	1 - 20 µl
Total Upper Layer Volume	10.0 µl	25.0 µl	50.0 µl

## The HotStart Storage and Reaction Tube Process

<p><b>Step 1</b> Add lower layer.</p> 	<p><b>Step 2</b> After thermocycler reaches 90°C, heat tubes for 30 seconds to melt wax.</p> 
<p><b>Step 3</b> After wax cools, add upper layer.</p> 	<p><b>Step 4</b> Complete all PCR cycles ensuring first cycle is above 90°C for one minute.</p> 

## 7.0 REFERENCES

- Chin, C., M. Russell, D. E. Birch, J. Raymond and W. Bloch. "Prevention of Pre-PCR Dimerization Improves Low Copy Number Amplifications." *Nucleic Acid Res* 20 (1992): 1717-1723.
- D'Aquila, R.T., L.J. Bechtel, J.A. Videler, J.J. Eron, P. Gorczyca and J.C. Kaplan. "Maximizing Sensitivity and Specificity of PCR by Pre-Amplification Heating." *Nucleic Acids res.* 19 (1991): 3749.
- Kwok, S. "Procedures to Minimize PCR Product Carryover." In: Innis, M.A., D.H. Gelfand, J.J. Sninsky and J.J. White. eds. *PCR Protocols: A Guide to Methods and Applications*. San Diego: Academic Press, (1990): 142-145.
- Higuchi, R. and S. Kwok. "Avoiding False Positives with PCR." *Nature* 339. (1989): 237-238.
- Mifflin, T.E. "Control of Contamination Associated with PCR and Other Amplifications Reactions." MBP (1994).
- Peters, R. "Elimination of PCR Carryover." *American Biotechnology Laboratory*, November, 1992.

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## SPECIAL PROTOCOL

### RT-PCR In One Tube Using HotStart 50

#### A. Introduction

Standard reverse transcription PCR\* protocols are typically time-consuming and extremely prone to contamination. This situation compromises an already highly sensitive reaction. However, with the development of thermostable reverse transcription enzymes and MBP's HotStart 50 Tube, RT-PCR has been made quicker, easier, and virtually contamination free.

By utilizing a wax barrier as a separation medium, RT-PCR can now be completed in one PCR tube. In doing so, the concern for sample loss and contamination involved in transferring reagents from tube-to-tube is eliminated. Also, because the enzyme used in this protocol is thermostable, reverse transcription time is cut in half. The specificity and sensitivity associated with RT-PCR is also heightened as the separation of reaction layers provides for a "hot start" PCR reaction.

#### B. Procedure

Using a HotStart 50<sup>®</sup> for a total reaction volume of 50  $\mu$ l, premix, or add individually, the reagents listed in TABLE 1 to the bottom of the tube.

Place in pre-heated thermal cycler at 70<sup>°</sup> C for 15 minutes, then cool to room temperature, completing the reverse transcription reaction. Then either pre-mix or add individually the reagents listed in TABLE 2 on top of the wax barrier.

Carry out the regular DNA amplification procedure involved with your template (30-35 cycles). Due to the separation of one's template DNA from the primer and Mg<sup>2+</sup> ion, false priming is discouraged. Simply remove your PCR product from the tube upon completing amplification cycling and analyze.

table 1

Reagent	50 $\mu$ l Reaction
DEPC Treated H <sub>2</sub> O	5.2 $\mu$ l
RNA	0.5 $\mu$ l (125 ng)
Reverse Transcription Buffer, 10X	1.0 $\mu$ l
10mM MnCl <sub>2</sub> Solution	1.0 $\mu$ l
dNTPs, 2.5mM each	0.8 $\mu$ l
tTH or rtTH DNA Polymerase**	1.0 $\mu$ l
oligo dT 12-18	0.5 $\mu$ l (0.25 $\mu$ g)
Total Volume	10.0 $\mu$ l

table 2

Reagent	50 $\mu$ l Reaction
Autoclaved dH <sub>2</sub> O	31 $\mu$ l
Chelating Buffer, 10X	4.0 $\mu$ l
25 mM MgCl <sub>2</sub>	4.0 $\mu$ l
First Primer	0.5 $\mu$ l (10 $\mu$ M)
Second Primer	0.5 $\mu$ l (10 $\mu$ M)
Total Volume	40.0 $\mu$ l

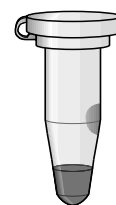
RT buffer: 100 mM tris-Cl pH 8.3, 900 mM KCl

Chelating buffer: 50% (v/v) glycerol, 100 mM tris-Cl pH 8.3, 1M KCl, 0.5% tween 20, 7.5 mM EGTA

\* The Polymerase Chain Reaction (PCR) process is covered by patents owned by Hoffman-La Roche, Inc. Use of this product does not confer a license for related Hoffman-LaRoche patents.

\*\* Perkin-Elmer DNA polymerase, part No. N808-0097 (has RT activity)

#### Reaction Tube Process

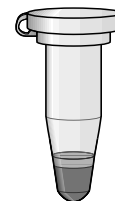


### Step 1

Add reverse transcription reagents.

### Step 2

Heat at 70<sup>°</sup>C for 15 minutes.  
(Then allow wax to cool)



### Step 3

Add PCR reagents.

### Step 4

Complete all PCR cycles.

