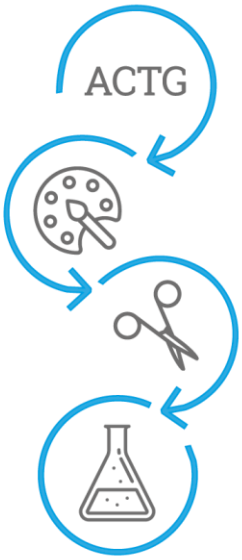

DNAdots™

Simple explanations
of modern genetic technologies

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Droplet Digital PCR

What it is:

Counting the needles in your haystack

When PCR (Polymerase Chain Reaction) was invented in the 1980s, it revolutionized biology. With tiny amounts of DNA, scientists could make billions of copies of a particular sequence that they were interested in, and with those billions of copies do all sorts of different experiments that were never before possible. In essence, scientists were given a metal detector that could not just find a needle in the haystack of DNA, but also make more of the rare needle it found. But scientists don't always just want to find a needle; sometimes they want to count the needles they find.

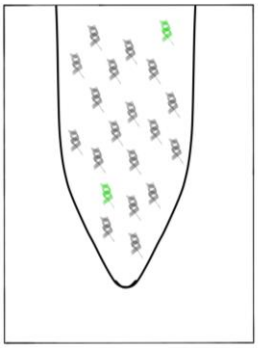
Imagine you are looking for needles in a haystack and you have a metal detector for a tool. Waving the detector over the haystack will tell you that there may be a needle there, but it can't tell you if there is one needle or 500. If you want to count needles with a metal detector, you need to spread the pile out enough that needles are unlikely to be near each other. Digital PCR works like this, but instead of using a metal detector that beeps, we use a molecular reaction that lights up. To count copies of DNA, instead of doing one single reaction, the PCR is broken up into many, many smaller reactions. When broken up enough, the small starting amount of DNA, your needles, will be spread so thin that some reactions will have the DNA you are interested in; others will not. Many of these small reactions will not be able to make copies of the DNA simply because there won't be any template molecule in the reaction to make copies of, but some reactions, those that have the template molecule, will make copies. To approximate the number of molecules you started with, you can simply count the number of reactions that were able to make copies. The term digital refers to something that can be counted in ones and zeros - reactions that worked and reactions that didn't.

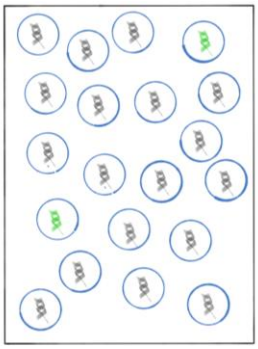
How it works:

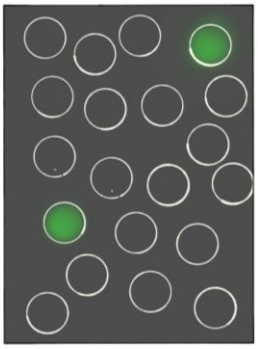
Breaking up a PCR into thousands of pieces

Digital PCR refers to any format where a single PCR is broken up to into many smaller reactions that can each be tracked individually. The power of digital PCR comes from the number of smaller reactions you can break your original PCR into; the more reactions that can be done simultaneously, the more precision digital PCR has. Droplet Digital PCR (ddPCR) takes this precision to a new level. ddPCR is done by suspending tens of thousands or even millions of tiny droplets of water in an oil emulsion, kind of like an extremely well shaken salad dressing. Each little droplet of water performs its own separate little PCR, with buffer, primer, dNTPs, polymerase, and fluorescent dye or probe distributed evenly among all the droplets. The fluorescent dye or probe makes the droplet light up only when there are a lot of copies of the target sequence in the droplet. The starting template DNA, however, will be spread thin enough that there won't be enough target DNA sequence to go around; only some of the droplets will have the right template DNA. If the target sequence is there, the PCR will work, and the reaction will make copies of the sequence you are interested in. If the target sequence is not there, the PCR will not work and no copies will be made. As more and more copies are made in the reactions that work, the dye or probe in those droplets will begin to fluoresce. To know how many molecules of interest you started with, simply count the droplets that glow.

To set up a reaction, a PCR is set up in a tube, like most any normal PCR. A machine is then used to break that larger reaction (still very small - usually about 20-50 microliters) into tens of thousands, or even millions, of smaller reactions depending on the exact technology used. Each droplet of water is its own reaction isolated by a sea of oil. The oil emulsion is then cycled through different temperatures like a normal PCR. At the end, a detector evaluates each droplet. If it fluoresces, the PCR worked, and the target sequence must have been present. If it does not fluoresce, the PCR failed and the target sequence must have been absent. The number of PCR reactions that worked is equal to the number of original reactions that contained a target sequence. Of course, even when the concentration of target DNA is fairly low, some reactions will have more than one starting molecule by chance, since there are so very many reactions. The number of molecules in the different reactions is expected to follow a Poisson distribution, so a simple statistical correction can be applied to arrive at a very precise count of starting template molecules.







Applications:

Who wants to count DNA?

Perhaps the most classic application that ddPCR has been applied to is measuring gene expression. By quantifying the *RNA* in a tissue, we can quantify how much a particular gene is being used under different circumstances. The more common way to do this is a related procedure known as quantitative PCR, or qPCR (another DNAdot). But where qPCR requires a greater than 2-fold differences in starting template to reliably quantify such differences, droplet digital PCR can detect changes of 10% or less in starting concentration. Scientists can now explore changes in gene expression that may be too subtle to observe with other methods.

Where ddPCR has really excelled, however, is in detecting and quantifying rare DNA sequences. Where, in other procedures, very rare signals can be obscured due to background effects, because ddPCR runs many separate small reactions, in each individual reaction the amount of background is very low. This means that any time you are trying to identify and quantify the amount of a rare sequence or mutation, ddPCR is an excellent choice. This has proved to be very useful in many different applications, a major one being cancer research. Identifying and quantifying early and rare cancer mutations can be very difficult because early and rare cancer cells are, by definition, rare cells surrounded by much more common healthy cells. ddPCR is able to identify and quantify the sequences from these rare cells at a rate that is many times more sensitive than other approaches, allowing for earlier and more precise molecular diagnoses. Similarly, when someone is infected with a virus, ddPCR can quantify quite accurately not only whether a virus is present, but also the viral load, or how many copies of the virus are present, even when the virus is present in numbers too low to detect by other methods. And when testing for environmental pathogens, often samples from water or soil may contain chemicals that work as inhibitors that interfere with the sensitivity of other approaches like qPCR. But because ddPCR only counts if the reaction worked or not, inhibitors that may affect the *rate* of the reaction won't affect ddPCR. This means that high quality quantification can be obtained even in difficult to process samples. In the future, you can expect scientists continue to apply ddPCR to more and more applications, searching more and more haystacks for rarer and rarer needles - and all because we learned to count in ones and zeros.

Learn more:

- Jankovic, Jalena. "Divide and Conquer: How to set up your first Ditial PCR" *BitesizeBio*
- Liao and Huang. "Digital PCR: Endless Frontier of Divide and Conquer" *Micromachines* 2017

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Questions

Review:

1. Why is digital PCR called "digital"?
2. Explain why the oil is used in ddPCR.
3. Based on how digital PCR works, compare the relative amounts of template DNA compared to primers and other reagents that must be present in the original reaction?
4. If 10,000 of the 20,000 droplets have a successful PCR, why do you think there are likely more than 10,000 actual starting template molecules? Why are Poisson statistics needed to get the actual number?
5. Why may ddPCR be better at measuring pathogen loads than qPCR?

Critical thinking:

1. Digital PCR equipment is still relatively new and fairly expensive, while qPCR equipment is much more widely available in labs today. Under what conditions may a researcher elect that doing a ddPCR is not necessary and regular qPCR is sufficient.
2. First generation digital PCR procedures required breaking a sample up into hundreds of different reactions that were then each performed in their own tube in PCR machine. Compare and contrast this approach to digital PCR with droplet digital PCR.

Discussion:

1. As technologies such as ddPCR become more and more advanced, they require more and more expensive, specialized equipment. Only the best funded labs can afford such technology today. How do you think this affects scientific progress? Is there any responsibility of the government or other organizations to control the prices of such equipment or to see that such expensive equipment is more widely available to different researchers?

Answer key:

Available to teachers upon request: dnadots@minipcr.com