International Journal of Latest Trends in Engineering and Technology
Vol.(14)Issue(1), pp.034-040
DOI: http://dx.doi.org/10.21172/1.141.07
e-ISSN:2278-621X

DETECTION OF AUTOIMMUNE DISEASEGENES ON MEMBRANE BASED LATERAL FLOW STRIPS

Jui-Chuang Wu[1], Shih-En Jha[2], Hsun-Yu Huang[3], Guan-Ting Lin[4]

Abstract- This study utilizes antibody-antigen immunoreactionsto detect ligand-labeled gene targets on membrane-based lateral-flow (MBLF) strips. Alleles HLA-Cw1602 and HLA-B5801, which are respectively responsible to Behçet's diseaseand medicine allergy, wereselected to demonstrate on our detection platform.These two genes were first amplified by a Polymerase Chain Reaction (PCR), of whichprocessing time was accelerated by employing a gold-copper alloy tubewith anexcellentthermal conductivity, and then PCR samples were applied on the deletion platform of membrane assembly. Ligands digoxigenin, FITC and biotin cooperated with their immune counter-partners and receptors specifically distinguished these two genes on the detection device. Under the optimal detection condition, genes HLA-Cw1602 and HLA-B5801 received detection limits of 1.2 and 1.0 ng, respectively. The coefficients of variation, 2.7-9.8% for the intra assay and 9.9-14.9% for the inter assay, supportedusers a confidence on the detection stability. In the independence study, the results did not show any interferences between individual immunoreactions.
Keywords –autoimmune diseases, membrane-based lateral flow, nitrocellulose membrane, nano-gold particles, biosensors.

1. INTRODUCTION

From a given gene, anautoimmune disease-risk allele is a variant associating with altering human's autoimmune system[1]. Recent studies reported specific Human Leukocyte Antigen (HLA) alleles cause severe adverse drug reactions(ADRs). For instances, patients with HLA-B5701 allele would causeHypersensitivity Syndrome (HSS) to Abacavir, a medication for preventing and treating HIV/AIDS.Alleles HLA-B1502, B27 and A3101 causeimmune-mediated skin rashof Steven-Johnson Syndrome(SJS), Toxic Epidermal Necrolysis (TEN),orMaculopapular Eruption (MPE)to Carbamazepine, the medicine for curing epilepsy[2-3].

Allopurinol, a popular medicine for treatinghyperuricemia for gout patients, was reported to cause SJS and TEN when patients are with allele HLA-B5801. This special caseonly cover 5% of all ADRs medical anamnesis, but finally leads to 26~30% of high mortality rate[4], particularly discovered in Han(漢)nationality[5]. HLA-Cw1602 is another allelestrongly associating with Behcet's disease(BD), which is a rare disorder that causes blood vessel inflammation throughout body, particularly appearing at lips and eyes[6].

Membrane-based lateral-flow strips (MBLF) detection is one of the most important tools used for rapid medical diagnoses andpublic-health research activities. It allows untrained personnel to operate detection devices at caring sites withlimited or unavailable support of laboratory instrumentation[7]. Many researchers have reported applications of MBLF using immunoassay, for instances, thedetections ofbacteria Shigella[8] to ensure food safetyand alpha fetoprotein[9] forcontaminationcontrol in agricultural products.

This study develops detectionsof genes HLA-B5801 and Cw1602 on MBLF strips. This development will provide a clinic on-site diagnosis toquickly screen risk allelesforpatients and prevent medicine prescription from misusages. A high-thermal-conductivity gold-copper tube was usedfor the Polymerase Chain Reactions (PCR) to accelerate gene amplification process and cute down patient's waiting time.For a typical test, the analyte was added onto the sample pad and subsequently flowedonto its neighboring conjugate pad as shown in Figure 1. For the scenarios of allnegative-control genetic analytes, there was no immune-reaction occurred, so that the anti-digoxigenin antibody will move alone onto the control line. For thedetection of HLA-B5801 and Cw1602 genetic analyte, the gene-antibody complex migrated onto the NC membraneand then subjected a capillary flow in the interstitial space of the membrane. An absorbent pad placed at the distal end wicked fluid away from the membrane tokeep the flow continuous.

[1] Center for Circular Economy and Department of Chemical Engineering, Chung Yuan Christian University, Chung Li, Taiwan
[2,3,4] Department of Chemical Engineering, Chung Yuan Christian University, Chung Li, Taiwan, Taiwan

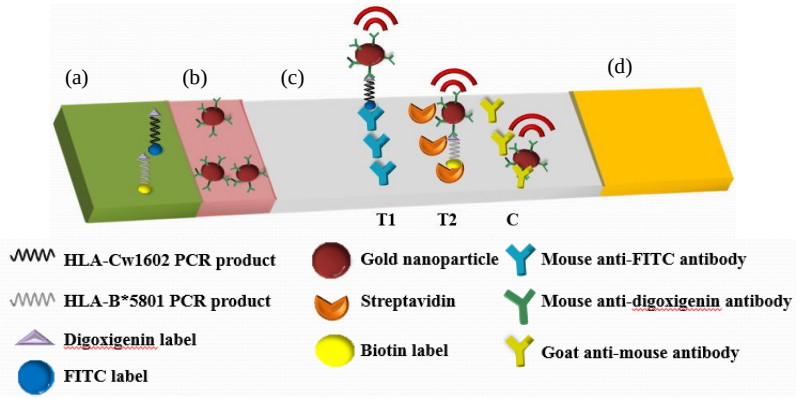


Figure 1. Detection Immunoassay and the MBLF Strips. (a) sample pad for loading PCR products. (b) conjugate pads for pre-loaded nano gold-labeled report antibody. (c) nitrocellulose membrane pre-dispensed with the capturing antibody at the test lines T1, at control line C and receptors at test line T2. (d) absorbent pad. The PCR products were tagged with FITC and biotin labels

2. EXPERIMENTAL

2.1 Material and Instrument –

Thesequences of the genetic targets and their corresponding PCR primers are listed in Table 1.

Table -2Primers of Target Templates

	Sequences
HLA-CW1602	
Forward	5′-digoxigenin-
Reverse	CCGAGTGAACCTGCGGAAA-3′
	5′-FITC-CCCTCCAGGTAGGCTCTCT-3′
HLA-B5801	
Forward	5’-digoxigenin-
Reverse	GGGCCGGAGTATTGGGACGG-3’
	5’-Biotin-
	GCCATACATCCTCTGGATGAT-3’

Arabidopsis thaliana plasmidwas adopted as the first species source of genetic negative control. Its PCR productwas cloned andnotated as pda13015(252b) in this study,It was the first plant genome to be sequenced and has become a popular model organism in plantbiology and genetics [10-11]. The second species source of control gene was Avian influenza H5(358b), which refers the H5 sequence from the subtype H5N1, a highly infectiouspathogen, generally spreading among poultry and birds. Several efforts have been made todetect this fatal pathogen [12].These two genetic targets are suitable asthe negative models, since they are two very different species from our target genes and their geneticsequences have already been well studied. The third negative control is human genes PSMA5 (432b). Our detection specificity will be also interested in checking genes from the same species. Areport indicated that PSMA5 exists mainly as tetramer [13].All these templates were provided by Biokit Biotech (Taiwan).The corresponding information of all genes can be found in Table 2.

Table 2. The DNA analytes and their gene information

Genetic target	Length (bases)	Definition (gene bank)
HLA-Cw1602	264	IMGT/HLA Acc No: HLA00386
	380	GenBank accession number AJ420241.2
HLA-B5801	252	Arabidopsis thaliana clone RAFL15-32-O04 (R20961) putative protein kinase (At1g76370) mRNA, partial cds.
pda13015		Riken Database BT003995.
H5	358	Avian Influenza, type H5, Gene Bank S68489, A/turkey/England/50-92/91
PSMA5	432	Human Proteasome (prosome, macropain) subunit, alpha type,5; NCBI Database gi 2311094, ref NM_002790.2

The essential materials for PCR are detailed as follows: Taq polymerase was purchased from NEB(Herts, UK) and dNTP from Amersham (Pittsburgh, PA, USA). Ethidium bromide for stainingagarose gel, and TBE buffer, for running the gel, both were obtained from BioBasic (Markham,Ontario, Canada). The PCR products were purified by using a QIAquick purification

kit (Qiagen,Valencia, CA, USA).The strip elements, including sample pads, conjugate pads, absorbent pads and plastic backing card were all provided by RegaBio (Taiwan). The nitrocellulose membrane was provided by Prisma Biotech (Taiwan, UniSart CN140). As other three-dimensional bio-compatiblematerials, e.g., an aerogel we developed [14-15], nitrocellulose provides a large internal surfacearea to adsorb much more biological analytes than the traditional 2D surface of biosensors.

Gold-Copper PCR tube was made in 80%-20% proportion and similar shape as a regular plastic tube to accelerate the PCR processing time. This alloy possesses a thermal conductivity around 1000 times of that of plastic tubes. The top three metals with high thermal conductivities are copper, silver and gold. Since copper and silver cannot be waived from oxidation during a long-term usage, gold is the best choice as the candidate. However, pure gold is unable to keep the same tube shape. Addition of a small proportion of copper could fix this problem.

Materials printed on nitrocellulose membrane to capture labeled ligands and report antibody are streptavidin (Jackson Laboratories, USA), mouse anti-FITC IgG antibody (Sigma-Aldrich, USA)and goat anti-mouse IgG antibody(Jackson Laboratories, USA). The report antibody, mouse anti-digoxigenin IgG antibody, wasfrom Jackson Laboratories (USA). Nano-gold particleswithan average size of 32 nmfor labelling thereport antibody were from RegaBio (Taiwan).

2.2. Instrument –

Capture antibodies were dispensedonto the membraneby theAgitest™ RP-1000A dispenser RegaBio (Taiwan).The final membrane-pads assembly was cutinto strips by the cutter, Model JS-101 (JihShuenn Electrical Machine, Taichun, Taiwan). The PCRmachine, Model PC-320 (Astec, Tokyo, Japan), duplicated genetic samples for detection. The signalbands of the agarose gel were read on GeneFlash (Syngene, Frederick, MD, USA). The concentrations of genetic analytes were measured by a NanoDrop1000 spectrophotometer (ThermoFisher Scientific, Hudson, NH USA). A low temperature centrifuge (Hettich 16R, Tokyo, Japan) wasused to separate the gold-labeled antibodies from buffer. A desiccator (D-60C, Moisture Buster,Taichun, Taiwan), was used to store the nitrocellulose membrane. A HP4800 Scanner scanned thedetection signals from the membrane. The quantitative analysis of the signal intensities was carried out by genepixPro□ software.

2.3. Procedure –

2.3.1.Preparation of Running Buffers and Report Antibody

This procedure is the same as that previously published [16].In a standard procedure, 20X SSC was prepared by dissolving 175.3 g of sodium chloride and 88.2 gof sodium citrate in ddH2O. The pH value of the solution was adjusted to 7.6 using HCl/NaOH. Thefinal solution was prepared by diluting the original to 1L using ddH2O.The report antibody, 0.2 mL of 50 □ g/mL mouse anti-digoxigenin antibody, was mixed with the goldsolution to label gold nanoparticles. After storage at 4 °C for 2 h, the solution was centrifuged at 4 °Cand 14,000 rpm for 10 min. After disposal of top clear solvent, the gold-labeled antibody was addedto 0.2 mL of 0.01 M Tris buffer as a wash. Another centrifugation and disposal cycle was thenperformed to obtain the final gold-labeled antibody, which was then saved in the lateral-flow buffer at4 °C.

2.3.2.Assembling of Test Strips

Nitrocellulose membrane was first adhered onto the plastic backing card. The capturing antibodies, mouse anti-FITC IgG antibodyand goat anti-mouse IgG antibody, and the receptor streptavidin were diluted into appropriate concentrations and then dispensed onto the membrane by the dispenser. Thefinish-dispensed NC membrane was then stored in a desiccator maintained at RH% of 30%–40%, for atleast one day.All elements were then assembled together inthe following order onto the plastic card: conjugate pads, absorbent pads, and samplepads. The assembly was then cut into 5-mm widestrips by the cutter.

2.3.3 Preparation of PCR Products and Electrophoresis of Agarose Gel

The primers, in a concentration of 10 μM and volume of 2 μL, were mixed with 1 μL of 50 ng/ μL template, 5 L of 10X PCR buffer,2.5 μL of 5 mMdNTP, and 36.5 μL ddH2O. After addition of 1 μL of 1.25U/μLTaq, the mixture wasquickly loaded into the PCR machine. Each PCR cycle was set for different tube materials. For plastic tubes, the standard condition was set as denaturization 95 °C for 5.5 min,annealing 50 °C for 1 min and extension 72 °C for 1 min and total 40 cycles.For metal tubes, different trials were conducted to minimize the process time of each step in one cycle and the cycle times.

Standard agarose gel in 2% of concentration was prepared by dissolving 0.6 g of agarose powderin 30 mL of 0.5X TBE. The solution was microwaved and then cast in an electrophoresis tray for20 min. The PCR products were mixed in 1:1 with an electrophoretic blue dye and loaded into the gelgrooves. 100 V was applied for 30 min to allow electrophoresis. The gel slabs were then stained withethidium bromide and images were acquired by exposing the gel to a UV light.

2.3.4. The Detection Assay and Image Acquisition

As the digoxigenin-labeled PCR product flowed through the conjugate pad, which was pre-loaded with gold-tagged mouse anti-digoxigenin antibody,the antibody would undergoa immunoreaction with thedigtoxigenin ligand to form a complex molecule. The resultant complexmolecule then kept flowing toward the capturing antibody immobilized on the test line. The

capturing antibody would capture its immune counter-partner on the PCR products. If it exists on the product, a signal would appear; otherwise, blank information would show at the test line.

In a typical run, a PCR product was first diluted to the desired concentration using therunning buffer and then applied onto the sample pad. After samples flew through the membrane and result were obtained. The detection signals were finally scanned by the HP scanner and saved asimages. The images were later retrieved to measure the signal intensity by the commercial softwaregenepixPro . The commercial scanner was found the best way to save qualified images for signal intensityanalysis in this study.

3. Experiment and Result

3.1. The Optimal Annealing Temperature –

Based on the formula for melting temperature [17]

$$T_m = 81.5 + 16.6\log\frac{[salt]}{1+0.7[salt]} + 41\times GC\% - 500 / L \quad (1)$$

We obtained63℃ as the melting temperature of the target genes. The annealing temperature is normally 5-7℃ lower than the melting temperature; therefore, test temperatures 63℃, 59℃ and 56℃ were set for test. As shown in Figure 2, the optimal annealing temperature was obtained as 59℃; therefore this temperature was adopted to proceed all followed tests.

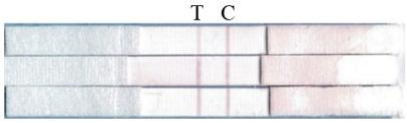
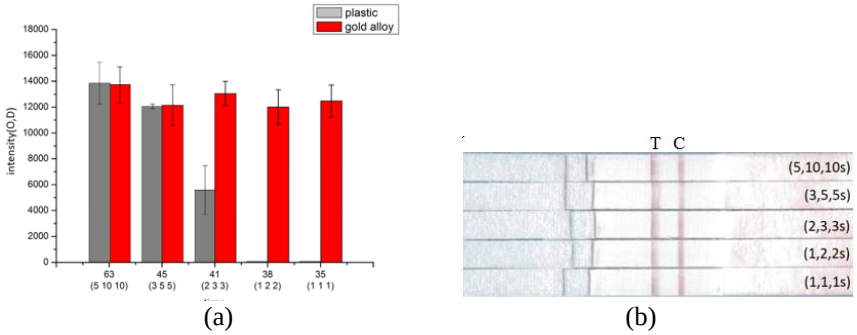


Figure 2. Test for Optimal Annealing Temperature. From top to bottom: 63℃, 59℃ and 56℃. Obviously 59℃received the best performance.

3.2. The Optimal PCR Processing Time –

In order to cut down the waiting time during clinic diagnosis, the PCR processing time was investigated whether or not can be saved. To achieve this goal, the gold-copper alloy PCR tube was employed to gain the heat conductivity between PCR machine and the tube. Three steps in PCR process, denaturation, annealing and extension, were set their time. Their corresponding PCR products were tested on MBLF strips, and the results are shown in Figure 3.

Form Figure 3, when the PCR processing time for the gold alloy tubes was lowered down to the minimum possible setting of (1 sec, 1 sec, 1 sec), the signals only reduced 9% from the control case of (5 sec, 10 sec, 10 sec), but the plastic tube did not show any signal due to its poor thermal conduction. A test was further condcted to investigate if any residual PCR product was carried over to next test. This result indicated the residual product can be entriely cleaned by 1-2 times of ddH2O washes.

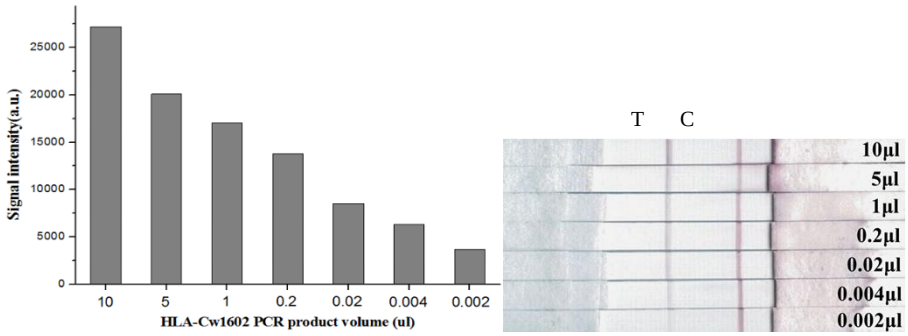


(a) (b)

Figure 3. Comparison of the performance using gold alloy and regular plastic PCR tubes.
(a) Signal intensities on MBLF strips in different PCR processing time. (b) Image of MBLF strips using gold alloy.

3.3. The Detection Limits on MBLF Strips –

Genes HLA-Cw1602were investigated their detection limits on MBLF strips. To save patient's waiting time, the PCR products were not purified and directly mixed with the lateral flow buffer and then loaded onto the sample pads of the strips. Volume of the unpurified PCR products of HLA-Cw1602in concentrations of 50 ng/μl was set from 10 to 0.002 l. The results reprted detection limits of 0.004 l (1.0 ng) of HLA-Cw1602 as shown in Figure 4.

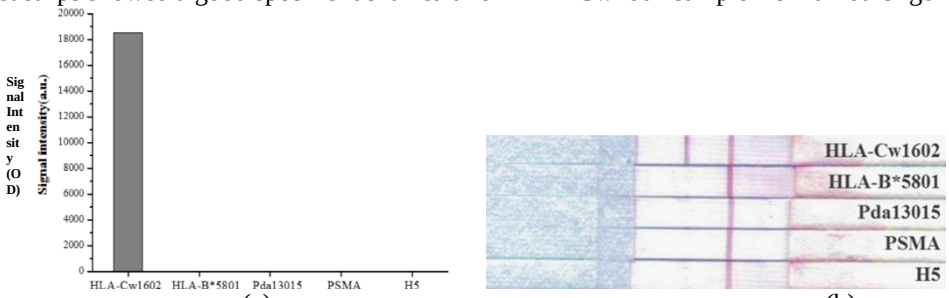


(a) (b)

Figure 4. Detection Limits of Unpurified HLA-Cw1602 PCR Products on MBLF Strips. (a) Signal plot. (b) Strip images. Letter "C" stands for control singal and "T" test signal.

3.4. Detection Specification –

Negative control genes and HLA-B5801were individually mixed with the primer of gene HLA-Cw1602 to check the amplification specification. Their PCR products were run on MBLF strips to test the detection specification. As indicated in Figure 5, test strips showed a good specific identificaitn on HLA-Cw1602 sample from all other genes.

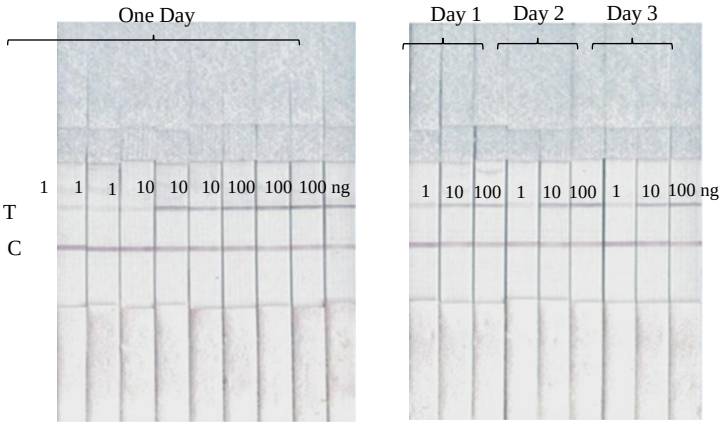


(a) (b)

Figure 5. Detection Specification on Gene HLA-Cw1602.(a) Signal plot. (b) Strip images.

3.5 The Detection Stability –

The detection stability was investigated in two approaches. The intra-assay test detected three analyte loadings, 100, 10, and 1 ngof HLA-Cw1602, in one day with three strip replicates for each loading; while the inter-assay test detected three analyte loadings in three consecutive days with only one strip for each loading in each day. As shown in Figure 6, all strips receive stable results. The coefficients of variation of two assays, 2.7-9.8%and 9.9-14.9%, respectively, support users a confidence of the detection repeatability.



(a) (b)

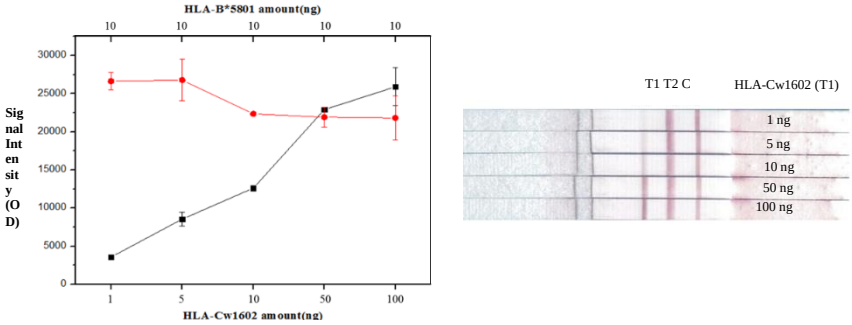
Figure 6. Detection Stability. (a) Theintra-assay testcompleted in one day. (b)Theinter-assay test completed in three consecutive days.

Three analyte loadings, 10, 1, and 0.1 ng of HLA-A3101were detected in one or three consecutive days.

3.6 The Independece Study –

One particle application is testing these two target genes of interest in one strip. This strategy is able to display more information in one single test at the time for the reference of medication treatment. As shown in Figure 7.,HLA-B5801 was

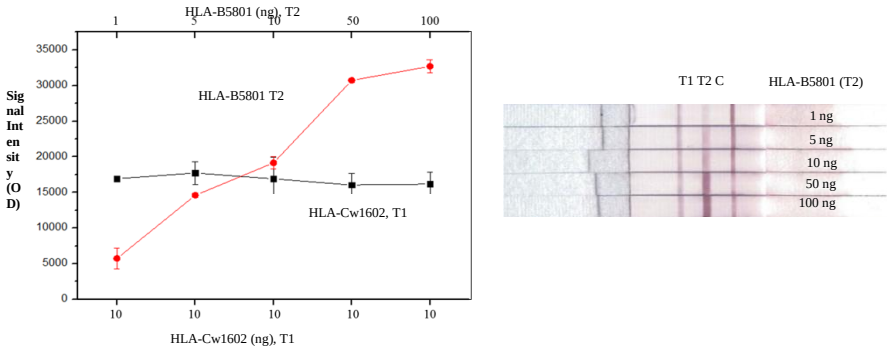
loaded in the same amount of 10 ng for each strip; while HLA-Cw1602 varied its loading in 1to 100 ng on different strips. Signals on test line T2 (for HLA-B5801) almost remained quite in a constant. Signals on test line T1 (for HLA-Cw1602), varied along the trend of loading amountas expected.



(a) (b)

Figure 7. Detection Independence Study. (a) Signal intensities on MBLF strips. (b) Image of MBLF strips. Each strip has the same loading of 10 ng of HLA-B5801 (T2); while HLA-Cw1602 (T1) varied its loading from 1 to 100 ng on different strips.

Another investigation approach is reversing the loading amount of these two target genes. As shown in Figure 8., HLA-Cw1602 was loaded in the same amount of 10 ng for each strip; while HLA-B5801 varied its loading in 1 to 100 ng on different strips. Again, signals on test line T1 (for HLA-Cw1602) almost remained quite in a constant. Signals on test line T2 (for HLA-B5801), varied along the trend of loading amountas expected.



(a) (b)

Figure 8. Detection Independence Study. (a) Signal intensities on MBLF strips. (b) Image of MBLF strips. Each strip has the same loading of 10 ng of HLA-Cw1602 (T1); while HLA-B5801 (T2) varied its loading from 1 to 100 ng on different strips.

4. CONCLUSION

Autoimmune-disease genes HLA-Cw1602 and B5801 were detected on MBLF strips with a good specification. A gold alloy PCR tube was demonstrated to be able to lower down the process waiting time. Detection limit showed the strips can detect as low as 1 ng of PCR products. The intra- and inter-assay tests gave a good promise to the detection stability from their low coefficients of data variation. The independence study made sure that multiple gene detection did not generate interferences between one another genes on the strips. This detection platform can be applied on diagnoses conducted at clinics, particularly for care sites without much source of diagnosis instrument,for preventing medicine mistreatment. This application can be further expanded to more multiple target genes dispensed on one strip. The report patterns could be expanded in arrayed dots or letters in the small read-out window.

5. REFERENCES

[1] J. Ray and N. Hacohen, “Impact of autoimmune alleles on the immune system,” Genome Med., 7(1):57, doi: doi: 10.1186/s13073-015-0182-y.,2015.

[2] Q.Wang, Y.Zhang, X.Pan, W.Tan, and L.Chao, “The associations between idiosyncratic adverse drug reactions and HLA alleles and their underlying mechanism,”Acta Pharmaceutica Sinica, pp.799 −808, 2013.

[3] Y.Yang, L. Yuan,X. Fang,X.Liang, andF. Yang,“Detection of HLA-B*27 gene using a spectral plasmon resonance imaging system,”Biosens BioelectronVol. 46, pp. 80-83, 2013.

[4] S. Hung, W. Chung, L. Liou, C. Chu, M. Lin, H. Huang,Y. Chen, “HLA-B*5801 allele as a genetic marker for severe cutaneous adverse reactions caused by allopurinol,”PNAS, Vol. 102,pp. 4134–4139, 2005.

[5] J.Jung, W.Song, Y.Kim, K. Joo, K. Lee, S.Kim,H. Kang, “HLA-B58 can help the clinical decision on starting allopurinol in patients with chronic renal insufficiency”Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association vol. 26, pp. 3567-3572, 2011.

[6] L.Sanz, F.Gonzalez-Escribano, R. Pablo, A.NOiiez-Roldan, M. Kreisler and C.Vilches,“HLA-Cw*1602: a new susceptibility marker of Behcet's disease in southern Spain”Tissue Antigens, vol. 51, pp. 111-114, 1998.

[7] S.Wang, C. Zhang,J.Wang, Y. Zhang, “Development of colloidal gold-based flow-through andlateral-flow immunoassays for the rapid detection of the insecticide carbaryl”Anal. Chim. Acta., vol. 546, pp. 161–166, 2005.

[8] C.Song, C. Liu, S.Wu, H. Li, H. Guo, B. Yang and Q. Liu, “Development of a lateral flow colloidal gold immunoassay strip for the simultaneous detection of Shigella boydii and Escherichia coli O157:H7 in bread, milk and jelly samples”Food Control,vol. 59, pp. 345-351, 2016.

[9] R.Liang, X.Xu, T. Liu, J. Zhou,X. Wang, Z. Ren and Y. Wu, “Rapid and sensitive lateral flow immunoassay method for determining alpha fetoprotein in serum using europium (III) chelate microparticles-based lateral flow test strips”Anal Chim Acta, vol. 891, pp. 277-283, 2015.

[10] Z.Lorkovic,”Role of plant RNA-binding proteins in development, stress response and genome organization”Trends Plant Sci., vol. 14, pp. 229–236,2009.

[11] F. Sandoval, Y.Zhang and S.Roje, “Flavin nucleotide metabolism in plants monofunctional enzymessynthesize FAD in plastids,”J. Biol. Chem. vol. 283, pp.30890–30900, 2008.

[12] S. Lycett, M. Ward,F. Lewis, A. Poon,S. Kosakovsky,A. Pond and J.Brown, “Detection of mammalian virulence determinants in highly pathogenic avian influenza H5N1 viruses: multivariate analysis of published data”J. Virol., vol. 8319, pp. 9901-9910,2009

[13] Y.Han, H. Liu, H. Zheng, S. Li and R. Bi,“Purification and refolding of human α5-subunit(PSMA5) of the 20S proteasome, expressed as inclusion bodies in Escherichia coli”Protein Expr. Purif.,vol. 35, pp. 360–365,2004.

[14] Y. Li,Y. Chen, K. Jiang, J.Wu, Y. Chen-Yang,“Three-dimensional arrayed aminoaerogel biochips for molecular recognition of antigens”Biomaterials, vol 32, pp. 7347–7354, 2011.

[15] Y. Li,D.Yang, Y.Chen, H.Su, J.Wu, Y.Chen-Yang,“A novel three-dimensionalaerogel biochip for molecular recognition of nucleotide acids”Acta. Biomat. vol. 6, pp.1462–1470, 2010.

[16] J. Wu, C. Chen; G. Fu and H. Yang , “Electrophoresis-enhanced detection of deoxyribonucleic acids on a membrane-based lateral flow strip using Avian Influenza H5 genetic sequence as the model” Sensors, vol.14 , pp.4399-4415, 2014.

[17] Frank H. Stephenson. Calculations for Molecular Biology and Biotechnology. p.159. Acedmaic Press. UK., 2000.