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A PAM-free CRISPR induced DNA reorganization for multiplexed nucleic acid detection

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Abstract

Nucleic acid sensing powered by the sequence recognition of CRIPSR technologies has enabled major advancement toward rapid, accurate and deployable diagnostics. While exciting, there are still many challenges facing their practical implementation, such as the widespread need for a PAM sequence in the targeted nucleic acid, labile RNA inputs and limited multiplexing. Here we report FACT (**F**unctionalized **A**mplification **C**RISPR **T**racing), a PAM-free CRISPR-based nucleic acid barcoding technology

compatible with Cas12a and Cas13a, enabling diagnostic outputs based on *cis*- and *trans*-cleavage. Furthermore, we link the activation of CRISPR-Cas12a to the expression of proteins through a Reprogrammable PAIRing system (RePAIR). We then combine FACT and RePAIR to create FACTOR (FACT on RePAIR), a CRISPR-based diagnostic, that we use to detect infectious disease in a novel agricultural use case: honey bee viral infection. With high specificity and accuracy, we demonstrate the potential of FACTOR to be applied to the sensing of any nucleic acid of interest.

Introduction

Repurposing the components of life into the functional elements of engineered biological systems have led to great advances for the field of synthetic biology. The resulting applications have spanned domains as broad as health care[1] (diagnostics[2] and the development of synthetic probiotics[3]), commodities (biofuels or biomolecules[4,5]) and electronics or chemistry[6,7,8,9]. The recent development of CRISPR diagnostic strategies[10–15] based on the non-specific *trans*-cleavage activity of these enzymes, have demonstrated specific and sensitive detection of several infectious diseases, such as the SARS-CoV-2 and Human Papilloma Virus[10,16–18]. In these applications, and for most CRISPR enzymes, a protospacer adjacent motif (PAM) or a PAM-like motif (called protospacer flanking site (PFS), must typically be present in the amplified target nucleic acid for the pre-loaded complex CRISPR-CRISPR RNA (crRNA) to bind and induce sequence specific *cis*-cleavage activity[19,20]. This primary *cis*-cleavage event then induces the *trans*-cleavage activity used as the diagnostic output (e.g. fluorescence). Unfortunately, dependency of these CRISPR-based diagnostics on PAM availability limits their ability to target within the sequence space. Specifically, the vast majority of potential target nucleic acid sequences are PAM-free (94.8% in the reference human genome for the Cas9 PAM 'NGG'[21]). Therefore, most established nucleic acid-based molecular diagnostics (e.g., PCR, sequencing) are directed at sequences that lack PAM sites. For this reason, the development of a CRISPR nucleic acid detection technology that is independent of this PAM requirement would be a tremendous benefit to the

emerging CRISPR diagnostic field by broadening the target sites possible for each disease[22].

Further, it is increasingly important for de-centralized diagnostics to provide multiplexed detection, where a common underlying molecular machinery can simultaneously monitor multiple target sequences. This requires the generation of distinct reporter readouts for each sequence of interest, which is difficult with non-specific *trans*-cleavage. To date, CRISPR-based systems with multiplexing capacity have been a challenge[23,24], requiring various CRISPR enzymes[16], and/or separate wells[25,26]. Other gene circuit-based diagnostics have begun to deliver the capacity for decentralized and multiple readouts[27–29]. Taken together, if more practical multiplexing could be brought to a PAM-free, CRISPR nucleic acid sensing technology, it could enable greater capacity for disease monitoring and, perhaps, new readout possibilities (e.g., colorimetric, glucometer, fluorescent).

Here we describe FACT (**F**unctionalized **A**mplification **C**RISPR **T**racing), a PAM-free nucleic acid detection principle based on CRISPR-functionalized isothermal amplification outputs (Fig. 1). When detected, the target nucleic-acid is barcoded with a functional crRNA sequence in a single-step amplification strategy. Through downstream CRISPR *cis*-cleavage activation, the functionalized amplicon can then produce direct readouts or *trans*-cleavage induced signals. We have also developed a new CRISPR-based reporter system, called RePAIR (**Re**programmable **PAIR**ing system), that uses CRISPR-induced DNA reorganization to activate silent gene circuits and serve as a decoding mechanism. This led to FACTOR (FACT on RePAIR), which has capacity for multiple protein-based outputs, enabling fluorescent, colorimetric and electrochemical signals, that can be easily interchanged. FACTOR shows attomolar sensitivity to DNA target nucleic acids, and the ability for one-pot multiplexing. Moreover, FACT can be easily linked to established CRISPR diagnostic platforms through *trans*-cleavage activation. Finally, FACTOR can be rationally deployed to detect viral diseases. Here, we show that FACTOR can sense agriculturally important

viral infections in honey bees with an accuracy of 94.7 % and 100 % compared to RT-qPCR for DWV (Deformed Wing Virus) and IAPV (Israeli Acute Paralysis Virus).

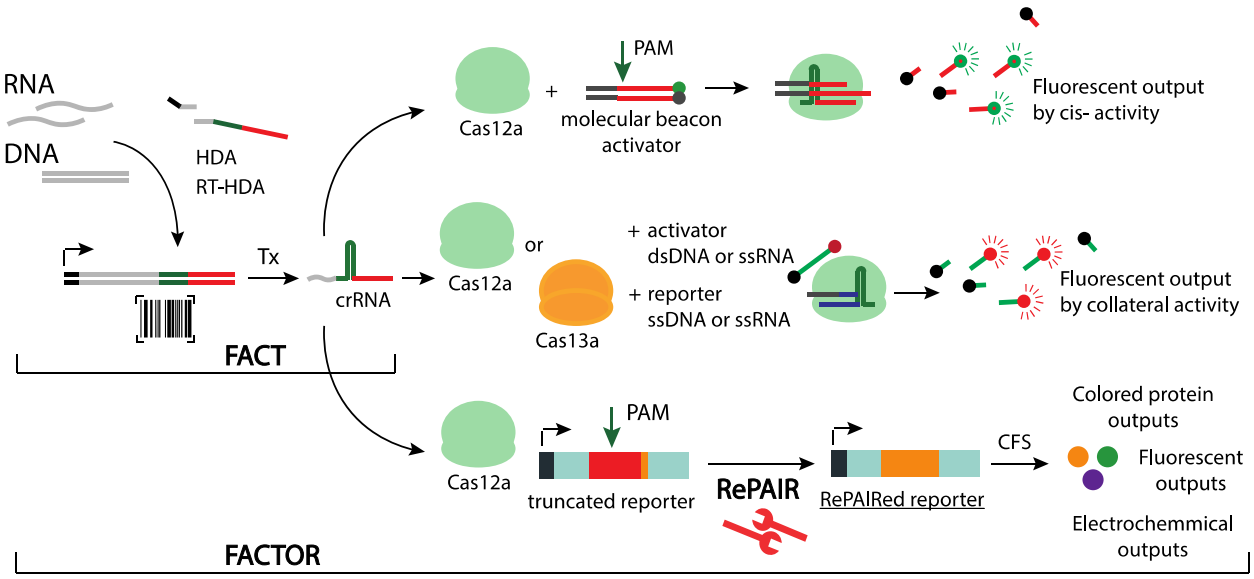


Fig. 1. Regulation of CRISPR gene circuits through PAM-free nucleic acid barcoding amplification. The isothermal amplification of target nucleic acid (HDA for DNA or RT-HDA for RNA), results in the generation of a barcoded, composite DNA encoding crRNA (DNAcrRNA). This functionalized CRISPR amplicon, upon transcription (Tx), produces a crRNA that can be self-cleaved and loaded by a Cas12a (or Cas13a) enzyme. The Cas12a/crRNA complex can now regulate gene circuit readouts using (*i*) sequence specific *cis*-cleavage activity (top), (*ii*) conventional diagnostic technologies based on *trans*-cleavage activity using Cas12a or Cas13a (middle), or (*iii*) CRISPR induced DNA reorganization (RePAIR system), enabling the generation of RNAs and proteins in a cell-free protein expression system (bottom).

Results

Regulation of CRISPR gene circuits through PAM-free nucleic acid amplification

CRISPR-based diagnostics typically rely on the isothermal amplification of a PAM-containing target nucleic acid[10,16]. This considerably limits sequence selection diversity for the nucleic-acid target of interest. And while CRISPR enzymes can be engineered to relax PAM recognition, broadening targeting capabilities[30–32], this can be a drawback in applications where high specificity is required. To address this challenge, we set out to develop a PAM-free nucleic acid amplification strategy that could regulate a CRISPR readout system. We hypothesized that the coding sequence of a crRNA could be incorporated into a barcode on a target nucleic acid during the isothermal amplification, enabling the regulation of a CRISPR-based system. We chose to use the helicase-

dependent amplification (HDA) isothermal method in combination with the well-defined Class II CRISPR enzyme *Francisella novicida* Cas12a (FnCas12a)[33,34], capable of sequence specific *cis*-cleavage, for validation (Fig. 2a).

Using synthetic nucleic acid targets (RNA and DNA), we first designed the barcoding primers. The target specific forward primer (P1) encodes an overhanging T7 promoter to enable downstream transcription using T7 RNA polymerase, while the target specific reverse primer (P2) encodes for an overhanging sequence coding for a crRNA (spacer and direct repeat) (Fig. 2a and S1). Following HDA (for DNA amplification) or RT-HDA (for RNA amplification), a composite DNA containing the PAM-free target sequence and the specific crRNA barcode is generated (DNAcrRNA). Upon downstream transcription (Tx), an RNA coding for a crRNA is produced, cleaved and loaded into FnCas12a (Fig. 2a). This nucleoprotein complex can now guide the sequence specific cleavage of reporter dsDNA, here a molecular beacon, leading to fluorescence increase.

To test our hypothesis, an RT-HDA reaction containing 5 pM of the target synthetic RNA sequence was run for 3 hours, to ensure amplification. The product of the amplification, incubated with FnCas12a CRISPR enzyme and a reporter dsDNA molecular beacon, was monitored in real time (Fig. 2b) and yielded fluorescent detection within 1 hour (Fig. 2c). This demonstrated that the barcoding strategy was functional and could subsequently induce sequence specific *cis*-cleavage of a molecular beacon, indicating the detection of the target nucleic acid. The same experiment was also performed using a synthetic target dsDNA (Fig. 2d,e) bearing the same result. We refer to the functionalization of the amplification output with the crRNA barcode sequence, in combination with the transcription of the crRNA and the CRISPR-induced readout, as FACT.

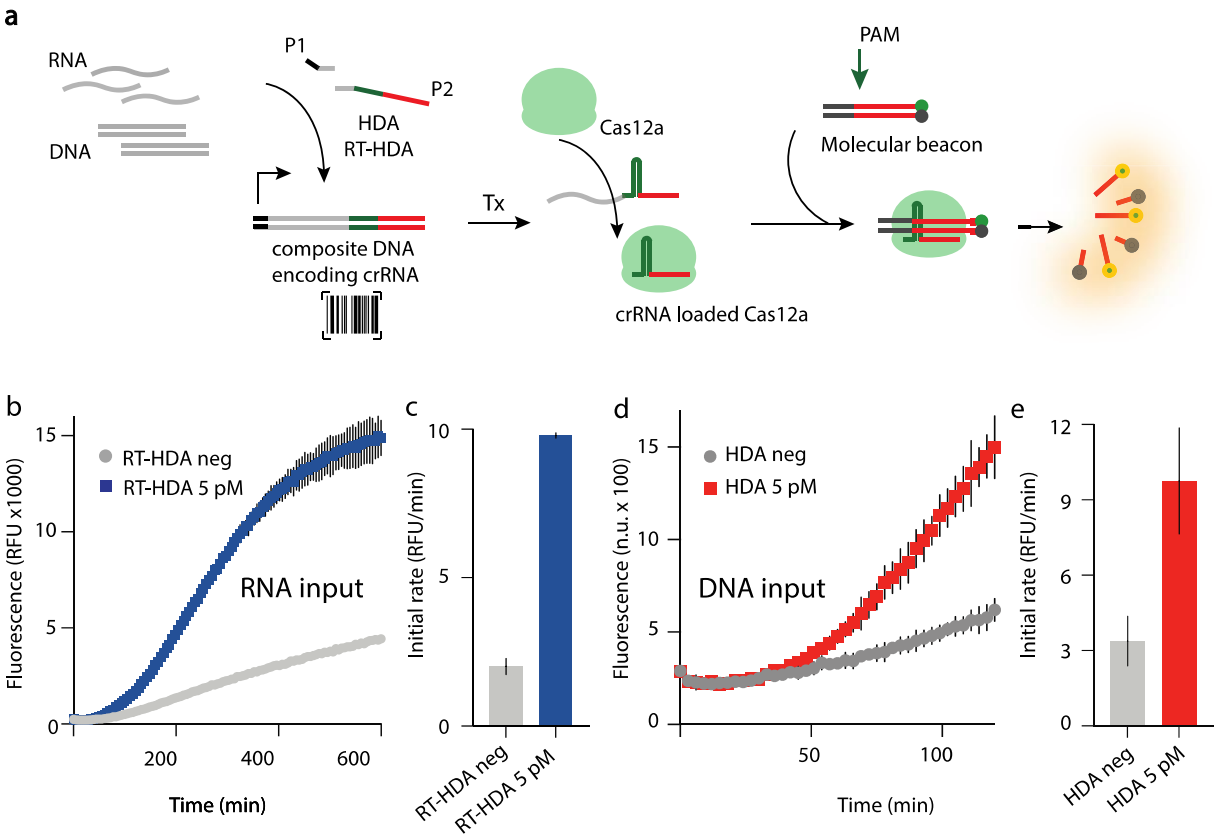


Fig. 2. PAM-free nucleic acid barcoding amplification leads to a CRISPR induced *cis*-cleavage output. a. Schematic of the generation of a composite DNA encoding crRNA (DNAcrRNA) upon target nucleic acid amplification using P1 and P2 primers. Following transcription (Tx), the functionalized CRISPR output product can be cleaved and loaded into Cas12a. The DNAcrRNA serves as an encoded barcode specifying which nucleic acid was amplified. The correct combination of the crRNA and molecular beacon (containing a PAM + protospacer), generates a fluorescent output. **b.** RT-HDA was performed using synthetic target RNA at 5 pM or H_2O (neg). The barcoded product was then transcribed, and combined with FnCas12a and the reporter dsDNA molecular beacon. TAMRA fluorescence (543/584 nm) was monitored in real time at 37 °C in a plate reader. **c.** Using the data from b. the initial rate of fluorescence increase within 1 hour (60 min) was plotted. **d.** HDA was performed using synthetic target DNA at 5 pM or H_2O (neg). The barcoded product was then transcribed, and combined with FnCas12a and the reporter dsDNA molecular beacon. TAMRA fluorescence was monitored in real time at 37 °C in a plate reader. **e.** Using the data from d. the initial rate of fluorescence increase within 1 hour (60 min) was plotted. Error bars: SD. Experiments displayed are representative of independent biological triplicates.

Reprogrammable PAIRing (RePAIR) enables a multiplexed output system

Validation of CRISPR-induced Reprogrammable PAIRing (RePAIR)

With the process of PAM-free crRNA encoded target amplification validated, we next sought to expand the capabilities of the functionalized CRISPR amplicon by linking cell-free protein expression system (CFS) to the sensing mechanism. CFS gene circuits have been widely used for bioproduction, rapid prototyping, and sensing applications

amongst others[5,35,36]. We decided to take advantage of the multiplicity of output capabilities provided by cell-free protein expression[2,28,37] and use FACT as regulator of rationally designed and programmable gene circuits to generate any desired protein-based output (i.e. electrochemical, fluorescent and enzymatic).

We first developed a Homologous Direct Recombination-based system (HDR), induced by a crRNA-Cas12a complex (Fig. S2a), building on previous work using Cas12a for the assembly of biological parts (C-Brick)[38] and homologous recombination via SLIC (sequence and ligation-independent cloning)[39]. To do so, we engineered a non-functional coding sequence for LacZα (by deleting a segment of the coding sequence) that contained a protospacer adjacent motif (PAM) close to the deletion site. In the presence of a crRNA targeting the protospacer, *cis*-cleavage should be induced and allow for a donor dsDNA fragment containing (*i*) left and right homologous arms (L-HA and R-HA) of 30 bp each to LacZα sequence and (*ii*) the missing fragment of LacZα to recombine with the truncated LacZα. The product of the recombination, now containing a functional LacZα sequence, could then be tested in a paper-based CFS reaction[35] for activity. Upon complementation of LacZα and LacZΩ (supplemented into the CFS) the resulting active LacZ complex should cleave its substrate, chlorophenol red-β-D-galactopyranoside (CPRG). The resulting color change from yellow to purple can be monitored using absorbance at 570 nm. As shown in Figure S2b, the proposed method successfully generated a LacZ signal, demonstrating effective homologous recombination induced by Cas12a. Moreover, the background signal and observed leakiness of the system was not significant (ns, Fig. S2b). We then optimized the system by varying the length of the HA and found that HA lengths of 40 bp significantly increased levels of *in vitro* HDR (Fig. S2c). Although we could successfully recapitulate the LacZ enzymatic signal, this strategy ultimately failed when we extended the demonstration from crRNA-induced recombination to DNAcrRNA. While the reasons for this are unclear, the general demonstration was a good proof of concept for readout generation and led us to rethink our recombination system. We then designed the Reprogrammable PAIRing system (RePAIR), which allows us to use both crRNA and DNAcrRNA molecules as inputs.

With RePAIR-based DNA reorganization, the reporter is engineered to be non-functional by removing a short segment of the coding sequence downstream of a FnCas12a PAM sequence (TTA). The deletion generates a synthetic protospacer (24 bp downstream the PAM) that is not found in the non-engineered LacZα reporter gene (Fig 3a, red). By programming the spacer of the crRNA to match the synthetic protospacer, sequence-specific *cis*-cleavage of the non-functional reporter should be induced in presence of FnCas12a. Furthermore, we take advantage of two additional features of the Cas12a enzyme. First, the ability of Cas12a to stay bound to the cleaved proximal side of the target dsDNA while releasing the distal side[40]. Second, the staggered cut generated by Cas12a[33] distal to the 5' PAM sequence on the targeted non-functional dsDNA reporter. We hypothesized that if the RePAIR reaction was supplemented with a new proximal donor containing the deleted base pairs, and a ligase to enable ligation of staggered cuts, a repaired functional reporter could be assembled.

We first tested the RePAIR system using a LacZα reporter as the output. Using a crRNA as the input of the gene circuit, and in the presence of all reaction components (ligase, proximal donor strand, crRNA and Cas12a), we detected an increase in absorbance over time. Sequential removal of key components of RePAIR and side-by-side comparisons of reactions, demonstrated the successful DNA reorganization of the LacZα reporter from a non-functional coding sequence to a functional state, including its concomitant expression in CFS well within 1 hour (Fig. 3b and Fig. S3a). Building on these results, and to link the RePAIR system to the output of the barcoding amplification (FACT), we then demonstrated that RePAIR could be induced using a synthetic DNAcrRNA input (Fig. 3c) with a signal clearly detected within the first hour (Fig. 3c, insert). We also tested the sensitivity of RePAIR to crRNA (Fig S3b, sensitivity to 600 pM) and DNAcrRNA inputs (Fig. 3d, sensitivity to 20 pM) in the absence of isothermal amplification, demonstrating that sensitivity of this recombination system equals other CRISPR nucleic acid sensing platforms[12]. Wanting to evaluate the speed of RePAIR, using crRNA or DNAcrRNA inputs at 100 nM, we incubated RePAIR reactions for 5-,

15-, 30- min or 1 hour at 37 °C. An aliquot from each time point was then added to CFS to determine maximum initial rates within the first hour, with results showing all the RePAIR reaction timepoints yielded productive LacZα reorganization (Fig. S4a). Monitoring CFS reactions for 120 min, all RePAIR reactions induced by crRNA (blue) or DNAcrRNA (red) yielded comparable absorbance reads (Fig. S4b) allowing us, if needed, to significantly shorten the RePAIR reaction without any detection loss over time. It is also worth noting that at equal input concentrations, the DNAcrRNA performed better than the crRNA in the RePAIR reaction, possibly due to the amplification of the signal through the generation of additional crRNA from the T7 promoter on the dsDNA. We refer to this combination of FACT and RePAIR DNA reorganization as FACTOR (FACT on RePAIR).

Comprehensive output capabilities

To broaden output capabilities, we designed four supplementary constructs, three fluorescent proteins and one enzyme enabling electrochemical detection. For all fluorescent proteins (Fig. 3e), we successfully detected the repaired reporter within 1 hour of the CFS reaction. For the electrochemical output, and building upon our recent demonstration of the glucose meter as an interface for point-of-care gene circuit diagnostics[28], we designed a RePAIR system for the *trehalase* gene. This enzyme converts a trehalose molecule into two molecules of glucose. Upon correct DNA reorganization of the *trehalase* gene, glucose was generated and detected on the glucose meter (Fig. 3f). Taken together, these demonstrations show how RePAIR-mediated DNA organization can be adapted to virtually any sequence, here showing three different outputs mechanisms (colorimetric, fluorescent and electrochemical) with protein outputs of LacZα, BFP, GFP, RFP and trehalase.

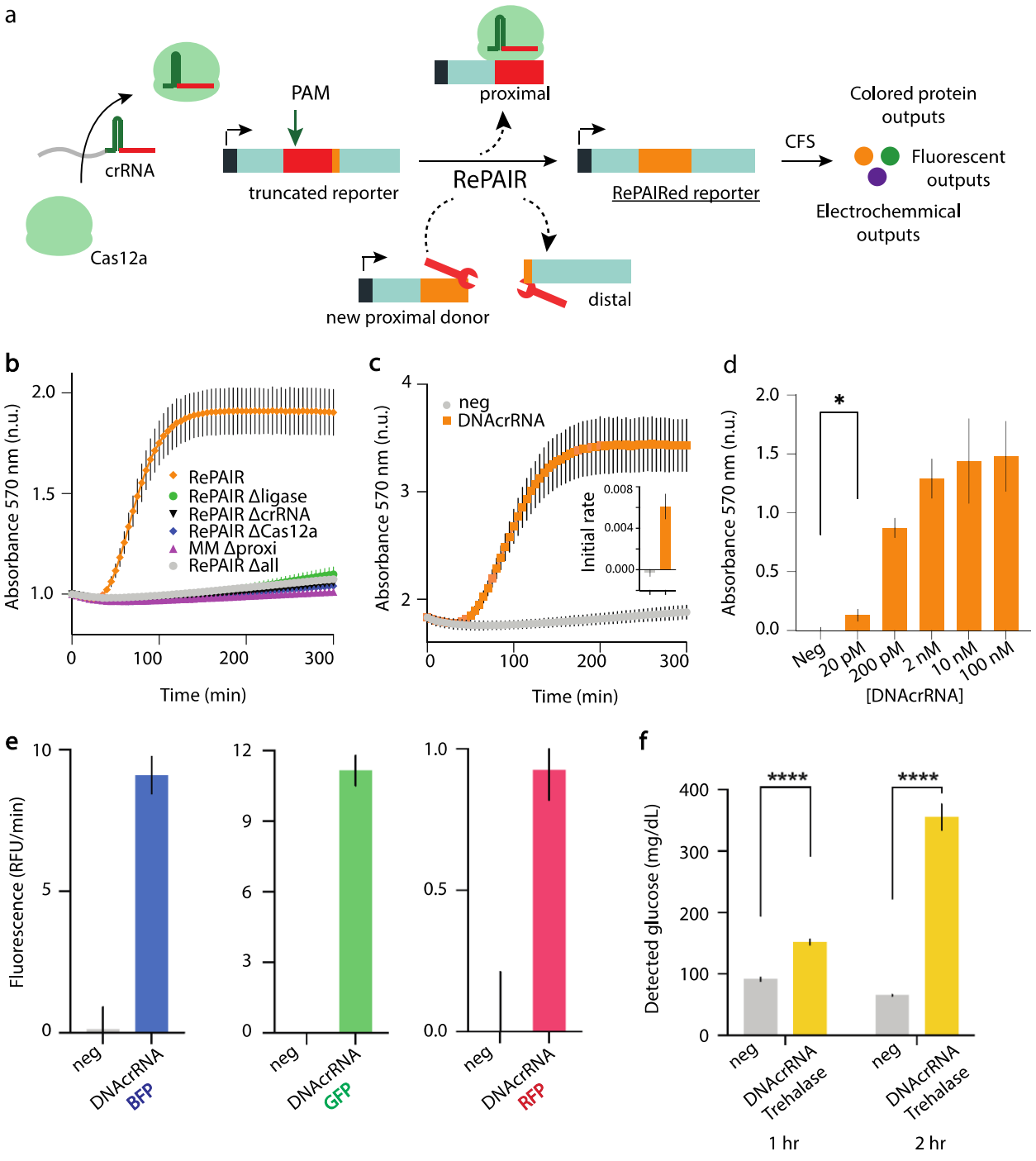


Fig. 3. Reprogrammable PAIRing (RePAIR) enables a multiplexed output system. **a.** Schematic of RePAIR, a CRISPR induced DNA reorganization process for multiplexed outputs in a cell-free protein expression system (CFS). **b.** Validation of RePAIR, using a crRNA. Here, reactions contained a truncated LacZ**α** coding sequence and were supplemented with all the components needed for RePAIR (orange) or, alternatively, selected components were removed (**Δ**component). The product of RePAIR was added to a CFS reaction. Absorbance was monitored at 570 nm in a plate reader to reflect functional RePAIRed LacZ**α**. **c.** Validation of RePAIR using a synthetic DNAcrRNA. Truncated LacZ**α** and all the

components needed for RePAIR were added to the reactions, with (orange) or without (neg-H_2O, grey) the addition of the DNAcrRNA. The product of RePAIR was added to a CFS reaction. Absorbance was monitored at 570 nm in a plate reader to reflect functional RePAIRed LacZ**α**. Insert shows the initial rate of absorbance within 1 hour. **d.** Various concentrations of DNAcrRNA, or H_2O (neg) were added to the RePAIR reaction to test sensitivity. Products of RePAIR were added to a CFS and absorbance was monitored at 570 nm in a plate reader. *: p=0.0188. **e.** RePAIR reactions containing truncated blue fluorescent protein (BFP), truncated green fluorescent protein (GFP), or truncated red fluorescent protein (RFP) were supplemented with H_2O (neg) or with the respective DNAcrRNA. Products of RePAIR were added to a CFS. Fluorescence was monitored at 399/456 nm for BFP, 485/530 nm for GFP and 584/607 nm for RFP. **f.** RePAIR reactions containing truncated trehalase coding sequence were supplemented with H_2O (neg) or with the matching DNAcrRNA. Products of RePAIR were added to a CFS. Production of glucose was monitored at 1hr and 2hr on a glucometer. ****: p<0.0001. Error bars: SD. Experiments displayed are representative of independent biological triplicates.

One-pot multiplexing capabilities

We next tested whether RePAIR was compatible with one-pot, multiplexed DNA reorganization for parallel output detection. A single mix reaction was set up containing the three non-functional fluorescent reporters (truncated RFP, BFP and GFP), along with all of the respective donor proximal strands and enzymes (Cas12a and ligase). Synthetic DNAcrRNAs were added to the mix to induce sequence-specific RePAIR, individually or in combination, incubated for 1 hour, and then added to the CFS to enable protein expression. After a 2-hour incubation, clear fluorescence signals were detected in each of the corresponding channels where the related DNAcrRNAs were added, demonstrating high specificity and capacity for one-pot RePAIR-based multiplexing (Fig. 4a).

Functionalized CRISPR amplification products as global connector to alternative CRISPR diagnostic platforms

Having established that functionalized barcode amplification products can be used directly for *cis*-cleavage outputs (Fig. 2) and induce RePAIR as an output for nucleic acid sensing (Fig. 3), we next examined whether barcode amplicons could also be linked to *trans*-cleavage-based CRISPR diagnostics[10,11]. Using synthetic target RNA or DNA, we began by performing isothermal barcoding amplification. The CRISPR functionalized products were then added to collateral cleavage fluorescent output reactions. In this context, we tested both Cas12a (Fig. 4b, c, d) and Cas13a (Fig. 4e, f, g). Using Cas12a, the reaction contained an activator dsDNA as well as the quenched

fluorophore ssDNA (Fig. 4b). We observed a rapid fluorescent increase in the Cas12a reactions containing either target DNA or RNA, compared to their negative controls well before 1 hour (Fig. 4c and initial rate within 45 min Fig. 4d). This confirmed that, as with previous CRISPR-based sensors, collateral cleavage provides a suitable readout to FACT barcoded nucleic acids. We then moved to Cas13a, another widely utilized enzyme in CRISPR diagnostic platforms. Primer overhangs containing the direct repeat and spacer length characteristic of this enzyme were designed. Here again, a functionalized CRISPR amplicon was used in a collateral cleavage reaction containing Cas13a enzyme and its substrate, a reporter ssRNA containing a quenched fluorophore. In the presence of RNA or DNA target sequences, a rapid fluorescence increase was detected in less than 10 minutes (Fig. 4f and initial rate within 14 min Fig. 4g).

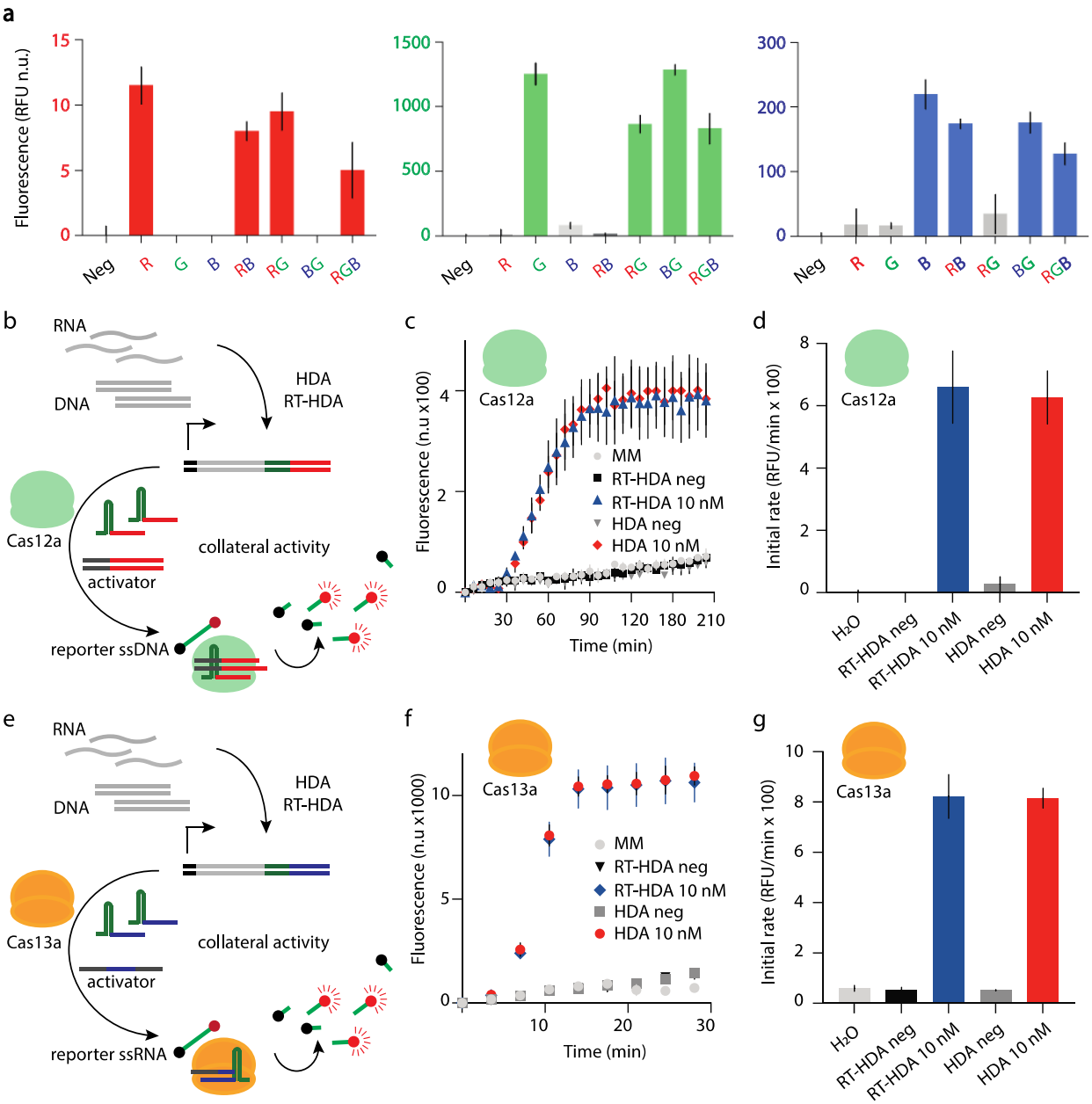


Fig. 4. One-pot multiplexing and compatibility with *trans*-cleavage-based CRISPR outputs. a. A one-pot RePAIR reaction was set up containing all truncated coding sequences, proximal donor strands and enzymes. DNAcrRNA(s) inducing RePAIR were added alone or in combination (R or G or B or RB or RG or BG or RGB). Products of RePAIR reactions were added to a CFS in technical duplicate to a 384-well plate. Each well was monitored in the three fluorescent channels (red, green, blue) to record signal increase induced by RePAIR for all the combinations. Results were plotted at time point 2 hr of CFS. **b.** Schematic of nucleic acid sensing using the functionalized amplification (FACT) product with Cas12a enzyme in *trans*-cleavage activity. The activator is a dsDNA and reporter is a ssDNA linked to a quenched fluorophore. **c.** Products of the isothermal amplification were directly added into the *trans*-cleavage readout reaction and fluorescence was monitored real time at 536/576 nm. **d.** Using data in c. initial rates were plotted within the first 45 min. **e.** Schematic of nucleic acid sensing using the

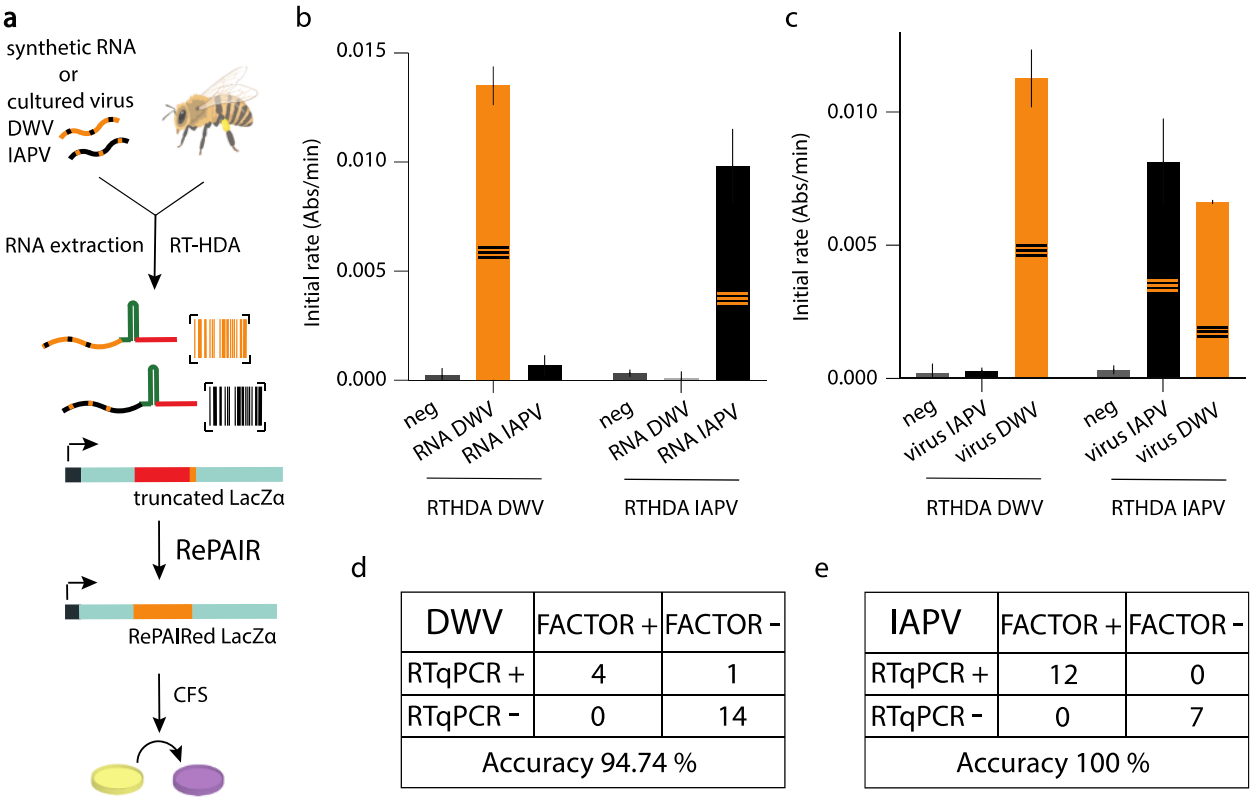
functionalized amplification (FACT) product with Cas13a enzyme in *trans*-cleavage activity. The activator is a ssRNA and reporter is a ssRNA linked to a quenched fluorophore. **f.** Products of the isothermal amplification were directly added into the *trans*-cleavage readout reaction and fluorescence was monitored in real time at 490/520 nm. **g.** Using data in f. initial rate was plotted within the first 14 min. Error bars: SD. Experiments displayed are representatives of independent biological triplicates

Diagnostic application of FACTOR

Finally, we sought to evaluate the potential of FACTOR for infectious disease detection in a novel agricultural use case. We applied our nucleic acid sensing technology to honey bee virus detection. Honey bees are critical pollinators worldwide, and, with an estimated 30 % of the agricultural crops depending on them, their economic and environmental impact is tremendous[41]. Unfortunately, bees have been heavily affected by pathogenic infections, such as viruses, that impacts their productivity, ability to sense direction, life span and can cause sudden collapse of entire colonies[42,43]. Further, these viruses can 'spillover' from honey bees to infect native bee populations[44]. Currently definitive diagnosis of viral infection in bees is laboratory-based RT-qPCR[45], making surveillance impractical and expensive for bee managers, such as beekeepers. To demonstrate the potential of FACTOR in agricultural diagnostics, we focused on two critical honey bee viruses: Deformed Wing Virus (DWV) and Israeli Acute Paralysis Virus (IAPV) (Fig. 5a).

Using serial dilutions of synthetic RNA or DNA encoding for DWV, we began by testing the sensitivity of FACTOR. After a 90 min isothermal amplification (RT-HDA or HDA), followed by a 1-hour RePAIR reaction with Cas12a, we were able to detect the synthetic target sequences at 500 fM and 50 aM, respectively (Fig. S5). Using synthetic RNA sequences from DWV and IAPV (at 10 nM), we then tested the specificity of FACTOR (Fig. 5b). Using the maximum initial rate of LacZ signal within the first hour, the reaction provided clear selective detection of IAPV and DWV RNAs. Finally, to provide a proof-of-concept for the viral detection, we used RNA extracted from cultured viruses (Fig. 5c) and from whole bees (Fig. 5d and 5e). Isolating pure honey bee virus (only DWV or only IPAV) is challenging and samples are often cross-contaminated with multiple virii[46,47]. Additionally, wild-caught honey bees are often infected with DWV and therefore inoculation with IAPV will lead to joint infections. We therefore compared the

performance of FACTOR to RT-qPCR (being the current diagnostic gold-standard). As shown Fig. 5c and as confirmed by RT-qPCR (Fig. S6a), IAPV cultured virus contained only IAPV RNA, however DWV cultured virus contained both DWV and IAPV RNAs. Finally, 19 bees were individually analyzed for DWV and IAPV infections using the two methods. Results compared to RT-qPCR (Fig. S6b) demonstrated an accuracy of FACTOR DWV and IAPV diagnostic of 94.7 % and 100 %, respectively.



d

DWV	FACTOR +	FACTOR -
RTqPCR +	4	1
RTqPCR -	0	14
Accuracy 94.74 %		

e

IAPV	FACTOR +	FACTOR -
RTqPCR +	12	0
RTqPCR -	0	7
Accuracy 100 %		

Fig. 5. Diagnostic application of FACTOR. **a.** Schematic of FACTOR workflow using synthetic RNA, cultured viruses or whole bees for detection of DWV and IAPV nucleic acids. Following RNA extraction, isothermal RT-HDA amplification was performed, followed by RePAIR using LacZ**α** as reporter. RePAIR products were added into CFS in technical triplicates and absorbance at 570 nm was monitored over time. **b.** Using synthetic *in vitro* transcribed RNA, isothermal amplification was performed in solutions containing H_2O (neg) or DWV synthetic RNA or IAPV synthetic RNA. Following RePAIR, initial rate of the absorbance within 1 hour of CFS is plotted. Biological triplicate experiments were performed in technical triplicate, with b. showing one representative experiment. **c.** Using cultured viruses, isothermal amplification specific for DWV or IAPV was performed on solutions containing H_20 (neg) or extracted RNA

from both cultured viruses. Following RePAIR, initial rate of the absorbance within 1 hour of CFS is plotted. RT-qPCR were performed in parallel (Fig. S6). Error bars: SD. **d and e.** RNA extracted from whole bee lysates were used for FACTOR-based diagnosis of DWV (d.) or IAPV (e.) infections in with parallel RT-qPCR to determine the accuracy. The honey bee design in Fig. 5 was created using BioRender.com account.

Discussion

Here, we report the development of FACT, a CRISPR-based nucleic acid tracing system, relying on an engineered CRISPR functionalized isothermal amplicon. Importantly, this system is independent of any PAM sequence requirements in target nucleic acids. The output does not serve as the substrate of CRISPR, but rather acts as a regulatory element for programmable gene circuit applications. This addresses a key limitation of conventional CRISPR diagnostics[48], where the presence of a PAM in the target nucleic acid is a pre-requisite and often limits the number of detection sites in a target nucleic acid. By barcoding the nucleic acid of interest with the sequence of a crRNA, we can now choose any sequence within diagnostic targets, which we anticipate will further enable the field of CRISPR-based diagnostics. The flexibility of the system also allows users to choose the CRISPR enzyme that best suits their needs (Cas12a or Cas13a) while maintaining their target site of interest, simply by reprogramming the crRNA sequence with the appropriate characteristics. We further demonstrated that FACT can guide and regulate the *cis*- and *trans*- cleavage activity of established CRISPR diagnostic platforms (Cas12a/Cas13a)[10,11], providing a global linker between systems [10–12]. Moreover, FACT and the subsequent induced cleavage do not rely on the presence of crRNAs in the solution (loaded or in complex with a CRISPR enzyme). This is an important consideration for the stability of CRISPR-based diagnostics during storage and deployment to point-of-care applications.

RePAIR similarly introduces new capabilities to *in vitro* CRISPR-based circuits with a *cis*-cleavage based mechanism enabling sequence-specific DNA reorganization of theoretically any protein-based output. In conjunction with FACT, we see RePAIR as a global converter to link CRISPR activity to a broad range of gene circuit-based outputs

for portable diagnostics. As we demonstrate, the direct output of amplification can be used to control *in vitro* transcription and translation, catalyzing fluorescent, colorimetric and electrochemical signals (GFP, RFP, BFP, LacZ and Trehalase, Fig. 2b-f).

Demonstrating the simplicity of the system, we also show that RePAIR can enable one-pot, multiplexed gene reorganization events using a single CRISPR enzyme (Fig. 3a). Further, by combining the systems to create FACTOR, a simple shift of the crRNA sequence can enable a shift from fluorescence or colorimetric outputs to formats compatible with portable systems like the glucometer[28]. This has potential in the design-test process where gene circuit prototyping can be done using a simple fluorescent assay and then outputs changed for practical deployment. In the application of FACTOR to honey bee diagnostics, we found that the nucleic acid tracing technology can provide specificity and accuracy comparable to RT-qPCR, suggesting it may be useful for in-field diagnostics and the detection of other nucleic acids-based diseases.

While we demonstrate a series of new capabilities, and strong performance of the technology, these new applications for CRISPR-mediated regulation are still at the proof-of-concept stage. To enable point-of-care deployment, we see opportunities in optimizing the speed of some steps, like target amplification or the CFS, perhaps through buffer optimization, as well as a potential to reduce the number of steps needed to perform the test. Beyond *in vitro diagnostic* applications, and looking toward the future, we are also curious about the potential of linking these concepts to monitor gene expression *in vivo* by integrating a spacer/direct repeats into the UTR of mRNAs for Cas-protein-mediated expression of reporter proteins via RePAIR.

We also envision the use of DNAcrRNA as a molecular barcode, where FACT and/or RePAIR could facilitate the use of DNA to label objects. In such applications, DNA labels can be used to authenticate high-value commodities like art, food, textiles, oil or drugs[49,50]. The concept is analogous to optical barcoding systems, such UPC or QR codes, but with the advantage that a DNA barcode is more challenging to replicate and can be embedded in or on any product, making tampering or fraud more difficult.

Moreover, as a DNA molecule, it encompasses high stability, and contains high combinatorial capabilities. It would also be interesting to merge the optical and DNA label concepts, with the development of a molecular QR code as an accessible and multiplexed readout system.

On a technical level, FACT and RePAIR expand the field of CRISPR-based diagnostics with the concepts of target barcoding and decoding, respectively. While FACT links sequence recognition to established CRISPR reporter methods, such as collateral cleavage induced fluorescence, FACTOR introduces the ability to control multiplexed protein expression in response to upstream sequences. There are many contexts where such control of *in vitro* protein synthesis could be exploited, including, possibly in the future, where diagnostic outcomes could direct the production of a protein-based therapeutic treatment. More broadly, the ability of FACT to serve as a global connector, converting sequence inputs and into molecular signals (cleavage, RNA, proteins) provides the community with a new lever for engineering biology. We are keen to see how this work will integrate into the ever-growing toolbox of technologies promoting low-cost and decentralized health care.

Material and methods

Plasmids and oligonucleotides:

Oligonucleotides

All oligonucleotides were ordered through Eurofin or Integrated DNA Technologies (IDT), as Gblocks, ultramers or primers. Molecular beacons were ordered as ssDNA with the fluorophore (IDT, 6-FAM) or quencher (IDT, IowaBlack FQ) attached respectively to the 3’ or the 5’ end. Bottom strands of the RePAIR donor proximal strand were ordered with 5’-phosphate to enable ligation during RePAIR.

Synthetic DNA for DWV, IAPV and rpl8 sequences were ordered and amplified by PCR (NEB Q5 M0491L) using their corresponding primers (Table 1).

For *in vitro* transcription, synthetic DNA were ordered with a T7 promoter appended to their sequences or the T7 promoter was added by the forward primer used during PCR (Table 1).

Plasmids

pBR939b (NovoPro, V009103) was a gift from the Collins lab. LacZα and LacZΩ were gifts from the Green lab. LacZα coding sequence was inserted into a pET15b backbone (Invitrogen). RFP (http://parts.igem.org/Part:BBa_E1010) (group USAFA iGEM 2019) and BFP (http://parts.igem.org/Part:BBa_K592100)[51] coding sequences were parts from iGEM and inserted into pET15b (Invitrogen) backbone. pY002 was a gift from Feng Zhang (Addgene plasmid # 69975)[33] and the coding sequence for FnCas12a was amplified by PCR and inserted in a pET15 backbone. Trehalase was purchased from DNASU (BGH37_B01a).

Chemicals:

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) or BioShop Canada Inc. (Burlington, ON, Canada).

RNA synthesis and extraction:

Target synthetic RNAs and crRNAs were generated using the T7 HighScribe InVitro transcription kit (NEB, E2040S), following the manufacturer's instruction, overnight at 37°C. RNA from bees or cultured viruses were extracted using TRIzol reagent (Thermofisher #15596026) following manufacturer's instructions and resuspended in water.

(RT)-HDA amplification

Reactions were assembled following manufacturer's protocol, with minor modifications. Briefly, 9 µL reactions were assembled with 3 mM of $MgSO_4$, 40 mM NaCl, 2% RNAse inhibitors (NEB, M0314L), 100 nM of each primer (see Table 1). If RT-HDA was run, 0.1 µL (20 U) of Superscript IV RT enzyme (Invitrogen, #18090010) was supplemented. 1 µL of samples containing synthetic RNA/DNA or 500 ng of extracted RNA from cultured viruses or from crushed bees was then added for a final volume of 10 µL and 10 µL of

oil was layered on top of the reaction. Reactions were incubated between 30 min and 3 hours at 65°C.

If the output of the amplification was to be used for RePAIR or *cis*-cleavage applications, following amplification, 2 µL of the product was treated with 0.6 µL of Proteinase K (Thermofisher, EO0491) for 10 min at 37 °C and heat inactivated for 5 min at 90 °C. Heat inactivated products were used for downstream experiments at 5% v/v.

If the output of the amplification was to be used for *trans*-cleavage application, the product of the amplification was directly used in the downstream experiment at 5% v/v.

CRISPR Cas12a *cis*-cleavage

Cis-cleavage experiments were performed using the proteinase K treated outputs of isothermal amplification. Briefly, reactions of 9 µL were assembled in KGB buffer[34] (100 mM potassium glutamate, 25 mM Tris-acetate (pH 7.5), 500 µM 2-mercaptoethanl, 10 µg/mL BSA), containing: 5 % v/v of the output amplification, 2 µM FnCas12a, 1 µM dsDNA reporter molecular beacon, 2 mM NTPs (NEB, N0450S), 1 U/µL of T7 RNA polymerase (Thermofischer, EP0111), 2 U/µL of RNAse inhibitors (NEB, M0314L). Technical duplicate of 4 µL for each reaction were run in a 384-well plate (Corning #3544), monitoring TAMRA fluorescence (543/584 nm) on a plate reader (BioTek, Fisher Scientific BTNEO2M).

Homologous direct recombination-based RePAIR

Truncated linear pET15a (lipET15trca) (20 nM) was incubated with FnCas12a (2 µM), MgCl2 (10 mM), repair fragment containing the homologous arms (HA) of 30 or 40 bp: HA30trca or HA40trca (60 nM), crRNAtrca (6 µM), HiFi DNA assembly master mix (NEB, #E2621S) at 0.5X final concentration, in KGB buffer. Reactions of 5 µL were assembled and incubated for 1 hour at 37 °C. Following HDR, 0.5 µL of the reaction was added to an 8 µL CFS reaction and absorbance was monitored real-time at 570 nm, using a plate reader at 37 °C.

RePAIR

RePAIR reactions were assembled in a 5 µL final volume in KGB buffer (see above): 2 µM Cas12a, 10 mM $MgCl_2$, 20 nM of truncated reporter, 60 nM of RePAIR proximal donor strand, 2 U/µL of RNAse inhibitors, 1.25 µL of ligase (final concentration of 0.5X, NEB, M0370S). If RePAIR was performed using a synthetic DNAcrRNA or using the output of an isothermal amplification, 1 U/µL of T7 RNA polymerase (Thermofischer, EP0111) and 2 mM NTPs (NEB, N0450S) were supplemented.
Finally, 100 nM of DNAcrRNA for LacZa/BFP/RFP/GFP or 57.5 nM of DNAcrRNA for Trehalase or 0.25 µL of crRNA/DNAcrRNA (at specified concentrations for sensitivity) or 5 % v/v outputs of isothermal amplification were added to the RePAIR mix and incubated for 1 hour at 37°C unless specified otherwise.
RePAIR proximal donor strands (top and bottom) were ordered as ssDNA, resuspended in water at 100 µM and annealed together in a 1X PBS solution to a final concentration of 20 µM, before use.

Cell-free system

NEB PURExpress (#E6800L) was used as the only source of cell-free system (CFS) in this paper. Cell-free reactions where prepared according to the manufacturer's protocol (40 % solution A, 30 % solution B), supplemented with 0.5 % v/v RNase inhibitor (NEB M0314S).

For colorimetric output reactions

All colorimetric CFS reactions were supplemented with 0.2 µL of 25 mg/mL CPRG (chlorophenol red-b-D-galactopyranoside, Roche #10884308001) and 0.5 µL of LaZΩ in each 8 µL. LaZΩ was produced by an overnight (o/n) CFS reaction, containing 5 ng/µL of plasmid encoding the omega subunit of LacZ (LaZΩ), and supplemented directly in the CFS reactions for colorimetric outputs.
Filter papers (Whatman #Z241067) were blocked in 5 % BSA overnight, then dried. A 2 mm biopsy punch (Miltex, VWR CA-95039-098) was used to cut a paper-disc from the prepared filter paper, which was then placed into reaction wells of the 384-well plate.
1.8 µL of each reaction in technical triplicate were added on paper, the plate was sealed with a clear plastic film (Sarstedt 95.1994) and incubate at 37 °C in the plate reader for absorbance monitoring at 570 nm.

For fluorescent output reactions

0.5 µL of the outputs reaction of RePAIR was added to an 8 µL CFS reaction set as described previously. The reaction was divided into technical duplicates of 3.8 µL, loaded into the 384-well plate and incubated at 37°C in the plate reader for fluorescence monitoring at Ex: 399/20 nm and Em: 456/20 nm for BFP, at Ex: 584/10 nm and Em: 607/10 nm for RFP and at Ex: 485/20 nm and Em: 530/20 nm for GFP.

For electrochemical output reactions

0.5 µL of the outputs reaction of RePAIR was added to an 8 µL CFS reaction set as described previously and supplemented in trehalose (the trehalase enzyme substrate (BioShop, TRE222)) at 20 mM. Reactions were incubated for 1 or 2 hours at constant temperature of 37°C in a thermocycler. Following incubation, 0.7 µL was pipetted and measured with a glucose meter (Bayer Contour Blood Glucose Monitoring System - Model 9545C) as described previously[28]. Each measurement was performed three times to achieve technical triplicate measurements for each reaction.

CRISPR FnCas12a *trans*-cleavage

RT-HDA or HDA amplification were set up as previously described and incubated for 90 min prior to the readout reaction. DNAseAlert (IDT #11-04-03-04), used as reporter ssDNA, was rehydrated in the 10X buffer at 3.33 µM. Reactions were set up in 13 µL final volume, containing: 100 nM of the activator dsDNA, 150 nM of the reporter ssDNA, 10 mM of $MgCl_2$, 2 mM NTPs, 1 U/µL of T7 RNA polymerase, 2 U/µL of RNAse inhibitors, 2 µM FnCas12a. Product of the amplification was added at 5% v/v and reaction volume was completed to 13 µL with KGB buffer. 4 µL of each reaction was dispensed in triplicate in 384-well plate and monitored over time using fluorescence at 536 nm / 576 nm.

CRISPR LwCas13a *trans*-cleavage

RT-HDA or HDA amplification were set up as previously described and incubated for 90 min prior to the readout reaction. RNaseAlert_v2 (ThermoFisher #4479768), used as reporter ssRNA, was rehydrated in the 10X buffer at 3.33 uM. Reactions were set up in 13 µL final volume, containing: 100 nM of the activator RNA, 150 nM of the reporter

RNA, 10 mM of $MgCl_2$, 2 mM NTPs, 1 U/µL of T7 RNA polymerase, 2 U/µL of RNAse inhibitors, 2 µM FnCas12a. Product of the amplification was added at 5% v/v and reaction volume was completed to 13 µL with KGB buffer. 4 µL of each reaction was dispensed in triplicate in 384-well plate and monitored over time using fluorescence at 490 nm / 520 nm.

Statistical Analysis

Unless otherwise indicated, experimental data sets were compared using two-tailed unpaired t tests. All statistical analysis and graphing were done using Graphpad Prism 7 software.

Cultured viruses and Bees

Cultured viruses and infected bees were produced exactly as previously described[46,47].

Cas12a purification

pET15-FnCas12a plasmid was transformed into BL21 cells (DE3, NEB, C2527I). 1 L of media [LB Lennox Broth, 50 µg/mL Ampicillin, 1% glucose] in a 2 L baffled flask (Tunair; SS-6001C No-Baffle Flask Kit (Dri-Gauze)) was inoculated with a starter culture and grown in a shaker (New Brunswick™; Innova® 44/44R) at 37°C - 200 RPM until OD600 0.6 was reached (ThermoScientific Spectrophotometer). 0.5 mM IPTG () was then added to the culture for induction, followed by 18 hours at 18°C - 200 RPM (New Brunswick, Innova® 44/44R). Culture was then spun down for 25 min at 4°C - 8000 RPM using a JLA8.100 rotor (Beckman Coulter; Avanti JXN-26) and the supernatant was decanted. The pellet was resuspended in 30 mL filter-sterilized Lysis Buffer, pH 7.5 [0.005 M imidazole, 0.5 M NaCl, 0.05 M HEPES, 5% (v/v) glycerol, 0.5 mM TCEP, 0.1 mM PMSF], homogenized by vortexing, sonicated (Qsonica Sonicator) on ice using a 6.4 mm probe for 6 min (1 sec ON, 1 sec OFF, 50% amplitude), and spun down using a JA-14.50 rotor (Beckman Coulter; Avanti JXN-26) for 45 min at 4°C – 17000 RCF. The supernatant was then passed through a 0.45 µm filter (MilliporeSigma Syringe Filters), followed by a 0.22 µm filter and kept at 4°C. The lysate was then purified using the AKTA Pure Chromatography System (GE Healthcare, FPLC) on a 1 mL HisTrap FF

Column. The column was first equilibrated with 5 columnar volumes (CVs) of filter-sterilized Binding Buffer, pH 8.2 [0.015 M imidazole, 0.5 M NaCl, 0.02 M HEPES, 5%(v/v) glycerol]. Lysate was loaded onto the column, the washed using 20 CVs of Binding Buffer, and eluted using 10-20 CVs of filter-sterilized Elution Buffer, pH 8.2 [0.5 M imidazole, 0.5 M NaCl, 0.02 M HEPES, 5% (v/v) glycerol]. The elution fractions corresponding to the chromatography peak were tested using Coomassie Brilliant Blue staining of 10% SDS-PAGE gels. Fractions showing expression were consolidated in an Amicon Ultra-15 Centrifugal Filter Unit 50 kDa (MilliporeSigma) and washed four times in Storage Buffer, pH 7.5 [0.5 M NaCl, 0.02 M HEPES, 5% (v/v) glycerol]. The purified protein was aliquoted and flash frozen in liquid nitrogen.

RT-qPCR

To estimate reactions efficiency, DNA templates were *in vitro* transcribed with the HiScribe T7 Quick High Yield RNA synthesis kit (NEB, E2040S) according to the manufacturer's instruction. 100 ng of RNA was used as template for cDNA synthesis using the Superscript IV VILO kit (ThermoFisher #11756050) and 10x serial dilutions were performed and ran alongside samples being tested.

Extracted RNAs (whole bees or viral aliquots) were quantified using a nanodrop (ThermoFisher, #ND-ONE-W), diluted in RNase-free water and 100 ng RNA input was used for cDNA synthesis. For RT-qPCR setup, PowerUP SYBR green master mix (ThermoFisher #25742) and appropriate primer sets (Table 1) were prepared for 10uL total reactions in a 384-well plate format, with 2 µL of cDNA input, or controls, added to each well. Real-time PCR was performed on a Bio-Rad CFX-384 system with standard cycling and melt curve analysis.

Competing interests

M.K, P.S.M and K.P. are co-inventors of the molecular sensor technology. Other authors have no conflict of interest.

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Authors contribution

M.K. developed the concept, designed and performed the experiments and wrote the manuscript. E.A designed and performed experiments, X.Y. contributed to experimental designs and writing of the manuscript. J.D. performed the qPCR experiments. P.S.M helped review the manuscript, P.H., P.S. and S.L. purified FnCas12a, W.C participated in literature review. A.D. and A.C. provided the bees. B.H provided bees, reviewed the manuscript and co-supervised the project. K.P supervised the project and reviewed the manuscript.

References < 50

1. Tan, X., Letendre, J. H., Collins, J. J. & Wong, W. W. Leading Edge Synthetic biology in the clinic: engineering vaccines, diagnostics, and therapeutics. *Cell* **184**, 881–898 (2021).
2. Pardee, K. *et al.* Rapid, Low-Cost Detection of Zika Virus Using Programmable Biomolecular Components. *Cell* **165**, 1255–1266 (2016).
3. Mao, N., Cubillos-Ruiz, A., Cameron, D. E. & Collins, J. J. Probiotic strains detect and suppress cholera in mice. *Sci. Transl. Med* **10**, 2586 (2018).
4. Georgianna, D. R. & Mayfield, S. P. Exploiting diversity and synthetic biology for the production of algal biofuels. *Nat. 2012 4887411* **488**, 329–335 (2012).
5. Pardee, K. *et al.* Portable, On-Demand Biomolecular Manufacturing. *Cell* **167**, 248-259.e12 (2016).
6. Lin, G. M., Warden-Rothman, R. & Voigt, C. A. Retrosynthetic design of metabolic pathways to chemicals not found in nature. *Curr. Opin. Syst. Biol.* **14**, 82–107

(2019).
7. Xu, S. & Li, Y. Yeast as a promising heterologous host for steroid bioproduction. *J. Ind. Microbiol. Biotechnol.* **47**, 1 (2020).
8. Choi, Y. & Lee, S. Y. Biosynthesis of inorganic nanomaterials using microbial cells and bacteriophages. *Nat. Rev. Chem. 2020 412* **4**, 638–656 (2020).
9. Cui, Y. Engineered phages for electronics. *Biosens. Bioelectron.* **85**, 964–976 (2016).
10. Chen, J. S. *et al.* CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity. *Science (80-.).* **360**, 436–439 (2018).
11. JS, G. *et al.* Nucleic acid detection with CRISPR-Cas13a/C2c2. *Science* **356**, 438–442 (2017).
12. Kaminski, M. M., Abudayyeh, O. O., Gootenberg, J. S., Zhang, F. & Collins, J. J. CRISPR-based diagnostics. doi:10.1038/s41551-021-00760-7.
13. Azhar, M. *et al.* Rapid and accurate nucleobase detection using FnCas9 and its application in COVID-19 diagnosis. *Biosens. Bioelectron.* **183**, (2021).
14. Ding, X. *et al.* Ultrasensitive and visual detection of SARS-CoV-2 using all-in-one dual CRISPR-Cas12a assay. *Nat. Commun. 2020 111* **11**, 1–10 (2020).
15. Li, S. Y. *et al.* CRISPR-Cas12a-assisted nucleic acid detection. *Cell Discov. 2018 41* **4**, 1–4 (2018).
16. Gootenberg, J. S. *et al.* Multiplexed and portable nucleic acid detection platform with Cas13, Cas12a, and Csm6. *Science* **360**, 439–444 (2018).
17. Parinaz Fozouni, A. *et al.* Amplification-free detection of SARS-CoV-2 with CRISPR-Cas13a and mobile phone microscopy. *Cell* **184**, 323-333.e9 (2021).
18. JP, B. *et al.* CRISPR-Cas12-based detection of SARS-CoV-2. *Nat. Biotechnol.* **38**, 870–874 (2020).
19. Kellner, M. J., Koob, J. G., Gootenberg, J. S., Abudayyeh, O. O. & Zhang, F. SHERLOCK: nucleic acid detection with CRISPR nucleases. *Nat. Protoc. 2019 1410* **14**, 2986–3012 (2019).
20. Burmistrz, M., Krakowski, K. & Krawczyk-Balska, A. RNA-Targeting CRISPR-Cas Systems and Their Applications. *Int. J. Mol. Sci.* **21**, (2020).
21. Scherer, S. *A short guide to the human genome*. (Cold Spring Harbor Laboratory

Press, 2008).

22. Kaminski, M. M., Abudayyeh, O. O., Gootenberg, J. S., Zhang, F. & Collins, J. J. CRISPR-based diagnostics. *Nat. Biomed. Eng. 2021 57* **5**, 643–656 (2021).
23. Bruch, R., Urban, G. A. & Dincer, C. CRISPR/Cas Powered Multiplexed Biosensing. *Trends Biotechnol.* **37**, 791–792 (2019).
24. Li, Y., Liu, L. & Liu, G. CRISPR/Cas Multiplexed Biosensing: A Challenge or an Insurmountable Obstacle? *Trends Biotechnol.* **37**, 792–795 (2019).
25. Ackerman, C. M. *et al.* Massively multiplexed nucleic acid detection with Cas13. *Nat. 2020 5827811* **582**, 277–282 (2020).
26. Jiao, C. *et al.* Noncanonical crRNAs derived from host transcripts enable multiplexable RNA detection by Cas9. *Science (80-.).* **372**, 941–948 (2021).
27. Hajian, R. *et al.* Detection of unamplified target genes via CRISPR–Cas9 immobilized on a graphene field-effect transistor. *Nat. Biomed. Eng. 2019 36* **3**, 427–437 (2019).
28. Amalfitano, E. *et al.* A glucose meter interface for point-of-care gene circuit-based diagnostics. *Nat. Commun. 2021 121* **12**, 1–10 (2021).
29. Sedlmayer, F., Aubel, D. & Fussenegger, M. Synthetic gene circuits for the detection, elimination and prevention of disease. *Nat. Biomed. Eng. 2018 26* **2**, 399–415 (2018).
30. Leenay, R. T. & Beisel, C. L. Deciphering, Communicating, and Engineering the CRISPR PAM. *J. Mol. Biol.* **429**, 177–191 (2017).
31. Kleinstiver, B. P. *et al.* Broadening the targeting range of Staphylococcus aureus CRISPR-Cas9 by modifying PAM recognition. *Nat. Biotechnol. 2015 3312* **33**, 1293–1298 (2015).
32. Kleinstiver, B. P. *et al.* Engineered CRISPR-Cas9 nucleases with altered PAM specificities. *Nat. 2015 5237561* **523**, 481–485 (2015).
33. B, Z. *et al.* Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* **163**, 759–771 (2015).
34. Fonfara, I., Richter, H., Bratovič, M., Le Rhun, A. & Charpentier, E. The CRISPR-associated DNA-cleaving enzyme Cpf1 also processes precursor CRISPR RNA. *Nat. 2016 5327600* **532**, 517–521 (2016).

35. Pardee, K. *et al.* Paper-Based Synthetic Gene Networks. *Cell* **159**, 940–954 (2014).
36. Garenne, D. *et al.* Cell-free gene expression. *Nat. Rev. Methods Prim. 2021 11* **1**, 1–18 (2021).
37. Mousavi, P. S. *et al.* A Multiplexed, Electrochemical Interface for Gene Circuit-Based Sensors. *Nat. Chem.* **12**, 48 (2020).
38. SY, L., GP, Z. & J, W. C-Brick: A New Standard for Assembly of Biological Parts Using Cpf1. *ACS Synth. Biol.* **5**, 1383–1388 (2016).
39. Li, M. Z. & Elledge, S. J. Harnessing homologous recombination in vitro to generate recombinant DNA via SLIC. *Nat. Methods 2007 43* **4**, 251–256 (2007).
40. Jeon, Y. *et al.* Direct observation of DNA target searching and cleavage by CRISPR-Cas12a. *Nat. Commun. 2018 91* **9**, 1–11 (2018).
41. Gallai, N., Salles, J. M., Settele, J. & Vaissière, B. E. Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. *Ecol. Econ.* **68**, 810–821 (2009).
42. Tantillo, G. *et al.* Virus Infections of Honeybees Apis Mellifera. *Ital. J. Food Saf.* **4**, 157–168 (2015).
43. O, B., T, B., I, D., H, K. & N, N. Occurrence of six honeybee viruses in diseased Austrian apiaries. *Appl. Environ. Microbiol.* **72**, 2414–2420 (2006).
44. Gusachenko, O. N., Woodford, L., Balbirnie-Cumming, K., Ryabov, E. V. & Evans, D. J. Evidence for and against deformed wing virus spillover from honey bees to bumble bees: a reverse genetic analysis. *Sci. Reports 2020 101* **10**, 1–10 (2020).
45. de Miranda, J. R. *et al.* Standard methods for virus research in Apisï¿¿mellifera. *J. Apic. Res.* **52**, 1–56 (2013).
46. Hsieh, E. M., Carrillo-Tripp, J. & Dolezal, A. G. Preparation of Virus-Enriched Inoculum for Oral Infection of Honey Bees (Apis Mellifera). *JoVE (Journal Vis. Exp.* **2020**, e61725 (2020).
47. Carrillo-Tripp, J. *et al.* In vivo and in vitro infection dynamics of honey bee viruses. *Sci. Reports 2016 61* **6**, 1–12 (2016).
48. Collias, D. & Beisel, C. L. CRISPR technologies and the search for the PAM-free nuclease. *Nat. Commun. 2021 121* **12**, 1–12 (2021).

49. SelectaDNA Forensic Coding | SelectaDNA. https://www.selectadna.co.uk/.
50. University at Albany Launches i2M Standards: Key Advances in Systemic Solutions to Solve Art Industry Fraud - University at Albany-SUNY. https://www.albany.edu/news/63269.php.
51. OM, S. *et al.* Conversion of red fluorescent protein into a bright blue probe. *Chem. Biol.* **15**, 1116–1124 (2008).