Title: Rapid detection of rifampicin-resistant *Mycobacterium tuberculosis*, based on isothermal DNA amplification and DNA chromatography

Authors;

Yutaka Takarada [1),*,$], Takuya Kodera [1),$], Kumi Kobayashi [1)], Chie Nakajima[2),3)], Mitsuo Kawase[1)], Yasuhiko Suzuki [2),3),*]

1) TBA Co., LTD, T-Biz 307, 6-6-40 Aramaki-Aza-Aoba, Aoba-ku, Sendai, 980-8579 Japan

2) Hokkaido University Research Center for Zoonosis Control, Sapporo, Japan

3) Hokkaido University, GI-CoRE Global Station for Zoonosis Control, Sapporo, 001-0020 Japan.

E-mail addresses:

y-takara@t-bioarray.com , t-kodera@ecei.tohoku.ac.jp , k-kobaya@t-bioarray.com , cnakajim@czc.hokudai.ac.jp, m-kawase@ecei.tohoku.ac.jp, suzuki@czc.hokudai.ac.jp

[$]YT and TK equally contributed to this work.

*Corresponding author:

Yutaka Takarada

TBA Co.,LTD, T-Biz 307, 6-6-40 Aramaki-Aza-Aoba, Aoba-ku, Sendai, 980-8579 Japan

E-mail: y-takara@t-bioarray.com

Telephone: +81-22-795-7204; FAX: +81-22-795-7204

Co-corresponding author:

Yasuhiko Suzuki

Kita 20, Nishi 10, Kita-ku, Sappro, 001-0020, Japan

E-mail: suzuki@czc.hokudai.ac.jp

Telephone: +81-11-706-9503; FAX: +81-11-706-7310

Abstract

Rapid and easy detection of nucleotide point mutations in bacterial pathogens associated with drug resistance is essential for the proper use of antimicrobials. Here, we developed a rapid and simple method for the detection of mutations using Loop-mediated isothermal amplification (LAMP) combined with the single-tag hybridization (STH) chromatographic printed array strips (PAS) method. This procedure is able to detect four mutations (C1349T, A1295C, G1303T, A1304T) in Rifampicin Resistance Determining Region (RRDR) of rifampicin-resistant *Mycobacterium tuberculosis* (RR-TB), simultaneously.

LAMP reactions contained a LAMP primer and eight allele-specific primers for each mutation. The allele-specific primers products were detected by nucleic acid chromatography using PAS. Four detection lines were detected there, one of which was detected at different positions depend on the wild type and the mutant type. We carried out the four mutations detection using 31 genomic DNA (2 A1295T, 1 G1303T, 6 A1304T, 22 C1349T) from clinical isolate. The mutations have been confirmed by sequence analysis. The detection results were completely consistent with the sequence analysis. In the present study, four mutations could be detected, but only 60% of RR-TB could be detected with these four. It is expected that the detection rate will increase by adding more mutant primers.

The combined LAMP and STH chromatographic PAS method is a simple and rapid method for detecting point mutations in clinical isolates as a point-of-care testing (POCT) technique. In addition, it does not require special equipment and can meet the demand in areas where drug-resistant bacteria are endemic, such as developing countries.

Keywords

60 *Mycobacterium tuberculosis*, Rapid detection, Loop-mediated isothermal amplification,
61 DNA-Chromatography, Mutation detection
62

Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (MTB) is an infectious disease that affects more than 10 million people worldwide every year, mainly in developing countries (WHO, 2019). In addition, MTB is still a global leading cause of death (WHO, 2019). Although in general TB cases have gradually decreased annually, the incidence of MTB resistant to rifampicin and multi-drug treatments (RR-TB/MDR-TB) have increased five-fold in the past decade (WHO, 2019). Therefore, measures are taken to prevent drug-resistant tuberculosis from spreading. Of special importance is that diagnosis and treatments be properly conducted in developing countries.

For the treatment of MTB, rifampicin is the first-line drug in a combination supported by 3-4 other drugs. Therefore, the presence or absence of rifampicin resistance is a very important factor an appropriate TB treatment. Approximately 78-100% of rifampicin-resistant MTB (RR-MTB) has mutations in the 81 bp region (Rifampicin Resistance Determining Region: RRDR) of the RNA polymerase subunit β (*rpoB*) gene (Telenti et al., 1993; Rahim et al., 2012; Aye et al., 2016).

Typically, diagnosis of mycobacterial infections, including drug-resistance MTB, is carried out by culturing, but these bacteria are slow growing and thus culturing is time-consuming. Therefore, a method to quickly identify drug resistance is needed. Molecular diagnostic methods can quickly identify drug resistance, which can help expedite the development of new drugs for the treatment of drug-resistant MTB. The Word Health Organization (WHO) recommended Xpert MTB/RIF® assay as rapid diagnostic test for the detection of TB and RR-TB (WHO, 2017). This assay is a single-use sample-processing cartridge system with integrated multicolor real-time PCR to detect majority of RR associated mutations. WHO states that the system requires no additional experimental equipment and is suitable for use at all levels of the health system, but because of its sophisticated system, it requires a stable, uninterrupted

electrical supply. And, this system cannot be used to monitor treatment and requires traditional cultures and drug susceptibility test to detect resistance to anti-tuberculosis drugs other than rifampicin. In addition, this system has low throughput, it was unable to meet the demands from high TB endemic regions (Tam et al., 2017).

Loop-mediated isothermal amplification (LAMP) is an isothermal amplification method that uses four primers and a DNA polymerase with strand displacement activity (Notomi et al., 2000; Mori et al., 2001; Nagamine et al., 2001). The four primers recognize six regions and therefore show very high specificity compared to conventional PCR. The amplification product has a structure with several loops and an inverted repeat sequence. Additional primers designed to the loop position, the efficiency and specificity can be further increased. Since reaction can be carried out at a fixed temperature, the reaction products can be detected by the naked eye, no expensive instruments are needed, LAMP is recognized as a powerful tool for point-of-care genetic testing. Detection of mutations using LAMP has been reported, which was either allele-specific LAMP (Badolo et al., 2012; Badolo et al., 2015) or PNA clamp method using a PNA probe (Itonaga et al., 2016; Sakai et al., 2017). Nonetheless, multi-mutations were difficult to detect simultaneously by these reported methods.

The single-tag hybridization (STH) chromatographic printed array strip (PAS) method is also an innovative multi-detection method. The PCR products using primer-attached tag sequences and primers with biotin are mixed with streptavidin-bound colored beads. The resulting mixture then binds chromatographically to the anti-tag probe on the strip. Different anti-tag probes are located at different sites on the strip, so that multi-detection is possible. In this method, individual amplicons of multiplex PCR products can be easily detected with high sensitivity within 10-15 min, with the naked eye (Monden et al., 2014; Tian et al., 2014; Tian et al., 2016; Saito et al., 2018). As for the detection of drug resistant genes using this method, there are cases in which the

115 *mecA* and *blaZ* of the *Staphylococcus aureus* were detected in blood culture samples
116 (Ohshiro et al., 2016) and the carbapenemase gene of carbapenem-resistant bacterium
117 was detected in fecal samples (Shanmugakani et al., 2017). More recently, the gene for
118 pork in beef meatballs (Riztyan et al., 2018) and the gene for genetically modified crops
119 in soybeans (Takabatake et al., 2018) were detected.
120 In the present study, we developed a rapid and simple method for the detection of
121 mutations using LAMP combined with the STH chromatographic PAS method. We
122 were able to detect four mutations (C1349T, A1295C, G1303T, A1304T) for RRDR of
123 RR-TB, simultaneously.
124
125 1. **Materials and Methods**
126 **1.1** DNA preparation
127 For evaluation of LAMP primers, extract DNA were used from T702 (*M.*
128 *tuberculosis*), KPM1001 (*M. kansasii*), KPM3011 (*M. avium*) and KPM2783 (*M.*
129 *intracelluare*).
130 To verify the detection of mutations, 31 DNAs (2 of A1295C, 1 of G1303T, 6 of
131 A1304T and 22 of C1349T) extracted from clinical isolates obtained in Bangladesh
132 were used. The drug resistance has been confirmed by drug susceptibility test, and their
133 mutations have been confirmed by a sequence analysis. (Rahim et al., 2012).
134
135 **1.2** LAMP primer and allele-specific primer
136 The MTB-specific LAMP primer sets for Refampicin Resistance Determining Region
137 (RRDR) of rpoB gene were designed using online software (PrimerExplorerV4,
138 http://primerexplorer.jp/v4_manual/index.html). The F1 region of forward inner primer
139 (FIP) and B1 region of backward inner primer (BIP) were designed on the outer side of
140 RRDR. The allele-specific primers to detect mutation were designed between the F1

and B1 region (Fig. 1). The primers for individual mutations or wild types were tagged with different tags. The LAMP primer set and allele specific primers are listed in Table 1 and the assigned positions are shown in Fig. 2.

1.3 LAMP reaction

LAMP reactions were carried out in a 10-μl reaction mixture consisting of 0.8 μM biotinylated FIP primer, 0.8 μM biotinylated BIP primer, 0.2 μM of each outer primer (F3 and B3), 0.8 μM of each loop primer (FLP and BLP), 1.4 mM dNTP, 1 μl 10× reaction buffer (Nippon gene KK), 4.8 U of Bst DNA polymerase (Nippon gene KK), 1 μl of 3,000-fold diluted SYBR Green I and 1 μl of extracted DNA. Fluorescence of SYBR Green I was measured every minute for 50 min at 68 ℃ during the ongoing reaction, using a thermal cycler Dice real time system (TAKARA Bio Inc.). To terminate the reaction, the mixture was incubated for 2 min at 80 °C.

For multiple mutant detection, allele-specific primers for detecting mutations were added to the reaction mixture at a concentration of 0.2 μM each. In individual mutation detection, the reaction was carried out with two primers (wild and mutant) at the mutation position. Eight primers were used in the reaction for simultaneous detection of four positions. The LAMP reaction condition was same as above.

1.4 Detection design of STH chromatographic PAS

Fig. 2 shows the detection design of PAS for mutations. On the individual detection line of C-PAS, 8, 8 oligonucleotide complementary tags (named c-tags 1 - 8) with distinct nucleotide sequences were previously immobilized from the lower end of PAS. In Table 1, c-tags attached to their respective allele-specific primer are shown.

1.5 Detection of mutations from LAMP products using STH chromatographic PAS

After the LAMP reaction, 10 µl of the products were diluted 100-fold with distilled water and mixed with 2 µl of streptavidin coated blue latex beads and 10 µl of a developing solution (TBA Co., Ltd.). Subsequently, a C-PAS8 membrane strip (TBA Co., Ltd.) was dipped into the mixture for 15-20 min. The appearing blue line was dependent on the type of the tag bound to the primer and hence, it determined what mutations were present. To prevent contamination, reagent preparation and detection sites were strictly isolated. In fact, it took place on the 1st and 4th floors of the building.

2. Results

2.1 LAMP reaction for RRDR region of TB

One FIP, three BIP, two FLP and four BLP primers were examined to amplify the 81bp of the RRDR region. To select the optimal primer combinations, the concentration, the reaction temperature and time were examined by real time amplification using SYB Green I. The selected primers and conditions were able to amplify the TB-specific target region. The detection limit was 200 fg/test using the extracted DNA from isolate strain. (Fig. 3)

2.2 Detection of the individual mutations using STH chromatographic PAS by LAMP

The detection reaction of individual mutations was conducted using the extracted genomic DNA (10 pg/test) . Sequences of the allele-specific primers are shown in Table 1. LAMP reactions were carried out containing LAMP primers and two allele-specific primers (wild and mutant-types) of each target sequence in RRDR. After the LAMP reaction, elongation products of each specific primer were detected by nucleic acid chromatography using PAS. The results are shown in Fig. 4. Genotypes of C1349 (wild type: C), 1349T (mutation: T), A1295 (wild type: A), 1295C (mutation: C), A1304

193 (wild type: A), 1304T (mutation: T), G1303 (wild type: G) and 1303T (mutation: T)
194 were detected by tags 1, 2, 3, 4, 5, 6, 7 and 8, respectively. According to the analysis by
195 Rahim et al., the mutation of nucleotide position 1349 was the most frequent, followed
196 by 1333 to 1334 and 1303 to 1304, followed by 1295,1345,1355 with low frequency.
197 Since there are many types of mutations in 1333 to 1334 and they are close to 1349, so
198 in order to accurate detection of the 1349, we do not select mutations from 1333 to
199 1334, and the following 1303 to 1304 and 1295 was selected.
200
201 **2.3** Detection of the multi-mutations using STH chromatographic PAS by LAMP
202 Reactions of the multi-mutation detection were carried out using 31 genomic DNA
203 from analyzed mutations and 1 wild type DNA. Moreover, LAMP reactions contained a
204 LAMP primer and eight allele-specific primers for each mutation. After the LAMP
205 reactions, elongation products of each allele-specific primer were detected by nucleic
206 acid chromatography using PAS. Four detection lines were detected there, one of which
207 was detected at different positions depend on the wild type and the mutant type. The
208 results are shown in Fig. 5 and summarized Table 2. In samples with a mutation, a
209 positive line was shifted from the wild type to the mutant type corresponding to the
210 specific mutation. This result indicated that each specific primer underwent a mutation-
211 specific elongation reaction and could be accurately detected by nucleic acid
212 chromatography using PAS.
213
214 **3. Discussion**
215 The isothermal amplification method is extremely useful for genetic point of care
216 testing (POCT) because special equipment such as PCR is not needed. However, many
217 of the known isothermal amplification methods (NASBA, SDA, RCA) are complicated
218 and costly, using heat sensitive enzymes and requiring multiple enzymes. Primers are

complex, but once the primer design is completed, the LAMP method is comparable in sensitivity and reproducibility to PCR reactions. Moreover, only one type of thermostable enzyme is used, and hence the cost is reduced. By contrast, the LAMP method is not good for multi-detection. As a strand displacement enzyme is used, neither the Taqman probe method nor the Molecular Beacon probe can be applied to LAMP. Furthermore, since the LAMP product is very complicated, multi-detection by electrophoresis is difficult to achieve.

STH chromatographic PAS is a rapid and easy detection method for multi-amplification products. It has been reported that multi-LAMP reaction products can be easily detected (Takabatake et al., 2018). In this study, allele-specific primers were used to detect mutations using the LAMP amplification method. The principle of LAMP reaction using allele-specific primers is shown in Fig. 6a. First, LAMP primers were designed to squeeze in between the mutation site of RRDR. In LAMP amplification, a dumbbell structure is generated after the first few steps. An allele-specific primer with tag anneals to the target sequence in RRDR, and DNA strands are only synthesized when the primer sequences fully match with the target sequences. The product is then displaced by the loop primer elongation. The replacement product gets looped and extends, with the biotin-LAMP primer reacting at the loop part. Next, the allele-specific primer reacts and elongates from the single-stranded portion of the product, and the biotin-LAMP primer product is displaced. Subsequently, the allele-specific primer reacts and expands to the biotin-LAMP primer product, after which the reaction is terminated. At this point, the terminated product has biotin and a tag. Detection of the product with biotin and a tag is shown in Fig. 6b (Monden et al., 2014; Tian et al., 2014; Tian et al., 2016; Saito et al., 2018).

In the present study, it was possible to detect 60% or more of RR-MTB by detecting the aforementioned four mutations. With this new method, it was proved that it is

245 possible to simultaneously react multiple mutagenic primers with one LAMP reaction.
246 To detect additional mutations, it is possible to add a mutation primers or perform
247 another reaction with the same LAMP primer and different mutation primers.
248 Xpert MTB/RIF® assay is a useful method to detect the RR-TB (Helb et al., 2010).
249 According to its principle, when a wild type sequence is present, the signal of the
250 molecular beacon probe is detected and the signal disappears when a mutated sequence
251 is present (El-Hajj et al., 2001). In other words, the detection rate would differ
252 depending on the abundance ratio of the susceptible and the resistant bacteria in the
253 specimen. If the majority of the samples were susceptible bacteria and only small
254 amounts of resistant bacteria were present, there would be a possibility that no resistant
255 bacteria could be detected.
256 Line probe assay is also a useful method to detect RR-TB (Beenhouwer et al., 1995).
257 As this method positively detects the mutant sequence, it is less affected by the
258 abundance ratio of the resistant bacteria. However, the line probe assay requires 3 hours
259 or longer and 7 or more detection steps to detect mutant sequence after PCR, and it
260 needs heating equipment. In contrast, mutant sequences can be detected by STH
261 chromatographic PAS method at room temperature in 15-20 minutes and following only
262 two steps after LAMP.
263 Since the combined LAMP and STH chromatographic PAS method does not require
264 special equipment and complicated work after the Xpert assay or Line probe assay is
265 completed, it can be implemented anywhere. For example, it can be conducted and
266 tested on site in a remote location and or in areas with unstable power supply. However,
267 there are some issues in the combined LAMP and the STH chromatographic PAS
268 method that need to be addressed. There is a risk of contamination as the lid of LAMP
269 reaction tube needs to be opened after LAMP is completed. Further improvement of the
270 method could eliminate the risk of contamination. A simple device with LAMP reaction

chamber connected to the STH chromatographic PAS cassette enables chromatographic development without opening the lid after LAMP reaction. This will be reported in our future publication.

In conclusion, the combined LAMP and STH chromatographic PAS method does not require special equipment and readily detects several mutations in a quick manner, which meets the demand for it in areas with high prevalence of drug-resistant bacteria such as those in developing countries. In addition, it is suitable for POCT.

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382 Table1. Nucleotide sequence of LAMP primers for RRDR and allele-specific primers for mutation
383 detection

Use	Name	Sequence
LAMP	bFIP	biotin-CTTGATCGCGGCGACCACCGGACGTGGAGGCGATCACA
	F3	AGCGGATGACCACCCAG
	FLP	GGATGTTGATCAACGTCTGCG
	bBIP	biotin-ACGTGCACCCGTCGCACTTGTTGGGCCCCTCAGG
	B3	AGCGAGCCGATCAGACC
	BLP	CGGATGTGCCCGATCGAAA
Allele-detection	C1349	tag1-CCACAAGCGCCGACTGTC
	1349T	tag2-CCACAAGCGCCGACTGcT
	A1295	tag4-GGCACCAGCCAGCTGAGCgA
	1295C	tag3-GGCACCAGCCAGCTGAGCgC
	A1304	tag5-ACAGCGGGTTGTTCTGGT
	1304T	tag6-ACAGCGGGTTGTTCTGGA
	G1303	tag7-CCAGCTGAGCCAATTCATGG
	1303T	tag8-CCAGCTGAGCCAATTCATGT

384 The small character shows artificial mutations.

385

386 Table2. Characteristics of samples used for this study and reactivities to tags

No.	Name	Mutation position	tag 1	tag 2	tag 3	tag 4	tag 5	tag 6	tag 7	tag 8
1	MTB	Wild	+	-	-	+	+	-	+	-
2	1-70	A1295C	+	-	+	-	+	-	+	-
3	8-11		+	-	+	-	+	-	+	-
4	2P-14	G1303T	+	-	-	+	+	-	-	+
5	11-72	A1304T	+	-	-	+	-	+	+	-
6	OM-4		+	-	-	+	-	+	+	-
7	OM-5		+	-	-	+	-	+	+	-
8	OM-10		+	-	-	+	-	+	+	-
9	OM-19		+	-	-	+	-	+	+	-
10	OM-95		+	-	-	+	-	+	+	-
11	9-8	C1349T	-	+	-	+	+	-	+	-
12	OM-2		-	+	-	+	+	-	+	-
13	OM-6		-	+	-	+	+	-	+	-
14	OM-7		-	+	-	+	+	-	+	-
15	OM-9		-	+	-	+	+	-	+	-
16	OM-12		-	+	-	+	+	-	+	-
17	OM-13		-	+	-	+	+	-	+	-
18	OM-14		-	+	-	+	+	-	+	-
19	OM-16		-	+	-	+	+	-	+	-
20	OM-18		-	+	-	+	+	-	+	-
21	OM-20		-	+	-	+	+	-	+	-
22	OM-21		-	+	-	+	+	-	+	-
23	OM-25		-	+	-	+	+	-	+	-
24	OM-26		-	+	-	+	+	-	+	-
25	OM-29		-	+	-	+	+	-	+	-
26	OM-31		-	+	-	+	+	-	+	-
27	OM-35		-	+	-	+	+	-	+	-
28	OM-37		-	+	-	+	+	-	+	-
29	OM-38		-	+	-	+	+	-	+	-
30	OM-42		-	+	-	+	+	-	+	-
31	OM-55		-	+	-	+	+	-	+	-
32	OM-57		-	+	-	+	+	-	+	-

387

Figure legends

Figure 1. Design of the LAMP primer and the allele-specific primer

DNA sequence of partial rpoB gene containing RRDR region and position of mutations, and LAMP and allele-specific primers designed for this study. The mutation nucleotide is shown in red and bolded. The nucleotides shown in blue are the region of RRDR. The LAMP primers were designed to amplify the region of RRDR . The 3' end nucleotide of the allele-specific primers were assigned to the base corresponding to the wild type and the mutant type, respectively.

Figure 2. Detection design of STH chromatographic PAS for four mutations of RRDR.

Prior to development, C-PAS8 had eight orange lines of immobilized c-tag and 1 flow control. The orange lines disappeared after development. During the development, each tag in the primer hybridized to the each c-tag independently and streptavidin coated blue latex beads bound to the biotin conjugate primer.

Figure 3. Real time detection for LAMP reaction of RRDR .

The sensitivity and specificity of the LAMP reaction was measured with a real-time PCR instrument (Dice real time system). The results showed a detection limit of 200fg/test for the RRDR gene from the MTB genome DNA and no cross reactivity for *Mycobacterium avium*, *Mycobacterium intracellulale* and *Mycobacterium kansacii*.

Figure 4. Detection results of individual mutation.

Individual mutation was detected using the allele-specific primer for each mutation. No.1 and 2 used 531 wild and mutation primers, respectively. The wild type genome used No.1 reaction and the mutation type genome used No. 2 reaction. The results showed No.1 detected only wild type primer signal (tag1) and No. 2 detected only

mutation type signal (tag2). No. 3 and 4 used 513 wild and mutation primers, respectively. The wild type genome used No. 3 reaction and the mutation type genome used No. 4 reaction. The results showed No. 3 detected only wild type primer signal (tag4) and No. 4 detected only mutation type signal (tag3). No. 5 and 6 used 516 wild and mutation primers, respectively. The wild type genome used No. 5 reaction and the mutation type genome used No. 6 reaction. The results showed No. 5 detected only wild type primer signal (tag7) and No. 6 detected only mutation type signal (tag8). No.7 and 8 used 516 wild and mutation primers, respectively. The wild type genome used No.7 reaction and the mutation type genome used No.8 reaction. The results showed the No.7 detected only wild type primer signal (tag5) and No. 8 detected only mutation type signal (tag6).

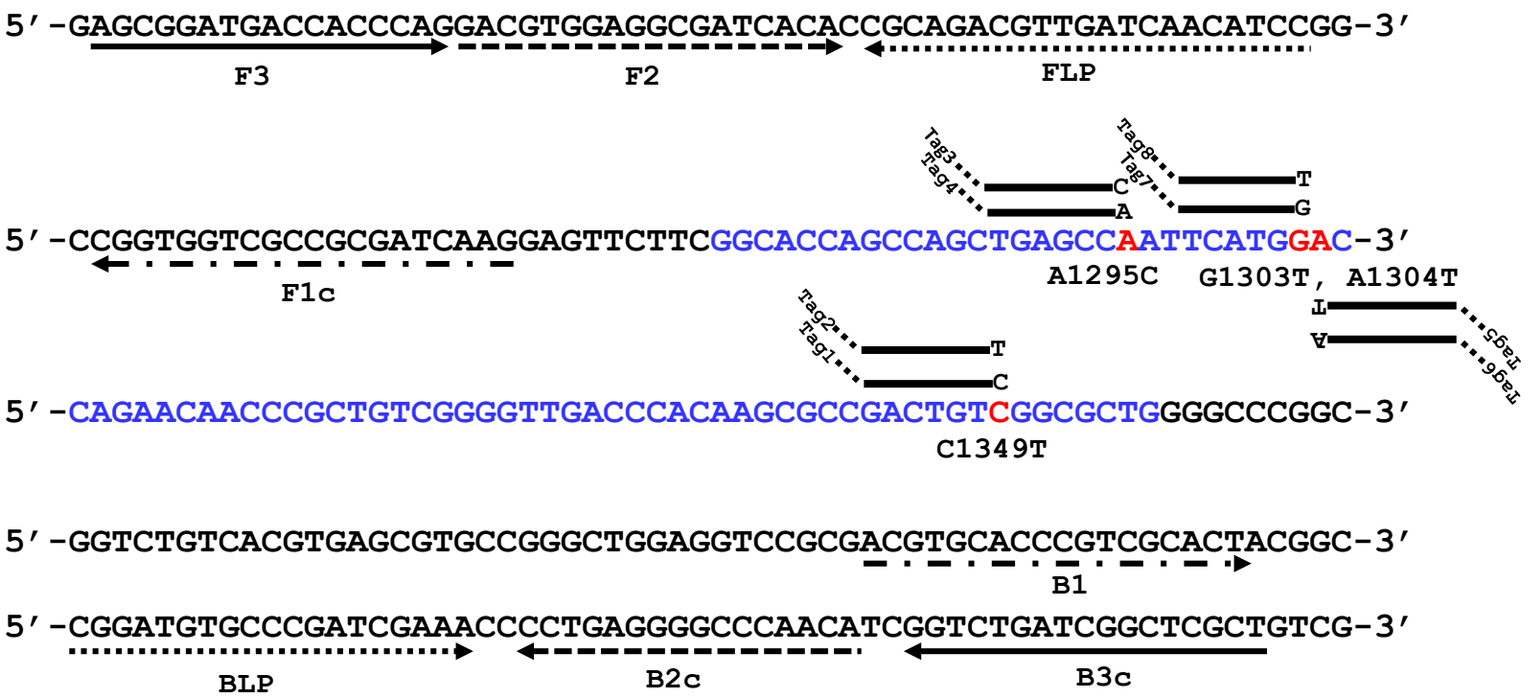
Figure 5. Detection results of multi-detection

Multi-mutations were detected using the eight allele-specific primers for each mutation. The extracted genome sample are summarized in Table 2. The results show that four mutations were precisely detected.

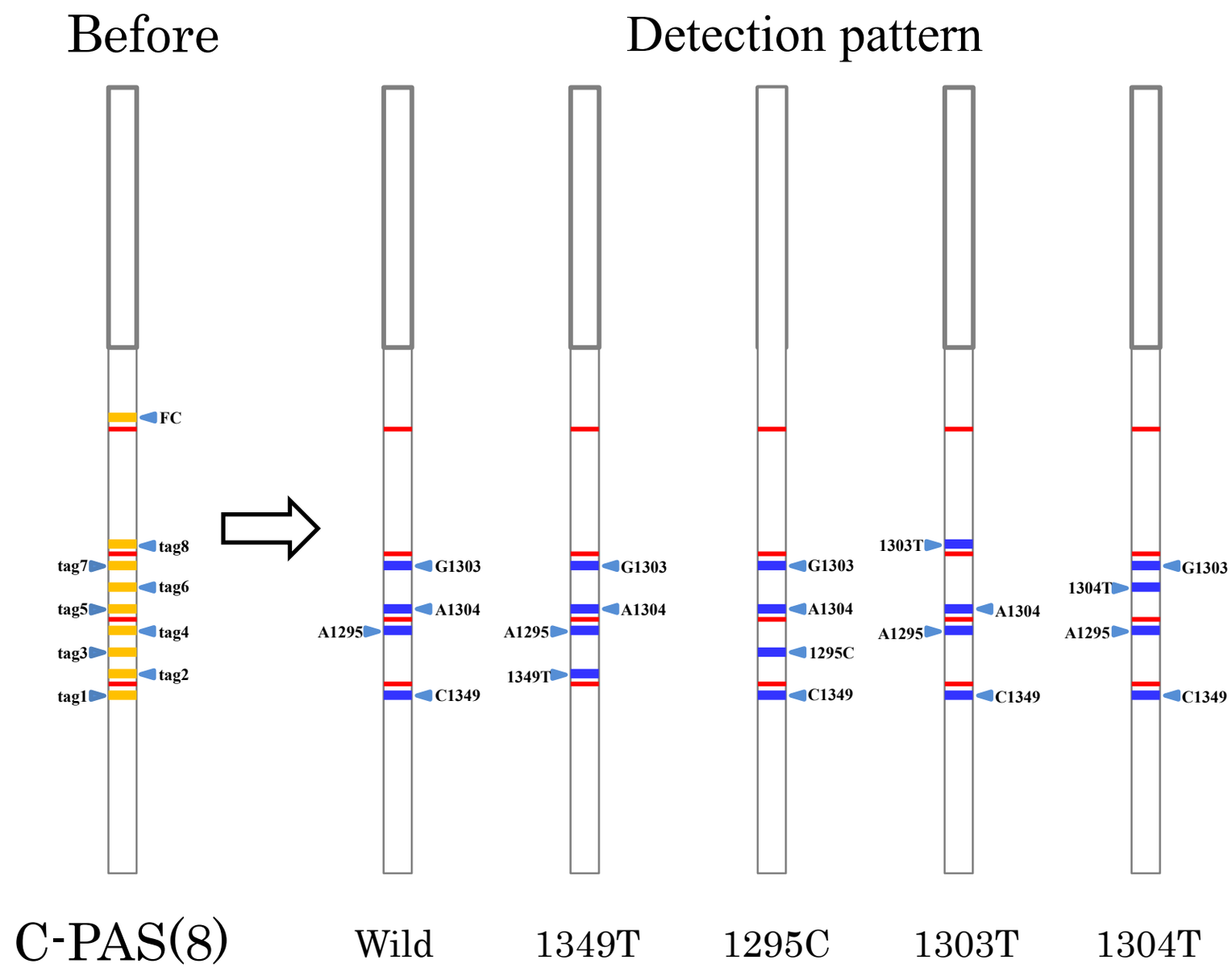
Figure 6. Detection principle for STH chromatographic PAS using LAMP

(A) To detect with the STH chromatographic PAS method, an amplification product with a tag and biotin will be synthesized by isothermal amplification. In this scheme, the extension product of tag-attached allele-specific primer forms a double strand with biotin-attached to the elongation product of FIP. (B) Biotin labeled LAMP products are labeled by avidin-coated blue beads. Blue beads labeled LAMP products are trapped by anti-tag oligonucleotides printed on the strip membrane via the tag at the end of LAMP products.

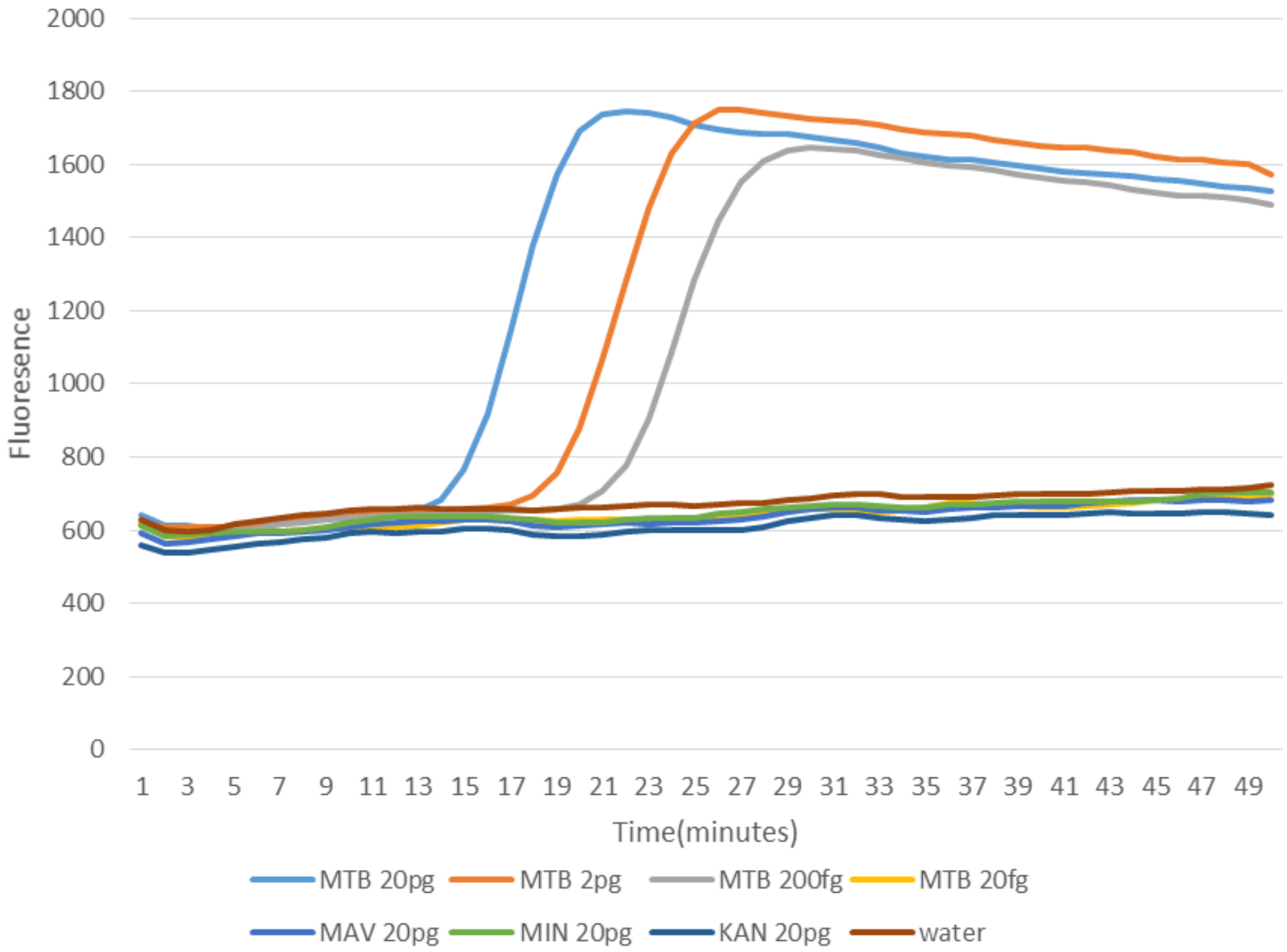
Takarada et al. Figure 1



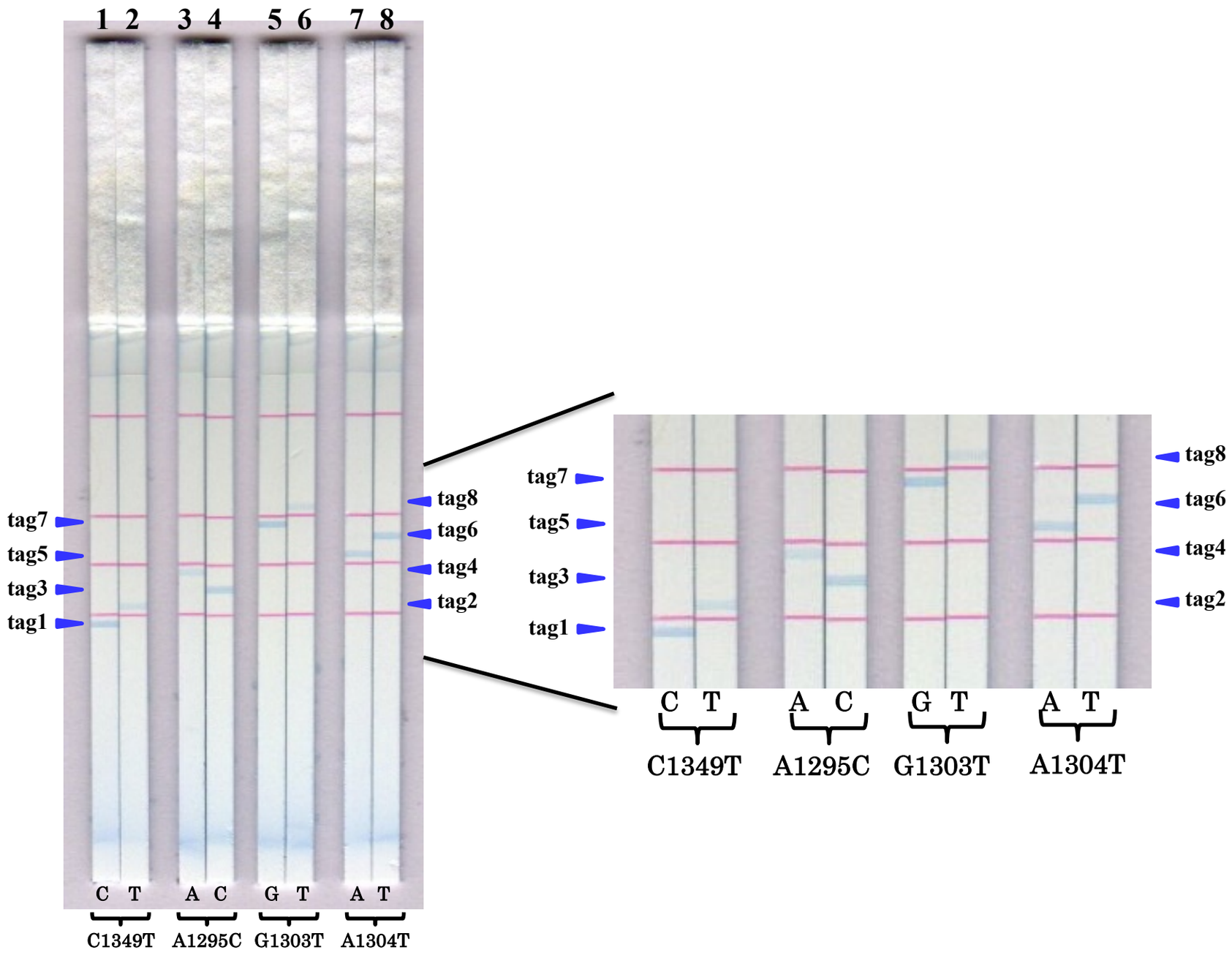
Takarada et al. Figure 2



Takarada et al. Figure 3

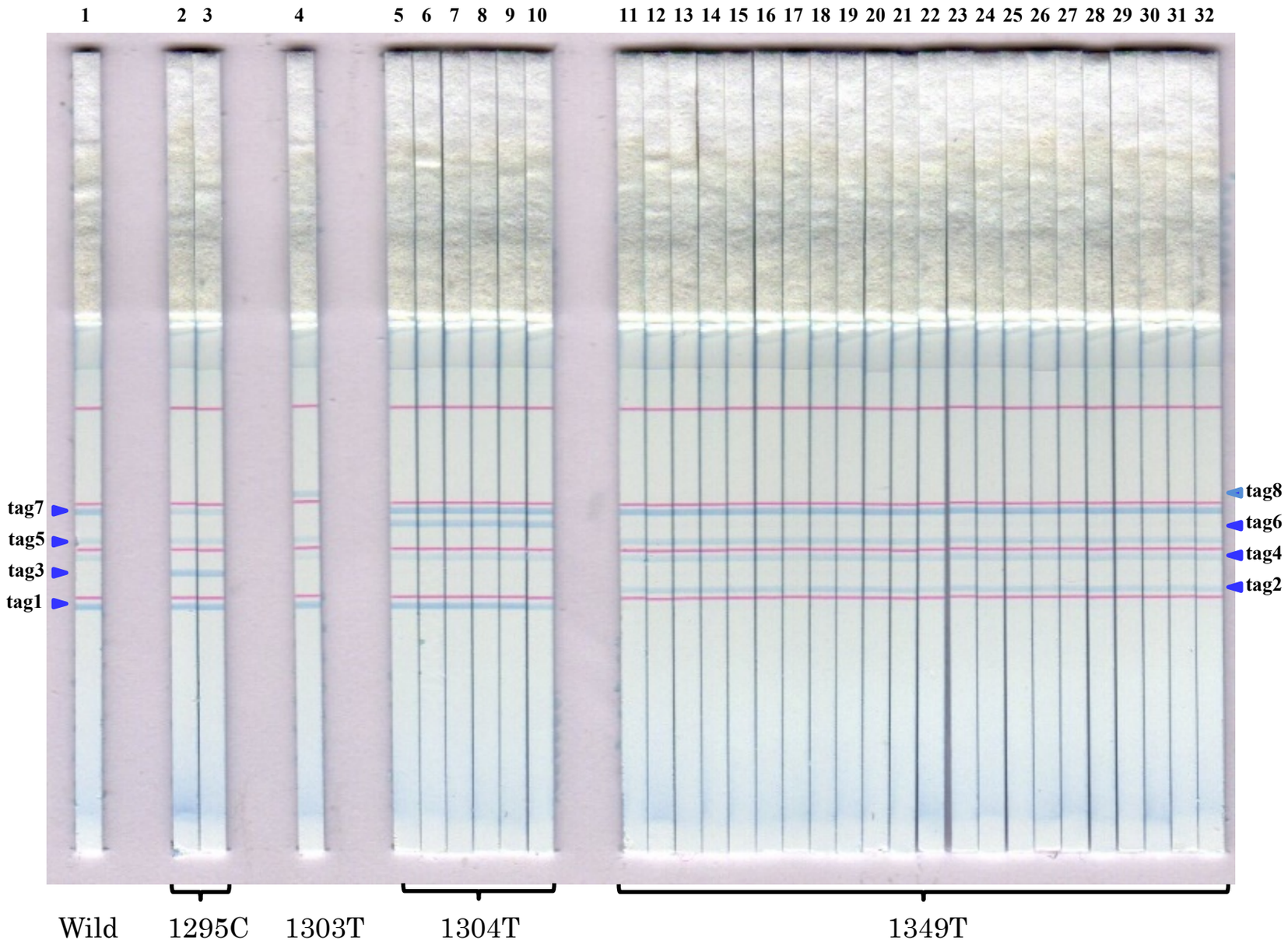


Takarada et al. Figure 4



S R S R S R S R

Takarada et al. Figure 5



Takarada et al. Figure 6