Automated mixing of isothermal RAA assays using the Axxin T8-ISO instrument

Related products

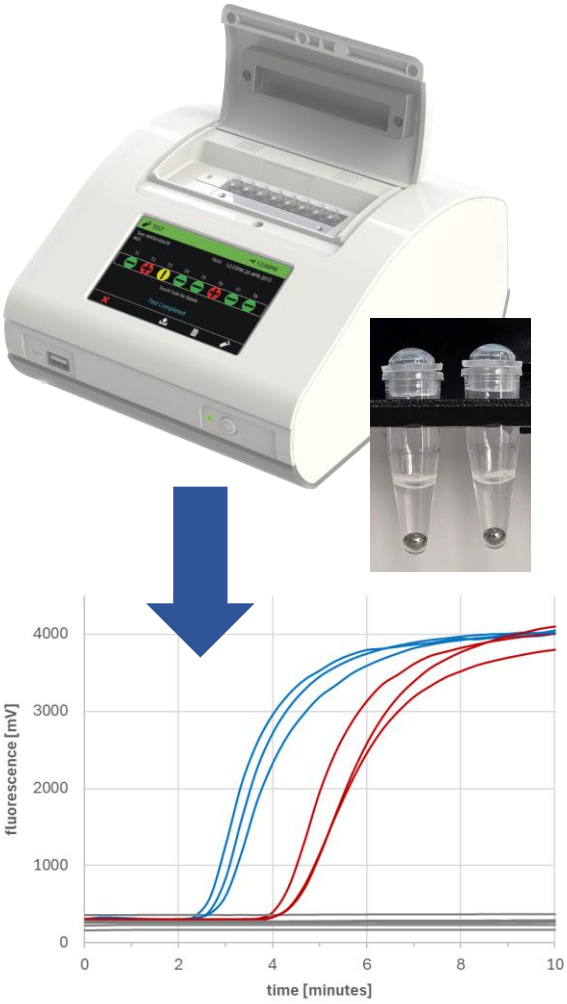
This application note is related to:

- **Axxin T8-ISO** (cat.no. P003843) and **External power pack** (cat.no. P004052)
- **Recombinase-aided amplification (RAA) reagents** for rapid isothermal amplification and detection of nucleic acids (cat.no. F00001 and F00R01)
- **High-grade stainless-steel balls** for automated reagent mixing (cat.no. SSB-50)

Summary

Here we report our practical experience with a Recombinase-Aided Amplification (RAA) assay for the rapid isothermal **real-time detection of West-Nile Virus (WNV) RNA**. We conducted the assays using the compact, portable fluorescence reader system T8-ISO by Axxin (Australia).

To unlock the full performance of recombinase-based assays, a second manual reagent mixing step is required a few minutes after the initial start of the detection reaction. Utilizing the unique automated mixing option of the T8-ISO instrument and by employing high-grade stainless-steel balls, we demonstrated probe-based detection of WNV RNA **in as little as 3 minutes with enhanced usability** and without negative impact on the RAA assay performance compared to the traditional mixing mode.



Introduction

Isothermal technologies for the amplification of DNA and RNA are established alternatives to PCR-based nucleic acid amplification testing (NAAT). Technologies such as LAMP, NASBA, RPA, and TMA have been on the market for more than two decades and are as mature as PCR. They feature two main advantages over PCR-based NAAT: speed and simplicity of required instrumentation [1]. Since the amplification cycles of isothermal methods do not require changes in reaction temperature, the instruments for detection of molecular diagnostic results are much more compact and energy-efficient than rapid PCR cyclers. An example for this type of devices is the T8-ISO fluorescence reader by Axxin. The instrument allows for simultaneous measurement of up to 8 samples and comprises a two-channel fluorescence optics. It supports full stand-alone operation and provides stable off-grid power supply through an external powerpack.

Recombinase-based isothermal amplification such as RPA and RAA is among the fastest commercially available amplification chemistries. Formulations are available which include reverse transcriptase for seamless one-step amplification and detection of DNA and RNA signatures in as little as 3 minutes [2, 3]. However, to unfold their full sensitivity and speed, recombinase-based chemistry needs a second mixing step a few minutes after the reaction has been started [2]. Usually, this requires the user to open the detection instrument after the run has been started to remove the amplification tubes, vortex and spin them, and place them back into the instrument.

This cumbersome and error-prone procedure can be automated by the Axxin T8-ISO instrument. It features a mechanism that allows for seamless mixing of reagents within the amplification tubes. The continuous mixing is facilitated through a magnet moving in a horizontal direction along the amplification tubes thereby actuating a stainless-steel ball place inside each amplification tube.

Experimental details

For RT-RAA amplification and detection of West-Nile Virus (WNV) RNA, the primer set described by Tomar *et al.* (4) was used together with Recombinase-aided amplification reagents from Jiangsu Qitian Gene Biotechnology, which are distributed by Xpedite Diagnostics (cat.no. F00R01). The WNV RNA target was purchased from TIB Molbiol (Germany). Incubation and fluorescence detection was performed in the T8-ISO instrument by Axxin, which is distributed by Xpedite Diagnostics (cat.no. P003843). For automated mixing, high-grade stainless-steel balls with a 2mm diameter (cat.no. SSB-50) were used.

The reactions were set up as follows: 40µL mastermix (25µL Rehydration Buffer VI, 2µL primer/probe-mix (final concentration in the amplification reaction: 400nM primers, 50nM probe), 13µL H_2O) was added to the freeze-dried enzyme pellet in the amplification tube without mixing. Then 5µL Magnesium acetate as well as 5µL Template was pipetted into the cap of the amplification tube and the cap was carefully closed while avoiding that “cap liquids” mix with the mastermix in the bottom part of the tube. The tubes were then vortexed for 5 seconds and spun down briefly with a mini centrifuge. The tubes were immediately placed inside the T8-ISO reader and the amplification run was started without delay.

The standard procedure requires a brief interruption of the fluorescence detection for a second mixing of the amplification mixture. For that, the run protocol is programmed to open the lid of instrument after 3 minutes together with a prompt for manual mixing. For that, the tubes are taken out of the instrument, vortexed for a second and spun down briefly before they are placed back into the instrument.

Results

Comparison of different reagent mixing methods

In a first step, we have compared amplification results under 3 conditions: (a) with a second manual mixing step interrupting the fluorescence measurement at 3 minutes after start of the reaction, (b) without any second mixing step, and (c) applying the automated reagent mixing feature of the Axxin T8-ISO instrument. We used a West-Nile-Virus (WNV) assay published by Tomar *et al.* with a synthetic RNA target at $5{\cdot}10^5$ and $5{\cdot}10^2$ copies per reaction. Whereas in the original publication RPA reagents (Twist Dx, UK) were used, we adapted the assay to high quality RAA reagents. The experience from us and our customers shows that assays designed for RPA amplification can easily be adapted to RAA reagents.

The results are summarized in Figure 1. Most amplification curves show a sigmoidal shape with a steep increase in fluorescence upon template amplification. The short drop in fluorescence around 3 minutes of reaction time is caused by the opening of the device for the second mixing step and is iconic for RPA/RAA real-time detection nowadays.

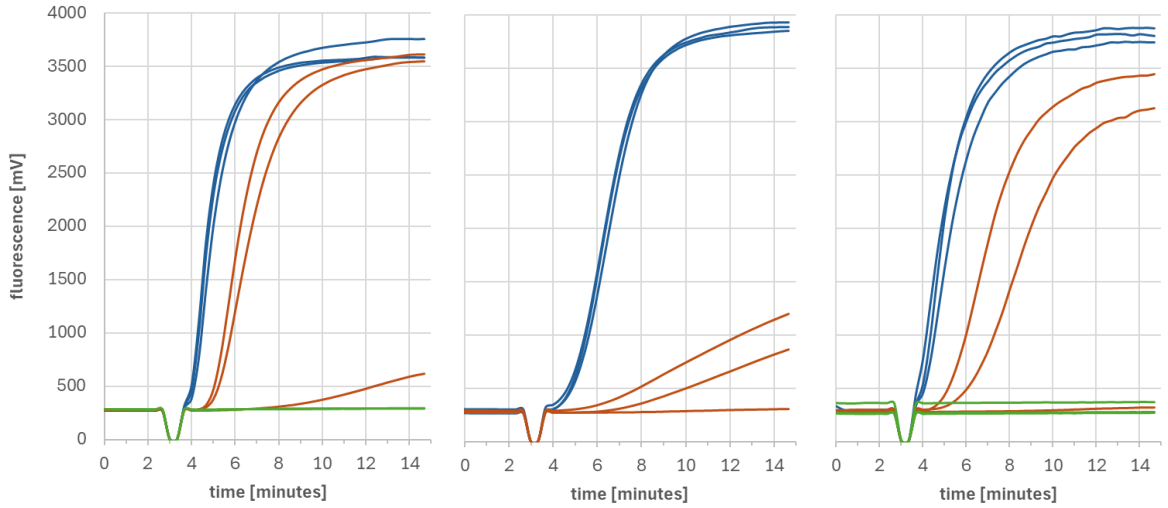


Figure 1. Comparison of amplification curves of a West-Nile Virus Assay using RT-RAA reagents. **Left:** manual mixing step 3 minutes after amplification start; **Middle:** no second mixing step; **Right:** automated mixing using steel ball. Each graph summarizes amplification data from 3 independent runs. **Blue curves:** $5{\cdot}10^5$ copies of template RNA; **Orange curves:** $5{\cdot}10^2$ copies of template RNA; **Green curves:** non-template control reactions.

As expected, at high template concentration, the mixing of RAA reagents has a smaller impact on detection results. The only difference between the three mixing approaches is that the time-to-threshold is delayed by about 1 minute without mixing. At the lower template concentration of 500 copies per reaction, this shift is much more prominent. Furthermore, the chosen concentration was close to the supposed Limit-of-detection (LoD) of the assay, because in all three setups the variation of detection signal is much larger than at high template concentration and, furthermore, in case of “no mixing” and “automated mixing” 1 out of 3 replicates returned a negative amplification result.

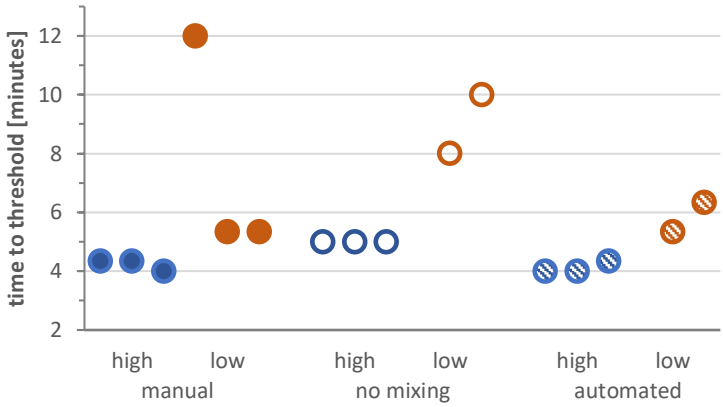


Figure 2: Overview of amplification signals of the different mixing approaches with high ($5{\cdot}10^5$ copies per reaction, **blue circles**) and low ($5{\cdot}10^2$ copies per reaction, **orange circles**) template concentrations. Signal threshold was set to 200mV above the initial background fluorescence in each reaction tube.

RNA template dilution series using automated mixing

After demonstrating that the automated mixing approach can replace the current manual mixing step in real-time RAA reactions, we examined the fluorescence signals of a dilution series of the WNV RNA template from $5{\cdot}10^7$ to $5{\cdot}10^2$ copies per reaction.

Figures 3 and 4 show that the amplification signals displayed only small variations at higher template concentrations above 10^4 copies per reaction. Below this concentration, the variation between replicates increased and at $5{\cdot}10^2$ copies per reaction again 1 out of 3 replicates returned a negative signal.

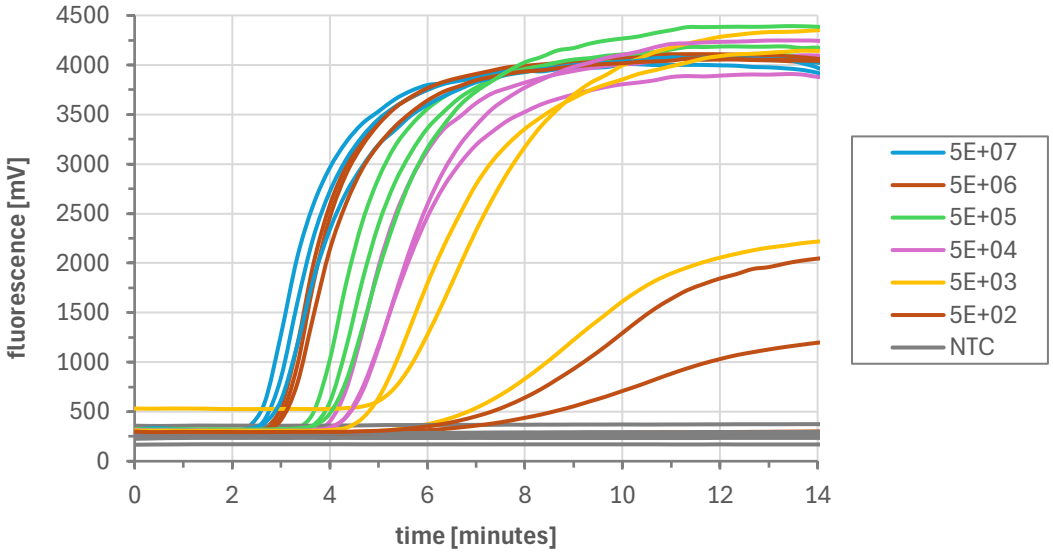


Figure 3. Amplification curves of a West-Nile Virus Assay using RT-RAA reagents with a dilution series of a WNV RNA template using the automated mixing function of the T8-ISO instrument. Amplification data from 3 independent runs are summarized. Numbers given in the legend indicate template copies per reaction.

A particularly positive impact of using the automated mixing function of the T8-ISO instrument is that the second manual intervention after the start of the RAA reaction is not required anymore. Not only

does this simplify conducting RAA-based assays for rapid DNA/RNA detection but also avoids the interruption of fluorescence acquisition during the amplification-detection (compare Figure 1 with Figure 3).

The time-to-threshold data (Figure 4) indicate a very consistent performance of the automated mixing process. The increasing variability at lower template concentrations is typical for RAA/RPA assays and appears with manual reagent mixing as well.

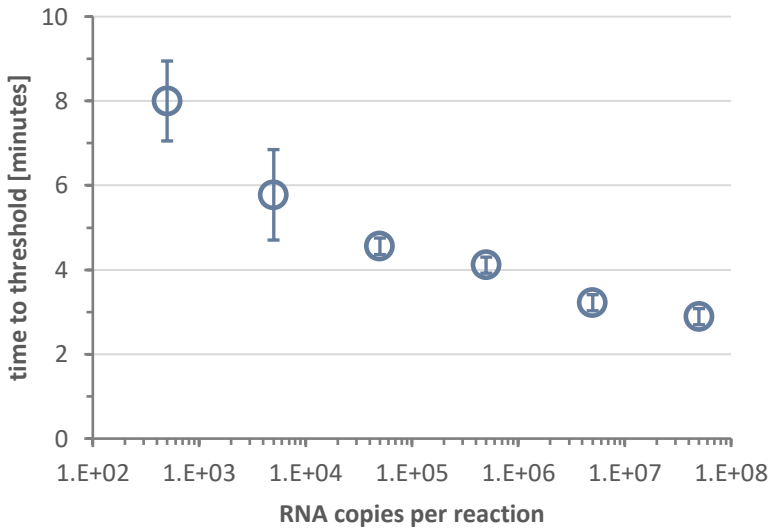


Figure 4: Summary of time-to-signal values of the dilution series shown in Figure 3. Signal threshold was set to 200mV above the initial background fluorescence in each reaction tube.

It can be concluded that switching from manual to automated mixing does not affect the performance of the assay. To achieve that, we highly recommend the use of high-grade stainless-steel balls to avoid inhibition of (RT-)RAA reagents due to leakage or other interactions between steel balls and amplification reagents. Since we could not observe any degradation of the steel balls or leakage of chemicals into the amplification mixes (Figure 5), it is evident that the steel balls recommended by Xpedite Diagnostics match these requirements.

Figure 5: Photograph of RAA reactions in PCR tubes containing the stainless-steel balls after the RAA amplification has been finished.

Discussion

To date, RAA/RPA-based diagnostic assays require a second mixing step after the initial start of the reaction to perform optimally. This has several disadvantages: it renders the detection process more complex and prone to user-errors while it also compromises the continuous fluorescence acquisition for data interpretation.

This application note provides evidence that the "traditional" manual mixing step can be replaced by a continuous automated mixing of reagents during the assay run. For that, we utilized the mixing functionality of the of the Axxin T8-ISO portable fluorescence reader. Based on the presented data it can be concluded that automated mixing does not compromise the assay performance but circumvents the need for another manual intervention during the run, reducing the potential for operator mistakes or inconsistencies & avoids the dip in fluorescence signal typically seen in publications using RPA technology.

It shall be pointed out that it is important to use steel balls with a high-grade of "cleanliness" and chemical resistance to ensure that there is no interference with the assay chemistry and amplification results. We also recommend using fresh steel balls for every reaction to avoid any possibility of cross-contamination.

Literature

1. Gloekler *et al.* (2021) Isothermal amplifications – a comprehensive review on current methods. Critical Rev Biochem Mol Biol 56: 543-586
2. Lobato & O'Sullivan (2018) Recombinase polymerase amplification: Basics, applications and recent advances. Trends Anal Chem 98: 19-35
3. Chen *et al.* (2018) Use of a rapid reverse-transcription recombinase aided amplification assay for respiratory syncytial virus detection. Diagn Microbiol Infect Disease 90: 90-95
4. Tomar *et al.* (2021) Development and Evaluation of Real-Time Reverse Transcription Recombinase Polymerase Amplification Assay for Rapid and Sensitive Detection of West Nile Virus in Human Clinical Samples. Front Cell Infect Microbiol Vol 10 Article 619071