A new method for detection of single nucleotide polymorphism in a female reproduction-associated gene, *tmigd1,* of *Anas platyrhynchos* using a strip biosensor with gold nanoparticles

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ABSTRACT In this study, we first reported a lateral flow assay combined with primer extension (**PEXT**) and gold nanoparticles for single-nucleotide polymorphism (**SNP**) genotyping of the *tmigd1* gene of the Tsaiya ducks (*Anas platyrhynchos*), which has the advantages of simplicity of operation, cost-effectiveness, and time-saving. Gold nanoparticles were tailed with thiol-thymine oligodeoxyribonucleotides (thiol-$(dT)_{30}$) using the salt-aging method at 25°C and used as a label in a lateral flow assay. The lateral flow device was composed of test and control zones on a nitrocellulose membrane containing streptavidin and adenosine oligodeoxyribonucleotides ($(dA)_{30}$), respectively. When the specific SNP existed, the corresponding primers were extended, and the reaction product was captured by streptavidin at the test zone owing to the introduction of biotin-deoxyuridine triphosphate (biotin-dUTP) into the reaction product during PEXT. Gold nanoparticles hybridized with the reaction product to render it visible. Here, we developed a new system for detection of single nucleotide polymorphism in a female reproduction-associated gene, *tmigd1,* of *Anas platyrhynchos* using the strip biosensor, and identified the optimized parameters for the concentration of Mg^{2+} in the PEXT reaction and the amount of streptavidin used on membranes for signal specificity.

Key words: lateral flow assay, gold nanoparticle, *tmigd1* gene, single nucleotide polymorphism (SNP), Tsaiya ducks (*Anas platyrhynchos*)

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INTRODUCTION

Single nucleotide polymorphism (**SNP**) is a variance of one base, which occurs one in a thousand of base pair when one compares 2 human genomes (Kwok and Chen, 2003). Thus, SNPs can be used as biomarkers for the detection of genetic predispositions to diseases (Hofker et al., 2014; Dunaif, 2016; Jones et al., 2016) and economic traits of farm animal (Huang et al., 2011a,b, 2013a,b; Jin et al., 2013; Huang and Cheng, 2014; Guo et al., 2016). In addition, this variation can be used as a genetic marker for application in genetic mapping, in which genes are located and their functions are identified (Baird et al., 2008; Mousavi et al., 2016; Zhang et al., 2016a,b).

Single nucleotide polymorphism genotyping method is composed of 2 main steps: polymerase chain reaction amplification and allele discrimination. Allele discrimination approaches include restriction fragment polymorphism analysis, hybridization with allele-specific oligonucleotide probes, melting curve analysis, oligonucleotide ligation, and primer extension (**PEXT**) reactions (Litos et al., 2007). However, enzyme-based genotyping methods are preferred owing to their high specificity (Toubanaki et al., 2009; Yamada et al., 2016; Zhang et al., 2016a,b). PEXT, an enzyme-based method, is most commonly employed for genotyping because of its high accuracy of nucleotide incorporation via deoxyribonucleic acid (**DNA**) polymerase.

Mass spectrometry, fluorescence resonance energy transfer, flow cytometry (Pickering et al., 2004), microtiter well-based chemiluminometric assays (Zerefos et al., 2006; Glynou et al., 2007; Konstantou et al., 2007), microarray analysis (Lu et al., 2005), restriction fragment length polymorphism (**RFLP**), etc., are SNP genotyping methods. However, all of the above detection techniques require expensive specialized equipment, reagents or massive lab work. Lateral flow assay (**LFA**) is a useful alternative method for SNP detection owing to the low cost, disposability, simplicity, potential for visual detection using only a few assay steps, and minimal requirement for qualified personnel (Boulware et al., 2014). Furthermore, the chemical, physical, and optical properties of gold nanoparticles

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allow them to bind oligonucleotide probes, and to be utilized as a visual tool in detection techniques without special equipment, which have been applied in various fields (Hurst et al., 2006; Hou et al., 2007; Dykman and Khlebtsov, 2012). Those particles even can be visualized with naked eye when they are in a specific aggregation-distance (Elghanian et al., 1997).

Our previous studies (Huang et al., 2011a, 2013a,b; Huang and Cheng, 2014) reported that SNPs in a series genes were associated with good duck reproductive performance, and can be used to increase reproductive ability in Tsaiya ducks (*Anas platyrhynchos*), the major egg-laying duck in Taiwan (Poivey et al., 2001; Cheng et al., 2002). However, we have been looking for simpler methods for detection of SNPs. The present work first demonstrated a novel DNA biosensor for the detection of SNP in reproduction-associated gene (Huang et al., 2011b), transmembrane, and immunoglobulin domain containing 1 (***tmigd1***), of Tsaiya ducks.

MATERIALS AND METHODS

Animals and DNA

Tsaiya ducks involved in this study were raised in the Livestock Research Institute, Council of Agriculture, Taiwan. The care and use of these research animals met the guidelines approved by the institutional animal care and use committee. Genomic DNA was isolated from the blood of Tsaiya ducks using the method described in a previous study (Huang and Cheng, 2014) for strip assay.

Materials

Vent (exo-) DNA polymerase and ThermoPol II (Mg^{2+}-free) reaction buffer packs were purchased from New England Biolabs (Beverly, Massachusetts, USA). DyNAzyme II DNA polymerase with optimized DyNAzyme buffer was purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). PCR primers were obtained from Mission Biotech (Taipei, Taiwan). PEXT primers, 2′-deoxyribonucleoside 5′-triphosphates (**dNTPs**), 5′-thiol-thymine oligodeoxyribonucleotides (5′-Thiol-$(dT)_{30}$), adenosine oligo deoxyribo nucleotides (**$(dA)_{30}$**), and 10 × phosphate buffer saline (**PBS**) were obtained from Protech Technology Enterprise (Taipei, Taiwan). Biotin-16-deoxyuridine triphosphate (**Biotin-16-dUTP**) was obtained from Biotium (Fremont, California, USA), and streptavidin was purchased from Amresco (Solon, Ohio, USA). Gold nanoparticle (**AuNP**) solution (40 nm), tris(2-carboxyethyl)phosphine (**TCEP**), sucrose, and Tween-20 were obtained from Sigma-Aldrich (Saint Louis, Missouri, USA). Glycerol and sodium dodecyl sulfate (**SDS**) were purchased from Avantor Performance Materials (Center Valley, Pennsylvania, USA). Sodium chloride (**NaCl**) was obtained from Showa Chemical Industry (Tokyo, Japan). Methanol was purchased from Echo Chemical (Miaoli, Taiwan). The sequence and use of each oligonucleotide are presented in Table 1.

Instrumentation

PCR and PEXT reactions were performed using an Applied Biosystems 2720 thermal cycler (Brockton, Massachusetts, USA). A dry bath incubator purchased from Major Science (Saratoga, California, USA) was used for denaturation of the PEXT product. A DELTA ultrasonic cleaner DC-150H from Delta New Instrument (New Taipei, Taiwan) and a Microfuge16 microcentrifuge from Beckman Coulter (Brea, California, USA) were used for the preparation of oligonucleotide-conjugated gold nanoparticles. A Canon Powershot G16 digital camera (Canon Inc., Tokyo, Japan) was used to record the experimental results.

PCR of the tmigd1 Gene and PEXT Reaction

To conduct the following experiments, 30 tests of samples, known-genotype ducks examined by genotyping of real-time PCR or restriction fragment length polymorphism in an unpublished data, were used. PCR amplifications were performed in a final volume of 25 μL consisting of 25 ng genomic DNA, 300 nM of each primer, 200 μM of each dNTP, 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, and 1 unit DyNAzyme II DNA polymerase. The PCR cycling conditions were as follows: denaturation at 95°C for 2 min, then 30 cycles at 94°C for 30 s, annealing at 53°C for 15 s, and extension at 72°C for 11 s.

Primer extension reactions were performed in a total volume of 20 μL containing 1 μL of above PCR product; 1 pmol of the G or A-targeted PEXT primers of 50 base pair containing $(dA)_{27}$; 2.5 μM of dATP, dCTP, and dGTP; 1.25 μM of dTTP and biotin-16-dUTP; 20 mM Tris-HCl (pH 8.8); 10 mM $(NH4)_2SO_4$; 10 mM KCl; 1 or 2 mM $MgSO_4$; 0.1% Triton X-100; and 0.4 units of Vent (exo-) DNA polymerase. The PEXT conditions were as follows: denaturation at 95°C for 5 min, then 3 to 7 cycles at 95°C for 15 s, annealing at 60°C for 10 s, and extension at 72°C for 15 s.

Preparation of Oligonucleotide-Conjugated Gold Nanoparticles

5′-thiol-$(dT)_{30}$ was in disulfide form. The mixture of 83.3 μM 5′-thiol-$(dT)_{30}$ and 8.3 mM TCEP was therefore incubated at 25 °C for one hour to reduce the disulfide bond. Subsequently, 0.61 μL of the mixture was added to 500 μL gold nanoparticle solution (40 nm, approximately 0.6 fmol of AuNP) and incubated at room temperature for 20 min. After incubation, 25 μL of 2 molar NaCl was added to the mixture, which was sonicated for 10 to 20 s

Table 1. Oligonucleotides used in this study.

Purpose	Name	Sequence (5′-3′)	Size (b.p.)[a]
PCR primers	TMi2F1[b]	CCTGAGGATTGCCTAAAAGC	20
	TMi2R5[c]	TGGGAAAACAGTATGGGAA	19
PEXT primers	G_Primer	$(A)_{27}$GTGCCTTTATATTCTGCAATGTG	50
	A_Primer	$(A)_{27}$GTGCCTTTATATTCTGCAATGTA	50
Oligonucleotide on nano-gold	Thiol-$(dT)_{30}$	Thiol-$(dT)_{30}$	30
Control zone	$(dA)_{30}$	$(dA)_{30}$	30

[a]b.p. is base pair.
[b]TM is tmigd1, i2 is intron 2, and F is forward.
[c]R is reverse.

and incubated for another 20 min. This process was repeated 10 times until the final concentration of NaCl was 0.67 M at room temperature for 24 h. Subsequently, the solution was centrifuged at 13,000 rpm for 20 min, and the supernatant was removed. The remaining particles were resuspended in 24 μL storage solution containing 30% sucrose, 45 mM NaCl, 0.25% SDS, and 0.25% Tween-20.

Preparation of Dry Reagent Strips and Genotyping Test

The lateral flow assay in this work was obtained from the Easypack Developer's Kit produced by Advanced Microdevices (Ambala, India). The strip is composed of a glass fiber sample pad (**GF-S**), a polyester conjugate pad (**PE-C**), a nitrocellulose membrane (**NC**), and an adsorbent pad (**AD**) supported by a plastic backing. The 5 parts of strip were assembled as follows. Firstly, streptavidin solution (0.4 μL) containing 2% of sucrose was loaded onto the NC membrane using a Pipetman as a "test spot (T)". Furthermore, 0.2 μL of $(dA)_{30}$ solution containing 1 × PBS, 5% methanol, and 2% of sucrose was loaded onto the NC membrane using a Pipetman as a "positive control spot (C)". Secondly, gold nanoparticle-$(dT)_{30}$ (Au-$(dT)_{30}$) solution (4 μL) obtained by the above-described procedure was loaded onto the conjugate pad (PE-C) using the Pipetman. Finally, after drying the NC and PE-C parts of strip with a hair dryer for approximately 30 s, the 5 parts of strip were then assembled.

For genotyping, the sequence of the tested samples is "GTGCCTTTATATTCTGCAATGT[G/A]", and [G/A] is the site of polymorphism. The PEXT reaction products were denatured at 95°C for 5 min and on ice for 2 min. The denatured PEXT products (10 μL) were applied onto the sample pad (GF-S), following which the strip was dipped into a 1.5-mL centrifuge tube holding 220 μL of running buffer containing 1 × PBS, 3% glycerol, and 1% SDS for 10 min. The direction of flow was from GF-S, PE-C (where Au-$(dT)_{30}$ can conjugate the extended PEXT-biotin product which matched the SNP; for the non-extended PEXT product which did not matched the SNP, it can conjugate Au-$(dT)_{30}$, but was without biotin to be captured by the latter position of test spot), NC, to AD of strip. The signal (the pink color of gold nanoparticles: Au) in positive control spot (C) indicated that our lateral flow system of *tmigd1* genotyping was successful. The experiment was recorded using a Canon Powershot G16 digital camera, and the intensity of the test spot was analyzed using ImageJ software.

RESULTS AND DISCUSSION

Effect of the Amount of Streptavidin on the Specificity of tmigd1 Genotyping

Figure 1 is the composition of the strip and figure 2 reveals a preliminary experiment of background signal of the strip system. In this experiment, we applied only streptavidin to the test zone of NC pad with no test sample of the PEXT product during strip preparation step. This test is based on the fact that streptavidin is a protein, which can adsorb onto colloidal gold through a combination of electrostatic and hydrophobic interactions (Konstantou et al., 2007). Figure 2 shows the effect of streptavidin amounts on the signal intensity of the test zone without test sample of the PEXT product. Decreasing the amount of streptavidin from 2 to 0.25 μg resulted in an decreasing signal intensity of the test zone. The result indicated that streptavidin of 0.25 μg cannot react with gold nanoparticles. Therefore, when preparing test zone of NC pad for the strip system, the amount of streptavidin should be equal to or below 0.25 μg.

Since the main factors affecting the specificity of the strip result are the concentrations of streptavidin and Mg^{2+}, we search literatures and found that the optimal concentration of Mg^{2+} for DNA polymerase should be 1 to 2 mM (Harris and Jones, 1997; Lorenz, 2012). Here, we started from 2 mM of Mg^{2+} in PEXT reaction. Considering the addition of PEXT mixture, Figures 3–5 show the detection of G/G, A/A, and G/A genotypes of *tmigd1* gene using streptavidin of 0.25 to 0.12 μg at test spot during strip preparation step. In the case of 0.25 μg (Figure 3), all 3 genotypes showed signals for both the G and A primers. In theory, no signal at test spot can be detected for G/G genotyping with the A primer extension and for A/A genotyping with the G primer extension in PEXT reaction. Hence, Figure 3 indicated streptavidin amount of 0.25 μg could be too high. When the streptavidin amount was decreased

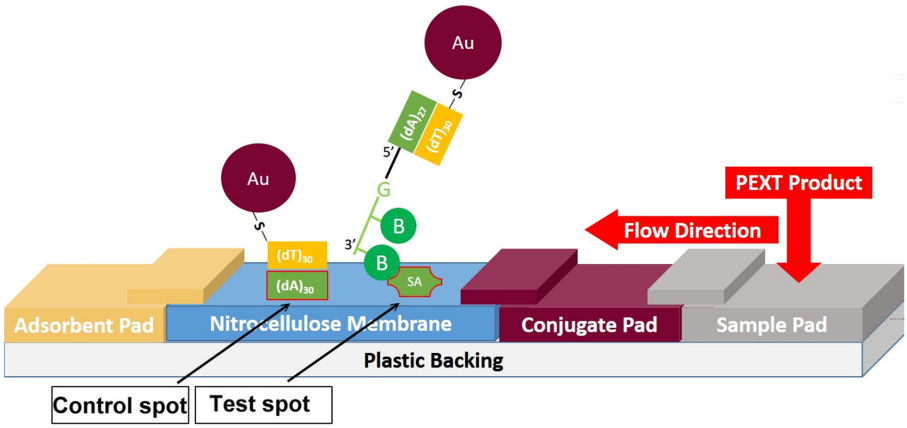

Figure 1. The composition of lateral flow assay. The PEXT reaction product was applied to the sample pad (GF-S) and then the strip was immersed in running buffer. As running buffer moved along the membrane, PEXT product with biotin and gold nanoparticles were captured by streptavidin at test spot. "SA" and "B" represent streptavidin and biotin, respectively.

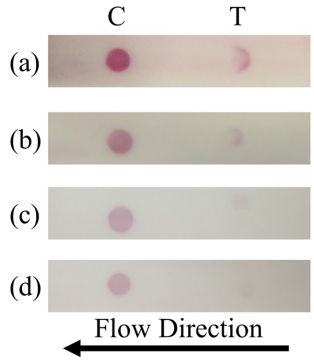

Figure 2. Effect of the amount of streptavidin ((a) 2, (b) 1, (c) 0.5, and (d) 0.25 μg) for strip preparation step on the detectable signal intensity of the representative test zone without primer extension (PEXT) products. T and C represent the test and control zones, respectively.

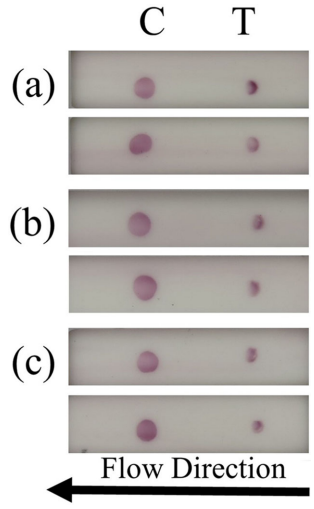

Figure 3. Representative lateral flow-based detection of (a) G/G, (b) A/A, and (c) G/A genotypes of ducks with 0.25 μg of streptavidin for strip preparation and 2 mM Mg^{2+} in PEXT reaction. The upper and bottom strips represent the G and A primer extensions for PEXT, respectively. Symbols T and C represent the test and control spots, respectively.

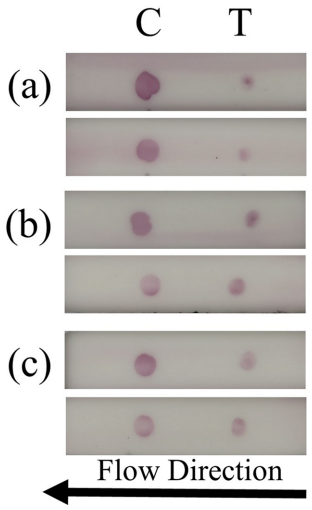

Figure 4. Representative lateral flow-based detection of (a) G/G, (b) A/A, and (c) G/A genotypes of ducks with 0.16 μg of streptavidin and 2 mM Mg^{2+}. The upper and bottom strips represent the G and A primer extensions for PEXT, respectively. The abbreviations of T and C in this figure are the same as in Figure 3.

from 0.16 to 0.12 μg (Figures 4 and 5), the detected signals of G/G, A/A, and G/A genotypings were also reduced in the cases of both the G and A primers at test spot. However, the difference in the detected signals of G/G and A/A at strips was not humungous between the G and A primer extension in most of the cases from Figures 3 to 5, with the exception of the A/A genotyping with 0.12 μg of streptavidin (Figure 5b). Those result until Figure 5 indicated that only A/A genotyping was feasible with 0.12 μg streptavidin for test zone preparation and 2 mM of Mg^{2+} in PEXT reaction in this strip system. It is worth noting that in this study, we intended not to purify the PCR or PEXT products in order to simplify the experimental process and reduce the duration and cost of the strip assay.

After the denaturation, we obtained single strands of extended PEXT product containing dA_{27} and biotin. These single strands were labeled with Au-dT_{30} when

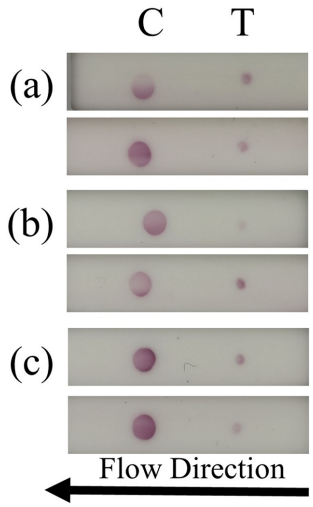

Figure 5. Representative lateral flow-based detection of (a) G/G, (b) A/A, and (c) G/A genotypes of ducks with 0.12 μg of streptavidin and 2 mM Mg^{2+}. The upper and bottom strips represent the G and A primer extensions for PEXT, respectively. The abbreviations of T and C in this figure are the same as in Figure 3.

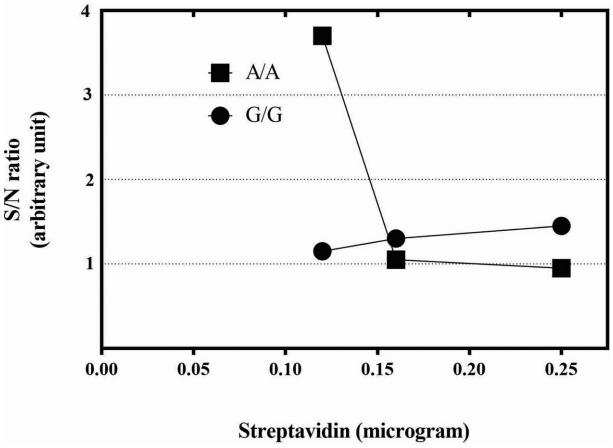

Figure 6. Effect of streptavidin on the signal-to-noise (S/N) ratio obtained from the representative lateral flow assay of the PEXT product using 2 mM Mg^{2+} for the G/G genotype DNA sample. The data were calculated from Figure 3 (a and b), Figure 4 (a and b), and Figure 5 (a and b).

they moved through PE-C pad, and later were captured by streptavidin and observed at the test spot of NC pad. Hypothetically, taking the G/G genotyping as an example, the signal intensity of the test spot was resulted from specific annealing of the G primer with SNP leading to successful PEXT extension; for the A primer, if there was any signal at the test spot, it would be referred to as non-specific products. Therefore, the signal obtained from the specific PEXT product with SNP and that arising from non-specific PEXT products were considered as signal (S) and noise (N), respectively, at test spot.

To convert signal display from spot intensities (Figures 3 to 5) to a number, as shown in Figure 6, we calculated the signal-to-noise (S/N) ratio at test spot of strip of either G or A primer extension in the PEXT reaction. The result confirmed the above description

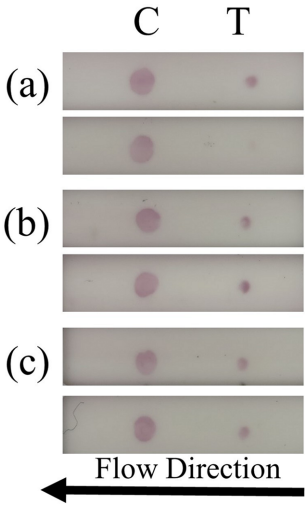

Figure 7. Representative lateral flow-based detection of (a) G/G, (b) A/A, and (c) G/A genotypes of ducks with 0.25 μg of streptavidin for strip preparation and 1 mM Mg^{2+} in PEXT reaction. The upper and bottom strips represent the G and A primer extensions for PEXT, respectively. T and C represent the test and control spots, respectively.

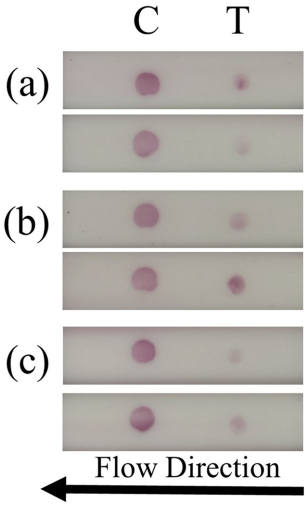

Figure 8. Representative lateral-flow based detection of (a) G/G, (b) A/A, and (c) G/A genotypes of ducks with 0.16 μg of streptavidin and 1 mM Mg^{2+}. The upper and bottom strips represent the G and A primer extensions for PEXT, respectively. The symbols of T and C in this figure are the same as in Figure 7.

which related to the effect of 0.25 to 0.12 μg streptavidin on genotyping: for the G/G genotyping, the S/N ratios were all close to a value of one, which indicated no vast differences in the signals between the G and A primer extensions at all streptavidin levels. For the A/A genotyping, the S/N ratio was approximately 4-fold higher at 0.12 μg of streptavidin than at 0.16 or 0.25 μg of streptavidin.

Effect of Mg^{2+} Concentration on the Specificity of tmigd1 Genotyping

We also examined effect of Mg^{2+} concentration of 1 mM in PEXT reaction on the specificity of genotyping. Figures 7–9 show detection of the G/G, A/A, and

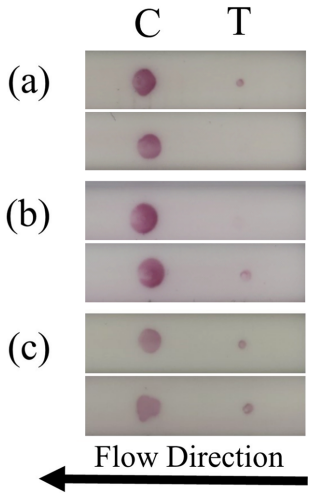

Figure 9. Representative lateral flow-based detection of (a) G/G, (b) A/A, and (c) G/A genotypes of ducks with 0.12 μg of streptavidin and 1 mM Mg^{2+}. The upper and bottom strips represent the G and A primer extensions for PEXT, respectively. The symbols of T and C in this figure are the same as in Figure 7.

G/A genotypings for *tmigd1* gene with 0.25 to 0.12 μg streptavidin and 1 mM Mg^{2+}. In the case of the G/A genotyping, when the streptavidin amount was used from 0.25 to 0.12 μg, the signals were almost equal for both the G and A primer extensions. As for the G/G genotyping, a signal was strongly detected for the G primer, and, on the contrary, no signal was detected for the A primer at test spots of 0.12 μg streptavidin (Figure 9a). In the case of the A/A genotyping, a signal was strongly detected for the A primer, and signal was barely detected for the G primer at test spots of 0.12 μg streptavidin (Figure 9b). The results proved the specificity and evenness for the G/G, A/A, and G/A genotyping signals of *tmigd1* gene (Figure 9). On the basis of the above results, we use 0.12 μg of streptavidin for strip preparation combined with 1 mM Mg^{2+} for PEXT reaction as the optimized setup for our system.

In this work, we successfully developed a SNP detection strip of *tmigd1* gene for Tsaiya ducks. Optimal results for the G/G and A/A genotyping were obtained under the experimental condition of 1 mM Mg^{2+} combined with 0.12 μg of streptavidin. The steps for optimization is only for the establishment of the method. It will be easier to spot sample to a pre-assembled pad with prepared C and T zones (we will use our system as a ready-to-use strip for a future commercial product, also because tmigd1 can be used as a DNA marker to detect good reproductive performance in Huang et al. (2011b), and an unpublished data), and dip the strip in a prepared solution to wait 10 min for the result, than waiting enzyme digestion, preparing gels, and performing electrophoresis for 2 h for RFLP. A detector and image analyzer were only for the analysis of our detect method. Because the strip method to detect tmigd1 polymorphism has been created and tested as described in the manuscript, a detector and image analyzer are not required for practical application of our method: our test strip is for naked-eye observation. According to our calculations, the price for one strip is approximately US$1.31. This new, low-cost, and fast method for identifying genotypes of *tmigd1* gene, can be used to assist the improvement of the reproductive ability of Tsaiya ducks.

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