

Novel molecular diagnostic methods for seed transmitted pathogens of tomato



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- EPPO-TESTA Meeting, Angers, France
- 1 December 2015



WAGENINGEN **UR**
For quality of life

Agricultural products in danger



Current status for control phytosanitary quality of seeds worldwide



Possibilities new detection methods for seed:

- TaqMan PCR
- Multiplex: Luminex
- Next Generation Sequence
- On-site: LAMP
- Live - Dead



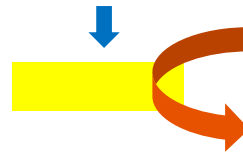
DNA Extraction Experiment



10.000 uninfected tomato seeds

Soaking 16 hr o/n at 4°C in SEB (150 ml)

Addition of Bacteria



Spike in different concentrations:
4 species of Xanthomonas,
1 Pseudomonas,
1 Clavibacter mm

8X

Stomacher step on seed



50 ml Slow Centrifugation



sup

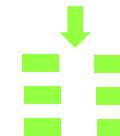
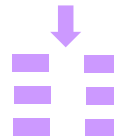
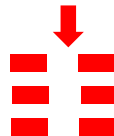
45 ml Fast Centrifugation



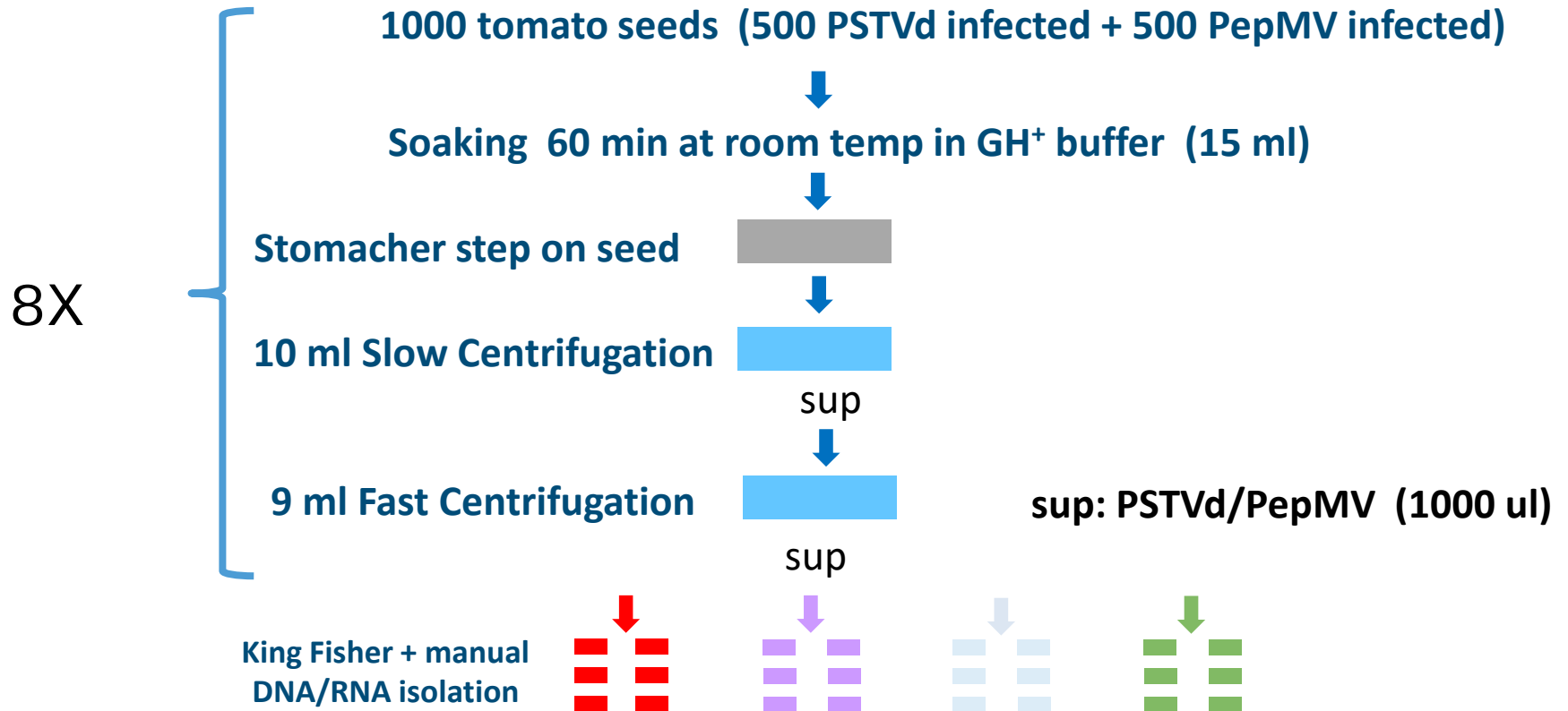
pellet

Bacterial pellet in 1000 ul

3x King Fisher +
manual DNA
isolation



RNA Extraction Experiment



Detection: TaqMan PCR



■ Pathogens:

- Pospiviroids: PSTVd

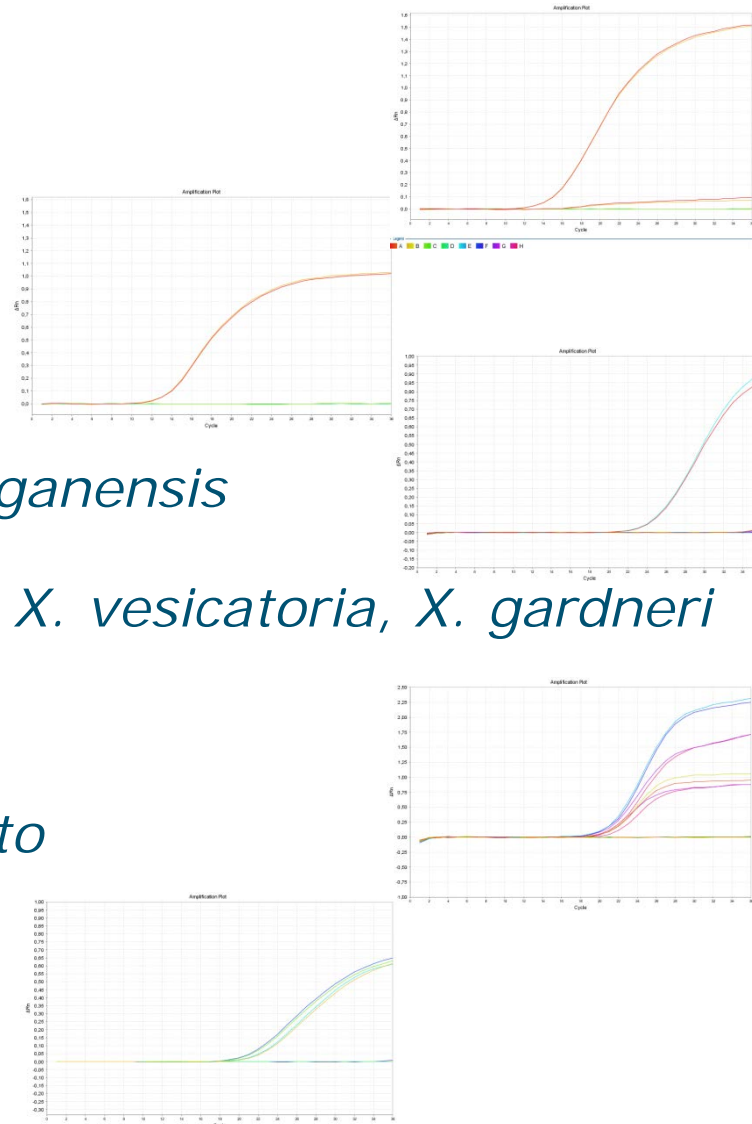
- Viruses: PepMV

- Bacteria:

- *Clavibacter michiganensis michiganensis*

- *Xanthomonas* (*X. euvesicatoria*, *X. vesicatoria*, *X. gardneri*
and *X. perforans*)

- *Pseudomonas syringae* pv *tomato*



Performance characteristics:

- Analytical sensitivity
 - 3 dilution series
- Analytical specificity
 - In TaqMan PCR development
- Selectivity
 - 8 different tomato seed lots, healthy seed lots
- Repeatability
 - Within lab, 8 seed bags, 8 extractions
- Reproducibility
 - Different persons

Reproducibility / Repeatability



Cmm: all positive

Repeatability/reproducibility																
person	First Taqman								Second Taqman							
	x		y		x		y		x		y		x		y	
	bag	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7
1	25.05	25.54	26.92	26.94	26.02	25.38	25.43	25.45	24.12	24.72	25.9	25.92	25.19	24.6	24.87	24.79
2	25.31	26.09	26.03	26.02	25.69	25.05	25.71	25.81	24.4	25.04	25.12	25.48	24.82	24.66	25.19	25.07
3	24.03	24.13	25.62	25.81	25.07	25.21	25.8	24.71	23	23.37	24.81	25.17	24.41	24.32	24.81	24.03
4	23.36	24.11	24.91	25.21	25.04	25.39	25.46	24.11	22.83	23.51	24.34	24.38	24.16	24.37	24.52	23.21
5	23.37	24.14	24.3	24.54	24.44	24.7	25.27	23.97	22.65	23.42	23.83	23.85	23.72	23.95	24.42	23.28
6	22.95	24.1	23.94	24.44	24.77	24.87	25.27	23.59	22.27	23.26	23.63	23.89	23.82	23.68	24.39	22.83
7	22.83	24.01	22.93	24.42	23.68	23.76	25.78	23.66	22.26	22.93	22.42	24.04	22.92	23.12	24.69	22.8
8	23.23	23.27	23.65	22.97	23.21	23.16	23.32	23.38	22.24	22.54	22.91	22.39	22.01	22.37	22.34	22.44

PepMV: all positive

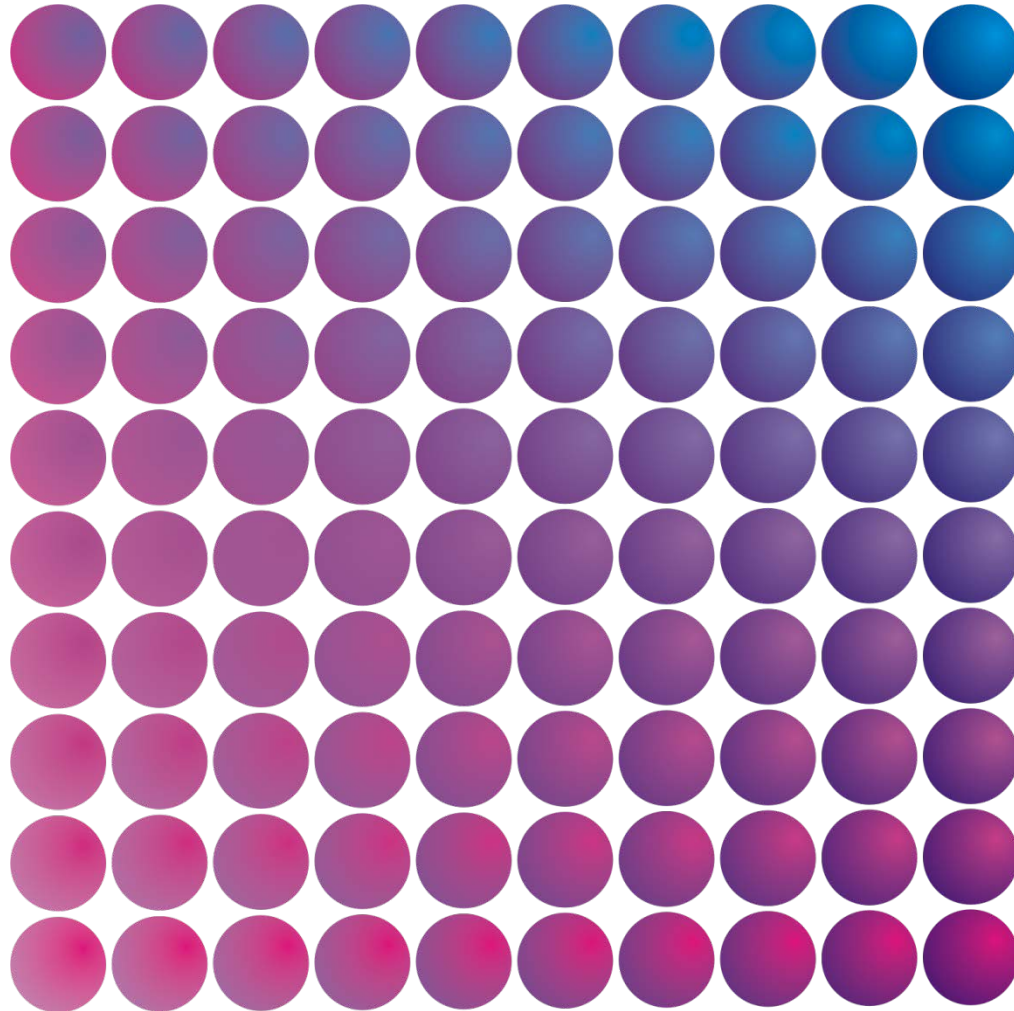
Repeatability/reproducibility																			
person	bag	First Taqman								Quanta	Second Taqman								Quanta
		1	2	3	4	5	6	7	8		1	2	3	4	5	6	7	8	
x	1	19.94	19.88	20.51	20.52	20.6	20.7	20.47	20.45	18.33	19.41	19.64	19.84	20.02	20.06	20.19	19.91	20.03	18.97
x	2	19.57	19.43	19.63	19.6	19.86	20.16	20	19.68	17.8	19.19	18.89	18.96	19.58	19.72	19.5	19.42	19.43	18.63
y	3	20.66	20.34	20.64	20.82	20.94	21.09	21.43	21.16	18.74	20.17	19.81	20.15	20.79	20.64	20.61	20.83	20.59	19.45
y	4	21.12	20.06	20.51	20.55	20.66	21.16	20.94	20.56	18.32	20.35	19.53	19.85	20.33	20.35	20.7	20.5	20.39	19.15
x	5	21.98	21.97	21.98	21.49	23.25	23.76	22.21	22.69	20.2	21.45	21.6	21.4	21.17	22.76	23.25	21.47	22.17	20.94
x	6	20.46	20.64	20.7	21.36	20.86	21.56	21.58	21.11	18.14	19.93	20.19	20.22	21.08	20.55	20.99	20.62	20.7	18.85
y	7	20.13	20.14	20.15	20.29	20.64	20.99	20.95	20.71	18.2	19.5	19.62	19.67	19.77	20.11	20.37	20.29	20.11	18.87
y	8	20.45	20.42	20.42	20.71	20.8	20.86	20.69	20.69	18.12	19.96	19.6	19.73	20.18	20.19	20.31	20.18	20.21	18.76

Validation: Conclusions



- Specificity ✓
- Sensitivity ✓
- Selectivity ✓
- Reproducibility ✓
- Repeatability ✓
- Robustness ✓

Detection Multiplex: Luminex



**100 Color-codes =
100 Simultaneous Tests**

Using this method, over 100 distinct microsphere sets can be created.

**Microspheres in a
Fluid Stream**

Precision fluidics align the microspheres in single file, and pass them through the lasers one at a time.

Multiplex detection: Luminex

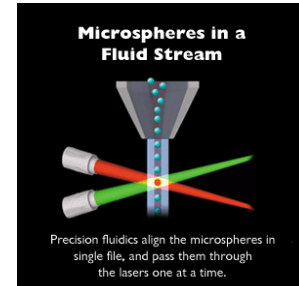
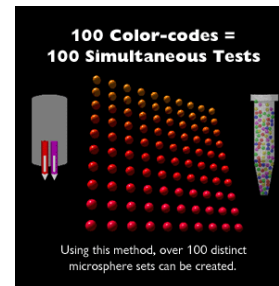
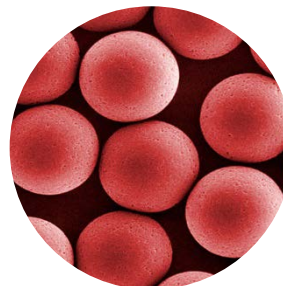
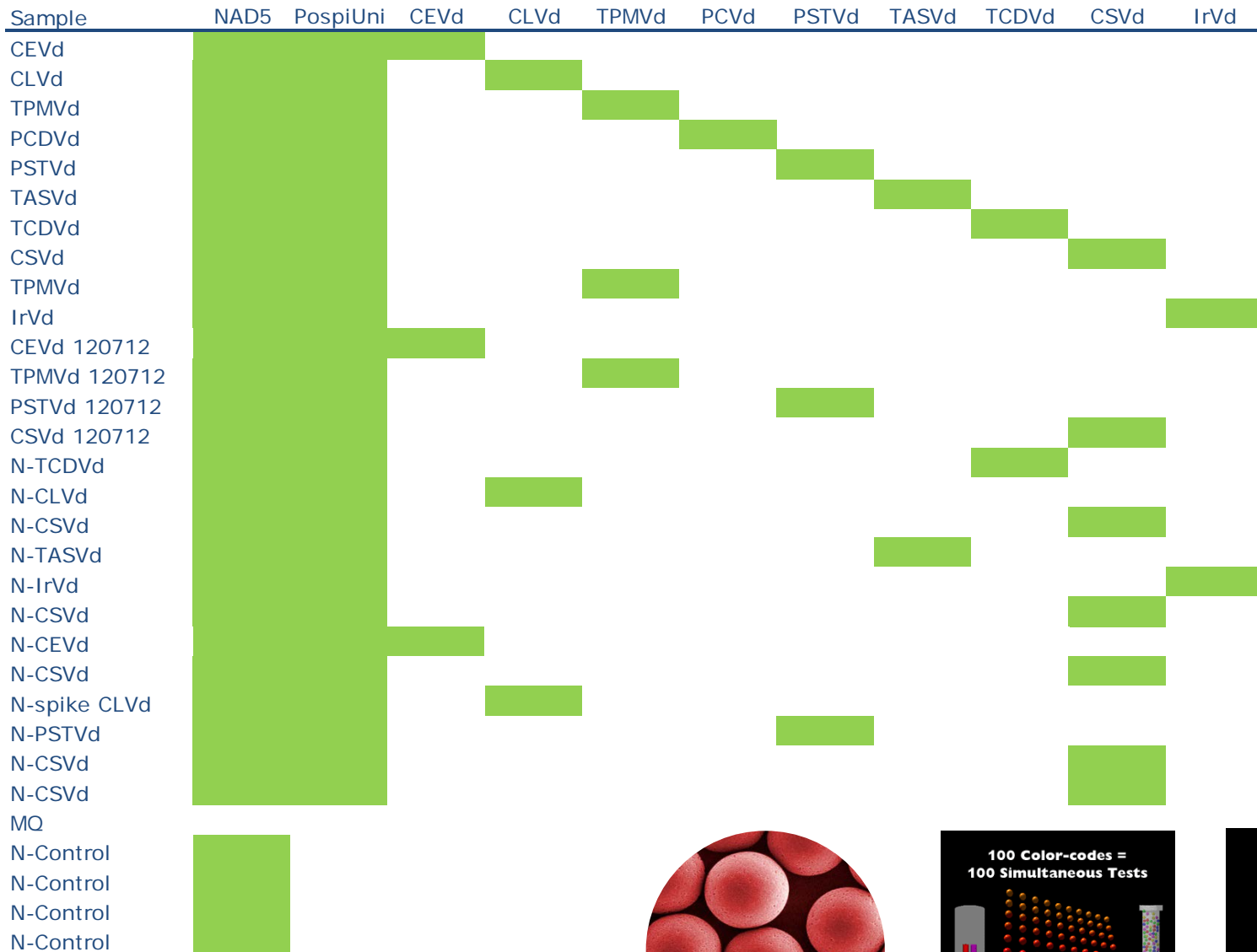


- Pospiviroid assay: PSTVd, CEVd, CLVd, TPMVd, PCVd, TASVd, TCDVd, CSVd, IrVd, general Pospi, NAD5
- Clavibacter spp: Cmm, Cms, Cmt, Cmn, Cmi, LAL, general Cm
- PepMV: several strains

MLGT: Multi Locus Gene Targeting

- Different genes for different targets (SNP's)
- Generic amplification (semi specific)
- Specific detection using Target Specific Primer Extension (TSPE)

Results xTAG: Pospiviroid 11-plex

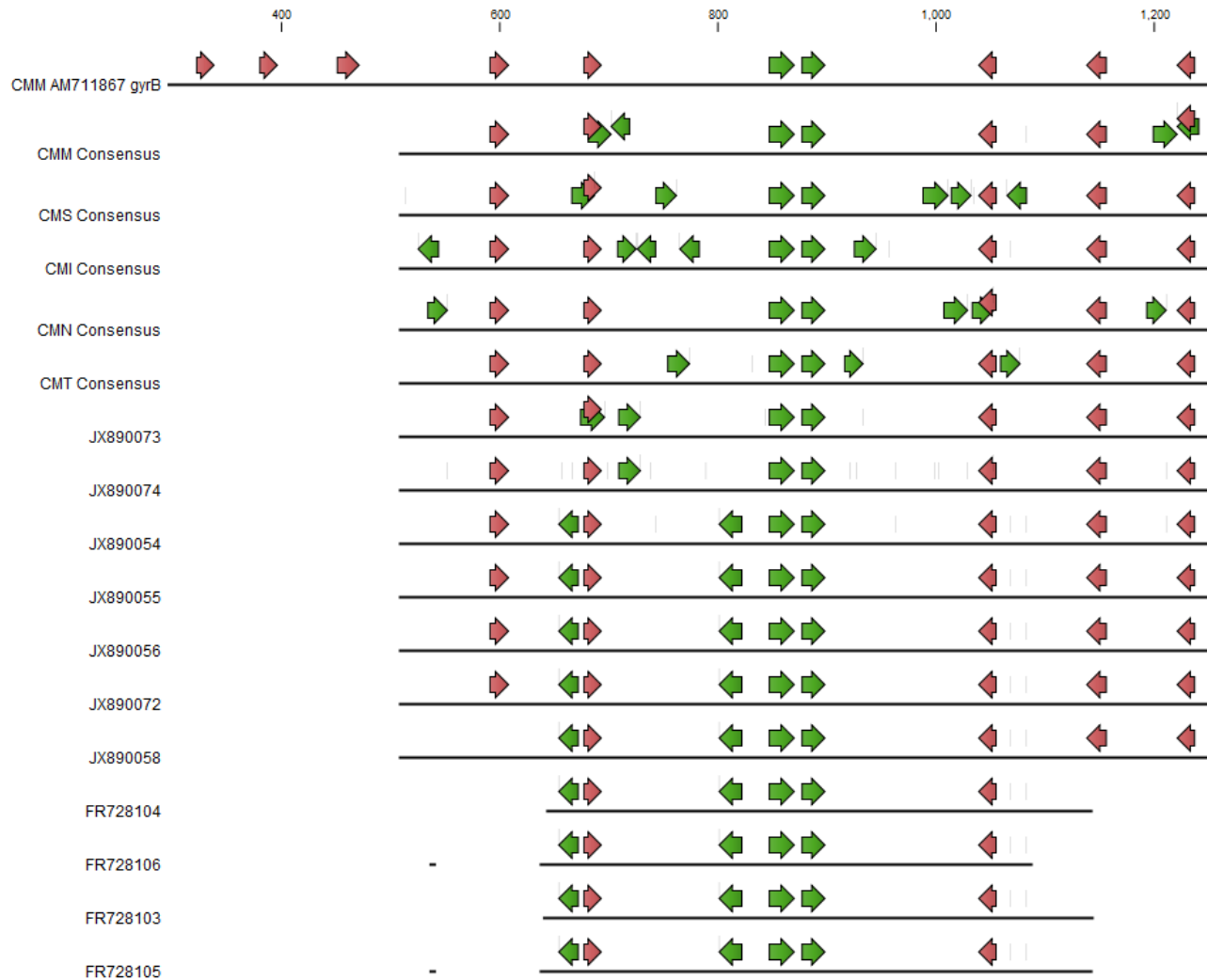


Clavibacter michiganensis subspecies

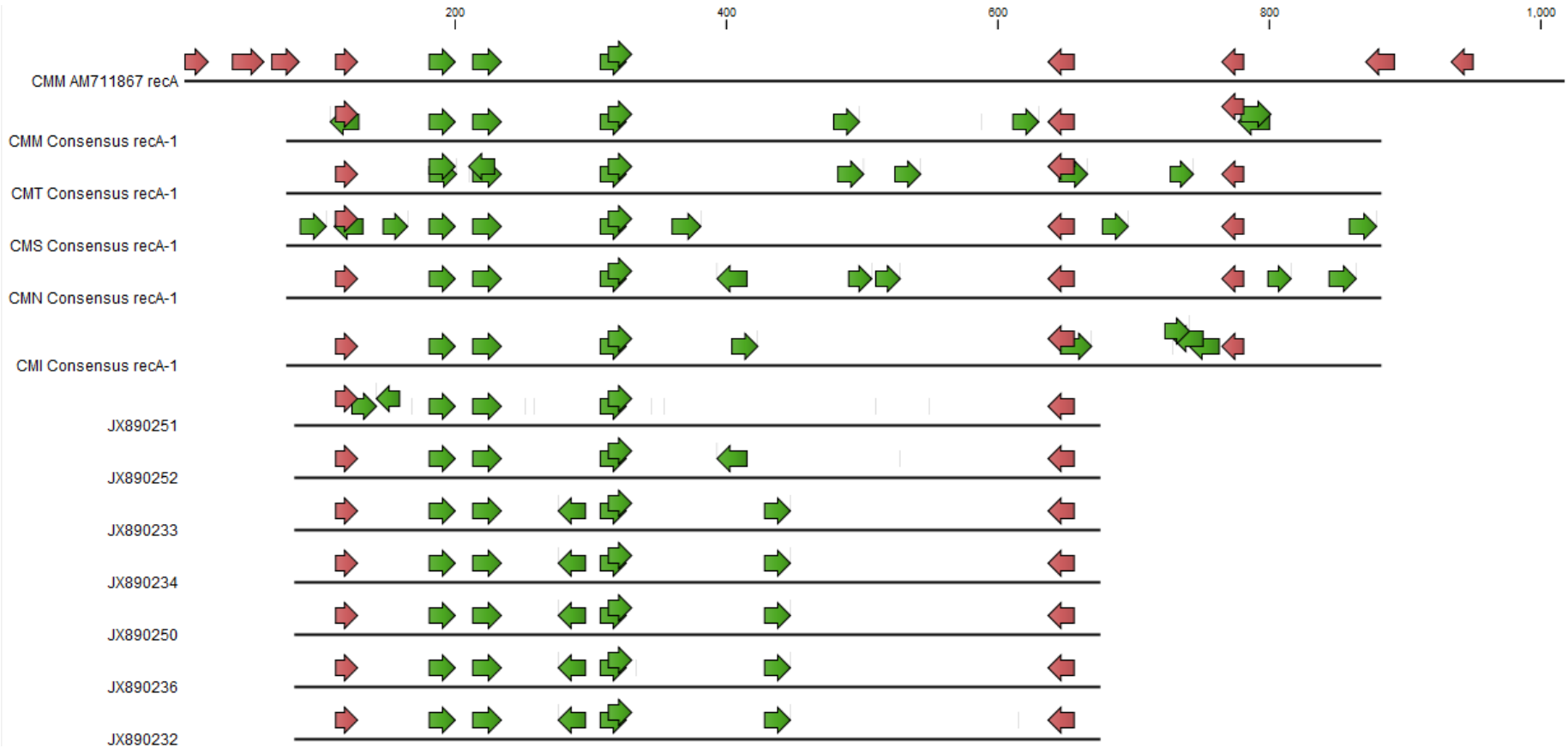
- *Clavibacter michiganensis* subsp. *michiganensis* (**Cmm**)
- *Clavibacter michiganensis* subsp. *insidiosus* (**Cmi**)
- *Clavibacter michiganensis* subsp. *nebraskensis* (**Cmn**)
- *Clavibacter michiganensis* subsp. *sepedonicus* (**Cms**)
- *Clavibacter michiganensis* subsp. *tessellarius* (**Cmt**)
- Non-pathogenic strains:
 - *Clavibacter*-like saprophytes (**SAP**)
 - Cmm look-alikes (**LAL**)



Outer and TSPE primers tested on *gyrB*



Outer and TSPE primers tested on *recA*



Results on *gyrB* and *recA* amplicons

Selection of 7 TSPE primers:

2 generic TSPE primers for all *Clavibacter michiganensis*

5 subspecies specific TSPE primers for:

Cmm, Cms, Cmt, Cmn and Cmi



Detection NGS



454

Illumina HiSeq2000

Pacbio RS

Illumina MiSeq

2008

2011

2012

2014



<900
< 1 Gb/run

100
< 300 Gb/run

>3000
< 500 Mb/run

100
< 8.5 Gb/run

De-novo
Amplicon
Metagenome

De-novo
RNA seq
genome reseq
Metagenome
Epigenome Bisulphite
Chip-Seq

De-novo
Amplicon
Epigenome

genome reseq
Amplicon
Metagenome

NGS: Illumina HiSeq 2500



Longer Reads in Rapid Mode:
Max Read Length
2x125 bp

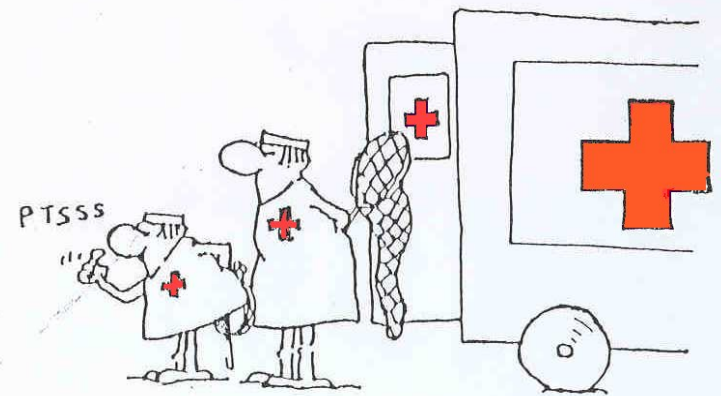
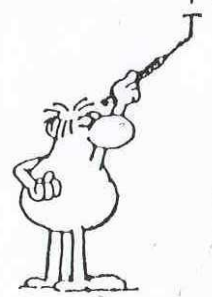
Increased output:
Max Output
1000 Gb

Increased number:
Max Read Number
4000 M

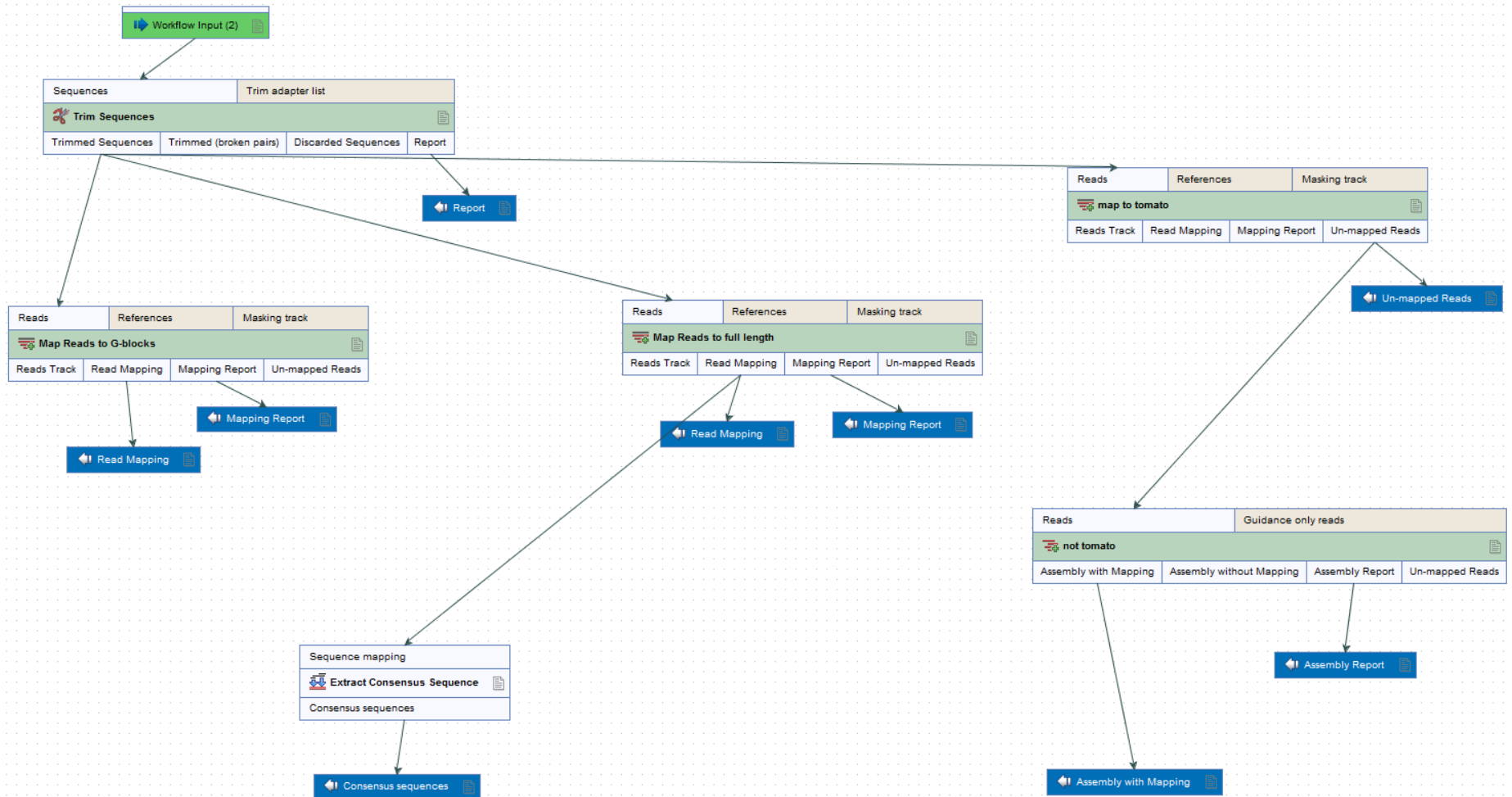


A DNA sequence in itself is useless

C T A T T G A H A L C H T A G C H L H A H A C G T G C H G L A H T A T G G G G G T A H G T A T C A A G G A H A C C G T C A H A H G T G C C C C T T A T
T G T A T C C A C T C C C A C A C C C A C A T C T A G G C G G T G A G C C T T G A A A G G T G T C T C G T A T T G G A C A C T T A G C A C G C T C
T G A T C G A A G G A G T T T A G C C C G A T T T A G C T T G G C C T T C G G T T A A T A G T A A T C T C T T A T A A G C A A G C C A G A G G
C G T G G C A C G G C T G A G A T C C G A T G T G A T C C A A T T C C A T G C T T T T A A G G T T G T T A C T C C T T A G T A G A G T G G G A G
G C G C A A C C A A C G A A A C A C T C G G A A C A A G T T C A T C G T T T T G T C A C G G A T A G C C C T A G A G T A G T C A G C G A T A C
C T T T A C G C T T G A G G G T G T A T A A G G T C T A C A C T T G C G C A T G T C T C T C C T T T T A T C C A A C A C A C A C T T C G T T G
A A C A G G C C T C A C C G A T G C T A C G C G A T G G T G A G T T T T T C C A A A G C G A C T T C G C A A A C A C T G G C G G C G T G T T A
T G G G T C T C A T T A G T G A T G A A T G C T T C C A C G C A T G T T C A A G G T C C T G G C T T C A C C G A T G A G T G T C C G G C G T A C
G G T C C T A C C A C C G A T A C C A G T T A C A T T C A T G G C A C G T T C C A C G G A G C C G C A A C A G A A G G T G A C C G A A G C A A
C A A A C G C A T G T C T T T G T C C G A C A C A C C T A C G T T C A G C G T G C C G T T T A A C A A T A G C C T C G C G T T G C C G T G
C G T T G C G G G G A A G G A A C T G C G C G G C C T C T T T C C A A C T T A T A C C T T T G T T C C G C C A G A G A G G A T T T G A G
T A G T A G T T C T C C C A T A T G C G T A C C G A A A G G C A A T A T T A A A A T A G T T C C G C T C A G T A G G T C G T T G A T
C T T A T G A C T T C T T C C G C G T C T G C C G A T C A T A T A G G T T T C C G T A C C G C A T A A G A C A T G C G C T T T
A G A G G G G T C A A T A C G G C A C C G T A A A G G G C T G C G T G A C G T T C C C C B T T C G G C T A A A T G
T C A T C G G G G A A G A C G T C C G A C G T G G T G G A T C T C A C C T C T A C C A C C T A G
A G G C A T C T T G A A T T C G G G G G C C T A A T A G C T
G G C C G C T G G T C A C C A T T T A
T G T A C C G A T G A C C
A T A C G A G
T G A



DNA extraction – CLC pipeline



Annette Dullemans, Wednesday 2 December, 9.40 hrs

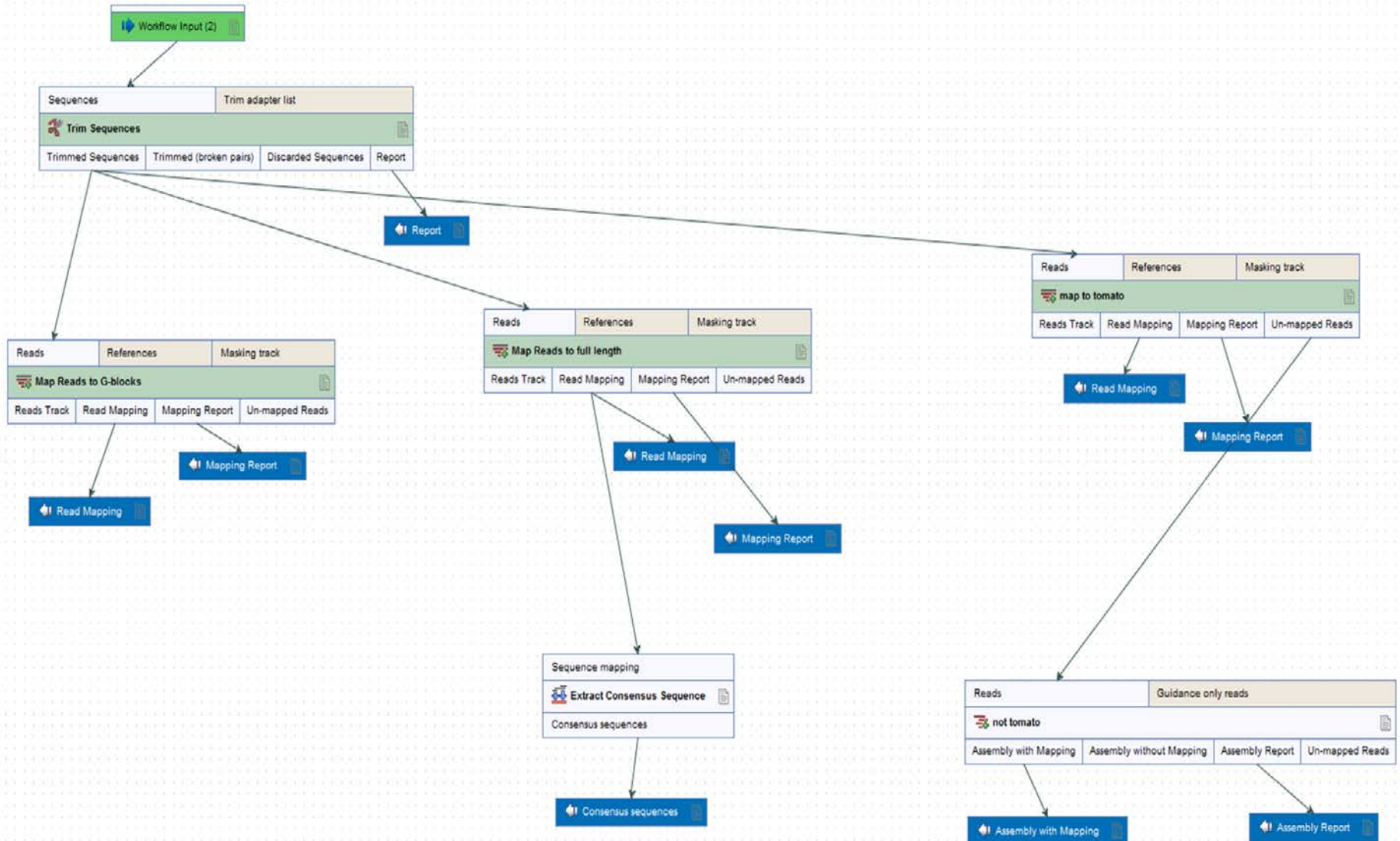
DNA extraction - results

All spiked bacteria could be detected:

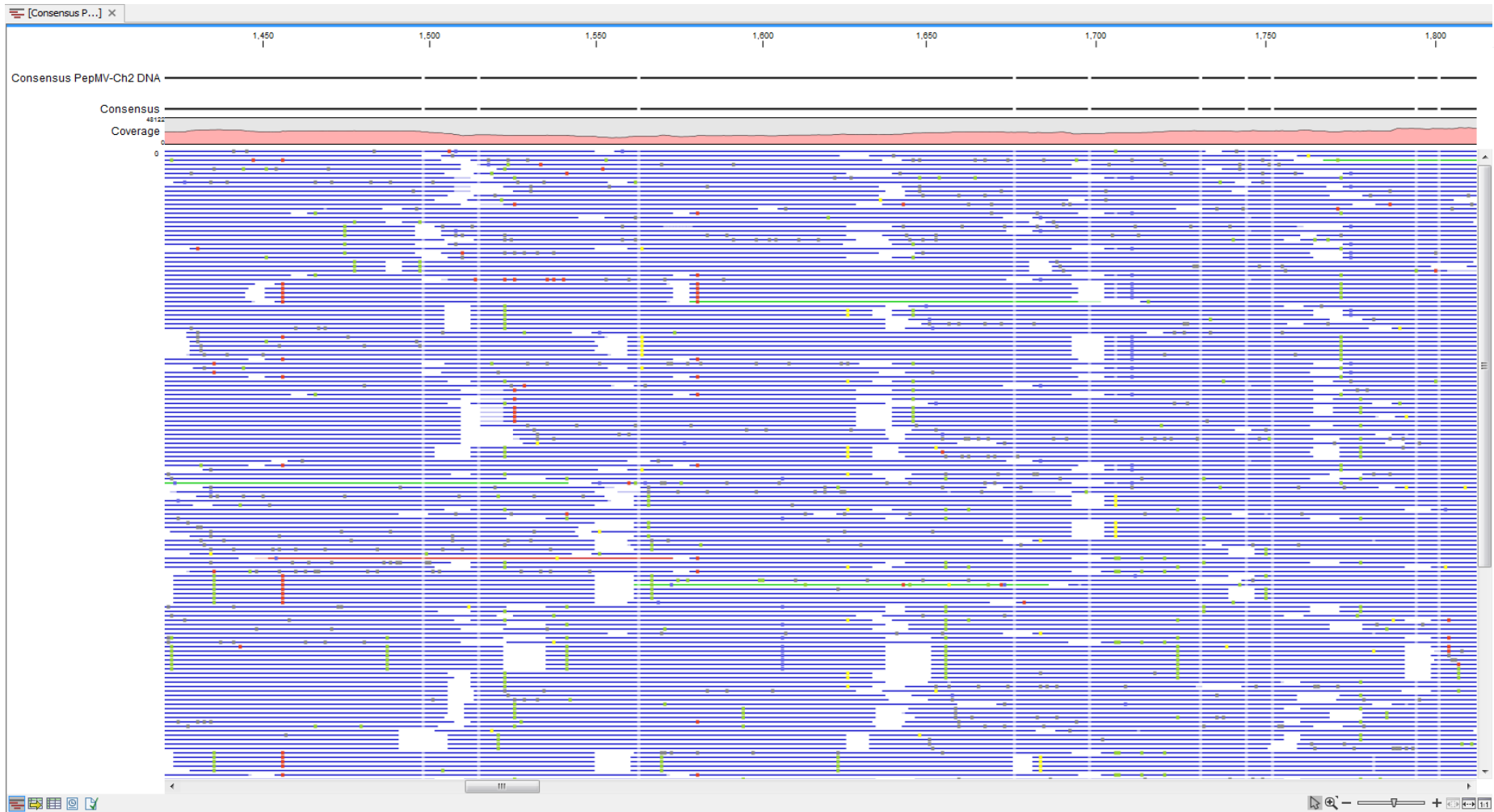
- *Clavibacter michiganensis michiganensis*
- *Xanthomonas euvesicatoria*
- *Xanthomonas vesicatoria*
- *Xanthomonas gardneri*
- *Xanthomonas perforans*
- *Pseudomonas syringae pv tomato*



RNA extraction – CLC pipeline



NGS RNA analysis shows PepMV-Chili 2 strain- High coverage (24766x)



RNA extraction - results

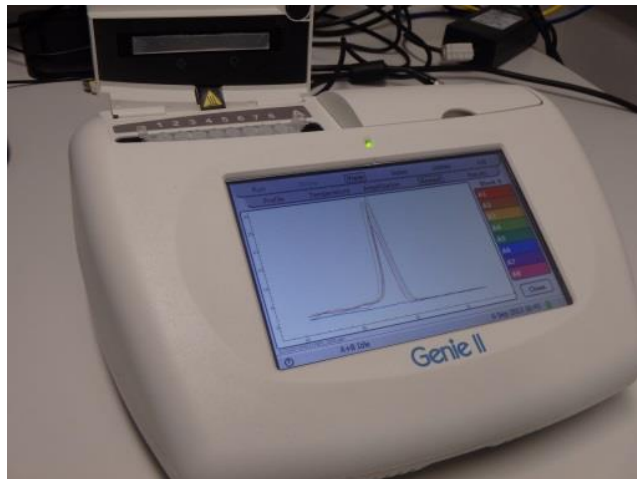
- PepMV positive
- PSTVd positive



On-site detection: LAMP Cmm



- 3 seed lots
- 3 subsamples (1500 seeds/treatment)
- Spiked with 5 contaminated seeds (highly and slightly contaminated with Cmm)
- Seeds were incubated on “seed extract buffer”, blended using a stomacher machine, afterwards the seed extract was analysed at 0 and 3 days after incubation at 25°C.
- LAMP reaction



LAMP reaction was positive with both slightly and highly contaminated seeds

Conclusions



- TaqMan PCR for individual pathogens
- Multiplex detection: Luminex for antibodies and nucleic acids
- On-site detection: LAMP
- Live-dead: Luminex, TaqMan PCR, PMA PCR
- Next Gen Sequencing: known and unknown targets
- Reference material from reference collections
- Reference data in reference database (Q-bank: www.q-bank.eu)

Acknowledgements



- Wageningen UR
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 - Marjon Krijger
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 - Maaïke Bruinsma
 - Harrie Koenraad
- Suppliers of seed:
 - Bejo Seeds
 - Enza Seeds
 - Bayer / Nunhems
 - Rijk Zwaan
 - Fera
- EU funding: TESTA project
- NL funding: PPS Diagnostics