COVID19 Molecular Diagnostics Briefing

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Abstract: This document is designed for novices to biotechnology and pandemic response. The goal is to introduce various categories and molecular methods for diagnosing COVID19, outline companies and academic groups developing diagnostics, and to inform decision-making about maintaining the resilience of nations against the rapidly spreading viral disease.

COVID diagnostics are a diverse bunch and not all tests are created equal. Tests can vary in their turnaround time, cost, specificity, accuracy, and scalability. Here follows a crash course in the various molecular detection methods of note for COVID-19, and their suitability for use in a large- scale diagnostic programme.

The authors assume the following recommendations are under consideration by the readers in government and healthcare settings, which the content has been designed to support discussion around:

1. Consider **temporary regulatory waivers of validated kit components** of supply limited assays and domestic manufacture of alternates.

2. Consider at the minimum the **parallel usage of multiple alternative diagnostic technologies** for surveillance purposes while collecting a data package and regulatory fast path to consider their usage for diagnostic purposes as well.

3. Strongly consider supply chain risks in solutions and **invest in domestic manufacturing capacity**, including the potential temporary repurposing of existing infrastructure at the university or commercial level. That may require temporary waivers or reductions of containment level, facility accreditation, training, insurance, and documentation requirements.

Readers should be aware of the non-exhaustive list of diagnostic tests available and known to authors at this link

Note: this is a LIVE document, a composite of several parallel works by the authors, which remains in progress and will be updated regularly based on redlined elements.

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0. OVERVIEW

Types of Test

COVID diagnostics are a diverse bunch and not all tests are created equal. Tests can vary in turnaround time, cost, specificity, accuracy, and scalability. Here follows a crash course in the various molecular detection methods of note for COVID-19, and their suitability for use in a large- scale diagnostic programme.

Broadly speaking there are three functional categories of testing for COVID19:

1) In-house lab-based diagnostic testing (lengthy, up to several days for a test, often used for clinical confirmation and clinical decision-making)
2) In hospital rapid clinical diagnostic tools (fairly fast, but detect the symptoms, not the virus itself) and require secondary lab confirmation
3) On the spot rapid diagnostic testing (RDT) (provides results within a few minutes to hours, often used for decision-making by healthcare and public workers)

It should be noted that these represent a spectrum, and that hybrid systems exist which lie somewhere between the two former categories (explained below).

Several molecular methods for detecting COVID19 in patient samples are available for use:

1) Polymerase Chain Reaction (PCR)
2) Serological testing for antibodies
3) RNA sequencing
4) CRISPR-based diagnostics
5) Additional PCR techniques

Some of the molecular methods are more commonly used for in-house diagnostics, whilst others are typically used as the preference for RDT. The suitability depends largely on the workflow used to obtain a sample, extract relevant elements from the sample, prepare it for a molecular assay, and then obtain the assay readout.

Choosing a Test

For each kind of test, the following questions are important for both the clinical response and the contact-tracing response efforts:

- What patient samples are needed to run the test?
    - Nasal / buccal swab, blood, sputum
- What does this test tell you?
    - Simple “positive” or “negative” result?
    - Information about viral load or immune response?
    - Information about the progression of the infection?
- How long does it take to get a result?
- How soon after infection can you get a true positive result?
- How long after infection can you get a positive result?
- What are the false negative and false positive rates?
- What equipment or personnel are needed to administer the tests?
- What are the bottlenecks to testing capacity?
- How complex is the test to perform at scale?

Simple tests are ideal, and the greater the number of steps required, the greater the requirement for specialised equipment and staff, and the more complex the method is.

A rule of thumb is that more complex methods require dedicated equipment and a lab, and more work for a user, who may need training or would benefit from having automated sample prep and testing to achieve high-throughput diagnostic capability. More complex does not mean better in terms of information provided by the test.

However, the rule of thumb has exceptions - for instance there has been extensive work done to miniaturise some complex processes that are traditionally considered lab-based into field-portable products. Unfortunately market penetration of field-portable products of lab-based molecular diagnostics remains limited for many types of test. The majority of installed capacity remains in labs.

What Would We Like to See for COVID Diagnostics and Their Use?

Ideally, field-based RDT would be available for COVID, with the tests having a high SENSITIVITY to the presence of the pathogen and being able to detect infected patients before they show symptoms, with low complexity so that minimal training and equipment would be needed.

Ideally a lab-based in house protocol would be available with high SPECIFICITY to detect which strain of the virus a patient has, in order for triage.

In a retrospective analysis of the Ebola epidemic, it was found that use of dual screening methodology with both a highly sensitive RDT, followed by a highly specific PCR assay, could have reduced the epidemic (source). To this end, having both would be an asset to responding to the COVID pandemic.

RDT tests would be used more and should be cheap and easy to use. They should not require cold-chain reagents, and would be used as a means to ‘rule out’ infection (high sensitivity, but with possibly lower specificity) and as a triage tool at community health posts and by contact tracing teams, requiring no laboratory infrastructure and implementable by large numbers of relatively low-skilled personnel. Patients who flag positive would then be referred to larger health centers with laboratory and powerful in-house facilities to confirm infection.

If RDTs are not available then it would be necessary to screen the entire population for COVID-19 using lab-based in-house methodologies, whilst simultaneously restricting movement of the population, until potentially uninfected patients could be ruled-in to public contact as healthy and non-infectious. This could take time, and calls for the investment, manufacture and targeted use of RDTs in order to encourage their use in a variety of important settings: hospitals, airports, critical service jobs etc.

SECTION 1: IN HOUSE MOLECULAR DIAGNOSTIC METHODS

Whilst all the molecular methods of all COVID diagnostics can be performed in a laboratory, in-house molecular diagnostics focuses heavily on PCR. This is because PCR is generally considered the gold standard for diagnosing pathogens in the patient thanks to their high sensitivity.

Q. On a high level, how does PCR work?

PCR-based tests amplify small sections of RNA that are characteristic of SARS-CoV-2. This amplification is done through temperature-mediated cycles of replication by leveraging the ability of enzymes to replicate specific genomic (nucleic acid) elements. When combined with a fluorescent dye that glows in the presence of DNA, PCR can also show how much amplified RNA is present.

Several variants exist, and the nomenclature is not used consistently[1]. You may see references to the following approaches:

- Polymerase chain reaction (PCR)
- Reverse transcriptase polymerase chain reaction (RT-PCR)
- Quantitative Real time Polymerase chain reaction or quantitative (qPCR although sometime qRT-PCR)
- The "correct" terminology is the RT-qPCR, but you may hear about variants above used to denote this test. It is able to detect the presence and level of target COVID-19 sequences in RNA purified from the patient sample, which tells you if someone is infected and, if so, for approximately how long.

These protocols and variants of the PCR share commonality in requiring the use of several standard elements including a thermocycler instrument and several critical enzymes and chemicals, which require cold chain logistics and typically require laboratory facilities for use. Most installed systems cannot easily be deployed for on the spot testing in the field.

Alongside these standard elements, variable reagents are also required, namely customised DNA probes (AKA primers) that are used to target a specific gene in the virus for amplification and detection. The custom DNA is synthesised in a lab by a company or a dedicated foundry in a facility for DNA synthesis.

Probes vary based on the target (a particular gene product), as well as the PCR method variant they are being designed for. The probe must be designed around one or more specific target viral genes, and they must be designed so as not to produce inaccurate results through "dimerisation" (self-binding) of the primers to one another rather than the target viral gene, which might provide inaccurate amplification of non-target genes and provide false positives or negatives.

Variants of the PCR allow detection time to be reduced by directly measuring (quantifying) the amplified viral product in a special thermocycler in real time (RT-qPCR), but this requires additional capability to design and manufacture the probes and adds complexity in manufacturing.

Q. What patient samples are needed to run PCR test?

- As of March 13, the CDC guidelines for clinical specimens recommend only a single nasopharyngeal swab (i.e. a swab of the nostril).
- Most protocols had been recommending that two samples from each patient be run: a nasopharyngeal swab (i.e. the nostril) and an oropharyngeal swab (i.e. the throat). Some test lower-respiratory samples (i.e. coughed-up sputum), which have also been used with some tests. The goal is to collect material that's recently been in the lungs, where the virus is believed to replicate.

[1] Since SARS-CoV-2 is an RNA virus, the PCR tests involve *reverse transcription* PCR (RT-PCR). Accurate testing also requires *real-time* PCR (confusingly, also sometimes called RT-PCR) or *quantitative* PCR (qPCR) - rather than just running the reaction for a certain number of cycles and measuring the total amount of RNA amplified, that measurement is made at each cycle.

- Clinical nasopharyngeal samples cannot be directly tested with PCR as the RNA from the sample must be separated from the other contents before loading into the thermocycler instrument. The RNA extraction step is critical to the success of the PCR,

Q. What does a PCR test tell you?

- Whether there was RNA matching the PCR primers in the sample taken. The tests have typically targeted multiple locations in the SARS-CoV-2 genome, and will be considered inconclusive if the test amplifies some, but not all, locations.
- Each patient sample is run alongside a negative and positive control. The test will also be inconclusive if anything is amplified in the negative control, or if nothing is amplified in the positive control.

Q. What are the false negative and false positive rates?

- It's been hard to find data on this, since we don't have a *better* test for SARS-CoV-2. PCR has been proposed as a gold standard for sensitivity as is the case for many infectious diseases.
- False positives can be caused by contamination and false negatives can be caused by poor sample collection. There have been some reports of many false positives from people in frequent in-person contact with infected individuals, perhaps because RNA from the infected person is being picked up, but unclear how credible this is.

Q. How soon after infection can you get a true positive result? How long after infection can you tell that someone was infected?

- A study from Germany, described in STAT, "said that while people with mild infections can still test positive by throat swabs for days and even weeks after their illness, those who are only mildly sick are likely not still infectious by about 10 days after they start to experience symptoms."
- One January study of patients in Wuhan found that "the mean interval time between the initial negative to positive RT-PCR results was 5.1 ± 1.5 days; the initial positive to subsequent negative RT-PCR result was 6.9 ± 2.3 days".

How long does it take to get a result?

- This depends a bit on whether a high-throughput system is available. Most places have reported turnarounds of 24-48 hours, though personnel and supply chain bottlenecks may lengthen these.
- In real-world settings reporting times are often several days and factors like specimen transport, human resources, and supply chain are more important causes of delay than hands-on analysis time (source)
- In the USA, the fastest turnaround is available via the integrated (and very expensive) Roche *cobas* system, which can provide results from patient samples in around 3.5 hours. It's not possible to reduce the time much lower than that, because the RNA extraction involves several incubation steps (i.e. "let the sample marinate with these chemicals for 15 minutes at 37 C") and the PCR reaction requires repeated thermal cycles, each of which lasts on the order of tens of minutes.
- RT-qPCR assays typically require cold-chain reagents and take 1–3h, depending on throughput and batching
- Specialised field-based PCR systems, which are not widely available at this time, can provide gold standard results outside of a lab, but their installed base is likely low and challenging to increase.

Q. What equipment or personnel are needed to administer the tests?

- This depends greatly on the test kit being used. All of them will require an incubator and a combined thermal cycler and plate reader, but not all test kits are compatible with all equipment.

- Different vendors provide slightly different ways of measuring amplification at each cycle. It is possible to do RNA extraction *without* a vendor-specific kit, but this isn't approved by the CDC/FDA and is more complicated / easier to mess up.
- Here's some information about the highly-integrated Roche system, the use of OpenTrons robots to speed up sample preparation (12 hours/sample) (covered in more detail below), and supply chain challenges for Qiagen test kits, and scaleup plans.
- Different protocols use a diverse set of variable reagents (primers for targeting viral genes for amplification), but may also have slight variations in the standard elements (for instance the PCR master mix that is used for incubation of samples in the thermocycler. There is uncertainty about which protocol is the best, and how to measure performance amongst existing kits and methods.

- PCR reagents as well as RNA extraction reagents are critical. Some (but not all) reagents may be produced directly in a lab with the appropriate capability. Generally speaking laboratories are able to purchase pre-made kits that contain RNA extraction and PCR reagents, which simplify steps for the user.

Q. What are the bottlenecks to testing capacity?

- At first, it was approval of tests. Now it appears the bottlenecks are high-throughput machinery for sample preparation and supply chains for reagents, nasal swabs, test kits and validated control samples.

- *Challenge 1: Validation, Troubleshooting & Practicality*
    - New assays are not being widely validated for false-positive and false-negative rates, or for the efficacy of the primer designs.
    - Validation require reagents including inactivated virus, live viral constructs, extraction kits, instrumentation including RT-PCR kits, as well as positive and negative controls
    - Lack of clinical specimens for viral material to clinically validate existing and new tests before use (less of an issue as cases increase, but not a solved problem)
    - It is not clear how reagents from different protocols could be mixed and matched
    - Victor Shi, CEO of Adicon Clinical Laboratories Inc. notes that every step in the PCR testing process is problematic, in China (source)
    - It takes skill to take nasal or throat samples, and because the samples are "relatively messy compared to a blood draw" there is potential for contamination.
    - Once a sample has been obtained, it must be stored and transported to a lab. The SARS-CoV-2 virus that causes COVID-19 "is an RNA virus, it is very unstable, so storage conditions are critical," Shi said.
    - As a result of the compressed development time and difficulty accessing samples, there was a "very limited amount of clinical validation" of PCR kits, Shi reported

- *Challenge 2: Supply Chain Continuity*
    1) There is a major problem of supply shortages for both standard and variable elements of the PCR reaction. Those that are critical to maintain continuity are:
        a) Enzymes, which are typically sourced from microbes engineered to synthesise the enzyme + requiring bioproduction facilities for scaleup.
        b) Single stranded DNA (ssDNA) probes (AKA primers), typically sourced from DNA synthesis companies
        c) Thermocyclers and their parts
        d) Sample collection materials and vials, including medical grade sterile cotton swabs
        e) RNA extraction reagents

As of March 17th, 2020, 125K tests have been ordered by PHE from Primerdesign, a UK based kit manufacturer, and reports are they have materials to scale to 3.5 million.

If diagnosis is limited by kit manufacturing, then 'pooled' testing options can be considered. In pooled testing, multiple patient samples are mixed and tested together. If the test returns positive, then the patient group is split into subgroups and pooled testing is performed again. This is repeated until the positive sample(s) is (are) uniquely identified. If the frequency of positive samples is low (on the order of 1 in N, where N is the initial pool size), then pooling reduces the number of tests required from N to log(N). Concretely, it would mean 13 tests would be needed to check 64 patients (instead of 64 tests). This approach might therefore be particularly useful for general population testing, where the incidence of COVID-19 is low. It would not be useful for targeted testing of suspected cases as incidence of true positives would be too high, making pooled testing less efficient than standard testing. In academic studies, pooled testing has been found to save resources, for example for HIV and Influenza testing. Pooled strategies are also commonly used in veterinary surveillance programmes. Finally, a collaborative effort in Israel appears to have developed a pooled testing protocol for SARS-CoV-2.

- *Challenge 3: Extraction & Scaling*

Performing PCR requires genomic material to be extracted from a physical sample (blood/sputum) in a multi-step process where reagents and liquids are mixed, transferred into a thermocycler, and then processed for readouts.

Given the volume of tests that need to be done, this process must be automated. Automation is typically seen as a luxury amongst scientists working in the majority of academic laboratories and there is limited installed automated RT-PCR capability. Robotic liquid handling systems are important for scaleup, but are typically very expensive and out of reach for most labs.

The key issues being faced in this category include:

1. Lack of extraction capability and instrumentation bottlenecks
2. Lack of trained staff, skilled technicians, and training capability for existing and new tests, especially for high-throughput platforms which have complex protocols
3. Lack of large-scale high complexity physical testing capacity (facilities, automation platforms/robotics) in public service (much of the installed base is operating in commercial settings)

The previously mentioned supply chain limitations on approved clinically validated viral RNA kits also require broadening the approved viral RNA methodologies and kits. For example, on March 10, Politico reported that all validated viral RNA extraction kits (i.e. the ones from Qiagen) were on backorder.

Multiple labs have reported successful changes to alternative unapproved solutions. Current short list of reported alternates includes (but additional validation of quality required):

1. OmegaBiotek Mag-Bind Viral RNA 96 kits (and amenable to liquid handling automation)
2. Switching to vet rated Qiagen kits (limited stock available)
3. Ambion vRNA kits (limited EU stock)
4. Zymo Research Direct-zol + Trizol inactivation (limited EU availability, available in USA)
5. Analytic Jena Innuprep kits + FeliX robot for automated 96 well extractions

Case Study: Qiagen

Qiagen is looking to steeply ramp up its worldwide production of the RNA test reagents used to perform certain COVID-19 diagnostics. Previously, the Dutch diagnostics manufacturer turned out enough of the chemical mixture to supply about 1.5 million coronavirus tests per month. As of March 18th, Qiagen plans to scale up to support more than 6.5 million tests by the end of April, and more than 10 million per month by the end of June. By the end of the year, the company hopes to provide for more than 20 million monthly tests through its manufacturing sites in the U.S. and Europe.

(Source)

Case Study: Salis Lab

Recently published: "A Massively Parallel COVID-19 Diagnostic Assay for Simultaneous Testing of 19200 Patient Samples" Protocol at https://docs.google.com/document/d/1kP2w_uTMSep2UxTCOnUhh1TMCjWvHEY0sUUpkJHPYV4…. Primers & Spike-in Controls at https://docs.google.com/spreadsheets/d/1y_Bf0Zz4FJRx53oSkX59u0kfouDOdrtr8E26M5NINFs

SECTION 2: RAPID CLINICAL DIAGNOSTIC (RCD) METHODS

Due to the generic nature of the initial clinical symptoms (fever and dry persistent cough), clinical presentation alone is challenging to use as an effective benchmark. However, SARS-CoV-2 causes particular pathologies in the lungs that can be identified on images and that distinguish it from other respiratory viruses.

Note: technically these are not molecular diagnostic methods, but we include them here for the convenience of the reader.

RCD Method 1: CT scans

While heavily overloaded with patients in Wuhan, China adopted medical imaging based (CT) diagnostics, including a modified CT protocol which was capable of 10 minute runs and over 200 patients per day per CT. This method only detects later stage infections. There are also only ~700 CT machines in the UK, indicating major bottlenecks on clinical diagnostic throughput. These strategies ideally are then confirmed with a nucleic acid based diagnostic (lab validated vs clinically identified).

RCD Method 2: Ultrasound based imaging

Desk portable ultrasound machines exist in most hospital contexts, which can be wheeled into patient rooms for initial assessments. That makes it a potentially far more scalable clinical tool than CT, but does have a lower resolution of imaging quality requiring more expert interpretation.

Butterfly Network

A UK based SME with a handheld, tablet or phone connected ultrasound unit. They have demonstrated similar diagnostic imaging capabilities to CT, at a far lower price point per unit.

SECTION 3: RAPID DIAGNOSTIC TEST (RDT) METHODS

The primary issue with any PCR based approach is the requirement for testing in a laboratory using a lengthy PCR workflow. Point-of-care testing, or rapid diagnostic tests (RDT) could be achieved through use of alternative molecular diagnostic approaches which dispense with complex workflows. Here we list several technologies and products well-suited for RDT purposes, or could be developed toward RDT[2].

RDT Method 1: Antibody based assays (Immunological assays)

Antibody based diagnostics can be from blood samples (which are safer to draw than nasal swabs), are rapid (15minute readouts) and can be deployed in a point of care context i.e. no central lab needed. Additionally, they can provide a clinically useful staging diagnostic based on the ratio of different antibodies in the form of IgM vs IgG.

This is useful in differentiating between patients at different stages of COVID disease, including patients who have resolved and have immunity (see Annex 1, figure 1). Currently however they do not appear to detect early stage infections, but do detect recovered patients, which may be helpful to identify immune individuals for volunteer duties. There are reportedly over 200 companies with serological home tests in development

Q. On a high level, how do antibody tests work?

- These measure antibodies present in the bloodstream, specifically IgM and IgG. IgM is a protein that provides immediate immune response, while IgG is made later in infection and drives a secondary immune response.
- Antibody-based tests check whether anything from a blood sample binds to a known *antigen* (in this case, something that looks like SARS-CoV-2). You might coat the wells of a microplate with the antigen and then read absorbance on a plate reader (this is how ELISA often works) or have the antigen on a colloidal test strip (similar to a pregnancy test).
- For SARS-CoV-2, the antigen of choice is the spike protein; it's the main surface protein, and most people's immune systems will end up using it to recognize and neutralize the virus. Scientists are working on designing an effective recombinant antigen (see e.g. Twitter summary).

Q. What patient samples are needed to run the test?

- A blood draw (or in some cases, finger prick) from a patient.

Q. What does this test tell you?

- Antibody tests can tell you when someone has developed antibodies against SARS-CoV-2 as part of their immune response.
- Usefully, you can get some read on how far along someone is in their infection by comparing different types of antibody (IgG and IgM)

Q. How long does it take to get a result?

- Usually less than half an hour. Antibody-based tests are fast, and simple.

[2] In essence the line between in-house test and RDT is fuzzy.

Q. How soon after infection can you get a true positive result? How long after infection can you get a positive result?

- A study from Germany, described in STAT, "noted that people who are infected begin to develop antibodies to the virus quickly, typically within six to 12 days. The rapid rise of antibodies may explain why about 80% of people infected with the virus do not develop severe disease."
- A rapid positive IgM result may be detectable more or less indefinitely after infection.

Q. What are the false negative and false positive rates?

- Unclear but perhaps not great at this time. In a small sample of the Biomedomics test, the manufacturer found a sensitivity of 88.66% and a specificity of 90.63%. ELISA-based tests could potentially do a bit better than that, but they are expected to be worse than rRT-PCR.
- False negatives may be caused by natural variation between the antibodies produced by individual immune systems in response to an infection; not all spike-protein-targeting antibodies will have the same domains.
- Some false positives are caused by similarities between the spike proteins of SARS-CoV-2 and other coronaviruses. The FDA policy issued on March 16 requires antibody-based tests to have the following caveat: "Positive results may be due to past or present infection with non-SARS-CoV-2 coronavirus strains, such as coronavirus HKU1, NL63, OC43,or 229E."
- In general, IgM has many false positives. A 2016 review paper states that "when an accurate diagnosis is essential for therapy, prognosis, infection control, or public health, when the patient is sick enough to be hospitalized, or when the clinical or epidemiologic findings do not fit, IgM detection should not be accepted as a stand-alone test. Rather, whenever possible, the diagnosis should be confirmed by other means, including testing of serial samples and the application of additional test methods."

Q. What equipment or personnel are needed to administer the tests?

- Equipment can be lab based and might need an incubator and plate reader similar to a PCR, or for antibody-specific equipment necessary for an ELISA test.
- Antibody technologies can also be used as low cost paper-based diagnostics and lateral flow assays (like pregnancy tests)

Q. What are the bottlenecks to testing capacity?

- The FDA has made a general exception for use of these tests in clinical settings without requiring Emergency Use Authorizations.[3]
- These tests are being produced at scale, so the chief bottleneck is their importation and distribution.

Case Study: Biomedomics (North Carolina, USA)

BioMedomics is a point-of-care diagnostics company that aims to provide novel, rapid point-of-care tests to aid in the diagnosis of critical diseases.

[3] See section D https://www.fda.gov/media/135659/download

BioMedomics has developed and launched one of the world's first rapid point-of-care lateral flow immunoassays for the diagnosis of coronavirus infection. The test has been used widely by the Chinese CDC to combat infections and is now available globally. This test detects both early marker and late marker, IgM/IgG antibodies in human finger-prick or venous blood samples. It can be used for rapid screening of carriers of the virus that are symptomatic or asymptomatic and has been shown to be both sensitive and specific. With the tests taking around 15 minutes to generate results, their ease of use and lack of need for additional equipment makes the test ideally suited for hospitals, clinics and test laboratories. The test can also be effectively deployed in businesses, schools, airports, seaports and train stations, etc. The USFDA guidance issued on March 16, 2020, allows the distribution of this product for diagnostic use in laboratories or by healthcare workers at the point-of-care while their FDA EUA application is being reviewed.

Case Study: Aytu Biosciences (Colorado, USA)

A small specialty pharma company which has deployed a rapid point of care lateral flow immunoassay with the Chinese CDC as well. On march 17, 2020 they announced acceleration of availability under the FDA's new rapid access rules (but not yet approved).

Case Study: Reagent Genie (Ireland)

An Irish company is preparing to release rapid Covid-19 testing kits, which can provide results in 15 minutes and potentially act as a "clinical weapon" against coronavirus.
The tests have been developed with the same technology contained in pregnancy tests and although they are in a pilot phase, they could reduce testing times dramatically from four hours to just 15 minutes.
Assay Genie, a Reagent Genie brand, will be releasing the rapid POC (Point of Care) kit within weeks globally and already some Irish hospitals have been in touch to sample the product, according to Colm Ryan, biochemist and chief executive of Assay Genie.

RDT Method 2: Direct RNA sequencing based assay

RNA/DNA sequencing-based diagnostics have been field deployed before in Ebola and Zika outbreaks. They capture viral DNA/RNA from a sample and directly measure it by passing it through a pore in a membrane that reads the charge on the DNA/RNA to identify its sequence.

AREA OF IMPROVEMENT - EXPAND THIS TO FOLLOW STYLE OF OTHER DIAGNOSTICS SUCH AS CELL FREE AMPLIFICATION STRATEGIES OR NANOPARTICLE BASED TESTS

Oxford Nanopore (Oxford, UK)

The world leader for that is in the UK with Oxford Nanopore, including domestic manufacturing capacity. They are however more expensive per test than PCR based protocols but provide a critical capacity for using molecular epidemiology to estimate transmission chain volume and dynamics, as has been demonstrated in Seattle.

Oxford Nanopore is working with public health laboratories around the world, to support the rapid sequencing of the novel coronavirus that was first seen in Wuhan, China. A large number of scientists from large centralised labs - and also smaller decentralised ones - are now using nanopore sequencing to support rapid data sharing of genomic data. Rapid data sharing has been key to the public health response, and researchers all over the world have been fast to share the genomes they have sequenced on public databases such as GISAID, GenBank or elsewhere.

Sequencing the virus can support 'genomic epidemiology'- characterising the virus and helping public health authorities to understand the identity of the virus, whether it is changing and how it is being transmitted - all in conjunction with other epidemiological data.

The scientific community has previously developed methods for the rapid, near-sample nanopore sequencing of pathogens in multiple outbreak situations including Zika, Ebola, Yellow Fever and Swine Flu and a range of other pathogens. This experience has supported the rapid deployment of nanopore sequencing for the current outbreak.

At this time, Oxford Nanopore staff are working with the community to support the development and sharing of best practice and protocols for the sequencing of this virus. We are offering technical support to public health authorities, and working to understand the needs of public health staff so that we can continue to provide the most useful support to the community. If you are a public health laboratory/scientist in the microbiology community and wish to discuss how we can support you in the current outbreak please get in touch

RDT Method 3: CRISPR based assays

CRISPR based diagnostics have a higher limit of detection than gold standard CDC RT-PCR, are 30-60 minutes to outcome, and can be performed with simple equipment, and could be easily automated for higher throughput. See Annex 1, figure 2 and 3 for comparison of known viral loads from throat and nasal swabs vs the known limit of detection for RT-PCR vs CRISPR protocols.

There are currently at least two CRISPR tests in development that would greatly improve our testing capabilities through increased accuracy, turn-around times, and lower equipment and training costs than PCR systems: Mammoth's DETECTR test and MIT's SHERLOCK test.

Note: although CRISPR technology is listed as an RDT, currently the technology appears to be more relevant to lab-based testing. In order to leverage the power of this technology, there are aspirations to develop paper-based CRISPR detection systems for field use.

Both test methods should be developed with great urgency, and further methods should be explored.

Q. On a high level, how do CRISPR diagnostics work?

- Both the solutions proposed by Sherlock Biosciences (SHERLOCK) and Mammoth Biosciences (DETECTR) are based on Cas enzymes that, after recognizing a target, begin indiscriminately cutting surrounding single-stranded nucleic acids. You can have the indiscriminate cleavage produce a rapid readout (for example, you can link a chromophore with a quencer using RNA, so that indiscriminate cleavage will produce a color signal).
- In more detail:
    - DETECTR, or DNA Endonuclease Targeted CRISPR Trans Reporter, uses CRISPR-Cas12a with a guide RNA that matches SARS-CoV-2.Since Cas12a cleaves dsDNA, they recommend RT-LAMP to amplify the sample.
    - SHERLOCK, or Specific High Sensitivity Enzymatic Reporter UnLOCKing, uses CRISPR-Cas13a, which binds to RNA, rather than DNA, so they amplify the sample using RT-RPA.
- In both cases, the RNA must be amplified before detection is attempted. Isothermal amplification is possible, and is recommended by both whitepapers.

Q. What patient samples are needed to run the test?

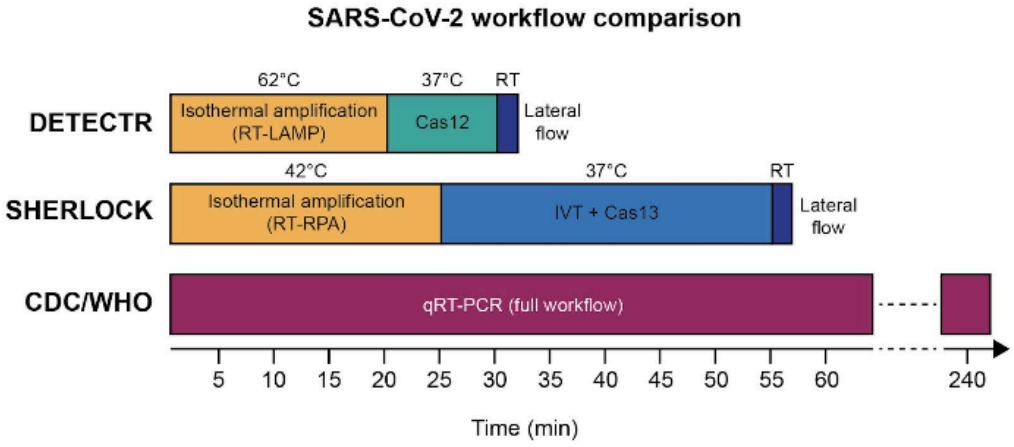
- Both theMammoth and Sherlock whitepapers recommend using patient RNA that has been extracted according to CDC guidelines, which are described in the PCR section above.

Q. What does this test tell you?

- This test tells you whether SARS-Cov-2 RNA was present in the patient sample. It's a simple positive or negative result based on a band appearing on a lateral flow test strip.
- The Mammoth whitepaper reports a detection limit of 70-300 copies of viral RNA per µl of input (n = 7)., while the Sherlock whitepaper reports detecting 10-100 copies per µl of input.

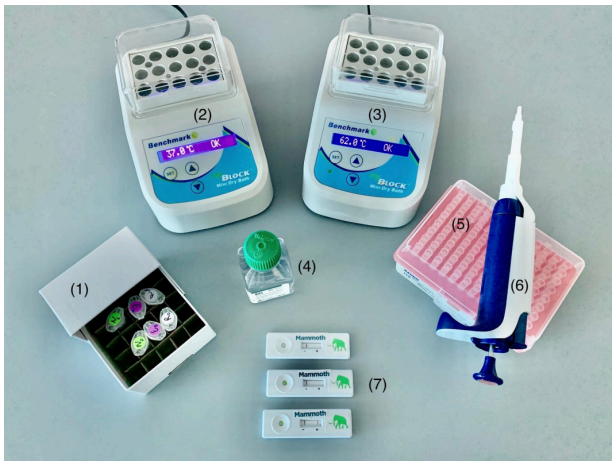
Q. How long does it take to get a result?

- Below is a comparison graph from the Mammoth whitepaper. They estimate that DETECTR takes around 35 minutes and SHERLOCK takes around 60.



Q. What equipment or personnel are needed to administer the tests?

- Mammoth intends to distribute the equipment along with the reagents in a single kit which can be used inside a fairly routine for a simple lab setup requiring a heat block.
- Given patient RNA extracted according to CDC requirements, the test can be run by anyone trained in pipetting and simple molecular biology reactions. This makes the test suitable for "off-site" deployment, for example at airports or community hospitals (no need to send the test to a remote testing facility) [2].



Minimal sample equipment
(1) DETECTR reagents
(2) 37°C heat block
(3) 62°C heat block
(4) Nuclease-free water
(5) Pipette tips
(6) Pipette
(7) Lateral flow strips

Q. How soon after infection can you get a true positive result? How long after infection can you tell that someone was infected?

- x

Q. What are the false negative and false positive rates?

- X
- As with PCR, false positives can be caused by contamination and false negatives can be caused by poor sample collection. There have been some reports of many false positives from people in frequent in-person contact with infected individuals, perhaps because RNA from the infected person is being picked up, but unclear how credible this is.

Q. What are the bottlenecks to testing capacity?

- Validation and testing with more samples
    - Trevor Martin, founder of Mammoth Biosciences has noted that CRISPR tests "could possibly scale up to millions of tests in a matter of months". The scaleup timeline might depend on supply chain issues, but he thinks that the supply chains problems we've been seeing with COVID-19 are unlikely to affect the materials necessary for this test.
- In the initial whitepapers, DETECTR was run on real patient samples, while SHERLOCK was tested on synthetic RNA,

Other notes:

- Faster than qPCR: rather than sending results to a lab, you can get a simple visual readout in a few hours or less (a lateral flow strip)
- Since the readout is visual, it's easier for a less-trained person to interpret
- The above two features means that it's well-suited for "off-site" deployment at e.g. community hospitals, airports, potentially even home kits.

Company Case Study: Mammoth Biosciences

Mammoth Biosciences, was spun out of the laboratory of Jennifer Doudna, one of the inventors of CRISPR. Mammoth has been working on its diagnostic system called DETECTR to be a robust platform to test for diseases. They have reconfigured their DETECTR platform to rapidly and accurately detect SARS-CoV-2 in humans using a visual lateral flow strip format within 30 minutes from sample to result. DETECTR couples CRISPR detection with isothermal pre-amplification using primers based on protocols validated by the US CDC and WHO. Currently in the United States, the CDC SARS-CoV-2 real-time RT-PCR diagnostic panel has a laboratory turnaround time of

approximately 4-6 hours, with results that can be delayed for >24 hours after sample collection due to shipping requirements. In addition, these tests are only available in CDC-designated public health laboratories certified to perform high-complexity testing. Mammoth is working to enable point of care testing (POCT) solutions that can be deployed in areas at greatest risk of transmitting SARS-CoV-2 infection, including airports, emergency departments, and local community hospitals. Leveraging an “off-the-shelf” strategy to enable practical solutions within a short time frame, the protocol is fast (<30 min), practical (available immediately from international suppliers), and validated using contrived samples. However, this process is still in development, currently both Mammoth and Sherlock’s approaches would initially require centralised lab based testing.

RDT Method 4: Lateral Flow Immunoassay

Lateral flow immunoassay test, similar to a pregnancy test (results available within minutes) Hybrid systems may be used in either situation, each bringing their own advantages and disadvantages.

Lateral flow immunoassay tests, while incredibly quick (within minutes), have higher errors rates than other test methodologies.

SECTION 4 - HYBRID AND EMERGING METHODS OF NOTE

Several companies or research groups have developed field-portable PCR instruments and molecular diagnostic methods that build off of PCR methods that are more suitable for centralised laboratories.

Hybrid Method 1: RT-LAMP

Reverse transcription loop-mediated isothermal amplification (RT-LAMP) is a one-step nucleic acid amplification method using an isothermal enzymatic amplification that has been used to diagnose infectious diseases. RT-LAMP has several advantages including that it has high specificity and sensitivity, can be done in less than an hour, can work at various pH and temperature ranges which is advantageous for clinical samples, does not require a thermocycler, and that the reagents are relatively low cost and can be stable at room temperature. Importantly RT-LAMP requires only a single temperature and thus does not require a thermocycler and provide a readout by a change in colour in the tested sample upon detection of the pathogen.

A group at the University of Oxford has developed an RT-LAMP based test for SARS-CoV-2 with clinical validation from China in a small sample. A group in Beaumont School of Medicine (US) have previously used this method to detect zika virus in clinical serum and urine samples as well as mosquitos and have demonstrated its use for COVID-like viruses.

Conventional qRT-PCR, while specific and sensitive, must be done by trained personnel on specialized equipment at a qualified laboratory. Since this disease is spreading rapidly, centralized labs may have trouble keeping up with testing demands or may need an alternative approach if qRT-PCR kits are not available. This feasibility study demonstrated that RT-LAMP allows rapid detection of COVID-19 in a variety of common human specimens collected for clinical testing, including serum, urine, saliva, oropharyngeal swabs, and nasopharyngeal swabs.

LAMP-based test for early-stage infection: Dyes such as SYBR green, can be used to create a visible color change that can be seen with the naked eye without the need for expensive equipment, or a response that can more accurately be measured by instrumentation. Dye molecules intercalate or directly label the DNA, and in turn can be correlated with the number of copies initially present. Hence, LAMP can also be quantitative. In-tube detection of DNA amplification is possible using manganese loaded calcein which starts fluorescing upon complexation of manganese by pyrophosphate during in vitro DNA synthesis.

Possible issues with LAMP: the pH dyes are not very stable have a very short window between which a user is able to detect the positive and negative.

Hybrid Method 2: Field-Portable PCR / PCR Workflow Improvements

Semi-Portable PCR Case Study - Cepheid (France / California)

The company produces GeneXpert® Xpress systems for detecting pathogens, and provides base units which take receipt of consumable PCR test cartridges designed to specifically detect a pathogen. Cepheid is currently looking to adapt their system to detect COVID. Base units come in multiple sizes, with some suitable for on-the-spot RDT in the field, and larger units that can take receipt of multiple test cartridges simultaneously.

The company is endorsed by WHO for rapid test capability for different diseases, ranging from 20 minutes to an hour. To this end the system combines ideal attributes from in-house testing with attributes for RDT.

The company's Flu/RSV cartridge technology sees cartridges loaded with primers which target multiple regions of the viral genome to provide rapid detection of current viruses, and could be repurposed against pandemic coronavirus strains.

The largest variant of the system would be able to run up to 1,152 tests in 24 hours. The smallest would test 72 in 24 hours. The machine requires a laptop and a power source for use in the field.

Source 1, Source 2, Source 3, Source 4

Point-of-care PCR Case Study - Mesa-Biotech (San Diego CA)

Mesa Biotech designs, develops, manufactures and commercializes next generation molecular diagnostic tests, bringing diagnostic performance of nucleic acid PCR amplification to the point-of-care (POC).Mesa Biotech Inc., a privately-held, molecular diagnostic company that has developed an affordable, sample-to-answer molecular testing platform designed for point-of-care (POC) infectious disease diagnosis, announced the addition of the novel coronavirus (SARS-CoV-2) to its active influenza clinical trial in China. The coronavirus test development and clinical trial is in collaboration with Dr. Wang Guangfa, head of the Department of Pulmonary Medicine at Peking University First Hospital in Beijing. The clinical trial results will be submitted under an 'emergency use' authorization in both China and the United States.

Technology development started at Los Alamos National Lab supported by NIH grants from the National Institute of Allergy and Infectious Diseases (NIAID) and the Western Regional Centers for Excellence in Biodefense and Emerging Infectious Disease program. Since the beginning we have focused on technology suited for emergency defense and rapid deployment for SARS, Ebola and other emerging infectious diseases. Mesa's platform was specifically designed for use outside the lab to enable rapid responses to global pandemics, such as COVID-19.

If successful, Mesa's coronavirus test may be the first molecular POC test to enable care providers to obtain laboratory-quality results in approximately 30 minutes, facilitating more immediate response to the spread of the coronavirus.

Source

Automation Layer Case Study - Opentrons (USA & China)

Opentrons is a robotics and software company that has developed a low cost platform to automate laborious PCR sample preparation using largely off-the-shelf components designed for 3D printers. The system is useful for sample preparation and can be modified to suit a variety of PCR protocols. It can be used with an integrated thermocycler.

Opentrons' COVID-19 Testing System is designed for labs running COVID-19 public health surveillance projects that need to immediately scale up to automated operations. Opentrons can install systems that automate up to 2,400 tests per day within days of an order being placed. They are currently deploying this surveillance capability at the Open Medicine Institute in Palo Alto, California, with more surveillance projects soon to follow.

The robotic system and software are entirely open source, meaning that anyone can produce the product and modify it as they see fit. Nonetheless the company is the sole producer of bona fide OpenTrons products at this time. The OT-2 system is portable but requires 2 people to lift it. It would also need to be combined with other reagents providers, but a series of hardware, software and wetware protocols could be developed and distributed to many users nationally and internationally if production could be ramped up.

The company has partnered with BP Genomics, *to provide the robots and reagents necessary to ramp to a total capacity of at least 250k tests every week,* most likely to the US market.

The founder of the company reports that they have capability to produce at least several hundred more robots in the US, and that the system could potentially be modified for field testing by deploying the systems into mobile laboratories. The founder also notes the potential to begin production of robotic systems (but not reagents) in other geographies outside of the US and China, however this would be challenging. It should be noted that the Opentrons system may have issues with reproducibility as they are effectively custom built low cost systems and require appropriate calibration. However they can be easily and cheaply produced.

Source

Case Study: Abbott Diagnostics ID Now

Abbott Diagnostics has gained approval for a rapid (approximately 15 minute) point-of-care test called ID NOW which is based on NEAR: Nicking Enzyme Amplification Reaction. NEAR is an isothermal nucleic acid detection technology requiring two enzymes: a polymerase possessing strand-displacement and reverse transcriptase activity and a nickase. Type IIS restriction enzymes are typically heterodimers which recognize a non-palindromic site; by mutating one subunit to remove DNA cleaving ability a nickase is created which cuts only on one strand. In NEAR, probe targeting the genome of interest contain 5' tags with a nickase site. Extension of the probes ultimately results in two extended molecules containing the target region of interest flanked by nickase sites. Nicking creates a priming site for polymerase, which upon extension is available to be nicked again, creating a rapid cycle of polymerization of the target. Fluorescently labeled nucleic acid hairpins, or molecular beacons, hybridize with amplified material to generate signal; in the absence of amplified material the beacon hairpins bring a quenching residue near the fluorescent label, suppressing background signal.

Case Study - Adding Automation Layers to Traditional Equipment

AREA OF IMPROVEMENT - ADD DETAIL ON ADDITIONAL ROBOTIC PLATFORM COMPANIES

AREA OF IMPROVEMENT - ADD MORE BRITISH / EU COMPANIES / ORGS THAT COULD GET INVOLVED

SECTION 5 - Conclusions and Takeaways

PCR tests are currently best poised to provide the most accurate testing at scale in the United States and in many other countries. Antibody tests are useful, but their lack of early detection capability is a significant drawback. Nevertheless, antibody tests are better than no test, and as they are far easier to administer, they serve an important purpose in initial front-line diagnostic use. Crucially, when combined with rtrPCR, antibody tests can provide additional information in clinical settings, for instance, by informing the disease state of the patient (early infection, late infection, immune). Both antibody and PCR of tests should be used at scale as soon as possible.

While there are many producers of rtPCR kits (see spreadsheet), the main bottleneck is the production of test kits, because most types of rtPCR tests require the use of a specific kit of reagents, primers, and materials assembled into kits. Due to recent approval of tests by Roche and Thermo Fisher, the bottleneck will soon shift from testing kits to testing supply chains (reagents, swabs, sites, etc). Supply chains for both RNA extraction and PCR reagents will need to be maintained, especially in cases where production companies may be nationalised as the pandemic progresses.

A key drawback of PCR tests at present is their limited accuracy and the requirement of PCR machines (thermocyclers) and trained operators. While many machines are inexpensive or quite portable, much of the installed base is in laboratories and is not being put to use on COVID yet. This installed capacity could be repurposed at scale, especially as universities and many companies are shutting down non-essential operations. While many PCR tests are more accurate than antibody tests, we do not yet have good statistical data on their accuracy at scale. In time, it is likely the accuracy of rtPCR tests can be improved. In addition, we need to push

forward the efforts to design new tests that can achieve greater accuracy at scale without expensive equipment. Another key issue for PCR is the laborious nature of sample prep for both RNA extraction and PCR setup. Automation layers (robotics systems) could aggressively reduce the workload of individual lab technicians conducting testing. There are opportunities to automate existing PCR systems, or to otherwise massively scale up use of installed base PCR systems across laboratories in universities, companies, hospitals and elsewhere.

New technologies offer attractive improvements on well-established molecular testing methods. There are currently at least three tests in development that would greatly improve our testing capabilities through increased accuracy, turn-around times, and lower equipment and training costs. Of these, two are CRISPR-based tests: Mammoth's DETECTR test and MIT's SHERLOCK test. These systems are attractive as they can achieve greater specificity than rtPCR tests, and they can be performed in ~1 hour without very specialized machinery, for instance the Covid19 RAMP test. CRISPR-based testing systems could thus provide testing capability in laboratory and point-of-care or field testing situations. To this extent, all three of these test methods should be developed with great urgency.

ANNEX 0

Summary table of available RT-PCR protocols

Different countries have chosen different gene targets; you can read about these on the WHO website. Labs are exercising a range of options in developing the primers and probes for a test.

Country	Institute	Gene targets	Notes
China	China CDC	ORF1ab and N	
Germany	Charité	RdRP, E, N	This is the currently recommended WHO protocol. Pinksy group at Stanford, validated a modified version of the test developed by the Drosten group at Charité Virology in Germany, which was the first to be publicly available. The test, which is the basis for the WHO test, targets the SARS-CoV-2 envelope gene, or E gene, and the RNA-dependent RNA polymerase gene, or RdRp gene, Pinsky said. For this protocol, Pinsky said the Stanford group in the USA has not yet encountered reagent supply issues. "However, as testing increases globally, we anticipate reagents may be more difficult to obtain," he added. The WHO assay was found to be very sensitive by the Greninger group
Hong Kong	HKU	ORF1b-nsp14, N	
Japan	National Institute of Infectious Diseases, Department of Virology III	Pancorona and multiple targets, Spike protein	

Thailand	National Institute of Health	N	
USA	US CDC Research Only (IDT-manufactured)	Three targets in N gene	IDT has also said that sufficient primer and probe kits have been manufactured and validated to enable over one million tests, and that the company expects to manufacture enough kits to enable 2.5 million tests this week, and five million tests each week starting March 16th.
USA	US CDC Multiplex Kit (LGC Biosearch)	Uncertain to authors at this time	LGC has said that it has manufactured 625 kits and has capacity to make 1,000 kits per week going forward, with each kit supporting 1,000 tests.

Note: the US CDC primers for amplification have been found to be defective by several groups. Novel sequences required for testing. At this time it is not clear to the authors of this document which CDC protocol suffers from primer issues, or if it is both.

ANNEX 1

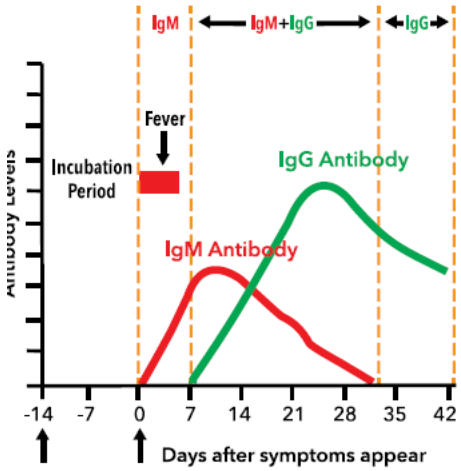


Figure 1 Antibody Detection Timeline (Biomedomics)

Protocol Name	**CRISPR DETECTR**	**CRISPR SHERLOCK**	**CDC RT-PCR (US standard)**
Limit of Detection	70-300 copies/ul	10-100 copies/ul	3.16-10 copies/ul
Assay Reaction Time	30 minutes	60 minutes	120 minutes
Heavy Equipment?	No	No	Yes

Figure 2 CRISPR Detection Summary (MammothBio)

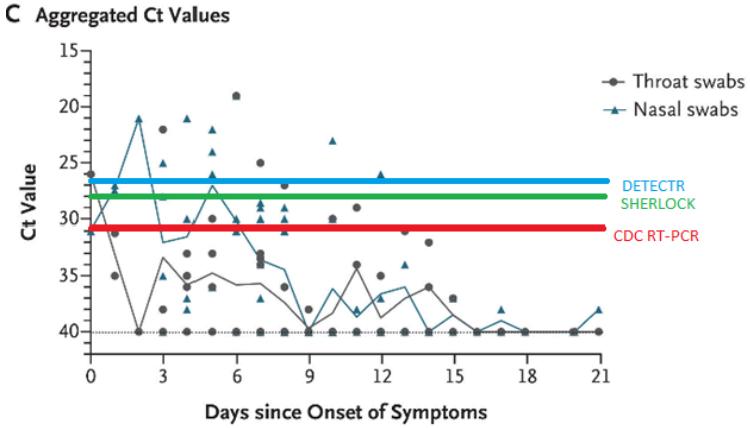


Figure 3 SARS-CoV-2 Viral Load in Upper Respiratory Specimens of Infected Patients (NEJM). Samples above the coloured bars are detectable as positive. Estimates are based on known levels of detection, as no direct comparative study of the technologies has been performed, and even gold standard protocols indicate likely a high error rate given variations in viral load over time.

Additional Resources

Biomedomics data: https://www.biomedomics.com/products/infectious-disease/covid-19-rt/

Info about Roche system being fast:
https://www.wsj.com/articles/fda-grants-new-coronavirus-test-emergency-approval-11584090078?mod=e2tw

MIT's Open Access SHERLOCK protocol development
https://mcgovern.mit.edu/2020/02/14/enabling-coronavirus-detection-using-crispr-cas13-an-open-access-sherlock-research-protocol/

https://www.biocentury.com/article/304556/covid-19-could-give-crispr-diagnostics-their-first-proof-of-principle
Sherlock should give results in <30 minutes

Chemrxiv "A Single and Two-Stage, Closed-Tube, Molecular Test for the 2019 Novel Coronavirus (COVID-19) at Home, Clinic, and Points of Entry"
https://chemrxiv.org/articles/A_Single_and_Two-Stage_Closed-Tube_Molecular_Test_for_the_2019_Novel_Coronavirus_COVID-19_at_Home_Clinic_and_Points_of_Entry/11860137?

Great article explaining both DETECR and SHERLOCK CRISPR systems
https://www.biocentury.com/article/304556/covid-19-could-give-crispr-diagnostics-their-first-proof-of-principle

Daily tracking of Covid19 Test Capacity in the United States: https://twitter.com/COVID2019tests

FDA Announcement - Thermo Fisher 1.5 million tests (claims of 5 million per week in April) and 400,000 from Roche (claim of 400,000 more per week)
https://www.wired.com/story/fda-approves-the-first-commercial-coronavirus-tests-in-the-us/

FDA Emergency authorizations for Covid19 testing
https://www.fda.gov/medical-devices/emergency-situations-medical-devices/emergency-use-authorizations#covid19ivd

FDA Authorization of Antibody tests without EUA (Section D)
https://www.fda.gov/media/135659/download
Community Crash Course on Molecular Diagnostics for COVID19
https://docs.google.com/document/d/1ra3L84yKwz3TU1xdRgDMQU3A0ZCGZmljeyqCI179KtQ/edit

Read more about PCR tests and their limitations and utility
https://www.theverge.com/2020/3/17/21184015/coronavirus-testing-pcr-diagnostic-point-of-care-cdc-techonology?fbclid=IwAR1qCsHPByHyLL9ZB5uGE7HC0BML2oZVz-nKl6VwnkxkitJWnNceVgILH_Q

WHO Coronavirus disease (COVID-19) technical guidance: Laboratory testing for 2019-nCoV in human
https://www.who.int/emergencies/diseases/novel-coronavirus-2019/technical-guidance/laboratory-guidance

A (more comprehensive?) list of tests
https://www.finddx.org/covid-19/pipeline/

ASM Expresses Concern about Coronavirus Test Reagent Shortages
https://www.asm.org/Articles/Policy/2020/March/ASM-Expresses-Concern-about-Test-Reagent-Shortages

Wired on Testing
https://www.wired.com/story/everything-you-need-to-know-about-coronavirus-testing/

Reagents as potential bottlenecks
https://www.linkedin.com/posts/jennifer-wilkins-153a26aa_hello-bay-area-biotech-community-today-activity-6645682866770587648-jHPb/

RNA extraction kits very limited. Previously there was just one supplier, Qiagen, and as of March 11th there were two, Qiagen and Roche.
https://www.the-scientist.com/news-opinion/rna-extraction-kits-for-covid-19-tests-are-in-short-supply-in-us-67250

Mail order tests, self administered nasal swabs, 30,000 initial availability, currently limited by number of (cotton?) swabs. The main manufacturer being located in Italy.
https://time.com/5805953/home-covid-19-test-everlywell/