Creating CRISPR-responsive smart materials for diagnostics and programmable cargo release

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EDITORIAL SUMMARY This Protocol describes the design and synthesis of CRISPR-responsive smart hydrogels, and their actuation for both the controlled release of cargos (small molecules, enzymes, nanoparticles, and living cells) and diagnostic applications.

TWEET A new Protocol describes CRISPR-responsive hydrogels for the programmable actuation of materials in diagnostics and cargo delivery applications.

COVER TEASER CRISPR-responsive smart materials

Up to three primary research articles where the protocol has been used and/or developed.

1. English, M. A. *et al.* Programmable CRISPR-responsive smart materials. *Science* **365**, 780-785, doi:10.1126/science.aaw5122 (2019)
2. Gootenberg, J. S. *et al.* Nucleic acid detection with CRISPR-Cas13a/C2c2. *Science* **356**, 438-442, doi:10.1126/science.aam9321 (2017)
3. Pardee, K. *et al.* Rapid, low-cost detection of Zika virus using programmable biomolecular components. *Cell* **165**, 1255-1266, doi:10.1016/j.cell.2016.04.059 (2016).

Abstract (250 words)

Materials that sense and respond to biological signals in their environment have a broad range of potential applications in drug delivery, medical devices, and diagnostics. Nucleic acids are important biological cues, encoding information about organismal identity and clinically relevant phenotypes such as drug resistance. We have recently developed a strategy to design nucleic acid-responsive materials using the CRISPR-associated nuclease Cas12a as a user-programmable

sensor and material actuator. This approach improves on the sensitivity of current DNA-responsvie materials, while also allowing their rapid re-purposing towards new sequence targets. Here, we provide a comprehensive resource for the design, synthesis and actuation of CRISPR-responsive hydrogels. First, we provide guidelines for the synthesis of Cas12a guide RNAs (gRNAs) for *in vitro* applications. We then outline methods for the synthesis of both poly(ethylene glycol)-DNA (PEG-DNA) and polyacrylamide-DNA (PA-DNA) hydrogels, and their controlled degradation using Cas12a for the release of cargos, including small molecules, enzymes, nanoparticles, and living cells within hours. Finally, we detail the design and assembly of microfluidic paper-based devices that use Cas12a-sensitive hydrogels to convert DNA inputs into a variety of visual and electronic readouts for use in diagnostics. Following the initial validation of the gRNA and Cas12a components (1 day), the synthesis and testing of either PEG-DNA or PA-DNA hydrogels require 3 days of laboratory time. Optional extensions, including the release of primary human cells or the design of the paper-based diagnostic, require an additional 2-3 days each.

INTRODUCTION

Smart materials that respond to biologically relevant signals play an important role in emerging biotechnologies ranging from drug delivery systems to diagnostics. These materials can provide dynamic structural supports, serve as depots for therapeutic compounds, and respond autonomously to biological cues[1,2]. For example, indirect physiological signals such as pH[3] and redox states[4] have been harnessed for the targeted delivery of therapeutics to inflamed tumor microenvironments. Materials that respond to direct biological information, in the form of enzyme activity for instance, have the potential to expand the repertoire of physiological states that can be probed and acted upon[5,6].

Nucleic acids are information-rich molecules that can be used as the input for bio-responsive materials[1]. Genomic sequences reliably identify different organisms and are routinely used to track clinically relevant markers such as antibiotic-resistance genes[7]. Similarly, knowledge of the abundances of RNA transcripts in a sample can aid clinical decision-making through the prediction of treatment responsiveness[8] and microbial virulence states[9-11]. Materials that detect and respond to the presence of specific DNA or RNA sequences are therefore of particular interest in the development of next-generation diagnostics[12], as well as for applications that require the context-dependent release of therapeutics[2,13]. Incorporating DNA into materials as both a structural and information-encoding element is emerging as a promising approach to address these needs[14].

In a recent study, we demonstrated the use of programmable CRISPR-associated (Cas) nucleases as both sensors and actuators in nucleic acid-responsive hydrogels[15]. In this protocol, we provide detailed instructions to design and test a representative set of CRISPR-actuated materials. We detail how this sensing platform can be used for the biomarker-dependent release of a range of cargos from hydrogel scaffolds. We also describe how CRISPR-responsive materials can be incorporated in the development of paper-based nucleic acid diagnostics with colorimetric, analog and wireless digital readouts.

Development of the protocol

Microbial CRISPR-Cas systems have been successfully adapted into powerful genetic engineering tools, owing to the facile modular programmability of their effector proteins[16]. The target sequence of a Cas nuclease is largely defined by the sequence of the guide RNA (gRNA) to which it is complexed[17]. Beyond the canonical double-stranded DNA (dsDNA) cleavage activity characteristic of the effectors most commonly used in gene editing, the Type V and VI family of Cas enzymes are able to hydrolyze single-stranded DNA (ssDNA) and RNA (ssRNA), respectively[11].

The discovery of these alternative activities has fueled the rapid development of a new class of diagnostic platforms, such as SHERLOCK[11,18-20], HOLMES[21], and DETECTR[22], that generate fluorescent or lateral flow readouts[11,22]. The last-named method uses Cas12a, a programmable nuclease guided by a single guide RNA. Cas12a targeting requires both a gRNA that matches 18-24 nt of the target dsDNA, and a T-rich protospacer adjacent motif (PAM) on the DNA strand opposite to the target site. Upon activation, Cas12a exhibits two distinct – but coupled – activities (Fig. 1a). When the Cas12a-gRNA complex encounters a dsDNA molecule matching the sequence of its gRNA (a "trigger" DNA molecule), the enzyme cleaves the DNA in a sequence-specific way (termed "targeted" or "cis" cleavage) and remains bound to one of the resulting DNA fragments. This first cleavage event causes a protein conformational shift enabling a second, non-specific nuclease activity (termed "collateral" or "trans" cleavage); the activated Cas12a-gRNA complex cleaves nearby ssDNA molecules with a very high turnover, at rates approaching the diffusion limit[22-25].

We reasoned that this programmability and intrinsic amplification mechanism would make Cas12a well suited to both the detection of biologically relevant nucleic acid cues and the subsequent large-scale actuation of materials containing structural nucleic acid elements[15]. Our approach expands the range of downstream processes that can be coupled to the original molecular recognition event, from changes in physical properties of the material, like stiffness and conductivity, to the stimulus-triggered release of bioactive cargos. To confirm the compatibility of our technology with various polymer chemistries, we tested the ability of Cas12a-gRNA to control the properties of two different materials in response to biological cues: (1) in poly(ethylene glycol) (PEG) hydrogels harboring pendant groups anchored through ssDNA linkers, Cas12a-gRNA controlled the release of active cargos without altering material structure; and (2) in polyacrylamide hydrogels cross-linked with DNA (PA-DNA), Cas12a-gRNA mediated the release of entrapped nanoparticles and cells through the complete degradation of the material (Fig. 1b).

To further expand the range of functional outputs that can be generated by a Cas12a sensor, we used the macro-scale actuation of PA-DNA hydrogels to control a microfluidic paper-based analytical device (μPAD). As a demonstration of the versatility of CRISPR-hydrogel actuation, we customized the μPAD to allow for the electronic detection of nucleic acids and used this feature to wirelessly transmit diagnostic data through a radiofrequency identification (RFID) tag. Cas12a-gRNA *cis* activity preferentially targets dsDNA substrates[22], so we incorporated reverse-transcription (RT) and isothermal amplification steps into the diagnostic workflow to allow the

detection of RNA molecules. With these additional steps, we built a µPAD-based diagnostic with attomolar sensitivity for Ebola virus RNA.

Overview of the procedure

The procedure starts with the design, synthesis, and *in vitro* validation of Cas12a gRNAs specific to a chosen DNA target (Steps 1-18). These gRNAs form an essential part of the CRISPR sensing platform. The user then selects the architecture of the CRISPR-responsive material (e.g., PEG-DNA or polyacrylamide-DNA) based on the downstream application. The options for PEG-DNA gels include the release of tethered molecules such as fluorophores (Step 19 Option A) or enzymes (Step 19 Option B), while polyacrylamide-DNA gels can be used to release entrapped cargos such as nanoparticles (Step 19 Option C) or cells (Step 19 Option D), or in µPAD diagnostics (Step 19 Option E). Within each different Option, we also provide instructions for the actuation of the synthesized CRISPR-responsive hydrogels using Cas12a-gRNA complexes and dsDNA triggers.

Comparison to other approaches

Nucleic acid-responsive materials. Typical DNA- and RNA-sensing materials rely on strand hybridization for nucleic acid detection, and incorporate DNA molecules in the material itself. Interest in this approach is driven, in part, by the decreasing cost of DNA synthesis[26] and the ease with which target recognition can be predicted and programmed at the sequence level[27-29]. Methods to develop DNA-responsive materials generally use strand displacement of cross-linking DNA elements to change the properties of the material. However, the one-to-one stoichiometry of this hybridization event can severely limit the sensitivity of these systems. As the recognition of target nucleic acids is mediated by DNA strands that also play a structural role in the material[27,29,30], decoupling the tuning of design parameters such as target sequence recognition from the gel properties can prove challenging. In our approach, the user can rapidly reprogram the specificity of Cas12a across a broad sequence space by simply altering the sequence of the gRNA, which can then be produced and validated *in vitro* in a few hours. This modularity greatly facilitates the process of redesigning and optimizing DNA-responsive materials to respond to different nucleic acid cues. Additionally, the efficient *trans* cleavage activity of Cas12a-gRNA rapidly converts the detection of low concentrations of trigger DNA into macroscopic changes in hydrogel properties.

CRISPR-based diagnostics. In keeping with the previously described SHERLOCK[11,18-20], HOLMES[21], and DETECTR[22] platforms, we are able to detect both RNA and dsDNA molecules by incorporating a reverse-transcription step in the workflow of a hydrogel-controlled µPAD diagnostic platform (Step 19 Option E). The sensitivity of our assay, in the low attomolar range[15], matches that previously demonstrated for lateral flow-based SHERLOCK[11,20]. Our strategy offers the option of generating readouts that are not only informational, but also functional. The electronic modalities of our µPAD-based diagnostic turn the device into a passive component that can be easily integrated into frameworks for data processing and transmission. While some technologies have successfully converted sequence-specific Cas

binding into direct electronic signals, for example, by immobilizing the enzyme on a field-effect transistor[31], the detection of the hybridization event typically requires advanced electronic equipment that may not be widely accessible. The complexity of such a system may also increase manufacturing costs. Our CRISPR-controlled μPAD uses only low-cost materials (e.g., paper, wax) without the need for advanced equipment or specialized training, making it well suited for deployment in low-resource environments. This illustrates the ease with which this technology could be adopted and adapted by the broader scientific community.

Limitations

In our implementations of CRISPR-responsive materials, the Cas12a-gRNA complex acts as a mobile sentinel programmed to detect specific dsDNA triggers. The long-term (> 24h) stability and activity of this complex in solution has not been investigated in detail. Applications that require sensing over longer periods are therefore outside of the scope of this work. Additionally, these responsive materials are restricted to applications in which environmental conditions fall within the range tolerated by Cas12a and its gRNA; for instance, the Cas ortholog used here requires at least 5mM of Mg^{2+} cofactor in the medium, and is most active around 37°C [15,21,22]. The stability of the DNA materials themselves is also important, and can be affected by the presence of contaminating enzymes in undefined media (Fig. 8).

The ability to rapidly reprogram the target specificity of the system at the level of the gRNA represents a major advantage of CRISPR-responsive materials. While this facilitates reprogramming across a very broad sequence space, possible targets are limited to molecules that possess the correct protospacer-adjacent motif (PAM). The required PAM varies depending on the Cas12 ortholog[37]. The *Lachnospiraceae bacterium* Cas12a ortholog used here requires a TTTV motif (where V indicates a non-T DNA base); these may be less frequent in targets from low-GC content genomes, for example. There are ongoing efforts to expand the target space of commonly used Cas nucleases through protein engineering[38].

Here, we present a series of demonstrations using two established material chemistries that illustrate CRISPR actuation. The literature provides many examples of how the composition, geometry and functionalization of these blank-slate materials can be modified to meet the needs of specific applications[33,39-41]. While we expect our CRISPR-based actuation strategy to be generalizable across many types of hydrogels, it is important to note that any change to the material properties (e.g., porosity, amount of DNA linkers, geometry) can affect the response behavior[15]. For this reason, we recommend re-testing the behavior of Cas12a actuation (e.g., rate of cargo release) in any new material context.

Directions for future extension of the approach

We have demonstrated the application of Cas12a to the control of material properties, but the repertoire of Cas nucleases is steadily growing. The application space of CRISPR-responsive materials could be expanded by using the rapid, collateral degradation of ssRNA elements afforded by Cas13a[11,18,24]. Similarly, the recently described Cas14a (also known as Cas12f1) is

much smaller than Cas12a, but demonstrates a similar collateral cleavage of ssDNA after target recognition[42]. As the toolbox of characterized Cas enzymes grows, so does the space of potential biological inputs and material actuation strategies.

Our recent work suggests that CRISPR-Cas sensing elements can be interfaced with a range of materials. Our proof-of-concept experiments were based on a pair of well-defined synthetic scaffolds (PEG and PA) in which the DNA cross-linkers were the only functional elements. Existing examples of multi-input responsive materials suggest that CRISPR-based sensors for nucleic acid cues could be incorporated alongside other orthogonal sensing modalities within the same materials[28]. Further applications might require the use of alternative materials[43] or the functionalization of hydrogels with additional biomolecules, such as cell adhesion signals[5,39,43]. Directly tethering the functional enzyme to the material could therefore be one avenue of further investigation, as other experiments may require the Cas12a enzyme to remain co-localized with the material for prolonged periods. Despite the limited nature of the set of cross-linking chemistries that we use in this work, we expect the Cas12a-mediated actuation platform to be compatible with other similar strategies to build and functionalize materials[33,40-41].

EXPERIMENTAL DESIGN

All of the experimental designs and conditions described in this article focus on a previously described set of CRISPR-responsive hydrogels (Fig. 1)[15]. Whenever possible, critical steps have been added to specify how certain steps may be modified to adapt this protocol to other experimental situations. For users interested in investigating other material systems, the necessary controls, such as *in vitro* activity validations (Steps 15-18), should be performed first to verify the activity of the CRISPR reagents in anticipation of a modified experimental design.

Design and synthesis of gRNA (Steps 1-18). One of the fundamental steps when developing CRISPR-Cas assays is the choice of a dsDNA target and the preparation of the cognate gRNA[11,18,22] (Fig. 2a). The gRNA molecule for Cas12a consists of two main parts: the constant scaffolding "handle" region which the Cas protein recognizes and binds to, and a user-defined spacer region that determines specificity to the target of interest (Fig. 2b). Table 2 provides examples of sequences successfully used for CRISPR-material actuation[15]. In the first part of the procedure, we detail how to select (Steps 1-5) and synthesize (Steps 6-14) a gRNA sequence based on a DNA target sequence of interest (Fig. 2a). If the target dsDNA is purchased as two strands of complementary ssDNA, we provide a brief procedure describing how to hybridize them efficiently (see Reagent Setup). Alternatively, the dsDNA can be purified from natural sources or purchased directly as dsDNA. We also detail the workflow for assessing the performance of a chosen gRNA-dsDNA pair in solution (Steps 15-18). The test is similar to the DETECTR assay described by Chen *et al.*[22]: Cas12a is complexed to its gRNA and subsequently mixed with the dsDNA target in the presence of a reporter probe, consisting of a fluorophore bound to a quencher via a ssDNA linker. The collateral activity of the Cas12a-gRNA complex can be observed by measuring the increase in fluorescence as the ssDNA linker is hydrolyzed (Fig. 1b).

CRISPR-responsive PEG hydrogels. In Step 19 Options A and B, we detail the assembly and actuation of a class of CRISPR-responsive hydrogels for which the activation of Cas12a by a gRNA-defined dsDNA trigger results in the release of molecular cargos tethered to the hydrogel scaffold by ssDNA linkers. The structural integrity of the material itself is thus independent from the DNA oligonucleotides and is unaffected by the Cas enzyme (Fig. 1b). In the original version of this technology, we assemble PEG hydrogels decorated with labeled ssDNA anchors by first reacting sub-stoichiometric amounts of oligonucleotides onto multi-arm PEG macromers in order to functionalize the PEG backbone (Step 19 Option A). We then use the remaining chemical handles of the PEG to polymerize the hydrogel by adding macromers with complementary chemical-end functionalization. To achieve this, we describe the use of an established Michael-type thiol-ene click chemistry (Fig. 3, Box 1), widely used to synthesize PEG-based biomaterials[32]. We expect this general synthesis strategy to be adaptable to other crosslinking chemistries that do not cross-react with the DNA anchors or the molecular cargos[33]. In Step 19 Option B, we also illustrate how to graft biomolecules, such as enzymes (streptavidin-horseradish peroxidase), onto ssDNA anchors after the polymerization of PEG hydrogels. For both options, we detail methods to track the release of cargos from the gel upon material actuation by Cas12a-gRNA, in response to a defined dsDNA signal (Fig. 4).

CRISPR-responsive polyacrylamide-DNA hydrogels. In Step 19 Options C and D, we outline the design and synthesis of acrylamide-DNA hydrogels for use in our Cas12a-based sensing framework, following a modified version of the protocol described by Previtera and Langrana[34]. DNA oligos functionalized with 5'-methacryl groups can be incorporated into polyacrylamide chains through a radical-catalyzed polymerization reaction (Fig. 5). Here, we outline the production of gel precursors with either 4% or 7% w/v acrylamide. PA-DNA hydrogels can be formed on a flat surface or in a tube to verify that the polymerization was successful, and to assess their macroscopic properties. The general cross-linking protocol provided here can be used to incorporate various cargos by steric entrapment. As an example, we detail in Step 19 Option C how to prepare gold nanoparticles (AuNPs) according to the method developed by Turkevich *et al.*[35], and use these as molecular cargos in PA-DNA hydrogels (Fig. 6).

As a proof-of-concept demonstration for the use of CRISPR-responsive materials in tissue culture applications, we provide a method to entrap human cells in polyacrylamide-DNA hydrogels and then release them on-demand using Cas12a-gRNA and a dsDNA trigger molecule (Step 19 Option D; Fig. 7). This protocol has been validated with two non-adherent human cell lines: primary peripheral blood mononuclear cells (PBMCs)[15] and the K562 leukemia cell line. As an illustration, we outline a procedure involving PBMCs. For applications involving living cells, it is important to test the growth medium for background ssDNA cutting activity. The choice of the medium may affect the long-term stability of materials containing DNA (Fig. 8).

CRISPR-mediated µPAD diagnostics. In Step 19 Option E, we present a modified version of the protocol described by Wei *et al.*[36] to design µPADs for the detection of synthetic Ebola virus ssRNA (Fig. 9). We provide a protocol for the construction of the µPAD platform that can either be used as is for visual readouts or modified further by the user (Fig. 10). To expand the capabilities of the device for ssRNA detection as well as dsDNA, we detail a method to incorporate an RT-RPA step into the workflow of the CRISPR-actuated µPAD device (Fig. 9).

The µPAD device used for the colorimetric detection of ssRNA and dsDNA can be modified to enable the analog electronic measurement of buffer flow through the lateral channel (Fig. 11). The basic µPAD design used for colorimetric readouts can also be modified to allow for the wireless transmission of diagnostic results via an RFID tag (Fig. 12).

Materials

Biological materials

- Nonadherent cells: In the example described in this protocol (Step 19 Option D), we use purified primary peripheral blood mononuclear cells or K562 cells (ATCC, Cat# CCL-243, RRID:CVCL_0004), maintained in suspension culture by conventional methods as described by Masters & Stacey[44] [Optional, Step 19 Option D]. **CAUTION** Any cell lines used in your research should be regularly checked to ensure they are authentic and are not infected with mycoplasma.

Reagents

General reagents

- Cas12a enzyme, LbCas12a 100µM (New England Biolabs, M0653T)
- Single-strand or double-strand DNA (ssDNA or dsDNA) containing the target sequence. If the target DNA is ordered as two single-strand DNAs, hybridize them as described in the Reagent Setup.
- HiScribe T7 Quick High Yield RNA Synthesis Kit (New England Biolabs, E2050S)
- RNA Clean and Concentrator Kit (Zymo, R1017)
- DNAse I (RNase free) 2,000 units/ml (New England Biolabs, M0303S)
- Mung Bean Nuclease (New England Biolabs, M0250S)
- Bovine serum albumin, molecular biology grade (New England Biolabs, B9000S)
- Custom synthesized oligonucleotides, including primers (Integrated DNA Technologies), see Table 1
- Ethanol 200 proof (Koptec, VWR, 71002)
- Sodium chloride (Sigma Aldrich 71376)
- Tris-EDTA (TE) Buffer 20X, pH 7.5 (VWR PAA2651)
- Tris 1M, pH 8, nuclease-free (Invitrogen AM9855GG)
- Molecular biology-grade water (VWR, 02-0201-1000)
- Magnesium chloride, 2M solution (Millipore Sigma, 68475)
- Magnesium acetate tetrahydrate (ThermoFisher Scientific, BP215-500)
- 2.1 buffer, 10X (New England Biolabs, B7202), or home-made equivalent (0.1M Tris-HCl, 0.1M $MgCl_2$, 0.5M NaCl, 100µg/ml bovine serum albumin, adjusted to pH 7.9)

Optional reagents

- Triethanolamine, TEA (Sigma Aldrich, T58300) [Optional, Step 19 Options A and B]
- Tris(2-carboxyethyl)phosphine hydrochloride, TCEP, 0.5M solution (Sigma Aldrich, 646547) [Optional, Step 19 Options A and B]

- 4-arm poly(ethylene glycol), PEG-SH, pentaerythritol core, thiol-functionalized, 10kDa (JenKem USA, 4ARM-SH) [Optional, Step 19 Options A and B]
- 8-arm poly(ethylene glycol), PEG-VS, tripentaerythritol core, vinyl sulfone-functionalized, 10kDa (JenKem USA, 8ARM(TP)-VS) [Optional, Step 19 Options A and B]
- Thiol-conjugated poly(ethylene glycol), 5kDa, linear (Nanocs, PG1-TH-5k) [Optional, Step Options A and B]
- Dithiothreitol (ThermoFisher Scientific, BP172-5) [Optional, Step 19 Options A and B]
- Streptavidin-horseradish peroxidase (HRP) conjugate (ThermoFisher Scientific, 21130) [Optional, Step 19 Option B]
- Sulfuric acid (ThermoFisher Scientific, A300-212) [Optional, Step 19 Option B] **Caution**: Concentrated sulfuric acid is corrosive and should be manipulated under a fume hood with proper protective equipment.
- Tetramethylbenzidine solution, TMB/E Ultra Sensitive, Blue, Horseradish Peroxidase Substrate (Millipore Sigma, ES022-500ML) [Optional, Step 19 Option B]
- Gold chloride trihydrate (Sigma Aldrich, 520918) [Optional, Step 19 Option C]
- Sodium citrate (Millipore Sigma, W302600) [Optional, Step 19 Option C]
- 500kDa MW FITC-Dextran (Millipore Sigma, FD500S) [Optional, Step 19 Option C]
- Low molecular weight DNA ladder (New England Biolabs, N3233L) [Optional, Step 19 Options C and D]
- OmniPur Acrylamide, 40% (w/v) solution (Calbiochem, 1185-500ML) [Optional, Step 19 Options C and D]. Caution: Acrylamide is toxic. Make sure you wear proper protective equipment and process waste according to local regulations.
- Ammonium persulfate, APS (Millipore Sigma, A3678-25G) [Optional, Step 19 Options C and D]
- N,N,N',N'-Tetramethylethylenediamine, TEMED (Millipore Sigma, T9281-25ML) [Optional, Step 19 Options C and D]
- NorthernMax-Gly Kit (ThermoFisher Scientific, AM1946) [Optional, Step 19 Options C and D]
- SeaKem LE agarose (Lonza, 50004-500G) [Optional, Step 19 Options C and D]
- 50X TAE buffer (BioRad, 1610743) [Optional, Step 19 Options C and D]
- Heat Inactivated Fetal Bovine Serum, FBS (Gibco, 10082147 Lot #2086998) [Optional, Step 19 Option D]
- Interleukin 2, IL-2, recombinant (Gibco, CTP0021) [Optional, Step 19 Option D]
- Calcein Blue AM, cell-permeant dye, (ThermoFisher Scientific, C1429) [Optional, Step 19 Option D]
- Ethidium Homodimer-1 (EthD-1), (ThermoFisher Scientific, E1169) [Optional, Step 19 Option D]
- Growth medium, RPMI 1640 GlutaMAX™ supplement (ThermoFisher Scientific, 61870036) [Optional, Step 19 Option D]
- Reduced serum medium, Opti-MEM GlutaMAX™ supplement (ThermoFisher Scientific, 51985091) [Optional, Step 19 Option D]
- Dulbecco′ s modified PBS, without calcium or magnesium (Gibco A1285601) [Optional, Step 19 Option DSuperscript IV Reverse Transcriptase (Invitrogen, 18090010) [Optional, Step 19 Option E]

- TwistAmp Basic (TwistDx TABAS03KIT) [Optional, Step 19 Option E] Silver nanoparticle ink (Mitsubishi NanoBenefit 3G Series) [Optional, Step 19 Option E]

Table 1 - Custom synthesized oligonucleotides designed and used in this study. All of the molecules are single-stranded unless described otherwise.

Name/description	Sequence (5'-3')*	Modifications †
Acrydite oligo X	TTATTCTTGTCTCCCGAGAT	5' Acrydite
Acrydite oligo Y	TTATT*TCACAGATGAGTATC*	5' Acrydite
Linker-15 (i.e. L-15)	*GATACTCATCTGTGA*TTATTTTATTTTATTATCTCGGGAGACAAG	N/A
Linker-15-F (L-15F)	T*GATACTCATCTGTGA*TTATTTTATTTTATTATCTCGGGAGACAAG	5'-6FAM
ssDNA anchor-bound Cy3 cargo for PEG gels	TTTTTATTTATCTATCTGACGA	5'-C6-thiol, 3'-Cy3
ssDNA-biotin anchor for PEG gels	TTATTATTACTATCTATTATCATTATCATT	5'-C6-thiol, 3'-biotin
mecA gRNA IVT template	**CCACATACCATCTTCTTTAA**ATCTACACTTAGTAGAAATTACCCTATAGTG AGTCGTATTAGCGC	N/A
ermA gRNA IVT template	**TCCATCTCCACCATTAATAG**ATCTACACTTAGTAGAAATTACCCTATAGTG AGTCGTATTAGCGC	N/A
ermC gRNA IVT template	**ACATGCAGGAATTGACGATT**ATCTACACTTAGTAGAAATTACCCTATAGTG AGTCGTATTAGCGC	N/A
spa gRNA IVT template	**ACAAAGCTCAAGCATTACCA**ATCTACACTTAGTAGAAATTACCCTATAGTG AGTCGTATTAGCGC	N/A
vanA gRNA IVT template	**CTCGACTTCCTGATGAATAC**ATCTACACTTAGTAGAAATTACCCTATAGTG AGTCGTATTAGCGC	N/A
T7 promoter sequence for hybridization	GCGCTAATACGACTCACTATAGGG	N/A
Scrambled *mecA* dsDNA trigger	TAGTAGTGATTATGTTAGATAGTGAATAGGTTTAATGTAT	N/A
Fluorophore-quencher reporter (FQ)	TTATT	5'-6FAM, 3'-IowaBlackFQ
mecA dsDNA trigger	TTTAATTTTGTTAAAGAAGATGGTATGTGGAAGTTAGATT	N/A
ermA-dsDNA trigger	GCTTTGGGTTTACTATTAATGGTGGAGATGGATATAAAAA	N/A
ermC dsDNA trigger	TAATATTGTTTAAATCGTCAATTCCTGCATGTTTTAAGGA	N/A
spa dsDNA trigger	TTCACCAGTTTCTGGTAATGCTTGAGCTTTGTTAGCATCT	N/A
vanA dsDNA trigger	ACGGAATCTTTCGTATTCATCAGGAAGTCGAGCCGGAAAA	N/A
ZEBOV gblock (dsDNA)	GTGCGCGTTCCTACTGTATTTCATAAGAAGAGAGTTGAACCATTAACAGT TCCTCCAGCACCTAAAGACATATGTCCGACCTTGAAAAAAGGATTTTTGT	N/A

	GTGACAGTAGTTTTTGCAAAAAAGACCACCAGTTAGAAAGTTTAACTGA TAGGGAATTACTCCTACTAATCGCCCGTAAGACTTGTGGATCAGTAGAAC AACAATTAAATATAACTGCACCCAAGGACTCG	
ZEBOV RPA-1 (forward primer)	CTACTGTATTTCATAAGAAGAGAGTTGAACC	N/A
ZEBOV RPA-2 (reverse primer)	AATTGTTGTTCTACTGATCCACAAGTCTTAC	N/A
PCR primer for the ZEBOV gblock, with T7 for *in vitro* transcription	GCGCTAATACGACTCACTATAGGGTGCGCGTTCCTACTGTATT	N/A
qPCRT-ZEBOV-R (RT reverse primer)	GAGTCCTTGGGTGCAGTTATATT	N/A

† Integrated DNA Technologies naming conventions. *Underlined regions indicate complementarity between the Acrydite oligo X and Linker-15 or Linker-15-F of the hydrogel; italics indicate complementarity between the Acrydite oligo Y and Linker-15 or Linker-15-F of the hydrogel; bold indicates sequences in the gRNA templates that correspond to the pathogen detection targets *mecA, ermA, ermC, spa* and *vanA*.

Equipment

- Thermal cycler (Bio-Rad T100)
- Gel imager (G:Box mini 9 gel imager, Syngene USA)
- Microplate reader: Synergy NEO HTS (BioTek) / SpectraMax M5 (Molecular Devices)
- Freeze-dryer (Freezemobile, SP scientific) [Optional, Step 19 Option E]
- Inverted microscope: EVOS FL (Thermo Scientific) [Optional, Step 19 Option D]
- Digital stirring hot plate (Cole Parmer, IKA C-Mag HS)
- Benchtop microcentrifuge (Corning LSE)
- Wax (solid ink) printer (Xerox Phaser 8560) [Optional, Step 19 Option E]
- Hotpress (Cricut EasyPress) [Optional, Step 19 Option E]
- Modified PIXMA iP7220 Inkjet Photo Printer (Canon) [modified as described by Lee *et al.*[45]] with QY6-0082 printhead and refillable cartridges [Optional, Step 19 Option E]
- Benchtop Digital Multimeter (Keysight 34411A) [Optional, Step 19 Option E]
- UHF RFID Antenna (Sparkfun WRL14131) [Optional, Step 19 Option E]
- Simultaneous RFID Reader - M6E Nano SEN14066 (SparkFun) [Optional, Step 19 Option E]
- Arduino Uno Rev3 microcontroller (Arduino #8058333490090) [Optional, Step 19 Option E]
- Incubator (37ºC)
- Orbital rocker (Boekel 201100)
- Automated Cell Counter (ThermoFisher Scientific, A27974) [Optional, Step 19 Option D]
- BL2 tissue culture facility with laminar flow hood and standard incubator (37ºC, 5% CO_2) [Optional, Step 19 Option D]
- Microvolume Spectrophotometer (Thermo Scientific, ND2000C)

- Rainin Pipet-Lite LTS Pipette L-1000XLS+, L-200XLS+, L-20XLS+, L-2XLS+ (Mettler Toledo, 17014382, 17014391, 17014392, 17014393)
- Rainin pipette Tips LTS 1000µL 768/4 SS-L1000, LTS 250µL 960/5 SS-L250 and LTS 10µL 960/5 SS-L10 (Mettler Toledo,17007090, 17005875, 17005873)
- Conical centrifuge tubes, 15mL and 50mL (Corning 352097,3520980)
- 384-well microplates, low volume, non-binding surface, black with clear flat bottom (Corning, 3544)
- 96-well microplates, black with clear flat bottom, non-tissue culture-treated (Corning, 3631). **Critical**: Tissue culture-treated microplates are hydrophilic and can make hydrogel casting more difficult.
- Microplate aluminum sealing tape (Corning, 6570)
- Press-to-Seal silicone isolators (round, 2.0mm diameter, 1.7mm depth, ThermoFisher Scientific, USA, GBL665208-25EA) [Optional, Step 19 Option C]
- Microseal 'B' Seals (Bio-Rad, MSB1001)
- 24-well cell culture plate (flat bottom with lid, tissue culture-treated, Corning, 3526)
- Tru-Punch Sterile Disposable Biopsy Punch 5mm (Sklar, 96-1120) [Optional, Step 19 Option C]
- Axygen PCR Tubes with Flat Cap, 0.2ml (Fisher Scientific, PCR02C)
- 1.7mL microcentrifuge tubes (VWR, 87003-294)
- Ultrahigh-frequency (UHF) RFID tag (Sparkfun, WRL-14147) [Optional, Step 19 Option E]
- Amicon ultra 0.5mL centrifugal spin filters, 10KDa MWCO (Millipore Sigma, UFC501008)
- Nickel/copper conductive fabric tape (Laird Technologies, 87580) [Optional, Step 19 Option E]
- Chromatographic filter paper, Whatman Grade 1 (ThermoFisher Scientific) [Optional, Step 19 Option E]
- Vacuum filter/storage bottle system, 500mL, 0.22µm pore size (Corning, 431097)

Reagents setup (3 hours)

Hybridizing two single-stranded target DNAs into dsDNA: If the target DNA is ordered as two complementary single-stranded DNA molecules, hybridize them as follows:

1. Dilute each of the ssDNA molecules to be hybridized (ssDNA1 and ssDNA2) to a final concentration of 100µM in nuclease-free water.
2. Prepare the hybridization mixture by combining the reagents as follows:

Reagent	Volume (µL)
5M NaCl	0.5
10X TE buffer	5
ssDNA 1 (100µM)	22.5
ssDNA 2 (100µM)	22.5
Total	50.5

3. Anneal the ssDNA target sequences by performing a 5-min denaturation, then slowly cool the reaction to 4°C in a PCR thermocycler. For the slow cooling of the samples, adjust the ramp rate to 0.04°C/s.

   Pause point: The hybridized DNA can be used immediately, or aliquoted and stored at -20ºC for several months.

FITC-Dextran stock solution (Optional, Step 19 Option B). Dissolve FITC-Dextran particles in nuclease-free water to a final concentration of 2.5mg/ml. Aliquot this stock into microcentrifuge tubes and store them at -20ºC until needed.

Polyacrylamide hydrogel reagents (Optional, Step 19 Option C). Dilute the 50X TAE buffer and mix it with magnesium acetate to make a 10X TAE, 125mM magnesium acetate solution, which will be a 10X concentration of the reaction buffer. The 10X TAE buffer can be stored at 4°C for 2 weeks. Prepare fresh 20% v/v TEMED and 2% w/v APS solutions in nuclease-free water before each polymerization reaction.

24-well plates for acrylamide gel release assays (Optional, Step 19 Option C). Using a 5mm diameter tissue biopsy punch, cut Press-to-Seal silicone isolators into rings such that the wells are at the center. Using tweezers, stick the silicone isolators in 24-well plates so that their centers align with the middle of the wells. Confirm that the isolators do not obstruct the optical path of the plate reader by measuring the absorbance of each well at an arbitrary visible spectrum wavelength; the absorbance value should be identical to that of an empty well. Do not use obstructed wells.

Cell culture media (Optional, Step 19 Option D). Make R10 medium by combining RPMI-1640 with 1X GlutaMAX supplement, 10% (v/v) FBS, and 2ng/ml recombinant IL-2. Make O10 medium by mixing Opti-MEM with 10% (v/v) FBS. Prepare sterile working solutions of $MgCl_2$ 200mM by diluting and filtering a 2M $MgCl_2$ stock. Sterile media can be stored at 4°C for up to 2 weeks. Warm up all media that will be in contact with cells in a 37ºC bath before use.

PROCEDURE

Procedure A: Designing and producing gRNAs and dsDNA triggers (1 day)

1. ***Designing the guide RNA (Steps 1-5):*** Choose an 18-24 nt target sequence within the marker that is immediately in 3' of a TTTV (where V indicates a non-T DNA base) protospacer-adjacent motif (PAM) region, which is specific for Cas12a. The target sequence should not contain additional TTTV motifs.
   CRITICAL STEP There are several publicly available resources to help design efficient gRNAs: Benchling (https://benchling.com/pub/cpf1), Broad Institute GPP sgRNA designer (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design), Zhang Lab - Guide Design Resources (https://zlab.bio/guide-design-resources)
   CRITICAL STEP dsDNA markers can be bound and detected by the Cas12a-gRNA complex with high sensitivity. Cas12a is also able to bind ssDNA in a sequence-specific manner, but the level of activation of collateral *trans*-cleavage is lower than with dsDNA

(see Fig. 2 of Ref. 22). ssRNA markers require a reverse-transcription and amplification step to convert the signal into dsDNA (see Step 19 Option E.xix).
CRITICAL STEP Different Cas enzymes require distinct PAM sites. It should be noted that the gRNA design and assembly described here uses a negative-sense template and a short T7 promoter adapter oligo for the production of the gRNA through *in vitro* transcription (IVT, Fig. 2a). Extension of the oligo is not needed, as T7 RNAP can actively transcribe dsDNA or ssDNA templates[46].
CRITICAL STEP As a negative control in all downstream Cas12a activation experiments, we recommend using a synthetic, sequence-randomized ("scrambled") dsDNA molecule.

~~1.~~2. Start the gRNA design (5' to 3') by taking the reverse-complement of a nuclease-specific gRNA direct repeat (the underlined sequence in the gRNA in Table 2). This is the same for all the LbaCas12a gRNAs used in our experiments. **TROUBLESHOOTING.**
CRITICAL STEP Different Cas enzymes may require distinct direct repeats in the gRNA.

~~2.~~3. In the position immediately upstream (5') of the sequence from Step 2, include the reverse-complement of the 18-24 nt target-specific RNA sequence as determined in Step 1 (shown in blue in Table 2).

~~3.~~4. Check that the PAM (red in Table 2) is immediately on the 5' end of the target DNA sequence that matches the guide RNA (Fig. 2b).

~~4.~~5. For the production of the gRNA *in vitro* using T7 transcription (Steps 6-14), add the reverse-complement of the T7 promoter at the 3' end of the designed sequence. We included the three guanines present in the T7 promoter consensus sequence (Table 2 and Fig. 2a) to increase transcription yield. Order the resulting T7 adapter oligonucleotide and matching gRNA template oligonucleotide sequences from regular vendors such as Integrated DNA Technologies; a standard scale (25nmol) and purity (desalted) are sufficient. **TROUBLESHOOTING.**

Table 2 - Design of gRNA molecules and their transcription templates for five different example DNA target sequences derived from staphylococcal antibiotic-resistance genes. Lowercase: protospacer-adjacent motif; bold: target sequence; italics: T7 promoter; underlined: Cas12a gRNA handle.

Target name	gRNA IVT template (5'-3')	gRNA (5'-3')	target (5'-3')
mecA	**CCACATACCATCTTCTTTAA**AT CTACACTTAGTAGAAATTACCC*CT ATAGTGAGTCGTATTAGCGC*	GGGUAAUUUCUACUAAGU GUAGAU**UUAAAGAAGAU GGUAUGUGG**	TTTAATtttg**TTAAAGAAGAT GGTATGTGG**AAGTTAGATT
ermA	**TCCATCTCCACCATTAATAG**AT CTACACTTAGTAGAAATTACCC*CT ATAGTGAGTCGTATTAGCGC*	GGGUAAUUUCUACUAAGU GUAGAU**CUAUUAAUGGU GGAGAUGGA**	GCTTTGGGttta**CTATTAATG GTGGAGATGGA**TATAAAA A
ermC	**ACATGCAGGAATTGACGATT**A TCTACACTTAGTAGAAATTACCC *TATAGTGAGTCGTATTAGCGC*	GGGUAAUUUCUACUAAGU GUAGAU**AAUCGUCAAUUC CUGCAUGU**	TAATATTGttta**AATCGTCAA TTCCTGCATGT**TTTAAGGA
spa	**ACAAAGCTCAAGCATTACCA**A TCTACACTTAGTAGAAATTACCC *TATAGTGAGTCGTATTAGCGC*	GGGUAAUUUCUACUAAGU GUAGAU**UGGUAAUGCUU GAGCUUUGU**	TTCACCAGtttc**TGGTAATGC TTGAGCTTTGT**TAGCATCT
vanA	**CTCGACTTCCTGATGAATAC**AT CTACACTTAGTAGAAATTACCC*CT ATAGTGAGTCGTATTAGCGC*	GGGUAAUUUCUACUAAGU GUAGAU**GUAUUCAUCAG GAAGUCGAG**	ACGGAATCtttc**GTATTCATC AGGAAGTCGAG**CCGGAAA A

5.6. ***Synthesizing the gRNA by in vitro transcription (Steps 6-14):*** Dissolve the gRNA template oligonucleotide in nuclease-free water at a final concentration of 50µM (e.g., 80µL for a 4nmol tube) by pipetting the water volume into the tube containing the gRNA 2-3 times, gently shaking and centrifuging to bring all the liquid to the bottom for collection. **CRITICAL STEP**: Use nuclease-free reagents for all procedures that involve the manipulation of RNA.

6.7. To synthesize the gRNA *in vitro*, the two oligonucleotides are assembled as illustrated in Fig. 2a. Prepare the annealing reaction in a PCR tube by combining the following components:

Reagent	Volume (µL)
5M NaCl	0.5
1X TE buffer	47
T7 adapter oligonucleotide (100µM)	0.55
gRNA template oligonucleotide (50µM)	1
Total	49.05

7.8. Anneal the gRNA template and T7 adapter oligonucleotide by performing a 5-min denaturation at 95ºC, then slowly cool the reaction to 4°C in a PCR thermocycler at a ramp rate of 0.04°C/s.

PAUSE POINT: The annealed IVT gRNA templates can be stored at -20ºC for several weeks.

8.9. Assemble the IVT reaction using the NEB HiScribe T7 Quick High Yield RNA Synthesis Kit in 200µL PCR tubes:

Reagent	Volume (µL)
Annealed gRNA template	8
HiScribe Quick master mix (10X)	10
T7 polymerase	2
Total	20

9.10. Incubate the reaction at 37ºC for 4h or overnight.

10.11. Digest the DNA template with DNase I by adding 30µL of water and 2µL of DNase I to the reaction mixture. Incubate the samples at 37ºC for 15min to allow the digestion to complete.

11.12. Heat-inactivate the DNase I by incubating the samples at 75ºC for 10min.

12.13. Proceed with RNA cleanup using a Zymo RNA Clean and Concentration kit, according to the manufacturer's instructions.

13.14. Measure the concentration of the gRNA on a microvolume spectrophotometer. The absorbance ratio A260/A280 should be above 1.8 and the ratio A260/A230 should be above 2.0.

Pause point: The RNA can be used immediately, or aliquoted and stored at -80ºC for several weeks.

14.15. **In vitro *activity assays with Cas12a (Steps 15-18):*** Assemble the Cas12a-gRNA complex by incubating 200nM Cas12a with 250nM gRNA (from Step 14) in 1X NEB 2.1

buffer at 37ºC for 10min. Prepare 1µL for each point of the calibration curve in the next steps. Make sure to account for technical replicates.

~~15.~~16. Prepare a calibration curve with dsDNA trigger (from Step 15-18) present at decreasing concentrations between 100nM to 10pM. The reaction mixture should also contain 1X NEB 2.1 buffer and 1µM quenched, fluorescently labeled reporter ssDNA (hereafter referred to as "FQ reporter", see Table 1). Prepare a minimum of 3µL for each point of the calibration curve.

~~16.~~17. Mix 1µL of the Cas12a-gRNA complex from Step 15 with 3µL of the trigger-FQ reporter mix from Step 16 to obtain final concentrations of 50nM Cas12a, 62.5nM gRNA, and 750nM FQ reporter in 1X NEB 2.1 buffer.
CRITICAL STEP We routinely use mung bean nuclease (MBN) as a positive control for the degradation of the FQ reporter by a known ssDNA-specific endonuclease. Mix 0.8µL of MBN with a 3.2µL solution of 940nM FQ reporter in 1X NEB 2.1 buffer. The fluorescence signal observed in the MBN reaction should be similar to the fluorescence obtained with Cas12a when using a high concentration of dsDNA trigger (50nM).

~~17.~~18. Incubate the 3µL reactions in a 384-well microplate and place the plate in the plate reader (Biotek NEO HTS). Record fluorescence readings every 5min (excitation: 485nm; emission: 535nm) for 120min at 37°C.
TROUBLESHOOTING.

Creating CRISPR-responsive DNA hydrogels

~~18.~~19. For fluorophore or enzyme-release from CRISPR-responsive PEG hydrogels, follow Option A or B respectively. For CRISPR-responsive polyacrylamide hydrogels encapsulating entrapped nanoparticles or cells follow Option C or D respectively. For CRISPR-responsive µPAD diagnostics follow Option E. See Experimental Design for details.

Option A: Fluorophore release from CRISPR-responsive PEG hydrogels (3 days)

i. ***PEG-DNA gel synthesis (Steps i-xii):*** Design and purchase Cas12a-sensitive, AT-rich (>70%) ssDNA linkers (20-40 nt) that exhibit little secondary structure. *In silico* tools to verify the thermodynamic properties of interacting nucleic acid strands such as the IDT OligoAnalyzer (www.idtdna.com/oligoanalyzer) can be used to confirm the absence of secondary structure. Pick sequences such that all predicted secondary structures have a melting temperature below 30°C. To avoid unpredicted intermolecular interactions, make sure the ssDNA linkers show no complementarity to the DNA target and gRNA designed in Steps 1-5. Include a protected 5'-end thiol modification for the attachment to the PEG-vinyl sulfone groups (see examples in Table 1) and a 3'-chemical handle that does not react with thiols or vinyl sulfones for the attachment of the cargo molecules. In the example used here, the molecular cargo (cyanine dye Cy3) was grafted onto the 3' end of the oligos during DNA synthesis by the manufacturer.

ii. Resuspend the pellet of oligonucleotide linkers at 500µM in nuclease-free water. If the cargo is not pre-attached, attach the molecule of interest on the DNA using the chemistry

of choice, making sure to thoroughly purify the functionalized product, and adjust its molarity. **TROUBLESHOOTING**.

iii. Mix 10µL of oligonucleotides (5nmol) with 0.5µL of TCEP (250nmol). Incubate the reaction mix for 4h at room temperature (20-23°C) in the dark to chemically reduce the protecting groups on the ssDNA.

iv. Resuspend an aliquot of PEG-VS powder at a concentration of 4% w/v in 1M TEA buffer. Make sure to consider the volume of the PEG powder based on the density indicated by the manufacturer. **CRITICAL STEP**: The TEA acts as a buffer, and also as a catalyst in the subsequent Michael-type thiol-ene addition reaction with the PEG macromers. If the use of TEA is detrimental for your application, consider testing another tertiary amine catalyst, or using free-radical addition chemistry.

v. Add 62.5µL of PEG-VS solution to the reduced oligonucleotides from Step iii and incubate overnight (18h) in the dark at room temperature. **CRITICAL STEP**: This incubation ensures that the majority of the ssDNA is grafted onto the PEG-VS precursors. Any unreacted DNA (and attached cargos) will diffuse away from the gels during the wash step that follow the hydrogel formation (Step xii).
PAUSE POINT: The functionalized PEG-VS can be stored at 4°C for an additional 24h.

vi. In the tube containing the DNA-functionalized PEG-VS, add an additional 62.5µL of PEG-VS solution. This should be diluted in 1M TEA to the appropriate concentration (e.g., 2% w/v to make a 1.5% PEG-DNA gel; see Box 1) from the stock prepared in Step iv.

vii. Resuspend an aliquot of PEG-SH powder in water at the appropriate concentration (e.g., 3% w/v to make a 1.5% PEG-DNA gel; see Box 1). **TROUBLESHOOTING**.

viii. On ice, add 125µL of the PEG-SH solution from Step vii to the solution of functionalized PEG-VS macromers from Step vi. Adjust the volume to 500µL with water (239.5µL).

ix. Dispense the hydrogel precursor solution into the final containers while it is still liquid. The polymerization time will depend on the hydrogel concentration and should take at least 5-10min if the solution is kept cold. For the subsequent real-time monitoring of the reactions in a 96-well plate using a plate-reader, tilt a non-treated, flat-bottom plate at an angle of about 45°, and cast 5µL of the precursor on the lower edges of the wells (see Fig. 4a). **CRITICAL STEP**: The kinetics of in-gel Cas12a actuation are affected by diffusion, and therefore by the shape of the hydrogels. It is important to cast the gels with a consistent size and shape. This will help to maximize the reproducibility of the experiment. **TROUBLESHOOTING**.

x. Seal the wells of the 96-well plates using light- and gas-impermeable microplate sealing tape. Leave the gels in the tilted plate at room temperature overnight to allow for the polymerization of the PEG macromers and the formation of the hydrogels.

xi. Block any unreacted vinyl sulfone groups by incubating the gels for 4h at 37°C in a large excess of 20mM dithiothreitol freshly dissolved in 1X NEB 2.1 buffer.

xii. Wash the gels in a large volume (at least 30-fold excess) of 1X NEB 2.1 buffer at 37°C. Replace the buffer at least three times, incubating the gels for no less than 4h each time to ensure complete equilibration between the gels and supernatant. **PAUSE POINT**: If the plate is properly sealed to prevent dehydration, the hydrogels can be stored in 1X NEB 2.1 buffer at 4°C for several days. Bring the gels back to a temperature of 37°C for several hours before use, as the equilibrium swelling volume is temperature-dependent.

xiii. ***Cas12a-mediated fluorophore release from PEG-DNA hydrogels (Steps xiii-xvii):*** Prepare a fresh stock of gRNA-primed Cas12a enzyme (1µM Cas12a with a two-fold molar excess of gRNA from Step 14) in 1X NEB 2.1 buffer. Incubate the stock for 10min at 37°C to allow the enzymes and the gRNAs to form functional complexes.

xiv. Overlay the 5µL cargo-functionalized PEG-DNA hydrogels (from Step xii) with 95µL of 1X NEB 2.1 buffer pre-warmed to 37°C.

xv. Add 5µL of warm Cas12a-gRNA solution from Step xiii to the supernatant of each hydrogel to reach a final enzyme concentration of 50nM. From this point on, incubate the gels at 37°C on a rocker bed. For experiments monitoring the release of a fluorophore in real-time in a microplate reader, set the instrument to shake the plate for 1s every minute. **CRITICAL STEP**: Include reference samples that lack Cas12a enzyme but contain unattached cargo at the theoretical concentration expected for the scenario in which 100% of it is released. For example, for a 5µL hydrogel made from precursors containing 10µM of oligonucleotide-bound fluorophore, make a control overlaid with 100µL of buffer containing 476nM of cargo.

xvi. Start monitoring the release of the cargo from the gels (see Fig. 4b). For the real-time detection of a fluorescent reporter, pick wavelengths that maximize the signal-to-noise ratio rather than the absolute amplitude (for Cy3, excitation: 555nm; emission: 625nm), and minimize the frequency of the measurements to limit photobleaching. If the cargo is not suitable for in-well reaction monitoring (e.g., enzymes), sacrifice wells at each timepoint by sampling the supernatant and testing for activity using the appropriate assay. **TROUBLESHOOTING**.

xvii. At the desired time point, spike in 1µL of a 5µM stock of the dsDNA trigger (in water, Tris, or PBS) from Step 16 to reach a 1:1 enzyme-to-target molar ratio. **CRITICAL STEP**: Include appropriate controls in your experiment, namely hydrogels exposed to Cas12a-gRNA only and hydrogels exposed to non-target dsDNA fragments.

Option B: Enzyme release from CRISPR-responsive PEG hydrogels (3 days)

i. ***PEG-DNA gel synthesis (Steps i-x):*** Design and purchase Cas12a-sensitive, AT-rich (>70%) ssDNA linkers (20-40 nt) that exhibit little secondary structure. Include a 5'-end thiol modification for their attachment to the PEG-vinyl sulfone groups (see examples in Table 1) and a 3'-end biotin modification for the attachment of the enzyme. *In silico* tools to verify the thermodynamic properties of interacting nucleic acid strands such as the IDT OligoAnalyzer (www.idtdna.com/oligoanalyzer) can be used to confirm the absence of secondary structure. Pick sequences such that all predicted secondary structures have a melting temperature below 30°C. To avoid unpredicted intermolecular interactions, make sure the ssDNA linkers show no complementarity to the DNA target and gRNA designed in Steps 1-5.

ii. Obtain streptavidin-tagged enzymes that can be attached to the material. In the example illustrated here, we use horseradish peroxidase (HRP). **TROUBLESHOOTING**.

iii. Synthesize 3µL PEG-DNA hydrogels in PCR tubes using the same method as outlined in Step 19 Option A.ii-ix, with a final PEG concentration of 1.5% w/v, and 5µM of ssDNA anchors.

iv. After casting the gels, wash them overnight in 100µL 1X NEB 2.1 buffer to remove unreacted ssDNA.

v. Make a fresh stock of 20mM dithiothreitol in 1X NEB 2.1 buffer, accounting for 200µL of buffer per gel and a small degree of pipetting error.

vi. Block the unreacted vinyl sulfone groups by incubating the gels with 200µL of 20mM dithiothreitol in 1X NEB 2.1 buffer for 4h at 37˚C.

vii. Rinse the gels three times in 200µL 1X NEB 2.1 buffer, incubating for 1h at 37˚C each time.

viii. After the final rinse, incubate the gels for 16h at room temperature in a 200µL solution of 1X NEB 2.1 buffer containing 55µg/ml of streptavidin-conjugated HRP from Step ii.

ix. Wash the enzyme-conjugated gels through successive incubations of no less than 2h in room-temperature 1X NEB 2.1 buffer until there is no residual HRP activity in the supernatant. To measure the HRP in the supernatant, take a 20µL aliquot and dilute it 5-fold in a TMB substrate solution (80µL). Incubate the reaction for 20min at 37˚C before checking for the appearance of the blue TMB oxidation product, as per the manufacturer's guidelines, making sure to include negative controls. Typically, this requires 5-10 rounds of washing.

~~ix.~~x. Confirm the immobilization of the active enzyme in the gel by overlaying the gel with 50µL of TMB substrate. The substrate should be rapidly oxidized in contact with the gel.

~~x.~~xi. ***Cas12a-mediated enzyme release from PEG-DNA hydrogels (Steps xi-xvi):*** Prepare a reaction mix on ice in 1X NEB 2.1 buffer containing 10nM Cas12a, 20nM gRNA (from Step 14), and 10nM trigger or scrambled dsDNA (Step 16, Table 1). Pre-warm the reaction mix to 37˚C immediately before the assay.

~~xi.~~xii. Overlay 20µL of the reaction mix onto each 3µL gel sample from Step 15. Incubate the gels at 37˚C on a rocker bed. The supernatant volume can be increased to 50µL if desired.

~~xii.~~xiii. At the desired time points, sacrifice replicate samples by removing their supernatants and store these at 4˚C until all time points have been collected.

~~xiii.~~xiv. Once the final time point has been reached, pipette 10µL of each supernatant sample into 40µL TMB substrate in a 96-well plate (a five-fold dilution).

~~xiv.~~xv. Incubate the plate at 37˚C with shaking for 10min and then block the reaction with 50µL of 1M sulfuric acid.

~~xv.~~xvi. Measure the absorbance at 450nm using a plate reader. **TROUBLESHOOTING**.

Option C: Nanoparticle release from CRISPR-responsive polyacrylamide-DNA hydrogels (3 days)

i. ***Designing and ordering the DNA components (Steps i-ii).*** For the preparation of acrylamide-DNA gels, three single-stranded DNA oligos are required: two 5'-methacryl functionalized strands that are separately incorporated into polyacrylamide chains (designated X and Y), and a bridging "linker" strand (designated L-15) that can be

functionalized with fluorescent dyes (resulting in L-15F) (see Table 1). Design each 5'-methacryl oligo X and Y to have a 5nt TTATT sequence, followed by a 15bp sequence that binds to the linker strand. The linker strand has two 15bp regions that bind to X and Y separated by a 15bp sequence consisting of a $(TTATT)_3$ motif (Fig. 5) See Table 1 for example sequences. We outsourced the 1µmol scale synthesis of HPLC-purified ssDNAs to IDT. **TROUBLESHOOTING**.

CRITICAL STEP L-15F should be used in place of L-15 when observing the gel itself using fluorescence imaging.

~~i.~~ii. Resuspend oligos X, Y, L-15F and L-15 to a stock concentration of 3mM in nuclease-free water and vortex them for 10s to ensure the pellet dissolves.

PAUSE POINT: The DNA stocks can be used immediately or stored at -20˚C for up to 2 months.

~~ii.~~iii. ***Polymerizing the gel precursors (Steps iii-x).*** Prepare a 10X concentration stock of hydrogel buffer (TAE/Mg^{2+}) in advance as follows:

Reagent	Volume (mL)
50X TAE buffer	20
1M Magnesium acetate	12.5
Nuclease-free water	67.5
Total	100

~~iii.~~iv. For a typical preparation of the gel precursor strands X and Y, perform 180µL reactions in 1.7ml microcentrifuge tubes. This protocol can be scaled down to 50µL and up to 400µL. For 4% w/v acrylamide gels, add the reagents below in the following order. Prepare one gel precursor for the strand X and one for strand Y:

Reagent	Dilution	Final concentration	Volume (µL)
10X TAE/Mg^{2+} buffer	10X	1X	18.0
3mM X or Y	3X	1mM	60.0
40% w/v acrylamide	10X	4%	18.0
Nuclease-free water			75.0
		Total	171

CRITICAL STEP For 7% w/v acrylamide gels, instead add 31.5µL 40% w/v acrylamide and 55.5µL nuclease-free water.

~~iv.~~v. Vortex the microcentrifuge tubes briefly and spin them down for 3s at 2000×g (room temperature) in a centrifuge to collect their contents.

~~v.~~vi. Place the open tubes under a vacuum for 10min to de-gas the solutions. We use a vacuum desiccator chamber with the desiccant removed.

~~vi.~~vii. During this incubation period, prepare fresh stocks of 2% w/v ammonium persulfate (APS) in 1mL nuclease-free water, and 20% v/v tetramethylethylenediamine (TEMED) in 0.5mL of nuclease-free water (see Reagent Setup).

~~vii.~~viii. Add 4.5µL of the APS stock to the polymerization reaction from Step vi, invert the tube a few times to mix the reagents, and then spin it down briefly to collect the contents of the tube. **CRITICAL STEP**: This step is time-sensitive, proceed rapidly to the next step.

viii.ix. Add 4.5µL of the TEMED stock to the polymerization reaction, invert the tube a few times to mix the reagents, and then spin it down briefly to collect contents of the tube. **CRITICAL STEP**: This step is time-sensitive, proceed rapidly to next step.

ix.x. Place the microcentrifuge tubes in the vacuum chamber again to allow the polymerization to proceed under vacuum for 15min at room temperature. **PAUSE POINT:** The gel precursors PA-X and PA-Y can be stored in TAE/Mg^{2+} buffer at 4˚C for up to a month. **TROUBLESHOOTING.**

x.xi. ***Cross-linking and handling macroscopic polyacrylamide-DNA gels (Steps xi-xii).*** To generate the cross-linked hydrogel, mix equal volumes of the polymer solutions PA-X and PA-Y (e.g., 10µL each) from Reagent Setup with 6µL of the linker DNA (3mM stock) for a final ratio of 1:1:0.6. **TROUBLESHOOTING**.

xi.xii. After adding the linker DNA, mix the gel gently with the tip of a pipette and incubate it at room temperature for 10min. The gelation process should begin immediately.

xii.xiii. ***Qualitatively assessing DNA incorporation into polyacrylamide chains (Steps xiii-xix).*** Prepare a 2% w/v agarose gel containing 1X NorthernMax-Gly running buffer for electrophoresis.

xiii.xiv. As standards for the unreacted methacryl oligos (X and Y), mix 4µL aliquots containing 10pmol of each oligo from Step ii with 4µL of NorthernMax-Gly gel loading dye.

xiv.xv. Take 2µL samples from the polymerized precursors (4% or 7% PA-X and PA-Y, Step xii), mix them with 2µL of nuclease-free water, and add 4µL of NorthernMax-Gly gel loading dye.

xv.xvi. Heat the samples from Step xiv and xv with NorthernMax™-Gly gel loading dye in PCR tubes at 50℃ for 30min.

xvi.xvii. Submerge the 2% w/v agarose gel in NorthernMax-Gly gel running buffer.

xvii.xviii. Load a low molecular weight DNA ladder, the unreacted oligo standards , and the polymerized gel precursors into the agarose gel.

xix. Run the gel for 1.5h at 80V, and image the gel under UV light using a standard gel imager. If the incorporation of ssDNA into the polyacrylamide chains was successful, the lanes containing PA-X and PA-Y should appear as high molecular weight smears, while the low molecular weight bands of the unreacted oligonucleotides should be absent or very faint.

xviii.xx. ***Preparing PEG-functionalized gold nanoparticles (AuNPs) (Steps xx-xxvi).*** Prepare 50mL of aqueous solution, containing 0.1mg/mL of gold (III) chloride trihydrate. **CRITICAL STEP:** The procedure in this section describes how to probe the release of PEG-functionalized gold nanoparticles (AuNPs) from polyacrylamide-DNA hydrogels. The procedure can easily be adapted to study the release of FITC-Dextran. In order to do so, skip steps xxi-xxvi and directly proceed to 'preparing the Cas12a reaction master mix' in Step xxvii.

xix.xxi. Heat the solution and stir it vigorously on a plate heater until it boils.

xx.xxii. Add 1ml of 20mg/mL sodium citrate to the reaction while continuing to heat and stir for 15min. **CRITICAL STEP:** You should see a color change from faint yellow to deep red indicating AuNP formation.

xxi.xxiii. Turn off the heat and continue to stir the reaction until the solution reaches room temperature.

~~xxii.~~xxiv. Add 0.33ml of 2mM, 5kDa thiol terminated PEG. Mix the reaction well and leave it overnight at room temperature to allow PEG conjugation to the AuNPs.

~~xxiii.~~xxv. Use a UV-Vis spectrophotometer to determine the concentration of the NPs based on an extinction coefficient of $5.7\times10^{8}M^{-1}cm^{-1}$ at 520nm.

~~xxiv.~~xxvi. To wash and concentrate the AuNPs to the desired level, use a centrifuge to spin down the solution in microcentrifuge tubes at 13000×g for 30 minutes (room temperature). Remove most of the supernatant and replace it with the wash buffer, or adjust the final volume to the desired concentration.

PAUSE POINT: The PEG-stabilized AuNPs can be stored for months at room temperature or at 4˚C. Do not freeze solutions containing gold nanoparticles, as they will be irreversibly aggregated. **TROUBLESHOOTING**.

~~xxv.~~xxvii. ***Preparing the Cas12a reaction master mix (Steps xxvii-xxvii).*** In a PCR tube on ice, prepare the Cas12a-gRNA master mix, containing NEB Cas12a (10µM) and gRNA from Step 14 (15µM) in 1X NEB 2.1 buffer. Each hydrogel sample being assayed will require 1µL of the Cas12a-gRNA master mix. Remember to account for pipetting error by preparing a slight excess of the master mix. For example, 15µL of the Cas12a-gRNA reaction master mix is prepared by combining the following components in order:

Reagent	Dilution	Final concentration	Volume (µL)
Nuclease-free water			10.87
10X NEB 2.1. buffer	10X	1X	1.50
NEB Cas12a 100µM	10X	10µM	1.50
gRNA (200µM stock)*	13X	15µM	1.13
		Total	15

*This component is produced in Step 14. and can be adjusted to the desired concentration after the *in vitro* transcription process.

CRITICAL STEP Prepare the Cas12a master mix immediately before preparing the acrylamide-DNA hydrogels for gel release experiments.

~~xxvi.~~xxviii. ***Releasing AuNPs or FITC-Dextran from polyacrylamide-DNA hydrogels (Steps xxviii-xxxvi).*** Prepare a 24-well tissue culture plate for the AuNP release measurement according to the diagram in Fig. 6.

~~xxvii.~~xxix. Prepare the gels in volumes of 9µL in PCR tubes at room temperature by combining the following reagents in order:

Reagent	Prepared in Step:	Volume (µL)
PA-X (4% or 7%)	23.C.x	3
PA-Y (4% or 7%)	23.C.x	3
10X NEB 2.1 buffer	Reagent Setup	0.67
PEGylated AuNPs (5µM)	23.C.xxvi	1
Cas12a-gRNA master mix	23.C.xxvii	1
Trigger or scrambled dsDNA	Reagent Setup (Table 1)	0.33
	Total	9

CRITICAL STEP: Mix the contents of the PCR tube thoroughly by pipetting up and down gently, and mixing with the pipette tip. The 9µL volume can be scaled up to create enough volume to aliquot multiple gels.

CRITICAL STEP: The procedure can be adapted to probe the release of FITC-Dextran. In order to do this, replace the PEGylated AuNPs by 1µL 2.5mg/ml 500kDa MW FITC-Dextran.

~~xxviii.~~xxx. Transfer 9µL of the pre-gel mixture into the Press-to-Seal silicone isolators at the center of the wells of a 24-well tissue culture plate.

~~xxix.~~xxxi. To cross-link each gel, pipette 1µL of the 3mM ssDNA linker L-15 (from Step ii) directly into the 9µL pre-gel mix.

~~xxx.~~xxxii. Stir the gels briefly with a pipette tip to ensure the ssDNA linker is mixed, and incubate the plate at room temperature for 20min.

~~xxxi.~~xxxiii. After gelation, gently add 850µL of 1X NEB 2.1 buffer to each well and ensure the gels are fully submerged in the supernatant. If necessary, rock the plate gently by hand to cover the gels.

CRITICAL STEP: When adapting the approach to probe FITC-Dextran release, incubate the tubes at 37˚C without shaking. At the desired time-points (every 4h in our case), invert the tubes once and image them using a gel imager (excitation: 385nm; emission: 525nm). Steps xxxiv-xxxvi can be omitted. **TROUBLESHOOTING**

~~xxxii.~~xxxiv. Seal the plate using an optically clear plate seal, ensuring that the seal adheres across the whole plate to prevent evaporation.

~~xxxiii.~~xxxv. Using a plate reader, record the release of gold nanoparticles by measuring the absorbance at 520nm at the center of each well of the 24-well plate (Fig. 6). Maintain the plate at a constant temperature of 37˚C and record the absorbance at 2-min intervals for at least 15h.

~~xxxiv.~~xxxvi. To complement the absorbance reading of the gel itself, readings of the supernatant can be taken at the final time point. Take a 200µL aliquot of the supernatant from each sample and transfer it to a 96-well plate for measurement. As a standard for 100% nanoparticle release, include wells containing a 1:850 dilution of the AuNP starting stock from Step xx. Measure the absorbance at 520nm for each sample. **TROUBLESHOOTING**.

Option D. Human primary cell release from CRISPR-responsive polyacrylamide-DNA hydrogels (2 days)

i. ***Pre-culturing human primary cells (Steps i-v).*** First follow Step 19 Option C.i-ii to design and order the DNA components.

ii. Expand the PBMCs in R10 medium until they reach a density of about 0.5×10^6 /ml .

iii. Detach the PBMCs from the plate through gentle pipetting with a 10mL serological pipet.

iv. Centrifuge the cell suspension in a 15ml falcon tube at 300×g for 5min (room temperature) to collect the cells.

v. Resuspend the cells in R10 medium to a final concentration of 10^7cells/mL.

vi. ***Preparing reagents for cell encapsulation (Steps vi-xii).*** Follow Step 19 Option C.iii-x to create the PA-X and PA-Y gel precursors. Prepare pre-gel (partially crosslinked) PA-X and PA-Y stocks by mixing 10µL of PA-X, 10µL of PA-Y and 6µL of 10µM ssDNA linker (L-15).

vii. Mix the reaction well and let it crosslink for 10min at room temperature.

viii. To remove any unreacted acrylamide monomer that may compromise the viability of the cells, clean the pre-gelled solution using a centrifugal spin filter (Amicon, 10kDa 0.5mL spin filter). Wash the pre-gel sample with 0.45ml of 1X PBS and spin it down at 14,000×g for 15min (room temperature).

ix. Repeat the washing and filtering process (Step vii) two additional times.

x. Measure the concentration of ssDNA in the pre-gels with a microvolume spectrophotometer (Nanodrop). Dilute the sample in 1X PBS to reach a final concentration of ssDNA of 1.8mg/mL. In the upcoming steps, we will refer to this mixture as PA-XYb.

xi. Filter a 2M $MgCl_2$ stock solution using a 0.22µm membrane (e.g., steri-flip or syringe filter) inside a laminar flow hood while maintaining standard sterile technique.

xii. Prepare sterile working stocks of 200mM $MgCl_2$ in the laminar flow hood.

xiii. ***Generation of cell-encapsulating PA-DNA hydrogels (xiii-xviii).*** Prepare a 30µL aliquot of a suspension of ssDNA linker, $MgCl_2$, and human cells by combining the follow reagents in order:

Reagent	**Volume (µL)**
ssDNA linker L-15 (3mM)	3.6
Fluorescent ssDNA linker L-15F (3mM)	0.4
Cells at 10^7cells/ml [from step 19 D.v]	8
$MgCl_2$ (200mM) [from step 19 D.xii]	6
10X PBS	3
Nuclease-free water	9
Total	30

CRITICAL STEP: An intercalating DNA gel stain (e.g., EvaGreen) can be used instead of a fluorescent ssDNA linker L-15F.

xiv. Prepare a 30µL aliquot of clean PA-XYb pre-gel mixture (from Step ix).

xv. In a black, clear-bottomed 96-well plate, fill the wells immediately surrounding the well being used for the experiment with water. This helps to reduce evaporation from the gel during the upcoming incubation periods.

xvi. Just before depositing the gels, thoroughly mix 30µL of the filtered PA-XYb pre-gel (from Step xiv) with 30µL of the ssDNA linker/ $MgCl_2$/cell suspension (from Step xiii) until the gel is homogeneous and the flow is highly viscous.

xvii. Deposit 2µL droplets of the suspension containing human cells and the various gel components at the center of the wells of a 96-well plate. **CRITICAL STEP**: The black, glass-bottomed 96-well plate being used must be sterile and not be tissue-culture treated. Tissue-culture treated plates have a hydrophilic surface treatment that causes the gel droplets to spread.

xviii. Incubate the plates at 37ºC in a tissue culture incubator for 1-15min (2min is ideal; see Fig. 13) to allow the gels to cross-link. **TROUBLESHOOTING**.

xix. ***Depositing media on top of cell-loaded PA-DNA hydrogels (Steps xix-xx).*** Add 100 µL pre-warmed (37°C) O10 medium to the hydrogel and incubate for 5min at 37°C.
Critical: Mix the growth medium and deposit it into each well from the side, making sure not to dislodge the gel itself.

~~xix.~~xx. Observe the gels under a microscope to assess their integrity before any further testing. Gels should be homogeneous, with no signs of mechanical disruption.

~~xx.~~xxi. ***Releasing cells from PA-DNA hydrogels (xxi-xxiii).*** Make a 26µL aliquot of the Cas12a-gRNA master mix by combining the reagents below, and then add this gently to the well containing the PA-DNA hydrogel and cell culture media.

Reagent	**Volume (µL)**
Cas12a (100µM)	2
gRNA from Step 14	2
10X NEB 2.1 buffer	2
Trigger DNA (100µM) from Step 17	20
Total	26

CRITICAL STEP: Negative controls can be generated by substituting the trigger DNA with scrambled DNA. The concentration of the trigger dsDNA can be modified.
CRITICAL STEP: Avoid the mechanical disruption of the cell-encapsulating hydrogels by minimizing fluid shear and excessive movement.

~~xxi.~~xxii. Record the fluorescence of the gels (excitation: 490nm; emission: 535nm) on an EVOS FL inverted fluorescence or equivalent microscope to measure their degradation at 37ºC. We recommend imaging the gels every 1h to assess their degradation kinetics.

~~xxii.~~xxiii. Image the morphology of the PA-DNA hydrogels once the desired period of Cas12a degradation at 37˚C has elapsed. Acquire the images using a 4X magnification objective, 30% LED illumination intensity and 50% contrast. Compare these images with images of the initial state of the gels at the beginning of the experiment.

~~xxiii.~~xxiv. ***Assessing the viability of cells released from PA-DNA hydrogels (xxiv-xxv).*** Once the gel degradation experiment is complete, assess the viability of the cells by staining with ethidium homodimer-1 and calcein blue-AM, following the manufacturer's instructions.

~~xxiv.~~xxv. Observe viable PBMCs released from the hydrogels using the digital inverted microscope in DAPI mode (excitation: 360nm; emission: 447nm) with a 20X objective, 40% LED illumination intensity, and 90% contrast. Dead cells can also be imaged using the RFP mode (excitation: 530nm; emission: 593nm) with 50% LED illumination intensity and 90% contrast.

Option E: CRISPR-mediated µPAD diagnostics (2 days)

i. ***Fabricating the µPAD base construct (Steps i-xi).*** First follow Step 19 Option C.i-ii to design and order the DNA components.
ii. Prepare PA-X and PA-Y gel precursors as described in Step 19 Option C.iii-x.
iii. Align a sheet of chromatographic filter paper on the Xerox Phaser 8560 wax printer.

iv. Print side A of the provided pattern (Supplementary Data 1).

v. Turn the printed paper and print side B of the provided pattern (Supplementary Data 2), ensuring that the printed design is aligned on both sides of the paper. **CRITICAL STEP**: Validate µPAD alignment from both sides of the paper by checking against a light source. **TROUBLESHOOTING**.

vi. Wax reflow the printed µPADs to ensure wax penetration through the paper fibers by pressing each printed sheet for 15s at 125°C using a hot press. **CRITICAL STEP**: Different hot press equipment and substrates will require the optimization of reflow conditions to achieve similar results.

vii. Allow the µPADs to cool down to room temperature.

viii. Cut and fold the individual µPADs as shown (Fig. 10) so that the layers overlap like an accordion when pressed together. **CRITICAL STEP:** Ensure that layers 1 to 4 of the µPADs exhibit circular hydrophilic paper regions of approximately 1.5mm in diameter, surrounded by an evenly distributed hydrophobic wax coating to prevent undesired lateral flow. Layer 5 of the µPADs contains a 1.5x30mm lateral flow channel with marked lengths. **TROUBLESHOOTING.**

ix. Extend the µPAD reaction layers and fill layer 3 with 0.5µL of a mixture containing PA-X and PA-Y (1:1 v/v).

x. Fill layer 4 with 0.5µL of 1X PBS containing food color dye diluted 5X in PBS.

xi. Freeze-dry the paper µPADs with the deposited reagents for 4-8h using a lyophilizer. **PAUSE POINT:** The lyophilized µPADs can be stored for several days in airtight bags with desiccant pouches.

xii. ***Preliminary µPAD operation tests (Steps xii-xiv).*** Test the basic operation of freeze-dried µPADs by adding 0.3µL of either 1mM or 100µM ssDNA linker (L-15) to layer 2. Next, fold all of the layers together and deposit 10µL of PBS on top of layer 1. The folded layers can be held together using a standard paperclip or other clamp. In the case where 1mM L-15 is used, there should be no flow in the lateral channel due to crosslinking of the PA gel impeding capillary flow. Similarly, when using 100µM L-15, minimal flow should be observed in the lateral channel. This method can be used to validate the effective formation of gels in the µPAD at high concentrations of the ssDNA linker. **CRITICAL STEP**: Visible flow beyond 5mm on the lateral channel indicates ineffective gelation or defects in the fabrication of the µPAD. **TROUBLESHOOTING**.

xiii. Once both the processes of flow and gel polymerization have been validated in the µPAD, colorimetric tests can be conducted. To layer 2 of the µPAD, add 0.3µL of a 100µM solution of ssDNA linker (from Reagent Setup and Table 1) that has been pre-incubated for 4h at 37ºC with 300nM Cas12a, 1µM gRNA (from Step 7), and dsDNA trigger (from Reagent Setup and Table 1) in 1X NEB Buffer 2.1. To assess the sensitivity of the device, use varying concentrations of the dsDNA trigger (e.g., 0nM, 0.4nM, 2nM, 10nM, and 50nM).

xiv. Assess the degree of cleavage of the ssDNA bridge by Cas12a by measuring the length of flow of the buffer through the lateral channel of the µPAD at an endpoint time of 5min. The distance traveled by the dye-containing buffer should be correlated with the initial concentration of the dsDNA trigger in the pre-incubation reaction.

CRITICAL STEP. The µPAD design can be modified to generate analog and wireless digital electronic readouts. To do so, follow the instructions presented in Box 2 and Box 3, respectively. Then, proceed to the amplification and detection steps (steps xv-xxiii).

~~xiv.~~xv. ***RT-RPA CRISPR-µPAD tests for EBOV ssRNA (Steps xv-xxiii).*** Obtain a dsDNA fragment coding for the VP30 protein of the Zaire strain of the Ebola virus from a commercial manufacturer.
CRITICAL STEP: The RT-RPA section (Steps xv-xx) is only required for ssRNA targets, such as the Ebola virus. When the approach is used to detect a dsDNA target, Steps xv-xx can be omitted.

~~xv.~~xvi. Amplify the fragments by PCR and perform *in vitro* transcription using a HiScribe T7 Quick High Yield RNA Synthesis Kit (NEB) to produce synthetic ssRNA (see Table 1 for the relevant primers).

~~xvi.~~xvii. Purify the transcript using an RNA Clean and Concentrator Kit (Zymo), following the manufacturer's instructions.

~~xvii.~~xviii. Dilute the RNA by serial dilution. We recommend starting with a range of concentrations in the femtomolar to attomolar range.

~~xviii.~~xix. Perform reverse transcription reactions on the diluted RNA samples using a Superscript kit (Invitrogen). Follow the manufacturer's instructions for the kit and include the EBOV RPA-2 reverse primer (see Table 1) in the reaction.

~~xix.~~xx. Add 5µL of the reverse-transcribed RNA to lyophilized, 50µL RPA Basic reactions (TwistDx). Each reaction should contain 480µM of each RPA primer (see Table 1), and 14mM magnesium acetate. Perform the RPA reactions as per the manufacturer's instructions.

~~xx.~~xxi. Incubate the RPA reaction for 40min at 37ºC. Once the amplification is complete, add the ssDNA linker L-15 (from Step 19 Option C.ii), gRNA (from Step 14), and Cas12a to final concentrations of 300µM, 0.9µM, and 0.5µM, respectively.

~~xxi.~~xxii. Incubate the samples for an additional 4h, then dilute the reactions 1:1 v/v with nuclease-free water. We refer to the product of this step as the RT-RPA/linker mix.

~~xxii.~~xxiii. Test the µPADs by depositing 0.3µL of the RT-RPA/linker mix in layer 2. The µPAD should also contain 0.3µL of 4% (v/v) PA-X and PA-Y in layer 3, and 0.3µL of 1X PBS with red dye in layer 4. Once the reagents have been added to layers 2-4, they should be air-dried for 2min. To maintain contact between the hydrophilic sections of the device, ensure the µPAD is fully collapsed throughout the remainder of the experiment using a paperclip as a clamp. Finally, add 2µL of 1X PBS running buffer to layer 1 (the top) of the µPAD to initiate either colorimetric or electrical readings. **TROUBLESHOOTING**.

Troubleshooting
Troubleshooting guidance can be found in **Table 3.**

Table 3. Troubleshooting Table

Step	Problem	Possible reason	Solution

2	A low affinity of Cas12a for its target when designing new gRNA sequences.	The choice of direct repeat sequence can affect target binding performance, as has been discussed in genome editing contexts[47,48].	Check that the gRNA contains a direct repeat sequence that results in high binding performance.
5	IVT yield is lower than expected	T7 transcription can be affected by the bases that immediately follow the promoter sequence	If the yield of the transcript is low, 2G bases or a GCG motif can be tested instead of the 3G bases at the end of the T7 RNA polymerase promoter. This should not affect downstream Cas12a activity.
18	The negative control has a high background.	The gRNA may be contaminated with DNase I that was neither inactivated nor washed out in the Zymo spin column.	Repeat IVT steps 6-14 to produce fresh gRNA.
18	High concentrations of trigger do not lead to high fluorescence signals, as compared to a positive control with MBN.	The gRNA might be contaminated with RNases.	Repeat IVT steps 6-14 to produce fresh gRNA. If the problem persists, try selecting a new dsDNA target; it may be a target-specific issue.
18	Reactions performed with MBN show fluorescence, but there is no fluorescence in those with Cas12a.	The gRNA or Cas12a might be degraded.	Check that the gRNA sequence includes the direct repeat sequence motif and the target contains a PAM site (TTTV). Make a fresh stock of Cas12a-gRNA master mix, keeping it on ice and using it within 15min of preparation. Do not freeze and reuse aqueous dilutions containing Cas12a.

19.A.ii	The reporter molecules do not leave the gel after Cas12a activation.	The cargo presents exposed chemical moieties that may have reacted with the PEG precursors (e.g., surface cysteine residues of proteins may react with vinyl sulfones).	If the cargo cannot be modified to avoid cross-reactivity, selectively graft the cargo onto the DNA *in situ* after forming and blocking the PEG gels (e.g., by cycloaddition on 3'-azide modified DNA anchors, or as described for HRP).
19.A.vii	The gel polymerizes very slowly.	The PEG-SH macromers are oxidized.	Use freshly-made PEG-SH stocks.
19.A.ix	The gels are too dense to pipette.	The gels polymerized in the tube before they could be added to the microtiter plate wells.	Prepare fresh gels. Keep them on ice while transferring them to the microtiter plate.
19.A.xvi	The degradation behavior of the gel is highly variable across technical replicates.	Gel was deposited with inconsistent pipetting, which results in irregular gel shapes with different surface areas exposed to the degradation bulk.	Deposit your gels on the distal side of the wells, in the columns furthest from the center of the 96-well plate. Cover and spin the plate slowly (e.g., at 500×g) during the polymerization to flatten the gels and homogenize their shapes.
19.A.xvi	The degradation behavior of the gel is slower or faster than expected.	The use of containers with different material and geometries than the ones specified may led to differences in gel shape, exposed surface areas exposed to the bulk, which can affect the expected Cas12a diffusion and DNA degradation profiles.	Ensure the use of the specified materials, wells and tubes in all experiments.
19.A.xvi	Some of the gels exhibit a very high baseline release of cargo in the absence of trigger.	The gels were cast too close to the center of the wells, where the plate reader reads the optical signal. The instrument is therefore reading hydrogel fluorescence rather than signal from the solution.	Define custom plate dimensions in the plate reader software to ensure that the instrument reads fluorescence near the edge of the well, opposite to the hydrogels (see Fig. 4b).

19.B.ii	There are issues with the enzymatic activity when testing enzymes other than HRP.	If you are extending this protocol to test the release of other functional enzymes, then these will also need to be modified for DNA attachment. This may partially alter their functionality.	Validate that the attachment chemistry does not affect enzyme function.
19.B.xvi	There is no signal in the supernatant after incubating with Cas12a.	The ssDNA linkers were not cut.	Perform steps 15-18 to validate the activity of Cas12a (i.e., that the gRNA and Cas12a are not degraded).
19.C.i	The gel polymer strands are too viscous to handle.	The sequences and length of the complementary regions (15bp in our case) can be tuned according to the user's needs. However, we attempted experiments with an 18bp overlap for each of X and Y and the resulting gel polymer strands were too viscous to handle effectively.	Re-design the sequences and lengths of the complementary regions. Polymerized gel precursors can be heated to 37ºC or even up to 70˚C for short periods to help reduce viscosity while pipetting; however, avoid the repetitive heating and cooling of the same stock of PS-X or PS-Y.
19.C.x	There is no visible polymerization of the acrylamide.	While the polymerization does not lead to optical changes, it is possible to assess it by changes in viscosity or by comparing the size of DNA-stained bands on an agarose gel.	To check that polymerization has occurred, use a 10µL pipette tip to check for an increase in the viscosity of the reaction by pipetting up and down. Alternatively, run a 1µL aliquot of the reaction on an agarose gel and look for a very slow-moving band.

19.C.x	The polymerization is not successful.	APS and TEMED are sensitive to moisture and air.	Buy new APS and TEMED vials. These chemicals are sensitive to both moisture and air, and should be stored at 4˚C with a desiccant. Over time, the solutions become inactive. De-gas the water you use to make the APS and TEMED stocks. Oxygen can also be removed by bubbling an inert gas through the water for some time.
19.C.xi	Precursor strands are too viscous to pipette accurately.	The precursor strands can be highly viscous after storage at 4˚C, particularly in the case of the 7% acrylamide gels.	To ensure accurate pipetting, warm the stocks of PA-X and PA-Y to 50-70˚C in a heating block before handling. If the mixture is still too viscous to pipette, a sharp razor can be used to remove the tip of the disposable pipette tip, which increases the diameter of the opening.
19.C.xxvi	The nanoparticles change color to blue/gray or black.	A change in color from the characteristic red/purple of well-dispersed gold nanoparticles to grey/black indicates aggregation.	Repeat the synthesis of the AuNPs. Ensure that the glassware and the stirrer bar are cleaned with ultrapure water before synthesizing AuNPs. If necessary, use aqua regia to clean gold deposited on glassware. *CAUTION: Aqua regia is highly corrosive, reacts violently with organic compounds, and should be handled in a chemical hood with appropriate personal protective equipment.*
19.C.xxxiii	The activated Cas12a does not cleave ssDNA.	See troubleshooting for step 18.	

19.C.xxxiii	The level of background leakage is unexpectedly high.	Background leakage can occur due to the mesh size of the hydrogel and the mechanism of AuNP entrapment.	Modify the particle size to reduce diffusion out of the gel. Possible strategies include PEGylation, attachment to the matrix, or the alteration of the properties of the gel to make it stiffer.
19.C.xxxvi	The degradation behavior of the gel is slower or faster than expected.	The use of containers with different material and geometries that the ones specified may led to differences in gel shape, exposed surface areas exposed to the bulk, which can affect the expected Cas12a diffusion and DNA degradation profiles.	Ensure the use of the specified materials, wells and tubes in all experiments.
19.D.xviii	The gels have heterogeneous morphologies.	Different crosslinking times at this stage can affect the shape and final size of the hydrogel drop due to the combined effects of bulk crosslinking and evaporation.	Control the drying time of the gels (see Fig. 13).
19.E.v	There is no flow in the µPAD after printing.	Misalignments between the layers of as little as 1mm can cause unreliable flow in the µPADs.	The front and back of the chromatographic paper wax patterns should be perfectly aligned.
19.E.viii	There is no flow in the µPAD after printing.	The degree of wax reflow might have been excessive.	Repeat the printing and reflow the wax at a lower temperature for longer.
19.E.viii	There is flow outside of the wax channel.	The wax reflow was ineffective.	Repeat the reflow at higher temperatures for longer to ensure that the wax penetrates pores from both sides of the chromatographic paper.

19.E.viii	There is no flow in the µPAD due to poor alignment.	The hydrophilic regions are poorly aligned.	Confirm the alignment of the hydrophilic sections below 0.5mm using a light source and visual inspection. Discard any µPADs with visible printing, reflowing, or alignment defects. Ensure that layers 1-4 of the µPADs exhibit circular hydrophilic paper regions approximately 1.5mm in diameter, surrounded by an evenly distributed hydrophobic wax coating to prevent undesired lateral flow.
19.E.xii	There is ineffective gelation in the channels, or no flow through the µPAD.	The gel reagents should be tested separately in a PCR tube to confirm that the precursors crosslink rapidly when combined with the ssDNA bridge.	Successful precursor cross-linking can be determined by visually inspecting the gel flow in a pipette. This should be visibly more viscous 2-5s after the mixing of the reagents. If the gel components polymerize as described, defects in the µPAD pattern alignment or insufficient wax reflow are the most likely reasons for the device failure (troubleshooting step 19 E viii).
19.E.xxiii	There is ineffective gelation in the channels or too much flow through the µPAD.	The use of different equipment to print and reflow the wax might cause differences in pore and channel sizes.	Ensure the µPAD channel size and geometry does not differ due to changes in printing equipment. Apply the specified volume of PBS to characterize the flow in the channel.
Box 3, vii	The IDE is not connected to the UHF-RFID.	There may be issues with the electrical conductivity of the components or with the attachment of the insulating materials.	If the IDEs are not electrically connected to the UHF-RFID chip, try pressing the tape and ensuring that both the tape and the conductive silver film in the UHF-RFID are clean and free of insulating materials.

Timing

Reagent setup *3h*

Steps 1-18 Designing and producing gRNAs and dsDNA triggers **1d**

Steps 1-5, Design of gRNA and DNA components	30min each
Steps 6-14, *In vitro* transcription and preparation of DNA targets	6-12h
Steps 15-18, *In vitro* validation of gRNAs	3h

Step 19 Creating CRISPR-responsive DNA hydrogels

Step 19(A) Fluorophore release from CRISPR-responsive PEG hydrogels *3d*

Steps i-xii, PEG gel synthesis	2-3d
Steps xiii-xvii, Release of cargos	30min (setup) + 12h

Step 19(B) Enzyme release from CRISPR-responsive PEG hydrogels *3d*

Steps i-x	2-3d
Steps xi-xvi	30min (setup) + 6h

Step 19(C) Nanoparticle release from CRISPR-responsive PA-DNA hydrogels *3d*

Steps i-xix Polymerization and synthesis validation	3h
Steps xx-xxvi, Synthesis of PEGylated gold nanoparticles	1d
Steps xxvii-xxxvi, Synthesis and actuation of AuNP-containing gels	30min (setup) + 15h
Steps xxvii-xxxvi Synthesis and actuation of FITC-dextran-containing gels	30min (setup) + 18h

Step 19(D) Primary cell release from CRISPR-responsive PA-DNA hydrogels *3d*

Steps i-v, Harvesting cells from cultures	30min
Steps vi-xii Preparation of precursors for cell experiments	3h (synthesis) + 2h (washing)
Steps xiii-xx, Embedding of cells in hydrogels	3h
Steps xxi-xxiii, Monitoring of cell release	8h
Steps xxiv-xxv, Post-release viability assay	1h

Step 19(E) CRISPR-mediated µPAD diagnostics *1-2d*

Steps i-xiv, Fabrication and testing of base µPAD	6h
Steps xv-xxiii, RT-RPA and µPAD detection	2h

Boxes 1 and 2, Modification of µPAD design	2h each

Anticipated results

Based on the experimental results described in our previous work[15] and presented in Fig. 14, we expect this protocol to lead to the following key observations.

For any gRNA-dsDNA pair, we expect the rate of collateral cleavage of ssDNA upon binding the trigger to depend on the dsDNA concentration, within the limits of stoichiometric Cas12a saturation (Fig. 14a)[15,22]. It is important to validate the gRNA sequence for the chosen dsDNA target, as the relative performance of gRNAs and the ability of a dsDNA sequence to activate Cas12a are quantitatively variable. When actuating hydrogels similar to those described here (on the millimeter scale), response times are typically on the order of hours[15]. In new material contexts, response times might differ from those presented here, which may require adjustments to the frequency of measurements to best capture the kinetics of actuation.

In both PEG-DNA and PA-DNA hydrogels, cargos trapped in the hydrogels should be released upon activation of Cas12a by the gRNA-defined dsDNA trigger, but not by non-matching sequences (Fig. 14b,c)[15]. When Cas12a acts as a sentinel and the dsDNA is added later, we observed that pre-exposure to non-trigger DNA did not noticeably compromise Cas12a activation by dsDNA triggers at later timepoints[15].

Cas12a is a large molecule, and can be partially slowed down by the chains of the hydrogels. As a consequence, the kinetics of ssDNA cleavage are also affected by the physical properties of the DNA-containing material in cases where the diffusion of the enzyme through the gel is partially limiting (Fig. 14b)[15]. We also observed a reduction in the background release of nanoparticles (Step 19 Option C) when using 7% polyacrylamide-DNA gels compared to 4% gels, consistent with an improvement in the retention of physically enmeshed cargos[15].

Despite some basal ssDNA cleavage activity of serum-rich media (Fig. 8), we observed that PA-DNA hydrogels were stable for at least several hours in the absence of Cas12a activation, and only released encapsulated cells (Step 19 Option D) in the presence of a dsDNA trigger. The encapsulation and release of the cells were not cytotoxic, as even sensitive primary cells were 80-90% viable after Cas12a-mediated release[15].

In µPAD-based diagnostics (Step 19 Option E), both the visual and analog resistive modes provided continuous readouts dependent on the wicking length of the buffer inside the lateral flow channels (Fig. 14d, top), whereas the short-circuiting of the RFID antenna typically produced sharp signal transitions and binary behaviors within minutes (Fig. 14d, bottom). For the gRNA-dsDNA pairs that we used, the limit of detection of the hydrogel-controlled µPAD diagnostic after an RT-RPA was comparable to other CRISPR-based technologies, with a limit of detection of about 11aM. Without pre-amplification, the µPAD routinely detected target sequences down to 400pM[15].

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Author contributions

R.V.G., H.P., M.A.E., L.R.S., P.Q.N., A.S.M., N.A.M. designed and conducted experiments described in this article, and wrote the manuscript. J.J.C. directed overall research and edited the manuscript.

Competing interests

R.V.G., H.P., M.A.E., L.R.S., P.Q.N., A.S.M., N.A.M. and J.J.C. are inventors on U.S. Patent Application No. 16/778,524 that covers CRISPR-responsive materials. J.J.C. is a co-founder and director of Sherlock Biosciences.

Data availability

The data generated or analyzed during this study are included in this published article and the accompanying publication[15]. The original data files can be obtained from the corresponding author upon reasonable request.

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Supplementary Information

- **Supplementary Data 1:** Printing template of the µPAD, side A
- **Supplementary Data 2:** Printing template of the µPAD, side B
- **Supplementary Data 3:** Printing template of the IDEs used to modify the µPAD for remote data transmission
- **Supplementary_Software_1:** Arduino code to record the RFID signal of the modified µPAD

Box 1: Example computations for PEG hydrogel synthesis

We work from a concentrated stock of each PEG macromer (4% w/v) and prepare the gels by mixing pure PEG-VS stock in TEA, diluted PEG-VS in TEA, and a solution of PEG-SH in water, while the remaining volume is used by other components (DNA, TCEP, water, etc.). The recipe below details how to prepare gels with PEG concentrations ranging from 1 to 2% w/v; in all formulations, the molar ratio of the two types of macromers is identical (by number of cores). We alter the concentration of the diluted PEG-VS while always using the same concentration of undiluted PEG-VS stock during DNA grafting, in order to modulate the final gel concentration without affecting the functionalization.

Example calculation:

Final precursor volume:	V_f = (enough for a 96-well plate)
Desired PEG concentration:	C_f = 1.5 % w/v

For both PEG-VS and PEG-SH, make a fresh stock at a concentration of 4% w/v, respectively in TEA and water:

Chosen stock concentration	C_{stock} = 4 % w/v
PEG density:	d = 1.1g/cm^3
PEG aliquot mass:	m = 50mg
Volume for a 4% stock:	$V = 100*(m/C_{stock})-(m/d)$ = 1204.5µL

To make a volume Vf of gel with a concentration C_f, we use a total of three PEG solutions: one unit volume ($V_{VS1}=V_f/8$=62.5µL) of PEG-VS stock that we functionalize with ssDNA; one unit volume ($V_{VS2}=V_{VS1}=V_f/8$=62.5µL) of PEG-VS diluted as necessary from the stock; and two unit volumes ($V_{SH}=V_f/4$=125µL) of PEG-SH diluted from a stock. Concentrations are computed as follows:

PEG-VS undiluted, for DNA grafting	$C_{VS1} = C_{stock}$ = 4 % w/v in TEA
PEG-VS to adjust gel concentration	$C_{VS2} = 4*C_f-C_{stock}$ = 2 % w/v in TEA
PEG-SH, to cross-link the gel	$C_{SH1} = 2*C_f$ = 3 % w/v in water

This leaves four volumes ($V_f/2$=250ul) for DNA, water and other aqueous components.

From these solutions, we can compute the overall molarity of cross-links in the final hydrogels, based on the number of arms of the macromers and their molecular weight:

Number of arms on PEG-VS	K_{VS} = 8
Number of arms on PEG-SH	K_{SH} = 4
Molecular weight of PEG-VS	M_{VS} = 10000g/mol
Molecular weight of PEG-SH	M_{SH} = 10000g/mol
Molarity of vinyl sulfones in gels	$X_{VS} = 10*K_{VS}*1000*(C_{VS1}*V_{VS1}+C_{VS2}*V_{VS2})/(V_f*M_{VS})$ = 6mM
Molarity of thiols in gels	$X_{SH} = 10*K_{SH}*1000*(C_{SH1}*V_{SH}/V_f)$ = 3mM

The 3mM excess of vinyl sulfones ensures that the DNA functionalization reaction does not significantly alter the subsequent reaction of the macromers with the PEG-SH by competing for functional groups. It also explains the need to block the gels with free thiols after polymerization to prevent cross-reactions with amines in downstream experiments.

- **END OF BOX 1 -**

Box 2: Modification of the µPAD for conductivity measurements.

The µPAD can be adapted for conductivity measurements. In order to do so, follow the instructions below:

i. Cover layer 5 of the µPAD with 3x30mm strips of conductive tape placed along the top and bottom sides of the lateral flow channel. These act as parallel conductive planes for the measurement of electrical resistance across the channel, as a function of the buffer wicking distance.
ii. Laminate both the conductive tape and wiring over layer 5 to ensure robust electrical connection between the components. Avoid covering the fluid inlet in layer 1. To laminate the device, use clear adhesive tape cut to exceed the perimeter of the µPAD, allowing the top and bottom layers of the adhesive tape to come into contact with each other around its perimeter. This provides a seal that prevents detachment of the electrical contacts, while maintaining a fluidic connection between the µPAD layers. At this stage, leave layers 1 and 2 of the µPAD uncovered to receive the conductive buffer and ssDNA linker during testing.
iii. At the time of testing, fill layer 2 of the µPAD with 0.3µL of the Cas12a-gRNA assay reaction. The Cas12a-gRNA mix assay should contain 100µM ssDNA linker (from Step 19 Option C.ii) 300nM Cas12a, 1µM gRNA (from Step 14), and dsDNA (from Reagent Setup, Table 1) at varying concentrations (e.g., 0nM, 1nM, 10nM, 100nM, etc.) in 1X NEB 2.1 buffer. This reaction should be prepared separately and incubated for 4h at 37ºC prior to testing with the µPAD. Negative control reactions can be performed with scrambled dsDNA.
iv. After the pre-digested ssDNA linker (L-15) solution has been deposited and air-dried for 1min, fold the µPAD layers to fluidically connect all of the hydrophilic regions. In this state, layer 1 acts as a protective cover.
v. Add 2µL of 1X PBS to layer 1 and measure the conductivity across the channel.
vi. Obtain electronic measurements from the µPAD by monitoring channel resistance using a digital multimeter. The 5min endpoint resistance values for experiments with different concentrations of dsDNA trigger can be collated to delineate the titration curve.

- END OF BOX 2 -

Box 3: RFID integration into the CRISPR-mediated stop-flow µPAD

The µPAD can be adapted to wirelessly transmit diagnostic data through a radiofrequency identification (RFID) tag. In order to do so, follow the instructions below:

i. Acquire commercial RFID tags with an exposed printed antenna (Fig. 12).
ii. Inspect the RFID tags to confirm that they function as expected.
iii. Print the flexible, interdigitated electrodes (Supplementary Data 3) on a poly(ethylene terephthalate) substrate using silver nanoparticle ink. Deposit the ink using a modified printer with refillable cartridges according to the protocol developed by Lee *et al.*[45].
iv. Cut each printed interdigitated electrode into 20x20mm squares as outlined in the file (Supplementary Data 3).
v. Place the IDE on top of the conductive side of the UHF-RFID tag, with both conductive IDE terminals coplanar and in close proximity to the first conductive antenna loop (<10mm away from the UHF-RFID chip). Double-sided tape can be used to prevent movement between the IDE and UHF-RFID tag (Fig. 12).
vi. Connect the IDE terminals to the UHF-RFID antenna loop with two 2x4mm strips of conductive tape.
vii. Verify electrical conductivity between each of the IDE terminals and each side of the UHF-RFID chip should using a multimeter. **TROUBLESHOOTING**.
viii. Cut the lateral flow channel of a multi-layer µPAD stack to 20mm in length, and place it on top of the interdigitated electrode/UHF-RFID tag arrangement.
ix. Align the bottom of the lateral hydrophilic region in layer 5 to be in direct contact with the conductive side of the IDE.
x. Test the reagent placement and activation for the µPAD by performing conductivity readings across the flow channel as described in Step 19 Option E.xxiv-xxix .
xi. Measure the relative received signal strength indicator (RSSI) of each µPAD RFID tag in the presence or absence of target DNA. Place two RFID tags (one modified to incorporate the µPAD, and one unmodified) at a distance of 0.5m from a UHF-RFID antenna. Connect the UHF-RFID antenna to a simultaneous RFID tag reader and an Arduino microcontroller using the M6E-NANO RFID Arduino library. The Arduino code used to record this signal can be downloaded as Supplementary Software 1. A measurable decrease in the absolute RSSI values during the test indicates that the power level of the received radio signal has decreased due to the flow of conductive buffer through the device, and the resulting RFID tag antenna short-circuiting.

- END OF BOX 3 -

Figure captions

Fig. 1 | Activation of Cas12a collateral ssDNA cleavage activity by trigger dsDNA. a, Cas12a probes dsDNA molecules for complementarity based on the sequence of its guide RNA (gRNA, orange). Upon recognition of a matching trigger sequence (blue) flanked by a protospacer-adjacent motif (PAM, pink), the Cas12a-gRNA complex generates staggered cuts in the dsDNA molecule (yellow arrows) and remains bound on the fragment bearing the PAM. The resulting complex then indiscriminately cuts nearby ssDNA molecules. **b**, In this protocol, we use the trigger-induced collateral cleavage activity of Cas12a to cut ssDNA (orange) in multiple contexts: to validate the activity of user-defined gRNA-dsDNA pairs, we observe the fluorescence signal generated by the dissociation of a fluorophore (F, green) from a quencher moiety (Q, black) bound through a ssDNA linker (Steps 15-18); in inert hydrogels, the cleavage of ssDNA tethers by Cas12a results in the release of molecular cargos (M, pink), such as small molecules and proteins, without altering the structure of the material (Step 19 Option A and B); and in materials crosslinked by ssDNA molecules, the collateral activity of Cas12a induces bulk hydrogel depolymerization and the release of enmeshed cargos (C, blue), such as cells and nanoparticles (Step 19 Option C and D).

Fig. 2 | Design and synthesis of guide RNA molecules (Steps 1-14). a, The *in vitro* transcription (IVT) of gRNAs is performed by hybridizing an adapter (pink) with a template oligonucleotide (grey) upstream of the guide sequence. This generates a double-stranded promoter that efficiently recruits the T7 RNA polymerase. **b**, In the Cas12a ribonucleoprotein, the target-specific gRNA sequence (orange) hybridizes with the DNA target molecule. The "TTTV" protospacer-adjacent motif (PAM, pink) is immediately 5' of the sequence that matches the guide sequence. The sequences shown in both panels correspond to those used to activate Cas12a in response to dsDNA fragments (blue) derived from the *S. aureus mecA* gene.

Fig. 3 | Synthesis of PEG hydrogels harboring ssDNA-tethered molecules (Step 19 Option A). First, oligonucleotides (blue) carrying molecules of interest (red) on their 3' terminus must be treated with a non-thiol reducing agent (TCEP) in order to deprotect the 5'-thiol reactive groups. The 8-arm vinyl sulfone-functionalized PEG macromers (orange) undergo three successive rounds of TEA-catalyzed thiol-ene Michael additions: first with the DNA anchors to graft the cargo molecules, then with thiol-functionalized 4-arm PEG macromers (pink) to polymerize the gels, and finally with a blocking thiol.

Fig. 4 | Monitoring the Cas12a-mediated release of fluorophores from PEG hydrogels (Step 19 Option A). a, In our setup, the measurement plate is tilted on a support to ensure consistency when depositing precursors on the sides of the wells. **b**, The release of cargo can be observed in real time by measuring the fluorescence increase in the hydrogel supernatant. The fluorimeter is calibrated to measure signals on the side of the well opposite from the fluorophore-loaded material, to ensure that the signal recorded is only generated by molecules released into solution.

Fig. 5 | Synthesis of polyacrylamide hydrogels crosslinked by DNA molecules (Step 19 Option B). Methacryl-functionalized DNA is incorporated into polyacrylamide chains (PA-X,

PA-Y) during macromer polymerization. In one example of gel actuation, the Cas12a-gRNA is added to the gel precursor with the nanoparticle cargo, prior to the addition of dsDNA cues and ssDNA cross-linker. In another example, to encapsulate cells into hydrogels, a small amount of ssDNA bridge cross-linker is added to mixed macromers to thicken the pre-gel solution and minimize losses during the washing step. More ssDNA linker is then added at the same time as the cells to fully cross-link the hydrogels. Finally, the experiment is initiated by exposing the gels to gRNA-complexed Cas12a and dsDNA. **Insert:** Additional details of the cross-linking strategy – the two ends of the DNA bridge hybridize with distinct ssDNA anchors incorporated in polyacrylamide macromers, while the central AT-rich portion remains single-stranded and sensitive to Cas12a collateral activity.

Fig. 6 | Monitoring the Cas12a-mediated release of nanoparticles through bulk PA-DNA gel degradation (Step 19 Option C). a, The PA-DNA gel precursor is cast in a circular silicone mold placed in the center of the microplate well. **b**, Nanoparticles contained in the intact gel absorb light. Upon the detection of a trigger dsDNA by Cas12a, gel degradation causes the dispersion of the particles away from the optical path and into the surrounding solution, thereby causing a decrease in absorbance.

Fig. 7 | Workflow for the crosslinking and Cas12a-mediated actuation of PA-DNA hydrogels containing encapsulated cells (Step 19 Option D). Cells are added to the DNA-polyacrylamide macromers at the same time as the cross-linking ssDNA bridge, after which the solution must be dispensed quickly before the precursor thickens. Cas12a can be activated by the gRNA-defined dsDNA trigger in the cell growth medium, resulting in the degradation of the PA-DNA gel and the release of cells with minimal cytotoxicity.

Fig. 8 | Basal ssDNA cleavage activity of cell culture media . We synthesized small PEG hydrogels harboring ssDNA-anchored cyanine dye. After washing the hydrogels, we overlaid them with various cell culture media and reagents, and kept the tubes at 37°C for 48h before observing fluorescence. While common cell passaging reagents (PBS, Trypsin-EDTA) did not exhibit notable signs of ssDNA degradation, the performance of cell culture media was variable and highlights the need to test one's cell culture conditions when designing CRISPR-hydrogel actuation experiments in complex media (e.g., for tissue engineering application). Blue: no visually detectable cargo release. Orange: significant basal ssDNA cleavage activity in the absence of Cas12a nuclease.

Fig. 9 | Multi-modal detection of DNA and RNA targets using CRISPR-sensitive hydrogels in paper-based fluidic devices. a, A schematic of the generation of RNA targets for the experimental validation of RNA sensors. The RNA sequence of interest (blue), ordered as a DNA element, is amplified by PCR with primers that generate a terminal T7 promoter (pink). This PCR product then serves as the template for *in vitro* transcription. **b**, A schematic of the workflow for the detection of DNA and RNA targets using CRISPR-responsive materials as fluidic valves in paper devices. RNA targets are first converted into a DNA signal, as Cas12a is preferentially activated by dsDNA targets. Cas12a then probes input sequences for gRNA-matching dsDNA triggers and conditionally digests the ssDNA linkers. If sufficient degradation occurs, the linkers are unable to cross-link PA-DNA hydrogels in subsequent steps. In the folded fluidic device, buffer applied on the top layer (L1) brings the product of the pre-incubation (in

L2) in contact with the PA-DNA macromers (in L3), thereby polymerizing a gel within the pores of the paper, which obstructs the channel. In the case of a successful Cas12a-gRNA complex activation by dsDNA, the digestion of ssDNA bridges prevents gel formation, and buffer proceeds through L4, where it dissolves salts and dyes before reaching the lateral flow channel in L5. There, the buffer can be detected by various means: visually, or electronically, either by measuring the increase in electrical conductance through the channel (analog signal) or by recording the short-circuiting of an RFID antenna (digital signal).

Fig. 10 | Design and assembly of the microfluidic paper-based analytical device (Step 19 Option E ii-xii). a, The μPAD assembly starts with a piece of filter paper (1) to which a hydrophobic mask is applied by wax printing (2). The structure is then folded (3-5) to generate the assembled device, prior to buffer application (6). **b**, The stacked μPAD structure is assembled by folding paper layers on top of each other so as to align the hydrophilic regions and create a continuous channel that ends in the final lateral flow channel. Adapted with permission from Ref. 15.

Fig. 11 | Modification of the paper-fluidic device for analog signal recording. This design is based on the μPAD used for visual readouts. Both sides of the final lateral flow channel of the folded device (layer 5 in Fig. 9) are uniformly pressed against conductive fabric tape interfaced with standard electric connectors. As a result of Cas12a-mediated valve actuation, buffer wicks through the channels and causes a measurable increase in device conductance. Adapted with permission from Ref. 15.

Fig. 12 | Modification of the paper-fluidic device for remote transmission of digital signals. In this design, as a result of dsDNA-dependent Cas12a-gRNA complex activation, conductive buffer reaches the final layer of the μPAD and closes a conductive loop that short-circuits the printed RFID antenna. This results in a discrete modification of the strength of the transmitted radio frequency signal monitored at a distance by an RFID reader. Adapted with permission from Ref. 15.

Fig. 13 | Time-dependent drying of PA-DNA hydrogels. Structural alterations caused by hydrogel drying after 2min define this as the optimal incubation time for gel cross-linking before overlaying the supernatant (cell growth medium).

Fig. 14 | Anticipated Cas12a activation by dsDNA triggers and subsequent material actuation. a, The rate of collateral ssDNA cleavage correlates with the amount of activated Cas12a, and therefore the amount of gRNA-matching dsDNA input, both in solution and in hydrogels. We monitored the presence of Cy3 in the supernatant of 2% PEG hydrogels upon exposure to either a *mecA* dsDNA trigger or a scrambled control (Step 19 Option A). Traces indicate mean ± SD for 4 technical replicates. **b**, The Cas12a-gRNA complex is inactive until it encounters a specific trigger dsDNA, after which it penetrates the PEG hydrogels and cuts ssDNA linkers. Material properties affect the kinetics of Cas12a-mediated dsDNA-specific PEG hydrogel actuation. Results were obtained as in panel a, with 50nM of Cas12a-gRNA complex and 10nM of dsDNA. **c**, When all components are pre-loaded in the PA-DNA hydrogel, sequence-specific Cas12a-gRNA complex activation results in a sudden disruption of the material and the release of its components in solution. We measured the absorbance through 7%

PA-DNA hydrogels loaded with AuNPs, Cas12a-gRNA and dsDNA (Step 19 Option C). Traces indicate mean ± SD for 4 technical replicates. **d**, Top: digestion of ssDNA bridges by activated Cas12a prevents gel formation in the paper-based fluidic device, resulting in a dsDNA trigger-dependent lateral flow detectable by visual and resistive readouts. Bottom: detection of buffer flow by short-circuiting an RFID antenna results in a wirelessly detectable digital signal. Panel shows two representative signals recorded after RT-RPA and μPAD testing of samples containing either 0aM or 11aM synthetic Ebola ssRNA (Step 19 Option E). Adapted with permission from Ref. 15.