Technological Bottlenecks for PCR, LAMP, and Metagenomic Sequencing

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Executive Summary

A robust biosurveillance system would aim to identify and monitor new and known pathogens with the goal of reducing the severity of pandemics. From past pandemics, it's clear that modern biosurveillance systems have the potential to improve tremendously. To do so, a better understanding of the bottlenecks biosurveillance systems face is needed.

The report consists of two standalone parts. This is part II of the report that assumes a broad understanding of the biosurveillance and pathogen detection. We focus primarily on three technologies that are commonly used for biosurveillance: Polymerase Chain Reaction (PCR), Loop-Mediated Isothermal Amplification (LAMP) and Metagenomics Sequencing. If you would like to access an introduction to the biosurveillance landscape and an overview of the different technologies used, please see part I of the report here.

In this report, we explore the importance of technological bottlenecks and investigate a few bottlenecks to gain a deeper understanding of their core issues. We focused on identifying the primary technological bottlenecks in metagenomics sequencing, LAMP and PCR. Towards the end, we also did an exploratory analysis on the non-technological bottlenecks that biosurveillance systems face.

This report will be most useful if you would like to have a deeper understanding of PCR, LAMP, and metagenomics sequencing and how they can be applied in biosurveillance. We also highlighted some potential avenues for technological improvements that could be worthwhile for research and funding agencies to look into.

We used a combination of empirical research and expert interviews to gather information throughout the project. A list of the organizations that we have talked to is included in the Appendix. In total, we gathered the opinions of 25 experts and sourced over fifty peer-reviewed research papers.

Chapter I - Technological Bottlenecks for PCR, LAMP and Metagenomic Sequencing (link)

The key bottlenecks and some potential solutions for PCR, LAMP and metagenomics are listed in the table below.

PCR & LAMP

Technologies	Description	Key bottlenecks	Potential solutions
PCR	Nucleic-acid based diagnostic. Gold standard for pathogen amplification and detection due to its high sensitivity and accuracy	• Long sample-to-answer **time**: RNA extraction takes 50 minutes, amplification takes 70 minutes • **Costs** tens of thousands of USD	• Eliminate RNA purification step by direct-to-test addition
LAMP	A non-PCR, nucleic-acid based alternative that's gaining attention. Performs at a comparable level of sensitivity as PCR, while having inherently lower cost and a quicker time to detection	• **High temperature** needed for optimal performance • High risk of carry-over **contamination**	• Use phosphorothioated primers that allow for optimal performance at lower temperature • Improve CRISPR/Cas9 based methods or other methods to reduce crossover contamination

Metagenomic Sequencing (section)

Metagenomic sequencing enables researchers to sample the genes within a biological sample. It is currently used for oncology and whole genome sequencing but has potential to be used in biosurveillance. The ideal metagenomic sequencer diagnostic would be rapid, inexpensive, fully automated and miniaturized for ubiquitous use. In this report, we break down metagenomic sequencing into sample preparation, library preparation, and sequencing.

Process	Technological Bottlenecks
Sample Preparation	• Samples suffer from **low concentration rates** of the pathogen material and high contamination rates. • Need better sample concentration and filtration methods to remove **contaminants**
Library Preparation	• Current methods require lab personnel to use external equipment to prepare samples. • Requires **automation and integration** of library preparation methods with sequencers • **High in costs** due to aforementioned reasons.
Sequencing	• Need **miniaturization** of sequencers as current sequencers are large benchtop devices used in lab settings. • Depending on the read length, sequencers can take hours to days. A focus on decreasing the read out **time** of sequencers is needed for rapid detection. • The costs of these devices are too high today.

In doing this research, we have also noticed that metagenomic sequencing suffers from misalignment of incentives. Today, funding is driving innovation in metagenomic sequencing for oncology and whole

genome sequencing use. As the technology is developed for these applications, they become less suitable for its use as a diagnostic tool.

Chapter II - Discussion on Non-Technological Bottlenecks (link)

While we initially hypothesized that technological bottlenecks were crucial to biosurveillance systems, we gradually realized throughout research that non-technological bottlenecks are equally important and even more neglected. There are many stages involved in order to implement an effective and robust biosurveillance system - from funding, regulatory approval, R&D, manufacturing, implementation, distribution, adoption to ensuring that data is interoperable between different systems. We ranked the relative importance of each of these bottlenecks for PCR, LAMP, and metagenomic sequencing, but have relatively low confidence on the exact rankings as we explored it in shallow depth.

We suspect that although much work can be done to improve biosurveillance technologies, we also urgently need more people working on addressing the less tangible non-technological bottlenecks. We believe that it would be useful to explore deeper into these non-technological bottlenecks and their potential solutions.

Chapter I - Technological Bottlenecks in Biosurveillance System Technologies

In this section, we explored the technological bottlenecks in PCR, LAMP and metagenomic sequencing. We first provide an overview of the main bottlenecks associated with PoP, clinical and environmental surveillance. We will then discuss each of the prioritized technologies and their challenges.

Prioritized Technologies and Bottlenecks

Both PCR and LAMP are nucleic-acid-based technologies that could be used for sequence amplification and detection. These technologies are useful in a variety of settings. For instance, they can be used in Lab on Chip designs to offer real-time diagnosis. LAMP is especially promising for this purpose due to its simplicity, ease of performance, and low-cost. PCR can also be integrated with sequencing technologies in the sample preparation step in order to amplify genetic sequences.

PCR

Polymerase Chain Reaction is one of the most widely used methods for pathogen amplification and detection, and can detect pathogens in various sample types, including blood, saliva and both treated and untreated wastewater samples. PCR tests rely on heat denaturation or chemical lysis to separate DNA into single stranded RNA, addition of primers and polymerization to amplify the genome. The amplified product can then be detected via fluorescence or dyes.

Many of the disadvantages to traditional PCR-based detection such as low throughput and lack of differentiation between live and dead cells (Shannon *et al*., 2007) can be alleviated by specific forms of PCR. For instance, real-time quantitative PCR (qPCR) allows for accurate quantitative results that are displayed in real-time without the need for post-PCR processing steps such as gel electrophoresis. Real-time quantitative PCR can be combined with reverse-transcriptase PCR (RT-PCR) to allow for selective detection of viable cells only. Multiplex PCR addresses the traditional limitation of only being able to detect a single type of pathogen at a time. By introducing different primers, multiplex PCR can do targeted detection of hundreds and even thousands of microorganisms. The simultaneous detection can be done for different pathogen families, including virus, bacteria and protists (Aridgides *et al*., 2004).

However, several challenges remain that prevent PCR from being more widely used, especially in lower-resource settings. PCR is time-consuming, labor-intensive, expensive, and relatively slow to deliver results (Sheridan, 2020). Current state-of-the-art RT-PCR requires 70 minutes for RNA extraction and 90 minutes for amplification. The total cost of trained staff, equipment, and laboratories could cost tens of thousands of USD (Keong Wee *et al*., 2020). One potential solution is to improve or even eliminate the step of RNA extraction. Preliminary studies have shown that such a direct-to-test

addition is possible, although the main challenge lies in doing so without compromising on the specificity and sensitivity of the subsequent PCR tests. Morindol *et al* (2020) showed that some swab mediums are more suitable than others. They showed that direct addition of viral RNA from swabs stored in water or UTM at 4°C had equivalent RT-PCR amplification compared to RNA purified from the same swabs, but this doesn't apply if swabs were stored in saline solution or Hanks medium. Keong Wee *et al* (2020) developed DIRECT-PCR that conducted a single-tube homogeneous reaction within 36 minutes which can be carried out in commercial portable PCR thermocyclers. The DIRECT-PCR had a sensitivity of six RNA copies per reaction which is comparable to RT-PCR. While it is unclear which of these methods are the most optimal and how they could be extended to other pathogens, direct-to-test addition is an avenue worth looking into.

I personally think that it is unlikely that improving direct-to-test addition will significantly improve the usefulness of PCR. Performing such tests would still require quite advanced laboratory equipment. I believe that non-PCR methods could be more promising as they can achieve comparable sensitivity and accuracy as PCR while sidestepping many of the inherent limitations that PCR has. LAMP is one such technology, and is described below.

LAMP

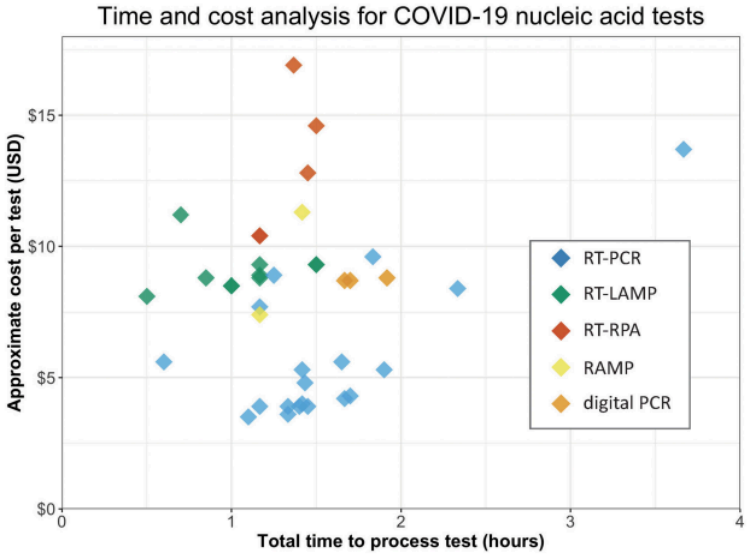

Diagram 4 - Comparing various nucleic acid tests on cost per test and time to process the test. RT-PCR and RT-LAMP seem the most promising. Graph taken from Esbin *et al* 2020.

Developed as an alternative to PCR, LAMP (Loop-Mediated Isothermal Amplification) is a sensitive and specific method to amplify DNAs and RNAs. The high selectivity is achieved by using primers that anneal to four to six unique target regions. Like PCR, there are different forms of LAMP that include conventional LAMP, reverse-transcription LAMP and multiplex LAMP. Amplification can be detected in

real-time via precipitation of magnesium pyrophosphate or fluorescent dye. LAMP does not require complex equipment and can be done in non-laboratory conditions.

LAMP addresses some of the bottlenecks seen with PCR-based methods so that it can operate at a stable temperature without the need for multiple temperature cycling, is much cheaper to operate and greatly reduces analysis time. Billions of copies of amplified DNA can be produced with this method in less than an hour, compared to only millions with PCR (Soroka *et al*., 2021). Yu *et al* (2020) developed the iLACO assay for detecting the SARS-CoV-2 virus that takes 15-40 minutes to complete. Furthermore, LAMP does not require the step of genetic material extraction.

LAMP can be combined with other isothermal methods like RPA (recombinase polymerase amplification) to further improve test accuracy. El-Tholoth *et al* (2020) was able to improve the detection sensitivity 100-fold compared to RT-PCR. This technique has not been applied to real-life patient samples yet, but if such an enhancement in sensitivity can be achieved, this technique could have great potential in analyzing diluted samples particularly in the area of targeted environmental surveillance.

However, LAMP also has various limitations. First, LAMP shows optimal performance at around 65 °C which could limit its applications in lower resource settings. Cai *et al* (2018) used phosphorothioated (PS-) primers in LAMP tests and brought the optimal temperature down to 40°C (Cai *et al*., 2018). The sensitivity of PS-LAMP is similar to regular LAMP. We suspect that not many groups are working on lowering the temperature as I only identified this one example. If we can bring the temperature down to ambient temperature, this could make LAMP-based detection a viable option for cheap and highly accurate point-of-person tests.

Another big challenge with LAMP is the high risk of carryover contamination (such as from materials present in the aerosol) that can lead to false positive results. In molecular terms, carryover contamination refers to the phenomenon where amplified DNA products from previous reactions become templates for re-amplification. Strict operational protocols such as analyzing different samples separately and clean working conditions such as ventilated rooms are recommended, but may not always be possible. Several methods have been developed to control such carryover contamination in LAMP assays. Currently the most recognized method is using uracil-DNA-glycosylase (UDG) to destroy the amplified carryover products (Tang *et al*., 2016). This can be accomplished by introducing two additional components (dUTP and UDG enzyme) into the LAMP tests. However, the limit of detection is somewhat lower than classic LAMP reactions, suggesting some degree of LAMP inhibition by the UDG enzyme (Hsieh et al., 2014). Ma *et al* (2017) introduced another method by using restriction endonucleases to eliminate cross-contamination so that the contaminants are removed prior to LAMP amplification. The main limitation with this method is the high temperatures (~70°C) required for such reactions to occur. More recently, Bao *et al* (2020) used CRISPR/Cas9 cleavage to remove contamination which can be done at room temperature. So far, this has lower efficiency of eliminating

carryover contamination compared to UDG, but more research would be very beneficial for improving the accuracy of LAMP tests and extending its utility.

I believe that carryover contamination is one of the major technological bottlenecks that currently limit the usefulness of LAMP, and if we can address this then LAMP could be much more widespread. However, technological bottlenecks are only one piece of the puzzle. There are many other bottlenecks such as regulatory bottlenecks especially if LAMP were to be used for PoP tests.

Metagenomic Sequencing

Metagenomic sequencing aims to sequence the genomes of known and novel pathogens. The development of this technology is primarily driven by oncology research, whole genome sequencing and environmental surveillance efforts but it also has immense potential to be a clinical diagnostic tool.

If metagenomic sequencing could be on-site, rapid and miniaturized, it could significantly improve the current biosurveillance system. Instead of diagnostics taking several days to weeks, patients could get their results on the same day, significantly limiting the spread of infectious disease if the detection of this pathogen is closely followed by appropriate outbreak response activities. Furthermore, having a diagnostic test that can detect novel pathogens will allow us to recognize novel diseases, further preventing outbreaks (Chiu, 2019).

Metagenomics Sequencing Pipeline

Step in Pipeline	**Description**
Sample Preparation	Acquire sample, filter out contaminants, and ensure there is a sufficiently high concentration of material of interest. This varies by sample type e.g. soil vs saliva sample.
Library Preparation	DNA is fragmented to the length required by the sequencing technology, adapters are ligated to the 3’ and 5’ ends.
Sequencing	DNA fragments are sequenced, this can be done using various methods both short read or long read. For example, lasers that activate fluorescence markers on nucleotide bases (Illumina), measuring the changes in the ion current using nanopores (Oxford Nanopore) among other methods.
Assembly	Varies by type of metagenomic sequencing, but reads containing genomic data are reconstructed to form a sequence.
Data Analysis	Computational methods for understanding the genomic data; binning algorithms are used to overlap segments of DNA that represent the genome to the point that it can be reconstructed.

Metagenomic sequencing techniques differ by numerous aspects including the length of read, the method of sequencing, accuracy, costs and more.

Metagenomic Sequencing Bottlenecks

It's important to note that the bottlenecks for metagenomic sequencing vary by its application. The metagenomic sequencing bottlenecks when applied to whole genome sequencing are very different. In this report, we are focusing on metagenomic sequencing bottlenecks for its use as an infectious disease diagnostic tool in clinics and as an environmental surveillance tool.

Below, we have included a list of bottlenecks in metagenomic sequencing in each step in the metagenomic sequencing pipeline. These bottlenecks were researched by synthesizing insights we gathered from reading literature reviews and papers on metagenomic sequencing, talking to experts who are working with metagenomic sequencing or build metagenomic sequencers and reading white papers from the primary metagenomic sequencing companies such as Oxford Nanopore, Illumina and Pacific Biosciences.

Sample Preparation

- There is often an insufficient concentration of viral material in samples. The concentration varies by the type of sample (blood, saliva, nasal etc.) and where the patient is in their infection cycle.
- It is also important to note that there is a high degree of contaminants in samples. This presents a secondary challenge which is filtering out contaminants and irrelevant materials; it's important to strike a balance where enough viral material is conserved without having contaminants alter the read out.

While this report does not dig further into the particular concentration and filtration methods and their associated challenges, we see this as a useful direction of research for the future.

Library Preparation

- There is a lack of automation of library preparation methods. Most companies such as Illumina, Pacific Biosciences among others use library preparation kits that require skilled professionals to work with samples for several hours. Hiring researchers to manually do library prep drives up the cost of using metagenomic sequencing. Therefore, automated solutions that require no further interaction once the sample and cartridge is loaded are more ideal.
    - Creating automated tools for library preparation will involve making library preparation workflows generalizable and pre-programmable to work for various sample types.
    - Oxford Nanopore has an automated library prep device VOLTRAX but currently, these devices are incredibly expensive with VOLTRAX costing £12,000. Once more automated library prep devices are created, driving down the costs of these devices is imperative.
- Library preparation methods require external equipment and/or are not integrated with sequencers. For metagenomic sequencers to be used on-site, the ideal solution would involve a device that can do all sample prep, library prep and sequencing in one step where genomic

data is then streamed to the database. In clinical surveillance in particular, this would allow pathogen agnostic diagnostics to take a lead in becoming much cheaper.
- Library preparation as a step in the metagenomic sequencing pipeline is very expensive relative to other steps in the pipeline. Further research needs to be done to better investigate the root causes but we believe one factor may be the high cost of reagent kits and cartridges

Sequencing

- Sequencers need to be miniaturized. Most sequencers on the market are large benchtop machines used in lab settings. For use in an environmental and point of person settings, they need to be miniaturized for easy transport. Likewise in clinical settings, further miniaturization would make them easier to integrate in clinics.
- Costs are too high. Modern-day sequencers are incredibly costly making them only suitable for medium-large lab use. For example, Oxford Nanopore's cheapest miniaturized sequencer MinION costs £800 with Illumina's cheapest sequencer MiniSeq costing £50,000. For sequencing to be viable in a point of person or point of care setting, the costs will need to be comparable to tests such as qPCR and LAMP.
- The time from sample input to read out is too long. Ideally, point of person and environmental sequencing tests are rapid and on-site. For many metagenomic sequencers, read-out times take several hours to days depending on the read-length

In doing this research, it has become clear that technological bottlenecks are very important in the development of a robust biosurveillance system. Without technological developments in the areas we mentioned above, it will be difficult to ensure humanity's readiness to future, potentially more deadly pandemics. While we believe it is true, we have come to learn that the task of creating a biosurveillance system is a multifaceted problem. Technological bottlenecks play a single role in this task, and while we have high confidence that they are very important, we don't believe they should necessarily be prioritized above all angles of working on biosurveillance systems. In the last section of our report, we briefly explore non-technological bottlenecks and our reasoning for believing that they are important to further investigate and prioritize.

Chapter II: Non-Technological Bottlenecks

Our initial hypothesis before starting the project was that technological bottlenecks were one of the main factors limiting our biosurveillance systems. However, as we did more research and talked to various experts (~25 experts) in the field, we have the impression that non-technological bottlenecks are equally, if not more neglected, than technological ones. Much more resources should be put into addressing these bottlenecks, and we have listed some below. This is a non-exhaustive list with the main purpose of demonstrating the complexity of the landscape and the need for non-technological solutions. We ranked the relative importance of the non-technological bottlenecks for each of the technologies, but have relatively low confidence on this as we explored it in shallow depth.

LOW PRIORITY
MEDIUM PRIORITY
HIGH PRIORITY

	PCR	**LAMP**	**Metagenomics***
Funding	PCR is widely recognized as the gold standard for amplification, so its importance is well recognized.	The usefulness of LAMP has been demonstrated in several cases and there is increasing attention on isothermal methods such as LAMP.	Metagenomic sequencing for use in PoP, PoC and PoP is underfunded and most funding goes for metagenomic sequencing use in oncology or WGS.
Regulatory Approval	PCR is already very widely used.	As it is a slightly newer technology, LAMP may be subjected to more regulatory approval than PCR. There would also be more regulations for using LAMP in clinical or Lab on Chip devices.	Regulatory approval will be a hurdle as using metagenomic sequencing in PoP or PoC for diagnostics is very novel; this will be a new device on the market which presents unique regulatory challengers
R&D	Research should go into reducing the cost and time for performing PCR.	One of the main problems that prevents LAMP from being more widely used is the high risk of cross-contamination. Much more research is needed to find the optimal solution to address this key bottleneck.	R&D for automated library prep, on-site more rapid sequencers among other areas need to be further developed.
Manufacturing	Multiple enzymes and reagents are needed, some of which need to be stored at low temperatures.	As multiple primers are needed, engineering and mass-producing these could be a challenge.	Manufacturing of reagent kits, cartridges and sequencers can be potentially further optimize to drive down costs
Implementation	Could be a problem in low resource settings due to	Doesn't need specific laboratories to perform	Getting metagenomic sequences in clinics once

	lack of laboratory and trained staff.	LAMP tests, but does need precautions to prevent cross-contamination.	they're sufficiently developed will be a large hurdle
Distribution	Sending the needed reagents and equipment to all locations could be an issue especially in low-resource settings.	Sending the needed reagents and equipment to all locations could be an issue especially in low-resource settings.	The distribution networks that support today's PoP and PoC devices could likewise support metagenomic sequences if they're sufficiently miniaturized.
Adoption	PCR is already widely adopted.	Due to problems with sensitivity/accuracy, it may be more challenging to convince the public to use this test.	New researchers and doctors will need to be trained on how to use metagenomic sequences if implemented in clinics
Data Interoperability	PCR outputs can be directly used for sequencing.	Current LAMP outputs cannot easily be used for sequencing.	Ideally, large databases that aggregate data about infectious disease spread in different populations will be useful. Since metagenomic sequencers have not been used in this application frequently, the issues around this are not concrete

Note: Metagenomic Sequencing & Incentive Misalignment

The metagenomic sequencing industry has primarily been developed out of its use in oncology research and whole genome sequencing. As a result, many of the core companies working on metagenomic sequencers are incentivized to further improve their devices for these use cases. Unfortunately, as metagenomic sequencers are further developed for oncology and whole genome sequencing, they become less practical for use in infectious disease diagnostics. For example, whole genome sequencing applications aim to minimize error rates of reads which further increases the cost of the device. In contrast, infectious disease diagnostics require low costs comparable to qPCR and can allow for higher error rates. If metagenomic sequencing companies do not actively work on decreasing the cost of sequencers, it's unlikely that metagenomic sequencing will ever be used as a clinical diagnostic tool.

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