QUANTITATIVE UTILITY OF THE AMPLIFYRP® ISOTHERMAL PLATFORM

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Abstract

Molecular detection methods for plant pathogens have advanced very quickly. The PCR-based methods were widely used in this community. However, PCR-based methods rely on the use of purified DNA or RNA as template, increasing cost and labor. In recent years, isothermal amplification has gained favor for field-test platform because of tolerance to host inhibitors and its use of a simple incubator.

The AmplifyRP® platform is an isothermal amplification system based on the recombinase polymerase amplification system. Detection of amplification can be performed in real-time using a fluorometer (XRT) similarly to real-time PCR, or as an end-point assay using lateral flow strips in an Amplicon Detection Chamber (Acceler8). Both formats are extremely sensitive and specific as qualitative assays. However, as with qPCR, the XRT has the potential to be quantitative. We present here, data from multiple tests, demonstrating that time of "onset of amplification" can be related to template concentration such that one may be predictive of the other.

Methods

AmplifyRP XRT reactions

All AmplifyRP XRT reactions were run according to standard protocols, using Total Reaction Pellets (include primers and probes). Pellets were rehydrated with 25ul of PD1 buffer containing template. Reactions were incubated in an ESE Quant T8 Tube Scanner with data collected using the Tube Scanner Suite program.

Data Analysis

Raw data was copied into Excel and normalized by subtracting initial background from the readings at each time point and amplification plots were generated. A threshold of 200mV was then assigned for Onset of Amplification (OT). For the purpose of quantification, this is the value at which exponential amplification was empirically determined to begin.

The reader scans at 20 second intervals, so it is highly unlikely that any reaction will exceed the threshold exactly at the moment of scanning. The difference between the two readings bracketing 200mV divided by 20 was used to calculate the rate of increase in mV/second for that time period. The Onset of Amplification for each reaction was then calculated to the nearest second.

Standard Curves

Three types of templates were used in these experiments; DNA purified from BBTV-infected banana leaves, plasmid containing cloned BBTV target region, and DNA purified from a Cmm bacterial culture. Reactions were run, in replicates, for multiple known template concentrations. The OTs for these replicates were averaged and the result used to produce standard curves for quantification. These curves were then utilized to back calculate the empirical data and compare those results with the expected results.

Data collected from two different assays is reported here.

Results

Figures 1A, B, and C show the results of serial dilutions of DNA isolated from BBTV-infected banana leaf. 2-fold differences in DNA concentration from 2.0ng to 500fg were tested in quadruplicate. **Figure 1A** is the amplification plot generated from the averages of the replicates.

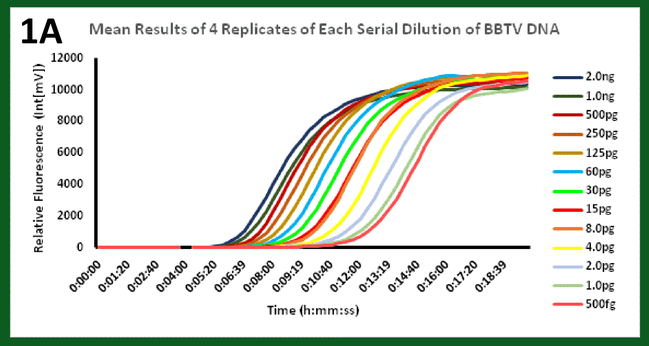


Figure 1B shows a plot of onset time and range against initial DNA concentration. Limited range of onset time for each DNA concentration suggests it may be possible to identify 2-fold differences in concentration, however very low or very high concentrations of template may require 5-fold or greater differences for accuracy.

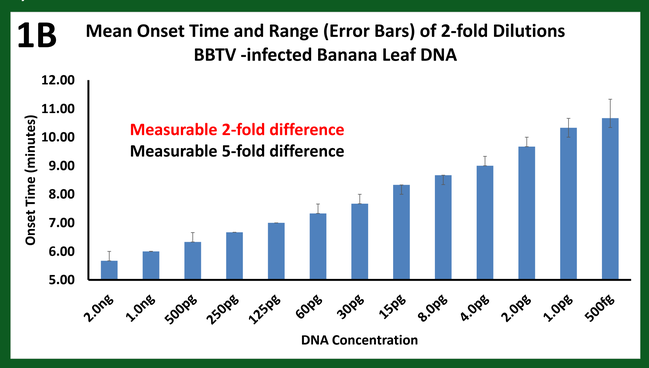


Figure 1C shows a linear regression of initial template concentration as a function of onset of DNA isolated from BBTV-infected banana leaf. Onset time of each reaction was determined and the mean onset time for each concentration of DNA was calculated. A strong correlation between onset time and initial template concentration (R^2 of 0.9977) is easily seen.

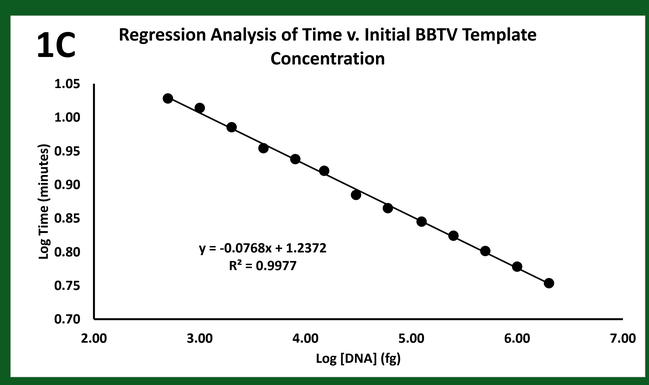
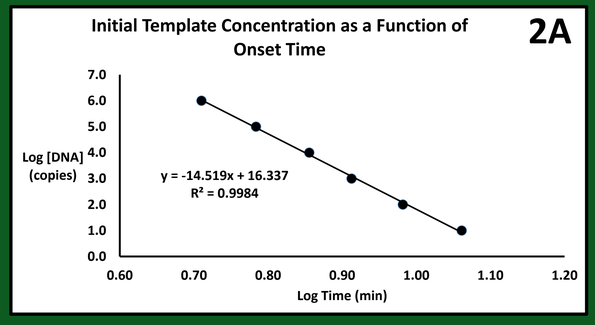
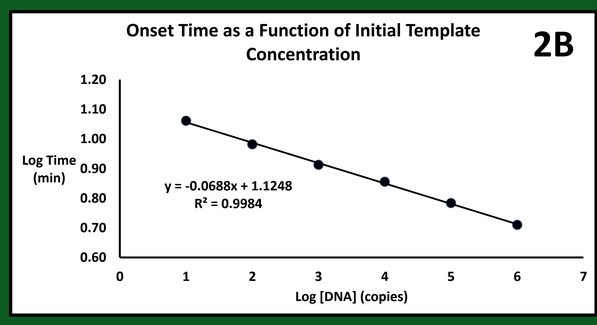


Figure 2 demonstrates the utility of the BBTV AmplifyRP XRT assay for predicting either Onset Time from template concentration (**2A**) or template concentration from Onset Time (**2B**). Log dilutions of plasmid containing cloned BBTV target were run in quadruplicate. The average Onset Time was determined for each dilution. Standard curves were generated and those curves were then used to back calculate and predict either template concentration or Onset Time. Comparisons of expected versus calculated values were done. Percent CV was determined for each data set. All CVs were below 15% , showing good predictability within the data sets.

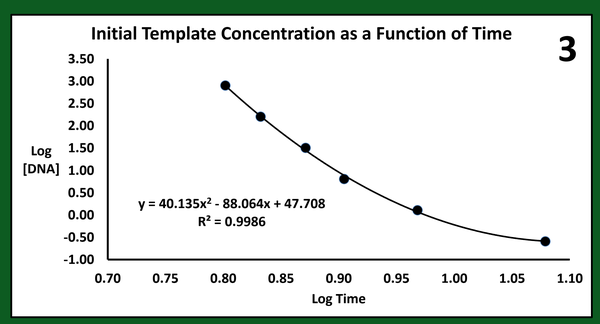


Back Calculations for Copy Number							
Mean OT (min)	[DNA] (copies)	Log OT (min)	Log DNA copies	Predicted copies	Mean copies	SD copies	%CV
5.13	1000000	0.71	6	1063119.6	1031559.8	44632.3	4.3
6.08	100000	0.78	5	90868.7	95434.4	6456.8	6.8
7.17	10000	0.86	4	8224.0	9112.0	1255.8	13.8
8.18	1000	0.91	3	1221.1	1110.6	156.4	14.1
9.59	100	0.98	2	120.8	110.4	14.7	13.3
11.51	10	1.06	1	8.5	9.3	1.1	11.4



Back Calculations for Onset Time							
[DNA] (copies)	Mean OT (min)	Log DNA copies	Log OT (min)	Predicted OT	Mean OT	SD Time	%CV
1000000	5.13	6	0.71	5.15	5.14	0.02	0.3
100000	6.08	5	0.78	6.04	6.06	0.03	0.5
10000	7.17	4	0.86	7.07	7.12	0.07	1.0
1000	8.18	3	0.91	8.29	8.23	0.08	0.9
100	9.59	2	0.98	9.71	9.65	0.08	0.9
10	11.51	1	1.06	11.38	11.44	0.10	0.8

Figure 3 shows data generated with Agdia's *Clavibacter michiganensis subsp michiganensis* XRT assay. A standard curve was generated, values back calculated, and CVs determined. Again, the resulting low CVs support a high degree of predictability for this assay.

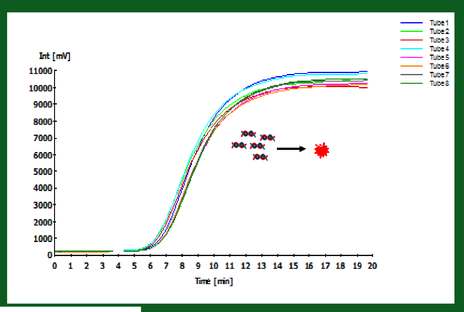


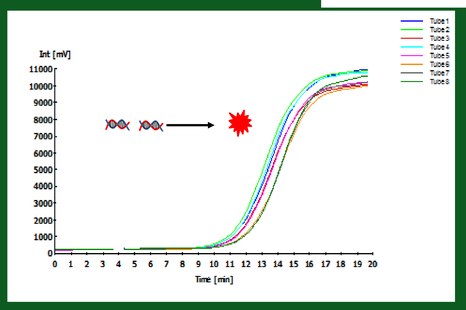
Back Calculations for DNA Concentration							
Mean OT (min)	[DNA] (pg)	Log OT (min)	Log DNA (pg)	Predicted (pg)	Mean (pg)	SD (pg)	%CV
6.34	800	0.80	2.90	795.37	797.68	3.28	0.41
6.80	160	0.83	2.20	163.81	161.90	2.69	1.66
7.43	32	0.87	1.51	28.02	30.01	2.81	9.38
8.03	6.4	0.90	0.81	7.72	7.06	0.93	13.20
9.29	1.28	0.97	0.11	1.17	1.22	0.08	6.38
11.99	0.256	1.08	-0.59	0.26	0.26	0.00	0.94

Conclusions

AmplifyRP XRT can be used to determine initial template concentration. Similar to real time PCR, initial nucleic acid concentration correlates to time of amplification onset.

Early onset time correlates to high concentration of nucleic acid





Late onset time correlates to low concentration of nucleic acid.