

# MOLECULAR DIAGNOSIS OF INFECTIOUS DISEASE AND CANCER VIA SYNTHETIC BIOLOGICAL METHODS



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## Abstract

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Synthetic biology is an emerging field that unveils a new dimension to biology, where the disciplines of engineering and biological sciences are brought together to program novel biological entities or re-wire existing ones. The ultimate goal is to achieve *de novo* engineering of biological systems to address the most pressing societal issues ranging from environmental and industrial to healthcare while accelerating the progress of fundamental biological research to answer important questions about life. However, the complications, instability and insecurity of a genetic network have hindered the translation of synthetic biological technologies into real-world applications. This thesis aims to contribute partly to these challenges and seeks to address two of the most urgent global health threats at the moment, the COVID-19 pandemic and cancer.

To support the testing strategy for COVID-19, I begin by re-purposing a traditional cell-free enzymatic reaction, RT-LAMP to detect multiple targets on the SARS-CoV-2 RNA genome. Reported here are proof-of-concept experiments to demonstrate the diagnostic sensitivity and specificity of this method and highlight the practicality of using RT-LAMP as a rapid diagnostic test for COVID-19. To refine the testing method, I introduce a molecular switch that reduces the false positive likelihood by preventing self- and off-target amplification. In particular, the adoption of molecular switch has seen a drop of the false positive rate from 60 % to 0 % in a RT-LAMP experiment with 20 replicates. Through a large-scale clinical validation, this optimised RT-LAMP is shown to provide COVID-19 diagnosis within a short turn-around time at point-of-care setting.

To design a diagnostic for colorectal cancer, I first develop a bacterial whole-cell biosensor by expressing a specific single-domain antibody on the outer membrane of *Escherichia coli* BL21 to confer binding specificity to a cancer biomarker, carcinoembryonic antigen. I then demonstrate the diagnostic sensitivity of the whole-cell biosensor in a biological agglutination assay format. To transform the whole-cell biosensor into a potential *in vivo* diagnostic and therapeutic agent, I adopt two different top-down approaches to generate synthetic biological chassis, SimCell and minicell, which are non-replicable, non-growing and metabolically active. These chassis allow the housing and actuation of synthetic circuits without any interference from the host genome, which can thus be exploited for a wide range of healthcare-related applications.

Overall, the research on the cell-free system and genome-less chassis described herein provides a genetically stable and secure system to help synthetic biology reach its translational potential.

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## Acronym

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|                |                                                               |
|----------------|---------------------------------------------------------------|
| RNA            | Ribonucleic Acid                                              |
| DNA            | Deoxynucleic Acid                                             |
| CRISPR         | Clustered, regularly interspaced short palindromic repeats    |
| Cas            | CRISPR-associated proteins                                    |
| <i>E. coli</i> | <i>Escherichia coli</i>                                       |
| RT-LAMP        | Reverse transcription, loop mediated isothermal amplification |
| mRNA           | Messenger RNA                                                 |
| tRNA           | Transfer RNA                                                  |
| siRNA          | Small interfering RNA                                         |
| sRNA           | Small RNA                                                     |
| miRNA          | Micro RNA                                                     |
| RBS            | Ribosome binding site                                         |
| PCR            | Polymerase chain reaction                                     |
| GFP            | Green fluorescence protein                                    |
| CFS            | Cell-free system                                              |
| SARS-CoV-2     | Severe acute respiratory syndrome coronavirus 2               |
| COVID-19       | Coronavirus disease 2019                                      |
| NAAT           | Nucleic acid amplification test                               |
| LFT            | Lateral flow test                                             |
| CRC            | Colorectal cancer                                             |
| WCB            | Whole-cell biosensor                                          |
| VHH            | Single domain antibody/nanobody                               |

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# 1. Introduction

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## 1.1 Context

The field of synthetic biology is at the interface of biological sciences and engineering, where the overarching goal is to use engineering-based approaches such as standardisation, modularisation and modelling in a biological context. The scope of synthetic biology encompasses both fundamental and translational research: through a forward engineering approach, the biological system is decoupled and reconstructed into synthetic circuits<sup>1</sup>, synthetic networks<sup>2</sup>, and even synthetic organisms<sup>3</sup> to parse the foundation and design blueprint underlying biological complexities. In addition to the learning-by-building element, synthetic biologists also adopt a build-to-deploy strategy to re-wire existing biological components for real-world applications, including environmental biosensing<sup>4</sup>, clinical diagnostics<sup>5</sup>, biotherapeutics<sup>6</sup>, and industrial biomanufacturing<sup>7</sup>, indicating the significance of synthetic biology as a new driving force for the next industrial revolution.

The origins of synthetic biology can be traced back to 1961 where the operon model was first described by Jacques Monod and François Jacob.<sup>8</sup> The model proposed a negatively controlled transcription system, where a repressor protein encoded by a regulatory gene binds to an operator sequence located upstream to a gene. This physical interaction between the repressor protein and the operator results in the occlusion of the binding sites of RNA polymerase and prevents transcription initiation. Despite the fact that it was later shown that operon could also be positively regulated<sup>9</sup>, the work done by Monod and Jacob unfolds the first conceptualisation of a genetic regulatory system, forming the pillar of modern synthetic biology. This discovery is considered a landmark event in the history of molecular biology, and it also earned Monod and Jacob (together with their colleague, André Michel Lwoff) the Nobel Prize in Physiology or Medicine in 1965.<sup>10</sup>

The idea of programmable gene expression soon started growing as our understanding of prokaryotic transcriptional regulatory networks deepened.<sup>11</sup> Meanwhile, the maturity of molecular techniques such as restriction digestion, polymerase chain reaction and

ligation around the 1970s and 1980s offered the technical tools for biologists to manipulate gene regulation in different organisms.<sup>12–14</sup> However, genetic engineers still lacked the necessary knowledge around the complexity of biological systems to engineer an artificial genetic network with a specific regulatory behaviour.

It was the introduction of automated DNA sequencing at the genomic level in the 1990s that constituted a paradigm shift not only to the field of synthetic biology, but the biology sector in a whole.<sup>15</sup> Together with high-throughput and multiplex methods, this era generated a vast amount of in-depth information spanning over different omics data types.<sup>16,17</sup> The reductionist approach successfully compartmentalised biological systems into a hierarchy of molecular building blocks, which closely resembles an electrical engineered system.<sup>18</sup> As the list of functional molecular parts continued to grow, scientists gradually recognised that by systematically re-organising these biological constituents within a mechanistic framework, one can forward-engineer an artificial genetic network both to analyse the underlying system properties and to tailor it for biotechnological applications.<sup>19</sup>

## **1.2 Motivation**

The two decades after the design of synthetic biology's first two devices<sup>20,21</sup> have seen the repertoire of synthetic genetic modules and their complexity undergoing an exponential growth. Some prominent examples include a genetic clock<sup>22</sup>, a cellular counter<sup>23</sup>, genetic circuits with memory element<sup>24</sup>, as well as some that are heavily inspired by electronics that incorporate control motifs, such as genetic logic gates<sup>25</sup>, genetic amplifiers<sup>26</sup>, and noise filters.<sup>27</sup> Meanwhile, the idea of circuit engineering also extends beyond the DNA based system. The arrival of clustered, regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated proteins (Cas) has driven the expansion of RNA-directed devices.<sup>28–30</sup> Post-translational or protein-based circuits that involve complex phenotypes such as three-dimensional shape and spatial localisation remain out of reach, even though substantial progress has been made in areas such as synthetic scaffold<sup>31</sup> and protein localisation.<sup>32</sup>

Nevertheless, the expansion of the field is hindered by multiple challenges. The bold attempt from scientists to further increase the complexity and number of parts within a synthetic genetic system is frequently faced with unpredictable and undesirable outcomes.<sup>33</sup> This does not come as a surprise: the composition and integration of new biological hardware relies heavily on the tools derived from systems and experimental biology. However, both systems and experimental biology have yet to reach the stage to underpin synthetic biology as solid-state physics underpins electrical engineering. The degree of interconnection and thus the functional organisation between biological parts is far more complicated compared to their non-living counterparts. Hence, most new systems need to be constructed iteratively within a design-build-test-learn cycle, in which the *ad hoc* process will inevitably introduce undefined and uncharacterised parts within the resulting circuit.<sup>34</sup>

Tremendous efforts have been made over the past two decades to tackle the parts standardisation challenge. As of 2018, the Registry of Standard Biological Parts located at the Massachusetts Institute of Technology in Cambridge MA in the United States of America physically houses and distributes more than 20,000 standardised biological parts.<sup>35</sup> To further improve the quality and usability of distributed parts, a tailored *in vivo* measurement kit was also introduced to reduce the variability across different testing contexts.<sup>36</sup> Meanwhile, the international Genetically Engineered Machine (iGEM) competition has become the largest synthetic biology event which not only helps to globalise the synthetic biology community, but also to promote and encourage the better curation of biological parts.<sup>37</sup>

Nevertheless, the bottleneck of synthetic biology is beyond an undefined or uncharacterised part. As the assembly of biological parts normally occurs within an existing cellular context, one cannot expect the assembly of parts to take place *ex nihilo* - instead the interactivity between parts and host environment needs to be taken into consideration when designing a synthetic system. Even within the same organism, the host's physiological state and its proximal environment can have a lasting impact on the synthetic circuit.<sup>38,39</sup> Similarly, for an organism that has evolved over the past century to optimally maintain its genetic integrity, the presence of a foreign genetic circuit can substantially perturb its behaviour.<sup>40</sup> This bottleneck leads to the first research question

this thesis seeks to address: **How can we isolate the foreign genetic software (synthetic circuitry) from the cellular operating system (host genome)?**

Similar to other fields of engineering, synthetic biology has been shown to follow Moore's Law, most notably seen in the cost of gene sequencing and synthesizing.<sup>41,42</sup> The dramatic drop in cost significantly speeds up the design-build-test-learn cycle, which reduces the entry barrier for a synthetic biomolecular system to move into the application stage. Taking advantage of this, academic and industrial groups begun to build synthetic systems with the objective to produce high-value compounds. Some notable examples include the production of bioplastics<sup>43</sup>, biodiesel<sup>44</sup>, and cannabinoids.<sup>45</sup>

The 2010s also witnessed the growth of synthetic biology companies vowing to tackle some of the greatest challenges humanity will face. One of the highest profile scientific success cases is the production of artemisinin, an antimalarial drug used to treat the multi-drug resistant *Plasmodium falciparum* strains<sup>46</sup>, at an industrial scale from engineered bacteria and yeast.<sup>47,48</sup> Amyris, the company pioneering the commercialisation of semi-synthetic artemisinin, plays a vital role in garnering interest in synthetic biology from private investors and the general public.<sup>49</sup> It was not long until the emergence of other synthetic biology unicorns, namely Ginkgo Bioworks and Zymergen, with the latter raising 500 million US dollars in its initial public offering on the Nasdaq Exchange.<sup>50</sup> As the second richest man of the world (as of 7<sup>th</sup> May 2021) once famously tweeted<sup>51</sup>:

*Prototypes are easy, production is hard.*

*-Elon Musk*

The accomplishments of these companies are ground-breaking: not only could they program cells to cater to commercial needs, but they also manage to scale-up the manufacturing process at a competitive cost. More importantly, public acceptance of the deployment of synthetic biology in bio-manufacturing goods continues to grow, as reflected in the €57 billion annual revenue of bio-based products in 2020 in the EU alone<sup>52</sup>, which signals a thrilling coming-of-age time to the field. Nevertheless, the rapid

growth of public interest in synthetic biology also calls for serious ethical, religious and philosophical discussions to prevent the misuse of knowledge. In contrast to nuclear technology which sparks a huge debate due to its destructive nature, the conversation around synthetic biology often begins with ‘creationism’, where human can create or manipulate completely new forms of life, and then followed by the risk of intentionally weaponizing a synthetic life form. The recent debate that hypothesises SARS-CoV-2 originates from a lab<sup>53</sup> serves as the perfect example that the field of synthetic biology needs to be continuously monitored and governed to reduce its underlying biosafety and biosecurity concerns. Meanwhile, synthetic biology has also made great strides in redefining the medical field. The term ‘drug’ is no longer restricted to a molecule with therapeutic effect but includes a living system with targeted delivery and sense-and-response machinery that can confer a more personalised treatment. One of the earliest attempts made in the cancer therapeutic scene is the report of an invasin-expressing *Escherichia coli* which was shown to invade different cancer cell types under a hypoxia or high cell density condition *in vitro*.<sup>54</sup> Another example that has made headlines is the use of engineered bacteria to treat phenylketonuria in a mouse model<sup>55</sup>, a metabolic disorder that can lead to mental related illness, by degrading phenylalanine (Phe) in the gastrointestinal tract using a synthetic genetic circuit encoding Phe-metabolizing enzymes.<sup>56</sup>

Nevertheless, most of the living therapeutics are still at their early phase of development, and many more have failed to reach the later stage of clinical trials.<sup>57,58</sup> In addition to their therapeutic potency in a human host, patient and environmental safety of an engineered living therapy remain the top concerns for both the recipients and the regulatory bodies. This has prompted the Food and Drug Administration (FDA) of the United States and the European Pharmacopoeia Commission (EPC) to issue a safety guideline on their common expectations of an engineered living biotherapeutic designed for human use.<sup>59,60</sup> Some of the most heavily discussed issues within the guidelines are the potential spread and horizontal gene transfer of engineered bacteria within a patient. Innovations such as a genetic kill-switches have been previously proposed<sup>61</sup>, but genetic stability and the inherent risk of genetic mutation that comes with every cycle of cell division have not been addressed.<sup>62,63</sup> This spurs the second important question of this

thesis: **How can we remove the effect of genetic instability and insecurity, once and for all?**

This thesis contributes to answering in part the two research questions mentioned above, with a strong build-to-deploy motivation to address two of the greatest current threats to global health: the COVID-19 pandemic and cancer.

### 1.3 Thesis Outline

Here I provide an overview of this thesis with a summary of the main outputs of each chapter.

**Chapter 2:** This Chapter begins with an overview of the molecular basis of synthetic biology, before describing the design principles and genetic tools which will be referred to throughout the thesis. I then review the past efforts made in addressing the above-mentioned research questions, namely the adaptation of cell-free system and synthetic biology chassis. Lastly, I discuss current methods and challenges in diagnosing COVID-19 and cancer, focussing primarily on colorectal cancer. Also highlighted in this chapter is the multivalency of a synthetic biological system that could transition from a cancer diagnostic to a cancer therapeutic.

**Chapter 3:** In this chapter I present a proof-of-concept for the development of reverse transcription, loop mediated isothermal amplification (RT-LAMP) for the detection of SARS-CoV-2 for COVID-19. I repurpose a traditional cell-free enzymatic reaction using simple primer design principles to target different locations on the SARS-CoV-2 genome. I show the practicality, scalability and effectiveness in using the RT-LAMP method to complement current testing strategy and to fulfil the demand of mass testing for the COVID-19 pandemic. This chapter is presented through publication at <sup>64</sup>.

**Chapter 4:** In this chapter I describe the design and testing of a molecular switch which aims to realise the deployment of RT-LAMP in a clinical setting. To reduce self- and non-specific amplification, I incorporate an additional temperature-dependant oligonucleotide to prevent one of the amplifying primers from binding to a non-specific target. Through a large-scale clinical validation, I demonstrate an optimised RT-LAMP

method that is rapidly deployable at different testing venues. This chapter is presented through the publication at <sup>65</sup>.

**Chapter 5:** In this chapter I describe the design of a whole-cell bacterial biosensor for the diagnostic and potential treatment of colorectal cancer. In contrast to the cell-free system discussed in the previous chapters, I aim to address the genetic stability issue with synthetic biology chassis that are genome-less, non-replicating and metabolically active. I design a diagnostic system that mimics the clinically used agglutination assay and re-programme the biosensor into a potential cancer therapeutic. This chapter is presented through a manuscript submitted to *Cell Reports*.

**Chapter 6:** This final chapter discusses and concludes the scientific findings observed in this thesis and provides a future outlook for follow-up studies.

## 1.4 Scientific Contributions and Impact

The following publications represent the main scientific outputs originating from the work described in this thesis:

- Huang, W.E.<sup>1</sup>, Lim, B.<sup>1</sup>, Hsu, C.C.<sup>1</sup> *et al.* RT-LAMP for rapid diagnosis of coronavirus SARS-CoV-2. *Microbial Biotechnology* **13**, 950-961 (2020).
  - (<sup>1</sup>joint first authors)
  - This paper is presented as Chapter 3 of this thesis.
- Lim, B. *et al.* Clinical validation of optimised RT-LAMP for the diagnosis of SARS-CoV-2 infection. *Sci Rep* **11**, 16193 (2021).
  - This paper is presented as Chapter 4 of this thesis.
- Lim, B. *et al.* Reprogramming Synthetic Biology Chassis for Targeted Cancer Therapy. (2021).
  - Submitted to *Communication Biology*.
  - This manuscript is presented as Chapter 5 of this thesis.

I have also contributed to the following studies which are not explicitly presented in this thesis:

- Cui, Z., Chang, H., Wang, H., Lim, B. *et al.* Development of a rapid test kit for SARS-CoV-2: an example of product design. *Bio-Design and Manufacturing* 1-4 (2020).
- Chen, JX., Lim, B. *et al.* Redesign of ultrasensitive and robust RecA gene circuit to sense DNA damage. *Microbial Biotechnology* 0(0), 1-16 (2021).
- Wang, Y.\*, Lim, B.\* *et al.* RT-LAMP performance for the Diagnosis of SARS-CoV-2 Infection. (2021).
  - Unsubmitted manuscript.
  - (\*joint first authors)
  - This work investigates the efficacy of Direct LAMP (i.e., LAMP reaction using nasopharyngeal specimen as testing sample without RNA extraction) and saliva LAMP (i.e., LAMP reaction using RNA extracted from saliva specimen).

In addition, the COVID-19 related work has led to the generation of the following inventions:

- Rapid test kit for COVID-19 (Oxford University Innovation Project Number: 17733)
- Optimised primer design to stabilise the performance of RT-LAMP (Oxford University Innovation Project Number: 17999)

These IPs also translate into an Oxford University's spin-out social enterprise, Oxsed, which supports the UK's first airport rapid testing facility at London Heathrow International Airport.<sup>66</sup> The project was awarded The President's Special Award for Pandemic Service by the Royal Academy of Engineering.<sup>67</sup>

On the other hand, the bacterial cancer diagnostic and therapeutic system described in Chapter 5 is currently used by Oxford University Innovation to support the international (PCT) application of a previously filed patent (WO2021079145A1) in which I will be named as one of the co-inventors.

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## 2. Background

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### 2.1 Molecular Basis of Synthetic Biology

In September 1957, Francis Crick gave one of the most renowned lectures that still frames the way we understand the logic of biology.<sup>68</sup> In his hour-long lecture, Crick first publicly introduced the concept of ‘Central Dogma’, which he later re-stated in a *Nature* publication<sup>69</sup>:

*The central dogma of molecular biology deals with the detailed residue-by-residue transfer of sequential information. It states that such information cannot be transferred back from protein to either protein or nucleic acid.*

In essence, the concept of central dogma is based upon the transfer of sequence information within a living system, and the three major biopolymers that partake in this flow of information are the deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein. This concept was concisely illustrated in one of Crick’s unpublished notes made in 1956 (Figure 2.1).<sup>70</sup> Even though Crick later admitted that this theory had a large speculating nature, his idea of the Central Dogma still lingers in the way we understand life and the information transfer principle remains at the core of synthetic biology to date. In the coming section, I will briefly outline the key takeaways from the Central Dogma of molecular biology. Uncited information from this section can be found in common biology textbooks such as *Molecular Biology of the Cell*.<sup>71</sup>

#### 2.1.1 Central Dogma of Molecular Biology: A Trailblazing Theory

Along with the idea of information relay proposed in Crick’s initial review of the Central Dogma, he also described the ‘Sequence Hypothesis’ which stated DNA, RNA and protein as information storage modules essential to all known life, and their specificity is solely determined by the sequence of their monomers.<sup>68</sup>

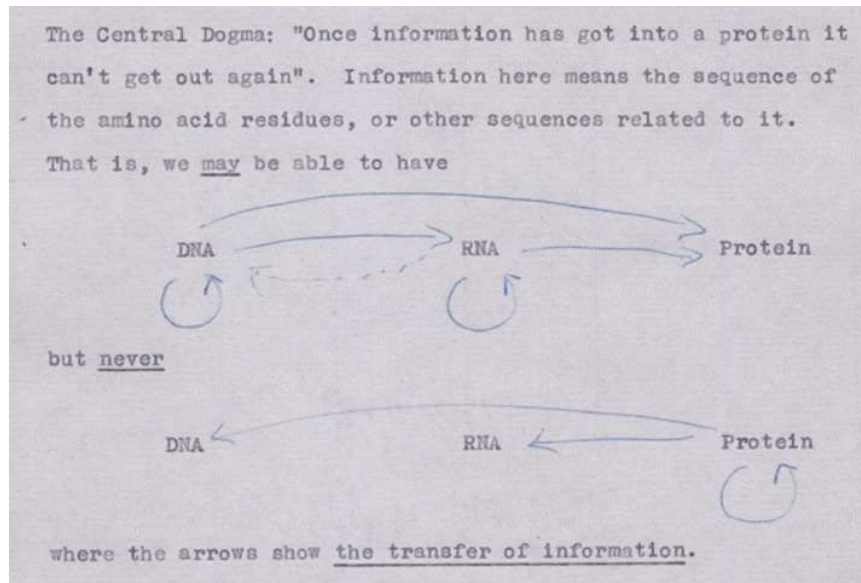


**Figure 2.1:** Crick's first illustration of the central dogma of molecular biology. The blue arrow indicates the direction of information transfer; The dotted lines refer to a step that Crick thought was possible but at the time without evidence. (adapted from <sup>70</sup>)

DNA is a polymer that consists of four bases, adenine (A), thymine (T), cytosine (C) and guanine (G). The specific one-dimensional arrangement of these bases stores information which could confer different molecular functions, ranging from initiating or terminating RNA synthesis to encoding the amino acid sequence of a protein. Information transfer involving DNA include DNA replication ( $\text{DNA} \rightarrow \text{DNA}$ ), transcription ( $\text{DNA} \rightarrow \text{RNA}$ ) and reverse transcription ( $\text{RNA} \rightarrow \text{DNA}$ ), which at the time was perceived only as structurally possible due to the lack of evidence but was later confirmed in Rous Sarcoma Virus.<sup>72</sup>

RNA, similar to DNA, consists of four bases, adenine (A), uracil (U), cytosine (C) and guanine (G). Despite carrying similar nitrogenous bases, the backbone of RNA is made up of ribose instead of deoxyribose which renders the RNA more susceptible to hydrolysis compared to a DNA. As a product of the transcription process ( $\text{DNA} \rightarrow \text{RNA}$ ), RNA serves as an important intermediate to produce protein during translation ( $\text{RNA} \rightarrow \text{Protein}$ ). Meanwhile, the information relay involving RNA also includes RNA-dependant RNA replication ( $\text{RNA} \rightarrow \text{RNA}$ ), a process commonly found in viruses.<sup>73</sup> In contrast to DNA which naturally exists in a double stranded helix form, RNA is found to possess multiple structure motifs, including a single-stranded form

found in messenger RNA (mRNA) that serves as the template to create protein; an L-shaped three dimensional structure found in transfer RNA (tRNA) which is an adaptor that physically links the mRNA to an amino acid, the monomer of a protein<sup>74</sup>; a double stranded form found in small interfering RNA (siRNA) that can interfere with the translation process.<sup>75</sup> This gives an important indication that apart from information storage, sequence specificity of a biopolymer could also dictate the structural motifs which confer additional functions.

Protein or polypeptide is a polymer made up of 20 common amino acids produced from mRNA through translation (RNA  $\rightarrow$  Protein). Protein is the workhorse of a living cell, driving a wide range of functions such as metabolising nutrients, regulating gene expression, importing and exporting molecules of interest. The function of a protein is conferred by its three-dimensional structure, which is derived from its primary amino acid sequence. Sequence specificity of the amino acids within a polypeptide is defined by its mRNA sequence which, in turn, is contributed by the arrangement of DNA bases. This completes the sequential transfer of information within the Central Dogma which connects the essential link between information stored in nucleic acids to the biochemical processes conducted by proteins that define a living system.

Several findings have revoked, at least partly, what Crick considered impossible 64 years ago (Figure 2.1, bottom). For example, the discovery of chaperone proteins, which are appointed to assist the conformational folding of other protein species from their primary to tertiary structure, elucidating a protein-protein interaction that could represent the information transfer of Protein  $\rightarrow$  Protein.<sup>76</sup> In addition, enzymes such as DNA methylases can affect the gene expression level by regulating the methylation states without altering the underlying DNA sequence.<sup>77</sup> In this case, the resulting information encoded by a DNA sequence is amended by a protein, which could act as an example of Protein  $\rightarrow$  DNA information transfer. Nevertheless, the main arguments of the Central Dogma still stand: once the genetic information has shifted from the DNA into a protein, it could never go back to modify the DNA sequence.

Crick's proposition of the Central Dogma of Molecular Biology continues to exert its influence on modern biology. In the next section I will explain how the Central Dogma frames the way scientists think about engineering biology.

### 2.1.2 Synthetic biology: A Nature Engineer

Synthetic biology is poised to be the engineering branch of biology for rewiring biological system in a more predictive and effective manner. Using the Central Dogma as a foundation, synthetic biology is essentially engineering multiple 'nodes' within the framework through internal or external regulators (Figure 2.2).

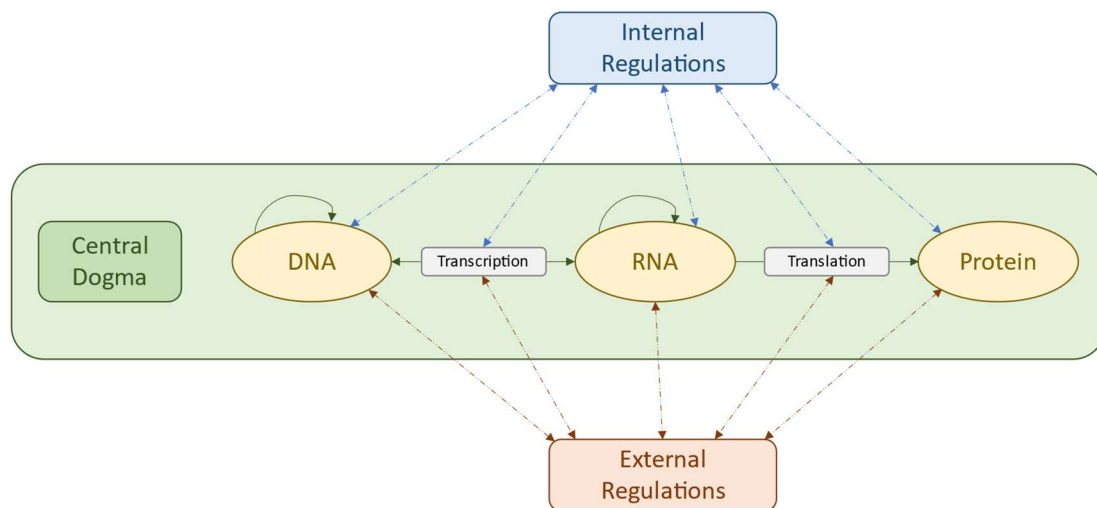


**Figure 2.2:** Engineering biology through the Central Dogma framework. The content in the green rectangle depicts the Central Dogma of Molecular Biology. Each component (DNA, RNA and Protein) and the central processes (Transcription and Translation) are connected as illustrated by the green solid arrows. All units in the Central Dogma (yellow circles and grey rectangles) are nodes that are subjected to internal (blue dashed arrows) or external (red dashed arrows) regulations through synthetic biological tools.

From this viewpoint, synthetic biologists can programme a biological system through multiple access points, or nodes, within the Central Dogma. Specifically, each node can be engineered through an internal regulator, i.e., components involve the intrinsic control mechanisms within a cellular system; or an external regulator, i.e., an environmental signal. Below I will outline the design principles of using internal and

external regulators to manipulate the Central Dogma. This section is by no means exhaustive but aims to mainly cover the components fundamental to this thesis.

**Internal Regulation:** The manipulation of intrinsic cellular mechanisms began around the 1980s, when recombinant technology such as restriction digestion, polymerase chain reaction (PCR) and ligation took over the biotechnology industry.<sup>78,79</sup> Metabolic engineering of microbial systems advanced expeditiously during this period, with the concept of ‘cell factory’ being introduced through the ground-breaking innovation of *Escherichia coli* (*E. coli*)-produced human insulin.<sup>79</sup> Despite early success, recombinant methods are inherently limited by their heavy reliance on pre-existing DNA templates. Nevertheless, the arrival of low-cost, high throughput DNA synthesis methods have once again revolutionised the internal regulating process by allowing *de novo* synthesis of DNA ranging from short genetic fragments to an entire synthetic genome.<sup>80,81</sup> In conjunction with the dramatic decline of DNA sequencing cost<sup>41</sup>, the DNA node within the Central Dogma offers the largest operational freedom for synthetic biologists as the turn-around time to design and validate a DNA-based synthetic system is the shortest.

*Promoter Engineering:* One of the most commonly used DNA-based strategies is promoter engineering to control the level of gene expression.<sup>82,83</sup> To transcribe mRNA from a DNA template, RNA polymerase first needs to be recruited to a specific region via the native transcriptional machinery. The regions where RNA polymerase and other transcription regulators (termed transcription factors) bind depend upon the underlying DNA sequence of the binding regions. Thus, tuning the strength of a promoter and thereby the level of transcription can be easily achieved through altering the specific promoter or enhancer (region where transcription factor binds) sequence. For example, replacing some of the promoter region with AT-repeats can result in a different twist angle within the minor groove of the DNA, leading to a stricter controlled promoter with lower leakiness.<sup>83,84</sup>

*RBS Engineering and Codon Optimisation:* The example above illustrates the effect of DNA node engineering to the transcription process. Along with promoter engineering, other DNA-based methods that are routinely adopted include ribosome binding site (RBS) engineering and codon optimisation. RBS is a region within the mRNA in which

the ribosome, a cellular machine that is central to the translation process, binds to perform protein synthesis. Owing to computation tools such as sequence modelling and optimisation algorithms, synthetic biologists can alter the sequence of RBS through its DNA sequence to modulate the translation initiation rate, and hence the level of protein expression.<sup>85</sup> Upon binding, the ribosome will read the mRNA three nucleotides at once to link to a corresponding amino acid through a tRNA. This tri-nucleotide combination is known as codon and the usage probabilities of a codon vary among different genetic functions or different organisms, a phenomenon known as codon usage bias.<sup>86</sup> Therefore, to design a heterologously expressed protein, synthetic biologists can opt for a computational method such as deep learning to optimise codons within the mRNA through their DNA sequence to enhance the translation efficiency within a specific host organism.<sup>87</sup> Together, the engineering of RBS and codon at the DNA level enables the modulation of translation process for rewiring cells. Overall, re-programming at the DNA node affords a higher level of control to the Central Dogma in contrast to the conventional recombinant over-expression method.

*RNA interference:* In addition to tRNA and mRNA mentioned previously, the RNA family also includes small, non-translational RNA (sRNA) within the length of 200 nucleotides that plays vital regulatory roles especially at translation. For example, the double stranded small interfering RNA (siRNA) can mediate gene silencing by degrading complementary mRNA through a multiprotein complex known as RNA induced silencing complex (RISC).<sup>75</sup> Another sRNA example, micro RNA (miRNA), regulates gene expression by binding to complementary mRNA sequence, resulting in physical occlusion of RBS or shortening of poly(A) tail, both of which reduce translation rate.<sup>88</sup> Understanding the molecular mechanisms of sRNA is one of the most significant advances in engineering the RNA node. Since the sequence of sRNA can be designed artificially, synthetic biologists can model, predict and tune the expression of target genes based on the binding strength of sRNA and mRNA.<sup>89</sup> The novel capabilities and ease of implementation of synthetic sRNA offers substantial advantages to fine-tune target gene expression over traditional knock-out strategies as the modification is done at RNA level without the need to modify their underlying DNA sequence. In addition, sRNA has also found its niche in medical applications, for example siRNA was

developed into a nucleic acid medicine to repress disease-causing genes that are previously regarded as non-druggable by conventional therapeutics.<sup>90</sup> Similarly, miRNA was discovered to have both tumour-causing and tumour-suppressing roles under different conditions which implicates miRNA as a promising candidate for cancer diagnosis, prognosis and treatment.<sup>91</sup> A recent paper also reported the potential of miRNA as a diagnostic biomarker for central nervous system disorders, including Alzheimer's disease and Parkinson disease.<sup>92</sup>

**External regulations:** Cellular communication modules comprise multiple elements, namely signal detecting elements and signal transducing molecules, to modulate the signal transmission from the extracellular environment onto different nodes within the Central Dogma to generate a response. The Central Dogma has therefore evolved a myriad of sense-and-response circuits to allow an organism to survive optimally in a wide range of environmental conditions. This provides a unique opportunity for synthetic biologists to manipulate the signal transmission pathway to construct a genetic circuit that can respond to a plethora of environmental signals, such as temperature<sup>93</sup>, metabolites<sup>94</sup> and light.<sup>95</sup>

*Environment-responsive Promoter:* One of the earliest attempts made in genetic engineering is the adaptation of an environment-responsive promoter that comprises both sensing and transducing elements to regulate gene expression. For example, the combination of promoter and operator in the well-known *lac operon* found in *E. coli* can regulate their downstream gene expression based on the presence of lactose within its surrounding environment.<sup>8</sup> The environmental sensing capability of these naturally evolved promoters are also exploited by synthetic biologists to design circuits with therapeutical functions. A great example includes the insertion of hypoxia-inducible promoter such as *pepT* upstream of a drug-encoding gene to govern the location and timing of drug expression *in vivo*.<sup>96</sup> To confer additional control to the synthetic genetic system, multiple environment-responsive promoters can be coupled within a single genetic circuit to generate an AND gate response, i.e. an output will only result in the simultaneous presence of both environmental signals.<sup>97</sup> Such a synthetic system can allow strict control and precise expression of therapeutic effectors to minimise the potential side effects of a synthetic biological medicine.

*Quorum Sensing:* Quorum sensing is a natural strategy used by many species of bacteria to achieve cell-cell communication, whereby cells coordinate their gene expression according to the local density of their population. In 1970, the first quorum sensing system using N-acyl-homoserine lactone (AHL) inducible bioluminescence circuit was reported, where the synthesis of luciferase will only be activated once the bacteria population reaches a certain threshold.<sup>98</sup> For synthetic biologists, the quorum sensing system serves as a powerful tool to control a cell population and to drive complex behaviour. In an ingenious example, a bacterial light sensing system was combined with a quorum sensing circuit to program a genetic edge detection algorithm within *E. coli*.<sup>99</sup> As for a real-world medical use, a proof-of-concept was established by combining the AHL quorum sensing circuit with invasins as the output module to enable the invasion of *E. coli* inside cancer cells once the bacterial cell density reached a certain threshold.<sup>54</sup> These examples clearly illustrate the potential real-world impact of complicated genetic algorithms made from wiring together simple genetic circuits.

*Riboswitch:* A riboswitch is the non-coding domain found in several mRNA that recognises small molecules through its specific folded structure. Upon the binding of a small metabolite, a riboswitch can modulate the translation of mRNA through allosteric conformational changes that hinder the binding of ribosome to the RBS region.<sup>100</sup> In addition to metabolite binding, certain riboswitches were also reported to respond to temperature changes.<sup>101</sup> The modular nature of these riboswitches presents a novel approach for synthetic biologists to augment the environmental control of a synthetic genetic system via the RNA node, which is demonstrated through the success of different riboswitch-based biosensors.<sup>102–104</sup> To exert further control and flexibility, the riboswitch can also be linked to a sRNA module to engineer complex genetic networks in response to cellular and environmental stimuli.<sup>105</sup>

For both internal and external regulations of the Central Dogma, engineering at the protein node is still beyond the state-of-the-art. The protein folding problem that has puzzled the biology community for more than half a century remains unsolved and thus the *de novo* creation of an entirely synthetic protein with desired functions is still deemed impossible. Methods such as directed evolution are effective to fulfil some of the protein engineering needs but the outputs are not as predictable as their DNA and RNA

counterparts.<sup>106,107</sup> Nevertheless, substantial progress has been made in this scene to uncover the sequence-structure-function relationship of a protein molecule<sup>108</sup>, hinting an exciting coming of age time for the synthetic biology community.

## **2.2 Circuit-Host Interaction**

The depiction of biology through Central Dogma is elegant, simple and straightforward. However, to rationally construct a biological system is still a long-envisioned goal and synthetic biologists have yet to reach the state where the traditional engineering disciplines, such as computer science and electrical engineering, are. The stochastic behaviour and the integrative complexity of biological components within the Central Dogma make even the simplest synthetic circuit hard to predict.<sup>109</sup> One of the biggest goals of synthetic biology is to catalogue a list of biological parts that can be assembled in a plug-and-play fashion to achieve user-defined functions, in which a huge effort has been made through the BioBricks Foundation to compile a detailed and well characterised cellular blueprint.<sup>110</sup> Nevertheless, predicting the behaviour of such synthetic circuits remains challenging due to the profound impacts of biological noise. In this section I will briefly discuss the variabilities and challenges faced in manipulating a biological system.

### **2.2.1 Biological Noise: An Internal Conflict**

In a standard synthetic biology design process, the genetic circuit is typically treated as an isolated entity that has no interaction with its surrounding cellular context, both as a simplification and also due to the lack of understanding of the host model. In reality, a synthetic circuit is intimately connected to its surrounding cellular environment. This interaction has been evidenced since the introduction of the first synthetic biological device, where the activity of the synthetic repressilator halted upon the transition of the host cell into stationary phase.<sup>21</sup>

Cells possess a limited supply of resources to maintain their cellular activity. Important cellular contexts such as the availability of enzymes, nutrients and energy need to be allocated appropriately to optimise their fitness.<sup>111</sup> As a synthetic genetic construct also consumes the same resources to maintain its biochemical processes, the fine balance of

the resource allocation in the host cell will be disrupted to support the newly introduced synthetic system.<sup>112</sup> Consequently, this additional load will affect the fundamental processes such as protein synthesis and cell growth within the host cell, thereby further modifying the cellular physiological state which then recursively affects the synthetic circuit. This phenomenon, commonly known as metabolic burden, is found to impair each and every node within the Central Dogma.<sup>112,113</sup>

The sharing of resources between the synthetic construct and the host also gives rise to undesirable crosstalk between their components. This unintended interaction often triggers a chain reaction that could jeopardise the host fitness. For example, in an experiment where two independent synthetic circuits with no existing link between their biological parts were inserted into *E. coli*, a synchronised behaviour due to over-burdening of the downstream protein-degrading enzyme was observed.<sup>114</sup> Overloading of the protease inadvertently increases the concentration of sigma factor  $\sigma^S$ , which is actively degraded at stationary phase and the surge of  $\sigma^S$  will hence activate the stress response.<sup>115</sup> In more extreme cases, the synthetic construct might confer cellular toxicity when the burden is enormous. This is frequently seen when a genetic construct is introduced from a different kingdom or species. For example, a report showed that more than 15,000 gene products derived from cloning gaps, including restriction enzymes and non-coding toxic sRNA, can inhibit *E. coli* growth.<sup>116</sup> However, it is important to note that the inhibitory effects of these biological parts are host-dependant, as a part that is lethal to one organism might display no visible toxicity to another.<sup>117</sup>

In addition to the circuit-host compatibility issues, a synthetic circuit naturally operates in a stochastic way due to the probabilistic nature of a biochemical reaction.<sup>118</sup> The stochastic production and degradation of individual biological molecules collectively limit the precision of the Central Dogma, resulting in random fluctuations and phenotypic heterogeneity.<sup>109</sup> For example, by fusing a green fluorescence protein (GFP) downstream of the glycolysis and gluconeogenesis related promoters in *Bacillus subtilis*, their transcription profiles revealed random bursting of 30 to 250 GFP especially in the glycolytic promoter.<sup>119</sup> As most proteins are present within the tens or hundreds of range, this transcriptional bursting magnifies the relative order of fluctuations within a cell that could alter both the performance of a synthetic circuit and host fitness.

Overall, a synthetic circuit is constantly influenced by the availability of cellular machinery at a certain physiological state (extrinsic noise), as well as its inherent randomness even if all relevant rate constants are fixed (intrinsic noise). Often it is unrealistic to quantify the individual contribution of intrinsic and extrinsic noise to a synthetic biological system due to the interplay and embedment of multiple sources in a shared environment, but in most cases the extrinsic noise plays a greater role in influencing the dynamics of a biological system.<sup>109,120,121</sup>

### 2.2.2 Evolution: A Foe or a Friend?

The renowned American microbiologist, Carl Richard Woese once stated in his 2001 review<sup>122</sup>:

*The dark side or Achilles' heel of molecular biology – that is, its failure to embrace evolution.*

The birth of synthetic biology was determined by a distinct purpose: to resolve the functional biological challenge through rational design rather than random exploration, as seen in evolution. However, it is a common nature, especially within the prokaryotes, that a biological output is selected based on its function but not its specific molecular instructions.<sup>123</sup> Given that all living entities are evolutionary programmed to reproduce for their best benefit, one of the biggest hurdles synthetic biology needs to overcome is to outrun evolution.

As mentioned previously, cells that are transformed with a synthetic genetic circuit are undergoing metabolic burden, which in turn puts them at a fitness disadvantage. In the presence of environmental stress, cells will be selectively pressured to escape the burden by replicating at a higher rate. As the synthetic circuit is a non-essential entity of the cell, mutations are prone to accumulate which ultimately result in loss-of-function.<sup>124</sup> To the benefit of evolution, cells that have lost of the synthetic system will be compensated with less burden and rewarded with a higher probability of survival in the stressful environment.

The above example also indicates the relevance of environmental conditions to both the synthetic circuit and the host endogenous modules. Even in a confined and controlled

lab setting, the quality of growth media can have a significant impact on the performance of the construct, a phenomenon known as growth-rate dependence.<sup>125</sup> For example, two different synthetic circuits (an AND gate and a NOR gate) were tested in *E. coli* growing in a rich media or a minimal media. While the NOR gate exerted no variation, the AND gate was found to behave in a stark contrast between the two media.<sup>126</sup> The situation was worsened when the experiment was upscaled to an industrial relevant conditions.<sup>127</sup>

Despite being notorious for complicating the implementation of synthetic biology, evolution actually inspires several tools and methods to complement the design process of engineering biology. After all, as the legendary designer Alexander McQueen once quoted<sup>128</sup>:

*There is no better designer than nature.*

Nature seeks solutions through random exploration, and occasionally phenotypes arise from natural selection that coincide with human needs. Recently, to access these useful phenotypes at a higher frequency, scientists adopted directed evolution to accelerate the occurrence of useful genetic diversification. In conjunction with the rise of high-throughput screening and selection, directed evolutionary approaches have proven to be robust and effective in optimising synthetic devices, particularly in generating useful proteins such as enzymes<sup>129</sup> and antibodies.<sup>130</sup> With the increased demand of rapid and effective strain optimisation, scientists now opt for ‘evolutionary engineering’ in which a desired biological system is obtained through evolutionary driven methods to overcome the lack of rational design knowledge. Evolutionary engineering is beyond the scope of this thesis but is comprehensively reviewed in <sup>131,132</sup>.

For synthetic biology to become a true engineering discipline, a synthetic system needs to faithfully propagate over time without collapsing the host. As the famous evolutionary biologist, Theodosius Dobzhansky once said<sup>133</sup>:

*Nothing in biology makes sense except in the light of Evolution.*

As long as there is cell growth, the intrinsic risk of genetic mutation that comes with every cell division will always obstruct the engineering of biology. In the coming section,

I will discuss strategies to address this bottleneck to realise the promise of synthetic biology.

## **2.3 From Lab to Field**

As synthetic biologists became more aware of the circuit-host interactions, novel tools have been incorporated into the design process of a synthetic system to ensure its maintenance over time. Novel strategies such as resource allocators<sup>134</sup> and *in vivo* burden monitors<sup>40</sup> have improved the performance of a synthetic system, but just as any synthetic construct designed at the DNA node, these strategies are at risk of loss-of-function mutation. Hence, to eliminate this limitation once and for all, synthetic biologists started adopting host systems which are decoupled from cell growth.

### **2.3.1 Cell-Free System: Think Outside the Cell**

A cell-free system (CFS) is generally referring to an *in vitro* transcription and translation system operating outside a living cell.<sup>135</sup> Strictly speaking, CFS constitutes a wider range of biological processes within the Central Dogma (e.g. DNA replication and reverse transcription) using purified or crude cellular extracts without the constraint of maintaining cell growth. The cellular extract or reaction mixture can then be customised with different cofactors or pH according to their purpose, offering an unprecedented flexibility to manipulate the Central Dogma while bypassing evolutionary risk.

The plasticity of CFS has offered a unique approach to decipher molecular biology mechanisms. One of the greatest examples of using CFS to answer basic research question is the use of *E. coli* cell-free extracts to unravel the information transfer from DNA to RNA for which the research was awarded the Nobel Prize in Physiology or Medicine in 1968.<sup>136</sup> In addition, many fundamental biological processes were also firstly depicted through a CFS, such as the mechanism underlying DNA replication that was first recapitulated in *E. coli* cell-free extracts.<sup>137</sup>

Owing to their flexibility, biologists started probing into the engineering potential of CFS for translational applications. Similar to the rapid expansion of synthetic biology over the past decades, the field of CFS has seen an explosive growth in recent years,

where the low-cost high titre of protein production<sup>138</sup>, new class of protein such as monoclonal antibody<sup>139</sup>, and refined recipe for high yielding extract<sup>140</sup> have been achieved in a CFS.

In the context of synthetic biology, CFS is an excellent cellular model for prototyping a synthetic system. Acting as a cellular ‘breadboard’, CFS can be used to rapidly test, characterise and validate the out-of-cell behaviours of a synthetic circuit, circumventing the labour intensive cloning and transformation process at the testing stage.<sup>141</sup> Below I highlight the key advantages of using CFS for synthetic biological purposes:

*Accessibility:* The core difference between CFS and a cell-based system is the lack of a physical barrier (i.e., cell wall or plasma membrane); its open nature allows the programming and modification of reaction conditions at the experimenter’s discretion. The synthetic system can be augmented through the direct addition of small molecules or proteins at a desired concentration, and the kinetic progress can be profiled in a real-time, *in situ* manner without being obscured by the endogenous metabolism of a living cellular system.<sup>142,143</sup> The consistency of system parameters and operational environment thus enables CFS to close the loop of the design-build-test cycle.

*Simplified Reaction Environment:* In contrast to a cell-based system, CFS operates in a relatively homogenous, well-mixed environment, which reduces the probability of aggregation of macromolecules. Such an environment is beneficial for the study of rationally designed biosystems, as technically the behaviour that occurs in a simpler environment can be elucidated through a quantitative and deterministic model.<sup>144</sup> The lack of circuit-host interaction also allows the study of cellular pathways at a higher resolution which further optimises the design process of a cell-based synthetic system.<sup>145,146</sup>

*Genetic Stability:* Without proper molecular instruction, CFS will not replicate its genetic material. This implies a synthetic circuit in a cell-free environment will not undergo loss-of-function mutation as none of its components can be replicated or regenerated.

Not surprisingly, these properties of CFS have called for its application in commercialised settings. In particular, CFS have found its niche in biosensing.<sup>147</sup> The lack of cell wall and cell membrane allows the detection of cell-impermeable analytes. In addition, most CFS matrices can be lyophilized without significantly affecting their performance which allows cheaper storage and distribution at ambient temperatures. The deployment of CFS is also facilitated by the less strict regulation in comparison to its whole-cell counterpart. Some of the early successes of CFS in biosensing include the detection of pathogens such as Zika virus<sup>148</sup>, Ebola virus<sup>147</sup> and *Clostridium difficile*.<sup>149</sup>

Despite its practical advantages, several fundamental challenges remain in a CFS. Without any homeostatic machinery, a synthetic circuit within a CFS is ultrasensitive to additional components present in the matrix, especially when a foreign sample such as blood needs to be added for a biosensing assay. The lack of homeostasis and self-regeneration also implies any reaction within a CFS has a limited lifetime, where the length depends on the enzyme stability and resource availability. Even though advances have been made to maintain the steady state of CFS up to several days<sup>150</sup>, its robustness and cost are compromised.<sup>151</sup> Indeed, economic consideration is of the biggest concern in scaling up CFS, especially when purification is required.<sup>135</sup> In addition, the lack of compartmentalisation means it is hard to mimic a multicellular context and to express membrane protein efficiently. Nevertheless, as its technical edge continues to expand, CFS will complement the development of synthetic biology by offering an ideal platform for rapid prototyping and technology translation.

### **2.3.2 Synthetic Cell: A Bottom-Up Approach**

Cell is the fundamental unit of life. To create a cell *de novo*, and thus create life, represents the most ambitious but challenging goal of synthetic biology. The archetype of a synthetic cell involves encapsulation of the components of Central Dogma into a phospholipid bilayer, normally a liposome, at a cellular scale (<30  $\mu\text{m}$ ) to carry out biological processes.<sup>152</sup> This process, which can be portrayed as entrapping the CFS within a lipid vesicle, actually predates most cell-free technologies and leads the research in synthetic cell.<sup>153</sup> In this regard, a synthetic cell with a phospholipid bilayer, overcomes many of the shortcomings faced by CFS mentioned previously.

In addition to the advantages of CFS, synthetic cell resembles a biological cell in its natural state, thus allowing the hosting of more sophisticated bioprocesses, such as cell-cell communication<sup>154</sup>, colony formation<sup>155</sup>, and spatial organisation.<sup>156</sup> Another promising advance made in synthetic cell is the ability to construct membrane proteins and organelles efficiently. For example, a synthetic cell allows the engineering of G-protein-coupled receptors (GPCR) to study this signalling pathway in-depth.<sup>157</sup> With GPCR being the most studied drug targets, including for central nervous system disorders<sup>158</sup>, a synthetic cell offers a novel avenue to support the GPCR drug discovery process. In another spectacular example, an artificial photosynthetic machinery including an ATP synthase and two photoconverters were constructed within a synthetic cell to control complex cellular activities such as carbon fixation and actin polymerisation through optical induction.<sup>159</sup>

The capability of synthetic cell to house and mimic complicated cellular functions within an enclosed entity indicates its potential in medical applications that are not achievable by CFS, such as *in vivo* therapeutics.<sup>160</sup> However, unlike CFS which is compatible with rapid and high-throughput processes, the incorporation of lipid membrane is technically challenging and the lack of high-throughput method makes the screening and selection process far more laborious. In addition, despite having an established method with few successes<sup>157,159</sup>, current understanding on the biochemical and biophysical activities of membrane protein is still inadequate, making the programming of membrane protein in a synthetic cell a major bottleneck.

Thus far, the discussion of synthetic cell in this section focuses on the development of a functional system by confining CFS within a lipid bilayer. However, the notion of synthetic cell is beyond the Central Dogma: the defining hallmark of cell, or life, is being able to self-regenerate and self-replicate. Thus, the quest for a synthetic cell with sufficient complexity while being programmed with regeneration and replication properties remains at the forefront of synthetic biology.

One of the most prominent examples in this scene is the creation of *Mycoplasma mycoides* JCVI-syn1.0, in which a chemically synthesized replica of the wild-type *M. mycoides* genome was transplanted into recipient cells to make viable synthetic cells

capable of self-replication.<sup>81,161</sup> Continuous refinement of this synthetic genome coupled with a rigorous design-build-test cycle culminated in JCVI-syn3.0 in 2016, which has a substantially smaller genome and faster doubling rate than syn1.0.<sup>3</sup>

The basic principles at the core of life sciences are only marginally uncovered in current biological research. Despite being far from synthesizing a living system *de novo*, synthetic cell research has successfully elucidated several key components of life, such as quasi-essential genes for robust growth<sup>3</sup> and essential genes for cell division.<sup>162</sup> Pioneered by synthetic cell researchers and institutions (namely Craig Venter at J. Craig Venter Institute<sup>163</sup>, Kate Adamala at the University of Minnesota and Build-A-Cell foundation<sup>164</sup>, MaxSynBio from the Max Planck Society<sup>165</sup> and more), the bottom-up approach works towards answering fundamental questions about life while fostering novel applications to address societal issues. Even though the study of synthetic cell is not extensively covered in this thesis, it is vital to encourage ethical and philosophical discussions relating to synthetic life which have a broader societal impact beyond scientific research.

### **2.3.3 Synthetic Biology Chassis: A Top-Down Approach**

The development of molecular biology and system biology is built upon a small group of model organisms, notably *E. coli* and *Saccharomyces cerevisiae*. Particularly, *E. coli* is termed the ‘workhorse of molecular biology’ in which several Nobel Prizes were awarded to findings developed in this model microbe, namely the discovery of DNA and RNA synthesis<sup>166,167</sup> (awarded to Severo Ochoa and Arthur Kornberg in 1959), the control of gene expression<sup>8</sup> (awarded to François Jacob, André Lwoff and Jacques Monod in 1965), restriction enzymes<sup>168</sup> (awarded to Werner Arber, Daniel Nathans and Hamilton O. Smith in 1978) and more. As the field of synthetic biology grows out of molecular biology and genetic engineering, *E. coli* continues to play a pivotal role and remains the cornerstone of many significant findings.

In synthetic biology, an organism that hosts and supports synthetic components is known as chassis, a term borrowed from mechanical engineering to describe the circuit- or motor- bearing framework.<sup>169</sup> Undoubtedly, as the most studied and best understood

organism on the planet, *E. coli* is set to be the most prominent synthetic biology chassis with the largest available genetic toolkit, parts and delivery methods.<sup>170</sup>

As mentioned previously, the intrinsic propensity for an organism to mutate is highly undesirable within synthetic biology. While the programming aspect of synthetic biology is gaining massive technical gains, immense research progress is also being made on the optimisation of chassis to streamline, stabilise and maintain the performance of a synthetic system. This leads to the foundation of one of the most significant approaches used in the top-down aspect: genome reduction.<sup>171</sup>

Low-cost gene sequencing and synthesis along with comparative bioinformatic analysis provide an exhaustive mean to study the genomes derived from a wide range of organisms. While identifying indispensable genes required for innate cellular activities such as those involved in DNA replication<sup>162</sup>, systematic genome-wide analysis also reveals disposable genes that could potentially sabotage or reduce the efficiency of a synthetic system, such as insertion sequences and prophages.<sup>172</sup> Using established methods such as  $\lambda$  Red-flippase recombinase<sup>173</sup> or CRISPR-Cas assisted multiplex automated genome engineering<sup>174</sup>, the genome architecture of a synthetic biology chassis can be further streamlined by shedding the non-essential genes to enhance the programmability of the chassis. For example, the genome-reduced *E. coli* K12 MDS42 with a deletion of 663kb (14.3% of the original *E. coli* K12 genome) contains no insertion sequence and was shown to have significant higher electroporation efficiency.<sup>175</sup> In another example, all prophages and insertion sequences were efficiently removed from *E. coli* BL21(DE3), a strain commonly used in heterologous and industrial protein expression, resulting in 9% reduced genome and improved transformability.<sup>174</sup> Specifically, the error-prone DNA polymerase was also eliminated from this strain to reinforce its genetic stability during stressful conditions.

For downstream biotechnological applications, several strains have inherent superiority due to their innate genome structure. For example, *E. coli* Nissle 1917 (EcN), a probiotic member with similar genetic background to the *E. coli* family, is a promising strain that can be re-programmed for medical applications.<sup>176</sup> Using clinically relevant synthetic circuits, EcN has been engineered to express tumstatin (an angiogenesis inhibitor)<sup>177</sup>, to

mediate chemoprevention (i.e., to inhibit tumorigenesis)<sup>178</sup> and to trigger anti-tumour immunity<sup>6</sup>. The safety profile of EcN renders it an optimal synthetic biological chassis for clinical applications and with further optimisation EcN is poised to be the go-to chassis against challenging diseases.

One of the main drawbacks of the top-down approach is that system biology has yet to reach the stage to functionally define the entire genome architecture. As most methodologies adopted in genome reduction are target specific, i.e., only recognised genetic fragments can be removed, the range of applications is substantially restricted as undesired stability consequences might arise when unknown regions are removed. To overcome this bottleneck, two relatively extreme methods were proposed to circumvent the genetic instability issue – by removing the genome of chassis entirely (Figure 2.3).

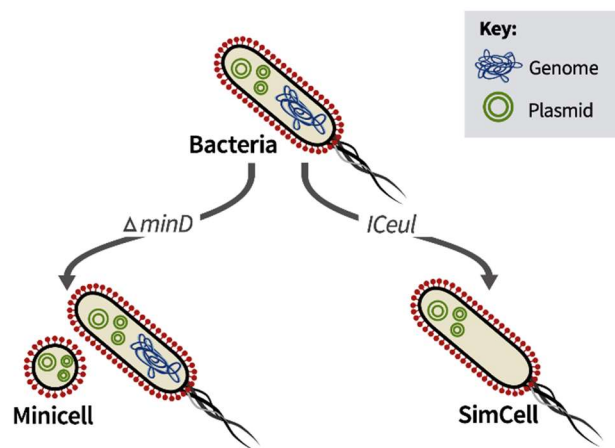


**Figure 2.3:** Synthetic biological chassis without the bacterial genome. To eliminate the genetic instability issue, the genome of chassis is removed through *minD* deletion ( $\Delta minD$ ) to generate minicell or through *ICeuI* endonuclease expression to generate SimCell. Both minicell and SimCell can carry and maintain their plasmids without the presence of bacterial genome.

*Minicell:* Genome-wide characterisation reveals the synergy between a multi-protein family, the Min system, to mediate equal cell division in bacteria. The Min system comprises three proteins, MinC, MinD and MinE, which dictate the placement of cell division machinery solely at the midcell.<sup>179</sup> By mutating a member within this protein family, particularly the *minD* gene, the septum responsible for cell division will be

placed randomly resulting in an aberrant split. When the division occurs at the pole ends instead of the mid-cell, small chromosome-free minicell will be produced.<sup>180</sup> The lack of genome stops minicells from replicating and growing, but the presence of vital cellular components allows minicells to carry out other cellular processes such as transcription, translation and ATP synthesis.<sup>181</sup> As a plasmid is previously revealed to be preferentially positioned at the poles<sup>182</sup>, minicell offers a great synthetic biology chassis to carry customised plasmids to perform programmed function such as biosensing where its finite energy resource and lifespan can be seen as an advantage with regards to biocontainment risk.<sup>183</sup>

*SimCell*: Simple cell or SimCell is a term used to describe a chromosome-free chassis capable of expressing synthetic circuits.<sup>184</sup> By inducing the heterologous expression of ICEuI, an endonuclease with multiple target sites within the *E. coli* genome<sup>185</sup>, the bacterial chromosome can be abolished through the combined action of ICEuI and endogenous nucleases. As native cellular machineries and plasmids remain unaffected, SimCells can be reprogrammed to carry out designed functions without the inherent risk of replication and mutation. To further extend the longevity of synthetic systems, the glycolysis pathway can be engineered into SimCell to prolong the essential enzymatic activities by rejuvenating ATP and NADH.<sup>184</sup> Similar to minicell, SimCell offers a non-replicating synthetic biology chassis to host synthetic circuits without interference from the native genetic network.

Cell-free, bottom-up and top-down approaches set the scene for synthetic biology to be moved from lab benches into fieldable technologies. In the next section, I will introduce two major global health issues, namely the COVID-19 pandemic and colorectal cancer, and the potential impact synthetic biology could have on addressing these challenges.

## **2.4 The COVID-19 Pandemic**

In late 2019, a previously unreported beta-coronavirus was discovered in a cluster of patients with pneumonia at Wuhan, China.<sup>186</sup> The virus was soon recognised to form a sister clade with severe acute respiratory syndrome coronaviruses (SARS-CoVs) and

hence was designated as SARS-CoV-2, with the underlying disease presented as coronavirus disease 2019 (COVID-19).<sup>187</sup> Within a month, cases of COVID-19 were no longer limited to Wuhan but were reported internationally.<sup>186</sup> In 11<sup>st</sup> March 2020, the World Health Organization (WHO) declared COVID-19 as a global pandemic<sup>188</sup> and as of 18<sup>th</sup> May 2021 the world recorded more than 163 million confirmed cases and 3.4 million COVID-19 related deaths.<sup>189</sup>

A sustainable testing strategy for COVID-19 is of utmost importance for alleviating the impact of the pandemic on public healthcare systems while ensuring economies can operate continuously. A positive case detected before the symptom arises can allow early and targeted quarantine to break the transmission chain of SARS-CoV-2, which reduces the probability of severe cases and hence hospitalisation.

To date, reverse transcription, real-time Polymerase Chain Reaction (RT-qPCR) remains the gold-standard for the diagnosis of COVID-19.<sup>190</sup> This approach represents a paradigm of cell-free system used in a real-world setting, where the principles of the testing method are based on the reverse transcription of RNA into complementary DNA (cDNA) and the amplification of the target nucleic acid sequence using a set of primers (short oligonucleotides complement to a part of the target sequence) and a DNA polymerase, in which the process mimics RNA to DNA reverse transcription and DNA replication within the Central Dogma. Below I will describe the enzyme-based nucleic acid amplification method and briefly introduce the antibody-based lateral flow test.

#### **2.4.1 Nucleic Acid Amplification Test (NAAT)**

Following the outbreak, the whole genome of SARS-CoV-2 was swiftly isolated and characterised to fuel the development of molecular diagnostic assays. SARS-CoV-2 is a positive-sense single-stranded RNA virus with a nucleic acid length of roughly 30 kilo bases.<sup>191</sup> Similar to other coronaviruses, the genome of SARS-CoV-2 is constituted almost entirely by protein-coding sequences (Figure 2.4).<sup>192</sup>

The first RT-qPCR assay developed to diagnose the original patient cluster from Wuhan uses the Spike (*S*) gene as target.<sup>193</sup> Despite a high specificity (i.e., the assay is managed to differentiate SARS-CoV-2 from other respiratory virus including SARS-CoV W1V1

with 95% similarity), its sensitivity is relatively mediocre as the virus was no longer detected at second sampling from the same patients. To improve the diagnostic sensitivity, WHO recommends the combination of RNA-dependant RNA polymerase (*RdRp*, encoded within *ORF1b*), Envelope (*E*), Nucleocapsid (*N*) and *S* genes for the diagnosis of COVID-19 with high specificity and sensitivity.<sup>194</sup>

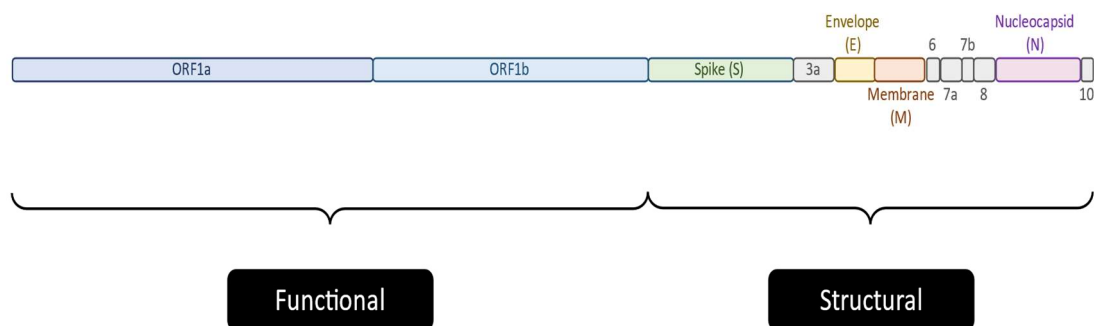


**Figure 2.4:** Schematic representation of the SARS-CoV-2 RNA genome. Annotated are the open reading frames (ORF). The first half of the genome are two ORF encoding functional proteins, *ORF1a* and *ORF1b*. The second half of the genome encodes primarily structural proteins which include the Spike (S), Envelope (E), Membrane (M) and Nucleocapsid (N) proteins. The remaining transcripts (3a, 6, 7a, 7b, 8 and 10) have unknown function but could contribute to recombination activity within host cells.<sup>192</sup>

A RT-qPCR reaction consists of multiple steps: the reverse transcription of RNA into cDNA by a reverse transcriptase; the denaturation of double-stranded DNA; the complementary binding of primer onto a specific target; and the elongation of target through a thermostable DNA polymerase, normally the Taq polymerase. As each step requires a different optimal temperature, the RT-qPCR assay necessitates a thermal cyclor which is a specialised machine to facilitate the temperature-sensitive assay for a precise duration. This inevitably complicates the testing strategy demanding for a decentralised process with a short turn-around time. In this regard, an isothermal nucleic acid amplification method is a potential alternative for routine testing with better accessibility.

One of the most characterised isothermal nucleic acid amplification methods is the loop-mediated isothermal amplification (LAMP) assay.<sup>195</sup> Using two or three sets of primers

(in contrast to one set of primer in conventional PCR or qPCR) and a polymerase with high strand-displacement activity (e.g. Bst polymerase), the LAMP method uses a single temperature (between 60 to 65 °C) to cycle between different steps: the priming of target sequence through inner primers; the hybridisation of outer primers; the displacement of DNA strand synthesized by inner primers; the self-priming of inner primers to the synthesized DNA; and the formation of dumb-bell shape DNA.<sup>195,196</sup> Combining with a reverse transcriptase (which can also function between 60 to 65 °C), the LAMP method allows the amplification of target RNA sequence at a sensitivity and specificity comparable to the gold-standard within 30 minutes without the need of a specialised instrument. Since the onset of the COVID-19 pandemic, several groups have reported the use of reverse-transcription LAMP (RT-LAMP) for the detection SARS-CoV-2 RNA, predominantly targeting the *ORF1a*, *S*, and *N* genes.<sup>64,197–199</sup> For results interpretation, most groups adopted a colorimetric RT-LAMP readout using a pH indicator such as phenol red or a fluorescent readout using an intercalating dye such as SYTO-9, with several groups employing modified CRISPR-Cas system for RT-LAMP product identification.<sup>200,201</sup>

#### **2.4.2 Lateral Flow Test (LFT)**

Lateral flow test (LFT), also known as immunochromatographic assay, represents a diagnostic test format that can detect the presence of target analyte almost instantaneously without the need of any complex instrument. One of the most well-known examples, the pregnancy test strip, uses specific antibody in a LFT format to detect human chorionic gonadotropin within an urine sample.<sup>202</sup> In principle, LFT resembles the enzyme-linked immunosorbent assays (ELISA) which both utilize a solid-phase antigen or antibody to bind the target-of-interest and consequently visualised by a coloured signal, normally a conjugated gold nanoparticle on the secondary antibody.<sup>203</sup>

LFT for COVID-19 can generally be divided into two categories: antigen LFT or antibody LFT. Antigen LFT refers to the detection of viral particles directly from a sample. To recognise the surface antigen of SARS-CoV-2, the antibody embedded on the LFT device is normally raised against its structural proteins, such as the nucleocapsid protein.<sup>204</sup> The diagnosis of COVID-19 will then be based on the presence of a coloured

line formed by an antibody-viral particle-antibody sandwich.<sup>204,205</sup> In contrast to antigen LFT, antibody LFT utilises a small drop of blood to identify the presence of specific immunoglobins, IgG and IgM, responding to SARS-CoV-2 under similar assay format.

In a global pandemic, both NAAT and LFT play a vital role in the testing regime to contain the spread of the virus and to study the epidemiological effect of the disease. Nevertheless, both display certain downsides that need to be strongly considered before their implementation in a nation-wide mass testing. NAAT, especially RT-qPCR, remains the reference method for all COVID-19 diagnosis because of its high sensitivity and quantifiability. The ability to detect viral particle down to a single copy offers a valuable tool to study the effect of viral load on different stages of COVID-19 and to compare the efficacy of different sampling methods.<sup>206,207</sup> However, the requirement of trained personnel and laboratory settings strictly limit the scalability of NAAT and its deployment as a point-of-care-testing economically. In addition, the need of rigorous sample preparation steps such as RNA extraction also further complicates the testing process and increases the cost per test.<sup>208</sup> The introduction of an isothermal amplification method such as RT-LAMP simplifies the testing process and shortens the turnaround time, but the stability and efficacy of RT-LAMP, especially the occurrence of false positives<sup>209</sup>, need to be improved prior to the decentralisation of NAAT outside of standard diagnostic laboratories.

In contrast to NAAT, LFT offers better scalability and significantly lower cost per test to fulfil the demand of a population-wide COVID-19 diagnosis. Despite having a relatively lower diagnostic sensitivity, antigen LFT is approaching the clinical performance of RT-qPCR while retaining the advantages of LFT.<sup>210</sup> Antibody LFT is essential to study the humoral immunity towards SARS-CoV-2 and to obtain epidemiological information of the disease, such as rate of infection and magnitude of outbreak.<sup>207</sup> However, the early rollout of rapid antigen test in late 2020 had been heavily criticized for their subpar diagnostic sensitivity.<sup>211</sup> The study of antibody profile in response to SARS-CoV during the 2003 SARS epidemic revealed that the amount of IgG and IgM only peak at week 3 after the onset of symptoms, with IgM gradually decline until disappearing at week 12.<sup>212</sup> This leaves a relatively narrow testing window for antibody LFT and calls for a higher testing frequency per individual to make an

informed diagnosis. Another critical drawback of LFT is the impact of viral mutation on the efficacy of the test. As both antigen and antibody LFT are based upon the epitopes of a target analyte, mutation at the structural proteins of SARS-CoV-2 could potentially render both LFTs invalid. In fact, mutation is reported to occur predominantly at the structural half of the SARS-CoV-2 genome (Figure 2.4), particularly at the *S* gene that will generate new strains with modified spike protein.<sup>213</sup>

Overall, the existing methods for COVID-19 diagnosis are the quintessential medical applications adopting a cell-free system, which should be improvised continuously to achieve decentralise, low-cost, rapid turn around and accurate testing. The execution of mass diagnostic testing for COVID-19 also needs to be assessed critically to avoid false results, which otherwise could have disastrous impact to the socio-economic and health wellbeing of the population.

## **2.5 Colorectal Cancer**

### **2.5.1 Colorectal Cancer: A Silent Killer**

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the fourth leading cause of cancer death worldwide.<sup>214</sup> In the coming 10 years, the global incidence rate of CRC is expected to rise by 60% with a continuous decline in mortality rate.<sup>214</sup> Specifically, the incidence and mortality rate of CRC are rising considerably in many developing countries, whereas reduced and stabilised trends have been observed in developed countries, though the rates are still among the highest worldwide.<sup>215</sup>

95% of CRC cases are formed by adenocarcinomas originate from the mutation of oncogenes, tumour suppressor genes or DNA repair machineries.<sup>214</sup> Depending on the source of mutation, CRC can be grouped into three different categories: sporadic, inherited and familial. Sporadic form of CRC represents 70 % of all CRC, which is derived from non-inherited point mutations commonly occur at the adenomatous polyposis coli (*APC*), *k-ras* and *p53* genes over a 10-year time span.<sup>216</sup> Other CRC cases are inherited, where roughly 5 % roots from germline mutations found in *APC* and *p53* genes and the remaining has unidentified cause.<sup>217</sup>

As adenomas may take up to a decade to progress into carcinomas, this long tumorigenesis process provides a unique time window for early detection of CRC. Several studies showed that the high reduction in CRC mortality and incidence rates observed in the US over the past decade is the result of a more organised and universal CRC screening programme.<sup>218,219</sup> Currently, most screening strategies are based on the detection of early and precancerous adenomas via invasive (e.g. colonoscopy, computed tomographic colonography) or non-invasive (e.g. faecal occult blood test, faecal immunochemical test) methods. Invasive methods are comparatively more sensitive, with colonoscopy being the gold standard for CRC screening.<sup>214</sup> Nevertheless, even with the best performance among all screening methods, colonoscopy could potentially still miss 20% of all adenomas.<sup>220</sup> In addition, most invasive methods require the insertion of medical apparatus into the patient's body, which increases the risk of other complications such as bowel tears and infections.<sup>221</sup> Most non-invasive methods are based on the detection of blood in stool samples. Despite being less intrusive and simpler to operate, these methods are less sensitive and require the patient to follow special dietary restrictions prior to the test.<sup>222</sup> In general, most screening programmes follow a two-step approach, where a non-invasive method is followed by an invasive test.

Recently, liquid biopsy was introduced as a novel and less intrusive screening method that can analyse biomarkers found in body fluids such as blood, sweat or urine to characterise and trace the progress of tumour development.<sup>223</sup> Advances in molecular techniques and bioinformatics allow the isolation of cancer-relevant biological molecules, such as cell-free DNA (cfDNA) and miRNA, to signal cancer status and to monitor the efficacy of cancer treatments. Several studies have reported a significant increase of specific cfDNA and miRNA in the blood plasma of a CRC patient which can be attributed to tumour cell apoptosis and secretion.<sup>224–226</sup> Even though both DNA and RNA screening methods show promising results in CRC diagnosis and prognosis, nucleic acid retrieval methods, especially the isolation of RNA from a complex sample, need to be improved to maintain the quality of these biomarkers.

In addition to nucleic acid biomarkers, protein biomarkers are also an important indicator to CRC. Two of the best-studied protein biomarkers for CRC are carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9). CEA is a

glycoprotein produced in a developing foetus but not in a healthy adult. Elevated CEA level (more than 5 ng/ml) was previously identified in certain types of cancer, including gastric, pancreatic, lung and CRC.<sup>227</sup> Recommended by multiple organisations including the American Society of Clinical Oncology<sup>228</sup>, the National Academy of Clinical Biochemistry<sup>229</sup> and the European Group on Tumour Markers<sup>230</sup>, measuring CEA level is considered the gold standard and the most cost-effective way in determining the progression and estimating the potential recurrence of CRC. CA19-9 is a glycoprotein most frequently associated with pancreatic and CRC, however its clinical relevance to CRC is reported to be less significant compared to CEA.<sup>231</sup> Nonetheless, combining the detection of CEA and CA19-9 in the blood serum can have a complementary effect to increase the sensitivity of CRC diagnosis.<sup>231</sup>

Overall, instead of restricting to a single approach, liquid biopsy could thrive from the synergy of different biomarkers together with a refined bioinformatical algorithm to profile, diagnose and characterise CRC while guiding treatment options.

### **2.5.2 Whole-Cell Biosensor: A Pioneer of Translational Synthetic Biology**

Traditional analytical approaches using chemical and physical traits of a sample can provide highly accurate and sensitive information. Methodologies such as mass spectrometry, gas chromatography, and high-performance liquid chromatography are well-established for decomposing healthcare-related specimens including samples from cancer patients. Nevertheless, such methodologies are tedious, costly and require specialized instruments operated by trained personnel, which consequently fuels the demand for liquid biopsy using biosensor. As described previously, cell-free system such as RT-qPCR has gained a lot of attention as it promises trace-level detection of specific nucleic acid biomarkers. Meanwhile, whole-cell biosensor (WCB) which uses an intact cellular system offers a cheaper alternative compared to other biosensors with the potential to achieve point-of-care monitoring.

First generation WCB uses live cells in their natural state to measure important parameters such as bioavailability and genotoxicity which are not achievable by other molecular or chemical methods. For example, bacteria such as *Vibrio fischeri* and *Pseudomonas fluorescens* are commonly used in ecotoxicological assessment by

measuring their bioluminescence in which its reduction is directly corresponding to the concentration of cytotoxic compound presents in the tested sample.<sup>232</sup> Despite its low complexity and cost structure, first generation WCB lacks specificity and struggles to find an application under medical settings.

The rise of genetic engineering and subsequently synthetic biology have fuelled the development of next generation WCB (as described in Section 2.1.2). In essence, current WCB translates biochemical detection into a quantifiable signal by coupling sensing elements to a reporter gene. In most cases, the sensing elements consist of a regulatory protein and a promoter which control the expression of a reporter gene. Upon the recognition of a target analyte, the regulatory protein will undergo conformational changes and bind to the promoter sequence, which subsequently trigger the transcription of the reporter gene. Eventually, the mRNA of the reporter gene will be translated into a detectable output signal (e.g., fluorescence<sup>233</sup> or electrical<sup>234</sup>) which confers the system its sensitivity and detection limit.

Bacteria have a huge repertoire of sensing elements essential for their survival. Using simple genetic engineering tools, these regulator protein-promoter pairs can be easily shifted between different bacteria to further expand their sensing potential. Common targets of WCB range from toxic compounds such as heavy metals<sup>235</sup>, antibiotics<sup>236</sup> to nutrients such as glucose.<sup>237</sup> Among these sensing elements, some have tremendous medical relevance, particularly the oxygen<sup>96</sup>, pH<sup>238</sup> and glucose<sup>239</sup> sensors which are sensitive to tumour microenvironment *in vivo*. As most WCBs are constructed by re-engineering existing bacterial sensing systems, their scope of detection could be limited as the sensing element of a target of interest might not be found in the nature. To expand the sensing repertoire of WCB, antibody, particularly the single-domain antibody (also known as nanobody or VHH) is adopted as its sensing element to offer modularity and specificity to molecules with no naturally existing bacterial receptor. For example, VHH coupled with a bacterial Cad system can translate the binding of caffeine into the expression of a reporter gene.<sup>240</sup> For large molecules such as protein cancer biomarkers that cannot penetrate the bacterial outer membrane, VHH can be expressed and anchored on the surface of WCB to bind these molecules.<sup>241,242</sup> Integrating synthetic biological components (discussed in Section 2.1.2 and 2.3.3) can further improve the overall

performance of WCB and potentially translates its application into cancer diagnostic and treatment.

### **2.5.3 Bacterial Cancer Therapy: A Wonder or a Blunder?**

Over the last 40 years, the global cancer survival rates have increased significantly due to the improvements in conventional cancer therapies (e.g., chemotherapy and radiotherapy) and novel immunotherapy (e.g., immune checkpoint inhibitor).<sup>243</sup> However, increased survival rate does not necessarily improve the quality of life of cancer patients as many of the cancer treatments are accompanied by adverse side effects.

Recent studies have unveiled the role of symbiotic microorganisms in cancer progression and their vast impact in cancer diagnosis and treatment.<sup>244</sup> For example, the microenvironment within a tumorous tissue is highly favourable to strictly anaerobic bacteria. As the blood supply around cancerous tissue is limited, the tumour core is normally deprived of nutrients and oxygen.<sup>245</sup> Therefore, strict anaerobes, especially those with germination features such as *Clostridium* species, are highly effective at colonizing and proliferating within the hypoxic and necrotic regions of tumour.<sup>246</sup> This offers an opportunity to treat metastases at poorly vascular regions that are notorious for their resistance to traditional cancer treatment.<sup>247</sup>

With the rise of synthetic biology, bacteria are gradually returning to the forefront of cancer research as an engineerable anti-tumour agent. Scientists can now engineer bacteria that have attenuated virulence<sup>248</sup>, high tumour specificity<sup>249</sup>, strict control of drug expression coupled with precise delivery<sup>250</sup> to unleash the full potential of bacterial therapy in cancer treatment. A more recent example includes the development of a probiotic strain, *Escherichia coli* Nissle 1917 (*EcN*) to produce cyclic di-nucleotides, a STimulator of INterferon Genes (STING) agonist, intratumorally to activate the antigen-presenting cells to produce type I interferons for subsequent antitumour immune response.<sup>6</sup>

Despite the high anticipation, several clinical trials of bacterial cancer therapy have shown that the prominent tumour-targeting and anti-tumour effects observed in a mouse model are hardly replicable in human and canine.<sup>251,252</sup> To enhance tumour targeting,

specific motifs such as affibody and ligand are engineered as the sensing element to achieve cell-specific delivery.<sup>178,253</sup> Yet, not all cancer cell specific markers have a naturally available ligand that can be readily folded and expressed onto the bacterial surface. In addition, as described previously (in Section 2.2) each bacterial cell division carries the risk of gene mutation which could default the targeted delivery and drug expression system through loss-of-function mutation.<sup>254</sup> These challenges culminate in the main research objective I aim to pursue in this thesis: to employ synthetic biological methods in a stabilised genetic environment to construct diagnostic and potentially treatment systems with immediate translational applications.

## **2.6 Conclusion**

The emergence of synthetic biology at the interface of biology and engineering domains has yielded significant research advances, both in understanding the fundamental principles of life and in addressing pressing global problems. Upon the introduction of high-throughput, automated DNA sequencing and synthesizing technologies, biologists have successfully generated a vast amount of data spanning over multiple omics types while exploiting the systematically acquired information to re-wire existing biological components for biotechnological applications.

Nevertheless, unlike an electrical engineered system, the stochastic behaviour and integrative complexity of a biological system make even the simplest synthetic circuit hard to predict. Despite the enormous effort made to catalogue and characterise biological parts in a plug-and-play fashion, predicting the behaviour of a synthetic biological circuit remains challenging due to the profound impacts of biological noises.

Overall, a synthetic circuit is constantly influenced by its inherent randomness even if all relevant rate constants are fixed (intrinsic noise), as well as the availability of cellular machineries at a certain physiological state (extrinsic noise). Meanwhile, given that all living entities are evolutionary programmed to reproduce for their best benefit, one of the biggest hurdles synthetic biology need to overcome is to outrun evolution, as the presence of a foreign genetic circuit can substantially perturb the genetic integrity of an organism.

On the other hand, while making great strides in re-defining the medical field, patient and environmental safety of a synthetically engineered product remains the top concern for both the recipients and the regulatory bodies, as some of the most heavily discussed issues such as genetic stability, uncontrollable spread and horizontal gene transfer of engineered bacteria within a patient and the environment have yet to be wholly addressed.

The next three chapters of this thesis aim to answer in part the main challenges faced in the materialisation of synthetic biological innovations into healthcare applications, namely, to isolate the foreign genetic software (synthetic circuitry) from the cellular operating system (host genome); and to eradicate the effect of genetic instability and insecurity of an engineered system. In particular, Chapter 3 and 4 introduces reverse-transcription, loop-mediated isothermal amplification (RT-LAMP) as a cell-free system and Chapter 5 introduces whole-cell biosensor (WCB) as a synthetic biology chassis to contribute solutions to two of the most urgent healthcare threats faced by humanity at the very moment.

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### 3. RT-LAMP for Rapid Diagnosis of Coronavirus SARS-CoV-2

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In this chapter, I re-purpose a traditional cell-free enzymatic system, reverse transcription, loop-mediated isothermal amplification (RT-LAMP) for the detection of SARS-CoV-2 as a proof-of-concept COVID-19 diagnostic nucleic acid amplification test (NAAT). Specifically, I design LAMP primers to target the *ORF1ab*, *S* and *N* gene within the SARS-CoV-2 genome and achieve a diagnostic sensitivity of 80 copies of viral RNA per mL sample. Also, I show the potential of RT-LAMP to circumvent the RNA extraction step to simplify and shorten the entire diagnostic process.

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# RT-LAMP for rapid diagnosis of coronavirus SARS-CoV-2

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## Summary

**The pandemic coronavirus SARS-CoV-2 in the world has caused a large infected population suffering from COVID-19. To curb the spreading of the virus, WHO urgently demanded an extension of screening and testing; thus, a rapid and simple diagnostic method is needed. We applied a reverse transcription-loop-mediated isothermal amplification (RT-LAMP) to achieve the detection of SARS-CoV-2 in 30 min. We designed four sets of LAMP primers (6 primers in each set), targeting the viral RNA of SARS-CoV-2 in the regions of orflab, S gene and N gene. A colorimetric change was used to report the results, which enables the outcome of viral RNA amplification to be read by the naked eye without the need of expensive or dedicated instrument. The sensitivity can be 80 copies of viral RNA per ml in a sample. We validated the RT-LAMP method in a hospital in China, employing 16 clinic samples with 8 positives and 8 negatives. The testing results are consistent with the conventional RT-qPCR. In addition, we also show that one-step process without**

RNA extraction is feasible to achieve RNA amplification directly from a sample. This rapid, simple and sensitive RT-LAMP method paves a way for a large screening at public domain and hospitals, particularly regional hospitals and medical centres in rural areas.

## Introduction

There is an urgent need for rapid diagnosis of SARS-CoV-2 infected COVID-19 patients even before an immune response can occur and for asymptomatic carriers. This is critical in making decisions on public health measures, such as movement restrictions, and quarantine duration. Nucleic acid testing, that is detecting the viral RNA, is a feasible and practical method. Currently, the most common method for nucleic acid diagnosis is based on real time RT-PCR (Drosten et al., 2002; Mackay et al., 2002; Espy et al., 2006). For example, BGI in China (<https://www.bgi.com/kit>) and the US CDC (<https://www.cdc.gov/coronavirus/2019-ncov/about/testing.html>) provide reagents, primers and probes to support RT-PCR diagnosis for SARS-CoV-2. Although real-time RT-PCR is sensitive and reliable, it is time-consuming (~2 h) and requires a specific detection device or instrument, which limits its broad application to current huge demand for the global pan-demic of COVID-19.

To address this challenge, a fast and simple-to-operate test kit would be highly desirable. With such a test, virus infected patients could be identified at an early stage and quarantined to prevent the spread of the infection, whilst the non-infected individuals could carry on with their life as usual. Ideally, the test kit is mobile with-out the need of any complicated instrument and the test result can be read by the naked eye. Hence, it can be used at airports, railway stations and hospitals, particularly regional hospitals and medical centres in rural areas.

Loop-mediated isothermal amplification (LAMP) is a rapid technology of DNA amplification (Notomi et al., 2000; Tomita et al., 2008), which has been applied to pathogen detection such as virus, bacteria and malaria (Thai et al., 2004; Boehme et al., 2007; Mori and Notomi, 2009; Law et al., 2015; Reboud et al., 2019). The LAMP reaction generally takes place in a constant temperature, and the target DNA can be amplified in 30 min (Tomita et al., 2008). The LAMP method employs

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4 or 6 primers to bind six regions of a target DNA, and the specificity is extremely high (Notomi et al., 2000; Tomita et al., 2008). Since the LAMP method only needs one constant temperature (usually 65°C), the device can be simple. Initially, the LAMP uses 4 primers (Notomi et al., 2000; Tomita et al., 2008), later it is found that the addition of two loop primers can shorten half of the time required for the original LAMP reaction (Nagamine et al., 2002). The availability of WarmStart RTx Reverse Transcriptase (New England Biolabs, UK) makes it possible to combine both reverse transcription and LAMP in one reaction. Since SARS-CoV-2 is a RNA virus with the length about 30 kb (Wang et al., 2020), the single reaction of reverse transcription (RT) and LAMP together can significantly shorten the reaction time without the DNA purification step from RT, thus a rapid detection of SARS-CoV-2 can be achieved.

In this work, we developed a COVID-19 diagnosis kit for the rapid detection of SARS-CoV-2, using one-step reverse transcription and loop-mediated isothermal amplification (RT-LAMP). The whole reaction can be as short as 20 min at a constant 65°C. The detection limit is 80 copies of viral RNA per ml sample. A simple colour change indication can be visualized by the naked eye to confirm the result of viral RNA amplification. The kits were validated by 16 clinical COVID-19 samples. This new technique can also provide a rapid and feasible approach for the detection of various virus.

## Results

### *Specificity of O117, S17, N1 and N15 primers to amplify synthesized DNA of SARS-CoV-2*

We designed four sets of LAMP primers O117, S17, N1 and N15, which target RNA encoding Orf1ab, spike glycoprotein and two regions in nucleocapsid protein of SARS-CoV-2 respectively (Fig. S1). Each set has 6 primers (Table 1). SARS-CoV-2 is a single stranded RNA virus with the length of about 30 kb. Orf1ab is about 21kb long, encoding the replicase polyprotein (Woo et al., 2010). O117 primers were designed to cover the conserved region of 5'-end of the viral RNA in Orf1ab (Fig. S1). S17 primers target the S gene, which encodes spike glycoprotein, a key for this SARS-CoV-2 virus to bind human ACE2 protein and to invade human cells (Menachery et al., 2015; Cui et al., 2019; Walls et al., 2020). The N gene for nucleocapsid protein is at the 3'-end of the viral RNA, conserved to SARS-like coronavirus (Cui et al., 2019). During sampling and RNA extraction processes, the viral RNA might be attacked by RNase, degrading from 5'- to 3'-, so we designed N1 and N15 to ensure the detection of 3'-end of the viral RNA of SARS-CoV-2, even though it is partially degraded. The targeted SARS-CoV-2 RNA fragments of these four sets of primers (Fig. S1 and Table 1) are about 240–260 bp, long

enough to be targeted by 6 primers and short enough to achieve a rapid amplification.

In all experiments of LAMP and RT-LAMP, the experiments were carried out with more than three biological replicates and the results were consistent.

To verify their specificity, we first tested these primers using synthetic target DNA of SARS-CoV-2. N1 and N15 primers were able to amplify DNA fragments of the N gene, but not human genomic DNA (Fig. 1A and B). By adding the fluorescent dye provided by NEB (New England Biolabs, UK), positive results can be visualized directly from the PCR tubes under UV exposure. The fluorescent intensity in the amplification-positive tubes was stronger than that in the amplification-negative tubes, although fluorescence background was also present in the negative tubes (Fig. 1A and B). The fluorescent read-outs are consistent to the results by electrophoresis gel. However, the fluorescent signal background might cause ambiguous reading and it requires fluorescent sensitive instrument to analyse the result. So we changed it to colorimetric reading later in this study.

Similarly, O117 and S17 primers can also specifically amplify the synthesized DNA fragments of Orf1ab gene and S gene, respectively, but they were unable to amplify human genomic DNA (Fig. 1C and D). The addition of human genomic DNA to the viral DNA in the reaction mixture did not interfere with the amplification performance (lane 4 in Fig. 1A and B, lane 5 in Fig. 1C and D), which is essential for using RT-LAMP to detect viral RNA in a human sample. The LAMP reaction time for all experiments was 30 min. Positive amplification products were obtained even for 2 copies of the synthetic viral DNA fragment template in 30 min when using the S17 primers (lane 4 in Fig. 1D), demonstrating that the LAMP reaction was rapid and sensitive.

### *RT-LAMP and sensitivity to amplify synthesized RNA of SARS-CoV-2*

To assess the potential of RT-LAMP in detecting RNA virus of SARS-CoV-2, we then tested the performance of these primers with synthesized RNA fragments of the N gene, S gene and Orf1ab gene obtained from in vitro transcription (Appendix S1). It shows that reverse transcription and LAMP can be integrated into one single RT-LAMP reaction to specifically amplify viral RNA fragments (Fig. 2).

We evaluated the sensitivity of RT-LAMP by diluting the copy number of RNA target from 200 to 2 copies per reaction (in total 25 µl reaction solution) and examined the efficiency by sampling the reaction mixture at 15, 20

**Table 1.** Primers for the detection of SARS-CoV-2.

| N1                                               | Sequence 5'-3'                                |
|--------------------------------------------------|-----------------------------------------------|
| F3                                               | TGGACCCCAAAATCAGCG                            |
| B3                                               | GCCTTGCTCTCGAGGGAAT                           |
| FIP <sup>a</sup>                                 | CCACTGCGTTCTCCATTCTGGTAAATGCACCCCGCATTACG     |
| BIP                                              | CGCGATCAAAACAACGTCGGCCCTTGCCATGTTGAGTGAGA     |
| LF                                               | TGAATCTGAGGGTCCACAAA                          |
| LB                                               | GGTTTACCCAATAATACTGCGTCTT                     |
| N15                                              | Sequence 5'-3'                                |
| F3                                               | AGATCACATTGGCACCCG                            |
| B3                                               | CCATTGCCAGCCATTCTAGC                          |
| FIP <sup>a</sup>                                 | TGCTCCCTTCTGCGTAGAAGCCAATGCTGCAATCGTGCTAC     |
| BIP                                              | GGCGGCAGTCAAGCCTCTTCCCTACTGCTGCCTGGAGTT       |
| LF                                               | GCAATGTTGTTCTTGAGGAAGTT                       |
| LB                                               | GTTCTCATCACGTAGTCGCAACA                       |
| S17                                              | Sequence 5'-3'                                |
| F3                                               | TCTTTCACACGTGGTGTT                            |
| B3                                               | GTACCAAAAATCCAGCCTC                           |
| FIP <sup>a</sup>                                 | CATGGAACCAAGTAACATTGGAAAACCTGACAAAGTTTTTCAGA  |
| BIP                                              | TCC                                           |
| LF                                               | CTCTGGGACCAATGGTACTAAGAGGACTTCTCAGTGGAAGCA    |
| LB                                               | GAAAGGTAAGAACAAGTCCTGAGT                      |
|                                                  | CTGTCCTACCATTTAATGATGGTGT                     |
| O117                                             | Sequence 5'-3'                                |
| F3                                               | CCCCAAAATGCTGTTGTT                            |
| B3                                               | TAGCACGTGGAACCCAAT                            |
| FIP <sup>a</sup>                                 | GGTTTTCAAGCCAGATTTCATTATGGATGTCACAATTCAGAAGTA |
| BIP                                              | GGA                                           |
| LF                                               | TCTTCGTAAGGGTGGTTCGAGCACACTTGTTATGGCAAC       |
| LB                                               | TCGGCAAGACTATGCTCAGG                          |
|                                                  | TTGCCTTTGAGGCTGTGT                            |
| Human $\beta$ -actin primers (Poon et al., 2005) |                                               |
| $\beta$ -actin                                   | Sequence 5'-3'                                |
| F3                                               | ACTGGGACGACATGGAGAA                           |
| B3                                               | TCACCGGAGTCCATCACG                            |
| FIP                                              | GTGGCCTTGGGGTTCAGGGTTCTACAATGAGCTGCGTGT       |
| BIP                                              | TTGAGACCTTCAACACCCAGCGAGGCGTACAGGGATAGCA      |

a. The 5'- of FIP primer was labelled by FAM for the application to hospital.

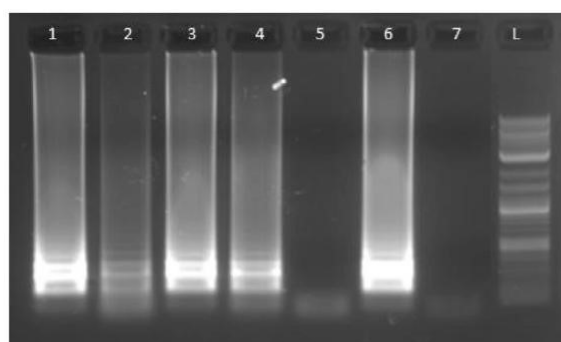
and 30 min after the reaction started. It shows that 2 copies of target RNA can be amplified to detectable level using N1 primers within 20 min, whilst it requires 30 min using N15 primers (Fig. 2A–C). The sensitivity and efficiency of RT-LAMP were also tested on S17 and O117 primers. As shown in Figure 2, 2 copies of S gene RNA can be detected using S17 primers in 30 min, whilst the detection limit using O117 primers is 20 copies of Orflab gene RNA in 30 min. The results suggest that RT-LAMP is sensitive enough to detect viral RNA within a 30-min reaction and the sensitivity can be 2 copies in 25  $\mu$ L reaction (80 copies viral RNA per mL) by employing primers N1 and S17.

It is also observed that the higher copies of RNA, the shorter time is needed to obtain the products (Fig. 2). When the copy

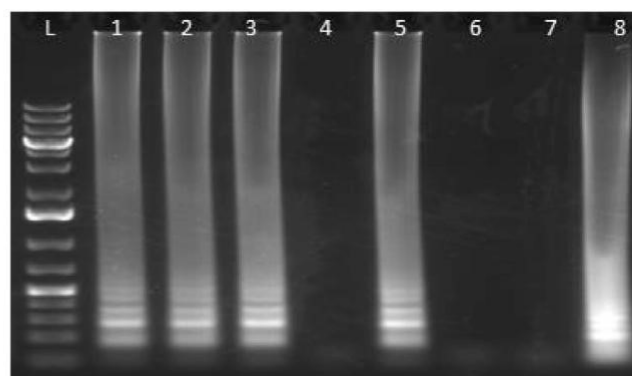
number of RNA is 200 per reaction, the amplification result can be observed as short as 15 min (Fig. 2).

#### *Colour change of LAMP reaction mixture provides semi-quantitative results*

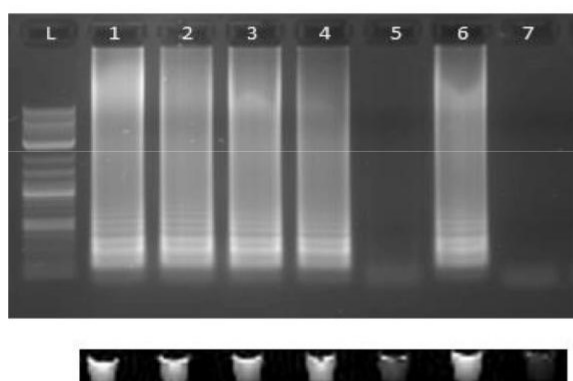
Since result-reporting using fluorescent dye requires a specific instrument (Fig. 1), we also tried to read the results using the naked eye. Since the nucleic acid amplification releases pyrophosphate and hydrogen ion, which decreases the pH of reaction solution, a sensitive pH indicator can be used to show the positive or negative result of RT-LAMP (Tanner et al., 2015; Poole et al., 2017). Hence, RT-LAMP result can



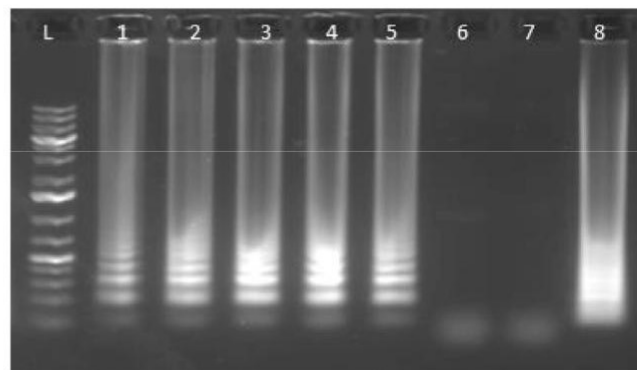
(A) The performance of N1 primers.



(C) The performance of O-117 primers.



(B) The performance of N15 primers.



(D) The performance of S-17 primers.

**Fig. 1.** LAMP results of four different sets of primers targeting the DNA of N, S and Orflab gene of SARS-CoV-2.

**A.** Performance of N-1 primer set using N gene DNA sequence. (1) N-1 primers + N gene DNA (200k copies)\*; (2) N1 primers + N gene DNA (200 copies); (3) N1 primers + N gene DNA (200k copies) + Human genome; (4) N1 primers + N gene DNA (200 copies) + Human genome; (5) N1 primers + Human genome; (6) Human  $\beta$ -actin primers + Human genome; (7) Human  $\beta$ -actin primers + N gene DNA (200k copies); L, Ladder. LAMP results using fluorescent dye were visualized under UV exposure, which is consistent to the result of gel electrophoresis.

**B.** Performance of N-15 primer set using N gene DNA sequence. (1) N-15 primers + N gene DNA (200k copies); (2) N15 primers + N gene DNA (200 copies); (3) N15 primers + N gene DNA (200k copies) + Human genome; (4) N15 primers + N gene DNA (200 copies) + Human genome; (5) N15 primers + Human genome; (6) Human  $\beta$ -actin primers + Human genome; (7) Human  $\beta$ -actin primers + N gene DNA (200k copies); L, Ladder. LAMP results using fluorescent dye were visualized under UV exposure, which is consistent to the result of gel electrophoresis.

**C.** Performance of O-117 primer set using Orflab gene DNA sequence. (1) O-117 primers + Orflab gene DNA (200k copies); (2) O-117 primers + Orflab gene DNA (200 copies); (3) O-117 primers + Orflab gene DNA (20 copies); (4) O-117 primers + Orflab gene DNA (2 copies); (5) O-117 Primers + Orflab gene DNA (200 copies) + Human Genome; (6) O-117 Primers + Human Genome; (7) O-117 Primers + water (8) Human  $\beta$ -actin primers + human genomic DNA; (L) Ladder.

**D.** Performance of S-17 primer set using S gene DNA sequence. (1) S-17 primers + S gene DNA (200k copies); (2) S-17 primers + S gene DNA (200 copies); (3) S-17 primers + S gene DNA (20 copies); (4) S-17 primers + S gene DNA (2 copies); (5) S-17 Primers + S gene DNA (200 copies) + Human Genome; (6) S-17 Primers + Human Genome; (7) S-17 primers + water (8) Human  $\beta$ -actin primers + human genomic DNA; (L) Ladder. \*200k (200,000) 200, 20 and 2 represent the total copy number of DNA sequence in the reaction mix.

be visualized by a pH indicator such as phenol red, which shows pink 8.2–8.6 and becomes yellow when pH drops. Using the WarmStart™ Colorimetric LAMP 29 Master Mix (DNA & RNA) from New England Bio-labs, a visible colour change from pink to yellow could be observed if the amplification of the target sequence has taken place (Fig. 3). We show that the colorimetric RT-LAMP assay can reliably detect N genes of SARS-CoV-2 viral RNA, and the colour change could indicate the number of target sequence semi-quantitatively (Fig. 3).

#### *Both colorimetric and fluorescent reading in one RT-LAMP reaction*

Colorimetric reading is dependent on pH change during RT-LAMP. However, in clinical application, many factors might interfere pH change. For example, patient

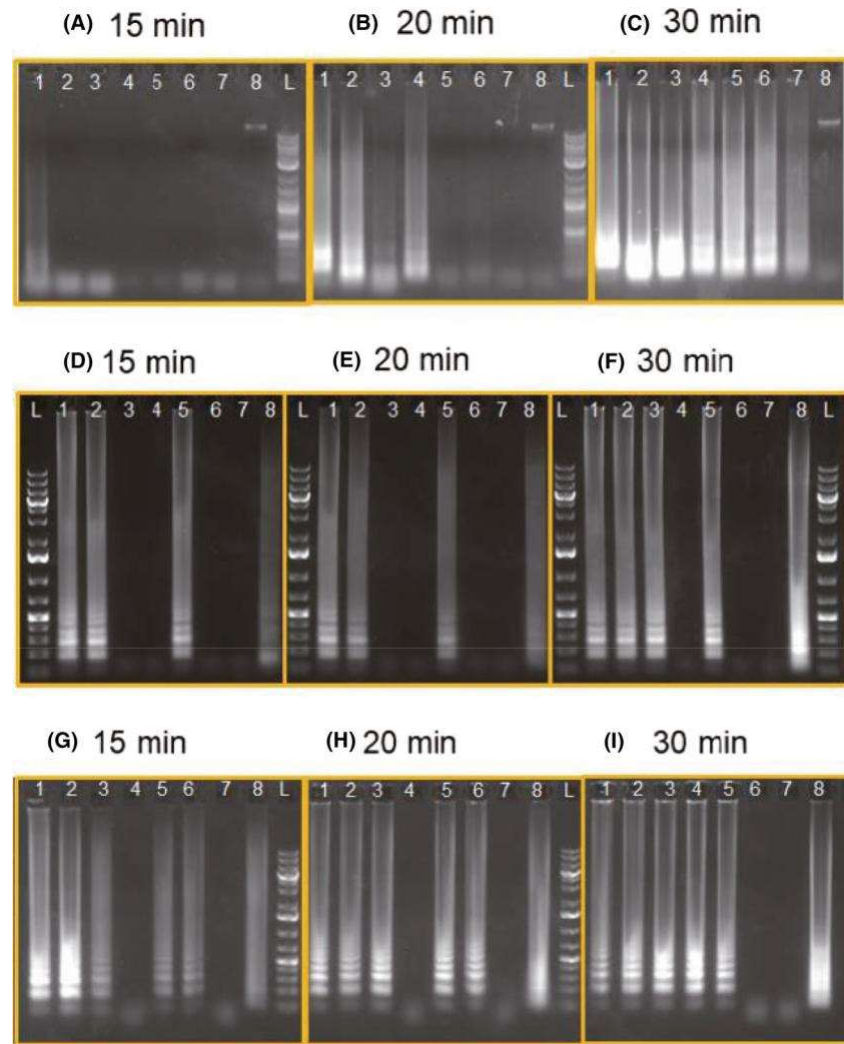


**Fig. 2.** Sensitivity and efficiency of RT-LAMP using N1 and N15 primers (**A, B, C**). RNA sequence of N gene was derived from in vitro transcription and used as the target. Reaction mixture was sampling at (**A**) 15 min, (**B**) 20 min and (**C**) 30 min. (1) N1 Primers + N gene RNA (200); (2) N1 Primers + N gene RNA (20); (3) N1 Primers + N gene RNA (2); (4) N15 Primers + N gene RNA (200); (5) N15 Primers + N gene RNA (20); (6) N15 Primers + N gene RNA (2); (L) Ladder. Sensitivity and efficiency of RT-LAMP using O-117 (**D, E, F**) and S-17 primers (**G, H, I**). RNA of orflab and S17 was derived from in vitro transcription and used as the targets. Reaction mixture was sampling at (**D, G**) 15 min, (**E, H**) 20 min and (**F, I**) 30 min. (1) Viral Primers + RNA target (200 k); (2) Viral Primers + RNA target (200); (3) Viral Primers + RNA target (20); (4) Viral Primers + RNA target (2); (5) Viral Primers + RNA target (200) + human genome; (6) Viral Primers + human genome; (7) Viral Primers + Water; (8) Human Primers + human genome.

sample conditions can be different, introducing uncertain factors to pH change in the RT-LAMP reaction. In addition, viral RNA of SARS-CoV-2 might be eluted by various buffers, which might be compatible to RT-LAMP reaction but could significantly affect the colorimetric reading (Fig. S2). To overcome the uncertainty and ensure the reliability of the diagnosis results, we also designed another set of FIP primers, which 5'-end was conjugated with fluorescent FAM (6-Carboxyfluorescein; Table 1). Figure 4 shows that the application of 5'-FAM-FIP primers was able to display both colorimetric and fluorescent readings, and both results were consistent.

#### *Clinical test validates the performance of RT-LAMP*

To validate the performance of RT-LAMP in diagnosing COVID-19, a test kit consisting of three tubes (#1: O117 primers, #2: N15 primers, #3: human b-actin primers; See Materials and Methods) was sent to Shenzhen Luohu People's hospital for clinical validation. RT-qPCR and RT-LAMP tested the 16 clinical samples (8 positive and 8 negative samples) in parallel, which were taken by swab from patients in a standard way. The samples were initially tested by conventional RT-qPCR; then, RT-LAMP was used to test the same samples. Using RT-LAMP, tubes #1 and #2 of each testing kit turned yellow

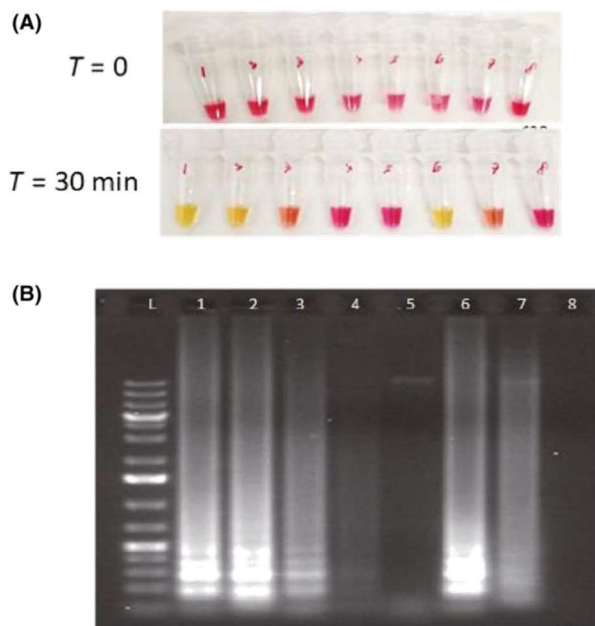


**Fig. 3.** Colorimetric RT-LAMP assay using N15 primers. **A.** Colour of reaction mixtures before RT-LAMP. **B.** Colour of reaction mixtures after RT-LAMP. **C.** Gel electrophoresis of reaction mixtures. The intensity of RT-LAMP products in the gel tallies with the colour of reaction mixtures and provides a semi-quantitative result. (1) N15 Primers + N gene RNA (200k); (2) N15 Primers + N gene RNA (200); (3) N15 Primers + N gene RNA (20); (4) N15 Primers + N gene RNA (2); (5) N15 Primers + human genome; (6) Human primers + human genome; (7) Human Primers whole human RNA; (8) Colorimetric MasterMix only.

which indicate the presence of target viral RNA in all 8 positive samples, whilst tube #1 and #2 remained pink for all 8 negative samples (Figure 5). This shows the RT-LAMP assay has a good agreement with the conventional RT-PCR results in diagnosing COVID-19 samples (Fig. 5 and Fig. S3). Tube #3 of all test kits remained pink in both positive and negative samples, which indicates the human  $\beta$ -actin primers are not able to detect the  $\beta$ -actin gene transcripts within the patient samples. Comparing the results between RT-qPCR and RT-LAMP, it shows that the RT-LAMP was able to detect viral RNA with  $C_t = 36$  by RT-qPCR (Table 2), suggesting that RT-LAMP with visually colour change reading is very sensitive.

#### *One-step process of nucleic acid detection using RT-LAMP*

Cell samples can be processed in one single step to obtain nucleic acid amplification. Heating cells at the same temperature of RT-LAMP 65°C should lyse cells or virus and release RNA. In this study, we tested the one-step process using human Episomal induced pluripotent stem cells (iPSCs), which were suspended in 0.85% saline. Figure 6 shows that the nucleic acids of  $\beta$ -actin

gene in iPSC cells can be directly amplified using RT-LAMP within 40 min. After 30 min, only the purified human RNA can be amplified, showing the colour change (Fig. 6A). However, after 40 min, the tube with human cells changed the colour, whilst the tube without human cells and blank water control remained unchanged pink colour (Fig. 6B). The one-step process is sensitive enough to detect human 10 cells per 25  $\mu$ l reaction. This one-step process took 10 min longer than RT-LAMP to RNA, because it takes about 5–10 min time to lyse cells and release nucleic acids. The result suggests that it should be possible to use one single step to achieve the detection of specific nucleic acids in cells. Further test is needed to validate its practical feasibility on clinical swab samples containing SARS-CoV-2.

## **Discussion**

Here, we described a fast and simple-to-operate method to diagnose COVID-19. We applied RT-LAMP to achieve a rapid testing of SARS-CoV-2, using different primers (O117, S17, N1 and N15) to target Orflab gene, S gene and N gene. We showed that a 30 min reaction could detect down to 2 copies of target RNA per 25  $\mu$ l reaction (80 copies per ml; Figs 1 and 2). However, this extreme sensitivity is also a double-edged sword: carry-over contamination was common in LAMP reactions, which usually cause false positive results (Hsieh et al., 2014; Ma et al., 2017). We also observed carry-over contamination after the RT-LAMP reactions were performed several times in the laboratory. We suspect the aerosol formed from the RT-LAMP products were able to spread everywhere in the laboratory, contaminating lab-coats, pipettes, reagents, materials and equipment used for the next reaction, thus for clinical use all components required in the RT-LAMP reaction need to be prepared in a separate laboratory space with good practice of molecular biology.

The colorimetric display of the testing outcome is easy without the need of expensive or complicated instrument. However, the colour change of RT-LAMP reaction is based on pH indicator phenol red (Tanner et al., 2015; Poole et al., 2017). The elution buffers of the various RNA extraction kits will significantly affect the result. Hence, in all of our experiments including the clinical tests, the RNA samples were eluted in DNase/RNase-free water to ensure the RNA elution buffer has a minimal impact of pH indication in RT-LAMP reactions. The viral RNA clinical tests work well to match the results of RT-qPCR. To tube #3, all samples were negative, suggesting that RNA of human  $\beta$ -actin gene transcript was not amplified in the samples (Fig. 5). The four-primer design for human  $\beta$ -actin (Poon et al., 2005) might require a longer reaction time than 30 min in RT-LAMP assay. In the future, the better human control should be

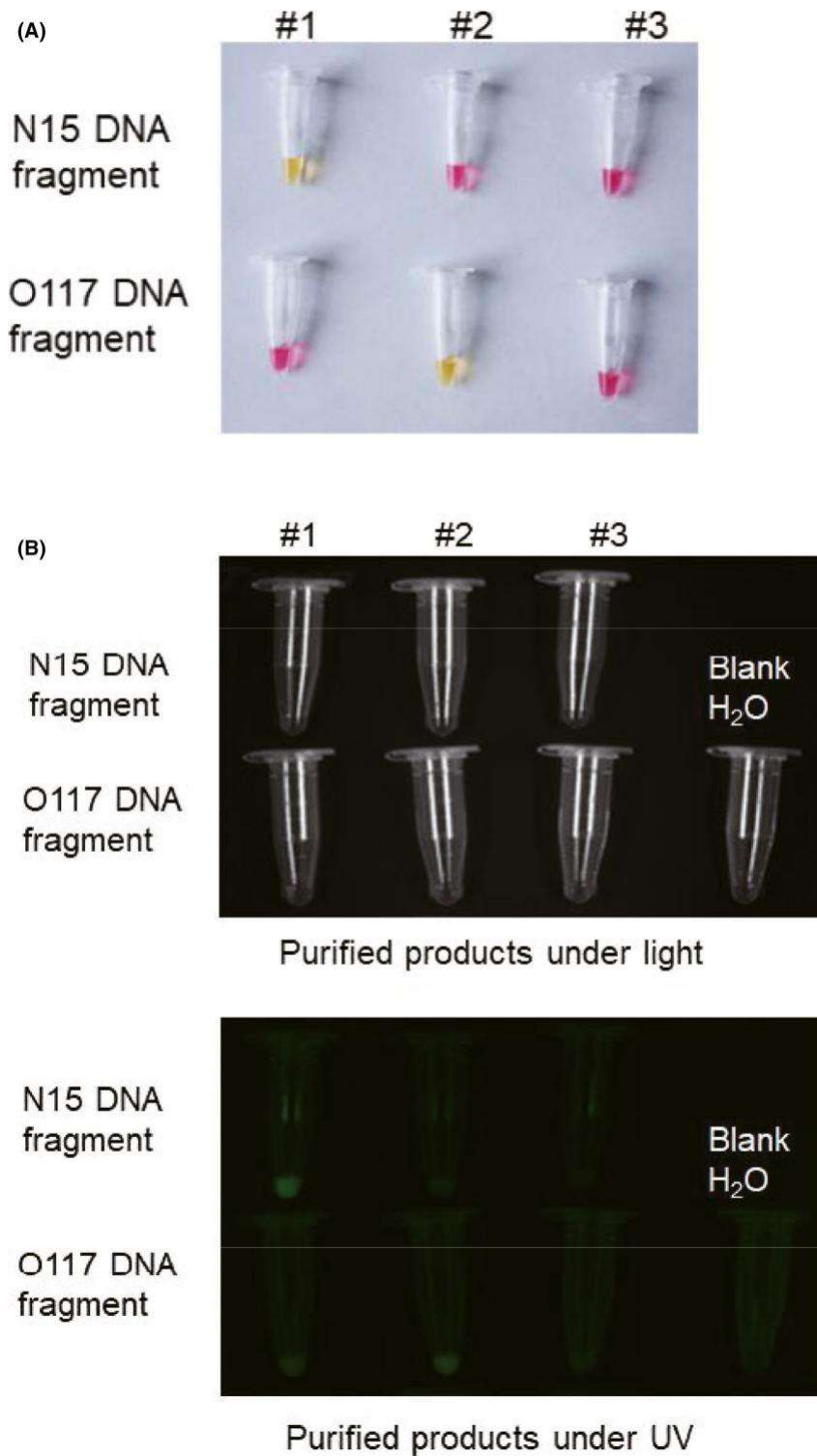


**Fig. 4.** The products of RT-LAMP can be read by both colour change and fluorescent signal, which are consistent.

**A.** Colorimetric display of the RT-LAMP products.

**B.** Fluorescent image under the UV lamp of the RT-LAMP products. #1, #2 and #3 samples separately contained N15, O117 and  $\beta$ -actin primers.

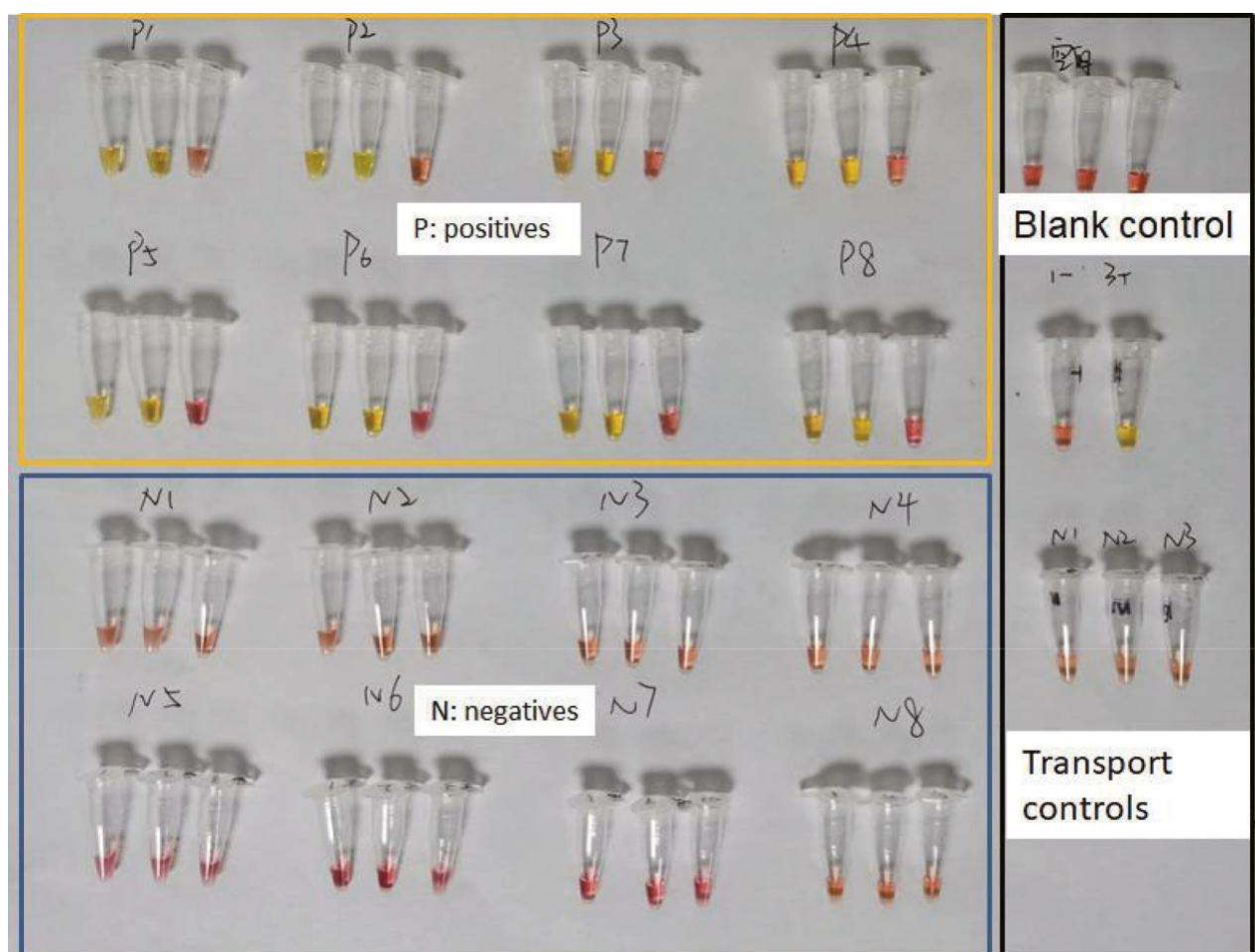


**Fig. 5.** Validation of RT-LAMP using 16 clinical COVID-19 samples from Shenzhen Luohu People's hospital. Samples were first tested with conventional RT-PCR (Table 2), and eight positive samples were labelled 'P', and eight negative samples were labelled 'N'. The components of each test kit are listed in Experimental procedures. Three tubes in each RT-LAMP assay are #1, #2, #3 in order from left to right. Tube #1 and #2 separately contain O117 and N15 primers targeting the Orflab gene and N gene of SARS-CoV-2. Tube #3 contain human b-actin primers. To tube #1 and 2, all positive samples turned yellow, whilst all negative samples remained pink after 30-minute reaction. The results of RT-LAMP method are consistent to the results of conventional RT-PCR (Table 2 and Fig. S1).

applied. For example, the primers for human RNase gene can be used as a positive control.

The ultimate aim is to develop an enclosed device that integrates RNA extraction, purification, reverse transcription (RT) and loop-mediated isothermal amplification (LAMP) to detect the SARS-CoV-2 virus directly from a throat swab sample. It has been well-known that colony PCR can be done directly using cells (Huang et al., 2005). Heating cells can cause cell lysis and nucleic acid release, which can be used as template for the amplification of nucleic acids. We tested one-step RT-LAMP for nucleic acid amplification and it worked well (Fig. 6). The advantage is that the same temperature 65 °C for RT-LAMP was able to lyse cells and the cell lysis in pure water (or with low salt) did not affect the reaction of RT-LAMP. This preliminary work suggests that it is possible to combine RNA extraction, RT and LAMP in

one single step, which will significantly simplify the detection process and shorten the detection time. Current criteria of the test in China are 300 copies ml<sup>-1</sup>; the preliminary results of one-step RT-LAMP should meet this requirement.

Proving the practicability of RT-LAMP in diagnosing COVID-19 is the first step towards developing a more accessible diagnostic kit. Testing is crucial not just for getting treatment to those in need, but also to curb the spread of disease within a region. As we face a global shortage of testing kits amid this global pandemic, our work will be vital to address this need in clinical practice as well as to extend towards a home-based or personal diagnostic system.

With this proof-of-concept work, it is possible to develop a simple, low cost and one-step testing kit which can be used to screen individuals for SARS-CoV-2 virus

**Table 2.** Comparison of conventional RT-qPCR and LAMP results for clinical samples.

| Sample number | RT-qPCR result (Ct value) |       |       |       | LAMP result |       |
|---------------|---------------------------|-------|-------|-------|-------------|-------|
|               | ORF1ab                    | N     | E     | IC    | N-15        | O-117 |
| P1            | 35.78                     | 34.84 | 34.9  | 25.94 | +           | +     |
| P2            | 31.14                     | 29.24 | 28.87 | 25.86 | +           | +     |
| P3            | 34.48                     | 32.13 | 30.37 | 25.84 | +           | +     |
| P4            | 36.64                     | 34.91 | 31.18 | 26.15 | +           | +     |
| P5            | 31.34                     | 31.11 | 27.53 | 25.7  | +           | +     |
| P6            | 31.61                     | 30.49 | 26.92 | 25.88 | +           | +     |
| P7            | 29.93                     | 20.98 | 25.51 | 26.07 | +           | +     |
| P8            | 33.15                     | 33.09 | 31.55 | 28.95 | +           | +     |
| N1            | —                         | —     | —     | 26.33 | —           | —     |
| N2            | —                         | —     | —     | 25.89 | —           | —     |
| N3            | —                         | —     | —     | 26.65 | —           | —     |
| N4            | —                         | —     | —     | 25.04 | —           | —     |
| N5            | —                         | —     | —     | 26.31 | —           | —     |
| N6            | —                         | —     | —     | 26.56 | —           | —     |
| N7            | —                         | —     | —     | 26.89 | —           | —     |
| N8            | —                         | —     | —     | 26.11 | —           | —     |

Ct, cycle threshold; E, E gene for envelop protein; IC, RNase gene as internal control; N, N gene for nucleocapsid protein; ORF1ab, orf1ab gene.

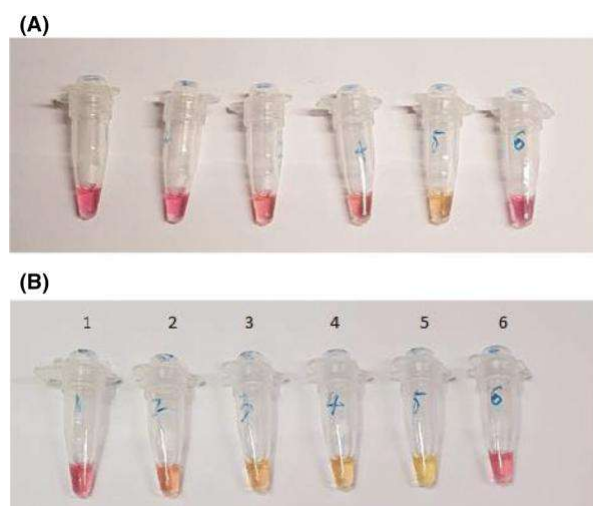


**Fig. 6.** One-step process to get the products of nucleic acid amplification. One microlitre of iPSC cells in 0.85% sterile saline was directly added into the LAMP reagents in each tube and incubate for 30 min (A) and 40 min (B). The whole process was 40 min. (1) 0 cells + Human b-actin primers. (2) 10 cells + Human  $\beta$ -actin primers. (3) 50 cells + Human b-actin primers. (4) 100 cells + Human b-actin primers. (5) Extracted human RNA + Human b-actin primers. (6) H<sub>2</sub>O + Human  $\beta$ -actin primers.

at the point-of-care testing (POCT). There is a need for an on-site rapid test for the infection which can be operated easily with minimal training and without the risk of getting infected. Existing methods to detect SARS-CoV-2 are either based on detecting the virus RNA itself using reverse transcription real-time polymerase chain reaction (RT-qPCR) technologies or based on specific IgM and IgG from patients' blood generated several days after the infection. Currently, these tests would require a

laboratory with specific machines operated by skilled scientists and technicians that take at least 2 h to perform. The ultimate goal will be development of a rapid (30–45 min) enclosed test device with one-step operation for the detection of SARS-CoV-2 virus. Multiple sites on the viral RNA are detected to ensure specificity and sensitivity, and an internal control is embedded to prevent false positives and false negatives. This study will pave the way to move forward for rapid and large-scale screening and diagnosis, where patients exhibiting suspected symptoms can all be tested in the early stages.

## Experimental procedures

### Cell lines and clinical samples

Human Episomal iPSC Line was purchased from Thermo Fisher (UK) and used as the source of human genomic DNA and RNA.

Shenzhen Luohu People's hospital is authorized by Chinese CDC for the detection of clinical samples for SARS-CoV-2 virus. All clinical samples were sampled by clinical throat swab, stored and transported by virus transport media (VTM). Virus deactivation steps were done following the standard guidelines for detection of nucleic acid of SARS-CoV-2 in clinical samples. The research on rapid diagnostic technique for COVID-19 using clinical samples has been approved by the ethical committee at Shenzhen Luohu People's hospital.

### Design of primers and DNA/RNA synthesis for RT-LAMP

Four sets of LAMP primers were designed targeting the Orf1ab, S and N genes of SARS-CoV-2 published at NCBI

(GenBank NC\_045512.2). Primers were designed using LAMP primer designing software, PrimerExplorer (<http://primerexplorer.jp/e/>; Tomita et al., 2008). six primers including forward primer F3, backward primer B3, forward inner primer FIP, backward inner primer BIP, forward loop primer LF and backward loop primer LB were designed to accelerate the LAMP reaction (Nagamine et al., 2002). Table 1 shows all the primer sequences used in this work. In some experiments, 5'-end of FIP primers were labelled by FAM. To carry out mock experiment, we also designed four DNA fragments, each containing a T7 promoter for in vitro transcription (Appendix S1). All designed primers and DNA fragments, as well as a 2019-nCoV\_N\_Positive Control plasmid, were ordered from Integrated DNA Technologies (IDT, UK).

#### *DNA and RNA purification*

The DNA fragments of N, S and Orflab target genes were synthesized by IDT and reconstituted to 50 ng  $\mu\text{l}^{-1}$  according to the manufacturer's instruction. The copy number of each target gene was determined from their molecular weight and diluted into 200 000, 200, 20 and 2 copies  $\mu\text{l}^{-1}$  for subsequent experiments.

T7\_N, T7\_S, and T7\_O DNA synthesized by IDT were reconstituted to 50 ng  $\mu\text{l}^{-1}$  according to the manufacturer's instruction. These DNA sequences were subjected to in vitro transcription using HiScribe™ T7 High Yield RNA Synthesis Kit (New England Biolabs, UK). Transcription products were purified using RNeasy Mini Kit (Qiagen, UK), and the concentration and quality of RNA were measured using Nanodrop. The copy number of each target RNA was determined from their molecular weight and diluted into 200 000, 200, 20 and 2 copies  $\mu\text{l}^{-1}$  for subsequent experiments.

Human whole-genome was purified from human iPSC cells using Fast DNA™ SPIN Kit from MP Biomedicals followed by followed by DNA Cleanup Kit (New England Biolabs, UK). Human whole-RNA was purified from human iPSCs using RNeasy Mini Kit (Qiagen, UK).

#### *LAMP and RT-LAMP for SARS-CoV-2*

All equipment, laminar-flow cabinet and working bench were sprayed with RNaseZap™ prior to experimental work. Filtered pipette tips were used to prevent cross-contamination. All experiments of LAMP and RT-LAMP were run for at least three replicates.

A 10X primer mix (FIP, 16  $\mu\text{M}$ ; BIP, 16  $\mu\text{M}$ ; F3, 2  $\mu\text{M}$ ; B3, 2  $\mu\text{M}$ ; LF, 4  $\mu\text{M}$ ; LB, 4  $\mu\text{M}$ ) was made before the reactions. Both LAMP and RT-LAMP reactions were carried out using WarmStart™ LAMP 2X Master Mix (DNA & RNA) or WarmStart™ Colorimetric LAMP 2X Master Mix (DNA & RNA) from New England Biolabs (NEB).

A 25  $\mu\text{l}$  reaction mixture (2X MasterMix, 12.5  $\mu\text{l}$ ; 10X primer mix, 2.5  $\mu\text{l}$ ; DNA/RNA target, 1  $\mu\text{l}$ ; DNase & RNase-free molecular grade water, 9  $\mu\text{l}$ ) was mixed homogeneously and centrifuged for 1 s. LAMP or RT-LAMP was performed in a thermocycler at 65°C for 30 min. Colour change can be observed directly by the naked eye, and gel electrophoresis was performed to confirm the result.

#### *LAMP and RT-LAMP for human cells.*

All equipment, laminar-flow cabinet and working bench were sprayed with RNaseZap™ prior to experimental work. Filtered pipette tips were used to prevent cross-contamination.

Human Episomal iPSCs obtained from Thermo Fisher Scientific (UK) were dissociated with 0.5 mM EDTA (pH 8.0; Thermo Fisher Scientific) in sterile PBS. The cells were then counted, resuspended and diluted into 100, 50, and 10 cells  $\mu\text{l}^{-1}$  in sterile 0.85% NaCl (Sigma-Aldrich) solution.

A 10X  $\beta$ -actin primer mix (FIP, 16  $\mu\text{M}$ ; BIP, 16  $\mu\text{M}$ ; F3, 2  $\mu\text{M}$ ; B3, 2  $\mu\text{M}$ ) was made before the reactions, and LAMP and RT-LAMP were carried out using WarmStart™ Colorimetric LAMP 2X Master Mix (DNA & RNA) from New England Biolabs (NEB). For the one-step amplification of nucleic acids, cells in 200  $\mu\text{l}$  0.85% NaCl were first heated at 65°C for 10 min and a 25- $\mu\text{l}$  reaction mixture (2X MasterMix, 12.5  $\mu\text{l}$ ; 10X primer mix, 2.5  $\mu\text{l}$ ; the boiled cell sample, 1  $\mu\text{l}$ ; DNase & RNase-free molecular grade water, 9  $\mu\text{l}$ ) was prepared. On the other hand, for the cell in LAMP experiments, cells were directly added into the LAMP reaction reagents, where a 25- $\mu\text{l}$  reaction mixture (2X MasterMix, 12.5  $\mu\text{l}$ ; 10X primer mix, 2.5  $\mu\text{l}$ ; 0.85% NaCl solution with or without iPSCs, 1  $\mu\text{l}$ ; DNase & RNase-free molecular grade water, 9  $\mu\text{l}$ ) was prepared. LAMP/RT-LAMP was performed in a thermocycler at 65°C for 30 or 40 min. Colour change was observed directly by the naked eye.

#### *RNA extraction from clinical samples*

Respiratory specimens (throat swabs) collected from patients were immediately placed into sterile tubes containing 3 ml of VTM (Health Gene Technologies, Ningbo, China). In total, 16 clinic samples were collected from patients. The swab samples were deactivated by heating at 56°C for 30 min in a biosafety level 2 (BSL 2) medical laboratory of Shenzhen Luohu People's Hospital in China. RNA was extracted from swab samples using RNA extraction kit (Health Biomed, China) on a Smart LabAssist-32 platform (Taiwan Advanced Nanotech Inc, Taoyuan, China). To avoid the interference of TE buffer to RT-LAMP reaction, RNA was eluted by RNase-free and DNase water here.

A commercial 2019-nCoV RT-PCR kit (Shanghai ZJ BioTech, China) was used to determine if the samples are positive or negative to SARS-CoV-2 virus. in an ABI 7500 Real-Time PCR System (Thermo Fisher Scientific Inc., Waltham, USA) according to the manufacturer's instructions. A 25 µl reaction mixture contained 5 µl of RNA as the template, 19 µl of 2019-nCoV RT-PCR Buffer, 1 µl of RT-qPCR enzyme mix. The thermal cycling condition was 10 min at 45°C for reverse transcription, 3 min at 95°C for PCR initial activation and 45 cycles of 15 s at 95°C and 30 s at 58°C.

#### RT-LAMP for clinical RNA samples

RT-LAMP assays on clinical samples were performed in pre-mixed test kits. Each test kit consists of three tubes #1, #2 and #3, which contain O-117, N-15 and human β-actin primers respectively (Table 1). The three tubes were first filled up with 5 µl of RNase-free water (Sigma-Aldrich), 12.5 µl of WarmStart Colorimetric Lamp 29 Master Mix (New England Biolabs, UK), and 2.5 µl of 10x primer mix. The test kits were first prepared in OSCAR and delivered to Shenzhen Luohu People's hospital in an ice box. For the detection of SARS-CoV-2 virus, 5 µl of extracted RNA from each patient sample was added into tube #1, #2 and #3 respectively. The test kit was incubated at 65°C for 30 min.

#### Acknowledgements

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#### Conflict of interest

None declared.

#### References

Boehme, C.C., Nabeta, P., Henostroza, G., Raqib, R., Rahim, Z., Gerhardt, M., et al. (2007) Operational feasibility of using loop-mediated isothermal amplification for diagnosis of pulmonary tuberculosis in microscopy centers of developing countries. *J Clin Microbiol* 45: 1936–1940.

Cui, J., Li, F., and Shi, Z.L. (2019) Origin and evolution of pathogenic coronaviruses. *Nat Rev Microbiol* 17: 181–192.

Drosten, C., Götting, S., Schilling, S., Asper, M., Panning, M., Schmitz, H., and Gunther, S. (2002) Rapid detection and quantification of RNA of Ebola and Marburg viruses, Lassa virus, Crimean-Congo hemorrhagic fever virus, Rift Valley fever virus, Dengue virus, and Yellow fever virus by real-time reverse transcription-PCR. *J Clin Microbiol* 40: 2323–2330.

Espy, M.J., Uhl, J.R., Sloan, L.M., Buckwalter, S.P., Jones, M.F., Vetter, E.A., et al. (2006) Real-time PCR in clinical microbiology: Applications for a routine laboratory testing. *Clin Microbiol Rev* 19: 165.

Hsieh, K., Mage, P.L., Csordas, A.T., Eisenstein, M., and Soh, H.T. (2014) Simultaneous elimination of carryover contamination and

detection of DNA with uracil-DNA-glycosylase-supplemented loop-mediated isothermal amplification (UDG-LAMP). *Chem Commun* 50: 3747–3749.

Huang, W.E., Wang, H., Zheng, H.J., Huang, L.F., Singer, A.C., Thompson, I., and Whiteley, A.S. (2005) Chromosomally located gene fusions constructed in *Acinetobacter* sp. ADP1 for the detection of salicylate. *Environ Microbiol* 7: 1339–1348.

Law, J.W.F., Ab Mutalib, N.S., Chan, K.G., and Lee, L.H. (2015) Rapid methods for the detection of foodborne bacterial pathogens: principles, applications, advantages and limitations. *Front Microbiol* 5: 770.

Ma, C.P., Wang, F.X., Wang, X.D., Han, L.Z., Jing, H., Zhang, H., and Shi, C. (2017) A novel method to control carryover contamination in isothermal nucleic acid amplification. *Chem Commun* 53: 10696–10699.

Mackay, I.M., Arden, K.E., and Nitsche, A. (2002) Real-time PCR in virology. *Nucleic Acids Res* 30: 1292–1305.

Menachery, V.D., Yount, B.L., Debbink, K., Agnihothram, S., Gralinski, L.E., Plante, J.A., et al. (2015) A SARS-like cluster of circulating bat coronaviruses shows potential for human emergence. *Nat Med* 21: 1508.

Mori, Y., and Notomi, T. (2009) Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases. *J Infect Chemother* 15: 62–69.

Nagamine, K., Hase, T., and Notomi, T. (2002) Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Mol Cell Probes* 16: 223–229.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., and Hase, T. (2000) Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res* 28: e63.

Poole, C.B., Li, Z.R., Alhassan, A., Guelig, D., Diesburg, S., Tanner, N.A., et al. (2017) Colorimetric tests for diagnosis of filarial infection and vector surveillance using non-instrumented nucleic acid loop-mediated isothermal amplification (NINA-LAMP). *PLoS ONE* 12: e0169011.

Poon, L.L.M., Leung, C.S.W., Chan, K.H., Lee, J.H.C., Yuen, K.Y., Guan, Y., and Peiris, J.S.A. (2005) Detection of human influenza A viruses by loop-mediated isothermal amplification. *J Clin Microbiol* 43: 427–430.

Reboud, J., Xu, G.L., Garrett, A., Adiko, M., Yang, Z.G., Tukahebwa, E.M., et al. (2019) Paper-based microfluidics for DNA diagnostics of malaria in low resource underserved rural communities. *Proc Natl Acad Sci USA* 116: 4834–4842.

Tanner, N.A., Zhang, Y.H., and Evans, T.C. (2015) Visual detection of isothermal nucleic acid amplification using pH-sensitive dyes. *Biotechniques* 58: 59–68.

Thai, H.T.C., Le, M.Q., Vuong, C.D., Parida, M., Minekawa, H., Notomi, T., et al. (2004) Development and evaluation of a novel loop-mediated isothermal amplification method for rapid detection of severe acute respiratory syndrome coronavirus. *J Clin Microbiol* 42: 1956–1961.

- Tomita, N., Mori, Y., Kanda, H., and Notomi, T. (2008) Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nat Protoc* 3: 877-882
- Walls, A.C., Park, Y., Tortorici, M.A., Wall, A., McGuire, A.T., and Veasler, D. (2020) Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein. *Cell* 180: 1–12.
- Wang, C., Horby, P.W., Hayden, F.G., and Gao, G.F. (2020) A novel coronavirus outbreak of global health concern. *Lancet* 395: 470–473.
- Woo, P.C.Y., Huang, Y., Lau, S.K.P., and Yuen, K.Y. (2010) Coronavirus genomics and bioinformatics analysis. *Viruses-Basel* 2: 1804–1820.

## Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

## Appendix S1. Synthesised DNA with T7 promoter.

**Fig. S1.** The RNA map of SARS-CoV-2 and the locations of four sets of primers: O-117, S-17, N1 and N-15, which target the regions encoding Orflab, Spike protein, and N protein.

**Fig. S2.** The impact of virus transport media to the reading of RT-LAMP results. All tubes contain N15 primers and WarmStart Colorimetric Lamp Master Mix. The tubes were incubated at 65°C for 30 min.

**Fig. S3.** Real-time fluorescence RT-qPCR for clinical samples using the commercial 2019-nCoV RT-PCR kit. (A) The reaction amplification curve of the orflab gene of 8 positive samples. (B) The reaction amplification curve of the E gene of 8 positive samples. (C) The reaction amplification curve of the N gene of 8 positive samples. (D) The reaction amplification curve of the RNase P gene as internal control of 8 positive samples. (E) The reaction amplification curve of the positive control. (F) The reaction amplification curve of the negative control.

## Supplementary information

### RT-LAMP for rapid diagnosis of coronavirus SARS-CoV-2

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## Synthesised DNA with T7 promoter

### *N1-T7*

CCATGGgtTAATACGACTCACTATAGGGAGATgataatggaccccaaatcagcgaaatgcaccccgatt  
acgtttggtggaccctcagattcaactggcagtaaccagaatggagaacgcagtggggcgcgatcaaaacaacgtcgccccc  
aaggtttaccaataatactgcgtcttggttcaccgctctcactcaacatggcaaggaagacctaaattccctcgaggacaaggc  
gttccaattaacaccaatagcagtcagatggatccact

### *N15-T7*

CCATGGgtTAATACGACTCACTATAGGGAGATgcaactgaggagccttgaatacaccaaaagatcacatt  
ggcaccgcgaatctgctaacaatgtgcaatcgtgctacaacttcctcaaggaacaacattgccaaaaggcttctacgcagaa  
gggagcagaggcggcagtcgaagcctcttcgcttcctcatcacgtagtcgaacagttcaagaaattcaactccaggcagcagt  
aggggaacttctcctgctagaatggctggcaatggcggatgatgcggatccact

### *S17-T7*

CCATGGgtTAATACGACTCACTATAGGGAGAcactaattctttcacacgtggtgtttattaccctgacaaagtttc  
agatcctcagttttacattcaactcaggacttgttctacctttctttccaatgttacttggtccatgctatacatgtctctgggaccaatg  
gtactaagagggttgataaccctgtcctaccatttaatgatggtgtttatgttgcctcactgagaagttaacataataagaggctgga  
ttttggtactactggatccact

### *O117-T7*

CCATGGgtTAATACGACTCACTATAGGGAGAGttacttaccctaaatgctgttgtaaaattattgtccagcat  
gtcacaattcagaagtaggacctgagcatagcttgcgaataaccataatgaatctggcttgaaaaccattcttcgtaagggtggtc  
gcactattgcctttggaggctgtgtgtctcttatgttggttgccataacaagtgtgcctattgggttccacgtgctagcgtacatagatccact

\*yellow highlight is the sequence of T7 promoter.

**Figure S1**

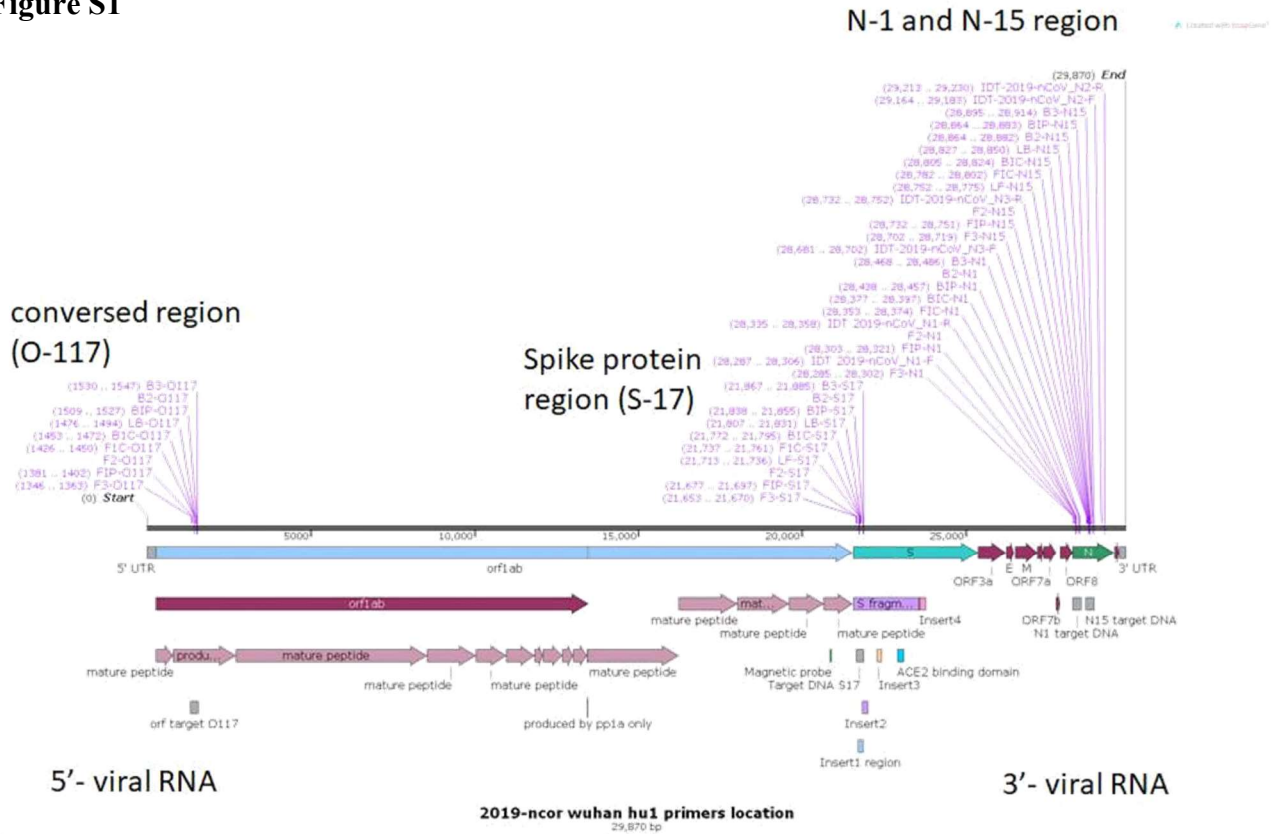
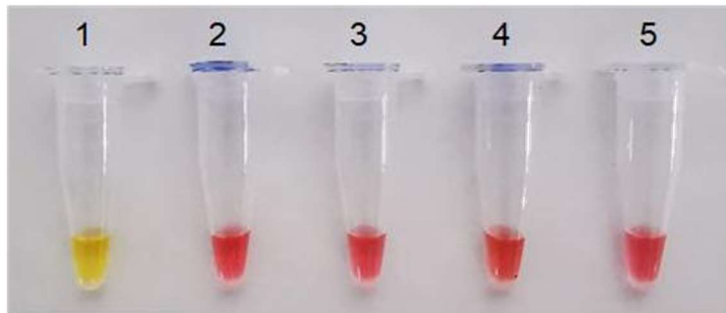


**Figure S2**



1. 4  $\mu$ l H<sub>2</sub>O + 1  $\mu$ l N15 DNA fragment
2. 4  $\mu$ l virus transport media (Yocon Ltd, Beijing, China) + 1  $\mu$ l N15 DNA fragment
3. 4  $\mu$ l H<sub>2</sub>O + 1  $\mu$ l human RNA
4. 5  $\mu$ l virus transport media (Yocon Ltd, Beijing, China)
5. 5  $\mu$ l H<sub>2</sub>O

**Figure S2.** The impact of virus transport media to the reading of RT-LAMP results. All tubes contain N15 primers and WarmStart Colorimetric Lamp Master Mix. The tubes were incubated at 65 °C for 30 min.

**Figure S3**

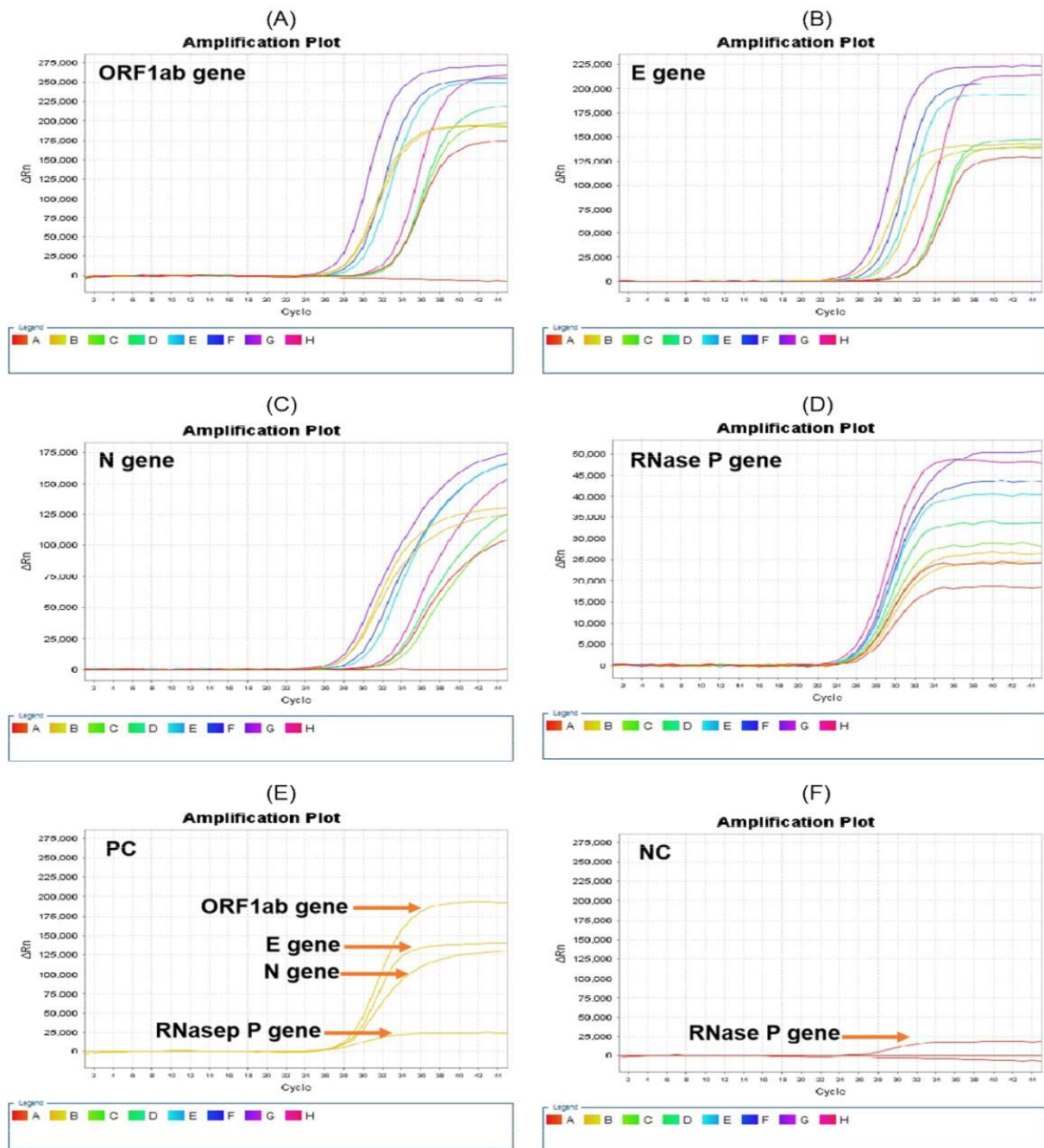


**Figure S3.** Real-time fluorescence RT-qPCR for clinical samples using the commercial 2019-nCoV RT-PCR kit. (A) The reaction amplification curve of the orf1ab gene of 8 positive samples. (B) The reaction amplification curve of the E gene of 8 positive samples. (C) The reaction amplification curve of the N gene of 8 positive samples. (D) The reaction amplification curve of the RNase P gene as internal control of 8 positive samples. (E) The reaction amplification curve of the positive control. (F) The reaction amplification curve of the negative control.

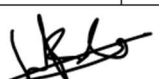
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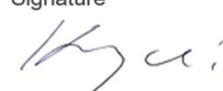
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### Student Confirmation

|                           |                                                                                                                                                       |      |                          |
|---------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------|------|--------------------------|
| Student Name:             | Boon Lim                                                                                                                                              |      |                          |
| Contribution to the Paper | Performed or supported the implementation of all experiments; Reviewed and analyzed all collected data; Drafted, revised and reviewed the manuscript. |      |                          |
| Signature                 |                                                                    | Date | 8 <sup>th</sup> May 2021 |

### Supervisor Confirmation

By signing the Statement of Authorship, you are certifying that the candidate made a substantial contribution to the publication, and that the description described above is accurate.

|                                                                                                                                                                                                                                                                                                                                                                                      |      |                          |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------|--------------------------|
| Supervisor name and title: Professor Wei Huang                                                                                                                                                                                                                                                                                                                                       |      |                          |
| Supervisor comments<br><br>I confirm that Boon made significant contribution to this paper. He shares the first authorship with me and Dr Hsu. His main contributions include: 1. Optimise the LAMP reaction and have intelligent input to the experiment design, 2. Perform experiment. 3. Generate experimental data and carry out data analysis. 4. Help to write the manuscript. |      |                          |
| Signature                                                                                                                                                                                                                                                                                                                                                                            | Date | 8 <sup>th</sup> May 2021 |
|                                                                                                                                                                                                                                                                                                   |      |                          |

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## 4. Clinical Validation of Optimised RT-LAMP for the Diagnosis of SARS-CoV-2 infection

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In this chapter, I introduce an additional oligonucleotide as a molecular switch to reduce self- and non-specific amplification commonly seen in a LAMP reaction. The idea of this molecular switch originates from the action of miRNA, which binds to complementary mRNA sequence to occlude the interaction of ribosome with RBS. The incorporation of this molecular switch optimises the RT-LAMP method and enable its deployment at different testing venues, which is further validated in a large-scale clinical validation.

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OPEN

## Clinical validation of optimised RT-LAMP for the diagnosis of SARS-CoV-2 infection

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We have optimised a reverse transcription-loop-mediated isothermal amplification (RT-LAMP) assay for the detection of SARS-CoV-2 from extracted RNA for clinical application. We improved the stability and reliability of the RT-LAMP assay by the addition of a temperature-dependent switch oligonucleotide to reduce self- or off-target amplification. We then developed freeze-dried master mix for single step RT-LAMP reaction, simplifying the operation for end users and improving long-term storage and transportation. The assay can detect as low as 13 copies of SARS-CoV2 RNA per reaction (25-μL). Cross reactivity with other human coronaviruses was not observed. We have applied the new RT-LAMP assay for testing clinical extracted RNA samples extracted from swabs of 72 patients in the UK and 126 samples from Greece and demonstrated the overall sensitivity of 90.2% (95% CI 83.8–94.7%) and specificity of 92.4% (95% CI 83.2–97.5%). Among 115 positive samples which Ct values were less than 34, the RT-LAMP assay was able to detect 110 of them with 95.6% sensitivity. The specificity was 100% when RNA elution used RNase-free water. The outcome of RT-LAMP can be reported by both colorimetric detection and quantifiable fluorescent reading. Objective measures with a digitized reading data flow would allow for the sharing of results for local or national surveillance.

The rapid spread of SARS-CoV-2 has challenged the testing capacity of many countries. In the United Kingdom, the testing strategy early on in the outbreak prioritized identifying

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and protecting essential workers and vulnerable patients and, during the height of the outbreak, only those who were symptomatic were tested<sup>1</sup>. However, to effectively control localized outbreaks as governments begin to ease lockdown policies, a wider testing strategy achieved by decentralised and rapid testing capacity will be of the utmost importance. Currently, reverse transcription, real-time quantitative polymerase chain reaction (RT-qPCR) detection of SARS-CoV-2 RNA remains the gold standard for the diagnosis of COVID-19<sup>2</sup>. Whilst largely reliable and able to evaluate samples through high-throughput processes, RT-qPCR requires trained personnel, RNA extraction, and sophisticated instrumentation, all of which limit the use of RT-qPCR test for decentralized testing. In addition, the rapid rise in the global demand for RT-qPCR testing has resulted in a global shortage in necessary supplies<sup>3</sup>.

We propose a reverse transcription, loop mediated isothermal amplification (RT-LAMP) assay for the detection of SARS-CoV-2 RNA. The RT-LAMP assay is a colorimetric nucleic acid amplification assay—successful amplification of the target sequence results in a colour change from pink to yellow. The reaction takes place in a single-tube format and simply requires heating at 65 °C for 30 min for the reaction to proceed. The RT-LAMP assay is rapid, single-step, and ideal for point-of-care-testing (POCT) as the only equipment required is a heating platform. The assay endpoint can be assessed using a variety of outputs including colour change, fluorescence and absorbance<sup>4</sup>. By shortening the sample collection, testing, and readout, the POCT RT-LAMP assay is particularly suited to settings where real-time results would directly impact on patient care, including mobile testing centres, emergency departments, primary care facilities, residential homes, and airports<sup>5</sup>.

Several groups have reported RT-LAMP assays for detecting SARS-CoV-2 RNA<sup>6–10</sup>. Our RT-LAMP assay method has been previously presented<sup>11</sup>. There, we demonstrated the ability of our O117 primer set to reliably detect 20 copies per reaction of an Orf1ab RNA transcript. In this project, we have extended on our previous formulation in several ways to further streamline and stabilise the performance of this assay as a POCT outside of standard diagnostic laboratories. We validate the new formulation using several commercial SARS-CoV-2 transcripts, residual oropharyngeal clinical samples and saliva clinical samples.

## Materials and methods

**Primer design.** Primers were designed as previously described in Huang et al.<sup>11</sup>. The primer set O117 targeting Orf1ab of SARS-CoV-2 was designed using PrimerExplorer (<http://primerexplorer.jp/e/>)<sup>12</sup> and each primer were synthesised by Integrated DNA Technologies (IDT, UK). The Switch was synthesised by Integrated DNA Technologies (IDT, UK) with an Iowa Black Dark Quencher at the 3' end (See Supplementary Table S1).

10× O117\_N primer mix was prepared by mixing equal volumes of 16 µM FIP, 16 µM BIP, 2 µM F3, 2 µM B3, 4 µM LF and 4 µM LB.

10× O117\_Q primer mix was prepared by mixing equal volumes of 16 µM FIP, 16 µM BIP, 2 µM F3, 2 µM B3, 4 µM LF, 4 µM LB and 24 µM Switch.

**RT-LAMP reaction mix preparation and generalized procedure.** All equipment, laminar-flow cabinets, and working benches were sprayed with 70% w/v ethanol (Sigma-Aldrich Co., UK) and RNaseZap (Sigma-Aldrich Co. UK) prior to reaction mix preparation. All reagents and PCR tubes were kept on ice during kit preparation.

For wet reaction mix, 12.5 µL of WarmStart Colorimetric LAMP 2× Master Mix (DNA & RNA) from New England Biolabs (New England Biolabs, UK), 5 µL of DNase & RNase-free molecular grade water (Thermo Fisher Scientific Ltd), and 2.5 µL of 10× O117\_N or 10× O117\_Q primer mix was added into PCR tubes and mixed homogeneously.

For dried reaction mix, 12.5 µL 2× Master Mix was supplemented with 7.5 µL drying protective agent, containing sucrose, dextran, lactose and trehalose. 2.5 µL of 10× O117\_N or 10× O117\_Q primer mix was then added into the PCR tubes. The PCR tubes with caps open were dried with a freeze dryer (VirTis Genesis Pilot Lyophilizer, SP Scientific). After desiccation nitrogen gas was imported to release the vacuum. Seal the caps of the PCR tubes under nitrogen environment. The dried reaction mixes were stored at – 20 °C immediately.

The RT-LAMP reaction was run by adding 5 µL of sample of interest to a 20 µL wet reaction mix or a dried reaction mix resuspended in 20 µL of DNase/RNase-free water and heating the reaction at 65 °C for 30 min. A positive result was confirmed through a pH-dependent colorimetric change or confirmation of the production of LAMP product using absorbance or fluorescence.

**Full-length transcripts.** Three full-length SARS-CoV-2 RNA transcripts were used in this study. Research reagent 19/304 (NIBSC, UK) was the kind gift of Giada Mattiuzio. Synthetic RNA Control 1—MT007544.1 (Twist Bioscience Ltd) and Synthetic RNA Control 2—MN908947.3 (Twist Bioscience Ltd) were the kind gifts of John Taylor (Oxford Medical Genetics Laboratories).

**Clinical samples.** This study is to evaluate the optimised RT-LAMP by comparing it with conventional RT-qPCR. This is not a clinical trial and this study is not used as clinical diagnosis. We confirm that that all methods were carried out in accordance with relevant guidelines and regulations. All experimental protocols were approved by the Joint Research Office study classification group in Oxford University. The informed consent was obtained from all subjects or, if subjects are under 18, from a parent and/or legal guardian. Residual samples from clinical testing of 72 symptomatic patients from the Oxford University Hospitals NHS Foundation Trust, Department of Microbiology, were accessed for the evaluation. This study did not require formal ethical approval as is classified as a service evaluation. The study was registered and accepted as a service evaluation on Oxford University Hospitals Governance System Ulysses register (CSS-MICRO-6330). The 126 clinical samples from University Hospital of Alexandroupolis in Greece. The study protocol was approved by the local committee of ethics and deontology in accordance with the Declaration of Helsinki (Number 44/30-7-2020). All samples have informed consent to be used for research and validation studies on an anonymous basis after deidentification.

**RT-qPCR.** Briefly, for the patient samples oro-nasopharyngeal swabs were stored in Universal Transfer Medium (COPAN Diagnostic, USA) or 0.9% saline and extracted using the QIAAsymphony system (Qiagen Co., UK). 200 µL of sample solution was mixed with 430 µL of Buffer OBL and carried into the extraction phase. RNA was eluted into 60 µL of buffer AVE and 10 µL of the elution was carried into a RT-qPCR amplification using the Rotor-Gene Q (Qiagen Co., UK). RT-qPCR reactions were set up using an RNA polymerase gene target validated by Public Health England (PHE, UK) and the Altona RealStar SARS-CoV-2 RT-PCR Kit targeting the E and S genes.

The deep-throat saliva samples were taken from 198 participants and diluted with PBS in 1:1 ratio. Diluted DTS of 200  $\mu$ L was added to the binding buffer of MagMAX Viral/Pathogen Nucleic Acid Isolation Kit (Thermo Fisher Scientific) and viral RNA extraction was carried out according to the manufacturer protocol with the exception that RNA was eluted in 60  $\mu$ L RNase-free water instead of the elution buffer. The entire isolation process was done in Kingfisher Flex automated system (Thermo Fisher Scientific). RT-PCR for detecting extracted RNA of SARS CoV-2 was carried out according to the US CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RTPCR Diagnostic Panel Instructions for Use. RT-PCR targeted N1, N2 of SARS-CoV-2 genes and human RNaseP gene. Briefly, 5  $\mu$ L of extracted RNA or control, 1.5  $\mu$ L combined primer/probe mix (IDT, US), 5  $\mu$ L 4 $\times$  TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher Scientific) and 8.5  $\mu$ L RNase-free water were mixed (total volume 20  $\mu$ L) and loaded into real-time PCR system (Applied Biosciences ViiA7, Thermo Fisher Scientific). The data were analysed using the QuartStudio Real-Time PCR software (Thermo Fisher Scientific).

*RT-LAMP.* RT-LAMP for detecting SARS CoV-2 was carried out according to the previous description<sup>11</sup>. For the wet reaction mix, 5  $\mu$ L of the eluted RNA sample was carried into a reaction containing O117\_Q for RTLAMP assay. For dry reactions, 5  $\mu$ L of extracted RNA was mixed with 20  $\mu$ L RNase-free water to reconstitute lyophilized RT-LAMP master mix. The reaction was incubated at 65 °C for 30 min and the colour was recorded.

**Analytic stability, sensitivity, and specificity.** The stability of the wet reaction mix containing O117\_N or O117\_Q was assessed. 5  $\mu$ L of 40 copies/ $\mu$ L of Synthetic RNA Control 2 was added as a positive control and 5  $\mu$ L of 0.2  $\mu$ g/ $\mu$ L human genome cDNA was added as a negative control. Human genome cDNA was reverse transcribed from whole RNA extracted from human bone marrow derived MSC line (Lonza, UK). RNA extraction was performed as previously described<sup>13,14</sup> and reverse transcribed using the QuantiTect Reverse Transcription kit (Qiagen, Manchester, UK). 0.5–1  $\mu$ g whole RNA was converted into cDNA per manufacturer's instructions including a genomic DNA wipe-out step. Final cDNA sample was diluted to 0.2  $\mu$ g/ $\mu$ L in RNase/ DNase free water and stored at – 20 °C until used for RT-LAMP assay as negative control. The reaction was run as described above and the colour change was assessed at 0, 30, and 60 min.

To obtain an estimate of analytic sensitivity, the 50% endpoint of the LAMP assay was assessed using the three full-length SARS-CoV-2 transcripts. 19/304 was extracted using the viral RNA mini kit (Qiagen Co., UK) and eluted in buffer AVE. Serial dilutions of 5  $\mu$ L of RNA in buffer AVE were used to spike 20  $\mu$ L of solution from a 50  $\mu$ L negative throat swab resuspended in 450  $\mu$ L of RNase-free water. 25  $\mu$ L of solution was added to a dried kit containing O117\_N or O117\_Q primers and were tested in parallel and with five replicates. The LAMP assay was run as described above and assessed via colour change and confirmed via UV–Vis of LAMP product on a 2% agarose gel stained with Sybr-Safe (Thermo Fisher Scientific). The 50% endpoint was calculated using the Reed-Muench method<sup>15</sup>.

The specificity of the LAMP assay for SARS-CoV-2 RNA against other human-infective coronaviruses was assessed. RNA was extracted from biobanked respiratory samples positive for OC43, HKU1, NL63, and 229E (matched samples were previously collected and processed as described in Gaunt et al.<sup>16</sup>) using the QIAamp Viral RNA Mini Kit (Qiagen Co., UK) according to the manufacturer's instructions. 20  $\mu$ L of solution from a 50  $\mu$ L negative throat swab resuspended in 450  $\mu$ L of RNase-free water was spiked with 5  $\mu$ L of the RNA eluate. 25  $\mu$ L was added to a dried kit containing O117\_N or O117\_Q primers

and were tested in parallel. The LAMP assay was run in duplicate at 65 °C for 40 min and assessed via colour change.

**Quantitative detection methods.** Three methods for converting the qualitative colour change output of RT-LAMP reactions into a quantitative output were assessed. The absorbance spectrum of negative and positive samples was read using a Fluorostar Omega microplate reader (BMG Labtech, UK), and the reaction outcome was assessed using the ratio of 430/560 nm. Detection of the LAMP product was also performed using an intercalating fluorescent dye, SYTO 9, at a final concentration of 3 µM (Thermo Fisher Scientific), added to the reaction mix once the 30 min RT-LAMP reaction was completed. Excitation and emission were performed at 485 nm and 500 nm, respectively by Fluorostar Omega microplate reader (BMG Labtech, UK). Alternatively, 5 µL of the reaction product was carried into the Qubit dsDNA BR Assay Kit (Invitrogen, UK). The assay was performed according to the manufacturer's instructions and read using a Qubit 2.0 fluorometer. Cut-off for positive results for absorbance and SYTO 9 quantitative assays were determined to be three times the standard deviations above the negative controls. For Qubit, three times the standard deviation was calculated using negative samples and negative controls.

## Results

**Temperature dependent oligo switch stabilises RT-LAMP assay.** Several reports have indicated that carryover contamination and off target amplification can result in false positives when conducting RT-LAMP assays<sup>17–20</sup>. To address the problem of self- and off-target amplification, we introduced a short oligonucleotide designated as a switch, whose sequence is complementary to one of the primers used in the LAMP reaction and 3'-end was modified by a dark quencher molecule—Iowa Black RQ (IBRQ). The switch serves as a temperature-dependent switch to bind an essential primer of LAMP (e.g. FIP), limiting non-specific amplification and the formation of primer dimers. A working hypothesis for the mechanism of action is that when the temperature is below the working temperature of LAMP (e.g. 65 °C), the FIP primer is bound to the switch oligonucleotide. The switch likely prevents non-specific amplification of non-SARS-CoV-2 RNA eluted during sample processing by binding the FIP primer with higher affinity than contaminating nucleic acids. At the working temperature, the switch competes with the sample RNA for binding, allowing FIP to bind target RNA/DNA for reverse transcription and amplification of target nucleic acids. The mechanism should be further defined with additional experimentation but that is out of the scope of this study.

The O117 primer set<sup>11</sup> was used to evaluate the performance of the switch. Two preparations of the primer mix were used throughout this study: O117\_N contains the original six LAMP primers, and O117\_Q contains the six LAMP primers and a switch oligonucleotide (Table 1). The blank controls without target template have been previously shown to result in false positive due to non-template amplification<sup>21</sup>. In this study, a 12-bp switch with 3'-end modification with IBRQ (IDT, UK) was designed to bind FIP and block self or off-target amplification when the temperature is below the RT-LAMP working temperature. O117\_N or O117\_Q primers were used to test Synthetic RNA Control 2—MN908947.3 (Twist Bioscience, US) and human cDNA (Sigma-Aldrich UK) through a prolonged incubation of up to 1 h. WarmStart Colorimetric Master Mix (New England Biolabs, UK) was used for the RT-LAMP assay. After incubating at 65 °C for 30 min, both O117\_N and O117\_Q LAMP primers, showed positive (yellow) for 200 copies of Synthetic RNA Control 2, and negative (pink) for the human cDNA control (Fig. 1a). However, the O117\_N primer mix turned yellow within 1 h of heating, representing a

| O117 primer | Sequence (5'-3')                                              |
|-------------|---------------------------------------------------------------|
| FIP         | GGT TTT CAA GCC AGA TTC ATT ATG GAT GTC ACA ATT CAG AAG TAGGA |
| BIP         | TCT TCG TAA GGG TGG TCG CAG CAC ACT TGT TAT GGC AAC           |
| F3          | CCC CAA AAT GCT GTT GTT                                       |
| B3          | TAG CAC GTG GAA CCC AAT                                       |
| LF          | TCG GCA AGA CTA TGC TCA GG                                    |
| LB          | TTG CCT TTG GAG GCT GTG T                                     |
| Switch      | GGC TTG AAA ACC -IBRQ                                         |

**Table 1.** Primer sequences used in O117\_N and O117\_Q primer mix. *IBRQ* Iowa Black Dark Quenchers.

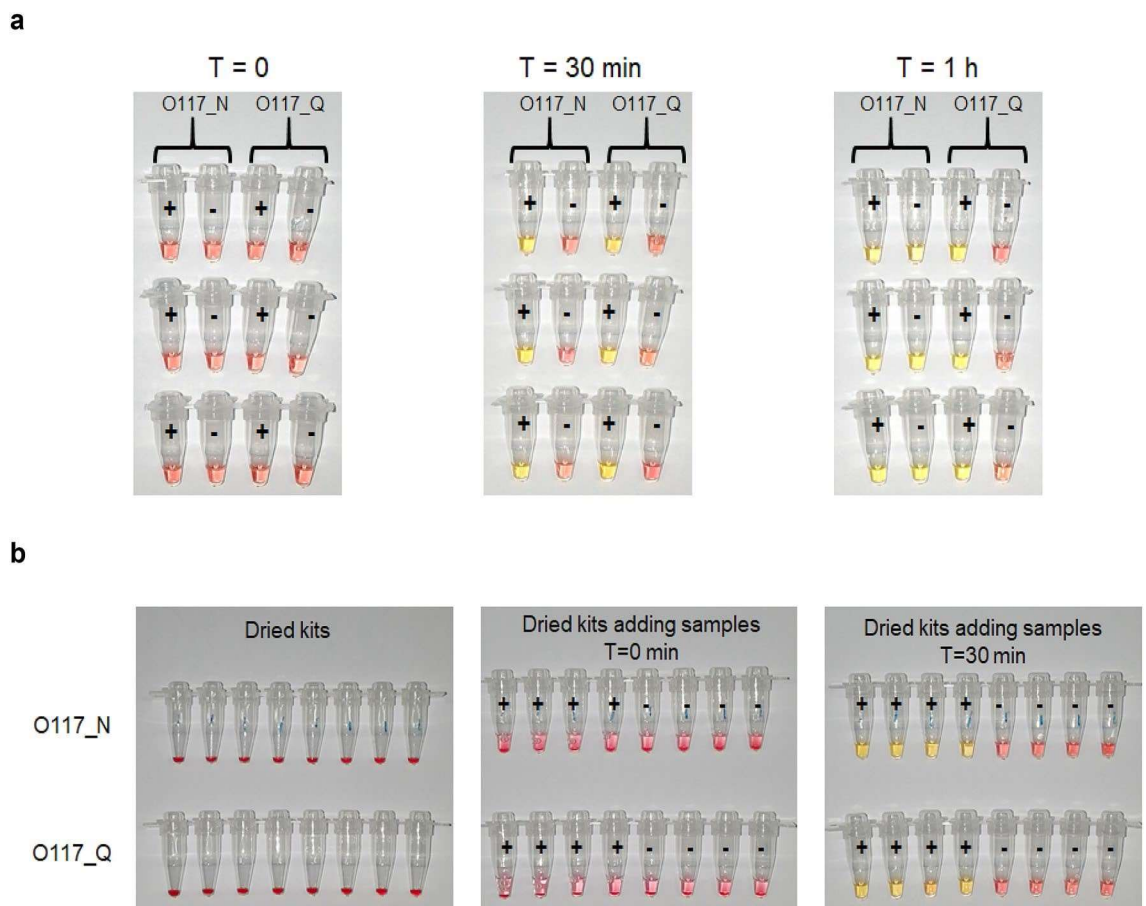


**Figure 1.** Stability test of improved RT-LAMP reaction mix. **(a)** Stability test of RT-LAMP reaction mix containing O117\_N and O117\_Q. Tubes were added with Synthetic RNA Control 2—MN908947.3 ('+') or human genome cDNA ('-') followed by heating at 65 °C for the time indicated by T. Each case has three replicates. **(b)** Dried reaction mixes stored at room temperature up to 3 days. Then they mixed with 20  $\mu$ L DNase/RNase free water and 5  $\mu$ L of Synthetic RNA Control 2—MN908947.3 ('+') or human genome cDNA ('-'). The tubes were incubated at 65 °C for 30 min. Top row: Dried reaction mixes containing O117\_N; Bottom row: Dried reaction mixes containing O117\_Q.

| Full-length transcript  | Dried kit with O117_Q <sup>a</sup> | Dried kit with O117_N <sup>a</sup> |
|-------------------------|------------------------------------|------------------------------------|
| 19/304                  | 71                                 | 89                                 |
| RNA control 1           | 131                                | 224                                |
| RNA control 2           | 60                                 | 13                                 |
| RNA control 2 (wet kit) | 71                                 | 42                                 |

**Table 2.** The 50% end-point of detection using full-length transcript standards. <sup>a</sup> The total copy number per 25-μL reaction.

false positive, while the O117\_Q mix maintained a pink colour throughout the experiment (Fig. 1a). We also showed that O117\_N primer set could generate up to 60% false positive result, whilst O117\_Q can perform reliably after incubating at 65 °C for one hour (Supplementary Fig. S1). The results suggest that a switch in O117 primers reduces off-target amplification at lower temperature and stabilises the LAMP assay at reaction temperature.

**Freeze-drying improve reaction mix storage and transport.** One limitation of the deployability of RT-LAMP to developing countries and rural areas is the need for cold-chain transport of reagents. To overcome this limitation, we freeze-dried a complete reagent mix, including Bst2.0 DNA polymerase, WarmStart reverse transcriptase, colorimetric indicator, dNTPs, primers, and buffer in one PCR tube to make a dry and ready-use reaction mixture. The stability of the dried reaction mix was assessed by storing at room temperature for 15 days before RT-LAMP assay was performed (Fig. 1b and Figs. S2 and S3). On day 3, the dried mixes were first re-suspended in 20 μL DNase/RNase free water followed by the addition of positive control (Synthetic RNA Control 2) and negative control (1 μg human genome cDNA) (Fig. 1b v). After the RT-LAMP reaction, a clear colour difference was shown between the positive controls and negative controls, indicating a high retention of enzymatic activity after 3 days of dried storage at room temperature (Fig. 1b vi). We found freeze-dried samples were more stable than vacuum-dried samples. Freeze-dried reaction mixes stored at room temperature for 15 days showed as good performance as day 0 mixes, indicating the dried kits can be stored for at least 15 days (Supplementary Fig. S3). The further test shows that the dried kits can be stored at room temperature for at least two months.

**Optimised RT-LAMP reaction mix maintains high sensitivity and specificity.** The 50% endpoints of detection (50EP) of dried kits of the RT-LAMP assay containing O117\_N and O117\_Q were determined using three full-length transcripts serially diluted in buffer AVE (Table 2). The RNA templates were research reagent 19/304 (NIBSC, UK), Synthetic RNA control 1—MT007544.1 and Synthetic RNA control 2 MN908947.3 (Twist BioScience, US). The testing results are summarised in Table 2, Fig. 2a and Supplementary Fig. S4. It shows that 50EP against 19/304 were 71 (O117\_Q) and 89 (O117\_N) copies per reaction (25-μL). The 50EP of Synthetic RNA Control 1 was 131 and 224 copies per reaction for O117\_Q and O117\_N, respectively. Synthetic RNA Control 2 demonstrated 50EP of 60 and 13 copies per reaction for O117\_Q and O117\_N, respectively. Additionally, we also tested the 50EP for detection of Synthetic RNA control 2 in wet reaction mixes. The level of detectable RNA for O117\_Q and O117\_N in wet reaction mix were 71 and 42 copies per reaction. The 50EP for dried reaction mixes containing O117\_Q targeting 19/304 and Synthetic RNA Control 2 had a mean of 65 copies per reaction (95% CI 57.3–72.9). The

50EP for dried reaction mixes containing O117\_N and targeting 19/304 and Synthetic RNA control 2 had a mean of 51 copies (95% CI – 1.6 to 104.0). These results suggest that the oligo switch in O117 primers has a minimal impact on the LAMP sensitivity for viral RNA detection and the 50EP is 60–131 RNA copies per 25- $\mu$ L reaction.

The ability of the optimised RT-LAMP assay to discriminate SARS-CoV-2 from human-infective seasonal coronaviruses was assessed using RNA extracted from stored clinical samples previously demonstrated to be infected with respiratory pathogens. The RNA samples from infected patient samples positive for betacoronaviruses (OC43 and HKU1) and alphacoronaviruses (229E and NL63) were used for specificity testing of O117\_Q LAMP assay. Neither replicate of the seasonal coronaviruses tested by O117\_Q produced a positive result, even using an extended reaction time (40 instead of 30 min) (Fig. 2b,c). This result suggests that O117\_Q primer set had a high specificity for SARS-CoV-2 and can discriminate other human-infective coronaviruses.

**Quantitative assessment of colorimetric readout.** Reading the colorimetric RT-LAMP assay has an element of subjectivity. To overcome any ambiguity and potential for inter-user and inter-lab variation when using a visually assessed colorimetric readout, we sought to establish quantitative measurements for differentiating positive and negative samples (Fig. 3). The first method uses the ratio of the absorbance at 430 nm and 560 nm to quantitatively assess the color change of the 72 swab samples (Supplementary Fig. S5). The result is consistent with the colorimetric readout by the naked eye (Fig. 3a,b). Fluorescent dyes SYTO 9 and Qubit were also used to detect the products of the RT-LAMP reaction. Specifically, RT-LAMP products were stained with SYTO 9 and read using a microplate reader (Fig. 3c,d) or stained with Qubit BR DNA dye and read with a Qubit fluorometer (Fig. 3e,f). Positive samples were defined as having values greater than three times the standard deviation of negative samples ( $n = 18$ ). As the Qubit is a portable, compact, and gives an accurate readout of fluorescence, we randomly selected a set of ten positive and negative samples as identified previously using RTqPCR to verify its applicability for POCT. Overall, all three methods demonstrated good agreement with the pH-dependent colorimetric RT-LAMP readout, validating their use as a quantitative measurement to indicate RT-LAMP outcomes. Specifically, absorbance, SYTO9, and Qubit revealed a 2.1, 2.1 and 9.4 fold change between SARS-CoV-2 positive and negative patient samples, respectively. Qubit reagents and fluorometer reading demonstrated a good performance.

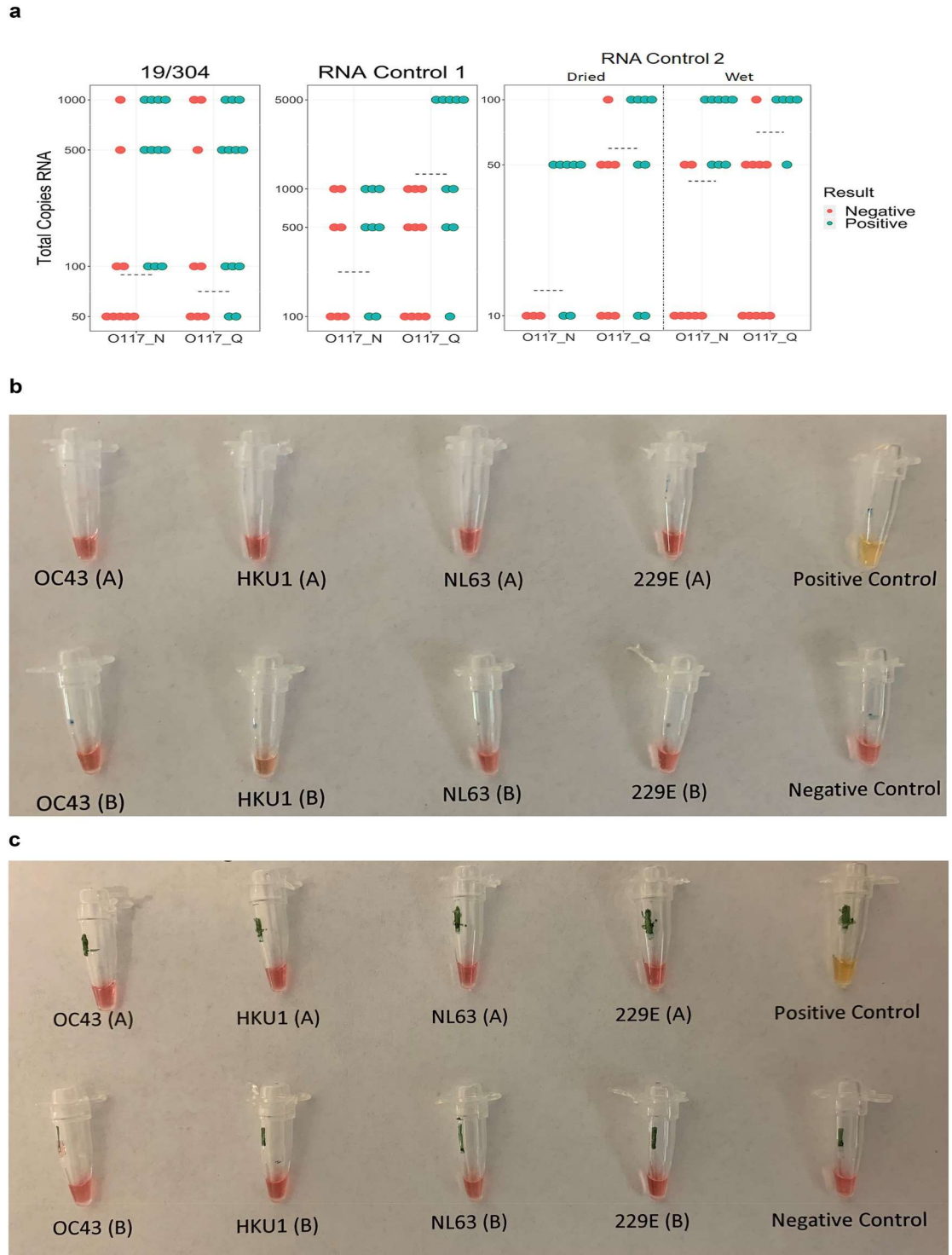


**Figure 2.** Sensitivity and specificity of improved RT-LAMP assay. **(a)** Determination of 50% endpoints for O117\_Q and O117\_N preparations of RT-LAMP assay. Full length transcripts were serially diluted in buffer AVE to achieve the total RNA input/reaction indicated on the y axis. Each dot represents one experimental replicate. Dashed lines indicate 50% endpoint as calculated by the Reed-Muench method. Kits were dried except where indicated for RNA Control 2. Full length transcript indicated on figure. Dried reaction mix containing **(b)** O117\_N and **(c)** O117\_Q were tested against non-SARS human-infective coronaviruses. A and B represent two technical replicates.

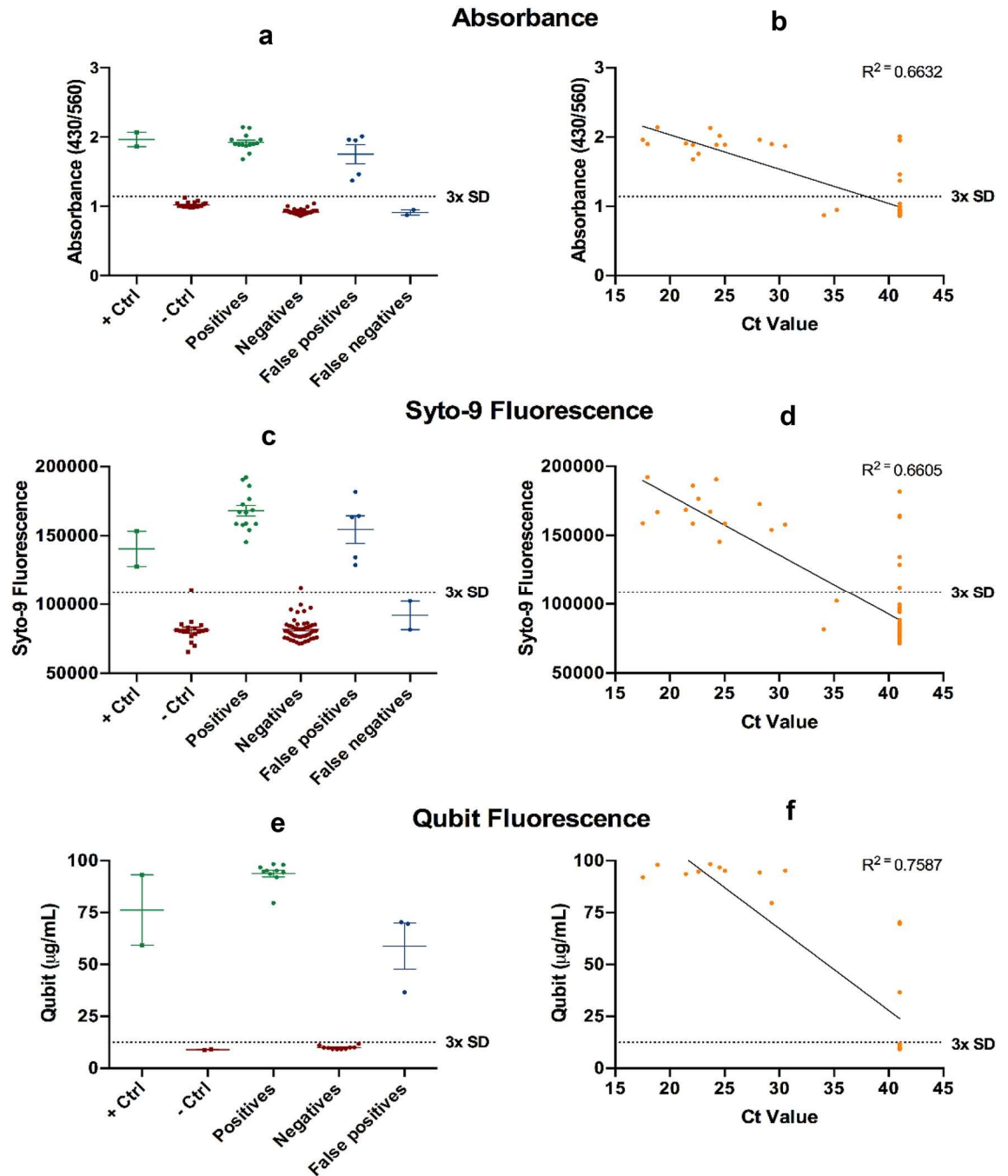


**Figure 3.** Quantitative evaluation of pH-dependent colorimetric RT-LAMP readout. Three quantitative methods (a) Absorbance using 430/560 nm ratio; (b) SYTO 9 fluorescence using 485 nm (excitation) and 500 nm (emission); and (c) Qubit fluorescence using Qubit 2.0 fluorometer were used to assess the RT-LAMP result. (d–f) are the correlation analysis between RT-qPCR and the three quantitative evaluations, respectively. Each dot represents one experimental replicate. Dotted lines indicate 3× standard deviations above the negative controls.

|                                    | Clinical RNA testing (Swab and Saliva)                                                                                                                    |
|------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------|
| Samples                            | 198 (72 UK and 126 Greece RNA extracts)<br>Positive RT-PCR: 132 (16 UK + 116 Greece RNA extracts)<br>Negative RT-PCR: 66 (56 UK + 10 Greece RNA extracts) |
| Overall sensitivity (95% CI)       | 90.2% (83.8–94.7)                                                                                                                                         |
| Overall specificity (95% CI)       | 92.4% (83.2–97.5)                                                                                                                                         |
| Overall percentage agreement (OPA) | 91%                                                                                                                                                       |
| Detection time                     | 15–30 min                                                                                                                                                 |

**Table 3.** Summary of Clinical testing results. The raw data of the clinical samples can be found in Supplementary Table S1.

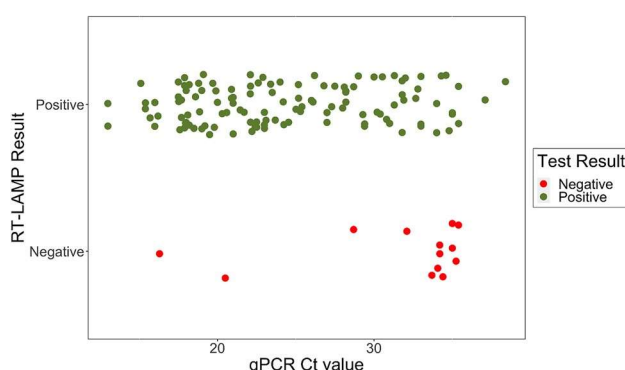


**Figure 4.** Detection of SARS-CoV-2 from RNA extracts from swab and saliva specimen. Comparison of RT-LAMP for detecting SARS-CoV-2 RNA in extract vs RT-qPCR Ct value. RT-LAMP results were read by colorimetric changes (see Supplementary Fig. S5).

**Clinical validation with RNA extract from patient samples.** The performance of wet reaction mix containing O117\_Q was evaluated using SARS-CoV-2 RNA extracts from 198 nasopharyngeal swab samples. The clinical RNA samples were assessed by both RT-qPCR and RT-LAMP in parallel and the results were shown in Supplementary Table S1. Negative controls and positive controls show the expected results (Table S1 and Supplementary Fig. S5).

Compared to RT-qPCR, RT-LAMP demonstrated an overall sensitivity of 90.2% (119 detected/132 positive samples; 95% CI 83.8–94.7%) and specificity of 92.4% (61 detected/66 negative samples; 95% CI 83.2–97.5%) (Table 3). There were five false positives detected by RT-LAMP when elution buffer was AVE (Qiagen, UK). After we changed the elution buffer to RNase free water, the false positive has been completely eliminated in RT-LAMP assay (Supplementary Table S1). In total, there were 115 positive samples which Ct values were less than 34, the RT-LAMP assay was able to detect 110 of them with 95.6% sensitivity (Fig. 4 and Supplementary Table S1). The 13 false negative samples may have been due to the degradation of extracted RNA or the low SARS-CoV-2 copy numbers in the samples (Fig. 4).

## Discussion

The rapid spread of SARS-CoV-2, including to resource limited areas, requests the development of novel, rapid methods for detecting infection in individuals. The standard diagnostic tool, RT-qPCR, cannot be readily deployed outside of large diagnostic laboratories due to the necessary technical expertise, sophisticated instrumentation, and

costly reagents required for sample preparation and processing. The RT-LAMP assay is an ideal POCT as the only required equipment is a simple heating platform, which could be a heating block, dry incubator, water bath or thermal cycler. The assay is simple to perform, delivering an accurate result rapidly that can be easily interpreted by several different detection systems including fluorescence and absorbance detection, or simple visual inspection in a POC setting. Effective surveillance depends on the frequency of testing and test turnaround time, which has been shown to be only marginally improved by using a test with a high sensitivity<sup>22</sup>.

**A short oligo switch to minimise false positives.** We have introduced several adaptations to improve on the performance of our previously reported RT-LAMP assay<sup>11</sup> that differentiate us from other published RT-LAMP assays for SARS-CoV-2 detection. To minimize the risk of false positive due to unspecific amplification or primer dimers in RT-LAMP<sup>23–25</sup>, we introduce a short oligo switch with sequence complementary to the FIP primer, which ‘locks’ FIP from random self-amplification in the absence of the target RNA. In the presence of the target RNA sequence of SARS-CoV-2, the switch is competitively inhibited for binding the FIP primer and the RT-LAMP reaction proceeds. The addition of the switch decreased the risk of self- or off-target amplification (Fig. 1) and did not demonstrably impair the sensitivity of the assay (Fig. 2a).

**Detection limit of this RT-LAMP assay.** We have demonstrated that the RT-LAMP assay is sensitive and highly specific for SARS-CoV-2 RNA. Neither O117\_N nor O117\_Q primer set produced false positive results over eight reactions of samples containing the four seasonal coronaviruses. The 50% endpoint values for transcripts 19/304 and RNA control 2 are largely in agreement and demonstrate that the RT-LAMP assay can readily detect < 100 copies per reaction of SARS-CoV-2 RNA. The substantially higher 50% endpoint for RNA control 1 may be due to improper initial quantification during shipment or transcript degradation. While a trend towards increased sensitivity in O117\_N as one may expect is noted, no statistically significant difference between the O117\_N and O117\_Q preparations was found when averaged across the 19/304 and RNA Control 2 transcripts, but this may be a consequence of the low sample size.

The RT-LAMP assay demonstrated high concordance with a standard RT-qPCR assay in a laboratory setting. RNA extracts from clinical samples were tested using the RT-LAMP assay and showed reasonably high level of overall percentage agreement (OPA) 91% compared to RT-qPCR.

The five false positives detected by our RT-LAMP assay from the RNA extract samples raise the concerns of self-amplification and carryover contamination repeatedly observed in RT-LAMP<sup>23–25</sup>. We have sought to mitigate these risks through the addition of the switch oligonucleotide which demonstrably increases the stability of the reaction. Through freeze-drying the kit components, we also simplify the process for end users and limit the opportunities for onsite carryover contamination.

RT-LAMP assay detects the vast majority of samples from patients who are likely to be very infectious and this assay could be used for rapid identification of individuals with medium to high viral loads to divert these samples away from overburdened diagnostic laboratories. Some previous data has reported that a threshold of Ct = 33 was threshold for which a patient is no longer considered infectious, as viable virus isolated from patients with a Ct value beyond 33 did not generate a positive culture growth<sup>26,27</sup>. More recent data, which remains to be confirmed, suggests that infectious virus was not

recovered at a Ct above 24<sup>28</sup>. Since this RT-LAMP assay was able to get 95.6% sensitivity to Ct < 34, it has a clinical value in practice.

An important consideration in this discussion is that Ct values are not directly comparable and may differ by as much as five cycles when compared directly<sup>29,30</sup>. We also found that two sets of primers including O117\_Q and S17<sup>11</sup> can significantly enhance sensitivity (Supplementary Fig. S6). The switch in O117\_Q also prevents false positive results using these two sets of primers. This new design will be applied to future clinical validation.

**Drying mixes enables room temperature transport and storage.** Drying the master mix simplifies the process for the end user—all that is required is the addition of water and the patient sample. A further advantage of the dried reagent form is its long-term durability and storage at room temperature. Performance of the dried O117\_N and O117\_Q kits was not compromised by storage at room temperature for 14 days, although the color of the kits might change to yellow after 5 days due to the change of pH. However, pH and the colour of the kits can be re-adjusted to the original pink colour by adding 2.5 µL of 10 mM KOH. The pH corrected kits still worked as normal in response to water control and full viral RNA transcript (Supplementary Fig. S2). A fluorescence quantitative reading showed that the dry kits responded well to viral RNA after the dry kits were stored at room temperature for at least 14 days (See Supplementary Fig. S3), suggesting that Bst 2.0 DNA polymerase and WarmStart reverse transcriptase in NEB mastermix remain active for a long duration under drying storage condition. The dried kits can be transported without cold chain constraints, reducing transport and storage costs, increasing the resilience of the supply chain and providing access to testing for resource limited settings and POCT outside of standard diagnostic laboratories.

**Colorimetric and fluorescent double display to report RT-LAMP results.** Colorimetric RT-LAMP detection methods rely on the qualitative assessment of a pH-dependent colour change of phenol red as an indirect indication of polymerase amplification of the target sequence. As visual interpretation of the experimental results could be subjective, we have established three different quantitative methods to overcome these limitations. The first method exploits the absorbance changes associated with phenol red as a function of pH and the other two methods utilized fluorescent dyes and functions independent of pH. The fluorescent methods are particularly attractive as they function independent to the pH changes and could potentially overcome the problem associated with buffers present in the sample collection media affecting the readouts associated with phenol red. Although RT-LAMP reactions have been conducted in a qPCR machine with real time fluorescence measurement, this necessitates the use of a qPCR machine. Here we show that these readouts can be performed accurately using relatively inexpensive equipment that is readily available to many laboratories. Furthermore, these methods provide additional flexibility to the end users such that the quantification methods can be chosen according to equipment availability and/or throughput requirements. Where higher throughput is required, the RT-LAMP reaction can be conducted in a multi-well format optimised for 96 or 384 well plates using a fluorescent dye such as SYTO9 and read with a microplate reader. On the other hand, the Qubit 2.0 is a relatively inexpensive desktop fluorometer requiring only a power source and can be readily integrated into the testing workflow in a variety of diverse settings, including universities, schools, GP clinics and airports. The additional advantage of Qubit allows

electronic transfer of data which could be linked to local and national surveillance programmes.

Thus, given the high accuracy of our LAMP assay, the potential use of multiple detection systems (including portable options such as Qubit), ease of assay performance and the rapid turn around time, we believe this assay could be used as an effective and highly practical first-line screening tool. This RT-LAMP assay shows high accuracy, acceptable sensitivity, and rapid turnaround time, thereby potentially providing a strategic and affordable way to manage surveillance of the SARS-CoV-2 public health crisis.

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## References

1. Coronavirus (COVID-19): Getting tested. *GOV.UK*. <https://www.gov.uk/guidance/coronavirus-covid-19-getting-tested>. Accessed 21 December 2020.
2. CDC. Information for Laboratories about Coronavirus (COVID-19). *Centers for Disease Control and Prevention*. <https://www.cdc.gov/coronavirus/2019-ncov/lab/virus-requests.html> (2020). Accessed 21 December 2020.
3. Babiker, A., Myers, C. W., Hill, C. E. & Guarner, J. SARS-CoV-2 testing. *Am. J. Clin. Pathol.* **153**, 706–708 (2020).
4. Becherer, L. *et al.* Loop-mediated isothermal amplification (LAMP)—Review and classification of methods for sequence-specific detection. *Anal. Methods* **12**, 717–746 (2020).
5. Cui, Z. *et al.* Development of a rapid test kit for SARS-CoV-2: An example of product design. *Bio-Des. Manuf.* **3**, 83–86 (2020).
6. Broughton, J. P. *et al.* CRISPR–Cas12-based detection of SARS-CoV-2. *Nat. Biotechnol.* **38**, 870–874 (2020).
7. Park, G.-S. *et al.* Development of reverse transcription loop-mediated isothermal amplification assays targeting severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). *J. Mol. Diagn. JMD* **22**, 729–735 (2020).
8. Yang, W. *et al.* Rapid detection of SARS-CoV-2 using reverse transcription RT-LAMP method. *medRxiv* <https://doi.org/10.1101/2020.03.02.20030130> (2020).
9. Yu, L. *et al.* Rapid detection of COVID-19 coronavirus using a reverse transcriptional loop-mediated isothermal amplification (RT-LAMP) diagnostic platform. *Clin. Chem.* **66**, 975–977 (2020).
10. Yan, C. *et al.* Rapid and visual detection of 2019 novel coronavirus (SARS-CoV-2) by a reverse transcription loop-mediated isothermal amplification assay. *Clin. Microbiol. Infect.* **26**, 773–779 (2020).
11. Huang, W. E. *et al.* RT-LAMP for rapid diagnosis of coronavirus SARS-CoV-2. *Microb. Biotechnol.* **13**(4), 950–961 (2020).
12. Tomita, N., Mori, Y., Kanda, H. & Notomi, T. Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nat. Protoc.* **3**, 877–882 (2008).
13. Brinkhof, B. *et al.* Improving characterisation of human Multipotent Stromal Cells cultured in 2D and 3D: Design and evaluation of primer sets for accurate gene expression normalisation. *PLoS ONE* **13**, e0209772 (2018).
14. Brinkhof, B., Spee, B., Rothuizen, J. & Penning, L. C. Development and evaluation of canine reference genes for accurate quantification of gene expression. *Anal. Biochem.* **356**, 36–43 (2006).
15. Reed, L. J. & Muench, H. A simple method of estimating fifty percent endpoints. *Am. J. Epidemiol.* **27**, 493–497 (1938).
16. Gaunt, E. R., Hardie, A., Claas, E. C. J., Simmonds, P. & Templeton, K. E. Epidemiology and clinical presentations of the four human coronaviruses 229E, HKU1, NL63, and OC43 detected over 3 years using a novel multiplex real-time PCR method. *J. Clin. Microbiol.* **48**, 2940–2947 (2010).
17. Baek, Y. H. *et al.* Development of a reverse transcription-loop-mediated isothermal amplification as a rapid early-detection method for novel SARS-CoV-2. *Emerg. Microbes Infect.* **9**, 998–1007 (2020).
18. Thi, V. L. D. *et al.* A colorimetric RT-LAMP assay and LAMP-sequencing for detecting SARS-CoV-2 RNA in clinical samples. *Sci. Transl. Med.* **12**(556), eabc7075 (2020).
19. Hsieh, K., Mage, P. L., Csordas, A. T., Eisenstein, M. & Soh, H. T. Simultaneous elimination of carryover contamination and detection of DNA with uracil-DNA-glycosylase-supplemented loop-mediated isothermal amplification (UDG-LAMP). *Chem. Commun.* **50**, 3747–3749 (2014).
20. Kim, E.-M. *et al.* Uracil-DNA glycosylase-treated reverse transcription loop-mediated isothermal amplification for rapid detection of avian influenza virus preventing carry-over contamination. *J. Vet. Sci.* **17**, 421–425 (2016).
21. Tanner, N. A., Zhang, Y. & Evans, T. C. Visual detection of isothermal nucleic acid amplification using pH-sensitive dyes. *Biotechniques* **58**, 59–68 (2015).
22. Larremore, D. B. *et al.* Test sensitivity is secondary to frequency and turnaround time for COVID-19 screening. *Sci. Adv.* **7**(1), eabd5393 (2020).
23. Suleman, E., Mtshali, M. S. & Lane, E. Investigation of false positives associated with loop-mediated isothermal amplification assays for detection of *Toxoplasma gondii* in archived tissue samples of captive felids. *J. Vet. Diagn. Investig. Off. Publ. Am. Assoc. Vet. Lab. Diagn. Inc.* **28**, 536–542 (2016).

24. Postel, A. *et al.* Evaluation of two commercial loop-mediated isothermal amplification assays for detection of avian influenza H5 and H7 hemagglutinin genes. *J. Vet. Diagn. Investig. Off. Publ. Am. Assoc. Vet. Lab. Diagn. Inc.* **22**, 61–66 (2010).
25. Abbasi, I., Kirstein, O. D., Hailu, A. & Warburg, A. Optimization of loop-mediated isothermal amplification (LAMP) assays for the detection of Leishmania DNA in human blood samples. *Acta Trop.* **162**, 20–26 (2016).
26. Walsh, K. A. *et al.* SARS-CoV-2 detection, viral load and infectivity over the course of an infection. *J. Infect.* **81**, 357–371 (2020).
27. Scola, L. *et al.* Viral RNA load as determined by cell culture as a management tool for discharge of SARS-CoV-2 patients from infectious disease wards. *Eur. J. Clin. Microbiol. Infect. Dis.* **39**(6), 1059–1061 (2020).
28. Binnicker, M. J. Can the severe acute respiratory syndrome coronavirus 2 polymerase chain reaction cycle threshold value and time from symptom onset to testing predict infectivity?. *Clin. Infect. Dis.* **71**, 2667–2668 (2020).
29. Nalla, A. K. *et al.* Comparative performance of SARS-CoV-2 detection assays using seven different primer-probe sets and one assay kit. *J. Clin. Microbiol.* **58**(6), e00557–20 (2020).
30. Rodino, K. G. *et al.* Evaluation of saline, phosphate-buffered saline, and minimum essential medium as potential alternatives to viral transport media for SARS-CoV-2 testing. *J. Clin. Microbiol.* **58**(6), e00590–20 (2020).

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## Author contributions

Z.C., W.E.H. and M.A. designed research; B.L., J.R., D.A.N., Y.Y., H.R.S., C.H., L.P., S.E., S.D. and S.R. performed research; S.H.H., S.R.V., P.S. and H.C. contributed new reagents/analytic tools; S.A., A.M., M.P., C.E.X. performed clinical trial in Greece. B.L., J.R., D.A.N., K.P., H.R.S., S.R.V., Z.C., W.E.H. and M.A. analyzed data; and B.L., J.R. and W.E.H. drafted the manuscript; all authors revised and reviewed the manuscript.

## Competing interests

The authors declare no competing interests.

## Additional information

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## Supplementary information

### Clinical Validation of Optimised RT-LAMP for the Diagnosis of SARS-CoV-2 infection

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**Supplementary Table S1.** Performance of the improved RT-LAMP assay in detecting SARS- CoV-2 from RNA extract isolated from patient samples.

| <b>RT-LAMP, May-June.2020 UK</b> |                       |                 |                       |               |                       |                 |                              |
|----------------------------------|-----------------------|-----------------|-----------------------|---------------|-----------------------|-----------------|------------------------------|
| <b>Sample</b>                    | <b>RT-qPCR Result</b> | <b>Ct Value</b> | <b>RT-LAMP Result</b> | <b>Sample</b> | <b>RT-qPCR Result</b> | <b>Ct Value</b> | <b>RT-LAMP Result</b>        |
| <b>B1</b>                        | NOT DETECTED          | -               | NEGATIVE              | <b>E1</b>     | NOT DETECTED          | -               | NEGATIVE                     |
| <b>B2</b>                        | NOT DETECTED          | -               | NEGATIVE              | <b>E2</b>     | NOT DETECTED          | -               | NEGATIVE                     |
| <b>B3</b>                        | NOT DETECTED          | -               | NEGATIVE              | <b>E3</b>     | NOT DETECTED          | -               | NEGATIVE                     |
| <b>B4</b>                        | NOT DETECTED          | -               | NEGATIVE              | <b>E4</b>     | NOT DETECTED          | -               | NEGATIVE                     |
| <b>B5</b>                        | NOT DETECTED          | -               | NEGATIVE              | <b>E5</b>     | NOT DETECTED          | -               | NEGATIVE                     |
| <b>B6</b>                        | NOT DETECTED          | -               | NEGATIVE              | <b>E6</b>     | NOT DETECTED          | -               | POSITIVE<br>(False positive) |
| <b>B7</b>                        | DETECTED              | 24.54           | POSITIVE              | <b>E7</b>     | NOT DETECTED          | -               | NEGATIVE                     |
| <b>B8</b>                        | NOT DETECTED          | -               | NEGATIVE              | <b>E8</b>     | NOT DETECTED          | -               | NEGATIVE                     |
| <b>B9</b>                        | DETECTED              | 21.45           | POSITIVE              | <b>E9</b>     | DETECTED              | 28.2            | POSITIVE                     |
| <b>B10</b>                       | DETECTED              | 29.29           | POSITIVE              | <b>E10</b>    | DETECTED              | 34.07           | NEGATIVE<br>(False negative) |
| <b>B11</b>                       | DETECTED              | 25.02           | POSITIVE              | <b>E11</b>    | NOT DETECTED          | -               | NEGATIVE                     |
| <b>B12</b>                       | NOT DETECTED          | -               | NEGATIVE              | <b>E12</b>    | NOT DETECTED          | -               | NEGATIVE                     |

|            |              |       |                              |            |              |       |                              |
|------------|--------------|-------|------------------------------|------------|--------------|-------|------------------------------|
| <b>C1</b>  | NOT DETECTED | -     | NEGATIVE                     | <b>F1</b>  | NOT DETECTED | -     | NEGATIVE                     |
| <b>C2</b>  | NOT DETECTED | -     | NEGATIVE                     | <b>F2</b>  | NOT DETECTED | -     | NEGATIVE                     |
| <b>C3</b>  | NOT DETECTED | -     | NEGATIVE                     | <b>F3</b>  | NOT DETECTED | -     | NEGATIVE                     |
| <b>C4</b>  | NOT DETECTED | -     | NEGATIVE                     | <b>F4</b>  | NOT DETECTED | -     | NEGATIVE                     |
| <b>C5</b>  | NOT DETECTED | -     | NEGATIVE                     | <b>F5</b>  | NOT DETECTED | -     | NEGATIVE                     |
| <b>C6</b>  | NOT DETECTED | -     | NEGATIVE                     | <b>F6</b>  | NOT DETECTED | -     | NEGATIVE                     |
| <b>C7</b>  | NOT DETECTED | -     | NEGATIVE                     | <b>F7</b>  | NOT DETECTED | -     | NEGATIVE                     |
| <b>C8</b>  | NOT DETECTED | -     | NEGATIVE                     | <b>F8</b>  | DETECTED     | 22.11 | POSITIVE                     |
| <b>C9</b>  | DETECTED     | 23.69 | POSITIVE                     | <b>F9</b>  | DETECTED     | 35.24 | NEGATIVE<br>(False negative) |
| <b>C10</b> | DETECTED     | 17.53 | POSITIVE                     | <b>F10</b> | DETECTED     | 17.96 | POSITIVE                     |
| <b>C11</b> | DETECTED     | 30.54 | POSITIVE                     | <b>F11</b> | NOT DETECTED | -     | NEGATIVE                     |
| <b>C12</b> | NOT DETECTED | -     | NEGATIVE                     | <b>F12</b> | NOT DETECTED | -     | NEGATIVE                     |
| <b>D1</b>  | NOT DETECTED | -     | NEGATIVE                     | <b>G1</b>  | NOT DETECTED | -     | POSITIVE<br>(False positive) |
| <b>D2</b>  | NOT DETECTED | -     | NEGATIVE                     | <b>G2</b>  | NOT DETECTED | -     | NEGATIVE                     |
| <b>D3</b>  | NOT DETECTED | -     | NEGATIVE                     | <b>G3</b>  | NOT DETECTED | -     | NEGATIVE                     |
| <b>D4</b>  | NOT DETECTED | -     | POSITIVE<br>(False positive) | <b>G4</b>  | NOT DETECTED | -     | NEGATIVE                     |
| <b>D5</b>  | NOT DETECTED | -     | POSITIVE<br>(False positive) | <b>G5</b>  | NOT DETECTED | -     | NEGATIVE                     |

|            |              |       |                              |            |              |       |          |
|------------|--------------|-------|------------------------------|------------|--------------|-------|----------|
| <b>D6</b>  | NOT DETECTED | -     | NEGATIVE                     | <b>G6</b>  | NOT DETECTED | -     | NEGATIVE |
| <b>D7</b>  | NOT DETECTED | -     | NEGATIVE                     | <b>G7</b>  | NOT DETECTED | -     | NEGATIVE |
| <b>D8</b>  | NOT DETECTED | -     | POSITIVE<br>(False positive) | <b>G8</b>  | NOT DETECTED | -     | NEGATIVE |
| <b>D9</b>  | DETECTED     | 18.86 | POSITIVE                     | <b>G9</b>  | DETECTED     | 24.24 | POSITIVE |
| <b>D10</b> | DETECTED     | 22.61 | POSITIVE                     | <b>G10</b> | DETECTED     | 22.11 | POSITIVE |
| <b>D11</b> | NOT DETECTED | -     | NEGATIVE                     | <b>G11</b> | NOT DETECTED | -     | NEGATIVE |
| <b>D12</b> | NOT DETECTED | -     | NEGATIVE                     | <b>G12</b> | NOT DETECTED | -     | NEGATIVE |

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| RT-LAMP, May-June 2021, Greece |                |          |                           |        |                |          |                |
|--------------------------------|----------------|----------|---------------------------|--------|----------------|----------|----------------|
| Sample                         | RT-qPCR Result | Ct Value | RT-LAMP Result            | Sample | RT-qPCR Result | Ct Value | RT-LAMP Result |
| 57942                          | DETECTED       | 15.1     | Positive                  | 58999  | NOT DETECTED   | -        | NEGATIVE       |
| 57813                          | DETECTED       | 17.5     | Positive                  | 58998  | NOT DETECTED   | -        | NEGATIVE       |
| 58030                          | DETECTED       | 17.9     | Positive                  | 58982  | NOT DETECTED   | -        | NEGATIVE       |
| 58348                          | DETECTED       | 22.1     | Positive                  | 58979  | NOT DETECTED   | -        | NEGATIVE       |
| 57981                          | DETECTED       | 22.5     | Positive                  | 58978  | NOT DETECTED   | -        | NEGATIVE       |
| 58405                          | DETECTED       | 26.8     | Positive                  | 58977  | NOT DETECTED   | -        | NEGATIVE       |
| 58071                          | DETECTED       | 27       | Positive                  | 58976  | NOT DETECTED   | -        | NEGATIVE       |
| 58063                          | DETECTED       | 28.1     | Positive                  | 58975  | NOT DETECTED   | -        | NEGATIVE       |
| 58620                          | DETECTED       | 19.5     | Positive                  | 60009  | NOT DETECTED   | -        | NEGATIVE       |
| 58349                          | DETECTED       | 22.1     | Positive                  | 60010  | NOT DETECTED   | -        | NEGATIVE       |
| 58285                          | DETECTED       | 25.17    | Positive                  | 53323  | DETECTED       | 16.2     | Positive       |
| 58036                          | DETECTED       | 31.3     | Positive                  | 53443  | DETECTED       | 17.7     | Positive       |
| 58017                          | DETECTED       | 34.2     | NEGATIVE (False negative) | 54216  | DETECTED       | 31.8     | Positive       |

|       |          |      |                                 |       |          |       |                                 |
|-------|----------|------|---------------------------------|-------|----------|-------|---------------------------------|
| 58356 | DETECTED | 34.4 | NEGATIVE<br>(False<br>negative) | 59295 | DETECTED | 33.7  | NEGATIVE<br>(False<br>negative) |
| 57991 | DETECTED | 35   | NEGATIVE<br>(False<br>negative) | 53004 | DETECTED | 15.4  | Positive                        |
| 58038 | DETECTED | 35.4 | NEGATIVE<br>(False<br>negative) | 53002 | DETECTED | 16    | Positive                        |
| 58800 | DETECTED | 13   | Positive                        | 52977 | DETECTED | 19    | Positive                        |
| 58801 | DETECTED | 15.7 | Positive                        | 53003 | DETECTED | 19.8  | Positive                        |
| 58799 | DETECTED | 17.9 | Positive                        | 53834 | DETECTED | 21    | Positive                        |
| 58802 | DETECTED | 20.9 | Positive                        | 53056 | DETECTED | 25.18 | Positive                        |
| 58811 | DETECTED | 34.2 | NEGATIVE<br>(False<br>negative) | 53148 | DETECTED | 26    | Positive                        |
| 58789 | DETECTED | 18.6 | Positive                        | 53140 | DETECTED | 34    | Positive                        |
| 57981 | DETECTED | 22.5 | Positive                        | 52831 | DETECTED | 18    | Positive                        |
| 58951 | DETECTED | 23.1 | Positive                        | 52835 | DETECTED | 18    | Positive                        |
| 53505 | DETECTED | 25.4 | Positive                        | 52829 | DETECTED | 18.8  | Positive                        |
| 58405 | DETECTED | 26.2 | Positive                        | 52833 | DETECTED | 19    | Positive                        |
| 58320 | DETECTED | 28.7 | NEGATIVE<br>(False<br>negative) | 52832 | DETECTED | 20    | Positive                        |

|       |          |      |          |       |          |      |                              |
|-------|----------|------|----------|-------|----------|------|------------------------------|
| 53579 | DETECTED | 34.6 | Positive | 52876 | DETECTED | 20.3 | Positive                     |
| 53516 | DETECTED | 35   | Positive | 52830 | DETECTED | 21   | Positive                     |
| 54471 | DETECTED | 18.1 | Positive | 53124 | DETECTED | 22.4 | Positive                     |
| 54256 | DETECTED | 19.1 | Positive | 52566 | DETECTED | 13   | Positive                     |
| 54512 | DETECTED | 19.2 | Positive | 52824 | DETECTED | 17.7 | Positive                     |
| 54172 | DETECTED | 22.2 | Positive | 52567 | DETECTED | 18.2 | Positive                     |
| 54472 | DETECTED | 28.2 | Positive | 52825 | DETECTED | 20.5 | NEGATIVE<br>(False negative) |
| 54254 | DETECTED | 29.4 | Positive | 52826 | DETECTED | 27.5 | Positive                     |
| 54473 | DETECTED | 31.3 | Positive | 52828 | DETECTED | 29.4 | Positive                     |
| 54217 | DETECTED | 17.5 | Positive | 52823 | DETECTED | 30   | Positive                     |
| 49321 | DETECTED | 18.2 | Positive | 52803 | DETECTED | 33   | Positive                     |
| 58755 | DETECTED | 24.2 | Positive | 52549 | DETECTED | 16   | Positive                     |
| 58680 | DETECTED | 25.3 | Positive | 52542 | DETECTED | 18.5 | Positive                     |
| 54195 | DETECTED | 28   | Positive | 52407 | DETECTED | 23   | Positive                     |

|       |          |       |                                 |       |          |       |          |
|-------|----------|-------|---------------------------------|-------|----------|-------|----------|
| 54475 | DETECTED | 28.7  | Positive                        | 52408 | DETECTED | 23    | Positive |
| 54025 | DETECTED | 29    | Positive                        | 52410 | DETECTED | 24    | Positive |
| 53201 | DETECTED | 32.67 | Positive                        | 52406 | DETECTED | 27    | Positive |
| 53622 | DETECTED | 16.3  | NEGATIVE<br>(False<br>negative) | 52548 | DETECTED | 28.2  | Positive |
| 53359 | DETECTED | 17.9  | Positive                        | 52409 | DETECTED | 35    | Positive |
| 53988 | DETECTED | 19.7  | Positive                        | 52179 | DETECTED | 20.59 | Positive |
| 54016 | DETECTED | 19.9  | Positive                        | 52007 | DETECTED | 23    | Positive |
| 53978 | DETECTED | 20.9  | Positive                        | 59322 | DETECTED | 31.8  | Positive |
| 53629 | DETECTED | 26.1  | Positive                        | 59920 | DETECTED | 32.8  | Positive |
| 53625 | DETECTED | 30.8  | Positive                        | 59642 | DETECTED | 34    | Positive |
| 53826 | DETECTED | 31.9  | Positive                        | 52037 | DETECTED | 34.3  | Positive |
| 53744 | DETECTED | 17.6  | Positive                        | 52019 | DETECTED | 35.4  | Positive |
| 53569 | DETECTED | 21.7  | Positive                        | 52031 | DETECTED | 35.4  | Positive |
| 53823 | DETECTED | 23.5  | Positive                        | 52029 | DETECTED | 15.4  | Positive |

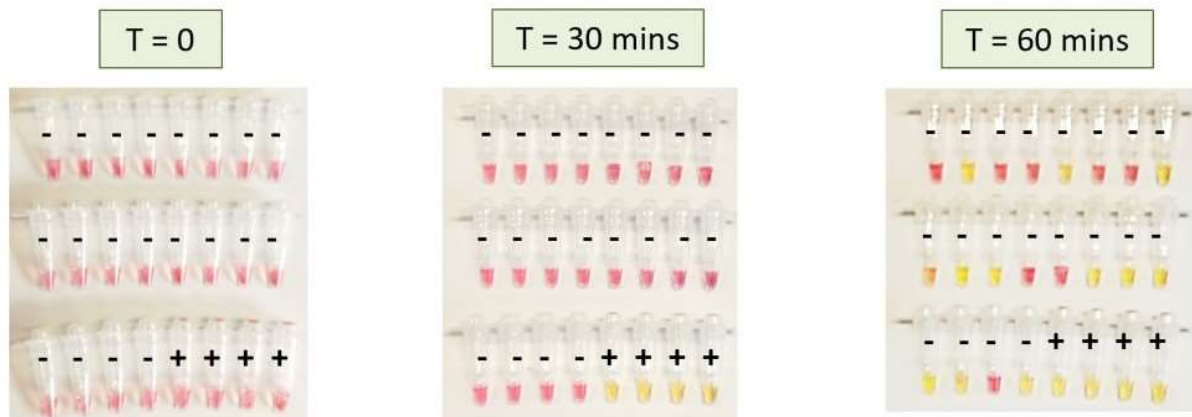
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|       |          |      |                                 |       |          |      |          |
|-------|----------|------|---------------------------------|-------|----------|------|----------|
| 53787 | DETECTED | 30.2 | Positive                        | 52182 | DETECTED | 21   | Positive |
| 53762 | DETECTED | 30.4 | Positive                        | 52178 | DETECTED | 22.9 | Positive |
| 53512 | DETECTED | 33   | Positive                        | 59823 | DETECTED | 27.3 | Positive |
| 53977 | DETECTED | 34.8 | Positive                        | 52180 | DETECTED | 31   | Positive |
| 53767 | DETECTED | 35   | NEGATIVE<br>(False<br>negative) | 59324 | DETECTED | 31.8 | Positive |
| 52381 | DETECTED | 23.4 | Positive                        | 52354 | DETECTED | 32   | Positive |
| 60197 | DETECTED | 32.1 | NEGATIVE<br>(False<br>negative) | 59528 | DETECTED | 33   | Positive |
| 51951 | DETECTED | 37.1 | Positive                        | 52009 | DETECTED | 38.4 | Positive |

3

4 (a)

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6

7 (b)

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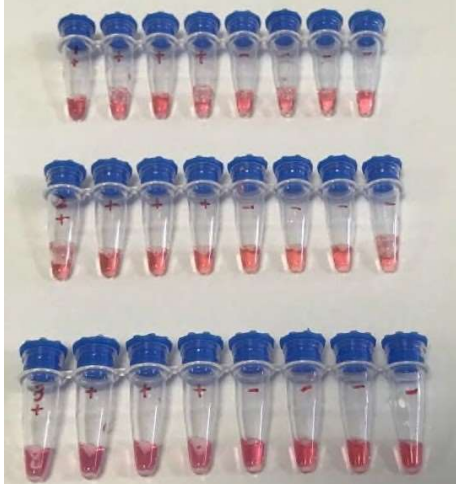
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11 **Supplementary Figure S1. The switch can stabilise RT-LAMP performance within**  
12 **an hour of incubation.** O117\_N generated 60% false positive results (12/20 reactions)  
13 due to self- and off-target amplification in human RNA control sample after incubating at  
14 65 °C for 60 minutes. O117\_Q still shows negative to human RNA control as long as 60  
15 minutes at 65 °C reaction.

16 Day 0 in room temperature (20 °C)

17 Before reaction T= 0 min

|         | Before reconstitution                                                             | After reconstitution                                                               |
|---------|-----------------------------------------------------------------------------------|------------------------------------------------------------------------------------|
| Batch 1 |  |  |
| Batch 2 |                                                                                   |  |
| Batch 3 |                                                                                   |  |
|         | 1 2 3 4 5 6 7 8                                                                   | 1 2 3 4 5 6 7 8                                                                    |

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Day 0

After reaction T= 30 min

|         | Before 65°C for 30 minutes                                                          | After 65°C for 30 minutes                                                             |
|---------|-------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|
| Batch 1 |  |   |
| Batch 2 |                                                                                     |  |
| Batch 3 |                                                                                     |  |
|         | 1 2 3 4 5 6 7 8                                                                     | 1 2 3 4 5 6 7 8                                                                       |

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28     **Day 15 in room temperature**

29     **(20 °C) Before reaction T= 0**

30     **min**

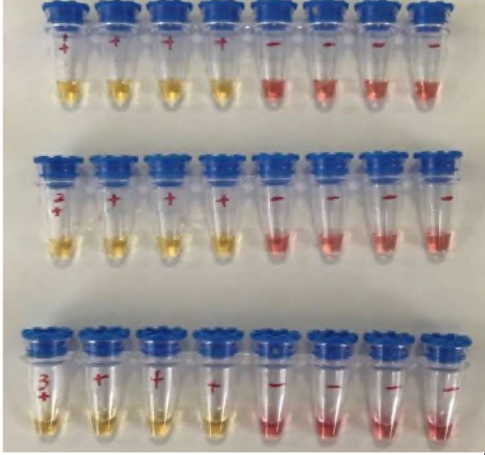
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|         | Before reconstitution                                                              | After reconstitution                                                                |
|---------|------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|
| Batch 1 |  |  |
| Batch 2 |                                                                                    |                                                                                     |
| Batch 3 |                                                                                    |                                                                                     |
|         | 1 2 3 4 5 6 7 8                                                                    | 1 2 3 4 5 6 7 8                                                                     |

33

**Day 15**  
**After reaction T= 30 min**

|         | Before 65°C for 30 minutes                                                        | After 65°C for 30 minutes                                                          |
|---------|-----------------------------------------------------------------------------------|------------------------------------------------------------------------------------|
| Batch 1 |  |  |
| Batch 2 |                                                                                   |                                                                                    |
| Batch 3 |                                                                                   |                                                                                    |
|         | 1 2 3 4 5 6 7 8                                                                   | 1 2 3 4 5 6 7 8                                                                    |

**Supplementary Figure S3. Long-term storage of optimized RT-LAMP dried reaction mixes at room temperature.** Dried mixes were stored for day 0 (A) and day 15 (B) at room temperature. Dried mixes were reconstituted with 20 µL DNase/RNase free water followed by the addition of 5 µL of Synthetic RNA Control 2 - MN908947.3 (40 copies/µL, Twist Bioscience) or human genome DNA as negative control. (iii) Tubes were incubated at 65 °C for 30 minutes. Each time three batches and each batch three replicates were carried out.

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O117\_N

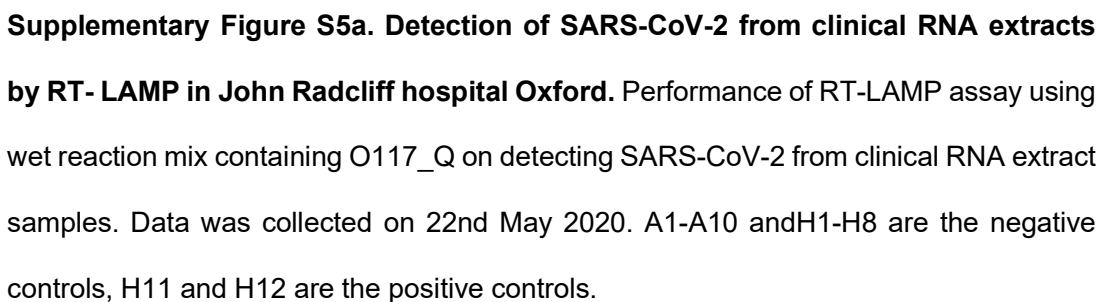
O117\_Q

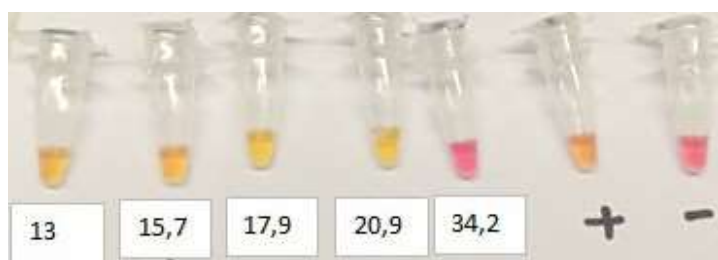
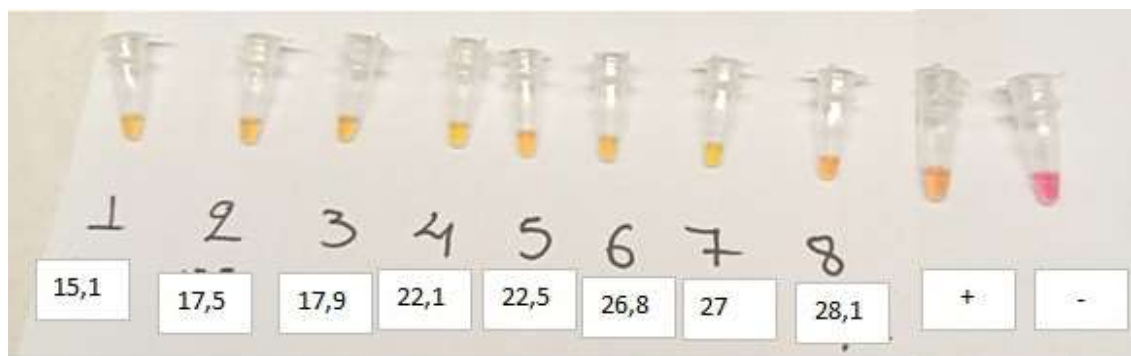


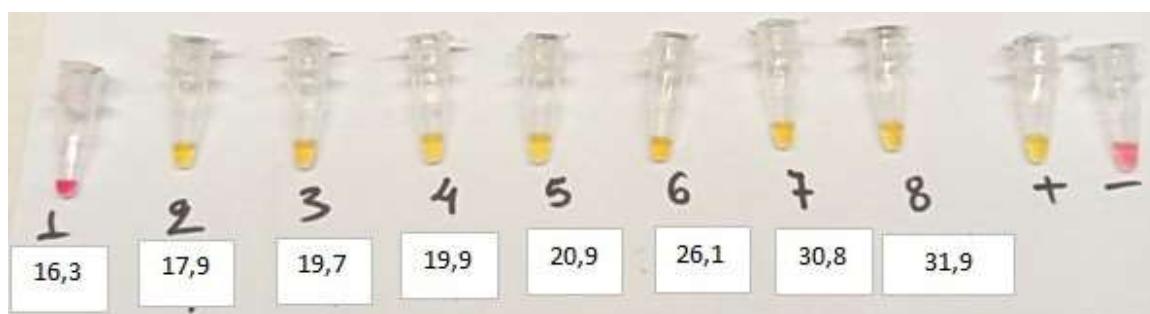
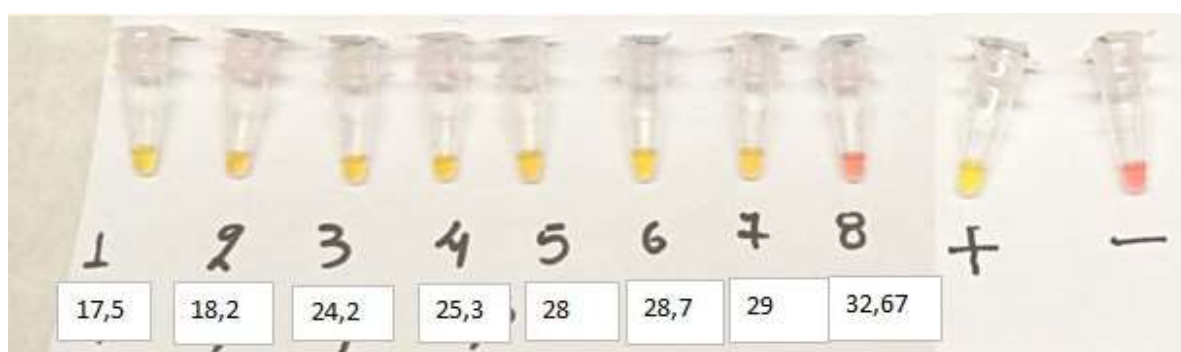
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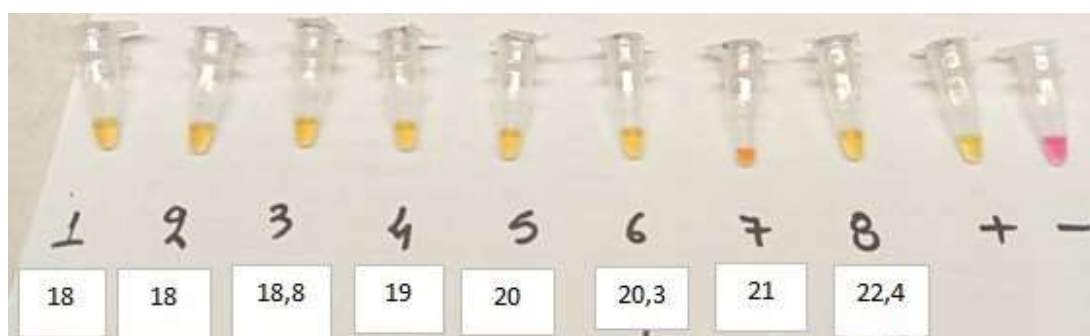
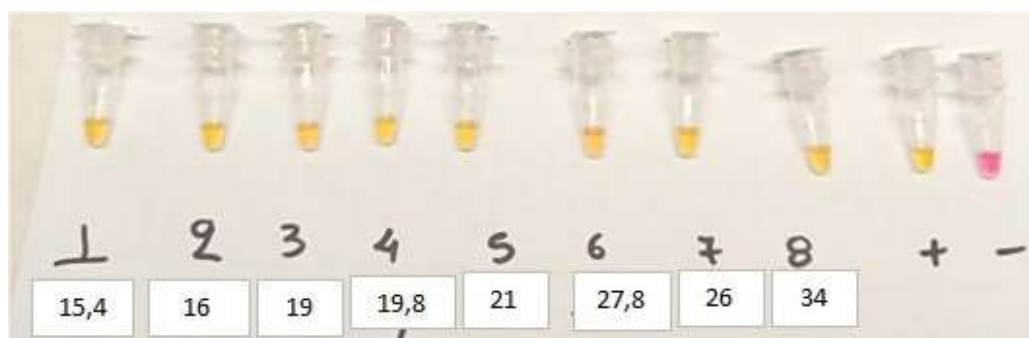
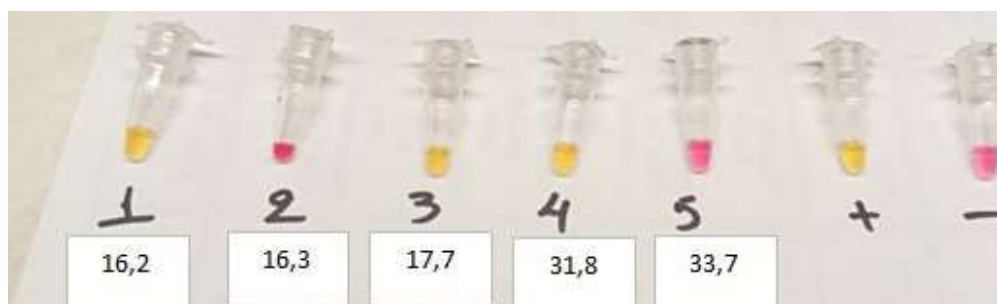
**Supplementary Figure S4. Sensitivity analysis of improved RT-LAMP assay.** SARS-CoV-2 RNA transcript and reaction mix used in each experiment are labelled on the top right white box of each image. '+' indicates a positive result while '-' indicates a negative result. A, B, C, D, E represents five technical replicates.

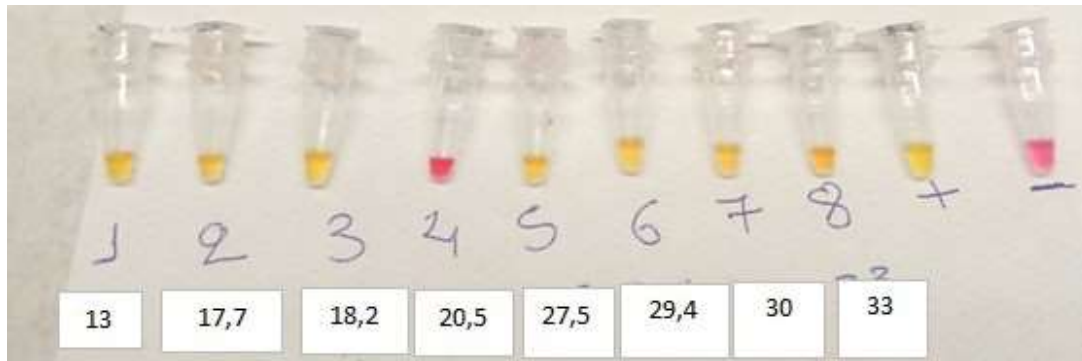
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**Supplementary Figure S5b. Detection of SARS-CoV-2 from 126 RNA extracts (116 positives and 10 negatives) by RT-LAMP in University Hospital of Alexandroupolis, Greece.** Performance of RT-LAMP assay using freeze drying reaction mix containing O117\_Q on detecting SARS-CoV-2 from RNA extract from saliva samples. PC: positive control and NC: negative control.



**Supplementary Figure S6. Two sets of primers (O117\_Q and S17) in one reaction enhanced detection sensitivity of SARS-CoV-2 virus in RT-LAMP assay.** Two sets of primers with the switch showed enhanced sensitivity and no false positive results, whilst those without the switch resulted in false positive results.

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## 5. Reprogramming Synthetic Biology Chasses for Targeted Cancer Therapy

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In this chapter, I develop a bacterial whole-cell biosensor (WCB) by expressing specific nanobody on the outer membrane of *E. coli* BL21 to target carcinoembryonic antigen (CEA), a cancer biomarker commonly used for colorectal cancer diagnosis and prognosis. The diagnostic sensitivity of this whole-cell biosensor to CEA is assessed in a biological agglutination assay which mimics the clinical agglutination test. To improvise for *in vivo* application, I engineer the WCB via two different top-down approaches, ICeul endonuclease expression and *minD* deletion, to generate synthetic biological chassis SimCell and minicell, respectively. These chassis can be functionalised by different synthetic circuits while remain genome-less, non-replicating and metabolically active, which hold enormous potential to help translate synthetic biological system into healthcare and clinical applications.

This chapter is presented in a manuscript submitted to *Communication Biology*.

# Reprogramming Synthetic Biology Chassis for Targeted Cancer Therapy

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Key words: synthetic biology, SimCells, bacterial therapy, chromosome-free, I-CeuI endonuclease, chassis, synthetic cell, artificial cell, minimal cell, drug delivery, catechol, cancer

## Abstract

The evidence of bacteria harboured within most cancers has prompted the application of bacteria therapy for cancer treatment. Advances in synthetic biology enable the reprogramming of bacteria as a safe and smart agent to specifically target tumour and locally release anti-cancer drugs in a highly controlled manner. However, the bench-to-bedside translation of engineered bacteria is often impeded by genetic instability and potential spread of living therapeutics inside the patient. Here we describe the reprogramming of minicells and chromosome-free SimCells for targeted cancer therapy. We engineer a surface display nanobody system to detect carcinoembryonic antigen (CEA), an important biomarker found commonly in colorectal cancer cells. We show that a chassis transformed with this system can achieve cancer cell specific binding *in vitro*. Without the addition of active cancer-killing circuit, these cancer-targeting minicells and SimCells can induce cancer cell death *in vitro* by compromising the plasma membrane of cancer cell. This cancer killing effect can be further enhanced from 19.3% cytotoxicity to 26.4% cytotoxicity by the addition of a synthetic circuit to deliver anticancer catechol to cancer cells. This work highlights the potential of minicells and SimCells for targeted cancer therapeutics and lays the foundation for synthetic biological therapy for other diseases.

## Introduction

This year marks the 130<sup>th</sup> anniversary of the first reported use of living bacteria in treating cancers<sup>1</sup>. At the Memorial Hospital in New York, a bone sarcoma surgeon, William B. Coley, used *Streptococcus pyogenes* and other bacterial products to treat inoperable bone and soft-tissue sarcomas. This method came under withering criticism from medical practitioners due to its inconsistency in therapeutic results, and the concurrent rise of radiotherapy and chemotherapy soon diminished the clinical application of bacterial therapy in cancer treatment<sup>2</sup>. Nevertheless, a century of advances in microbiology, immunology and cancer biology have shed new light on the underlying mechanisms used in Coley's method.

Recently, a rigorous and comprehensive survey of 1526 human tumour samples across seven tumour types showed that each tumour type harboured a diverse microbiome composition<sup>3</sup>. This evidence, together with the growth of synthetic biology in biotechnology, has stimulated the application of bacteria to cancer therapy (reviewed in<sup>4,5</sup>). Scientists can now engineer bacteria that have attenuated virulence<sup>6</sup>, high tumour specificity<sup>7</sup>, strict control of drug expression coupled with precise delivery (reviewed in<sup>8</sup>) to unleash the full potential of bacterial therapy in cancer treatment. A more recent example includes the development of a probiotic strain, *Escherichia coli* Nissle 1917 (EcN) to produce cyclic di-nucleotides, a Stimulator of INterferon Genes (STING) agonist, intratumorally to activate the antigen-presenting cells to produce type I interferons for subsequent antitumour immune response<sup>9</sup>.

Thus far, most of the bacteria-based strategies rely on the intrinsic ability of bacteria to colonise and proliferate at the tumour microenvironment, especially within the hypoxic and necrotic tumour core<sup>9-11</sup>. However, previous clinical trials showed that the prominent tumour colonisation effect observed in a mouse model does not always translate to another host, such as human and canine<sup>12,13</sup>. To enhance tumour targeting, specific motifs such as affibody and ligand are engineered onto the bacterial outer membrane to achieve cancer cell specific delivery, for example the expression of histone-like protein A (HlpA) on the surface of EcN can result in colorectal cancer cell specific attachment in a murine model with colorectal carcinoma<sup>14</sup>. Yet, there is no

clinically proven ligand-equipped therapeutic with specific cancer targeting activity, indicating the challenge of achieving specific tumour targeting in a clinical setting. On the other hand, not all cancer cell specific markers have a naturally available ligand that can be readily folded and expressed onto the bacterial surface. In addition, each bacterial cell division carries the risk of gene mutation which could default the targeted delivery and drug expression system through loss-of-function mutations <sup>15</sup>.

We aim to overcome these challenges using an engineering approach: we adopted a modular surface display system reported previously to express a cancer-cell targeting nanobody <sup>16</sup> on the outer membrane of engineered *Escherichia coli* BL21(DE3) and two chromosome-free synthetic cells. Specificity of this system can be modulated by raising the nanobody against a marker of interest through an immune library <sup>17</sup>. In this study, we used carcinoembryonic antigen (CEA), one of the most thoroughly studied tumour biomarkers in the cancer diagnostic and monitoring history, as our marker of interest <sup>18</sup>. We engineered a strain of *E. coli* that binds CEA in a clinical diagnostic setting which is then translated into *in vitro* specific binding of Caco2, a high CEA expressing colorectal cancer (CRC) cell line <sup>19</sup>. Next, we re-programmed two genome-free, non-replicating and metabolically active synthetic biology chassis: minicells<sup>20</sup> and SimCells<sup>21</sup>, using the CEA-binding machinery to develop a targeted cancer therapy (Fig. 1). We evaluated both engineered chassis as stand-alone cancer therapeutics *in vitro* with and without the additional synthetic circuit for anti-cancer compound (catechol) synthesis. Importantly, both chassis show their therapeutic potential without inherent biological risk which makes them ideal for further manipulation as a cancer therapy.

## Results

### A biological agglutination test confirms the binding of engineered *E. coli* to CEA

Surface displays of recombinant protein using bacterial outer membrane protein <sup>22</sup>, lipoprotein <sup>23</sup>, autotransporter protein <sup>24</sup> and ice nucleation protein<sup>25</sup> have successfully led to the development of multiple biotechnological applications. Nevertheless, the binding of *E. coli* to a target surface will require a surface display system that has high expression efficiency and precise protein folding without compromising cell viability.

In this study, we adopted a previously reported system using  $\beta$ -intimin domain as the anchor<sup>16,26–28</sup> to express nanobodies on the surface of *E. coli* which has shown specific binding to CEA.

We first designed two surface display systems: (i) pNV\_2 with low expression promoter J23105 and (ii) pNV\_3 with high expression promoter K1758100 (<http://parts.igem.org/Catalog>) (Fig. 2a) to accommodate the anti-CEA nanobody candidates, C17<sup>[29]</sup> and C43<sup>[30]</sup>. For control, we used an anti-spike protein nanobody, CYT<sup>[31]</sup>, which targets the receptor binding domain of the SARS-CoV-2 virus. Surface display of the nanobodies in *E. coli* BL21(DE3) and the difference in expression level of pNV\_2 and pNV\_3 was confirmed by flow cytometry using a primary anti-Myc antibody that binds to the Myc tag downstream of the nanobody (Fig. 2a) and a secondary Alexa Fluor 488 conjugated antibody (Fig. 2b). The nanobody expression of C17, C43 and CYT on the surface showed strong fluorescent signals compared to the control *E. coli* BL21(DE3).

To investigate CEA binding ability of the nanobodies, we employed a biological agglutination test which resembles the clinically used latex agglutination test<sup>27,28</sup>. Briefly, in the presence of target analyte/antigen, such as CEA in this case, surface-displayed nanobodies will cross-link with the target molecules resulting in agglutination through a bacteria-target-bacteria sandwich interaction. Without binding, bacteria will create a cloudy or clear suspension; when surface nanobodies bind with target analyte/antigen, bacteria will form cell pellets which can be visually examined in a round-bottom 96-well plate. We set up the biological agglutination test following a standard diagnostic hemagglutination assay: a series of concentrations of human CEA protein ranging from 1 nM to 200 nM were added to the same concentration of bacterial cells (100  $\mu$ L of OD<sub>600</sub> = 0.5) carrying the pNV plasmids in a round-bottom 96-well plate. For ease of visualisation, super folder green fluorescence protein (sfGFP) was cloned downstream to the nanobody in all pNV plasmids. After overnight incubation, cell agglutination was observed in cultures carrying pNVC17 and pNVC43 within the range of 5 to 200 nM of CEA in pNV\_2 and 10 to 200 nM of CEA in pNV\_3 system, whilst none of the pNVCYT controls showed any agglutination (Fig. 2c). This result demonstrates the binding capacity of engineered *E. coli* surface-displaying C17 and C43

nanobody to human CEA protein. Consistent with the previously reported model, modifying the surface expression level of nanobody will shift the equivalence zone of the assay which results in different diagnostic sensitivity<sup>27,28</sup>. For all *in vitro* cancer cell culture experiments, the pNV\_3 system fused with sfGFP was chosen (named pNV\_sfGFP from here onwards) to increase the probability of binding and a sandwich assay is not required for the binding of bacteria to targeted cancer cells expressing CEA.

### **Engineered *E. coli* specifically binds to CEA-expressing colorectal cancer cells in vitro**

We tested the *in vitro* binding efficacy of engineered *E. coli* to two colorectal cancer cell lines with different CEA expression strengths: (i) Caco2 with high CEA expression level and (ii) SW480 with low CEA expression level<sup>19</sup>.

For proof-of-concept experiments, a monolayer of both cancer cell lines, Caco2 or SW80 was incubated with engineered *E. coli* (multiplicity of infection, MOI 300:1 determined by incubating  $1.5 \times 10^7$  CFU of bacterial cells in a well seeded with 50,000 cancer cells) up to 8 hours in 24-well plates (Fig. 3 and Supplementary Fig. S1-S3). All wells were washed three times with fresh media to reduce non-specific binding prior to microscopic imaging. All engineered *E. coli* also expressed strong GFP, enabling the visualisation of the bacteria on cancer cells. The results show that *E. coli* pNVC17\_sfGFP and pNVC43\_sfGFP specifically bound to the high CEA-expressing colorectal cancer cell line Caco2 throughout the incubation period (Fig. 3 and Supplementary Fig. S1-S3). However, the control strains expressing a non-targeting nanobody (pNVCYT\_sfGFP) or without a nanobody (proD\_sfGFP) did not bind to Caco2 even with a prolonged incubation of up to 8 hours (Fig. 3 and Supplementary Fig. S1-S3). None of the engineered *E. coli* strains showed any binding to the low CEA expressing cell line SW480 (Fig. 3 and Supplementary Fig. S1-S3). The results demonstrate that specific binding of engineered *E. coli* to targeted Caco2 cells was determined by the nanobodies displayed on the surface. Testing of the knockdown of such binding upon inclusion of competitive concentrations of anti-CEA antibody or following siRNA treatment of Caco2 cells would fully verify the selectivity of the binding event.

## Purification and engineering of minicells to target colorectal cancer cells

Along with *minC* and *minE* proteins, *minD* plays a crucial role in bacterial cell division by confining the division septum at mid-cell region<sup>32</sup>. A common strategy to generate a chromosome-deficient minicells is to knock out the *minD* gene to create an aberrant cell division, in which minicells can then be purified through sequential centrifugation<sup>33</sup>. To generate minicells, we first created the *minD* deletion *E. coli* BL21(DE3) mutant using the lambda Red recombineering system<sup>34</sup>. The successful mutant was confirmed via colony PCR (Supplementary Fig. S4a) and observation of minicell production (Fig. 4a). We then transformed the  $\Delta minD$  strain with pNVC17\_sfGFP for nanobody surface display. Once the culture reached their late log phase ( $OD_{600} = 0.6$ ), we removed the parental cells by centrifuging at 2,000 g followed by the addition of 100  $\mu\text{g/mL}$  of ceftriaxone<sup>35</sup>, cefotaxime<sup>36</sup> and penicillin G<sup>37</sup>. These antibiotics exerted their bactericidal action through the inhibition of peptidoglycan cell wall synthesis, resulting in the cell lysis of dividing parental cells while the non-dividing minicells remained intact. This has substantially improved the yield and purity of minicells. Fig. 4a shows the highly purified minicells after the purification steps. Overnight  $OD_{600}$  reading and agar plate incubation of the purified minicells showed no further growth (Supplementary Fig. S4b, c), indicating that all parent cells have been removed.

To investigate the binding efficacy of engineered minicells, we repeated the *in vitro* cancer cells binding experiment with minicells. As shown in Fig. 4b and Supplementary Fig. S5, specific binding can only be achieved between the high-CEA expressing Caco2 and minicells engineered with pNVC17\_sfGFP. No adhesion was found on the low-CEA expressing SW480 or minicells transformed with unspecific nanobody pNVCYT\_sfGFP. These results indicate that minicells expressing C17 nanobody can also bind to cancer cells with CEA with high specificity.

## Generation and re-programming of SimCells to target cancer cells

We recently reported the production and characterisation of an ICEuI endonuclease-induced, chromosome-free SimCell as a programmable synthetic biology chassis<sup>21</sup>. The generation of SimCell is based upon the recognition of ICEuI endonuclease on a defined 26-bp sequence ubiquitously found in most bacterial genomes that leads to a RecBCD-

initiated double strand break<sup>38</sup>. We obtained an optimised version of plasmid, pYB1 to produce SimCells which shows superior performance compared to the previously reported TetR monitored pJKR-L-TetR system<sup>21</sup>. pYB1 showed no visible basal expression and upon induction, a complete halt of growth can be achieved within 90 minutes, indicating high magnitude of SimCell generation (Fig. 5a).

To reprogramme SimCells for cancer cell targeting, we transformed both pYB1 and pNVC43\_sfGFP plasmids into *E. coli* BL21(DE3). Once the culture reached their early log phase ( $OD_{600} = 0.3$ ), we induced the conversion of SimCell pNVC43\_sfGFP/pYB1 into a high purity, non-dividing SimCells culture (see *Methods*). Overnight agar plate and  $OD_{600}$  reading showed the lack of growth of SimCells compared to their uninduced counterpart (Supplementary Fig. S6).

We then assessed the binding capacity of programmed SimCells to targeted cancer cells (MOI 300:1 determined by incubating 0.5 mL of SimCells at  $OD_{600} = 0.3$  in a well seeded with 50,000 cancer cells; An  $OD_{600} = 0.3$  represents  $3 \times 10^7$  CFU/mL by plating BL21(DE3) on LB agar with respective antibiotic). As shown in Fig. 5b and Supplementary Fig. S7-S9, adhesion of high CEA expressing Caco2 was achieved by both induced and uninduced SimCells carrying the pNVC43\_sfGFP plasmid within 2 hours of incubation. It is unclear why positive staining is confined to a single Caco2 cell and not its neighbours, but this may reflect the division, viability or CEA status of the cell. For controls, no binding was observed on the low CEA expressing SW480. Similarly, induced and uninduced SimCells carrying the pNVCYT\_sfGFP plasmid did not bind to any of the colorectal cancer cells, which shows the specificity of re-programmed SimCells to targeted cancer cell line.

### **Binding of re-programmed minicells and SimCells can induce targeted cancer cell death in vitro**

Previous attempts to treat cancers using bacterial therapy relied on the colonisation and proliferation of bacteria at the tumour site, followed by the expression of different

therapeutic payloads such as immunomodulators <sup>9</sup> and enzymes <sup>14</sup> to enhance the anti-cancer property. As minicells and SimCells were re-programmed to bind directly onto specific cancer cells, we investigated the cytotoxic effect of this interaction to assess the potential of minicells and SimCells as stand-alone therapeutics without any additional anti-cancer circuits.

We first incubated Caco2 with minicells and SimCells for 2 hours, followed by three-times washing to remove any unbound bacteria. The Caco2 culture was then incubated at ideal conditions until a specific time-point to run the cytotoxicity assay. As shown in Fig. 6a, minicells and SimCells carrying pNVC17\_sfGFP and pNVC43\_sfGFP, respectively can induce cancer cell killing effect from 8 hours of incubation after the removal of unbound SimCells, with a higher cytotoxicity effect observed in SimCell at 24 hours (18.3% cancer cell damage) and 48 hours (19.3% cancer cell damage) of incubation. In contrast, no cytotoxicity was observed in non-specific minicells and SimCells (pNVCYT\_sfGFP) post-washing despite the initial 2-hour incubation. Despite the relatively low cytotoxicity effect, this result suggests that the specific binding of minicells and SimCells can exert their cancer-killing effect gradually without any additional active cancer-killing circuits.

To further examine the killing mechanism, we supplemented the Caco2 culture incubated with minicells and SimCells with ethidium homodimer, a membrane-impermeable nucleic acid dye that is commonly used for dead-cell staining <sup>39</sup>, and subjected the culture to time-lapse fluorescence microscopy after 2 hours of incubation followed by three-times washing (Fig. 6b and Supplementary Movie S10). Caco2 bound by targeted minicells (pNVC17\_sfGFP) and SimCells (pNVC43\_sfGFP) starts showing red fluorescence from 2 hours of microscopic imaging post-washing, indicating a compromised cancer cell membrane due to minicells and SimCells binding. In contrast, incubation with non-specific minicells and SimCells does not generate substantial membrane damage on the cancer cell within 5 hours post-washing, which confirms that the cytotoxicity effect is exerted by specific binding of minicells and SimCells. As shown at T = 5h, minicells and SimCells are unbound from the dead cancer cells and dissipate to their surrounding. This result potentially reflects the gradual cell-damaging effect seen in Fig. 6a, in which the increase in cytotoxicity from 8h to 24h of incubation

could be a result of bystander effect caused by minicells and SimCells binding from one cancer cell to another. However, it is important to note that microscopic imaging was carried out in less than ideal conditions (no temperature and CO<sub>2</sub> control), which accounts for the shorter time scale (5 hours in the microscopy experiment compared to 8 hours in the cytotoxicity assay) for cancer cell killing.

As a proof-of-concept to enhance its anticancer property, we cloned *sala*, the gene encodes for salicylate hydroxylase<sup>21,40</sup>, upstream of *sfGFP* in the pNVC43\_sfGFP plasmid. SalA and indigenous esterase of *E. coli* can convert salicylate/aspirin into catechol, which has been previously shown to exert apoptotic effect towards selective cancer cell lines<sup>21,40-42</sup>. Using similar protocol, we built SimCells pNVC43\_SalA\_sfGFP/pYB1 and repeated the *in vitro* cancer killing experiments with the addition of 500  $\mu$ M salicylic acid. As shown in Fig. 6c, the addition of salicylic acid improves the cancer killing effect of engineered SimCells compared to SimCells binding alone. No cytotoxic effect was observed in cancer cell cultures contained only salicylic acid, including when it was pre-incubated with SimCells pNVCYT\_SalA\_sfGFP for 2 hours. Despite further improvement will be necessary for its clinical relevance, these results still demonstrate the therapeutic potential of targeted minicells and SimCells *in vitro*, and with further engineering these chassis will possess enormous advantages over conventional bacterial therapy with their high specificity and biological safeness.

## Discussion

An important lesson from past clinical trials using living bacteria for cancer treatment is that their intrinsic ability to colonise the tumour microenvironment does not always translate from model organisms to human (reviewed in<sup>43</sup>). This gives rise to another important question: in the cases where tumour regression was observed, is the colonisation and proliferation of bacteria at the tumour microenvironment accountable for the therapeutic effect, or is it a mere bacteria-incited cytokine storm? To realise the potential of bacterial cancer therapy, the ability of an engineered cancer cell to target cancer cells with high specificity is a prerequisite. In addition, one of the biggest hurdles to translate living therapeutics for clinical use is the regulatory considerations in deploying replication-competent bacteria in patients.<sup>43</sup> In this study, we addressed both

concerns by engineering the non-replicating synthetic biology chassis, minicells and SimCells, to develop a targeted cancer therapeutic.

We first described a CEA diagnostic system using engineered *E. coli* surface-displaying anti-CEA nanobodies in a biological agglutination test setting. In contrast to a conventional bacterial biosensing system which requires active production of signal protein such as GFP or Lux protein, this simple platform allows the detection of a cancer biomarker through the physical binding between engineered bacteria and target analyte. Further modulation on the sensitivity of the test is possible through the tuning of expression strength of anti-CEA nanobody on the bacterial surface. Previous reports also showed the usability of the biological agglutination test on more complex samples such as urine and blood.<sup>27,28</sup> Ideally, the CEA diagnostic system should be built to reach a sensitivity of 2.2 ng/mL (~12 pM) to have clinical significance.<sup>44</sup> Nevertheless, to quantitatively validate the binding of engineered *E. coli* to CEA, flow cytometry should be used to test the labelled CEA antigen along with the anti-myc tagged surface-expressed nanobodies.

We showed the modularity of the pNV system in conferring cell binding specificity to engineered bacteria. With simple molecular cloning steps, we could modify the nanobody sequence to expand the cell binding repertoire. As shown in the *in vitro* cancer cell binding experiment, anti-CEA nanobodies C17 and C43 can be used interchangeably without affecting the binding capacity of engineered bacteria. To further enhance the binding strength or to identify a new target of interest, we could adopt a phage surface display library or induce an immune response in llama to generate new nanobody variants.<sup>45,46</sup> However, a better control such as a complete CEA gene knock-out of the Caco2 cell line should be tested to exclude non-specific binding of the engineered bacteria. As the spatiotemporal expression of CEA varies during tumorigenesis<sup>47,48</sup>, the *in vitro* cancer cell binding experiment would also benefit from including more fields-of-view of the same experimental well, increasing the number of biological repeats and experimenting against different cell lines that also express CEA.

Recent advances in synthetic biology have promised controllable, tumour specific and user-defined therapeutic payloads for bacterial cancer therapy (reviewed in <sup>4,5,8,43,49</sup>).

While the collection of synthetic biology circuits (such as quorum lysis<sup>50</sup> and genetic kill switches<sup>51</sup>) for *in vivo* therapeutic applications continues to grow, the genetic stability and spreading of living therapeutics within a patient remain one of the biggest concerns to regulatory bodies.<sup>49,52</sup> With minicells and SimCells, we remove the growth aspect of the living bacteria and thereby the inherent risk of gene mutation that comes with every doubling cycle. In addition to the plating experiment (Fig. S4c and Fig. S6b), the purity of minicells and SimCells should be further validated through quantifiable methods, for example primers can be designed to bind at multiple positions within the bacterial genome, and the action of ICEuI endonuclease and minicell formation should result in the loss of product when the culture is subjected to a PCR reaction. Another key measurement that is missing in this manuscript is the quantification of minicell, which could potentially be calibrated by measuring the amount of plasmid in a minicell culture through qPCR.

We showed the capability of both minicells and SimCells as stand-alone anti-cancer agents through *in vitro* experiments, and by combining a user-defined therapeutic circuit, in this case the *salA* gene and salicylic acid, SimCells can exert a higher cytotoxic effect to achieve targeted cancer cell killing. The uptake of salicylate into a bacterial cell is likely to involve the non-specific porins at the outer membrane, where a small compound within 600 Da could permeate through the outer membrane with negligible interaction.<sup>53</sup> Previous attempt to construct an aspirin biosensor also proved that aspirin can be detected intracellularly by *E. coli*<sup>54</sup>, but the conversion of salicylate to catechol, which is brown when exposed to air and light<sup>21</sup>, should be quantified prior to the *in vitro* cancer cell killing experiment to measure the catechol conversion rate of SimCells. On the other hand, alternative methods should be used to quantify live/dead cells on top of the LDH assay to better determine the percentage of cells undergoing different pathways of cell death.

As the binding and cancer killing mechanism can be delivered through independent genetic circuits, these chassis can be further customised and expanded to target other diseases. More importantly, the circuits dictating the formation of minicells and SimCells can be transformed into a probiotic (e.g. *E. coli* Nissle 1917<sup>[ref 9,14]</sup>) or attenuated strains to reduce the pathogenicity of resulting minicells and SimCells. The

modularity, scalability and reliability of these chassis can accentuate the impact of synthetic biology in the medical field, and we envision the deployment of minicells and SimCells to unlock this new class of bacterial therapeutics.

Nevertheless, it is important to recognise that the success of bacterial cancer therapy relies on striking the perfect balance between safety and therapeutic benefit. Even though the attenuation of bacteria through minicell or SimCell conversion could potentially address part of the safety issue, the therapeutic effect of such therapy could only be achieved if the constructs reached their target sites without immune clearance upon administration. For example, the innate immune system, which is mediated by the germline encoded pattern-recognition receptors (PRRs), does not differentiate between pathogenic microorganisms and symbiotic microorganisms.<sup>55</sup> Meanwhile, if the patient was previously infected with a similar pathogen, their adaptive immune system might neutralise the constructs with circulating antibodies. This challenge could potentially be overcome by administering the constructs intratumorally, but this will inevitably reduce the overall usability of a user-designed, synthetic live therapeutic. It would thus be crucial to demonstrate the practicality of minicells and SimCells therapeutics in an *in vivo* experiment on tumour-bearing mice to find the balance between their safety and effectiveness.

## **Methods**

### **Bacterial strains, plasmids, primers and routine cell growth conditions**

A list of bacterial strains and plasmids used in this study is listed in Table S1. In general, *Escherichia coli* DH5 $\alpha$  was used for routine cloning and plasmid maintenance while *E. coli* BL21(DE3) was used for the rest of the study unless stated otherwise.

Primers and gene blocks used in this study are listed in Table S2. All primers and gene blocks were ordered from Sigma Aldrich. Parts and backbones were amplified using Q5 High Fidelity DNA Polymerase (New England Biolabs, NEB) and plasmids were constructed using NEBuilder Hifi DNA Assembly (NEB).

For all routine bacterial cell growth and strain selection, bacteria were grown in Luria–Bertani (LB) media (with agitation, 250 rpm) or plated on LB agar (static) with corresponding antibiotics: kanamycin (Kan, 50 µg/mL), carbenicillin (Carb, 100 µg/mL) or chloramphenicol (Cm, 25 µg/mL) and incubated at 37 °C, for 16 hours.

All strains were transformed via heat-shock unless stated otherwise. Chemically competent cells and transformation were conducted according to methods described previously.<sup>56,57</sup> For strains containing double plasmid, transformation was done sequentially by first making the single-plasmid strain chemically competent again.

### **Cancer cell lines and routine cell growth conditions**

Human colorectal cancer cell lines Caco2 and SW480 were kindly gifted by Professor Adrian Harris from the Department of Oncology, University of Oxford. Both cell lines were routinely grown as a monolayer in fresh media (Dulbecco's modified Eagle's medium (DMEM, high glucose, pyruvate) supplemented with 10% fetal bovine serum (FBS) and Penicillin-Streptomycin solution (100 U/mL)) at 37 °C with 5% CO<sub>2</sub>. Cells were generally grown to 70% confluency before sub-culturing or transfer to 24-well plate or 96-well plate.

### **Quantification of bacterial surface expressing nanobody using flow cytometry**

Overnight BL21(DE3) culture carrying pNV\_2 or pNV\_3 plasmid (5 mL) was centrifuge at 4 °C, 2,000 g for 5 minutes. 1 mL non-fat milk blocking buffer (1 %) in PBS was added to resuspend the cell pellet. Culture was incubated at room temperature for 1 hour. 1.5 mL of the culture was transferred to an Eppendorf tube and were centrifuged at 4,000 g for 2 minutes. The pellet was washed twice and resuspended in 1 mL of PBS. 2 µL of primary anti-Myc antibody (Abcam, ab9106) was added to the 1 mL cell suspension and incubated at room temperature for 1.5 hours. The suspension was centrifuge at 1,000 g for 5 minutes and washed twice with 1 mL of PBS. The pellet was resuspended in 500 µL of PBS and added with 0.5 µL of secondary Alexa Fluor 488 conjugated antibody (Abcam, ab150077). Culture was incubated at room temperature for 1 hour. Finally, the culture was centrifuged at 1,000 g for 5 minutes, washed twice and resuspended in 1 mL of PBS. Flow cytometry was done using FACS Calibur (BD

Biosciences) and analysed using CellQuest. The FL1 filter used to detect Alexa Fluor 488 has an excitation/emission at wavelength 488/530 nm.

### **Biological agglutination test**

Overnight culture carrying pNV\_2\_sfGFP or pNV\_3\_sfGFP plasmid was diluted in PBS to OD<sub>600</sub> = 0.5. 100 µL of the diluted culture was transferred to a clear, round-bottom 96-well plate, supplemented with a series of concentration of human CEACAM5 protein (SinoBiological) ranging from 1 nM to 200 nM (volume added 0.5 µL to 2.5 µL). The plate was incubated statically at room temperature overnight before a top-view image was taken with VersaDoc Imaging system (Bio-rad) under FITC channel.

### **Generation of BL21(DE3) ΔminD**

Kanamycin resistance gene cassette (Kan<sup>R</sup> cassette) was amplified using Q5 High Fidelity DNA Polymerase (NEB) using primers minD-HR-Kan-for and minD-HR-Kan-rev, followed by PCR purification using QIAquick PCR purification kit (QIAGEN).

All cloning and plasmid maintenance of pSIJ8 were done at 30 °C due to its temperature sensitivity. Overnight BL21(DE3) culture transformed with pSIJ8 was sub-cultured 1:100 in 150 mL fresh LB-Carb media and incubated at 30 °C, 250 rpm for 3 hours. Arabinose (15 mM) was added to induce the recombination machinery (*exo*, *bet* and *gam*) and incubated for a further 45 minutes at 30 °C, 250 rpm. The bacterial culture was then made electrocompetent following methods described previously.<sup>58</sup>

Kan<sup>R</sup> cassette was transformed into BL21 pSIJ8 via electroporation: 50 µL of electrocompetent BL21 pSIJ8 was added with ~250 ng Kan<sup>R</sup> cassette (<2 µL) and mixed by pipetting up and down. The mixture was transferred into a 0.2 cm electroporation cuvette (Bio-rad) and pulsed at 2.5 kV using Cellject Uno (ThermoFisher Scientific). 1 mL of fresh LB was added immediately into the cuvette and mixed by pipetting up and down. The mixture was transferred to an Eppendorf tube and incubated at 30 °C, 250 rpm for 4 hours. The mixture was centrifuged at 4,500 g for 10 minutes and the supernatant was removed until ~100 µL left in the Eppendorf tube. Cell pellet was

resuspended in the left-over media and plated on LB-Carb-Kan agar at 30 °C, overnight. On the next day, colony PCR was done using primers HR-check-for and HR-check-rev for success recombination. Colony was inoculated in LB-Kan and incubated at 37 °C, 250 rpm, overnight to cure the pSIJ8 plasmid and make BL21  $\Delta minD::Kan^R$  culture. This culture was subsequently made chemically competent to be transformed with the pNV plasmids.

### **Purification of minicells**

Overnight BL21  $\Delta minD$  culture was diluted 1:100 in 300 mL of LB-Kan-Cm and incubated at 37 °C, 250 rpm for 3~4 hours until  $OD_{600} = 0.6$ . Culture was centrifuged at 2,000 g for 10 minutes at 4 °C and the supernatant was retained for further centrifugation at 12,000 g for 15 minutes at 4 °C. The pellet was resuspended in 1 mL of fresh LB, pooled together, and incubated at 37 °C, 250 rpm for 45 minutes. Then, ceftriaxone (100 µg/mL), penicillin G (100 µg/mL) and cefotaxime (100 µg/mL) were added to the culture and further incubated at 37 °C, 250 rpm for 2 hours. The culture was first centrifuged at 500 g for 10 minutes at 4 °C to remove cell debris. The supernatant was retained and further centrifuged at 12,000 g for 15 minutes at 4 °C. The final pellet was resuspended in 1 mL of PBS and stored at 4 °C until further use. This purified culture is the minicells.

5 µL of pre-purified and post-purified culture were plated on LB-Cm-Kan agar plate and added into 195 µL of LB-Cm-Kan media in a 96-well plate, incubated overnight at 37 °C to collect  $OD_{600}$  readings.

### **Generation of genome-less SimCells**

To characterise SimCell conversion, *E. coli* K12 MDS42 was transformed with pJKR-L-TetR-ICEuI (TetR controlled system) and compared to SimCell pYB1 (obtained from Oxford SimCell Ltd.). Overnight cultures were diluted 1:100 in 200 µL of LB-Kan and transferred into a flat 96-well plate. The plate was sealed with Breath Easy sealing membrane and was incubated in the Synergy 2 microplate reader (BioTek) at 37 °C with constant orbital shaking at 1,000 rpm.  $OD_{600}$  measurements were taken every 15 minutes for 18 hours. At the 4<sup>th</sup> hour, 0.2 µL of inducer cocktail X (obtained from Oxford

SimCell Ltd.) and anhydrotetracycline (final concentration 1  $\mu$ M) were added into SimCell pYB1 and *E. coli* pJKR-L-TetR-ICeuI culture, respectively.

To re-programme SimCells to surface-display nanobody, overnight culture of SimCell pYB1 (Oxford SimCell Ltd, UK) transformed with pNVC43\_sfGFP or pNVCYT\_sfGFP plasmid was diluted 1:100 in LB-Cm-Kan (50 mL) and incubated at 37 °C, 250 rpm for 3 hours until OD<sub>600</sub> of ~0.3. The culture was split into half: 25 mL of ‘Uninduced’ culture was rested at 4 °C until further use; 25 mL of ‘Induced’ culture was added with 25  $\mu$ L of inducer cocktail compound A (Oxford SimCell Ltd, UK) to induce the conversion of SimCells. ‘Induced’ culture was incubated at 37 °C, 250 rpm for another 2 hours before storing at 4 °C until further use. This ‘Induced’ culture is the genome-less SimCells.

5  $\mu$ L of ‘Uninduced’ and ‘Induced’ culture were plated on LB-Cm-Kan agar plate and added into 195  $\mu$ L of LB-Cm-Kan media in a 96-well plate, incubated overnight at 37 °C to collect OD<sub>600</sub> readings using plate reader (BioTek Synergy 2).

### ***In vitro* cancer binding**

Cancer cells were seeded in 24-well tissue culture plate (~50,000 cells/well) with 0.5 mL fresh media and grown at 37 °C with 5% CO<sub>2</sub> to 70% confluency.

For BL21(DE3) strains, overnight bacteria culture was diluted to OD<sub>600</sub> = 0.3 (~3 x 10<sup>7</sup> CFU/mL by plating on LB agar with corresponding antibiotic) in PBS and centrifuged at 5,000 g for 5 minutes. After removing the supernatant, cell pellet was resuspended in fresh media supplemented with Hoechst dye (0.5  $\mu$ M) at the same volume (i.e., 10 mL diluted bacteria culture in PBS resuspended into 10 mL of supplemented media after centrifugation and supernatant removal). For SimCell strains, ‘Induced’ culture was centrifuged at 5,000 g for 5 minutes and followed by similar steps as the BL21(DE3) strains. For minicell strains, Hoechst dye (0.5  $\mu$ M) was added into purified minicells in PBS directly.

Media for cancer cell culture in 24-well plate was removed and washed once with fresh media. For an infection at MOI of 300:1, 0.5 mL of BL21(DE3) at OD<sub>600</sub> = 0.3 (~1.5 x

$10^7$  CFU/mL according to previous plating), SimCells or minicells were added into each well seeded with ~50,000 cancer cells/well for incubation at 37 °C with 5% CO<sub>2</sub> for 0, 2, 4 and 8 hours. For time point 0, 0.5 mL fresh media supplemented with Hoechst dye (0.5 µM) was added and incubated for 15 minutes prior to the addition of bacteria. At each timepoint, each well was washed thrice and added with 0.5 mL of fresh media before subjecting to fluorescence microscope imaging under 10x magnification using Eclipse Ti fluorescence microscope (Nikon). Fields were viewed under brightfield or fluorescent illuminance (excitation/emission for sfGFP: 488/510 nm; Hoechst dye: 361/497 nm) at 10x or 20x objective.

### ***In vitro* cancer killing**

For plate-reading experiment, Caco2 cells were first seeded in 96-well tissue culture plate (~10,000 cells/well) with 100 µL of fresh media and grown at 37 °C with 5% CO<sub>2</sub> to 70% confluency. For an infection at MOI of 300:1, minicells and SimCells were prepared as described in the previous section, and 100 µL was added into each well for incubation at 37 °C with 5% CO<sub>2</sub> for 2 hours. After 2 hours incubation, each well was washed thrice and added with 100 µL of fresh media, followed by further incubation at 37 °C with 5% CO<sub>2</sub>. For experiments using SimCells pNVC43\_SalA\_sfGFP, 100 µL of fresh media supplemented with salicylic acid (final concentration 500 µM) was added after the washing step. At T=8h, 24h and 48h (and T=72h and 96h for experiments using SimCells pNVC43\_SalA\_sfGFP), cytotoxicity assay was carried out using LDH Cytotoxicity Assay Kit (Abcam) following manufacturer's instruction. Briefly, 5 µL of supernatant from each well was added into 95 µL of LDH reaction mix in another white, flat-bottom 96-well plate, and shake gently at room temperature for 10 minutes. Fluorescence (excitation/emission: 535/587 nm) was measured using a plate-reader (SpectraMax i3x). The percentage cytotoxicity is calculated using the formula below:

$$Cytotoxicity \% = \left( \frac{Fluo_{Test\ Sample} - Fluo_{Negative\ Control}}{Fluo_{Lysate\ Control} - Fluo_{Negative\ Control}} \right) \times 100$$

Cell culture was returned to 37 °C, 5% CO<sub>2</sub> incubation after each time point. For time-lapse experiment, Caco2 cells were first seeded in 24-well tissue culture plate (~50,000 cells/well) with 0.5 mL of fresh media and grown at 37 °C with 5% CO<sub>2</sub> to 70%

confluency. Minicells and SimCells were prepared as described in the previous section, and 0.5 mL was added into each well for incubation at 37 °C with 5% CO<sub>2</sub> for 2 hours. After 2 hours incubation, each well was washed thrice and added with 0.5 mL of fresh media supplemented with Ethidium Homodimer (4 µM). Cultures were subjected to fluorescence microscope imaging under 10x magnification using Eclipse Ti fluorescence microscope (Nikon) for 5 hours with an interval of 5 minutes. Fields were viewed under brightfield for T=0 and fluorescent illuminance (excitation/emission for sfGFP: 488/510 nm; Ethidium homodimer: 528/617 nm) for the time-lapse.

### **Author contributions**

W.E.H conceived original idea; B.L. and W.E.H designed research; B.L. and Y.Y. performed research; H.Y., Z.C. and A.P. contributed experimental tools and ideas; B.L. and Y.Y. analysed data; B.L. drafted the manuscript; all authors reviewed and revised the manuscript.

Data Availability Statement: All data discussed in the paper will be made available to readers.

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## References

1. Coley, W. B. II. Contribution to the Knowledge of Sarcoma. *Ann. Surg.* **14**, 199–220 (1891).
2. McCarthy, E. F. The Toxins of William B. Coley and the Treatment of Bone and Soft-Tissue Sarcomas. *Iowa Orthop. J.* **26**, 154–158 (2006).
3. Nejman, D. *et al.* The human tumor microbiome is composed of tumor type–specific intracellular bacteria. *Science* **368**, 973–980 (2020).
4. Duong, M. T.-Q., Qin, Y., You, S.-H. & Min, J.-J. Bacteria-cancer interactions: bacteria-based cancer therapy. *Exp. Mol. Med.* **51**, 1–15 (2019).
5. Sedighi, M. *et al.* Therapeutic bacteria to combat cancer; current advances, challenges, and opportunities. *Cancer Med.* **8**, 3167–3181 (2019).
6. Ozdemir, T., Fedorec, A. J. H., Danino, T. & Barnes, C. P. Synthetic Biology and Engineered Live Biotherapeutics: Toward Increasing System Complexity. *Cell Syst.* **7**, 5–16 (2018).
7. Park, S.-H. *et al.* RGD Peptide Cell-Surface Display Enhances the Targeting and Therapeutic Efficacy of Attenuated Salmonella-mediated Cancer Therapy. *Theranostics* **6**, 1672–1682 (2016).
8. Chien, T., Doshi, A. & Danino, T. Advances in bacterial cancer therapies using synthetic biology. *Curr. Opin. Syst. Biol.* **5**, 1–8 (2017).
9. Leventhal, D. S. *et al.* Immunotherapy with engineered bacteria by targeting the STING pathway for anti-tumor immunity. *Nat. Commun.* **11**, 2739 (2020).
10. Zhang, Y. *et al.* Escherichia coli Nissle 1917 Targets and Restrains Mouse B16 Melanoma and 4T1 Breast Tumors through Expression of Azurin Protein. *Appl. Environ. Microbiol.* **78**, 7603–7610 (2012).
11. Zheng, J. H. *et al.* Two-step enhanced cancer immunotherapy with engineered Salmonella typhimurium secreting heterologous flagellin. *Sci. Transl. Med.* **9**, (2017).
12. Thamm, D. H. Systemic Administration of an Attenuated, Tumor-Targeting Salmonella typhimurium to Dogs with Spontaneous Neoplasia: Phase I Evaluation. *Clin. Cancer Res.* **11**, 4827–4834 (2005).
13. Toso, J. F. *et al.* Phase I Study of the Intravenous Administration of Attenuated Salmonella typhimurium to Patients With Metastatic Melanoma. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **20**, 142–152 (2002).
14. Ho, C. L. *et al.* Engineered commensal microbes for diet-mediated colorectal-cancer chemoprevention. *Nat. Biomed. Eng.* **2**, 27–37 (2018).
15. Sleight, S. C. & Sauro, H. M. Visualization of Evolutionary Stability Dynamics and Competitive Fitness of Escherichia coli Engineered with Randomized Multigene Circuits. *ACS Synth. Biol.* **2**, 519–528 (2013).

16. Piñero-Lambea, C. *et al.* Programming Controlled Adhesion of *E. coli* to Target Surfaces, Cells, and Tumors with Synthetic Adhesins. *ACS Synth. Biol.* **4**, 463–473 (2015).
17. Muyldermans, S. A guide to: generation and design of nanobodies. *FEBS J.* **288**, 2084–2102 (2021).
18. Tong, G. *et al.* The role of tissue and serum carcinoembryonic antigen in stages I to III of colorectal cancer—A retrospective cohort study. *Cancer Med.* **7**, 5327–5338 (2018).
19. Rama, A. R. *et al.* Specific Colon Cancer Cell Cytotoxicity Induced by Bacteriophage E Gene Expression under Transcriptional Control of Carcinoembryonic Antigen Promoter. *Int. J. Mol. Sci.* **16**, 12601–12615 (2015).
20. Rampley, C. P. N. *et al.* Development of SimCells as a novel chassis for functional biosensors. *Sci. Rep.* **7**, 7261 (2017).
21. Fan, C. *et al.* Chromosome-free bacterial cells are safe and programmable platforms for synthetic biology. *Proc. Natl. Acad. Sci.* **117**, 6752–6761 (2020).
22. Lång, H. Outer membrane proteins as surface display systems. *Int. J. Med. Microbiol.* **290**, 579–585 (2000).
23. van Bloois, E., Winter, R. T., Kolmar, H. & Fraaije, M. W. Decorating microbes: surface display of proteins on *Escherichia coli*. *Trends Biotechnol.* **29**, 79–86 (2011).
24. Detzel, C., Maas, R., Tubeleviciute, A. & Jose, J. Autodisplay of nitrilase from *Klebsiella pneumoniae* and whole-cell degradation of oxynil herbicides and related compounds. *Appl. Microbiol. Biotechnol.* **97**, 4887–4896 (2013).
25. Li, L. *et al.* A selective and sensitive d-xylose electrochemical biosensor based on xylose dehydrogenase displayed on the surface of bacteria and multi-walled carbon nanotubes modified electrode. *Biosens. Bioelectron.* **33**, 100–105 (2012).
26. Salema, V. *et al.* Selection of Single Domain Antibodies from Immune Libraries Displayed on the Surface of *E. coli* Cells with Two  $\beta$ -Domains of Opposite Topologies. *PLOS ONE* **8**, e75126 (2013).
27. Kylilis, N. *et al.* Whole-Cell Biosensor with Tunable Limit of Detection Enables Low-Cost Agglutination Assays for Medical Diagnostic Applications. *ACS Sens.* **4**, 370–378 (2019).
28. Riangrunroj, P., Bever, C. S., Hammock, B. D. & Polizzi, K. M. A label-free optical whole-cell *Escherichia coli* biosensor for the detection of pyrethroid insecticide exposure. *Sci. Rep.* **9**, 12466 (2019).
29. Kaliberov, S. A. *et al.* Adenoviral targeting using genetically incorporated camelid single variable domains. *Lab Invest* **94**, 893–905 (2014).
30. Behar, G. *et al.* Llama single-domain antibodies directed against nonconventional epitopes of tumor-associated carcinoembryonic antigen absent from nonspecific cross-reacting antigen. *FEBS J.* **276**, 3881–3893 (2009).
31. Hanke, L. *et al.* An alpaca nanobody neutralizes SARS-CoV-2 by blocking receptor interaction. *Nat. Commun.* **11**, 4420 (2020).

32. de Boer, P. A. J., Crossley, R. E. & Rothfield, L. I. A division inhibitor and a topological specificity factor coded for by the minicell locus determine proper placement of the division septum in *E. coli*. *Cell* **56**, 641–649 (1989).
33. Ebersbach, G., Galli, E., Møller-Jensen, J., Löwe, J. & Gerdes, K. Novel coiled-coil cell division factor ZapB stimulates Z ring assembly and cell division. *Mol. Microbiol.* **68**, 720–735 (2008).
34. Jensen, S. I., Lennen, R. M., Herrgård, M. J. & Nielsen, A. T. Seven gene deletions in seven days: Fast generation of *Escherichia coli* strains tolerant to acetate and osmotic stress. *Sci. Rep.* **5**, 17874 (2015).
35. Rawls, S. M. Antibiotics,  $\beta$ -Lactam. in *Encyclopedia of the Neurological Sciences (Second Edition)* (eds. Aminoff, M. J. & Daroff, R. B.) 207–209 (Academic Press, 2014). doi:10.1016/B978-0-12-385157-4.00250-5.
36. LeFrock, J. L., Prince, R. A. & Leff, R. D. Mechanism of action, antimicrobial activity, pharmacology, adverse effects, and clinical efficacy of cefotaxime. *Pharmacotherapy* **2**, 174–184 (1982).
37. Yocum, R. R., Rasmussen, J. R. & Strominger, J. L. The mechanism of action of penicillin. Penicillin acylates the active site of *Bacillus stearothermophilus* D-alanine carboxypeptidase. *J. Biol. Chem.* **255**, 3977–3986 (1980).
38. Liu, S. L., Hessel, A. & Sanderson, K. E. Genomic mapping with I-Ceu I, an intron-encoded endonuclease specific for genes for ribosomal RNA, in *Salmonella* spp., *Escherichia coli*, and other bacteria. *Proc. Natl. Acad. Sci.* **90**, 6874–6878 (1993).
39. Sanfilippo, S. *et al.* Viability assessment of fresh and frozen/thawed isolated human follicles: reliability of two methods (Trypan blue and Calcein AM/ethidium homodimer-1). *J. Assist. Reprod. Genet.* **28**, 1151–1156 (2011).
40. Jones, R. M., Pagmantidis, V. & Williams, P. A. sal Genes Determining the Catabolism of Salicylate Esters Are Part of a Supraoperonic Cluster of Catabolic Genes in *Acinetobacter* sp. Strain ADP1. *J. Bacteriol.* **182**, 2018–2025 (2000).
41. de Oliveira, D. *et al.* Catechol cytotoxicity in vitro: Induction of glioblastoma cell death by apoptosis. *Hum. Exp. Toxicol.* **29**, 199–212 (2010).
42. Schweigert, N., Zehnder, A. J. B. & Eggen, R. I. L. Chemical properties of catechols and their molecular modes of toxic action in cells, from microorganisms to mammals. *Environ. Microbiol.* **3**, 81–91 (2001).
43. Zhou, S., Gravekamp, C., Bermudes, D. & Liu, K. Tumour-targeting bacteria engineered to fight cancer. *Nat. Rev. Cancer* **18**, 727–743 (2018).
44. Tan, E. *et al.* Diagnostic precision of carcinoembryonic antigen in the detection of recurrence of colorectal cancer. *Surg. Oncol.* **18**, 15–24 (2009).
45. Tanha, J., Dubuc, G., Hiram, T., Narang, S. A. & MacKenzie, C. R. Selection by phage display of llama conventional VH fragments with heavy chain antibody VHH properties. *J. Immunol. Methods* **263**, 97–109 (2002).

46. van der Linden, R. *et al.* Induction of immune responses and molecular cloning of the heavy chain antibody repertoire of Lama glama. *J. Immunol. Methods* **240**, 185–195 (2000).
47. Huang, J. Q., Turbide, C., Daniels, E., Jothy, S. & Beauchemin, N. Spatiotemporal expression of murine carcinoembryonic antigen (CEA) gene family members during mouse embryogenesis. *Dev. Camb. Engl.* **110**, 573–588 (1990).
48. Guadagni, F. *et al.* Quantitative analysis of CEA expression in colorectal adenocarcinoma and serum: Lack of correlation. *Int. J. Cancer* **72**, 949–954 (1997).
49. Sieow, B. F.-L., Wun, K. S., Yong, W. P., Hwang, I. Y. & Chang, M. W. Tweak to Treat: Reprogramming Bacteria for Cancer Treatment. *Trends Cancer* **7**, 447–464 (2021).
50. Din, M. O. *et al.* Synchronized cycles of bacterial lysis for in vivo delivery. *Nature* **536**, 81–85 (2016).
51. Chan, C. T. Y., Lee, J. W., Cameron, D. E., Bashor, C. J. & Collins, J. J. ‘Deadman’ and ‘Passcode’ microbial kill switches for bacterial containment. *Nat. Chem. Biol.* **12**, 82–86 (2016).
52. Aggarwal, N., Breedon, A. M. E., Davis, C. M., Hwang, I. Y. & Chang, M. W. Engineering probiotics for therapeutic applications: recent examples and translational outlook. *Curr. Opin. Biotechnol.* **65**, 171–179 (2020).
53. Prajapati, J. D., Kleinekathöfer, U. & Winterhalter, M. How to Enter a Bacterium: Bacterial Porins and the Permeation of Antibiotics. *Chem. Rev.* **121**, 5158–5192 (2021).
54. Chen, J. X. *et al.* Development of Aspirin-Inducible Biosensors in Escherichia coli and SimCells. *Appl. Environ. Microbiol.* **85**, e02959-18.
55. Medzhitov, R. Recognition of microorganisms and activation of the immune response. *Nature* **449**, 819–826 (2007).
56. Chang, A., Chau, V., Landas, J. & Pang, Y. Preparation of Calcium Competent Escherichia coli and Heat-Shock Transformation. *JEMI Methods* **1**, 22–25 (2017).
57. Untergasser, A. Transformation of Chemical Competent Cells. *Untergasser’s Lab* [http://www.untergasser.de/lab/protocols/competent\\_cells\\_chemical\\_trafo\\_v1\\_0.htm](http://www.untergasser.de/lab/protocols/competent_cells_chemical_trafo_v1_0.htm) (2008).
58. Untergasser, A. Preparation of Electro-Competent Cells. *Untergasser’s Lab* [http://www.untergasser.de/lab/protocols/competent\\_cells\\_electro\\_v1\\_0.htm](http://www.untergasser.de/lab/protocols/competent_cells_electro_v1_0.htm) (2008).

## Figures:

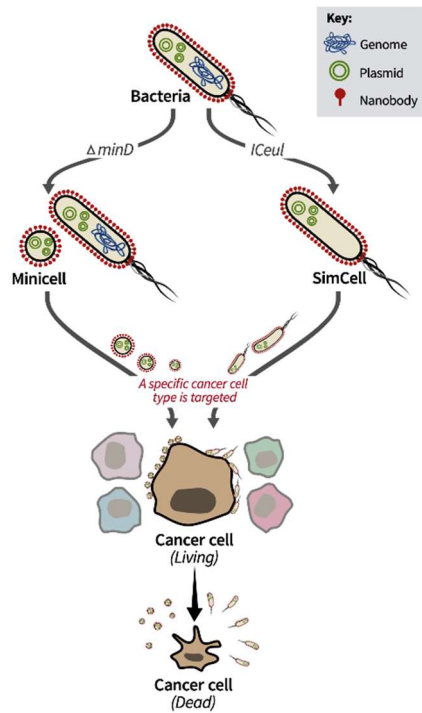


**Figure 1.** An overview of the engineering of synthetic biology chassis, minicell and SimCell, for targeted cancer therapy. A surface-displayed nanobody confers binding specificity to a targeted cancer cell. *minD* mutation and ICeul endonuclease expression induce the production of minicell (Fig. 4) and SimCell (Fig. 5), respectively, which are both anucleate, non-replicating but metabolically active. Combined with a prodrug conversion genetic circuit or used as a stand-alone therapeutic, these synthetic biology chassis can be developed into a targeted cancer therapy for tumour clearance.

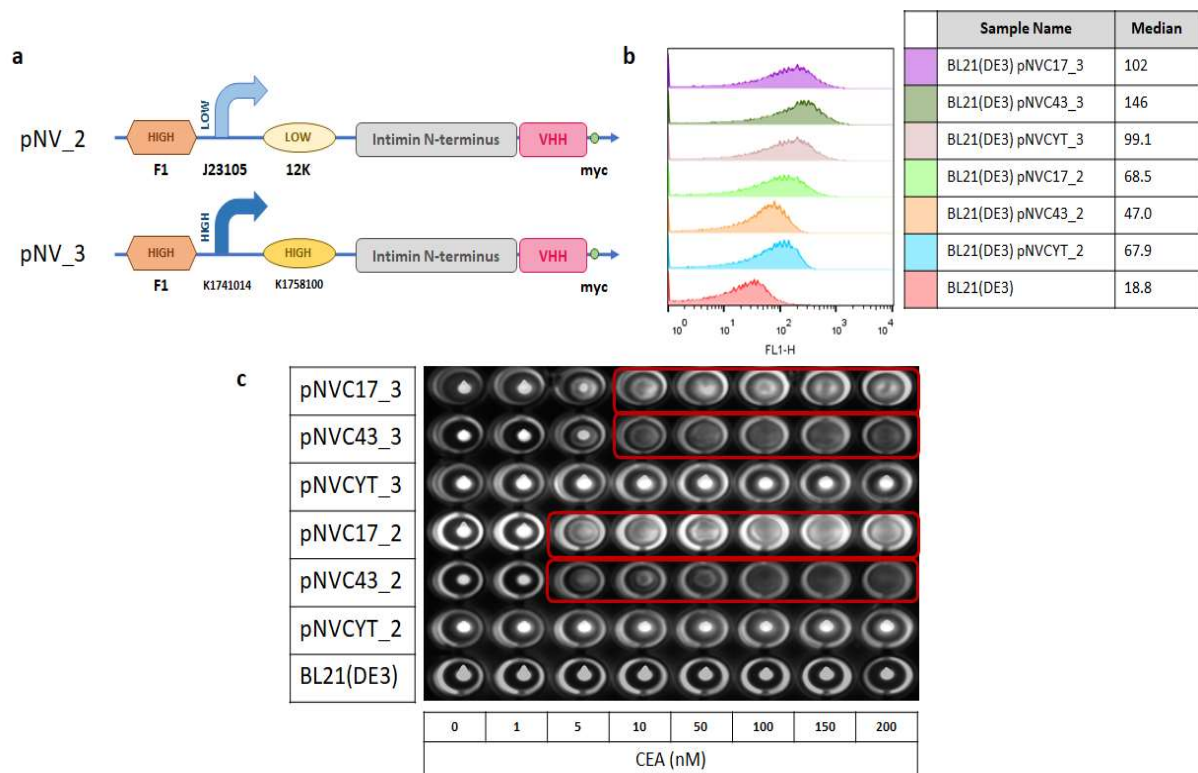


**Figure 2.** Engineered *E. coli* expressing nanobody through the surface display pNV system. **(a)** Schematic representation of pNV\_2 with low expression profile and pNV\_3 with high expression profile. F1 is a high copy number origin of replication; J23105 has low promoter activity whereas K1741014 has high promoter activity; 12k is a low strength Ribosome Binding Strength (RBS) whereas K1758100 is a high strength RBS; Intimin is the outer membrane anchor; VHH is the nanobody used in this study, which includes anti-CEA, C17 and C43 and anti-spike protein, CYT; Myc is the tag used for flow cytometry analysis. **(b)** Flow cytometry analysis of engineered *E. coli* carrying pNV systems. Histograms indicate the fluorescence intensity of bacteria probed with primary anti-Myc antibody and secondary Alexa Fluor 488 antibody. The median fluorescence intensity of each sample is also presented. **(c)** Binding of engineered *E. coli* to CEA in a biological agglutination test. Binding between surface displayed nanobody and target antigen CEA results in agglutination (red box, clear suspension) while no binding results in a cell pellet. The image was taken using VersaDoc Imaging system under FITC channel.

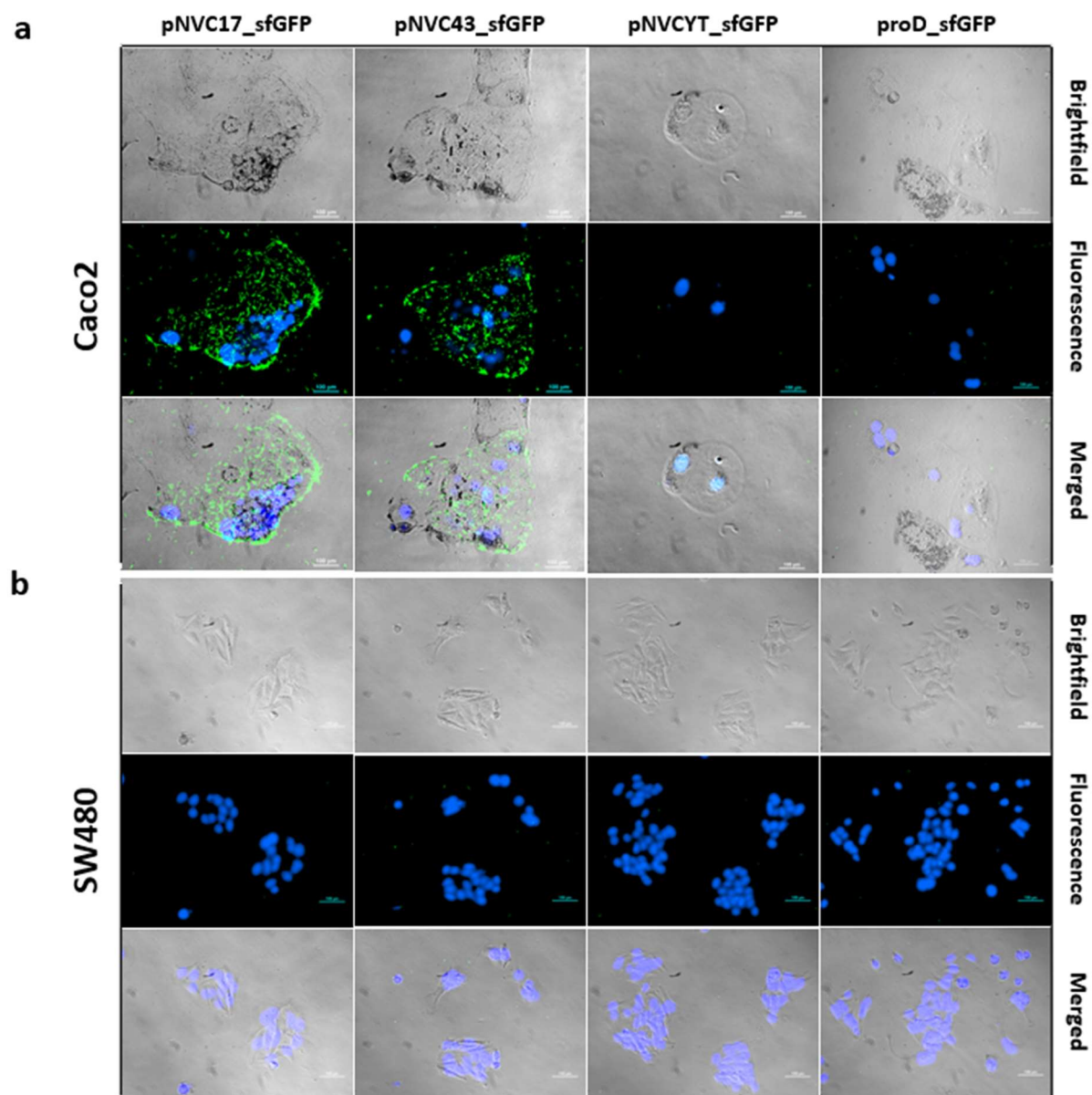


**Figure 3.** Specific adhesion of engineered *E. coli* to targeted cancer cell after 2 hours of incubation. Engineered *E. coli* carrying different pNV plasmids with sfGFP (green) were incubated with two different colorectal cancer cell lines (MOI 300:1): **(a)** Caco2, a high CEA expressing cell line and **(b)** SW480, a low CEA expressing cell line in which both are stained with Hoechst dye (blue). Microscopic images at different time points throughout the 8 hours incubation can be found in Supplementary Figure S1-S3. Scale bar is 100  $\mu$ m. Each image represents one field-of-view of one experimental well.

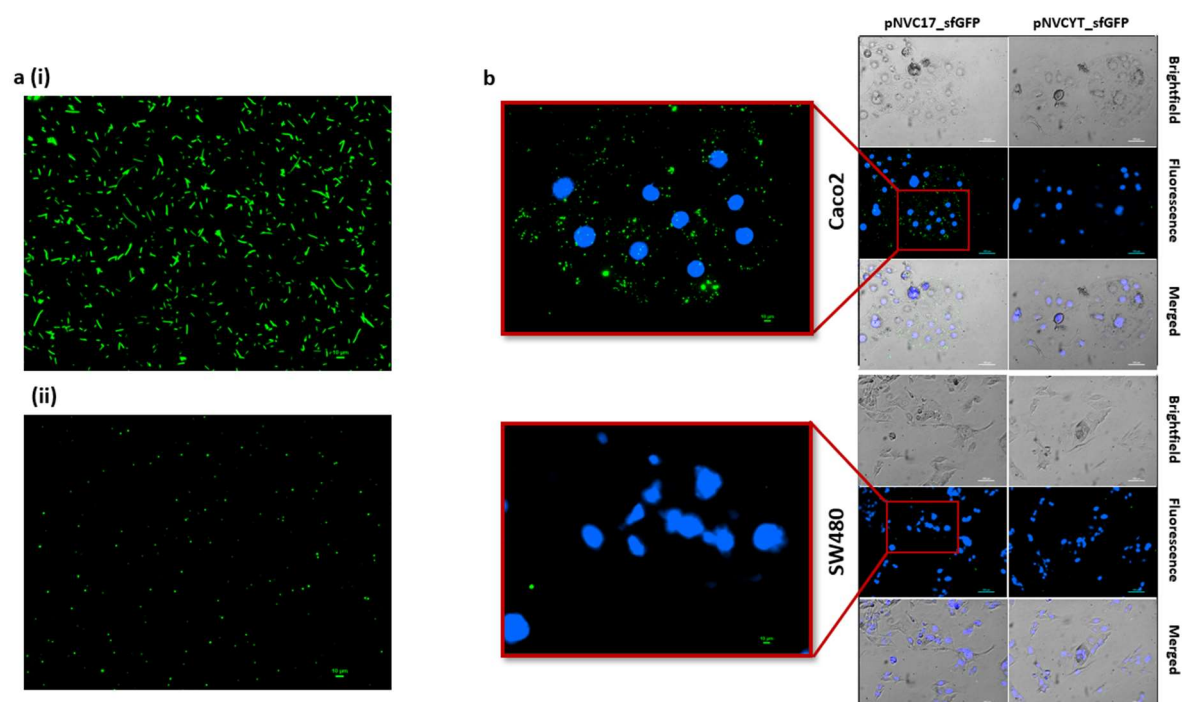
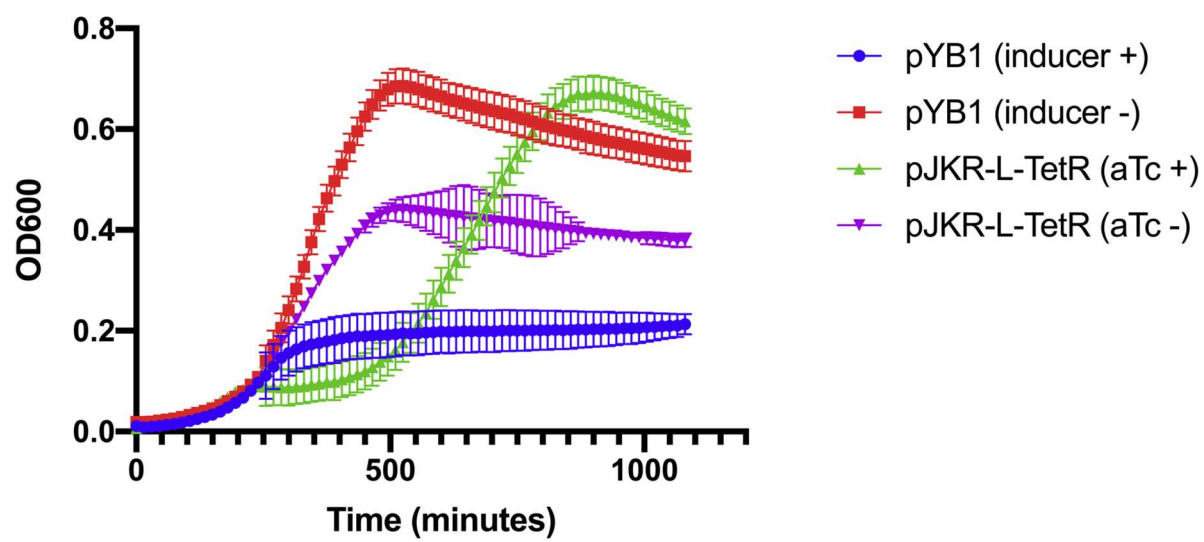


**Figure 4.** Engineering of *E. coli* minicells for specific cancer cells adhesion. **(a) (i)** Pre- and **(ii)** post-purification of pNVC17\_sfGFP transformed *E. coli* minicells (green). Scale bar is 10 μm. **(b)** 2 hours incubation of pNVC17\_sfGFP or pNVCYT\_sfGFP transformed minicells with high CEA expressing Caco2 and low CEA expressing SW480. Red box shows the zoom in region using 20x magnification. Nuclei were stained with Hoechst dye (blue). Microscopic images at different time points throughout the 8 hours incubation can be found in Supplementary Figure S5. Scale bar is 100 μm and 10 μm for zoom-in region. Each image represents one field-of-view of one experimental well.

a

### Efficiency of SimCell Conversion



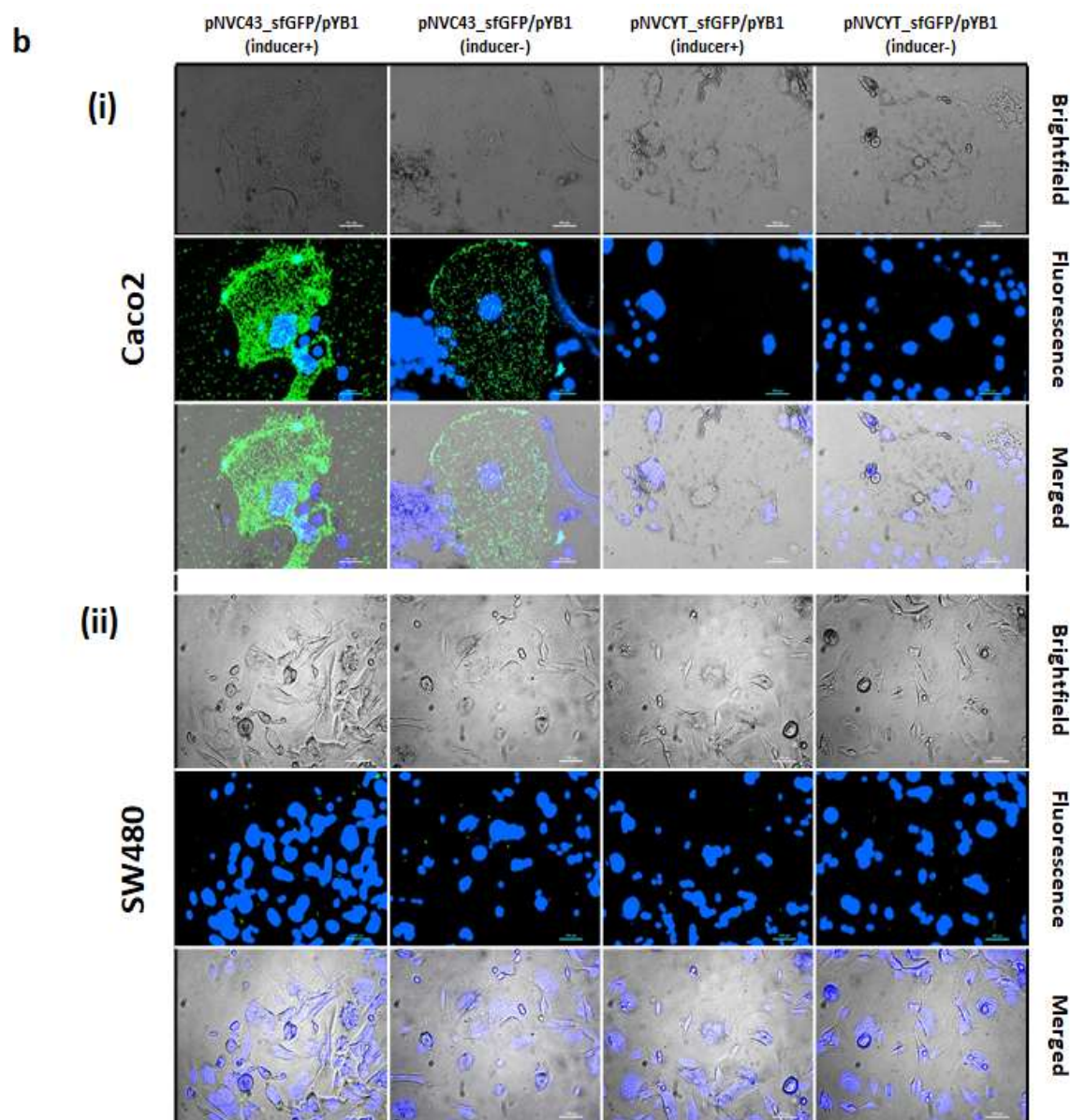
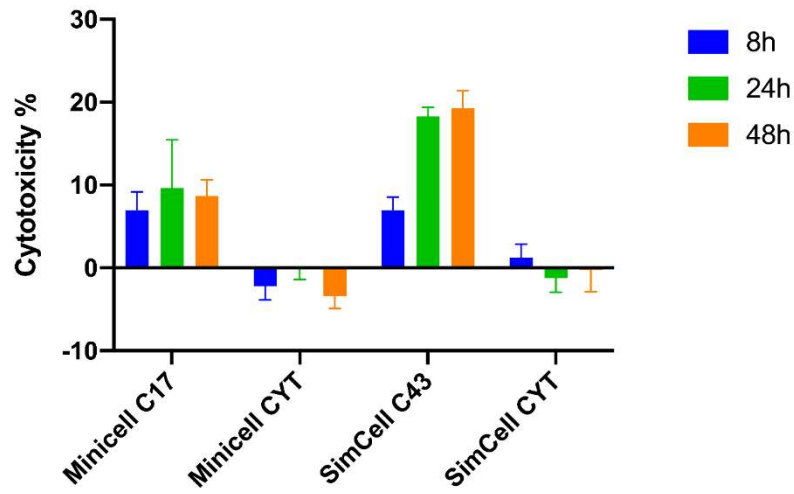


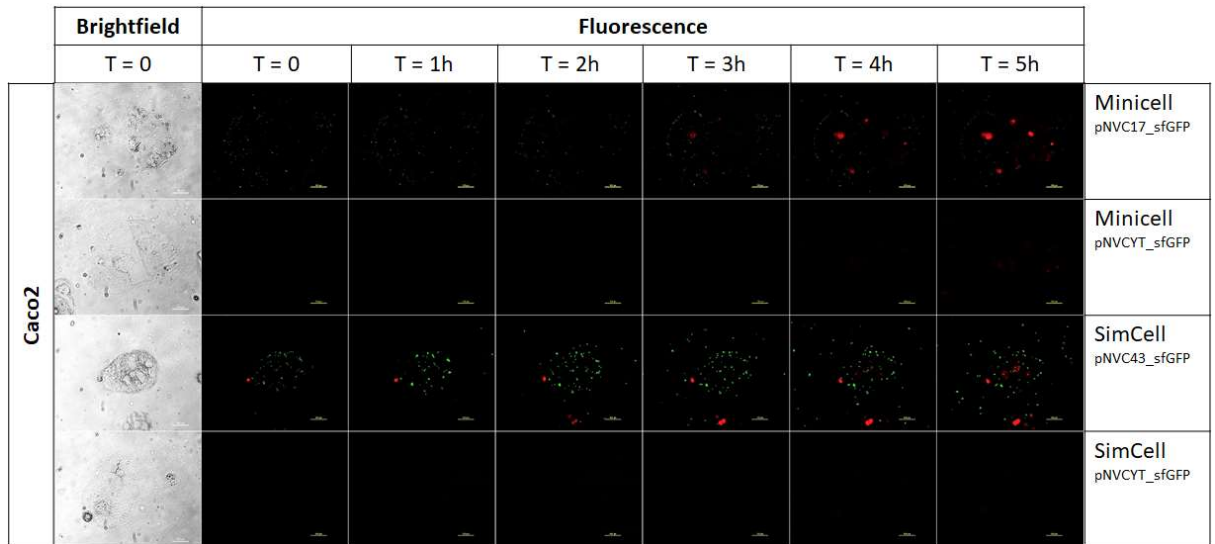
**Figure 5.** Re-programming genome-less, non-replicating SimCells for targeted cancer cell adhesion. **(a)** OD<sub>600</sub> reading of SimCell pYB1 and *E. coli* carrying pJKR-L-TetR (TetR controlled) plasmid for SimCell conversion. Red arrow indicates the time point of the addition of inducer cocktail X (inducer +) and anhydrotetracycline (aTc+) to pYB1 (blue line) and pJKR-L-TetR (green line) culture, respectively. Inducer - (red line) and aTc - (purple line) are the control cultures without the addition of inducer cocktail X and aTc. Error bars represent the standard deviation from three biological repeats. **(c)** 2 hours incubation of programmed SimCell pYB1 carrying pNVC43\_sfGFP or pNVCYT\_sfGFP with (+) and without (-) the addition of inducer cocktail X (green) (MOI 300:1). Bacterial cells under inducer + are genome less and non-dividing SimCells (Fig. S6). **(i)** Caco2 and **(ii)** SW480 are the high CEA expressing and low CEA expressing colorectal cancer cell lines, respectively. Nuclei were stained with Hoechst

dye (blue). Microscopic images at different time points throughout the 8 hours incubation can be found in Supplementary Figure S7. Scale bar is 100  $\mu\text{m}$ . Each image represents one field-of-view of one experimental well.

a



b



c

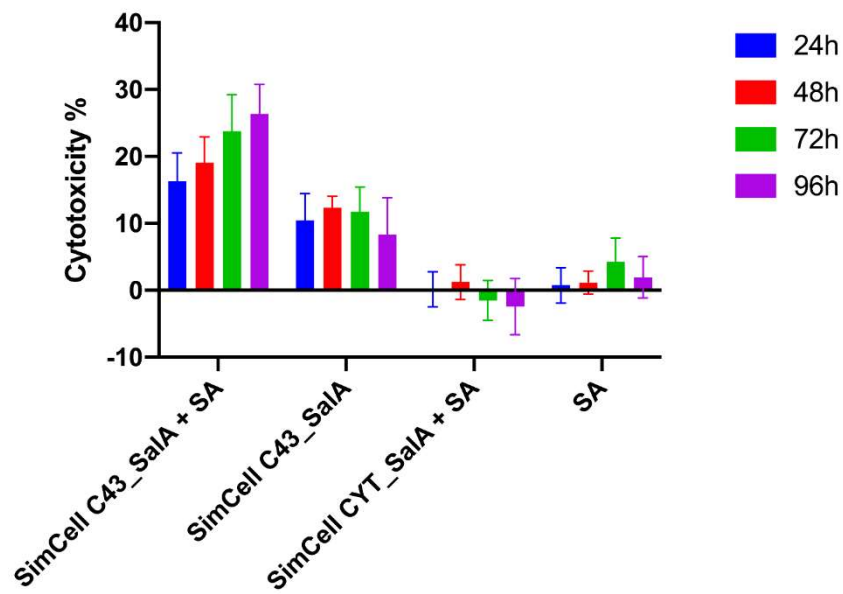


**Figure 6.** Induction of targeted cancer cell death via SimCell and minicell *in vitro*. **(a)** Cytotoxicity of targeted minicell C17 and SimCell C43 (representing pNVC17\_sfGFP and pNVC43\_sfGFP, respectively) towards Caco2 compared to their non-specific counterparts CYT (representing pNVCYT\_sfGFP). Caco2 was first incubated with minicells and SimCells for 2 hours, followed by three-times washing with fresh media prior to further incubation at 37 °C with 5% CO<sub>2</sub>. LDH assay was used to measure cytotoxicity at T=8h, 24h and 48h. Error bars represent the standard deviation from eight biological repeats. **(b)** Time-lapse images of Caco2 at time point 0, 1, 2, 3, 4 and 5 hour after 2 hours incubation with minicell (pNVC17\_sfGFP and pNVCYT\_sfGFP and SimCell (pNVC43\_sfGFP and pNVCYT\_sfGFP). All cultures were washed thrice and added with fresh media supplemented with Ethidium homodimer prior to imaging. Remaining SimCells and minicells are in green and the nuclei of Caco2 with compromised membrane were stained by Ethidium homodimer (red). Scale bar is 100 µm. The full 5-hour time-lapse video can be found in Supplementary Movie S10. **(c)** Cytotoxicity of targeted SimCell C43\_SalA (representing pNVC43\_SalA\_sfGFP) towards Caco2 with and without the addition of salicylic acid (SA), in comparison to pre-incubation with non specific SimCell CYT\_SalA (representing pNVCYT\_SalA\_sfGFP). Caco2 was first incubated with SimCells for 2 hours, followed by three-times washing with fresh media supplemented with 500 µM of SA prior to further incubation at 37 °C with 5% CO<sub>2</sub>. LDH assay was used to measure cytotoxicity at T= 24h, 48h, 72h and 96h. Error bars represent the standard deviation from eight biological repeats. Each image represents one field-of-view of one experimental well.

## Supplementary Info

### Reprogramming Synthetic Biology Chassis for Targeted Cancer Therapy

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**Table S1**

#### Bacterial strains and plasmids used in this study.

| Strains                      | Genotype or description                                                                       | Source         |
|------------------------------|-----------------------------------------------------------------------------------------------|----------------|
| <i>Escherichia coli</i> DH5α | <i>fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i> | Lab collection |
| <i>E. coli</i> BL21(DE3)     | <i>fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS</i>                                               | Lab collection |
| <i>E. coli</i> K12 MDS42     | <i>fhuACDB(del) endA(del)</i> and deletion of all IS and cryptic prophage <sup>1</sup>        | Lab collection |
| DH5α pNVC17                  | BL21(DE3): pNVC17 (for cloning)                                                               | This study     |
| BL21(DE3) pNVC17_2           | BL21(DE3): pNVC17_2 (low expression profile)                                                  | This study     |
| BL21(DE3) pNVC43_2           | BL21(DE3): pNVC43_2 (low expression profile)                                                  | This study     |
| BL21(DE3) pNVCYT_2           | BL21(DE3): pNVCYT_2 (low expression profile)                                                  | This study     |
| BL21(DE3) pNVC17_3           | BL21(DE3): pNVC17_3 (high expression profile)                                                 | This study     |
| BL21(DE3) pNVC43_3           | BL21(DE3): pNVC43_3 (high expression profile)                                                 | This study     |
| BL21(DE3) pNVCYT_3           | BL21(DE3): pNVCYT_3 (high expression profile)                                                 | This study     |
| BL21(DE3) control            | BL21(DE3): proD_sfGFP (high expression profile control with sfGFP)                            | This study     |

|                           |               |                                                                                                                  |            |
|---------------------------|---------------|------------------------------------------------------------------------------------------------------------------|------------|
| BL21(DE3) pNVC17_sfGFP    |               | BL21(DE3): pNVC17_sfGFP (high expression profile with sfGFP downstream)                                          | This study |
| BL21(DE3) pNVC43_sfGFP    |               | BL21(DE3): pNVC43_sfGFP (high expression profile with sfGFP downstream)                                          | This study |
| BL21(DE3) pNVCYT_sfGFP    |               | BL21(DE3): pNVCYT_sfGFP (high expression profile with sfGFP downstream)                                          | This study |
| SimCell pYB1/pNVC43_sfGFP |               | High expression profile with sfGFP downstream; SimCell conversion.                                               | This study |
| SimCell pYB1/pNVCYT_sfGFP |               | High expression profile with sfGFP downstream; SimCell conversion.                                               | This study |
| BL21(DE3) pSIJ8           |               | BL21(DE3): pSIJ8 (for recombination engineering)                                                                 | This study |
| BL21(DE3) $\Delta minD$   |               | BL21(DE3) $\Delta minD::Kan^R$ (for minicells generation)                                                        | This study |
| BL21(DE3) pNVC17_sfGFP    | $\Delta minD$ | BL21(DE3): pNVC17_sfGFP, $\Delta minD::Kan^R$ (for minicells with high expression profile with sfGFP downstream) | This study |
| BL21(DE3) pNVC43_sfGFP    | $\Delta minD$ | BL21(DE3): pNVC43_sfGFP, $\Delta minD::Kan^R$ (for minicells with high expression profile with sfGFP downstream) | This study |
| BL21(DE3) pNVCYT_sfGFP    | $\Delta minD$ | BL21(DE3): pNVCYT_sfGFP, $\Delta minD::Kan^R$ (for minicells with high expression profile with sfGFP downstream) | This study |

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#### Plasmids

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|        |                                                                                                                                                             |                                           |
|--------|-------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------|
| pNVgfp | Plasmid with anti-GFP nanobody attached to $\beta$ -intimin, constitutively expressed by the promoter BBa_J23106 and a low strength RBS (12K TIR). $Cm^R$ . | A gift from Dr Karen Polizzi <sup>2</sup> |
| pNVC17 | Anti-GFP nanobody of pNVgfp replaced by anti-CEA nanobody. $Cm^R$ .                                                                                         | This study                                |

|                   |                                                                                                         |                                |
|-------------------|---------------------------------------------------------------------------------------------------------|--------------------------------|
| pNVC43            | Anti-GFP nanobody of pNVgfp replaced by another variant of anti-CEA nanobody. Cm <sup>R</sup> .         | This study                     |
| pNVC17_2          | BBa_J23106 of pNVC17 replaced by BBa_J23105. Cm <sup>R</sup> .                                          | This study                     |
| pNVC43_2          | BBa_J23106 of pNVC43 replaced by BBa_J23105. Cm <sup>R</sup> .                                          | This study                     |
| pNVCYT_2          | Anti-CEA nanobody of pNVC17_2 replaced by anti-spike protein nanobody. Cm <sup>R</sup> .                | This study                     |
| pNVC17_3          | BBa_J23106 and RBS of pNVC17 replaced by BBa_K1741014 and BBa_K1758100, respectively.                   | This study                     |
| pNVC43_3          | Anti-CEA nanobody of pNVC17_3 replaced by another anti-CEA nanobody variant. Cm <sup>R</sup> .          | This study                     |
| pNVCYT_3          | Anti-CEA nanobody of pNVC17_3 replaced by anti-spike protein nanobody. Cm <sup>R</sup> .                | This study                     |
| pNVC17_sfGFP      | BBa_K1758100 and <i>sfGFP</i> cloned downstream to the anti-CEA nanobody in pNVC17_3. Cm <sup>R</sup> . | This study                     |
| pNVC43_sfGFP      | Anti-CEA nanobody of pNVC17_sfGFP replaced by another anti-CEA nanobody variant. Cm <sup>R</sup> .      | This study                     |
| pNVCYT_sfGFP      | Anti-CEA nanobody of pNVC17_sfGFP replaced by anti-spike protein nanobody. Cm <sup>R</sup> .            | This study                     |
| prod_sfGFP        | A lab plasmid with BBa_K1741014 and BBa_K1758100 upstream of a <i>sfGFP</i> . Cm <sup>R</sup> .         | Lab collection                 |
| pJKR-L-TetR-ICeuI | Plasmid with I-CeuI endonuclease controlled by TetR. Carb <sup>R</sup> .                                | <sup>3</sup>                   |
| pYB1              | Plasmid for high efficiency SimCell conversion. Kan <sup>R</sup> .                                      | Provided by Oxford SimCell Ltd |

|       |                                                                                                                                               |   |
|-------|-----------------------------------------------------------------------------------------------------------------------------------------------|---|
| pSIJ8 | Plasmid with lambda Red recombineering genes ( <i>exo</i> , <i>bet</i> and <i>gam</i> ) and a flippase (FLP) recombinase. Carb <sup>R</sup> . | 4 |
|-------|-----------------------------------------------------------------------------------------------------------------------------------------------|---|

**Table S2**

**Primers and gene blocks used in this study.**

| Primers             | Sequence (5' → 3')                                                                    | Part/Purpose                             | Plasmid                                                  |
|---------------------|---------------------------------------------------------------------------------------|------------------------------------------|----------------------------------------------------------|
| VHH-for             | GAACCGGCCCAGCCGGCCGAAGTTCAACTGGT<br>TGAAAG                                            | Anti-CEA<br>nanobody                     | pNVC17,<br>pNVC43,<br>pNVC17_2,<br>pNVC43_2              |
| VHH-rev             | GATGAGTTTTTGTTCGGCGGCCGCGCTGCTAAC<br>TGTAACCTG                                        |                                          |                                                          |
| pNV-for             | GCTCGAGCCGAACAAAACTCATCTCAGAAGA<br>GGATGCAGCTGC                                       | pNVgfp<br>backbone                       | pNVC17,<br>pNVC43,<br>pNVC17_2,<br>pNVC43_2,<br>pNVCYT_2 |
| pNV-rev             | GGCCGGCTGGGCGGTTCCAGC                                                                 |                                          |                                                          |
| pNV-3-for           | ATGATTACTCATGGTTGTTATACCCGG                                                           | pNVC17<br>backbone                       | pNVC17_3,<br>pNVC43_3,<br>pNVCYT_3                       |
| pNV-3-rev           | GCACACGGTCACACTGCTTC                                                                  |                                          |                                                          |
| pNV-sfGFP-for       | GCATGGATGAGCTCTACAAAAAGCTTGACCTG<br>TGAAGTG                                           | pNVC17_3<br>backbone                     | pNVC17_sfGFP,<br>pNVC43_sfGFP,<br>pNVCYT_sfGFP           |
| pNV-sfGFP-rev       | ATGGTACCTTTCTCCTCTTTTATGCAGCTGCA<br>TCCTC                                             |                                          |                                                          |
| sfGFP-for           | AAAGAGGAGAAAGGTACCATGAGCAAAG                                                          | sfGFP                                    | pNVC17_sfGFP,<br>pNVC43_sfGFP,<br>pNVCYT_sfGFP           |
| sfGFP-rev           | TTTGTAGAGCTCATCCATGCCATGTG                                                            |                                          |                                                          |
| minD-HR-<br>Kan-for | GTTGGCTGTGTTTTTCTTCCGCGAGAGAAAGA<br>AATCGAGTAATGCCATAACTTAGAAAACTCA<br>TCGAGCATCAAATG | Kanamycin<br>resistance gene<br>cassette | -                                                        |

|                  |                                               |                                   |
|------------------|-----------------------------------------------|-----------------------------------|
| minD-HR-         | CGCTTTGACCGTTCAACCGTTAAATTGATCCCT             | -                                 |
| Kan-rev          | TTTAAACAAGGAATTTCTATGAGCCATATTCAA<br>CGGGAAAC |                                   |
| HR-check-<br>for | GACCTCAAGAATATCTTTACGCAACTG                   | Check for success recombination - |
| HR-check-<br>rev | CTGTTTGTAATTGTCCTTTTAACAGCG                   | -                                 |

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### Gene blocks

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|         |                                                                                                                                                                                                                                                                                                                                                                                                                      |                                          |
|---------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------|
| VHH-C17 | GAAGTTCAACTGGTTGAAAGCGGTGGTGGTTT<br>TGTTCAAGGCAGGCGAAAGTCTGACCCTGAGCT<br>GTACCAGCAGCACCTGACCTTTACACCGTAT<br>CGTATGGCATGGTATCGTCAGGCACCGGGTAA<br>ACAGCGTGATCTGGTTGCAGATATTAGCAGCG<br>GTGATGGTCGTACCACCAATTATGCAGATTTTG<br>CCAAAGGTCGTTTTACCATTAGCCGTGACAAT<br>ATCAAAAACACCGTTTTTCTGCGTATGACCAAT<br>CTGAAACCGGAAGATACCGCAGTGTATTATTG<br>TAATACCTTTGTGAGCTTTGTGGGTATTGCACG<br>TAGCTGGGGTCAGGGCACCCAGGTTACAGTTA<br>GCAGC | Anti-CEA nanobody <sup>5</sup>           |
| VHH-C43 | GAAGTTCAACTGGTTGAAAGCGGTGGTGGTCT<br>GGTTCAGGCAGGCGGTAGTCTGACCCTGAGCT<br>GTACCAGCAGCACCTGACCTTTACACCGTAT<br>CGTATGGGTGGTATCGTCAGACACCGGGTAA<br>ACAGCGTGATCTGGTTGCAGATATTAGCCCTG<br>GTGATGGTAGCACCAAAAACCTATGCAGGTTTT<br>GCACAGGGTCGTTTTACCATTAGCCGTGATAA<br>TATCAAAAACACCGTTTACCTGCAGATGAACG<br>ATCTGAAACCGGAAGATACCGCAGTGTATTAT<br>TGTAATACCTATGTTGCCTTTGTTGGTTCGTGCA<br>CGTACCTGGGGTCAGGGCACCCAGGTTACAGT<br>TAGCAGC | Anti-CEA nanobody <sup>6</sup>           |
| VHH-CYT | CAGGTGCAGCTCGTGGAGACGGGGGGAGGCTT<br>GGTGCAGCCTGGGGGGTCTCTGAGACTCTCCT<br>GTGCAGCCTCTGGATTCACCTTCAGTAGCGTCT<br>ACATGAACTGGGTCCGCCAGGCTCCAGGGAAG<br>GGGCCCCAGTGGGTCTCGCGTATTAGTCCGAA<br>TAGTGGTAATATTGGGTATACAGACTCCGTGA<br>AGGGCCGATTACCATCTCCAGAGACAACGCC<br>AAGAACACACTGTATCTGCAAATGAATAACCT                                                                                                                         | Anti-Spike protein nanobody <sup>7</sup> |

GAAACCTGAGGACACGGCCCTGTATTACTGTG  
CGATTGGTTTGAATTTGAGTAGTAGCTCCGTTA  
GGGGCCAGGGGACCCAGGTCACCGTCTCCTCA

|          |                                                                                                                                                                                                              |                               |
|----------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------|
| proD-g10 | CACAGCTAACACCACGTCGTCCCTATCTGCTGC<br>CCTAGGTCTATGAGTGGTTGCTGGATAACTTTA<br>CGGGCATGCATAAGGCTCGTATAATATATTCA<br>GGGAGACCACAACGGTTTCCCTCTACAAATAA<br>TTTTGTTTAACTTTGAATTCAAAAGATCTTTAA<br>CTTTAAGAAGGAGATATACAT | BBa_K1741014 and BBa_K1758100 |
|----------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------|

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**Figure S1**

0 hour (within ~5 minutes) incubation of engineered *E. coli* carrying different pNV plasmids with sfGFP (green) with two different colorectal cancer cell lines: (a) Caco2, a high CEA expressing cell line and (b) SW480, a low CEA expressing cell. Nuclei of cancer cells are stained with Hoechst dye (blue). Scale bar is 100  $\mu$ m.

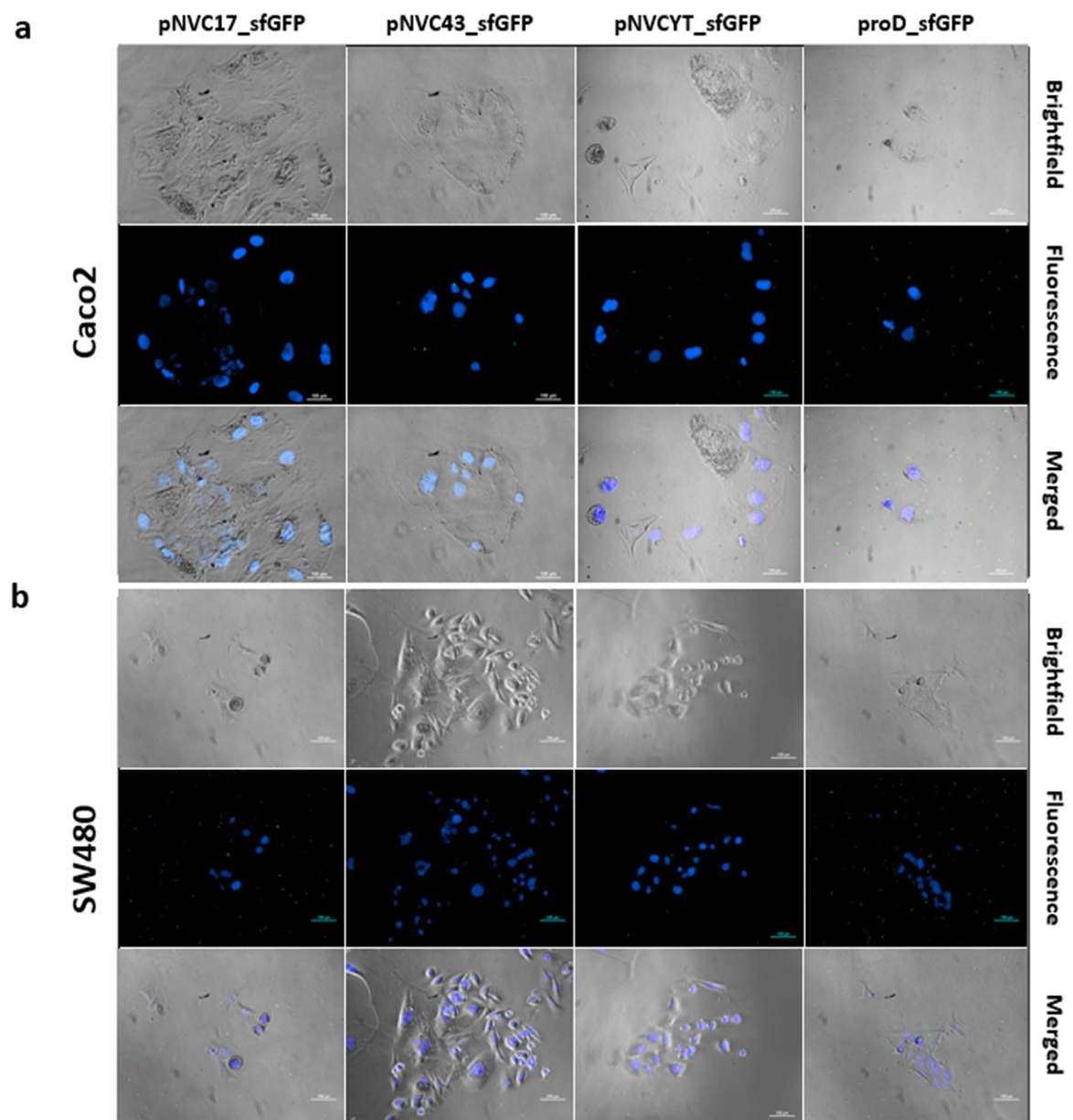


**Figure S2**

4 hours incubation of engineered *E. coli* carrying different pNV plasmids with sfGFP (green) with two different colorectal cancer cell lines: (a) Caco2, a high CEA expressing cell line and (b) SW480, a low CEA expressing cell. Nuclei of cancer cells are stained with Hoechst dye (blue). Scale bar is 100  $\mu$ m.

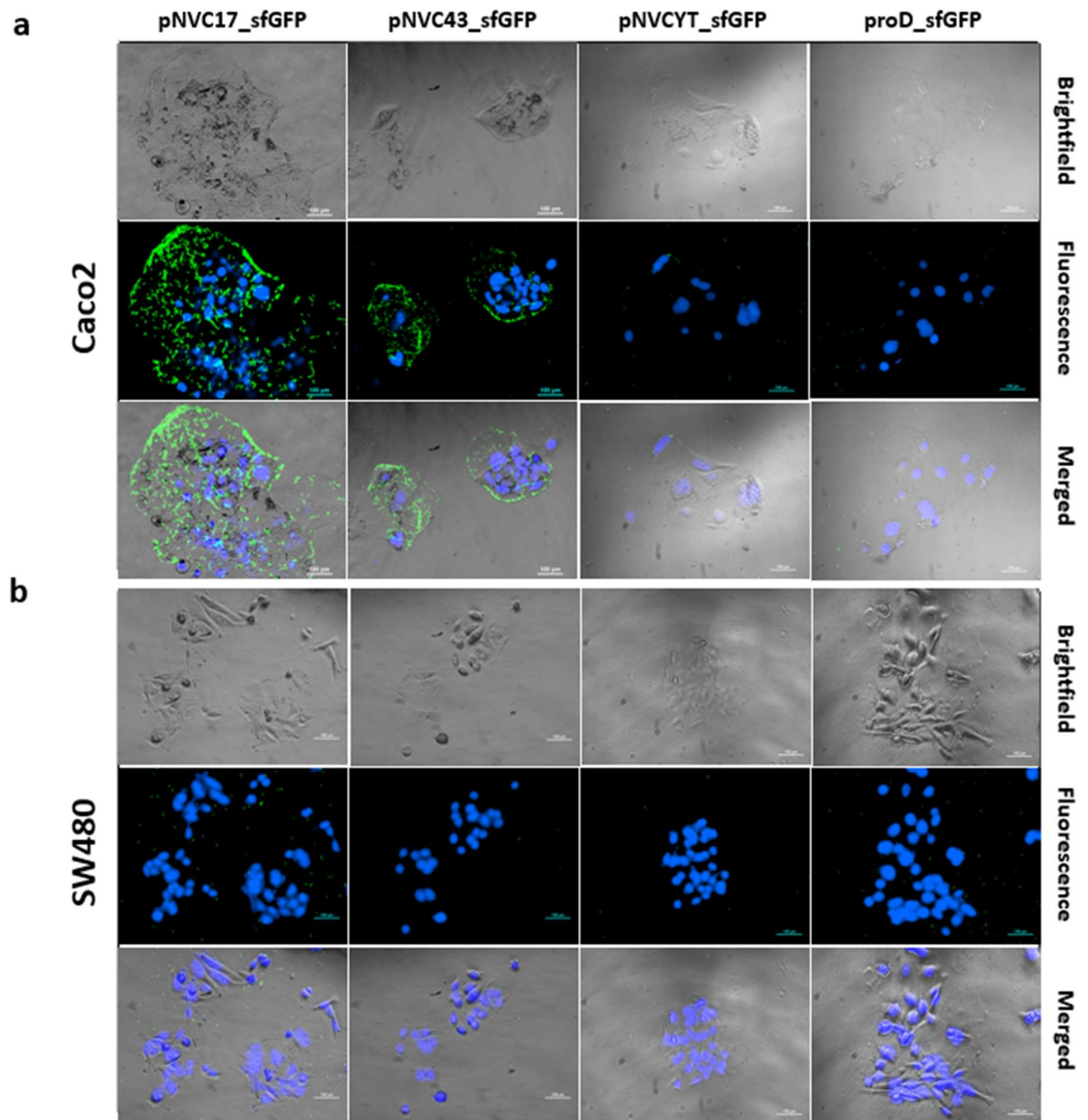


**Figure S3**

8 hours incubation of engineered *E. coli* carrying different pNV plasmids with sfGFP (green) with two different colorectal cancer cell lines: **(a)** Caco2, a high CEA expressing cell line and **(b)** SW480, a low CEA expressing cell. Nuclei of cancer cells are stained with Hoechst dye (blue). Scale bar is 100  $\mu$ m.

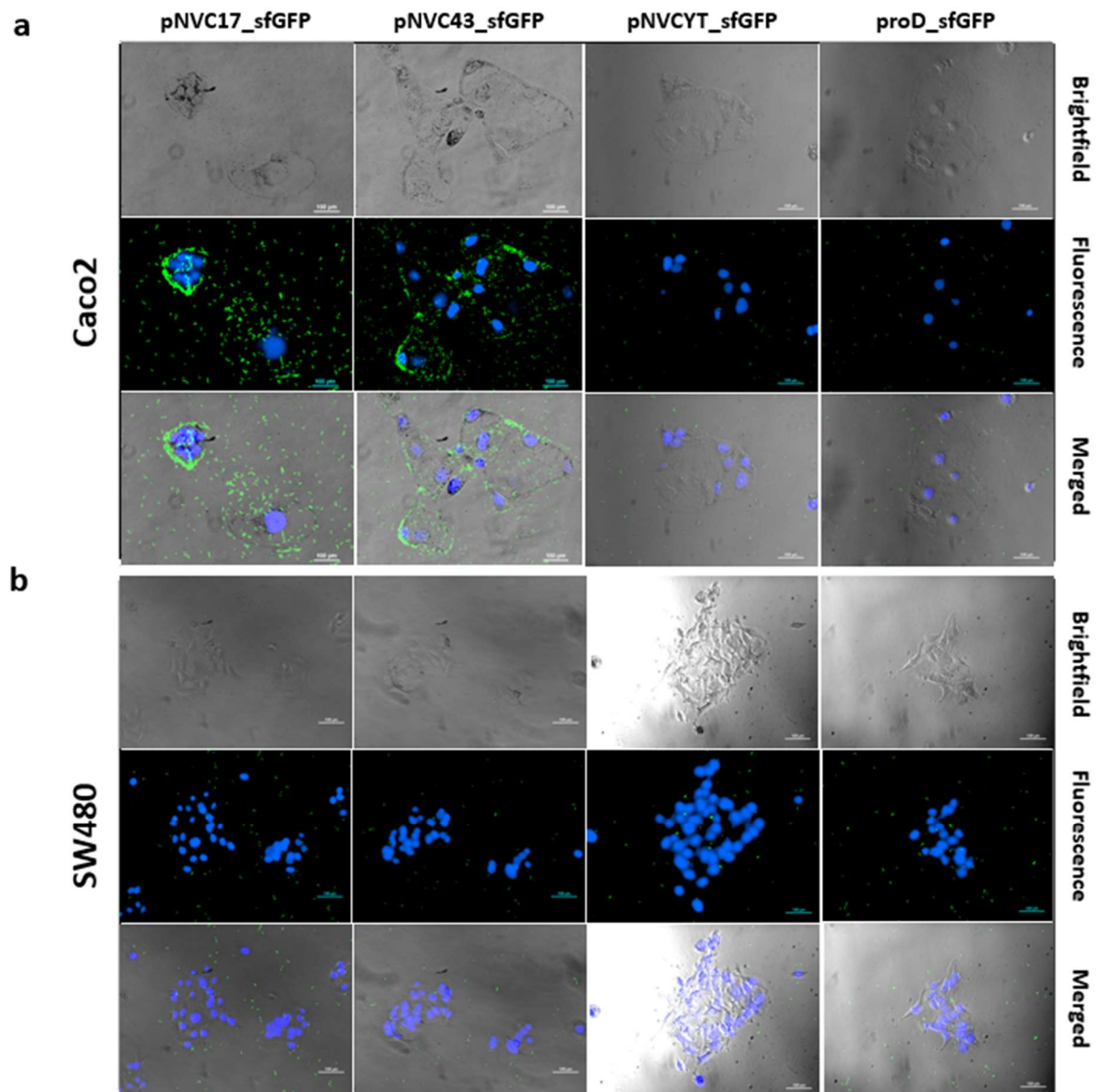


**Figure S4**

**(a)** Gel electrophoresis of PCR products from five different colonies (C1-C5) with successful *minD* knock out. The primers used were minD-HR-Kan-for and minD-HR-Kan-rev (Table S2), which anneal to the inserted Kanamycin cassette and the *minE* gene within the bacterial genome, respectively. The length of PCR product is 563 bp. The ladder used is a 1kb plus DNA ladder from NEB. **(b)** Overnight OD<sub>600</sub> reading of pre-purification culture (pre 1, 2, 3) and post-purification culture (post 1, 2, 3) of minicells. Each line represents a single biological repeat. **(c)** Pre-purification and post-purification minicell culture plated on LB-Kan-Cm agar for 24 hours (top), 48 hours (mid) and 72 hours (bottom) at 37 °C.

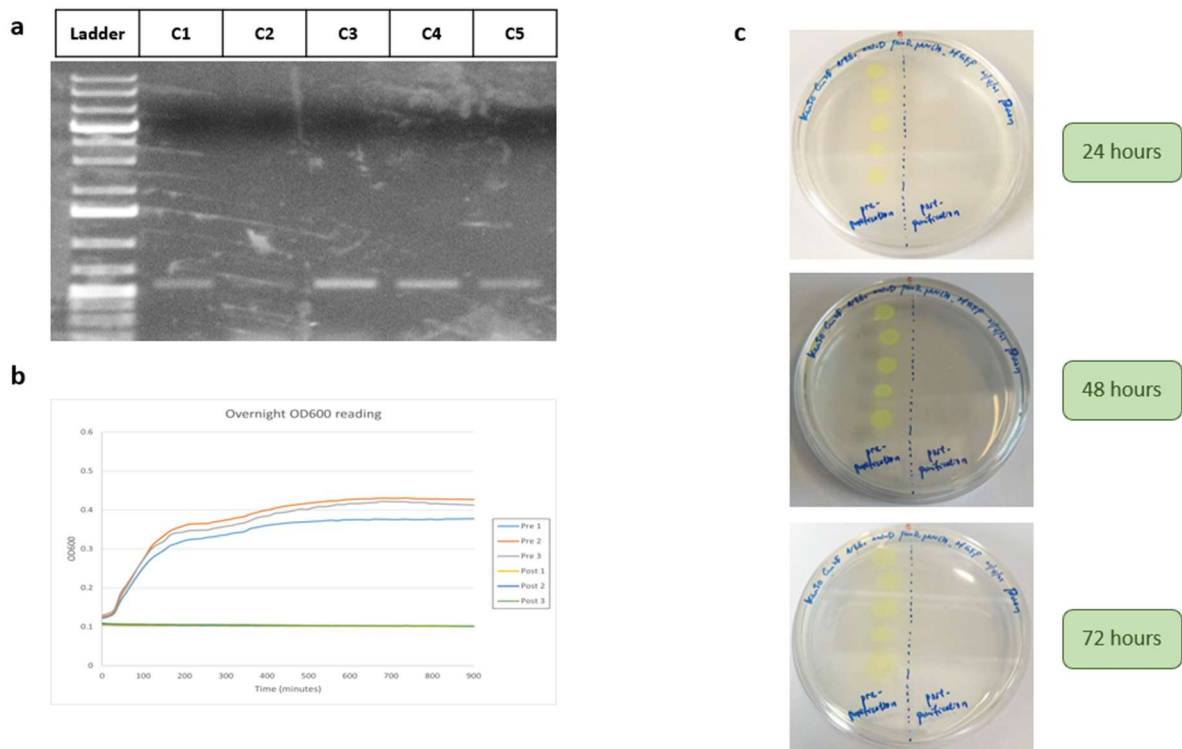


**Figure S5**

0 (left), 4 (mid) and 8 (right) hours incubation of engineered minicells carrying pNVC17\_sfGFP or pNVCYT\_sfGFP plasmids (green) with two different colorectal cancer cell lines: **(a)** Caco2, a high CEA expressing cell line and **(b)** SW480, a low CEA expressing cell. Nuclei of cancer cells are stained with Hoechst dye (blue). Scale bar is 100  $\mu\text{m}$ .

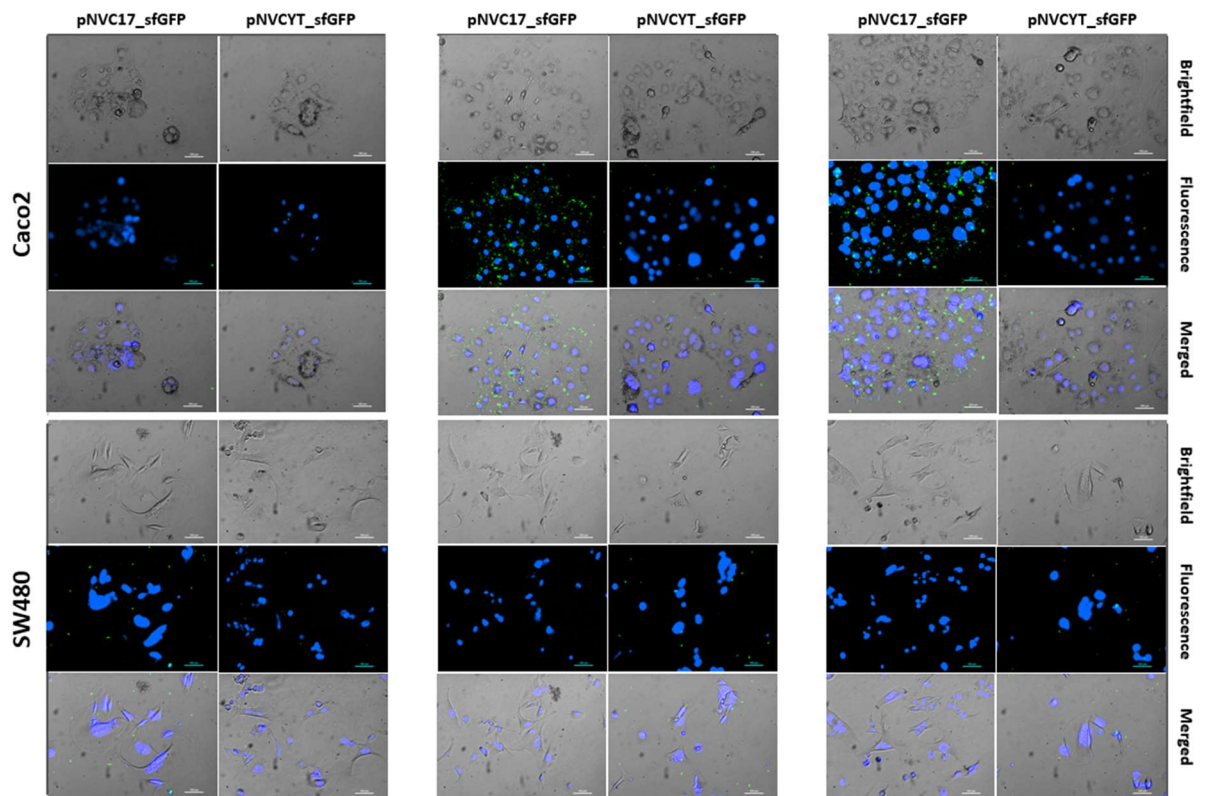


**Figure S6**

**(a)** Overnight OD<sub>600</sub> reading of SimCell pYB1 carrying pNVC43\_sfGFP after two hours induction of inducer cocktail X (inducer +) or without inducer (inducer -). Each line represents a single biological repeat. **(b)** Induced and uninduced SimCell pYB1 culture plated on LB-Kan-Cm agar for 24 hours (top), 48 hours (mid) and 72 hours (bottom) at 37 °C.

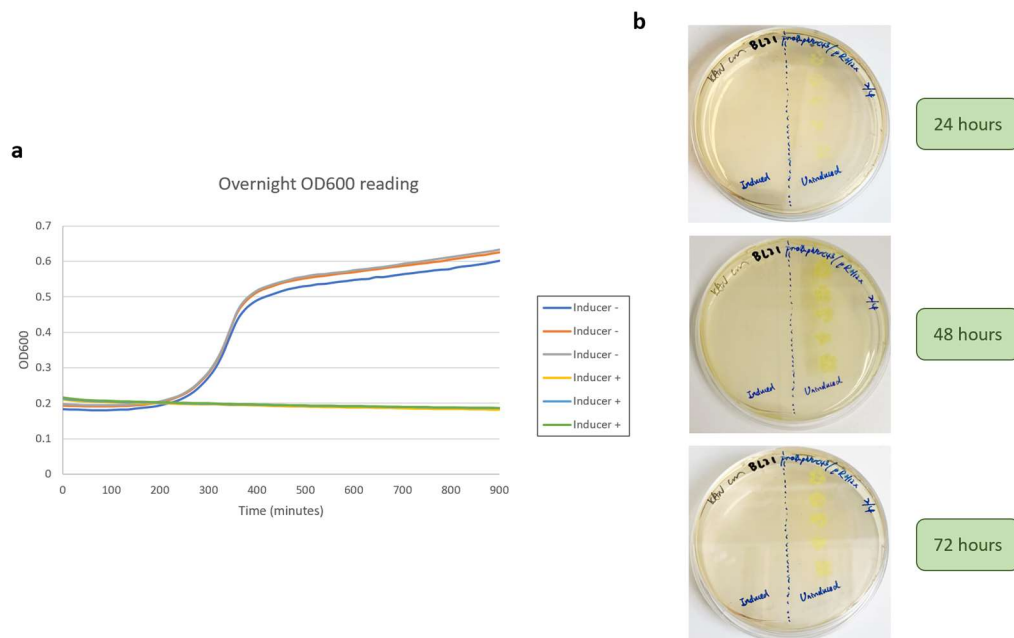


**Figure S7**

0 hour (~within 5 minutes) incubation of programmed SimCell pYB1 carrying pNVC43\_sfGFP or pNVCYT\_sfGFP with (+) and without (-) the addition of inducer cocktail X (green). Bacterial cells under inducer+ are genome less and non-dividing SimCells. **(a)** Caco2 and **(b)** SW480 are the high CEA expressing and low CEA expressing colorectal cancer cell lines, respectively. Nuclei were stained with Hoechst dye (blue). Scale bar is 100  $\mu$ m.

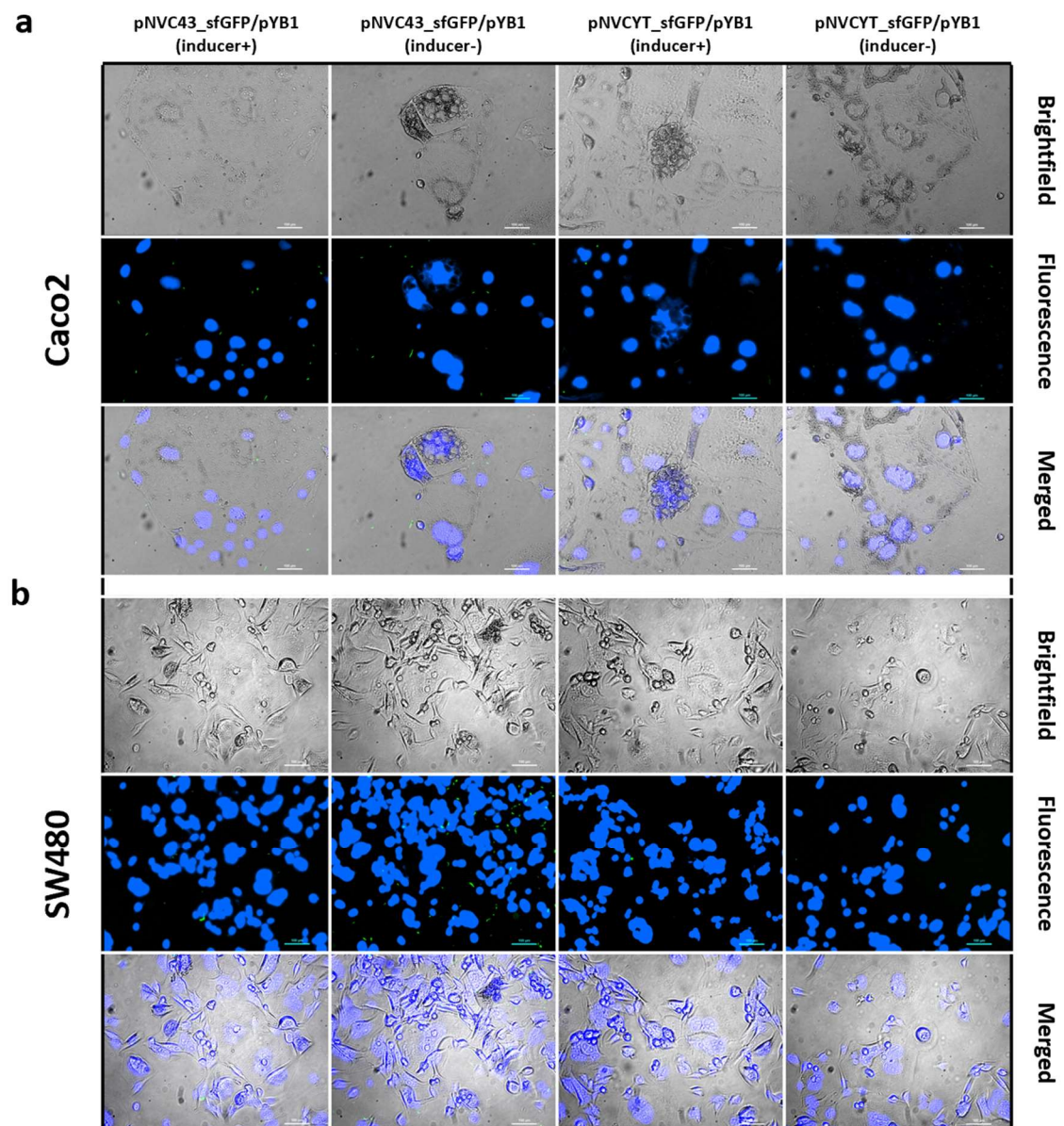


**Figure S8**

4 hours incubation of programmed SimCell pYB1 carrying pNVC43\_sfGFP or pNVCYT\_sfGFP with (+) and without (-) the addition of inducer cocktail X (green). Bacterial cells under inducer+ are genome less and non-dividing SimCells. **(a)** Caco2 and **(b)** SW480 are the high CEA expressing and low CEA expressing colorectal cancer cell lines, respectively. Nuclei were stained with Hoechst dye (blue). Scale bar is 100  $\mu$ m.

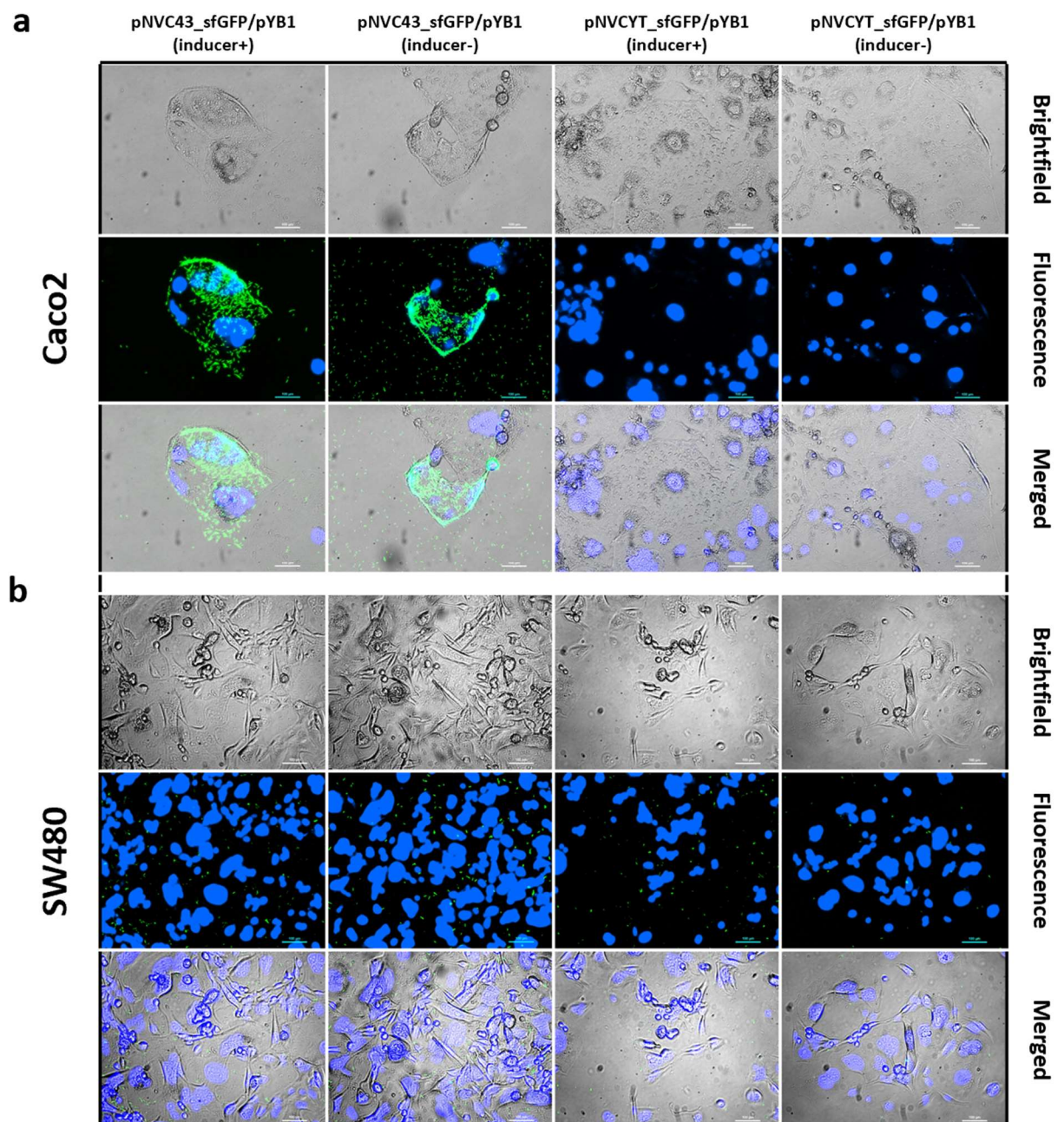
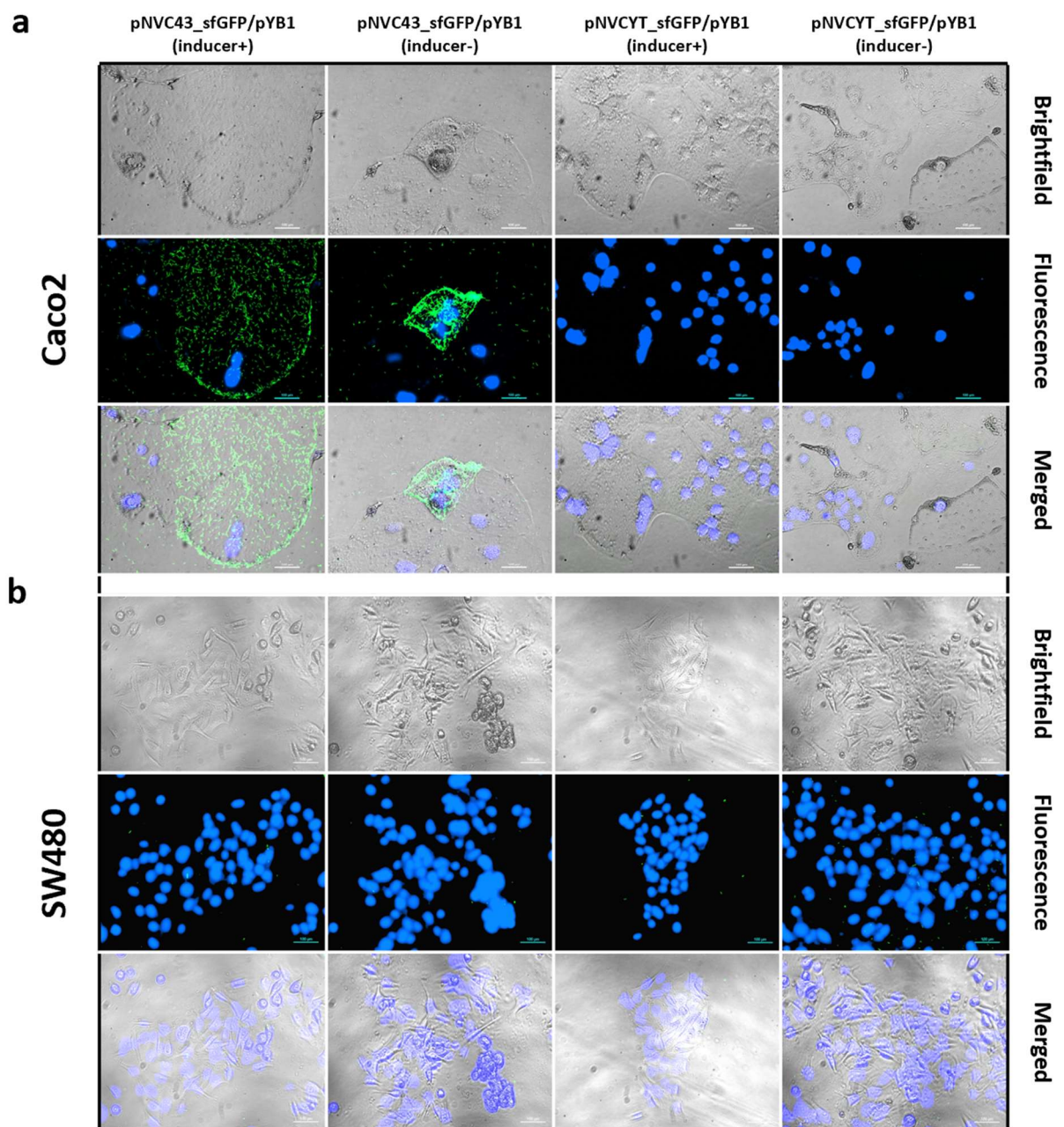


**Figure S9**

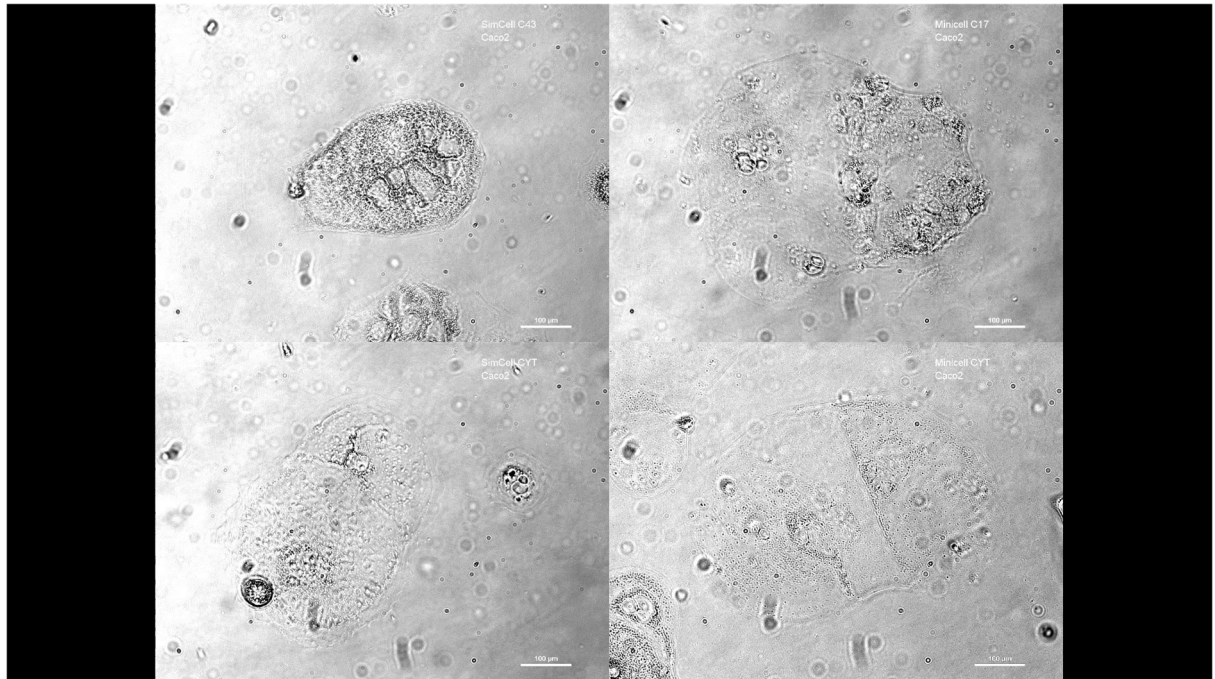
8 hours incubation of programmed SimCell pYB1 carrying pNVC43\_sfGFP or pNVCYT\_sfGFP with (+) and without (-) the addition of inducer cocktail X (green). Bacterial cells under inducer+ are genome less and non-dividing SimCells. **(a)** Caco2 and **(b)** SW480 are the high CEA expressing and low CEA expressing colorectal cancer cell lines, respectively. Nuclei were stained with Hoechst dye (blue). Scale bar is 100  $\mu$ m.



## Movie S10

Time lapse video of Caco2 after 2 hours incubation with SimCell + pNVC43\_sfGFP (top left); SimCell + pNVCYT\_sfGFP (bottom left); minicell + pNVC17\_sfGFP (top right); minicell + pNVCYT\_sfGFP (bottom right). All cultures were washed thrice and added with fresh media supplemented with Ethidium homodimer prior to imaging. The duration of the experiment is labelled in hh:mm:ss format on the top left. SimCell and minicell are in green and the nuclei of Caco2 with compromised membrane were stained by Ethidium homodimer (red). Scale bar is 100  $\mu$ m.

(Please access the video here:  
<https://1drv.ms/v/s!Atd6LwNkjPrwx158BYJ5FBiO6hxn?e=aladcC>)



## References

1. Pósfai, G. *et al.* Emergent Properties of Reduced-Genome *Escherichia coli*. *Science* **312**, 1044–1046 (2006).
2. Kylilis, N. *et al.* Whole-Cell Biosensor with Tunable Limit of Detection Enables Low-Cost Agglutination Assays for Medical Diagnostic Applications. *ACS Sens.* **4**, 370–378 (2019).
3. Fan, C. *et al.* Chromosome-free bacterial cells are safe and programmable platforms for synthetic biology. *Proc. Natl. Acad. Sci.* **117**, 6752–6761 (2020).
4. Jensen, S. I., Lennen, R. M., Herrgård, M. J. & Nielsen, A. T. Seven gene deletions in seven days: Fast generation of *Escherichia coli* strains tolerant to acetate and osmotic stress. *Sci. Rep.* **5**, 17874 (2015).
5. Kaliberov, S. A. *et al.* Adenoviral targeting using genetically incorporated camelid single variable domains. *Lab Invest* **94**, 893–905 (2014).
6. Behar, G. *et al.* Llama single-domain antibodies directed against nonconventional epitopes of tumor-associated carcinoembryonic antigen absent from nonspecific cross-reacting antigen. *FEBS J.* **276**, 3881–3893 (2009).
7. Hanke, L. *et al.* An alpaca nanobody neutralizes SARS-CoV-2 by blocking receptor interaction. *Nat. Commun.* **11**, 4420 (2020).

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## 6. Discussion and Concluding Remarks

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The translation of synthetic biological technologies into real-world applications, particularly in the healthcare settings, is heavily reliant on the stability of both the synthetic system and the environment in which the system is operating. The overarching goal of this thesis is to develop and improvise two different synthetic biological methods, namely reverse transcription, loop mediated isothermal amplification (RT-LAMP) and whole cell biosensor (WCB) that represent a cell-free system (CFS) and a top-down approach, respectively, to address two of the most pressing global health issues faced by humanity.

In Chapter 3 and 4, I re-purposed a traditional cell-free enzymatic reaction, RT-LAMP, to target different sites within the SARS-CoV-2 RNA genome as a mean to diagnose COVID-19. To further stabilise the assay for its deployment outside of a standard diagnostic laboratory, I introduced a short oligonucleotide complementary to the FIP primer to occlude the reaction from self-amplification or non-specific amplification. This modification reduces the risk of false positive which was frequently reported in a RT-LAMP assay<sup>209,255</sup> with minimal influence on the diagnostic sensitivity. In addition to other adaptations such as lyophilisation, the improvised RT-LAMP assay is ideal for the diagnosis of SARS-CoV-2 infection with high sensitivity, short turn-around time at a point-of-care setting.

As RT-LAMP has been previously developed to detect different infectious pathogens such as influenza viruses<sup>255</sup> and *Plasmodium falciparum*<sup>256</sup>, these assays can be quickly improvised through the addition of a molecular switch described herein. Another feature should be considered is the development of a multiplex RT-LAMP, where multiple targets can be identified within one RT-LAMP reaction. For example, different LAMP primers can be labelled with specific chemical compounds such as biotin to target different sequences, which can be subsequently isolated using an ELISA assay.<sup>257</sup> As the main symptoms of COVID-19 resemble a seasonal flu, such multiplex assay will enable the differentiation between multiple infectious agents and prevent the healthcare system from overloading.

The addition of a molecular switch to the RT-LAMP assay can be further optimised to generate a more predictable outcome. As this idea is inspired by the mode of action of miRNA, we can model the efficacy of the molecular switch by incorporating the thermodynamic of nucleic acid binding and folding into the design process<sup>258</sup>, which can be easily achieved through the modification of length, sequence and complementary target (i.e., FIP or BIP primer) of the molecular switch. Furthermore, the secondary structure of the molecular switch can also be optimised to construct a synthetic system with more complex behaviours.<sup>259</sup>

On the other hand, the diagnostic process also requires streamlining beyond the RT-LAMP assay. Currently, most healthcare professions adopt nasopharyngeal specimen followed by RNA extraction to diagnose respiratory infections.<sup>260</sup> Although generally considered safe, a small number of adverse events were reported from the use of nasopharyngeal swab such as the leakage of cerebrospinal fluid resulting from nasal swabbing procedures.<sup>261</sup> Although the occurrence of such iatrogenic event is extremely rare, a more user-friendly and less intrusive sampling method compatible with the downstream molecular assay can simplify the diagnostic process and ease the implementation of a nation-wide testing. In this regard, we are trialling the use of gargling sample for the detection of SARS-CoV-2 while investigating different additives such as polyvinylsulfonic acid<sup>262</sup> to preserve the quality of RNA in the sample. As described in section 2.3.1, CFS is noticeably sensitive to components present within the reaction matrix and thus every modification prior to the RT-LAMP assay needs to be enforced meticulously to ensure its effectiveness.

Nevertheless, it should be recognised that no test is completely fool proof, and even the ‘gold standard’ assay, RT-qPCR could succumb to false test results due to sample mishandling, sample degradation or cross contamination. Both types of false results can carry serious repercussions: a high false positive rate might overestimate the extent of the disease which could consequently restricts societal activities that could further lead to huge mental and economical impacts; A high false negative rate might give a false sense of security that could promote the transmission of disease which could lead to further outbreaks. In general, a nucleic acid amplification test (NAAT) has a better testing performance in both regards compared to an antigen- or antibody-based lateral

flow test (LFT). For example, high incidence of COVID-19 false positive was reported in Japan when an antibody LFT was used due to its cross-reactivity to the antibody against a human common cold coronavirus.<sup>263</sup> Meanwhile, a study also reported that an antibody LFT that uses the combination of IgG/IgM to detect the presence of SARS-CoV-2 only have a sensitivity of 30.1% during the first week since the onset of symptoms, which then increases to 72.2% and 91.4% on the second week and third week, respectively.<sup>264</sup> Despite its cost advantage, these studies indicated the inherent inferiority of a LFT when compared to a NAAT, where its detection method (by using primers and nucleic acid amplification) and detection target (by targeting pathogen's genome) can reduce both the false positive and false negative rates.

In chapter 5, I proposed a WCB using nanobody (VHH) as its sensing element to detect carcinoembryonic antigen (CEA), a prominent protein biomarker found in 70% of all human cancers.<sup>265</sup> This diagnostic format mimics a clinically used agglutination assay where the cross-linking between VHH and target analyte can be visualised through naked eyes.<sup>266</sup> More importantly, WCB circumvents the process of VHH production and purification which are indispensable in the manufacturing process of a conventional agglutination or ELISA assay, hence reduces the cost significantly.

An immediate improvement step for the biological agglutination test is the removal of bacterial genome to generate SimCell or minicell from the WCB. This modification can enhance the biosafety of the assay to allow field-site deployment. Besides, the assay needs to be further refined to accelerate results visualisation. Currently, the biological agglutination test requires around 16 hours for the WCB to sediment, which could potentially be shortened via low-speed centrifugation. Other parameters such as pH and temperature of the medium should also be considered as they could affect the interaction between WCB and its target analyte which thereby influences the assay duration.

WCB can be adapted into SimCell or minicell to fit different purposes. For minicell, its dimension at the nano-meter range can be exploited for high-resolution microscopic imaging. In contrast to nanoparticle- or fluorescein-conjugated antibody, minicell can be modified to produce fluorescence or luminescence through simple genetic engineering. More importantly, once the underlying DNA sequence of an ideal VHH

candidate is identified, the expression of VHH on minicell requires only engineering at the DNA node which avoids the laborious procedure of antibody production, purification and conjugation. Different synthetic circuits can be engineered to further functionalise the minicell for *in situ* imaging purposes, for example minicell binding to its target could express different reporters in response to its proximal environment to indicate the *in situ* biological state.

In contrast to minicell, SimCell has a comprehensive cellular content to process more complicated or energy intensive genetic circuits. In this regard, SimCell is well-suited to be developed as a controllable and programmable *in vivo* therapy. Using different responsive promoters and reporter genes (as described in section 2.5.2), the binding of SimCell onto targeted cancer cells can be combined with user-defined therapeutic payloads to achieve tumour clearance. By decoupling growth and thereby removing the inherent risk of genetic mutation, SimCell addresses the main concern of most regulatory bodies to prevent the uncontrollable spread of living therapeutics within a patient.<sup>267,268</sup> As each functionality is contributed by an individual genetic circuit, SimCell represents a modular and customisable synthetic biological chassis that has enormous potential to be the new frontier of bacterial therapeutic.

## **Future Outlook**

The overarching goal of this thesis is to improve the genetic stability of a synthetic biological system for its translational applications. This objective is highlighted in the two research questions outlined in Chapter 1 which are addressed throughout the thesis with a strong build-to-deploy theme. As the renowned Nobel Physicist Richard Feynman once said:

*What I cannot create, I do not understand.*

It is through the rigorous design-build-test-learn cycle we can challenge our understanding of the biology underpinning a synthetically constructed system. Contributing to this objective while developing translational technologies to tackle pressing societal challenges is what this thesis has strived to achieve.

Lastly, I propose multiple studies to follow up the research described herein:

Study of regulatory RNA can provide more insights into the engineering of RT-LAMP. As described in Chapter 4, the idea of adopting a molecular switch is inspired by the mode of action of miRNA. To exert a more complex computation for the cell-free enzymatic reaction, other regulator RNA component such as riboswitch<sup>269</sup> or toe-hoe switch<sup>270</sup> can be introduced. For example, the current RT-LAMP diagnostic system for COVID-19 is single-plex and lacks an internal human control, unlike RT-qPCR which uses several primer sets to identify multiple targets on the SARS-CoV-2 genome together with a human target (usually the human *RNase P*<sup>190</sup>). To overcome this shortcoming, a toe-hoe switch can be designed to sequester the FIP or BIP primer when the *RNase P* is absence. Only when *RNase P* is present, and thus confirming the sample coming from a human, FIP or BIP primer will be released from the toe-hoe switch and proceed to initiate the LAMP reaction. By attaching a fluorescence probe and a quencher to these toe-hoe switches, presence of the *RNase P* can be elucidated via fluorescence signal even without the nucleic acid amplification process, indicating a human sample without SARS-CoV-2 RNA. Different sRNA-based feedback systems (e.g., a synthetic negative feedback circuit<sup>271</sup>) can be incorporated to further modulate the diagnostic test, i.e. a negative feedback system to reduce false positive and a positive feedback system to increase sensitivity.

The deployment of LAMP should not be restricted to infectious diseases. As discussed previously (in Section 2.5.1), the extraction and analysis of biological molecules from human samples, especially miRNA and cfDNA, can aid in the diagnosis and prognosis of different diseases.<sup>91,92,224,225</sup> However, in contrast to infectious diseases in which a qualitative output is sufficient, most diseases such as metabolic disorders and cancers require a quantitative analysis of selected biomarkers to provide a comprehensive diagnosis. In this regard, LAMP needs to be modified to provide real-time or quantitative information to support the diagnosis of these diseases. A quick solution will be the incorporation of a fluorescence dye (e.g., SYTO-9) and a reader to mimic the generation of threshold cycle (Ct) value as seen in a qPCR. By calibrating the LAMP reaction using disease-specific primers, it is possible to correlate the production of LAMP amplicons

to the amount of starting material which could then provide an indication to the disease status.

The WCB described in Chapter 5 uses a biological agglutination test format to detect specific protein biomarker semi-quantitatively. To increase its accessibility and usability, this assay format can be modified into a lateral flow test (LFT) since the principle of both assays is based on the formation of antibody-target-antibody sandwich. The functionality of this LFT can then be improvised through different genetic circuits, for example to produce luminescence from lux operon or blue stain from  $\beta$ -galactosidase and X-gal. As the assay specificity is defined by the VHH expressed on the WCB, this LFT can be modified to target different diseases by changing the underlying VHH sequence, including COVID-19 by using a spike protein targeting nanobody.<sup>272</sup> Similarly, a LFT detecting CEA and CA19-9 from a biological sample will be beneficial for the prognosis and assessment of cancer treatment.

For *in vivo* therapeutic, it is essential to remove the remaining endotoxin (i.e., lipopolysaccharide (LPS)) on the outer membrane of a SimCell. As the expression of ICEuI is controlled by an isolated circuit, it is possible to transform the probiotic, LPS deficient strain, *E. coli* Nissle 1917 (EcN) (described in Section 2.3.3) with the ICEuI system to generate a SimCell with anti-inflammatory activity.<sup>273</sup> However, as the expression and folding of nanobody need to be precisely maintained to ensure the accurate binding of SimCell to its *in vivo* target, EcN will need to be further manipulated (e.g., deletion of protease genes such as *ompT* and *lon* which are commonly found in a BL21 strain<sup>274</sup>) to improve the quality of a recombinant protein expressed in this strain. Alternatively, the BL21 strain used in Chapter 5 can be attenuated by deleting its LPS production pathway<sup>275</sup> to detoxify the SimCell produced later. Notably, modification of LPS will not mitigate innate complement driven and reticuloendothelial cell mediated neutralisation and clearance of bacterial constructs and thus strategies to enhance pharmacokinetics and pharmacodynamics of bacteria-based therapy are required before its translation into intravenous delivery applications such as metastatic cancer.

In addition, it is vital to characterise the mechanism underlying the cancer killing effect achieved by SimCell. Recently, natural invasion of intratumoral bacteria into melanoma

tumours was reported<sup>276</sup>, suggesting the possibility of cancer killing achieved by bacteria inside the cancer cells. It is also possible to engineer the bacteria to actively invade a cancer cell at its close proximity<sup>54</sup>, in which the surface-displayed nanobody system could potentially enhance its effectiveness. Multiple cancer-killing circuits have been reported previously, and thus their compatibility with SimCell and their efficacy on different cancers need to be thoroughly investigated to find the best combination. On the other hand, instead of binding cancer cells, SimCell could also target immune cells such as antigen presenting cell to activate the adaptive immune response. Moving forward, the use of 3D tumoroid as well as macrophage culture need to be prioritised to examine the efficacy and immunogenicity of SimCell-based bacterial cancer therapy before heading into animal or human study.

In the longer term, the combination of diagnostic and therapeutic aspects of WCB will be instrumental in developing personalised medicines. A diagnostic panel to identify the most relevant biomarkers of a disease can direct the construction of a SimCell therapy. These advances will facilitate the development of synthetic biology to provide novel medicines to many of the 21<sup>st</sup> century's most challenging diseases.

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## References

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1. Sprinzak, D. & Elowitz, M. B. Reconstruction of genetic circuits. *Nature* **438**, 443–448 (2005).
2. Süel, G. M., Garcia-Ojalvo, J., Liberman, L. M. & Elowitz, M. B. An excitable gene regulatory circuit induces transient cellular differentiation. *Nature* **440**, 545–550 (2006).
3. Hutchison, C. A. *et al.* Design and synthesis of a minimal bacterial genome. *Science* **351**, (2016).
4. Wan, X. *et al.* Cascaded amplifying circuits enable ultrasensitive cellular sensors for toxic metals. *Nat. Chem. Biol.* **15**, 540–548 (2019).
5. Courbet, A., Endy, D., Renard, E., Molina, F. & Bonnet, J. Detection of pathological biomarkers in human clinical samples via amplifying genetic switches and logic gates. *Sci. Transl. Med.* **7**, 289ra83–289ra83 (2015).
6. Leventhal, D. S. *et al.* Immunotherapy with engineered bacteria by targeting the STING pathway for anti-tumor immunity. *Nat. Commun.* **11**, 2739 (2020).
7. Shi, T., Han, P., You, C. & Zhang, Y.-H. P. J. An in vitro synthetic biology platform for emerging industrial biomanufacturing: Bottom-up pathway design. *Synth. Syst. Biotechnol.* **3**, 186–195 (2018).
8. Monod, J. & Jacob, F. General Conclusions: Teleonomic Mechanisms in Cellular Metabolism, Growth, and Differentiation. *Cold Spring Harb. Symp. Quant. Biol.* **26**, 389–401 (1961).
9. Kimata, K., Takahashi, H., Inada, T., Postma, P. & Aiba, H. cAMP receptor protein–cAMP plays a crucial role in glucose–lactose diauxie by activating the major glucose transporter gene in *Escherichia coli*. *Proc. Natl. Acad. Sci.* **94**, 12914–12919 (1997).
10. The Nobel Prize in Physiology or Medicine 1965. *NobelPrize.org* <https://www.nobelprize.org/prizes/medicine/1965/summary/>.
11. Madan Babu, M. & Teichmann, S. A. Evolution of transcription factors and the gene regulatory network in *Escherichia coli*. *Nucleic Acids Res.* **31**, 1234–1244 (2003).
12. Kleppe, K., Ohtsuka, E., Kleppe, R., Molineux, I. & Khorana, H. G. Studies on polynucleotides. XCVI. Repair replications of short synthetic DNA's as catalyzed by DNA polymerases. *J. Mol. Biol.* **56**, 341–361 (1971).
13. Danna, K. & Nathans, D. Specific cleavage of simian virus 40 DNA by restriction endonuclease of *Hemophilus influenzae*. *Proc. Natl. Acad. Sci.* **68**, 2913–2917 (1971).
14. Lehman, I. R. DNA ligase: structure, mechanism, and function. *Science* **186**, 790–797 (1974).
15. Hunkapiller, T., Kaiser, R. J., Koop, B. F. & Hood, L. Large-scale and automated DNA sequence determination. *Science* **254**, 59–67 (1991).
16. Uetz, P. *et al.* A comprehensive analysis of protein–protein interactions in *Saccharomyces cerevisiae*. *Nature* **403**, 623–627 (2000).
17. Scherf, M., Klingenhoff, A. & Werner, T. Highly specific localization of promoter regions in large genomic sequences by PromoterInspector: a novel context analysis approach. Edited by J. Karn. *J. Mol. Biol.* **297**, 599–606 (2000).

18. Hartwell, L. H., Hopfield, J. J., Leibler, S. & Murray, A. W. From molecular to modular cell biology. *Nature* **402**, C47–C52 (1999).
19. Benner, S. A. Synthetic biology: Act natural. *Nature* **421**, 118–118 (2003).
20. Gardner, T. S., Cantor, C. R. & Collins, J. J. Construction of a genetic toggle switch in *Escherichia coli*. *Nature* **403**, 339–342 (2000).
21. Elowitz, M. B. & Leibler, S. A synthetic oscillatory network of transcriptional regulators. *Nature* **403**, 335–338 (2000).
22. Atkinson, M. R., Savageau, M. A., Myers, J. T. & Ninfa, A. J. Development of Genetic Circuitry Exhibiting Toggle Switch or Oscillatory Behavior in *Escherichia coli*. *Cell* **113**, 597–607 (2003).
23. Friedland, A. E. *et al.* Synthetic Gene Networks That Count. *Science* **324**, 1199–1202 (2009).
24. Siuti, P., Yazbek, J. & Lu, T. K. Synthetic circuits integrating logic and memory in living cells. *Nat. Biotechnol.* **31**, 448–452 (2013).
25. Tamsir, A., Tabor, J. J. & Voigt, C. A. Robust multicellular computing using genetically encoded NOR gates and chemical ‘wires’. *Nature* **469**, 212–215 (2011).
26. Wang, B., Barahona, M. & Buck, M. Engineering modular and tunable genetic amplifiers for scaling transcriptional signals in cascaded gene networks. *Nucleic Acids Res* **42**, 9484–9492 (2014).
27. Hooshangi, S., Thiberge, S. & Weiss, R. Ultrasensitivity and noise propagation in a synthetic transcriptional cascade. *Proc. Natl. Acad. Sci.* **102**, 3581–3586 (2005).
28. Wiedenheft, B., Sternberg, S. H. & Doudna, J. A. RNA-guided genetic silencing systems in bacteria and archaea. *Nature* **482**, 331–338 (2012).
29. Bikard, D. *et al.* Programmable repression and activation of bacterial gene expression using an engineered CRISPR-Cas system. *Nucleic Acids Res.* **41**, 7429–7437 (2013).
30. Larson, M. H. *et al.* CRISPR interference (CRISPRi) for sequence-specific control of gene expression. *Nat. Protoc.* **8**, 2180–2196 (2013).
31. Whitaker, W. R., Davis, S. A., Arkin, A. P. & Dueber, J. E. Engineering robust control of two-component system phosphotransfer using modular scaffolds. *Proc. Natl. Acad. Sci.* **109**, 18090–18095 (2012).
32. Chau, A. H., Walter, J. M., Gerardin, J., Tang, C. & Lim, W. A. Designing Synthetic Regulatory Networks Capable of Self-Organizing Cell Polarization. *Cell* **151**, 320–332 (2012).
33. Cardinale, S. & Arkin, A. P. Contextualizing context for synthetic biology – identifying causes of failure of synthetic biological systems. *Biotechnol. J.* **7**, 856–866 (2012).
34. Kwok, R. Five hard truths for synthetic biology. *Nature* **463**, 288–290 (2010).
35. parts.igem.org. [http://parts.igem.org/Main\\_Page](http://parts.igem.org/Main_Page).
36. Kelly, J. R. *et al.* Measuring the activity of BioBrick promoters using an in vivo reference standard. *J. Biol. Eng.* **3**, 4 (2009).
37. Smolke, C. D. Building outside of the box: iGEM and the BioBricks Foundation. *Nat. Biotechnol.* **27**, 1099–1102 (2009).

38. Nyström, A., Papachristodoulou, A. & Angel, A. A Dynamic Model of Resource Allocation in Response to the Presence of a Synthetic Construct. *ACS Synth. Biol.* **7**, 1201–1210 (2018).
39. Dai, X. *et al.* Reduction of translating ribosomes enables *Escherichia coli* to maintain elongation rates during slow growth. *Nat. Microbiol.* **2**, 1–9 (2016).
40. Ceroni, F., Algar, R., Stan, G.-B. & Ellis, T. Quantifying cellular capacity identifies gene expression designs with reduced burden. *Nat. Methods* **12**, 415–418 (2015).
41. The Cost of Sequencing a Human Genome. *Genome.gov* <https://www.genome.gov/about-genomics/fact-sheets/Sequencing-Human-Genome-cost>.
42. Hughes, R. A. & Ellington, A. D. Synthetic DNA Synthesis and Assembly: Putting the Synthetic in Synthetic Biology. *Cold Spring Harb. Perspect. Biol.* **9**, (2017).
43. Yim, H. *et al.* Metabolic engineering of *Escherichia coli* for direct production of 1,4-butanediol. *Nat. Chem. Biol.* **7**, 445–452 (2011).
44. Zhang, F., Carothers, J. M. & Keasling, J. D. Design of a dynamic sensor-regulator system for production of chemicals and fuels derived from fatty acids. *Nat. Biotechnol.* **30**, 354–359 (2012).
45. Luo, X. *et al.* Complete biosynthesis of cannabinoids and their unnatural analogues in yeast. *Nature* **567**, 123–126 (2019).
46. Krishna, S., Bustamante, L., Haynes, R. K. & Staines, H. M. Artemisinins: their growing importance in medicine. *Trends Pharmacol. Sci.* **29**, 520–527 (2008).
47. Paddon, C. J. *et al.* High-level semi-synthetic production of the potent antimalarial artemisinin. *Nature* **496**, 528–532 (2013).
48. Westfall, P. J. *et al.* Production of amorphadiene in yeast, and its conversion to dihydroartemisinic acid, precursor to the antimalarial agent artemisinin. *Proc. Natl. Acad. Sci.* **109**, E111–E118 (2012).
49. Amyris. <https://amyris.com/>.
50. ‘Biofacturing’ Firm Zymergen Raises \$500 Million in Expanded IPO. *Bloomberg.com* (2021).
51. Elon Musk. @utsavtechie Prototypes are easy, production is hard. @elonmusk <https://twitter.com/elonmusk/status/1389102532706848768> (2021).
52. Bio-based products. [https://ec.europa.eu/growth/sectors/biotechnology/bio-based-products\\_en](https://ec.europa.eu/growth/sectors/biotechnology/bio-based-products_en).
53. Maxmen, A. & Mallapaty, S. The COVID lab-leak hypothesis: what scientists do and don’t know. *Nature* **594**, 313–315 (2021).
54. Anderson, J. C., Clarke, E. J., Arkin, A. P. & Voigt, C. A. Environmentally Controlled Invasion of Cancer Cells by Engineered Bacteria. *J. Mol. Biol.* **355**, 619–627 (2006).
55. de Groot, M. J., Hoeksma, M., Blau, N., Reijngoud, D. J. & van Spronsen, F. J. Pathogenesis of cognitive dysfunction in phenylketonuria: Review of hypotheses. *Mol. Genet. Metab.* **99**, S86–S89 (2010).
56. Isabella, V. M. *et al.* Development of a synthetic live bacterial therapeutic for the human metabolic disease phenylketonuria. *Nat. Biotechnol.* **36**, 857–864 (2018).
57. Duong, M. T.-Q., Qin, Y., You, S.-H. & Min, J.-J. Bacteria-cancer interactions: bacteria-based cancer therapy. *Exp. Mol. Med.* **51**, 1–15 (2019).

58. Synlogic scraps ammonia-lowering drug after early phase fail. *FierceBiotech* <https://www.fiercebiotech.com/biotech/synlogic-scraps-ammonia-lowering-drug-after-early-phase-fail>.
59. Early Clinical Trials with Live Biotherapeutic Products: Chemistry, Manufacturing, and Control Information; Guidance for Industry. 20.
60. Live Biotherapeutic Products (LBPs): unprecedented quality requirements by the Ph. Eur. Commission | EDQM - European Directorate for the Quality of Medicines. <https://www.edqm.eu/en/news/live-biotherapeutic-products-lbps-unprecedented-quality-requirements-ph-eur-commission>.
61. Chan, C. T. Y., Lee, J. W., Cameron, D. E., Bashor, C. J. & Collins, J. J. 'Deadman' and 'Passcode' microbial kill switches for bacterial containment. *Nat. Chem. Biol.* **12**, 82–86 (2016).
62. Denamur, E. & Matic, I. Evolution of mutation rates in bacteria. *Mol. Microbiol.* **60**, 820–827 (2006).
63. Giraud, A. Costs and Benefits of High Mutation Rates: Adaptive Evolution of Bacteria in the Mouse Gut. *Science* **291**, 2606–2608 (2001).
64. Huang, W. E. *et al.* RT-LAMP for rapid diagnosis of coronavirus SARS-CoV-2. *Microb. Biotechnol.* **13**, 950–961 (2020).
65. Lim, B. *et al.* Clinical validation of optimised RT-LAMP for the diagnosis of SARS-CoV-2 infection. *Sci. Rep.* **11**, 16193 (2021).
66. Rapid COVID-19 test, Oxsed RaViD Direct, now in use at Heathrow and trials at Hong Kong International Airport | University of Oxford. <https://www.ox.ac.uk/news/2020-11-04-rapid-covid-19-test-oxsed-ravid-direct-now-use-heathrow-and-trials-hong-kong>.
67. Test showing if someone is infectious could help halt pandemic spread - Royal Academy of Engineering. <https://www.raeng.org.uk/grants-prizes/prizes/prizes-and-medals/awards/presidents-special-awards-pandemic-service/test-infectious>.
68. Crick, F. H. C. ON PROTEIN SYNTHESIS. *Mol. Biol.* **27** (1956).
69. Crick, F. Central Dogma of Molecular Biology. **3** (1970).
70. Cobb, M. 60 years ago, Francis Crick changed the logic of biology. *PLoS Biol.* **15**, (2017).
71. Alberts, B. *et al.* *Molecular Biology of the Cell*. (Garland Science, 2002).
72. Temin, H. M. & Mizutani, S. Viral RNA-dependent DNA Polymerase: RNA-dependent DNA Polymerase in Virions of Rous Sarcoma Virus. *Nature* **226**, 1211–1213 (1970).
73. Ahlquist, P. RNA-Dependent RNA Polymerases, Viruses, and RNA Silencing. *Science* **296**, 1270–1273 (2002).
74. Raina, M. & Ibba, M. tRNAs as regulators of biological processes. *Front. Genet.* **5**, (2014).
75. Dana, H. *et al.* Molecular Mechanisms and Biological Functions of siRNA. *Int. J. Biomed. Sci. IJBS* **13**, 48–57 (2017).
76. Ellis, R. J. Discovery of Molecular Chaperones. *Cell Stress Chaperones* **1**, 155–160 (1996).
77. Moore, L. D., Le, T. & Fan, G. DNA Methylation and Its Basic Function. *Neuropsychopharmacology* **38**, 23–38 (2013).

78. Nagata, S. *et al.* Synthesis in *E. coli* of a polypeptide with human leukocyte interferon activity. *Nature* **284**, 316–320 (1980).
79. Goeddel, D. V. *et al.* Expression in *Escherichia coli* of chemically synthesized genes for human insulin. *Proc. Natl. Acad. Sci.* **76**, 106–110 (1979).
80. Smith, H. O., Hutchison, C. A., Pfannkoch, C. & Venter, J. C. Generating a synthetic genome by whole genome assembly:  $\phi$ X174 bacteriophage from synthetic oligonucleotides. *Proc. Natl. Acad. Sci.* **100**, 15440–15445 (2003).
81. Gibson, D. G. *et al.* Complete Chemical Synthesis, Assembly, and Cloning of a *Mycoplasma genitalium* Genome. *Science* **319**, 1215–1220 (2008).
82. Ruegg, T. L. *et al.* Jungle Express is a versatile repressor system for tight transcriptional control. *Nat. Commun.* **9**, 3617 (2018).
83. Chen, J. X. *et al.* Redesign of ultrasensitive and robust RecA gene circuit to sense DNA damage. *Microb. Biotechnol.* (2021) doi:10.1111/1751-7915.13767.
84. Zhang, A. P. P., Pigli, Y. Z. & Rice, P. A. Structure of the LexA–DNA complex and implications for SOS box measurement. *Nature* **466**, 883–886 (2010).
85. Salis, H. M., Mirsky, E. A. & Voigt, C. A. Automated design of synthetic ribosome binding sites to control protein expression. *Nat. Biotechnol.* **27**, 946–950 (2009).
86. Fiers, W. & Grosjean, H. On codon usage. *Nature* **277**, 328–328 (1979).
87. Fu, H. *et al.* Codon optimization with deep learning to enhance protein expression. *Sci. Rep.* **10**, 17617 (2020).
88. Bartel, D. P. MicroRNA Target Recognition and Regulatory Functions. *Cell* **136**, 215–233 (2009).
89. Na, D. *et al.* Metabolic engineering of *Escherichia coli* using synthetic small regulatory RNAs. *Nat. Biotechnol.* **31**, 170–174 (2013).
90. Soutschek, J. *et al.* Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature* **432**, 173–178 (2004).
91. Peng, Y. & Croce, C. M. The role of MicroRNAs in human cancer. *Signal Transduct. Target. Ther.* **1**, 1–9 (2016).
92. van den Berg, M. M. J. *et al.* Circulating microRNAs as potential biomarkers for psychiatric and neurodegenerative disorders. *Prog. Neurobiol.* **185**, 101732 (2020).
93. Waldminghaus, T., Kortmann, J., Gesing, S. & Narberhaus, F. Generation of synthetic RNA-based thermosensors. **389**, 1319–1326 (2008).
94. Fung, E. *et al.* A synthetic gene–metabolic oscillator. *Nature* **435**, 118–122 (2005).
95. Liu, Z. *et al.* Programming Bacteria With Light—Sensors and Applications in Synthetic Biology. *Front. Microbiol.* **9**, (2018).
96. Javan, B. & Shahbazi, M. Hypoxia-inducible tumour-specific promoters as a dual-targeting transcriptional regulation system for cancer gene therapy. *Ecancermedicalscience* **11**, 751 (2017).
97. Anderson, J. C., Voigt, C. A. & Arkin, A. P. Environmental signal integration by a modular AND gate. *Mol. Syst. Biol.* **3**, 133 (2007).

98. Neelson, K. H., Platt, T. & Hastings, J. W. Cellular Control of the Synthesis and Activity of the Bacterial Luminescent System I. *J. Bacteriol.* **104**, 313–322 (1970).
99. Tabor, J. J. *et al.* A Synthetic Genetic Edge Detection Program. *Cell* **137**, 1272–1281 (2009).
100. Mandal, M. & Breaker, R. R. Gene regulation by riboswitches. *Nat. Rev. Mol. Cell Biol.* **5**, 451–463 (2004).
101. Chung, B. Y. W. *et al.* An RNA thermoswitch regulates daytime growth in Arabidopsis. *Nat. Plants* **6**, 522–532 (2020).
102. Muranaka, N., Sharma, V., Nomura, Y. & Yokobayashi, Y. Efficient Design Strategy for Whole-Cell and Cell-Free Biosensors based on Engineered Riboswitches. *Anal. Lett.* **42**, 108–122 (2009).
103. Serganov, A. & Nudler, E. A Decade of Riboswitches. *Cell* **152**, 17–24 (2013).
104. Wieland, M., Benz, A., Klauser, B. & Hartig, J. S. Artificial Ribozyme Switches Containing Natural Riboswitch Aptamer Domains. *Angew. Chem. Int. Ed.* **48**, 2715–2718 (2009).
105. Bayer, T. S. & Smolke, C. D. Programmable ligand-controlled riboregulators of eukaryotic gene expression. *Nat. Biotechnol.* **23**, 337–343 (2005).
106. d’Oelsnitz, S. & Ellington, A. Continuous directed evolution for strain and protein engineering. *Curr. Opin. Biotechnol.* **53**, 158–163 (2018).
107. Behar, G. *et al.* Llama single-domain antibodies directed against nonconventional epitopes of tumor-associated carcinoembryonic antigen absent from nonspecific cross-reacting antigen. *FEBS J.* **276**, 3881–3893 (2009).
108. Senior, A. W. *et al.* Improved protein structure prediction using potentials from deep learning. *Nature* **577**, 706–710 (2020).
109. Elowitz, M. B., Levine, A. J., Siggia, E. D. & Swain, P. S. Stochastic Gene Expression in a Single Cell. *Science* **297**, 1183–1186 (2002).
110. BioBricks Foundation | Biotechnology in the public interest. <https://biobricks.org/>.
111. Peebo, K. *et al.* Proteome reallocation in Escherichia coli with increasing specific growth rate. *Mol. Biosyst.* **11**, 1184–1193 (2015).
112. Lynch, M. & Marinov, G. K. The bioenergetic costs of a gene. *Proc. Natl. Acad. Sci.* **112**, 15690–15695 (2015).
113. Gyorgy, A. *et al.* Isocost Lines Describe the Cellular Economy of Genetic Circuits. *Biophys. J.* **109**, 639–646 (2015).
114. Cookson, N. A. *et al.* Queueing up for enzymatic processing: correlated signaling through coupled degradation. *Mol. Syst. Biol.* **7**, 561 (2011).
115. Cavaliere, P. *et al.* The stress sigma factor of RNA polymerase RpoS/σS is a solvent-exposed open molecule in solution. *Biochem. J.* **475**, 341–354 (2018).
116. Kimelman, A. *et al.* A vast collection of microbial genes that are toxic to bacteria. *Genome Res.* **22**, 802–809 (2012).
117. Saïda, F. Overview on the expression of toxic gene products in Escherichia coli. *Curr. Protoc. Protein Sci.* **Chapter 5**, Unit 5.19 (2007).

118. Novick, A. & Weiner, M. ENZYME INDUCTION AS AN ALL-OR-NONE PHENOMENON\*. *Proc. Natl. Acad. Sci. U. S. A.* **43**, 553–566 (1957).
119. Ferguson, M. L. *et al.* Reconciling molecular regulatory mechanisms with noise patterns of bacterial metabolic promoters in induced and repressed states. *Proc. Natl. Acad. Sci.* **109**, 155–160 (2012).
120. Singh, A. & Soltani, M. Quantifying Intrinsic and Extrinsic Variability in Stochastic Gene Expression Models. *PLOS ONE* **8**, e84301 (2013).
121. Hilfinger, A. & Paulsson, J. Separating intrinsic from extrinsic fluctuations in dynamic biological systems. *Proc. Natl. Acad. Sci.* **108**, 12167–12172 (2011).
122. Woese, C. R. Translation: in retrospect and prospect. *RNA* **7**, 1055–1067 (2001).
123. Cases, I. & de Lorenzo, V. The black cat/white cat principle of signal integration in bacterial promoters. *EMBO J.* **20**, 1–11 (2001).
124. Rugbjerg, P., Myling-Petersen, N., Porse, A., Sarup-Lytzen, K. & Sommer, M. O. A. Diverse genetic error modes constrain large-scale bio-based production. *Nat. Commun.* **9**, 787 (2018).
125. Klumpp, S., Zhang, Z. & Hwa, T. Growth Rate-Dependent Global Effects on Gene Expression in Bacteria. *Cell* **139**, 1366–1375 (2009).
126. Cardinale, S., Joachimiak, M. P. & Arkin, A. P. Effects of Genetic Variation on the E. coli Host-Circuit Interface. *Cell Rep.* **4**, 231–237 (2013).
127. Moser, F. *et al.* Genetic Circuit Performance under Conditions Relevant for Industrial Bioreactors. *ACS Synth. Biol.* **1**, 555–564 (2012).
128. Baker, L. How fashion can stop ruining the planet. <https://www.bbc.com/culture/article/20180419-how-fashion-can-stop-ruining-the-planet>.
129. Coelho, P. S., Brustad, E. M., Kannan, A. & Arnold, F. H. Olefin Cyclopropanation via Carbene Transfer Catalyzed by Engineered Cytochrome P450 Enzymes. *Science* **339**, 307–310 (2013).
130. Salema, V. & Fernández, L. Á. Escherichia coli surface display for the selection of nanobodies. *Microb. Biotechnol.* **10**, 1468–1484 (2017).
131. Packer, M. S. & Liu, D. R. Methods for the directed evolution of proteins. *Nat. Rev. Genet.* **16**, 379–394 (2015).
132. Yang, J., Kim, B., Kim, G. Y., Jung, G. Y. & Seo, S. W. Synthetic biology for evolutionary engineering: from perturbation of genotype to acquisition of desired phenotype. *Biotechnol. Biofuels* **12**, 113 (2019).
133. Dobzhansky, T. Nothing in Biology Makes Sense except in the Light of Evolution. *Am. Biol. Teach.* **35**, 125–129 (1973).
134. Segall-Shapiro, T. H., Meyer, A. J., Ellington, A. D., Sontag, E. D. & Voigt, C. A. A ‘resource allocator’ for transcription based on a highly fragmented T7 RNA polymerase. *Mol. Syst. Biol.* **10**, 742 (2014).
135. Silverman, A. D., Karim, A. S. & Jewett, M. C. Cell-free gene expression: an expanded repertoire of applications. *Nat. Rev. Genet.* **21**, 151–170 (2020).

136. Nirenberg, M. W. & Matthaei, J. H. The dependence of cell-free protein synthesis in *E. coli* upon naturally occurring or synthetic polyribonucleotides. *Proc. Natl. Acad. Sci.* **47**, 1588–1602 (1961).
137. Fuller, R. S., Kaguni, J. M. & Kornberg, A. Enzymatic replication of the origin of the *Escherichia coli* chromosome. *Proc. Natl. Acad. Sci.* **78**, 7370–7374 (1981).
138. Calhoun, K. A. & Swartz, J. R. An Economical Method for Cell-Free Protein Synthesis using Glucose and Nucleoside Monophosphates. *Biotechnol. Prog.* **21**, 1146–1153 (2005).
139. Thoring, L., Dondapati, S. K., Stech, M., Wüstenhagen, D. A. & Kubick, S. High-yield production of “difficult-to-express” proteins in a continuous exchange cell-free system based on CHO cell lysates. *Sci. Rep.* **7**, 11710 (2017).
140. Kwon, Y.-C. & Jewett, M. C. High-throughput preparation methods of crude extract for robust cell-free protein synthesis. *Sci. Rep.* **5**, 8663 (2015).
141. Sun, Z. Z., Yeung, E., Hayes, C. A., Noireaux, V. & Murray, R. M. Linear DNA for Rapid Prototyping of Synthetic Biological Circuits in an *Escherichia coli* Based TX-TL Cell-Free System. *ACS Synth. Biol.* **3**, 387–397 (2014).
142. Vilkhovoy, M., Dai, D., Vadhin, S., Adhikari, A. & Varner, J. D. Absolute Quantification of Cell-Free Protein Synthesis Metabolism by Reversed-Phase Liquid Chromatography-Mass Spectrometry. *JoVE J. Vis. Exp.* e60329 (2019) doi:10.3791/60329.
143. Didovyk, A., Tonooka, T., Tsimring, L. & Hasty, J. Rapid and Scalable Preparation of Bacterial Lysates for Cell-Free Gene Expression. *ACS Synth. Biol.* **6**, 2198–2208 (2017).
144. Laohakunakorn, N. Cell-Free Systems: A Proving Ground for Rational Biodesign. *Front. Bioeng. Biotechnol.* **8**, (2020).
145. Borkowski, O. *et al.* Cell-free prediction of protein expression costs for growing cells. *Nat. Commun.* **9**, 1457 (2018).
146. Halter, W., Allgower, F., Murray, R. M. & Gyorgy, A. Optimal Experiment Design and Leveraging Competition for Shared Resources in Cell-Free Extracts. in *2018 IEEE Conference on Decision and Control (CDC)* 1872–1879 (2018). doi:10.1109/CDC.2018.8619039.
147. Pardee, K. *et al.* Paper-Based Synthetic Gene Networks. *Cell* **159**, 940–954 (2014).
148. Pardee, K. *et al.* Rapid, Low-Cost Detection of Zika Virus Using Programmable Biomolecular Components. *Cell* **165**, 1255–1266 (2016).
149. Takahashi, M. K. *et al.* A low-cost paper-based synthetic biology platform for analyzing gut microbiota and host biomarkers. *Nat. Commun.* **9**, 3347 (2018).
150. Korman, T. P., Ogenorth, P. H. & Bowie, J. U. A synthetic biochemistry platform for cell free production of monoterpenes from glucose. *Nat. Commun.* **8**, 15526 (2017).
151. Lewis, D. D., Villarreal, F. D., Wu, F. & Tan, C. Synthetic Biology Outside the Cell: Linking Computational Tools to Cell-Free Systems. *Front. Bioeng. Biotechnol.* **2**, (2014).
152. Stanó, P. & Luisi, P. L. Semi-synthetic minimal cells: origin and recent developments. *Curr. Opin. Biotechnol.* **24**, 633–638 (2013).
153. Ishikawa, K., Sato, K., Shima, Y., Urabe, I. & Yomo, T. Expression of a cascading genetic network within liposomes. *FEBS Lett.* **576**, 387–390 (2004).

154. Stano, P. *et al.* Semi-synthetic minimal cells as a tool for biochemical ICT. *Biosystems* **109**, 24–34 (2012).
155. Carrara, P., Stano, P. & Luisi, P. L. Giant vesicles ‘colonies’: a model for primitive cell communities. *ChemBiochem Eur. J. Chem. Biol.* **13**, 1497–1502 (2012).
156. Elani, Y., V. Law, R. & Ces, O. Protein synthesis in artificial cells: using compartmentalisation for spatial organisation in vesicle bioreactors. *Phys. Chem. Chem. Phys.* **17**, 15534–15537 (2015).
157. Shaw, W. M. *et al.* Engineering a Model Cell for Rational Tuning of GPCR Signaling. *Cell* **177**, 782–796.e27 (2019).
158. Hauser, A. S., Attwood, M. M., Rask-Andersen, M., Schiöth, H. B. & Gloriam, D. E. Trends in GPCR drug discovery: new agents, targets and indications. *Nat. Rev. Drug Discov.* **16**, 829–842 (2017).
159. Lee, K. Y. *et al.* Photosynthetic artificial organelles sustain and control ATP-dependent reactions in a protocellular system. *Nat. Biotechnol.* **36**, 530–535 (2018).
160. Xu, C., Hu, S. & Chen, X. Artificial cells: from basic science to applications. *Mater. Today* **19**, 516–532 (2016).
161. Gibson, D. G. *et al.* Creation of a Bacterial Cell Controlled by a Chemically Synthesized Genome. *Science* **329**, 52–56 (2010).
162. Pelletier, J. F. *et al.* Genetic requirements for cell division in a genomically minimal cell. *Cell* **184**, 2430–2440.e16 (2021).
163. J. Craig Venter. *J. Craig Venter Institute* /about/j-craig-venter.
164. Protobiology. <http://www.protobiology.org/people.php>.
165. Home. <https://www.maxsynbio.mpg.de/home>.
166. Grunberg-Manago, M. & Ochoa, S. ENZYMATIC SYNTHESIS AND BREAKDOWN OF POLYNUCLEOTIDES; POLYNUCLEOTIDE PHOSPHORYLASE1. *J. Am. Chem. Soc.* **77**, 3165–3166 (1955).
167. Lehman, I. R., Bessman, M. J., Simms, E. S. & Kornberg, A. Enzymatic synthesis of deoxyribonucleic acid. I. Preparation of substrates and partial purification of an enzyme from *Escherichia coli*. *J. Biol. Chem.* **233**, 163–170 (1958).
168. Arber, W. Restriction Endonucleases. *Angew. Chem. Int. Ed. Engl.* **17**, 73–79 (1978).
169. Adams, B. L. The Next Generation of Synthetic Biology Chassis: Moving Synthetic Biology from the Laboratory to the Field. *ACS Synth. Biol.* **5**, 1328–1330 (2016).
170. Blount, Z. D. The unexhausted potential of *E. coli*. *eLife* **4**, e05826 (2015).
171. Leprince, A., van Passel, M. W. & dos Santos, V. A. M. Streamlining genomes: toward the generation of simplified and stabilized microbial systems. *Curr. Opin. Biotechnol.* **23**, 651–658 (2012).
172. Kolisnychenko, V. *et al.* Engineering a Reduced *Escherichia coli* Genome. *Genome Res.* **12**, 640–647 (2002).
173. Jensen, S. I., Lennen, R. M., Herrgård, M. J. & Nielsen, A. T. Seven gene deletions in seven days: Fast generation of *Escherichia coli* strains tolerant to acetate and osmotic stress. *Sci. Rep.* **5**, 17874 (2015).

174. Umenhoffer, K. *et al.* Genome-Wide Abolishment of Mobile Genetic Elements Using Genome Shuffling and CRISPR/Cas-Assisted MAGE Allows the Efficient Stabilization of a Bacterial Chassis. *ACS Synth. Biol.* **6**, 1471–1483 (2017).
175. Pósfai, G. *et al.* Emergent Properties of Reduced-Genome *Escherichia coli*. *Science* **312**, 1044–1046 (2006).
176. Sonnenborn, U. & Schulze, J. The non-pathogenic *Escherichia coli* strain Nissle 1917 – features of a versatile probiotic. *Microb. Ecol. Health Dis.* **21**, 122–158 (2009).
177. He, L. *et al.* *Escherichia coli* Nissle 1917 engineered to express Tum-5 can restrain murine melanoma growth. *Oncotarget* **8**, 85772–85782 (2017).
178. Ho, C. L. *et al.* Engineered commensal microbes for diet-mediated colorectal-cancer chemoprevention. *Nat. Biomed. Eng.* **2**, 27–37 (2018).
179. Rowlett, V. W. & Margolin, W. The Min system and other nucleoid-independent regulators of Z ring positioning. *Front. Microbiol.* **6**, (2015).
180. Ebersbach, G., Galli, E., Møller-Jensen, J., Löwe, J. & Gerdes, K. Novel coiled-coil cell division factor ZapB stimulates Z ring assembly and cell division. *Mol. Microbiol.* **68**, 720–735 (2008).
181. Farley, M. M., Hu, B., Margolin, W. & Liu, J. Minicells, Back in Fashion. *J. Bacteriol.* **198**, 1186–1195 (2016).
182. Million-Weaver, S. & Camps, M. Mechanisms of plasmid segregation: Have multicopy plasmids been overlooked? *Plasmid* **75**, 27–36 (2014).
183. Rampley, C. P. N. *et al.* Development of SimCells as a novel chassis for functional biosensors. *Sci. Rep.* **7**, 7261 (2017).
184. Fan, C. *et al.* Chromosome-free bacterial cells are safe and programmable platforms for synthetic biology. *Proc. Natl. Acad. Sci.* **117**, 6752–6761 (2020).
185. Liu, S. L. & Sanderson, K. E. I-CeuI reveals conservation of the genome of independent strains of *Salmonella typhimurium*. *J. Bacteriol.* **177**, 3355–3357 (1995).
186. Zhu, N. *et al.* A Novel Coronavirus from Patients with Pneumonia in China, 2019. *N. Engl. J. Med.* **382**, 727–733 (2020).
187. The species Severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2. *Nat. Microbiol.* **5**, 536–544 (2020).
188. Cucinotta, D. & Vanelli, M. WHO Declares COVID-19 a Pandemic. *Acta Bio Medica Atenei Parm.* **91**, 157–160 (2020).
189. WHO Coronavirus (COVID-19) Dashboard. <https://covid19.who.int>.
190. CDC. Information for Laboratories about Coronavirus (COVID-19). <https://www.cdc.gov/coronavirus/2019-ncov/lab/virus-requests.html> (2020).
191. V'kovski, P., Kratzel, A., Steiner, S., Stalder, H. & Thiel, V. Coronavirus biology and replication: implications for SARS-CoV-2. *Nat. Rev. Microbiol.* **19**, 155–170 (2021).
192. Singh, D. & Yi, S. V. On the origin and evolution of SARS-CoV-2. *Exp. Mol. Med.* **53**, 537–547 (2021).

193. Zhou, P. *et al.* A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* **579**, 270–273 (2020).
194. Organization, W. H. Laboratory testing for coronavirus disease 2019 (COVID-19) in suspected human cases: interim guidance, 2 March 2020. (2020).
195. Notomi, T. *et al.* Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res.* **28**, e63–e63 (2000).
196. Nagamine, K., Hase, T. & Notomi, T. Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Mol. Cell. Probes* **16**, 223–229 (2002).
197. Thi, V. L. D. *et al.* A colorimetric RT-LAMP assay and LAMP-sequencing for detecting SARS-CoV-2 RNA in clinical samples. *Sci. Transl. Med.* **12**, (2020).
198. Yu, L. *et al.* Rapid Detection of COVID-19 Coronavirus Using a Reverse Transcriptional Loop-Mediated Isothermal Amplification (RT-LAMP) Diagnostic Platform. *Clin. Chem.* **66**, 975–977 (2020).
199. Baek, Y. H. *et al.* Development of a reverse transcription-loop-mediated isothermal amplification as a rapid early-detection method for novel SARS-CoV-2. *Emerg. Microbes Infect.* **9**, 998–1007 (2020).
200. Broughton, J. P. *et al.* CRISPR–Cas12-based detection of SARS-CoV-2. *Nat. Biotechnol.* **38**, 870–874 (2020).
201. Ooi, K. H. *et al.* An engineered CRISPR-Cas12a variant and DNA-RNA hybrid guides enable robust and rapid COVID-19 testing. *Nat. Commun.* **12**, 1739 (2021).
202. Gnoth, C. & Johnson, S. Strips of Hope: Accuracy of Home Pregnancy Tests and New Developments. *Geburtshilfe Frauenheilkd.* **74**, 661–669 (2014).
203. Pan, R. *et al.* Gold nanoparticle-based enhanced lateral flow immunoassay for detection of *Cronobacter sakazakii* in powdered infant formula. *J. Dairy Sci.* **101**, 3835–3843 (2018).
204. Albert, E. *et al.* Field evaluation of a rapid antigen test (Panbio™ COVID-19 Ag Rapid Test Device) for COVID-19 diagnosis in primary healthcare centres. *Clin. Microbiol. Infect.* **27**, 472.e7–472.e10 (2021).
205. INNOVA SARS-CoV-2 Antigen Lateral Flow Rapid Test Kits. Box of 20. *Primary Care Supplies* <https://www.primarycaresupplies.co.uk/innova-sars-cov-2-antigen-lateral-flow-rapid-test-kit-box-of-20/>.
206. Zou, L. *et al.* SARS-CoV-2 Viral Load in Upper Respiratory Specimens of Infected Patients. *N. Engl. J. Med.* **382**, 1177–1179 (2020).
207. Caruana, G. *et al.* Diagnostic strategies for SARS-CoV-2 infection and interpretation of microbiological results. *Clin. Microbiol. Infect.* **26**, 1178–1182 (2020).
208. Babiker, A., Myers, C. W., Hill, C. E. & Guarner, J. SARS-CoV-2 Testing. *Am. J. Clin. Pathol.* **153**, 706–708 (2020).
209. Suleman, E., Mtshali, M. S. & Lane, E. Investigation of false positives associated with loop-mediated isothermal amplification assays for detection of *Toxoplasma gondii* in archived tissue samples of captive felids. *J Vet Diagn Invest* **28**, 536–542 (2016).

210. Grant, B. D. *et al.* SARS-CoV-2 Coronavirus Nucleocapsid Antigen-Detecting Half-Strip Lateral Flow Assay Toward the Development of Point of Care Tests Using Commercially Available Reagents. *Anal. Chem.* (2020) doi:10.1021/acs.analchem.0c01975.
211. Covid-19: government must urgently rethink lateral flow test roll out. *The BMJ* <https://blogs.bmj.com/bmj/2021/01/12/covid-19-government-must-urgently-rethink-lateral-flow-test-roll-out/> (2021).
212. Li, G., Chen, X. & Xu, A. Profile of Specific Antibodies to the SARS-Associated Coronavirus. *N. Engl. J. Med.* **349**, 508–509 (2003).
213. outbreak.info. *outbreak.info* <https://outbreak.info/>.
214. Street, W. Colorectal Cancer Facts & Figures 2020-2022. 48.
215. Arnold, M. *et al.* Global patterns and trends in colorectal cancer incidence and mortality. *Gut* **66**, 683–691 (2017).
216. Fearon, E. R. & Vogelstein, B. A genetic model for colorectal tumorigenesis. *Cell* **61**, 759–767 (1990).
217. *Molecular Pathogenesis of Colorectal Cancer*. (Springer New York, 2013). doi:10.1007/978-1-4614-8412-7.
218. Levin, B. *et al.* Screening and surveillance for the early detection of colorectal cancer and adenomatous polyps, 2008: a joint guideline from the American Cancer Society, the US Multi-Society Task Force on Colorectal Cancer, and the American College of Radiology. *Gastroenterology* **134**, 1570–1595 (2008).
219. Europe is falling behind America in the fight against colorectal cancer due to low screening uptake say experts. <https://ueg.eu/a/192>.
220. Heresbach, D. *et al.* Miss rate for colorectal neoplastic polyps: a prospective multicenter study of back-to-back video colonoscopies. *Endoscopy* **40**, 284–290 (2008).
221. Pickhardt, P. J., Hassan, C., Halligan, S. & Marmo, R. Colorectal cancer: CT colonography and colonoscopy for detection--systematic review and meta-analysis. *Radiology* **259**, 393–405 (2011).
222. Kaur, K. & Adamski, J. J. Fecal Occult Blood Test. in *StatPearls* (StatPearls Publishing, 2021).
223. Alix-Panabières, C. The future of liquid biopsy. *Nature* **579**, S9–S9 (2020).
224. Frattini, M. *et al.* Quantitative and qualitative characterization of plasma DNA identifies primary and recurrent colorectal cancer. *Cancer Lett.* **263**, 170–181 (2008).
225. Ng, E. K. O. *et al.* Differential expression of microRNAs in plasma of patients with colorectal cancer: a potential marker for colorectal cancer screening. *Gut* **58**, 1375–1381 (2009).
226. Yong, F. L., Law, C. W. & Wang, C. W. Potentiality of a triple microRNA classifier: miR-193a-3p, miR-23a and miR-338-5p for early detection of colorectal cancer. *BMC Cancer* **13**, 280 (2013).
227. Li Destri, G. *et al.* Monitoring carcinoembryonic antigen in colorectal cancer: is it still useful? *Surg. Today* **28**, 1233–1236 (1998).
228. Locker, G. *et al.* ASCO 2006 Update of Recommendations for the Use of Tumor Markers in Gastrointestinal Cancer. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **24**, 5313–27 (2006).

229. Sturgeon, C. M. *et al.* National Academy of Clinical Biochemistry Laboratory Medicine Practice Guidelines for Use of Tumor Markers in Testicular, Prostate, Colorectal, Breast, and Ovarian Cancers. *Clin. Chem.* **54**, e11–e79 (2008).
230. Duffy, M. *et al.* Tumor markers in colorectal cancer, gastric cancer and gastrointestinal stromal cancers: European group on tumor markers 2014 guidelines update. *Int. J. Cancer J. Int. Cancer* **134**, 2513–2522 (2014).
231. Zhang, S.-Y., Lin, M. & Zhang, H.-B. Diagnostic value of carcinoembryonic antigen and carcinoma antigen 19-9 for colorectal carcinoma. *Int. J. Clin. Exp. Pathol.* **8**, 9404–9409 (2015).
232. Girotti, S., Ferri, E. N., Fumo, M. G. & Maiolini, E. Monitoring of environmental pollutants by bioluminescent bacteria. *Anal. Chim. Acta* **608**, 2–29 (2008).
233. Cotlet, M., Goodwin, P. M., Waldo, G. S. & Werner, J. H. A comparison of the fluorescence dynamics of single molecules of a green fluorescent protein: one- versus two-photon excitation. *Chemphyschem Eur. J. Chem. Phys. Phys. Chem.* **7**, 250–260 (2006).
234. Liu, X. *et al.* Power generation from ambient humidity using protein nanowires. *Nature* **578**, 550–554 (2020).
235. Gutiérrez, J. C., Amaro, F. & Martín-González, A. Heavy metal whole-cell biosensors using eukaryotic microorganisms: an updated critical review. *Front. Microbiol.* **6**, (2015).
236. Reder-Christ, K. & Bendas, G. Biosensor Applications in the Field of Antibiotic Research—A Review of Recent Developments. *Sensors* **11**, 9450–9466 (2011).
237. Hu, H. *et al.* Glucose monitoring in living cells with single fluorescent protein-based sensors. *RSC Adv.* **8**, 2485–2489 (2018).
238. Flentje, K. *et al.* A Bioluminescent Transposon Reporter-Trap Identifies Tumor-Specific Microenvironment-Induced Promoters in Salmonella for Conditional Bacterial-Based Tumor Therapy. *Cancer Discov.* **2**, 624–637 (2012).
239. Panteli, J. T. & Forbes, N. S. Engineered bacteria detect spatial profiles in glucose concentration within solid tumor cell masses. *Biotechnol. Bioeng.* **113**, 2474–2484 (2016).
240. Chang, H.-J. *et al.* A Modular Receptor Platform To Expand the Sensing Repertoire of Bacteria. *ACS Synth Biol* **7**, 166–175 (2018).
241. Kylilis, N. *et al.* Whole-Cell Biosensor with Tunable Limit of Detection Enables Low-Cost Agglutination Assays for Medical Diagnostic Applications. *ACS Sens.* **4**, 370–378 (2019).
242. Piñero-Lambeck, C. *et al.* Programming Controlled Adhesion of *E. coli* to Target Surfaces, Cells, and Tumors with Synthetic Adhesins. *ACS Synth. Biol.* **4**, 463–473 (2015).
243. Worldwide cancer mortality statistics. *Cancer Research UK* <https://www.cancerresearchuk.org/health-professional/cancer-statistics/worldwide-cancer/mortality> (2015).
244. Sepich-Poore, G. D. *et al.* The microbiome and human cancer. *Science* **371**, (2021).
245. Barisam, M., Saidi, M. S., Kashaninejad, N. & Nguyen, N.-T. Prediction of Necrotic Core and Hypoxic Zone of Multicellular Spheroids in a Microbioreactor with a U-Shaped Barrier. *Micromachines* **9**, (2018).

246. Heap, J. T. *et al.* Spores of *Clostridium* engineered for clinical efficacy and safety cause regression and cure of tumors in vivo. *Oncotarget* **5**, 1761–1769 (2014).
247. Fares, J., Fares, M. Y., Khachfe, H. H., Salhab, H. A. & Fares, Y. Molecular principles of metastasis: a hallmark of cancer revisited. *Signal Transduct. Target. Ther.* **5**, 1–17 (2020).
248. Ozdemir, T., Fedorec, A. J. H., Danino, T. & Barnes, C. P. Synthetic Biology and Engineered Live Biotherapeutics: Toward Increasing System Complexity. *Cell Syst.* **7**, 5–16 (2018).
249. Park, S.-H. *et al.* RGD Peptide Cell-Surface Display Enhances the Targeting and Therapeutic Efficacy of Attenuated *Salmonella*-mediated Cancer Therapy. *Theranostics* **6**, 1672–1682 (2016).
250. Chien, T., Doshi, A. & Danino, T. Advances in bacterial cancer therapies using synthetic biology. *Curr. Opin. Syst. Biol.* **5**, 1–8 (2017).
251. Thamm, D. H. Systemic Administration of an Attenuated, Tumor-Targeting *Salmonella typhimurium* to Dogs with Spontaneous Neoplasia: Phase I Evaluation. *Clin. Cancer Res.* **11**, 4827–4834 (2005).
252. Toso, J. F. *et al.* Phase I Study of the Intravenous Administration of Attenuated *Salmonella typhimurium* to Patients With Metastatic Melanoma. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **20**, 142–152 (2002).
253. Gujrati, V. *et al.* Bioengineered Bacterial Outer Membrane Vesicles as Cell-Specific Drug-Delivery Vehicles for Cancer Therapy. *ACS Nano* **8**, 1525–1537 (2014).
254. Sleight, S. C. & Sauro, H. M. Visualization of Evolutionary Stability Dynamics and Competitive Fitness of *Escherichia coli* Engineered with Randomized Multigene Circuits. *ACS Synth. Biol.* **2**, 519–528 (2013).
255. Postel, A. *et al.* Evaluation of two commercial loop-mediated isothermal amplification assays for detection of avian influenza H5 and H7 hemagglutinin genes. *J. Vet. Diagn. Investig. Off. Publ. Am. Assoc. Vet. Lab. Diagn. Inc* **22**, 61–66 (2010).
256. Zhang, Y. *et al.* Development of loop-mediated isothermal amplification with *Plasmodium falciparum* unique genes for molecular diagnosis of human malaria. *Pathog. Glob. Health* **111**, 247–255 (2017).
257. Nkouawa, A., Sako, Y., Okamoto, M. & Ito, A. Simple Identification of Human *Taenia* Species by Multiplex Loop-Mediated Isothermal Amplification in Combination with Dot Enzyme-Linked Immunosorbent Assay. *Am. J. Trop. Med. Hyg.* **94**, 1318–1323 (2016).
258. Mann, T., Humbert, R., Dorschner, M., Stamatoyannopoulos, J. & Noble, W. S. A thermodynamic approach to PCR primer design. *Nucleic Acids Res.* **37**, e95 (2009).
259. Singh, J., Hanson, J., Paliwal, K. & Zhou, Y. RNA secondary structure prediction using an ensemble of two-dimensional deep neural networks and transfer learning. *Nat. Commun.* **10**, 5407 (2019).
260. Meerhoff, T. J. *et al.* Detection of multiple respiratory pathogens during primary respiratory infection: nasal swab versus nasopharyngeal aspirate using real-time polymerase chain reaction. *Eur. J. Clin. Microbiol. Infect. Dis.* **29**, 365–371 (2010).
261. Sullivan, C. B. *et al.* Cerebrospinal Fluid Leak After Nasal Swab Testing for Coronavirus Disease 2019. *JAMA Otolaryngol. Neck Surg.* **146**, 1179–1181 (2020).

262. Earl, C. C., Smith, M. T., Lease, R. A. & Bundy, B. C. Polyvinylsulfonic acid: A Low-cost RNase inhibitor for enhanced RNA preservation and cell-free protein translation. *Bioengineered* **9**, 90–97 (2018).
263. Shibata, S. *et al.* High incidence of false-positive results of IgG antibody against SARS-CoV-2 with rapid immunochromatographic antibody test due to human common cold coronavirus infection. *Respir. Med. Case Rep.* **31**, 101180 (2020).
264. Deeks, J. J. *et al.* Antibody tests for identification of current and past infection with SARS-CoV-2. *Cochrane Database Syst. Rev.* (2020) doi:10.1002/14651858.CD013652.
265. Diamandis, E. P., Bast, R. C., Gold, P., Chu, T. M. & Magnani, J. L. REFLECTION ON THE DISCOVERY OF CARCINOEMBRYONIC ANTIGEN, PROSTATE-SPECIFIC ANTIGEN AND CARBOHYDRATE ANTIGENS CA125 and CA19.9. *Clin. Chem.* **59**, 22–31 (2013).
266. Gupta, A. & Chaudhary, V. K. Whole-Blood Agglutination Assay for On-Site Detection of Human Immunodeficiency Virus Infection. *J. Clin. Microbiol.* **41**, 2814–2821 (2003).
267. Sieow, B. F.-L., Wun, K. S., Yong, W. P., Hwang, I. Y. & Chang, M. W. Tweak to Treat: Reprogramming Bacteria for Cancer Treatment. *Trends Cancer* **7**, 447–464 (2021).
268. Aggarwal, N., Breedon, A. M. E., Davis, C. M., Hwang, I. Y. & Chang, M. W. Engineering probiotics for therapeutic applications: recent examples and translational outlook. *Curr. Opin. Biotechnol.* **65**, 171–179 (2020).
269. Waters, L. S. & Storz, G. Regulatory RNAs in Bacteria. *Cell* **136**, 615–628 (2009).
270. Hoang Trung Chau, T., Hoang Anh Mai, D., Ngoc Pham, D., Thi Quynh Le, H. & Yeol Lee, E. Developments of Riboswitches and Toehold Switches for Molecular Detection—Biosensing and Molecular Diagnostics. *Int. J. Mol. Sci.* **21**, 3192 (2020).
271. Kelly, C. L. *et al.* Synthetic negative feedback circuits using engineered small RNAs. *Nucleic Acids Res.* **46**, 9875–9889 (2018).
272. Esparza, T. J., Martin, N. P., Anderson, G. P., Goldman, E. R. & Brody, D. L. High affinity nanobodies block SARS-CoV-2 spike receptor binding domain interaction with human angiotensin converting enzyme. *Sci. Rep.* **10**, 22370 (2020).
273. Güttsches, A.-K. *et al.* Anti-inflammatory modulation of immune response by probiotic *Escherichia coli* Nissle 1917 in human blood mononuclear cells. *Innate Immun.* **18**, 204–216 (2012).
274. Ono, B., Kimiduka, H., Kubota, M., Okuno, K. & Yabuta, M. Role of the *ompT* Mutation in Stimulated Decrease in Colony-Forming Ability Due to Intracellular Protein Aggregate Formation in *Escherichia coli* Strain BL21. *Biosci. Biotechnol. Biochem.* **71**, 504–512 (2007).
275. Mamat, U. *et al.* Detoxifying *Escherichia coli* for endotoxin-free production of recombinant proteins. *Microb. Cell Factories* **14**, 57 (2015).
276. Kalaora, S. *et al.* Identification of bacteria-derived HLA-bound peptides in melanoma. *Nature* **592**, 138–143 (2021).