

## **SharCo**

### **Containment of Sharka virus in view of EU-expansion**

Small Collaborative project of the 7<sup>th</sup> Framework Programme

Theme 2

Food, Agriculture, Biotechnologies

### **DE.1.4**

#### **Second generation arrays for genome-wide analysis of PPV diversity**

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Concerned workpackage leader	Miroslav Glasa
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## Deliverable report structure

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# 1. General Presentation

## 1.1. Context

SharCo project aims to develop strategies and current tools for *Plum pox virus* containment in the European Union. For these reasons multidisciplinary approaches organized in several work packages were planned. WPE1 entitled “Large scale analysis of *Plum pox virus* current diversity worldwide”, includes different tasks and objectives. In the first SharCo meeting, Task TE 1.1 was divided in two sub-tasks, the development of a Snplex approach, led by Partner 1, being a very promising strategy for characterization of point mutations, and the development of conventional microarrays, led by Partner 6. The microarray development was divided in two phases, the construction of a first generation of oligo-arrays and the construction of a second one, taking into account the experience gained in the first phase and sequencing results obtained in other tasks of WPE1. The first oligo-array generation was successfully assayed and demonstrated to be able to characterize inter-strain variability among PPV isolates. Here we report the delivery of the second generation of the chip. It has been successfully assayed, and shown to be able to discriminate between known PPV isolates. The main drawback of the method is the impossibility to find suitable probes to unambiguously discriminate very small nucleotide changes, due to stringency and specificity problems. In this regard the Snplex would be expected to cover these deficiencies, since it is able to detect single point mutations. The recent improvements in the sequencing methods allowed us to design a novel alternative approach, based on deep sequencing of small RNAs, which was already raised in our report of Milestone ML2, and has been successfully assayed now. Here, we describe the construction of the second generation of oligo-arrays and the first results of the deep sequencing approach, which is able to characterize not only intra-strain variability but also intra-isolate variability. The results of this SharCo task represent a dramatic improvement in the tools available for the study of the natural variability of viruses, including *Plum pox virus*.

## 1.2. Rational

Serological and molecular tests available for typing of *Plum pox virus* to date were not able to perform a genome-wide survey of PPV diversity. The identification of virus strains and characterization of isolates are necessary for the control of viral spread, mainly if different strains or isolates have different biological aggressiveness. Within this deliverable, this objective has been achieved through



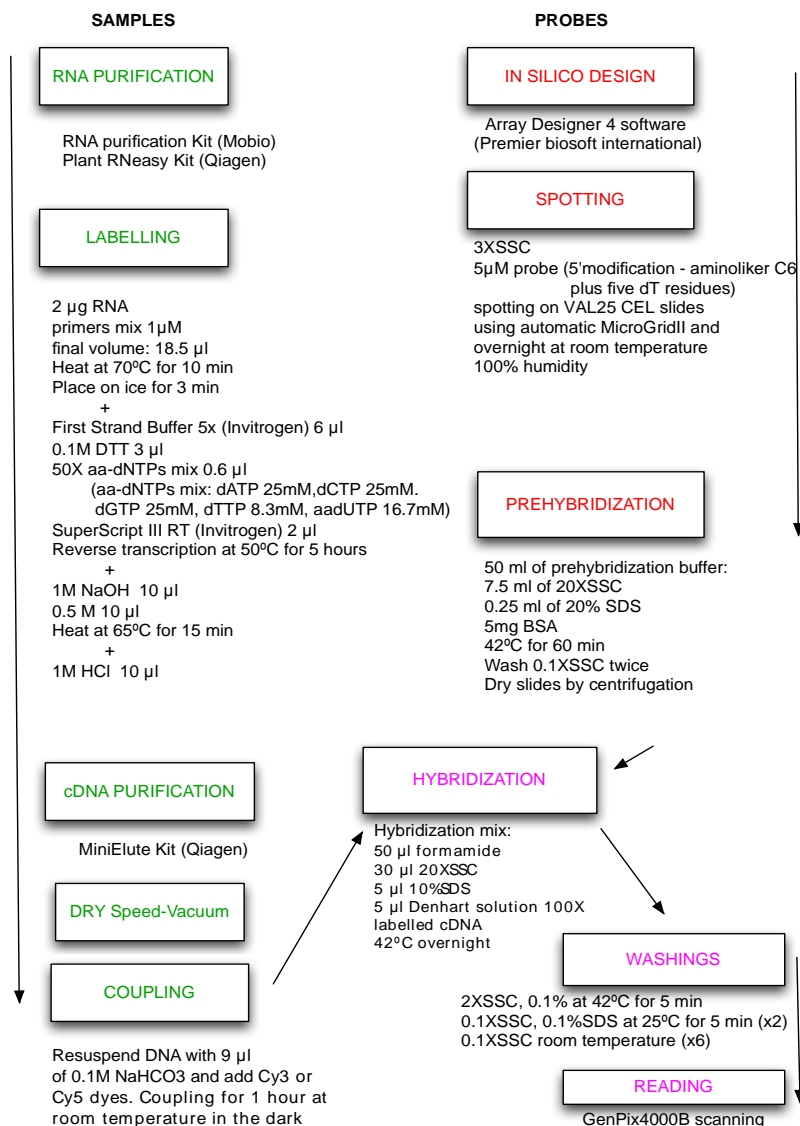
the development of PPV isolate high throughput typing tools which will be useful for early warning systems as well as for studies focusing on the understanding of PPV outbreaks and epidemiology.

## 2. Detailed description

### DESIGNING

#### A) Second Generation of OligoChip

Figure 1 summarizes the protocol developed for the design of the oligo probes and their spotting in the chip slides, as well as the labelling of viral cDNA and its hybridization to the oligo arrays. The universal and strain-specific primers used for RT-PCR amplification and labelling of PPV cDNA are listed in Table 1. Oligo probes plotted in the second generation PPV chips are listed in Table 2.



**Figure 1. Construction and use of second generation PPV chips**

**Table 1.- PPV-strain and PPV universal primers used for reverse transcription**

Primer name	Nucleotide sequence 5' – 3'
1	CTGTGTCCTCTTCTTGTTCC
2	TTCAATATACGCTTCAGCCACG
3	AAGAGCCATTCCACACTCCTT
4	GGTGTCTGTCTGCCTCAAT
5	CCCATCCATCATCACCCACA
6	CGAGGAATGGAGTGGATGTCT
7	TCTCCGATCAAGTCCTCTGC
8	GACTACAACCTGACCTTCTCCA
9	ATGAACAACAACCTGACCATCC
10	GACTCACGCTTGTTCTGATGAA
11	GGCTTCTATGTAGTCTTGTCG
12	AAGTTGTAGTATGCCTCGGA
13	Oligo-dT

**Table 2.- Oligoprobes for the second generation of the chip**

Probes	Nucleotide sequence 5' – 3'
1	NH2-CCCCCCCCCCCCCTTTTTATCTTGTGTTGGACGAGGCA
2	NH2-CCCCCCCCCCCCCTTTTTGACGAGGCAACCAAGAAGATT
3	NH2-CCCCCCCCCCCCCTTTTTAGCACATCTGACCTGGACG
4	NH2-CCCCCCCCCCCCCTTTTTAGGAGTGTGGTGTGTCGC
5	NH2-CCCCCCCCCCCCCTTTTTGAGTGTGGTTATGTCGCAGC
6	NH2-CCCCCCCCCCCCCTTTTTATCGCACGGAGAACATTAGGA
7	NH2-CCCCCCCCCCCCCTTTTTATCGCACGGAGAACATTAGGA
8	NH2-CCCCCCCCCCCCCTTTTTTGCAACCTTGTTATGTCAAC
9	NH2-CCCCCCCCCCCCCTTTTTGAAGACGGTACACCACTGG
10	NH2-CCCCCCCCCCCCCTTTTTACTCATTGGAACACTGTCAAC
11	NH2-CCCCCCCCCCCCCTTTTTTCGGATCACTGTCAACTGGA
12	NH2-CCCCCCCCCCCCCTTTTTAAGCGAACACAATCAATCAGC
13	NH2-CCCCCCCCCCCCCTTTTTAGCGAACACAATCAACCAGC
14	NH2-CCCCCCCCCCCCCTTTTTGGTTGGAGTTCTGAAGTGG
15	NH2-CCCCCCCCCCCCCTTTTTCGTTGGCAGCGATCACATC
16	NH2-CCCCCCCCCCCCCTTTTTGCCAAAGTTTACCCGCAA
17	NH2-CCCCCCCCCCCCCTTTTTCACTGGCAAGTACACTGAATG
18	NH2-CCCCCCCCCCCCCTTTTTGCAGATTTAGGAGGCGGGTA
19	NH2-CCCCCCCCCCCCCTTTTTGTGGCTCTTGTTATGATGCTGT
20	NH2-CCCCCCCCCCCCCTTTTTGGCTCACATCAAGACATCCT
21	NH2-CCCCCCCCCCCCCTTTTTGCGAGGTCAACCATTCAAC
22	NH2-CCCCCCCCCCCCCTTTTTCGAGGTCAACCATTCAATGTCA
23	NH2-CCCCCCCCCCCCCTTTTTACTGTGATGACAAGCGGTTAC
24	NH2-CCCCCCCCCCCCCTTTTTACTGTGATGACAAGCGGTTAC
25	NH2-CCCCCCCCCCCCCTTTTTAATCGGCAATGTTGAGATACCA
26	NH2-CCCCCCCCCCCCCTTTTTCACTCAGGCTAAACCGCATT
27	NH2-CCCCCCCCCCCCCTTTTTCAGGCTAAACCGCATTTCGTA
28	NH2-CCCCCCCCCCCCCTTTTTCGATACACGAAGAAGAGCATCA
29	NH2-CCCCCCCCCCCCCTTTTTCTAACGGTGTGTAACGAGC
30	NH2-CCCCCCCCCCCCCTTTTTGTCAACGAGCCTACTAGCGA
31	NH2-CCCCCCCCCCCCCTTTTTACTGTCAAGCAAGCACGGA
32	NH2-CCCCCCCCCCCCCTTTTTTCTACACAGTGCCGTTGGT
33	NH2-CCCCCCCCCCCCCTTTTTGCGTGACTCAGAGGTAATCTT
34	NH2-CCCCCCCCCCCCCTTTTTGCGTGACTCAGAGGTAATCTT

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35	NH2-CCCCCCCCCCCCCTTTTTGGACTTGGATGAGAACATTCG
36	NH2-CCCCCCCCCCCCCTTTTTAGAGAATATGGCAAGCGGTAGA
37	NH2-CCCCCCCCCCCCCTTTTTGACGCACTGTTGGAGCA
38	NH2-CCCCCCCCCCCCCTTTTTGCCAAAGACCATACGGAAGAAA
39	NH2-CCCCCCCCCCCCCTTTTTAATGCTTGGAGTCTTGGTGG
40	NH2-CCCCCCCCCCCCCTTTTTGTTACCAAGGCTTCAATCGT
41	NH2-CCCCCCCCCCCCCTTTTTTCAGGCAAGCACGAGATAAC
42	NH2-CCCCCCCCCCCCCTTTTTGGTGACGATTCAACTATGGAGG
43	NH2-CCCCCCCCCCCCCTTTTTCAGAAAGCCCTCAAAGTGGAC
44	NH2-CCCCCCCCCCCCCTTTTTCCGACGACTGAGGACAGG
45	NH2-CCCCCCCCCCCCCTTTTTCGACGACTGAGGACAGAGTT
46	NH2-CCCCCCCCCCCCCTTTTTGGATTAGCACGAAGGATGGT
47	NH2-CCCCCCCCCCCCCTTTTTAAGCAGTGGCGATAACAAC
48	NH2-CCCCCCCCCCCCCTTTTTAACTGCTAACGGATCTTGATGG
49	NH2-CCCCCCCCCCCCCTTTTTAACCTACCTGTTGACTCATCCA
50	NH2-CCCCCCCCCCCCCTTTTTATGGAGCGTTGGGATGGC
51	NH2-CCCCCCCCCCCCCTTTTTAGAGCACTGCCAGAAGGATG
52	NH2-CCCCCCCCCCCCCTTTTTGAGAAGACGAGGAGGAAGTTGA
53	NH2-CCCCCCCCCCCCCTTTTTAGGACGAGGAGGAAGTTGATG
54	NH2-CCCCCCCCCCCCCTTTTTGCCAGGACCTCAACTGC
55	NH2-CCCCCCCCCCCCCTTTTTCGTAGTCAACACGAACAGAG
56	NH2-CCCCCCCCCCCCCTTTTTCTCTGCCAAAGGTGAAGGG
57	NH2-CCCCCCCCCCCCCTTTTTCTCTGCCAAAGGTGAAGGG
58	NH2-CCCCCCCCCCCCCTTTTTAAGCGAGATTATGATGTCACGG
59	NH2-CCCCCCCCCCCCCTTTTTAAGCGAGATTATGATGTCACGG
60	NH2-CCCCCCCCCCCCCTTTTTAGTGGTCTCGGTATCTATCGTA
61	NH2-CCCCCCCCCCCCCTTTTTCTGGTGAGAGTCTAATCATCC
62	NH2-CCCCCCCCCCCCCTTTTTGCAAGTCAAGATGTCAACCATT
63	NH2-CCCCCCCCCCCCCTTTTTCAATAATCGCAACAGAAGCAGC
64	NH2-CCCCCCCCCCCCCTTTTTTGAAAGGGCTGGAAGAGAA
65	NH2-CCCCCCCCCCCCCTTTTTCGTAAGCGTGTAGTCGGTAAC
66	NH2-CCCCCCCCCCCCCTTTTTAACACCAGGAATGAGCGGAT
67	NH2-CCCCCCCCCCCCCTTTTTGTTGACTCACGGTGTAAAGGT
68	NH2-CCCCCCCCCCCCCTTTTTGGAACCAAGCCATACGCATC
69	NH2-CCCCCCCCCCCCCTTTTTCGGTACTCAGACAGGCAAC
70	NH2-CCCCCCCCCCCCCTTTTTATCGTGTTCCGGTCTCTTGCT
71	NH2-CCCCCCCCCCCCCTTTTTCGACCAGGCTGTTTCATCATC
72	NH2-CCCCCCCCCCCCCTTTTTACAGAAGGAGGTGAAGGTCATT
73	NH2-CCCCCCCCCCCCCTTTTTCTCCAATCTCGGCACAGGT
74	NH2-CCCCCCCCCCCCCTTTTTGAGCAGCACTTCGATTGGATT
75	NH2-CCCCCCCCCCCCCTTTTTAGTCAATCCTGCTCAAGTTCAA
76	NH2-CCCCCCCCCCCCCTTTTTATGGCGAAGTCTCAGTTGCT
77	NH2-CCCCCCCCCCCCCTTTTTCTTATGGACATCAACTTGGTGC
78	NH2-CCCCCCCCCCCCCTTTTTACTGGATTAGCACGAAGGA
79	NH2-CCCCCCCCCCCCCTTTTTAACTACACATTGGCTCAGAGAT
80	NH2-CCCCCCCCCCCCCTTTTTGCTACACCAGATGGCACTATTG
81	NH2-CCCCCCCCCCCCCTTTTTCAAGGGCAACAATAGTGGTC
82	NH2-CCCCCCCCCCCCCTTTTTGCGGAGACAGCACTGAAGA
83	NH2-CCCCCCCCCCCCCTTTTTCACTGACACTGAAGCATCTGAG
84	NH2-CCCCCCCCCCCCCTTTTTACGACGACATTAACGATGATGG
85	NH2-CCCCCCCCCCCCCTTTTTGCAAGCCGATTGTAGTTACTG
86	NH2-CCCCCCCCCCCCCTTTTTTCAACCACCTCCAGTCATACA
87	NH2-CCCCCCCCCCCCCTTTTTCAGCAACAACCTCAACCAGCA
88	NH2-CCCCCCCCCCCCCTTTTTCGTGGTGATGTTAATCGCA
89	NH2-CCCCCCCCCCCCCTTTTTCCGAGGCATCACTACAACCT
90	NH2-CCCCCCCCCCCCCTTTTTGTCCAGAGAACCGCCAAGT
66	NH2-CCCCCCCCCCCCCTTTTTAACACCAGGAATGAGCGGAT
67	NH2-CCCCCCCCCCCCCTTTTTGTTGACTCACGGTGTAAAGGT
68	NH2-CCCCCCCCCCCCCTTTTTGGAACCAAGCCATACGCATC
69	NH2-CCCCCCCCCCCCCTTTTTCGGTACTCAGACAGGCAAC
70	NH2-CCCCCCCCCCCCCTTTTTATCGTGTTCCGGTCTCTTGCT
71	NH2-CCCCCCCCCCCCCTTTTTCGACCAGGCTGTTTCATCATC
72	NH2-CCCCCCCCCCCCCTTTTTACAGAAGGAGGTGAAGGTCATT
73	NH2-CCCCCCCCCCCCCTTTTTCTCCAATCTCGGCACAGGT



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74	NH2-CCCCCCCCCCCCCTTTTTGAGCAGCACTTCGTATTGGATT
75	NH2-CCCCCCCCCCCCCTTTTTAGTCAATCCTGCTCAAGTTCAA
76	NH2-CCCCCCCCCCCCCTTTTTATGGCGAAGTCTCAGTTGCT
77	NH2-CCCCCCCCCCCCCTTTTTCTTATGGACATCAACTTGGTGC
78	NH2-CCCCCCCCCCCCCTTTTTACTGATTAGCACGAAGGA
79	NH2-CCCCCCCCCCCCCTTTTTAACTACACATTGGCTCAGAGAT
80	NH2-CCCCCCCCCCCCCTTTTTGCTACACCAGATGGCACTATTG
81	NH2-CCCCCCCCCCCCCTTTTTTCAAGGGCAACAATAGTGGTC
82	NH2-CCCCCCCCCCCCCTTTTTGCGGAGACAGCACTGAAGA
83	NH2-CCCCCCCCCCCCCTTTTTCACTGACTGAAGCATCTGAG
84	NH2-CCCCCCCCCCCCCTTTTTACGACGACATTAACGATGATGG
85	NH2-CCCCCCCCCCCCCTTTTTGCAAGCCGATTGTAGTTACTG
86	NH2-CCCCCCCCCCCCCTTTTTCAACCACCTCCAGTCATACA
87	NH2-CCCCCCCCCCCCCTTTTTCAGCAACAACCTCAACCAGCA
88	NH2-CCCCCCCCCCCCCTTTTTCGCTGGTGATGTTAATCGCA
89	NH2-CCCCCCCCCCCCCTTTTTCCGAGGCATCACTACAACCTT
90	NH2-CCCCCCCCCCCCCTTTTTGTCCAGAGAACCGCCAAGT

B) Deep sequencing of PPV isolates based on Illumina Technology

Illumina's sequencing by synthesis technology is one of the most successful and widely-adopted new-generation sequencing platform worldwide. In fact new-generation sequencing is fastly evolving and has provided a tremendous great output for this deliverable. Query millions of small RNA sequences have been analyzed. The critical step resulted in the purification of total RNA.

Purification of total RNA was performed as follows:

1. Grind flash-frozen tissues in liquid nitrogen using mortar and pestle
2. Transfer approximately 200 mg of frozen tissue powder directly into 1 ml of ice, immediately vortex for 20 seconds and shake for 5 min. at room temperature (RT).
3. Centrifuge at 21,000 x g for 2 min. Transfer the supernatant to a new tube on ice.
4. Add 200 µl of cold 5M NaCl and centrifuge at 21,000g for 2 min.
5. Transfer the supernatant to new tube, add 500 µl of chloroform and mix by inverting. Centrifuge at 21,000 x g for 2 min. and transfer the aqueous/top layer to a pre-chilled 2 ml tube. Repeat the chloroform extractions 2-3 times until the aqueous phase is clear.
6. After the final chloroform extraction step, transfer the aqueous layer to a pre-chilled tube, add 0.8 volumes of 2-propanol and precipitate RNA for 10 min. at RT.
7. Centrifuge at 21,000 x g for 10 min., remove supernatant and wash RNA pellet with cold 80% ethanol.
8. Air-dry the pellet for approximately 5 min. and re-suspend RNA in 200 µl, store at -80°C.

Total RNA was sent to a specialized company to prepare and sequence a cDNA library of small RNAs Millions of reads were obtained, which were analyzed using several softwares such as Geneious and CLC Genomic Benchmark.



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*ANALYSIS OF RESULTS*

In order to evaluate the oligo-chip approach, full sequenced PPV genomes of the Spanish isolate ES91 and the Romanian isolate RO22 were used. Results showed that oligoprobes require more than four point mutations to discriminate sequences and this situation is not easy to find in the 18-20 nucleotides of the probe length among isolates within a strain such as type D. In addition it requires specific conditions of melting temperature. In fact all sequences were successfully detected by both isolates. Additional probes would be required to improve the discrimination of closely related isolates.

In contrast, deep sequencing allowed the analysis of intra-strain and intra-isolate variability as demonstrated in collaborative effort of Partners 1, 4 and 6. Full length genome sequences could be assembled with a coverage of a large number of reads for most of the nucleotide positions, allowing a real genome wide analysis of PPV diversity.

### **3. Original specifications and actual achievements**

A second generation of mini oligochips including 90 selected probes covering different regions of the PPV genome has been produced and assayed. In this deliverable we detailed the construction of the second generation of the oligo-chip, probes included and final protocol for hybridizing and reading. The main drawback derived from the probes that required several mutations to discriminate sequences while keeping specific  $T_m$  conditions. Further validation in different laboratories will be performed later this summer and an update of the implementation of this micro-array technology will be submitted by Autumn 2011.

This deliverable also includes a deep sequencing approach that allowed overcoming the limitations of the microarray technology and allowed for the first time the genome-wide analysis of intra-isolate variability.

### **4. Use and dissemination of the results**

Publication(s) on the characterisation of PPV diversity and on PPV typing based on minioligo arrays and deep sequencing will be done.



