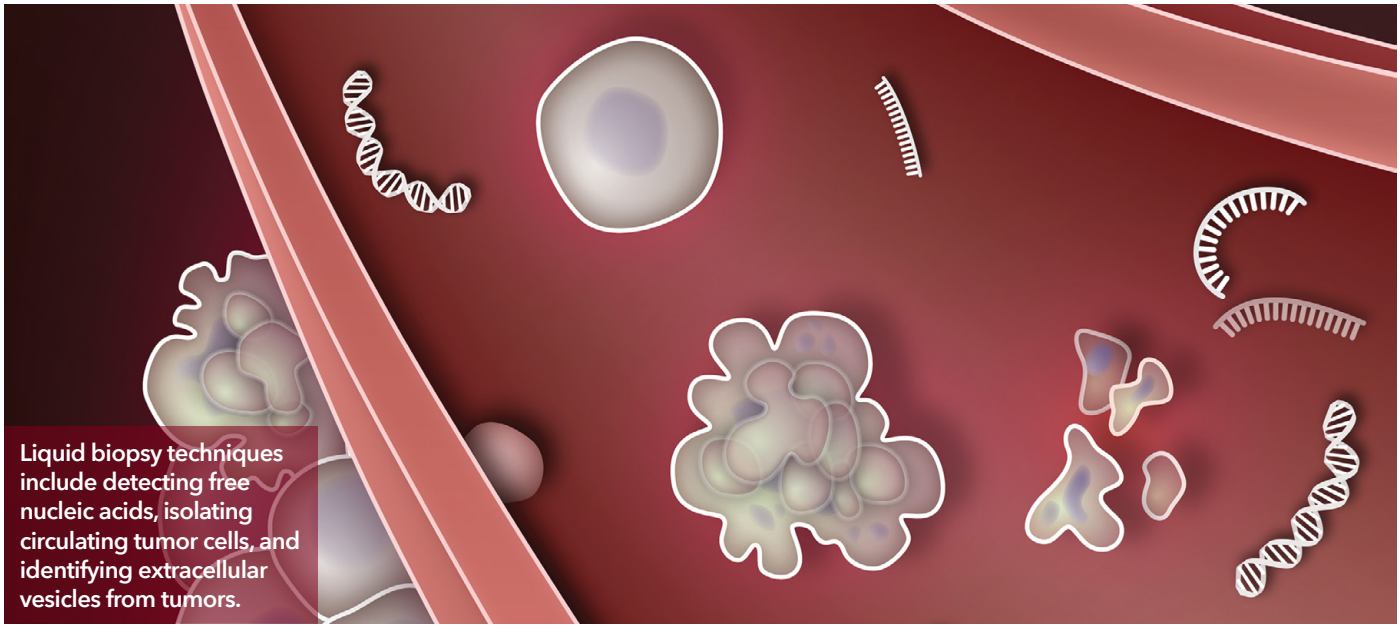


Liquid biopsy techniques include detecting free nucleic acids, isolating circulating tumor cells, and identifying extracellular vesicles from tumors.

Pumping up liquid biopsies

Researchers have long dreamed of developing simple blood tests to monitor and diagnose solid tumors, but the scarcity of circulating cancer biomarkers has held the field back. Highly sensitive assays and innovative strategies are finally changing that, and bringing a new era of liquid biopsies closer than ever. **By Alan Dove**

Beginning to circulate

Tumor biopsies are among the most cumbersome, risky, and expensive tests in all of medicine, so it's no surprise that researchers have been trying for decades to improve them. While more sensitive assays have enabled doctors to take smaller samples of some types of solid tumors, oncologists have long dreamed of avoiding traditional biopsies altogether. The focus of those hopes is the liquid biopsy: a test that could detect and analyze a solid tumor from biomarkers in the bloodstream or other easily sampled body fluids, such as saliva.

Tumors often shed cells and subcellular components into the bloodstream, but these are exceedingly hard to detect. Even in advanced diseases, the background level of healthy cells and free biomolecules remains several orders of magnitude higher than the signals from most tumors. Using novel purification and analytical techniques, though, investigators are now pushing several types of liquid biopsies toward the clinic.

The field segregates into three major strategies: detecting free nucleic acids, isolating intact circulating tumor cells, and identifying extracellular vesicles from tumors. Each comes with its own advantages and drawbacks. With a few liquid biopsies already approved for clinical use and many more in clinical trials, experts in the field agree that the approach is on the cusp of a major breakthrough.

Tiny reactions, big impact

Of the three methods, nucleic acid–based liquid biopsies appear to be taking an early lead. "I think that ultimately [cell-free DNA] will become a little bit more widely used, mostly because it's very easily applied in the clinic," says Cloud Paweletz, head of the Translational Research Laboratory at the Belfer Center for Applied Cancer Science in the **Dana-Farber Cancer Institute** in Boston, Massachusetts. He explains that DNA-based liquid biopsies typically use standard blood collection tubes, and those based on polymerase chain reaction (PCR) are particularly easy for clinical laboratorians to understand and implement.

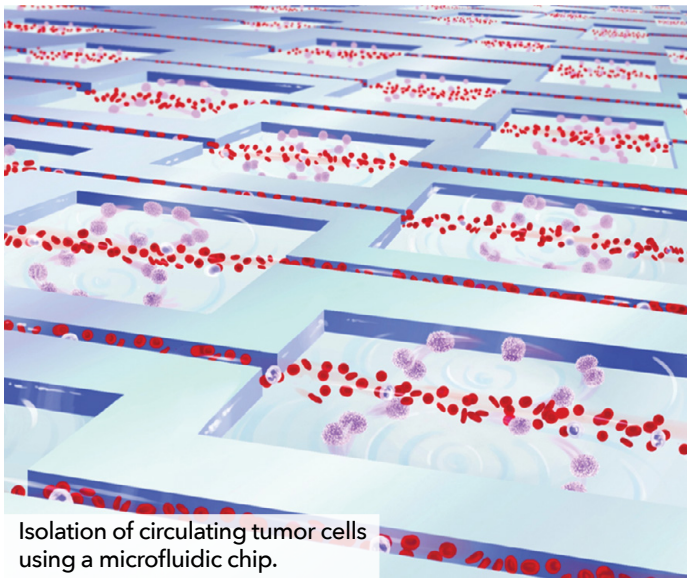
Paweletz and his colleagues are now developing a PCR-based test to detect drug resistance mutations in lung tumors. The test can be performed from a standard blood sample in just two days, and identifies any of three mutations covering 40 percent of all lung cancer patients. The team is now building on actual clinical experience with approved liquid biopsies to identify the assays oncologists will find most useful.

Geoff Oxnard, an assistant professor of medicine at **Dana-Farber Cancer Institute** and **Harvard Medical School** in Boston and one of Paweletz's collaborators, remarks that "I had a hospitalized patient last week, she's sick with metastatic lung cancer, and she's exactly the kind of patient who might have an [epithelial growth factor receptor (EGFR)] mutation, but I simply didn't have enough tissue to ask those questions yet." He says that using a liquid biopsy test that was developed and now offered at Dana-Farber Cancer Institute, he was able to determine that the patient did indeed have the mutation, making her a good candidate for drugs that target it.

"The clinical need drives the technology, and the technology empowers the clinic," says Paweletz. In addition to their PCR-based tests, his group is also designing liquid biopsies that use next-generation sequencing (NGS) to identify a broader range of tumor types and mutations. cont.>

Upcoming features

DNA/RNA: Single-Cell DNA Sequencing—November 3 ■ Proteomics: Biomarkers—December 8 ■ Biotechnology: Biosensors and Microfluidics—March 16



Isolation of circulating tumor cells using a microfluidic chip.

"Circulating tumor cells have the advantage that they are ... intact living cells." – Michael Kazinski, Qiagen

For PCR-based tests, Paweletz, Oxnard, and many other liquid-biopsy developers have found conventional quantitative PCR (qPCR) techniques problematic. Even after initial purification of the sample, liquid biopsies typically contain much more nucleic acid from healthy cells than from tumors. That early bias in the bulk qPCR sample only gets amplified in subsequent cycles, drowning out the tumor's signal. "The problem with plasma cell-free liquid biopsy in particular is that the amount of wild-type [DNA] can be very, very high, and so keeping that really rare signal from being swamped out by the wild-type ... is key," says Dawne Shelton, R&D manager for in vitro diagnostic products at **Bio-Rad** in Hercules, California.

To circumvent that issue, investigators have turned to a family of techniques called digital PCR, which separates free DNA in a sample into individual reactions that each contain a single template. In Bio-Rad's droplet digital PCR system, for example, a sprayer partitions a sample into thousands of nanoliter-size droplets before the amplification step. The PCR reactions occur inside the individual droplets, which the machine then sorts with fluorescent markers to detect target sequences.

Compared to NGS, digital PCR is relatively easy and cheap, making it a popular choice for researchers who are just starting to work on liquid biopsies. Nonetheless, Shelton cautions that the technique requires careful thought about issues biologists may not be accustomed to considering. "Most of what we are all doing with liquid biopsies is dealing with very small numbers [of molecules], so using a poor sample extraction or nonoptimized kit can really get rid of a lot of the signal," she says. She adds that the small numbers mean researchers must also use robust statistical techniques to distinguish meaningful tumor signals from random mutations and errors.

Sweating the small stuff

Besides planning their data analysis, researchers working on liquid biopsies need to think carefully about their controls. "It's difficult to make contrived samples behave like actual cell-free nucleic acids, so that's been quite a challenge," says Kelli Bramlett, director of R&D in clinical sequencing and oncology at **Thermo Fisher Scientific** in Waltham, Massachusetts.

Bramlett's team works on liquid biopsy technologies for both DNA and RNA in human blood. While the latter has a reputation for being hard to isolate, she explains that even degraded RNA generally contains enough intact sequence to analyze—provided investigators can detect the scarce tumor signals against the immense background of other RNA molecules in a sample.

The exceptionally low signal-to-noise ratio raises other problems for scientists and reagent providers. "Now that you're looking for these needles in a haystack, you start finding a lot of other needles that you didn't want to find, like variability in manufacturing or slight lot-to-lot differences in the controls that you purchased," says Bramlett.

Extensive quality testing and a diverse market have made good commercial reagents and control specimens expensive. Bramlett explains that manufacturers initially focused on providing large panels of control samples to cover as many types of liquid biopsy experiments as possible. Researchers targeting a small subset of tumor markers would end up paying for extra controls they didn't need. Recently, though, Thermo has begun developing more focused panels, and hopes that these will allow researchers to perform cost-effective liquid biopsies.

Before the experiments even begin, though, labs need to develop good protocols for sample collection and processing. "A lot of times people underestimate the importance of the preanalytical stuff," says Bramlett, advising that researchers should "think through all of that before you jump in, and make sure you have a good sample going into whatever technology you're going to use to evaluate your liquid biopsy."

Given the challenges of dealing with freely circulating DNA and RNA fragments, it's no surprise that many scientists are looking for other things to analyze in liquid biopsies. The other two major targets in the field, circulating tumor cells and exosomes, come with their own challenges.

"Circulating tumor cells have the advantage that they are ... intact living cells," says Michael Kazinski, senior director and head of global product management for sample technologies at **Qiagen** in Hilden, Germany. Isolating both the genomic DNA and RNA from such cells allows researchers to develop complete transcriptional profiles from the liquid biopsy. That, in turn, should provide deep insights into the tumor's biology, at least in theory.

However, researchers have long thought that tumors don't release intact cells into the bloodstream until relatively late in the disease, and then only in vanishingly small numbers. A successful test would therefore require isolating enough of these scarce cells to make statistically valid inferences about the tumor, often at a stage when the tumor itself is growing and changing rapidly. Newer work suggests that these circulating tumor cells may show up much earlier, but finding them then requires extremely sensitive assays.

The third and newest approach to liquid biopsies, exosome screening, relies on circulating subcellular vesicles. "There are these microvesicles called 'exosomes' that are actually released

Featured participants

Bio-Rad
www.bio-rad.com

Dana-Farber Cancer Institute
www.dana-farber.org

Harvard Medical School
hms.harvard.edu

Menarini Silicon Biosystems
www.siliconbiosystems.com

Purdue University
www.purdue.edu

Qiagen
www.qiagen.com

Thermo Fisher Scientific
www.thermofisher.com

Vortex Biosciences
vortexbiosciences.com

from every living cell in the body. They have natural functions, but also act as a preserving container for RNA," says Kazinski. Besides messenger RNA, microvesicles also contain microRNA and proteins, providing information about both the transcriptional and regulatory environments of their parent cells.

Regardless of the approach researchers are taking, Kazinski reiterates the importance of careful sample handling: "What is massively underestimated is the impact of the trivial question: How do you take a blood draw, and how do you store it and how do you ship it?" To address that, Qiagen has introduced a line of specially treated blood collection tubes that chemically stabilize samples for sensitive liquid biopsies.

Going with the flow

For investigators studying circulating tumor cells, the next step after sampling is to isolate the desired cells as quickly and gently as possible. Many tumors release cells with distinctive antigens on their surfaces, and affinity-based techniques can pull these cells out of the blood sample relatively easily. Indeed, that's the basis of the first FDA-approved liquid biopsy test, CellSearch**,** developed by former Johnson & Johnson subsidiary Veridex (the test is now owned by **Menarini Silicon Biosystems** in San Diego, California).

Unfortunately, CellSearch also became a cautionary tale for liquid biopsy developers. The FDA approved the test for determining prognoses in breast, prostate, and colon cancers in 2008. When subsequent studies found that CellSearch results had no influence on clinical outcomes, though, insurers lost interest in paying for it.

While many researchers are still developing affinity-based techniques for isolating circulating tumor cells, others are exploiting the physical differences between normal and tumor cells. "[Picture] when you walk near a river and there are some ... whirlpools in the river that will trap leaves and branches; [we use] exactly the same flow phenomenon, but at a much smaller scale," says Elodie Sollier-Christen, vice president of R&D at **Vortex Biosciences** in Menlo Park, California.

Vortex's system uses a microfluidic chip to generate tiny vortices that trap larger, more deformable cancer cells from a blood plasma sample. The chip itself is disposable, and the system is completely automated. Users simply collect a blood sample from a patient, feed it into the device, and retrieve any purified tumor cells a few hours later. The cells "have not been bound or squeezed, so they're really kept intact during the flow, and then [researchers] can just bring the [circulating tumor cells] to whatever platform they want to perform whatever assay they want," says Sollier-Christen. While the Vortex system is designed specifically for blood, the company is working with academic researchers to develop versions that could be used to isolate cells from other body fluids as well, such as cerebrospinal fluid and urine.

Besides keeping the cells intact, the flow-based system also allows investigators to isolate them for single-cell sequencing or other analyses. That type of fine-grained control is also revealing that at least some tumors may begin releasing cells sooner than scientists had realized. "There was this initial thought that [circulating tumor cells] are only present at late stage," says Sollier-Christen, but she notes that in the past year, several studies using more sensitive techniques have found such cells much earlier in tumor development, even before the tumor becomes visible by conventional imaging techniques.

Spinning into the future

The newest trend in liquid biopsy research, looking for circulating extracellular vesicles, grew partly from researchers' frustrations with protein biomarkers. "We believe there are at least 10,000 proteins in our blood," says W. Andy Tao, professor of biochemistry at **Purdue University** in West Lafayette, Indiana. Tumors undoubtedly contribute distinctive proteins to this vast pool. Unfortunately, the high background of normal proteins, along with the presence of circulating phosphatases and other degradation enzymes, consistently stump researchers searching for these rare tumor biomarkers.

To address that, Tao and his colleagues focused on the subcellular vesicles released by the tumors. Because a majority of tumors exhibit phosphorylation changes at some point in their development, his lab looked specifically at protein phosphorylation in vesicles.

Many investigators use immunological markers to isolate extracellular vesicles from blood; Tao prefers the tedious but unbiased approach of differential centrifugation. His protocol uses progressively higher centrifuge speeds to remove blood cells and debris, and then isolate two different sizes of vesicles.

The next challenge is to identify the proteins in the isolated vesicles, and determine their phosphorylation states. Antibody-based tests can also work for this purpose, but only after researchers know what proteins and modifications they're looking for. Scientists doing more open-ended searches often use mass spectrometry, generating lists of the extracellular vesicle–associated proteins in samples from diseased and healthy patients or animals.

Although these surveys have recently revealed several promising avenues for new liquid biopsy development, Tao warns that translating the results to clinical use will require some major changes. "In the discovery stage, it's perfectly fine using differential centrifugation followed by mass spectrometry, but when you go on to validation and verification [in] clinical samples, I think this is certainly not practical." Instead, he plans to narrow the list of useful biomarkers, then develop antibody-based tests for clinical use.

Regardless of whether they're looking at free DNA, intact cells, or extracellular vesicles, researchers and physicians are optimistic about the field's prospects. Oxnard anticipates that "over the next five years it will become routine, and just be something every [hospital] needs to offer to every cancer patient."

Alan Dove is a science writer and editor based in Massachusetts.