



Title	Rapid detection of rifampicin-resistant Mycobacterium tuberculosis, based on isothermal DNA amplification and DNA chromatography
Author(s)	Takarada, Yutaka; Koder, Takuya; Kobayashi, Kumi; Nakajima, Chie; Kawase, Mitsuo; Suzuki, Yasuhiko
Citation	Journal of microbiological methods, 177, 106062 https://doi.org/10.1016/j.mimet.2020.106062
Issue Date	2020-10
Doc URL	http://hdl.handle.net/2115/83154
Rights	© 2020. This manuscript version is made available under the CC-BY-NC-ND 4.0 license http://creativecommons.org/licenses/by-nc-nd/4.0/
Rights(URL)	http://creativecommons.org/licenses/by-nc-nd/4.0
Type	article (author version)
File Information	MS-Takarada-1_Main Text_2020.09.14R2.pdf



[Instructions for use](#)

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

3

4 Authors;

5 Yutaka Takarada ^{1),*,§}, Takuya Koderu ^{1),§}, Kumi Kobayashi ¹⁾, Chie Nakajima^{2),3)},
6 Mitsuo Kawase¹⁾, Yasuhiko Suzuki ^{2),3),*}

7

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

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

11 3) Hokkaido University, GI-CoRE Global Station for Zoonosis Control, Sapporo, 001-
12 0020 Japan.

13

14 E-mail addresses:

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

17

18 [§]YT and TK equally contributed to this work.

19

20 *Corresponding author:

21 Yutaka Takarada

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

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

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

26

27 Co-corresponding author:
28 Yasuhiko Suzuki
29 Kita 20, Nishi 10, Kita-ku, Sapporo, 001-0020, Japan
30 E-mail: suzuki@czc.hokudai.ac.jp
31 Telephone: +81-11-706-9503; FAX: +81-11-706-7310
32
33

34 **Abstract**

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

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

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

58

59 **Keywords**

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

63 **Introduction**

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

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

78 Typically, diagnosis of mycobacterial infections, including drug-resistance MTB, is
79 carried out by culturing, but these bacteria are slow growing and thus culturing is time-
80 consuming. Therefore, a method to quickly identify drug resistance is needed.

81 Molecular diagnostic methods can quickly identify drug resistance, which can help
82 expedite the development of new drugs for the treatment of drug-resistant MTB.

83 The World Health Organization (WHO) recommended Xpert MTB/RIF® assay as rapid
84 diagnostic test for the detection of TB and RR-TB (WHO, 2017). This assay is a single-
85 use sample-processing cartridge system with integrated multicolor real-time PCR to
86 detect majority of RR associated mutations. WHO states that the system requires no
87 additional experimental equipment and is suitable for use at all levels of the health
88 system, but because of its sophisticated system, it requires a stable, uninterrupted

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

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

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

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 Rifampicin 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

141 and B1 region (Fig. 1). The primers for individual mutations or wild types were tagged
142 with different tags. The LAMP primer set and allele specific primers are listed in Table
143 1 and the assigned positions are shown in Fig. 2.

144

145 **1.3 LAMP reaction**

146 LAMP reactions were carried out in a 10- μ l reaction mixture consisting of 0.8 μ M
147 biotinylated FIP primer, 0.8 μ M biotinylated BIP primer, 0.2 μ M of each outer primer
148 (F3 and B3), 0.8 μ M of each loop primer (FLP and BLP), 1.4 mM dNTP, 1 μ l 10 \times
149 reaction buffer (Nippon gene KK), 4.8 U of Bst DNA polymerase (Nippon gene KK), 1
150 μ l of 3,000-fold diluted SYBR Green I and 1 μ l of extracted DNA. Fluorescence of
151 SYBR Green I was measured every minute for 50 min at 68 $^{\circ}$ C during the ongoing
152 reaction, using a thermal cycler Dice real time system (TAKARA Bio Inc.). To
153 terminate the reaction, the mixture was incubated for 2 min at 80 $^{\circ}$ C.

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

159

160 **1.4 Detection design of STH chromatographic PAS**

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

165

166 **1.5 Detection of mutations from LAMP products using STH chromatographic PAS**

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

174

175 **2. Results**

176 **2.1 LAMP reaction for RRDR region of TB**

177 One FIP, three BIP, two FLP and four BLP primers were examined to amplify the
178 81bp of the RRDR region. To select the optimal primer combinations, the
179 concentration, the reaction temperature and time were examined by real time
180 amplification using SYB Green I. The selected primers and conditions were able to
181 amplify the TB-specific target region. The detection limit was 200 fg/test using the
182 extracted DNA from isolate strain. (Fig. 3)

183

184 **2.2 Detection of the individual mutations using STH chromatographic PAS by** 185 **LAMP**

186 The detection reaction of individual mutations was conducted using the extracted
187 genomic DNA (10 pg/test) . Sequences of the allele-specific primers are shown in Table
188 1. LAMP reactions were carried out containing LAMP primers and two allele-specific
189 primers (wild and mutant-types) of each target sequence in RRDR. After the LAMP
190 reaction, elongation products of each specific primer were detected by nucleic acid
191 chromatography using PAS. The results are shown in Fig. 4. Genotypes of C1349 (wild
192 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

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

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

243 In the present study, it was possible to detect 60% or more of RR-MTB by detecting
244 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

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

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

278

279 **Acknowledgment**

280

281 We thank Dr. Aki Tamaru for providing DNA extracted from clinically isolated *M.*
282 *tuberculosis*.

283

284 **Fund**

285 This study is based on previous results obtained from a project subsidized by the
286 New Energy and Industrial Technology Development Organization (NEDO). The
287 research was supported in part by a grant from the Ministry of Education, Culture,
288 Sports, Science and Technology, Japan(MEXT), the Joint Research Program of the
289 Research Center for Zoonosis Control, Hokkaido University to YS, and in part by Japan
290 Agency for Medical Research and Development (AMED) under Grant Number
291 JP20jk0210005, JP20jm0110021 and JP20wm0125008 to YS.

292

293 **References**

- 294 1. Aye KS, Nakajima C, Yamaguchi T, Win MM, Shwe MM, Win AA, Lwin T,
295 Nyunt WW, Ti T, Suzuki Y. Genotypic characterization of multi-drug-resistant

- 296 *Mycobacterium tuberculosis* isolates in Myanmar. J Infect Chemother.
297 2016;22:174-9. DOI: [10.1016/j.jiac.2015.12.009](https://doi.org/10.1016/j.jiac.2015.12.009)
- 298 2. Badolo A, Okado K, Guelbeogo WM, Aonuma H, Bando H, Fukumoto S, Sagnon
299 N, Kanuka H. Development of an allele-specific, loop-mediated, isothermal
300 amplification method (AS-LAMP) to detect the L1014F *kdr-w* mutation in
301 *Anopheles gambiae s.l.* Malar J. 2012;11:227. DOI: [10.1186/1475-2875-11-227](https://doi.org/10.1186/1475-2875-11-227)
- 302 3. Badolo A, Bando H, Traore A, Ko-ketsu M, Guelbeogo WM, Kanuka H, Ranson
303 H, Sagnon N, Fukumoto S. Detection of G119S *ace-1^R* mutation in field-collected
304 *Anopheles gambiae* mosquitoes using allele-specific loop-mediated isothermal
305 amplification (AS-LAMP) method. Malar J. 2015;14:477. DOI: [10.1186/s12936-](https://doi.org/10.1186/s12936-015-0968-9)
306 [015-0968-9](https://doi.org/10.1186/s12936-015-0968-9)
- 307 4. De Beenhouwer, H., Lhiang, Z., Jannes, G., Mijs, W., Machtelinckx, L., Rossau,
308 R., Traore, H., Portaels, F. Rapid detection of rifampicin resistance in sputum and
309 biopsy specimens from tuberculosis patients by PCR and line probe assay. Tuber
310 Lung Dis. 1995;76:425-30. DOI: [10.1016/0962-8479\(95\)90009-8](https://doi.org/10.1016/0962-8479(95)90009-8)
- 311 5. Global Health Observatory (GHO) data repository. Geneva, Switzrland: World
312 Health Organization (WHO).
313 <http://apps.who.int/gho/data/node.main.1315?lang=en>
- 314 6. Itonaga M, Matsuzaki I, Warigaya K, Tamura T, Shimizu Y, Fujimoto M, Kojima
315 F, Ichinose M, Murata S. Novel methodology for rapid detection of KRAS
316 mutation using PNA-LNA mediated loop-mediated isothermal amplification. PLoS
317 ONE. 2016;11:e0151654. DOI: [10.1371/journal.pone.0151654](https://doi.org/10.1371/journal.pone.0151654)
- 318 7. Monden Y, Takasaki K, Futo S, Niwa K, Kawase M, Akitake H, Tahara M. A
319 rapid and enhanced DNA detection method for crop cultivar discrimination. J
320 Biotechnol. 2014;185:57-62. DOI: [10.1016/j.jbiotec.2014.06.013](https://doi.org/10.1016/j.jbiotec.2014.06.013)

- 321 8. Mori Y, Nagamine K, Tomita N, Notomi T. Detection of loop mediated isothermal
322 amplification reaction by turbidity derived from magnesium pyrophosphate
323 formation. *Biochem Biophys Res Commun.* 2001;289:150-4.
324 DOI: [10.1006/bbrc.2001.5921](https://doi.org/10.1006/bbrc.2001.5921)
- 325 9. Nagamine K, Watanabe K, Ohtsuka K, Hase T, Notomi T. Loop-mediated
326 isothermal amplification reaction using a nondenatured template. *Clin Chem.*
327 2001;47:1742–3. PMID: 11514425
- 328 10. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase
329 T. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res.*
330 2000;28,:e63. DOI: [10.1093/nar/28.12.e63](https://doi.org/10.1093/nar/28.12.e63)
- 331 11. Ohshiro T, Miyagi C, Tamaki Y, Mizuno T, Ezaki T. Development of a rapid
332 diagnostic method for identification of *Staphylococcus aureus* and antimicrobial
333 resistance in positive blood culture bottles using a PCR-DNA-chromatography
334 method. *J Infect Chemother.* 2016;22:372-6. DOI: [10.1016/j.jiac.2016.02.013](https://doi.org/10.1016/j.jiac.2016.02.013)
- 335 12. Rahim Z, Nakajima C, Raqib R, Zaman K, Endtz HP, van der Zanden AG, Suzuki
336 Y. Molecular mechanism of rifampicin and isoniazid resistance in *Mycobacterium*
337 *tuberculosis* from Bangladesh. *Tuberculosis* 2012;92:529-34.
338 DOI: [10.1016/j.tube.2012.07.005](https://doi.org/10.1016/j.tube.2012.07.005)
- 339 13. Takasaki, K., Yamakoshi, Y., Futo, S., 2018. Single-Laboratory Validation of
340 Rapid and Easy DNA Strip for Porcine DNA Detection in Beef Meatballs. *J*
341 *AOAC Int.* 2018;101:1653-6. DOI: [10.5740/jaoacint.17-0394](https://doi.org/10.5740/jaoacint.17-0394)
- 342 14. Saito T, Kikuchi A, Kaneko A, Isozumi R, Teramoto I, Kimura M, Hirasawa N,
343 Hiratsuka M. Rapid and sensitive multiplex single-tube nested PCR for the
344 identification of five human Plasmodium species. *Parasitol Int.* 2018;67:277-83.
345 DOI: [10.1016/j.parint.2018.01.005](https://doi.org/10.1016/j.parint.2018.01.005)

- 346 15. Sakai J, Maeda T, Tarumoto N, Misawa K, Tamura S, Imai K, Yamaguchi T, Iwata
347 S, Murakami T, Maesaki S. A novel detection procedure for mutations in the 23S
348 rRNA gene of *Mycoplasma pneumoniae* with peptide nucleic acid-mediated
349 isothermal amplification assay. J Microbiol Methods. 2017;141:90-6.
350 DOI: [10.1016/j.mimet.2017.08.009](https://doi.org/10.1016/j.mimet.2017.08.009)
- 351 16. Shanmugakani RK, Akeda Y, Yamamoto N, Sakamoto N, Hagiya H, Yoshida H,
352 Takeuchi D, Sugawara Y, Kodera T, Kawase M, Laolerd W, Chaihongsa N,
353 Santanirand P, Ishii Y, Hamada S, Tomono K. PCR-Dipstick Chromatography for
354 Differential Detection of Carbapenemase Genes Directly in Stool Specimens.
355 Antimicrob Agents Chemother. 2017;61:e00067-17. DOI: [10.1128/AAC.00067-](https://doi.org/10.1128/AAC.00067-17)
356 [17](https://doi.org/10.1128/AAC.00067-17)
- 357 17. Takabatake R, Kagiya Y, Minegishi Y, Futo S, Soga K, Nakamura K, Kondo K,
358 Mano J, Kitta K. Rapid Screening Detection of Genetically Modified Crops by
359 Loop-Mediated Isothermal Amplification with a Lateral Flow Dipstick. J Agric
360 Food Chem. 2018;66:7839-45. DOI: [10.1021/acs.jafc.8b01765](https://doi.org/10.1021/acs.jafc.8b01765)
- 361 18. Tam KK-G, Leung KS-S, To SW-C, Siu GK-H, Lau TC-K, Shek VC-M, Tse CW-
362 S, Wong SS-Y, Ho P-L, Yam W-C. Direct detection of *Mycobacterium*
363 *tuberculosis* and drug resistance in respiratory specimen using Abbott Realtime
364 MTB detection and RIF/INH resistance assay. Diagn Microbiol Infect Dis.
365 2017;89,:118-24. DOI: [10.1016/j.diagmicrobio.2017.06.018](https://doi.org/10.1016/j.diagmicrobio.2017.06.018)
- 366 19. Telenti A, Imboden P, Marchesi F, Lowrie D, Cole S, Colston MJ, Matter L,
367 Schopfer K, Bodmer T, Detection of rifampicin-resistance mutations in
368 *Mycobacterium tuberculosis*. Lancet 1993;341:647-50. DOI: [10.1016/0140-](https://doi.org/10.1016/0140-6736(93)90417-f)
369 [6736\(93\)90417-f](https://doi.org/10.1016/0140-6736(93)90417-f)

- 370 20. Tian L, Sato T, Niwa K, Kawase M, Tanner ACR, Takahashi N. Rapid and
371 sensitive PCR-dipstick DNA chromatography for multiplex analysis of oral
372 microbiota. Biomed Res Int. 2014: 180323. DOI: [10.1155/2014/180323](https://doi.org/10.1155/2014/180323)
- 373 21. Tian L, Sato T, Niwa K, Kawase M, Mayanagi G, Washio J, Takahashi N. PCR-
374 dipstick DNA chromatography for profiling of a subgroup of caries-associated
375 bacterial species in plaque from healthy coronal surfaces and periodontal pockets.
376 Biomed Res Int. 2016;37:29-36. DOI: [10.2220/biomedres.37.29](https://doi.org/10.2220/biomedres.37.29)
- 377 22. World Health Organization (WHO). Global tuberculosis report 2019. Geneva,
378 Switzerland: World Health Organization; 2019.
379 [https://apps.who.int/iris/bitstream/handle/10665/329368/9789241565714-](https://apps.who.int/iris/bitstream/handle/10665/329368/9789241565714-eng.pdf?ua=1)
380 [eng.pdf?ua=1](https://apps.who.int/iris/bitstream/handle/10665/329368/9789241565714-eng.pdf?ua=1)
381

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-ACGTGCACCCGTCGCACTTGTGGGCCCTCAGG
	B3	AGCGAGCCGATCAGACC
	BLP	CGGATGTGCCCGATCGAAA
	Allele-detection	C1349
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

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		-	+	-	+	+	-	+	-

388 **Figure legends**

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

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

396

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

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

402

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

404 The sensitivity and specificity of the LAMP reaction was measured with a real-time
405 PCR instrument (Dice real time system). The results showed a detection limit of
406 200fg/test for the RRDR gene from the MTB genome DNA and no cross reactivity for
407 *Mycobacterium avium*, *Mycobacterium intracellulare* and *Mycobacterium kansasii*.

408

409 Figure 4. Detection results of individual mutation.

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

414 mutation type signal (tag2). No. 3 and 4 used 513 wild and mutation primers,
415 respectively. The wild type genome used No. 3 reaction and the mutation type genome
416 used No. 4 reaction. The results showed No. 3 detected only wild type primer signal
417 (tag4) and No. 4 detected only mutation type signal (tag3). No. 5 and 6 used 516 wild
418 and mutation primers, respectively. The wild type genome used No. 5 reaction and the
419 mutation type genome used No. 6 reaction. The results showed No. 5 detected only wild
420 type primer signal (tag7) and No. 6 detected only mutation type signal (tag8). No.7 and
421 8 used 516 wild and mutation primers, respectively. The wild type genome used No.7
422 reaction and the mutation type genome used No.8 reaction. The results showed the No.7
423 detected only wild type primer signal (tag5) and No. 8 detected only mutation type
424 signal (tag6).

425

426 Figure 5. Detection results of multi-detection

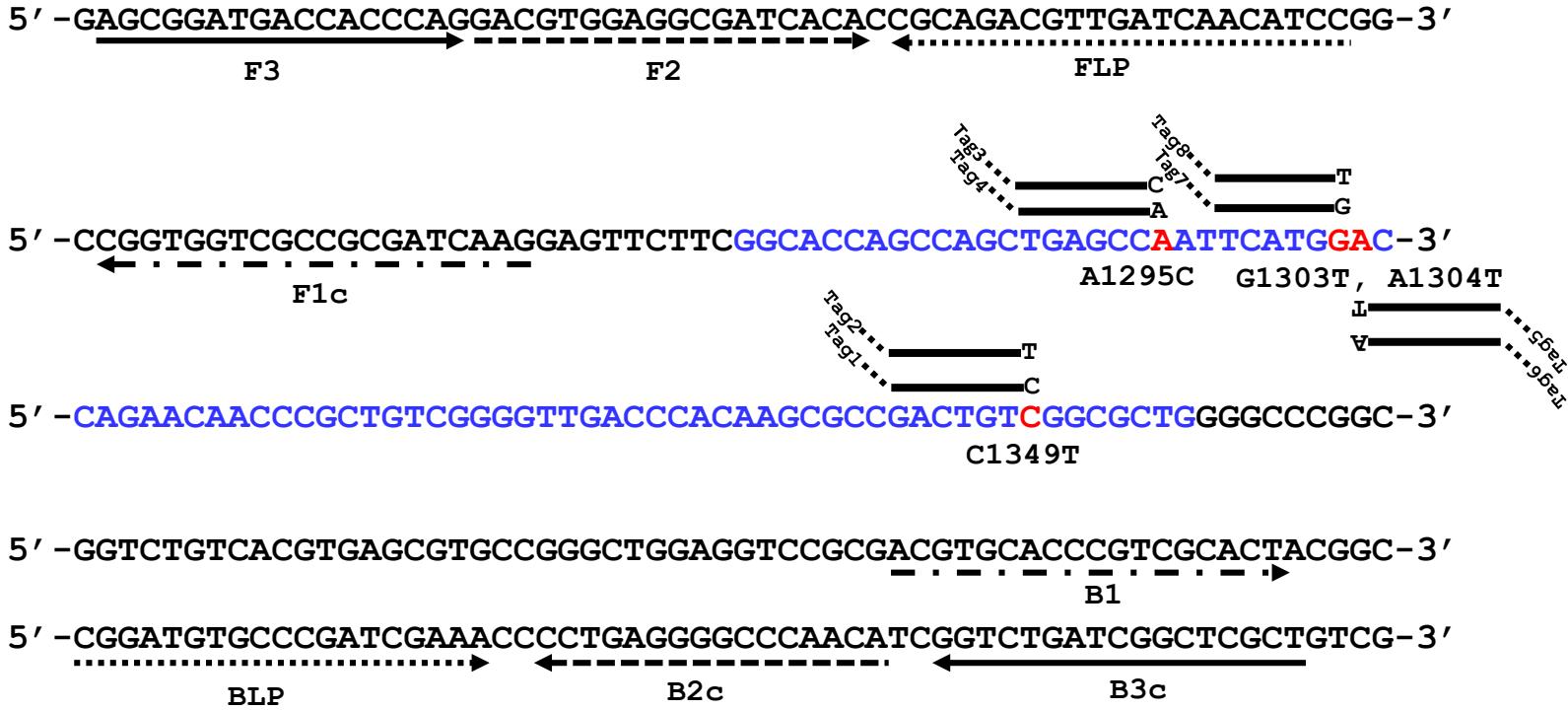
427 Multi-mutations were detected using the eight allele-specific primers for each mutation.
428 The extracted genome sample are summarized in Table 2. The results show that four
429 mutations were precisely detected.

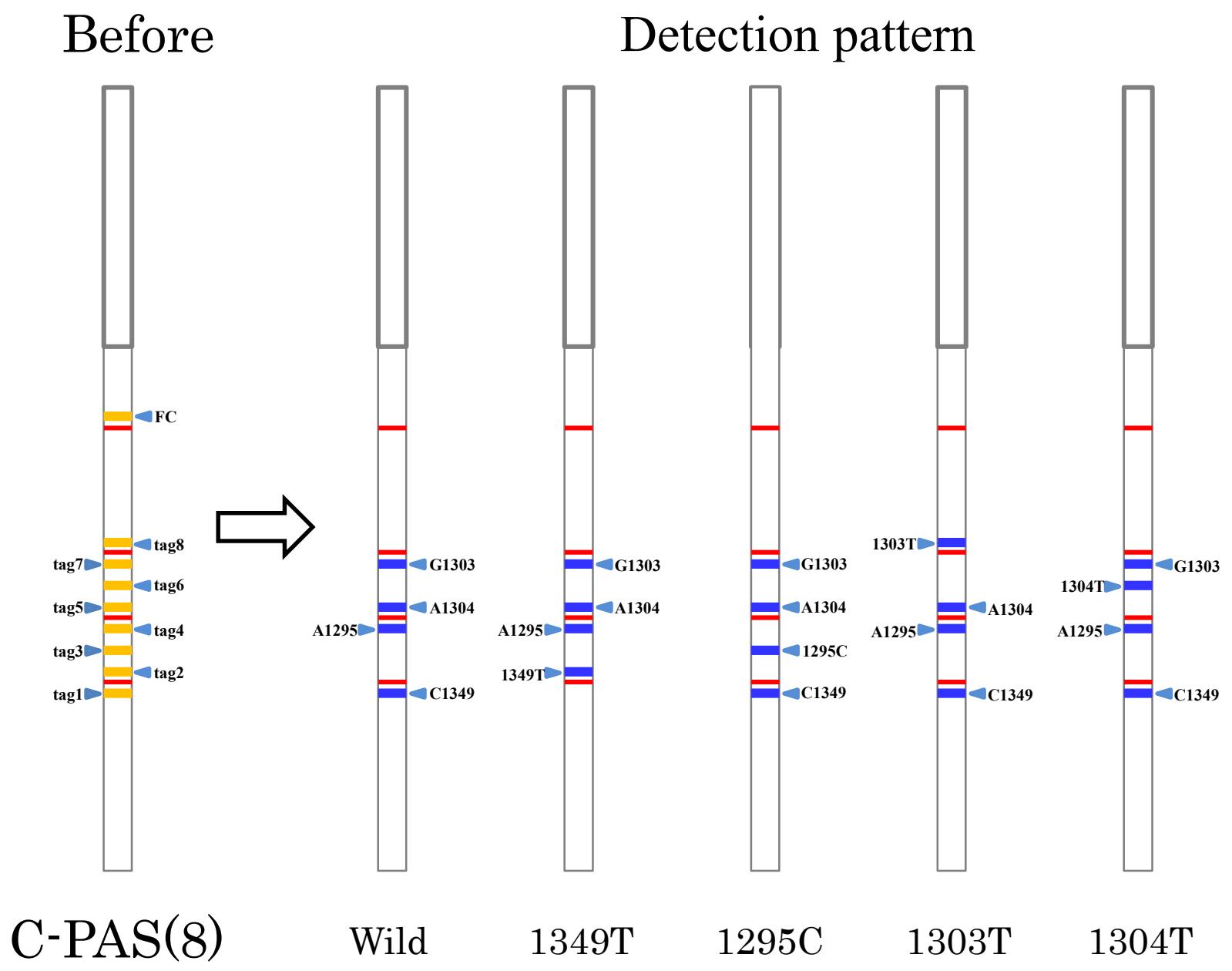
430

431 Figure 6. Detection principle for STH chromatographic PAS using LAMP

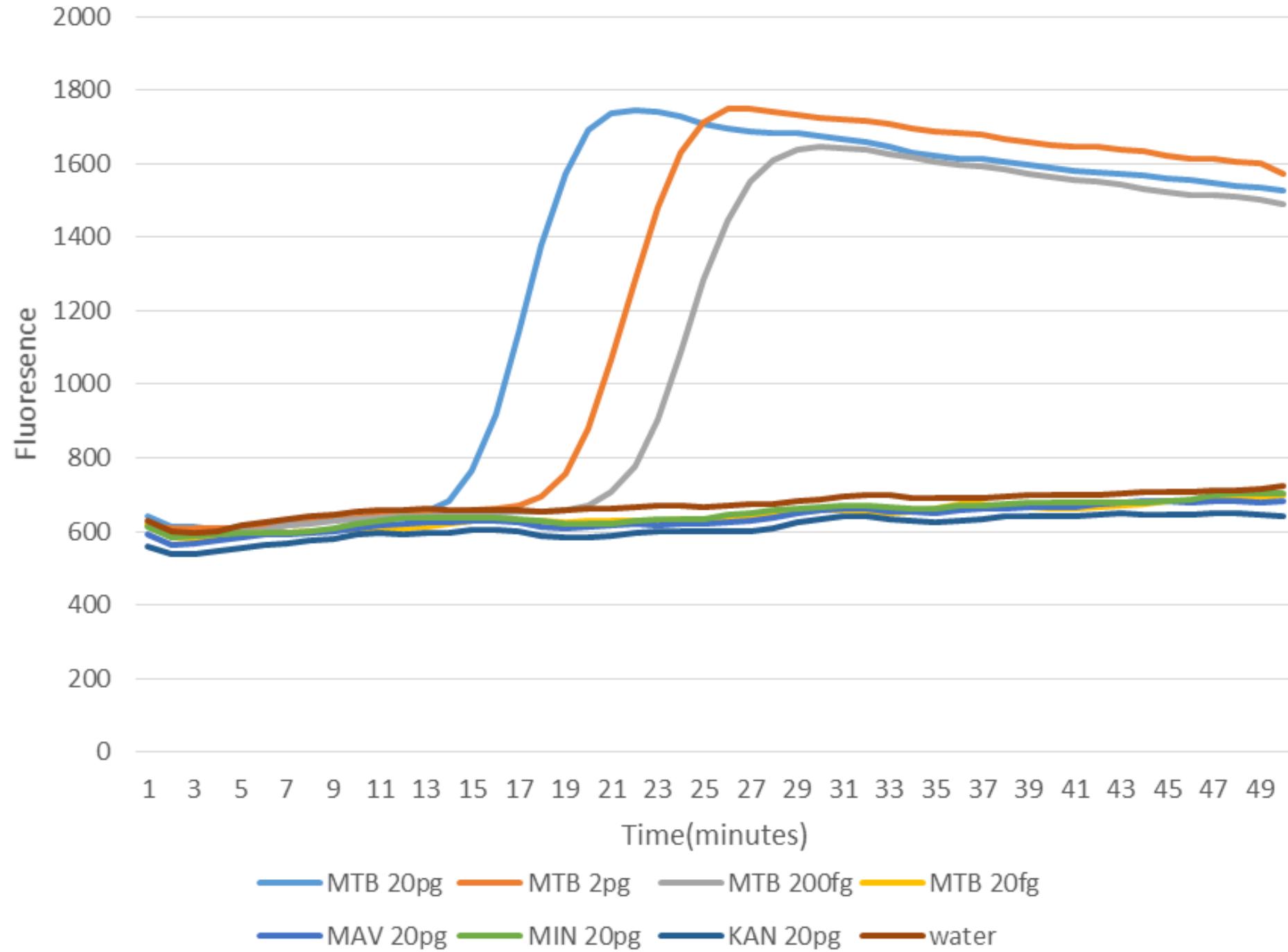
432 (A) To detect with the STH chromatographic PAS method, an amplification product
433 with a tag and biotin will be synthesized by isothermal amplification. In this scheme, the
434 extension product of tag-attached allele-specific primer forms a double strand with
435 biotin-attached to the elongation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

Takarada et al. Figure 1

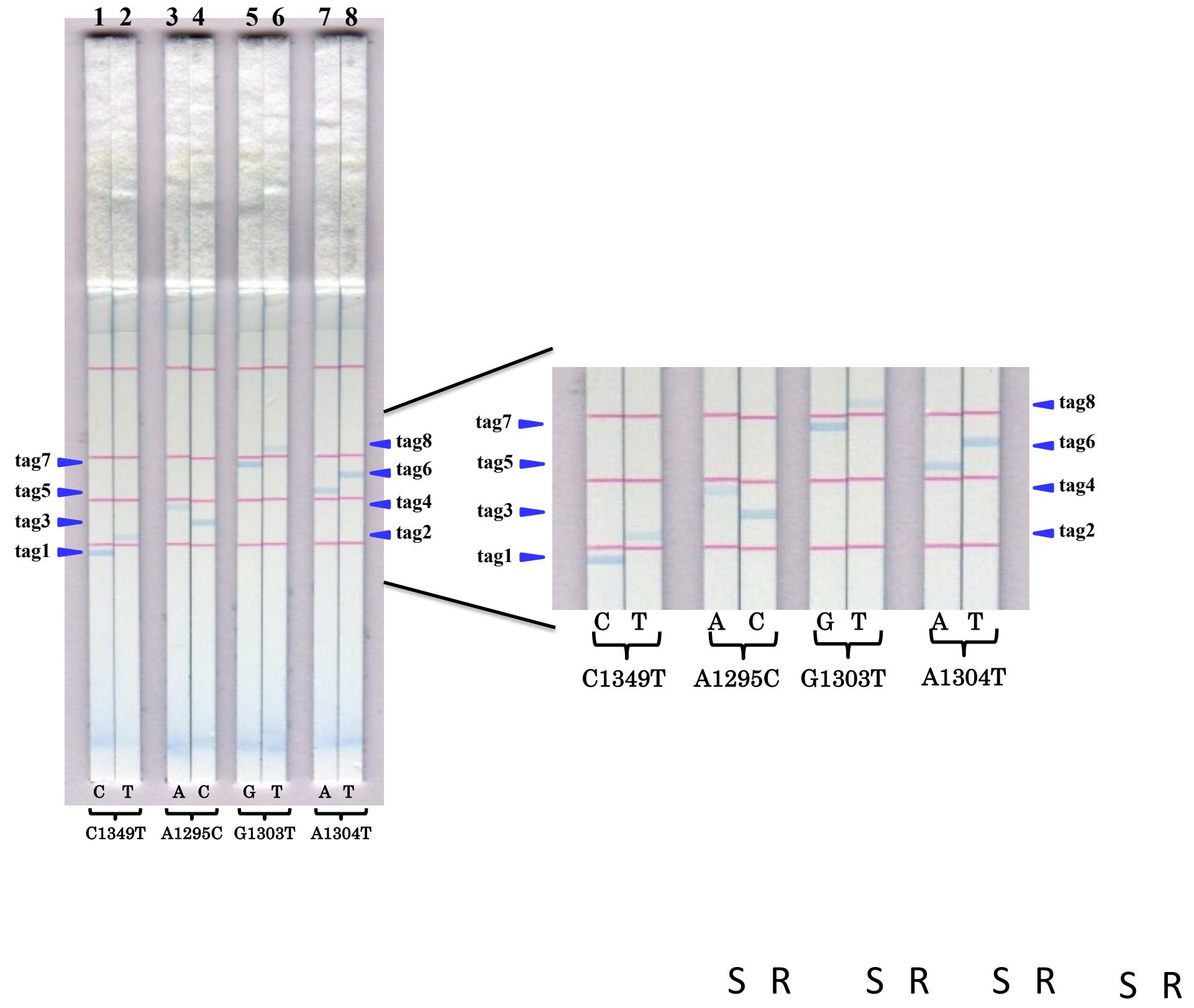




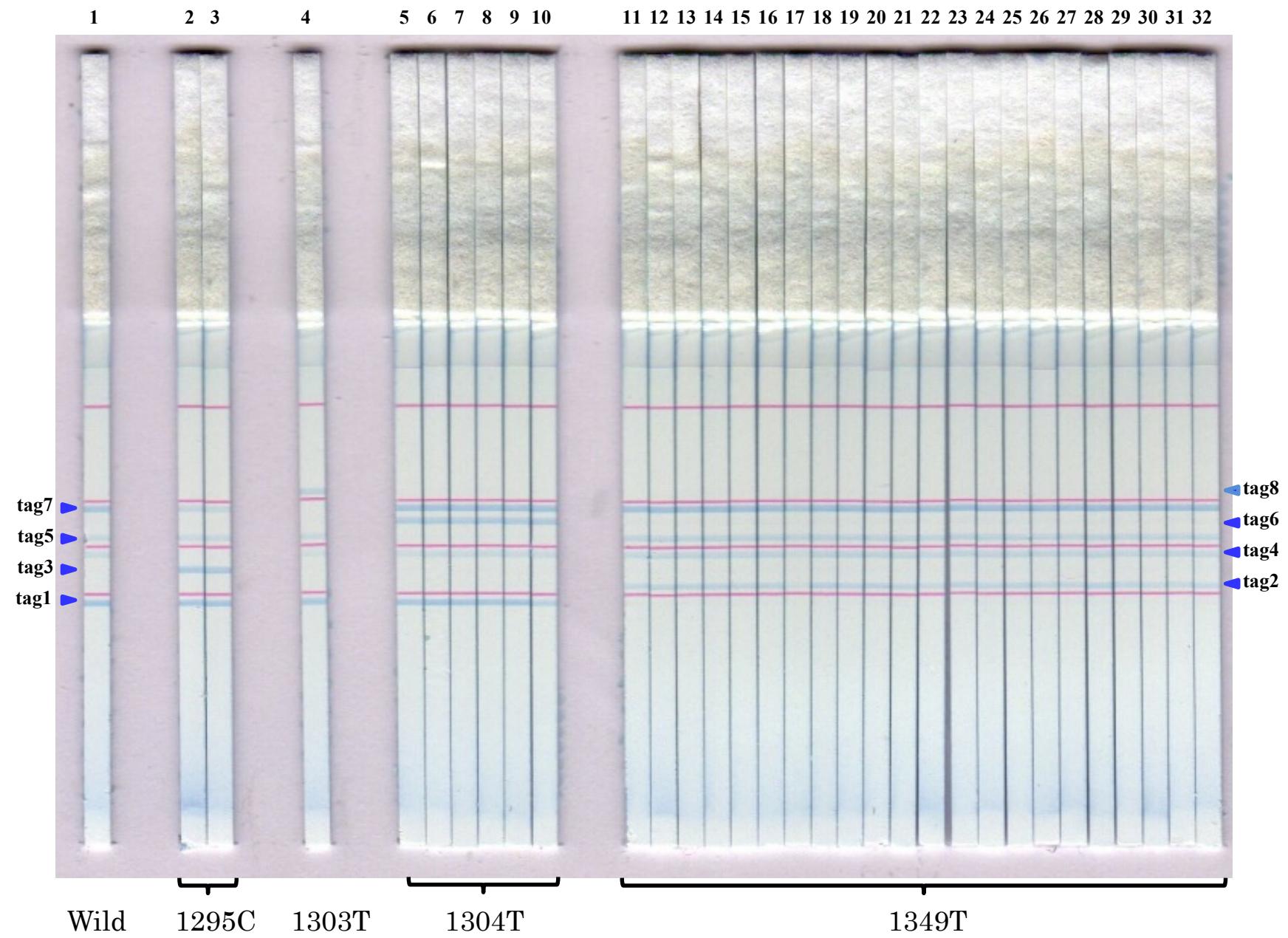
Takarada et al. Figure 3



Takarada et al. Figure 4

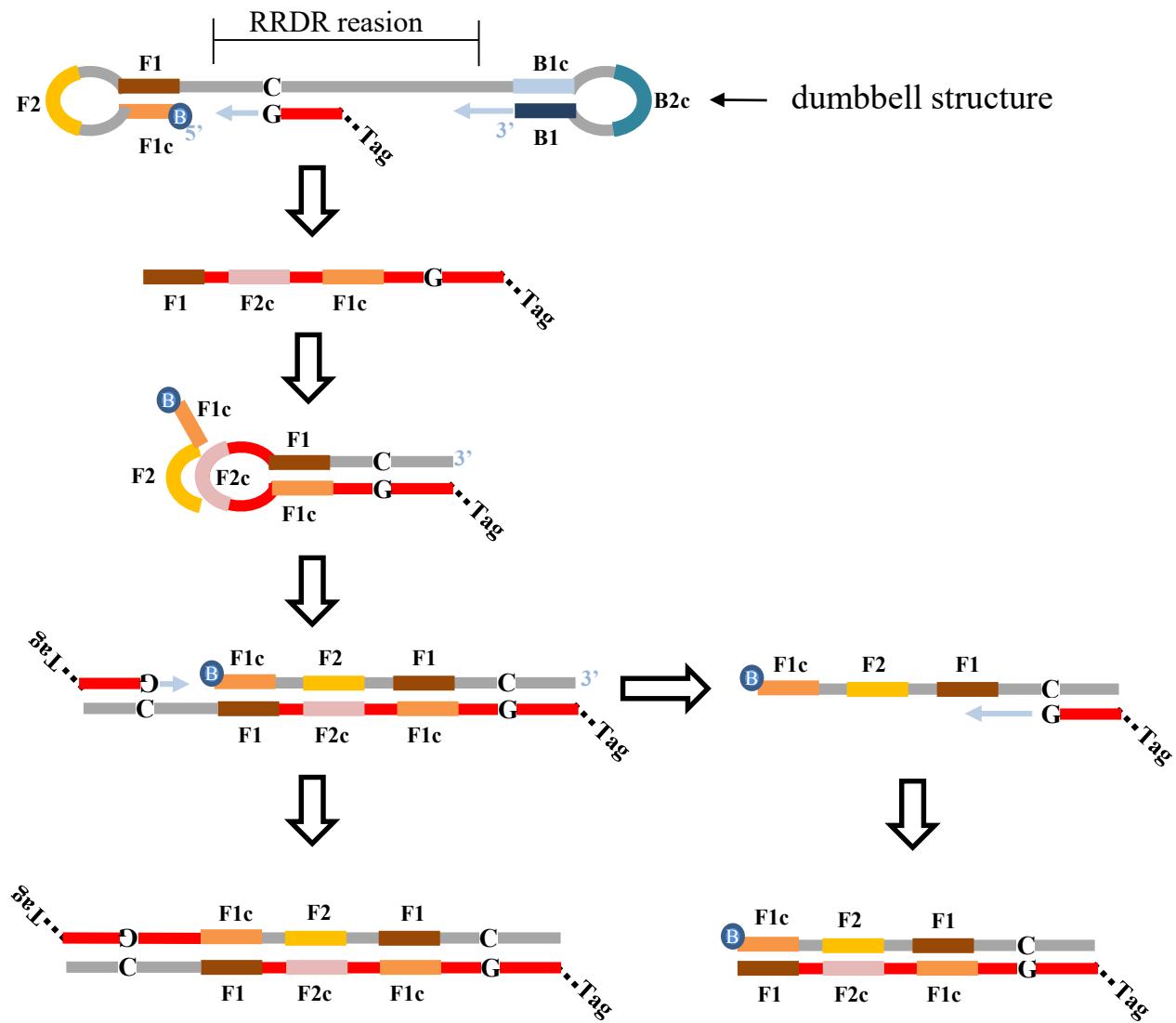


Takarada et al. Figure 5



Takarada et al. Figure 6

A



B

