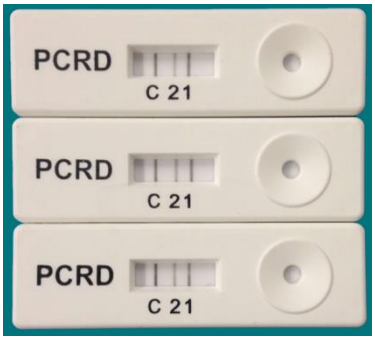
Isothermal Amplification – LAMP

Isothermal amplification: Introduction

Isothermal amplification is used to describe a suite of DNA amplification methods which unlike PCR, can be used to amplify a specific DNA target by incubation of the reaction at a single temperature. There are a number of different techniques such as Nucleic Acid Sequence Based Amplification (NASBA), Helicase-Dependent Amplification (HDA) and Rolling-Circle Amplification (RCA), however, Loop mediated AMPlification (LAMP) and Recombinase Polymerase Amplification (RPA) are the two methods that have been adopted most frequently for diagnostic applications. Some of the isothermal methods share advantages over PCR for diagnostic purposes. Frequently the enzymes used (e.g. BST1) are more robust and do not become inhibited by small increases in proteins, polysaccharides or polyphenolic compounds present in plant material. Secondly, the enzymes copy DNA faster than Taq DNA polymerase, resulting in shorter reaction times and finally, because no precise thermal cycling is required, the assays can be run with much simpler equipment. Taken together these advantages identify with isothermal DNA amplification techniques being suitable for performing DNA diagnostics in the field or in remote locations with limited resources. However, they also provide many benefits over PCR in the routine testing laboratory, being faster, more robust and requiring less sophisticated equipment to run.

Isothermal amplification methods can be used in conjunction with a range of methods for the resolution of positive and negative results. Due to the large amount of product generated by the highly processive enzymes used, the difference between a negative and a positive reaction is generally unambiguous, so colour change reactions (see example from NEB - https://international.neb.com/products/m1800-warmstart-colorimetric-lamp-2x-master-mix-dna-rna#Product%20Information) can be used to give a simple read-out, although weak positive results can be difficult to interpret. Lateral-flow devices for detection of labelled products (see the example below from Abingdon Healthcare https://www.abingdonhealth.com/other-products/) also have the advantage of



providing a read-out which is unambiguous and easy to interpret. Whilst the large amount of DNA produced can be an advantage it also poses a significant risk in the diagnostic laboratory, that is the risk of post-amplification, carry-over contamination. The risk of contamination can be reduced by minimising the number of manipulations required to perform the test, and by separation of pre- and post-amplification steps. Real-time detection can also be used, the closed-tube nature of which greatly reduces the risk of contamination. Instruments including the OptiGene Genie III have been developed for real-time isothermal amplification. The Genie III is particularly suitable for use in a diagnostic lab or in remote locations due to its small size and portability, and its ability to run on battery power for several hours.

Genie III instrument (OptiSense Ltd)

Loop mediated AMPlification (LAMP): Introduction

One approach to isothermal amplification is to generate products containing self-complementary regions which form single-stranded loops to which primers can bind. Loop-mediated isothermal amplification (LAMP) is the most common method to use this approach and was first described by Notomi et al. (2000). LAMP uses two pairs of primers (internal and external) and a DNA polymerase with strand displacing activity to generate amplification products containing loops. The internal primers target two different binding sites of opposite orientations such that the extension product contains self complementarity and forms a loop. In the early stages of the reaction, this extension product is displaced from the template strand by extension of the adjacent external primer, but this is not required later in the reaction. The amplification product consists of structures composed of differing numbers of alternately oriented repeats of the target sequence, resulting in a ladder of concatenated amplified product when visualized by gel electrophoresis. Amplification can be accelerated by the addition of loop primers (Nagamine et al., 2002) which bind to loops in the amplification product which are in the incorrect orientation for the internal primers to bind. Whilst loop primers are not essential for the amplification mechanism to proceed, assays with two loop primers typically amplify target DNA in a shorter period of time.

References

Nagamine, K., Hase, T. and Notomi, T. (2002) Accelerated reaction by loop-mediated isothermal amplification using loop primers. Molecular and Cellular Probes 16, 223-229.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. and Hase, T. (2000) Loop-mediated isothermal amplification of DNA. Nucleic Acids Research 28, e63.

LAMP protocol

Because the enzymes used in a LAMP reaction are more robust and less affected by compounds that inhibit PCR it is possible to perform sample preparation using very simple methods, one example is detailed below.

Alkaline PEG DNA extraction

1. Place a small piece (1-2cm^2) of leaf material to be tested into a screw cap tube containing 1ml PEG buffer (60% PEG 200 (Sigma) plus 20 mM KOH (or NaOH), pH 13.3-13.5) and a stainless-steel ball-bearing.
2. Replace cap and shake bottle for 1 – 2 minutes, or until the sample is a mid-green colour.
3. Dilute the PEG extract by a factor of one in ten by transferring 10 µl PEG extract into a tube containing 90 µl molecular biology grade water and shake or vortex to mix.
4. The DNA sample is now ready to be used for LAMP amplification and ideally would be prepared just prior to amplification.

Real-Time LAMP amplification

Real-time LAMP reactions can be set up in advance and kept on ice for several hours before use, taking care to avoid DNA contamination

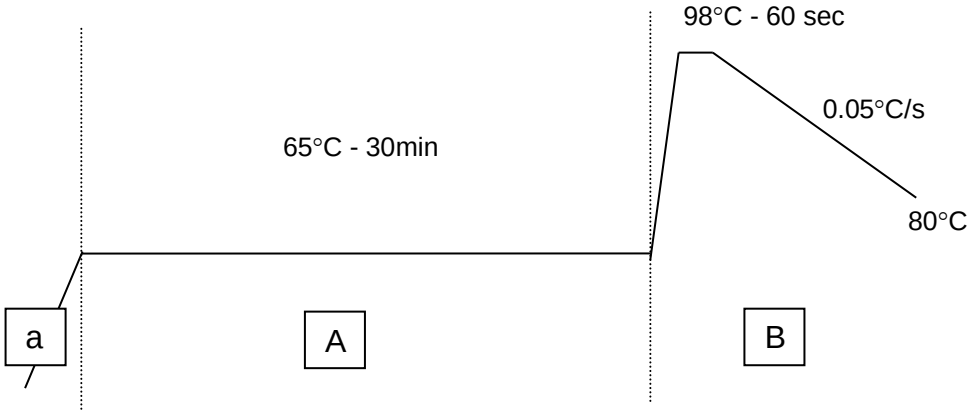
1. The primer mix has been made up in advance as follows. The primer sequences are presented in table 1:

Primer	Starting concentration	Final concentration	Volume per reaction (µl)	Volume for x reactions (µl)
Primer F3	10 µM	200 nM	0.5	
Primer B3	10 µM	200 nM	0.5	
Primer FIP	100 µM	2 µM	0.5	
Primer BIP	100 µM	2 µM	0.5	
Primer F-loop	100 µM	1 µM	0.25	
Primer B-loop	100 µM	1 µM	0.25	

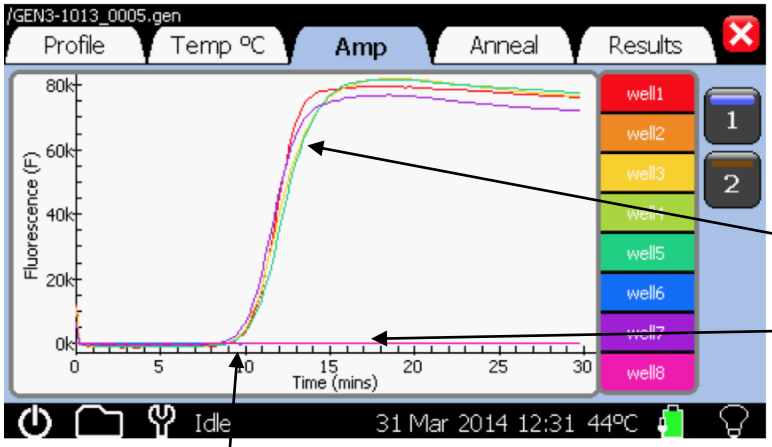
2. The LAMP master mix is made up as follows.

Reagent	Volume per reaction (µl)	Volume for x reactions (µl)
Isothermal Master Mix	15	
Primer mix	2.5	
Water	6.5	

3. Pipette 24 µl aliquots of master mix into each tube in a Genie 8-well strips, partially close the lids and store on ice until ready to use.
4. Add 1 µl of diluted PEG extract to each tube and close the lids firmly. Include at least one negative control reaction in each run, where DNA is replaced with molecular biology grade water.
5. Place the tubes into the real-time instrument (e.g. Genie III).
6. Run the LAMP by (a) preheating reactions to 65°C, (A) incubating for 30 minutes for amplification with real-time data capture and (B) finally generating an annealing profile as detailed below.



7. The results can be observed as (i) amplification plots along with (ii) the annealing profile of the amplified DNA as detailed below.

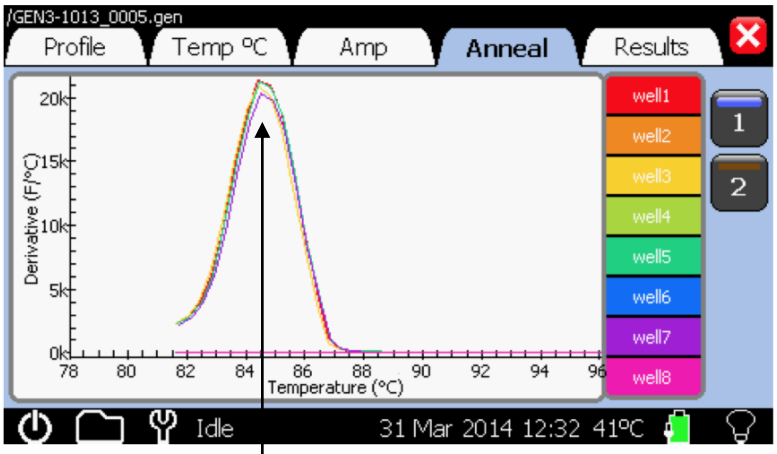


TTP = 9 minutes

i. Amplification plots following a 30- minute incubation giving

Positive
&
Negative results

Enabling an estimation of the time to positive TTP of the samples



Annealing temperature = 65°C

ii. Fluorescent derivative data following annealing of the amplification product enabling an estimation of the annealing temperature

(**IMPORTANT**: Do not open the tubes after the completion of the amplification reaction, as this carries the risk of contamination with amplification product!).

Table 1: Primer sequences for LAMP assays for the detection and discrimination of strains of PepMV (Ling K. S., Li R. & Bledsoe M. (2013). Pepino mosaic virus genotype shift in North America and development of a loop-mediated isothermal amplification for rapid genotype identification. Virology Journal, 10, 117-128).

Strain	Primer	Sequence
CH2	F3	CGATGAAGCTGAACAACATTTCC
	FIP	CTTAATGGGTTGATCTTGGTGGAAGCTGTGAGAAAGCTTCACAAAC
	BIP	GGGTTAAGTTTTCCCCAGTTTGAAAATTCCTTCAGTGTTAATCTTGTG
	B3	TCCAGCAATTCCGTGCACAACAA
	Loop F	GGCCTCGCCTTGATGGA
	Loop B	TGGAAAGATCAACTTTGATCAATT
EU	F3	ACCAAGAAGATACAAAATTTGC
	FIP	TRAGACCATCAGCAGGCTGCTGCATTTGACTTCTTCGATG
	BIP	TCAGGCARCCAAATGAGAAAGAAACCTGTGGAGATCTTTT GC
	B3	TGACTTCTCCAAGTGTGG
	Loop F	TGGCAGGGTTGGTGACTC
	Loop B	CTAGCTGCTCACTCCGTAGCTAA
US1	F3	GCATTCATACCAAATGGGAG
	FIP	TGCGAACAGCCAAGAAATATAAATTGCATGAATACCTTACTCC
	BIP	TTGCACAAACTCCACCAAGGACTTAACCCGTCAATGTGTT
	B3	CCATTTCGAACAGGGGAA
	Loop F	TGCTCAGCTTCATCA
	Loop B	TGAAGCCATGAGACTT