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Development and validation of a real-time PCR assay for the identification of *Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* isolates

Validation report, July 2016

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ISHI VALIDATION REPORTS

This ISHI validation study has been conducted to determine the fitness of the described method for its intended purpose according to common practices in effect at the time.

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Published by:

International Seed Federation (ISF)

Reposoir 7, 1260 Nyon, Switzerland

Developed by ISHI

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Introduction

The current ISTA method for the detection of *Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* (Xap) on bean seeds is described in method 7-021 version 3.0. The identity of a suspect isolate is confirmed by either a pathogenicity test or a PCR test. A comparison study of the pathogenicity test and specific primers (Audy et al., 1994) was previously carried out in a collaboration between ISTA, ANSES, INRA and ISHI-veg. This study demonstrated that the ISTA 7-021v3.0 pathogenicity test and the Audy et al. (1994) primers are both suitable confirmation tools. The Audy et al. (1994) p7X4c and p7X4e primers amplify a 730bp fragment present in the plasmid and genomic DNA of Xap strains.

According to the ISHI-veg Best Practices for Molecular Techniques in Seed Health Tests (ISF, 2014) an internal amplification control is essential for identification of single isolate cultures by PCR. An internal amplification control (IAC) is a non-target nucleic acid sequence present in the same sample reaction tube which is co-amplified simultaneously with the target sequence. IAC controls are already used in other ISTA and ISHI-veg methods.

One approach is to combine a pathogen specific Taqman probe with an IAC Taqman probe specific to conserved bacterial DNA. An example of this approach is described in the ISHI-veg method v4.3 for the detection of Clavibacter on tomato seed. Suspect isolates are identified with Cmm specific Taqman probes and an IAC is included in each PCR tube with a probes adapted from Wu et al. (2008). The use of a real-time PCR assay facilitates the interpretation of results and removes the added complexity and safety hazards of gel electrophoresis with ethidium bromide.

Project objectives

- Design a new Taqman assay based on the same target of the primers described by Audy *et al.* (1994)
- Integrate the IAC Taqman assay adapted from Wu et al. (2008)
- Validate the combined Xap-Taqman/IAC assay on the DNA extracts of isolates used in the ISTA/ISHI comparative test (Grimault et al., 2012).

Materials and Methods

Conventional PCR

An Eppendorf Mastercycler gradient was programmed for the conventional PCR : 94°C, 2 minutes (min) ; 35 x (94°C, 30 seconds (sec); 56,5°C - 63,2°C, 30 sec; 72°C, 30 sec); 72°C, 1min. All PCR reactions were done in 25µl reactions: 5µl buffer, 1.5µl MgCl2, 0.2µl Go Taq polymerase (Promega, USA), 0.25µl dNTPs (GE Healthcare, UK), 1µl of each primer at 10µM, and 11.05µl water. 5µl of isolate suspension was added to each PCR.

Taqman reaction conditions

The initial validation Real-time PCR reactions were done in a final volume of 15µl containing : 7.5µl Taqman® Universal Master Mix II (Life Technologies, USA), 0.6µl of each primer at 10µM (0.4µM final concentration), 0.24µL probe 5µM (final concentration 0.08µM) and water to 13µl. 2µl of each isolate suspension was added to each PCR. The cycling program was applied as described in the mastermix protocol: 95°C, 10 min ; 40 x (95°C, 15 sec ; 60°C, 1 min).

The first series of reactions with the Wu IAC were also done in final volume of 15µl containing: 7.5µl Taqman® Universal Master Mix II (Life Technologies, USA), 0.6µl of each Xap primer at 10µM (0.4µM final concentration), 0.24µL Au1FAM probe 5µM (final concentration 0.08µM), 0.3µl of each Wu primer and probe at 10µM (final concentration 0.2µM) and water to 13µl. 2µl of each isolate DNA extract was added to each PCR. The cycling program was applied as described in the mastermix protocol: 95°C, 10 min ; 40 x (95°C, 15 sec ; 60°C, 1 min).

The second series of PCR reactions on fresh isolates were done as described above and additionally in final volumes of 25µl containing: : 12.5µl Taqman® Universal Master Mix II (Life Technologies, USA), 1µl of each Xap primer at 10µM (0.4µM final concentration), 0.4µL Au1FAM probe 5µM (final concentration 0.08µM), 1µl of each Wu primer and 0.25µl of each Wu probe at 10µM (final concentrations of 0.4µM and 0.1µM for the primers and probes respectively) and water to 20µl. 5µl of each isolate suspension was added to each PCR. The cycling program was applied as described in the mastermix protocol: 95°C, 10 min ; 40 x (95°C, 15 sec ; 60°C, 1 min).

Oligonucleotides

Table Primer and probe sequences

Primer Name	Sequence (5'-3')	5' Modification	3' Modification	Amplicon size	Reference
p7X4c	GGCAACACCCGATCCCTAAACAGG			730 bp	Audy et al. 1994
p7X4e	CGCCGGAAGCACGATCCTCGAAG				
AuF1	ACGGCCGGCGTCTTGTCTCT			148 bp	This study
AuR1	GCCGAGGTCCGCGAGATTCT				
Au1FAM	CGTCTCTGGCTTGACTGCGGTCGC	FAM	BHQ1		
WuF	CAACGCGAAGAACCTTACC				Wu et al. 2008
WuR	ACGTCATCCCCACCTTCC				
WuPr1	ACGACAACCATGCACCACCTG	Yakima Yellow	QSY		
WuPr2	ACGACAGCCATGCAGCACCT				

Results

Sequencing of Xap isolates on the Audy target and design of a Taqman assay

The p7X4c and p7X4e primers were used to amplify 730bp fragment from 4 independent Xap isolates isolated from seed and from plants. The PCR products were checked for specific amplification by gel electrophoresis and were sequenced in both directions with the same PCR primers. After trimming and alignment 624bp of sequence data were obtained for each isolate. The identity of sequences was 100% between the 4 isolates.

The 624bp sequence was used to identify suitable sequences for Taqman primers and probes. Two 20bp primers were designed and named AuF1 and AuR1 which have annealing temperatures of 60°C and amplify a 148bp fragment. A 24bp Taqman probe named Au1FAM was also designed with a predicted melt temperature of 68°C. The primers without the probe were first tested in conventional PCR at three annealing temperatures on three Xap isolates and three lookalike non-Xap isolates from bean seeds. Amplification was specific at all the tested temperatures (Figure 1).

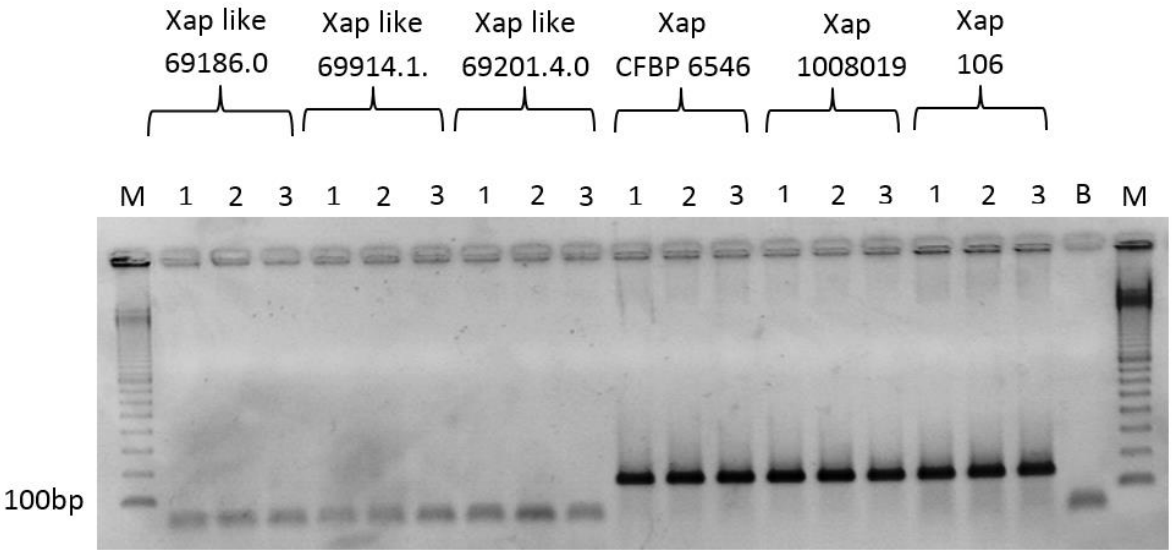

Figure 1 Agarose gel electrophoresis of DNA amplified with the primers AuF1 and AuR1

M: 100bp marker; B: Water control; Annealing temperatures 1: 58°C; 2: 60°C; 3: 63°C

A real-time PCR was done with the addition of the Au1FAM Taqman probe on a larger collection of 15 Xap look-alike isolates from bean seeds, 7 other *Xanthomonas* pathogens and 25 Xap isolates from the Vilmorin collection. All the Xap isolates were identified before Ct20, two lookalike strains showed Ct values between 35-40 cycles. With the application of a Ct35 cut-off, these real-time PCR results demonstrate the 100% diagnostic and analytical sensitivity of the Taqman assay (Table 1).

Table 1 Taqman real-time PCR results on a collection of Xap and lookalike isolates

Isolate ID	Ct	Isolate ID	Ct
xap like 69186.0	37.54	xap 1008019	15.62
xap like 69914.1.3	No Ct	xap 1007014	14.86
xap like 69201.4.0	No Ct	xap 1008017	13.54
xap like 69253.5.1	No Ct	xap J81747.1.2	15.54
xap like 69276.10.0	No Ct	xap I70296.1.0	13.99
xap like 69193.2.0	No Ct	xap i72066.3	13.5
xap like 69189.2	No Ct	xap 717626.1	17.62
xap like 69208.6.1	No Ct	xap 45.5	14.13
xap like 69270.0.2	No Ct	xap J426542.2	13.78
xap like 69176.1.1	39.74	xap 716806.1	15.34
xap like 69284.6.0.1	No Ct	xap 15.1p	13.88
xap like 77037.4.0	No Ct	xap 7.1 p	14.37
xap like 69297.0	No Ct	xap 59102.2	12.92
xap like 69311.0	No Ct	xap 3.1 p	13.63
xap like 70357.5.0	No Ct	xap 8.2 p	17.87
CFBP Xe 6864	No Ct	xap 4.2 p	12.9
CFBP Xp 7293	No Ct	xap 277401	13.72
CFBP Xv 4645	No Ct	xap 58.6 d2b	14.18
CFBP Xg 6822	No Ct	xap 20.1 p	14.03
CFBP Xcr 5829	No Ct	xap 17.1 p	13.85
Xhc 539	No Ct	xap 106	19.6
Xcc 645.2	No Ct	xap 107	13.89
xap CFBP 6546	16.52	xap 108	14.99
xap J95292 6.1.1	14.65	Negative control	No Ct

Validation on the collection of comparative test DNA extracts of the Xap specific Taqman assay with a Wu-derived IAC

The 60 isolate DNA extract collection tested in the ISTA/ISHI comparative test and stored in the bio-GEVES laboratory was sent to Vilmorin in order to validate the Audy derived Taqman assay. An internal amplification control based on the primers and probes described by Wu et al. (2008) was added to the reaction mix. The amplification curves obtained with the Au1FAM Taqman probe and the Wu-Yakima Yellow Taqman probes are illustrated in Figure 2a and 2b.

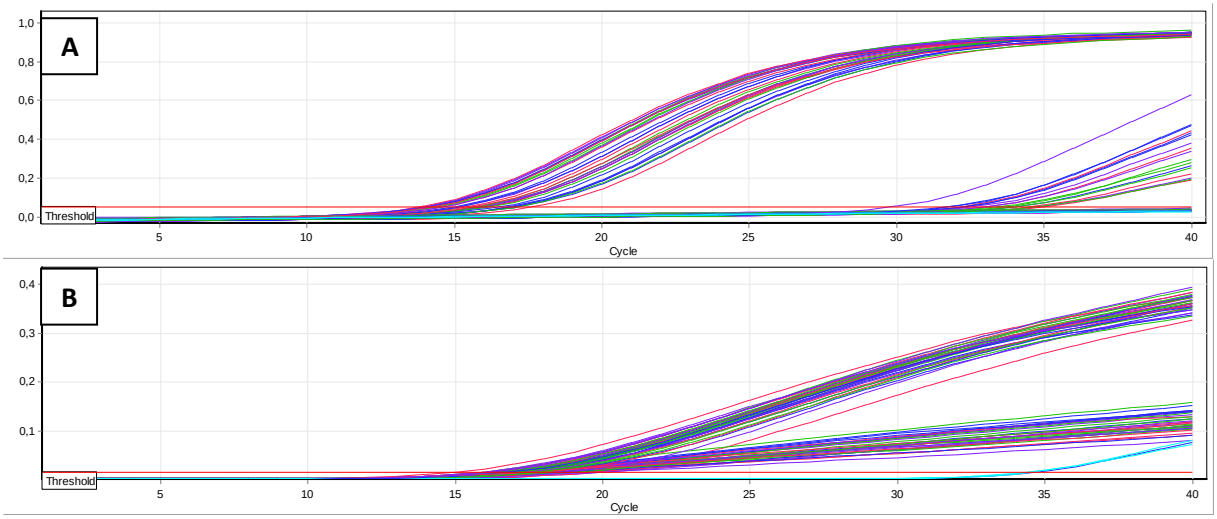

Figure 2 Au1FAM (A) and Wu-Yakima Yellow (B) amplification curves

The results obtained with the duplex Taqman reactions are presented in Table 2. Samples X1-30 are target Xap DNA extracts, X31 is a *X. axonopodis* pv. *dieffenbachiae* isolate also detected with the Audy primers of the ISTA method. All but one of the target extracts were detected at fewer than 20 cycles (<Ct20). The extract X9 was only detected with the new Taqman assay at Ct34.9. The DNA extract X9 was also not detected with the original PCR assay. The DNA extract X24 was not detected with the original Audy PCR assay, but is detected with the new Taqman assay. However, in the ISTA validation report it is indicated that extracts X9 and X24 were excluded from the analysis because X9 was not pure and there was a mistake during DNA preparation with X24 (Grimault et al. 2012).

Samples 32-60 are non-target DNA extracts. Several of the non-target extracts show a late amplification between Ct30-35. Traces of amplifications were also observed in the comparative test on some of the non-target DNA extracts (Appendix 1 comparison with the 2011 comparative test results), this may be due to non-specific amplification or traces of contamination in the extract solutions.

Table 2 Ct values obtained from real-time PCR tests on the comparative test DNA collection

	Audy-Wu Duplex	
Name	Audy Ct	WU
X1	16.04	16.82
X2	13.98	15.77
X3	14.16	16.08
X4	15.06	17.54
X5	17.66	16.73
X6	17.04	18.75
X7	15.9	16.86
X8	16.88	17.17
X9	34.93	18.61
X10	15.2	15.6
X11	15.86	15.33
X12	16.94	15.55
X13	15.43	17.74
X14	15.87	16.73
X15	14.5	16.83
X16	17.13	16.28
X17	16.06	15.88
X18	15.99	15.85
X19	16.17	16.42
X20	16.52	15.67
X21	13.85	16.45
X22	14.16	17.71
X23	14.28	15.66
X24	15.17	15.39
X25	14.62	15.72
X26	13.93	15.36
X27	15.71	17.0
X28	14.12	16.84
X29	15.65	15.78
X30	14.57	16.38
X31	17.12	17.2
X32	32.25	15.66

	Audy-Wu Duplex	
Name	Audy Ct	WU
X33	32.18	15.28
X34	34.76	15.89
X35	NA	15.79
X36	NA	16.95
X37	32.97	17.21
X38	32.53	17.77
X39	33.42	16.86
X40	33.89	16.24
X41	NA	13.89
X42	NA	14.89
X43	NA	14.85
X44	32.2	15.83
X45	34.54	15.62
X46	33.06	16.27
X47	34.08	15.75
X48	31.8	16.14
X49	NA	15.53
X50	NA	15.23
X51	NA	15.94
X52	NA	16.19
X53	NA	14.91
X54	NA	14.95
X55	NA	15.63
X56	NA	16.1
X57	NA	16.49
X58	NA	16.59
X59	34.78	16.18
X60	31.72	15.85
Xap	14.26	15.72
Xcc	29.8	15.23
Xcv	33.74	16.69
Water	NA	33.73

NA= No amplification

The 14 non-target isolates showing late amplification results (X32, X33, X34, X37, X38, X39, X40, X44, X45, X46, X47, X48, X59, X60) were re-tested on fresh isolate cultures. PCR reactions were done in reactions of 15µl and 25µl. In both reaction conditions the PCR results demonstrate the specificity of the Audy Taqman assay. None of the non-target isolates were amplified with the Audy Taqman assay (Figures 3A and 3C; Table 3). The amplification curves obtained with the Wu assay were superior in the 25µl reaction conditions (Figure 3B) when compared with the 15µl reaction conditions (Figure 3D).

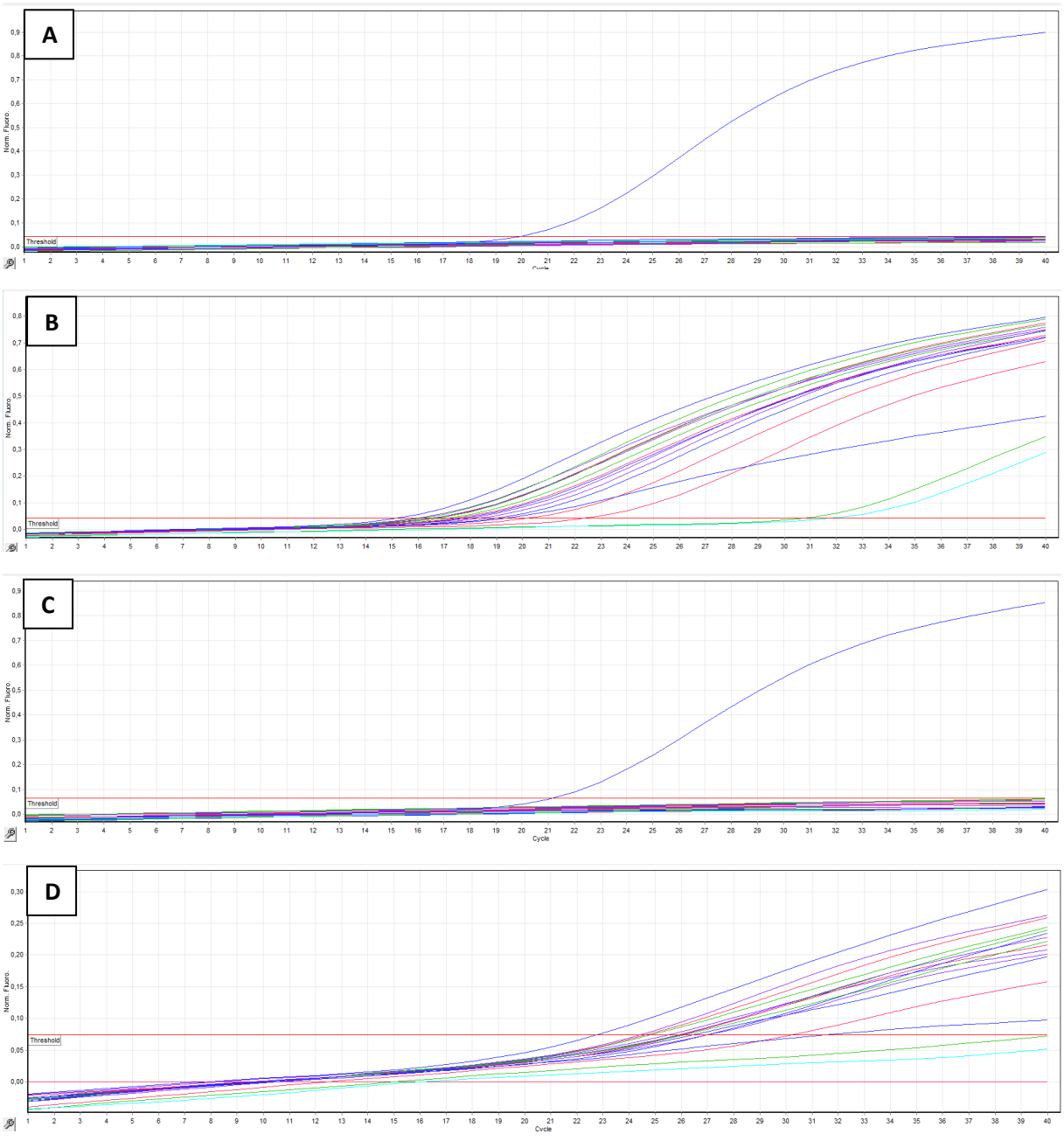

Figure 3 Au1FAM (A) and Wu-Yakima Yellow (B) amplification curves from 25µl reactions; Au1FAM (C) and Wu-Yakima Yellow (D) amplification curves from 15µl reactions

Table 3 Ct values obtained from real-time PCR tests on fresh isolates from the comparative test isolate collection

	Audy-Wu Duplex (25µl)	
Name	Audy Ct	WU
X32	NA	16.58
X33	NA	15.93
X34	NA	16.15
X37	NA	15.19
X38	NA	22.40
X39	NA	16.47
X40	NA	17.17
X44	NA	17.66
X45	NA	17.44
X46	NA	17.88
X47	NA	16.73
X48	NA	18.92
X59	NA	20.18
X60	NA	18.37
Xap	19.93	19.32
Water	NA	30.71

NA= No amplification

Conclusion

The newly designed Audy derived Taqman assay in combination with an IAC appears to be a potentially suitable replacement for the PCR primers described in the current ISTA method 7.021.

Acknowledgements

The development of the Audy derived Taqman assay was done by Laëtitia Larvor during her Master degree project in the Vilmorin laboratory. The DNA extracts and the data from the comparative test were supplied by Mathieu Rolland (bio-GEVES). The isolates were supplied by Valérie Grimault (SNES-GEVES).

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Appendix 1 Comparison of the Audy derived Taqman-Wu duplex results with the comparative test results

		Audy-Wu Duplex		Test Audy 2011						Test Toth 2011
Name	Type	Audy Ct	WU	Lab1	Lab2	Lab3	Lab4	Lab5	Lab6	
X1	Unknown	16,0	16,8	1	1	1	1	1	1	0
X2	Unknown	14,0	15,8	1	1	1	1	1	1	1
X3	Unknown	14,2	16,1	1	1	1	1	1	1	1
X4	Unknown	15,1	17,5	1	1	1	1	1	1	1
X5	Unknown	17,7	16,7	1	1	1	1	1	1	0
X6	Unknown	17,0	18,8	1	1	1	1	1	1	0
X7	Unknown	15,9	16,9	1	1	1	1	1	1	1
X8	Unknown	16,9	17,2	1	1	1	1	1	1	0
X9	Unknown	34,9	18,6	0	0	0	0	0	0	0
X10	Unknown	15,2	15,6	1	1	1	1	1	1	0
X11	Unknown	15,9	15,3	1	1	1	1	1	1	0
X12	Unknown	16,9	15,6	1	1	1	1	1	1	0
X13	Unknown	15,4	17,7	1	1	1	1	1	1	0
X14	Unknown	15,9	16,7	1	1	1	1	1	1	0
X15	Unknown	14,5	16,8	1	1	1	1	1	1	0
X16	Unknown	17,1	16,3	1	1	1	1	1	1	0
X17	Unknown	16,1	15,9	1	1	1	1	1	1	0
X18	Unknown	16,0	15,9	1	1	1	1	1	1	0
X19	Unknown	16,2	16,4	1	1	1	1	1	1	0
X20	Unknown	16,5	15,7	1	1	1	1	1	1	0
X21	Unknown	13,9	16,5	1	1	1	1	1	1	1
X22	Unknown	14,2	17,7	1	1	1	1	1	1	1
X23	Unknown	14,3	15,7	1	1	1	1	1	1	1
X24	Unknown	15,2	15,4	0	0	0	0	0	0	1
X25	Unknown	14,6	15,7	1	1	1	1	1	1	1
X26	Unknown	13,9	15,4	1	1	1	1	1	1	1
X27	Unknown	15,7	17,0	1	1	1	1	1	1	1
X28	Unknown	14,1	16,8	1	1	1	1	1	1	1
X29	Unknown	15,7	15,8	1	1	1	1	1	1	1
X30	Unknown	14,6	16,4	1	1	1	1	1	1	1
X31	Unknown	17,1	17,2	1	1	1	1	1	1	0
X32	Unknown	32,3	15,7	0	trace	0	0	0	0	0
X33	Unknown	32,2	15,3	0	trace	0	0	0	0	0
X34	Unknown	34,8	15,9	0	0	0	0	0	0	0
X35	Unknown		15,8	0	0	0	0	0	0	0
X36	Unknown		17,0	0	0	0	0	0	0	0
X37	Unknown	33,0	17,2	0	0	0	0	0	0	0
X38	Unknown	32,5	17,8	0	0	0	0	0	0	0
X39	Unknown	33,4	16,9	0	trace	0	0	0	0	0
X40	Unknown	33,9	16,2	0	0	0	0	0	0	0
X41	Unknown		13,9	0	0	0	0	0	0	0
X42	Unknown		14,9	0	0	0	0	X	0	0
X43	Unknown		14,9	0	0	0	0	0	0	0
X44	Unknown	32,2	15,8	0	trace	0	0	0	0	0
X45	Unknown	34,5	15,6	0	0	0	0	0	0	0
X46	Unknown	33,1	16,3	0	trace	0	0	0	0	0
X47	Unknown	34,1	15,8	0	trace	0	0	0	0	0
X48	Unknown	31,8	16,1	0	trace	0	0	0	0	0
X49	Unknown		15,5	0	0	0	0	0	0	0
X50	Unknown		15,2	0	0	0	0	0	0	0
X51	Unknown		15,9	0	0	0	0	0	0	0
X52	Unknown		16,2	0	0	0	0	0	0	0
X53	Unknown		14,9	0	0	0	0	0	0	0
X54	Unknown		15,0	0	0	0	0	0	0	0
X55	Unknown		15,6	0	0	0	0	0	0	0
X56	Unknown		16,1	0	0	0	0	0	0	0
X57	Unknown		16,5	0	0	0	0	0	0	0
X58	Unknown		16,6	0	0	0	0	0	0	0
X59	Unknown	34,8	16,2	0	0	0	0	0	0	0
X60	Unknown	31,7	15,9	0	0	0	0	0	0	0
T+ Xap	Unknown	14,3	15,7	1	1	1	1	1	1	1
T- Xcc	Unknown	29,8	15,2	0	trace	0	0	0	0	0