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Rapid RNase inhibitor production to enable low-cost, on-demand cell-free protein synthesis biosensor use in human body fluids

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Abstract

Human body fluids contain biomarkers which are used extensively for prognostication, diagnosis, monitoring, and evaluation of different treatments for a variety of diseases and disorders. The application of biosensors based on cell-free protein synthesis (CFPS) offers numerous advantages including on-demand and at-home use for fast, accurate detection of a variety of biomarkers in human fluids at an affordable price. However, current CFPS-based biosensors use commercial RNase inhibitors to inhibit different RNases present in human fluids and this reagent is approximately 90% of the expense of these biosensors. Here the flexible nature of *Escherichia coli*-lysate-based CFPS was used for the first time to produce murine RNase Inhibitor (m-RI) and to optimize its soluble and active production by tuning reaction temperature, reaction time, reduced potential, and addition of GroEL/ES folding chaperons. Furthermore, RNase inhibition activity of m-RI with the highest activity and stability was determined against increasing amounts of three human fluids of serum, saliva, and urine (0%–100% v/v) in lyophilized CFPS reactions. To further demonstrate the utility of the CFPS-produced m-RI, a lyophilized saliva-based glutamine biosensor was demonstrated to effectively work with saliva samples. Overall, the use of CFPS-produced m-RI reduces the total reagent costs of CFPS-based biosensors used in human body fluids approximately 90%.

KEYWORDS
biosensor, body fluids, cell-free protein synthesis, RNase, RNase inhibitor

1 | INTRODUCTION

Human body fluids such as blood, plasma, serum, saliva, and urine contain myriad biomarkers which enable medical diagnosis, streamline and enhance drug discovery and approval, facilitate monitoring of treatment progress, and increase fundamental understanding of a variety of diseases and disorders (Group et al., 2001; Strimbu & Tavel, 2010). Unfortunately, detection of biomarkers often requires (1) expensive, centralized equipment, (2) large sample volumes, and (3) lengthy assays which escalate medical costs and constrains utility. Economic, rapid, and point-of-care biosensors could help transform medical care.

Cell-free protein synthesis (CFPS) is a rapid, high-yielding, and flexible platform for producing a variety of products including pharmaceuticals and biosensors (Carlson et al., 2012; Slomovic et al., 2015; Smith et al., 2015; Voyvodic & Bonnet, 2020). Lyophilized

Abbreviations: CFPS, cell-free protein synthesis; MSO, methionine sulfoximine; m-RI, murine RNase inhibitor; RI, RNase inhibitor.

CFPS reactions in a single-pot are shelf-stable at room temperature for months (Gregorio et al., 2020; Hunt et al., 2017) until activated by the addition of an aqueous sample for point-of-care applications (Green et al., 2014; Slomovic et al., 2015; Soltani et al., 2018). For example, CFPS is a promising platform for distributed therapeutic production (Dondapati et al., 2020; Hunt, Wilding, et al., 2020; Wilding et al., 2019; Zhou et al., 2020). Additionally, lyophilized biosensors may enable facile and economic point-of-care detection of a variety of targets in human fluids including Ebola (Pardee et al., 2014), Zika (Pardee et al., 2016), Norovirus (Ma et al., 2018), microRNAs (Wang et al., 2019), natural and synthetic hormones (Salehi et al., 2017, 2018), and amino acids (Hunt, Barnett, et al., 2020; Jang et al., 2017). The overall cost of CFPS biosensors is substantially reduced by using *Escherichia coli*-lysate based systems prepared in-house instead of commercially available PURE or lysate-based CFPS reagents (Dopp & Reuel, 2018; Hunt, Wilding, et al., 2020); however, there have been no reports for economic, in-house production, and utilization of an effective RNase inhibitor (RI) in CFPS systems. Here, we report an optimized platform for the expression of highly soluble and active RI in an *E. coli*-lysate-based CFPS system to reduce the reagent costs of CFPS biosensors by approximately 90% (cost analysis of CFPS biosensors, online Supporting Information).

Because of its inexpensive, fast growing and yet robust nature, *E. coli* is one of the most convenient and widely used hosts for recombinant protein production (Gutiérrez-González et al., 2019). However, high-yielding recombinant expression of properly folded RI in *E. coli* has been proven challenging due to the highly reduced and hydrophobic core of RI proteins. In vivo expression of RI in *E. coli* has resulted in high aggregation and low soluble yields of 0.25–15 μg/ml (Klink et al., 2001; F. S. Lee & Vallee, 1989). Furthermore, solubilization of these insoluble aggregates is inefficient, time and labor intensive, and difficult (Singh et al., 2015). Fusion partners may improve in vivo expression, but these decrease RI activity and require postexpression processing for removal of the tag (Šiurkus & Neubauer, 2011a; Šiurkus et al., 2010). Likewise, lower fermentation temperatures and reducing environments may increase soluble yield, but these cripple *E. coli* growth kinetics. (Šiurkus & Neubauer, 2011b) Improved techniques for the synthesis of recombinant RI are highly desirable to enable economic, point-of-care detection of biomarkers in human fluids with cell-free protein synthesis.

Here, we report an optimized platform for the expression of highly soluble and active recombinant murine RNase inhibitor (m-RI) in an *E. coli*-lysate-based CFPS system to reduce the reagent costs of human-fluid CFPS biosensors by approximately 90%. This platform leverages the open nature of the CFPS reaction environment and residual cytosolic reduction pathways (Knapp et al., 2007) to incorporate folding chaperons and manipulate redox potential for optimal m-RI folding and achieved soluble titers approaching 750 μg/ml in 2 h. Then, CFPS reactions expressing sfGFP reporter protein containing 30% human saliva (which contains high concentrations of RNases) were utilized to assess the expression-dependent activity of m-RIs to ensure optimal RNase-inhibition as well as expression yield. Next, the productivity of lyophilized CFPS reactions incorporating highly active and stable in-house m-RI was assessed in presence of three human fluids (saliva, serum, and urine) relevant in diagnosis and treatments (Senf et al., 2020; Zhang et al., 2019). Lastly, a lyophilized glutamine biosensor was developed as a proof-of-concept application for an on-demand saliva-based biosensor. Glutamine is a biomarker for a variety of disorders and diseases including diabetes, cancer, kidney disease, chronic intestinal pseudo-obstruction, brain dysfunction, Alzheimer's disease, major depression, and anorexia nervosa (Chen et al., 2019; Madeira et al., 2018; Rhee et al., 2018). This study demonstrates the utility of CFPS systems to elucidate and overcome complex protein folding requirements, and to provide a distributable, economic platform for rapid, point-of-care biomarker detection for a variety of disorders and diseases.

2 | MATERIALS AND METHODS

2.1 | Extract preparation

E. coli extracts were prepared using strain BL21-Star (DE3) (Invitrogen) as described previously (Smith, Hawes, et al., 2014) without any additional transformed plasmids (normal extract) or by transforming GroEL/ES in it (pOFX extract), a kind gift from Dr. Dong-Myung Kim. GroEL/ES chaperones have been used for proper folding of the target proteins without aggregation or degradation (Georgescauld et al., 2014). All cell cultures were grown at 37°C and 280 RPM. First, overnight cultures of 5 ml LB media containing no antibiotic and spectinomycin for the normal and pOFX extracts, respectively, were added to 100 ml of 2xYT media in 500 ml flasks and grown until an OD_{600} of about 2.0. Then, 100 ml cultures were added to 900 ml fresh 2xYT media in 2.5 L Tunair baffled shake flasks (IBI Scientific). At OD_{600} of 0.6, both cultures were induced by 1 mM final concentration of β-d-1-thiogalactopyranoside. Cells were harvested at the late log phase at OD_{600} of 3.7 and 3.0 for the normal and pOFX extracts, respectively. Cells were centrifugated at 8000 RCF for 20 min, washed (10 ml/g wet cells) then resuspended (0.7 ml/g wet cells) in cold Buffer A. Buffer A contains 14 mM magnesium acetate, 10 mM tris, 60 mM potassium glutamate, and 1 mM dithiothreitol (DTT), pH 8.2. Cells were then homogenized with the Emulsiflex B-15 French press homogenizer (Avestin), three passes at 21,000 PSI, with 1 min cooling between the second and the third pass. Lysed cells were centrifuged at 12,000 RCF for 30 min and the supernatant was aliquoted, flash-frozen, and stored at −80°C until single-use.

2.2 | DNA plasmid preparation

pTwist m-RI plasmid harboring the codon-optimized m-RI sequence (Protein Data Bank ID: 3TSR) for expression in *E. coli*, was designed and submitted for synthesis by Twist Bioscience containing no tag (plasmid sequence in Online Supporting Information). It was transformed into XL1-blue chemically competent cells (Agilent Technologies) and the plasmid insertion was later confirmed by sequencing.

A single colony on solid culture media was inoculated in 5 ml LB media containing ampicillin and grown overnight at 37°C in a shaker incubator. Then, 5 ml culture was added to 200 ml of Terrific Broth media (24 g/L yeast extract, 20 g/L tryptone, and 4 ml/L glycerol) in a 500 ml flask and left for at least 16 h before harvesting in 8000 RCF for 20 min. QIAGEN Plasmid Maxi Kit was used for plasmid DNA isolation according to the manufacturer's instructions. QuikChange II site-directed mutagenesis (Agilent Technologies) was used per manufacturer's instructions to insert 6x-His tag into either N or C-terminal of the m-RI insert gene and successful insertions were confirmed by sequencing. All primers were designed according to an efficient method by Liu and Naismith (2008) and all primer sequences are available in Table S1. The Plasmid PY71-sfGFP was used for sfGFP expression and its sequence was reported previously (Bundy & Swartz, 2010).

2.3 | CFPS reaction and m-RI activity assessment

CFPS reactions used PANOx-SP system for energy regeneration as described previously with some modifications (Bundy & Swartz, 2010). Reaction volumes for expressing m-RI and testing m-RI activity were 30 µl and 50 µl, respectively in 2 ml round-bottom microcentrifuge tubes (GeneMate, BioExpress). For m-RI expression, each reaction contained 35% (v/v) *E. coli* extract (normal or pOFX), 25% (v/v) small molecules master mix as previously described (Hunt et al., 2019), 10.5 µM ^{14}C-labeled Leucine (Moravek Inc.), 9–12 mM magnesium glutamate (concentration adjusted based on extract for optimum protein expression), 36 nM pTwist m-RI plasmid as the DNA template, and the balance ultrapure water. Reducing potential for each reaction was adjusted by DTT or glutathione. Reactions ($n = 3$) were incubated at 15–30°C for 2–8 h and 280 RPM. Liquid scintillation counting was used as described previously (Bundy & Swartz, 2010) for quantifying total and soluble protein expression yields. "Soluble" portion describes the supernatant of the CFPS reaction after centrifugation at 16,000 RCF for 10 min at 4°C.

NuPAGE 10% Bis-Tris Gel (Invitrogen) was used per manufacturer's instructions followed by autoradiography for 3 days to confirm the correct size of in-house, ^{14}C-labeled m-RIs, and compare with the standard commercial m-RI (NEB). These in-house m-RIs were expressed at 24°C and 2 h under reduced, oxidized, and native CFPS conditions with pOFX extract.

sfGFP was employed as an indicator protein in CFPS reactions to test m-RI activity. Each CFPS reaction for sfGFP expression composed of 25% (v/v) normal BL21-Star (DE3) extract, 25% (v/v) small molecules master mix, 18 mM $Mg(Glu)_2$, 2 nM PY71-sfGFP DNA plasmid, 30% (v/v) saliva (provided by volunteers following IRB approved protocols), and ultrapure water for the remaining 50 µl reaction volume. The activity of the CFPS-produced m-RI was determined by adding 1–3 µg/ml soluble unpurified m-RI expressed at different conditions, to CFPS expressing sfGFP. As a standard for comparison, 1 U/µl reaction commercial m-RI (NEB) was used. As the positive control (no saliva), deionized water was added instead of saliva and m-RI. sfGFP expression after 3 h of reaction at 37°C and 280 RPM was quantified by fluorescence analysis. The 20 µl of CFPS reaction and 45 µl deionized water were added to a black 96-well plate and fluorescence was measured by a BioTek Synergy MX (Biotek Instruments) plate reader at 480/510 nm excitation/emission. sfGFP expression was reported either as relative fluorescence units (RFU) or µg/ml (as determined by comparing RFU levels of known concentrations of C^{14}-radiolabeled sfGFP).

To determine m-RI stability, similar sfGFP expression reactions in presence of 30% (v/v) saliva were performed by adding 1–3 µg/ml of unpurified CFPS-produced m-RI after one and two freeze-thaw cycles. For freeze-thaw cycles, m-RI CFPS reactions were flash-frozen by liquid nitrogen and kept at −80°C for at least 1 day before thawing at room temperature and activity assessment.

To test m-RI activity in presence of varying amounts (0%–100% (v/v)) of three human fluids of saliva, serum, and urine, CFPS reactions for sfGFP expression were assembled without adding deionized water or any human fluids. Reactions were flash-frozen by liquid nitrogen and placed in a freeze-dryer (Labconco FreeZone 2.5 L) and lyophilized at −50°C overnight. Lyophilized CFPS reactions were stored at −20°C for at least one week before rehydration with 0%–100% human fluids.

Saliva and urine samples were donated by volunteers and after aliquoting in smaller (single-use) volumes, were flash-frozen and stored at −80°C for consistency purposes. Human serum samples were obtained from Gemini Bio Products, aliquoted in smaller volumes, flash-frozen, and kept at −80°C for single use.

2.4 | Glutamine biosensor

For testing glutamine concentration in saliva in the presence or absence of externally added glutamine, CFPS reactions were assembled similar to m-RI activity assessment by sfGFP expression as previously reported (Hunt, Barnett, et al., 2020) with some modifications. First, CFPS reagents were deficient in glutamine amino acid. To eliminate background glutamine synthesis by the inherent glutamine synthetase (GLNS) in *E. coli* extract, methionine sulfoximine (MSO, 70 mM final concentration) was added to PANOx-SP to inhibit GLNS as has been recently reported in detail. To include the effect of lyophilization on m-RI activity, CFPS biosensing reactions which included the CFPS-produced m-RI as an unpurified CFPS-product were assembled and lyophilized in a freeze-dryer overnight as described previously (Smith, Berkheimer, et al., 2014). Lyophilized CFPS reactions were stored at −20°C for at least 1 week before rehydration with diluted saliva or ultrapure water (control). Glutamine was added (0–600 µM final concentration) to the lyophilized CFPS reactions in presence of deionized water or 20%–70% (v/v) saliva and sfGFP expression yields were measured after 3 h reaction at 37°C.

3 | RESULTS AND DISCUSSION

3.1 | m-RI soluble expression yields

Eukaryotic RNase inhibitors have internal hydrophobic leucine-rich cores and contain 30-32 reduced cysteine residues which make its soluble and active expression by prokaryotic systems challenging (Šiurkus & Neubauer, 2011a). To address this challenge m-RI was expressed with *E. coli*-lysate-based CFPS. Preliminary expression results at 30°C showed higher soluble expression of m-RI by using a BL21 Star DE3 *E. coli* extract which contains GroEL/ES chaperons (expressed from the pOFX plasmid), compared with the BL21 Star DE3 *E. coli* extract. This is in accordance with previous results for in vivo expression of m-RI by *E. coli* in presence of GroEL/ES chaperons (Šiurkus & Neubauer, 2011a). The exact mechanism for superior folding of m-RI in presence of GroEL/ES compared with the native folding machinery of *E. coli* has not been explored in detail but is postulated to be m-RI protection from oxidation or aggregation within the chaperone cage (passive cage), or involvement of the cage in folding by limited mobility and interaction of the cage (active cage), or unfolding the misfolded m-RI within the cage and refolding inside or outside the chaperone cage (Georgescauld et al., 2014; Šiurkus & Neubauer, 2011a).

All cysteines in m-RI are reduced and interact with RNase substrates (Šiurkus & Neubauer, 2011a). Thus, the reducing environment of native CFPS reactions was expected to favor this requirement. Nevertheless, the accessible nature of the CFPS reaction environment facilitated the systematic investigation of the protein synthesis redox potential that yielded optimal soluble expression at three reaction temperatures and for three reaction durations. Figure 1 reports soluble expression yields of m-RI with two ratios of oxidized (reducing agent) and reduced glutathione (8 mM GSH/0.5 mM GSSG and 4 mM GSH/1 mM GSSG), two concentrations of the reducing agent DTT (4 and 12 mM), and the native CFPS reaction environment. Each redox condition was tested at three reaction temperatures of 15°C, 24°C, and 30°C and three batch reaction durations of 2, 6, and 8 h.

Interestingly, a CFPS reaction temperature of 24°C yields the highest soluble titer of m-RI across all redox conditions (Figure 1). This observation corroborates several studies which recommend lower reaction temperature for higher solubility and activity of different recombinant protein expression in *E. coli* (Schein & Noteborn, 1988; Vasina & Baneyx, 1997) and yeast cells (Li et al., 2001). Surprisingly, the 30°C reaction temperature is less favorable for high soluble protein expression than the two lower reaction temperatures despite anticipated faster reaction kinetics. Indeed, faster expression of the target protein may overwhelm GroEL/ES folding machineries and lead to more aggregation or misfolding than correct folding of the target protein (Ignatova & Gierasch, 2006; Kiefhaber et al., 1991). Higher reaction temperature also reduces the solubility of oxygen in the CFPS liquid, a condition that has been shown to reduce CFPS expression yield (Nelson et al., 2020; Zhou et al., 2020).

Reaction duration also affected soluble m-RI yield in interesting and explicable ways. At all conditions tested, soluble yields remained constant or decreased between 6 h and 8 h reaction duration. This decrease could be attributed to increased time for unproductive protein–protein interactions that lead to aggregation. The rate of this aggregation is likely more pronounced as the protein synthesis rate reaches a maximum then subsequently slows as a result of DNA and mRNA degradation, depletion of high energy compounds, and accumulation of toxic side products such as inorganic phosphates (Hodgman & Jewett, 2013; Kim & Swartz, 1999; Schoborg et al., 2014). The decline in soluble expression yield after 6 h could also be attributed to the activity of

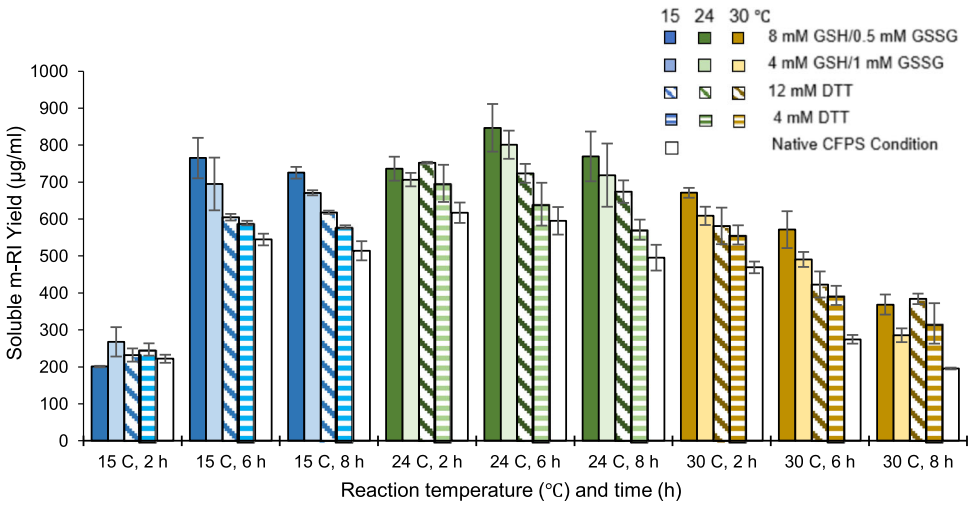

FIGURE 1 Soluble expression yields of m-RI at 15°C (blue), 24°C (green), and 30°C (brown) after 2, 6, and 8 h reaction, expressed by CFPS in a GroEL/ES enriched extract. Different reduced conditions are shown in solid (8 mM GSH/0.5 mM GSSG), pale (4 mM GSH/1 mM GSSG), diagonal stripes (12 mM DTT), horizontal stripes (4 mM DTT), and transparent (Native) using the reaction temperature color scheme. CFPS, cell-free protein synthesis; DTT, dithiothreitol; m-RI, murine RNase inhibitor [Color figure can be viewed at wileyonlinelibrary.com]

native *E. coli* proteases. Notably, protein synthesis reaction rate is significantly slower at 15°C. Consistent with these explanations, the decline in soluble yield over time was most significant for the higher reaction temperature of 30°C (Figure 1, brown bars).

Importantly, soluble expression of m-RI was higher in CFPS reactions supplemented with a reducing buffer (Figure 1, colored/patterned bars) for all times and temperatures except for 15°C and 2 h which had comparable soluble yields regardless of reducing potentials. Among the two reductants, GSH/GSSG combination provided higher soluble expression yields compared to DTT for most conditions.

The highest soluble m-RI expression was achieved by 8 mM GSH/0.5 mM GSSG at 24°C after 6 h (847 ± 64 μg/ml). Nearly 87% of this expression was achieved at the same temperature after 2 h which demonstrates the rapid expression capabilities of CFPS systems (K.-H. Lee & Kim, 2018).

Interestingly, follow-up studies expressing m-RI in BL21 Star DE3 *E. coli* extract that did not express GroEL/ES were also effective in expressing m-RI at similar yields and expression trends with the GSH/GSSG and DTT buffers (Figure S1). This suggests that the CFPS environment is more conducive to high soluble titers of m-RI than the *E. coli* cytoplasm (Šiurkus & Neubauer, 2011a, 2011b) even without GroEL/ES overexpression, possibly because it is more dilute by more than an order of magnitude (Underwood et al., 2005). Overall, the clear advantages of reducing agents suggest that insoluble aggregates may result directly from incorrect intramolecular disulfide bond formation, and that soluble conformation are preserved by the presence of reducing agents.

In anticipation that purification of the soluble m-RI is desirable, a 6xHis-tag was added to either N or C-terminals of the insert m-RI gene by QuikChange II mutagenesis protocol, and insertions were confirmed by sequencing. m-RI did not express well after adding 6xHis-tag to the N-terminal of m-RI (Figure S2a). The soluble expression yield for the C-terminal 6xHis-tag (expressed at 24°C and 2 h by pOFX extract in presence or absence of 12 mM DTT) was comparable (75%–80%) to the no tag m-RI (Figure S2a). Full-length m-RI expression (with and without 6xHis-tag) was confirmed using SDS-PAGE and autoradiography (Figure S3).

3.2 | m-RI activity

The primary purpose of m-RI expression in this study is to provide a low-cost option for supplying m-RI to CFPS biosensors that require m-RI for use with human body fluid samples. The activity of soluble m-RI expressed at different conditions was thus assessed by adding 1–3 ng/μl soluble unpurified m-RI to a second CFPS reaction producing biosensor reporter protein sfGFP in presence of 30% human saliva (Figure 2). Saliva was selected as the target human fluid as it contains different RNases (including RNase 1, RNase 3, and RNase H) at significant concentrations (Potenza et al., 2006) and contains biomarkers for diagnosis or treatment purposes. Most importantly, saliva is easily collected in a noninvasive manner and does not require trained personnel or special equipment for its collection, making it ideal for at-home testing (K. Lee et al., 2020).

The temperature and time CFPS conditions tested for m-RI activity were 24°C for 2 h as most m-RI synthesis at 24°C was

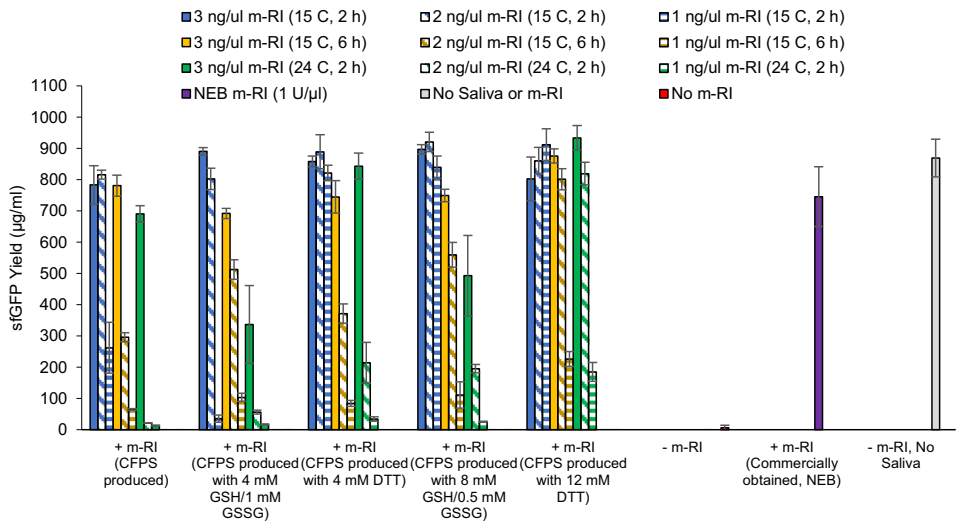

FIGURE 2 RNase inhibition of three m-RI preparation expressed at 15°C-2h (blue), 15°C-6h (yellow), and 24°C-2 h (green) with 3 ng/μl (solid colors), 2 ng/μl (diagonal stripes), and 1 ng/μl (horizontal stripes) soluble m-RI concentrations. All m-RIs were expressed by CFPS in an extract with GroEL/ES overexpressed and added directly without purification or freeze-thawing to a second CFPS reaction expressing sfGFP in the presence of 30% human saliva. RNase inhibition activity of 1 U/μl reaction of commercially obtained -m-RI from NEB is shown in purple and a control sfGFP CFPS reaction without saliva or m-RI addition is shown in gray. Near complete sfGFP production inhibition is demonstrated when 30% saliva is added to the sfGFP-producing CFPS reaction without the addition of m-RI (red). CFPS, cell-free protein synthesis; DTT, dithiothreitol; m-RI, murine RNase inhibitor [Color figure can be viewed at wileyonlinelibrary.com]

complete after 2 h (Figure 1) and 15°C for 2 h and 6 h where m-RI synthesis at 15°C appeared complete after 6 h (Figure 1). The lower temperatures and times were especially considered important due to previous in vivo reports where temperatures as low as room temperature and shorter induction periods results in significantly higher m-RI specific activities (Šiurkus & Neubauer, 2011b). While the temperature and time ranges were constricted, the original range of GSH/GSSG and DTT redox buffers were maintained and Figure 2 reports sfGFP CFPS expression in 30% saliva where unpurified CFPS-produced m-RI is provided as a reagent at 1, 2, and 3 ng/µl (horizontal stripes, diagonal stripes, and solid colors, respectively). As a comparison, sfGFP expression in absence of saliva and m-RI (gray bar), sfGFP expression in presence of 30% saliva and 1 U/µl reaction of the commercial m-RI (NEB, purple bar), and sfGFP expression in the presence of 30% saliva and absence of any RNase inhibitors (red bar) are included in Figure 2.

As expected, CFPS reaction in the presence of 30% saliva without m-RI resulted in undetectable amounts of sfGFP protein (red bar, Figure 2), and the addition of commercial m-RI recovers most sfGFP expression (purple bar compared with the gray bar, Figure 2). Adding 3 ng/µl of m-RI produced at 15°C by CFPS (blue and yellow solid bars, Figure 2) performed equivalently or better at restoring sfGFP production in the presence of saliva as compared to 1 U/µl of commercial m-RI. When the concentration of CFPS-produced m-RI is reduced to 1 ng/µl, only the m-RI produced at 15°C for 2 h with 4 or 12 mM DTT or 8 mM GSH/0.5 mM GSSG buffers were effective at recovering all the sfGFP production capabilities. When comparing the data in aggregate, 12 mM DTT appeared to be the best performing buffer (which also has the most reducing redox potential) for all m-RI-producing CFPS temperatures and time conditions tested. This result of optimal CFPS expression of m-RI at 2 h, 15°C and 12 mM DTT is consistent with the high quantity of unpaired cysteines (30) in m-RI and previously reported in vivo expression trends where lower temperature, strongly reducing redox conditions, and shortened expression windows were most effective at producing active m-RI (Šiurkus & Neubauer, 2011b).

It should also be noted, that adding either C- or N-terminal 6xHis-tags to m-RI caused a complete loss of activity (Figure S2b), thus using unpurified m-RI is particularly important as using a purified product would require not only purification steps but also cleavage of the tag which would significantly increase its costs and justifies the higher costs of commercially available purified m-RI.

3.3 | Stability of m-RI

Producing m-RI with CFPS immediately before incorporation with CFPS biosensor reagents is more cumbersome than stockpiling CFPS-produced m-RI as a frozen, ready-made reagent when assembling CFPS biosensors. Thus, CFPS-produced and unpurified m-RI activity was assessed after one and two freeze-thaw cycles as detailed in Section 2. After freeze-thawing, m-RI activity was analyzed using the same procedure as described above (i.e., a second CFPS reaction expressing sfGFP in the presence of 30% (v/v) saliva) and the result is reported in Figure S4 (after one freeze-thaw cycle) and in Figure 3 (after two freeze-thaw cycles).

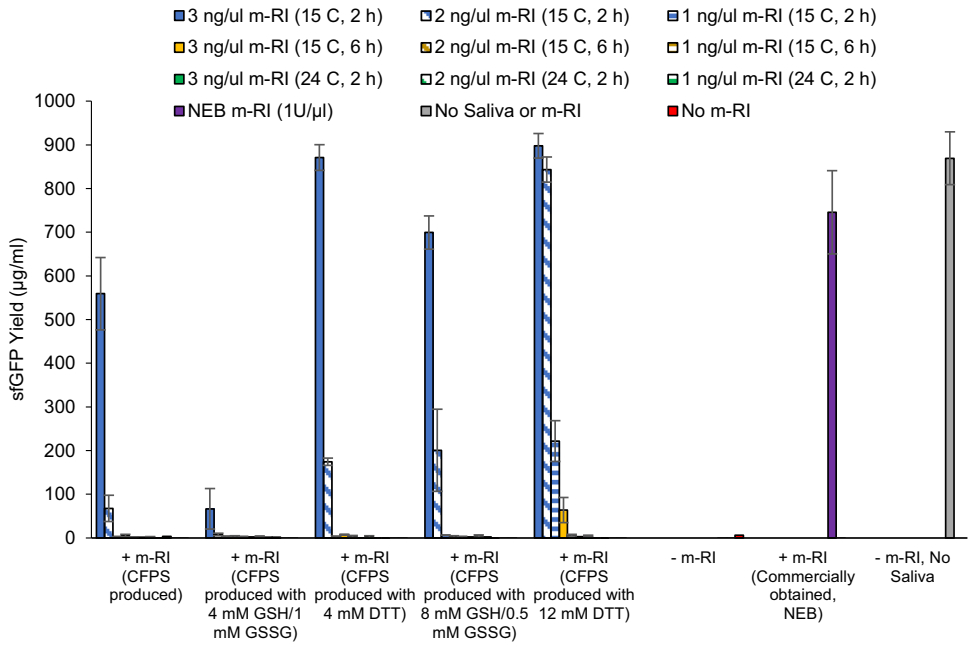

FIGURE 3 m-RI activity after two freeze-thaw cycles. m-RIs expressed at 15°C- 2 h (blue), 15°C- 6 h (yellow), and 24°C- 2 h (green) with 3 ng/µl (solid colors), 2 ng/µl (diagonal stripes), and 1 ng/µl (horizontal stripes) soluble m-RI concentrations. All m-RIs were expressed by pOFX extract. RNase inhibition activity of NEB-m-RI is shown in purple and the No saliva/m-RI control is in gray. CFPS, cell-free protein synthesis; DTT, dithiothreitol; m-RI, murine RNase inhibitor [Color figure can be viewed at wileyonlinelibrary.com]

After two freeze-thaw cycles it is abundantly clear that m-RI expressed at 15°C for 2 h is much more active than m-RI expressed at 24°C or 15°C after 6 h where m-RI expressed at these conditions did not restore sfGFP expression (Figure 3). Also, 12 mM DTT was confirmed as the best buffer for both active and stable m-RI expression. Most importantly, this result verifies that simply adding 2 ng/μl of CFPS-produced m-RI at 15°C, 2 h, and 12 mM DTT is sufficient to account for m-RI activity losses due to freeze-thawing and completely restore CFPS activity in the 30% human saliva samples tested. Accordingly, m-RI was expressed at 15°C for 2 h in presence of 12 mM DTT and added to 50 μl CFPS reactions (with 6 ng/μl final concentration) to inhibit RNase activity in varied amounts of three human fluids of saliva, serum, and urine (Section 3.4) or as a saliva-based glutamine biosensor (Section 3.5).

In all activity and stability data presented so far, m-RI was expressed using an *E. coli* extract that was enriched for GroEL/ES (pOFX plasmid overexpresses GroEL/ES during fermentation of *E. coli* used to produce extract). However, previously mentioned m-RI expression data suggested similar yields of soluble m-RI could be obtained in the same *E. coli* lysate without GroEL/ES enrichment (without pOFX, Figure S1). The activity of m-RI expressed at 15°C and 2 h (highest activity and stability condition) in both extracts were assessed for activity after one freeze-thaw cycle (Figure S5). m-RI expressed in extract with overexpressed levels of GroEL/ES had higher activity, justifying the use of this extract condition and agrees with prior in vivo m-RI expression results (Šiurkus & Neubauer, 2011a, 2011b).

3.4 | CFPS-produced m-RI restores CFPS production in samples containing up to 100% human body fluids of saliva and serum and up to 40% urine

To demonstrate the utility of CFPS-produced m-RI beyond 30% saliva samples, the impact of different concentrations of human serum, urine, and saliva samples on CFPS reactions producing sfGFP as the reporter were assessed (Figure 4a). Simply adding these fluids to CFPS at concentrations as low as 0.5% (v/v) had a statistically significant negative impact on CFPS. At 8% (v/v) of these samples, greater than 95% of the CFPS production capability was lost (Figure 4a). However, in-house, CFPS m-RI added to the sfGFP-producing reaction effectively inhibited sample RNases sufficient to restore most of the sfGFP production capability (Figure 4b–d).

A "just add sample" biosensor format is advantageous in point-of-care applications but requires CFPS to retain activity in 100% (v/v) human sample fluid. To test m-RI performance in CFPS with human fluid volume percent up to 100%, the sfGFP-producing CFPS reaction was lyophilized after unpurified m-RI was added to it. Lyophilization has been reported as an excellent way to facilitate long-term shelf-stable storage and on-demand use of CFPS biosensors (Gregorio et al., 2020; Hunt et al., 2017) and these results also demonstrate the ability of CFPS-produced m-RI to be included in this format. As shown in Figure 4b, 1 ng/μl of CFPS-produced m-RI (freshly expressed at 15°C, 2 h, in presence of 12 mM DTT) was sufficient to restore most CFPS activity in up to 40% saliva and 10% urine. Increasing the m-RI concentration to 6 ng/μl improved the recovery of CFPS activity at even higher percentages of human samples and appears to be sufficient to recover most of the CFPS activity with 100% saliva (Figure 4c). At 15 ng/μl, approximately 80% of CFPS activity was recovered when the lyophilized reagents were rehydrated with 100% saliva, approximately 25% of CFPS activity was recovered in 100% serum samples, and approximately 50% of CFPS activity was recovered in 30% urine (Figure 4d). While inhibitory effects beyond RNases likely exist in these samples, it appears that RNase inhibitor is a primary limiting factor, and m-RI is effective in restoring CFPS activity in these samples, potentially enabling detection of myriad biomarkers in these fluids using CFPS biosensors.

3.5 | CFPS-produced m-RI enables glutamine CFPS-biosensor application in human saliva

To further demonstrate the utility of CFPS-produced m-RI with on-demand CFPS biosensors using human fluids, a recently reported CFPS-glutamine biosensor was tested (Hunt, Barnett, et al., 2020). Previously, this biosensor required commercial m-RI (NEB) and compatibility with lyophilization or with human saliva samples has not been demonstrated. As the most abundant amino acid, glutamine is an important biomarker for a variety of diseases or disorders including diabetes, cancer, kidney disease, chronic intestinal pseudo-obstruction, brain dysfunction, and Alzheimer's disease (Chen et al., 2019; Madeira et al., 2018; Rhee et al., 2018). Determining glutamine concentration with saliva is less invasive and has been postulated as a better alternative to blood or serum for infants, disabled people, or people with hemophilia (Malon et al., 2014).

CFPS-based glutamine biosensing works by omitting glutamine as a CFPS reagent and inhibiting endogenous *E. coli* glutamine synthetase activity in the cell lysate with MSO (Hunt, Barnett, et al., 2020). The absence of glutamine in a test sample results in undetectable levels of full-length fluorescent sfGFP synthesis, while the concentration of full-length sfGFP is proportional to the amount of glutamine in the sample. Indeed, a linear correlation between glutamine concentrations and sfGFP production has been reported over the physiological range of glutamine concentrations in the body (Hunt, Barnett, et al., 2020). To demonstrate the utility of in-house m-RI for CFPS biosensing in human fluids, the CFPS-glutamine biosensor was constructed as reported previously (Hunt, Barnett, et al., 2020), with two exceptions (1) 6 ng/μl CFPS-produced m-RI (freshly expressed at 15°C, 2 h, in presence of 12 mM DTT) replaced commercial m-RI and (2) all CFPS-reagents were lyophilized together to create a "just-add-sample" single-pot batch biosensor. Approximately 40% (v/v) human saliva and water was spiked with increasing concentrations of glutamine and used to rehydrate the lyophilized CFPS glutamine biosensor (Figure 5a). Additionally, the performance of the

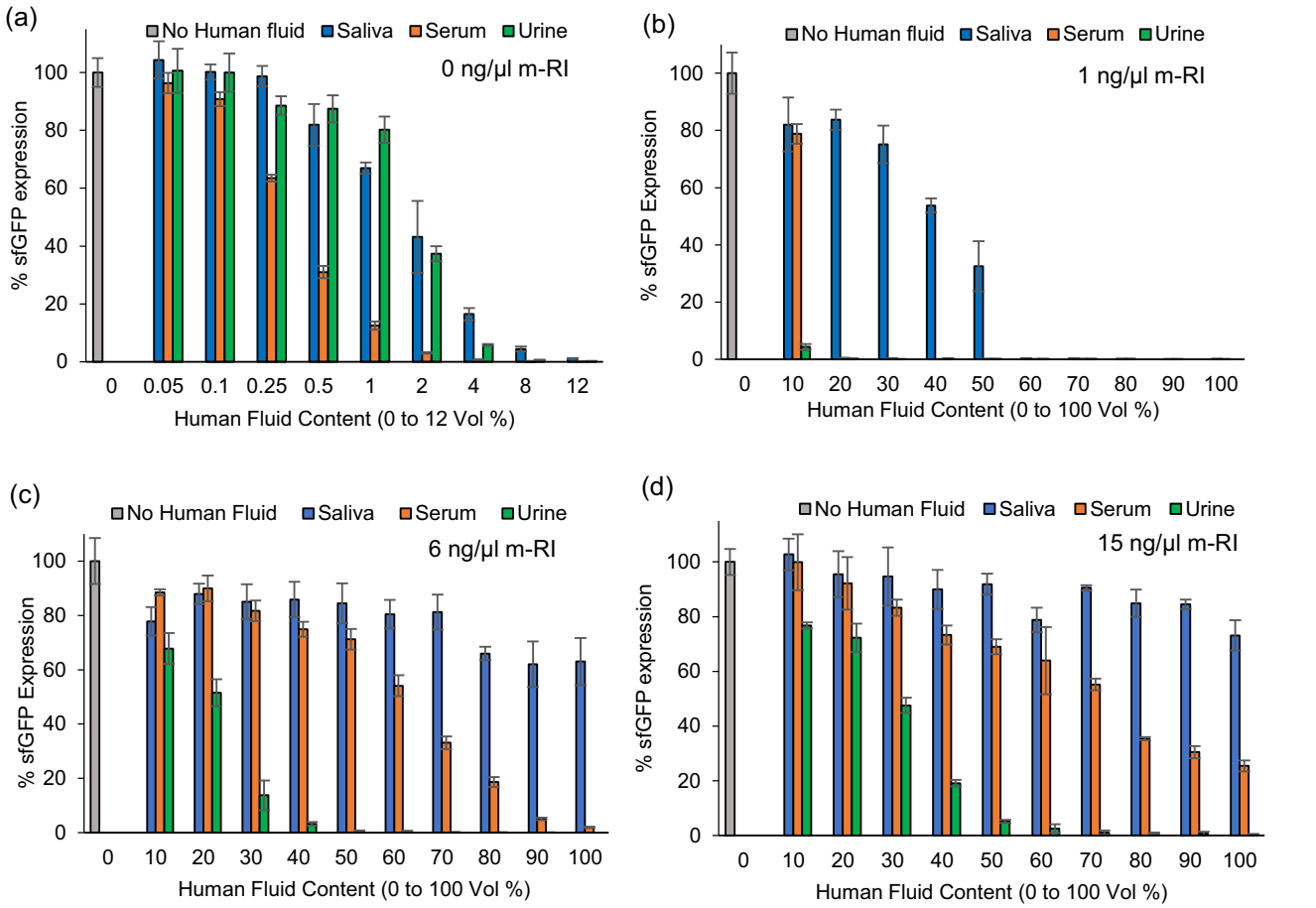

FIGURE 4 (a) Relative sfGFP expression capability in presence of three human fluids of saliva (blue), urine (green), and serum (orange) in absence of any RNase inhibitors. sfGFP expression in presence of 0.05–12 volume percent human fluids are normalized relative to the reaction without human fluid (gray, 100%). All reactions were performed at 37°C, 3 h and error bars represent one standard deviation for $n = 3$ reactions. (b) Relative sfGFP expression of the rehydrated CFPS reactions by (10–100) vol% human fluids of saliva (blue), serum (orange), and urine (green) supplemented with 1 ng/µl freshly prepared in-house m-RI. (c) Relative sfGFP expression of the rehydrated CFPS reactions by (10–100) vol% human fluids of saliva (blue), serum (orange), and urine (green) supplemented with 6 ng/µl freshly prepared in-house m-RI. (d) Relative sfGFP expression of the rehydrated CFPS reactions by (10–100) vol% human fluids of saliva (blue), serum (orange), and urine (green) supplemented with 15 ng/µl of freshly prepared in-house m-RI. m-RIs were expressed at 15°C for 2 h, in presence of 12 mM DTT and GroEL/ES chaperons. For consistency, all human fluids were treated with one freeze-thaw cycle before use and sfGFP was expressed at 37°C for 3 h. All sfGFP expression yields are normalized relative to the control (water) CFPS reaction at the indicated m-RI concentration. CFPS, cell-free protein synthesis; DTT, dithiothreitol; m-RI, murine RNase inhibitor [Color figure can be viewed at wileyonlinelibrary.com]

lyophilized glutamine biosensor in the presence of higher concentrations of saliva was assessed (Figure 5b).

As is shown in Figure 5a, a strong linear correlation ($R^2 > 0.98$) between the concentration of glutamine added to a control water sample (gray) and the saliva sample (blue) were observed with similar slopes and the higher sfGFP results for saliva samples relative to water samples are expected due to glutamine levels naturally in saliva samples (x-axis reports glutamine added to these samples). To further verify glutamine detection, saliva was added at increasing volume percentages (20%, 40%, and 70%) to the lyophilized CFPS biosensor (Figure 5b), which increases the overall effective concentration of glutamine in the biosensor with the glutamine naturally in saliva (Masoudi Rad et al., 2014). A strong linear correlation between sfGFP-production in the glutamine-depleted CFPS biosensor to the saliva concentration was observed. The range of 20%–70% saliva was selected as sfGFP production with 6 ng/µl m-RI used in the test was essentially constant in sfGFP-producing CFPS reactions that were not initiated without glutamine (Figure 4b blue bars). Thus, CFPS-produced m-RI enables the detection of glutamine in human saliva as a lyophilized "just-add-sample" biosensor. Importantly, in-house m-RI added directly to the CFPS glutamine biosensor decreases the overall reagent cost of the sensor by approximately 90% (Online Supporting Information) as it provides m-RI without expensive purification or other post-translational processing. It is important to note that this estimated reagent cost does not include labor, equipment, and m-RI activity validation, however, these costs would decrease significantly with scale-up (Zawada et al., 2011).

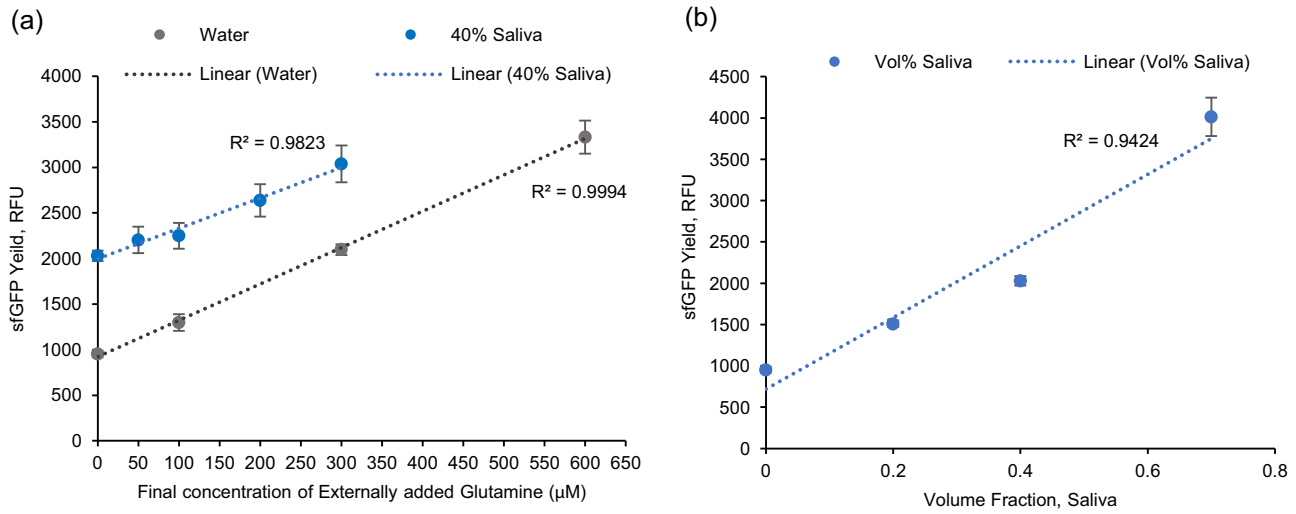

FIGURE 5 (a) Lyophilized glutamine biosensor CFPS reactions containing 6 ng/μl m-RI (expressed at 15°C, 2 h with 12 mM DTT) rehydrated with water (gray dots/line) or 40% (v/v) saliva (blue dots/line). Rehydration samples were spiked with glutamine to achieve the indicated CFPS glutamine concentrations. (b) Lyophilized glutamine biosensor CFPS reactions containing 6 ng/μl m-RI (expressed at 15°C, 2 h with 12 mM DTT) rehydrated with 0%, 20%, 40%, and 70% (v/v) saliva (blue dots/line). CFPS, cell-free protein synthesis; DTT, dithiothreitol; m-RI, murine RNase inhibitor [Color figure can be viewed at wileyonlinelibrary.com]

4 | CONCLUSION

Analysis of human fluids often requires significant sample volumes (≥ 1 ml), labeling, freezing/cooling, and transportation to the lab before analysis (Malon et al., 2014). These cumbersome requirements highlight the need for point-of-care, "just-add-sample" biosensors. This study leverages the flexible nature of CFPS systems to optimize the production of highly soluble and active m-RIs. Reaction temperature, reaction time, reducing potential, and presence of chaperons were manipulated to achieve soluble m-RI titers of nearly 1 g/L. Among the expression conditions tested, 24°C and 6 h in presence of 12 mM DTT and GroEL/ES folding chaperons produced the highest soluble yield. However, m-RI with the highest specific activity and stability after freeze-thawing was produced in CFPS reactions at 15°C for 2 h in the presence of 12 mM DTT and GroEL/ES folding chaperons (Figures 2 and 3). The concentrations of CFPS-produced m-RI required to inhibit the RNases native in human body samples of blood, urine, and saliva, and enable the production CFPS-based biosensor reporters, was also determined (Figure 4). CFPS-produced m-RI lyophilized with CFPS reagents enabled CFPS biosensor reporter production in the presence of 100 volume percent human samples. Finally, a CFPS-based glutamine biosensor was created for the first time that (1) accepts saliva as the test sample and (2) is lyophilized in a "just-add-sample" format for on-demand at-home use, and (3) employs CFPS-produced m-RI rather than commercial m-RI to inhibit RNases, thus reducing the total reagent cost of the glutamine biosensor by approximately 90%. This study demonstrates that m-RI produced with CFPS has the potential to enable CFPS biomarker detection in human serum, urine, and saliva, thus improving diagnosis and treatment of many debilitating disorders and diseases.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Mehran Soltani and Bradley C. Bundy conceived the project. Mehran Soltani designed and carried out the experiments and wrote the manuscript. J. Porter Hunt consulted regarding some experimental design. Mehran Soltani, J. Porter Hunt, and Bradley C. Bundy analyzed the data and edited the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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