Optimized Integration of New England Biolabs® Loop-mediated Isothermal Amplification (LAMP) Reagents with Axxin ISO Instruments

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Introduction

Molecular diagnostics requires the identification of a specific DNA or RNA sequence of interest from a given sample, which has long been enabled by the polymerase chain reaction (PCR). As diagnostic methods move out of clinical laboratory settings and to the point of care, solutions for rapid and simple molecular detection become increasingly important to avoid the cost and complexity of PCR systems. Most prominently, isothermal amplification methods have become a powerful and flexible option for building simple and fieldable molecular diagnostics (1,2). As isothermal amplification requires incubation at only a single temperature, power requirements and the degree of sophistication of instrument design can be significantly reduced, as compared to PCR thermal cycling. In the absence of amplification limits defined by thermal cycles (i.e., a maximum of doubling of DNA copies per PCR cycle), isothermal reactions can proceed extremely rapidly, amplifying to a detectable amount in 20 minutes or less in standard reaction vessels (e.g., PCR strip tubes). Detection options for isothermal reactions also vary, with the large amount of synthesis enabling visual detection, in addition to the more standard fluorescence detection methods employing intercalating dyes or probes. The fluorescence methods are well-suited for quantitative analysis, multiplexing, and approaches seeking CLIA waivers.

The most commonly used and powerful isothermal amplification method is loop-mediated isothermal amplification (LAMP), which utilizes a unique primer design to create self-priming "loop" structures to facilitate rapid and extensive amplification (3,4). In contrast to methods like recombinase polymerase amplification (RPA) and helicase dependent amplification (HDA), no additional enzymes are necessary to enable amplification; LAMP is powered by only a strand-displacing polymerase for DNA targets and the addition of a reverse transcriptase for RNA targets. LAMP primers are complex but are easily designed using free software tools (e.g., Primer Explorer V5; 5).

For use in LAMP, an instrument must provide consistent heating to the desired reaction temperature, and sensitive, real-time fluorescence measurement for quantitative assays. Axxin has developed flexible benchtop and field-deployable isothermal platforms with these capabilities. This application note provides optimized settings and considerations for performing LAMP reactions on the Axxin T8-ISO and T16-ISO instruments. These instruments are readily compatible with LAMP reactions utilizing DNA or RNA targets in singleplex or multiplex format.

Axxin T8 Isothermal Instrument

Reagents from NEB Developed for Isothermal Amplification:

- **Novel engineered enzymes**

  Bst 2.0 DNA Polymerase (NEB #M0537) for faster and more robust amplification of DNA targets

  Bst 3.0 DNA Polymerase (NEB #M0374) for reactions requiring a single RT and DNA polymerase enzyme

  WarmStart® RTx Reverse Transcriptase (NEB #M0380) for faster and more robust amplification of RNA targets (used with *Bst* 2.0 or *Bst* 3.0)

- **WarmStart control of isothermal enzymes**

  Bst 2.0 WarmStart DNA Polymerase (NEB #M0538) and WarmStart RTx for utilizing unique WarmStart control of enzyme activity for room-temperature reaction setup

- **Optimized master mixes and kits**

  WarmStart LAMP Kit (DNA & RNA) (NEB #E1700) utilizes WarmStart RTx and *Bst* 2.0 WarmStart

  WarmStart Colorimetric LAMP 2X Master Mix (DNA & RNA) (NEB #M1800) utilizes pH-based visual detection for simple, non-instrumented detection of LAMP assays (6)

- **Enabling methods that extend the power of isothermal diagnostics**

  Multiplex LAMP via labeled DARQ primers (7)

  Carryover prevention *via* Antarctic Thermolabile UDG (NEB #M0372) (8)

  Reduction of nonspecific amplification *via Tte* UvrD Helicase (NEB #M1202) (9)

Example 1: **Singleplex LAMP with Intercalating Dye**

The basic LAMP assay design uses DNA-binding fluorescent dyes to detect amplification through an increase in the amount of double-stranded DNA. This approach is widely used for laboratory and field LAMP, and the best performance for real-time and field detection is provided by the SYTO® family of dyes (10), notably SYTO-9 for compatibility with the FAM/SYBR channel common to real-time fluorimeters. NEB utilizes a dye with similar properties in our WarmStart LAMP Kit (DNA & RNA) (NEB #E1700), and we tested its compatibility with LAMP reactions on the T8-ISO. Typical concentrations of the dye used in standard LAMP assays on qPCR fluorimeters saturated the T8-ISO detector using default conditions and settings. To measure amplification, we adjusted the concentration to 0.1X final (dye is provided as a 50X stock) and reduced the FAM Test Channel level to 7%. Reactions were incubated at 65°C for 20–30 minutes, and fluorescence measured at 10 or 20 second intervals. An initial 30 second delay before the first reading is suggested, as the background fluorescence level of the dye will stabilize to a lower level once the reaction is heated to 65°C. For algorithmic settings, any desired parameter set can be used but the rapid nature of the LAMP reaction allows for gradient settings of 10 mV/s and amplitude requirements of 2000 mV fluorescence increase with background correction (4500 mV without due to higher background fluorescence). Figure 1 shows an example RT-LAMP reaction detecting ACTB from Jurkat total RNA, with positive judgment to 1 pg of RNA input in ~20 minutes. As seen in the graph, some non-template amplification occurred, but this can be distinguished from positive amplification based on settings in the test channel algorithm, here using amplification time. This discrimination can be used for well-characterized assays (validated with melt curve analysis, agarose gel electrophoresis, or other secondary method) to eliminate false positives and maximize detection sensitivity

Materials:

	Standard	Optimized
WarmStart LAMP Kit (DNA & RNA), (NEB #E1700)	1X	1X
LAMP Fluorescent Dye, (NEB #E1700) (50X)	1X	0.1X
Jurkat Total RNA, Biochain	1 pg–100 ng	1 pg–100 ng
Primer Mix*	1X	1X

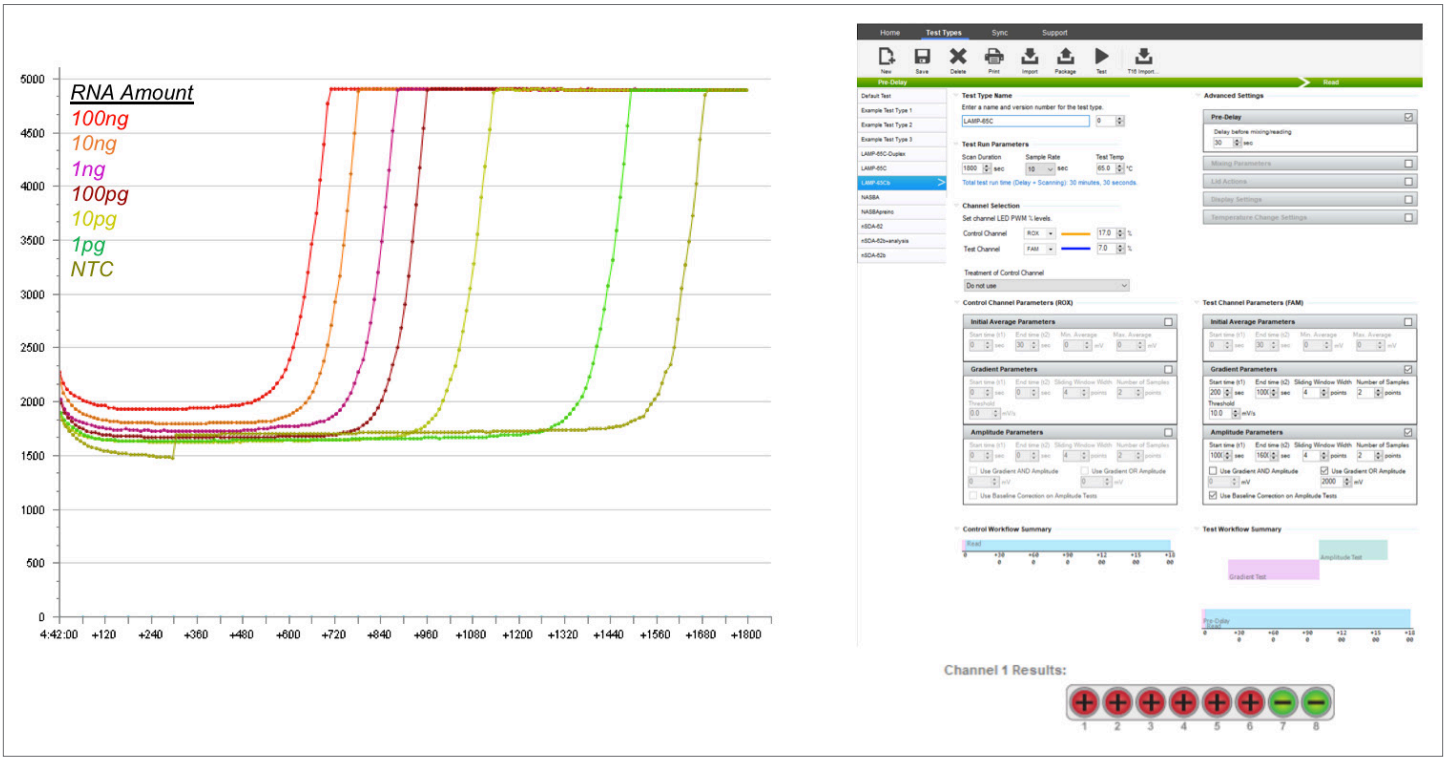
*1.6 µM FIP, BIP; 0.2 µM F3, B3; 0.4 µM Loop F, Loop B

T8-ISO Instrument Settings:

	Default	Optimized
Temperature	40°C	65°C
Time	15 minutes	20–30 minutes
Read Interval	10 seconds	10 seconds
Read Start Delay	0 seconds	30 seconds
FAM Channel LED PWM%	20%	7%

Amplitude Test: Start 100 s–End 1600 s; 4 point width, 2 point sample; 2,000 mV Amplitude with Baseline Correction

FIGURE 1:
Detection of ACTB in an RT-LAMP reaction using NEB's WarmStart LAMP Kit and Axxin's T8-ISO instrument



Example 2: **Multiplex LAMP with DARQ Probes**

In order to include an internal control in an amplification reaction, some type of target specific detection is typically employed to distinguish between the different amplicon targets. For PCR methods hydrolysis probes are commonly used, with the 5´→3´ flap endonuclease activity of *Taq* DNA Polymerase producing target-specific fluorescence from a dual-labeled quencher-fluorophore probe that can be detected with multiple channels of a real-time fluorimeter. This approach is incompatible with LAMP due to the requirement for 5´→3´ strand displacement activity from the DNA polymerase used in the reaction, with *Bst* or engineered forms used here truncated to the "large fragment" form, removing the natural 5´→3´nuclease domain and promoting efficient strand displacement. Accordingly, for multiplex LAMP an alternative probe design must be used. NEB developed one approach for multiplex LAMP detection which uses a 5´ dark quencher-modified FIP or BIP annealed to a corresponding 3´ fluorophore-labeled oligonucleotide ("Fd") using the inherent tailed design of the FIP/BIP LAMP primers (termed "DARQ"; 7). As the duplex primer is used for LAMP amplification, synthesis from the opposite direction by the polymerase will displace the Fd oligo from the duplex primer, releasing the quenching and producing fluorescence. This method requires no additional probe design and can be adapted to any LAMP assay or primer set, with the existing unlabeled primer sequence modified with 5´ quencher and synthesis of a labeled Fd probe oligo. Q-FIP and Fd should be pre-annealed prior to use in the LAMP reaction to create a quenched duplex, with this duplex primer used in combination with the standard FIP or BIP for maximum speed and performance. Other than labeled primers, the only adjustment to a standard LAMP protocol is to reduce the primer concentration by a factor of the number of targets in the reaction (e.g., for 2 targets, use 50% standard primer concentration) to avoid inhibition due to high oligonucleotide primer concentration (7). Figure 2 shows a 2-plex reaction detected on the T8-ISO using a FAM control assay (λ phage DNA) and a ROX test assay with either RNA (Jurkat total, HMBS) or *C. elegans* gDNA (lec-10). Control Channel was set to the FAM assay and Test Channel to ROX, with similar algorithm settings for both. Primer sets are as described in Reference 7. While 2 target detection allows for use of an internal control, if a third target is desired the T16-ISO instrument can be used, with the third channel dedicated to a HEX or similar flourophore-labeled primer set and target.

Materials:

	Standard	Optimized
WarmStart Colorimetric LAMP 2X Master Mix (DNA & RNA), (NEB #M1800)	1X	1X
DNA 1 (λ DNA), (NEB #N3011)	500 fg–5 ng	500 fg–5 ng
DNA 2 (*C. elegans* DNA)	10 pg–100 ng	10 pg–100 ng
RNA 1 (Jurkat Total RNA), BioChain	1 pg–100 ng	1 pg–100 ng
Primer Mix 1	1X*	0.5X**
Primer Mix 2	N/A	0.5X**

* 1.6 µM FIP, BIP; 0.2 µM F3, B3; 0.4 µM Loop F, Loop B

** 0.4 µM FIP, Q-FIP:Fd; 0.8 µM BIP; 0.1 µM F3, B3; 0.2 µM Loop F, Loop B

T8-ISO Instrument Settings:

	Default	Optimized
Temperature	40°C	65°C
Time	15 minutes	20–30 minutes
Read Interval	10 seconds	10 seconds
Read Start Delay	0 seconds	30 seconds
FAM Channel LED PWM%	20%	7%
ROX Channel LED PWM%	30%	17%

Gradient Parameters:
Control (FAM): Start 240 s–End 1200 s; 4 point width, 2 point sample; 10 mV/s

Test (ROX): Start 800 s–End 2400 s; 4 point width, 2 point sample, 5 mV/s

Amplitude Parameters:
Control (FAM): Start 1200 s–End 3000 s; 4 point width, 2 point sample; 4500 mV Amplitude

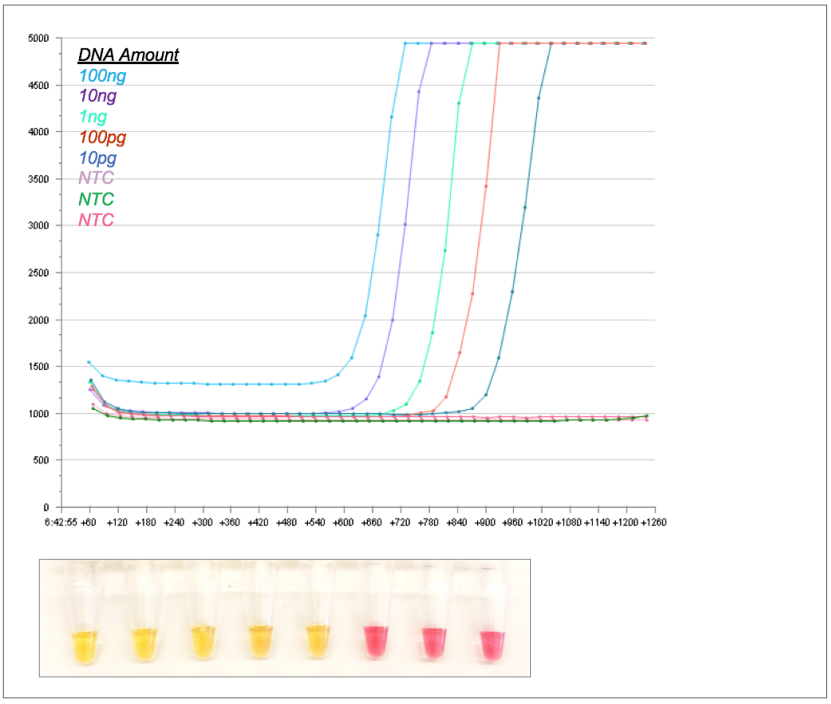
Test (ROX): Start 2000 s–End 3600 s; 4 point width, 2 point sample; 3500 mV Amplitude

FIGURE 2:
2-plex LAMP reaction using NEB's WarmStart Colorimetric LAMP 2X Master Mix and Axxin's T8-ISO instrument, using the FAM control assay

Example 3: **Colorimetric LAMP**

LAMP detection can be performed using a variety of strategies, with a high degree of amplification and tolerance of the reaction suitable to use of metal-binding indicators (e.g. calcein, hydroxynaphthol blue), fluorescent dyes, or even magnesium pyrophosphate precipitation as the readout. NEB developed a clear visual approach to LAMP detection utilizing the inherent production of hydrogen ions by a DNA polymerase as it incorporates dNTP into nascent DNA. Synthesis in a LAMP reaction can produce pH changes of 2+ units, and by pairing a minimal-buffered LAMP reaction with pH-sensitive dye we created a rapid visual format for LAMP detection-the WarmStart Colorimetric LAMP 2X Master Mix (DNA & RNA) (NEB #M1800) (6). This colorimetric format is particularly well suited for simple field and point-of-care assays, for example detecting Zika virus directly in urine samples (11). As a complement to visual readouts, the Colorimetric Master Mix can still support the fluorescence readouts described previously, and we tested the compatibility of ISO instruments with fluorescence detection. Figure 3 shows Colorimetric LAMP on the T16-ISO and intercalating dye detecting human BRCA1 with HeLa genomic DNA. Both a fluorescence judgment of amplification and a visual color change were measured in under 20 minutes, with no interference by the colorimetric dye. The flexibility of both the instrument and amplification chemistry enable a range of applications, with suitability for accurate molecular tests and rapid field diagnostics.

FIGURE 3:
Detection of BRCA1 using NEB's WarmStart Colorimetric LAMP 2X Master Mix and Axxin's T16-ISO instrument, using intercalating dye



Summary

LAMP is a powerful isothermal amplification method enabling simple and rapid molecular diagnostics. But to fully realize the potential of the amplification chemistry, it must be paired with instrumentation capable of performing and detecting the reaction. As described here, the Axxin ISO instruments are a such platform, compatible with robust NEB LAMP reagents to help bring molecular diagnostics to the point of care.

Materials:

	Standard	Optimized
WarmStart Colorimetric LAMP 2X Master Mix (DNA & RNA), (NEB #M1800)	1X	1X
LAMP Fluorescent Dye, (NEB #M1800) (50X)	1X	0.1X
HeLa Genomic DNA, (NEB #N4006)	30 pg–100 ng	30 pg–100 ng
Primer Mix	1X*	1X

* 1.6 µM FIP, BIP; 0.2 µM F3, B3; 0.4 µM Loop F, Loop B

T8-ISO Instrument Settings:

	Default	Optimized
Temperature	40°C	65°C
Time	15 minutes	20–30 minutes
Read Interval	10 seconds	10 seconds
Read Start Delay	0 seconds	30 seconds
FAM Channel LED PWM%	20%	7%

Amplitude Test: Start 100 s–End 1600 s; 4 point width, 2 point sample; 2,000 mV Amplitude with Baseline Correction

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