

TaqMan low-density arrays for simultaneous detection of multiple pathogens

Nupur Rani Agrawal¹, Anupam Patra², Bolaji F. Oyeyemi³ and Gajraj Singh Kushwaha⁴

¹Jawaharlal Nehru University, New Delhi, India ²Transcription Regulation Group, International Centre of Genetic Engineering and Biotechnology (ICGEB), New Delhi, India ³Molecular Biology Research Group, Department of Science Technology, The Federal Polytechnic Ado-Ekiti, Ado-Ekiti, Nigeria ⁴KIIT-Technology Business Incubator, Kalinga Institute of Industrial Technology (KIIT-DU), Bhubaneswar, Odisha, India

5.1 Introduction

Polymerase chain reaction (PCR) has been proven to be a revolutionary technology in molecular diagnostics; therefore its variation has been developed to expand its applications particularly in molecular diagnostics. The most important milestone in its variation journey is development of quantitative PCR (qPCR), also called real-time PCR (RT-PCR). The most useful feature of RT-PCR is that the amplification can be quantified using fluorescence in real-time thus it removes the time-consuming step of amplicon detection by electrophoresis.

In conventional PCR, the amplification is carried out by a thermostable DNA polymerase enzyme, Taq from *Thermus aquaticus*.

Interestingly, Taq polymerase has exonuclease activity in addition to polymerase activity and this activity is utilized to cleave a fluorescent probe during amplification process. The amount of released fluorescence probe is detected to quantify amplification. This method is called TaqMan assay.

Being a highly sensitive technique, several technological advancements have been made in TaqMan assay to make it more versatile. TaqMan low-density array (TLDA) is an advancement of TaqMan assay which offers high throughput and simultaneous PCR setup into 384 wells, even without using liquid handling system thus avoiding multichannel pipetting that may lead to handling errors. The TLDA, a microfluidic card, is developed to

carry out up to 384 simultaneous RT-PCR (qPCR) reactions and it can support 1–8 samples, 11–380 assay targets, and 1–4 replicates of each assay per card. These properties along with ease of handling procedure make RT-PCR a great molecular tool for high-throughput and rapid analysis of a large number of samples. There are four commercial formats of TLDA available, including inventoried (preconfigured), made-to-order flexible, custom, or specialty-designed arrays.

5.2 TaqMan low-density array

Accurate detection of infectious diseases is a challenging task and clinicians often rely on classical syndromic approaches for diagnosis. Currently available diagnostic procedures identify single pathogen per sample, resulting in delay in commencement of treatment. Further delay in appropriate treatment may happen if an individual is infected with less common pathogens. All this takes a toll on a patient's health resulting in extended hospital stay, exposure to broad-spectrum antibiotics and undue increase in drug toxicity.

Broadly, diagnosis deals with the reason behind disease while further detection helps in identifying causal organisms present in the test sample. It becomes very difficult to spot causative pathogen if the host does not display distinguished symptoms. Additionally, the diagnosis is further complexed if a host is attacked by several pathogens either simultaneously or progressively, the symptoms show huge variations, which makes treatment even more difficult. In such scenario, disease management depends primarily on speedy and accurate spotting of causal pathogens. Molecular biology methods are making ways in development of techniques for easy identification of pathogens. Pulsed-field gel electrophoresis, multilocus sequence typing, culture, and colony-counting methodologies, along with immunology-based approaches are the most

commonly utilized pathogen identification techniques. These methods rely on fundamental principles including DNA analysis, bacterial counting, and antigen–antibody interactions. However, these approaches often show lower sensitivity, reliability, and reproducibility. Moreover, traditional pathogen detection procedures are laborious, tedious, less sensitive, and frequently necessitate specific expertise and/or facilities. The limitations of these conventional approaches led to development of easy and affordable molecular tools for the precise and rapid detection of infectious pathogens. In this series, PCR has emerged as an important tool in clinical laboratories aiding in rapid pathogen identification in past decades. It presents enhanced sensitivity and specificity, decreases risk of contamination, and enhances speed of detection, along with ease of handling and reduced hands-on time. Later on, RT-PCR has been included in the routine molecular diagnosis of infectious pathogens and over the time the desired features have made RT-PCR an attractive diagnostic method in the medical world [1].

5.3 Design of TaqMan low-density array card

TLDA cards are designed using microfluidic technology to accommodate very small quantities of reaction samples. As shown in Fig. 5.1, it contains 384 wells connected with 8 sample loading channels. The sample loading parts of TLDA include vent ports, fill port, fill reservoir and it is connected to feeder channels through main channels. Finally the feeder channels supply samples to reaction wells. The convenient design of sample loading ports allows easy and rapid setup of reaction because of easy loading of samples without multichannel pipettes and liquid-handling robotics. Hence, it makes cost-effective and low volume precious clinical samples. Another major benefit of TLDA cards is flexible design which can be customized or it can be preconfigured

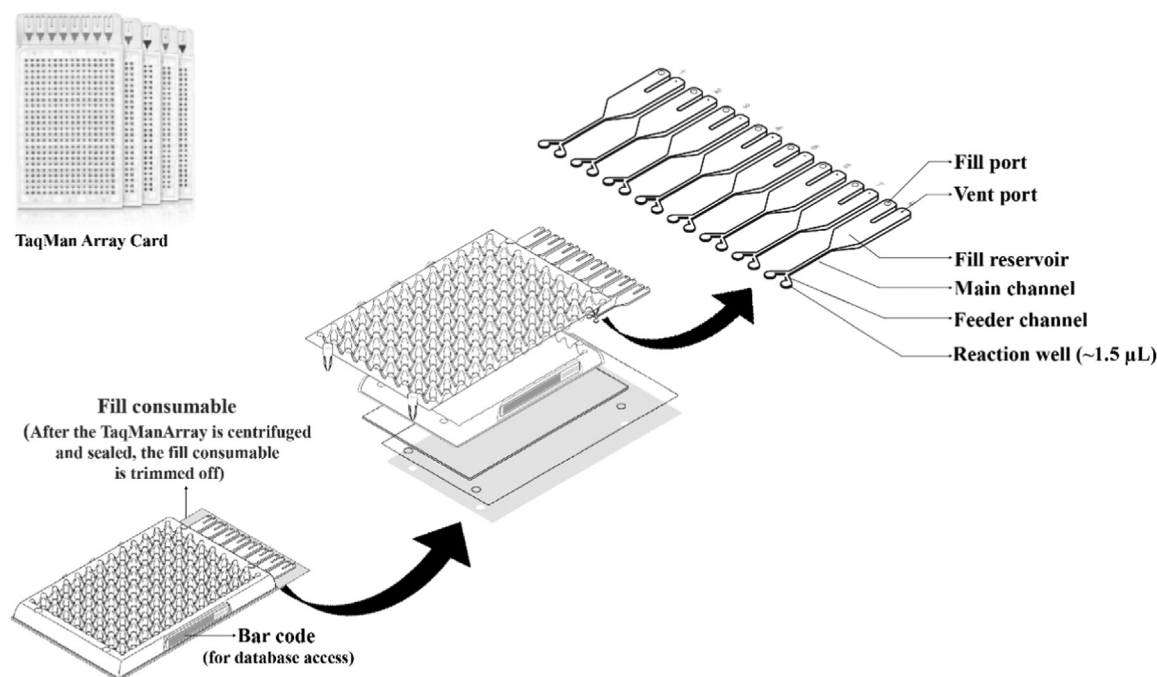


FIGURE 5.1 Design of TaqMan low-density array card.

according to target assays. Since each sample is flows separately in microfluidic channel thus it reduces the chances of cross contamination and variability. Overall design of TLDA cards provides reproducibility, convenience, flexibility, and consistency along with high-throughput reaction setup.

5.4 TaqMan low-density array workflow

TLDA, a microfluidic card, designed in a RT-PCR single plate format with 384 wells in which 8 samples can be loaded on 48-well setup with a single target being analyzed in every 48 wells simultaneously (Fig. 5.1). The wells in this card are preloaded with lyophilized probes and primers. The lyophilization of both primers and probes increases shelf-life of the card by almost 2 years at low temperature

without any loss of sensitivity. Microfluidic channel distribution mechanism helps in distribution of samples in the well where reactions take place.

Application of samples on TLDA for identification of pathogens begins with extraction of nucleic acid from the pathogen. It is then added to a reaction mixture containing enzymes, buffers, and nucleotides. In a typical reaction setup, the reaction volume is adjusted to 100 μL by addition of nuclease-free water. The reaction mixture is then loaded on a single reservoir/port. Likewise, all eight samples are prepared and loaded into the wells, thereafter the TaqMan array card (TAC) plate is centrifuged at 1200 RPM twice. Ports/reservoirs of the plate in which samples were loaded are clipped with scissors. Finally, the TLDA plate is then loaded on an RT-PCR instrument with a run time of around 53 minutes (Fig. 5.2).

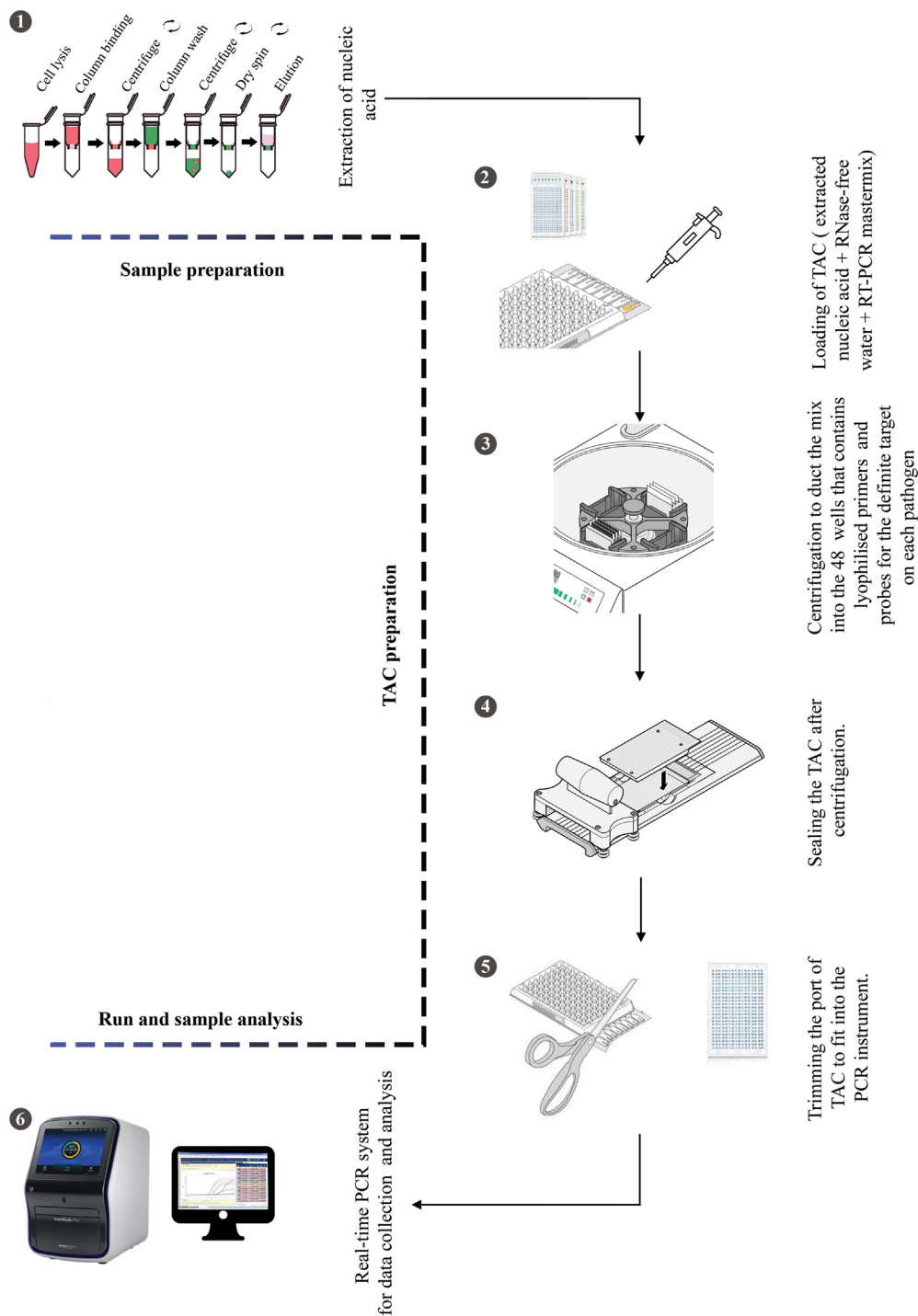


FIGURE 5.2 Schematic diagram showing workflow for TaqMan low-density array reaction setup.

5.5 Advantages and disadvantages of TaqMan low-density array

Working with TLDA is quite simple hence its handling does not require special training. Technicians/scientists working on PCR can easily work on TLDA too, even the results generated by TLDA are easy to interpret. Its user-friendly approach helps to reduce the cost of manpower in clinical diagnosis. Presently, TLDAs are the only available PCR-based array that can identify up to 48 targets in a single specimen. Confirmatory assays can be taken into account by detection of multiple targets per pathogen on the TLDA plate, it can increase assurance of diagnosis. However, reduced sensitivity is reported with TLDA as compared to monoplex assays [2] but it can be resolved by using more competent nucleic acid extraction methods or by increasing the extraction volume [3]. Another pitfall of TLDA is limitation of eight samples at a time; this adds constraint to the number of samples that can be acquired in a single run, further lack of automation and unavailability of PCR product for further analysis. Single annealing temperature is required for all PCR experiments and all assays should be optimized to work under universal conditions. Narrow sensitivity of TLDA compared to monoplex assay does not have much impact if the aim of assay is detection rather than quantification of pathogens. Cycle threshold (C_t) value helps clinicians in getting an approximate estimate of the

relative amount of an organism present in the test sample. Several organisms can be detected in a single run in TLDA and identification of causal organisms requires careful clinical interpretation. The major advantages and disadvantages are summarized in Table 5.1.

5.6 Expanding RT-PCR assay to TaqMan low-density array

Development of any diagnostic test or assay follows certain regulatory guidelines; likewise TLDA assays follow the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) and in vitro diagnostics (IVD) guidelines [4]. Because all tests in TLDA are conducted under the same conditions, every monoplex and multiplex RT-PCR are modified in such a way that should be suitable to a single format that uses the same reagents, probes with the identical reporter fluorophore, and quencher. Similarly, the primer modification to increase T_m to 55°C–60°C is achieved by addition of target-matched extensions so that all 48 assays amplify at one temperature. Correspondingly, the preferred length of amplicon should be between 60 and 150 nucleotides. The TLDA designs also include probes with a single fluorescent 5' dye (FAM) and “minor groove binder” (MGB) at the 3' end nonfluorescent

TABLE 5.1 Advantages and disadvantages of TaqMan low-density array card assay.

Advantages of TLDA	Disadvantages of TLDA
Ease of data interpretation	Only eight samples can be acquired in a single run
No specialized training is needed	Lack of automation
Easy to handle	PCR product cannot be used for further analysis
Multiple organisms can be detected in single run	Single annealing temperature is required for all assays
TLDA card has long shelf-life of almost 2 years	Reduced sensitivity compared to monoplex assays
User friendly	It is not helpful in quantification of organism

quencher. All these modifications increase specificity and sensitivity. Incorporation of MGB moiety also proves beneficial in allelic discrimination, designing AT-rich probes for the genotyping, and programming the test assays to identify drug-resistant mutants. The modified PCR experiments impeded by inhibitors present in test samples also show high sensitivity in TLDA.

During development of assay protocol, accuracy which comprises both sensitivity and specificity parameter, productivity, and fluorescence-dependent intensity should be compared to both standards as well as patient samples. Assay development and comparison with standards should be carried on diverse sample type such as plasma, stool, nasal swabs, bronchoalveolar lavage, naso-pharyngeal aspirates, CNS, fresh biopsy tissue and formalin-fixed, and paraffin-embedded specimens. Internal control should be included in case of low pathogen load [5], even increasing extraction volume also serves the purpose. Optimization and validation of assays will open new doors in the clinical diagnostic world as TLDA is a robust PCR-based platform capable of detecting up to 48 targets in a specimen in single run.

5.7 Importance of TaqMan low-density array in biomedical science

TLDA cards hold the potential to revolutionize the clinical diagnostic industry as it can help in detection of multiple pathogens simultaneously. It could make detection easier by eliminating the several middle experiment steps, including transport, enroll, aliquot, and pretreatment of sample for several pathogenic agents in the same sample in a particular sequence. It will reduce the cost of diagnosis as it requires lesser handling thereby also reducing chances for human error. A single run that takes almost 3 hours to complete

during which TLDA can detect 48 pathogens in a sample whereas individual RT-PCR (IRTP) for detection of 48 pathogens in a specimen would be more time-consuming and labor intensive. In one study, Rachwal et al. explored the potential of TACs for detection of multiple biological agents. Ten PCR assays targeting five biological agents *Bacillus anthracis*, *Burkholderia mallei*, *Burkholderia pseudomallei*, *Francisella tularensis*, and *Yersinia pestis* were integrated into array cards [6]. Kodani et al. explored the ability of TLDA for simultaneous detection of multiple respiratory pathogens [2]. It was used by O'Brien K for microRNA profiling for biomarker and on cosuppressor discovery by isolating miRNAs from cell lines [7]. In the coming years, TLDA could prove a boon for fast and continuous detection of several infectants during the epidemic surge, which helps inspections and disease spread monitoring.

5.8 TaqMan array card in multipathogen assays

Pioneer work with TLDA comparison on respiratory pathogens was conducted by Kodani et al. In pneumonia and upper respiratory tract infections, the sensitivity and specificity of TLDA cards were compared and assessed on 192 viral positive specimens versus IRTPs utilizing identical methodologies, primers, and probes [2]. TLDA results have a sensitivity and specificity of 95% and 98%, accordingly, in this case.

Based on this comparison study, the following points are summarized as:

1. It enables the identification of many infections at the same time, eliminating the need to collect, enroll, fraction, and analyze diagnostic samples for distinct pathogens linearly.

2. TLDA lowers the manual handling requirement as well as the potential for human error.
3. In the event of an epidemic, a single technician may identify 21 pathogens from a variety of patient samples in three hours, whereas IRTP procedures would take significantly longer.
4. The TLDA card uses far less nucleic acids than the IRTP; for identifying 21 pathogens utilizing separate assays in duplicate, more than 210 μL of DNA is required, whereas the TLDA uses just 20 μL .
5. The necessity to sort between putative pathogens is eliminated by evaluating many infectants, even when the diagnostic sample provided is limited.
6. The TLDA card has a single plex, presorted channel that avoids the need for lengthy assay tuning and reduces cross-contamination.

Although TLDA cards require relatively more nucleic acid concentrations than IRTPs, the accuracy of the TLDA, contrasted to the IRTP, was similar under the given conditions. At the same time, the sensitivity of the CT threshold of 43 was 4% lower. On the other hand, the TLDA's sensitivity for infections increased by 33% when the CT cut off was dropped to 35. The sensitivity of TLDA may be less due to the dilution of the sample; however, in an epidemic, this problem may be overlooked because disease manifestation is always associated with a large pathogen load. When all factors are considered, TLDA results are fairly similar to IRTP in terms of sensitivity and specificity. Another extensive study was done with 942 specimens by Weinberg et al. and the sensitivities values were 83% and 99%, respectively. Individual RT-qPCR and TLDA assays were used to analyze 942 clinical specimens for the 10 target viruses [8]. This research also revealed annealing temperature-dependent sensitivity difference on adenovirus, rhinovirus, and HPIV1, as indicated in the prior work.

The consistency of cycle threshold (C_t) value regarding TLDAs was also good in these two studies, with a mean coefficient of variation with less than 2.91%.

A subsequent TLDA study found that improving the whole testing strategy, which included assessing alternative enzyme systems, modifying oligonucleotide dosages, and expanding the number of copies from two to four, might improve sensitivity even more near the limit of detection (LoD) [3].

Besides two previous, another large-scale study conducted by Harvey et al., where the Association of Public Health Laboratories (PHLs) and the Centers for Disease Control and Prevention (CDC) collaborated with six PHLs to undertake a multicenter appraisal of TLDA technology using viral and bacterial respiratory infection detection. The main objectives were to compare 21 individual respiratory pathogens, based on CDC-generated reference assays' analytical performance on TLDAs with individual CDC "laboratory-developed tests" (LDTs), LDTs from two separate state, and three trending multiple target control panels [9]. This study is also consistent with Kodani et al. [2] regarding the sensitivity of TLDA as it was one log lower, where nonoptimized nucleotide for smaller reaction volume could be the explanation. Despite information on LoD determination in terms of infectious dose 50% or copies/mL, results show the TLDA's sensitivity, specificity, and repeatability comparable to existing multipathogen detection technologies, regardless of the specimen type tested.

Following the previous comparison studies, recently, a research group developed a large-scale TLDA that could detect 28 common lung infections in China and compare its performance to "gold-standard" approaches like bacterial culture as well as RT-PCR [10]. This TLDA test has a sensitivity and specificity of 95.4% and 96.6%, respectively, which was greater than in prior studies. A respiratory disease might spread swiftly [11] and lead to catastrophic pandemics, where timely detection of lung infections is crucial in disease

management. Based on satisfactory TLDA results, one can conclude that in 3 hours, the TLDA assay may detect numerous respiratory tract pathogens, including pretreatment, column isolation, and amplification.

5.9 Comparison of TaqMan array card with RT-PCR assay and commercial multipathogen assays

In any pathogenic disease, diagnosis is the most crucial step that needs fast results for many pathogens at a time regardless of compromising its sensitivity. Methods for detecting viral and bacterial pathogens evolve with time, and RT-PCR, in addition to culture, has become the preferred method in molecular diagnostics. Traditional approaches are expensive, time-consuming, less sensitive, and frequently necessitate specialized expertise and/or equipment. Nevertheless, those are useful in circumstances when culture-independent assays have low sensitivity. Modern-day science develops multiple detection systems by attaching mass spectrometry, bead sorting, dye-labeled probes, and microarray technology [12–15] with the multiplex PCR facility. First, molecular techniques all need to evaluate their primer-specific sensitivity for the probe as they deal with multiple additional primers. With the growing data science field, all possible essential upgrades require a longer period but high sensitivity. Luminex xTAG Respiratory Viral Panels (Luminex Corporation, Austin, TX, United States), Qiagen ResPlex v2.0 (Qiagen, Hilden, Germany), and TessArae RPM 3.1 Flu (TessArae LLC, Potomac Falls, VA, United States) [16–19], among others, are commercial platforms and assays for the concurrent detection of multiple respiratory pathogens in a specimen. The type and number of studies conducted (viral, bacterial, mixed, influenza-specific), detection technique and the need for specialist instrumentation, complexity, sensitivity, throughput, and cost per specimen are all differences between these platforms.

However, several of these panels have been evaluated and shown to have good specificity and different sensitivity levels across pathogens.

The TAC (Life Technologies, Carlsbad, California) is a low-density microfluidic card with an 8-sample, 48-assay array, and 384 IRTPs reactions. The experiments described in this publication were carried out on a TAC partitioned into an 8-by-24 array, with each sample being unique and tests being run twice. TACs have been designed to detect respiratory infections [20], hepatitis genomes [21], enteropathogens [22], and bioterrorism agents [23], as well as associated positive control materials [24].

The microfluidic-based TLDA provides a practical, field-supportive system for multiple-pathogen detecting. Besides pathogenic diseases, TLDA was extensively used in cancer transcriptomics for genes as well as mi-RNA expression also [25–28]. Recently, mi-RNA biomarker specificity was investigated in a drug-induced toxicology study using TAC applications [29]. So, this technique is useful for both pathogenic DNA and RNA trace. In addition, the TLDA platform has the advantage of being a closed system with validated single-plex PCR technology, allowing for additional primers and probes without having to recalibrate the others currently on the cards. Furthermore, this format is simple to use because the compounds for each experiment are preallocated to the reaction wells, and the sample only needs to be introduced once. The TLDA card architecture also makes it easier to handle specimens and uses less input template nucleic acid than a same number of separate PCR processes. One internal positive control, two nucleic acid quality controls, and 21 pathogen assays are available on the TLDA card, each with real-time semi-quantitative output.

5.10 Conclusion

The TLDA card could prove a useful tool based on these findings for pathogen

identification, especially in monitoring an epidemic scenario. Furthermore, rather than individual clinical therapy, the data can be comprehended in a wider population for public health studies.

Cases where more than one pathogenic agent tests positive should be given special attention. Developing techniques to combat numerous positive infection responses to determine which pathogens are mostly responsible for the disease, as pathogens can function synergistically to cause illness, and which pathogens indicate carriage, will be a barrier to TLDA use. Clinical assessment is crucial in distinguishing between transport and disease, as it is with any multipathogen detection technique. Furthermore, the appearance of a novel type or variant that is resistant to molecular characterization, as well as the combined effect of genetic drift, could diminish detection accuracy.

The TLDA card can be an effective and promising resource for quick screening of various respiratory pathogens when the causative agent of pulmonary illness is unknown or multiple pathogens are presumed. TLDA offers several advantages over other commonly used diagnostics, however, more research is needed to assess the TLDA's performance in a clinical setting. Other than respiratory diseases, TLDA cards could help with dysentery, septic, and other inflammatory conditions, in which several bacteria can cause the same symptoms.

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