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Determining the Detection Limits of a LAMP Assay

Analysis of the assay needs to be performed using Genie® Explorer. It is important that the amplification rate is analysed as well as the amplification curves. Genie® explorer is available on the OptiGene website http://www.optigene.co.uk/support/

In order to find the detection limit of an assay you will need to run a dilution series of decreasing template concentrations. If you have a required detection limit you need to ensure your dilution series goes beyond this level.

A 1 in 10 dilution series will give a rough estimate of the detection limit and an indication of the DNA concentration where the assay is close to the limit. Ideally each template dilution should be run in at least duplicates.

A 1 in 2 dilution series will more accurately show the detection limit of the assay. Ideally each template dilution should be run in at least triplicates. 1 in 2 dilution series are often quite crowded and difficult to read and therefore should be based on the detection limit determined by a 1 in 10 serial dilution first. It will likely be necessary to toggle off certain wells to more clearly see the results, you can also zoom in on graphs using Genie® Explorer

Sensitivity of an assay can sometimes be improved by increasing primer concentrations

- F3/B3 @ 0.2µM
- FIP/BIP @ 2.0µM
- LF/LB @ 1.0µM

These concentrations are final per primer in a 25µl LAMP reaction

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Determining the Detection Limits of a LAMP Assay

Mastermix

A mastermix should be made to include all components of the reaction, but not template. Enough mastermix should be made to cover all reactions with an excess of 1-2 reactions

	1 LAMP rxn	1x strip of 8 rxn	2x strip of 8 rxn
	1x25µl	10x 25µl	18x 25µl
LAMP reagent (e.g ISO-001) 10x primer mix Template	15µl 2.5µl 5µl	150µl 25µl Add individually	270µl 45µl Add individually
Water	2.5µl	25µl	45µl

For RNA based assays RT will need to be added as per protocol with the final reaction volume remaining at 25µl

Dispense 20µl of each reaction into wells. Always start from the lowest dilution, add 5µl of appropriate dilution into each well and close cap securely before proceeding to the next dilution. Ensure a new tip is used for every template addition. Run an isothermal reaction as per standard settings

For ISO-001 run the isothermal amplification step for no longer than 30mins or 20mins for ISO-004. Amplification for longer than these recommended times will give false identification of the detection limit of the assay

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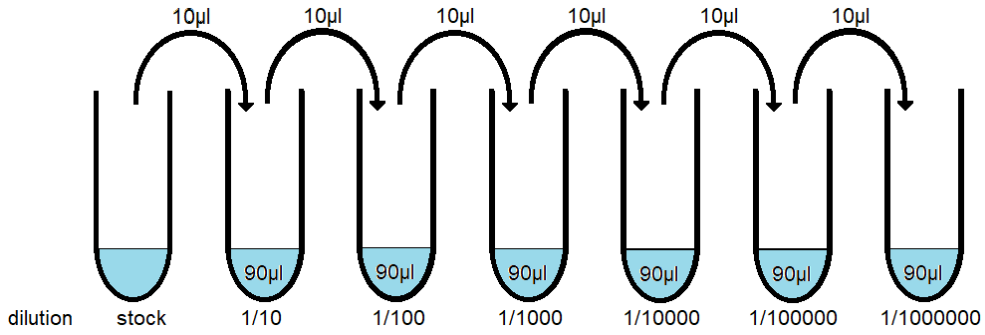
Determining the Detection Limits of a LAMP Assay

1 in 10 Serial Dilutions

A 1 in 10 dilution series is where each subsequent dilution is 1/10th of the concentration of the previous dilution. This is made with 1part DNA solution and 9 parts nuclease free water

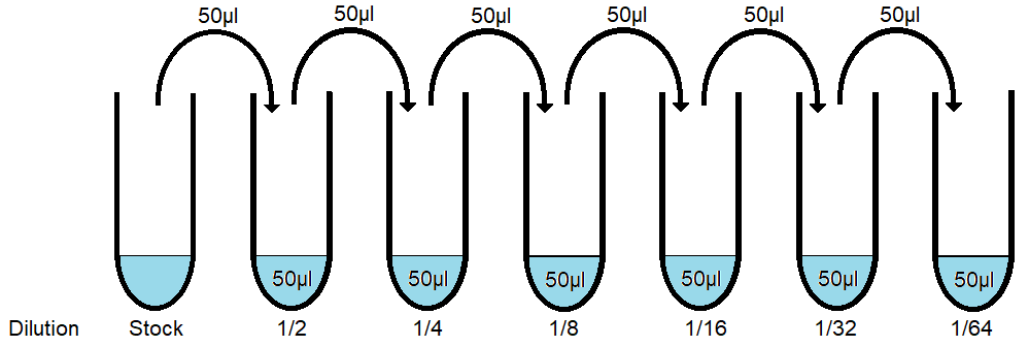
1. Dispense 90µl into each tube required for a dilution
2. Transfer 10µl of the stock DNA into the first dilution tube containing the pre-dispensed 90µl water and mix thoroughly. This creates a 1 in 10 dilution of the original stock
3. Transfer 10µl of the 1/10 dilution into the next dilution tube containing the pre-dispensed 90µl water and mix thoroughly. This creates a 1 in 100 dilution of the original stock
4. Transfer 10µl of the 1/100 dilution into the next dilution tube containing the pre-dispensed 90µl water and mix thoroughly. This creates a 1 in 1000 dilution of the original stock
5. This pattern should be repeated to complete the full dilution series required



1 in 2 Serial Dilutions

1 in 2 dilution series is where each subsequent dilution is 1/2th of the concentration of the previous dilution. This is made with 1part DNA solution and 1 part nuclease free water

1. Dispense 50µl into each tube required for a dilution
2. Transfer 50µl of the stock DNA into the first dilution tube containing the pre-dispensed 50µl water and mix thoroughly. This creates a 1 in 2 dilution of the original stock
3. Transfer 50µl of the 1/2 dilution into the next dilution tube containing the pre-dispensed 50µl water and mix thoroughly. This creates a 1 in 4 dilution of the original stock
4. Transfer 50µl of the 1/4 dilution into the next dilution tube containing the pre-dispensed 50µl water and mix thoroughly. This creates a 1 in 8 dilution of the original stock
5. This pattern should be repeated to complete the full dilution series required



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Determining the Detection Limits of a LAMP Assay

General Rules for Determining Detection Limit

*****The colours in this table are used throughout this document to indicate a specific rule*****

Amplification Curve Observations	Amplification Rate Observations	Amplification of replicates	Amplification Time	Conclusion
Steep increase in fluorescence after initial detection. Reaches plateau quickly. Even spacing of amplification curves between dilutions. Replicates look the same	High, sharp peaks with an even shape. Replicates look the same	All amplified	Consistent for all replicates	Reliable - Within detection limits
Slower increase in fluorescence as seen by a shallower amplification curve. No longer following the spacing pattern of earlier dilutions. usually reaches plateau but amplification curves often look different in each replicate	Replicates starting to look different from each other. Peak often lower and wider	All amplified	Consistent for all replicates	Reliable - Approaching detection limits
Slow increase in fluorescence, amplification with often very shallow amplification curve. Unlikely to reach plateau. Amplification curves look different in each replicate. Last dilution were all replicates are amplified	Replicate peaks no longer match. Peak height is lower than previous dilutions and often rounded at the top. The peaks can often be irregular in shape and my not be a typical peak at all. They can be long and not smooth. Despite detection of fluorescence sometimes no amplification time can be called as it is below the detection threshold or no true peak can be seen	All amplified	Varies in each replicate	Reliable - Detection limit
Often very shallow, often uneven amplification curve when seen. Where replicates amplify the curves look difference in appearance to each other. Does not reach plateau	Where replicates amplify the peaks are no longer matching. Peak height is lower than previous dilutions and often rounded at the top. The peaks can often be irregular in shape and my not be a typical peak at all. They can be long and not smooth. Despite detection of fluorescence sometimes no amplification time can be called as it is below the detection threshold or no true peak can be seen	Some amplified	None or varies in each replicate	Unreliable - Beyond detection limit
No amplification seen	No amplification seen	None amplified	No amplification	Unreliable - Beyond detection limit

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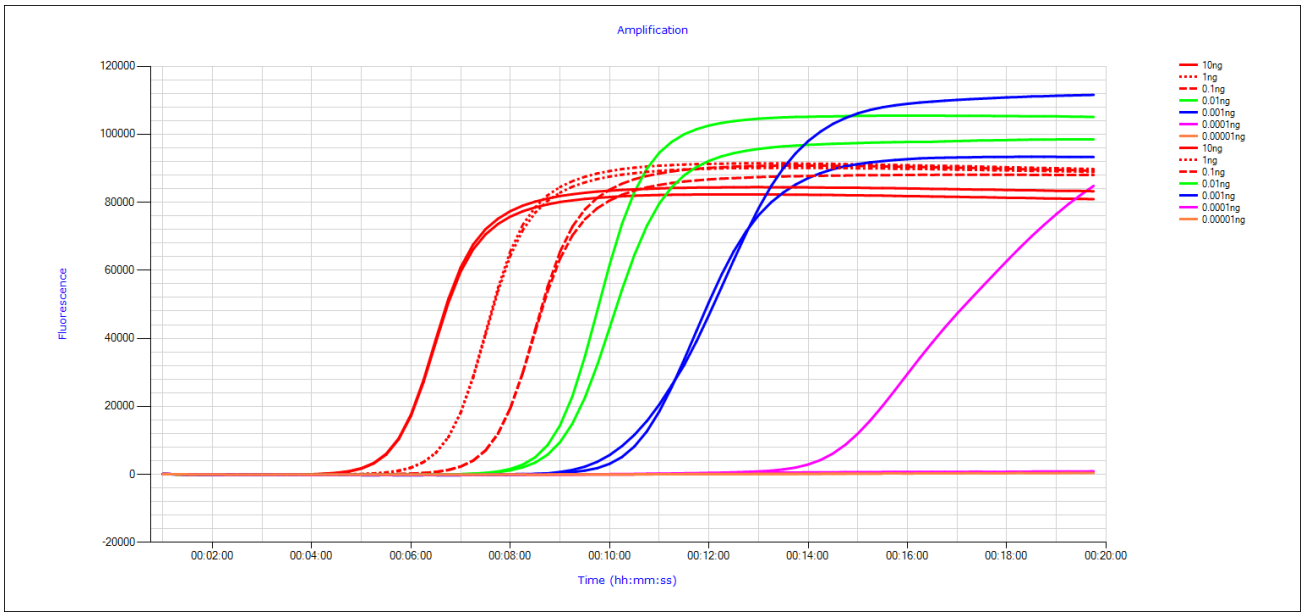
Determining the Detection Limits of a LAMP Assay

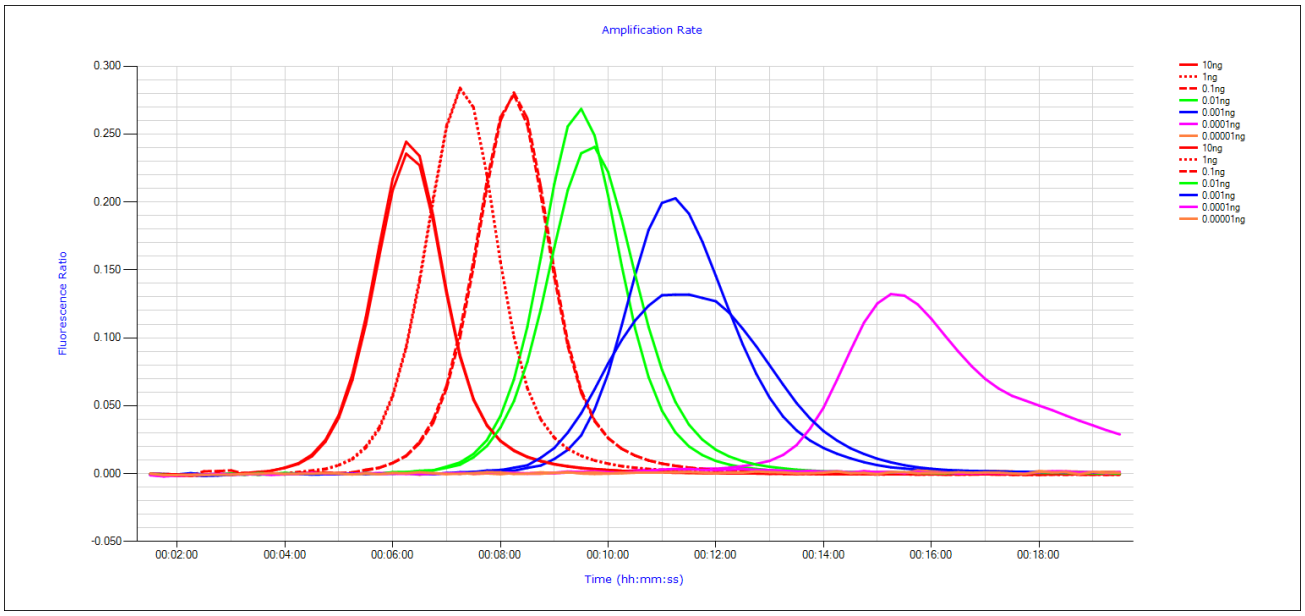
Typical 1 in 10 Dilution Series Assay

1 in 10 dilutions typically display a pattern similar to those seen below. As long as dilutions are well within the detection limit you should always see replicates which reliably amplify at the same time with equal spacing between dilutions. The cut off between reliable and non-reliable detection can be quite sudden as the difference in DNA concentrations is quite large compared to a 1 in 2 dilution. This assay can be used to determine what dilution to start with for a 1 in 2 serial dilution to more precisely ascertain the detection limit of the assay. If an assay is not following a typical dilution series as outlined below, even for large concentrations of DNA, then this is likely an assay design fault (poorly designed primers for example) or a template issue (contamination/inhibitors present).

Isothermal Amplification for a 1 in 10 Dilution Series Assay



Amplification Rate for a 1 in 10 Dilution Series Assay



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Determining the Detection Limits of a LAMP Assay

Results, Observations and Conclusions for a 1 in 10 Dilution Series Assay

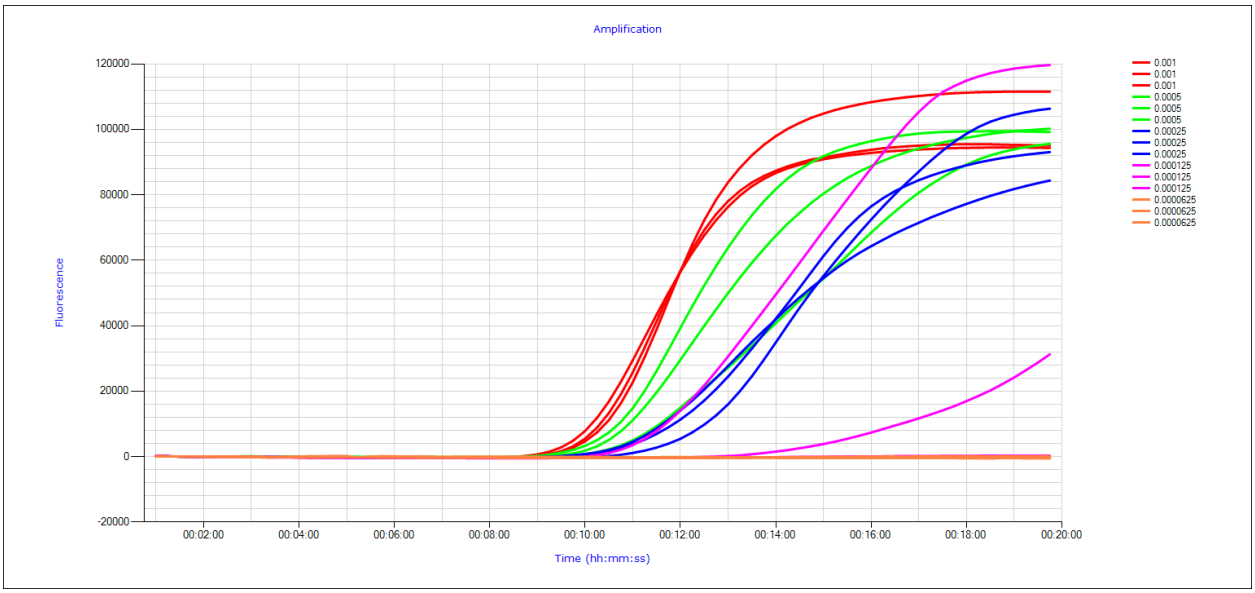
Dilution	DNA conc. (ng)	Replicate Amp Time	Amplification Curve Observations	Amplification Rate Observations	Conclusion
1	10	06:15 06:15	Amplification curve increases rapidly from initial detection and reaches plateau quickly. Both duplicates detected	High sharp peaks, even shaped, replicates look the same	Reliable - Within detection limits
1 in 10	1	07:15 07:15	Consistent spacing of detection times between dilutions (1minute). Amplification curve increases rapidly from initial detection and reaches plateau quickly. Both duplicates detected	High sharp peaks, even shaped, replicates look the same	Reliable - Within detection limits
1 in 100	0.1	08:15 08:15	Consistent spacing of detection times between dilutions (1minute). Amplification curve increases rapidly from initial detection and reaches plateau quickly. Both duplicates detected	High sharp peaks, even shaped, replicates look the same	Reliable - Within detection limits
1 in 1000	0.01	09:30 09:45	Consistent spacing of detection times between dilutions (1minute). Amplification curve increases rapidly from initial detection and reaches plateau quickly. Both duplicates detected	Replicates slightly different, peak height getting lower. Still has a defined and even peak	Reliable – approaching detection limit
1 in 10000	0.001	11:15 11:30	Time from previous dilution is now greater than 1minute. Amplification curve is shallower, not seeing such a steep increase in fluorescence. It is not uncommon to see amplification times that are quite different at the detection limit. Both duplicates detected.	Replicate peaks no longer matching. Peaks are less defined and rounded. Peak now more irregular in shape. Peak height lower than previous dilutions	Reliable - Detection limit reached
1 in 100000	0.0001	15:15 None	Only 1 out of the 2 duplicates amplified. The duplicate that does amplify has a very shallow amplification curve that never reaches plateau. It is detected late and the amplification time spacing follows no typical spacing as seen in higher dilutions	Only one amplified, peak height is low and is now irregular shaped	Unreliable - Beyond detection limit
1 in 1000000	0.00001	None None	No amplification seen	No amplification seen	Beyond detection limit

Determining the Detection Limits of a LAMP Assay

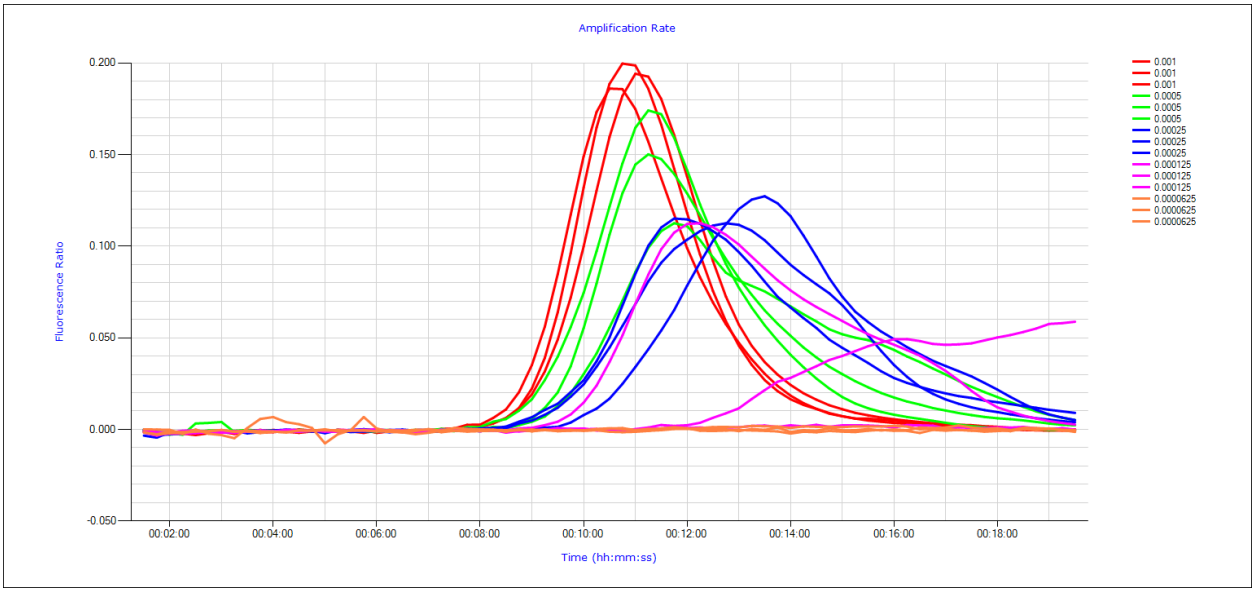
Isothermal Amplification Curve for a Typical 1 in 2 Serial Dilution

1 in 2 dilution series are typically more muddled and confusing than a 1 in 10 dilution series. This is because the difference between each dilution is much smaller, each dilution is run in triplicates and you are approaching the detection limit of the assay; as determined by a 1 in 10 dilution series. It is not uncommon for the amplification times to be quite varied as you approach the detection limit and sometimes a lower dilution can be detected earlier than a higher dilution as amplification becomes more random and can jump around a lot. It is likely you will need to zoom in on the image and toggle off certain wells to properly analyse the appearance of the curves and ascertain the detection limit cut off for your assay. As a general rule the last dilution where replicates are consistently amplified and detected can be determined as the lowest reliable detection point of the assay.

Isothermal Amplification for a 1 in 2 Dilution Series Assay



Amplification Rate for a 1 in 2 Dilution Series Assay



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Determining the Detection Limits of a LAMP Assay

Results, Observations and Conclusions for a 1 in 2 Dilution Series Assay

Dilution	DNA Conc. (ng)	Replicate Amp Time	Amplification Curve Observations	Amplification Rate Observations	Conclusions
1	0.001	10:45 10:30 11:00	Fluorescence increases rapidly after initial detection and reaches plateau quickly. All replicates amplified and look the same.	High sharp peaks with an even shape. Replicates look the same.	Reliable - Within detection limits
1 in 2	0.0005	11:45 11:15 11:15	Slower increase in fluorescence leading to shallower amplification curve. Still reaches plateau but different amplification timers starting to differ from one replicate to another. All replicates amplify	Still a defined peak but replicates starting to look different. Peaks often more uneven and can be much broader	Reliable - Approaching detection limit
1 in 4	0.00025	13:30 11:45 12:45	Amplification curve become shallower as fluorescence increase is slower. Often does not reach plateau. Replicates can look different to each other as some are more shallow and uneven than others. All replicates amplify. This is the last dilution where all replicates amplify	Peaks becoming a lot more uneven with different amplification times detected. Often lower peaks that can be quite broad. An amplification time may not be given, even though fluorescence detected, as the peak may not be a defined or falls under the calling threshold set on the Genie® instrument	Reliable - Detection limit
1 in 8	0.000125	16:15 None 12:15	Shallow amplification curve, usually later than others but the randomness of detection does mean some are detected earlier though the amplification curve does not look a typical shape as those seen in the reliable range. Usually does not reach plateau. Not all replicates amplify. Replicates that do amplify look different to each other. Amplification times will differ	Very uneven peaks, often not even completing a full peak as they are irregular and broad. An amplification time may not be given, even if fluorescence detected, as the peak may not be a defined or falls under the calling threshold set on the Genie® instrument. Replicates that do amplify look different to each other	Unreliable - Beyond detection limit
1 in 16	0.0000625	None None None	No amplification seen	No amplification seen	Unreliable - Beyond detection limit

Below are the individual amplification curves for each dilution

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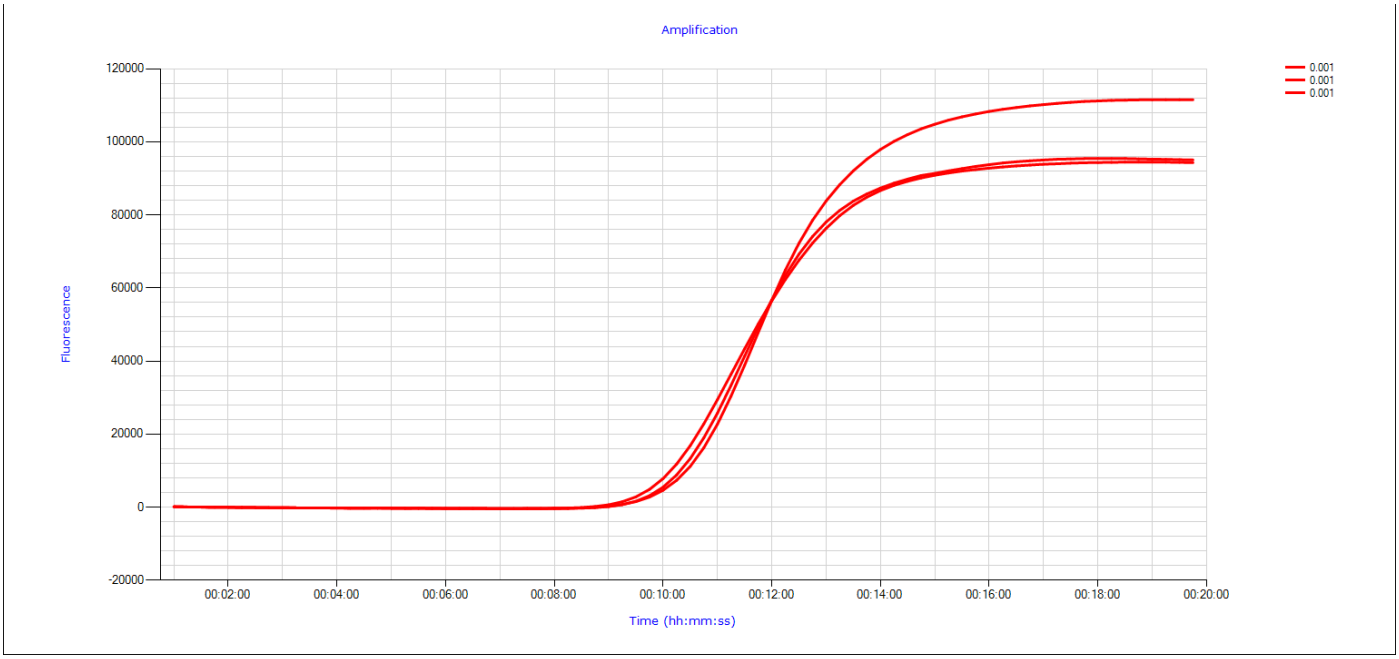
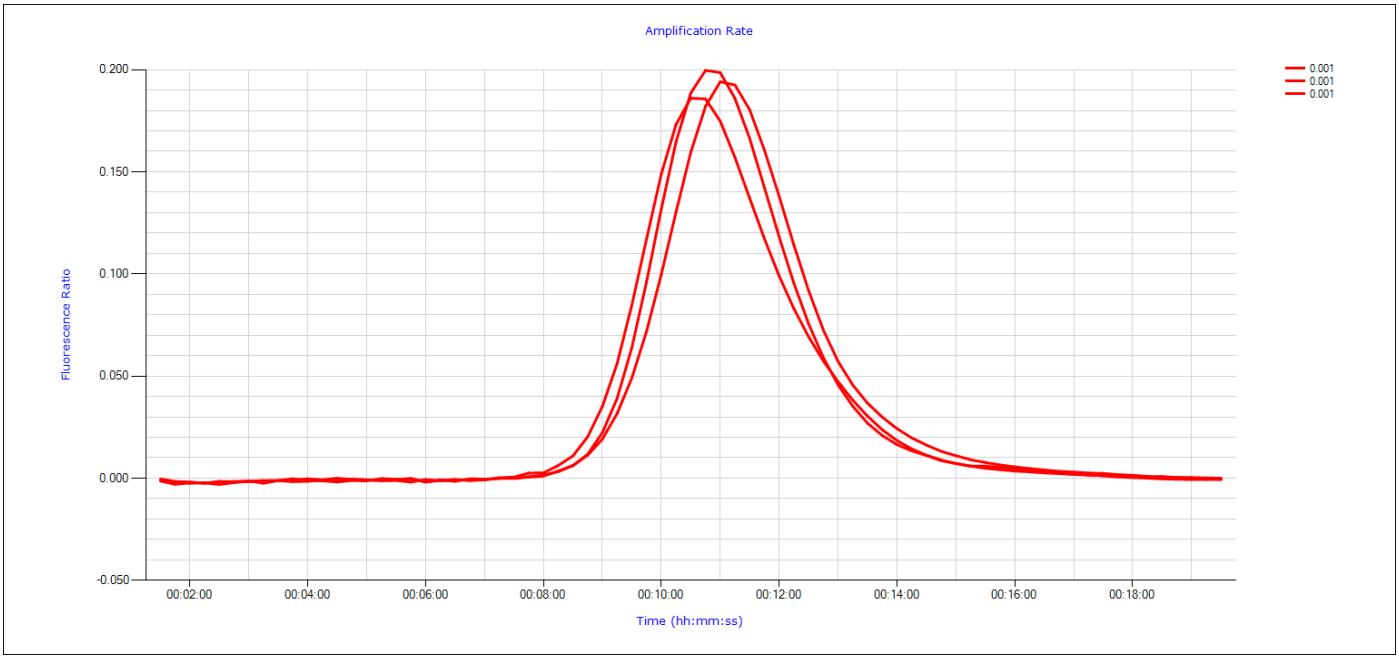
Determining the Detection Limits of a LAMP Assay

Initial DNA concentration (Stock DNA)





Amplification Curve Observations	Amplification Rate Observations	Conclusions
Fluorescence increases rapidly after initial detection and reaches plateau quickly. All replicates amplified and look the same.	High sharp peaks with an even shape. Replicates look the same.	Reliable - Within detection limits

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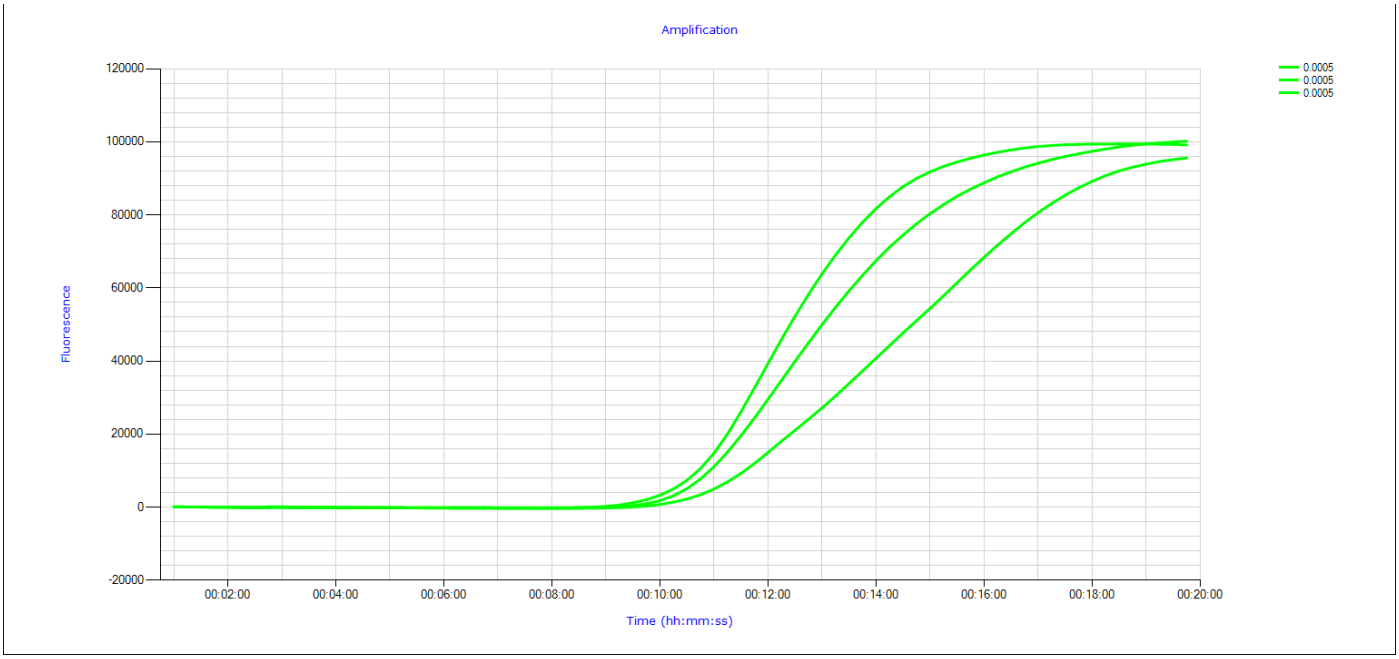
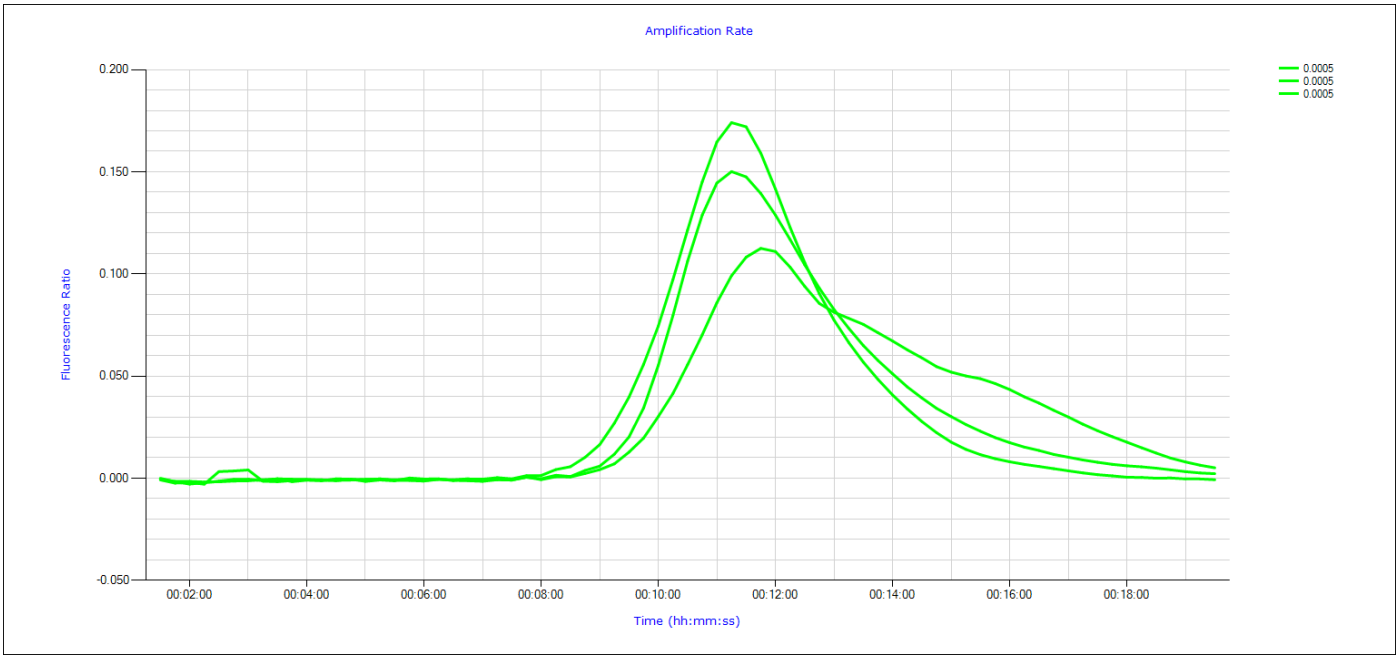
Determining the Detection Limits of a LAMP Assay

1 in 2 dilution of Stock DNA





Amplification Curve Observations	Amplification Rate Observations	Conclusions
Slower increase in fluorescence leading to shallower amplification curve. Still reaches plateau but different amplification timers starting to differ from one replicate to another. All replicates amplify	Still a defined peak but replicates starting to look different. Peaks often more uneven and can be much broader	Reliable - Approaching detection limit

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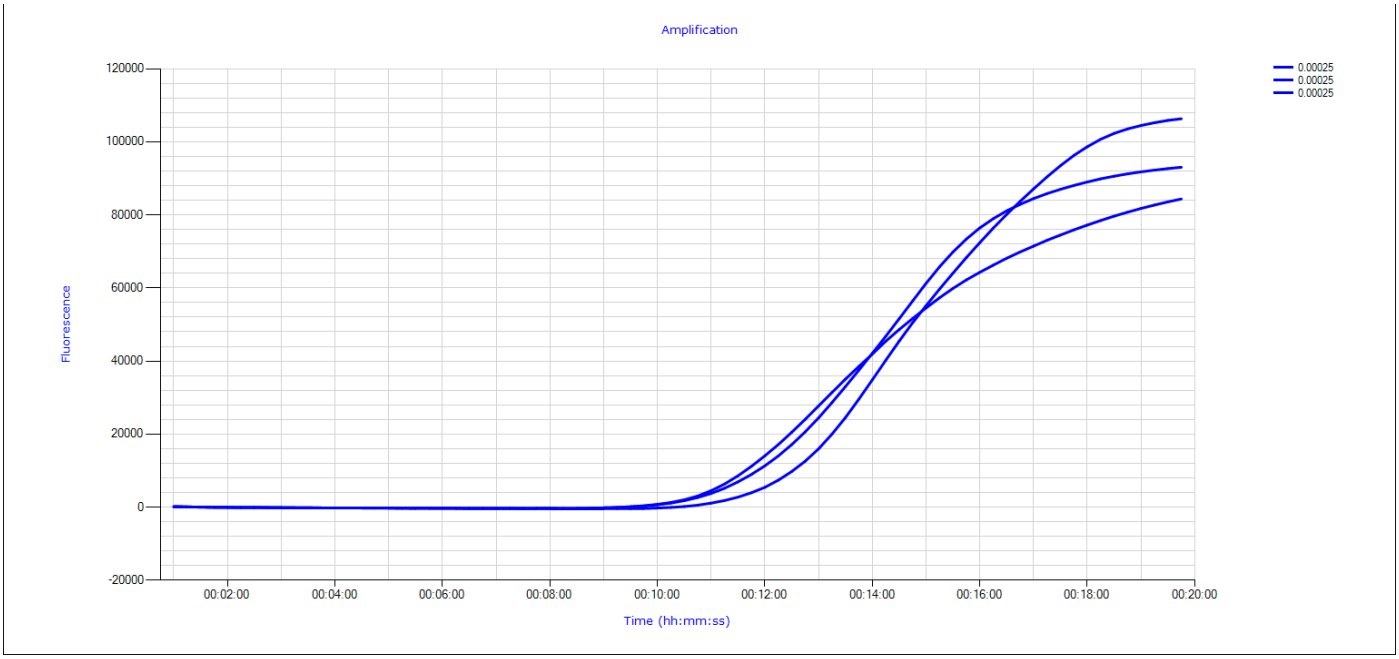
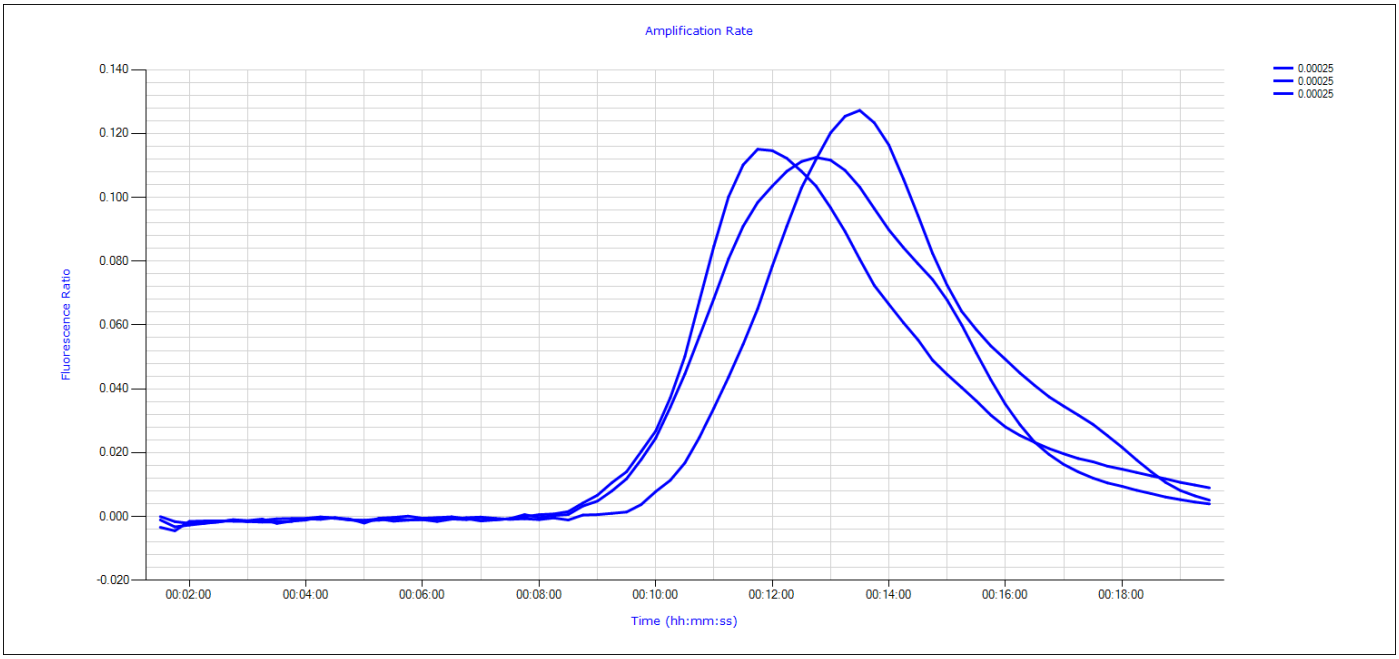
Determining the Detection Limits of a LAMP Assay

1 in 4 dilution of Stock DNA





Amplification Curve Observations	Amplification Rate Observations	Conclusions
Amplification curve become a lots shallower as fluorescence increase is slower. Often does not reach plateau. Replicates can look different to each other as some are more shallow and uneven than others. All replicates amplify. This is the last dilution where all replicates amplify	Peaks becoming a lot more uneven with different amplification times detected. Often lower peaks that can be quite broad. An amplification time may not be given, even though fluorescence detected, as the peak may not be a defined or falls under the calling threshold set on the Genie® instrument	Reliable - Detection limit

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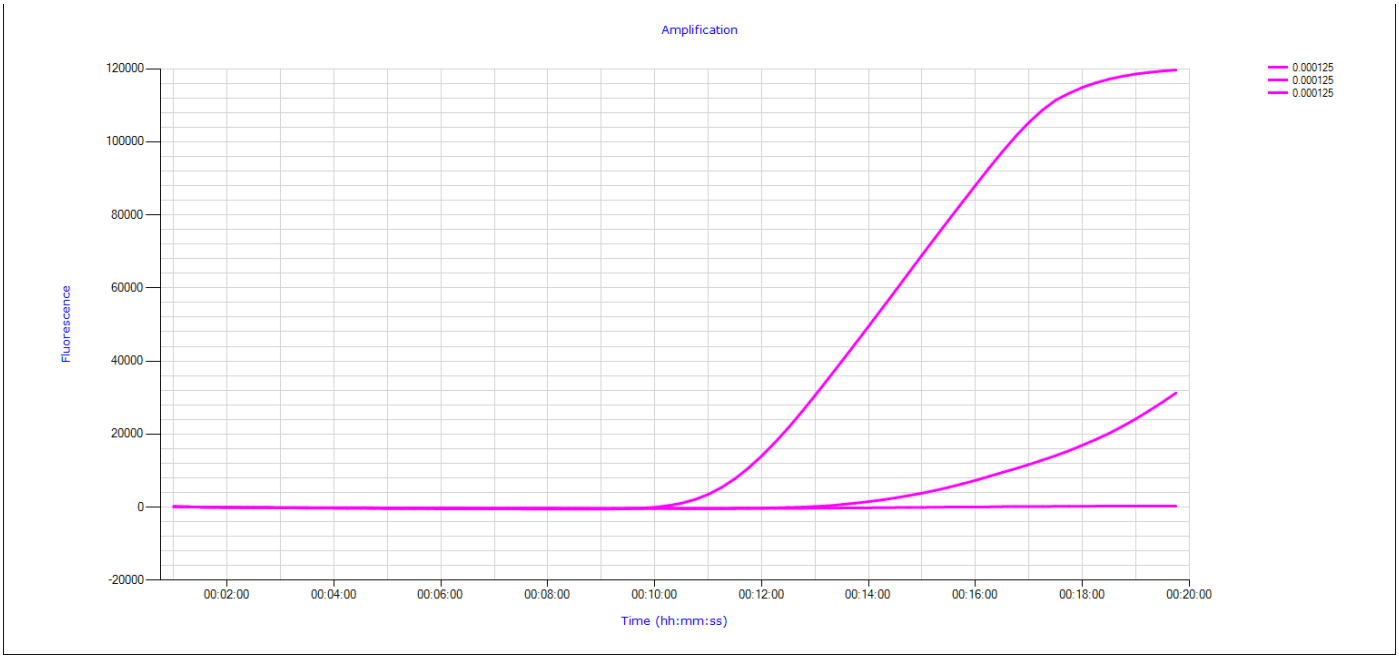
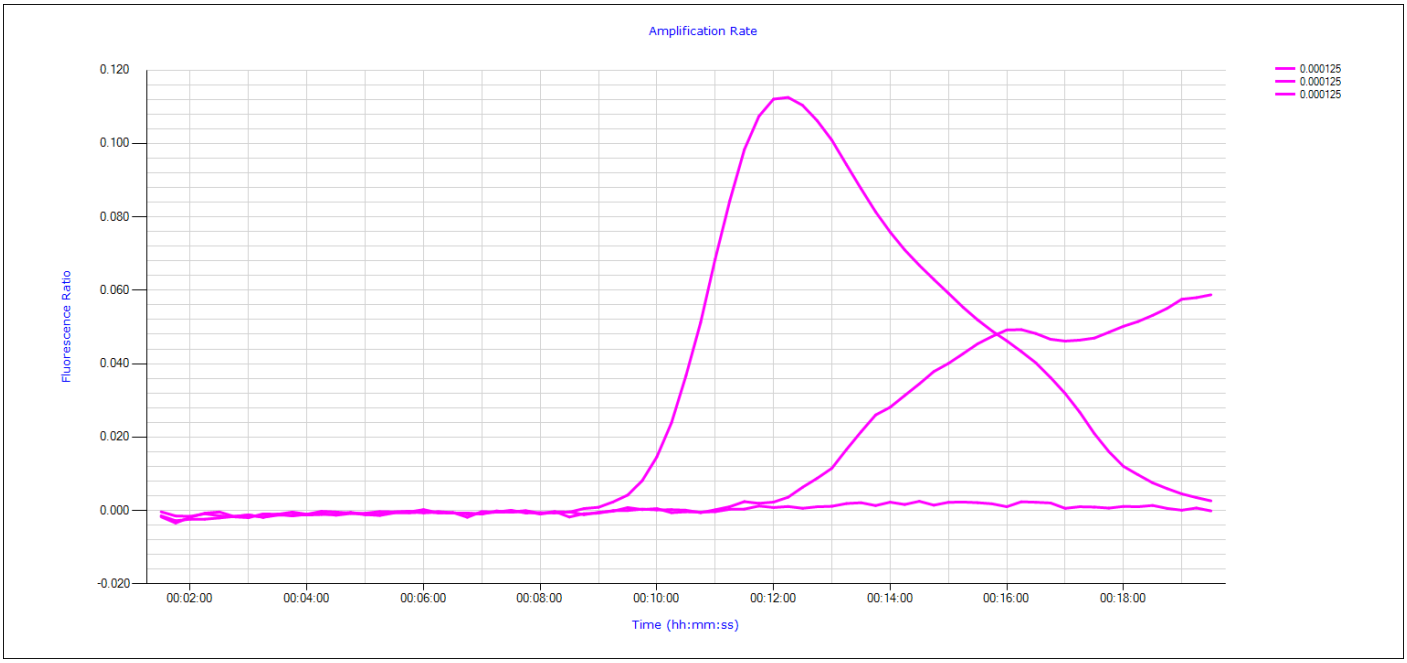
Determining the Detection Limits of a LAMP Assay

1 in 8 dilution of Stock DNA





Amplification Curve Observations	Amplification Rate Observations	Conclusions
Shallow amplification curve, usually later than others but the randomness of detection does mean some are detected earlier though the amplification curve does not look a typical shape as those seen in the reliable range. Usually does not reach plateau. Not all replicates amplify. Replicates that do amplify look different to each other. Amplification times will differ	Very uneven peaks, often not even completing a full peak as they are irregular and broad. An amplification time may not be given, even if fluorescence detected, as the peak may not be a defined or falls under the calling threshold set on the Genie® instrument. Replicates that do amplify look different to each other	Unreliable - Beyond detection limit

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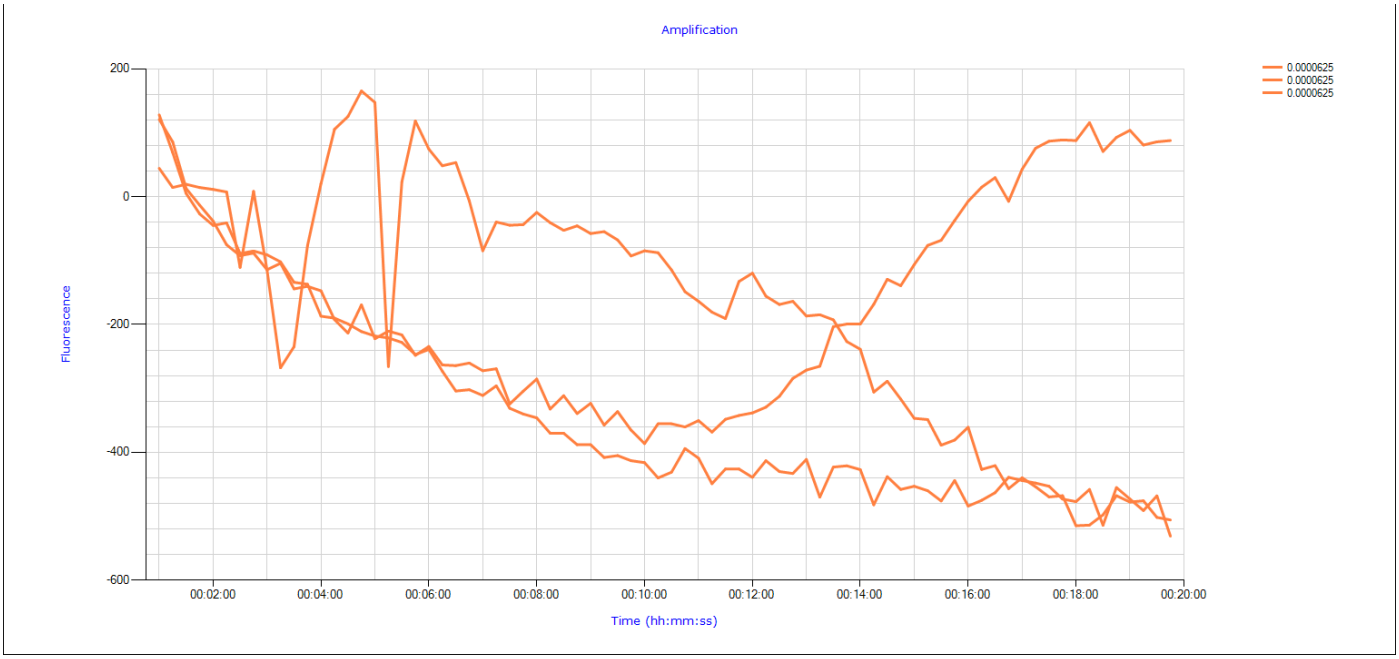
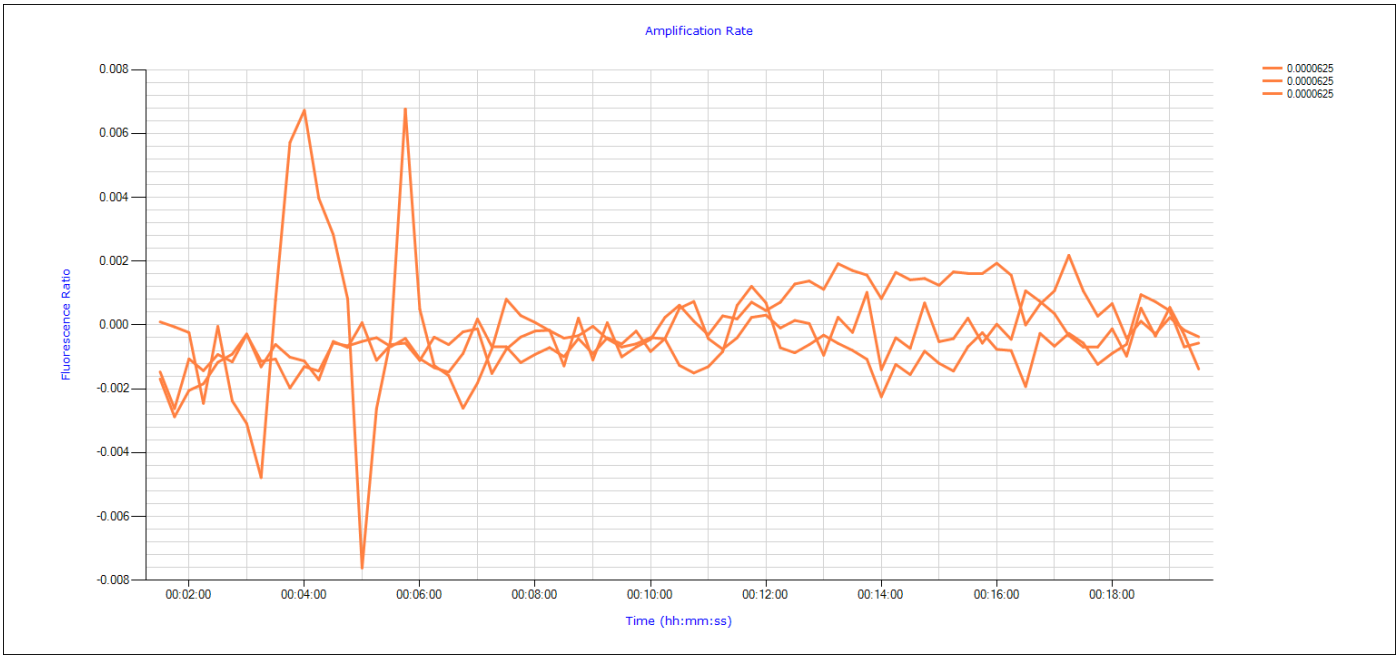
Determining the Detection Limits of a LAMP Assay

1 in 16 dilution of Stock DNA





Amplification Curve Observations	Amplification Rate Observations	Conclusions
No amplification seen	No amplification seen	Unreliable - Beyond detection limit

When looking at samples with no amplification the plots are very noisy with no true fluorescence being detected. When plotted alongside a positive amplification these actually appear less noisy and the lack of fluorescence is more obvious as shown by the two plots below

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Determining the Detection Limits of a LAMP Assay

1 in 16 dilution of Stock DNA (orange) alongside positive amplification (Red)