CIP
INTERNATIONAL POTATO CENTER
A CGIAR RESEARCH CENTER

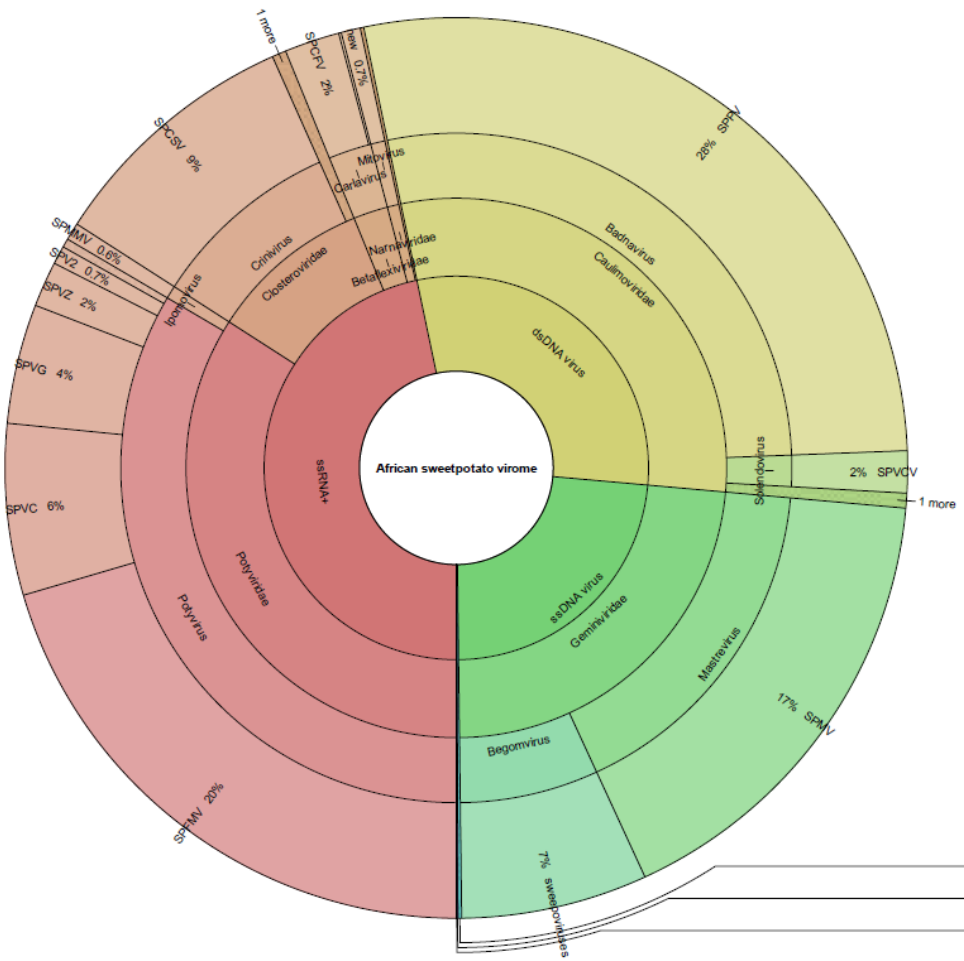
Validating small RNA sequencing and assembly to replace classical virus indexing in root and tuber crops

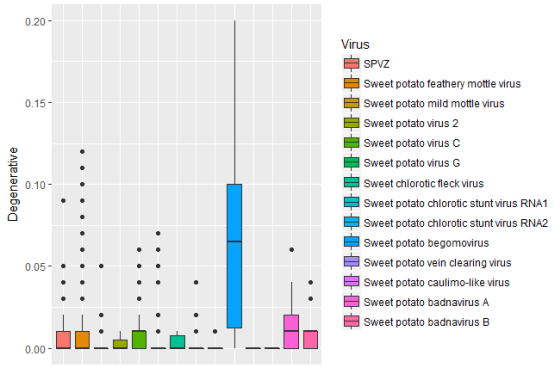
November 22, 2017
Bari

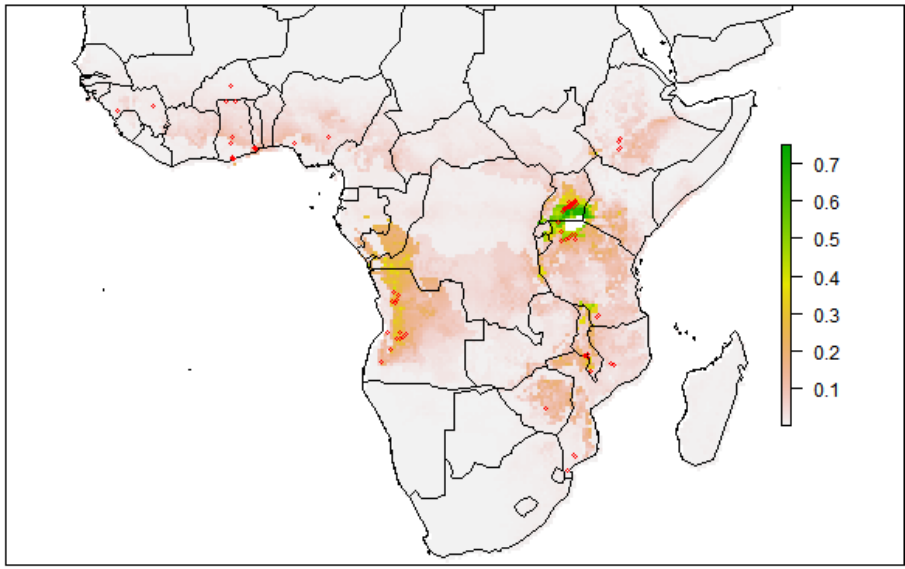
C O S T E U P H R E S C O 2 0 1 7

Small RNA sequencing and assembly: a generic virus identification method for plants

Sweetpotato virome: 3193 viruses from 1168 samples, >15 new species







ssDNA satellite 0.1%

dsRNA 0.03%

Viroid 0.03%

Current indexing process

sweetpotato

In vitro plantlets → PROPAGATION → "A"

Growing in jiffy strips (30 days)

Growing in pots (30-45 days)

10—15 NODES

Symptoms

High probability of detection

Symptoms, NCM-ELISA, PCR (Begomovirus)

GRAFTING

Held grafted plants for a minimum of 30 days

Ipomoea setosa (3 weeks old or 15-20 cm height)

IF NEGATIVE

Prune and allow regrowing (30-45 days)

10—15 NODES AGAIN

Symptoms

High probability of detection

Symptoms, NCM-ELISA PCR (Begomovirus)

GRAFTING

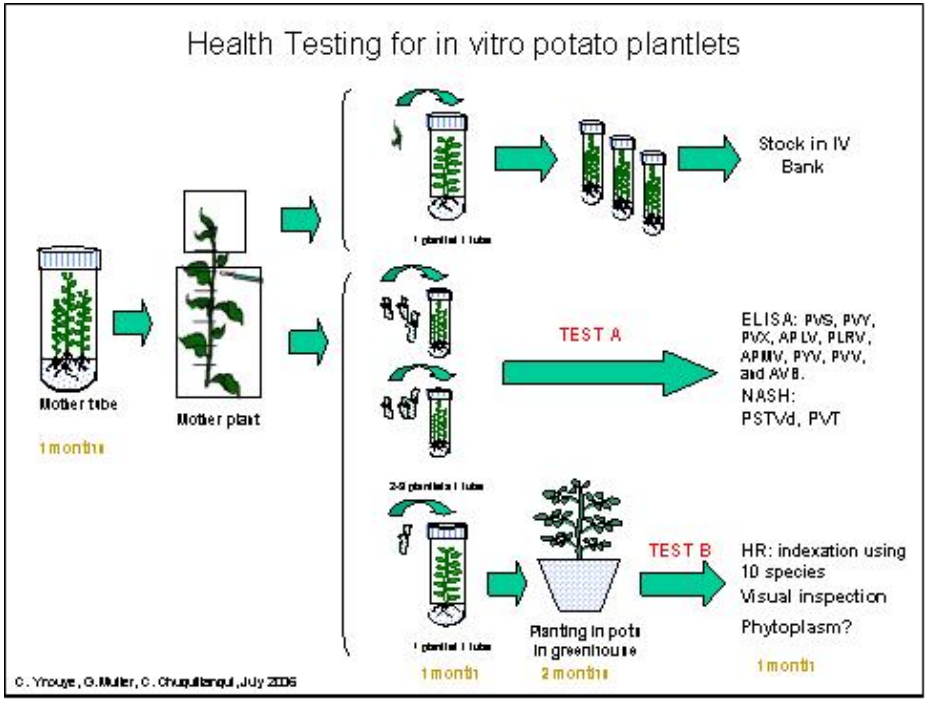
30 days

Ipomoea setosa (3 weeks old or 15-20 cm height)

IF NEGATIVE MULTIPLY AND DISTRIBUTE FROM IN VITRO "A"

5- 6 months (US$ 110- 120)

potato

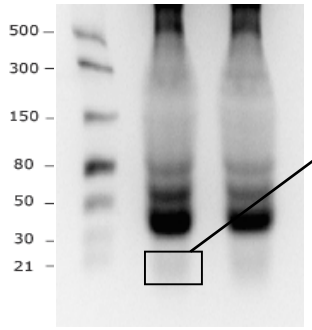


NCM-ELISA is performed for 10 viruses (SPFMV, SPLV, SPVG, SPMSV, SPMMV, SPCSV, SPCFV, SPC6V, SPCV, and CMV).

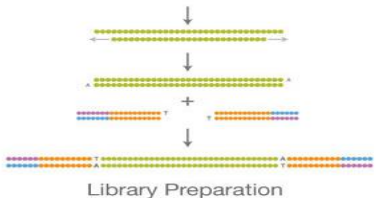
2 weeks, 48 samples

~ 30 US$/sample

Extract RNA
& run in 4%
agarose gel



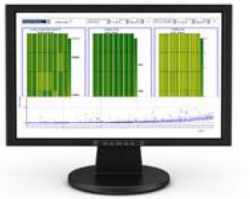
Cut and purify 20-30 nt
band, prepare library



3-5 days

<1 US$/sample

Send to sequencing
provider



Bio-informatics:
VirusDetect v1.0

<1 day

cue + 3 days

~20 US$/sample

Small scale validation for routine detection in potato: side by side comparison to standard virus indexing

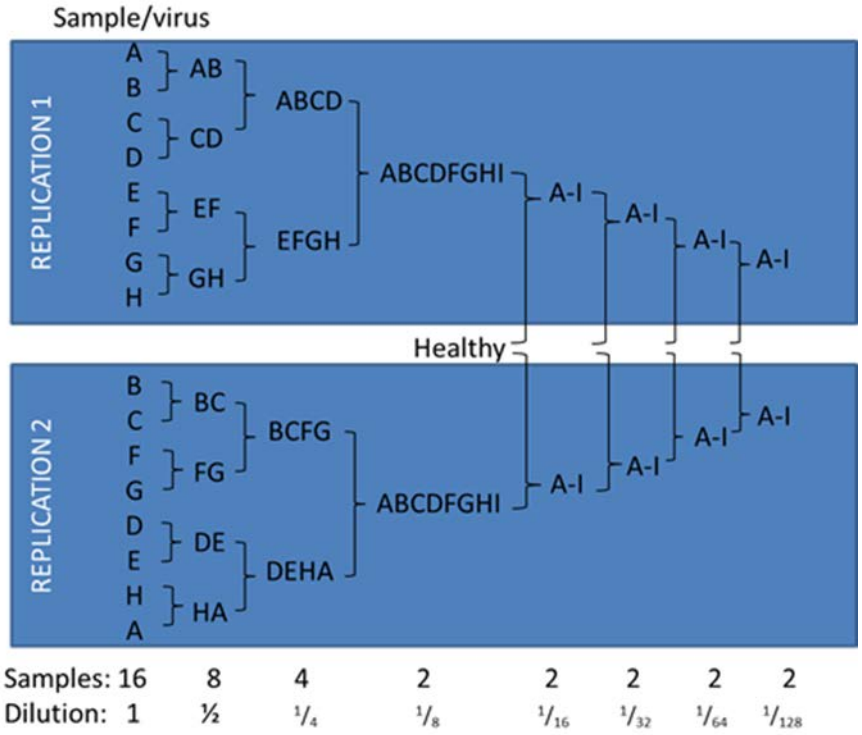
Library ID	Sample (CIP number)	Country	Standard Indexing (from potato and/or indicator plants grown in greenhouse)	sRSA[4] (from *in vitro* potato plant extractions)	PCR confirmation (from *in vitro* potato plant extractions)
GAF318-1	706735	Argentina	PVX[1,2,3]	PVX, **PVA**[5]	PVX, PVA
GAF318-2	396009.258	Peru	–	–	–
GAF318-3	703471	Peru	PVS[1]	PVS	PVS
GAF318-4	705268	Ecuador	PLRV[1], PVX[1,2,3]	PLRV, PVX	PLRV, PVX
GAF318-5	700744	Peru	PVS[1,2,3], PVT[1]	PVS, PVT	PVS, PVT
GAF318-6	706851	Peru	PVX[2,3], PVS[1]	PVX, PVS, **PVT**	PVX, PVS,PVT
GAF318-7	703518	Colombia	PVS[1]	PVS	PVS
GAF318-8	704832	Bolivia	PLRV[3], APLV[1,3], **PVX**[3]	PLRV, APLV, **PVT**	PLRV, APLV, PVT
GAF318-9	703573	Colombia	–	–	–
GAF318-10	308328.32	Peru	–	–	–
GAF318-11	398098.20	Peru	–	–	–
GAF318-12	396272.12	Peru	PVS[1,3]	PVS	PVS
GAF318-13	396063.1	Peru	PLRV[3]	PLRV	PLRV
GAF318-14	598198.4	Peru	–	–	–
GAF318-15	304413.45	Peru	–	–	–
GAF318-16	393046.7	Peru	PVX[1,2,3], PVS[1,2]	PVX, PVS	PVX, PVS

Validation experiment for potato

Interlaboratory testing

LAB	SAMPLES	
	SASA 1-48 (1-48)	**CIP 1-48 (49-96)**
SASA	SASA 1-24	CIP 1-24 (49-72)
UNALM	SASA 1-24	CIP 1-24 (49-72)
CIP	SASA 25-48	CIP 25-48 (73-96)
SENASA	SASA 25-48	CIP 25-48 (73-96)

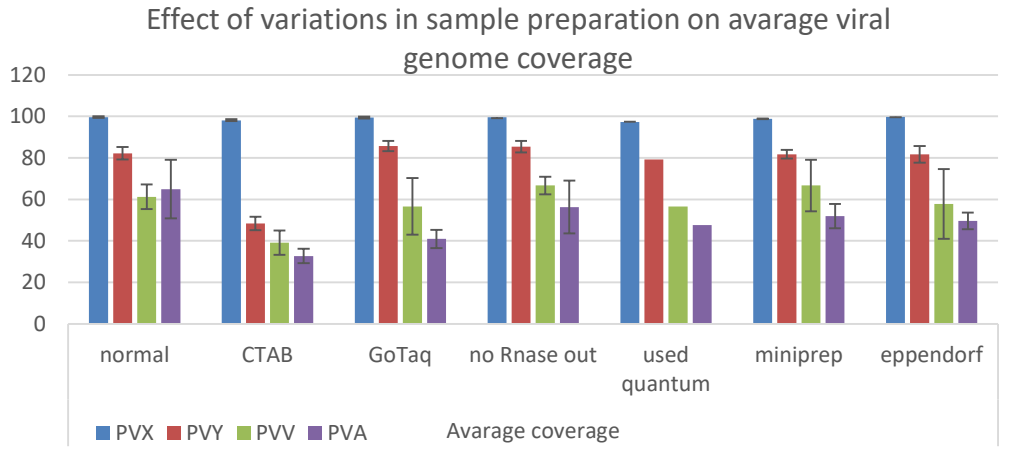
Sensitivity analysis

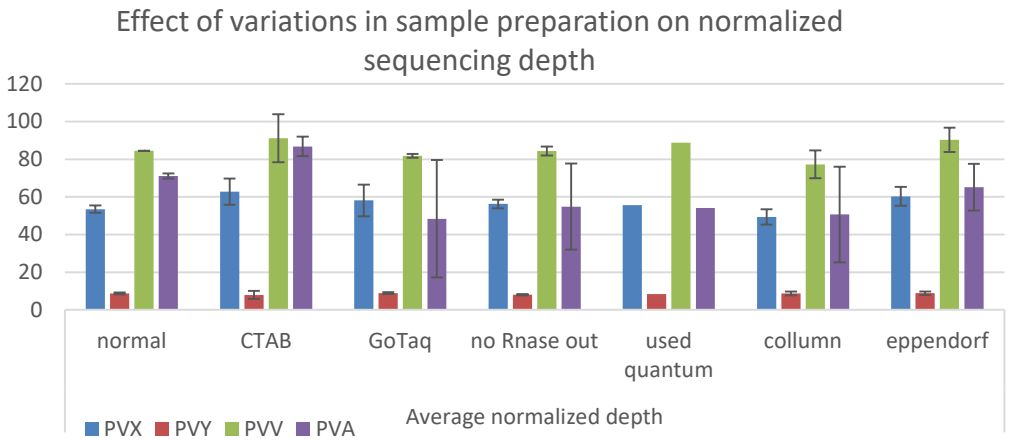


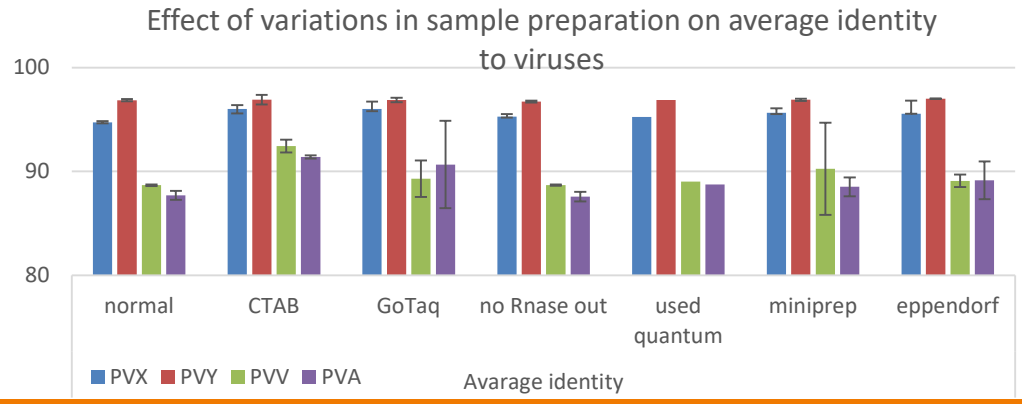
Sources of variation: protocol variations

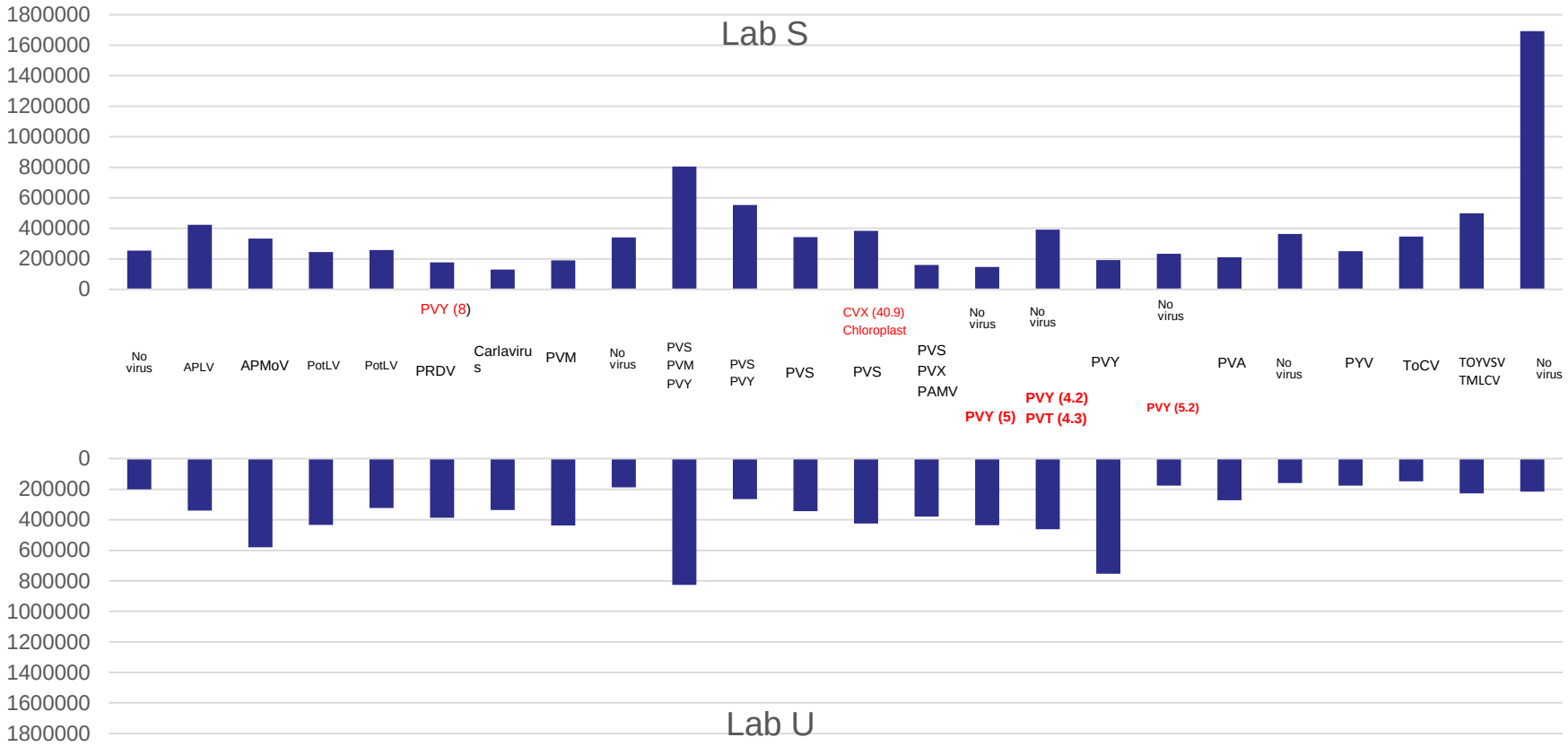
Sources of variation from potato:

- Same conclusion, whatever method used (except where reads were very low). Overall quite similar results.
- CTAB seems to reduce average coverage, but increase sequencing depth, and average sequence identity (sufficiently to make a difference?)
- Quantum prep columns can be re-used after washing without cross-contamination
- RNAse-Out has no effect and can be left out (=cheaper protocol)
- GoTaq had little effect (only PVA) and could replace more expensive phusion.
- Quality of gel is most critical factor (from results other results).







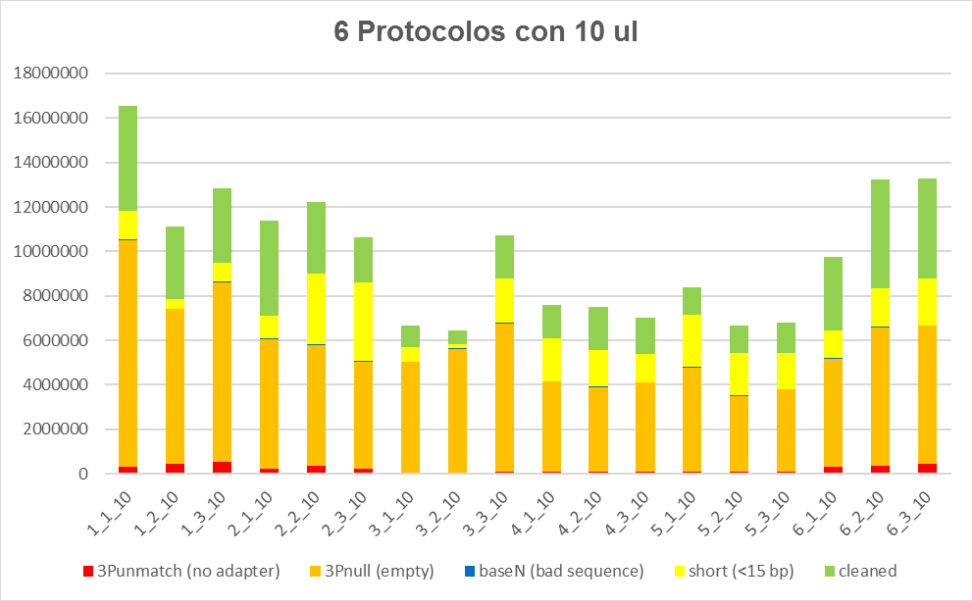

Lab S
1800000
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
No virus
APLV
APMoV
PotLV
PotLV
PRDV
PVY (8)
Carlaviruses
PVM
No virus
PVS PVM PVY
PVS PVY
PVS
PVS
CVX (40.9) Chloroplast
PVS PVX PAMV
PVY (5)
No virus
No virus
PVY (4.2) PVT (4.3)
PVY
No virus
PVY (5.2)
PVA
No virus
PYV
ToCV
TOYVSV TMLCV
No virus
0
200000
400000
600000
800000
1000000
1200000
1400000
1600000
1800000
Lab U

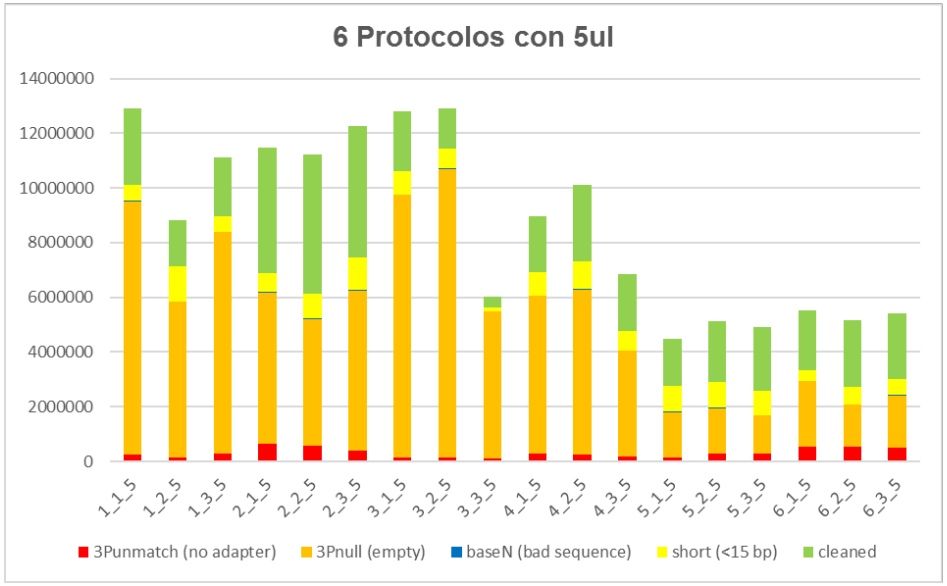
2.1. sRSA is established as a standard virus indexing method for sweetpotato, yam, and cassava and protocols are available for application in other crops

Complementing Output 1.1, the certification of virus-free status in regenerated plants will be done within one month in germplasm exchange tissue culture laboratories through the development of sRSA technology, ensuring release of certified virus-free plants at the end of the four month *in vitro* regeneration cycle.

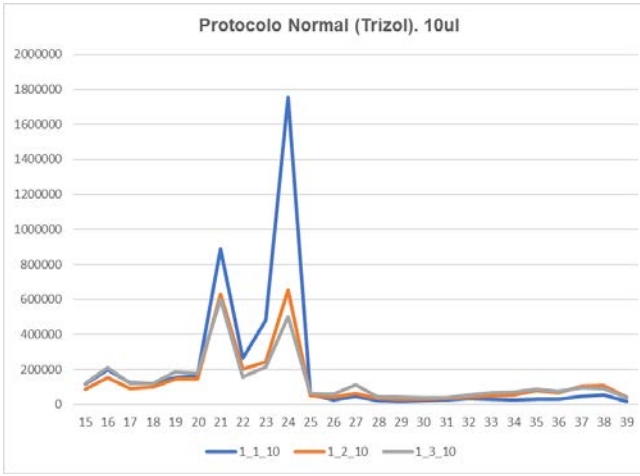
2.1	**The sRSA process standardized at IITA**	**30/9/2017**
2.2	New version of VirusDetect, including drop-down menus, automated siRNA library quality control and traffic light classification of results and reduced hardware requirements	30/9/2018
2.3	Diagnostic sensitivity, specificity and accuracy determined for up to 16 different viruses in Yam and sweetpotato.	30/9/2018
2.4	192 (4 x 48) accessions for all three crops processed by sRSA and standard indexing independently and correlations determined	30/9/2019
2.5	Standard operating procedures for sRSA for virus indexing in-vitro and in-vivo plants available	30/9/2019

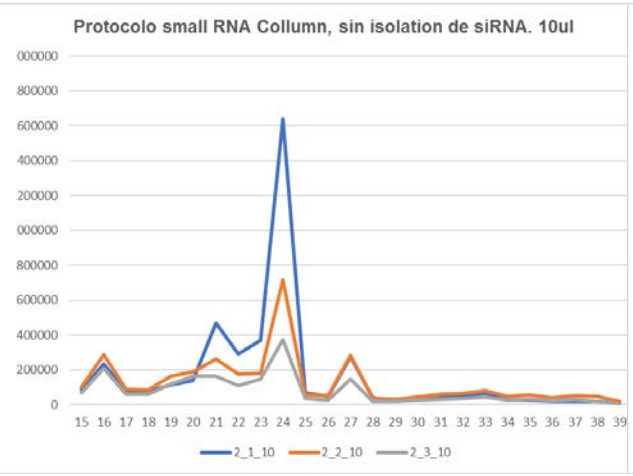
Effect of different protocols on library quality:

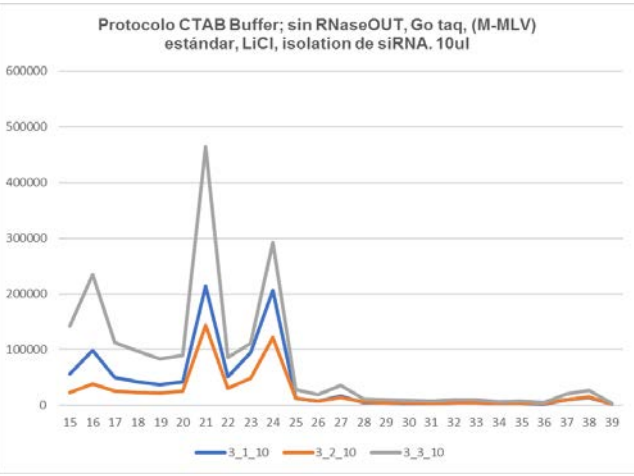


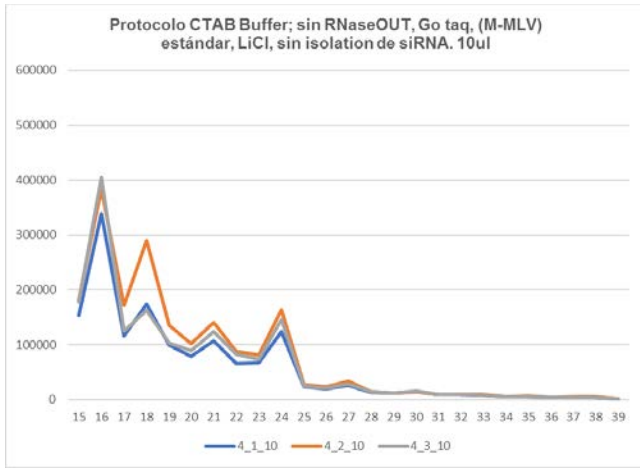


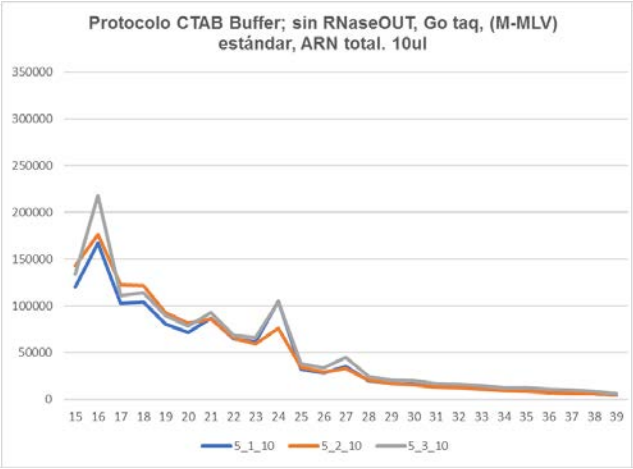
Effects different protocols on read size: 10 ql RT-reaction M-MLV 50 ºC

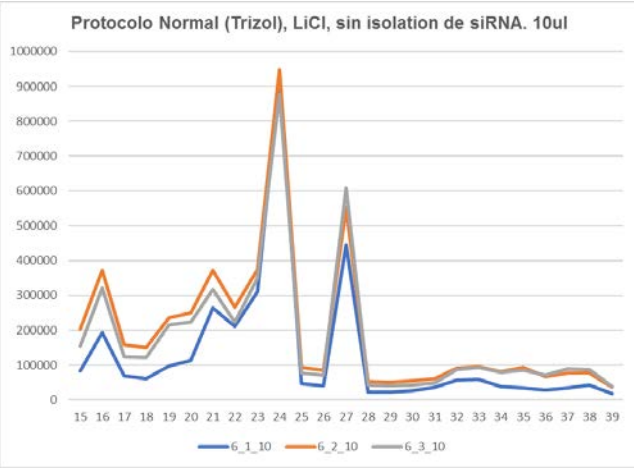




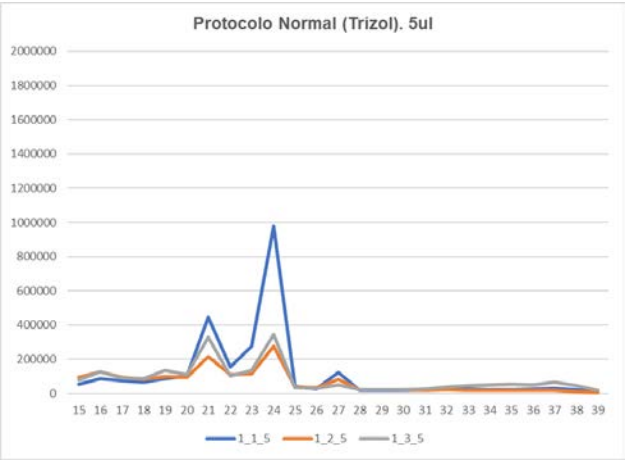


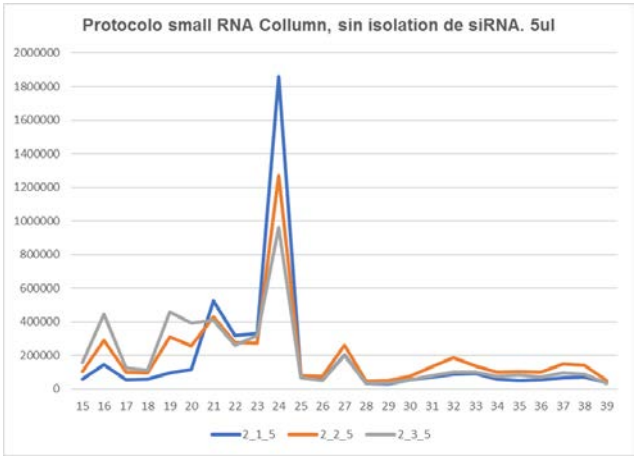


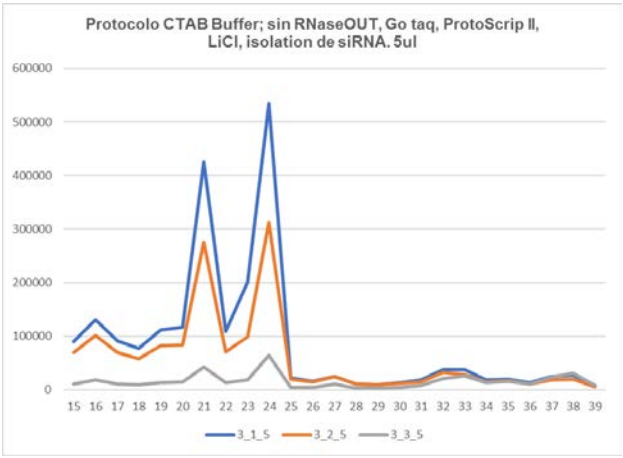


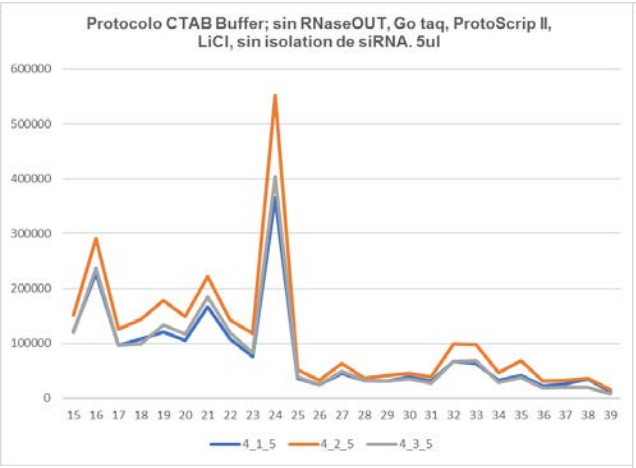


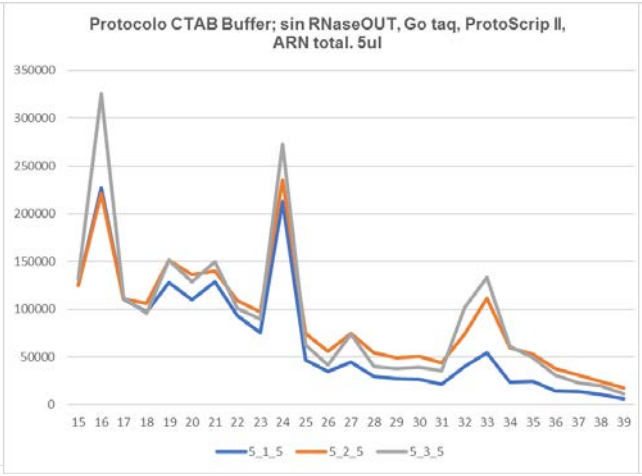
Effects different protocols on read size: 5 ql RT-reaction Protoscript 50 ºC

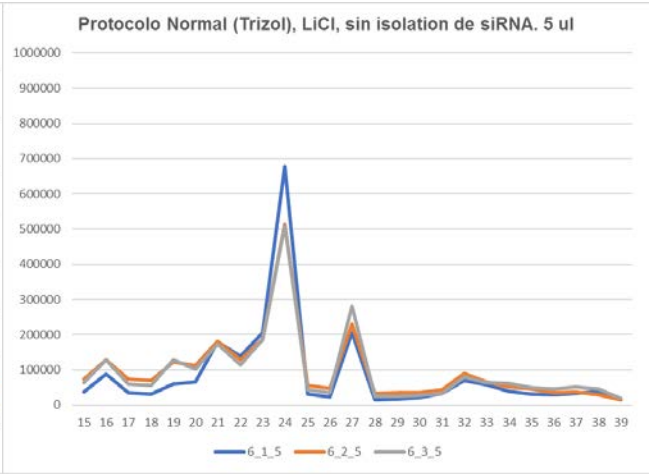


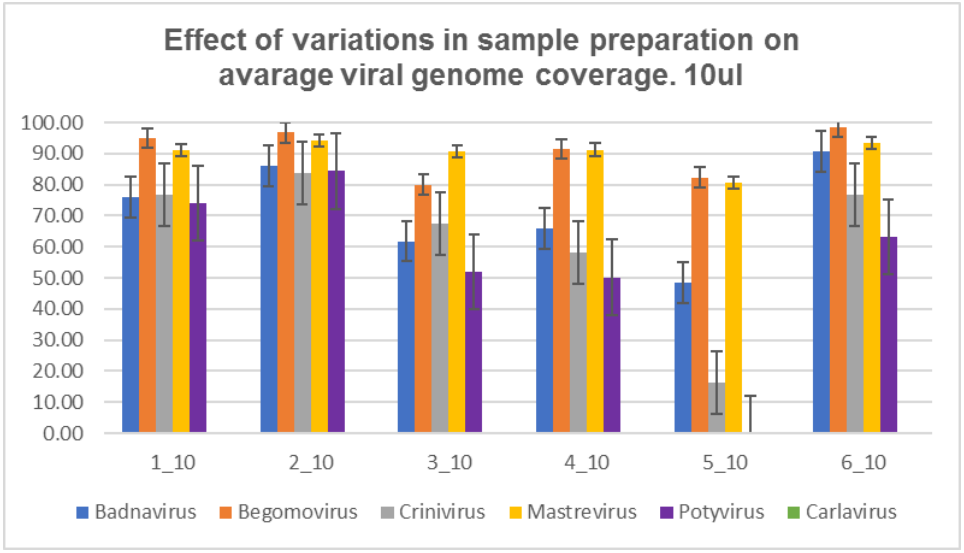


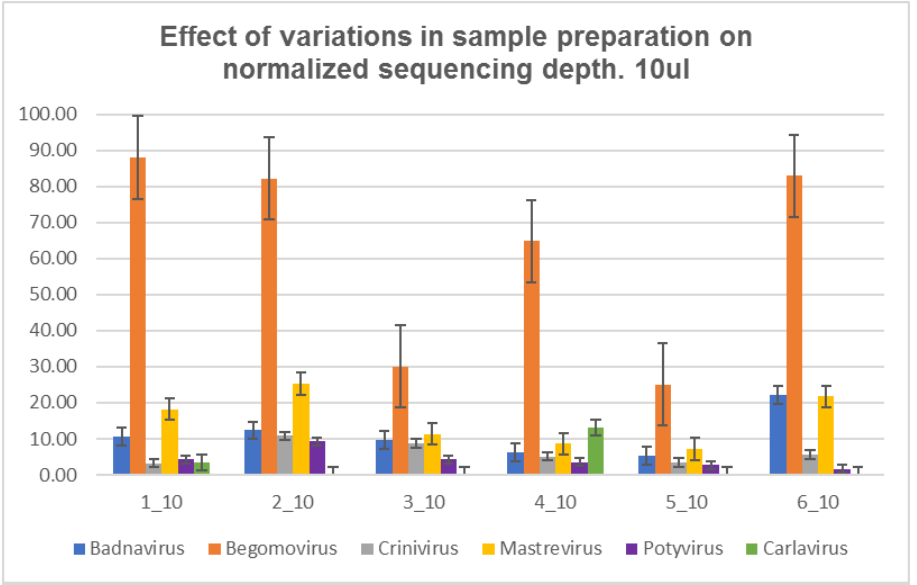


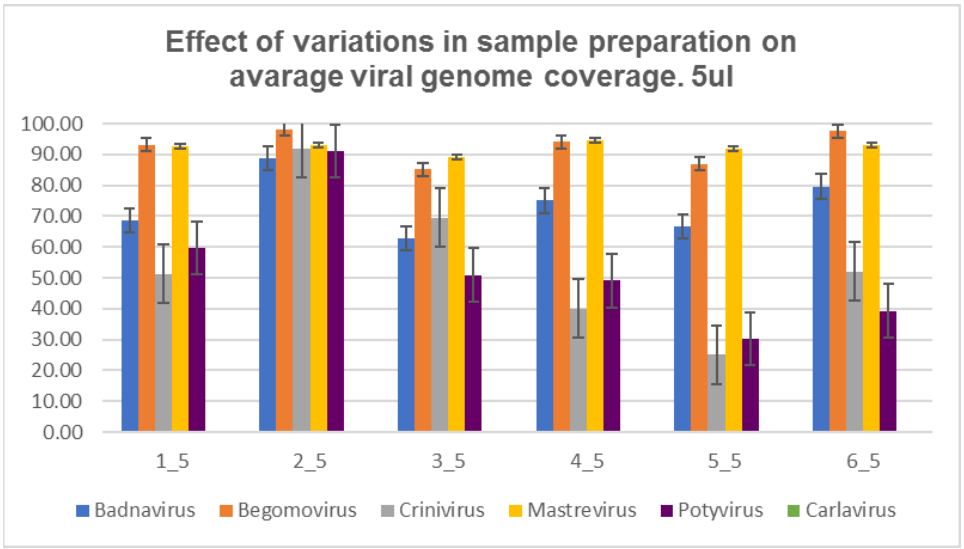












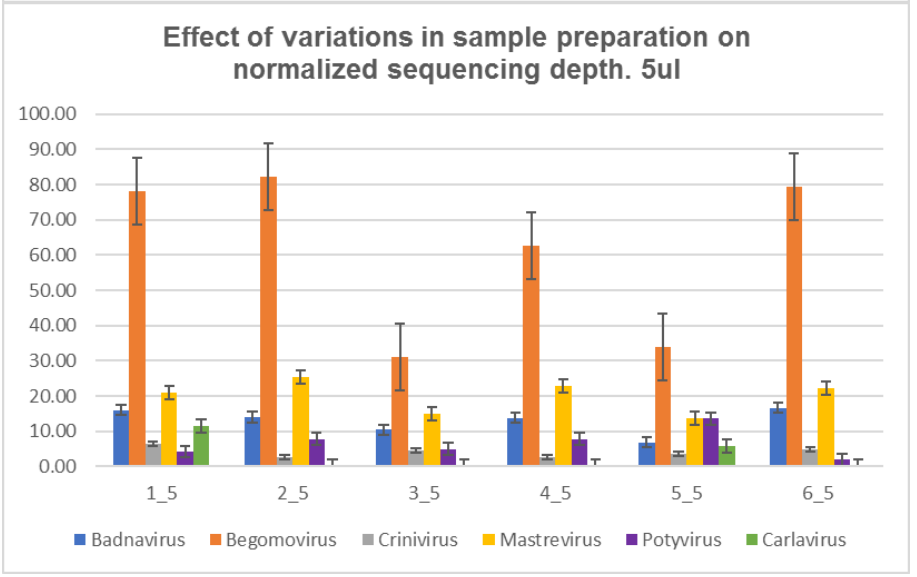
sRNA column no cut

No cut

No cut
No LiCl

No cut

CTAB MLV GoTaq



sRNA column no cut

No cut

No cut
No LiCl

No cut

CTAB MLV GoTaq

Conclusions/suggestions

- Anything works ☺
- It is not worthwhile cutting sRNA from the gel
- M-MLV/protoscript seem to have reduced template transcription compared to superscript
- Columns show best results!
- Analysis of simulated sequencing depth on coverage and depth pending
- Include positive and negative controls in each bulk.
- Will include bulk of 48 samples for ribosomal RNA depleted total RNA to evaluate if siRNA is missing anything.