Immunochromatography—Application Example and POCT Type Genetic Testing

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Received February 19, 2021

Membrane-based rapid test reagents including immunochromatography are widely used in clinical practice. Recently, high-sensitive reagents based on the immunochromatography method, such as silver amplification method and time resolved fluorescence method for influenza testing, have been developed and early confirmation of infection can be achieved. Furthermore, genetic testing, automated all the steps from extraction till detection, is getting popular. Genetic testing of mycoplasma by Smart Gene Myco system and Coronavirus disease 2019 (COVID-19) test is a good example of membrane-based rapid test reagents. This system uses silica particle-containing membrane filter and enable to shorten the assay time by automates pre-treatment process for removing contamination substances in the sample which affect polymerase-chain-reaction amplification. We hope utilized genetic testing application will help quick confirmation of COVID-19 positive patient and prevent the collapse of medical system under COVID-19 development.

Key words immunochromatography; point-of-care testing; PCR; infection; rapid test reagent; Coronavirus disease 2019

1. Introduction

Coronavirus disease 2019 (COVID-19) outbreaks and developed global pandemic rapidly in 2020.\(^\text{1,2}\) Infected population increased dramatically and medical system collapse has been occurred in many countries. Increasing COVID-19 PCR test and identify positive patient is required.

Under this situation, measuring soluble urokinase plasminogen activator receptor (suPAR) by lateral-flow (immunochromatography) kit attracts a lot of attention.\(^\text{3}\) suPAR is a biomarker which indicates activation of various immune systems. It is also a risk prediction marker for mortality from a wide variety of causes such as infectious diseases.\(^\text{4}\) Denmark uses suPAR for triage, to determine hospitalization patients and this helps under COVID-19 pandemic to prevent medical collapse.\(^\text{5}\)

Immunochromatography method is the laboratory test method, developed as lateral flow immunoassay long time ago, and widely used for infectious diseases which needs to diagnose rapidly.\(^\text{6,7}\) The result is judged visually from the judgement line which appears by the antigen-antibody immunoreaction between the reagent and sample, collected from the patient’s nose or throat. This is very useful to prevent patient aggravation and secondary infections, since assay time is short and rapid diagnose is possible.\(^\text{8}\) However, lower sensitivity than PCR was its disadvantage. Newly developed high-sensitive immunochromatography method achieves early diagnosis of influenza and medication before serious symptoms appeared.\(^\text{9}\)

PCR method was devised by Kary Mullis in 1983. Amplified large amount of specific region on the DNA sequence using thermostable DNA polymerase\(^\text{10}\) and real-time PCR method,\(^\text{11}\) RT-PCR method\(^\text{12}\) and DNA sequencing method\(^\text{13}\) etc. are developed. PCR method is applied to various diagnoses and tests, since it is possible to amplify a specific region if the template DNA exist even in a very small amount. On the other hand, the disadvantage of PCR test is the complicated pretreatment and long assay time.\(^\text{8}\)

COVID-19 require nasopharyngeal swab sample and it is necessary to remove impurities that inhibit PCR reaction.\(^\text{14}\) Assay time can be shortened with simple procedure by adopting chaotropic effects of the silica particle-containing membrane filter into a pre-treatment step on genetic test.\(^\text{15}\)

Here, we introduce the basic principle of membrane-based rapid test reagents focusing on immunochromatography and application examples of a pretreatment step for PCR testing.

2. Clinical Laboratory Tests and Trends

Clinical laboratory tests can be broadly divided into sample tests and biopsy. Sample tests examine abnormalities using samples excreted from the human body, such as urine, stool, blood and spinal fluid flowing through the human body, cells and organs that constitute human body. In MedlinePlus, a biopsy is defined as the removal of a small piece of tissue for laboratory examination.\(^\text{16}\)

Doctor uses those clinical laboratory test data and the patient’s symptoms, whether illness exist or not and its severity, to decide the treatment method. It is also used for judgement of therapeutic effect, administration, dose reduction, and discontinuation of medication. Therefore, the role of labora-
tory tests is expanding to prevent and detect diseases, early treatment, and prognosis (prevention of recurrence).8)

Laboratory diagnostic test reagents and laboratory diagnostic equipment have been progressing remarkably these days.18,19) With these progresses, laboratory diagnostic test reagent achieves highly specific new measurement principles that can measure only the target substance using enzymes and antibodies, improvement of accuracy and sensitivity, expansion of measurement range, reagent stability, and elimination of interfering substances. Laboratory diagnostic equipment helps to achieve shortening measurement time, improvement of through-put, accuracy, elimination of cross-contamination, and less requirement of sample and reagent volume.

Recent progress of biotechnology and life science have been applied to the medical and diagnostic fields. Currently reagents applying PCR method (mainstream of gene amplification method) are available but also reagents applying another new methods are available; loop-mediated isothermal amplification (LAMP) method,20) and strand displacement amplification (SDA) method,21) which does not require reaction temperature change during nucleic acid amplification; transcription-reverse transcription concerted reaction (TRC) method22) and transcription mediated amplification (TMA) method23) which is using RNA as labeled gene. In recent years, companion diagnostics for determining the effects of cancer therapeutic agents has been performing, and technology and reagent development for detecting single nucleotide polymorphism (SNPs) gene mutations are progressing.24) Furthemore, point of care testing (POCT) helps to improve QOL and patient satisfaction by shortening assay time and on-site testing. Therefore, genetic test system has been equipped in addition to the conventional immunochromatography test and attracts attention.8)

3. Immunochromatography

The principal technique is the immunoassay using capillarity that sample is spread slowly over cellulose membrane with dissolving reagents. When sample is applied, the antigen in a sample forms immune complexes with labelled antibody and moves over cellulose membrane, then react with capture antibody and enable to judge by color (Fig. 1). This system requires low volume of samples, less than 0.1 mL, easy operation, short assay time around several minutes to 30 min and do not require special facilities.25) Easy and rapid is the biggest advantage of this system and applied to pregnancy test, influenza test and further to food and environmental test.26) Variety of rapid test kit for infectious disease such as bacteria, virus, fungus, protozoan disease was developed.27)

4. Influenza Virus Rapid Test Reagent

There are at least 6 representative test reagents used in the world.28) Rapid antigen detection kit requires over 10^3–10^7 pfu/mL viruses in a sample.28) The kit design is either device type or the dip stick type and gold colloid, color latex particle and platinum is used for color development. Assay time is less than 15 min. More than 20 kinds of commercial kits are available in Japan and improvement of sensitivity, specificity and user friendliness has been done; — e.g. simultaneous detection of influenza and RS virus, using same extraction buffer for influenza, RS and adenovirus detection, short assay time and improvement of the cotton swab for sampling.27)

Recently, test kit with detection instrument is getting common. The instrument detection improves the variation of the result judgement by eye and the automation simplify the step.29) Although using detection instrument, if the sample contains low virus, near the detection limit of the kit, gives week positive or negative result and if sample contains low virus, below the detection limit, gives negative result despite of infectious of Influenza.30) Therefore accurate high-sensitive assay is desired.

The silver amplification improved sensitivity by arranged photograph technology which turned small material to large silver particle.31) Silver ion and reducing reagent only react with 0.05 μm diameter of gold colloid when existence of gold colloid, reducing reagent (ammonium sulfate iron) and silver ion (silver nitrate) and metallic silver, approx. 6 μm diameter, appears (Fig. 2). Using this principle, capture antibody at the detection line capture the antigen reacted with gold colloid labelled antibody and metallic silver appears only at the gold colloid exist. High-sensitivity was achieved to improvement of judgement by using approx. 100 times large diameter particle.24,31) Specificity decrease by higher sensitivity is prevented by the following.26,32) (1) Using F(ab')2) format reduce
non-specific reaction by rheumatoid factor and the heterophil antibody which react with Fc fragment of immunoglobulin G (IgG). (2) Reduce false-positive result by adding animal immunoglobulin for blocking nonspecific reaction. (3) Reducing reagent before a silver amplification step works as a washing and reduce background. Instrumentation runs assay at proper timing and mix silver amplification reagents in the cartridge automatically and form immune complexes. The judgment line image is saved and judged by intensity of the line automatically. The judgment is more sensitive and objective than eye judgement.33)

Time resolved fluorescence method (TRF) uses europium (Eu) that has 200000 times longer fluorescence life time than conventional fluorescent substance. TRF starts measurement after conventional fluorescence disappeared and measures fluorescence at certain period. Broad range of stokes shift is one of the features and this contributes to minimize the background by conventional fluorescence.34) TRF implemented high-sensitive fluorescence detection and enable to diagnose with low virus volume samples on early infectious stage. Early diagnose leads to early treatment and contribute to reduce transmission. The conventional detection instrument run only 1 sample but recently launched TRF high-sensitive instrument can runs several samples simultaneously with minimum 1.5 min detection time.35)

The advantage of influenza virus rapid test reagent as a POCT kit is visual judgement by eye without instrumentation.36) However recently, reagents with judgment instrument have been developed successively and achieved prevention of judgement variation by visual inspection, automatic judgment simplify time setting and high-sensitivity detection by silver amplification technology and fluorescent substances.36) Processable sample numbers differ by instrument on silver amplification method and TRF method, but both methods enable to detect influenza virus with high sensitivity and contribute to reduce unnecessary medication and implementation of appropriate infection control measures.

5. Procalcitonin Rapid Test Reagent

Procalcitonin (PCT) is a peptide consisting of 116 amino acids with 13 kDa molecular weight, normally synthesized in thyroid C cells as a precursor of calcitonin.37) In serious infections (sepsis) caused by bacteria, parasites, and fungi, inflammatory cytokines such as tumor necrosis factor (TNF)-α are produced by the effect of bacterial cells and toxins. Inflammatory cytokines stimulate producing PCT all over the body and secrete into blood. PCT increases in bacterial infections but hard to increase in viral infections. One of the reasons is that PCT production is suppressed by interferon (INF)-γ, which increases during virus infection.37,38)

PCT measurement is semi-quantitative detection by immunochromatography with anti-human calcitonin antibody. Short detection time with 30 min is the biggest advantage.39) Recently, quantitative assay test by automated immunofluorescence measuring instrument based on the chemiluminescence immunoassay method is available.40)

PCT attracts a lot of attention as a specific bacterial infection marker,37) but sensitivity and specificity in Sepsis is not so significant compare to C-reactive protein (CRP).41) Antimicrobial treatment with high PCT value in suspected sepsis is considered a safe choice. No antimicrobial treatment for low PCT in sepsis may increase a risk. PCT test can be a useful biomarker as a monitoring infectious severity and antibiotic treatment if tested depending on the situation, however, PCT should be considered as a reference value because it is not a single diagnosis marker. On the other hand, low PCT value at serious non-bacterial infection case may be useful to judge the case is not by infectious disease.42)

6. Smart Gene Myco System

Membrane coated with silica particle is welding with supporting plate and locates in-between sample spot area and absorption pad. Sample applied on the membrane is absorbed by absorption pad and nucleic acid in the sample is adsorbed on the surface of the silica particles. Impurities in the sample and components which inhibit PCR reaction is removed by washing step before measurement. Actual operation on the first step is to move forward the washing buffer chamber, and washing buffer is added to the sample spot area according this movement. Liquid sensor confirms washing steps and supporting plate is moved forward and inserted into the reaction tube. Commercially available washing buffer in the pretreatment kit normally inhibit amplification and detection, so washing buffer should be removed by drying or centrifugal treatment.

![Image](image_url)
This system applied the boom method for the pretreatment,\(^\text{15}\) which nucleic acids in a chaotropic salt solution are adsorbed on the materials with hydrophobic surface such as silica (chaotropic effect), so no need of drying or centrifugal treatment that washing buffer and silica particles are prepared which do not inhibit amplification and detection (Fig. 3).

Smart Gene Myco system (SG Myco) applies PCR amplification method and QProbe detection method.\(^\text{29}\) QProbe method is based on ‘fluorescence quenching phenomenon’ which decreases fluorescence emission when guanine base comes closer to fluorescent dye and detect the presence or absence of this quenching by monitoring. QProbe has a fluorescent substance bound to cytosine at the probe end and emit fluorescence when it does not bind to the target gene. Quenching occurs when QProbe binds. This system can identify single nucleotide polymorphisms by melting curve analysis.\(^\text{43,44}\) Identification of mycoplasma-resistant bacteria is one of the applications of this system.\(^\text{45,46}\)

The target genome on SG Myco system is a mycoplasma-specific QProbe which encoding 23S ribosome RNA. 2063th and 2064th bases which located in domain V are adenine in resistant strains and 2062th base which located in domain V is adenine in susceptible bacteria. Approximately 90% of resistant strains have substituted 2063th base with guanine, and other resistant strains have substituted 2064th base with guanine.\(^\text{46,47}\) PCR temperature profile at SG Myco system repeats liquid temperatures of 55, 95, 66 and 95°C degree. \(T_m\) value of the primer is set to around 66°C degree. Sensitive strains bind at 66°C degree and continue to emit fluorescence when lower the temperature to 55°C degree. The drug-resistant strain of mycoplasma can be identified by the difference of binding ability by temperature. SG Myco system change the liquid temperature to 55°C degree once every two cycles and resistant strain QProbe is quenching. Mycoplasma can be measured with or without mutation by monitoring fluorescence emission change at 55°C degree.

COVID-19 test using SG Myco system has been developed and is now available.\(^\text{49}\) Table 1 is the comparison data against real-time one-step RT-PCR method for COVID-19 close contact by National Institute of Infectious Diseases (NIID). This real-time RT-PCR method by NIID is recognized as a standard method. SG Myco for COVID-19 corelate 100% and this confirms the kit can be used at clinically with reliable result. The kit will contribute to expand diagnose ability at medical clinic by confirming the result immediately.

### 7. Conclusion

Developing high-sensitive antigen-antibody reaction or new assay format is desired because Rapid test is high specific but the sensitivity is not sufficient. Recent advances in biotechnology and life science have been applied to the medical and diagnostic fields and helps to achieve high sensitivity of immunochromatography and application of genetic testing system as POCT. Under COVID-19 outbreak situation, we hope utilized genetic testing application helps quick confirmation of COVID-19 positive patient and prevent the collapse of medical system.

**Conflict of Interest** The authors declare no conflict of interest.

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### Table 1. Comparison of COVID-19 Detection by Real-Time RT-PCR and Smart Gene

| Real-Time RT-PCR | Smart gene |
|------------------|------------|
| Pos   | Neg   |
| Positive | 10    | 0    |
| Negative | 0     | 15   |
| Total   | 10    | 15   |
| Concordance (%) | 100  |

Legend: Neg, negative; Pos, positive. The RNA in the positive sample is quite distributed in the range from about 10 to 200000 copies, the range concerned to become equal. Also, among 10 samples, at least one sample includes 100–200 copies and at least two samples includes 10–20 copies.
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