

Rapid Detection of Gene HLA-A3101 on Membrane Based Lateral-Flow Strips

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Abstract: Gene Human leukocyte antigen (HLA)-A3101 is known responsible of a medicine allergy which may cause a fatal outcome. General clinics need a quick test to determine if a patient is engaged with the related gene defect and avoid the medical prescription from suspicious drugs. In this study, Gene HLA-A3101 is detected on membrane-based lateral-flow (MBFL) strips, incorporated with an immune reaction and color readouts developed by gold nanoparticles. The HLA-A-3101 DNA modified with biotin and digoxigenin labels was amplified by the Polymerase Chain Reaction (PCR) and then detected on the MBFL strips. The optimal condition of antibody concentration was at least 10 μ g/ml, to avoid the antibody-gold complex from aggregation. The signal can be observed as low as 0.49 ng of the PCR product by naked eyes. The specificity of PCR primers was also investigated their annealing validity. The detection specificity from other species genes, including a plant, a virus, and a human gene, were also verified. The coefficient of variation values of the intra assay, which tests the same sample in the same day, and the inter assay, which tests three samples in three consecutive days, are in satisfactory ranges of 4.6% and 6.5%, respectively.

Key words: Gene HLA-A3101, membrane based lateral-flow detection, gold nanoparticles, medicine allergy.

1. Introduction

Human leukocyte antigens (HLA) are responsible with many human self-immune diseases [1]. Recent researches indicate that several HLA allele genes may cause adverse drug reactions (ADR's) [2] and drug-induced liver injury (DILI) [3]. These pairs of HLA genes and their corresponding allergic medicines include HLA-B5701/Abacavir [4], HLA-B5801/Allopurinol [5], [6], HLA-A3101/Carbamazepine [3], and so on. This study will develop a membrane-based lateral-flow (MBLF) test to perform a rapid detection on HLA-A3101.

The MBLF test is one of the most efficient diagnosis tools for public health at care sites. Starting from the rapid detection on human chorionic gonadotropin (HCG) in a urine sample to determine if a woman is pregnant, the MBLF test has been widely applied in different fields. These applications cover the detections of pesticide residue in agriculture products [7]-[9], drug abuses [10], health care in husbandry [11], [12], and so on.

The MBLF technology has ever been also applied on the detection of various Polymerase Chain Reaction (PCR) products, for instances, allergens [13], [14], bacteria [15], pathogens [16], antibodies [17], and antigens [18], [19]. Our recent study revealed that an electric field could enhance the detection performance of single-trended Deoxyribonucleic acids (DNA) on the MBLF strips [20].

The MBLF offers lots of advantages in medical early diagnoses. It provides a cost-effective platform to manipulate samples. Secondly, it gives quick detection readouts without instrumental aids and a flexible temperature range for the application. Since the test operators do not need much training, homecare and clinics are its ideal application sites.

This study adopts the MBLF test to detection HLA-A3101 by labeling two ligands on its PCR products. One ligand is recognized by the report antibody loaded on the conjugate pad of the MBLF strip, and the other by the capture reagent on the capturing zone. The gold nanoparticles serve as the readout color, which can be directly read by naked eyes.

The detection principle of this assay is shown as Figure 1. The HLA3101 gene is first amplified by a PCR with labeling two ligands. This PCR product is then applied on the sample pad. As it flows through the conjugate pad, which is deposited with the report antibody, the PCR product will interact with the report antibody, since they have an affinity with each other. After the immune reaction, the PCR product-antibody complex flows ahead through the nitrocellulose membrane by a capillary force induced by the porous membrane material of nitrocellulose, of which porosity is around 5 μm . At the capture zone, the complex is again recognized on the other ligand by the capturing reagent deposited in advance on the membrane to form the test line. A readout color will appear on the capture zone to indicate a positive result, otherwise negative. An absorbent pad is placed at the other end of the strip to absorb excess fluid and keeps the flow continuous.

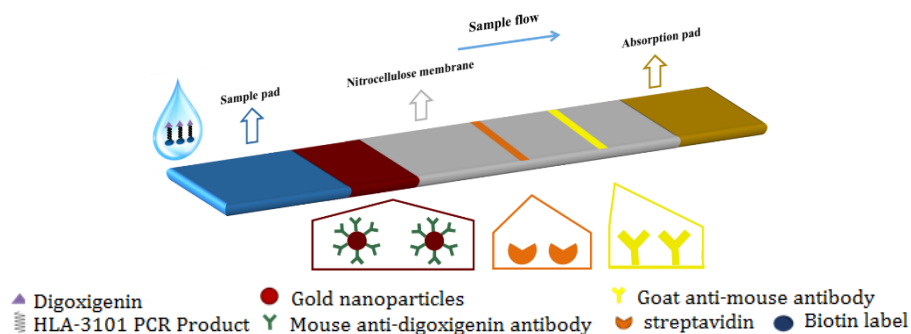


Fig. 1. The detection principle of the MBLF on HLA3101. Gene HLA3101's PCR product is induced to laterally flow through the membrane and encounters the report antibody and the capturing reagent, which respectively recognize tow ligands labeled on the PCR product. The result is identified by the gold color appearing or not at the capture zone as the test line.

2. Experimental

2.1. Materials

HLA-A3101 template (GenBank accession number: M84375.1) has a length of 124 bp. Its forwarding and reversing primers were 5'-digoxigenin-GTTCTCACACC ATCCAGATG-3' and 5'-biotin-GAGCGCAGGTCCT CGTTCAA-3', respectively. The ligands digoxigenin and biotin were respectively labeled on two primers at the 5' ends.

For the specificity test, human genes of HLA-B5801 and PSMA5, plant gene of Arabidopsis thaliana Pda13015, Avian Influenza gene of H5, and their corresponding primers were also employed. The human gene PSMA5 is a popular genetic model. It was recently reported that it exists mainly as a tetramer [21]. All templates and PCR primers were synthesized by Biokit Biotech (Taiwan).

2.2. Report and Control Antibodies

Mouse anti-digoxigenin IgG antibody serviced as the report antibody. This monoclonal antibody was

labeled with 30nm gold particles before being deposited on the conjugate pad.

Goat anti-mouse IgG antibody served as the control antibody and was directly deposited on the control zone, which was just next to the capture zone by 5 mm apart. If the control line does not appear, the test is invalid and needs to run again. Both antibodies were purchased from Biomate (Taiwan).

2.3. Elements of MBLF Strips

The nitrocellulose membrane with 5µm porosity served as the detection platform. Nitrocellulose has an excellent affinity with protein and provides a permanent binding after 24-hr post-dispensing desiccation in a 40%RH dry box. The membrane was first adhered onto a adhesive plastic card and then deposited with the capture reagent and the control antibody.

The sample pad served as a sample reservoir to slowly release sample onto the conjugate pad. The conjugate pad, right next to the sample pad with a partially overlapping, was deposited with the gold-labeled report antibody. The absorbent pad was placed at the other end of the strip to provide a continuous capillary flow induced by the absorption of excess sample fluid from the pad. All membrane and pad elements were purchased from Rega (Taiwan).

2.4. Other Reagents

Streptavidin, which is deposited on the membrane as the test line, serves as the capturing reagent to capture biotin ligand labeled on the PCR product. If a red-color line appears at the capturing zone, the result is identified as positive, otherwise negative.

Nano-gold particles serve as the readout indicator. Its size is about 40nm and appears in red. Rega (Taiwan) offered this commercial product.

2.5. Instrument

Agitest™ RP-1000 (Rega, Taiwan) served as the antibody and reagent dispenser to plot lines on the designated zones. Strip cutter (Jihshuenn JS-101, Taiwan) cut pad-membrane assembly into 5mm-wide strips. PCR machine (Astec PC-320, Japan) was used as the gene amplifier. Gel electrophoresis (BioBasic, Mupid-exU, Canada) was performed to verify the PCR validation. The gel electrophoresis was read at GeneFlash (Syngene, USA). The concentrations of antibody were measured by NanoDrop 1000.

2.6. Procedures

Before printing the control antibody (control line) and the capturing reagent (test line) on the membrane, the nitrocellulose membrane was first adhered on a plastic card. The test line is designed to be within 2~2.5cm from the sample application zone and the control line is 5mm apart from the test line. After printing finished, the plastic card is preceded for adhering other three pads: the sample, conjugate and absorbent pads. Before this adhesion step, the report antibody is deposited on the conjugate pad and dried at 37°C for 2 hr. The finished membrane-pad-card assembly is finally cut into 5mm-wide and ready-to-use strips.

For detections on PCR products for a typical run, 3µl of the sample was added into 67µl of PBS buffer and the mixed solution was then added onto the sample pad. The room humidity was set at 40%RH and 26°C. High room humidity causes moisture deposition from the atmosphere onto nitrocellulose membrane and slowing down the lateral flow of fluid. The detection time will be then delay. The sample-antibody complex will also be retarded on membrane to form a signal background.

The optimal concentration and pH of mouse anti-digoxigenin antibody for nano-gold conjugation were set at 10µg/ml and 8.6, respectively. Other pH conjugation conditions will cause gold-particle aggregation. As a nano-size material is aggregated, it loses its original physical property. In our case, the nano-gold

particles became dark purple and smeared the membrane if they are forced to apply.

3. Result and Discussion

3.1. Validation of PCR Annealing Temperature

The general formula [22] for the optimal PCR's annealing temperature is

$$T_m = 81.5 + 16.6 \log \frac{[salt]}{1 + 0.7[salt]} + 41 * GC\% - 500 / L.$$

Therefore, $59 \pm 3^\circ\text{C}$ was set as the PCR condition, and the products were detection on MBLF strips after PCR was complete. As shown in Fig. 2, 59°C , obtained from the formula, gave the best performance.

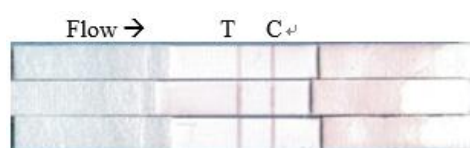


Fig. 2. Optimization of the PCR's annealing temperature. From top to down, 62°C , 59°C , and 56°C were set.

3.2. Verification of PCR Products

The PCR products were verified by running 2% agarose gel electrophoresis to see if they were successfully amplified. As shown in Figure 3, PCR products of HLA-A3101 (124bp), HLA-Cw1602 (264bp), Avian Influenza H5 (358bp), human PSMA5 (433bp), and Arabidopsis thaliana Pda13015 (252bp) were all located at their size-corresponding positions of the gel, indicating all PCR products were correctly amplified.

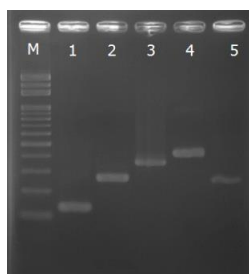


Fig. 3. Agarose gel electrophoresis to verify the PCR products. Lane M: the ladder; 1: HLA-A3101(124bp); 2: HLA-Cw1602(264bp); 3: H5(358bp); 4: PSMA5(433bp); 5: Pda13015(252bp).

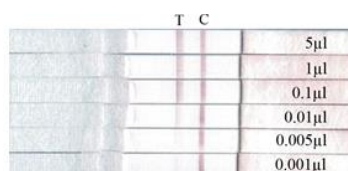


Fig. 4. The detection limit of HLA-A3101 PCR product on MBLF strips. The detection signal can be recognized as low as $0.01\mu\text{l}$.

3.3. Detection Limit of HLA-A3101 on MBLF Strips

Different volumes of HLA-A3101 PCR product were added onto MBLF strips in $5\mu\text{l}$, $1\mu\text{l}$, $0.1\mu\text{l}$, $0.01\mu\text{l}$, $0.005\mu\text{l}$, and $0.001\mu\text{l}$. The result is shown in Figure 4, the detection limit was as low as $0.01\mu\text{l}$, and of which

corresponding amount was about 0.49 ng. Since the real detection limit will actually depend on the sampling gene template concentration during the application, the result here only provides a reference for the condition starting from 2ng of gene template mixed into total 50 μ l of PCR 25-cycle batch.

This result was also scanned their optical density to digitize the eye readings as shown in Fig. 5.

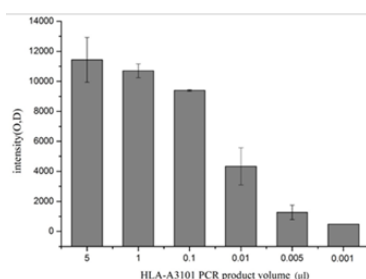


Fig. 5. Digitized plot for the detection limit of HLA-A3101 PCR product on MBLF strips. The detection signal threshold can be recognized at around 4000 OD in our analysis approach.

3.4. Detection Specificity of HLA-A3101 on MBLF Strips

The primers of HLA-A3101 were added to other four genes' PCR batches: HLA-Cw1602 (264bp), H5 (358bp), PSMA5 (433bp), and Pda13015 (252bp) to see if a non-specific PCR amplification occurred. Figures 6 to 8 show their PCR products run on MBLF strips and agarose gel.



Fig. 6. Specification test of HLA-A3101 primers with other gene templates on MBLF strips. HLA-A3101's primers show a loyalty to their own template.

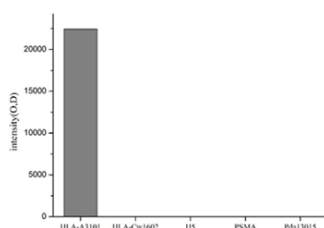


Fig. 7. Digitized plot of the Specification test of HLA-A3101 primers with other gene templates on MBLF strips.

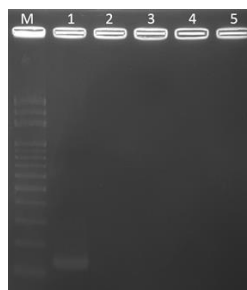


Fig. 8. Agarose gel electrophoresis for the specification test. Lane M: the ladder; HLA-A3101 primers run with Lane 1: HLA-A3101; 2: HLA-Cw1602; 3: H5; 4: PSMA5; 5: Pda13015. Only HLA-A3101 was correctly amplified.

3.5. Test Reproducibility of HLA-A3101 on MBLF Strips

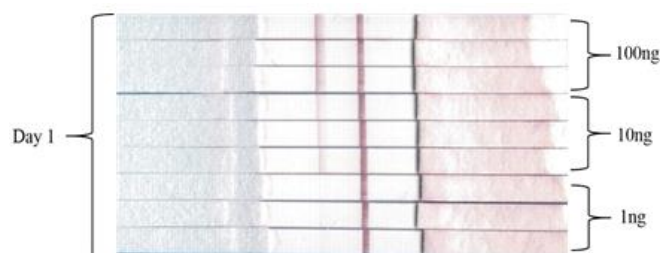


Fig. 9. The Intra-assay reproducibility test for HLA-A3101 on MBLF strips. Three amounts of samples, 100ng, 10ng, and 1ng of the PCR product were loaded in three replicate strips at the same time.

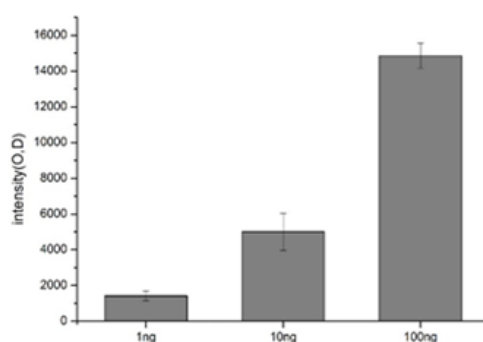


Fig. 10. The digitized plot of the Intra-assay reproducibility test for HLA-A3101 on MBLF strips. The Inter-assay test run on MBLF strips and its digitized plot are also shown in Figures 11 and 12, respectively. The statistic data shows the strip-to-strip (or day-to-day) variation keeps a good range within 6.3%, indicating this detection approach gives a stable result for continual tests. It is very meaningful for a clinic to ensure a quick and reliable diagnosis always robustly provided by this detection method.

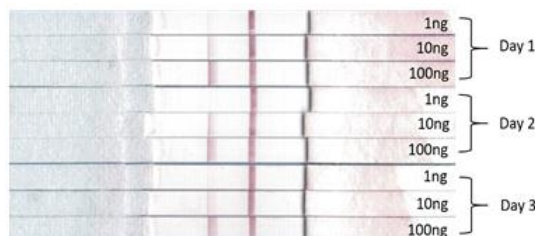


Fig. 11. The Inter-assay reproducibility test for HLA-A3101 on MBLF strips. Three amounts of samples, 100ng, 10ng, and 1ng of the PCR product were loaded in three consecutive days.

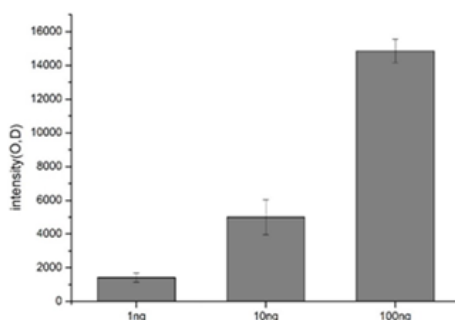


Fig. 12. The digitized plot of the Inter-assay reproducibility test for HLA-A3101 on MBLF strips.

The test reproducibility of HLA-A3101 on MBLF strips was conducted by loading 100ng, 10ng, and 1ng of

its PCR product in two sets. For the Intra-assay test set, three replicates of each sample amount were run at the same day; while the Inter-assay test set repeated the same test in three consecutive days. The Intra-assay test run on MBLF strips and its digitized plot are shown in Figures 9 and 10, respectively. The statistical data shows the strip-to-strip variation keeps a good range within 4.6%, indicating the reagent-dispensing process and pad-assembling procedure of MBLF strips had a low manipulation variation. As well, the test operator had a sophisticated skill, though operation training might not be necessary for the MBLF technology.

4. Conclusion

This study successfully amplified HLA-A3101 gene in optimized PCR condition and conducted rapid tests of the products on MBLF strips in 5 minutes. The readouts can be interpreted by naked eyes without gel electrophoresis and other instrument aids. The specification test indicates the HLA-A3101 primers gave an excellent loyalty to identify their own template from others. The reproducibility test indicates our samples were run in a stable experimental skill and the detection approach gave steady readouts.

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