Activity-Based Diagnostics: An Emerging Paradigm for Disease Detection and Monitoring

Ava P. Soleimany1,2,3 and Sangeeta N. Bhatia1,2,4,5,6,7,8,*

Diagnostics to accurately detect disease and monitor therapeutic response are essential for effective clinical management. Bioengineering, chemical biology, molecular biology, and computer science tools are converging to guide the design of diagnostics that leverage enzymatic activity to measure or produce biomarkers of disease. We review recent advances in the development of these ‘activity-based diagnostics’ (ABDx) and their application in infectious and noncommunicable diseases. We highlight efforts towards both molecular probes that respond to disease-specific catalytic activity to produce a diagnostic readout, as well as diagnostics that use enzymes as an engineered component of their sense-and-respond cascade. These technologies exemplify how integrating techniques from multiple disciplines with preclinical validation has enabled ABDx that may realize the goals of precision medicine.

Noninvasive Diagnostics: Methods, Challenges, and Possibilities

Accurate detection and diagnosis of disease are essential for effective clinical management and treatment. Rapid diagnostics for highly infectious diseases such as Ebola can enable early case detection and intervention, thereby informing impactful public health interventions [1]. Similarly, sensitive and specific diagnostics for noncommunicable diseases such as cancer offer the promise of improved therapeutic outcomes through early diagnosis of localized disease and accurate classification of high-risk patients [2–4]. Parallelizing therapeutic development with diagnostics that can measure treatment response could facilitate personalized treatment regimens tailored specifically to the disease state of each patient. Effective diagnostics improve patient outcomes by providing actionable information on the presence, prognosis, or progress of disease.

Traditional noninvasive diagnostic strategies rely on a combination of imaging tests and assays for endogenous biomarkers. Imaging tests, such as computed tomography for lung cancer and magnetic resonance imaging (MRI) for brain scanning, remain the clinical standard for noninvasive diagnostics and enable detection and localization of disease. However, they often suffer from poor specificity [5,6] and require investment in costly infrastructure and specialized personnel to interpret the findings. Recent advances in molecular diagnostics have yielded promising assays for endogenous disease biomarkers, such as nucleic acids for viral infections [7], stool-based tests for colon cancer screening [8], and circulating tumor DNA (ctDNA) [9–12] for cancer, that can be used in conjunction with or as an alternative to imaging.

An ideal molecular test for infectious diseases would be simple, rapid, inexpensive, and accurate; such a test would enable specific disease detection and isolation directly at the point of care and would have tremendous implications for global health [13]. In oncology, ctDNA has emerged as a promising tool for noninvasive disease detection and evaluation of treatment response in patients with advanced cancers whose tumors shed ample cell-free DNA (cfDNA) into the bloodstream [12]. In this context, ctDNA has enabled comprehensive reconstruction of patient-specific mutation profiles via whole-exome sequencing [14], and can be used for longitudinal monitoring of cancer, that can be used in conjunction with or as an alternative to imaging.
treatment efficacy or disease relapse because ctDNA levels are thought to scale with tumor burden [12]. However, this correlation with tumor burden presents fundamental sensitivity limits for early-stage, localized disease [12,15]. Multi-analyte blood tests that measure both ctDNA and protein biomarkers, such as CancerSEEK [10], have recently been developed as a means to improve detection rates. The median sensitivity of CancerSEEK was 70% for the eight common cancers tested, but the median sensitivity for stage I cancers was only 43%. Although multi-analyte tests can improve specificity and resolution, strategies that detect endogenous biomarkers in circulation face intrinsic sensitivity limitations for minimal residual or early-stage disease owing to low analyte concentration, high background signal, and biomarker clearance [12]. As a complementary strategy, engineered diagnostics that are selectively activated in disease states to generate amplified readouts may complement existing tests to help address these challenges and realize more accurate and accessible diagnostics.

Convergent efforts from bioengineering, chemical biology, and molecular biology have inspired a new class of smart diagnostics that leverage enzymatic activity to measure or produce biomarkers of disease. These engineered activity-based diagnostics (ABDx; see Glossary) offer the potential to overcome the limitations faced by current standard tests because they harness the specialized substrate recognition and signal amplification properties of enzymes to achieve specific and sensitive disease detection (Figure 1). This Review broadly organizes ABDx into two classes, and focuses on recent efforts towards their preclinical development. First, we discuss molecular and chemical probes that monitor dysregulated enzyme activity as a functional biomarker of disease. Second, we highlight molecular and biological tools that use enzymatic activity as a means of sensing, measuring, or reporting on disease state. Throughout, we highlight the enabling technologies that have catalyzed the emergence of these diagnostics, the performance advantages afforded by ABDx, as well as strategies for enhancing ABDx specificity (Box 1) and supporting clinical translation through dialogue with regulatory agencies. Continued efforts to engineer and validate ABDx will encourage their use as next-generation tests for precision medicine.

Enzyme Activity as a Functional Biomarker of Disease

Biomarkers such as proteins or nucleic acids are biological indicators of disease progression or therapeutic response [16]. Because enzymes such as proteases play crucial roles in several biological processes that contribute to disease progression, many ABDx measure enzyme activity as a functional biomarker of disease [17] (Figure 1A). Furthermore, by leveraging the catalytic nature of enzymes for signal amplification, activity measurements may offer sensitivity advantages relative to endogenous blood biomarkers. Advances in chemistry and nanotechnology have enabled new molecular probes that are selectively activated by disease-associated enzymatic activity to generate a measurable diagnostic readout (Figure 1A).

Substrate and Activity-Based Probes for Molecular Imaging In Vivo

Several ABDx function by converting the activity of enzymes involved in disease progression into an imaging readout (Figure 2) [17,18]. For cancer in particular, accurate in vivo visualization of malignant tissues can aid in early diagnosis, surgical planning and resection, and monitoring of treatment response. Because proteases play direct functional roles in all cancer hallmarks [17], imaging the proteases involved in cancer progression has emerged as a promising detection strategy.

Advances in chemistry and nanotechnology have spawned both substrate cleavage- and binding-based probes for molecular imaging of enzymes involved in cancer (Figure 2). Substrate-based probes that fluoresce in the near-IR upon proteolytic cleavage have been extensively used for
imaging-based tumor detection [19–24]. For example, the cathepsin-activated probe 6QC has shown preclinical promise in labeling lung, breast, and colon tumors [25], and has also been utilized for in vivo surgical guidance, where it can be detected intraoperatively with the da Vinci Si Surgical System [25,26]. Cathepsin-responsive fluorescent probes [23,24] are currently under commercial development for in vivo tumor detection. These probes and similar technologies, such as topical probes activated by transpeptidases [27], could enable high-resolution intraoperative molecular imaging for guided tumor resection and debulking. In another strategy, a cell-penetrating peptide, a short, positively charged peptide that can facilitate cellular delivery of associated cargo, is released from a probe following proteolysis [28]. When functionalized with fluorescent acceptor–donor pairs [29,30] or MRI contrast agents [31], these activatable cell-penetrating peptides (ACPPs) have enabled visualization of primary tumors and metastases in several mouse models [30–32]. Improved ACPPs are currently under clinical development for intraoperative evaluation of lymph node metastases. Further, several enzyme-responsive nanoparticle systems have been developed for optical imaging of cancer in mouse models [31,33–37]. As an alternative to cleavable substrates, quenched activity-based probes (ABPs) [17,18,38] that covalently react with enzyme active sites through a chemical warhead have also been used to visualize tumors in vivo in mice [39–41]. Although covalent labeling enables localization of proteolysis, the resulting enzyme inactivation prevents signal amplification, an important consideration for probe sensitivity.

In addition to optical approaches, analogous techniques utilize enzymatic activation for specific tumor detection via other imaging modalities such as positron emission tomography (PET) [42] and MRI [35,37]. Dual-readout probes for multimodal assessment have also been developed [31]. One recent approach described a magnetism-based nanoscale phenomenon in which the

physiological compartments (i.e., blood, tissue, or organ compartments).

**Paper-based test (also, paper test):** a simple paper-based device that can detect a target analyte in a sample without any specialized equipment beyond the test itself. Lateral flow assays are a prominent example, such as pregnancy tests.

**Predictive classification:** the process of arranging samples into distinct classes or categories using a predictive statistical model.

**Protease:** an enzyme that hydrolyzes peptide bonds.

**Reporter enzyme:** an enzyme that is produced by an engineered diagnostic in response to activation. Its activity can be queried in vivo or in vitro/ex vivo, depending on the nature of the sensor, to provide a diagnostic readout.

**Sensory histidine kinase:** an enzyme involved in signal transduction that modulates the phosphorylation of a response regulator as a result of changes in an input signal.

**Smart materials:** engineered materials with properties that can be controlled by input stimuli such as small molecules, pH, or temperature.

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**Figure 1. Principles of Activity-Based Diagnostics (ABDx).** ABDx leverage enzymatic activity to measure or produce biomarkers of disease. (A) Molecular and chemical probes can be used to measure dysregulated enzyme (orange Pacman) activity, either in vivo or ex vivo, as a functional biomarker of disease. Before catalysis, probes remain off (grey star). Enzyme-specific probe activation generates a measurable output as a diagnostic readout (green star). (B) Biological sensors, such as engineered bacteria or mammalian cells, carry enzyme-driven genetic circuits that enable them to sense and report on disease state. Synthetic reporter enzymes produced following sensor activation can generate amplified diagnostic readouts as output upon cleavage of reporter probes. (C) Guided, programmable nucleases, such as CRISPR-associated (Cas) effector enzymes, can be exploited for sequence-specific nucleic acid detection. Select nucleases can cleave synthetic reporter probes upon nucleic acid target binding to produce a signal-amplified readout for specific nucleic acid detection.
distance between a paramagnetic enhancer and a superparamagnetic quencher tunes the MRI signal [37]. Based on this method, a matrix metalloprotease (MMP)-cleavable substrate can be used to link the enhancer and quencher such that proteolysis by MMPs in the tumor microenvironment led to MRI contrast enhancement.

Despite these advances, there are several potential limitations to imaging-based ABDx. In regards to target specificity, cleavage promiscuity can hinder the discriminative power of single-substrate agents [17]. Further, several probes incorporate substrates that are responsive to enzymes, such as cathepsins [25,26], that are highly expressed in malignancy, benign diseases, inflammation, and even healthy tissues. Potential false positives could be attenuated by rational protease target nomination through omic analyses and downselection of highly
expressed candidates (Box 1). Disease specificity could also be improved through active targeting (e.g., integrin [43] or receptor [44] docking) or multiplexing, although the multiplexing capacity of imaging is fundamentally limited by the paucity of orthogonal readouts [22] (Box 1).

Thus, imaging probes that require multiple steps of enzymatic processing, such as those responsive to **Boolean logic operations** [45,46], may improve specificity. Furthermore, probe deployment in a focused clinical context, such as intraoperative visualization of tumor margins in reduction surgery, can help to eliminate false positives. Other significant challenges facing imaging-based ABDx include tissue penetrance, probe biocompatibility and clearance, and sensitivity, particularly for low levels of disease. Because these are novel agents, the track through regulatory agencies is not yet established. Specifically, there is no defined path towards FDA approval of optical contrast agents, nor are there established precedents regarding desired outcomes in Phase II and III trials, and product submissions continue to be considered on a case-by-case basis [18]. Completed and ongoing clinical trials for imaging-based ABDx will inform the design of future studies and help to improve the efficiency and consistency of regulatory constructs.

**Activity-Based Nanosensors for Noninvasive Detection of Disease**

As an alternative to imaging approaches that rely on contrast agents, Bhatia and colleagues have engineered a class of enzyme-responsive nanoparticles, termed **activity-based nanosensors** (ABNs), that respond to dysregulated protease activity *in vivo* to generate urinary reporters of disease (Figure 3) [47]. Following ABN administration, proteolytic cleavage of peptide substrates carried by ABNs liberates reporters that are small enough to be cleared by the kidney and detected in the urine. ABNs provide signal amplification advantages because of local enzymatic turnover and concentration of reporters in urine. Highly multiplexed detection can be achieved by leveraging orthogonal isotope-encoded reporters that can be read by mass spectrometry [47–49]. In a mouse model of colorectal cancer, a broadly MMP-responsive ABN panel with
this encoding scheme exhibited superior diagnostic performance to the clinically approved blood biomarker carcinoembryonic antigen (CEA) because the urinary reporter signature enabled accurate detection [area under the curve (AUC) = 0.94] of tumors ~60% smaller than those detectable with CEA (130 mm³ vs. 330 mm³ for ABNs and CEA, respectively) [47]. In addition to mass encoding, urinary detection can also be achieved with ligand-encoded reporters measurable by immunoassays [50–53].

Both experimental and computational tools from seemingly disparate fields have yielded key advances in the evolution of the ABN platform. In work spanning nanoparticle engineering and transplant immunology, ABNs were recently engineered to measure the activity of granzyme B, a protease released by cytotoxic T cells as they engage and kill target cells, and were deployed as sensors for the early diagnosis of acute transplant rejection in an allogenic skin graft model [54]. The demonstrated capacity of ABNs to measure immune-related protease activity could enable immunotherapy response monitoring and assessment of T cell-mediated autoimmune diseases. Recent work has leveraged inorganic catalytic nanomaterials [55,56], specifically
renal-clearable gold nanoclusters (AuNCs), to design ABNs wherein the peroxidase-mimicking catalytic activity of proteolytically liberated AuNCs could be monitored in urine by producing a visible color change as a diagnostic readout [57]. Paper-based tests [51,53] and colorimetric [57] readouts encourage point-of-care deployment of the ABN technology in resource-limited settings for both infectious [58] and non-communicable diseases.

The sensitivity of the ABN platform for in vivo tumor detection has been described through a multi-compartment pharmacokinetic model [59]. This in silico model informed the development of ultrasensitive ABNs that combined improved substrate presentation and active targeting for accurate detection of millimeter-sized tumors in mice [43]. In an orthotopic model of ovarian cancer, the accuracy of this urinary diagnostic exceeded that of the blood biomarker human epididymis protein (HE4) – ABNs achieved an AUC of 0.99 at an average tumor burden of 36 mm³ whereas HE4 was not elevated to a detectable level at this timepoint (AUC_{HE4} = 0.51). Further, both transcriptomic analysis to identify proteases that are upregulated in disease, and in vitro cleavage assays against peptide libraries to nominate substrates, have been used to inform the design of multiplexed ABN panels [48,49]. Finally, preliminary work that couples ABN multiplexing with machine learning has yielded statistical algorithms for prospective diagnosis based on urinary reporter signatures [49].

Although these results encourage the potential of ABNs, there are several limitations that must be considered. Namely, systemic (i.e., intravenous) delivery leaves ABNs susceptible to nonspecific activation in off-target organs, which can reduce the signal-to-noise ratio. Preliminary work in the context of lung cancer detection suggests that intrapulmonary ABN delivery may provide a means of maximizing delivery to the target organ and increasing signal, while eliminating off-target activation to reduce noise [49]. However, even in the context of intrapulmonary delivery, ABNs are unable to precisely localize proteolysis and, by extension, the site of disease. Thus, ABNs may have clinical utility as companion tests to imaging approaches, for example as a rapid and cost-effective follow-up to diagnostic imaging to confirm the presence of malignancy or infection. In addition, ABNs have largely been tested in cell transplant models of cancer. Although intrapulmonary ABNs were recently deployed in a genetically engineered mouse model of lung cancer [49], the model used only represents a subset of human disease. Clinical studies will be necessary to establish the utility of ABNs for detecting lung cancer in humans and for discriminating malignancy from benign comorbidities.

The specificity of ABNs for a disease of interest, particularly in the context of concomitant comorbidities, presents another significant challenge. Similarly to imaging probes, ABNs are limited by the cleavage specificity of their substrates and the expression levels of protease targets. Because proteases are dysregulated not only in cancer but also in infection, inflammation, and fibrosis, ABN panels must be rationally designed with this in mind, and thorough in vivo validation in preclinical models and human trials will be necessary to ensure that ABNs are not confounded by benign disease etiologies or environmental insults such as alcohol consumption and tobacco smoke. Protease target nomination, peptide substrate engineering, and multiplexing may provide a concrete means to improve specificity (Box 1). Although mass-encoded ABNs enable multiplexing, the cost and equipment requirements of mass spectrometry may limit the accessibility of ABNs for routine use, particularly in resource-poor settings. Finally, the biocompatibility and clearance properties of nanosensor formulations must be established in clinical studies.

Continued efforts towards improved understanding of protease biology in both health and disease, methods for high-throughput substrate screening, and ex vivo assays for sensor
validation in human specimens will be crucial for designing ABNs that are specific and responsive to human disease. Currently under clinical development for noninvasive monitoring of fatty liver disease, the ABN technology provides an example of how advances in nanomedicine, disease biology, and computational science can synergize to yield ABDx with clinical promise. Several of the described approaches, such as substrate optimization for specific enzymes [54], bioinformatics mining for candidate targets [48,49], and machine learning for predictive classification [49], are broadly applicable to the development of next-generation ABDx for human disease.

**Ex Vivo Activity Assays in Clinical Biospecimens**

In addition to in vivo probes for diagnostic and intraoperative assessment, ex vivo measurements of disease-specific enzyme activity (Figure 1A) may enable differential diagnosis, relapse prediction, and treatment response monitoring. Active enzymes such as proteases [60] have been found in a variety of human specimens, including tissue sections [61,62], blood [63], urine [60,64], and sputum [65]. Although several diagnostics are run on patient specimens, such as blood-based tests for cancer [9–11,66] and sputum-based tests for tuberculosis [67], assays and probes that rely on catalytic processing by disease-relevant enzymes could offer performance advantages driven by catalytic signal amplification.

Bridging molecular imaging and anatomic pathology, in situ zymography assays are a class of methods that measure and localize protease activities directly in tissue [43,44,61,62]. Chemical probes such as ABPs [62] and substrate-functionalized nanoparticles [43] have shown potential for imaging proteolytic activity in frozen tissue sections. Similarly, topically applied probes for dipeptidyl peptidase [68] and cathepsin [69] activity have been applied ex vivo on resected tissues to identify tumor margins. More broadly, in situ and topical enzyme activity measurements could be used to inform the development of ABDx or, with further validation, to serve as stand-alone diagnostics.

Approaches to bulk activity profiling have leveraged microfluidic [70] or mass spectrometry [71,72] techniques to measure protease activity in patient-derived biospecimens for diagnostic applications. In recent work, screening pancreatic cyst fluid samples against a multiplexed synthetic substrate library identified two aspartic proteases with increased activities in malignant, mucinous cyst fluid relative to benign specimens [72]. The activity of one identified protease, gastricsin, detected malignancy with 100% specificity and 93% sensitivity, significantly outperforming the widely used molecular biomarker CEA that exhibited 94% specificity and 65% sensitivity [72]. Emerging approaches that couple bulk activity profiling with mass tag [47,73] or DNA barcoding [74] schemes could enable quantitative substrate or ABP screens for diagnostic classification.

The convergence of discovery efforts in microbiology and of bio-orthogonal synthesis strategies from chemistry has led to the development of activatable molecular probes for pathogen detection and infectious disease diagnosis [75–78]. A striking example is in the design of enzyme-activatable fluorogenic probes for active tuberculosis (TB), a disease responsible for an estimated 1.6 million deaths in 2017 [79]. Standard diagnostics for active TB rely on sputum stains for the causative bug *Mycobacterium tuberculosis* (Mtib), and these require extensive sample processing, have widely variable sensitivity (32–94%), and cannot distinguish live from dead bacteria, thereby precluding their ability to report on treatment efficacy or drug resistance [67,80]. Chemical probes whose signal is specifically activated and amplified by pathogen-specific enzymatic processing could overcome these limitations and enable next-generation ABDx for TB.
Biochemical investigations have yielded promising targets and pathways for new activity-based TB diagnostics. For example, trehalose mycolates, a class of glycolipids that are essential for Mtb viability, are the major constituents of the mycobacterial outer membrane. Accordingly, organic synthesis techniques can be used to design chemical probes that measure the activity of mycolyltransferases that convert trehalose to trehalose mycolates to assemble this outer membrane [77,78]. In a recent diagnostic application, a fluorogenic trehalose analog, DMN-Tre (4-N,N-dimethylamino-1,8-naphthalimide-conjugated trehalose), was developed to directly detect live Mtb in patient sputum [77]. Enzymatic conversion of DMN-Tre by mycobacterial acyl transferases enables its incorporation into the hydrophobic membrane, resulting in a dramatic increase in the fluorescence of the attached solvatochromic dye. Although DMN-Tre can label live Mtb in human sputum following a single incubation step, it lacks specificity for Mtb relative to other bacterial strains that express mycolyltransferases.

Earlier work has established that the resistance of Mtb to β-lactam antibiotics is due to the expression of β-lactamase [81], pointing to new enzymatic targets such as DprE1 for anti-TB drugs [82]. A recent study leveraging these insights described an activatable dinitrobenzene (DNB)-based fluorogenic probe, CDG-DNB3, that requires two steps of enzymatic processing for specific detection of live Mtb [76]. CDG-DNB3 is first cleaved by a conserved Mtb β-lactamase to activate fluorescence [75,81], and is then covalently modified by the Mtb essential enzyme DprE1 [82] to retain signal within the membrane. The combined use of two enzymes as diagnostic markers enabled the detection of viable bacteria with specificity for Mtb over non-tuberculosis mycobacteria [76]. Future efforts that continue to bridge microbiology, genetics, and organic chemistry could enable rapid target identification and probe design for new ABDx for TB and other infectious diseases.

Leveraging Enzymatic Activity To Engineer Molecular and Cellular Diagnostics

Advances in synthetic and molecular biology have spawned new tools, such as genetic memory circuits [83] and CRISPR/Cas systems [84], that have precipitated the emergence of diagnostic innovations that use enzymatic activity as a way to monitor for and report on disease state. In this section we review recent work focusing on (i) activity-based probiotic and cellular biosensors (Figure 1B), and (ii) CRISPR-based diagnostics (Figure 1C), and specifically highlight how integrative research can continue to accelerate the development of these ABDx for precision health applications.

Engineering ABDx with Synthetic Biology

Synthetic biology is a forward engineering approach that integrates experimental manipulation, computational modeling techniques, and principles of circuit design to produce specific and controlled behaviors in biological systems. The programmability, circuit design principles, and fast design-to-production cycles of synthetic biology afford the potential for diagnostics that can be rationally engineered and rapidly deployed to meet clinical needs. Recent advances in synthetic biology have promoted the design and use of bacteriophages [85], molecular recorders [86], mammalian cells [87–89], and programmable probiotics [90,91] for diagnostic applications.

Bacteria offer unique advantages as a chassis for programmable ABDx because they can be readily manipulated to sense disease markers through enzyme-driven circuits or signaling cascades, as well as to generate amplified, noninvasive diagnostic readouts. By bringing insights from microbiology and genetic circuit design together, researchers have engineered commensal bacteria to sense disease biomarkers and to report on their presence after in vivo interrogation.
Diverse bacterial species possess sensing circuits that are responsive to disease-relevant signals such as cytokines, metabolites, inflammatory markers, heme, hormones, pH, and temperature, as previously reviewed [91]. Two-component systems include a class of sensing circuits wherein signal transduction is mediated by the activity of sensory histidine kinases [92]. These circuits can be exploited to engineer probiotic diagnostics that respond to specific disease biomarkers via kinase-driven signal amplification, such as in sensing transient markers of inflammation in the gastrointestinal (GI) tract [93,94]. As a recent example, commensal mouse E. coli were engineered with a kinase-mediated sensing circuit that was synthetically linked to a memory element [95] and delivered orally to record exposure to the gut inflammatory marker tetrahionate [94]. Similar systems based on other enzyme-mediated signaling cascades could be used for the diagnosis of a diversity of disease states, for example fucose-sensing probiotics for detecting GI pathogens [96]. Furthermore, computational mining of genomic data [97] and library-based

Disease biomarker

Reporter enzyme expressed and secreted

Sensing, memory, and output

Administer

Sense and respond

Diagnostic readout

Pathogen

V. cholerae

CAI-1: quorum sensing molecule

CqsS: sensory histidine kinase

NisR signal transduction domain

Engineered probiotic

L. lactis

TetR

TetR

β-Lactamase

ON

AP

Engineered probiotic diagnostics can then be administered in vivo, for example orally for diagnostic applications in the gastrointestinal tract. (B) Quorum sensing through cholera autoinducer 1 (CAI-1, red hexagon) in Vibrio cholerae has been used to induce expression of the reporter enzyme β-lactamase in Lactococcus lactis engineered as a cholera diagnostic [100]. The engineered V. cholerae-sensing circuit consists of a hybrid two-component system composed of the sensory histidine kinase CqsS linked to the NisR signal transduction domain, and a TetR/Ptet reporter module. Upon CAI-1 binding to CqsS (i), phosphorylation through the two-component system is halted, preventing TetR expression and leading to derepression of the Tet promoter (P_{tet}) (ii). In the absence of TetR, the β-lactamase reporter is freely expressed and secreted (iii). (C) An activity assay for the reporter enzyme can be used to produce a diagnostic readout, for example directly in collected fecal samples [100].
screening strategies [98] could be used to identify new bacterial biosensors for the design of more sensitive and specific circuits.

Owing to the programmability of genetic circuit design, in vivo bacterial diagnostics can be engineered to produce a synthetic reporter enzyme that generates an activity-based readout of disease (Figure 1B). This approach offers three potential modes of signal amplification: bacterial expansion in vivo, high levels of enzyme production from stably maintained plasmids, and enzymatic substrate turnover in generating the final readout. One study explored the capacity of orally administered probiotic E. coli to preferentially expand in metastatic liver tumors, where the bacteria produced high levels of a reporter enzyme that could cleave a systemically delivered substrate, which ultimately led to a color change that was detectable in the urine [99]. In addition to liver metastases, this technology may also enable the detection of tumors in other organs exposed to bacterial loads from the GI tract, such as colorectal cancer.

Although this system achieved high accuracy (AUC = 0.93) in a mouse model of liver metastases, it lacked any disease-specific sensing circuitry upstream of the enzymatic readout. Engineered sense-and-respond probiotics that link synthetic reporter enzymes downstream of biomarker sensing circuits have potential for improved diagnostic performance. For example, a probiotic strain was recently engineered to detect quorum-sensing molecules specific for the pathogen Vibrio cholerae via sensory histidine kinase-based signal transduction and, after in vivo interrogation in a mouse model of cholera, to generate a reporter enzyme whose activity could be readily queried in fecal samples (Figure 4) [100]. The signal amplification and substrate flexibility afforded by synthetic reporter enzymes encourage the deployment of these bacterial systems at the point of care for early detection of cancer and infectious diseases.

Although engineered probiotics are perhaps the most widely used scaffold for applying synthetic biology to diagnostic design, genetically encoded molecular recorders, such as DNA vectors that leverage a tumor-specific promoter to drive expression of a reporter enzyme [86], and mammalian cell biosensors [87–89] have also been applied as engineered ABDx. One example of the latter involved a subcutaneous cell implant that, in response to blood hypercalcemia associated with mouse models of colon and breast cancer, formed a visible ‘tattoo’ via enzymatic production of the black pigment melanin [88].

An alternative strategy is the systemic delivery of engineered mammalian cells that are selectively activated in vivo to produce an activity-based readout. In a novel integration of ideas from synthetic biology and cell therapy, macrophages were engineered to express a synthetic reporter enzyme in response to adopting a tumor-associated (M2 macrophage) metabolic profile [89]. In cell transplant models of colorectal cancer in immunodeficient or syngeneic mice, this system of ‘diagnostic adoptive cell transfer’ achieved greater sensitivity and specificity than clinically used cancer biomarkers. Namely, in a xenograft model that sheds the protein biomarker CEA, subcutaneous tumors of average volume 45 mm³ were discriminated more accurately by the macrophage sensor relative to CEA (AUCs of 0.914 and 0.829, respectively). The sensitivity of this cellular ABDx was also benchmarked to cfDNA in the syngeneic CT26 subcutaneous tumor model, where the macrophage sensor could detect 25–50 mm³ tumors whereas tumor-specific cfDNA mutations were only detectable for tumor volumes of 1500–2000 mm³. This 50-fold improvement in limit of detection highlights the potential of disease-activatable probes to circumvent the biological and mathematical limitations of endogenous biomarkers for early cancer detection [15].
The specificity of the macrophage sensor was also characterized in the context of co-occurring acute inflammation in a lung metastasis mouse model established via intravenous injection of the 4T1 breast cancer cell line, and no significant differences in accuracy were observed in the absence (AUC = 0.975) or presence (AUC = 1.00) of acute lung inflammation [89]. However, in models of lung and muscular inflammation, the macrophage sensors were activated during

Figure 5. Guided, Programmable Nucleases for Activity-Based Diagnostics (ABDx). (A) Nucleic acid detection with CRISPR/Cas-augmented toehold sensing. The presence of a specific protospacer adjacent motif (PAM; orange) in a DNA input results in cleavage by Cas9 with a PAM-specific guide RNA. Following transcription, this differential cleavage leads to either truncated or full-length trigger RNAs that can be designed to differentially activate an engineered toehold sensor. Because only full-length triggers can activate the toehold sensor, a downstream signal can be used to discriminate the two inputs [111]. (B) SHERLOCK diagnostic test. Nucleic acids, either double-stranded DNA (dsDNA) or RNA, are either amplified or reverse-transcribed (RT) and amplified, respectively. Following transcription, amplified target RNAs and fluorescently quenched cleavage reporters (grey star, fluorophore; blue circle, quencher) are incubated with the Cas13a–CRISPR RNA (crRNA) complex. Detection and binding of the RNA target unleashes the collateral activity of Cas13a, resulting in reporter cleavage, fluorophore activation (green star), and signal amplification [114]. (C) DETECTR diagnostic test. Target dsDNA are amplified and, together with fluorescently quenched cleavage reporters, incubated with Cas12a–crRNA complex. Detection and binding of the dsDNA target triggers the collateral activity of Cas12a, resulting in reporter cleavage, fluorophore activation, and signal amplification [115]. (D) CRISPR-based diagnostics achieve high specificity driven by crRNA guide–target base pairing (left), detection sensitivity from the highly efficient collateral cleavage of reporter molecules (middle), and diagnostic programmability owing to facile guide RNA design for nucleic acid targets of interest (right). Abbreviation: cfDNA, cell-free DNA.
the wound healing and inflammation resolution phases, which are characterized by M2 macrophage influx. In light of these results, the specificity of this cellular ABDx for malignancy relative to confounding benign comorbidities must be validated. Greater signal multiplicity may enhance specificity because the sensor in its current state measures a single M2-associated marker [89]. Further characterization will also be necessary to determine whether these M2-polarized macrophage sensors have an impact on tumor progression.

Tools from synthetic biology offer great promise for the future development of ABDx with improved functionality, clinical relevance, and performance. Enzyme-driven synthetic circuits, such as DNA recombinase-based logic gates [101–103] and state machines [104] or protease-based circuits [105,106], could provide the ability to logically integrate combinations or sequences of several signals for improved diagnostic specificity. For engineered probiotics, importing circuits optimized in model organisms into clinically relevant strains is another significant consideration [107]. Future efforts to characterize the stability, safety, and performance of sense-and-respond circuits in commensal strains and in relevant disease models will be indispensable for clinical translation of probiotic diagnostics. These studies will be crucial for regulatory approval of engineered bacterial strains or cell sensors in anticipation of clinical implementation. Finally, automated circuit design with computational techniques such as machine learning could enable rapid development of more sensitive systems [108].

Another intriguing possibility is the integration of synthetic biology with other engineering disciplines to create hybrid diagnostics that interface more directly with the body. For example, an ingestible bacterial–electronic microcapsule was recently developed and deployed for accurate in situ diagnosis of GI bleeding in swine [109]. Bacteria engineered to sense heme and to produce a luciferase reporter enzyme in response were integrated with luminescence readout electronics that could transmit signal wirelessly to an external device. Alternatively, hybrid materials hosting genetically engineered bacteria could enable diagnostic wearables composed of both synthetic and living materials [110]. Advances in synthetic biology, low-power electronics, and soft materials may open new frontiers for novel ABDx devices for noninvasive health monitoring.

Harnessing CRISPR/Cas Biology for Accurate, Rapid, and Accessible Diagnostic Tools

The discovery of microbial clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) adaptive immune systems has transformed the life sciences. These systems consist of sequence-specific RNA-guided nucleases, known as CRISPR-associated Cas effectors, that can recognize and cleave nucleic acids that match the guide sequence. The programmable and efficient activity of CRISPR/Cas has been extensively exploited for gene editing in basic science and therapeutic applications [84]. Recent insights into the biology of CRISPR/Cas have led to new molecular diagnostics that leverage the activity of Cas effectors for sensitive, specific, and rapid nucleic acid detection (Figure 1C).

In a platform driven by engineered RNA biosensors, cell-free synthetic gene networks were coupled with a CRISPR/Cas9 module to yield a low-cost paper test for strain-specific Zika virus detection [111]. In this system, these engineered RNA sensors, termed toehold switches [112], detect ‘trigger’ viral RNAs to ultimately produce a differential color change on paper. Strain-specific resolution is achieved by leveraging the requirement for an NGG protospacer adjacent motif (PAM) for Cas9 endonuclease activity (Figure 5A). Specifically, the presence or absence of a strain-specific PAM within the target RNA results in differential Cas9-mediated cleavage, such that active trigger molecules are only produced in the absence of Cas9 activity.
This integration of guided Cas9 endonuclease activity with toehold switch RNA sensing enabled visual discrimination of American and African Zika viral strains with single-base resolution.

Fundamental discoveries into the structure, function, and diversity of CRISPR/Cas systems have led to the emergence of next-generation CRISPR-based diagnostics. For example, Cas13a is an RNA-guided, RNA-targeting CRISPR effector that exhibits promiscuous RNase activity upon target recognition [113]. One technique, termed Specific High-Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK; Figure 5B), couples this collateral activity with isothermal amplification to achieve detection of target nucleic acids with attomolar sensitivity and single-base mismatch specificity [114]. Detection of a target sequence by a guided Cas13a unleashes its promiscuous RNase activity, leading to activation of reporter molecules and robust signal amplification. This triggered collateral activity was exploited for the detection of Zika virus in human samples and for cancer mutation identification in cell-free DNA [114]. An orthogonal CRISPR-based diagnostic system utilizes a distinct effector, Cas12a, that exhibits target-activated, non-specific single-stranded DNA endonuclease (ssDNase) activity [115]. Similarly to SHERLOCK, this diagnostic platform, termed DNA Endonuclease-Targeted CRISPR Trans Reporter (DETECTR; Figure 5C), leverages the target-specific ‘unlocking’ of the highly efficient, indiscriminate ssDNase activity of Cas12a to produce an amplified detection signal only after Cas12a has been activated by its cognate DNA target, and DETECTR achieved specific detection of cancer-associated human papilloma virus in human anal swab samples.

Both these platforms leverage the guided programmability of Cas enzymes for highly specific nucleic acid detection and exploit the promiscuous nuclease activity of two particular effectors for robust signal amplification. However, both SHERLOCK and DETECTR lack precise nucleic acid quantification, rely on a fluorescent readout, and are limited in their multiplicity. SHERLOCKv2

Table 1. Comparison of ABDx Strategies

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<th>Technology</th>
<th>Advantages</th>
<th>Disadvantages</th>
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| In vivo imaging with cleavage- and binding-based probes | • Ability to localize disease  
• Utilty for both preoperative and intraoperative evaluation  
• Clinical development of several probes | • Limited multiplexing capacity owing to the paucity of orthogonal imaging readouts  
• Binding-based probes covalently modify targets, preventing signal amplification |
| Activity-based nanosensors (ABNs)        | • Multiplexing capacity  
• Generalizability to extracellular proteases  
• Modularity in substrate selection, delivery method, and urinary readout | • Inability to localize the site of disease, which may necessitate follow-up imaging  
• Cost and accessibility of mass spectrometry analysis  
• Need for injected probes |
| Ex vivo probes and activity assays        | • No need to administer a probe or sensor into a patient  
• Specimens can be collected as part of standard diagnostic or therapeutic workflows  
• Can be used to validate new diagnostics on human tissue | Unclear how ex vivo measurements correspond to in vivo microenvironment  
Sample processing, such as blood collection or tissue homogenization, may increase background signal |
| Engineered probiotics and cellular sensors | • Programmability governed by the methods of synthetic biology  
• Use of characterized genetic circuits to carry out Boolean logic and signal multiplexing  
• Potential to generate synthetic reporter enzymes for amplified diagnostic readouts | • Unclear path to regulatory approval; safety and stability considerations for in vivo cell sensors  
Lack of ABDx characterization in clinically relevant probiotic strains  
Limited repertoire of characterized biosensors |
| CRISPR/Cas diagnostics                   | • High specificity owing to guide RNA/target recognition  
• High sensitivity owing to collateral cleavage of reporter molecules by Cas effectors  
• Programmability owing to guide RNA design | • Can only be applied to nucleic acid biomarkers  
• Lack of benchmarking against standard tests and vetting at the point of care  
• Multiplicity limited by the collateral cleavage properties of Cas effectors |
addresses these limitations by achieving improved quantification through optimization of the isothermal amplification step, point-of-care compatibility with a paper-based test, and increased multiplicity via the use of orthogonal Cas effectors [116]. Finally, to support diagnostic deployment in any context, a protocol that enables direct detection of target molecules in clinical specimens was coupled with SHERLOCK for rapid visual diagnosis of viral infection in body fluids [117].

The CRISPR/Cas9 system has also been combined with next-generation sequencing (NGS) technologies to enrich for low-abundance sequences in clinical samples [118]. In this approach, a sample of DNA or cDNA is subjected to Cas9 digestion of target genes, producing cleavage products to which adapters are attached for subsequent amplification, enrichment, and sequencing of the target DNA. This approach was applied to clinical respiratory fluid samples to identify antimicrobial resistance-associated genes, with sub-attomolar sensitivity and up to five orders of magnitude greater enrichment relative to NGS alone. Future efforts that combine the enzymatic activity of CRISPR/Cas effectors with NGS may prove useful for the detection of tumor mutations and for diagnostic molecular profiling in clinical samples.

The programmability of Cas enzymes offers tremendous potential for the development of responsive smart materials that can be actuated via nucleic acid sensing. Notably, DNA-embedded hydrogels were recently designed to respond to signal-triggered CRISPR-associated nuclease activity [119]. When integrated into a microfluidic device linked to an electronic circuit, one such actuated hydrogel modulated a conductive flow channel in response to Cas12a collateral activity, enabling the detection of nucleic acid biomarkers such as Ebola virus RNA and antibiotic resistance genes. Wireless electronic monitoring of this ABDx readout was achieved by incorporating a microelectronic radio transmission module into the device. In exploiting the enzymatic properties of Cas12a for actuated smart materials that directly interface with electronic monitoring systems, this work highlights the promise of CRISPR/Cas for programmable ABDx that can be applied globally.

CRISPR-based technologies harness the unique enzymatic properties of Cas effector enzymes to achieve highly specific, sensitive, and programmable molecular diagnostics (Figure 5D). Strategies to combine the programmable activity of Cas enzymes with biomaterial, microfluidic, and bioelectronic interfaces may lead to new point-of-care diagnostic devices, similar to glucose monitors or home pregnancy kits, for cancer and infectious diseases. Future efforts that apply CRISPR-based diagnostics to clinical specimens from different diseases and rigorously benchmark performance against standard tests will support their clinical translation.

Concluding Remarks
A variety of enabling technologies, including bio-orthogonal chemistry, CRISPR/Cas, and synthetic gene circuits, have armed researchers with the tools to design a powerful new class of diagnostic tests that leverage enzymatic activity to measure or produce biomarkers of disease. As an emerging class of technologies, these ABDx must be evaluated in terms of both their relative advantages and disadvantages (Table 1). Looking forward, continued preclinical validation and technical development of ABDx will be crucial to reach the performance necessary for clinical use (see Outstanding Questions). Several technical strategies, including probe and substrate optimization through high-throughput screening or targeted chemistry and signal multiplexing, may enable the design of more selective tests with the classification power necessary for high disease specificity (Box 1). Furthermore, applying machine learning to the chemical or biological signals generated by ABDx, alone or in conjunction with other modalities, will provide prospective statistical classifiers for validation in independent cohorts.
The emerging diagnostic tools described in this Review must be rigorously benchmarked, at both the preclinical and clinical levels, against standard tests to ensure sensitivity and specificity (see Outstanding Questions). Head-to-head benchmarking against imaging and molecular diagnostics, including ctDNA and multi-analyte blood biomarkers, will help to establish the concrete advantages that ABDx afford, identify specific clinical use cases for their deployment, and suggest opportunities for integration with other diagnostic modalities or medical devices. Further, studies that assess the ability of ABDx to monitor treatment response will clarify their potential as integrated diagnostics for precision health (see Outstanding Questions).

There are several considerations to keep in mind on the path towards regulatory approval for ABDx (see Outstanding Questions). First, projects should be designed with clear clinical motivation, components that are generally regarded as safe (GRAS), and proposed metrics for diagnostic success. For ABDx designed as in vivo sensors, such as imaging probes, nanoparticles, and engineered probiotics, in vivo efficacy experiments should be conducted in appropriate disease models and constructed with clinical use cases in mind. In addition, the dosage regimens and safety of these in vivo sensors must be established both preclinically and clinically. The need to assess ABDx performance relative to standard and other emerging diagnostics cannot be emphasized enough, particularly in regards to establishing success and outcomes metrics for future clinical trials.

For many ABDx, such as optical imaging agents [18], there is no pre-established pathway to FDA approval. Similarly to other emerging technologies, current FDA frameworks consider these product submissions on a case-by-case basis, often as combination products. As with any new modality, increased examples of performance and utility will establish more efficient regulatory frameworks for clinical translation. As these technologies grow in scope and number, sustained collaborations between researchers, clinicians, and regulatory officials will be crucial to achieve clinical deployment. With further development, ABDx may provide an accurate way to intercept and manage disease and ultimately improve patient outcomes.

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