# Protocol for IsoAmp tHDA Kit

## Overview

The tHDA reaction can be performed using a two-step protocol (heat denaturation at 95°C, followed by amplification at 64°C) or a one-step protocol (the entire reaction at 64°C). Sensitivity of the tHDA reaction may increase approximately 10-fold with the two-step protocol. If possible, increase the amount of initial template DNA when following the one-step protocol.

## Introduction

**A. Two-step tHDA protocol**

1. To set up a 50 µl tHDA reaction, prepare a 25 µl premix of DNA template and primers in a 0.5 ml micro centrifuge tube in a sterile hood or a PCR workstation by combining:
10X Annealing Buffer: 2.5 µl
DNA template: __ µl
Forward primer (5 µM): 1 µl [a]
Reverse primer (5 µM): 1 µl [a]
$MgSO_4$ (100 mM): 1.25 - 2.5 µl [b]
$dH_2O$: __ µl
Total volume: 25 µl [c]

2. Incubate the premix at 95°C for 2 minutes, and then 64°C for 3 minutes using a water bath, an incubator or a thermocycler. Place the premix on ice.

3. Add an equal volume (25 µl) of 2X tHDA Mix (thawed and kept on ice) to the premix and gently mix the reaction by brief vortexing or by pipetting followed by brief centrifugation. Overlay the reaction mixture with mineral oil when needed to prevent evaporation during the reaction.

4. Incubate the tube at 64°C for 75 minutes (to enhance sensitivity, the incubation time can be extended to 90 minutes). Use 10 - 25 µl of the reaction for gel electrophoresis.

**B. One-step tHDA protocol**

For one-step tHDA, please skip step 2 from the two-step tHDA protocol above and proceed directly with step 3 after preparation of the premix as outlined in step 1.

**Notes:**

a. Primer concentrations affect the efficiency of the amplification reaction. The working range of primer concentration is from 0.05 to 0.2 µM. Use 0.1 µM for an initial test (1 µl of 5 µM stock in a 50 µl reaction volume).
b. Due to the sensitivity of the tHDA reaction to $MgSO_4$, it is necessary to experimentally determine its optimal concentration for each set of primers and DNA template. Concentrations between 2.5 mM to 5 mM have proven effective. Please see the table below to determine the amount of $MgSO_4$ (100 mM) to add to the assay. In general use the 3.5 mM and 4 mM $MgSO_4$ (highlighted in bold type) for the initial test:

**100 mM $MgSO_4$ (for a 50 µl tHDA reaction)**

Volume -- Final Concentration
1.25 µl -- 2.5 mM
1.50 µl -- 3.0 mM

**1.75 µl -- 3.5 mM**
**2.00 µl -- 4.0 mM**
2.25 µl -- 4.5 mM
2.50 µl -- 5.0 mM

c. Addition of NaCl to a final concentration of 5 - 50 mM may enhance the performance of tHDA for certain amplicons. After step 2, add varying concentration of NaCl (0.5 - 5 µl of the 500 mM NaCl) to the premix to experimentally determine the optimal NaCl concentration.

Caution: The tHDA reaction is extremely sensitive to alterations in temperature as well as magnesium and salt concentrations. The recommended temperature is 64°C; however, the tHDA reaction can be performed at temperatures ranging from 62°C - 65°C. Avoid introduction of any substances that may alter the concentration of magnesium and salt in the reaction.