biohelix®

next generation HDA nucleic acid analysis

IsoAmp® III Universal tHDA Kit

(tHDA : thermophilic Helicase-Dependent Amplification)

Catalog # H0120S

Store at -20°C

INSTRUCTION MANUAL

Thermophilic Helicase-Dependent Amplification (tHDA) is a novel method for isothermal amplification of nucleic acids. Like PCR, the tHDA reaction selectively amplifies a target sequence defined by two primers. However, unlike PCR, tHDA uses an enzyme called a helicase to separate DNA, rather than heat. This allows DNA amplification without the need for thermocycling. The tHDA reaction can also be coupled with reverse transcription for RNA analysis.

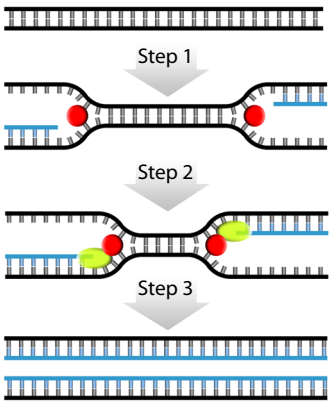

Figure 1. HDA technology

Step 1. Helicase unwinding and primer binding.

Step 2. DNA polymerization

Step 3. DNA amplification

IsoAmp® III Universal tHDA kit is based on a third-generation tHDA formula for more robust and specific amplification with reduced non-specific products due to primer-dimmer. In some cases, rapid amplification may be achieved by adding more IsoAmp® III Enzyme Mix into the reaction (unpublished observation). The volume of IsoAmp® III Enzyme Mix has been reduced to 2 µL per reaction, which allows the user to add more enzymes (2 X or 3 X) for possible high-speed amplification. IsoAmp® III Enzyme Mix (Cat# H1020S) can be purchased separately.

IsoAmp® III Universal tHDA kit supports all reactions which are supported by IsoAmp® II Universal tHDA kit. Selected examples are included in this manual, including end-point tHDA and real-time quantitative HDA (qHDA) in both one-step and two-step formats. A Control Template and a set of specific primers are supplied for the positive control reaction.

KIT COMPONENTS

IsoAmp® III Enzyme Mix (50 reactions)	100 µl
IsoAmp® dNTP Solution (50 reactions)	175 µl
10X Annealing Buffer II	0.5 ml
$MgSO_4$ (100 mM)	1 ml
NaCl (500 mM)	1 ml
pCNG1 (Control Template: 1 ng/µl)	30 µl
NGF3 (Forward Primer: 5 µM)	30 µl
NGR3 (Reverse Primer: 5 µM)	30 µl

STORAGE CONDITION

Reommended storage conditions for the tHDA kit are -20°C for storage shorter than 6 months and -80°C for storage greater than 6 months. Please avoid repeated freeze-thaw cycles as this may decrease performance of the kit.

REAGENTS FOR REAL TIME HDA (not included)

Recommended for use with the IsoAmp® III Universal tHDA kit when applicable:

EvaGreen (Biotium, Cat# 31000)
ROX Reference Dye (Invitrogen, Cat# 12223-012)

POSITIVE CONTROL ASSAYS

The Control Template and Control Primers provided in this kit can be used as a positive control in HDA (Fig. 2). This control reaction amplifies a 102 bp target sequence. The expected melting temperature (Tm) of the amplicon in the melt-curve analysis is 78°C ± 1°C. To set up a positive control, follow the one-step or two-step tHDA or qHDA protocols using 1 µl of pCNG1 (Control Template: 1 ng/µl), 0.75 µl of NGF3 (Forward Primer: 5 µM) and 0.75 µl of NGR3 (Reverse Primer: 5 µM).

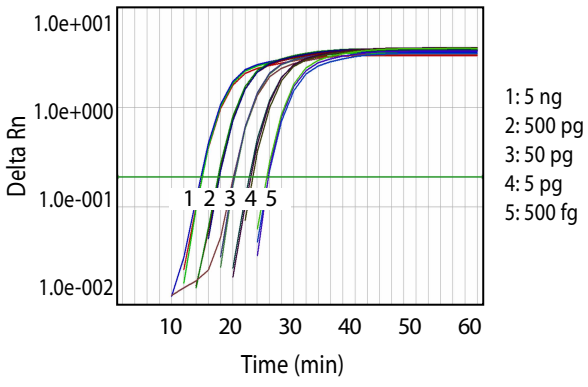

Figure 2. Positive control assays with varying amount of Control Template. Positive control assays were conducted following the one-step qHDA protocol. Control Template was used at 5 ng - 500 fg per assay in triplicate.

PROTOCOLS

All reactions supported by the IsoAmp® III Universal tHDA kit can be performed using either a one-step (the entire reaction at 65ºC) or a two-step protocol (template denaturation at 95ºC, followed by amplification at 65ºC) . For maximal sensitivity, the two-step protocol is recommended. The sensitivity of the tHDA and qHDA reactions may be up to 10-fold higher using a two-step protocol than using a one-step protocol for some amplicons and/or primer sets.

One Step Protocols

A. One-Step tHDA (thermophilic HDA)

A.1. Set up a 50 µl reaction in a 0.2-ml or a 0.5-ml micro centrifuge tube in a sterile hood or a PCR Workstation.

H_2O	X µl
10X Annealing buffer II	5.0 µl
$MgSO_4$ (100 mM)*	2.0 µl
NaCl (500 mM)*	4.0 µl
IsoAmp® dNTP Solution	3.5 µl
DNA template	X µl
Forward Primer (5 µM)*	0.75 µl
Reverse Primer (5 µM)*	0.75 µl
IsoAmp® III Enzyme Mix	2.0 µl
Total volume	50.0 µl

A.2. Mix the reaction by brief vortexing or by pipetting followed by brief centrifugation. Overlay the reaction mixture with 50 µl mineral oil. Place the tubes on ice.

A.3. Incubate at 65ºC for 90 minutes using a thermocycler, a water bath or an incubator.

A.4. Load 10 µl of the tHDA product on a 2% agarose gel.

B. One-Step qHDA (Real-time quantitative tHDA)

The following protocols are intended for real-time detection using the Applied Biosystems 7300 Real-Time PCR System.

The kit also can be coupled with real-time detection methods to conduct real-time quantitative tHDA (qHDA) to monitor amplification as it progresses. For optimal performance, use EvaGreen as a reporter dye and ROX as a passive reference dye. Sequence-specific probes can also be designed for qHDA experiments.

B.1. Set up a 50 µl reaction in a MicroAmp optical tube (Applied BioSystems) in a sterile hood or a PCR Workstation.

H_2O	X µl
10X Annealing buffer II	5.0 µl
$MgSO_4$ (100 mM)*	2.0 µl
NaCl (500 mM)*	4.0 µl
IsoAmp® dNTP Solution	3.5 µl
DNA template	X µl
Forward Primer (5 µM)*	0.75 µl
Reverse Primer (5 µM)*	0.75 µl
IsoAmp® III Enzyme Mix	2.0 µl
EvaGreen (20X, Biotium)	0.5 µl
ROX Reference Dye (50X, Invitrogen)	1.0 µl
Total volume	50.0 µl

B.2. Mix the reaction by brief vortexing or by pipetting followed by brief centrifugation. Overlay the reaction mixture with 50 µl mineral oil. Place the tubes on ice.

B.3. Real-time detection is carried out on a 7300 Real-Time PCR System (ABI) with the following well inspector setting: reporter dye: SYBR; quencher: none; passive reference dye: ROX. Use the following program:

Stage 1: (60 X)
- Step1: 66ºC for 0:05
- Step2: 65ºC for 1:55
- Data collection and real-time analysis enabled

Stage 2: (1 X)
- Dissociation Stage (default settings of the machine)
- Melt curve data collection and analysis enabled

Two Step Protocols

C. Two-Step tHDA (thermophilic HDA)

C.1. To set up a 50 µl tHDA reaction, prepare a 25 µl Mix A in a 0.5-ml micro centrifuge tube in a sterile hood or a PCR Workstation.:

H_2O	X µl
10X Annealing buffer II	2.5 µl
DNA template	X µl
Forward Primer (5 µM)*	0.75 µl
Reverse Primer (5 µM)*	0.75 µl
Total volume of Mix A	25 µl

In addition, prepare a 25 µl Mix B in a separate 0.5-ml micro centrifuge tube in a sterile hood or a PCR Workstation:

H_2O	11.0 µl
10X Annealing buffer II	2.5 µl
$MgSO_4$ (100 mM)*	2.0 µl
NaCl (500 mM)*	4.0 µl
IsoAmp® dNTP Solution	3.5 µl
IsoAmp® III Enzyme Mix	2.0 µl
Total volume of Mix B	25.0 µl

C.2. Gently mix each of the mixes by brief vortexing or by pipetting followed by brief centrifugation. Overlay Mix A with 50 µl mineral oil. Place the tubes on ice.

C.3. Incubate Mix A at 95ºC for 2 minutes and place promptly on ice. Add 25 µl of Mix B into Mix A underneath the oil layer and gently mix the reaction by pipetting. Place the tubes on ice.

C.4. Incubate at 65ºC for 90 minutes using a thermocycler, a water bath or an incubator.

C.5. Load 10 µl of the tHDA product on a 2% agarose gel.

D. Two-Step qHDA (Real-time quantitative tHDA)

The following protocols are intended for real-time detection using the Applied Biosystems 7300 Real-Time PCR System.

The kit also can be coupled with real-time detection methods to conduct real-time quantitative tHDA (qHDA) to monitor amplification as it progresses. For optimal performance, use EvaGreen as a reporter dye and ROX as a passive reference dye. Sequence-specific probes can also be designed for qHDA experiments.

D.1. To set up a 50 µl qHDA reaction, prepare a 25 µl Mix A in a 0.2-ml MicroAmp optical tube (ABI) and a 25 µl Mix B in a 0.5-ml micro centrifuge tube in a sterile hood or a PCR Workstation.

H_2O	X µl
10X Annealing buffer II	2.5 µl
DNA template	X µl
Forward Primer (5 µM)*	0.75 µl
Reverse Primer (5 µM)*	0.75 µl
Total volume of Mix A	25.0 µl

H_2O	8.0 µl
10X Annealing buffer II	2.5 µl
$MgSO_4$ (100 mM)*	2.0 µl
NaCl (500 mM)*	4.0 µl
IsoAmp® dNTP Solution	3.5 µl
IsoAmp® III Enzyme Mix	2.0 µl
EvaGreen (20X, Biotium)	0.5 µl
ROX Reference Dye (50X, Invitrogen)	1.0 µl
Total volume of Mix B	25.0 µl

D.2. Gently mix each of the mixes by brief vortexing or by pipetting followed by brief centrifugation. Overlay Mix A with 50 µl mineral oil. Place the tubes on ice.

D.3. Incubate Mix A at 95ºC for 2 minutes and place promptly on ice. Add 25 µl of Mix B into Mix A underneath the oil layer and gently mix the reaction by pipetting. Place the tubes on ice.

D.4. Real-time detection is carried out on a 7300 Real-Time PCR System (ABI) with the following well inspector setting: reporter dye: SYBR; quencher: none; passive reference dye: ROX. Use the following program:

Stage 1: (60 X)
Step1: 66ºC for 0:05
Step2: 65ºC for 1:55
Data collection and real-time analysis enabled

Stage 2: (1 X)
Dissociation Stage (default settings of the machine)
Melt curve data collection and analysis enabled

* The condition of tHDA reactions can be further optimized by titering the following components:

Components	Recommended concentration	Recommended concentration for titering
$MgSO_4$	3.5 to 4 mM	3 to 4.5 mM
NaCl	30 to 40 mM	20 to 50 mM
Primer	75 to 100 nM	50 to 200 nM

PRIMER DESIGN AND AMPLICON SELECTION

tHDA primers can be designed using either the PrimerQuest program (*http://www.idtdna.com/Scitools/Applications/Primerquest/Advanced.aspx*) or the Primer3 program (*http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).* Recommended parameter settings:

Product size: 80 – 120 bp**
Product Tm: Min. 68; Opt. 71; Max. 75†
Primer size: Min. 24; Opt. 27; Max. 33‡
Primer Tm: Min. 60; Opt. 68; Max. 74§
Primer GC%: Min. 35; Opt. 44; Max. 60

(**) tHDA works most efficiently with a product size around 100 bp. Successful tHDA amplifications were achieved with a product as short as 85 bp and as long as 129 bp.

(†) The Tm of an amplicon with a product size around 100 bp and a G + C content around 40% is approximately 71°C from the calculation of Primer3. Successful tHDA amplifications were achieved with a product Tm as low as 68 °C and as high as 77°C.

(‡) The optimal primer size may be set at 26 bases when the G + C content of target sequence is larger than 45%. Successful tHDA amplifications were achieved with a primer size as short as 22 bases and as long as 32 bases.

(§) The optimal primer Tm may be set at 64 - 66°C when the G + C content of target sequence is smaller than 37.5% and at 70 - 72°C when the G + C content of target sequence is larger than 45%. Successful tHDA amplifications were achieved with a primer Tm as low as 60 °C and as high as 75 °C.

Other considerations:

1) Amplicons (regions to be amplified) containing a G + C content of approximately 40% are preferable (the G + C content of amplicons and target sequences can be calculated using the Oligonucleotide Properties Calculator program found at: http://www.basic.northwestern.edu/biotools/oligocalc.html).

2) Primer sets obtained using Primer3 with an average G + C content closest to the G + C content of the corresponding amplicons are preferable.

3) To obtain ideal amplification performance, test several amplicon regions for each target gene and design several primer sets for each selected region. The efficiency of the tHDA reactions may vary dramatically for different amplicons as well as for different primer sets within the specific region. Using primers optimized for PCR reactions may not guarantee similar performance in tHDA reactions.

4) Once a set of primers yields a positive amplification result, serial primer sets longer or shorter than this set may be analyzed to generate optimal primers for increased amplification efficiency.

5) Due to the sensitivity of tHDA to changes in salt concentrations in the reaction, the use of desalted primers is recommended. Successful tHDA reactions were achieved using salt-free primers synthesized by Operon Biotechnologies.

GENERAL RECOMMENDATIONS

As tHDA and qHDA are exponential amplification reactions, extra attention should be paid to avoid sample cross-over and carry-over contamination. Reaction assembly, amplification, and gel electrophoresis-based detection steps should be carried out in physically separated locations. Always wear gloves. Open the reaction tubes only when adding reagents into them during reaction setup and keep them closed at any other time. Never open the tubes after qHDA reactions are done.

Performance of tHDA and qHDA is extremely sensitive to changes in the magnesium and salt concentrations of the reaction. Avoid introducing any substances that may affect these concentrations.

Every freshly thawed reagent should be gently vortexed and spun down before being added to the reaction. Autoclaved Milli-Q water should be used for tHDA and qHDA reactions. Use sterilized filter tips only and change tips after each pipetting step.

For technical support or to place an order, please contact BioHelix:

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