# Development of field diagnostics for cassava

Jimmy Botella, Mike Mason & Jess Hintzsche
School of Agriculture and Food Sciences, University of Queensland, Australia

j.botella@uq.edu.au

# Goals for the cassava diagnostic method

- Can be used in the field
- Easy to use
- Minimal equipment
- Low cost
- Robust

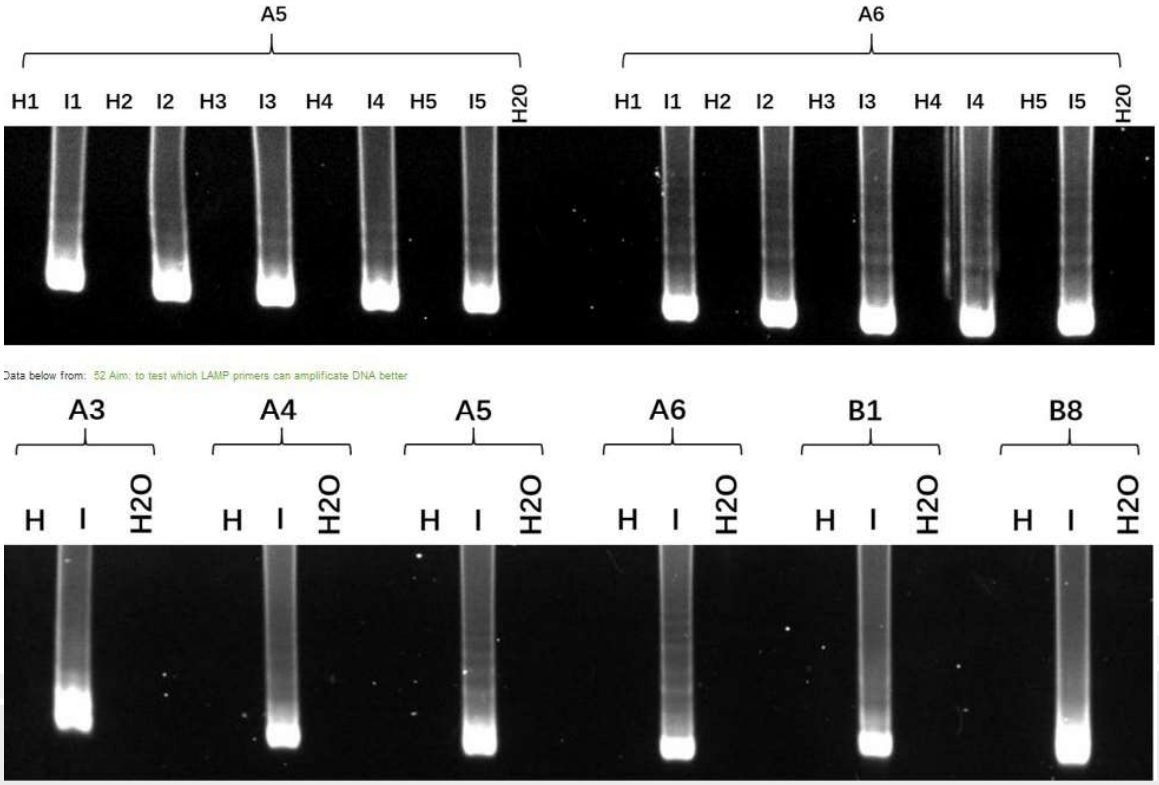
# Goals

To produce

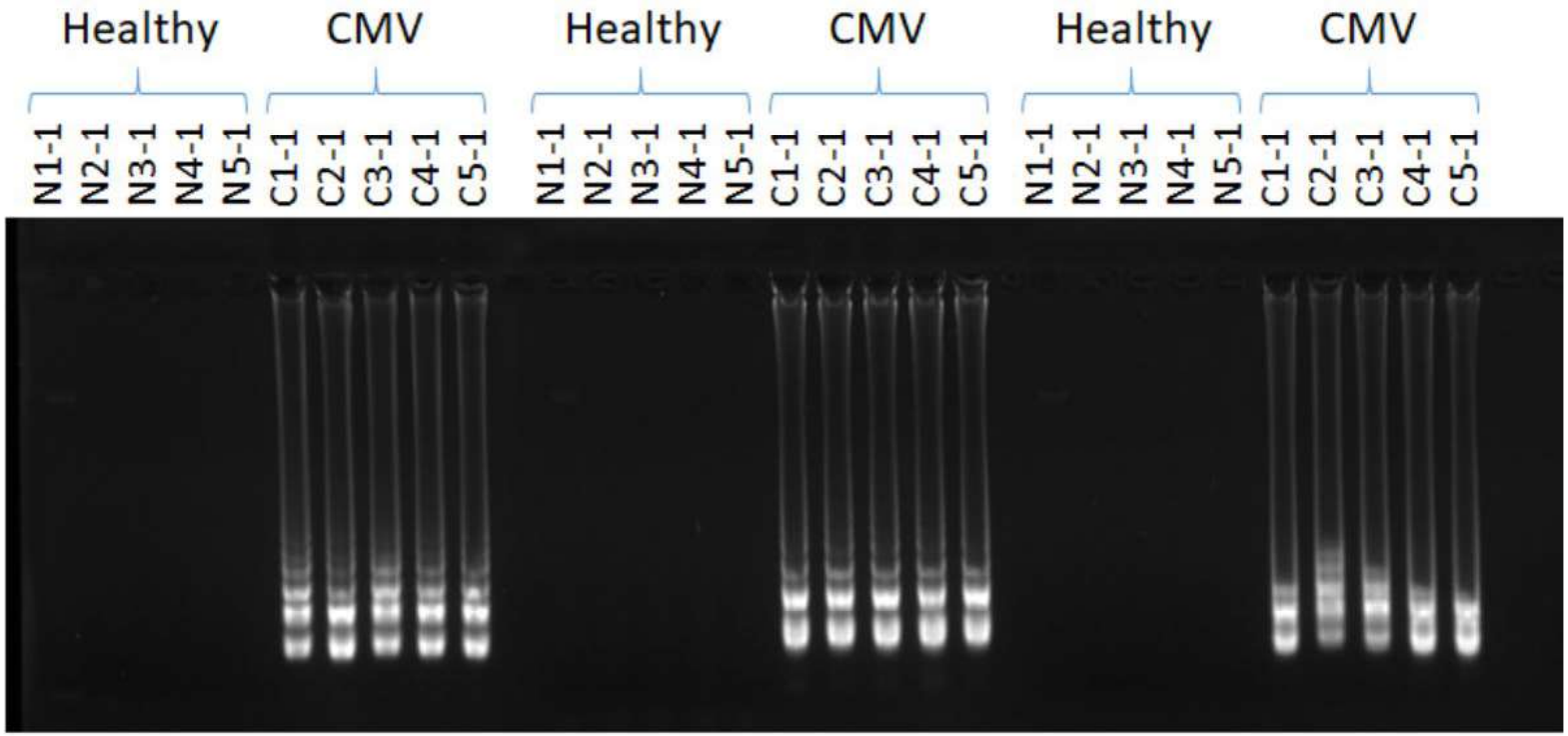
- Something useful
- Something that local partners trust and use
  - It works for them
  - They are comfortable using the method
  - They participate in its development
- Something that does not die at the end of the project

# Optimisation of LAMP for CMV

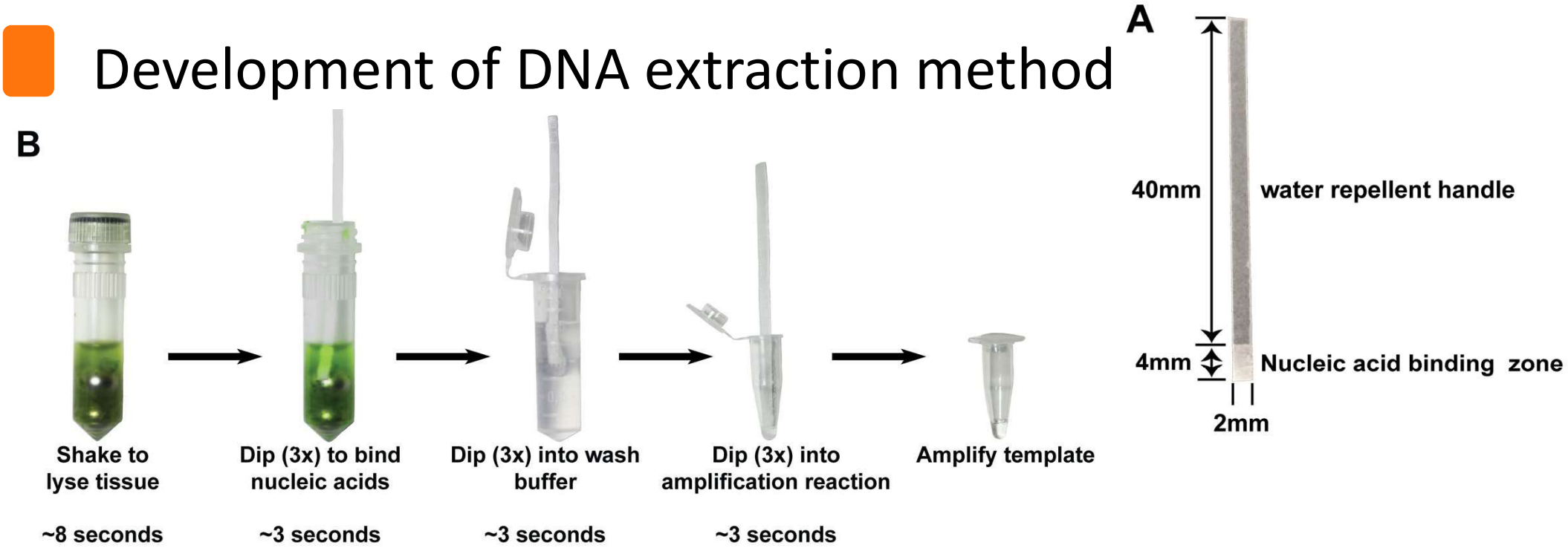
## Design and testing of primer sets



# Optimisation of LAMP for CMV



# Development of DNA extraction method




PLOS BIOLOGY

advanced search

OPEN ACCESS PEER-REVIEWED

METHODS AND RESOURCES

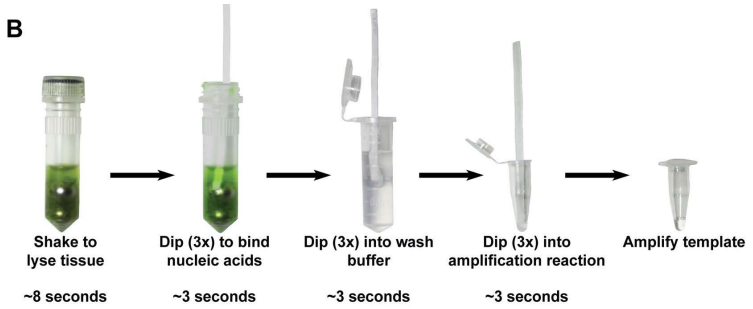
## Nucleic acid purification from plants, animals and microbes in under 30 seconds

Yiping Zou, Michael Glenn Mason, Yuling Wang, Eugene Wee, Conny Turni, Patrick J. Blackall, Matt Trau, Jose Ramon Botella

# Development of DNA extraction method

**GOALS**

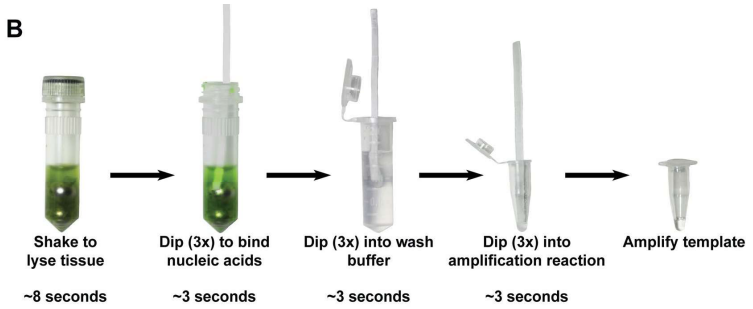
- Simple
- Fast
- ↓ # steps
- Cheap
- Minimize risk of cross-contamination



# Development of DNA extraction method

## Methods

- Optimise buffers
- Optimise tissue amounts
- Optimise maceration techniques
  - Ball bearings
  - Sand
  - Tube types
- Optimise maceration times

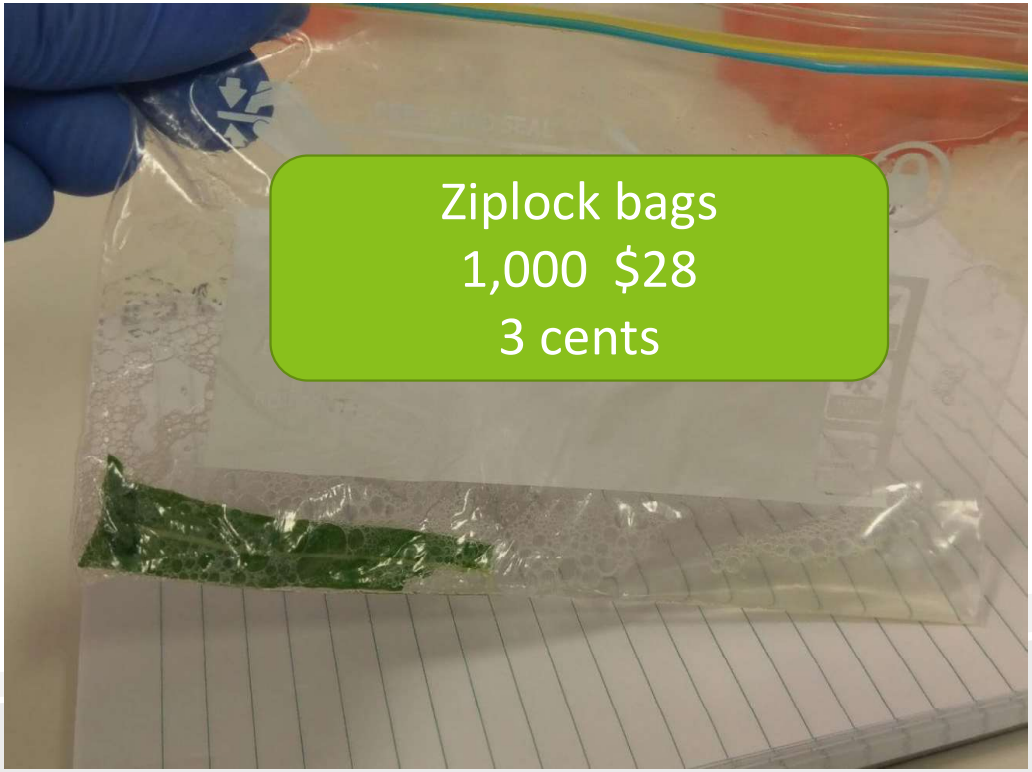


# Development of DNA extraction method

Tubes

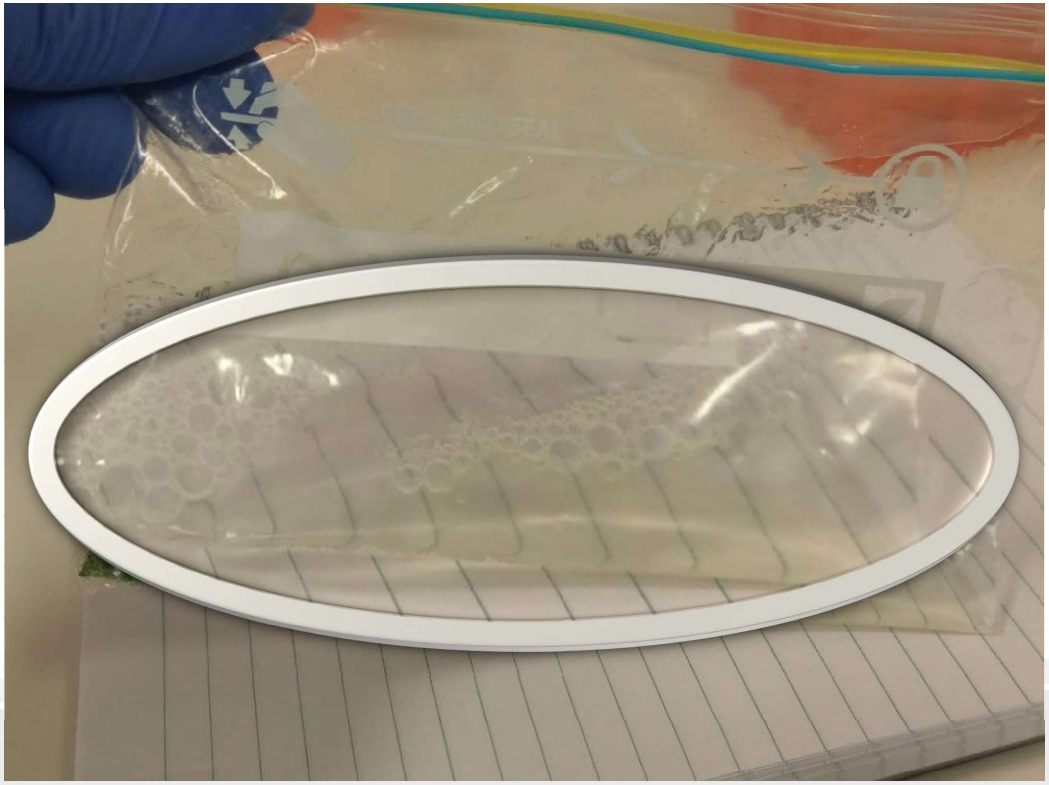

2 ml

Disposable bags

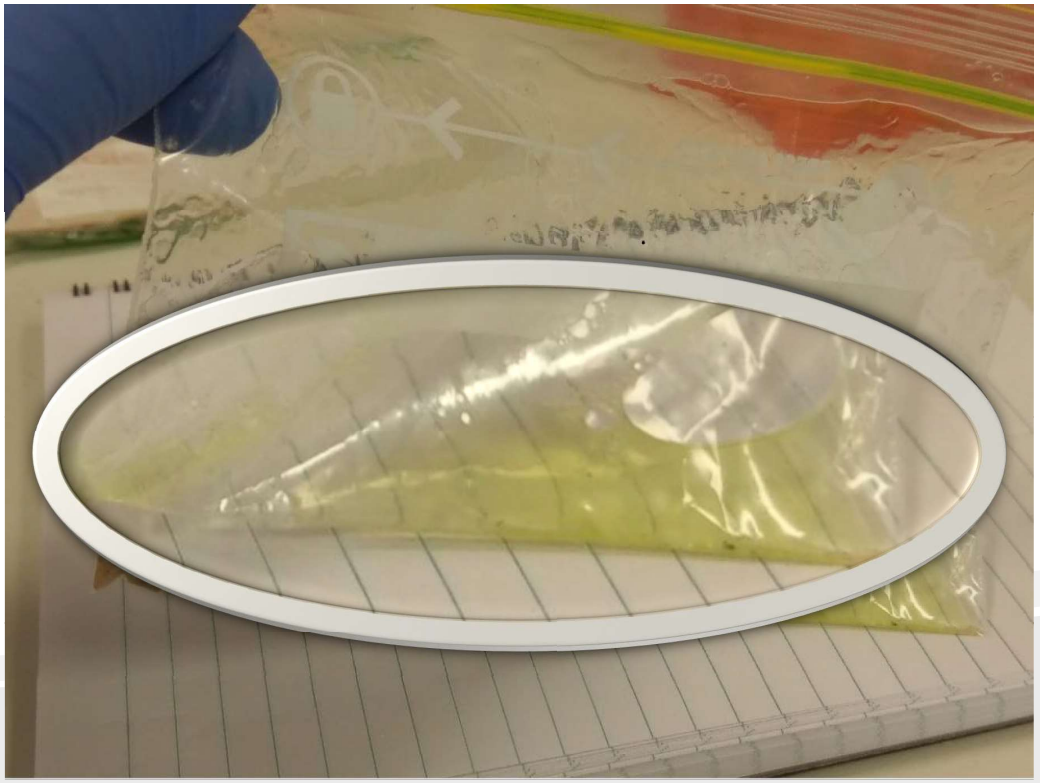


# Development of DNA extraction method

No sand

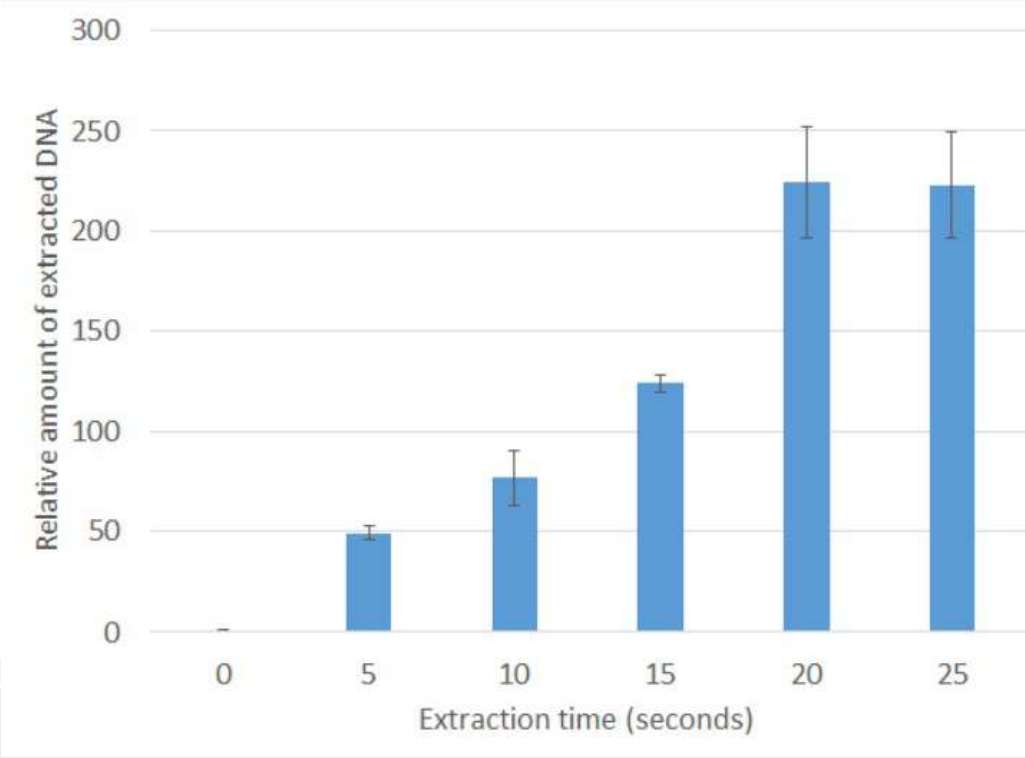


Sand



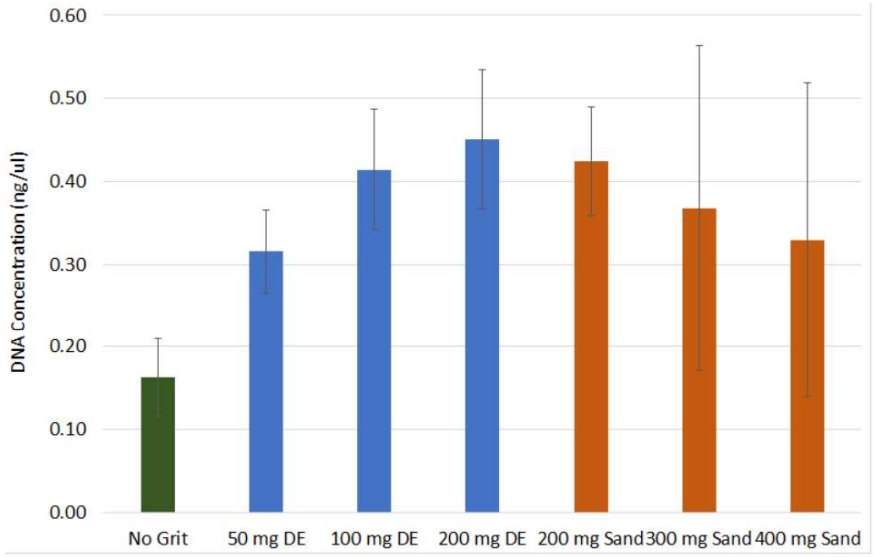
# Development of DNA extraction method

How long?



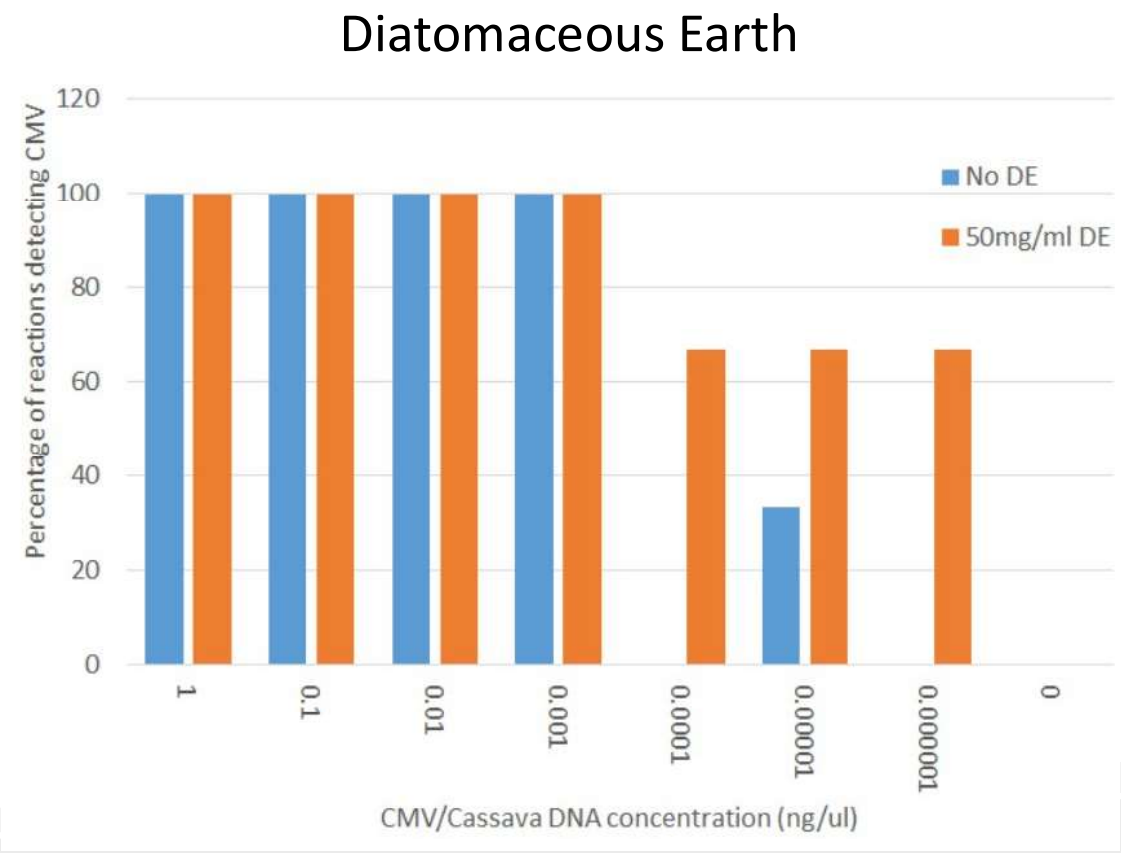
# Development of DNA extraction method

Diatomaceous Earth vs Sand

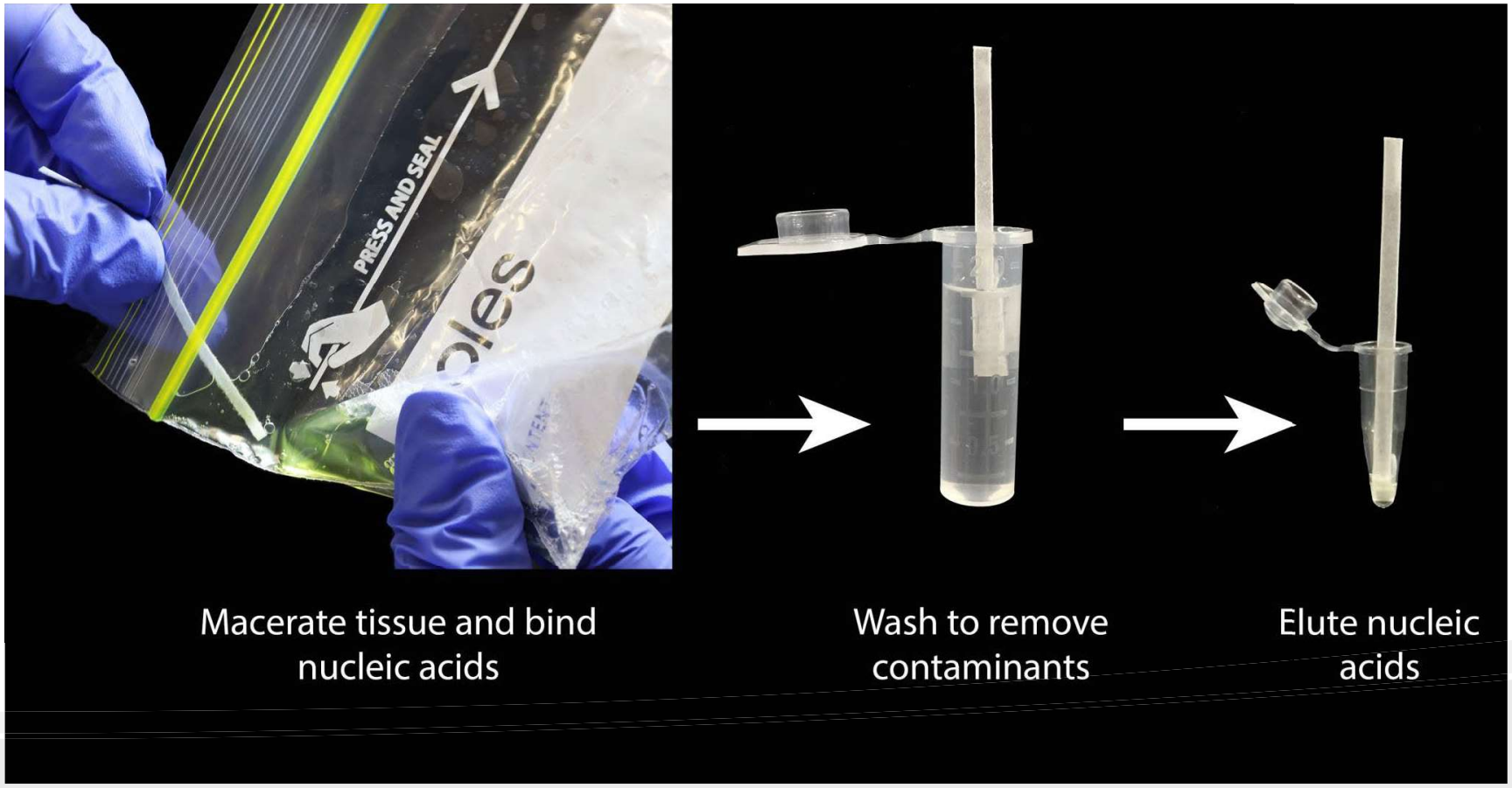



Diatomaceous Earth better than sand as it stays in solution making grinding easier

# Development of DNA extraction method

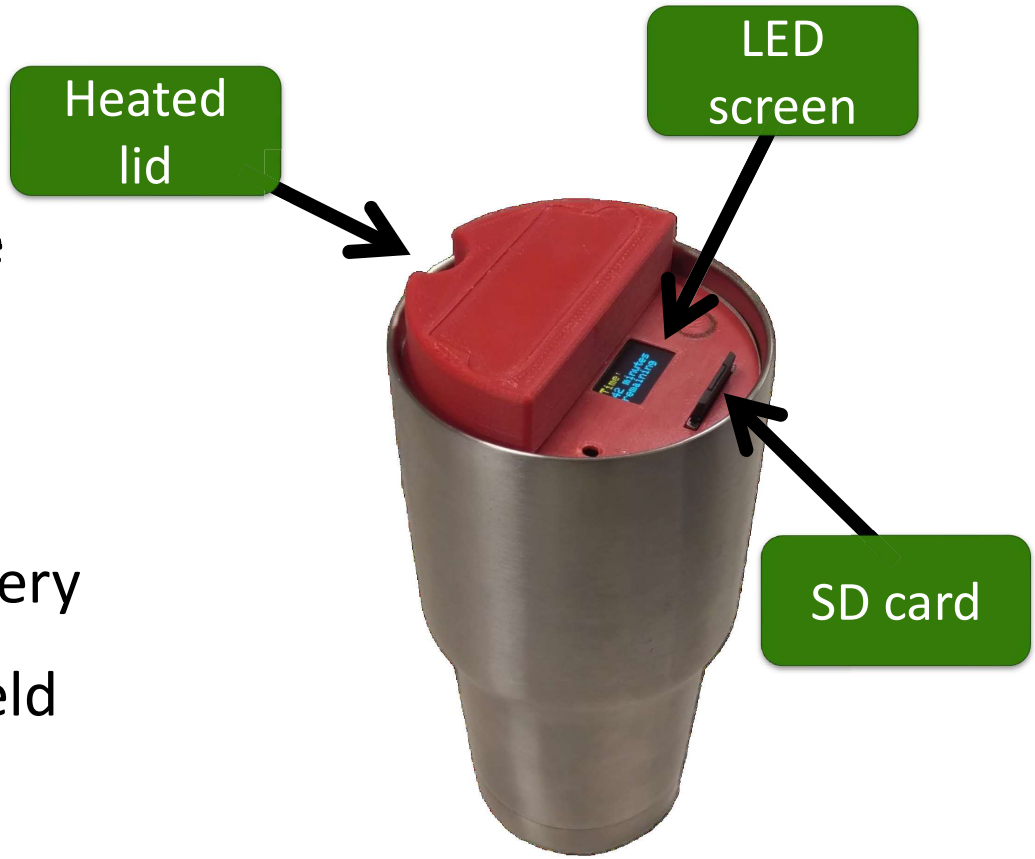


# Rapid Cassava DNA extraction

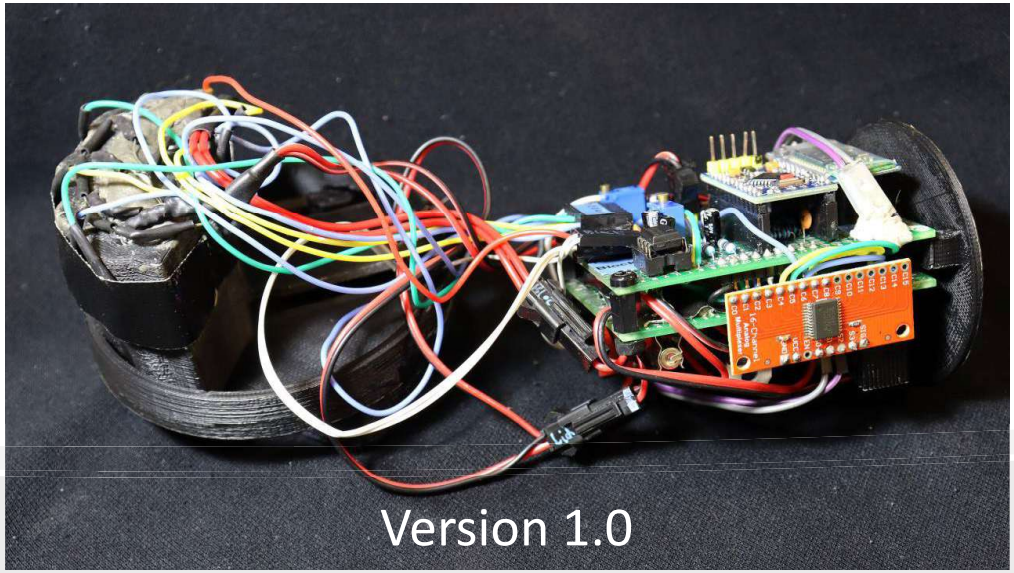


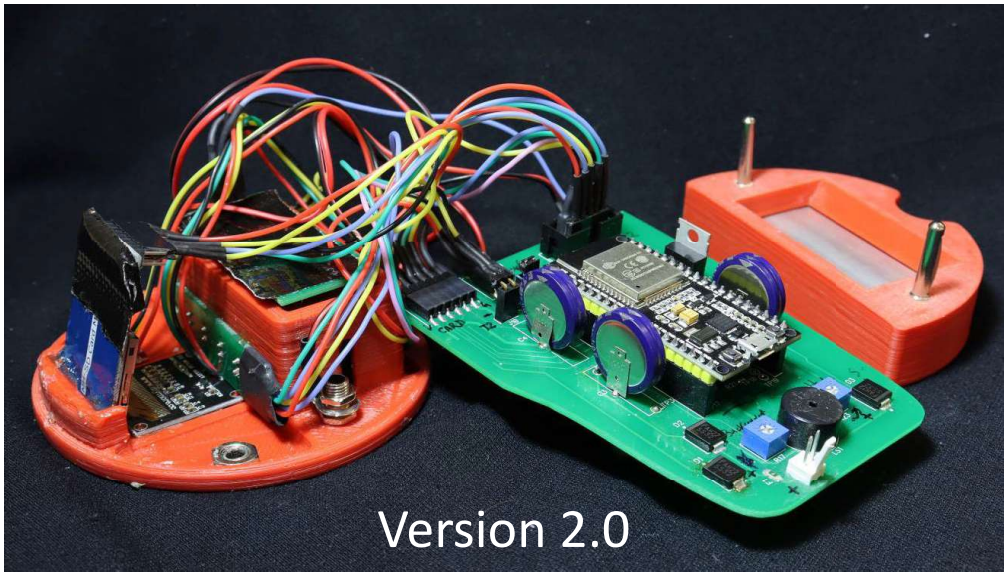
# Optimise Diagnostic Droid

- Portable DNA amplification device
- Easy to use
- Eliminates human bias
- Run from mains power or car battery
- Can be used in the lab or in the field
- Integrated artificial intelligence



# Optimise Diagnostic Droid




Version 3.0

Engaged design/development company

Being reviewed by electronic engineer

Will redesign for easy assemblage

# Eliminate cold storage for reagents

- Optimised freeze dry of reagents
  - No available method for LAMP enzymes
- Tested activity of reactions after freeze dry
- Optimal configuration
  - Individual tubes with 8.5x reactions
  - Can be shipped anywhere at room temperature
  - Can be rehydrated on the day of use
  - Leftovers can be stored

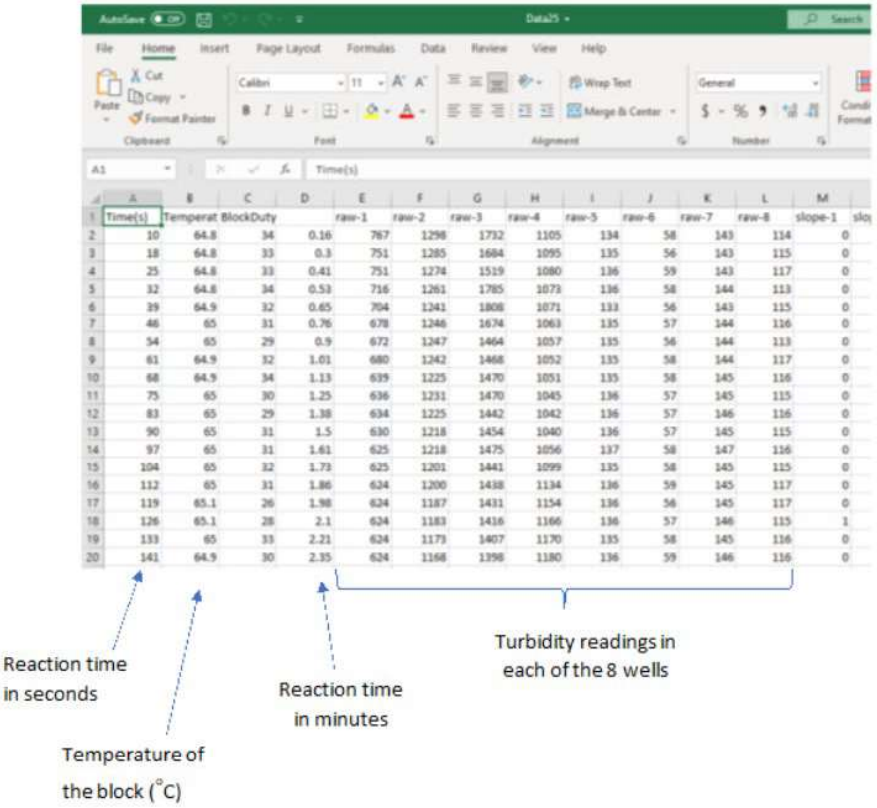
# Technology transfer to partners

- Sent droids
- Sent reagent kits
- Produced detailed instruction protocols
  - Reaction preparation
  - Sample/DNA preparation
  - Droid use
- Produced video material

## Data produced by the Diagnostic Droid

In addition to the on-board display, the raw data recorded by the Droid can also be viewed and analysed manually. Below are instructions on how to do this.

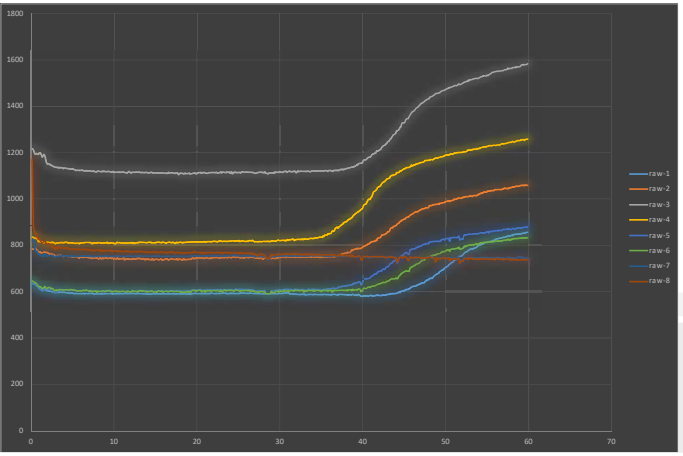
- Insert the SD card into your computer
- Access the last dataset recorded by the droid.
  - The filename will be something like 'Data01.csv'
  - Each new data file recorded by the Droid will have a new number (from 0 to 99) following the word 'Data'
- Open the file in Microsoft Excel or equivalent
  - The data will look like the image below



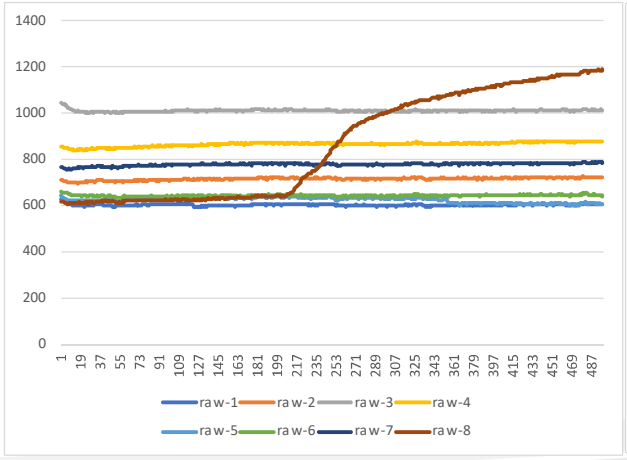
# Technology transfer to partners

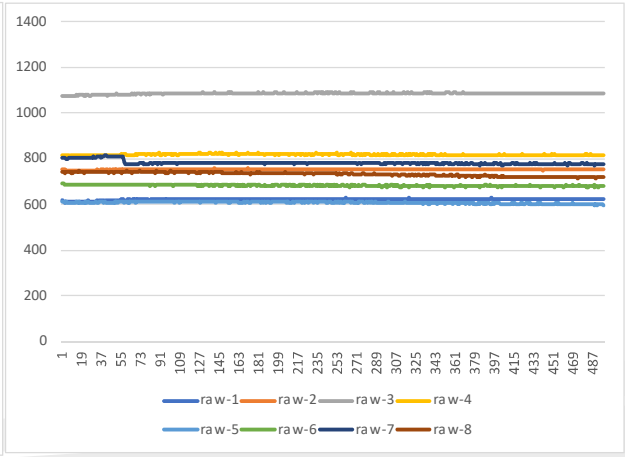
- Liaised with partners for technology establishment
- Train collaborators (COVID)… Training video
- Reviewed initial results

First results perfect




Then less than perfect

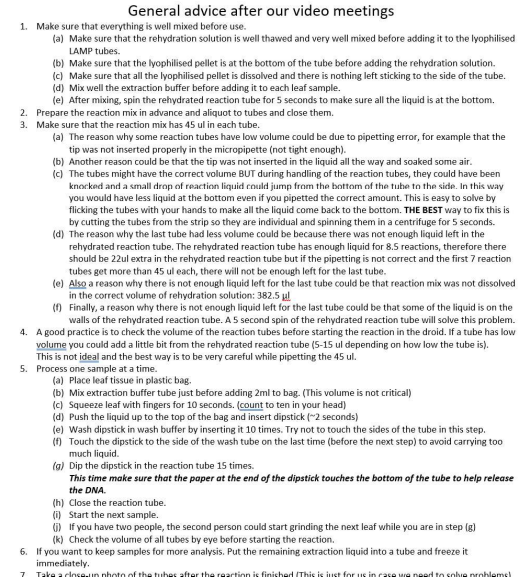



Two repeats same samples
5-8 symptomatic

# Technology transfer to partners

- Liaised with partners for technology establishment
- Train collaborators (COVID)… Produced training video
- Reviewed initial results
- Reviewed videos of partners in action (thanks to Jono Spielberg)
- Zoom meetings to troubleshoot
  - Last tube never worked (had very little mix)
- Live zoom sessions to observe partners live
- Produced additional documents to address problems
- Better results afterwards



General advice after our video meetings

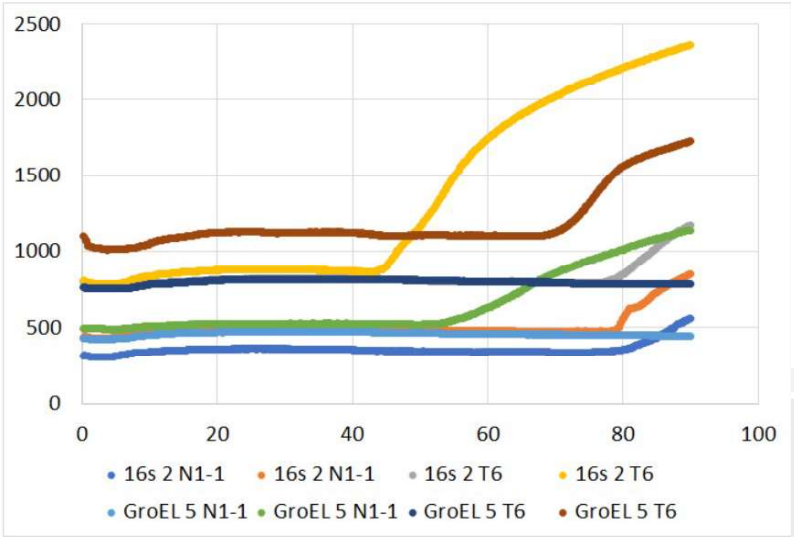
1. Make sure that everything is well mixed before use.
   (a) Make sure that the rehydration solution is well thawed and very well mixed before adding it to the lyophilised LAMP tubes.
   (b) Make sure that the lyophilised pellet is at the bottom of the tube before adding the rehydration solution.
   (c) Make sure that all the lyophilised pellet is dissolved and there is nothing left sticking to the side of the tube.
   (d) Mix well the extraction buffer before adding it to each leaf sample.
   (e) After mixing, spin the rehydrated reaction tube for 5 seconds to make sure all the liquid is at the bottom.
2. Prepare the reaction mix in advance and aliquot to tubes and close them.
3. Make sure that the reaction mix has 45 ul in each tube.
   (a) The reason why some reaction tubes have low volume could be due to pipetting error, for example that the tip was not inserted properly in the micropipette (not tight enough).
   (b) Another reason could be that the tip was not inserted in the liquid all the way and soaked some air.
   (c) The tubes might have the correct volume BUT during handling of the reaction tubes, they could have been knocked and a small drop of reaction liquid could jump from the bottom of the tube to the side. In this way you would have less liquid at the bottom even if you pipetted the correct amount. This is easy to solve by flicking the tubes with your hands to make all the liquid come back to the bottom. **THE BEST** way to fix this is by cutting the tubes from the strip so they are individual and spinning them in a centrifuge for 5 seconds.
   (d) The reason why the last tube had less volume could be because there was not enough liquid left in the rehydrated reaction tube. The rehydrated reaction tube has enough liquid for 8.5 reactions, therefore there should be 22ul extra in the rehydrated reaction tube but if the pipetting is not correct and the first 7 reaction tubes get more than 45 ul each, there will not be enough left for the last tube.
   (e) Also a reason why there is not enough liquid left for the last tube could be that reaction mix was not dissolved in the correct volume of rehydration solution: 382.5 µl
   (f) Finally, a reason why there is not enough liquid left for the last tube could be that some of the liquid is on the walls of the rehydrated reaction tube. A 5 second spin of the rehydrated reaction tube will solve this problem.
4. A good practice is to check the volume of the reaction tubes before starting the reaction in the droid. If a tube has low volume you could add a little bit from the rehydrated reaction tube (5-15 ul depending on how low the tube is). This is not ideal and the best way is to be very careful while pipetting the 45 ul.
5. Process one sample at a time.
   (a) Place leaf tissue in plastic bag.
   (b) Mix extraction buffer tube just before adding 2ml to bag. (This volume is not critical)
   (c) Squeeze leaf with fingers for 10 seconds. (count to ten in your head)
   (d) Push the liquid up to the top of the bag and insert dipstick (~2 seconds)
   (e) Wash dipstick in wash buffer by inserting it 10 times. Try not to touch the sides of the tube in this step.
   (f) Touch the dipstick to the side of the wash tube on the last time (before the next step) to avoid carrying too much liquid.
   (g) Dip the dipstick in the reaction tube 15 times.
   ***This time make sure that the paper at the end of the dipstick touches the bottom of the tube to help release the DNA.***
   (h) Close the reaction tube.
   (i) Start the next sample.
   (j) If you have two people, the second person could start grinding the next leaf while you are in step (g)
   (k) Check the volume of all tubes by eye before starting the reaction.
6. If you want to keep samples for more analysis. Put the remaining extraction liquid into a tube and freeze it immediately.
7. Take a close-up photo of the tubes after the reaction is finished (This is just for us in case we need to solve problems)

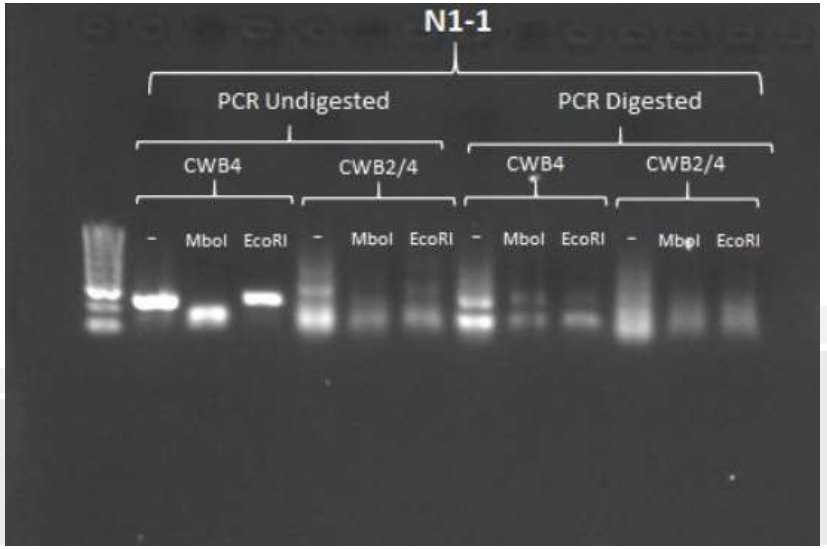
# Cassava witches' broom

- Phytoplasma
- Few reports with limited sequence information
- Phylogenetic analysis place them in different subgroups
- Heavily dependent on geographical location instead of pathogen/host

# Cassava witches' broom

- Tried published LAMP and PCR methods
- A report does 3x nested PCR!!
- Inconsistent results
- Problems with sequence similarity to cassava



# Cassava witches' broom

- Can't develop reliable method with available sequence information
- Remember that witches broom sequences are strongly related to geographical location
- Need additional sequence information
  - Willmer at CIAT is trying to obtain the data

# Cost of reagents

| Reagents | Manufacturer | Cat No | Price | Amount | Delivery (to UQ) | Total | MW (g/mol) | [Conc] (mM) | 1000x reactions (45 ml) | unit | $ for x1000 rxn |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Tris | Bio-rad | 1610716 | $123.00 | 500 g | $0.00 | $123.00 | 121.14 | 20 | 0.0009 | g | 0.0002 |
| (NH4)SO4 | Sigma alrdrich | A5132-1KG | $69.67 | 1 kg | $0.00 | $69.67 | 132.13 | 10 | 0.00045 | g | 0.0000 |
| KCl | Sigma Alrdrich | PHR1329-5( | $38.72 | 5 g | $0.00 | $38.72 | 74.55 | 50 | 0.00225 | g | 0.0174 |
| MgSO4 | Novachem | 135-00425 | $48.00 | 500 g | $35.00 | $83.00 | 120.37 | 8 | 0.00036 | g | 0.0001 |
| Tween 20 | Thermo Fisher | FSBBP337-1 | $32.26 | 100 ml | $0.00 | $32.26 | | 0.10% | 0.045 | ml | 0.0145 |
| Betaine | Chem Supply A | B0455-100( | $88.80 | 100 g | $35.00 | $123.80 | 117.15 | 80 | 0.0036 | g | 0.0045 |
| Bst polymerase (1.8ul) (8units/ml) | NEB | M0538L | $488.07 | 8000 U (1 r | $0.00 | $488.07 | | | 1.8 | ml | 878.5260 |
| dNTPs (10 mM) | NEB | N0447L | $390.15 | 4 ml | $0.00 | $390.15 | | | 6 | ml | 585.2250 |
| LAMP primers | | | | | | $0.00 | | | | | -- |
| Total x1000 rxn | | | | | | | | | | | **$1,463.7877** |
| Per reaction | | | | | | | | | | | **$1.46** |