For my daughters: August 22, 2020

The Coronavirus Pandemic

Finally, a COVID-19 Test

While the pandemic continues unabated, the news this week is much better than in weeks past. On the horizon are COVID-19 tests that are inexpensive, rapid, and point-of-contact. When they are rolled out, testing can become universal, and this pandemic hopefully brought under control.

How was this done, after so many months of testing difficulties? Well, the science behind this very important news is a bit hairy for civilians. As your father and also a science teacher, I am torn between wanting to quickly inform you what is going on, and wanting to explain to you how a test this important works. Your mother suggests I do both, and so I shall: first a short letter giving you the news, then, in the hope you are interested, a sort of appendix explaining the science.

COVID-19 Testing Has Been a Disaster

The single greatest problem we have encountered in combatting the coronavirus pandemic in the United States is the lack of a quick, cheap way to test for the COVID-19 virus. To see if a person is infected with COVID-19, today's tests basically look to see if there is any of the virus's genetic material to be found deep in a person's nose. A sample taken from the patient is sent to a lab, where it is put into a fancy machine that adds the COVID-19 "spike" gene to the sample. If the sample has any genetic material that matches the COVID-19 spike gene, the matching portion starts to multiply. We test for COVID-19 by looking for the burst of gene synthesis.

Sounds straightforward. So what has been the problem? Four issues arise that have made COVID-19 testing a major problem in controlling our coronavirus pandemic: 1. The machines are expensive and complicated; 2. The lab procedures involve expensive reagents and require a technical staff; 3. The analysis takes a considerable period of time, often as much as a week; 4. People avoid being tested because of the uncomfortable nature of the up-your-nose sample collection.

Saliva Test for COVID-19

New tests are being announced that address and solve all four problems. They eliminate the awkward procedure of collecting a sample by running a brush far up your nose, instead collecting a little saliva – you simply spit into a tube! There are far fewer COVID-19 viruses in saliva than in nasal passages (and so, much less of the virus RNA to detect), but these new saliva tests use fancy molecular chemistry that makes them hyper-sensitive, able to generate super-large bursts of gene synthesis from even the tinyest amount of virus. The new tests are fast, the reagents used are cheap, and so is the equipment.

The FDA has so far issued emergency use authorization for five so-called "spit tests." The first, approved on May 8, was developed at Rutgers University and is available commercially now. You send your spit sample to their lab, results guaranteed in two days.

The other four FDA emergency-use approved saliva tests won't be far behind in reaching the commercial market. Many other saliva tests are in the pipeline awaiting approval as well, including ones developed at Columbia University, University of Illinois, University of Chicago, Cornell Medical School, and even one developed here at Washington University.

The Yale University saliva COVID-19 test approved by the FDA for emergency use on August 16 is especially nice. By using very stable reagents unaffected by the enzymes and other chemicals in spit, the Yale test is the first spit test that eliminates the need to extract the virus RNA from the saliva sample at the beginning of the test. This makes the Yale test so simple it can be carried out on-site – you don't need a lab, just an inexpensive machine that can maintain hot coffee temperatures and a cartridge of the reagents. Getting a result takes less than 3 hours.

Yale press releases say its SalivaDirect test will cost only about $10 per spit, and should be available to the public in a matter of weeks. Even more fun is the fact that the Yale test was developed with funding from the National Basketball Association, who want to be able to quickly test all players right before a game.

A Less Lonely Future

This saliva test promises to be the answer to quick and affordable COVID-19 testing for everyone, eliminating the need for the sort of laboratory analysis that currently takes weeks for many of us. The ability to test and quickly get negative results would let a granddad like me visit my wee granddaughter Jed safely (that's her below, waving at me) and allow my children to visit their mother and me.

So. That's the news for this week.

Now, in case you are interested, I am adding a "science" appendix to this week's letter that explains how the new saliva COVID-19 test works. I want to explain the new test to you so you know a bit about how something this important works, and also because the development of this saliva test is a beautiful example of how science works in the real world, each researcher building on earlier discoveries.

I am going to have to do a little more chemistry than I usually do in my letters, but bear with me – it's worth putting up with a few diagrams to understand this really beautiful science.

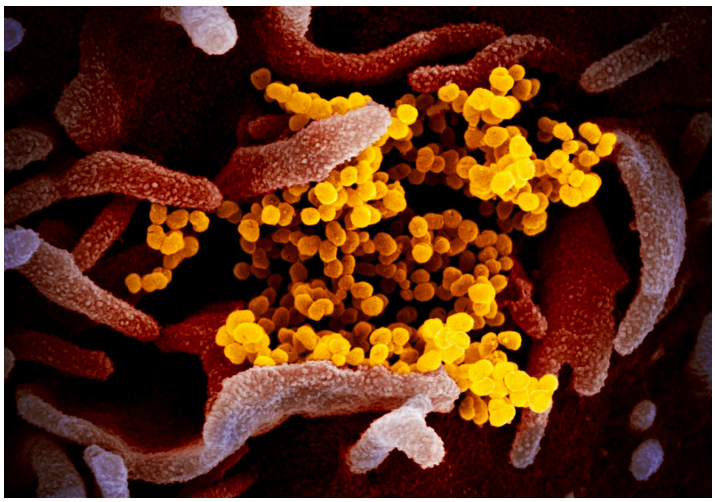
APPENDIX

The Saliva Loop-mediated Isothermal Amplification COVID-19 Test

That mouthful is the name of the new test. To explain how it works, I am going to start at the end of the name and work backwards.

COVID-19 Test

The key to success of any COVID-19 test will be its ability to detect very small amounts of the COVID-19 virus. What you need is a way to detect its genes (like many but not all viruses, COVID-19 genes are made of RNA rather than the DNA of your cells – it makes a difference, because although the chemical difference is slight, DNA is double-stranded but RNA is single-stranded). The widely-used "antigen" tests do NOT detect the virus or its genes. Rather, they tell you if a patient has antibodies in his or her blood to the virus, which if present indicate the virus has infected you in the past. As the immune response that produces these antibodies can take weeks to do so, a positive antigen test tells you nothing about whether or not the virus is present now. Anyone who has ever been infected but now recovered will give a positive. A useful COVID-19 test must test for the presence of the virus itself (the yellow spheres in the photo).



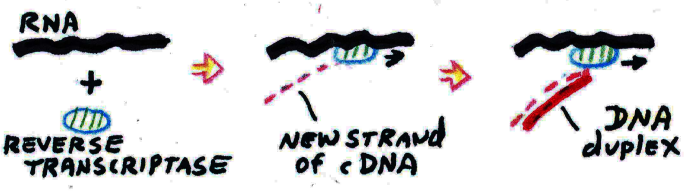
The first step in testing for the presence of the virus in a sample is to extract the sample's RNA. You want the RNA you are going to analyze to be free from enzymes and impurities that might inhibit the test you wish to perform. This is a relatively simple chemical procedure, but needs to be done in a lab.

Amplification

A sample obtained from a virus-infected patient with a nasal swab or saliva will contain only a small amount of COVID-19 virus, not enough to analyze. Thus the first thing an investigator needs to do is to increase this tiny amount of RNA. You do this the same way the virus does when it multiplies within a cell, by making copies of the virus genome.

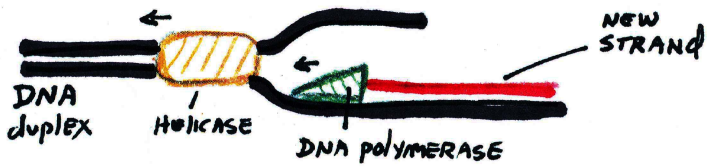
1. **Reverse Transcription**. Because COVID-19 is a virus with genes of single-stranded RNA, and all of the analytic procedures you will want to use employ double-stranded DNA, you typically first want to make a DNA version of the virus. To do this, you employ an enzyme called *reverse transcriptase*. This enzyme reads along the RNA

strand and matches each RNA letter of the virus (called a "base") with the DNA equivalent, assembling a cDNA (complementary DNA) strand as it moves down the RNA. As it does so, it adds the matching base to the cDNA, creating a DNA duplex with the same sequence as the RNA it was reading:



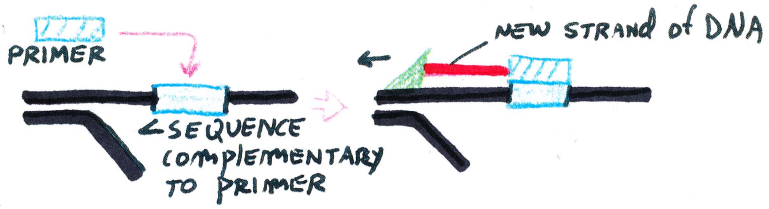
REVERSE TRANSCRIPTASE

2. **DNA Replication**. It is now necessary to make copies of this DNA (to "amplify" the sample). How do we do it? In a living cell, the DNA duplex is first unwound by an enzyme called a *helicase*. In a test tube, the investigator mimics this same reaction. Then a second enzyme called *DNA polymerase* (the green triangle in the diagram below) starts at one end of a strand and moves down it, adding on DNA letters to the growing new strand (red in the diagram) one base at a time. How does it know which letters to add? It looks at the strand it is moving down to see which ones to place next, always A pairing with T, and G with C:



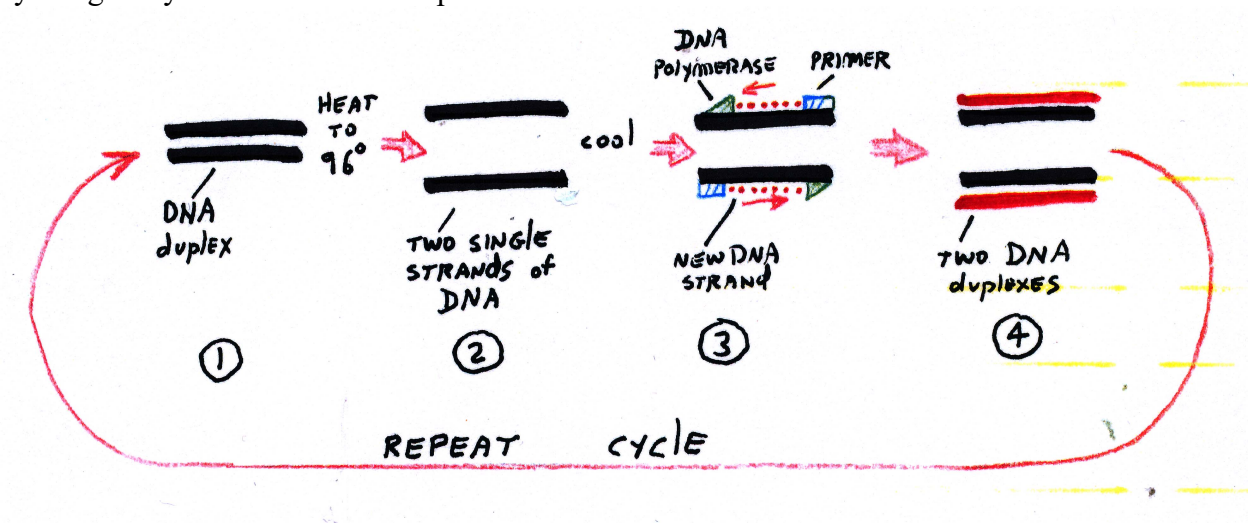
DNA REPLICATION

3. **Priming**. BUT the chemistry of life introduces a complication here. It turns out that, because of the chemistry of the reaction, the enzyme DNA polymerase can only add DNA bases to the end of an existing strand. The polymerase needs a place to start, an existing strand to which to add bases. Living cells (and viruses) solve the problem by providing just that. They manufacture a short nucleotide fragment called a "primer" (the blue segment in the diagram below) that is exactly complementary to a specific base sequence located at the start of the DNA to be replicated. The primer, because it matches that sequence, will sit down on the DNA there and only there. The DNA polymerase enzyme now has a place to start. It can proceed to add nucleotides to the end of this primer, moving down and replicating the DNA strand. The new strand it is assembling (red in the diagram) gets ever longer:



ROLE OF PRIMER

The first practical procedure ever developed for amplifying DNA is called the *Polymerase Chain Reaction,* or PCR. In PCR, the need for a helicase enzyme to dissociate the strands of a double-stranded DNA fragment is avoided by simply heating the DNA solution to 96 degrees C. The heat causes the two strands of the DNA duplex molecule – held together only by weak hydrogen bonds – to dissociate into single strands. The solution is then cooled, allowing the enzyme DNA polymerase (using primers to get started) to replicate each strand, so producing two double-stranded fragments. The fragments are heated again to separate the two duplexes into four strands. The mix is then cooled, and the four strands copied by the polymerase again to produce four double-stranded fragments. Then we do the heating and cooling again, to get 8 duplex molecules. Then again… In a typical PCR procedure the heat/cool cycle is repeated many times, each time doubling the number of copies. Think about it. It's a chain reaction -– doing 20 cycles gives you over a million copies!



Isothermal

The problem with using PCR in a clinical test, obvious to anyone tracking our pandemic, is that it requires an expensive machine to do all the temperature cycling. The cheapest with the ability to carry out dozens of tests simultaneously cost $25,000, and many of the better ones cost much more. Wouldn't it be nice if we didn't need all that temperature cycling? What would be ideal is a test that is *isothermal* – that is, carried out at a single temperature.

The road to such a test starts in 1920, when scientists discovered an unusual bacteria growing in hot thermal pools. Called thermophiles, these heat-loving microbes thrive at temperatures of 60-65 degrees C (the temperature of a hot Starbucks coffee).

Researchers studying a thermophile called *Bacillus stearothermophilus* found it had a most unusual DNA polymerase, one that was able by itself to open up the DNA duplex to replicate its DNA. You see the point? No need to heat the duplex to separate the DNA strands!

Two decades ago, a team of Japanese researchers at Osaka University Medical School used this unique DNA polymerase to create a brand new kind of isothermal DNA amplification procedure. Their goal was to avoid the need for expensive PCR machinery. They developed a way to use the *B. stearothermophilus* polymerase to dramatically increase the amount of DNA being made without cycles of heating it. Quick, powerfully, and at very little cost, the procedure they came up with amplifies DNA in a single tube at a uniform temperature of 63 degrees C (the temperature loved by *B. stearothermophilus*). How did they do it? With loops.

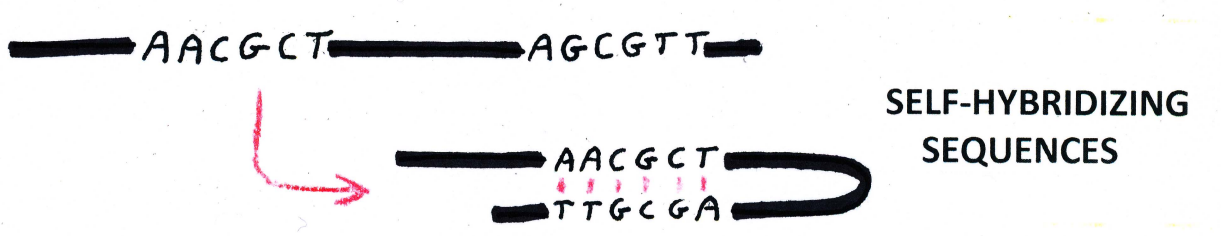
Loop-mediated

Before we get into loops, we need to ask in a more general way how we are going to test for COVID-19. Then we will use loops to do it better. How do we test? By being stingy with the primers we provided the polymerase. Remember, there can be no DNA synthesis without primers to provide a place for synthesis to start. Controlling what primers are provided lets the researcher allow the polymerase to amplify the DNA when, and only when, a specific sequence is present (say, a COVID-19 spike protein gene). The burst of polymerase activity that would occur if such a virus gene were present provides a very powerful signal that COVID-19 is present in the sample being analyzed. We test for COVID-19 by looking for bursts of DNA synthesis.

So, what is this loop-mediated procedure the Japanese scientists developed to do it better? The researchers identify portions of the COVID-19 DNA where a single strand of DNA could bend back on itself to form a loop; they then use a segment of the DNA within the loop to provide a new place for DNA synthesis to start.

How do loops help? Said simply, making lots of loops provides the polymerases lots of extra places to start DNA synthesis.

Loops? Isn't DNA double-stranded? Not all the time. When the *B. stearothermophilus* polymerase replicates a DNA duplex, it displaces the strand it is not copying. That dangling strand is single-stranded and so able to form a loop. Why would it do so? Because one portion of the strand, say for example, AACGCT, has nearby a similar-sized segment with the complementary sequence reading backwards. In this instance, that would be AGCGTT (remember, A pairs with T and G with C). Twisting back on itself, the strand forms a hairpin loop as one of the opposite segments base-pairs with the other, just as they would in a DNA duplex!

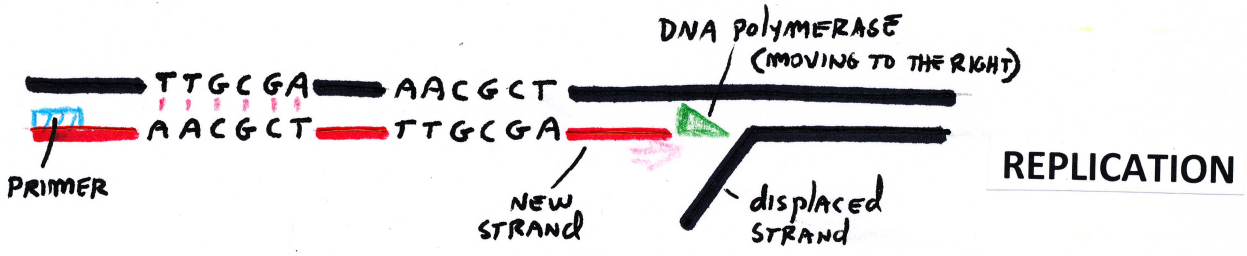


The key to identifying loops which the investigators can use as replication origins is that we know the entire sequence of the COVID-19 virus. Because we know the complete sequence, we can search it for ANY six-base sequence with a nearby complementary sequence. With the help of computers, it is not difficult to search through the COVID-19 genome and identify one.

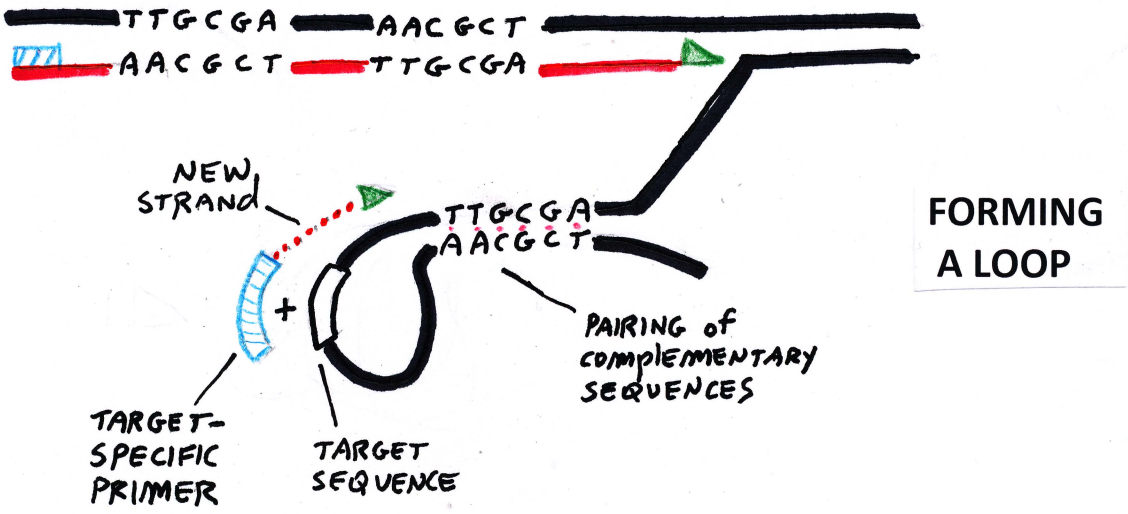
Now – and we're almost home – look at the DNA between the two complementary sequences and select some short sequence of bases located there. I will call this the target sequence.

The Japanese research team now did a very simple thing: they prepared a "primer" (using chemistry, they manufactured a specific DNA fragment) that is complementary to the target sequence.

Now they are ready to amplify: They add the *B. stearothermophilus* polymerase and primers for fragment ends as used in PCR to a solution containing the virus DNA and the four bases – and also add a primer for the target sequence. What happens? The polymerase chugs through the long fragment, starting at one end to replicate it and then replicate the copies, as in PCR:



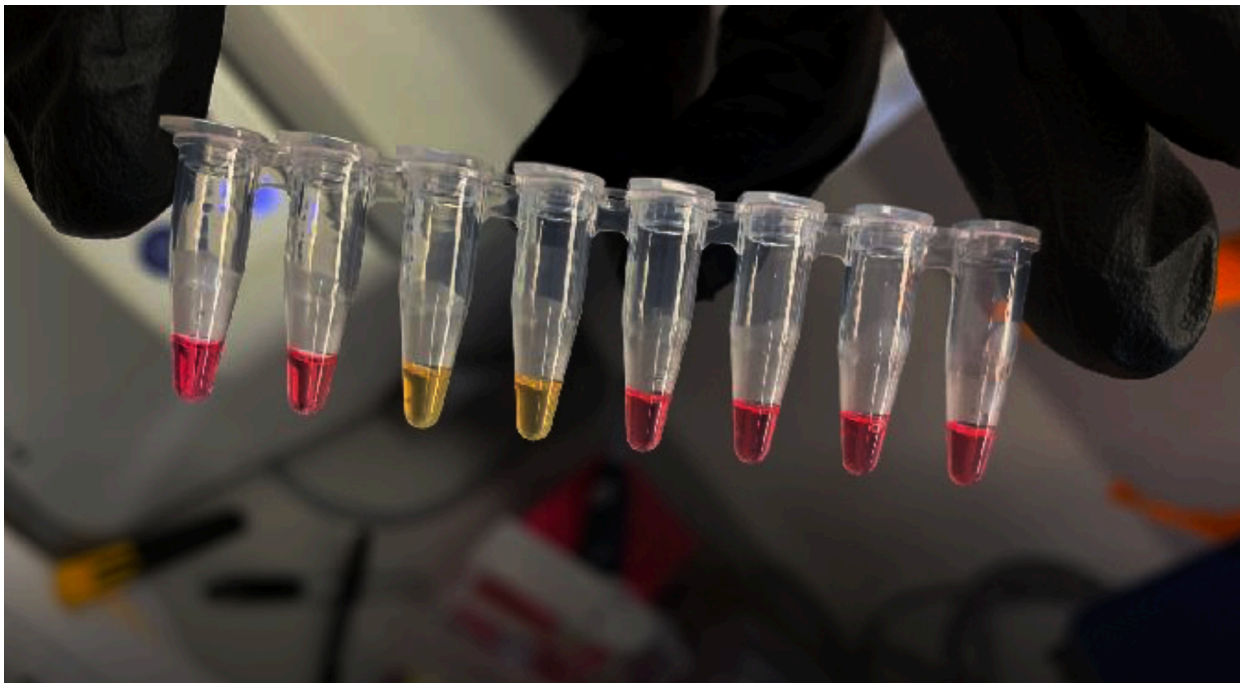
As it does so, the polymerase displaces the strand it is not reading, which forms a loop due to its internal complementary sequences. The polymerase then starts also replicating from the primer that is now able to bind to the target sequence sticking out single-stranded from the loop! A whole new replica of the strand begins to be created there, in addition to the one started at the end of the fragment. You are now doing twice as much DNA synthesis.



In practice, two or more target sequences are used, each embedded within complementary sequences so that they will form loops. As you can imagine, all this replication creates a swarm of interconnected DNA loops and fragments, many connected to each other and all furiously replicating. I have skimmed over a lot of details about the primers, not telling you about a bunch

of them and how they all interact. What you need to know is that there is a whole lot of DNA replication going on.

How do you know when the test result is positive, without complex analysis of the results? Well, if the sample being tested does not contain COVID-19 RNA there will be no sites matching the primers, and so no DNA synthesis can occur in the reaction tube. If the tube does contain COVID-19 RNA, there will be a lot of DNA synthesis. How do you know which has happened? The simplest way, which doesn't involve any machines, turns out to be adding a pH-sensitive dye to the reaction tube. DNA is deoxyribonucleic acid, after all. As more and more of this acid is produced, the pH of the solution falls, turning the red dye yellow. It's that simple.



This loop-mediated DNA amplification (called loop-amp or LAMP for short) is easy to run, using cheap reagents and inexpensive equipment, and LAMP tests are just as reliable and sensitive as those from the far more expensive and complex-to-perform PCR tests. Researchers claim the ability to detect a single copy of COVID-19 virus in one microliter of sample!

Spit

As I described in this letter, the new test is cheap and works very well on samples of saliva, eliminating both the awkward sample collection and the need for the sort of laboratory analysis that currently takes a week or more for many of us. Positive cases could quarantine far sooner, slowing the spread of the virus within our communities. People with no symptoms could be routinely tested, identifying asymptomatic individuals within hours of the test so they can be quickly isolated from others (the CDC says some 30% of all COVID-19 infected individuals are asymptomatic).

All in all, this is just the COVID-19 test we have for so long been seeking.

So. Now you know. Stay Safe. Dad