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Review

A review of molecular biology detection methods for human adenovirus

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Abstract: In humans particularly in children, adenovirus is one of the most common viruses that cause respiratory illnesses. Knowing how to detect adenovirus proficiently and rapidly will help reinforce surveillance of adenovirus infections, detect epidemic situations in real-time, and understand the trend of virus epidemics, which will allow effective actions to be taken quickly. The rapid detection of antiviral antibodies or viral antigens in clinical samples can be achieved by molecular diagnostic techniques like PCR, Real-Time PCR, LAMP, mPCR-RLB, PCR-ELISA, Tem-PCR, Gene Chip, and so on. Some of the molecular diagnostic methods are relatively economical, exceedingly sensitive and explicit. There are several commercially accessible molecular diagnostic techniques that enable their use in clinical laboratories all over the world. In this review, the principles, characteristics, and applications of molecular biology surveillance methods commonly used in labs and clinics for the detection of human adenoviruses are examined and highlighted.

Keywords: adenovirus; PCR; real-time PCR; LAMP; mPCR-RLB; PCR-ELISA; Tem-PCR; gene chip; NGS technology; MLPA

1. Introduction

Adenoviruses are a type of non-enveloped DNA virus with a diameter of 70–100 nm, an icosahedral symmetrical arrangement, and 252 capsomers [1]. Out of the 252 capsomers, 240 non-vertex capsomers are arranged in trimers and are known as hexon. As the foremost neutralizing antibodies producing protein of adenoviruses, hexon stimulates antibody production, inhibits virion conformational changes, and neutralizes them [2]. Aside from the hexon, the icosahedron's capsomers consist of 12 penton bases, each consisting of a base and a tagged fiber with a protruding apicosphere

at its end [3]. In 1953, Rowe WP isolated the human adenovirus (HAdV) from the tonsils of children [4]. It belongs to the family Adenoviridae and to the genus Mastadenovirus. It is a common pathogen that infects the human body. Research data shows that around 105 adenovirus types have been found so far. The International Committee on Taxonomy of Viruses divides human adenoviruses into seven species from A to G. Types A and F mainly cause digestive tract infections. E, C and some B species are the main causes of respiratory diseases, other B species cause urinary tract infections, D species are the chief causes of keratitis and conjunctivitis [4], F and G species are intestinal ADV, and adenovirus can also cause transplant immunodeficiency disease, encephalitis and obesity and other diseases [5,6]. Due to its high sensitivity, high specificity, and rapidity, molecular biology detection technology is currently becoming more and more established. Various molecular diagnosis technologies mainly based on nucleic acid amplification have greatly shortened the detection time, and have gradually replaced traditional methods for virus detection in many laboratories and clinics. Nucleic acid amplification-based diagnostic technologies, such as polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP), have greatly improved the speed and accuracy of virus detection. The great sensitivity of nucleic acid amplification-based detection, which enables the detection of very low quantities of viral genetic material, is one of its key benefits. This is crucial for identifying viral infections early, when the viral load could still be low. These techniques are versatile and adaptable to many testing environments since they may also be used to find viral genetic material in a range of sample types, including blood, urine, respiratory and oral swabs, and swabs from the mouth and nose. Moreover, PCR and LAMP are also widely used in various settings including hospitals, laboratories and even at home, and they are relatively easy to perform, with minimal equipment required. PCR and LAMP are widely used for the detection of a wide variety of viruses, including SARS-CoV-2, which causes COVID-19. These methods allow for the rapid and sensitive detection of viral genetic material, even in small amounts. This is in contrast to traditional methods, such as cell culture or serological assays, which can be time-consuming, labor-intensive, and less sensitive. In the meantime, gene chips, next-generation sequencing, and other technologies have made rapid, sensitive, specific, and high-throughput detection possible [3]. In order to understand the most effective and fast detection methods for human adenoviruses at present, I am reviewing the most frequently used detection techniques in molecular biology of human adenoviruses.

2. Conventional PCR technology

2.1. General PCR

A potent method for amplifying specific DNA sequences, polymerase chain reaction (PCR) is a very sensitive and specific approach for identifying the presence of a specific disease, such as the human adenovirus. Polymerase Chain Reaction (PCR) was discovered in 1983 by Dr. Kary Mullis, a biochemist working at Cetus Corporation, a biotechnology company based in Emeryville, California [7]. He was awarded the Nobel Prize in Chemistry in 1993 for his discovery. By using the target gene as a template, PCR technology synthesizes DNA in vitro. The double-stranded DNA is heated to a high temperature (typically around 95-98 °C) to separate the two strands. The temperature is lowered to around 55-65 °C, which allows specific primers (short DNA sequences) to bind to the ends of the target sequence. The temperature is raised to around 72–75 °C, which allows the polymerase enzyme to extend the primers, synthesizing new DNA strands complementary to the target sequence. The process is then repeated for

several cycles, each time doubling the amount of the target DNA sequence. After a few cycles, the amount of the target sequence becomes exponentially increased (Figure 1). Using complementary oligonucleotide primers to both ends of the target sequence, the specificity of the PCR results can be attained rapidly. It is conceivable to detect adenovirus using variability of clinical specimens, including throat swabs, nasopharyngeal lavage fluid, stool, and blood samples [3]. The main principle amplification regions are the preserved region gene of the penton base, hexon, and fiber knob which are used as the amplification target sequence to achieve the purpose of further amplifying [8,9]. As an example, ordinary PCR amplification can be used to identify adenovirus hexon nucleic acids. The amplified product is electrophoresed in agarose gel after several cycles of denaturation, annealing, and extension, and the size band is observed under ultraviolet light, and the final sequencing outcome confirms its type by BLAST sequence analysis [7]. With the research of universal primer PCR, a primer that is universal and explicit to adenovirus and whose amplified product has adenovirus type-specificity has been efficaciously designed as it is sensitive which is appropriate for clinical routine diagnosis of adenovirus infection [8,9]. Several studies have demonstrated the high sensitivity and specificity of PCR-based methods for adenovirus detection in respiratory tract samples. For example, Harrington et al.2004 showed that PCR-based detection of the hexon gene was more sensitive than traditional cell culture methods for detecting adenoviruses in respiratory tract samples [10]. Similarly, Siyabi et al. 2013 found that PCR-based detection of the fiber gene had a sensitivity of 100% and a specificity of 69.2% for detecting adenovirus in respiratory tract samples [11]. Adenoviruses can also be found in feces and blood samples using PCR-based techniques. According to Casas et al. 2005, finding adenoviruses in feces samples using PCR-based hexon gene detection was 100% sensitive and 98.8% specific [12].

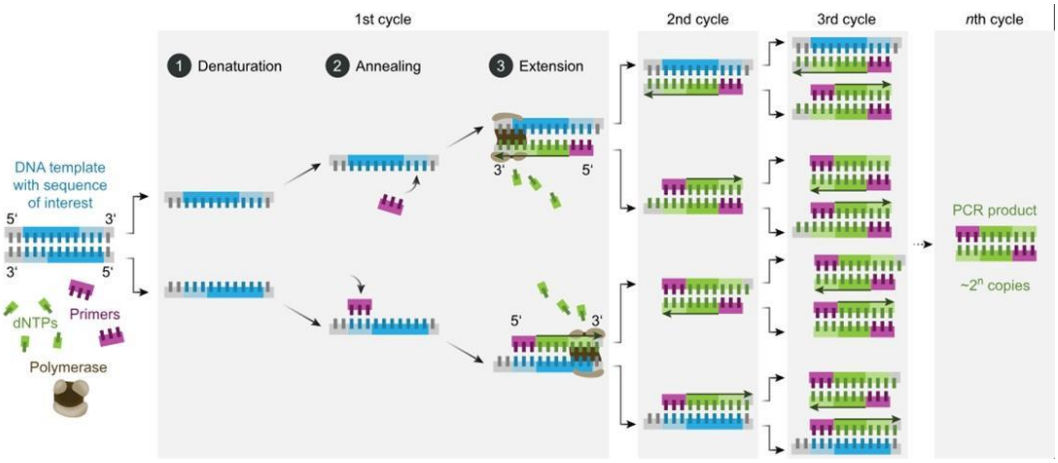

Figure 1. Schematic Representation of PCR. PCR works in three steps: denaturation, annealing, and extension. a) Denaturation: In this step, the double-stranded DNA is heated to a high temperature to separate the two strands. b) Annealing: The temperature is then lowered to allow primers, short DNA sequences that are complementary to the ends of the target DNA sequence, to bind to the single-stranded DNA. c) Extension: The temperature is then raised again to activate a heat-stable polymerase enzyme, which extends the primers along the template DNA, synthesizing a new complementary strand. This process is repeated multiple times, leading to exponential amplification of the target DNA sequence. **Source**:https://en.wikipedia.org/wiki/Polymerase_chain_reaction#/media/File:Polymerase_chain_reaction-en.svg

As a result of its simplicity and budget, PCR technology is the foundation for modern molecular biology detection technology, which is effortlessly available to most laboratories and very easy to endorse. The target sequence begins to multiply exponentially as a result of the PCR process. The beginning quantity of the target sequence present in the sample can only be determined by extrapolating backward during the exponential phase of the PCR process. The PCR reaction eventually stops amplifying the target sequence at an exponential rate, leading to a "plateau effect," which makes the endpoint quantification of PCR products unreliable. This plateau effect is caused by inhibitors of the polymerase reaction that are present in the sample, reagent limitation, and self-annealing of the accumulating product. Also due to its long detection time, easy contamination, and incapacity to quantify accurately, it is gradually being substituted by other derivative methods as technology improves.

2.2. Real-time PCR

Real-time polymerase chain reaction (real-time PCR) is a variation of the PCR technique that allows for the detection and quantification of specific DNA sequences in real-time, making it a powerful tool for the detection of human adenoviruses. Almost similar to conventional general PCR in its fundamentals, real-time PCR additionally uses fluorescent probes or primers that bind to certain sections of the target DNA sequence. During Real-Time PCR, a small amount of the DNA sample is mixed with primers (short DNA sequences) that are complementary to the target DNA sequence. The mixture is then heated to denature the DNA into single strands. Then, the temperature is lowered to allow the primers to anneal to the target DNA strands. Next, a special enzyme called Taq polymerase copies the target DNA sequences by adding complementary nucleotides to the primers. As the Taq polymerase copies the DNA, the fluorescent dyes or probes that are specific to the target DNA sequence emit a signal that can be detected in real-time. The amount of DNA in the sample is measured based on the amount of fluorescence emitted during each cycle of the PCR reaction (Figure 2). When these probes or primers attach to the target DNA, they release a fluorescent signal that enables the detection and real-time measurement of the target DNA. The fluorescent signals are monitored and analyzed constantly during DNA amplification reactions, and a standard curve is drawn to determine the amount of amplified products at different intervals after the PCR cycle. There are several types of fluorescent dyes and probes that are commonly used in real-time PCR like SYBR Green, TaqMan probes, Molecular Beacons and Scorpions. To determine the initial amount of DNA in the sample, a standard curve is created. A standard curve is a plot of the fluorescence signal versus the known concentration of a target DNA. The standard curve is created by analyzing a series of known concentrations of the target DNA. The standard curve is then used to determine the initial amount of DNA in the sample by comparing the fluorescence signal of the sample to the standard curve. This technique allows researchers to quantify the amount of DNA present in the sample in real-time and determine the exact time at which the amplification reaches a certain threshold. The adenovirus sample for real-time PCR can be collected from various sources, such as clinical specimens, environmental samples, or cell cultures. The collected sample is subjected to appropriate treatment, such as filtration, centrifugation, or viral lysis, to release the adenovirus particles. The released adenovirus particles are subjected to DNA extraction, which involves the purification of the viral DNA from the other cellular components and contaminants. This can be achieved using various commercial kits or manual methods. The extracted DNA is quantified and its quality is assessed using methods such as spectrophotometry,

gel electrophoresis, or Qubit fluorometry. The extracted DNA is diluted to an appropriate concentration for use as a template in Real-Time PCR. The template DNA is added to a reaction mixture that contains the necessary reagents, such as Taq polymerase, primers, and fluorescent probes. The reaction is performed in a specialized Real-Time PCR machine that measures the fluorescence signal produced by the reaction in real-time. The data generated by Real-Time PCR is analyzed using software that calculates the cycle threshold (CT) value, which is a measure of the amount of DNA present in the reaction. The CT value can be used to determine the presence and quantity of the adenovirus in the sample.

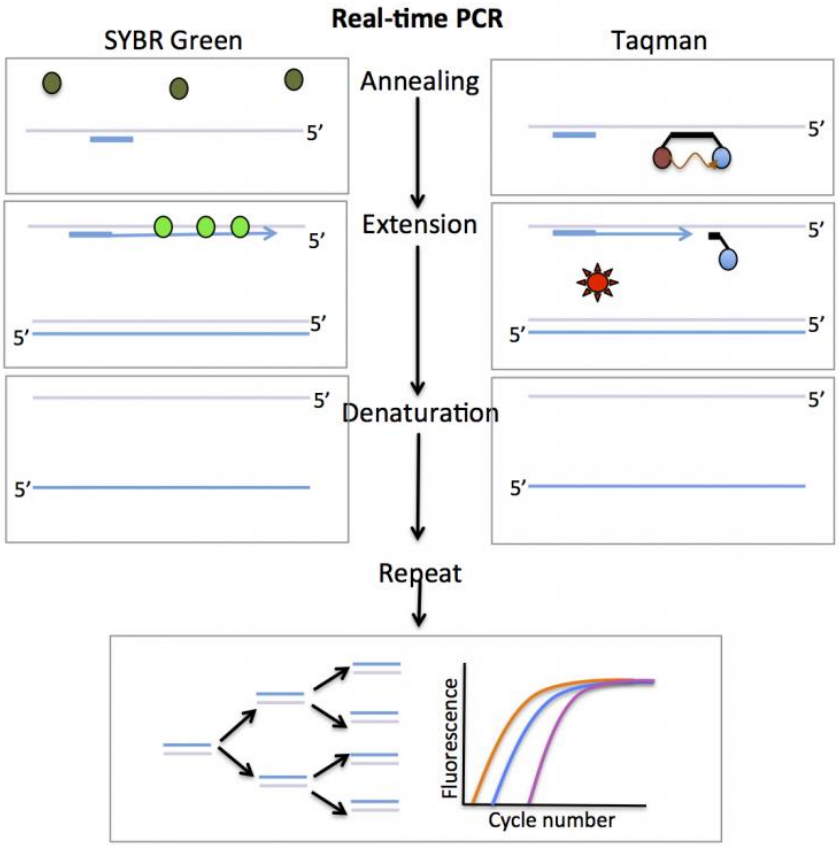

Figure 2. Schematic diagram of Real-Time PCR: The following steps describe Real-Time PCR: a) The sample containing the target DNA is extracted, and the DNA is purified and quantified. b) Primers are designed and synthesized. c) A reaction mix is prepared with the purified DNA, primers, Taq polymerase, and other reagents. d) The reaction mix is subjected to thermal cycling, with repeated cycles of denaturation, annealing, and extension. During each cycle, the fluorescence generated by a fluorescent probe like SYBR green that bind to the amplified DNA and emit light as the reaction progresses, is measured and recorded, providing a quantitative measurement of the amount of target DNA present. e) The fluorescence data is analyzed to determine the quantity of target DNA present in the sample.

Source: Huerta L, Burke M (2020) Functional genomics II: Common technologies and data analysis methods; https://www.ebi.ac.uk/training/online/courses/functional-genomics-ii-common-technologies-and-data-analysis-methods/real-time-pcr/

The great sensitivity and specificity of real-time PCR is one of its most significant benefits. In a 2005 research, the sensitivity and specificity of a real-time PCR test targeting the viral hexon gene were compared to that of conventional cell culture techniques [13]. With a detection limit of 10 virus particles per response, the real-time PCR test was shown to be more sensitive than cell culture techniques, which had a detection limit of 103–104 viral particles per milliliter [14]. Furthermore, the real-time PCR test demonstrated 100% specificity, properly detecting both all adenovirus-positive and adenovirus-negative samples. Another advantage of real-time PCR is its ability to quantitatively measure the amount of viral DNA in a sample, which can provide valuable information for the diagnosis and management of adenovirus infections. For example, a study published in the *Pediatric Infectious Disease Journal* in 2005, used real-time PCR to quantify the amount of adenovirus DNA in respiratory tract samples from patients with acute respiratory infections [15]. The study found that patients with higher levels of adenovirus DNA were more likely to have severe disease and required hospitalization. To increase the sensitivity and specificity of viral detection, real-time PCR has also been combined with other methods including loop-mediated isothermal amplification (LAMP). In order to identify the virus in samples from the respiratory tract, Choi and Jiang, 2005 used LAMP and real-time PCR to target the adenovirus hexon gene [16]. With a detection limit of 10 virus particles per response, the study discovered that the combination of LAMP and real-time PCR was more sensitive than either approach used alone. Morozumi et al., [17] used this method to promptly recognize pediatric respiratory adenovirus-infected patients, validating that this technique not only has very great sensitivity, but also circumvents the use of unnecessary antibiotics and averts HAdV-related disease epidemics. As a consequence of its ease of use, good repeatability, high sensitivity and specificity the method is perfect for monitoring human adenoviruses on a regular basis and diagnosing epidemics during emergencies [18,19]. At the same time, due to the restrictions of the type of fluorescein and the detection light source and the high experimental cost, its extensive application is inadequate.

2.3. Loop-mediated iso-thermal amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification technique that allows for the detection and quantification of specific DNA sequences at a constant temperature using heat-stable enzymes to amplify a target DNA sequence in a single tube reaction. LAMP amplifies DNA by exploiting the properties of Bst polymerase and the unique structure of the target DNA to generate a characteristic fluorescence or turbidity change, which makes it a powerful tool for the detection of human adenoviruses. Recent years have seen an outpouring in the use of LAMP which consists of the design of four specific primers for six regions of the target gene, including two inner primers and two outer primers. The basic LAMP process involves the use of primers that specifically bind to regions of the target DNA sequence, allowing for the amplification of the target DNA at a constant temperature. In this method, a set of primers is designed to target specific regions of the target DNA sequence, which leads to the formation of a loop structure. The DNA sample is mixed with the primers and a special enzyme called Bst polymerase, which has strand displacement activity. The mixture is then incubated at a constant temperature, which is maintained throughout the reaction. The LAMP reaction starts with the denaturation of the target DNA, followed by the annealing of the primers to the target DNA. The primers bind to the target DNA and initiate the amplification reaction. Bst polymerase extends the primers, displacing the original strands and generating multiple copies of the target DNA sequence. The amplification reaction produces a characteristic ladder-like structure (amplicon), which

can be visualized by adding a DNA-specific dye or by monitoring the turbidity of the reaction mixture (Figure 3). The high-fidelity polymerase then extends the primers, leading to the formation of many copies of the target DNA. The amplified DNA strands then serve as templates for the next round of extension and amplification. As the amplification process continues, the amount of target DNA increases, leading to the increase in visible turbidity in the reaction mixture, due to the accumulation of the amplicon. The presence of the amplicon can be visualized by the naked eye, using a lateral flow test strip, or by using a fluorescent probe. In a special strand displacement due to the action of DNA polymerase, it is amplified at a constant temperature of 60 ℃~65 ℃ [20]. It uses one strand in the double-stranded DNA as a template to synthesize a new strand, and replace the other strand, about 15~60min, and the efficiency of the process can reach 10^9~10^{10} orders of magnitude. This method requires simple equipment, high amplification efficiency, short time-consuming, strong specificity, high sensitivity, no special instruments and reagents, and high visualization of results which makes this method ideal for the prompt recognition of pathogenic microorganisms in laboratories with poor experimental conditions [21,22].

Adenovirus samples used for LAMP analysis can be obtained from various clinical specimens, such as nasal, ocular, and respiratory secretions, or tissue samples. The collection and storage of adenovirus samples are crucial steps in preserving the virus and ensuring its integrity for downstream analysis. Samples should be collected in sterile tubes and stored at 4 °C until further processing. The initial processing steps for LAMP analysis of adenovirus samples include viral extraction and purification. The extracted viral DNA is then used as a template for the LAMP reaction. In LAMP, a set of primers is designed to bind specifically to the target DNA, allowing for the amplification of the target sequence. The primers bind to a unique sequence in the target genome and result in the formation of a DNA ladder that is amplified in a temperature-controlled environment. In the LAMP reaction, the target sequence is amplified in a reaction mixture that contains the LAMP primers, DNA polymerase, dNTPs, and buffer. The reaction is carried out in a single-tube format at a constant temperature, usually 60-65 °C, and the amplified products are monitored in real-time by visual inspection or by using a fluorescent probe. LAMP is a highly specific and sensitive method for the detection of human adenoviruses, with a detection limit of approximately 10 copies/reaction. Its simplicity, speed, and ability to detect low-level virus copies make it a valuable tool for the rapid and accurate diagnosis of adenovirus infections.

Maarseveen et al., 2010, compared the sensitivity and specificity of a LAMP assay targeting the hexon gene of an adenovirus to that of real-time PCR [23]. The LAMP assay was found to be more sensitive, with a detection limit of 10 viral particles per reaction, compared to the detection limit of 10^2 viral particles per reaction for real-time PCR [24]. Additionally, the LAMP has a specificity of 100%, correctly identifying all adenovirus-positive samples and correctly identifying all adenovirus-negative samples. LAMP has the additional benefit of being done at a constant temperature, which eliminates the requirement for thermal cycling equipment and makes it more appropriate for settings with limited resources or in the field. In a research that was published in the BMC Biotechnology in 2020, the LAMP is shown to be a good substitute for conventional PCR-based approaches for adenovirus detection in resource-constrained situations since it was simple to use and needed little equipment and it has the ability to amplify DNA at a constant temperature, and in a short time [25]. However, LAMP technology also needs to be further improved and perfected, such as the primer design being complex and easily contaminated, as well as the storage and transportation of reaction reagents.

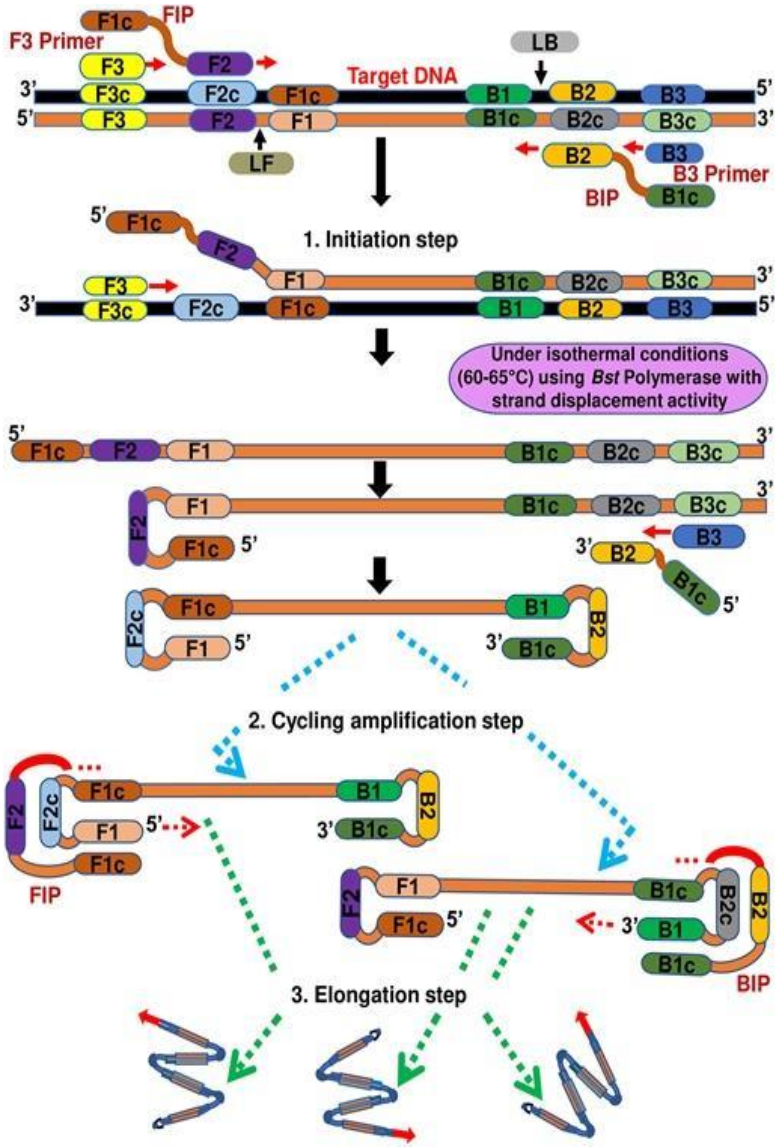

Figure 3. Illustration of mechanism of LAMP. a) The double-stranded target DNA is denatured into single-stranded DNA. b) Primers specific to the target DNA sequence are added and anneal to the single-stranded DNA. c) The Bst polymerase uses the primers to initiate strand displacement synthesis and generate multiple copies of the target DNA. d) The extension phase continues until the reaction reaches a plateau, resulting in an exponential increase in the amount of target DNA.

Source: Nzelu CO, Kato H, Peters NC. (2019). Loop-mediated isothermal amplification (LAMP): An advanced molecular point-of-care technique for the detection of Leishmania infection. *PLoS neglected tropical diseases*, 13(11), e0007698. https://doi.org/10.1371/journal.pntd.0007698

3. PCR derivation techniques

3.1. Multi-Plex Pcr-based reverse line blot hybridization (mPCR-RLB)

Multi-Plex PCR-Based Reverse Line Blot Hybridization (mPCR-RLB) is a powerful tool for the detection and typing of human adenoviruses. This technique combines the sensitivity and specificity of PCR with the high-throughput capabilities of reverse line blot hybridization (RLB) to detect and identify multiple adenovirus types simultaneously. A swift detection method for common respiratory viruses established at the gene level is multi-plex PCR combined with reverse dot blot hybridization. The basic mPCR-RLB process involves the use of multiple pairs of primer sets that specifically bind to conserved regions of the adenovirus genome. These primer sets are used in a multiplex PCR reaction that allows for the simultaneous amplification of multiple target DNA sequences in a single reaction. It is accomplished by using a mixture of different pairs of primers, each specific to a different target sequence. During the PCR reaction, the primers anneal to their corresponding target sequences and are extended by the polymerase enzyme, resulting in the simultaneous amplification of multiple DNA or cDNA sequences in a single reaction. This allows for the simultaneous amplification of multiple adenovirus types. The amplified DNA is then hybridized to a reverse line blot (RLB) membrane that contains probes for different adenovirus types. The probes are specific for each adenovirus type and are designed to bind to the amplified DNA, allowing for the identification of the specific adenovirus type present in the sample (Figure 4). The probes are labeled with different colored tags, and they will only hybridize to the complementary target amplicons generated by the multiplex PCR. The presence or absence of the hybridization signal on the membrane allows the identification of adenovirus present in the sample. Grounded on adenovirus DNA homology, specific primers can be constructed based on its conserved gene sequence (such as the hexon protein gene), and the primer's 5' end is labeled with biotin; the probe's 5' end is labeled with biotin. Streptavidin is a commonly used protein to capture biotinylated probes. It is a naturally occurring protein that binds with high specificity and affinity to biotin, a small molecule that is often covalently attached to probes used in mPCR-RLB. The role of streptavidin in mPCR-RLB is to provide a stable and specific interaction between the biotinylated probes and the target DNA sequences on the nitrocellulose or nylon membrane. This interaction allows for the efficient transfer of fluorescence signals from the probes to the target DNA, providing a clear indication of the presence or absence of specific target sequences. Streptavidin is commonly used in mPCR-RLB because of its high binding specificity and stability, which ensure consistent and reliable results. Furthermore, streptavidin is easily conjugated to other materials and can be easily obtained in high purity, making it a convenient and cost-effective tool for molecular biology applications. As part of the amino-labeled probe procedure, the probe is immobilized on nylon (or nitrocellulose) membranes, a primer bound to the probe contains a biomarker, and the hybridization result is presented by a non-radioactive enzyme color reaction [3]. As a result of reverse dot hybridization, the reaction efficiency and reaction time can be greatly improved, the interference can be reduced, and the specificity and sensitivity can be improved, allowing for high-throughput detection of mixed infection pathogens [26,27].

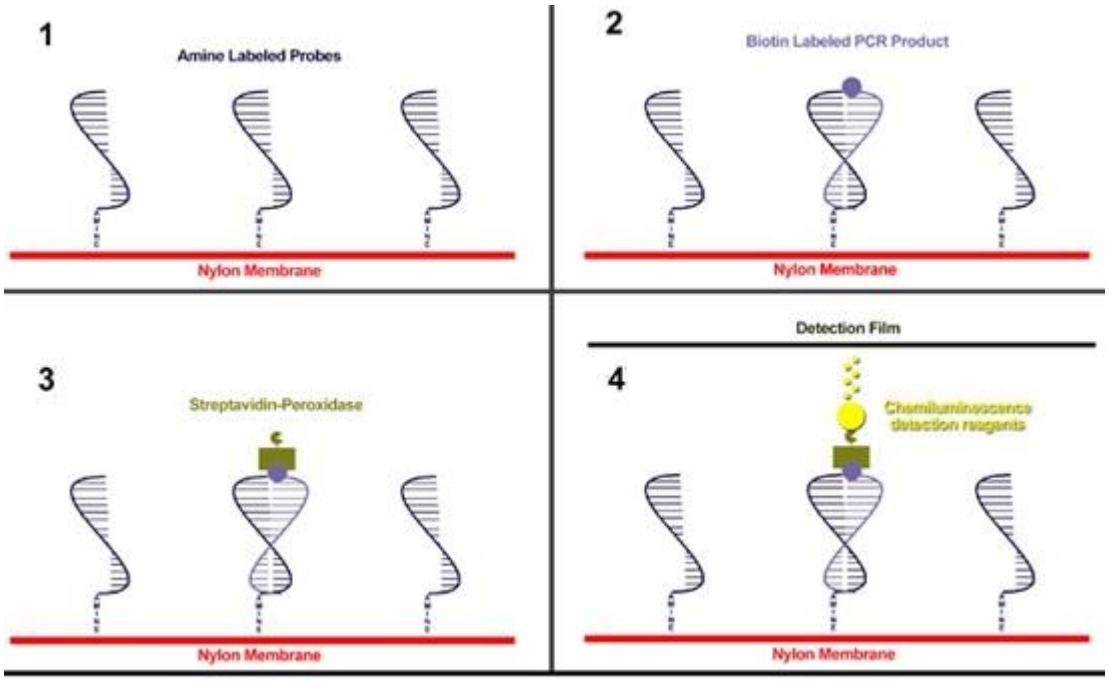

Figure 4. Schematic illustration of multi-Plex Pcr-based reverse line blot Hybridization (mPCR-RLB): The following steps describe the working of mPCR-RLB: a) Adenovirus samples are collected from clinical specimens (e.g. respiratory secretions) and stored at appropriate conditions to preserve the virus. b) The collected samples are processed to extract the adenoviral RNA or DNA. c) The extracted adenoviral RNA or DNA is amplified using multiplex PCR (simultaneously amplifying multiple targets). d) The amplified products are hybridized to a line blot strip that contains multiple probes for different adenovirus serotypes. d) The probes on the line blot strip specifically bind to the amplified products, indicating the presence of specific adenovirus serotypes. The detection can be done using fluorescent methods where each of the specific probes is labeled with a different fluorescence dye. e) The results are interpreted based on the presence or absence of specific adenovirus serotypes on the line blot strip.
Source: O'Sullivan MV, Zhou F, et al., (2011). Multiplex PCR and reverse line blot hybridization assay (mPCR/RLB). *Journal of Visualized Experiments*: JoVE, (54), 2781. https://doi.org/10.3791/2781

Adenovirus samples used for this method can be obtained from various clinical specimens such as blood, urine, nasopharyngeal swabs, and throat swabs, depending on the site of infection. To preserve the virus, the collected sample should be stored at −70 °C or in liquid nitrogen. The initial processing steps for mPCR-RLB include virus lysis and DNA extraction. The collected sample is treated with lysis buffer to release the viral DNA, which is then extracted using a commercial DNA extraction kit. The extracted DNA is then subjected to multiplex PCR, where forward and reverse primers specific to different adenovirus species are used to amplify the viral DNA. The amplified products are then subjected to reverse line blot hybridization, where they are hybridized with specific probes immobilized on a solid support. The hybridization reaction is then visualized by streptavidin-

horseradish peroxidase conjugate, which recognizes the biotinylated probes, resulting in a positive signal. The positive signal indicates the presence of the targeted adenovirus species in the sample. Adenovirus detection and typing in samples from the respiratory tract were tested for sensitivity and specificity in a research that was published in the *Journal of Clinical Microbiology* in 2012. The research discovered that the mPCR-RLB test accurately identified all adenovirus-positive samples and all adenovirus-negative samples with a sensitivity of 98.4% and a specificity of 100% [28]. Another advantage of mPCR-RLB is its ability to detect and identify multiple adenovirus types simultaneously. A study published in Molecular Microbiology: Diagnostic Principles and Practice in 2011, used an mPCR-RLB assay to detect and type adenoviruses in stool samples from patients with diarrhea. The study found that the mPCR-RLB assay was able to detect and identify multiple adenovirus types simultaneously, allowing for the rapid and accurate identification of the specific adenovirus responsible for diarrhea [29]. mPCR-RLB has also been used in combination with other techniques such as fluorescent antibody-based assays to improve the sensitivity and specificity of adenovirus detection. Kuypers et al., 2006 used an mPCR-RLB assay in combination with fluorescent antibody-based assays to detect and type adenoviruses in respiratory tract samples. The study found that the combination of mPCR-RLB and fluorescent antibody-based assays was more sensitive and specific than either technique alone, with a sensitivity of 100% and a specificity of 99.4% [30]. Due to the binding of non-amplification primers and primer-dimer products to the probe, false positive signals result, and mutations of the probe's binding point may result in weak or non-existent signals, so special attention should be paid during the experiment [3].

3.2. PCR-enzyme linked immunosorbent assay (PCR-ELISA)

PCR-Enzyme Linked Immunosorbent Assay (PCR-ELISA) technology is a detection technology fashioned by combining immunological reaction and PCR technology. It is a highly sensitive and specific method for the detection of human adenoviruses. This technique combines the specificity of PCR with the sensitivity of an enzyme-linked immunosorbent assay (ELISA) to detect and identify adenovirus in a variety of clinical samples. The basic PCR-ELISA process involves the use of specific primer sets that bind to conserved regions of the adenovirus genome. These primer sets are used in a PCR reaction to amplify the adenovirus DNA. In PCR-ELISA, the target DNA is first amplified using PCR in the presence of digoxigenin-11-dUTP (DIG-dUTP), and the amplified products are then captured and immobilized on a solid surface, such as a well in a microplate. The captured DNA is then probed with a specific antibody that binds to the target sequence, creating a sandwich-like structure between the DNA and the antibody. The amplified DNA is then captured by specific antibodies bound to a solid microplate. The next step involves the addition of a secondary antibody that is conjugated with an enzyme, such as horseradish peroxidase. The secondary antibody binds to the primary antibody, creating a complex that is bound to the target DNA. After incubation, the unbound detection antibodies are washed away, and a chromogenic or fluorogenic substrate is added to the wells. The presence of the complex is then detected by adding a substrate that is specifically cleaved by the enzyme, resulting in a color change or fluorescence signal that is proportional to the amount of target DNA present in the sample (Figure 5). The captured DNA is then detected by the enzyme-conjugated antibody, which binds to the captured DNA and generates a visible signal. It assimilates the high specificity of the antigen-antibody reaction with the exponential amplification ability of the polymerase chain reaction.

Adenovirus samples are typically collected from clinical specimens such as respiratory secretions, stool, or blood. The collected samples are stored at appropriate temperatures (usually −80 °C) to preserve the viral particles. The initial processing steps to expose the adenoviral DNA or RNA involve viral lysis, which breaks open the virus particles, and purification of the viral DNA or RNA. This can be done through techniques such as centrifugation, column-based purification, or bead-based purification. In the first step of the PCR-ELISA assay, the purified adenoviral DNA or RNA is subjected to PCR amplification using primers specific to the adenovirus genome. The amplified product is then used as the target in the ELISA step. In the ELISA step, the amplified product is immobilized onto a solid surface and incubated with an adenovirus-specific antibody conjugated to an enzyme, such as horseradish peroxidase (HRP). If the amplified product is specific to adenovirus, the antibody will bind to the target and can be detected through the addition of a substrate that is specific to the conjugated enzyme. The amount of product that is bound to the antibody can be quantified by measuring the amount of substrate that is converted by the enzyme.

PCR-ELISA is a prompt, sensitive, and consistent method for detecting enteric adenoviruses [3]. This method entails less equipment and can be performed as long as an amplification instrument and the microplate is present, involves a small sample, saving time, and is easy to automate. Comparing these methods with other conventional methods, the sensitivity, specificity and accuracy are greatly improved, making them a viable platform for early virus detection [3]. A 2004 research that appeared in the Journal of Clinical Virology assessed the sensitivity of a PCR-ELISA technique for adenovirus identification in samples from the respiratory tract [31]. According to the study, the PCR-ELISA test exhibited a sensitivity of 96.2% and successfully identified all samples that were adenovirus positive [31]. The simultaneous detection and identification of numerous viral types is another benefit of PCR-ELISA. Adenoviruses were identified and categorized in respiratory tract samples using the PCR-ELISA test in a 2013 research that was published in the Infection. The study discovered that the PCR-ELISA assay was able to simultaneously detect and identify various viral types, enabling the quick and precise identification of the particular adenovirus inflicting the illness [32]. It has the disadvantage of causing environmental and cross-contamination issues due to the use of reagents and equipment that may have an impact on the environment, and it has an inclination of producing false positives due to various factors, including contamination of reagents or samples, cross-reactivity of antibodies with non-specific targets, and incorrect interpretation of results. For example, the use of low-quality or contaminated reagents can result in the presence of non-specific signals in the assay, leading to false positive results. Similarly, the cross-reactivity of antibodies with non-specific targets can result in the detection of false positive signals. Each link disturbs the sensitivity and specificity of the test [3].

Poor PCR amplification, weak antibody binding, low sensitivity of the detection system, and cross-reactivity of antibodies with non-specific targets can all lead to reduced sensitivity. Contamination of reagents or samples and incorrect interpretation of results can also lead to false positive results, reducing specificity. To maintain sensitivity and specificity, it is important to optimize each step in the protocol and to follow strict quality control measures. In order to make it easy to use, optimal reaction conditions are explored, steps are simplified and standardized, and marketable production is implemented, making it easy for routine laboratories to use. PCR-ELISA has also been used in combination with other techniques such as fluorescent antibody-based assays to improve the sensitivity and specificity of adenovirus detection. Smit et al., 2014 used a PCR-ELISA assay in combination with fluorescent antibody-based assays to detect and type adenoviruses in respiratory tract samples. The study found that the combination of PCR-ELISA and fluorescent antibody-based assays

was more sensitive and specific than either technique alone, with a sensitivity of 100% and a specificity of 99.4% [33].

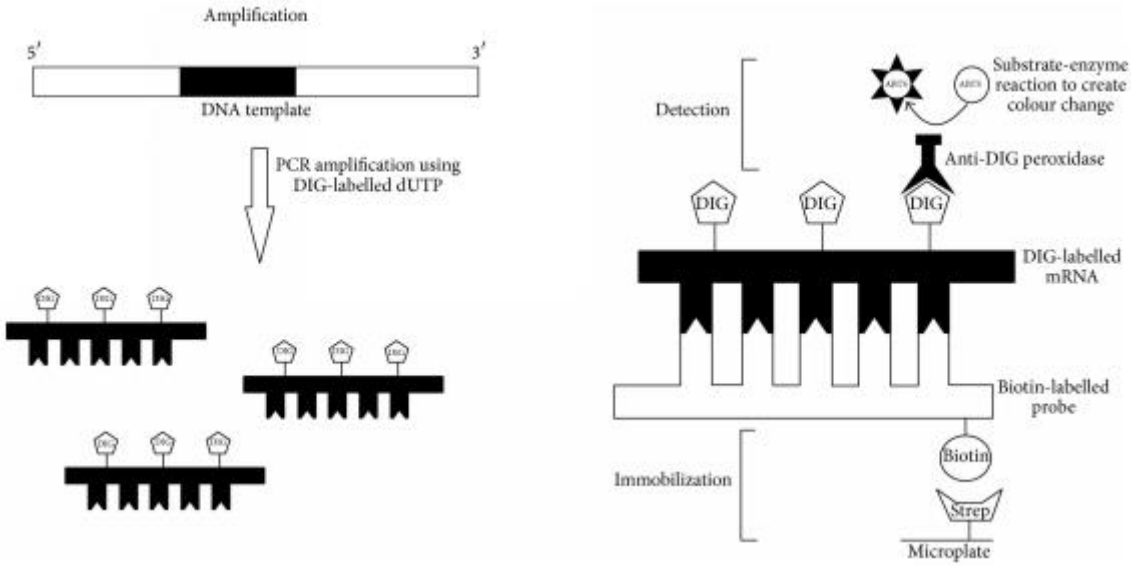

Figure 5. Schematic Representation of PCR-ELISA. a) The adenovirus sample is collected and the DNA is extracted from the sample. b) The extracted DNA is amplified using PCR, with primers designed to specifically target the adenovirus sequence and initiate the replication of the DNA by the polymerase enzyme in the presence of DIG-dUTP. c) The amplified product is then immobilized by the complementary probe on a solid support, typically a microplate, and specific antibodies are used to detect the presence of the amplified target DNA. d) Antibodies that recognize the amplified target DNA are linked to an enzyme. This enzyme-linked antibody-antigen complex is incubated with a substrate that is converted into a colorimetric signal by the enzyme, indicating the presence of the target DNA. e) The intensity of the colorimetric signal is proportional to the amount of amplified target DNA and can be quantified using a spectrophotometer or a chemiluminescent reader.
Source: Sue MJ, Yeap S K, Omar A R, and Tan SW, (2014). Application of PCR-ELISA in molecular diagnosis. *BioMed research international*, 2014, 653014. https://doi.org/10.1155/2014/653014

3.3. Target enriched-multiplex PCR (Tem-PCR)

Target Enriched-Multiplex PCR (Tem-PCR) is a highly sensitive and specific method for the detection of human adenoviruses. This technique combines the specificity of PCR with the sensitivity of target enrichment to detect and identify adenovirus in a variety of clinical samples. As a result of tem-PCR technology, multiple target genes can be amplified concurrently in one reaction system with high sensitivity and specificity. First, the template is enriched, and target sequences are augmented by using a very small amount of specific primers to reduce primer dimers and nonspecific background to escalate the specificity of the assay [3]. The target enrichment step involves the selective amplification of specific regions of interest from the sample DNA. This is achieved by hybridizing the sample DNA

to a set of specific probes, which serve as bait for the target DNA sequences. These probes can be designed to hybridize to specific regions of interest, such as exons or disease-associated variants, and are immobilized on beads or a solid support. The hybridized probes are then captured and selectively amplified using PCR. Once the target DNA has been enriched, the multiplex PCR reaction is performed. In this step, multiple sets of primers (forward and reverse primers) are used to amplify different targets in the same reaction, resulting in the simultaneous detection of multiple targets. The forward primers are designed to bind to the 5' end of the target DNA sequence, and the reverse primers are designed to bind to the 3' end of the target DNA sequence. When the PCR reaction is carried out, the Taq polymerase enzyme synthesizes a new strand of DNA complementary to the target DNA. The forward and reverse primers serve as the starting points for this synthesis process. The multiplex PCR reaction is performed at a constant temperature, which is known as the isothermal amplification step (Figure 6). This allows the simultaneous amplification of multiple targets without the need for temperature cycling. The amplified targets can then be analyzed using various downstream applications, such as gel electrophoresis, sequencing, or hybridization or microarray analysis, to identify the specific regions of interest in the sample.

In order to improve sensitivity, perform high-efficiency exponential amplification, and carry biomarkers on the products, super primers with high affinity and high concentration are used after template enrichment [3]. Super primers contain RNA-dependent DNA polymerase and reverse transcriptase activities, allowing them to produce cDNA from RNA templates, which are then amplified using PCR. The use of super primers allows Tem-PCR to be performed without the need for reverse transcription, simplifying the assay and increasing its sensitivity and specificity. Super primers can be designed to specifically target a certain RNA species, such as a viral RNA, to increase the specificity of Tem-PCR. Finally, liquid chip technology is used to perform high-throughput multiplex detection. In a liquid chip, individual reaction chambers are fabricated on a microfluidic chip, allowing for multiplex detection of different target RNAs. The Tem-PCR reaction is performed in these individual reaction chambers, with each chamber containing a specific set of super primers and reaction conditions optimized for the detection of a specific RNA species. Liquid chip technology allows for high-throughput analysis of multiple target RNAs in a single reaction, improving the efficiency and speed of the assay. The microfluidic design of the chip also reduces the volume of reagents and samples required for each reaction, reducing costs and minimizing waste. It is a fast and accurate method for detecting common respiratory viruses, as well as viruses that can't be detected using traditional virus culture methods like tissue culture, embryonated egg culture, PCR and RT-PCR [34].

The specificity of Tem-PCR is maintained by using specific probes and primers that bind only to the target DNA sequences of interest. The sensitivity of the assay is enhanced by the enrichment step, which increases the concentration of the target DNA and reduces the background noise from non-target DNA. Adenovirus samples used in Tem-PCR are typically collected from clinical specimens such as respiratory secretions, urine, or blood. The samples should be collected and stored in a manner that preserves the viability of the virus, such as using virus transport media or RNA stabilizing agents. The initial processing steps for Tem-PCR include the extraction of the adenoviral DNA from the sample. This is usually done by using commercial kits or manual protocols that employ methods such as phenol-chloroform extraction, spin-column purification, or silica-based spin-columns. Once the viral DNA has been extracted, the next step is to enrich the target regions of the genome by hybridization with specific probes. This is done by mixing the extracted DNA with a pool of biotinylated probes that have been designed to target specific regions of the adenoviral genome. The hybridization reaction is

then performed under conditions that allow the probes to specifically bind to the target regions of the genome. After the hybridization step, the enriched targets are amplified by multiplex PCR. This is performed by adding a set of primers specific to the target regions of the genome and a thermocycler to amplify the DNA. The multiplex PCR reaction allows the simultaneous amplification of multiple targets in a single reaction, increasing the efficiency and specificity of the test. Finally, the amplified products are detected by a variety of methods, such as gel electrophoresis, agarose gel electrophoresis, or real-time PCR. The results of the test are interpreted based on the presence or absence of specific amplification products, which can be used to determine the presence of adenovirus in the sample.

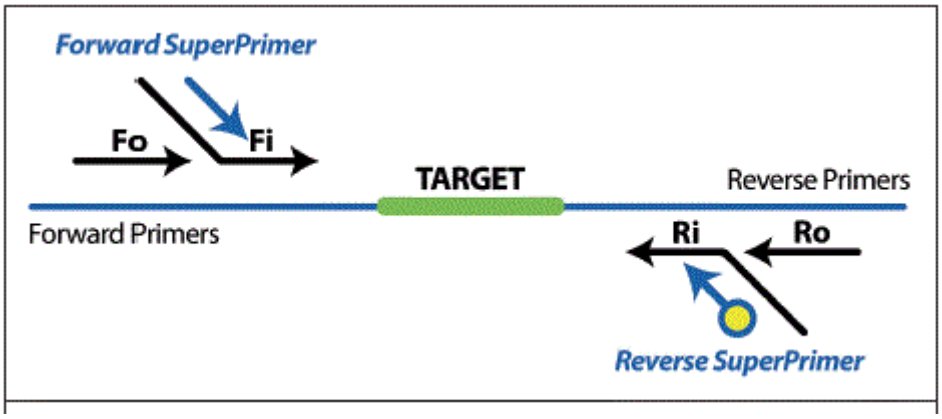

Figure 6. Schematic Representation of Tem-PCR: The following steps describe the working of Tem-PCR: a) Adenovirus samples are typically collected from clinical specimens such as nasal or throat swabs, stool, or other bodily fluids. These samples are then processed to extract the adenoviral RNA or DNA. b) This step involves the amplification of specific regions of the adenoviral genome using biotinylated forward (F#) and reverse (R#) primers. The reverse primers are made to bind to the 3' end of the target DNA sequence, whereas the forward primers are made to connect to the 5' end. The enriched targets are then captured onto streptavidin-coated beads. c) The enriched targets are then subjected to multiplex PCR using fluorescently labeled primers. The amplified products are then analyzed using a real-time PCR instrument to detect the presence of the target sequences. d) The results of the Tem-PCR are analyzed to determine the presence and quantity of the targeted adenovirus sequences in the sample.
Source: Lazas D, Ber LC, Grigorenko E et al., (2015). Multiplex PCR test for detection of enteropathogens in an infant. Cap Today. https://www.captodayonline.com/multiplex-pcr-test/

With Tem-PCR, respiratory diseases like adenovirus are detected without the restrictions of traditional multiplex PCR like general PCR, such as target incompatibility, difficulties in designing and optimizing multiple primer sets, potential competition between different amplicons, limitations in the number of targets that can be amplified in a single reaction, and uneven amplification. These limitations can affect the sensitivity and specificity of the test, and may also make it challenging to detect low-abundance or divergent strains of a virus. An advantage of Tem-PCR is its ability to detect and identify multiple adenovirus types simultaneously. A study published in the *Journal of Clinical*

Microbiology in 2005, used a Tem-PCR assay to detect and type adenoviruses in respiratory tract samples. The study found that the Tem-PCR assay was able to detect and identify multiple adenovirus types simultaneously, allowing for the rapid and accurate identification of the specific adenovirus responsible for the infection [35]. Tem-PCR has also been used in combination with other techniques such as next-generation sequencing (NGS) to improve the sensitivity and specificity of adenovirus detection. Researchers found that the combination of Tem-PCR and NGS was more sensitive and specific than Tem-PCR alone, with a sensitivity of 100% and a specificity of 99.4% [36]. It is important to note that there are still a number of shortcomings. For example, opening the PCR reaction tube during detection may cause laboratory contamination and false positives, and the nucleotide sequences of the conserved regions of the virus vary to varying degrees. Therefore, further optimization is obligatory [37].

4. Other techniques

4.1. Gene chip

Gene chip technology, also known as DNA microarray, is a powerful tool that can be used for the detection and characterization of human adenoviruses. This technology allows for the simultaneous detection and analysis of multiple adenovirus genes, providing a comprehensive view of the viral load and genetic diversity of adenoviruses in a given sample. Gene chip is a novel technology industrialized in the field of biological high-tech in contemporary years. In this method, a sample of genomic DNA or cDNA is labeled and hybridized to the chip surface. If the target sequence is present in the sample, it will bind to the complementary probe on the chip (microarray), resulting in the formation of fluorescent complexes. The microarray is a glass slide or silicon wafer that is coated with thousands of DNA probes that are complementary to specific regions of the adenovirus genome. This binding can be detected by a variety of methods, such as fluorescent labeling, colorimetric detection, or mass spectrometry (Figure 7). The signal generated is proportional to the amount of target sequence in the sample, providing information on the relative expression levels of the genes in the sample [38]. After hybridization, the microarray is scanned to detect the fluorescence signals. The resulting image is then analyzed using specialized software that quantifies the fluorescence signals and normalizes the data. The normalized data can then be used to identify differentially expressed genes between samples or to group samples based on their gene expression profiles. As a result of collecting the hybridization signal intensity from each collection point, and using computer software to perform image analysis and processing, Shaffer et al., 2015. established a gene chip to perceive adenovirus, rhinovirus, and respiratory syncytial virus low titers in samples instantaneously and specifically, providing a clinical basis for diagnosis. Due to its high quantification, micro quantification, and rapidity, gene chip technology is extensively used to screen and diagnose viral diseases.

Adenovirus samples used for gene chip analysis are typically collected from human or animal tissues and are stored in a suitable preservation medium, such as RNA stabilizing solution or a freezing solution at −80 °C, to prevent degradation of the viral DNA. The initial processing steps to isolate the adenoviral DNA involve homogenization of the tissue samples followed by purification of the DNA. This can be done using commercial kits or manual methods, such as phenol-chloroform extraction or column purification. Once the DNA is purified, it is used as a template for cDNA synthesis using reverse transcriptase. The cDNA is then used as a target for the hybridization on the gene chip. The

gene chip contains thousands of probes, each specific for a single target gene, including the genes of interest (e.g. genes specific for adenovirus detection). The cDNA from the sample is hybridized into the gene chip, and fluorescence-based detection is used to determine which probes bind to the cDNA. This information is then analyzed using specialized software to identify the presence and abundance of the adenovirus in the sample. The gene chip was designed to detect all known human adenovirus serotypes and the results showed that adenovirus was present in all the samples analyzed, demonstrating the utility of gene chip technology in adenovirus detection. The great sensitivity and specificity of Gene Chip technology are some of its benefits. An analysis of the sensitivity and specificity of a Gene Chip test for the detection of human adenoviruses in samples from the respiratory tract was reported by Bogaerts et al. 2016 [40]. The research discovered that the Gene Chip test has 100% sensitivity and 99.5% specificity, properly identifying all adenovirus-positive samples as such and correctly identifying all negative samples as such [40]. It has incomparable advantages over conventional virus detection technology in terms of accuracy and speed. It has several advantages over traditional molecular biology techniques, such as PCR, because it allows parallel analysis of thousands of genes in a single experiment, and has high sensitivity and specificity. This technology has been widely used in many areas of biology, including human disease research, drug discovery, and biotechnology. In addition to its high cost and complex chip manufacturing technology, it is not appropriate for grassroots promotion and use by small businesses, especially in small communities.

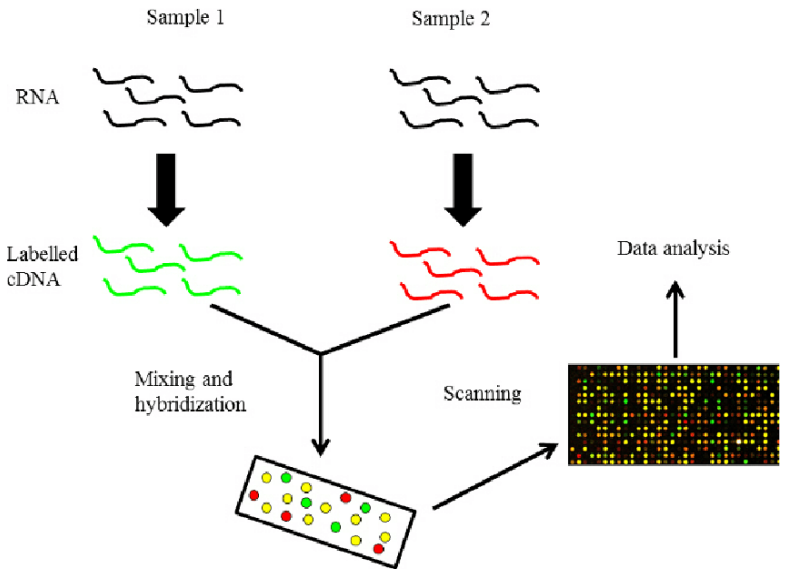

Figure 7. Schematic Representation of Gene Chip Technology using DNA Microarrays. a) The first step is the hybridization of fluorescently labeled nucleic acid probes to specific target sequences of both samples on a DNA microarray. b) The fluorescently labeled nucleic acid probes are derived from the sample being analyzed and are hybridized to the DNA probes on the microarray. c) The resulting fluorescence signal is used to detect the presence and quantity of the target sequences in the sample.
Source: Afzal M, Manzoor I, Kuipers OP. (2015). A Fast and Reliable Pipeline for Bacterial Transcriptome Analysis Case study: Serine-dependent Gene Regulation in Streptococcus pneumoniae. *Journal of Visualized Experiments*: JoVE. (98), 52649. https://doi.org/10.3791/52649

4.2. Next generation sequencing technology

Next generation sequencing (NGS) technology has revolutionized the field of molecular biology and has become an important tool for the detection and characterization of human adenoviruses. NGS technology allows for the high-throughput sequencing of entire viral genomes, providing a comprehensive view of the viral diversity and evolution of adenoviruses in a given sample. NGS technology is based on the parallel sequencing of millions of short DNA fragments, known as reads, which are then assembled into contiguous sequences, known as contigs. The basic principle of NGS is to parallelize the sequencing process by simultaneously sequencing millions of DNA fragments in a single reaction. The DNA sample is fragmented into smaller pieces, and the resulting fragments are attached to a solid surface and amplified using polymerase chain reaction (PCR) or by bridge amplification, resulting in a cluster of identical DNA fragments. Each cluster is then sequenced in parallel using fluorescently labeled nucleotides that are incorporated into the growing DNA strand, allowing the sequence of the DNA or RNA fragment to be determined. The DNA fragments are ligated to adapters that allow the fragments to be amplified and sequenced on a sequencer. The adapters contain sequences that are recognized by the sequencer, allowing the fragments to be captured and sequenced in parallel. The amplified DNA is then sequenced using one of several sequencing by synthesis (SBS) techniques. In SBS, the DNA is incorporated with nucleotides (the building blocks of DNA), and the fluorescence of the nucleotides is detected as the DNA is synthesized (Figure 8). This allows for the determination of the DNA sequence in real-time. The resulting sequence data is then aligned to a reference genome, and the presence of mutations or variations in the DNA can be determined.

Library preparation for NGS involves the fragmentation of genomic DNA into smaller pieces and the addition of specific barcodes and adapters to the ends of the fragments. The barcodes and adapters allow for the identification and tracking of individual fragments during the sequencing process. The adapters typically include a specific sequence that can be recognized and amplified by polymerase chain reaction (PCR) in the next step of the library preparation process. The fragments are amplified by clonal amplification by bridge-PCR after they have been tagged with barcodes and adapters. Bridge PCR is a technique used to amplify specific DNA regions to facilitate their sequencing by NGS. Clonal ligation is a process used to amplify DNA fragments and create libraries suitable for NGS. Pyrosequencing is a type of sequencing by synthesis that uses light signals to determine nucleotide incorporation in real-time. Sequencing by ligation and synthesis is a method that uses ligase enzymes to join the DNA fragments and synthesize new DNA strands. Adaptive focused acoustic shearing is a technique used to mechanically break large DNA molecules into smaller fragments suitable for NGS. The PCR reaction results in the creation of many copies of each fragment, which allows for an increased yield of DNA for sequencing. The most common NGS platforms are Illumina, PacBio and Nanopore. The choice of the NGS platform depends on the specific application, with Illumina being the most widely used for viral detection due to its high-throughput and cost-effectiveness. Using next-generation sequencing technology, people can analyze genome, transcriptome, and protein collaboration data more expansively and deeply. Short oligonucleotides are used as primers to synthesize complementary DNA strands to templates in next-generation sequencing technology. As chain termination reagents, dideoxynucleotides (ddNTPs) are used to produce polynucleotide fragments of various lengths by primer extension of polymerase, which are then detached. During the sequencing reaction, ddNTPs are incorporated into the growing DNA strand, and the incorporation of

a ddNTP terminates the extension of the strand. By using different ddNTPs, the different nucleotides can be differentiated, and the sequence of the genomic fragment can be determined. Different distinct fluorescent labels are applied to four different ddNTPs using next-generation sequencing technology [3]. When DNA polymerase makes complementary chains, each added ddNTP will release different fluorescence [3].

Adenovirus samples can be collected from various sources such as blood, stool, urine, respiratory specimens, and tissue biopsies. The samples are usually stored in a temperature range between −70 °C to −80 °C to preserve the virus. The initial processing step involves exposure of the adenoviral DNA or RNA by extraction. RNA extraction is commonly used in NGS-based adenovirus detection as it is a more sensitive method compared to DNA extraction. The extracted RNA is converted into cDNA using reverse transcription. This cDNA is then fragmented and ligated with sequencing adapters to form a library of DNA fragments. The library is then amplified and sequenced using NGS technology. Specific computer software processing is used in order to obtain the sequence information of the DNA to be tested. The detection results can comprehensively reflect the virus contamination status of clinical patients with this high-throughput detection technology with high sensitivity and specificity. The capability of NGS technology to recognize and classify new viral types is one of its benefits. Illumina NGS was utilized in a study that was published to find and classify new viral types in samples from the respiratory tract. The study discovered that a new viral type that was not picked up by traditional PCR-based techniques could be recognized and identified using NGS technology [41]. The capability of NGS technology to identify low-frequency viral variations is another benefit. Illumina NGS was utilized in a study to find low-frequency viral mutations in samples from the respiratory tract. According to the study, NGS technology can identify low-frequency viral variants that are resistant to detection by traditional PCR-based techniques [42].

Additionally, the development and dissemination of adenoviruses in various populations have been studied using NGS technology. Illumina NGS was utilized in a 2017 study to examine the evolution and transmission of adenoviruses in a pediatric population [43]. The study discovered that the use of NGS technology allowed researchers to understand the evolutionary dynamics of adenoviruses in this population and to pinpoint potential transmission pathways [43,44]. NGS can detect a wide range of adenoviruses and can identify multiple adenoviruses present in a single sample. The technology is capable of accurately detecting low levels of adenovirus in a sample, making it a valuable tool for the diagnosis of adenovirus-related infections. NGS technology has been widely used in the characterization of adenovirus outbreaks, the development of new diagnostic methods, and the identification of new adenovirus strains. It has broad presentation visions in the exposure of various pathogens and epidemiological outbreak investigations. Its disadvantages comprise a high detection cost for a single sample, limited accuracy, and challenges in data analysis and interpretation, a cumbersome experimental procedure, and a computer prone to misreading when several identical bases appear consecutively.

The cost of sequencing can be a barrier for some researchers, and the accuracy of the technology is dependent on the quality and quantity of the starting DNA sample. In addition, the vast amount of data generated by NGS requires specialized expertise and computational resources for analysis and interpretation, which can also present challenges. However, due to obvious defects in the conventional virus detection technology, for example, even though virus isolation technology is the highest standard for virus infection diagnosis, it takes days or even weeks to detect a virus infection, and virus isolation success is closely related to the quality of clinical specimens and the source of specimens. In addition,

the results of separation must be observed by experienced laboratory personnel, so using this method as a clinical treatment guide is difficult. In the event of an epidemic of acute infectious diseases, it does not permit timely cure of patients and timely regulation of the scope of infection [3]. The advancement of real-time rapidity, safety, and pollution-free adenovirus detection methods with strong specificity, high sensitivity, easy operation, economics, and practicality is therefore a problem worthy of contemplation and in-depth study.

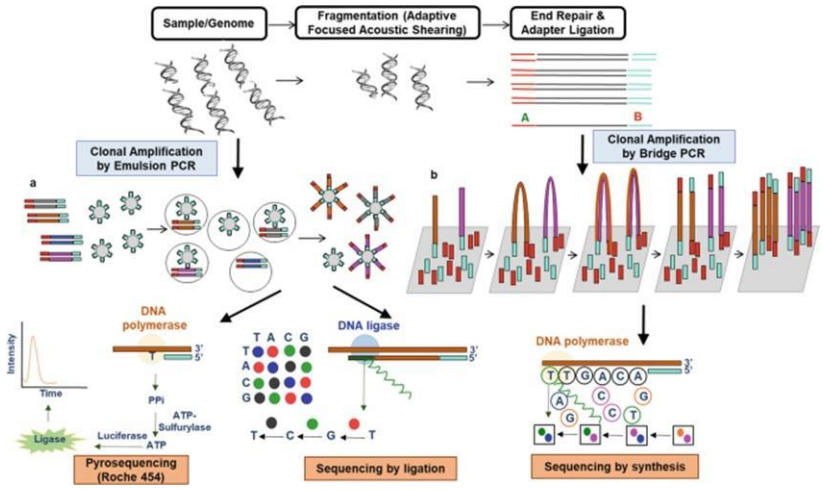

Figure 8. Schematic illustration of next generation sequencing technology. a) The DNA is fragmented into small pieces and then randomly ligated to adapter sequences to prepare for sequencing. Adaptive focused acoustic shearing is a method of fragmenting the DNA in a way that ensures even representation of the genome. b) The fragments are amplified by bridge PCR and attached to a sequencing platform, where they form clusters of identical fragments. c) The amplified DNA is then subjected to sequencing by ligation and synthesis or pyrosequencing. In sequencing by ligation and synthesis, DNA strands are cleaved and ligated with fluorescently labeled probes. d) The probes are then incorporated into a sequencing reaction and imaged to determine the sequence of the DNA. e) The platform reads the DNA sequence in each cluster, producing short reads. f) The short reads are then assembled into longer contiguous sequences, called contigs, which can be aligned to a reference genome to determine the sequence of the original DNA sample.
Source: Gupta N, Verma VK. (2019). Next-Generation Sequencing and Its Application: Empowering in Public Health Beyond Reality. *Microbial Technology for the Welfare of Society,* 17, 313–341.

4.3. Multiplex ligation-dependent probe amplification (MLPA)

Multiplex Ligation-Dependent Probe Amplification (MLPA) is a technique that can be used to detect and quantify specific sequences in a sample. It is a powerful tool that can be used to detect human adenoviruses, a group of viruses that can cause a wide range of illnesses in humans. In gene diagnosis, ligation-dependent multiple probe amplification is used to precisely detect nucleic acid target sequences, and is now extensively used. Its distinctive feature is that the amplification is only achieved for the ligated probe rather than the target sequence of the sample. The principle of MLPA is

based on the ligation of two probes, one specific for the target sequence and the other for a control sequence, followed by PCR amplification. The probes are designed to bind to specific sequences within the target DNA, and the ligation reaction results in the formation of a specific probe-target DNA hybrid. During an MLPA experiment, the DNA sample is first denatured and then hybridized to the MLPA probes, which consist of two oligonucleotide probes that are ligated together if they hybridize to adjacent target sequences. The ligated probes are then amplified by PCR using universal primers, resulting in a pool of amplified fragments that are proportional to the amount of DNA in the sample. The PCR amplification step then amplifies the probe-target DNA hybrids, generating a product that can be quantified by gel electrophoresis or real-time PCR or capillary electrophoresis (Figure 9). The resulting peak heights are analyzed to determine the relative copy numbers of the target sequences in the DNA sample. Changes in copy number can be indicative of genetic disorders or disease-associated variants. As long as the specific probe is complementary to the target sequence, ligase can bind the two probes together to generate a single-stranded amplification, and each pair of amplified products is diverse in length, and capillary electrophoresis can be used to isolate and identify the amplified products, safeguarding high specificity.

Human adenoviruses have been found with MLPA in a range of materials, including feces, blood, and secretions from the respiratory tract. Before initiating the MLPA process, the sample must undergo initial processing steps to isolate and purify the adenoviral DNA. This typically involves homogenization of the sample, followed by lysis of the cells to release the viral DNA. The isolated nucleic acids are then purified using methods such as column-based purification or magnetic bead-based purification. Once the purified adenoviral nucleic acids have been obtained, they can be subjected to MLPA. MLPA works by using a set of specific probes, each of which is designed to bind to a specific target sequence within the adenoviral genome. These probes are labeled with a specific fluorescent dye and are then ligated to the target sequence. After ligation, the probes are amplified using PCR to generate a large amount of the labeled probe. The amplified probes are then separated by electrophoresis and the fluorescence signals are analyzed using a fluorescent reader. The pattern of fluorescence signals produced can be used to identify and quantify the presence of specific adenoviral sequences in the sample. A work by Reta et al. 2020 using MLPA to identify and measure human adenovirus DNA in respiratory tract secretions from individuals with acute respiratory infections serves as an illustration of its application in human adenovirus detection [45]. The study discovered that MLPA could identify human adenovirus DNA in a greater percentage of samples than conventional PCR techniques. This can be useful for identifying the presence of multiple adenovirus infections in a single patient, or for characterizing the genetic diversity of a given adenovirus population. In research by Salez et al. 2015, human adenovirus DNA was found and quantified using MLPA in blood samples from individuals with acute respiratory infections [46]. This is another illustration of the application of MLPA in human adenovirus detection. The study found that MLPA was able to detect human adenovirus DNA in a higher proportion of samples than traditional PCR methods.

In addition to its use in human adenovirus detection, MLPA has also been used to detect other viruses, including human papillomavirus and Epstein-Barr virus. It has also been used to detect chromosomal abnormalities and genetic disorders. The capability of MLPA to identify many targets in a single response is one of its benefits. Given the wide variety of adenoviruses that may infect people, this is helpful in the identification of human adenoviruses. The success of the therapy and the severity of the illness may both be assessed using MLPA to quantify the viral load in the sample. However, one limitation of MLPA is that it requires a high amount of starting material, which can be problematic for

some types of samples. In addition, the probes and primers need to be designed specifically for each target, which can be time-consuming and costly. Respi Finder assay, an MLPA-based recognition method that is presently being used in Europe, is able to detect 15 respiratory viruses concurrently with ideal sensitivity and specificity, including adenovirus, influenza, parainfluenza, respiratory syncytial viruses, etc [36,37]. In assessment to other molecular diagnostic techniques, MLPA is extremely sensitive and precise, easy to use, repeatable and does not necessitate distinctive equipment. In terms of uncovering time, the results are only positive to be available within 24 hours, which means they cannot be used for prompt diagnosis.

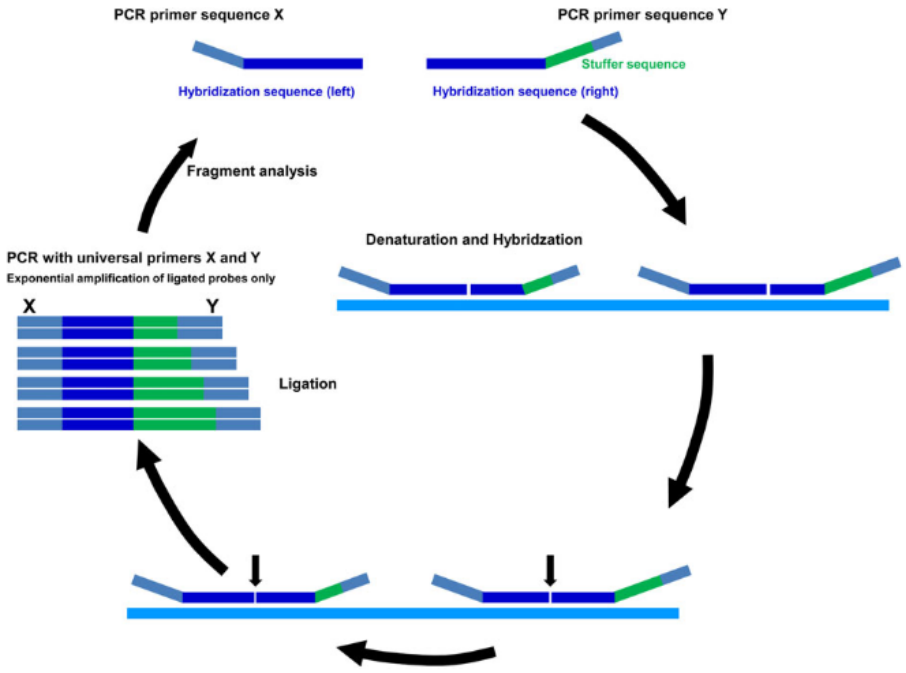

Figure 9. Schematic Representation of MLPA. The following steps describe the working of MLPA: a) The first step is to extract DNA from the sample. The extracted DNA is then fragmented into smaller pieces. b) Specific probes are ligated to the target DNA fragments. The probes have unique sequences and are complementary to the target DNA. c) The ligated probes are then denatured. d) Primers specific to the probes are then added, and they initiate the extension of the probes. e) The extended probes are then amplified using the polymerase chain reaction (PCR) method. During the PCR process, the probes get amplified in a multiplex manner, i.e., multiple probes are amplified simultaneously. d) The amplified products are then separated by electrophoresis, and the presence of specific bands indicates the presence or absence of the target DNA sequences.
Source: Talley PJ, Chantry AD, Buckle CH. (2015). Genetics in myeloma: genetic technologies and their application to screening approaches in myeloma. *British medical bulletin*, 113(1), 15–30. https://doi.org/10.1093/bmb/ldu041

5. Conclusions

Since molecular diagnostic tests are exceedingly delicate and precise, they play an important role

in detecting and controlling human adenovirus infections. The detection of adenovirus is crucial in understanding and controlling its spread. Accurate identification and recognition of the penton base, hexon, and fiber regions of the virus are essential for correct diagnosis. Recombination has been shown to be a significant evolution mechanism for the emergence of novel human adenovirus pathogens. There are several molecular biology-based methods for the detection of human adenovirus, including Next Generation Sequencing Technology, Target Enriched-Multiplex PCR, PCR-ELISA, Multi-Plex PCR-Based Reverse Line Blot Hybridization, Loop-Mediated Iso-Thermal Amplification, Real-Time PCR and general PCR. These methods use various techniques such as amplification and detection of viral DNA or RNA, hybridization, and antibody detection to determine the presence of adenovirus in a sample. By detecting viral RNA or DNA, these molecular diagnostic methods can identify infected individuals before antibodies are raised against the virus are mounted. The adenovirus samples used in these methods are typically collected from a patient's clinical specimens, such as saliva, stool, blood, or throat swabs. To preserve the virus, the samples must be stored at appropriate temperatures and in the proper storage medium, such as a viral transport medium. The initial processing steps to expose the adenoviral DNA or RNA to initiate the detection method vary depending on the specific technique being used. In some cases, such as with Next Generation Sequencing Technology, the samples are sequenced directly to determine the presence of adenovirus. In other cases, such as PCR-ELISA, the samples are first subjected to viral extraction or purification to isolate the adenovirus and then amplified before being subjected to detection. As a result of their high cost, the complexity of instrumentation, and requirement for technical expertise, these techniques are out of reach of resource-limited countries, especially among young, elderly, and immunosuppressed persons. Although these methods are easy to execute and economical, they aren't widely available in low-income countries. Therefore, scientists in low-income nations are developing inexpensive quality tests. Additionally, many countries in the developing world are opening postgraduate departments and institutes to train their citizens. In conclusion, while the initial processing steps to detect adenovirus can vary, the use of sophisticated techniques may be a challenge for many resource-limited countries. However, the development of affordable and accessible detection methods, as well as the provision of appropriate training, can help to overcome these challenges and contribute to the control and prevention of adenoviral infections in these regions. With continued efforts and collaborations among scientists, governments, and other stakeholders, we can work towards a future where accurate and affordable adenoviral detection is accessible to all.

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